Title
Growth and Genetic Manipulation of Entamoeba histolytica

Journal
Current Protocols, 2(1)

ISSN
2691-1299

Authors
Suleiman, Rene L
Ralston, Katherine S

Publication Date
2022

DOI
10.1002/cpz1.327

Peer reviewed

HHS Public Access

Author manuscript

Curr Protoc. Author manuscript; available in PMC 2023 January 01.

Published in final edited form as:

Curr Protoc. 2022 January ; 2(1): e327. doi:10.1002/cpz1.327.

Growth and genetic manipulation of *Entamoeba histolytica*

Rene L. Suleiman[1], **Katherine S. Ralston**[1]

[1]Department of Microbiology and Molecular Genetics, University of California, Davis, USA

Abstract

Entamoeba histolytica is a parasitic protozoan and the causative agent of amoebiasis in humans. Amoebiasis has a high incidence of disease, resulting in ~67,900 deaths per year, and it poses a tremendous burden of morbidity and mortality in children. Despite its importance, *E. histolytica* is an understudied parasite. These protocols describe the *in vitro* growth, maintenance, cryopreservation, genetic manipulation and cloning of axenic *E. histolytica* trophozoites. There has been significant progress in genetic manipulation of this organism over the past decade, and these protocols outline the ways in which these advances can be implemented.

Keywords

Entamoeba histolytica ; cryopreservation; culture; parasite; transfection; RNAi

INTRODUCTION

Entamoeba histolytica is the causative agent of amoebiasis, a parasitic infection of the gastrointestinal tract that is found predominantly in developing countries without water sanitation facilities. *E. histolytica* spreads from host to host as a dormant cyst, and grows and divides as a motile trophozoite in the intestinal tract. Repeated diarrheal illness caused by *E. histolytica* has been associated with stunting and malnutrition in children (Petri Jr et al., 2009). In studies of endemic areas, ~80% of infants are infected with *E. histolytica* (Gilchrist et al., 2016). Clinical presentation includes asymptomatic infection, mild diarrhea, bloody diarrhea with intestinal ulceration, and potentially fatal extra-intestinal abscesses.

*Address correspondence to Katherine S. Ralston, ksralston@ucdavis.edu.

COMPETING INTERESTS

The authors declare that they have no competing interests.

Amoebiasis causes 15,500 deaths per year in children under 5, and 67,900 deaths per year among people of all ages (Wang et al., 2016). Treatment options are limited to a single class of therapeutic agents, and currently there is no vaccine available.

Despite its high incidence and impact on human health, *E. histolytica* is understudied relative to many other parasites. This highlights the need for greater efforts to study the basic biology of *E. histolytica* and the pathogenesis of amoebiasis. Historically, the genome has been difficult to manipulate and genetic tractability has been limited. *E. histolytica* is tetraploid (Willhoeft & Tannich, 1999), the genome is 75% A + T, and expanded gene families comprise 56% of the proteome (Lorenzi et al., 2010; Wilson et al., 2019). Given the challenges of the genome, the endogenous RNAi pathway has generally been the most useful tool for genetic manipulation. Older approaches for RNAi knockdown in *E. histolytica* affected the expression of off-target genes (Bracha et al., 2003, 2006), but newer approaches to characterize the endogenous pathway in detail have led to the more modern "trigger" approach for gene knockdown (Morf et al., 2013).

This unit provides several protocols ranging from basic cell culture to genetic manipulation of *E. histolytica*. Initial procedures outline the process of growing *E. histolytica* trophozoites (referred to as "amoebae") and creating frozen stocks. Support protocols detail the recipe for culture media and how to test media components that exhibit lot-to-lot variation, to ensure that they are suitable to support cell growth. A method for stable transfection of *E. histolytica* using Attractene is provided. Since a limitation of standard transfection approaches is that the resultant transfectants are heterogeneous, a protocol for obtaining clonal lines is generally useful. Details are provided for best practices in using the trigger-induced RNA interference (Morf et al., 2013) approach for gene knockdown. Support protocols are provided to describe screening mutants for gene knockdown using either conventional reverse transcriptase PCR (RT-PCR) or quantitative RT-PCR. Finally, a procedure for conducting growth curves to assess mutant phenotypes is included.

CAUTION: E. histolytica is classified as Biosafety Level 2 (BSL2), and all precautions associated with BSL2 classification should be strictly followed. Guidelines for BSL2 practices can be found in the latest edition of *Biosafety in Microbiological and Biomedical Laboratories* (BMBL, 5th Edition) at the following CDC website: https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF.

NOTE: All materials and reagents coming into contact with live cells must be sterile, and aseptic technique should be carefully applied at every relevant step.

BASIC PROTOCOL 1

Culturing *E. histolytica* trophozoites

This protocol is an adaptation of Louis S. Diamond's original work (Diamond, 1983), and contains updated information about best practices in *E. histolytica* cultivation. *E. histolytica* is adherent and grows on surfaces, but only requires chilling to facilitate detachment unlike other adherent cell lines which require trypsinization. *E. histolytica* is microaerophilic, and thus the amount of air left in any culturing vessel is also an important consideration.

Included below are detailed instructions for how to harvest and passage *E. histolytica* trophozoites.

Materials—Frozen stock of *Entamoeba histolytica* Schaudinn strain HM-1:IMSS (ATCC, cat. no. 30459)

Complete TYI-S-33 medium (see Support Protocol 1), warmed in 35°C water bath

Disposable 16×125mm borosilicate glass tubes with threaded end (Fisher, cat. no. 14–959-35A)

Kimble black phenolic screw-thread closures GPI thread 15mm-415 (Fisher, cat. no. 05–569-5)

25cm² Rectangular Canted Neck Cell Culture Flask with Plug Seal Cap (Corning, cat. no. 430168)

Slanted tube racks (Fisher, cat. no. 14–796-1)

Inverted microscope capable of 400x magnification

Centrifuge equipped with a swinging bucket rotor, such as Allegra X-14R (Beckman Coulter, cat. no. B08861)

Sterile 15ml polypropylene conical centrifuge tubes (Corning, cat. no. 352196)

35°C incubator

35°C water bath

Maintenance of E. histolytica HM1:IMSS *trophozoites in glass culture tubes*

1. Once cells are ~80% confluent (Figure 1a) in the glass culture tube, they should be split into a new culture tube to prevent overgrowth (Figure 1b).

   In order to maintain cultures in active growth, it is necessary to passage cells at least twice a week (every 3–4 days). If cells are not ready to passage after a week, it is necessary to replace the culture media with fresh media (see step 6, below), since TYI-S-33 media is only stable for one week.

   If cells do not grow quickly enough in order to passage at the desired frequency of every 3–4 days, it may be necessary to seed new culture tubes with a greater number of cells. In general, when cultures are 80% confluent, 1ml should be an appropriate volume for passaging into a new tube. Seeding with roughly 5×10^4 to 1×10^5 cells will result in an ~80% confluent culture after 3–4 days. If these parameters have been used, and cultures are still not ready to passage every 3–4 days, it will be important to carefully evaluate the cell culture medium, as E. histolytica is sensitive to lot-to-lot variation in components of TYI-S-33 medium, and is sensitive to the preparation of cell culture glassware (see Support Protocols 1 and 2, and Commentary, Critical Parameters and Troubleshooting).

2. Prepare a new glass culture tube with 13ml warm complete TYI-S-33 medium.

   Before use, glass culture tubes must be rinsed and autoclaved. First, tubes must be rinsed with deionized water three times and set upside down in a plastic test tube basket until dry. Once tubes are dry, caps should be loosely attached to tubes, and then capped tubes can be autoclaved.

   Glass culture tubes hold 16ml of media; since E. histolytica *is microaerophilic, the culture tubes may be filled with as much as 14ml of media; overfilling could result in overflow and provide a potential source of contamination via liquid contact with tube threading and lid closure. Amoebae also grow best when tubes are not entirely filled – too little air can be as detrimental as too much air* (Diamond, 1983).

3. To detach cells, place culture tube on ice for 5–10 minutes.

   E. histolytica *cells are adherent, and placing culture tubes and flasks on ice is an effective method for detaching cells from surfaces. Slightly melted ice (or a mixture of ice and water) works best in cooling culture tubes and flasks, because a water interface transfers temperature more efficiently to the culture tube than an air interface. Incubation on ice is a stressor, and cells should only be iced for up to 5–10 minutes. To avoid contamination, be sure to use a paper towel to wipe any remaining ice off of the culture tube, and then spray the tube with 70% ethanol and wipe with a paper towel, before opening the tube in the biosafety cabinet.*

4. Gently invert the tube several times to detach adherent cells. It is critical at this step to evaluate cell detachment by using microscopy to visualize the culture tube. Successful detachment is observed when the majority of cells have been detached, and only a handful of cells are seen per imaging field (Figure 1c).

   If cells have not been successfully detached, incubate on ice again, until most cells have detached.

5. Invert glass tube to resuspend amoebae; transfer 1ml cells to the new culture tube and invert to mix.

   E. histolytica *cells tend to settle quickly in solution, thus it is important to resuspend cells every time aliquots are taken from a cell suspension.*

   If cultures are 80% confluent, 1ml should be an appropriate volume for passaging into a new tube. Seeding with roughly 5×10^4 to 1×10^5 cells will result in an ~80% confluent culture after 3–4 days.

6. Incubate tubes at a 10° angle at 35°C (Diamond, 1968b).

   Position tubes in the slanted tube rack with their labels facing upwards. This provides a reference point for where cells will be located, since the amoebae will attach and grow along the lowest glass surface, which will be opposite from the label.

E. histolytica *cultures can be maintained in glass culture tubes indefinitely, as long as they are split at least twice a week and before reaching 80% confluency. If cells require more than a week to grow to confluency, either carefully replace approximately half of the volume of spent TYI-S-33 medium with fresh complete TYI-S-33 medium or use centrifugation to remove spent TYI-S-33 medium in order to fully replace the spent media with fresh media. For the latter option, detach cells from the culture tube as above by incubating on ice* (Basic Protocol 1 Section 3), *transfer to a 15 ml conical tube, and centrifuge at 200xg for 5 minutes. Resuspend the cell pellet with 14 ml fresh complete TYI-S-33 medium and transfer to a new glass culture tube.*

7. To grow up many cells for experimental purposes, a 25cm² (T25) or 75 cm^2 (T75) flask may be used to culture larger quantities of amoebae. The number of cells required to seed a T25 is dependent on the concentration of the starter culture and the desired time to confluency.

   As a general rule, seeding a T25 flask with about 9×10^5 cells will result in confluency after 24 hours.

   As with the glass culture tubes, T25 and T75 flasks may be filled up to a few ml below their maximum capacity, since a small amount of air is necessary for optimal growth.

   Cultures grown in T25 or T75 flasks can also be detached by incubating on ice for up to 5–10 minutes, and gently inverting several times. Check on a microscope to confirm that most cells have detached.

SUPPORT PROTOCOL 1

Preparation of TYI-S-33 medium

This support protocol provides detailed instructions for the preparation of TYI-S-33, which stands for Trypticase-Yeast Extract-Iron-Serum. The recipe for TYI-S-33 medium was created and tested by L.S. Diamond in 1978, ten years after he achieved axenization of *E. histolytica* (Diamond et al., 1978). Of particular importance is the observation that distilled water is preferred over water from purification systems such as Milli-Q, and media made with distilled water promotes more growth than water from other sources (Diamond, 1968a). The primary rich media component, Biosate peptone, may be substituted with yeast extract and tryptone at a 1:2 ratio. This is possible because Biosate peptone is in fact a combination of yeast extract and tryptone. TYI-S-33 medium can be made in large batches and stored at −20°C indefinitely. Individual bottles of TYI-S-33 can be thawed as needed and stored at 4°C. However, once a bottle of TYI-S-33 has been thawed it can only be stored at 4°C for up to one week, and should be discarded after that point.

Materials—BBL Biosate Peptone (Gibco, cat. no. 211862)

D-(+)-Glucose (Sigma, cat. no. G8270)

NaCl (Sigma, cat. no. S7653)

K_2HPO_4 (Sigma, cat. no. 60353)

KH_2PO_4 (Sigma, cat. no. P5655)

L-Cysteine hydrochloride monohydrate (Sigma, cat. no. C7880)

L-Ascorbic acid (Sigma, cat. no. A0278)

Ferric Ammonium Citrate (Sigma, cat. no. F5879)

Diamond Vitamin Tween 80 Solution 40x (Sigma, cat. no. 58980C)

Penicillin-Streptomycin (10,000 U/ml) (Gibco, cat. no. 15140–122)

Heat-inactivated Adult Bovine Serum (Gemini Biosciences, cat. no. 100–101)

Distilled water

Plastic beakers (Thermo Scientific, cat. no. 02–591-10)

Graduated cylinders

Stir bars and stir plate

Scale and microscale

Glass stirring rod (30–40cm long)

pH meter

Media storage bottles, 1L (Corning, cat. no. 1395–1L)

Bottle top vacuum filters, 0.2μm PES membrane, 0.5–1L (Thermo Scientific, cat. no. 5974520)

35°C water bath

Batch Preparation of Incomplete TYI-S-33 medium

1. Dissolve BBL Biosate Peptone in a 4L plastic beaker with stir bar (Table 1).

   Start by adding 1L distilled water and use a glass stirring rod to bring peptone into solution. Continue adding distilled water until the volume is roughly 1L from initial listed volume in Table 1 (for example, only add up to 2L to the 3x preparation). The stir bar will not function until a decent amount of peptone is dissolved. A transfer pipette may also be used to rinse peptone off of the sides of the plastic beaker to get as much peptone into solution as possible. It can take as long as one hour to bring peptone into solution with 3x and 4x batches.

   The Biosate peptone can be substituted with yeast extract and tryptone in a 1:2 ratio – for example, one bottle of TYI-S-33 medium would contain 8.0g yeast extract (Gibco, cat. no. 212750) and 16.0g tryptone (Gibco, cat. no. 211921).

2. Slowly add other ingredients to dissolved peptone solution (Table 1).

   Do not add other ingredients until the peptone is fully dissolved.

3. Add distilled water up to initial volume listed in Table 1. Ensure that all other ingredients are in solution.

4. Measure the pH and adjust to 6.8 using 10M NaOH.

   For a 3x batch add up to ~14ml 10M NaOH; for a 4x batch add up to ~19ml 10M NaOH. The amount of 10M NaOH needed may vary, so it is important to add NaOH slowly.

5. Bring up to final volume in Table 1 with distilled water.

6. Filter sterilize a 725ml aliquot into each 1L bottle, using bottle top vacuum filters.

   Media can also be sterilized by autoclaving – however, variables including autoclave age and heat can potentially cause variation that may affect growth.

7. Freeze at −20°C and store for future use.

Preparation of Complete TYI-S-33 medium

8. Thaw a 1L bottle (containing 725 ml) of Incomplete TYI-S-33 medium in a 35°C water bath.

   This will take 1–2 hours. Periodically swirl the bottle while the media is thawing to help dissolve any media components that have come out of solution. If media bottle caps were not properly rinsed right after use, flakes of dried media from the bottle cap might be observed.

9. Thaw a 20ml aliquot of Diamond Vitamin Tween 80 Solution 40x (Vitamins) and a 7ml aliquot of Penicillin-Streptomycin (Pen-Strep).

   When receiving a new Diamond vitamins order, prepare 20ml sterile aliquots and freeze at −20° C to avoid multiple freeze/thaw cycles. The supplement is light-sensitive and should be stored in the dark. The same applies to Pen-Strep solution, which should be stored in 7ml aliquots at −20° C.

10. Add the following to the 1L bottle (containing 725 ml) of Incomplete TYI-S-33 medium:

    130ml Heat-inactivated Adult Bovine Serum

    20ml Vitamins

    7ml Pen-Strep

    Heat inactivation of Adult Bovine Serum can be performed upon request by Gemini Biosciences, thus, we purchase serum that has already been heat-inactivated. The protocol that is used by Gemini Biosciences is outlined below.

11. Swirl the bottle to mix components; store at 4°C and use within a week of thawing.

Heat-inactivation of Adult Bovine Serum

12. Serum is received frozen and should be stored at −20°C. A control bottle containing water should be stored alongside the serum.

The control bottle should be prepared from an empty serum bottle, so that it will be of the identical composition to the serum bottles, and it should be filled with the same volume of water as the volume of serum contained in the serum bottles.

13. Thaw serum thoroughly and swirl to homogenize. Thaw the control bottle at the same time.

14. Prepare a 56°C water bath, with a sufficient amount of water. The water level should be high enough to be above the level of the serum, but lower than the level of the serum bottle lid.

For the most consistent results, a circulating water bath is preferable to a water bath that lacks circulation, as circulation provides more even heating to the bottles.

15. Place a thermometer in the control bottle and secure it so that it does not touch the bottom or sides of the bottle. Place the control bottle and serum bottle into the prepared water bath.

16. Swirl the bottles every 3–5 minutes. When the control bottle temperature reaches 56°C, start a timer. Continue swirling every 3–5 minutes until the timer reaches 30 minutes.

17. Allow the bottles to cool slowly to room temperature. If aliquoting will be performed, store the serum overnight at 4°C, and aliquot it the next day.

18. Heat-inactivated serum should be stored at −20°C. Once thawed, it should be stored at 4°C, and should not be frozen again.

Maintenance of Glassware for TYI-S-33 medium

19. Once a bottle of TYI-S-33 is used, immediately rinse the glass bottle and cap with deionized water at least three times to completely remove residual media.

Deionized water is sufficient for rinsing of amoeba specific glassware, though distilled or MilliQ water would also serve the same purpose.

E. histolytica *is very sensitive to detergents and other cleaning reagents, so avoid washing glassware used for amoeba media with dish soap.*

If media bottle caps are not thoroughly rinsed right after use, flakes of dried media from the bottle cap might be observed in future batches of TYI-S-33 medium.

20. Place bottles and caps upside down on a clean absorbent surface to dry, and autoclave before using again to store media.

SUPPORT PROTOCOL 2

Lot Testing of Biosate Peptone and Adult Bovine Serum for TYI-S-33 Medium

The culturing of *E. histolytica* requires the use of two rich media components that can vary greatly from lot to lot – biosate peptone and adult bovine serum. *E. histolytica* is a fastidious microorganism that is sensitive to these variations. Biosate peptone is a combination of 65% yeast extract and 35% tryptone. The yeast extract is the component for which lot-to-lot variation can be problematic for *E. histolytica*, while lot-to-lot variation in tryptone is generally not an issue. As an alternative to biosate peptone, the separate products (yeast extract and tryptone) can be used at the appropriate ratio. Initial growth in media containing a new lot of biosate peptone may appear similar to normal growth, but growth defects can manifest in the second or third week; therefore new batches of biosate peptone should be tested for 2–3 weeks. *E. histolytica* is less sensitive to changes in adult bovine serum, and testing need only be carried out for up to 2 weeks. Below is a protocol for how to test different lots of serum and biosate peptone, and an example of lot testing data is shown in Figure 2.

Materials—~80% confluent T25 flask of wildtype HM1:IMSS *E. histolytica*

TYI-S-33 medium ingredients (see Support Protocol 1)

Biosate peptone (Gibco, cat. no. 211862), or yeast extract (Gibco, cat. no. 212750) and tryptone (Gibco, cat. no. 211921)

Disposable borosilicate glass tubes with threaded end (Fisher, cat. no. 14–959-35A)

Kimble black phenolic screw-thread closures GPI thread 15mm-415 (Fisher, cat. no. 05–569-5)

Benchtop microcentrifuge (such as Beckman, cat. no. B30137)

Inverted microscope capable of 400x magnification

Cell counter (Bio Rad TC20 Automated Cell Counter, cat. no. 1450102), or hemocytometer

Centrifuge equipped with a swinging bucket rotor, such as Allegra X-14R (Beckman Coulter, cat. no. B08861)

Sterile 15ml polypropylene conical centrifuge tubes (Corning, cat. no. 352196)

35°C incubator

35°C water bath

1. Obtain several available lots for testing.

   Since there is a possibility that one or more of the lots being tested will not support normal amoebic growth, it is useful to test as many lots as possible at

once. Once a suitable lot has been identified, purchase as much as possible while also taking into account usage and expiration dates.

2. Make TYI-S-33 medium for each new component for testing. Prepare a control batch of TYI-S-33 medium containing the existing media components that are already in use, as a control for normal *E. histolytica* growth.

   Biosate Peptone (Gibco, cat. no. 211862) *can be substituted with 35% yeast extract (Gibco, cat. no. 212750) and 65% tryptone (Gibco, cat. no. 211921).*

   If different lots of biosate peptone are under evaluation, this will require preparing individual batches of TYI-S-33 medium from scratch. One bottle per lot may be frozen down into aliquots to be used each week of the lot testing process, which takes 2–3 weeks.

   If different lots of adult bovine serum are under evaluation, a bottle of premade TYI-S-33 medium for each week of the experiment can be thawed and divided into aliquots for each lot of adult bovine serum under evaluation (including the current lot of adult bovine serum). Add an appropriate amount of adult bovine serum, vitamins, and pen-strep depending on the volume of each aliquot.

3. Detach healthy wildtype amoebae from an ~80% confluent T25 flask (Basic Protocol 1, Step 7), centrifuge (Basic Protocol 2, Step 2), and count cell number using a cell counter or hemocytometer (Basic Protocol 2, Step 3). Resuspend cells at anywhere between 5×10^4 and 1×10^5 cells/ml, deciding based on the amount of cells that are needed to seed each tube to reach ~80% confluency within 3–4 days.

4. Transfer 1ml of cell suspension into culture tubes with 13ml complete TYI-S-33 medium from each lot, invert tubes to mix, and incubate on a 10° angle at 35°C.

5. After 3–4 days, ice the tubes for 10 minutes and transfer cells to a 15ml conical centrifuge tube.

6. Spin down the cells at 200x*g* for 5 minutes, and resuspend the pellet in 1–2ml lot-specific complete TYI-S-33 medium.

7. Count the amoebae from each lot. Resuspend at the same concentration that was used in Step 3, by adding an appropriate amount of media to the 1–2ml of cells.

8. Transfer 1ml of each sample into another culture tube of lot-specific complete TYI-S-33 medium, invert to mix and incubate on a 10° angle at 35°C.

9. Repeat steps 5–8 for 2–3 weeks, tracking the rate of growth over time (Figure 2).

BASIC PROTOCOL 2

Cryopreservation of *E. histolytica* trophozoites

This protocol originates primarily from work conducted by Louis S Diamond, who developed a method for cryopreservation based off of years of observation and experience (Diamond, 1995). Axenically cultured trophozoites are particularly difficult to preserve

compared to xenic and monoxenic trophozoites. Diamond and his colleagues determined that several parameters were essential for successful creation and recovery of *E. histolytica* frozen stocks. Establishing a cooling rate of 1°C per minute is extremely important in reducing the harshness of transitioning from liquid to solid form, and can be facilitated using simple freezing containers. The growth phase of trophozoite cultures should also be taken into consideration. Cells transitioning from a logarithmic phase into a stationary phase of growth are less vulnerable to environmental stressors, and thus should be fully confluent prior to cryopreservation. The composition of the cryoprotectant solution is also crucial for cell preservation. In addition to freezing agents such as DMSO and glucose, the cryoprotectant solution contains high levels of peptone and a cysteine/ascorbic acid solution. Methods for the creation and recovery of *E. histolytica* cryostocks are detailed below.

Materials—Confluent T25 flask of *E. histolytica* cells

Complete TYI-S-33 medium (see Support Protocol 1), warmed in 35°C water bath

Cryoprotectant solution (see Support Protocol 3)

Recovery media (see Support Protocol 3)

100% Isopropyl alcohol

25cm² Rectangular Canted Neck Cell Culture Flask with Plug Seal Cap (Corning, cat. no. 430168)

Externally threaded NUNC cryogenic vials, 1.8ml (Corning, cat. no. 430659)

Freezing container (Thermo Scientific, cat. no. 5100–0001)

Inverted microscope capable of 400x magnification

Centrifuge equipped with a swinging bucket rotor, such as Allegra X-14R (Beckman Coulter, cat. no. B08861)

Sterile 15ml and 50ml polypropylene conical centrifuge tubes (Corning, cat. no. 352196 and cat. no. 352070)

Cell counter (Bio Rad TC20 Automated Cell Counter, cat. no. 1450102) or hemocytometer

35°C incubator

35°C water bath

−80°C freezer

Liquid nitrogen tank for cell storage (such as Thermo Scientific, cat. no. CY509108)

Dry Ice

Preparing frozen stocks of E. histolytica

1. Detach a single confluent T25 flask of amoebae by incubating on ice for up to 5–10 minutes, and gently inverting several times. Check on a microscope to confirm that most cells have detached.

   The culture should be more confluent than it would be for standard cell passaging or for most experiments. Here, the cells should ideally be at the transition between logarithmic growth and stationary growth, because logarithmically growing cells are more susceptible to the stress of the cryopreservation process.

2. Transfer cells to sterile conical centrifuge tubes. Centrifuge cells at 200x*g* for 5 minutes.

   A T25 flask contains ~68ml total media volume. This step may be done in two ways: After incubating on ice to detach cells from the flask, one T25 flask can be split into two 50ml conical tubes, centrifuged, combined and centrifuged once more to obtain one pellet of cells. To save time, before incubating on ice to detach cells from the flask, some media (at least 20ml) can be aspirated from the T25 to reduce the total volume to slightly less than 50ml. If there is any cellular debris in the flask, it can be set vertically for a few minutes and debris that collects at the bottom can be easily aspirated along with the media. After removing media, the flask can be placed horizontally in the ice to make sure all cells are covered with media.

3. Aspirate the supernatant and resuspend cells in warm complete TYI-S-33 medium. Transfer the resuspended cells to a 15ml conical, take an aliquot out to count the cell density using an automated cell counter or hemocytometer, and centrifuge 5 minutes at 200x*g*.

   15ml conical centrifuge tubes are preferable in general since E. histolytica *forms a fairly loose pellet, and the smaller dimensions of a 15ml conical enable the formation of a somewhat tighter pellet.*

   At this point the expected cell density of a confluent T25 resuspended in 10ml TYI-S-33 medium is between 2×10^5 and 3×10^5 cells/ml.

   E. histolytica *settles out of solution fairly quickly. When counting or pipetting amoebae, make sure to gently mix the cell suspension to resuspend any cells that have settled to the bottom of the tube. It is not necessary to keep the tube cold.*

4. Resuspend cells in cryoprotectant at 2×10^6 cells/ml. Equilibrate for 15 minutes at room temperature to allow for osmotic equilibration.

   The time between resuspending the cells in cryoprotectant and freezing at −80°C (step 7) should not exceed 30 minutes.

5. Aliquot 0.5ml portions into labeled cryotubes.

6. Place tubes in a freezing container filled with 100% isopropyl alcohol.

Make sure the cryogenic tubes are tightly closed to prevent N_2 gas from entering tubes and causing tubes to explode upon thawing.

7. Place containers at −80°C for 24–48 hours.

   Cells should be transferred to liquid nitrogen storage tanks within 48 hours of cryopreservation, since −80°C is not cold enough for cells to be properly preserved for long term storage.

8. Transfer cryotubes quickly on dry ice to liquid nitrogen tank for storage.

   Placing tubes on dry ice while transferring will prevent unnecessary thawing during transport that could decrease cell viability.

9. After at least one week, perform a test thaw of cryostocks to make sure cells are able to recover.

   Perform this test thaw before killing off any cell lines that are no longer needed for active culture. Amoebae can be disposed of by treating with 10% bleach for at least 20 minutes.

Thawing frozen stocks of E. histolytica

10. Pre-warm complete TYI-S-33 medium (see Support Protocol 1) in a 35°C water bath.

11. Prepare a glass culture tube with 13.5ml Recovery Medium (see Support Protocol 3) or complete TYI-S-33.

    Recovery media is the preferred choice over complete TYI-S-33 medium.

12. Retrieve cryotube from liquid nitrogen and thaw for 2 minutes in a 35°C water bath without agitation.

    Make sure the cap of the cryotube remains above the water to prevent contamination.

13. Add an equal volume of Recovery medium or complete TYI-S-33 medium from the pre-filled glass culture tube to the cryogenic tube, dropwise (for example, if the frozen stock contains 0.5ml cells, add 0.5ml Recovery medium or complete TYI-S-33 medium).

    Thawed amoebae lyse after recovery when kept longer than 10 minutes in cryoprotectant. Therefore, Recovery media or complete TYI-S-33 medium is added to dilute the cryoprotectant to a non-lethal concentration of DMSO (below 2%), as soon as the cells are thawed.

14. Mix and transfer cells to the glass culture tube dropwise, and invert to mix.

15. Incubate cells at a 10° angle for 2 hours at 35°C.

16. Ice culture tube for 2 minutes, then transfer cells to a sterile 15ml conical centrifuge tube and centrifuge for 5 minutes at 200x*g*.

Incubation on ice is a stressor. Here, only 2 minutes is used, in order to reduce stress on recovering cells.

17. Remove and discard supernatant carefully in order to not disturb cell pellet.

18. Replace with 14ml warm complete TYI-S-33 medium, invert to mix and transfer to a glass culture tube. Incubate at a 10° angle at 35°C.

19. *E. histolytica* takes 1–2 weeks to recover from thawing. Viable, dividing cells should be visible in the culture within 2–3 days of thawing.

SUPPORT PROTOCOL 3

Preparation of Cryoprotectant Solutions

E. histolytica trophozoites require very specific media for successful freezing and thawing of specific cell lines. Several solutions are required, including a peptone rich basal broth solution, a cysteine/ascorbic acid solution, and complete cryoprotectant which includes these solutions in addition to other freezing agents and rich media components. This support protocol provides detailed instructions for the preparation of cryoprotectant solution and recovery medium for cryopreservation and thawing of *E. histolytica* trophozoites (Diamond, 1995).

Materials—K_2HPO_4 (Sigma, cat. no. 60353)

KH_2PO_4 (Sigma, cat. no. P5655)

NaCl (Sigma, cat. no. S7653)

BBL Biosate Peptone (Gibco, cat. no. 211862)

Ferric Ammonium Citrate (Sigma, cat. no. F5879)

Heat-inactivated Adult Bovine Serum (Gemini Biosciences, cat. no. 100–101)

D-(+)-Glucose (Sigma, cat. no. G8270)

Dimethylsulfoxide (DMSO) (Sigma, cat. no. D2650)

L-Cysteine hydrochloride monohydrate (Sigma, cat. no. C7880)

L-Ascorbic acid (Sigma, cat. no. A0278)

Distilled water

250ml glass beaker

100ml graduated cylinder

stir bar and stir plate

Sterile 15ml and 50ml polypropylene conical centrifuge tubes (Corning, cat. no. 352196 and cat. no. 352070)

Steriflip filter unit, 0.22μm pore size (Millipore, cat. no. SCGP00525)

Bottle top vacuum filter, 0.2μm PES membrane, 0.5L (Thermo Scientific, cat. no. 5974520)

pH meter

35°C water bath

Preparation of Basal Broth

1. Add ~80ml distilled water to a beaker and mix with a stir bar.
2. Add the following to the beaker:

   0.1g K_2HPO_4

   0.06g KH_2PO_4

   0.2g NaCl

   6.0g Biosate Peptone

   Biosate Peptone can be substituted with 2.0g yeast extract (Gibco, cat. no. 212750) and 4.0g tryptone (Gibco, cat. no. 211921). Use the same lot of Biosate peptone or yeast extract that has been tested for use in TYI-S-33 medium (Support Protocol 2).
3. Separately, in a 15ml conical tube, add 0.00228g ferric ammonium citrate to 10ml distilled water. Dissolve by vortexing and transfer 1ml into the beaker.

   The ferric ammonium citrate solution must be made fresh on the day of basal broth preparation.
4. Once all reagents are dissolved, transfer the solution to a 100ml graduated cylinder and add distilled water up to 100ml.
5. Filter sterilize and aliquot 6ml into 15ml conical tubes. Store at −20°C for up to 6 months.

Preparation of Cryoprotectant

6. Thaw a 6ml basal broth aliquot in a 35°C water bath.
7. Prepare a cysteine/ascorbic acid solution by adding the following to a 15ml conical tube and dissolve by vortexing:

   0.5g L-cysteine hydrochloride monohydrate

   0.05g ascorbic acid

   Q.S. with distilled water to 5ml

The cysteine/ascorbic acid solution must be made fresh on the day of cryoprotectant preparation. Do not store this solution.

8. Adjust the pH of the cysteine/ascorbic acid solution to 7.0–7.2 with 10M NaOH.

   The cysteine/ascorbic acid solution will be highly acidic initially. Before measuring the pH, add ~0.31ml 10M NaOH to the solution and mix well by inverting. Once the pH is ~6.0 adjust dropwise with 1M NaOH to carefully bring the solution to the target pH.

9. Prepare a 2.5M glucose solution with distilled water and D-(+)-Glucose.

   The 2.5M glucose solution can be prepared and stored at 4° C for up to 12 months.

10. Combine the following in a 50ml conical tube:

    6.0ml basal broth

    2.0ml Heat-Inactivated Adult Bovine Serum

    0.8ml 2.5M glucose solution

    1.0ml DMSO

    0.2ml cysteine/ascorbic acid solution

11. Filter sterilize with a 50ml steriflip filter unit and leave at room temperature until ready to use for cryopreservation.

    Cryoprotectant should be used on the day that it was prepared. Do not store this solution.

Preparation of Recovery Medium

12. Thaw two 6ml basal broth aliquots in a 35°C water bath.

13. Prepare a cysteine/ascorbic acid solution by adding the following to a 15ml conical tube and dissolve by vortexing:

    0.5g L-cysteine hydrochloride monohydrate

    0.05g ascorbic acid

    Q.S. with distilled water to 5ml

    The cysteine/ascorbic acid solution must be made fresh on the day of recovery medium preparation.

14. Adjust the pH of the cysteine/ascorbic acid solution to 7.0–7.2 with 10M NaOH.

    The cysteine/ascorbic acid solution will be highly acidic initially. Before measuring the pH, add ~0.31ml 10M NaOH to the solution and mix well by inverting. Once the pH is ~6.0 adjust dropwise with 1M NaOH to carefully bring the solution to the target pH.

15. Combine the following in a 50ml conical tube:

11.7ml basal broth

3.0ml Heat-Inactivated Adult Bovine Serum

0.3ml cysteine/ascorbic acid solution

16. Filter sterilize with a 50ml steriflip filter unit and leave at room temperature until ready to use for recovery of frozen cells.

Recovery medium should be used on the day that it was prepared. Do not store this solution.

BASIC PROTOCOL 3

Transfection of *E. histolytica* trophozoites with Attractene reagent

This protocol describes one way to introduce plasmid DNA into *E. histolytica* trophozoites. Attractene is a nonliposomal lipid that enables transfection of cells without high toxicity. An advantage of this strategy for *E. histolytica* transfection is that it uses less plasmid DNA and fewer trophozoites than available electroporation protocols. The stable transfection efficiency of *E. histolytica* using this method is approximately one in a thousand cells (Bettadapur et al., 2020), though this is expected to vary depending on the plasmid construct.

In order for cells to be most amenable to plasmid uptake, they should be healthy and in exponential growth phase. Therefore, it is necessary to passage them one to two days before transfection. Refer to the commentary section for troubleshooting ideas if difficulties are encountered in recovering stable transfectants.

Materials—~80% confluent T25 flasks of *E. histolytica* cells – approximately one T25 per transfection condition (i.e., for each plasmid or control)

M199s (without heat inactivated adult bovine serum) (see Reagents and Solutions) – *prepare fresh the day of transfection*

M199s (with heat-inactivated adult bovine serum) (see Reagents and Solutions) – *prepare fresh the day of transfection*

Purified plasmid DNA (such as from Basic Protocol 5)

Attractene transfection reagent (Qiagen cat. no. 301007)

Complete TYI-S-33 medium (see Support Protocol 1), warmed in 35°C water bath

Selective antibiotic

Geneticin™ Selective Antibiotic (G418 Sulfate) (such as Gibco, cat. no. 11811023)

Hygromycin B (50mg/ml) (such as Gibco, cat. no. 10687010)

25cm² Rectangular Canted Neck Cell Culture Flask with Plug Seal Cap (Corning, cat. no. 430168)

Cell counter (Bio Rad TC20 Automated Cell Counter, cat. no. 1450102), or hemocytometer

Centrifuge equipped with a swinging bucket rotor, such as Allegra X-14R (Beckman Coulter, cat. no. B08861)

Sterile 15ml and 50ml polypropylene conical centrifuge tubes (Corning, cat. no. 352196 and cat. no. 352070)

35°C incubator

35°C water bath

1. Prepare 10μg of plasmid DNA in a total of 200μl M199s (without adult bovine serum) in a 2ml cryotube.

   Plasmid DNA should ideally be from a plasmid maxi prep rather than a mini prep, as maxi prepped DNA is generally more pure than mini prepped DNA. It may also be useful to use either ethanol precipitation or magnetic bead-based purification to further purify plasmid DNA and to increase its concentration. Best results are obtained with plasmid DNA that hasn't been through numerous freeze-thaw cycles or stored at 4°C.

   Prepare two cryotubes per plasmid. Also prepare a negative control with no DNA to test the effectiveness of antibiotic selection.

2. Add 37.5μl of Attractene to each tube. Mix by pipetting up and down or vortexing. Incubate samples for 10–15 minutes to allow DNA transfection complexes to form.

   This step should be kept at 15 minutes maximum, and not extended beyond this length of time.

3. While transfection complexes are forming, prepare amoebae for transfection. Detach T25 flasks by incubating on ice for up to 5–10 minutes, and gently inverting several times. Check on a microscope to confirm that most cells have detached.

4. Transfer cells to sterile conical centrifuge tubes. Centrifuge cells at 200x*g* for 5 minutes.

   A T25 flask contains ~68ml total media volume. This step may be done in two ways: After incubation on ice to detach cells from the flask, one T25 flask can be split into two 50ml conical tubes, centrifuged, combined and centrifuged once more to obtain one pellet of cells. To save time, before incubation on ice to detach cells from the flask, some media (at least 20ml) can be aspirated from the T25 to reduce the total volume to slightly less than 50ml. If there is any cellular debris in the flask, it can be set vertically for a few minutes and debris that collects at the bottom can be easily aspirated along with the media. After removing media, the flask can be placed horizontally in the ice to make sure all cells are covered with media.

5. Aspirate the supernatant and resuspend cells in M199s (with adult bovine serum). Transfer the resuspended cells to a 15ml conical, take an aliquot out to count the cell density, and centrifuge 5 minutes at 200x*g*.

   15ml conical centrifuge tubes are preferable in general since E. histolytica *forms a fairly loose pellet, and the smaller dimensions of a 15ml conical enable the formation of a somewhat tighter pellet.*

6. Resuspend cells in M199s (with adult bovine serum) at 2.5×10^5 cells/ml.

   E. histolytica *settles out of solution fairly quickly. When counting or pipetting amoebae, make sure to gently mix the cell suspension to homogenize any cells that have settled to the bottom of the tube.*

   E. histolytica *is microaerophilic and does not survive well in 1xPBS or other commonly used media for transfection. M199 supplemented with L-cysteine, ascorbic acid, HEPES, and Adult Bovine Serum (M199s with adult bovine serum) has been used successfully.*

7. Add 1.8ml amoebae to each cryotube, and incubate horizontally at 35°C for 3 hours.

8. Add two cryotubes (4 ml total) to each T25 flask that contains 64ml warm complete TYI-S-33 medium.

9. Incubate for 24 hours at 35°C.

10. After 24 hours, add selective antibiotic to all T25 flasks and continue incubation at 35°C.

    Transfected plasmid DNA is maintained episomally in E. histolytica, *and thus there is no initial transient population that is replaced by a stable population. The initial population of amoebae that survive drug selection can be considered stable transfectants, and can be cloned out as soon as they have recovered and expanded.*

    If stable transfectants are difficult to recover for a particular plasmid transfection, it can be useful to begin antibiotic selection at 48 hours instead of 24 hours. It can also be useful to begin selection at a slightly lower antibiotic concentration, and to gradually increase the concentration to the typical level, once stable transfectants start growing. In either case, it is important to have a negative control transfection that did not receive plasmid DNA, and to carefully monitor growth of the negative control and experimental transfections to ensure that the negative control cells have been killed under the selection conditions that were used.

11. After 2–3 days, replace half the media in each flask while also cleaning up as much cell debris as possible.

    Incubate flasks standing vertically for 5–10 minutes to allow dead cell debris to settle to the bottom of the flask. Carefully remove the debris using aspiration,

and remove approximately half of the media volume. Replace the media as soon as possible, taking care not to disturb attached cells.

12. Continue observing cells and replacing media as needed. The cells in the negative control flask should all die while the cells in the flasks should recover within a week.

    After cells have been incubated in the same flask for about a week, growth generally begins to stagnate. Transfected cells will generally recover more easily if passaged to a new flask within about a week of transfection.

    Transfected cells often grow in a more “patchy” distribution over the area of the flask, rather than in an even confluent layer. Thus, they do not need to reach the benchmark of ~80% confluency to be passaged into a new flask. After transfected cells have been passaged to a new flask, growth will no longer have a “patchy” distribution.

BASIC PROTOCOL 4

Creating Clonal Lines using Limiting Dilution

This protocol describes a method for the creation of clonal cell lines using limiting dilution. It is sometimes useful to generate clonal lines from otherwise heterogeneous populations of cells. This is of particular importance after stably transfecting amoebae with an expression plasmid or RNAi knockdown plasmid, since gene expression can be variable from cell to cell. This can be seen in Figure 5a-5b, where differing RNAi knockdown levels are apparent in individual clonal lines, compared to heterogeneous transfectants. In this approach, a small number of *E. histolytica* trophozoites are added to the first column of a 96-well plate full of media, and half of those cells are transferred to the next column of media. This dilutes the cell concentration by half, and continuing this process of halving theoretically results in one cell per well by the ninth transfer (see Table 2). The most dilute wells that lead to cell growth can be considered clonal, because subsequent dilutions did not yield any viable cells.

Materials—~80% confluent T25 flask of *E. histolytica* cells

Complete TYI-S-33 medium (see Support Protocol 1), warmed in 35°C water bath

25cm² Rectangular Canted Neck Cell Culture Flask with Plug Seal Cap (Corning, cat. no. 430168)

96-well Clear Flat Bottom Polystyrene TC-treated Microplates (Corning, cat. no. 3596)

24-well Clear Flat Bottom TC-treated multiple well plates (Corning, cat. no. 3526)

Disposable borosilicate glass tubes with threaded end (Fisher, cat. no. 14–959-35A)

Kimble black phenolic screw-thread closures GPI thread 15mm-415 (Fisher, cat. no. 05–569-5)

GasPak EZ Anaerobe Pouch System (BD, cat. no. 260683)

Disposable Reagent Reservoir, sterile (Corning, cat. no. RES-V-25-S)

20–200µl multichannel pipette

Inverted microscope capable of 400x magnification

Cell counter (Bio Rad TC20 Automated Cell Counter, cat. no. 1450102), or hemocytometer

Centrifuge equipped with a swinging bucket rotor, such as Allegra X-14R (Beckman Coulter, cat. no. B08861)

Sterile 15ml and 50ml polypropylene conical centrifuge tubes (Corning, cat. no. 352196 and cat. no. 352070)

35°C incubator

35°C water bath

1. Aliquot 100µl warm complete TYI-S-33 medium into a sterile 96-well microplate using the multichannel pipette. If cells are under antibiotic selection, make sure to include the proper concentration of antibiotic in the media.

   Each row of the 96 well plate represents a potential clonal line – filling all 8 rows would yield a maximum of 8 clonal lines. An explanation of why a row might not yield a clonal line is included in the following steps.

2. Transfer cells to sterile conical centrifuge tubes. Centrifuge cells at 200x*g* for 5 minutes.

   A T25 flask contains ~68ml total media volume. This step may be done in two ways: After incubation on ice to detach cells from the flask, one T25 flask can be split into two 50ml conical tubes, centrifuged, combined and centrifuged once more to obtain one pellet of cells. To save time, before incubation on ice to detach cells from the flask, some media (at least 20ml) can be aspirated from the T25 to reduce the total volume to slightly less than 50ml. If there is any cellular debris in the flask, it can be set vertically for a few minutes and debris that collects at the bottom can be easily aspirated along with the media. After removing media, the flask can be placed horizontally in the ice to make sure all cells are covered with media.

3. Aspirate the supernatant and resuspend cells in warm complete TYI-S-33 medium. Transfer the resuspended cells to a 15ml conical, take an aliquot out to count the cell density, and centrifuge 5 minutes at 200x*g*.

   15ml conical centrifuge tubes are preferable in general since E. histolytica *forms a fairly loose pellet, and the smaller dimensions of a 15ml conical enable the formation of a somewhat tighter pellet.*

4. Resuspend cells in warm complete TYI-S-33 medium at 5×10^3 cells/ml.

E. histolytica *settles out of solution fairly quickly. When counting or pipetting amoebae, make sure to gently mix the cell suspension to homogenize any cells that have settled to the bottom of the tube.*

5. Using a single channel pipette, add 100µl cells to each of the wells in column 1 and mix by gently pipetting up and down.

6. Using the multichannel pipette, transfer 100µl of column 1 into column 2 and mix by gently pipetting up and down.

7. Using the multichannel pipette, transfer 100µl of column 2 into column 3 and mix by gently pipetting up and down.

8. Continue transferring 100µl from one column to the next all the way to column 12.

9. Remove and discard 100µl from column 12. The plate has now been seeded with the number of amoebae indicated in Table 2.

10. Carefully place the 96 well plate into a GasPak plastic bag and add a GasPak pouch with indicator; incubate on a flat surface at 35°C.

Position the GasPak pouch in the bag so that it does not overlap with the 96 well plate – this will allow for easy monitoring of growth without exposing the plate and GasPak pouch to air (Figure 3). Make sure the bag is fully closed and do not open unless absolutely necessary.

11. Monitor and record growth in all wells daily. Take note of media levels in wells and add media as necessary to avoid wells drying out.

Column 1 should quickly overgrow, and column 12 should not contain any cells. It will take several days to observe growth in the more dilute columns.

12. After about a week of incubation, cells in the most dilute wells should be nearly confluent and ready to expand into a 24-well plate. Examine each row and identify the most dilute well that contains any growth. All wells after this well should not contain any live cells.

If there is growth in column 12, do not use that particular row. Any cell line coming from column 12 is not truly clonal as there is no verification that a lower dilution would not contain growth.

13. Fill a 24-well plate with 2ml of warm complete TYI-S-33 medium (one well per clonal line). Place 96-well plate horizontally on ice for 10 minutes.

Do not beat or agitate plate to detach cells as this may result in cross well contamination.

14. Transfer the most dilute well containing growth from each row into one well of the 24-well plate. Mix and use complete TYI-S-33 medium from the 24-well plate to ensure all cells are transferred.

15. Carefully place the 24-well plate into a GasPak plastic bag and add a GasPak pouch with indicator; incubate on a flat surface at 35°C.

Position the GasPak pouch in the bag so that it does not overlap with the 24-well plate – this will allow for easy monitoring of growth without exposing the plate and GasPak pouch to air. Make sure the bag is fully closed and do not open unless absolutely necessary.

16. Monitor growth and transfer clonal lines from 24-well plates to 16ml culture tubes when wells are nearly confluent. ~80% confluent culture tubes can then be passaged into T25 flasks and cryostocks can be made once T25 flasks reach confluency (see Basic Protocol 2).

BASIC PROTOCOL 5

Knockdown of 1–2 genes with trigger-induced RNA interference

The genetic manipulation of *Entamoeba histolytica* has been an area of constant growth and innovation. As discussed in the Commentary section, the trigger-based RNA interference knockdown system is a modern strategy available to researchers for knockdown of gene expression (Morf et al., 2013). This method takes advantage of endogenously expressed small RNAs that correspond to an endogenously knocked down gene called the “trigger” gene. A fragment of the trigger gene is cloned into an expression plasmid adjacent to a fragment of a gene of insert, and this leads to spreading of silencing, such that the endogenous gene of interest is knocked down.

The plasmid described in the following protocol as “pTrigger” is a modified version of pKT-04T (Morf et al., 2013). pTrigger contains 132 base pairs of the trigger gene EHI_048600 directly before a SmaI restriction enzyme cut site added to make cloning inserts easier (Figure 4) (Bettadapur et al., 2020). It may be necessary to check your specific *E. histolytica* culture for endogenous knockdown of this particular trigger gene. In order for the spreading mechanism that allows this RNAi silencing method to work, sRNAs complimentary to the trigger gene must be present in the cell lines being used for knockdown. If there is expression of the trigger gene fragment used in pTrigger, another trigger gene has been successfully used (Khalil et al., 2016; Morf et al., 2013). This protocol provides step by step instructions on choosing an insert, cloning into pTrigger, transfecting trophozoites and screening for knockdown.

Materials—*E. histolytica* genomic DNA

pTrigger plasmid (Bettadapur et al., 2020; Morf et al., 2013)

Nuclease-free Water (Millipore Sigma, cat. no. W4502)

KAPA HiFi HotStart PCR kit (Roche, cat. no. KK2502)

10mM dNTPs (Applied Biosystems cat. no. N8080260)

SmaI restriction enzyme (NEB cat. no. R0141S)

XhoI restriction enzyme (NEB cat. no. R0146S)

10X CutSmart buffer (included with NEB SmaI and XhoI)

PCR Purification kit (Qiagen cat. no. 28104)

Forward and reverse primers with homology to insert and backbone (for Gibson assembly)

Gibson Assembly Ultra Kit (Synthetic Genomics, cat. no. GA1200)

DH5α competent *E. coli* (NEB cat. no. C2987H)

Miniprep kit (QIAprep Spin Miniprep Kit, cat. no. 27104)

Maxiprep kit (Sigma GenElute HP Plasmid Maxiprep kit, cat. no. NA0310–1KT)

LB plates + ampicillin

Agarose

1xTAE buffer

DNA stain for gel visualization (such as ethidium bromide)

Gel loading dye, purple (6X) (included with NEB SmaI and XhoI)

CS5 sequencing primer

5'-TCAGTCTTACCACGTCATAAAGT-3'

CS3 sequencing primer

5'-TGCAAGAAGATGTTACAAAGCA-3'

PCR strip tubes (such as USA scientific, cat. no. 1402–3900)

NanoDrop spectrophotometer (Thermo Fisher NanoDrop 2000)

Thermocycler (BioRad C1000 Touch Thermal Cycler)

Benchtop microcentrifuge (Beckman, cat. no. B30137)

Bunsen burner

Shaker (250 rpm)

−20°C freezer

−80°C freezer

Additional reagents and equipment for agarose gel electrophoresis (see Current Protocols article: (Voytas, 2000)).

Selection of gene fragment and cloning into the pTrigger plasmid

1. Identify the accession number of the gene of interest (GOI) and locate it on AmoebaDB (amoebadb.org).

2. Use the NCBI Nucleotide Basic Local Alignment Search Tool (BLAST) to search for *E. histolytica* genes that share regions of homology with the mRNA sequence of the GOI.

   Narrow the search by specifying Entamoeba histolytica *HM-1:IMSS (taxid:294381) as the organism, and include the most potential regions of overlap by choosing "somewhat similar sequences (blastn)" in program selection.*

3. Choose the fragment of the GOI to use as a knockdown trigger, taking several parameters into consideration:

   a. Roughly 500–1000bp in length (Bettadapur et al., 2020; Khalil et al., 2016; Morf et al., 2013)

   b. Low homology to other genes to minimize off-target silencing

   c. Smaller than the size of the GOI if possible (which helps at a later step, by allowing for the design of primers that sit outside of the GOI fragment for RT-PCR analysis of gene knockdown)

   d. Whether the GOI fragment will be cloned into pTrigger in the forward or reverse orientation (Bettadapur et al., 2020; Morf et al., 2013)

   e. Whether the GOI fragment will be cloned into pTrigger in or out of frame (Bettadapur et al., 2020; Morf et al., 2013)

   Ideally, the GOI fragment will be large enough to create a sufficient amount of small RNAs for knockdown, and is likely that as the GOI fragment gets larger, more small RNAs will be produced. At the same time, smaller GOI fragments help to minimize off-target silencing, since in general, smaller fragments will allow for less sequence homology to related gene sequences. The potential for off-target silencing can be predicted by using BLAST to identify related gene sequences, and checking for stretches of homology longer than ~20nt, which can potentially lead to off-target silencing of related genes.

   It is also useful if the GOI fragment is smaller than the size of the entire GOI, because this makes it possible to design primers for RT-PCR that sit outside of the GOI fragment. This is useful because having the RT-PCR primers sit outside of the GOI fragment prevents challenges in RT-PCR that arise from unintentionally amplifying contaminating plasmid DNA.

   Although the original description of the pTrigger plasmid described using GOI fragments that were both in the forward orientation and in frame, we have empirically determined that the insert may be either in or out of frame, and it may be in either the forward or reverse orientation for silencing to be effective (Bettadapur et al., 2020).

4. Design primers to amplify the GOI fragment while also adding regions of homology with pTrigger to the 5' and 3' ends of the cloning site, to the 5' ends of the primers.

   Gibson primers can be anywhere from 30bp to 80bp long – with the 3' half of the primer containing homology to the insert, and the 5' half containing homology to the backbone. We generally use 20bp of homology to pTrigger. The length of the region of homology to the GOI fragment depends on the primer sequences that are determined by using a primer design program, such as Primer3.

5. Use KAPA HiFi HotStart PCR kit to test the GOI fragment primers, following manufacturer guidelines and the cycling program outlined in Table 3.

   Use 10ng E. histolytica *HM1:IMSS genomic DNA as a template for amplification; since* E. histolytica *does not have as many introns as other eukaryotic organisms, amplification of gene inserts can generally be done directly from genomic DNA. Determine the estimated melting temperature (Tm) using a Tm calculator (such as tmcalculator.neb.com) and use that to set an annealing temperature gradient. Test a range of annealing temperatures and run the resulting product on a DNA gel to check for presence and length of product.*

   Primer design can potentially involve several troubleshooting steps in terms of identifying the correct annealing temperature and polymerase. If the initial approach with KAPA does not work, troubleshoot by switching to Phusion High-Fidelity DNA polymerase (Thermofisher cat. no. F530L), or AccuPrime Taq (Invitrogen, cat. no. 12346086). Primers can also be designed in a "nested" fashion, where two sets of primers can be used sequentially. For example, the first set can include the insert sequence without adding regions of homology to pTrigger, which allows for easier amplification of the insert from genomic DNA. The PCR product can then be used for a second PCR amplification, using primers that are the same as in the first PCR amplification, except that they also have regions of homology to pTrigger at their 5' ends.

6. Amplify the chosen GOI fragment from *E. histolytica* genomic DNA with KAPA or another polymerase based on empirical optimization performed in step 5; purify the PCR reactions using a PCR purification kit, and then use as the insert in the following Gibson reaction.

   Since E. histolytica *does not have as many introns as other eukaryotic organisms, amplification of gene inserts can generally be done directly from genomic DNA. Six 50ul PCR reactions should generate enough for cloning, after PCR purification using 2–3 purification columns.*

7. Linearize the pTrigger silencing plasmid with the restriction enzyme SmaI; PCR purify and use as the backbone in the following Gibson reaction.

   Dephosphorylation of the backbone with a phosphatase (such as rSAP) is not required, but may reduce background from self-ligation of the backbone. Empty backbone background can also be reduced by ensuring that pTrigger is fully linearized.

The pTrigger backbone may also be amplified with PCR, using primers that add additional homology to the insert 5' and 3' sequence. However, since pEhEx contains UTRs that are AT rich, it is often difficult to use PCR for this step and may take some optimization. We find that Gibson cloning into pTrigger is generally more efficient when the backbone is prepared by restriction digestion rather than through PCR.

8. Use the Gibson Assembly Ultra Kit to clone the GOI fragment with overlapping ends into the pTrigger backbone, following manufacturer guidelines.

Although use of the Gibson Assembly Ultra Kit (Synthetic Genomics) is described, other Gibson Assembly kits should work equally well for cloning.

Before beginning the cloning process, make sure to run aliquots of the purified backbone and insert on a gel to make sure the products are robust and that they are at the expected size.

9. Transform the Gibson product into DH5α chemically competent *E. coli*, and screen potentially positive clones by miniprep and double digest. Confirm any positive colonies with sequencing.

We recommend miniprep and restriction digest instead of colony PCR due to the increased difficulty of PCR with E. histolytica *sequences. Since Gibson Assembly is relatively efficient, most likely, only 4–8 colonies need to be screened for the presence of insert. Make sure to prepare glycerol stocks for each colony that was selected (190μl 80% sterile glycerol + 810μl culture) and store in a −80°C freezer until positive colonies are verified.*

Use of restriction enzymes that flank the insert is a simple way to confirm successful cloning – in this case using XhoI and SmaI to screen colonies will result in either a linearized backbone or a backbone + insert.

Use the primers CS5 and CS3 to sequence the junctions of pTrigger and the insert – this will confirm that the insert was cloned into pTrigger correctly, while the restriction digest will confirm the size and cut sites of the plasmid as a whole.

10. Prepare a plasmid maxiprep and ethanol concentrate the plasmid DNA to produce enough pTrigger+insert plasmid to be able to transfect in the following steps.

In parallel with pTrigger+insert, make sure to prepare enough pTrigger to use as a vector control for use in future experiments.

Transfection, clonal lines, and screening for knockdown

11. Follow Basic Protocol 3 to transfect wildtype amoebae with pTrigger+insert and pTrigger plasmids, using G418 to select for transfectants.

Make sure to prepare a negative control that does not receive plasmid, to verify that antibiotic selection fully kills untransfected cells. In addition, the pTrigger transfectants will act as a vector control to use in screening for knockdown and in downstream phenotyping experiments.

12. As transfectants grow and recover, expand both vector control and knockdown cell lines into three T25s for steps 13–15.

13. Use the first ~80% confluent T25s to make cryostocks (Basic Protocol 2).

    It is important to make cryostocks as soon as possible after transfection, so that knockdown cell lines are preserved.

14. Use the second ~80% confluent T25s to extract RNA (Support Protocol 4).

15. Use the third ~80% confluent T25 of knockdown cells to create clonal lines via limiting dilution cloning (Basic Protocol 4).

    It is good practice to make clonal lines from the heterogeneous population. Prepare 4–8 clonal lines for each knockdown plasmid transfected.

16. While clonal lines are growing up, use conventional and/or quantitative RT-PCR (Support Protocol 4) to screen RNA from heterogeneous populations of cells for expression of the gene of interest, using the vector control for comparison.

    It is possible that initially transfected populations will appear to have somewhat incomplete knockdown, due to the heterogeneity of stable transfectants. Making clonal lines generally allows for the isolation of clones that have more complete levels of knockdown.

17. Once clonal lines are expanded into T25s, make cryostocks and extract RNA from each clone. Use extracted RNA to screen for knockdown (Support Protocol 4).

18. As soon as knockdown is verified in one or multiple clonal lines, perform phenotyping assays to compare the effect of silencing the gene of interest to the vector control condition.

    While trigger induced knockdown is stable and selected for with G418, we have observed that detectable phenotypes can be time sensitive and any assays to be used on knockdown cell lines should be already optimized and performed as soon as knockdown clonal lines are verified.

Cloning two gene fragments into pTrigger for a double knockdown cell line

19. To silence two genes using a single plasmid, the above steps apply to both gene inserts with the following modifications.

20. Each GOI fragment can be ~500 base pairs long, such that the combined insert length is approximately 1000 base pairs. Note that this is based on limited empirical data from our laboratory and from published work (Khalil et al., 2016), and it is possible that it would be more effective to use larger GOI fragments.

21. Instead of designing Gibson primers that add homology to pTrigger on both ends of an insert, the primers must instead be designed to create overlap between the two inserts as well as the pTrigger backbone, as described in the Gibson Assembly instructions for cloning two or more inserts.

22. Screen for knockdown of both genes separately using RT-PCR (Support Protocol 4).

SUPPORT PROTOCOL 4

Evaluation of RNAi Knockdown with RT-PCR

The effectiveness of RNAi knockdown can be evaluated by using Western blotting or RT-PCR. If antibodies are not available to allow for Western blotting analysis, knockdown can be evaluated by using either conventional or qRT-PCR. In conventional RT-PCR, PCR products are evaluated by using agarose gel electrophoresis. Since this is an endpoint assay, conventional RT-PCR does not allow for quantitative evaluation of gene knockdown. Thus, this approach is not useful for distinguishing subtle levels of gene knockdown, and is most useful when mutants have nearly complete gene knockdown. By contrast, qRT-PCR approaches use fluorescent dyes that allow for the abundance of PCR product to be measured at the end of each PCR cycle. This allows for quantitative evaluation of the relative level of expression in RNAi knockdown mutants versus controls. qRT-PCR using SYBR green dye allows for relatively straightforward detection of a variety of gene products, without the need to design custom probes for each gene. Both conventional RT-PCR and qRT-PCR can be challenging in *E. histolytica*, due to the high A/T content in the genome. Examples of RT-PCR and qRT-PCR analysis of pTrigger transfectants are shown in Figure 5.

Materials—~80% confluent T25 of *E. histolytica* knockdown cells

~80% confluent T25 of *E. histolytica* vector control cells

E. histolytica genomic DNA

Direct-zol RNA Miniprep Plus kit (Zymo Research, cat. no. R2071)

RNase ZAP (Invitrogen, cat. no. AM9780)

TURBO DNase 2U/µl (Invitrogen, cat. no. AM2238)

10X TURBO DNase buffer (included in TURBO DNase kit)

0.5M EDTA (see recipe); diluted to 300 mM

SuperScript II Reverse Transcriptase kit (Invitrogen, cat. no. 18064022)

5X First-Strand Buffer (included in SSII RT kit)

0.1M DTT (included in SSII RT kit)

Oligo$(dT)_{18}$ primer 500 µg/ml (Thermo Scientific cat. no. SO132)

10mM dNTPs (Applied Biosystems cat. no. N8080260)

Taq DNA Polymerase (NEB cat. no. M0273)

10X Standard *Taq* Reaction Buffer (included with *Taq*)

KAPA SYBR FAST qPCR Master Mix (2X) Universal (Roche cat. no. KK4601)

Nuclease-free Water (Millipore Sigma, cat. no. W4502)

GAPDH primers:

Forward: 5'-CGTCCACAGACAATTCGAAGGAAC-3'

Reverse: 5'-AAGGCAGTTGGTTGTGCATGA-3'

RPL21 primers:

Forward: 5'-CCTTGAAAGAAAGGCTGCTGTT-3'

Reverse: 5'-GTTCAGCTGGTCTTGGTTGTTT-3'

VTP primers:

Forward: 5'-TCCAAGTCAACCATCTGTACAGT-3'

Reverse: 5'-ACTGGTTCGTCAAGAGTAGTCTC-3'

Nonstick, RNase-free Microfuge Tubes, 1.5 ml (Ambion, cat. no. AM12450)

PCR strip tubes (USA scientific, cat. no. 1402–3900)

20–200μl digital repeater micropipette

qPCR microplates and plate cover film (PCR microplate for Roche 480 LightCycler with UC-500 film, Roche cat. no. AXYPCR96LC480W)

Centrifuge equipped with a swinging bucket rotor and adapters for 96-well plates, such as Allegra X-14R (Beckman Coulter, cat. no. B08861)

NanoDrop spectrophotometer (Thermo Fisher NanoDrop 2000)

Thermocycler (BioRad C1000 Touch Thermal Cycler)

qPCR machine (Roche LightCycler 480)

Additional reagents and equipment for agarose gel electrophoresis (see Current Protocols article: (Voytas, 2000)).

RNA extraction and cDNA synthesis for Conventional RT-PCR

1. Detach T25 flasks of ~80% confluent *E. histolytica* RNAi knockdown and vector control transfectants by incubating on ice for 5–10 minutes, and gently inverting several times. Centrifuge cells at 200x*g* for 5 minutes.

2. Dust is a major source of RNAse, thus it is important to maintain a clean working environment during RNA extraction. Additionally, RNase ZAP or an equivalent product can be used to treat benchtop, micropipettes, and tube racks that will be used during the RNA extraction process. Always wear gloves and use filter tips while working with RNA.

3. Extract RNA from vector control and knockdown cells with the Zymo Direct-zol RNA Miniprep Plus Kit according to the manufacturer's instructions.

   Resuspend the cell pellet in 300µl TRI Reagent and mix thoroughly to lyse cells. Perform the recommended DNase I treatment. Elute RNA in 50µl nuclease-free water into an RNase-free microfuge tube.

   Note that the quality of RNA isolated using the Zymo Direct-zol RNA Miniprep Plus Kit is of sufficient quality for RT-PCR analysis of gene expression, as outlined below. This kit is less expensive than some other kits that are available for RNA extraction. Other kits or protocols can likely be used, though they would need to be empirically evaluated. For other downstream applications besides RT-PCR, the optimal method for RNA extraction would also need to be empirically determined.

4. RNA can be stored indefinitely at −80°C until ready to use for cDNA synthesis in the following steps.

5. Quantify RNA concentration with a NanoDrop spectrophotometer.

6. In a PCR tube, prepare 2µg of RNA in 16µl RNase-free water.

   Each PCR tube of RNA will produce 20µl of +RT cDNA. Before continuing, consider the amount of cDNA that will be needed for downstream applications. The qRT-PCR protocol in particular can require more than 20µl cDNA during primer validation, therefore it may be wise to prepare multiple tubes at this step.

7. Divide each 20µl reaction into two PCR tubes, labeled +RT and -RT.

   +RT tubes will receive reverse transcriptase enzyme, and -RT tubes will not. The -RT tube acts as a control for downstream PCR reactions, since PCR amplification in this sample would indicate the presence of DNA contamination.

8. Add the following reaction components to each tube:

   1µl Oligo$(dT)_{18}$ primer

   1µl 10mM dNTPs

9. Incubate at 65°C for 5 minutes.

10. Quickly chill on ice and briefly centrifuge, then add the following reaction components to each tube:

    4µl 5X First-Strand buffer

    2µl 0.1M DTT

11. Mix contents gently, then incubate at 42°C for 2 minutes.

12. Add 1μl SuperScript II Reverse Transcriptase to +RT tubes only.

13. Incubate at 42°C for 50 minutes, and inactivate the reaction by heating at 70°C for 15 minutes.

14. Store cDNA at −20°C until ready to perform conventional PCR.

Conventional RT-PCR

15. Design primers that will amplify a segment of the gene of interest.

    In order to avoid contamination of the PCR reaction with plasmid DNA, try to design a primer set that amplifies a PCR product that lies outside of the region of the gene that was inserted into pTrigger. Even having one primer that sits outside of the insert sequence will greatly decrease contamination from the plasmid being used for knockdown (pTrigger+insert). At the same time, avoid sequences that share homology with other genes to minimize non-specific amplification.

    The size of the PCR product is not important, as long as it is an appropriate size to visualize on an agarose DNA gel; roughly 200bp to 2kb would be a reasonable size. A primer design program such as Primer3 can be helpful in optimizing primer melting temperature and reducing secondary structure.

    GAPDH primers (Table 4) *can be used as a control for cDNA loading. Any constitutively expressed* E. histolytica *gene may be used.*

16. To empirically establish the appropriate annealing temperature, test RT-PCR primers on *E. histolytica* genomic DNA, employing an annealing temperature gradient in the PCR reaction. Use NEB Taq DNA polymerase according to manufacturer instructions and the cycling conditions outlined in Table 5. Run the product on an agarose gel and visualize with a DNA stain such as ethidium bromide.

    Using genomic DNA for primer testing will ensure that the designed primers amplify as expected without the added variable of expression level. Since E. histolytica *has very few introns, it is unlikely that the genomic DNA template would differ greatly from the mRNA template, and this can be checked during primer design.*

    Use a melting temperature (Tm) calculator (such as tmcalculator.neb.com) to predict the Tm of the primer set, and use that to set a range of temperatures to test.

17. Amplify cDNA with NEB Taq DNA polymerase, as in Step 16, and use the annealing temperature that was empirically established. Analyze RT-PCR samples by gel electrophoresis. Examples of typical data are shown in Fig. 3a-3c.

    The amount of template cDNA is dependent on the expression level of the gene being amplified. A 1:10 dilution of cDNA is generally effective, but some reactions may require undiluted cDNA or further dilution. Start with a 1:10 dilution and evaluate the apparent saturation of the PCR product band on the stained agarose gel. If a band is particularly bright, it may be saturated, and

there is likely room for further dilution of cDNA. This is important because this analysis is qualitative, and any signal difference between knockdown and vector control expression will not be detectable with saturation. If 1:10 is too concentrated, try running 1:20, 1:50, and 1:100 dilutions alongside a 1:10 dilution, to empirically determine the appropriate dilution.

Make sure to run the -RT negative control alongside the sample reactions. This cDNA does not need to be diluted. In addition, include the GAPDH positive control primers to evaluate cDNA loading.

RNA extraction and cDNA synthesis for qRT-PCR

18. Detach T25 flasks of ~80% confluent *E. histolytica* RNAi knockdown and vector control transfectants by incubating on ice for 5–10 minutes, and gently inverting several times. Centrifuge cells at 200x*g* for 5 minutes.

19. Dust is a major source of RNAse, thus it is important to maintain a clean working environment during RNA extraction. Additionally, RNase ZAP or an equivalent product can be used to treat benchtop, micropipettes, and tube racks that will be used during the RNA extraction process. Always wear gloves and use filter tips while working with RNA.

20. Extract RNA from vector control and knockdown cells with the Direct-zol RNA Miniprep Kit according to the manufacturer's instructions.

Resuspend the cell pellet in 300µl trizol and mix thoroughly to lyse cells. Perform the recommended DNase I treatment. Elute RNA in 50µl nuclease-free water into an RNase-free microfuge tube.

21. RNA can be stored indefinitely at −80°C until ready to use for cDNA synthesis in the following steps.

22. Quantify RNA concentration with a NanoDrop spectrophotometer.

23. In a PCR tube, prepare 2µg of RNA in 16µl RNase-free water.

Each PCR tube of RNA will produce 20µl of +RT cDNA. Before continuing, consider the amount of cDNA that will be required in downstream applications. The qRT-PCR protocol in particular can require more than 20µl cDNA during primer validation, therefore it may be wise to prepare multiple tubes at this step.

24. Add the following reaction components (from TURBO DNase kit):

2µl 10X TURBO DNase buffer

2µl TURBO DNase (2 U/µl)

25. Incubate at 37°C for 30 minutes.

Incubation steps may be carried out in a thermal cycler for convenience.

26. Inactivate DNase by adding 1µl 300mM EDTA and incubating at 75°C for 10 minutes.

27. Divide each 20µl reaction into two PCR tubes, labeled +RT and -RT.

+RT tubes will receive reverse transcriptase enzyme, and -RT tubes will not. The -RT tube acts as a control for downstream PCR reactions, since PCR amplification in this sample would indicate the presence of DNA contamination.

28. Add the following reaction components to each tube:

1µl Oligo$(dT)_{18}$ primer

1µl 10mM dNTPs

29. Incubate at 65°C for 5 minutes.

30. Quickly chill on ice and briefly centrifuge, then add the following reaction components to each tube:

4µl 5X First-Strand buffer

2µl 0.1M DTT

31. Mix contents gently, then incubate at 42°C for 2 minutes.

32. Add 1µl SuperScript II Reverse Transcriptase to +RT tubes only.

33. Incubate at 42°C for 50 minutes, and inactivate the reaction by heating at 70°C for 15 minutes.

34. Store cDNA at −20°C until ready to perform qPCR.

qRT-PCR

35. Design primers that will amplify a segment of the gene of interest (GOI), with the following parameters:

- **a.** 20–25bp primer length
- **b.** 75–150bp product
- **c.** ~62–65°C melting temperature (Tm)
- **d.** Tm of forward and reverse primers are very similar
- **e.** At least one primer sits outside of the insert (if measuring gene expression of trigger-induced RNAi knockdown cell lines)
- **f.** Primer sequence is not common to other genes to minimize off target amplification

Design two or three sets of primers to test simultaneously, since not all primer sets will pass empirical quality testing.

36. Empirically evaluate the GOI primer sets by performing steps 37–43 with a dilution series of wildtype or vector control cDNA (as in Figure 6). Suggested dilutions are: 1:1 (undiluted), 1:10, 1:100, 1:1,000, and 1:10,000. Include two technical replicates per sample (Figures 6a, 6c). Plot the resulting Ct values

against the log of the cDNA concentration. Calculate efficiency and percent efficiency using the linear best fit line equation (Figures 6b, 6d).

Efficiency = 10^(−1/slope); Percent efficiency = (Efficiency-1) x 100. The primer efficiency should be in between 90–110%.

Evaluate the primer efficiency for housekeeping gene primer sets RPL21 and VTP (Table 4) alongside GOI primer sets.

Note that the annealing temperature may need to be empirically determined for the GOI primer sets. Ideally, an annealing temperature of 60°C would be effective for a GOI primer set, since if a different annealing temperature is needed, GOI PCR reactions would have to be amplified in the qPCR machine separately from the housekeeping gene PCR reactions, in separate qPCR plates.

37. Dilute cDNA to appropriate concentration, or use undiluted cDNA.

Make sure to include an undiluted -RT and a water control for every primer set. Also include both housekeeping primer sets and GOI primer set. Wild-type or vector control transfectants must be evaluated alongside RNAi knockdown mutants in order to establish the relative gene expression level in the knockdown. Include two technical replicates per sample.

38. Calculate and prepare master mixes for each primer set, using the following reagent recipe. Exclude cDNA from the master mixes.

Reagent	**Volume**
cDNA	2µl
Forward Primer (10µM)	0.5µl
Reverse Primer (10µM)	0.5µl
KAPA SYBR FAST qPCR Master Mix (2X) Universal	10µl
Nuclease-free Water	7µl
Total	**20µl**

Prepare enough master mix for 1.25 reactions per treatment plus two extra. For example, if the GOI primer is needed for 26 wells, prepare (26 × 1.25) + 2 reactions, or 34.5 reactions for a total of 621µl master mix.

39. Aliquot master mix and cDNA into the wells of a 96-well qPCR microplate. Cover microplate with clear adhesive plastic film, and seal very well.

Seal across plate over wells first, then top and bottom edges of plate, then sides. This is very important as an incomplete seal could result in sample evaporation.

40. Spin plate down at 200x*g* for 1 minute.

41. Run plate in qPCR machine with the settings outlined in Table 6.

42. Examine -RT and H_2O amplification curves and check for the presence of amplification, which reflects the level of DNA contamination in the reaction.

43. Evaluate the specificity of the primer sets by examining the melt curve data.

Samples amplified with the same primer set should have nearly identical melting temperatures, and the melt curve should have only one peak. The presence of multiple peaks in melt curve data may indicate non-specific amplification and the presence of multiple PCR products. If multiple peaks are observed, run a portion of the reaction on a DNA gel and look for multiple bands.

44. Export Ct values and calculate percent expression using the ΔΔCt method for relative quantification of gene expression (Livak & Schmittgen, 2001). An example of typical data is shown in Fig. 3d.

Calculate percent expression separately using both housekeeping genes, and use the average of those two values for the final percent gene expression. Ideally, qRT-PCR analysis should be performed on more than one independent RNA sample, and the final data should be presented as the average gene expression, with the standard deviation from the independent RNA samples.

BASIC PROTOCOL 6

E. histolytica Growth Curves

Measuring growth rates of *E. histolytica* trophozoites is one way to phenotype different cell lines, and it is a way to ensure that wild-type cell lines are growing appropriately. This protocol outlines a method for measuring the growth rate of *E. histolytica* trophozoites over six days. Cells are seeded in six separate tubes so that growth is not disturbed during data collection, which requires incubation on ice to detach cells from tubes, and then collecting amoebae for cell counting. For the best results, it is important for cultures to be in exponential growth prior to the start of the growth curve. Therefore, cultures should be passaged within 1–2 days before the start of a growth curve.

Materials—~80% confluent T25 flask of wildtype or vector control *E. histolytica* cells

~80% confluent T25 flask of transfected *E. histolytica* cells (one per cell line)

Complete TYI-S-33 medium (see Support Protocol 1), warmed in 35°C water bath

Disposable borosilicate glass tubes with threaded end (Fisher, cat. no. 14–959-35A)

Kimble black phenolic screw-thread closures GPI thread 15mm-415 (Fisher, cat. no. 05–569-5)

Benchtop microcentrifuge (Beckman, cat. no. B30137)

Inverted microscope capable of 400x magnification

Cell counter (Bio Rad TC20 Automated Cell Counter, cat. no. 1450102), or hemocytometer

Centrifuge equipped with a swinging bucket rotor, such as Allegra X-14R (Beckman Coulter, cat. no. B08861)

Sterile 15ml and 50ml polypropylene conical centrifuge tubes (Corning, cat. no. 352196 and cat. no. 352070)

35°C incubator

35°C water bath

1. Add 13ml warm complete TYI-S-33 medium to 12 tubes for each cell line.

   Sufficient culture tubes for six days, with two replicate tubes for each day, are needed. This is a total of 12 tubes per cell line.

   Since these tubes will be grown without media replacement for up to six days, make sure to use recently thawed and completed TYI-S-33 medium.

2. Transfer T25 flasks of cells to sterile conical centrifuge tubes. Centrifuge cells at 200x*g* for 5 minutes.

   A T25 flask contains ~68ml total media volume. This step may be done in two ways: After incubation on ice to detach cells from the flask, one T25 flask can be split into two 50ml conical tubes, centrifuged, combined and centrifuged once more to obtain one pellet of cells. To save time, before incubation on ice to detach cells from the flask, some media (at least 20ml) can be aspirated from the T25 to reduce the total volume to slightly less than 50ml. If there is any cellular debris in the flask, it can be set vertically for a few minutes and debris that collects at the bottom can be easily aspirated along with the media. After removing media, the flask can be placed horizontally in the ice to make sure all cells are covered with media.

3. Aspirate the supernatant and resuspend cells in warm complete TYI-S-33 medium. Transfer the resuspended cells to a 15ml conical, take an aliquot out to count the cell density, and centrifuge 5 minutes at 200x*g*.

   15ml conical centrifuge tubes are preferable in general since E. histolytica *forms a fairly loose pellet, and the smaller dimensions of a 15ml conical enable the formation of a somewhat tighter pellet.*

4. Resuspend cells in warm complete TYI-S-33 medium at 8×10^3 cells/ml, and add 1ml cell suspension to each tube.

   E. histolytica *settles out of solution fairly quickly. When counting or pipetting amoebae, make sure to gently mix the cell suspension to homogenize any cells that have settled to the bottom of the tube.*

5. Incubate tubes at 35°C.

6. After 24 hours, ice the “day 1” tubes and transfer to a 15ml conical tube.

7. Spin the cells down and remove all media but 1ml; resuspend cells and transfer to a microcentrifuge (epi) tube.

8. Spin the epi tubes again in a table top microcentrifuge and resuspend in 100µl media.

The volume of media used to resuspend cells depends on both the number of cells and the concentration limits of the hemocytometer being used – on day 1 of the growth curve there will be less cells than on day 4, so less media should be used to resuspend day 1 cells than day 4 cells.

9. Count cells using a TC20 Cell Counter or a hemocytometer, taking two measurements per tube.

10. Repeat this process each day of the growth curve, adjusting the resuspension volume as necessary given the approximate concentration of the culture.

REAGENTS AND SOLUTIONS

M199s (without adult bovine serum), for transfection

Combine the following with 50ml Medium 199 (Gibco cat. no. 11043023).

0.045g L-Cysteine hydrochloride monohydrate (Sigma, cat. no. C7880)

0.3g HEPES (Sigma, cat. no. H4034)

0.005g L-Ascorbic acid (Sigma, cat. no. A0278)

Adjust pH to 6.8 with 1M NaOH or 1M HCl and filter sterilize.

M199s (with adult bovine serum), for transfection

Add heat-inactivated Adult Bovine Serum (Gemini Biosciences, cat. no. 100–101) to M199s (above recipe) for 15% final concentration: 42.5ml M199s + 7.5ml adult bovine serum.

M199s, for phenotyping assays

Combine the following with 100ml Medium 199 (Gibco cat. no. 11043023).

0.1g L-Cysteine hydrochloride monohydrate (Sigma, cat. no. C7880)

0.6g HEPES (Sigma, cat. no. H4034)

0.5g Bovine Serum Albumin (Gemini Biosciences, cat. no. 700–107P)

Adjust pH to 6.8 with 1M NaOH or 1M HCl.

0.5M EDTA (pH 8.0)

Add 186.1g of Ethylenediaminetetraacetic acid disodium dihydrate (EDTA) (Sigma cat. no. E4884) to 800ml DI water. Stir vigorously with a magnetic stir bar. Adjust the pH to 8.0 by the addition of NaOH (either 10M NaOH solution or about 20g NaOH pellets). The EDTA will not go into solution until the pH is adjusted to 8.0 with NaOH. Add DI water to 1000ml and filter sterilize or autoclave (Green et al., 2012).

COMMENTARY

Background Information

The axenic culturing of *E. histolytica* was achieved in 1961 by Louis S. Diamond (Diamond, 1961). Diamond advanced the field of parasitology greatly with his research into *Entamoeba* and other luminal parasites such as *Giardia* and *Trichomonas* (Clark & Diamond, 2002). Diamond developed several medium formulations that ultimately led him to TYI-S-33, a liquid medium containing trypticase, yeast extract, iron, bovine serum, and a vitamin-Tween 80 mixture (Diamond et al., 1978). This was critical in that it allowed for easier and more streamlined culturing of amoebae, and when coupled with axenization, made the study of *E. histolytica* much more accessible.

After *E. histolytica* was axenized, genetic manipulation of trophozoites for *in vitro* studies followed. Electroporation was used to perform the first transient transfections of plasmid DNA into *E. histolytica* cells (Nickel & Tannich, 1994; Purdy et al., 1994). Stable transfection came soon after, and this enabled researchers to modify *E. histolytica* gene expression over long enough periods of time to study the effect of such modifications (Hamann et al., 1995; Vines et al., 1995). Transfected plasmid DNA is maintained episomally in *E. histolytica.* Additional transfection methods were also tested and adopted, such as transfection with lipofectamine reagent (Olvera et al., 1997). Iterations of these first transfection methods are still in use today.

Alongside the innovation in plasmid delivery, plasmids were developed specifically for expression in *E histolytica*. The same groups that contributed to initial transfection of trophozoites also developed tetracycline-inducible gene expression systems (Hamann et al., 1997; Ramakrishnan et al., 1997). These provided researchers with the ability to switch expression of certain constructs on and off with the addition of tetracycline. Just as overexpression systems are useful, downregulation or knockout systems are likewise important. Gene knockouts have not been achieved in *E. histolytica*, but the robust, endogenous RNAi pathway has been exploited for gene knockdown. Many iterations of RNAi knockdown approaches have been used. An early example was the expression of an antisense copy of an entire gene (Ankri et al., 1998; Bracha et al., 1999). Later, epigenetic silencing of the amoebapore A gene was achieved through expression of its 5’ region, which includes the amoebapore A promoter in addition to a repetitive retrotransposon element (Anbar et al., 2005; Bracha et al., 2003). This strain was named “G3,” and it can be used to silence other genes in the context of an amoebapore A knockdown background (Bracha et al., 2006; Zhang et al., 2011). Expression of double-stranded RNAs (dsRNAs) (Kaur & Lohia, 2004) and expression of shRNAs (Linford et al., 2009) have also been used as strategies for gene knockdown.

A more recent innovation in RNAi technology in *E. histolytica* has been the introduction of the “trigger-induced” knockdown system, where the expression of an endogenously silenced region of DNA fused to a gene of interest can induce the synthesis of small RNAs (sRNAs) mapping to the gene of interest (Khalil et al., 2016; Morf et al., 2013). This trigger-induced RNAi knockdown can be used to silence 1–2 genes, as discussed in Basic Protocol 5. We have also developed a genome-wide RNAi knockdown library to facilitate forward

genetic screening, which takes advantage of the trigger silencing approach (Bettadapur et al., 2020). Additional tools are still needed to fully study the pathogenesis and basic biology of *E. histolytica*. Recently, a proof of concept study demonstrated successful CRISPR/Cas9-mediated editing of episomal DNA (Kangussu-Marcolino et al., 2021).

Note that the strain HM1:IMSS is the strain that is generally used for all experiments and genetic manipulations. While there are a few other laboratory strains available, such as the less virulent Rahman strain, these other strains are currently rarely used in practice. Other *E. histolytica* strains have historically been used for limited comparative studies (e.g., microarrays, proteomics (Davis et al., 2007, 2009; MacFarlane & Singh, 2006)) between strains. Other strains besides HM1:IMSS are not routinely cultured, genetically modified, etc. The protocols and troubleshooting tips that we present in this article are based on the literature and our experience with HM1:IMSS.

Critical Parameters and Troubleshooting

Media Preparation—*E. histolytica* trophozoites are sensitive to variations in some components of TYI-S-33 medium. One variable in medium preparation is the source of water and the way in which it is purified or processed. Early in the development of *Entamoeba* media, Diamond specifies his use of “glass-distilled water” in all culture media (Diamond, 1968a). In contrast to water processed by distillation, most modern laboratories routinely use purification systems such as Milli-Q systems to prepare purified water for a variety of laboratory applications. Some laboratories have been successful in using water purified through Milli-Q systems for TYI-S-33 medium preparation. The ability to successfully use Milli-Q water in TYI-S-33 medium may be related to the municipal water supply in the region, as there appears to be a general correlation with the level of mineral content, i.e., the hardness of the water. In our experience, when comparing TYI-S-33 medium batches in which the only variable is the water source, distilled water leads to a quantitatively higher *E. histolytica* growth rate. Therefore, in our laboratory, we use distilled water for the preparation of TYI-S-33 medium, and for the preparation of any other media or buffers that will be used with live cultures.

Another variable in medium preparation is the lot to lot variation in yeast extract. Biosate peptone, which is a combination of yeast extract and tryptone, is more commonly used for TYI-S-33 medium preparation than the two separate components. Lot to lot variation has not been observed for tryptone (Diamond et al., 1978); hence, the yeast extract in biosate peptone is the problematic component. Before using a new lot of biosate peptone or yeast extract, it is necessary to test the new lot, as outlined in Support Protocol 2. It is important to grow cells for at least 2 weeks, and up to 3 weeks, since the impacts on *E. histolytica* growth are often not seen immediately. It is also important to test lots of adult bovine serum, though issues with serum lots are rarely seen, while issues with yeast extract lots are fairly common.

The storage containers and sterilization methods used during medium preparation can also impact the growth of *E. histolytica* cultures. Thus, these factors are critical during the preparation of TYI-S-33 medium, as well as M199s medium, and its variations. Media should be stored in sterile bottles that have not been exposed to detergent or bleach, to prevent the introduction of these components into the media. Used medium bottles

should not be treated with bleach, washed by hand with soap, or washed in a dishwasher. Instead, used bottles should be washed extremely well with distilled water, allowed to dry thoroughly, and autoclaved. It is also useful to use bottles that are distinct from general lab glassware (e.g., wide mouth bottles with orange lids), so that bottles for TYI-S-33 medium are visibly distinct and are not used for the storage of other laboratory solutions. Similarly, in our laboratory, large plastic beakers are used exclusively for the preparation of TYI-S-33 medium, and they are also washed with water only. Autoclaving media before use is the fastest and most affordable choice for sterilization, but it can introduce additional variation if autoclave temperatures are not consistent. If the available autoclaves are not consistent, it is worth the extra time and expense to use bottle top filter units to sterilize media batches.

Molecular Biology—Studying *E. histolytica* with genetics requires amplification and manipulation of genomic sequences. The *E. histolytica* genome is ~75% A + T, which can make some aspects of molecular biology more challenging (Lorenzi et al., 2010). Primer design is a consideration for PCR. It is a good practice to use a primer design program such as Primer3. In general, primer length can be increased for *E. histolytica*, which can increase primer melting temperature. For PCR amplification of *E. histolytica* sequences, the choice of polymerase is also important. Phusion is commonly used for high-fidelity PCR amplification, but with *E. histolytica*, we have found that KAPA HiFi HotStart DNA polymerase is often a better choice. The KAPA polymerase has increased DNA affinity and is generally more suitable for A/T rich sequences. Similarly, for qPCR, we have empirically compared commonly used reagents (e.g., Roche LightCycler 480 SYBR Green I Master mix and BioRad SsoAdvanced Universal SYBR Green Supermix) with KAPA SYBR FAST qPCR Master Mix (Figure 6). The KAPA polymerase resulted in significantly lower Ct values than the other polymerases, and thus, it allows for a greater dynamic range of detection.

Transfection—While only one method for transfection of *E. histolytica* is presented in Basic Protocol 3, there are many different techniques for the introduction of plasmid DNA. If necessary, the parameters of drug selection can be varied during troubleshooting of this protocol. Many *E. histolytica* plasmids carry the neomycin gene for selection of stable transfectants. The timing and the level of antibiotic added to trophozoites after transfection can be varied, depending on the transfection efficiency and the particular plasmid. Transfected plasmid DNA is maintained episomally in *E. histolytica*. Drug selection is used to maintain selection for plasmid DNA, though in practice, once stable transfectants have been recovered, it is somewhat difficult to remove plasmid DNA (Foda & Singh, 2015; Huguenin et al., 2010; Morf et al., 2013). Thus, drug selection is maintained to safeguard against plasmid loss, but plasmid loss is fairly unlikely.

When performing transfection experiments, it is critical to have a negative control (no DNA) sample, so that the viability of the negative control can be assessed under the drug selection conditions used in each experiment. While G418 is often used for selection at 6μg/ml, nontransfected amoebae can be effectively killed by 3μg/ml G418, and this level of antibiotic can be used to select stable transfectants (Vines et al., 1995), though a large range of antibiotic concentrations have been reported in the literature (Kangussu-Marcolino et al.,

2021; MacFarlane & Singh, 2007). If a plasmid proves difficult to stably transfect, it can be effective to start selection relatively gently at 1μg/ml, and to increase the concentration after ~3–6 days (Olvera et al., 1997; Saito-Nakano et al., 2004). It can also be effective to begin selection 48 hours after transfection, as opposed to 24 hours (Rastew et al., 2015).

Understanding Results

Populations of transfected amoebae are heterogeneous. This is apparent when assessing knockdown of the GOI in amoebae that have been stably transfected with pTrigger. Sometimes, there can appear to be relatively low levels of knockdown. Once clonal lines are obtained through limiting dilution, the levels of expression of the GOI will often vary between clonal lines. It has not yet become standard practice in the *E. histolytica* community to obtain clonal lines of stably transfected cells, and hence, the reason for variation in the level of knockdown between clonal lines has not been investigated experimentally. It is possible that variation in the level of knockdown could be due to differences in plasmid copy number. In our hands, by performing limiting dilution, clones with very good levels of knockdown can typically be obtained, while avoiding cells with lower levels of knockdown. This highlights the importance of making clonal lines, in order to avoid inconsistencies in phenotype caused by heterogeneity.

In the same vein, phenotyping RNAi knockdown cell lines can be time sensitive. We have observed that as knockdown amoebae are cultured for long periods of time, they may lose silencing despite continued antibiotic selection. Because RNAi knockdown is constitutive in the pTrigger approach, knockdown that causes a decrease in fitness can be selected against over time. Thus it is best to phenotype knockdown mutants as soon as possible. It can be prudent to set aside some cells using a reagent like RNAprotect (Qiagen, cat. no. 76526) when performing a phenotypic assay, so that gene expression can be checked if phenotypic results are inconsistent from one experiment to another. If possible, qRT-PCR should be used when assessing knockdown, since the readout is quantitative, so changes in knockdown over time would be detectable.

Time Considerations

Cells that have been thawed from frozen stocks generally take 1 week to reach ~80% confluency, and should be treated with care until they have been passaged several times and are growing normally. Stably transfected cells under drug selection are generally ready to be passaged within ~1 week, and similarly should be treated with care until they have been passaged at least once. The preparation of clonal lines by limiting dilution leads to wells of cells that are ready to be passaged within 1 week, and after gradually scaling up the culture volume, ~80% confluent T25 flasks are generally obtained within another week. Making multiple large batches of TYI-S-33 can take 6–8 hours, and is most efficient with the combined efforts of several people working together. By contrast, preparation of one bottle takes about two hours. Lot testing of biosate peptone or yeast extract takes 2 to 3 weeks.

ACKNOWLEDGMENTS

We thank the members of our laboratory for their efforts towards refining these protocols and for critical evaluation of this article. We thank Emily M. Smith for performing the qRT-PCR experiments. We thank Dr. Upinder Singh (Stanford University) for the pTrigger plasmid, and the RPL21 and VTP primer sequences for qPCR. Work in K.S.R.'s laboratory is funded by NIH grants AI146914 and AI154163, and a Pew Scholarship awarded to K.S.R.

LITERATURE CITED

Anbar M, Bracha R, Nuchamowitz Y, Li Y, Florentin A, & Mirelman D (2005). Involvement of a short interspersed element in epigenetic transcriptional silencing of the amoebapore gene in Entamoeba histolytica. Eukaryotic Cell, 4(11), 1775–1784. [PubMed: 16278444]

Ankri S, Stolarsky T, & Mirelman D (1998). Antisense inhibition of expression of cysteine proteinases does not affect Entamoeba histolytica cytopathic or haemolytic activity but inhibits phagocytosis. Molecular Microbiology, 28(4), 777–785. [PubMed: 9643545]

Bettadapur A, Hunter SS, Barbieri CG, Settles ML, & Ralston KS (2020). Establishment of quantitative RNAi-based forward genetics in Entamoeba histolytica and identification of genes required for growth. BioRxiv, 2020.05.28.121780. 10.1101/2020.05.28.121780

Bracha R, Nuchamowitz Y, Anbar M, & Mirelman D (2006). Transcriptional silencing of multiple genes in trophozoites of Entamoeba histolytica. PLoS Pathogens, 2(5), e48. [PubMed: 16733544]

Bracha R, Nuchamowitz Y, Leippe M, & Mirelman D (1999). Antisense inhibition of amoebapore expression in Entamoeba histolytica causes a decrease in amoebic virulence. Molecular Microbiology, 34(3), 463–472. [PubMed: 10564488]

Bracha R, Nuchamowitz Y, & Mirelman D (2003). Transcriptional silencing of an amoebapore gene in Entamoeba histolytica: Molecular analysis and effect on pathogenicity. Eukaryotic Cell, 2(2), 295–305. [PubMed: 12684379]

Clark CG, & Diamond LS (2002). Methods for Cultivation of Luminal Parasitic Protists of Clinical Importance. Clinical Microbiology Reviews, 15(3), 329–341. 10.1128/CMR.15.3.329-341.2002 [PubMed: 12097242]

Davis PH, Chen M, Zhang X, Clark CG, Townsend RR, & Stanley SL (2009). Proteomic Comparison of Entamoeba histolytica and Entamoeba dispar and the Role of E. histolytica Alcohol Dehydrogenase 3 in Virulence. PLoS Neglected Tropical Diseases, 3(4), e415. 10.1371/journal.pntd.0000415 [PubMed: 19365541]

Davis PH, Schulze J, & Stanley SL (2007). Transcriptomic comparison of two Entamoeba histolytica strains with defined virulence phenotypes identifies new virulence factor candidates and key differences in the expression patterns of cysteine proteases, lectin light chains, and calmodulin. Molecular and Biochemical Parasitology, 151(1), 118–128. 10.1016/j.molbiopara.2006.10.014 [PubMed: 17141337]

Diamond LS (1961). Axenic cultivation of Entamoeba histolytica. Science, 134(3475), 336–337. [PubMed: 13722605]

Diamond LS (1968a). Improved Method for the Monoxenic Cultivation of Entamoeba histolytica Schaudinn, 1903 and E. histolytica-like Amebae with Trypanosomatids. The Journal of Parasitology, 54(4), 715–719. JSTOR. 10.2307/3277027 [PubMed: 4319344]

Diamond LS (1968b). Techniques of axenic cultivation of Entamoeba histolytica Schaudinn, 1903 and E. histolytica-like amebae. The Journal of Parasitology, 1047–1056. [PubMed: 4319346]

Diamond LS (1983). Lumen Dwelling Protozoa: Entamoeba, Trichomonads, And Giardia. In In Vitro Cultivation of Protozoan Parasites (pp. 65–109). CRC Press.

Diamond LS (1995). Cryopreservation and Storage of Parasitic Protozoa in Liquid Nitrogen. Journal of Eukaryotic Microbiology, 42(5), 585–590. 10.1111/j.1550-7408.1995.tb05911.x

Diamond LS, Harlow DR, & Cunnick CC (1978). A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Transactions of the Royal Society of Tropical Medicine and Hygiene, 72(4), 431–432. [PubMed: 212851]

Foda BM, & Singh U (2015). Dimethylated H3K27 Is a Repressive Epigenetic Histone Mark in the Protist Entamoeba histolytica and Is Significantly Enriched in Genes Silenced via the RNAi Pathway*. Journal of Biological Chemistry, 290(34), 21114–21130. 10.1074/jbc.M115.647263

Gilchrist CA, Petri SE, Schneider BN, Reichman DJ, Jiang N, Begum S, Watanabe K, Jansen CS, Elliott KP, & Burgess SL (2016). Role of the gut microbiota of children in diarrhea due to the protozoan parasite Entamoeba histolytica. The Journal of Infectious Diseases, 213(10), 1579–1585. [PubMed: 26712950]

Green MR, Hughes H, Sambrook J, & MacCallum P (2012). Molecular cloning: A laboratory manual. In Molecular cloning: A laboratory manual (pp. 1890–1890).

Hamann L, Buß H, & Tannich E (1997). Tetracycline-controlled gene expression in Entamoeba histolytica. Molecular and Biochemical Parasitology, 84(1), 83–91. [PubMed: 9041523]

Hamann L, Nickel R, & Tannich E (1995). Transfection and continuous expression of heterologous genes in the protozoan parasite Entamoeba histolytica. Proceedings of the National Academy of Sciences, 92(19), 8975–8979. 10.1073/pnas.92.19.8975

Huguenin M, Bracha R, Chookajorn T, & Mirelman D (2010). Epigenetic transcriptional gene silencing in Entamoeba histolytica: Insight into histone and chromatin modifications. Parasitology, 137(4), 619–627. [PubMed: 19849886]

Kangussu-Marcolino MM, Morgado P, Manna D, Yee H, & Singh U (2021). Development of a CRISPR/Cas9 system in Entamoeba histolytica: Proof of concept. International Journal for Parasitology, 51(2), 193–200. 10.1016/j.ijpara.2020.09.005 [PubMed: 33264648]

Kaur G, & Lohia A (2004). Inhibition of gene expression with double strand RNA interference in Entamoeba histolytica. Biochemical and Biophysical Research Communications, 320(4), 1118–1122. 10.1016/j.bbrc.2004.06.064 [PubMed: 15249205]

Khalil MI, Foda BM, Suresh S, & Singh U (2016). Technical advances in trigger-induced RNA interference gene silencing in the parasite Entamoeba histolytica. International Journal for Parasitology, 46(3), 205–212. [PubMed: 26747561]

Linford AS, Moreno H, Good KR, Zhang H, Singh U, & Petri WA (2009). Short hairpin RNA-mediated knockdown of protein expression in Entamoeba histolytica. BMC Microbiology, 9(1), 38. 10.1186/1471-2180-9-38 [PubMed: 19222852]

Livak KJ, & Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCT method. Methods, 25(4), 402–408. [PubMed: 11846609]

Lorenzi HA, Puiu D, Miller JR, Brinkac LM, Amedeo P, Hall N, & Caler EV (2010). New Assembly, Reannotation and Analysis of the Entamoeba histolytica Genome Reveal New Genomic Features and Protein Content Information. PLoS Neglected Tropical Diseases, 4(6), e716. 10.1371/journal.pntd.0000716 [PubMed: 20559563]

MacFarlane RC, & Singh U (2006). Identification of Differentially Expressed Genes in Virulent and Nonvirulent Entamoeba Species: Potential Implications for Amebic Pathogenesis. Infection and Immunity, 74(1), 340–351. 10.1128/IAI.74.1.340-351.2006 [PubMed: 16368989]

MacFarlane RC, & Singh U (2007). Identification of an Entamoeba histolytica serine-, threonine-, and isoleucine-rich protein with roles in adhesion and cytotoxicity. Eukaryotic Cell, 6(11), 2139–2146. [PubMed: 17827347]

Morf L, Pearson RJ, Wang AS, & Singh U (2013). Robust gene silencing mediated by antisense small RNAs in the pathogenic protist Entamoeba histolytica. Nucleic Acids Research, 41(20), 9424–9437. [PubMed: 23935116]

Nickel R, & Tannich E (1994). Transfection and transient expression of chloramphenicol acetyltransferase gene in the protozoan parasite Entamoeba hitolytica 4.

Olvera A, Olvera F, Vines RR, Recillas-Targa F, Lizardi PM, Dhar S, Bhattacharya S, Petri W Jr, & Alagon A (1997). Stable transfection of Entamoeba histolytica trophozoites by lipofection. Archives of Medical Research, 28, 49–51. [PubMed: 9033009]

Petri WA Jr, Mondal D, Peterson KM, Duggal P, & Haque R (2009). Association of malnutrition with amebiasis. Nutrition Reviews, 67, S207–S215. 10.1111/j.1753-4887.2009.00242.x [PubMed: 19906225]

Purdy JE, Mann BJ, Pho LT, & Petri WA (1994). Transient transfection of the enteric parasite Entamoeba histolytica and expression of firefly luciferase. Proceedings of the National Academy of Sciences, 91(15), 7099–7103. 10.1073/pnas.91.15.7099

Ramakrishnan G, Vines RR, Mann BJ, & Petri WA Jr (1997). A tetracycline-inducible gene expression system in Entamoeba histolytica. Molecular and Biochemical Parasitology, 84(1), 93–100. [PubMed: 9041524]

Rastew E, Morf L, & Singh U (2015). Entamoeba histolytica rhomboid protease 1 has a role in migration and motility as validated by two independent genetic approaches. Experimental Parasitology, 154, 33–42. [PubMed: 25889553]

Saito-Nakano Y, Yasuda T, Nakada-Tsukui K, Leippe M, & Nozaki T (2004). Rab5-associated Vacuoles Play a Unique Role in Phagocytosis of the Enteric Protozoan Parasite Entamoeba histolytica. Journal of Biological Chemistry, 279(47), 49497–49507. 10.1074/jbc.M403987200

Vines RR, Purdy JE, Ragland BD, Samuelson J, Mann BJ, & Petri WA (1995). Stable episomal transfection of Entamoeba histolytica. Molecular and Biochemical Parasitology, 71(2), 265–267. 10.1016/0166-6851(95)00057-8 [PubMed: 7477110]

Voytas D (2000). Agarose Gel Electrophoresis. Current Protocols in Molecular Biology, 51(1), 2.5A.1–2.5A.9. 10.1002/0471142727.mb0205as51

Wang H, Naghavi M, Allen C, Barber RM, Bhutta ZA, Carter A, Casey DC, Charlson FJ, Chen AZ, Coates MM, Coggeshall M, Dandona L, Dicker DJ, Erskine HE, Ferrari AJ, Fitzmaurice C, Foreman K, Forouzanfar MH, Fraser MS, … Murray CJL (2016). Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: A systematic analysis for the Global Burden of Disease Study 2015. The Lancet, 388(10053), 1459–1544. 10.1016/S0140-6736(16)31012-1

Willhoeft U, & Tannich E (1999). The electrophoretic karyotype of Entamoeba histolytica. Molecular and Biochemical Parasitology, 99(1), 41–53. 10.1016/S0166-6851(98)00178-9 [PubMed: 10215023]

Wilson IW, Weedall GD, Lorenzi H, Howcroft T, Hon C-C, Deloger M, Guillén N, Paterson S, Clark CG, & Hall N (2019). Genetic Diversity and Gene Family Expansions in Members of the Genus Entamoeba. Genome Biology and Evolution, 11(3), 688–705. 10.1093/gbe/evz009 [PubMed: 30668670]

Zhang H, Alramini H, Tran V, & Singh U (2011). Nucleus-localized Antisense Small RNAs with 5′-Polyphosphate Termini Regulate Long Term Transcriptional Gene Silencing in Entamoeba histolytica G3 Strain*,. Journal of Biological Chemistry, 286(52), 44467–44479. 10.1074/jbc.M111.278184

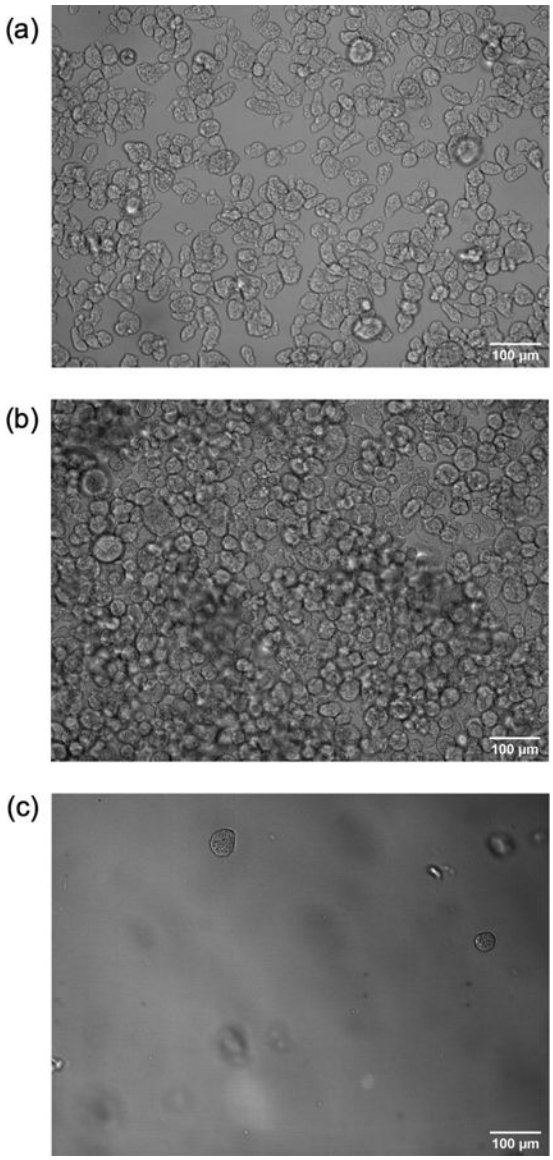

Figure 1: Example images of confluent, overgrown, and detached amoebae in T25 flasks.
(a) A healthy *E. histolytica* culture at approximately 80% confluence. (b) An overgrown culture of *E. histolytica.* Note the rounded morphology of individual cells and the appearance of cells that are "floating," detached and in the plane above the plane of cells attached to the surface of the flask (c) A confluent culture after icing and inversion. It is important to visualize the focal plane that corresponds to the surface of the flask, where attached cells would be found. It is common for a few cells per field to remain attached after a flask or tube has been incubated on ice, but the vast majority of cells should be detached. T25 flasks were imaged on an Intelligent Imaging Innovations hybrid spinning-disk confocal microscope using a 10x objective lens.

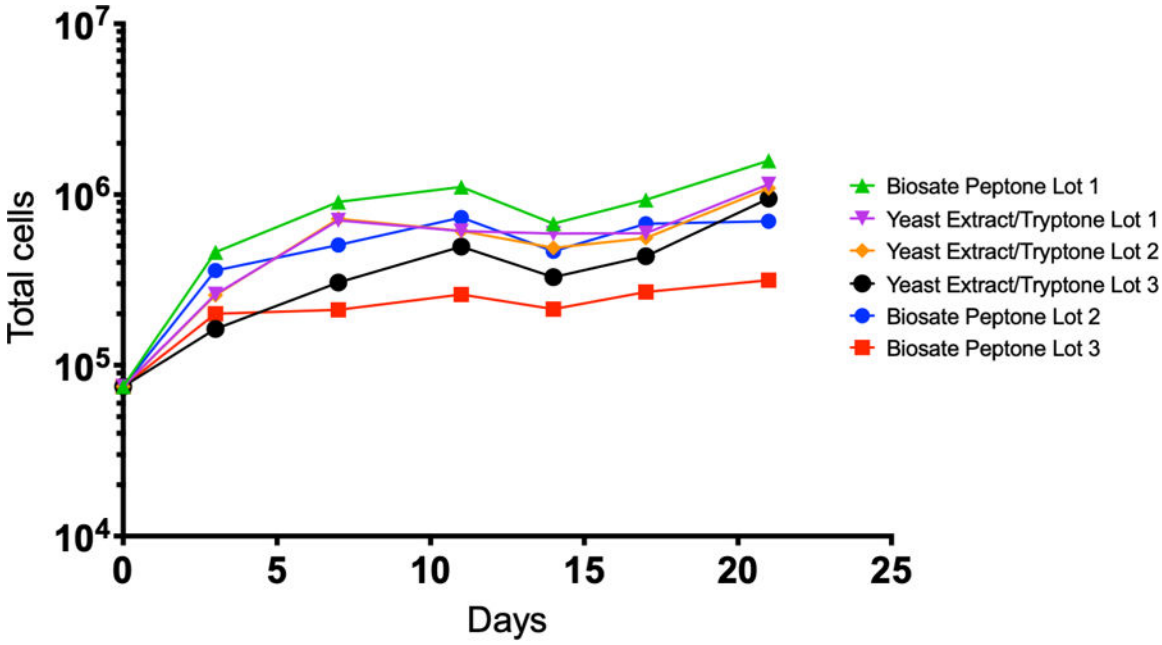

Figure 2: Growth of *E. histolytica* in different lots of Biosate peptone and yeast extract/tryptone over 21 days.

E. histolytica was grown in complete TYI-S-33 made with different lots of Biosate peptone or yeast extract/tryptone during the process of lot testing (Support Protocol 2). All yeast extract/tryptone TYI-S-33 batches were made with the same lot of tryptone, while different lots of yeast extract were tested. Glass culture tubes of each lot were seeded with 7.5×10^4 cells/tube and allowed to grow for 3 or 4 days. After 3 or 4 days, cells were harvested and counted, and new tubes were seeded at 7.5×10^4 cells/tube. Lot testing occurred for a total of 21 days.

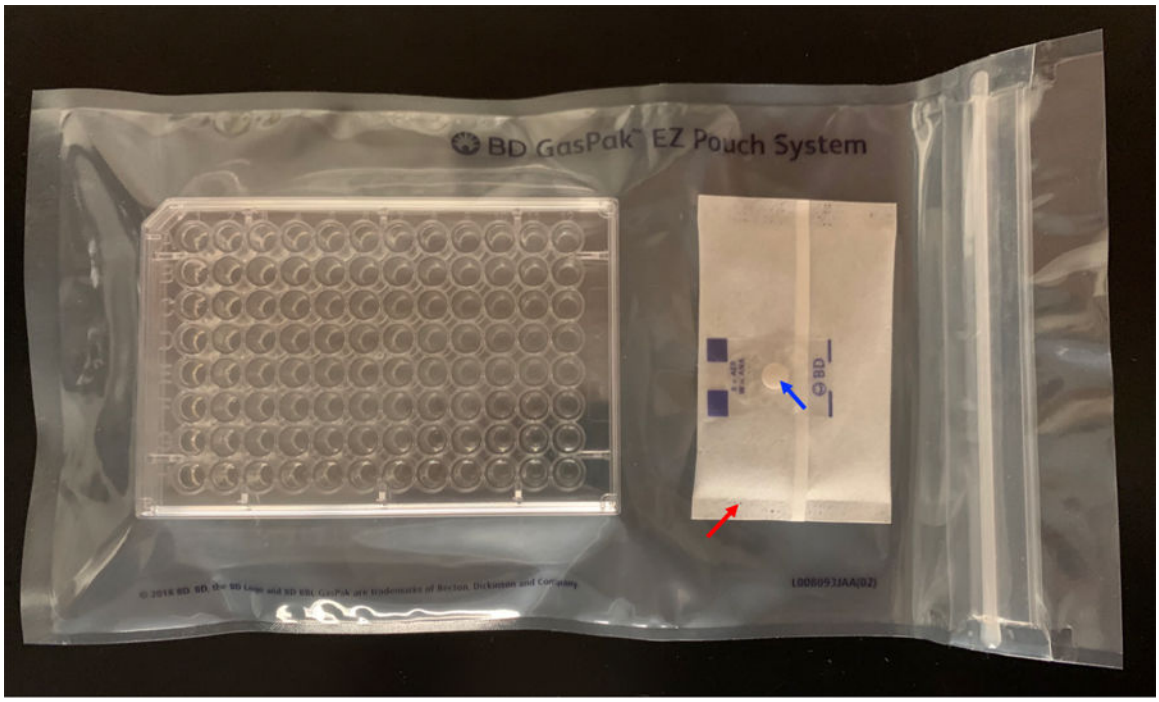

Figure 3: 96-well plate and GasPak set up for making clonal lines using limiting dilution.
A 96-well plate with 100μl TYI-S-33 media in each well was carefully placed into a GasPak plastic bag, followed by a GasPak pouch (red arrow) with indicator (blue arrow). The GasPak pouch is positioned beside the plate to allow for monitoring of growth using an inverted microscope (Basic Protocol 4). As long as the interior of the GasPak remains anaerobic, the indicator should remain white.

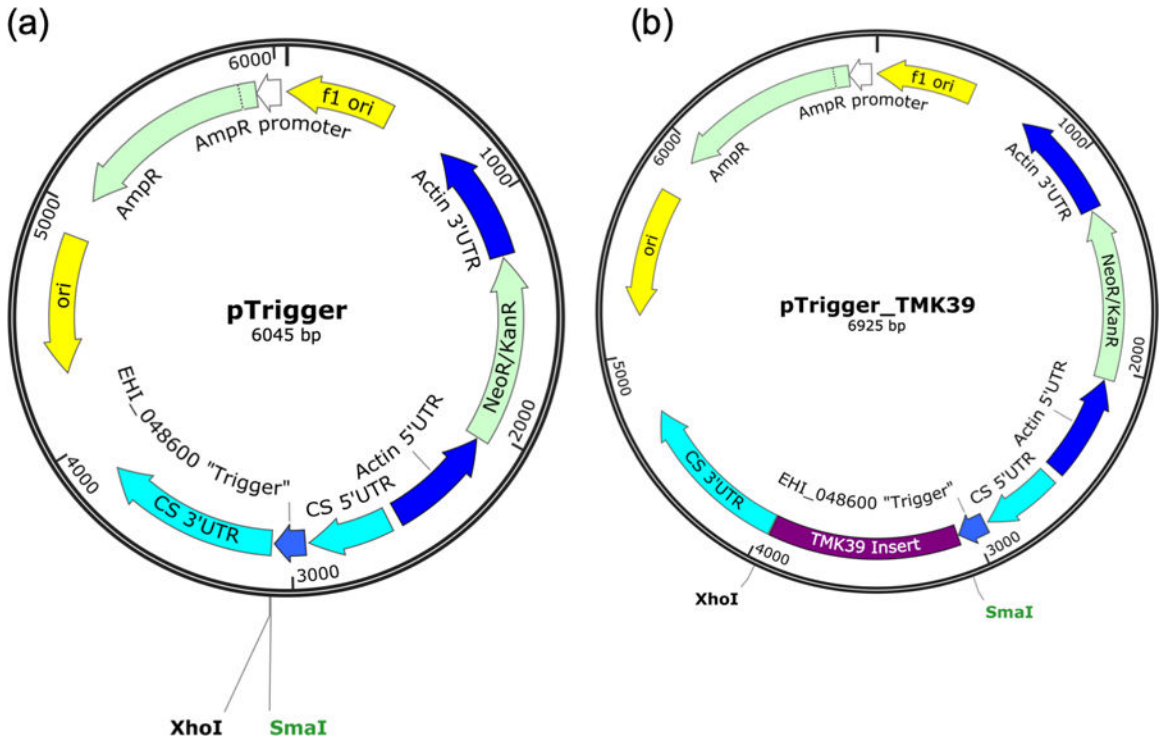

Figure 4: Trigger induced RNA interference expression plasmids.

(a) Map of pTrigger showing *Xho*I and *Sma*I sites used for cloning of gene of interest (GOI) fragments, and "Trigger" sequence from trigger gene EHI_048600 (Basic Protocol 5). (b) Example map of pTrigger with 880bp of TMK39 (EHI_037140) inserted between *Xho*I and *Sma*I sites. CS: cysteine synthase; UTR: untranslated region.

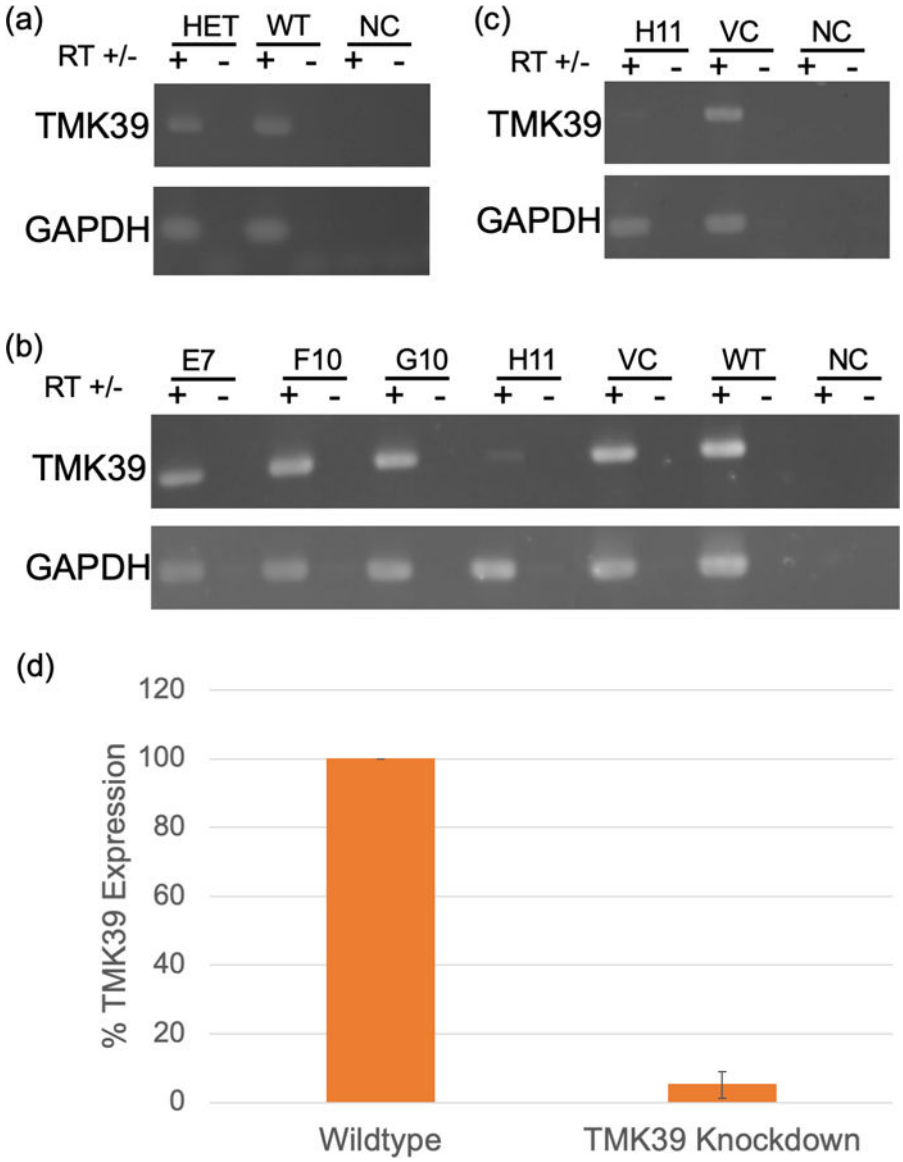

Figure 5: RT-PCR analysis of heterogeneous knockdown transfectants and clonal lines.

(a) Conventional RT-PCR was performed on RNA samples from heterogeneous (HET) transfectants of pTrigger_TMK39 and compared to wildtype (WT) and negative control (NC) samples (Support Protocol 4). Primers to amplify TMK39 and housekeeping gene GAPDH were used. Knockdown of TMK39 was not apparent in the heterogeneous transfectants. (b) Clonal lines of heterogeneous pTrigger_TMK39 transfectants were obtained by limiting dilution. Conventional RT-PCR analysis of four clonal lines compared to both vector control (VC) and WT samples showed that clonal lines had differing levels of knockdown of TMK39, with the greatest apparent knockdown in clone H11. (c) Conventional RT-PCR analysis of an independent RNA sample from clone H11, compared to a VC sample. (d) Quantitative RT-PCR analysis was performed on RNA samples from the TMK39 knockdown clone H11, compared to a wildtype sample. Percent expression of TMK39 was calculated using the ΔΔCt method for relative gene expression with both

RPL21 and VTP as housekeeping or reference genes. Error bars represent the standard deviation of expression calculated with RPL21 and VTP as reference genes. RT: reverse transcriptase; HET: heterogeneous; WT: wildtype; VC: vector control; NC: negative control

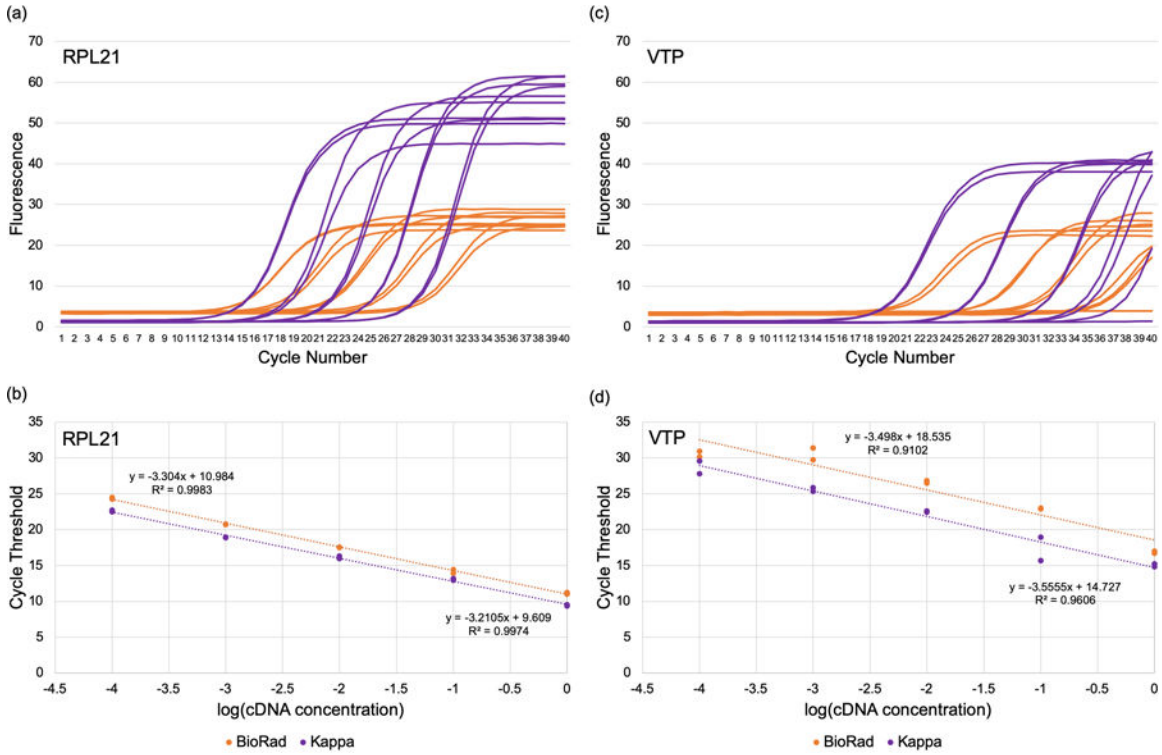

Figure 6: Comparison of BioRad and Kappa polymerases on amplification of RPL21 and VTP housekeeping genes using quantitative RT-PCR

(a, c) Quantitative RT-PCR was performed on wildtype RNA samples with two different polymerases: BioRad SsoAdvanced Universal SYBR Green Supermix (BioRad, cat. no. 1725270) shown in orange, and KAPA SYBR FAST qPCR Master Mix (Roche cat. no. KK4601) shown in purple. Primer sets for RPL21 (a) and VTP (c) were used to amplify five different concentrations of cDNA: 1:1, 1:10, 1:100, 1:1,000, and 1:10,000. Fluorescence values from each sample were plotted against cycle number for a total of 40 cycles. (b, d) RPL21 (b) and VTP (d) primer sets were empirically evaluated for efficiency by plotting the cycle threshold values against the log of the cDNA concentration. The threshold value was determined automatically by the LightCycler analysis software. Detailed instructions for how data were collected and analyzed can be found in Support Protocol 4, step 36.

Table 1.

Reagents required for 1 bottle and 1x-4x TYI-S-33 medium preparations.

	1 bottle	**1x**	**2x**	**3x**	**4x**
BBL Biosate Peptone	24g	144g	288g	432g	576g
D-(+)-Glucose	8g	48g	96g	144g	192g
NaCl	1.6g	9.6g	19.2g	28.8g	38.4g
K_2HPO_4	0.8g	4.8g	9.6g	14.4g	19.2g
KH_2PO_4	0.48g	2.88g	5.76g	8.6g	11.52g
L-Cysteine hydrochloride monohydrate	0.9g	5.4g	10.8g	16.2g	21.6g
L-Ascorbic acid	0.16g	0.96g	1.92g	2.88g	3.84g
Ferric ammonium citrate	0.018g	0.108g	0.216g	0.324g	0.432g
Distilled water (initial volume)	400ml	1000ml	2000ml	3000ml	4000ml
Final volume	725ml	4350ml	8700ml	13050ml	17400ml
Number of bottles	1	6	12	18	24

Table 2.

Number of cells seeded in each well after completing steps 1–9.

Column	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
#cells	250	125	63	31	16	8	4	2	1	0.5	0.25	0.13

Table 3.

Thermocycler program for PCR using KAPA HiFi HotStart PCR Kit.

Stage	Temperature (°C)	Time (min:sec)	Cycles
Initial denaturation	95°C	3:00	1
Denaturation	98°C	0:20	35
Annealing	Tm specific	0:15	
Extension	72°C	1:00/kb	
Final extension	72°C	1:00/kb	1
Hold	4°C	Hold	1

Table 4.

RT-PCR housekeeping gene primer sets.

Primer Name	Reaction Type	Product Size	Gene ID	Annealing temperature	Primer Sequence
GAPDH	Conventional PCR	319bp	EHI_187020	58–65°C	**F:** 5’-CGTCCACAGACAATTCGAAGGAAC-3’
					R: 5’-AAGGCAGTTGGTTGTGCATGA-3’
RPL21	qPCR	122bp	EHI_069110	60°C	**F:** 5’-CCTTGAAAGAAAGGCTGCTGTT-3’
					R: 5’-GTTCAGCTGGTCTTGGTTGTTT-3’
VTP	qPCR	84bp	EHI_092149	60°C	**F:** 5’-TCCAAGTCAACCATCTGTACAGT-3’
					R: 5’-ACTGGTTCGTCAAGAGTAGTCTC-3’

Table 5.

Thermocycler program for PCR using NEB Taq DNA polymerase.

Stage	Temperature (°C)	Time (min:sec)	Cycles
Initial denaturation	95°C	5:00	1
Denaturation	95°C	0:30	30
Annealing	Tm specific	1:00	
Extension	68°C	1:00/kb	
Final extension	68°C	5:00	1
Hold	4°C	hold	1

Table 6.

Thermocycler program for qPCR using KAPA SYBR FAST qPCR Master Mix.

Stage	Analysis Mode	Acquisition Mode	Temperature	Time (min:sec)	Ramp Rate	Cycles
Pre-amplification	none	none	50°C	2:00	1.6°C/s	1
		none	95°C	2:00		
Amplification	quantification	none	95°C	0:15	1.6°C/s	40
		single	60°C*	0:30		
Melt curve	melting curves	none	95°C	0:15	1.6°C/s	1
		none	60°C	1:00	1.6°C/s	
		continuous	95°C	none	0.14°C/s	
Cool down	none	none	50°C	0:30	1.6°C/s	1

*60°C is a suitable annealing temperature for RPL21 and VTP primer sets. The annealing temperature may need to be optimized for the GOI primer set.