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Electrotransformation and clonal isolation of *Rickettsia* species

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Abstract

Genetic manipulation of obligate intracellular bacteria of the genus *Rickettsia* is currently undergoing a rapid period of change. The development of viable genetic tools, including replicative plasmids, transposons, homologous recombination, fluorescent protein-encoding genes, and antibiotic selectable markers has provided the impetus for future research development. This unit is designed to coalesce the basic methods pertaining to creation of genetically modified *Rickettsia*. The unit describes a series of methods, from inserting exogenous DNA into *Rickettsia* to the final isolation of genetically modified bacterial clones. Researchers working towards genetic manipulation of *Rickettsia* or similar obligate intracellular bacteria will find these protocols to be a valuable reference.

Keywords

Transformation; cell sorting; intracellular bacteria; clonal isolation; plaque assay; limiting dilution; immunofluorescence

Introduction

This unit is designed to take the researcher from possession of DNA, *Rickettsia*, and host to cryopreservation of genetically modified *Rickettsia* clones. The choice of DNA, *Rickettsia* species, and host cell type are each specific to the goal of the researcher. However, transformation, clonal isolation, and validation of transformation follow the specific set of protocols described in this unit. As shown in Figure 1, the workflow begins with a universally applicable electroporation protocol. This process is followed by three different methods that all result in the isolation of clonal populations of *Rickettsia*. Subsequently, the analysis of transformed *Rickettsia* clones in Basic Protocols 3, 4, and 5 is again universally applicable. By performing the procedures described in this unit, the researcher will achieve successful isolation of transformed *Rickettsia*.

As shown in Table 1, there are three known methods for creating clonal populations of bacteria. These methods are described in Basic Protocol 2, Alternate Protocol 1, and Alternate protocol 2. Each of these methods has different strengths and weaknesses as will be described later in this unit, but all results in possession of the materials for determining validity of clones as described in Basic Protocols 3 and 4. Therefore, the researcher's

strategy and protocol choice will be based on available time, tools, experience, and investigator's discretion.

Some *Rickettsia* species necessitate biosafety level 3 (BSL-3) containment. Extensive standard operating procedures must be developed, approved, and strictly followed during manipulation of these organisms. All manipulation of live organisms should be performed in a class II biosafety cabinet within an approved BSL-3 laboratory, as suggested in the 5th edition of the Biosafety in Microbiological and Biomedical Laboratories (BMBL).

Genetic manipulation of *Rickettsia* is a challenging and time-consuming process. These bacteria are notoriously difficult to utilize. These hurdles have undoubtedly contributed to the publication of only 17 peer-reviewed reports in the 17 years since the first successful genetic manipulation of *Rickettsia* (Rachek et al., 1998). The investigator will be unable to assess final results of *Rickettsia* genetic manipulation for at least a month, so it is advisable to repeat this protocol frequently and not wait until the final results to start another round of transformation. The commitment required to complete these protocols has also placed a strong impetus on the creation of this unit. We have analyzed the literature pertaining to this topic, compared these methods to our current protocols, and created this unit containing the methods with the greatest possible chance of success.

Basic Protocol 1: Electrotransformation of *Rickettsia* species

First described in 1998 (Rachek et al., 1998), the process of introducing exogenous DNA into *Rickettsia* and subsequent observation of the growth of transformed bacteria is similar to common bacterial electrotransformation techniques (Seidman and Struhl, 2001). Transformation of *Rickettsia* species has developed from this initial report, and has been successful in multiple different pathogenic and non-pathogenic species, including *R. bellii* (Oliver et al., 2014), *R. parkeri* (Welch et al., 2012), *R. prowazekii* (Driskell et al., 2009; Liu et al., 2007; Qin et al., 2004; Rachek et al., 2000; Rachek et al., 1998; Wood et al., 2012), *R. rickettsii* (Clark et al., 2011a; Clark et al., 2011b; Kleba et al., 2010; Noriega et al., 2015), *R. montanensis* (Baldrige et al., 2010), *R. monacensis* (Baldrige et al., 2005; Baldrige et al., 2007), and *R. typhi* (Troyer et al., 1999). Additionally, related obligate intracellular bacteria of the genera *Ehrlichia*, *Coxiella*, and *Anaplasma* have been successfully transformed (Beare et al., 2011). As such, transformation is likely to be successful in *Rickettsia* species that have yet to be transformed.

One limiting factor for successful transformation is the quantity of bacteria that is needed for this protocol. Reported transformation efficiencies are between 10^{-7} to 10^{-8} , so each transformation requires at least 3×10^8 bacteria (Baldrige et al., 2005; Clark et al., 2011b). With regard to transforming DNA, 4 different construct types have been demonstrated. To date, genetic manipulation of *Rickettsia* has been achieved through: 1. single crossover chromosomal insertion (Rachek et al., 2000; Rachek et al., 1998); 2. double crossover chromosomal insertion (Driskell et al., 2009; Noriega et al., 2015; Troyer et al., 1999); 3. transposon mutagenesis (Baldrige et al., 2005; Baldrige et al., 2007; Baldrige et al., 2010; Clark et al., 2011a; Clark et al., 2011b; Kleba et al., 2010; Qin et al., 2004; Welch et al., 2012); and 4. episomal plasmid insertion (Burkhardt et al., 2011; Oliver et al., 2014;

Wood et al., 2012). Recovery of mutants that require insertion into the chromosome will likely be less efficient, as these constructs rely on homologous recombination by the bacterium. The protocol specifically describes transformation using the plasmid pRam18dRGA[AmTrCh] (Burkhardt et al., 2011) but is applicable to all transforming DNAs. pRam18dRGA[AmTrCh] contains the gene for the expression of Rpaar2, which confers Rifampicin resistance. Importantly, the protocol can easily be modified for other antibiotic selection processes.

Electrotransformation is divided into 4 major processes as will be described below: 1. preparation of *Rickettsia*, DNA, and host cells (steps 1–3); 2. creation of electrocompetent *Rickettsia* (steps 4–5); 3. electrotransformation (steps 6–12); and 4. selection of transformed *Rickettsia* (steps 13–15).

Materials

10 μ L DNA at 0.5–2mg/mL in H₂O
 3×10^8 – 3×10^{10} *Rickettsia* sp.
 175cm² flask of confluent Vero cells
 HBSS++
 250mM sucrose
 Ice
 trypsin-EDTA
 0.1cm-gap electroporation cuvette
 Complete (c) DMEM
 three 175cm² flasks cDMEM with 200ng/mL Rifampicin

Equipment

Electroporator
 tissue culture incubator
 rotator or shaker
 swinging bucket centrifuge

1. Grow and purify 3×10^8 *Rickettsia* by filter, sucrose or Renografin methods.

Propagation and purification of Rickettsia is described in detail in Ammerman, et.al. 2008 (Ammerman et al., 2008; UNIT 3A.5). Growth and purification of Rickettsia is a time consuming process that takes about 2 weeks to accomplish.

2. Create your DNA construct. Plasmid, PCR, or restriction fragment DNA are all applicable as long as it is provided at 0.5–2mg/mL in H₂O.

Currently, the largest DNA successfully transformed is the 27,588 base pair pRAM18/Rif/GFPuv plasmid (Burkhardt et al., 2011). This is unlikely to be the maximum size of transforming DNA as R. felis naturally maintains the 63kbp pRF plasmid (Ogata et al., 2005), implying that bacteria of this genus are capable of maintaining larger episomal DNAs. DNA designed for transposon mutagenesis, single crossover events, or extrachromosomal maintenance can all remain circular. Only DNA designed for double crossover events needs to be linearized by restriction digestion or PCR.

3. One day prior to transformation, seed 4.9×10^6 Vero cells into a 175cm² tissue culture flask containing 30mL cDMEM. Incubate overnight at 34°C 5%CO₂.

Other host cells can be used. This protocol has been successful in utilizing ISE6 and L929 host cells. The cell line must support fulminant growth of the bacteria.

4. Wash bacteria thrice by resuspending in 5mL ice-cold 250mM sucrose followed by centrifugation at 10,000× gravity(g) for 5 minutes at 4°C.

Sucrose is a non-ionic osmoprotectant. The goal of this step is to remove all traces of salt, while preserving bacterial membrane integrity.

5. Fully resuspend the bacteria in 90μL cold 250mM sucrose.
6. Add in 10μL (5–20μg) cold DNA sample in H₂O to the resuspended bacteria from step 5.

It is vitally important to remove all traces of salt from the bacteria/DNA mixture. Excess salt will cause arcing during electroporation and will drastically lower transformation efficiency.

7. Transfer the 100μL DNA/bacteria mixture to pre-cooled 0.1cm-gap electroporation cuvette.
8. Incubate the filled cuvette on ice for 10 minutes.
9. During this incubation, remove the media from the 175cm² flask. Dissociate the Vero cells by adding 6mL of trypsin-EDTA and incubating for 10 minutes at 34°C.
10. Remove the disassociated cells and centrifuge at 500× g for 5 min in a 50mL polypropylene tube. Resuspend the pellet in 1mL HBSS++. The final concentration of Vero cells will be approximately 2×10^7 cells/mL.
11. Place the filled cuvette in the electroporation apparatus. Electroporate using an exponential decay program with the settings: voltage= 17kV/cm, resistance= 150Ω, capacitance= 50μF.

The resulting Time Constant (TC) for an exponential decay program should be >4ms. Shorter TC values or arcing indicate high salt

concentrations and inefficient electroporation. Alternatively, a square wave protocol can be utilized at 4–6ms time constant.

12. Immediately after transformation, gently pipette the bacteria from the electroporation cuvette and transfer the bacteria into the 1mL HBSS++ containing Vero cells. Incubate this mixture for 1 hour at 34°C with rotation at approximately 30 rpm.

This step is designed to maximize contact between the transformed bacteria and host cells in order to promote maximum bacterial invasion.

13. Divide the mixture from step 11 into three aliquots. Place 333μL of the infected Vero cells into each of three 175cm² flasks containing uninfected Vero cells with 30mL cDMEM. Gently swirl the mixture and at 34°C 5% CO₂ to allow Vero cell attachment to the flask.

14. After 24 hours, the Vero cells will have adhered to the tissue culture flask. Replace the media in each of the flasks with 30mL cDMEM containing 200ng/mL Rifampicin.

This starts the process of selection of transformed *Rickettsia*. The non-transformed bacteria will be unable to grow in the presence of Rifampicin.

15. Change the media every 3–4 days to maintain antibiotic selection. Due to the bacteriostatic nature of Rifampicin, there will be untransformed *Rickettsia* in the selection until approximately day 7. Expect observable growth of antibiotic resistant bacteria after approximately 11–21 days.
16. Observe growth of *Rickettsia* by taking a sample of the infected cells by scraping a small section of Vero cells. Place the scraped cells into PBS and mount onto a slide using a cytospin centrifuge. Stain the *Rickettsia* by Diff-Quik or Gimenez-stain and observe under a light microscope equipped with a 100× oil objective.
17. Upon observation of outgrowth of bacteria, proceed to clonal isolation protocols (Basic Protocol 2, Alternate Protocol 1, or Alternate Protocol 2).

Basic Protocol 2: Clonal isolation of *Rickettsia* species by cell sorting

This method for isolation of *Rickettsia* clones relies on a cell sorter to place a single bacterium in each well of a 96-well plate. The transformed *Rickettsia* are labeled with a fluorescent-tagged antibody and are individually sorted based on fluorescent properties. This method requires the most training and start-up costs, as use of the cell sorter is quite complicated. However, this method is the least time consuming and ensures that the isolated populations are clonal. The use of a cell sorter for clonal isolation relies on extensively understanding the physics of cell sorting as well as respect for proper usage of this powerful tool in achieving specific tasks. The usual goal of cell sorting is to provide the largest yield of a specific cellular population. However, this specific protocol is designed to achieve purity at the cost of total yield. This logic is derived from the fact that a fully recovered *Rickettsia* transformation from Basic Protocol 1 will contain millions of bacteria; rather than purifying all of these bacteria, the goal is to place a single bacterium in each well of a tissue

culture plate. To achieve this goal, the cell sorter is calibrated towards a high rejection percentage of non-rickettsial events and droplets that contain more than one cell. Basic cytometry, cell sorting, and quality control protocols are covered in the Current Protocols in Cytometry Units 1.24, 11.4, and 11.17 (Arnold and Lannigan, 2010; Harkins, 2001; Harkins and Harrigan, 2004). Because every manufacturer has different idiosyncrasies to the cell sorter startup protocol, these methods will not be covered in this protocol.

This protocol describes a very specific set of reagents, but can be adequately performed with substitute reagents. First, we describe Rifampicin-resistant *Rickettsia* expressing the fluorescent protein GFP_{uv}, and use an anti-rickettsia PerCP/Cy5.5-tagged primary antibody (RcPFA) (Chan et al., 2011). *Rickettsia* resistant to other antibiotics or lacking an intrinsic fluorescence can be sorted. Similarly, it is possible to utilize any antibody that recognizes the surface of the bacterium conjugated to a fluorochrome that is complementary to the laser and filter settings of your cell sorter. We have found that extrinsic labeling of the bacteria is more efficient than utilizing the fluorescent properties of GFP_{uv}, because of greater separation of background and labeled events. Despite the fact that antibody-coated *Rickettsia* may have decreased invasion (Feng et al., 2004), the recovery of antibody-tagged bacteria is more efficient than sorting based on size, granularity, or GFP_{uv} fluorescence (unpublished data).

The last consideration for performance of this protocol is biosafety. Some *Rickettsia* species are handled in a biosafety level 3 (BSL-3) setting, and the sorting process potentially generates infectious aerosols. Appropriate biosafety protocols should be developed and implemented prior to performance of this protocol (Oberyszyn, 2002). With proper safety precautions this protocol will achieve the experimental goals with speed and precision.

Materials

Transformed *Rickettsia* from Basic Protocol 1
75cm² flask of confluent Vero cells
four 5mL syringes
15G, 19G, 25G needles
2µm syringe filter
fluorophore-conjugated anti-*Rickettsia* antibody (RcPFA-PerCP/Cy5.5)
96-well tissue culture plate of confluent Vero cells
Trypsin-EDTA

Equipment

Cell sorter
tissue culture incubator
swinging-bucket centrifuge

1. One day prior to sorting, prepare host cells for the sorting process. Gather a 75cm² flask and one confluent 96-well plate. Seed the 75cm² flask with 2.1×10^6 Vero cells in cDMEM and every well of a 96-well plate with 8×10^3 Vero cells in cDMEM supplemented with 200ng/mL Rifampicin. Incubate these plates overnight at 34°C 5%CO₂.
2. Prior to starting the protocol, place PBS on ice.
3. Begin the cell sorter startup and daily quality control process. Include a cell sorter sterilization step by running approximately 1L of 70% ethanol through the sorter.

Since cell sorters are quite different, individual expertise is needed. A general summary of cell sorting is found in Arnold and Lannigan 2010 (Arnold and Lannigan, 2010). For completion of this protocol, the stream alignment, drop cutoff, drop charging, and sort delay must be properly calculated and stable.

4. During this incubation, remove the media from the 75cm² flask of Vero cells and the 175cm² of *Rickettsia*-infected Vero cells. Dissociate the Vero cells by adding 4–6mL of trypsin-EDTA and incubating for 10 minutes at 34°C. Perform parallel manipulation of infected and uninfected Vero cells for steps 5–11.

We have found that the partial purification described later in this protocol generally produces better results than performing full sucrose purification. The extremely small Rickettsia pellet is frequently lost during sucrose purification. As such, we will require an uninfected control (Vero cells) to define the fluorescent properties of host cell debris, such that we can efficiently remove these contaminants.

5. Pipette lifted cells into 50mL falcon tubes and centrifuge at 500× g for 5 min to pellet host cells.
6. Remove supernatant, and resuspend the *Rickettsia*-infected Vero and uninfected Vero cell pellets separately in 5mL cold PBS+1:1000 RcPFA-PerCP/Cy5.5 antibody.

This protocol describes the use of the fluorophore PerCP/Cy5.5. This fluorophore is readily excited at 488nm and emits into the 692/30 channel. Any fluorophore can be used for your analysis apart from a fluorophore that emits into the FITC channel, as this will conflict with GFP_{uv} expressing *Rickettsia*.

7. Lyse infected and uninfected Vero cells separately to liberate the *Rickettsia*. Use a 5mL syringe to pass the liquid through an 18g needle approximately 15 times. Repeat with 20g and 25g needles.
8. Use a 5mL syringe to pass lysed cells through a 2µm filter thrice to remove unbroken host cells.
9. Incubate on ice, or at room temperature (RT) if cold incubator is unavailable, for 1 hour with agitation to allow antibody binding to the liberated *Rickettsia*.

10. Wash the bacteria thrice with 5mL cold PBS by centrifuging at 10,000× g 5 min followed by resuspension.
11. After washing, resuspend the samples in 3mL cold PBS.
- If the solution is cloudy, further dilute in PBS so that the cytometer does not become clogged.
12. Finish cell sorter startup and daily quality control.
13. Set up cytometer workspace with forward scatter (FSC) threshold/trigger and two heat-map graphs. Set the axes of the first graph to side scatter (SSC) and FSC. Set up the second graph with SSC and log 488nm excitation 692/30nm emission axes.
- If you have chosen a different fluorophore for analysis, use FSC, SSC, and the appropriate excitation and emission filters.
14. Apply a solution of PBS to the cell sorter. Increase the FSC photomultiplier tube (PMT) voltage such that the cytometer registers impurities in the PBS solution. This occurs when the event rate is in the thousands per second range. With the cytometer registering these multiple events, decrease the FSC PMT voltage to the highest voltage where no events are registered.
- Rickettsia* are small organisms. For most cytometers, the *Rickettsia* will be near the lower limit of detection for forward scatter (FSC) and side scatter (SSC) parameters. This is why the cytometer must be setup to select extremely low voltage FSC/SSC events.
15. After applying the uninfected Vero lysate suspension to the cytometer, many registering events appear. Adjust the SSC and 692/30 PMT voltages such that most of the observed events are off of the x- and y-axes. Record approximately 10,000 events.
16. Remove the uninfected Vero lysate and apply the solution containing the infected Vero lysate containing *Rickettsia*. Record 10,000 events.
17. Display the recorded uninfected and *Rickettsia* infected Vero lysate cytometry plots side by side (Figure 2). A distinct population only present in the *Rickettsia* infected sample should be evident. This population will be found at the lower right quadrant of the SSC × 692/30 graph. As seen in Figure 2, the fluorescently-tagged *Rickettsia* have a much higher 692/30 fluorescence associated with the antibody binding.
18. Create a sort gate that contains very few of the events present in both of the input samples. These events common to both samples have a very Vero cell lysate content. Instead, choose the events that are unique to the *Rickettsia*-infected sample. The events in the right portion of the heat map will contain the free *Rickettsia*.
- Inevitably, there will be some overlap between the two populations. This is to be expected, considering the presence of both miniscule Rickettsia and larger Vero contaminants that possess some autofluorescence. It is*

recommended to gate no more than 5% of the total events of the partially-purified Rickettsia sample.

19. Create a sort program that will place a single event into each well of the Vero cell seeded 96-well plate from step 1. Do not sort into the last three wells of the plate (H10–12). These last three wells will be utilized for control samples in step 22. Because the overarching goal of this protocol is to sort a single *Rickettsia*-gated event into each well, adjust your sort decision settings to the most stringent setting possible. The sorter should be put into single sort mode with 0.5 drop mask. This mode will reject any drops that contain more than one cell.
20. Place the 96-well plate of Vero cells in the sort chamber, remove the lid, and close the sort chamber. With the *Rickettsia* on the sample port, turn on the sample flow, and decrease the sample offset so the cytometer is observing <50 events per second. Use the cytometer to observe the samples in real time. Ensure that there are indeed events contained within your chosen gate.
21. Perform the sort.

Upon conclusion of the sort program, do not immediately open the sort chamber. The aerosols will need approximately 30 seconds to settle or evacuate the sort chamber.
22. Remove the 96-well plate from the sort chamber. Place 20µL of the partially purified *Rickettsia* input from step 11 into well H10. This control is to ensure the presence of *Rickettsia* in your sort input. Do not put *Rickettsia* in well H11. Put 1µL purified Rifampicin-resistant *Rickettsia* into well H12 (if available) as a positive control for later analyses.
23. Centrifuge the 96-well plate at 500× g for 5 minutes to induce *Rickettsia* contact with the host cell monolayer.
24. Place the 96-well plate in a tissue culture incubator. Incubate at 34°C 5%CO₂ for 5 days.
25. Centrifuge the remainder of the two input samples at 10,000× g for 5 min. Remove the supernatant and resuspend in 1mL SPG. Freeze these remaining samples as described in Basic Protocol 5.
26. Four days post sorting seed three full 96-well plates with Vero cells and Rifampicin as done in step 1.
27. On the fifth day post sort, remove the media from the infected plate. Add 40µL of trypsin-EDTA to the cells and incubate for 10 minutes or until the monolayer is disassociated from the well.
28. Using a multichannel pipette, create three identical replicas of the original plate. Do this by pipetting 10µL of each well from the original plate into each corresponding well of the three clean 96-well plates.
29. Centrifuge the newly infected plates at 500× g for 5 min, and place back into the incubator.

30. Incubate for five additional days at 34°C 5% CO₂.

31. Proceed to analysis protocol (Basic Protocol 3).

Alternate Protocol 1: Clonal isolation of *Rickettsia* species by limiting dilution

Limiting dilution of the *Rickettsia* provides an inexpensive method for creating clones. The antibiotic resistant bacteria from Basic Protocol 2 are subjected to serial dilution such that the concentration of bacteria is decreased to the point where a single bacterium is likely placed into each well. After allowing the bacteria to grow, the process is repeated two more times to ensure that the isolated bacteria are clonal. This protocol requires the fewest reagents and the lowest amount of training, but required a considerable length of time to complete the protocol. Three rounds of limiting dilution isolation will take in excess of 45 days to fully isolate a *Rickettsia* clonal population.

Materials

Transformed *Rickettsia* from Basic Protocol 1
 cDMEM supplemented+ 200ng/mL Rifampicin
 Trypsin-EDTA
 four 5mL syringes
 15G, 19G, 25G needles
 2µm syringe filter
 96-well tissue culture plate of Vero cells
 filter-sterilized SPG.

Equipment

- Tissue culture incubator
 swinging-bucket centrifuge
1. One day before performing this protocol, seed 6×10^5 Vero cells per well into a 96-well plate with cDMEM + 200ng/mL Rifampicin. Incubate overnight at 34°C 5% CO₂.
 2. Remove the media from the infected Vero cells created in Basic Protocol 1. Add 6mL trypsin-EDTA to each of these wells. Incubate 10 min at 34°C to disassociate the cells from the plate.
 3. Pipette lifted cells into 50mL falcon tubes and centrifuge at 500× g for 5 min to pellet host cells.
 4. Remove supernatant, and resuspend the *Rickettsia*-infected Vero and uninfected Vero cell pellets separately in 5mL PBS.

5. Lyse Vero cells to liberate the *Rickettsia* using a 5mL syringe by passing the liquid through an 18g needle approximately 15 times. Repeat with 20g and 25g needles.
6. Use a 5mL syringe to pass lysed cells through a 2µm filter three times to remove unbroken host cells.
7. Prepare 10-fold dilutions of *Rickettsia* in 100mL cDMEM+ 200ng/mL Rifampicin. Each sample will require 90µL liquid.
8. Remove media from the new 96-well plate.
9. Add 10µL of each dilution to eight wells (one column) of the 96-well plate. Repeat for each dilution.
10. From step 7, place 10µL of the partially purified *Rickettsia* into well H10. This control is to ensure that there were actually *Rickettsia* in your input. Do **not** place *Rickettsia* in well H11 as a negative control. Put 1µL purified Rifampicin-resistant *Rickettsia* into well H12 (if available) as a positive control for later analyses.
11. Centrifuge at 500× g for five min at RT to induce bacteria- host contact.
12. Incubate 15 min at 34°C.
13. Add 190µL cDMEM+200ng/mL Rifampicin to all wells.
14. Incubate 34°C 5% CO₂ for five days.
15. During the fourth day after sorting, seed three full 96-well plates with 6×10⁵ Vero cells in cDMEM+200ng/mL Rifampicin as done in step 1.
16. On the fifth day post sort, remove the media from the infected plate. Add 40µL of trypsin-EDTA to the cells and incubate for 10 minutes at 34°C or until the monolayer is disassociated from the well.
17. Using a multichannel pipette, create three identical replicas of the original plate. Do this by pipetting 10µL of each well from the original plate into each of the three new 96-well plates.
18. Incubate for an additional 5 days at 34°C 5% CO₂.
19. Proceed to Basic Protocol 3.

Alternate Protocol 2: Clonal isolation of *Rickettsia* species by plaque assay

This classic method for isolation of *Rickettsia* clones relies on a soft-agar overlay on top of lightly infected host cells (Ammerman et al., 2008). Because the bacteria are unable to disperse through the soft agar, *Rickettsia* form foci of extremely heavy infection, which are referred to as plaques. At a high dilution, each plaque represents a population of cells that grew from a single parent bacterium. This protocol has historically been used by rickettsiologists to isolate rickettsial strains from various hosts (Cory et al., 1975; Weinberg et al., 1969). This protocol lacks the removal of un-transformed bacteria and lack of maintenance of selection without being able to refresh the culture medium. Again, this protocol requires reagents and limited training, but required a considerable length of time to

complete the protocol. Three rounds of limiting dilution isolation will take in excess of 45 days to fully isolate a *Rickettsia* clonal population.

Materials

Transformed *Rickettsia* from Basic Protocol 1
six 6-well plates of Vero cells
cDMEM supplemented+200ng/mL Rifampicin
cDMEM supplemented+400ng/mL(2×) Rifampicin
Trypsin-EDTA
four 5mL syringes
15G, 19G, 25G needles
2µm syringe filter
1% agarose in water, autoclaved and kept at 50°C
cDMEM+ Neutral red solution
sterile Pasteur pipette

Equipment

- 37°C water bath
50°C water bath
tissue culture incubator
inverted light microscope
1. One day before performing this protocol, seed 6×10^5 Vero cells per well into six 6-well plates.
 2. Warm cDMEM to 37°C and the liquid agarose solution to 50°C.

Both of these liquid solutions need to be kept at this temperature. When the samples are combined, they will be at an appropriate temperature to coat the Vero cells.
 3. Remove the media from the infected Vero cells created in Basic Protocol 1. Add 6mL trypsin-EDTA to each of these wells. Incubate 10 min at 34°C to disassociate the cells from the plate.
 4. Pipette lifted cells into 50mL falcon tubes and centrifuge at $500 \times g$ for 5 min to pellet host cells.
 5. Remove supernatant and resuspend the *Rickettsia*-infected Vero cells in 5mL PBS.
 6. Lyse infected Vero cells to liberate the *Rickettsia* using a 5mL syringe by passing the liquid through an 18g needle approximately 15 times. Repeat with 20g and 25g needles.

7. Use a 5mL syringe to pass lysed cells through a 2µm filter three times to remove unbroken host cells.
8. Perform 10-fold serial dilutions of the lysed host cells in 1mL cDMEM+200ng/mL Rifampicin from 10^{-1} to 10^{-10} . In the end, each dilution should contain at least 900µL.
9. Remove the growth medium from the 6-well plates.
10. Add 150 µL of each *Rickettsia* dilution to each well of a 6-well plate. Repeat for other dilutions (10^{-5} – 10^{-10}) such that there are six full 6-well plates containing a different dilution on each plate.
11. Gently swirl the plates such that the liquid covers the entire well.
12. Incubate the inoculated plates for one hour at 37°C to allow *Rickettsia* invasion.
13. Wash all wells twice with 2mL cDMEM+200ng/mL Rifampicin.
14. In a 15mL tube, combine 7mL 1% agarose and 7mL cDMEM +400ug/mL (2×) Rifampicin for each plate.
15. Mix thoroughly by pipetting and add 2mL of this mixture to each well. Gently swirl to ensure equal coverage.
16. After solidification, add 1mL cDMEM + 200µg/mL Rifampicin to the top of the agar.
17. Incubate plates at 34°C 5% CO₂.
18. Check cells daily using an inversed microscope.

Plaques will appear as small cleared areas in the monolayer. The loss of Vero cells will be apparent after 5–7 days of culture.

19. When plaques are detected, replace liquid portion with 2mL Neutral Red solution. This solution will aid in defining the plaque borders.
20. Find a well-defined and isolated plaque. Utilizing the inverted microscope, place the plaque in the center of a 20× magnification field. Use an elongated sterile pasture pipette with the bulb depressed, place the tip through the agarose into the plaque all the way to the bottom of the culture plate. Release the end of the bulb to suck the bored hole into the pipette. Repeat for every well-isolated plaque.
21. Place each agarose plug in a separate microcentrifuge tube containing 200µL cDMEM + 200ng/mL Rifampicin. Elute the bacteria out of the agarose by rotating the tube overnight at 4°C.
22. Seed every well of a 96-well plate with 8×10^3 Vero cells in cDMEM. Incubate overnight at 34°C 5%CO₂.
23. Remove the media from each well of the 96-well plate. Place plug contents into individual wells of the 96-well plate.

24. During the fourth day after sorting, seed three 96-well plates with Vero cells and Rifampicin as done in step 1.
25. On the fifth day post-infection, remove the media from the infected plate. Add 40µL of trypsin-EDTA to the cells and incubate for 10 min at 34°C or until the monolayer is disassociated from the well.
26. Using a multichannel pipette, create three identical replicas of the original plate. Do this by pipetting 10µL of each well from the original plate into each of the three new 96-well plates.
27. Centrifuge the plates at 500× g for five min, and place back into the incubator.
28. Incubate for five additional days at 34°C 5% CO₂.
29. Proceed to basic Protocol 3.

Basic Protocol 3: Confirmation of *Rickettsia* infection by immunofluorescence

After the electrotransformation and clonal isolation protocols, the isolated *Rickettsia* need to be further analyzed. This process is classified into three major steps: 1. identify wells containing *Rickettsia*; 2. confirm the DNA manipulation; and 3. propagate the clones. This protocol is a continuation of Basic Protocol 2 (cell sorting), Alternate Protocol 1 (limiting dilution), or Alternate Protocol 2 (plaque). The three replica plates containing potential clones created in the above protocols will each be utilized for a different function. The first plate will be analyzed for the presence of *Rickettsia* by immunofluorescence (Basic Protocol 3). In the second plate, wells containing *Rickettsia* will be analyzed for the presence of a gene from the transforming DNA (Basic Protocol 4). Finally, the third plate will be utilized for propagation of the clones (Basic Protocol 5).

Importantly, Basic Protocol 3 simply provides instructions for confirmation of the presence of transformed clones. Presence of *Rickettsia* in individual wells does not indicate the recovery of a clone containing the genetic manipulation. Positive results from this protocol confirm the presence of antibiotic resistant clones. As there is a potential for isolation of spontaneous resistant bacteria, each mutation must be confirmed through direct analysis of target DNA in Basic Protocol 4.

Materials

Three 96-well plate containing infected Vero cells from Basic Protocol 2

Paraformaldehyde

permeabilization buffer (PBS, 2% BSA, 0.1% Triton X100)

Primary antibody solution (PBS, 2% BSA, 1:1000 RcPFA)

secondary antibody solution (PBS, 2% BSA, 1:1000 donkey anti rabbit-Alexafluor488)

Equipment

Multichannel Pipetter

Confocal Microscope

1. Take one of the three identical 96-well plates containing potential clones.
2. Using a multichannel pipetter, gently remove the growth media from all of the cells on the plate.
3. Replace the media with 50 μ L 4% paraformaldehyde. Incubate for 20 minutes at RT.

Many *Rickettsia* sp. are BSL-3 pathogens. Paraformaldehyde incubation will kill the bacteria in the samples. If your standard operating procedure allows, the plate can be removed from the BSL-3 laboratory for analysis.

4. Remove the paraformaldehyde and dispose of appropriately. Using a multichannel pipetter, wash once with 200 μ L PBS.
5. Remove the PBS by inverting the plate and tapping it on a stack of paper towels. All liquid can be removed by this method for the remainder of this protocol.
6. Fill the wells with 50 μ L permeabilization buffer. Incubate 45 min at RT with shaking.
7. Remove the permeabilization buffer. Fill the wells with 50 μ L primary antibody solution. Incubate 1 hour at RT with shaking.

The anti-RcPFA antibody was created through vaccination with formaldehyde fixed R. conorii (Chan et al., 2011). Any similar primary antibody can be used so long as the antibody recognizes the surface of the Rickettsia species being analyzed.

8. Wash the plate by removing the primary antibody solution and add 200 μ L PBS. Incubate the plate for five min. Repeat four times.
9. Remove the final wash and add 50 μ L secondary antibody solution. Incubate in the dark for 1hr at RT with shaking.
10. Wash the plate by removing the secondary antibody solution and adding 200 μ L PBS. Incubate the plate for five min. Repeat four times. Keep the plate in the dark as much as possible for the remainder of this protocol.
11. Analyze the plate using an inverted fluorescence microscope with a 20 $\times$ objective. Excite with blue light and observe under a green filter.
12. First observe the difference between wells H11 (uninfected) and H12 (positive control). Well H12 will contain bright green bacteria and H11 will be nearly black.
13. Observe Well H10. If this well contains *Rickettsia*, then the investigator has achieved transformation and selection (but not clonal isolation) in Basic Protocol 1.

- a. Annotation: This control is valuable because it provides evidence that you started with some *Rickettsia* in the beginning of the clonal isolation process.
14. Analyze each of the other wells (A1-H9). Note the wells containing green fluorescent *Rickettsia*.

Basic Protocol 4: Confirmation of DNA transformation

Before beginning this protocol, *Rickettsia*-infected Vero cells must have been identified in specific wells of the 96-well plate. Next, characterization of transformed *Rickettsia* is verified by the presence of the inserted DNA. To that end, this protocol describes additional *Rickettsia* culture, DNA isolation, and PCR. To this point in the unit, the *Rickettsia* are antibiotic resistant and clonal if derived from Basic Protocol 2. Alternate Protocol 1 and 2 require completion of this protocol three times before clonality is assured.

Materials

DNA isolation kit
standard PCR buffers and polymerase
gfp_{uv}-specific DNA primers
DNA loading dye
1% agarose DNA gel
Ethidium bromide

Equipment

- Tissue culture incubator
thermal cycler
electrophoresis apparatus
UV light box
1. Retrieve the second replicate plate. This plate will be used to isolate DNA from the wells containing *Rickettsia*.
 2. Remove the media from the wells that contain *Rickettsia* as identified in Basic Protocol 3.
 3. Add 50 μ L trypsin-EDTA to each of these wells. Incubate 10 min at 34°C to disassociate the cells from the plate.
 4. Pipette the cells into 1.5mL microcentrifuge tubes.
 5. Centrifuge for 5 min at 500 $\times$ g to pellet the infected Vero cells.
 6. Remove the supernatant.
 7. Isolate the DNA.

This is done using a genomic DNA isolation kit. Because each of these kits are specific to the manufacturer, the protocol will not be described here. It is important to perform the full DNA isolation, as attempting to perform PCR directly on the samples promotes highly variable results.

8. Elute the DNA into 20–30µL water.

If your biosafety protocol allows, the sterile DNA can be removed from containment.

9. Perform a standard PCR using the primers GFP_{uv}F (5'-TTCTGTCAGTGGAGAGGGTGAAGGTGAT) and GFP_{uv}R (5'-CCATCCTTTTGTGTCTGCCGTG). Cycle at 95°C 1min, 55°C 1 min, 72°C 1 min for 30 cycles.

Alternative genes can be used for PCR amplification, so long as the gene is present in the target DNA. The primers should be designed to amplify approximately 150–400 base pairs (b.p.) of this specific DNA.

10. Add DNA loading dye, run each PCR sample on a 1% agarose gel, and stain with ethidium bromide.
11. Visualize the gel under UV light. The presence of the *gfp_{uv}* gene will produce a band of approximately 400 b.p.
12. Note the PCR-positive wells and proceed to Basic Protocol 5 (cryopreservation).

If you performed Basic Protocol 2, proceed directly to Basic Protocol 5. Alternate Protocols 1 and 2 (limiting dilution and plaque assay) require three rounds of isolation to ensure that the isolated bacteria are indeed clonal.

Basic Protocol 5: Expansion and cryopreservation of *Rickettsia* clones

After verification of the presence of transforming DNA, the *Rickettsia* need to be preserved for further phenotypic analysis. It is useful to freeze large batches of *Rickettsia* because purification is labor-intensive and all follow-up analysis can be performed on identical bacteria. This protocol describes cryopreservation of partial-, sucrose-, or Renografin-purified *Rickettsia*. For in-depth description of culture and purification of *Rickettsia* see unit 3A.5 (Ammerman et al., 2008).

Materials

Cell-free *Rickettsia*
filter-sterilized SPG
cryovials

Equipment

–80°C freezer

1. Seed one 175cm² flask with 5×10⁶ Vero cells and 30mL cDMEM+200ng/mL Rifampicin for every PCR positive clone from Basic Protocol #4. Incubate overnight at 34°C 5% CO₂.
2. The next day, remove the media from the specific PCR-positive wells in the last replica plate.
3. Add 50μL trypsin-EDTA to each of these wells. Incubate 10 min at 34°C to disassociate the cells from the plate.
4. Add the liberated cells directly to each 175cm² flask. Incubate for 7–10 days at 34°C 5% CO₂.
5. After outgrowth of the *Rickettsia*, perform partial-, sucrose-, or Renografin-purification as described in Unit 3A.5.
6. Resuspend *Rickettsia* in 1–2mL cold SPG.
7. Transfer 50–500μL *Rickettsia* to each cryovial.

Small aliquots of *Rickettsia* are necessary because each episode of thawing and re-freezing will severely decrease bacteria viability.

8. Place the *Rickettsia* vials directly into the –80°C freezer for preservation.

Reagents and Solutions

1. cDMEM–	445mL DMEM, high glucose, pyruvate 50mL heat inactivated FBS 5mL Non-essential Amino Acids
2. HBSS++	Hanks balanced salt solution 5 mM glutamic acid 0.1% gelatin filter sterilize
3. Trypsin-EDTA	Hanks balanced salt solution 0.25% Trypsin 0.1% EDTA filter sterilize
4. 4% Paraformaldehyde	10mL 10× PBS 4g Paraformaldehyde 4g Sucrose Adjust to 100mL with H ₂ O
5. PBS with 0.1% Triton X-100	500mL PBS 0.5mL Triton X-100 filter sterilize
6. PBS/BSA	500mL PBS 10mL Bovine Serum Albumin filter sterilize
7. Rifampicin (1000× solution)	10mL DMSO, methanol, acetone, or chloroform

	2mg Rifampicin
	filter sterilize
8. SPG	218mM Sucrose
	3.8mM KH_2PO_4
	7.2mM K_2HPO_4
	4.9mM L-glutamate of glutamic acid
	pH7.2, filter sterilize

Commentary

Background Information

For decades, the intractability of obligate intracellular bacteria severely inhibited analysis of these fascinating and medically pertinent bacteria. This genetic recalcitrance was largely a consequence of complications from the requirement of infecting a eukaryotic cell for growth, the lack of an axenic growth medium, and the relative lack of usable antibiotic resistance markers. Over the last two decades, however, great strides have been undertaken to moving the field of rickettsiology from the development of protocols and tools, to venturing into the world of functional *Rickettsia* forward genetics. The research community now possesses the genetic tools for transposon mutagenesis, site-directed mutagenesis, and shuttle plasmids (Beare et al., 2011). While each of these tools is linked to a desired specific outcome, insertion of all of these DNA sources into the bacterium proceeds through a limited set of protocols that is described in this unit.

In bacterial genetics, it is important to create a specific bacterial population that universally contains the desired DNA manipulation. In order to achieve this goal, the mixed bacterial population must be cultured such that an entire new population is derived from a single parent bacterium in a process called clonal expansion. For bacteria that form colonies on agar plates, this process is as simple as plating on selective media and choosing a single isolated colony for further characterization. Unfortunately, many bacterial species, including *Rickettsia*, cannot be cultured on agar-containing artificial media and thus, do not form distinct colonies. Currently, three methods have been developed to perform clonal expansion in *Rickettsia*: agar overlay, limiting dilution, and cell sorting with a common theme of extensive expenditure of labor and time. This difficulty is confounded by the slow replication rate of these bacteria (12–18 hours) and reliance on the host cell for growth of the bacteria. Furthermore, the most common antibiotic used in *Rickettsia* selection, Rifampicin, is bacteriostatic, meaning that there will typically be a small population of non-transformed, non-resistant bacteria mixed in with the desired transformant. Unfortunately, these contaminating bacteria will begin to grow if the selection antibiotic is removed. Despite these profound difficulties, various researchers have successfully developed the basic genetic tools for manipulating these bacteria (Baldrige et al., 2005; Baldrige et al., 2007; Baldrige et al., 2010; Burkhardt et al., 2011; Clark et al., 2011a; Clark et al., 2011b; Driskell et al., 2009; Kleba et al., 2010; Oliver et al., 2014; Qin et al., 2004; Racheck et al., 2000; Racheck et al., 1998; Troyer et al., 1998; Troyer et al., 1999; Welch et al., 2012; Wood et al., 2012). Each of the methods of forward genetics described in this unit have advantages

and disadvantages but careful and deliberate performance of the protocols in this unit will provide a strong basis for success.

Critical Parameters

Spontaneous resistance to Rifampicin was noted very early in the development of *Rickettsia* genetic tools (Rachek et al., 1998; Troyer et al., 1998). This finding complicates every step of this unit. Spontaneous Rifampicin resistant bacteria cannot be eliminated from clone candidacy until PCR screening in Basic Protocol 4. Currently, only two solutions are available to account for the existence of these contaminating bacteria. The first method is simply a numbers game. Since this unit describes the isolation of many independent clones, it is the hope that at least one clone will contain the desired DNA, even if spontaneous Rifampicin-resistant bacteria are also isolated. The second solution is to frequently restart and complete the methods described in this protocol to ensure eventual success.

A confounding factor in all bacterial genetics is the presence of restriction/modification systems (Matic et al., 1996). These bacteria-encoded systems are defense mechanisms whereby the bacterium will digest DNA of foreign origin. To date, no restriction/modification systems have been described in *Rickettsia*, but the bacteria do contain genes with limited homology to restriction systems found in other bacteria. *Rickettsia* also likely scavenges all of the building blocks of DNA from the host cell (Andersson et al., 1998), so the bacteria are constantly exposed to foreign DNA. While exogenous DNA can be transformed into multiple *Rickettsia* species, the current efficiency of transformation is low. More efficient *Rickettsia* genetics will develop only after analysis of these putative restriction/modification systems and development of protocols for more efficient transformation.

Yet another difficulty associated with *Rickettsia* genetics is the potential for loss of virulence after repeated passage outside of a mammalian host (Perez Gallardo and Fox, 1948). Indeed, completion of this unit requires 3–10 passages of the bacteria in tissue culture cells. This process can potentially attenuate the bacteria (Perez Gallardo and Fox, 1948). In some cases, the bacteria can be cultured within egg yolk sacks to re-introduce infectivity. However, we must be extremely careful with this process; if the genetic manipulation affects the fitness of the bacteria in any way, then introduction into an egg yolk sack or animal system will actually select for secondary mutations that restore the infectivity of the mutants. It is suggested that transformation is done on *Rickettsia* sp. recently passed through a vertebrate host or arthropod vector.

Anticipated Results—Completion of this unit will result in antibiotic-resistant *Rickettsia* clones containing the DNA of choice. These bacteria can now be analyzed for phenotypic changes associated with the genetic manipulation.

Time Considerations—This unit describes a series of extremely time-consuming protocols. Even prior to performing Basic Protocol 1, at least a month is needed to create the specific transforming DNA and a full propagation of most *Rickettsia* species will take two weeks. The other protocols are expected to take:

Basic Protocol 1: Transformation can be completed in a single day, but selection and outgrowth takes between 9–21 days.

Basic Protocol 2: Sorting and outgrowth of clones takes 11 days; one day to purify and sort, five days for the initial propagation, and five more days for the replica plate propagation.

Alternate Protocol 1: Performance of limiting dilution takes approximately the same amount of time as Basic Protocol 2. The dilution takes one day with 10 days for complete outgrowth. However, limiting dilution must be repeated thrice to ensure clonality.

Alternate Protocol 2: Outgrowth from the initial seed until detection of plaques takes 5–7 days. This is followed by 10 days of outgrowth in 96-well plates. Again, plaque isolation must be performed thrice to ensure clonality.

Basic Protocol 3 and 4: These protocols can and should be completed on two successive days.

Basic Protocol 5: Outgrowth of the bacteria will take 7–10 days, but the freezing protocol only takes approximately one hour.

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Figure 1.
Schematic flow of methods

Figure 2. Heat map of uninfected Vero lysate (left) and *Rickettsia*-infected Vero lysate (right) with the approximate *Rickettsia* positive sort gate.

Table 1

Factors influencing choice of clonal isolation protocol

	Positives	Negatives
Cell Sorting	Speed of results Ensured clonality	Extensive experience needed Large equipment cost Biosafety concerns
Plaque Assay	Well documented	No continuous selection Penetrance of non-transformed bacteria Need multiple rounds to ensure clonality Time Difficulty of techniques
Limiting Dilution	Easiest technique Continuous selection	No confidence in clonality Need for repeated isolation to ensure clonality