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Forward and Reverse Genetic Analysis of *Chlamydia*

Mateusz K dzior, Robert J. Bastidas*

Department of Molecular Genetics and Microbiology, Duke University, Durham, N.C, USA

Abstract

Chlamydia is a major etiological agent of human disease that affects millions of individuals worldwide. Historically, our understanding of the mechanisms that contribute to its pathogenesis has been limited. However, the recent development of powerful genetic tools for manipulating *Chlamydia* has resulted in significant gains in our ability to dissect its virulence mechanisms. These tools have overcome several barriers for manipulating intracellular pathogens and are amenable for the routine genetic engineering of *Chlamydia*. Here, we provide several detailed protocols for performing genetic analysis in *Chlamydia trachomatis* allowing investigators to elucidate how this obligate intracellular pathogen causes disease.

Keywords

Chlamydia genetics; *Chlamydia* chemical mutagenesis; *Chlamydia* DNA isolation; *Chlamydia* linkage analysis; *Chlamydia* plaque assay; *Chlamydia* plasmid transformation

Introduction

Chlamydia is an obligate intracellular pathogen that survives and thrives within human cells and is a major cause of sexually transmitted human disease [1–3]. Its life cycle alternates between an infectious elementary body (EB) and a vegetative, non-infectious reticulate body (RB), which replicates within the confines of a membrane bound pathogenic vacuole called the inclusion [4]. Understanding the mechanisms by which *Chlamydia* infections lead to pathogenesis is a topic of active investigation, and in the last few years, several factors important for its virulence and intracellular parasitism have been identified in part due to the development of genetic based approaches tailored for use in *Chlamydia*.

Several groups have harnessed chemical mutagenesis to generate diverse populations of *Chlamydia* mutant strains [5–9] that have been screened by TILLING [10] and Whole Genome Sequencing (WGS) based approaches. These screens have led to the identification of a *C. trachomatis* virulence factor (PmpD), a transcription regulator (ChxR) of virulence-associated genes, and a metabolic factor (TrpB) required by *C. trachomatis* for avoiding IFN- γ -induced tryptophan starvation [5, 11, 12]. In addition, over 8,000 single nucleotide variants, including nonsense inducing mutations in 80 *C. trachomatis* L2 ORF's, have been identified and mapped [7]. Forward genetic screens have also led to the discovery of *C. trachomatis* gene products required for *Chlamydia*'s intracellular growth (GlgB and GspE)

*Corresponding author. robert.bastidas@duke.edu.

[6], survival (CpoS) [13], and maintenance of its intracellular niche (CPAF, InaC) [7, 14]. Likewise, temperature sensitive conditional mutations [8] and *C. trachomatis* genes required for its survival during IFN- γ -driven persistence [15] have been discovered from forward genetic screens.

Tools for targeted genetic analysis have also been developed and are particularly useful for validating candidate loci identified from genetic screens. These include the use of a *C. trachomatis* adapted type II intron (TargeTron[®], Sigma Inc) for targeted insertional mutagenesis [16, 17] and targeted gene replacement by Fluorescence Reported Allelic Exchange Mutagenesis (FRAEM) [18]. An upgrade to the FRAEM approach with a Cre-*loxP* system now allows for recycling of selectable markers during mutagenesis and disruption of individual genes within operons without incurring polar effects on transcription of downstream genes [19].

The development of tools for genetic analysis of *Chlamydia* now enables investigators to systematically map virulence factors in any *Chlamydia* species without major investments in time and resources. The genomes of *Chlamydia* species are relatively small (~ 1 Mb) and mutations introduced by chemical mutagens into their genomes can be readily mapped with Next Generation Sequencing technologies. *Chlamydia* genetic exchange has been demonstrated in a coinfection setting with two different antibiotic resistant strains giving rise to dual resistant recombinant progeny [20, 21]. Thus, lateral gene transfer events between wild type and mutant strains allows strong associations to be made between segregating mutations in a gene or genetic pathway and a phenotype. Genetic associations can be verified by either TargeTron or FRAEM based targeted mutagenesis. Here, we describe a methodology for performing genetic analysis in *Chlamydia* by both forward and reverse genetic approaches. In the following sections, we provide detailed protocols for generating populations of chemically mutagenized *Chlamydia*, mapping and linkage analysis of chemically induced mutations in strains of interest, and protocols for targeted mutagenesis that can be used to validate genetic linkages.

Note: *Chlamydia trachomatis* is a Biosafety Level 2 (BSL2) designated organism and handling should be performed according to BSL2 safety guidelines.

2. Materials

2.1 Chemical mutagenesis

1. Vero cells (ATCC[®] CCL-81).
2. Cell culture flasks for routine growth of Vero cells, 25 cm² cell culture flasks, and 6-well cell culture plates.
3. *C. trachomatis* L2 434 Bu rifampin resistant (Rif^R) strain [6].
4. High-glucose Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and sodium pyruvate, ThermoFisher Scientific Inc.
5. Fetal Bovine Serum (FBS), ThermoFisher Scientific Inc.
6. Ethyl methanesulfonate (EMS).

7. 1M NaOH.
8. Phosphate buffered saline (PBS) supplemented with 0.9 mM calcium chloride and 0.49 mM magnesium chloride.
9. Cycloheximide (100 mg/mL in DMSO), Sigma-Aldrich Inc.
10. UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher Scientific Inc.
11. Sucrose-Phosphate-Glutamic acid (SPG) buffer (5X): 1.25 M sucrose, 50 mM sodium phosphate, 25 mM *L*-glutamic acid, pH 7.4. Filter sterilize. Store at 4 °C.
12. Sucrose-Phosphate-Glutamic acid (SPG) buffer (1X): Dilute 5X stock with distilled water to 1X concentration and filter sterilize. Store at 4 °C.
13. DMEM supplemented with sodium bicarbonate. Dissolve 13.5 g of DMEM high glucose, pyruvate, *L*-glutamine powder (ThermoFisher Scientific Inc.) and 1.85 g of sodium bicarbonate in 500 mL of distilled water to obtain 2X DMEM supplemented with 44 mM sodium bicarbonate, filter sterilize, and store at 4 °C.
14. MEM Non-Essential Amino Acids (100X), Sigma-Aldrich Inc.
15. Gentamicin solution (50 mg/mL), ThermoFisher Scientific Inc.
16. Ofloxacin (1 mg/mL). Dissolve in 1 N NaOH, aliquot into single use aliquots, and store at –20 °C.
17. SeaKem® LE agarose (1.5% w/v), Lonza. Dissolve 7.5 g of agarose in 500 mL of distilled water, autoclave, and store at 4 °C. Melt agarose in a microwave prior to use.

2.2 Isolation of *Chlamydia* enriched DNA

1. Vero cells (ATCC® CCL-81).
2. Cell culture flasks for routine growth of Vero cells and 6-well culture plates.
3. Chemically mutagenized *Chlamydia* strains.
4. High-glucose Dulbecco's Modified Eagle Medium (DMEM) with *L*-glutamine and sodium pyruvate, ThermoFisher Scientific Inc.
5. Fetal Bovine Serum (FBS), ThermoFisher Scientific Inc.
6. UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher Scientific Inc.
7. Sucrose-Phosphate-Glutamic acid (SPG) buffer (5X): 1.25 M sucrose, 50 mM sodium phosphate, 25 mM *L*-glutamic acid, pH 7.4. Filter sterilize. Store at 4 °C.
8. Sucrose-Phosphate-Glutamic acid (SPG) buffer (1X): Dilute 5X stock with distilled water to 1X concentration and filter sterilize. Store at 4 °C.
9. DNase I, New England Biolabs.
10. DNeasy Blood and Tissue Kit, Qiagen Inc.
11. Qubit dsDNA BR Assay Kit, ThermoFisher Scientific Inc.

12. Qubit fluorometer, ThermoFisher Scientific Inc.

2.3 Mapping single nucleotide variants by Whole Genome Sequencing.

1. Adaptive Focused Acoustics S220 instrument (Covaris) or NEBNext dsDNA Fragmentase (New England Biolabs).
2. Library prep kit (e.g. NEBNext Ultra II DNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina, New England Biolabs) and sequencing instrument (e.g. MiSeq, Illumina Inc.).

2.4 Linkage analysis

1. Vero cells (ATCC® CCL-81).
2. Cell culture flasks for routine growth of Vero cells and 24-well cell culture plates.
3. High-glucose Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and sodium pyruvate, ThermoFisher Scientific Inc.
4. Fetal Bovine Serum (FBS), ThermoFisher Scientific Inc.
5. Titered *C. trachomatis* Rif^R mutant strain and a titered *C. trachomatis* Spec^R strain [6].
6. UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher Scientific Inc.
7. Sucrose-Phosphate-Glutamic acid (SPG) buffer (5X): 1.25 M sucrose, 50 mM sodium phosphate, 25 mM L-glutamic acid, pH 7.4. Filter sterilize. Store at 4 °C.
8. Sucrose-Phosphate-Glutamic acid (SPG) buffer (1X): Dilute 5X stock with distilled water to 1X concentration and filter sterilize. Store at 4 °C.

2.5 Clonal isolation of strains by plaque assay

1. Vero cells (ATCC® CCL-81).
2. Cell culture flasks for routine growth of Vero cells, 6-well, and 96-well cell culture plates.
3. High-glucose Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and sodium pyruvate, ThermoFisher Scientific Inc.
4. Fetal Bovine Serum (FBS), ThermoFisher Scientific Inc.
5. Crude lysates from cells coinfecting with *C. trachomatis* Rif^R mutant strain and a *C. trachomatis* Spec^R strain [6] (from 3.4).
6. UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher Scientific Inc.
7. DMEM supplemented with sodium bicarbonate. Dissolve 13.5 g of DMEM high glucose, pyruvate, L-glutamine powder (ThermoFisher Scientific Inc.) and 1.85 g of sodium bicarbonate in 500 mL of distilled water to obtain 2X DMEM supplemented with 44 mM sodium bicarbonate, filter sterilize, and store at 4 °C.

8. MEM Non-Essential Amino Acids (100X), Sigma-Aldrich Inc.
9. Gentamicin solution (50 mg/mL), ThermoFisher Scientific Inc.
10. Rifampin (25 mg/mL). Dissolve in DMSO, aliquot into single use aliquots, and store at -20°C protected from light.
11. Spectinomycin (50 mg/mL). Dissolve in distilled water, filter sterilize, aliquot into single use aliquots and store at -20°C .
12. SeaKem® LE agarose (1.5% w/v), Lonza. Dissolve 7.5 g of agarose in 500 mL of distilled water, autoclave, and store at 4°C . Melt agarose in a microwave prior to use.
13. Sucrose-Phosphate-Glutamic acid (SPG) buffer (5X): 1.25 M sucrose, 50 mM sodium phosphate, 25 mM *L*-glutamic acid, pH 7.4. Filter sterilize. Store at 4°C .
14. Sucrose-Phosphate-Glutamic acid (SPG) buffer (1X): Dilute 5X stock with distilled water to 1X concentration and filter sterilize. Store at 4°C .

2.6 Genotyping recombinant progeny and linkage analysis

1. DNase Free distilled water.
2. Crude *C. trachomatis* lysates (from 3.5).
3. Primers designed to amplify each locus harboring a single nucleotide variant.
4. High fidelity PCR amplification reagents and thermocycler.

2.7 Reverse Genetic Analysis

1. Primers required for re-targeting the intron in pDFTT3-*aadA* for insertion at a specific genomic site (IBS, EBS1, and EBS2 primers). Re-targeting primers can be designed using software available at Targetronics, LLC (www.targetronics.com/targetron_pLtrB.php) (see Note 17).
2. *C. trachomatis* adapted Targetron vector pDFTT3-*aadA* [17] (see Note 18).
3. EBS universal primer: 5'-AATTAGAACTTGCGTTCAGTAAACACAACCTTATAC-3'.
4. High fidelity PCR amplification reagents and thermocycler.
5. QIAquick PCR Purification Kit, Qiagen Inc.
6. *HindIII* and *BsrGI*, restriction enzymes.

¹⁷Targetronics provides two different algorithms for designing targeting primers for LtrB and EcI5 introns. The LtrB intron in pDFTT3-*bla* and pDFTT3-*aadA* recognizes different target sequences than the EcI5 intron, and the appropriate algorithm (www.targetronics.com/targetron_pLtrB.php) should be used when designing re-targeting primers. Primer design requires a paid subscription (http://www.targetronics.com/targetron_buy.php). Design several primer sets. Choose primer sequences based on the highest probability of insertion (reported p-value) and predicted location of integration. Output primer sequences will contain *HindIII* and *BsrGI* restriction site sequences.

¹⁸pDFTT3-*aadA* [17] is a derivative of pDFTT3-*bla* [16] which contains a Group II intron expressing a *bla* cassette under the control of a *C. trachomatis* promoter. In pDFTT3-*aadA*, the *bla* cassette has been replaced with the spectinomycin resistance cassette *aadA*. Both plasmids are available upon request.

7. DNA cloning reagents.
8. Qubit dsDNA BR Assay Kit, ThermoFisher Scientific Inc.
9. Qubit fluorometer, ThermoFisher Scientific Inc.
10. Chloramphenicol (25 mg/mL). Dissolve in ethanol and protect from light.
11. Titered *C. trachomatis* wild type strain.
12. Transformation buffer (5X): 250 mM CaCl₂, 50 mM Tris, pH 7.4. Filter sterilize. Store at 4 °C.
13. UltraPure™ DNase/RNase-Free Distilled Water, ThermoFisher Scientific Inc.
14. Vero cells (ATCC® CCL-81).
15. Cell culture flasks for routine growth of Vero cells and 6-well cell culture plates.
16. High-glucose Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and sodium pyruvate, ThermoFisher Scientific Inc.
17. Fetal Bovine Serum (FBS), ThermoFisher Scientific Inc.
18. Spectinomycin (50 mg/mL). Dissolve in distilled water, filter sterilize, aliquot into single use aliquots and store at –20 °C.
19. Sucrose-Phosphate-Glutamic acid (SPG) buffer (5X): 1.25 M sucrose, 50 mM sodium phosphate, 25 mM L-glutamic acid, pH 7.4. Filter sterilize. Store at 4 °C.
20. Sucrose-Phosphate-Glutamic acid (SPG) buffer (1X): Dilute 5X stock with distilled water to 1X concentration and filter sterilize. Store at 4 °C.

3. Methods

3.1 Chemical mutagenesis

Currently, chemical mutagenesis remains as the only available tool for surveying *Chlamydia* gene function at a genome wide resolution due to the low transformation efficiency of *Chlamydia* with exogenous DNA. Chemical mutagens bypass this limitation and can yield large number of mutant strains containing a wide spectrum of mutations in their genomes. Mutagenized strains can be screened by defined assays for virulence traits either as pools or individually from arrays of individual clonal strains. The latter can be generated by plaque purification of clonal isolates our protocol described in section 3.5. In this protocol we describe a procedure for generating pools of chemically mutagenized *Chlamydia* strains. This protocol is based on a previously established protocol [22].

Note: Ethyl methanesulfonate (EMS) is a powerful mutagen and carcinogen. Use appropriate personal protective equipment. EMS is a volatile liquid and should always be handled in a chemical safety hood. All EMS exposed waste (solid and liquid) should be treated with 1M sodium hydroxide to neutralize EMS. Dispose of treated waste according to appropriate and approved procedures.

1. Seed Vero cells in DMEM supplemented with 10% FBS, at a density of 1×10^6 cells per well, in a 25 cm² flask, and culture overnight at 37 °C in a 5% CO₂ humidified incubator (see Note 1).
2. Use a titrated stock of rifampin resistant (Rif^R) *C. trachomatis* [6] and infect confluent Vero cell monolayers at a multiplicity of infection of 5 (see Note 2).
3. Spin down infected Vero monolayers at 3,500 rpm for 30 minutes at 10 °C to synchronize infection cycles (see Note 3).
4. Culture infected cell monolayers at 37 °C for 18 hours in a 5% CO₂ humidified incubator.
5. Prepare fresh 20 mg/mL EMS in PBS supplemented with calcium chloride and magnesium chloride.
6. Wash infected Vero cell monolayers with 3 mL of PBS supplemented with calcium chloride and magnesium chloride.
7. Add 3 mL of EMS solution (step 5) to Vero cell monolayers and incubate cells in a chemical safety hood at room temperature for 1 hour (see Note 1).
8. Remove EMS solution and wash cell monolayers 3 times with 3 mL of PBS supplemented with calcium chloride and magnesium chloride. All washes should be performed in a chemical safety hood. Treat all EMS exposed waste with 1 M sodium hydroxide.
9. Add 6 mL of DMEM supplemented with 10% FBS, 200 ng/mL cycloheximide, and 25 µg/mL gentamicin (see Note 4).
10. Culture infected cell monolayers at 37 °C for 72 hours in a 5% CO₂ humidified incubator.
11. Harvest bacteria from infected cells by quickly washing infected cell monolayers with 10 mL of UltraPure water. Lyse cells by adding 1 mL of UltraPure water and incubating cells for 20 minutes at room temperature with gentle agitation. Collect cell lysates in an Eppendorf tube and add 0.250 mL of 5X SPG buffer. Store lysates at –80°C (see Note 5).
12. Determine the titer of viable *C. trachomatis* strains.
13. Repeat procedure to establish independent pools of mutagenized *C. trachomatis* strains.

¹ Vero cells can tolerate high levels of EMS mutagenesis and can survive short periods of incubation outside of a humidified CO₂ incubator [22]. Follow proper sterile technique and approved safety procedures for handling cell cultures. All work should be done in a sterile laminar flow hood.

² We recommend performing EMS mutagenesis on a *Chlamydia* antibiotic resistant strain for facilitating downstream linkage analysis.

³ Centrifugation of *Chlamydia* is required for optimal and synchronized development of bacterial inclusions. Some mutant strains are unable to infect host cells in the absence of centrifugation.

⁴ Inhibition of eukaryotic protein translation by cycloheximide can increase recovery of slow growing mutant *Chlamydia* strains.

⁵ EMS mutagenesis will decrease recovery of viable bacteria. At 72 hours post EMS treatment, roughly 10% of *C. trachomatis* inclusions will have a wild type appearance while remaining inclusions will be devoid of bacteria.

14. Assess rates of mutagenesis by plaquing 7×10 -fold serial dilutions of lysates containing 1×10^8 EMS mutagenized *C. trachomatis* inclusion forming units in the presence of 4 µg/mL ofloxacin (see Note 6). See section 3.5 for plaquing protocol. Determine frequency of ofloxacin resistant *C. trachomatis* plaque forming units and divide by total number of bacteria plated.
15. Screen mutants for phenotypes of interest.

3.2 Isolation of *Chlamydia* enriched DNA

The relatively small size of *Chlamydia* genomes (~ 1 Mb) allows for physical mapping of single nucleotide variants by WGS in any mutant strain of interest. In this protocol, we describe a procedure for isolation and enrichment of *Chlamydia* DNA directly from crude lysates of Vero cells infected with a *Chlamydia* strain can yield several micrograms of DNA suitable for preparations of high-quality sequencing libraries for WGS.

1. Seed Vero cells in DMEM supplemented with 10% FBS, at a density of 6×10^5 cells per well, in all wells of two six well plates, and culture overnight at 37 °C in a 5% CO₂ humidified incubator (see Note 7).
2. Use a titrated stock of a *C. trachomatis* mutagenized strain of interest and infect confluent Vero cell monolayers at a multiplicity of infection of 3.
3. Spin down infected Vero monolayers at 3,500 rpm for 30 minutes at 10 °C to synchronize infection cycles (see Note 3).
4. Culture infected cell monolayers at 37 °C for 40-48 hours in a 5% CO₂ humidified incubator.
5. Assess efficiency of infection using an inverted light microscope. The majority of Vero cells should contain visible inclusions with mobile bacteria (see Note 8).
6. Harvest bacteria from infected cells by quickly washing infected cell monolayers with 3 mL of UltraPure water per well. Lyse cells by adding 400 µL of UltraPure water per well and incubating cells for 20 minutes at room temperature with gentle agitation. Collect cell lysates in Eppendorf tubes and centrifuge at 14,000 rpm for 15 minutes at 4 °C. Discard supernatants and resuspend bacteria containing pellet in 1 mL of 1X SPG buffer.
7. Store crude lysates at –80 °C or proceed with DNA enrichment and isolation.
8. Sonicate crude lysates for 10 seconds to promote complete lysis of host cells.
9. Centrifuge crude lysates at 14,000 rpm for 15 minutes at 4 °C and resuspend pellet in 100 µL of 1X NEB DNase I buffer.

⁶. If ofloxacin resistant *C. trachomatis* plaque forming units are not observed, determine ofloxacin minimum inhibitory concentration and repeat plaque assay.

⁷. Vero cells can be cultured for extended periods of time without passaging and are ideal for routine growth and transformation of *Chlamydia* mutant strains.

⁸. Vero cells occasionally form large vacuoles upon stressful conditions that resemble *C. trachomatis* inclusions. Typically, these vacuoles are devoid of moving bacteria. In some instances, *Chlamydia* mutant strains can also form inclusions with non-mobile bacteria.

10. Deplete host (Vero) DNA by adding 4 units of NEB DNase I and incubating at 37 °C for 30 minutes (*see* Note 9).
11. Add an additional 4 units of NEB DNase I and incubate at 37 °C for 30 minutes.
12. Remove DNase I by centrifuging crude lysates at 14,000 rpm for 15 minutes at 4 °C and removing supernatant.
13. Wash pellet with 0.5 mL of PBS (removal of carry over DNase I).
14. Centrifuge crude lysates at 14,000 rpm for 15 minutes at 4 °C and remove supernatant.
15. Resuspend pellet in 180 µL of buffer ATL (DNeasy Blood and Tissue Kit).

Remainder of protocol follows instructions from Qiagen DNeasy Blood and Tissue Kit: Purification of Total DNA from Animal Tissues. All reagents are included in the kit.

16. Add 20 µL of proteinase K. Mix by vortexing and incubate at 55 °C overnight (*see* Note 10).
17. Add 4 µL of RNase A (100 mg/mL), mix by vortexing and incubate for 2 minutes at room temperature.
18. Vortex for 15 seconds and add 200 µL of buffer AL. Mix thoroughly by vortexing and incubate at 70 °C for 10 minutes.
19. Add 200 µL of 100% ethanol and mix by vortexing.
20. Pipet mixture into a DNeasy spin column and centrifuge at 8,000 rpm for 1 minute.
21. Place column into new collection tube, add 500 µL of buffer AW1, and centrifuge at 8,000 rpm for 1 minute.
22. Place column into new collection tube, add 500 µL of buffer AW2, and centrifuge at 14,000 rpm for 3 minutes.
23. Place column into an Eppendorf tube, add 50 µL of buffer AW1, and centrifuge at 8,000 rpm for 1 minute.
24. Discard column and measure total DNA concentration (*see* Note 11).
25. Run a 5 µL aliquot on a 1 % agarose gel to assess integrity of isolated DNA.
26. Store isolated total DNA at –80 °C.

3.3 Mapping single nucleotide variants by Whole Genome Sequencing.

Several platforms can be used to sequence *Chlamydia* genomes. We routinely use the MiSeq (Illumina Inc.) platform which enables sequencing of up to 5 *Chlamydia* genomes at a time

⁹DNase I should only target host genomic DNA. *Chlamydia* EBs are osmotically stable and intact at this junction of the protocol.

¹⁰Overnight incubation is optimal for proteinase K induced lysis of EBs and degradation of residual DNase I. Alternatively, incubate for a minimum of 3 hours.

¹¹Accurate measurement of total DNA is critical for preparing high quality sequencing libraries. Quantify DNA by Qubit fluorometric quantification or with a Bioanalyzer.

by multiplexing. Output reads provides over 25X coverage, which is more than adequate for genome assembly. Below we describe a basic protocol for DNA sequencing library preparation and single nucleotide variant mapping.

1. Fragment DNA to appropriate size (400-600 b.p for Illumina based sequencing) using an Adaptive Focused Acoustics S220 instrument (Covaris Inc.) according to manufacturer's settings. Fragmentation can also be achieved using Fragmentase (New England Biolabs) according to manufacturer's instructions.
2. Genomic sequencing library preparations can be constructed using commercially available kits (see Note 12). Libraries can be indexed using multiple barcoded primers, pooled, and sequenced simultaneously.
3. Sequencing is usually performed by core facilities or commercial services.
4. Illumina FASTQ reads can be used for reference-based assembly and single nucleotide variant mapping by using open source genome assemblers such as MAQ [23] and BWA [24]. Alternatively, user friendly commercial software programs are also available such as Geneious Prime from Biomatters Ltd. (<https://www.geneious.com>).

3.4 Linkage analysis

Chemical mutagenesis can lead to the accumulation of multiple single nucleotide variants in the genomes of mutagenized strains. To identify causal variants in *Chlamydia* mutant strains, we exploit the high frequency at which *Chlamydia* strains exchange DNA [20, 21]. Coinfection of host cells with two antibiotic resistant strains leads to production of recombinant progeny that are selected for by isolating strains exhibiting dual antibiotic resistance [20, 21]. Recombinant strains can then be screened for segregating variants that are linked to a phenotype. In this approach, chemical mutagenesis is performed in a rifampin resistant (Rif^R) *C. trachomatis* L2 parental strain [6]. Mutants of interest are then coinfecting with a spectinomycin resistant (Spec^R) *C. trachomatis* L2 strain [6] according to the protocol detailed below.

1. Seed Vero cells in DMEM supplemented with 10% FBS, at a density of 5×10^4 cells per well in 1 well of a twenty-four well plate. Culture overnight at 37 °C in a 5% CO₂ humidified incubator (see Note 7).
2. Use a titrated *C. trachomatis* Rif^R mutant strain and a titrated *C. trachomatis* Spec^R strain [6] to coinfect the confluent Vero cell monolayer at a multiplicity of infection of 2 for each strain.
3. Spin down infected Vero cells at 3,500 rpm for 30 minutes at 10 °C to synchronize infection cycles (see Note 3).
4. Culture the infected cell monolayer at 37 °C for 36-40 hours in a 5% CO₂ humidified incubator.

12. Sequencing library preparation kits from New England Biolabs (NEB) and from Kapabiosystems yield high quality sequencing libraries that are compatible with Illumina sequencing platforms.

5. Assess infection efficiency using an inverted light microscope. The majority of Vero cells should contain visible inclusions with mobile bacteria (*see* Note 8).
6. Harvest bacteria from infected cells by quickly washing infected cell monolayer with 1 mL of UltraPure water. Following wash, lyse cells by adding 400 μ L of UltraPure water and incubating cells for 20 minutes at room temperature with gentle agitation. Add 100 μ L of 5X SPG buffer and store crude lysates at -80°C or proceed with clonal isolation of doubly antibiotic resistant strains.

3.5 Clonal isolation of strains by plaque assay

A plaque assay enables the isolation of a clonal bacterial population derived from a single EB. In this protocol, we utilize a plaque assay to isolate individual doubly antibiotic resistant recombinant progeny strains. Clonal isolation by limiting dilution is an alternative assay that can also be employed. A detailed protocol has been previously published [25] and will not be discussed here.

1. Seed Vero cells in DMEM supplemented with 10% FBS, at a density of 6×10^5 cells per well, in all wells of two six well plates, and culture overnight at 37°C in a 5% CO_2 humidified incubator (*see* Note 7).
2. Infect confluent Vero cell monolayers in one plate with 10 μ L of $6 \times 1:10$ serial dilutions of crude cell lysates from co-infected cells. In the second plate, infect cell monolayers in one well with the Rif^R mutant strain, and cell monolayers in a second well with the Spec^R strain. Infect each strain at a multiplicity of infection of 2 (*see* Note 13).
3. Spin down the infected cell monolayers at 3,500 rpm for 30 minutes at 10°C to synchronize the infection cycles (*see* Note 3).
4. Culture infected cell monolayers at 37°C for **2 hours** in a 5% CO_2 humidified incubator to allow for bacterial internalization.
5. Remove growth medium and add 6 mL of DMEM/agarose overlay media (Table 1) per well (*see* Note 14).
6. Cool plates outside of laminar flow hood at room temperature for 10 minutes (*see* Note 15).
7. Place plates inside laminar flow hood, remove lids, and dry overlays for 15 minutes.
8. Culture the cells at 37°C for 10-14 days in a 5% CO_2 humidified incubator until plaques become visible by light microscopy.

¹³. Infection with the antibiotic resistant strains serves as a control for antibiotic selection efficacy. Plaques should not be observed if antibiotics are active during plaque assay.

¹⁴. Agarose cools and solidifies quickly. To avoid solidification during preparation of overlay, remove growth medium from infected Vero cell monolayers prior to melting agarose. Boil agarose in a microwave oven and quickly add to DMEM/agarose overlay already containing all other components (Table 1). Do not let agarose cool. Mix and pour immediately.

¹⁵. Airflow inside laminar flow hood causes surface vibrations in the DMEM/agarose overlay which disturbs solidification of the overlay.

9. Select a well with clearly separated plaques and using an inverted light microscope and a permanent ink pen, circle 24 *Chlamydia* bearing plaques for harvesting and expansion in Vero cells.
10. Seed a 96-well plate with Vero cells at a density of 2×10^4 cells per well and culture overnight at 37 °C in DMEM supplemented with 10% FBS.
11. When cells are fully confluent, replace growth medium with DMEM supplemented with 10% FBS and 200 ng/mL rifampin plus 150 µg/mL spectinomycin.
12. Core individual plaques using a p1000 pipette tip and transfer to one well of confluent Vero cell monolayers seeded in a 96-well plate (*see* Note 16).
13. Spin down infected Vero cells at 3,500 rpm for 30 minutes at 10 °C to synchronize the infection cycles.
14. Culture the infected cells at 37 °C for 48 hours in a 5% CO₂ humidified incubator.
15. Harvest monoclonal *Chlamydia* populations from infected cells in each well of the 96-well plate by washing cell monolayers with 200 µL of UltraPure water per well. Lyse cells by adding 80 µL of UltraPure water per well and incubating cells for 20 minutes at room temperature with gentle agitation. Add 20 µL of 5X SPG buffer and store crude lysates at –80 °C.
16. Save a small aliquot of crude lysate for segregation analysis of single nucleotide variants by PCR.

3.6 Genotyping recombinant progeny and linkage analysis

1. Prepare a 1:10 dilution of crude lysates from infected cells with DNase free distilled water.
2. Use 1 µL of diluted crude lysate as template in a standard PCR reaction containing primers designed to amplify each locus harboring a single nucleotide variant (PCR amplicon should be between 600 and 1,000 b.p in length for optimal amplification).
3. During PCR cycling, include an initial step at 94 °C for 10 minutes for lysing *Chlamydia* and exposing DNA.
4. Verify if each amplified locus harbors a single nucleotide variant by Sanger sequencing.
5. Screen each recombinant strain for desired phenotype and use genotyping results to establish genetic linkage.

¹⁶.To core a desired marked plaque, use a p1000 pipette with an attached 1 mL sterile tip, align the tip perpendicularly to the plate surface, and insert into the agarose/DMEM overlay directly over the plaque until reaching the bottom of the plate. Gently pipet up and down and transfer the agarose plug to a well of a 96-well plate containing a confluent Vero cell monolayer. Resuspend the plug briefly in the growth medium by pipetting up and down.

3.7 Reverse genetics analysis

Genetic links can be validated by generating an independent *Chlamydia* strain harboring a single null allele of the locus exhibiting linkage in an otherwise wild type background. For single nucleotide variants residing in essential genes, linkage can be validated by complementing recombinant strains with *C. trachomatis*-ColE1 shuttle vectors [26] expressing a wild type ORF under native or inducible promoters. Null alleles can be generated by gene replacement using Fluorescence-Reported Allelic Exchange Mutagenesis (FRAEM) [18]. A detailed protocol describing gene exchange by FRAEM in *Chlamydia* has previously been published [25] and will not be mentioned here. Null alleles within operons can be generated without incurring polar effects on downstream genes by utilizing a modified Cre-*loxP*FRAEMs approach [19]. Here, we describe a protocol for generating loss of function alleles in non-essential ORFs using a *C. trachomatis* adapted Group II intron (TargeTron system) [17].

1. Design IBS, EBS1 , and EBS2 re-targeting primers (*see Note 17*).
2. Prepare 100 μ M working stocks of IBS and EBS1 re-targeting primers and 20 μ M working stocks of EBS2 and universal EBS primers (*see section 2.6 for EBS primer sequence*).
3. Combine 2 μ L of IBS primer stock, 2 μ L of EBS1 primer stock, 2 μ L of EBS2 primer stock, 2 μ L of EBS universal primer stock, and 12 μ L of UltraPure water.
4. Generate re-targeting DNA fragments by mixing 1 μ L of the four-primer master mix prepared in step 3 and 1 μ L of pDFTT3-*aadA* plasmid DNA [17] (~50 ng/ μ L) (*see Note 18*) in a standard high fidelity PCR reaction mix. Amplify fragments by PCR based amplification.
5. Verify amplification by gel electrophoresis. Re-targeting amplicons should be 350 b.p in size.
6. Isolate re-targeting fragments by gel extraction.
7. Digest re-targeting amplicons and pDFTT3-*aadA* plasmid with *Hind*III and *Bsr*GI.
8. Purify digested products using a QIAquick PCR Purification Kit.
9. Measure DNA concentrations of each purified sample.
10. Ligate digested re-targeting amplicons and digested pDFTT3-*aadA* and transform into competent *E. coli* cells. Select transformants by plating cells on LB-agar supplemented with 25 μ g/mL chloramphenicol. Protect plates from light (chloramphenicol is light sensitive) and incubate at 37 °C until colonies are observed.
11. Screen for plasmids containing the re-targeted 350 b.p insert in pDFTT3-*aadA*.

The next step consists of transforming *Chlamydia* with suicide TargeTron vectors to generate null alleles. The transformation protocol described below can also be used to transform FRAEM based suicide plasmids and *Chlamydia* shuttle plasmids for complementation analysis.

12. Seed Vero cells in DMEM supplemented with 10% FBS, at a density of 6×10^5 cells per well, in all wells of two six well plates, and culture overnight at 37 °C in a 5% CO₂ humidified incubator.
13. Mix 1×10^6 *C. trachomatis* IFUs, 10 µg of re-targeted pDFTT3-*aadA* plasmid DNA, 50 µL of transformation buffer, and UltraPure water up to 250 µL (*see* Note 19).
14. Prepare a mock transformation control by repeating step 2 and replacing pDFTT3-*aadA* plasmid DNA with UltraPure water.
15. Incubate transformation mixtures for 30 minutes at room temperature.
16. Use entire transformation mixture to infect confluent Vero cell monolayers seeded in all wells of a six well plate. Distribute transformation mixture equally among all wells.
17. Spin down infected Vero cell monolayers at 3,500 rpm for 30 minutes at 10 °C to synchronize the infection cycles.
18. Culture infected cell monolayers at 37 °C for 12-14 hours in a 5% CO₂ humidified incubator.
19. Replace growth medium with DMEM supplemented with 10% FBS and 150 µg/mL spectinomycin (*see* Note 20).
20. Culture cell monolayers at 37 °C for an additional 28-34 hours in a 5% CO₂ humidified incubator.
21. Harvest bacteria by quickly washing infected cell monolayers with 3 mL of UltraPure water. Lyse cells by adding 400 µL of UltraPure water per well and incubating cells for 20 minutes at room temperature with gentle agitation. Collect cell lysates in Eppendorf tubes and centrifuge at 14,000 rpm for 15 minutes at 4 °C. Discard supernatants and resuspend pellet in a total of 1 mL of 1X SPG buffer. Use lysates immediately in step 11. Do not freeze or store.
22. Replace growth medium on Vero cell monolayers previously seeded as described in step 1 with DMEM supplemented with 10% FBS and 150 µg/mL spectinomycin. Infect cell monolayers with crude cell lysates from step 10. Distribute entire cell lysates equally among all wells.
23. Spin down the infected Vero cells at 3,500 rpm for 30 minutes at 10 °C to synchronize the infection cycles.
24. Culture infected cell monolayers at 37 °C for 40-48 hours in a 5% CO₂ humidified incubator (*see* Note 21).

¹⁹. *Chlamydia* preparations from crude cell lysates of infected host cells exhibit higher transformation efficiencies relative to gradient purified EB preparations. Plasmid DNA isolated from common *E. coli* laboratory strains such as DH5alpha is perfectly suitable for transformation.

²⁰. Determine optimal antibiotic concentration for selection by performing a growth curve assay.

²¹. This step is considered passage number 1.

25. Check for presence of inclusions using an inverted light microscope. If inclusions are not observed, repeat steps 10–13 until inclusions are observed (*see* Notes 8 and 22).
26. Once inclusions are observed, harvest bacteria as described in step 10 and store at -80°C (*see* Note 23).
27. Verify integration of Group II intron at targeted locus (*see* Note 24).
28. Isolate clonal populations by plaque purification and complement.

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²²-Lack of observable inclusions is typical at passage number 1. Repeat passaging infected cells at most until passage number 6. If inclusions are not observed by then, repeat transformation.

²³-Presence of inclusions in the mock control can result from growth of spontaneously spectinomycin resistant *C. trachomatis* strains. In this case, repeat transformation with new source of *Chlamydia* parental strain.

²⁴-Determine integration of Group II intron by PCR genotyping (*see* Figure 1) and single genomic integration by Southern blot analysis.

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Fig. 1. PCR based strategy for verifying insertional inactivation of an ORF by TargetTron based mutagenesis.

A. Use a gene specific forward primer and an intron specific reverse primer (e.g., EBS universal primer) to amplify across the 5' gene-intron junction. **B.** Use an intron specific forward primer (e.g., EBS2 primer) and a gene specific reverse primer to amplify across the 3' gene-intron junction. **C.** Use gene specific primers flanking the insertion site to verify intron insertion and ORF disruption. **D.** Use *aadA* cassette specific primers to detect the spectinomycin resistance marker.

Table 1:

Recipe for DMEM/agarose overlay media (*see* section 2.1 for preparation of reagents).

Reagent	Final concentration
2X DMEM with Sodium Bicarbonate	1X
FBS	10%
MEM Non-Essential Amino Acids (100X)	1X
Gentamycin solution (50 mg/mL)	0.025 mg/mL
Rifampin (25 mg/mL)	200 ng/mL
Spectinomycin (50 mg/mL)	150 µg/mL
UltraPure water	Up to final volume
SeaKem® Agarose (1.5% w/v)	0.54%