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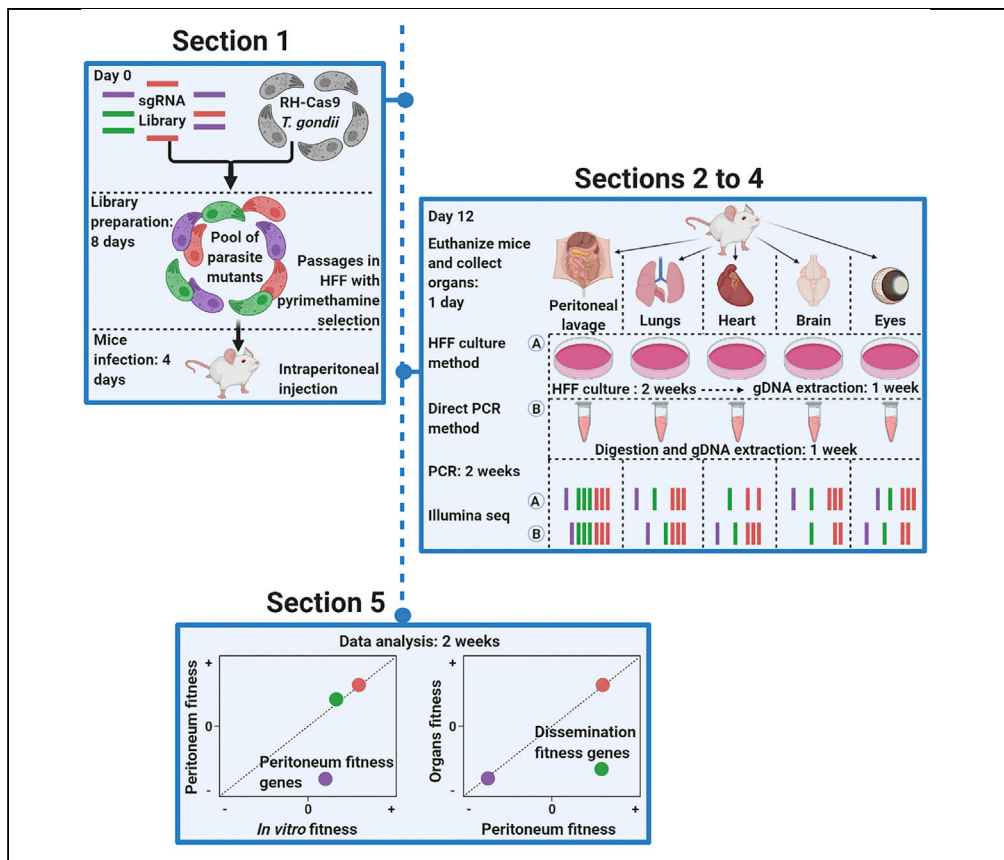
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Protocol

CRISPR screen to determine the *in vivo* fitness of *Toxoplasma* genes



The virulence of eukaryotic parasites like *Toxoplasma gondii* depends on their capacity to escape from the host immune response and disseminate throughout the host organism. However, *Toxoplasma* gene products essential for its *in vivo* pathogenesis remain uncharacterized. Here, we present the complete workflow of a CRISPR-Cas9 *in vivo* loss-of-function screen to identify *Toxoplasma* fitness-conferring genes. This protocol can be used to uncover gene products that play a role in *Toxoplasma* immune evasion, nutrient acquisition, dissemination, and tissue colonization.

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Highlights
Generation of a
Toxoplasma gondii
mutant library with
CRISPR-Cas9

Intra-peritoneal
injection of mice with
mutant library

Collection of infected
organs, genomic
DNA isolation, and
amplification of
sgRNAs

Analysis of Illumina
sequencing reads
and calculation of
gene fitness scores

Protocol

CRISPR screen to determine the *in vivo* fitness of *Toxoplasma* genes

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SUMMARY

The virulence of eukaryotic parasites like *Toxoplasma gondii* depends on their capacity to escape from the host immune response and disseminate throughout the host organism. However, *Toxoplasma* gene products essential for its *in vivo* pathogenesis remain uncharacterized. Here, we present the complete workflow of a CRISPR-Cas9 *in vivo* loss-of-function screen to identify *Toxoplasma* fitness-conferring genes. This protocol can be used to uncover gene products that play a role in *Toxoplasma* immune evasion, nutrient acquisition, dissemination, and tissue colonization.

For complete details on the use and execution of this protocol, please refer to Sangaré et al. (2019).

BEFORE YOU BEGIN

The following table (Table 1) lists critical resources: mouse strain, cell line, parasite strain, culture media, and plasmid need to be ordered before starting this protocol.

Cell culture and mice ordering

⌚ Timing: 1–2 h (does not include the time for cell culture or expected delivery time of the mice)

One week before Day 0

1. Prepare fifty 150-mm dishes (150 × 25 mm) with 25 mL of HFF medium and five 100-mm dishes (100 × 15 mm) with 10 mL of medium containing human foreskin fibroblasts (HFFs) and let them form a confluent monolayer. The dishes will be used for the inoculation with organ suspensions and peritoneal lavage from infected mice at step 46 in section 3.
2. Prepare three 24-well plates containing HFFs (with 1 mL of HFF medium) and let them form a confluent monolayer. These plates will be used to determine parasite viability before, after electroporation, and before mice's infection.
3. Order fifteen CD1 female mice at age six to seven weeks old. All fifteen mice will be intraperitoneally injected in section 2; however, only ten mice will be used for genomic DNA extraction in section 3.

⚠ **CRITICAL:** Order six to seven-week-old mice at least one week before the library preparation. At the time of infection, the mice will be eight to nine weeks old. Consider



Table 1. Critical resources needed before beginning

Resources	Details
Human Foreskin Fibroblasts	Passage number between 8 to 15
HFF culture media	DMEM containing 10% FBS, 2 mM L-Glutamine, 100 µg/mL of Penicillin/Streptomycin
RH-Cas9 <i>T. gondii</i> (HA-FLAG tagged/ chloramphenicol acetyltransferase resistant)	(Sidik et al., 2016)
Parasite culture media	DMEM containing 1% FBS, 2 mM L-Glutamine, 100 µg/mL of Penicillin/Streptomycin, 40 mg/mL Chloramphenicol (M1 medium)
pU6-DHFR + GRA sgRNAs library	See Data and Code Availability
CD1 mice	Order fifteen CD1 female mice at age six to seven weeks old

More details for these resources can be found in the [key resources table](#).

requirements for acclimatization time. Young mice are more susceptible and will die too early after injecting a high dose of parasites, which will affect parasite dissemination.

Two days before Day 0

- Use freshly lysed out RH-Cas9 *Toxoplasma gondii* (*T. gondii*) parasites from three T25 flasks to infect two 150-mm dishes of HFFs (with 25 mL of parasite culture medium) and allow them to replicate. These parasites should be ready to lyse out on Day 0 and be used for transfection in section 1.

△ **CRITICAL:** The RH-Cas9 *T. gondii* parasites can be kept in culture for more than a month as long as they are in the parasite culture medium with Chloramphenicol (M1 medium).

- Prepare 200 µg of pU6-DHFR + GRA sgRNAs library from bacteria liquid culture.

Plasmid linearization

⌚ **Timing:** 5 h (does not include the bacteria culture for plasmid isolation).

One day before Day 0

- In two 1.5 mL Eppendorf tubes, linearize 200 µg (100 µg each tube) of sgRNA-containing plasmid with restriction enzyme AseI for 3 h at 37°C (Table 2).
 - Inactivate the enzyme by incubating the samples at 65°C for 5 min.
 - Fill two 100-mm dishes with 20 mL (each) of UltraPure water and lay the dialysis membranes on the liquid surface (floating).
 - Pipette the digestion product (~100 µL) directly onto the middle of the floating dialysis membrane.
 - After 20 min, retrieve the digestion product in a new 1.5 mL Eppendorf tube.
 - Measure the DNA concentration.
 - Sterilize the linearized plasmid by incubating the sample at 65°C for 10 min.

Table 2. Digestion reaction to linearize the sgRNA-containing plasmid (pU6-DHFR + GRA sgRNAs library)

Reagent	Amount per reaction	Final concentration
sgRNA-containing plasmid	100 µg	1 µg/µL
AseI	50 units	0.5 unit/µL
10× NE Buffer (3.1)	10 µL	1×
UltraPure water (DNase free)	up to 100 µL	n/a

△ **CRITICAL:** It is crucial to remove the salt from the restriction enzyme buffer because it can affect the CytoMix composition and lead to electric arc and decreased transfection efficiency. Therefore, it is necessary to dialyze using a commercial pre-cut membrane (see reference in the [key resources table](#)). It is expected that 20 to 30% of the initial DNA amount will be lost.

CytoMix buffer and cell culture medium

To prepare CytoMix buffer, combine the reagents in order as listed in [Table 3](#). Adjust pH to 7.6 using KOH and sterilize using a 0.2 µm filter. It can be stored at 4°C for up to three months. The ATP and GSH stocks should be stored at –20°C. Complete CytoMix is obtained by adding fresh ATP (2 mM final concentration) and GSH (5 mM final concentration) to the volume of CytoMix stock needed for transfection. Further details on CytoMix preparation can be found in *Toxoplasma gondii* in Methods and Protocols: (Tonkin, 2020). For cell culture media (M1 and M2-M3) preparation, please refer to [Table 4](#) and [Table 5](#), respectively.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: cell lines		
Human foreskin fibroblasts	A gift from John Boothroyd	N/A
Experimental models: organisms/strains		
RH-Cas9 <i>T. gondii</i> (HA-FLAG tagged/ chloramphenicol acetyltransferase resistant)	(Sidik et al., 2016)	N/A
CD1 mice	Charles River Laboratories	Strain Code: 022
Recombinant DNA		
pU6-DHFR (sgRNA-containing plasmid)	Addgene	Plasmid#80329
Pooled library containing pU6-DHFR	Addgene	Plasmid#80636
pU6-DHFR + GRA sgRNAs library	(Sangaré et al., 2019)	See Data and Code Availability
Critical commercial assays		
T25 Flask cell culture	Fisher	Cat#353108
Tissue culture dish 100 × 20 mm	Corning	Cat#353003
Tissue culture dish 150 × 25 mm	Fisher	Cat#353025
24-Well plate	Fisher	Cat#353047
Asel - 2,000 units (10,000U/mL)	NEB	Cat#R0526S
Molecular Biology Grade Water (UltraPure water, DNase free)	Corning	Cat#46-000-CV
Dialysis membrane	Millipore	Cat#165-2089
5 mL Syringe	BD	Cat#309646
Needle 27 G	Fisher	Cat#305109
Needle 30 G	Fisher	Cat#305106
Cuvettes 0.2 mm Gene Pulser	Bio-Rad	Cat#1652086
DNaseI	Sigma-Aldrich	Cat#DN25-100MG
Reagent Reservoir (50 mL)	Genesee	Cat#28-125
Single Edge Blades	AccuTec	Cat#61-0045
70 µm Cell strainer	Sigma-Aldrich	Cat#CLS431751
DNeasy Blood and Tissue Kit	QIAGEN	Cat#69504
Q5 High-Fidelity DNA Polymerase - 500 units	NEB	Cat#M0491L
QIAquick Gel Extraction Kit	QIAGEN	Cat#28704
Chemicals, peptides, and recombinant proteins		
Penicillin/Streptomycin	Life Technologies	Cat#15140-122
Gentamicin	Life Technologies	Cat#15710072
DMEM Medium (high glucose/high L-glutamine)	Life Technologies	Cat#11965-118
L-Glutamine	Life Technologies	Cat#25030-081
HEPES	Life Technologies	Cat#15630-080

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Non-Essential Amino Acids	Thermo Fisher	Cat#11140-050
Sodium Pyruvate	Life Technologies	Cat#11360-070
Fetal Bovine Serum (FBS)	Thermo (Gibco)	Cat#26400036
Phosphate Buffer Saline (PBS) pH 7.4	Fisher	Cat#10010-049
Ethanol	VWR	Cat#89125-180
Deposited data		
(Sangaré et al., 2019)	N/A	N/A
Oligonucleotides		
Table S5: primer sequences	(Sangaré et al., 2019)	N/A
Table S1: GRA sgRNAs library	(Sangaré et al., 2019)	N/A
Software and algorithms		
R	R Core Team, 2013	http://www.r-project.org/
Microsoft Excel	N/A	Office.com
Machine and device		
Electroporator Gene Pulser Xcell	Bio-Rad	Cat#1652660
Power supply PowerPac	Bio-Rad	Power Pac Basic

STEP-BY-STEP METHOD DETAILS

Section 1: library preparation

⌚ Timing: 8 days

Day 0: Preparation of RH-Cas9 T. gondii parasites (from two 150-mm dishes of HFFs, infected two days before)

1. Use a cell scraper to remove the parasite-infected cells from the surface of the 150-mm dishes.
2. Pool the parasite-infected cells from the two 150-mm dishes in one 50 mL conical tube and centrifuge at $570 \times g$ for 7 min at 4°C .
3. Aspirate the medium and resuspend the parasite-infected cells in 5 mL CytoMix.
4. Pass the parasite-infected cells through two successive syringes (5 mL volume) with 27 G needles to mechanically break the cells and release the parasites. Collect in a 15 mL conical tube.
5. Determine the number of parasites using a hemocytometer (Neubauer Chamber).
6. Transfer the volume of parasites necessary to obtain 5×10^7 parasites total to a new tube and centrifuge at $570 \times g$ for 7 min at 4°C .
7. Resuspend the parasite pellet in 800 μL of Complete Cytomix + digested sgRNA-containing plasmid library.

Table 3. Recipe for CytoMix (500 mL total), storage at 4°C

Reagent	Final Concentration	Volume
KPO ₄ (0.2 M) pH 7.6	10 mM	25 mL
KCl (2.5 M)	120 mM	24 mL
MgCl ₂ (0.1 M)	5 mM	25 mL
CaCl ₂ (1 M)	0.15 mM	75 μL
HEPES (1 M)	25 mM	12.5 mL
EGTA (0.1 M) pH 7.6	2 mM	10 mL
KOH (1 N)	titration to get pH 7.6	N/A
ddH ₂ O	N/A	N/A
Total	N/A	500 mL
ATP (100 mM)	2 mM	N/A
GSH (100 mM)	5 mM	N/A

Table 4. Parasite medium after transfection (M1 medium), storage at 4°C

Reagent (stock concentration)	Storage temperature	Final Concentration	Volume
DMEM	4°C	N/A	484.5 mL
FBS	-20°C	1%	5 mL
L-Glutamine (200 mM)	-20°C	2 mM	5 mL
Penicillin/Streptomycin (10,000 µg/mL each)	-20°C	100 µg/mL	5 mL
Chloramphenicol (40 mg/mL)	-20°C	40 µg/mL	0.5 mL
Total	N/A	N/A	500 mL

8. Collect 10 µL in a 15 mL conical tube containing 1 mL of **M1 medium**. Those parasites will be used for plaque assay to determine parasite viability before transfection.
9. Split remaining parasites evenly between two electroporation cuvettes (400 µL each).
10. Place the cuvettes in the electroporator and electroporate each following this setup:
 - a. Voltage (V): 1250
 - b. Capacitance (µF): 25
 - c. Resistance (Ω): ∞
11. Let the transfected parasites recover in the cuvettes for 15 min at room temperature. During this time, remove four 150-mm dishes of HFFs from the incubator and change the medium to **M1 medium** (25 mL per dish).
12. Pool parasites from both cuvettes in a 15 mL conical tube.
13. Collect 10 µL in a 15 mL conical tube containing 1 mL of **M1 medium**. Those parasites will be used for plaque assay to determine parasite viability after transfection.
14. Use the transfected parasites (~800 µL) to infect four 150-mm dishes of HFFs, each with ~200 µL.

Note: For the viability before transfection (in **step 8**), dilute the parasites from 10 µL (6×10^5 parasites) in **M1 medium** until a concentration of 100 parasites/mL (in a total volume of 10 mL). Then, add 1 mL of this dilution into four individual wells of a 24-well plate of HFFs. For the viability after transfection (in **step 13**), dilute the parasites from 10 µL (6×10^5 parasites) in **M1 medium** until a concentration of 5,000 and 1,000 parasites/mL (in a total volume of 10 mL). Then, add 1 mL of each dilution into four individual wells of a 24-well plate of HFFs. Incubate both plates at 37°C.

Day 1: Selection

15. Remove the **M1 medium** from the 24-well plate at step 8, add 1 mL of **M3 medium** into each well, and incubate the plate at 37°C undisturbed for five days. Remove the **M1 medium** from the

Table 5. Parasite Selection Medium (M2 medium), storage at 4°C

Reagent (stock concentration)	Storage temperature	Final Concentration	Volume
DMEM	4°C	N/A	423.5 mL
FBS	-20°C	10%	50 mL
L-Glutamine (200 mM)	-20°C	2 mM	5 mL
Penicillin/Streptomycin (10,000 µg/mL each)	-20°C	100 µg/mL	5 mL
HEPES (1 M)	-20°C	10 mM	5 mL
Non-Essential Amino Acids (100x)	4°C	N/A	5 mL
Sodium Pyruvate (100 mM)	4°C	1 mM	5 mL
Gentamicin (10 mg/mL)	4°C	10 µg/mL	1 mL
^a Chloramphenicol (40 mg/mL)	-20°C	40 µg/mL	0.5 mL
^a Pyrimethamine (100 mM)	4°C	1 µM	5 µL
Total	N/A	N/A	500 mL

^aM3 medium= M2 medium without chloramphenicol and pyrimethamine, storage at 4°C.

24-well plate at step 13, add 1 mL of **M2 medium** into each well, and incubate the plate at 37°C undisturbed for five days.

△ **CRITICAL:** Do not move the infected 24-well plates during the incubation time. Otherwise, parasites from a formed plaque could generate new plaques and skew parasite viability. For example, 3% out of 1×10^7 parasites means that 2.1×10^5 parasites incorporated the sgRNA-containing plasmid into the genome and acquired resistance to pyrimethamine. Because the library contains ~2,000 sgRNAs, the parasite mutant coverage will be $(2.1 \times 10^5)/2,000$ sgRNAs = 105 × coverage. *In vitro*, a minimum of 50 × coverage is required to maintain library diversity (Sidik et al., 2018).

16. Start the pyrimethamine selection the following day after electroporation.
 - a. Aspirate the **M1 medium** from each 150-mm dish of infected HFFs.
 - b. Add 25 mL of **M2 medium** (+ 10 µg/mL of DNaseI) in each 150-mm dish of infected HFFs.

△ **CRITICAL:** For the first passage of the parasites after electroporation, add 10 µg/mL of DNaseI (10 mg/mL stock) to the **M2 medium**. The enzyme will digest any remaining extracellular sgRNA-containing plasmid, thereby preventing its amplification during the PCR steps of section 4.

Day 3: (Passage of parasites to new 150-mm dishes of HFFs. Parasites are mostly in big vacuoles or starting to lyse out):

17. Scrape the 150-mm dishes and combine two 150-mm dishes into one 50 mL conical tube.
18. Centrifuge at $570 \times g$ for 7 min at 4°C and resuspend in 10 mL of **M2 medium**.

Optional: There is no need to add DNaseI to the **M2 medium** at this point.

19. Pass the parasites through two syringes (10 mL) with 27 G needles and collect them in a new 50 mL conical tube.
20. Count parasites as indicated in step 5.
21. Collect 1×10^7 parasites as the **first lysis sample**.
22. Pellet down the parasites in a 1.5 mL Eppendorf tube at $15,000 \times g$ for 1 min.
23. Aspirate supernatant and freeze the tube at -80°C.
24. With the rest of the parasites, infect two to four 150-mm dishes of HFFs (containing 25 mL of **M2 medium**), each with 1×10^7 parasites.

Note: Always use 1×10^7 parasites to infect 150-mm dishes of HFFs. 1×10^7 theoretically represent 5,000 × coverage of a 2,000-sgRNA library. This number will be used for the inoculum because a high coverage is necessary to prevent random drop-out of mutants due to the strong bottleneck during the *in vivo* infection.

Day 5: Parasites viability before and after electroporation

25. To determine the viability, take out the plates, and use a microscope to count the number of lysis plaques formed on the HFF monolayer.
26. For the viability before transfection: for 100 parasites/well, if all of them were alive (100% viability), 100 plaques would be observed. Therefore, if the four wells have an average of 70 plaques, the viability would be 70%.
27. For the viability after transfection: for 1,000 parasites/well, if all of them were alive (100% viability), 1,000 plaques would be observed (technically, it is impossible to count 1,000 plaques in a 24-well). Therefore, if the four wells have an average of 30 plaques, the viability would be 3%.

Note: There is no need to fix or stain the 24-well plate with a crystal violet solution to count the plaques. The plaques are well visible by direct observation of live cells on the bright field of the microscope.

Day 6: (Passage of parasites to new 150-mm dishes of HFFs)

28. Repeat steps 17 to 23 and collect 1×10^7 parasites as the **second lysis sample**.
29. With the rest of the parasites, infect four to eight 150-mm dishes of HFFs (containing 25 mL of **M2 medium**), each with 1×10^7 parasites.

Section 2: Mouse infection

⌚ **Timing:** 4 days

Day 8: The parasites from Day 6 are mostly in big vacuoles or starting to lyse out.

30. Prepare the parasites for peritoneal injection of mice by repeating steps 17 to 20 in section 1.

Note: Perform an additional centrifugation step to have all the parasites in the same 50 mL conical tube, and resuspend them with 10 mL of **M1 medium**.

31. After counting, dilute the parasites to 5×10^7 parasites/mL.
32. Collect 1×10^7 parasites (200 μ L) as the **Inoculum sample** and follow steps 22 to 23 in section 1.
33. Pour the parasites into a reagent reservoir and load fifteen Insulin syringes, each with 200 μ L of **M1 medium** containing 1×10^7 parasites.
34. Inject fifteen mice intraperitoneally, each with 1×10^7 parasites (200 μ L).

⚠ CRITICAL: Be careful when loading the syringes and during the intraperitoneal injection. Follow safety measures to avoid any direct exposure to infected needles. Prepare extra syringes in case injection needs to be repeated or needles are defective. Ten mice will be used in section 3; however, infect fifteen mice to anticipate unexpected deaths before the final time point or mistakes during the mice injection.

Note: To determine the inoculum's viability, bring an extra 1×10^7 parasites (200 μ L) aliquot in a 15 mL conical tube to the mouse facility, and then back to the lab, add 9,800 μ L of **M1 medium** to have 1×10^6 parasites/mL. Then perform serial 10-fold dilutions until reaching a concentration of 100 parasites/mL. Infect four wells of a 24-well plate, each with 1 mL (100 parasites).

35. Every day, two times a day, monitor the condition of the mice. Mice usually present the first signs of infection (rough hair coat, inappetence, lethargy) around day two post-infection.

Note: The goal is to maximize the parasite's dissemination throughout the mice before euthanasia. However, with the inoculum of 1×10^7 parasites, the mice will likely need to be euthanized around four days (96 h) post-infection. It is essential to avoid using death as an endpoint by ensuring that mice are euthanized as soon as their condition significantly deteriorates (unable to reach food/water, inability to move, patent distress, etc.).

Day 12: Euthanize the infected mice.

36. Stop the infection when the condition of the mice requires it.

△ **CRITICAL:** Between three- and four-days post-infection. 1) The coat of the mice is rough. 2) Mice show reduced activity and cuddle together. 3) Mice stop eating and drinking (less food and water consumption are indirect effects of the previous condition cited). 4) The abdomen of the mice is swollen. Make sure not to reach the final stage before death, which is complete body lethargy with slow breathing. For more information about euthanasia, see (Avma, 2020).

37. Place the ten mice into a CO₂ chamber and monitor until no movement is detected. Maintain the CO₂ for one more minute before removing them from the chamber. The death should be confirmed by a total absence of breathing, then direct palpation of either the pulse in the carotid or femoral artery or direct cardiac palpation to assess the heartbeat.

Note: Apply the following steps on each of the ten mice.

38. Soak the dead mouse entirely into 70% ethanol for about 30 sec.
39. With scissors, make a small midline incision in the abdomen, and enlarge the incision with your fingers to remove the skin from the peritoneal membrane.
40. Inject 5 mL of PBS into the peritoneal cavity (5 mL syringe + 30 G needle). Vigorously shake the mouse to homogenize the peritoneal lavage.
41. Use a new 5 mL syringe + 27 G needle to retrieve the peritoneal lavage and transfer it to a 15 mL conical tube.
42. Open the peritoneal cavity and collect any organ of interest.
43. With scissors, open the thoracic cavity, collect the heart and lungs, and transfer them to different 50 mL conical tubes containing 10 mL of PBS.
44. Using forceps, collect the eyes and open the skull with scissors to collect the brain. Transfer both eyes to the same 50 mL conical tube and the brain to a separate 50 mL conical tube containing 10 mL of PBS.

△ **CRITICAL:** Soaking each mouse into 70% ethanol reduces the chance of contamination with bacteria, fungi, or yeasts. Be careful when taking peritoneal lavage as it contains a high number of parasites. Follow safety measures to avoid any direct exposure to infected needles. It is crucial to prevent the transfer of the peritoneal parasites to the thorax or skull. Use different sets of tools to collect each organ or wash the tools with 70% ethanol in between. Discard the carcass in an adequate biohazard container.

Optional: Our initial experiments showed that liver and spleen sgRNA profiles were almost identical to the peritoneal lavage profile.

△ **CRITICAL:** Section 3 starts as soon as all the organs and peritoneal lavages are collected on Day 12.

Section 3: Genomic DNA extraction

⌚ **Timing:** 3 weeks

Note: Section 3 contains only two steps with subheadings. Step 45 will detail how to amplify the sgRNAs by PCR (direct PCR method) using the genomic DNA extracted from the organs and peritoneal lavages from five mice. This method is limited by the amount of parasite genomic DNA available in the total amount of DNA. Step 46 will explain how to culture the organ suspensions and peritoneal lavage from five mice, allowing parasite replication, genomic DNA extraction, and PCR amplification of the sgRNAs (HFF culture method). This method introduces an extra *in vitro* step in the screen. We found that for organs with higher

Table 6. Organ digestion

Sample	Digestion	Expected genomic DNA yield (ng/μL)
Peritoneal lavage	Easy and rapid	60 to 389 (100 μL total)
Brain	Difficult, requires overnight incubation	31 to 95 (200 μL total)
Eye	Easy and rapid	133 to 227 (100 μL total)
Heart	Difficult, requires overnight incubation	25 to 78 (200 μL total)
Lung	Difficult, requires overnight incubation	26 to 60 (200 μL total)

parasite loads (e.g., peritoneal lavage, lung), the sgRNA profiles were similar for both methods, indicating that both methods are reliable. However, for organs with low parasite loads, the best method to obtain the largest sgRNA diversity was previously described in (Sangaré et al., 2019).

45. Genomic DNA extraction from organs and peritoneal lavages of five mice (Direct PCR method). A summary of the digestion process and expected DNA yields for each organ is shown in Table 6.
- Centrifuge the peritoneal lavages from step 41 in section 2 (peritoneal lavages for only five mice) at $570 \times g$ for 7 min at 4°C. Use the pellets for DNA extraction.
 - Next, aspirate the PBS from the 50 mL conical tubes containing the organ suspension from step 44 in section 2 (organs for only five mice) and place each of them in a 100-mm dish.
 - Cut the organs into small pieces using razor blades. Use a separate blade for each organ.
 - Use the blade to scoop up the organ pieces and transfer them to 1.5 mL Eppendorf tubes.
 - Use a scale to weigh each organ.

Pause point: All the organs and peritoneal lavages for the direct PCR method can be stored at -80°C for several months.

Note: The genomic DNA extraction will follow QIAGEN's protocol (DNeasy Blood and Tissue Kit, Cat#69506). If an organ's weight exceeds the maximum amount of tissue per digestion with Proteinase K, split it into different reactions to digest the whole organ.

CRITICAL: Before starting step 46, remove the medium from all the 150-mm and 100-mm dishes of HFFs and add fresh M3 medium (20 mL and 10 mL, respectively). This is very important because HFF cells need a rich medium when organ suspensions or peritoneal lavage are added on top of them.

46. Genomic DNA extraction from parasites after HFF culture of the organs and peritoneal lavages of five mice (HFF culture method). A summary of the organ suspension culture procedure is shown in Table 7.
- Centrifuge the peritoneal lavage from step 41 in section 2 (for the five other mice) at $570 \times g$ for 7 min at 4°C.
 - Resuspend the pellets with 1 mL of M3 medium.
 - Inoculate 150-mm dishes of HFFs (one peritoneal lavage for one 150-mm dish) with 1 mL of the resuspended peritoneal lavage.

Table 7. Organ suspension culture on HFF

Organ suspension	Number of dishes/organ	Expected parasites lysis time (days post-infection)
Peritoneal lavage	One 150-mm dish	4 to 5
Brain	Two 150-mm dishes	8 to 15
Eyes	One 100-mm dish	8 to 9
Heart	Two 150-mm dishes	8 to 15
Lungs	Two 150-mm dishes	3 to 5

- d. Next, aspirate the PBS from the 50 mL conical tubes containing the organ suspension from step 44 in section 2 (for the five other mice) and place each organ on top of a cell strainer (70 μm).
- e. Place the cell strainer + organ on top of a new 50 mL conical tube.
- f. Take a 5 mL syringe, remove the plunger.
- g. Use the hard edge of the plunger to crunch the organ inside the cell strainer.
- h. When the organ is completely crunched, rinse (with up to 10 mL of **M3 medium**) the plunger and cell strainer to collect the organ suspension into the 50 mL conical tube.
- i. Use the organ suspension to inoculate 150-mm or 100-mm dishes of HFFs as follows:

Optional: It is possible to increase the number of 150-mm dishes per organ; however, the expected lysis time will be longer.

- j. Regularly (at least once daily) check the dishes and gently stir them to resuspend the organ debris.

△ CRITICAL: Regularly move the dishes to avoid too much organ debris clamping on top of HFFs and to spread the newly lysed parasites all over the dish (see [Troubleshooting 1](#)).

- k. When the parasites are mostly in big vacuoles or starting to lyse the cells, follow steps 17 to 23 in section 1, collect 1×10^7 parasites from each organ and peritoneal lavage cultures.

▮▮▮ Pause point: Here, all the parasite pellets can be stored at -80°C for several months.

Note: Prepare several parasite pellets from each culture and store them at -80°C . The genomic DNA extraction will follow the QIAGEN DNeasy Blood and Tissue Kit (Cat#69506). For 1×10^7 parasites, the genomic DNA yield can vary between 50 to 200 ng/ μL in 100 μL .

Section 4: Amplification of sgRNAs

⌚ Timing: 1 to 2 weeks

Day 33: Start the PCR steps

Note: The sgRNAs amplification was adapted from steps 57 to 59 from the published protocol ([Sidik et al., 2018](#)). The minimal amount of genomic DNA recommended (from 1×10^7 parasites) is 1 μg in a total reaction volume of 100 μL . The primers listed for each sample is shown in Table S5 ([Sangaré et al., 2019](#)).

△ CRITICAL: Be aware of cross-contamination between samples. To avoid that: make small aliquots of UltraPure water in Eppendorf tubes used for the PCR reactions. Do PCR reaction in groups according to each type of organ (for example, only brain samples at the same time). Start with organs expected to have less diversity (e.g., eyes first, then brain, heart, lung, and peritoneum). The library plasmid should be the last reaction. Do not set PCR reactions on a bench where the sgRNA-containing plasmid was purified, digested, dialysis, etc. If possible, use a specific PCR station with a hood. Clean with a DNase solution (e.g., diluted bleach), and perform ultraviolet treatment for a set of pipettes between each group of PCR reactions. Gloves are an important source of cross-contamination; change them between each group of PCR reactions. Always include a negative PCR control, which adds UltraPure (DNase-free) water instead of template DNA to know if the primers or other reagents are contaminated.

47. [Table 8](#) details the number of PCR reactions necessary according to the origin of the genomic DNA.
48. Prepare the PCR reaction mix according to [Table 9](#).

Table 8. Number of PCR reactions per sample

Origin of genomic DNA	Number of PCR reactions	Genomic DNA amount per reaction
sgRNA-containing plasmid	2	1 µg
Parasites from the first lysis in HFFs	6	1 µg
Parasites from the inoculum	6	1 µg
Organ/peritoneal lavage	Use all genomic DNA available	1 µg
Parasites from organ/peritoneal lavage cultures on HFFs	3	1 µg

Note: Primer 1 (P1) contains the P5 Illumina adaptor, and it is the common primer for all the samples. Primer 2 (P2) contains the P7 Illumina adaptor and a specific barcode. Each sample should have a specific P2 primer for sgRNA amplification. See Table S5 (Sangaré et al., 2019) for P1, P2, and barcode sequences.

49. Perform PCR following the cycling conditions detailed in Table 10.
50. Pool together PCR reactions from the same sample and analyze 10 µL of the pool using standard gel electrophoresis. The expected band size is between 368 to 369 bp (Figure 1).

⚠ **CRITICAL:** If there is no band for direct PCR organ and peritoneal lavage samples, see Troubleshooting 2.

51. Then for each sample, run 100 to 200 µL of the pool using standard gel electrophoresis.
 - a. Place the gel on a glass cutting board with blue light (do not use UV light).
 - b. Use a clean blade to cut out the band corresponding to the amplified sgRNA fragments (see Figure 1).
52. Purify the PCR product using a QIAquick Gel Extraction Kit (Cat#28704), following the manufacturer's instructions.

⚠ **CRITICAL:** The manufacturer's protocol indicates melting the gel at 50°C for about 10 min. We recommend melting the gel at room temperature. The process will take more time but will avoid amplicon denaturation and PCR bubble formation (PCR bubbles contain considerable amounts of single-stranded DNA that will not be quantified). Here, it is not recommended to keep the PCR products for a long time before sequencing. The products can be kept at 4°C for a maximum of one week but do not freeze/thaw them.

Section 5: Illumina sequencing and analysis

⌚ **Timing:** 2 weeks (Time for the Illumina sequencing is not included)

53. Use the samples from step 52 in section 4 for next-generation sequencing.

Table 9. PCR reaction mix

Reagent	Final concentration	Final volume
5 × Q5 buffer	1 ×	20 µL
dNTP (10 mM)	200 µM	2 µL
P1 (10 µM)	0.5 µM	5 µL
^a P2 (10 µM)	0.5 µM	5 µL
Template DNA	1 µg of genomic DNA or 500 ng of sgRNA-containing plasmid	N/A
Q5 High-Fidelity DNA Polymerase	N/A	1 µL
UltraPure (DNase free) water	N/A	up to 100 µL

^aEach sample has a specific P2.

Table 10. PCR reaction condition

Cycle	Denaturation	Annealing	Extension	Final
1	98°C, 30 s			
2 - 27	98°C, 10 s	60°C, 30 s	72°C, 15 s	
28			72°C, 2 min	
29				12°C, Hold

Note: The samples for next-generation sequencing should be prepared following the corresponding sequencing facility's protocol. We used a NextSeq (Illumina 2500) with single-end reads using primers P150 and P151 to sequence the sgRNAs and barcodes, respectively (See Table S5 (Sangaré et al., 2019)). From here, the peritoneal lavage will be mentioned as peritoneum and the sgRNA-containing plasmid as the library.

Day 48: Start of the Analysis

54. Analysis of sgRNA sequencing reads and calculation of fitness scores for each gene.
 - a. Extract the Illumina sequencing reads and match them against the sequences of the sgRNA library. Follow Steps 61 to 66 from the published protocol by Sidik et al., 2018.
 - b. Count the number of exact matches and consider these as raw read numbers.

Note: Due to reduced sgRNA coverage (Sangaré et al., 2019), we decided to pool the reads of the five individual mice for the heart, brain, and eye to have three different samples. We kept ten separate samples for lung and peritoneum.

Note: We followed steps 61 to 66 of the published protocol (Sidik et al., 2018) to get each gene's fitness score from each sample. Here the fitness score corresponded to the average log₂ fold-change in sgRNAs abundance between organ/peritoneum vs. inoculum.

55. Fitness scores calculation.
 - a. HFF fitness score = Inoculum vs. Library
 - b. Peritoneum fitness score = Peritoneum (for each peritoneum) vs. Inoculum
 - c. Lung fitness score = Lung (for each lung) vs. Inoculum
 - d. Heart fitness score = Heart (pooled hearts) vs. Inoculum
 - e. Brain fitness score = Brain (pooled brains) vs. Inoculum
 - f. Eyes fitness score = Eye (pooled eyes) vs. Inoculum
56. Rank the genes from the lowest to the highest fitness score.
 - a. Rank the genes in each of the ten peritoneum samples.
 - b. Rank the genes in each of the ten lung samples.
57. Calculate the Z-score for the gene ranks in peritoneum and lung. Gene Z-score = (100.5 - gene average rank)/ gene average rank standard deviation. 100.5 represents the population ranking mean = (R1 + R2 + + R200)/200. Where 200 is the number of genes in the population.

Note: The Z-score will identify genes with a consistent top ranking in the peritoneum and lung, which means those genes have the lowest fitness scores in each organ.

58. Criteria to identify genes that confer fitness in the peritoneum and/or organs.
 - a. Genes with a Z-score ≥ 2 in the peritoneum were considered as peritoneum fitness-conferring genes. Those genes will also have a high Z-score in the lung and a low fitness score in the heart, brain, and eye.
 - b. Genes (excluding peritoneum fitness conferring genes) with a Z-score ≥ 2 in the lung and/or with a fitness score of ≤ -1 (2-fold decrease in abundance of sgRNAs) in at least three other organs (heart, brain, and eye) were considered as organ fitness conferring genes.

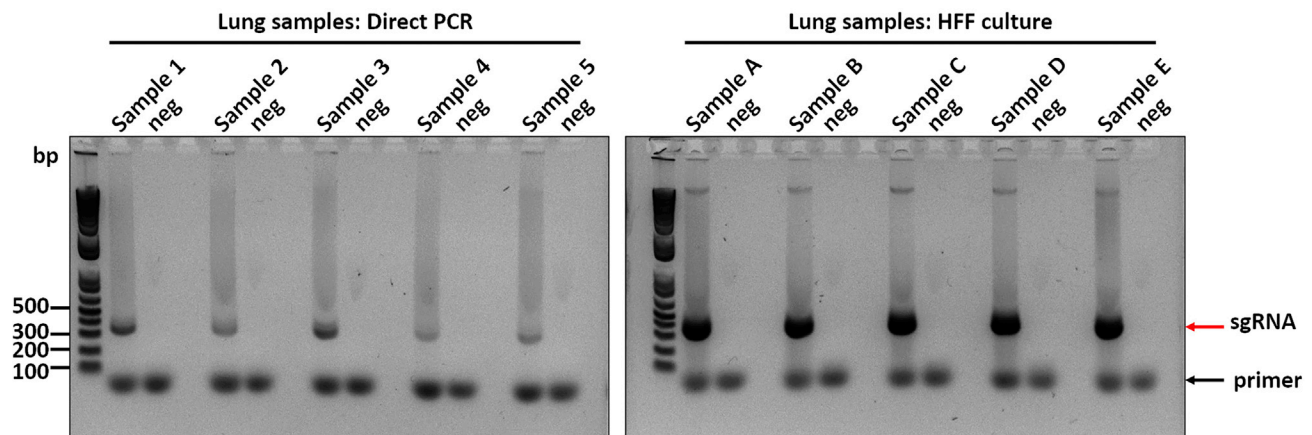


Figure 1. sgRNA amplification

Left, genomic DNA was extracted from mice's lungs and used as a template to amplify sgRNAs (Samples 1 to 5). Right, lungs were put in HFF cell culture to allow the parasites to replicate. Subsequently, parasites were isolated, genomic DNA extracted, and used as a template to amplify sgRNAs (samples A to E). The red arrow indicates sgRNA bands 368–369 bp, and the black arrow indicates remaining primers from the PCR reaction. neg= negative control for PCR reactions.

EXPECTED OUTCOMES

The HFF fitness score = Inoculum vs. Library is expected to have a high correlation with the published phenotype score (Sidik et al., 2016). This indicates the reproducibility of the *in vitro* part of the screen (from step 14 in section 1 to step 30 in section 2) compared to published data. It is expected to find a high correlation between fitness scores in peritoneum and lungs obtained by the amplification of sgRNAs by direct PCR vs. amplifying parasites in organ suspensions on HFFs first. This indicates that both methods are reliable and maintain library coverage during all processes. It is expected to see a high correlation between the peritoneum and lung fitness scores in the same mouse. Parasite mutants that do not survive in the peritoneum will generally be absent from the lungs. The fitness scores derived from the pooled organs (heart fitness, brain fitness, eye fitness) correlate with the average peritoneum and lung fitness scores. The high correlations between organs indicate the reproducibility of the *in vivo* screen between animals. The Pearson correlations between samples are summarized in Table S2 (Sangaré et al., 2019).

LIMITATIONS

The protocol was designed to overcome the *in vivo* bottleneck effect on a library containing 2,000 sgRNAs. However, for many samples from the heart and brain, the protocol failed to amplify all the sgRNAs, consistent with a severe bottleneck for parasites colonizing these organs within 90 h. To counteract this bottleneck, we pooled the sgRNA counts from all mice for samples from the brain, heart, and eye. Considering that information, it will be challenging to obtain reproducibility between mice for a larger library. As a recommendation, we suggest using a less complex library. Fewer parasites need to be injected to keep the mutant coverage, resulting in mice surviving longer and increased parasite dissemination.

TROUBLESHOOTING

Problem 1

The cells are dying after inoculation with organ suspensions.

Potential solution

After infecting 150-mm dishes with organ suspension in step 46 in section 3, it is essential to look at the cells regularly. Due to the significant amount of tissue and blood cells, the HFF monolayer could become less confluent as the cells are dying. This scenario is more probable with the heart suspension containing abundant erythrocytes and brain suspensions that form a compact cloud on top of the HFF monolayer. If that is the case, remove the medium (containing organ suspension) and put it

on top of one or two new 150-mm dishes. Add new medium to the previous dish (this dish may contain intracellular parasites); the cells will recover rapidly.

Problem 2

No sgRNA was amplified from the genomic DNA of an organ.

Potential solution

For the direct PCR method, due to the high amount of the murine tissue DNA compared to the lower amount of parasites DNA, there is a high chance of having a PCR reaction without any sgRNA amplification, step 50 in section 4. The amount of total genomic DNA could reduce or inhibit the amplification. One solution could be to perform a nested PCR using the first PCR reaction as a template for the second PCR reaction. Another solution is to reduce the amount of genomic DNA per reaction and perform more reactions with less genomic DNA as a template.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Jeroen Saeij (jsaeij@ucdavis.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The library sgRNAs and primer sequences are available in Tables S1 and S5 ([Sangaré et al., 2019](#)). The pU6-DHFR plasmid to clone sgRNAs libraries and the Pooled library containing pU6-DHFR plasmid are available on Addgene (respectively Plasmid#80329 and Plasmid#80636). The pU6-DHFR + GRA sgRNAs library will be provided upon request. The Pearson correlation data are in Table S2 ([Sangaré et al., 2019](#)). The data for Illumina sequencing reads, fitness scores, and Z-scores are available in Table S4 ([Sangaré et al., 2019](#)).

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AUTHOR CONTRIBUTIONS

Conceptualization, L.O.S. and J.P.J.S.; investigation, L.O.S., Y.W., and D.A.S.; [Figure 1](#), L.O.S. and Y.W.; writing and review, L.O.S., J.P.J.S., Y.W., and D.A.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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