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1 # Assays for monitoring *Toxoplasma gondii* infectivity in the laboratory mouse

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10 Running title: *Mouse toxoplasmosis models*

11

12 **Key Words**

13 virulence, bioluminescence, dissemination, central nervous system, pathogenesis,

14

15 ## Abstract

16 *Toxoplasma* is a widespread parasite of animals including many rodents that are a

17 natural part of the transmission cycle between cats, which serve as the definitive host.

18 Although wild rodents, including house mice, are relatively resistant, laboratory mice

19 are highly susceptible to infection. As such, laboratory mice and have been used to

20 compare pathogenesis of natural variants, and to evaluate the contributions of both

21 host and parasite genes to infection. Protocols are provided here for evaluating acute

22 and chronic infection with different parasite strains in laboratory mice. These protocols

23 should provide uniform standards for evaluating natural variants and attenuated

24 mutants and for comparing outcomes across different studies and between different

25 laboratories.

26

27 ## 1 Introduction

28 *Toxoplasma gondii* is an extremely widespread parasite of animals that also causes

29 zoonotic infections in humans (1). Strains of *T. gondii* have been grouped into three

30 major clonal lineages that predominate in North America and Europe (2-4). These

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31 lineages differ in their acute virulence in laboratory mice due to the presence of
32 polymorphic secretory effectors, many of which are derived from rhoptry secretion (5)
33 or released from dense granules (6). Representatives of all three genotypes have
34 been reported as part of a comparative genomes project for *T. gondii*, which provides a
35 framework for comparing strain types within and between major lineages (7). Strains
36 of *T. gondii* from South America, which differ genetically, also show high levels of
37 virulence in laboratory mice, in part due to similar virulence factors that mediate
38 differences among the clonal lineages (8). Here we will present protocols for
39 monitoring infectivity and pathogenesis of the three major clonal lineages in laboratory
40 mice, although similar methods could be adapted to study other more diverse strains.

41 Transmission of *T. gondii* normally occurs between cats, which serve as the
42 definitive host, and rodents and many other animals that serve as intermediate hosts
43 (1). Mice are a natural host for *T. gondii* and as such the laboratory mouse provides an
44 excellent model to study innate and adaptive immunity (9, 10). Type I strains are
45 acutely virulent and a single viable organism is uniformly lethal in all strains of
46 laboratory mice (11, 12). Type I strains do not readily differentiate to bradyzoites in
47 mice and their high level of virulence makes it difficult to obtain tissue cysts in
48 chronically infected animals. As a consequence, infections with Type I strains are
49 typically administered by intraperitoneal (IP) injection of tachyzoites. Acute infections
50 progress rapidly by expansion of parasite numbers and dissemination to all organs of
51 the body, leading to death within the first 10-12 days (13). Parasite expansion and
52 dissemination are prominent features of acute infection, leading to cytokine shock (13,
53 14), which is likely a contributing cause of death. Genetic crosses have been used to
54 map genes that contribute to acute virulence of Type I strains in laboratory mice (15)
55 and the roles of specific genes in pathogenesis have been confirmed using a variety of
56 techniques to disrupt or modify genes (16).

57 By contrast, Type II strains display intermediate virulence in laboratory mice where
58 high doses lead to lethal outcome, while lower doses resolve and result in chronic
59 infection, allowing LD_{50} values to be established (13). Because of their ability to cause
60 nonlethal, chronic infection, Type II strains are often used to explore a range of

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61 immunological functions that control infection (9, 10). Infection protocols vary with
62 some investigators using IP injection of tachyzoites grown in vitro, while others isolate
63 tissue cysts from chronically infected mice and administer them by IP injection or by
64 oral gavage. Oral ingestions of tissue cysts follow the natural route of infection as *T.*
65 *gondii* is able to transmit between different intermediate hosts by omnivorous or
66 carnivorous feeding (17). High challenge doses delivered by the oral route result in
67 acute gastroenteritis that can lead to death, and the immunological basis of this form
68 of pathogenesis has been explored through numerous studies (18).

69 Type III strains are relatively common in animals in North America and yet they are
70 rarely encountered in human cases of toxoplasmosis (4, 19, 20). Type III strains are
71 highly avirulent in laboratory mice, with high challenge doses leading to low levels of
72 lethal infection (21). The Type III strain CTG (aka CEP) has been used to study sexual
73 phase transmission in the cat and to develop genetic mapping strategies for *T. gondii*
74 (22, 23). The basis for the lack of acute virulence in CTG was shown to be due to
75 under-expression of ROP18, a polymorphic virulence determinant of Type I strains
76 (24). Infection studies with another commonly used Type III strain called VEG revealed
77 that the stage used for infection (i.e. bradyzoite vs. oocyst challenge) greatly
78 influences pathogenesis (25).

79 Laboratory mice are derived from a few founder lines that represent mixtures of
80 the *Mus musculis musculis*, *M. m. domestica*, and *M. m. castaneus* lineages, with the
81 majority of loci coming from *M. m. domesticus* (26). These founders were used to
82 establish outbred Swiss Webster and CD-1 lines, which have been kept in closed
83 colonies and bred to maximize genetic heterozygosity (27). Inbreeding of founder
84 lineages to minimize heterozygosity gave rise to C57Bl/6 mice and other inbred lines
85 that differ by a small number of polymorphic loci, notably the major histocompatibility
86 complex (MHC). Such inbred lines have been extremely useful for studying the
87 association of genotype with phenotype. However the total variation within all inbred
88 laboratory mouse lines is far less than that seen in wild caught or wild-derived isolates
89 of *M. musculis* (28). Although both outbred and inbred strains of laboratory mice are
90 relatively susceptible to *T. gondii* infection, wild strains derived from *M. musculis* are

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91 much more resistant (29). Given their susceptibility to infection, laboratory mice have
92 been extremely useful to highlight pathogenesis differences among parasite strains
93 and to study immune mechanisms involved in control of infection.
94

95 2 Materials

96 2.1 Propagation of tachyzoite cultures in vitro

97 1. Commonly used strains for mouse challenge studies are shown in Table 1. For
98 type I strains we recommend the lab-adapted RH strain, or GT-1 which undergoes
99 the complete life cycle. Commonly used type II strains include ME49 and
100 Prugniaud (aka Pru). Type III strains include CTG and VEG. Estimates of the
101 pathogenicity in different mouse strains are also provided, although these can
102 vary with colony and source and so need to be determined locally.
103 2. Tissue culture incubator for culture at 37 °C, 5% CO_2 and BSL-2 biosafety cabinet.
104 3. Tissue-culture flasks (25 cm^2), 96-well plates, and cell scrapers.
105 4. Human foreskin fibroblasts (HFF) cells (ATCC, cat. # SCRC-1041).
106 5. D10 medium: For 1 liter, combine 1 package Dulbecco's Modified Eagle's Medium
107 (DMEM) powder (Thermo Fisher, Gibco cat. # 12100046), 3.7 g $NaHCO_3$, 100 ml
108 fetal bovine serum (FBS), 10 ml of 200 mM L-glutamine, 1 ml of 10 mg/ml
109 gentamycin, and dH_2O to 1 liter. Sterilize by 0.45 micron filtration.
110 6. HHE: 1X Hank's Balanced Salt Solution, 10 mM HEPES, 1 mM EGTA, sterilized
111 by 0.45 micron filtration.
112 **7.** Blunt-end needles (20, 23, and 25 gauge), bulb transfer pipets, 5 and 10 ml pipets,
113 5 and 10 ml syringes.
114 **8.** Hemocytometer (Thermo Fisher Scientific, cat. # 0267151B) or other cell counting
115 instrument.
116 **9.** Inverted tissue culture microscope equipped with 10x, 20x objectives.
117 10. Swin-Lok filter holder (Whatman, cat. # 420200) and polycarbonate filter
118 membranes (3 micron pore) (Whatman, cat. # 110612). Although larger pore
119 sizes can be used, there is more risk of contaminating host nuclei or debris.
120 **11.** Centrifuge capable of holding 15 conical tubes and spinning at 400 x *g.*

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121

122 **2.2 Challenge studies**

123 1. In vitro tachyzoite cultures of the *T. gondii* strains of interest.
124 2. Outbred CD-1 and various inbred lines of mice.
125 3. HHE: 1X Hank's Balanced Salt Solution, 10 mM HEPES, 1 mM EGTA,
126 sterilized by 0.45 micron filtration.
127 4. Tuberculin syringes for IP injection.
128 5. Oral gavage needles 22 g x 1 ½ inch, 2.4 mm tip (Patterson Veterinary, cat. #
129 07-809-7615).
130 6. Sterile PBS.
131 7. Sterile plastic 10 ml syringes equipped with 16, 18, 20 g needles.
132 8. Sulfadiazine (Thermo Fisher Scientific, cat. # S-6387) solution (0.1 – 0.2 gr/L).

133

134 **2.3 Plaquing assay**

135 1. Tissue culture incubator for maintaining cell cultures at 37 °C, 5% CO_2 and BSL-2
136 biosafety cabinet.
137 2. Human foreskin fibroblasts (HFF) cells (ATCC, cat. # SCRC-1041).
138 3. D10 medium: For 1 liter, combine 1 package Dulbecco's Modified Eagle's Medium
139 (DMEM) powder (Gibco, cat. # 12100046), 3.7 g $NaHCO_3$, 100 ml FBS, 10 ml of
140 200 mM L-glutamine, 1 ml of 10 mg/ml gentamycin, and dH_2O to 1 liter. Sterilize
141 by 0.45 micron filtration.
142 4. Tissue culture plates (6 well) containing confluent monolayers of HFF cells.
143 5. Solution of 1% Crystal violet solution water.
144 6. Inverted tissue culture microscope, equipped with 4x, 10x, 20x objectives.

145

146 **2.4 ELISA for monitoring infection status**

147 1. High binding ELISA plates (Disposable Sterile ELISA Plates, cat. # 25801).
148 2. Model 500 Sonic Dismembrator with microprobe (Thermo Fisher Scientific).
149 3. Tachyzoite culture of *T. gondii*. Most antigens cross-react so RH (Type I) or ME49
150 (Type II) strains can be used interchangeably for detecting infection with multiple

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151 different strain types.

152 4. Control mouse serum (previously infected positive and non-infected negative animals
153 for reference). Store in aliquots at -80°C until use.

154 5. Serum samples from infected mice. BD Microtainer tube with serum separator gel
155 (Thermo Fisher Scientific, cat. # 02-675-185). Typically small volumes (10-100 uL)
156 can be collected from saphenous vein or cheek vein puncture. Store in aliquots at
157 -80°C until use.

158 6. Horse radish peroxidase (HRP)-conjugated secondary antibody (HRP goat
159 anti-mouse IgG) (Thermo Fisher Scientific, cat. # 62-6520).

160 7. Phosphate buffered saline (PBS).

161 8. Wash solution: PBS/ 0.05% Tween-20. Plastic squirt bottle for dispensing wash
162 solution.

163 9. BSA blocking solution: PBS/ 0.05% Tween-20, 1.0% bovine serum albumin (BSA).

164 10. BSA incubation solution: PBS/ 0.05% Tween-20, 0.1% BSA.

165 11. Substrate: BD OptEIA Substrate Reagent A, Substrate Reagent B (BD Biosciences,
166 cat. # 51-2606KC).

167 12. Plate reader for absorbance reading at 450 nm.

168

169 **2.5 Tracking infection by bioluminescence**

170 1. D-Luciferin, potassium salt (Gold Biotechnology, cat # Luck-1G).

171 2. Isoflurane (Henry Schein Animal Health, cat. # SKU 029405).

172 3. IVIS Spectrum BL imager (Perkin Elmer) or equivalent instrument capable of
173 detecting bioluminescence.

174 4. Tuberculin syringes for injection.

175

176 **2.6 Cyst harvesting and staining**

177 1. *Dolichos biflorus* lectin conjugated with FITC (Vector Labs, cat. # FL-1031).

178 2. Fixating and Permeabilizing Solution: 2X stock consisting of 6% formaldehyde,
179 0.2% Triton-X-100 in PBS.

180 3. Blocking solution: 10% normal goat serum in PBS.

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181 4. Glass slides and coverslips.
182 5. Epifluorescence microscope equipped with phase contrast (10X, 40X) and filter
183 set for detecting FITC.
184 9. Sterile PBS.
185 10. Sterile plastic 5, 10 ml syringes equipped with 16, 18, 20 g needles.
186 6. Polystyrene 15 ml tubes and centrifuge capable of spinning at 400 g.
187

188 **3 Protocols**

189 **3.1 In vitro propagation of tachyzoites**

190 *T. gondii* are most easily propagated in human foreskin fibroblasts (HFFs) because
191 the host cells reach confluency and stop dividing. These features facilitate passage at
192 high MOI that leads to natural egress at 2-3 day intervals, and also allow for plaque
193 formation on preformed monolayers. Procedures for propagation of HFF monolayers,
194 serial passage of *T. gondii* lines, and harvest of viable tachyzoites have been defined
195 previously (30) and are only briefly summarized here.

196 1. Passage *T. gondii* tachyzoites by serial passage on HFF monolayers. Typically,
197 strains are inoculated serially using parasites from a freshly egressed culture to
198 inoculate a new monolayer of HFF cells. The flask is inoculated at a high MOI (1:1
199 or 2:1) to assure uniform infection and host cell lysis in a single round. For Type I
200 stains, natural egress occurs ~2 days after the initial inoculation (or slightly less),
201 while for Type II and III strains it is often 3 days post-inoculation.
202 2. To passage strains, disperse the contents of a recently egressed culture using a 5
203 ml pipet to resuspend the culture material and remove cells from the surface (it is
204 also possible to mechanically release as described below).
205 3. Gently pipet the material using 5 ml pipet to draw the liquid in and out several
206 times to disperse and break up clumps. Dispense parasites using 5-10 drops from
207 a bulb transfer pipet (1 drop ~ 50 μL) to inoculate a new confluent monolayer of
208 HFF cells grown in a $T25^2$ flask. For Type I strains, this method results in a 1:10 to
209 1:20 split by volume (based on a starting volume of 5 mls for culture in a $T25^2$ flask)
210 every 2 days. Type II and III strains may require a higher inoculum from 10-20

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211 drops, equating to a 1:10 to 1:5 split by volume. Return the flask to the incubator,
212 37°C, 5%CO_2, for 2-3 days.
213 4. Prior to inoculating mice, it is important to establish the parasites on a consistent
214 passage cycle every 2-3 days, otherwise viability will be compromised.
215 5. To prepare parasites for inoculation into animals, harvest tachyzoites at the peak
216 of their natural intracellular replication cycle, either at the point of natural egress or
217 shortly before. Any cells remaining on the monolayer at this time point should be
218 heavily infected and will be easily disrupted by gentle pipetting. If necessary,
219 scrape the monolayer to remove cells
220 6. Resuspend the contents of the flask and passage sequentially through 20, 23, and
221 25 g blunt needles attached to a 10 ml syringe. When expelling the material from
222 the needle, keep it submerged below the surface to avoid aerating the sample.
223 7. Filter to separate host cell debris from tachyzoites using 3 micron polycarbonate
224 filter unit. Flush the filter with 10 ml of HHE collecting the flow-through in a 15 ml
225 conical tube.
226 8. Centrifuge the filtered culture at 400 x *g* for 10 min at 18°C, resuspend the pellet in
227 10 ml HHE.
228 9. Count the parasites using a hemocytometer.
229 10. Dilute the parasites to an appropriate concentration so that injection volumes
230 (typically 0.1 – 0.2 ml) will contain the desired number of parasites.
231 11. Maintain the parasite at room temperature throughout, chilling them does not
232 result in better viability. Rather it is important to perform these procedures quickly
233 and immediately before you go the facility to inject animals. Different strains of *T.*
234 *gondii* also vary on how well tachyzoites survive outside of culture, with type I
235 being the most robust. Type II and III strains loose viability much faster and should
236 be used immediately after harvest. We also find the use of Hank's Balanced Salt
237 Solution based medium is better for resuspending the parasites in compared to
238 PBS, as the former is better for maintaining parasite viability.
239
240 **3.2 Estimating viability by plaquing**

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241 1. After returning from the animal facility, use a portion of the unused parasite
242 suspension to perform a plaquing assay and establish the viability of the inoculum.
243 2. Inoculate 200 – 500 parasites per well of a 6 well plate containing HFF cells
244 growing in DMEM-10% FBS. For a uniform suspension, it is best to place the
245 parasites in 2 ml of D10 medium and then use this to replace the medium that is in
246 each well. Allow parasites to settle by gravity being careful not to swirl the plate
247 (this action creates a vortex that brings the parasites to the center and skews the
248 count). Use triplicate wells per strain.
249 3. Incubate at 37C, 5% CO_2 for 7-9 days, depending on the strain. Do not move the
250 plate during this time period.
251 4. Remove the plate from the incubator, rinse in PBS, and stain with 1% Crystal
252 violet (made in dH_2O) followed by rinsing in H_2O.
253 5. Plaques will appear as clear zone on the stained background. Count by eye or
254 under low power (2-5 X). In the event that plaques have not fully lysed, they can
255 be difficult to visualize. In this case, score foci of infection by examining the
256 stained monolayer under low power using an inverted microscope (5-20 X
257 depending).
258 6. Expect maximum viability of 50%, however it can also be as low as 5%, especially
259 if tested more than 1-2 hr after initial harvest of the parasites.
260

261 **3.3 Acute virulence model**

262 The following protocol is used to establish "acute virulence" based on serial dilution of
263 parasites and challenge into outbred CD-1 mice. Cumulative survival (or inversely %
264 mortality) is used to evaluate the degree of pathogenicity. This definition of acute
265 virulence has been used to establish the difference between clonal types (11), and
266 map virulence differences between them (12, 24, 31). Additional readouts that are
267 useful to obtain include weight loss, time to death, and tissue burden by
268 bioluminescence (Fig. 1).
269 1. Harvest tachyzoites from freshly egressed cultures, count, and dilute in HEE as
270 described above.

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271 2. Infect separate groups of 5 CD-1 mice by IP inoculation with serial dilutions of
272 tachyzoite, (i.e. 10, 100 or 1,000 tachyzoites / mouse using Type I parasites).
273 Experiments should be repeated 2-3 times on different days to account for
274 possible variation in the viability of the inoculum.
275 3. Monitor the animals for weight loss and signs of illness. Use an appropriate end
276 point prior to death, depending on institutional approved protocol.
277 4. Animals can be monitored for expansion of parasites using luciferase tagged
278 strains and bioluminescence (see **Imaging infection by bioluminescence**
279 protocol).
280 5. At 30 days post-infection, determine the number of surviving animals. The time
281 point of 30 days is somewhat arbitrary as acutely virulent lineages will generally
282 lead to death prior to day 20.
283 6. Bleed animals form the saphenous or cheek vein, collecting a small volume (100
284 -200 µL) in a microtainer with serum separator gel. Spin the separator to extract
285 the serum (top) layer from the red cells. Store serum at -20°C before use.
286 7. Perform ELISA (see **ELISA for monitoring infection** protocol below). to
287 determine the titer in comparison to controls. Seropositivity is an outcome of
288 successful infection. At low inoculum, or low viability of the inoculum, some
289 animals may not become infected and they remain serologically negative. Such
290 non-infected animals are removed from the calculation of % survival.
291 8. Survival % is calculated as: # infected animals that survive / the number of
292 infected animals (dead animals plus seropositive survivors) x 100. Lower survival
293 equates with higher virulence.
294

295 **3.4 Imaging infection by bioluminescence**

296 Bioluminescence provides a powerful way to track infection *in vivo* with the advantage
297 that the same animals can be imaged over time (32). Strains of *T. gondii* have been
298 transfected with various luciferase proteins including firefly (33) and click beetle
299 luciferase (34). These reporters can be imaged by injecting the substrate luciferin *in*
300 *vivo* just prior to imaging with very sensitive bioluminescence imagers.

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301 1. Resuspend 1 gram of luciferin into 66.7 ml of PBS, filter, dispense in 1.5 ml
302 aliquots, and store in -80°C. One tube should be sufficient for ~5 mice.
303 2. Weigh mice to determine how much luciferin to inject. Before beginning the
304 experiment, thaw luciferin aliquots.
305 3. Lightly anesthetize the mice using isoflurane. Inject mice using 10 µL of luciferin
306 (15 mg/ml stock concentration) per gram of weight. For example, if the mouse
307 weighs 25 grams, you will inject 250 µL into the mouse. Injections are performed
308 IP using a tuberculin syringe. Luciferin is readily soluble and rapidly partitions
309 throughout the body, allowing imaging of essentially al tissues, although the
310 penetrance of the signal varies by tissue density.
311 4. Place the animals in the IVIS chamber using the isoflurane manifold to keep them
312 under light anesthesia to prevent movement during imaging. Image the mice
313 within 20 min of injection for best results.
314 5. Image the bioluminescence signal following the manufacturers instructions for
315 operation of the IVIS unit.
316 6. Analyze your data using Living Image ® (Perkin Elmer) and graph results using
317 Prism (Graphpad) or Excel. Appropriate statistical analyses typically involve
318 non-parametric tests using adjustments for multiple comparisons.
319

320 **3.5 ELISA for monitoring infection**

321 **Antigen preparation**

322 1. Harvest parasites from a freshly egressed culture grown in HFF cells into a 50 ml
323 polystyrene tube. Centrifuge at 400 *g* for 10 min, room temperature. Resuspend the
324 pellet in 10 ml HHE.
325 2. Count the parasites and resuspend in PBS at a final concentration of 10^{8} cells / ml.
326 Sonicate using 15 sec on/45 sec off cycle for 2 minutes, power 2.8 unit (Fisher Model
327 500 Sonic Dismembrator with microprobe).
328 3. Add glycerol to a final concentration of 10%, aliquot, and store aliquots in -80□C.

329 **Perform the ELISA**

330 1) Take an aliquot of parasite lysate from the freezer and dilute in PBS ($1x10^{6}$/ ml

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331 parasites). Add 100 µl of antigen per well and incubate the plate for 1 hr at 37°C or at
332 4°C overnight. Cover the plates with lid or parafilm.
333 2) After incubation, rinse wells 3 times with PBS/ 0.05% Tween-20, using a squirt bottle.
334 3) Add 250 µl of 1% BSA blocking solution in each well and incubate for 1 hr at room
335 temperature. After incubation, rinse all the wells with PBS/ 0.05% Tween-20 three
336 times.
337 4) Dilute primary antibody (unknown mouse sera, positive, or negative controls) to
338 appropriate concentration in BSA incubation solution (1:500 – 1:5,000). Add 100 µl of
339 primary antibody to each well and incubate for 1 hr at room temperature. Test each
340 sample and positive and negative controls in duplicate wells. Rinse 3 times with PBS/
341 0.05% Tween-20.
342 5) Dilute HRP-conjugated secondary antibody to 1:2,500 – 1: 10,000 in PBS/ 0.05%
343 Tween-20, 0.1%BSA. Add 150 µl of secondary antibody per well for 1 hr at RT°C.
344 Rinse 4-5 times with PBS/ 0.05% Tween-20. It is important to wash very carefully with
345 PBS after this incubation to avoid unspecific staining because of free
346 peroxidase-conjugated antibodies.
347 6) Mix equal volume of substrate-A and substrate-B and immediately add 100 µl of this
348 mixture to each well and incubate for 20-30 min at dark. Stop the reaction with 50 µL of
349 2M H_2SO_4 added to each well.
350 7) Measure the Absorbance at 450 nm with a plate reader.
351 8) The results of ELISA assays from sample of infected mice are compared to known
352 positive and negative controls. Comparisons can be made by ANOVA to compare
353 the average values of positive and negative controls to individual samples.
354 Alternatively, cutoffs for positive values can be determined as described
355 previously (35).
356
357 **3.6 Chronic tissue cyst bank**

358 Outbred mice are useful to maintain chronic infections that are characterized by low
359 cyst burdens (i.e. 50-200 / animal). Cyst numbers can be amplified in strains of inbred
360 mice that develop higher cysts counts (i.e. 500-3,000 / animal). It is advisable to

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361 "bank" strains in CD-1 outbred mice and pass them by sub-inoculating every ~ 6 mos.
362 Cysts are then amplified in susceptible inbred strains (e.g. BALB/c, CBA/J) followed
363 by harvest of tissue cysts over a 1-3 mos period for use in experiments. Although this
364 protocol takes longer to establish, it avoids increases in virulence that can occur with
365 frequent passage and also allows production of high number of cysts for challenge
366 experiments.
367

368 1. Inject mice IP with 100-1,000 tachyzoites that have been grown in tissue culture,
369 harvested, purified by filtration, and resuspended in HHE. Viability can be a major
370 issue since it will take you some time to prepare the inoculum and walk to the
371 animal house. Use only freshly egressed parasites that have been grown under
372 optimum conditions (i.e. 2-3 day synchronous cultures).
373 2. Intermediately virulent strains (i.e. ME49) have LD_{50}s around 10^3-10^5 in outbred
374 mice. Therefore it is usually not necessary to treat the mice to prevent death. You
375 want the mice to be heavily infected without dying. Use animals that survive the
376 highest dose possible to obtain higher cyst counts.
377 3. Virulent Type I strains like GT-1 are more problematic as they will invariably cause
378 death even at low doses. Use an inoculum of 50-100 tachyzoites IP (at lower
379 doses some mice will not become infected).
380 4. To prevent accidental death with high virulence strains, it is necessary to treat the
381 mice with sulfadiazine (dissolved in the drinking water). Begin treatment with a
382 dose of 0.4-0.5 g/L on day 3 or 4 and treat for 6-10 days or until they recover. Even
383 for less virulent strains (i.e. ME49) it is sometimes necessary to treat with
384 sulfadiazine at lower doses (0.1 or 0.2 g/L as above).
385 5. Harvest the brain of chronically infected mice at 1-3 months post-infection and
386 determine the cyst burden by homogenizing and counting a fraction of the
387 homogenate (see **Cyst harvesting and staining** protocol below). It is possible to
388 obtain a sufficient number of cysts from a single animal to use in subsequent
389 challenge studies, depending in the number of animals and desired inoculum.
390 6. To maintain chronic cysts, establish a bank of CD-1 mice and passage them at 6

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391 mos intervals. Infected animals should be humanely sacrificed, brains removed
392 and homogenized. After staining and counting a proportion of the brain (see **Cyst**
393 **harvesting and staining** protocol below) serial passages are done by oral
394 gavage of 5-10 cysts per animal into naïve mice.

395 7. To expand cyst numbers, inoculate 5-10 cysts IP or PO into inbred CBA/J or
396 BALB/c mice, which are more susceptible and will lead to higher cysts counts.
397 Infections can be passed sequentially for 2-3 times in inbred mice, but do not use
398 this for long-term passage as the strain will increase in virulence.

399

400 **3.7 Cyst harvesting and staining**

401 This protocol is designed to help visualize tissue cysts that form in the brain of
402 chronically infected animals. Tissue cysts can be recognized by their appearance in
403 phase contrast microscopy (refractive, slightly amber cyst wall with internal granular
404 material). However, it is much easier to identify them based on positive staining with
405 FITC-conjugated *Dolichos biflorus* lectin (DBL) (36).

406

407 1. Sacrifice chronically infected animals using an approved method.
408 2. Remove the brain and place in a sterile conical 15 ml tube containing ~ 2 ml PBS.
409 Gently mince the brain using a 16 g needle. Draw the tissue into a 5 ml syringe
410 and gently expel. Repeat 3 times using 16 and 18 g needles to homogenize the
411 tissue.
412 3. Add an aliquot of brain lysate (typically ¼ of the total or 0.5 ml) to an equal volume
413 of Fixing and Permeabilizing Solution in a 15 ml polystyrene centrifuge tube.
414 Incubate for 20 min at 4°C.
415 4. Spin at 400 g, 4°C, 5 min. Resuspend in 4 ml PBS/10% goat serum. Spin down
416 pellet at 400 g for 5 min and remove supernatant.
417 5. Retain the pellet and add 1 ml of 10% goat serum in PBS, containing 2~4 µL of
418 FITC-conjugated-lectin stock solution. Incubate at room temperature for 45-60
419 min.

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420 6. Wash twice by centrifugation at 400 g, 15°C for 5 min. Resuspend the pellet each
421 time in 4 ml PBS + 10% goat serum.
422 7. After the final wash, re-suspend cysts in PBS at the original volume of tissue
423 homogenate used.
424 8. Add 12.5 μL of sample to a microscope slide, cover the sample with coverslip, and
425 screen under fluorescence microscope using a 10X objective. Scan the slide at
426 10X and when you see a possible cyst, confirm by examining at 40X. Measure the
427 diameter of the cysts using a calibrated ocular micrometer. Scan the entire slide
428 and determine the cyst number per 12.5 μL. For each sample, count 4 aliquots
429 and determine the total number of cysts. Calculate the number of cysts per brain
430 (total number of cysts in 4 aliquots (50 μL) x 40 = total number per brain).
431

432 **3.6 Chronic infection model**

433 There are many advantages to working with less virulent strains that readily produce
434 chronic infections in mice as both the acute and chronic phases can be studied
435 (Figure 2). Type II strains, such as ME49 (popular clones include the B7 clone, and
436 PTG) and Pru, provide convenient lab-adapted strains for this purpose. Susceptibility
437 of different mouse strains to infection with Type II strain parasites varies in large part
438 due to differences in MHC with BALB/c (H-2d) and C3H/HeN (H-2k) mice being more
439 resistant than C57Bl/6 (H-2b) (37). Hence, the combination of Type II strains with
440 different mouse backgrounds can be used to study acute virulence, chronicity and
441 reactivation. Genetic mutants in the Type II strain are particularly useful for studying
442 attenuation as they are more likely to reveal partial phenotypes that may be masked
443 by the extremely high virulence of Type I strains.

444 1. Inject mice IP with tachyzoites that have been grown in tissue culture, harvested,
445 and resuspended in HHE. Refer to Table 1 for approximate doses depending on
446 the mouse strain being used. Viability can be a major issue since it will take you
447 some time to prepare the inoculum and walk to the animal house. Use only freshly
448 egressed parasites that have been grown under optimum conditions (i.e. 2-3 day
449 synchronous cultures).

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450 2. When testing genetic mutants vs. wild type, it is useful to bracket the inoculum
451 starting at the LD_{50} and increased by half log or log intervals. Typically 5 mice are
452 used per group. It is generally necessary to repeat the experiment at least once
453 since the outcome is influenced by differences in viability. Statistical analysis of
454 Kaplan Meier survival curves is readily performed in Prism (GraphPad).
455 3. Plaque an aliquot of the parasite suspension after injecting them to assure that the
456 different parasite isolates had approximately equal viability.
457 4. During the acute phase (day 4-20) monitor expansion of the parasites using the
458 **Imaging infection by bioluminescence** protocol. It is also useful to monitor
459 weight loss and regain as the mice recover following acute infection.
460 5. Animals are generally followed for 30-60 days, and percent survival is calculated
461 as above. In most cases lethal outcomes will be apparent by day 20, although in
462 rare occasions mutants will show delayed kinetics of death. Use an appropriate
463 end point prior to death, depending on institutionally approved protocol.
464 6. At the end of 30-60 days, sacrifice the animals using an approved method.
465 Remove the brain and perform the **Cyst harvesting and staining protocol.**
466 Statistical analyses of tissue cyst numbers is usually performed using
467 non-parametric tests with adjustment for multiple comparisons.
468 7. It is also possible to test the oral infectivity of tissue cysts by oral gavage (or IP
469 injection) into naive animals. Administer tissue cysts in 100-200 µL PBS
470 suspension, inoculating a dose of 5-10 cysts per animal. Progression of infection
471 can be monitored by sero-conversion using the **ELISA** protocol.

472 **4 Notes**

473 **Strain origins and derivatives:** The type I RH strain was originally isolated from an
474 adolescent who succumbed to fatal encephalitis (38). This lab-adapted strain has
475 been modified to express various transgene reporters including luciferase for
476 bioluminescence imaging (32). One isolate commonly used for genetic studies has
477 been modified to delete the hypoxanthine, xanthine, guanosine phosphoribosyl
478 transferase gene (*HXGPRT* also known as *HXG*), allowing for positive and negative
479 selection (39), as well as disruption of the *KU80* gene, which results in increased

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480 levels of homologous recombination (40, 41). The RHΔ*hxg*Δ*ku80* strain maintains the
481 full virulence of the wild type RH strain. Another commonly used Type I strain is GT-1,
482 which was isolated from a goat (42), and which maintains the entire life cycle unlike
483 the commonly used RH strain (12). Type II strains are also widely used for creating
484 chronic infections and for virulence studies where the LD_{50} can be titrated in inbred
485 mice. The most common of these is the ME49 strain, originally isolated from a sheep
486 in California (43): it readily generates chronic infection in mice and undergoes the
487 entire life cycle in cats (23). The ME49 strain is also available as a Δ*hxgprt* knockout
488 for facilitating selection and is tagged with firefly luciferase (FLUC) (33). Another
489 commonly used Type II strain is Prugniaud (aka Pru), originally isolated from a
490 congenital human infection in France, and including a variant that is lacking both
491 *KU80* and *HXGPRT* (PruΔ*hxg*Δ*ku80)* (44). Although less commonly used, Type III
492 strains also generate chronic infections in mice but are rarely used for infection
493 studies due to their relative avirulence (4). The Type III strain CTG was originally
494 isolated from a cat in New Hampshire (22) and the VEG strain was isolated from an
495 immunocompromised patient (45).
496 **Acute virulence:** Infection with Type I strains is always lethal in laboratory mice, and
497 any animals surviving at low inoculum are sero-negative (i.e., never infected). We
498 favor the use of outbred mice, which are more resistant, as they clearly discriminate
499 from the high virulence of Type I strains in comparison to strains that have
500 intermediate virulence that depends on the mouse strain. This definition of acute
501 virulence has been used to establish the difference between clonal types (11), and
502 map virulence differences between them (12, 24, 31). With Type II strains, the LD_{50} in
503 outbred and inbred mice differs substantially (Table 1) and aspects of pathogenesis
504 during chronic infection can be evaluated in surviving mice. The LD_{50} of Type II
505 strains is also somewhat dependent on the local mouse colony, so it needs to be
506 titrated in each instance.
507 **Bioluminescence:** The sensitivity of bioluminescence detection *in vivo* is not easy to
508 relate directly to parasite number and it is likely that high tissue burdens (i.e. > 10^5
509 parasites / gram) are needed to detect infection. Additionally, it can be more

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510 challenging to detect infection in deep tissues in particular in the CNS due to the fact
511 that the cranium partially blocks the signal. Nonetheless, this method has proven
512 highly useful for tracking acute infection and dissemination (8, 31, 46), as well as
513 reactivation of infection in immunocompromised mice (47).
514 **Alternatives for monitoring tissue burdens:** Other approaches for monitoring the
515 number of parasites in tissue following acute or chronic infection have been
516 developed based on PCR (48) or plaquing (13). These methods have the advantage
517 of being quantitative in terms of relating to genome equivalents or infectious units.
518 However, PCR can over-estimate the number of parasites due to remnant DNA that is
519 not derived from viable organisms (48). Using an RNA target and performing
520 qRT-PCR can partially compensate for this possibility (49), although neither method
521 measures viable parasites. The main limitations of these methods are they are time
522 consuming, and do not allow the possibility of sampling the same mouse repeatedly.
523

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531

532 **Figure Legends**

533 **Figure 1.** Acute virulence model in outbred CD-1 mice. Following IP challenge with
534 different doses of tachyzoites, mice are followed for 30 days post-infection. Useful
535 end points include weight loss, bioluminescence imaging for luciferase expressing
536 parasites, percent mortality, and time to death. Example shows control and infected
537 mouse imaged for firefly luciferase (FLUC) expression.
538

539 **Figure 2.** Chronic infection model in inbred mice. Infections can be administered

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540 either by IP injection of tachyzoites or by oral feeding of tissue cysts derived from
541 chronically infected animals. Useful end points include dose-dependent mortality, cyst
542 burden, and cyst size. It is also possible to use bioluminescence imaging to evaluate
543 differences in parasite numbers during the acute phase. The infectivity of tissue cysts
544 can also be tested by sub-inoculation into naive animals. Example shows tissue cyst
545 stained with *Dolichos biflorus* lectin. Scale bar = 10 microns.

546

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704

Table 1 Representative strains of *T. gondii* useful for mouse infection

		LD50 values				
Types	Strains	CD-1[a]	C57BL/6[a]	BALB/c[a]	ATCC #	Ref
I	RHΔhxgΔku80 GT-1	1 1	1 1	1 1	PRA-319 50853[b]	40, 41 42
II	ME49Δhxg::FLUC Pru ΔhxgΔku80	10^3 10^5	100-200 500-1,000	200-500 500-1,000	50611[b] NA	33 44
III	CTG (CEP) VEG	$>10^6$ $>10^6$	$>10^4$ $>10^4$	$>10^4$ $>10^4$	50842[b] 50861	22 45

[a] These values will vary with local mouse colony and need to be tested.

[b] Reference lines are wild type, although FLUC lines can be obtained on request

Figure 1

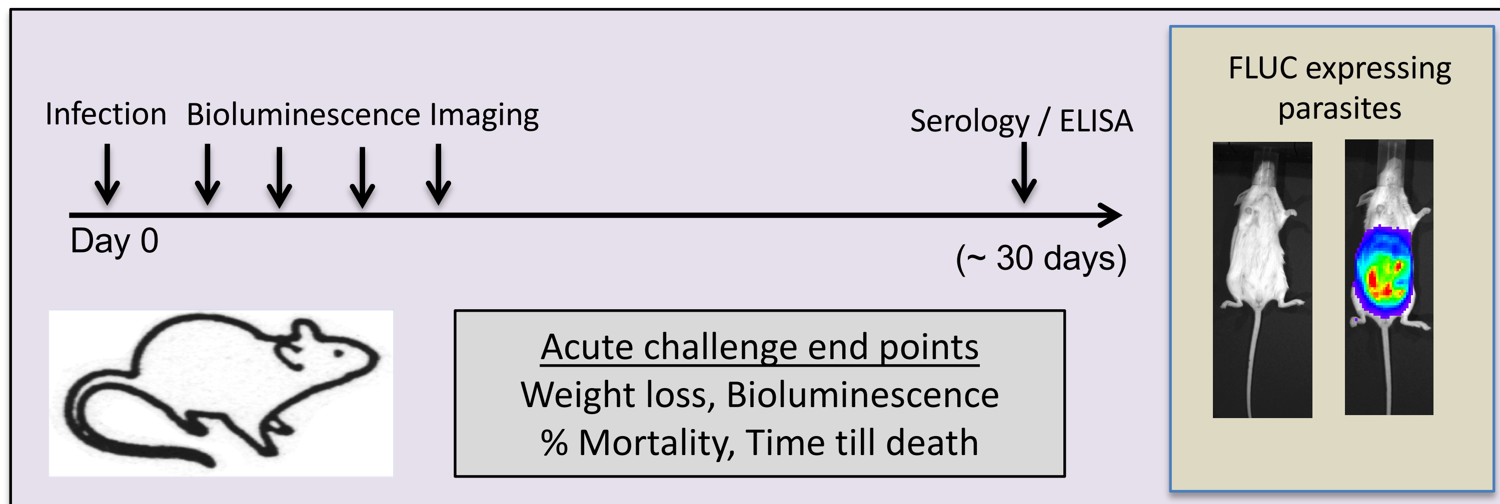

Figure 2