# *Verifying and Quantifying Helicobacter pylori Infection Status of Research Mice [Chapter 19]*



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# Verifying and Quantifying *Helicobacter pylori* Infection Status of Research Mice

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## Abstract

Mice used to model helicobacter gastritis should be screened by PCR prior to experimental dosing to confirm the absence of enterohepatic *Helicobacter* species (EHS) that colonize the cecum and colon of mice. Natural infections with EHS are common and impact of concurrent EHS infection on *Helicobacter pylori*-induced gastric pathology has been demonstrated.

PCR of DNA isolated from gastric tissue is the most sensitive and efficient technique to confirm the *H. pylori* infection status of research mice after experimental dosing. To determine the level of colonization, quantitative PCR to estimate the equivalent colony-forming units of *H. pylori* per μg of mouse DNA is less labor-intensive than limiting dilution culture methods. Culture recovery of *H. pylori* is a less sensitive technique due to its fastidious in vitro culture requirements; however, recovery of viable organisms confirms persistent colonization and allows for further molecular characterization of wild-type or mutant *H. pylori* strains. ELISA is useful to confirm PCR and culture results and to correlate pro- and anti-inflammatory host immune responses with lesion severity and cytokine gene or protein expression. Histologic assessment with a silver stain has a role in identifying gastric bacteria with spiral morphology consistent with *H. pylori* but is a relatively insensitive technique and lacks specificity. A variety of spiral bacteria colonizing the lower bowel of mice can be observed in the stomach, particularly if gastric atrophy develops, and these species are not morphologically distinct at the level of light microscopy either in the stomach or lower bowel. Other less commonly used techniques to localize *H. pylori* in tissues include immunohistochemistry using labeled polyclonal antisera or in situ hybridization for *H. pylori* rRNA. In this chapter, we will summarize strategies to allow initiation of experiments with helicobacter-free mice and then focus on PCR and ELISA techniques to verify and quantify *H. pylori* infection of research mice.

### Keywords

*Helicobacter pylori*; Mice; ELISA; Quantitative PCR

## 1. Introduction

Gastric helicobacter infections modeled in mice include mouse-adapted *Helicobacter pylori* SS1 (1), *Helicobacter felis* for its ability to induce robust gastritis (2) and *Helicobacter heilmannii* for its induction of MALT lymphoma (3). To most closely model *H. pylori* gastritis and the sequelae of gastric atrophy and cancer in humans, *H. pylori* SS1 is commonly used due to the restricted scope of *H. pylori* strains that will colonize mice. Included here are PCR and serology methods within the capability of most laboratories for verifying the helicobacter-free infection status of research mice pre-dosing and verifying and

quantifying *H. pylori* SS1 infection post-dosing. PCR and serology techniques are similar for EHS as well as gastric helicobacters with the exception that fecal PCR has not been a reliable technique in our laboratory for detecting *H. pylori* infection of mice. The value of histologic techniques, such as silver stain, immunohistochemistry and in situ hybridization, and culture as screening techniques is summarized but details of these latter techniques are not within the scope of this chapter.

### 1.1. Strategy to Verify Helicobacter Infection Status of Research Mice

Investigators need to be aware that the *Helicobacter* genus currently includes at least nine formally named enterohepatic species (EHS), as well as a number of novel isolates yet to be formally named, that colonize the cecum and colon of mice unless specific precautions are taken to exclude them from research colonies (4, 5). Helicobacter-free mice are routinely available from commercial vendors but endemic EHS infection of mice maintained in academic facilities is common (4). Investigators using *H. pylori* mouse models should confirm that their mice are free of EHS as impact of concurrent helicobacter-associated disease has been demonstrated in at least two mouse models (6, 7). Commercial vendors maintain health profiles of their mouse colonies on their Web sites. Note that specific pathogen free (SPF) health status for *Helicobacter* spp. should be verified using *Helicobacter* genus PCR primers and not be limited to *Helicobacter* species-specific exclusion. Mice naturally infected with EHS should be embryo transfer rederived, confirmed helicobacter-free by genus-inclusive PCR, and subsequently maintained in a helicobacter-free barrier facility.

To verify that research mice are helicobacter-free, feces (for example, from live mice, prior to *H. pylori* experimental infection) or a mucosal scraping of the cecal–colic junction (for example to confirm the status of EHS infection at the completion of an experiment) collected aseptically at necropsy should be PCR amplified using *Helicobacter* genus-specific primers (8). If helicobacter speciation is important, PCR products can be sequenced or nested PCR performed. Using *Helicobacter* species-specific primers to amplify the PCR product from the initial genus-level PCR will confirm the presence or absence of specific EHS as well as enhance the overall sensitivity of the PCR screen for EHS (9). For additional evidence and further characterization of EHS infection, feces or cecal scrapings should be stored at −70°C in Brucella broth containing 30% glycerol pending microaerobic culture by an appropriately experienced microbiologist (see Chapter on culture techniques).

Warthin-Starry or Steiner silver stains can be used to localize EHS in the liver parenchyma and biliary system of susceptible mouse strains or gastric helicobacters (*H. pylori, H. felis*) in the stomach crypts of experimentally challenged mice. Silver staining of lower bowel to screen for EHS is unrewarding because other morphologically similar bacteria may also be visualized with the silver stain. In the liver of EHS-infected mice, helicobacters such as *H. hepaticus* or *H. bilis* are localized between hepatocytes within biliary cuniculi and can be difficult to visualize if the inflammation is significant. Immunohistochemistry using polyclonal antibodies to detect EHS in mouse livers (10) and *H. pylori* in mouse stomachs have been described (11) as has in situ hybridization for whole cell detection of *H. pylori* in mouse stomach (11) using probes developed for detecting rRNA of *H. pylori* in human gastric biopsies (12).

Commercial serology for helicobacter infection of rodents is not available and lack of seroconversion is not definitive evidence of a helicobacter-free health status, particularly in transgenic mice with potential immunodeficiency. In contrast, successful experimental infection of immunocompetent mice is readily supported by “in-house” positive ELISA serology to helicobacter antigens and has been shown to be an assay with sensitivity exceeding 90%, although with variable specificity, particularly if mice are infected

concurrently with multiple EHS (13). The ELISA methods described here are intended to confirm seroconversion of experimentally dosed mice and to characterize the IgG subclass response to chronic *H. pylori* (or *H. felis*) colonization, as B cell isotype class switch recombination reflects polarization of the pro- and anti-inflammatory T cell subset responses. IgG1 and IgG2a/2c (14) subclass responses will typically support pathology findings and cytokine profiles in helicobacter studies (6, 7). As a research tool, serology is inexpensive, noninvasive and allows for serial sampling of individual animals.

## 2. Materials

### 2.1. Helicobacter Genus-Specific 16S rDNA-Based PCR Assays for Detecting Helicobacters in Feces and Tissues

1. High Pure PCR Template Preparation Kit (Roche Applied Science Part No.: 11 796 828 001).
2. QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, CA Part No.: 51504).
3. RNase-Free disposable pellet pestles with microtubes.
4. Fresh feces or murine tissues which have been stored in sterile 2 mL microtubes at −20°C prior to DNA extraction.
5. Lysozyme at 10 mg/mL in 10 mM Tris–HCl (pH 8.0).
6. Sterile PBS (adjust pH to 7.4 with HCl): 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$.
7. PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare).
8. Expand High Fidelity PCR System (Roche Applied Sciences, Indianapolis, IN): Expand High Fidelity Enzyme mix with 10× Reaction Buffer.
9. Agarose.
10. 1× TAE buffer: 40 mM Tris-acetate, 1 mM EDTA (pH 8.0).
11. 1× TE buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0).
12. Ethidium bromide.
13. Prepare a solution containing both forward and reverse primers (5 μM of each primer) as 10× stock (see Note 1).
14. Ure-B primers (Applied Biosystems). Original stocks of primers and probe at 100 μM; 20× stock: a solution containing 4 μM of both primers and the probe (see Note 2).
15. TaqMan 7500 FAST detection system.

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[1] *Helicobacter* genus-specific PCR primers C97 (Forward: 5′-GCTATGACGGGGTATCC-3′ corresponding to positions 276–291) and C05 (Reverse: 5′-ACTTCACCCCAGTCG CTG-3′ corresponding to positions 1,478–1,495) were derived from the 16S rRNA gene for amplifying a ~1,200 bp *Helicobacter*-specific PCR product as previously described (8). A pair of internal primers, C98F (Forward: 5′-TGGTGTAGGGGTAA AATC-3′ corresponding to positions 681–698) and H3A-20 (Reverse: 5′-GCCGTGCAGCACCTGTTTTC-3′ corresponding to positions 1,007–1,026), are used for nested PCR based on the C97/C05-amplified products (see Note 12) (16). Positions of the primer sequences are in reference to the *H. pylori* 16S rRNA sequence. Primers are synthesized and SDS-PAGE-purified by Integrated DNA Technologies, Inc. Store the primers at 100 μM in sterile deionized $ddH_2O$ as stock at −70°C.

[2] *ureB*-based primers and a probe were developed and used for quantification of *H. pylori* SS1 as previously described (17). Forward primer, 5′-CAAAATCGCTGGCATTGGT-3′; Reverse primer, 5′-CTTCACCGGCTAAGGCTTCA-3′; probe 5′-AACAAAGACATGCAAGATGGCGTTAAAAACA-3′, which is labeled with six FAM dye at 5′-end and with MGB (quencher) at 3′-end.

16. MicroAmp® Fast Optical 96-Well Reaction Plate.
17. TaqMan® Fast Universal PCR Master Mix (2×).
18. Adhesive film.
19. FAM-labeled 18S rRNA gene-based primer/probe solution.
20. Electrophoresis loading buffer (6×): 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol in $ddH_2O$.

### 2.2. ELISA Materials

1. Bacterial sonicator (Misonix Ultrasonic Processor Model S-4000, QSonic LLC, Newtown, CT, or equivalent).
2. Phase microscope to confirm adequate cell lysis (see Notes 3 and [4]).
3. *N*-octyl-beta-glucopyranoside.
4. Ultracentrifuge capable of generating 100,000 × *g* for 1 h at 4°C (Sorvall Ultra Pro 80, Thermo Scientific) or equivalent.
5. Compatible ultracentrifuge tubes.
6. Mineral oil for balancing centrifuge tubes.
7. 4 in. section of dialysis tubing with a molecular weight cutoff of 12–14,000 kDa.
8. Two locking, compression clamps designed to seal dialysis tubing.
9. Spectrophotometer.
10. Lowry kit.
11. Sorvall RC-5B Refrigerated Superspeed Centrifuge (or equivalent).
12. Bovine serum albumen (BSA).
13. ELISA plate blocking buffer (volumes of 1 and 2% BSA in PBS).
14. Timer.
15. 37°C incubator.
16. Antigen coating buffer: 1.59 g $Na_2CO_3$, 2.93 g $NaHCO_3$, in 1 L of $ddH_2O$, pH of 9.6 (see Note 5).
17. Immulon II High Binding (protein) (designated as 2HB) 96-well plates (Thermo Scientific).
18. Biotinylated secondary (detection) antibodies including polyclonal goat anti-mouse IgG and monoclonal rat anti-mouse antibodies produced by clones A85-1, R19-15

[3]Preparation of antigens from *H. pylori* cultures should be performed in a biohazard containment hood (except for centrifugation steps). Reagents inclusive of secondary (detection) antibodies can be prepared and used at room temperature for the assay duration but otherwise should be stored at 4°C to avoid potential freeze–thaw damage. Sample sera should be stored at −20°C and then thawed and diluted in 1% BSA in PBS just prior to the assay. Consider avoiding freeze–thaw damage to sample sera by aliquoting samples when first collected.

[4]Sonication produces a whole cell lysate whereas the OMP method theoretically selects for outer cell wall antigens and discards the more highly conserved, cytosolic proteins. In theory, OMP should yield an assay with higher specificity but this does not have practical significance when seroconversion is being evaluated in mice experimentally infected with a gastric helicobacter and are otherwise SPF for EHS. The protein yield from the OMP method is lower due to the nature of detergent digestion in a 4 mL volume and sample loss inherent in centrifugation steps.

[5]This has a 2-week shelf life.

and 5.7 (BD PharMingen) for detecting IgG1, IgG2a, and IgG2a$^b$ (also known as IgG2c), respectively (see Note 6).

19. Extravidin peroxidase (Sigma) diluted 1:2,000 in 1% BSA in PBS.
20. 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS®) substrate.
21. Automated ELISA plate washer with six cycle wash of 300 μL per well.
22. PBS-Tween (0.05%).
23. ELISA plate reader such as Dynatech MR7000 (Dynatech Laboratories, Inc., Chantilly, VA) capable of reading wavelength matched to color substrate (such as 405 nm for ABTS®).
24. Plastic wrap.
25. 1% sodium dodecyl sulfate (SDS).

## 3. Methods

### 3.1. PCR for Helicobacter Infection in Animals

**3.1.1. Preparation of PCR DNA Templates (see Note 7)—**Unless otherwise specified, all procedures are conducted at room temperature and all reagents and solutions are prepared with deionized dd$H_2O$.

1. DNA from murine tissues should be isolated using the High Pure PCR Template Preparation Kit following protocol 2.4 in the kit manual. Bacterial DNA from cultivated helicobacter cells should be isolated using the High Pure PCR Template Preparation kit following protocol 2.6 included in the kit directions (see Note 8).
2. Isolate bacterial DNA from cultivated helicobacter as a control (see Note 9).
3. Prepare DNA from feces for screening mice for EHS (see Note 10) according to the protocol described in the QIAamp DNA Stool Mini Kit (see Note 11).

**3.1.2. PCR Detection of Helicobacter DNA Using the Genus-Specific Primers (see Note 12) Using Ready-to-Go PCR Beads (see Note 13)**

1. Add ~50–500 ng of DNA template, 2.5 μL of 10× primer stock for C97/C05 or C98F/H3A-20 and dd$H_2$ O up to a final volume of 25 μL in a 0.5-mL microtube

[6]Based on mouse genetics, specific detection antibodies must be used to accurately quantify Th1-associated subclass responses because C57BL/6, C57BL/10, and NOD mice do not express the IgG2a isotype and instead produce the IgG2a$^b$ isotype, also known as IgG2c (14).
[7]Preparation of DNA from feces or tissues used to screen mice for EHS or from *H. pylori*-infected tissues should be performed in a biohazard containment hood (except for centrifugation steps). Some EHS of mice may have zoonotic potential (18, 19).
[8]For increasing DNA yield from tissue, homogenize each sample with a pestle in a 1.5 mL microtube prior to incubation with proteinase K as described in protocol 2.4 included in the kit directions.
[9]Our laboratory uses High Pure PCR Template Preparation kit following protocol 2.6.
[10]Minor modifications to the kit directions include: In step 1 of kit manual, we use 2–3 fecal pellets instead of the suggested 180–220 mg of feces; the fecal pellets are homogenized in 1.4 mL of kit-supplied buffer ASL. In step 6 of kit manual, one-half of an inhibitEX tablet is used rather than the suggested whole tablet. Note that fecal PCR to detect *H. pylori* has not been a reliable diagnostic technique in our laboratory.
[11](Pages 15–18 for isolation of pathogen DNA from stool) with minor modifications (see Note 10).
[12]Measure quality of DNA samples based on a ratio of spectrophotometry wavelengths (usually A260/A280 ≥1.6) using a standard spectrophotometer or the more recently available Nanodrop technology (Thermo Scientific) that measures DNA quality using very small volumes (0.5 μL). Adjust tissue or fecal DNA concentrations to approximately 25–50 ng/μL with TE buffer. Thaw and mix DNA samples before loading them onto the plate. For increasing helicobacter detection sensitivity, nested PCR can be performed following the protocols described in Subheadings 3.1.2 or 3.1.3 by using 2 μL of the C97/C05-amplified mixture (obtained using Subheadings 3.1.2 or 3.1.3) as nested PCR template and 10× primer stock of C98F/H3A-20.

containing PCR beads. Mix gently with pipetting and centrifuge the reaction mixture for 10 s at 9,000 × *g*.

2. Place the samples into a PCR machine fitted with a heated lid and run the assay using an initial incubation step of 3 min at 94°C followed by cycle program of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C for 35 cycles followed by 8 min at 72°C and holding at 6°C pending removal of the samples.

3. Mix 10 μL of each PCR sample with 2 μL of 6× electrophoresis loading buffer and load samples on a 1.2% agarose gel. PCR products are separated by electrophoresis at 100 V for ~60 min in 1× TAE buffer followed by staining in $ddH_2O$ containing 0.5 μg/mL ethidium bromide for 15–30 min. After destaining with $ddH_2O$ for at least 15 min, DNA bands can be visualized under UV light.

### 3.1.3. PCR with High Fidelity PCR System (see Note 14)

1. Set up the reaction mixture in a total volume of 50 μL: template DNA (generally 5 μL containing 50–500 ng template DNA), 1 μL of dNTP mixture (10 mM each of dATP, dCTP, dGTP, dTTP), 5 μL of 10× primer stock for C97/C05 or C98F/H3A-20, 5 μL of Expand High Fidelity Buffer 2, 0.75 μL Expand High Fidelity Enzyme mix (2.6 U/reaction), with balance to 50 μL with $ddH_2O$. The thermocycling program is the same as described step 2 of Subheading 3.1.2. *Using Ready-to-Go PCR beads*.

### 3.1.4. Quantitative PCR (qPCR) for Estimating H. pylori Strain SS1 Colonization Levels in Tissues of Experimentally Infected Mice (see Note 15)

1. Add 5 μL of each template DNA into duplicate wells of a MicroAmp® Fast Optical 96-Well Reaction Plate (see Note 16). In two non-template control (NTC) wells, add 5 μL $ddH_2O$ in place of DNA for ruling out DNA contamination.

2. Create a standard curve using serial tenfold dilutions of *H. pylori* SS1 genomic DNA (see Note 17). 5 μL of each standard (equal to $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, $10^1$, respectively, of SS1 genome copies) is added in duplicate to the MicroAmp® Fast Optical 96-Well Reaction Plate.

3. Carefully transfer 15 μL of a reaction mixture containing 10 μL of 2× Master Mix, 1 μL of 20× Primers/Probe mix, and 4 μL $ddH_2O$ (see Note 18) into each well containing controls or samples (see Note 19).

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[13] PCR beads contain all required reagents for PCR except for primers and template DNA and are supplied in individual PCR tubes. Advantages of using PCR beads include minimizing variability in reagent preparation and minimizing DNA contamination. PCR beads are generally used for production of amplicon products <2 kb in size. In some samples, nonspecific amplicons may be generated along with the amplicon of interest.

[14] High Fidelity PCR System yields PCR products up to 5 kb in size with high specificity and sequence fidelity. PCR products used for subsequent cloning and sequencing should be produced using this system to minimize introduction of error nucleotides during PCR. Disadvantages of the High Fidelity PCR System include increased expense, time for preparing PCR reactions and increased risk of DNA contamination.

[15] Due to extensive nucleotide sequence diversity within allelic genes among *H. pylori* strains, these primers and probe may not adequately hybridize to non-SS1 *H. pylori* strains.

[16] The manufacturer recommends use of quadruple replicates per sample. We found that use of duplicate samples reduced cost without compromising results.

[17] Based on the average size (~1.66 mb) of two sequenced *H. pylori* strains 26695 (3) and J99 (4), two fentograms of *H. pylori* SS1 DNA is approximately equal to one genome copy.

[18] When pipetting samples into wells, avoid creating air bubbles that will interfere with fluorescence readings. Carefully pipette the reaction mixture along the wall of each well and allow the mixture to settle to the bottom by gravity.

[19] The reaction mixture of primers/probe for all samples can be prepared in a single tube and distributed into individual wells using an eight-channel pipette. Prepare extra reaction mixture volume to compensate for pipetting error, e.g., one extra reaction volume per eight wells.

4. Seal the plate with the kit-supplied adhesive film and compression pad. Centrifuge plates briefly at 1,050× *g* in a Labofuge 400 (Heraeus Instruments) before loading it into the TaqMan 7500 FAST detection system.

5. Warm the TaqMan 7500 for at least 15 min then follow the manufacturer's software instructions for setting up an assay plate, including defining baseline and data analyses. Run the assay in the 7,500 Fast using the default setting (requires about ~40 min).

6. To normalize genomic copies of *H. pylori* SS1 between samples, quantities of mouse DNA in each sample are first measured using the 18S rRNA gene-based primer/probe mixture supplied by Applied Biosystems as described in steps 1–5. A standard curve is generated from serial dilution of DNA isolated from the cecum (tissue) of *Helicobacter*-free mice as follows in pg: $10^5$, $10^4$, $10^3$, $10^2$, $10^1$. Genomic copies of *H. pylori* SS1 may then be reported as copies of *H. pylori* per μg mouse DNA.

## 3.2. Antigen Preparation

### 3.2.1. Sonication of Helicobacter Cells for Antigen

1. Harvest helicobacter cells from ten confluent blood agar culture plates or from a 250 mL broth suspension. Pellet the broth suspension by centrifugation using Sorvall RC-5B Refrigerated Superspeed Centrifuge (or equivalent) set at 6,000 rpm (to achieve approximately 5,000 × *g*) for 10 min at 4°C. Resuspend harvested cells in 10 mL sterile PBS (see Note 20). Repeat PBS wash two more times with final resuspension in approximately 1 mL sterile PBS (should be turbid). Confirm culture purity by gram stain and phase microscopy.

2. Sonicator instrument (use in a biocontainment hood) settings for duration, amplitude, and number of cycles need to be empirically established. The goal is to lyse the majority of cells without overheating the suspension. Keep the suspension in an ice bath during the entire sonication cycle to prevent overheating of proteins. Using the Misonix sonicator, an example of a sonication cycle is 15–30 s of sonication using the Microtip™ set at an amplitude of 40%, chilling the suspension for an additional 20–60 s on ice and then repeating the entire cycle 4–5 more times. Turbid bacterial suspensions will increase in clarity as cells are effectively lysed which should be confirmed by phase microscopy. Sterile technique is not essential but sterile PBS and tubes should be used and the sonicator probe should be cleaned with 70% ethanol prior to use. Measure total protein concentration (Lowry kit) and store sonicate at −20°C and aliquot into 1 mL maximum volumes (to avoid freeze–thaw damage). Typical protein concentrations achieved are up to 1,500 μg/mL.

**3.2.2. Preparation of Outer Membrane Proteins for Antigen—**This technique was adapted from a previous publication (15).

1. Perform step 1 as under Sonication method except resuspend the final pellet (following the last PBS wash) in 4 mL of 1% *N*-octyl-beta-glucopyranoside (Sigma) (40 mg in 4 mL of PBS).

2. Incubate suspension at room temperature for 30 min. Vortex every 10 min to encourage membrane digestion.

[20]To prepare antigen from broth culture, inoculate 250 mL of Brucella broth containing 5% fetal calf serum with helicobacter cells harvested from one confluent blood agar plate. Incubate overnight at 37°C to achieve a turbidity reading measured by spectrophotometry of 1.0 at 600 nm wavelength. 250 mL will generate a cell pellet equivalent to approximately $2 \times 10^{11}$ cells.

3. Pipette suspension into ultracentrifuge tubes and bring tube volume close to the top of the tube with mineral oil (to prevent tube collapse during ultracentrifugation) and balance tube total weights with mineral oil.
4. Ultracentrifuge at 100,000 × *g* for 1 h at 4°C.
5. In the interim, soak a 4 in. section of dialysis tubing in PBS at room temperature to wet membrane.
6. After ultracentrifugation, pipette off the majority of the mineral oil and discard. Insert pipette tip down through any remaining mineral oil and remove the middle, clear to amber, fluid layer which contains the solubilized OMPs. The pelleted material consists of cytosolic debris and should be discarded.
7. Approximately 1 in. from the end of a 4 in. section of prewetted dialysis tubing, firmly apply a locking, compression clamp. Gently pipette (to avoid membrane tear) the outer membrane proteins (OMP) solution into the dialysis tubing followed by firmly applying a second clamp just above the fluid layer. Leave 1 in. of dialysis tubing protruding from both clamped ends to ensure a tight seal.
8. Place dialysis tubing containing OMP solution into 2 L of PBS for 12 h at 4°C. Decant and replace the PBS and dialyze for an additional 6 h. Times and temperatures are empirical and using a stir bar is optional. Slight modifications are not known to alter ELISA performance.
9. Measure total protein concentration (Lowry kit) and aliquot into 1 mL maximum volumes for use or storage at −20°C (to avoid freeze–thaw damage). Typical protein concentrations achieved are lower than by the sonication method but optimally will be 400–1,000 μg/mL.

### 3.3. Mouse Serum ELISA to Detect Helicobacter-Specific IgG or Isotypes

The following method describes an endpoint ELISA performed at a serum dilution of 1:100. It can be adapted to measuring titers by serial twofold dilution of sera.

One Day Prior to Performing the Assay

1. Design a plate map (see Note 21).
2. Prepare 2% BSA in PBS blocking solution (see Note 22) and carbonate buffer.
3. Coat 96-well Immulon 2 HB plate(s) with 1 μg/mL (for IgG) or 10 μg/mL (for IgG isotypes) of sonicate or OMP antigens in carbonate buffer. Requires 10 mL of either protein concentration per 96-well plate mixed in carbonate. Pipette 100 μL

[21]Design a plate map to maximize use of multichannel pipettes to efficiently apply reagents. Assign plate wells for triplicate control (i.e., blank or background wells without sera) and triplicate samples. For a total IgG assay, designate well 1–3 in row A as blanks and designate the remaining 93 wells for samples to be pipetted in triplicate, oriented either in a horizontal or vertical direction to fill the plate (can thus accommodate 31 test sera). For IgG isotype measurements, each isotype requires its own triplicate blank wells reserved in row A. An efficient design is to designate row A wells 1–3 for IgG blanks, row A wells 4–6 for IgG1 blanks, and row A wells 7–9 for IgG2a or IgG2c blanks (see Note 6). Each sample can then be pipetted horizontally across nine wells using a multichannel pipette without having to change pipette tips. Remaining samples are then similarly pipetted into rows B through H under their respective blanks. In this design, columns 10–12 would be left empty. The plate map should indicate the antigen coating concentration (1 μg/mL for IgG, 10 μg/mL for isotypes) and where each individual detection antibody (anti-IgG, anti-IgG1, or anti-IgG2a/2c) will be pipetted. After application of detection antibodies, every well receives the same reagents (i.e., Extravidin peroxidase, ABTS).

[22]Because BSA is hydrophobic, make 2% BSA in PBS blocking buffer on the day prior to the ELISA by adding 2 g of BSA to every 100 mL of PBS required and store at 4°C overnight. Mixing is not necessary and unnecessarily creates protein foam. The blocking step will require 20 mL of blocking buffer per plate. Make excess blocking solution to conveniently create 1% BSA in PBS during the blocking incubation step the following day to accommodate dilution of sera and other reagents (simply adding equal volume of PBS to the 2% BSA in PBS left over from the blocking step to create 1% BSA in PBS).

into every well according to the plate map. Cover plate(s) with plastic film to prevent dehydration and incubate at 4°C overnight.

The Following Morning (the Day that ELISA is Performed)

4. Wash plates 6× with 300 μL per well of PBS-0.05% Tween washing buffer. After last wash cycle, invert plate(s) with a sharp motion onto a fresh paper towel to absorb residual wash buffer.
5. Pipette 200 μL of 2% BSA in PBS blocking buffer into every well, Cover plate(s) with plastic film and incubate at 37°C for 1 h.
6. During blocking step, dilute sera 1:100 in 1% BSA in PBS. Make sufficient volume of diluted sera (100 μL of diluted sera per well) to accommodate the plate map with 100–200 μL extra volume of each sample to adjust for pipetting error. For example, add 5 μL serum sample into 495 μL 1% BSA in PBS so each sample can be tested in triplicate (requires 300 μL of diluted sera), leaving 200 μL for pipetting error.
7. Wash plates 6× with 300 μL per well of PBS-0.05% Tween washing buffer. After last wash cycle, invert plate(s) with a sharp motion onto a fresh paper towel to absorb residual wash buffer.
8. Apply 100 μL of samples (or 1% BSA in PBS to control blank wells) following the plate map. Cover plate(s) with plastic film and incubate at 37°C for 1 h.
9. Wash plates 6× with 300 μL per well of PBS-0.05% Tween washing buffer. After last wash cycle, invert plate(s) with a sharp motion onto a fresh paper towel to absorb residual wash buffer.
10. Following plate map, apply 100 μL of biotinylated detection antibody diluted 1:2,000 in 1% BSA in PBS (goat anti-mouse IgG, monoclonal rat anti-mouse IgG1, 2a, or 2c). Note that secondary (detection) antibodies are also pipetted into blank wells according to plate map. Cover plate(s) with plastic film and incubate at 37°C for 1 h.
11. Wash plates 6× with 300 μL per well of PBS-0.05% Tween washing buffer. After last wash cycle, invert plate(s) with a sharp motion onto a fresh paper towel to absorb residual wash buffer.
12. Apply 100 μL of Extravidin-peroxidase diluted 1: 2,000 to every well designated as blank or sample. Cover plate(s) with plastic film and incubate at 37°C for 30 min.
13. During this incubation (step 9), mix sufficient ABTS solutions labeled A and B (1:1 ratio) to yield 100 μL per well (10 mL per plate). Mix in a glass bottle, store ABTS in the dark and let reach room temperature before use.
14. Wash plates 6× with 300 μL per well of PBS-0.05% Tween washing buffer. After last wash cycle, invert plate(s) with a sharp motion onto a fresh paper towel to absorb residual wash buffer.
15. Apply 100 μL ABTS to every well designated as blank or sample and incubate uncovered (no plastic film) at room temperature for 30 min in the dark.
16. Optionally, add 100 μL 1% SDS as a stop solution; does not change wavelength reading. This stop solution is specific to ABTS and other stop solutions do change the required reading wavelength for other color substrates.
17. Using an ELISA reader, measure optical densities at 405 nm (specific for ABTS substrate) at 30 min. Some ELISA readers have dual wavelength capability and will simultaneously read a distant wavelength such as 590 nm to correct for background

optical distortions. Adjust time based on color development, record OD at different time points if indicated.

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