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Protocol

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Identification of immunogenic commensal antigens using phage display

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Abstract

Humoral immunity plays a major role in the establishment and maintenance of host–microbiota commensalism and immunity to pathogenic microorganisms. However, identification of antigens eliciting adaptive immune responses within barrier and systemic tissues represents a significant hurdle to further understanding this host–microbe dialogue. Here, we provide a protocol to identify immunogenic protein antigens expressed by commensal and pathogenic microbes by using bacteriophage (phage) display-mediated antibody/antigen biopanning. The procedure entails generation of an M13-phagemid library, production of an ORFeome (totality of open reading frames) phage library followed by multiple rounds of affinity-based immunoprecipitation and subsequent antigen validation by ELISA, ELISPOT and/or B cell tetramer generation. The protocol is optimized to identify antigens eliciting both IgA and IgG isotype responses and can use either circulating or intestinal antibodies for antigen identification. Generation and isolation of monoclonal phage encoding putative protein antigens enable simple identification of immunogenic antigens by Sanger sequencing, often providing protein domain-level resolution of epitope-bearing regions. Our protocol can be carried out by a trained molecular biologist and enables antigen identification and validation in the timeframe of weeks.

Key points

- This Protocol identifies immunogenic protein antigens expressed by commensal and pathogenic microbes by phage display-mediated antibody/antigen biopanning and subsequent antigen validation by ELISA, ELISPOT and/or B cell tetramer generation.
- This enables a priori identification of antigen-specific B cells from the polyclonal repertoire, allowing users to bypass a number of laborious experimental approaches common to previously available methods.

Key reference

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Introduction

The commensal microbiota plays a fundamental role in the function of the immune system[1]. B and T cell responses to commensal microbes are essential for barrier tissue homeostasis, but it is increasingly apparent that dysregulation of commensal-specific immunity is also associated with systemic inflammatory disorders[2]. B cells and antibodies are key components of the intestinal immune response to commensals, contributing to intestinal homeostasis, microbiome compartmentalization, diversity and gut function. Commensal-specific IgA is present in high concentrations in intestinal tissue and is secreted into the gut lumen, where it influences bacterial adherence, growth and function[3–5]. Commensal-specific IgG is also present in the intestine and is key for defense against commensals that translocate to systemic tissues during infection and inflammation[6]. By contrast, commensal-reactive T cells and antibodies are elevated in the blood of patients with inflammatory bowel disease (IBD), and accumulation of IgG^+ colonic plasma cells is a hallmark of severe IBD[7–10]. Circulating antibodies to gut commensals are elevated in children with genetic predisposition to type-1 diabetes[11] and in patients with rheumatoid arthritis[12], demonstrating that aberrant immunity to commensal microbes is a common feature of immune-mediated disorders. A better mechanistic understanding of how commensal-specific B cell responses are induced, regulated and maintained is important for studies of intestinal homeostasis, immunity and inflammation and for future work to develop therapies to manipulate these cells in the context of inflammatory disorders.

The identification of antigen-specific B cells is key to studies of host immunity. However, a dearth of tools to identify microbe-specific B cells has hampered the field. Bacteriophage (phage) display technology is a high-throughput screening technique used to identify ligands and binding partners for proteins and other macromolecules[13,14]. This approach also enables identification of antibody binding to thousands of potential antigens in parallel, with previous successful use identifying immunogenic antigens derived from pathogenic microbes[15–17]. Here, we present a detailed protocol for generating antigen phage display libraries for identification of immunogenic microbial antigens. To date, our research and examples provided in this protocol have focused on investigating immunity to commensal microbes. We have used this approach to successfully identify immunogenic antigens from the common murine commensal bacterium segmented filamentous bacteria (SFB)[18]. However, this protocol is broadly applicable for identification of immunogenic antigens from a range of sources from which genomic DNA (gDNA) or cDNA can be isolated, including bacteria, viruses, parasites, protozoa, food, environmental samples and autoantigens. This approach enables downstream utilization of identified proteins as antigens for analysis of B cell frequency and effector function by enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunosorbent spot (ELISPOT) or generation of B cell antigen tetramers. B cell tetramers are growing in their utilization as an approach by which to profile antigen-specific B cells upon vaccination and exposure to infectious pathogens[19–21]. Broader application of B cell tetramers will enable substantial advances in research to understand host–microbiota symbiosis.

Development of the protocol and comparison with other approaches

Multiple approaches exist to identify immunogenic microbes or antigens eliciting B cell responses, including but not limited to IgA-seq/IgG-seq, monoclonal antibody generation, culture collection screening and mass spectrometry[22–25]. The experimental strengths and limitations of each technique should be considered before designing experiments. For example, microbial colonization can elicit induction of humoral immune responses to the microbiota, particularly that of IgA production. Endogenous coating of gut bacteria by IgA can be used to identify microbial species eliciting IgA^+ B cell responses; this is particularly powerful when combined with 16S rRNA or metagenomic sequencing in a technique termed 'IgA-seq'[23,25–27]. More recently, analogous approaches to identify IgG-bound microbes have also revealed bacterial taxa eliciting IgG responses to commensal microbes during homeostasis and

in disease states[6]. Such methods have identified gut microbes that can elicit cognate adaptive immune responses and provoke inflammation in models of human disease. These approaches enable rapid identification of immunogenic microbes eliciting IgA and/or IgG responses, although the antigens targeted in these responses cannot be determined. In addition, identification of the anatomical locale and cellular phenotype of B cells responding to these microbes is difficult to achieve.

B cell responses to intestinal microbes can also be identified by cloning of B cell receptors isolated from B cells present within gut-associated lymphoid tissues[24,28] or the intestinal lamina propria[29,30]. By amplifying B cell receptor sequences, and through subsequent generation of monoclonal antibodies, Ig-binding–based assays such as ELISA, bacterial flow cytometry and probing of glycan arrays can be conducted. This approach requires multiple labor-intensive steps, particularly when using B cells elicited by a complex bacterial community that may encode many thousands of potential antigens. Although B cell phenotype can be accounted for, in the absence of monocolonization gnotobiotic systems, this becomes a significant exercise in culture collection panning[24,28]. Even when using monoclonal antibodies derived from monocolonized animals, complex screening approaches are required to identify immunogenic antigens, including assaying glycan arrays and using transposition-based gene disruption of genetically amenable bacterial isolates[31]. Many of these approaches require considerable skill, technical expertise and, in some instances, prohibitively expensive and complex instrumentation. Here, we describe a means by which to enable a priori identification of antigen-specific B cells from the polyclonal repertoire, allowing users to bypass a number of these laborious experimental approaches.

Phage display technology is a high-throughput screening technique that enables identification of ligands and binding partners for proteins and oligopeptides. Specifically relevant to this protocol, phage display library screening enables antibody-mediated identification of thousands of potential antigens in parallel, including identification of immunogenic antigens derived from pathogenic bacteria and viruses[15–17]. We designed a protocol for the high-resolution detection of immunogenic commensal bacterial antigens by using phage display (Fig. 1). Phage display library generation involves the shotgun cloning of genomic DNA into a phagemid expression vector that fuses potential antigens ‘in-frame’ to the bacteriophage pIII surface coat protein. The pIII protein of bacteriophage, which is readily accessible on the phage surface, provides an ideal site for displaying fused antigens. Random blunt-end cloning of gDNA fragments into the phagemid expression vector results in few inserts being cloned in the correct orientation and without stop codons or missense mutations. Therefore, genetic complementation of pIII-deficient ‘Hyperphage’ with pIII-antigen–fused phagemid vectors enables selection and enrichment of an ORFeome (totality of open reading frames (ORFs)) phage library[32,33]. Phage libraries bearing putative antigens are generated by infection of F′ *Escherichia coli* followed by affinity-based immunoprecipitation using serum or intestinal antibodies of IgA or IgG isotypes. Multiple rounds of affinity-based selection (or biopanning) ensure robust selection of high-affinity interactions, and subsequent isolation, generation and screening of monoclonal phage bearing single putative antigens allow quantification of library enrichment and determination of immunogenic epitopes by simple Sanger sequencing. Subsequent validation of antigens can be experimentally determined by using ELISA, ELISPOT or generation of B cell tetramers; a protocol detailing the generation and utilization of these reagents was recently described[34].

Applications of the method

This protocol is designed to identify immunogenic antigens allowing downstream usage of these proteins to assay, stimulate and detect commensal-specific B cell responses. Phage display-based antigen discovery has been performed in a number of experimental and clinical contexts, including in pathogen infection and autoimmunity, and here we extend that utility to the identification of immunogenic antigens derived from the commensal microbiota. Phage display library screening can be conducted on both eukaryotic and prokaryotic organisms that elicit a detectable serum antibody response upon colonization or infection,

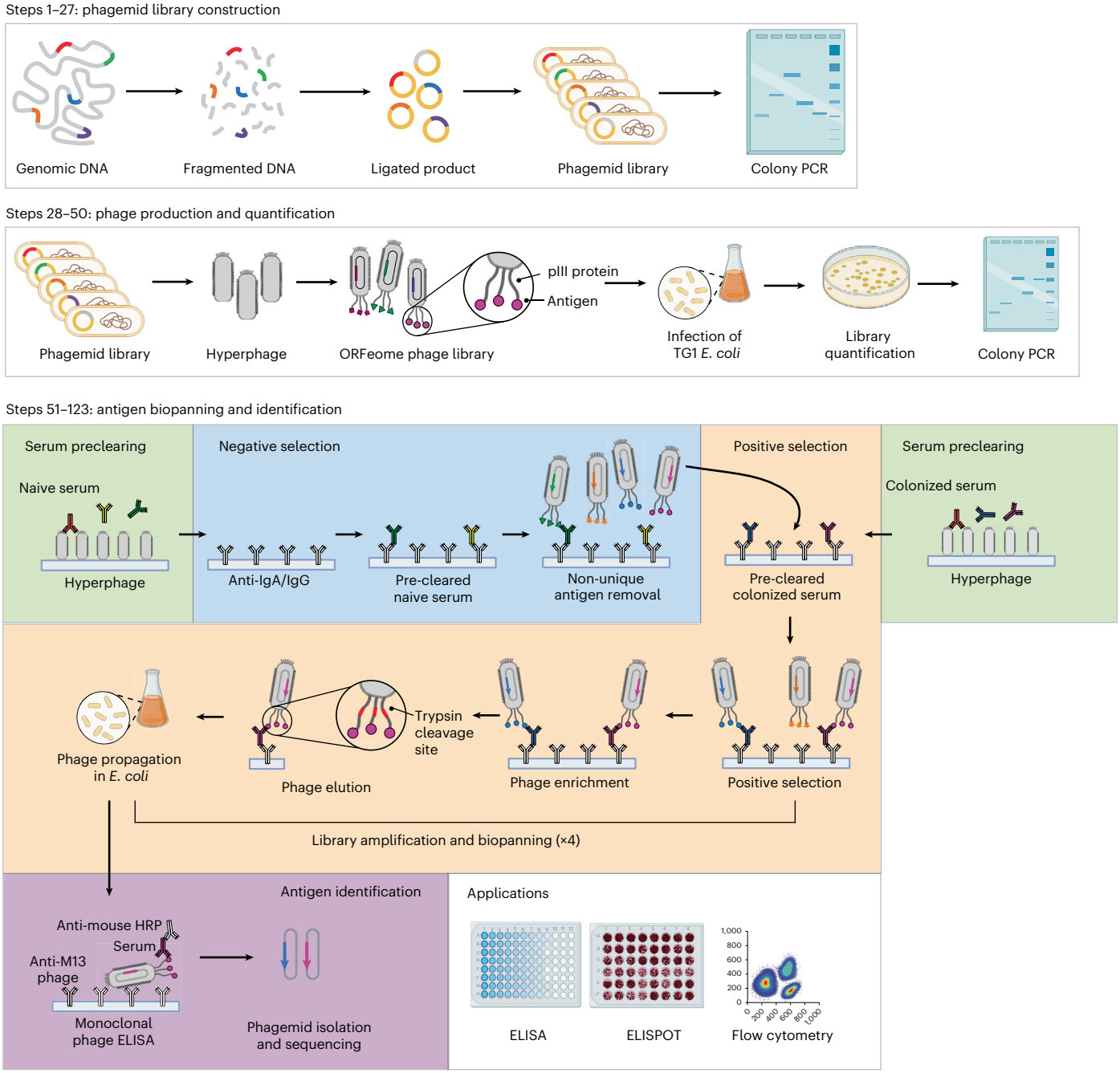


Fig. 1 | Schematic overview of phage display screening. Summary of steps with procedure step numbers for phage library generation, packaging, biopanning and antigen identification. Potential downstream applications of the method are highlighted. ORFeome, totality of open reading frames. Figure created with BioRender.com.

and although this protocol details antigen discovery from commensal bacteria, bacterial pathogens and non-bacterial components of the intestinal microbiota, including viruses, fungi and parasites, are also probably amenable to investigation by using this approach. Although the detection of antigens is detailed here by using murine serum and intestinal lavage antibodies, this approach is not host species specific and could be applied to other species, including humans.

The immunogenic proteins identified by phage display can be used in various applications. By identifying bacterial proteins from the microbiome that elicit an immune response, we can pinpoint specific antigens that are recognized by the host's immune system. For example, in IBD, excessive immune responses to the gut microbiota are thought to promote inflammation. If certain bacterial proteins are found to consistently trigger a strong antibody response in IBD patients, these proteins could be used to develop promising biomarker candidates.

Limitations

Phage display screening using polyclonal Ig is inherently biased toward identifying high-affinity antibody/antigen interactions. This method tends to identify antigens that provoke strong antibody responses and may overlook those that induce low-affinity interactions. Although highly mutated and high-affinity B cells play a role in gut homeostasis[35], the significance of low-affinity responses remains unclear. Although some commensal microbes like SFB elicit detectable serum antibody responses, this is not true for all, meaning that serum antibodies may not be suitable for phage display screening of all target microbes. In addition, phage display is limited by its requirement for DNA-encoded antigens, restricting its ability to discover carbohydrates, lipids or post-translationally modified proteins. Similarly, generation of phage in laboratory *E. coli* may not faithfully represent all post-translational modifications to antigens that may occur in their microbes of origin. The size constraints of gDNA fragments used in library construction also pose challenges in detecting complex antigens from multimeric or conformational epitopes. Phage display library screening is most effective for culturable microbes or those that can be isolated with relative purity in gnotobiotic conditions. We have not yet systematically assessed whether sufficiently large and complex phage display libraries can be generated from diverse metagenomes to enable comprehensive identification of immunogenic antigens across commensal species. Related to this, it is worth noting that the isolation of gDNA directly from feces will yield poor provision of gDNA from bacteria present at low abundance, despite these microbes still potentially evoking strong immune responses. This protocol does not directly assess the genome coverage of the phage libraries generated, instead relying on efficient production of large phage libraries.

Expertise needed to implement the protocol

All steps in this protocol can be carried out by individuals with training in molecular biology, particularly molecular cloning and protein production.

Overview of the procedure

The procedure overview is illustrated in Fig. 1. The protocol consists of three main stages, summarized as follows:

1. *Library preparation and packaging (Steps 1–50)*. This stage details the strategy for creating a phage display library by using gDNA from bacteria of interest (Steps 1–27). It includes a step-by-step guide for DNA fragmentation, library construction using a phagemid vector and packaging of the library with Hyperphage (Steps 28–50).
2. *Antigen biopanning (Steps 51–90)*. This section outlines the protocol for screening the phage library against targets of interest. It includes detailed procedures for serum preclearance, phage library incubation, removal of non-specific phages and elution of positive binders followed by their amplification.
3. *Monoclonal phage ELISA and antigen identification (Steps 91–123)*. This section details the process of making monoclonal phage in 96-well plates, performing phage binding analysis by ELISA and identifying positive binders.

Experimental design

Selection of gDNA and antibody source/input material

Broadly, this approach entails the utilization of DNA to encode an arrayed protein library upon the surface of bacteriophage as targets for binding and enrichment based on affinity.

Here, we detail the use of gDNA from a bacterium; however, other sources of DNA, including those generated by synthesis, have been described[36,37]. By contrast, in the case of identification of antigens bound by antibodies, polyclonal antibodies may be isolated from circulation or the intestinal lumen, but monoclonal antibodies may also be used, as discussed later.

Of the highest priority is an experimental demonstration that the microbe of interest elicits a detectable antibody response. For example, some commensal microbes elicit little to no detectable antibody response and would thus be unsuitable candidates for antigen discovery using phage display. Likewise, being able to isolate, culture or by other means obtain sufficient microbial biomass to isolate gDNA from the microbe of interest is essential. Some immunogenic commensal microbes are poorly represented among the microbiota and often require significant microbiological expertise to isolate in pure culture and in sufficient quantity. However, in the case of limited gDNA, commercially available DNA amplification kits can be used to increase starting material. These steps will need to be determined in each case by the user, given their experimental system of choice.

Phagemid library preparation and packaging

With sufficient gDNA and detectable circulating or intestinal antibody responses in hand, consideration should then be given to the process of phage display, which can be broadly divided into several key steps, including phagemid library generation, phage production and quantification and antigen biopanning and identification. Initially, isolated gDNA is fragmented, most usually by sonication, before blunt-end repair and random cloning into a recipient plasmid. Sonication tends to produce a more uniform distribution of fragments across the entire genome compared to restriction digestion, wherein the number and size of the fragments depend on the frequency and location of the restriction sites. In this case, shotgun cloning occurs into a phagemid vector that enables display of putative antigens fused onto the phage surface protein pIII. The phagemid vector pHORF3 includes an M13 phage origin of replication, which is crucial for the packaging of the vector into phage particles during assembly. Given the random nature of the insertions into the vector when using non-directional cloning, many sequences will not encode functional proteins because the DNA fragments are not in the correct orientation or reading frame. As such, only a small fraction of cloned DNA fragments will result in a functional ORF, leading to a small fraction of in-frame phage particles being displayed on the surface of the bacteriophage[16]. To address this, the enrichment of correct ORFs is achieved by using the specialized helper phage Hyperphage[32,33]. Hyperphage lack the pIII gene and can generate infectious phages only when complemented by the pHORF3 phagemid, which provides the pIII protein. For functional phage production, the DNA fragments inserted upstream of the pIII gene must be correctly in-frame and devoid of stop codons. If the inserts are out of frame or incorrectly oriented, they will create premature stop codons, leading to the failure of pIII fusion proteins and, consequently, non-infective phage particles. The selection of ORFs enhances library quality and facilitates the identification of binding partners among a large pool of non-binding partners. From here, infectious phage can be produced, and subsequently phage-bearing *E. coli* libraries can be generated and propagated to generate phage libraries.

Biopanning control conditions

The procedure to isolate specific targets from phage display libraries is termed ‘biopanning’. Several key steps are incorporated into our modified version of phage display, and they are important for reduction of background signals that can arise because of non-relevant or non-specific antibody binding including, (i) pre-clearing, (ii) negative selection and (iii) positive selection. ‘Pre-clearing’ aims to minimize library enrichment by antibodies specific to endogenous bacteriophage within the gut microbiota. In this instance, we ‘pre-clear’ circulating or intestinal antibodies by binding to immobilized, ‘empty’ Hyperphage before removal of unbound antibodies for use in downstream biopanning. Second, negative selection refers to the use of circulating or intestinal antibodies from uncolonized (or uninfected) control subjects, in this case, mice. Initial binding of the phage library to pre-cleared circulating or intestinal

antibodies from control mice aims to remove antigens bound by either 'polyreactive' antibodies that are mounted in response to commensal colonization[38] or antigens that are not unique to the microbe of interest and present in multiple members of the microbiota (e.g., flagellin). Finally, a phage library that has undergone negative selection with pre-cleared circulating or intestinal antibodies can undergo positive selection using pre-cleared circulating or intestinal antibodies from mice colonized or infected with the microbe of interest.

Biopanning and downstream assays

Here, circulating or intestinal antibodies are immobilized by using anti-mouse IgA or IgG reagents, and the phage library is introduced for binding. Phage bearing putative antigens of interest are immobilized while non-specific phage are washed away, enriching target phage within the library. Bound phage are eluted by trypsin-mediated digestion of the pIII protein before infection of F′ *E. coli* and regeneration of an *E. coli* library. pHORF vectors are designed with a trypsin cleavage site, typically placed upstream of the pIII protein. This allows trypsin to specifically cleave at this site, leading to the release of the protein of interest from the phage particles. Elution using low or high pH is also used in some phage display protocols, but it can be less efficient than trypsin, especially if the pH conditions do not perfectly match the stability requirements of the phage or the displayed peptide. After each round of biopanning, phage are amplified and then used for the subsequent round of selection. After three to four rounds of biopanning, colonies from titration plates are picked, and the phage particles are amplified in microtiter plates. Verification of specific binding is achieved by using monoclonal phage ELISAs followed by phagemid isolation, Sanger sequencing and alignment to the microbial genome to identify immunogenic antigens and epitopes for downstream validation and utilization.

Materials

Biological material

- gDNA from the microbe of interest (≥10 µg): provided by the user. The user is responsible for provision and culture of the microbe of interest and isolation of gDNA according to approved institutional guidelines.
- TG1 competent cells (Lucigen, cat. no. 60502-1)
- Hyperphage M13 KO7ΔpIII (Progen Biotechnik, cat. no. PRHYPE)
  ▲ **CAUTION** Follow the recommended biosafety precautions and protocols when working with infectious organisms. In our case, we followed biosafety level 1 practices because SFB is a biosafety level 1 agent.
- Serum from mice colonized with the microbe of interest (≥100 µl)
- Serum from mice not colonized with the microbe of interest (≥100 µl)
- Intestinal wash from mice colonized with the microbe of interest (5 ml)
- Intestinal wash from mice not colonized with the microbe of interest (5 ml)
  ▲ **CAUTION** Follow appropriate safety protocols for biohazardous samples.
  ▲ **CAUTION** Animal work must be approved by institutional ethics review committees. The mice used for this protocol were housed under specific-pathogen-free (SPF) conditions and handled according to protocols approved by the Benaroya Research Institute Animal Care and Use Committee.

Reagents

Kits/enzymes/antibodies

- EconoTaq PLUS GREEN 2× Master Mix (Lucigen, cat. no. 30033-1)
- PmeI restriction enzyme (New England Biolabs, cat. no. RO560S)
- Quick calf intestinal alkaline phosphatase (CIP) kit (New England Biolabs, cat. no. M0525S)
- T4 ligase (New England Biolabs, cat. no. M0202S)

- DNA clean and concentrator-5, capped columns (Zymo Research, cat. no. D4013)
  ▲ **CRITICAL** Using a DNA purification kit that allows for DNA elution in small volumes is important. This will ensure that the DNA is concentrated enough to prevent the reaction volume from being too large in downstream applications.
- Fast DNA end repair kit (Thermo Scientific, cat. no. K0771)
- Goat anti-mouse IgG, human ads-horseradish peroxidase (HRP) (Southern Biotech, cat. no. 1030-05; RRID: AB_2619742)
- Goat anti-mouse IgA-HRP (Southern Biotech, cat. no. 1040-05; RRID: AB_2714213)
- M13 phage coat protein polyclonal antibody (Thermo Scientific, cat. no. PA1-26758; RRID: AB_795743)
- TMB (3,3', 5,5'-tetramethylbenzidine) substrate reagent set (BD, cat. no. 555214)
- Goat anti-mouse IgG, human ads (unconjugated) (Southern Biotech, cat. no. 1030-01; RRID: AB_2794290)
- Goat anti-mouse IgA (unconjugated) (Southern Biotech, cat. no. 1040-01; RRID: AB_2314669)
- (Optional) REPLI-g mini kit (Qiagen, cat. no. 150023)

Plasmids/oligonucleotides

- pHORF3 phagemid vector (acquired from Michael Hust, Technische Universität Braunschweig, under an institutional Material Transfer Agreement)
- Primers for colony PCR for library validation and sequencing; LacZ F 5′-GGCTCGTATGTTGTGTGG-3′ and pIII R 5′-CTAAAGTTTTGTCGTCTTTCC-3′

Reagents/chemicals

- PBS (e.g., 1× PBS; Corning, cat. no. 21-040-CV)
- Agar (Sigma-Aldrich, cat. no. A1296-500G)
- Agarose (VWR, cat. no. 0710-500G)
- Ethidium bromide solution, 10 mg ml^{-1} (Sigma-Aldrich, cat. no. E1510-10ML)
  ▲ **CAUTION** Ethidium bromide is highly mutagenic. Avoid inhalation and contact with skin.
- Recovery medium (Biosearch Technologies, cat. no. F98226-1)
- Tryptone (VWR, cat. no. 97063-390)
- Yeast extract (VWR, cat. no. 97063-370)
- Sodium chloride (VWR, cat. no. BDH9286)
- PEG 6000 (Sigma-Aldrich, cat. no. 81260)
- Glycerol, molecular biology grade (Fisher Scientific, cat. no. BP229-1)
- D-Glucose anhydrous (VWR, cat. no. VWRC0188)
- Kanamycin sulfate from *Streptomyces kanamyceticus* (Sigma-Aldrich, cat. no. K1377)
- Ampicillin sodium salt (VWR, cat. no. 0339-EU)
- HyClone HyPure water, molecular biology grade (VWR, cat. no. 82007-334)
- Trizma base (Sigma-Aldrich, cat. no. T1503)
- Corning 0.5 M EDTA, pH 8.0 (Corning, cat. no. 46-034-CI)
- Glacial acetic acid (VWR, cat. no. BDH3094-2.5LG)
  ▲ **CAUTION** Glacial acetic acid is corrosive and combustible. Avoid contact with skin and keep away from ignition sources.
- Sodium phosphate monobasic (Sigma-Aldrich, cat. no. S0751)
- Potassium phosphate monobasic (Sigma-Aldrich, cat. no. P5655)
- Ammonium chloride (Sigma-Aldrich, cat. no. A9434)
- Magnesium sulfate (Sigma-Aldrich, cat. no. M7506)
- Calcium chloride (Sigma-Aldrich, cat. no. C1016)
- Thiamine hydrochloride (Sigma-Aldrich, cat. no. T4625)
- Tween-20 (Sigma-Aldrich, cat. no. P1379)
- Difco skim milk (BD, cat. no. 232100)
- Trypsin from bovine pancreas (Sigma-Aldrich, cat. no. T1426)
- GeneRuler 100-bp DNA ladder (Thermo Scientific, cat. no. SM0244)

- Sulfuric acid, reagent grade (Sigma-Aldrich, cat. no. 470045-604)
  ▲ **CAUTION** Sulfuric acid is highly corrosive. Avoid contact with skin and eyes.
- cOmplete EDTA-free protease inhibitor cocktail (Sigma-Aldrich, cat. no. 11873580001)
  ▲ **CAUTION** Protease inhibitors are corrosive/irritants. Avoid contact with skin and eyes.
- RPMI-1640 medium (Sigma-Aldrich, cat. no. R8758)
- MEM non-essential amino acids solution (Thermo Scientific, cat. no. 11140050)
- HEPES buffer, 1 M (Corning, cat. no. 25-060-CI)
- Sodium pyruvate, 100 mM (Thermo Scientific, cat. no. 11360070)
- 2-Mercaptoethanol (Thermo Scientific, cat. no. 21985023)
  ▲ **CAUTION** 2-Mercaptoethanol is toxic and combustible. Avoid inhalation or contact with skin and keep away from ignition sources.
- L-Glutamine, 200 mM (Thermo Scientific, cat. no. 25030081)
- Penicillin-streptomycin, 10,000 U ml^{-1} (Thermo Scientific, cat. no. 15140122)
- FBS, USA origin (Sigma-Aldrich, cat. no. F2442)

Equipment

- Corning square bioassay dish, 245 mm × 245 mm style (Corning, cat. no. 431111)
- Petri dishes, 100 mm × 15 mm (e.g., VWR, cat. no. 25384-094)
- MicroTUBE AFA fiber pre-slit snap cap cuvettes, 6 mm × 16 mm (Covaris, cat. no. 520045)
  ▲ **CRITICAL** For accurate fragmentation of DNA, use microtube cuvettes that are optimized for your sonication device.
- M220 Focused-ultrasonicator (Covaris, cat. no. 500295)
- VWR centrifuge tube, 50 ml (VWR, cat. no. 21008-178)
- Micropulser electroporator (BioRad, cat. no. 1652100)
- Shaking incubator (e.g., New Brunswick Scientific Excella E24; Eppendorf, cat. no. M1352-0010)
- Cryovials (e.g., Fisher Scientific, cat. no. 09-761-71)
- Micropipettes
- Pipette tips
- Refrigerated ultracentrifuge (e.g., Beckman J2-21)
- QIAvac 24 Plus vacuum manifold (Qiagen, cat. no. 19413)
- DNA gel electrophoresis chamber system (e.g., BioRad Sub-Cell GT cell horizontal electrophoresis system, cat. no. 1704483) and power supply
- Rocker
- Microtubes (e.g., Eppendorf, cat. no. 022364120)
- Glass conical flasks (125 ml and 1 liter)
- Conical tubes (e.g., Fisher Scientific, cat. no. 14-432-22)
- Refrigerated microcentrifuge (e.g., Centrifuge 5424 R; Eppendorf, cat. no. 5406000240)
- Nanodrop spectrophotometer (e.g., ND-1000 spectrophotometer; Thermo Scientific)
- DNA gel imager (e.g., Chemidoc MP; BioRad, cat. no. 12003154)
- Microplate reader (e.g., VERSAmax; Molecular Devices)
- Cell spreaders (e.g., L-shape cell spreaders; VWR, cat. no. 76207-748)
- 0.45-µm syringe filters (e.g., Millex-HV syringe filter unit; Sigma-Aldrich, cat. no. SLHVR33RS)
- 1-ml syringe
- 96-well flat-bottom high-bind microplate (Corning, cat. no. 9018)
- 96-well round-bottom microplate (Greiner, cat. no. 650261)
- Thermal cycler (e.g., C1000 Touch thermal cyler; BioRad, cat. no. 1851196)
- Sterile disposable filter units with polyethersulfone (PES) membrane, 0.2 µm (Thermo Scientific, cat. no. 5660020)
- MicroPulser electroporation cuvettes, 0.1-cm gap (BioRad, cat. no. 1652083)
- Heated water bath
- Dissection scissors
- Forceps

Software

- SnapGene (www.snapgene.com) or other appropriate DNA analysis software

Reagent setup

2× YT medium

Store at room temperature (RT; 22–25 °C) for ≤6 months

Component	Concentration (wt/vol)	Amount (for 1 liter)
Tryptone	1.6%	16 g
Yeast extract	1%	10 g
Sodium chloride	0.5%	5 g
Deionized water	–	≤1 liter
Autoclave to sterilize		

2× YT-GA medium

Use immediately after the addition of antibiotics and glucose.

Component	Concentration	Amount (for 1 liter)
Tryptone	1.6% (wt/vol)	16 g
Yeast extract	1% (wt/vol)	10 g
Sodium chloride	0.5% (wt/vol)	5 g
Deionized water	–	≤1 liter
Autoclave to sterilize and allow to cool. Before use, add the components below		
Ampicillin	100 μg ml^{-1}	–
Glucose	100 mM	–

2× YT-GA agar plates

Store at 4 °C for ≤1 month.

Component	Concentration	Amount (for 1 liter)
Tryptone	1.6% (wt/vol)	16 g
Yeast extract	1% (wt/vol)	10 g
Sodium chloride	0.5% (wt/vol)	5 g
Agar	1.5% (wt/vol)	15 g
Deionized water	–	≤1 liter
Autoclave to sterilize. When the medium has cooled to 55 °C, add the components below and gently swirl the flask to mix. Pour the plates		
Ampicillin	100 μg ml^{-1}	–
Glucose	100 mM	–

2× YT-AK medium

Use immediately after the addition of antibiotics.

Component	Concentration	Amount (for 1 liter)
Tryptone	1.6% (wt/vol)	16 g
Yeast extract	1% (wt/vol)	10 g
Sodium chloride	0.5% (wt/vol)`	5 g
Deionized water	–	≤1 liter
Autoclave to sterilize and allow to cool. Before use, add the components below		
Ampicillin	100 μg ml^{-1}	–
Kanamycin	50 μg ml^{-1}	–

2× YT glycerol
Store at RT for ≤6 months.

Component	Concentration	Amount (for 1 liter)
Tryptone	1.6% (wt/vol)	16 g
Yeast extract	1% (wt/vol)	10 g
Sodium chloride	0.5% (wt/vol)	5 g
Deionized water	–	≤1 liter
Autoclave to sterilize and allow to cool. Then, add the components below		
Glycerol	16% (vol/vol)	160 ml

PEG-sodium chloride (PEG-NaCl) solution
Store at 4 °C for ≤3 months.

Component	Concentration	Amount (for 1 liter)
PEG6000	20% (wt/vol)	200 g
Sodium chloride	2.5 M	146.1 g
Deionized water	–	≤1 liter
Mix well and filter-sterilize with a 0.2-µm filter		

TAE buffer (50×)
Store at RT indefinitely.

Component	Concentration (M)	Amount (for 1 liter)
Tris base	1.0	242 g
Glacial acetic acid	2.0	57.1 ml
EDTA	0.5	100 ml
Deionized water	–	≤1 liter
Start with 600 ml of deionized water. Add tris base and mix until dissolved. Add glacial acetic acid and EDTA and then bring the volume to 1 liter. Dilute to 1× before using		

PBST
Store at RT and use within 1 week.

Component	Concentration (vol/vol)	Amount (for 1 liter)
Tween-20	0.05%	500 µl
PBS	–	≤1 liter
Mix well before use		

Blocking buffer
Prepare fresh each time.

Component	Concentration (wt/vol)	Amount (for 1 liter)
Skim milk	2%	20 g
PBS	–	≤1 liter
Mix to dissolve and use immediately		

M9 salts (5×)
Store at RT for ≤1 year.

Component	Concentration (mM)	Amount (for 1 liter)
Sodium phosphate dibasic	240	64 g
Potassium phosphate monobasic	110	15 g
Sodium chloride	42.8	2.5 g

Component	Concentration (mM)	Amount (for 1 liter)
Ammonium chloride	93	5 g
Deionized water	–	≤1 liter
Start with 600 ml of deionized water, add the components and mix to dissolve and then adjust the volume to 1 liter. Autoclave to sterilize		

Minimal medium plates

Store at 4 °C for ≤6 months.

Component	Concentration	Amount (for 1 liter)
Agar	1.5% (wt/vol)	15 g
Deionized water	–	800 ml
Add agar to 800 ml of deionized water and autoclave. When the medium has cooled to 55 °C, add the components below and gently swirl the flask to mix. Pour the plates		
5× M9 salts	1×	200 ml
20% glucose	0.2% (vol/vol)	10 ml
1 M magnesium sulfate	1 mM	1 ml
1 M calcium chloride	0.1 mM	100 µl
1 mg ml^{-1} thiamine hydrochloride	1µg ml^{-1}	1 ml

Complete RPMI

Store at 4 °C for ≤1 month.

Component	Concentration	Amount (for 500 ml)
RPMI-1640	–	500 ml
1 M HEPES	50 mM	10 ml
2-Mercaptoethanol (1,000×)	1×	500 µl
FBS	3% (vol/vol)	15 ml
100 mM sodium pyruvate	10 mM	5 ml
200 mM L-glutamine	20 mM	5 ml
Penicillin-streptomycin	1% (vol/vol)	5 ml
MEM nonessential amino acids (100×)	1×	5 ml
Combine the above components and mix. RPMI is best if used when fresh		

Gut wash buffer

Prepare fresh each time.

Component	Concentration	Amount
EDTA-free protease inhibitor (50×)	1×	1 tablet
PBS	–	50 ml
Use immediately		

1 M H_2SO_4

Store in an acid cabinet at RT indefinitely.

Component	Concentration (M)	Amount (for 100 ml)
Sulfuric acid (1.83-g ml^{-1} density)	1	2.80 ml
Deionized water	–	97.2 ml
Slowly add 2.80 ml of sulfuric acid to 25 ml of deionized water. Adjust the final volume to 100 ml		

Serum preparation

Process serum by following your laboratory's preferred protocols or as follows. Euthanize the mouse as per institutional animal care and use committee guidelines. Collect blood via cardiac puncture by using a 25-gauge needle and transfer the blood to a 1.5-ml microcentrifuge tube. Allow the blood to clot by leaving it undisturbed at RT for 30–60 min or leave it overnight at 4 °C.

Remove the clot by centrifuging the blood at 3,000*g* for 15 min. Carefully transfer the supernatant to a fresh 1.5-ml microcentrifuge tube. Serum can be stored at −20 °C or at −80 °C for long-term storage (≤6 months).

Antibody isolation

For isolation of luminal antibodies, lavage the entire small intestinal contents with 7 ml of sterile PBS supplemented with 1× protease inhibitor into a 50-ml centrifuge tube. Centrifuge at 4,000*g* for 10 min to clear the luminal contents. Store the supernatant in small aliquots at −80 °C until use.

Procedure

Part 1: phagemid library construction

● **TIMING 9 d**

Phage library cloning

● **TIMING 5 d**

1. Grow bacteria of interest at optimum culture conditions to isolate gDNA. For non-culturable bacteria like SFB, feces of SFB-monocolonized germ-free mice can be used as the source of DNA.
   ▲ **CRITICAL STEP** The volume of culture required for gDNA preparation depends on the bacterial growth and can vary. It must be determined empirically by the user through testing.
2. Purify gDNA by using a DNeasy PowerSoil kit according to the manufacturer's instructions or any commercial kit suitable for the gDNA preparation.
3. Measure the concentration of the gDNA by using a Nanodrop spectrophotometer. Use 2 µl of water (or the buffer in which DNA is eluted, if not water) to blank the machine. Blot the fluid and clean the Nanodrop sensor with water before adding DNA.
4. Add 2 µl of DNA to the Nanodrop sensor, lower the arm and quantify the DNA concentration. Around 10 µg of gDNA is required for the library construction.
   ▲ **CRITICAL STEP** DNA quality is assessed by using a Nanodrop spectrophotometer by measuring the A_{260}/A_{280} ratio, with an ideal range of 1.8–2.0 for pure DNA.
   ▲ **CRITICAL STEP** If the initial gDNA amount is low, it can be amplified by multiple displacement amplification using the REPLI-g Mini kit. The recommended starting amount of DNA for this kit is ~10 ng, with an expected yield of 10 µg from a single reaction.
5. To check the integrity of the gDNA, prepare a 1% (wt/vol) agarose gel by using 1× TAE buffer. Load ~100 ng of sample and run the gel at 100 V for 40 min. gDNA appears as a distinct, thick band at the top of the agarose gel (Fig. 2a, lane 3).
6. To prepare for fragmentation with the Covaris M220 Focused-ultrasonicator, dilute the gDNA with ultrapure water if required so that it fits in a Covaris microTUBE. Start with 10 µg of gDNA and set the target fragment size to 300 bp. The starting concentration of the DNA can vary depending on the source of gDNA.
   ▲ **CRITICAL STEP** These steps may vary depending on the model of sonicator; consult the operating manual of your instrument.
7. To analyze the fragmented DNA, load ~200 ng of the sample onto a 1.5% (wt/vol) agarose gel and run it at 100 V for 40 min. This will ensure that the fragment size falls within the 200–600-bp range.
   ▲ **CRITICAL STEP** Fragmented DNA should appear as a smear on the gel with a concentrated band at the expected size defined by the user (Fig. 2a). DNA fragments longer than 400–500 bp may be infrequently displayed on phage and could be lost during the library packaging step.
   ◆ **TROUBLESHOOTING**
8. Purify the sonicated DNA by using the Zymo Research DNA clean and concentrator kit and elute in 20 µl of elution buffer.
   ▲ **CRITICAL STEP** DNA fragments can be purified by using any DNA cleanup kit. The Zymo Research DNA clean and concentrator kit that we used is column based, ensuring a high concentration of DNA into minimal volume. Follow the manufacturer's recommended protocol for using the Zymo DNA cleanup kit.

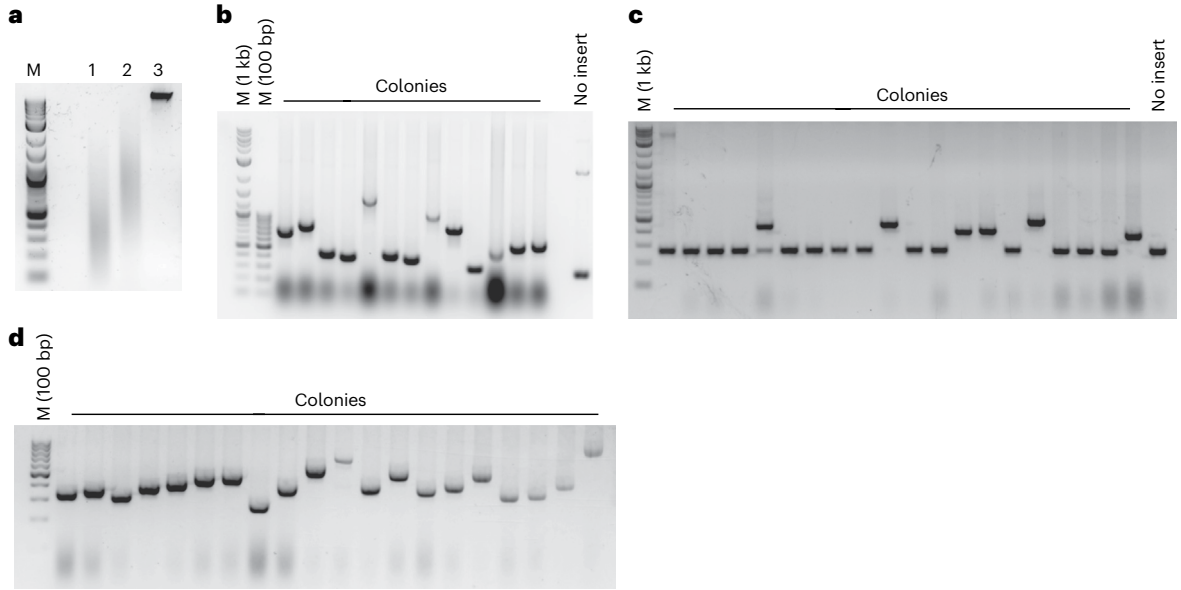


Fig. 2 | Phagemid library generation. Agarose gels representing the sequential steps of phage library preparation. **a**, Fragmentation pattern of SFB gDNA. M = 1-kb DNA marker. Lanes 1 and 2 represent 200- and 500-bp fragmentation, respectively. Lane 3 is SFB gDNA. **b**, Colony PCR results of 13 randomly picked colonies before library enrichment. Lane 14 is empty, and Lane 15 is a negative control (no insert). **c**, Colony PCR results from a poor library generation with low insert rate, considered suboptimal for further downstream steps. **d**, Colony PCR of 20 randomly picked colonies after ORFeome enrichment.

9. Repair the ends of the fragmented DNA by using the Thermo fast end DNA repair kit as detailed below. Any PCR-grade tube can be used for this step. The repair kit blunts cohesive ends generated during sonication and phosphorylates the blunt ends. Incubate the reaction for 15 min at 20 °C.

Component	Volume (µl)
Fragmented DNA (from Step 8)	X
10× end-repair mix	5
End-repair enzyme	2.5
H_2O	≤50 µl

10. Purify the end-repaired fragmented DNA by using the Zymo Research DNA clean and concentrator kit (following the manufacturer's recommended protocol) and elute DNA in 20 µl of elution buffer.
11. Linearize the pHORF3 phagemid vector by doing a restriction digest with PmeI enzyme as indicated below. Incubate at 37 °C for 1 h.

Component	Volume (µl)
pHORF3 (~5 µg)	X
CutSmart buffer (10×)	5
PmeI (10 U μl^{-1})	2.5
Nuclease-free H_2O	≤50 µl

12. Purify the linearized phagemid by using the Zymo Research DNA clean and concentrator kit (following the manufacturer's recommended protocol) and elute in 20 µl of elution buffer. Verify linearization by running a 1% (wt/vol) agarose gel at 100 V for 40 min.
▲ **CRITICAL STEP** The vector digestion reaction can also be heat-inactivated instead of performing purification before proceeding for CIP treatment. However, we always purify the digested DNA.

13. Incubate the linearized phagemid with quick CIP enzyme to dephosphorylate the DNA 5′-ends and prevent re-ligation events. Incubate at 37 °C for 30 min.

Component	Volume (µl)
pHORF3 (~4 µg) (from Step 12)	X
CutSmart buffer (10×)	2
Quick CIP (10 U μl^{-1})	4
Nuclease-free H_2O	≤20 µl

14. Purify the linearized phagemid by using the Zymo Research DNA clean and concentrator kit to get rid of residual enzyme or buffer components that may inhibit the ligation reaction. Elute DNA in 20 µl of elution buffer.
15. Measure the concentration of the purified phagemid and the fragmented DNA by using a Nanodrop spectrophotometer.
16. Ligate the fragmented DNA and the linearized and dephosphorylated pHORF3 phagemid vector at a 1:10 ratio of vector to insert. The amount of fragmented DNA needed for ligation varies depending on the size of fragmentation. Incubate the reaction for 16 h at 16 °C.

$$\text{Insert (ng)} = \frac{\text{nanograms of phagemid} \times \text{insert size (bp)} \times \text{n (fold excess insert)}}{\text{vector size (bp)}}$$

Component	Volume (µl)
T4 DNA ligase buffer (10×)	5
pHORF3 (4,144 bp)	X (1,000 ng)
Fragmented DNA (300 bp)	X (723.94 ng)
T4 DNA ligase	1
Nuclease-free H_2O	≤50 µl

▲ **CRITICAL STEP** The volume of T4 DNA ligase may vary depending on the source used. The T4 DNA ligase that we used has a concentration of 400,000 U ml^{-1}.

17. Purify the ligated product by using the Zymo Research DNA clean and concentrator kit and elute in 20 µl of nuclease-free H_2O.
18. Place electroporation cuvettes and microcentrifuge tubes on ice. Thaw TG1 electrocompetent cells on ice (~10 min) and resuspend the cells by gently flicking the bottom of the tube with your finger. Distribute 25-µl aliquots of TG1 cells into chilled microcentrifuge tube and add 5 µl of the ligated product. Do not pipette up and down to mix, because it can introduce air bubbles and warm the cells.
    ▲ **CRITICAL STEP** Using >5 µl of ligation mix may cause electrical arcing during electroporation. The volume of electrocompetent cells used for electroporation may vary depending on the manufacturer's recommendation, with the recommendation for TG1 cells being 25 µl.
19. Carefully transfer the DNA mixture into a chilled cuvette, avoiding bubble formation. Gently tap the cuvette to settle the cells at the bottom. Electroporate by using the pre-set program for *E. coli* at 1.8 kV.
20. Immediately after electroporation, add 1 ml of pre-warmed recovery medium to the cuvette and pipette up and down to resuspend the cells. Transfer the cells to a microcentrifuge tube. Place the tube at 37 °C for 1 h with shaking at 250 rpm.
21. To determine the transformation rate, take 10 µl of transformed bacteria and make 10-fold dilutions in 2× YT medium until 10^{-5} and plate 100 µl of the 10^{-3}, 10^{-4} and 10^{-5} dilutions on 2× YT-GA agar plates (10 cm). Plate the remaining cells on 2× YT-GA agar plates (24.5 cm × 24.5 cm) and incubate at 37 °C overnight.
22. Add 20 ml of 2× YT 16% (vol/vol) glycerol to the 24.5 cm × 24.5 cm plate and incubate on a rocker for 10 min. Scrape the cells and prepare aliquots of 1 ml in cryovials. Flash-freeze the cryovials in dry ice and store them at −80 °C.

▲ **CRITICAL STEP** Ensure that you get a bacterial lawn on the 24.5 cm × 24.5 cm plate. Quantification of the bacterial culture is not necessary for preparing the aliquots. From a single plate, we can prepare seven to eight vials of glycerol stock.

■ **PAUSE POINT** The phagemid stocks can be stored long term (≤6 months) at −80 °C.

23. Count the colonies from the 10-cm plate (Step 21) to calculate the initial library size on the basis of transformation rate, as follows:

$$\text{Library size} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume plated}}$$

▲ **CRITICAL STEP** The expected c.f.u. yield should be in the range of 10^7–10^8.

◆ **TROUBLESHOOTING**

24. Pick ≥20 colonies from the 10-cm plates (Step 21) and perform colony PCR to determine the insert rate of the library. For colony PCR, use a pipette tip to pick individual bacterial colonies from the plate and transfer them into the PCR reaction mix as detailed below. Use an empty vector as a negative control.

Component	Volume (µl)
EconoTaq Master Mix	10
LacZ forward primer (10 µM)	0.5
pIII reverse primer (10 µM)	0.5
DNA	–
Nuclease-free H_2O	≤20 µl

▲ **CRITICAL STEP** Any DNA polymerase can be used for colony PCR because the PCR product is not intended for downstream applications. The EconoTaq polymerase that we regularly use does not have proofreading capacity, although users may wish to use proofreading polymerases if they have downstream applications beyond those detailed in this protocol. Polymerases with proofreading include Pfu DNA polymerase, Phusion high-fidelity DNA polymerase, Q5 high-fidelity DNA polymerase and others.

25. Amplify target DNA by using the following thermocycler conditions or conditions compatible with your polymerase master mix of choice.

Step	Temperature (°C)	Time (min)	Cycle
Initial denaturation	95	5	1×
Denaturation	95	0.25	30×
Annealing	56	0.5	
Extension	72	0.5	
Final extension	72	5	1×
Hold	4	∞	–

▲ **CRITICAL STEP** With the primers detailed in this protocol, the empty vector will yield a 225-bp product, whereas positive clones will yield products larger than this size. The forward primer is located within the LacZ promoter sequence, 125 bp upstream of the restriction site, while the reverse primer is situated in the pIII protein, which is 100 bp downstream of the restriction site.

26. To check the size of each fragment, prepare a 1.5% (wt/vol) agarose gel, load 20 µl of PCR product (with an appropriate amount of loading dye if not present in the PCR master mix) and separate the DNA by electrophoresis at 150 V for 30 min.

▲ **CRITICAL STEP** Optimizing the gel running conditions by altering the agarose gel concentration may be required to detect small size differences between the empty vector and short inserts.

27. To determine the insert size of the phagemid library, calculate the number of positive clones that have larger amplicons compared to the negative control (Fig. 2b). Achieving a high insert rate is essential for ensuring a well-sized library. Ideally, the insert rate would be >90%, although a >80% insert rate is acceptable. If the insert rate is <80%, consider

repeating the previous steps. An example of a gel with an acceptable insert rate is shown in Fig. 2b, and an example with a low insert rate is shown for comparison in Fig. 2c.

$$\text{Percentage of positive clones} = \frac{\text{Number of clones with larger amplicons} \times 100}{\text{Total number of colonies picked}}$$

◆ **TROUBLESHOOTING**

Phage library packaging

● **TIMING 4 d**

28. Thaw an aliquot of phagemid library stock prepared in Step 22 on ice. Inoculate 400 ml of pre-warmed 2× YT-GA medium in a 2-liter shake flask with the library stock to an optical density at 600 nm (OD_{600}) of ~0.1.
29. Incubate the bacterial culture at 37 °C with shaking at 250 rpm until the OD_{600} reaches 0.5, ~1.5–2 h.
    ▲ **CRITICAL STEP** It is essential that the bacteria are in log phase for efficient phage infection. The OD_{600} should not exceed 0.5, to ensure optimal conditions.
30. Take out a lyophilized vial of M13K07ΔpIII helper phage 'Hyperphage' and resuspend in 2 ml of sterile ultrapure water.
    ▲ **CRITICAL STEP** The concentration of Hyperphage particles per ml (plaque-forming units (p.f.u.) ml^{-1}) after reconstitution is indicated on the label and varies from lot to lot.
31. Transfer 25 ml (1.25×10^{10} cells) of culture by using a sterile serological pipette to a 50-ml Falcon tube. Infect the bacterial cells with a multiplicity of infection of 20 by adding 2.5×10^{11} p.f.u. of Hyperphage.
32. Incubate the tube at 37 °C for 30 min without shaking, followed by an additional 30 min at 37 °C with shaking at 250 rpm to enable expression of antibiotic resistance.
33. Centrifuge the tube at 3,000*g* for 10 min at RT. Discard the supernatant by using a serological pipette and resuspend the pellet in 200 ml of 2× YT medium supplemented with 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin (2× YT-AK).
    ▲ **CRITICAL STEP** Do not include glucose in the medium during the phage production step.
    ▲ **CRITICAL STEP** Hyperphage has kanamycin resistance, which allows for selection of double-infected clones.
34. Incubate the flask at 30 °C with shaking at 250 rpm for 20–24 h. In parallel, streak a plate of TG1 bacteria on minimal medium and incubate at 37 °C (for use in Step 51).
    ▲ **CRITICAL STEP** The growth on minimal medium is slow. It takes ~24–36 h to grow bacteria on the plates. The TG1 bacterial plate can be stored at 4 °C for 1 month.
35. Transfer the overnight culture to centrifuge tubes and centrifuge at 10,000*g* for 1 h at 4 °C. Collect the supernatant and add 1/5 volume (~40 ml) of PEG-NaCl solution to 160 ml of supernatant. Incubate the tube on ice for ≥4 h, ideally overnight at 4 °C. Meanwhile, prepare a 15-ml culture tube by inoculating 3 ml of 2× YT medium with a single TG1 colony. Incubate overnight at 37 °C with shaking at 250 rpm.
    ▲ **CRITICAL STEP** The addition of PEG-NaCl solution precipitates the phage out of solution. To achieve a high concentration of phages for the first round of biopanning, it is essential to perform PEG precipitation for an extended time.
36. Centrifuge the supernatant containing the precipitated phages at 10,000*g* for 1 h at 4 °C.
37. Discard the supernatant and resuspend the phage-containing pellet in 8 ml of sterile, cold 1× PBS and transfer it into a 50-ml centrifuge tube. If using multiple tubes, it is better to combine phage pellets at this step.
38. Centrifuge at 10,000*g* for 15 min at 4 °C to remove any residual bacterial debris and collect the supernatant.
39. Add 1/5 volume (~2 ml) of PEG-NaCl solution to the supernatant and incubate for 30 min on ice, mixing every 5 min.
40. Centrifuge at 10,000*g* for 1 h at 4 °C and discard the supernatant.
41. Resuspend the pellet in 1 ml of PBS and centrifuge at 11,600*g* for 10 min at 4 °C in a microcentrifuge to remove any remaining bacterial debris. Transfer the supernatant containing the phagemid library to a microcentrifuge tube and store at 4 °C for short-term

storage. For long-term storage, distribute aliquots of the phage into PBS with 16% (vol/vol) glycerol and store at −80 °C. Retain 10 µl separately for library titration.

■ **PAUSE POINT** Phage libraries can be stored long term at −80 °C (≤6 months).

42. Inoculate 25 ml of 2× YT medium in a 100-ml shake flask with 250 µl of the primary TG1 culture prepared in Step 35. Incubate at 37 °C with shaking at 250 rpm until the OD_{600} is ~0.5 (~2 h).
43. When the OD reaches 0.5, take 10 µl of the phage suspension prepared in Step 41 and make 10-fold serial dilutions in PBS, up to a dilution of 10^{-11}.
44. Prepare four 1.5-ml centrifuge tubes, each containing 50 µl of the TG1 culture. Add 10 µl of each of the last four phage dilutions (10^{-8}, 10^{-9}, 10^{-10} and 10^{-11}) to the respective tubes.
45. Incubate the tubes at 37 °C for 30 min in a water bath without shaking.
46. Divide one 2× YT-GA agar plate into four parts and spot three 10-µl droplets per dilution (one dilution per part of the plate) for calculating library size.
47. Plate the remaining ~30 µl from 10^{-9} and 10^{-10} dilutions on 2× YT-GA plates to get single colonies for sequencing.
48. Allow the plates to dry in a biosafety cabinet for 10 min or until the spots have dried and then incubate at 37 °C for 16 h.
49. Count the colonies for each dilution spot and calculate the phage library titer as c.f.u. ml^{-1} as follows:

$$\text{c.f.u. ml}^{-1} = \frac{\text{average no. of colonies per three droplets} \times \text{dilution factor}}{\text{Volume plated}}$$

▲ **CRITICAL STEP** The expected library titer should be between 10^{11} and 10^{12} c.f.u. ml^{-1}. If the titer is significantly below this range, consider repeating the phage production steps (Steps 28–49). For the first round of biopanning, a minimum of 10^{10} c.f.u. of phages is required.

◆ **TROUBLESHOOTING**

▲ **CRITICAL STEP** In addition to quantifying phage library size as above, phages can also optionally be quantified by using spectrophotometry, to assess both their purity and concentration. This method estimates the number of phages by measuring the absorbance of UV light by the virions. The correlation between the virion count and absorbance is determined by using the following formula:

$$\text{Number of phage particles/ml} = \frac{(A_{269} - A_{320}) \times 6 \times 10^{16}}{\text{Number of DNA bases in the M13 phage genome}}$$

At 320 nm, phage chromophores exhibit minimal absorption, and this wavelength is typically used to correct for light scattering caused by both phage particles and non-phage contaminants. The M13 phage genome contains ~7,220 DNA bases. However, for a more accurate determination of c.f.u., we recommend performing colony counting, because it provides a more direct and reliable measure compared to spectrophotometry.

50. Pick ≥20 colonies from the other plate (Step 47) and analyze the insert rate and size by colony PCR as discussed in Step 20. An example gel demonstrates that all colonies contain inserts, as well as the loss of high-molecular-weight inserts above 500 bp (Fig. 2d). Send ~5–10 colonies for Sanger sequencing using the LacZ forward primer to check that inserts are in-frame. In-frame fusion proteins can be identified by maintenance of the ORF from the putative antigen to the pIII phage protein.

Part 2: biopanning of phage library

● **TIMING 16 d**

51. Using the minimal medium plate previously streaked in Step 34, pick a single colony and inoculate an overnight culture of TG1 in 15 ml of 2× YT medium in a 50-ml flask.
52. Coat two wells of a 96-well ELISA plate with 4×10^{10} p.f.u. of Hyperphage in 300 µl of PBS.
53. In the same plate, coat four wells with 100 µl of the unconjugated anti-mouse antibody of interest (anti-IgG or anti-IgA) at a concentration of 10 µg ml^{-1} in PBS. Cover the plate with plastic adhesive to prevent evaporation. Incubate the plate at 4 °C overnight.

▲ **CRITICAL STEP** Any unconjugated anti-mouse antibodies can be used; however, we have found that Southern Biotech cross-absorbed polyclonal antibodies are highly specific in detecting mouse antibody isotypes.

54. Remove the coating solution from the wells and wash the plate by filling the wells completely with PBS by using a wash bottle. Flick the liquid out vigorously over the sink. Invert the plate and tap forcefully on absorbent paper towels to remove excess liquid.
55. Block the wells by adding 300 µl of blocking buffer (2% skim milk in PBS) and incubate for 1 h at RT.
56. Dilute the serum (both negative and positive selection) to 1:200 by using blocking buffer. Intestinal content can be used directly without dilution.
57. Remove the blocking buffer from the wells coated with Hyperphage. Add 300 µl of serum or intestinal content to each well (use one Hyperphage-coated well for the negative selection and the other for the positive selection) to preclear any phage-specific antibodies. Incubate for 2 h at RT.
58. Transfer 150 µl of the precleared serum or intestinal content to the wells previously coated with anti-mouse antibody of interest. Use two wells for the negative selection step and the other two wells for the positive selection step. Incubate for 2 h at RT.
59. Wash the wells three times with PBS containing 0.05% (vol/vol) Tween 20 (PBST) to remove excess antibodies, as previously described in Step 54.
60. Dilute the phage library (prepared in Step 41) to a concentration of 5×10^{11} c.f.u. in 150 µl of blocking buffer. Incubate the library with the immobilized serum or intestinal content used for negative selection. Add 150 µl of blocking buffer in the wells used for positive selection.
61. Incubate the library for 1 h at RT without shaking.
62. After 1 h, remove the blocking buffer from the positive selection wells. Transfer the library from the negative selection wells to the positive selection wells containing the specific serum or intestinal content.
63. Incubate for 2 h at RT without shaking.
64. Meanwhile, inoculate 15 ml of 2× YT medium with 150 µl of TG1 overnight culture in a 50-ml flask. Incubate at 37 °C with shaking at 200 rpm until OD_{600} reaches 0.5.
65. Wash the wells 10 times with PBST to remove unbound phage.
66. Shake out the excess PBST by flicking the plate.
67. The phagemid-encoded pIII fusion protein contains a trypsin cleavage site. Elute bound phage with 200 µl of phage elution buffer (10 µg ml^{-1} trypsin in PBS) per well for 30 min at 37 °C without shaking. Pool the elution from both the wells.
68. For phage titration, take 10 µl of eluted phage from Step 67 and make 10-fold dilutions up to 10^{-5} by using 2× YT medium.
69. Add 50 µl of TG1 bacteria in each dilution and incubate at 37 °C for 30 min in a water bath.
70. Divide one 2× YT-GA agar plate into four equal sections and spot three 10-µl drops of the 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilution, placing one dilution per section. Incubate overnight at 37 °C to titer the phage.
71. Take 5 ml of TG1 at an OD_{600} of 0.5 and add the remaining 380 µl of pooled eluted phage from two wells from Step 67.
72. Incubate for 30 min at 37 °C in a water bath without shaking.
73. Centrifuge the cells at 1,500*g* in a microcentrifuge for 5 min. Resuspend the pelleted bacteria in 200 µl of 2× YT medium and plate on a 2× YT-GA agar plate.
74. Incubate the plates at 37 °C overnight.

◆ **TROUBLESHOOTING**

▲ **CRITICAL STEP** For the selection of specifically bound oligopeptides, perform three to four rounds of panning. The first round of selection is very important, because any errors made at this point will be amplified in subsequent rounds of selection. Ideally, each round should yield ≥100 infectious phages, and a lower count suggests a potential mistake. Expected elution titers are 10^3–10^4 c.f.u. in round 1 of panning and 10^5–10^6 c.f.u. in round 2 and all subsequent rounds.

75. Count the colonies for each dilution spot plated in Step 70 and calculate the output phage titer from the first round of biopanning by using the same formula as outlined in Step 49.

76. Add 2 ml of 2× YT 16% (vol/vol) glycerol to the plate from Step 74 and scrape the bacteria off the plate. Mix thoroughly to ensure uniform suspension. Either make a glycerol stock aliquot of bacterial culture and store it at −80 °C for future use or proceed directly to Step 77 with scraped bacterial suspension.
    ■ **PAUSE POINT** The bacterial stock from biopanning can be stored at −80 °C for long-term storage (≤6 months).
77. Inoculate 50 ml of 2× YT-GA medium with the bacterial suspension or glycerol stock from Step 76 to achieve an OD_{600} of 0.1. Freeze remaining glycerol stock at −80 °C for future use.
78. Incubate the culture at 37 °C with shaking at 250 rpm until the OD_{600} is 0.5.
79. Transfer 25 ml of culture (1.25×10^{10} cells) to a 50-ml Falcon tube and infect the bacterial cells with Hyperphage at a multiplicity of infection of 20, using a total of 2.5×10^{11} p.f.u.
80. Incubate the culture in a 37 °C water bath without shaking for 30 min and then continue incubation at 37 °C with shaking at 250 rpm for an additional 30 min.
81. Centrifuge at 3,000*g* for 10 min. Resuspend the pellet in 50 ml of 2× YT medium containing 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin.
82. Incubate the culture overnight at 30 °C with shaking at 250 rpm.
83. Centrifuge the overnight culture at 3,000*g* for 15 min.
84. Add 10 ml of PEG-NaCl solution to 40 ml of supernatant. Mix well and leave for 1 h on ice.
    ▲ **CRITICAL STEP** The incubation step should be carried out for ≥1 h on ice, but it can be extended overnight.
85. Centrifuge at 10,000*g* for 1 h at 4 °C. Carefully discard the PEG-NaCl solution and aspirate any remaining dregs.
86. Resuspend the pellet in 1 ml of PBS and centrifuge at 11,600*g* for 10 min in a microcentrifuge to remove any remaining bacterial debris. Titrate the phages as described in Steps 68–70.
87. Store the amplified phages at 4 °C for the short term and use them for the next round of selection.
    ▲ **CRITICAL STEP** Repeat the process (Steps 52–87) for a total of four panning rounds. Use 3×10^{11} c.f.u. of phage library for input in the second, third and fourth panning rounds. Increase the stringency of washing before the elution step with each round: perform 10 washes in the first round (Step 65), 20 washes in the second round and 30 washes in the third round). If insufficient phages are produced to meet the required input, use 150 μl of the amplified phage from the previous round for the next round or repeat the preceding biopanning round.
88. During the third and fourth rounds of biopanning, make a 10-fold serial dilution of 10 μl of eluted phages, diluting up to 10^{-6}. Use 10 μl of each dilution to infect 50 μl of TG1 bacteria at an OD_{600} of 0.5.
89. Incubate for 30 min at 37 °C in a water bath.
90. Plate the entire 60 μl of each dilution on 2× YT-GA agar plates and incubate overnight at 37 °C to obtain single colonies, which will be used for screening of monoclonal phage.

Part 3: monoclonal phage production and ELISA

● **TIMING 5 d**

Monoclonal phage production

● **TIMING 2 d**

91. Pipette 150 μl of 2× YT-GA medium into each well of a 96-well U-bottom propylene plate.
92. Pick individual colonies from the third and fourth panning round plates (47 colonies from each round) by using 200-μl pipette tips. Include two wells with medium-only controls.
    ▲ **CRITICAL STEP** The number of monoclonal phages selected for screening depends on the study's goals. For epitope mapping, fewer colonies may suffice, whereas for identifying immunogenic proteins, a larger number is preferable. As a general practice, begin by picking colonies from one 96-well plate. The bacterial culture plates can be stored at 4 °C for ≤1 month, allowing flexibility to return and pick additional colonies if needed.
93. Add a breathable membrane over the plate.
94. Incubate overnight at 37 °C with shaking at 250 rpm. This plate will be considered the master plate.

95. To make glycerol stocks of the master plate, pellet the cells at 3,000*g* for 10 min. Resuspend the pellet in 200 µl of 2× YT 16% (vol/vol) glycerol and store the plate at −80 °C.
    ■ **PAUSE POINT** The glycerol stock master plate can be stored at −80 °C for long-term storage (≤6 months).
96. For phage production, take 10 µl from the master plate and inoculate a new 96-well U-shaped propylene plate containing 195 µl of 2× YT-GA medium per well.
97. Incubate for 2 h at 37 °C with shaking at 300 rpm until the cells reach logarithmic growth (OD_{600} of 0.5).
98. After 2 h of incubation, infect the cells with 5×10^9 p.f.u. of Hyperphage per well. Incubate for 30 min at 37 °C without shaking followed by 30 min with shaking at 300 rpm.
    ▲ **CRITICAL STEP** Dilute the Hyperphage stock in 2× YT medium to a concentration of 5×10^{11} p.f.u. ml^{-1}. Add 10 µl to each well of the 96-well plate by using a multichannel pipette, achieving a final concentration of 5×10^9 p.f.u. of Hyperphage per well.
99. Centrifuge the cells in the plate at 3,000*g* for 10 min at RT and resuspend them in 200 µl of 2× YT-AK medium for phage production.
100. Incubate the plate overnight at 30 °C with shaking at 300 rpm.
101. The next day, pellet the cells and transfer 150–180 µl of the supernatant to a new polypropylene plate. Store the phage supernatant at 4 °C. The supernatant containing phages can be used directly for monoclonal phage ELISA in the steps below.
    ■ **PAUSE POINT** The phage containing supernatant from Step 101 can be stored at 4 °C for short-term storage (≤7 d).

Monoclonal phage ELISA

● **TIMING 1 d**

102. Coat two 96-well ELISA plates with 100 µl of rabbit anti-M13 antibody at a 1:1,000 dilution in PBS. Incubate overnight at 4 °C. Cover the plate with plastic adhesive or aluminum foil to avoid evaporation.
    ▲ **CRITICAL STEP** The anti-M13 antibody used in ELISA is polyclonal, elicited by immunization with intact bacteriophage. The optimal dilution may vary by brand, so always refer to the manufacturer's guidelines for best results.
103. Remove the antibody solution by carefully inverting the plates and discarding the liquid. Wash the wells three times with 1× PBS.
104. Saturate the plate with 300 µl of blocking buffer and incubate at RT for 2 h.
105. Wash the plates three times with 1× PBS.
106. Add 50 µl of blocking buffer to each well, followed by 50 µl of the prepared monoclonal phages from Step 101. Mix the solutions thoroughly in each well by using a multichannel pipette.
107. Incubate the plates at RT for 2 h.
108. Wash the plates three times with PBST to remove any unbound phages.
109. Dilute control serum (used for negative selection) as well as serum containing the antibody of interest to 1:500. Add 100 µl of the diluted control serum to one plate and 100 µl of the diluted serum containing the antibody of interest to the other plate.
110. Incubate the plates for 2 h at RT.
111. Wash the plates three times with PBST.
112. Prepare a 1:10,000 dilution of anti-IgG-HRP- or IgA-HRP-conjugated antibodies in PBST. Add 100 µl per well and incubate for 1 h at RT. Wash the plates three times with PBST.
    ▲ **CRITICAL STEP** The dilution of HRP-conjugated antibody may vary depending on the brand specifications.
113. Prepare TMB substrate by mixing equal volumes of substrate A and B up to the desired total volume. Add 100 µl of the substrate solution to each well and leave at RT for 12–15 min in the dark. A blue color should develop.
114. Stop the reaction by adding 50 µl of 1 M sulfuric acid. The blue color will turn yellow.
115. Read the optical density at 450 nm by using an ELISA plate reader.
    ◆ **TROUBLESHOOTING**

Identification and sequencing of positive hits

● **TIMING 2 d**

116. In the monoclonal phage ELISA, each phage clone is tested with both control serum and serum containing the antibody of interest. Select and identify the clones that exhibit an OD >0.5 with the antibody of interest.
117. Retrieve the master plate prepared in Step 94 and use it as a source for DNA extraction for the selected clones.
118. Inoculate the selected positive clones in 4 ml of 2× YT with ampicillin (100 μg ml^{-1}) and incubate overnight at 37 °C with shaking.
119. Isolate plasmid DNA by using the QIAprep Spin Miniprep kit and following the manufacturer's instructions.
▲ **CRITICAL STEP** To ensure DNA stability, after selecting positive binders, the plasmids can be retransformed into DH5α, Stbl3, XL1 or other strains commonly used for plasmid propagation.
120. Send the isolated plasmid DNA from the positive clones for Sanger sequencing using the LacZ F primer to determine the sequence of inserted oligopeptide.
121. Perform a BLASTn search (nucleotide alignment) on the sequenced data to ensure the presence of oligopeptides specific to the target of interest.
122. Perform an in-frame translation analysis of the selected sequences by using the Expasy translate website or other software for assessing protein translation from DNA sequence input.
123. Conduct a BLASTx (protein alignment) to annotate the selected sequences, analyze the insert size and check for sequence redundancy with other microbes/antigens. The identified oligopeptide sequences can be used to produce full-length recombinant protein. This can be achieved either by amplifying the complete protein-coding sequence from gDNA and cloning it into an expression vector or by commercially purchasing the recombinant protein. The immunogenic proteins identified through phage display can be used for binding assays such as ELISA and ELISPOT and/or to develop novel B cell tetramers to track B cells in vivo.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting

Step	Problem	Possible reason	Solution
7	Fragmented DNA is not concentrated around the expected size	The sonicator needs to be calibrated	A higher number of sonication cycles or an optimization of the sample preparation and sonication protocol should be considered
23	There are few colonies after electroporation	The end repair treatment of the fragmented DNA did not work	Try repeating Steps 10–23 with fresh reagents and eliminate one potential issue at a time
		The CIP treatment of the vector did not work	
		The ligase enzyme was compromised	
		The use of old electrocompetent cells affected the electroporation efficiency	
27	The colony insert rate is <80%	Ineffective vector-to-insert ratio	Try changing the ligation ratio of vector to insert
49	No colonies after library packaging, or the library titer is not optimal	Subpar Hyperphage infection of the bacteria	Ensure that the OD_{600} of the bacteria does not pass 0.5 before infecting
			Use fresh Hyperphage stocks
			Repeat the packaging and amplification steps
74	No phages after biopanning elution	Selection for positive binders did not occur	Re-examine the serum being used for positive and negative selection
115	High background in monoclonal phage ELISA	Not enough stringency when washing during biopanning	Washing cycles can be increased with each panning round (first round: 10 cycles, second round: 20 cycles, third round: 30 cycles)
		Using too high a dilution of serum could result in background	Consider diluting serum to 1:500 or 1:1,000 for biopanning

Timing

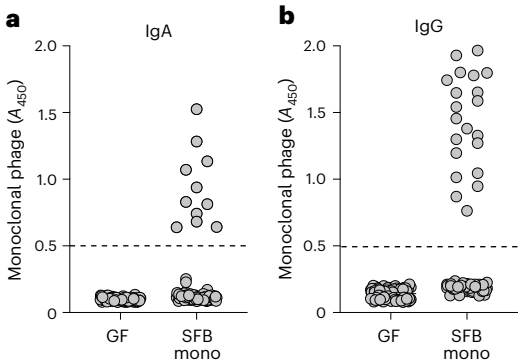
Part 1: library preparation and packaging: ~9 d
Steps 1–27, library preparation: 5 d
Steps 28–50, library packaging: 4 d

Part 2: antigen biopanning: ~16 d
Steps 51–75, single biopanning round: 2 d
Steps 76–90, amplification of phage: 2 d (the process in part 2 is repeated four times for a total of four biopanning rounds)

Part 3: monoclonal phage ELISA and antigen identification: ~5 d
Steps 91–101, monoclonal phage production: 2 d
Steps 102–115, monoclonal phage ELISA: 1 d
Steps 116–123, sequencing positive hits: 2 d

Anticipated results

To identity the immunogenic proteins targeted by SFB-specific B cells, we created an SFB phage display library and antibody-based biopanning screen[18]. SFB is not readily culturable, so DNA was isolated from fecal pellets of SFB monocolonized germ-free mice. The gDNA was randomly fragmented by sonication, and the fragmented DNA appeared as a smear on the agarose gel as shown in Fig. 2a. Fragments ranging from 200 to 600 bp of SFB gDNA were cloned into a phagemid library vector pHORF3, which fuses potential antigens to the M13 phage outer surface protein pIII. The initial size of the library based on the transformation



c Monocolonized serum biopanning

Antigen	SFB3340	SFB5460	SFB3310	SFB15470
Size (aa)	1,060	1,172	719	1,918
IgA bound	3	3	0	5
IgG bound	2	16	3	0
Total hits	5	19	3	5
Unique epitopes	3	1	1	1

d SPF luminal IgA biopanning

Antigen	SFB3340	SFB2450	SFB15470
Size (aa)	1,060	690	1,918
Total hits	2	26	7
Unique epitopes	1	1	1

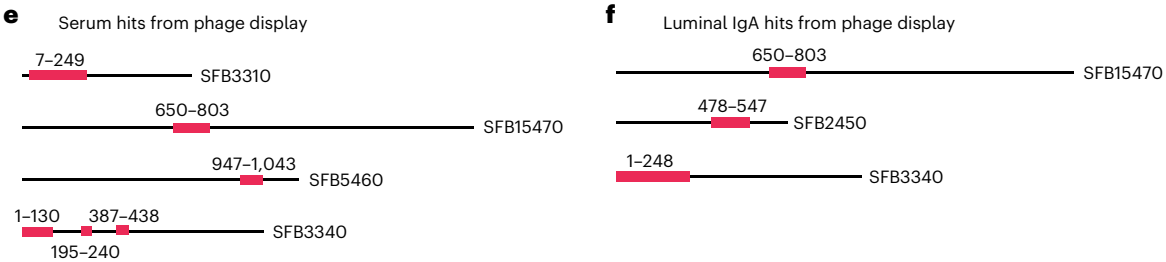


Fig. 3 | Phage display biopanning identifies immunogenic SFB antigens. **a**,**b**, Identification of immunogenic antigens using serum from germ-free (GF; $n = 3$) or SFB monocolonized (n = 3) mice, assessed by binding of enriched monoclonal phage to immobilized IgA (**a**) or IgG (**b**). The dashed line indicates the absorbance cutoff threshold for positive binders IgA or IgG. **c**,**d**, Features of the immunogenic SFB proteins identified by phage display using serum from SFB monocolonized mice (**c**) or intestinal IgA from B6 wild-type mice from Taconic Farms (SFB colonized) (**d**). aa, amino acid. **e**,**f**, Schematics of four antigens and targeted epitopes (red) identified by SFB-specific phage library screening in combination with Sanger sequencing, using serum antibodies from SFB monocolonized mice (**e**) or intestinal IgA from B6 wild-type mice from Taconic Farms (SFB colonized) (**f**). A_{450}, absorbance at 450 nm. Reprinted with permission from ref. 18, Elsevier.

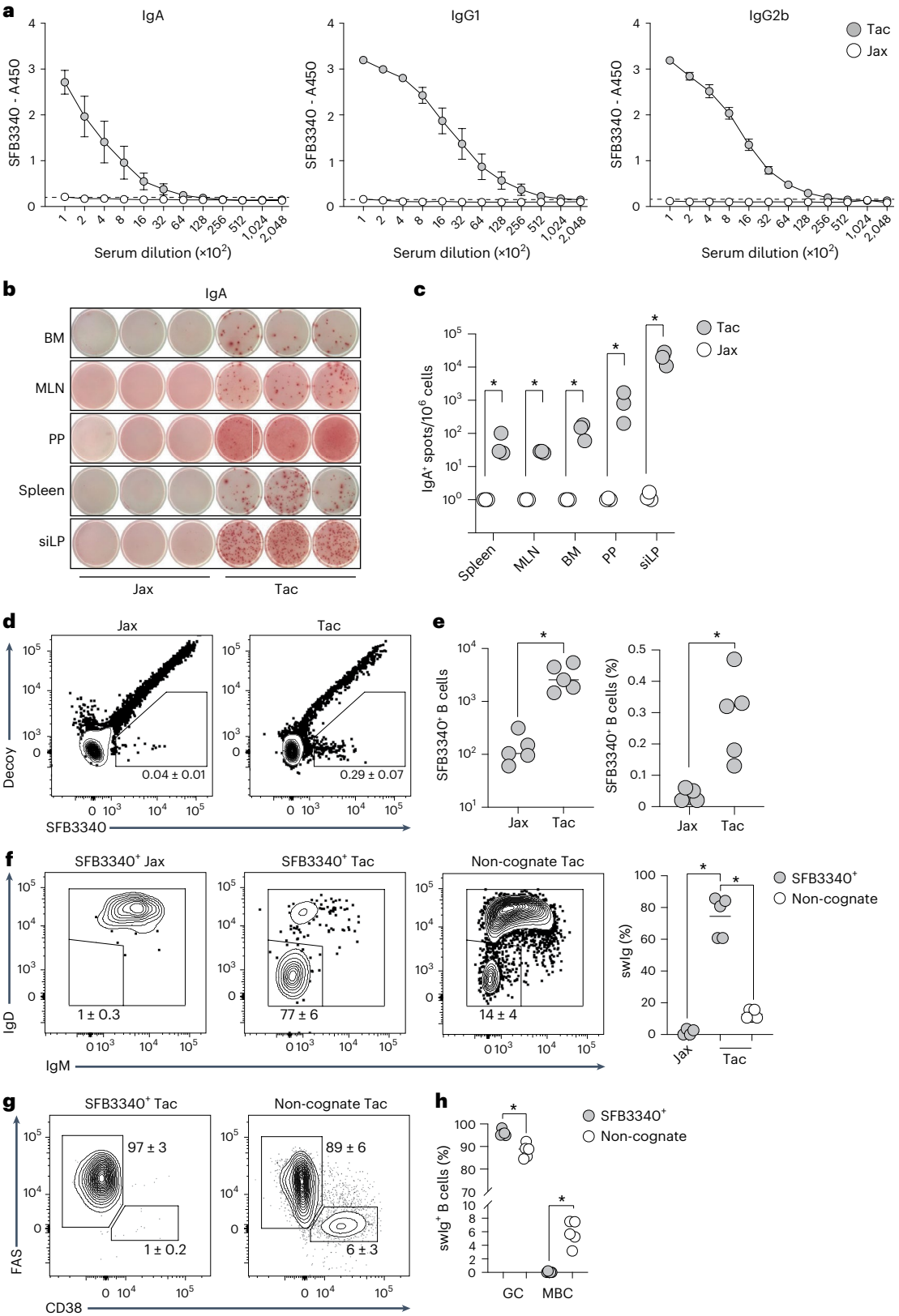

a
IgA
IgG1
IgG2b
Tac
Jax
SFB3340 - A450
Serum dilution (×10²)
b
IgA
BM
MLN
PP
Spleen
siLP
Jax
Tac
c
IgA⁺ spots/10⁶ cells
Tac
Jax
Spleen
MLN
BM
PP
siLP
d
Jax
Tac
Decoy
SFB3340
0.04 ± 0.01
0.29 ± 0.07
e
SFB3340⁺ B cells
SFB3340⁺ B cells (%)
Jax
Tac
f
SFB3340⁺ Jax
SFB3340⁺ Tac
Non-cognate Tac
IgD
IgM
1 ± 0.3
77 ± 6
14 ± 4
swIg (%)
SFB3340⁺
Non-cognate
g
SFB3340⁺ Tac
Non-cognate Tac
FAS
CD38
97 ± 3
1 ± 0.2
89 ± 6
6 ± 3
h
SFB3340⁺
Non-cognate
swIg⁺ B cells (%)
GC
MBC

Fig. 4 | Validation of SFB3340 protein targeting in SFB colonized mice. **a**, ELISA for SFB3340-specific serum antibody responses in C57BL/6 WT mice from Taconic Biosciences or Jackson Laboratories (Jax). The limit of detection is represented as a dashed line. **b**,**c**, Representative images (**b**) and quantification (**c**) of IgA ELISPOT responses upon recombinant SFB3340 restimulation of leukocytes isolated from bone marrow (BM), mesenteric lymph node (MLN), PP or siLP. **d**, Representative cytometry plots of decoy tetramer and SFB3340 B cell tetramer staining in total B cells among cells isolated from PPs of naive WT B6 mice from Jax or Tac. **e**, Quantitation of SFB3340$^+$ B cell numbers and frequencies among total B cells in PPs of WT Jax or Tac B6 mice. **f**, Representative cytometry plots of IgM and IgD expression and frequencies of swIg$^+$SFB3340$^+$ PP B cells in WT Jax or Tac B6 mice or of non-cognate SFB3340$^-$Decoy$^-$ B cells in Tac B6 WT mice. **g**,**h**, Representative cytometry plots (**g**) and frequencies (**h**) of germinal center (GC) and memory B cell (MBC) populations among SFB-specific or non-cognate B cells in PPs of SFB-colonized Tac B6 WT mice. For ELISA and ELISPOT data (**a**–**c**), data shown represent one of at least three independent experiments each with three mice per group. For cytometry data (**d**–**h**), data shown represent one of at least three independent experiments each with three to five mice per group. *$P < 0.05$ calculated by using Student's t test. ELISA data are represented as mean ± s.d. Reprinted with permission from ref. 18, Elsevier.

rates was 5×10^7 independent clones. Insert rates were >90% as analyzed by colony PCR (Fig. 2b). To form infectious phage, the phagemid library was packaged by using Hyperphage, resulting in selection of in-frame pIII-fusion ORFs. A range of insert sizes is observed during phagemid library preparation, with larger fragments being lost from the packaged phage. This demonstrates a preferential retention of smaller inserts (<500 bp) (Fig. 2d). Colony PCR analysis confirmed that the insertion rates were 100% after enrichment with Hyperphage (Fig. 2d).

Circulating antibodies from germ-free and SFB-monocolonized gnotobiotic mice were pre-cleared by using plate-bound Hyperphage to remove phage-reactive antibodies before being bound to plate-bound anti-IgA and anti-IgG antibodies to bind circulating antibodies. These pre-cleared antibodies were subsequently used for negative and positive selection of SFB antigen-bearing phage from the library. Bound phages were released by trypsin-based elution and amplified by infection of TG1 *E. coli*. Four biopanning cycles of increasing stringency were performed to identify high-affinity binders. After four rounds of biopanning, 96 individual monoclonal phage were generated and assessed for binding by ELISA using serum from SFB-monocolonized gnotobiotic mice. From this, we identified 32 monoclonal phage with OD > 0.5, all encoding epitopes aligning to the SFB genome (Fig. 3a,b). Among these high-affinity hits, we identified epitopes from four SFB-derived antigens (Fig. 3c). Among these antigens, SFB5460, SFB3310 and SFB15470 harbored a single immunogenic domain that was identified multiple times, whereas SFB3340 was targeted in three distinct N-terminal epitopes (Fig. 3e). Although SFB3340 and SFB5460 were targets for both IgA and IgG, SFB3310 and SFB15470 were bound only by IgG or IgA, respectively (Fig. 3c).

To investigate whether phage display screening could also be conducted with luminal IgA, and if so, if this could be achieved by using luminal IgA from wild-type (WT) mice with a complex microbiota, we screened our phage library by using small intestinal luminal antibodies isolated from WT C57BL/6 SPF mice from Taconic Biosciences (Tac) that are naturally colonized with SFB. Indeed, SFB3340 and SFB15470 antigens could be identified by using luminal IgA, as well as an additional SFB-derived protein SFB2450 (Fig. 3d,f). This demonstrates that both local and circulating antibodies can be used to conduct phage display screening for commensal-derived immunogenic antigens.

To identify the most-promising protein candidates for use in downstream applications (ELISA, ELISPOT and B cell tetramers) from the list of antigens identified by monoclonal phage ELISA, in silico predictions can provide preliminary insights, but these must ultimately be validated through experimental approaches, particularly functional assays tailored to the specific research objectives. Several factors should be considered when selecting protein candidates, including protein size, the presence of transmembrane domains and homology to proteins in closely related or unrelated microbes. The size of the protein, for instance, can significantly affect its functional potential and the feasibility of its production and purification. Proteins that are excessively large may be challenging to express and may not be suitable for further studies because of technical limitations in protein production.

Among the immunogenic SFB antigens identified by phage display screening, SFB3340 has also been identified as a target of Th17 and Tfh cell responses elicited by SFB in the small intestine lamina propria (siLP) and Peyer's patches (PPs), respectively[39]. Thus, we focused on SFB3340 to investigate commensal-specific B cells within systemic and gut-associated lymphoid tissues. To validate the SFB3340 antigen as a target of mucosal B cells and assess

the extent of SFB3340 targeting under homeostatic conditions in SPF mice, we did a series of experiments. Our approach involved using antigen-specific assays to examine both serum and luminal antibodies as well as tissue samples from naive WT B6 mice that were either naturally colonized with SFB (purchased from Taconic Biosciences) or not colonized (obtained from Jackson Laboratories). By ELISA (Supplementary Methods), SFB3340-specific serum and luminal IgA was detectable in the SFB-colonized Tac B6 mice but was absent in the non-colonized Jax B6 mice (Fig. 4a). In addition, in Tac mice, we identified the presence of SFB3340-specific serum IgG1 and IgG2b (Fig. 4a). These results substantiate that SFB3340 is indeed a specific target of the mucosal immune response in SPF mice. Using ELISPOT (Supplementary Methods), we found the presence of SFB3340-specific antibody secreting cells producing IgA (Fig. 4b,c), IgG1 and IgG2b (data not shown) in multiple tissue sites including the spleen, PPs, mesenteric lymph nodes, siLP and bone marrow of Tac mice. These results highlight the tissue distribution and functional profile of SFB3340-specific B cells within the context of a natural commensal microbiota, providing valuable insights into the mucosal immune response to SFB.

Phelps et al. have recently provided a thorough and detailed explanation of the procedure for producing B cell tetramers[34]. We generated phycoerythrin-conjugated B cell tetramers containing most of the SFB3340 protein (Supplementary Methods). In naive Jax B6 mice, which are not colonized with SFB, the few SFB3340$^+$ B cells found in PPs were naive follicular B cells (IgD$^+$CD38$^+$GL-7$^-$FAS$^-$, representing the naive immune repertoire) (Fig. 4d,e). By contrast, in the PPs of Tac B6 mice colonized with SFB, we observed activated SFB3340$^+$ B cells. Specifically, a mean (± s.d.) of 77% ± 6% of SFB3340$^+$ B cells were IgM$^-$ IgD$^-$ class switched (swIg$^+$), compared with 14% ± 4% of non-cognate B cells in these mice (Fig. 4f). Furthermore, nearly all swIg$^+$ SFB3340$^+$ B cells were germinal center (GC) B cells, identified as FAS$^+$CD38$^-$, with few, if any, FAS$^-$CD38$^+$ memory B cells detectable (Fig. 4g). Conversely, memory B cells were present among the non-SFB3340-specific repertoire of swIg$^+$ PP B cells (Fig. 4g,h). Overall, our data[18] demonstrate that B cell tetramers enable direct identification of SFB-specific B cell activation and demonstrate that SFB colonization is necessary and sufficient to elicit SFB3340$^+$ GC B cell responses in the context of complex microbiota.

Data availability

All data are represented within the paper. Raw data files are available upon request. The corresponding author has recombinant SFB3340 protein used for antigen tetramer construction, which can be shared upon reasonable request.

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Author contributions

S.V. and O.J.H. conceived the study and acquired funding. S.V., S.K. and O.J.H developed the methodology, provided formal analysis and wrote the original and revised drafts. S.V. and S.K. conducted the investigation. O.J.H. provided resources, performed visualization and supervised the study.

Competing interests

The authors declare no competing interests.

Additional information

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