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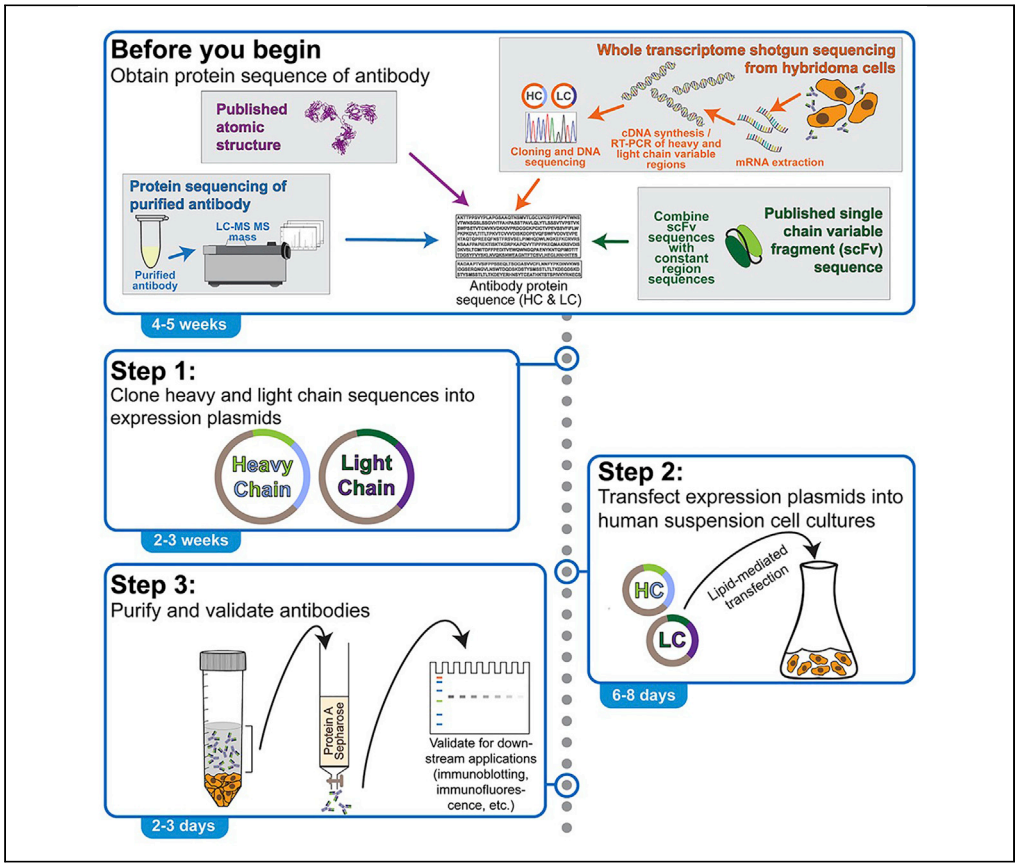
Production and purification of recombinant monoclonal antibodies from human cells based on a primary sequence



There are challenges to using commercially available antibodies generated in animals, including concerns with reproducibility, high costs, and ethical issues. Here, we present a protocol for generating and purifying recombinant antibodies from human HEK293 suspension culture cells from a primary sequence. We describe the steps to generate antibody heavy and light chain plasmids, followed by transfection of the plasmids into cells and purification of antibodies. This protocol can produce high-yield recombinant monoclonal antibodies at a relatively low cost.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Antibody sequences are used to produce heavy and light chain expression plasmids

Recombinant antibody expression plasmids are transfected into HEK293 suspension cells

Antibodies are purified using low-cost methods to produce high-yield reagents

Antibody sequences can be diversified to increase experimental flexibility

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Production and purification of recombinant monoclonal antibodies from human cells based on a primary sequence

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SUMMARY

There are challenges to using commercially available antibodies generated in animals, including concerns with reproducibility, high costs, and ethical issues. Here, we present a protocol for generating and purifying recombinant antibodies from human HEK293 suspension culture cells from a primary sequence. We describe the steps to generate antibody heavy and light chain plasmids, followed by transfection of the plasmids into cells and purification of antibodies. This protocol can produce high-yield recombinant monoclonal antibodies at a relatively low cost.

For complete details on the use and execution of this protocol, please refer to DeLuca et al. (2021).[1]

BEFORE YOU BEGIN

An alternative to using traditional, commercially available antibodies produced in animals is to generate and purify recombinant antibodies from primary sequences. In recent years, technologies for sequencing monoclonal antibodies have become readily available. For example, sequences can be obtained from purified monoclonal antibodies using mass spectrometry approaches, monoclonal hybridoma cell lines using whole transcriptome shotgun sequencing, atomic structures, and clonal selection of sequence-defined antibody fragments.[2–9] Once the sequences of an antibody are in-hand, the DNA encoding for the antibody can be cloned into parent plasmids for expression and purification from a variety of cells. We have recently generated a bank of such parent plasmids for the purpose of expressing and purifying full-length monoclonal antibodies from human cells[1]; however, the antibody sequences can be cloned into any plasmid appropriate for expression of high levels of recombinant protein in mammalian cells. Here we describe a protocol for producing full-length, recombinant monoclonal antibodies from protein sequences, and we also briefly describe how to generate antibody derivatives (e.g., antibodies with alternate species specificity and antibody fragments).

Obtaining a monoclonal antibody protein sequence

Timing: 4–5 weeks

The protein sequence of a monoclonal antibody of interest must first be obtained. It is important to note that if the sequence is obtained through mass spectrometry of purified antibody or from an atomic structure, the signal peptide sequence (also sometimes referred to as a leader sequence), which directs an antibody to the secretory pathway, will not be present. If the sequence is obtained

through whole shotgun transcriptome sequencing of monoclonal hybridoma cells, the native signal peptide will be included in the sequence.

1. Obtain sequences of monoclonal antibody heavy and light chains.

Note: Many monoclonal antibody sequences are available from public databases or from published atomic structures. Some examples are the ABCD (Antibodies Chemically Defined) database at the Geneva Antibody Facility[2] (https://web.expasy.org/abcd); the Kabat database[10] (http://bigd.big.ac.cn/databasecommons/database/id/5425); SAbDab (http://opig.stats.ox.ac.uk/webapps/newsabdab/sabdab/); abYbank(http://www.abybank.org/sacs/); and the IMGT (International Immunogenetics Information System) monoclonal antibodies database (http://www.imgt.org/mAb-DB/doc). A second option is to obtain the sequence through whole transcriptome shotgun sequencing of hybridoma cell lines producing monoclonal antibodies. Many companies and facilities offer this as an "add-on" service when producing monoclonal hybridoma cells lines, or alternatively, companies will provide the sequence as a stand-alone service from a sample of the cells, provided by the customer. Finally, protein sequences can be obtained through mass spectrometry analysis of a purified monoclonal antibody. Numerous commercial companies offer such antibody protein sequencing in which case the customer provides ~100 μg of purified antibody.

Preparation and assessment of HEK293 suspension cells

Timing: 1–2 weeks

2. Maintain 30 mL of Expi293F cells, a type of human HEK293 suspension culture cells, in Expi293F expression media at 36.5°C in 8% CO_2 in 125 mL spinner flasks on an orbital shaker, rotating at 125 rpm. Cells should be passaged less than 30 times, after which a new vial should be thawed.
   a. Cells should be split 2–3 times after initial seeding from a frozen aliquot ("recovery" period) before seeding for transfection.
   b. When cells reach 3–4 × 10^6 cells/mL, split cells to a new density of 0.3 × 10^6 cells/mL. For example, for a 30 mL culture at a density of 4 × 10^6 cells/mL, remove 2.25 mL of cells and place in a new flask containing 27.75 mL of fresh media.

Note: Expi293F cells have been adapted to grow at high density in suspension, and they typically produce higher levels of expressed proteins when compared to regular HEK293 suspension culture cells.

Alternatives: Regular HEK293 cells can also be grown in suspension and used for this protocol; however, since they grow at lower density than Expi293F cells, we suggest scaling up for higher yields.

⚠ CRITICAL: Maintaining precise rotation speed, temperature, and CO_2 levels are important for maintaining cell health, successful transfection, and protein expression. We find that Expi293F cells are particularly sensitive to temperature fluctuations, therefore maintaining the temperature at 36.5°C is critical, since we have found that cells quickly die at temperatures even slightly above 37°C. In addition, the cell density should not exceed 5 × 10^6 cells/mL.

Preparation of key reagents and supplies

Timing: 1–2 days

3. Prepare isothermal assembly enzyme mix,[11] and store in 15 μL aliquots at −80°C.

Alternatives: Gibson assembly enzyme mix may alternatively be purchased commercially.

Alternatives: We describe cloning using the Gibson assembly method[11,12]; however, alternative cloning methods may be used.

4. Prepare Protein A Sepharose beads (perform steps a-e at a temperature between 20°C to 25°C).
   a. Dissolve 1.5 g of powdered Protein A Sepharose into 45 mL of filtered 1 × TBS (pH 7.5) in a 50 mL conical tube.
   b. Gently invert 10 times.

⚠ CRITICAL: Do not vortex, as this may damage the beads.

   c. Allow the Protein A Sepharose slurry to settle for 30 min, and then carefully decant off supernatant.
   d. Add 45 mL of 1 × TBS (pH 7.5) to the 50 mL conical tube, gently invert 10 times, and allow the Protein A Sepharose slurry to settle for 30 min.
   e. Repeat step (d) two more times.

Note: Wash steps are necessary to remove all impurities. Small amounts of debris will be visible in the supernatant after each wash, but the bulk of the Protein A Sepharose should remain compacted at the bottom of the tube.

   f. Resuspend the compacted Protein A Sepharose in a final volume of 40 mL 1 × TBS (pH 7.5). Do not allow the Protein A Sepharose to dry out, as this will permanently damage the beads. The resuspended Protein A Sepharose can be stored at 4°C for at least up to 6 months.
   g. To prepare a column, clamp a 10 mL chromatography column (height of 50 cm) equipped with a stopcock to a column stand. Run 20 mL 1 × TBS (pH 8.0) through the column via gravity to wash. Loosely cover the top of the column with parafilm during this step.
   h. Place a 250 mL funnel reservoir on top of the column and run another 20 mL 1 × TBS (pH 8.0) via gravity through the column to wash. Loosely cover the top of the funnel reservoir with parafilm during this step. Close the column stopcock.
   i. Resuspend the Protein A Sepharose slurry by inverting the 50 mL conical tube multiple times. Remove 3 mL of the slurry and add to the column. Allow the slurry to settle for at least one hour at 4°C.

Note: Some antibodies produce higher yields than others, and the Protein A Sepharose amount can be increased to accommodate higher-yield antibody preparations.

Note: The prepared Protein A columns can be re-used up to 5 times for multiple preparations of the same antibody. After use, columns should be washed as described in the main protocol section "purification of antibodies" (step 18) and stored in 20% EtOH. When reusing a column, the EtOH should be drained and the column washed 3 times with 20 mL 1 × TBS (pH 8.0) before use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
NEB 5-alpha (DH5α) competent *E. coli*	New England Biolabs	Cat# C2987
Chemicals, peptides, and recombinant proteins		
Polyethylenimine (PEI)	Polysciences, Inc.	Cat# 24765-1
Valproic acid	Sigma-Aldrich	Cat# PHR1061

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Glycerol	MP Biomedicals	Cat# 800687
20% EtOH	Sigma	Cat# 459828
Coomassie Brilliant Blue R-250 Staining Solution	Bio-Rad	Cat# 1610436
Coomassie Brilliant Blue R-250 Destaining Solution	Bio-Rad	Cat# 1610438
NaCl	Fisher	Cat# S271
KCl	Fisher	Cat# P217
Sodium phosphate dibasic heptahydrate (Na_2HPO_4*$7H_20$)	Fisher	Cat# S373
Potassium phosphate dibasic anhydrous (KH_2PO_4)	Fisher	Cat# P288
TRIS	Sigma	Cat# T6066
Agarose LE, molecular biology grade	Thermo Fisher	Cat# J32802.22
Ethylenediaminetetraacetic acid (EDTA)	Sigma	Cat# E9884-500G
Glacial acetic acid	Sigma	Cat# AX0073
Gel loading dye, Purple (6×), included with REs	NEB	Cat# B7024S
SYBR Safe DNA gel stain	Invitrogen	Cat# S33102
PCRBio VeriFi mix	Genesee Scientific	Cat# 17-208
Restriction enzyme Sac1-HF	NEB	Cat# R3156S
Restriction enzyme Not1-HF	NEB	Cat# R3189S
DTT, molecular biology grade	GoldBio	Cat# DTT25
dNTP Mix, 10 mM each	Thermo Fisher	Cat# 18427088
Magnesium chloride	Sigma	Cat# M8266
Polyethylene glycol 8000, powder	Sigma	Cat# 89510
Nicotinamide adenine dinucleotide (NAD)	Sigma	Cat# N8410
T5 Exonuclease	NEB	Cat# M0663S
Phusion High-Fidelity DNA Polymerase	NEB	Cat# M0530S
Taq DNA Ligase	NEB	Cat# M0208L
NEB Gibson assembly master mix (alternative to making in-house isothermal assembly enzyme mix)	NEB	Cat# E2611S
Luria broth agar	Genesee Scientific	Cat# 11-122
Luria broth	Genesee Scientific	Cat# 11-120
Kanamycin	Gold Biotechnology	Cat# K-120-50
Critical commercial assays		
Zymo Clean Gel DNA Recovery kit	Genesee Scientific	Cat# 11-301C
ZymoPURE II Plasmid Miniprep kit	Genesee Scientific	Cat# 11-553B
ZymoPURE II Plasmid Maxiprep kit	Genesee Scientific	Cat# 11-555
Experimental models: Cell lines		
Expi293F	Thermo Fisher	Cat# A14527
Recombinant DNA		
pEGFP-N1	Clontech (now available at Addgene)	Cat# 6085-1
Other		
GE Imaging System (ImageQuant)	GE Healthcare	ImageQuant LAS 500
Spinner flasks	Sigma	Cat# CLS4985125
New Brunswick S41i Incubator/Shaker	Eppendorf	Cat# S41I120010
Optimem media	Gibco	Cat# 31-985-070
Protein A Sepharose	Sigma	Cat# P3391
Peristaltic pump	Bio-Rad	Model # EP-1
Chromatography column: 50 cm high	Bio-Rad	Cat# 7311551
Funnel reservoir, 250 mL	Bio-Rad	Cat# 7310003

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Column adaptor	Bio-Rad	Cat# 7311555
Funnel reservoir adaptor	Bio-Rad	Cat# 7310003
NuPAGE 4%–12% Bis-Tris SDS-PA gel (pre-poured)	Invitrogen	Cat# NP0321
Expi293 Expression Medium	Gibco	Cat# A14351
Trypan blue	Corning	Cat# 25-900
Amicon Ultra-4 Centrifugal Filter 30 kDa MW Cutoff	Millipore	Cat# UFC903024
Vacuum filter (0.2 μm)	Genesee Scientific	Cat# 25-233
25 kDa MW Cutoff, Pre-wetted Dialysis membrane	Spectrum Labs	Cat# 132554
Dialysis clips	Thermo Fisher	Cat# 10341444
BioRad PowerPac Basic Electrophoresis Supply	Bio-Rad	Cat# 1645050
Mini-Sub Cell GT Horizontal Electrophoresis System	Bio-Rad	Cat# 1704406
Microcentrifuge 5425	Eppendorf	Cat# 5405000107
T100 Thermal Cycler	Bio-Rad	Cat# 1861096
Parafilm	Thermo Fisher	Cat# 11747487
50 mL conical tubes	Genessee Scientific	Cat# 28-106
15 mL conical tubes	Genessee Scientific	Cat# 28-103
NanoDrop Spectrophotomer	Thermo Scientific	Cat# ND-2000
Hemacytometer	Sigma	Cat# Z359629
Eppendorf tubes, 0.5 mL	Genessee Scientific	Cat# 24-272
Eppendorf tubes, 1.2 mL	Genessee Scientific	Cat# 24-281

MATERIALS AND EQUIPMENT

1 × PBS, pH 7.2 (1 L)

Reagent	Final concentration	Amount
NaCl	0.137 M	8.01 g
KCl	0.0027 M	0.2 g
Na_2HPO_4*$7H_20$	0.01 M	2.68 g
KH_2PO_4	0.0018 M	0.24 g
ddH_2O	–	Up to 1 L

*Filter and store at 4°C for up to 6 months.

1 × TBS, pH 8.0 (1 L)

Reagent	Final concentration	Amount
TRIS	0.1 M	12.11 g
NaCl	0.15 M	8.77 g
ddH_2O	–	Up to 1 L

*Adjust pH to 8.0, filter, and store at 4°C for up to 6 months.

1 × TBS, pH 7.5 (1 L)

Reagent	Final concentration	Amount
TRIS	0.1 M	12.11 g
NaCl	0.15 M	8.77 g
ddH_2O	–	Up to 1 L

*Adjust pH to 7.5, filter, and store at 4°C for up to 6 months.

Elution buffer, pH 2.95 (1 L)		
Reagent	Final concentration	Amount
NaCl	0.15 M	8.77 g
Glycine	0.1 M	7.5 g
ddH_2O	–	Up to 1 L

*Adjust pH to 2.95, filter, and store at 4°C for up to 1 month (make sure to check and adjust pH before using each time).

TRIS-HCl, pH 7.4 or TRIS-HCl, pH 7.5 (1 L)		
Reagent	Final concentration	Amount
TRIS	1 M	121.1 g
ddH_2O	–	Up to 1 L

*Adjust pH to 7.4 or 7.5 by adding concentrated HCl. Filter and store at 4°C for up to 6 months.

10 × TAE (1 L)		
Reagent	Final concentration	Amount
TRIS	0.4 M	48.5 g
Glacial Acetic Acid	0.2 M	11.4 mL
0.5 M EDTA pH 8.0	0.01 M	20 mL
ddH_2O	–	Up to 1 L

*Dilute to 1 × for use. Store between 20°C–25°C for up to 9 months.

5 × isothermal reaction buffer (6 mL)		
Reagent	Final concentration	Amount
TRIS-HCl, pH 7.5	0.5 M	3 mL 1 M TRIS-HCl, pH 7.5
$MgCl_2$	0.05 M	300 µL 1 M $MgCl_2$
dNTPs	0.001 M	600 µL 10 mM dNTP mix
DTT	0.05 M	300 µL 1 M DTT
PEG-8000	25%	1.5 g
NAD	0.005 M	20 mg
ddH_2O	–	Up to 6 mL

*Store in 320 µL aliquots at −80°C for up to 1 year.

Isothermal assembly enzyme mix (1.2 mL)		
Reagent	Final concentration	Amount
5 × isothermal reaction buffer	1×	320 µL
T5 Exonuclease	0.01 U/µL	1.2 µL
Phusion Polymerase	0.03 U/µL	20 µL
Taq DNA Ligase	5.3 U/µL	160 µL
H_2O		700 µL

*Store in 15 µL aliquots at −80°C for up to 1 year.

STEP-BY-STEP METHOD DETAILS

Generation of antibody heavy and light chain plasmids from primary sequence

Timing: 2–3 weeks

The purpose of this section is to generate two separate expression plasmids, one encoding the sequence for the antibody heavy chain and one encoding the sequence of the antibody light chain.

1. With antibody heavy and light chain sequences in-hand, design and order two separate gene fragments, one containing the entire heavy chain sequence, and one containing the entire light chain sequence.

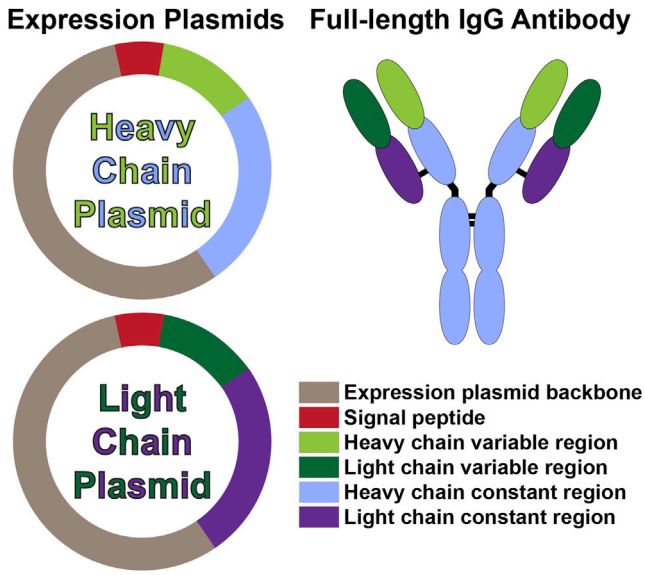

Figure 1. Heavy and light chain expression plasmids for production of full-length IgG antibodies

Shown on the left is a schematic indicating the major components of the two expression plasmids containing the heavy chain and light chain sequences. Light brown indicates the expression plasmid backbone (typically containing a CMV promotor for high expression in human cells). Red indicates the signal peptide sequence, which directs the expressed protein through the secretory system and into the cell media. The two shades of green indicate the heavy and light chain variable regions, and the two shades of purple indicate the heavy and light chain constant regions. Shown on the right is a schematic of a full-length IgG antibody, which is also color-coded.

Note: When ordering the gene fragments, use a codon optimization algorithm that optimizes the sequence for expression in human cells. Also, make sure the gene fragments contain the sequence elements required for downstream cloning (e.g., appropriate overhangs for Gibson assembly).

⚠ CRITICAL: Signal peptides target proteins through the secretory pathway, thus it is critical to include signal peptide sequences in both heavy and light chain gene fragments so that antibodies will be secreted into the cell growth media. If native signal peptides are included in the sequence (e.g., sequences are obtained through transcriptome sequencing), include these in the gene fragments. If they are not (e.g., sequences are obtained through mass spectrometry analysis of purified antibodies), make sure to add signal peptide sequences, located N-terminal to the variable region of each chain[13,14] (Figure 1).

Alternatives: A cost-saving alternative to ordering gene fragments encoding for the *entire* heavy and light chain sequences is to only order the variable regions of each of the chains and clone them into parent plasmids along with constant regions of choice (described below in step 5).

2. Upon receipt of the gene fragments, follow the manufacturer's provided protocol to suspend the DNA in nuclease-free water. We order gene fragments from Integrated DNA Technologies (IDT) and follow their protocol for resuspension of the plasmids as follows:
   a. Centrifuge the tube for 3–5 s at 3,000 × *g* in a microcentrifuge to pellet material to bottom of the tube.
   b. Add nuclease-free water to the tube to reach the desired concentration (e.g., 25 µL for a final concentration of 20 ng/µL from a synthesis scale of 500 ng).
   c. Briefly vortex, and spin down again.

d. Determine DNA fragment concentration (e.g., using a NanoDrop spectrophotometer) and store at −20°C when not in use.

3. Prepare parent plasmids for gene fragment insertion.
   a. Digest 1 μg of the pEGFP-N1 plasmid (or other expression plasmid) with appropriate restriction enzymes (e.g., we digest the pEGFP-N1 plasmid with Sac1 and Not1 to remove the sequence encoding for EGFP and to linearize the plasmid).
   b. Run the digest on a 1% agarose gel in 1× TAE buffer.
   c. Using a clean razor blade, cut out the band corresponding to digested plasmid, place in an Eppendorf tube and purify from the agarose gel using a DNA gel recovery kit (e.g., Zymo Clean Gel DNA Recovery Kit).
   d. Determine DNA digest concentration (e.g., using a NanoDrop spectrophotometer).
   e. Use digested plasmid for both heavy and light chain "parent" plasmids as needed in downstream cloning reactions. Store at −20°C when not in use.

Note: Any plasmid designed for a high level of protein expression in mammalian cells (e.g., driven by a CMV promotor) can be used to generate the parent heavy and light chain plasmids for this protocol.

4. Clone entire heavy and light chain DNA from the gene fragments into separate, digested heavy and light chain parent plasmids. The Gibson assembly method that we use is described here:
   a. To a single 15 μL aliquot of isothermal enzyme mix (or commercially purchased Gibson enzyme mix), add 50–100 ng of digested parent plasmid and the gene fragment of interest using a 1:2 molar ratio (digest:fragment). In a typical Gibson reaction, for the heavy chain, we use 100 ng parent plasmid (3,952 bp) and 70 ng heavy chain DNA fragment (1,400 bp), and for the light chain, we use 100 ng parent plasmid (3,952 bp) and 35 ng light chain DNA fragment (700–800 bp).
   b. Bring the reaction volume to 20 μL with H_2O, mix, and incubate at 50°C for 20 min.
   c. Transform 3 μL of each cloning reaction into DH5α competent *E. coli* cells, and grow on appropriate LB (Luria broth) + antibiotic agar plates (e.g., our parent plasmids are Kanamycin resistant).
   d. Select and expand individual colonies for screening by inoculating 3 mL LB plus antibiotic, and shake at 225 rpm for 16 h at 37°C.
   e. Purify plasmid DNA clones using a DNA miniprep kit.
   f. Screen for correct plasmid clones by Sanger or nanopore sequencing.
   g. Store positive clones as miniprep DNA at −20°C.

⚠ CRITICAL: The Gibson assembly cloning method requires 20 base pair overlapping sequence regions between gene fragments and digested parent plasmids. Information on Gibson cloning can be found in Gibson et al.[11] and Gibson.[12]

Alternatives: As an alternative to generating in-house isothermal assembly enzyme mixes, Gibson enzyme mixes can be purchased commercially.

5. An alternative to cloning the entire heavy and light chain sequences into parent plasmids is to order gene fragments containing only the heavy and light chain variable regions. These are then combined with heavy and light chain constant regions (amplified by PCR), and cloned into parent plasmids. The Gibson assembly method for this approach is described here:
   a. Following steps 1 and 2 above, design, order, and prepare two separate gene fragments, one containing the heavy chain variable sequence, and one containing the light chain variable sequence. Again, be sure to include signal peptide sequences and any necessary overlap sequences for downstream cloning.

b. Using standard PCR methodologies, design primers and perform PCR to obtain DNA fragments of the heavy chain constant region and light chain constant region of choice (using PCRBio VeriFi Mix or similar), being mindful to ensure that the fragments will be compatible with your downstream cloning method of choice.
c. For both the heavy and light chains (in separate reactions), to a single 15 μL aliquot of isothermal assembly enzyme mix (or commercially purchased Gibson enzyme mix), add 50–100 ng of digested parent plasmid, the variable region gene fragment, and the corresponding constant region PCR fragment using a 1:2:2 molar ratio (digested parent plasmid:variable region gene fragment:constant region PCR fragment). For example, in a typical Gibson reaction for assembling the heavy chain expression plasmid, we would use 100 ng of digested parent plasmid (3,952 bp), 20 ng heavy chain variable region fragment (400 bp), and 50 ng heavy chain constant region PCR fragment (1,000 bp).
d. Follow steps 4b–4g above to complete the cloning process.

6. Prepare purified DNA (e.g., Maxipreps) of the appropriate expression plasmids containing both the heavy and light chain antibody sequences.

⚠ CRITICAL: Ensure that DNA preparations contain an endotoxin removal step.

Transfection of expression plasmids into human suspension cell cultures

Timing: 6–8 days

The purpose of this section is to transfect the two antibody expression plasmids, one encoding the heavy chain and one encoding the light chain, into human suspension cell cultures.

7. Twenty-four hours prior to transfection, seed 30 mL cells at 1×10^6 cells/mL for a total of 30×10^6 cells. The viability should be greater than > 90%.

Note: Depending on the desired yield, the number of cells can be scaled up. Irrespective of the total number of cells, seed at 1×10^6 cells/mL.

8. On the day of transfection, count cells and ensure that > 90% of the cells are viable. Pellet 45×10^6 cells by centrifugation at 2,000 × *g* for 4 min at 4°C.
9. Aspirate off the supernatant and gently resuspend cell pellet in 15 mL fresh Expi293F media. Return resuspended cells to spinner flasks and incubate in the orbital shaker while the transfection reagent reactions are prepared.
10. Prepare the transfection reactions.
    a. Mix 100 μL of polyethylenimine (PEI) at 1 mg/mL plus 1 mL serum-free Optimem media.
    b. In a second tube, mix 50 μg of the light chain plasmid, 35 μg of the heavy chain plasmid (3:2 of light chain plasmid to heavy chain plasmid), in 1 mL of Optimem media.
    c. Incubate contents of the two separate tubes for 5 min with occasional flicking, at a temperature between 20°C–25°C.
    d. After 5 min, combine the contents of the two tubes and incubate for 15 min at a temperature between 20°C–25°C, flicking the tube approximately every 2 min to ensure thorough mixing.
11. Remove cells from the orbital shaker, add the transfection reaction, place the cells back in the shaker, and incubate for 24 h, rotating at 125 rpm.
12. Remove cells from the shaker and add 15 mL fresh Expi293F media to the 17 mL culture plus 300 μL of 220 mM valproic acid, which has been shown to increase recombinant protein production. Return cells to the shaker and incubate for 4 days.
13. Four days after addition of the valproic acid, it is advisable to test the cell media for positive antibody reactivity using an assay that the purified antibody is intended to be used for (e.g., immunofluorescence, immunoblotting). To test, remove 0.5 mL of the media, spin at 2,000 × *g* for 4 min at 4°C, and collect the supernatant/clarified media. We suggest testing a range of

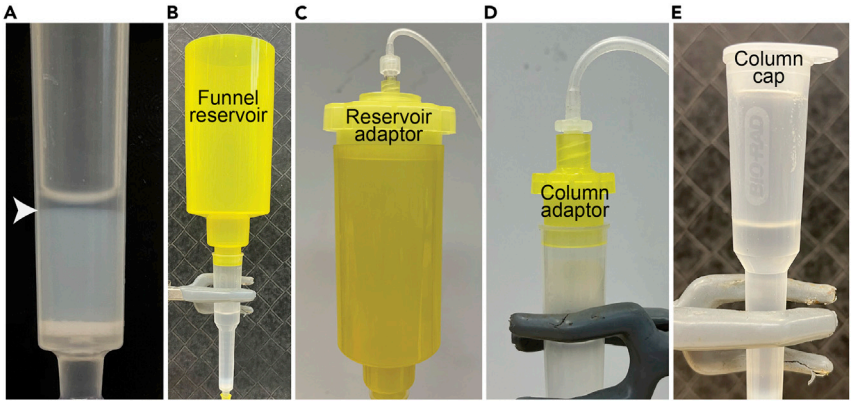

Figure 2. Images of the column apparatus used for protein purification

(A) The white arrow indicates the top of the Protein A Sepharose bed, and the image shows the liquid approaching, but not entering, compacted bed.

(B) The funnel reservoir is attached to the top of the column.

(C) The reservoir adaptor is placed on top of the funnel reservoir and then attached to the peristaltic pump through tubing.

(D) The column adaptor is placed on top of the column and attached to the peristaltic pump through tubing.

(E) The column cap is placed on top of the column for storage.

dilutions of the clarified media (from undiluted to 1:1000). We typically test the media at days 4, 5, and 6 post-transfection to determine the optimal time window in which to harvest the antibody. This may vary between antibody preparations and should be tested for each different antibody individually.

Purification of antibodies

Timing: 2 days

After transfection of the expression plasmids, Expi293F cells will produce the antibody heavy and light chains and secrete the folded, bi-valent antibody into the cell media. The purpose of this section of the protocol is to harvest and purify the antibody for use in downstream applications.

14. Four to six days post-transfection, and ideally after confirming positive reactivity of the cell media (see step 13), remove cells from the incubator, transfer to a 50 mL conical tube, and centrifuge at 2,000 × *g* for 4 min at 4°C to pellet the cells. Remove the antibody-containing supernatant without disturbing the pellet, and pass through a 0.2 μm filter into a sterile 100 mL glass bottle. Measure the volume of the filtered supernatant, and add 1.0 M Tris-HCL (pH 7.4) to bring the pH between 7.4–7.7 (adding ~10% of the measured volume, or 3 mL to a 30 mL prep). Store the filtered supernatant at 4°C until purification, which is optimally within 24 h of harvesting.
15. Remove a prepared Protein A Sepharose column from storage at 4°C (see "before you begin / preparation of key reagents and supplies" section above). Attach to a column stand and open the stopcock to drain storage buffer. Wash the column with 30 mL (approximately 3 column volumes) 1 × TBS (pH 8.0). Drain the wash buffer until it approaches, but does not enter the compacted Protein A Sepharose (Figure 2A).

Note: The binding affinity of Protein A for the constant region of IgG antibodies varies depending on the specific subclass of IgG. For our purifications, we have used Protein A beads for all antibodies thus far, which include the following subclasses: mouse IgG1, mouse IgG2a, mouse IgG2b and rabbit IgG kappa. For these subclasses of antibodies, Protein G has a similar affinity as Protein A, therefore Protein G could also be used. In the cases where the constant

region of a particular antibody subclass has a higher affinity for Protein A vs. G, this should be taken into consideration when choosing between the two.

16. Apply the antibody-containing cell media to the column.
    a. Close the stopcock and place a funnel reservoir on the top of the column (Figure 2B).
    b. Move the column apparatus to a 4°C walk-in cold room.
    c. Slowly add the filtered cell media to the column. The cell media should fill the column and partially fill the funnel reservoir.
    d. Allow any disrupted Protein A Sepharose to settle for 1 h.
    e. Once the Protein A Sepharose has settled, place a reservoir adaptor (with an inlet for tubing) on the funnel reservoir, and attach the apparatus to a peristaltic pump with tubing (Figure 2C).
    f. Open the stopcock, turn on the peristaltic pump and collect the flow-through at a rate of 1 mL/min into a sterile 100 mL glass bottle.
    g. Once the liquid approaches the compacted Protein A Sepharose bed, close the stopcock.
    h. Remove the reservoir adaptor, and gently add back the collected flow-through to the funnel reservoir.
    i. Allow the Protein A Sepharose to re-settle for 1 h.
    j. Open the stopcock and collect the flow-through at a rate of 1 mL/min.
    k. Stop the pump and close the stopcock when the liquid approaches the compacted Protein A Sepharose.
    l. Remove the funnel reservoir, slowly add 3 mL of 1 × TBS (pH 8.0) to the column, and let settle for 15 min.
    m. Gently attach a column adaptor with a threaded valve on top of the column and connect via tubing to the peristaltic pump (Figure 2D).
    n. Open the stopcock and collect the flow-through at a rate of 1 mL/min.
    o. When the buffer approaches the top of the compacted Protein A Sepharose, close the stopcock.
17. Elute the antibody from the Protein A Sepharose.
    a. Slowly add 9 mL of elution buffer to the column.
    b. Open the stopcock and turn on the peristaltic pump adjusted to a flow rate of 5 mL/min.
    c. Collect the eluate in a 15 mL conical tube containing 0.9 mL 1 M Tris-HCl (pH 8.0).
    d. Once all of the elution buffer has entered the Protein A Sepharose, close the stopcock, and stop the pump.
    e. Immediately cap and invert the 15 mL conical tube to mix the solution and neutralize the low pH of the elution buffer.
18. Immediately clean the Protein A Sepharose column to avoid any damage to the Sepharose beads.
    a. Gently fill the column with elution buffer and set the peristaltic pump to 5 mL/min.
    b. Wash with two column volumes of elution buffer (~20 mL) followed by two column volumes (~20 mL) of 1 × TBS (pH 8.0).
    c. After the second 1 × TBS (pH 8.0) wash, stop the pump and close the stopcock when the 1 × TBS (pH 8.0) approaches the compacted Protein A Sepharose bed.
    d. Fill the column with 20% EtOH (~7 mL), place a cap on the column (Figure 2E), and store upright at 4°C. Columns can be used for up to 5 rounds of purification of an individual antibody before being discarded.
19. Transfer the eluate from step 17 into pre-soaked dialysis membrane, and seal with dialysis clips. Place the dialysis membrane into a beaker containing 1 L of 1 × PBS (pH 7.2) and a 6 cm stir bar. Gently stir at 4°C for 4 h. After 4 h, replace the 1 × PBS (pH 7.2) with 1 L fresh 1 × PBS and stir for 14–16 h at 4°C.
20. Soak a 30 kDa cutoff centrifugal filter concentrator in 1 × PBS for 1 min, and then spin at 2000 x g for 1 min to remove PBS. Retrieve purified, dialyzed antibodies from the tubing and place into the concentrator (should be approximately 10 mL). Spin at 2,000 × *g*, and check the volume of

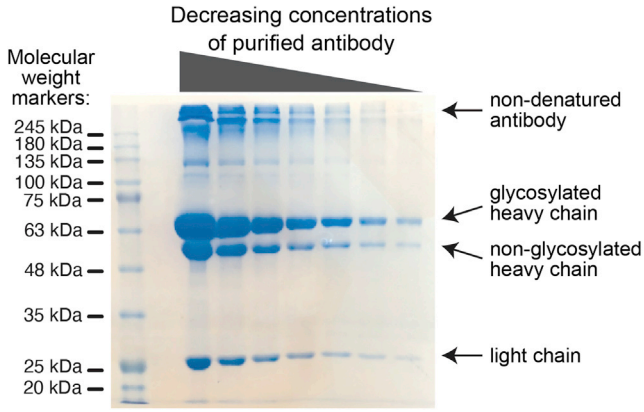

Figure 3. Image of protein gel in which decreasing concentrations of a purified, recombinant, monoclonal IgG antibody was applied

Starting from the left, 40–0.01 μg purified rMAb-Hec1^{ms} antibody was run on a 4%–12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Antibody chains are labeled in the figure. Gel shown was originally published in DeLuca et al.[1]

the concentrated antibody solution every 20 min, until the final, concentrated volume reaches between 100 and 200 μL.

21. Remove the purified antibody from the concentrator and measure the protein concentration (e.g. using a NanoDrop spectrophotometer). Dilute the antibody to 1–2 mg/mL in 1 × PBS (pH 7.2) plus 15% glycerol. Aliquot the antibody into tubes and store at −20°C.

Note: We typically make small-volume aliquots (2–5 μL), in order to avoid multiple freeze/thaw events), as well as large-volume, "stock" aliquots (100–200 μL). Once the stock aliquots are thawed, we re-aliquot the antibody into smaller, 2–5 μL aliquots.

22. Serially dilute a sample of the purified antibody and load a 4%–12% SDS-polyacrylamide gel with the samples (e.g., ~0.01 μg–40 μg). Run the gel according to standard SDS-PAGE protocols, and stain/destain with Coomassie Brilliant Blue or other appropriate protein stain. A typical gel showing the expected bands is shown in Figure 3 (gel shown is from DeLuca et al.[1]).

Note: After purification, antibodies should be validated for the intended application (e.g., immunofluorescence, immunoblotting, immunoprecipitation). It is advised to first test a wide range of concentrations and adjust the range accordingly after obtaining initial results.

Note: In addition to purifying an antibody using the sequences of the heavy and light variable regions paired with sequences of constant regions corresponding to the species the antibody was originally generated in, it is also straightforward to generate plasmids containing the heavy and light chain variable regions combined with the constant regions from a different species. By generating species specificity variants of an original antibody, this overcomes a common limitation in indirect immunofluorescence or immunohistochemical experiments, where the combination of antigens researchers wish to detect is limited by the species specificity. Sequences of plasmids containing constant regions specific to mouse, rabbit, and human can be found in DeLuca et al.[1]

Note: Once an antibody sequence is in-hand, it is also possible to generate single chain antibody fragments including native scFv (single chain variable fragments) and native scFvC (single chain variable fragments plus a truncated constant region). "Native" is used to indicate that the scFv or scFvC fragments are derived from the original primary sequence of the

full-length antibody. Such fragments can be designed such that they are genetically encoded and tagged (e.g., with a fluorescent protein) for expression and visualization in cells. In brief, to generate a native scFv, the following PCR fragments are produced: (1) the heavy chain variable region; (2) a flexible linker, and (3) the light chain variable region. PCR fragments are then cloned into a parent plasmid containing the sequence for GFP, resulting in a final single scFv plasmid. For construction of a native scFvC plasmid, the following PCR fragments should be generated: (1) heavy chain variable region; (2) a flexible linker, (3) the light chain variable region, and (4) heavy chain constant regions CH2 and CH3, specific to the species of preference. Similar to the scFv protocol, PCR fragments are then cloned into a parent plasmid containing the sequence for GFP, resulting in a final, single scFvC plasmid. Plasmids can then be transfected into a cell line of choice and imaged via fluorescence microscopy. All relevant plasmid sequences for this protocol can be found in DeLuca et al.[1]

Note: There are growing numbers of scFv sequences available to the public due to advances in approaches such as antibody phage display and hypervariable domain grafting.[9,15–19] Using the techniques described in this protocol, it is possible and straightforward to reverse-engineer full-length, bivalent antibodies from an scFv sequence. In brief, to convert an scFv sequence into a full-length antibody, PCR fragments are generated corresponding to the heavy chain and light chain variable regions of the scFv, which both include an N-terminal signal peptide. PCR fragments corresponding to the light chain and heavy chain constant regions (chosen from the species of choice) are also generated and, together with the heavy and light chain variable regions, are cloned into an expression plasmid for transfection into HEK293 cells for downstream purification.

EXPECTED OUTCOMES

Here we describe a protocol for producing low-cost antibodies from a primary amino acid sequence. Typical yields for full-length, bi-valent antibody preparations from ~30 mL of HEK293 Expi293F cells cultured in suspension are in the range of 0.5 mg–1.5 mg, although this can vary. Our highest yield to date is 2.0 mg of purified antibody, while our lowest yield is 0.1 mg per 30 mL of starting culture. We have also tested both sensitivity and specificity of the recombinant monoclonal antibodies generated according to this protocol compared to the original, traditionally-generated antibodies. As described in DeLuca et al.,[1] both the sensitivity and specificity are typically increased for the recombinant versions, with only one case to date where these parameters were not statistically different. Importantly, we have not yet encountered a situation where the original antibody was successfully used for a particular application and the corresponding recombinant antibody was not.

In addition to using this protocol to obtain purified full-length antibodies, the methods described here can be used for diversification of sequenced antibodies. One practical example is altering the species specificity by cloning the variable regions of a given antibody into plasmids containing constant regions from a different species. Many cell biological experiments are limited in regard to the combination of antigens that can be detected by indirect immunofluorescence or immunohistochemistry by the species specificity of the antibodies. After a monoclonal antibody sequence has been obtained, the protocol provided here can be used to produce species variants, which increases experimental flexibility and provides a solution to previous limitations.

Another outcome of the methodology described here is the ability to generate different types of antibody fragments from a primary sequence. There are numerous advantages of using fragments, including more efficient binding to poorly-accessible epitopes; ability to bind targets without inducing antigen cross-linking; preventing or significantly reducing steric effects that might arise from using large, full-length antibodies; and tracking protein modifications and specific protein conformations in living cells.

If a researcher sequences a monoclonal antibody from a sample of purified protein, a significant portion of the costs for carrying out the protocol comes from the protein sequencing itself. To date, the approximate costs of sequencing a purified monoclonal antibody ranges from ~$5,000–$12,000. Alternatively, the costs for obtaining sequences from hybridoma cell lines producing monoclonal antibodies is much lower (~$500–$1,500), and is offered through commercial companies and non-profit facilities as a stand-alone service, or in many cases is included with the costs for producing a custom monoclonal antibody. We hope in the future that researchers submit their existing hybridoma cell lines for sequencing and provide this information publicly. This open sharing of sequences would provide unprecedented access to low-cost, sequence-defined, sustainable antibodies and antibody fragments, which would benefit the entire research community.

LIMITATIONS

This protocol has been successfully used to generate recombinant monoclonal antibodies from primary sequences; however, it is important to note that the yields may differ, depending on the antibody. We have not yet defined the source of yield variation, but we are working to optimize the cloning, expression, and purification conditions for lower-yield reagents.

Another limitation we have encountered with this protocol is in regard to the success rates of genetically-encoded antibody fragments. While the success rate of generating native scFvCs in our hands is 100%, that of generating native scFvs is ~20%. We have not determined the reason for this low rate; however, there are alternative methods that can be used to obtain scFvs from a primary sequence. A recently published study demonstrated that genetically encoded single chain antibody fragments can be generated by cloning the hyper-variable domains, obtained from the primary sequence of a monoclonal antibody, into optimized scFv scaffolds which are known to function in cells.[19]

TROUBLESHOOTING

Problem 1

Gibson cloning reactions do not yield colonies on transformation plate (step 4).

Potential solution

- Ensure that all necessary fragments are included in the Gibson reaction, and ensure that all fragments have the appropriate 20 bp overlapping sequences.
- Doublecheck the molar ratios of DNA fragments in the Gibson reaction.
- Ensure that the correct antibiotic is used on transformation plates.

Problem 2

Cell viability is low (step 7).

Potential solution

- Make sure that the temperature during cell shaking does not increase above 37°C. We place at least two thermometers at different locations within the shaker and check these at least twice a day. We also set the incubator temperature to 36.5°C to help ensure the temperature does not rise above 37°C.
- Cell viability could also decrease if the incubator intermittently stops and restarts shaking. To address this, make sure the incubator is equipped with a monitoring device that is activated and set up to send alerts to the user when shaking has stopped.

Problem 3

No antibody production from cells (step 13).

Potential solution

- Ensure that a signal peptide was added to the original gene fragments. If not, antibody will be produced in cells but not secreted into the media.
- No antibody production could also result from poor cell viability and/or low cell density. Ensure that cells are at least 90% viable and at a density of 3 million cells per mL at time of transfection.
- A third cause of no or low antibody production could be the amount of DNA added to the transfection reactions. Typically, to troubleshoot this, we begin by adding 50% more DNA, and then try doubling the amount.

Problem 4

Antibody is detected in cell media, but there is a low yield of purified antibody (step 21).

Potential solution

- Antibody yields can typically be increased by running the flow-through from the Protein A column (step 16) back through the column for up to two additional rounds (total of three applications).

Problem 5

Generation of native, genetically-encoded, fluorescently-labeled scFv is not successful (step 22, final note).

Potential solution

- One potential solution is to use alternate algorithms to identify the hyper-variable domains (also known as complementarity determining regions) vs. what is provided with the annotated sequence,[20–24] and combine these domains with published scFv "scaffolds," as described, for example, in Zhao et al.[19]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jennifer DeLuca (jdeluca@colostate.edu).

Materials availability

All plasmid sequences relevant to this protocol will be available through Addgene or by request from the lead contact.

Data and code availability

This study did not generate any datasets.

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AUTHOR CONTRIBUTIONS

K.F.D., J.E.M., and J.G.D conceived the study and designed experiments. J.G.D. wrote the manuscript with inputs from K.F.D. and J.E.M. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. DeLuca, K.F., Mick, J.E., Ide, A.H., Lima, W.C., Sherman, L., Schaller, K.L., Anderson, S.M., Zhao, N., Stasevich, T.J., Varma, D., et al. (2021). Generation and diversification of recombinant monoclonal antibodies. Elife *10*, e72093.

2. Lima, W.C., Gasteiger, E., Marcatili, P., Duek, P., Bairoch, A., and Cosson, P. (2020). The ABCD database: a repository for chemically defined antibodies. Nucleic Acids Res. *48*, D261–D264. https://doi.org/10.1093/nar/gkz714.

3. Cosson, P., and Hartley, O. (2016). Recombinant antibodies for academia: a practical approach. Chimia *70*, 893–897. https://doi.org/10.2533/chimia.2016.893.

4. Vazquez-Lombardi, R., Nevoltris, D., Rouet, R., and Christ, D. (2018). Expression of IgG monoclonals with engineered immune effector functions. Methods Mol. Biol. *1827*, 313–334.

5. Gavilondo, J.V., and Larrick, J.W. (2000). Antibody engineering at the millennium. Biotechniques *29*. 128-32, 134-136, 138 passim. https://doi.org/10.2144/00291ov01.

6. Saeed, A.F.U.H., Wang, R., Ling, S., and Wang, S. (2017). Antibody engineering for pursuing a healthier future. Front. Microbiol. *8*, 495.

7. Alfaleh, M.A., Alsaab, H.O., Mahmoud, A.B., Alkayyal, A.A., Jones, M.L., Mahler, S.M., and Hashem, A.M. (2020). Phage display derived monoclonal antibodies: from bench to bedside. Front. Immunol. *11*, 1986.

8. Almagro, J.C., Pedraza-Escalona, M., Arrieta, H.I., and Pérez-Tapia, S.M. (2019). Phage display libraries for antibody therapeutic discovery and development. Antibodies *8*, 44. https://doi.org/10.3390/antib8030044.

9. Laustsen, A.H., Greiff, V., Karatt-Vellatt, A., Muyldermans, S., and Jenkins, T.P. (2021). Animal immunization, in vitro display technologies, and machine learning for antibody discovery. Trends Biotechnol. *39*, 1263–1273.

10. Johnson, G., and Wu, T.T. (2000). Kabat database and its applications: 30 years after the first variability plot. Nucleic Acids Res. *28*, 214–218.

11. Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods *6*, 343–345.

12. Gibson, D.G. (2011). Enzymatic assembly of overlapping DNA fragments. Methods Enzymol. *498*, 349–361.

13. Burton, D.R., Pyati, J., Koduri, R., Sharp, S.J., Thornton, G.B., Parren, P.W., Sawyer, L.S., Hendry, R.M., Dunlop, N., and Nara, P.L. (1994). Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science *266*, 1024–1027.

14. Yu, X., Gilden, D.H., Ritchie, A.M., Burgoon, M.P., Keays, K.M., and Owens, G.P. (2006). Specificity of recombinant antibodies generated from multiple sclerosis cerebrospinal fluid probed with a random peptide library. J. Neuroimmunol. *172*, 121–131.

15. Panikar, S.S., Banu, N., Haramati, J., Del Toro-Arreola, S., Riera Leal, A., and Salas, P. (2021). Nanobodies as efficient drug-carriers: progress and trends in chemotherapy. J. Control. Release *334*, 389–412.

16. Valldorf, B., Hinz, S.C., Russo, G., Pekar, L., Mohr, L., Klemm, J., Doerner, A., Krah, S., Hust, M., and Zielonka, S. (2022). Antibody display technologies: selecting the cream of the crop. Biol. Chem. *403*, 455–477.

17. Shim, H. (2017). Antibody phage display. Adv. Exp. Med. Biol. *1053*, 21–34.

18. Shukra, A.M., Sridevi, N.V., Chandran, D., and Maithal, K. (2014). Production of recombinant antibodies using bacteriophages. Eur. J. Microbiol. Immunol. *4*, 91–98.

19. Zhao, N., Kamijo, K., Fox, P.D., Oda, H., Morisaki, T., Sato, Y., Kimura, H., and Stasevich, T.J. (2019). A genetically encoded probe for imaging nascent and mature HA-tagged proteins in vivo. Nat. Commun. *10*, 2947.

20. Chiu, M.L., Goulet, D.R., Teplyakov, A., and Gilliland, G.L. (2019). Antibody structure and function: the basis for engineering therapeutics. Antibodies *8*, 55. https://doi.org/10.3390/antib8040055.

21. Dondelinger, M., Filée, P., Sauvage, E., Quinting, B., Muyldermans, S., Galleni, M., and Vandevenne, M.S. (2018). Understanding the significance and implications of antibody numbering and antigen-binding surface/residue definition. Front. Immunol. *9*, 2278. https://doi.org/10.3389/fimmu.2018.02278.

22. Wu, T.T., and Kabat, E.A. (1970). An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. J. Exp. Med. *132*, 211–250. https://doi.org/10.1084/jem.132.2.211.

23. Chothia, C., Lesk, A.M., Tramontano, A., Levitt, M., Smith-Gill, S.J., Air, G., Sheriff, S., Padlan, E.A., Davies, D., Tulip, W.R., et al. (1989). Conformations of immunoglobulin hypervariable regions. Nature *342*, 877–883. https://doi.org/10.1038/342877a0.

24. Sela-Culang, I., Kunik, V., and Ofran, Y. (2013). The structural basis of antibody-antigen recognition. Front. Immunol. *4*, 302. https://doi.org/10.3389/fimmu.2013.00302.