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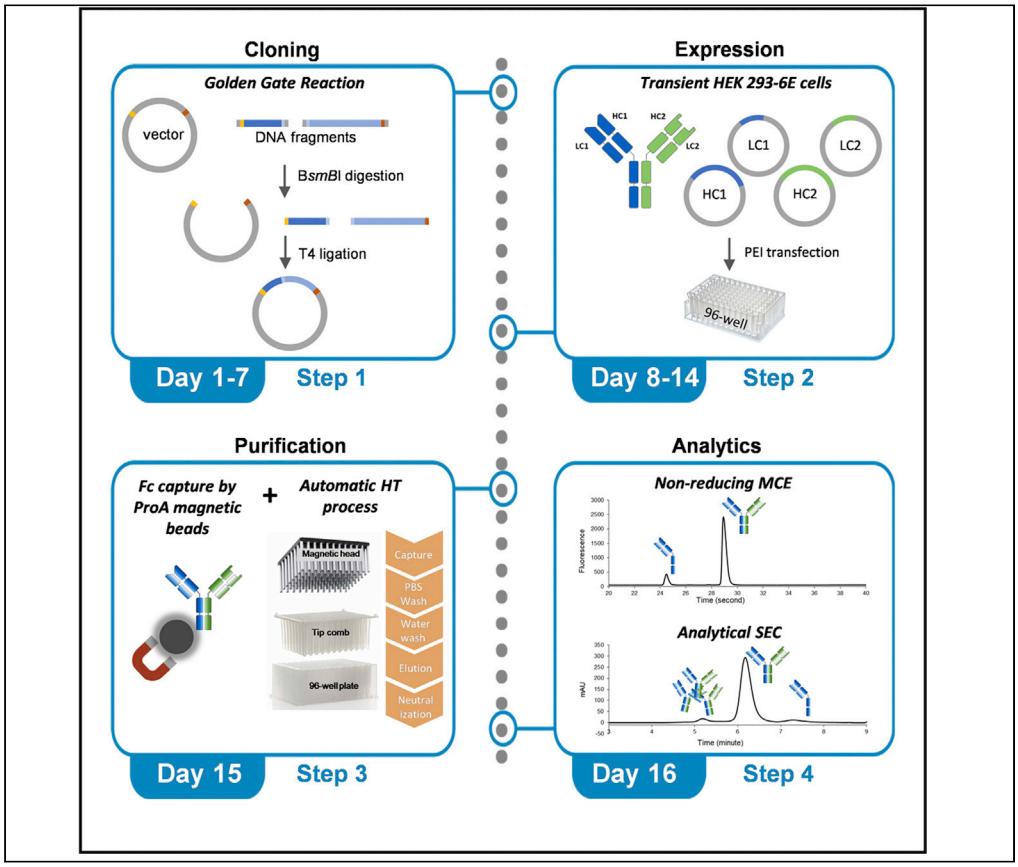
Protocol for high-throughput cloning, expression, purification, and evaluation of bispecific antibodies



Bispecific antibodies are a powerful new class of therapeutics, but their development often requires enormous amounts of time and resources. Here, we describe a high-throughput protocol for cloning, expressing, purifying, and evaluating bispecific antibodies. This protocol enables the rapid screening of large panels of bispecific molecules to identify top candidates for further development.

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

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Highlights

A streamlined protocol to screen large panels of bispecific antibodies in 16 days

Automated process to increase throughput and minimize errors

Optimized method to transiently express challenging molecules

Combined evaluation to measure yield and product quality

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Protocol for high-throughput cloning, expression, purification, and evaluation of bispecific antibodies

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SUMMARY

Bispecific antibodies are a powerful new class of therapeutics, but their development often requires enormous amounts of time and resources. Here, we describe a high-throughput protocol for cloning, expressing, purifying, and evaluating bispecific antibodies. This protocol enables the rapid screening of large panels of bispecific molecules to identify top candidates for further development.
For complete details on the use and execution of this protocol, please refer to Estes et al. (2021).

BEFORE YOU BEGIN

Experimental considerations

Timing: 2 h

1. DNA fragments and construct design.

Golden Gate Assembly provides a seamless and orderly strategy to clone multiple DNA fragments into a mammalian expression vector (Figure 1) (Engler et al., 2008, 2009; Estes et al., 2021; Gong et al., 2021). The pTT5 vector is a suitable vector for both bacterial cloning as well as protein expression in mammalian hosts. It contains a CMV promoter to drive robust expression and an oriP *cis*-element to facilitate replication in human embryonic kidney (HEK) 293-6E cells (Lufino et al., 2008). DNA fragments covering antibody variable and constant domains are designed using a software application such as Geneious (Biomatters). Scientific suppliers such as Twist Bioscience or IDT can separately synthesize the DNA fragments of unique variable and constant domains, which normally takes ~7 business days. The vector stock can be prepared with a QIAGEN Plasmid Maxi Kit.

2. Cell line considerations.

HEK 293-6E suspension cells (National Research Council of Canada) are an ideal tool to transiently express recombinant protein in a short time frame (1 week) with minimal handling (Fang et al., 2017; Vink et al., 2014; Jäger et al., 2015). Compared to Chinese hamster ovary (CHO) stable cell line expression, which often requires about one month, HEK 293-6E system offers a substantially reduced turnaround time. Though protein yields from a HEK 293-6E expression may be slightly

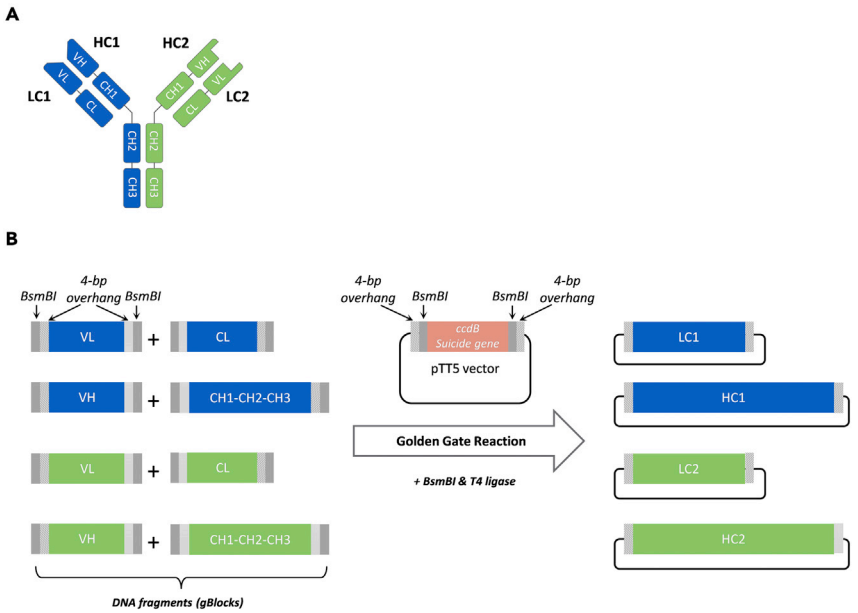


Figure 1. Schematic illustration of a bispecific Hetero-IgG molecule and corresponding Golden Gate Reaction

(A) Schematic illustration of a bispecific Hetero-IgG molecule. HC, heavy chain; LC, light chain; VH, variable heavy region; VL, variable light region; CL, constant light region; CH1, constant heavy region 1; CH2, constant heavy region 2; CH3, constant heavy region 3.

(B) Schematic illustration of DNA fragment design and Golden Gate Reaction. BsmBI, a type IIS restriction enzyme that cuts DNA outside of its recognition site; ccdB, a toxic gene that targets *E.coli* DNA gyrase.

lower than that from a CHO stable cell line, there is typically sufficient yield needed to perform the initial characterization and downstream analytics during early development (i.e., purity assessment, binding and functional analysis). Due to its reduced cycle time, the HEK 293-6E transient system is a preferred tool for high-throughput expression of bispecific antibodies.

3. Cell freezing, recovery and passaging.
   a. Prepare HEK 293-6E stocks.
      i. A cell stock could be obtained from a research cell bank (National Research Council of Canada).
      ii. Expand cell stock culture to 700 mL using cell culture medium, and centrifuge cells in the log growth phase (0.8–1.2 × 10^6 cells/mL) at 200 × *g* for 5 min at 20°C–25°C. Cell culture medium can be prepared using the table in the materials and equipment section.

      Note: Typically, a 700 mL culture with a viable cell density (VCD) of 1.0 × 10^6 cells/mL can be expected to yield approximately 60–70 vials of cell stocks.

      iii. Resuspend cell pellets with 0.1 volume of freezing medium (90% v/v FreeStyle F-17 medium plus 10% v/v DMSO), and aliquot into cryogenic tubes. Each aliquot should contain 1 × 10^7 viable cells (in a volume of approximately 1 mL).
      iv. Freeze using a controlled-rate freezing apparatus (Thermo Scientific) and store at −80°C for at least 24 h. For long term storage, transfer cryovials to a liquid nitrogen tank (vapor phase).
      v. After two to three days, evaluate the viability of frozen cells by thawing a test vial via the procedure below.
   b. Recover cell stock.

i. To recover cells from liquid nitrogen storage, thaw a cryovial in a 37°C water bath, and thoroughly sanitize with 70% ethanol before opening.
ii. In a biological safety cabinet, transfer cells into a 125 mL shake flask containing 19 mL of fresh cell culture medium (i.e., at an initial cell density of 5×10^5 cells/mL) and then place on a shaking platform set to 120 RPM in a humidified incubator controlled to 37°C with 5% CO_2.
iii. Three days post-thawing, measure cell viability using the trypan blue method, using an automated analyzer (for example, the Vi-CELL XR automated cell viability analyzer (Beckman Coulter)), or using a hemocytometer and light microscope. A cell viability of > 98% indicates a successful recovery.

Note: In the trypan blue method, nonviable cells are distinguished from live cells through their uptake of dye.

c. Maintaining cells.
i. Subculture every 2 or 3 days and dilute to a density of 0.35×10^6 or 0.2×10^6 cells/mL.
ii. During cell culturing, frequently check cell density to ensure it does not exceed 2.2×10^6 cells/mL.
iii. Discard cells after the 30^{th} passage and prepare a fresh cell culture from frozen stocks.

4. Analytical SEC instrumentation, column selection and software considerations.
a. Instrument configuration. A high-performance liquid chromatography (HPLC) system, such as the Infinity LC system (Agilent) or the Vanquish system (Thermo Fisher Scientific), can be utilized for high-throughput analytical size-exclusion chromatography (aSEC) analysis. The following configuration is based on the Agilent 1260 Infinity Series (Agilent):
i. Agilent 1260 Infinity Series High Performance Autosampler (G1367E) with temperature control module.
ii. Agilent 1260 Infinity Series Quaternary Pump (G1311B).
iii. Agilent Infinity Thermostatted Column Compartment (G1316C).
iv. Agilent 1200 Series Multiple Wavelength Detector SL (G1365C).
v. Agilent 1200 Series Fraction Collector (G1364C).
b. Column selection. A Zenix-C SEC column (300 Å, 3 micron, 4.6 × 300 mm) (Sepax Technologies) is an ideal choice for bispecific antibodies, as it displays minimal nonspecific interactions with proteins and enables separation of a molecular weight range of 5,000–1,250,000 Daltons. An isocratic mobile phase consisting of 100 mM sodium phosphate, 250 mM NaCl, pH 6.8 (made in HPLC-grade water), at a flow rate of 0.4 mL/min, is recommended in this protocol; however, the Zenix-C SEC column is compatible with a broad range of aqueous buffers. A BEH C200 column (1.8 micron, 4.6 × 300 mm) (Waters), which utilizes an alternative stationary phase chemistry, is also a suitable option for antibody characterization. A suitable mobile phase for this column consists of 100 mM sodium phosphate, 250 mM NaCl, 7.5% ethanol, pH 6.9 (made in HPLC-grade water), at a flow rate of 0.4 mL/min.

Note: Organic solvents such as ethanol or isopropanol (5%–15% v/v final concentration) can be added to the mobile phase to further reduce secondary interactions between proteins and the stationary phase of the column.

c. Chromatography software. The Chromeleon Chromatography Data System (Thermo Fisher Scientific) is routinely used for instrument control, data acquisition and data analysis. Alternatively, software packages such as the OpenLab Software Suite (Agilent) may be used.

5. Laboratory reagent preparation.
a. Prepare 0.1% w/v linear PEImax transfection reagent:
i. Add 450 mL Milli-Q water to a 500 mL glass beaker containing a magnetic stir bar.
ii. Weigh 500 mg PEImax and then slowly add to beaker while stirring at 200 rpm.
iii. Stir until dissolved. PEImax dissolves easily after 5–15 min of stirring.

iv. Adjust pH to 6.9–7.1 using 1 M NaOH dropwise.
v. Transfer solution into a 500 mL cylinder; adjust final volume to 500 mL with Milli-Q water.
vi. To sterilize, vacuum-filter the solution using a 0.22 μm cellulose acetate (CA) membrane.
vii. Aliquot to desired volumes and store at 4°C. PEImax aliquots can be stored at 4°C for up to two years.

b. Prepare the ProA magnetic beads (GenScript, 25% slurry in PBS):
i. Calculate the amount of bead slurry required based on the following formula:

Bead slurry volume = (number of samples) × 200 μL (50 μL settled beads) × 1.2.

Note: The calculation above provides a 20% excess of slurry to account for loss due to pipetting error.

ii. Transfer bead slurry into a 50 mL conical tube or other straight edge containers depending on the volume.
iii. Place the container onto a magnetic separation rack to collect the beads. Incubate for 5 min, then discard the liquid.

Note: All bead collection steps involving the magnetic separation rack require a 5-minute incubation unless otherwise indicated.

iv. Add 5–10 volumes (settled beads) of deionized water into container, and resuspend beads by swirling. Incubate mixture with the magnetic separation rack, then discard the water. Repeat 3 times.
v. Add 5–10 volumes (settled beads) of 0.1 M NaOH into container and resuspend beads by swirling. Incubate for 5 min, then collect the beads by a magnetic separation rack and discard the supernatant.

Note: 0.1 M NaOH is used to remove all protein binding to beads.

vi. Add 5–10 volumes (settled beads) of DPBS into container, resuspend beads by swirling, collect the beads by a magnetic separation rack and discard the supernatant. Repeat 3 times.

Note: After this step, beads can be stored in DPBS for up to 2 months at 4°C.

vii. Add 1 volume (settled beads) of cell culture medium (see table in the materials and equipment section) to container to create a 50% v/v bead slurry.

Note: Adding cell culture medium to the beads should be done freshly prior to each experiment. Storing the beads in cell culture medium for long periods of time is not recommended.

viii. Strip and recover used beads as needed: Pool used beads into a clean beaker, use a magnetic separation rack to collect the beads and discard the supernatant. Add 5–10 volumes of 0.1 M NaOH, swirl and transfer to an appropriately sized bottle. Mix for 10 min on a tube roller. Use the magnetic separation rack to collect the beads and discard the supernatant. Repeat 2 times. Add 5–10 volumes of DPBS into container, resuspend beads by swirling, collect the beads on the magnetic separation rack and discard the DPBS. Using water, repeat the previous step an additional 3 times. Add 1 volume of 20% v/v ethanol to the beads to make a 50% slurry, and store at 4°C.

Note: ProA magnetic beads can be used up to 30 times. Beads used beyond the recommended limit will exhibit reduced binding capacity due to damage during stripping and recovery.

Note: The manufacturer states that the beads have a shelf life of 2 years when stored unopened at 4°C.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Esp3I (BsmBI)	Thermo Scientific	Cat# FD0454
T4 DNA ligase	Thermo Scientific	Cat# EL0011
Top10 chemically competent *E. coli*	Lucigen	Cat# 60000-PQ805-G
PEImax	Polysciences	Cat# 24765-1
FreeStyle F-17 medium	Gibco	Cat# A1383502
Kolliphor P188	Sigma-Aldrich	Cat# K4894
G418 (antibiotic)	Gibco	Cat# 10131027
Carbenicillin Disodium Salt	Gibco	Cat# 10177012
L-glutamine	Gibco	Cat# 25030149
Tryptone-N1	Organotechnie	Cat# 19553
Glucose (200 g/L)	Gibco	Cat# A2494001
DPBS	Gibco	Cat# 14190136
DMSO	Sigma-Aldrich	Cat# D2650
Sodium valproate	MP Biomedicals	Cat# 0215206480
Octet Sample Diluent	Sartorius	Cat# 18-1104
Iodoacetamide	Sigma-Aldrich	Cat# A3221
Critical commercial assays		
QIAGEN Plasmid Maxi Kit	QIAGEN	Cat# 12165
QIAGEN Plasmid plus 96 Kit	QIAGEN	Cat# 16181
Vi-CELL sample vial	Beckman Coulter	Cat# 383721
Trypan blue-based Vi-CELL XR reagent	Beckman Coulter	Cat# 383722
Protein Express Assay Reagent Kit	PerkinElmer	Cat# 760328
Protein Express Assay LabChip	PerkinElmer	Cat# 760528
Protein Express Sample Buffer	PerkinElmer	Cat# 760518
Gel Filtration Standard	Bio-Rad	Cat# 1511901
Experimental models: Cell lines		
HEK 293-6E	National Research Council of Canada	NRC File 11565
Recombinant DNA		
pTT5	National Research Council of Canada	N/A
Synthesized DNA fragments	Twist Bioscience	N/A
Other		
Octet ProA biosensors	Sartorius	Cat# 18-5010
ProA magnetic beads	GenScript	Cat# L00695
Kingfisher 96-well plates	Fisher Scientific	Cat# 95040450
Kingfisher Flex 96-well magnetic head	Fisher Scientific	Cat# 24074430
FLEX 96-Tip Comb	Fisher Scientific	Cat# 97002534
96-well 0.22 µm filter plates	Pall Corporation	Cat# 8175
Controlled-rate freezing apparatus	Thermo Scientific	Cat# 5100-0001
Drop plate 96 (High Lunatic Plates, OD 200)	Fisher Scientific	Cat# 50-112-5955
VIAFLO 96 channel electronic pipette	Integra	https://www.integra-biosciences.com/united-states/en/electronic-pipettes/viaflo-96-viaflo-384
Tecan Freedom EVO	Boston Industries	https://lifesciences.tecan.com/freedom-evo-packages
DropSense96 (or similar)	PerkinElmer	https://www.unchainedlabs.com/lunatic/

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Multiskan GO	Thermo Scientific	https://www.fishersci.pt/shop/products/multiskan-go-microplate-spectrophotometer/p-4530546
KingFisher Flex Purification System	Thermo Scientific	Cat# 5400620
Caliper LabChip GXII	PerkinElmer	Part# CLS138160
HPLC Infinity 1260 Series (or similar)	Agilent	https://www.agilent.com/en/product/liquid-chromatography/hplc-systems/analytical-hplc-systems/1260-infinity-ii-lc-system#systemcomponents
Vi-CELL XR Cell Viability Analyzer	Beckman Coulter	https://www.beckman.com/cell-counters-and-analyzers/vi-cell-xr
Octet QK Instrument (or similar)	ForteBio	https://www.sartorius.com/en/products/protein-analysis

MATERIALS AND EQUIPMENT

Golden Gate Reaction master mix:		
Reagents	Final concentration	Amount per sample
gBlocks (variable region) 5 ng/μL	1 ng/μL	2 μL
gBlocks (constant region) 5 ng/μL	1 ng/μL	2 μL
Vector 5 ng/μL	1 ng/μL	2 μL
BsmBI	n/a	0.5 μL
T4 DNA ligase	n/a	0.5 μL
10× Fast Digest Buffer with 5 mM ATP (supplied with BsmBI)	1×	1 μL
Water	n/a	2 μL
Total	n/a	10 μL

Note: Golden Gate Reaction master mix should be prepared freshly.

Cell culture medium		
Reagents	Final concentration	Amount
FreeStyle F-17 medium	n/a	959.5 mL
Kolliphor P188 (10%)	0.1%	10 mL
G418 (50 mg/mL)	25 μg/mL	0.5 mL
L-glutamine (200 mM)	6 mM	30 mL
Total	n/a	1000 mL

Note: Cell culture medium can be stored at 4°C for up to one year after preparation.

Cell culture supplemental medium		
Reagents	Final concentration	Amount (per well)
Casein hydrolysate TN1 (20% w/v)	1% w/v	25 μL
Glucose (20% w/v)	0.9% w/v	22.5 μL
Cell culture medium	n/a	443.5 μL
Total	n/a	0.5 mL

Note: Supplemental medium can be stored at 4°C for up to 2 months after preparation.

nrMCE Sample Buffer		
Reagents	Final concentration	Amount
Tris-HCL, pH 7.0 (1 M)	6.0 mM	3 mL
Glycerol (50% w/v)	5.7% w/v	57 mL
EDTA (0.5 M)	1.7 mM	1.7 mL
SDS (10% w/v)	2.0% w/v	100 mL
Iodoacetamide (1 M) (added immediately prior to use)	23 mM	11.5 mL (see **Note** below)
Ultrapure water	n/a	326.8 mL
Total	n/a	500 mL

Note: nrMCE sample buffer stock without iodoacetamide can be stored at 20°C–25°C. An iodoacetamide stock (1 M, in ultrapure water) can be prepared in advance, aliquoted and stored at −20°C for long-term use. A freshly thawed aliquot(s) of iodoacetamide should be added immediately prior to sample preparation.

STEP-BY-STEP METHOD DETAILS

Cloning

Timing: 1 week

High-throughput cloning is performed in 5 sequential steps: Golden Gate Reaction, transformation, bacterial culture and DNA purification, sequencing verification, and DNA mixing.

1. Golden Gate Reaction.
   a. Resuspend synthesized DNA fragments (gBlocks) in deionized water to a final concentration of 5 ng/μL.
   b. Dilute vector in deionized water to a final concentration of 5 ng/μL.
   c. Set up Golden Gate Reactions in 96-well PCR plates. Master mix can be prepared using the table in the materials and equipment section. Prepare enough master mix for 1.2× number of samples, to account for loss due to pipetting error.

Note: Master mix is prepared and aliquoted into PCR plates using a multichannel pipette. Required gBlocks can be added using an automatic liquid handler (Tecan Freedom EVO, Boston Industries) following manufacturer's instructions.

Note: Positive and negative controls are not routinely used for Golden Gate cloning. However, these controls can be useful for troubleshooting failed reactions, and are described in the troubleshooting section.

   d. Seal the PCR plates with a thermal plate sealer. Mix thoroughly and quickly spin plates at 500 g for 1 min.
   e. Set up the Golden Gate thermocycler reaction as follows:

Steps	Temperature	Time	Cycles
Digestion	37°C	2 min	15–25 cycles
Ligation	16°C	3 min	
Final digestion	37°C	5 min	1
Denaturation	80°C	5 min	1
Hold	4°C	Forever	

Note: 15 cycles of digestion/ligation are recommended for constructs assembled with fewer than 3 DNA fragments; 25 cycles are preferred for constructs with 3 or more DNA fragments.

Note: Holding the thermocycler at 4°C is recommended for product stability. A heated lid (104°C) should be used to mitigate condensation during Golden Gate Reaction, including the Hold step.

2. Transformation.
   a. Remove pre-dispensed chemically competent bacterial cells (Lucigen, 15 μL of cells pre-dispensed in 96-well plate or 8-tube strip) from −80°C storage and thaw on ice for 5 min.
   b. Use a multichannel pipet to add 1.5 μL of Golden Gate Reaction to each well of competent cells.
   c. Gently mix by tapping the wall of the tube, then incubate on ice for 20 min.
   d. Using a pre-warmed thermocycler, heat shock cells at 42°C for 1 min, and then place on ice for 1 min.
   e. Add 80 μL recovery medium (medium supplied with competent cells or SOC broth) to cells. Seal plate and incubate at 37°C for 30 min.
   f. Plate cells onto standard LB agar plates containing 100 μg/mL carbenicillin, and plate using sterile glass beads or another preferred method.
   g. Incubate the agar plates at 37°C overnight.
3. Bacterial culture and DNA purification.
   a. Prepare growth medium (LB broth + 100 μg/mL carbenicillin). Use a multichannel pipette or VIAFLO 96 channel electronic pipette (Integra) to aliquot growth medium into 96-well deep well blocks (1 mL per well).
   b. Inoculate each well with a single colony from a successful transformation.
   c. Grow bacterial culture overnight in a 37°C incubator with a shaking platform set to 1,200 RPM.
   d. Purify transfection-grade DNA (~30–50 μg of DNA per sample) using a QIAGEN Plasmid plus 96 Kit (96-well format) following manufacturer's recommendation.
   e. Assess concentration of the purified DNA by measuring absorbance at 260 nm (A_{260}).

   Note: A DropSense96 instrument (PerkinElmer) or similar instrument is recommended for high-throughput measurements, as DNA samples can be measured in a 96-well format using Drop plate 96 (Unchained Labs).

   Note: On average, analyzing 4 colonies per construct is sufficient. However, if constructs are complex and expected to be difficult-to-clone, more colonies should be screened to ensure success.

4. Sequencing verification.
   a. Prepare 96-well DNA sequencing plates by adding 10 μL of DNA, diluted with 30 μL deionized water to a final concentration of 50–200 ng/μL, to each well using a multichannel pipette or VIAFLO 96 channel electronic pipette (Integra).
   b. Use Sanger sequencing to verify construct identity. Sequencing can be done in-house or through third-party suppliers such as Genewiz or Eurofins. The following primers are frequently used for pTT5 cloning:
      i. Vector forward primer: 5′- ACGAGGAGGATTTGATATTCACC-3′.
      ii. Vector reverse primer: 5′- CTTCCGAGTGAGAGACAC-3′.
      iii. Heavy chain CH2 reverse primer: 5′- TTGTACTCCTTGCCATTCAGC-3′.
   c. To identify successful clones, analyze Sanger sequencing results using a software application such as Geneious (Biomatters).
5. DNA mixing.
   a. For each construct, pool sequencing-confirmed DNA using an automatic liquid handler (Tecan Freedom EVO, Boston industries) according to manufacturer instructions.
   b. To prepare DNA for transfection, mix plasmid DNA encoding each chain of a bispecific antibody at defined ratios (i.e., HC1:LC1:HC2:LC2 = 1:1:1:1 for a Hetero-IgG), and then normalize concentration of the mixed DNA to 100 ng/μL. DNA mixing and normalization can be performed using a Tecan liquid handler.

Note: The 1:1:1:1 ratio mentioned above is recommended for high-throughput screening. However, the optimal DNA ratios for multi-chain molecules are largely sequence-dependent, and it is recommended to test other ratios for top candidates. Optimizing the DNA ratio is critical for balancing chain expression, which can lead to higher yields and lower levels of impurities.

c. Validate the concentration of each sample by measuring A_{260} of the mixed DNA. A DropSense96 (PerkinElmer) or similar instrument allows for rapid testing of 96-well plates. Mixed DNA can be stored at 2°C–8°C for several weeks, or at −20°C for long-term storage.

Expression

Timing: 7 days

The HEK 293-6E expression protocol shown below was developed in our laboratory and consists of 3 key steps: cell seeding, DNA transfection and supplementation (including histone deacetylase inhibitor (HDACi) supplementation), and measuring titers.

Note: As a control, include a mixed DNA containing genes for a high-expressing monoclonal antibody (cloned into the same vector types as the experimental samples) in every transfection.

6. Cell seeding.
   a. Twenty-six hours before transfection, and using a biological safety cabinet, seed cells into a shake flask at a density of 1×10^6 cells/mL in fresh cell culture medium, so that the cells reach optimal cell density ($\sim 2 \times 10^6$ cells/mL) at the time of transfection.

   Note: Viable cell density (VCD) and cell viability are measured using a Vi-CELL XR viability analyzer, Vi-CELL sample vials and Trypan blue based Vi-CELL XR reagents (Beckman Coulter), following instrument manual instructions.

   b. On the day of transfection, take culture medium and DNA from 4°C and warm to 20°C–25°C.
   c. Measure cell density and viability using the Vi-CELL XR viability analyzer. Cell density should be between 1.8 to 2.2×10^6 cells/mL at the time of transfection, with >98% viability.

   ⚠ CRITICAL: Carefully monitor cultures to ensure cell density does not exceed 2.2×10^6 cells/mL. Achieving optimal cell density and high viability is critical for efficient transfection.

   d. Gently aliquot cells into 96-well deep-well blocks (0.45 mL per well) and place the cell plate in the 37°C incubator on a shake rack (Kuhner AG) at 1,000 RPM.
7. Transfection and supplementation.
   a. To make the DNA-PEI complex, use a multichannel pipette to aliquot 50 μL Freestyle F17 medium into each well of a 96-well plate, add 5 μL of DNA (100 ng/μL) and 2 μL of PEImax (0.1% w/v, pH 7.0, Polysciences; see before you begin section), and then immediately swirl to mix.
   b. After incubation at 20°C–25°C for 10 min, add DNA-PEI mixture to cells, gently mix by swirling, and place cell plates in the 37°C incubator on a shake rack at 1,000 RPM.
   c. 24 h post-transfection, add 0.5 mL of cell culture supplemental medium (pre-warmed to 37°C) to each well of cells. Supplemental medium can be prepared using the table in the materials and equipment section.

   Note: Adding supplemental medium can be performed as soon as 4 h post-transfection, although a 24-hour interval is recommended and routinely performed.

d. 4 days post-transfection, add 7.5 μL of 500 mM sodium valproate (prepared using deionized water) to each well of cells (final concentration = 3.75 mM).

Note: Sodium valproate solution can be stored at −20°C for at least 2 years, or at 4°C for 3 months.

8. Measure antibody titers.
   a. 6 days post-transfection, using a multichannel pipette or VIAFLO 96-channel electronic pipette (Integra), transfer 50 μL of each well of transfected cells into a 96-well plate.
   b. Centrifuge the plate at 3,000 × *g* for 30 min to pellet cells and cellular debris.
   c. Carefully transfer 30 μL of the supernatant (i.e., the conditioned medium) to a new 96-well plate and add 120 μL Octet Sample Diluent using a multichannel pipette or VIAFLO 96-channel electronic pipette (Integra).
   d. To generate a standard curve dataset, use 2-fold serial dilution (in triplicate) of a purified antibody of known concentration (e.g., a commercially available antibody) to prepare a dilution series ranging from 250 to 1.9 mg/L.
   e. Measure antibody binding curves using an Octet QK instrument (ForteBio) and with Octet ProA biosensors (Sartorius).

Note: ProA biosensors are ready for use and are automatically conditioned as part of the titering run. Reusing biosensors is not recommended.

   f. Calculate antibody concentration estimates based on a standard curve generated from the serially diluted purified mAb.

Purification

Timing: 20 h (~16 h incubation followed by ~4 h hands-on time).

Protein A (ProA) affinity capture is a widely established method for purification of antibodies, for both small- and industrial-scale workflows. ProA magnetic beads, in conjunction with the KingFisher Flex Purification System (Thermo Scientific), provide a high-throughput and automated solution for purifying antibodies from conditioned medium.

9. ProA magnetic bead incubation.
   a. Six days post-transfection, in a biological safety cabinet, use a multichannel pipette to add 100 μL of the 50% bead slurry to each cell culture (see before you begin section).
   b. Return cells to 37°C shake rack in incubator for an overnight (~16 h) binding step.

⚠ CRITICAL: Beads in the source container may settle over time, especially when a large number of samples are being prepared. While aliquoting beads into cell cultures, frequently swirl the bead source container to achieve a consistent distribution.

10. ProA magnetic bead washing and elution using Kingfisher.
    a. For each 96-well plate containing the cell/bead mixture, prepare the following Kingfisher 96-well plates with wash and elution buffers in each well:
       i. 5 plates containing 1 mL 1×DPBS (pH 6.8).
       ii. 2 plates containing 1 mL deionized water.
       iii. 1 plate containing 0.4 mL elution buffer (100 mM Sodium acetate, pH 3.6).

⚠ CRITICAL: Ensure each plate is properly labeled and oriented to avoid mix-ups.

b. Collect ProA magnetic beads from cell culture using Kingfisher Flex 96-well magnetic head and FLEX 96-Tip Comb, and then carefully transfer beads to 96-well plates containing 1×DPBS.
c. Place the plates on the Kingfisher instrument in the following order: Elution plate, 2 water plates, 3 DPBS plates, beads plate (in DPBS), and the final DPBS plate assembled with Tip Comb.
d. Run Kingfisher purification using the following protocol:

Steps	Time	Number of cycles
DPBS wash	1 min	3
Water wash	1 min	2
Elution	10 min	1
Recover beads in DPBS	1 min	1

e. After Kingfisher purification, immediately neutralize the eluted samples by adding 8 μL 3 M Tris-HC (pH 11).
f. Transfer neutralized samples to 96-well 0.22 μm filter plates, and then place the filter plates on top of 96-well collection plates. Filter by centrifuging at 1,200 × *g* for 3 min.

⚠ CRITICAL: Neutralization must be performed immediately after elution, to minimize damage to the samples. However, if residual ProA beads are present in the neutralized elution plates, they can potentially re-bind the eluted proteins. Thus, neutralized samples should be filtered as soon as possible to maximize recovery.

g. Samples in collection plates are ready to be used for downstream analytics. Samples can be stored at 4°C for approximately 4 weeks, or at −80°C for longer-term storage.

Analytics

Timing: 1 day

With the purified material, first assess the total recombinant expression level by measuring the absorbance at 280 nm (A_{280}). In addition to the correctly assembled bispecific antibodies, many undesired species, such as aggregates and half-antibody fragments ($^1/_2$ Ab), can be present in the conditioned medium (cell supernatant), and any species containing an Fc region can be recovered by Kingfisher ProA purification. To assess product quality, analyze ProA samples with non-reducing Microchip Capillary Electrophoresis (nrMCE) and Analytical Size-exclusion Chromatography (aSEC).

11. Calculate ProA yield by A_{280} measurement.
    a. Transfer 140 μL of each sample into A_{280}-compatible plates using a multichannel pipette or VIAFLO 96 channel electronic pipette (Integra).
    b. Measure absorbance (A_{280}) with an instrument such as Multiskan Go (Thermo Scientific) following instrument manual instructions. Samples used for A280 nm can subsequently be used for other analytics assays if desired.
    c. Calculate protein concentration using a rearrangement of the Beer-Lambert Law:

    Concentration (mg/mL) = A_{280} / molar extinction coefficient ($M^{-1}cm^{-1}$).

    Note: Molar extinction coefficient is sequence-dependent and can be predicted using online tools such as the ProtParam Tool (https://web.expasy.org/protparam/). Molar extinction coefficient of the correct species should be used for calculation.

    d. Calculate ProA yield: ProA yield (mg/L) = concentration (mg/mL) × elution volume (mL) / cell culture volume (L).

12. Non-reducing Microchip Capillary Electrophoresis (nrMCE).

The Caliper LabChip GXII (PerkinElmer) is a Microcapillary Electrophoresis system used for rapid characterization of protein samples. The microfluidics chip technology automatically stains, electrophoretically separates, destains and analyzes protein samples from 96- or 384-well microplates (Le-Saout et al., 2016). The separation observed in nrMCE is analogous to that of nrSDS-PAGE, in which high molecular weight (MW) species (such as covalent aggregates) migrate at a slower rate compared to low-MW species (such as $^{1}/_{2}$ mAb).

⚠ CRITICAL: Sample protein concentrations should be within the range of 0.05–2 mg/mL. The Caliper LabChip GXII is capable of separating proteins between 14 and 200 kDa. High molecular weight (HMW) covalent aggregates may not be resolved by this system.

a. Allow Protein Express Reagent Kit and one Protein Express Assay LabChip to equilibrate to 20°C–25°C (~30 min).
b. Calculate the amount of nrMCE sample buffer needed to distribute 21 µL to each sample, plus an additional 20% excess to account for pipetting error. A stock solution of nrMCE buffer (without iodoacetamide) can be prepared in advance using the table in the materials and equipment section.
c. Based on the calculation in the previous step, transfer the required volume of nrMCE sample buffer stock to a separate container.
d. Thaw an aliquot(s) of iodoacetamide, add the appropriate amount to the separate container (based on the nrMCE sample buffer recipe table), and gently mix.
e. Transfer 6 µL samples to 96-well microplates, add 21 µL nrMCE sample buffer from the reservoir, cover with adhesive foil, gently vortex to mix, and centrifuge at 2,200 × *g* for 30 s.
f. Denaturation: Heat microplates at 85°C for 10 min, and then centrifuge at 2,200 × *g* for 30 s.
g. Remove foil, add 105 µL water to each sample, pipet up and down to mix, cover with adhesive foil, and then centrifuge at 2,200 × *g* for 30 s.
h. Remove foil and visually inspect wells to ensure no air bubbles are present in the wells.

⚠ CRITICAL: Air bubbles can cause spikes in the electropherogram profiles and interfere with analysis. All wells should be carefully checked for air bubbles after centrifugation. If bubbles are present, re-centrifuge samples and if necessary, break bubbles using clean pipet tips.

i. Place the microplate in the instrument platform, aligning the plate with the "A1" marking on the platform edge.
j. After warming to 20°C–25°C, gently vortex the HT Protein Express Ladder tube (supplied in the Protein Express Assay Reagent Kit), and pulse centrifuge (using a mini centrifuge at approximately 2,000 × *g*).
k. Transfer 12 µL to a 0.2 mL microtube (provided in Protein Express Assay Reagent Kit), and then add 120 µL water followed by pipetting up and down to mix.
l. Reverse pipette 520 µL of Protein Express Gel Matrix (supplied in the Protein Express Assay Reagent Kit) to the top basket of the provided spin filter.
m. Gently vortex the HT Protein Express Dye Solution (supplied in the Protein Express Assay Reagent Kit), centrifuge briefly at 2,000 × *g*, and transfer 20 µL to the spin filter containing the Protein Express Gel Matrix.
n. Cap the filter, invert, and vortex until the solution is well mixed.
o. Centrifuge at 9,000 × *g* for 6 min at 20°C–25°C, discard filter, label and minimize light exposure to the tube.

Note: Reverse pipetting is a technique used to minimize pipetting error caused by loss of residual material in the tip, and can improve pipetting accuracy for highly viscous samples. Depress the pipette plunger beyond the first stop, insert the tip into the liquid, and

completely release the plunger while aspirating. Dispense the liquid by depressing the plunger to the first stop. Residual material in the tip should be discarded.

p. Reverse pipette 250 μL of Protein Express Gel Matrix (supplied in the Protein Express Assay Reagent Kit) to a separate spin filter. Centrifuge at 9,000 × *g* for 6 min at 20°C–25°C, discard filter, label and minimize light exposure to the tube.
q. Open chip storage box and remove parafilm covering from the wells. Using a pipette tip attached to a vacuum line, aspirate liquid from active wells (only touch the sides of the well; do not touch the center region of the bottom of the well).
r. Wash each active well with ultrapure water 2–3 times, using the vacuum line to aspirate after each wash.

Note: After the final wash, proceed immediately to the next step. Do not allow the wells to dry out.

s. In accordance with the instrument manual and desired number of runs, load the recommended amount of Ladder solution, Gel-Dye Solution and Destain solution to the corresponding chip wells.
t. Transfer 750 μL of HT Protein Express Wash Buffer (supplied in the Protein Express Assay Reagent Kit) to a provided 0.75 mL Buffer Tube.
u. Place the prepared chip and Buffer Tube in the instrument platform and then run the assay using default settings.
v. Using the Caliper LabChip software, quantify the area under the curve for pre-main peak (pre-MP, low-MW species), main peak (MP), and post-main peak (post-MP, high-MW species) as a percent of total area for each sample.

13. HPLC-based Analytical Size-exclusion Chromatography (aSEC).

An aSEC workflow enables rapid and automated characterization of bispecific antibodies in solution, with run times of approximately 12 min or less. aSEC is routinely used to quantify relative abundances of pre-MP, MP, and post-MP. However, in contrast to nrMCE, the pre-MP in aSEC generally contains species of higher hydrodynamic radius, including aggregates; likewise, the post-MP generally indicates the presence of low molecular-weight species.

Prior to running experimental samples, several control runs are recommended. First, a blank sample (i.e., sample buffer) can be used to identify any signals originating from buffer or the HPLC system; second, a Gel Filtration Standard (Bio-Rad, prepared according to the manufacturer's instructions) (Figure 4C) as well as a control antibody (e.g., a commercially available purified mAb) can be run and analyzed to ensure the column and instruments are functioning properly. In addition, Standards and Control Abs can be run at the end of a sample sequence to ensure consistent column performance throughout the sequence. Multiple replicates of sample runs are not routinely performed.

The following steps describe the use of a 1260 Series HPLC system (Agilent) and the Chromeleon Chromatography Data System (Thermo Fisher Scientific) for aSEC:

a. Install hardware and column(s) according to manufacturer's instructions (see before you begin section above).
b. Create and save an instrument method using the Instrument Method Wizard. Specify run time, flow rate, inlets, and a description.
c. Create and save a processing method using the Processing Method Wizard. Specify components to be used for annotation (i.e., pre-MP, MP and post-MP).
d. Select and save a report template layout using on-screen instructions.
e. Prior to initializing a run, navigate to the instrument control console and manually switch on all devices required for the method. The status of all devices must read as "Ready" before starting a run.

f. Create a workflow sequence using the eWorkflow Wizard containing the desired number of runs.
g. Navigate to the Data tab and enter sample information, including sample ID, injection volume, sample position, instrument method, processing method, and any other relevant information.

Note: When using a 300 mm Zenix-C column, injected mass should be no greater than 32 µg, in a volume of no greater than approximately 50 µL. If necessary, samples can be diluted with SEC running buffer prior to injection.

h. An example of an aSEC workflow sequence is shown below:
   i. Blank.
   ii. Gel Filtration Standard.
   iii. Control Molecule.
   iv. Sample 1.
   v. Sample 2.
   vi. (All sample runs).
   vii. Gel Filtration Standard.
   viii. Control Molecule.
i. Start run sequence. During initial Standard run, monitor run progress to ensure proper performance of the system (Figure 4C).
j. After the run sequence is complete, use the Chromeleon analysis tool suite to quantify the area under the curve for pre-MP, MP and post-MP as a percent of total area for each sample.

EXPECTED OUTCOMES

This protocol describes a high-throughput method for cloning, expression, purification, and the initial evaluation of bispecific antibodies.

The Golden Gate cloning method utilizes a Type IIS restriction enzyme (BsmBI in this protocol) and T4 DNA ligase to allow simultaneous and directional assembly of multiple DNA fragments. This strategy enables the high-throughput production of plasmids without scars, and with high fidelity—typically, a success rate of over 80% is achieved.

HEK 293-6E cells allow for expression of large panels of bispecific antibodies in a timely and high-throughput manner. Typically, expression titers of up to 300 mg/L can be obtained, as exemplified in Figure 2. From the conditioned medium, ProA capture using the Kingfisher magnetic bead system shows efficient and consistent recovery of bispecific antibodies. As shown in Figure 2, there should be a tight correlation between estimated expression titer and ProA yield.

Many bispecific antibody formats are composed of multiple polypeptide chains, which often have the potential to form species with undesired quaternary structures (Brinkmann and Kontermann, 2017; Gong et al., 2021; Labrijn et al., 2019). Taking a Hetero-IgG as an example, impurities can come from Heavy Chain (HC) mispairs (two identical HCs paired with LCs, often lacking covalent linkage in the hinge regions), Light Chain (LC) mispairs (two different HCs paired with the same LC), $^{1}/_{2}$ Ab (one HC + one LC), and (covalent and non-covalent) aggregates (Figure 3). Therefore, in addition to expression level, the quantitation of correct and undesired species is required for analyzing protein quality and selecting top candidates. Routinely, we analyze purified ProA samples with nrMCE and aSEC. nrMCE quantitates the relative levels of IgG species, $^{1}/_{2}$ Ab (including monomeric $^{1}/_{2}$ Ab and non-covalent homodimers) and some covalent aggregates (< 200 kDa) (Figure 4A), while aSEC measures the relative levels of IgG species (though it often fails to distinguish between the correct species vs. homodimeric mispaired species, or IgG lacking covalent linkage in the hinge), aggregates and monomeric $^{1}/_{2}$ Ab (Figure 4B).

Note: In aSEC, both covalent and non-covalent aggregates migrate differently from the desired species and generally appear as a pre-MP; conversely, in nrMCE, non-covalent

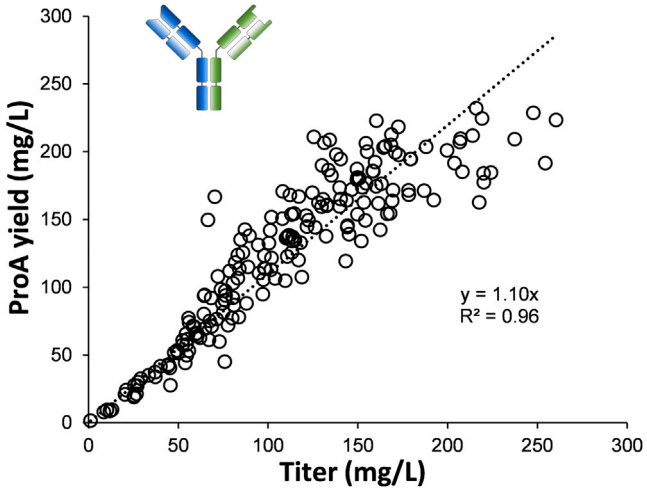


Figure 2. Correlation of titer and ProA yield

A total of 192 molecules in Hetero-IgG format were high-through expressed in HEK 293-6E cells. Expression titers were measured with conditional medium at day 6 post-transfection. ProA purification yields were determined by A_{280} at day 7 post-transfection.

aggregates are separated by SDS, and only some covalent aggregates (< 200 kDa) will appear as high-MW species, in the post-MP area. In many cases of Hetero-IgG purification, we have observed that HC mispairs are not covalently linked in the hinge region. These homodimers appear as $^1/_2$ Ab species in nrMCE (pre-MP), but migrate as an IgG (*i.e.*, MP) in aSEC.

LIMITATIONS

This high-throughput protocol can be used to produce up to 300 μg protein from a 1 mL HEK 293-6E cell transfection. Yields at this level enable numerous downstream analytics such as MCE, aSEC, liquid chromatography–mass spectrometry (LC-MS), analytical cation exchange chromatography (aCEX), binding characterization, and functional evaluation. However, if more materials are required for downstream analysis, a larger-scale expression will be necessary. In such cases, the protocol outlined here can easily be linearly scaled to 4-mL expression using 24-well deep-well-blocks.

Combining nrMCE and aSEC analyses enables the identification and quantification of most undesired species (including $^1/_2$ Ab, aggregates, and HC mispairs) (Creamer et al., 2014; Goyon et al., 2018). However, certain formats can contain mispaired contaminants (i.e., LC mispairs) that may appear very similar to the target species in both nrMCE and aSEC. In these cases, LC-MS can serve as a complementary method for identifying and distinguishing these species, provided that their MWs are sufficiently distinct from one another (Campuzano et al., 2019; Gong et al., 2021). Importantly, because different species exhibit unique ionization efficiencies, absolute quantification of contaminants using LC-MS is a major challenge due to over or underestimation of impurity abundance (i.e., $^1/_2$ Ab species have significantly higher ionization efficiencies compared to IgG species leading to over-representation in LC-MS) (Sawyer et al., 2020). However, in some cases, relative quantification can be useful tool for characterization of species with similar MWs.

TROUBLESHOOTING

Problem 1

No or few colonies for all constructs (step 3).

Potential solution

Repeat Golden Gate Reaction or transformation with fresh reagents. Troubleshooting Golden Gate reactions and transformation can be performed using the positive controls described below:

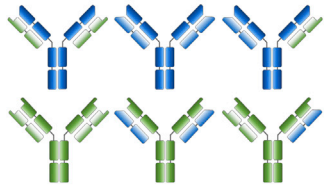
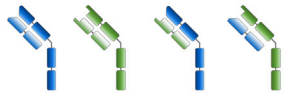
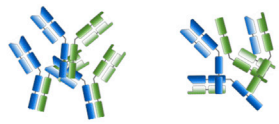
Species	Schematics	Abundance*	Appearance in nrMCE	Appearance in SEC
Correct		High	MP	MP
HC mispairs		Medium	Often pre-MP, Sometimes MP	MP
LC mispairs		Medium	MP	MP
½ Ab		Low	Pre-MP	Post-MP
Covalent aggregates		Low	Unable to detect	Pre-MP
Non-covalent aggregates		Low	MP or pre-MP	Pre-MP

Figure 3. Correct species and potential undesired species for Hetero-IgG molecules

MP, main peak; pre-MP, pre main peak; post-MP, post-main peak; HC, heavy chain; LC, light chain. HC mispairs are often lack of a covalent linkage in the hinge region. Therefore, HC mispairs are often dissolved by SDS in nrMCE and migrate like $^{1}/_{2}$ species (pre-MP), while appear as MP in SEC. *, Abundance of species is sequence- and engineering-dependent.

A pUC19 plasmid can be used as a positive control for transformation. Transfect 1 ng of pUC19 and plate 1% of the transformed bacteria onto an LB agar plate containing 100 μg/mL carbenicillin as described in the cloning section. Transformation efficiency, defined as the number of colony forming units (CFUs) per μg of DNA transformed, should be greater than 10^7 CFUs (i.e., 100 colonies observed on the plate). Observing fewer than 100 colonies in the pUC19 control could indicate an issue with transformation.

A validated gBlock DNA fragment can be used as a positive control for the Golden Gate reaction. Observing good transformation efficiency with the pUC19 but not with the validated gBlock control could indicate an issue with the Golden Gate reaction. For constructs having 4 or more DNA fragments, increasing cycle number (up to 25) of the Golden Gate Reaction can improve efficiency.

Problem 2

No or few colonies for some constructs (step 3).

Potential solution

Check construct design, especially BsmBI overhangs, to ensure constructs are correctly designed.

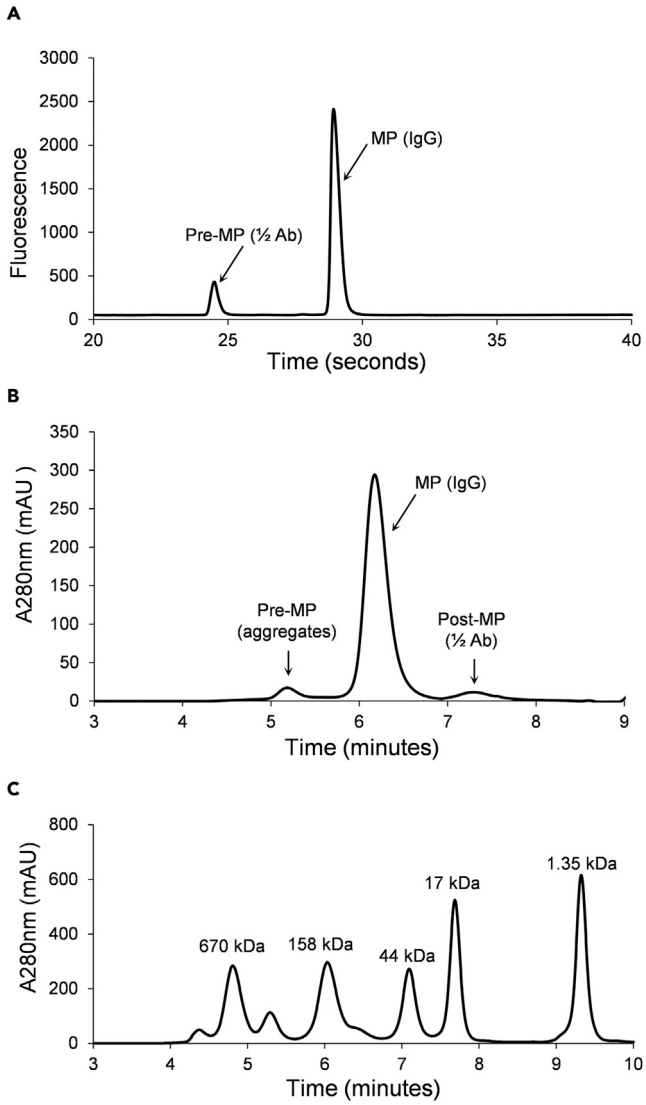


Figure 4. Representative examples of nrMCE and aSEC methods for bispecific antibodies

(A and B) A hetero-IgG was prepared and run on nrMCE (A) and aSEC (B) as described in steps 12 and 13, respectively. (C) aSEC profile showing Gel Filtration Standards (Bio-Rad). Approximately 18 μg of Standard was injected into a Zenix-C 300 (300 Å, 3 micron, 4.6 × 300 mm) SEC column as described in step 13.

Occasionally, multichannel pipettes and automatic liquid handlers may erroneously transfer incorrect amounts of reagents for some samples. Repeat Golden Gate Reaction and transformation for the failed constructs.

Problem 3

All picked clones for some constructs have mutations (step 4).

Potential solution

Pick more colonies for miniprep and sequencing.

In some cases, gBlocks ordered from vendors may have quality issues and contain unexpected mutations. Reordering gBlocks may offer a solution if mutations are repeatedly observed during sequence validation.

Problem 4

Both the control (a high-expressing monoclonal antibody with stock DNA) and bispecific antibodies (using newly purified DNA) show low expression levels (step 8).

Potential solution

Troubleshoot transfection reagent (PEImax) and HEK 293-6E cells using control DNA encoding a monoclonal antibody, and/or a GFP construct. If necessary, use freshly prepared PEImax and/or recover a new batch of cells.

Problem 5

Control (a high-expressing monoclonal antibody with stock DNA) shows high expression, but bispecific antibodies (using newly purified DNA) express at low levels (step 8).

Potential solution

Some bispecific formats (i.e., long and complex polypeptide chains) are expected to have low expression levels compared to monoclonal antibodies.

If the bispecific antibodies are expected to have higher expression, re-purify and mix the required DNA, repeat expression.

> *Note:* IgG-like molecules, i.e., hetero-IgG, are expected to have good expression. Complex molecules, especially those with long polypeptide chains (i.e., IgG-scFv, IgG-scFab), could have lower expression. In general, we observe that unnatural long polypeptide chains, containing engineered linkers between domains, can pose challenges during expression, folding, and assembly.

Problem 6

Low elution efficiency during purification (step 10).

Potential solution

To improve elution efficiency, perform a second round of elution with fresh buffer, consisting of 0.4 mL of 100 mM Sodium acetate, pH 3.6.

Problem 7

ProA yields are significantly lower than predicted by titer measurements (step 11).

Potential solution

Double-check the standard curve generated for titer measurements. Human error during standard measurements or standard curve calculation may be responsible for unexpectedly low titers.

If titer measurements are correct, the reduced ProA yields may be due to reduced binding capacity caused by damaged or dirty beads. Consider repeating the purification using a new batch of magnetic beads.

Problem 8

Peaks are not well separated in nrMCE (i.e., samples show multiple overlapping peaks) (step 12).

Potential solution

This indicates insufficient denaturation and resolution of proteins. Make sure protein concentration is below 2 mg/mL. Increase SDS concentration (from 2% to 4%) and heating temperature (from 85°C to 94°C) during sample preparation.

Problem 9

Bispecific peaks in aSEC show broadening or fronting (step 13).

Potential solution

Broadening may indicate an excessive injection volume was used, while fronting can signify mass overload. To address volume or mass overload, reduce sample injection volume or concentration, respectively.

Problem 10

Main peak in aSEC exhibits a substantially delayed elution position, impairing analysis (step 13).

Potential solution

In some cases, samples can interact with the stationary phase of certain aSEC columns, leading to delayed or broadened elution profile. Change the salt concentration and/or pH of the running buffer or use a column with an alternative stationary phase. Additionally, consider using an additive such as ethanol in the aSEC running buffer to reduce nonspecific interactions (see step 4 in before you begin section).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Danyang Gong (dgong@amgen.com).

Materials availability

There are restrictions to the availability of plasmid and cell line used in this study due to the lack of an external centralized repository for its distribution and our need to maintain the stock.

Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Z.W., F.G., T.P.R., and D.G. conceptualized and designed the project. D.L., A.C.P., D.G., and T.P.R. performed the experiments and interpreted the results. D.L., A.C.P., L.Z., and D.G. wrote the manuscript. Z.W., F.G., T.P.R., I.C., and M.L.M. reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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