Golden Gate assembly of designed antibody repertoires

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Abbreviations

CDR – Complementarity-determining region
PCR – Polymerase Chain Reaction
GGA – Golden Gate Assembly[1]
bp – base pair
Q5 - High-Fidelity DNA Polymerase (NEB M0491L)
DPNI – DPNI restriction enzyme (NEB R0176L)
Monarch kit – Monarch Spin PCR and DNA Cleanup Kit (NEB T1130L)
BbsI – BbsI-HF type IIs restriction enzyme (NEB R3539L)
rSap – Shrimp alkaline phosphatase (NEB M0371L)
SapI – SapI type IIs restriction enzyme (NEB R0569L)
Chlor – chloramphenicol
Kan – kanamycin
2YT – 2x Yeast Extract Tryptone medium
ExoSAP – 1:1 mix Exonuclease I (NEB M0293L) and Shrimp Alkaline Phosphatase (NEB M0371L)
EcoRV – EcoRV-HF restriction enzyme (NEB R3195L)
SPRi beads - SPRIselect DNA size selection reagent (Beckman B23318)

BsaI - BsaI-HFv2 restriction enzyme (NEB R3733L)

Primers used

Primer name	Sequence 5'-3'	Description	Steps used
OATC67	GAGATAGAAGACCCGCTAGCACC	Linearize plasmid Fwd	1
OATC68	ACTGGTGAAGACTTGGCAGCCTGG	Linearize plasmid Rev	1
OATF162	GTAAAACGACGGCCAG	M13 Fwd	2.3
OATF163	CAGGAAACAGCTATGAC	M13 Rev	2.3
OATC74	GACATTGGTCTCCTAGGAGGAATTTA AAATGAAATACCTATTGCCTACGGCAG	Amplify heavy chain pools FD	3.4
OATC77	CTCATAACGCCTGTTCGACTCG	Amplify heavy chain pools Rev	3.4, 4
OATC66	CCAGCCTGATAAGTAGCACCG	Amplify full product Fwd	4

Overview

Current DNA synthesis methods do not enable the synthesis of repertoires comprising billions of antibodies. To address the problem of synthesizing a repertoire of this size, we exploit the fact that antibody diversity is generated in the CADAbRe repertoire through the combination of a small number of V and J gene segments (under 100 genes for the entire designed repertoire) and hundreds of thousands of short, designed CDR H3s. This built-in modularity enables rapid, accurate, and cost-effective assembly from oligo pools (for H3) and custom-synthesized V and J genes. Furthermore, the fragments can be reused in future assemblies, lowering assembly cost. An added benefit of modular gene assembly is that it minimizes the use of polymerase chain reactions (PCRs), thereby reducing undesired mutations, recombination, and large deviations from the uniform representation of the designs in the repertoire. In this protocol, the V and J genes were flanked by suitable sites for Golden Gate cloning[1] and cloned into plasmids by Twist Bioscience. The light and heavy chains were assembled separately, amplified, and then ligated. The resulting product, encoding the designed antibodies in Fab format, was then ligated into the pComb3x phagemid[2]. The protocol can be easily modified to accommodate other antibody formats, such as single-chain variable fragments (scFvs). This protocol enables the assembly of billions of designs at a DNA cost of less than $30,000 (under $10^{-4} per variant) in under 10 days.

Background on Golden Gate Assembly

Golden Gate Assembly[1] (GGA) is a scarless method for assembling DNA fragments in a one-pot reaction. GGA utilizes type IIs restriction enzymes, which have a recognition site distinct from their cleavage site, resulting in 5' or 3' overhangs (gates) consisting of any nucleotides. Therefore, 256 different gates can be created using a type IIs enzyme that creates a four-basepair (bp) gate. Alternatively, some type IIs enzymes create three-bp gates, meaning a single conserved amino acid can be used to ligate two pieces together. Gates differ in their propensities to ligate to their cognate gates and off-target gates, and these propensities have been leveraged to generate sets of gates that combine with high fidelity[3,4]. In a typical GGA, linear DNA fragments or plasmids containing inserts flanked by a type IIs restriction site and the desired gates are mixed with a type IIs restriction enzyme and T4 DNA ligase. The mixture is cycled between 37°C (ideal for the type IIs restriction enzyme) and 16°C (ideal for the ligase). In each cycle, the fragments are digested and ligated together. Incorrect or incompletely ligated fragments are then digested in the next cycle, as they contain the type IIs recognition site. The final product contains the DNA fragments ligated together in a programmable order with no scars.

Suggested timeline

Note: Day 1 can be done before ordering DNA from Twist
Note: Days 2 and 3 can be done while waiting for H3 cloned oligo pools to arrive
Day 1: Step 1 (preparation of plasmid for library insertion)
Day 2: Steps 2.1, 2.2, 2.3 day 1 (light chain GGA)
Day 3: Steps 2.3 day 2, 2.4 (preparation of full light chain pools for GGA)
Day 4: Step 3.1-3.3 (preparation of heavy chain inserts for GGA)
Day 5: Step 3.4 (heavy chain GGA)
Day 6: Step 4
Day 7: Step 5

Step 1: Preparation of plasmid for library insertion

In this section, the plasmid is prepared for library insertion. The plasmid is linearized using PCR, the closed plasmid is digested using DPNI, and the linearized plasmid is pre-digested with BbsI. The digested plasmid is then treated with rSap to prevent unwanted ligations. In this example, the plasmid (pATF51) is 4536 bp before PCR, 3648 bp after PCR, and 3616 bp after BbsI digestion (link to an example plasmid map).

1) Prepare 6-8 of the following PCR reactions with OATC67 and OATC68 to linearize the plasmid.

Reagent	Volume (µL)
Q5 reaction buffer (5X B9027S)	10
dNTPs (N0447L) (10 mM)	1
OATC67 10 uM	2.5
OATC68 10 uM	2.5
1 ng plasmid	X
Q5 polymerase (M0491L)	0.5
Nuclease-free water	Up to 50
Total	**50**

2) Run the following PCR program (ATQ5, but need to adjust parameters**).**

Step	Temp (ºC)	Duration
1	98	30 sec
2	98	10 sec
3	67	30 sec
4	72	2 min 30 sec
	Go to 2	24X
5	4	Forever

3) Combine the reactions into one tube, mix, and run 5 µL on a gel.

4) If the product looks like a clean band at the correct size, split into 45 µL aliquots.

5) Add 5 µL DPN1 to each aliquot. Incubate 37°C for 3 hours.

6) Combine the reactions into 100 µL aliquots and purify with the Monarch kit. Elute each in 10 uL nuclease-free water pre-heated to 50°C and mix the elutions.

7) Prepare enough digestion reactions with BbsI to digest the entire product. The maximum amount of input DNA for each reaction is 2 µg.

8) For 1x digestion reaction, prepare the following mix:
*Keep BbsI in a cooling tub while using.

Reagent	Volume (µL)
rCutSmart Buffer (B6004S) (10X)	5.0
BbsI-HF (R3539L) (20U/ µL)	1.0
Linearized plasmid (max 2 µg)	X
Nuclease-free water	Up to 50
Total Volume	**50 µL**

9) Incubate at 37°C for 1 hour.

10) Add 1.5 uL rSap to each reaction and incubate for another 30 min at 37°C.

11) Combine the reactions into 100 µL aliquots and purify with the Monarch kit. Elute each in 10 uL nuclease-free water pre-heated to 50°C and combine the elutions.

12) Quantify DNA concentration by Nanodrop.

13) Quantify DNA length by Tapestation.

Step 2: Assembly of light chain pools from cloned genes

Light chains are formed by ligating together two parts. The first part consists of the light V genes, and the second part consists of the light J genes, each connected to the same light constant sequence. This protocol relies on a constant gate between the light V and light J genes, which is a conserved proline at the end of the light V gene. This is only conserved in kappa light chains, and another solution will be necessary for lambda light chains. This section results in pools of light chain plasmids, where all light V genes have been ligated into several plasmids. Each plasmid contains one light J gene and the light constant region. The resulting plasmids are transformed into bacteria, amplified, and extracted.

Step 2.1: Digestion of light J plasmids with SapI to open the plasmids

In this section, the light J gene plasmids are digested with SapI and treated with rSap to prepare them for GGA. This section can likely be skipped; however, skipping it may result in lower ligation fidelity and a higher frequency of colonies containing the original plasmids.

In this example, the light J plasmids are 2685 bp (link to an example light J plasmid map). These genes were ordered and cloned into the pTwist Kan High Copy v2 plasmid by Twist Bioscience.

1) Resuspend plasmids from Twist to 100 ng/µL in nuclease-free water. If there is at least 3 µg of DNA of each light J plasmid, proceed directly to the digestion step. If not, the plasmids must be transformed into bacteria and minipreped.

2) Prepare two of the following digestion reactions for each light J plasmid (each plasmid in a separate reaction). The total number of reactions is 2 * (number of light J plasmids).

   * Mix SapI gently by pipetting before use, and keep it in a cooling tub while using.

Reagent	Volume (µL)
rCutSmart Buffer (B6004S) (10X)	5.0
SapI (R0569L) (10U/ µL)	1.0
Light J gene plasmids (100 ng/µL)	10
rSap (M0371) (1U/uL)	1
Nuclease-free water	33
Total Volume	**50**

3) Run the following program in a thermal cycler (37C1H).

Temp (ºC)	Duration
37ºC	60 min
4ºC	Hold

4) Combine the two digestion tubes for each light J.

5) Purify the digested plasmids using the Monarch kit and elute in 12 µL nuclease-free water.

6) Quantify DNA concentration by Nanodrop. Ensure there is at least 0.25 pmol of DNA for each light J. Amount of DNA needed for 0.25 pmol can be calculated using the NEBiocalculator. The maximum yield from this reaction is very close to the amount of DNA added to the reaction, since the dropout is quite small.

7) Quantify DNA length using Tapestation.

Step 2.2: Digestion of light V plasmids with SapI to produce linear fragments (can be skipped)

In this section, the light V gene plasmids are digested with SapI, and the light V gene linear fragments are purified to prepare them for GGA. This section can likely be skipped, as the light V gene plasmids contain Chlor resistance, and transformed bacteria will not survive under Kan selection in Step 1.3.

In this example, the light V plasmid backbone is 2095 bp, and the light V inserts are 342 bp (link to an example light V plasmid map. These genes were ordered and cloned into the pTwist Chlor High Copy plasmid by Twist Bioscience.

1) Resuspend plasmids from Twist to 100 ng/µL in nuclease-free water. If the total amount of DNA exceeds 10 µg, proceed directly to the digestion step. If not, the plasmids must be transformed into bacteria and minipreped.

2) Mix all light V gene plasmids in an equal ratio, ensuring there is at least 10 µg total DNA.

3) Prepare eight of the following digestion reactions.

   * Mix SapI gently by pipetting before use, and keep it in a cooling tub while using.

Reagent	Volume (µL)
rCutSmart Buffer (B6004S) (10X)	5.0
SapI (R0569L) (10U/ µL)	1.0
Light V gene plasmids mix (100 ng/uL)	10
Nuclease-free water	34
Total Volume	**50.0**

4) Run the following program in a thermal cycler (37C1H).

Temp (ºC)	Duration
37	60 min
4	forever

5) Combine two digestions into a single 100 µL mix (resulting in four 100 µL mixes in total) and purify the digestion products using the Monarch kit (four columns in total). Elute in 8 µL nuclease-free water from each column and mix the elutions (32 µL total).

6) Quantify DNA concentration by Nanodrop.

7) Quantify DNA length by Tapestation.

8) Purify the digested product using a BluePippin 2% agarose cassette (link to cassettes) with a selection range of 200-500 bp. It may be necessary to use two lanes, as each lane can accommodate a maximum of 5 µg of DNA. The purification can be combined with the purification of heavy Vs and heavy J (steps 2.1 and 2.2).

9) Purify the elution using the Monarch kit. Elute in 12 µL nuclease-free water.

10) Quantify DNA concentration by Nanodrop.

11) Quantify DNA length by Tapestation.

12) Estimate the fraction of the correct product using the Tapestation results and adjust the concentration accordingly. The maximum yield is ~14% of the original plasmid DNA digested. Ensure there is at least 0.25 pmol of the correct size DNA for each light J. Amount of DNA needed for 0.25 pmol can be calculated using the NEBiocalculator.

Step 2.3: Golden Gate assembly of light Vs and light Js to form light chain pools

The previous two steps prepared the light V inserts and the light J plasmids for GGA. In this step, the light V genes are ligated into the light J plasmids, and the assemblies are transformed into bacteria. The bacteria are then miniprepped to extract large amounts of ligated DNA without the use of PCR. This results in one pool of plasmids per light J.

In this example, the light chain plasmids are 2978 bp (link to example light assembly plasmid map).

1) Prepare four of the following reactions for each light J.
   * Mix SapI gently by pipetting before use, and keep it in a cooling tub while using.
   * Amounts of DNA needed can be calculated using the NEBiocalculator.

Reagent	Volume (µL)
NEBridge ligase master mix (M1100L) (3X)	5.0
SapI (R0569L) 10U/ µL	1.0
Light V mix	X (0.05 pmol)
Light J plasmids	Y (0.05 pmol)
Nuclease-free water	Up to 15
Total Volume	**15**

2) Run the following program in the thermal cycler (37C1H60).

Temp (ºC)	Duration
37	60 min
60	5 min
4	forever

3) Combine the four GGAs for each light J and purify with the Monarch kit. Elute in 7 µL ultrapure water.

4) Transform 5 µL of each reaction into E.cloni bacteria. The number of transformations should equal the number of light Js. Use 2YT as recovery media.

5) Titrate a few 10 µL aliquots of the transformations. Plate titrations on 2YT + Kan plates and incubate for 16 hours at 30°C. In the morning, move the plates to 37°C so the colonies grow enough to be counted.

6) Centrifuge the remaining recovered bacteria (~950 µL) for 10 min at 5,000 xg. Discard the supernatant and resuspend the pellet in 100 µL 2YT.

7) Plate the resuspended bacteria on 140 mm round 2YT + Kan plates. Incubate for 16 hours at 30°C.

The following day:

8) Count the number of colonies on each titration plate and calculate the transformation efficiency. This should be at least 100 times the number of light Vs.

9) Perform colony PCR on 5 colonies per light J pool using OATF162 and OATF163. In this example, the length of the PCR product is 865 bp.

10) While PCRs are running, remove all bacteria from 140 mm plates with 2YT + Kan for each light J pool. Add 3 mL of 2YT + Kan to each plate and scrape the bacteria off using a spreader. If a large number of bacteria remain, add an additional 2 mL of 2YT + Kan and scrape the remaining cells.

11) Dilute 10 µL of bacteria in 990 µL of 2YT + Kan and measure OD600. Calculate the final OD600 as 100 * the measured value.

12) Calculate the volume of bacteria needed to spin down for minipreps. You would like to have around $3x10^9$ cells to match the number of cells used in a typical miniprep. The number of cells can be estimated as 1 OD600 = $2x10^8$ cells/mL. Typically, ~100-150 µL of bacteria are needed.

13) Take several aliquots of this calculated volume for each light J. Spin down for 5 min at 10,000 xg in a tabletop centrifuge.

14) Make glycerol stocks of the remaining bacteria for each light J. Make at least two glycerol stocks for each light J.

15) Miniprep the light chain pools for each light J. Elute in nuclease-free water. Ensure there is at least 15 µg for each light chain pool.

16) Quantify the DNA concentration of each light chain pool by Nanodrop.

17) Run each colony PCR on a gel to verify size.

18) Treat the colony PCRs with ExoSap and send for sequencing with OATF162 and OATF163.

Step 2.4: Digestion of the light chain pools with EcoRV to produce linear fragments

In this section, the light chain pools are digested with EcoRV to produce linear fragments. These fragments will then be ligated to the heavy chains created in Step 2. In this example, the length of the linear fragments is 714 bp (link to an example light pool EcoRV digestion product), and the length of the rest of the plasmid backbone is 2174 bp. EcoRv is used because it leaves a blunt end. This results in one pool of linear fragments per light J.

1) Prepare 6 of the following digestion reactions for each light J

   *Keep EcoRV in a cooling tub while using.

Reagent	Volume (µL)
rCutSmart Buffer (B6004S) (10X)	5.0
EcoRV-HF (R3195S) (20U/ µL)	1.0
Light plasmid pool (2 µg)	X
Nuclease-free water	Up to 50
Total Volume	**50**

2) Run the following program in a thermal cycler (37C1H).

Temp (ºC)	Duration
37	60 min
4	forever

3) Combine the digestion reactions into three tubes of 100 µL each for each light J and purify with the Monarch kit. Elute each in 12 µL nuclease-free water and combine elutions for each KJ. This should result in one tube of 36 µL per light J.

4) Quantify the DNA concentration of each digested light J pool by Nanodrop.

5) Quantify the DNA length of each digested light J pool by Tapestation

6) Purify each light J pool with BluePippin 1.5% agarose cassette (link to cassettes) using a selection of 600 - 800 bp. The maximum capacity for each lane is 2 ug, so you might need to run several lanes per light J.

7) Purify each elution with the Monarch kit. Elute in 12 uL nuclease-free water.

8) Quantify DNA concentration by Nanodrop.

9) Quantify DNA length by Tapestation. Estimate the fraction of correct product and adjust the concentration accordingly. Ensure there is at least 0.5 pmol of each light J pool. The maximum yield of this reaction is ~24% of the total DNA added to the reaction.

Step 3: Assembly of heavy chain pools from cloned genes and oligo pools

Heavy chains are formed by ligating together three parts. The first part consists of the heavy V genes, the second part consists of CDR H3 sequences, and the third part consists of one heavy J gene. We decided to use only one heavy J gene, as most of the diversity in the antigen-binding site is contained in H3, according to our definitions. However, additional heavy J genes could easily be added if desired. This protocol relies on a constant gate between the heavy V and H3 sequences (a conserved cysteine at the end of the heavy V gene) and between the H3 and heavy J gene. We used a threonine in the heavy J, but several other gates could be chosen here. This section results in pools of heavy chain fragments, where all heavy V genes have been ligated with all H3 sequences and the heavy J gene. The heavy constant region is not included since it is already present in our vector. The resulting fragments are amplified using PCR, in which part of the heavy leader sequence and the Shine-Dalgarno sequence are added. We have found that including these in plasmids leads to inconsistent results.

Step 3.1: Digestion of heavy V plasmids with EcoRV to produce linear fragments

In this section, the heavy V gene plasmids are digested with EcoRV to prepare them for GGA. EcoRV is chosen because it is a blunt-end cutter. In this example, the heavy V gene inserts are 378 bp, and the rest of the plasmid backbone is 2171 bp (link to an example heavy V plasmid). These genes were ordered and cloned into the pTwist Kan High Copy v2 plasmid by Twist Bioscience. This section results in a pool of linear heavy V fragments.

1) Resuspend plasmids from Twist to 100 ng/µL in nuclease-free water. If there is at least 15 µg of total DNA, proceed directly to the digestion step. If not, the plasmids must be transformed into bacteria and minipreped.

2) Mix the heavy V genes at an equal ratio.

3) Prepare 6 of the following digestion reactions.

*Keep EcoRV in a cooling tub while using.

Reagent	Volume
rCutSmart Buffer (B6004S) (10X)	5.0 µL
EcoRV-HF (R3195S) (20U/ µL)	1.0 µL
Heavy V genes mix 2 µg	20 µL
Nuclease-free water	24 µL
Total Volume	**50.0 µL**

4) Run the following program in a thermal cycler (37C1H).

Temp (ºC)	Duration
37	60 min
4	forever

5) Combine the six tubes into three tubes of 100 µL and purify each with the Monarch kit. Elute each in 23 µL nuclease-free water and combine. This should result in a tube of 69 µL.

6) Quantify the DNA concentration by Nanodrop.

7) Quantify the DNA length by Tapestation.

8) Purify with a BluePippin 2% agarose cassette (link to cassettes) using a selection range of 100-500 bp. The maximum capacity of each lane is 5 µg, so you may need to use more than one lane. Can be combined with the purifications of the light Vs and heavy J (Steps 1.2 and 2.2).

9) Purify the elution with the Monarch kit. Elute in 12 uL nuclease-free water.

10) Quantify DNA concentration by Nanodrop.

11) Quantify DNA length using Tapestation and adjust the concentration accordingly based on the fraction of correctly sized product. Ensure there is at least 0.5 pmol of the correct size product.

Step 3.2: Digestion of the HJ plasmid with EcoRV to produce a linear fragment

In this section, the heavy J gene plasmid is digested with EcoRV to prepare it for GGA. EcoRV is chosen because it is a blunt-end cutter. In this example, the heavy J insert is 294 bp, and the rest of the plasmid backbone is 2261 bp (link to an example heavy J plasmid). Random DNA is appended to the end of the heavy J gene to increase the final product's size and thus make it easier to manipulate. This gene was ordered and cloned into the pTwist Kan High Copy v2 plasmid by Twist Bioscience. This section results in a linear fragment for the heavy J gene.

1) Resuspend the plasmid from Twist to 100 ng/µL in nuclease-free water. If there is at least 15 µg of DNA, proceed directly to the digestion step. If not, the plasmid must be transformed into bacteria and minipreped.

2) Prepare 6 of the following digestion reactions.

*Keep EcoRV in a cooling tub while using.

Reagent	Volume (µL)
rCutSmart Buffer (B6004S) (10X)	5.0
EcoRV-HF (R3195S) 20U/ µL	1.0
Heavy J plasmid 2 µg	20
Nuclease-free water	24
Total Volume	**50.0**

3) Run the following program in the thermal cycler (37C1H).

Temp (ºC)	Duration
37	60 min
4	forever

4) Combine the six tubes into three 100 µL tubes and purify them using the Monarch kit with three columns. Elute each in 23 µL nuclease-free water and combine. This should result in one tube with 69 µL.

5) Quantify the DNA concentration by Nanodrop.

6) Quantify the DNA length by Tapestation.

7) Purify with BluePippin 2% agarose cassette (link to cassettes) using a selection range of 100 - 500 bp. It may be necessary to use two lanes, as each lane can accommodate a maximum of 5 µg of DNA. The purification can be combined with the purification of light Vs and heavy Vs (steps 1.2 and 2.1).

8) Purify the elution with the Monarch kit. Elute in 12 uL nuclease-free water.

9) Quantify DNA concentration by Nanodrop.

10) Quantify DNA length by Tapestation and adjust the concentration according to the fraction of the correct size product. Ensure there is at least 0.5 pmol of the correct size product.

Step 3.3: Preparation of the H3 cloned oligo pool

In this section, the H3 oligo pool cloned into a plasmid is resuspended to prepare it for the GGA. The inserts can be extracted from the plasmid using restriction digest and purified using BluePippin, but this would likely result in significant sample loss.

1) Resuspend the plasmid from Twist to 100 ng/µL in nuclease-free water. Ensure there is at least 0.5 pmol of the plasmid.

Step 3.4: Golden Gate assembly of heavy Vs, H3s, and heavy J to form heavy chain pools

The previous three steps prepared the heavy V fragments, the H3 oligo pool, and the heavy J fragment for GGA. In this step, the three pieces are ligated together and amplified using PCR. The PCR reaction also adds part of the heavy linker and the Shine-Dalgarno sequence. The heavy J fragment contains extra DNA after the heavy J sequence that is removed in the PCR reaction. This additional DNA enables clear visualization of the ligation in Tapestation.

In this example, the assembled heavy chain pools are 674 bp (link to an example assembled heavy chain pool). After PCR, the heavy chain pools are 479 bp (link to an example heavy chain pool after PCR).

1) Prepare 8 of the following reactions:

   *Mix SapI gently by pipetting and keep it in the cooling tub while using.
   *Need many more ng of H3 than HV or HJ because it is still in a plasmid.

Reagent	Volume (µL)
NEBridge ligase master mix (M1100L) (3X)	5.0
SapI (R0569L) (10U/ µL)	1.0
Heavy V pooled fragments	X (0.05 pmol)
H3 cloned oligo pool	Y (0.05 pmol)
Heavy J	Z (0.05 pmol)
Nuclease-free water	Up to 15
Total Volume	**15.0**

2) Run the following program in a thermal cycler (ATSAP1).

Step	Temp (ºC)	Duration
1	37	5 min
2	16	5 min
	Go to step 1	29X
3	60	5 min
4	4	forever

3) Collect the 8 15 uL reactions (120 uL total) into 3 40 uL tubes.

4) Add another 1 µL SapI to each tube and incubate 1 hour at 37C.

5) Combine the three reactions into a single tube and purify using the Monarch kit. Elute in 12 uL nuclease-free water.

6) Quantify DNA concentration by Nanodrop.

7) Quantify DNA length by Tapestation and adjust the concentration according to the fraction of correct product.

8) Amplify the assembled heavy chain pool by PCR using OATC74 and OATC77. First prepare one of the following test reactions.

Reagent	Volume (µL)
Q5 reaction buffer (B9027S) (5X)	10
OATC74 10 uM	2.5
OATC77 10 uM	2.5
dNTPs (N0447L) (10 mM)	1
1 ng correct-sized product	X
Q5 polymerase (M0491L)	0.5
Nuclease-free water	Up to 50
Total	**50**

9) Run the following PCR program (ATQ5, but need to adjust parameters).

Step	Temp (ºC)	Duration
1	98	30 sec
2	98	10 sec
3	69	30 sec
4	72	10 sec
	Go to 2	11X
5	72ºC	2 min
6	4ºC	

10) Run 5 µL of the product on a gel. If it appears to be a single band of the correct size, perform enough additional reactions to have at least 10 times (we recommend 100 times) the number of molecules of correct size template DNA as the number of variants in this heavy chain library. Use the NEBio calculator to help with the calculation. This will likely require at least 8 reactions.

11) Combine the additional reactions into one tube and run 5 µL of the product on a gel.

12) If it looks like a single band of the correct size, add the test PCR to the tube.

13) Purify the PCR products with SPRi beads using a 1.8X ratio of beads to DNA. Elute in 30 µL nuclease-free water. Alternatively, you can purify using the Monarch kit, but this will require many columns.

14) Quantify DNA concentration by Nanodrop.

15) Quantify DNA length by Tapestation and adjust the concentration according to the fraction of the correct size product. Ensure there is at least 0.5 pmol of the correct size product for each light J.

Step 4: Assembly of heavy and light chain pools

The light chain pools for each light J (produced in step 2) are combined with the heavy chain pools (produced in step 3). The products are then amplified using PCR and purified. After PCR, the light J pools are combined. In this example, the length of the full product is 1160 bp (link to an example full product) and the length of the PCR-amplified product is 1157 bp (link to an example full product after PCR).

1) Prepare 8 of the following reactions for each light J. If the volume of fragments exceeds 9 µL, you need to move to a larger reaction volume.
   *Keep BsaI in a cooling tub while using.

Reagent	Volume (µL)
NEBridge ligase master mix(M1100L) (3X)	5.0
BsaI-HFv2 (R0535)	1.0
Light chain pool for each light J	X (0.05 pmol)
Heavy chain pool	Y (0.05 pmol)
Nuclease-free water	Up to 15
Total Volume	**15.0**

2) Run the following PCR program.

Temp (ºC)	Duration
37	1 hour
60	5 min
4	forever

3) Combine all ligation reactions for each light J. This should result in one 120 µL tube for each light J.

4) Purify full product for each light J with the Monarch kit. Elute each in 12 µL nuclease-free water. This should result in one tube of 12 µL for each light J.

5) Quantify DNA concentration by Nanodrop.

6) Quantify DNA length by Tapestation and adjust the concentration according to the fraction of correct size product.

7) Prepare the following reaction to amplify the product for each light J using PCR with OATC66 and OATC77.

Reagent	Volume (µL)
Q5 reaction buffer (5X B9027S)	10
dNTPs (N0447L) (10 mM)	1
OATC66 10 uM	2.5
OATC77 10 uM	2.5
1 ng correct-sized product	X
Q5 polymerase (M0491L)	0.5
Nuclease-free water	Up to 50
Total	**50**

8) Run the following PCR program (ATQ5, but need to adjust parameters).

Step	Temp (ºC)	Duration
1	98	30 sec
2	98	10 sec
3	69	30 sec
4	72	20 sec
	Go to 2	11X
5	72ºC	2 min
6	4ºC	Forever

9) Run 5 µL of the product for each light J on a gel. If they appear to be single bands of the correct size, perform enough additional reactions to have at least 10 times (we recommend 100 times) the number of molecules of correct size template DNA as the number of variants in each light chain pool. Use the NEBio calculator to help with the calculation. This will likely require at least 8 reactions.

10) Combine the additional reactions into one tube for each light J and run 5 µL of the product on a gel.

11) If the products look like a single band of the correct size, add the test PCRs to each tube.

12) Purify the PCR products with SPRi beads using a 1.8X ratio of beads to DNA. Elute in 30 µL nuclease-free water. Alternatively, you can purify using the Monarch kit, but this will require many columns.

13) Quantify DNA concentration by Nanodrop for each light J.

14) Quantify DNA length by Tapestation for each light and adjust the concentrations according to the fraction of the correct size products. Ensure there is at least 1 µg of the correct size product for each light J.

15) If the tapestation results look fairly clean and/or consistent between different light Js, combine 1 µg of the full product for each light using the adjusted concentration from Tapestation. This should result in one tube containing 1 µg of DNA for each light J pool.

16) Purify the mix or individual light Js with a BluePippin 1.5% agarose cassette (link to cassettes) using a selection range of 600 - 1500 bp (or another appropriate range based on the Tapestation results). It may be necessary to use several lanes, as each lane can accommodate a maximum of 2 µg of DNA

17) If the volume is small enough, combine the elutions and purify with the Monarch kit and elute in 20 uL nuclease-free water. Otherwise, purify with two columns, elute each in 12 µL nuclease-free water, and combine them.

18) Quantify DNA concentration by Nanodrop.

19) Quantify DNA length by Tapestation and adjust the concentration according to the fraction of correct size product. Ensure there is at least 2 pmol of product.

Step 5: Ligation of full product into pre-digested vector

In this section, the full product prepared in Step 4 is ligated into the pre-digested vector prepared in step 1. This represents the final step of the library assembly process. After this, the library is transformed into electrocompetent bacteria.

1) Prepare 10 of the following reactions.
   *Keep BsaI in a cooling tub while using.
   *Do not use less insert than this even if it is tempting

Reagent	Volume (µL)
NEBridge ligase master mix (M1100L) (3X)	5.0
BbsI-HF (R3539L) (20U/ µL)	1.0
Full product	X (0.15 pmol)
Pre-digested vector	Y (0.05 pmol)
Nuclease-free water	Up to 15
Total Volume	15.0

2) Run the following program in a thermal cycler (ATSapI).

Step	Temp (ºC)	Duration
1	37	5 min
2	16	5 min
	Go to step 1	29X
3	60	5 min
4	4	forever

3) Combine the ten reactions into two tubes of 75 µL and purify each with the Monarch kit. Elute each in 12 µL nuclease-free water pre-heated to 50°C and combine the two elutions into one tube.

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Competing interests

S.J.F. and A.T. are named inventors on patents related to antibody design, including the methods of this paper. S.J.F. is a paid advisor on protein design.

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