



May 16, 2023

Generating rabbit rAbs from heterohybridomas

DOI

<https://dx.doi.org/10.17504/protocols.io.eq2ly7k4mlx9/v1>

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Protocol Citation: Chun'e Zhang, Yu Peng 2023. Generating rabbit rAbs from heterohybridomas. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.eq2ly7k4mlx9/v1>

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Protocol status: Working

We use this protocol and it's working

Created: May 08, 2023

Last Modified: May 16, 2023

Protocol Integer ID: 81623

Keywords: rabbit rabs from heterohybridoma, generating rabbit rab, rabbit recombinant antibody, mrna extraction to rab production, recombinant antibody, rab production, existing heterohybridoma, heterohybridoma, rab, mrna extraction

Abstract

This protocol describes the materials and methods used to generate rabbit recombinant antibodies (rAbs) from existing heterohybridomas, from mRNA extraction to rAb production.

Materials

1) cells, enzymes and kits

DH5a competent cells (CB101), TIANGEN, Cat#CB101-02

DNA Loading buffer, EX-VISION, Cat#N313-1ML

DNA Ladder marker, Fermentas, Cat#SM0311

RNaseZap™ Wipes, Invitrogen, Cat#AM9786

RNeasy Micro Kit (50), QIAGEN, Cat#74004

iScript™ cDNA Synthesis Kit, Bio-Rad, Cat#1708891

AxyPrep™ PCR Cleanup Kit, Axygen, Cat#AP-PCR-250

Phusion DNA polymerase, Thermo, Cat#F-530L

La Taq, TAKARA, Cat#RR52AG

Taq 2x Master Mix, BioLabs, Cat#M0270S

Gibson Assembly® Master Mix, NEB, Cat#E2611L

2) DNA oligos as primers

5-terminal primer (5'→3'):

Up-pcDNA3.4-rab-IgLCgaacccttgctagc ccAcc ATGGACACGAGGGCCCCCACTCAG

Up-pcDNA3.4-rab-IgHtcgaacccttgctagc ccAcc ATGGAGACTGGGCTGCGCTGGCTT

rab-IgLV ATGGACACGAGGGCCCCCACTCAG

rab-IgHV ATGGAGACTGGGCTGCGCTGGCTT

3-terminal primer (5'→3'):

rab-IgL-V-p1CAGTTGTTTGGGTGGTGCCATCCAC

rab-IgH-V-p1GCTGGCTGCTTGAGGTCACGCTCACCAC

rab-IgL-V-PexTTCGCCACACACACGATGGTGACT

rab-IgH-V-Pexggcagcccagggtcaccgtggagct

3) cell expression system

Expi293 cell

Expi293™ Expression Medium, Life, Cat#A1435101

Opti-MEM, Life, 31985062, 100mL

ExpiFectamine™ 293 Transfection Kit, Life, Cat#A14524

AxyPrep™ Plasmid Kit, Axygen, Cat#AP-96-P-4ExpiFectamine™ 293 Transfection Kit, Life, A14524 (including ExpiFectamine, enhancer 1, enhancer 2)

4) Buffer and solution for antibody purification

Protein A binding buffer: 25mM Tris-HCl, 25mM NaCl, pH7.2

Elution buffer: Bio-Rad Cat#1536161, prepared following the manufacturer's instruction

Neutralizing buffer: 1M Tris-HCl, pH9.0

5) regular instruments

Thermal cycler (PCR machine), DNA electrophoresis system, 37°C incubator, 37°C orbital shaker, TC-10 cell counter, centrifugate, pH meter, magnetic stirrer, vortex shaker.

6) Key DNA sequences

C_H

GGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACACACCCAGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGGCTACCTCCCGGAGCCAGTGACCGTGACCTGGAACCTCGGGCACCCCTACCAATGGGGTACGCA



CCTTCCCGTCCGTCCGGCAGTCCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCGTGACCTCAAGCAGCCAGCCC
GTCACCTGCAACGTGGCCCAACCCAGCCACCAACACCAAAGTGGACAAGACCGTTGCGCCCTCGACATGCAGCAAGCC
CAGGTGCCCAACCCCTGAACTCCTGGGGGGACCGTCTGTCTTCATCTTCCCCC AAAACCCAAGGACACCCTCATGA
TCTCACGCACCCCGAGGTACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGGTGCAGTTCACATGGTACA
TAAACAACGAGCAGGTGCGCACCGCCCCGGCCGCCGCTACGGGAGCAGCAGTTCAACAGCACGATCCGCGTGGTCAG
CACCCTCCCCATCGCGCACCCAGGACTGGCTGAGGGGCAAGGAGTTCAAGTGCAAAGTCCACAACAAGGCACTCCCGG
CCCCATCGAGAAAACCATCTCCAAAGCCAGAGGGCAGCCCCTGGAGCCGAAGGTCTACACCATGGGCCCTCCCCG
GGAGGAGCTGAGCAGCAGGTCCGTCAGCCTGACCTGCATGATCAACGGCTTCTACCCTTCCGACATCTCGGTGGAGTG
GGAGAAGAACGGGAAGGCAGAGGACA ACTACAAGACCACGCCGGCCGTGCTGGACAGCGACGGCTCCTACTTCCTCT
ACAGCAAGCTCTCAGTGCCACGAGTGAGTGGCAGCGGGGCGACGTCTTCACCTGCTCCGTGATGCACGAGGCCTTG
CACAACCACTACACGCAGAAGTCCATCTCCCGCTCTCCGGGTAAATGA

C_L

ATGGTGACCTTACCCTACTACTTTAACTTGTGGGGCCAAGGCACCCTGGTCACCGTCTCCTCAGGGCAACCTAAGGCT
CCATCAGTCTTCCCCTACTGGCCCCCTGCTGCGGGGACACACCCAGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGG
CTACCTCCCGGAGCCAGTGACCGTGACCTGGA ACTCGGGCACCCCTCACCAATGGGGTACGCACCTTCCCGTCCGTCC
GGCAGTCCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCGTGACCTCAAGCAGCCAGCCCGTCACCTGCAACGTG
GCCACCCAGCCACCAACACCAAAGTGGACAAGACCGTTGCGCCCTCGACATGCAGCAAGCCCATGTGCCACCCCC
TGA ACTCCTGGGGGGACCGTCTGTCTTCATCTTCCCCC AAAACCCAAGGACACCCTCATGATCTCACGCACCCCCG
AGGTACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGGTGCAGTTCACATGGTACATAAACAACGAGCAGG
TGCGCACCGCCCCGGCCGCCGCTACGGGAGCAGCAGTTCAACAGCACGATCCGCGTGGTCAGCACCCCTCCCCATCGC
GCACCAGGACTGGCTGAGGGGCAAGGAGTTCAAGTGCAAAGTCCACAACAAGGCACTCCCGGCCCCCATCGAGAAAA
CCATCTCCAAAGCCAGAGGGCAGCCCCTGGAGCCGAAGGTCTACACCATGGGCCCTCCCCGGGAGGAGCTGAGCAG
CAGGTCCGTGAGCCTGACCTGCATGATCAACGGCTTCTACCCTTCCGACATCTCGGTGGAGTGGGAGAAGAACGGGAA
GGCAGAGGACA ACTACAAGACCACGCCGGCCGTGCTGGACAGCGACGGCTCCTACTTCCTCTACAGCAAGCTCTCAG
TGCCACGAGTGAGTGGCAGCGGGGCGACGTCTTCACCTGCTCCGTGATGCACGAGGCCTTGACAACCACTACACG
CAGAAGTCCATCTCCCGCTCTCCGGGTAAATGA

1. mRNA extraction

- 1 Before extracting mRNA, use RNaseZap™ Wipes to wipe the outer surface of the centrifuge tube, tube rack, pipette, centrifuge, operating table, etc. to remove possible RNase to prevent mRNA degradation. Among them, using an RNase-free pipette tip with a filter within. The operator should wear a mask, and wipe gloves and items with wet wipes from time to time during the operation. Before mRNA is reverse-transcribed into cDNA, protection is required to prevent RNase from contaminating experimental samples.
RLT buffer is used as cell lysis buffer, and β -mercaptoethanol needs to be added before use. In an RNase-free centrifuge tube, add 10 μ L of β -mercaptoethanol per 1 mL of RLT buffer, add mercaptoethanol, mix well, and mark the name (RLT +) and date. The mixture can be placed at room temperature to ensure that it can be used within one month
- 2 Gently pipette the cell clones. Then move the cell suspension into a clean RNase-free 1.5 mL centrifuge tube, centrifuge at 500 g for 4 min, and discard all the supernatant. If the number of cells does not exceed 1×10^5 , add 75 μ L of RLT buffer (with mercaptoethanol); if the number of cells is less than 500, add an additional 5 μ L carrier RNA solution to each sample to reduce the loss during mRNA extraction (Preparation and use of carrier RNA solution: Take 5 μ L 310 ng/ μ L stock solution and add 34 μ L RLT buffer, mix gently, then take 6 μ L and add 54 μ L RLT buffer, this is 4 ng/ μ L working solution; take 5 μ L working solution for use). After adding the RLT buffer, shake on the vortex shaker at the maximum speed for more than 15 sec to help the cells to lyse completely.
- 3 Use absolute ethanol and RNase-free water to prepare 70% ethanol. Add one volume of 70% ethanol to the sample, pipette, and mix well. Then transfer it to an RNeasy MinElute spin column, centrifuge at $>8000g$ for 30 sec, and discard the flow-through.
- 4 Add 350 μ L RW1 buffer to the RNeasy MinElute spin column, centrifuge at $>8000g$ for 30 sec, and discard the flow-through.
- 5 Mix 10 μ L DNase I stock solution with 70 μ L RDD buffer, add it to the RNeasy MinElute spin column, and let it stay at room temperature (20-30°C) for 15-20 min.
- 6 Add 350 μ L RW1 buffer to the RNeasy MinElute spin column, centrifuge at $>8000g$ for 30 sec, and discard the flow-through.
- 7 Add 500 μ L RPE buffer to the RNeasy MinElute spin column, centrifuge at $>8000g$ for 30 sec, and discard the flow-through.
- 8 Use absolute ethanol and RNase-free water in the kit to prepare in proportion to obtain 80% ethanol. Add 500 μ L 80% ethanol to the RNeasy MinElute spin column, centrifuge



at >8000 g for 2 min, and discard the flow-through and collection tube. Put the RNeasy MinElute spin column into a new 2 mL collection tube, open the lid, and centrifuge at the highest speed for 5 min to allow the alcohol to evaporate.

- 9 Put the RNeasy MinElute spin column into a new 1.5 mL collection tube, add 14 μL 37°C preheated RNase-free water, and centrifuge at 15,000 rpm for 1 min to elute the mRNA.
- 10 Measure the mRNA concentration on the Nanodrop. Avoid the degradation of mRNA caused by repeated freezing and thawing of mRNA.

2. Reverse transcription

- 11 Mix the sample with primers following the design,

Total RNA	5.05 μL
rab-IgL-V-p1	0.5 μL
rab-IgH-V-p1	0.5 μL

3 min at 72°C and 2 min at 42°C, to generate the pre-mix sample

- 12 Mix the samples with primers following the design,

5x first strand buffer	2 μL
DTT (100mM)	0.2 μL
dNTP(20mM)	0.5 μL
RNase inhibitor (40U/ μL)	0.25 μL
SMART reverse transcriptase (100U/ μL)	1 μL
Pre-mix sample	6.05 μL

90 min at 42 °C , and 10 min at 72 °C to generate the cDNA sample

- 13 Add 50 μL Tricine-EDTA buffer I to the cDNA sample. it can be stored at -20°C for up to 3 months before being applied to V_H and V_L amplification.

3. V_H and V_L amplification and subclone

- 14 Amplify V_H and V_L with primers following the design,

H_2O	5.1 μL
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5x Phusion HF Buffer	2 μ L
dNTP (2mM)	1 μ L
Up-pcDNA3.4-rab-IgL or	
Up-pcDNA3.4-rab-IgH	0.5 μ L
rab-IgL-V-Pex or	
rab-IgH-V-Pex	0.5 μ L
DMSO	0.3 μ L
Phusion DNA polymerase	0.1 μ L
cDNA	0.5 μ L

The reaction starts with 30 sec at 98 °C, proceeds with 35 cycles (10 sec at 98 °C, 30 sec at 65 °C , and 20 sec at 72 °C), and ends with 5 min at 72 °C

- 15 Amplify V_H and V_L in 10 μ L PCR reaction respectively, mount the PCR product to 1% agarose gel for DNA electrophoresis (5%-10% Loading buffer), and observe the brightness of the target band. If the reaction runs well, scale it up to a 50 μ L system. Purify the PCR product with a PCR purification kit and quantify it using Nanodrop.

- 16 Mix the samples for ligation,

Gibson Assembly® Master Mix	2.5 μ L
Linearized modified	
pcDNA3.4(carrying C_H or C_L)	0.5 μ L
rab-VH/VL PCR product	50 ng
Make the reaction to 5 μ L with H_2O	

The reaction lasts 15 min at 50 °C to generate ligation products

- 17 Add 5 μ L of the ligation product to 30-50 μ L of DH5 α competent cells, ice-bath for 30 min; heat shock at 42°C for 1.5 min, and ice-bath for 2 min. Then, add 300-500 μ L of LB medium (antibiotics-free), and shake at 150 rpm for 45-60 min at 37°C.

- 18 After the transformation, so in down the cells and discard most supernatant. Spread the pellet (bacteria cells) evenly on the AmpR plate (with 50 μ g/mL Amp). Incubate the plate overnight at 37°C. The next day, pick clones into 200 μ L LB medium (containing 50 μ g/mL Amp) and shake the tubes at 37°C for 4 hours. Then screen clones using the following reaction,

Taq 2x Master Mix	5 μ L
H_2O	3 μ L
rab-IgLV	
or rab-IgHV	0.5 μ L
rab-IgL-V-Pex	

or rab-IgH-V-Pex 0.5 µL
Bacteria suspension 1 µL

The reaction starts with 5 min at 98 °C, proceeds with 25 cycles (20 sec at 94 °C, 20 sec at 60 °C, and 40 sec at 68 °C), and ends with 5 min at 68 °C

Analyze the clones by resolving the PCR products in DNA electrophoresis. Subject the positive clones to Sanger sequencing.

4. Sequence analysis (Sequence Priority by Residual Preference)

19 Translate the DNA sequences of clone H chain and L chain into protein sequences, and identify their CDR3 region using any regular bioinformatic tools (i.e. <http://imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi>)

20 Open the online tool (<https://www.ebi.ac.uk/Tools/msa/muscle/>), input the protein sequences in FASTA format of all H chains from the same clone, run sequence alignment, and generate a phylogenetic tree. Pick chains following the rules below to generate a prior pool of H chains,

selecting a V_H sequence that comprises a CDR3 that is 8-20 residues in length; and (i) does not contain greater than 3 consecutive residues of the same amino acid; (ii) comprises at least one aromatic residue; (iii) does not comprise three of one, or a combination of, the following amino acids: P, C, K or Q;

21 Open the online tool (<https://www.ebi.ac.uk/Tools/msa/muscle/>), input the protein sequences in FASTA format of all L chains from the same clone, run sequence alignment, and generate a phylogenetic tree. Pick chains following the rules below to generate a prior pool of L chains,

selecting a V_L sequence that comprises a CDR3 that is 8-20 residues in length; and (i) does not contain greater than 3 consecutive residues of the same amino acid; (ii) comprises at least one aromatic residue; (iii) does not comprise three of one, or a combination of, the following amino acids: P, C, K or Q;

5. screening the prior pools

22 Select the H and L chains in prior pools, use the preserved bacteria clones to inoculate 2 mL of LB medium, and incubate them overnight. Extract the plasmids using a regular plasmid preparation kit, and use Nanodrop to determine the concentration.

- 23 **Cell transfection:** Add 25 μL Opti-MEM to each well of a 24-well plate, add 0.167 μg H plasmid and 0.333 μg L chain plasmids, and shake the 24-well plate gently to mix well. Add 25 μL Opti-MEM in a 1.5 mL centrifuge tube, add 1.325 μL ExpiFectamine into it, and mix well. Let the tube stay at room temperature for 5 min, then take 26 μL of the mixture into each well of the 24-well plate. Pipette and mix well, and place it at room temperature for 20–30 min. Add 0.425 mL of Expi293 cells with a density of 3.3×10^6 cell/mL into each well of the 24-well plate and place it on an orbital shaker at 125 rpm in an incubator.
- 24 After 4–5 days of transfection, centrifuge the cell culture at 8000 rpm for 10 min, and collect the supernatant for subsequent Bio-plex screening. The pair of H and L chains with high detection signal was used for subsequent 30 mL rAb expression and purification.

6. Recombinant antibody expression and purification

- 25 For each antibody clone, take two 15 mL centrifuge tubes and add 1.5 mL of OptiMEM each. Then, add 80 μL of ExpiFectamine to one centrifuge tube and mix well, add 10 μg of IgH plasmid and 20 μg of IgL plasmid to the other centrifuge tube and mix well. After standing for 5 min, gently mix two tubes into one tube, and place it at room temperature for 20–30 min to prepare the transfection solution.
- 26 During the 20–30 min waiting, dilute the cells with medium to adjust the density to 3×10^6 cell/mL. Take 25.5 mL cell suspension and add it to a 125 mL flask, and slowly and gently add the transfection solution to the cell culture, placed the flask in a 37°C incubator on an orbital shaker at 125 rpm overnight.
- 27 On the next morning (almost 16 hours after transfection), add 1.5 mL of Enhancer 2 and 150 μL of Enhancer 1 to each shake flask, and continue the cell culture on an orbital shaker in the 37°C incubator at 125 rpm.
- 28 After 5 days of transfection, centrifuge the culture medium at 8000 rpm for 10 min, collect the supernatant, and store it in a refrigerator at 4°C for purification.
- 29 Remove the upper and lower seals of a 1 mL gravity purification column, pour off the residual ethanol, wash the column with 20 mL water first, and then equilibrate the column with 10 mL of binding buffer.
- 30 Filtrate the culture medium in step 28 with a 0.45 μM filter to remove cell debris. Then load the medium to the gravity column. Discard the flow through. Then gradually add 10 mL of binding buffer.
- 31 Prepare eight 1.5 mL centrifuge tubes, and add 30 μL neutralizing buffer into each tube.

- 32 Place the column directly above the first centrifuge tube, add 350 μ L of elution buffer, and collect the eluent. Mix the eluent well with the neutralizing buffer. Then replace it with the second centrifuge tube and repeat the operation until all 8 centrifuge tubes are completely filled.
- 33 Take a piece of parafilm and put it on white paper, place eight 30 μ L drops of Coomassie chromogenic solution on the piece, and take 3 μ L of solution from each tube and mix with the chromogenic solution. Estimate the antibody concentration in the eluent by the chromogenic reaction of the droplet (turning blue). Collect 2-4 tubes with high antibody concentration, centrifuge at 12000 rpm for 1 min, and transfer the supernatant to a new centrifuge tube.
- 34 Dialyze the samples in 1000 mL PBS at 4°C overnight. Then determine the antibody concentration using Nanodrop.

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