MAY 16, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.eq2ly7k4mlx9/v1

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

Protocol status: Working
We use this protocol and it's working

Created: May 09, 2023

Last Modified: May 16, 2023

PROTOCOL integer ID: 81623

Generating rabbit rAbs from heterohybridomas

Zhang[1], Yu Peng[1]

[1]Bio-Rad (Shanghai) Life Science Research and Development Co., Ltd

Yu Peng

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℃ incubator，37℃ orbital shaker, TC-10 cell counter，centrifugate，pH meter，magnetic stirrer, vortex shaker.

6) Key DNA sequences

C_H

GGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACACACCC
AGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGGCTACCTCCCGGAGCCAGTGACC
GTGACCTGGAACTCGGGCACCCTCACCAATGGGGTACGCACCTTCCCGTCCGTCCGG
CAGTCCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCGTGACCTCAAGCAGCCAG
CCCGTCACCTGCAACGTGGCCCACCCAGCCACCAACACCAAAGTGGACAAGACCGTT
GCGCCCTCGACATGCAGCAAGCCCACGTGCCCACCCCCTGAACTCCTGGGGGGACCG
TCTGTCTTCATCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCACGCACCCCCG
AGGTCACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGGTGCAGTTCACAT
GGTACATAAACAACGAGCAGGTGCGCACCGCCCGGCCGCCGCTACGGGAGCAGCAGT
TCAACAGCACGATCCGCGTGGTCAGCACCCTCCCCATCGCGCACCAGGACTGGCTGA
GGGGCAAGGAGTTCAAGTGCAAAGTCCACAACAAGGCACTCCCGGCCCCCATCGAGA
AAACCATCTCCAAAGCCAGAGGGCAGCCCCTGGAGCCGAAGGTCTACACCATGGGCC
CTCCCCGGGAGGAGCTGAGCAGCAGGTCGGTCAGCCTGACCTGCATGATCAACGGCT
TCTACCCTTCCGACATCTCGGTGGAGTGGGAGAAGAACGGGAAGGCAGAGGACAACT
ACAAGACCACGCCGGCCGTGCTGGACAGCGACGGCTCCTACTTCCTCTACAGCAAGC
TCTCAGTGCCCACGAGTGAGTGGCAGCGGGGCGACGTCTTCACCTGCTCCGTGATGC
ACGAGGCCTTGCACAACCACTACACGCAGAAGTCCATCTCCCGCTCTCCGGGTAAAT
GA

C_L

ATGGTGACCTTACCCTACTACTTTAACTTGTGGGGCCAAGGCACCCTGGTCACCGTCT
CCTCAGGGCAACCTAAGGCTCCATCAGTCTTCCCACTGGCCCCCTGCTGCGGGGACA
CACCCAGCTCCACGGTGACCCTGGGCTGCCTGGTCAAAGGCTACCTCCCGGAGCCAG
TGACCGTGACCTGGAACTCGGGCACCCTCACCAATGGGGTACGCACCTTCCCGTCCG
TCCGGCAGTCCTCAGGCCTCTACTCGCTGAGCAGCGTGGTGAGCGTGACCTCAAGCA
GCCAGCCCGTCACCTGCAACGTGGCCCACCCAGCCACCAACACCAAAGTGGACAAGA
CCGTTGCGCCCTCGACATGCAGCAAGCCCATGTGCCCACCCCCTGAACTCCTGGGGG
GACCGTCTGTCTTCATCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCACGCAC
CCCCGAGGTCACATGCGTGGTGGTGGACGTGAGCCAGGATGACCCCGAGGTGCAGTT
CACATGGTACATAAACAACGAGCAGGTGCGCACCGCCCGGCCGCCGCTACGGGAGCA
GCAGTTCAACAGCACGATCCGCGTGGTCAGCACCCTCCCCATCGCGCACCAGGACTG

GCTGAGGGGCAAGGAGTTCAAGTGCAAAGTCCACAACAAGGCACTCCCGGCCCCCAT
CGAGAAAACCATCTCCAAAGCCAGAGGGCAGCCCCTGGAGCCGAAGGTCTACACCAT
GGGCCCTCCCCGGGAGGAGCTGAGCAGCAGGTCGGTCAGCCTGACCTGCATGATCAA
CGGCTTCTACCCTTCCGACATCTCGGTGGAGTGGGAGAAGAACGGGAAGGCAGAGGA
CAACTACAAGACCACGCCGGCCGTGCTGGACAGCGACGGCTCCTACTTCCTCTACAG
CAAGCTCTCAGTGCCCACGAGTGAGTGGCAGCGGGGCGACGTCTTCACCTGCTCCGT
GATGCACGAGGCCTTGCACAACCACTACACGCAGAAGTCCATCTCCCGCTCTCCGGG
TAAATGA

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 µL 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 µL of RLT buffer (with mercaptoethanol); if the number of cells is less than 500, add an additional 5 µL carrier RNA solution to each sample to reduce the loss during mRNA extraction (Preparation and use of carrier RNA solution: Take 5 µL 310 ng/µL stock solution and add 34 µL RLT buffer, mix gently, then take 6 µL and add 54 µL RLT buffer, this is 4 ng/µL working solution; take 5 µL 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 350µL RW1 buffer to the RNeasy MinElute spin column, centrifuge at >8000g for 30 sec, and discard the flow-through.

5 Mix 10 µL DNase I stock solution with 70 µL 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 µL RW1 buffer to the RNeasy MinElute spin column, centrifuge at >8000g for 30 sec, and discard the flow-through.

7 Add 500 µL 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 µL 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 ℃ , and 10 min at 72 ℃ to generate the cDNA sample

13 Add 50 µL Tricine-EDTA buffer I to the cDNA sample. it can be stored at -20℃ 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
5x Phusion HF Buffer	2 µL
dNTP (2mM)	1 µL
Up-pcDNA3.4-rab-IgL or Up-pcDNA3.4-rab-IgH	0.5 µL
rab-IgL-V-Pex or rab-IgH-V-Pex	0.5 µL
DMSO	0.3 µL
Phusion DNA polymerase	0.1 µL
cDNA	0.5 µL

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

15 Amplify V_H and V_L in 10 µL 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 µL 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 µL
Linearized modified	

pcDNA3.4(carrying C_H or C_L)	0.5 µL
rab-VH/VL PCR product	50 ng
Make the reaction to 5 µL with H_2O	

The reaction lasts 15 min at 50 ℃ to generate ligation products

17 Add 5 µL of the ligation product to 30-50 µL of DH5α 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 µL of LB medium (antibiotics-free), and shake at 150 rpm for 45-60 min at 37°C.

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

Taq 2x Master Mix	5 µL
H_2O	3 µL
rab-IgLV or rab-IgHV	0.5 µL
rab-IgL-V-Pex or rab-IgH-V-Pex	0.5 µL
Bacteria suspension	1 µL

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

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 Prefer...

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 µL 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 µL drops of Coomassie chromogenic solution on the piece, and take 3 µL 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.