IgG sequencing of rat hybridoma

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ABSTRACT

The purpose of this protocol is to amplify IgG antibody variable regions derived from rat hybridoma RNA, using RT-PCR and Sanger sequencing.

Materials needed:

- RNA extraction: Qiagen RNEasy mini kit (74104)
- Reverse Transcription Kit: SMARTScribe Reverse Transcriptase (639537)
- DNA Polymerase: Invitrogen Platinum SuperFi II PCR Master Mix (12368010)
- Invitrogen's PureLink™ PCR Purification Kit #K310001

The layout of this protocol was adapted from a protocol composed by Andrew McGuire's laboratory at the Fred Hutchinson Cancer Center, which in turn was adapted from Meyer et al 2019 "A simplified workflow for monoclonal antibody sequencing." See attachment for the respective manuscript.

For this protocol, rat-specific primers were designed, as the immunization campaign was executed in rats. See attachment for the primer design strategy.

ATTACHMENTS

Meyer et al 2019 A simplified workflow for monoclonal antibody sequencing.pdf

20211119 primer design.xlsx

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

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Primers

1 TS pF is ordered as RNA oligo; the remainder as DNA oligo

universal TS pF AAGCAGTGGTATCAACGCAGAGTACATrGrGrG

ISPCR pF aagcagtggtatcaacgcagag

rat IGHG RT pR GGACAGGGCTCCAGAGTTCC

rat IGHG PCR pR GACTGGCTCAGGGAAATAGCC

rat IGKC RT pR CTGATCAGTAACACTGTCCAGGAC

rat IGKC PCR pR CACTGATGTCTCTGGGATAGAAGTTG

rat IGLC1 RT pR GGGAGATAGGTGCACCATTTGC

rat IGLC1 PCR pR GGCCACTTCCACATCACTCG

rat IGLC2 RT pR TCCACACCCTGAGTGATAGGG

rat IGLC2 PCR pR CTTCCAGACCACTGTCATAACACC

Procedure

2 **RNA extraction:** Extract RNA from the hybridoma sample using RNeasy total RNA kit, according to the manufacturer's instructions.

3 **Reverse Transcription:** On ice, prepare the 1st reaction mix in PCR tubes for gamma chain and kappa chain (or lambda chain) cDNA synthesis according to the following recipe:

3.1 Gamma

Component	Volume per rxn

IGHG RT pR (10 uM)	1 uL
dNTP (10 mM)	1 uL
RNA sample (50 ng/uL)	2 uL
Total volume	4 uL

3.2 Kappa

Component	**Volume per rxn**
IGKC RT pR (10 uM)	1 uL
dNTP (10 mM)	1 uL
RNA sample (50 ng/uL)	2 uL
Total volume	4 uL

3.3 Lambda *(include later if Kappa does not amplify)*

A	B
Component	**Volume per rxn**
IGLC1 (or C2) RT pR (10 uM)	1 uL
dNTP (10 mM)	1 uL
RNA sample (50 ng/uL)	2 uL
Total volume	4 uL

3.4

On ice, prepare a 2nd mastermix in Eppendorf tubes. This recipe if for one reaction regardless of chain type. *Scale up for the number of samples as needed.*

2nd reaction mix

A	B
Component	**Volume per rxn**
5x SMARTScribe buffer	2 uL
DTT (20 mM)	1 uL
Universal TS pR (100 uM)	0.3 uL
H2O	1.70 uL

	A	B
	Total volume	5.00 uL

3.5 After preparing the 2nd mastermix, incubate each of the 1st reaction mixes in a thermocycler for **3 minutes at 72°C.**

3.6 While the incubation reaction is proceeding, add the following to the 2nd reaction mix:

	A	B
	Component	**Vol. per rxn**
	RNAse inhibitor (40 U/uL)	0.50 uL
	SMARTScribe Rev. Transcriptase (100 U/uL)	0.50 uL
	Total volume	1.00 uL

3.7 Once the incubation of the 1st reaction mix (from step 3.5) finishes, add 6 uL of the 2nd reaction mix to each tube of the 1st reaction mix.

3.8 With the 2 reaction mixes now combined, incubate each according to the following conditions:

	A	B	C
	Temperature	**Time**	**Cycles**
	42°C	60 min	1
	70°C	5 min	1
	4°C	hold	-

Proceed to PCR amplification step immediately after incubation has finished (once samples reach 4°C hold step).

4 **PCR amplification:** Prepare mastermix for PCR reaction according to following 2-step recipe:

4.1 Add the following components to each PCR tube.

Gamma

	A	B
	Component	**Volume per rxn**
	Platinum SuperFi II PCR MM	25 uL
	ISPCR pF (10 uM)	2.5 uL
	IGHG PCR pR (10 uM)	2.5 uL
	cDNA from RT step (5-100ng)	3 uL
	Water, nuclease-free	17 uL
	Total volume	50 uL

4.2 **Kappa**

	A	B
	Component	**Volume per rxn**
	Platinum SuperFi II PCR MM	25 uL
	ISPCR pF (10 uM)	2.5 uL
	IGK PCR pR (10 uM)	2.5 uL
	cDNA from RT step (5-100ng)	3 uL
	Water, nuclease-free	17 uL
	Total volume	50 uL

4.3 **Lambda** *(if Kappa doesn't amplify)*

	A	B
	Component	**Volume per rxn**
	Platinum SuperFi II PCR MM	25 uL
	ISPCR pF (10 uM)	2.5 uL
	IGLC1 (or C2) PCR pR (10 uM)	2.5 uL

	A	B
	cDNA from RT step (5-100ng)	3 uL
	Water, nuclease-free	17 uL
	Total volume	50 uL

4.4 Cap each tube, then mix and briefly centrifuge the PCR tubes.
Place PCR tubes in thermocycler, and run according to the following conditions:

Gamma

	A	B	C
	Temperature	**Time**	**Cycles**
	98°C	30 sec	1
	98°C	15 sec	35
	63°C	30 sec	
	72°C	25 sec	
	72°C	5 min	1
	4°C	hold	-

Kappa/Lambda

	A	B	C
	Temperature	**Time**	**Cycles**
	98°C	30 sec	1
	98°C	15 sec	35
	56°C	30 sec	
	72°C	25 sec	
	72°C	5 min	1
	4°C	hold	-

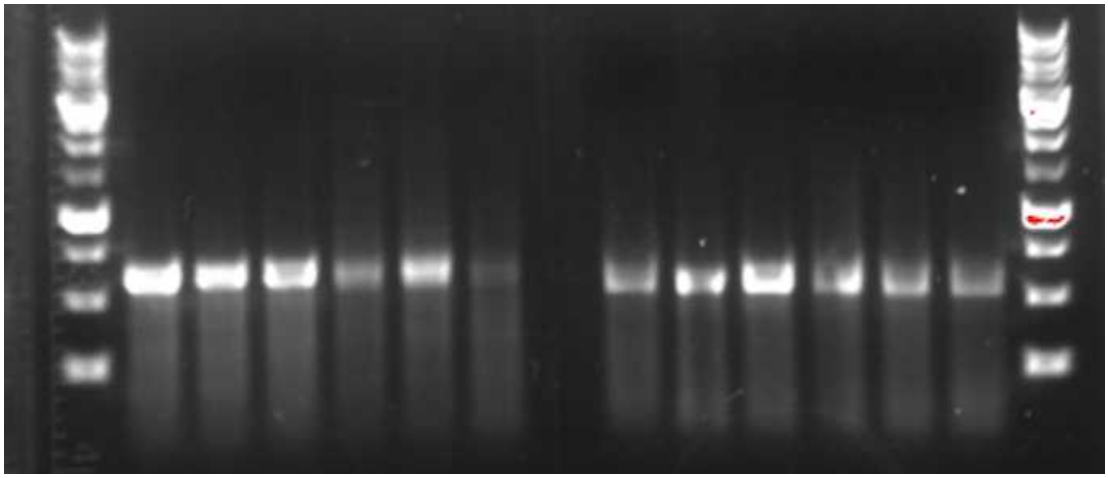
5 Verify the gel bands

After PCR reaction completes, set up 1% agarose gel according to the following conditions:
In a flask, add the following components:
0.5 g – Agarose powder
50 mL – 1X TAE buffer

Put flask, with added reagents, in microwave and heat up until all the powder is fully dissolved (about 1 minute).
After microwaving is finished, remove flask with hot pad; add 5 uL of Sybrsafe to flask, swirl to mix. Pour into a tray with appropriate plastic comb, preferably one with wells that accommodate 20uL volumes.

Prepare a fraction of each PCR products to verify the band size on the gel. Aliquot 5 uL from each PCR tube into a new PCR strip, and add 1 uL of DNA Loading Dye to each sample.
Load the gel and run it for 25-30 minutes at 110 V. *Modify these settings if needed.*
Remove gel, image, and save an image copy for the records.

Note: There should be a gamma chain, and either a kappa or lambda chain.
Amplified rat antibody products: 550-600 bp.
A significant majority of mouse antibody light chains will be kappa; if kappa not present, repeat with IGLC1 and IGLC2 samples.



6 PCR cleanup

PCR samples that produce a band of the expected size can then be finalized using a PCR purification kit (e.g. Invitrogen's PureLink™ PCR Purification Kit #K310001), and eluted in 25 uL volume. Verify cDNA concentration by nanodrop.

7 **Sanger sequencing** should be performed (e.g. GeneWiz or Genomic Core).

8 Analyze the antibody nucleotide sequence

Open the website: https://www.imgt.org/IMGT_vquest/vquest

Copy and paste sequence into the IMGT webpage, in the section “sequence submission”.

Select “Rattus norvergicus” for species, and “IGH” as receptor type or locus.

Click on "Start".