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We use this protocol and it's working

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TCR sequencing and activation

Zorea
Jonathan[1]

[1]Shraga Segal Department of Microbiology, Immunology, and Genetics, Ben-Gurion University of the Negev, 8410501, Beer-Sheva, Israel

J Zorea Jonathan

ABSTRACT

This protocol is a procedure we follow to isolate tumor infiltrating T cells and try to activate them with unique peptides and to characterize their TCR repertoire

PROTOCOL integer ID:
85701

Tumor implantation

1 Grow sufficient amount of cells (5*10^6 per mouse). At the day cells are ready to be inject, thaw a vial of Basement Membrane Extract (Biotest, 3433-010-01) on ice. Keep 200ul tips in -20° as well. 1w

2 Once the BME is thawed, trypsinize the cells, count them using trypan blue, and resuspend them in PBS in the concentration of 5M cells in 40ul PBS. add 10ul of BME per 40ul of cells suspension and keep pn ice. 1h

**** BME should be always kept on ice**

3 Inject 50ul of cells solution to the lip of each mouse using 300ul syringe. 1h

4 Allow cells to grow until tumor size reach the size of 4X4 (5-8 days) 1w

Mice treatment 1d

5 A day before treatment initiation, suspend GDC-0077 in MCT buffer to reach a dose of 25mg/kg. 1d

Note

average mouse weight 25g. It means 25mg of drug will be sufficient for 40 mice. each mice will be orally gavaged with 200ul of drug solution. So, for example, for 40 mice you will need 25mg of drug and 8ml of MCT buffer

MCT buffer recipe

Smart component allows you to create a key-value table that will appear to be filled out during the run.

0.5% Methylcellulose	
0.2% tween-80	
DI water	

Keep The drug on a magnetic stirrer over night.

6 The next day, gavage each mice with 200ul of the drug solution daily.
Measure tumor size using the caliper at the first day of treatment and every 3-4 days.

7 Keep treating the mice for 7-10 days, until the effect of the drug can be seen.

Tumor + lymph node collection

8 Arrange enough plain RPMI medium and ice before start sacrificing mice!

9 Sacrifice the mice, cage by cage, and collect tumors and mandibular lymph bodes using scissors and forceps. Keep the correct attribution for each set of tumor + lymph nodes. Place everything on ice in the RPMI medium.

Single cell isolation

30m

10 **Tumors:** Chop 1-2 tumors on a 60mm petri plate using scalpel, until all big chunks are gone.

10.1 Collect all the content of the plate using plain RPMI. Once you finished chopping all of the tumors, centrifuge all the tubes, waste the supernatant, and resuspend the tumors in 5ml of an enzymatic dissociation cocktail:

Smart component allows you to create a key-value table that will appear to be filled out during the run.

collagenase II 1mg/ml	
hyaluronidase 1mg/ml	
DNAse I 1mg/ml	

10.2 Transfer all the content to a gentleMACS™ C Tubes, place the C tubes on the gentleMACS machine, and run an appropriate protocol

Note

In general, for mice solid tumors we use the 37C_m_LIDK_1 protocol

10.3 Transfer all the C tube content to a 15 or 50ml tube, centrifuge for 5min on 300g, and waste the supernatant. Resuspend the tumor cells in 4ml RPMI.

10.4 Insert a glass pipet to the bottom of the 15ml tube, blocking air tranfer with your thumb. With a syringe, add 2ml of Ficoll-Paque PLUS, making sure not to mix it.

10.5 Centrifuge the tubes for 10min, 350g with **no brakes**.

10.6 Collect **only** the lymphocytes phase to a new 15 ml tube and wash it with PBS. Aspirate the supernatant and resuspend the cells in PBS.
Count the cells and resuspend up to 10^7 cells in 40ul MACS buffer. (same amount for each sample)
Keep the pellet of **CD8+ isolation**.

MACS buffer
0.5% bovine serum albumin (BSA)
2 mM EDTA
in PBS

11 **Lymphs:** Place a 70um strainer on top of a 50ml tube. Place the lymph nodes on top of the strainer together with RPMI. Using the "leg" of a 3ml syringe, press the lymph nodes over the mesh. Collect all the single cells by washing the mesh with RPMI.

11.1 Centrifuge for 5min on 300g, and collect the pellet.

12 Resuspend the cells pellet with 3ml of sterile AKC buffer for 3 min.
Add 9ml of PBS and centrifuge for 5min at 300g. Aspirate the supernatant and resuspend the cells in T-cells medium. **Count the cells.**

T-cells medium

Component	Final concentration	Amount
RPMI 1640	n/a	To 500 mL
L-glutamine	1x	5 mL
Penicillin-streptomycin solution (10×)	1×	5 mL
2-mercaptoethanol (1 M)	50 µM	25 µL
FBS	10% (v/v)	50 mL
HEPES 1M	25mM	12.5 mL
sodium pyruvate	1x	5 mL
MEM non-essential amino acids	1x	5 mL

ACK Lysis Buffer

	Reagent	Quantity (for 1000 mL)	Final concentration
	NH4Cl	8.02 g	150 mM
	KHCO3	1 g	10 mM
	Na2EDTA	37.2 mg	0.1 mM

CD8+ isolation

13 *Following the CD8a+T Cell Isolation Kit mouse Order no. 130-104-075 protocol of Miltenyi :*

Add 10 µL of Biotin-Antibody Cocktail per 10^7 total cells.

14 Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).

15 Add 30 µL of buffer per 10^7 total cells.

16 Add 20 µL of Anti-Biotin MicroBeads per 10^7 total cells.

17 Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).

18 Proceed to subsequent magnetic cell separation.
▲ Note: A minimum of 500 µL is required for magnetic separation. If necessary, add buffer to the cell suspension.

19 Place LS Column in the magnetic field of a suitable MACS Separator. For details refer to the respective MACS Column data sheet.

20 Prepare column by rinsing with 3 mL of buffer.

21 Apply cell suspension onto the column. Collect flow-through containing unlabeled cells, representing the enriched CD8a+ T cells.

22 Wash column with 3 mL of buffer. Collect unlabeled cells that pass through, representing the enriched CD8a+ T cells, and combine with the effluent from step 21.

23 Remove column from the separator and place it on a suitable collection tube. Pipette 5 mL of buffer onto the column. Immediately flush out the magnetically labeled nonCD8a+ T cells by firmly pushing the plunger into the column.

24 Centrifuge both labeled and unlabeled cells 5min at 300g, and resuspend both in 1ml of T-cell medium. Take 50ul of each for the Flow cytometry validation.

Analyze positive population by Flow

25 Fc-block cells with 25ul TruStain fcX™ (anti-mouse CD16/32) fc blocker(1:1000) diluted in FACS buffer for 5-10 minutes on ice.

FACS buffer
1% FCS
1mM EDTA
0.05% Na-Azid
in PBS

26 Add 25ul of appropriately conjugated fluorescent primary antibodies at predetermined optimum concentrations (1:300 by default) and incubate on ice for 15-20 minutes in the dark.

** dilute antibodies in FACS buffer. It is recommended to create a stock solution of each antibody following the next calculation:
2ul of AB stock will be diluted in a total of 25ul, so stock should be 12.5X. 300/12.5 = 24. If we take 1ul of an antibody and dilute it with 23ul of FACS buffer, we then create a stock we can use 2ul of it per sample. This will allow us to mix up to 12 antibodies and no exceeding the 25ul.

27 prepare DAPI solution by diluting DAPI in FACS buffer to a concentration of 3ug/ml, and add 100ul of this solution per sample.

28 Analyze the positive and negative population over the Flow cytometer.

CD8+ T cells RNA isolation

29 Spin cells for 5 min at 300 X g. Remove media and resuspend cells in ice cold PBS. Pellet cells by spinning at 300 X g for 5 min. Lyse cells with TRIZOL Reagent by repetitive pipetting or by passing through syringe and needle. Use 1 ml of the reagent per 5-10 X 10^6 of animal cells.

30 Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Centrifuge to remove cell debris. Transfer the supernatant to new tube.

31 Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes.

32 Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 80C.

Note

Following centrifugation, the mixture separates into lower red, phenolchloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase

33 Transfer upper aqueous phase carefully without disturbing the interphase into fresh tube. Measure the volume of the aqueous phase (The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization).

34 Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at 15 to 30oC for 10 minutes and centrifuge at not more than 12,000 x g for 10 minutes at 2 to 4oC. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

35 Remove the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the samples by vortexing and centrifuge at no more than 7,500 x g for 5 minutes at 2 to 8 oC. Repeat above washing procedure once. Remove all leftover ethanol.

36 Air-dry or vacuum dry RNA pellet for 5-10 minutes. Do not dry the RNA pellet by centrifuge under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/A280 ratio < 1.6. Dissolve RNA in DEPC-treated water by passing solution a few times through a pipette tip.

CFSE staining of lymph cells

37 Prepare a 2 µM working solution by diluting 1 µL of 5 mM CFSE stock solution in 2.5 mL PBS

38 Spin down and resuspend cells at 10-100 x 10^6 cells/mL in the CFSE working solution.

39 Incubate cells for 20 minutes at room temperature and keep protected from light.

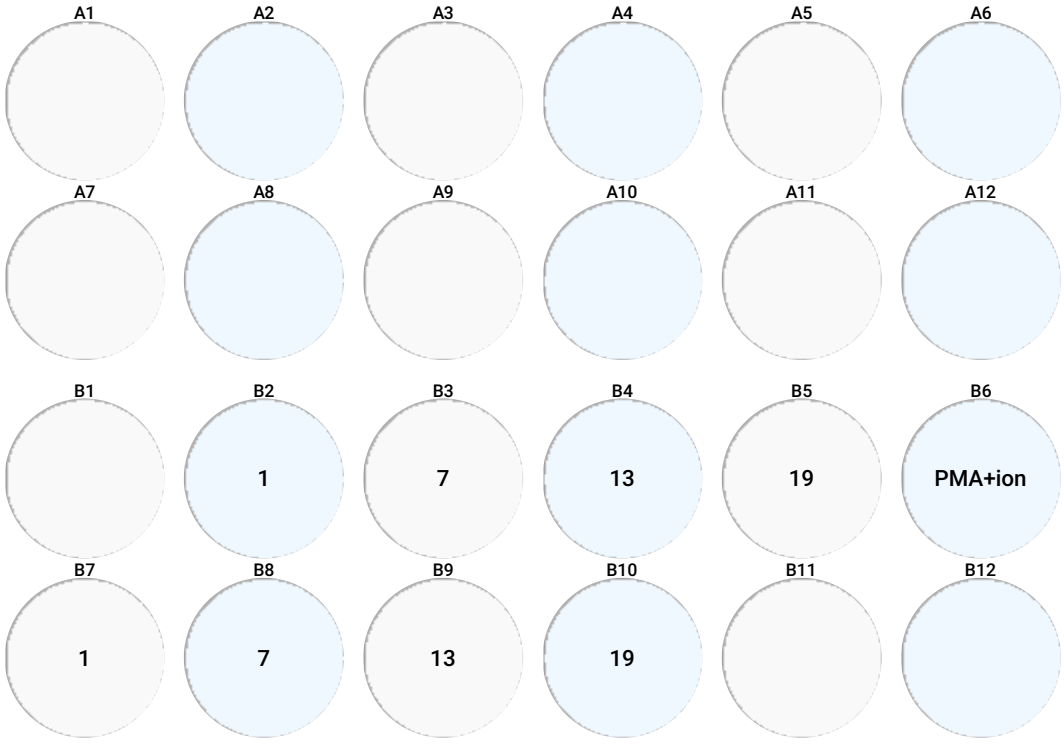
40 Quench the staining by adding 5 times the original staining volume of cell culture medium containing 10% FBS.

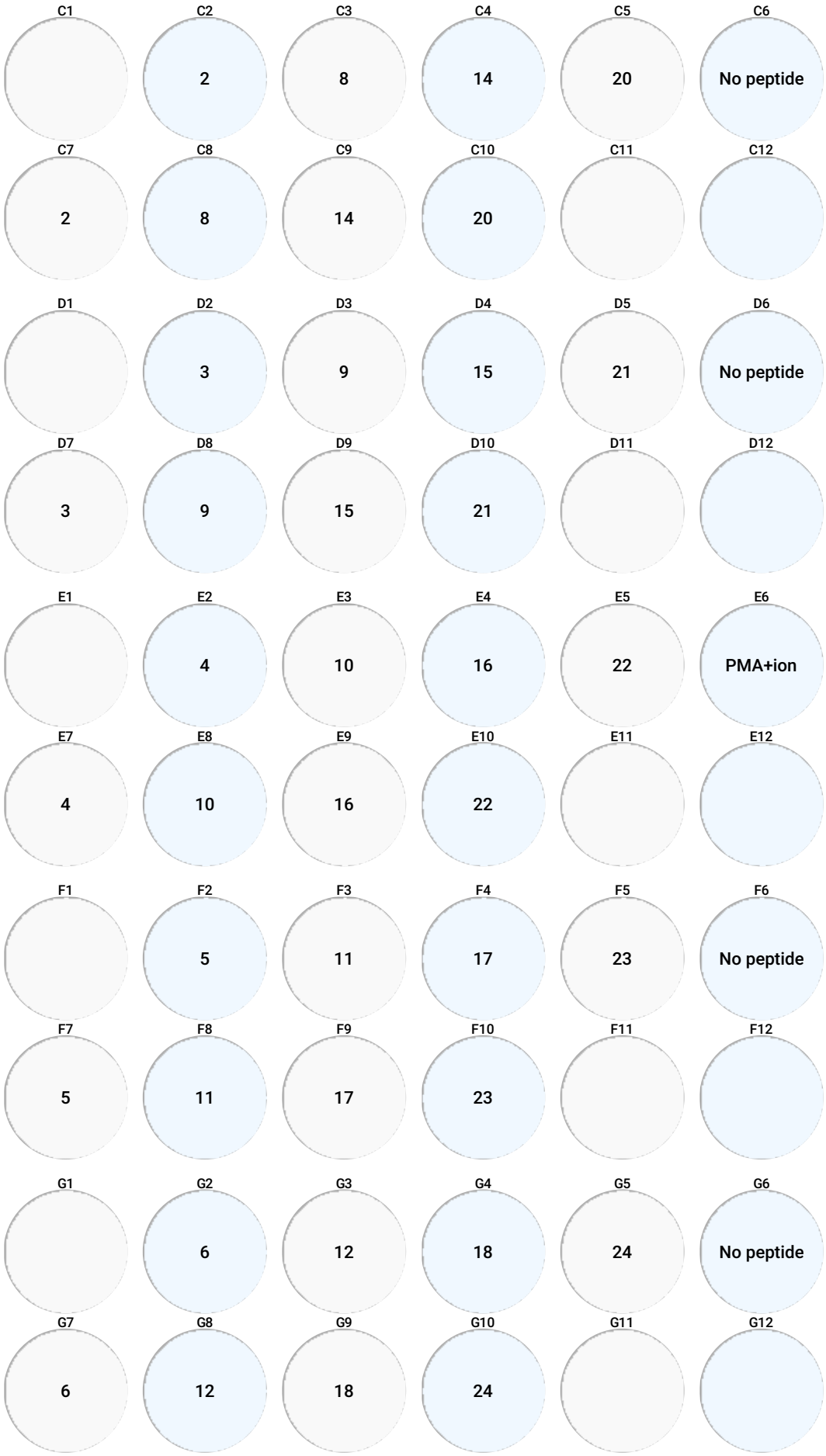
41 Pellet cells and resuspend in pre-warmed cell culture medium.

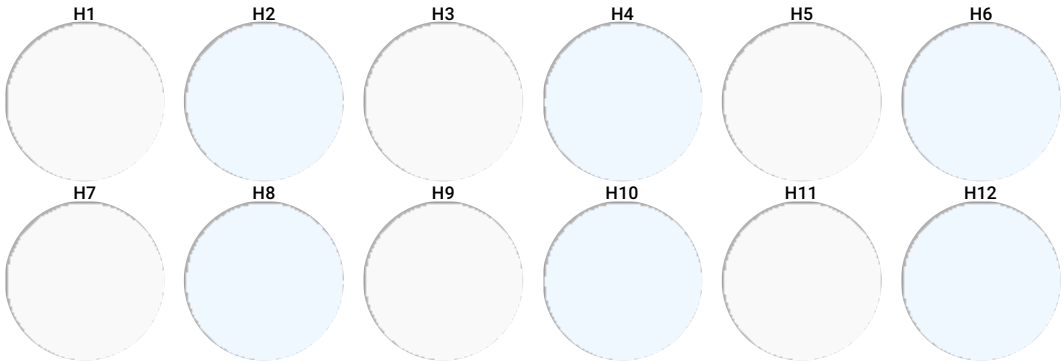
42 Count the cells and dilute them to 200k/100ul in T-cells medium.

Incubate peptides with lymph node cells

43 The 24 peptides are in a stock concentration of 2mM. Dilute the peptides 1:100 in T-cells medium and place them according to the map below :




C1
C2
2
C3
8
C4
14
C5
20
C6
No peptide
C7
2
C8
8
C9
14
C10
20
C11
C12
D1
D2
3
D3
9
D4
15
D5
21
D6
No peptide
D7
3
D8
9
D9
15
D10
21
D11
D12
E1
E2
4
E3
10
E4
16
E5
22
E6
PMA+ion
E7
4
E8
10
E9
16
E10
22
E11
E12
F1
F2
5
F3
11
F4
17
F5
23
F6
No peptide
F7
5
F8
11
F9
17
F10
23
F11
F12
G1
G2
6
G3
12
G4
18
G5
24
G6
No peptide
G7
6
G8
12
G9
18
G10
24
G11
G12



44 Seed the cells from step 42 in the 24 wells of the peptide + 2 No peptides well + PMA+ION well as a positive control.

45 Allow cells to grow 48-72 hours. Quantify CFSE together with CD8+ using FACS.