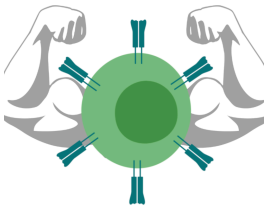
Jan 13, 2020

Measuring relative reactivity of mouse TCRs against a mouse cancer cell line



In 1 collection

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

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Abstract

This protocol repurposes Promega's T Cell Activiation Bioassay workflow to be able to test relative mouse TCR reactivity against a cell line. This specific protocol uses MC38 as the target as it doesn't normally present SIINFEKL and have good H2Kb and H2Db expression levels. The reactivity will be in relative to the positive control (OT-I reactivity against SIINFEKL-pulsed cells) and the negative control (OT-I reactiviy against unpulsed cells).

Materials

MATERIALS

- EcoRI-HF - 10,000 units **New England Biolabs Catalog #**R3101S
- NotI-HF - 2,500 units **New England Biolabs Catalog #**R3189L
- HiScribe T7 ARCA mRNA Kit (with Tailing) - 20 rxns **New England Biolabs Catalog #**E2060S
- UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) **Thermo Fisher Scientific Catalog #**15593031
- Nuclease-Free Water
- Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #**BP2818500
- T Cell Activation Bioassay (NFAT) **Promega Catalog #**J1621
- SpectraMax i3 Multi-Mode Microplate Detection Platform **Molecular Devices Catalog #**i3x
- Corning™ RPMI 1640 Medium (Mod.) 1X with L-Glutamine **Fisher Scientific Catalog #**MT10041CV
- Fetal Plus® **Atlas Biologicals Catalog #** FP-0500-A
- Penicillin-Streptomycin (10,000 U/mL) **Thermo Fisher Scientific Catalog #**15140122
- Neon™ Transfection System **Thermo Fisher Scientific Catalog #**MPK5000
- Chloroform **Fisher Scientific Catalog #**C298-4
- Neon™ Transfection System 100 µL Kit **Thermo Fisher Catalog #**MPK10096
- pcDNA3.1( )-OTI-TCRA **addgene Catalog #**131035
- pcDNA3.1( )-OTI-TCRB **addgene Catalog #**131036
- Cd8a (NM_001081110) Mouse Tagged ORF Clone **OriGene Catalog #**MR227539
- Cd8b1 (NM_009858) Mouse Tagged ORF Clone **OriGene Catalog #**MR225204
- CELL CULTURE MICROPLATE 96 WELL PS F-BOTTOM (CHIMNEY WELL) WHITE CELLSTAR® TC LID WITH CONDENS **greiner bio-one Catalog #**655083

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Protocol materials

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T Cell Activation Bioassay (NFAT) **Promega Catalog #**J1621

SpectraMax i3 Multi-Mode Microplate Detection Platform **Molecular Devices Catalog #**i3x

Before start

Make sure you have some familiarity with

- Plasmid propagation, midi-prepping, restriction, and purification
- In vitro transcription and RNA handling
- Electroporation
- Basic cell cell culture maintenance

Preparation of electroporation material

1 Order, clone, and midi-prep all the TCR and mouse CD8 plasmids:

pcDNA3.1( )-OTI-TCRA **addgene Catalog #**131035

pcDNA3.1( )-OTI-TCRB **addgene Catalog #**131036

Cd8a (NM_001081110) Mouse Tagged ORF Clone **OriGene Catalog #**MR227539

Cd8b1 (NM_009858) Mouse Tagged ORF Clone **OriGene Catalog #**MR225204

and make sure they are of good quality for further applications.

2 Linearize plasmids using the corresponding enzymes right at the end of their inserts

Preferred enzyme for the OT-I plasmids is **EcoRI**:

EcoRI-HF - 10,000 units **New England Biolabs Catalog #**R3101S

and the preferred enzyme for the mouse CD8s is **NotI**:

NotI-HF - 2,500 units **New England Biolabs Catalog #**R3189L

We recommend the following restriction reaction:

- 50 µg of plasmid DNA
- 25 µL of the corresponding restriction enzyme
- 25 µL of the CutSmart Buffer (10X)
- Top the reaction with nuclease-free water to 250 µL

Incubate the reaction at 37 °C for at least 01:00:00 .

> Note
>
> It is very important to fully linearize the plasmid to prevent potential off-running mRNAs. Based on the incubation time, the amount of enzyme can be reduced but the linearization should always be quality-checked via running the product on agarose gel when in doubt.

3 Extract the linearized DNA via the standard phenol:chloroform extraction protocol:

1. Add 250 µL of nuclease-free water so that the final volume for the restriction reaction is 500 µL
2. Add 500 µL of phenol:chloroform and vortex well
3. Spin at 14000 rpm, 4°C, 00:05:00
4. Transfer the (top) aqueous layer to a new tube, add 500 µL chloroform, and vortex well
5. Spin at 14000 rpm, 4°C, 00:05:00
6. Transfer the (top) aqueous layer to a new tube, add 1000 µL absolute EtOH, and mix well by inverting the tube a few times
7. Keep the sample at -20 °C for at least 00:30:00
8. Spin at 14000 rpm, 4°C, 00:30:00
9. Discard the supernatant without losing the pellet and add 500 µL 70% EtOH to wash the pellet
10. Spin at 14000 rpm, 4°C, 00:10:00
11. Discard the supernatant, remove all the residual alcohol, and resuspend the pellet in 50 µL nuclease-free water
12. Quality check the final DNA solution and estimate the concentration via Nanodrop.

4 In vitro transcribe mRNA using the linearized templates using NEB's **mRNA synthesis** and LiCl isolation protocols.

> Note
>
> We recommend starting with 10 µg of linearized template for each product and scaling the NEB's recommended reaction by 10X. Our preferred final elution volume is 250 µL, which should yield **1.5 - 2 ug/uL** mRNA.

Store the IVT'ed mRNA at -80 °C for future use.

Culturing and expanding effector and target cells (Day -3)

5 Thaw the Jurkat-NFAT cells that come with the T cell bioactivity kit:
T Cell Activation Bioassay (NFAT) **Promega Catalog #**J1621

and culture them by seeding 5 million cells in 50 mL of Jurkat media within a T75 flask for at least 3 days or until they reach a density of **1.5 million cells per mL.**

Note

Jurkat media:

- 500 mL of **Corning™ RPMI 1640 Medium (Mod.) 1X with L-Glutamine**
- 50 mL of **Fetal Plus®**
- 5 mL of **Penicillin-Streptomycin (10,000 U/mL)**

6 Thaw and start culturing MC38 cells. Seeding ~2 million cells in a T75 flask and culturing them for at least three days should yield enough cells for the co-culture.

Note

We have been using MC38 cells as our effectors but the choice of cell line is up to the experimenter. Ideally, the cell line doesn't present the SIINFEKL peptide on its own (without pulsing) so that we can use it as a negative control when co-cultured with the OT-I TCR. This protocol assumes, the cell line is of adherent nature so any suspension cell line could require some customization.

Co-culture setup (Day 0)

7 Electroporate Jurkats with mouse CD8 and OT-I subunits

7.1 Fill 4 wells of a 6-well culture plate with 6 mL of warm Jurkat media. We will be using this plate as our recovery plate after the electroporation.

7.2
1. Collect 20 million Jurkats
2. Spin them down at 350 x g, 4°C, 00:05:00
3. Re-suspend them in 25 mL of PBS (first wash)
4. Spin them down at 350 x g, 4°C, 00:05:00
5. Re-suspend them in 25 mL of PBS (second wash)

6. Re-suspend them in 900 µL of R buffer

7.3 Add

1. 25 µL of mouse CD8A mRNA (~ 40 µg)
2. 25 µL of mouse CD8B mRNA (~ 40 µg)
3. 25 µL of mouse OT-I alpha mRNA (~ 40 µg)
4. 25 µL of mouse OT-I beta mRNA (~ 40 µg)

onto the 900 µL Jurkat cell suspension in R buffer and make sure you mix them well

7.4 Using Neon's the 100 uL tips, electroporate cells at **1350 V 10 ms 3 pulse** setting. Use one tip for one reaction. Change the E2 solution every 6 electroporation reactions. Recover at most two reactions within each 6-well-plate well. Electroporate at least 8 reactions, which should yield 16 million Jurkat cells.

Equipment

new equipment	NAME
Thermo Fisher Scientific Neon™ Transfection System	BRAND
MPK5000S	SKU

7.5 Let the electroporated Jurkat cells recover for at least 12 hours.

8 Seed MC38s into two solid-bottom white plates through 2-fold dilutions:

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	R1	R1	R1	R1	R1	R1	R1	R1	-	-
C	-	-	R2	R2	R2	R2	R2	R2	R2	R2	-	-
D	-	-	R3	R3	R3	R3	R3	R3	R3	R3	-	-
E	-	-	R4	R4	R4	R4	R4	R4	R4	R4	-	-
F	-	-	R5	R5	R5	R5	R5	R5	R5	R5	-	-
G	-	-	R6	R6	R6	R6	R6	R6	R6	R6	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

MC38B	100K	50K	25K	12.5K	6.3K	3.2K	1.6K	0.8K	

Plate set up for the target cells. Have at most 6 replicates (row-wise) and prepare 2-fold serial dilutions for each replicate. We will assume that the cells will replicate once overnight so the numbers will double on the day of the co-culture.

Note

Although the final volume doesn't matter that much since we will be aspirating the media before setting the co-culture, when in doubt you can go with 60 µL of media per well. To have 100K cells in 60 µL of media, the serial dilution should start roughly at 3.3 million cells per mL concentration and we will be needing roughly 1.5 million cells per plate.

8.1 Let the targets cells attach and settle down for at least 12 hours.

Assay luciferase activity (Day 1)

9 Replenish Jurkats with fresh media:

1. Collect and combine all electroporated cells into a single 50-mL falcon tube
2. Spin 350 x g, 4°C, 00:05:00
3. Discard the supernatant
4. Re-suspend in 10 mL of fresh Jurkat media (~ 1.3 million cells per mL)
5. Split the cell suspension into two (5 mL each) 15-mL falcon tubes
6. Label and pulse one of the tubes with the **SIINFEKL** peptide at [M] 10 micromolar (µM) (9.63 ug/mL)

10 Aspirate the media from the MC38-seeded plates

11 Add 75 µL (~100K) of the electroporated Jurkats onto each well. Label the plates as **pulsed** or **unpulsed** accordingly.

Note

To reduce the chances of cross-contamination, always start by setting up the co-culture for the unpulsed condition.

This should give us the following plate setup:

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	R1	R1	R1	R1	R1	R1	R1	R1	-	-
C	-	-	R2	R2	R2	R2	R2	R2	R2	R2	-	-
D	-	-	R3	R3	R3	R3	R3	R3	R3	R3	-	-
E	-	-	R4	R4	R4	R4	R4	R4	R4	R4	-	-
F	-	-	R5	R5	R5	R5	R5	R5	R5	R5	-	-
G	-	-	R6	R6	R6	R6	R6	R6	R6	R6	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

MC38B	200K	100K	50K	25K	12.5K	6.3K	3.2K	1.6K	
Jurkat	100K	100K	100K	100K	100K	100K	100K	100K	

Plate setup for the coculture condition. Jurkat concentration is kept fixed but the target cells are titrated down from 2:1 target:effector ratio using 2-fold serial dilution.

12 Co-culture for at least 06:00:00 .

13 Take the plates and the luciferase substrates out and let them equilibrate at Room temperature for 00:10:00 .

14 Add 75 µL of the luciferase reagent onto each well and let the reactions run for at 00:10:00 .

15 Measure the luciferase activity using a standard plate reader with luminescence reading capability

SpectraMax i3 Multi-Mode Microplate Detection Platform **Molecular Devices Catalog #**i3x

Dataset

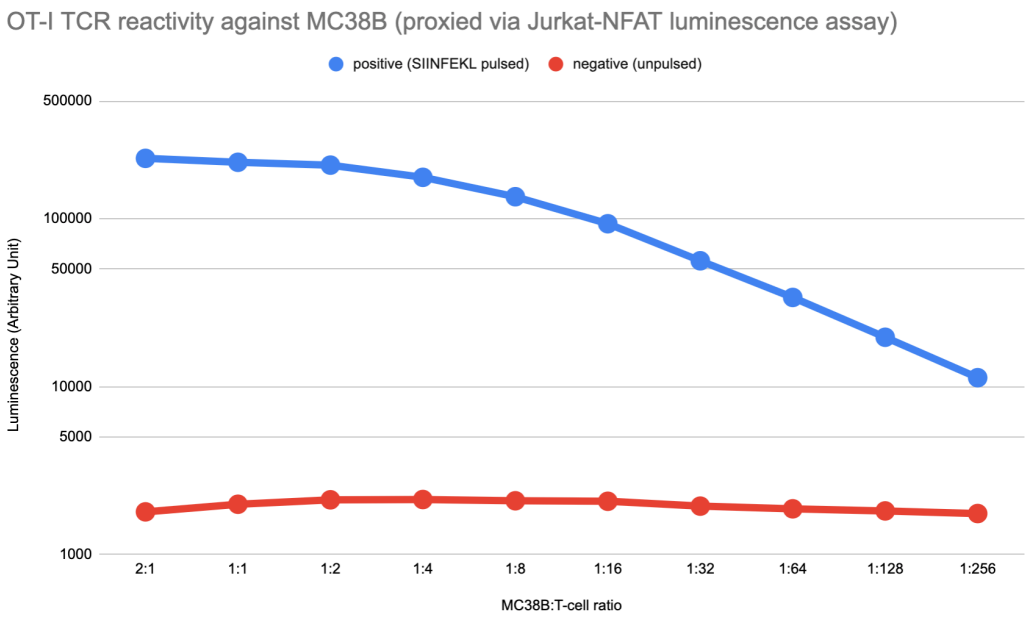
NAME

OT-I TCR reactivity against SIINFEKL-pulsed or -unpulsed MC38s

LINK

https://docs.google.com/spreadsheets/d/1exKy0eE89bW9RymnPyf7ww-TIs1-V3EMyZ224h-xs58/edit?usp=sharing

Expected result



Median luminescence at different target:effector ratios across pulsed (positive) and unpulsed (negative) control samples. Each TCR that will be tested will produce a reactivity metric relative to these two controls.