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

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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

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

STEPS MATERIALS

NAME	CATALOG #	VENDOR

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pcDNA3.1( )-OTI-TCRA	131035	addgene
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T Cell Activation Bioassay (NFAT)	J1621	Promega
SpectraMax i3 Multi-Mode Microplate Detection Platform	i3x	Molecular Devices

BEFORE STARTING

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
by addgene
Catalog #: 131035

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

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

Cd8b1 (NM_009858) Mouse Tagged ORF Clone
by 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
by New England Biolabs
Catalog #: R3101S

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

NotI-HF - 2,500 units
by 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** .

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.

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)
by 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.**

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.

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.

Thermo Fisher Scientific Neon™ Transfection System	MPK5000S

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.

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 **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.

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** .

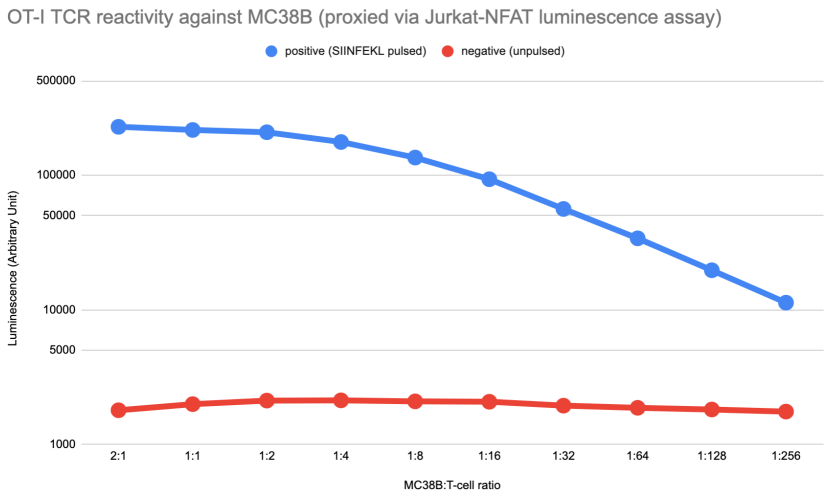
13 Take the plates and the luciferase substrates out and let them equilibrate at **Room temperature** for **00:10:00** .

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
by Molecular Devices
Catalog #: i3x

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



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.