Cambridge Institute for Medical Research

INFECTION, IMMUNITY
IMMUNOPHENOTYPING

# STANDARD OPERATING PROCEDURE
# SANGER SCREEN

## Plate Stimulation Cytotoxicity Screen

## PRINCIPLE OF PROCEDURE

CD8+ T cells kill target cells through the polarised release of cytotoxic granules which initiates apoptosis and the subsequent release of LDH. Stimulation of effector T cells is achieved in this case, through culture with plated αCD3e and αCD28 antibodies, resulting in the expansion of the desired CTL population. FACs analysis ascertains the ratio of CD4+:CD8+ cells post stimulation, allowing identification of T subset dominance, while a killing assay identifies the cytotoxic potential of the T cell sample through the measurement of LDH release from successfully killed target cells. This is useful as a screening test in order to provide high-throughput analysis of mutant mouse lines in order to identify mutations with a possible link to T cell cytotoxicity.

Lymphocytes are isolated from the splenic tissues of mutant mouse lines and left to stimulate for 48 hours, after which the cells are split for maintenance. Cells are stained for FACs analysis with anti-CD-8a conjugated to APC and anti-CD4a conjugated to PE. P815 cells are an immortalised cell line and are used as target cells in the killing assay. This protocol has an 8 day cycle, with killing assays performed on day seven, post stimulation.

## SPECIMEN REQUIREMENTS

This requires a whole spleen from each mutant mouse line. The assay requires ~10,000 P815/well, depending on the number of available CTL and a starting effector to target ratio of 10:1. A normal spleen will yield around 25,000 responders.

### Equipment

- Centrifuges
- Class II safety cabinet
- Discard container
- Eppendorfs
- FACS Calibur
- Gilson pipettes

- Haemocytometer
- Pipette aid
- Tube racks
- Universal rack

## Consumables

- Sanger Spleen (s)
- 70 µM cell strainers (BD Falcon, Order number:352350, Company: SLS)
- 50ml Falcon tubes (Order number: 352070, Company: SLS)
- 15ml conical Falcon Tubes (Order number: 734-0450, Company: VWR)
- 2 ml syringes (BD Plastipak)
- 96 well plates, round bottom (BD Falcon)
- 96 well plates, flat bottom polystyrene lidless (BD Falcon)
- 6 well plates (BD Flacon, Item code:353046)
- 3ml graduated Pasteur pipettes (sterile) IND (Order number: E1414-0311, Company: Starlab)
- BD Falcon 25cm2 50ml Tissue Culture Flask Canted Neck with Vented Cap (Order number: 353108, Company: SLS).
- TC Falcon 75 cm2 flasks (Order number:353136, Company: VWR)
- 5ml sterile serological pipette (Order number: 86.1253.001, Company: Sarstedt)
- 10ml sterile serological pipette (Order number:86.1254.001,Company : Sarstedt)
- 25ml sterile serological pipet (Order number: 734-0343 Company : VWR)
- Sterile Gilson pipette tips (blue and yellow)
- 50ml Corning Costar reagent reservoirs (Order number: PMP-331-010C)
- 5ml BD Falcon polystyrene Round-Bottom FACS tubes (Ref: 352058, Company :)
- Vacuum Filter Unit Stericup Sterile 500Ml Funnel 500Ml Receiver 0.22 Micron Pore Size Millipore (Order number: SCGPU05RE, Company : Fisher Scientific)
- Trigene Spray
- 70% Ethanol
- Virkon Tablets
- Autoclave Tape
- Medium gloves 10x100 Supreno Microflex (order number: SU-INT-M, Company : Starlab)

## Reagents

- B-mercaptoethanol (GIBCO Invitrogen: tissue culture fridge or frozen-stock vials in tissue culture fridge)
- Purified Hamster Anti-Mouse CD3e Clone: 145-2c11
- Anti-Mouse CD28 Clone: 37.51
- APC anti mouse CD8a Clone5-6.7 (Cat 100712 Biolegend)
- PE anti-mouse CD4 Clone RM4-5 (Cat 100511 Biolegend)
- Heat inactivated FCS (Hyclone laboratories: frozen 50ml aliquots in tissue culture freezer)
- 2mM L-Glutamine (GIBCO Invitrogen: frozen 5ml aliquots in pips freezer)
- PBS
- Penicillin/Streptomycin (GIBCO Invitrogen: tissue culture fridge stock)

- RPMI - Pheno Red
- RPMI-1640 medium with glutamine (GIBCO Invitrogen: cold room)
- 1mM Sodium pyruvate (GIBCO Invitrogen: tissue culture fridge stock)
- Trypan blue (Sigma)

## Media Recipes

### T cell Media

- 500ml RPMI
- 500ul B-ME
- 5ml NaPyr
- 5ml pen/strep
- 5ml L-glut
- 50ml FCS
- 100µl IL-2

### P815 cell media

- 500ml DMEM
- 10% heat inactivated FCS (50 ml)

### Killing Assay media

- 500 ml RPMI-pheno red
- 10 ml FCS
- 5 ml pen/strep

### FACS Buffer

- 500ml PBS
- 5ml FCS

### FACS Master Mix staining solution

- FACS buffer
- 1:200 anti-CD8a conjugated to APC
- 1:200 anti-CD4a conjugated to PE

## METHOD

### *Plate setup*

1. Use anti-murine CD3ϵ (1mg/ml) and anti-murine CD28 (1mg/ml).
2. Final concentration: 1µg/ml αCD3e, 2µg/ml αCD28 (per ml dPBS add 1µl αCD3e and 2µl αCD28).
3. Coat one well of a 6 well plate per sample (1701µl dPBS + 1.7ul αCD3e + 3.4µl αCD28).
4. Incubate for 1 hour at 37˚C in the incubator.
5. After that point the plate can be stored in the fridge until use.

### *Prep Samples*

1. Make single cell suspension from spleens using cell strainers and T cell media + IL-2.
2. Spin cells at 193 x g for 10 minutes.

### *Cell Count and Plating up*

1. 13.6 million cells in 8ml/well
2. Dilute/re-suspend cell pellet appropriately.
3. Remove dPBS and plate samples.

## *48 hours post stimulation*

1. Wash cells by centrifuging at 193 x g for 8 minutes.
2. Re-suspend pellet in 8ml media and re-plate onto new well.

## *FACS analysis*

1. Gently re-suspend cells and take at least 500µl into round bottom polystyrene FACS tubes.
2. Top up with FACS buffer solution and centrifuge at 433 x g for 4 minutes.
3. Make master mix: 100µl/sample. FACS buffer solution + 1:200 (Anti-CD8a conjugated to APC + Anti-CD4a conjugated to PE).
4. Discard supernatant and re-suspend cell pellet in 100ml master mix.
5. Incubate in the dark for 7 minutes at room temperature.
6. Top up with FACS buffer solution and centrifuge at 433 x g for 4 minutes.
7. Discard supernatant and re-suspend cell pellet in 200µl FACS buffer solution.
8. Analyse on FACS machine (PE signal through FL-2 and APC signal through FL-4).

## *Killing Assay*

*(P815 need to be split on the day prior to testing)*

1. Gently re-suspend cells and count.
2. Take required number of cells, top up with Killing assay media and centrifuge at 433 x g for 5 minutes at room temperature.
3. Remove last drops of supernatant using a p200 pipette and re-suspend cell pellet in killing assay media, at a starting E:T ratio of 10:1.
4. Require 4.5ml of target p815 cells/killing assay plate.
5. Require 1.6ml/sample of effector CTLs.
6. Add purified hamster anti-mouse CD3e to P815 target cells (1:2000).

*The killing assay plate is setup as in Figure 1, page 8*

1. Half fill a reagent reservoir with killing assay medium and put 100 µl of killing assay medium in rows B-F and row H in columns 1-12 using the multichannel pipette.

2. Plate control CTL directly from the 15 ml tube into the 96 well round bottomed plate as shown with 200 µl per well in row A 1-6 using a P200 pipette and experiment CTL in row A 7-12.

3. Pour the P815 target cells into a reagent reservoir, as well as put killing assay medium in a separate reagent reservoir.

4. Make a 1:1 dilution series by setting the multichannel to 100 µl and transferring 100 µl from row A to row B, then row B to row C etc, mixing before each transfer by pipetting up and down 4-5 times.

   *NB check the multichannel – sometimes not every channel works properly after each transfer!*

5. Add 100µl P815 (10,000 cells per well) to each well in columns 4-6 and 10-12 from the reagent reservoir and medium from the reagent reservoir to columns 1-3 and 7-9 so that each well has a final volume of 200 µl

6. For controls: rows H1-6 add 100µl P815; rows H7-12 add another 100 µl medium only

7. Briefly spin plate at 193 x g for less than 1 min to encourage cells to settle, then incubate 96 well plate at 37°C for 3 hours.

8. After 2 hours and 15 minutes add 20 µl lysis buffer to H4-9, mixing thoroughly.

9. *NB Make sure the lysis buffer has been bought to RT and vortexed prior to use.*

10. *NB Thaw assay buffer from the kit approximately 30 min before you need it.* The white bottle is the one which you need to make up the Substrate mix in the small vials – 12 ml of assay buffer is added to the Substrate mix vial for reconstitution.

11. Briefly spin the 96 well plate at 193 x g for less than 1 min to pellet cells

12. Tilt the plate and, using the multichannel pipette, transfer 50 µl of supernatant to a flat bottom polystyrene 96 well plate (transferring supernatant so that the two plates match i.e. A1 to A1, B1 to B1 etc).

13. Add 50µl substrate (made up with 12ml solution per vial as above) and leave to develop for 30 minutes in the dark.

14. Add 50µl stop solution to each well after 3 hours, pop bubbles and then read at **490 nm** using a spectrophotometer.

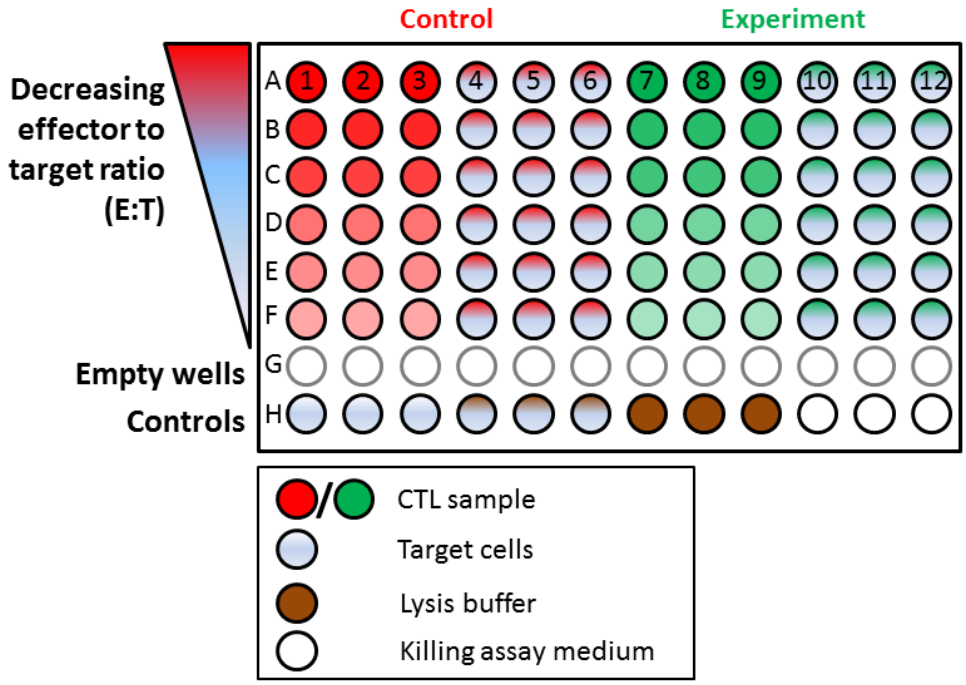


**Figure 1: Setting up a killing assay plate**

## INTERNAL QUALITY CONTROL

1) Record any equipment problems on the worksheets and the troubleshooting log.
2) Refer any problems to an appropriate senior member of staff.
3) Check that the controls are within their specified range and record the normal CD8:CD4 ratio in the FACs file (with compensation noted).
4) Refer any failed QC to an appropriate senior member of staff.
5) All actions and outcomes should be recorded.
6) All results interpreted.
7) All results entered into the Sanger pipeline database as per Sanger paperwork SOP.

## LIMITATIONS

The FACs analysis is performed on day 8 post stimulation and cells counted before use in experiments. The killing Assay is also performed on day 8 post stimulation. The staining intensity may alter in the samples if left too long before FACs analysis, even if fixed. Once stained, samples should not be left longer than overnight before acquiring results if possible.

## NOTES

Routinely, the first time a mutant line is screened using this protocol, a lysate is not required. A lysate should be made on any repeat tests, where the results were abnormal on initial analysis.

## HEALTH AND SAFETY INFORMATION

| **PROCEDURE** |
|---|
| Generation of Mouse CTL, Analysis by FACs, Killing Assay. |
| **Procedure Details** |
| Screening mutant mouse lines for CTL cytotoxicity through FACs and analysis of LDH release. |

## PROCEDURE ASSESSMENT

| **Substances Used** | **Type of Hazard** | **HAZARD Category** | | | **EXP.POT** | | | **RISK NO** |
|---|---|---|---|---|---|---|---|---|
| | | H | M | L | H | M | L | |
| RPMI 1640 contains Gentamycin | Harmful (R42/43) | | X | | | | X | 1 |
| Fetal calf serum | - | | | X | | | X | 1 |
| IL2 | - | | | X | | | X | 1 |
| Anti CD4 and Anti CD8 | - | | | X | | | X | 1 |
| FACS Flow | - | | | X | | X | | 1 |
| 1% Virkon | Irritant (R36/38) | | X | | | | X | 1 |
| Mouse spleen tissue | Biohazard (ACDP Category 2) | | | X | | | X | 1 |

| | |
|---|---|
| PPE | GLP : Nitrile Gloves/ Lab. Coat / Class 11 Cabinet |
| Spillage/Disposal | Biohazard's: Use 1% Virkon or Virkon powder and dispose of in autoclave bag<br><br>Chemicals: Dilute as much as possible with water, wash away down the sink or mop up with paper towels or unisafe and place for incineration. Larger amounts can be flushed down sink with plenty of running water |
| Accidents | Eyes: Irrigate immediately with water for at least 10 minutes<br><br>Skin: Wash site immediately with water<br><br>Mouth: Wash out mouth immediately with water<br><br>Lungs: Remove to fresh air immediately |

| | |
|---|---|
| Additional hazards | Unbalanced centrifuge: Make sure buckets are balanced before use.<br><br>Electrical: Electrical safety checks are made annually by the Work's Department.<br><br>In case of an electrical emergency, push the red button situated on the wall. This will cut off the current to the room.<br><br>Splashing: Use cabinet or splash guards if necessary. Be careful when emptying supernatants down the sink.<br><br>Twisting: Move chair to turn rather than twisting body. Stand on step stool if necessary to turn machines on and off or reach equipment.<br><br>Observe Manual Handling techniques when lifting 20L Facsflow containers. |
| | **Discontinue working if you feel ill or unstable. Always inform the first aid officer or seek medical advice after an accident.** |

Refer to COSHH file, Health and Safety Policy and General Risk assessment for more information