

*Staining tissue sections with MHC multimers has been previously demonstrated, but may require optimisation. This protocol is designed to visualize Pentamer staining with Texas Red and anti-CD8 staining with FITC. However, suitable alternative Fluorescent molecules may be substituted to adapt to the wavelengths available in your Fluorescent microscope.*

*This protocol has been optimized with murine tissue using the reagents suggested in the 'Materials Required' section. However, suitable alternatives should also work.*

### ***Materials Required***

Tissue upon which staining is to be performed (e.g. murine spleen)

O.C.T. Freezing compound (e.g. Tissue-Tek<sup>®</sup>; Sakura Finetek)

Pro5<sup>®</sup> Recombinant MHC Pentamer conjugated to R-PE. **Ensure that the stock Pentamer is stored consistently at 4° C in the dark, with the lid tightly closed.**

FITC\*-labeled anti-CD8 antibody

Rabbit anti-PE antibody (e.g. AbD Serotec #7374-2304)

Texas Red\* conjugated goat anti-rabbit IgG antibody (e.g. Invitrogen #T2767)

Glucose oxidase (e.g. Sigma #49180) stock at 10,000 U/ml (in 100mM sodium acetate, 40mM potassium phosphate, 250mM potassium chloride, pH 4.5). Store as aliquots at -20°C.

100x Glucose oxidase buffer (100mM Sodium Azide, 1M β-D-Glucose). Store as aliquots at -20°C.

Phosphate buffered saline (PBS)

PBS-buffered 2% formaldehyde

Mouse serum (e.g. AbD Serotec #C11SA)

Fetal bovine serum (Heat-inactivated; e.g. Invitrogen #26140)

Goat serum (e.g. AbD Serotec #C07SA)

Tissue mounting medium (e.g. Vectashield<sup>®</sup> Mounting medium with DAPI; Vector Laboratories #H-1200)

Silica gel desiccant

### ***Equipment Required***

Mold for freezing tissue in

Microscope slides

Coverslips

Cryostat

37°C water bath or incubator

Humidified chamber

\* Or suitable alternative Fluorescent molecule - please contact us if you need assistance with determining suitable Fluorochromes.

### *Preparation of tissue sections*

1. **Isolate tissue/organ from animal** and, if desired, keep in PBS on ice, otherwise proceed immediately to step 2.
2. **Embed tissue/organ into O.C.T. Freezing compound and place on dry ice.** Wait until O.C.T. is frozen. If desired, samples may be stored at -80°C prior to sectioning, otherwise proceed to step 3.
3. **Cut 10µm tissue sections using a Cryostat and mount onto microscope slides.** Store slides in a desiccated box at -20°C until required for staining.

### *Staining protocol*

**n.b. Keep samples shielded from light as far as possible.**

1. **Equilibrate slides in a desiccated box to 4°C for at least 30 minutes.**
2. **Remove box to room temperature and equilibrate for 15 minutes.**
3. **If desired, circle each tissue section with hydrophobic marker.** This allows you to stain sections on the bench (in a humidified chamber) with as little as 50 µl volume per section.
4. **Rehydrate tissue with 3 washes (2 minutes each) with PBS.**
5. **Meanwhile, add 500 ml Glucose Oxidase buffer and 5 ml Glucose Oxidase (1 U/ml final) to 50 ml PBS in a Koplín jar.**
6. **Place slides in the Koplín jar and incubate at 37°C for 30 minutes.** This step quenches any endogenous peroxidase activity in the tissue due to hydrogen peroxide generation as glucose oxidase oxidizes the glucose.
7. **Wash sections with 3 washes (5 minutes each) with PBS.**
8. **Block sections with 10% mouse serum / 5% FBS at room temperature for 15 minutes.**
9. **Aspirate serum and apply 50 ml R-PE-labeled Pentamer (5 tests) per section. Incubate at 4°C overnight in a humidified box.**
10. **Wash sections with 3 washes (5 minutes each) with PBS.**
11. **Fix sections in PBS-buffered 2% formaldehyde at room temperature for 30 minutes.**
12. **Wash sections with 3 washes (5 minutes each) with PBS.**
13. **Apply rabbit anti-R-PE (1:1000 dilution in 2% goat serum in PBS). Incubate at 4°C for 3 hours.**
14. **Wash sections with 3 washes (5 minutes each) with PBS.**
15. **Apply Texas Red conjugated goat anti-rabbit (1:100 in 2% goat serum in PBS) plus FITC-conjugated anti-CD8 antibodies. Incubate at 4°C for 3 hours.**
16. **Wash sections with 3 washes (5 minutes each) with PBS.**
17. **Mount slides and store in the refrigerator in the dark until analysis.**