Optimized Multicolour Immunofluorescence Panel for Cattle T Cell

Phenotyping by an 8-Colour, 10-Parameter Panel

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Purpose and appropriate sample types:

This multiplex staining panel was developed to differentiate cattle T cells into conventional (CD4 and CD8) and unconventional ($\gamma\delta$ -TCR) subsets as well as their stage of differentiation and activation. The combination of CD45RO and CD62L allows the identification of naïve ($T_{Naïve}$), central memory (T_{CM}), effector memory (T_{EM}) and terminal effector (T_{TE}) T cells. Activated cattle T cells (T_{AV}) can be identified by the cell surface expression of CD25. This panel was developed using cryopreserved cattle peripheral blood mononuclear cells (PBMCs) and tested on fresh as well as stimulated PBMCs. Therefore, this 8-colour, 10-parameter flow cytometry panel simultaneously identifies cattle $T_{Naïve}$, T_{AV} , T_{CM} , T_{EM} , T_{TE} and $\gamma\delta$ -TCR cells. This panel will improve our ability to examine T cell response to pathogens and vaccines in cattle including the potential to identify previously undescribed subpopulations. Furthermore, this panel can be readily optimised for other bovid species as many of these reagents are likely to cross react.

Key terms:

26 Flow cytometry; cattle PBMC; T cells; naïve T cells; effector memory T cells; central
27 memory T cells; activated T cells; $\gamma\delta$ T cells; T cell subsets

28

29 **Background:**

30 Robust T cell responses are critical in the response to pathogen infection both for
31 clearance and the formation of strong and broad memory responses (1). Cattle, like
32 several other species, have a much higher proportion of $\gamma\delta$ T cells compared to CD4
33 and CD8 (2–4). Consequently, it is important to study the entire T cell compartment
34 simultaneously to fully characterise how immune protection arises and persists.
35 Furthermore, as the research climate focusses on One Health approaches, the ability
36 to study the immune response at high resolution in species that underpin global
37 food security is essential.

38

39 Common to several non-model species, the first mAbs to study CD molecules on
40 cattle T cells were derived from mouse immunizations with whole cattle PBMC
41 populations or PBMC lysates. Antibodies were characterized in three international
42 workshops on ruminant antigens (2,5,6). Together with identification of cross-
43 reactive mAbs, this allowed the establishment of a basic toolbox to study cattle T
44 cells and various subsets within them (7). However, several limitations still exist for
45 the establishment of polychromatic flow cytometry staining panels. For example,
46 many of the current 371 human CD molecules do not have an antibody that cross
47 react with cattle. Another major limitation is the lack of useful mAbs that are
48 labelled to a wider range of fluorochromes. This makes it difficult to expand panels

49 beyond the three most common fluorochromes FITC (or AF488), PE and APC (or
50 AF647). By conjugating existing T cell markers in-house we were able to develop a
51 cattle T cell panel that utilises eight colours excluding PE and APC-conjugated
52 antibodies. This allows the addition of specific antibodies, such as for cytokines or
53 transcription factors, that maximises the broader utility of this panel for individual
54 research needs. Additionally, if more of the available mAbs would be conjugated to
55 fluorochromes that are excited by the violet laser, the panel can be further expanded.

56

57 This OMIP identifies all three main cattle T cell subsets (CD4, CD8 and $\gamma\delta$), as well
58 as their subsets that are activated (T_{AV}) or in the distinct differentiation states of
59 naïve ($T_{Naïve}$), central memory (T_{CM}), effector memory (T_{EM}) and terminal effector
60 (T_{TE}). The gating strategy we used initially identifies the two $\alpha\beta$ T cell subsets CD4
61 (mAb clones CC8/CC30) and CD8 (mAb clone CC63) as well as the $\gamma\delta$ T cells (mAb
62 clone GB21A) (2,8) (Fig. 1). Like in swine and chickens, $\gamma\delta$ T cells constitute a major
63 T cell subset in cattle blood and can comprise more than 50% of circulating T cells
64 (3,9). To identify activated T cells, CD25 (mAb clone IL-A111) can be used (8,10–12),
65 whereas the memory state of the cells can be defined using the CD45RO (mAb clone
66 IL-A116) and CD62L (mAb clone CC32) cell surface markers (6,8,13–15) (Fig. 1).

67 Using this gating strategy, the following known subsets can be identified for the
68 helper T cells, $T_{Naïve}$ ($CD3^+\gamma\delta^-TCR^+CD4^+CD25^-CD45RO^-CD62L^+$), T_{CM} ($CD3^+\gamma\delta^-TCR^+$
69 $CD4^+CD25^-CD45RO^+CD62L^+$), T_{EM} ($CD3^+\gamma\delta^-TCR^+CD4^+CD25^-CD45RO^+CD62L^-$), T_{TE}
70 ($CD3^+\gamma\delta^-TCR^+CD4^+CD25^-CD45RO^-CD62L^-$) and T_{AV} ($CD3^+\gamma\delta^-TCR^+CD4^+CD25^+$).

71 Similarly, the cytotoxic T cells can be separated into $T_{Naïve}$ ($CD3^+\gamma\delta^-TCR^+$

72 CD8 α ⁺CD25⁻CD45RO⁻CD62L⁺), T_{CM} (CD3⁺ $\gamma\delta$ -TCR-CD8 α ⁺CD25⁻CD45RO⁺CD62L⁺),
73 T_{EM} (CD3⁺ $\gamma\delta$ -TCR-CD8 α ⁺CD25⁻CD45RO⁺CD62L⁻), T_{TE} (CD3⁺ $\gamma\delta$ -TCR-CD8 α ⁺CD25⁻
74 CD45RO⁻CD62L⁻) and T_{AV} (CD3⁺ $\gamma\delta$ -TCR-CD8 α ⁺CD25⁺). Furthermore, $\gamma\delta$ T cells can
75 also be identified by (CD3⁺ $\gamma\delta$ -TCR⁺) (Fig. 1; Online Table 3). A major improvement
76 by this panel is the simultaneous analysis of all these T cell subsets in a single
77 sample, hence reducing the variation between replicates and the number of samples
78 needed per animal.

79

80 The panel was designed and optimised on a BD LSRFortessa and was tested on a BD
81 Aria IIIU. The BD Aria IIIU allows for sorting of the T cell subsets. Further
82 adaptations to the panel are enabled by having both the PE and APC channel empty,
83 for which many antibodies are commercially available. If more reagents become
84 available in the violet channel, they can easily be added to the panel with only minor
85 influence on compensation requirements.

86

87 In conclusion, this cattle T cell panel will advance the understanding of the cattle
88 immune response as it allows the measurement of all major T cell subsets and their
89 differentiation stage within a single sample.

90

91 **Similarity to published OMIPs:**

92 None to date.

93

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Figure 1 Gating strategy of the cattle T-cell panel into CD4⁺, CD8⁺ and γδ-TCR⁺ subsets. A) Sub-gating of the CD3⁺γδ-TCR⁺ subset with CD45RO and CD62L. B) Sub-gating of the CD3⁺ γδ-TCR-CD8α⁺ subset with CD25. C) Sub-gating of CD3⁺ γδ-TCR-CD8α⁺CD25⁺ subset with CD45RO and CD62L. D) Sub-gating of CD3⁺ γδ-TCR-CD4⁺ with CD25. E) Sub-gating of CD3⁺ γδ-TCR-CD4⁺CD25⁺ subset with CD45RO and CD62L. Sub-gating of the CD4 and CD8α with CD45RO and CD62L allows the identification of effector memory (T_{EM}), central memory (T_{CM}), terminal effector (T_{TE}) and naïve (T_{Naïve}) T cells. The CD25 identifies the activated T (T_{AV}) cells, that include the T regulatory cells.