CIP Assay guidelines

Following guidelines and assay recommendations are essentially derived from results obtained by the CIP proficiency panel program. For details, please refer to CIP publications or contact us.

Guideline for analyzing antigen-specific $CD8^+$ T cells with HLA-multimer staining

1. Establish a protocol in your lab and generate a SOP for the test that has to be strictly followed by every staff member who performs the assay.

2. Make sure that every new staff member who is introduced to the assay fully understands each step of the SOP, strictly follows the SOP and is capable to reproducibly perform the assay before being allowed to test material from clinical trials. You may test this by assaying a number of healthy donor-derived PBMC against known (viral) antigens. Thaw a number of PBMC vials (3-4) at one day and treat every vial as an individual sample. Repeat this at 3-4 different days. Compare the variation per day and between days.

3. Since some anti-CD8 mAbs interfere with multimer binding, we propose a list – non exhaustive- of anti-CD8 mAbs that have been used by panel participants:

- Anti-CD8-FITC, clone SK1 from BD cat n° 345772 / diluted 1:30
- Anti-CD8-PerCP, clone SK1 from BD cat n° 345774 / diluted 1:20
- Anti-CD8-APC, clone DK25 from Dako Cytomation cat n° C7227 / diluted 1:200
- Anti-CD8-PE-Cy7, clone SFCI21Thy2D3 from Beckman Coulter cat n° 737661/diluted 1:100
- Anti-CD8-FITC, clone RPA-T8 from BD cat n° 555366 diluted 1/10 or BioLegend 301006 diluted 1/10

Please, titrate the Ab for your own assay and cytometer.

4. Flow-cytometry analysis for 3 colour stainings.

Express the results as:

- A dot-plot showing Forward scatter/Side scatter and the lymphocyte gate used (region R1)
- For CD3/CD8/multimer staining: a dot-plot showing CD3 vs. CD8 or SSC, or a histogram-plot showing CD3 in region R1 and a new gate on $CD3^+$ cells (region R2).
- A dot-plot showing CD8 vs. multimer among the $CD3^+$ lymphocyte gate (regions R1 + R2)

Calculate the % of $CD8^+$ $multimer^+$ cells among the $CD8^+$ population: give this % as a number with two decimals (0.00).

Crucial issues for assay performance

The lower the frequency of cells to be detected is, the more cells should be included in the analysis, for this reason:

5. Use **NOT LESS than 1 x 10^6 cells / staining** (for ex vivo staining).

6. Perform the acquisition on **ALL cells** present in the test-tube (this means until the tube is empty).

7. **Use a negative control staining** for setting analysis gates: FMO (= all Ab without multimer) or better, include a staining with a negative control HLA-multimer.

How to keep up the performance of your analyses

8. Check the performance of your cytometer regularly and audit the results

9. Perform intra-center validation of your test performance at least once a year. Here you can make repetitive use of donor PBMC samples with known T-cell reactivities.

10. Participate regularly in inter-laboratory testing projects in order to obtain an external validation of your test performance.

Guidelines for analyzing antigen-specific $CD8^+$ T cells with interferon-gamma ELISPOT assay

1. Establish a protocol in your lab and generate a SOP for the test that has to be strictly followed by every staff member who performs the assay.

2. Make sure that every new staff member who is introduced to the assay fully understands each step of the SOP, strictly follows the SOP and is capable to reproducibly perform the assay before being allowed to test material from clinical trials. You may test this by assaying a number of healthy donor-derived PBMC against known (viral) antigens. Thaw a number of PBMC vials (3-4) at one day and treat every vial as an individual sample. Repeat this at 3-4 different days. Compare the variation per day and between days.

3. Test each condition at least in triplicate. Note that it useful to have the medium control in six wells.

4. Use 4-5x10^5 cells per well for ex vivo ELISPOT.

5. Let the PBMC rest after thawing to increase the proportion of living cells seeded.

- Calculate the number of PBMC that are required for the test and add 25% extra to count for dead cells.
- Resuspend PBMC in culture medium and transfer the cells to an appropriately labeled tissue culture flask or tube.
- Incubate PBMC at 37° C in a CO_2 incubator several hours up to overnight.
- Wash the cells and count them before seeding them in the ELISPOT plate with antigen.

6. Use media that support ELISPOT assays in which no serum is used (e.g. X-vivo, Optmizer), as it is difficult to maintain a good stock of serum batches that support ELISPOT assays for longer periods.

7. Use an automated spot counter to obtain spot counts with optimal pre-settings. Audit the plate and outcomes.

8. Determine the number of antigen-specific spots by subtracting the number of background spots from the number of spots found after addition of the antigen.

9. Responses detected with ≤ 6 spots per 100,000 PBMC should be viewed with caution.

Crucial issues for assay performance

10. **Do not use allo-APC** because this can lead to enhanced background.

11. If you use serum in your test note that some sera do not support IFN-gamma production, while others may generate high background cytokine secretion. Make sure to select the best serum available.
As demonstrated in CIP panels, media without serum do lead to equivalent performance than media with serum in terms of cell viability and functionality.

12. The amount of antigen used in the assay can vary, good starting concentrations are: minimal CTL peptide 1 μg/ml and protein 10 μg/ml. Other antigen preparations can also be used (e.g. infected/transfected APC) and should be pretested for an optimal effector:stimulator ratio.

How to keep up the performance of your analyses

13. Perform intra-center validation of your test performance at least once a year. Here you can make repetitive use of donor PBMC samples with known T-cell reactivities.

14. Participate regularly in inter-laboratory testing projects in order to obtain an external validation of your test performance.