



Addendum B cell ELISPOT assay

Guidelines and troubleshooting for B cell ELISPOT analyses

Isolation and handling of human and non-human primate blood cells

Venous or arterial blood should be collected from humans or non-human primates, fasted for at least 6 hours and using heparin as anti-coagulant. After being drawn, blood is kept at room temperature for maximally 16 hours.

Peripheral blood mononuclear cells (PBMCs) are isolated from venous blood by density gradient centrifugation and washed twice in serum-free medium (RPMI-1640 + L-glutamin + penicillin/streptomycin).

Specimen collection from humans and non-human primates should be carried out in accordance with NCCLS document M29-T2. No known test method can offer complete assurance that human- or non-human primate-derived blood or tissue samples will not transmit infection. Therefore, all human and non-human primate specimens should be considered potentially infectious.

Directions for cell culture of human or non-human primate PBMCs

PBMCs are suspended in RPMI-1640 culture medium supplemented with 2 mM L-glutamine, penicillin/streptomycin and 10% fetal calf serum (R10 medium). When the frequency of recently *in vivo* activated B cells is determined, cells can directly be brought into the wells of the ELISPOT plate and incubated at 37°C.

For the enumeration of memory B cells, R10 medium is supplied with the B cell stimulatory reagent (provided with the kit) and the cells are incubated for 5 days under conditions as described in the manual. R10 is the medium of choice for the B cell ELISPOT assay. No more than 2×10^5 cells/well should be suspended in the ELISPOT plate.

Directions for washing of polystyrene-bottomed plates

- All washing must be performed with Wash buffer (PBS containing 0.05% Tween-20).
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into each well. Take care not to scratch the bottom of the well. After aspiration, fill the wells with at least 250 μ l of wash buffer and then aspirate the liquid. After washing, the wells of the plate are emptied by a firm shake-out action followed by tapping both sides of the plate on absorbent tissue.
- Alternatively, the Wash buffer may be put into a squirt bottle (use a squirt bottle with a wide spout). If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After washing, the wells of the plate are emptied by a firm shake-out action followed by tapping both sides of the plate on absorbent tissue.
- If using an automated washing device, the operating instructions should carefully be followed.

Directions for washing of PVDF membrane-bottomed plates

- All washing must be performed with Wash buffer (PBS containing 0.05% Tween-20).
- For effective washing of PVDF membranes, a squirt bottle with a wide spout has shown to produce the best results. The bottle should be used to thoroughly flush all wells of the plate with Wash buffer. While flushing, the wells are completely filled with Wash buffer and subsequently emptied by a firm 'shake-out' action. After washing, the plate is emptied by tapping both sides on absorbent tissue.
- Additional washing of the underside of the PVDF membrane is needed after the incubation steps with detection antibody and conjugate to further reduce background staining. To do so, remove the plastic underdrain of the plate and use the squirt bottle to flood the underside of the membrane with Wash buffer. After washing, the Wash buffer is removed by a gentle 'shake-out' action.

Recommended reagents

- Ficoll-Paque: Amersham biosciences (GE Healthcare) cat. no. 17-1440-02 (for isolation of PBMCs by density gradient centrifugation)
- RPMI 1640 medium: Invitrogen cat. no. 52400-025
- L-glutamine: Invitrogen cat. no. 35030-024
- Penicillin/Streptomycin: Invitrogen cat. no. 15140-122

Troubleshooting

No or low frequency of spots

- To obtain a single cell suspension, it is critical to resuspend the cells thoroughly before they are brought into the wells of the ELISPOT plate.
- Do not use human or non-human primate serum as growth supplement. Primate antibodies will interfere with spot formation.
- PBMCs isolated from blood and kept for more than 16 hours at room temperature may produce less spot forming cells.

Faintly stained spots

- PBS tablets should not be used for the preparation of the coating antibody or antigen solution. The filler in the tablets interferes with the coating process.
- The AEC stock solution can lose activity when it is exposed to light or prolonged stored at temperatures $\geq 0^{\circ}\text{C}$ (enzymatic staining procedure).

- For optimal coloring, the AEC substrate solution can be best applied to the wells at temperatures of 25-30°C (enzymatic staining procedure).
- The Activator I and II solutions can lose activity when they are exposed to air and/or light, are not properly stored or have been cross-contaminated (silver-staining procedure).

Artifactual spots and/or high background staining

- Just prior to spot counting, it is important to clean the underside of the polystyrene-bottomed wells with 70% ethanol and to remove dust particles by blowing 4-5 bar compressed air into the wells (dust particles can be the cause of spot-like structures).
- The reconstituted antibody solutions should not be used if there is an indication of bacterial growth or if the solutions have become turbid.
- Inadequate post-coating of the ELISPOT well or insufficient washing between the different incubation steps may be the cause of artifactual spots or high background staining.
- When the cells are pre-stimulated for 5 days with B cell stimulatory reagent, wash the cells thoroughly before they are transferred to the ELISPOT plate to avoid the carryover of antibodies released in the pre-incubation medium.
- Complete drying of the PVDF membranes (overnight at room temperature and in the dark) after the completion of the assay, is important to obtain optimal spot intensity and low background staining.

Other

- Do not stack the plates during incubation.
- Do not puncture the PVDF membrane by pipetting/washing procedures. The membrane is fragile and may easily be damaged.
- To identify the optimal cell concentration for spot formation, include a wide range of cell concentrations in the first experiment.
- Spots may become irregular and ambiguous when the ELISPOT plate is moved during incubation. Even minor vibrations caused by closing the door of the incubator can affect spot formation.
- During incubation with blocking solution, membrane-leakage occasionally occurs. This phenomenon, however, does not negatively affect assay results (PVDF membrane-bottomed plates only).

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