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Addendum
B cell ELISPOT assay

Guidelines and troubleshooting for B cell ELISPOT analyses

Specimen collection and handling

PBMC from human and non-human primate origin
Venous or arterial blood should be collected from humans or non-primates, preferably fasted for at least 6 hours and using heparin as anti-coagulant. After being drawn, blood is kept at room temperature for maximum 16 hours. Peripheral blood mononuclear cells (PBMCs) are isolated from venous blood by density gradient centrifugation (using e.g. Ficoll) and washed twice in serum-free medium. This involves two centrifugation/resuspension steps (8 minutes, 200 x g, RT).

PBMC can be used directly or frozen for later use in the B cell ELISPOT assay.

Spleen cells from mouse origin
Spleen cells are aseptically removed from rodents and collected in RPMI-1640 medium. A single cell suspension is prepared by gently teasing the spleen tissue through a sterile stainless steel or nylon screens into medium and then washed twice with serum-free medium. This involves two centrifugation/resuspension steps (8 minutes, 200 x g, RT).

If necessary, red blood cells can be lysed by a brief hypotonic stock in between the two wash steps, using 4.5 ml ice-cold sterile water for 15 seconds. Red blood cell lysis is stopped by adding 500 µl sterile 10x concentrated PBS (ice-cold).

Spleen cells can be used directly or frozen for later use in the B cell ELISPOT assay.

Cryopreserved cells
Both PBMC and spleen cells can be frozen using 10% dimethyl sulfoxide (DMSO) and 20% fetal calf serum (FCS) in culture medium. The recommended cell concentration is $\geq 2x10^7$ cells/ml for PBMC and $\geq 5x10^7$ cells/ml for spleen cells. Freeze the cells slowly by reducing the temperature at approximately 1 ºC per minute and store the cells at -80 ºC. For long-term storage the cells are thereafter transferred to -170 ºC.

The thawing procedure is stressful to frozen cells. Using proper techniques and working quickly ensures that a high proportion of the cells survive the procedure.

The expected recovery of PBMC shall be around 80% whereas the recovery of spleen cells can be as low as 25%. A low recovery percentage of cells does not interfere with the B cell ELISPOT assay. The cell viability is determined using trypan blue.

Rapidly thaw the cells by gently swirling the vial in a 37 ºC water bath until there is just a small bit of ice left. Dropwise add 1 volume of fresh cell culture medium containing 50% FSC. Subsequently add gently 10 volumes of culture medium containing 10% FSC for the first wash step. To remove DMSO in the thawing procedure the cells are washed twice. This involves two centrifugation/resuspension steps (8 minutes, 200 x g, RT) with fresh culture medium containing 10% FCS. Since cell dead may not be immediately visible after thawing, it is recommended to let the cells rest for 15 minutes to one hour at RT in between the wash steps.

NOTE: 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.

Recommended reagents

- Dimethyl sulfoxide: Sigma-Aldrich cat. no. D2650.
- Fetal calf serum: Life Technologies cat. no. 16000-044.
- Ficoll-Paque: GE Healthcare cat. no. 17-1440-02 (for isolation of PBMCs by density gradient centrifugation).
- RPMI-1640 medium: Life Technologies cat. no. 52400-025.
- L-glutamin: Life Technologies cat. no. 35030-024.
- Penicillin/Streptomycin: Life Technologies cat. no. 15140-122.
- Trypan Blue Solution: Sigma-Aldrich cat. no. T8154

The recommended serum-free culture medium is RPMI-1640 supplemented with 2 mM L-glutamin, 100 units/ml penicillin and 100 µg/ml streptomycin.

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 µl of wash buffer and then aspirate the liquid. Repeat this as many times as mentioned in the manual. After washing, the wells of the plate are emptied by a firm shake-out action followed by tapping the plate upside down 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, empty the wells by a firm 'shake-out' action and then flood the plate with wash buffer, completely filling all wells. Repeat this as many times as mentioned in the manual. After washing, the wells of the plate are emptied by a firm 'shake-out' action followed by tapping the plate upside down on absorbent tissue.

When 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 emptied 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. Repeat this as many times as mentioned in the manual. After washing, the plate is emptied by tapping it upside down 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.

NOTE: Do not puncture the PVDF membrane by pipetting/washing procedures. The membrane is fragile and may easily be damaged.

During incubation with blocking solution, membrane-leakage occasionally occurs. This phenomenon, however, does not negatively affect assay results.

Troubleshooting B cell ELISPOT

Problem	Possible cause	Solution
High background	Incomplete washing	- Follow the “Directions for washing” in this Addendum carefully.
	Too many cells in ELISPOT well	- Lower cell concentration on ELISPOT plate, by making a series of dilutions that will result in formation of distinct spots (± 50 spots/well).
	Aspecific binding	- Do not use human, non-human primate or rodent serum as growth supplements. The antibodies in the serum will interfere with spot formation. - Serum in the culture medium should be selected on low background staining.
	Contaminated working solutions	- Solutions should not be used when they have become turbid or there is an indication of bacterial growth. - Use a clean container for the transfer of solutions into the wells of the ELISPOT plate.
	Carryover of antibodies released during preincubation of memory B cells	- Wash cells thoroughly with fresh culture medium before they are transferred to the ELISPOT plate.
	Overdeveloped plate	- Reduce incubation time of color development.
	Incomplete drying of PVDF membranes after completion of the ELISPOT assay Ω	- Allow the PVDF membranes to dry completely (at RT in the dark) prior to spot counting.
Faintly stained spots	Incorrect incubation times or temperature	- Ensure sufficient incubation times. - Reagent solutions should reach RT before use.
	Use of PBS tablets for preparing coating antibody or antigen solution	- The filler in tablets interferes with the coating process. Use sterile liquid PBS instead.
	Improper handling of reconstituted Streptavidin-HRP conjugate ●	- Avoid prolonged exposure to light and heat. - Avoid storage at RT.
	Improper handling of AEC stock solution ●	- Avoid exposure to light or prolonged storage at ≥ 0 °C.
	Improper handling of Activator I and II solutions Δ	- Avoid exposure to light and/or air. - Avoid cross contamination between the two vials. - Ensure proper shaking of the vials before use.
	Inaccurate pre-wetting of the PVDF membrane Ω	- Do not allow PVDF membrane to dry after pre-wetting. If this occurs, repeat the pre-wetting step.
	Inaccurate color development	- Increase time for color development.
	Bleaching of enzymatic stained spots ●	- Store ELISPOT plates at a dry place in the dark. Enzymatic stained spots will always bleach eventually.

Problem	Possible cause	Solution
Low spot frequency	Clumping of cells	- Resuspend cells gently but thoroughly, to gain a good homologous cell suspension, before they are brought into the wells of the ELISPOT plate.
	Reduced viability of cells	- Blood or spleens kept for more that 16 hours at RT may result in less viable cells.
	Not enough cells were added per ELISPOT well	- Increase cells concentration on ELISPOT plate, by making a series of dilutions that will result in formation of distinct spots (± 50 spots/well). - Blood or spleen did not contain sufficient numbers of *in vivo* activated B cells. Collect cells at a different time point after *in vivo* exposure to the antigen. - Ensure that memory B cells are stimulated properly before addition to ELISPOT plate. See "Cell preparation" in the B cell ELISPOT manual for more information. - Determine the optimal preincubation time of the memory B cells by increasing or decreasing the preincubation time.
	Inadequate incubation time of cells in the ELISPOT plate	- Increase incubation time of cells on the ELISPOT plate.
Poor consistency of replicates	Inaccurate pipetting	- Ensure accurate pipetting. - Check pipettes.
	Clumping of cells	- Resuspend cells gently but thoroughly, to gain a good homologous cell suspension, before they are brought into the wells of the ELISPOT plate.
	Evaporation of solutions	- Ensure proper sealing of the plate during incubation steps in 37 °C incubator.
	Inaccurate temperature distribution during incubation steps	- Do not stack plates during incubation.
	Inadequate washing	- Follow the "Directions for washing" in this Addendum carefully.
Small spot size	Inadequate incubation time	- Increase incubation time of cells on ELISPOT plate and/or prolong the preincubation time.
Large spots size	Too long incubation time	- Decrease incubation time of cells on ELISPOT plate.
Confluent spots or poorly defined spots	Too many cells in ELISPOT well	- Lower cell concentration on ELISPOT plate, by making a series of dilutions that will result in formation of distinct spots (± 50 spots/well).
	Moving ELISPOT plate during cell incubation	- Prevent the plate from being moved during the cell incubation step. Even minor vibrations caused by closing the door of the incubator can affect spot formation.
	Dust particles ‡	- Prior to spot counting, clean the underside of the plates (polystyrene-bottomed) wells with 70% ethanol and remove dust particles by blowing 4-5 bar compressed air into the wells.

• Accounts only for enzymatic staining procedure on PVDF membrane-bottomed plates.
Δ Accounts only for silver staining procedure.
Ω Accounts only for procedure with PVDF membrane-bottomed plates.
‡ Accounts only for silver staining procedure on transparent polystyrene-bottomed plates.

Problem	Possible cause	Solution
Blank areas	Cells are unevenly distributed	- Resuspend cells gently but thoroughly, to gain a good homologous cell suspension, before they are brought into the wells of the ELISPOT plate.
	Inaccurate pre-wetting of the PVDF membrane Ω	- Do not allow PVDF membrane to dry after pre-wetting. If this occurs, repeat pre-wetting step.
	Foam formation during washing ‡	- The spout of the squirt bottle is too narrow and should be wider, or the automated washing device does not operate properly and should be adjusted.
	Washing problem	- The aspiration tubes of the washing device may be too close to the bottom of the ELISPOT wells and/or the flow rate may be too high. Change settings.

• Accounts only for enzymatic staining procedure on PVDF membrane-bottomed plates.
Δ Accounts only for silver staining procedure.
Ω Accounts only for procedure with PVDF membrane-bottomed plates.
‡ Accounts only for silver staining procedure on transparent polystyrene-bottomed plates.

Abbreviations

AEC	3-amino-9-ethylcarbazole
DMSO	Dimethyl sulfoxide
ELISPOT	Enzyme-linked immunoSPOT
FCS	Fetal calf serum
HRP	Horse Radish Peroxidase
PBMC	Peripheral blood mononuclear cell
PVDF	Polyvinylidene fluoride
RT	Room temperature

Notes

Technical assistance

If you require assistance, information or have questions, please contact our company:

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On our website (www.ucytech.com/manuals) you can find: Manuals, Typical data, Addendum and MSDS of our B cell ELISPOT kits.