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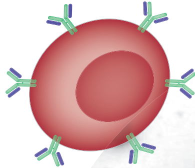
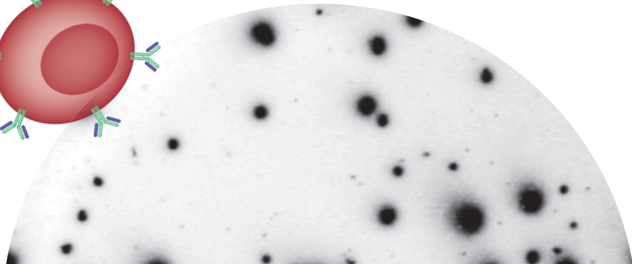
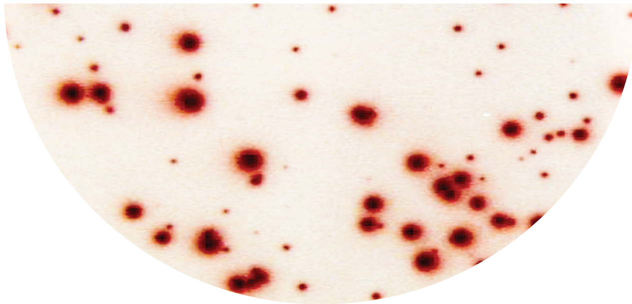
Addendum
B cell ELISPOT assay

Guidelines and troubleshooting



Cell collection and handling

PBMCs derived from human and non-human primates
In general, cell samples from humans and non-human primates are collected as Peripheral blood mononuclear cells (PBMCs) but may also be obtained from lymphoid organs or mucosal sites. For PBMCs whole blood samples are collected, using heparin or citrate as anti-coagulant. After being drawn, blood samples are kept at room temperature (RT) for up to 8 hours. PBMCs are isolated by density gradient centrifugation (using e.g. Ficoll) and washed twice in culture medium. Washing involves two centrifugation/resuspension steps (8 min, 200 x g, RT). PBMCs can be used directly or frozen for later use in the B cell ELISPOT assay. B cells normally constitute a minor fraction of the cells (5-12% of PBMC). Therefore, the samples can be enriched by using e.g. negative selection by depleting non-B cells or by cell sorting.

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.

Cryopreservation of PBMCs
PBMCs can be safely frozen in culture medium with 10% DMSO and 30% to 90% Fetal FCS with a recovery of more than 80%. The recommended cell concentration is ≥ $2x10^7$ cells/ml. Cool the freezing medium to 0 °C before use. Freeze the cells by using a Nalgene™ cryo 1 °C freezing container and by placing the container in a -80 °C freezer overnight. The vials with cells are subsequently stored in liquid nitrogen. Alternatively, also serum-free cell freezing medium can be used for the ELISPOT (e.g. BAMBANKER™).

The thawing procedure is stressful to frozen cells. Using a proper and fast procedure ensures that a high proportion of cells will survive. In brief, the cells are rapidly thawed by gently swirling the cryovial in a 37 °C water bath until there is just a small bit of ice left. Dropwise add 1 volume of fresh and cold cell culture medium containing 50% FCS (4 °C). Subsequently, 10 volumes of cold culture medium containing 10% FSC (4 °C) is added (slowly and under constant swirling) for the first wash step (8 min, 200 x g, 4 °C). Thereafter, the cells are washed for a second time with fresh culture medium (RT) containing 10% FCS (8 min, 200 x g, RT).

Spleen cells from murine origin
Murine B cells are ususally obtained from the spleen or other lymphoid organs but can also be collected from peripheral blood. However, spleen cells are relatively easily collected in great numbers and contain a larger proportion of B cells compared to blood. Spleens are aseptically removed from mice and collected in culture medium. It is recommended to start isolating spleen cells within 8 hours after spleen removal. A single cell suspension is prepared

by gently teasing the spleen tissue through a sterile stainless steel or nylon screens into culture medium and by washing with culture medium. Washing involves two centrifugation/ resuspension steps (8 min, 200 x g, RT). Spleen cells can now be used in the ELISPOT assay.

Cryopreservation of murine spleen cells

Cryopreservation of murine spleen cells is complicated and has in general a negative impact on cell viability and function. By using BAMBANKER™ serum-free cell freezing medium, it is possible to obtain a recovery of 70-80% viable cells and B cell function at levels similar to freshly isolated spleen cells. To thaw the cells, follow the procedure described in the section "Cryopreservation of PBMCs". Overall, it is recommended to work with fresh spleen cells.

Recommended reagents

- BAMBANKER™: Nippon Genetics Europe cat. no. BB03
- Dimethyl sulfoxide (DMSO): Sigma-Aldrich cat. no. D2650.
- Fetal Calf Serum (FCS): Life Technologies cat. no. 16000.
- Ficoll-Paque PLUS: GE Healthcare cat. no. 17-1440-02 (for isolation of PBMCs).
- Culture medium RPMI-1640: Thermo Fisher Scientific cat. no. 52400.
- L-glutamine: Thermo Fisher Scientific cat. no. 25030-024.
- Penicillin/Streptomycin: Thermo Fisher Scientific cat. no. 15140-122.
- Trypan Blue Solution: Sigma-Aldrich cat. no. T8154

The recommended culture medium is RPMI-1640 supplemented with 2 mM L-glutamine, 100 units/ml Penicillin and 100 µg/ml Streptomycin.

Directions for washing of polystyrene-bottomed plates

All washing must be performed with Wash buffer.

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 paper.

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 paper.

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.

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 paper.

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
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 ‡	- Avoid exposure to light and/or air. - Avoid cross contamination between the two vials. - Ensure gently, but thorough shaking of the vials before use.
	Drying out of the PVDF membrane ●	- Do not allow PVDF membrane to dry after pre-wetting. If this occurs, repeat pre-wetting step.
	Poor 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.
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 ●	- Allow the PVDF membranes to dry completely (at RT in the dark) prior to spot counting.

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

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	- Whole blood samples or spleens kept for more than 8 hours at RT may result in less viable cells. - Improper freezing/thawing procedure.
	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 (± 50-100 spots/well). - No sufficient numbers of *in vivo* activated B cells in the blood or spleen. Collect cells at a different time point after *in vivo* exposure to the antigen. - Ensure that memory B cells are properly stimulated before addition to ELISPOT plate. Read “Cell sample preparation” in the B cell ELISPOT manual. - Determine the optimal preincubation time of the memory B cells by increasing or decreasing the preincubation time.
	Inadequate incubation time of the cells in the ELISPOT plate	- Increase incubation time of cells in 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.
	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 in ELISPOT plate and/or prolong the preincubation time.
Large spots size	Inadequate incubation time	- Shorten incubation time of cells in ELISPOT plate.
Confluent spots or poorly defined spots	Too many cells in ELISPOT well	- Lower cell concentration by making a series of dilutions that will result in formation of distinct spots (± 50-100 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.

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 ●	- 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.
‡ 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
min	minute(s)
PBMC	Peripheral blood mononuclear cell
PVDF	Polyvinylidene fluoride
RT	Room temperature
sec	seconds

Technical assistance

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

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E-mail: info@ucytech.com

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