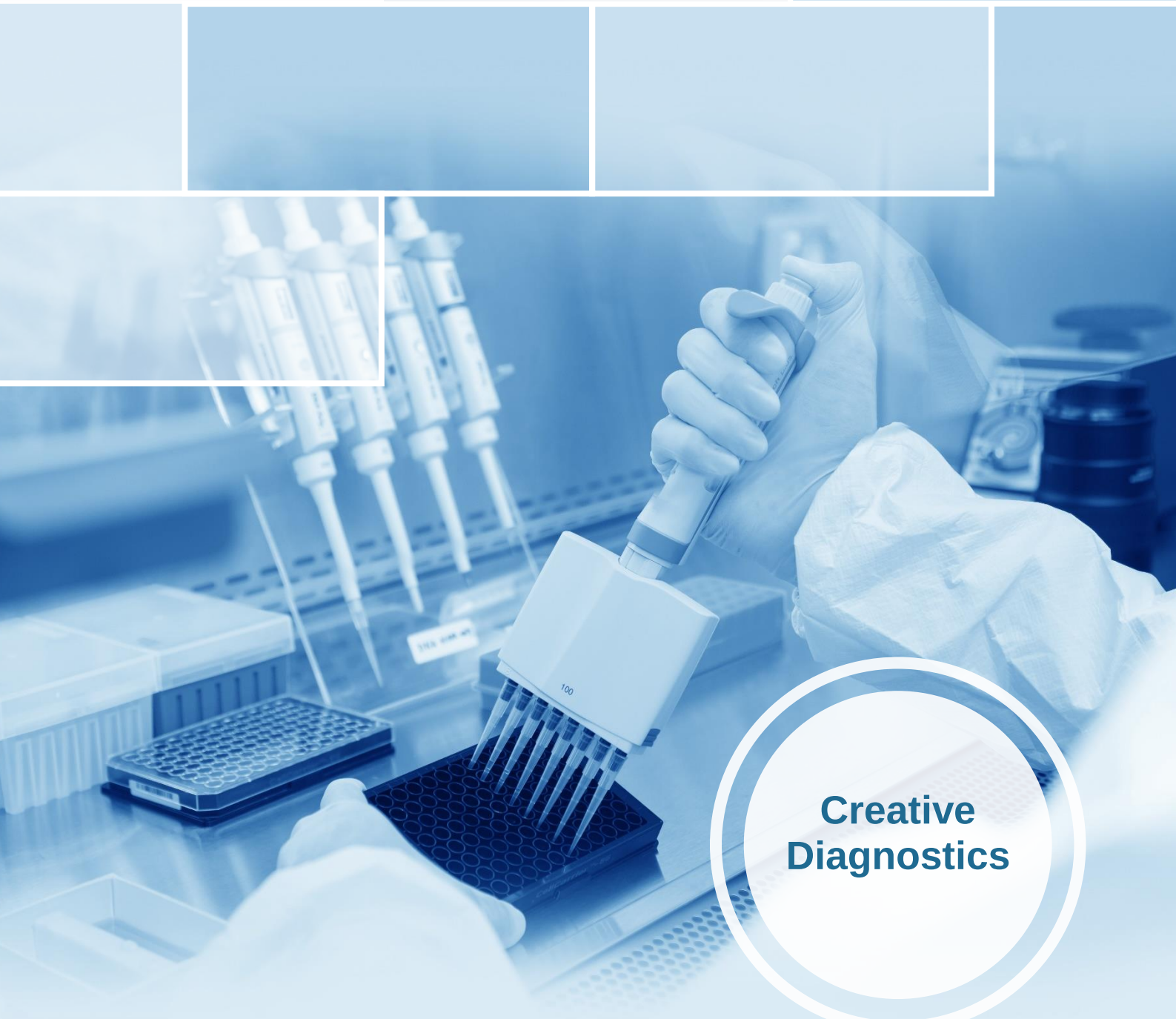
Creative Diagnostics®

ELISPOT TROUBLESHOOTING TIPS



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ELISPOT troubleshooting tips

Solve your ELISPOT problems with these troubleshooting tips, covering common causes of no spots or rare spots, blank areas (Edge effects) or blank centre, confluent spots and more.

Problems Index

➢ High background

- Possible causes and corresponding solutions

1. Wells are ineffectually washed

Some reagents may leak through the membrane into the base of the plate. Increase number of washes and wash more carefully. Soak and wash the plate with PBS (without Tween) prior to adding substrate (Tween-20 from the wash buffer can interfere with the substrate development, causing high background). Wash both sides of the membrane with distilled water before and after color development.

2. Overdeveloping plate after stop solution added

Observe color development carefully and do not overdevelop. Developing plate over 1 hour may bring about increased background color.

3. Substrate incubation carried out in light

Substrate incubations should be carried out in the dark.

4. Reaction not stopped

Use stop solution to prevent overdevelopment.

5. Plate membrane not dried enough

Microplates won't be analyzed accurately until PVDF filter membranes are completely dry. Dry the plate longer if necessary, usually 15-30 minutes at 37° C or 1-1.5 hours at RT, or overnight at 4° C may help increase the contrast between background and spots. Do not dry the plate at a temperature higher than 37°C to avoid cracking of the membrane filters.

6. Cells secreting cytokine/protein of affinity too many

Reduce and optimize the number of cells added. The concentration of stimulant used may also require optimization.

7. Contaminated wash buffer

Prepare fresh wash buffer.

➢ No spots or rare spots

- Possible causes and corresponding solutions

1. Cells are not been stimulated effectively

a) Ensure that reagents used to stimulate the cytokine expression from the cells kept their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation. Use a positive stimulation control (a stimulant that you know will induce release of your cytokine/protein) will also be useful.

b) Since cells may take time to respond to stimulant. Make sure cells not incubated for long enough or use indirect method to stimulation (pre-treat cells with stimulant).

2. Too few cells secreting target cytokine/protein

Make a wide range dilutions of cells (i.e., 1 x 106, 1 x 105, 1 x 104 cells/well) in the first experiment to determine the optimal cell concentration that will result in formation of distinct spots (usually range between 1-2 x 105 cells per well).

3. Insufficient color development

Increase color development time properly. Make sure that the BCIP/NBT Chromogen have correctly been stored and have not deactivated.

4. Not enough antibody used

Try increase primary and/or secondary antibody concentration for optimal results.

5. The membrane filters damaged.

The membranes in the ELISPOT microplates are fragile. Take care not to puncture the membrane on the bottom of the plate wells. Do not touch the bottom of the wells with the ends of the pipet tips when adding cells or reagents and when washing plates.

➢ Blank areas (Edge effects) or blank centre

- Possible causes and corresponding solutions

1. Cells distributed unequally

   Mix cells gently but sufficient to obtain even suspension before pipetting the cells into wells. Do not stack the plates in the incubator. Place each ELISPOT plate individually on the shelf to allow an even distribution of heat to each microwell and to avoid edge effects.

2. Forget to pre-treat membrane

   Membrane should be adequately pretreated with 35% ethanol and washed with PBS 3x afterwards.

3. Wash membrane inadequately after ethanol treatment

   Sometimes ethanol may be trapped between the membrane and the bottom of the plate (leakage). Wash the membrane thoroughly.

4. Membrane dried out at some stage

   Avoid membrane dry out.

5. Pipette tip touched the membrane and caused damage

   Take care with pipetting steps, particularly with washing. Flow rate on an automated washer can’t be too high; manual pipetting can’t be too harsh. A gentler washing procedure required.

6. Formation of foam

   Using squirt bottles with narrow spouts produce excessive foam during washing, preventing an effective and uniform wash. Try remove the bubbles.

➢ Confluent spots

- Possible causes and corresponding solutions

1. Too much primary antibody result in poor coating

   Reduce primary antibody concentration.

2. Cells over-stimulated

Over-stimulation will result in a lot of cytokine or protein being secreted by the cell. Thus causing spots merge and become indistinguishable. The longer the cells are incubated, the more and larger cytokine/protein they will secrete. Reduce the amount of stimulant in the culture media or reduce cell culture step incubation time (It is generally advised not to exceed 24 hours).

➢ Poorly defined spots

- Possible causes and corresponding solutions

1. Membrane not pre-treated

The membrane must be pre-treated with ethanol or the spots may be ambiguous and poorly defined.

2. Coating antibody not enough

Increase coating antibody concentration. Spots will hard to define if the capture antibody is too diluted or if there is a problem during the coating step.

3. Plate movement during cell incubation

Cells moved during incubation will create more than one spot. Do not disturb the incubator or ELISPOT plate during cell incubation to avoid streaks and fuzzy spots. If possible use a dedicated incubator that will not be opened during incubation. Do not tap the plate after adding cells.

➢ False positive

- Check for false positive by running a negative control media Possible causes and corresponding solutions

1. Cells not thoroughly washed off from the membrane

   Wash membrane with PBS Tween 20 adequately before secondary antibody incubation. Make sure all cells be washed off.

   Cells left on the membrane will result in irregular shaped spots.

2. Secondary antibody aggregates

   Filter the secondary antibody.

3. Platelets contaminated (when using PBMC prepared from blood samples)

   PBMC preparation needs to be efficient. Wash the plate well after cell culture stage.

4. Cell culture contaminated by dust or microbes

   Keep reagents and cell culture techniques as aseptic and clean as possible. Reagents can be filter-sterilized using a low protein binding syringe (0.2 μm).

5. Mitogens and other factors in the serum are stimulating the cells

   Heat inactivate the serum

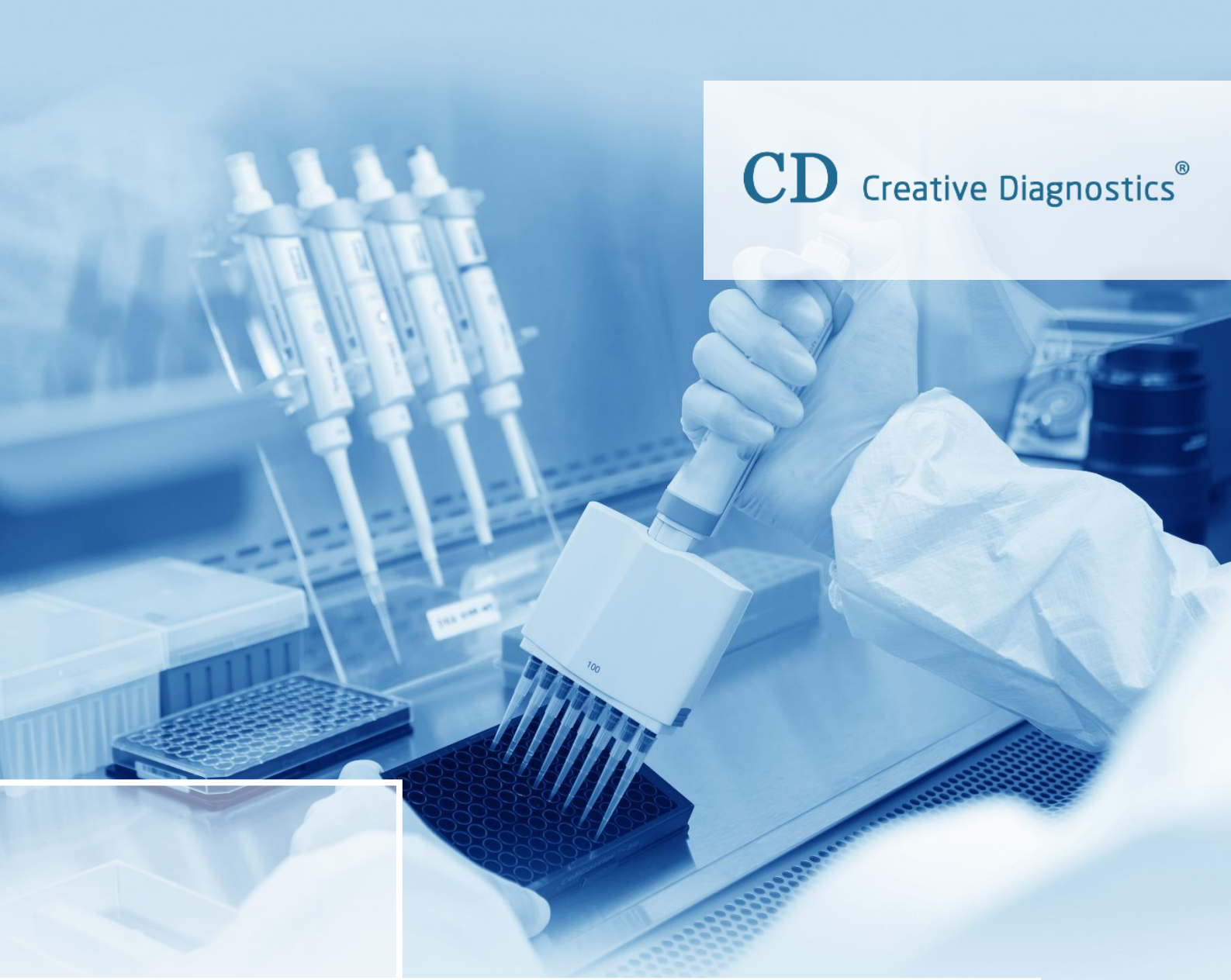
➢ White spots

Using enzymatic detection method, sometime there may appear white spots in the middle of a normal spot. This usually implies the enzymatic conjugate has run out of substrate. Increase the concentration of second antibody and substrate.

For fluorescence: increase the antibody concentrations.

➢ Inconsistent results between wells

1. Do not stack the plates during cells incubation. Stacking may cause plate temperature to distribute uneven, bringing about an edging effect.
2. Ensure a well-mixed single cell suspension of sample is used when adding cells to the wells.



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