Newsletter

The Dot-Spot Test

a simple method to monitor immunoreagent activity and influence of fixation on antigen recognition

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This newsletter focuses on the ImmunoGold Silver Staining method. However, the dot-spot test can be used for every immunodetection system giving signals that are macroscopically visible. Examples are amongst others immuno-enzymatic methods using peroxidase or alkaline phosphatase as marker molecule and carbon or latex coated immunochemicals.

• Introduction

The success of an immunodetection experiment depends on the reactivity and quality of the reagents employed on the one hand and on the degree the antigen can be recognised on the other hand. Negative results may be caused by using reagents with no or low reactivity and by absence, alteration or masking of antigens (by fixation and specimen processing). When a different set of reagents with the same primary antibody gives good results on specimens prepared in the same way, clearly the cause for negative results is in the set of reagents and not at the level of the antigen. On the other hand when the reagents give good results with a different primary antibody (from the same animal species), it is obvious that the problem is either at the level of antigen preservation or at the level of primary antibody activity. When such comparative data are not available the reason for negative results is unclear.

By working backwards through the protocol it is easy to pinpoint the problem. For instance in ImmunoGold Silver Staining the first step to test would be the silver enhancement reagents used. If this step works appropriately, the next step would be to test the reactivity of the immunogold reagent for the primary antibody and so on.

Since detection experiments and specimen preparation techniques are time consuming, a simple and fast method to test the relevant steps in an immunodetection experiment should be helpful. The dot-spot test described in this Newsletter fulfils these requirements.

The dot-spot test is a model system that makes it possible to pinpoint trouble-shooting to a defined part of the immunodetection experiment. A strip of nitro-cellulose membrane, with strong protein binding capacity, is used as a support for binding one of the components needed to check the reactivity of the succeeding step in the immunoincubation protocol.
For instance, (i) to test the silver enhancement reagents, immunogold reagent is applied to the membrane strip. The nitro-cellulose membrane is then incubated with the silver enhancement reagents,

(ii) to test the reactivity of the immunogold reagent, a dilution series of the primary antibody is applied to a nitrocellulose strip and incubation follows with the immunogold reagent.
The results obtained from a dot-spot test give direct information on the quality and reactivity of the reagent tested. A step-by-step approach backwards through the incubation protocol will elucidate the reason for the negative results.

The left hand panel in figure 1 shows the relevant steps in an ImmunoGold Silver Staining (IGSS) set-up. The right hand panel shows the relevant dot-spot test options.

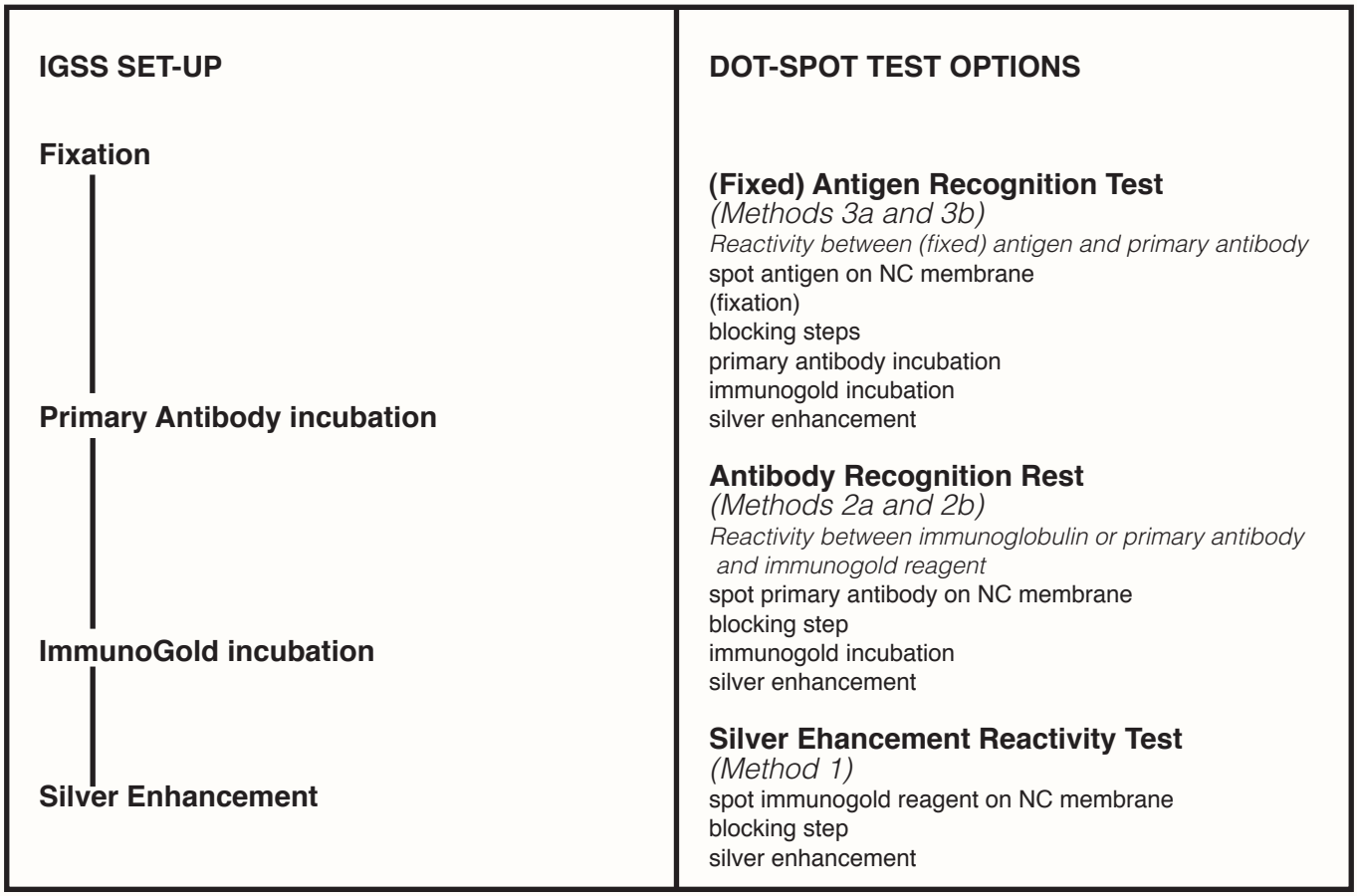

Figure 1: Standard two-step IGSS set-up related to the relevant dot-spot test options.

• Materials

- 0.5x6 cm strips of nitro-cellulose (NC) membrane, pore size 0.45 µm (blotting membrane)
- micropipet with glass capillary tips
- disposable screw cap sealed tubes
- tilting apparatus

• Buffers and Solutions

PBS-buffer, pH 7.4
10 mM phosphate buffer (Na_2HPO_4, KH_2PO_4)
150 mM NaCl

Dot-spot buffer, pH 7.4
PBS buffer with
50 µg/ml Bovine Serum Albumin, Fraction V

Aldehyde block buffer, pH 7.4
PBS buffer with 50 mM glycine

Protein block solution, pH 7.4
PBS buffer with
5% Bovine Serum Albumin, Fraction V

Incubation solution with BSA-c™
0.1% acetylated Bovine Serum Albumin
(BSA-c™, Aurion)
check pH and adjust to 7.4 with 0.1M HCl if necessary

• Procedure

For application of the spots to the nitro-cellulose membrane, it is advisable to use a micropipet with glass capillary tip. The dot-spot strip is an artificial immunospecimen. Blocking, incubation and washing steps are similar to those used in an immunodetection experiment. The strips are processed in disposable screw cap sealed plastic tubes on a tilting apparatus.

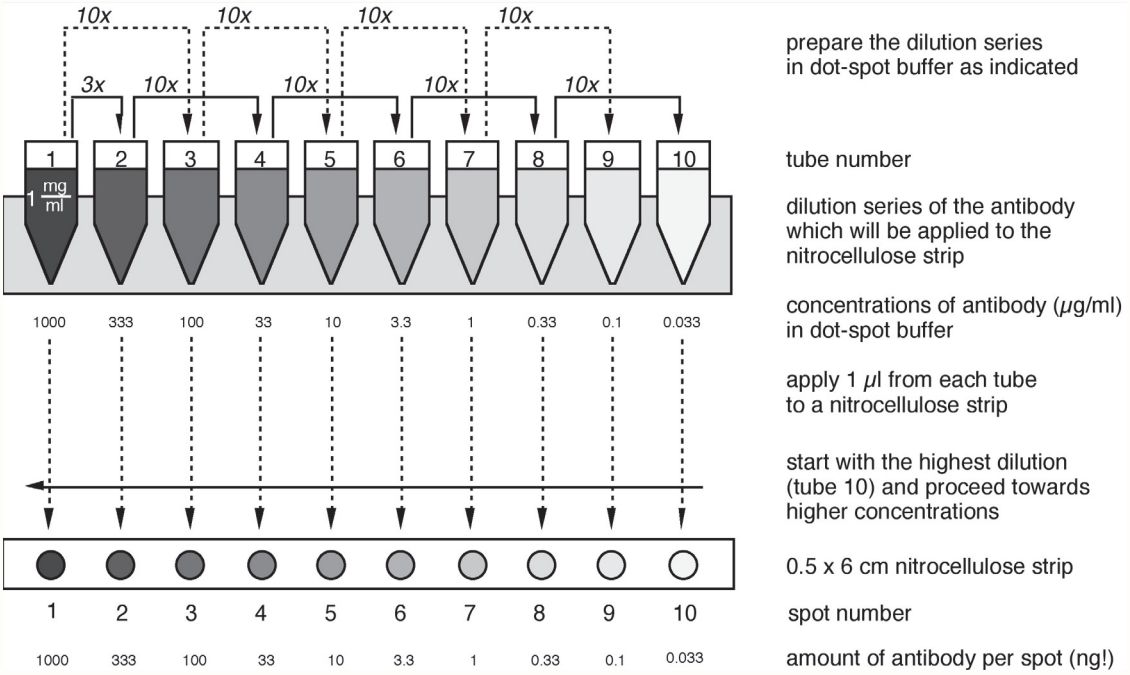

Figure 2: Preparation of an immuno dot-spot strip

• Method 1

Reactivity of the Silver enhancement reagents

1.1. Prepare a 1/10-1/100-1/1000 dilution series of the immunogold conjugate in dot-spot buffer
1.2. Apply 1 µl of each dilution on a strip of nitro-cel lulose (NC) membrane. Start with the highest dilu tion
1.3. Allow the spots to dry and transfer the strip into a plastic tube
1.4. Block the strip with protein block solution for 15 min.
1.5. Wash 3x5 min. in distilled water
1.6. Proceed with silver enhancement

• Evaluation

The enhancement time depends on the initial gold particle size: ultra small particles require a longer enhancement time than for instance 10 nm particles.
The least concentration spot should be visible after 20-30 minutes when using Aurion R-Gent SE-LM and 45-60 min using Aurion R-Gent SE-EM^{plus}.

• Method 2a

Immunoglobulin Recognition Test

Bio-activity of the ImmunoGold Conjugate

2a.1. Prepare a dilution series of purified immunoglobulin (e.g. mouse IgG when testing a Goat-anti-Mouse IgG gold conjugate) in dot-spot buffer, as shown in Fig. 2.
2a.2. Apply 1 µl of each dilution on a strip of NC, starting with the highest dilution.
2a.3. Allow the spots to dry.
2a.4. Wash briefly in PBS.
2a.5. Block in protein block solution for 30 min.
2a.6. Wash in BSA-c™ buffer, pH 7.4 for 5 min.
2a.7. Incubate in 1 ml of immunogold conjugate diluted in BSA-c™ buffer for 2 hrs.
2a.8. Wash in BSA-c™ buffer 3x10 min.
2a.9. Wash in PBS 3x5 min.
2a.10. Wash 3x5 min. in distilled water
2a.11. Proceed with silver enhancement

Evaluation

After silver enhancement the 1 ng spot (spot nr. 7) should be clearly visible.

Remarks and tips

- The dilution of the immunogold conjugates is the same as for incubation on tissue sections, i.e.:
Aurion Conventional reagents diluted 1/20-1/40,
Aurion Ultra Small reagents diluted 1/50-1/100.
- Immunoconjugates, especially those with Protein A and Protein G, may become uncoupled during the washing step in distilled water. It is advisable therefore to include a post-fixation step in 1% glutaraldehyde in PBS for 10 minutes before washing with distilled water.
- Bio-activity of conventional reagents can also be tested without silver enhancement. Dilute 1/10 and incubate for 4 hours. The 10 ng spot (spot nr. 5) should be detected.

Conclusion

If the results meet the criteria, the quality of the immunogold silver reagents is not the cause for negative results in the immunodetection experiment. Continue with Method 2b to see if there is a case-specific recognition problem between the specific primary antibody and the immunogold conjugate.

• Method 2b

Antibody Recognition Test

Case-specific bio-activity of the ImmunoGold Conjugate

Make sure primary antibody and gold conjugate match!

2b.1. Prepare a dilution series of the primary antibody in dot-spot buffer as described in Fig. 2.
2b.2. Apply 1 µl of each dilution on a strip of NC, starting with the highest dilution.
2b.3. Allow the spots to dry.
2b.4. Wash briefly in PBS.
2b.5. Block in protein block solution for 30 min.
2b.6. Wash in BSA-c™ buffer, pH 7.4 for 5 min.
2b.7. Incubate in 1 ml of immunogold conjugate diluted in BSA-c™ buffer for 2 hrs.
2b.8. Wash in BSA-c™ buffer 3x10 min.
2b.9. Wash in PBS 3x5 min.
2b.10. Wash 3x5 min. in distilled water
2b.11. Proceed with silver enhancement

Evaluation

After silver enhancement the 1 ng spot (spot nr. 7) should be clearly visible.

Remarks and tips

- Unfortunately the primary antibody concentration is not always known. However, the working dilution gives an indication of this concentration as it is normally between 1 and 10 µg/ml. Alternatively the concentration can be estimated by measuring the optical density of the antibody stock solution at 280 nm using the buffer as a reference. A 1 mg/ml stock has an OD_{280} of 1.3-1.4 (10 mm light path). The stock solution should only contain antibody as protein and not for instance BSA or other proteins. In such a case measurement will not be reliable.
- Immune serum contains a high amount of immunoglobulin, only part of which is the specific antibody. In this case the primary antibody recognition test will not give a reliable result. The immunogold conjugate will detect all matching immunoglobulin present in the spots. Using an immune serum poses however no restriction to the antigen recognition test (see Methods 3a and 3b).
- Immunoconjugates, especially those with Protein A and Protein G, may become uncoupled during the washing step in distilled water. It is advisable therefor to include a post-fixation step in 1% glutaraldehyde in PBS for 10 minutes before the distilled water wash step.
- Binding of Protein G conjugates is pH dependent. The Antibody Recognition Test can be used to determine the optimal incubation pH for every new primary antibody.

Conclusion

If the results in the dot-spot tests, described in Methods 1 and 2a or 2b match the criteria, the immunogold/silver reagents are not causing signal intensity problems in the immunodetection experiment. If the purified antigen is available, continue with Method 3a or 3b. Otherwise go on with positive control LM specimens, e.g., cryostat sections.

• Method 3a

Native Antigen Recognition Test

3a.1. Prepare a dilution series of the purified antigen in dot-spot buffer as described in Fig. 2.
3a.2. Apply 1 µl of each dilution on a strip of NC, starting with the highest dilution.
3a.3. Allow the spots to dry.
3a.4. Wash briefly in PBS.
3a.5. Block in protein block solution for 30 min.
3a.6. Wash in BSA-c™ buffer, pH 7.4 for 5 min.
3a.7. Incubate in 1 ml of primary antibody diluted in BSA-c™ buffer (concentration between 0.5 and 5 µg/ml) for 2 hrs.
3a.8. Wash in BSA-c™ buffer, pH 7.4 for 5 min.
3a.9. Incubate in 1 ml of immunogold conjugate diluted in BSA-c™ buffer for 2 hrs.
3a.10. Wash in BSA-c™ buffer 3x10 min.
3a.11. Wash in PBS 3x5 min.
3a.12. Wash 3x5 min. in distilled water
3a.13. Proceed with silver enhancement

Evaluation
After silver enhancement the 1 ng spot (spot nr. 7) should be clearly visible.

Remarks and tips
- If the purified antigen is not available, a dot-spot test with partially purified antigen can indicate the quality of the primary antibody.
- Another option is incubation of antigen containing strips cut from a Western blot. However, SDS denaturation destroys the three-dimensional structure of native proteins and thus maybe also antigen recognition.

Conclusion
When the antigen recognition test gives good results the immunoreagents are not the cause for low (or no) signal intensity. Remaining probable causes are now:
- The antigen is not available or masked. This can be checked with positive control specimens.
- The fixation or embedding procedure is not compatible with your primary antibody.
The Fixed Antigen Recognition Test (see Method 3b) may clarify the effect of fixation on antigen recognition.

• Method 3b

Fixed Antigen Recognition Test

Test fixatives should keep the NC support intact. It is therefore not possible to test the effect on antigen recognition of fixatives like methanol and acetone using the dot-spot test.

3b.1. Prepare a dilution series of the purified antigen in dot-spot buffer as described in Fig. 2.
3b.2. Apply 1 µl of each dilution on a strip of NC, starting with the highest dilution.
3b.3. Allow the spots to dry.
3b.4. Wash in PBS.
Optional: change to the buffer used in fixation
3b.5. Incubate in fixative for the same time as planned for specimen fixation.
Optional: Wash in buffer used in fixation
3b.6. Wash in PBS 3x5 min.
3b.7. Block free aldehyde groups in aldehyde block buffer for 15 min.
3b.8. Block in protein block solution for 30 min.
3b.9. Wash in BSA-c™ buffer, pH 7.4 for 5 min.
3b.10. Incubate in 1 ml of primary antibody diluted in BSA-c™ buffer (concentration between 0.5 and 5 µg/ml) for 2 hrs.
3b.11. Wash in BSA-c™ buffer, pH 7.4 for 5 min.
3b.12. Incubate in 1 ml of immunogold conjugate diluted in BSA-c™ buffer for 2 hrs.
3b.13. Wash in BSA-c™ buffer 3x10 min.
3b.14. Wash in PBS 3x5 min.
3b.15. Wash 3x5 min. in distilled water
3b.16. Proceed with silver enhancement

Evaluation
Compare results with the non-fixed strip (see Method 3a).

Remarks and tips
- The degree of fixation changes with time and concomitantly antigen recognition may be affected progressively.
- Do not use $NaBH_4$ to quench free aldehyde groups. The interaction between $NaBH_4$ and the nitro-cellulose membrane interferes with silver enhancement.
- The purified antigen is not surrounded by other proteins as in the specimen. The effect of cross-linking fixatives is not truly mimicked.
- The antigen dot-spot test is a time-saving test to compare the effect of different fixation methods on antigen recognition. Incubate in the different test fixatives (e.g. for 1 hour). An example of a fixation series is:
4% paraformaldehyde (in buffer)
4% paraformaldehyde+0.01% glutaraldehyde
4% paraformaldehyde+0.1% glutaraldehyde
4% paraformaldehyde+1% glutaraldehyde.

Conclusion
The Fixed Antigen Recognition Test shows the direct effect of fixation on antigen recognition. Fixatives that give no or only a very weak signal can not be used.
This test minimises the amount of work needed to find an acceptable fixation protocol. If a fixation method has no or only minor effect on antigen recognition and negative results persist, troubleshooting has to be focused on other steps in the specimen preparation protocol (e.g. the embedding procedure).

General Discussion

Dot-spot tests are short-time experiments. These tests show at which levels in an immunodetection experiment signal intensity problems arise. They demonstrate whether the immunodetection system is to blame or whether the specimen preparation method has to be adapted. With the antigen recognition test a pre-selection of fixation regimes can be made. This again limits workload needed to obtain a positive immunolabeling. Figure 3 is a schematic representation summarising the different steps needed to find the cause for negative results in an immunodetection experiment based on the IGSS method.

Checking Antigen Recognition: Alternatives

When free antigen is not available for testing it is of course not feasible to use a dot-spot test for evaluating antigen recognition and/or sensitivity of antigens to fixatives. As an alternative cryostat sections of unfixed tissue (and even cultured cells grown on coverslip) contraining the antigen can be used. A series of specimens can be exposed to different fixatives and reactivity can be compared with levels obtained in unfixed material.

-When the antigen can be detected with a different detection method (e.g. immunofluorescence) using the same primary antibody the problem is either in the IGSS reagents or in the degree of exposure of the antigen. Use method 1 and 2b to check the reagents.

-When the IGSS reagents work well with a different primary antibody the problem is at the level of the primary antibody or at the level of antigen recognition. Use method 2a to check the bioactivity of the conjugate and methods 3a and 3b to check antigen recognition.

-When the IGSS reagents work well with a the case-specific primary antibody on a different specimen type the problem is at the level of the antigen recognition. Use method 3b to check the influence of fixation on antigen recognition.

-In all other cases check activities of the reagents starting with method 1. When the test is positive continue with the next method and so on.

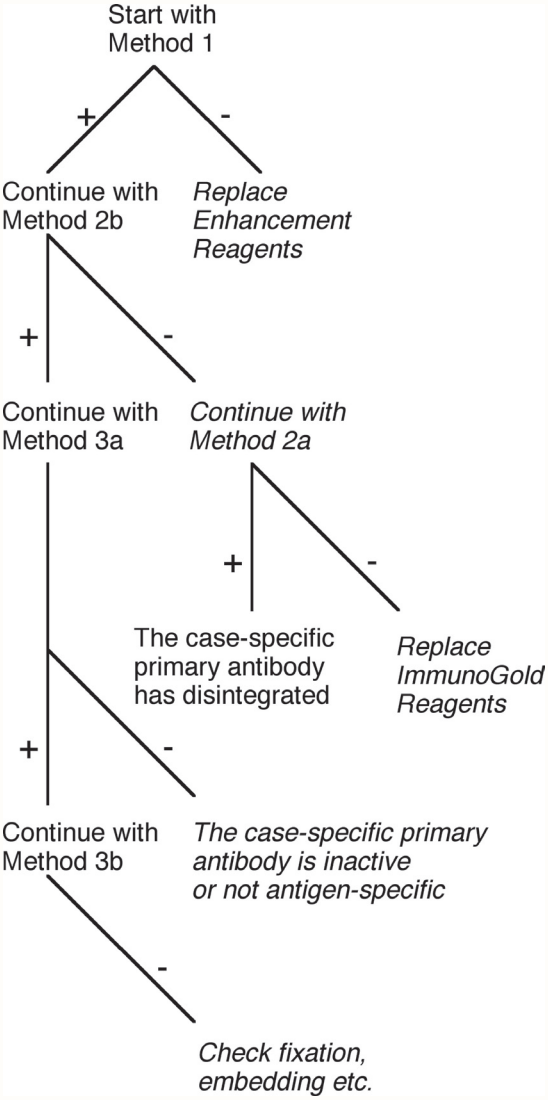

Figure 3: Trouble shooting planner.

+ indicates path to follow when preceeding tests results are positive. Comment text plain.
- indicates path to follow when preceeding tests results are negative. Comment text in italics.