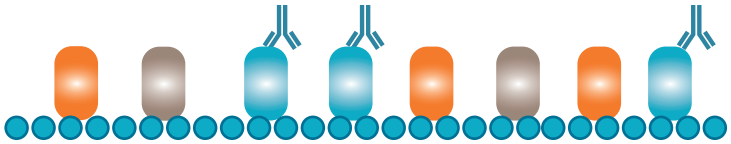
Immunohistofluorescence Multiplexing with Two Mouse Primary Antibodies and Pan IgG Specific Secondary Antibodies

Staining of mutually exclusively expressed proteins cytokeratin 19 and PGP9.5 on FFPE sections of human pancreas.

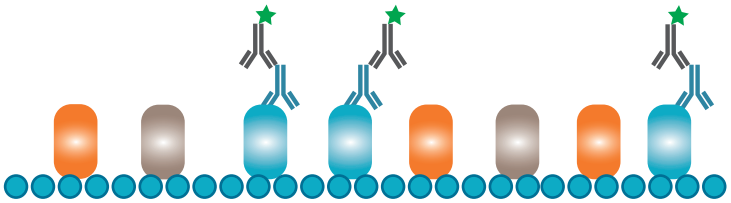
Step 1

First the mouse primary antibody (anti-cytokeratin 19) is added and binds to the target protein.



Step 2

The green fluorescent labeled polyclonal anti-mouse IgG secondary antibody is then added and binds to the target primary antibody.



The resulting green fluorescence is shown in Figure 1.

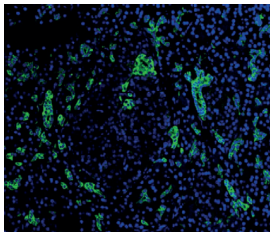
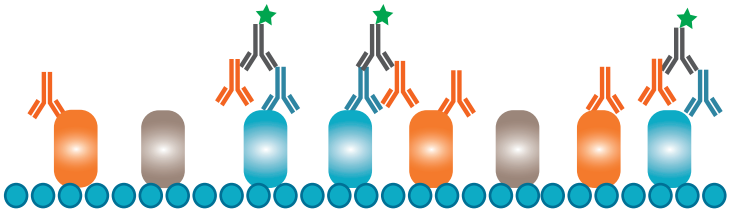


Fig. 1. Image showing the specific green stain of cytokeratin 19.

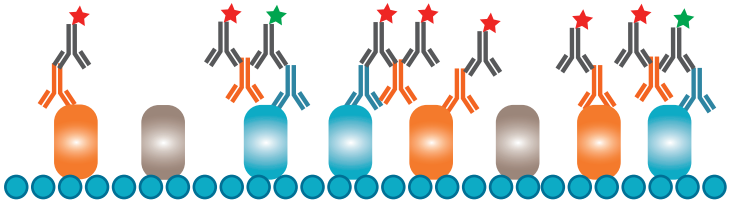
Step 3

The second mouse primary antobody (anti-PGP9.5) is added and binds to the protein target, but is also captured by the secondary antibody resulting in nonspecific binding.



Step 4

The red fluorescent labeled polyclonal anti-mouse IgG secondary antibody is added and binds to all anti-PGP9.5 primary antibodies including those that have bound nonspecifically as shown in step 3.



The resulting red fluorescence is shown in Figure 2.

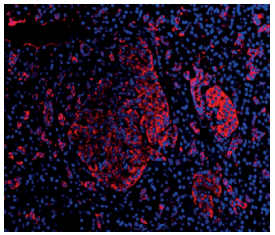


Fig. 2. Image showing combined specific red stain of PGP9.5 and nonspecific staining; not obvious unless compared to a single stain.

Result

Double stained cells detected (yellow), as shown in Figure 3, but these are artifactual.

The common solution is to block the free binding sites on the first secondary antibody. But does this prevent false positives?

Read on to find out..

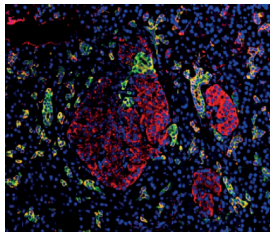


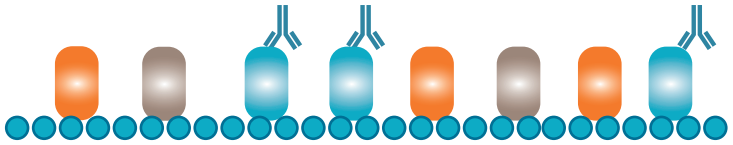
Fig. 3. Figures 1 and 2 merged showing incorrect double staining visible due to nonspecific red staining.

Key

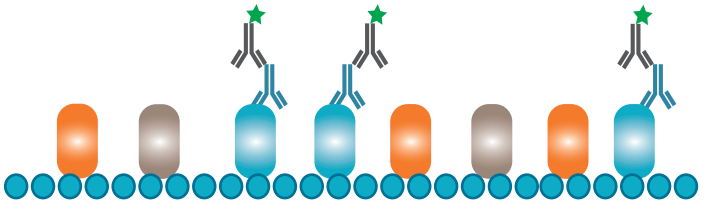
BIO-RAD

Immunohistofluorescence Multiplexing with Two Mouse Primary Antibodies - Adding a Blocking Step

Step 1 The first mouse primary antibody (anti-cytokeratin 19) is added and binds to the target protein.



Step 2 The green fluorescent labeled polyclonal anti-mouse IgG secondary antibody is added and binds to the target primary antibody.



The resulting green fluorescence is shown in Figure 1.

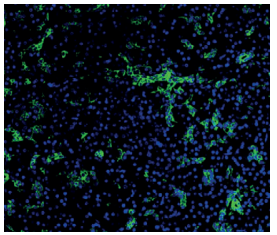
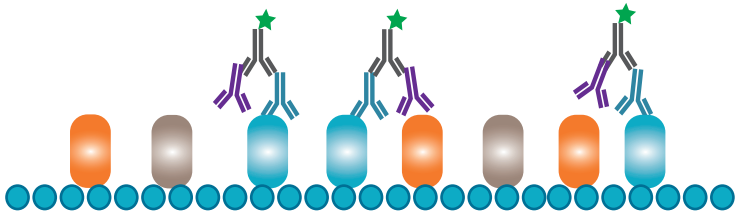
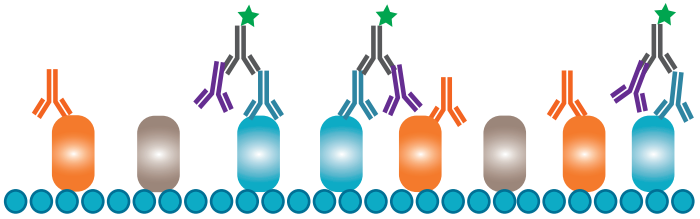


Fig. 1. Image showing the specific green stain of cytokeratin 19.

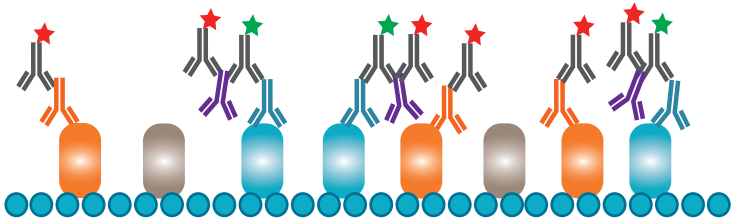
Step 3 An irrelevant anti-mouse IgG is added to block vacant binding sites on the green fluorescent secondary antibody.



Step 4 The second mouse primary antibody (anti-PGP9.5) is added, binds to the target protein but is not captured by the green fluorescent secondary antibody.



Step 5 The red fluorescent labeled polyclonal anti-mouse IgG binds to anti-PGP9.5 but also to the blocking antibody captured by the green fluorescent secondary antibody resulting in nonspecific binding.



The resulting red fluorescence is shown in Figure 2.

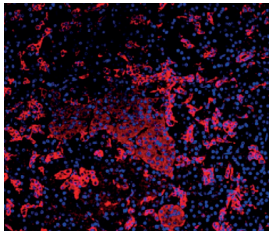


Fig. 2. Image showing combined specific red stain of PGP9.5 and nonspecific staining; not obvious unless compared to a single stain.

Result

Double stained cells detected (yellow), but are artifactual as shown in Figure 3.

How can you have confidence in your experiments?

Read on to find out...

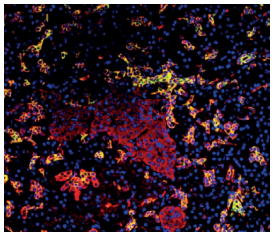
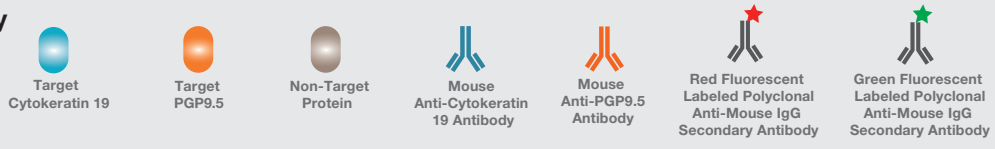


Fig. 3. Figures 1 and 2 merged showing incorrect double staining, visible due to nonspecific red staining.

Key

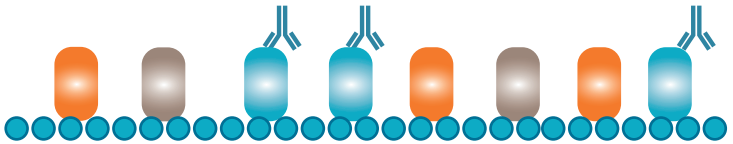


BIO-RAD

Immunohistofluorescence Multiplexing with Two Mouse Primary Antibodies -Using Serial Isotype Specific Secondary Antibodies

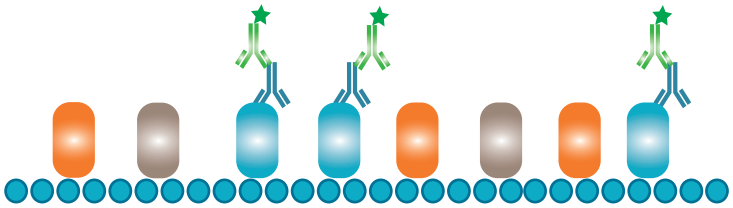
Step 1

The first mouse primary antibody (IgG1 anti-cytokeratin 19) is added and binds to the target protein.



Step 2

The green fluorescent labeled monoclonal, isotype specific anti-mouse IgG1 secondary antibody is added and binds to the target primary antibody.



The resulting green fluorescence is shown in Figure 1.

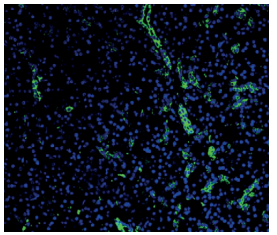
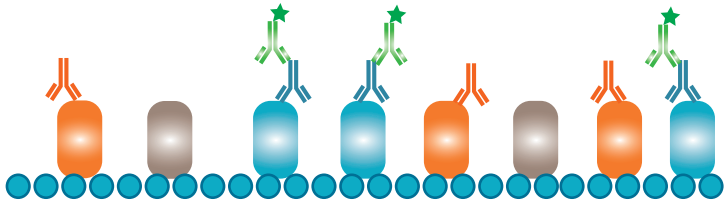


Fig. 1. Image showing the specific green stain of cytokeratin 19.

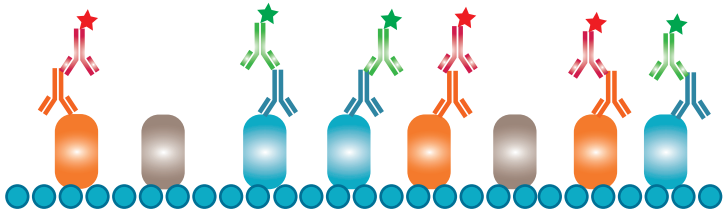
Step 3

A second mouse primary antibody (IgG2a anti-PGP9.5) is added and binds to the target protein only.



Step 4

The red fluorescent labeled monoclonal, isotype specific anti-mouse IgG2a secondary antibody is added and only binds to the anti-PGP9.5 primary antibody.



The resulting red fluorescence is shown in Figure 2.

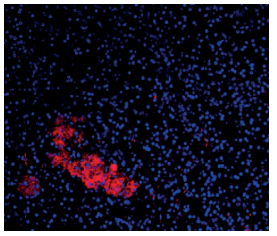


Fig. 2. Image showing the correct specific staining of PGP9.5.

Result

Using these isotype specific secondary antibodies you can be sure that false positive staining (yellow) is not present.

Using isotype specific secondary antibodies not only prevent false positives but can also save you time.

Read on to find out...

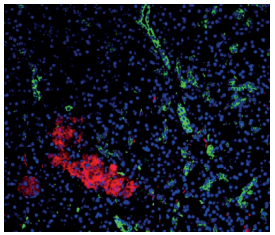


Fig. 3. Figures 1 and 2 merged showing no false positives.

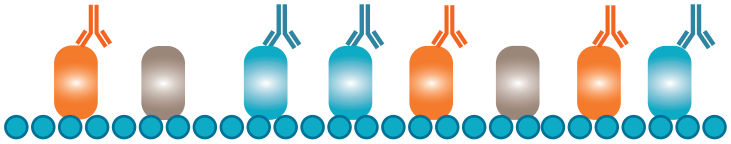
Key

BIO-RAD

Immunohistofluorescence Multiplexing with Two Mouse Primary Antibodies - Parallel Isotype

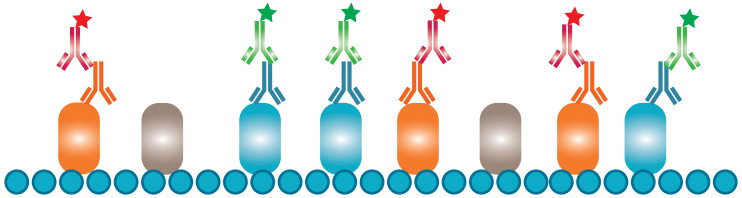
Step 1

Both mouse primary antibodies (IgG1 anti-cytokeratin 19 and IgG2a anti-PGP9.5) are added, and bind to target proteins.



Step 2

Both secondary (green fluorescent isotype specific anti-mouse IgG1 and red fluorescent isotype specific anti-mouse IgG2a) are added and bind specifically to target primary antibodies.



The resulting red and green fluorescence are shown in Figures 1 and 2.

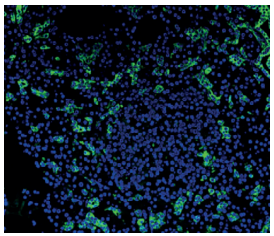


Fig. 1. Image showing the correct specific green stain of cytokeratin 19.

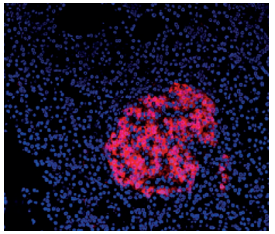
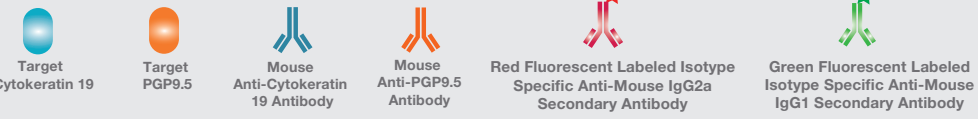


Fig. 2. Image showing the correct specific red stain of PGP9.5.

Things to note before you start this protocol:

- The two primary antibodies need to be different isotypes
- The secondary antibody needs to be targeted to (specific for) the isotype of its target primary antibody
- You need to know the isotypes of your primary antibodies
- You need to know that your secondary antibodies are isotype specific

Key



Result

Trust your results - no false positives.

Save time - two-step procedure.

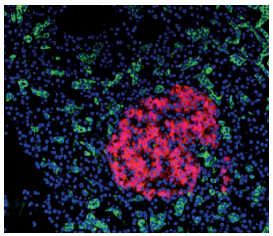


Fig. 3. Figures 1 and 2 merged showing no false positives.

Visit **bio-rad-antibodies.com/isotype-secondaries** to learn more about isotype specific secondary antibodies.

BIO-RAD