ZytoChem Plus 2-Step Double Stain Polymer Kit

REF / Cat. No.: POLD2S-006 60 tests, 6 ml
POLD2S-100 1000 tests, 100 ml

Instructions for use

Intended use
ZytoChem Plus 2-Step Double Stain Polymer Kit is designed for qualitative detection of antigens in fixed paraffin-embedded tissue sections, in frozen tissue sections, and in cytological samples. It is especially developed for double colour immunostaining with pairs of antibodies, one from mice, and one from rabbit.
The kit can be used for examining tissues fixed in different solutions, e.g. formalin (neutrally buffered), B5, Bouin, ethanol, or HOPE.
It is intended for research use only.

Summary and Explanation
The purpose of immunohistochemical staining is to make tissue and cell antigens visible.
ZytoChem Plus 2-Step Double Stain Polymer Kit is a highly sensitive detection kit intended for use in immunohistochemistry and immunocytochemistry. The enzyme polymers in this kit consist of several molecules of secondary antibodies covalently bound to several enzyme molecules.
The Double Stain Polymer Kit includes two types of enzyme polymers: (1.) horse radish peroxidase (HRP) bound to anti-mouse secondary antibodies and (2.) alkaline phosphatase (AP) bound to anti-rabbi secondary antibodies.
Visualisation occurs via two consecutive enzyme-substrate reactions in the presence of colourising reagents which permit microscopical analysis.
The first enzyme-substrate reaction is HRP in combination with DAB/H_2O_2. All bound primary mouse antibodies are stained in dark brown colour. The second reaction is AP in combination with a suitable chromogenic substrate detecting all bound primary antibodies from rabbit. The chromogenic substrate for this step should result in a good contrast to DAB. We recommend using Permanent AP Red (ZUC001).
The test system is suitable for the detection of mono- and polyclonal primary antibodies and sera obtained from mice and rabbits. In contrast to other detection techniques, which often use the streptavidin-biotin technique all ZytoChem Plus Polymer Kits avoid the problem of background staining caused by endogenous biotin in the tissue.

Principle of the method
Paraffin-embedded tissue sections are first deparaffinised and rehydrated.
Endogenous peroxidase activity in the tissue may cause non-specific staining. This enzyme activity can be blocked by incubation with 3% H_2O_2-solution (peroxide block).
Background staining caused by unspecific binding of the primary antibody or the secondary antibody in the HRP-polymer is minimized by incubation with a protein blocking solution. This step can be omitted if the primary antibodies are diluted in an appropriate buffer.
The next step is the incubation with the specific primary antibodies; one must be from mice and the other from rabbit. The antibodies can be incubated simultaneously (as a mixture or “cocktail”) or one after another.
After washing, the HRP-Polymer anti-mouse is applied and incubated. Any excess of unbound polymer is thoroughly washed away thereafter. Now, the AP-Polymer anti-rabbit is applied and incubated. Again, the excess of unbound polymer is thoroughly washed away thereafter.
The addition of DAB/H_2O_2 solution starts the enzymatic reaction of the peroxidase which leads to colour precipitation where the primary mouse antibody is bound. The colour can be observed with a light microscope.
After another wash step the AP reaction is started by adding the second chromogenic substrate. The formed precipitate detects all bound primary rabbit antibodies.
The chromogen DAB forms a dark brown precipitate at the place of the target antigen. Permanent AP Red (compatible with AP) leads to the formation of a magenta-red product of reaction at the place of the target antigen.

Reagents provided

REF / Cat. No. POL2DS-006
6 ml **HRP-Polymer x Mouse** (ready-to-use)
6 ml **AP-Polymer x Rabbit** (ready-to-use)

REF / Cat. No. POL2DS-100
100 ml **HRP-Polymer x Mouse** (ready-to-use)
100 ml **AP-Polymer x Rabbit** (ready-to-use)

Substrate systems recommended:

DAB Substrate Kit	*Cat. No. DAB057*	*500 tests*
(2 components)	*Cat. No. DAB530*	*5000 tests*
Permanent AP Red	*Cat. No. ZUC001-125*	*1250 tests*
	Cat. No. ZUC001-500	*5000 tests*

Other available substrate systems:

DAB High Contrast Kit	*Cat. No. DAB500 plus*	*500 tests*
(2 Components)	*Cat. No. DAB5000 plus*	*5000 tests*
AEC Single Solution	*Cat. No. ZUC037-008*	*80 tests*
	Cat. No. ZUC037-125	*1250 tests*
AEC Substrate Kit	*Cat. No. ZUC042-050*	*500 tests*
(2 Components)	*Cat. No. ZUC042-500*	*5000 tests*
Permanent AEC Kit	*Cat. No. ZUC054-200*	*2000 tests*
(4 Components)		
Permanent HRP Green	*Cat. No. ZUC070-100*	*1000 tests*
(2 Components)		

Materials required but not supplied
Positive and negative control tissue
Xylene or suitable substitutes
Ethanol, distilled or deionised H_2O
Reagents for enzyme digestion or heat pre-treatment
Wash buffer PBS or TBS (Cat. No. ZUC020)
PAP Pen (Cat. No. LP0001)
Primary antibodies or antibody cocktail (user-defined)
Primary antibody diluent (Cat. No. ZUC025)
Negative control reagent
Chromogenic substrate (1 x HRP-compatible, 1 x AP-compatible)
Counterstain solution
Mounting medium
Cover slips

Storage and handling
The solutions should be stored at 2-8°C without further dilution. Please store the reagents in a dark place and do not freeze them. Under these conditions the solutions are stable up to the expiry date indicated on the label. They should not be used after the expiry date.
A positive and a negative control have to be carried out in parallel to the test material. If you observe unusual staining or other deviations from the expected results which could possibly be caused by the kit reagents, please contact Zytomed Systems' technical support or your local distributor.

Precautions
Use by qualified personnel only.
Wear protective clothing to avoid eye, skin or mucous membrane contact with the reagents. In case of a reagent coming into contact with a sensitive area, wash the area with large amounts of water. Sodium Azid and ProClin 300 are used for stabilisation. Microbial contamination of the reagents must be avoided, since otherwise non-specific staining might appear. Material safety data sheets (MSDS) are available upon request.

Typical staining procedure

1.	Blocking Solution (protein block) *(This step is optional.)*	**5 min.**
2.	Rinse with wash buffer	**1 x 2 min.**
3.	First primary antibody (optimally diluted) or negative control reagent	**30-60 min.**
4.	Rinse with wash buffer	**3 x 2 min.**
5.	Second primary antibody (optimally diluted) *(Both antibodies can be incubated in one step)*	**30-60 min.**
6.	Rinse with wash buffer	**3 x 2 min.**
7.	HRP-Polymer anti-mouse	**30 min.**
8.	Rinse with wash buffer	**3 x 2 min.**
9.	DAB (Controlling the colour intensity via light microscope is recommended.)	**10 min.**
10.	Rinse with wash buffer	**3 x 2 min.**
11.	AP-Polymer anti-rabbit	**30 min**.
12.	Rinse with wash buffer	**3 x 2 min.**
13.	Permanent AP Red (*Controlling the colour intensity via light microscope is recommended*.)	**20 min.**
14.	Stop the reaction with distilled H_2O when the desired colour intensity is attained	
15.	Counterstaining and blueing	
16.	Mounting: aqueous or permanent depending on the chromogenic substrates used.	

Quality control

We recommend carrying out a positive and a negative control with every staining run. The positive control permits the validation of appropriate processing of the sample. If the negative control has a positive result, this points to unspecific staining.

Expected results

During the reaction of the substrates with the enzyme polymers (HRP or AP) in presence of chromogens, coloured precipitates are formed at the location of the bound primary antibodies. These reactions only take place if the target antigens are existent in the tissue. The chromogens used determine the colours of the precipitates. The analysis is carried out using a light microscope.

Limitations of the procedure

Immunohistochemistry is a complex method in which histological as well as immunological detection methods are combined. Tissue processing and handling prior to immunostaining, for example variations in fixation and embedding or the inherent nature of the tissue can cause inconsistent results (Nadji and Morales, 1983).
The reagent system is especially developed for double colour staining with pairs of primary antibodies. One antibody has to be from mouse, one from rabbit. Primary antibodies from other species have to be detected via different detection systems. The same applies for two primary mouse antibodies or two primary rabbit antibodies.
It is essential to check if both antibodies are compatible with the same epitope retrieval technique.
Endogenous peroxidase or pseudoperoxidase activity may cause non-specific staining. The enzyme activity is blocked by incubation with hydrogen peroxide solution. Tissues containing Hepatitis B Surface Antigen (HBsAg) may give false positive results with HRP (horse radish peroxidase) detection systems (Omata et al, 1980).
Endogenous alkaline phosphatase activity may cause non-specific staining too. The enzyme activity can be blocked by incubation with levamisole. However, neither intestinal nor placental alkaline phosphatase can be blocked with levamisole.
Inadequate counterstaining and mounting can influence the interpretation of the results. The colour intensity of the reaction product can decrease with time, especially when exposed to light.
Zytomed Systems guarantees that the product will meet all requirements described from its shipping date until its expiry date, as long as the product is correctly stored and utilized. No additional guarantees can be given. Under no circumstances shall Zytomed System be liable for any damages arising out of the use of the reagent provided.

Troubleshooting

If you observe unusual staining or other deviations from the expected results which could possibly be caused by the reagents, please read these instructions carefully, contact Zytomed Systems' technical support or your local distributor.

No staining on an actually positive control slide:

1. Reagents were not used in the proper order.
2. Chromogenic substrate solution was too old.
3. Bleaching because chromogen and mounting medium are incompatible.
4. The antigen/epitope in the tissue was insufficiently accessible to the primary antibody. Try a pre-treatment such as heat pre-treatment or enzyme digestion. If you used a pre-treatment it should be extended.
5. Primary antibody not from mouse or rabbit, but from a different species.
6. The antigen/epitope was not stable in the fixation and/or pre-treatment procedure used. Try another fixation or pre-treatment.

Weak staining:

1. Inadequate fixation or overfixation.
2. Incomplete deparaffinisation.
3. The antigen/epitope in the tissue was insufficiently accessible to the primary antibody. If you used heat pre-treatment or enzyme digestion it should be extended.
4. Excessive incubation with Blocking Solution or insufficient washing after this step.
5. Too much wash buffer remains on the slides after washing, diluting the reagents applied in the next step.
6. If you are using PBS-based wash buffer: the activity of alkaline phosphatase in the reagents is blocked if too much wash buffer remains on the slides.
7. Incubation times were too short or primary antibody concentration too low.
8. Chromogenic substrate solution was too old.

Non-specific background staining or overstaining:

1. Incomplete deparaffinisation.
2. Excessive tissue adhesive on slides.
3. Insufficient washing especially after the incubation steps with enzyme polymer or the chromogenic substrate solution. These washings are critical.
4. Tissue was allowed to (partially) dry out with reagents on.
5. Unspecific binding of the primary antibodies. Please use the Blocking Solution provided with this kit or dilute the primary antibodies in appropriate diluents.
6. Incubation times of the primary antibodies were too long or primary antibody concentration too high.
7. Incubation times of the chromogenic substrate solutions were too long or reaction temperature too high (e.g. if temperature in the laboratory is high).
8. The substrate for the horse radish peroxidase is metabolised by endogenous HRP in the tissue. Maybe the hydrogen peroxide solution used for blocking was inactivated.
9. The substrate for the alkaline phosphatase is metabolised by endogenous AP in the tissue. This undesired activity can often be suppressed using levamisole (see section Limitations of the Procedure).

Performance characteristics

Zytomed Systems has conducted studies to evaluate the performance of the kit reagents. The product has been found to be suitable for the intended use.

Bibliography

Elias JM Immunohistopathology – A practical Approach to Diagnosis ASCP Press 2003
Nadji M and Morales AR Ann N.Y. Acad Sci 420:134-139, 1983
Omata M et al. Am J Clin Pathol 73: 626-632, 1980

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1. Explanations of the symbols on the product label

Symbols are used in accordance with ISO 15223-1. Further symbols on the product label might be:


GSH02: Flammable


GSH05: Caustic


GSH07: Attention / Warning


GSH08: Systemic health hazards

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