HRP cytochemistry

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A range of substrates is available for the cytochemical staining of peroxidase activity. The most utilized substrates are *diaminobenzidine* for brownish color, *aminoethylcarbazole* for reddish reactions and *chloronaphthol* to give a blue-black product. Thus, in double or multiple staining experiments, the respective antigens can be differently stained in the same tissue section. A selection of enzyme markers and substrates is given in chapters *Enzymes as marker molecules* and *Enzyme cytochemical substrate solutions*.*

For the detection of peroxidase activity at light and electron microscopic levels, the method of RC GRAHAM and MJ KARNOVSKY (1966) is the most widely employed procedure which is based on the oxidation of diaminobenzidine.

Diaminobenzidine technique

Enzyme substrate (HRP)	**Cytochemical feature**
0.05% Diaminobenzidine* (DAB) and 0.01% H_2O_2 • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20ºC (stable for years) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Working solution: 500 µL DAB stock dissolved in 4.5 mL Tris-HCl buffer (0.2 M pH 7.2-7.4)** *plus* 50.0 µL H_2O_2 stock * 3,3'-Diaminobenzidine tetrahydrochloride ** PBS can be also used	**Brown color** Due to the very insoluble reaction product in both aqueous solutions and organic solvents this peroxidase substrate is the most used chromogen for light and electron microscopic studies **Electron dense product (electron microscope)** Enzyme reaction product is further intensified by postfixation in OsO4 (osmium black)
General procedure for immunohistological staining (paraffin sections): – Deparaffinize in xylene: 2 x 5 min – rehydrate with ethanol: 2 x 5 min absolute ethanol; 1 x 1 min 95% ethanol; 1 x 1 min 70% ethanol – distilled water: 2 x 1 min – washing buffer (e.g.PBS/BSA): 2 x 2min	

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* Chromogens and other cytochemical reagents can be toxic and carcinogenic. They must be handled with care

– blocking solution	endogenous peroxidases
– blocking solution	background reactions
– primary antibody	up to 24 hours depending on antibody concentration
– washing buffer	3 x 5 min
– HRP labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
– PBS	3 x 5 min
– 0.2 M Tris-HCl buffer pH 7.2-7.4	rinse
– enzyme substrate, chromogen	10-20 min (variable incubation)
– PBS	2 x 2 min
– distilled water	2 x 2 min,
– postfixation with 0.1% OsO_4	1-3 min (optional)
– counterstain	haematoxylin or other (optional)
– dehydration, mounting	ascending series of ethanol, xylene, mounting medium

Note: intensity of HRP staining can be enhanced at an acid pH or at a neutral pH by the addition of imidazole to the DAB/H_2O_2 substrate mixture. The nitrogenous compound was found to raise the enzyme activity of HRP in test tube assays by about 30%.

Furthermore, the addition of cobalt chloride and nickel ammonium sulfate will intensify the DAB reaction product

Aminoethylcarbazole technique

Enzyme substrate (HRP)	Cytochemical feature
0.05% Aminoethylcarbazole* (AEC) and 0.01% H_2O_2 • AEC stock solution: 0.1 g AEC are dissolved in 10 mL DMF** aliquoted and stored at 4°C (stable for years) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Working solution: 250 µL AEC stock dissolved in 4.7 mL acetate buffer (0.05 M pH 5.5) *plus* 50.0 µL H_2O_2 stock * 3-Amino-9-ethylcarbazole ** N,N-Dimethylformamide	**Red color** The substrate is also useful in combination with other substrates for double stainings

General procedure for immunohistological staining (paraffin sections):

– Deparaffinize in xylene	2 x 5 min
– rehydrate with ethanol	2 x 5 min absolute ethanol 1 x 1 min 95% ethanol 1 x 1 min 70% ethanol

–	distilled water	2 x 1 min
–	washing buffer (e.g.PBS/BSA)	2 x 2min
–	blocking solution	endogenous peroxidases
–	blocking solution	background reactions
–	primary antibody	up to 24 hours depending on antibody concentration
–	washing buffer	3 x 5 min
–	HRP labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
–	PBS	3 x 5 min
–	0.05 M acetate buffer pH 5.5	rinse
–	enzyme substrate, chromogen staining intensity is controlled under the microscope	5-15 min at 37ºC in the dark
–	distilled water	3 x 2 min
–	counterstain	haematoxylin or other (optional)
–	mounting	glycerol/gelatine or other mounting medium

Chloro-naphthol technique

Enzyme substrate (HRP)	**Cytochemical feature**
0.03% Chloro-naphthol (CN)* and 0.01% H_2O_2 • CN stock solution: 0.3 g CN are dissolved in 10 mL absolute ethanol aliquoted and stored at -20ºC (stable for months) • H_2O_2 stock solution: 100 µL 30% H_2O_2 dissolved in 3.0 mL distilled water • Working solution: 100 µL CN stock dissolved in 9.8 mL Tris-HCl buffer (0.05 M pH 7.4-7.6) *plus* 100 µL H_2O_2 stock Substrate solution is stable for about 60 min and must be filtered prior to use * 4-Chloro-1-naphthol	**Blue color** The substrate is also useful in combination with other substrates for double stainings

General procedure for immunohistological staining (paraffin sections):

–	Deparaffinize in xylene	2 x 5 min
–	rehydrate with ethanol	2 x 5 min absolute ethanol 1 x 1 min 95% ethanol 1 x 1 min 70% ethanol
–	distilled water	2 x 1 min
–	washing buffer (e.g.PBS/BSA)	2 x 2min
–	blocking solution	endogenous peroxidases

–	blocking solution	background reactions
–	primary antibody	up to 24 hours depending on antibody concentration
–	washing buffer	3 x 5 min
–	HRP labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
–	PBS	3 x 5 min
–	0.05 M Tris-HCl buffer pH 7.4-7.6	rinse
–	enzyme substrate, chromogen staining intensity is controlled under the microscope	5-20 min at 37°C in the dark
–	distilled water	3 x 2 min
–	counterstain	haematoxylin or other (optional)
–	mounting	glycerol/gelatine or other mounting medium

HRP-GOD coupled technique

In neuroanatomical tracing technique, modifications of the original HRP-DAB method have been described. One proposed way is the use of glucose oxidase (GOD) and D-glucose in the substrate medium by which a continuous release of hydrogen peroxide is generated instead of direct addition of hydrogen peroxide for the marker enzyme HRP (ITOH K et al., 1979; OLDFIELD BJ et. al., 1983; SAKANAKA M et al., 1987; SHU SY et al, 1988). It was suggested that the continuous release of hydrogen peroxide wouldfavor the deposition of DAB reaction products around HRP molecules. This coupled enzyme method together with metal intensification was used successfully in bringing out histochemical and immunoreactive structures.

Enzyme substrate (HRP) (HRP-GOD coupled method)	**Cytochemical feature**
0.05% Diaminobenzidine (DAB), 0,2% β -D-glucose 0.04% ammonium chloride containing 0.3-0.5 mg glucose oxidase (GOD) per 100 mL solution • *0.5% Cobalt acetate for pretreatment: 0.5 g cobalt acetate dissolved in 100 mL Tris buffer pH 7.6* • Ammonium chloride stock solution: 0.4 g NH_4Cl dissolved in 100 mL distilled water • DAB stock solution: 50 mg DAB are dissolved in 10 mL distilled water, aliquoted and stored at -20°C (stable for years) • β-D-Glucose stock solution: 200 mg D-glucose dissolved in 10 mL distilled water, aliquoted and stored at 4°C • Glucose oxidase (GOD, e.g. 250 U/mg) solution: 3 mg GOD dissolved in 10 mL 0.1 M phosphate	**Dark blue/black color** Method to increase the sensitivity of the HRP method, useful in combination with other substrates

buffer pH 7.3

- Working solution:
  1 mL DAB stock *plus*
  5 mL mL phosphate buffer pH 7.3 *plus*
  1 mL glucose stock *plus*
  1 mL ammonium chloride stock *plus*
  100 µL GOD solution *plus*
  1.9 mL 0.1 M phosphate buffer pH 7.3

Procedure for immunohistological staining (paraffin sections):

– Deparaffinize in xylene	2 x 5 min
– rehydrate with ethanol	2 x 5 min absolute ethanol 1 x 1 min 95% ethanol 1 x 1 min 70% ethanol
– distilled water	2 x 1 min
– washing buffer (e.g.PBS/BSA)	2 x 2min
– blocking solution	endogenous peroxidases
– blocking solution	background reactions
– primary antibody	up to 24 hours depending on antibody concentration
– washing buffer	3 x 5 min
– HRP labeled secondary antibody (other detection principle, e.g. biotin-avidin)	30 min
– PBS	3 x 5 min

DAB intensification by metal salts such as cobalt acetate or nickel chloride is frequently in use. In this case, incubation is continued as follows:

– rinse in 0.05 M Tris-HCl pH 7.6	1 x 15 min
– 0.5% cobalt acetate solution	10 min
– wash in 0.1 M Tris buffer pH 7.6	3 x 10 min
– wash in 0.1 M phosphate pH 7.3	2 x 10 min
– enzyme substrate solution several hours at 4°C (optimal incubation time and optimal temperature will depend on experimental conditions)	1-2 hours at room temperature or
– 0.1 M phosphate buffer pH 7.3	3 x 10 min
– distilled water	2 x 2 min dehydrated and mounted as usual
– distilled water	2 x 2 min
– counterstain	haematoxylin or other (optional)
– dehydration, mounting	ascending series of ethanol, xylene, mounting medium

References for further readings

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Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

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