Staining enhancement and signal amplification

WOLF D. KUHLMANN

Laboratory Diagnostics & Cell Science, 56112 Lahnstein

Tyramine amplification

The method of tyramine amplification is based on the ability of HRP to catalyze the deposition of large amounts of biotinylated tyramine in the vicinity of the antigen-antibody reaction (i.e. the cellular site which contains the peroxidase detection complex). The method is based on earlier biochemical observations on the action of peroxidase upon tyramine and other related compounds (GROSS AJ and SIZER IW, 1959). However, the use of peroxidase to catalyze the deposition of phenols as principle for signal amplification in immunoassays was first reported by MN BOBROW et al. (1989). This signal amplification method was called *catalyzed reporter deposition* (CARD). The novel method made use of an *analyte-dependent reporter enzyme* (ADRE) to catalyze the deposition of additional reporter on the surface in a solid-phase immunoassay; horseradish peroxidase was used as ADRE to catalyze the deposion of biotin labeled phenols. The deposited biotins were then reacted with streptavidin-labeled enzyme resulting in deposition of enzyme. Consequently, the deposition of additional enzyme resulted in an amplification of the signal of the ADRE alone which improved the detection limit of the assay.

Tyramine is a phenolic compound and HRP can catalyze the dimerization of those compounds when present in high concentrations. In lower concentrations such as those used in the signal amplification reaction, dimerization is reduced whereas the binding of the highly reactive intermediates to electron-rich moieties of proteins (tyrosine) near the site of the peroxidase binding site is favored. Thus, a large number of hapten molecules can be located at the antigen-antibody site. The CARD signal amplification of MN BOBROW et al. (1989) was first used in immunoblotting and ELISA assays. Subsequently, the tyramine signal amplification using the catalized reporter deposition (CARD) was adapted to immunohistology (ADAMS JC, 1992) and in situ hybridization (SPEEL EJM et al., 1997; SPEEL EJM et al., 1999) in order to increase the sensitivity of antigen and DNA detection, respectively, which may not be possible by other means.

Incubations in primary antibody, linking antibody and HRP conjugated avidin (strepavidin) detection complex are done as usual, followed by incubation with biotinylated tyramine. HRP from the detection complex will then catalyze the deposition of biotinylated tyramine at the site of the antigen-antibody reaction; this will increase the number of biotin in that region. Then, another incubation with HRP conjugated avidin detection complex follows, and additional detection complex will bind to biotin deposited in that area. Finally, the number of HRP molecules has increased at the site of antigen, and upon chromogen incubation the detection signal is stronger than with the routine method.

Background reactions, however, can be a serious problem, especially with tissues containing high amounts of endogenous biotin that will bind the detection complex. It is generally necessary to block endogenous peroxidases as well as biotin and avidin activities. Also, incubation times and tyramine concentrations must be optimized for each tissue.

Another modified Tyramide Signal Amplification System of high sensitivity consists in incorporating a signal amplification method based on the HRP catalyzed deposition of a fluorescein labeled phenolic compound, followed by a secondary reaction with a HRP conjugated anti-fluorescein. In a typical staining procedure, the bound primary antibody is first detected with HRP conjugated secodary antibody. Then, the bound HRP is used to catalyze the oxidation of a fluorescein labeled phenol (fluorescyl-tyramide) which precipitates onto the specimen. The staining procedure is continued with the detection of the bound fluorescein by a HRP labeled anti-fluorescein. The cytochemical procedure is completed by incubation in enzyme substrate (diaminobenzidine/hydrogen peroxide). Tyramide signal amplification systems are commercially available (e.g. Dako CSA Systems).

Polymer-based detection strategy

The method is based on a reagent in which multiple molecules of linking antibodies (secondary antibodies) and marker enzyme are conjugated to a polymer backbone; polymer based detection systems are commercially available (e.g. Dako EnVision™). Tissue specimens are first incubated with primary antibody, then, in a second step, the polymeric reagent is applied. With the second step, the usual sequential incubations in link antibody and detection complex are combined into a single step.

When comparing the polymer based detection system with other enhancing techniques such as the labeled streptavidin-biotin system (LSAB) or the avidin-biotin complex system (ABC), then it turned out that the polymer based principle is an easy to use system which was at least as sensitive as the other mentioned enhancing methods (SABATTINI E et al., 1998; VYBERG M and NIELSEN S, 1998; SHI SR et al., 1999). The polymer based sytem allowed up to fivefold higher dilutions of primary antibodies than the ABC, LSAB or APAAP methods. Thus, the polymer based detection principle combines simplicity of immunostaining procedure in the routine laboratory with high sensitivity (equal or higher sensitivity than the avidin-biotin procedures). Finally, with the polymer based detection system false-positive staining reactions (background) can be avoided and overcomes problems with endogenous biotin or avidin.

Another polymerized reporter enzyme staining system is realized by Vector Laboratories Inc. (ImmPRESS™) that is based on polymerizing enzymes and attaching these polymers ("micropolymers") to antibodies. This approach avoids the drawback of using large dextrans or other macromolecules as backbones inasmuch as attaching the "micropolymers" with their high density of active enzyme to secondary antibodies generates a reagent with enhanced accessibility to the target and outstanding sensitivity and specificity.

Enzyme metallography

Enzyme metallography is a quite new and simple method for immunohistology and in situ hybridization. in which a targeted enzyme conjugate mediates the deposition of metal from a solution to give a dense, sharply defined black signal in the light microscopy. The reaction product is equally useful for electron microscopy. Enzyme metallography is an excellent tool for high-resolution differentiation of cellular compartments (HAINFELD JF and POWELL RD, 2000; MAYER G et al., 2000; POWELL RD et al., 2002; FURUYA FR et al., 2004; DOWNS-KELLY E et al., 2005; TUBBS R et al., 2005; POWELL R et al., 2006).

In contrast to the technique of autocatalytic metal deposition such as silver enhancement staining of gold structures, the enzymatic activity of redox enzymes, in particular horseradish peroxidase (HRP) is used to catalyze the selective deposition of metal from solution. Thus, in the enzyme metallography process, HRP conjugates are used as probes, which in the presence of a metallographic substrate (an appropriate metal source, e.g. silver ions, and activating agents) selectively deposit metal at the site of the targeted substance. The resultant stain is highly localized and electron dense providing higher resolution than most organic chromogens. Background reactions are very low as compared to reactions of typical enzyme cytochemical substrates. Enzyme metallographic reagents can be obtained from Nanoprobes Inc. (EnzMet™ Kit). The exact composition of EnzMet™ ist not known. Fundamentally, the kit contains a silber salt solution, an acid buffer and H_2O_2 as substrate for HRP; electrons for the reduction of silver ions are provided by oxidation of hydrogen peroxide.

Staining enhancement with cobalt and nickel salts

The intensity of immunostainings can be improved by metal salts. In some methods, cobalt or nickel salts are added to the DAB substrate solution (details in chapter *Enzyme cytochemical substrate solutions*). Also, post-chromogenic enhancement is popular. This involves placing the slides in solutions which enhance the DAB reaction product. Currently we use osmium tetroxide with good results, but copper sulfate is also good for this purpose.

Rolling circle amplification (RCA) reporter system

The rolling circle amplification is a combined reaction. The immunologic reaction of the specific antibody (conjugated with RCA primers) with tissue antigen is done in a first step. Then, the bound conjugate is hybridized with a circularized oligonucleotide probe homologous to the RCA primer; RCA reactions are then run in an isothermal nucleic acid amplification mode with DNA polymerase and nucleotides. The enzymatic synthesis yields a high molecular weight DNA which remains attached to the primary antibody. Finally, the amplified DNA is stained depending on the labeled decorater probes, i.e. oligonucleotides labeled for example with biotin, fluorochrome or enzyme such as HRP. In the case of biotin labeling, the complex is visualized by incubation with streptavidin-HRP conjugates followed by enzyme substrate. In the case of oligonucleotides-HRP conjugates, the final complex is revealed by incubation in the appropriate enzyme substrate.

The difference between RCA and PCR is that the former amplified nucleic acid sequences remain connected to the target molecule. A hallmark of linear RCA is also the capability to localize multiple biomolecules simultaneously. The specificity of immuno-RCA is derived from the antigen-antibody interaction (in the tissue section), and its signal amplification from nucleic acid hybridization and synthesis. Problems of multiple steps of antibody mediated labelings and amplification are avoided. The linear mode of RCA can produce a high signal amplification during a brief enzyme reaction, and the achievable sensitivity is high enough to enable single antigen-antibody complexes to be detected. The main advantage of RCA is the significant signal increase that is achieved through the linear mode of enzymatic amplification of a nucleic acid substrate in an exponential mode to generate 10^5-fold signal amplification (SCHWEITZER B et al., 2000; GUSEV Y et al., 2001). The increased specific signals by the immuno-RCA technique will not increase the background as will be often observed with the tyramine amplification method where a multilayered amplification process is needed.

Selected publications for further readings

Gross AJ and Sizer IW (1959)
Bobrow MN (1989)
Adams JC (1992)
Speel EJM *et al*. (1997)
Sabattini E *et al*. (1998)
Vyberg M and Nielsen S (1998)
Shi SR et al. (1999)
Speel EJM *et al*. (1999)
Hainfeld JF and Powell RD (2000)
Mayer G *et al*. (2000)
Schweitzer B *et al*. (2000)
Gusev Y *et al*. (2001)
Powell RD *et al*. (2002)
Furuya FR *et al*. (2004)
Downs-Kelly E *et al*. (2005)
Tubbs R *et al*. (2005)
Powell R *et al*. (2006)

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

 11.08.2008