Methods and reagents

Chemiluminescent detection methods

Methods and reagents is a unique monthly column that highlights current discussions in the newsgroup bionet.molbio.methds-reagnts, available on the Internet. This month's column discusses some tips for using chemiluminescent detection methods. For details on how to partake in the newsgroup, see the accompanying box.

Detecting labeled biomolecules, such as DNA, RNA and proteins, is an essential part of everyday lab bench work. Although radioactive isotopes have traditionally been used for this, there is an ever-growing need for researchers to break those traditional bonds. Apart from the obvious concern over health risks involved with constant exposure or radioactive contamination by spills and other mishaps, there are also the cost considerations associated with storage, handling, collection and disposal of radioactive materials.

In the past, researchers have been slow to adopt non-radioactive methods because of low sensitivity compared with isotopic detection, and most likely because they often encountered variable results when they were first introduced to the commercial market. For example, initial attempts at using an alkaline phosphatase-based chromogenic detection method proved frustrating. The precipitation of an insoluble blue compound obtained through the interaction of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium salt (NBT) onto membranes[1] did not always give the best hands-on experimental results.

There's no excuse anymore. Having been thoroughly researched and modified to eliminate the variable precipitation steps, the newer chemiluminescent detection methods are sensitive and dependable enough to challenge conventional radioactive labeling and detection methods.

New kits on the block

The most commonly used systems for chemiluminescent detection are: (1) the luminol-based 'enhanced' chemiluminescence (ECL)[2–4] catalysed by horseradish peroxidase (HRP) in the presence of peroxides; and (2) the destruction of dioxetane compounds such as DPP, AMPPD, CSPD or CDP-Star, catalysed through dephosphorylation by alkaline phosphatase (AP)[5,6]. The breakdown reactions of these two systems each give off light, which is easily detected by imaging onto standard X-ray film. Both HRP- and AP-detection methods can be used in combination with either affinity conjugation systems of streptavidin plus biotin, or digoxigenin plus anti-digoxigenin antibody.

One problem remaining with the use of non-radioactive methods is that the materials for the detection are usually only available in kit form. Because the detection substrates comprise approximately 90% of the cost of the entire procedure, and the cost is nearly US$3–5 per Southern or western blot, buying a new kit just for the detection components or substrate is just too expensive.

Do-it-yourself kits

Thomas Cameron (cameron@risotto.mit.edu) recently wrote to the methods newsgroup asking about how he might make his own reagents for the ECL reactions, and several people posted their preferred method for preparing homemade chemiluminescent detection solutions.

David Shire (david.shire@tls1.elfsanofi.fr) wrote that obtaining good results with the ECL system mainly relies on the purity of the *p*-iodophenol (PIP) and the luminol. The PIP is needed for enhancing the visible light reaction by acting as a co-factor for peroxide activity toward luminol, rather than participating directly in the luminescence itself. When phenolic enhancers are used in combination with HRP, the level of light is said to be increased about 100-fold (Ref. 7). This is why the reaction has been dubbed 'enhanced' chemiluminescence[2] or 'ECL' (not to be confused with electrochemiluminescence, which has also been called by the same acronym ECL)

David Shire suggests that the cheapest PIP be purchased from Aldrich or Fluka and then sublimed in-house at 85°C under vacuum. He wrote that this is extremely easy to do and results in beautiful white crystals of pure iodophenol as compared to the mucky yellow powder obtained from the chemical supply company. He says that the commercially available apparatus to do this is expensive, but that a cheaper homemade version can be made for little cost.

Purifying your own PIP can be dangerous and some recommend simply buying the highest grade of purified PIP instead. Netters warn, however, that not all batches of purified PIP work as well as others, and some have complained about poor results from PIP obtained from various chemical companies.

To prepare luminol, David Shire says to suspend 3 g of the cheapest luminol you can find (available from Merck–Schuchard, Fluka or Lancaster Synthesis) in 90 ml of 1-butanol and to add 40 ml of 1M NaOH. After the luminol is dissolved by warming and agitating, 30 ml of methanol is added and the recipient cooled in ice before adding 40 ml of 1M HCl. This should then be left at −20°C for 30 min for precipitation. The crystals are then filtered onto a Whatman No. 1 filter, rinsed with dichloromethane and dried under vacuum. To protect the crystals from light, the bottle or tube should be wrapped with a piece of aluminum foil and placed in a dessicator.

The two solutions for the ECL detection protocol are prepared as follows. Solution 1 contains 780 μg ml^{-1} of luminol and 950 μg ml^{-1} of PIP in 0.1M Tris-HCl, pH 9.35. Solution 2 contains 100 μl 30% hydrogen peroxide in 1 l of 0.1M Tris-HCl, pH 9.35. Just before use, equal volumes of each solution are mixed together. One other person wrote that a cocktail composed of 1 ml luminol (4 mg ml^{-1} in DMSO), 1 ml *p*-iodophenol (1 mg ml^{-1} in DMSO) 0.6 ml of 1M Tris-HCl (pH 7.5), 5 μl of 30% H_2O_2, and 7.5 ml dH_2O works equally as well.

Netters say that homemade reagents produce excellent results and cost much less than buying a complete pre-packaged kit. In addition, side-by-side comparisons

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