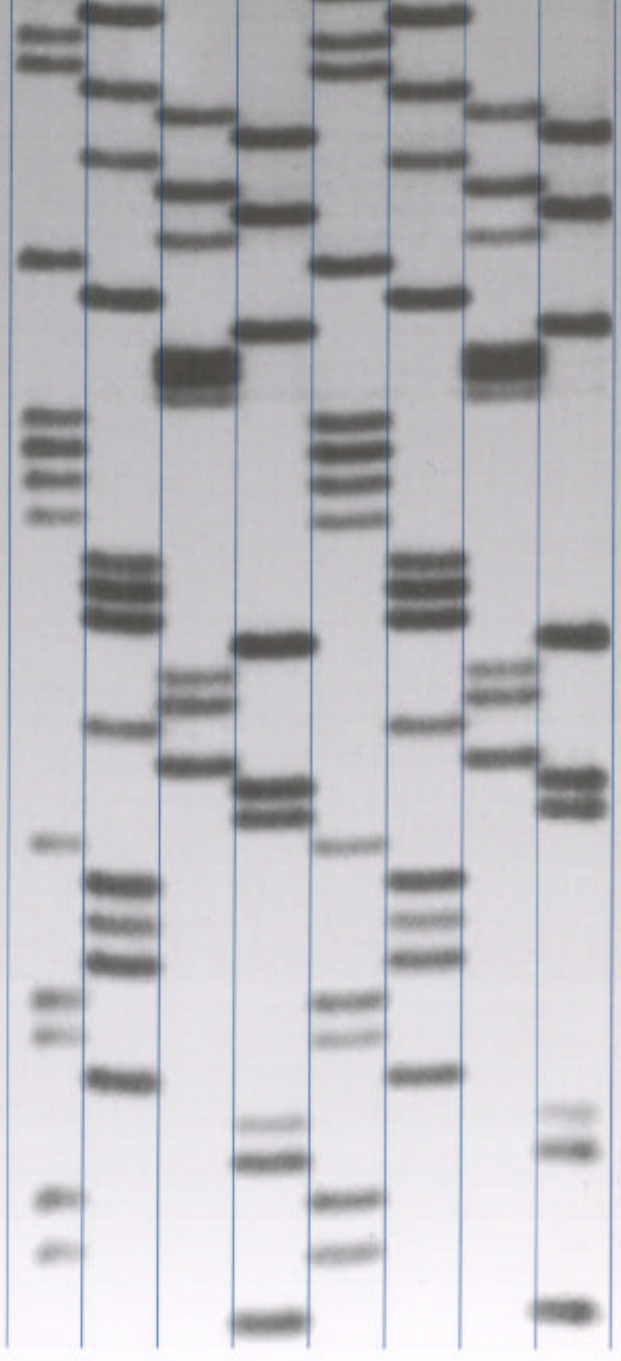
Review 23

Efficient Detection of Biomolecules by Autoradiography, Fluorography or Chemiluminescence

by R A Laskey



Amersham Biosciences

Efficient Detection of Biomolecules by Autoradiography, Fluorography or Chemiluminescence

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Methods of detecting biomolecules by autoradiography, fluorography and chemiluminescence

Choice of film and conditions for exposing it to the sample

Direct autoradiography is most efficient on `direct' types of film.

`Direct' films of this type are relatively insensitive to the blue to ultraviolet light emitted by intensifying screens, scintillators or the ECL chemiluminescence reaction. Therefore when emissions arc converted to light, a `screen type' radiographic film should be used such as Hyperfilm-MP from Amersham. As their generic name implies, these films are specifically designed to respond efficiently to the blue to ultraviolet light emitted from radiographic intensifying screens. Scintillators, such as Amplify and PPO or ECL chemiluminescence systems emit light of similar wavelengths to screens (figure 9) hence the same film is suitable for fluorography, intensifying screens and chemiluminescence.

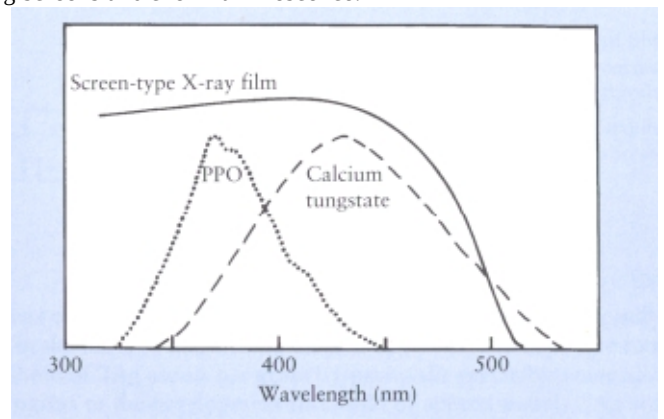

Figure 9. Sensitivity spectrum of a standard screen-type radiographic film compared to the emission spectra of the scintillator PPO (in solution), and a calcium tungstate intensifying screen.
The emission spectrum of the ECL detection system also falls within the film sensitivity spectrum, with an emission maximum at 428nm.

Films which have been produced for direct autoradiography of ^{3}H are not good substitutes for Hyperfilm-MP for detecting the light emitted from organic scintillators, intensifying screens or the ECL system. Since they lack the surface anti-scratch layer they detect any weak tritium β-particles which emerge from the sample relatively efficiently, but the main problem limiting the efficiency of tritium autoradiography is self-absorption within the sample. This problem is greatest for thick samples like polyacrylamide gels for which conversion of tritium β-particles to light remains essential for efficient detection.

For optimal sensitivity and resolution it is important for the film to be pressed into close contact with the sample or intensifying screen. The most convenient method for achieving this is by enclosing the film and the sample inside a radiographic film cassette such as Amersham's Hypercassette™ range. These are light-proof and they stack conveniently in -70°C freezers (although stacking is not advisable for samples containing ^{32}P or ^{125}I). When intensifying screens are used they should be positioned as shown in figure 10.

Figure 10. Relative positions of film and sample for using (a) a single intensifying screen[2] or (b) two intensifying screens with a translucent sample such as a gel supported by white filter paper[13]. While use of two screens in this way increases sensitivity, it decreases resolution. Note: It is important to ensure that the correct face of the intensifying screen is in contact with the film and sample - see manufacturer's instructions.

When cassettes are not available, the film and sample (with a screen when appropriate) can be clamped between glass plates, using strong spring clips, and sealed into an opaque envelope.

Whenever possible, attaching adhesive tape to the film should be avoided since its removal generates light which exposes the film artefactually. However, attachment of Amersham's Tracker Tape™ to the sample provides a convenient means of aligning the sample with the film.

Excessive exposure to dark-room safelights should be avoided, especially after film has been hypersensitized by pre-flashing.

The importance of pre-flashing and low temperature exposure for detecting light emitted from intensifying screens and scintillators is emphasized in part I. After exposure, film should be developed according to the manufacturer's instructions. Most blue-sensitive, 'screen-type' films are suitable for automated processors and several compact, bench-top processors are currently available such as Amersham's Hyperprocessor. To avoid artefacts, it is advisable to allow films to warm before processing if they have been exposed at -70°C.

Use of intensifying screens to visualize γ-rays or high-energy β-particles

Most of the energy of γ-rays or strong β-particles is wasted by passage through and beyond the film. Fortunately materials have already been produced to solve this problem in medical radiography. To increase the efficiency of detection of X-rays, and thus to minimize the exposure of patients to X-rays, the film is placed between two dense inorganic fluorescent screens. The screens absorb emissions more efficiently than film and their fluorescence superimposes a photographic image over the radiographic image on the film.

To apply intensifying screens to isotope detection, it is necessary to overcome the inefficient response of film to the extremely low intensities of light produced by low levels of radioactivity in autoradiographic exposures. As explained in part I, this problem is partly overcome by cooling the film to -70°C or by pre-exposing film to a flash of light[2]. It is completely overcome by the combination of low temperature and pre-exposure. This combination gives optimal sensitivity and allows quantitation of the image because it gives a linear response of film to the amount of radioactivity (see part I).

A single calcium tungstate intensifying screen placed beyond the film, as in figure 10a, can increase sensitivity 10-fold for ^{32}P and 16-fold for ^{125}I compared to direct autoradiography (table 3). However, this effect is achieved at the expense of some resolution loss (figure 2). Greater sensitivity can be achieved with translucent samples, including gels dried on filter paper, by placing a

second screen beyond the sample (figure 10b) so that the photons this screen produces can pass back through the sample to reach the film[13]. This method gives optimum sensitivity, but poor resolution because the film is not in close contact with that screen.

Note that the widespread practice of inserting two screens into each cassette sacrifices resolution compared to the less expensive alternative of using only one screen per cassette.

Intensifying screens can be used for any type of flat sample containing ^{32}P or γ-emitting isotopes. Screens do not improve detection efficiency of ^{3}H, ^{14}C or ^{35}S since these less energetic β-particles do not penetrate through film[2, 13]. Screens have no effect on chemiluminescence.

Screens made from calcium tungstate appear to be the most satisfactory for radioisotope detection (for example, Hyperscreen). Although other materials may be available some alternative screen materials have been found to contain traces of endogenous radioisotopes which blackened hypersensitized film spontaneously at -70C without requiring a sample[2]. Although these levels of endogenous radioactivity are insufficient to be detected in very short radiographic exposures at ambient temperature they blacken pre-flashed film at up to 0.4 absorbance units per week at -70°C. Amersham's Hyperscreen range has been introduced specifically for radioisotope detection rather than for medical radiography. It is essential that a 'screen-type' film (such as Hyperfilm-MP) should be used. Direct films such as Hyperfilm β-max are less sensitive to the blue or ultraviolet light emitted by intensifying screens.

When wet gels are frozen at -70°C ice crystal formation can distort the gel and decrease resolution. Drying the sample overcomes this problem. Alternatively, screens can be used at ambient temperature provided that the film is pre-exposed[2]. The efficiency is approximately 50% of that obtained at -70°C. The response of the film shows slight deviation from linearity, but it is very much more linear than untreated film at -70°C [2].

Impregnation of samples with scintillators for fluorographic detection of weak β-emitters

Weak β-particles such as those emitted by ^{3}H, ^{14}C, ^{35}S, and to a lesser extent ^{33}P, are absorbed within the sample so they are recorded inefficiently by film. In 1958 Wilson[7] showed that this problem can be overcome by incorporating a scintillator into the sample to convert the emissions to light. Since then this principle has been applied to thin-layer chromatograms, nitrocellulose sheets

and gels consisting of polyacrylamide or agarose. For samples containing water, such as acrylamide gels, it is necessary either to use an aqueous scintillator such as Amersham's Amplify or to replace the water by a suitable solvent for the scintillator.

After impregnation the sample is dried and exposed to a blue-sensitive, screen-type film such as Hyperfilm-MP under the conditions described in preceding sections.

Fluorography of acrylamide gels

The most convenient method for impregnating acrylamide gels with a scintillator is by soaking in Amplify. Compared to alternative methods, Amplify is safer, more convenient and requires only a small fraction of the time. Compared to direct autoradiography, impregnation of the gel with a scintillator increases the detection efficiency over 1000-fold for ^{3}H and over 10-fold for ^{35}S or ^{14}C[1].

After impregnation with Amplify, gels are dried by heating under vacuum and exposed to a pre-flashed screen-type radiographic film such as Hyperfilm-MP at -70°C.

Alternative earlier methods exist for impregnating acrylamide gels with a scintillator. The first used dimethyl sulphoxide as a solvent to carry the scintillator PPO (2, 5 diphenyloxazole) into the gel[12] . This method requires complete removal of water from the gel by equilibration with changes of dimethyl sulphoxide, or PPO will be precipitated without entering the gel. Hence the method is laborious. It is also potentially hazardous since dimethyl sulphoxide passes through skin and the consequences of subcutaneous deposition of PPO are unknown.

Other methods which have been developed include impregnation of gels with sodium salicylate[15] or with PPO dissolved in glacial acetic acid[16]. The sodium salicylate method suffers the disadvantage of a slightly mottled image. A possible cause of this problem is reflection of light from the surfaces of large crystals of salicylate. Salicylate also offers potential health hazards[15].

All of the methods for impregnating acrylamide gels with scintillators achieve roughly similar detection efficiencies (references 15, 16 and unpublished observations). It appears unlikely that there will be further substantial increases in efficiency arising from improved impregnation procedures since existing procedures already approach the quantum emission efficiency of liquid scintillation counting[12]. In contrast there is scope for further improvements in the quantum detection efficiency of photographic emulsions.

Fluorography of agarose gels

Agarose gels are as easy to impregnate with scintillators as acrylamide gels, provided that excess physical agitation is avoided and provided that the impregnation procedure removes ethidium bromide from stained gels. Unless it is removed, ethidium bromide heavily quenches the ultraviolet light emitted from scintillators, drastically decreasing efficiency. Soaking in ethanol can remove ethidium bromide and then gels can be impregnated by Amplify. Alternatively uniform impregnation of agarose gels can be achieved using 3% PPO in ethanol as described in reference 17. Ethidium bromide is removed at the same time. Dimethyl sulphoxide cannot be used as the solvent because it dissolves the gel.

Fluorography of nitrocellulose filters and paper or thin-layer chromatograms

Fluorography gives little gain over the sensitivity of direct autoradiography for ^{14}C, ^{35}S or ^{33}P on very thin samples such as filters or chromatograms. However, fluorography substantially increases sensitivity for ^{3}H in these samples. The published methods use a range of organic solvents to deliver the scintillator PPO to nitrocellulose filters[18], paper chromatograms[19] or thin-layer chromatograms [11].

Procedure for hypersensitizing film by pre-exposure to a flash of light ('pre-flashing')[1]

For optimal sensitivity in long exposures and for quantitation of film images obtained with intensifying screens, fluorography or chemiluminescence, it is essential to by-pass the reversible stage of latent image formation[1].

This is achieved by pre-exposing film to an instantaneous flash of light (duration approximately 1msec). Longer flashes increase the background without hypersensitizing the film (see part I). A suitable presensitizing flash is provided by Amersham's Sensitize unit which is supplied fitted with suitable attenuation filters.

Alternatively, conventional electronic photograph flash units or stroboscopes, but not most photographic enlargers, can be adapted. The intensity of the flash is adjusted to increase the absorbance of the developed film to 0.15 (A_{540}) above that of unexposed film. The wavelength of light used is unimportant, but adjusting the wavelength provides a convenient means of regulating the effective

light intensity, since screen-type radiographic films are most sensitive to the blue end of the spectrum (figure 9). Therefore orange filters (for example Wratten number 21 or 22) decrease the light output from photographic flash units to approximately the correct level. The Sensitize unit is already fitted with appropriate filters.

The exact light intensity required varies with the type of flash unit and film, and therefore must be determined experimentally. Apart from wavelength, adjustments are easily made by varying the distance of the flash unit from the film, or the diameter of an aperture in an opaque mask, or by adding neutral density filters. Long distances between the film and the flash unit give the most uniform pre-exposure. Trial exposures under varied conditions are most easily made by placing an opaque mask with a clear window at different positions on a single film. The fog levels achieved can be monitored either by a densitometer or by placing film sections in a spectrophotometer.

Note that linearity of the film response is obtained only when the fog level has been raised between 0.1 and 0.2 (A_{540}) above that of untreated film by a single flash and only when the flash is approximately 1 msec or less (see part I).

Procedures for double-isotope experiments

Double-isotope experiments are the only application for which gel slicing may still have advantages over fluorography. However, it is possible to distinguish ^{3}H from other isotopes in a fluorograph by varying the exposure conditions [20]. Thus fluorographic exposure of pre-flashed film at -70°C will efficiently detect scintillator fluorescence from ^{3}H as well as from other isotopes, but exposure of untreated film at ambient temperature will not record the fluorographic tritium image efficiently, though it will still record the direct autoradiographic image from higher energy radioisotopes. Since fluorography on pre-exposed film at -70°C and direct autoradiography on untreated film at ambient temperature are both linear (see part I), comparison of the two densitometer traces resolves the distribution of the two isotopes.

An alternative method has been described for discriminating between two populations of newly synthesized proteins using the combination of [^{35}S]methionine and [^{75}Se]selenomethionine[21].

Optimizing sensitivity and resolution

The resolution obtained in a film image from radioisotopes depends on the path length of the radioactive emission and thus on the energy and type of emission from the isotope. Tritium gives excellent resolution regardless of whether a fluorographic scintillator is used. In contrast, γ-emitters such as ^{125}I give relatively poor resolution with or without an intensifying screen. As shown in figure 2 an intensifying screen significantly lowers resolution for ^{32}P. Use of two screens, as in figure 10(b), lowers resolution much further. Furthermore those films and screens that give highest sensitivity give lowest resolution. In the case of ^{32}P optimal sensitivity and resolution are mutually exclusive. The shorter path length of β-particles from ^{35}S has led to this isotope partly replacing ^{32}P for DNA sequencing where resolution is more important than sensitivity. Similarly, ^{33}P may partly replace ^{35}S by offering an even better combination of sensitivity and resolution.

Both sensitivity and resolution of all detection methods which use film are increased by maintaining close contact between the film and the sample and by keeping the sample as thin as possible. When intensifying screens are used they should also be pressed close to the film.

Resolution and sensitivity when using intensifying screens or scintillators both depend critically on the temperature of exposure and on pre-exposure of the film as discussed in detail in part I. Although images obtained on 'non-pre-flashed' film cannot be quantitated accurately, they offer exaggerated resolution because the base of each peak is preferentially suppressed (figure 7). In spite of this phenomenon, resolution of ^{32}P remains higher by direct autoradiography.

Quantitation of film images

Images obtained by direct autoradiography or on pre-exposed film using intensifying screens, scintillators or chemiluminescence can be quantitated accurately by densitometry. However, as explained in part I, pre-exposure is essential for accurate quantitation of light produced from screens, fluorographs or chemiluminescence. Without correct pre-exposure low levels will be under-represented[1, 2]. In contrast direct autoradiography without screens or scintillators is inherently linear, allowing accurate quantitation without pre-exposure. In these circumstances blackening of the film, and hence the area under each peak of the densitometer profile, is proportional to the distribution

of radioactivity. If necessary this relationship can be confirmed by physical fractionation of the sample, for example slicing gels or scraping thin-layer plates and preparing each fraction for scintillation counting. Destructive methods of this sort cannot match the resolution of film detection but they can defend against mistakes in quantitation. By far the most common mistake is densitometry of images from chemiluminescence or intensifying screens on film which has not been pre-flashed. This mistake is responsible for frequent misinterpretations (see part I).

Although pre-flashing film can correct the non-linear response of film to the light emitted from intensifying screens, scintillators or chemiluminescence, a linear film response is obtained only when the fog level is raised to between 0.1 and 0.2 absorbance units above the fog levels of unexposed film and only when the flash is instantaneous (approximately 1 msec). Longer flashes raise the background without hypersensitizing. The Sensitize unit is designed for this purpose. Raising the fog level above 0.2 reverses the deviation from linearity, over-representing small amounts of radioactivity (figure 7 and part I), rather than under-representing them.

Accurate quantitation also requires levels of exposure which do not saturate the film. Absorbancies above 1.5 should be avoided for this reason.

Peak areas are more reliable than peak heights for quantitative comparisons.

Avoiding technical problems

Problem - High background

	Possible cause	Remedy
1.	Darkroom not light-tight	Identify leak by standing in unlit darkroom with eyes closed for 30 seconds then look for light sources. Plug gaps.
2.	Light source in darkroom (e.g. instrument panels)	Cover or remove.
3.	Safelight filter incorrect	Replace with compatible filter.
4.	Safelight too bright	Replace with lower power bulb.
5.	Safelight too close to work area	Reposition either safelight or work area - alternatively work without a safelight using Amersham's Hypertorch™.
6.	Film exposed to safelight for too long	Minimize delay before placing in autoradiography cassette.
7.	Film stored or used near radioactive source	Check work/storage area with appropriate monitor. Remove or shield source.
8.	Film stored at incorrect temperature	Check storage temperature and rectify problem.
9.	Film past recommended storage time	Order film in smaller packs.
10.	Processing procedure	Check manufacturer's recommendations and rectify fault.
11.	Processing chemicals exhausted	Check replenishment rate and establish 'housekeeping' rota.
12.	Preflash too intense	Recalibrate flash unit positioning.
13.	Phosphorescent emission from intensifying screens	Adapt screens to dark for 30 seconds prior to use.
14.	Film previously exposed to light	Check other sheets of film from the box and discard if exposed.

Problem - Image too faint

	Possible cause	Remedy
1.	Wrong detection procedure selected	See table 3.
2.	Wrong sensitivity expected	See table 2.

3.	Wrong film type	See table 3.
4.	Exhausted developer	Replace with fresh; check replenishment rate.
5.	Wrong exposure temperature	Use -70°C for fluorography or intensifying screens.
6.	Pre-flash omitted	Check procedures.
7.	Pre-flash too long	Purchase flash unit with duration < 1 m second (e.g. Sensitize).
8.	Stain in gel quenching emitted light	Do not prestain gels to be used for fluorography if sensitivity is a problem.
9.	Gel not dry	Dry gel thus reducing thickness.
10.	Sample not close enough to film	Use autoradiography cassette.

Problem - Poor resolution

	Possible cause	Remedy
1.	Poor contact between film sample or intensifying screen	Use specialised autoradiography cassette.
2.	Ice crystals distorting frozen wet gels	See table 3. Dry the gel or use pre-flashed film at ambient temperature.
3.	Diffusion of bands by immersing in scintillator solution too long	Keep to appropriate protocol.
4.	Resolution lost through use of intensifying screens. This problem is made worse when two screens are used	Minor inherent problem with use of intensifying screens.
5.	Urea in gel	Remove by soaking in 7% acetic acid.

6.	Scintillator concentration too high, especially for low gel concentrations such as agarose	Keep to appropriate protocol.
7.	Sample too thick	Cast thinner gels.

Problem - Artefactual local blackening

	Possible cause	Remedy
1.	Contaminated cassette	Wipe cassette out after each use.
2.	Light leaking into cassette or film box	Check with sheet of film, dispose of faulty cassette.
3.	Adhesion of film to adhesive tape, 'cling film' or damp samples (especially gels containing residual dimethyl sulphoxide). Detaching film generates static electricity and light	Avoid adhesive tape (except Tracker Tape). Keep sample as dry as possible; avoid condensation. Avoid cling film or, where necessary, use Saranwrap™, a form of cling film which sticks less to radiography film.
4.	Roller marks from use of automatic processor with very cold or damp film	Allow cassette to warm before removing film after exposure at -70°C.
5.	Physical damage to film (bending, scratching)	Exercise more care when handling film.
6.	Contamination by chemicals	Keep area clean.

Problem - Image patchy

	Possible cause	Remedy
1.	Poor contact between film and sample or intensifying screen	Use specialised autoradiography cassette.
2.	Dirt on intensifying screen absorbing light	Keep screens clean; wipe with mild detergent.

3.	Incomplete impregnation with fluorographic scintillator	Ensure agitation of gel.

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Exposure conditions

Expose direct autoradiographs at ambient temperature without pre-flashing film. For exposures with intensifying screens or fluorographs use pre-flashed film at -70°C for optimal sensitivity and quantitative accuracy (see part I). For chemiluminescence use pre-flashed film at ambient temperature.

Table 3. **Key to selecting detection method**

Label	Application	Objective	Method	Materials
^{32}P	Southern, Northern blots, most gels Plaque, colony lifts	Max sensitivity	Pre-flash and screens at -70°C	Hyperfilm-MP Hyperscreens
	DNA sequencing	Max resolution	Direct autoradiography	Hyperfilm-MP
^{35}S ^{14}C ^{33}P	Protein synthesis, lysates, most gel uses	Max sensitivity	Pre-flash and fluorography at -70°C	Amplify Hyperfilm-MP
	Southern, Northern and Western blots, DNA sequencing *In situ* hybridization	Max resolution	Direct autoradiography	Hyperfilm-βmax
	Thin layer/paper chromatograms	Max resolution	Direct autoradiography (slight sensitivity enhancement by fluorography)	Hyperfilm-βmax
^{3}H	Protein synthesis, lysates, all gel uses	Max sensitivity	Pre-flash and fluorography at -70°C	Amplify Hyperfilm-MP
	In situ hybridization	Max resolution	Direct autoradiography	Hyperfilm-^{3}H
^{125}I and other γ emitters	Western blots	Max sensitivity	Pre-flash and screens at -70°C	Hyperfilm-MP Hyperscreens
	In situ hybridization	Max resolution	Direct autoradiography	Hyperfilm-^{3}H/ βmax
Chemi-luminescence	Southern, Northern, Western blots	Max sensitivity and resolution	Pre-flash and expose at room temperature	Hyperfilm-ECL Hyperfilm-MP

Glossary

Amplify: reagent for efficient detection of weak β-emitting radioisotopes by fluorography of gels and similar samples.

Autoradiography: detection of radioisotopes by formation of an image on film.

Blotting: (Southern, Northern, Western). Detection of specific protein or nucleic acid molecules from within a mixture, by their affinity for a labelled probe (usually an antibody or a complementary nucleic acid sequence). Detection usually involves transfer of electrophoresed molecules from a gel to a membrane filter by 'blotting'.

Cassette: flat, light-proof, metal (or plastic) container used to hold radiographic film in close contact with flat sample and intensifying screen.

Chemiluminescence: emission of light as a result of a chemical reaction.

Densitometry: measurement of the density of a film image by scanning the film with a light beam.

Direct autoradiography: detection of radioisotopes by direct interaction of radioactive emissions with the film in the absence of scintillators or intensifying screens.

Fluorography: detection of weak β-emitters by converting emissions to light within the sample, so that the light produces a photographic image on the film.

Hypersensitizing: see pre-flash.

Intensifying screens: flat plastic screens impregnated with an inorganic scintillator such as calcium tungstate. Originally designed for medical radiography, they absorb strong radioactive emissions more efficiently than film and emit light which superimposes a photographic image over the autoradiographic image

Pre-flash: pre-exposure of film to a 1msec flash of light to bypass the reversible stage of latent image formation. It has no effect on direct autoradiography but increases sensitivity and allows quantitation of images from fluorography and intensifying screens.

Probe:	a labelled molecule which recognises and binds specific molecules within a mixture. It can be labelled radioactively or chemiluminescently.
Scintillator:	a molecule which emits light in response to ionizing radiation.