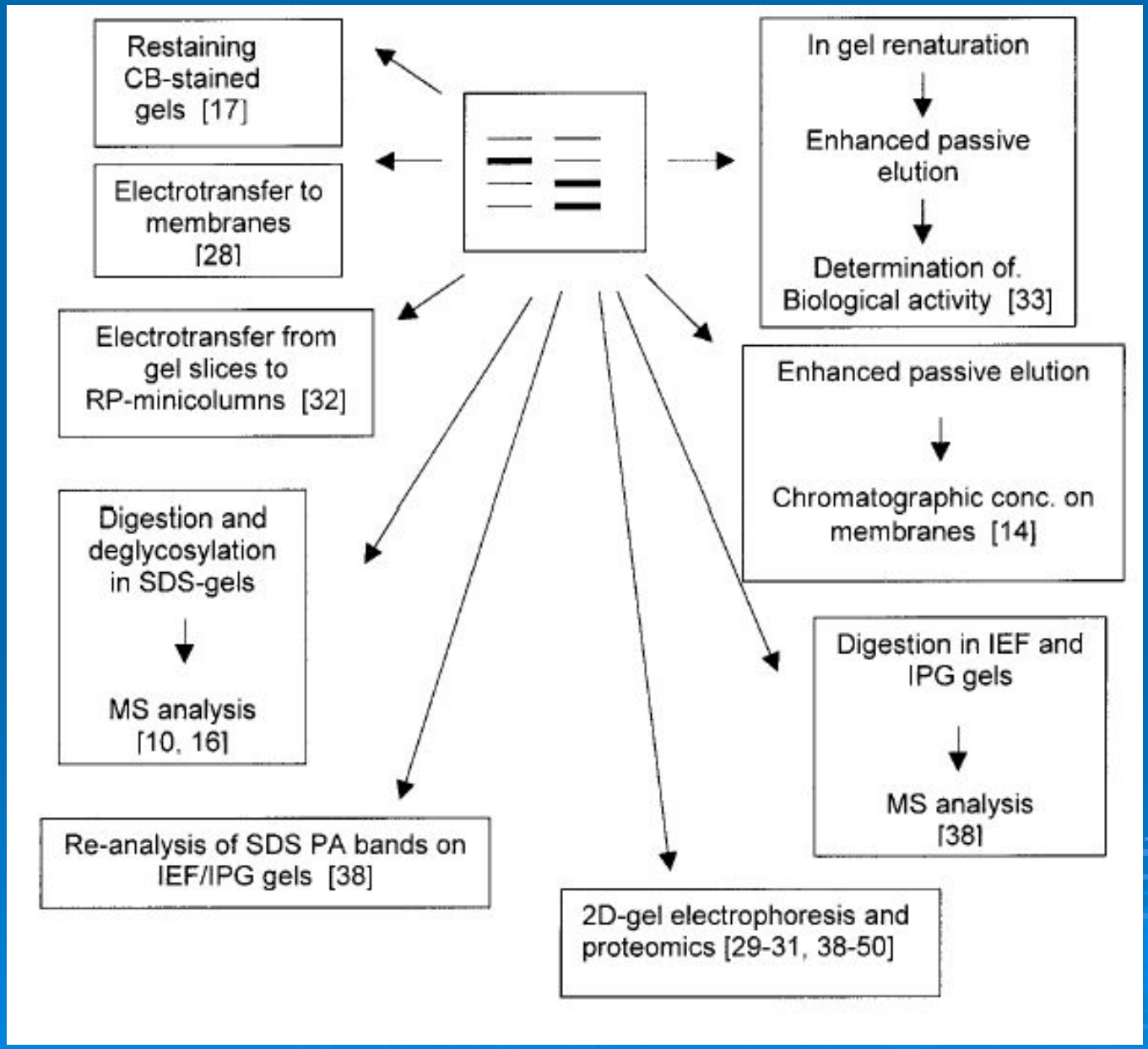
Table 9.1 A comparison of reagents for detecting and quantitating proteins in solution.

Assay	Detection Wavelengths (nm) †	Sensitivity and Effective Range	Mechanism of Action	Notes
NanoOrange protein quantitation assay (N-6666)	485/590	10 ng/mL to 10 µg/mL	Binds to detergent coating on proteins and hydrophobic regions of proteins; the unbound dye is nonfluorescent	• High sensitivity • Little protein-to-protein variation • Rapid and accurate assay with a simple procedure • Compatible with reducing agents
Bradford assay REF (Coomassie Brilliant Blue)	595	1 µg/mL to 1.5 mg/mL	Directly binds specific amino acids and protein tertiary structures; the dye's color changes from brown to blue	• High protein-to-protein variation • Not compatible with detergents • Rapid assay • Useful when accuracy is not crucial

BCA method (bicinchoninic acid)	562	0.5 µg/mL to 1.2 mg/mL	Cu^{2+} is reduced to Cu^{+} in the presence of proteins at high pH; the BCA reagent chelates Cu^{+} ions, forming purple-colored complexes	• Compatible with detergents, chaotropes and organic solvents • Not compatible with reducing agents • The sample must be read within 10 minutes
Lowry assay (biuret reagent plus Folin–Ciocalteu reagent)	750	1 µg/mL to 1.5 mg/mL	Cu^{2+} is reduced to Cu^{+} in the presence of proteins at high pH; the biuret reagent chelates the Cu^{+} ion, then the Folin–Ciocalteu reagent enhances the blue color	• Lengthy procedure with carefully timed steps • Not compatible with detergents or reducing agents
CBQCA protein quantitation assay (C-6667)	450/550	10 ng/mL to 150 µg/mL	Reacts with primary amine groups on proteins in the presence of cyanide or thiols; the unbound dye is nonfluorescent	• Sensitivity depends on the number of amines present • Not compatible with buffers containing amines or thiols • High sensitivity

Fluorescamine REF (F-2332)	390/475	0.3 µg/mL to 13 µg/mL	Reacts with primary amine groups on proteins; unbound dye is nonfluorescent	• Sensitivity depends on the number of amines present • Reagent is unstable • Not compatible with Tris or glycine buffers
OPA REF (*o*-phthaldialdehyde) (P-2331)	340/455	0.2 µg/mL to 25 µg/mL	Reacts with primary amine groups on proteins in the presence of β-mercaptoethanol; unbound dye is nonfluorescent	• Sensitivity depends on the number of amines present • Not compatible with Tris or glycine buffers • Low cost
UV absorption REF	205/280	10 µg/mL to 50 µg/mL or 50 µg/mL to 2 mg/mL	Peptide bond absorption Tryptophan and tyrosine absorption	• Sensitivity depends on number of aromatic amino acid residues present • Nondestructive • Low cost


Restaining CB-stained gels [17]
Electrotransfer to membranes [28]
Electrotransfer from gel slices to RP-minicolumns [32]
Digestion and deglycosylation in SDS-gels
MS analysis [10, 16]
Re-analysis of SDS PA bands on IEF/IPG gels [38]
2D-gel electrophoresis and proteomics [29-31, 38-50]
Digestion in IEF and IPG gels
MS analysis [38]
Enhanced passive elution
Chromatographic conc. on membranes [14]
In gel renaturation
Enhanced passive elution
Determination of. Biological activity [33]

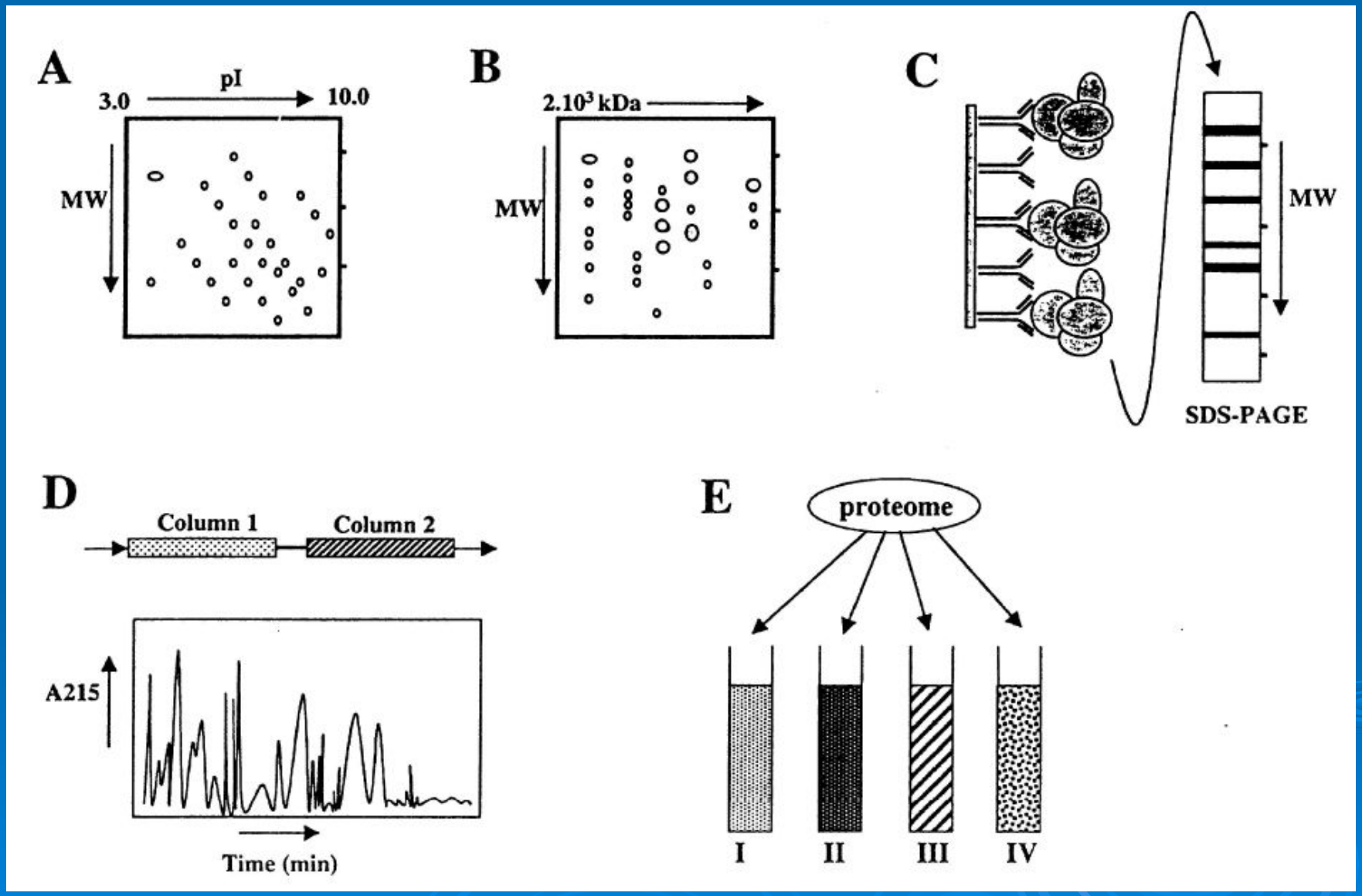

A
pI
3.0
10.0
MW
B
2.10³ kDa
MW
C
MW
SDS-PAGE
D
Column 1
Column 2
A215
Time (min)
E
proteome
I
II
III
IV

Figure 6. Tank (wet) transfer system.

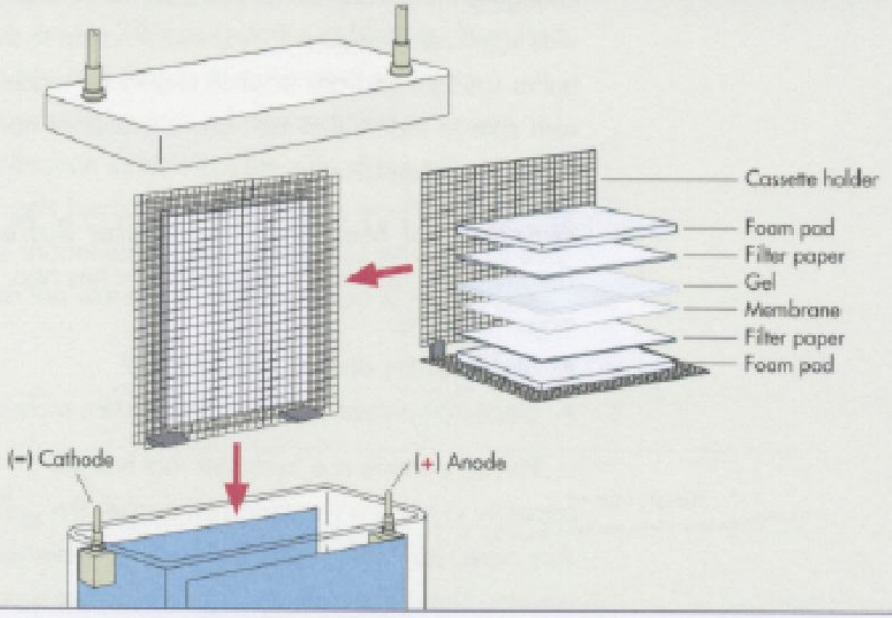
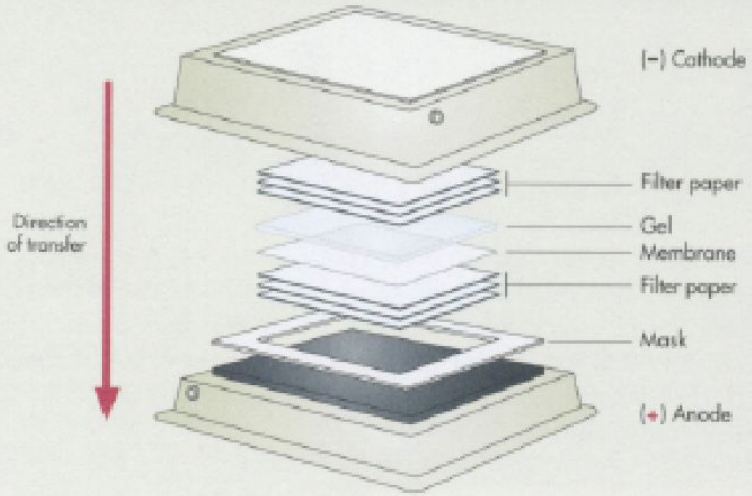
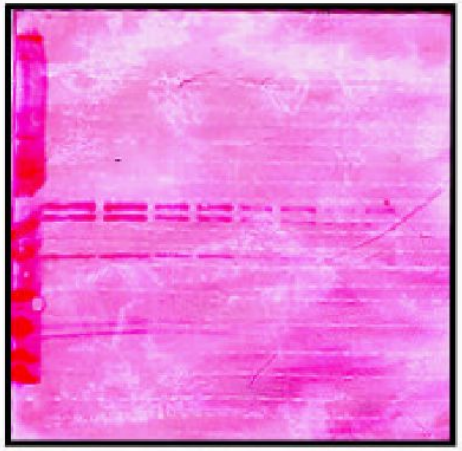


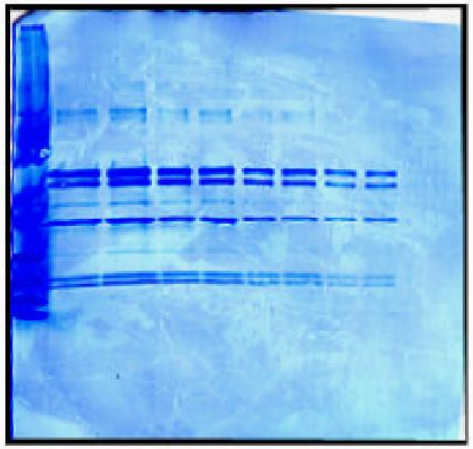
Figure 7. Semi-dry transfer system.



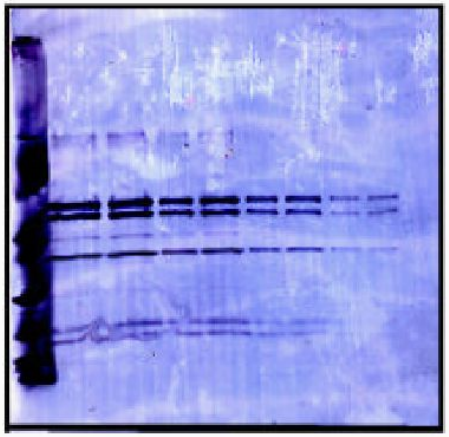
Ponceau S



CBB G-250



Amido Black



CBB R-250

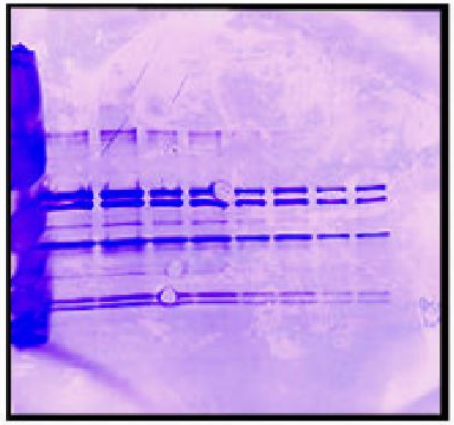


Figure 10. Calf liver proteins are visualized after electroblotting to Immobilon-P membranes: (A) Transillumination, (B) Coomassie Brilliant Blue, (C) Ponceau-S red, (D) Amido black and (E) CPTS total protein stains. Left to right, molecular weight standards and 12.2 µg, 6.1 µg, 3.1 µg of the lysate per lane.

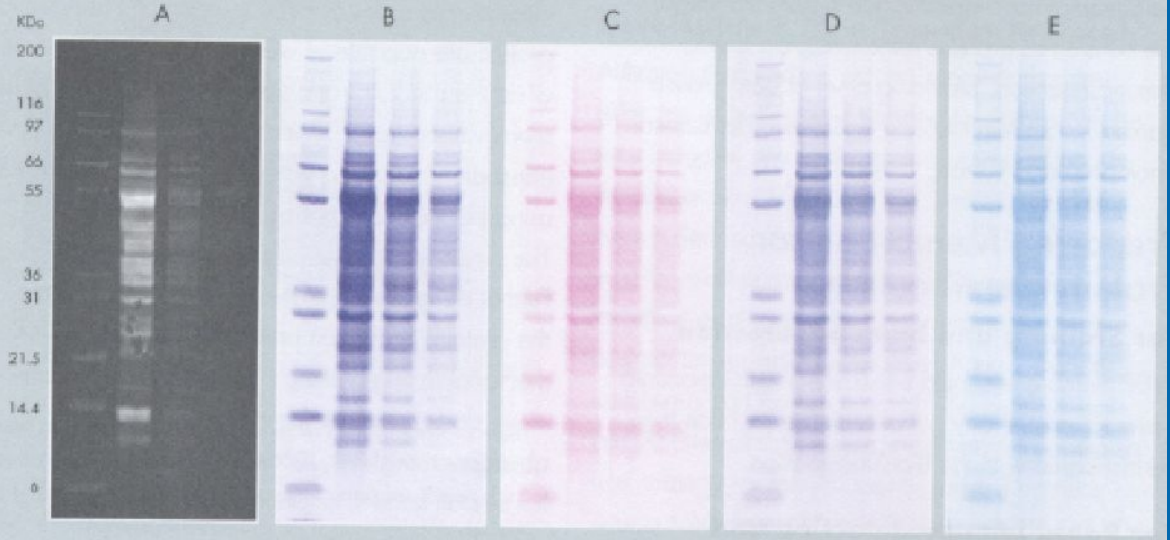


Table 5. *Common stains used in western blotting and their attributes.*

	Detection Reagent	Approximate Sensitivity (protein per spot)	Reference
Reversible	Ponceau-S red	5 µg	Dunn *et al.*, 1999
	Fast green FC	5 µg	Dunn *et al.*, 1999
	CPTS	1 µg	Bickar *et al.*, 1992
	Sypro Ruby	1–2 ng	Haugland, 2002
	Sypro Rose	1–2 ng	Haugland, 2002
Irreversible	Amido black 10B	1 µg	Dunn *et al.*, 1999
	Coomassie Brilliant Blue R-250	500 ng	Dunn *et al.*, 1999
	India ink	100 ng	Dunn *et al.*, 1999
	Colloidal gold	4 ng	Dunn *et al.*, 1999

Protein blotting membranes - recommended applications

	Hybond-C pure	Hybond ECL	Hybond-P	Hybond-C extra
Western Blotting Detection System				
Radioactive	✔✔✔	✔✔	✔✔	✔✔
Enhanced chemiluminescence	✔✔	✔✔✔	✔✔✔	✔
Chemifluorescence	✔	✔	✔✔✔	✘
Chromogenic	✔✔✔	✔✔	✔✔	✔
Colloidal gold	✔✔✔	✔✔	✔✔	✔
Specialty Labelling				
ECL Western blotting kit	✔✔	✔✔✔	✔✔	✔
ECL Plus Western blotting kit	✔	✔✔	✔✔✔	✔
ECF Western blotting kit	✔	✔	✔✔✔	✘
Glycoprotein detection kit	✔✔	✔✔	✔✔✔	✔
Expression screening	✔	✔	✔	✔✔✔
Reprobing	✔	✔	✔✔✔	✔✔

✔✔✔	Highly recommended
✔✔	Recommended
✔	Can be used
✘	Not recommended

Hybond Hybridization Membranes

Amersham Biosciences Hybond™ protein membranes are renowned for their excellent reproducibility, optimisation for various applications and careful packaging to maximise shelf life.

Hybond-C pure

- 100% pure unsupported nitrocellulose
- Excellent signal:noise across all labelling and detection systems—radioactive, non-radioactive or chromogenic

Hybond ECL

- 100% pure unsupported nitrocellulose
- Validated for use with ECL™ Western blotting system

Hybond-P, PVDF

- Ideal for reprobing
- Hydrophobic polyvinylidene difluoride (PVDF) membrane optimised for protein transfer
- Higher physical strength for significant handling advantages over unsupported nitrocellulose
- Particularly recommended for use with the ECL Plus™ and ECF™ Western blotting and ECL glycoprotein systems

Hybond-C extra

- High physical strength, supported mixed-ester nitrocellulose
- Protein binding capacity 80 - 100 µg/cm^2
- Highly recommended for expression screening with its low background, high signal:noise and handling ease

Table 1. *Comparison of PVDF and nitrocellulose membrane attributes and applications*

Attributes/Applications	Nitrocellulose	PVDF
Physical strength	Poor	Good
Protein binding capacity	80 – 100 µg/cm²	100 – 300 µg/cm²
Solvent resistance	No	Yes
Western transfer	Yes	Yes
Total protein stain	Colloidal gold Ponceau-S red Amido black India ink Sypro® blot stains	Colloidal gold Ponceau-S red Amido black India ink Coomassie™ Blue dye
Detection	Chromogenic Chemiluminescent Fluorescent Radioactive	Chromogenic Chemiluminescent Fluorescent Chemifluorescent Radioactive
Double-blotting method	No	Yes
Rapid immunodetection	No	Yes
Western reprobing	Yes	Yes
Edman sequencing	No	Yes
Amino acid analysis	Yes	Yes
Binding in the presence of SDS	Poor	Good
On-membrane digestion for mass spectrometry	No	Yes
Direct MALDI-TOF MS analysis	No	Yes
Data can be archived	No	Yes

Table 3. Protein blotting membranes – recommended applications

Application	Hybond-P	Hybond ECL	Hybond-C extra
Western blotting			
Detection method			
ECL	*highly recommended*	*highly recommended*	*suitable*
ECL Plus	*highly recommended*	*recommended*	*suitable*
chromogenic	*recommended*	*highly recommended*	*suitable*
colloidal gold	*recommended*	*highly recommended*	*not recommended*
ECF	*highly recommended*	*suitable*	*not recommended*
radioactive	*suitable*	*highly recommended*	*recommended*
Reprobing Westerns	highly recommended	not recommended	suitable
Glycoprotein	highly recommended	recommended	suitable
Expression screening	suitable	not recommended	highly recommended

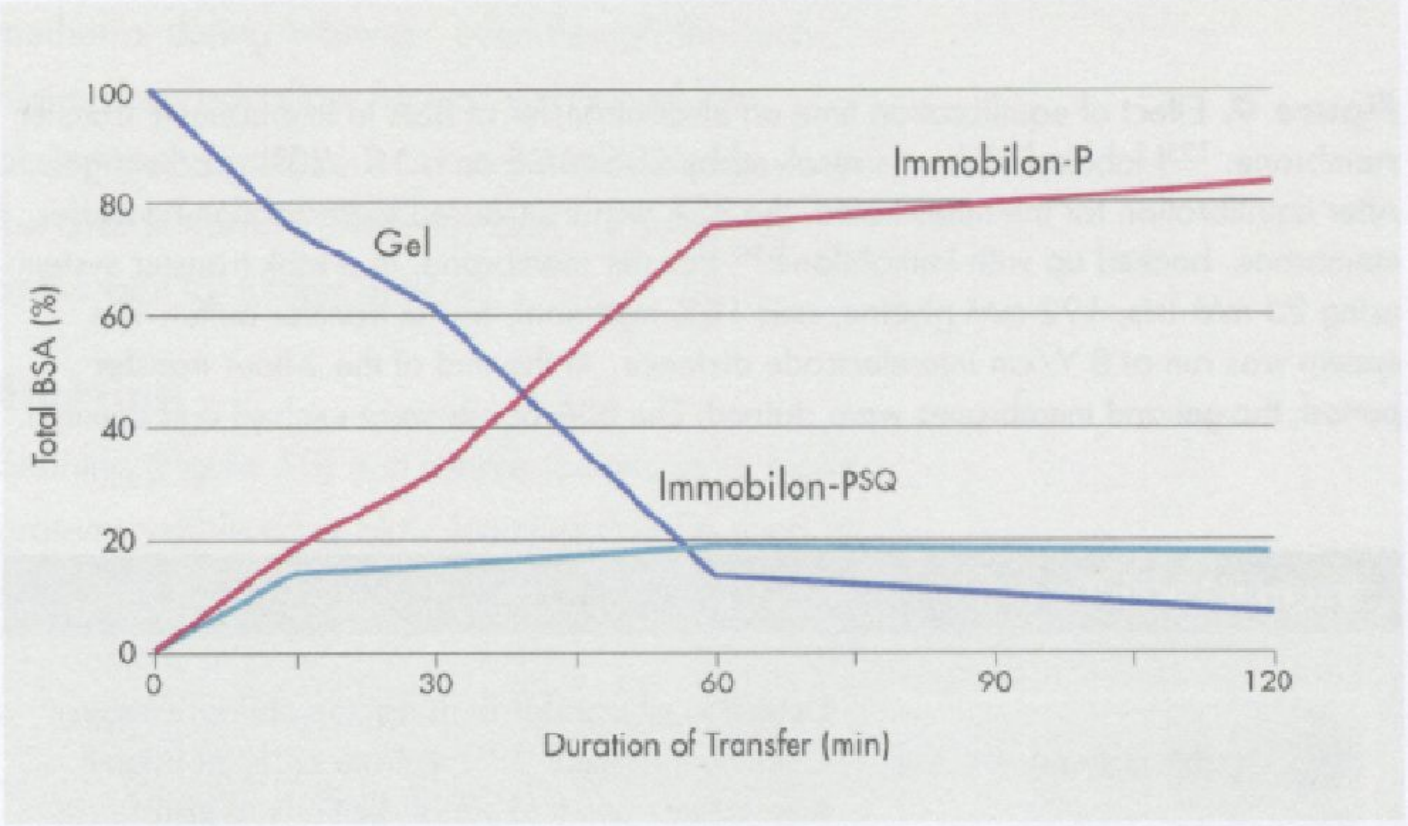
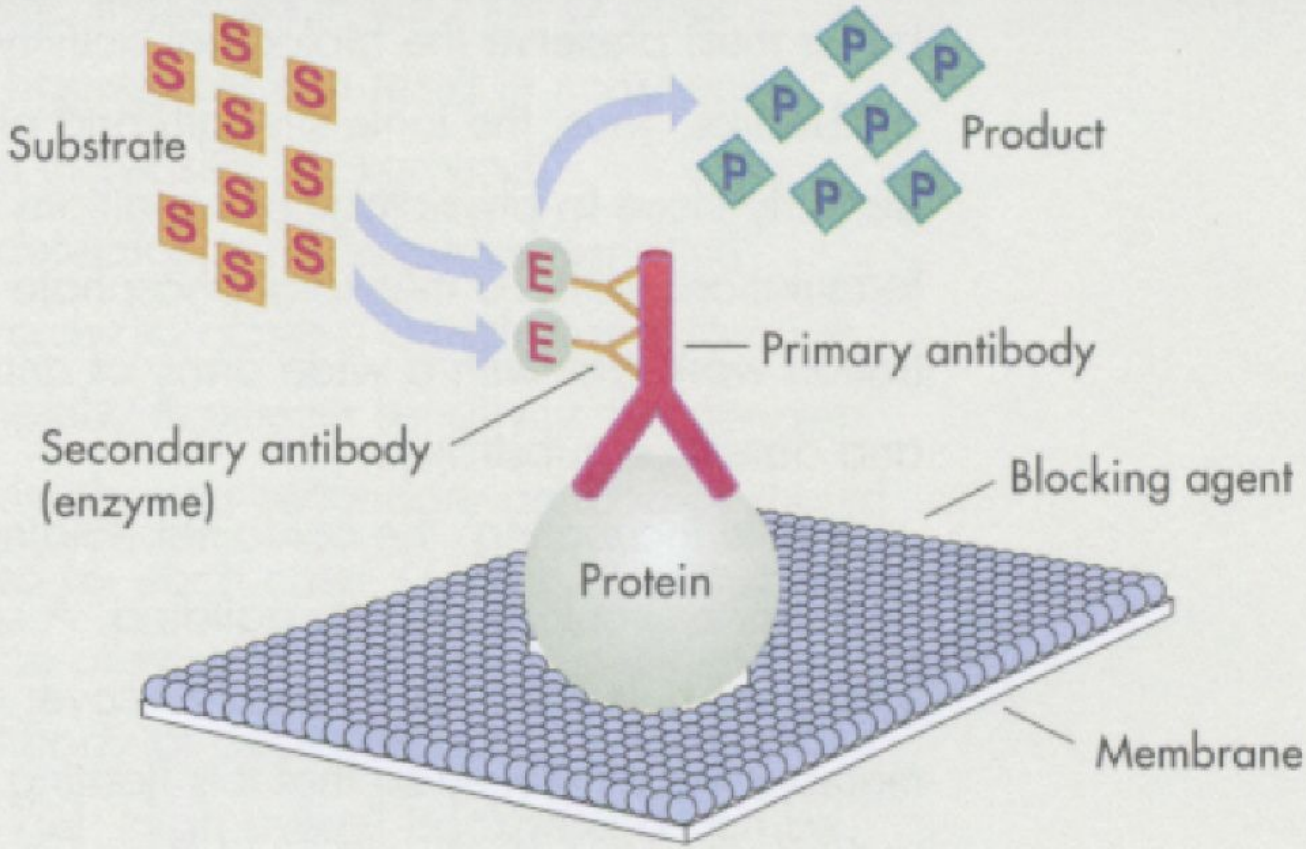


Figure 8. Electrotransfer of BSA. 25 picomoles of ^{125}I-labeled BSA were resolved by SDS-PAGE on a 10–20% gradient gel. After equilibration for 5 minutes, the BSA was transferred to Immobilon-P transfer membrane, backed up with Immobilon-P^{SQ} transfer membrane, in a tank transfer system using 25 mM Tris, 192 mM glycine, and 10% methanol, as the transfer buffer. The system was run at 8 V/cm interelectrode distance. At 15, 30, 60, and 120 minutes, a gel/membrane cassette was removed and stained. The BSA bands were excised and counted.

Figure 11. Membrane-based immunodetection.



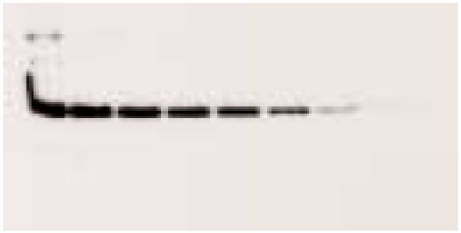


Fig 28. Detection of actin using ECL Western Blotting System. Ten-fold serial dilutions of actin (from 100 ng to 195 pg) were separated by electrophoresis and proteins were transferred to Hybond-P membrane. Primary antibody was mouse anti-actin (product code N350). Detection was performed using ECL detection reagents and Hyperfilm ECL.

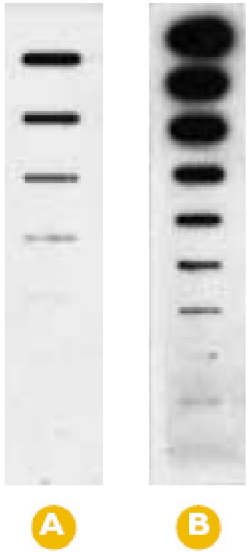


Fig 27. Target: mouse IgG slot blotted onto PVDF (Hybond-P, RPN2020F); loadings: doubling dilutions starting at 5ng; detection: 1:2500 dilution of anti-mouse Ig HRP conjugate (NA931) using (A) ECL detection reagents (RPN2106) and (B) ECL Plus detection reagents (RPN2132); exposure: on Hyperfilm ECL (RPN2103) for 5 minutes.

Table 3. Protein blotting membranes – recommended applications

Application	Hybond-P	Hybond ECL	Hybond-C extra
Western blotting			
Detection method			
ECL	*highly recommended*	*highly recommended*	*suitable*
ECL Plus	*highly recommended*	*recommended*	*suitable*
chromogenic	*recommended*	*highly recommended*	*suitable*
colloidal gold	*recommended*	*highly recommended*	*not recommended*
ECF	*highly recommended*	*suitable*	*not recommended*
radioactive	*suitable*	*highly recommended*	*recommended*
Reprobing Westerns	highly recommended	not recommended	suitable
Glycoprotein	highly recommended	recommended	suitable
Expression screening	suitable	not recommended	highly recommended

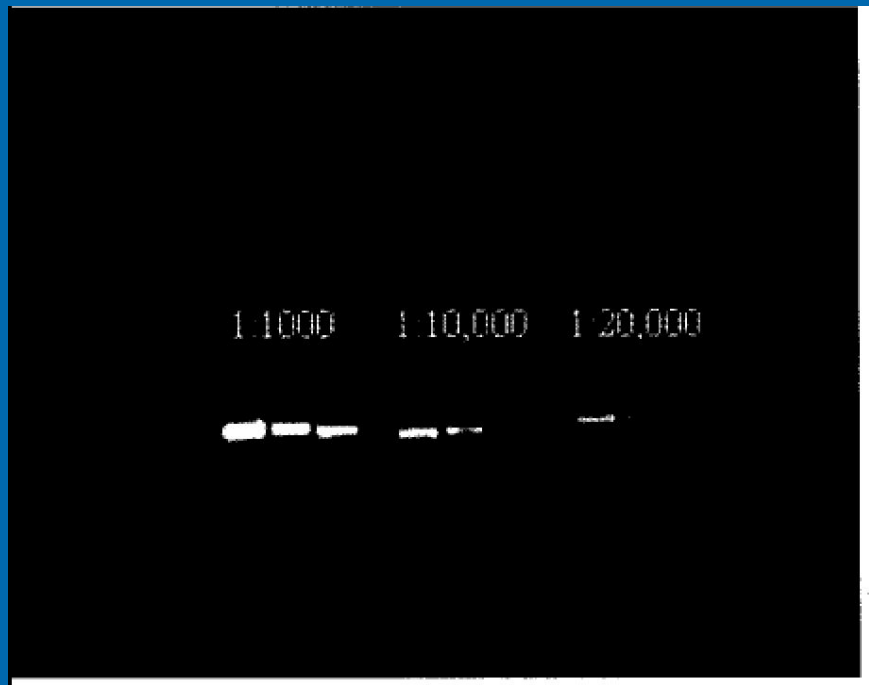


Fig. 1 *The effect of anti-chaperonin peroxidase conjugate on signal intensity. Chaperonin loaded in triplicate at 40, 20 and 10 ng/well (left to right), blotted to nitrocellulose and treated with antibody conjugate as noted. The 1:1000 gave enhanced signal and good intensity at the lower chaperonin concentration; however, background on film was relatively high (not shown). The middle dilution of 1:10,000 proved to be a good concentration for both cooled CCD camera and film detection.*

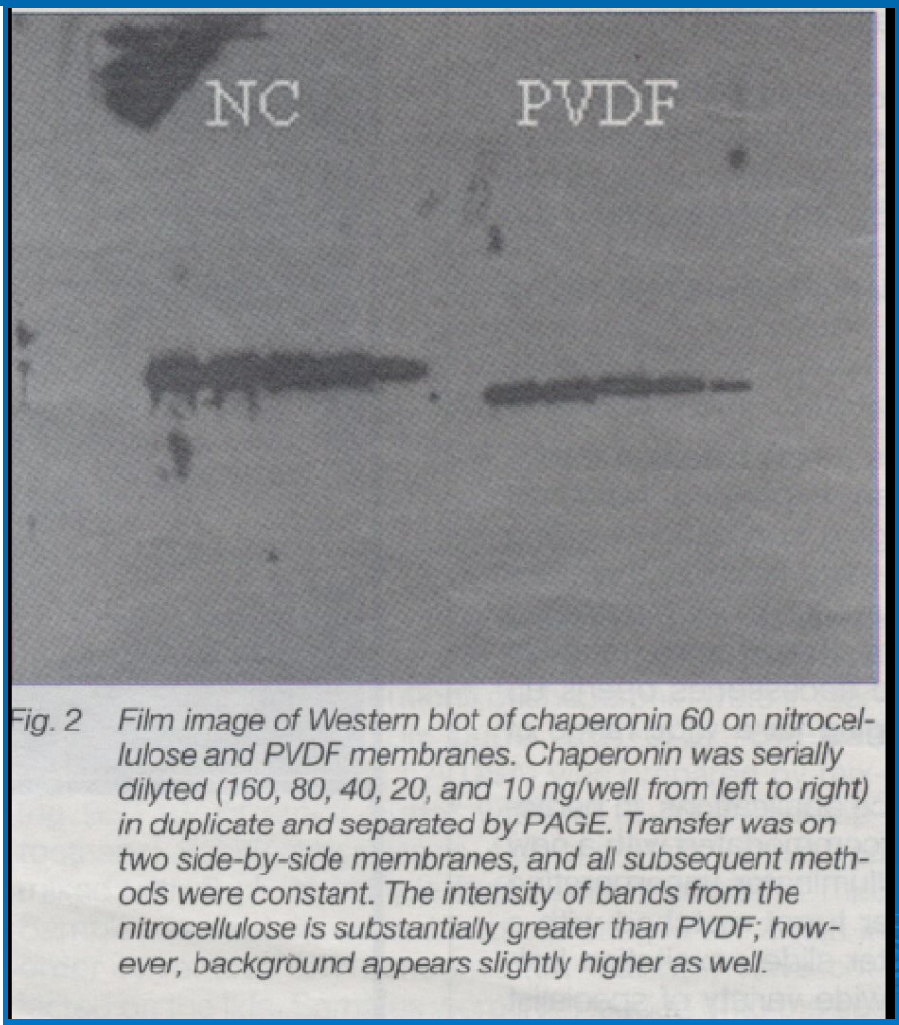


Fig. 2 *Film image of Western blot of chaperonin 60 on nitrocellulose and PVDF membranes. Chaperonin was serially dilyted (160, 80, 40, 20, and 10 ng/well from left to right) in duplicate and separated by PAGE. Transfer was on two side-by-side membranes, and all subsequent methods were constant. The intensity of bands from the nitrocellulose is substantially greater than PVDF; however, background appears slightly higher as well.*

Table 2.8. Comparison of Blocking Reagents

Blocking Reagent	Membrane Compatibility	Recommended Concentration	Notes
Gelatin	Nitrocellulose	1–3%	Requires heat to solubilize
Non-fat dry milk, BLOTTO, Blotting Grade Blocker	Nitrocellulose, PVDF, Nylon	0.5–5%	PVDF and nylon require higher concentrations of non-fat dry milk than nitrocellulose
BSA	Nitrocellulose, PVDF, Nylon	1–5%	PVDF and nylon require higher concentrations of BSA than nitrocellulose
Tween-20	Nitrocellulose	0.05%–0.3%	May strip some proteins from the blot

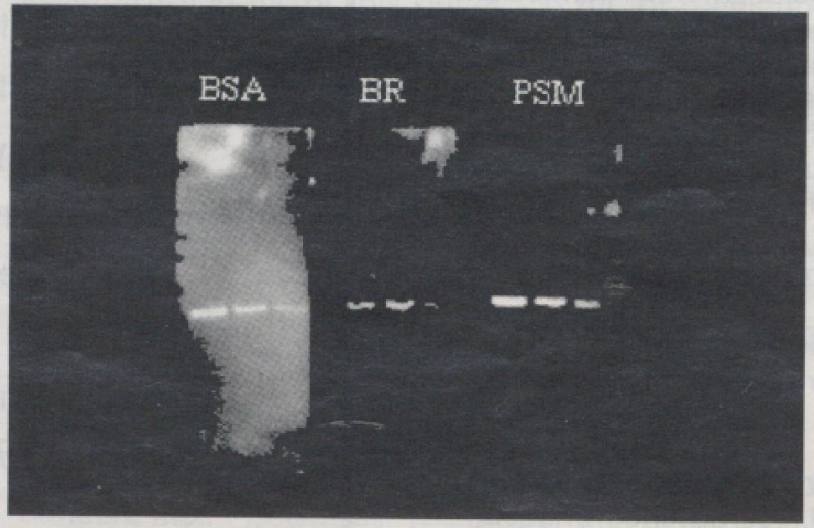


Fig. 3 Comparison of chaperonin Western blots blocked with BSA, blocking reagent and powdered skimmed milk. Chaperonin was loaded in triplicate at 40, 20 and 10 ng/well, separated and transferred to nitrocellulose. The membrane was cut in thirds and blocked with 3% BSA, 1x blocking reagent and 3% powdered skimmed milk (store brand). Powdered skimmed milk generated the strongest signals while significant background is seen with BSA blocking.

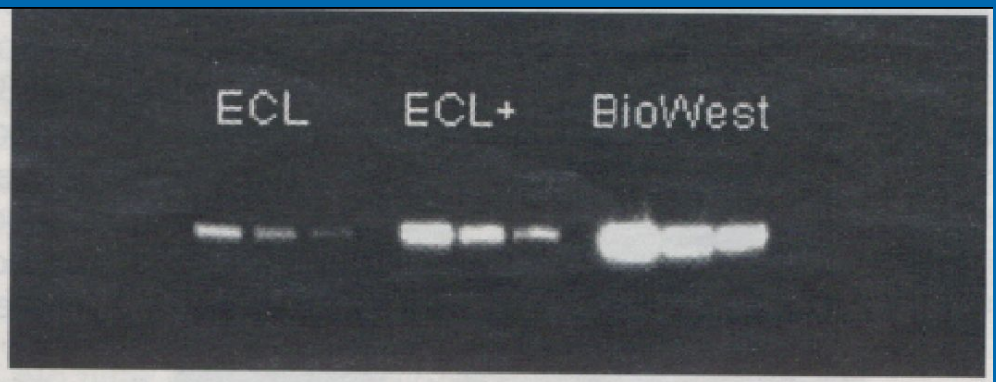


Fig. 4 Comparison of BioWest, ECL and ECL Plus chemiluminescent peroxidase substrates. Three concentrations of chaperonin 60 (40, 20 and 10 ng/well) were loaded in triplicate on a 4–20% polyacrylamide gel, separated and transferred to nitrocellulose. Prior to the addition of substrate, the membrane was cut into thirds and each of the chemiluminescent substrates was applied separately. The BioWest substrate generated a signal 3.5 times greater than ECL Plus and 14 times greater than ECL. This membrane was subsequently imaged for 150 min to assess the duration of the signal.

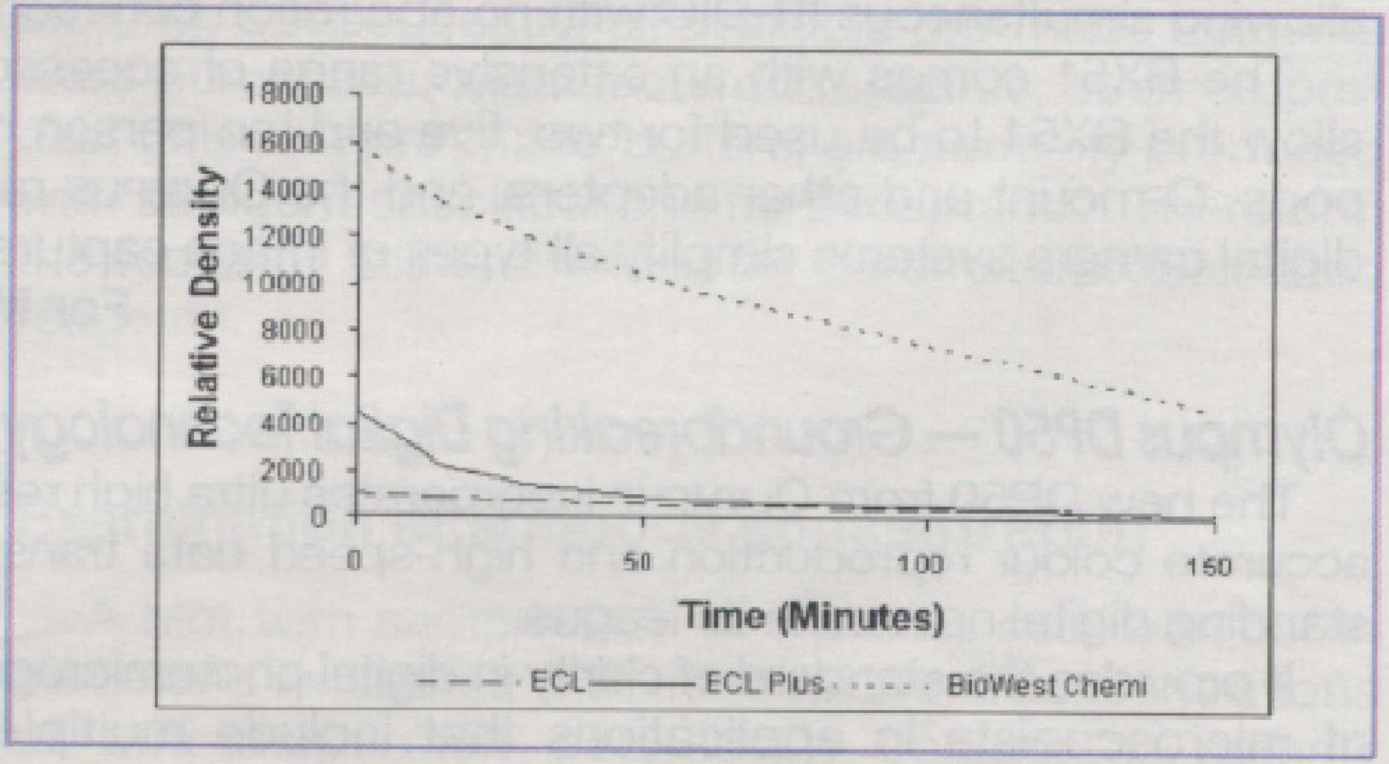


Fig. 5 Time course analysis of band intensities for ECL, ECL Plus and BioWest substrates. Parallel blots of chaperonin 60 were imaged at 0, 15, 30, 60 and 150 min and then measured for relative density. Initial intensity of the BioWest substrate was 3.4 times greater than ECL Plus and 14 times greater than ECL. In addition, the signal duration of BioWest greatly exceeded the other substrates.

Table 2.10. Comparison of Detection Reagent Systems

	Horseradish Peroxidase	Alkaline Phosphatase	Gold
Sensitivity	500 pg	100 pg (Immun-Blot) 10 pg (Immun-*Star*) 5 pg (Amplified AP)	100 pg 10 pg (Enhanced)
Substrates	4CN – purple DAB – brown	BCIP/NBT – purple CDP-*Star* – emits light	none
Comparative cost	least expensive	more expensive most expensive (Immun-*Star*)	more expensive
Stability of stored blots	poor	good	good
Restrictions	azide, endogenous peroxidase activities	endogenous phosphatase activities	none