British Journal of Anaesthesia **112** (2): 213–16 (2014)
doi:10.1093/bja/aet293

BJA

EDITORIAL SERIES ON METHODOLOGY 5

Radioimmunoassay, enzyme and non-enzyme-based immunoassays

R. D. Grange, J. P. Thompson and D. G. Lambert*

Department of Cardiovascular Sciences, Division of Anaesthesia, Critical Care and Pain Management, Leicester Royal Infirmary, University of Leicester, Leicester, UK

* E-mail: dgl3@le.ac.uk

The ability to quantify the amount of a specific protein in a complex sample has been a valuable addition to laboratory science, allowing the development of diagnostic tests, allergen detection in the food industry, and screening for immunity. This is particularly important in anaesthesia, intensive care, and pain research for the quantification of mediators (cytokines, peptides, and analytes) involved in inflammation, pain, and other pathways.

Immunoassays use the high specificity of antibodies, along with their enormous diversity, to target specific molecules of interest and analyse their concentration in a sample. The first immunoassay developed was described by Yalow and Berson[1] in 1959.[2] They used radiolabelled insulin to assess the concentration of insulin in human plasma, and thus developed the first radioimmunoassay (RIA). In 1971, Engvail and Perlman[3] described a technique whereby antigens were immobilized on a microplate well, incubated with antiserum, and then the concentration of antibody in the antiserum was quantified using an enzyme-linked anti-immunoglobulin antibody. This method is the enzyme-linked immunosorbent assay (ELISA). Enzyme immunoassays (EIAs) are very similar to ELISAs, and as such, the terms are often used interchangeably. The EIA was developed by Van Weemen and Schuurs[4] (independently of Engvail and Perlman) for the quantification of antigen rather than antibody. For the purpose of this article, EIA and ELISA should be considered interchangeable.

The majority of RIA assay formats recommend sample cleaning and concentration (particularly when analyte concentration and assay sensitivity is low), although a large number of ELISA assays can cope with direct use of unprocessed plasma. The cleaning and concentration process usually involves ion exchange chromatography followed by some form of freeze drying/lyophilization. We would recommend users to determine if sample cleaning is required for their analyte.

Often, there are differences in measured analyte concentration when comparing RIA and ELISA. This can result from specificity of the antibody (e.g. the cardiovascular peptide urotensin II)[5 6] or the fluid in which the analyte is suspended interfering with only one type of assay (e.g. the opioid-related peptide Nociceptin/Orphanin FQ).[7–11] Discordance has also been demonstrated between RIAs and EIAs measuring cortisol and carcinoembryonic antigen.[12 13] The selection of assay format is therefore critical and the remainder of this article covers the main formats currently available.

Radioimmunoassay

An RIA requires the following: a sample containing the antigen of interest, a complementary antibody, and a radiolabelled version of the antigen. The sample antigen and antibody are incubated together, allowing the sample antigen to bind with the antibody. The radiolabelled antigen is then added. The radiolabelled antigen competes with the sample antigen and displaces it from the antibody. The more sample antigen present, the less the radiolabelled antigen is able to bind to the antibody. A second antibody that binds the primary antibody can then be added, along with serum from the species of the primary antibody, to cause the solution to flocculate and allow for separation of the primary antibody from solution. Since solution containing antigen–antibody complex is more dense than that containing free-antigen, centrifuging this mixture allows separation, resulting in a pellet containing the bound sample antigen/radiolabelled antigen. By measuring the radioactivity of the pellet, it is possible to determine the amount of radiolabelled antigen that has bound to antibody, and therefore the concentration of antigen in the sample (Fig. 1). The drawbacks of RIA relate to the use of a radiolabel (usually [^{125}I]) and hence short shelf life. These assays do not use enzymes and thus reduces the risk of interference from the sample itself.

Enzyme-linked immunosorbent assay

There are a variety of ELISA methods. The important variations are described below (Fig. 2).

Direct ELISA

This is the simplest of the ELISA techniques. The sample is first added to the microplate well and incubated. The sample will contain the antigen of interest. The antigen becomes adsorbed onto the surface of the well. The wells are then washed thoroughly, leaving only the absorbed antigen. Remaining binding sites on the well are then blocked. An antibody, complementary to the antigen of interest, is then added to the wells where it binds to the antigen. The well is again washed. This leaves a bound antigen–antibody complex on the surface of

the well. The bound antibody will have attached to it an enzyme. A substrate is then added which will be converted by the enzyme into a detectable product. Detection may be based on colour, fluorescence, or luminescence.

This method has the advantage of being quicker and simpler than the other ELISA methods, with fewer steps, and just one antibody. It does, however, have some limitations. In complex samples, containing a range of different proteins, there will be a variety of proteins adsorbed onto the well that are not the antigen of interest. This proves problematic when the antigen of interest is in low abundance as the sensitivity of the test is reduced. Another issue is that the antibody needs to have an enzyme attached to it. This costly and time-consuming process has to be repeated for each individual ELISA, a problem avoided by the other methods. Also, conjugating the antibody with an enzyme has the potential to reduce the affinity of the antibody to the antigen, and thus reduce sensitivity once more.

Indirect ELISA

Sample containing the antigen of interest is adsorbed onto the wells of a microplate, followed by blocking of remaining sites on the well. A complimentary antibody (primary antibody) is then added, which binds to the antigen forming a complex. This method differs from the direct method in that the antibody binding to the antigen does not have attached to it an enzyme or any other signal-generating substance. Instead, the purpose of this antibody is to act as a bridge between the antigen and a secondary (enzyme-linked) antibody. This secondary antibody will have been raised in an animal different from that of the origin of the primary antibody and will target the Fc region of the primary antibody.

The secondary antibody is often polyclonal (originates from different B cells) and as such will be responsive to different epitopes on the primary antibody. This allows multiple secondary antibodies to bind to the same primary antibody, thereby amplifying the signal and increasing the sensitivity of the test (although there is still the issue of complex samples having multiple proteins adsorbed onto the surface of the well).

Another advantage of this method is the exclusion of the need to conjugate the primary antibody, avoiding the problems described above. Secondary antibodies can therefore be made commercially available at a much lower price, and with a variety of signal-producing conjugates (i.e. all ELISAs using a rabbit-derived primary antibody could use the same anti-rabbit IgG secondary antibody).

Sandwich ELISA

The direct and indirect methods both suffer from the fact that complex samples will reduce the sensitivity of the experiment due to a variety of proteins adsorbing to the well. The sandwich method overcomes this. An antibody complementary to that of the antigen (capture antibody) is first added to the plate where it is adsorbed to the well. A blocking agent is added as before and a sample is then added. Only the antigen of interest can remain on the plate since it is able to bind to the antibody. The rest of the experiment can now proceed in the same way as a direct or an indirect ELISA.

The clear benefit of this method is improved sensitivity. It does however come at a cost. For this method to work, two antigen-specific antibodies are required. They need to bind to different epitopes on the antigen, and these need to be far enough away from each other as to not hinder the binding of one another. If a secondary antibody is used (as in indirect ELISA), it is important that the capture and primary antibodies are raised in different species. This is because the secondary antibody will be raised against the species of the primary antibody. If both capture and primary antibody were from the same species, then the secondary antibody would bind to both and not reflect differences in bound antigen.

Competitive ELISA

This method requires two ligands to compete with each other for a limited number of antibody sites. One ligand will be the antigen of interest, and one will be a similar molecule that is able to bind to the antibody, but has a variation that allows a further molecule to exclusively bind to it. This is often achieved by adding biotin to the antigen of interest. The antigen and the biotinylated antigen will compete for the same site on the antibody. The signal generated by this assay will be inversely proportional to the amount of antigen in the sample.

As mentioned, biotin is often added to the competing antigen. It is a useful molecule since it is small, and thus does not appreciably reduce the affinity of the antigen for the antibody. It also binds readily and specifically to streptavidin.[14] Streptavidin is a protein that is easily conjugated to a variety of molecules, allowing signal generation from a variety of sources such as colour changes, chemiluminescence (immunoluminometric assay),[15] and fluorescence (immunofluorometric assay).[16] The biotin–streptavidin complex can also be used as a signal amplifier.

Other immunoassays

The use of enzymes in an assay can be advantageous since this allows for the use of a variety of substrates that can generate different signals. Enzymes are, however, open to interference. For example, horseradish peroxidase and alkaline phosphatase are the most frequently used enzymes and are inhibited by buffers containing sodium azide (a commonly used preservative) and phosphate, respectively. Endogenous sample peroxidases and phosphates may also interfere with the assay.

Immunoassays that do not require the use of enzymes and radionuclides are now being developed. These assays include competition assays using fluorescent peptides, and also a variety of labelled streptavidin compounds for use with biotinylated antibodies or peptides.

The above assay formats are heterogeneous immunoassays (assays that require separation of bound and unbound antibody/antigen before signal recording). Other assays, such as Enzyme multiplied immunoassay technique (EMIT)[17] and Fluorescence polarization immunoassays (FPIA)[18] do not require this

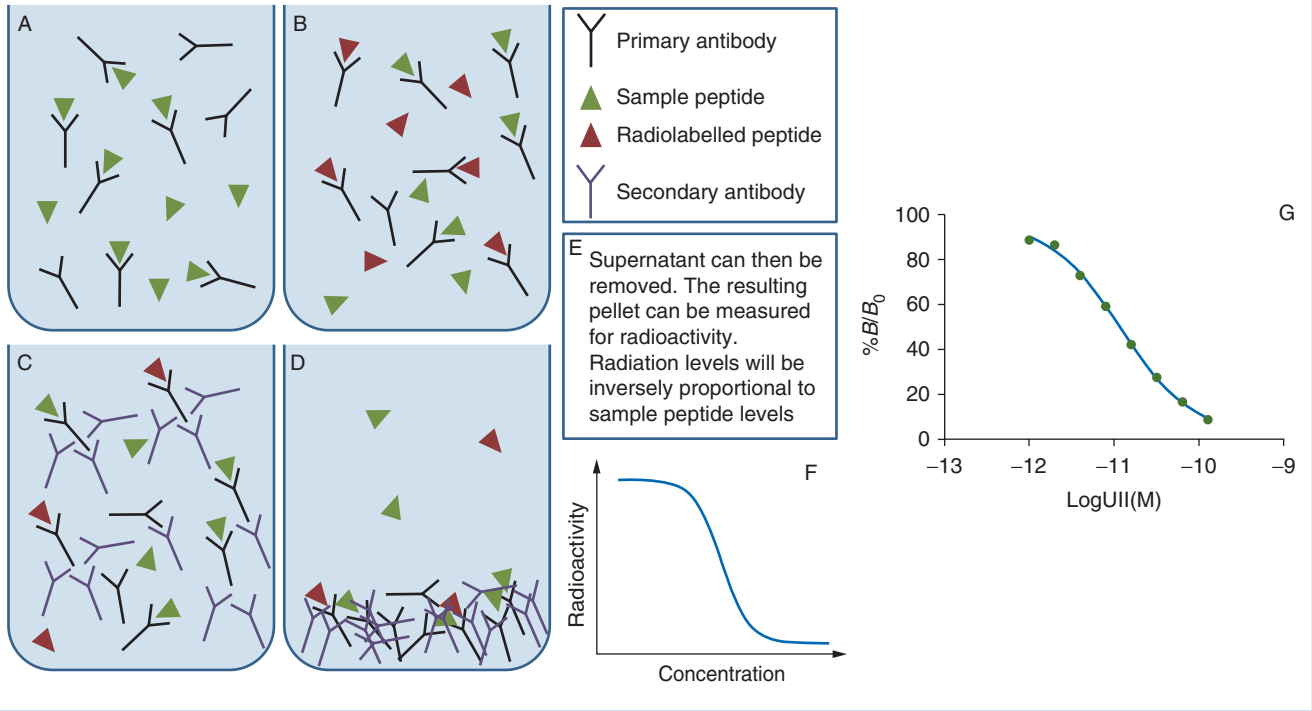

Fig 1 (A) Sample peptide is incubated with primary antibody. (B) Radiolabelled peptide is then added. It competes with sample peptide and displaces it. (C) Secondary antibody binds to primary antibody and causes it to precipitate out of solution. (D) Centrifugation causes the antibody–antigen complex to form a pellet. (F) Example of a typical standard curve. (G) Actual standard curve for urotensin-II (UII) where amount of radioactive iodine bound is expressed as B/B_0 which is the ratio of binding at each standard concentration, B to that bound in the absence of displacer, B_0. Analyte samples in biological specimens should lie on the straight part of the curve.

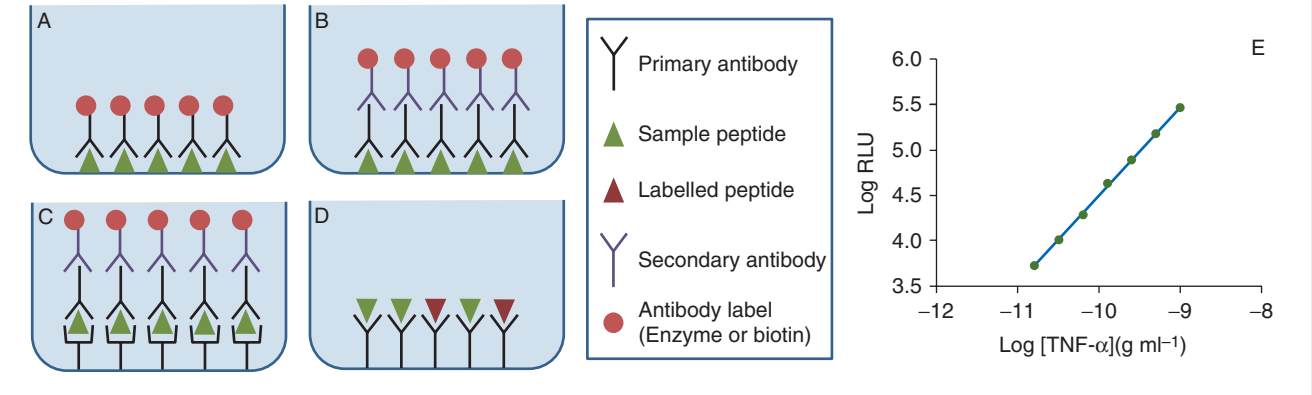

Fig 2 Schematic showing the differences between direct (A), indirect (B), sandwich (C), and competitive (D) EIA methods. (E) Actual standard curve for a sandwich TNF-α assay. Note the way the standard curve is presented varies with the RIA in Figure 1, but analyte samples in biological specimens should lie on the straight part of the curve. RLU, relative light units signal from the enzyme reaction.

separation, and are classified as homogenous immunoassays. EMIT requires an enzyme-linked antigen that will compete with sample antigen for antibody binding. The enzyme is designed so as to become deactivated by antibody binding. FPIA works similarly, with fluorescein-conjugated antigens competing. Bound and unbound fluorescein-conjugated antigens emit fluorescence of different intensities and can therefore be distinguished. Some recent *British Journal of Anaesthesia* RIA/ELISA data are summarized in Table 1.

Declaration of interest

D.G.L. holds a consultancy with Grunenthal GmbH, but this is not directly related to the content of this article. D.G.L. is the

Table 1 Some ELISA (Sandwich)/RIA assay formats used in studies published recently in *British Journal of Anaesthesia*. *Sensitivity quoted

Analyte	Manufacturer	Method	Range	Reference
Human IL-1β	R&D Systems	Sandwich	3.9–250 pg ml^{-1}	19
Human IL-6	R&D Systems	Sandwich	3.12–300 pg ml^{-1}	
Human IL-8	R&D Systems	Sandwich	31.2–2000 pg ml^{-1}	
Human IL-10	R&D Systems	Sandwich	7.8–500 pg ml^{-1}	
TNF alpha	R&D Systems	Sandwich	0.5–32 pg ml^{-1}	
Neural growth factor	Promega	Sandwich	3.9–250 pg ml^{-1}	20
Heat shock protein 70	Enzo Life Sciences	Sandwich	780–50 000 pg ml^{-1}	21
Heat shock protein 90	Enzo Life Sciences	Sandwich	62.5–4000 pg ml^{-1}	
Heat shock protein 60	Enzo Life Sciences	Sandwich	3.125–100 ng ml^{-1}	
β-Endorphin*		RIA	10 pg tube^{-1}	22

administration director and a board member of *BJA*, and J.P.T. is an editor and board member of *BJA*.

References

1 Yalow RS, Berson SA. Assay of plasma insulin in human subjects by immunological methods. *Nature* 1959; **184**(Suppl. 21): 1648–9

2 Annesley TM. It's about the journey, not the destination: the birth of radioimmunoassay. 1960. *Clin Chem* 2010; **56**: 671–2

3 Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* 1971; **8**: 871–4

4 Van Weemen BK, Schuurs AH. Immunoassay using antigen-enzyme conjugates. *FEBS Lett* 1971; **15**: 232–6

5 McDonald J, Batuwangala M, Lambert DG. Role of urotensin II and its receptor in health and disease. *J Anesth* 2007; **21**: 378–89

6 Aiyar N, Guida B, Ao Z, *et al.* Differential levels of 'urotensin-II-like' activity determined by radio-receptor and radioimmuno-assays. *Peptides* 2004; **25**: 1339–47

7 Lambert DG. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. *Nat Rev Drug Discov* 2008; **7**: 694–710

8 Barnes TA, Lambert DG. Editorial III: Nociceptin/orphanin FQ peptide-receptor system: are we any nearer the clinic? *Br J Anaesth* 2004; **93**: 626–8

9 Spadaro A, Ajello A, Luigiano C, *et al.* Low utility of plasma Nociceptin/orphanin FQ in the diagnosis of hepatocellular carcinoma. *World J Gastroenterol* 2006; **12**: 4716–20

10 Kumar N, Smart D, Mason S, McKnight AT, Rowbotham DJ, Lambert DG. Neither nociceptin nor its receptor are present in human synovial fluid or tissue. *Br J Anaesth* 1999; **83**: 470–1

11 Williams JP, Thompson JP, Young SP, *et al.* Nociceptin and urotensin-II concentrations in critically ill patients with sepsis. *Br J Anaesth* 2008; **100**: 810–4

12 Raff H, Homar PJ, Burns EA. Comparison of two methods for measuring salivary cortisol. *Clin Chem* 2002; **48**: 207–8

13 Fleisher M, Nisselbaum JS, Loftin L, Smith C, Schwartz MK. Roche RIA and Abbott EIA carcinoembryonic antigen assays compared. *Clin Chem* 1984; **30**: 200–5

14 Tech tip #65: ELISA technical guide and protocols. Available from http://www.piercenet.com/browse.cfm?fldID=EE79C527–5056-8A76-4E92-2E2C1E1643AB

15 Bhandari SS, Davies JE, Struck J, Ng LL. Influence of confounding factors on plasma mid-regional pro-adrenomedullin and mid-regional pro-A-type natriuretic peptide concentrations in healthy individuals. *Biomarkers* 2011; **16**: 281–7

16 Hemmila I. Fluoroimmunoassays and immunofluorometric assays. *Clin Chem* 1985; **31**: 359–70

17 Schneider RS, Lindquist P, Wong ET, Rubenstein KE, Ullman EF. Homogeneous enzyme immunoassay for opiates in urine. *Clin Chem* 1973; **19**: 821–5

18 Nielsen K, Lin M, Gall D, Jolley M. Fluorescence polarization immunoassay: detection of antibody to brucella abortus. *Methods* 2000; **22**: 71–6

19 Theusinger OM, Baulig W, Seifert B, Emmert MY, Spahn DR, Asmis LM. Relative concentrations of haemostatic factors and cytokines in solvent/detergent-treated and fresh-frozen plasma. *Br J Anaesth* 2011; **106**: 505–11

20 Yue W, Guo Z. Blockade of spinal nerves inhibits expression of neural growth factor in the myocardium at an early stage of acute myocardial infarction in rats. *Br J Anaesth* 2012; **109**: 345–51

21 Sulyok I, Fleischmann E, Stift A, *et al.* Effect of preoperative fever-range whole-body hyperthermia on immunological markers in patients undergoing colorectal cancer surgery. *Br J Anaesth* 2012; **109**: 754–61.

22 Groppetti D, Pecile AM, Sacerdote P, Bronzo V, Ravasio G. Effectiveness of electroacupuncture analgesia compared with opioid administration in a dog model: A pilot study. *Br J Anaesth* 2011; **107**: 612–8