

Although the techniques involved in modern assay development may not appear new, they are being applied with ever-increasing sophistication, resulting in major improvements in assay quality and performance.

Here, Mike Minihan outlines the process of automated enzyme immunoassay development and highlights specific techniques that are key to this process.

Rising challenge of immunoassay development

Automated enzyme immunoassays are continuously evolving to meet the ever-increasing demands put on them, such as the need for increased sensitivity, precision and measuring range. The time and expertise invested in the development of such assay systems is often underestimated. The companies involved have to work within a strict regulatory framework that also imposes a duty to undertake extensive testing and validation procedures, before any assay can be brought into routine use. However, far from holding back development, this framework has in many ways had a positive effect.

Backbone of the modern clinical laboratory

Immunoassays are probably one of the most advanced methods available for routine clinical diagnostic use. One example is thyroid function testing and here immunoassay not only enables the delivery of accurate test results but also provide for efficient therapy management and control. With a huge range of applications, the latest technologies have been coupled with advanced immunochemistry to create automated systems with high-throughput capabilities. They are well suited for use with large sample numbers and can detect small amounts of target analyte. Additionally, quantification is accurate using known concentrations of analyte as controls.

Today, the more advanced immunoassay analysers can accommodate various assay types (eg sandwich or competitive) that may have one or two incubation steps (Fig 1). This means that many patient samples can be tested simultaneously for a multitude of

analytes using different assay set-ups. However, producing assays for a high-throughput system of this magnitude can mean additional design challenges. This article examines how the development process works to overcome these challenges, maximise assay performance and finally release a quality product that is fit for the market.

In brief, the immunoassay development process is normally split into four main stages: feasibility, optimisation, verification and validation. There are two key points that prevail through each step of the development process: the techniques and materials used must provide maximum reproducibility and minimum lot-to-lot variability (there is also design control at every stage); and the product must meet specific criteria and regulations set by the US Food and Drug Administration (FDA) and the *in vitro* diagnostics directives (IVDD) for the US and European markets.

Feasibility

Initially, feasibility of the immunoassay is assessed using market research to establish assay design and performance goals. The result being that the assay concept is compared to those presently available. The design input specifications are proposed to define the performance of the assay and make it competitive. Thus, at this point, the main performance goals are set, such as precision, accuracy, stability, and sensitivity. These are then followed rigidly through the design and development process. The feasibility phase begins with the selection of:

- key raw materials for reagents, such as antibodies and solid-phase components
- key raw materials for calibrators and controls

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- methods for solid-phase coupling
- conjugation chemistries.

Selection of key raw materials

In the preliminary stage of selection, suppliers are assessed and raw materials put through basic quality control tests to assess consistency and reproducibility between batches. Modern high-throughput analysers and the assays they employ predominantly use microparticle-based solid phases (eg latex microspheres, paramagnetic microparticles [PMPs]) and enzyme-based chemiluminescent signal-generating systems using enzymes such as alkaline phosphatase (ALP). Such analysers, with their demand for shortened incubation times, and assays with higher sensitivity and specificity require selection of antibodies, enzymes and PMPs of the highest performance and quality.

Antibody selection

To a large extent the performance of the assay is heavily dependent on the antibodies used. With the increased number of antibodies available currently, it is becoming easier to select antibodies that provide assay

performance characteristics that are state of the art. They are carefully selected for sensitivity, specificity and stability. Both sensitivity and specificity can be tested using enzyme-linked immunosorbent assay (ELISA). A simple, yet efficient method for preliminary testing, ELISA offers rapid results without the need for expensive or elaborate equipment.

Solid-phase selection

Among the solid phases available today, PMPs are becoming the material of popular choice with system manufacturers. This is largely due to improvements in PMP technology and the ability of manufacturers to generate large lots reproducibly. In the selection of PMPs, several physical and functional features are important, in addition to the quality and security of supply. These include:

- the ability to be easily magnetised and resuspended
- the monodispersity of the particles (ie the PMPs are of uniform size and shape)
- low magnetic remanence (ie where there is no magnetism in the PMP after removal of the magnetic field).

These features are critical for handling PMPs at large-scale manufacture and also for assay washing efficiency on the analyser to avoid adverse effects on assay precision and sensitivity. Furthermore, the ability of manufacturers to provide smaller, monodispersed PMPs, resulting in increased surface area, allows assay developers to produce assays with high signal-to-noise ratios.

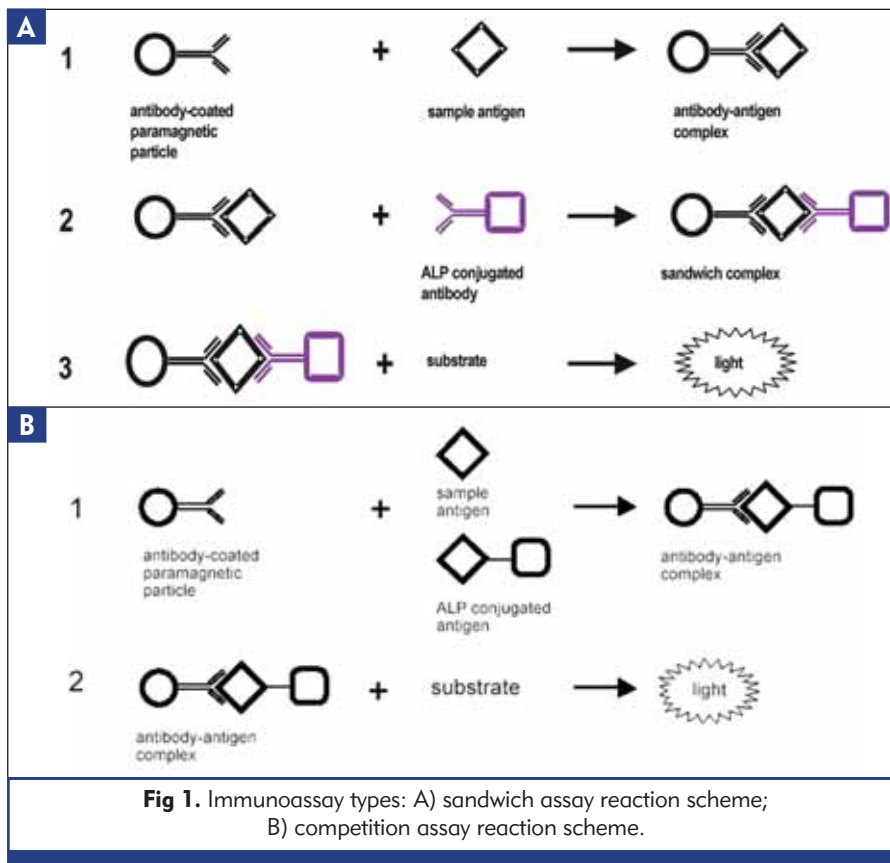
Solid-phase coupling

Depending on the type of assay, different molecules will be attached to the solid phase. For example, a typical sandwich assay format requires an antibody on the solid phase. Therefore, once the appropriate solid phase and antibody are selected, a method of attaching the antibody to the solid phase is determined from a variety of possible covalent or passive attachment methods. This, together with the coating concentration, is investigated to provide an assay with feasible performance and stability. Normally, the selection of solid phase, antibody, method of attachment and antibody coating concentration are a single process in the feasibility phase of development.

Conjugate development

During feasibility studies, a signal-generating system is selected that, together with the other assay components, must meet the performance requirements of the assay. Such signal-generating systems employ, for example, conjugates of an enzyme with an antibody. The conjugation chemistry allows the reagent developer to create antibody-enzyme complexes that maintain both the activity of the enzyme and the immunological reactivity of the antibody.

A range of conjugation chemistries can be used depending on the type of conjugate required. For example, in sandwich assays



the enzyme is conjugated to an antibody or other macromolecule, but in competitive assays the enzyme is conjugated directly to an assay-specific antigen, normally a small molecule. Furthermore, the conjugates must be stable over the product shelf life. At this stage, a conjugation method is selected, which the reagent developer will refine and optimise in the later stages.

Additional activities during feasibility studies include the first check of buffers to minimise the risk of interference within the assay and improvements to reagent stability. Basic calibration of the assay is performed. Methods for the inspection of raw materials commence development at this stage, and techniques such as high-performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) are used for these purposes.

Fast protein liquid chromatography

Size exclusion chromatography (SEC) is used in research and development (R&D) for the purification of conjugated antibody from unreacted antibody and enzyme. Also known as gel permeation chromatography (GPC), SEC separation, as the name implies, is based on the molecular size or hydrodynamic volume of the analyte.

The stationary phase or packing material contains uniform silicon or polymer particles of a defined diameter range and pore size. This is normally packed in a suitably sized column, typically made of glass or plastic. Small molecules and solvent diffuse in and out of the pores of these particles and therefore have a longer retention time on the

column than do larger molecules, which are forced to take an alternative route around the particles. Thus, all molecules larger than the pore size will elute first. In this way, any unreacted enzyme or antibody will have an increased retention time and be separated from the conjugate materials, which elute earlier.

Size exclusion chromatography is not a new method, but the technology behind it has improved dramatically over the last 10 years. The recent development of new packings or solid-phase gels for SEC has revolutionised this field and solidified FPLC as a key method for the development of immunoassay reagents. The efficient separation of proteins is achieved using the high-resolution, non-adsorptive columns that are now available.

In addition to the improved columns are modern FPLC systems with highly accurate and precise pumps that can now work efficiently at lower voltages. pH meters and ultraviolet (UV) detectors are today all linked to one centralised online monitor, enabling improved experimental control and set up.

When purifying the conjugate, the SEC column is connected to the fully automated online FPLC system with pre-programmed settings for each assay conjugate. This allows a controlled environment in which every parameter (pH, flow rate, UV detector functionality, conductivity etc) is predetermined and this means there is lot-to-lot traceability, as each conjugate run is compared against reference chromatograms. The R&D-scale FPLC columns can be accurately and efficiently scaled up, following

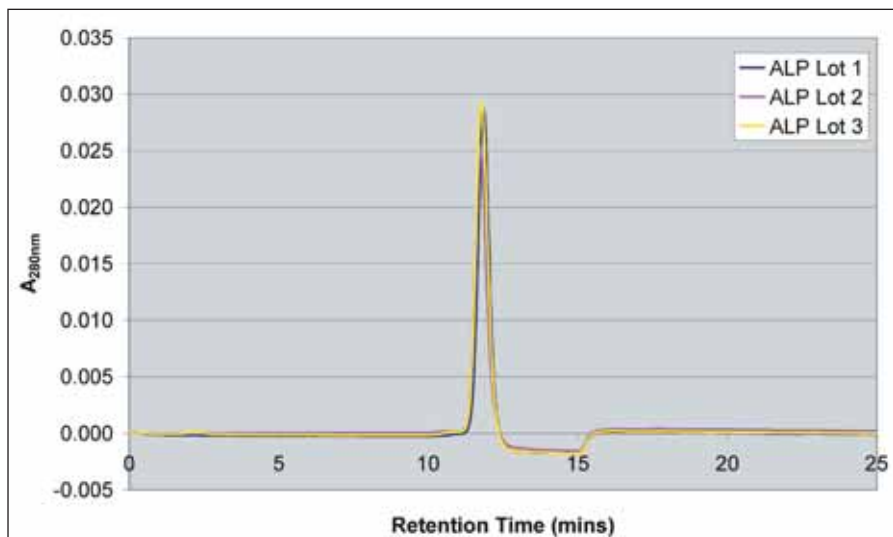


Fig 2. High-performance liquid chromatograms: three alkaline phosphatase (ALP) lots.

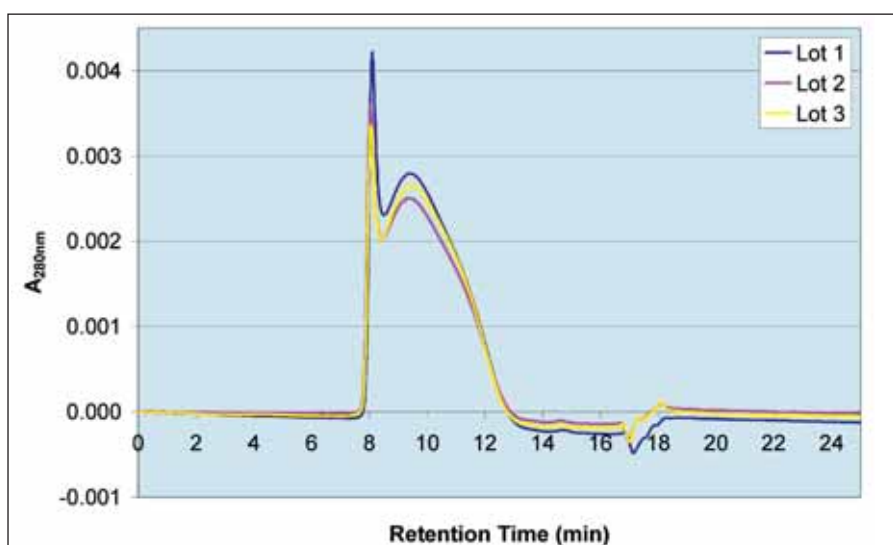


Fig 3. High-performance liquid chromatograms: lot-to-lot comparison of three AFP conjugates.

optimisation, due to the wide operating range of the FPLC system.

High-performance liquid chromatography

High-performance liquid chromatography can be used effectively to establish whether or not the conjugation reaction has worked and how successful it has been. The HPLC analysis of pre- and post-purification samples of conjugates provides an early indication of product quality and reproducibility of the reaction process. The chromatograph of pre-purification analysis represents the chromatograph produced by FPLC SEC, but with higher resolution. Post-purification chromatographs have a distinct peak, which is easily identifiable and represents the purified product. Therefore, HPLC is used to ensure that post-purification fractions do not contain unreacted species.

High-performance liquid chromatography is also utilised for the quality control (QC)

testing of raw materials such as antibodies and the chosen enzyme (eg ALP; Fig 2)

This, of course, would take place in the initial stages of assay development and would be vital in confirming the reliability of a raw material source. As in the case of ALP and antibodies, it enables the detection of contaminants or aggregates.

Process and method validation

Once a method is developed by FPLC/HPLC it must then be validated. This involves rigorous testing of the method using predefined experimental conditions that test the accuracy, sensitivity, precision, specificity and robustness of the method. The outcome of such experiments results in the setting of method operating limits within which any future run must fall.

Before method development can be performed, the FPLC/HPLC systems themselves must be qualified. The columns used are also qualified and must meet

predefined specifications when used on the systems to validate any runs that follow within a defined time period. This later qualification is sometimes known as 'system suitability'. This may also involve running standards to ensure accurate separation.

Imperative to the life cycle of a column is the need for cleaning after use. Such cleaning processes will vary depending on whether the column experiences multiple-product or single-product use. This limits the possibility of product cross-contamination. The cleaning process can be automated using software programmes on the FPLC/HPLC systems that enable total process control.

The development of methods begins in the feasibility phase and culminates in the validation of such methods during the optimisation phase of the project.

Optimisation

Following the first line of basic preliminary testing, the assay components are analysed intensively. The reagent developer typically refines the parameters developed during feasibility. For example, a range of antibody concentrations is checked to assess changes in conjugation ratios and optimal antibody binding for solid-phase coupling. As part of this process, consideration should also be given to system parameters such as temperature, incubation time and reagent and sample volumes. Consequently, extensive antibody performance checks ensue for sensitivity and specificity. Reagent stability is investigated in real time.

Where international standards (IS) are available these are used to ensure that the assays report results that are accurate and traceable to a higher order reference material. Where an IS is not available, traceability can be to a higher order reference method. The assay should meet the performance design goals at R&D scale and is therefore ready for performance verification on large-scale batches prepared under manufacturing conditions.

The processes for production and purification of reagents are optimised to ensure that lot-to-lot variation is minimised and production quality is maintained, from the initial stages of development, all the way through to manufacturing (Fig 3). The optimisation phase includes preparation for the manufacturing of components on a larger scale. Thus, at this point, each step of the process should have been analysed thoroughly and methods validated fully, as previously described, ready for scale up and pilot batch production.

Verification

The pilot batches prepared during this phase undergo complete assay performance testing and must meet the performance goals set in feasibility. Much of this testing will follow guidelines and protocols laid down by the Clinical and Laboratory Standards Institute (CLSI, previously NCCLS).^{1,2} It will also incorporate any special requirements from the

regulatory authorities governing the different markets in which the products are intended for sale. Scale up of the manufacturing processes is performed during verification and minor changes to protocols may be necessary, depending on the results obtained.

Real-time stability studies are conducted on these lots in preparation for market introduction. All manufacturing equipment is qualified prior to preparation of the pilot lots. This involves performing installation qualification (IQ) and operation qualification (OQ), and successful preparation of product using the equipment constitutes a performance qualification (PQ). Data generated from these studies are used to make the performance claims of the assay in the market. Furthermore, data are also used in the process of setting specifications for in-process and release testing of the product.

Validation

Product validation usually involves information that the test is fit for use with real patient samples and is performed preferably in the clinical laboratory environment against an approved competitor system. Typically, the accuracy, precision, linearity and potential sample-related interferences are tested.³ Such validation occurs on reagent lots manufactured under final conditions with current Good Manufacturing Practice (cGMP). Regulatory submissions are filed to the appropriate authorities during this phase. All technical trials for CE markings are completed, filed and the CE mark applied to the product in

preparation for launch in the European market.

There is additional validation of the manufacturing equipment and a last intense step of process validation, during which all the methods (eg antibody coating, conjugation, mixing, filling and cleaning) are tested within operating limits, specified during optimisation and verification. Where assays are intended for use on a new automated analyser, the analyser and reagents system undergoes comprehensive validation. This normally involves testing the system's capabilities under normal routine laboratory workflow. Features such as throughput, robustness and walkaway time are validated.

Future of immunoassay

Automated enzyme immunoassay has grown rapidly and developed in complexity over the last 10 years. The process of development has been dissected and evaluated constantly for the production of refined assay systems that offer improved sensitivity and reproducibility. The innovative technologies used to manufacture raw materials and equipment has become more sophisticated and, most importantly, highly controlled.

The methods of process, product and system validation, as explained above, are extensive but are key to the quality and high performance of assays released for routine diagnostic use. Today, the quality of immunoassays on the market far exceeds that seen previously, but we wait with

anticipation to see what measurement capabilities future assays will achieve. ■

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- 3 Selby C. Interference in immunoassay. *Ann Clin Biochem* 1999; **36**: 704-21.

SUGGESTED FURTHER READING

- Hage DS. Chromatographic approaches to immunoassays. *J Clin Ligand Assay* 1997; **20**: 293-301.

Mike Minihan is team leader for standardisation and verification in the Olympus R&D Group, Ireland. He has worked extensively on the recent development of the Olympus AU3000i, a high- specification, automated enzyme immunoassay system to be launched this summer.

Dade Behring Scholarships

The Emil von Behring scholarship programme awards \$250,000 a year to students worldwide who are pursuing advanced laboratory technology degrees. Scholarships will be awarded in the USA and several key European countries as well as in Canada, Asia and Latin America through partnerships with selected agencies and universities. The total contribution in countries outside the USA over a four-year period will be \$660,000.

In the UK, five scholarships, each worth £1000, will be awarded annually to selected students pursuing a co-terminus degree in biomedical science. Dade Behring has chosen the IBMS as its administering partner for this scholarship programme in the UK.

Eligibility

Applicants must be registered as Student Members of the IBMS and have successfully progressed from the penultimate year to the final year of their IBMS-accredited co-terminus biomedical science degree.

Selection

Eligible students must produce a PowerPoint presentation entitled "The Role of Biomedical Science in Healthcare Delivery". This should

comprise a maximum of 10 slides aimed at a subject audience of 16- to 18-year-old students. Presentations should be submitted to the programme leader for the co-terminus degree, who, with employer representatives, will select the best presentation.

The presentation (PowerPoint format plus presentation script) chosen by the university will be submitted to the IBMS Education and Professional Standards committee during August. The committee will review the presentations and produce a shortlist of the top 10 presentations. Shortlisted students will then be asked to give their presentation to the committee and representative(s) from Dade Behring in time for the awards to be made in September. Students giving the best five presentations will be awarded £1000.

Winning presentations must be made available both to the IBMS and to Dade Behring for use in promoting the profession. Students may also be asked to give their presentations at the time of the scholarship award (eg IBMS Biomedical Science Congress or HUCBMS conference).

Further information will be made available direct to university liaison officers from the IBMS Executive Head of Education.

Committee on Publication Ethics Seminar 2006

Friday 10 March, BMA House, London

This year's COPE seminar will take an international perspective and address publication ethics and research in several European countries and beyond, with interactive workshops on common ethical and editorial dilemmas. The manipulation of impact factor, and whether or not it is unethical, will also be considered. The seminar is for editors, authors and all those interested in increasing the standard of publication ethics.

The seminar is free to COPE members and £30+VAT for non-members. Numbers are limited and early booking is advisable. For registrations or more information please contact the COPE secretary at cope@bmjgroup.com or call 020 7383 6602.

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