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Review

Process analytics for purification of monoclonal antibodies☆

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Abstract

The application of appropriate analytical methods is an essential requirement for the purification of therapeutic antibodies. A range of analytical methods need to be employed to effectively determine the purity, identity, integrity and activity of these important class of pharmaceuticals. These include notably electrophoresis, high performance liquid chromatography and immunoassays. Regulatory and industry demands in recent years have brought the need for improvements and many have been successfully implemented. This article reviews the current analytical methods applied to support the purification of monoclonal antibodies.

Keywords: Antibodies; Purification; Analytical methods; Process analytical technology

Contents

Antibody products have become one of the important types of pharmaceutical that are able to successfully address certain diseases. Their production requires extensive and complex processes to generate a quality product. To satisfactorily understand any process, it is necessary to have suitable measurement systems. This is certainly true for production of monoclonal antibodies. Effective measurement systems are an absolute requirement for successful process development and manufacture of antibody products. Whether the aim is to determine the product quality or characteristics, the process consistency or operational parameters or the process efficiency and economics reliable measurement systems are essential. This article reviews the methods required to successfully develop and operate a purification process for a monoclonal antibody.

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The application of appropriate test methods has always been at the centre of regulatory requirements for therapeutic products. However in recent years, the introduction of the strategy of process analytical technology (PAT) has heightened the need for this approach. Recent developments in methodologies have been to improve their capabilities to provide more detailed results, to increase sample throughput and shorten analysis time. These developments have enabled the regulatory authorities to consider the PAT strategy for antibody production processes [1,2].

The objective for testing can be defined as the following areas; product characterisation, lot release testing, process monitoring (including development, validation and control). It is essential to understand the purpose of a specific measurement. The products and the processes are complex and indeed the process impurities may be complex molecules, i.e. heterogeneous in their composition. Therefore, it is necessary to understand and define precisely the specific aspect of a product that a particular test method will determine.

The test methods are either directed to determine characteristics of the product or measure the operational performance of the process. A large set of test methods is employed to measure operating parameters to be able to monitor and control purification processes, e.g. pH, conductivity, flow rate, pressure, temperature. These measurements are equally important particular with respect to PAT. The test methods are an inherent part of current purification systems, using integral detection devices, and often are incorporated into the control mechanisms for process control. The control of such operating conditions is essential for process consistency and product quality. Also included in this category is the measurement of product concentration through the use of integrated spectrophotometers, although the validation of such instruments is not straightforward. Their application is not discussed further in this article.

Product test methods are targeted at certain properties of the product and fall into two general categories. Those aimed at defining the physical and chemical characteristics of the antibody molecule and secondly those defining the impurity profile. Examples are provided in Table 1. This article reviews the application of these various tests, and recent developments in each area particularly with respect to purification development, process monitoring and control.

Table 1
Test methods applied for production of monoclonal antibodies

Product characteristic	Analysis properties	Test method platform
Physical and chemical characteristics	Purity	Electrophoresis Reverse phase HPLC Size exclusion HPLC
	Integrity/molecular weight	Electrophoresis Mass spectrometry Size exclusion HPLC Light scatter
	Identity	Isoelectric focussing Peptide mapping Ion exchange HPLC
Potency/activity	Antigen binding	Immunoassay
	Biological methods	Cell proliferation Complement mediated cytotoxicity Reporter-gene assays
Product related impurities	Aggregation/fragments	Electrophoresis Size exclusion HPLC
Process related impurities and contaminants	Host cell protein	Immunoassay
	Host cell DNA	DNA hybridisation QPCR DNA binding-threshold Fluorescent—picogreen
	Protein A Cell culture medium proteins	Immunoassay Immunoassay
	Viruses	QPCR Electron microscopy In vivo/in vitro assays
	Microorganisms	Bioburden Endotoxin—LAL test
	Others: column/vessel leachates/extractables cell culture medium components, purification reagents/chemicals	Various e.g. reverse phase HPLC, ion chromatography, GC-MS

1. Electrophoresis technologies for antibody identity, purity and integrity

The identity and integrity of monoclonal antibodies must be tested at all stages of the process, from cell culture through downstream purification (process monitoring), product characterisation and lot release. At each step, it must be confirmed that both the correct product is being produced, it is of appropriate purity and intact, and that no unwanted modifications to the antibody have occurred. Antibody products are heterogeneous mixtures of closely related size and charged isoforms. Electrophoretic methods are ideally suited for purity, identity and integrity testing of antibody products. The electrophoresis methods most commonly employed are isoelectric focussing (IEF) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). IEF separates proteins based on their net charge whilst SDS-PAGE separates on the basis of size (molecular weight). IEF is used for identity and SDS-PAGE mainly for integrity and purity tests.

2. Charge separations by electrophoresis

Electrophoresis methods of therapeutic proteins have historically been performed using polyacrylamide slab gel methods [3]. Commercially available pre-cast slab gels offer considerable time savings and consistency for slab gel IEF analysis. The assay is still relatively labour intensive and assays can take greater than 1 day if quantification by densitometry of the gel is required. The method is not well suited to rapid analysis for pro-

cess monitoring and control. In principal the same techniques performed by traditional slab gel electrophoresis can also be performed by capillary electrophoresis (CE), but with potentially increased sensitivity and resolution. The key characteristic of CE methods is that they provide the added benefit of rapid at/on-line measurement through the use of auto samplers for unattended operation. CE instruments are ideally equipped for automation and are very similar to high performance liquid chromatography (HPLC) systems in function. This provides significant automation advantages over traditional slab gel electrophoresis. CE was first introduced in 1988 [4], but has only recently gained the widespread acceptance in the biopharmaceutical industry that was first anticipated. The amount of sample material and chemical reagents required are extremely low, and the sample amount requirement is approximately 1000-fold less than that required for slab gel methods.

As previously mentioned, during process monitoring, characterisation, and finally bulk release, identity of the product needs to be confirmed at all stages, and this is most commonly achieved by analysing the product by isoelectric focussing. Protein products are a mixture of related isoforms with varying charge densities due to slight difference in glycosylation, minor modifications to amino acid residues within the protein sequence, as well as amino acid clipping and modification events at the terminals of the protein sequence. The minor charge density difference between isoforms can be separated by IEF and it is used as a fingerprint method to identify that the correct form of the antibody is being produced. Test samples are analysed alongside a reference sample and the charge profile between the two samples are compared. When monitored through a process, shifting of the banding pattern can indicate chemical modifications of the sequence such as deamidation of asparagine and glutamine residues.

Separation by CE is typically much quicker than by slab gel electrophoresis. However, once separation is achieved, the contents of the capillary need to be mobilised (pushed through the capillary) such that a flow passed a point detector is achieved. Detection of analytes is achieved by point detection using UV absorbance or laser-induced fluorescence (LIF) near the terminal end of the capillary. The need to mobilise the capillary contents to pass the point detector is the main source of method variation and particularly affects resolution.

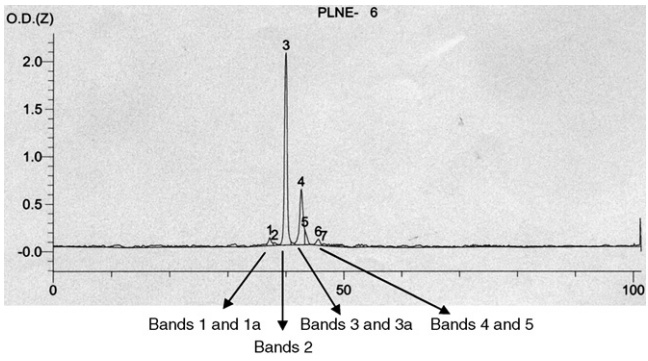

Fig. 1. Agarose IEF gel densitometry profile of an IgG.

Recent developments in capillary IEF (cIEF) have addressed the need for mobilisation. Of particular note is imaged capillary isoelectric focussing (icIEF) using Convergent Biosciences icIEF system with 280 nm detection (iCE280)[2]. Isoelectric focussing of proteins by icIEF (and all CE IEF methods) uses the same ampholytes as conventional slab gel electrophoresis, hence the separation technique used by all the methods are essentially the same. However, once the proteins have been focussed, the iCE280 system uses whole-column imaging detection to monitor the IEF pattern of proteins within the capillary column and eliminates the mobilisation process necessary with conventional CE instruments. While improving the resolution and method variation compared to conventional CE, elimination of the mobilisation process also considerably decreases the assay execution time and consequently sample throughput is increased three-fold or more. Furthermore, method development time is minimised since optimisation of the mobilisation process is not required. Results using the iCE280 instrument are directly comparable to slab gel IEF [5] and are achieved in a fraction of the time and cost (see Figs. 1 and 2).

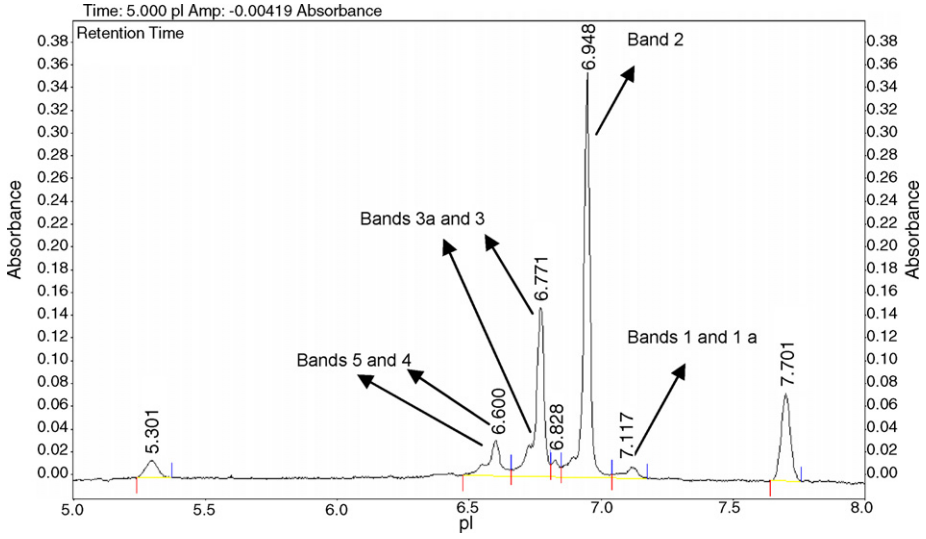

Fig. 2. Imaged cIEF profile of an IgG.

3. Size separations by electrophoresis

In addition to confirmation of product identity using IEF, product integrity and purity is often monitored by electrophoresis. For product integrity, the antibody product is analysed using both reducing and non-reducing SDS-PAGE. In non-reducing SDS-PAGE, samples are analysed for size and purity. Similarly in reducing SDS-PAGE, the individual protein subunits are analysed for size and purity first treating the protein product with a reducing agent in order to reduce any intramolecular and intermolecular disulphide bonds in the protein. Dissociation of the intact antibody is readily detected by non-reducing SDS-PAGE. Reducing SDS-PAGE is very effective at determining fragmentation (proteolysis) of the antibody heavy and light chain subunits.

As in IEF, slab gel methods have been the traditional analytical technique used for SDS-PAGE analysis. This method is time consuming and labour intensive, even with the use of commercially available pre-cast gels. The needs for quicker, automated and less labour intensive methods have led to the switch to CE type methods that provide the same purity and size information. The Agilent Bioanalyser is one of several recent technologies for rapid SDS-PAGE type analysis [6]. This system uses miniaturised capillary electrophoresis (lab-on-a-chip) technology where sample preparation, sample handling and biochemical analysis is carried out within a microchip. The Agilent Bioanalyser is available with a 96 well microtitre plate autosampler that significantly increases the throughput of this method. Using this system allows the automated analysis of size, concentration and purity of proteins in the range 14–210 kDa in less than 1 h, and since most IgG products and their subunits lie in the 20–150 kDa range, this method is ideally suited to rapid at/on-line monitoring during process monitoring of monoclonal antibody products. The chip consists of a network of miniature glass channels for the movement of sample fluid. Electrokinetic forces move sample fluid through selected gel filled pathways. This mimics the fluidic movement between electrodes in capillary electrophoresis. The proteins are separated by molecular sieving mechanism in the gel matrix in the separation channel of the chip. Staining and destaining occurs within the chip. Destaining decreases the fluorescent background and proteins are detected by laser-induced fluorescence at the detection point. The results are recorded and analysed by on-line software. The system automatically calculates protein size and purity in 30 min. Sample results can be visualised on the software screen as an electropherogram or a digital gel image during analysis, and thus can be compared to traditional slab gel methods.

The lab-on-a-chip technology has obvious key advantages over traditional slab gels. For monitoring antibody integrity (i.e. the molecule is intact and composed of two heavy and light chains) it is the method of choice. However, to date the detection and quantitation limits of the lab-on-a-chip method are approximately 0.5–1.0% for an impurity compared to 0.1–0.2% for slab gels. Therefore, for purity measurements the slab gels are still the method of choice.

4. HPLC technologies for antibody identity, purity and integrity

There are a number of established HPLC technologies available for testing proteins [7,8]. These include ion exchange HPLC and peptide mapping for primarily identity and reverse phase HPLC and size exclusion HPLC for purity measurements. The most commonly employed is size exclusion HPLC for determining purity and in particular the levels of product aggregation.

Size exclusion chromatography is employed as a non-destructive method for monitoring aggregates of native monoclonal antibodies that result from covalent or non-covalent interactions [9]. The method should be able to resolve a dimer of the antibody from the monomer and more highly aggregated species. Dissociation and fragmentation products may also be measured, although the level of resolution achieved between these species is very much inferior to that of SDS-PAGE. Elution of proteins from the columns is normally monitored with conventional spectrophotometric detectors set at a selected wavelength (e.g. 215 and 280 nm). The linear range for monomeric antibody response is around 0.1–500 μg. Aggregates have a response factor similar to that of their respective monomers. The limit of detection of aggregates is approximately 0.1% using current systems.

The major problem encountered with this method is interaction of the antibody molecules with the column matrix, resulting in non-ideal chromatography that is observed as peak broadening and elution at apparently lower molecular sizes. Resolution of monomer from other species is greatly affected under such conditions. Interactions occur primarily through two mechanisms involving hydrophobic and/or ionic forces. Hydrophobic interactions can be minimized by the inclusion of small amount of detergents or organic solvent in the mobile phase, while ionic interactions are principally controlled by altering the pH or ionic strength. Due to the weakly acidic nature of many column support materials basic-hydrophobic antibodies present the main problems. The addition of charges (basic) chaotropic agent can help minimize both types of forces. In this respect amino acids have been found to be most suitable (e.g. lysine and arginine [10]). Typically separation takes over 30 min per sample and therefore the technique is not suitable for rapid process analysis.

Reverse phase HPLC can be employed as a general purity test for antibodies. The impurities detected will include proteins (i.e. product related fragments or aggregates) and also non-protein derived from the manufacturing process (e.g. chemical leachates and extractables). The specificity of the technique should be considered for interpreting purity values. Separations can be achieved in a few minutes, particularly using current automated fast chromatography systems. Therefore, the method can be considered for process control.

Peptide mapping usually involves reverse phase separation of proteolytic peptides (e.g. tryptic peptides) of the product. The profile may consist of over 60 peaks representing the individual peptides and their derivatives. Peptide mapping is a very powerful technique for confirmation of the chemical composition of an antibody product, most often relative to a reference standard [11]. For this reason, it has become the method of choice as

an identity test for monoclonal antibodies. However currently it is an extensive technique that takes at least a few hours. There are a number of developments ongoing investigating alternative approaches to peptide mapping, e.g. direct mass spectrometry of proteolytic peptides, however these are not yet available for routine use. Therefore, the technique is currently not suitable for rapid process analysis.

Ion exchange HPLC, either cation or anion, is used as an alternative to charged based electrophoresis separations (e.g. IEF) of antibodies. It has the advantage over IEF of being able to detect differences in the surface charge distribution of antibody molecules and the peak elution can be quantified directly by the HPLC detector. However the overall resolution of charge isoforms is sometimes inferior to IEF and satisfactory separations can take somewhat longer than those by capillary based methods such as the imaged capillary isoelectric focussing described earlier.

5. Technologies for measurement of antibody physical characteristics

The process of production and formulation of therapeutic monoclonal antibodies exposes them to non-ideal matrices which may lead to a change in conformation and the formation of aggregates. It is imperative that products are characterised in order to assess the native structure and any changes in conformation. Transformation of the native nascent structure may affect the potency of the product by causing undesirable clinical effects to patients, e.g. immunogenic response.

Several types of aggregates may form during the above mentioned processes, which include aggregation consisting of non-covalent and covalent (often disulphide-linked) dimers and trimers. The detection of the aggregates at an early stage is important since this could influence the purification strategy. In addition characterisation studies of therapeutic antibodies are required to determine the composition of the aggregate species.

The most commonly used and very effective method for aggregate measurement is size exclusion HPLC, as described earlier. However this is not suitable for rapid process monitoring and control. Example methods that can be employed for analysis of confirmation and aggregation are summarized in Table 2.

There is currently no ideal analytical method for process monitoring of aggregation or conformation of antibodies [12]. For aggregate measurement it is a compromise between sensitivity for detecting low amount (e.g. <1%) of aggregates and speed of analysis. For example, SE HPLC is quite sensitive but is not a rapid technique for aggregate measurement. Alternatively, dynamic light scatter (DLS) can provide results in a few minutes but does not have the sensitivity of SE HPLC for aggregate detection.

Most of the methods for determining conformation (or relative conformation) are relatively insensitive [13]. Of the methods currently available Fourier transformed infra-red (FTIR) [14] and ultrasound [15] may have the greatest potential for process control. Both are relatively rapid techniques that require minimal sample manipulation and have been shown to detect changes due to aggregation and conformational changes to proteins. The changes detected by FTIR can be assigned to definitive structural changes to a protein. This is not currently possible for the changes detected by ultrasound where further work is required to understand what aspects of protein structure can be monitored. However ultrasound is a non-invasive measurement that can be made in-line and therefore is a format that can be readily applied to process monitoring and control.

Table 2
Potential techniques useful for aggregate and conformational analysis

Technique	Application	Advantages	Limitation
Fourier transformed infra-red (FTIR)	Conformation, secondary structure, tertiary structure	Rapid technique with minimal sample preparation requirements	Technique may not show minor changes in structure
Circular dichroism (CD)	Secondary structure	Minimal sample preparation requirements	The buffer matrix may interfere with analysis, requiring dilutions or buffer exchange
Micro-rheometry	Aggregation	Measures product viscosity and transition temperature. Minimal sample preparation requirements	The technique requires dedicated pressure supply and is not a rapid analytical tool
Differential scanning calorimetry (DSC)	Conformational aggregation	No sample preparation required. Multiple absolute parameters generated per single analysis	The technique is not a rapid system, recent development could increased throughput
Raman spectroscopy	Conformation, secondary structure tertiary structure	Minimal sample preparation required	High protein concentration required. Laser heat may induce changes in the sample.
Dynamic light scatter (DLS)	Aggregation	Rapid detection of aggregates	Sensitivity at very low aggregate levels
Static light scatter (SLS)	Aggregation	Absolute aggregate and monomer size measurement	This is not a rapid technique
SE HPLC	Aggregation	Aggregate and monomer size and contents measurements	Does not detect large aggregates Not rapid
Analytical ultracentrifugation (AUC)	Monomer and aggregate	Accurate aggregate size and contents determination	Protracted assay period
Ultrasound	Conformational aggregation	Non-invasive in-line for rapid analysis	Need to develop understanding of results

6. Impurities in antibody products

Process-related impurities for antibody products would include host cell proteins (HCP), DNA, cell culture medium components, chromatographic medium leachates (e.g. Protein A) used during purification, solvents and buffer components. These impurities should be minimised by use of appropriate well designed and controlled manufacturing processes.

To enable the efficient development of the required purification process for the purpose of the removal of these impurities and subsequent safe release of antibody products a panel of test methods are utilised to monitor the removal of impurities. For the impurities, the choice and optimisation of analytical procedures should focus on the separation of the desired product and product-related substances from impurities. Here we discuss the requirements and improvements which have occurred for the analysis of three key process related impurities, HCP, DNA and Protein A, in antibody production.

7. Host cell DNA

The DNA present within in-process and final product samples arise from the rupture of the cells during fermentation processes and may vary in level depending on the cell number, viability and other fermentation conditions. The DNA content in antibody products is required to be below a specified limit. It is suggested that, whenever possible, the final product contain no more than 100 pg cellular DNA per dose. It is also expected that the method used to determine DNA content will have a sensitivity of 10 pg to allow accurate DNA quantification. From a review of the WHO guidelines [16], the WHO has concluded that levels up to 10 ng of residual host cell DNA per purified dose can now be considered acceptable. However, it has been stated that instances do occur where DNA is considered to pose a greater risk, e.g. where it could include infectious retroviral provirion sequences.

The use of the DNA slot blot hybridisation method [17] to determine the residual DNA was routinely used for this analysis by the biopharmaceutical industry. The main alternative has been a method based on DNA binding proteins (e.g. Threshold system [18]). The DNA hybridisation method provided adequate sensitivity, however was subjected to lengthy analysis time, issues with robustness and low precision. These factors and the need to further challenge the turnaround time to return data to facilitate more efficient purification process development and operation lead to the evaluation of an alternative DNA analysis system based on the quantitative polymerase chain reaction (QPCR) technology.

QPCR is now the standard method applied for host cell DNA measurement [19]. For example, we have developed a QPCR residual DNA assay on the ABI 7700 platform to replace the standard DNA slot blot methodology. This resulted in significant improvements to the assay performance and quality of data provided for more rapid optimisation of purification process conditions (see Table 3).

The resulting improvement to the limits of quantification (LOQ) and assay robustness has allowed for higher precision in the measurement of low levels of DNA. This has delivered an increase in accuracy of the assignment of DNA per dose of product to meet a regulatory requirement and also has facilitated the rapid optimisation of purification conditions due to the delivery of DNA data in a short time frame compared to the previous methodology. Trends in DNA clearance were observed which previously would have not been apparent due to limitation in the hybridisation assay sensitivity (see Fig. 3).

Table 3
Comparison of DNA methods

Measured parameter	DNA hybridisation	QPCR
Working range	2 log	5 log
LOD (pg/ml)	2	1
LOQ (pg/ml)	4	2
DNA (pg/mg)	0.8	0.08
Precision (% CV)	<30	<15
Accuracy (%)	50–250	70–130
Robustness	High fail rate (20%)	Low fail rate (<2%)
Automated	No	Yes
Response to low molecular weight DNA	Negative bias	No bias
Usage of solvents/radio-isotopes	Required	Not required

The QPCR technology does not in this form allow for at line data delivery which would be required for rapid process monitoring and control. An alternative may come in the form of an enhanced immunoassay platform application, e.g. using DNA binding proteins, which could overcome the challenges of automation whilst maintaining an appropriate detection limit (e.g. at the pg level of sensitivity) and sample recovery.

It is also suggested that DNA of greater molecular weight >1 kb poses a higher risk than DNA of <1 kb due its potential oncogenic properties [20,21]. For this reason it is required to establish a method which allows for the isolation and characterisation of DNA from in-process samples. This assay can be performed on the Agilent Bioanalyser (as described for protein separation) and is based on chip based microfluidics technique for the characterisation of DNA. The method is able to accurately size the DNA present in samples after performing an initial isolation step for the DNA (see Fig. 4).

The method can also quantify the concentration of DNA. However the detection limit is approximately 1000-fold greater (ng DNA/mg product) than that of QPCR (pg DNA/mg product). The assay does have the potential to be used rapidly at/on-line with the development of rapid isolation methods. Therefore, it

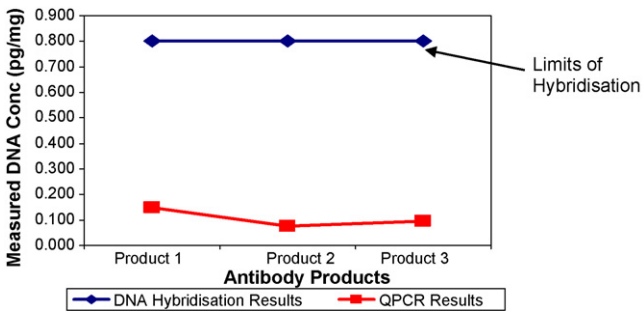

Fig. 3. Comparison of sensitivity between hybridisation and QPCR methods.

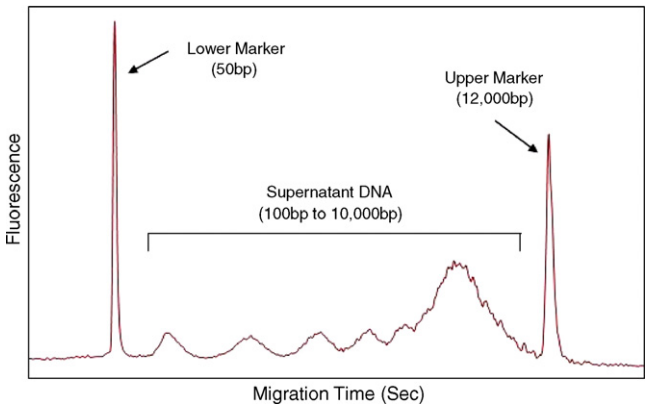

Fig. 4. DNA size determination using the Agilent Bioanalyser.

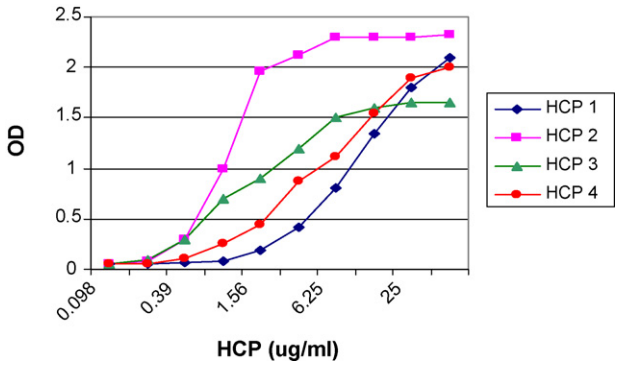

Fig. 5. Response factor for individual HCPs.

offers a strategy of being able to demonstrate DNA concentration and composition within a specified level at a defined point of the purification process. Supported with appropriate DNA removal data this can provide a justification for omitting the need for final product testing for DNA.

8. Host cell proteins

Host cell proteins (HCP) are a complex set of analytes that are required to be measured at low concentrations (ppm or ng HCP per mg antibody product). Development of an appropriate test method can be the greatest analytical challenge for process development. The assay system has to specifically and sensitively measure a wide array of analytes. Therefore, HCPs are most effectively assayed using immunoassay methods [22].

Typically antisera are raised against an antigen preparation, composed of all the expressed proteins, derived from the host cell line that lacks the specific gene coding for the product (a null cell line) [23]. The null cell line is cultured and the proteins extracted by processes equivalent to the manufacturing process. Partially purified preparations of the antigen, using steps from the manufacturing purification process, may also be used for the preparation of antisera where applicable.

There are two mainly used formats of the immunoassays (Table 4). One is based on a conventional ELISA format. The second is a Western blot format and involves separation of HCPs from the product by SDS-PAGE followed by electro-blotting onto a membrane and immuno-detection of the HCPs using the prepared anti-HCP antiserum. The relative merits of the two methods are defined in Table 4.

The Western blot method is very useful for process characterisation studies. It provides details of the effectiveness of individual process steps for the removal of particular HCPs. However, the Western blot is a relative extensive and labour intensive technique that is very difficult to automate and therefore is not suitable as an in-process control method.

The ELISA is the method of choice for overall quantification of HCP levels. The greatest challenged for the ELISA is to accurately quantify each HCP from a diverse large range within the same assay. For example, the calibration standard most often comprises the mixed antigen preparation that was used to prepare the antisera for the assay. The range of HCPs in this preparation will induce different immune responses and number of antibodies with differing affinities for individual HCPs within a polyclonal antibody preparation. Thus we have the potential for individual HCPs giving different response factors in the assay as demonstrated by the scenario depicted in Fig. 5. Therefore, the results of the ELISA may not be absolutely accurate. However in practise the results can be compared between samples (i.e. from different process steps) and can be used to set process control limits.

The procedure for the ELISA is a standard immunoassay format that can be readily automated and modified to fit into any of the rapid immunoassay systems in development (e.g. the Gyrolab system from Gyros AB and the Octet system from Forte Bio). It therefore has the potential to be applied as an at/on-line process control where necessary.

9. Protein A

Protein A affinity chromatography is a very efficient process step utilised commonly within purification processes for the production of monoclonal antibodies. It facilitates high purification of the product in a single step. However any Protein A leaching from the column matrix will associate strongly with the product antibody and it is necessary to demonstrate the removal of the Protein A by the subsequent process steps.

The measurement of Protein A is most usually achieved via a conventional immunoassay. The method must include a procedure to dissociate the Protein A from the product for accurate

Table 4
Comparison of the ELISA and Western blot HCP assay formats

Format	Direct quantification	Identification	Limit of detection (ppm)	Use
ELISA	Yes	No	1–100	Lot release testing and process control
Western blot	Semi	Yes	20–200	Process characterisation

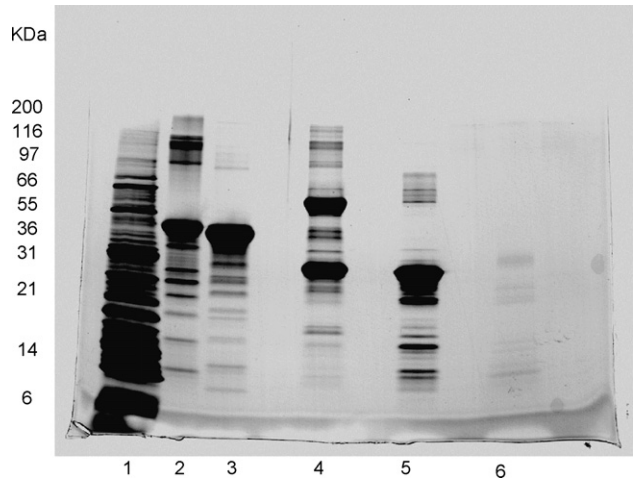

Fig. 6. Analysis of four sources of Protein A by SDS-PAGE.

measurement. This is often achieved by including a low pH (<4) incubation or detergents within the method. This is needed for accurate quantification. The target for the method should be to measure to a limit of detection of less than 1 ppm (1 ng Protein A per mg product) to ensure acceptable removal of Protein A.

Various types of Protein A ligand are available and used in the industry. Examples of four Protein A ligands analysed by SDS-PAGE are shown in Fig. 6. Each Protein A has a distinct molecular weight distribution and each is heterogeneous, e.g. for each there is some amount of fragmentation. The consequence of this is that each will have a different response factor within the assay. Thus a specific assay standard is required for each type of Protein A. Also the main aim of the test must be to accurately quantify the Protein A leaching from the purification matrix (e.g. lane 6 in Fig. 6). Therefore, the response of this preparation must be confirmed relative to the standard.

Similar to the HCP assay, the ELISA for Protein A is a standard immunoassay format that can be readily automated and modified to fit into any of the rapid immunoassay systems in development. It therefore has the potential to be applied as an at/on-line process control where necessary.

10. Potency/activity assays

Apart from the biochemical assessment of the produced antibody a key test is to demonstrate the actual biological functionality to confirm the desired in vivo effect can be achieved. This is required as an antibody can loose its activity because of the physical stresses and are intrinsically susceptible to chemical modifications due to the chemical environment that are part of purification processes. There is a requirement to test activity using a bioassay for product release testing and this can be performed using three routes. The first is a simple binding assay (immunoassay or cellular based) to measure the ability of the antibody to bind its target site (antigen); the second is the measure of potency which determines the product ability to elicit the required response within a representative biological model (cell based assay); the third is the use of animal based assays to measure biological activity, these latter assays will not be discussed as they are not applicable to process development.

11. Binding assays

The binding assay can be measured using two parameters, one relates to the actual amount of a product bound to the target site and is often expressed as a percentage compared to an antibody reference standard. This can be performed either on an isolated antigen by various immunoassay means, including using conventional microtitre plates, or by a cell based assay most often using flow cytometry as a measurement system. Both methods represent a robust and accurate measure of the ability of the antibody to bind to its target site with acceptable throughput with usually an approximate 1 day turn around. The use of automated immunoassay platforms can be utilised for more rapid at/on-line (<1 h) monitoring of antibody activity and provides information that can be used to influence processing decisions if required.

The second measure is of the binding rate or affinity of the antibody to its target site. This can be determine by measuring the binding kinetics rate of the antibody and target interaction using technique such as microcalorimetry, Biacore using surface plasmon resonance (SPR, which measure the change in angle of light from a gold surface during binding of an antibody to the capture molecule) [24] or new systems such as the Octet^{TM} from Fortebio^{TM} (which detects a wavelength shift of white light on the surface of multiple optical fibres during binding) [25]. The kinetic application is not widely used in the industry for process decisions and has yet to be established as a common requirement in the production of antibodies. This technology can be utilised for rapid analysis for a limited number of samples (<5) and could be used to deliver results with 2–3 h, however this application has not been utilised by the industry. It is potentially a more sensitive test for the effect of process conditions on antibody function.

12. Cellular assay (functional)

The measure of antibody potency is carried out using cellular based assays to determine the antibodies ability to elicit the required immune response, using a biological model. Typically cell proliferation, complement mediated cytotoxicity, reporter-gene and signal transduction assays are used for antibody activity testing. These assays are generally more relevant and sensitive to the measure of antibody function compared to the binding assays. However they are often less accurate, have lower precision and a longer time is required for method development and optimisation.

The use of a bioassay to test functionality is essential for product release, characterisation, immunogenic and comparability studies and stability assessment. For this reason regulatory bodies are recommending the use of two different types of assay, including binding assays with a second more relevant functional assay. Generally there is no real requirement to provide the data for at-line process decisions, which would be a particulate challenge due to the involvement of cell reagents and complexity of the test methods. It is sufficient to employ the tests for lot release

and to ensure they are accurate, stability indicating and sensitive to changes in the product.

13. Conclusion

A range of analytical methods are required for successful development and operation of antibody production processes. The majority of methods are available to monitor all aspects of process operation and product characteristics. The important current developments are in the areas of miniaturisation and automation of many of the analytical methods. This facilitates the possibility of at-line or real time measurements and consequently the opportunity for effective process control. The exciting concept of process analytical technology is being accepted by the biopharmaceutical industry as a means of improving manufacturing process to better control product quality and process efficiency. The antibody purification processes of the future will be well controlled.

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