PERSPECTIVE: ANALYTICAL BIOTECHNOLOGY

Quantitation of DNA and Protein Impurities in Biopharmaceuticals

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The development of drugs and biologicals for human injection generated from recombinant DNA and hybridoma technologies has resulted in new standards for product purity. We discuss the regulatory position relative to impurities in these biopharmaceuticals, focusing on the analytical goals for quantitation. Current methods for making these measurements are reviewed, and a new system designed for improved analysis is described. Assay results for both contaminating DNA and proteins are presented.

INTRODUCTION

Although the development and manufacture of drugs and biologicals have long been regulated, particularly for nonorally administered agents, recent advances in technology have heightened awareness of potential safety hazards. Corresponding advances in measurement and purification technology have allowed more stringent purity standards. Therapeutic biopharmaceuticals and in vivo diagnostics made by recombinant DNA and monoclonal antibody based processes (referred to in the present discussion as biopharmaceuticals) are typically proteins synthesized in and harvested from cultures of genetically modified cells. Thus, the starting material for the purification of each biopharmaceutical is complex, heterogeneous, and potentially unsafe. Potentially dangerous impurities and contaminants include host cell molecules (e.g., DNA, protein, or lipopolysaccharide), viruses infecting the host cell, and components introduced during processing (e.g., proteins and hormones in the cell culture medium or ligands used in immunoaffinity purification). Development of suitable purification processes and appropriate analytical tools for process validation and quality control requires reliable assays for such impurities and contaminants (*1*). It is helpful to distinguish impurities from contaminants. Impurities are undesired substances normally present in the starting biological material (such as the array of cell constituents in the supernatant). Contaminants are undesired substances accidentally introduced during the processing of the product, such as protein A leached from columns during purification of monoclonal antibodies. As such, DNA and host cell protein are impurities, as they are native to the process. The analytical problems posed by DNA and host cell protein are more complicated than those posed by identified, discrete contaminants; the former are heterogeneous collections of molecules, requiring broad screening assays. The present discussion focuses on the quantitation of DNA and protein impurities, with some discussion of specific contaminants.

Before the emergence of biotechnology, it was very unusual to purify and then detect impurities in pharmaceuticals at low parts per billion levels. This is now the case for DNA in products of recombinant DNA and hybridoma technology. Given the current pressures on a conservative FDA, it is likely that such standards will be maintained. We address several critical issues concerning trace contaminants and impurities in recombinant or hybridoma based pharmaceutical products. What are the bases, scientific and other, for declaring that DNA and contaminating protein in a product compromise the safety of that product? What do the FDA and WHO recommend for the measurement and interpretation of testing for impurities and contaminants in biologicals? How does one reliably detect such small amounts of these substances?

BASIS FOR THE REGULATORY POSITION

The consistency of the safety, potency, efficacy, and purity of the injectable product is ultimately the responsibility of the manufacturer and forms the basis of regulatory evaluation. Once a product is developed and approved, it is assumed that the appropriate bioprocess is in place to assure a consistent product. Tests for impurities and contaminants are critical in the development and validation of the purification process as well as in final product testing, where the test results provide on-going assurance that the bioprocess remains under control. Hence, these tests are a key element of good manufacturing practice and regulatory evaluation.

The identification of impurities is based on real or theoretical concern for risk to the recipient. It is recognized that there has been and will continue to be scientific debate about the basis of such concern. Indeed, specific experiments have demonstrated little or no risk associated with some of these impurities (e.g., genomic DNA) (*2*). However, because the mechanisms by which both DNA and protein impurities may put the recipient at risk are not well understood (e.g., the triggering of tumorigenicity or an allergic response), safety issues must frequently be based on theoretical considerations. Furthermore, at present no defined set of ethically permissible experiments demonstrating no adverse effects upon injecting DNA or nonrelevant proteins can totally remove the presumed risk that some of this material may be dangerous under some conditions.

In summary, the regulatory basis for concern over DNA and protein contamination in biopharmaceuticals is 2-fold. First, validating and assuring the removal of nonrelevant material is a primary part of demonstrating good manufacturing procedures. Second, these impurities present at least theoretical risks to the patient. These risks will be discussed.

Contaminating DNA. The theoretical concerns about contaminating DNA as a health risk have been with us since the mid-1950s. The need to produce massive amounts of viral

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vaccine, the most urgent being for polio, focused FDA concern upon this topic. Continuously proliferating cell lines seemed to be an ideal substrate for controlled manufacture of vaccines but shared several critical features with cancer cells. The most alarming shared feature was tumor formation upon injection of cells into an appropriate animal. In addition, there was the potential for the cell line to be infected with virus. In retrospect, this turned out to be a valid concern as evidenced by actual viral contamination of several vaccines (*3*). So in the 1950s the concern was a safety risk in biologicals due to viruses and tumor-forming agents.

In those days, there was no possibility of identifying the tumorgenic or oncogenic agent, so of course there was no required testing for it. The solution adopted by the FDA in 1962 was to test and control at the level of the cellular substrate. Primary cell cultures, but not diploid cell strains nor continuous cell lines, could be used to grow vaccine virus. It is of more than historical interest to note that these restrictions were gradually relaxed, first to diploid cell strains for the production of vaccines. More recently, transformed cell lines, such as the lymphoblastoid line for α-interferon and CHO for tissue plasminogen activator, have been approved for the production of biopharmaceuticals. This relaxation does not represent diminished FDA concern about potentially oncogenic impurities. The causes of tumors are now better understood, including the fact that oncogenes may be present in an individual's genome. Hence, the primary concern with contaminating DNA is that it may contain an oncogene, or cause an oncogene to be activated, or cause a tumor inhibitory gene to be turned off. Also, there is now increased confidence that products can be purified to an appropriate extent and that the offending impurity, DNA, can now be directly monitored. The magnitude of the perceived risk remains dependent upon the cell line used in the process. For example, there is greater concern relative to DNA contamination in products derived from mammalian cells, for theoretical reasons. First, the DNA of a continuous cell line is likely to be more tumorigenic, that is, contain oncogenes (*4*). Second, mammalian cell cultures are more likely to harbor a virus that is infectious in humans.

Data relative to the risk associated with DNA were generated by administering high doses of DNA in animals and measuring the frequency of tumor induction. The source of DNA was critical; oncogenic viruses of monkeys (SV40) (*5*), chickens (RSV) (*6*), and mice (polyoma) (*7*), cloned DNA from hepatitis B virus (which is associated with a higher incidence of human liver cancer) (*8*), and even cloned oncogenes (*6*, *9*) caused tumors in animals. A WHO Study Group concluded that viral DNA is tumorigenic at very substantial amounts of DNA (1–10 μg) (*10*). However, it is unclear what the implication is of these results when extrapolated to the human situation, where the DNA may be introduced at much lower levels and possibly infrequently. A quantitative estimate for the risk of tumor induction from an injection of DNA is a complex issue, requiring several assumptions. The 100-pg value proposed by the same WHO Study Group (*10*) assumes that 2 μg (2×10^6 pg) of oncogenic virus DNA is a "tumor-inducing dose" (TID) since it induces tumors in about 50% of animals injected, and chromosomal DNA (of the cell substrate containing an activated oncogene) has only one copy of the oncogene per genome, representing about 10^{-6} of the total DNA. If one makes other facilitating assumptions, such as the validity of extending the animal model to humans and that risk remains linear even at very low doses, one can estimate that a dose of therapeutic protein containing 100 pg of genomic DNA has 100×10^{-6} pg of oncogenes, or 5×10^{-11} TID. This study group concluded that the risk associated with 100 pg of heterogeneous contaminating DNA "is so small it can be safely regarded as being negligible"; more detailed calculations are presented elsewhere (*11*). It is important to note that the WHO value is corrected for the estimated frequency of oncogenes in genomic DNA; 100 pg reflects the mass of total DNA deemed safe.

Contaminating Proteins. The concern about contaminating proteins is based upon years of experience with conventional biologics. The adverse immune reaction caused by host cell protein in various generations of rabies vaccines is a classic illustration of this issue (*12*). The neurologic reactions were significantly reduced by changing the biological substrate for virus propagation as well as improving purification. Hence, the primary concern is the possibility of generating an immune response to contaminating protein by the recipient of the biopharmaceutical. The immune response could either be acute, as in an allergic response such as anaphylactic shock, or chronic, such as autoimmune disease. It is difficult to estimate the minimum immunogenic dose or a protein. The immune response to a particular protein is a complex phenomenon dependent on the protein and recipient. Only for the sake of illustration, low microgram doses of pure protein vaccines can generate an immune response in a high percentage of human recipients (*14*, *15*). Hence, it is possible that as little as 1 ng of a highly immunogenic protein might give an allergic response, representing only 1 ppm of a 1-mg dose, and even lower relative impurity levels in high doses of 100 mg or more.

A secondary concern is the possibility that a contaminating protein will exercise a biological response in the recipient. Such biological effects would be anticipated if the contaminating protein were a toxin, hormone, or cytokine with physiological effects in humans. In contrast to the long-term consequences of either genetic alteration by incorporation of foreign DNA or of induction of immune memory to contaminating protein, the biological effects of contaminating protein are expected to be transient (*13*). Proteins with intracellular biological activity (such as enzymes and structural proteins) need to be transported into cells to elicit biological effects. It is unlikely that cell uptake of such low level proteins would be efficient enough to generate sufficient intracellular concentrations for biological activity. Hence the risk associated with a biological response from such proteins is low. Unlike intracellularly active proteins, contaminating hormones, cytokines, and toxins which act on the cell membrane may be perceived to be of higher risk.

Altered forms of the product protein which differ in immunogenicity or potency may be considered to be impurities. These could include products with altered amino acids, glycosylation, etc. Though the analysis of alterations in the product protein is an important topic (*1*), it will not be considered in the present discussion. Other contaminating proteins may come from the cell growth, product purification, or the product modification steps. If serum is used in the medium for mammalian cell lines, it may be the source of contaminants. Antibodies coupled to toxins for targeted therapy present other contamination issues; free toxin or toxin in immunologically inactive conjugates may increase the nonspecific toxicity of the therapy. Finally, proteins introduced to the bioprocess during product purification may leach into the final product as a contaminant. Examples are protein A used in affinity purification of antibodies and monoclonal antibodies used in affinity purification of proteins.

THE REGULATORY RESPONSE

The FDA executes its policy with regard to testing for impurities and contaminants not in codified laws but instead as a series of opinions titled "Point to Consider...". These represent the current consensus at the Center of Biologicals Evaluation and Review (CBER) and formerly the Office of

Biologics Research and Review (OBRR). The FDA intends these to be flexible and evolving and to remain perpetually in a "draft" status, to be updated periodically with input from industry, academia, and other regulatory agencies worldwide (*16*).

DNA Contamination. The popular belief is that the FDA says that all biopharmaceutical products must contain less than 10 pg of DNA per dose. The actual situation is more subtle. All Points to Consider recommend tests for contaminating DNA in the final product. In addition, it is stated that the "method ought to provide sensitivity on the order of 10 picograms per dose" (*17*). There is no specification of what an acceptable or tolerable level of contaminating DNA might be. The statement of 10 pg of DNA per dose is an analytical goal, not a purity standard. It reflects what the FDA considers to be an achievable level of sensitivity (*18*). The distinction between an analytical goal and a purity standard is significant. The former is intended to prompt manufacturers to put in place the best methods for quantitation of impurities. If the WHO value of 100 pg per dose (*10*) is interpreted as a purity guideline, then it is appropriate for it to be higher that the analytical goal, and the two statements, so viewed, are not in conflict.

Another point reinforces the distinction between analytical goal and purity standard. It is the FDA's stated intention to evaluate each product on a case-by-case basis (*16*). With respect to contamination with putative oncogenes, the critical variables include the cell substrate, assessment of the balance between risks and benefits to the patient, and the size of the therapeutic dose as it impacts DNA quantitation in the presence of a huge excess of protein. Hence, the FDA permits latitude with respect to DNA contamination allowed in a given product. Though it is still early in the regulatory history of biopharmaceuticals, precedent remains an important consideration of the conservative process of FDA regulation, and its is known that the FDA has approved recombinant products with flexibility relative to amount of DNA allowed per dose.

Protein Impurities and Contaminants. The Points to Consider are vague with regard to analytical and purity goals for potentially contaminating proteins. "Monoclonal antibodies intended for in vivo human use should be as free as possible of extraneous immunoglobulin and non-immunoglobulin contaminants" (*19*). In an earlier document "Western blots, radioimmunoassays and enzyme-linked immunosorbant assays using high affinity antibodies raised against the product, host cell lysates, appropriate subcellular fractions, and medium constituents" are all mentioned as appropriate measurement techniques (*17*). In a still earlier draft, it is stated that "such methods can provide sensitivity in the range of 1 to 100 ppm", providing the only indication of an analytical goal (*20*). The 1985 Points to Consider recommend that "patients given large or repeated doses of a product should be monitored for the production of antibodies to contaminating antigens" (*17*).

ANALYTICAL METHODS

The essential problem is the measurement of trace impurities and contaminants in the presence of a relatively massive amount of product protein. The impact of interference by the product protein on assay performance (e.g., sensitivity, precision, and specificity) is critical in the choice of analytical methods.

DNA Probe Hybridization. DNA probe hybridization is an established methodology; Keller and Manak provide a recent review (*21*). Hybridization assays are sensitive enough to detect 10 pg of DNA (*22, 23*). The assay is based upon the specific annealing of labeled DNA probe to complementary sequences of target (contaminating) DNA. The probe may be generated from cellular or plasmid DNA, for example, complementary to repeating sequences (*23*). The standard procedure is to denature the sample DNA and to nonspecifically capture it on a microporous membrane, usually in a slot blot apparatus (*21–24*). Intermediate steps in the procedure fix the DNA to the membrane and then block additional DNA binding sites on the membrane in order to limit the nonspecific retention of the labeled probe. The membrane is then soaked in a solution containing the labeled probe, which hybridizes to the target DNA on the membrane. After washing away unbound probe, the amount of labeled probe is determined, usually visually.

Several options in the assay procedure can influence performance and convenience. First, the choice of membrane determines the preferred fixation method and influences the small fragment size cut-off for the assay. Traditionally nitrocellulose membrane was used for the capture of DNA in the sample. Here the preferred fixation protocol is baking the membrane for 2 h. It has been reported that capture and hybridization with nitrocellulose membranes is only efficient for DNA fragments longer than 400–600 bases (*24*). Nylon has largely replaced nitrocellulose, first for reasons of convenience; it is easier to handle, and it can be reprobed many times. Also, because DNA is more tightly bond to nylon, superior sensitivity (*25*) and possibly a lower fragment size cut-off are obtained.

The second significant variable in assay methodology involves the choice of labeling strategy. Traditionally, ^{32}P is incorporated in the probe enzymatically by nick translation using radiolabeled deoxynucleotide monomers (*26*). An alternative labeling method using random primers may be preferred because it provides longer probes with greater specific activity in shorter labeling times (*27*). Labeling may also be accomplished through chemical modification of the probe, resulting in the incorporation of a hapten (e.g., biotin or digoxigenin) (*28*). After hybridization, a second reaction is performed with an anti-hapten antibody–enzyme conjugate. The enzyme then is used to generate signal. Reporter enzyme may also be directly linked to the probe (*29*). Signal generation with an enzyme provides amplification as well as avoiding the hazard and instability of radioisotopic reagents. The labeling strategy is coupled to the read-out method for the assay. Traditionally ^{32}P decay is recorded by autoradiography on photographic or X-ray film (*21*). After the film is developed, the darkness of spots for samples is visually compared to concurrently run standards; assays with such detection means are semiquantitative and have a limited dynamic range. Scanning densitometers may be used to rapidly evaluate spot darkness, removing subjectivity; complete systems for the image analysis of ^{32}P-labeled DNA on filters or gels are now available (*30*). With enzyme labeling, colorimetric detection may be used. Here the enzyme hydrolyses a substrate that deposits a colored precipitate, which may be read visually or quantitatively.

Traditional hybridization assays may be relatively time consuming and labor intensive. For example, if autoradiography is used with ^{32}P labeling, film development may take from 24 to 28 h. The useful life of ^{32}P-labeled probe is 2 weeks or less, requiring the periodic regeneration of fresh probe, and there are the usual problems of reagent handling and waste disposal associated with radioisotopes. Without tight control of the assay procedures, hybridization assays may be technique dependent. For example, the efficiency of radiolabeling the probes influences the useful assay range, and nonspecific capture of the labeled probe influences assay sensitivity. Finally, hybridization assays only detect contaminating DNA which is complementary to the probe that is used. For example, if genomic DNA from the host cell is used to generate the probe, then the assay will miss DNA from adventitious infectious agents, plasmid DNA, or the DNA of specific

promoters or enhancers used in the process.

In recent years, several technical improvements, such as nylon membranes and scanning densitometers, have facilitated hybridization assays. In addition, there are now commercially available labeling kits. Genius (Boehringer Mannheim Corp., Indianapolis, IN) allows labeling wtih digoxigenin and detection with an antibody–enzyme conjugate (either alkaline phosphatase or horseradish peroxidase conjugated to *Fab* fragments of an anti-digoxigenin antibody). The manufacturer claims assay sensitivity allowing the detection of 1 pg of homologous DNA after 1 h of color generation by the enzyme. Chemiprobe (Orgenics International Corp., Columbia, MD) markets a kit with reagents to chemically modify probes. Sulfonyl groups are introduced at cytosine residues. An antibody against sulfonylcytosine is provided, and a second antibody is used to incorporate an enzyme label.

Polymerase chain reaction (PCR) when coupled to hybridization offers exquisite sensitivity and specificity (*31*). It could be used to amplify one or perhaps several specific sequences of potentially contaminating DNA. Recent reports deal with the quantitation of PCR amplified targets (*32*, *33*).

Separation and Detection of Contaminating Proteins. The classical method is to electrophorese the purified product, separating it from protein impurities and contaminants, followed by staining. This technique is fast and relatively easy; it also provides size information about the spectrum of impurities. However, the method is not suitable for the detection of impurities that comigrate with the therapeutic protein. With silver staining, 0.5–2.0 ng of protein can be detected if it migrates independently from the product protein (*34*). If a starting sample is 5 μg, this corresponds to 100–400 parts of an individual impurity per million parts of the relevant protein. One of the most useful aspects of electrophoresis is the ability to visualize a pattern of impurities. Constancy of this pattern from lot to lot then indicates that the process is under control. The analysis can be enhanced by using antibody based staining, as in immunoblots (*35*). The precision of reading gels is improved with image analysis (*36*). Alternative separation methods include isoelectric focusing (IEF) (*37*) and HPLC (*1*, *38*). As with electrophoresis, individual protein impurities must be separable from the product protein.

Immunoassays for Protein Impurities and Contaminants. Immunoassays have several features complementary to methods based on separation. First, they are much more sensitive and quantitative, allowing objective assessment of product purity. Second, they are based on antibody recognition; this specificity allows the measurement of copurifying protein impurities and contaminants in the presence of the product protein. Such immunoassays utilize antibodies raised against representative antigen(s). An immunoassay constructed against a specific contaminant, which may accidentally be introduced during processing, uses an antibody against that defined molecule. In addition, manufacturers of biopharmaceuticals use immunoassays that screen for a broad range of protein impurities and contaminants by using a mixture of antigens derived from the process.

Lucas et al. report the use of conventional enzyme-linked immunosorbent assays for the quantitation of contaminating proteins introduced during the processing of a monoclonal antibody (*39*). Bovine IgG from fetal bovine serum used in cell culture was measured in the 0.2–0.7% range, and protein A from the affinity purification of the monoclonal was measured at 64 ppm or less. Bloom et al. describe an ELISA for protein A with subnanogram sensitivity in the presence of a 100-fold excess of monoclonal IgG_1 (*40*).

For immunoassays that deal with impurities and contaminants collectively, the critical component is the diagnostic antibody. The task is to obtain antibodies against the full range of proteins, given that some proteins may be poorly immunogenic or occurring at subimmunogenic concentrations and allowing for those proteins that are most likely to come through the product purification regimen. Generation of an immunogen by purifying a sample that contains all possible antigens except the product protein (*41*) has become the generally accepted strategy. In the process-specific case, the entire bioprocess is followed, except that host cells without the gene for the expression of the product protein are used; this is sometimes referred to as a "blank" or "gene minus" run. A sample is typically extracted from the purification process, at the point that if the product protein was present, it would be approximately 90–99% pure. This material is used to make the immunogen that generates the diagnostic antibodies. The same material is used to calibrate the assay. In this way, an immunoassay that is specific for a particular cell line, media, and purification scheme is obtained, and one assay will screen for a range of copurifying proteins, originating from any part of the bioprocess. It is possible that a contaminant or impurity that is weakly bound to the protein product could be missed by this technique.

An analytical group at Genentech reported an ELISA for *E. coli* proteins (ECP) in human growth hormone (hGH) (*42*). The immunogen and reference samples for the assay were generated from the purification of processed material from cells lacking the hGH gene. The purification was taken to the stage where hGH, when present, is 99% pure, according to SDS PAGE with silver stain. A sandwich ELISA using antibodies affinity purified on immobilized ECP had a lower limit of detection of 2 ng/mL in the presence of hGH at 2 mg/mL, corresponding to 1 ppm. In a similar fashion, reference ECP was generated from a process for γ-interferon. Interestingly, the γ-interferon ECP was essentially undetectable (0.002% response) in the hGH ECP ELISA, demonstrating that this method for generating an assay for copurifying host cell proteins is truly process specific. A concern about this method is saturation of antibody to poorly immunogenic, major constituents. This may result in spuriously low detection of such proteins when they are in a state of antigen excess; this so-called prozone effect may be minimized by dilution of the sample. The same group evaluated two immunization procedures designed to improve the antibody response to weakly immunogenic components in complex antigen mixtures (*43*). The general strategy was to eliminate the most immunogenic proteins from the boosting doses given to the recipient animal. This so-called cascade immunization employs the in vitro depletion of the immunodominant ECP by absorption with antibodies from an already generated antiserum; the remaining antigens are injected into a suitable host animal and the antisera collected. A second method uses in vivo blocking of the dominant antigens. Analysis of the resultant antisera by two-dimensional SDS PAGE immunoblots demonstrated that the combination of the cascade immunization with a longer series of immunizations provided a superior immune response to minor components without diminution of the response to immunodominant components.

Immunoassays have the required sensitivity to detect proteins to 1 ppm, or lower, but critically depend on having antibodies to each protein that is a potential impurity or contaminant. Thus, only through the use of polyclonal antibody is one able to detect the presence of a spectrum of proteins in a single assay. In addition, there are several limitations of an immunoassay based on the gene minus method. First, it detects only those proteins that elicit an immune response in the animal that generates the diagnostic antibody. Second, among those proteins, the immunoassay response is governed by the combination of the amount of a given type of contaminating protein, its immunogenicity, the relative

concentration of diagnostic antibodies to that particular protein in the mix of polyclonal antibody, and the affinity of those antibodies. In other words, the meaning of an assay response is difficult to interpret. Third, immunoassays provide no information about the spectrum of individual impurities, as do the complementary techniques of electrophoresis and chromatography. Finally, reliance on process-specific immunoassays requires the development of new assays for new or significantly altered processes.

The Need for Improved Assays. We have distinguished an analytical goal from a guideline for purity in the regulatory position with regard to impurities and contaminants. We also recognize that in testing for potentially oncogenic DNA and potentially immunogenic proteins, the FDA is likely to take conservative positions in order to minimize health risks. Hence, the FDA tends to promote analytical goals that challenge the state-of-the-art for bioanalytical measurements. There are several areas of improvement that directly impact the ability to quantitate impurities at low levels. The primary aspects of assay performance (sensitivity, precision, dynamic range, and reproducibility) need to be considered, particularly for assays performed with real samples. In this section, we discuss a few areas of potential improvement.

First, a quantitative assay for a impurity is always preferred over a semiquantitative or qualitative assay, simply because a quantitative assay produces numbers with more significance. One presumes that when the bioprocess is under control, the product is adequately and reproducibly pure; the purpose of the assay is to demonstrate that the process is actually under control. The results of a quantitative assay are more accurate and precise, allowing finer control of the process, and in general making the assay validation procedure more straightforward. This argument in favor of a quantitative assay applies to final product quality control but may be even stronger when the assay supports process development and validation. Here it is useful to be able to quantitate the effects of perturbations to the purification process by obtaining statistically valid information in an efficient fashion.

Second, a rigorous analytical approach on the part of manufacturers, as well as a conservative regulatory position, favors progressive improvements in sensitivity. Consider the situation where measurements for a particular contaminant in actual samples are variable around the detection limit of the assay. Obviously the assay has limited value with regard to assuring that the bioprocess remains under control, and the situation will be improved with a lower limit of detection or higher precision. This is an argument in favor of making the analytical goal more stringent than the actual standard for purity. Also, it is likely that the regulatory position will include not only the general screening of classes of impurities (e.g., total DNA, host cell DNA, or co-purifying proteins) but also the testing for specific agents (e.g., oncogenes, pathogenic viruses, or a ligand used in immunoaffinity purification which is known to cause an adverse effect). In the latter case, assays with the lowest possible detection limit are desirable.

In addition assays should be robust enough to handle different types of samples, from purified product with 10^6–10^9-fold excess of protein, to various extraction and purification buffers, to biological fluids such as serum. These different sample matrices often present special interferences or inhibitions, requiring time-consuming assay development and optimization for each type of sample. High sensitivity allows one to deal with matrix issues simply by diluting the sample. The preparation of assay reagents also impacts assay development time. Radioimmunoassays depend upon isotopic labeling, producing reagents with limited shelf life and handling and disposal issues. It is desirable to have assays that utilize prelabeled and stable components.

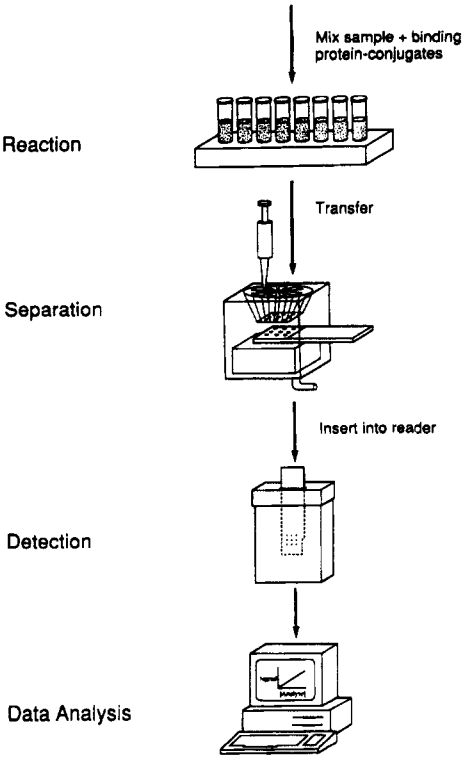

Figure 1. Four stages of a Threshold assay: formation of specific complexes in liquid phase, the capture and concentration of the complexes on a membrane, detection with a silicon sensor, and data analysis resulting in quantitation. Reprinted with permission from ref 47. Copyright 1990 Eaton.

MEASUREMENT SYSTEM FOR THE QUANTITATION OF DNA AND PROTEINS IN BIOPHARMACEUTICALS

Threshold Operating System. The Threshold system (Molecular Devices Corp., Menlo Park, CA) includes an instrument, computer, reagents, and disposables for running assays. All components of the system have been designed to work together in an highly integrated fashion. The four stages of running an assay are shown in Figure 1. The first stage is the formation of reaction complexes in liquid phase based on specific binding events involving the target analyte; for example, between DNA binding protein and a strand of DNA, antibody and antigen, or DNA probe and a specific DNA target. Two components, streptavidin and urease, are ultimately incorporated into the complexes. Streptavidin is used for the specific capture of the complexes onto a biotinylated membrane; urease is used for enzymatic signal generation.

The second stage of the procedure is capture and concentration of the complexes by the filtration of the liquid mixture through small capture sites on a biotinylated membrane (*44*). This filtration step is done in an eight-channel filtration unit, processed four at a time on the workstation. After the membrane is washed on the same filter unit, each capture site contains an amount of urease that is quantitatively related to the amount of analyte in the sample.

The third stage of the procedure is detection. The core of the detector is a silicon based light-addressable potentiometric sensor (LAPS) (*45*, *46*). Briefly, the membrane is pressed against a silicon sensor in the reader, which contains a solution of urea. Each capture site on the membrane is registered with a coincident sensing site on the silicon, with the detection volume being about 0.5 μL. As the urease hydrolyzes the urea to produce ammonia in this microvolume, a significant pH change occurs. This drives a change of surface potential on the sensor that is monitored kinetically by the electronics. The rate of change of the surface potential is a direct measure of

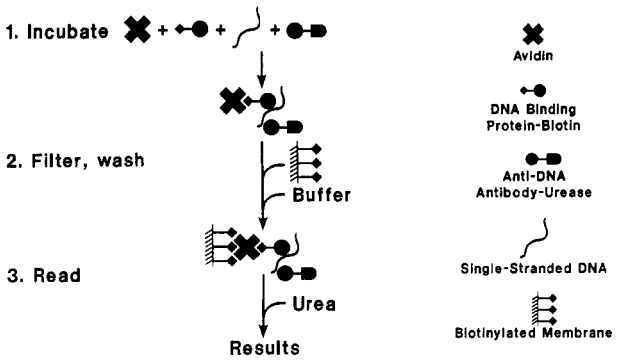

Figure 2. Assay format for total DNA, involving two conjugates of DNA binding proteins. Reprinted with permission from ref 48. Copyright 1990 Academic Press.

the enzyme activity (or number of enzyme molecules when the enzyme–substrate is in excess). The efficiency by which this sensor converts an enzyme reaction in a microvolume to an electrical response contributes to high sensitivity. Only about 10^8 urease molecules are required to produce in 1 min a response significantly above the background drifts of the complete detector circuitry in the Threshold reader (*46*).

The quantitative aspects of this system are due to coupling precise chemical modulation with sensitive and reproducible signal detection (*47*). The formation of the analyte-specific reaction complexes in liquid phase is completed with rapid kinetics and with the binding components retaining native configuration. The subsequent filtration and capture step concentrates essentially all of the signal producing complexes onto a small and precisely controlled area. After washing, the number of urease molecules at each measurement site is quantitatively related to the amount of analyte in the starting sample, thereby resulting in precise chemical modulation. By using a very small detection volume, few urease molecules can drive a significant change in local pH over a short measurement time, providing high sensitivity. Zero and positive calibrators on every membrane are used to compensate for membrane-to-membrane variability in the background and gain of the assay, thus increasing precision and reproducibility. Finally, the detector has an inherent dynamic range of greater than 3 logs of signal. Even allowing for assay background levels dominated by the chemistry (i.e., nonspecific retention of the urease conjugate on the membrane), this translates into dynamic ranges for typical assays of greater than 2 logs of analyte level.

Total DNA Assay. The first Threshold application is a quantitative assay for picogram amounts of total DNA, with the assay format given in Figure 2 (*48*). After DNA in the sample is denatured, the sample is combined with a single reagent containing conjugates of two DNA binding proteins plus streptavidin. A monoclonal anti-DNA antibody is conjugated directly to urease; *E. coli* single-stranded binding protein is conjugated to biotin. Both binding proteins have high affinity for single-stranded DNA with weak sequence specificity. During the liquid-phase incubation, complexes containing DNA, streptavidin, and urease are formed. These complexes are captured on a biotinylated membrane and read with the sensor, as described above. Assay response is substantially equivalent for all types of DNA longer than about 800 bases. Because the assay is based on the binding of proteins to DNA, there are limitations with respect to the ranges of ionic strength and pH, as well as susceptibility to interferents and inhibitors, associated with samples.

Calf thymus DNA is used to create calibrators, and the resulting standard curve has a quantitation range from 2 to 200 pg of DNA, Figure 3. To evaluate the detection limit of the assay, 11 replicates of calf thymus DNA in buffer were made at levels of 0–5 pg, at increments of 1 pg (only 10 replicates were made at 1 pg). These samples were run and quantitated by using a standard curve generated with eight singlet calibrators distributed between 0 and 200 pg. The mean quantitated levels are plotted in Figure 4 against the actual levels (as determined by absorbance and dilution), with the precision indicated by errors bars that span −1 to +1 standard deviation. The mean quantitated level at 2 pg is separated from zero by more than 2 standard deviations.

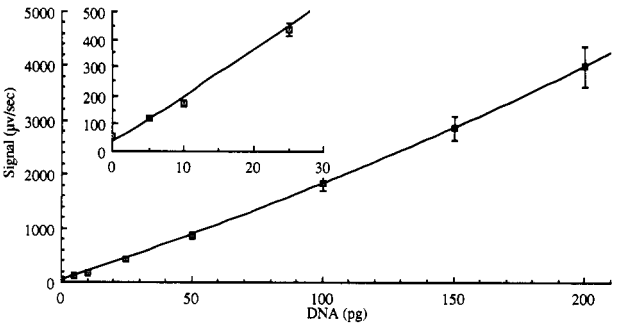

Figure 3. Standard curve for total DNA using calf thymus DNA calibrators, allowing quantitation from 2 to 200 pg of DNA.

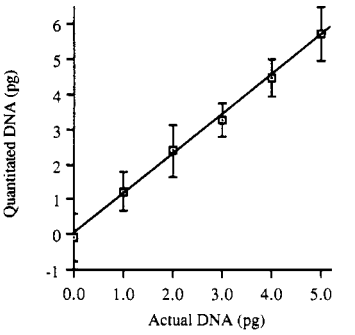

Figure 4. Quantitation of low picogram quantities of DNA in buffer. The starting concentration of calf thymus DNA was determined by absorbance. Dilutions were made to obtain 11 replicate samples at each picogram level from 0 to 5 pg (only 10 replicates were run at 1 pg), and these were quantitated by using a standard curve generated from separate calibrators, extending from 0 to 200 pg. The mean quantitated level is plotted versus the actual level (based on absorbance and dilution), and the error bars span −1 to +1 standard deviation.

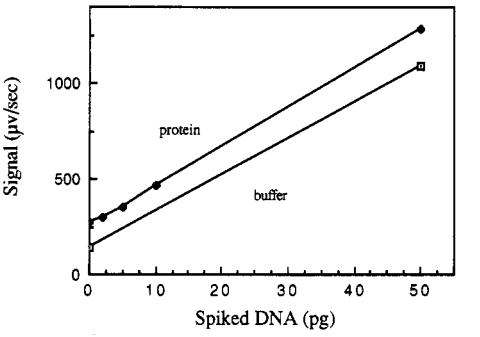

Figure 5. Example of good spike recovery of DNA in 1 mg of a recombinant protein, where the only required pretreatment was a proteinase K digestion followed by boiling the sample to inactivate the proteinase and denature the DNA.

The quantitation of contaminating DNA in biopharmaceuticals necessitates screening for DNA in samples containing very high amounts of protein. In these situations, it is very important to use rigorous analytical techniques to develop a pretreatment method to remove inhibitors or interferents associated with the protein, in a way that is compatible with the assay. Critical to the validation of a pretreatment method for a given protein is the demonstration of quantitative recovery of exogenous DNA added into protein samples, as

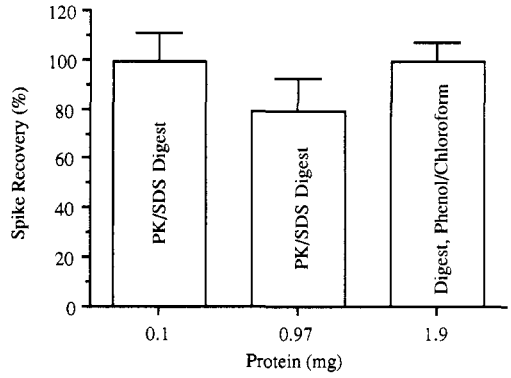

Figure 6. Essentially complete spike recovery (percentage relative to buffer) of 50 pg of DNA in various amounts of murine monoclonal IgG, always in a 0.5-mL sample. At the highest IgG level shown, a phenol extraction and chloroform separation step was incorporated to remove most of the digested protein before performing the total DNA assay. The error bars represent 1 standard deviation in the variation of recovery (%) among four replicate samples at the lowest protein level and eight replicates at the two higher levels.

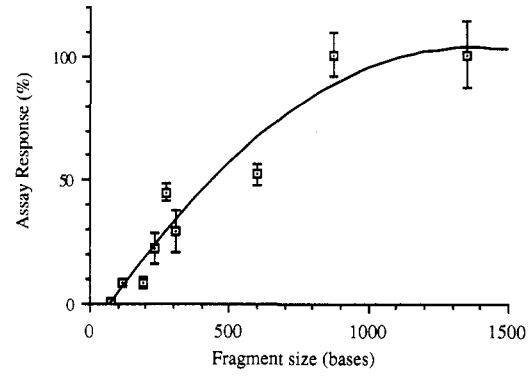

Figure 7. Short fragment cut-off for the total DNA assay. As the fragment size decreases, there is a decreasing probability that a given fragment will bind both DNA binding protein conjugates required for the generation of signal in the assay. Approximately 10% of the full response (relative to calf thymus DNA) is obtained with a purified fragment of 118 bases. The error bars represent −2 to +2 standard deviations among 6–12 replicates. Reprinted with permission from ref 49. Copyright 1991 Elsevier.

compared to identical amounts of DNA in buffer ("spike recovery"). Figure 5 shows the spike recovery for DNA in 1 mg of a recombinant protein, where the pretreatment involved proteinase K digestion followed by boiling to denature the DNA and to inactivate the proteinase. The upward shift of the response with added protein implies the presence of contaminating DNA. Shown in Figure 6 is the spike recovery of signal from 50 pg of DNA, relative to a pretreated buffer sample, for increasing amounts of a monoclonal IgG (in all cases, the absolute amount of protein was contained in a 0.5 ml sample). At the lower two protein levels, the assay was run directly after proteinase K digestion (with 0.1% SDS). At the highest protein level, a phenol extraction and chloroform separation step was incorporated after digestion. In each case, the error bar represents 1 standard deviation in the variation in recovery (%) among replicate assays, where four replicates were run at the lowest protein level and eight replicates were run at the two higher levels. The decreasing recovery with increasing protein (up to 1 mg with the simple protocol), though within the experimental uncertainty, is consistent with an assay inhibition associated with the digested protein.

Because various steps of the bioprocess are likely to shear DNA, assay response to short fragment DNA is an issue. Figure 7 shows the assay response to short fragment DNA, relative to calf thymus DNA (greater than 1 kbase). Measurements were made on samples generated by purification of restriction fragments. Six to 12 replicates were run at each size, and the error bars represent −2 to +2 deviations. The reduced response with decreasing fragment size is due to the decreasing probability that a given fragment can bind both binding protein conjugates. Because purified homogeneous fragments were used, the assay response at a particular fragment size is subject to the secondary structure and nucleotide content of that particular fragment. This probably contributes to the nonuniform decrease in assay response shown in Figure 7. One would expect these effects to average out in samples containing randomized fragments. At a fragment size of 118 bases, the assay response is approximately 10% of the maximum, and essentially full response is obtained with fragments greater than 800 bases. King and Panfili discuss DNA fragment size issues, including the size spectrum of bioprocessed DNA (*49*). A comparative study relates DNA determinations in samples of monoclonal antibody, based on this total DNA assay versus a standardized hybridization procedure (*50*). Sensitivity, precision, and accuracy are compared.

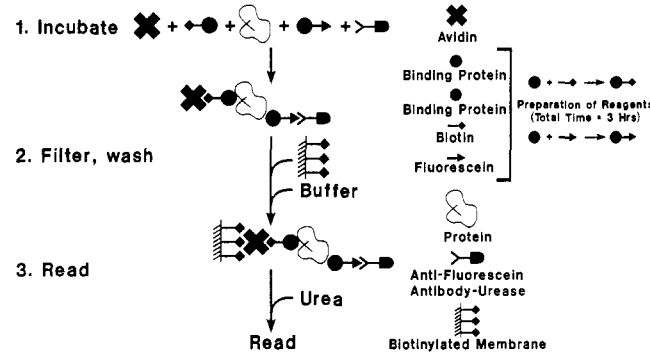

Figure 8. Assay format for a sandwich immunoassay for a protein. Two antibodies are haptenated with biotin and fluorescein, respectively. The addition of streptavidin and an anti-fluorescein–urease conjugate completes the complex, allowing capture and detection.

The fact that the total DNA assay measures all the DNA in a sample opens the question of background signal levels due to DNA introduced via buffers, vials, etc. Recommendations with respect to the selection of sample containers and the running of assay controls are not onerous but need to be followed to assure reliable measurements. There is a related issue of potential significance. There is detectable DNA in a variety of highly purified waters, including water for injection and samples of highly purified water used in the processing of semiconductors. Most of such background DNA is molecular, as opposed to particle borne, with a typical DNA level being a few picograms per milliliter of water. Routine ultrafiltration of water with positively charged nylon filters reduces the DNA level. Presumably the source of this background DNA is bacterial. The total DNA assay provides a convenient way of monitoring the DNA levels of buffers and purified water.

Immuno-Ligand Assay. A variety of assays based on specific binding reactions can be constructed with the Immuno-Ligand Assay (ILA). The format for a sandwich immunoassay for a protein is given in Figure 8. Two kits are provided, the first for labeling proteins with the haptens biotin and fluorescein. The second kit contains the detection reagent, an anti-fluorescein–urease conjugate plus streptavidin, and biotinylated membrane along with buffers and calibrators. The sandwich assay format shown here is for the detection of a large analyte molecule (e.g., a protein contaminant), which can bind two binding proteins (e.g., antibodies). After the binding proteins have been labeled with haptens, they are combined with the sample and detection reagent to be incu-

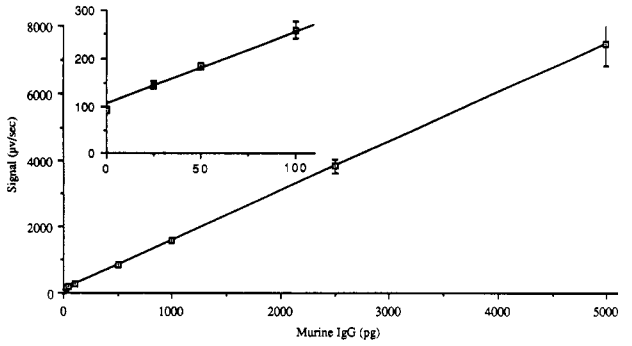

Figure 9. Standard curve for an assay for murine IgG, with a quantitation range that extends from 25 to 5000 pg. The error bars represent −1 to +1 standard deviation among four replicate calibrators. Reprinted with permission from ref 47. Copyright 1990 Eaton.

Table I. Precision and Accuracy of the Murine IgG Assay in Buffer[a]

spiked mIgG level, pg	quantitated level, pg	std dev, pg	coeff of var, %	no. of reps
100	103	13	13	12
500	515	36	7	12
1000	1060	64	6	12

[a] Quantitation used a standard curve generated from separate calibrators. Reprinted with permission from ref 47. Copyright 1990 Eaton.

bated in liquid phase. During this incubation, the complexes incorporating the analyte, streptavidin, and urease are formed. For a competitive assay, antigen and a single antibody are labeled with haptens. Here sample analyte competes against labeled analyte for one antibody and the signal is inversely related to the amount of analyte. Briggs et al. discuss assay performance and the results from a broad range of model analytes relevant to biopharmaceuticals, including the murine IgG and *E. coli* protein results discussed below (*47*).

Murine immunoglobulin (mIgG) was selected as a model because of its broad interest to the biopharmaceutical industry as a therapeutic, an in vivo diagnostic, or in some situations as a potential contaminant when used in immunoaffinity chromatography. A sandwich assay was constructed for mIgG by using two polyclonal anti-murine IgG antibodies directed against the *Fc* and *Fab* regions, respectively, labeled separately with biotin and fluorescein. A single incubation step of 2 h was used to form the sandwich complexes in liquid phase; total assay time was $2^1/_2$ h. Figure 9 shows the standard curve for this assay, which extends from 25 to 5000 pg of mIgG. The precision and accuracy of this assay is demonstrated in Table I. The given CVs are for the quantitated levels of mIgG, including assay-to-assay variations, and are less than 10% except at the low end of the quantitation range. The mean quantitated values at the three levels tested vary by no more than 6% from the actual level of mIgG. For a comparison to a standard method, the same antibodies were used to construct a kinetic ELISA. The ILA system obtained higher sensitivity to low levels of murine IgG, with a shorter assay time (*47*).

An assay was constructed for *E. coli* protein (ECP) by labeling separate aliquots of an anti-ECP polyclonal antibody. Figure 10 presents the standard curve, which allows quantitation from 10 to 2000 pg of ECP. This assay was used to quantitate various levels of ECP spiked into up to 5 mg of a recombinant protein; the results in Table II are the recovery, the quantitated level of ECP in the presence of the recombinant protein as a percentage of the expected spiked level. Note that at 5.0 mg of the recombinant protein, there was greater than 80% recovery of a 250-pg spike of ECP; this

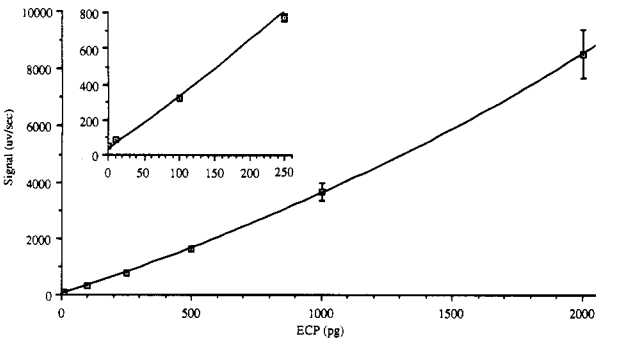

Figure 10. Standard curve for an assay for *E. coli* protein (ECP), with a quantitation range that extends from 10 to 2000 pg. The error bars represent −1 to +1 standard deviation among 2 replicate calibrators. Reprinted with permission from ref 47. Copyright 1990 Eaton.

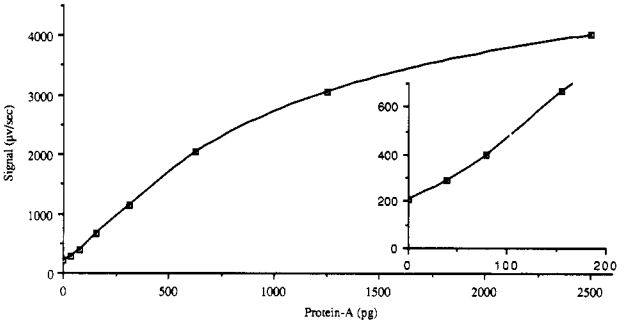

Figure 11. Quantitation of picogram quantities of protein A in the presence of 75 μg of IgG_1.

Table II. Quantitation of *E. coli* Protein (ECP) in the Presence of Various Levels of Recombinant Protein[a]

recombinant protein, μg	spiked ECP, pg	recovered ECP, %
5000	250	83
2500	250	85
1250	125	82
50	50	92
5	50	96
0.5	50	104

[a] Recovery is expressed as the percentage of the quantitated amount of ECP, relative to the spiked amount. Reprinted with permission from ref 47. Copyright 1990 Eaton.

corresponds to 0.05 ppm. In a separate on-going study, ECP assays are being compared in the ILA and ELISA formats (*50*).

The use of protein A for the purification of monoclonal antibodies for injection is currently limited due to the difficulty of demonstrating the absence of protein A in the final product. An assay for contaminating protein A is complicated by the fact that the sample will contain an excess of IgG, some of which will be complexed with the protein A, potentially inhibiting the binding of a diagnostic anti-protein A antibody. The ILA system was used to construct an assay for protein A, which functions in the presence of a murine monoclonal IgG_1, by using a polyclonal anti-protein A antibody that binds at pH 5. Figure 11 shows the assay response to subnanogram levels of protein A in the presence of 75 μg of IgG_1. The low end of the curve corresponds to detecting protein A at about 1 ppm IgG_1. Assays for contaminating protein A in other types of IgG will have to deal with differences in the binding characteristics of protein A for the immunoglobulin (e.g., pH dependence).

Several additional comparative studies are on-going (*50*). In two cases the high sensitivity of the ILA system is being used to construct fast assays, compared with HPLC, for monitoring levels of the product protein from the bioreactor

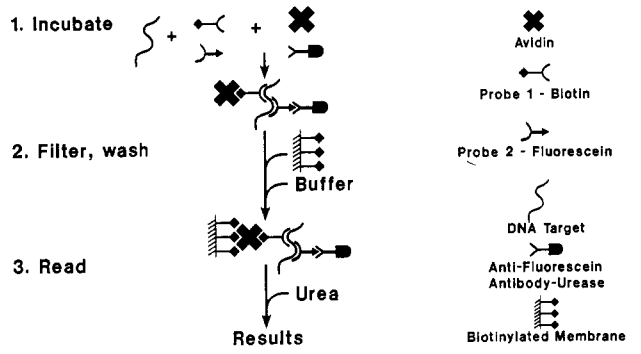

Figure 12. Assay format for a dual DNA probe hybridization assays, requiring probes that have been labeled with biotin and fluorescein, respectively.

in the first case, and through the purification process in the second case.

Future DNA Probe Assays. With the ILA system, a dual-probe approach may be used to develop quantitative DNA probe assays, as shown in Figure 12. Probes to adjacent sequences of a specific target were separately labeled with biotin and fluorescein (*51*). After liquid-phase hybridization, ILA capture and detection is used to complete the assay. Only one fluorescein label was used per probe, meaning that signal generation was limited to a maximum of only one urease conjugate per target. This probe assay has sensitivity to 20 $\times 10^6$ target molecules, with a total assay time of less than 2 h and coefficients of variation of less than 10%. Finally, this probe format can be coupled to PCR amplification of the target. In the future, this assay format, with or without target amplification, will be useful in the quantitation of specific DNA sequences of high risk as a potential contaminant to biopharmaceuticals.

CONCLUSION

As biotechnolgy advances, bioprocesses of inherently greater risk have been shown to be of value with respect to producing better therapeutics. Hence, advancing technology promotes the need for more stringent testing for potential impurities and contaminants in biopharmaceuticals. Corresponding advances in measurement technology allow for more stringent testing. Greater emphasis is being placed on quantitation of potential impurities, both during the development of purification processes and in the final product testing, and there will always be a need for measurements of high sensitivity for specific contaminants of significant risk. The regulatory agencies recognize these trends of biotechnology and hence promote the use of the best analytical methods to assure product purity. Some traditional methods are recognized as being particularly useful, for example, the use of immunoblots to visualize the pattern of protein impurities. The gene minus method represents a significant enhancement of traditional immunoassays for the purpose of quantitating a broad range of process-specific protein impurities and contaminants. The regulatory agencies are also receptive to new technology that can be demonstrated to enhance the quality of a therapeutic. We have described an integrated quantitative measurement system that features flexibility and sensitivity. Screening assays for picogram quantities of total DNA and immunoassays for picogram quantities of proteins have been described. In addition, the system can be adapted to run DNA probe assays for specific sequences of nucleic acid.

ACKNOWLEDGMENT

The Threshold measurement system and assays are the products of a large and multidisciplinary research and development team as indicated by the authors of several cited articles (*36–41*). We acknowledge the special contributions of Drs. Paul Johnston and Robert King in dealing with the difficult issues of quantitating impurities and contaminants in concentrated protein solutions, as well as contributing to the dialogue with regulatory agencies, worldwide. In addition, Dr. Vartan Ghazarossian and Baltazar Gomez provided the protein A results, working with reagents kindly supplied by Sepracor, and Dr. Edward Sheldon and John Olson generated the DNA probe results.

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ARTICLES

Comparison of Experimental and Calculated Results in Overloaded Gradient Elution Chromatography for a Single-Component Band

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The elution profiles of high-concentration bands of pure compounds in gradient elution chromatography are investigated. Emphasis is placed on the influences of the amount of sample injected and of the gradient program. At moderate gradient rates, the band profiles recorded for 2-phenylethanol agree very well with those predicted by numerical integration of the mass balance equation, following a procedure widely used in isocratic chromatography which is now conventional. For steep gradients, the agreement is still good but differences appear, probably related to the higher importance of the equilibrium isotherm data at high solute concentrations.

INTRODUCTION

Since its introduction nearly four decades ago (*1*, *2*), gradient elution liquid chromatography has become one of the most widely used techniques in analytical chromatography. A large number of theoretical studies have investigated the relationships between the retention times or volumes of analytes or their resolution and the gradient profile used for different chromatographic systems (*3-7*). Such relations allow the prediction of the chromatograms obtained under different experimental conditions and facilitate their optimization and the development of new analytical procedures. Furthermore, rapid developments in the field of analytical instrumentation and the design of advanced programmable solvent delivery systems have led to the commercial availability of numerous chromatographic instruments that provide the precision and the reproducibility required for quantitative analytical procedures. The technique has become a popular analytical method. Gradient elution liquid chromatography has found one of its most current areas of application in the analysis of the complex mixtures of clinical, biochemical, or environmental origins (*8*, *9*). Some of the most important advantages of this mode of chromatography are a reduction of the analysis time, an enhancement of the detection sensitivity, and an increase in the useful column peak capacity.

Recently, with the increase in the need of high-purity bioactive compounds and of other high-value-added chemicals, chromatography has established itself as a method of choice in extracting or preparing high-purity products (*10-14*). This is especially due to the high degree of flexibility provided by the many different modes available and by the numerous implementations available for each mode. Whereas gradient elution does not seem attractive in the field of large-scale industrial separations and purifications by preparative chromatography, its use for laboratory scale applications has drawn much interest and has become an important area of investigation (*15-18*). In industrial applications, the cost of regenerating the chemicals used to prepare a mobile phase of constantly changing composition is higher than for an

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