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The use of particle concentration fluorescence immunoassay technology for the analysis of rDNA products

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SUMMARY

The electrophoretic and immunological techniques typically used to detect potentially useful biopharmaceutical proteins are sensitive with detection limits in the nanogram range. However, quantitation of a recombinant protein can be cumbersome, and involve large numbers of samples throughout process optimization schemes. Although electrophoretic methods (i.e., SDS–PAGE and Western blots) now avail themselves to quantitation by densitometry, these techniques are time consuming because of the lack of appropriate automated systems. Biological activity assays, when available, often require relatively pure material and are not suitable for analyzing and quantitating impure or semi-purified samples, typical of the fermentation milieu. The optimization of several rDNA-derived protein systems from both prokaryotic and eukaryotic hosts has been completed using PCFIA, a rapid, sensitive system with high throughput. The development of Particle Concentration Fluorescence Immunoassay (CFIA) procedures for several of these rDNA-derived proteins of interest as potential biopharmaceuticals (e.g., α-1-antitrypsin, tPA, soluble CD4, and a malaria vaccine candidate) are discussed.

INTRODUCTION

The success of any bioprocess development effort relies upon the ability of the development staff to precisely and accurately quantitate the concentration of product synthesized. In the pharmaceutical industry, a variety of HPLC methods and bioassays have been successfully applied to the process development effort [6]. The use of such sensitive and precise assay systems often allows for the identification of relatively small (i.e., 10–20%) process improvements. As the fermentation development process continues and the scale of operation increases, such small increases in productivity can have a major impact on the overall process economics.

Recent advances in recombinant DNA technologies have made possible the production of a variety of potentially useful peptides and proteins. The electrophoretic and immunological techniques typically used to detect these proteins are very sensitive and can detect proteins at the nanogram level. However, quantitation of the amount of a recombinant protein produced can be cumbersome, as is the analysis of large numbers of samples. Typically, this is not a significant problem for molecular geneticists since successful cloning strategies can result in order of magnitude increases in the amount of protein produced and relatively few samples need be processed. However, when processes are transferred out of the laboratory and into the pilot plant (where smaller percent changes in product concentrations are quite significant) the need for accurate and precise quantification becomes critical. So too does the ability to process large numbers of samples, typical of most bioprocess development efforts. Once developed, these procedures must be amenable to validation in order to be transferred into manufacturing.

PARTICLE CONCENTRATION FLUORESCENCE IMMUNOASSAY

In an attempt to analyze production levels of several recombinant DNA-derived products (in both prokaryotic and eukaryotic expression systems), several methods were developed with a Particle Concentration Fluorescence Immunoassay (PCFIA™). This technique is a sensitive, rapid and highly automated system that can detect small changes in product levels. This system is capable of handling ten 96-well plates with a completed assay time of 2 h for one plate compared to 4 h for a typical ELISA procedure. At full capacity (i.e., 10 plates), the total output is 235 samples in 11 h.

A schematic model for PCFIA is shown in Fig. 1. First, an antibody (usually monoclonal) is hydrophobically

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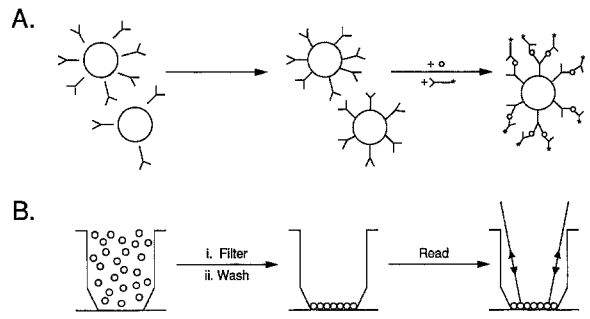

Fig. 1. Schematic diagram of PCFIA™ technology [7]. (A) Monoclonal antibodies (>–) are hydrophobically bound to polystyrene particles, followed by addition of analyte (○) and FITC-labelled second antibody (>–*). (B) Cross-section of a single well showing the dispersed particle bound complex which is filter concentrated to the well-bottom followed by an epifluorescence reading.

bound to submicron particles (typically 0.8 μm polystyrene beads). The resultant solid phase is dispersed in the sample containing a second antibody (either monoclonal or polyclonal) labelled with fluorescein. Because of the Brownian motion of the particles and their large surface area, the analyte rapidly binds to the antibody on the surface of the particles with concomitant (or subsequent) binding of labelled second antibody [7]. When the reaction is complete, the mixture of reactants is filtered, washed, and the total particle-bound fluorescence is determined.

ADVANTAGES AND DISADVANTAGES OF PCFIA

PCFIA has several advantages over other conventional analytical procedures, such as HPLC, ELISA, or Western blot analysis. Usually, activity assays and HPLC procedures require relatively pure material and are not suitable for analyzing impure or semi-purified samples. To detect subtle changes in product concentrations (~20%) by SDS–PAGE and/or Western blot analysis, it is necessary to run multiple dilutions and large numbers of gels. Although ELISA procedures typically produce similar results to PCFIA, the limited dynamic range and assay time have diminished their usefulness.

Conversely, degradation products containing immune-specific epitopes are recognized and not differentiated from full length products by this PCFIA [8]. Further, PCFIA reveals no information about biological activity of the product, since the method measures total immunoreactive product.

SANDWICH PROCEDURE

The majority of PCFIA procedures were developed using a sandwich procedure, with the critical issue being availability of reagents. Generally, the solid phase consists of a monoclonal antibody directed against the product of interest, this antibody is hydrophobically bound to polystyrene latex particles. Detection is achieved through a fluorescently labelled second antibody, typically fluorescein isothiocyanate (FITC). This second antibody may either be a polyclonal or a monoclonal which recognizes an epitope separate from the first.

COMPETITIVE PROCEDURE

When reagents are not readily available (i.e., polyclonal and monoclonal antibodies, or two monoclonal antibodies) and the protein of interest is smaller than 20 kDa, a competitive procedure may be chosen for development. Samples and standards are mixed with a rabbit polyclonal antibody directed against the protein product, followed by the addition of the product bound polystyrene particle. The order of reagent addition establishes competition between the particle-bound product and soluble protein for the rabbit polyclonal antibody. A goat anti-rabbit polyclonal antibody labelled with FITC is added for detection.

PRECISION AND ACCURACY OF PCFIA

During the development of an analytical procedure, assay accuracy and precision must be established. Validation of these critical parameters is presented for the detection of alpha-1-antitrypsin (A1AT). A1AT is an elastase inhibitor that has been investigated as a potential therapy for emphysema [3,4]. Expression of A1AT from *Escherichia coli* is under the control of the nalidixic acid induction system. The assay consists of a dual monoclonal sandwich procedure, that is, two monoclonal antibodies directed at separate epitopes of the A1AT molecule. Table 1 shows A1AT standard precision for the concentration range of 0–5000 ng/ml. Curve prozoning (i.e., bending of the curve at the upper range of the standard) is seen between 2500 and 5000 ng/ml in Table 1. Coefficients of variation exceeded 10% at the low (<31.2 ng/ml) and high (>2500 ng/ml) range of the concentrations tested. These results can be used to define a useful range for the assay of 30–2500 ng/ml with a mean precision of 5.1%.

Quadruplicate assays of crude *E. coli* lysates containing A1AT also resulted in a highly precise response pattern. The measurement of A1AT from cell lysates (Table 2) revealed a mean precision level of 5.4%, which was in excellent agreement with the precision of the A1AT

TABLE 1

Precision of replicate standards

Concentration (ng/ml)	Mean response ± S.E.M.[a] (Flour. units)	Coefficient of variation[b]
5000.0	72887 ± 4105	11.3
2500.0	43060 ± 1228	5.7
1250.0	24063 ± 913	7.6
1000.0	19876 ± 213	2.1
500.0	10547 ± 121	2.3
250.0	4812 ± 82	3.4
125.0	2232 ± 50	4.4
62.5	1108 ± 33	5.9
31.2	543 ± 26	9.6
15.6	225 ± 35	29.8
7.8	100 ± 25	50.0
0.0	0 ± 0	0.0

[a] Mean of four separate determinations.
[b] Ten percent coefficient of variation is the precision cut-off.

TABLE 2

Precision of replicate samples

Sample	Mean conc. ± S.E.M.[a] (μg/ml)	Coefficient of variation[b]
J852 T-1	1.81 ± 0.06	5.8
J852 T-2	2.23 ± 0.08	7.1
J852 T-3	4.36 ± 0.16	7.2
J852 T-4	6.22 ± 0.07	2.1
J852 T-5	7.43 ± 0.18	4.7

[a] Mean of four separate determinations.
[b] All samples were below the 10% precision cut-off.

standard. Accuracy for these studies was determined by spiking lysates of induced *E. coli* containing the appropriate plasmid without the A1AT gene [9]. A yeast-derived human A1AT standard and three representative crude *E. coli* lysates not containing A1AT were used for the spiking studies (Table 3). Interferences from non-specific cellular proteins did not appear to affect the binding of the monoclonal antibodies to A1AT, and accuracy levels were found to be within a mean of 94.5%. Accuracy results which are greater than 90% suggest that interferences from buffers and non-specific proteins do not affect the assaying system.

COMPARISON OF ASSAYS

Comparisons with other established procedures is an important aspect of validating an analytical method. A

TABLE 3

Spiking table for the determination of assay accuracy

Sample	Spike conc. (ng)	Measured conc.[a] (ng)	Error[b] (%)
Blank[c]	10.0	10.5	5
T-1[d]	10.0	10.4	4
T-3	10.0	10.8	8
T-5	10.0	10.9	9
Blank	5.0	5.2	4
T-1	5.0	5.1	2
T-3	5.0	5.3	6
T-5	5.0	5.4	8
Blank	1.0	1.0	0
T-1	1.0	1.1	10
T-3	1.0	0.9	10
T-5	1.0	1.0	0

[a] Measured conc. is calculated using the linear regression from Table 1.
[b] Error is ((measured conc. – spike conc.)/spike conc.) × 100.
[c] Lysis buffer alone with stated amounts of A1AT.
[d] Sample from a typical fermentation run, T-1 (1 h post-induction), etc.

comparison between ELISA and PCFIA is presented for the detection of tissue plasminogen activator (tPA). TPA is a thrombolytic molecule licensed for the treatment of thromboembolic disorders [11], and is expressed in chinese hamster ovary (CHO) cells. The assay is a similar format to A1AT, that is, a dual monoclonal antibody sandwich procedure. CHO supernatants containing various concentrations of tPA were assayed by both ELISA and PCFIA. The results of this study are shown in Fig. 2, where concentration (μg/ml) of samples quantitated by ELISA are plotted versus concentrations (μg/ml) by PCFIA. A regression line was fit to the points and shows that the two methods are in close agreement.

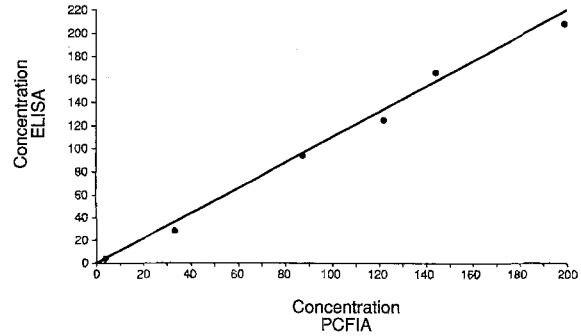

Fig. 2. Tissue plasminogen activator sample concentration comparison. Culture supernatants were analyzed by ELISA and PCFIA and subjected to regression analysis.

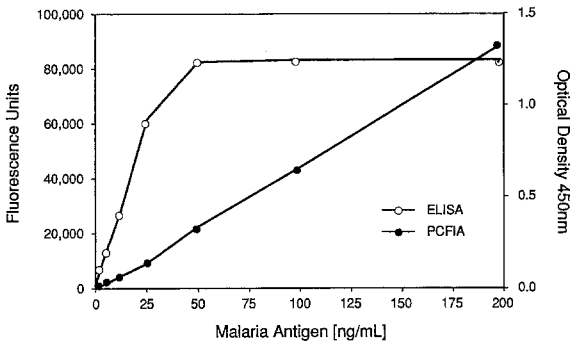

Fig. 3. Comparison of ELISA vs PCFIA standard curves for a malaria antigen.

Upon further investigation of differences between ELISA and PCFIA, there is a major discrepency in the linear sensitivity ranges which are depicted in Fig. 3. The linear standard curve range for a malaria antigen expressed in *E. coli* using an ELISA procedure was 3–25 ng/ml [2,10]. The same standard analyzed by PCFIA revealed a linear range of 3–200 ng/ml, which translates to a 10-fold increase in sensitivity. The reason for this discrepancy may be that the spherical solid phase of PCFIA has increased surface area which results in higher levels of sensitivity [7].

SAMPLE SENSITIVITY

Another parameter which is required for validation of an analytical method is the sensitivity of the procedure to unknown samples. For this purpose, a soluble form of CD-4 (sCD-4) expressed in CHO is presented. CD-4 serves as the cellular receptor for the human immunodeficiency virus (HIV) and soluble forms of CD-4 are potent inhibitors of HIV infection in vitro [5]. This assay consists of a monoclonal/polyclonal sandwich procedure, that is, a monoclonal antibody is bound to the particle (solid phase) and a polyclonal antibody is conjugated to an FITC label (detector). The linear range of this assay is 20–1250 ng/ml, which is very similar to the range for A1AT (data not shown). Various dilutions of an unknown CHO supernatant sample were then plotted on a linear curve to determine the relative range of sensitivity (Fig. 4). This result shows the wide range of linearity for PCFIA, that is, a 32-fold difference in dilution is within the linear range of this assay. Consequently, relatively few dilutions are required to obtain accurate results.

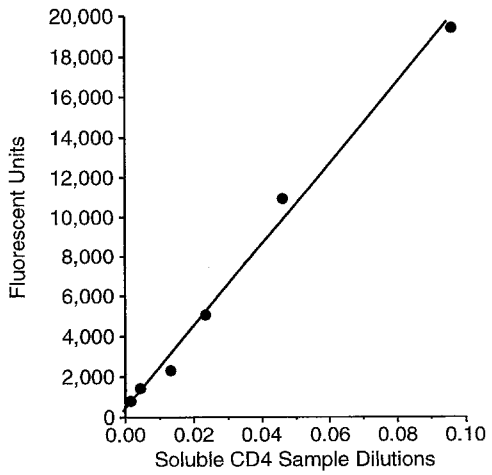

Fig. 4. Chinese hamster ovary derived soluble CD-4 sample sensitivity curve.

COMPETITIVE PCFIA FORMAT

As stated previously, when reagent availability is limiting and the protein of interest is smaller than 20 kDa, a competitive PCFIA may be warranted. An example of this strategy is presented for an sCD-4 derivative expressed in *E. coli* [1]. The low molecular weight of the protein ($\sim$ 16 kDa) coupled with the availability of a single rabbit polyclonal antibody raised against full length sCD-4 were compelling reasons for developing and validating a competitive PCFIA procedure for this molecule. Fig. 5 shows a typical standard curve using this format. Because of the nature of competition assays (i.e., product on the bead vs product in the sample competing for the same polyclonal epitopes), the protein concentration is inversely related to the fluorescent response. The differences between this and the sandwich format are that a semi-log plot is necessary to linearize the curve, and that the linear range of the upper end of the curve is significantly reduced. Taking the anti-log of the X-axis, the assay range is 35–600 ng/ml. These factors taken together result in a reduced level of accuracy, and make this format less appealing than the sandwich procedures discussed previously.

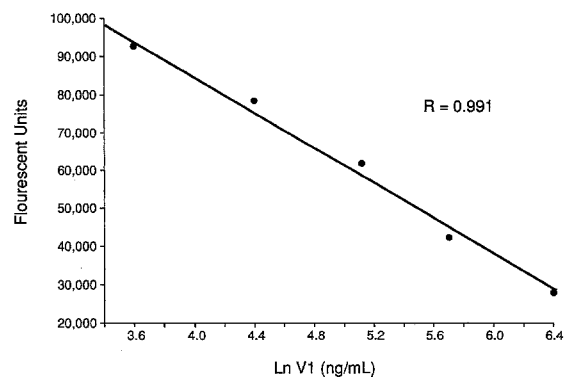

Fig. 5. Soluble CD-4 standard curve utilizing a competitive PCFIA procedure.

CONCLUSIONS

The work in this communication demonstrates a number of positive attributes of PCFIA for the analysis of rDNA products. The wide range of linearity for these assays decreases the number of dilutions necessary to obtain accurate results. The large sample capacity (i.e., 235 at full capacity) and speed of the analyzer significantly decrease total processing time. However, as with any assay, care must be taken when interpreting the results of this antibody based system. That is, since the antibodies employed recognize only a small portion of the protein of interest, any degradation product that still contains immune-specific epitopes will be recognized [8]. The results of these assays also do not provide any information as to the biological activity of the protein of interest. The high levels of accuracy ($>90\%$) and precision ($<6\%$) of this system allow the detection of 5–10% changes in product concentrations in the presence of large amounts of cell protein. Because of the degree of automation available with this system, these PCFIA procedures are easily transferred into a manufacturing setting.

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