Molecular Devices

Threshold® Immunoassay System

ILA Application Note

Applications of rapid immunoassays (FAST ILA) in cell culture/fermentation and process development using the Threshold System

INTRODUCTION

Accurate measurement of the concentration of a biological product is important in bioreactor/fermenter monitoring, downstream processing and final product quality control. Currently, electrophoretic, chromatographic and immunochemical methods are the most commonly used. Electrophoretic methods typically provide qualitative results; chromatographic methods are quantitative and provide information on all sample constituents, but may be subject to sample interference problems and also may not be specific for the product of interest; and finally, immunochemical methods are specific for the product of interest, but can be lengthy and may not be quantitative. Thus, these three methods are currently used in combination to provide the desired sample information. Any analytical method that can provide a rapid, quantitative measurement of a desired product or contaminant will be useful in monitoring and optimizing bioreactors/fermenters and individual purification steps.

Typical sample turnaround time, for optimal sensitivity, using the Threshold Immunoligand Assay (ILA) system is approximately 4 hours. This includes the sample preparation, incubation, filtration and read steps. A standard ILA employs a sample incubation time of two hours. This incubation generally provides sufficient time for the antibody-antigen reactions to reach equilibrium. In most cases, the initial binding kinetics of the antibody-antigen complexes are relatively fast (1 - 1000 seconds). A shorter sample incubation time 1– 10 minutes) should still provide enough time for sufficient antibody-antigen complex formation, even though the reactions have not reached equilibrium. In general, assay performance characteristics will be optimal if high affinity, fast binding antibodies are used. A reduction in sample incubation time now allows a total assay time of less than one hour.

This application note describes one example of a rapid sandwich immunoassay (FAST ILA) developed to detect murine IgG using the Threshold ILA system. This assay provides picogram sensitivity with a total turnaround time of less than one hour with a capability of assaying multiple samples in the same experiment.

MATERIALS

1 ***Threshold® System*** from Molecular Devices Corporation (catalog #0200-0500), 1311 Orleans Drive, Sunnyvale, CA 94089, tel: 408-747-1700 or 800-635-5577.

2 ***Immuno-Ligand Assay Labeling Kit*** from Molecular Devices Corporation (catalog #R9002).

3 ***Immuno-Ligand Assay Detection Kit*** from Molecular Devices Corporation (catalog #R9003).

4 ***Immunoaffinity purified goat anti-mouse IgG (F_c specific)*** was purchased from Sigma Chemical Company (catalog # M2650).

5 ***Immunoaffinity purified goat anti-mouse IgG (F_{ab} specific)*** was purchased from Accurate Chemical & Scientific Corporation (catalog # JGM-0572), Westbury, NY, tel: 516-333-2221.

6 ***Purified mouse IgG_1*** was purchased from Zymed Laboratories, Inc. (catalog # 02-6100), South San Francisco, CA, tel: 415-871-4494.

METHODS

Labeling of binding proteins

The *molar coupling ratio* (MCR) is defined as the moles of hapten (biotin or fluorescein) per mole of binding protein used in the labeling procedure (see the ILA section of the *Threshold System Operator's Manual*). The *molar incorporation ratio* (MIR) is a measure of the extent of labeling (protein haptenation) and is defined as the average moles of hapten covalently bound per mole of binding protein. The theoretical maximum MIR is determined by the total number of available amine groups on the binding protein. Labeling of the binding proteins will typically require three hours, including a two hour incubation.

Anti-mouse IgG (F_{ab} specific, "anti-F_{ab}"), and anti-mouse IgG (F_c specific, "anti-F_c"), were quantitatively labeled with fluorescein and biotin, respectively, at a MCR of 20:1 according to the protocol provided in the ILA section of the *Threshold System Operator's Manual*. The resulting MIR's were 5.0 for fluoresceinated anti-F_{ab} and 4.4 for biotinylated anti-F_c.

Antibody loading

In order to drive the antibody-antigen reaction towards complex formation, the loading of each antibody was increased over that used in a standard ILA (two hour incubation). The upper limit of each labeled antibody that can be used is determined by the resulting background level for a negative sample. Acceptable background levels are usually less than or equal to 150 μV/sec. A loading study will typically require one or two assays (one-half to one day's experiments).

With these considerations in mind, the optimal loading was determined to be approximately 50 ng of each labeled antibody/100 μL test volume. Since an excess of fluoresceinated antibody is used, a sequential format is required to avoid saturating the fixed quantity of anti-fluorescein:urease conjugate present in the Enzyme Reagent (i.e., adding Enzyme Reagent in a second filtration step after the first filtration capture step as outlined in the ILA section of the *Threshold System Operator's Manual*). The steps below outline the protocol used in the sequential FAST ILA format.

Assay protocol for sequential FAST ILA

Step 1 Prepare sample dilutions and standard curve in Assay Buffer.

Step 2 Prepare a mixture of biotin and fluorescein labeled antibodies in a single polypropylene tube.

Step 3 Combine 100 μL of sample with 100 μL of antibody mixture.

Step 4 Incubate 10 minutes at room temperature.

Step 5 Reconstitute the Capture Reagent with 25 mL of Assay Buffer. Prepare a 1:5 dilution of reconstituted Capture Reagent (1 volume of Capture Reagent + 4 volumes of Assay Buffer). When the incubation is complete, dispense 0.5 mL of the diluted Capture Reagent into each tube with an Eppendorf Repeater Pipetter and a Combitip.

Step 6 Transfer the reaction mixtures to the filtration units. Filter on low vacuum.

Step 7 When the wells are empty, add 2 mL Wash Buffer to each well and filter on high vacuum.

Step 8 During the filtration step, reconstitute the Enzyme Reagent with 4 mL of Assay Buffer. Prepare a 1:5 dilution of the reconstituted Enzyme Reagent (1 volume of Enzyme Reagent + 4 volumes of Assay Buffer).

Step 9 Turn the vacuum off and dispense 0.5 mL of diluted Enzyme Reagent to each well with a Repeater Pipetter and Combitip. Filter on low vacuum.

Step 10 When the wells are empty, add 2 mL Wash Buffer to each well and filter on high vacuum.

Step 11 When the wells are empty, turn off the vacuum and read the sticks.

Total assay time: 1 hour.

ASSAY CHARACTERIZATION

Standard curve

Figure 1 compares standard curves generated for murine IgG using the FAST ILA (10 minute incubation) format and the standard ILA (two hour incubation) format. The dynamic range of the FAST ILA is 200 pg - 10 ng murine IgG_1/test or 2- 100 ng murine IgG_1/mL, while the dynamic range of the standard ILA is 5 pg to 1 ng murine IgG_1/test or 50 pg to 10 ng murine IgG_1/mL. Loading used in the FAST ILA format was 50 ng of each labeled antibody per test. ILA sample volume is typically 100 μL per test.

As in other sandwich ILAs previously developed, the assay precision is in the range of 5 - 8%, within assay. Assay-to-assay variation is usually under 10%.

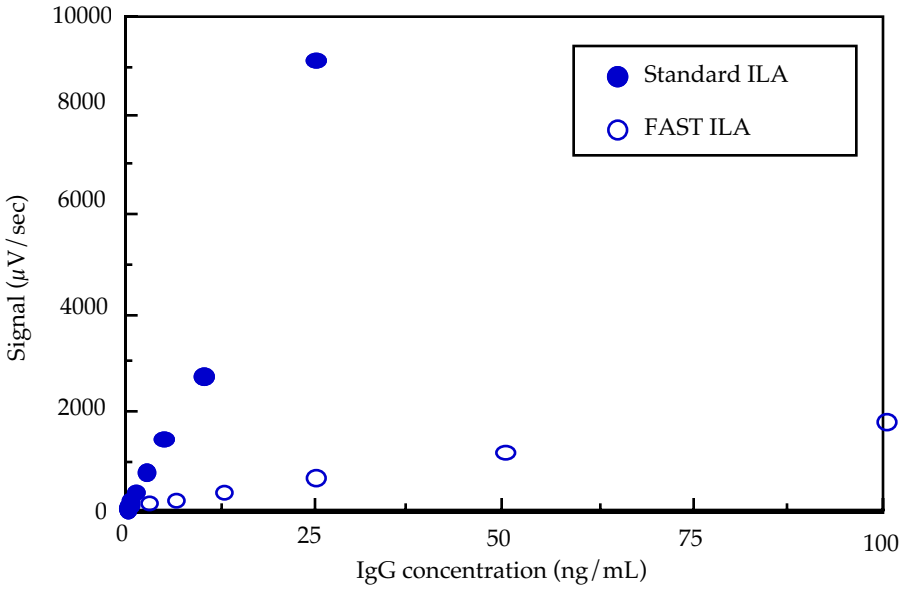

Figure 1: Comparison of standard curves generated for murine IgG using the FAST ILA format and the standard ILA format.

APPLICATIONS

The characteristics of the FAST ILA format make it ideal for applications in media optimization, clone selection, bioreactor monitoring and process development where quantitation, sensitivity and real-time or same day results are important.

Media optimization

Table 1 presents a comparison of small-scale cell culture experiments designed to determine optimum media conditions for production of monoclonal antibody. Cells were cultured for three days, after which the supernatant was recovered, diluted and tested for IgG concentration. In a second experiment, the cell culture step and immunoassays were repeated. The results obtained using the murine IgG assay in the FAST ILA format are compared to those obtained using a conventional ELISA requiring two to three days to generate an answer. The results agree closely.

		IgG_1 concentration ($\mu g/mL$)	
Medium	Run #	FAST ILA	ELISA
A	1	10.8	11.5
A	2	12.6	12.0
B	1	12.9	11.8
B	2	15.1	12.6
C	1	11.9	11.8
C	2	12.4	10.9
D	1	6.2	6.4
D	2	12.7	12.5
E	1	11.9	12.4
E	2	10.4	10.5
F	1	13.7	13.9
F	2	15.9	13.1
G	1	13.0	13.0
G	2	11.5	9.5

Table 1: Comparison of results of FAST ILA and ELISA assays to determine media optimization

Initially, sample interference was evident in that poor recovery of exogenously added IgG ("spike") and variable quantitation as a function of sample dilution were obtained with the FAST ILA format. However, the interference problem was solved by increasing the loading of both labeled antibodies to 100 ng/test and adding unlabeled anti-F_c and anti-F_{ab} at a concentration of 250 ng/test. It is known that free light chain and other proteolytic antibody fragments may be present in cell culture supernatants. It is possible that the interference may have been due to a large excess of free light chain and other proteolytic products which the labeled antibodies recognize and bind. These fragments effectively compete with intact murine IgG for the labeled antibodies. If the labeled antibodies are not present in excess, inhibition of spike recovery will occur. Increasing the loading of the two labeled antibodies alone will increase background of a negative sample and overall signal; this in turn affects detection limit and reduces the dynamic range. Addition of the unlabeled antibodies and more labeled antibodies effectively provides an excess of total binding antibodies without appreciably increasing the background (in fact, the background signal actually decreases). This allows the FAST ILA format to be utilized without interference from antibody fragments. Alternatively, the sample could be diluted further and tested in a standard ILA format.

Process development

The FAST ILA format can also be used to rapidly quantitate product recovery in downstream processing steps. Practical applications of the FAST ILA format in process design and optimization are now possible. Table 2 presents an application of the FAST ILA to determine murine IgG recovery through a TRIO™ hollow fiber IgG purification system (a product of Sepracor, Inc.). In this example, two different cell culture supernatants are purified using the TRIO system. The IgG concentration in each step of each process is determined using both FAST ILA and Protein A HPLC. The results obtained agree closely.

Sample		IgG concentration (μg/mL) FAST ILA	HPLC	Volume (mL)
Clone # 1	Culture Supernatant	59.0	50.0	194
	Wash # 1	1.1	2.0	152
	Wash # 2	5.8	7.0	96
	Wash # 3	0.1	2.0	194
	Eluate	773.0	~700.0	7
	Product recovery	47.3%	50.5%	
Clone # 2	Culture Supernatant	3.3	5.0	193
	Wash # 1	0.7	N/A	194
	Wash # 2	1.2	N/A	84
	Eluate	51.0	58.0	12
	Product recovery	96.1%	72.1%	

Table 2: FAST ILA for use in process development

These results show that the percent recovery of IgG_1 in the eluate (relative to the supernatant) is different for the two examples. The difference may be due to varied affinity of the IgG_1s for Protein A, differences in the wash schemes employed, or the different starting concentrations of IgG in the two supernatants. Testing the effects of wash schemes and supernatant IgG concentration on antibody recovery can now be rapidly performed using the FAST ILA format.

SUMMARY

When high affinity, fast binding antibodies are combined with the Threshold ILA system, rapid immunoassays can be developed in a matter of days. A FAST ILA for murine IgG_1, with a dynamic range of 2 ng to 100 ng IgG_1/mL and a total assay time of ≤ 1 hour was developed in 3 days. The accuracy, precision and rapid turnaround time, both for development and routine assays, make the FAST ILA format useful for a number of practical biopharmaceutical applications including

media optimization, process development, validation, clone selection and bioreactor optimization and monitoring. Rapid assays for other biological products and/or contaminants (e.g. host cell protein, insulin, transferrin, etc.) can be developed in a similar fashion. Thus, the Threshold FAST ILA format is a useful new analytical tool to be used in conjunction with existing methods such as electrophoresis and chromatography.

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