

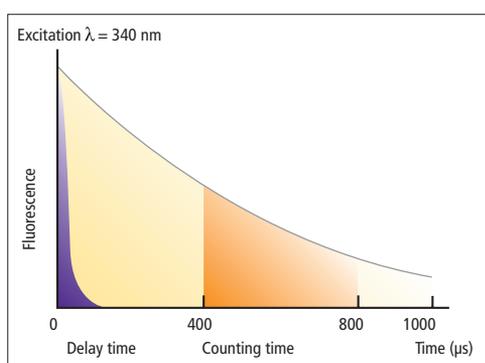
TRF Technology
for BiotherapeuticsPerkinElmer, Inc.
Hopkinton, MA USADELFLIA Assays:
Flexible and Sensitive
Tools for Monoclonal
Antibody Development

Figure 1. DELFLIA time-resolved fluorescence immunoassays dramatically improve signal detection (orange) relative to background (purple).

Introduction

DELFLIA® immunoassays are particularly well suited for discovery of specific, high-affinity monoclonal antibodies (mAbs), especially at the low concentrations that may be present in hybridoma supernatants. An assay used in these conditions must be sensitive, specific, reliable, reproducible and easy enough to handle 1,000 to 1,500 samples in one batch. DELFLIA time-resolved fluorescence (TRF) assay chemistry provides high sensitivity with inherently low fluorescent background. TRF-based detection relies on reagents labeled with lanthanide chelates containing Europium (Eu), Terbium (Tb) or Samarium (Sm). These possess both long fluorescence decay times and large Stokes shifts, properties that allow delayed signal measurement at a wavelength with little background interference. In addition, lanthanides emit a stable fluorescent signal that exhibits a sharp emission peak and high fluorescence intensity (Figure 1).

DELFLIA assay design is very flexible, enabling effective screening in cases where either purified antigen or polyclonal antibodies are in limited supply. The wide dynamic range of DELFLIA immunoassays also make them ideal for post-screening applications such as characterization of antibody affinity and specificity, and for quantifying mAb production in cell culture, ascites fluid and *in vitro* large scale production. The flexibility of the DELFLIA immunoassay also points to novel applications such as screening of hybridoma supernatant reactivity for two antigens/epitopes at the same time or detection of monoclonal antibodies specific for antigen in complexes.

Here we present an overview of these several strategies for mAb discovery using DELFIA immunoassays. As an example we also describe results generated using a DELFIA immunoassay to screen for and subsequently monitor stable production of mAbs directed against prostate specific antigen (PSA). This protein, a serine protease with molecular mass of 33 kDa, has been found to be the best and most reliable marker of prostate cancer.¹ A small portion of this marker exists free in human serum but the majority is coupled to α 1-antichymotrypsin (ACT).²

DELFIA Assay Configurations for Hybridoma Screening

The selection of the best assay configuration depends to a large extent on the availability, amount and quality of the purified target antigen or of polyclonal antibodies to that antigen. Some antigens may be available in large amounts, such as unpurified cell extracts. However, other antigens may be available only in limited amounts or they may be small molecules with only one epitope or with an epitope that would be blocked by chemical conjugation. Similarly, experimental design may be impacted by antibody reagents, which may be available either in very limited amounts or commercially available in large quantities. Solid-phase, microplate-based assays allow the use of an excess of reagents and are generally preferred for primary screening applications.

When a Polyclonal Antibody is Available

When an antigen-specific polyclonal antibody is available in relative abundance, the assay setup is fairly straightforward. If the specific polyclonal antibody is very abundant, it can be used to coat assay plates (0.5-1.0 μ g/well) as shown in Figure 2A. Antigen (either purified or partially purified) is then captured, followed by capture of mouse monoclonal antibodies from hybridoma supernatants. Detection is mediated by Eu-labeled anti-mouse IgG (Cat No. AD0124/AD0207). A benefit of this approach is that no custom-labeled reagents are needed.

The alternative configuration shown in Figure 2B conserves polyclonal antibody (only 50-100 ng needed/well). Here, the antigen-specific polyclonal antibody is Eu-labeled and used as the detection reagent. Capture of the mAb/antigen sandwich is accomplished using microplates coated with anti-mouse IgG. These plates are commercially available (Cat No. 4007-0010), which saves time and helps ensure consistent results.

When Antigen Availability is Limited: Use of Labeled Antigen

A DELFIA assay can be configured using antigen labeled with Eu chelate or biotin (Figure 2C and 2D), and this approach was used to generate the example data provided in this application note. A labeled antigen assay configuration is recommended when antigen-specific antibodies are not available or are in limited supply, or in cases where the compound is so small that two separate epitopes are not available for binding. Labeling of small antigens can be somewhat risky because it changes the molecular structure which may lead to decreased antibody binding. Fortunately, the Eu chelate is small and thus is unlikely to have a major effect on antigenicity. Use of Eu-labeled antigen also results in a simpler assay protocol, as no subsequent streptavidin binding step is needed. Eu labeling can be performed under gentle reaction conditions using Labeling Reagent (Cat No. 1244-301) or by PerkinElmer OnPoint® Custom Services (www.perkinelmer.com/onpoint). One mg of antigen (at a concentration of 1-5 mg/mL) is commonly used for a Eu or biotin labeling procedure, but only 20-30 ng of labeled antigen is needed per well.

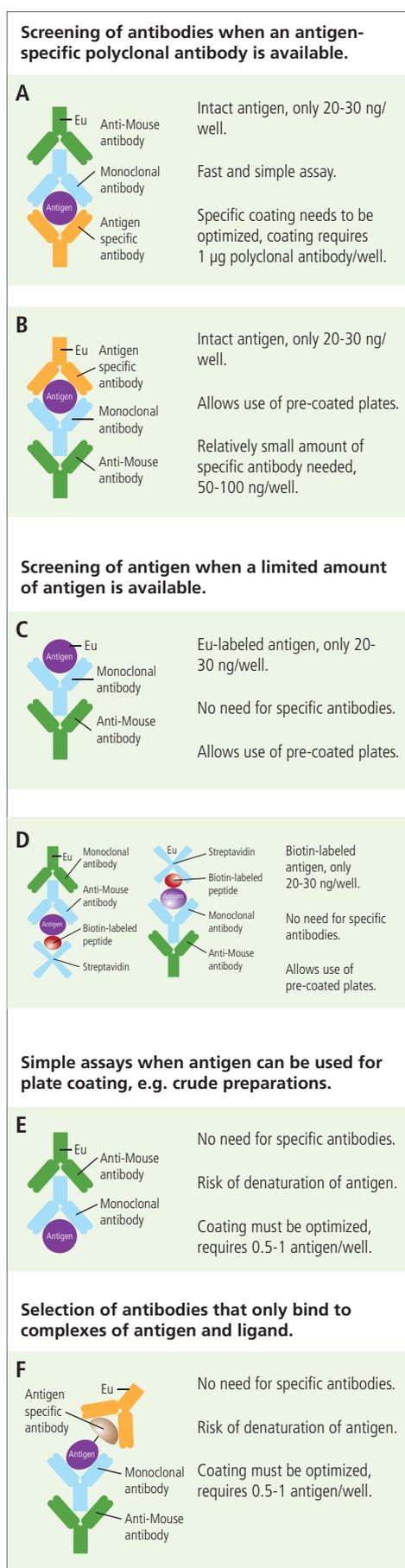


Figure 2. mAb screening assay configurations.

If a biotin-labeled antigen is used, two assay configurations are possible (Figure 2D). The best configuration should be determined empirically. Capture and detection can be performed with commercially available plates (Streptavidin-coated plates, Cat No. 4009-0010 or anti-mouse coated plates, Cat No. 4007-0010) and reagents (Eu-labeled anti-mouse IgG, Cat No. AD0124/AD0207 or Eu-labeled streptavidin, Cat No. 1244-360).

When Antigen is Available in Large Amounts

When antigen is available in large amounts, often as a crude cell lysate or a partially purified recombinant gene expression product, the mAb assay can be configured very simply, as outlined in Figure 2E. Antigen is coated directly on plate wells (0.5-1.0 µg/well) and the captured mAb is detected by a Eu-labeled anti-mouse IgG.

Detection of mAb: Antigen Complexes

In some cases it is beneficial to select for monoclonal antibodies that recognize antigens in a particular conformation, such as that of the antigen bound in a complex. Figure 2F illustrates how such a DELFIA assay would be configured.

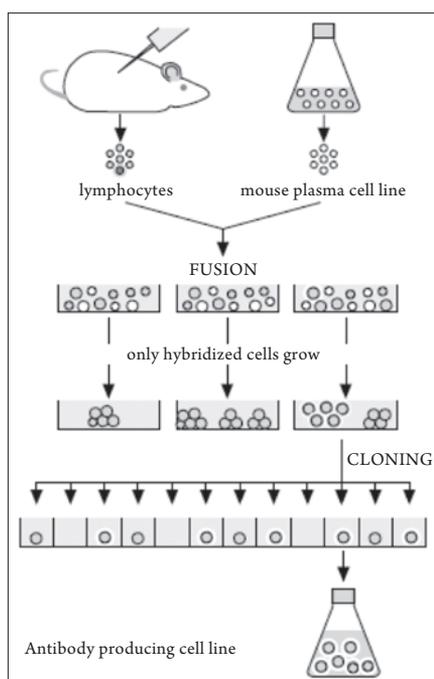


Figure 3. Hybridoma development and production.

Materials and Methods

Monoclonal Antibody Production

The hybridoma generation procedure is summarized in Figure 3. Briefly, several adult mice were intraperitoneally immunized with PSA peptide antigen. Plasma samples were monitored with the DELFIA immunoassay described below, and mice with a high immune response were splenectomized. Lymphatic cells of the spleen or, in some cases, lymph nodes from immunized mice were fused to a mouse myeloma cell line with fusogen and harvested in HAT media (containing hypoxanthine, aminopterin, and thymidine) which enables selective growth of hybridomas. Antibody production by hybridoma culture supernatants was tested 2-3 weeks after cell fusion. Cells that do not produce antibodies grow faster than antibody-producing cell lines. It is important to clone the antibody-producing cells before they experience toxicity due to pH changes caused by the faster growing, non-fused cells. Thus, the high sensitivity of a solid phase DELFIA assay is especially important for early detection of antigen-specific hybridomas in primary screens.

Following the initial screening for antigen-reactive mAbs, cells in positive wells were cloned by repeated dilution into 96 well cell culture plates. The master clones identified in this manner were then re-cloned and retested for antigen reactivity after 2-3 weeks. Some of these develop into stable antibody-producing cell lines, which can be frozen for storage.

DELFIA Assay Protocol

For the example presented here, mAbs against PSA were screened with a sandwich-type immunoassay (Figure 4). Mouse mAbs were captured on microplates coated with rabbit anti-mouse immunoglobulin (Cat No. 4007-0010) (Lövgren et. al., 1984). Detection of bound mouse monoclonal antibodies was performed using Europium (Eu) labeled PSA peptide antigen, prepared by PerkinElmer as described below. To perform the assay protocol (Figure 3), standards (ranging from 1.6-1000 ng/mL of anti-PSA antibody) were diluted in Assay Buffer (Cat No. 1244-106). Microplates pre-coated with anti-mouse Ig were pre-washed, loaded with 50 µL of Assay Buffer followed by 50 µL of standards or samples, and mixed by slow shaking for 5 min. The plates were then incubated 2 hrs at room temperature and overnight at 4 °C, and then washed 4 times with Wash Buffer (Cat No. 1244-114). The Eu-labeled antigen was then diluted to 300 ng/mL in Assay Buffer, added to each well (100 µL per well), and the plate was incubated for 60 min at room temperature with slow shaking. Following 4 washes in Wash Buffer, 200 µL per well of Enhancement Solution (Cat No. 1244-104) was added per well and the plate was read in a time-resolved fluorescence plate reader, either immediately or at any convenient time, hours or even days later.

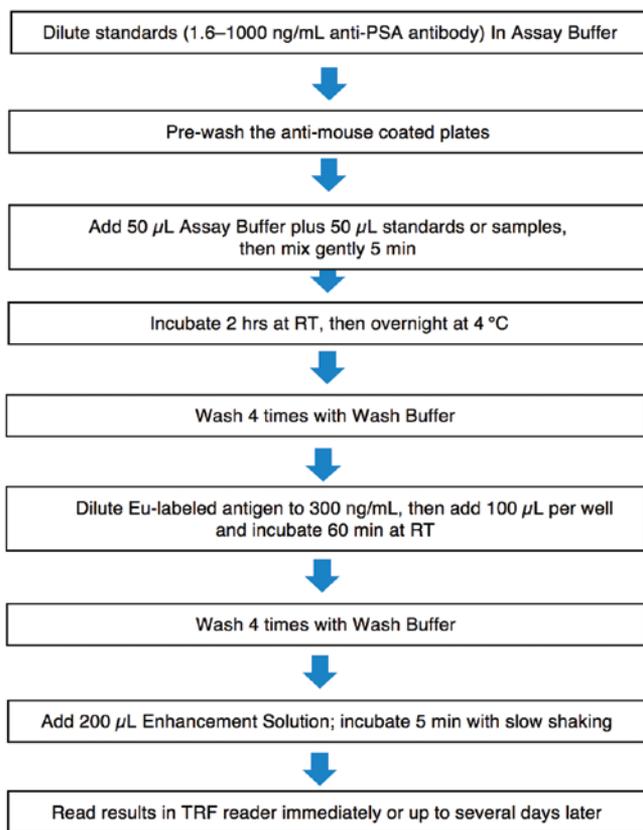


Figure 4. DELFIA monoclonal antibody screening protocol using Eu-labeled antigen.

Labeling of Antigen with Europium Chelate

The Eu-labeling reagent is very hydrophilic, which makes the labeling non-destructive to the antigen. A 25-fold molar excess of Eu Labeling Reagent (Cat No. 1244-301) was added to a solution 1 mg/mL solution of PSA (MW 33 kDa) in 0.15 M NaCl. The pH was adjusted to 9.8 by adding one tenth volume of 0.5 M sodium carbonate buffer, pH 9.8. Labeling was performed overnight at 4 °C. Unbound label was removed by gel filtration using an Ultrogel® AcA 54 column (1 x 50 cm) (IBF, France). Eu-labeled PSA was eluted in 50 mM Tris-HCl buffer, pH 7.8, containing 0.15 M NaCl. PSA yield was calculated using fluorescence intensity in the peak fractions relative to total fluorescence loaded. Evaluation of the Eu-labeling reaction and of the resultant DELFIA immunoassay can be readily performed using small amounts of anti-PSA serum from the immunized mice.

For Eu-labeling of antibodies rather than peptides, specific instructions for labeling are provided in the labeling reagent package insert.

Results

Assay Performance

The concentration of monoclonal antibodies in hybridoma culture supernatants can vary widely, from initial screening when few antibody-producing cells are present to subcultured, high-producing cell lines. The assay developed for this application (configuration in Figure 2C) exhibited a wide dynamic range of three orders of magnitude (Figure 5), typical of DELFIA assays.

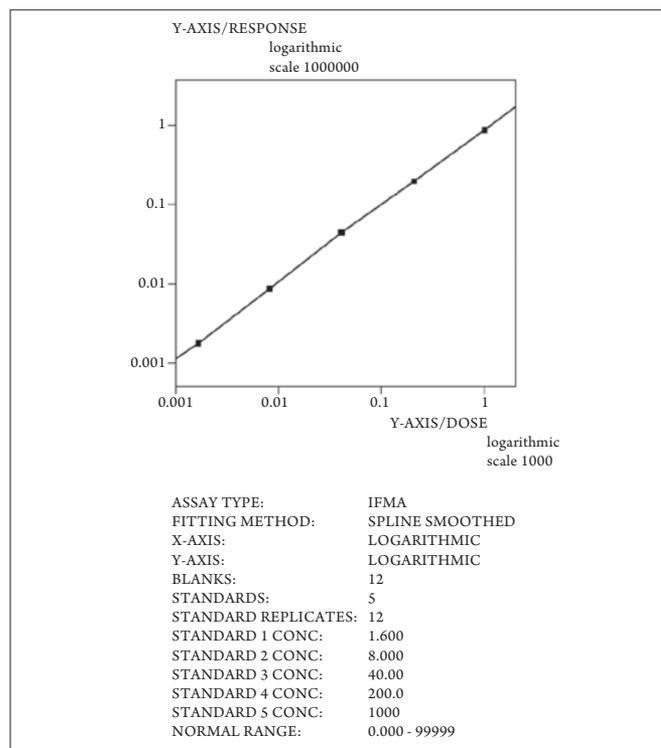


Figure 5. DELFIA monoclonal antibody detection assay standard curve extends over three orders of magnitude.

Screening for Positive Clones

Hybridoma clones producing monoclonal antibodies to PSA were quickly identified as microplate wells with signal far above background (Figure 6), visualized either as raw counts or as false color. Figure 7 illustrates that a more accurate quantification of antibody production in antibody-producing wells was obtained by counting cells in each of the hybridoma-producing wells assayed and then normalizing fluorescence intensity on the basis of cell number (counts per 10^6 cells). This provides a better basis for selection of clones to be subcultured for future analysis.

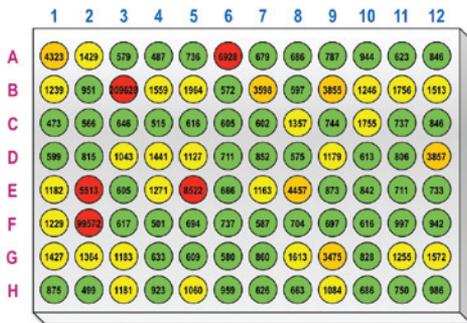


Figure 6. In very preliminary screening positive wells are quickly identified on the basis of raw counts and false color images.

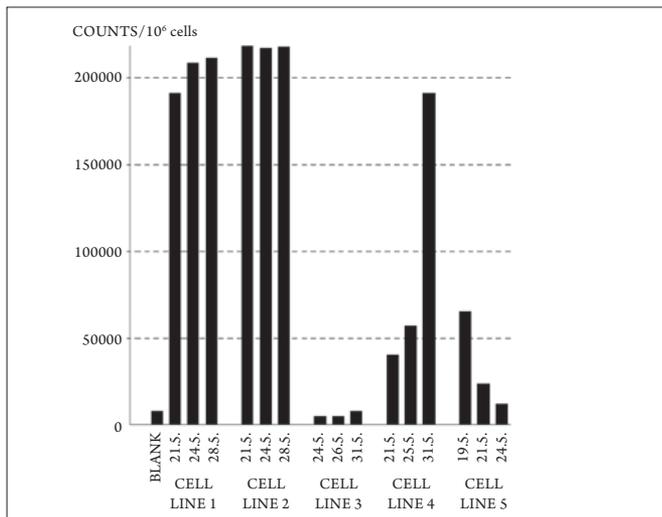


Figure 7. Measurements of immunoassay reactivity are more comparable when fluorescence intensity is normalized per 10^6 cells.

Stability of Monoclonal Antibody Production

Hybridoma clones vary in antibody production as well as antibody affinity and specificity, and antibody yields can increase or decrease over time. The most desirable clones will be steady producers of antibody. Figure 8 illustrates that two of five clones tested were steady producers at a high level, and that two other clones showed varying production, trending either up or down over time. Hybridoma clones selected for production of mono-specific antibodies should be monitored over time. Figure 9 shows the results of monitoring one such clone over a period of 90 days.

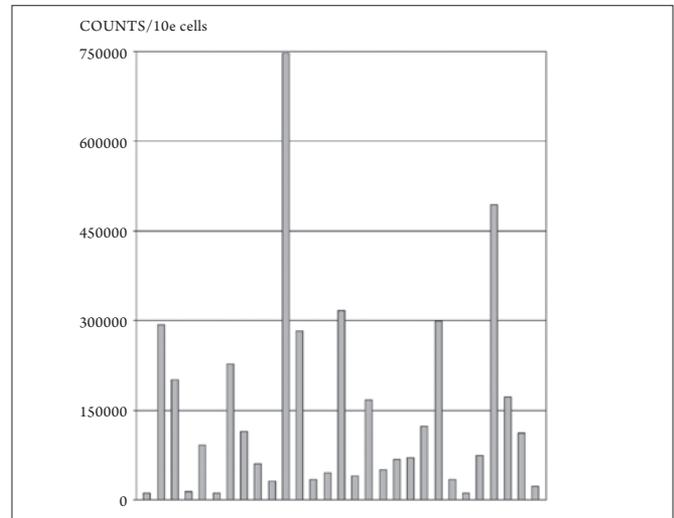


Figure 8. Antibody production trends for five hybridoma cell lines.

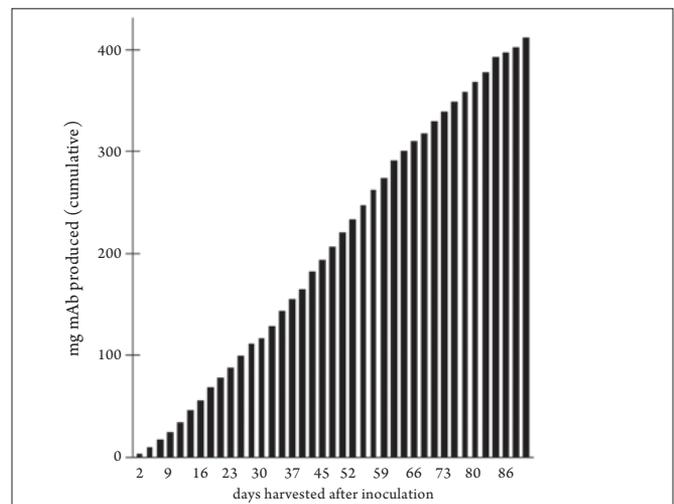


Figure 9. Antibody production in a cell culture can be easily monitored using DELFIA assays (configuration 2C). Multical™ software has been used to produce the standard curve.

Conclusions

Initial screening and subsequent monitoring of monoclonal antibody production in culture supernatants requires a sensitive assay with a low background. Furthermore, the differing constraints of each project demand a high degree of flexibility in assay configuration so that precious reagents, whether antigen or antibody, can be conserved. DELFIA immunoassays provide a highly flexible platform for development of assays for monoclonal antibody production, and exhibit the low background and wide dynamic range needed for this application. Where antigens must be labeled to allow detection, gentle labeling with the Eu chelate minimizes the potential for interference in antigen recognition. The availability of a wide range of stable Eu-labeled reagents (streptavidin, antibodies and expression tags) makes possible a wide range of assay designs, including selection for antibodies specific for antigens complexed with other ligands.

References

1. Lilja K., Christensson A., Dahlén U., Matikainen M.-T., Nilsson O., Pettersson K., and Lövgren T. Prostate-specific antigen in serum occurs predominantly in complex with α 1-antichymotrypsin. 1991. *Clinical Chemistry* 37: 1618-1625.
2. Christensson, A., Björk, T., Nilsson, O., Dahlén, U., Matikainen, M.-T., Cockett, A.T.K., Abrahamsson, P.-A., Lilja, H. (1993) Serum prostate specific antigen complexed to α 1- antichymotrypsin as an indicator of prostate cancer. *J. Urology* 150:100-105.
3. Hemmilä I., Bakudu S., Mikkala V.-M., Siitari H., Lövgren T. (1984) Europium as a label in Time-Resolved Immunofluorometric assays. *Anal. Biochem.* 137; 335-343.
4. Barnard, G., Kohen, F. (1990) Idiometric assay: Non-competitive immunoassay for small molecules typified by the measurement of estradiol in serum. *Clin. Chem.* 36:1945-1950.
5. Pettersson, K., Söderholm, J. (1991) Individual differences in lutropin immunoreactivity revealed by monoclonal antibodies. *Clin. Chem.* 333-340.
6. Shinohara, K., Kakiuchi, T., Kobori, M., Suzuki, M., Fukushima, T. (1993) Europium-linked immunoassay of IgM production by humanhuman hybridomas cultured with or without chicken egg-yolk lipoprotein. *Biosci.Biotech. Biochem.* 57(6):1012-1013.