Application Notes

DELFIA® assays bring convenience in monoclonal antibody development

INTRODUCTION

The DELFIA® system provides a method of discovering specific, high-affinity monoclonal antibodies (Mabs) even in low concentrations in hybridoma supernatants. The assay used in these conditions has to be very sensitive, specific, reliable, reproducible and easy enough to allow handling of about 1,000 to 1,500 samples in one batch.

By using sensitive DELFIA immunoassays for screening one can be confident that all the antibody-producing hybrids can be found. The ease, sensitivity and versatility of this assay makes it possible to test the hybridoma supernatants against different antigens in parallel. The assays can be optimized to fulfil specific needs for convenience; lanthanide-labelled compounds and buffers are commercially available or specific reagents can be prepared to meet users' individual needs.

As an example, production of Mabs against prostate specific antigen (PSA) is presented. This protein, a serine protease with molecular mass of 33 kDa, has been found to be the best and most reliable cancer marker of prostatic cancer (Lilja et al, 1991). A small portion of this marker exists free in human serum but the majority as coupled to α1-antichymotrypsin (ACT) (Christensson et al., 1993). Prior to splenectomy of mouse with high immune response the plasmas of several adult mice intraperitoneally immunized with the PSA were tested using immunofluorometric assay (IFMA) with Eu-labelled PSA.

APPLICATION AREAS OF DELFIA® SYSTEM

- Screening for hybridomas
- Quantitation of the production of Mabs in cell cultures, ascites fluid and *in vitro* large scale production
- Characterization of Mabs: affinity and specificity
- Epitope mapping with competitive DELFIA®

MONOCLONAL ANTIBODY PRODUCTION

Lymphatic cells of the spleen (alternatively lymph nodes) from an immunized mouse were fused to mouse myeloma cell line with fusogen and harvested in hypoxanthine, aminopterin, thymidine (HAT) containing medium which selectively allows the growth of hybridomas (Fig.1.). Antibody production of hybrids was tested in a couple of weeks from cell culture supernatants.

IMMUNOMETRIC METHODS

In the design the following factors need to be kept in mind:
- antigen used in immunization (purity, size, structure, amount)
- antibodies available, commercially or in-house
- other reagents suitable for screening

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IFMAs ARE SENSITIVE AND VERSATILE

IFMAs can be designed in many different ways depending on the amount and quality of reagents available. Decisive factors are the quality and amount of antigen, labelled reagents and the use of solid phase for binding of antibody or antigen. This assay design allows use of an excess of reagents and generally IFMAs are more suitable than FIAs as primary screening assays. FIAs are more suitable for epitope screening.

Mabs against PSA were screened with a Sandwich-type IFMA (fig. 3A, 3B, see next page) using microtitration wells (Nunc polysorb) coated with rabbit anti-mouse Ig (Lövgren et al., 1984) or commercially available microtitration wells (C120-105). Details of the procedure are described on the summary protocol sheet.

Cells that do not produce antibodies grow faster than antibody producing cell lines. It is important to be able to clone the antibody-producing cells before the positive cell lines get killed by the faster growing cell lines (pH decrease inhibits cell growth). In this primary screening the sensitive IFMA type assays should be used.

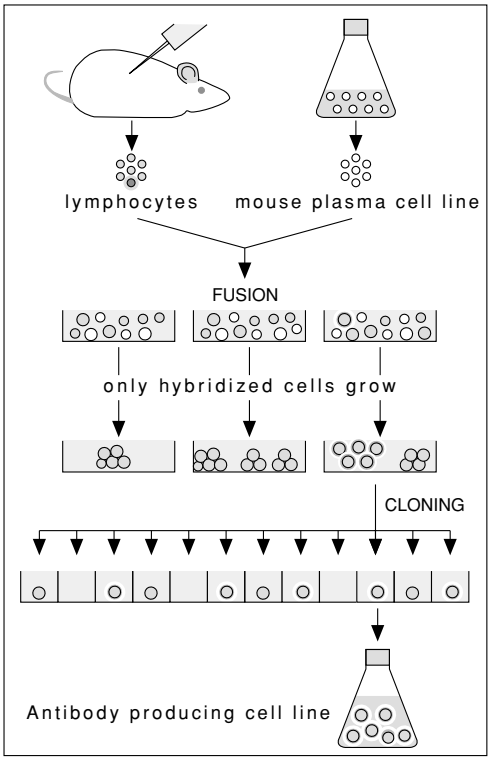


Fig. 1. Hybridoma development and production.

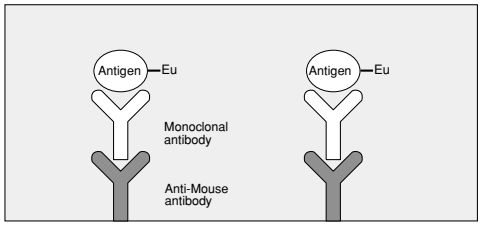
	1	2	3	4	5	6	7	8	9	10	11	12
A	4323	1429	579	487	736	6928	679	686	787	944	623	846
B	1239	951	209628	1559	1964	572	3598	597	3855	1246	1756	1513
C	473	566	646	515	616	605	602	1357	744	1755	737	846
D	599	815	1043	1441	1127	711	852	575	1179	613	806	3857
E	1182	5513	605	1271	8522	666	1163	4457	873	842	711	733
F	1229	99572	617	501	694	737	587	704	697	616	997	942
G	1427	1364	1183	633	609	580	860	1613	3475	828	1255	1572
H	875	499	1181	923	1060	959	626	663	1084	686	750	986

Fig. 2. In the very preliminary screening the intensity of fluorescence can be compared as counts.

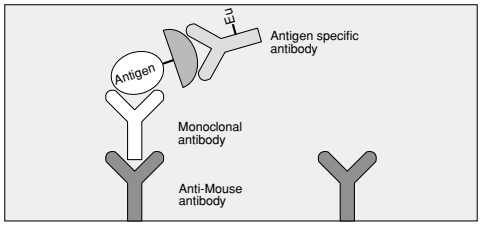
It is easy to locate samples producing antigen-specific antibodies from the primary screens even if no specific antibodies can be used for assay optimization. Cells in wells that produced antibodies are repeatedly diluted into 96 wells of cell culture plate to give single cell concentration (cloning). The master clones are recloned and retested after a couple of weeks (Fig.4). Some of them form cell lines continuously producing antibodies which can be frozen for storage (Fig.5.).

DESCRIPTION OF METHODS CONVENIENT IN PRELIMINARY SCREENING

POSSIBLE ASSAY DESIGNS WHEN ONLY LIMITED AMOUNT OF ANTIGEN IS AVAILABLE:

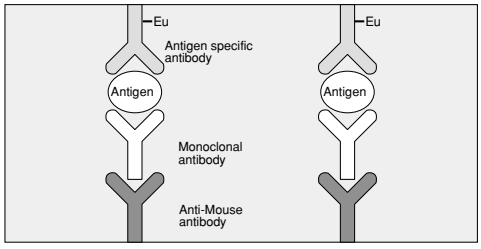


(3A) SCREENING OF ANTIBODIES USING ONLY 20-30 ng ANTIGEN PER WELL.
+ No need for specific antibodies
+ Allows use of precoated plates (C120-105)
– Labelling of antigen

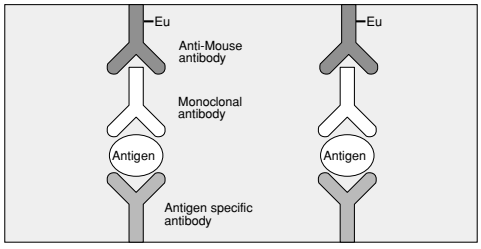


(3B) SELECTION OF ANTIBODIES THAT ONLY BIND TO COMPLEXED ANTIGEN.
+ Allows use of precoated plates (C120-105)
+ Intact antigen
– Labelled antibody against another part of the complex needed

SCREENING OF ANTIBODIES WHEN ANTIGEN SPECIFIC POLYCLONAL ANTIBODY IS AVAILABLE.

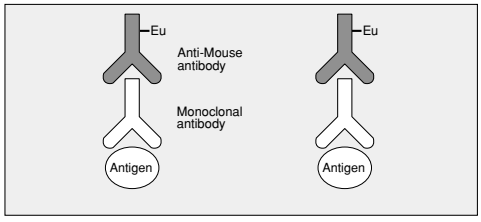


+ Intact antigen, only 20-30 ng/well
+ Allows use of precoated plates (C120-105)
+ Relatively small amount of specific antibody needed, 50-100 ng/well

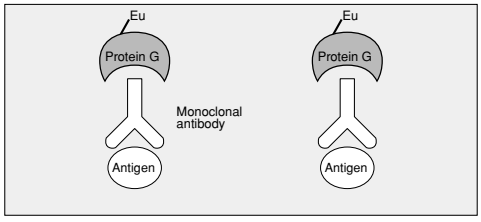


+ Intact antigen, only 20-30 ng/well
+ Fast and simple assay
- Specific coating needs to be optimized, coating requires 1 µg polyclonal antibody/ well

SIMPLE AND FAST ASSAYS CAN BE USED FOR COATING, E.G. CRUDE PREPARATIONS OF BACTERIA.

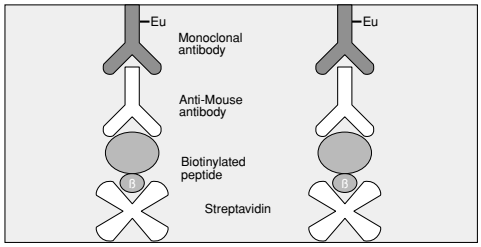


+ No need for specific antibodies
+ Allows use of Eu-labelled anti-mouse antibody (1244-130)
– Risk of denaturation of antigen
– Specific coating needs to be optimized, coating requires 0.5-1 µg antigen/ well

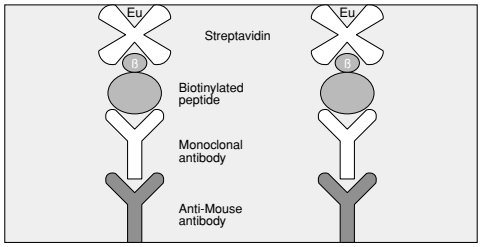


+ No need for specific antibodies
+ Allows use of Eu-labelled Protein G (1244-361)
– Risk of denaturation of antigen
– Specific coating needs to be optimized, coating requires 0.5-1 µg antigen/ well

SENSITIVE ASSAYS FOR SCREENING OF PEPTIDES WITH COMMERCIALLY AVAILABLE REAGENTS.



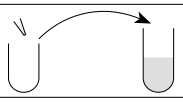
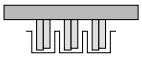
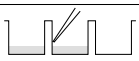
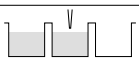
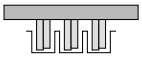
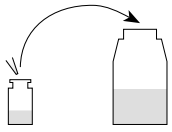
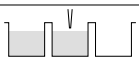
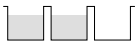
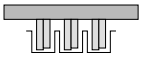
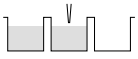
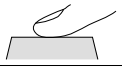
+ Only 20-30 ng/well of biotinylated antigen / well
+ Allows use of precoated plates (C122-105)
+ Allows use of Eu-labelled anti-mouse antibody (1244-130) or Protein G (1244-361)



+ Only 20-30 ng/well of biotinylated antigen / well
+ Allows use of precoated plates (C120-105)
+ Allows use of Eu-labelled streptavidin (1244-360)

DELFIA Mab Assay

Summary protocol sheet

Dilute standards.		In Assay Buffer (1244-106), 1.6 – 1000 ng/ml anti-PSA antibody
Wash.		Prewash the anti-mouse coated plates (C120-105)
Add Buffer.		50 µl of Assay Buffer (1244-106)
Add standards and supernatants.		50 µl
Incubate.		Slow shaking 5 min., 2 hours RT or overnight at + 4° C
Wash.		Four washes (B117-100)
Dilute tracer.		300 µg/ml in Assay Buffer
Add tracer.		100 µl
Incubate.		Slow shaking 60 min. RT
Wash.		Four washes (B117-100)
Enhance.		200 µl, slow shaking 5 min.
Count.		Create counting protocol.

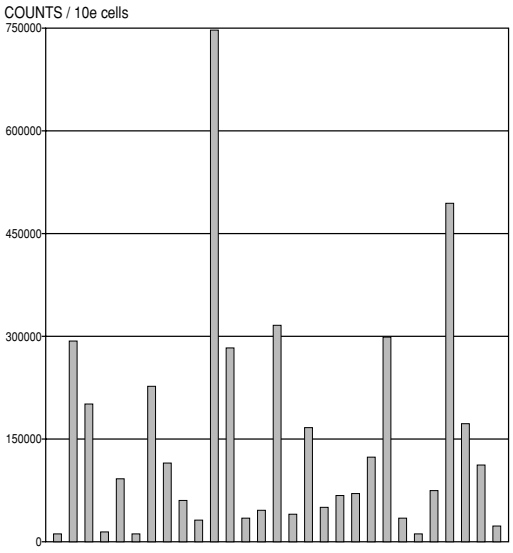


FIG.4. Intensity of fluorescence is more comparable when counts are given per 10^6 cells.

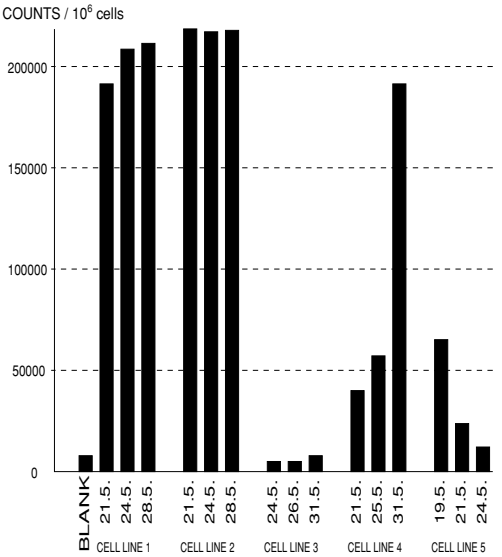
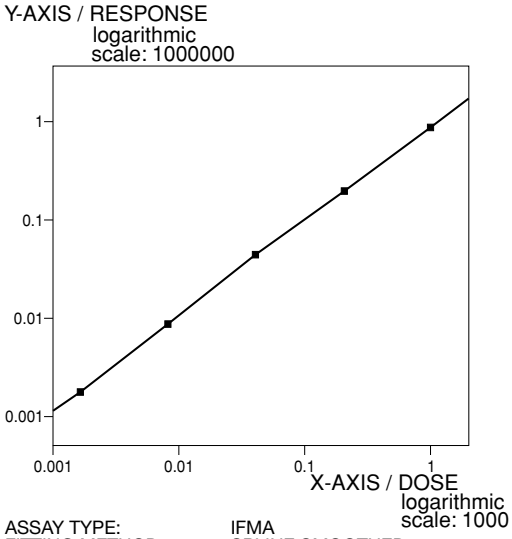


FIG.5. Stability of the production of five cell lines.



ASSAY TYPE:	IFMA
FITTING METHOD:	SPLINE SMOOTHED
X-AXIS:	LOGARITHMIC
Y-AXIS:	LOGARITHMIC
BLANKS	12
STANDARDS:	5
STANDARD REPLICATES:	12
STANDARD 1 CONC:	1.600
STANDARD 2 CONC:	8.000
STANDARD 3 CONC:	40.00
STANDARD 4 CONC:	200.0
STANDARD 5 CONC:	1000
NORMAL RANGE:	0.000 - 99999

Fig. 6. Standard curve of IFMA made using assay procedure 2A.

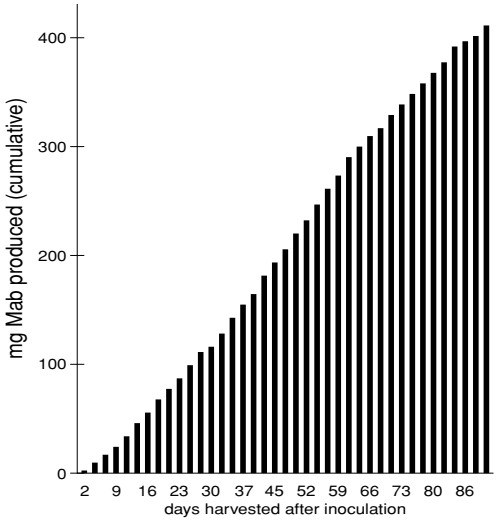


Fig. 7. Antibody production in a cell culture can be easily monitored using DELFIA® assays (design 2A). Multicalc™ software has been used to produce the standard curve.

GENTLE LABELLING OF PROTEINS

The labelling of small antigens is always risky because it changes the molecular structure which may cause decreased binding activity. However, this design has to be used when no antibodies are available against the antigen or the compound is so small that two epitopes are not available for binding. The Eu-labelling reagent is very hydrophilic which makes the labelling non-destructive to the antigen.

As an example of antigen labelling a protocol for labelling prostate specific antigen (PSA), molecular weight 33 kDa is given:

25 times molar excess of Eu-labelling reagent (product number 1244-301) was added to the PSA solution (1 mg/ml) in 0.15 mol/l NaCl and the pH was adjusted to 9.8 by adding one tenth of 0.5 mol/l sodium carbonate buffer, pH 9.8. The labelling was performed overnight at + 4°C. Unbound label was removed with Ultrogel AcA 54 column (1 x 50 cm) (IBF, France). The eluent was 50 mmol/l Tris-HCl buffer, pH 7.8 containing 0.15 mol/l NaCl. The PSA yield was calculated using the fluorescence intensity in the peak in relation to total fluorescence. The success of the labelling and the designed immunoassay for the screening can be tested using serum from the immunized animal.

When the specific antibodies are labelled with europium the guide lines given in the product inserts should be followed.

BENEFITS OF THE DELFIA® SYSTEM IN ANTIBODY SCREENING

- Simple assay designs
- Enzymes released from the dead cells do not increase the assay background even at 1:2 sample dilution or one-step assays.
- Denaturation of the antigen can be avoided by selecting a suitable DELFIA® assay design or by labelling with the non-destructive Eu-labelling reagent.
- Stabile Eu-labelled antigens and antibodies allow reliable comparison of different fusions and monitoring of antibody production.
- Use of multilabels, up to three analytes, considerably increases the overall test throughput.

PRODUCTS FOR ANTIBODY SCREENING

1244-301	Eu-labelling reagent, 1mg
1244-302	Eu-labelling kit, 0.2 mg
1244-303	Sm-labelling kit, 0.2 mg
1244-104 /105	Enhancement Solution 50 ml/250 ml
1244-106/111	DELFIA Assay Buffer, 50 ml/250 ml
B117-100	Wash concentrate, 8 x 250 ml
1244-130	Eu-labelled Anti-mouse antibody, 5 µg
1244-360	Eu-labelled Streptavidin, 0.25 mg
1244-361	Eu-labelled Protein G, 0.25 mg
1244-550	DELFIA microtitration plates, 8 x 12 wells, 20 plates
C120-105	Anti-mouse coated plates, 8 x 12 wells, 5 plates
C121-105	Anti-rabbit coated, 8 x 12 wells, 5 plates
C122-105	Streptavidin coated, 8 x 12 wells, 5 plates

INSTRUMENTATION

1420	VICTOR^2™ multilabel counter
1296-026	DELFIA Platewash
1296-003/004	DELFIA Plateshake
1296-014	Multipette
1296-016	Combitips, 5 ml, pack of 100
1296-017	Combitips, 2.5 ml, pack of 100

LABELLING SERVICE

Inquire for customized microtitration plate coatings and labelling of antibodies and antigens.

ACKNOWLEDGEMENT

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REFERENCES

Lilja, K., Christensson, A., Dahlén, U., Matikainen, M.-T., Nilsson, O., Pettersson, K., Lövgren, T. (1991) Prostate-specific antigen in serum occurs predominantly in complex with a1-antichymotrypsin. Clin. Chem. 37:1618-1625.

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 a1-antichymotrypsin as an indicator of prostate cancer. J. Urology 150:100-105.

Hemmilä I., Bakudu S., Mukkala V-M., Siitari H., Lövgren T. (1984) Europium as a label in Time-Resolved Immunofluorometric assays. Anal. Biochem. 137; 335-343.

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.

Pettersson, K., Söderholm, J. (1991) Individual differences in lutropin immunoreactivity revealed by monoclonal antibodies. Clin. Chem. 333-340.

Shinohara, K., Kakiuchi, T., Kobori, M., Suzuki, M., Fukushima, T. (1993) Europium-linked immunoassay of IgM production by human-human hybridomas cultured with or without chicken egg-yolk lipoprotein. Biosci.Biotech.Biochem. 57(6):1012-1013.

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