APPLICATION NOTE

DELFIA Technology for Immunogenicity Testing

Authors

Martin Boissonneault

Anja Rodenbrock

Chantal Illy

Gregory Cosentino

PerkinElmer, Inc.
Montréal, Québec, Canada

Qualification of a DELFIA Assay for the Detection of Anti-mouse IgG Antibodies in Human Serum

Introduction

Biotherapeutics, such as purified proteins and antibodies, are frequently used for the treatment of certain diseases. The main disadvantage of the use of these drugs is the immune response that they sometimes induce. The consequences of such immune reactions to a therapeutic protein range from transient appearance of antibodies without any clinical significance to severe life threatening conditions. Therefore, immunogenicity testing of therapeutic proteins is an essential step of the drug development process. There is a definite need for rapid and sensitive assay platforms for the detection of anti-drug antibodies (ADA), as current methods exhibit limitations related to assay sensitivity, robustness and drug tolerance.

We report here the development of a Dissociation Enhanced Lanthanide Fluorescence Immunoassay (DELFIA®) for the detection of ADA in serum samples. The use of DELFIA technology in immunogenicity testing was successfully demonstrated in a bridging format with a model antibody system. It is shown from the qualification study executed here that the DELFIA assay permits the sensitive detection of drug antibodies in the low ng/mL range, maintaining excellent performance in the presence of human serum and exhibiting good drug tolerance at µg/mL concentrations of free drug.

PerkinElmer®
For the Better

Materials and Methods

Materials

Mouse monoclonal IgG2b antibody (the drug) was purchased from AbD Serotec (Cat # MCA2472). The ADA positive control antibody (PC) used in these studies was a polyclonal goat anti-mIgG antibody obtained from Jackson ImmunoResearch (Cat # 115-005-062). The matrix consisted of pooled normal human serum (PNHS) and individual lots of normal human serum purchased from Bioreclamation (Cat # HMSRM). The following DELFIA reagents were obtained from PerkinElmer: Europium chelate N1 ITC (Cat # 1244-301), Streptavidin (SA)-coated 96-well plates (Cat # AAAND-0005), assay buffer (Cat # 1244-111), wash buffer (Cat # 1244-114), enhancement solution (Cat # 1244-104), and BSA stabilizer (Cat # CR84-100). Sephadex™ G50 columns were from GE Healthcare (Cat # 17-0042-02). Polypropylene 96-well plates were from Corning (Cat # 3365). The ChromaLink™ biotinylating reagent was obtained from SoluLink™ (Cat # B1001-105). Zeba desalting columns were from Pierce (Cat # 89883). The neutralization buffer was prepared by mixing the assay buffer with 1 M Tris-HCl pH 9.5 at a ratio of 60:40 (v/v).

Preparation of Europium Chelate Labeled Drug

Labeling of the drug molecule was performed using the Europium-N1 ITC reagent from PerkinElmer. A detailed labeling procedure is described in the Technical Data Sheet provided with the reagent. Briefly, the drug (1 mg at 1 mg/mL) and Europium chelate-N1 ITC (diluted to 15 mM) were mixed at a molar Europium chelate:drug ratio of 200:1. The reaction volume was completed to 1.7 mL with the reaction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% sodium azide) and the mix incubated for 16 hours at 4 ˚C. The Europium-labeled drug was then purified using a Sephadex G-50 column. The product was characterized by measuring the absorbance at 280 nm (for protein recovery) and the fluorescence at 615 nm against a Europium standard.

Drug Biotinylation

Drug biotinylation was performed with the ChromaLink™ biotinylating reagent using standard biotinylation and purification procedures. Briefly, 0.025 mg of antibody and 1.9 µL of biotinylating reagent (2 mg/mL) were mixed together at a 30:1 biotin/antibody ratio. The reaction volume was completed to 0.05 mL with PBS pH 7.4 and the reaction was incubated for 2 hours at 23 ˚C. Purification of the biotinylated antibody was performed using a Zeba 0.5 mL desalting column. The ratio of biotinylation of the final product and the protein recovery were determined from absorbance readings at 354 nm and 280 nm, respectively.

DELFIA ADA Assay

The DELFIA ADA assay was performed as follows:

In a polypropylene-96 microplate, 20 µL of samples were diluted 1/2 with acetic acid (600 mM) and then incubated for 60 minutes at room temperature. Then, 40 µL of neutralization buffer containing 3 µg/mL of biotinylated drug was added to the plate followed by 40 µL of Eu-chelate drug at 3 µg/mL diluted in assay buffer. The plate was then incubated for 60 minutes at room temperature. During this period, the 96-well SA-coated plate was incubated with 100 µL of assay buffer and finally washed twice with 200 µL of wash buffer. Then, 80 µL of the reaction mix was transferred from the polypropylene plate to the SA-coated plate for the capture step. The plate was then incubated for 60 minutes at room temperature. Finally, the plate was washed 7 times with 200 µL of wash buffer and 100 µL of the enhancement solution was added to each well. Following a 5 minute incubation time, the plate was read on an EnVision® Multilabel Plate Reader with excitation at 340 nm and time-delayed fluorescence readout at 615 nm.

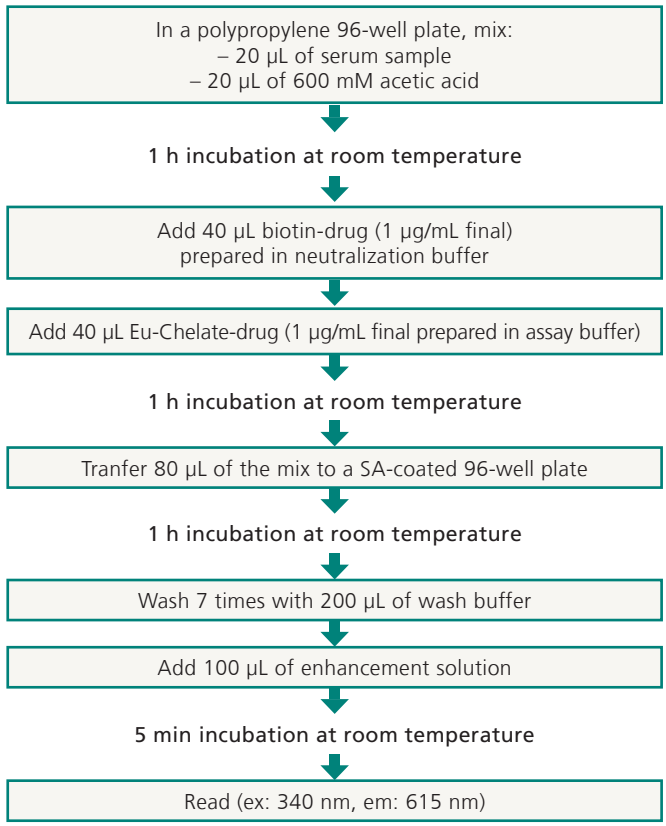

Figure 1. DELFIA assay protocol.

Assay Configuration

DELFIA technology exploits the unique fluorescence properties of lanthanide chelate labels. These labels have a long decay time as well as a broad Stokes shift, thus reducing background interference while providing high sensitivity and a wide dynamic range. The DELFIA ADA detection assay was developed using a bridging assay configuration, where the anti-drug antibodies to be detected act as a bridge between the biotin labeled drug and the Eu-chelate labeled drug. This complex is then immobilized on a SA-coated plate and unbound Eu-chelate drug removed by washing steps. The time-resolved fluorescence is then measured at 615 nm after addition of the enhancement solution. This method reflects very simple procedures, while delivering strong assay performance. Acid induced dissociation of the drug-ADA complexes was implemented during sample preparation in order to improve drug tolerance.

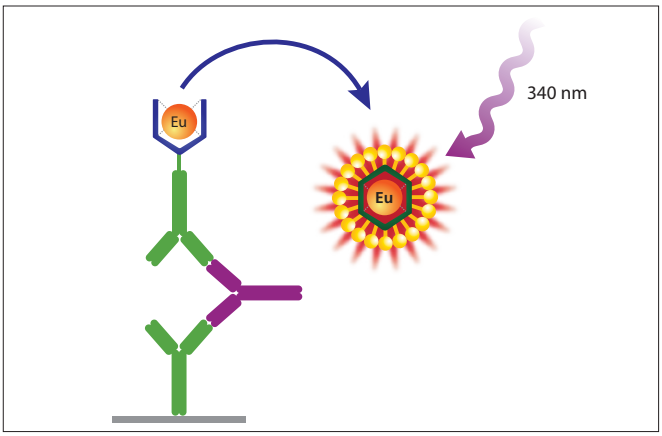

Figure 2. DELFIA bridging assay configuration.

Results

The DELFIA qualification study was performed using a standardized approach for qualification of immunogenicity assays. The analytical method was subjected to the following experimental assessments to ensure it was suitable for its intended use: determination of the screening cut-point (CP), determination of the confirmatory assay cut-point (CCP), intra- and inter-assay precision, sensitivity, specificity, selectivity, drug tolerance and prozone effect.

For this study, a model system was used in which the drug was mimicked by a monoclonal mouse IgG. The Positive Control (PC) used in this procedure was an affinity purified goat anti-mouse IgG antibody prepared at different concentration levels in neat pooled normal human serum (PNHS).

Screening Cut-point

The screening cut-point (CP) is defined as the level of signal at and above which the sample is considered positive and below which it is considered negative. The CP was statistically determined using 50 individual lots of normal human serum, each analyzed in duplicate on a total of four occasions by two analysts. On each occasion, twelve replicates of blank prepared with PNHS were included on each plate and a correction factor was calculated, corresponding to the ratio between the CP and the mean counts of PNHS. A fixed CP was used for this study and calculated based on the mean counts of the 50 individual lots of normal human serum plus 1.645xSD. This calculation represents the 95th percentile of a normal distribution and yields a 5 percent false positive rate. The normal distribution was assumed for the CP determination.

Table 1. Screening cut-point determination. The CP was determined using 50 individual lots of normal human serum, analyzed each in duplicate on a total of four occasions. It was calculated as the mean counts plus 1.645xSD.

Mean (counts)	501
n	50
SD	44
Cut-point (counts)	573
Mean Blank (counts)	538
Correction Factor	1.07

The CP was determined at 573 counts and the Correction Factor (CF) for the PNHS at 1.07. Several samples resulted in signals at or above the CP in only one or two out of the four experiments, indicating that the assay was showing some false positive rate. However, since no samples generated false positive signal on more than 50% of the occasions, no outliers were removed and all 50 samples were included in the CP calculation. The CF was used to calculate the plate specific cut-point (PSCP) in all subsequent experiments in the qualification using the following formula:

$$PSCP = mean\ PHNS\ counts \times 1.07$$

Confirmatory Cut-point

Confirmatory assays are designed to demonstrate the specificity of positive samples to the drug. We used a competitive inhibition test in which the sample was spiked with the drug and the % inhibition was calculated compared to the unspiked sample. A significant decrease in the level of counts is expected only if the sample contains specific ADA. The confirmatory cut-point (CCP) was determined using the same 50 lots as for the CP, except that each lot was spiked with 5 µg/mL of the drug. All samples were incubated for 1 hour at 23 °C before performing the analysis. This experiment was performed on 4 occasions by two analysts while calculating the CCP using the mean percentage of the inhibition plus 2.33xSD. This calculation was equivalent to the 99th percentile of a normal distribution and yielded a 1 percent false positive rate. The % signal inhibition was determined for each control and each serum lot, using the following formula:

% Signal Inhibition =
[1-(spiked sample/control unspiked sample)] x 100

Table 2. Confirmatory cut-point. The CCP was determined using 50 individual lots of normal human serum spiked with 5 µg/mL of the drug, analyzed each in duplicate on a total of four occasions. It was calculated as the mean percentage of inhibition plus 2.33xSD.

Mean (counts)	14.0
n	50
SD	8
Cut-Point (counts)	33

The mean CCP, calculated as the average of all CCP obtained from all occasions, was defined at 33%. Samples showing inhibition higher than the CCP were observed in less than 50% of the determination occasions, therefore no outliers were removed and all 50 samples were included in the calculation. The percentage of false positive signals (12 out of 4 x 50 lots tested; 6%) obtained during the determination of the screening CP and the CCP was in line with the recommended rate of 5% described by Mire-Sluis *et al.,* 2004.

To confirm the validity of the CCP value, two positive controls (LPC and HPC) and the PNHS were tested under the same conditions as for the CCP determination, i.e. spiked with 5 µg/mL free drug.

Acceptance Criteria

The PC spiked samples should generate a reduction (% signal inhibition) higher than or equal to the CCP whereas the blank samples (PNHS) should generate reduction (% signal inhibition) lower than the CCP.

Table 3. Controls for the CCP.

Samples	Mean counts Spiked	Unspiked	% inhibition	Status with regards to CCP
LPC	523	1149	54.4	≥ CCP
HPC	2952	16022	81.6	≥ CCP
PHNS	449	601	25.3	≥ CCP

Both positive control samples showed percentages of inhibition higher than the CCP confirming that these samples contain specific ADA, whereas the PNHS generated a reduction lower than the CCP. The acceptance criteria was therefore met for this parameter.

Assay Sensitivity and Prozone Effect

To investigate the possibility of assay deficiency at very high concentrations of the ADA, the positive control antibody was serially diluted (2-fold dilution) in PHNS to reach the PSCP level. The highest concentration of the positive control was ten-fold higher than the HPC. The lowest ADA concentration that produced a signal at or above the CP was indicative of the assay sensitivity.

Acceptance Criteria

The mean counts value obtained for each prozone dilution should be higher than or equal to the PSCP and should be greater than or equal to the overall mean relative counts value of the subsequent dilution.

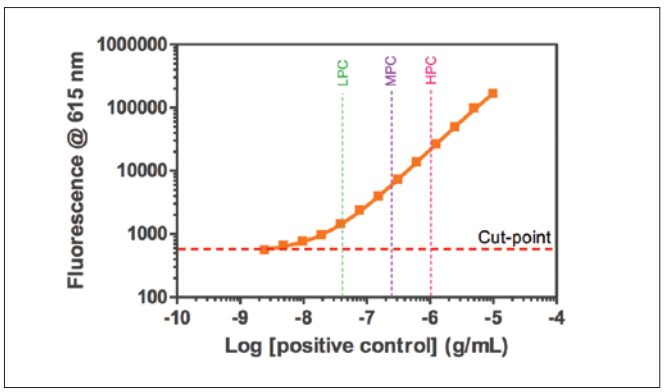

Figure 3. Prozone Effect and Assay Sensitivity. The PNHS was spiked with increasing concentrations of the PC antibody to generate the ADA standard curve. Three PC concentrations were selected for the study (LPC, low level PC: 40 ng/mL, MPC, medium level PC: 250 ng/mL and HPC, high level PC: 1000 ng/mL). For this experiment, the plate specific cut-point (PSCP) was calculated at 625 counts.

High signals and excellent sensitivity (4.9 ng/mL in neat serum) were obtained. The signal was still proportional to PC antibody at a level 10-fold higher than the HPC, indicative of a high assay dynamic range. The mean counts obtained for each prozone dilution was higher than the overall mean counts of the subsequent dilution, meeting the acceptance criteria for this parameter.

Drug Tolerance

Samples containing circulating free drug could exhibit assay interference, as a result of the competition between the free drug and the labeled drug used in the assay system for binding to ADA. The drug tolerance is defined as the lowest concentration of free drug that inhibits detection of the PC sample and brings the mean counts below the PSCP. In order to mimic biological samples, the PC antibody was diluted in PNHS and spiked with different amounts of drug (1, 5, 10, 20, 50, and 100 µg/mL). The samples were incubated 1 hour before analysis to allow for binding.

Acceptance Criteria

The drug tolerance was defined as the lowest concentration of drug that inhibits detection of the PC sample and brings the mean relative counts value below the PSCP.

Table 4. Drug tolerance. The PC antibody was diluted in PNHS and spiked with different amounts of drug (1, 5, 10, 20, 50, and 100 µg/mL). The samples were incubated 1 hour before analysis to allow for binding.

Samples	Final drug concentration in neat sample (µg/mL)	Mean counts	Status with regards to the PSCP
HPC (1000 ng/mL)	0	10972	–
	1	5360	≥
	5	2370	≥
	10	1400	≥
	20	715	≥
	50	471	≥
	100	386	<
LPC (40 ng/mL)	0	779	–
	1	556	≥
	5	414	≥
	10	368	<
	20	371	<
	50	348	<
	100	333	<

The HPC sample showed a signal above the PSCP even in the presence of 50 µg/mL of free drug, whereas the assay could tolerate up to 5 µg/mL of free drug at the LPC level.

This result demonstrated that the DELFIA ADA detection assay exhibited an excellent drug tolerance. It should be noted that the drug tolerance evaluated using the PC was highly dependent upon the affinity of the positive control.

Specificity and Selectivity

The specificity is defined as the ability of an assay to detect only the presence of a specific ADA. The specificity was determined using ten lots of normal human serum. These individual lots were analyzed unspiked and compared to the PSCP level.

Acceptance Criteria

For the specificity, at least 80% of the unspiked human serum lots should be below the PSCP.

Table 5. Assay specificity. Ten individual serum lots were analyzed unspiked and compared to the PSCP level.

Serum Lot	Mean counts	Status with regards to PSCP
PNHS	270	–
Serum 1	264	< PSCP
Serum 2	234	< PSCP
Serum 3	312	≥ PSCP
Serum 4	256	< PSCP
Serum 5	450	≥ PSCP
Serum 6	272	< PSCP
Serum 7	251	< PSCP
Serum 8	236	< PSCP
Serum 9	258	< PSCP
Serum 10	230	< PSCP

The acceptance criteria for the specificity was met, as 8 serum lots out of 10 generated a signal below the PSCP (289 counts for this experiment). This result indicates that the assay was not detecting unrelated components and presented good specificity.

The selectivity is defined as the ability of an assay to measure the ADA independently of any possible matrix effect. The selectivity was determined by calculating the recovery rate in ten individual lots of serum. Each lot was spiked with the LPC or HPC concentrations, respectively. In addition, PNHS was spiked with ADA at LPC and HPC levels to serve as reference for recovery determination. The mean counts were compared between individual serum lots and the reference samples and the % difference (recovery) was calculated using the following formula:

$$\%\ \text{difference (recovery)} = \frac{(\text{Mean RC of PC individual lot} - \text{Mean RC of PHNS}) \times 100}{\text{Mean RC of PC PNHS}}$$

Acceptance Criteria

For the selectivity, at least 80% of the individual lots of human serum spiked with the PC should be within ±25% difference of the corresponding PC prepared in PNHS.

Table 6. Assay selectivity. Ten individual serum lots were analyzed spiked with the LPC or HPC level and compared to the corresponding spiked PNHS level.

	LPC		HPC	
Serum Lot Number	**Mean counts**	**% Difference**	**Mean counts**	**% Difference**
PNHS	778	–	12098	–
Serum 1	637	-18.1	10898	-9.9
Serum 2	688	-11.6	12545	3.7
Serum 3	649	-16.6	11307	-6.5
Serum 4	656	-15.7	11123	-8.1
Serum 5	809	4.0	11429	-5.5
Serum 6	777	-0.1	11876	-1.8
Serum 7	582	-25.2	11069	-8.5
Serum 8	740	-4.9	12332	-1.9
Serum 9	717	-7.8	11531	-4.7
Serum 10	658	-15.4	12069	-0.2

We observed 19 samples out of 20 with count levels within a 25% difference of the corresponding PC prepared in PNHS, reflecting very good assay selectivity. Therefore, the acceptance criteria for selectivity was met.

Intra- and Inter-Assay Precision

Precision is a quantitative measure of the random variation between a series of measurements. To determine the reproducibility of the assay, twelve independent experiments using triplicate data points were performed by two analysts using a balanced design. The group mean, SD, and coefficient of variation (%CV) of the mean counts obtained for each assay were calculated and used to calculate the intra-assay precision. The group mean, SD, and %CV of all the intra-assay mean counts obtained over all occasions were calculated and used to determine the inter-assay precision. Inter- and intra-assay precision was evaluated by testing the three PC concentrations (LPC, MPC and HPC) and the PHNS.

Acceptance Criteria

For acceptable intra-assay precision, the %CV of the mean relative counts of intra-assay means (at all assay levels) should be less than or equal to 25%.

Table 7. Assay precision. Twelve independent experiments using triplicate data points were performed by two analysts using a balanced design to determine the intra- and inter-assay variability.

Samples	Mean counts	Mean intra-assay precision %CV	Inter-assay precision %CV (n=12)
PNHS	374	14.7	19.9
LPC	737	6.1	14.5
MPC	3104	12.5	22.3
HPC	11258	9.8	26.8

The intra-assay precision values for LPC, MPC, HPC and PHNS samples were all below 15%, indicating very good intra-assay reproducibility. The inter-assay %CV values were all within the acceptance criteria, except for the HPC level which was slightly higher; however, this value was still within an acceptable range and would typically meet the requirements for ADA screening assays. Overall, these results indicate that the DELFIA assay is robust and reproducible.

Conclusions

DELFIA is a well-established technology in drug development research. Providing high assay sensitivity and wide dynamic range of measurement, the method is characterized by the absence of sample interference during measurement. The flexibility of this technology allows its use for anti-drug antibody detection in a bridging format. A DELFIA immunogenicity assay follows a simple protocol with fewer wash steps than standard ELISA. The assay development is extremely easy with simple and inexpensive labeling procedures. Labeled molecules have high specific activity and good stability with the small label having minimal influence on its biological activity.

We report here the successful qualification of the DELFIA method for the detection of anti-drug antibodies in human serum using a model system and a standard qualification procedure. The results of this study prove the excellent performance of the assay, which offers significant advantages over current platforms for immunogenicity screening assays: 1) Simple assay protocol, 2) Superior sensitivity, 3) Excellent dynamic range, and 4) Good drug tolerance.

Therefore, this assay platform represents an important analytical tool offering distinct advantages for immunogenicity testing applications.

References

1. Mire-Sluis A.R., Barrett Y.C., Devanarayan V., Koren E., Liu H., Maia M., Parish T., Scott G., Shankar G., Shores E., Swanson S.J., Taniguchi G., Weirda D. and Zuckerman L.A. Recommendations for the Design and Optimization of Immunoassays Used in the Detection of Host Antibodies Against Biotechnology Products. 2004. Journal of Immunological Methods. 289: 1-16.

2. Shankar G., Devanarayan V., Amaravadi L., Barrett Y., Bowsher R., Finco-Kent D., Fiscella M., Gorovits B., Kirschner S., Moxness M., Parish Th., Quarmby V., Smith H., Smith W., Zuckerman L.A., Korren E. Recommendations for the Validation of Immunoassays Used for Detection of Host Antibodies Against Biotechnology Products. Journal of Pharmaceutical and Biomedical Analysis. Volume 48, issue 5, 15 Dec 2008 pp 1267-1281.

3. Koren E., Smith H.W., Shores E., Shankar G., Finco-Kent D., Rup B., Barrett Y-C., Devanarayan V., Gorovits B., Gupta S., Parish T., Quarmby V., Moxness M., Swanson S.J., Taniguchi G., Zuckerman L.A., Stebbins C.C., Mire-Sluis A. Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. Journal of Immunological Methods 333 (2008) pp 1-9.

4. FDA - Draft Guidance for Industry - Assay Development for Immunogenicity Testing of Therapeutic Proteins (Dec. 2009).

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

009765_01