

Development of Fluorescence-linked Immunosorbent Assay for High Throughput Screening of Interferon- γ

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ABSTRACT

Background: Human interferon-gamma (hIFN- γ) is produced by lymphocytes and has a variety of biological properties. Measurement of hIFN- γ is widely used for various immunological responses for allergic or autoimmune diseases. Enzyme-linked immunosorbent assay (ELISA) is an established immunoassay used to quantify cellular metabolites or cytokines. ELISA requires many incubation and wash steps and is not practically suitable for screening large numbers of samples.

Methods: We have developed a fluorescence-linked immunosorbent assay (FLISA) method for the detection of hIFN- γ . We measured the 50% inhibitory concentration (IC₅₀) value of the hIFN- γ production by interleukin (IL)-18 binding protein and anti-IL-18 monoclonal antibody. The IC₅₀ described by FLISA was compared with that by ELISA.

Results: We developed a new system for measuring hIFN- γ using Allophycocyanine (APC) fluorescent protein and compared it with the previous method using Cy5.5. The proposed FLISA had a smaller coefficient of variation than ELISA, and the means of coefficient of variation using the same samples measured by ELISA and FLISA were, respectively, 11.1% and 3.8%, suggesting that the edge effect often giving non-specific results may be smaller in FLISA than in ELISA.

Conclusions: The improved FLISA system proposed is ideally suited for efficient measurements of hIFN- γ . This homogeneous and multiplex method will be a powerful tool for high throughput screening for drug discovery research.

KEY WORDS

enzyme-linked immunosorbent assay, fluorescence-linked immunosorbent assay, fluorometric microvolume assay technology, high-throughput screening, interferon-gamma

INTRODUCTION

Several studies have shown that cytokines participate in the induction and effector phases of inflammatory responses in allergies. Studies of the cytokine network are important for identifying candidates for drugs or drug targets. In addition, measurement of cytokines is required for screening of drugs.¹ The regulation of human interferon-gamma (hIFN- γ) is particularly important for protective immunity in Th1 cells.² hIFN- γ is implicated with interleukin (IL)-12 in the adoption of a Th1 phenotype. On the other hand, Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13 af-

fect allergic responses.^{3,4} Many studies have shown that hIFN- γ secretion associated with IL-12 or 18 plays an important role in the Th1 and Th2 balance.^{5,6} Hence, measurement of hIFN- γ has been widely used for monitoring immune responses in allergic diseases such as atopic dermatitis.^{7,8}

Enzyme-linked immunosorbent assay (ELISA) is an established immunoassay used to quantify various cytokines. Conventional ELISA, which is not readily amenable for screening large numbers of samples in drug screenings, requires many processes and large quantities of antibody. For drug discovery and development, high throughput screening (HTS) would

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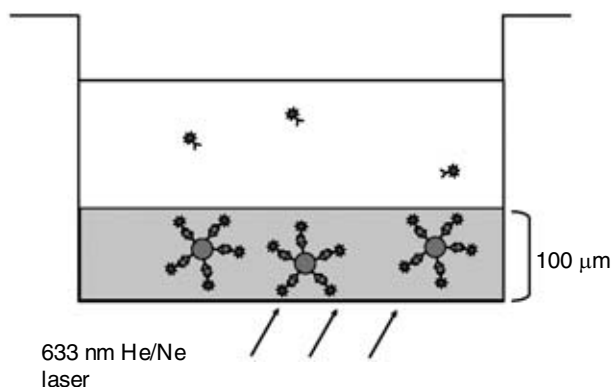


Fig. 1 Homogeneous assay is the main characteristic of FLISA. An FMAT® 8100 HTS has a macro confocal imaging system with a helium/neon laser, which automatically focuses on and scans fluorescent-bound beads resting on the bottoms of multiwell trays. Because the depth of focus, 100 μm , is small in comparison with the remaining volume, background fluorescence is minimal in relation to bead-bound fluorescence; thus, there are no washing steps to remove unbound fluorophores.

benefit from an immunoassay that requires minimal manipulation and uses just a small amount of the compounds required. Fluorescence-linked immunosorbent assay (FLISA) is particularly well suited for HTS.⁹

We have developed a FLISA method for the measurement of hIFN- γ . We constructed the assembly of capture antibody and fluorescent conjugated detection antibody for our FLISA system. This system enables high throughput measurement of hIFN- γ and could be applied to the assessment of other cytokines or chemical mediators in allergic responses.

METHODS

INSTRUMENTATION

A fluorescent microvolume assay technology (FMAT®) instrument consists of a detector (Applied Biosystems, Tokyo, Japan) integrated with a bar code reader and robotic plate handler (Zymark Corp, MA, USA) that can accommodate 60 plates. An FMAT® 8100 HTS has a macro confocal imaging system with a helium/neon (He/Ne) laser (633 nm) which automatically focuses on and scans fluorescent-bound beads resting on the bottoms of multiwell trays. The laser performs 256 scans across an area 1 mm $\times$ 1 mm $\times$ 100 μm deep (Fig. 1). Because the depth of focus, 100 μm , is small in comparison with the remaining volume, background fluorescence is minimal in relation to bead-bound fluorescence; thus, there are no washing steps to remove unbound fluorophores. The emitted fluorescence passes through the same optical path as the excitation beam and through a dichroic beam splitter for detection by photomultiplier

tubes through the filters (680–730 nm).

PREPARATION OF ANTIBODY COATED BEADS

200 μl of streptavidin beads (0.5% w/v; 6 μm bead diameter; SVP-60-55, Spherotech, were diluted in 800 μl of phosphate-buffered saline (PBS) + 0.01% sodium azide (NaN₃). After centrifugation at 10,000 g for 2 minutes, the supernatant was discarded and resuspended in 1 ml of PBS + 0.01% NaN₃. Next, a 4 μg portion of biotinylated mouse anti-hIFN- γ monoclonal antibody (mAb) (AHC4539, Biosource, California, USA) was added to the streptavidin beads solution and incubated with gentle mixing for 18 hours at room temperature. Washing was repeated 2 times and the capture antibody coated beads were stocked in PBS + 0.01% NaN₃ (8.4×10^6 beads/ml) at 4°C.

PREPARATION OF RECOMBINANT hIL-18 PROTEIN

We used recombinant wild-type hIL-18 for the inhibition assay. Expression and purification were carried out as described previously with minor modifications.¹⁰⁻¹² The concentration of purified hIL-18 protein was estimated using the absorbance constant (6160) for hIL-18.

PREPARATION OF hIFN- γ BIOLOGICAL SAMPLE

hIL-18 inhibition assay based on hIFN- γ induction was carried out as previously described.^{13,14} Briefly, human myelomonocytic KG-1 cells (ATCC CCL246) were grown in a culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/L), penicillin (100 U/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$). hIL-18 prepared at our laboratories (in a final concentration of 2 ng/ml) was preincubated with anti-hIL-18 mAb (Medical & Biological Laboratories, Nagoya, Japan) or hIL-18 binding protein Fc chimera (IL-18BP) (Research and Development Systems Inc, Minneapolis, USA) (in final concentrations between 0 and 500 ng/ml) at 37°C for 1 hour and then 100 μl of the mixture added to 100 μl of KG-1 cells (3×10^6 cells/ml) per well in a 96-well plate (Nunc, Roskilde, Denmark), followed by incubation at 37°C for 24 hours in 5% CO₂. The culture supernatants were collected, and the hIFN- γ production in each of the samples was determined by ELISA (Japan Immunoresearch Laboratories, Co. Ltd., Takasaki, Japan) and FLISA. Data are presented as the percent inhibition of total hIFN- γ production in the absence of hIL-18 antagonists. IC₅₀, the concentration of antagonists required to inhibit 50% of hIFN- γ production by KG-1 cells stimulated with hIL-18, was then calculated.

FLUOROMETRIC MICROVOLUME IMMUNOASSAY FOR hIFN- γ

A 50 μl aliquot of sample or hIFN- γ standard (Research and Development Systems Inc) was placed

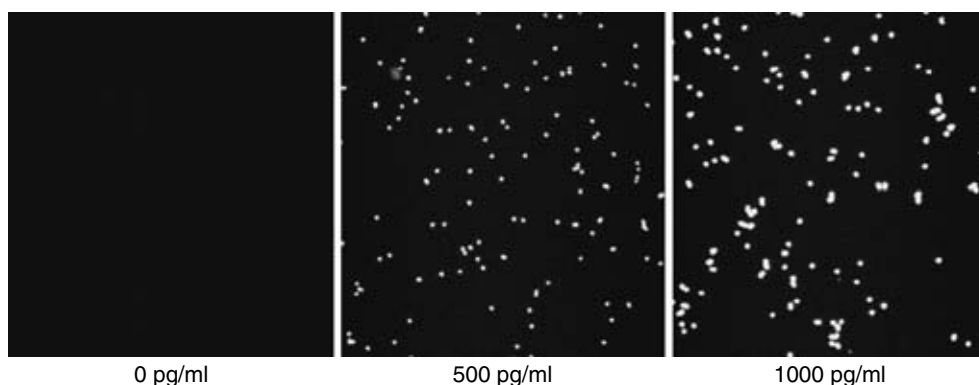


Fig. 2 Detection of fluorescence-linked beads in the wells with various concentrations of hIFN- γ . The intensity of the bead-bound fluorescence increased according to the hIFN- γ concentration.

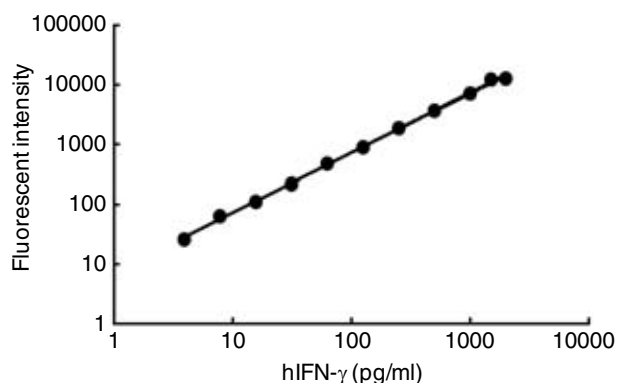


Fig. 3 The standard curve for hIFN- γ obtained with FLISA. The linear dynamic range for hIFN- γ was 3.9–1,500 ng/ml. Each data point represents the average of triplicate assays.

into a 96-well plate. 50 μ l of beads mixture (antibody beads:allophycocyanin (APC) conjugated mouse anti-hIFN- γ mAb (554702, Pharmingen, California, USA):FLISA buffer (PBS, 1% BSA, 0.35 M NaCl, 0.1% Tween-20, 0.01% NaN₃) = 100:6.4:6500) was then mixed with the sample or standard in each well and incubated for 4 hours overnight at room temperature in the dark. After incubation, the 96-well plate was scanned using the FMAT[®] scanner. The average fluorescence per bead was recorded.

RESULTS

FMAT[®] 8100 HTS includes a bead-based assay system and is designed to negate the unbound fluorescent dye-labeled antibody. At measurement, FMAT[®] scans for fluorescent-bound beads resting on the bottom of multiwell trays (Fig. 1). No beads are found in the upper portions of the wells and non-specific fluorescence is not involved in background fluorescence intensity. In the lower 100 μ m of the wells, the signals of unbound fluorescent dye-labeled antibody are deleted due to the limited size and form of the beads. Actually, the fluorescence intensity of negative con-

trol wells is 31.6 ± 2.1 and that of the wells, which have 3.9 pg/ml of the minimum IFN- γ is 77.2 ± 4.9 . This is an obvious difference between negative control wells and minimum IFN- γ wells.

A standard solution of hIFN- γ was two-fold serially diluted in RPMI-1640 + 10% FCS. The intensity of the bead-bound fluorescence increased according to hIFN- γ concentration (Fig. 2). A standard curve for hIFN- γ was generated using the FMAT[®] scanner (Fig. 3). The linear dynamic range of hIFN- γ by FLISA was 3.9–1500 pg/ml. These results indicate that our system can recognize minute changes of hIFN- γ values. With previously established methods for IL-6 and IL-8, the linear dynamic ranges were 15.6–1000 pg/ml and 15.6–2000 pg/ml, respectively. Our FLISA system has an equivalent dynamic range comparable to these results,⁹ a range that is sufficient to be applied in clinical use.

To test whether FLISA could accurately quantify hIFN- γ in a biological sample, inhibition assays of hIL-18 were performed.^{15,16} We measured about 150 samples using FLISA and about 90 samples using ELISA. Sample preparation time of FLISA was equal to that of ELISA. hIFN- γ produced by KG-1 cells was measured using ELISA and FLISA (Figs. 4A, 4B). hIL-18 was neutralized by anti-hIL-18mAb or IL-18BP before stimulation. The IC₅₀s for anti-hIL-18mAb and hIL-18BP using FLISA were 19.7 ng/ml and 5.2 ng/ml, respectively (Fig. 4B). Similarly, those using ELISA were estimated as 27 ng/ml and 5.4 ng/ml, respectively (Fig. 4A). However, as shown in Figure 4, the results of ELISA did not show a regression curve suitable for estimating precise IC₅₀ values. The means of the coefficient of variation using the same samples measured by ELISA and FLISA were respectively 11.1% and 3.8%, suggesting that the edge effect often giving non-specific results may be smaller in FLISA than in ELISA.

Our proposed FLISA method was modified to use a combination of capture antibody and fluorescent dye-labeled detection antibody. As shown in Figure 5,

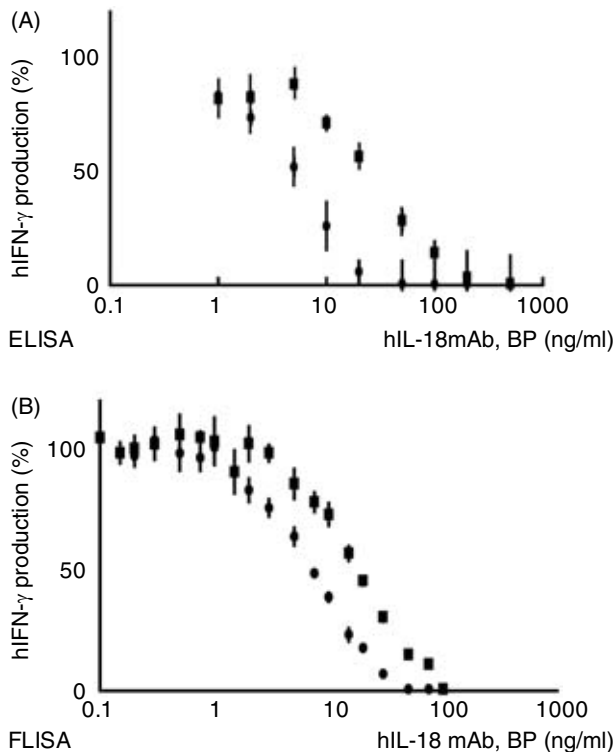


Fig. 4 Inhibition assay of hIL-18 by hIL-18BP or anti-hIL-18 mAb. hIL-18 (final concentration 2 ng/ml) was incubated with two-fold dilutions of anti-hIL-18 mAb or hIL-18BP for 1 hour at 37°C. After this incubation, the mixtures were added to KG-1 cells, and after 24 hours hIFN-γ was measured. The stimulation control of hIL-18 (2 ng/ml) was set at 100%, and the percent change was calculated for each concentration of anti-hIL-18mAb or hIL-18BP. Each data point represents an average of triplicate assays. (A) The inhibition curve measured by ELISA. (B) The inhibition curve measured by FLISA. The solid circle shows the inhibition of hIL-18 by hIL-18BP. The solid square shows the inhibition of hIL-18 by anti-hIL-18 mAb.

streptavidin beads and a biotinylated mouse anti-hIFN-γ mAb were used as IFN-γ capture particles and APC conjugated mouse anti-hIFN-γ mAb was used as the IFN-γ detection reagent. Previously, goat anti-mouse IgG (Fc) beads and mouse anti-hIFN-γ mAb have been used as the IFN-γ capture particles and Cy 5.5 conjugated mouse anti-hIFN-γ mAb as the IFN-γ detection reagent. Figure 6 shows our standard curve for IFN-γ estimated IFN-γ values in detail compared with that from the previously proposed FLISA method.

DISCUSSION

When measuring various cytokines, the effect of the environmental temperature is one of the factors influencing inter-experimental accuracy. ELISA requires washing and application of a detection antibody fol-

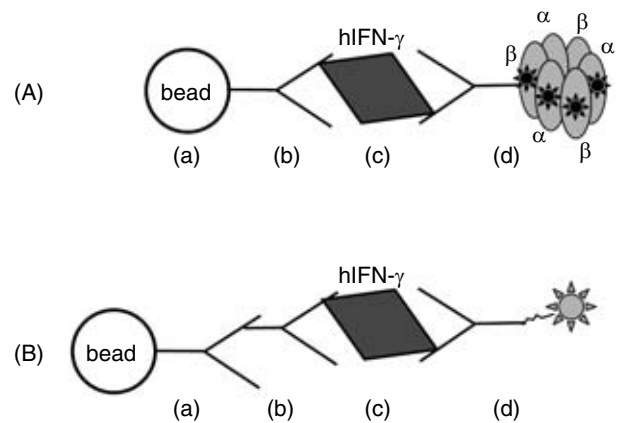


Fig. 5 Schematic diagram of our FLISA method (A) and a previous method (B). (A): (a) streptavidin beads. (b) biotinylated mouse monoclonal anti-hIFN-γ mAb. (c) hIFN-γ sample. (d) APC conjugated mouse monoclonal anti-hIFN-γ mAb beads. (B): (a) goat anti-mouse IgG(Fc) beads. (b) mouse anti-hIFN-γ mAb. (c) hIFN-γ sample. (d) Cy5.5 conjugated mouse anti-hIFN-γ mAb.

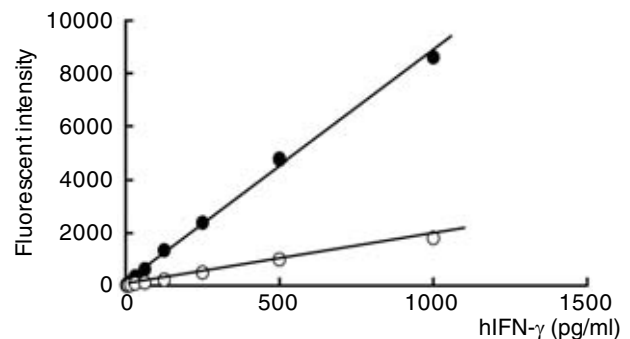


Fig. 6 The standard curve for hIFN-γ obtained with our newly proposed FLISA method (solid circles) and the previously reported FLISA method (open circles). The sensitivity of our method was about four-fold higher than that of the previously reported method.

lowing the first incubation, and washing and application of a detecting reagent following the second incubation. On the other hand, with FLISA, measurements can be performed following the first incubation. In all ELISA steps it is therefore very easy to expose the samples to environmental temperatures. The edge effect, which is the difference in temperature between the outer and inner wells, is therefore one of the factors resulting in inter-experimental errors.^{17,18} Accurate screenings are very much required in working toward drug discoveries.^{19,20}

We used two kinds of mouse hIFN-γ mAb in our FLISA method; one a capture antibody, the other an APC conjugated antibody as a detection antibody. Measurements of hIFN-γ in a previous report used a

goat anti-mouse IgG antibody as the bead-coating antibody and two kinds of mouse anti-hIFN- γ mAb, a capture antibody and a Cy5.5 conjugated antibody (Fig. 5).²¹ The sensitivity of our proposed FLISA method was about four-fold higher than that of previous methods (Fig. 6). That is, the minimum unit for measurement in our method is four times smaller; 0.5 pg/ml/fluorescence (FL) in the previous method by Komatsu *et al.*, 0.125 pg/ml/FL in our method. Therefore, we were able to obtain detailed IFN- γ values. The sensitivity of the FLISA method proposed in this paper allowed the discrimination between differences.

The sensitivity of each method may depend on the structural difference between APC and Cy5.5. One APC molecule bounds to one antibody. The APC is a trimer that consists of α and β subunits ($\alpha\beta$)₃. Each subunit has one fluorophore; so as a result an APC conjugated antibody contains six fluorophores (Fig. 5A).²² However, one Cy5.5 binds to one antibody, and the Cy5.5 is a single fluorophore itself. Thus an antibody conjugated Cy5.5 has one fluorophore (Fig. 5B). Although the fluorescent dye/antibody ratio of APC is equal to that of Cy5.5, an APC conjugated antibody has six times as many fluorophores as a Cy5.5 conjugated antibody. The difference in fluorescence intensity may also be a factor increasing sensitivity.

To quantify hIFN- γ in large amounts of sample, we developed a more efficient assay method than conventional ELISA, which can involve many incubation and washing steps, and requires large amounts of antibodies.⁹ In contrast, our FLISA method is a homogeneous bead-based immunoassay that requires no wash steps. Over 100-fold less capture antibody is needed in FLISA than in conventional ELISA.⁷ However, the most striking difference is the time required for the assay. In our study, the occupation time of the FLISA method was about 1 hour, demonstrating that FLISA can be performed in a much shorter time than conventional ELISA, which requires about 5 hours.²³ A minimal time requirement is a crucial factor for applications such as HTS in drug discovery research. FLISA is thus an attractive method as it involves less hands-on time and lower running costs.²¹

In immunological studies using FLISA, measurements of IL-6 and IL-8 and CD3+/CD4+ lymphocyte counts in whole blood have been reported.^{9,24} The bead-based system of the FLISA is readily applicable to any other plate-based assay, such as non-radioactive kinase, phosphatase, and protease assays. A multiplexed bead-based receptor-ligand binding assay has already been demonstrated using FLISA.^{25,26} In addition, FLISA is capable of detecting and quantifying fluorescence on live cells, allowing for such diverse assays as apoptosis and cytotoxicity, and cellular immunoassays, and receptor ligand binding assays.²⁵⁻²⁸ The FLISA system can be adapted for HTS

of large libraries of chemical and natural products, thus having a place in any laboratory that routinely performs multiple, repetitive assays.

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