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High-throughput miniaturized immunoassay for human interleukin-13 secreted from NK3.3 cells using homogenous time-resolved fluorescence

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Abstract

A miniaturized immunoassay for human interleukin-13 (IL-13) using homogeneous time-resolved fluorescence (HTRF®) has been developed. In this assay, IL-13 which was secreted from NK3.3 cells stimulated with interleukin-2 (IL-2) was detected by measuring the time-resolved fluorescence after adding a mixture of three reagents, biotinylated anti-IL-13 monoclonal antibody, europium cryptate (fluorescence donor)-labeled different anti-IL-13 monoclonal antibody and crosslinked allophycocyanin (fluorescence acceptor)-conjugated with streptavidin in a 384-well assay plate. The detection limit of IL-13 using this immunoassay was estimated to be less than 600 pg/ml and IL-13 levels measured by this method were very close to those measured by enzyme linked immunosorbent assay (ELISA; the correlation coefficient was 0.9535). The proposed assay requires only a fourth of the quantities of all reagents compared with the assay using a conventional 96-well microtiter plate. Furthermore, there is no need to transfer the culture supernatant to another assay plate and wash the plate. Therefore, this miniaturized immunoassay is economical and efficient and is particularly suitable for high-throughput drug screening.

Keywords: Rare earth cryptates; Homogeneous time-resolved fluorescence; Immunoassay; High-throughput screening; Interleukin-2; Interleukin-13; Natural killer cell

1. Introduction

High-throughput screening (HTS) with robotic systems is widely used for discovering drug candi-

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dates against novel biological targets. Although it enables the testing of more than hundred thousands of samples per day, it requires a high cost of reagents and much waste disposal. To solve these problems, miniaturizing HTS is highly desired. Recently, 384 and 1536-well assay plates have become available to reduce the amount of reagents needed and also to enable testing of more samples per plate than the usual 96-well assay plates. However, these plates are not usually used for immunoassays, which are very popular for measuring cytokines and many other biomolecules.

In the past, we have developed an immunoassay for human interferon-γ using homogeneous time-resolved fluorescence (HTRF®) and showed it to be very suitable for HTS [1]. It requires only four steps of reagent addition and two steps of incubation in contrast with the more than 20 steps of reagent addition, plate washing and incubation needed for conventional enzyme linked immunosorbent assay (ELISA) with colorimetric detection. However, that HTRF used 96-well assay plates. We have now developed a miniaturized HTRF immunoassay for human interleukin-13 (IL-13) using a 384-well assay plate and here compare its performance with conventional ELISA.

HTRF® [2,3] uses two fluorescence labels, europium cryptate (fluorescence donor, EuK) and crosslinked allophycocyanin (fluorescence acceptor, XL665). When both fluorescence molecules are in the proximity, the energy of EuK excited by nitrogen laser is transferred non-irradiatively to XL665 resulting in long-lived emission at 665 nm. Since the non-specific fluorescence from unbound XL665 and from some other components in media or plastic have short decay times, only the specific signal from the acceptor fluorophore in the immune complex can be detected by measurement of time-resolved fluorescence. In order to correct for media absorbance by colored samples and compensate for fluctuations from the excitation source, the ratio of fluorescence intensity at 665 nm to that at 620 nm, which is the EuK signal used as an internal standard, can be taken as an HTRF value. HTRF can be used for immunoassay, protein–protein binding assay, DNA hybridization, protease assay and tyrosine kinase assay. This homogeneous method is very useful for HTS as described above [4–7], and can be performed on a miniaturized scale because of its liquid-phase reaction without relation to the surface area of the assay plate and its sensitive detection of fluorescence.

IL-13, which is secreted from activated $CD4^+$ and $CD8^+$ T lymphocytes [8] and natural killer (NK) cells [9], has multiple biological activities including the up-regulation of CD23 antigen (FcεRII) and class II MHC molecules expression on monocytes and B cells and the production of IgG_4 and IgE from B cells [10,11]. IL-13 has been found to be a key factor in allergic asthma and infectious diseases. In mice, it has been reported that therapeutic administration of an IL-13 inhibitor could prevent allergic asthma [12,13]. Determination of cytokine such as IL-13 in tissue culture medium is often carried out by ELISA, which has many drawbacks for drug screening as described above. Our HTRF immunoassay for human IL-13 to screen IL-13 production inhibitors and to discover anti-allergic drugs can be miniaturized using a 384-well assay plate, optimized for cell stimulation and detection entirely within one assay plate, and consequently makes possible high throughput and low cost.

2. Materials and methods

2.1. Reagents

Recombinant human IL-2 and human IL-13 were obtained from Shionogi (Osaka, Japan) and R&D Systems (Minneapolis, MN, USA), respectively. Anti-IL-13 monoclonal antibody, # 32116.11, JES10-5A2 and B-P6, were purchased from R&D Systems, Biosource (Camarillo, CA, USA) and Bender Med Systems (Vienna, Austria), respectively. *N*-Succinimidyl-3-(2-pyridylthio) propionate (SPDP), sulfosuccinimidyl 4-[*N*-maleimidometyl]-cyclohexane-1-carboxylate (sulfo-SMCC) and sulfosuccinimidyl-6-(biotinamido) hexanoate (EZ-Link™ sulfo-NHS-LC-biotin) were acquired from Pierce (Rockford, IL, USA). Europium cryptate, XL665 and XL665-la-

beled streptavidin were provided by CIS bio international (Marcoule, France). All chemicals were of analytical grade, unless otherwise specified.

2.2. Cell culture

NK3.3 cells were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated human pooled AB serum (ICN Biomedicals, Inc., Aurora, OH, USA), 10% heat-inactivated fetal bovine serum (FBS) (Hyclone®, Logan, UT, USA), 10% Lymphocult-T (Biotest Diagnostics Corporation, Denville, NJ, USA), 50 u/ml penicillin G and 50 μg/ml streptomycin in a humidified incubator with 7% CO_2 at 37 °C.

2.3. Preparation of EuK-labeled anti-IL-13

To 100 μg of anti-IL-13 monoclonal antibody in 0.5 ml of phosphate buffer (pH 7.4; 0.1 M) was added 1.7 μg of SPDP in 5 μl of ethanol (the molar ratio of SPDP to IgG was 8). After incubation at room temperature for 20 min, 25 μl of 400 mM dithiothreitol in distilled water was added to the reaction mixture. After further incubation for 10 min, the SPDP-activated antibody was separated in phosphate buffer (pH 7.4; 0.1 M) from the reaction mixture by gel filtration using PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) and was concentrated to about 0.5 ml with a Centricon® centrifugal filter device, YM-30 (30 000 MW cut-off, Millipore Corporation, Bedford, MA, USA). To 100 μg of EuK in 0.2 ml of phosphate buffer (pH 7.4; 0.1 M)–dimethylformamide (9:1, v/v) was added 60 μg of sulfo-SMCC in the same buffer (the molar ratio of sulfo-SMCC to EuK was 2). After the incubation at room temperature for 30 min, the SMCC-activated EuK was purified in the same buffer from the reaction mixture by anion exchange chromatography using HiTrap®Q column (Amersham Pharmacia Biotech). Twenty-fold excess molar of the SMCC-activated EuK was added to the activated antibody above (SPDP activated antibody). After incubation at 4 °C for 16 h, EuK-labeled antibody was purified in PBS containing 0.05% NaN_3 by PD-10 column. The number of EuK introduced to one molecule of antibody was determined using the molecular extinction coefficient of EuK at 305 nm (30 000 M^{-1} cm^{-1}) and that of antibody at 280 nm (210 000 M^{-1} cm^{-1}). BSA (Fraction V, Difco, Detroit, MI) and Tween®20 (Nacalai tesque, Tokyo, Japan) were added to EuK-labeled antibodies to bring the concentrations to 0.1 and 0.1%, respectively, and EuK-labeled antibody was stored at −80 °C.

2.4. Preparation of XL-665-labeled anti-IL-13

To 0.98 mg of XL665 in 0.53 ml of phosphate buffer (pH 7.4; 0.1 M) was added 21 μg of sulfo-SMCC in 21 μl of phosphate buffer (pH 7.4; 0.1 M). After the incubation at room temperature for 30 min, the SMCC-activated XL665 was purified in the same buffer using PD-10 column and was concentrated to about 0.5 ml with Centricon® centrifugal filter device, YM-30. To the SPDP-activated antibody described in the previous section, was added 5-fold molar excess of the SMCC-activated XL665. After the incubation at 4 °C for 16 h, XL665-labeled antibody was purified from the reaction mixture by gel filtration using TSK-GEL® G-4000SW_{XL} (21.5 mm × 30 cm, Tosoh, Tokyo, Japan) column and PBS containing 0.05% NaN_3 as elution buffer. The number of XL665 introduced to one molecule of antibody was determined using the molecular extinction coefficient of XL665 at 650 nm (731 120 M^{-1} cm^{-1}) and that of antibody at 280 nm. XL665-labeled antibody was stored at −80 °C in the same manner as EuK-labeled antibody.

2.5. Preparation of biotinylated anti-IL-13

To 100 μg of anti-IL-13 monoclonal antibody in 0.5 ml of phosphate buffer (pH 7.4; 0.1 M), was added 3.7 μg of EZ-Link™ sulfo-NHS-LC-biotin in 10 μl of phosphate buffer (pH 7.4; 0.1 M) (molar ratio of NHS-LC-Biotin to IgG was 10). After incubation at 4 °C for 16 h, the biotinylated antibody was separated using PD-10 column. The biotinylated antibody was stored at −80 °C in the same manner as EuK-labeled antibody.

2.6. Miniaturized immunoassay for IL-13 using HTRF

NK3.3 cells suspended in the Lymphocult-T free culture medium (6.5×10^5 cells per ml, 20 μl) were added to each well of a 384-well assay plate (black color and flat bottom; Corning Incorporated, Acton, MA, USA). A 5-μl portion of 0.15–10 ng/ml IL-2 in the Lymphocult-T free culture medium was then added to each well and the plate was incubated at 37 °C in 7% CO_2 for 6 h. Next, 25 μl of HTRF reagent mixture of 160 ng/ml EuK-labeled antibody (B-P6), 1000 ng/ml biotinylated antibody (# 32116.11) and 2500 ng/ml XL665-labeled streptavidin in an HTRF buffer (PBS containing 0.8 M potassium fluoride and 0.5% BSA) was added to the well containing NK3.3 cells stimulated with IL-2 or 25 μl of IL-13 standard solution (0.13–20 ng/ml) in Lymphocult-T free culture medium. After the incubation for 24 h at room temperature, the HTRF value ([ratio of fluorescence at 665 nm to that at 620 nm] × 10 000) in each well was measured using a Victor^{2}™ multilabel counter (Wallac Oy and Perkin Elmer, Inc., Turku, Finland). When the culture supernatant was transferred, 40 μl of NK3.3 cells (3.3×10^5 cells per ml) and 10 μl of 0.15–10 ng/ml IL-2 were added to each well of a 384-well assay plate and 25 μl of the culture supernatant of each well was transferred to another well. Next, 25 μl of the HTRF reagent mixture was added to the well containing the stimulated NK3.3 cells or the well containing the culture supernatant only.

The HTRF immunoassay for IL-13 using a 96-well microtiter plate was also examined with the same assay reagents and conditions as the miniaturized assay using a 384-well assay plate but 4-fold volume of each solution was used.

2.7. Measurement of IL-13 levels by ELISA

Culture supernatants of NK3.3 cells stimulated with IL-2 in the well of a 384-well assay plate and IL-13 standard solution (0.13–20 ng/ml), described in the previous section, were diluted 20-fold with Lymphocult-T free culture medium. IL-13 levels in these diluted samples were measured with the BIOTRAK™ IL-13 human ELISA system (Amarsham Life Science, Buckinghamshire, UK) using 96-well microtiter plates. The absorbance at 450 nm was measured with a Victor^{2}™ multilabel counter.

3. Results and discussion

3.1. Development of miniaturized immunoassay using HTRF

We developed a miniaturized HTRF immunoassay for human IL-13 using a 384-well assay plate. First, three commercially supplied human IL-13 monoclonal antibodies, # 32116.11, JES10-5A2 and B-P6, were labeled with EuK using coupling reagents of SPDP and SMCC. The numbers of EuK introduced to one molecule of # 32116.11, JES10-5A2 and B-P6 were estimated at 2.9, 4.1 and 4.8, respectively. The optimal concentration of EuK-labeled antibody to be used for the measurement of HTRF is generally decided as the fluorescence at 620 nm of EuK that comes to at least 20 000 cps in order to correct efficiently for media absorbance by colored samples and compensate for fluctuations from the excitation source. Therefore, the concentrations of EuK-labeled # 32116.11, JES10-5A2 and B-P6 were decided to be 120, 60 and 80 ng/ml, respectively. Next, we prepared XL665-conjugates of the three antibodies in the same manner (molar ratios of XL665 to these antibodies were 1.5–1.8) and the best combination of the EuK-labeled antibody and the XL665-labeled antibody was investigated. IL-13 was detected most sensitively using a combination of EuK-labeled B-P6 antibody and XL665-labeled # 32116.11 antibody (Fig. 1).

We also tried indirect labeling of antibody with XL665 through biotin–avidin interaction because the biotinylation procedure is much simpler than the XL665-labeling procedure and the yield of biotinylated antibody tends to be higher than that of XL665-labeled antibody. We prepared biotinylated # 32116.11 antibody, which was combined with XL665-labeled streptavidin, and compared this indirect format with the direct format using XL665-labeled # 32116 antibody. There was no

difference between the direct and indirect formats in assay sensitivity (data not shown). Therefore, we decided to use EuK-labeled B-P6 antibody, biotinylated #32116.11 antibody and XL665-labeled streptavidin for the detection of IL-13 (HTRF reagent mixture). After a series of checkerboard-type experiments had been performed to optimize reagent concentrations, we selected the following concentrations, EuK-labeled B-P6 antibody, 80 ng/ml; biotinylated #32116.11 antibody, 500 ng/ml; XL665-labeled streptavidin, 1250 ng/ml.

IL-13 standard solution and the HTRF reagent mixture were added to a 384-well assay plate and the HTRF value of each well was measured after incubation. The HTRF value of the zero standard solution was 524 ± 16 ($n = 3$). Based on the standard curve for IL-13, the detection limit of IL-13 was estimated to be less than 600 pg/ml, corresponding to two standard deviations (S.D.) of the zero standard HTRF values (Fig. 2).

To compare the performance of this 384-well format with the 96-well format, we developed an HTRF immunoassay for IL-13 using a 96-well microtiter plate, where the assay reagents and conditions were the same as the 384-well assay format described above but the volumes of all reagents were increased to 4-fold from the 384-well assay format. The HTRF value of zero standard solution for the 96-well assay was 509 ± 14 ($n = 3$), and a standard curve superimposable onto that of 384-well assay format was obtained (Fig. 2). The detection limit of the 96-well assay format was less than 600 pg/ml, similar to the 384-well assay format. We found the HTRF immunoassay using the 384-well assay plate had almost the same performance as that using the 96-well assay plate and could be developed without any problem.

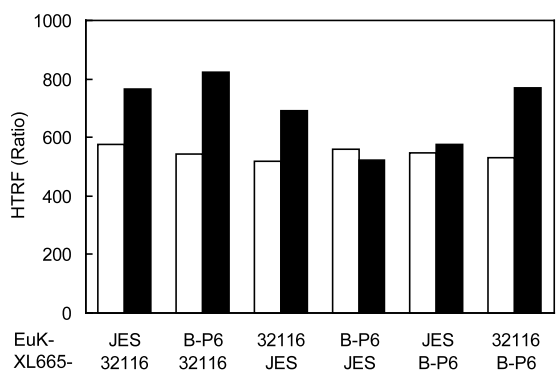

Fig. 1. Detection of IL-13 using several combinations of EuK-labeled and XL665-labeled anti-IL-13 monoclonal antibodies. To 0 pg/ml (open bars) or 2.5 ng/ml (closed bars) of IL-13 standard solutions in a well of a 384-well assay plate was added each combination of EuK-labeled and XL665-labeled monoclonal antibodies and then the HTRF value of each well was measured. The pairs of monoclonal antibodies are shown under the graph. Each bar represents a mean of two determinations.

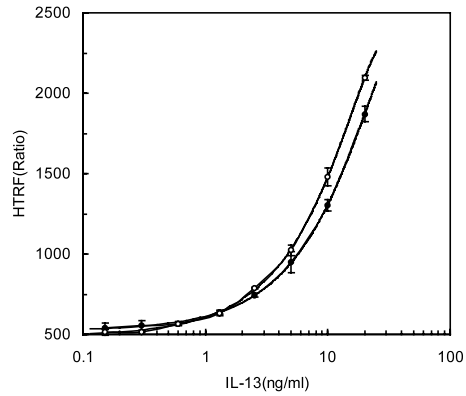

Fig. 2. Standard curves for human IL-13 using HTRF. HTRF reagent mixture (EuK-labeled B-P6, biotinylated #32116.11 and XL665-conjugated streptavidin) was added to a well of a 96-well microtiter plate (○) or a 384-well assay plate (●) containing diluted IL-13 with culture medium and then the HTRF value of each well was measured. Each point represents a mean ± S.D. of three determinations.

3.2. Application of miniaturized immunoassay to cell-based assay

To apply the immunoassay for human IL-13 to cell-based assay, we used NK3.3 cells established from human NK cells by Kornbluth [14,15], which are known to secrete IL-13 by stimulation with IL-2 [9]. First, we investigated the number of NK3.3 cells needed for this immunoassay. NK3.3 cells ($0.33–2.5 \times 10^4$ cells in 40 μl) were added to each well of a 384-well assay plate and were stimulated with 10 μl of 25 ng/ml IL-2 for 24 h. Next, 25 μl of the culture supernatant of each well was used for the miniaturized immunoassay.

When 1.3×10^4 per well of NK3.3 cells were used, the HTRF value reached the plateau (data not shown) and this number of the cells was thought to be sufficient to detect IL-13 by this immunoassay. We also decided to stimulate NK3.3 cells for 6 h with IL-2 because the HTRF value was remained at the plateau from 6 h until at least 48 h (data not shown).

We tried to omit the transfer of culture supernatants. HTRF values by direct addition of the HTRF reagent mixture to culture wells containing cells were compared with those where addition of the mixture to the assay well was done after the culture supernatants had been transferred following stimulation of NK3.3 cells in the culture well. Both procedures showed very close values at every concentration of IL-2 added (Fig. 3). This result shows that the miniaturized immunoassay could detect IL-13 in the presence of 1.3×10^4 per well of NK3.3 cells without transferring culture supernatants from culture well to assay well, which can raise assay throughput and reduce plate cost.

We investigated the incubation time after addition of HTRF reagent mixture and found that incubation at room temperature for at least 24 h was required to obtain steady HTRF values. Moreover, these HTRF values were stable even if the plate was left at room temperature for 4 days (data not shown). The robustness of the immunoassay was thought to result from the stable fluorescence probes for HTRF.

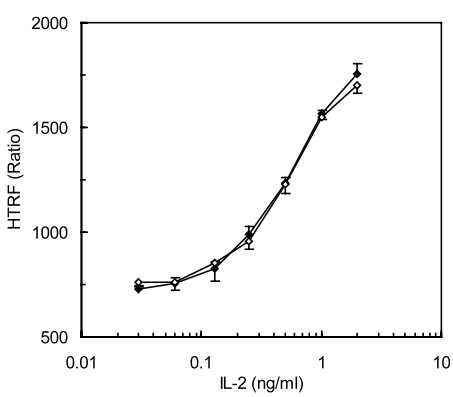

Fig. 3. IL-13 production in NK3.3 cells after IL-2 stimulation. NK3.3 cells (1.3×10^4 in 40 μl) and 10 μl of 0.15–10 ng/ml IL-2 were added to wells of a 384-well assay plate and the plate was incubated for 6 h. After the incubation, HTRF reagent mixture was added to the well where 25 μl of culture supernatants of NK3.3 cells had been transferred (◇) or the well where NK3.3 cells were stimulated with IL-2 (◆) and then HTRF values were measured. Each point represents a mean ± S.D. of three determinations.

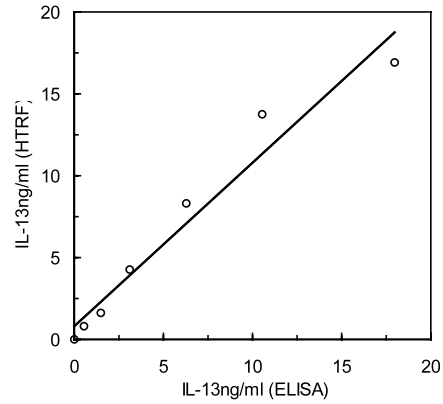

Fig. 4. Comparison of IL-13 levels measured by miniaturized immunoassay using HTRF with that measured by ELISA. Procedures of cell stimulation, miniaturized immunoassay, and ELISA are described in the text. IL-13 levels measured by miniaturized immunoassay vs. that measured by ELISA are plotted. Each point represents a mean of three determinations. The correlation coefficient was 0.9535 ($y = 0.993X + 0.8518$).

3.3. Comparison of miniaturized immunoassay with ELISA

We compared IL-13 concentrations measured by the miniaturized HTRF immunoassay with those measured by ELISA after 1.3×10^4 per well of NK3.3 cells were stimulated with IL-2 in a 384-well assay plate. As IL-2 concentrations increased from 0.03 to 2 ng/ml, IL-13 levels gradually increased from 0 ng/ml to 16.9 ± 1.6 ng/ml ($n = 3$) in a dose response manner, as shown in Fig. 3. IL-13 levels measured by both methods were very close at every concentration of IL-2 and the correlation coefficient was 0.9535 ($y = 0.993X + 0.8518$, Fig. 4). This result shows that IL-13 concentrations could be accurately determined by this miniaturized immunoassay using HTRF although cell stimulation and detection are done entirely within one 384-well assay plate. ELISA is very popular and widely used but requires many troublesome steps of reagent additions, washings, and incubations. Moreover, it

requires two types of microtiterplates, i.e. tissue culture plate and ELISA plate. Our HTRF assay requires only three steps and one 384-well assay plate.

4. Conclusion

We developed a high-throughput immunoassay for human IL-13 using 384-well assay plate in which both cell stimulation and HTRF detection can be done, successively. This miniaturized assay format gave similar assay performances to conventional 96-well microtiter plate assay and could reduce assay costs and increase assay throughput. Furthermore, in the present method, there was no need to transfer the culture supernatant to another assay plate and wash plate. Even if the cells were existed in the assay plate, the accuracy by this miniaturized assay was comparable to that by ELISA. Moreover, the signals measured by our HTRF assay were very stable for a long time. Our miniaturized HTRF assay is non-radioactive, homogenous, simple, robust and very suitable for robotic automation for screening IL-13 production inhibitors and discovering anti-allergic drugs.

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