Evaluation of α-Human IgG-Phycoerythrin Reporter Conjugate in a Model Human IgG Immunoassay

Bruce Amsden and Yasmin Imran

ProZyme, Inc., Hayward, CA, USA

Abstract

The most common embodiment of Luminex® xMAP® technology involves the use of biotinylated detection agents (antibodies or oligonucleotides) coupled with a streptavidin-RPE reporter conjugate. Different configurations are potentially useful in certain circumstances. In this study we examine the use of a different reporter conjugate, one that consists of polyclonal α-Human IgG conjugated directly to RPE (α-Human IgG-RPE). We show how this conjugate can be used to detect human IgG in a capture sandwich immunoassay.

Introduction

ProZyme has offered fluorescent conjugates for many applications under its PhycoLink® product line. For example, ProZyme has refined a selection of streptavidin-phycoerythrin reporter conjugates (SA-PEs) specifically optimized for xMAP applications. These conjugates rely on prior binding of biotinylated detection agents (biotinylated antibodies for immunoassays and biotinylated detection probes for oligonucleotide-based detection systems).

However, other binding interactions besides streptavidin-biotin are also potentially useful. In particular, detection antibodies directly conjugated to PE can be used in immunoassays instead.

One example of such an assay is an immune response characterization assay. In it, different antigens of interest would be coupled to different microspheres and the amount of antibody specific to each antigen in a sample could then be measured. This assay could be used to characterize such things as vaccine efficacy and patient immune competence. Since the analyte for each microsphere would be the same (IgG), the same detection agent could be used for all of them (α-human IgG).

In the present report we describe an assay for human IgG which uses an α-human IgG-RPE conjugate for direct detection of human IgG in a sandwich immunoassay format. The purpose of this assay is to evaluate the performance of α-human IgG-RPE conjugates in a way that is similar to an immune response characterization assay, but does not require the use of human samples.

Methods

Coupling of capture antibody to magnetic microspheres. Goat anti-Human IgG (Fab specific) polyclonal antibody (product number 109-005-006 from Jackson Immunoresearch [referred to as Supplier 2 in text], or product number 2085-01 from SouthernBiotech [referred to as Supplier 1 in text]) was covalently coupled to carboxylated magnetic microspheres (MagPlex™-C Microspheres) using a procedure recommended by Luminex Corporation (Sample Protocol For Two-Step Carbodiimide Coupling of Protein to MagPlex™-C Magnetic Carboxylated Microspheres, pp. 1-2, Luminex Corporation, April 2007).

Coupling of capture antibody to polystyrene microspheres. Goat anti-Human IgG (Fab specific) polyclonal antibody (product number 109-005-006 from Jackson ImmunoResearch) was covalently coupled to carboxylated polystyrene microspheres (MicroPlex™ Microspheres) using a procedure recommended by Luminex Corporation (Sample Protocols for Immunoassay using Luminex Microspheres, pp. 10-11, Luminex Corporation, April 2007).

Human IgG stock solutions. Human IgG in the form of gamma globulins was obtained from Biodesign International (catalog number A08400H). Concentrated stock solutions (100 mg/ml) were prepared in 0.2 *M* glycine + 0.05% NaN_3. Sub-stocks were prepared at 5 mg/ml in PBS + 0.05% NaN_3. The IgG concentration of the stocks was verified by absorbance. Stocks were stored at 2-8°C.

Evaluation of α-Human IgG-RPE using an IgG Standard Curve. Performance of α-Human IgG-RPE was evaluated by preparing 12-point standard curves of human IgG starting at 2 μg/ml (13 *nM*) and serially diluting in half-log (factor of 3.16) increments.

1. Antibody-coupled microspheres were diluted to 10^5/ml in PBS-TBN; 20 μl were mixed with 20 μl of diluted human IgG in 96-well microplates. The plate was incubated for 60 minutes in the dark at room temperature with shaking at 500 - 600 rpm.
2. α-Human IgG-RPE was diluted to a standard concentration (usually 20 μg/ml) in PBS-TBN; 20 μl were added to each sample and the plate was incubated for 15 minutes in the dark at room temperature with shaking at 500 - 600 rpm.
3. For magnetic microspheres, assay reagents were removed by pipetting after immobilizing the microspheres using a 96-well plate magnet (from PerkinElmer). For polystyrene microspheres, assay reagents were removed by vacuum filtration in pre-wetted wells of 96-well filter microplates (Multiscreen®$_{HTS}$ from Millipore Corporation). In either case, the microspheres were then washed twice with 200 μl of PBS-TBN. The microspheres were resuspended in 100 μl of PBS-TBN and read at low calibration (magnetic microspheres) or high calibration (polystyrene microspheres) on a Bio-Plex™ 200 instrument running the Bio-Plex Manager™ Software v5.0. Each test was performed in quadruplicate and averaged.

Treatment of outlying data points. In some cases, 3 of the 4 quadruplicate measurements were tightly grouped with the fourth point differing substantially from the other three. In such cases, the fourth point was eliminated from the calculated average if it was more than four times the standard deviation from the mean of the other three points.

Figure 1 **Log-log plot of fluorescence intensity *vs.* human IgG concentration using Fab-specific capture antibodies from different suppliers**

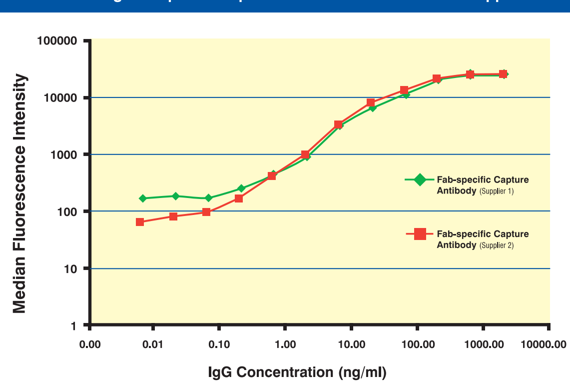


Figure 2 **Log-log plot of fluorescence intensity *vs.* human IgG concentration using different lots of PJ313 α-Human IgG(Fc)-RPE**

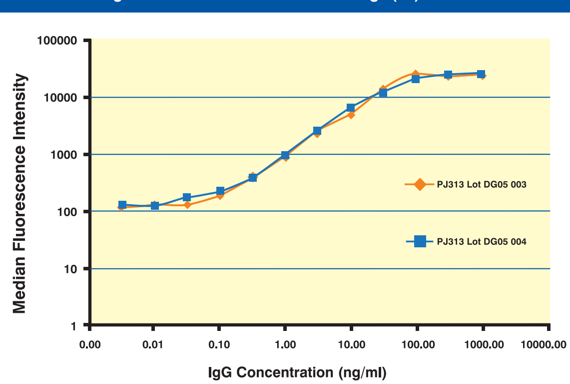
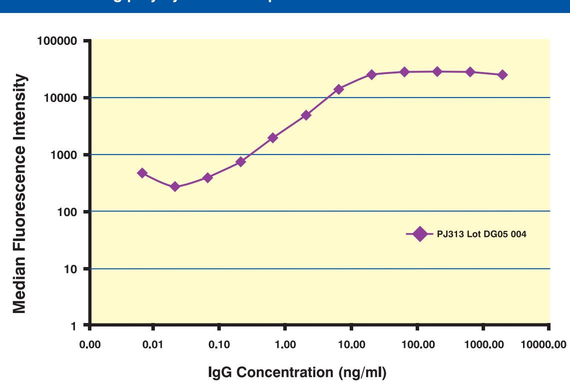


Figure 3 **Log-log plot of fluorescence intensity *vs.* human IgG concentration using polystyrene microspheres**



Results and Discussion

A model xMAP sandwich immunoassay for measuring human IgG was constructed as described in Methods. In this assay, a capture antibody which is specific for the Fab portion of human IgG was coupled to either magnetic or polystyrene microspheres. This specificity was chosen in order to most closely mimic antibody-antigen binding in that the Fc portion of the captured human IgG would be directed outward (away from the microsphere and into the solvent).

The purpose of this assay is to provide a means of characterizing and monitoring the performance of the α-human IgG-RPE conjugate which is the detection reagent in this assay.

1. Selection of Capture Antibody

Two different Fab-specific polyclonal α-human IgG antibodies were tried (see Methods). Each antibody was coupled to a different magnetic microsphere as described in Methods.

The two microsphere preparations were combined in a single assay and tested with a 12-point standard curve of human IgG. The starting concentration of the human IgG was 2 μg/ml (13 *nM*), with half-log dilutions down to 6.3 pg/ml (42 *fM*).

Figure 1 shows the results of this test. The two curves were similar, but the dynamic range was slightly higher, and the background lower, for Supplier 2 than for Supplier 1. As a result, the Supplier 2 capture antibody was used for the remainder of the study.

2. Assay Range

The plots show that signal (MFI) is proportional to IgG concentration from about 0.1 ng/ml (0.6 *pM*) up to 100 ng/ml (0.6 *nM*). Different IgG concentrations are reliably distinguishable throughout this range. At higher IgG concentrations, the signal is at or near the maximum which can be measured.

3. Reproducibility of Fluorescence Intensity

Figure 2 shows IgG assay curves generated using 2 different lots of PJ313 α-human IgG-RPE. The signal strengths throughout the curve are similar for both lots

4. Assay Precision

Each curve was performed in quadruplicate. For each IgG concentration, the four measurements were averaged and the coefficient of variation (%CV) was determined. The %CV values for measurements made at IgG concentrations from 0.01 - 10 ng/ml averaged less than 10%.

5. Polystyrene Microspheres

The capture antibody from Supplier 2 was also coupled to MicroPlex polystyrene microspheres as described in Methods. The same IgG standard curve was performed with these microspheres.

A similar result was obtained as with the MagPlex microspheres, but in this case the dynamic range of the assay was lower due to a high background. This background is not a constant feature of the assay, but the cause has not yet been determined.

Summary

The IgG assay described here will be useful for evaluating the performance of α-human IgG-RPE in immunoassays. We expect that it will be a suitable tool for evaluating lot-to-lot consistency as well as stability.

ProZyme will utilize this and other xMAP assays to continue to ensure high quality, demonstrate long-term stability and provide continuous improvement of its line of reporter conjugates.

References

Amsden, B and Imran, Y. Room Temperature Stability Study of Streptavidin-Phycoerythrin Reporter Conjugate Using a Model TSH Immunoassay. Presented at Planet xMAP Europe, Amsterdam (October, 2009). Also available at http://www.prozyme.com/documents/tnpj200_8.pdf.

Amsden, B, Imran, Y and Baker, HN. Evaluation of Streptavidin-Phycoerythrin Reporter Conjugates in a Model TSH Immunoassay. Presented at Planet xMAP USA, Austin (May, 2009). Also available at http://www.prozyme.com/documents/tnpj200_7.pdf.

Wegstein, EJ, Amsden, B, Chen, A and Oliver, K. New SA-PE Conjugates Improve Critical Assay Parameters in Sandwich Immunoassays Performed Using Luminex® xMAP® Technology. Presented at Planet xMAP Europe, Amsterdam (October, 2008). Also available at http://www.prozyme.com/documents/tnpj200_5.pdf.

Amsden, B, Chen, A and Dunbar, SA. New SA-PE Conjugates Reduce Capture-antibodyspecific Background in Sandwich Immunoassays. Presented at Planet xMAP USA, Boston (March, 2008). Also available at http://www.prozyme.com/documents/tnpj200_4.pdf.

Amsden, B, Oliver, K and Harding, H. Pushing the Limit of Detection: Signal Amplification and Screening for the Optimal Reporter Achieves Sub-attomole Sensitivity. Presented at Planet xMAP Europe, Amsterdam (October, 2007). Also available at http://www.prozyme.com/documents/tnpj200_3.pdf.

Luminex®, xMAP®, MagPlex™ and MicroPlex™ are trademarks of Luminex Corporation, Austin, TX USA
ProZyme® and PhycoLink® are registered trademarks of ProZyme, Inc., Hayward, CA USA
MultiScreen® is a registered trademark of Millipore Corp., Billerica, MA USA
Bio-Plex™ and Bio-Plex Manager™ are trademarks of Bio-Rad Laboratories, Inc., Hercules, CA USA

PROzyme®