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Research paper

Development of a custom pentaplex sandwich immunoassay using Protein-G coupled beads for the Luminex® xMAP® platform

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ABSTRACT

Multiplex bead-based assays have many advantages over ELISA, particularly for the analyses of large quantities of samples and/or precious samples of limited volume. Although many commercial arrays covering multitudes of biologically significant analytes are available, occasionally the development of custom arrays is necessary. Here, the development of a custom pentaplex sandwich immunoassay using Protein G-coupled beads, for analysis using the Luminex® xMAP® platform, is described. This array was required for the measurement of candidate biomarkers of vaccine safety in small volumes of mouse sera. Optimisation of this assay required a stepwise approach: testing cross-reactivity of the antibody pairs, the development of an in-house serum diluent buffer as well as heat-inactivation of serum samples to prevent interference from matrix effects. We then demonstrate the use of this array to analyse inflammatory mediators in mouse serum after immunisation. The work described here exemplifies how Protein G-coupled beads offer a flexible and robust approach to develop custom multiplex immunoassays, which can be applied to a range of analytes from multiple species.

1. Introduction

Vaccines are of global importance to public health because they are a cheap, safe and efficient means of combating infectious diseases, cancer and chronic diseases (Nossal, 2011). In order to accelerate the development and introduction of safer and more effective new vaccines, novel approaches to identify and predict adverse reactions to experimental vaccines early in the development cycle are essential (Kaufmann et al., 2014; Rappuoli et al., 2014). This will require the continued cooperation between the pharmaceutical, academic and regulatory sectors. BioVacSafe (Biomarkers for enhanced Vaccines immunoSafety) is an Innovative Medicine initiative (IMI)-funded consortium consisting of 19 partner organisations from leading European industrial, regulatory and academic institutions (www.biovacsafe.eu) (Lewis and Lythgoe, 2015). This 5-year project aims to identify biomarkers that will speed up, improve as well as reduce the cost of testing and monitoring vaccine safety, both before and after release to the market. As part of this project, many human and animal studies are being carried out and are generating a vast number of samples for analyses. xMAP® Technology is being employed as a high throughput approach to screen these samples for multiple potential biomarkers simultaneously, especially using small sample volumes.

The Luminex® xMAP® Technology (Houser, 2012; Vignali, 2000) is based on microscopic (5.6 or 6.5 μm) beads, known as microspheres, which are filled with different ratios of two or three dyes resulting in 500 unique fluorescent profiles or bead regions for the development of up to 500-plex assays. Analytes bound to the beads via "capture" molecules (e.g., antibodies, antigens, oligonucleotides, enzyme substrates, receptors, etc.) are detected by the reporter molecule phycoerythrin (PE) conjugated to streptavidin or a secondary or "detection" molecule (e.g., antibody). Multiplexing (i.e. simultaneous detection of many analytes in the same sample) is possible because one capture molecule to a specific analyte is attached to a specific bead region (i.e. a set of beads with the same fluorescent profile). Luminex® analyser instruments are essentially two laser flow cytometers: the first laser allows identification of the bead region by excitation of the bead dyes, whilst the second enables quantification of the analyte bound to the bead by excitation of the PE reporter molecule (Houser, 2012; Vignali, 2000). Analyte concentration is determined by measuring the median fluorescence intensity (MFI) of the reporter dye and interpolating from the standard curve.

It is not always possible to obtain a commercial xMAP® array that can detect all analytes of interest, and the use of multiple ELISAs is not a viable alternative for large numbers of small volume samples. Consequently, development of home-brew xMAP® assays is necessary. MagPlex® Microspheres are superparamagnetic beads with a surface

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Table 1
Catalogue numbers and stock concentrations of R&D systems DuoSet ELISA reagents utilised, as well as final concentrations of the capture antibodies used to coat the Protein G-coupled beads.

Mouse analyte	Cat. No.	Top standard (ng/ml)	Capture antibody (μg/ml)	Detection antibody (μg/ml)	Capture antibody coating concentration (μg/ml)
IL-2Ra	DY2438	0.5	144	108	2.88
TREM-1	DY1187	4	144	9	2.88
IL-1Ra	DY480	10	144	72	2.88
PTX3	DY2166	14	720	18	14.4
IP-10	DY466	4	360	108	7.2
CRP	DY1829	1.5	360	72	7.2
sTNFRII	DY4260	0.5	360	72	7.2

containing 100 million carboxyl groups on each bead, which facilitate covalent attachment of "capture" molecules during a two-step carbodiimide reaction (Staros et al., 1986). This chemistry involves activation of the carboxyl groups with the aid of EDC and sulfo-NHS to form amine reactive sulfo-NHS-ester intermediates. All proteins contain primary amine groups that readily form covalent amide bonds with activated carboxylated beads in the correct pH/ionic strength conditions. The most popular use of carboxylated xMAP® beads is as antibody-coupled reagents for use in sandwich immunoassays. However, the orientation of the coupled capture antibody is random (de Jager and Rijkers, 2006), and antibody immobilisation may mask antigen recognition epitopes (Schwenk et al., 2007). Consequently, only a fraction of the coupled antibodies will have the capacity to bind ligands.

In this study, Protein G-coupled MagPlex® Microspheres were generated as a universal reagent for the development of custom xMAP® arrays. Protein G is a cell wall protein of group G streptococci that binds immunoglobulins via Fc regions (Bjorck and Kronvall, 1984; Reis et al., 1984). Aside from ensuring the correct orientation of capture antibodies, the major advantage of coupling Protein G to xMAP® beads is the ease of producing "mix and match" bead regions for multiplex assays without further coupling reactions. Here, we describe the use of ELISA antibody pairs and Protein G-coupled beads for the development of a multiplex sandwich immunoassay for simultaneous quantification of interleukin 2 receptor alpha (IL-2Ra), IFN gamma inducible protein 10 (IP-10; also known as CXCL10), C-reactive protein (CRP), interleukin 1 receptor antagonist (IL-1Ra) and soluble Tumour Necrosis Factor Receptor II (sTNFRII) in small volumes of mouse sera. In determining optimal procedures for the multiplex assay, antibody cross-reactivity against Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) and Pentraxin 3 (PTX3) were identified, thereby ruling out their inclusion in the array. The analytes of interest are potential biomarkers of vaccine safety being investigated by the IMI-JU project BioVacSafe.

2. Materials and methods

2.1. Reagents, buffers and instruments

Bead coupling reactions were carried out using recombinant Protein G (AbD Serotec; 740601L), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; ThermoFisher Scientific) and N-hydroxysulfosuccinimide (Sulfo-NHS; ThermoFisher Scientific). Handheld tube (Life Technologies or Qiagen) or plate (Merck Millipore) magnetic separators were used when washing magnetic beads. Luminex analyses were carried out using a Bio-Plex®/LiquiChip 100 (Bio-Rad or Qiagen) instrument.

R&D systems Duoset® ELISA kits containing standards, capture antibodies and biotinylated detection antibodies (Table 1) were utilised. Also employed were biotinylated rabbit anti-Protein G (Abcam; ab7251), Streptavidin-PE (eBioscience; 12-4317-87, BD Pharmingen; 554,061, or Biolegend; 405245) as well as PE-conjugated species-specific secondary antibodies: anti-hamster IgG (R&D Systems; F0120), anti-goat IgG (R&D Systems; F0107) and anti-rat IgG (R&D Systems; F0105B). Commercial buffers utilised were General serum diluent (ImmunoChemistry Technologies; 647), Plasma sample diluent (ImmunoChemistry Technologies; 694) and Neptune diluent (ImmunoChemistry Technologies; 6124). The following buffers were also prepared: activation buffer [0.1 M Monobasic Sodium Phosphate, pH 6.2], PBS [pH 7.4; containing 138 mM NaCl, 2.7 mM KCl], PBS-TBN# [PBS, 0.1% IgG-free BSA (Jackson ImmunoResearch), 0.02% Tween-20, 0.05% Azide], PBS-BN# [PBS, 1% IgG-free BSA, 0.05% Azide], PBS-BSA [PBS, 1% BSA] and J Buffer [PBS, 20 mM Tris–HCl, 1% Goat Serum, 1% Sheep Serum, 0.05% Tween-20]. In addition, bovine serum albumin (BSA), foetal bovine serum (FBS; Gibco), normal mouse serum, non-fat dried milk powder (Panreac Applichem) and Tween-20 were employed as blocking agents in PBS, where appropriate. All buffers were filter-sterilised and stored at 4 °C. Unless otherwise stated, buffer reagents were obtained from Sigma Aldrich.

2.2. Coupling Protein G to MagPlex® microspheres

Protein G used in the coupling reactions was free of sodium azide, BSA, glycine, Tris and amine-containing additives. Prior to use, all buffers were brought to room temperature, EDC and Sulfo-NHS were desiccated at room temperature for approximately 1 h, and

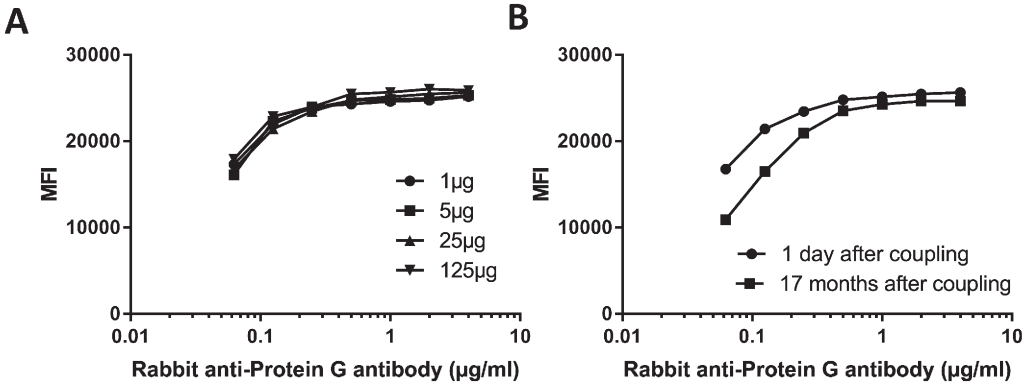

Fig. 1. Coupling and stability of Protein G on xMAP® beads. (A) MagPlex® Microspheres were conjugated to varying amounts of Protein G, ranging from 1 to 125 μg per 1.25×10^6 beads, in a carbodiimide reaction. Coupling was confirmed by labelling with dilutions of PE-conjugated rabbit anti-Protein G antibody. Optimal coupling is achieved when the curve saturates at ≥10,000 MFI. (B) The stability of the Protein G-coupled beads was determined by comparing coupling confirmations carried out 1 day or 17 months post coupling.