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Technical note

Comparison of carboxylated and Penta-His microspheres for semi-quantitative measurement of antibody responses to His-tagged proteins

Nelianne Verkaik [a,*], Eric Brouwer [b], Herbert Hooijkaas [c], Alex van Belkum [a], Willem van Wamel [a]

[a] *Department of Medical Microbiology and Infectious Diseases, Erasmus MC, 's Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands*
[b] *Department of Neurology, Lab. Neuro-Oncology, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands*
[c] *Department of Immunology, Erasmus MC, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands*

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ABSTRACT

The Luminex system is a flow cytometry based tool that permits the simultaneous measurement of many analytes from just a single serum sample. The technology uses microspheres, which are available in different colors and can be coated with different kinds of biomolecules. For the immobilisation of His-tagged proteins, two types of beads can be used: chemically activated carboxylated beads or Penta-His beads, which have antibodies against His-tags on their surface. In this study, we compared carboxylated and Penta-His beads. For carboxylated as compared to Penta-His beads, the non-specific background is lower (Median Fluorescence Intensity; MFI>250, 0% versus 15%), the specific signal intensity is higher (mean MFI 2860 versus 722) and not dependent on the configuration of the protein. Above all, the protein coupled carboxylated beads are useful over longer periods of time. Therefore, we conclude that for developing a multiplex assay for semi-quantitative measurement of antibody responses against His-tagged proteins the best microspheres to use are the carboxylated ones.
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1. Introduction

Conventional immunological techniques such as Enzyme-Linked ImmunoSorbent Assays (ELISA) allow for the detection of single antigens or antibodies at a time. The newer microsphere (bead) based flow cytometry technique (xMap, Luminex Corporation, Austin, TX, USA) permits the simultaneous analysis of antibodies for up to 100 different antigens from a single serum sample in a single run (Fulton et al., 1997). Microspheres are internally color-coded with a pair of fluorescent dyes. Through precise and balanced concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which can be coated with a specific reagent, usually a (poly)peptide or (poly)saccharide antigen. After the bead captures an analyte from a sample, a reporter molecule, labelled with a fluorescent dye, is introduced to complete the reaction on the surface of each microsphere. The analyser that is subsequently used resembles a flow cytometer with two lasers. The first (red) laser identifies the microsphere by its internal fluorescence signature and hence determines which antigen is carried by that bead. The second (green) laser excites the fluorescent reporter dye and determines the qualitative and quantitative result of the assay. Studies have demonstrated the ability to effectively multiplex a range of assays including those for antibody detection and quantitation (Martins et al., 2006).

We are interested in developing a multiplex assay to study antibody responses against His-tagged *Staphylococcus aureus* (*S. aureus*) proteins. Besides the frequently used carboxylated

Abbreviations: MFI, Median Fluorescence Intensity; CV, coefficient of variation; IsdA and IsdH, Iron-regulated surface determinant A and H; FnbpB, Fibronectin-binding protein B; SdrD, Serine-asparate dipeptide repeat protein D; PBS, phosphate buffered saline; BSA, bovine serum albumin; ELISA, Enzyme-Linked ImmunoSorbent Assay; RT, room temperature; MSCRAMMS, microbial surface components recognising adhesive matrix molecules.

* Corresponding author. Tel.: +31 10 7032218; fax: +31 10 7033875.
E-mail address: n.j.verkaik@erasmusmc.nl (N. Verkaik).

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microspheres, LiquiChip Penta-His beads can also be used for His-tagged protein coupling. Penta-His beads have Penta-His antibodies (mouse monoclonal IgG1) coated on the surface of the beads, which recognise and bind to the His-tags on recombinant proteins. At the bead surface, antibodies are immobilised in a specific manner through a spacer that couples to their Fc domain.

In the present study we compared carboxylated beads and Penta-His beads to determine which bead type is best used for development of a multiplex assay involving His-tagged proteins.

2. Materials and methods

2.1. Antigens

The constructs to produce recombinant *S. aureus* proteins were obtained from T. Foster (Moyne Institute of Preventive Medicine, Dublin, Ireland; Roche et al., 2003). The proteins were expressed in *Escherichia coli* XL1Blue, purified under denaturing conditions with Ni-NTA agarose recognising the His-tag and quality controlled by SDS-PAGE and mass spectrometry. The *S. aureus* recombinant proteins were the Iron-regulated surface determinant A and H (IsdA and IsdH), Fibronectin-binding protein B (FnbpB) and Serine-asparate dipeptide repeat protein D (SdrD). They belong to the group of staphylococcal "microbial surface components recognising adhesive matrix molecules" (MSCRAMMS), proteins that are generally considered to be important for host colonisation (Foster and Hook, 1998).

2.2. Serum samples

Venous blood was collected from 20 healthy volunteers and serum samples were stored at −80 °C until use. Volunteers had given informed consent and the local Medical Ethics Committee of the Erasmus MC Rotterdam approved the sampling (MEC-2007-106).

2.3. Coupling methods

The four proteins were coupled to Penta-His beads (Qiagen, Venlo, The Netherlands) and SeroMAP beads (Luminex Corporation, Austin, TX, USA), a carboxylated bead that is developed for serological applications. The coupling reactions were carried out according to the manufacturer's protocol. All centrifugation steps were carried out at 12.000 g for 2 min at room temperature (RT).

4 µg protein per million beads was added to 2.5×10^5 Penta-His microspheres and mixed by vortexing for 30 s at full speed. The microspheres were then incubated overnight at 4 °C in the dark. 1 ml PBS, 0.1% BSA was then added to the bead suspension after which they were centrifuged immediately. This step was repeated twice. The beads were resuspended in 400 µl blocking-storage buffer (PBS-BN; PBS, 1% BSA, 0.05% sodium azide, pH 7.4). The suspension was adjusted to 125 beads/µl and stored at 4 °C in the dark. A coupling concentration of 12 µg protein per million beads was investigated also, but showed no increase in Median Fluorescence Intensity (MFI) (results not shown). Therefore, we used the former concentration.

The coupling procedure for carboxylated beads was as described before (Martins et al., 2006). To 1.0×10^6 microspheres, 4 µg protein per million beads was added as well. As activation buffer we used 100 mM monobasic sodium phosphate, pH 6.2. For activation of the carboxyl groups on the surface of the beads, 10 µl of 50 mg/ml of *N*-hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) was used (Pierce Biotechnology, Rockford, IL, USA). The coupling buffer was 50 mM 2-[*N*-morpholino] ethanesulfonic acid (MES), pH 5.0 (Sigma–Aldrich, Zwijndrecht, The Netherlands). The final concentration of microspheres was adjusted to 500 beads/µl with blocking-storage buffer. The microspheres were protected from light and stored at 4 °C until use.

For uncoupled beads the coupling procedure for Penta-His or carboxylated beads was followed, only no *S. aureus* protein was added.

2.4. Assay for comparison of non-specific IgG microsphere binding

Twenty human serum samples were diluted 1/50 in PBS-BN. 50 µl per diluted serum sample was incubated with 10 µl uncoupled Penta-His or 10 µl uncoupled carboxylated microspheres and 40 µl PBS-BN in separate wells of a 96-well 1,2 µm PVDF filter microtiter plate (Millipore Corporation, Billerica, MA, USA) for 30 min at RT on a thermomixer plate shaker (Eppendorf, Hamburg, Germany). The plate was washed twice with assay buffer (PBS, BSA1%, 0.05% sodium azide, pH 7.4) which was aspirated by vacuum manifold. The microspheres were resuspended in 50 µl assay buffer and 50 µl of a 1/200 dilution of R-Phycoerythrin (RPE)-conjugated AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG, Fc_y Fragment Specific (Jackson Immuno Research, Suffolk, UK) was added. The plate was incubated for an additional 30 min at RT on the plate shaker and washed. The microspheres were resuspended in 100 µl of assay buffer. Measurements were performed on the Luminex 100 instrument (BMD, Croissy Beaubourg, France) with Luminex IS 2.2 software. The assay was performed twice and the MFI values, reflecting antibody levels, were averaged.

2.5. Assay for comparison of antigen-specific results

Because we wanted to determine and compare the antigen-specific results without any interference of non-specific background we decided to use the eleven serum samples with low non-specific background in both types of beads. The procedure was the same as described above. In this assay, antigen-coupled microspheres were used. The samples were tested in three separate experiments with a short time interval of 2 weeks and a longer time interval of 3 months with the same batch of antigen-coupled Penta-His and carboxylated beads. When possible, interassay variation was calculated and averaged per *S. aureus* protein. Otherwise, the percentage decline of the MFI values was calculated. Intra-assay variation was determined from testing eleven sera, each sample assayed on four wells within a plate. The percentage CV was calculated between each of these results and averaged.

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