

- erythematosus. Occurrence as multimeric complexes bound to histone. *J Clin Invest* 1990;86:69–74.
14. Chan KC, Zhang J, Chan AT, Lei KI, Leung SF, Chan LY, et al. Molecular characterization of circulating EBV-DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. *Cancer Res* 2003;63:2028–32.
15. Luger K. Structure and dynamic behavior of nucleosomes. *Curr Opin Genet Dev* 2003;13:127–35.
16. Ng EK, Tsui NB, Lam NY, Chiu RW, Yu SC, Wong SC, et al. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. *Clin Chem* 2002;48:1212–7.
17. Holdenrieder S, Stieber P, Bodenmueller H, Fertig G, Fuerst H, Schmeller N, et al. Nucleosomes in serum as a marker for cell death. *Clin Chem Lab Med* 2001;39:596–605.

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Relative Quantification of Experimental Data from Antigen Particle Arrays, Susan Pang,^{1*} Julie Reeve,² Michael Walker,² and Carole Foy¹ (¹ LGC Ltd, Teddington, Middlesex, United Kingdom; ² Genesis Diagnostics Ltd., Littleport, Cambridgeshire, United Kingdom; * address correspondence to this author at: LGC Ltd, Queens Road, Teddington, Middlesex, TW11 0LY, United Kingdom; e-mail Susan.Pang@lgc.co.uk)

Protein arrays typically consist of a capture protein on a matrix, e.g., glass slide, silicon chip, or coded microparticle (1). The latter minimizes steric constraints and enhances reaction kinetics (2). Microarray technologies have been used for detecting allergens (3) and cytokines (4). A primary advantage of microarray technologies over conventional immunoassays is the ability to multiplex assays.

Currently, ELISA is the method of choice for autoantibody detection (5). This method, however, is labor-intensive and requires comparatively large sample and reaction volumes. Nonetheless, ELISAs are currently performed to aid differential diagnosis of certain autoimmune diseases. Dermatomyositis (6) is characterized by detection of anti-Jo-1 IgG in patient sera. Both anti-Sm and anti-RNP/Sm IgGs are indicative of systemic lupus erythematosus (7). The presence of anti-Scl70 IgGs aids diagnosis of systemic sclerosis (8). Anti-SSB and -SSA IgGs are present in systemic lupus erythematosus (9) or Sjögren syndrome (10). With microarray technologies, all these autoantibodies can be screened simultaneously.

The Luminex particle array platform comprises a hundred microparticles, each possessing a distinct fluorescent signature generated by a blend of two internal fluorescent dyes. Capture protein is conjugated to the bead surface, to assay for the cognate entity within a single reaction vessel. The instrument includes a microfluidics system and two lasers. A 635 nm laser excites the red and infrared classifier fluorophores that form the particular signature of each bead set. The second laser (523 nm) excites phycoerythrin dye used as a molecular tag for detection. Detection of cytokines (11), cancer markers, and allergens (12) has been reported.

Use of internal calibration curves for cytokine quantification has been documented (13), but with antigen arrays, quantification is more difficult because of the time and

expense required for antibody synthesis. Where quantification of antibodies has been cited, competitive immunoassays are described that use labeled forms of the target analyte specifically tailored for the particular assays described (14). Assays for quantifying total immunoglobulin content have also been described (15), but not in the context of creating a reference material for a specific antibody within the same class of immunoglobulins.

Commercial Luminex-based assays for autoantibodies are available, e.g., from Linco Research and Zeus Scientific. These tests are qualitative, however, and do not allow for direct comparisons between assays. In this report we describe a set of internal standards for antigen arrays that enable interassay comparisons by creating a point of reference for the detection of human IgG. Relative quantification would enable monitoring of treatment administered to combat disease.

We illustrate the use of an internal IgG calibration curve and the detection of six autoantibodies: Jo-1 IgG, Sm IgG, Scl-70 IgG, RNP/Sm IgG, SSB/La IgG and SSA/Ro IgG in patient serum samples. Recombinant forms of the cognate antigens (5 mg/L in phosphate-buffered saline, pH 7.4; AroTec Diagnostics Ltd.) were coupled to the surface of Luminex xMapTM carboxylated microspheres according to the manufacturer's instructions. Within the multiplex, ~10 000 of each antigen-coupled bead set were challenged with serum diluted 1 in 300 (50 μ L), obtained from Genesis Diagnostics. To assess the scope for quantification, we used 22 serum samples. Each reaction mixture was agitated at room temperature for 1 h. Tubes were microcentrifuged for 1 min, and the resulting supernatant was discarded. Beads were washed three times with protein array wash buffer [50 μ L; phosphate-buffered saline (pH 7.4), containing 10 g/L bovine serum albumin, 0.2 g/L Tween 20, and 0.2 g/L sodium azide; Sigma]. The beads were incubated with biotinylated sheep anti-human IgG antibody (Amersham Pharmacia Biotech UK) diluted 1 in 10 000 (100 μ L) and mixed for 1 h at room temperature. Beads were washed before incubation with streptavidin-conjugated phycoerythrin (400 ng/100 μ L; Molecular Probes) for 30 min at room temperature. Tubes were foil-wrapped to prevent photobleaching of beads. Beads were washed before injection into the Luminex instrument, in which a minimum of 100 events per bead set were analyzed. Serum was designated as positive if the fluorescent output was greater than the upper 95% confidence interval of the single "normal" (nondisease state) serum sample included in each assay. The negative control contained antigen-conjugated bead sets treated with protein array buffer.

To quantify the analytes relative to a reference point, 11 sets of calibration beads were synthesized and incorporated into the assay. These comprise microspheres conjugated to known quantities of purified human IgG, ranging from 10 ng/L to 250 mg/L, to construct the calibration curve.

Three experiments were performed, each with triplicate determinations. Mean values, 95% confidence intervals, and CVs were determined with Microsoft Excel 97. Two-way ANOVAs (Statistica, Ver. 6; StatSoft) were applied.

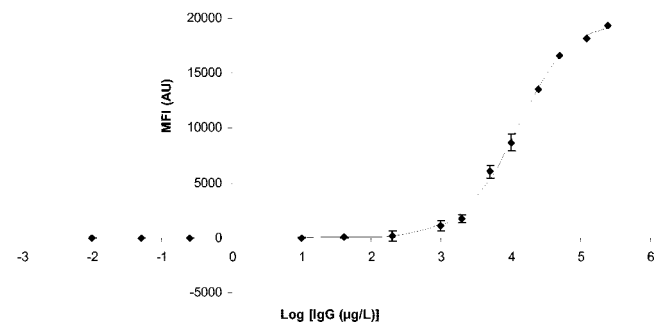


Fig. 1. IgG calibration curve.

Data points represent mean values of triplicate determinations, and the error bars indicate 95% confidence intervals. AU, arbitrary units.

Plotting the logarithm of known IgG concentrations against observed fluorescence output (Fig. 1) produced a highly robust sigmoidal trendline with a correlation coefficient exceeding 0.95 ($n = 12$). The antibody–antigen interaction is known to exhibit this trend, as demonstrated by other immunodetection methods (16). Median fluorescent intensities (MFIs) from multiplexed antigen arrays were interpolated from the IgG calibration curve constructed from a distinct multiplexed antibody array assay with all 11 concentrations of IgG-coupled bead sets within a separate reaction vessel. This enabled conversion of MFIs (ranging

from 0 to 15 000 arbitrary units) to conventional units of measure ($\mu\text{g/L}$) relative to the known concentration of IgG coupled to the calibration bead sets.

Interpolation of each experimental data point from the IgG calibration curve within the linear portion of the curve lowered the majority of CVs for the assay of each sample (Table 1). The MFI from the SSA/Ro IgG assay of serum sample 7 exceeded the range of the calibration curve; therefore, no relative concentration could be determined for this sample. CVs in Table 1 highlighted in bold denote values that increased on interpolation. Increases in the CVs $>5.73\%$ were because the MFIs lay within the plateau of the curve. Pairwise analysis of the platform and samples showed the extent of the variation when these high-scoring positives were interpolated (Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at <http://www.clinchem.org/content/vol51/issue6/>).

Trends in the observed MFIs for the three experiments were consistent for all six IgG assays. For data presented as MFI, the highest values were observed during the first experiment, 4 days after coupling of antigen to the beads. A significant decrease in fluorescent emission was apparent by the time experiment 2 was performed 7 days after coupling. An additional slight decrease in fluorescent output was seen between experiments 2 and 3; the latter

Table 1. Differences (%) between the CVs obtained by subtracting the CVs of the fluorescent output in MFI from the data interpolated from the IgG calibration curve.^a

Sample	Assay					
	Jo IgG	Sm IgG	Sci70 IgG	RNP/Sm IgG	SSB/La IgG	SSA/Ro IgG
Blank	0	−0.99	−2.09	−1.26	0	−0.75
Negative	−0.42	−0.06	−1.21	−0.44	−0.59	−0.17
1	−0.33	−0.90	−1.36	−1.05	−1.32	−0.75
2	−0.75	1.57	−0.88	0.23	−0.44	0.16
3	−0.99	−0.75	−0.69	−0.90	−0.78	−0.90
4	−1.32	1.00	−0.80	0.23	−0.89	−0.25
5	−0.46	−0.39	−0.19	−0.70	−0.20	−0.47
6	−0.54	1.95	−1.13	2.04	−0.29	0.70
7	−0.56	−0.52	−0.83	−1.05	4.58	NA ^b
8	−0.18	−0.25	−0.89	−0.59	−0.70	−0.58
9	−3.12	−2.27	−1.53	−2.24	5.73	12.28
10	−2.36	−1.42	−0.14	−1.57	−1.39	−0.42
11	−0.84	−1.37	−5.90	−1.85	4.53	2.41
12	−0.57	1.34	−0.18	0.41	−0.58	4.12
13	−0.94	−0.73	−0.09	−0.96	−0.97	−1.25
14	−0.39	−0.61	−0.39	−0.60	−0.33	−0.05
15	−29.53	−14.03	11.53	−26.63	−29.83	−20.58
16	−0.53	1.43	−0.79	−0.07	−0.25	0.88
17	−0.60	−1.15	−0.46	−1.54	−0.39	−0.57
18	−0.61	−0.76	−0.63	−1.41	−0.49	19.30
19	8.86	−2.52	−3.66	−3.41	−2.62	−2.43
20	−0.43	1.43	−1.44	1.33	−0.68	1.62
21	−0.14	−1.74	2.18	−0.56	−0.75	−0.42
22	−2.03	−0.82	−1.80	−2.23	10.16	24.82

^a Values in bold increased on interpolation.

^b NA, no relative concentration could be determined because the MFI in the assay exceeded the range of the calibration curve.

was performed 8 days after coupling (Fig. 2 in the online Data Supplement). However, on interpolation of data points from the calibration curve, the trend was reversed such that the highest signal was observed for experiment 3 and the lowest for experiment 1.

In parallel studies, antigen-conjugated beads stored at 4 °C gave consistent fluorescent emission within a period of 1 month, whereas antibody-conjugated beads showed diminished fluorescent emission (data not shown).

We identified two limitations of this methodology: The inherent instability of antibody-coupled beads and the occurrence of data points from test samples outside the linear portion of the semilogarithmic calibration curve. To resolve the latter issue, serum samples exhibiting fluorescent output within the plateau of the trendline should be reassayed after further dilution. Problems with long-term stability of protein-conjugated bead sets were evident when the beads were stored at 4 °C. On the Luminex platform, antibody-conjugated beads were viable for approximately 3 weeks. Antigen-conjugated beads exhibited slightly greater longevity, although decoding of the fluorescent signatures was problematic after storage at 4 °C beyond 1 month. The constituents of the storage buffer may have a detrimental effect on the fluorescent dyes within the microspheres.

The reversal of the signal output profile suggests that antibody-bound beads were more liable to degradation than antigen-coupled bead sets within the same timescale. The more elaborate structural complexity of antibodies compared with antigens may account for the greater instability of the former. Rapid freezing and lyophilization were procedures explored as alternative methods to prolong the shelf-life of protein-coupled beads, and both approaches appeared to be feasible (17). This provides the possibility of developing calibration bead sets as reference materials, thus enabling Luminex assay standardization.

This study illustrated the complexity of quantifying target analytes within antigen arrays. Production of purified antibodies is laborious and expensive. Methods that can be used for antibody purification, e.g., affinity chromatography, could theoretically be used to obtain material comparable to the target analyte of an antigen array. However, consistent antibody purity is paramount for quantification.

Although this approach has broad application for the comparison of any IgG, it will not measure absolute concentrations of target analyte. This is largely because of the presence of factors (e.g., soluble receptors, heterophilic antibodies, serum binding proteins, hemoglobin, and lipids) in sera that can interfere with antibody-based immunoassays (18). Nonetheless, this method has reduced intraassay variability and enables interassay comparisons for a wide range of antigen arrays.

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References

1. Zhou H, Roy S, Schulman H, Natan MJ. Solution and chip arrays in protein profiling. *Trends Biotechnol* 2001;19:S34–S39.
2. Cutler P. Protein arrays: the current state-of-the-art. *Proteomics* 2003;3:3–18.
3. Fall BI, Eberlein-König B, Behrendt H, Niessner R, Ring J, Weller MG. Microarrays for the screening of allergen-specific IgE in human serum. *Anal Chem* 2003;75:556–62.
4. Wiese R, Belosludtsev Y, Powdrill T, Thompson P, Hogan M. Simultaneous multianalyte ELISA performed on a microarray platform. *Clin Chem* 2001; 47:1451–7.
5. Alem M, Moghadam S, Malki J, Zaidi A, Nayak N, Li TM. Detection of autoantibodies to nuclear antigens by EIA and IF techniques. *Allerg Immunol (Paris)* 1997;188:191–4.
6. Arnett FC, Hirsch TJ, Bias WB, Nishikai M, Reichlin M. The Jo-1 antibody system in myositis: relationships to clinical features and HLA. *J Rheumatol* 1981;8:925–30.
7. Hildebrandt S, Weiner ES, Senecal JL, Noell GS, Earnshaw WC, Rothfield NF. Autoantibodies to topoisomerase I (Sci-70): analysis by gel diffusion, immunoblot, and enzyme-linked immunosorbent assay. *Clin Immunol Immunopathol* 1990;57:399–410.
8. Nakamura RM, Tan EM. Recent advances in laboratory tests and the significance of autoantibodies to nuclear antigens in systemic rheumatic diseases. *Clin Lab Med* 1986;6:41–53.
9. Chan EY, Mok TM, Lawton JW, Ko OK, Ho L, Lau CS. Comparison of counter immunoelectrophoresis with immunoblotting for detection of anti-extractable nuclear antigen antibodies in systemic lupus erythematosus. *Asian Pac J Allergy Immunol* 1999;17:275–9.
10. Ben-Chetrit E, Fischel R, Rubinow A. Anti-SSA/Ro and anti-SSB/La antibodies in serum and saliva of patients with Sjögren's syndrome. *Clin Rheumatol* 1993;12:471–4.
11. Carson RT, Vignali DA. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *J Immunol Methods* 1999;227:41–52.
12. Bacarese-Hamilton T, Mezzasoma L, Ingham C, Ardizzone A, Rossi R, Bistoni F, et al. Detection of allergen-specific IgE on microarrays by use of signal amplification techniques. *Clin Chem* 2002;48:1367–70.
13. de Jager W, te Velthuis H, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. *Clin Diagn Lab Immunol* 2003;10:133–9.
14. Martins TB. Development of internal controls for the Luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. *Clin Diagn Lab Immunol* 2002;9:41–5.
15. Gordon RF, McDade RL. Multiplexed quantification of human IgG, IgA, and IgM with the FlowMetrix system. *Clin Chem* 1997;43:1799–801.
16. Koertge TE, Butler JE. The relationship between the binding of primary antibody to solid-phase antigen in microtitre plates and its detection by the ELISA. *J Immunol Chem* 1985;83:283–99.
17. Pang S, Smith J, Onley D, Reeve J, Walker M, Foy C. A comparability study of the emerging protein array platforms with ELISAs. *J Immunol Methods*; in press.
18. Pantanowitz L, Horowitz GL, Upalakalin JN, Beckwith BA. Artifactual hyperbilirubinemia due to paraprotein interference. *Arch Pathol Lab Med* 2003; 127:55–9.

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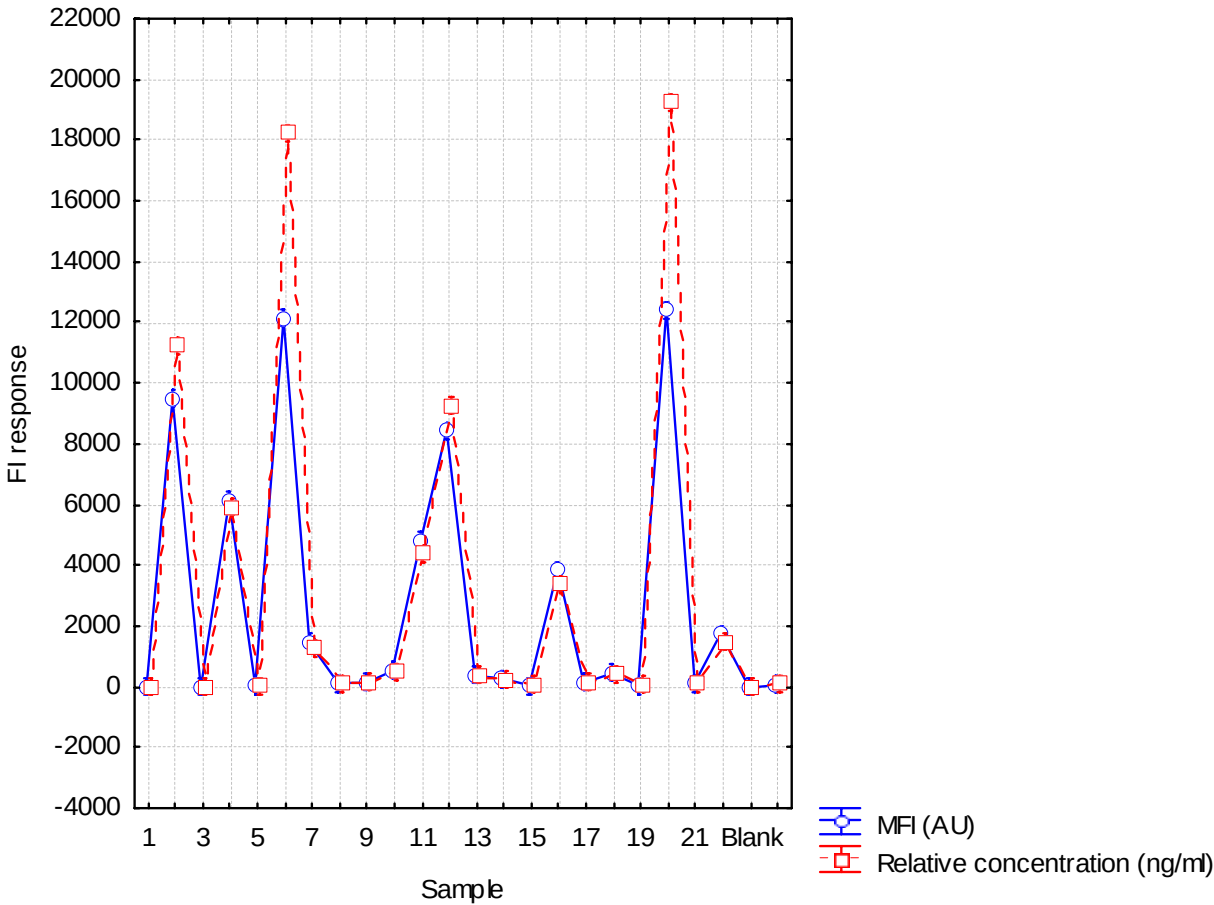
Lupus Anticoagulant: Performance of a New, Fully Automated Commercial Screening and Confirmation Assay, Barbara Montaruli,^{1*} Antonella Vaccarino,² Cristina Foli,² Cecilia Rus,² Cecilia Agnes,² Maddalena Saitta,¹ and Mario Bazzan² (¹ Laboratory Analysis and ² Department of Haematology and Thrombotic Disorders, Ospedale Evangelico Valdese–ASL-1, Turin, Italy; * address correspondence to this author at: Laboratorio Analisi, Ospedale-Evangelico Valdese–ASL-1, Via Silvio Pellico, 28, 10125 Turin, Italy; fax 39-11-6688640, e-mail b.montaruli@tin.it)

Lupus anticoagulants (LAs) are acquired circulating anti-coagulants that interfere with phospholipid (PL)-dependent coagulation tests and are frequently associated with thromboembolic disorders and obstetric complications.

Supplemental data figure 1 . Interaction graph illustrating the effects of platform and samples on the Sm IgG assays. Comparison of data presented as median fluorescent intensity (MFI) in arbitrary units (denoted by circles and solid lines) and the concentration relative to the IgG standards, in terms of ng/mL (represented by squares and dotted lines).

Supplemental data figure 2. Interaction graph illustrating the effects of platform and experiment on the Sm IgG assays. Data points represent the median fluorescent intensity (circles and solid lines) and the interpolated relative concentration (squares and dotted lines). The error bars denote 95% confidence intervals.

Supplemental data figure 1



Supplemental data figure 2

