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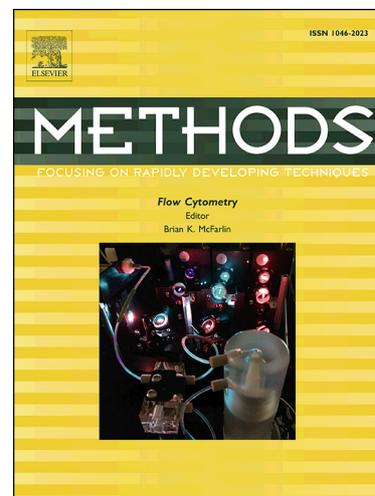
PII: S1046-2023(18)30172-5
DOI: <https://doi.org/10.1016/j.ymeth.2019.02.008>
Reference: YMETH 4633

To appear in: *Methods*

Received Date: 4 September 2018
Revised Date: 7 February 2019
Accepted Date: 11 February 2019

Please cite this article as: S.D. Dowall, V.A. Graham, T. Fletcher, R. Hewson, Use and reliability of multiplex bead-based assays (Luminex) at Containment Level 4, *Methods* (2019), doi: <https://doi.org/10.1016/j.ymeth.2019.02.008>

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Use and reliability of multiplex bead-based assays (Luminex) at Containment Level 4

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Abstract

In the UK, research on hazard group 4 (HG4) pathogens requires specialised Containment Level 4 (CL4) facilities. These differ from Biosafety Level 4 (BSL4) conditions in that work is conducted in class III microbiological safety cabinets for primary containment instead of using positive pressure suits. This presents unique challenges associated with the physical restrictions of working in a limited space, and prohibits the use of many techniques and specialist equipment. In consequence, detailed studies on the biology of HG4 pathogens and in particular their immunological relationships with the host are understudied in the UK; for example, the majority of immunological assays with which the immune system is interrogated require specialist equipment that is unsuitable for CL4.

Multiplexing to simultaneously measure multiple analytes is increasingly being used in immunological studies. This assay is attractive for CL4 work because it reduces the time spent in the laboratory whilst maximising the use of valuable sample volume. The Luminex microsphere approach allows for the determination of many cytokines and chemokines, however, the detection system uses fixed aligned lasers and integrated computer systems which are unsuitable for use at CL4. Therefore, we have developed an approach in which the Luminex assay is conducted within the CL4 laboratory and a formalin-fixation stage is introduced to allow for analysis to be undertaken outside of containment. Quality control preparations allow the assay characteristics to be monitored and analysis of assay performance to be evaluated. Our data demonstrate that Luminex is an applicable tool for use at CL4 and that assays can be run reliably to generate reproducible standardised data across different plates and individual experiments.

1 Introduction

The measurement of multiple analytes in a single sample has gained increasing popularity recently [1, 2]. Multiplexing has several advantages: (i) reduced sample volumes; (ii) reduced workload and shortened time spent performing multiple assays; (iii) assurance that all analytes are measured under the same conditions; and (iv) improved control over analytic quality and plausibility [1]. These properties make multiplex analysis attractive for use with material generated from clinical studies for immunological analysis with hazard group 4 (HG4) pathogen samples, especially as numerous cytokines and chemokines are involved in the pathogenesis of viral haemorrhagic fever infections [3].

One commonly used methodology for multiplex involves bead-based systems in which antibodies are bound covalently to fluorescent dyed beads instead of a solid carrier, as in ELISA [4]. The Luminex multi-analyte profiling (xMAP) technology uses digital signal processing, capable of classifying microsphere beads dyed with distinct ratios of red and near-infrared fluorophores, to define 'spectral addresses' for each bead population [5]. The replacement of conventional polystyrene bead suspension immunoassays (which necessitated several manual washes to prevent clogging of the filter plates used) with magnetic bead based immunoassays, enabling separation during washing steps, has significantly improved the execution of Luminex assays [2].

Work at Containment Level 4 (CL4) needs to follow strict safety and regulatory standards for high-hazard pathogens. The UK approach of containing the pathogen using a series of class III microbiological safety cabinets (MSC) arranged in a cabinet-line as primary containment for hazard group 4 (HG4) pathogens, and at the end of work undertaking decontamination with high concentrations of disinfectant and formaldehyde fumigation, restricts machine complexity and compatibility within this environment. Therefore, the integration of a Luminex analyser into the cabinet-line is not practical.

Opportunities for analysing chemokines and cytokines associated with disease caused by HG4 agents have been limited due to physical restrictions and the limited number of assays which can be employed in the CL4 environment. Previously, we have demonstrated a proof-of-principle concept using Luminex analysis for samples containing, or with the potential to contain, HG4 pathogens [6], by inactivating virus with formalin. However, since formalin alters cytokine and chemokine proteins such that their detection in immunoassays is impaired [7], we further developed a protocol that used formalin treatment after bead staining and demonstrated its suitability for use with highly pathogenic material [6]. One such HG4 agent is Crimean-Congo Haemorrhagic Fever virus (CCHFV), the most widespread tick-borne viral infection of humans. It occurs across a vast area from western China through southern Asia and the Middle East to south-eastern Europe and most of Africa [8]. Since the publication by the World Health Organisation (WHO) of its list of priority pathogens, [9], there is increased attention on this pathogen. One area of interest is vaccine development for CCHFV [10] including the analysis of immunological profiles associated with pathogenesis, requires the measurement of cytokines and chemokines. Whilst some limited work has been undertaken on measurement of cytokine levels during CCHFV infection [11-13], the ready availability of relevant samples, biocontainment facilities and assay technology has hampered progress. Similar limitations exist with other HG4 agents, such as Ebola virus where work on cytokine levels in human infection have been restricted to work during outbreaks [14-16]. Unlike Ebola virus which is characterised by

sporadic outbreaks, CCHFV is endemic within regions with more frequent case numbers, thus providing increased opportunities for analysis of factors involved with disease progression.

Our proof-of-concept paper demonstrated that formalin fixation could be utilised for fixing stained microbeads and the technique could be used at CL4 [6]. Here we refine the assay by utilising a magnetic bead microbead system and performing the assay within the CL4 laboratory alongside HG4 material. This improvement allowed us to assess the reproducibility of the method enabling precise validation of the assay performance [5]. For standardising results the internal control samples supplied with the kit were used in order to provide information of intra- and inter-assay variation [4].

2. Material and methods

2.1. Containment Level 4 (CL4) laboratory

The Luminex assay was conducted within the CL4 laboratory at Public Health England, Porton Down. The laboratory design consisting of multiple class III microbiological safety cabinets joined together to form a central spine with spurs of working cabinets on both sides (Figure 1). At one end of the spine is a dunk tank containing disinfectant which can be used to transfer small items into the cabinet line or to remove packaged virus or appropriately inactivated material. At the other end is an autoclave for removing waste. To remove waste from working cabinets or when changing cabinet use (e.g. between pathogens), cabinets are fumigated for at least 8 hours with formaldehyde vapour.



Figure 1: The CL4 laboratory at Porton Down showing the central spine with working cabinets at right angles.

2.2. Luminex staining

A 19-plex human cytokine/chemokine panel was used for this study which consisted of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon alpha-2 (IFN α 2), interferon-gamma (IFN γ), interleukin(IL)-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), IL-15, IL-17A, IFN γ -inducible protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), macrophage inhibitory protein (MIP)-1a, MIP-1b and tumor necrosis factor alpha (TNF α) (Millipore, Watford, UK). The assay was performed according to the manufacturer's instructions. As commercial multiplex kits are optimised to eliminate or minimise any artefacts from multiplexing, rigorous adherence to the

manufacturers' protocols is important [5]. Briefly, quality control and standard preparations supplied in the kit were reconstituted with deionised water. The two quality control preparations, QC1 and QC2, contain an expected amount of analytes in low and high concentration, respectively. These details are specific to the reagent lot number and values and expected ranges are supplied by the manufacturer. The standard preparation was further diluted with assay buffer to produce a range covering concentrations of 10,000, 2,000, 400, 80, 16 and 3.2 pg/ml. Within the CL4 laboratory, 25 μ l of standard and quality control preparations were added to the relevant wells followed by 25 μ l of serum matrix and 25 μ l of premixed beads supplied with the kit. Plates were incubated at 4°C overnight with continuous rocking before being washed twice with 200 μ l/well washing buffer using a hand held magnet. Washing was performed by allowing the beads to incubate in the washing buffer for 30 seconds, before securing the plate onto the magnet for 60 seconds. This allowed the beads to settle down onto the bottom of the well. The unbound contents were removed by gentle tapping onto absorbent material and more wash buffer was added. Following the wash steps, 25 μ l of detector beads were added to all wells and the plate incubator was fixed on a plate shaker for 1 hour at room temperature. Next, 25 μ l of streptavidin-phycoerythrin solution was added to each well and the plate was incubated for a further 30 minutes without any washing steps.

2.3 Microbead fixation

After completion of staining, the microbeads were washed twice with wash buffer. Beads were then resuspended with 100 μ l/well of 10% formalin solution made by dilution of 100% formalin (40% w/v formaldehyde solution) (Scientific Laboratory Supplies, Nottingham, England) 1:9 v/v with phosphate buffered saline solution (Thermo Fisher, Loughborough, England). Plates were fumigated with formaldehyde vapour overnight at room temperature for 16 hours with the lids left ajar to allow vapour to reach all surfaces. Following fumigation, plates were placed into a sealed bag and removed from the cabinet line for manipulation outside of the CL4 laboratory. Results were analysed within 24 hours of formalin solution of being added.

2.4 Acquisition and analysis of samples

Plates were washed twice with wash buffer, and once with sheath fluid in a Containment Level 2 laboratory to remove formalin solution before being resuspended in 150 μ l of sheath fluid. Results were acquired on a Luminex MAGPIX instrument using Exponent software (Invitrogen, Paisley, England). At least 50 events per region were collected and median fluorescence intensity (MFI) measured. MFI values were converted to concentrations using results from a standard cytokine preparation. On each plate, two quality control

preparations containing low and high concentrations of analytes were added, QC1 and QC2, respectively. Acceptance criteria of background wells being negative and bead aggregation being less than 20% was applied to all plates.

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3 Results

3.1 Quantified concentrations of analytes

For each batch of control preparations, a range of expected values is provided for each of the cytokines and chemokines. The actual values obtained in the assays aligned closely to the expected ranges for both the low concentration and high concentration control preparations, QC1 and QC2, respectively (Figure 2). None of the values were in excess of the expected concentrations, whereas some values were marginally below the expected limits.

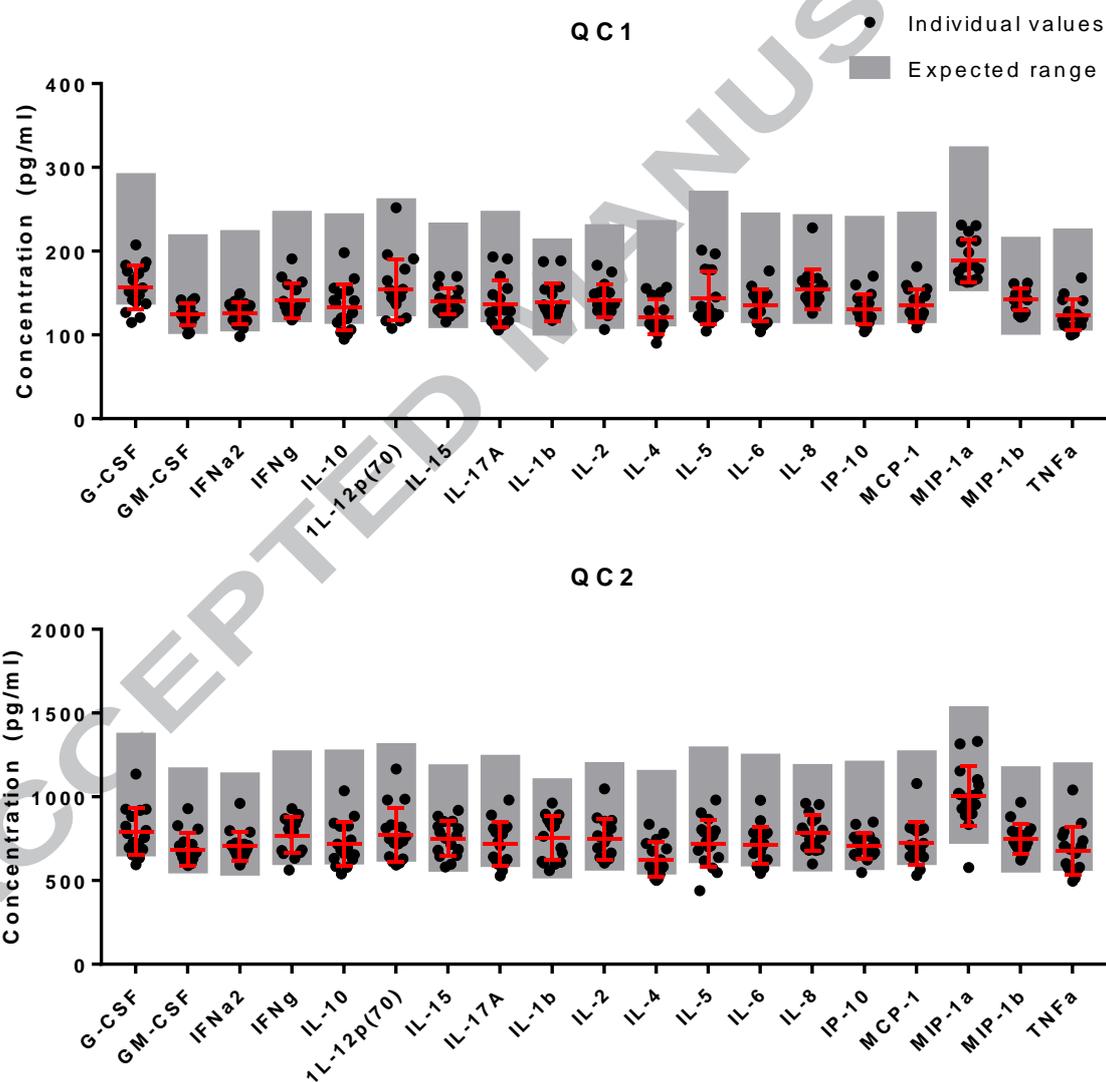


Figure 2: Concentrations of analytes in control preparations QC1 and QC2. Individual results from each Luminex plate are plotted. Grey bars represent the expected ranges provided. Red lines indicate the means with error bars denoting standard deviations. Results are determined from 16 individual plates.

3.2 Intra-assay variation

Each of the control preparations, QC1 and QC2, were run in duplicate on each plate and the coefficient of variation expressed as a percentage (%CV) was obtained (Figure 3). The mean %CV across the QC1 samples was 6.36% and that for QC2 was 10.19%; thus the level of intra-assay variation was greater when higher analyte concentrations were being measured.

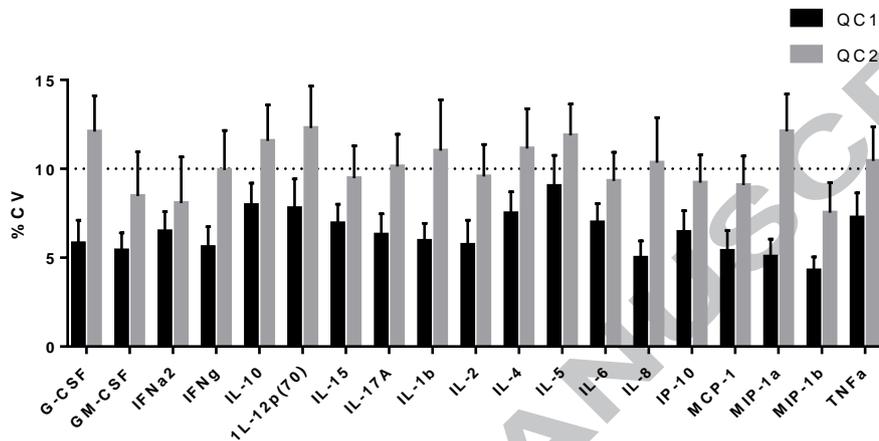


Figure 3: Intra-assay variations in the results between duplicate samples within plates. Results show the mean %CV for each of the analytes with error bars denoting standard error. Results are determined from 16 individual plates.

3.3 Inter-assay variation

The control standards were run on 16 independent plates on 6 separate occasions, allowing inter-assay variation to be assessed. The results displayed similar variations with both of the preparations used, QC1 and QC2 (Figure 4). The mean %CV for QC1 was 15.44 (range 9.24-23.78) and for QC2 it was 16.19 (range 11.04-20.85).

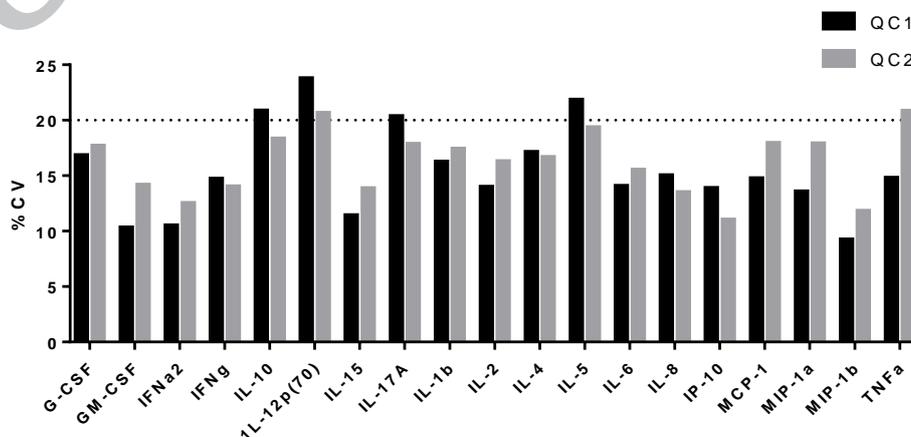


Figure 3: Inter-assay variations in the results between assays. Results show the mean %CV for each of the analytes. Results are determined from 16 individual plates run on 6 separate occasions.

4 Discussion

The Luminex assay using magnetic beads is well suited to work within the constraints of the CL4 laboratory. Unlike the polystyrene bead-system for which a vacuum arrangement is required for bead washing [6], our development of a manual washing approach with a hand held plate magnet is amenable to work with gauntlets, part of the necessary constraints at CL4. The simplicity of the approach brings additional benefits; for example it avoids the use of complex analytical machines in containment which would have inherent difficulties and additional risks when working with gauntlets (e.g. pinch points, sharps) including their intolerance to formaldehyde fumigation.

In our Luminex assays at CL4, virus inactivation with formaldehyde solution was employed post-bead staining. This avoided the detrimental effects on cytokines if inactivation is conducted prior to staining [7] and also enabled the bead-cytokine-antibody binding to be fixed so stabilising the reaction before cytokine degradation whilst materials were decontaminated via overnight fumigation. The expected ranges for the cytokine standards in the kits used in our studies have a range of $\pm 35\%$ of the median value, and the results demonstrated the recovery of cytokines from the standards were within these levels. Where others have used spike recovery, the frequency has differed by $\pm 25\%$ of expected values [17]. Whilst others have observed similarities in relative values but differences in absolute values [5, 18], our results fall within the expected ranges using standards that are prepared independently of the material used to generate the standard curves used for analysis.

To evaluate differences between assay performance, the coefficient of variation (CV) value was used. This is defined as the standard deviation (SD) divided by the mean; SDs of assays generally increase or decrease proportionally to the mean and the division accounts for this [19]. The CV is therefore a standardisation of the SD that allows comparison of variability estimates regardless of the magnitude of the analyte concentration [19]. CV analysis is thus applicable to Luminex analysis where values can vary across a large range.

To assess repeatability of results, two quality control preparations were run in duplicate on plates to determine the intra-assay %CV values. The mean %CV for the two standard preparations were 6.36% and 10.19%. Values in the region of 25% have been obtained by others [17, 20-23] but higher variances with some cytokine profiles of up to 30.3% [21] and 37.56% [24] have been reported. Luminex typically suggest values should be $\leq 10\%$ [25]. The calculation of %CV in our set-up relies on duplicates, as this is often the recommended practice for commercial assays [2].

Due to the number of Luminex assays conducted ($n=16$) over different days ($n=6$), the inter-assay variability could be assessed. Our results showed the largest range in the %CV was with the QC1 preparation containing the lower concentrations of cytokines and chemokines where values ranged from 9.24% to 23.78%. This is similar to other reported results where inter-assay %CV has been $\leq 20.84\%$ [21]. Our values fall within acceptable limits, where an

interbatch precision of %CV is recommended to be $\leq 20\%$ [2, 26], or 25% at the lower limit of quantification [26]. As concentrations of cytokines and chemokines are often altered in order of magnitude, these ranges are in line with similar assays such as ELISA [27].

It should be noted that the work performed for this manuscript used reagents with the same lot numbers. For other Luminex kits, lot-dependent differences have been observed [28]. In addition, the bead counts for each of the analytes was at least 50 to reduce variabilities between wells; lower quantities of beads result in greater CV values [29].

Internal calibrator controls along with the matrix-specific conversions are important to allow intra- and inter-assay variations to be assessed [22]. Controls to produce standard curves and the QC standard preparations used in the assay were diluted with serum matrix, since for data integrity, the accuracy of the assay requires the control samples to be measured in the same matrix type as the samples investigated [30]. The physical chemical properties (for example, pH and total protein content) of the different body fluids such as urine, tears and saliva differ, which may have an impact on the outcome of the cytokine assay [4].

Our results demonstrate the suitability of Luminex analysis for the measurement of cytokine and chemokines in samples infected, or potentially infected, with HG4 pathogens. Any study that involves sequential monitoring of patients, or other samples, should be performed using only a single technique, one platform, and one commercial supplier for all samples [5]. Due to the reliability of the Millipore kits used in this work at CL4 and the inclusion of two quality control specimens, this approach will be utilised at PHE in projects to determine the contribution of immunological markers in disease pathogenicity of viral haemorrhagic fever.

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Highlights

- Luminex analysis can be undertaken safely and effectively with material containing high containment biological pathogens.
- Formalin inactivation after bead staining allows data acquisition outside of the containment laboratory.
- Expected levels of cytokine and chemokines are detected when the assay is used within the Containment Level 4 laboratory using magnetic beads with manual washing.
- Intra- and inter-assay variations are acceptable.