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## Research paper

# Multiplex cytokine profiling with highly pathogenic material: Use of formalin solution in luminex analysis

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## ABSTRACT

Work with highly pathogenic material mandates the use of biological containment facilities, involving microbiological safety cabinets and specialist laboratory engineering structures typified by containment level 3 (CL3) and CL4 laboratories. Consequences of working in high containment are the practical difficulties associated with containing specialist assays and equipment often essential for experimental analyses. In an era of increased interest in biodefence pathogens and emerging diseases, immunological analysis has developed rapidly alongside traditional techniques in virology and molecular biology. For example, in order to maximise the use of small sample volumes, multiplexing has become a more popular and widespread approach to quantify multiple analytes simultaneously, such as cytokines and chemokines. The luminex microsphere system allows for the detection of many cytokines and chemokines in a single sample, but the detection method of using aligned lasers and fluidics means that samples often have to be analysed in low containment facilities. In order to perform cytokine analysis in materials from high containment (CL3 and CL4 laboratories), we have developed an appropriate inactivation methodology after staining steps, which although results in a reduction of median fluorescent intensity, produces statistically comparable outcomes when judged against non-inactivated samples. This methodology thus extends the use of luminex technology for material that contains highly pathogenic biological agents.

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## 1. Introduction

Determining the levels of cytokines produced during infectious disease can aid in the interpretation of pathogenic mechanisms and identify potential areas for clinical or therapeutic intervention. Of particular interest to our work is the identification and understanding of the fluxes of cytokines and chemokines produced during infections with viruses that cause haemorrhagic fevers in humans. Many of these viruses are thought to target and infect cells of the innate immune system (macrophages and dendritic cells) resulting in the release of proinflammatory cytokines and chemokines (reviewed by [Bray, 2005](#)). Of the most highly pathogenic microorganisms studied, Ebola virus has received the major

share of attention in part due to sampling and investigations during outbreaks, but also due to the availability of suitable animal models and high containment facilities in the USA. Thus, in human Ebola infection levels of interleukin (IL)-1 $\beta$ , IL-6, IL-8, IL-10, interferon (IFN)- $\gamma$ , IFN- $\alpha$ , tumour necrosis factor (TNF)- $\alpha$ , IFN- $\gamma$ -inducible protein (IP)-10, macrophage inflammatory protein (MIP)-1 $\beta$  and RANTES (regulated on activation, normally T cell-expressed and -secreted) have been monitored, showing differences in levels compared to control patients as well as between survivors and non-survivors ([Hutchinson and Rollin, 2007](#)). In addition to these cytokines, levels of granulocyte macrophage colony-stimulating factor (GM-CSF), IL-2, IL-4 and monocyte chemoattractant protein (MCP)-1 have been assessed in Ebola-infected cynomolgus macaques ([Hutchinson et al., 2001](#)).

Because so many cytokines and chemokines are potentially involved in the pathogenesis of viral haemorrhagic fevers, the ability to use multiplex assays to simultaneously assess numerous samples is extremely beneficial. This

Abbreviations: CL, containment level; MFI, median fluorescent intensity.

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application can also be extended to determine responses post-vaccination or after drug therapy, especially when assessing novel interventions. Such work with highly pathogenic material has only been possible by performing downstream assay work in high containment laboratories or by inactivating infectious material using gamma irradiation. Currently the practicalities of such down-stream assay work are difficult in the UK due to (i) a regulatory history of working with contained pathogens in biological safety cabinets, thereby using the concept of primary containment; and (ii) the consequential lack of amenable facilities including ready access to irradiation equipment. Additionally the availability of a contained (CL3/CL4) luminex reader presents difficulties: as there is an unknown risk of using of aligned lasers within microbiological safety cabinets and it is unclear what impact subsequent treatments with formaldehyde vapour during cabinet decontamination procedures will have. Thus, investigations into the role of many immunological processes associated with the pathogenesis of agents which require high containment (such as the viral haemorrhagic fever infections), necessitate an appropriate form of sample inactivation before such measurements are made.

One of the most simple and widely used methods to eradicate virus infectivity in samples is heat inactivation and this is often used in subsequent tests that require antibody detection. Although many viruses including the viral haemorrhagic fevers such as Lassa, Ebola and Marburg are inactivated by heating to 60 °C for 60 min (Mitchell and McCormick, 1984), cytokines are prone to heat degradation under these conditions so an alternative to allow measurement of these factors in clinical samples is required. Irradiation using gamma ( $\gamma$ ) rays (at approximately  $5 \times 10^6$  rad) is a suitable alternative which has been used to measure cytokines using the multiplex luminex analysis, (Hutchinson and Rollin, 2007). However, facilities for  $\gamma$  irradiation are not widely available and efficiencies of this treatment depend on the viral genome size, sera concentrations and the temperature of inactivation (Mahanty et al., 1999). Chemical inactivation of virus infectivity with formalin is another simple and routinely used method, but unfortunately such treatment alters cytokine and chemokine proteins so that their detection in immunoassays is impaired (Mahanty et al., 1999).

In this work we have investigated whether it would be possible to employ simple and routine chemical inactivation on samples after cytokine/chemokine and antibody binding to detector molecules, such that useful readout would be possible outside of high containment. We have employed the use of luminex microbeads in samples at high containment and developed standard staining procedures under these conditions, followed by an inactivation/fixation step using formalin treatment. By comparing the detection of the same molecules with and without formalin fixation we present statistically significant data that illustrates the utility of our methodology to the study of high containment pathogens.

## 2. Materials and methods

### 2.1. Sample preparation

Heparinised blood was collected from seven healthy UK volunteers. Blood was diluted at a 1:10 ratio with RPMI 1640

media (Sigma-Aldrich, Dorset, UK) supplemented with 2 mM L-glutamine (Sigma-Aldrich) and 100 U/ml penicillin plus 100  $\mu$ g/ml streptomycin (Sigma-Aldrich). 1.5 ml diluted blood was added to tubes along with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and 250 ng/ml ionomycin (Sigma-Aldrich) or 5  $\mu$ g/ml phytohemagglutinin (PHA) (Sigma-Aldrich) and 0.5  $\mu$ g/ml lipopolysaccharide (LPS) (Sigma-Aldrich). Samples were incubated at 37 °C in a 5% CO<sub>2</sub> incubator. Cells were harvested after 24 h (PMA and Ionomycin) and 5 days (PHA and LPS) by centrifugation at 300g for 5 min and collection of the supernatant. Samples were stored at –80 °C until required for analysis.

### 2.2. Luminex staining

A commercial human 26-plex luminex kit was used for this study (Millipore, Watford, UK). The assay was performed according to the manufacturer's instructions. Briefly, the wells of the 1.2- $\mu$ m filter membrane 96-well microtiter plates were pre-wetted with assay buffer. 25  $\mu$ l of sample, standard and quality control preparations were added to the relevant wells and incubated with pre-mixed microbeads for 2 h on an orbital plate shaker at room temperature. The plates were washed twice with assay wash buffer and 25  $\mu$ l biotinylated detector antibody added per well. Samples were incubated for 1 h at room temperature on the plate shaker. Without washing, 25  $\mu$ l/well streptavidin–phycoerythrin solution was added, and plates incubated for a further 30 min at room temperature on a plate shaker, protected from direct light.

### 2.3. Microbead fixation

After completion of staining, the microbeads were washed twice with assay wash buffer. Beads were then left overnight (17 h) with 100  $\mu$ l/well of 10% formalin, 4% formalin or phosphate buffered saline (PBS) solution. 10% formalin was made by diluting 100% formalin (40% w/v formaldehyde solution) (Scientific Laboratory Supplies, Nottingham, England) 1:10 with PBS. 4% formalin was made by a 1:25 dilution. Before analyzing, microbeads were washed twice in assay wash buffer and resuspended in 100  $\mu$ l/well of luminex sheath fluid.

### 2.4. Analysis of results

The luminex assay was acquired on a luminex-200™ instrument using Exponent software (Invitrogen, Paisley, England). An acquisition gate of between 8000 and 13,500 was set to discriminate against any doublet events and ensure that only single microbeads were measured. 100 events per region were collected and median fluorescence intensity (MFI) measured. MFI were converted to concentrations using results from a standard cytokine preparation. The cytokine standard was diluted 1:4 with a starting concentration of 10,000 pg/ml, giving a lower limit of detection of 3.2 pg/ml.

### 2.5. Statistical analysis

All statistical analysis was carried out using Minitab statistical software (version 15). To compare results between treatments

the data was screened for normality using the Anderson–Darling test. If data were normally distributed then a paired *t*-test was used. Where data was not normally distributed the nonparametric Mann–Whitney statistical test was used. A significance value of  $P < 0.05$  was applied to all tests.

### 3. Results

#### 3.1. Detection of cytokines in quality control samples with different formalin concentrations

Two quality control preparations supplied with the luminex kit, QC1 and QC2, had expected ranges of all cytokines between 88–305 and 461–1428 pg/ml, respectively. When cytokine levels were assessed with microbeads used with these samples, the ranges were very similar between control samples in PBS alone (0% formalin) and those treated with 4% and 10% formalin solution (Fig. 1). When results for each cytokine were compared individually between 0% vs. 4% formalin and 0% vs. 10% formalin, no significant differences were observed (Mann–Whitney statistical test,  $P > 0.05$ ). These results showed that formalin treatment after staining did not significantly affect the determination of the cytokine concentration.

#### 3.2. Effect of formalin solution on median fluorescent intensity

It was observed that after treatment with 4% and 10% formalin solution, the MFI readings were reduced for all of the cytokines tested. As an example the results from the standard

preparation at 2000 pg/ml is shown (Fig. 2), but the effect was seen in all preparations tested. This result was significantly different (Paired *t*-test,  $P < 0.001$ ) for both the 4% and 10% formalin concentrations compared to untreated samples.

#### 3.3. Effect of formalin solution on biological sample interpretation

To determine whether formalin treatment affected the measurement of clinical samples, human blood was stimulated with mitogenic preparations. Concentrations of cytokines/chemokines from mitogen-stimulated human PBMCs are shown in Table 1, showing variations in levels produced between conditions without treatment with formalin solution. Concentrations from the 4% and 10% formalin treatments were compared with untreated samples to determine whether the inactivation had any detrimental effects. The majority of cytokines tested showed that formalin inactivation did not significantly affect the yield compared to untreated samples (Fig. 3). Exceptions to this were IFN $\alpha$ 2 (4% formalin), IL-7 (4% and 10% formalin), IL-15 (4% formalin) and TNF- $\beta$  (4% formalin), where differences reached significance (Mann–Whitney statistical test,  $P < 0.05$ ).

### 4. Discussion

The results presented in this report provide evidence that luminex assays can be carried out on infectious material from CL3 and CL4 laboratories by treatment of microbeads with formalin solution post-staining. Formalin did not alter the

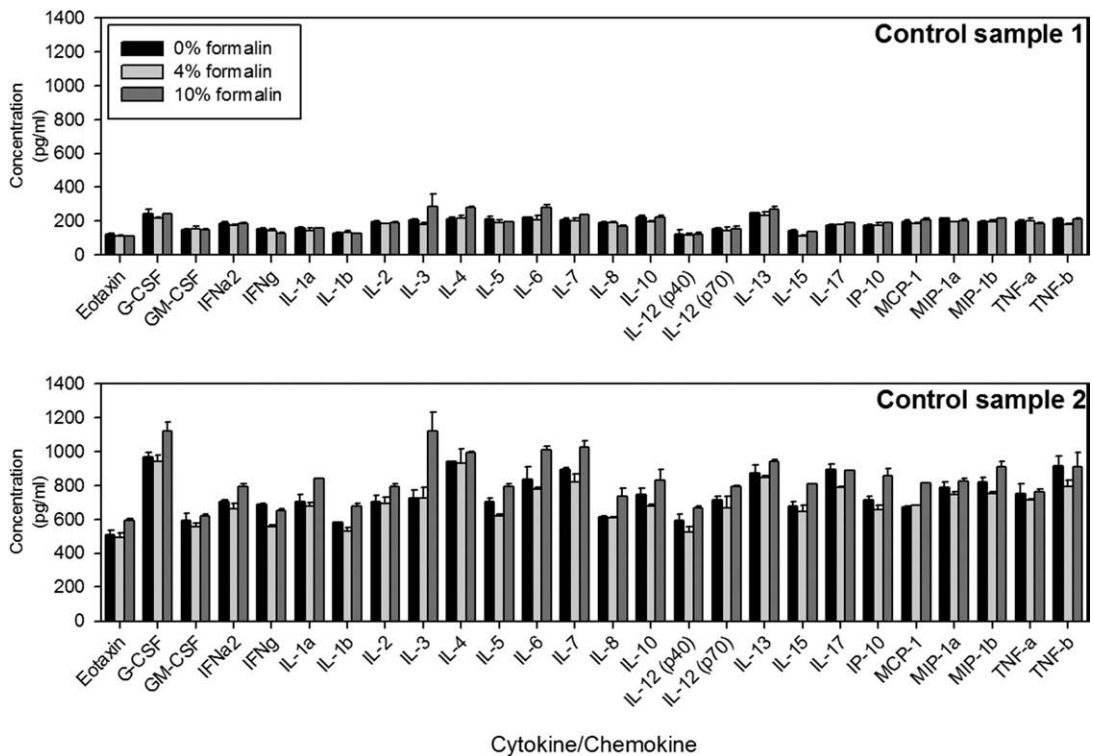
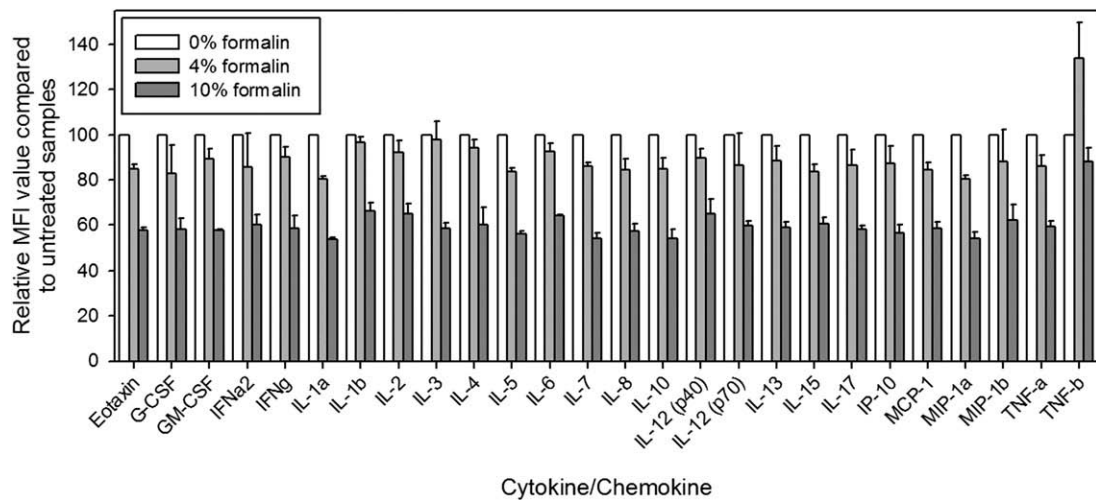


Fig. 1. Mean concentrations of 26 cytokines/chemokines in quality control preparations after treatment with 0%, 4% and 10% formalin solution (Error bars denote the standard deviation).



**Fig. 2.** Effect of 4% and 10% formalin treatment on the MFI values obtained using a preparation of 2000 pg/ml for all of the cytokines/chemokines tested (Error bars denote standard deviation. Untreated samples given a value of 100).

physical properties of the microbeads or the colorimetric dye ratios used for identifying individual bead sets (data not shown). This enabled data acquisition on the luminex-200™ analyzer using normal methodologies of setting bead regions given by the suppliers. It was observed that formalin treatment did significantly alter the MFI readings, by reducing

the intensities measured. This observation has also been seen in flow cytometry protocols, where formalin inactivation reduced MFI when cells were fixed prior (Maes et al., 2007) and after (McCarthy et al., 1994) staining with fluorescence antibodies. This effect of photobleaching by formalin has also been noted in cells fixed and mounted for immunofluorescent slide methodologies (Canete et al., 2001). The authors of this latter report suggest that an oxygen-scavenging solution may reduce the bleaching effect of fluorescent probes. However, as the reduced MFI had little effect on the concentration of cytokine detected, the use of different suspensions to increase MFI in formalin fixed samples was not further addressed.

**Table 1**

Concentrations (pg/ml) of cytokines/chemokines in mitogen-stimulated PBMCs without formalin treatment.

Cytokine/chemokine	Stimulation condition <sup>a</sup>	
	PMA + ionomycin	PHA + LPS
	(24 h)	(48 h)
Eotaxin	48.53 ± 8.97	71.86 ± 7.02
G-CSF	11.88 ± 6.58	1014.89 ± 477.29
GM-CSF	464.21 ± 126.73	335.33 ± 167.05
IFNα2	107.51 ± 36.33	162.12 ± 52.11
IFNγ	1321.98 ± 746.87	3054.56 ± 2072.15
IL-1a	12.42 ± 12.29	262.69 ± 55.12
IL-1b	50.13 ± 21.35	649.42 ± 333.29
IL-2	3168.91 ± 1816.89	43.41 ± 13.55
IL-3	8.86 ± 14.99	20.16 ± 33.00
IL-4	124.59 ± 66.17	38.10 ± 27.10
IL-5	60.53 ± 14.65	14.94 ± 18.98
IL-6	127.89 ± 47.59	5550.24 ± 3063.17
IL-7	75.20 ± 27.21	156.99 ± 17.61
IL-8	1471.12 ± 673.35	11013.27 ± 4814.11
IL-10	162.08 ± 82.55	306.56 ± 123.14
IL-12 (p40)	27.95 ± 45.03	142.75 ± 106.95
IL-12 (p70)	6.77 ± 3.42	15.70 ± 3.54
IL-13	251.64 ± 53.00	81.90 ± 35.28
IL-15	3.20 ± 0.00	3.12 ± 0.21
IL-17	74.17 ± 54.89	110.43 ± 112.83
IP-10	73.92 ± 32.30	6954.84 ± 3204.59
MCP-1	332.36 ± 131.56	6867.29 ± 2171.44
MIP-1a	1938.47 ± 596.44	6594.11 ± 4648.27
MIP-1b	2654.758 ± 630.22	5779.03 ± 2519.21
TNF-a	638.97 ± 137.06	453.98 ± 195.61
TNF-b	8.36 ± 5.20	15.77 ± 10.31

<sup>a</sup> Data are presented as average of group samples ( $n=7$ ) ± standard deviation.

Results in this report show that treating stained microbeads with formalin solution does not significantly affect the measurement for 22 of the 26 cytokines tested. To our knowledge, this is the first report showing formaldehyde fixation of luminex microbeads after prior staining. Due to constraints of working within enclosed microbiological safety cabinets and the complexity of the luminex analyser, the staining of plates can be undertaken within containment and without complication. However, the reading of results needs to be performed within lower containment facilities. Treating the beads with formalin overnight allows for inactivation of any pathogenic material and also for the sterilisation of the plates surfaces for analysis outside of containment, e.g. by fumigation with formaldehyde vapour. This is an improvement on a previous report which used 0.2% and 2% paraformaldehyde solution inactivation before quantifying cytokine levels, with a detrimental effect on most yields compared to untreated samples (Mahanty et al., 1999).

To investigate the effects on human samples, PBMCs were stimulated with PMA + ionomycin and PHA + LPS for 24 h and 48 h, respectively. In untreated samples, this resulted in a variation of cytokine/chemokine yields between mitogen treatments, and also between themselves. However, these differences would also be expected to be naturally occurring given that not all cytokines are secreted equally. Additionally, one mitogen treatment sometimes results in preferentially higher levels than the other. However, this enhances the data so as to allow a breadth of responses to be compared with

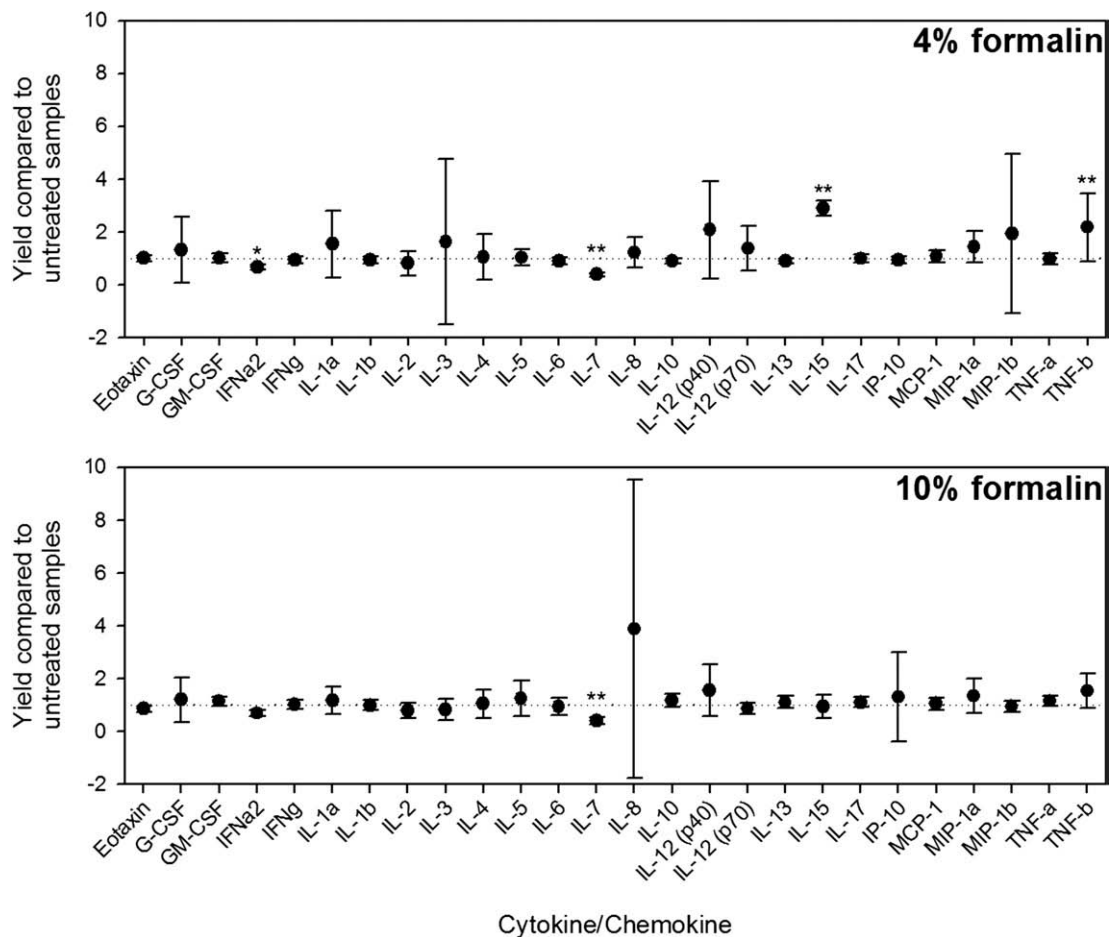
formalin treatment. As experimental needs are likely to be measuring fluxes in levels of cytokines/chemokines, the heterogeneity of data adds confidence that the observations will be application to future studies.

Of the 4 out of 26 cytokines that showed a difference with formalin treatment, only one (IL-7) was different at both concentrations (4% and 10%), whereas the remaining three were only non-significant using 4% formalin. These results suggest that using the higher concentration of 10% formalin resulted in only 1 of the 26 cytokines being statistically different to untreated sample. This observation is comparable to a study where inflammatory cytokines were measured by luminex analysis in supernatants inactivated with 0.2% and 2% paraformaldehyde prior to microbead staining. The results showed that with two cytokines of the five displayed (IL-1 $\beta$  and IL-10), responses were detected in the 2% paraformaldehyde-treated samples but not using the 0.2% concentration (Mahanty et al., 1999). Additionally, using 3% paraformaldehyde fixation for fluorescence *in situ* hybridisation resulted in cells with greater stability and integrity over time than fixation in 1% paraformaldehyde (Murrell-Bussell et al., 1998). Therefore, a higher concentration of formalin could be leading to an increased stability of the microbead that has bound with the cytokine and

detector antibodies. However, future work with differing levels of formalin solution is warranted to investigate this observation further.

The concentrations of formalin used for this analysis were 4% (1.6% formaldehyde) and 10% (4% formaldehyde). It has been shown that concentrations as low as 1% formalin can completely inactivate CL4 filoviruses such as Ebola and Marburg within 1 hour of exposure (Kuhn, 2008). Treatment with formaldehyde solutions has been reported to be effective for a wide range of viruses used in CL3 and above facilities, including: the coronavirus that induces severe acute respiratory syndrome (SARS) (Darnell et al., 2004) and Hantaan virus (Kraus et al., 2005) with concentrations of 0.009% and 1% formaldehyde, respectively. Additionally, 2% formaldehyde has been proven to be efficient in inactivation of bacteria, including *Mycobacterium tuberculosis* (Schwebach et al., 2001). As well as being used for human samples, the fact that luminex analysis is now routinely available for non-human primate material (Giavedoni, 2005) will allow for the testing of parameters using this assay in this important animal model.

Care should be exercised that the treatment with formalin sufficiently deactivates the agent being handled in the operating laboratory. Despite 0.37% (v/v) formaldehyde



**Fig. 3.** Effects of 4% and 10% formalin treatment on yield of cytokine in PHA + LPS and PMA + ionomycin stimulated PBMCs from human volunteers ( $n = 7$ ) compared to untreated samples (Cytokine yield determined by dividing the concentration of formalin-treated samples with untreated sample. Error bars denote standard deviation; Dotted line represents relevant value of untreated sample; Mann-Whitney statistical test: \* $P < 0.05$ ; \*\* $P < 0.01$ ).

being used to inactivate the human immunodeficiency virus (HIV) (Lifson et al., 1986), temperature also exerts a critical effect. For example, using 1% paraformaldehyde solution it was shown that after 18 h of incubation at 4 °C HIV was still detectable, but by incubating at 37 °C all virus had been inactivated within 6 h (Aloisio and Nicholson, 1990). In this report formalin inactivation was carried out at room temperature, as the purpose was to mimic overnight formaldehyde fumigation within a microbiological safety cabinet, where the temperature is difficult to standardise.

Other chemical methods for viral inactivation have been reported, including the use of  $\beta$ -propiolactone (Logrippo and Hartman, 1955), 3% acetic acid (Mitchell and McCormick, 1984) and aziridines (Brown, 2001). However, formaldehyde treatment has been so widely used and tested in many laboratories for the inactivation of highly pathogenic viruses that its use is better standardised and proven.

In summary, we describe an efficient method for the inactivation of luminex beads that allows the simultaneous quantification of multiple cytokines and chemokines in samples that require handling in high biological containment facilities. This work may help to elucidate further mechanisms of pathogenesis of CL4 viruses by providing an approach for multiplexing of assays, thus ensuring that maximal use is made of samples and available facilities. We acknowledge that this work forms an important proof-of-principal study and that further work may be required, including carrying out this work within class III microbiological safety cabinets.

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