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Research article

Analytical performance of a commercial multiplex Luminex-based cytokine panel in the rat

Tim Erkens*, Nick Goeminne, Ann Kegels, Martine Byloos, Petra Vinken

Mechanistic and Investigative Toxicology, Preclinical Development & Safety, Janssen Research & Development, A Division of Janssen Pharmaceutica NV, Turnhoutseweg 30, Beerse, Belgium

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ABSTRACT

Introduction: Multiplex immunoassays are an important tool in biomarker research during preclinical drug development. However, information regarding analytical performance of commercial multiplex assays for animal species is often limited. To be able to correctly interpret study results, a fit-for-purpose validation approach is recommended. The objective of our study was to provide a realistic example of what level of validation can be expected from this type of assay, using a rat cytokine panel.

Methods: The analytical performance of a commercial Luminex-based multiplex assay comprising IFN-γ, IL-6, IL-10, IL-12p70, IP-10 and TNF-α was evaluated in Sprague-Dawley rat plasma and serum. Calibration curve, working range, precision, accuracy, selectivity, parallelism, dilutional linearity, prozone effect and sample stability were assessed.

Results: Analytical performance in plasma and serum was comparable. Precision and accuracy results for all analytes were acceptable with coefficient of variation (CV) and relative error (RE) often below 15%, except for serum IL-6. Selectivity results varied per analyte with several cytokines showing CV > 30% and no single minimum required dilution (MRD) could be identified. In addition, some striking differences between recombinant and endogenous protein results were observed. A pronounced prozone effect was detected for IP-10. Analytes in samples stored at − 70 °C were stable (RE < 30%) from 1 up to 6 months depending on the analyte.

Discussion: The results illustrate the challenges encountered during validation of commercial animal Luminex-based multiplex assays, revealing analytical limitations such as matrix and prozone effects. The Milliplex rat cytokine panel under investigation was deemed suitable for relative quantification of exploratory type biomarkers.

1. Introduction

Immunoassays are an important tool in biomarker research and are of great value for generating pharmacodynamic and safety data during drug development. Besides the classical ELISA assays, multiplex immunoassays have become widely used in biomarker research. Multiplexing multiple analytes into one panel has several advantages over singleplex assays, such as reduction in time, cost and sample volume. The latter is of particular interest in preclinical (safety) research where available sample volumes are often limited, especially in the commonly used rodent models. In addition, multiplexing enables identification of consistent patterns in a single sample, instead of relying on individual biomarker data.

Although there are a number of different platforms available for multiplexing (Tighe, Negm, Todd, & Fairclough, 2013), the current paper focuses on animal Luminex-based assays using color-coded beads. Over the last couple of years, the number of commercial Luminex-based assays fit for animal use is rapidly expanding. However, the information manufacturers have available on the analytical performance of the assays is relatively limited in our experience, despite the fact that this information is critical for correct study result interpretation. This observation is supported by several other publications (Belabani, Rajasekharan, Poupon, Johnson, & Bar-Or, 2013; Khan et al., 2015).

Recommendations for immunoassay validation based on a fit-for-purpose approach have been described extensively (Andreasson et al., 2015; Findlay & Dillard, 2007; Khan et al., 2015; Lee et al., 2006; Nowatzke, Cole, & Bowsher, 2010; Valentin, Ma, Zhao, Legay, & Avrameas, 2011). Currently, there are no fixed acceptance criteria for these kind of biomarker assays and one should be cautious not to simply apply the criteria for bioanalytical pharmacokinetic assays (Tighe, Ryder, Todd, & Fairclough, 2015; Timmerman, 2016). In addition, combining multiple analytes into one assay is likely to have an impact

* Corresponding author.
E-mail address: terkens1@its.jnj.com (T. Erkens).

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on the analytical performance and adds a layer of complexity to immunoassay validation compared to singleplex assays (Chowdhury, Williams, & Johnson, 2009; Ellington, Kullo, Bailey, & Klee, 2009; Jani et al., 2016). Recently, an excellent white paper discussed the many challenges, such as minimum required dilution (MRD), cross-reactivity and selectivity, encountered when using commercial multiplex ligand binding assays (Jani et al., 2016).

The fit-for-purpose validation approach distinguishes between exploratory and decision-making biomarkers, the latter requiring the most stringent validation process (Lee et al., 2006; Valentin et al., 2011). However, since no formal criteria exist, in practice a wide range of different levels of validation are being used for (safety) biomarkers, including in good laboratory practice (GLP) settings. The objective of the current study was to provide an in-depth evaluation of the analytical performance of a commercial multiplex immunoassay for the measurement of IFN-γ, IL-6, IL-10, IL-12p70, IP-10 (CXCL-10) and TNF-α on a Luminex platform in both rat plasma and serum. The rat is an important species in preclinical drug development and this multiplex cytokine assay is a valuable tool, both for monitoring safety and for mechanistic investigations of immune modulatory compounds. Cytokine biomarkers also have great translational potential towards clinical studies. Hence it is critical to identify potential strengths and weaknesses in order to assess the level of validation that can be achieved with this type of commercial multiplex assay. The following aspects were evaluated: calibration curve, working range, intra- and inter-batch precision/accuracy, selectivity, prozone (high dose hook effect), parallelism, dilutional linearity, and sample stability.

2. Materials and methods

The Milliplex MAP Rat Cytokine/Chemokine Magnetic Bead 6-plex Panel with IFN-γ, IL-6, IL-10, IL-12p70, IP-10, TNF-α (RECYTMAG-65K; Merck-Millipore) was used according to the manufacturer's instructions, using an automated magnetic plate washer (Bio-Tek 405 LS Microplate Washer). Briefly, the assay uses 25 μl of sample to capture an analyte on analyte-specific color-coded magnetic beads coated with capture antibodies. In a next step biotinylated detection antibodies are added, followed by an incubation with streptavidin-phycoerythrin. All measurements were performed on a Magpix Luminex instrument, using xPonent 4.2 (Luminex) and Bio-Plex Manager 6.1 (Bio-Rad) software. Experimental work was conducted by 2 different analysts, each performing separate runs. Throughout the study EDTA-plasma and serum collected from the carotid artery from male Sprague-Dawley rats (Charles River Laboratories; approximately 2–4 months of age) was used, which was obtained from the on-site AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) approved rodent facility according to the applicable animal welfare guidelines and legislation. Samples were stored at approximately − 70 °C within 0.5–1 h after collection and kept at this temperature when not in use. Sample analysis started within 0.5–1 h after taking samples out of the freezer, except for stability analysis for which details are described below. Unless specified otherwise, matrix samples were diluted 1:2 in assay buffer as specified in the kit insert. In addition to the standard provided in the kit, the following external recombinant rat proteins were used for spiking of sample matrix: IFN-γ (585-IF-100, R&D Systems), IL-6 (506-RL-010, R&D Systems), IL-10 (522-RL-005, R&D Systems), IL-12 (1760-RL-010, R&D Systems), IP-10 (E-65340, PromoKine), TNF-α (510-RT-010, R&D Systems). Acceptance criteria detailed below were considered acceptable for our intended use of the assay, taking into account anticipated biologic variability and available information in literature (Defawe et al., 2012; DeSilva et al., 2003; Jani et al., 2016; Valentin et al., 2011).

2.1. Calibration curve

Each run contained a calibration curve (in duplicate) of lyophilized recombinant protein diluted in assay buffer, prepared as defined in the kit insert. Over 20 calibration curves were evaluated, using an acceptance criterion of 20% (25% at highest and lowest standard point) for precision (CV%) of duplicate concentrations and for relative error (RE %) of the mean back-calculated concentration of each standard point versus its nominal concentration.

2.2. Validation samples

In total 8 different validation samples (VS) were used to cover the working range of the assay: 2 kit quality control samples containing recombinant proteins dissolved in water, and 3 EDTA-plasma and 3 serum samples with endogenous levels of the analytes of interest. For each of these VS, a nominal concentration was established by calculating the mean of triplicate measurements from 3 independent assay runs.

2.3. Intra- and inter-batch precision/accuracy

The intra-batch concentration precision (CV%) and accuracy (RE%) of the method was determined in an assay batch in which each VS was analyzed 9-fold. For inter-batch precision and accuracy each VS was analyzed in triplicate in 5 additional assay batches, and CV% and RE% were calculated using data from all 6 runs. A maximum of 30% for precision and accuracy was considered acceptable.

2.4. Selectivity

In order to detect any differential matrix effects (endogenous matrix components that could interfere with assay performance), selectivity was evaluated using 8–10 independent rat plasma and serum samples. Each sample was spiked using either recombinant kit standard or using a sample containing endogenous levels of the analytes of interest. The volume of the spiked material did not exceed 5% of the total sample volume. A CV of maximum 30% between concentrations was considered acceptable.

2.5. Prozone (high dose hook effect)

In the presence of prozone or high dose hook effect, falsely lower concentrations of analyte are measured in samples that actually contain high levels of analyte. To investigate a potential prozone effect, plasma and serum was either spiked using recombinant protein to obtain high concentrations above the upper limit of quantification (ULOQ) or samples with high endogenous levels of the analytes of interest were used. Samples were serially diluted with kit assay buffer.

2.6. Dilutional linearity

Dilutional linearity evaluates potential matrix effects and demonstrates if analyte concentrations above ULOQ can be diluted into the validated range of the assay. At least 3 independent plasma and serum samples were spiked with recombinant kit standard and serially diluted with kit assay buffer. An accuracy (RE) of maximum 30% compared to the primary (least diluted) sample was considered acceptable.

2.7. Parallelism

Parallelism evaluates if the standard concentration–response curve is parallel to the sample dilution–response curve using samples with the endogenous analyte in sample matrix. Parallelism was assessed in at least 3 samples by serial dilution of those analytes with appropriate endogenous concentrations, using assay buffer as diluent. An accuracy (RE) of maximum 30% compared to the primary (least diluted) sample was considered acceptable.