# Multiplex profiling of human and mouse cytokines in humanized mouse models

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## Introduction

Multiplex immunoassay kits are essential for profiling a wide array of immunomodulatory proteins, including cytokines, chemokines, and growth factors in humans and animal models. Humanized mice enable the study of human immune responses, vaccine performance, and immune-related diseases. However, the cross-species homology of immune-related proteins complicates distinguishing between human and mouse homologues in the same sample. Reliable tools to differentiate these homologues are crucial for using humanized mouse models. We describe the development and validation of a multiplex assay with mouse- and human-specific antibodies against cytokines. Specificity was confirmed by testing individual mouse and human recombinant proteins. This kit was optimized for mouse samples, using mouse-analyte capture beads, while human-analyte capture beads showed no reactivity. To measure human cytokines, we spiked human PBMC supernatant into mouse serum samples, detecting the exogenous human analytes specifically only on the human beads. We validated this platform by analyzing biospecimen fluids from immunodeficient NSG mice bearing orthotopic human bladder cancer xenografts treated systemically or intravesically (locoregional) with tumor-targeting or non-targeting human CAR T cells. In this model, peripheral circulating levels of murine-derived cytokines were lower in cohorts treated with intravesical bladder CAR T cell administration compared to those receiving systemic administration. In contrast, human-derived inflammatory cytokines were higher in the urine of mice treated with intravesical bladder CAR T cell administration. These two contrasting findings highlight the applicability of this multiplex immunoassay kit in different visceral compartments and biospecimens (serum and urine). Taken together, this study demonstrates the feasibility and utility of measuring mouse and human proteins simultaneously, providing a tool for advancing research in humanized mouse models.

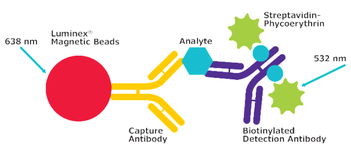
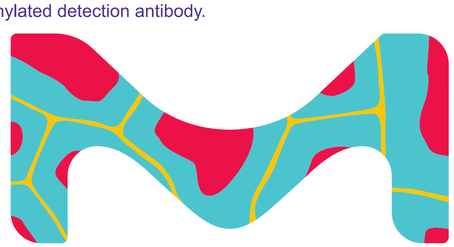



**Figure 1. MILLIPLEX® assay format.**
MILLIPLEX® assays use magnetic microspheres (beads) conjugated to capture antibodies. Each set of beads is distinguished by different ratios of two internal dyes yielding a unique fluorescent signature to each bead set, allowing researchers to simultaneously measure the analytes targeted by the capture antibodies. Native protein is analyzed by means of a "sandwich" immunoassay, pairing the capture beads with a biotinylated detection antibody.



## Methods

**Cell Culture**: Human PBMCs were stimulated with 1 µg/mL lipopolysaccharide (LPS) or Concanavalin A (ConA) for 48 hours or left untreated. PBMC supernatants were collected and used to spike mouse serum and plasma samples at a 1:10 ratio.

**Bladder Cancer Xenograft Model**: Immunodeficient NSG mice were orthotopically inoculated with human bladder cancer xenografts for 7 days. Tumor-bearing mice were adoptively transferred with tumor-targeting CAR or untransduced control T cells (UTD) via systemic intravenous (IV) or locoregional intravesical (bladder) routes. At 48 hours following adoptive transfer blood and serum were collected. Human and mouse proinflammatory cytokines were quantified using a multiplexed immunoassay.

**Multiplex Assays**: The MILLIPLEX® Humanized Mouse Magnetic Bead Panel (Cat. No. HUMU-210K) was assayed in 96-well plates according to the product manual. All assays were run on the Luminex® 200™ instrument and data was acquired via xPONENT® v. 4.3 software. Data analysis was performed for all immunoassays using the Belysa® Immunoassay Curve Fitting Software (Cat. No. 40-122).

## Results

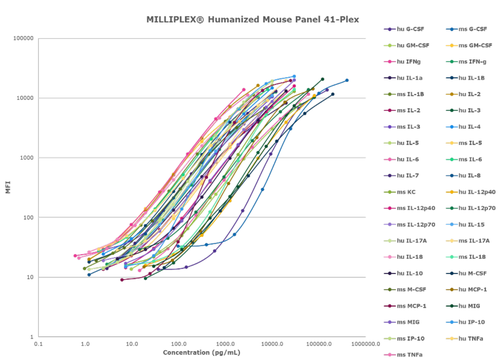



**Figure 2. 41-plex Humanized Mouse standard curves.**
MFI and concentration data were plotted from the standard curves of the 41 analytes included in the Humanized Mouse panel, according to kit instructions.

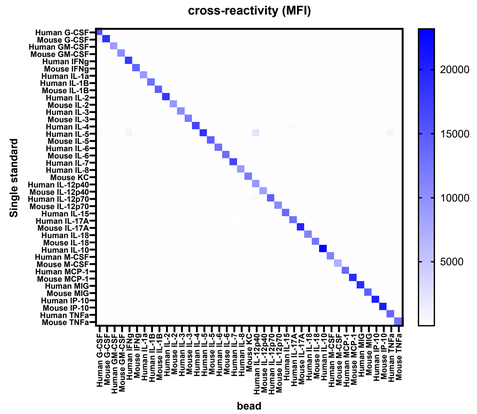



**Figure 3. Single standard cross-reactivity.**
Recombinant proteins for each analyte were tested with the 41-plex beads and 41-plex detection antibody cocktail to evaluate specificity.

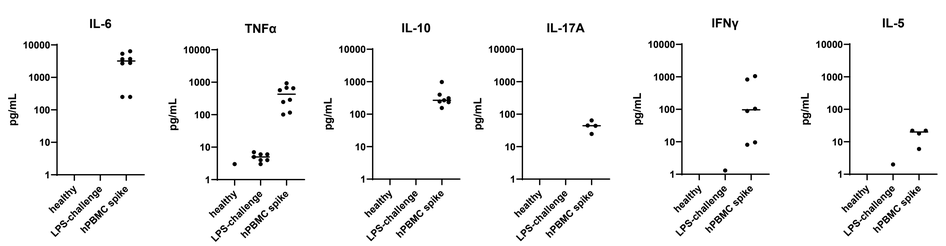



**Figure 4. Sample specificity.** Human analytes within the Humanized Mouse Panel were assessed in mouse serum and plasma samples, from healthy or LPS-stimulated individuals, to establish inter-species cross-reactivity. To establish the detection of human analytes in mouse samples, supernatant from LPS and ConA-stimulated PBMCs was spiked into mouse serum and plasma at a 1:10 ratio.

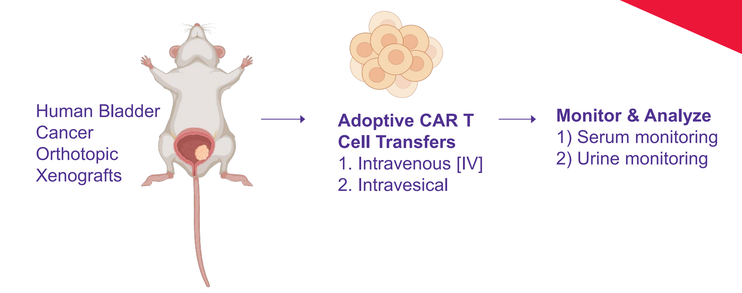



**Figure 5. Experimental schema.** Briefly, 7 days prior to adoptive transfer, NSG mice were orthotopically implanted with tumor cells. CAR T cells or control T cells were then administered either intravenously or intravesically (bladder). At 48 hours and 7 days, urine and sera were obtained from treated mice and subsequently analyzed using the humanized multiplex bead assay to identify human- and endogenous mouse-derived inflammatory cytokines.

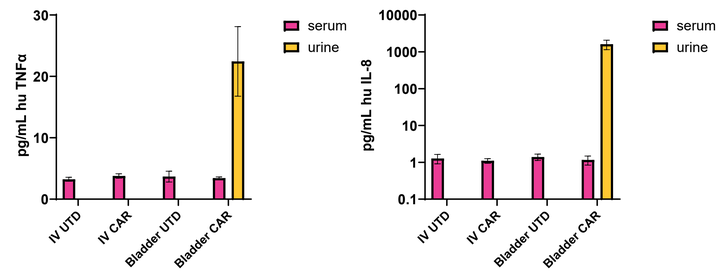



**Figure 6. Analysis of human-derived cytokines in the sera and urine of CAR T treated mice.** CAR T cells delivered intravesically (bladder) demonstrate significantly increased human-derived TNFα and IL-8 in the urine, but not sera, of mice, suggesting this route of administration leads to minimal systemic release of human-derived cytokines. Interestingly, systemic delivery of CAR T cells did not result in significant detection of human derived cytokines in the sera or the urine of orthotopic tumor-bearing mice.

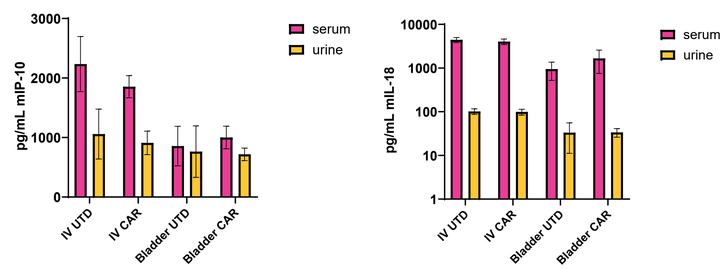



**Figure 7. Analysis of endogenous murine-derived cytokines in the sera and urine of CAR T treated mice.** CAR T cells delivered intravesically (bladder) demonstrate reduced mouse-derived IP-10 and IL-18 in the sera, suggesting this route of administration leads to reduced endogenous inflammatory cytokine release as compared to systemic delivery, and consistent with the analysis of human cytokines (Figure 6).

## Summary

Here we evaluated a novel CAR T model for the treatment of orthotopic bladder cancer, demonstrating locoregional intravesical (bladder) administration in a humanized mouse model without extravasation or cytokine release of human inflammatory cytokines into peripheral circulation of tumor-bearing mice and demonstrate reduced endogenous release of mouse cytokines with locoregional delivery.



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