Detection and Quantitation of Cytokines Using a Simple, 10-minute Assay

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Abstract

Cytokines are a broad category of proteins that regulate the responses of specific immune cell populations by binding to cell receptors and modulating intracellular signaling pathways. Recently, CAR-modified CD4+ and CD8+ T cells were reported to be equally effective in direct killing of target tumor cells, and the cytotoxic activity of these populations was associated with increased expression of both Th1 and Th2 signature cytokines. It has been demonstrated that the differing cytokine profiles of engineered T cells and native T cell receptor-expressing cells reflect varying mechanisms of activation, underscoring the importance of cytokine measurement and related assays as means for characterizing the functional properties of CAR-T cell products. Current methods for measuring cytokines include bead platforms (such as Luminex and CBA), solid-phase arrays, and intracellular cytokine staining, but the most ubiquitous means of cytokine analysis is the ELISA. ELISAs enable accurate, precise, and sensitive measurement of specific cytokines in an array of different biological samples, but are both labor intensive and time consuming, taking from 90 minutes to 4 hours to complete. In addition, cytokine ELISAs are formatted in a way that compels researchers to collect and accumulate samples over days or weeks to minimize hands-on time and costs by analyzing several samples in parallel and occupying an entire ELISA plate, rather than analyzing samples as they are obtained. In this work, we present lateral flow-based immunoassays for the analysis of both Th1 (IL-2, IFN-γ, and TNF-α) and Th2 (IL-6, IL-4, IL-10) cytokines that employ an iOS- and Android-compatible smartphone application to deliver accurate, quantitative results in approximately 10 minutes. The simplicity of these assays facilitates quick measurement of cytokine levels to enable real-time monitoring during experiments, and they can also be used to minimize the number of samples subjected to further downstream processing and analysis using more traditional immunoassays. Each two-step assay involves adding a small amount of culture supernatant or diluted serum to a lateral-flow cassette, followed by a brief 10-minute incubation. Imaging and densitometric analysis of resulting assay bands on the cassette are then performed with a smartphone running the intuitive GoStix™ Plus software application, which compares the results to an automatically downloaded, lot-specific standard curve to determine the quantity of analyte present in the sample. The GoStix Plus app provides a unit value (pg/ml) that generally falls within the same range as ELISA-based measurements but is generated in a fraction of the time. Analysis of the performance of the assay using recombinant control proteins yielded R^2 values greater than 0.99 and coefficients of variation less than 15%, indicating comparable precision to ELISA-based approaches. To demonstrate the utility of GoStix Plus assays as compared to ELISAs, we used both methods to analyze T cell culture supernatants at various timepoints following activation with either anti-CD3/CD28 conjugated beads or anti-CD3/CD28 tetramers. Both analysis methods revealed differing cytokine profiles for T cells activated by the respective methods, providing evidence of a relationship between activation strength and the conformation in which primary and secondary signals are provided. In summary, by combining the convenience of lateral-flow technology with smartphone-based image processing, GoStix Plus assays enable accurate and precise cytokine quantitation in approximately 10 minutes. These assays provide researchers with a powerful alternative to existing methods, enabling a reduction in expenses associated with labor and materials while accelerating the pace of immunology research and the development of novel therapies.

1 Standard cytokine measurement timelines

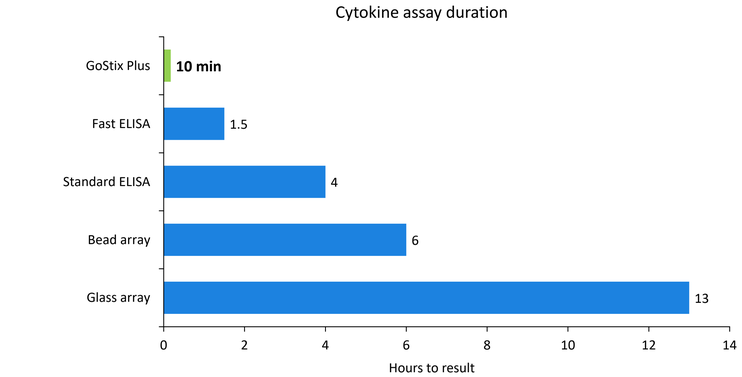

Figure 1. Commonly used methods for cytokine measurement and their associated timelines. GoStix Plus: a lateral-flow-based method for the detection of cytokines. **Fast ELISA:** measurement using targeted, preformulated ELISA reagents. **Standard ELISA:** measurement using standard direct or indirect sandwich assay. **Bead array:** multiparametric measurement by flow cytometry of bead-based, immunocapture of cytokines. **Glass array:** multianalyte measurement using quantitative, glass-slide multiplex ELISA microarray platform. Durations listed were gathered from protocols described by the manufacturer.

Conclusions

- GoStix Plus provide an easy method for quantifying Th1 and Th2 cytokines in just 10 minutes
- The GoStix Plus app yields cytokine measurements with high reproducibility and a low, consistent %CV across different mobile devices and for different GoStix Plus lateral flow tests (as was demonstrated previously for measurement of p24)
- The GoStix Plus App—with its improved user interface—provides an easy, quantitative means for consistent and effective analysis of cytokine-containing samples
- GoStix Plus results are in agreement with standard methods such as ELISA, but are delivered in a much shorter timeframe
- The speed and ease-of-use of GoStix Plus permit real-time monitoring of experiments and can serve as a complement or replacement for more standardized, but labor-intensive methods

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2 Rapid measurement of cytokines with GoStix Plus

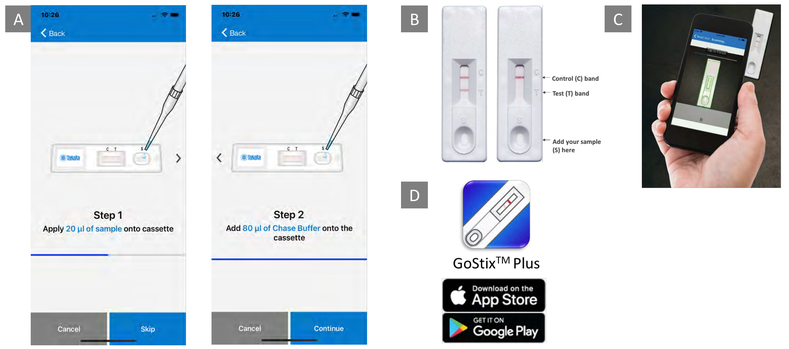


Figure 2. Rapid quantitation of cytokines using GoStix Plus. Panel A. This lateral-flow assay detects cytokines present in samples by simply applying 20 µl of culture medium or diluted serum, followed by the addition of Chase Buffer and incubation at room temperature for 10 minutes. **Panel B.** During the 10-minute incubation time, Test and Control bands will develop, indicating the presence of cytokine. **Panel C**. The results on the cassette can then be analyzed using the free GoStix Plus smartphone app, which quantifies cytokines by comparing intensities of the Test and Control bands. **Panel D.** The GoStix Plus App can be downloaded from the App Store or Google Play.

3 The GoStix Plus app's new user interface supports analysis of multiple analytes (IL-2, IFN-γ, TNF-α, IL-6, IL-10, IL-4, & lentiviral p24)

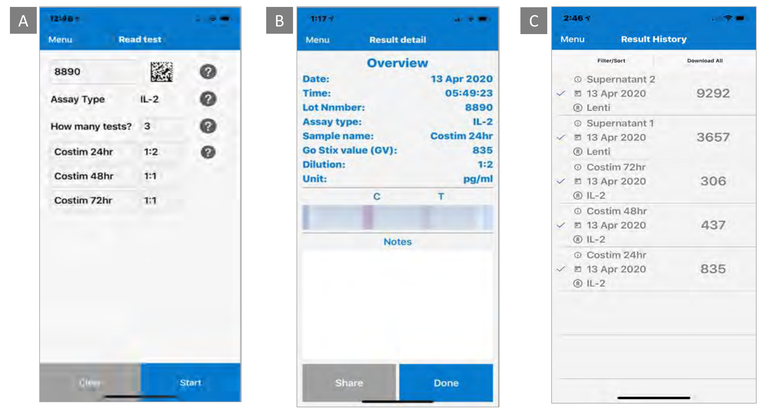


Figure 3. New GoStix Plus app user interface permits easier analysis of multiple test types. Panel A. Improvements in sample acquisition include lot-based assay designation, increased sample number size of up to 8, and an increased number of preset dilution factors. **Panel B**. The *Result detail* page includes time/date stamp, the lot number used for the control curve, sample name, GoStix Value (GV), dilution, and units of measure. An image of the read is also included, complete with a field for noting any special conditions related to the sample. These data can be distributed by email or SMS formats. **Panel C.** The *Result History* page allows entire data sets to be sorted based on assay run (IL-2, IFN-γ, etc.), and downloaded as well.

4 Establishment of control curve (iOS and Android)

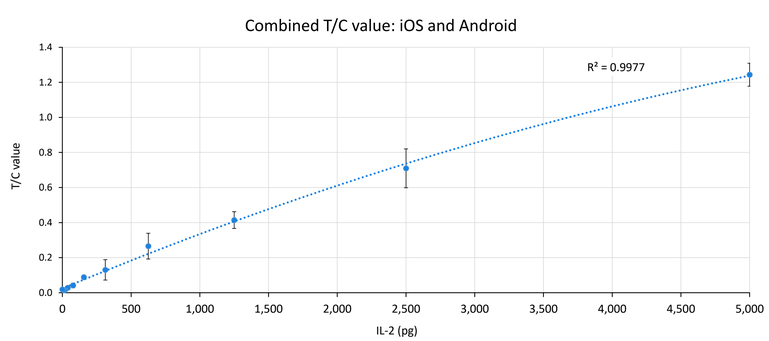


Figure 4. Generation of the internal standard curve. For each lot, a dilution series of recombinant interleukin 2 (IL-2) is prepared in tissue culture media and added to GoStix Plus in triplicate, developed for 10 minutes, and analyzed using the GoStix Plus App on both iOS (iPod 6th Gen) and Android (LG MP260) devices. T/C ratios for each amount of IL-2 are then plotted to generate the standard curve. The combined results of the two devices are then used to create the final control curve. This curve is downloaded to the application upon starting the app on any smartphone with an internet connection.

5 Low inter- and intra-assay variation with GoStix Plus

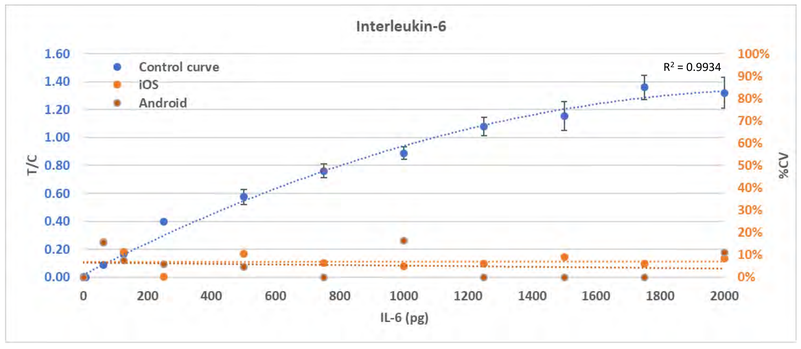


Figure 5. Low %CVs are observed using GoStixPlus. A dilution series of recombinant IL-6 was prepared in tissue culture medium and added to IL-6 GoStix Plus in triplicate, developed for 10 minutes, and then analyzed using the GoStix Plus smartphone app (v2.0.7) on both iOS (iPhone 11 Max) and Android devices (LG MP260). T/C ratios for each amount of IL-6 were then plotted to generate the standard curve. The reads were also analyzed for variance at each dilution (orange). The %CVs observed in the dilution series are in the same range as those expected for ELISA.

6 T-cell activation levels as measured by GoStix Plus & ELISA

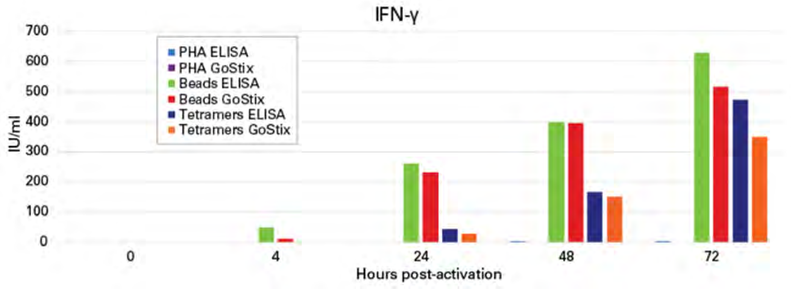
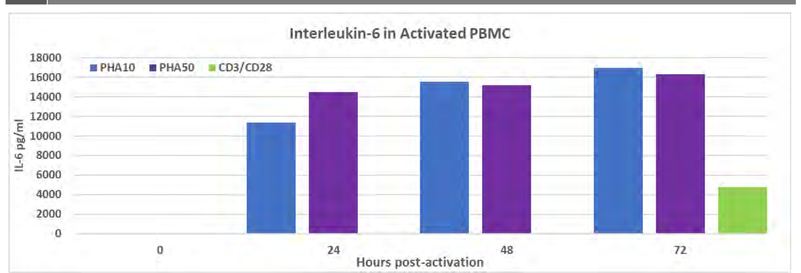


Figure 6. GoStix Plus provide results similar to ELISA. Comparison of T-cell activation methods using IFN-γ GoStix Plus and ELISA-based methods. Human primary T cells were isolated from peripheral blood (PB) mononuclear cells (MNCs) using negative selection. T cells were subsequently activated with either CD3/CD28-coated beads, CD3/CD28 tetramer complexes, or PHA (10 µg/ml). Supernatant samples were collected at the indicated timepoints and assayed for IFN-γ protein using IFN-γ GoStix Plus or an IFN-γ ELISA. Assay results were converted into IU/ml values using NIBSC reference standard 82/587.

7 Th2 cytokines in activated cell cultures measured by GoStix Plus



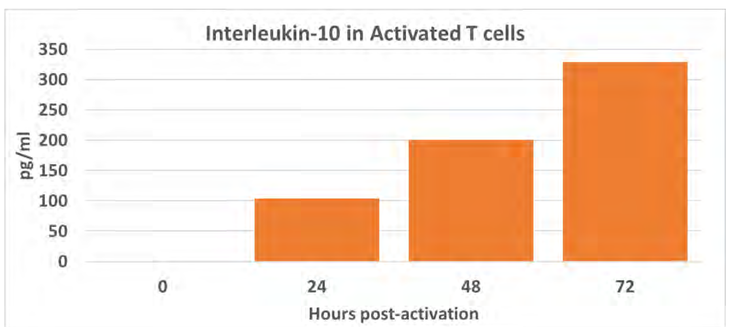


Figure 7. TH-2 cytokine detection in primary cell cultures using GoStix Plus. Panel A. Human primary peripheral blood mononuclear cells (PBMCs) were collected by apheresis and subsequently activated with either CD3/CD28-coated beads or PHA at either 10 µg/ml or 50 µg/ml, respectively. Cell culture supernatant was collected at the time points indicated after activation and assayed for IL-6 protein using IL-6 GoStix Plus. **Panel B**. Purified, human primary CD3+ T cells were activated with CD3/CD28-coated beads. Cell culture supernatant was collected at the time points indicated after activation and assayed for IL-10 protein using IL-10 GoStix Plus. All tests were allowed to develop for 10 minutes and then analyzed using the GoStix Plus app (iOS). Results are displayed in pg/ml.

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