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Automation and Analytical Techniques

Simultaneous Measurement of 25 Inflammatory Markers and Neurotrophins in Neonatal Dried Blood Spots by Immunoassay with xMAP Technology

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Background: **Inflammatory reactions and other events in early life may be part of the etiology of late-onset diseases, including cerebral palsy, autism, and type 1 diabetes. Most neonatal screening programs for congenital disorders are based on analysis of dried blood spot samples (DBSS), and stored residual DBSS constitute a valuable resource for research into the etiology of these diseases. The small amount of blood available, however, limits the number of analytes that can be determined by traditional immunoassay methodologies.**

Methods: **We used new multiplexed sandwich immunoassays based on flowmetric Luminex® xMAP technology to measure inflammatory markers and neutrophins in DBSS.**

Results: **The high-capacity 25-plex multianalyte method measured 23 inflammatory and trophic cytokines, triggering receptor expressed on myeloid cells-1 (TREM-1), and C-reactive protein in two 3.2-mm punches from DBSS. It also measured 26 cytokines and TREM-1 in serum. Standards Recovery in the 25-plex method were 90%–161% (mean, 105%). The low end of the working range for all 25 analytes covered concentrations found in DBSS from healthy newborns. Mean recovery of exogenous analytes added at physiologic concentrations in DBSS models was 174%, mean intra- and interassay CVs were 6.2% and 16%, respectively, and the mean correlation between added and measured analytes was r^2 = 0.91. In DBSS routinely collected on days 5–7 from 8 newborns with documented inflammatory reactions at birth, the method detected significantly changed concentrations of inflammatory cytokines. Measurements on DBSS stored at −24 °C for >20 years showed that most cytokines are detectable in equal concentrations over time.**

Conclusions: **The method can reliably measure 25 inflammatory markers and growth factors in DBSS. It has a large potential for high-capacity analysis of DBSS in epidemiologic case–control studies and, with further refinements, in neonatal screening.**

Newborn screening programs are based on analysis of a single sample of capillary blood collected by heel-prick from infants 3–14 days of age. The samples are usually stored as dried blood spot samples (DBSS)[5] on special filter paper that has been demonstrated to be a robust, convenient medium for collection, transport, and storage *(1)*. Most programs screen for phenylketonuria and con-

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[5] Nonstandard abbreviations: DBSS, dried blood spot sample(s); TREM-1, triggering receptor expressed on myeloid cells; CRP, C-reactive protein; IL, interleukin; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; NT-3 and -4, neurotrophin-3 and -4, respectively; MMP, matrix metalloproteinase; sTNF RI, soluble tumor necrosis factor receptor I; BDNF, brain-derived neurotrophic factor; sIL-6r, soluble IL-6 receptor; IFN-γ, interferon-γ; MCP-1, monocyte chemoattractant protein-1; GM-CSF, granulocyte/macrophage colony-stimulating factor; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; TGF, transforming growth factor; LOD, lower limit(s) of detection; and MFI, median fluorescence intensity.

genital hypothyroidism, and many laboratories screen for additional disorders. Because the blood samples are collected from populations with no selection and essentially universal coverage, residual DBSS constitute a valuable resource for retrospective screening projects and epidemiologic research. In Denmark, all residual DBSS have since 1982 been stored in a biological specimen bank at −24 °C, in accordance with regulations from the Ministry of Health *(2)*. Traditional technologies require at least one punch 3.2 mm in diameter, equivalent to ~3 μL of whole blood, for determination of a single analyte. This limits the number of analytes that can be determined in the limited amount of blood available in the DBSS; however, high-throughput multianalyte technologies such as tandem mass spectrometry and, now, the Luminex® xMAP technology open new analytical possibilities. Tandem mass spectrometry has already been shown to have value in newborn screening for a range of disorders of amino acid, fatty acid, and organic acid metabolism *(3)*.

The new xMAP technology is based on flowmetric analysis of microbeads that act as a solid support for individual assay reactions incorporating a common fluorophore reporter. Assays for several analytes can take place simultaneously on different sets of beads with unique fluorescence characteristics. The individual beads are identified, and their assay reactions are read quantitatively by the Luminex-100 instrument. Multiplexed immunoassays using the xMAP technology for determination of various biological compounds, including hormones, cytokines, and antibodies, in culture supernatants and serum have been reported *(4–11)*. The potential in newborn screening has been addressed by Bellisario et al. *(12, 13)* who described two multiplexed assays using DBSS: one for thyroxine/thyrotropin analysis and another for detection of antibodies to 3 HIV-1 antigens.

Cytokines are potent intercellular mediators that act in concert and play critical regulatory roles in inflammation and growth. In addition, they have significant systemic effects that may lead to fever, intravascular coagulation, and shock. Measurement of an array of cytokines in human body fluids is important for the study of many established inflammatory diseases and diseases of uncertain etiology *(14–17)*. The possibility of measuring a broad panel of cytokines in the small amount of blood available in archived newborn DBSS is of particular interest for the study of several congenital, neonatal, and early/late-onset diseases, including cerebral palsy, autism, and type 1 diabetes *(18–22)*. Recycling immunoaffinity chromatography, a multianalyte technique not suited for analysis of large numbers of samples, has produced tantalizing results with respect to cerebral palsy and autism *(16, 23–25)*. To date, these results have not been reproduced by the same technique or other methods.

We present a high-capacity 25-plex sandwich immunoassay method based on the xMAP technology for measurement of a panel of 23 inflammatory and trophic cytokines as well as triggering receptor expressed on myeloid cells-1 (TREM-1) and C-reactive protein (CRP) in DBSS. The method may be modified for other polypeptides and proteins and has potential for use in biobank case–control studies and, with further refinements, in neonatal screening. Details of assay development and performance on neonatal samples are provided.

Materials and Methods

ANTIBODIES AND CALIBRATORS

More than 100 antibodies from various sources were tested in different combinations. The sources of the antibodies that we found useful in this study, and of their corresponding recombinant antigens, are shown in Table 1.

ELISA REAGENT SETS

ELISA reagent sets for interleukin-6 (IL-6; cat. no. D6050), IL-8 (cat. no. D8050), tumor necrosis factor-α (TNF-α; cat. no. DTA50), and TNF-β (cat. no. DTB00) were from R&D Systems. The assays were performed according to the

Table 1. Sources of antibodies and antigens used in the xMAP assays.

Antibodies	Antibody source	Recombinant antigen source
IL-1β	R&D Systems	R&D Systems
IL-2	BD Biosciences Pharmingen	BD Biosciences Pharmingen
IL-4	BD Biosciences Pharmingen	BD Biosciences Pharmingen
IL-5	R&D Systems	R&D Systems
IL-6	BD Biosciences Pharmingen	BD Biosciences Pharmingen
IL-8	R&D Systems	R&D Systems
IL-10	BD Biosciences Pharmingen	BD Biosciences Pharmingen
IL-12	BD Biosciences Pharmingen	BD Biosciences Pharmingen
IL-17	R&D Systems	R&D Systems
IL-18	MBL	MBL
TNF-α	R&D Systems	R&D Systems
TNF-β	R&D Systems	R&D Systems
IFN-γ	BioSource	BioSource
RANTES	R&D Systems	R&D Systems
MCP-1	BD Biosciences Pharmingen	BD Biosciences Pharmingen
GM-CSF	R&D Systems	R&D Systems
MIP-1α	Biotrend/R&D Systems	Biotrend
MIP-1β	R&D Systems	R&D Systems
sIL-6ra	BD Biosciences Pharmingen	Peprotech
TGF-β	R&D Systems	R&D Systems
MMP-9	R&D Systems	R&D Systems
TREM-1	R&D Systems	R&D Systems
CRP	DAKO	DAKO
BDNF	R&D Systems	R&D Systems
NT-4	R&D Systems	R&D Systems
NT-3	R&D Systems	R&D Systems
MIF	R&D Systems	R&D Systems
sTNF RI	R&D Systems	R&D Systems

manufacturer's instructions. The assay detection limits, reported in the product inserts as the minimum detectable dose, were <0.70 ng/L for IL-6, <10 ng/L for IL-8, <4.4 ng/L for TNF-α, and <16 ng/L for TNF-β.

BUFFERS

The buffers used were as follows: phosphate-buffered saline (PBS; 140 mmol/L, pH 7.3); activation buffer (0.1 mol/L sodium phosphate, pH 6.2); coupling buffer (50 mmol/L MES, pH 5.0); washing buffer for bead coupling (PBS containing 0.5 mL/L Tween 20); blocking/storage buffer [PBS containing 10 g/L bovine serum albumin (BSA) and 0.5 g/L sodium azide]; extraction buffer [PBS containing "Complete protease inhibitor cocktail with EDTA" (Roche; 1 tablet dissolved per 25 mL of assay buffer)]; washing buffer for assay procedure (PBS containing 5 mL/L Tween); and assay buffer (PBS containing 5 mL/L Tween 20 and 10 g/L BSA).

COUPLING OF ANTIBODIES TO BEADS

Coupling of capture antibodies to carboxylated beads (Luminex Corp.) was performed according to the manufacturer's instruction: briefly, 2.5×10^6 beads were washed twice with activation buffer, resuspended in 80 μL of activation buffer, and sonicated until a homogeneous distribution of the beads was observed. Solutions (10 μL of each) of *N*-hydroxysulfosuccinimide (sulfo-NHS; Pierce) and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Pierce), both diluted in activation buffer to 50 g/L, were added to stabilize the reaction and activate the beads. After mixing, the beads were incubated with rotation for 20 min in the dark at room temperature. The activated beads were subsequently washed with coupling buffer, after which 500 μL azide-free solution of capture antibody (100 mg/L) was added and incubated with rotation for 2 h or overnight. The azide was removed from antibodies by dialysis (Slide-A-Lyzer® dialysis cassette; molecular weight cutoff, 10 000; Pierce) overnight at 4 °C against 3 L of PBS. After incubation, the beads were washed with washing buffer and resuspended in 75 μL of blocking/storage buffer. The beads were counted with a hemocytometer, adjusted to a concentration of 20×10^6 beads/mL with blocking/storage buffer, and stored protected from light at 2–8 °C.

The antibodies were coupled to bead sets as follows: TNF-α was coupled to bead 6, neurotrophin-3 (NT-3) to bead 8, matrix metalloproteinase-9 (MMP-9) to bead 9, soluble tumor necrosis factor receptor I (sTNF RI) to bead 12, TREM-1 to bead 15, brain-derived neurotrophic factor (BDNF) to bead 18, NT-4 to bead 19, IL-8 to bead 21, IL-17 to bead 25, IL-1β to bead 27, IL-18 to bead 29, soluble IL-6 receptor α (sIL-6rα) to bead 30, CRP to bead 33, IL-2 to bead 35, IL-10 to bead 42, interferon-γ (IFN-γ) to bead 44, monocyte chemoattractant protein-1 (MCP-1) to bead 46, granulocyte/macrophage colony-stimulating factor (GM-CSF) to bead 48, IL-5 to bead 50, macrophage migration inhibitory factor (MIF) to bead 52, RANTES (acronym for regulated upon activation, normal T cell expressed and presumably secreted) to bead 54, IL-12 to bead 56, macrophage inflammatory protein-1α (MIP-1α) to bead 59, transforming growth factor-β (TGF-β) to bead 61, IL-6 to bead 64, TNF-β to bead 68, MIP-1β to bead 70, and IL-4 to bead 77.

BIOTINYLATION OF ANTIBODIES AGAINST CRP

All detection antibodies, except anti-CRP, were purchased biotinylated. Before biotinylation of the CRP antibody, azide was removed by dialysis (Slide-A-Lyzer dialysis cassette, molecular weight cutoff, 10 000) overnight at 4 °C against 3 L of PBS. The solution was then diluted in PBS to a concentration of 150 mg/L, sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate [sulfo-NHS-LC-biotin; Pierce; diluted in MilliQ water (Millipore) to a concentration of 400 mg/L] was added, and the solution was incubated, with rotation, for 30 min at room temperature. PBS (500 μL) was then added, and the solution was dialyzed overnight at 4 °C against 3 L of PBS to remove uncoupled biotin. After dialysis, the volume was adjusted to 500 μL with PBS, and the solution was stored at 2–8 °C.

DEVELOPMENT OF MULTIPLEXED ASSAY

All assays were sandwich immunoassays using bead-coupled capture antibodies, biotinylated detection antibodies, and phycoerythrin-labeled streptavidin. Pairs of antibodies for each of the analytes were first evaluated separately in single-analyte xMAP setups by analysis of recombinant calibrators. Antibodies that worked properly in a single-analyte assay were sequentially added, pair by pair, into progressively larger multiplexed assays. The multiplexed assays were evaluated each time new pairs of antibodies were included. Interactions among antibodies and cross-reactions to other analytes in the multiplexed assays were observed directly from changes of the shapes of the multiplexed calibration curves or were revealed by use of different combinations of recombinant antigens or by omitting individual antibodies from the assay. Antibodies that interacted with other antibodies or cross-reacted with irrelevant analytes were excluded and, if possible, substituted with more suitable alternatives. Incubation conditions were systematically optimized for the multiplexed assays.

Occasionally sets of beads displayed very high fluorescence intensities, apparently because of nonspecific binding between biotinylated detection antibodies and unrelated capture antibodies immobilized on the beads. This phenomenon occurred primarily when measurements were performed on serum samples. Different blocking regimes were tested, and guinea pig/pig serum (1:1) added to a final concentration of 10 mL/L to the bead suspension in assay buffer before incubation with the samples gave satisfactory results.

PREPARATION OF CALIBRATORS

Various buffers and serum and blood from different animals were tested as solvents for the calibrators. A 1:1 mixture of pig (Dako) and guinea pig serum (Jackson ImmunoResearch) was empirically found to be the most suitable (see the *Results*). Mixtures of recombinant calibrators prediluted at suitable concentrations in extraction buffer were added to the pig/guinea pig serum in a volume constituting 5% of the total. Calibrators were prepared differently for analysis of cytokines in serum samples and in DBSS. The solutions were used as such as calibrators for analysis of serum samples, whereas for analysis of DBSS, they were spotted on filter paper and dried before use. Each of the calibrators was diluted to concentrations anticipated to be physiologically relevant. Because the area of a spot made from pig/guinea pig serum (200 μL) was 1.56 times the area of a spot from whole blood, this was taken into account during the calculations (see section on instrumentation and calculation). The calibrators were stored at −20 °C for several weeks without notable change in performance.

EXTRACTION OF ANALYTES FROM DBSS

Analytes were extracted from DBSS in extraction buffer with and without the presence of protease inhibitor. Extractions were performed on a microplate shaker (set at 600 rpm) in different volumes (50–200 μL), for 30, 60, 90, and 120 min, and at 4 °C, room temperature, and 45 °C. The combinations were tested on extractions each made from two 3.2-mm punches of filter paper containing dried blood from adults (see the section on human samples).

OPTIMIZED ASSAY PROCEDURE

All incubations were performed at room temperature on a microplate shaker (set at 600 rpm). Two filter-paper disks, 3.2 mm in diameter, were punched from DBSS or dried calibrators on filter paper and placed together in microtiter wells. Extraction buffer (180 μL) was added to each well, and the analytes were extracted at room temperature for 60 min. For analysis of serum samples, sera and calibrator solutions were diluted 1:10 in extraction buffer. The assay was subsequently set up for triplicate measurements of calibrators and samples. A 96-well filter plate (MultiScreen MABVN; 1.2 μm; Millipore) was prepared by prewetting with assay buffer. To each well we added 50 μL of sample (150 μL divided into 3 portions) and a 50-μL suspension of capture antibody–conjugated beads [1500 beads per analyte in assay buffer containing 1% guinea pig/pig serum (1:1)]. The capture antibodies were allowed to react with their corresponding antigens during a 1.5-h incubation, and unbound material was removed from the beads by filtration on a MultiScreen Vacuum Manifold (Millipore). The beads were washed twice with 200 μL of washing buffer per well. The captured antigens were subsequently reacted for 1.5 h with a mixture (50 μL) of biotinylated detection antibodies, each diluted 1:1000 in assay buffer. A 50-μL volume of streptavidin-phycoerythrin (20 mg/L) in assay buffer (Molecular Probes) was added to each well, and the incubation continued for an additional 30 min. The beads were finally washed twice with 200 μL of washing buffer and resuspended in 125 μL of washing buffer. After 15 min of shaking, the samples were analyzed on the Luminex 100™ according to manufacturer's instructions.

HUMAN SAMPLES

Adult whole blood, drawn from members of the research group who volunteered to give blood, was collected in EDTA tubes and cooled on ice. Occasionally it was enriched with different concentrations of recombinant analytes before it was spotted on filter paper, dried, and stored at −20 °C until use.

The serum pools consisted of normal sample residuals from anonymous women (n = 4) and men (n = 4). The samples were shipped as whole blood or serum by mail to Statens Serum Institut for analysis of serum steroid hormones. Samples from serum pools were enriched with different concentrations of recombinant analytes and stored at −20 °C until use.

Sixty-six DBSS obtained from the Danish newborn screening program were randomly and anonymously collected at arrival at the screening laboratory for use as reference material. The blood samples were taken 5–7 days after birth. For measuring increased concentrations of inflammatory markers, DBSS stored at −24 °C for 1 year in the Danish DBSS specimen bank were analyzed after informed consent was given by the parents (approved by the Scientific Ethical Committee of Denmark, protocols 11-035/01, 11-040/02, 11-042/02, and 11-048/02). Of 8 newborns affected at birth, 2 had a large placental infarct and 6 had positive bacterial cultures grown from the cord blood or placenta (Copenhagen University Hospital Hvidovre). Seven newborns with no signs of infection or infarct were used as controls. We have no postnatal clinical information about the infants.

For stability studies, DBSS stored for 23 years, 3 years, and 1 month, respectively, were taken anonymously from the Danish DBSS specimen bank. The mean concentration of each analyte from each period was calculated from 10 samples and compared with routinely collected anonymous DBSS that were stored in the laboratory for 2 weeks at −20 °C.

CHARACTERIZATION OF ASSAYS

Definitions. The LOD for each analyte was defined as the concentrations corresponding to the mean median fluorescence intensity (MFI) plus 2 SD of the 0 calibrator for each analyte. For both serum and DBSS, the values were based on 11 readings of the 0 analytes, after interpolation on the calibration curve to determine the corresponding concentrations. The working range for each analyte was assessed from the precision profile and defined as the concentration range in which the CV (SD/mean × 100%) was <20% *(26)*. Intra- and interassay imprecision was

defined as the CV of repeated measurements on series of identical samples performed the same day and different days, respectively. The correlation between added and measured analytes in samples was expressed as r^2 from the Pearson correlation coefficient calculated on basis of enrichment with different physiologic concentrations. The amounts of exogenous analytes recovered in enriched samples were expressed as: recovery (%) = [(concentration measured − endogenous concentration)/known concentration added] × 100%.

Serum. To assess the working range in serum, pig/guinea pig serum was enriched with the following concentrations of analytes: 0, 7.8, 31, 125 500, 1000, 2000 and 4000 ng/L IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IFN-γ, TNF-α, NT-3, and NT-4; 0, 19.5, 78.1, 312.5, 1250, 2500, 5000, and 10 000 ng/L IL-18, GM-CSF, TNF-β, MCP-1, MIP-1α, and MIP-1β; 0, 0.16 0.63, 2.5, 10, 20, 40, and 80 μg/L sTNF RI and BDNF; 0, 78, 312, 1250, 5000, 10 000, 20 000, and 40 000 ng/L TGF-β; 0, 1.95, 7.81, 12.5, 50, 100, 200, and 400 μg/L sIL-6rα; 0, 0.63, 2.5, 10, 20, 40, 80 and 160 μg/L RANTES; 0, 0.98, 3.9, 15.6, 52.5, 125, 250, and 500 μg/L MMP-9 and TREM-1; and 0, 0.195, 0.78, 3.13, 12.5, 25, 50, and 100 μg/L MIF. The CVs were calculated from 6 measurements of each concentration. To establish intraassay CVs, we assayed samples from the serum pool in duplicate 16 times within the same run; for interassay CVs, we measured samples from the pool in duplicate in 8 different runs. To determine the correlation between added and measured analytes, we enriched 5 samples from a serum pool with 0, 31, 63, 125, and 250 ng/L IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IL-18, IFN-γ, TNF-α, MIP-1α, GM-CSF, and NT-4; 0, 100, 200, 400 and 800 ng/L IL-8; 0, 31, 63, 500 and 2000 ng/L TNF-β, MCP-1, MIP-1β, and NT-3; 0, 1.0, 2.0, 4.0, and 8.0 μg/L MIF, sTNF RI, TGF-β, and TREM-1; 0, 1.0, 4.0, 8.0, and 16.0 μg/L RANTES; 0, 16.0, 32.0, 64.0 and 128.0 μg/L sIL-6rα; 0, 100, 200, 1600, and 64 000 μg/L MMP-9; and 0, 1.0, 2.0, 16.0, and 64.0 μg/L BDNF.

DBSS. Assessment of the working range in DBSS was based on measurements of filter-paper calibrators (see the section on preparation of calibrators) as well as of enriched DBSS models. Concentrations used in the filter-paper calibrator measurements were 0, 7.8, 31, 125, 500, 1000, 2000, and 4000 ng/L IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IFN-γ, TNF-α, and NT-4; 0, 19.5, 78.1, 312.5, 1250, 2500, 5000, and 10 000 ng/L IL-18, GM-CSF, TNF-β, MCP-1, MIP-1α, and MIP-1β; 0, 0.16, 0.63, 2.5, 10, 20, 40, and 80 μg/L BDNF and TGF-β; 0, 0.39, 1.56, 6.25, 25, 50, 100, and 200 μg/L sIL-6rα; 0, 0.63, 2.5, 10, 20, 40, 80, and 160 μg/L RANTES; 0, 0.98, 3.9, 15.6, 52.5, 125, 250, and 500 μg/L MMP-9 and TREM-1; and 0, 5, 20, 80, 320, 640, 1280, and 2560 μg/L CRP. The CVs were calculated from 6 measurements at each concentration. The DBSS models, made from blood from a healthy adult male, were also used to determine the correlation (r^2) between added and measured analytes and for determination of recovery. The DBSS models were enriched with 0, 31, 63, 125, 250, 500, 1000, and 2000 ng/L IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IFN-γ, TNF-α, MIP-1α, GM-CSF, and NT-4; 0, 125, 250, 500, 1000, and 2000 ng/L MIP-β and TNF-β; 0, 500, 1000, and 2000 ng/L MCP-1; 0, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 μg/L TGF-β, BDNF, and TREM-1; 0, 2.0, 4.0, 8.0, 32.0, and 128.0 μg/L RANTES; 0, 8.0, 16.0, 32.0, 64.0, and 128.0 μg/L sIL-6rα; 0, 100, 200, 400, 800, 3200, and 6400 μg/L MMP-9; and 0, 160, 320, 640, and 1280 μg/L CRP. The CVs were calculated from 9 measurements at each concentration.

To establish intra- and interassay imprecision, another set of DBSS models were enriched with the following concentrations: 125 ng/L (low) and 2000 ng/L (high) for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IFN-γ, TNF-α, TNF-β, MCP-1, MIP-1α, MIP-1β, GM-CSF, and NT-4; 4.0 μg/L (low) and 32 μg/L (high) for TGF-β, TREM-1, and BDNF; 8.0 μg/L (low) and 64 μg/L (high) for sIL-6rα and RANTES; 80 μg/L (low) and 640 μg/L (high) for CRP, and 80 μg/L (low) and 3200 μg/L (high) for MMP-9. For determination of intraassay CV, 16 DBSS models with no added analytes and 16 models enriched with high concentrations of analytes were measured in triplicate within one run on the same day. For determination of the interassay CV, models enriched with low and high concentrations of analytes were measured in triplicate on 12 different days.

INSTRUMENTATION AND CALCULATION

The Luminex 100 and the Luminex XYP™ platform were from Luminex Corp. The software, Bio-Plex™ 3.0, was from Bio-Rad. The array reader was calibrated with the high RP1 target value from the CAL2 calibration beads (Bio-Plex). For each region, 50 or 100 beads were counted, and the MFI values for each region were used. All samples and calibrators were measured in triplicate, and the mean MFI values were used for calculations of concentrations.

The calibration curves for each analyte were calculated by the Bio-Plex 3.0 software. Although a linear response was obtained for all analytes down to a certain concentration, use of a logistic regression method was preferred to obtain the most accurate determination of the analytes present in low concentrations in the extracts from the DBSS. The 5-parameter logistic equation (Logistic-5PL) was used *(27)*:

$$y = d + \frac{a - d}{\left[1 + \left(\frac{x}{c}\right)^b\right]^g}$$

where x is the concentration, y is the response, a is the estimated response at zero concentration, b is the slope of the tangent midpoint, c is the midrange concentration or midpoint, d is the estimated response at infinite concentration, and g is the asymmetry factor.

The goodness of fit for the 5-parameter logistic regression calibration curves in the multiplex assay is expressed as the Standards Recovery (Backcalculation of Standards) and is given as the means of all analytes with the highest and lowest values *(28)*.

All statistics analyses were performed with the software Prism 3.0.

Results

DEVELOPMENT OF MULTIPLEXED ASSAYS

Many pairs of antibodies that worked well alone were of no use in combination with other antibodies. Inclusion of an additional pair of antibodies to a panel could compromise the analysis of several analytes in an otherwise well-functioning multiplex assay. A frequent problem was binding between detection antibodies and capture antibodies immobilized on the beads, which gave a high background and low sensitivity. Various combinations of antibodies were evaluated. In general, monoclonal antibodies were less problematic than polyclonal, but inclusion of additional pairs of antibodies to a panel almost invariably impaired the sensitivity to some degree.

Initial experiments revealed that for calibrators dissolved in some buffers, the MFI readings for some analytes in the zero-concentration samples were actually higher than the corresponding readings obtained from samples of serum or DBSS extracts. This paradox was presumably caused by substances present in samples, but not in the buffers, that prevented nonspecific binding of detection antibodies to capture antibodies bound to the beads. Alternative solvents for calibrators were evaluated, and a mixture of pig and guinea pig serum (1:1) was empirically found to work well with respect to assay sensitivity, antibody interactions, reaction with endogenous cytokines, and recovery of exogenous recombinant cytokines. Systematic checks for cross-reactions, using various recombinant antigens alone and in combination, demonstrated that only the relevant pair of antibodies used in the multiplexed assays described below reacted with a certain analyte.

EXTRACTION OF DBSS

The presence of protease inhibitor in the extraction buffer generally led to significantly higher MFI readings for most analytes under all conditions, particularly long incubation times. Extraction for 60–90 min was optimal for the analytes investigated, and there was a clear decrease in fluorescence for some analytes after 120 min of incubation.

Extraction at 45 °C gave higher signals compared with room temperature and 4 °C; however, the beads tended to agglutinate at that temperature, which produced technical problems. We observed no significant difference between extractions at room temperature and 4 °C.

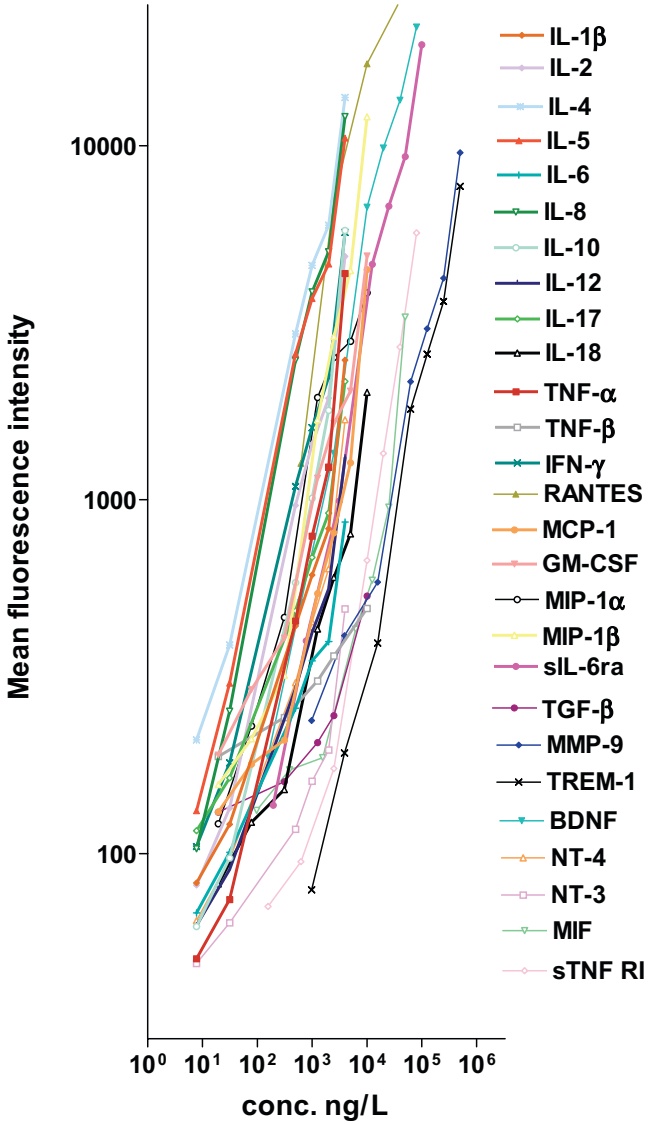

Fig. 1. Calibration curves for quantification of 27 analytes in human serum samples.

Calibrators were dissolved at appropriate concentrations in pig/guinea pig serum (1:1). Blank values are not subtracted.

PERFORMANCE OF THE OPTIMIZED ASSAYS

Calibration curves were first established for measurement of 26 cytokines and TREM-1 in serum (Fig. 1). The slopes of most curves were also fairly steep in the low (10–100 ng/L) concentration ranges. Standards Recovery was 104%–216% (mean, 118%). The performance of this 27-plex assay is shown in Table 2. The concentrations measured in a human serum pool were within the working range for all analytes. For comparisons with conventional ELISA, samples from the enriched serum pool were measured by the multiplexed xMAP assay and 4 commercial ELISAs for TNF-α, TNF-β, IL-6, and IL-8, respectively. In our hands, the ELISAs detected TNF-β and TNF-α only if these analytes were added at a relatively high concentration of 125 ng/L. The ELISA and the multiplex xMAP

Table 2. Characteristics of the 27-plex xMAP assay for analysis of serum.[a]

Analyte	Serum pool, ng/L	Working range, ng/L	LOD, ng/L	r^2 (enriched serum)	CV, %	
					Intraassay	Interassay
IL-1β	20	7.8–4000	54.0	0.99	10	17
IL-2	10	7.8–4000	30.9	0.87	16	23
IL-4	11	7.8–4000	7.1	0.98	6.7	10
IL-5	10	7.8–4000	7.8	0.98	11	18
IL-6	135	7.8–4000	60.4	0.90	9.3	23
IL-8	146	7.8–4000	8.1	0.97	11	13
IL-10	128	7.8–4000	46.1	0.96	13	21
IL-12	71	7.8–4000	57.1	0.95	12	7.3
IL-17	116	7.8–4000	39.7	0.95	12	21
IL-18	98	19.5–10 $\times$ 10^3	124.2	0.92	6.9	22
TNF-α	35	7.8–2000	54.1	1.00	13	25
TNF-β	0.61 $\times$ 10^3	19.5–10 $\times$ 10^3	1.18 $\times$ 10^3	0.99	6.9	18
IFN-γ	7	7.8–4000	14.4	0.99	12	12
RANTES	11.7 $\times$ 10^3	625–160 $\times$ 10^3	72.7	0.91	12	9.3
MCP-1	1.01 $\times$ 10^3	19.5–10 $\times$ 10^3	146.1	0.98	9.8	16
GM-CSF	75	19.5–10 $\times$ 10^3	85.4	0.93	10	17
MIP-1α	95	19.5–10 $\times$ 10^3	49.6	1.00	10	12
MIP-1β	341	19.5–10 $\times$ 10^3	47.8	1.00	12	18
SIL-6ra	0.43 $\times$ 10^6	1950–400 $\times$ 10^3	21.6	0.97	9.7	20
TGF-β	0.75 $\times$ 10^3	78–40 $\times$ 10^3	1.17 $\times$ 10^3	0.99	12	22
MMP-9	0.93 $\times$ 10^6	976–0.50 $\times$ 10^6	8.8 $\times$ 10^3	0.80	9.7	9.7
TREM-1	542	976–0.50 $\times$ 10^6	2.10 $\times$ 10^3	0.96	7.2	10.5
BDNF	22.5 $\times$ 10^3	156–80 $\times$ 10^3	82.6	0.92	9.5	11
NT-4	34	7.8–4000	58.7	0.99	10	16
NT-3	669	7.8–2000	132.9	1.00	12	19
MIF	1.06 $\times$ 10^3	195–50 $\times$ 10^3	0.55 $\times$ 10^3	0.99	12	14
sTNF RI	0.74 $\times$ 10^3	156–80 $\times$ 10^3	2.75 $\times$ 10^3	0.99	12	23
Mean				0.95	11	17

[a] Measurements were performed on a pool of human serum, and the intra- and interassay CVs were determined by repeated measurements. The working range is defined as the range of concentrations for which the CV was <20%. It is determined by repeated measurements of a mixture of animal serum enriched with different concentrations of the analytes. Correlations between added and measured analytes in the enriched samples are expressed by r^2 from the Pearson correlation coefficient. See *Materials and Methods* for further details.

assay gave generally comparable results for IL-6, whereas the concentrations of IL-8 reported by that ELISA were ~3 times higher than the amount reported by the xMAP assay.

Calibration curves for multiplex determinations of 25 analytes in DBSS are shown in Fig. 2. Three analytes that worked well for analysis of serum (NT-3, MIF, and sTNF RI) for some reason did not function together with the other analytes when the calibrators were spotted on filter paper. Another analyte, CRP, did however work well for analysis of DBSS and was consequently included in the panel. The slopes of the curves in the low concentration ranges for some analytes were not as steep as the corresponding regions of the curves for serum samples. Standards Recovery was 90%–161% (mean, 105%), indicating a good fit of the curves. We assessed the working range of the 25-plex assay on the basis of both calibrators dried on filter paper and added to DBSS models prepared with blood from a healthy adult. However, because of endogenous cytokines present in human blood, we could not assess the low end of the working range for the DBSS models down to the wanted concentrations. There was a variation in precision profiles among analytes, and the precision was generally better for filter-paper calibrators than for enriched DBSS models. Acceptable imprecision in the working range for both matrices was set to CV <20%. All tested concentrations of analytes in the filter-paper calibrators met this requirement. The low end of the working range for all 25 analytes covered the 25%–75% percentiles of analyte concentrations in DBSS obtained 5–7 days after birth from 66 random newborns (Table 3 and 4). In the case of DBSS models, all analytes met the requirement at low concentrations, including the endogenous concentrations present in the blood used for making the models. Enrichment of the DBSS models with high concentrations of analytes, however, gave a CV above the 20% limit for TGF-β, confining the upper end of the working range for this analyte slightly (Table 3).

Notably, the working range limits assessed by use of enriched DBSS models were within the working ranges assessed by the filter-paper calibrators. The lower limits of detection (LOD), defined as the concentrations corre-

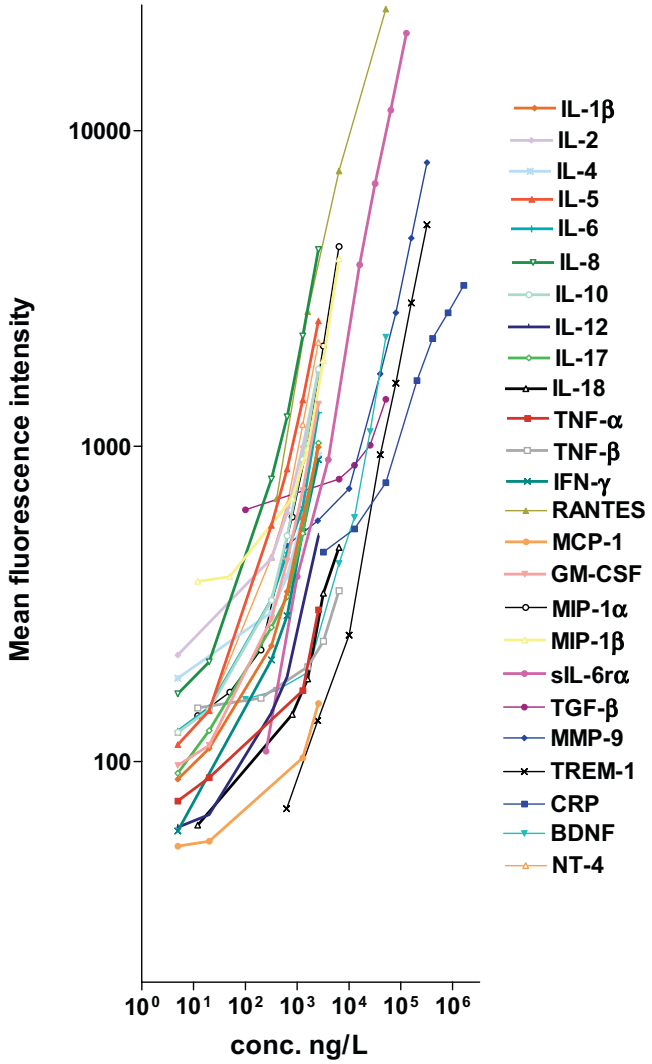

Fig. 2. Calibration curves for quantification of 25 analytes in DBSS.

Calibrators were dissolved at appropriate concentrations in pig/guinea pig serum (1:1), spotted on filter paper, and dried. Calibrators were extracted from two 3.2-mm punches and analyzed as described. Blank values are not subtracted.

sponding to the MFIs for the 0 calibrators plus 2 SD, were for all analytes lower than the median concentrations found in newborns except for IFN-γ (Table 3 and 4). The intraassay CVs calculated by repeated measurements of DBSS prepared from blood drawn from a healthy adult male were 0.6%–8.0% (mean, 5.7%), and if prepared from blood enriched with high concentrations of analytes, were 1.1%–12% (mean, 6.7%). Interassay CVs based on DBSS enriched with low concentrations of analytes were 7.8%–26% (mean, 15%), and if enriched with high concentrations, were 10%–25% (mean, 16%). It was evident that the accuracy of the work performed by the technician had a very significant impact on the imprecision of the multiplex assay. The correlation (r^2), calculated from the Pearson correlation coefficient, between added and measured concentrations in samples enriched with concentrations within the working range of each analyte ranged from 0.71–0.99 (mean, 0.91). The recovery of exogenous added cytokines was rather variable with recovery varying from 34% for the lowest amount of recovered analyte and 471% for the highest (mean recovery, 174%), indicating that exogenous cytokines may be either sequestered or not freely soluble in all blood compartments. The ELISAs used in this study were not sensitive enough in our hands to measure cytokines in DBSS at any of the concentrations added.

Measurement of inflammatory markers in neonatal DBSS

To further evaluate the reliability of the multiplexed assay, we analyzed DBSS from 8 newborns in whom changes in concentrations of inflammatory markers might be expected because of inflammatory conditions at birth (Table 5). IL-8, IL-12, IL-17, IL-18, TNF-α, TNF-β, IFN-γ, MCP-1, TREM-1, and NT-4 concentrations were significantly increased, and TGF-β were significantly decreased compared with a group of 7 healthy control infants. The pro-inflammatory cytokines IL-2 and IL-6 were also noticeably increased, but not significantly.

Stability of analytes in DBSS stored at −24 °C

The measurable amounts of most cytokines were nearly constant in DBSS stored up to 23 years. A marked decrease over time was observed in measurable concentrations of IL-1β, IL-8, sIL-6rα, MMP-9, TREM-1, CRP, BDNF, and NT-4 in stored DBSS (Table 6).

Discussion

Inflammatory reactions are believed to be important in the pathogenesis of certain congenital and early-onset disorders. The prospect of detecting inflammation by measurement of specific markers in neonatal blood samples has been explored in several studies *(16, 18, 19, 21, 24, 29–32)*. Best suited for such ventures in terms of sample accessibility is capillary blood collected as DBSS, which are widely used for routine screening of newborns and are well suited for long-term storage *(1)*. The small amount of blood available in the DBSS, combined with the low concentrations of many analytes relevant for monitoring inflammation, however, places high demands on the analytical methods. Multiplexed measurements of cytokines and coagulation factors by recycling immunoaffinity chromatography has been reported, but the method is highly specialized and laborious *(24, 33)*. We have developed a 25-plex high-capacity method for simultaneous determination of 23 cytokines, TREM-1, and CRP in two 3.2-mm punches of routinely collected neonatal DBSS, based on the commercially available Luminex xMAP technology. The short processing time and the potential for automation make the method suitable for studies of large populations of newborns.

The 25-plex method demonstrated significantly increased concentrations of IL-8, IL-12, IL-17, IL-18, TNF-α, TNF-β, IFN-γ, MCP-1, TREM-1, and NT-4 and decreased

Table 3. Characteristics of the 25-plex xMAP assay for analysis of DBSS.[a]

	Filter-paper calibrators		Enriched DBSS models			CV, %	
Analytes	Working range, ng/L	LOD, ng/L	Working range, ng/L	Recovery, %	r^2	Intraassay	Interassay
IL-1β	7.8–4000	26	22 + (0–2000)	120	0.95	6.7/6.0	11/22
IL-2	7.8–4000	41	54 + (0–2000)	225	0.92	7.2/8.8	11/11
IL-4	7.8–4000	0	35 + (0–2000)	131	0.81	4.2/6.2	9.7/19
IL-5	7.8–4000	2	10 + (0–2000)	471	0.94	5.9/6.9	13/16
IL-6	7.8–4000	24	13 + (0–2000)	284	0.90	5.3/10	16/17
IL-8	7.8–4000	1	147 + (0–2000)	168	0.95	8.4/6.5	16/15
IL-10	7.8–4000	76	18 + (0–2000)	167	0.96	5.4/6.1	13/15
IL-12	7.8–4000	56	33 + (0–2000)	81	0.93	4.7/4.5	12/13
IL-17	7.8–4000	24	27 + (0–2000)	157	0.91	5.8/6.5	17/16
IL-18	19.5–10 × 10^3	177	1810 + (0–2000)	234	0.95	6.7/4.8	14/10
TNF-α	7.8–4000	34	38 + (0–2000)	141	0.90	5.5/5.6	16/20
TNF-β	19.5–10 × 10^3	49	481 + (0–2000)	341	0.92	5.5/5.5	14/13
IFN-γ	7.8–4000	50	34 + (0–2000)	242	0.90	6.3/7.0	26/23
RANTES	625–160 × 10^3	1	[262.7 + (0–128)] × 10^3	148	0.85	3.0/1.1	21/18
MCP-1	19.5–10 × 10^3	82	1623 + (0–2000)	320	0.91	5.8/5.3	21/23
GM-CSF	19.5–10 × 10^3	70	24 + (0–2000)	181	0.93	5.8/4.0	14/13
MIP-1α	19.5–10 × 10^3	10	72 + (0–2000)	134	0.95	2.9/5.6	13/19
MIP-1β	19.5–10 × 10^3	141	614 + (0–2000)	156	0.91	6.6/12	23/20
sIL-6ra	390–200 × 10^3	444	[50.6 + (0–128)] × 10^3	77	0.92	7.3/6.3	16/13
TGF-β	156–80 × 10^3	693	[0.6 + (0–32.0)] × 10^3	45	0.93	6.8/10	18/25
MMP-9	975–0.50 × 10^6	22.0 × 10^3	[1.24 + (0–6.4)] × 10^6	34	0.71	0.6/5.0	14/13
TREM-1	975–500 × 10^3	30	[3.42 + (0–64.0)] × 10^3	103	0.94	3.9/4.3	10/17
CRP	4992–2.56 × 10^6	1.1 × 10^3	[0.65 + (0–1.28)] × 10^6	32	0.99	6.2/8.8	7.8/10
BDNF	156–80 × 10^3	76	[11.0 + (0–64.0)] × 10^3	83	0.90	8.0/9.5	18/19
NT-4	7.8–4000	27	4 + (0–2000)	275	0.90	8.0/11	13/13
Mean				174	0.91	5.7/6.7	15/16

[a] The working range is defined as the range of concentrations in which the CV is <20%. It is assessed based on measurements of both filter-paper calibrators and enriched DBSS models made from blood from a healthy adult. The working range assessed from enriched DBSS is given as measured endogenous concentration + the range of added analyte. Correlations between added and measured analytes in the enriched DBSS are expressed by r^2 from the Pearson correlation coefficient. Assay variations are given as values based on repeated measurements of DBSS models with no added analyte/ DBSS models with low concentrations of added analytes (intraassay CV), and on measurements of DBSS models with low concentrations of added analytes/models with high concentrations of added analytes (interassay CV). See *Materials and Methods* for further details.

concentrations of TGF-β in DBSS taken 5–7 days after delivery of newborns with placental infections or infarcts compared with unaffected controls. IL-2, IL-6, IL-10, GM-CSF, and CRP were also increased but not significantly. This finding is consistent with several other studies on neonatal infections/inflammations, which showed significantly increased plasma concentrations of IL-2, IL-6, IFN-γ, and TNF-α in preterm infants with systemic infections *(34)*; increased plasma concentrations of IL-6, IL-8, IL-10, IL-12, IFN-γ, and TNF-α in newborns with sepsis *(35)*; and increased whole blood concentrations of IL-8 and CRP in newborns with bacterial infections *(36)*. TREM-1 is believed to be increased only in bacterial infections and not in noninfective inflammatory disorders *(37, 38)*.

Immunoassays for cytokines in serum and blood are complex to characterize. It is well established that cytokines interact with a host of blood proteins, including autoantibodies and soluble and membrane-bound receptors *(39–41)*. This may render a fraction of cytokines undetectable by a particular immunoassay if relevant epitopes are blocked by such interactions and hence are unavailable to the antibodies in the assay. The phenomenon is particularly prominent for sandwich immunoassays using monoclonal antibodies because 2 specific epitopes both must be available for detection of a given analyte. Relevant, but rarely answered, questions may thus be which subfractions of cytokines an assay detects, and what the biological relevance of possible undetectable cytokine pools may be.

In this report we have not attempted to address which fractions of cytokines the multiplex method detects. The high correlations between added and measurable analytes show that the relative amounts of each analyte detectable are fairly constant at different concentrations. Consequently, from a pragmatic point of view, comparison studies are feasible. However, the fraction of detectable cytokines probably varies among individuals and over time depending on interacting substances, which may complicate interpretation of the results. The >100% recoveries of some cytokines from blood prepared as DBSS indicate that these exogenous cytokines do not enter

Table 4. Concentrations of analytes in DBSS from newborns 5–7 days of age.[a]

	Median, ng/L	25th percentile, ng/L	75th percentile, ng/L
IL-1β	123	52	193
IL-2	45	22	72
IL-4	28	16	43
IL-5	30	20	40
IL-6	47	35	80
IL-8	302	252	463
IL-10	131	87	156
IL-12	123	71	193
IL-17	179	85	348
IL-18	1908	1299	2468
TNF-α	84	52	133
TNF-β	360	212	468
IFN-γ	33	12	76
RANTES	302.4×10^3	288.7×10^3	349.8×10^3
MCP-1	2382	1803	3059
GM-CSF	118	71	171
MIP-1α	189	147	285
MIP-1β	1038	902	1572
sIL-6ra	47.1×10^3	37.6×10^3	59.5×10^3
TGF-β	4.20×10^3	3.26×10^3	4.89×10^3
MMP-9	1.16×10^6	0.93×10^6	1.51×10^6
TREM-1	2.96×10^3	1.95×10^3	4.02×10^3
CRP	0.54×10^6	0.16×10^6	0.89×10^6
BDNF	13.2×10^3	9.32×10^3	16.5×10^3
NT-4	44	27	66

[a] Concentrations were measured in 66 randomly collected DBSS obtained from newborns for the national routine screening program.

Table 5. Results for DBSS from newborns with inflammatory conditions.[a]

	Concentration, ng/L		
Analyte	Bacterial infection/ Infarct	Controls	P^b
IL-1β	75	66	0.69
IL-2	32	44	0.61
IL-4	32	32	0.78
IL-5	27	21	0.54
IL-6	69	44	0.12
IL-8	444	214	0.03
IL-10	95	74	0.28
IL-12	137	85	0.01
IL-17	158	80	0.001
IL-18	1.86×10^3	1.32×10^3	0.05
TNF-α	73	29	0.01
TNF-β	391	46	0.0003
IFN-γ	95	46	0.01
RANTES	109.0×10^3	115.0×10^3	0.34
MCP-1	1329	468	0.01
GM-CSF	124	77	0.28
MIP-1α	280	269	0.54
MIP-1β	757	647	0.46
sIL-6ra	27.9×10^3	27.7×10^3	0.69
TGF-β	1.04×10^3	2.37×10^3	0.03
MMP-9	1.10×10^6	1.06×10^6	0.87
TREM-1	7.46×10^3	3.12×10^3	0.03
CRP	0.29×10^6	0.22×10^6	0.69
BDNF	12.1×10^3	12.3×10^3	0.61
NT-4	92	39	0.001

[a] DBSS collected 5–7 days after birth from infants with verified bacterial infection at birth (n = 6) or infarct in the placenta (n = 2) and comparable controls (n = 7) were analyzed by the 25-plex xMap assay. Mean values in each group are shown. Note that the assay reveals significant changes in IL-8, IL-12, IL-17, IL-18, TNF-α, TNF-β, IFN-γ, MCP-1, TREM-1, NT-4, and TGF-β in the affected infants.

[b] Mann–Whitney test.

erythrocytes or mix freely with hemoglobin from lysed cells. A pronounced sequestering of the cytokines in the filter-paper calibrators is also a possibility. The recoveries were calculated as if all exogenous analytes distribute evenly within the different blood compartments. If some cytokines, however, distribute entirely within the serum fraction of blood, which typically constitutes ~55% of the total blood volume, the effective concentration of added analytes will be 1.8 times higher than anticipated. Further investigations are currently underway to study this aspect in detail. The observed recoveries <100% are most probably attributable to interactions between the analytes and blood proteins and/or cells.

Interacting blood substances also play a critical role for preparation of calibrators used for construction of calibration curves. Ideally, calibrators should be prepared in a matrix identical to the sample matrix investigated. For the analysis of DBSS, calibrators should thus be diluted in cytokine-free whole blood, spotted on filter paper, and dried. It was not possible, however, for us to obtain suitable cytokine-free whole blood, but a mixture of pig/guinea pig serum spotted on filter paper was found to work well. From our experiments with different matrices, it was clear that the absolute concentrations of cytokines reported by the assay were dependent on the solvent used for preparation of calibrators. Altogether, the interactions between analytes and blood substances call for caution if results obtained from different assay setups are compared. They also make it difficult to evaluate some assay characteristics, including the limit of quantification for each analyte.

The working range of the 25-plex assay was defined as the concentration range for each analyte within which the CV was <20%. These assessments were based on measurements of filter-paper calibrators as well as of enriched DBSS models. Theoretically, working range assessments based on the DBSS models may be the most informative, but assessments at the low end of the range were limited by the presence of endogenous analytes. This is unfortunate because some analytes may actually be present in lower concentrations in normal neonatal DBSS. The filter-paper calibrators made of pig/guinea pig serum do not possess this limitation because endogenous cytokines from these species do not cross-react with the antibodies used in the 25-plex assay. When we used this matrix for

Table 6. Concentrations of analytes detectable in DBSS stored at −24 °C.[a]

	Relative concentration, %		
Analytes	**23 years**	**3 years**	**1 month**
IL-1β	44	43	93
IL-2	116	115	113
IL-4	91	91	107
IL-5	105	116	122
IL-6	95	101	108
IL-8	28	38	64
IL-10	124	103	129
IL-12	95	108	107
IL-17	94	100	107
IL-18	138	113	129
TNF-α	92	101	109
TNF-β	88	94	93
IFN-γ	117	119	121
RANTES	87	89	90
MCP-1	94	112	112
GM-CSF	102	107	108
MIP-1α	85	88	98
MIP-1β	59	76	79
SIL-6ra	48	101	113
TGF-β	111	100	95
MMP-9	57	49	93
TREM-1	68	84	129
CRP	73	123	110
BDNF	22	54	58
NT-4	54	63	111

[a] Results are expressed as percentage of concentration detectable in 2-week-old DBSS not yet put into storage in the PKU-biobank. Notice that detected concentrations of some analytes are decreased after prolonged storage.

the assessment, concentrations found in normal neonatal DBSS fell well within the working range of the 25-plex assay.

The overall imprecision of the multiplex assay is comparable to conventional monoplex immunoassays, but for certain analytes, including IFN-γ, RANTES, MCP-1, and MIP-1β, the interassay CVs are rather high. This may limit the usefulness of the 25-plex assay in its present form as a whole. Triplicate determinations and analysis of cases and corresponding controls within the same run may minimize the imprecision of results. In addition, we found that very accurate work of the technicians or use of a pipetting robot may contribute to lower CVs. Experiments using internal controls composed of cytokines from different species that do not cross-react with human cytokines are currently underway.

Because no reference methods have been published for the measurement of cytokines in serum and blood and because of the large variations in serum concentrations reported in the literature, it is difficult to evaluate the absolute concentrations reported by the present multiplex method *(8, 16, 34, 42–48)*. However, to the best of our knowledge the cytokine concentrations measured in sera from healthy people fell within the ranges reported by several other assays, i.e., for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18, IFN-γ, TNF-α, GM-CSF, and MCP-1 *(35, 45, 49–54)*; RANTES *(47, 51)*; TGF-β *(52, 55)*; MIP-1α and MIP-1β *(47)*; MMP-9 *(56)*; sTNF RI and sIL-6ra *(57)*; MIF *(58)*; NT-3 and NT-4 *(59)*; and BDNF *(60, 61)*.

The 25-plex sandwich immunoassays use pairs of different monoclonal antibodies for capture and detection. The specificity of the overall assay is therefore intrinsically high, which was also supported by the fact that we observed no cross-reaction within the panel or to other analytes tested. In principle, the method is more specific than recycling immunoaffinity chromatography, in which the specificity relies on reaction between usually polyclonal capture antibodies and fluorochrome-derivatized sample passing through the microcolumns *(33)*. Recycling immunoaffinity chromatography would be expected to detect a larger fraction of cytokines interacting with blood proteins, based on the same argument.

Measurements on DBSS stored at −24 °C for more than 20 years revealed that many of the analytes are well preserved over time if dried on filter paper. Some analytes in old DBSS were only detectable in relatively low concentrations, however, compared with measurements on newer DBSS. There could be several reasons for this apart from degradation of analytes over time. The extraction of analytes may be less complete from old, dry DBSS, or the concentrations of the analytes in question may actually have been lower in babies born at that time. The latter possibility is tantalizing because of the increasing incidence during recent decades of disorders in which neonatal inflammation is believed to play a significant role in the pathogenesis *(18–21 29, 30, 62)*.

In conclusion, it is feasible to measure a panel of 23 cytokines, TREM-1, and CRP simultaneously in neonatal DBSS by use of the xMAP technology. Because the interassay variation may be relatively high for some analytes, triplicate measurements and analysis of cases and corresponding controls within the same run is recommended. The method is accessible to most laboratories, and it opens up new possibilities in the study of diseases in which neonatal inflammatory reactions are suspected to play important pathogenetic roles. In its present form it is best suited for biobank case–control studies, but after further refinements, it may also have a potential in routine newborn screening.

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