

A Multiplex Immunoassay Using the Guthrie Specimen to Detect T-Cell Deficiencies Including Severe Combined Immunodeficiency Disease

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BACKGROUND: Severe combined immunodeficiency (SCID) fulfills many of the requirements for addition to a newborn screening panel. Two newborn screening SCID pilot studies are now underway using the T-cell receptor excision circle (TREC) assay, a molecular technique. Here we describe an immunoassay with CD3 as a marker for T cells and CD45 as a marker for total leukocytes that can be used with the Guthrie specimen.

METHODS: The multiplexing capabilities of the Luminox platform were used. Antibody pairs were used to capture and detect CD3 and CD45 from a single 3-mm punch of the Guthrie specimen. The assay for each biomarker was developed separately in identical buffers and then combined to create a multiplex assay.

RESULTS: Using calibrators made from known amounts of leukocytes, a detection limit of 0.25×10^6 cells/mL for CD3 and 0.125×10^6 cells/mL for CD45 was obtained. Affinity tests showed no cross-reactivity between the antibodies to CD3 and CD45. The multiplex assay was validated against 8 coded specimens of known clinical status and linked to results from the TREC assay that had identified them. All were correctly identified by the CD345 assay.

CONCLUSIONS: The performance parameters of the CD345 assay met the performance characteristics generally accepted for immunoassays. Our assay classifications of positive specimens concur with previous TREC results. This CD345 assay warrants evaluation as a viable alternative or complement to the TREC assay as a primary screening tool for detecting T-cell immunodeficiencies, including SCID, in Guthrie specimens.

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Severe combined immunodeficiency (SCID)⁴ screening presents an opportunity for newborn screening (NBS) because detection of this condition in early infancy can be effectively treated by bone marrow transplant (1). Currently, the only available screening tool for T-cell deficiencies, like SCID, is the T-cell receptor excision circle (TREC) assay (2–4). However, the TREC assay comes with some difficulties in that it is a first-tier screening assay using DNA and molecular technology, which at this time is not universally adopted by the NBS community. As noted by Green and Pass (5), “PCR contamination and PCR artifacts that arise with automated, multi-well sample handling would need to be minimized and routinely assessed.”

Low or absent T cells are a major characteristic of SCID and other T-cell immunodeficiencies (6). Because there are 2 case reports of CD3 deficiency causing T-cell immunodeficiency (7, 8) and CD3 is part of the T-cell receptor complex on mature T cells, it was surmised that CD3 could be used as a marker for the presence or absence of T cells (9). CD45 is a common antigen present on all differentiated leukocytes and serves as the internal control in this assay (10). Immunoassays are used routinely in NBS as first-tier screening protocols (11) and can be multiplexed, on certain platforms, to include several biomarkers (12–14). Here we report the technical feasibility of detecting T-cell immunodeficiency by a multiplex immunoassay that simultaneously quantifies T cells and total leukocytes in a single 3-mm punch from a Guthrie specimen.

Materials and Methods

SAMPLES

All specimens used for assay development were provided by the New York State Department of Health Newborn Screening Program. In compliance with New York State Institutional Review Board guidance, no

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⁴ Nonstandard abbreviations: SCID, severe combined immunodeficiency; TREC, T-cell receptor excision circle; NBS, newborn screening.

identifying information was transferred with the samples. Eight coded 3-mm punches from specimens with known TREC values (4) were provided by A.M. Comeau.

ANTIBODIES AND REAGENTS

The antihuman CD3 and CD45 capture and detector antibodies were purchased from USBiological. Other reagents used were as follows: antiphycoerythrin (Biolegend); sulfo-NHS-LC-biotin (Pierce); streptavidin-Phycoerythrin (Prozyme); phosphate-buffered saline + Tween 20; protease inhibitor cocktail, gelatin, and Histopaque 1077 (Sigma); whole and leuko-depleted blood units (Tennessee Blood Services); carboxylated xMAP microspheres (Luminex); low protein binding 96-well filter bottom plates (Millipore); flat-bottom microtiter plates (Corning); pooled human serum (BioResource Technology); triton-x114 (MP Bioscience); and Ahlstrom Grade 226 Specimen Collection Paper (ID Biological Systems).

REAGENT PREPARATION

Anti-CD3- and anti-CD45-specific capture monoclonal antibodies were coupled to Luminex xMAP microspheres following the protocol provided by Luminex (<http://www.luminexcorp.com/support/protocols/index.html>). By use of techniques previously described (12–14), 25–100 µg anti-CD3 capture monoclonal antibody was coupled to 5×10^6 Luminex microspheres, region 132 (L-100-C132–04). Similarly, 25–100 µg of anti-CD45 capture monoclonal antibody was coupled to 5×10^6 Luminex microspheres, region 133 (L-100-C133–04). The anti-CD3 polyclonal and anti-CD45 monoclonal detector antibodies were biotinylated with sulfo-NHS-LC-biotin according to the manufacturer's instructions (Pierce).

CALIBRATOR PREPARATION

Whole blood was used to prepare calibrators and controls, after determining leukocyte counts by flow cytometry. Leukocytes were collected from whole blood by using Histopaque 1077, according to the manufacturer's instructions, counted on a hemacytometer, and resuspended at a concentration of 30×10^6 cells/mL in human serum containing 2% protease inhibitor cocktail. Serial dilutions were carried out in human serum containing protease inhibitors to achieve a final leukocyte concentration of 0.125×10^6 /mL. Leukocyte-reduced blood was examined by flow cytometry to confirm the absence of CD3- and CD45-positive cells. The leukocyte-reduced blood was washed 4 times with phosphate-buffered saline, pH 7.4, and an equal volume of packed red blood cells was added to each dilution. The remaining packed red blood cells were stored at -80°C . The leukocyte-enriched blood (75 µL) was

spotted on Ahlstrom Grade 226 Specimen Collection Paper and left to dry overnight. Dried spots were wrapped in foil and stored in a sealable bag with desiccant at -20°C . Controls were made from whole adult blood, with lymphocytes previously measured by flow cytometry.

ASSAY PROTOCOL

The assay protocol consisted of 6 steps, with washing between each step, using the same wash buffer, prepared by using phosphate-buffered saline (pH 7.4), 0.055% Tween 20, 0.05% sodium azide. To make the assay buffer, 0.2% gelatin was added to the wash buffer. To make the elution buffer, 1% protease inhibitor cocktail and Triton-X 114 (0.1%) were added to the assay buffer. A single 3-mm punch from a standard, control, or newborn specimen was placed in an individual well of a flat-bottom microtiter plate and eluted for 12–18 h at room temperature in 100 µL elution buffer with gentle shaking. For the assay, a 96-well filter plate was wetted with wash buffer and aspirated by vacuum filtration. A mixture of CD3 and CD45 microspheres was resuspended to yield a concentration of 6×10^7 microspheres/L for each set. A total of 50 µL of the microsphere mixture was added to each well, and then 75 µL of the sample eluate was added to the appropriate wells. All incubations were carried out at 37°C in the dark, with gentle shaking, using the times noted. Microspheres were incubated for 3 h and then washed by vacuum filtration 3 times with 200 µL wash buffer. All subsequent washes were carried out as described. Microspheres were resuspended in 50 µL of a mixture of anti-CD3 (1:300) and anti-CD45 (2 mg/L) detector antibodies. The microspheres were incubated for 1 h, washed, resuspended in 50 µL of 4 mg/L streptavidin phycoerythrin, and incubated for 20 min. The microspheres were washed, resuspended in 50 µL of 0.2 mg/L antiphycoerythrin, and incubated for 30 min. Microspheres were washed, resuspended in 50 µL of 4 mg/L streptavidin phycoerythrin, and incubated for 20 min. Microspheres were washed and then resuspended in 110 µL of Luminex sheath fluid for analysis. Data collection and analysis were performed in multiplex acquisition mode on the Luminex 100 instrument. Results were calculated with Luminex software (LX100 IS 2.3) and were expressed as median fluorescence intensity of 100 microspheres of each set. LiquiChip Analyzer software, v. 1.0 (Qiagen), was used to analyze the raw data.

ANTIBODY SELECTION

There were 30 different CD3 antibody capture microsphere sets prepared and tested against the same 30 antibodies, but in a biotinylated sandwich format that allowed testing of over 700 antibody pairs to CD3.

Those pairs showing excellent results in a constructed calibration curve were used. CD45 antibodies were tested in the same way, with an additional stipulation in that the capture and detector antibodies must recognize all isoforms of CD45. The performance of the antibodies was optimized for concentration, following standard immunoassay procedures, using titer studies that evaluated affinity, sensitivity, and cross-reaction tests that evaluated their specificity. Each analyte immunoassay was developed independently in identical buffers and required optimization of the ratio of microspheres to capture antibody, the concentration of detector antibody, and the concentration of phycoerythrin reporter. Before combining the immunoassays into a duplex format, each capture antibody was tested against the opposite detection antibody to show the absence of cross-reactivity among the pairs. No cross-reactivity was observed between CD3 and CD45 antibodies. The immunoassays were combined, and the concentration of each microsphere set was adjusted to allow for 100 of each set to be counted by the Luminex instrument. The detector antibody concentrations were also optimization for use in the duplex format.

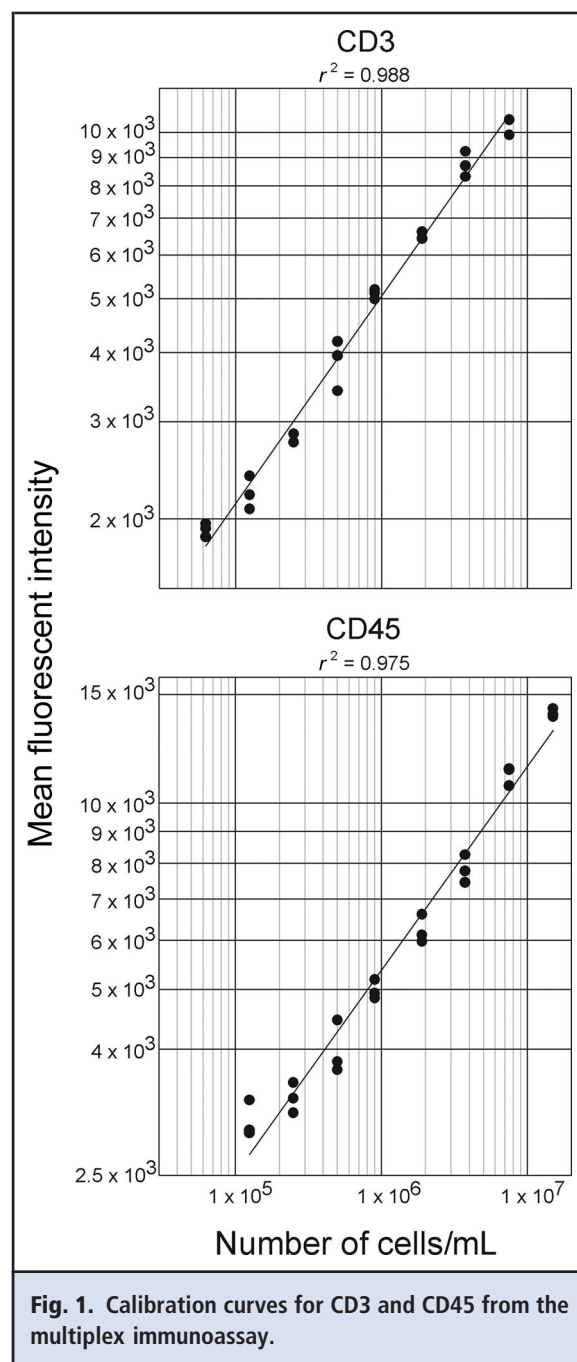
Results

CALIBRATION STUDY

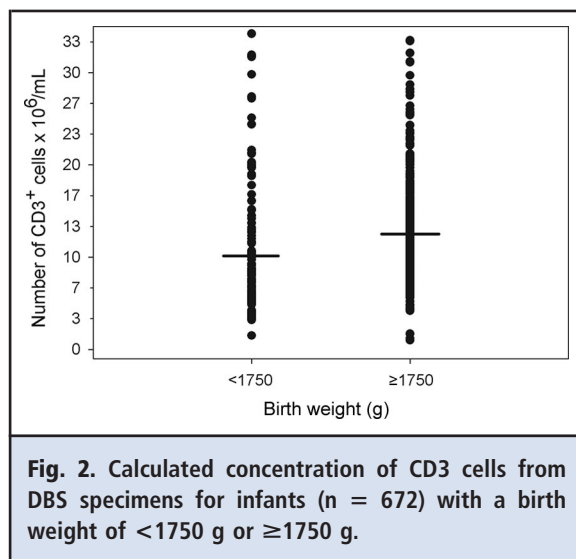
Fig. 1 shows calibration curves for CD3 and CD45 from dried blood calibration material. The analytical limit of detection was determined by using the mean plus 3 SD, from 12 replicates of the zero calibrator. The sensitivity of the antibodies was also tested by examining the mean median fluorescence intensity ± 3 SD. We found that there was adequate separation between calibration points. The analytical detection limit in the dry blood spot standard curve for CD3 was 0.25×10^6 cells/mL and 0.125×10^6 cells/mL blood for CD45. Using the mean of 12 independent measurements for each concentration of calibrators, we examined the assay imprecision profiles. The intraassay CVs ranged from 11% for the lower concentrations of CD3 to 3% for the higher concentration of CD3, and 12% for the lower concentrations of CD45 to 1% for the higher concentration. At none of the concentrations were the inter-assay CVs $>3\%$ for CD3 and $>1\%$ for CD45.

POPULATION STUDIES

A total of 672 Guthrie specimens from randomly chosen normal-weight newborns (≥ 1750 g; 525 specimens) and low-birth-weight newborns (< 1750 g; 147 specimens) were tested to determine a range for CD3 and CD45. The mean CD3 T-cell count was 12.5×10^6 cells/mL (range $3\text{--}34 \times 10^6$ cells/mL). For samples from low-birth-weight newborns the mean CD3 T-cell count was 10×10^6 cells/mL (range $2\text{--}35 \times 10^6$



cells/mL) (Fig. 2). Any values above 15×10^6 cells/mL are extrapolated numbers generated by the Liquichip program. Fifteen samples were labeled as “high,” all from the ≥ 1750 g category, and are not shown in Fig. 2. A value of “high” or “low” by the Liquichip software indicates that the measured median fluorescence intensity was too high or too low to extrapolate a concentration.



VALIDATION STUDIES

Because of the scarcity of positive SCID specimens, the 8 coded punches from the New England Newborn Screening Program were tested in singlicate. The 8 coded specimens were classified as positive or negative by the CD345 assay. These results and the white paper punches that remained after elution (ghost spots) were sent to A.M. Comeau for decoding and testing (Table 1). The 3 decoded control specimens (2 normal infants and 1 adult) showed CD3 values of $4 \times 10^6/\text{mL}$ or greater. In the remaining 5 decoded specimens, classified as positive by the TREC assay, 3 had undetectable CD3 and undetectable TREC. One of these 3 had maternal T-cell engraftment, and 1 infant showed unde-

tectable CD3 and detectable TREC, but the quantities were well below the TREC cutoff of 252 copies/ μL whole blood (lowest calibrator). The final infant showed detectable CD3, but at a 10-fold concentration below controls, and undetectable TREC (Table 1). Testing of the ghost samples with the TREC assay resulted in all designations remaining the same as originally assigned.

Discussion

In January 2010, The Secretary's Advisory Committee on Heritable Disorders in Newborns and Children, after a lengthy discussion and literature review, approved sending a recommendation to Dr. Kathleen Sebelius, the Secretary of Health and Human Services, and recommended that SCID should be included as part of the uniform panel of conditions (15). This is the first addition to the core panel recommended by the Advisory Committee and is expected to lead to rapid adoption of screening for SCID by US NBS programs. Sebelius accepted the recommendation. It is expected that this action will lead to rapid addition of SCID testing to NBS panels.

The literature is replete with reports of effective treatments for SCID (1, 16–18), thus making it a prime candidate for addition to NBS panels. Currently, the DNA-based TREC assay is the only assay that has been validated for detection of a variety of T-cell deficiencies using NBS specimens (3, 4, 19). Here, we report data from an alternative method—a multiplex immunoassay (CD345) that uses a T-cell marker to identify immunodeficient newborns. This is the first reported use of CD3 as a biomarker for T-cell lymphopenia in an immunoassay of Guthrie specimens. The CD45 biomarker provides an internal control for assay performance and confirms the presence of a punched sample in each well. It is important to note that the values for CD3 in control and affected infants from the New England Newborn Screening Program showed at least a 10-fold difference in CD3 concentration, thereby suggesting adequate separation between these 2 populations and potentially allowing for the identification of other T-cell lymphopenias (Table 1).

Given the limited number of positive specimens available for evaluation, it is impossible at this time to establish a cutoff for CD3. As discussed recently, other T-cell immunodeficiencies can be detected by screening for the TREC marker (3, 4, 20). A benefit to using the TREC assay is that it can identify the production of $\alpha\beta$ T cells in the presence of maternal engraftment, a condition that could potentially interfere with detecting T-cell absence in this immunoassay. However, a maternally engrafted specimen included among the coded specimens analyzed by the CD345 assay showed low concentrations of CD3 and thus was classified as

Table 1. Calculation of CD3 and CD45 cells in specimens from coded TREC-tested specimens and designation of CD345-positive specimens.

TREC-tested specimens and controls			
Number of CD3 cells $\times 10^6/\text{mL}$	Immunoassay classification	TREC ghost classification	Diagnosis
Low	Positive	Positive	ADA ^a
4.1	Negative	Negative	Control
10.8	Negative	Negative	Control
Low	Positive	Positive	PNP
5.5	Negative	Negative	Control
0.4	Positive	Positive	X-linked
Low	Positive	Positive	X-linked
Low	Positive	Positive	X-linked

^a ADA, adenosine deaminase deficiency; PNP, purine-nucleoside phosphorylase.

positive for T-cell deficiency. One sample is not conclusive evidence that this assay can identify T-cell deficiency with maternal engraftment, and we are working to distinguish these conditions.

The CD345 multiplex assay described here showed good concordance with TREC analysis on Guthrie specimens and thus suggests a potentially comparable assay that could be used as a primary screening tool. It is important to note that TREC can be amplified from the ghost spot previously used in the CD345 assay and can still correctly identify T cell-deficient specimens, thereby demonstrating that an initial immunoassay as a primary screen and a molecular assay as a secondary screen can be performed on a single DBS punch (4). In view of the limited availability of positive specimens for T-cell lymphopenia, we evaluated this assay with an admittedly small number of specimens. However, the number of confirming specimens used is not greatly different from that used by others in this field (21). We recognize that the CD345 assay, as described here, might be too time-consuming for routine testing of large numbers of specimens. However, this assay has the potential to be configured as a kit, as has been reported with other Luminex assays (22), which would greatly enhance its usefulness to large NBS programs. Additionally, the CD345 immunoassay has the potential to be multiplexed to include markers for B cells, NK cells, and, if maternal engraftment is still a concern, markers for naïve and memory T cells.

With this report, we provide an option for screening that has not previously existed. We believe the performance characteristics of the CD345 assay warrant its inclusion in population-based evaluations.

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quirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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