Use of Microsphere Immunoassay for Simplified Multianalyte Screening of Thyrotropin and Thyroxine in Dried Blood Spots from Newborns

To the Editor:
Early treatment of congenital hypothyroidism (CH) allows the normal development of an otherwise severely affected child. Because initial symptoms are scant and the onset of the disease is usually insidious, neonates are screened in developed countries for CH by measurements of either thyrotropin (TSH) or thyroxine (T4). Typically, increased TSH and decreased T4 are seen in CH (1).

Until recently, measurement of both analytes required two separate analytical procedures. In contrast, the Luminex MultiAnalyte Profiling Technology (LabMap®) allows the simultaneous measurement of TSH and T4. Briefly, the desired antibodies are immobilized on color-coded polymer beads. The amount of fluorescently marked analyte binding to the beads is read with a laser beam at 532 nm, comparable in principle to a flow cytometer. The difference is that an additional laser at 633 nm excites the bead color, so that each fluorescence measurement from the analyte can be assigned a unique bead set. Each color (usually hues of red) designates a certain assay.

We have modified the procedure of Bellissario et al. (2), which uses beads coated with either TSH or T4 and requires several washing steps between incubation periods. Our procedure uses a competitive inhibition assay for T4 and a sandwich capture assay for TSH. Mouse monoclonal anti-TSH and T4-bovine serum albumin were covalently bound as esters to two different bead sets (nos. 25 and 76) by a two-step method (3).

The bead sets were mixed equally and diluted in phosphate-buffered saline; 25 µL of the resulting solution was used for each assay. The washing steps in the original method rendered it difficult to perform and impossible to automate because beads measuring 5.6 µm had to be carefully filtered and rinsed before each step. In contrast, our optimized, patented procedure modifies the elution buffer in a way that cells are initially lysed and adds a signal-enhancing buffer that contains Tween 20 as well as bovine serum albumin and avoids washing steps (4).

We punched 3-mm diameter dried blood spots from filter paper cards and eluted the blood with 75 µL of the elution buffer. After the eluates were filtered through 0.45 µm filter plates (Millipore), we added 50 µL of a mixture of beads and biotinylated-anti-T4 antibody in phosphate-buffered saline (pH 7.4). The plate was incubated for 45 min at 37 °C. We then added 25 µL of biotinylated-anti-TSH to the wells and incubated the plate for 60 min at 37 °C. The reporter signals for both assays were generated by adding 50 µL of the fluorescent dye streptavidin-R-phycocerythrín (SRPE) in the final incubation, which amplifies the signal by forming a complex with four binding sites for biotin (excitation at 488 nm, and emission at 578 nm). Finally, we added 100 µL of phosphate-buffered saline at pH 7.4 directly before measurement to reduce background interference. The plate was read on a Luminex100 reader that aspirated 50 separate measurements. It took ~60 s to simultaneously obtain information on TSH and T4 concentrations in each sample. We added 5, 10, and 25 µL of human serum albumin (100 mL/L) to the assays and found that, unlike conventional fluorescence assays, high protein content did not influence results obtained on the Luminex (data not shown).

Linear regression for TSH yielded a curve with a slope of 5.6887 and an intercept of 36.057 MFI units ($R^2 = 0.992$; Fig. 1A). The dynamic range was 0–200 mIU/L of whole blood, which exceeds the physiologically relevant concentrations by 10-fold. Exponential regression for T4 yielded a curve with the equation: $y = 1095.4e^{-0.1519x}$ ($R^2 = 0.9882$; Fig. 1B). The dynamic range was 0–257 nmol/L of whole blood (0–20 µg/dL), which exceeds the physiologic range by approximately two-fold. The detection limits (mean plus 3 SD of the zero calibrator) for T4 and TSH in blood-spot samples were 12.6 nmol/L (0.98 µg/dL) and 1.36 mIU/L, respectively. For CDC Newborn Screening Quality Assurance Program materials, target/measured results were 37.3/45, 139/143, and 29.6/46.3 nmol/L (2.9/3.5, 10.8/11.1, and 2.3/3.6 µg/dL) for T4 and 70.3/74.6, 14.3/15.8, and 71.7/74.2 mIU/L for TSH. Within-run imprecision (CV) was 3.6% (high TSH values) to 16% (low TSH values) for TSH and 7% (normal T4) to 15% (low T4) for T4.

We tested 403 samples from healthy newborns previously assessed by the Northern German Newborn Screening Program in Hamburg. Results for TSH varied from 0 to 14.9 mIU/L with a mean (SD) of 5.1 (3.6) mIU/L. For T4, we observed concentrations of 25.7–154.4 nmol/L (2–12 µg/dL) with a mean (SD) of 92.7 (30.8) nmol/L. Therefore, all results were within the reference intervals (5). In addition, seven samples from newborns diagnosed with CH
were analyzed during the same period. All showed increased TSH (43–235 mIU/L). Four showed clearly decreased T4 [1.2–23.8 nmol/L (0.1–1.85 µg/dL)]. Another two had T4 concentrations below our cutoff of 30.8 nmol/L (2.4 µg/dL), and only one had a T4 concentration within the reference interval [67 nmol/L (5.2 µg/dL)]. This is in agreement with literature data that ~10–20% of all CH cases with increased TSH concentrations are not accompanied by low T4. Interestingly, the ratio of TSH to T4 was an excellent indicator for CH in neonates because deviations in TSH as well as T4 values will yield highly increased ratios of TSH to T4. For healthy newborns, we observed ratios of 0–4, whereas our seven patients as well as abnormal controls showed values >20.

In summary, our modified multiplex analyte assay for TSH and T4 simplifies the analytical protocol of the procedure to allow easier handling and more rapid analysis. Furthermore, future automation of the process becomes an option, which is indispensable for the high-throughput screening usually required in all larger screening laboratories. LabMap technology provides an alternative to mass spectrometry for the simultaneous analysis of metabolites present in samples at very low concentrations. Other endocrinologic metabolites, e.g., 17-hydroxyprogesterone, a marker for congenital adrenal hyperplasia, can also be incorporated in the analytical protocol. This would allow the simultaneous analysis of all endocrinologically relevant metabolites for newborn screening in one analytical run.

References

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Commentary on: Use of Microsphere Immunoassay for Simplified Multianalyte Screening of Thyrotropin and Thyroxine in Dried Blood Spots from Newborns

To the Editor:
In their letter, Lukacs et al. present an interesting modification and improvement of the procedure described previously by Bellisario et al. (1), using the Luminex Xmap system to simultaneously assay for thyroxine (T4) and thyrotropin (TSH) in a Guthrie specimen. Their innovation builds on the earlier work and, importantly, negates the necessity for multiple wash steps while maintaining acceptable sensitivity and specificity. This will greatly facilitate automation of these tests, an important aspect for newborn screening programs, in which thousands of specimens are processed each day. The concept of simultaneous measurement of T4 and TSH enhances the specificity of hypothyroidism screening in newborns, at the same time reducing the workload by combining two tests into a single procedure. Their comments concerning the use of a TSH:T4 ratio are interesting and worthy of follow-up.

The use of ratios such as this has been helpful in increasing the specificity of other newborn assays, such as for phenylketonuria (PKU) (2). With the addition of a bead-based test for 17-hydroxyprogesterone, the serum marker for congenital adrenal hyperplasia, and immunoreactive trypsinogen, the serum marker for cystic fibrosis, to the T4/TSH assay, a complete endocrine panel for newborn screening could be accomplished in this system in a single assay tube. This multiplex testing offers newborn screening programs tremendous opportunities for expanding the screening panel without increasing the workload.

References

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Comparison of Two Third-Generation Parathyroid Hormone Assays

To the Editor:
Parathyroid hormone (PTH) is an 84-amino acid peptide secreted by the parathyroid glands that is important in regulating the extracellular calcium concentration in the body. Because this hormone is rapidly metabolized into fragments, its measurement has always been problematic (1). During the last few years, it has been shown that the current intact PTH, or second-generation, assays will cross-react with a large C-terminal fragment, PTH (7–84), which lacks the six N-terminal amino acids (2, 3). Because of the lack of specificity in these second-generation systems, falsely increased PTH values have been observed (3). New third-