

Evaluation of UMELISA[®] T4 Neonatal and UMELISA[®] T4 using polystyrene plates coated with anti-thyroxine (T4) monoclonal antibodies.

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Resumen:

The Immunoassay Center has developed the UMELISA[®] T4 NEONATAL and UMELISA[®] T4 to determine neonatal thyroxine (T4) levels in dried blood and serum samples. Both reagent kits use the same polystyrene plates coated with anti-T4 polyclonal antibodies as solid phase. This work shows the re-standardization of the UMELISA[®] T4 NEONATAL and UMELISA[®] T4 using plates coated with anti-T4 monoclonal antibodies (T4Mabs).

Polystyrene plates of the modified assays were firstly coated with polyclonal IgG sheep-anti-mouse IgG for 18 hours. T4Mabs were added to the plates and incubated for 2 hours at room temperature. Different performance parameters were evaluated and correlation studies with the commercial kits done.

Using polystyrene plates coated with T4Mabs increases the slope of the calibration curve in the clinical interest zone. Recovery percentages (90,8-110,7 for UMELISA[®] T4 NEONATAL and 92,1-109,3 for UMELISA[®] T4) and intra (7,2-7,6 for UMELISA[®] T4 NEONATAL and 6,9-7,2 for UMELISA[®] T4) and inter (7,4-8,5 for UMELISA[®] T4 NEONATAL and 7,1-8,5 for UMELISA[®] T4) coefficients of variation were similar to the ones described for the commercial kits. Limits of detection and quantification were 9 and 21,1 nmol/L for UMELISA[®] T4 NEONATAL, and 8,9 and 20,5 nmol/L for UMELISA[®] T4 respectively. The results also showed high overall concordance between assays (n=244, r=0,92, pc= 0,91 for UMELISA[®] T4 NEONATAL and n=492, r=0,92, pc=0,9 for UMELISA[®] T4).

The analytical sensibility of UMELISA[®] T4 NEONATAL and UMELISA[®] T4 is improved by using polystyrene plates coated with T4Mabs, without affecting the precision and accuracy of the results.

Palabras clave: Congenital hypothyroidism, Thyroxine (T4), ultra-microELISA, SUMA Technology, monoclonal antibodies.

I. INTRODUCCIÓN

Congenital hypothyroidism (CH) is an inherited metabolic disorder, known as the most common preventable cause of mental retardation in children, with a worldwide incidence of 1:3000 to 1:4000 newborns (1, 2). In Cuba, the estimated incidence is 1:4382 live births (3).

The clinical features of CH are often subtle and many newborn infants remain undiagnosed at birth (4). The first American Academy of Pediatrics recommendations for newborn screening for CH were published in 1993 (5). During the past three decades, this screening with the vast majority of patients with CH achieving normal neurological outcome has been very successful (6, 7, 8).

The ultra-microanalytical system (SUMA), -created in the 1980s-, is a complete system of reagents and instrumentation to perform ultra-microassays combining the sensitivity of the micro-enzyme-linked immunosorbent assay tests with the use of ultra-micro volumes (UMELISA[®]). This technology has permitted establishing large-scale newborn screening programs for metabolic and endocrine disorders in Cuba (3). Since 1986, SUMA technology has been used in the Cuban newborn screening program for CH, initially studying thyroid hormone in cord serum samples.

UMELISA[®] T4 Neonatal and UMELISA[®] T4 are competitive ultra-micro methods for CH screening, designed for the determination of total thyroxine (T4) in dried blood samples and serum samples, where the natural antigen and the enzyme-labelled antigen compete for a limited number of binding sites on the antibody. Both assays use as solid phase ultra-microELISA strips coated with specific rabbit polyclonal anti-T4 antibodies (3, 9). The aim of this work was to re-standardize the UMELISA[®] T4 NEONATAL and UMELISA[®] T4 using polystyrene plates coated with anti-T4 monoclonal antibodies (modified assays).

II. MÉTODO

A. Equipment and accessories

The Ultra Micro Analytical System (SUMA, acronym in Spanish) technology was used. The system, manufactured by the Immunoassay Center, Havana, Cuba, includes reagents and instrumentation. The equipment comprises a fully computerized spectro-fluorimeter-photometer for the automatic reading, quantification, validation and interpretation of the results (PR-621) and a plate washer (MW-2001). The reagents (UMELISA[®] T4 Neonatal and UMELISA[®] T4) are placed in kits in quantities enough for 288 ultra-microtests (10 µL volumes of samples and reagents).

B. Immobilization of monoclonal anti-T4 antibodies

White opaque 96-well polystyrene ultra-microELISA plates (Tecnosuma International S.A, Havana) were coated with 18 µL/well of polyclonal IgG sheep-anti-mouse IgG at concentrations ranging from 8 µg/mL in 0,05 mol/L sodium carbonate/ bicarbonate buffer, pH 9,6, containing 3 mmol/L of NaN₃. The plates were placed for 18 hours in a humid chamber at room temperature (23-25 °C) and washed with 25 µL/well of 0,15 mol/L of phosphate-buffered saline solution containing 1,9 mol/L of NaN₃ and 1,1 mmol/L of Tween 20. Buffer was removed and the plates were treated with 18 µL of a solution containing 0,05 µg/mL of anti-T4 monoclonal antibodies, 14,5 µmol/L of BSA, 0,15 mol/L of saccharose and 0,45 mmol/L of Tween 20, for 2 hours at room temperature (23-25 °C). Finally, the ultra-micro plates

were dried and conserved with desiccant in polyvinyl sealed bags at 2–8 °C, where they are stable for at least 12 months under these conditions.

C. Ultra-micro enzyme-linked immunosorbent assays (UMELISA® T4 Neonatal and UMELISA® T4)

Calibrators of UMELISA® T4 Neonatal were prepared according to the previously described method (9). UMELISA® T4 Neonatal was used to determine T4 concentration in dried blood spots on filter paper by the previously described protocol. (9).

For UMELISA® T4 the calibration curve was prepared using T4-free serum. T4 sodium hydroxide solution at a concentration of 1 mg/mL was used for the preparation of 6 standards with concentrations between 25-400 nmol T4/L of serum. T4 standards were filtered, lyophilized, and stored at – 20 °C. For the measurement of T4 concentrations in serum samples, 5 µL of standards, controls, and samples were diluted with 100 µL of diluted T4-alkaline phosphatase conjugate in 0,015 mol/L Tris buffer solution with 0,151 mol/L of NaCl, 0,0246 mmol/L of NaN₃, 0,0375 mol/L of sodium salicylate and 0,407 mmol/L of Tween 20. 10 µL of each diluted specimen were transferred into the well of the reaction opaque polystyrene ultramicroplates coated with the specific polyclonal rabbit anti-T4 antibodies or monoclonal anti-T4 antibodies. The competitive reaction occurred for 2 hours at room temperature in a humid chamber and then, the plates were washed 4 times with 0,37 mol/L of Tris–HCl solution, pH 8, containing 3,76 mol/L of NaCl, 1.1 mmol/L of Tween 20 and 76,9 mmol/L of NaN₃. The fluorogenic reaction was performed by adding 10 µL of the substrate solution, pH 9,6, containing 5,07 mmol/L of 4-methylumbelliferil phosphate, 0,92 mol/L of diethanolamine–HCl, 0,7 mmol/L of MgCl₂ and 7 mmol/L of NaN₃. The ultra-microplates remained at room temperature in a humid chamber for 30 minutes. Finally, fluorescence was measured automatically in the fluorimeter-photometer reader. Automatic validation and interpretation of the results were done using an specific assay software.

UMELISA® T4 Neonatal and UMELISA® T4 kits use the same polystyrene plates coated with anti-T4 polyclonal antibodies as solid phase. For the evaluation of the modified assays the technical procedure of both tests did not change, except for the substitution of the plates.

D. Controls

Controls with a well-known T4 concentration that embraced a wide range of standard curve activity were used for assays evaluation. The controls were prepared by adding known T4 quantities to whole human blood with a 55% haematocrit value and dried on filter paper, or to T4 free serum. Moreover, three controls from the Center for Disease Control and Prevention (CDC, USA) and 3 controls from Randox laboratories Limited (RIQAS, United Kingdom) were used to evaluate the performance characteristics (precision, accuracy, linearity, limit of detection and quantification) of the modified UMELISA® T4 Neonatal and UMELISA® T4, respectively.

E. Determination of T4 levels in dried blood spots on filter paper in Cuban newborn using the new coating conditions. Correlation with the UMELISA® T4 Neonatal and UMELISA® T4

244 dried blood samples and 492 serum samples from the National Neonatal Screening Program were evaluated using the UMELISA® T4 Neonatal and the UMELISA® T4 respectively, using polystyrene plates coated with anti-T4 polyclonal or monoclonal antibodies. Prior to participating in the study, the patient identification of samples was removed.

Correlation between assays was established. We assessed the quantitative differences between the modified assays (plates coated with anti-T4 monoclonal antibodies), and the UMELISA[®] T4 Neonatal and UMELISA[®] T4 using two approaches: the Pearson correlation, and the concordance correlation coefficient (pc). The mean and standard deviation (SD) of T4 concentrations, the slope, y-intercept of the line-of-best-fit, and regression statistics to compare our modified assay results versus those obtained on the same dried blood samples using the UMELISA[®] T4 Neonatal or serum samples using UMELISA[®] T4, were obtained using Excel 2010 software. The concordance correlation coefficient was calculated as a measure of agreement between assays (10). Values between 0,2 and 0,7 indicate minor concordance; values between 0,7 and 0,85 indicate moderate concordance, and values > 0,85 indicate clear to high concordance.

III. RESULTADOS

A. Standard curve, Limit of Detection and Limit of Quantification

Typical standard curves for UMELISA[®] T4 Neonatal and UMELISA[®] T4 using polystyrene plates coated with anti-T4 monoclonal antibodies and anti T4 polyclonal antibodies are shown in Fig 1. The adjustments of the standard curves were automatically fitted to a linear function. The calculated values of the samples were interpolated in a graphic of the fluorescence quotient B/Bo (fluorescence for each calibrator over that of the standard zero calibrator of the curve; expressed in %) versus the T4 concentration, corresponding to the standard curve, getting the concentration values in nmol of T4/L blood or serum. In the resulting quotient, B/Bo was inversely proportional to the amount of T4 in the sample.

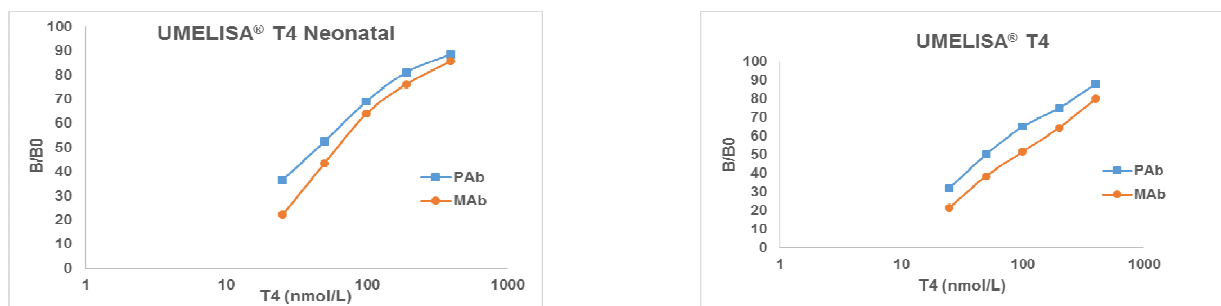


Fig. 1 Typical standard curves obtained with the UMELISA[®] T4 Neonatal and UMELISA[®] T4 using plates coated with anti T4 monoclonal antibodies (MAb) and anti T4 polyclonal antibodies (Pab).

Limit of detection (LOD) and limit of quantitation (LOQ) were determined according to CLSI Guidelines (11). The zero standard and 3 spiked blood or serum samples (8, 15 and 25 nmol/L) were analyzed 64 times to estimate LOD and LOQ. Means, SDs, CVs and the slope were calculated. LOD, defined as the lowest amount of T4 in a sample that can be detected with a probability of 95%, was 9 nmol/L for modified UMELISA[®] T4 Neonatal and 8,9 nmol/L for modified UMELISA[®] T4, and it was calculated by: $LOD = \text{limit of blank (LOB)} + 3 \times SDs$ (where $LOB = 95$ percentile of zero standard measurements; $SDs = \text{Pooled SD estimate of measurements on spiked samples}$).

LOQ, the lowest amount of T4 in a sample that can be quantitatively determined in our UMELISAs with an acceptable precision and a suitable accuracy, was 21,1 nmol/L and 20,5 nmol/L for modified

UMELISA[®] T4 Neonatal and modified UMELISA[®] T4, respectively. The spiked samples were studied to estimate the lowest concentration in the assay for which the CV is less than 20%, and the measured value is within 20% (80–120%) of true value. The LOQ was calculated by this formula: $LOQ = LOB + 10 \times (SDs / \text{slope})$.

B. Precision and Recovery

Precision and recovery were determined following CLSI Guidelines (12). For evaluating precision, samples representing 3 different levels of T4 concentrations were assayed. The repeatability, standard error of the daily means and within-laboratory precision were calculated from estimating T4 in 2 replicates of each sample, for 30 operating days. The intra and inter-assay coefficients of variation ranged between 7,2-7,6 % and 7,4-8,5 % respectively, for modified UMELISA[®] T4 Neonatal, and ranged between 6,9-7,2 and 7,1-8,5 for modified UMELISA[®] T4, depending on the T4 concentrations evaluated (Table 1).

Table 1. Precision for modified UMELISA[®] T4 Neonatal and UMELISA[®] T4

UMELISA [®] T4 Neonatal						
Sample T4 Concentration (nmol/L)	Repeatability		Between-day, between-run precision		Within-laboratory precision	
	S _r	CV (%)	S _{dd}	CV (%)	S _T	CV (%)
36,7	2,8	7,6	2,7	7,4	3,0	8,2
79,5	5,8	7,3	6,4	8,1	6,8	8,5
158,1	11,4	7,2	11,7	7,4	11,9	7,5
UMELISA [®] T4						
Sample T4 Concentration (nmol/L)	Repeatability		Between-day, between-run precision		Within-laboratory precision	
	S _r	CV (%)	S _{dd}	CV (%)	S _T	CV (%)
46,0	3,3	7,2	3,6	7,8	3,9	8,5
101,6	7,0	6,9	7,7	7,6	8,1	8,0
190,4	13,1	6,9	13,5	7,1	13,6	7,1

Table 2 shows the results obtained when controls were evaluated. The results were averaged of five replicates (13). For modified UMELISA[®] T4 Neonatal, percentage recoveries ranged 90,8-110,7% with a mean value of $99,8 \pm 8,7\%$. For modified UMELISA[®] T4 percentage recoveries ranged 92,1-109,3% with a mean value of $102,9 \pm 6,0\%$.

Table 2. Analytical recoveries of T4 measured by modified UMELISA® T4 Neonatal and UMELISA® T4

UMELISA® T4 Neonatal			UMELISA® T4		
Expected (nmol/L)	Determined (nmol/L)	% Recovery	Expected (nmol/L)	Determined (nmol/L)	% Recovery
Blood Controls			Serum Controls		
35,4	39,0	110,5	60	63,7	106,2
83,7	78,0	93,2	90	82,9	92,1
155,4	141,1	90,8	150	155,7	103,8
CDC Controls			Randox Controls		
25,74	28,5	110,7	42,5	46,5	109,3
90,09	88,1	97,8	90,9	95,7	105,2
141,57	135,9	96	205,6	206,6	100,5

C. Comparison of methods

Using polystyrene plates coated with anti-T4 monoclonal antibodies and anti-T4 polyclonal antibodies, dried blood samples and serum samples were evaluated in the UMELISA® T4 Neonatal and UMELISA® T4, respectively. All samples were obtained from apparently healthy full term neonates with birth weights above 2500 g. Distribution of T4 concentrations using both methods is shown in Fig.2.

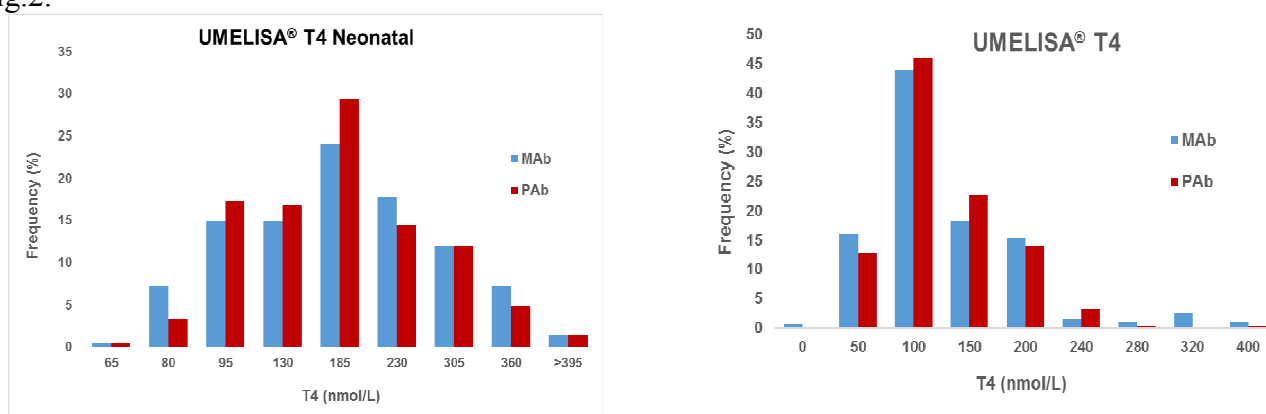


Fig. 2 Typical standard curves obtained with the UMELISA® T4 Neonatal and UMELISA® T4 using plates coated with anti T4 monoclonal antibodies (MAb) and anti T4 polyclonal antibodies (Pab).

Using plates coated with the anti-T4 monoclonal antibodies, T4 mean concentration in dried blood samples from 244 neonates (5-to-7 days-old) was 163,9 (SD 79,5), ranging 58,1- 400 nmol/L. The cut-off value (10th percentile) was 90 nmol/L, similar to the one used in the commercial UMELISA® T4 Neonatal. T4 mean concentration in serum samples from 492 neonates was 104,8 (SD 66,0), ranging 51,7- 400 nmol/L. The cut-off value (10th percentile) was 100 nmol/L, similar to the one used in the commercial UMELISA® T4.

The outcomes of the assays when using both solid phases were highly correlated by the Pearson correlation coefficient ($r = 0,92$ for modified UMELISA® T4 Neonatal and modified UMELISA® T4), where the slope, y-intercept and linear correlation coefficient were 0,8569, 19,01 and 0,851 for modi-

fied UMELISA[®] T4 Neonatal, and 0,7586, 24,1 and 0,8567 for modified UMELISA[®] T4, respectively. Values of Pearson correlation coefficient above 0,7 signify the existence of a high positive correlation, and values above 0,9 thus indicating that there is a very high positive correlation between the analysed variables (14). The absolute agreement among assays was quantified using the concordance correlation. The concordance correlation coefficient (ρ_c) combines precision and accuracy measurements. Values greater than 0,85 indicate the existence of a high correlation between the two methods evaluated (10). The results showed high overall concordance among assays when plates coated with anti-T4 monoclonal and anti-T4 polyclonal antibodies were used ($\rho_c = 0,91$ for UMELISA[®] T4 Neonatal and $\rho_c = 0,9$ for UMELISA[®] T4).

IV. CONCLUSIONES

This work shows the re-standardization of UMELISA[®] T4 Neonatal and UMELISA[®] T4, by using polystyrene plates coated with highly specific anti-T4 monoclonal antibodies. The use of MAbs to identify and characterize antigens contained in complex mixtures is peerless. MAbs are excellent tools as probes for identifying antigens empirically, as well as providing the means to isolate the molecule for characterization (15).

Overall evaluation of the results shows that modified UMELISA[®] T4 Neonatal and modified UMELISA[®] T4 using polystyrene plates coated with monoclonal antibodies increases the slope of the calibration curve in the zone of clinical interest.

Both competitive UMELISAs described exhibit similar characteristics to other commercially available assays, in terms of precision, accuracy and utility, thus, making them potentially useful in the neonatal screening for CH.

The total time required by the new assays using monoclonal antibodies is similar to commercial UMELISA[®] T4 Neonatal and UMELISA[®] T4 using polyclonal antibodies. Assays exhibited good within-run and between-run reproducibilities in the concentration range from 25 to 400 nmol/L, and the samples' linearity demonstrate its accuracy. Moreover, the results obtained following the evaluation of CDC and RANDOX controls demonstrated their accuracy. UMELISA[®] T4 Neonatal and UMELISA[®] T4 are intended for the quantitative measurement of T4 in blood spots dried on filter paper and serum samples for the detection of children with CH, where T4 levels are below 100 nmol/L. That is why the LOD and LOQ are more than adequate for population screening.

Modified UMELISA[®] T4 Neonatal and UMELISA[®] T4 with monoclonal antibodies as solid phase presented high correlation with the commercial kits. Additionally, the frequency distributions obtained with the new coating conditions, were similar to the ones with polyclonal antibodies in the Cuban newborn population and other populations as well (16, 17).

All in all, UMELISA[®] T4 Neonatal and UMELISA[®] T4 with strips coated with anti-T4 monoclonal antibodies, as part of SUMA technology, are simple, precise, accurate, and rapid assays that can be used to carry out screening programs in the newborns. Modifying both assays with monoclonal antibodies as solid phase, the analytical sensibility of them is improved without affecting precision and accuracy of the results.

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