CLINICAL INTEREST
Normal levels of thyroid hormones are indispensable for the correction of physical and mental development from the first moments of life (1). The assessment of hormone levels has an essential importance as an indicator of the functional status of the thyroid and may be specially used for early diagnosis of Congenital Hypothyroidism (2). This illness is the most frequent cause of mental retard, avoidable in children, with a frequency approximately of 1:4000 newborns (3). The deficit in the use or in the production of this hormone produces irreversible alterations in the development of the central nervous system, this can be avoided with a replacement therapy of thyroid hormone in the first moments of life (4,5). The clinical detection of Congenital Hypothyroidism in neonates is practically impossible because its symptoms are subjective and scarce, so the diagnosis is only possible by determining the concentration of thyroxine (T4) or thyroid stimulating hormone (TSH) in blood. Due to this reason, several Congenital Hypothyroidism screening programs are based on the determination of T4 in blood samples from the heel collected on filter paper (6).

PRINCIPLE OF THE TEST
The UMELISA T4 NEONATAL is a competitive enzyme immunoassay for the detection of total thyroxine in dried blood collected on filter paper, where the natural antigen and the enzyme-labelled antigen compete for a limited number of binding sites on the antibody (7). This assay uses as a solid phase ultramicroELISA strips (10 µL of solution per well) coated with anti-T4 antibodies, which guarantees specificity in the test. The blood spots are eluted with a solution that contains the T4/Alkaline Phosphatase (A.P.) conjugate and the eluate is placed in the reaction strip wells. When samples are incubated in the strip wells, an antibody-antigen-enzyme complex is formed. A posterior washing of the plates eliminates the unbound conjugate and other components of the samples. When the fluorogenic substrate is added to the wells, it is hydrolyzed by the conjugated enzyme so that the intensity of fluorescence will be inversely proportional to the thyroxin concentration present in the sample.
Contents:

Reaction strips: 12 strips x 8 wells                3
Blood spot calibrators and control                                         2 x 3 curves
R1: Buffer Solution                                          1 x 25 mL
R2: Conjugate Buffer             3 x 10 mL
R3: Conjugate                                           1 x 1 mL
R4: Substrate                                                                        1 x 2 mL
R5: Substrate Buffer             1 x 18 mL

The reaction strips lot is ubicated in the plastic container and it is composed by 5 digits. The four first ones indicate the expiry date and the 5th is an internal indicator of the production process.

The calibrators and the control have been prepared from whole human blood with an hematocrit value of 55 % dried on filter paper which has been homologated by the Immunoassay Center to be applied in neonatal screening using the Ultamicroanalytic System. All reagents contain sodium azide (0.2 g/L) as preservative.

The calibrators and the control have been tested for Anti-HIV 1+2, HBsAg and Anti-HCV by reliable methods and they were founded to be negative, anyway they should be handled as potentially infectious materials.

Preparation of working solutions:

R1: For 4 reaction strips, dilute 2 mL of R1 to 50 mL with distilled water. Mix gently to prevent excessive foaming.
R3: For 4 reaction strips, dilute 0.075 mL of R3 with 3 mL of R2. Homogenize the solution carefully.
R4: Dilute 1:10 with R5. Volume for 4 reaction strips: 2 mL (0.2 mL of R4 + 1.8 mL of R5).
R5: Prepare immediately before using.
STORAGE AND STABILITY
All reagents must be stored at 2 to 8 °C; under these conditions, they are stable in the unopened container until the expiry date. After using some of the content of vials, the remaining content is stable for 2 months, if kept at 2 to 8 °C in the closed original container and microbial contamination is avoided. Reconstituted reagents should not be stored after testing.

Unused reaction strips are stable during 2 months at 2 to 8 °C protected with desiccant in the provided bag carefully sealed.

MATERIALS REQUIRED BUT NOT PROVIDED
- Distilled water.
- Paper towels or absorbent paper.
- Sodium hypochlorite.
- Multichannel micropipette capable of accurately delivering 10 µL with disposable tips.
- Precision micropipettes in a range between 10 and 1000 µL.
- Graduated cylinder of 150 mL.
- Recipients for the elution of the spots.

CAUTIONS
- Before starting to work, make sure all the reagents had already been prepared according to the previous specifications, they must be completely homogenized and at room temperature.
- Handle the samples, calibrators and the control as potentially infectious. Wear disposable gloves. Place used materials in disinfecting solutions (5 % sodium hypochlorite) or sterilize in autoclave.
- Dried blood samples collected on filter paper which has been homologated by the Immunoassay Center to be applied in neonatal screening using the Ultamicroanalytic System must not remain at room temperature for more than a week. If we store them between 2 - 8 °C, they will be stable during 4 months.
- Consider equipment and accessories which have been in direct contact with samples and reagents as contaminated. Apply cleaning procedures recommended in relevant user's manuals.
- Bring the reaction strips to room temperature before removing the protective cover in order to prevent condensation.
- Ensure that the reaction strips are levelled in the strip holder.
- Use new or perfectly clean tips to work with reagents and samples.
- You must guarantee the right humidity control in every step of the assay. The reagents and samples must be kept in humidity chambers to avoid its evaporation because this can alter the results.
- Check regularly the accuracy and precision of the pipettes.
- Execute the manipulation norms of the instruments you have used and its right working, be careful during the operations of washing and pipetting.
- If you use the multipipette ERIZO to transfer the samples, calibrators and the control, you must wash carefully its tips to avoid contamination; at least a 5 cycles-wash with R1 working solution and at least a washing of 5 cycles with distilled water. Discard the solution and distilled water after each washing.
- Avoid possible contaminations with fluorescent materials.
- Vials containing fluorogenic substrate should be kept away from strong light.
- Do not use the kit beyond the expiry date.
- The components of UMELOSA T4 NEONATAL kit have been tested as a unit. Do not exchange components from other sources or from different lots.

**TECHNICAL PROCEDURE**

1. **Preparation of samples, calibrators and control**
   Cut with a puncher a 3 mm diameter disk from the central part of the spot. Elute this disk for at least one hour at room temperature (20-25°C) in an appropriate receptacle, with 60 µL of R3 working solution. Homogenize correctly.

2. **Addition of samples, calibrators and control into the reaction strips.**
   Transfer 10 µL of samples, calibrators and control into the reaction strips.
   Laboratories with SUMA readers requiring wells for setting 0 and 100 fluorescence values must use the following protocol:
Laboratories with SUMA readers not requiring wells for setting 0 and 100 fluorescence values must use the following protocol:

<table>
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<tr>
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</table>
These are the approximate concentration values that the calibrators of the assay should have. These values are determined for each lot.
Calibrator A: 0 nmol of T4/L serum
Calibrator B: 17-25 nmol of T4/L serum
Calibrator C: 40-54 nmol of T4/L serum
Calibrator D: 72-98 nmol of T4/L serum
Calibrator E: 144-196 nmol of T4/L serum
Calibrator F: 289-391 nmol of T4/L serum
CB Control: Assay control

**Duplicate determinations are recommended.** When using the UMEFLISA T4 NEONATAL software package for automatic interpretation of results, the first sample must be placed in positions 1 and 2, the second one in positions 3 and 4, and so on.

3.-Incubation of samples, calibrators and control.
Incubate the reaction strips during 2 hours at room temperature (20-25 °C) in humidity chamber.

4.-Washing.
Use a washer that belongs to the SUMA technology. Wash the reaction strips 6 times. Check that the wells were fully filled with R1 working solution (25 µL). This solution must remain at least 30 seconds in the wells during each wash. After the last aspiration, tap the inverted strips a few times on a clean paper towel.

5.-Addition of substrate.
Add 10 µL of properly diluted substrate into each well of the reaction strip. Do not add substrate to wells A1 and B1 when using readers that need wells on the strip for setting 0 and 100.

6.-Incubation of substrate.
Incubate at room temperature (20-25 °C) in humidity chamber. Normally a 30 minutes of incubation time is required to achieve a fluorescent signal of 100 to 150 units for Calibrator A. Nevertheless, the optimum incubation time should be established for each laboratory according to particular temperature conditions.

7.-Reading.
When using SUMA readers requiring positions for setting 0 and 100, add 10 µL of distilled water in A1 and 10 µL of Fluorescent Reference Solution (FRS), diluted 1:10 with distilled water, in B1.

Read intensity of fluorescence emitted in each determination using a SUMA reader.

**CALCULATION PROCEDURE**

Calculate the quotient F/F₀, where: F is the average fluorescence of the calibrators, the control or the samples, and F₀ is the average fluorescence of the Calibrator A. The result is expressed in (%). In the case of standard A: F/F₀ (%) = 100.

The calculated values of the samples are interpolated in a graphic of F/F₀ (%) versus T₄ concentration, correspondent to the calibration curve, getting the concentration values in nmol of T₄/L of serum.

![Calibration Curve](image)

Validation, interpretation and print-out of the results are done automatically, using the UMELISA T₄ NEONATAL software package.

**QUALITY CONTROL**

I. The Calibration Curve should fulfill the following condition:
The average of the two fluorescence values for each calibrator should produce a
decrease in the fluorescence proportional to its concentration, following a similar pattern
to the curve example; the discordant values are automatically discarded by the
software.
II. The concentration value calculated for the control should be in the range established
for the test.
III. Discard the results if the duplicates of a sample are discordant values.

INTERPRETATION OF THE RESULTS
In a study done on 410 neonates with a well defined normal thyroid function, was
determined the T4 concentration from blood pricked from the heel, collected on filter
paper on the fifth day of life, using the UMELISA T4 NEONATAL kit. Using the tenth
percentile of the distribution, the cut off level corresponded with 100 nmol of T4 /L of
serum, those inferior to this value, were considered as low ones.
Bearing in mind the various genetic and environmental factors acting on the population
of the different geographic locations, the international practice recommends that each
laboratory should establish its own reference values.
Conversion of Factor:

\[ \text{nmoL/L} \times 0.078 = \text{µg/dL} \]
\[ \text{µg/dL} \times 12.87 = \text{nmoL/L} \]
SPECIFIC PERFORMANCE CHARACTERISTICS

1-PRECISION.
Three samples, in three value ranges (high, medium and low) were evaluated in order to calculate precision.

<table>
<thead>
<tr>
<th></th>
<th>Intra-Assay (n=20)</th>
<th>Inter-Assay (n=20)</th>
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<tr>
<td></td>
<td>SD</td>
<td>CV (%)</td>
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<tr>
<td>37.48</td>
<td>3.8</td>
<td>10.14</td>
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<tr>
<td>56.22</td>
<td>4.7</td>
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<tr>
<td>86.55</td>
<td>5.4</td>
<td>6.89</td>
</tr>
</tbody>
</table>

SD: Standard Deviation  CV(%): Coefficient of Variation

Analytical recovery was evaluated using six CDC controls. Percentage recovery obtained was higher than 90%.

<table>
<thead>
<tr>
<th>CDC (Code)</th>
<th>Expected Value (nmol/L)</th>
<th>Obtained Value (nmol/L)</th>
<th>Recovered (%)</th>
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</thead>
<tbody>
<tr>
<td>2312</td>
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<td>109.8</td>
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<tr>
<td>3313</td>
<td>131.3</td>
<td>120.5</td>
<td>91.8</td>
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</tbody>
</table>

Dilutions of blood samples with high concentrations of T4 were done, before they were collected on filter paper. Calculated concentrations after correction with dilution factor were about ± 10 % of the original concentration in the undiluted sample.
3. SENSITIVITY.
The sensitivity of the UMELISA T4 NEONATAL is 17 nmol of T4/L of serum. This value was calculated as the concentration which was distinguishable from the zero calibrator, that is, two standard deviations below the fluorescence of the calibrator A.

4. SPECIFICITY.
The study of the specificity was carried out, evaluating the cross-reactivity with other substances related structurally, in the normal condition of the assay:

- (L)-T4: 100 %
- (D)-T4: 26 %
- (L)-T3: 1.5 %
- (D)-T3: 1.9 %
- (L)-T2: < 0.08 %
- Triiodopropionic acid: 2.9 %
BIBLIOGRAPHY


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UMEELISA T4 NEONATAL
Code UM 2125 (To use 3 mm discs)
Immunoassay Center. 25 Avenue and 134 Street, P.O.Box 6653, Havana, CUBA.
Phone: 2082929   Telex: 512439,   Fax: (537) 336514.