

# Immunoradiometric and Luminescence Immunoassay of Human Thyrotropin from Dried Blood Spots for Screening of Neonatal Hypothyroidism

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**Summary:** Two monoclonal antibodies to thyrotropin prepared in our laboratory were employed for the development of two-site immunometric assays in two modifications for the estimation of human thyrotropin in dried blood spots designed for the screening of neonatal hypothyroidism. The immunoradiometric assay using the second antibody labelled with <sup>125</sup>I is simple and fast (one incubation step lasting 2 h). The detection limit of 1 mU/l and the absence of the hook effect up to a concentration of more than 1000 mU/l are optimal for neonatal screening; the presence of other glycoprotein hormones does not interfere with the assay. In the luminescence immunoassay the second antibody is labelled with peroxidase. In spite of the two-step configuration the method is fast (4.5 h) and enables specific determination of thyrotropin levels in the range of 2.4–1100 mU/l. The conditions and properties of both immunometric assays described are comparable with the time-resolved immunofluorometric assay widely used in Europe.

The luminescence immunoassay was applied successfully in the screening of 3000 neonates for congenital hypothyroidism.

## Introduction

The incidence of neonatal hypothyroidism in the USA and in Europe is 1/4000, with differences in European countries from 1/3000 to 1/6000 (1). The determination of the level of thyrotropin in dried blood spots is now widely considered to be the optimal method for the screening of neonatal hypothyroidism (2). For this purpose a number of methods have been developed and immunometric assays based on two antibodies have been used almost exclusively in the past ten years. The combinations used involved two polyclonal antibodies (3–5), one monoclonal and one polyclonal antibody (6) and, most recently, a pair of monoclonal antibodies (7–10).

In this study we present a simple, rapid, specific and sensitive immunoradiometric assay and its non-isotopic variety – the luminescence immunoassay – for the measurement of thyrotropin in dried blood spots for the screening of neonatal hypothyroidism using our own monoclonal antibodies to thyrotropin.

## Materials and Methods

### Hormones

The starting material for the isolation of human thyrotropin was the glycoprotein fraction obtained from frozen pituitaries using the

method of Lumley Jones et al. (11). Following precipitation at a final ethanol volume fraction of 0.75, the precipitate was dissolved in 100 mmol/l NH<sub>4</sub>HCO<sub>3</sub> and applied to a column of Sephacryl S-100 HR. The glycoprotein hormone-containing fractions were pooled and freeze-dried. Thyrotropin was isolated from the freeze-dried material by consecutive chromatography on S-Sepharose Fast Flow, DEAE-cellulose (DE-52 Whatman), CM-cellulose (CM-52 Whatman), and Sephadex G-100 Superfine. All chromatographies were monitored using RIA for thyrotropin, follitropin, and lutropin. The resulting preparation of thyrotropin had an immunologic activity of 8.1 U/mg when measured in an IRMA system against the second IRP 80/558 and it contained only negligible traces of follitropin and lutropin.

Human somatotropin was isolated from frozen pituitaries by a modification of the method of Lumley Jones et al. (11). The resultant preparation of somatotropin had a specific potency of 3.1 U/mg when measured by RIA against the first IRP 66/217.

Human prolactin was isolated from frozen pituitaries by a modification of the method of Hwang et al. (12) and had a potency of 31.16 U/mg when measured by RIA against the third IS 84/500.

Second IS 80/552 was used as the standard for human lutropin, first IS 83/575 was used as the standard for human follitropin and third IS 75/537 was used as the standard for human chorionic gonadotropin.

### Monoclonal antibodies

Using methods found in the literature (13) we prepared 273 mouse monoclonal antibodies to thyrotropin. For the construction of the immunometric systems, two antibodies were chosen that could bind the thyrotropin (TSH) molecule simultaneously. The TSH-51

antibody of an IgG<sub>2<sub>a</sub></sub> subclass is strictly specific for thyrotropin with an association constant of  $5.5 \times 10^{10}$  l/mol and has a strong capacity to bind the hormone on the solid phase. The TSH-116 antibody of an IgG<sub>2<sub>a</sub></sub> subclass binds the  $\beta$ -subunit of thyrotropin with an association constant of  $1.1 \times 10^{11}$  l/mol. It cross-reacts considerably with other glycoprotein hormones, which is of no significance in the present configuration of the assay system (see below). Both antibodies have a high speed of reaction. The isolation of antibodies from ascitic fluid was performed by chromatography on a column of Protein-A Sepharose CL-4B (14).

#### Preparation of standards

Standards for immunoradiometric assay (IRMA) were prepared by adding the international reference preparation of thyrotropin (second IRP 80/558) in concentrations of 0, 5, 15, 30, 80 and 160 mU/l to heparinized blood with a thyrotropin content lower than 0.05 mU/l. After thorough mixing, blood-spots were prepared on Schleicher-Schuell No. 2992 filter paper. After drying at room temperature for 4 h the standards were stored at 4 °C.

For the investigation of the cross-reactions, spots of blood with 200 µg/l prolactin, 100 µg/l somatotropin, 100 U/l chorionic gonadotropin, 100 U/l lutropin and 100 U/l follitropin were prepared. These hormones were added both to blood with zero concentration of thyrotropin and to blood containing 36 mU/l thyrotropin.

Standards for luminescence immunoassay were prepared by adding thyrotropin in 10 mmol/l phosphate buffer, pH 7.4, to a special matrix consisting of human serum (with thyrotropin concentration lower than 0.03 mU/l) mixed with erythrocytes to give a physiological haematocrit ratio, the volume of the buffered thyrotropin standard not exceeding 5% of the matrix. Standards with thyrotropin concentrations of 0, 12, 30, 70 and 160 mU/l, respectively, were pipetted in a volume of 50 µl on Schleicher-Schuell No. 2992 filter paper, dried at room temperature and stored at 4 °C.

#### Immunoradiometric assay

Polystyrene tubes were pretreated with an aqueous solution of glutaraldehyde, 10 g/l, (Merck) for 4 hours. After washing with distilled water, 0.5 ml of a solution of monoclonal antibody TSH-51 in a concentration of 5 mg/l in phosphate-buffered saline, pH 7.5, was added and incubated overnight at room temperature. After subsequent washing with phosphate-buffered saline, the remaining binding sites were blocked by incubation with a 10% solution of bovine serum in phosphate-buffered saline.

A 4 mm disc cut out of the blood spot and 200 µl of a solution of <sup>125</sup>I labelled monoclonal antibody TSH-116 were added to these tubes. The labelled antibody was dissolved in phosphate-buffered saline containing 1 g/l bovine serum albumin to give a total activity of 60 000 counts per minute per tube. After an incubation for 2 h at room temperature, both the solution and the disc were discarded, the tubes were rinsed twice with phosphate-buffered saline and the bound radioactivity counted in a gamma-counter. Both the standards and the samples were run in duplicate.

The detection limit was estimated by measuring the 0 mU/l standard in 15 tubes and calculating from the standard curve the concentration corresponding to the mean enlarged by two standard deviations of the mean.

The standards containing 15, 30 and 80 mU/l of thyrotropin were measured in duplicate in five consecutive assays (inter-assay evaluation) and in twelve tubes in a single assay (intra-assay evaluation). The mean and standard deviation were calculated and the precision expressed as the coefficient of variation (standard deviation in percent of the mean).

#### Luminescence immunoassay

Monoclonal antibody TSH-51 was bound on the solid phase of 96-well white plates (Immuntotech, Prague) according to the

method of Immuntotech (European patent EP 0122 209 A1). A 4.8 mm disc cut out of the blood spot on filter paper and 100 µl of 200 mmol/l citrate buffer, pH 5.7, were added to each well. After shaking for 2 h at 37 °C, the discs were removed and the wells washed once. Phosphate-buffered saline containing the conjugate of the TSH-116 antibody with horseradish peroxidase (150 µl), was added to the wells. The conjugate of the antibody with horseradish peroxidase was prepared with the two-step glutaraldehyde technique (15). After shaking for 2 h at 37 °C and subsequent washing, 200 µl of the signal solution containing 1.95 mmol/l hydrogen peroxide, 290 µmol/l luminol and 45 µmol/l *p*-iodophenol was added (16, 17). The intensity of the light emitted was measured between 5 and 20 min on the LIANA luminometer (Immuntotech, Prague).

The detection limit was estimated by measuring the 0 mU/l standard in 15 tubes and calculating from the calibration curve the concentration corresponding to the mean enlarged by two standard deviations of the mean.

The standards containing 15, 30 and 70 mU/l of thyrotropin were measured in duplicate in twenty consecutive assays (inter-assay evaluation) and in twelve tubes in a single assay (intra-assay evaluation). The mean and standard deviation were calculated and the precision expressed as the coefficient of variation (standard deviation in percent of the mean). To estimate the precision profile, coefficients of variation of 960 duplicates (standards, control and unknown samples) measured in twenty assays were used. The mean coefficients of variation were calculated for individual 5 mU/l intervals of thyrotropin concentration and plotted against the thyrotropin concentration in the range of 1–200 mU/l. The curve was fitted using the polynomial function of the 6th degree.

## Results

### Immunoradiometric assay

A typical standard curve of the immunoradiometric assay of thyrotropin is shown in figure 1. When the calibration was extended to 1000 mU/l, no hook effect was present and the signal continued to increase.

The detection limit was 1.0 mU/l. The results with blood spots containing other hormones added are shown in table 1.

The intra-assay and inter-assay precision for three concentrations of thyrotropin is shown in table 2.

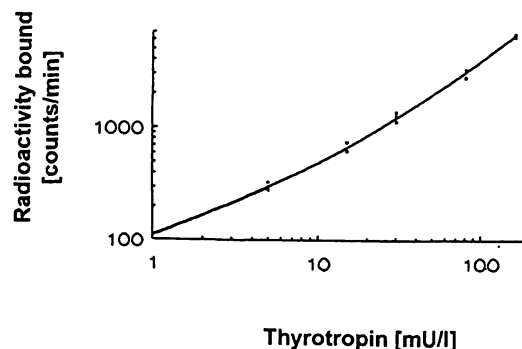


Fig. 1 Typical standard curve for the immunoradiometric assay of thyrotropin from dried blood spots.

The curve is fitted by the spline smoothed method. On the ordinate: measured radioactivity in counts per minute, logarithmic scale. On the abscissa: log of concentrations of thyrotropin standards.

### Luminescence immunoassay

A typical standard curve of the luminescence immunoassay of thyrotropin is shown in figure 2. The light intensity proved to increase up to a thyrotropin concentration of 1100 mU/l. The detection limit was 2.4 mU/l.

The intra-assay and inter-assay precision for three concentrations of thyrotropin is shown in table 2. The precision profile is shown in figure 3. The functional detection limit, defined as the concentration of thyrotropin measured with imprecision less than 15 percent, was 12 mU/l.

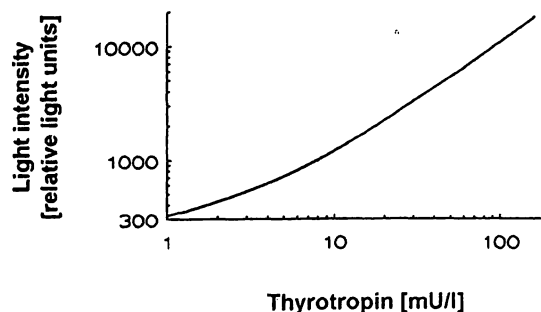
Thanks to the Prague Neonatal Screening Centre we were able to use the present luminescence immunoassay method on blood spots from 3000 neonates that had been screened for neonatal hypothyroidism by

**Tab. 1** Levels of thyrotropin determined in blood spots with the known concentration of hormone standards.

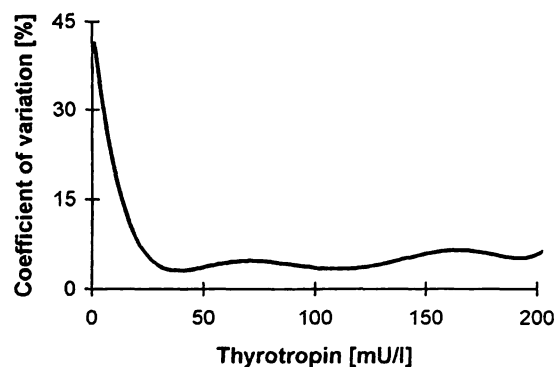
Possible interferent	Human thyrotropin (mU/l)	Measured value of human thyrotropin (mU/l)
Human chorionic gonadotropin		
100 U/l	0	<1.0
100 U/l	36	38.1
Human lutropin		
100 U/l	0	1.3
100 U/l	36	38.7
Human follitropin		
100 U/l	0	<1.0
100 U/l	36	37.0
Human prolactin		
200 µg/l	0	2.3
200 µg/l	36	35.6
Human somatotropin		
100 µg/l	0	<1.0
100 µg/l	36	34.8

**Tab. 2** The precision of measurement expressed as the intra-assay and inter-assay coefficients of variation.

Thyrotropin concentration (mU/l)	Coefficient of variation in %	
	Intra-assay	Inter-assay
<i>Immunoradiometric assay</i>		
15	8.3	9.3
30	8.1	8.7
80	7.0	5.0
<i>Luminescence immunoassay</i>		
15	8.6	12.4
30	6.8	8.2
70	7.1	5.5



**Fig. 2** Typical standard curve for the luminescence immunoassay of thyrotropin from dried blood spots. The curve is fitted by the spline smoothed method. On the ordinate: measured light intensities in relative light units, logarithmic scale. On the abscissa: log of concentrations of thyrotropin standards.



**Fig. 3** Precision profile of the present luminescence immunoassay.

The curve is fitted using the polynomial function of the 6th degree. On the ordinate: mean coefficient of variation in percent. On the abscissa: measured thyrotropin concentration.

methods including the Pharmacia Dry Spot TSH IRMA kit in the preceding three years. Positive samples were preferentially included. The concentration of thyrotropin in the vast majority of all samples under comparison proved to be lower than 20 mU/l (the cut-off level for positives used in the Screening Centre) by both methods. Higher concentrations were found in 15 cases by the present luminescence immunoassay method and in 16 cases by the Dry Spot TSH IRMA method. The only differing sample value could not be classified as controversial, because both values (20.2 mU/l for IRMA and 16.6 mU/l for luminescence immunoassay) were very close to the cut-off border value of 20 mU/l. To perform a more exact comparison of the two methods, various amounts of thyrotropin were added to 32 control blood samples up to a concentration of 200 mU/l, the samples were assayed simultaneously with both kits and the values obtained were studied by the x/y method. The result is presented in figure 4. The mean x/y was 0.982 and the coefficient of variation was 14.1 percent.

### Discussion

The immunoradiometric assay for the determination of thyrotropin from dried blood spots described in this pa-

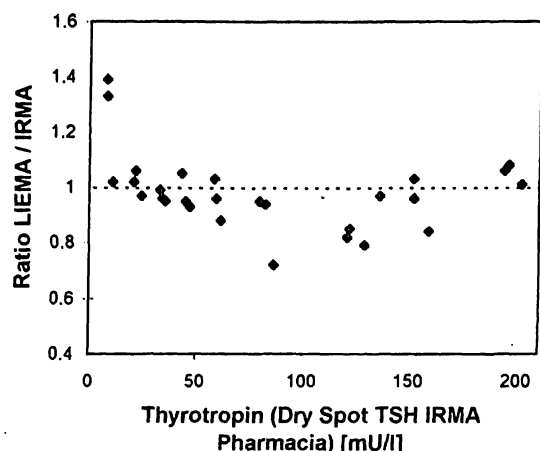


Fig. 4 Correlation between thyrotropin concentrations measured by our luminescence immunoassay (LIEMA) and by the Pharmacia Dry Spot TSH IRMA kit (IRMA). On the ordinate: ratio LIEMA/IRMA. On the abscissa: thyrotropin concentration measured by the Dry Spot TSH IRMA kit. The mean ratio was 0.982 and the coefficient of variation was 14.10.

per is simple, easy to perform and rapid. The detection limit of 1 mU/l is quite sufficient, as the levels below 20 mU/l are considered negative in the screening of neonatal hypothyroidism (18). A very low detection limit (0.037 mU/l) was described in an assay of Japanese researchers based on a combination of monoclonal antibody and polyclonal antibody (6). However, sensitivity in this range is not necessary for the screening of primary hypothyroidism. The continuing increase of the standard curve up to 1000 mU/l is more than adequate. In spite of the fact that the labelled antibody used cross-reacted with other glycoprotein hormones, the results were not influenced by the presence of 100 U/l each of lutropin, follitropin and chorionic gonadotropin. These levels are not exceeded in the blood of neonates (6).

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However, the immunoradiometric assay for thyrotropin served only as the springboard for the development of the luminescence immunoassay for clinical purposes and for this reason we have not examined any real patient samples.

The advantage of the luminescence immunoassay is the longer stability of the labelled antibody and that its use is not limited to isotope laboratories. It was not, however, possible to perform it in a one-step modification, because the enzymatic reaction was influenced by the presence of blood. Therefore it was necessary to perform a two-step method and to add one more wash. The precision seems to be comparable with the IRMA method; the detection limit is somewhat higher because of the higher signal of the zero standard (blank), but sufficient for the neonatal screening. In comparison with the immunochemiluminometric screening method used by Neto (10) the procedure described is simpler, because it is not necessary to pre-elute the disc, and more rapid, because no overnight incubation is necessary. There was no false positive result with real patient samples and the cases of neonatal hypothyroidism were properly recognized.

## Conclusion

The conditions and properties of the immunoradiometric and luminescence immunoassays described are adequate for the screening of neonatal hypothyroidism. They are comparable with the time-resolved immunofluorometric assay widely used in Europe (7, 8). The luminescence immunoassay was applied successfully in the screening of 3000 neonates for congenital hypothyroidism.

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*Received April 9/July 8, 1996*

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