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# Measuring free thyroxine levels in neonatal heel-prick samples ☆

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ABSTRACT

The Dutch neonatal screening scheme for Congenital Hypothyroidism (CH) is primarily based on the determination of thyroxine (T4) in filter paper blood spots. In the lowest 5% of T4 values, thyroxine binding globulin (TBG) is measured in order to be able to correct for occasional low TBG levels. However, because the commercial TBG kit has been withdrawn from the market, alternative strategies are needed to be explored including the assessment of free T4.

We evaluated the Neonatal Free Thyroxine (fT4) enzyme immunoassay (EIA) kit of Bio-Rad.

FT4 as measured in a daily run of random samples correlated with T4. We also observed a correlation between fT4 and T4, and between fT4 and T4/TBG ratio in blood spots with low T4 concentrations. The correlation between fT4 and T4 in blood spots of proven CH-patients was highly significant. ROC curves were constructed for the fT4 assay and the T4/TBG ratio based on 27 CH patients and 215 controls with a complete set of data. The curves of both assays seemed to be rather similar.

We conclude that the validity of the fT4 and the T4/TBG-approach seems to be the same. A study with a larger sample size giving the same or even more favorable results for the fT4-approach is necessary before we will change the present CH protocol.



## 1. Introduction

Congenital Hypothyroidism (CH) comprises a variety of disorders of either the thyroid gland itself or the regulatory system stimulating the thyroid gland consisting of the hypothalamus and the pituitary, and results in a lack of thyroid hormone (TH) in the neonatal period. TH is essential for brain development and the critical period for dependence of the central nervous system on TH is from fetal life until at least two years after birth. Therefore, the major risk of CH, if untreated, is irreversibly impaired cognitive and motor development [1]. For this reason, a neonatal screening program for CH was established in The Netherlands in 1981. Although many screening programs are based on TSH, the Dutch neonatal screening scheme for CH is primarily based on the determination of thyroxine (T4) in filter paper blood spots. The concentration of T4 is expressed as a standard deviation (T4SD) score of the daily mean. In the lowest 20% of T4 values, thyroid stimulating hormone (TSH) concentrations are measured additionally. In the lowest 5% of T4 values, thyroxine binding globulin (TBG) is measured in order to be able to correct for occasional low TBG levels. Combined T4, TSH and TBG determinations result in the detection of both primary and secondary hypothyroidisms (prevalence 1:15,000) [2,3]. However, because the TBG kit in use is no longer available per 2012, alternative strategies are needed to be explored. An alternative might be measuring fT4 levels in filter paper blood spots instead of T4 and TBG. We therefore tested the Neonatal Free Thyroxine (fT4) enzyme immunoassay (EIA) kit of Bio-Rad aiming to measure fT4 values initially and additional TSH in the lowest 20%.

## 2. Materials and methods

### 2.1. Methods

The neonatal screening program for CH is primarily based on a T4 measurement in filter paper blood spots. Sampling is performed between 72 and 168 h after birth. The T4 is expressed as standard deviation (T4SD) of the daily mean. If T4SD is $\leq -0.8$, TSH is additionally measured (20% of all samples). If T4SD is $\leq -1.6$ TBG concentration is also measured (5% of all samples). A T4SD/TBG ratio is calculated as follows: $(T4SD + 5.1) * 1000 / [TBG]$; (TBG expressed in nmol/L blood). If T4SD $\leq -3.0$ or [TSH] is $\geq 22$ mIU/L blood, children are referred to a pediatrician. In case of an equivocal result ($-3.0 < T4SD \leq -1.6$, combined with a T4SD/TBG ratio $\leq 17.0$ (TBG expressed in nmol/L blood) and/or $7 < [TSH] < 22$ mIU/L

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**Table 1**
Intra-assay variation, based on 10 measurements.

| | 1 | 2 |
|---|---|---|
| fT4 (pmol/L blood) | 16 | 34 |
| SD | 2.6 | 4.5 |
| % VC | 16.3 | 13.1 |

**Table 2**
Interassay variation, based on 13 runs.

| | Pool low | Pool medium |
|---|---|---|
| fT4 (pmol/L blood) | 14 | 35 |
| SD | 1.9 | 2.8 |
| % VC | 13.6 | 7.9 |

blood), a second heel puncture is performed and T4, TSH, and TBG measurements are repeated. For children born with a gestational age (GA) of 36.0 week or less in combination with a birth weight (BW) of 2500 g or less, the actions are based on TSH only: if [TSH] ≥ 22 mIU/L blood, the child is referred, and if 7 < [TSH] < 22 mIU/L blood, the result is considered equivocal and a second heel puncture is performed, after which the child is referred if the result is either equivocal again or positive [4].

### 2.2. Samples

A daily routine run (N = 126) for the neonatal screening in our laboratory was used to measure fT4 randomly. A selection of samples based on T4 concentration (N = 315) was made in order to study the relation between fT4 and T4 in samples with low T4 concentrations (T4SD ≤ −1.6). The same was done for TBG ([TBG] < 65 nmol/L blood) (N = 20). Finally, a cohort of proven CH patients (N = 56) was used to measure the fT4 levels in order to establish a cut-off value.

### 2.3. Assays

FT4 was measured using the Neonatal Free Thyroxin (fT4) enzyme immunoassay (EIA) kit (kindly provided by Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Briefly, the fT4 assay is a competitive EIA assay, for which dried blood spot samples are eluted directly into anti-rabbit IgG antibody-coated microtiter plate in a solution containing peroxidise labeled T4 and anti-T4 antibody. After incubation, the plate is washed free of unbound labeled T4 and antibody. Bound fT4 in the sample has been measured by the reaction with tetramethylbenzidine (TMB). The extinction measured in the well is reverse proportional to the fT4 concentration in the sample. Total T4 was measured by fluoro immunoassay (AutoDELFIA, Perkin Elmer, Waltham, MA) according to the manufacturer's protocol. TBG was measured using a radioimmunoassay kit (RIA; Eiken, Japan). This TBG kit is intended for measurement in serum and was modified for measurement in dried bloodspots [5]. The sample size for the three markers was a 1/8 punch from the bloodspots, corresponding with 3 μL blood. All measurements were done in duplicate.

### 2.4. Statistics

Inter- and intra-assay variations are presented as mean ± SD. Pearson's coefficient of correlation ($R_p$) was used for the evaluation of the association (linear dependence) between fT4 and T4 and T4/TBG ratio respectively when distributed normally. Spearman's coefficient of correlation ($R_s$) was used when the data were abnormally distributed. ROC curves were constructed in order to evaluate the validity of the fT4 assay and the T4/TBG ratio. The ROC analysis was based on 27 CH patients and 215 controls (all with T4 ≤ −1.6 SD) with a complete set of data. In this analysis children with a GA ≤ 36 weeks were excluded. All analyses were carried out in SPSS 12.5.1 (SPSS, Inc., Chicago, IL, USA). P-values less than 0.05 were considered statistically significant.

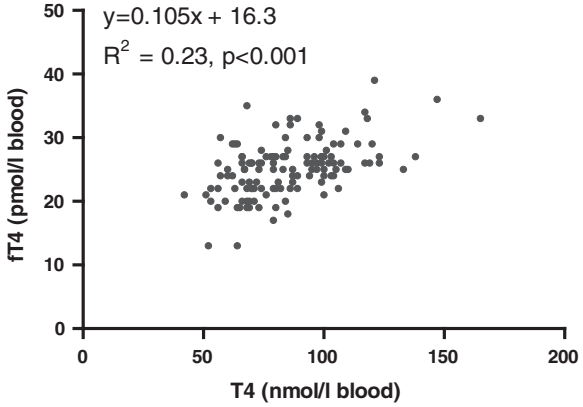


**Fig. 1.** Relationship between T4 and fT4 measured in filter blood spots of children sampled within the framework of the Dutch Neonatal Screening Program (n = 126, daily series). $R^2$ based on $R_p$ was given.

## 3. Results

### 3.1. Assay characteristics

The intra-assay variation for the FT4 kit was 16.3% in the lower range, 12.8% in the middle range and 13.1% measuring a sample with a high total fT4 concentration (Table 1). The inter-assay variation was 13.6% for a sample in the lower range of the assay while the inter-assay variation was 7.9% measuring a medium control sample included in the kit (Table 2).

### 3.2. Correlations (Figs. 1–5)

The average fT4 value of a daily run of random samples is 25.2 ± 4.3 pmol/L blood. fT4 concentrations correlated with total T4 (n = 126, $R_p$ = 0.50, P < 0.001) although the variability in fT4 was explained for only 23% by the variation in T4 ($R^2$ = 0.23) (Fig. 1). TSH has to be measured in 20% of the lowest T4 values of a daily run. Table 3 showed that only 8 samples, based on fT4 and T4 determinations were indicated for TSH while 19 and 24 samples needed a TSH determination based on fT4 or T4 values respectively.

We also observed a correlation between fT4 and T4 and between fT4 and the T4SD/TBG ratio in blood spots with a low T4 concentration (T4SD ≤ −1.6, n = 315, $R_p$ (fT4–T4) = 0.60, P < 0.001 (Fig. 2)

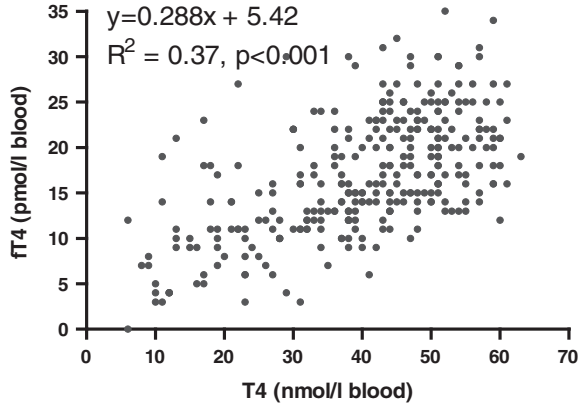


**Fig. 2.** Relationship between T4 and fT4 measured in a selection of filter blood spots of children sampled within the framework of the Dutch Neonatal Screening Program (n = 315, T4SD ≤ −1.6 SD). $R^2$ based on $R_p$ has been depicted.