ELISA kits available from ADI (see details at the web site)

#0010	Human Leptin		
#200-120-AGH	Human globular Adiponectin (gAcrp30)		
#0700	Human Sex Hormone Binding Glob (SHBG)		
#0900	Human IGF-Binding Protein 1 (IGFBP1)		
#1000	Human C-Reactive Protein (CRP)		
#100-110-RSH	Human Resistin /FIZZ3		
#100-140-ADH	Human Adiponectin (Acrp30)		
#100-160-ANH	Human Angiogenin		
#100-180-APH	Human Angiopoietin-2 (Ang-2)		
#100-190-B7H	Human Bone Morphogenic Protein 7 (BMP-7)		
#1190	Human Serum Albumin	**#1200**	Human Albumin (Urinary)
#1750	Human IgG (total)	**#1760**	Human IgM
#1800	Human IgE	**#1810**	Human Ferritin
#1210	Human Transferrin (Tf)	**#0020**	Beta-2 microglobulin
#1600	Human Growth Hormone (GH)		

#0060	Human Pancreatic Colorectal cancer (CA-242)		
#1820	Human Ovarian Cancer (CA125)	**#1830**	Human CA153
#1840	Human Pancreatic & GI Cancer (CA199)		
#1310	Human Pancreatic Lipase		
#1400	Human Prostatic Acid Phosphatase (PAP)		
#1500	Human Prostate Specific Antigen (PSA)	**#1510**	free PSA (fPSA)
#0500	Human Alpha Fetoprotein (AFP)		
#0050	Human Neuron Specific Enolase (NSE)		

#0030	Human Insulin	**#0040**	Human C-peptide
#0100	Human Luteinizing Hormone (LH)		
#0200	Human Follicle Stimulating Hormone (FSH)		
#0300	Human Prolactin (PRL)		
#0400	Human Chorionic Gonadotropin (HCG)	**#0410**	HCG-free beta

#0600	Human Thyroid Stimulating Hormone (TSH)		
#1100	Human Total Thyroxine (T4)	**#1110**	Human Free T4 (fT4)
#1650	Human free triiodothyronine (fT3)	**#1700**	Human T3 (total)

#1850	Human Cortisol	**#1860**	Human Progesterone
#1865	Human Pregnolone	**#1875**	Human Aldosterone
#1880	Human Testosterone	**#1885**	Human free Testosterone
#1910	Human Androstenedione	**#1920**	Human Estradiol
#1925	Human Estrone	**#1940**	Dihydrotestosterone (DHT)
#1950	Human DHEA-sulphate (DHEA-S)		
#3400	Human serum Neopterin		

#3000	Human Rheumatoid Factors IgM (RF)
#3100	Human anti-dsDNA
#3200	Anti-Nuclear Antibodies (ANA)

Instruction Manual #. M-1110

Free THYROXINE (fT4)

ELISA KIT Cat. # 1110

For Quantitative Determination of Free T4 In Human Serum

For In Vitro Research Use Only

ALPHA DIAGNOSTIC INTERNATIONAL

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DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

ELISA KIT Cat. No. 1110 (96 tests)

For Quantitative Determination of Free T4 in Human Serum

Kit Components(96 tests)	Cat #
Mouse Anti-fT4 Coated Strip plate, (96 wells)	1111
fT4 Standard A, 0.5 ml; 0 pg/ml	1112
fT4 Standard B, 0.5 ml; 1 pg/ml	1113
fT4 Standard C, 0.5 ml; 5 pg/ml	1114
fT4 Standard D, 0.5 ml; 20 pg/ml	1115
fT4 Standard E, 0.5 ml; 100 pg/ml	1116
fT4 Control 0.5 ml	1117
Exact values of stds and control (lot specific) are provided on the vials. Stability: Approx. 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquot and stored frozen. Avoid multiple freezing and thawing cycles.	
Assay buffer, 15 ml	1118
T4-HRP Conjugate, 300 ul (50X)	1119
Wash buffer 50 ml (10X)	W-10
TMB Substrate Soln, 16 ml	TMB-10
Stop Solution, 6 ml	T-10
Complete Instruction Manual	M-1110

Introduction

Thyroxine (T4), the principal thyroid hormone, circulates in blood almost completely bound to carrier proteins. However, only the free (unbound) fraction of thyroxine is considered to be biologically active. The main carriers of thyroxine are thyroxine-binding globulin (TBG), pre-albumin and albumin. The measurement of free thyroxine (fT4) levels correlate better with the clinical status than total thyroxine levels.

ADI's free T4 assay is a one step competitive ELISA system that is rapid and easy to perform compared to equilibrium dialysis and ultrafiltration methods, which are cumbersome and time-consuming. This system employs a highly specific monoclonal antibody and a non-analog tracer that was proved experimentally to have no significant binding to TBG and albumin.

In the euthyroid, normal population the free T4 concentration is 7 – 22 pg/ml. The level of free T4 is decreased in hypothyroidism while in thyrotoxic patients the level of free T4 is increased.

This assay is used at times with other thyroid tests for *in vitro* diagnostic purposes and for assessing patients who are receiving thyroid treatments (follow-up).

EFFECT OF HUMAN SERUM ALBUMIN (HSA)

Purified human serum albumin (HSA) was added to a patient sample at concentrations of 10, 20 and 40 mg/ml. Samples were assayed with the Direct fT4 ELISA kit. No binding of labelled fT4 to HSA was found at these concentrations.

EFFECT OF THYROXINE-BINDING GLOBULIN (TBG)

The zero calibrator was spiked precisely with purified TBG at concentrations ranging from 25-200 µg/ml and assayed with the Direct fT4 ELISA kit. Results are tabulated below:

Sample	TBG Added (ug/ml)	OD 450 nm
1	0	1.883
2	25	2.030
3	50	2.149
4	100	2.175
5	200	2.251

No significant binding of labelled fT4 to TBG was found at these concentrations.

EFFECT OF NON-ESTERIFIED FATTY ACIDS

Oleic acid was added to a patient sample at concentrations of 0.5, 5 and 20 mmol/L and assayed with the Direct fT4 ELISA kit. Results are tabulated below:

Sample	fT4 (pg/ml)
Unspiked	24.83
+0.5 mmol/L	20.53
+5 mmol/L	26.06
+20 mmol/L	83.64

At high concentrations of oleic acid, the free T4 level was significantly increased. This is due to the well-known effect that non-esterified fatty acids can dissociate T4 from its carrier proteins.

Species crossreactivity and List of Publications

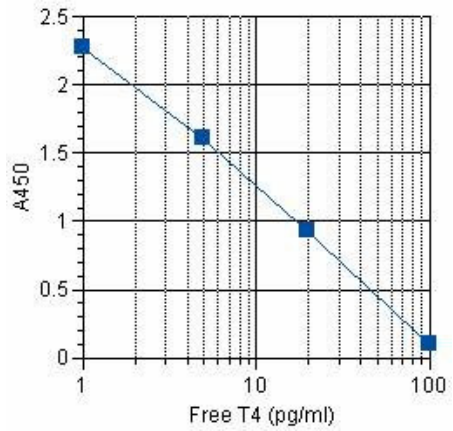
This kit has been optimized for use in human samples. We have not tested the kit in other species. Since T4 (fT4) hormones are the same in all species, this kit, in principle, should work in many species. In fact, ADI human T4 and T3 ELISA kit has been used in animals (see below).

T3 &T4, Huang W, 2005 J. Nutr., 135: 1631 – 1635,
T3 &T4; Xiao CW, 2004, J. Nutr., 134: 743 – 749, T3 and T4 in rat Plasma
T3 elisa Tsai Chen-En, 2002, Current Biol. 12: 1221-1226, T3 elisa kit
T4, Maglich, 2004, J. Biol. Chem., 279, 19832 - 19838., mice serum T4

WORKSHEET OF TYPICAL ASSAY

Wells	Stds/samples	Mean $A_{450\ nm}$	Calculated Concn (pg/ml)
A1, A2	**Std. A** (0 pg/ml)	2.516	
B1, B2	**Std. B** (1 pg/ml)	2.279	
C1, C2	**Std. C** (5 pg/ml)	1.973	
D1, D2	**Std. D** (20 pg/ml)	0.934	
E1, E2	**Std. E** (100 pg/ml)	0.098	
G1, G2	**Sample 1**	1.595	9.3

NOTE: These data are for demonstration purpose only. A complete standard curve must be run in every assay to determine sample values.



INTRA-ASSAY PRECISION

Three samples (3.79, 23.26, 70.60 pg/ml) were assayed ten times in a single assay. The values were SD 0.16, 1.1.4, 3.04 pg/ml and CV% 4.8, 4.9, and 4.3% respectively.

INTER-ASSAY PRECISION

Three samples (4.27, 20.54, 67.34 pg/ml) were assayed ten times at various times. The values were SD 0.53, 2.36, 6.67 pg/ml and CV% 12.3, 11.5, and 9.94, respectively.

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values. The following reference range (pg/ml) was established with 80 apparently healthy adults: Normal Euthyroid Samples (N=80; 7-22 pg/ml range).

EFFECT OF BILIRUBIN

Bilirubin was added to a patient sample at concentrations of 50 and 100 µg/ml and assayed with the Direct fT4 ELISA kit. Results were: Unspiked (8.78 pg/ml), + 50 µg/ml bilirubin (10.68 pg/ml), +100 µg/ml bilirubin (9.72%). No significant effect was observed at these concentrations.

PRINCIPLE OF THE TEST

Free T4 ELISA kit is based on competitive binding of human free thyroxine from serum samples and enzyme-labeled T_4 to T_4-specific antibodies immobilized on microtiter well plates. After a washing step, chromogenic substrate is added and color developed. The enzymatic reaction (blue color) is inversely proportional to the amount of T_4 present in the sample. The reaction is terminated by adding stopping solution (converts blue to yellow). Absorbance is then measured on a microtiter well ELISA reader at 450 nm. and the concentration of free T_4 in samples and control is read off the standard curve.

MATERIALS AND EQUIPMENT REQUIRED

Adjustable micropipet (20-100 ul) and multichannel pipet with disposable plastic tips. Reagent troughs, plate shaker (orbital shaker), plate washer (recommended) and ELISA plate Reader.

PRECAUTIONS

The Alpha Diagnostic International Free T4 ELISA test is intended for *in vitro research* use only. The reagents contain proclin-300 (0.1% v/v) as preservative; necessary care should be taken when disposing solutions. The standards and control serum may be prepared from human sera shown to be negative for HBsAg and HIV antibodies. Nevertheless, such tests are unable to prove the complete absence of viruses; therefore, sera should be handled with appropriate precautions.

Applicable **MSDS**, if not already on file, for the following reagents can be obtained from ADI or the web site.

TMB (substrate), H2SO4 (stop solution), and Prolcin-300 (0.1% v/v in standards, sample diluent and HRP-conjugates).

SPECIMEN COLLECTION AND HANDLING

Collect blood by venipuncture, allow clotting, and separating the serum by centrifugation at room temperature. Do not heat inactivate the serum.. If sera cannot be immediately assayed , these could be stored at -20ºC for up to six months. Avoid repeated freezing and thawing of samples. No preservatives should be added to the serum.

REAGENTS PREPARATION

Dilute Wash buffer (10x) in distilled water (50 ml stock in 450 ml water).

Prepare 1X solution of fT4-HRP conjugate. Dilute 20 ul stock conjugate per ml of assay buffer. Prepare 10 ml for a full plate assay (200 ul in 10 ml for complete 96-well plate). Do not store diluted conjugate and prepare only in required amounts.

STORAGE AND STABILITY

The microtiter well plate and all other reagents are stable at 2-8ºC until the expiration date printed on the label. The whole kit stability is usually 6 months from the date of shipping under appropriate storage conditions. The unused portions of the standards should be stored at 2-8ºC or stored frozen in small aliquots.

TEST PROCEDURE *(ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE BEFORE USE).*

1. Label or mark the microtiter well strips to be used on the plate. Dilute the enzyme conjugate (1:50) with assay buffer and wash buffer (1:10) with water. Dispense 200-300 ul of wash buffer or water to all wells. Mix for 5 seconds and discard or aspirate the solution. The step should be done just before adding the samples, do not allow the wells to dry at any time during the assay.

2. Pipet **25 ul of standards**, control, and serum samples into appropriate wells in *duplicate*.

3. Add **100 ul of diluted enzyme conjugate** into each well**.** Mix gently for 10 seconds. Cover the plate and incubate for **60 minutes at 37oC**.

4. Aspirate and **wash the wells 3 times** with 300 ul of 1x wash buffer. We recommend using an automated ELISA plate Washer for better consistency. Failure to wash the wells properly will lead to high blank or zero values. If washing manually, plate must be tapped over paper towel between washings to ensure proper washing.

5. Add **150 ul TMB substrate**. Mix gently for 10 seconds. Cover the plate and incubate for **10-15 minutes at 37oC** (incubation time may be decreased or increased by ~5 min to achieve optimal A450 of zero standard ~2.0-2.5).

6. Stop the reaction by adding **50 ul of stop solution** to all wells at the same timed intervals as in step 6. Mix gently for 10 seconds.

7. Measure the **absorbance at 450 nm** using an ELISA reader within 30 min. Yellow color will fade with time.

NOTES

Read instructions carefully before the assay. Do not allow reagents to dry on the wells. Careful aspiration of the washing solution is essential for good assay precision. Since timing of the incubation steps is important to the performance of the assay, pipet the samples without interruption and it should not exceed 5 minutes to avoid assay drift. If more than one plate is being used in one run, it is recommended to include a standard curve on each plate. The unused strips should be stored in a sealed bag at 4ºC. Addition of the HRP substrate solution starts a kinetic reaction, which is terminated by dispensing the stopping solution. Therefore, keep the incubation time for each well the same by adding the reagents in identical sequence. Do not touch the bottom of the wells.

Limitations

1. All the reagents within the kit are calibrated for the direct determination of fT4 in human serum. The kit is not calibrated for the determination of fT4 in other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Samples reading higher than 100 pg/ml should be reported as such and should not be diluted. Dilution will alter the existing equilibrium and may lead to false results.
5. The interpretation of free T4 results can be complicated by a variety of drugs, severe nonthyroidal illness and some rare conditions such as familial dysalbuminemic hyperthyroxinemia (FDH). For diagnostic purposes, the results of this assay should always be used in combination with the clinical examination, medical history and other findings.
6. Some individuals may have antibodies to mouse protein that can possibly interfere in this assay. Therefore, the results from any patients who have received preparation of mouse antibodies for diagnosis or therapy should be interpreted with caution.

CALCULATION OF RESULTS

1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.

PERFORMANCE CHARACTERISTICS

SENSITIVITY*:* The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the fT4 ELISA kit is **1. pg/ml.**

SPECIFICITY (CROSS REACTIVITY)

The following compounds were tested for cross-reactivity with the Direct fT4 ELISA kit with T4 cross-reacting at 100%: L-Thyroxine (100%), D-Thyroxine (94%), 3,3',5'-Triiodo-L-Thyronine (Reverse T3) (86%), 3,3',5-Triiodo-L-Thyronine (T3) (3.3%), 3,3',5'-Triiodo-D-Thyronine (1.8%), 3,3',5'-Triiodothyropropionic acid (0.6%).

The following compounds were tested but cross-reacted at less than 0.04%: Acetylsalicylic acid, 3,5-Diiodo-L-Thyronine, 3,5-Diiodo-L-Tyrosine and 3-Iodo-L-Tyrosine.