ASSAY PROCEDURE
Specimen Pretreatment: **None**.

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the free testosterone-HRP conjugate and wash buffer.
2. Remove the required number of well strips. Reseal the bag and return any unused strips to the refrigerator.
3. Pipette 25 µL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate.
4. Pipette 100 µL of the conjugate working solution into each well (We recommend using a multichannel pipette).
5. Gently shake the plate for 10 seconds.
6. Incubate the plate at 37°C for 1 hour.
7. Wash the wells 3 times with 350 µL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of a washer is recommended.)
8. Pipette 150 µL of TMB substrate into each well at timed intervals.
9. Incubate the plate at 37°C for 10–15 minutes (or until calibrator A attains dark blue colour for desired OD).
10. Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 8.
11. Read the plate on a microplate reader at 450 nm within 20 minutes after addition of the stopping solution.

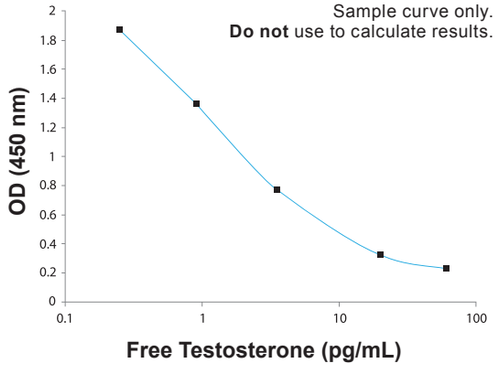
* If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.

CALCULATIONS
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 or 5-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.

TYPICAL TABULATED DATA
Sample data only. **Do not** use to calculate results.

Calibrator	Mean OD (450 nm)	Value (pg/mL)
A	2.292	0
B	1.680	0.1
C	1.181	1
D	0.780	5
E	0.426	20
F	0.214	60
Unknown	1.066	1.59
Unknown	0.441	19.6

TYPICAL CALIBRATOR CURVE
Sample curve only.
Do not use to calculate results.



PERFORMANCE CHARACTERISTICS
SENSITIVITY
The limit of detection (LoD) was determined from the analysis of 64 replicates of a low value sample and from the LoB.
LoD = LoB + 1.645σS,
where σS is the standard deviation of the low value sample.
σS was determined to be 0.0093 based on 64 measurements of a low value sample.
LoD = 0.0025 + (1.645*0.0093) = 0.018 pg/mL.

SPECIFICITY (CROSS-REACTIVITY)
The following compounds were tested for cross-reactivity with the Direct Free Testosterone ELISA kit with testosterone cross-reacting at 100%.

Steroid	% Cross Reactivity
Testosterone	100
5α-DHT	3.5
Androstenedione	0.17
Progesterone	0.007
Androsterone	0.075
Aldosterone	< 0.008
Cholesterol	< 0.0001
Cortisone	0.0025
DHEA	0.071
DHEAS	0.0014
17β-Estradiol	0.15
Estriol	< 0.008
Pregnenolone	0.028

INTRA-ASSAY PRECISION
Five samples were assayed 24 times each on the same calibrator curve. The results (in pg/mL) are tabulated below:

Sample	Mean	CV %
1	2.24	6.7
2	3.81	6.4
3	13.6	6.0
4	13.7	5.9
5	23.7	4.8

INTER-ASSAY PRECISION
Three samples were assayed twenty times in duplicate over a period of greater than ten days. The results (in pg/mL) are tabulated below:

Sample	Mean	CV %
1	3.53	8.1
2	13.8	11.5
3	23.3	6.9

COMPARATIVE STUDIES
The Direct Free Testosterone ELISA Kit (y) was compared with a competitors Free Testosterone Coated Tube RIA Kit (x). The comparison of 60 serum samples yielded the following linear regression results:
y (IBL) = 0.9362x (competitor) + 3.8794, r = 0.97

EFFECT OF SEX HORMONE BINDING GLOBULIN (SHBG)
The purpose of this study was to investigate a possible interference caused by the binding of SHBG to the free testosterone-HRP conjugate. A charcoal-stripped human serum pool was spiked precisely with SHBG at concentrations ranging from 6.25–200 µg/mL and was assayed with the the Free Testosterone ELISA Kit. Results tabulated below (in pg/mL):

SHBG Added	OD 450 nm	Percent B/B_o (%)
0	2.37	100.0
6.25	2.37	99.9
12.5	2.34	98.7
50	2.36	99.5
200	2.27	95.6

The results showed % binding values between 95–100% (Bo = unspiked serum) even at higher than normal SHBG levels. In conclusion, the results showed that there was no significant binding by SHBG on the free testosterone-HRP conjugate.

EXPECTED NORMAL VALUES
As for all clinical assays each laboratory should collect data and establish their own range of expected normal values. The results of an expected range study with apparently normal healthy subjects yielded the following results (all values are reported in pg/mL):

Cohort Group; Gender/Age	N	95% Confidence Range	Absolute Range
Males / < 13	44	–	ND–1.6
Males / 13–19	37	–	ND–22.3
Males / 20–39	120	9.1–32.2	–
Males / 40–59	120	5.7–30.7	–
Males / ≥ 60	120	5.9–27.0	–
Females / < 13	63	–	ND–1.3
Females / 13–19	17	–	0.2–2.0
Females / 20–39	120	0.1–6.3	–
Females 40–59	120	0.2–4.1	–
Females / ≥ 60	60	0.5–3.9	–

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SYMBOLS
CE European Comformity
IVD In vitro diagnostic device
Consult instructions for use
Contains sufficient for <n> tests
Storage Temperature
Legal Manufacturer
Use by
REF Catalogue Number
EC REP Authorized representative
LOT Lot number
X # Dilute 1: # Before use

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