PROBLEM	POSSIBLE CAUSE	SOLUTION
4. Poor reproducibility	a) Washing problems.	Check all 8 ports/ manifold for uniform flow of wash buffer. If there are blockage, clean the ports.
	b) Uncalibrated pipettes or tips not well fitted, improper pipetting/ dispensing.	Use only calibrated pipettes with well fitted tips & pipette carefully without bubbling.
	c) Reagent & sera not at room temperature or not well mixed before use.	Equilibrate reagents to room temperature and mix thoroughly before use
	d) Too long time for addition of calibrator, controls, samples or reagents, Inconsistency in time intervals.	Develop consistent and uniform technique.
	e) Interference in optical pathway due to Air bubbles.	Clean or dry the bottom of microwells, check for bubbles and repeat the readings.
5. False Positive	a) Beside 3a, b, c, d, e & f	Check the calculation
	incorrect interpretation and calculation of final results	part given in the insert and correctly interpret.
	b) High incubator temperature, incorrect timing or pipetting	Check incubator temperature, procedure & repeat assay.
	c) Use of turbid/ lipaemic or hemolyzed sample.	Centrifuge the sample at 5000 rpm for 30 minutes and re-run the test with clear sample.
6. False Negative/ low O.D. of Calibrator, Positive control / positive sample	a) Inadequate addition of substrate/conjugate solution.	Follow the procedure meticulously & repeat assay.
	b) Kit expired, reagent of different kit used.	Check the expiry of the kit before use.
	c) White particles in working substrate solution.	Discard the substrate and prepare the working substrate again in fresh tube.
	d) Uncalibrated pipettes, improper pipetting.	Use only calibrated pipettes with well fitted tips & pipette carefully without bubbling.
	e) Deterioration of Enzyme conjugate, TMB Substrate/ TMB Diluent.	Check storage of reagents. They shall be stored at 2-8ºC.
	f) Stop solution is added before 30 minutes. Reaction terminated before 30 minutes.	Follow the test procedure meticulously.
	g) O.D. taken at incorrect wavelength.	Read O.D. values at 450 nm and 630 nm.
	h) Incorrect incubator temperature, timing or pipetting	Check incubator temperature, procedure & repeat assay.
	i) Kit deterioration	Check storage of kit and it should be stored at 2-8ºC.
	j) Sample deterioration due to improper storage and / or microbial contamination.	Store the sample at 2-8ºC / -20ºC as recommended in the specimen collection & handling.

in vitro diagnostic Reagent, not for medicinal use

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T4 Quanti Microlisa

Microwell ELISA Immunoassay for the Quantitative Detection of Total Thyroxine (T4) in Human Serum/Plasma

1. INTRODUCTION

Thyroxine (T4) is one of the main thyroid hormones, and has a molecular weight of 777 Da. T4 enable to estimulate synthesis and energy metabolism, to increase basal metabolic rate and oxygen consumption, and to stimulate growth and development. In blood, 99.97% of T4 binds to thyroxine - binding globulin (TBG) and thyroxine - binding prealbumin (TBPA). T4's synthesis and decomposition are controlled by hypothalamus and pituitary. T4 is one of the indicators of diagnostics and efficacy evaluation of hyperthyroidism and hypothyroidism.

2. INTENDED USE

T4 Quanti Microlisa is designed for in-vitro quantitative determination of Total Thyroxine (T4) in human serum or plasma.

3. PRINCIPLE

T4 Quanti Microlisa is an enzyme immuno assay based on competitive ELISA. Microwells are coated with anti-thyroxine antibodies. Sample is added to the microwell followed by addition of enzyme conjugate (T4 labelled with HRPO). Binding of T4 is detected by Enzyme Conjugate. Incubation is followed by a washing step to remove unbound components. The color reaction is started by addition of substrate and stopped after a defined time. The color intensity is inversally proportional to the concentration of T4 in the sample.

4. DESCRIPTION OF SYMBOLS USED

The following are graphical symbols used in or found on J. Mitra diagnostic products and packing. These symbols are the most common ones appearing on medical devices and their packing. They are explained in more detail in European Standard EN ISO 15223-1:2021.

	Manufactured By	IVD	In vitro diagnostic medical device
Σ	No. of tests		Instruction for use
LOT	Lot Number Batch Number	2°C 8°C	Temperature Limitation
	Manufacturing Date		Caution, see instruction for use
	Expiry Date	REF	Catalogue Number
	Keep away from sunlight		Do not use if package is damaged
BIO	Contains biological Material of Human Origin	BIO	Contains biological Material of Animal Origin
IN	Country of Manufacture		Keep Dry

5. KIT PRESENTATION

- 96 Tests

6. KIT & ITS COMPONENTS

T4 Quanti Microlisa Strip Plates	Breakway microwells coated with anti-thyroxine antibodies antibodies packed in a pouch with dessicant.
Enzyme Conjugate Concentrate (11x)	Containing peroxidase conjugated to T4 with preservatives.
Conjugate Diluent	Buffer containing binding protein inhibitors.
Wash Buffer Concentrate (25x)	PBS with surfactant. Dilute 1:25 with distilled water before use.
TMB Diluent	Buffer solution containing H_2O_2 with preservative
TMB Substrate	To be diluted with TMB diluent before use.
Standard-1	0 µg/dl of T4 in Human Serum containing preservatives. *RTU
Standard-2	2 µg/dl of T4 in Human Serum containing preservatives. *RTU
Standard-3	5 µg/dl of T4 in Human Serum containing preservatives. *RTU
Standard-4	10 µg/dl of T4 in Human Serum containing preservatives. *RTU
Standard-5	15 µg/dl of T4 in Human Serum containing preservatives. *RTU
Standard-6	25 µg/dl of T4 in Human Serum containing preservatives. *RTU
Stop Solution	Ready to use, 1N sulfuric acid.
Plate Sealers	Adhesive backed sheets for sealing microwell plate/strips.

7. STORAGE AND STABILITY

Store the kit & its components at 2-8°C. Expiry date on the kit indicates the date beyond which kit should not be used.

8. ADDITIONAL MATERIAL AND INSTRUMENTS REQUIRED

- Micropipettes and microtips.
- Elisa reader
- Distilled or deionized water
- Graduated Cylinders, for reagent preparation
- Disinfectant Solution
- Paper towels or absorbent tissue
- Timer
- Elisa washer
- Incubator 37ºC
- Vortex Mixer
- Disposable gloves
- Disinfectant Solution

9. SPECIMEN COLLECTION & PREPARATION

1. Human serum or plasma samples should be used for the test. While preparing serum samples, remove the serum from the clot as soon as possible to avoid haemolysis. Fresh serum/plasma samples are preferred.
2. Specimens should be free of microbial contamination and may be stored at 2-8ºC for one week, or frozen at -20ºC or lower. Avoid repeated freezing and thawing.
3. Do not use heat inactivated samples as their use may given false results. Haemolyzed and Icteric hyperlipemic samples may give erroneous results.

10. SPECIMEN PROCESSING

(A) FROZEN SAMPLE

T4 Quanti Microlisa test is best used with fresh samples that have not been frozen and thawed. However most frozen samples will perform well if the procedure suggested below is followed.

Allow the frozen sample to thaw in a vertical position in the rack. Do not shake the sample. This allows particles to settle to the bottom. If a centrifuge is available, the sample should be centrifuged. (5000 rpm for 5 min.)

(B) TRANSPORTATION

If the specimen is to be transported, it should be packed in compliance with the current Government regulations regarding transport of aetiologic agents.

11. WARNING & PRECAUTION

CAUTION: THIS KIT CONTAINS MATERIALS OF HUMAN ORIGIN. NO TEST METHOD CAN OFFER COMPLETE ASSURANCE THAT HUMAN BLOOD PRODUCTS WILL NOT TRANSMIT INFECTION. NEGATIVE CONTROL, POSITIVE CONTROL & ALL THE SAMPLES TO BE TESTED SHOULD BE HANDLED AS THOUGH CAPABLE OF TRANSMITTING INFECTION.

1. The use of Disposable Gloves is RECOMMENDED while running the test.
2. In case there is a cut or wound in hand, DO NOT PERFORM THE TEST.
3. Do not smoke, drink or eat in areas where specimens or kit reagents are being handled.
4. Do not pipette by mouth.
5. Tests are for *in vitro* diagnostic use only and should be run by competent person only.
6. All materials used in the assay and samples should be decontaminated in suitable disinfectant solution for 30-60 min. before disposal. They should be disposed off in accordance with established biosafety guidelines for handling & disposal of potentially infective material.
7. Wash hands thoroughly with soap or any suitable detergent, after the use of the kit. In case of needle prick or other skin puncture or wounds, wash the hands with excess of water and soap.
8. Standards contain Sodium Azide as a preservative. If these materials are to be disposed off through a sink or other common plumbing systems, flush with generous amounts of water to prevent accumulation of potentially explosive compounds.
9. Spills should be decontaminated promptly with any suitable disinfectant.
10. ELISA Reader & micropipettes used in testing should be calibrated at regular interval to ensure accurate results.

12. PRECAUTIONS FOR USE

Optimal assay performance requires strict adherence to the assay procedure described in the instruction for use.

1. Do not use kit components beyond the expiration date which is printed on the kit.
2. Bring all the reagents & samples to room temperature (20 - 30oC) before use.
3. Do not combine reagents from different batches, as they are optimised for individual batch to give best results.
4. Avoid microbial contamination of reagents. The use of sterile disposable tips is recommended while removing aliquots from reagent bottles.
5. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid contamination of any sort.
6. Mark the test specimen with patient's name or identification number. Improper identification may lead to wrong result reporting.
7. Use freshly collected, clean serum samples for assay. Try to avoid turbid, lipemic serum samples.
8. Use a separate tip for each sample and then discard it as biohazardous waste.

9. All pipetting steps should be performed with utmost care and accuracy. Cross contamination between reagents and samples will invalidate results.
10. Do not allow microwells to dry once the assay has started.
11. Run all six standards, low & high controls in each assay to evaluate validity of the kit.
12. Incubation time should not vary by more than + 2 min.
13. Prevent evaporation during sample incubation by covering the strips with strip sealer. Remove sealer before washing.
14. Distilled or deionised water must be used for wash buffer preparation.
15. Thorough washing of the wells is critical to the performance of the assay. Overflowing of reagents or washing to adjacent wells must be prevented during washing, which may lead to incorrect results due to carry over effect.
16. Take care while preparing working substrate solution as Bottle of TMB Substrate & TMB Diluent are of same size.
17. Prepare working substrate solution just 10 minutes prior to adding in the wells.
18. Use separate tips for TMB Substrate and TMB diluent.
19. Avoid strong light exposure during the assay.
20. Ensure that the microwell strips are levelled in the strip holder. Before reading, wipe the bottom of the microwell strips carefully with soft, absorbent tissue to remove any moisture.
21. In case of any doubt the run should be repeated.

13. PREPARATION OF REAGENTS

Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned throughly and rinsed with distilled or deionized water. Prewarm the incubator to 37ºC.

- **T4 Quanti Microlisa strips :**
  Bring foil pack to room temperature (20-30ºC) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, taking into account that one each of negative & positive control & three calibrator should be included in the run while opening the fresh kit. However for one or two strips, one each of negative, positive control & two calibrator and for more strips one each of negative and positive control & three calibrator should be included in each subsequent runs.

b. Unused wells should be stored at 2-8ºC with desiccant in an aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC from the date of opening of sealed pouch, when stored with desicant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

- **Preparation of Working Wash Buffer:**

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37ºC until all crystals dissolve.

b) Prepare at least 50 ml (2 ml concentrated buffer with 48 ml water) of buffer for each strip used. Mix well before use.

c) Mix 30 ml of 25x wash buffer concentrate with 720 ml. of distilled or deionized water. **Working wash buffer is stable for 2 months when stored at 2-8ºC.**

- **Preparation of Working Conjugate:**

Dilute Enzyme Conjugate Concentrate 1:11 in conjugate diluent. **Do not store working conjugate.** Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from the table below. Mix solution thoroughly before use.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
Enzyme Conjugate Concentrate (ml.)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2
Conjugate Diluent in (ml.)	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0

- **Preparation of working substrate solution :**

Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
TMB Susbstrate (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
TMB Diluent (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use. Discard unused solution. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.

14. PROCEDURAL NOTES:

1. Material should not be used after the expiry date shown on the labels. Components and test specimen should be at room temperature (20-250C) before testing begins. Return the reagents to 2-80C after use.
2. All reagents must be mixed well before use.
3. To avoid contamination, do not touch the top or bottom of strips or edge of wells.
4. All pipetting steps should be performed with utmost care and accuracy. Cross contamination between reagents and samples will invalidate results.
5. Prevent evaporation during sample incubation by covering the strips with sealer; remove sealer before washing.
6. Routine maintenance of wash system is strongly recommended to prevent carry over from highly reactive specimens to non reactive specimens.
7. Check incubator temperature before starting the test. It should be set at 37ºC.
8. Mark the test specimen with patient's name or identification number. Improper identification may lead to wrong result reporting.

15. TEST PROCEDURE

The instructions of the procedure must be strictly followed.

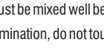
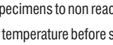
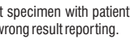
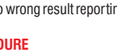
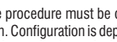
The sequence of the procedure must be carefully followed. Arrange the standards in a horizontal or vertical configuration. Configuration is dependent upon reader software. **It is recomended to perform all six standards and samples to run in duplicate.**

(i) Fit the stripholder with the required number of T4 Quanti Microlisa coated microwell strips.
(ii) Prepare working enzyme conjugate as specified in preparation of reagents
(iii) Add 25 μl of each standards and samples in respective wells. Use a separate tip for each sample and then discard as biohazardous waste.
(iv) Add 100 μl of working enzyme conjugate to each well.
(v) Gently mix the microplate for 20-30 seconds and cover.
(vi) Cover the plate and incubate in an incubator at 37oC + 1oC for 60 minutes.
(vii) Dilute the wash buffer concentrate with distilled water to 1:25 dilution.
(viii) Wash with working wash buffer.

WASHING: Washing can be performed either with WASHER or manually as follows:

a). Empty the wells.
b). Add 300-350μl of working washing solution into each well.
c). Empty the wells.
d). Wash each well 3 times in total.
(ix) After the third wash, tap dry the Microwells a few times on an absorbent tissue.
(x) Add 100 μl working substrate solution in each well.
(xi) Incubate at room temperature (20-30oC) in dark for 15 mins. and do not expose to light.
(xii) Add 50 μl of stop solution to each well.
(xiii) Read the absorbance at 450 & 630 nm within 15 minutes in ELISA reader.

16. SUMMARY OF PROCEDURE

Prepare Working Enzyme Conjugate		No. of Strips 1 2 3 4 5 6 7 8 9 10 11 12 Enzyme Conjugate Concentrate (ml) 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 Conjugate Diluent (ml) 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
Add Standards* and samples		25 μl
Add Enzyme Conjugate		100 μl
Cover the plate & incubate		60 mins. at 37ºC
Wash		3 Cycles
Prepare Working Substrate		No. of Strips 1 2 3 4 5 6 7 8 9 10 11 12 TMB Substrate (ml) 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 TMB Diluent (ml) 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
Add Substrate		100 μl
Cover the plate & incubate		15 mins. at Room Temp.
Add Stop Solution		50 μl
Read Results		450 nm./630 nm.

*(Ready to use)

17. CALCULATION OF RESULTS

1. Calculate the mean absorbance values for each set of standards and samples.
2. Construct a best fit curve by ploting the absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the absorbance value for each sample determine the corresponding concentration from the best fit curve.
4. Automated Method : The results have been calculated automatically using a 4 PL (4 parameter logistics) curve fit which is the preferred method. Other data reduction functions may give slightly different results.
5. The concentration of the sample can be read directly from the best fit curve. Sample with concentrations higher than that of the highest standard have to be futher diluted (1:0.5 or 1:1 with Standard-1) or reported as >25 μg/dl. For the calculation of the concentration, this dilution factor has to be taken into account.
6. For subsequent run, once master curve has been established in an ELISA Reader, calculate the results with stored master curve and absorbance of 3 standards with necessary data analytics.

Important Note: QC data sheet is batch specific and can be downloaded from company web site; www.jmitra.co.in

18. EXPECTED VALUES

Each laboratory should establish its own range of normal value. The values given below are only indicative.

Distribution of normal values ranges from 4.4 to 10.8 μg/dl in Male and from 4.8 to 11.6 μg/dl in Female.

19. PERFORMANCE CHARACTERISTICS

Precision :

Intra-Assay: Within precision have been determined by testing 10 replicates of 3 different samples with T4 concentration (low, medium and high value respectively) on the same lot on same day. The C.V (%) is < 10%.

Inter-Assay: Between precision have been determined by testing 10 replicates of 3 different samples with T4 concentration (low, medium and high value respectively) in 10 different run at different time interval. The C.V (%) is <15%.

Accuracy: The accuracy of T4 Quanti Microlisa was detected with clinical specimen and compared with reference immunoassay test. The co-relation co-efficient > 0.985.

Specificity :

The following cross reactanats were tested in the assay by diluting them in serum matrix at various concentration (0.01 μg/ml to 100 μg/ml) and calculated the cross reactivity (%CR) at 50% binding point.

Cross reactants	%CR
T4 L-Thyroxine	100%
Tri iodo L-thyronine	0.048%
L-Thyronine	0.025%
Tetraiodo thyroacetic acid	0.016%
L-Tyrosine	0.016%
D-Tyrosine	0.015%

Analytical Sensitivity :

The sensitivity is defined as being the lowest detectable concentration different from zero with a probability of 95%. The sensitivity of the assay is 0.4 μg/dl.

Linear Range :

T4 Quanti Microlisa is linear between 0.4 μg/dl to 25 μg/dl.

20. LIMITATIONS OF THE ASSAY

1. Any improper handing of samples or modification of this test might influence the results.
2. Samples which show turbidity, haemolysis, hyperlipemia or contain fibrin may give erroneous results.
3. No hook effect was observed in this test.
4. No substances (drugs) are known to us, which have an influence to the measurement of T3 in a sample.

21. LIMITED EXPRESSED WARRANTY DISCLAIMER

The manufacturer limits the warranty to the test kit, as much as that the test kit will function as an in-vitro diagnostic assay within the limitations and specifications as described in the product instruction for use, when used strictly in accordance with the instructions contained therein. The manufacturer disclaims any warranty expressed or implied including such expressed or implied warranty with respect to merchantability, fitness for use or implied utility for any purpose. The manufacture's liability is limited to either replacement of the product or refund of the purchase price of the product and in no case liable to for claim of any kind for an amount greater than the purchase price of the goods in respect of which damages are likely to be claimed.

The manufacturer shall not be liable to the purchaser or third parties for any injury, damage or economic loss, howsoever caused by the product in the use or in the application there of.

22. REFERENCES

1. Journal of Thyroid Research; Volume 2011, Article ID 905734, 7 pages, Free Thyroxine Level in the High Normal Reference Range Prescribed for Nonpregnant Women May Reduce the Preterm Delivery Rate in Multiparous.
2. Clinical Chemistry 47:8 1353–1363 (2001), Direct and Indirect Free Thyroxine Assay Methods: Theory and Practice.
3. SUPPLEMENT TO JAPI JANUARY 2011 VOL. 59, Laboratory Evaluation of Thyroid Function.
4. International Journal of Endocrinology; Volume 2014, Article ID 618572, 5 pages-8. Clinical Evaluation of Various Thyroid Hormones on Thyroid Function.

23. TROUBLE SHOOTING CHART

PROBLEM	POSSIBLE CAUSE	SOLUTION
1. Standards curve out of validation limit	a) Incorrect temperature timing or pipetting	Check procedure & repeat assay
	b) Improper preparation of reagents, error of dilution, improper mixing of reagents.	Check procedure & repeat assay
	c) Cross contamination of standards/ reagents	Pipette carefully and do not interchange caps. use separate tip for standards / reagents.
	d) Used components from different lots.	Do not use components from different lots as they are adjusted for each batch released.
	e) Expired Reagents	Check the kit expiry date. Use the kit with-in shelf life
	f) Use of non calibrated micropipette and/or ELISA Reader	Calibrate micropipette and ELISA Reader at defined interval.
2. No colour developed at the end of assay	a) Any one reagent has been added in wrong sequence.	Follow the procedure meticulously & repeat assay.
	b) Inactivated conjugate, improper storage	Check storage of enzyme conjugate and it should be free of any contamination and store at 2-8ºC.
	c) Microplate inactivated, due to improper storage	Keep unused strips in aluminium poly pouch with the dessicant pouch inside and proerly closed with clamp & rod.
	d) Inactivated substrate, improper storage or preparation	Use freshly prepared substrate solution and it should be free of any contamination. Recheck procedure and repeat assay.
	e) Omission of any step in test procedure	Follow the procedure meticulously & repeat assay.
	f) Incorrect temperature timing or pipetting	Check procedure & repeat assay
	g) Improper preparation of reagents, error of dilution, improper mixing of reagents.	Check procedure & repeat assay
	h) Kit expired/ deteriorated/ reagent of different kit used. stored at 2-8ºC.	Check storage and expiry of the kit before use and should be
3. Too much colour in all wells of the plate (high background)	a) Contaminated substrate	Check substrate (TMB Diluent) it should be colourless. If blue in colour then discard and use clean disposable container.
	b) Contaminated washing solution (1X). Poor quality of water used for diluting wash buffer conc.	Check the container and quality of water used for dilution. Use of distilled water is preferred.
	c) Over incubation of substrate and delay in addition of stop solution.	Follow the procedure meticulously.
	d) Insufficient washing. i) Washing not consistent due to blockage of probes	Check wash device and clean probes of manifold. fill the well close to the top.
	ii) Filling volume not sufficient.	After washing, blot the microwells on absorbent
	iii) Insufficient no. of wash cycles.	tissue. Follow wash protocol meticulously
	iv) Contaminated wash device	
	e) Use of wash solution from other manufacturer.	Use only T4 Quanti Microlisa wash solution.
	f) Working substrate not protected from light	Incubate the plate in dark after addition of substrate.