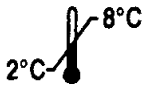
ERBA Thyrokit® T4

REF **E-TT4-1P**

IVD



CE

This package insert must be read carefully before product use.
Package insert instructions must be carefully followed.
Reliability of assay results cannot be guaranteed if there are any deviations from the instructions in this package.

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en

Symbols used on labels

				
In Vitro Diagnostic Medical Device	**Catalogue Number**	**Lot Number**	**See Instructions for Use**	**Use by**
				
Manufacturer	**Date of Manufacture**	**Temperature Limitation**	**Keep away from Sunlight**	**For Single Use Only**

EN *ENGLISH*

1.0 INTENDED USE

ERBA Thyrokit® T4 kit is a direct solid phase enzyme immunoassay for the quantitative determination of total Thyroxine (T4) in human serum or plasma.
The method can be used to measure T4 concentrations over the range of 4 to 300 ng/ml (0.4 to 30 µg/dL).

2.0 SUMMARY AND EXPLANATION OF THE TEST

Measurement of serum thyroxine concentration is generally regarded as an important in-vitro diagnostic test for assessing thyroid function. This importance has provided the impetus for the significant improvement in assay methodology that has occurred in the last three decades. This procedural evolution can be traced from the empirical protein bound iodine (PBI) test (1) to the theoretically sophisticated radioimmunoassay (2).
This microplate enzyme immunoassay methodology provides the technician with optimum sensitivity while requiring few technical manipulations.

3.0 PRINCIPLE OF THE METHOD

Sample of serum or plasma is pipetted into the well coated with Streptavidin. The addition of the Biotin-T4 conjugate (Biotin-T4) and of the immunological reaction starter Anti-T4 -HRP conjugate initiates the competitive assay. During the immunological incubation, the T4 of the sample competes with the Biotin-T4 conjugate for the anti-T4 binding sites of the HRP Anti T4 conjugate which, in turn, is bound by the streptavidin coated wells through the biotin moiety. After washing off the non-reacted species, the amount of the immunological complex remained bound to the wells

Streptavidin **:** Biotin-T4 **:** Anti-T4-HRP

is revealed by the incubation with the chromogen/substrate. The blue colour development is then stopped with sulphuric acid, turning the final solution to a yellow colour which is measured photometrically at 450 nm. The intensity of the colour is proportional to the bound HRP-T4 conjugate, and therefore inversely related to the amount of T4 in the sample.
By reference to a series of T4 standards, assayed in the same way, the concentration of T4 in the unknown sample is quantified.

4.0 REAGENTS - STORAGE AND HANDLING

The ERBA Thyrokit® T4 kit contains sufficient reagents for 96 wells. All the reagents are ready to use. On receipt, store the kit and each reagent at 2...8°C, stable up to the expiration date on the labels.
If not otherwise specified, also, after the first opening all the reagent are also stable up to the expiry date printed on the labels, provided they are stored as indicated and no contaminations occur during the pipetting.

4.1 Streptavidin Microwell Plate

The bag contains a microplate of 12 strips x 8 wells.
Each well is coated with Streptavidin and it may be used individually. Allow microplate to warn to room temperature (18...25°C) before use. After the first opening the unused strips are stable for 2 months at 2...8°C, provided they are stored in the plastic bag with the dessicant.

4.2 Total T4 Anti HRP Enzyme Conjugate

1 vial contains monoclonal anti-T4 HRP conjugate in TRIS buffer pH 7.8, a red dye, preservative and binding protein inhibitors**.** 8 ml.
Attention, see paragraph 6.2.

4.3 Total T4 Biotin Conjugate

1 Vial, contains Anti-T4 coniugate with biotin in TRIS buffer pH 7.8, preservative and a yellow dye**.** 8 ml.
Attention, see paragraph 6.2.

4.4 Total T4 Standards

6 vials containing 1 ml/each of serum standards for thyroxine. A preservative has been added (Sodium azide 0.09%).
Attention, see paragraph 6.2.
Ready to use.
Store at 2-8°C.

Standard	Concentration
Standard A	0 µg/dL (0 ng/ml)
Standard B	2.0 µg/dL (20 ng/ml)
Standard C	4.0 µg/dL (40 ng/ml)
Standard D	8.0 µg/dL (80 ng/ml)
Standard E	15.0 µg/dL (150 ng/ml)
Standard F	30.0 µg/dL (300ng/ml)

4.5 TMB Substrate

1 vial of TMB Substrate containing 0.26 mg/ml of 3,3',5,5' Tetramethylbenzidin (TMB) and 0.01% w/v of Hydrogen peroxide (H_2O_2), in citrate buffer. 16 ml. Mix gently before use.

NOTES:

1. The TMB/H_2O_2 single solution is colourless or slightly yellow-blue. If accidental contamination occurs, the solution starts to develop a blue colour and must therefore be discarded.
2. The TMB/H_2O_2 single solution is not sensitive to light. Direct sunlight can however oxidize the solution to a blue colour. Such a colour disappears after 4 hours storage in the dark after which the solution can again be used.

Attention: Avoid contact of this reagent with skin and mucous membranes. Should this occur, wash thoroughly with cold tap water.

4.6 Stop Solution

1 vial of Stop Solution containing Sulfuric acid (H_2SO_4) 0.3 mol/l. 15 ml. Mix gently before use.
In case of contact with eyes, rinse immediately with plenty of water.

4.7 Wash Solution 10X

The bottle contains 50 ml of borate citrate buffer and 2.5 µg/ml Amphotericin B. Dilute 1:10 with distilled or equivalent grade water.

5.0 MATERIALS AND EQUIPMENT REQUIRED

5.1 Materials Provided

The kit contains reagents for 96 tests (code E-TT4-1P).

Material for 96 tests	Quantity
Streptavidin Microwell Plate	One bag
Total T4 Standard A	1x1 ml
Total T4 Standard B	1x1 ml
Total T4 Standard C	1x1 ml
Total T4 Standard D	1x1 ml
Total T4 Standard E	1x1 ml
Total T4 Standard F	1x1 ml
Total T4 Biotin Conjugate	1x8 ml
Total T4 Anti-HRP Enzyme Conjugate	1x8 ml
TMB Substrate	1x16 ml
Wash Solution (10x)	1x50 ml
Stop Solution	1x15 ml

5.2 Materials and Equipment Not Provided

- Distilled or deionized water
- 50 and 100 µl micropipettes
- 0.1 ml repeating dispenser or positive displacement pipettes for addition of Conjugate, Substrate and Stop Solution.
- Automatic plate washer.
- Microtiter incubator.
- Microtiter plate reader, equipped for the measurement of the absorbance at 450 nm (reference filter at 620 nm).
- Adsorbent pad or paper.
- Control sera (recommended).

6.0 WARNING, PRECAUTIONS AND LIMITATIONS

For *in vitro* diagnostic use.

Only experienced laboratory personnel should use this test and handling should be in agreement with GLP.

Do not interchange reagents from different lots. Do not use kit components beyond their expiration date.

Attention

Stop Solution contains sulphuric acid <5% and is classified as Skin Corr. 1A, H314

Danger

Hazard statements:

H314 Causes severe skin burns and eye damage.

Precautionary statements:

P260 Do not breathe vapours/spray.

P303+P361+P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower.

P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

P501 Dispose of contents/container in accordance with local regulations.

Total T4 Anti HRP Enzyme Conjugate and Total T4 Biotin Conjugate contain Reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC no. 247-500-7] and 2-methyl-2H-isothiazol-3-one [EC no. 220-239-6] (3:1) and are classified as Skin Sens. 1, H317

Warning

Hazard statements:

H317 May cause an allergic skin reaction.

Precautionary statements:

P261Avoid breathing vapours/spray.

P280 Wear protective gloves/protective clothing/eye protection/face protection.

P333+P313 If skin irritation or rash occurs: Get medical advice/attention.

P302+P352 IF ON SKIN: Wash with plenty of water.

P501 Dispose of contents/container in accordance with local regulations.

6.1 Safety Precautions

- Do not pipet by mouth.
- Do not smoke, eat or apply cosmetics in areas in which patients samples or kit reagents are handled.
- Cuts, abrasions, and other skin lesions should be properly protected with an appropriate waterproof dressing.
- Take care to avoid self-inoculation, splashing of mucous membranes or generation of aerosols.
- Laboratory gloves should be worn while handling patient samples or disposing of solid or liquid wastes.
- Avoid microbial contamination of standards during pipetting by using disposable pipet tips.
- Disposal of all waste should be in accordance with local regulations.
- Some reagents contain less than 0.1% sodium azide. Sodium azide can react with copper and lead plumbing to form explosive metal azides. Regulations currently in use regarding dangerous waste elimination must be respected. If discharge in the canalisations, rinse with plenty of water.
- Read carefully the Safety Data Sheet (SDS) before product use.

6.2 Potential Biohazard Warning - Human Serum

Some reagents of ERBA Thyrokit® T4 system contain material of animal origin, even if they are certified as deriving from healthy animals, it is recommended to handle them with the same precaution used for potentially infectious samples.

Some reagents used may have been prepared from pools of human serum. Each unit of blood used to prepare these pools were tested and found non reactive for syphilis, for the presence of Hepatitis B Surface Antigen (HBsAG), HCV and for antibodies to Human Immunodeficiency Virus (HIV 1 and 2) using an FDA approved method. Because no test can offer complete assurance that Hepatitis B virus, HIV or other infectious agents are absent, these reagents should be considered as potentially biohazardous and handled with the same precautions as applied to any serum or plasma samples. Some reagents such as calibration standards and control

may contain materials of human tissue origin. At present there is no standard test method for the presence of HIV in such material. It is therefore recommended that these reagents are also considered as potentially biohazardous.
Such materials should be handled according to good laboratory practices, as described in CDC (Center for Disease Control, Atlanta U.S.) document: Universal precautions for prevention of transmission of human immunodeficiency virus, hepatitis B virus, and other blood bourne pathogens in healthcare setting "MMWR" 37:377-387, 1999.

6.3 Sodium Azide (NaN_3) Warning
Sodium azide is present as a preservative in the standard matrix at a concentration of no more than 0.09% w/w. Sodium azide may react with lead and copper plumbing to form explosive metal azides. Liquid and solid wastes should be disposed of safety, in accordance with local regulations. Azide at concentration higher than 0.1% w/w interfere in this assay, therefore the assay of control sera or samples containing the above compound may give overestimated results.

6.4 Sulphuric Acid (H_2SO_4) Warning
Sulphuric Acid is present in the Stop Solution at a concentration of no more than 0.3 mol/l.
Do not pipette by mouth.

6.5 3-3'-5-5' Tetramethylbenzidine (TMB) Warning
TMB (3-3'-5-5' Tetramethylbenzidine) is present in the Substrate TMB. Avoid contact of this reagent with skin and mucous membranes. Should this occur, wash thoroughly with cold tap water. Do not pipette by mouth.

6.6 Limitations
For diagnostic purposes, the results obtained from this assay should always be used in combination with the clinical examination, patient medical history, and other findings.
Procedural directions must be followed exactly as any modification of the procedure may change the results.
Use of reagents, disposables or spare parts other than those supplied by authorized distributor may produce incorrect results.

6.7 Indications of Substrate Deterioration
- The Substrate single solution is colourless or slightly yellow-blue. If accidental contamination occur, the solution starts to develop a blu colour and must therefore be discarded.
- The Substrate single solution is not sensitive to light. Direct sunlight can however oxidize the solution to a blue colour. Such a colour disappears after 4 hours storage in the dark after which the solution can again be used.
- On aging the substrate may became of slight yellow-orange colour. This does not affect its performances.
- Should only part of the Substrate vial content be used, in order to avoid contamination, transfer the volume needed into a clean plastic container which has previously been washed with ethanol and rinsed with high-quality distilled water.

7.0 SPECIMEN COLLECTION AND STORAGE
The following recommendations for the handling and storage of blood samples have been provided by the National Committee for Clinical Laboratory Standards (NCCLS - Doc. H18-A):
- Take blood samples in observance of the standard precautions for the withdrawal of biological fluids.
- In the case of serum samples, ensure adequate coagulation of the samples prior to centrifugation:
  Note: Centrifugation of the serum samples before coagulation is complete can produce fibrin. To avoid incorrect results due to the presence of fibrin, ensure that the coagulation process is complete before centrifuging the samples. Some samples, in particular those of patients undergoing treatment with anti-coagulants, may require longer coagulation times.
- Test tubes must be kept closed in a vertical position.
- Do not use samples that have remained at room temperature for more than 8 hours.
- Hermetically seal and refrigerate the samples (from 2°C...8°C) if dosage is not performed within 8 hours.
- Freeze the samples at -20°C or lower temperatures if dosage is not performed within 48 hours.
- Freeze samples only once and mix thoroughly after defrosting.

Use of an ultracentrifuge is recommended to clarify lipemic samples.
Hemolyzed samples may indicate unsuitable treatment of the sample prior to delivery to the laboratory; for this reason results must be interpreted with caution.

7.1 Serum
Collect 5 ml of venous blood in a glass tube without additives. Allow to clot at room temperature. Centrifuge, separate the serum fraction, and store.

7.2 Plasma
Collect 5 ml of venous blood in a glass or plastic tube containing heparin or citrate as an anticoagulant. Centrifuge, separate the serum fraction, and store.

7.3 Known Interference
Avoid using the following types of serum or plasma samples as these may give incorrect results:
Grossly hemolyzed samples;
Grossly lipemic samples:
Grossly icteric samples;
EDTA anticoagulated plasma samples with EDTA concentration higher than 5 g/l should not be used.

8.0 ASSAY PROCEDURE

8.1 Preparation for Assay
Bring all reagents and specimens to room temperature (20°C - 30°C) before beginning the assay. Swirl gently before use.

8.2 Pipetting and Incubation steps.
1. Pipette in duplicate 50 µl of each standard and 50 µl of each sample into the appointed wells.
2. Pipette 50 µl of the Biotin-Conjugate (Biotin-T4 conjugate) into all the wells
3. Pipette 50 µl of the Anti-T4-HRP Conjugate into all the wells
4. Shake the plate for 10 seconds on an orbital shaker or manually, by gently hitting the side of the microplate against your index finger; the

movement must be sideways to avoid spilling the well content.

5. Incubate the plate at room temperature for 1 hour.
6. At the end of the incubation period, wash the strips 3 times as described in par. 8.3, section 8.
7. Add 100 µl of TMB Substrate.
8. Incubate at room temperature for 15 min.
9. Stop the reaction by adding 100 µl of stop solution to each well in the same order followed for dispensing the substrate.
10. Measure the absorbance within 30 min. with microtiter reader at the wavelength of 450 nm (reference filter at 620 nm)

8.3 Procedural Notes

1. Room temperature is defined between 18° and 25°C.
2. A standard curve must be run in each assay to assure valid results.
3. Reagents from different Kits and lots should not be mixed.
4. Add the reagents in the same order as the standards and samples.
5. It is recommended to time the addition of the chromogen/substrate solution and stop solution until familiar with the method (i.e. if the chromogen/substrate solution is dispensed into the wells every 3 seconds one from each other, the stop solution should also be dispensed in the same order and at the same frequency).
6. The total dispensing time of standards, controls and speciments for a whole plate should not exceed 15 minutes.
7. **The shaking step after the addition of the conjugate reagents is critical and must be performed correctly.**
8. **Washing procedure:** For the washing procedure, the use of an automatic plate wash equipment is recommended. After the washing, tap the inverted plate on absorbent paper to remove any residual from the wells. Three washings are required.

   If an automatic plate washer is not available, the washing procedure can be carried out manually using a simple wash-bottle filled with the washing solution:
   - Empty the content of the wells by keeping the plate tight in the middle and turning it firmly upside-down.
   - Fill the wells with 300 µl/each of the washing solution contained in the wash-bottle and empty them as aforesaid.
   - Repeat this procedure twice more.
   - Firmly tap the inverted plate on absorbent paper to remove any residual from the wells.

9.0 QUALITY CONTROL

It is recommended that each laboratory routinely use quality control materials and establish its own control ranges. Multi-level controls should be used in each Total T4 run.

The Total T4 values obtained for the quality control material should not repeatedly fall outside the control ranges established in each laboratory.

10.0 CALCULATION OF RESULTS

10.1 Quality Control

Each laboratory should assay controls at levels in the hypothyroid, euthyroid and hyperthyroid range for monitoring assay performance.

These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. In addition, maximum OD should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

10.2 O.D. Conversion

The optical densities (O.D.s) of some standards and samples may be higher than 2.0 , in such a case, they could be out of the measurement range of the microplate reader. It is therefore necessary, for O.D.s higher than 2.0, to perform a reading at 405 nm (=wavelength of peak shoulder) in addition to 450 nm (peak wavelength) and 620 (reference filter for the subtraction of interferences due to the plastic).

For microplate readers unable to read the plate at 3 wavelengths at the same time,

it is advisable to proceed as follows:

- Read the microplate at 450 nm and at 620 nm.
- Read again the plate at 405 nm and 620 nm.
- Find out the wells whose ODs at 450 nm are higher than 2.0
- Select the corresponding ODs read at 405 nm and multiply these values at 405 nm by the conversion factor 3.0 (where OD 450/OD 405 = 3.0), that is: *OD 450 nm = OD 405 nm x 3.0*

Warning: The conversion factor 3.0 is suggested only. For better accuracy, the user is advised to calculate the conversion factor specific for his own reader.

10.3 Data Reduction – Automated Method

Use the 4 parameters logistic – preferred – or the smoothed cubic spline function as calculation algorithm.

NOTE: If computer controlled data reduction is used to calculate the results of the test, it is imperative that the predicted values for the standards fall within 10% of the assigned concentrations.

10.4 Data Reduction – Manual Method

A dose response curve is used to ascertain the concentration of thyroxine in unknown specimens.

Plot the ODs of standards versus the respective T3 concentration (use a linear or a semilogarithmic scale). Determine the T3 concentration of the sample by interpolation of the sample ODs on the calibration curve.

11.0 EXPECTED VALUES

The reported expected results were determined with the dosage ERBA Thyrokit® T4 analysing 136 samples.

It is the responsability of each laboratory to determine the reference ranges applicable to its own patient population and investigation procedures. As a guide only.

The ranges were calculated using data from serum samples from normal individuals. The mean values and reference ranges were calculated and are shown in Table 1:

Table 1		**T4 Value (µg/dL)**			
Category	**n**	**Mean**	**Range 2.5 ÷ 97.5 Percentile**	**Min**	**Max**
Normal subjects	**136**	**8.23**	**5.3 - 12.1**	**4.7**	**12.8**

It is important to keep in mind that establishment of a range of values which can be expected to be found by a given method for a population of "normal"-persons is dependent upon a multiplicity of factors: the specificity of the method, the population tested and the precision of the method in the hands of the analyst. For these reasons each laboratory should depend upon the range of expected values established by the Manufacturer only until an in-house range can be determined by the analysts using the method with a population indigenous to the area in which the laboratory is located.
Total serum thyroxine concentration is dependent upon a multiplicity of factors: thyroid gland function and its regulation, thyroxine binding globulin (TBG) concentration, and the binding of thyroxine to TBG (3, 4). Thus, total thyroxine concentration alone is not sufficient to assess clinical status.
Total serum thyroxine values may be elevated under conditions such as pregnancy or administration of oral contraceptives. A T3 uptake test may be performed to estimate the relative TBG concentration in order to determine if the elevated T4 is caused by TBG variation.
A decrease in total thyroxine values is found with protein-wasting diseases, certain liver diseases and administration of testosterone, diphenylhydantoin or salicylates. A table of interfering drugs and conditions, which affect total thyroxine values, has been compiled by the Journal of the American Association of Clinical Chemists.
NOT INTENDED FOR NEWBORN SCREENING.

12.0 PERFORMANCES

12.1 Analitycal range
4 - 300 ng/ml (0.4 - 30 µg/dL)

12.2 Sensitivity
The sensitivity limit of the assay, defined as the concentration of T4 equivalent to the mean absorbance of zero standard, assayed in 20 replicates, minus two standard deviations, is < 4 ng/ml (0.4 µg/dL)

12.3 Precision
The precision of the ERBA Thyrokit® T4 assay was assessed by the manual method and by the automated instruments Personal Lab and Nexgen.
Three controls sera were assayed in quadruplicate, one run per day, for a total of 20 runs and 80 replicates using manual method and a total of 5 runs and 20 replicates using automated instruments.
The results obtained are shown in the table below. The values are expressed in ng/ml.

Manual method

			Within-Run		Between-Run		Overall	
Sample	**nr**	**Mean**	**SD**	**CV**	**SD**	**CV**	**SD**	**CV**
1	80	48.98	3.49	7.1	2.67	5.5	4.4	9.0
2	80	104.08	6.59	6.3	6.82	6.6	9.49	9.1
3	80	152.36	10.25	6.7	7.65	5.0	12.79	8.4

Personal Lab method

			Within-Run		Between-Run		Overall	
Sample	**nr**	**Mean**	**SD**	**CV**	**SD**	**CV**	**SD**	**CV**
1	20	39.76	3.4	8.5	2.53	6.4	4.24	10.7
2	20	104.81	8.2	7.8	8.55	8.2	11.84	11.3
3	20	136.64	7.68	5.6	8.29	6.1	11.3	8.3

Nexgen method

			Within-Run		Between-Run		Overall	
Sample	**nr**	**Mean**	**SD**	**CV**	**SD**	**CV**	**SD**	**CV**
1	20	54.49	4.2	7.7	4.55	8.3	6.19	11.4
2	20	117.97	8.96	7.6	13.18	5.7	16.54	9.5
3	20	148.33	9.99	6.7	13.18	8.9	16.54	11.1

12.4 Accuracy
Dilution test
Three patient samples containing concentrations of T4 within the assay range were diluted in a human serum with low T4 content and measured using manual method. The results are shown in the table below:

Dilution Factor	**n**	**% Value Un-Diluted**	**SD**
1	3	NA	NA
2	3	99.4	1.4
4	3	107.8	7.3
8	3	102.9	3.59
Mean % Recovery		103.4	4.1

Recovery
Five samples were spiked with pure T4 and assayed: results are reported in the following table:

Sample	**Added (ng/ml)**	**Measured (ng/ml)**	**Recovery %**
1	0	64.4	ND
	50	111.21	97.2
2	0	54.92	ND
	50	108.03	103.0
3	0	64.38	ND
	50	110.06	96.2
4	0	109.85	ND
	50	142.35	89.05
5	0	75.96	ND
	50	120.47	95.64

ND : Non Detectable

12.5 Specificity
The cross-reactivity of the assay was assessed by measuring the apparent response of the assay to various potentially cross-reactants prepared gravimetrically at specified concentrations

Substance	Concentration measured	Cross Reactivity %
L-Tiroxina		100
D-Tiroxina		100
3,3',5 Triiodotironina	625	7.8
3,5-Diiodo-L-Tirosina	10000	Absent up to 10000 ng/ml
3 Iodo L Tyrosina	10000	Absent up to 10000 ng/ml
3,5 Diiodo L Tyronina	10000	Absent up to 10000 ng/ml
3,3',5 Triiodo Thyroacetic Acid	625	31

12.6 Method Comparison

The ERBA Thyrokit® T4 assay was compared to an established method. A linear regression analysis was performed and the following results were obtained:

$y = 0.936 x + 7.111$
$r^2 = 0.852$
x = Reference Method
y = ERBA Thyrokit® T4 Method
n = 57

13.0 AUTOMATION

Application protocols for the proper automation on the microplate analyzers are available upon request at ERBA GmbH directly.

14.0 SUGGESTIONS FOR TROUBLESHOOTING

Adherence to assay procedure and specifications, as well as a correct use of reagents and proper pipetting, may help to avoid the following kinds of errors.

ERROR	POSSIBLE CAUSES / SUGGESTIONS
OD very different (± 50%) from OD reported on QC	- incorrect dispensing volume of reagents (suggestion: check the correspondence between the volume dispensed by the pipette and the one required by the assay; re-calibrate again pipettes) -incorrect temperature or incorrect incubation time (suggestion: more care in the incubator maintenance; note down the beginning of the incubation) -error in washing or in photometer reading (suggestion: check operating or settings of respective instruments) -contamination of Substrate or Conjugate (suggestion: use only disposable and clean plastic containers)
Low reproducible results	-not constant dispensing volume of samples or reagents (suggestion: check the pipettes precision and the correspondence between the volume dispensed by the pipette and the one required by the assay; re-calibrate again pipettes) -error in washing or in reading (suggestion: check operating or settings of respective instruments) -contamination of Substrate (suggestion: use only disposable and clean plastic containers) -pollution or degradation of reagents (suggestion: use appropriate tips, disposable and clean plastic containers for reagents and high quality distilled or equivalent water)
no colorimetric reaction after addition of substrate	-some reagent not pipetted - strong contamination of Conjugate or Substrate -errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)
too low reaction (too low ODs)	-incubation time too short, incubation temperature too low -incorrect conjugate dilution
too high reaction (too high ODs)	-incorrect conjugate dilution -incubation time too long, incubation temperature too high -water quality for wash buffer insufficient (low grade of deionization) -insufficient washing (conjugates not properly removed)
unexplainable outliers	-contamination of pipettes, tips or containers -inconstant and insufficient washing (conjugates not properly removed)
too high within-run CV%	-reagents and/or strips not pre-warmed to Room Temp. prior to use - plate washer is not washing correctly (suggestion: clean washer head)
too high between-run CV%	-incubation conditions not constant (time, temperature) -controls and samples not dispensed at the same time (with the same intervals) (check pipetting order) -person-related variation

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