# Anti-TSH Receptor (TRAb) ELISA (IgG)
# Test instruction

| ORDER NO. | ANTIBODIES AGAINST | Ig CLASS | SUBSTRATE | FORMAT |
|---|---|---|---|---|
| EA 1015-9601 G | TSH Receptor (thyrotropin receptor) | IgG | Ag-coated microplate wells | 96 x 01 (96) |

**Indication:** Diagnosis or exclusion of the treatment of Graves' disease and therapy monitoring.

**Principle of the test:** The ELISA test kit provides a quantitative in vitro assay for human autoantibodies against thyrotropin (TSH) receptor (TRAb). The test kit contains microplate strips, each with 8 break-off reagent wells coated with TSH receptor. In the first reaction step, patient sera are incubated in the wells. If samples are positive, specific antibodies bind to the TSH receptors. Bound antibodies are able to inhibit the binding of biotin-labelled TSH, which is added in a second incubation step. To detect the bound TSH-Biotin, a third incubation is carried out using enzyme-labelled avidin (enzyme conjugate), catalysing a colour reaction. The intensity of the colour formed is inversely proportional to the concentration of antibodies against TSH receptor.

**Contents of the test kit:**

| Component | Colour | Format | Symbol |
|---|---|---|---|
| **1. Microplate wells** coated with antigens: 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use | --- | 12 x 8 | STRIPS |
| **2. Calibrator 1** 40 IU/l (IgG, human), ready for use | colourless | 1 x 1.0 ml | CAL 1 |
| **3. Calibrator 2** 8 IU/l (IgG, human), ready for use | colourless | 1 x 1.0 ml | CAL 2 |
| **4. Calibrator 3** 2 IU/l (IgG, human), ready for use | colourless | 1 x 1.0 ml | CAL 3 |
| **5. Calibrator 4** 1 IU/l (IgG, human), ready for use | colourless | 1 x 1.0 ml | CAL 4 |
| **6. Negative control** 0 IU/l (IgG, human), ready for use | colourless | 1 x 1.0 ml | NEG CONTROL |
| **7. Positive control** (IgG, human), ready for use | colourless | 1 x 1.0 ml | POS CONTROL |
| **8. Sample buffer**, ready for use | yellow | 1 x 10 ml | SAMPLE BUFFER |
| **9. TSH** biotin-labelled thyrotropin, lyophilized | colourless | 3 x 4.5 ml | TSH |
| **10. TSH buffer**, ready for use | red | 1 x 15 ml | TSH BUFFER |
| **11. Enzyme conjugate** peroxidase-labelled avidin, 20x concentrated | colourless | 1 x 0.75 ml | CONJUGATE 20x |
| **12. Conjugate buffer**, ready for use | colourless | 1 x 15 ml | CONJ BUFFER |
| **13. Wash buffer**, 10x concentrated | colourless | 1 x 100 ml | WASH BUFFER 10x |
| **14. Chromogen/substrate solution** TMB/$H_2O_2$, ready for use | colourless | 1 x 15 ml | SUBSTRATE |
| **15. Stop solution** 0.5 M sulphuric acid, ready for use | colourless | 1 x 10 ml | STOP SOLUTION |
| **16. Plastic foil** | --- | 1 piece | |
| **17. Quality control certificate** | --- | 1 protocol | |
| **18. Test instruction** | --- | 1 booklet | |

LOT Lot — CE — Storage temperature

IVD In vitro determination — Unopened usable until

**Storage and stability:** The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

**Waste disposal:** Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents are to be disposed of according to official regulations.

## Preparation and stability of the reagents

**Note:** All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the protective wrapping of the microplate. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C until the indicated expiry date.

- **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.

- **TSH:** Lyophilized. Reconstitute the contents of one vial with 4.5 ml TSH buffer. If more than one vial of TSH is going to be used, pool the contents of each vial after reconstitution and mix gently before use.
  The reconstituted TSH is stable for a maximum of 4 weeks at +2°C to +8°C. If the reconstituted TSH is not to be used completely it can be stored until the indicated expiry date at -20°C.

- **Enzyme conjugate:** The enzyme conjugate is a 20x concentrate. The quantity required should be removed from the vial using a clean pipette and diluted 1:20 with conjugate buffer (1 part reagent plus 19 parts conjugate buffer). Mix gently before use.
  The diluted enzyme conjugate is stable until the indicated expiry date at +2°C to +8°C.

- **Sample buffer:** Ready for use.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable until the indicated expiry date when stored at +2°C to +8°C and handled properly.

- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.

- **Stop solution:** Ready for use.

**Warning:** Calibrators and controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

## Preparation and stability of the patient samples

**Sample material:** Human serum.

**Stability: Patient samples** to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Do not use grossly haemolysed or lipaemic serum samples. **Do not use plasma samples.**

## Incubation

For **quantitative analysis** incubate the calibrators 1-4, negative control, positive control and patient samples.

**(Partly) manual test performance**

**Sample incubation:** (1st step)
Add **75 µl** of sample buffer into each of the microplate wells used. Transfer **75 µl** of the calibrators, negative and positive controls or patient samples into the individual microplate wells according to the pipetting protocol. Cover the frame and incubate for **2 hours** at room temperature (+18°C to +25°C) on a **microplate shaker set at 500-700 rpm**.

**Washing:**
Manual: Remove the plastic foil and empty the wells and add **once** 350 µl of working strength wash buffer for each wash.
Automatic: Wash reagent wells **once** with 450 µl working strength wash buffer (program setting: e.g. TECAN Columbus Washer “Overflow Modus”).

Leave the wash buffer in each well for 30 to 60 seconds, then empty the wells. After manual washing, thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

**TSH incubation:** (2nd step)
Pipette **100 µl** of TSH (biotin-labelled thyrotropin) into each of the microplate wells. Incubate for **25 minutes** at room temperature (+18°C to +25°C).

**Washing:**
Empty the wells. Wash as described above.

**Conjugate incubation:** (3rd step)
Pipette **100 µl** of enzyme conjugate (peroxidase-labelled avidin) into each of the microplate wells. Incubate for **20 minutes** at room temperature (+18°C to +25°C).

**Washing:**
Manual: Empty the wells and subsequently wash **twice** using 350 µl of working strength wash buffer for each wash followed **one wash** with 350 µl of **deionised or distilled water**.
Automatic: Wash reagent wells 3 times with 450 µl working strength wash buffer (program setting: e.g. TECAN Columbus Washer “Overflow Modus”).

Note: Residual liquid (>10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short reaction times) can lead to false high extinction values.

**Substrate incubation:** (4th step)
Pipette **100 µl** of chromogen/substrate solution into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°C) (protect from direct sunlight).

**Stopping the reaction:** Pipette **50 µl** of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

**Measurement:** **Photometric measurement** of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

**Test performance using fully automated analysis devices**

Sample dilution and test performance are carried out fully automatically using the analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the EUROIMMUN Analyzer I and this EUROIMMUN ELISA. Validation documents are available on inquiry.
Automated test performance using other fully automated, open system analysis devices is possible, however, the combination should be validated by the user.

## Pipetting protocol

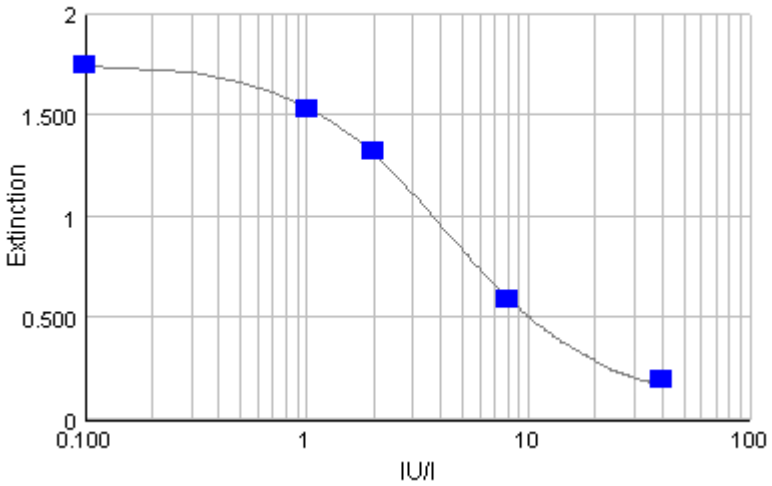
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | **C 1** | **P 3** | **P 11** | **P 19** | | | | | | | | |
| B | **C 2** | **P 4** | **P 12** | **P 20** | | | | | | | | |
| C | **C 3** | **P 5** | P 13 | **P 21** | | | | | | | | |
| D | **C 4** | **P 6** | **P 14** | **P 22** | | | | | | | | |
| E | **neg.** | **P 7** | **P 15** | **P 23** | | | | | | | | |
| F | **pos.** | **P 8** | **P 16** | **P 24** | | | | | | | | |
| G | **P 1** | **P 9** | **P 17** | **P 25** | | | | | | | | |
| H | **P 2** | **P 10** | **P 18** | **P 26** | | | | | | | | |

The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 26 patient samples (P 1 to P 26).

The calibrators (C 1 to C 4), the negative (neg.) and positive (pos.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

## Calculation of results

**Quantitative:** The calibration curve from which the concentration of TSH receptor antibodies in the serum samples can be taken is obtained by plotting the extinction values measured for the **4 calibrators and the negative control as zero calibrator** (linear, y-axis) against the corresponding concentrations (logarithmic, x-axis). The results for unknowns can be calculated using one of the following curve-fitting techniques: four-parameter logistic or spline fits. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of TSH receptor antibody concentrations in patient samples.




If the extinction of a serum sample lies below the value of calibrator 1 (40 IU/l), the result should be given as “>40 IU/l“. It is recommended that the sample be re-tested at a dilution of 1:10 in TRAb negative serum. The result in IU/l read from the calibration curve for this sample must then be multiplied by a factor of 10.

The upper limit of the normal range (**cut-off value**) recommended by EUROIMMUN is **2 international units per litre (IU/l)**. EUROIMMUN recommends interpreting results as follows:

| | |
|---|---|
| **<1.8 IU/l:** | **negative** |
| **≥1.8 to <2.0 IU/l:** | **borderline** |
| **≥2.0 IU/l:** | **positive** |

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

For diagnosis, the clinical symptoms of the patient should always be taken into account alongside the serological results.

## Test characteristics

**Calibration:** The calibration is performed in international units (IU) using the 1st International Standard for thyroid stimulating antibody (WHO, 1995, standard 90/672, National Institute for Biological Standards and Control, Hertfordshire, England). The NIBSC 90/672 standard contains 0.1 IU per ampoule by definition.

For every group of tests performed, the international units determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

**Antigen:** The microplate wells were coated with porcine TSH receptor. Mouse monoclonal antibodies specific for TSH receptor were used to immobilise the receptor onto microplate wells.
Human autoantibodies against thyrotropin (TSH) receptor show similar reactivity to porcine and to human TSH receptor.

**Linearity:** The linearity of the test was investigated by assaying serial dilutions of patient samples with high antibody concentrations. The ELISA is linear in the concentration range of 0 - 20 IU/l.

**Detection limit:** The lower detection limit is defined as the mean extinction minus two times the standard deviation of an analyte-free sample (competitive test configuration) and is the lowest clearly detectable antibody titre. The detection limit of the TSH receptor antibody ELISA was found to be 0.2 IU/l.

**Functional sensitivity:** The functional sensitivity, which was determined via the precision profile of the ELISA, is around the concentration of calibrator 4 (1 IU/l).

**Analytical specificity:** The ELISA specifically detects human autoantibodies against thyrotropin (TSH) receptor with the ability to inhibit the binding of TSH to the TSH receptor (TBII, TSH-binding inhibitory immunoglobulins). Analysis of 44 sera from patients with different autoimmune diseases (origin: Europe) indicated no interference in the ELISA from autoantibodies to thyroglobin, thyroid peroxidase, glutamic acid decarboxylase, 21-hydroxylase, acetylcholine receptors, dsDNA or rheumatoid factors.

**Interference:** No effect is observed with human LH up to 10 U/ml, hCG up to 160 U/ml, human FSH up to 70 U/ml, human TSH up to 3 U/l, haemoglobin up to 5 mg/ml, bilirubin up to 0.2 mg/ml and intralipid up to 30 mg/ml.

**Reproducibility:** The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 4 sera with values at different points on the calibration curve. The intra-assay CVs are based on 12 determinations and the inter-assay CVs on 6 determinations.

| ***Intra-assay variation, n = 12*** | | |
|---|---|---|
| **Serum** | **Mean value (IU/l)** | **CV (%)** |
| 1 | 1.0 | 15.5 |
| 2 | 3.0 | 3.9 |
| 3 | 8.0 | 2.4 |
| 4 | 22.0 | 5.5 |

| ***Inter-assay variation, n = 6*** | | |
|---|---|---|
| **Serum** | **Mean value (IU/l)** | **CV (%)** |
| 1 | 1.0 | 18.0 |
| 2 | 2.8 | 13.0 |
| 3 | 8.5 | 6.9 |
| 4 | 15.8 | 6.5 |

**Method comparison:** The TRAb content in 60 sera (origin: Europe) was assessed both by the 2nd generation ELISA (y) and by a 2nd generation $^{125}$I-RRA (x) (BRAHMS Diagnostica). The results of linear regression analysis showed the following correlation characteristics: y = 1.26x+0.1, r = 0.95. Of the 60 sera studied, 40 sera were positive and 14 sera were negative in both assays. Five sera were found positive by the ELISA only, and one serum by the comparison RRA only. Both assays are comparable.

**Clinical sensitivity and specificity:** In a study using a total of 415 clinically characterized sera (origin: Europe, USA), 96 of the 108 sera from patients with Graves´ disease of varying duration and at various stages of therapy reacted positively in the ELISA. All 168 sera from patients with other diseases and 139 sera from healthy blood donors were negative.
The results showed a sensitivity of 89%, and a specificity of 100% for the ELISA.

**Reference ranges:** In a study of 60 individual healthy blood donor sera (origin: Europe), 59 sera (98%) gave concentrations of less than 1IU/l (corresponds to an inhibition of TSH binding of less than 10%). However, each laboratory should establish its own reference ranges by use of representative sera.

## Clinical significance

The functions of the thyroid are controlled by the hypothalamus in the brain stem via the pituitary gland. The releasing and inhibition factors formed in the hypothalamus stimulate or slow down the emission of TSH (thyroidea stimulating hormone = thyreotropin) that is produced in the pituitary gland inducing the thyroid gland to release the thyroid hormones T3 (triiodothyronine) and T4 (tetraiodothyronine = thyroxine).

The free thyroid hormones T3 and T4 belong to the vitally important hormones that regulate the metabolism of almost all organs. On a cellular level they are responsible for the oxygen consumption, warmth production and also for the mental growth of the total organism [1].

An increase of T3 and T4 levels are in general an indication of a hyperthyroid functional disorder (hyperthyrosis), whereas low levels of T3 and T4 hormones in serum are allocated to a hypothyroid functional disorder (hypothyrosis) [2]. Independent from the cause of the varying thyroid hormone levels the clinical symptoms of hyper- and hypothyrosis are largely the same.

The symptoms of a hyperthyrosis are nervousness, irritablility, restlessness, trembling hands, insomnia, perspiration, warm damp hands, ravenous appetite, thirst, and weight loss despite a good appetite and in women menstruation cycle disorders (unregular or increased bleeding, or absence of the menstruation) [1].

The symptoms of a hypothyrosis are low body temperatures, increased sensitivity to coldness, edemas (particulary on eye lids, face and extremities), feeling of pressure on or in the throat, feeling of strangulation (also only sporadic), frequent clearing of the throat and coughing, hoarse or throaty voice (vocal cord edemas), depressive moods, listlessness, concentration and memory disorders, sleepiness, weak muscles, muscle hardening, dry, chapped skin and accompanying ichiness, dry mucosa, brittle hair and fingernails, high weight increase, decreased libido, menstrual cycle change in women, joint pain [1].

Aside from a disorder of the thyroid hormone regulation, thyreoiditis (thyroid inflammation) can be the cause of the symptoms of either hyper- or hypothyrosis [3]. These include several diseases: A differentiation is made between an acute (bacterial infection), a subacute (non infectious), and a chronic thyreoditis (autoimmune disease) [4]. Autoimmune thyreopathies are chronic inflammatory thyroid diseases that are caused by disregulation of specific immune defences (B-cells and T-cells) [5]. They occur most often after a virus infection and sometimes also after a subacute thyreoditis. Genetic factors play a role in their development. During an autoimmune process antibodies against one or several of the three autoantigens, thyroid perioxidase (TPO), thyreoglobulin (TG) and TSH receptor (TR), of the thyroid are formed [4, 6, 7, 8, 9].

TSH receptor autoantibodies (TRAk) are heterogeneous regarding their biological effect. There are antibodies that have a stimulating or blocking effect on the TR, antibodies that stimulate thyroid growth and others that inhibit the binding of TSH and TR [6, 7, 10]. The biological effect of TRAk for one individual patient can change during the course of the disease, e.g. from a blocking TR to a stimulating TR or the reverse, which is more rare [7, 10].

The determination of TRAk is mainly performed if there is the suspicion of **Grave's disease**, an autoimmune disease that shows not only the symptoms of a hyperthyreose, but additional symptoms such as struma, exophthalmus and tachycardia (Merseburg triad). Severe cases are characterised by weight loss, heart insufficiency and coma [2]. Approximately 2% of the female and 0.2% of the male population are affected by a manifest Grave's disease. Graves' disease often appears in women during hormonal changes (puberty, pregnancy, menopause). 60% of all cases of hyperthyroidism can be ascribed to Grave's disease.

TRAk determinations are performed for the confirmation of Grave's disease with a prevalence of 90-100%. Thus TRAk are considered to be diagnostic markers and are utilized for differential diagnostics compared to a disseminated autonomy of the thyroid gland [17]. Monitoring TRAk concentrations during the course of Graves' disease allows a prognostic statement and provides an important decision-making aid for management of therapy. High TRAk titers in patients with Grave's disease following a long thyreostatic therapy show an increased risk for reoccurrence of the disease [11]. Moreover increased TRAk concentrations in the third trimester of pregnant women with Grave's disease indicate a hyperthreosis in the fetus. Where normal values are found, the diagnosis can be supported by the determination of antibodies against TPO (thyroid peroxidase) with a prevalence of 60-70% [2, 8].

Additionally antibodies against TG in 20-50% of the cases are found [9]. Since there are associations with other autoimmune diseases, e.g. myasthenia gravis, pernicious anemia, chronic-atrophic gastritis and autoimmune polyendocrinopathies, it is likely that further autoantibodies are found (e.g. ANA in approx. 30% of cases, AMA, ASMA, PCA). TRAk determinations are indications for ophthalmology as many patients first visit the optometrist.

Hashimoto's thyroiditis is one of the most frequently found autoimmune diseases in humans and is the most frequent cause of primary thyroid hypofunction. **Hashimoto's thyroiditis** (autoimmune thyropathy type 1A and 2A with struma) is a chronic thyroiditis with progressive destruction of the thyroid tissue by T-lymphocytes [13]. Ord's thyroiditis (autoimmune thyropathy type 1B and 2B) is a special form of Hashimoto-Thyreoiditis and is characterised by an atrophy of the thyroid gland [14]. These two conditions (the hypertrophic and the atrophic form) lead to a thyroid hypofunction, with possible phases of hyperfunction (so-called hyperthyrodism, in extreme cases hashitoxicosis) at the onset of the disease due to the destruction of thyroid tissue.

There is a genetic predisposition for Hashimoto's thyroiditis. Women are affected significantly more often than men (approx. ratio 8:1 to 10:1). The disease can be triggered by stress, severe virus infections (e.g. infectious mononucleosis, shingles), dysfunction of the adrenal cortex or, as in patients with Graves' disease, high levels of iodine (iodine excess). So far, Hashimoto's thyroiditis cannot be cured; however, the thyroid hypofunction must be treated [15]. From a serological point of view, antibodies against TPO can be detected with a prevalence of 60-70%. Antibodies against thyreoglobulin are in 90-100% of the cases initially high [8, 16]. In Hashimoto's disease and myxoedema, blocking TRAb may cross the placenta in pregnant women and lead to transient neonatal hypothyroidism [13, 17].

Approximately 5% of women have postpartum thyroiditis, which is a transient hypothyrote autoimmune thyroiditis with a very high risk of a simultaneously present insulin-dependent diabetes mellitus. Due to the therapeutic consequences, all women who have just given birth should be tested for antibodies against TPO.

The determination of antibodies against TG is particularly important in the diagnosis of differentiated thyroid carcinoma, since the presence of these antibodies can interfere with the measurement of TG concentrations in serum.

Autoimmune test methods which have proven successful are the indirect immunofluorescence test (IIFT) and the EUROASSAY for the detection of autoantibodies against TPO and TG, the Enzyme Linked Immunosorbent Assay (ELISA) and the radioimmunoassay for the detection of autoantibodies against TPO, TG and TR. Today, enhanced ELISA tests which are highly sensitive and specific for the determination of autoantibodies against TR are available [6, 7, 10, 16, 18, 19, 20, 21].

## Literaturliste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# Pipetting Scheme for ELISA

| | Calibrators | Controls | Samples |
|---|---|---|---|
| Sample buffer | 75 µl | | |
| Calibrator 1 - 4 | 75 µl | | |
| Negative control | | 75 µl | |
| Positive control | | 75 µl | |
| Sample serum | | | 75 µl |

Incubate for 2 hours at room temperature on a shaker
set at 500-700 rpm

wash 1x

| | |
|---|---|
| TSH | 100 µl |

Incubate for 25 minutes at room temperature

wash 1x

| | |
|---|---|
| Enzyme conjugate | 100 µl |

Incubate for 20 minutes at room temperature

wash 3x

| | |
|---|---|
| Substrate | 100 µl |

Incubate for 30 minutes at room temperature in the dark

| | |
|---|---|
| Stop solution | 50 µl |

Shake briefly and read absorbance at 450 nm