Anti-IA2 ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG CLASS	SUBSTRATE	FORMAT
EA 1023-9601 G	Tyrosine phosphatase (IA2)	IgG	Ag-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a quantitative in vitro assay for human autoantibodies against tyrosine phosphatase (IA2) in serum or plasma for the diagnosis of Type 1 diabetes mellitus (insulin-dependent diabetes mellitus, IDDM).

Application: Autoantibodies against the insulinoma-associated antigen 2 (IA2) are detected in 50 to 80% of patients with a newly apparent diabetes mellitus type 1. In persons without diabetes, they have a high predicative value for the individual risk of type 1 diabetes. The prevalence of anti-IA2 antibodies is strongly associated with the patients' age – with increasing age, it decreases. In healthy children, the autoantibodies have a high diagnostic sensitivity with respect to a quick progression towards a manifest diabetes mellitus type 1.

Principle of the test: The test kit contains microplate strips, each with 8 break-off reagent wells coated with IA2. In the first reaction step, patient samples are incubated in the wells. If samples are positive, specific antibodies bind to the IA2. Bound antibodies are able to act divalently and form a bridge between IA2 on reagent wells and biotin-labelled IA2, which is added in a second incubation step. To detect the bound biotin, a third incubation is carried out using enzyme-labelled avidin (enzyme conjugate) catalysing a colour reaction. The intensity of the colour formed is proportional to the concentration of antibodies against IA2.

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 to 6 4000 / 400 / 250 / 75 / 20 / 10 IU/ml (IgG, human), ready for use	colourless	6 x 0.7 ml	CAL 1 - CAL 6
3. Negative control 0 IU/ml (IgG, human), ready for use	colourless	1 x 0.7 ml	NEG CONTROL
4. Positive control (IgG, human), ready for use	colourless	1 x 0.7 ml	POS CONTROL
5. Sample buffer, ready for use	red	1 x 4.0 ml	SAMPLE BUFFER
6. IA2, biotin-labelled IA2, lyophilised	colourless	3 x 4.5 ml	IA2
7. IA2 buffer, ready for use	blue	2 x 15 ml	IA2 BUFFER
8. Enzyme conjugate peroxidase-labelled avidin, 20x concentrated	colourless	1 x 0.7 ml	CONJUGATE 20x
9. Conjugate buffer, ready for use	colourless	1 x 15 ml	CONJ BUFFER
10. Wash buffer, 10x concentrate	colourless	1 x 125 ml	WASH BUFFER 10x
11. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 15 ml	SUBSTRATE
12. Stop solution 0.25 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
13. Plastic foil	---	3 pieces	FOIL
14. Quality control certificate	---	1 protocol	
15. Test instruction	---	1 booklet	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

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 for 4 months.

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

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

- **IA2:** Lyophilised. Reconstitute the contents of one vial with 4.5 ml IA2 buffer. If more than one vial of IA2 is going to be used, pool the contents of each vial after reconstitution and mix gently before use. Avoid air bubbles. The reconstituted IA2 is stable for a maximum of 1 day at +2°C to +8°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 thoroughly before use.
  The diluted enzyme conjugate is stable for a maximum of 20 weeks at +2°C to +8°C.

- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to +37°C and mix well before dilution. The quantity required should be removed from the bottle using a clean pipette and diluted 1:10 with deionised or distilled water (1 part reagent plus 9 parts water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks 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.

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 must be disposed of in accordance with local disposal regulations.

Warning: The calibrators and controls of human origin have 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 agent sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum or EDTA, heparin or citrate plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Samples can be stored at -20°C or at lower temperatures. Repeated freezing and thawing must be avoided.

Incubation

For **quantitative analysis** incubate **calibrators 1 to 6** along with the negative control, positive control and **undiluted** patient samples.

Sample incubation: (1st step)
Transfer **50 µl** of the calibrators, negative and positive controls or patient samples into the individual microplate wells according to the pipetting protocol. Add **25 µl** of sample buffer into each of the microplate wells used. Cover the frame and **shake for 5 seconds** (**microplate shaker set at 500 rpm**). Incubate for **16 to 20 hours at +4°C to +8°C**.

Washing:
Manual: Remove the plastic foil, empty the wells and subsequently wash 3 times using 350 µl of working strength wash buffer for each wash.
Automatic: Remove the protective foil and wash the reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Mode").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, 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.

IA2 incubation: (2nd step)
Pipette **100 µl** of cold IA2 (biotin-labelled IA2) into each of the microplate wells. Cover the frame and incubate for **1 hour at +4°C to +8°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. Cover the frame.
Incubate for **20 minutes** at room temperature (+18°C to +25°C) on a **microplate shaker set at 500 rpm**.

Washing:
Empty the wells. Wash as described above.

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 residence 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 **20 minutes** at room temperature (+18°C to +25°C), protect from direct sunlight.

Stopping:
Pipette **100 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, then 405 nm** and a reference wavelength between 620 nm and 650 nm **within 5 minutes of adding the stop solution**. Prior to measuring, carefully shake the microplate to ensure a homogeneous distribution of the solution.

Pipetting protocol

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

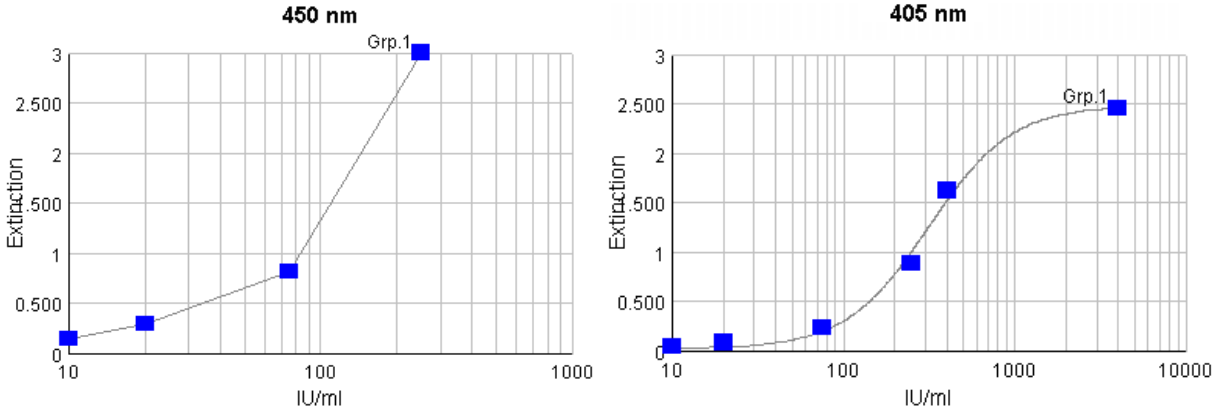
The pipetting protocol for microtiter strips 1 to 5 is an example for the **quantitative analysis** of 25 patient samples (P 1 to P 25).

The calibrators (C 1 to C 6), 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 curves from which the concentration of IA2 antibodies in the patient samples can be taken are obtained by plotting the extinction values measured for the **calibrators 3 to 6 (450 nm) or calibrators 1 to 6 (405 nm)** (linear, y-axis) against the corresponding concentrations (logarithmic, x-axis). For the computer-guided calculation of the calibration curve at 405 nm, the LogitLog method should be used, or alternatively point-to-point plotting or 4-parameter logistic, for 450 nm exclusively point-to-point. **Lower concentrations (<75 IU/ml, calibrator 4)** are to be evaluated using the **450 nm calibration curve**, and **higher concentrations (>75 IU/ml)** using the **405 nm calibration curve**. The following plots are examples of typical calibration curves. Please do not use these curves for the determination of IA2 antibody concentrations in patient samples.

Calibrator/control	*Extinction at 450 nm*	*Extinction at 405 nm*
Calibrator 1	>4.00	2.45
Calibrator 2	>4.00	1.62
Calibrator 3	3.00	0.89
Calibrator 4	0.82	0.24
Calibrator 5	0.29	0.09
Calibrator 6	0.15	0.05
Negative control	0.09	0.03



If the extinction for a patient sample lies below the value of calibrator 6 (10 IU/ml), the result should be reported as "<10 IU/ml". If the extinction of a patient sample lies above the value of calibrator 1 (4000 IU/ml), the result should be reported as ">4000 IU/ml". It is recommended that the sample be retested in a new test run at a dilution of e.g. 1:20 in anti-IA2 negative serum. The result in IU/ml read from the calibration curve for this sample must then be multiplied by a factor of 20.

The upper limit of the normal range (**cut-off value**) recommended by EUROIMMUN is **10 international units per millilitre (IU/ml)**. EUROIMMUN recommends interpreting results as follows:

<10 IU/ml: negative
≥10 IU/ml: positive

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: The calibration is performed in international units (IU) using the 1st WHO reference reagent for islet cell antibodies (WHO, 1999, reagent 97/550, National Institute for Biological Standards and Control, Hertfordshire, England). The NIBSC 97/550 reference reagent contains 100 IU of anti-IA2 per ampoule by definition.
125 IU/ml correspond to 1 U/ml (relative units per millilitre) used in EUROIMMUN`s Anti-IA2 RIA kit.

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: Human recombinant tyrosine phosphatase (IA2) was used for coating the microplate and preparation of the biotinylated IA2.

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of the Anti-IA2 ELISA is 1.04 IU/ml.

Analytical specificity: The ELISA specifically detects human autoantibodies against tyrosine phosphatase (IA2). No cross-reactions with other autoantibodies were observed. 5% (n = 20) of the samples which were positive for autoantibodies against thyroglobulin and thyroid peroxidase, as well as 5% (n = 20) of the samples which were positive for rheumatoid factors also showed anti-IA2 antibodies. These samples were also positive in the corresponding Anti-GAD ELISA.

Interference: Haemolytic and icteric samples showed no influence on the result up to a concentration of 5 mg/ml for haemoglobin and 20 mg/dl for bilirubin in this ELISA.
Addition of 1000 and 3000 mg/dl intralipid caused interference in lipaemic samples.

Reproducibility: The reproducibility of the test was investigated by determining the intra-assay coefficients of variation (CV) using 5 samples and inter-assay (CV) using 2 samples with values at different points on the calibration curve. The intra-assay CVs are based on 25 determinations and inter-assay CVs are based on 20 determinations each.

Intra-assay variation, n = 25		
Sample	**Mean value (IU/ml)**	**CV (%)**
1	6.1	2.1
2	12.0	3.1
3	41.0	1.3
4	80.0	1.6
5	296.0	2.1

Inter-assay variation, n = 20		
Sample	**Mean value (IU/ml)**	**CV (%)**
1	41.0	4.5
2	140.0	4.2

Method comparison: The IA2 antibody concentrations in 122 sera (origin: Germany) were assessed using the ELISA and a RIA (EUROIMMUN AG) as reference method. Of the 122 analysed sera, 52 sera were positive and 67 sera were negative in both assays. The remaining three sera were found positive by the RIA only. The sensitivity of the ELISA was 95% with a specificity of 100% referring to the RIA.

Clinical sensitivity and specificity: The sera n = 140 (50 sera from patients with newly diagnosed type 1 diabetes, and 90 sera from blood donors; origin: USA) from the Islet-Diabetes Autoantibody Standardisation Program (IASP, 2015) were assayed and results showed a sensitivity of 68%, and a specificity of 100% for the ELISA.

Reference ranges: For determination of the normal range, 153 sera from healthy blood donors were investigated. 100% of the determined anti-IA2 antibody concentrations of these sera were below the cut-off value of 10 IU/ml.
However, each laboratory should establish its own reference ranges by use of representative samples.

Clinical significance

Type I diabetes mellitus (T1DM, T1D) is an organ-specific autoimmune disease that is characterised by selective destruction of insulin-producing beta cells. Both occurrence and progression of the autoimmune reactions are influenced by three interacting components, namely genetic predisposition, disordered immune regulation and exogenic factors.

In most cases, T1D is a **polygenic disease** of genetically predisposed individuals. More than 20 different gene loci with T1D association have so far been described. The HLA genotype is the most influential factor in the development of diabetes. The high frequency of T1D in family members is due to the presence of certain HLA alleles in up to 50% of cases. Among these alleles, the genotypes HLA DR3-DQ2/DR4-DQ8 and HLA DR4-DQ8/DR4-DQ8 are associated with the highest diabetes risk. Approximately half of the children who develop T1D before the age of five exhibit one of these high-risk genotypes. Around 20% of children of parents who suffer from T1D and who carry a high-risk genotype develop autoantibodies against islet cells before they turn two.

The main targets (autoantigens) of T1D-specific autoimmune reactions are islet cells (endocrine part of the pancreas tissue, cytoplasmic islet antigens), the 65 kDa isoform of the enzyme glutamic acid decarboxylase (GAD65), the tyrosine phosphatase-homologue IA-2 proteins (IA2α and IA-2β), the zinc transporter 8 (ZnT8), insulin and the insulin precursor proinsulin. It may take months or years for the immune system to react to the proteins of the body's own insulin-producing beta cells. The fasting blood sugar level increases only when around 80% of the beta cells have been destroyed. For this reason, extended risk screening is indispensible for early identification of beta cell destruction and for establishing a prognosis.

“Noxious substances” (virus infections, e.g. with coxsackievirus type B, rubivirus, echovirus, cytomegalovirus, herpes virus; harmful chemical substances, e.g. bafilomycine, some foods etc.) and psychological criteria (stress, emotional distress etc.) are being discussed as exogenic factors. Children with familial T1D predisposition, for instance, develop autoantibodies against islet cells in 100% of cases if HLA DR3-DQ2/DR4-DQ8 is present and they were given cow's milk or gluten-containing products before the fourth month of life.

In 1965, diabetes was classified into different types by the WHO, which were modified in 1998. The German Diabetes Association (Deutsche Diabetes Gesellschaft, DDG) took them over in 2000 and established them, in a modified version, as a guideline in 2009:

- Type 1 diabetes mellitus:
  Destruction of the beta cells in the islets of Langerhans leads to complete insulin deficiency.
- Type 2 diabetes mellitus:
  This encompasses genetically caused insulin resistance with relative insulin deficiency as well as absolute insulin deficiency in later stages of the disease. This type is often associated with other problems of metabolic syndrome.
- Eight other specific diabetes types (e.g. gestational diabetes)

The number of diabetes mellitus patients worldwide is relatively well known. In 2010, 6.4% of the world population (285 million people) suffered from diabetes, 10% of which had T1D. The incidence (number of new cases) is increasing worldwide. It is estimated to be 3% per year. In 2030, 7.7% of the world population (around 640 million people) are expected to have diabetes.

In Germany, around 350,000 people suffer from T1D, of these around 15,000 are children and adolescents of up to 14 years. In this age group, 2,100 to 2,300 new cases are recorded each year. It is likely that approximately 500,000 assumed type 2 diabetes patients in fact suffer from T1D/LADA (latent autoimmune diabetes with onset in adults). Without investigation of antibodies, these patients are often wrongly diagnosed and, consequently, receive unsuitable therapy.

Clinical manifestation of diabetes mellitus late syndrome starts with polyuria, polydipsia, nocturia, weight loss and fatigue. The severity of the metabolic imbalances is characterised by apparent microangiopathies (diabetic arteriosclerosis). Further complications such as polyendocrinopathy, neuropathy, retinopathy, diabetic glomerulosclerosis, gangrene and diabetic coma are responsible for the frequently reduced life expectancy.

The immunological tool for the **serological diagnosis** of T1D is the detection of specific autoantibodies. In newly apparent diabetes, the autoantibody result is an important criterion in the differentiation of type 1 diabetes and non-autoimmune diabetes forms such as type 2 diabetes. Autoantibodies against beta cell proteins, so-called islet cell autoantibodies, are the best diagnostic markers for the identification of incipient or existing autoimmune processes and for monitoring the disease course.

With the use of highly specific and highly sensitive serological tests such as ELISA (with recombinant protein antigens), RIA (radioimmunoassay with radioactively labelled autoantigens) and IIFT (BIOCHIP Mosaic® with transfected cells) the main autoantibodies for the diagnosis of T1D can be analysed. These are autoantibodies against:

- **GAD65** (glutamic acid decarboxylase)
  The frequency in newly onset T1D is 70 to 90%.
  65 kDa glutamic acid decarboxylase is synthesised mainly in the islet cells of the pancreas.
  The prevalence is not dependent on the patient's age.
- **IA2** (tyrosine phosphatase IA-2)
  The frequency is 50 to 70% in children and adolescents and 30 to 50% in adults.
  The 105 kD transmembrane islet cell-specific antigen IA2 is the main antigen in T1D, together with GAD. The level of progression correlates with the titer level.
  The prevalence is not dependent on the patient's age.
- **ICA** (islet cells of the pancreas, cytoplasmic islet cell antigens)
  The frequency in newly onset T1D is 80%.
  In the course of the disease the titer decreases, so that ICA can be found in only 10% of patients after approx. 10 years.
  The prevalence decreases with increasing disease duration.

Note:
A high concentration of autoantibodies against GAD can be considered as an indication of stiff-person syndrome (formerly stiff-man syndrome), a disease with progressive muscle rigidity and secondary stiffening of almost all extremities, and of progressive encephalomyelitis with rigidity (PER).

The first T1D autoantibody screening in children, adolescents and young adults (up to 25 years old) should comprise the investigation of different autoantibodies using ELISA, RIA or IIFT. For evaluation of the antibody reactivity, these parameters should be monitored at regular intervals (1 to 3 years, depending on the age and risk of diabetes), particularly in children and adolescents, since their autoantibody responses change more frequently and rapidly.

Since in 90% of T1D cases one or several diabetes mellitus-associated autoantibodies can be detected in the serum before clinical manifestation, individuals with an increased risk of disease can be identified early. The earlier and stronger the autoantibody response (number of positive islet cell autoantibodies, autoantibody affinity, autoantibody titer level), the higher the risk of diabetes. High autoantibody titers are associated with T1D progression. If the immune response spreads to further target antigens, this can be interpreted as an indication of a qualitatively altered, more aggressive autoimmune destruction of beta cells. The younger the patient at the time of autoantibody detection, the higher the risk of de-veloping islet cell autoimmunity. Of those children who exhibit multiple autoantibodies in the first year of life, 50% develop T1D within two years.

By early detection and monitoring of the “prediabetic stage” using serological diagnostics, timely **intervention** can be achieved:

1. Primary prophylaxis for the prevention of islet cell autoimmunity in genetically predisposed children
2. Secondary prophylaxis for the prevention of diabetes manifestation in children and adults with islet cell autoimmunity
3. Tertiary prophylaxis for the prevention of late complications in T1D patients

New **therapy methods** embark on the therapeutic strategy of modulating the autoimmune process to achieve long-term tolerance of islet cell antigens and the protection of beta cells from destruction. Current therapies comprise temporarily limited general immune suppression, including depletion of activated autoreactive T-cells (e.g. anti-CD3 antibodies), and antigen-specific immunomodulation by vaccination with an autoantigen (e.g. oral/intranasal insulin).

Note:
As shown in a recent multicentre analysis of almost 30,000 cases (children, adolescents and young adults) from Germany and Austria, T1D patients often also suffer from other autoimmune diseases. In addition to T1D, autoimmune thyroiditis occurred in approx. 20% of patients, coeliac disease in approx. 11%, autoimmune adrenalitis in approx. 10% and autoimmune gastritis in approx. 6.5%. 1 to 2% of these T1D patients even had three or four of these autoimmune diseases.

Patent information

The following patents apply:
RSR patents: European patent EP 1 448 993 B1, Chinese patent ZL02822274.1, Indian patent 226484, Japanese patent 5711449 and US patent 8,129,132 B2.

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Pipetting Scheme for ELISA

	Calibrators	Controls	Samples
Calibrators 1 to 6	50 µl		
Negative control		50 µl	
Positive control		50 µl	
Patient sample			50 µl
Sample buffer	25 µl		

Shake for 5 sec at 500 rpm and incubate for 16 to 20 hours at +4°C to +8°C

wash 3x

Cold IA2	100 µl

Incubate for 1 hour at +4°C to +8°C

wash 3x

Enzyme conjugate	100 µl

Incubate for 20 minutes at room temperature on a shaker set at 500 rpm

wash 3x

Substrate	100 µl

Incubate for 20 minutes at room temperature in the dark

Stop solution	100 µl

Shake briefly and read absorbance at 450 nm and then 405 nm