Anti-Influenza A Virus ELISA (IgG)
Test instruction

ORDER NO.	ANTIBODIES AGAINST	IG-CLASS	SUBSTRATE	FORMAT
EI 2691-9601 G	Influenza A virus	IgG	Ag-coated microplate wells	96 x 01 (96)

Principles of the test: The ELISA test kit provides a semiquantitative or quantitative in vitro assay for human antibodies of the IgG class against Influenza A virus in serum or plasma. The test kit contains microtiter strips each with 8 break-off reagent wells coated with Influenza A virus antigens. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction.

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 1 200 RU/ml (IgG, human), ready for use	dark red	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (IgG, human), ready for use	red	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (IgG, human), ready for use	light red	1 x 2.0 ml	CAL 3
5. Positive control (IgG, human), ready for use	blue	1 x 2.0 ml	POS CONTROL
6. Negative control (IgG, human), ready for use	green	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	green	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	light blue	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrate	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Test instruction	---	1 booklet	
13. Quality control certificate	---	1 protocol	
LOT Lot description	CE	Storage temperature	
IVD In vitro diagnostics		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 shoud be handled as infectious waste. All reagents must be disposed of in accordance with local disposal 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 resealable protective wrapping of the microplate at the recesses above the grip seam. 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.

- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.

- **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 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.

Warning: Calibrators and controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. 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 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. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted **1:101** in sample buffer. For example: dilute 10 µl serum in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: Calibrators and controls are prediluted and ready for use, do not dilute them.

Incubation

For **semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation: (1st)
Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for **30 minutes** at room temperature (+18°C to +25°C).

Washing:
Manual: Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.
Automatic: Wash reagent wells 3 times 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 per washing cycle, then empty the wells. After washing (manual and automated tests), 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.

Note: Residual liquid (> 10 µl) remaining 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.
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.

Conjugate incubation: (2nd)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to 25°C).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd)
Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to 25°C) (protect from direct sunlight).

Stopping the reaction:
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 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, Analyzer I-2P or the DSX from Dynex 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 2**	**P 6**	**P 14**	**P 22**			**C 1**	**P 4**	**P 12**	**P 20**		
B	**pos.**	**P 7**	**P 15**	**P 23**			**C 2**	**P 5**	**P 13**	**P 21**		
C	**neg.**	**P 8**	**P 16**	**P 24**			**C 3**	**P 6**	**P 14**	**P 22**		
D	**P 1**	**P 9**	**P 17**				**pos.**	**P 7**	**P 15**	**P 23**		
E	**P 2**	**P 10**	**P 18**				**neg.**	**P 8**	**P 16**	**P 24**		
F	**P 3**	**P 11**	**P 19**				**P 1**	**P 9**	**P 17**			
G	**P 4**	**P 12**	**P 20**				**P 2**	**P 10**	**P 18**			
H	**P 5**	**P 13**	**P 21**				**P 3**	**P 11**	**P 19**			

The pipetting protocol for microtiter strips 1-4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7-10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) 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.
The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimizes reagent wastage.
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

Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2. Calculate the ratio accordingn to the following formula:

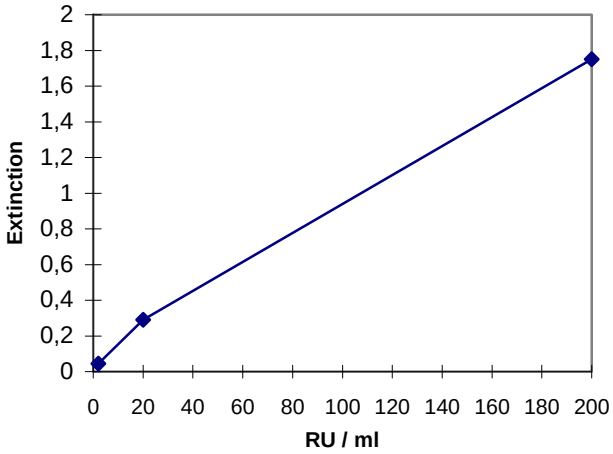
$$\frac{\textbf{Extinction of the control or patient sample}}{\textbf{Extinction of calibrator 2}} = \textbf{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <0.8:	**negative**
Ratio ≥0.8 to <1.1:	**borderline**
Ratio ≥1.1:	**positive**

In cases of borderline test results, an additional patient sample should be taken 7 days later and re-tested in parallel with the first patient sample. The results of both samples allow proper evaluation of titer changes.

Quantitative: The standard curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.



If the extinction of a serum sample lies above the value of calibrator 1 (200 RU/ml). The result shold be given as ">200 RU/ml". It is recommended that the sample be re-tested at a dilution of 1:400. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range of non-infected persons (**cut-off value**) recommended by EUROIMMUN is **20 relative units (RU)/ml**. EUROIMMUN recommends interpreting results as follows:

<16 RU/ml:	**negative**
≥16 to <22 RU/ml:	**borderline**
≥22 RU/ml:	**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 along with the serological results.

Test characteristics

Calibration: As no international reference serum exists for antibodies against influenza A, the calibration is performed in relative units (RU).

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratios 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.

The activity of the enzyme used is temperature-dependent and the extinction values may vary if a thermostat is not used. The higher the room temperature during substrate incubation, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largelycompensated in the calculation of the result.

Antigen: The antigen source is provided by inactivated allantois fluid of chicken embryos infected with the "Texas" (H3N2) strain of Influenza A virus, „Singapore" (H1N1) strain of influenza A virus and "California" (H1N1, Porcine Influenza).

Linearity: The linearity of the Anti-Influenza A Virus ELISA (IgG) was determined by assaying 4 serial dilutions of different patient samples. The coefficient of determination R^2 for all sera was > 0.95. The Anti-Influenza A Virus ELISA (IgG) is linear at least in the tested concentration range (18 RU/ml to 171 RU/ml).

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-Influenza A Virus ELISA (IgG) is 1 RU/ml.

Cross reactivity: This ELISA showed cross reactivity with antibodies against influenza B virus.

Antibodies against	n	Anti-Influenza A Virus ELISA (IgG)
Adenovirus	12	0%
Bordetella pertussis	12	0%
Chlamydia pneumoniae	12	0%
CMV	12	0%
EBV-CA	12	0%
Helicobacter pylori	12	0%
HSV-1	12	0%
Measles virus	12	0%
Mumps virus	12	0%
Mycoplasma pneumoniae	12	0%
Parainfluenza Viruses Pool	12	0%
Parvovirus B19	12	0%
RSV	12	0%
Rubella virus	12	0%
Toxoplasma gondii	12	0%
VZV	12	0%
Yersinia enterocolitica	12	0%

Interference: Haemolytic, lipaemic and icteric samples showed no influence at the result up to a concentration of 10 mg/ml for hemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation using 3 sera. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed on 6 different days.

Intra-assay variation, n = 20		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	64	3.4
2	142	2.7
3	142	3.3

Inter-assay variation, n = 4 x 6		
Serum	**Mean value (RU/ml)**	**CV (%)**
1	69	6.6
2	149	4.8
3	151	3.7

Specificity and sensitivity: Different serum cohorts were investigated using the EUROIMMUN Anti-Influenza A Virus ELISA (IgG). The clinical sensitivity and specificity with respect to patients with acute influenza infection and healthy children were 100%, respectively. The prevalence of influenza A specific IgG antibodies in vaccinated persons was 100%.

Sera from	Number (n)	Anti-Influenza A Virus ELISA (IgG) (borderline and positive results)
Vaccinated individuals	21	21 (100%)
Patients with acute influenza infection	10	10 (100%)
Healthy children	15	0 (0%)

Reference range: The levels of the anti-influenza A virus antibodies (IgG) were analyzed with this EUROIMMUN ELISA in a panel of 500 healthy blood donors. With a cut-off of 20 RU/ml, 90.6% of the blood donors were anti-influenza A virus positive (IgG).

Clinical Significance

Influenza, also commonly known as the flu, is an infectious disease occurring in humans which is caused by viral agents of the influenza species A or B. There are three different species of the influenza virus in total, all belonging to the orthomyxovirus family [1, 2].

- Influenza A viruses:
  The linear, single-stranded RNA of their genome has 8 segments. Compared to the other influenza species, the antigenic properties of influenza A viruses vary greatly due to a very high mutation rate and genetic rearrangement. These subtypes are generally host-specific [2, 3, 4]. Main hosts are humans and various mammals such as pigs, horses, minks, seals and whales as well as several bird species. Water birds are the primary reservoir of influenza A viruses [1,2].

  Influenza A virus H1N1 (A/H1N1) is an influenza A virus subtype and the causative agent of the Spanish influenza which claimed 20-50 million lives in 1918. “H1” and "N1” stand for the specific surface antigens of the virus (haemagglutinin H1 and neuraminidase N1) [2, 4, 5]. In spring 2009 another at the time still unknown subtype of the H1N1 virus emerged. This porcine influenza virus subtype A/California/7/2009 caused the pandemic H1N1 2009/10 (“swine flu” / “new flu”) [3, 4, 6, 7].

- Influenza B viruses:

  Their genome is also characterised by a linear, single-stranded RNA with 8 segments. Influenza B viruses are human pathogenic [3].

- Influenza C viruses:
  This virus type infects humans and pigs. But it is hardly ever associated with disease in humans [3, 8].

The ability of the influenza viruses to survive depends on the ambient temperature. At summer temperatures of around 20°C, dry viruses can survive for two to eight hours. At 22°C they can last for at least 4 days in excrement, tissues of dead animals and water. In ice their lifespan is almost unlimited [2, 4, 6, 7].

The virus is transmitted by aerosols from infected individuals and enters the body e.g. via mouth, nose or eyes [3, 4].

In the vernacular the term influenza is commonly used to refer to flu-like infections, which are usually other virus infections with a relatively mild course. Influenza viruses A and B, however, are the causative agents of “true” influenza, which is one of the most frequent infectious diseases in all age groups (e.g. 22,000 to 32,000 hospitalisations per year in Germany). The infection is characterised by high fever of up to 40°C, headache, joint pain and infection of the respiratory tract after an incubation period ranging from a few hours to five days [4]. Small children additionally experience gastrointestinal symptoms, such as abdominal pain and vomiting [6, 7, 9]. Among newborns, there is a higher risk of infection in premature babies and twins [6, 9]. The most frequent complications are influenza pneumonia, myocarditis and encephalitis [3, 9, 10, 11, 12, 13]. Clinical symptoms of acute necrotising

encephalopathy are high fever, cramp attacks and unconsciousness in combination with thalamic necrosis, which can be determined by magnetic resonance tomography [4, 6, 7, 9, 10, 11, 12, 13]. Influenza is epidemic during the cold season, e.g. in Germany between December and April [4].

If an organism is infected with two different influenza A virus types simultaneously (double infection), a rearrangement (genetic reassortment) of the two 8-genome segments of the involved influenza viruses can take place. During the process single or multiple RNA molecules can be exchanged between the influenza viruses in a doubly infected cell [1, 2, 4, 14]. The most important surface antigens of influenza A viruses are haemagglutinin H1, H2, H3 and H5, more rarely H7 and H9, and neuraminidase N1 and N2, more rarely N7 [2, 1, 14]. The creation of new subtypes by the process of genetic reassortment has already led to pandemics [1, 2, 3, 4, 5, 6, 7].

- 1918-19 (Spanish influenza) caused by the subtype H1N1,
- 1957 (Asian influenza) caused by H2N2,
- 1968 (Hongkong influenza) caused by H3N2,
- 1977 (Russian influenza) caused by reemergence of H1N1,
- 2005 (so-called avian influenza) caused by influenza A/H5N1
- 2009 (so-called swine flu/new influenza) caused by the porcine influenza virus subtype A/California/7/2009 H1N1

The most recent influenza is the “swine influenza” (“new influenza”), which broke out in 2009. Its severity was at first compared to hat of the Spanish influenza from 1918 [2, 5]. However, statistics showed that the symptoms of the swine flu rather resembled those of conventional influenza A virus infections, including known complications [4, 6, 7, 9, 10]. In 2010 further spread could be successfully prevented by comprehensive worldwide immunisation measures. Nevertheless, new life-threatening influenza variants created by mutations (i.e. reassortment of genetically related subvariants) of the influenza A virus subtype A/H1H1 cannot be excluded.

Because of the pathogen's high degree of genetic variability, infections with influenza viruses do not lead to life-long immunity [3, 16]. Persons belonging to high-risk groups (persons with a weakened immune system, older persons) can be given preventive immunisation with the appropriate virus strain at the beginning of each epidemic period [15, 16]. Protection is only against those subtypes which are used for immunisation [3, 6, 15, 16].

Beside the evaluation of clinical symptoms there are “influenza quick tests” for rapid orentiation diagnostics. But these tests can only differentiate between influenza A and B viruses and not between subtypes. Diagnostic methods such as cultivation of the influenza viruses collected from secretions by bronchoalveolar lavage (BAL) or detection of virus RNA by means of PCR are time-consuming and subject to errors. Consequently, the serological test methods IIFT and ELISA for the determination of antibodies against influenza A and B (including the influenza virus subtype A/California/7/2009 H1N1 antigens) play an important role.

However, it must be taken into account that a reliable clinical diagnosis can only be made on the basis of two serum samples taken and investigated at an interval of two to three weeks. Evaluation of a second serum sample to determine the titer course is important for restrospective confirmation of the diagnosis. One sample alone cannot provide sufficient information with respect to a primary, chronic or past infection or an individual’s specific immune status [16, 17, 18].

Since influenza viruses can cause severe accompanying or secondary diseases, e.g. pneumonia, myocarditis and encephalitis, a specific diagnostic procedure is indicated. Clinical diagnosis is supported by early virus detection using polymerase chain reaction (PCR) on a throat swab, haemaglutination inhibition test (HIT), virus culture for fine typing and sequence analysis and for confirmation by serological antibody detection using ELISA and IIFT [32]. Early diagnosis allows immediate appropriate therapy, including symptomatic/supportive and adjuvant therapy along with the administration of virostatics (adamantane derivates, neuraminidase inhibitors). The efficacy of antiviral drugs, however, is limited due to viral resistance [3, 6, 7, 10, 11, 12, 13, 16, 17, 18, 19, 31, 33]. The best prevention against epidemic/pandemic influenza virus infections is active immunisation. The seasonal composition of the influenza vaccine depends on the dominant circulating viruses [3, 6, 7, 33]. The WHO recommendation for the northern hemisphere for the year 2010, for example, was to use trivalent vaccines consisting of the influenza virus strains A/California/7/2009 (2009 H1N1), A/Perth/16/2009 (H3N2) and B/Brisbane/60/2008 (B/Victoria line) [3]. The serological determination of the immune status is very important with respect to potential pandemics [3, 6, 16, 23, 24, 25].

In Germany, laboratories and doctors are legally obligated to notify authorities of influenza virus infections.

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