



Zika virus infections

EUROIMMUN test systems for the diagnosis of Zika virus infections

Zika virus

Zika virus (ZIKV) belongs to the family of flaviviruses which encompasses more than 50 different viruses. Dengue, yellow fever and West Nile viruses are the most prominent flaviviruses. Zika virus was first isolated in 1947, but only became more known due to a series of epidemics in recent years.

Since 2013, an increasing number of Zika virus outbreaks in different regions has been registered, for example in South-east Asia, Polynesia and the Pacific region, some islands in the Caribbean, and in over 30 countries and regions in North, Central and South America.



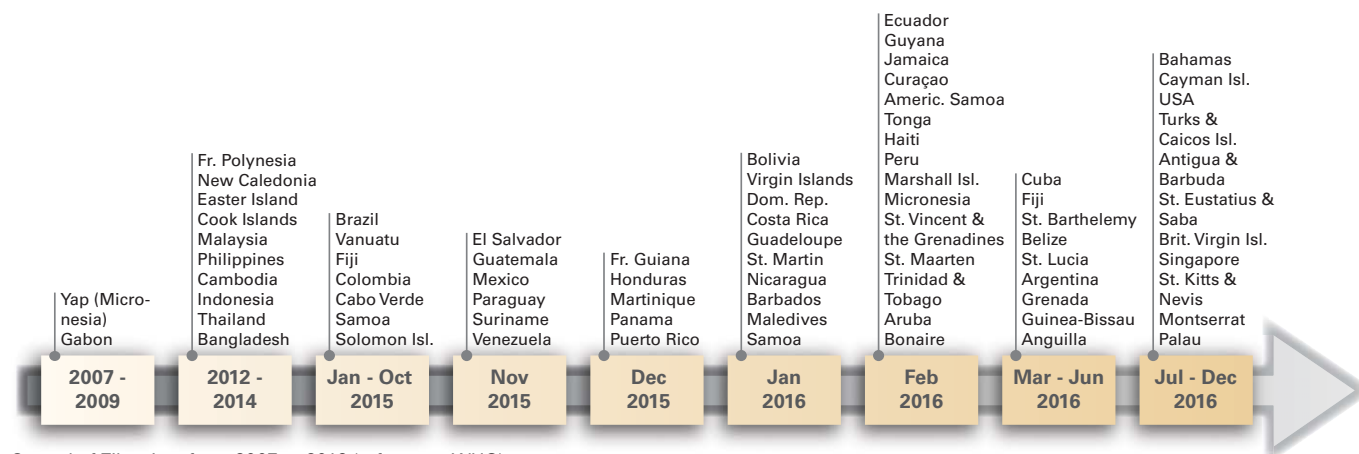
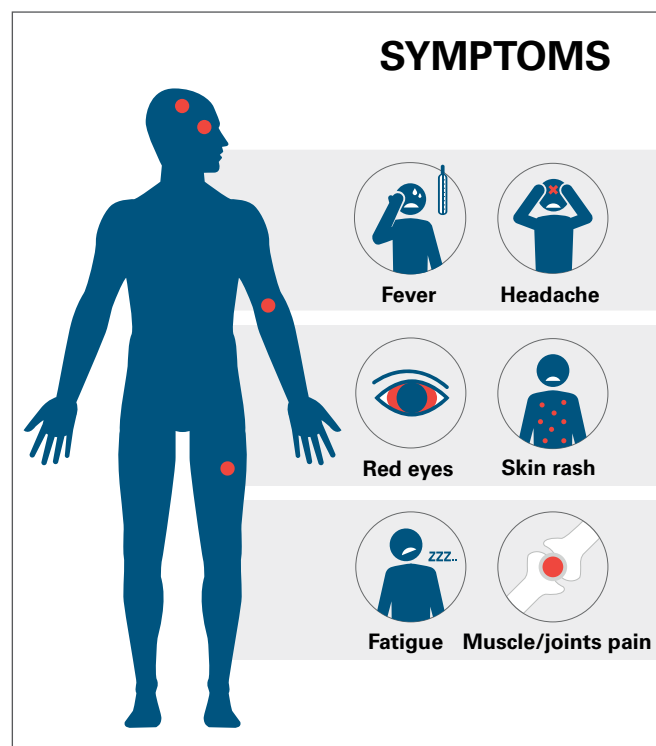
The virus is transmitted to humans by the bite of mosquitoes of the *Aedes* genus. Infected female mosquitoes transmit the virus whilst feeding on blood. Another mode of transmission is perinatal transmission, i.e. transmission from an infected mother to the unborn child. Transmission through sexual intercourse and blood transfusions has also been reported from different countries.

Symptoms

Zika virus infections proceed asymptomatically in most cases (approx. 80%). Only in 20% of cases do patients show symptoms, which usually encompass rash, fever, headache,

pains in the joints, and conjunctivitis. These symptoms usually occur 3 – 12 days after the mosquito bite and persist for 2 – 7 days. The disease course is usually mild and self-limiting. The symptoms are very similar to those of dengue or chikungunya virus infections.

In Brazil and several other countries, a significant increase in neurological disorders such as Guillain-Barré syndrome was registered during the Zika epidemic 2015/2016. Moreover, there was an exceptionally high number of babies with microcephaly. The link between Zika virus infection and the occurrence of neurological disorders and foetal malformations is considered as virtually proven. The topic is currently intensively investigated.



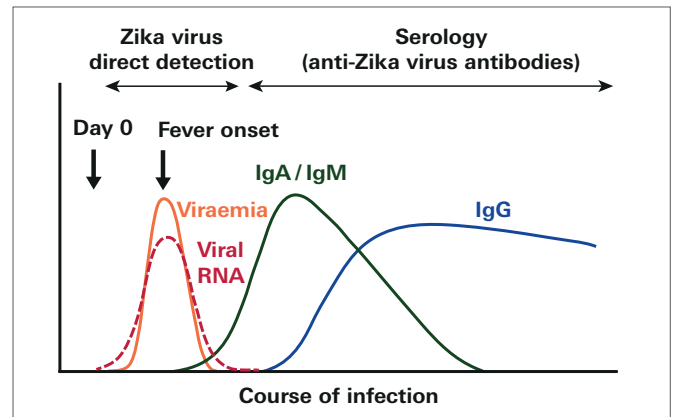
Spread of Zika virus from 2007 to 2016 (reference: WHO)

Diagnosis

Diagnosis of Zika virus infections can be made by direct detection of viral RNA or by indirect detection, i.e. determination of antibodies. Direct detection of the virus in the blood of the patient is possible for maximum one week after onset of symptoms. Specific antibodies are detectable for a longer period and can indicate both past and acute infections.

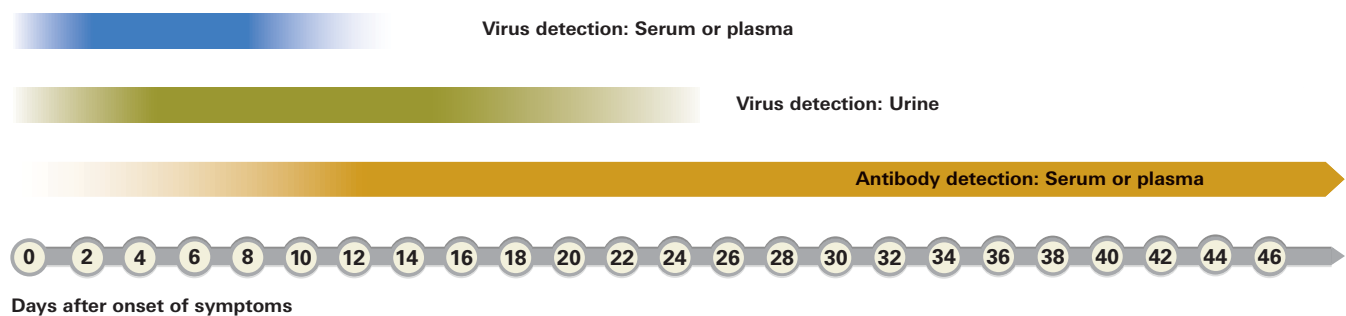
From approximately day 5 onwards, IgA and/or IgM antibodies can be detected in the serum of infected patients. Approximately 2 – 3 days after the appearance of IgA and/or IgM antibodies, also IgG antibodies are detectable. However, all antibody classes may also appear at the same time. Antibodies can be detected by means of serological tests such as ELISA or indirect immunofluorescence (IIFT). In the interpretation of serological results, the structural similarity of flaviviruses must be taken into account, as this can cause cross reactions of the specific antibodies. In patients without past contact with

other flaviviruses the cross reactivity is only minimal. In cases of prior infection or vaccination to another flavivirus, however, a significant cross reactivity must be expected. This can be virtually excluded by the use of highly specific NS1 antigen.



Time window for reliable diagnosis of Zika virus infections

The most suitable method for the detection of Zika virus infection depends on the disease stage. In the early phase, viral RNA can be determined. The Zika virus can be detected in blood by RT-PCR for up to one week after onset of symptoms. In infected pregnant women, the virus can be detected also several weeks after this in individual cases (according to Driggers et al., (2016)). Virus detection in urine by PCR is possible for a longer period of time than in serum or plasma. Here, positive results may occur up to 2 – 4 weeks after onset of symptoms. If the infection is older than 7 days, serological testing is recommended. Antibodies can be detected in the blood of the patient from day 5.

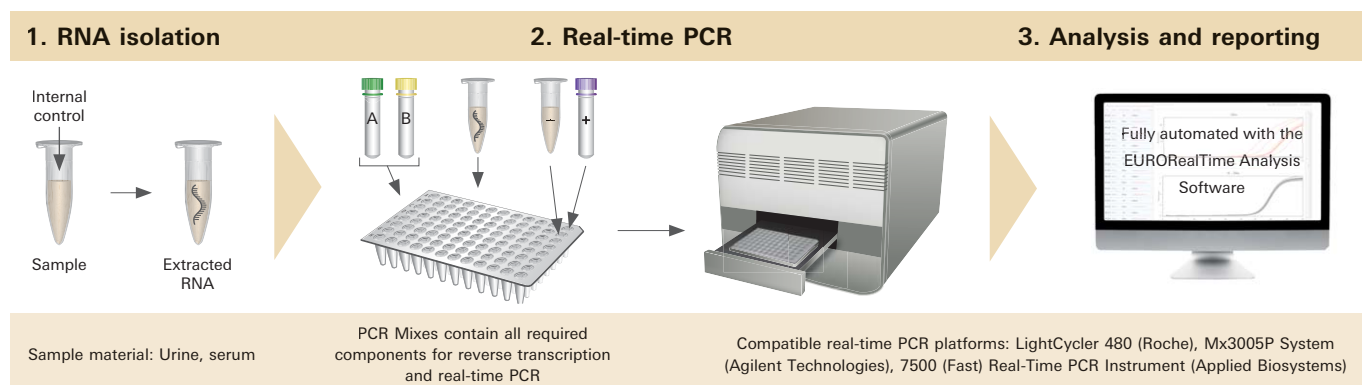


Days after onset of symptoms	Virus detection	Virus detection	Serology (IgA/IgG/IgM)
	RT-PCR serum	RT-PCR urine	ELISA or IIFT
1–7	+	+	–/+
8–27	–	–/+	+
from 28	–	–	+

Direct pathogen detection by real-time PCR

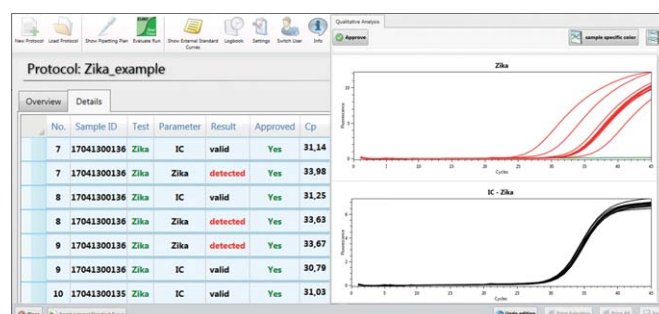
The EURORealTime Zika Virus PCR provides detection of viral RNA in the early phase of a Zika virus infection. This EURO-IMMUN test system includes reverse transcription of the viral RNA into complementary DNA (cDNA), followed by PCR amplification and fluorescence-based real-time detection of defined ZIKV genome sequences. The evaluation of results is performed fully automatically using the EURORealTime Analysis Software.

EURORealTime Zika Virus PCR



EURORealTime Analysis Software

- Standardised and automated raw data analysis for fast and objective result evaluation
- Fully automated report production and result documentation including all internal and external controls
- Convenient guidance through the entire workflow
- Automated layout creation for the PCR plates, including all required controls
- Supplements existing real-time PCR platforms, fully compatible with different LIS



Method comparison

The sensitivity of the EURORealTime Zika Virus PCR was compared to another CE-IVD-certified PCR test was investigated in a comparative study. For this, samples with 3 different concentrations of Zika virus template were analysed in both systems in triplicate determination. Zika virus RNA was detected with both tests in all three or in two out of three runs. Thus, both systems show an identical analytical sensitivity.

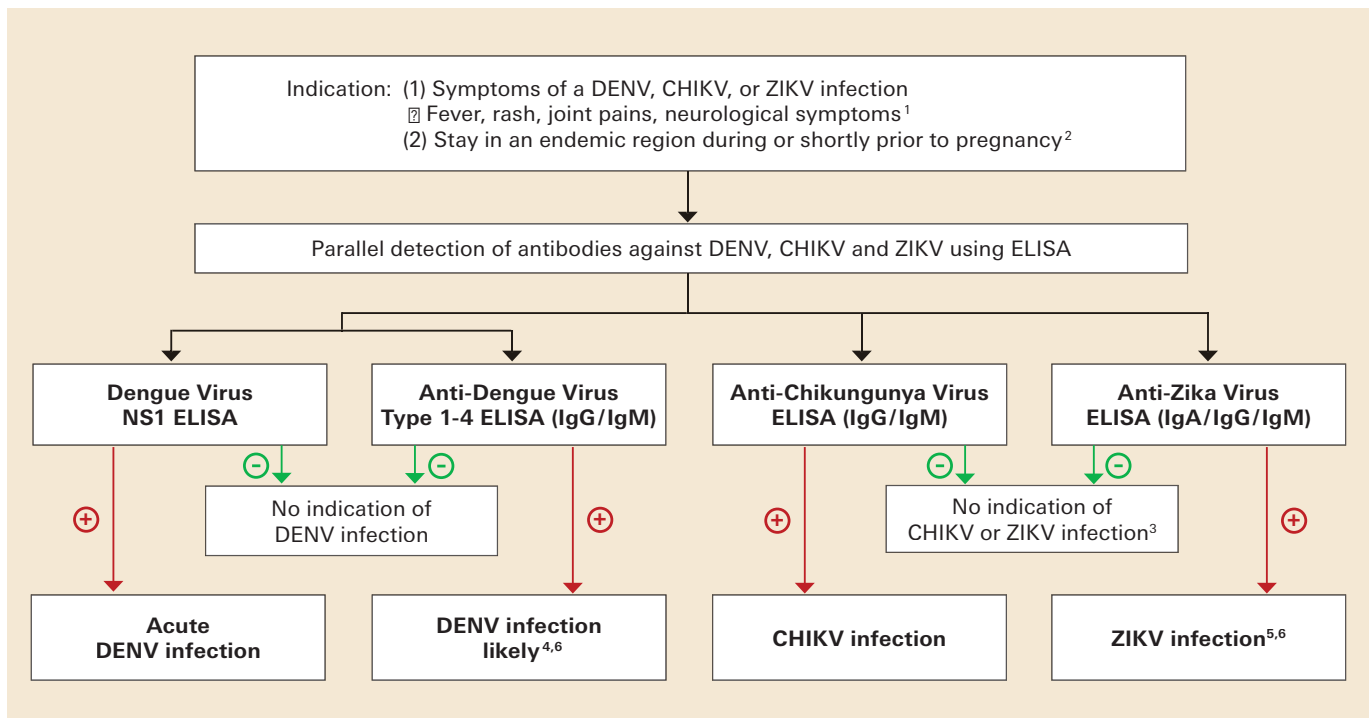
Concentration Zika RNA cp/μl template	EURORealTime Zika Virus PCR	Real-time PCR Zika test manufacturer A
3	detected	detected
	detected	detected
	detected	detected
1	not detected	detected
	detected	detected
	detected	not detected
0.5	detected	detected
	not detected	detected
	detected	not detected

In an external comparison study in a Brazilian laboratory, 29 clinical serum and 26 urine samples were analysed with the EURORealTime Zika Virus PCR and another CE-IVD-certified PCR test. There was a very high agreement of the positive and negative results from both test systems of 95.2% and 97.0%, respectively.

n = 55 (29 serum samples, 26 urine samples)		Real-time PCR Zika Test manufacturer A	
EURORealTime Zika Virus PCR	positive	20	1
	negative	1	33

Serological differential diagnosis with ELISA test systems

In suspected cases of dengue, chikungunya or Zika virus (DENV, CHIKV, ZIKV) infection, EUROIMMUN recommends differential diagnosis following the procedure described in the scheme below. If the blood sample was taken within 7 days after symptom onset, an additional RT-PCR test on serum or urine for direct pathogen detection should be performed.



¹ e.g. loss of mobility, numbness in the limbs, ascending pareses, facial pareses or loss of muscular reflex as a sign of Guillain-Barré syndrome (GBS).

² Men who have been to endemic regions and whose partner is pregnant should also be examined, since sexual transmission of Zika virus is possible.

³ In clinically-supported suspected cases and in diagnosis during pregnancy, a follow-up sample should be taken within 1 – 2 weeks; If this is also negative, an acute infection is highly unlikely.

⁴ Cross reactivity with other flaviviruses cannot be excluded. In secondary infections with other flaviviruses, the DENV IgG titer can be above the titer of the virus causing the acute infection.

⁵ False positive results can occur in sera from patients with acute Plasmodium infections.

⁶ Possible serological constellations and their relevance in flavivirus infections (e.g. DENV, ZIKV, tick-borne encephalitis virus, yellow fever virus, West Nile virus, etc.):

IgA*	IgM	IgG	IgG titer increase in follow-up sample after 1–2 weeks	Indication of
–/+	+	–/+**	yes	Acute infection without prior contact with flavivirus (primary infection)
–/+	–/+***	+	yes	Acute infection after prior contact with flavivirus (secondary infection)
–	–	+	no	Past infection or previous virus contact

* IgA antibody detection can support the diagnosis of acute ZIKV infections; ** IgG antibodies usually occur together with IgA and/or IgM antibodies or shortly after; *** In cases of previous contact with other flaviviruses, the IgM response can occur with a time delay or with reduced intensity.

Study data of ELISA test systems

Sensitivity and specificity

Anti-Zika Virus ELISA (IgG/M)

129 samples were investigated with the Anti-Zika Virus ELISA (IgG) and the Anti-Zika Virus ELISA (IgM). 29 samples originated from patients who had tested positive for Zika virus in examinations of the WHOCC. 100 serum samples of healthy pregnant women were used as reference group. Sensitivity of the Anti-Zika Virus ELISA amounted to 97%, and specificity to 100% (taking into account both immunoglobulin classes). When considering the immunoglobulin classes separately, the sensitivity was 73% for the Anti-Zika Virus ELISA (IgG) and 89% for the Anti-Zika Virus ELISA (IgM). Depending on the disease stage, it is possible that only one Ig class is present.

n = 129		WHOCC, Hamburg/ Routine laboratory, Germany*		
		positive	borderline	negative
EUROIMMUN Anti-Zika Virus ELISA (IgG and IgM) together	positive	28	0	0
	borderline	1	0	0
	negative	0	0	100

* 29 samples from patients with Zika virus infection: WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research (WHOCC), Hamburg, Germany; 100 samples from healthy pregnant women: Routine laboratory, Germany

For the determination of sensitivity and specificity, a second study was performed using 38 samples from patients whose first samples had been classified as positive by Zika Virus RT-PCR (origin: Dominican Republic, Colombia). The serological investigation was done with samples withdrawn > 5 days after onset of symptoms. 33 patients in whom a dengue virus infection could be confirmed in the first sample by direct detection were used as a reference. Due to the positive precharacterisation, the follow-up samples were used to determine the specificity (returning travellers, origin: Germany). Samples from 33 European returning travellers were investigated in the same way with the Anti-Zika Virus ELISA (IgG) and the Anti-Zika Virus ELISA (IgM). The sensitivity of the Anti-Zika Virus ELISA considering both immunoglobulin classes was 100% at a specificity of 94%. When observing the immunoglobulin classes separately, the sensitivity of the Anti-Zika Virus ELISA (IgG) amounted to 100% in both panels. The sensitivity of the Anti-Zika Virus ELISA (IgM) was 27 (Columbian panel), or 87% (panel of European returning travellers). The specificity was 97% for each.

n = 71		Confirmed Zika virus infection (RT-PCR positive at first withdrawal)/ Reference panel (confirmed dengue virus infection)		
		positive	borderline	negative
EUROIMMUN Anti-Zika Virus ELISA (IgG and IgM) together	positive	38	0	2
	borderline	0	0	0
	negative	0	0	31

n = 66		Confirmed Zika virus infection (RT-PCR positive at first withdrawal)/ Reference panel (confirmed dengue virus infection)		
		positive	borderline	negative
EUROIMMUN Anti-Zika Virus ELISA (IgG and IgM) together	positive	33	0	2
	borderline	0	0	0
	negative	0	0	31

To evaluate the specificity of the Anti-Zika Virus ELISAs a study was performed on 72 patient samples that were seropositive for rheumatoid factors and a variety of other autoantibodies (ANA). 22 additional samples originated from patients with acute EBV infection. Of the total of 94 samples, all sera were negative with the Anti-Zika Virus ELISA (IgG), and four sera were positive with the Anti-Zika Virus ELISA (IgM).

Possible influencing factors	n	Anti-Zika Virus ELISA (IgG) positive	Anti-Zika Virus ELISA (IgM) positive
Acute EBV infection	22	0%	4.5%
Various autoantibodies	35	0%	5.7%
Rheumatoid factor	37	0%	2.7%

The high specificity and low cross reactivity of the EUROIMMUN ELISA was confirmed in a study of the University Clinic Freiburg and the Bernhard-Nocht Institute for Tropical Medicine (Huzly et al., 2016). Serum samples from European patients with flavivirus infections or vaccinations, and samples from patients with acute virus infections were investigated. The study yielded a specificity of 100% for the Anti-Zika Virus ELISA (IgG) and 98% for the Anti-Zika Virus ELISA (IgM).

Cohort (Huzly et al.)	Anti-Zika Virus ELISA positive			
	n (IgG)	IgG	n (IgM)	IgM
TBE virus infection	21	0%	38	0%
Dengue virus infection	10	0%	16	0%
Yellow fever vaccine	15	0%	15	0%
Hepatitis C virus infection	16	0%	–	–
Polyclonal IgM	–	–	52	5.8%

Anti-Zika Virus ELISA (IgA)

For investigation of the sensitivity and specificity, a study was performed on 38 patients from ZIKV-endemic regions (origin: Dominican Republic, Colombia) and 11 patients from non-ZIKV-endemic regions (returning travellers, origin: Europe), whose first samples had been classified as positive using Zika-Virus RT-PCR. For the serological investigation, samples were used which were withdrawn > 5 days after onset of symptoms. The reference sera were from 33 patients in whom a dengue virus infection was confirmed by direct detection in the first sample. Due to the positive precharacterisation, the follow-up samples were used for the determination of sensitivity and specificity (returning travellers, origin: Germany). The sensitivity was 92%, with a specificity of 97%.

n = 82		Confirmed Zika virus infection (RT-PCR positive at first withdrawal)/ Reference panel (confirmed dengue virus infection)		
		positive	borderline	negative
EUROIMMUN Anti-Zika Virus ELISA (IgA)	positive	45	0	1
	borderline	0	0	0
	negative	4	0	32

For evaluation of the specificity of the Anti-Zika Virus ELISA (IgG) a further study was performed on 72 patient samples that were seropositive for rheumatoid factors and a variety of other autoantibodies (ANA). 22 additional samples originated from patients with acute EBV infection. All 93 sera showed a negative result using the Anti-Zika Virus ELISA (IgA).

Possible influencing factors	n	Anti-Zika Virus ELISA (IgA) positive
Acute EBV infection	22	0%
Various autoantibodies	35	0%
Rheumatoid factor	36	0%

Cross reactivity

Due to the use of a highly specific, recombinant protein as antigen, cross reactions are virtually ruled out in the EUROIMMUN ELISAs. The investigation of sera panels from clinically and serologically characterised patients with high antibody titers of class IgA, IgG and IgM against other flaviviruses showed a very reduced cross reactivity. The dengue samples used originate from patients with confirmed dengue infections as determined by positive PCR and/or positive NS1 determination.

Note: Double infection or infection with another flavivirus at an earlier time are possible, particularly in endemic areas. In this case, positive results are not caused by a cross reactivity of the corresponding antibodies.

Antibodies against (in total >450)	Anti-Zika Virus ELISA positive					
	n	IgA	n	IgG	n	IgM
Dengue virus						
Characterised by direct detection methods (RT-PCR and/or NS1 antigen positive)	86	0.0%	104	0.0%	103	0.0%
Clinically and serologically characterised	10	10% (1 sample)	42	2.4% (1 sample)	42	0.0%
TBE virus						
After vaccination	15	0.0%	135	0.0%	134	0.0%
Clinically and serologically characterised	54	0.0%	18	0.0%	18	0.0%
Yellow fever virus						
After vaccination (neutralisation test positive)	12	0.0%	12	0.0%	12	0.0%
Japanese encephalitis virus						
Clinically and serologically characterised	13	0.0%	25	4.0%	25	0.0%
West Nile virus						
Characterised by direct detection methods (RT-PCR positive)	40	2.5% (1 sample)	40	0.0%	40	0.0%
Clinically and serologically characterised (neutralisation test positive)	16	0.0%	34	0.0%	34	2.9% (1 sample)

Primary and secondary immune response in Zika virus infections

At the first contact with a virus, IgA and/or IgM antibodies are formed within 3–5 days after infection and remain detectable in the blood for some weeks. With a time delay, approximately from day 8 on, an increasing amount of IgG antibodies are secreted into the blood (primary immune response, case examples 1 and 2).

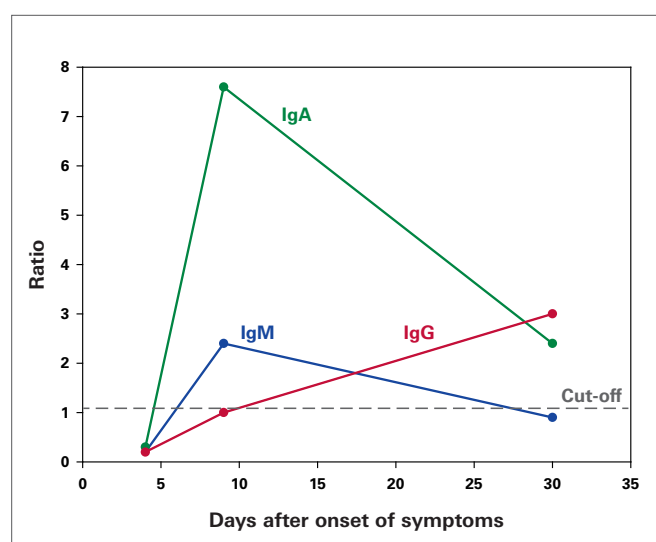
Case study 1

Patient: Returning traveller from Brazil (PCR positive)

■ Antibodies are not yet detectable in the blood in the first serum sample, withdrawn at day 4 after onset of symptoms. In this early phase, PCR diagnostics are indispensable.

■ In the follow-up sample (day 9), antibodies of class IgA and IgM are present. In the further course, also IgG antibodies will be detectable.

Days after onset of symptoms	EUROIMMUN Anti-Zika Virus ELISA		
	IgA (ratio)	IgM (ratio)	IgG (ratio)
4	0.3	0.2	0.2
9	7.9	2.4	1.0
30	2.4	0.9	3.0



Case study 2

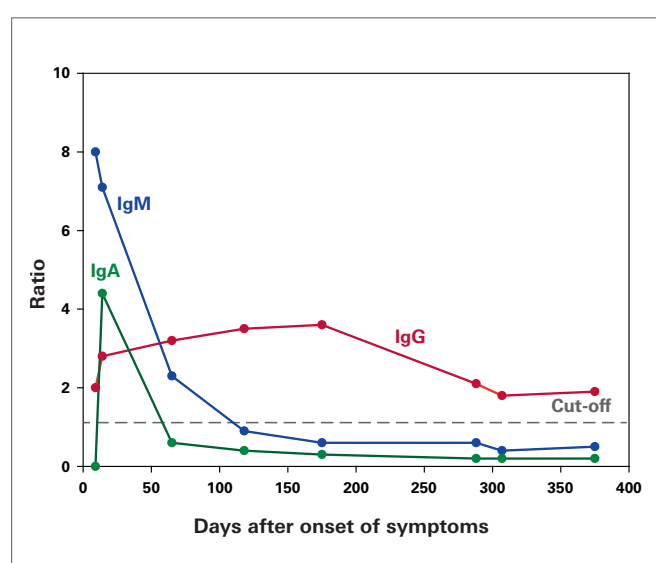
Patient: Returning traveller from Colombia

■ IgG levels positive after onset of symptoms. Slow titer increase over a longer time period. After approximately 175 days, the titer slowly decreases, but persists above the cut-off.

■ In the first serum sample after onset of symptoms, IgA antibodies cannot yet be detected. After a fast increase between the first and the second serum sample (day 9 and 14), the IgA titer decreases again.

Days after onset of symptoms	EUROIMMUN Anti-Zika Virus ELISA		
	IgA (ratio)	IgM (ratio)	IgG (ratio)
9	0.0	8.0	2.0
14	4.4	7.1	2.8
65	0.6	2.3	3.2
118	0.4	0.9	3.5
175	0.3	0.6	3.6
288	0.2	0.6	2.1
307	0.2	0.4	1.8
375	0.2	0.5	1.9

■ IgM levels are first positive, then decrease over the further course, and are negative after approximately 120 days.



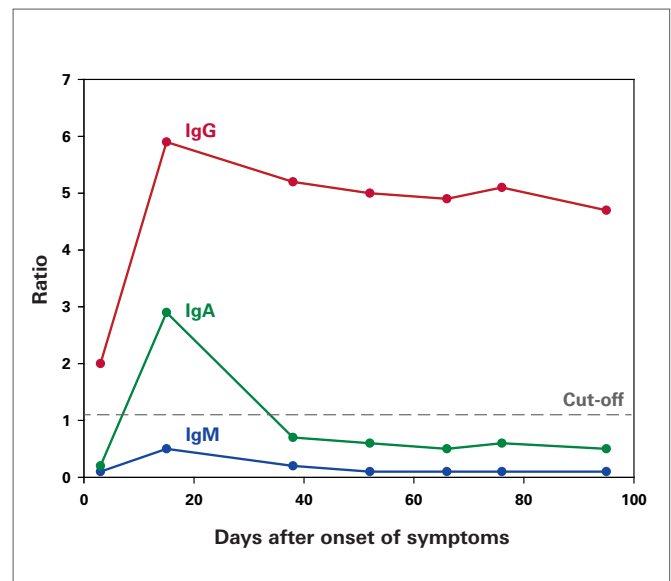
In the case of a second contact with the same virus, the IgG titer increases strongly within the first days of infection. Specific IgM antibodies, however, are only formed in reduced, partly not detectable quantities (secondary immune response, case examples 3 and 4). Within the immune response, antibodies of class IgA are often formed in parallel to the IgM antibodies. The determination of anti-Zika virus IgA antibodies may support diagnosis of an acute infection and contribute essentially to the clarification of ambiguous findings.

Case study 3

Patient: 18 years, male, Colombia

- IgG levels positive already on the 3rd day after onset of symptoms (literature: from day 5 – 6).
- IgM levels negative over the complete period of time.
- IgA positive from day 8 – 35 after onset of symptoms.

Days after onset of symptoms	EUROIMMUN Anti-Zika Virus ELISA		
	IgA (ratio)	IgM (ratio)	IgG (ratio)
3	0.2	0.1	2.0
15	2.9	0.5	5.9
38	0.7	0.2	5.2
52	0.6	0.1	5.0
66	0.5	0.1	4.9
76	0.6	0.1	5.1
95	0.5	0.1	4.7

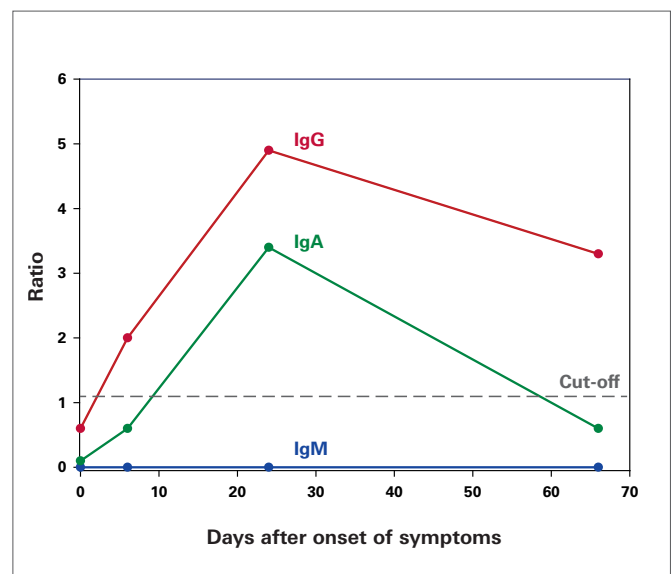


Case study 4

Patient: 51 years, female, Colombia

- IgG levels positive shortly after onset of symptoms (day 6). After further increase, the titer decreases but remains above the cut-off.
- IgM are never detectable.
- IgA titer is only positive at the 3rd sample withdrawal, three weeks after symptom onset, then decreases, and is negative approximately after day 60.

Days after onset of symptoms	EUROIMMUN Anti-Zika Virus ELISA		
	IgA (ratio)	IgM (ratio)	IgG (ratio)
0	0.1	0.02	0.6
6	0.6	0.01	2.0
24	3.4	0.03	4.9
66	0.6	0.02	3.3



Indirect immunofluorescence

Alongside the ELISA test systems, EUROIMMUN offers indirect immunofluorescence tests (IIFT) for the diagnosis of Zika virus infections. There are three products available:

- **Anti-Zika Virus IIFT:** For the screening of Zika virus antibodies
- **Arbovirus Fever Mosaic 2:** For differential diagnosis of Zika, dengue, and chikungunya virus infections, which cause similar symptoms.
- **Arbovirus Profile 3:** Ideally suited for the investigation of cross reactivity within the group of flaviviruses.

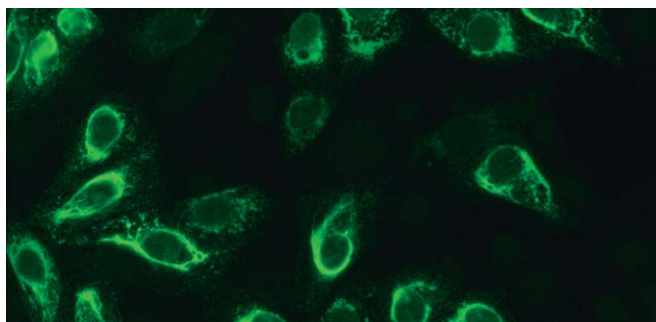
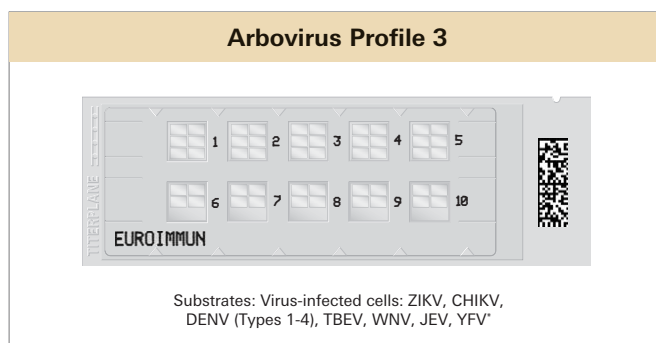
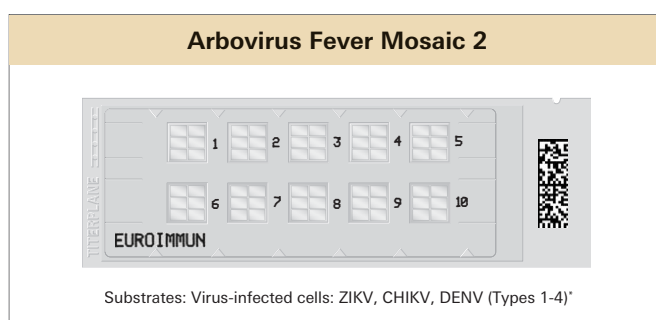
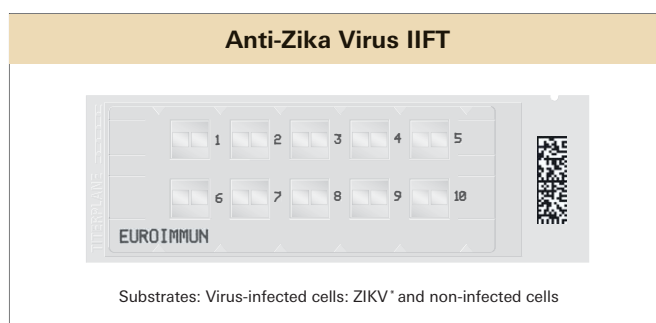
Cells infected with different arboviruses are used as test substrate. In a positive reaction, mainly the cytoplasmic regions fluoresce, showing fine- to coarse-granular structures (see figure below). Some cells also show a net-like fluorescence pattern with a dense, perinuclear reactivity.

With these flavivirus mosaics/profiles, several specific antibodies can be detected simultaneously. They can be used to support clarification of cross reactivities between different flaviviruses and enable reliable differential diagnosis in the case of similar clinical symptoms.

* ZIKV: Zika Virus, DENV: Dengue Virus, CHIKV: Chikungunya Virus, TBEV: Tick-borne encephalitis virus, WNV: West Nile virus, JEV: Japanese encephalitis virus, YFV: Yellow fever virus

Cross reactivity

Since complete virus particles are used as antigen in the IIFT products (contrary to the ELISA), cross reactivities are to be expected with the antibodies against viruses of the flavivirus family. In primary flavivirus infections, the dominant antibody titer of the virus which causes the infection can usually be identified by creating a dilution series of the patient sample. In secondary flavivirus infections, a high cross reactivity of the IgG antibodies must be expected. The final titers are equal or similar on all flavivirus substrates. In some cases, the determination of IgM antibodies may allow differentiation.



Fluorescence pattern: positive reaction: IgG antibodies against Zika virus.

Automation

ELISA

The Anti-Zika Virus ELISAs are suitable for processing on fully-automated analysis instruments. The tests are validated for the Analyzer I and Analyzer I-2P from EUROIMMUN and the DSX device from Dynex. Automated performance using other fully automated, open system analysis devices is possible. However, the combination should be validated by the user.

EUROIMMUN Analyzer I-2P



IIFT

The IIF tests for the diagnosis of a Zika virus infection can be processed by the automated laboratory devices Sprinter and Sprinter XL from EUROIMMUN. All steps from the dilution and assignment of samples to the incubation and washing of slides are performed fully automatically. Evaluation of the incubated slides at the microscopes is possible, supported by the computer-aided immunofluorescence microscope EUROPattern.

EUROIMMUN Sprinter XL



Publications

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11. Fourcade C, Mansuya JM, Dutertre MD, Delobel B. Viral load kinetics of Zika virus in plasma, urine and saliva in a couple returning from Martinique, French West Indies. *Journal of Clinical Virology*, Jun 2016.
12. Calleri G, Burdino E, Bonora S, Raso R. Zika virus infection in two travelers returning from an epidemic area to Italy, 2016: Algorithm for diagnosis and recommendations, *Travel Medicine and Infectious Disease* (2016).



Product overview

Test systems	Order number
Anti-Zika Virus ELISA (IgA)	EI 2668-9601 A
Anti-Zika Virus ELISA (IgM)	EI 2668-9601 M
Anti-Zika Virus ELISA (IgG)	EI 2668-9601 G
Anti-Zika Virus ELISA (IgAM)	EI 2668-9601 Q
Anti-Zika Virus IIFT (IgG or IgM)	FI 2668-#### G/M
Anti-Zika Virus IIFT EUROPattern (IgG or IgM)	FR 2668-#### G/M
Arbovirus Fever Mosaic 2 (IgG or IgM)	FI 2668-####-1 G/M
Arbovirus Fever Mosaic 2 EUROPattern (IgG or IgM)	FR 2668-####-1 G/M
Arbovirus Profile 3 (IgG or IgM)	FI 2668-####-3 G/M
EURORealTime Zika Virus PCR	MP 2668-####