Nonenzymatic electrochemical method for determination of the measles virus antigen using the synthesized IgG—(Fe_3O_4—SiO_2) conjugate as the signal label*

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An electrochemical method for determining viral antigens was developed in relation to the measles virus antigen. Using inverse adsorption voltammetry and the synthesized conjugates of antibodies with the Fe_3O_4—SiO_2 nanocomposite particles as the signal label, a sensitive, easily measurable analytical signal was obtained.

Key words: nanocomposite particles, immunoassay, inverse voltammetry, meeasles virus antigen.

The modern protocols of treatment and prevention of viral diseases by means of antiviral drugs, immunomodulators, and vaccines with different mechanisms of action require fast and accurate laboratory diagnosis techniques. Apart from elucidation of the disease ethiology, this is highly important for arrangement of antiepidemic measures (quarantine, hospitalization, vaccination, and so on[1]). Early diagnosis of the first cases of infections allows timely measures to be taken.

Measles is among the most contagious diseases. It is rapidly spread by airborne means and cause severe complications affecting the central nervous system (encephalites), respiratory system (pneumonias), and gastrointestinal tract. This infection has an almost 100% susceptibility. The significance of laboratory assays for measles diagnosis constantly increases. This is related, in particular, to the presence of atypical forms of the diseases with reduced latent period, mitigation of symptoms, change in the type of hives, and so on. Making the diagnosis is difficult for vaccinated patients having an attenuated form of the disease and in the case of hives appearing in response to injection of the live measles vaccine.[2,3]

The laboratory diagnosis of viral infections uses three principal approaches:[4] 1) serological diagnosis based on detection of the considerable increase in the level of viral antibodies during the illness; 2) virus isolation from the clinical material and identification; 3) direct investigation of the material for viral antigen or nucleic acids. The serological assay based on the antigen—antibody reaction can be used to determine both anti-measles IgM and IgG and may be significant for elucidation of the viral infection ethiology even if the result of virus isolation is negative. The specific IgG antibodies are produced in the body in a concentration sufficient for determination only a certain period of time (2 to 3 weeks) after getting the infection, which makes this method inapplicable for detecting the early stage of the disease. A serum examination of every patient suspected to have measles is often faced with problems related to the difficulty of serum delivery from remote out-of-the-way regions and the necessity to maintain "cold conditions" during the material transportation.[5]

Virus isolation is one of the oldest and labor-consuming diagnosis techniques. This is done using cell cultures, laboratory animals, or chicken embryos. The process is long-lasting, requiring, in some cases, conduction of several passages before the virus is detected and identified by one or several methods: neutralization and immunofluorescence reactions, enzyme immunoassay (EIA), or polymerase chain reaction (PCR). This approach provides the result as soon as 24—72 h after the tissue culture cells have been infected. The virological diagnosis method can be employed only in specialized laboratories, in rare cases, due to complexity of the assay.[6]

Direct methods (electron microscopy, immunofluorescence reaction, EIA, and molecular (PCR) and cytological methods), which are currently used in the top measles laboratories in Russia and in most other countries,

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make it possible to detect the virus, the viral antigen, or the viral nucleic acid directly in the clinical material, *i.e.*, these techniques are the fastest (2—24 h). However, due to some specific features of the infectious agents, direct methods have some limitations (the possibility of false positive or false negative results). The necessity of using expensive and unstable ingredients (enzymes) for conducting the immunological reactions reduces the availability of EIA.[7,8]

Currently, the vast majority of research works on the determination of viral agents are devoted to the development of new versions of PCR assay.[9—14] However, the proposed procedures are complicated, expensive, and not yet standardized enough for routine use. Therefore, development of methods and sensors for fast "on-site" diagnostic procedures to detect viral agents is fairly topical. Electrochemical methods using biosensors seem promising for diagnosis of hazardous infections. These methods are characterized by high sensitivity, good reproducibility and selectivity, simple implementation, the possibility of fabricating portable devices suitable for the use both in laboratory and in small clinics, and low cost of the assay as compared with other methods.[15—17]

The purpose of this work was to develop a non-enzymatic electrochemical immunoassay for determination of the measles virus antigen using a conjugate of secondary antibodies with nanocomposite particles based on silica-coated Fe_3O_4 nanoparticles as the signal label in model solutions.

Results and Discussion

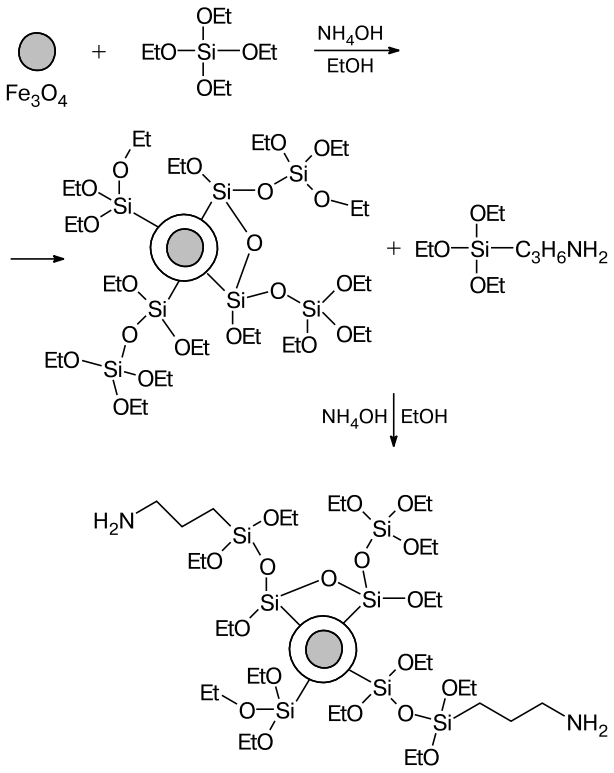
The developed immunoassay procedure was implemented using an antibody—nanocomposite particles conjugate (Schemes 1 and 2).

The synthesized conjugate represented an aqueous suspension of a finely dispersed powder, which exhibited magnetic properties upon application of a field.

Considering the proposed procedure of the assay (Fig. 1), including the formation of a sandwich immune complex comprising the secondary antibody—antigen conjugate with nanocomposite Fe_3O_4—SiO_2 particles on the surface of a thick-film graphite epoxy electrode (TGE) and subsequent acid treatment, one can expect that the Fe^{3+} reduction current would provide information on the presence and amount of the antigen in the sample.

When the test solution contained the antigen, the voltammogram exhibited an iron(III) reduction peak (Fig. 2, *a*). In the blank experiment (no measles virus antigen in the sample), the potential region $E = -0.6$—(-1.0) V exhibited no peaks (see Fig. 2, *b*) because the immune complex was not formed. The obtained results also indicated the absence of non-specific binding of the conjugates of secondary antibodies and nanocomposite particles to the working part of the antibody-modified TGE. Figure 3

Scheme 1



shows the dependence of the analytical signal on the time of sandwich formation between the immune complex on the TGE surface and the conjugate of antibodies with nanocomposite particles (stage 3 in Fig. 1).

The curve flattens out in 30 min, which is apparently due to filling of all binding sites. Therefore, in the subsequent investigations, the formation of sandwich immune complexes was conducted for 30 min.

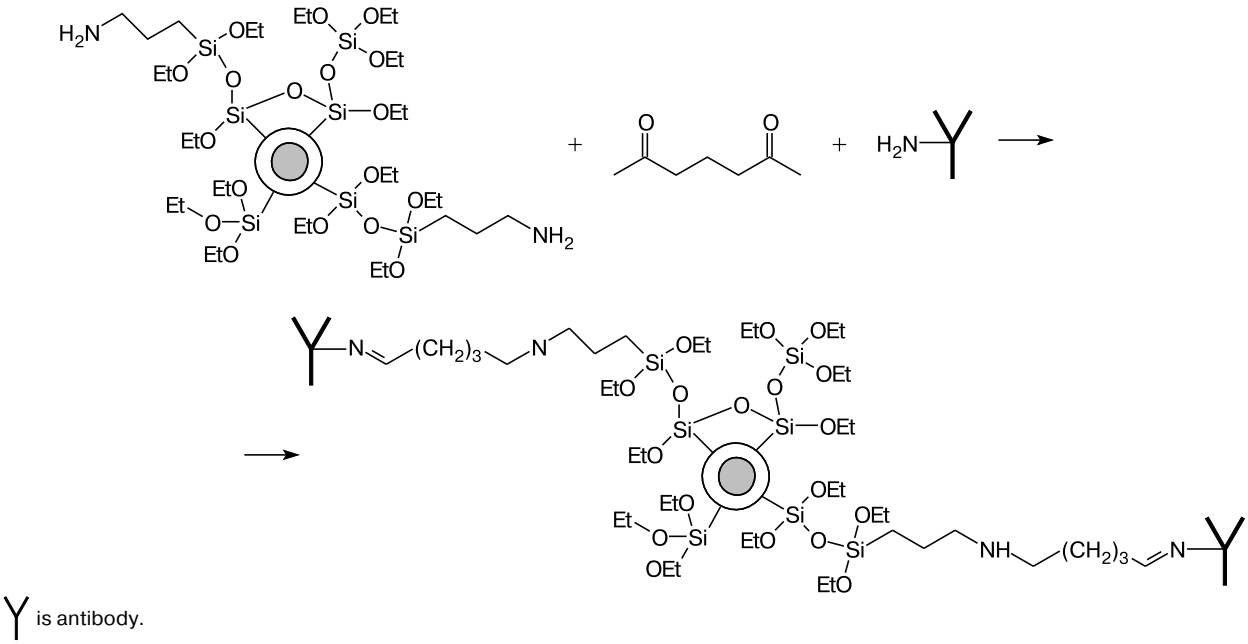
Figure 4 shows the plot of the analytical signal *vs.* the time of formation of the immune complex between the measles virus antigen and the antibodies immobilized on the TGE surface (stage 2 in Fig. 1).

The curve flattens out in the first 20 min of formation of the immune complex between the measles virus antigen and the antibodies. This is apparently due to the maximum filling of the TGE surface. Therefore, in the subsequent investigations, the formation of the immune complex was conducted for 20 min.

Then voltammograms were recorded using the selected immunoassay conditions. This gave a linear dependence on the analytical signal on the logarithm of the measles virus antigen concentration in the range of $2.33 \cdot 10^{-4}$—2.33 mg mL^{-1} according to the equation $dI/dE = 2.44 \cdot \log C + 11.6$ ($R = 0.995$). The detection limit calculated for the $3S$-criterion was $1.87\ 10^{-5}$ mg mL^{-1}.

Table 1 presents the analytical characteristics of measles virus antigen determination in different periods of time after preparation of immunoassay reagents. The repro-

Scheme 2



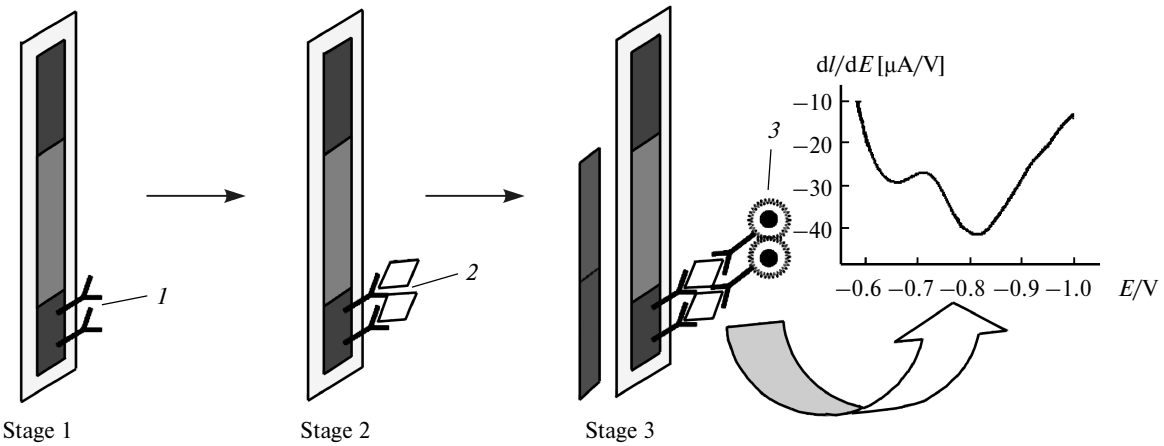


Fig. 1. Immunoassay protocol. (*1*) Anti-measles virus antibody immobilized on the working area of a TGE, (*2*) measles virus antigen, (*3*) conjugate of the secondary anti-measles virus antibodies with the Fe_3O_4—SiO_2 nanocomposite particles.

ducibility and repeatability factors do not exceed 0.01. Thus, the proposed method gives reproducible results on the measles virus antigen concentration in the range of $2.33 \cdot 10^{-4}$—2.33 mg mL^{-1}.

The specificity of the developed method was evaluated in the following way: the TGE with immobilized anti-measles virus antibodies were incubated in a solution containing the tick-borne encephalitis virus antigen. Then, according to the immunoassay protocol, the nanocomposite particle-labeled secondary anti-measles antibodies were added and the analytical signal was measured. After incubation in a solution containing no measles virus antigen, no signals were generated because the immune complex was not formed on the TGE surface. This indicates that non-specific interactions or adsorption do not affect the analytical signal.

Thus, the performed study demonstrated that the developed electrochemical immunoassay procedure is suitable for determining the measles virus antigen in a model solution in a $2.33 \cdot 10^{-4}$—2.33 mg mL^{-1} concentration range. Owing to the use of nanocomposite particles based on the magnetite Fe_3O_4 as the signal label, an easily measurable analytical response was obtained. Also, the assay principles and protocol suggest that the method could be

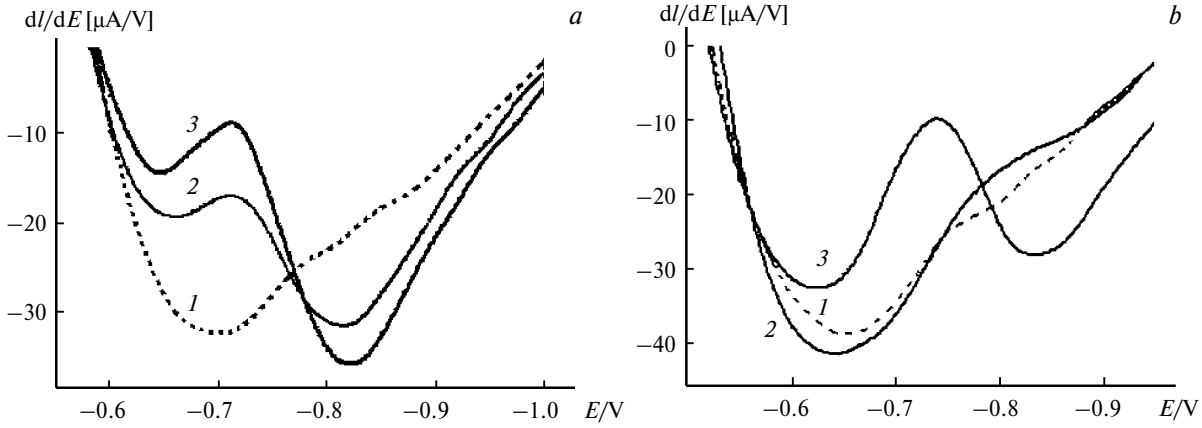


Fig. 2. Differential form of voltammograms of solutions with (*a*) and without the measles virus antigen (*b*): supporting solution (0.1 *M* solution of AcONa containing $5 \cdot 10^{-4}$ mol L^{-1} of pyrocatechol, pH 7.5) (*1*), sample (*2*), sample with Fe^{3+} additive (*3*). Antigen concentration: $2.33 \cdot 10^{-2}$ mg mL^{-1}. Accumulation potential 0.1 V, accumulation time 60 s, potential sweep rate 0.5 V s^{-1}.

extended to other viral agents. In the future, the developed method may be adapted for determining viral antigens directly in the biological objects such as saliva, nasal lavage, and blood.

Experimental

The following commercial products were used: sterile physiological saline solution (0.9% solution of NaCl, Research and Production Enterprise PanEco), measles virus antigen (NovO/96) in a 2.33 mg mL^{-1} concentration, anti-measles virus antibodies, secondary anti-measles virus antibodies (Federal State Research Institution State Scientific Center of Virology and Biotechnology

Table 1. Analytical characteristics of the electrochemical method for measles virus antigen determination

C^a /mg mL^{-1}	N, days	dI/dE /μA V^{-1}			CR^b	K^c
$2.33 \cdot 10^{-4}$	1	2.21	2.23	2.20	0.007	0.006
	2	2.22	2.20	2.24	0.009	
	3	2.21	2.20	2.22	0.005	
$2.33 \cdot 10^{-3}$	1	5.25	5.26	5.20	0.006	0.005
	2	5.22	5.20	5.24	0.004	
	3	5.27	5.25	5.20	0.007	
$2.33 \cdot 10^{-2}$	1	8.12	8.13	8.10	0.002	0.007
	2	8.21	8.21	8.05	0.010	
	3	8.17	8.22	8.14	0.005	
0.233	1	10.26	10.08	10.18	0.009	0.006
	2	10.17	10.10	10.08	0.005	
	3	10.20	10.15	10.14	0.003	
2.33	1	12.15	12.27	12.30	0.006	0.009
	2	12.32	12.00	12.25	0.010	
	3	12.31	12.13	12.31	0.009	

[a] Antigen coefficient.
[b] Reproducibility factor.
[c] Repeatability factor.

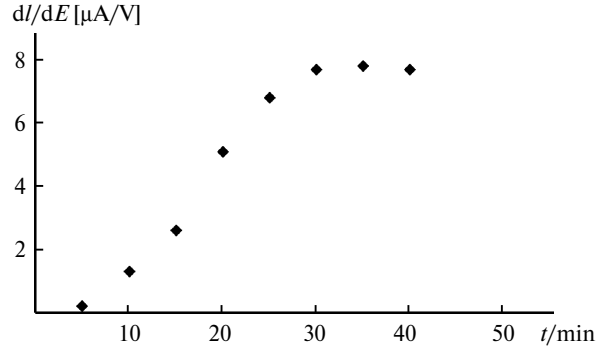


Fig. 3. dI/dE value found during the assay of solutions after acid treatment of the sandwich immune complex *vs.* the time of formation of the complex. Antigen concentration $2.33 \cdot 10^{-2}$ mg mL^{-1}. The average values for $n = 5$ are given.

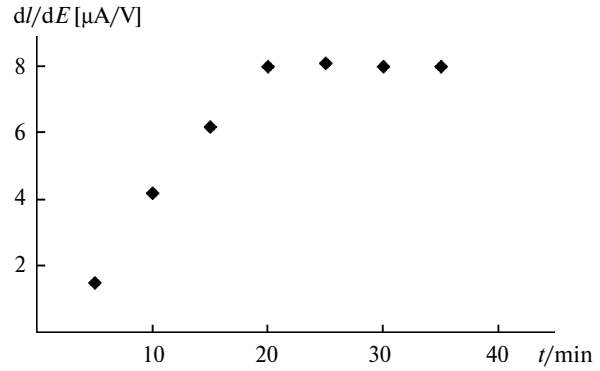


Fig. 4. dI/dE value found during the assay of solutions after acid treatment of the sandwich immune complex *vs.* the time of formation of the immune complex between the measles virus antigen and the antibodies immobilized on the TGE surface. Antigen concentration $2.33 \cdot 10^{-2}$ mg mL^{-1}. The average values for $n = 5$ are given.

В таблице чего-то не хватает.

Vector, Novosibirsk), 57% H_2SO_4, 57% HNO_3 (special purity grade, OJSC Giredmet, Moscow), medicine grade H_2O_2 (36—38%, LLC Lega, Dzerzhinsk), $CH_3COONa \cdot 3H_2O$ (special purity grade), pyrocatechol (special purity grade), certified reference sample of Fe^{III} ions, $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, $Si(OEt)_4$, glutaraldehyde (25%), and (3-aminopropyl)triethoxysilane (25%) (Sigma-Aldrich), $NH_3 \cdot H_2O$ (analytical grade, LLC Sigma TEK, Moscow), 95% ethanol (LLC Gippokrat, Samara). Deionized water for preparation of solutions was obtained on a DVS-M/1NA(18)-N combined membrane unit (Mediana-Filter, Moscow).

Suspensions of nanocomposite particles were prepared in an Ultrasonic processor 500W homogenizer (Sigma-Aldrich, USA). The stage of formation of the immune complex "secondary IgG antibodies—viral antigen—IgG/nanocomposite conjugate" was conducted using a GFL type 1010 incubator. The nanoparticles and nanocomposites were synthesized using a C-MAG HS 7 IKAMAG hot plate magnetic stirrer (IKA, Germany), an IKA Eurostar digital 2482000 overhead stirrer (IKA, Germany), and an MagneSphere magnetic stand for 12 Eppendorf microtubes (Promega, USA) with a magnetic field strength of $31.83 \cdot 10^3$ A m^{-1}. A thick-film graphite epoxy electrode (TGE) was used as the inert substrate for antibody immobilization.

The electrochemical measurements were conducted using an IVA-5 inverse voltammetric analyzer (IVA, Ekaterinburg). A calomel-modified thick-film graphite-containing (TMGE) working electrode and an Ag/AgCl reference electrode (Metrohm, Switzerland) were used, and a glass carbon rod (Metrohm, Switzerland) served as the auxiliary electrode.

Synthesis of nanocomposite particles and conjugation of the particles with antibodies. The Fe_3O_4 nanoparticles were prepared by co-precipitation.[18] A 25% solution of $NH_3 \cdot H_2O$ was added dropwise to an aqueous solution containing 4.5 wt.% $FeCl_3$ and 2.25 wt.% $FeCl_2$. The reactants were taken in stoichiometric amounts (except for ammonia, which was taken in a slight excess) according to the equation

$$Fe^{2+} + 2\ Fe^{3+} + 8\ OH^- = Fe_3O_4 + 4\ H_2O$$

The nanocomposite particles were obtained by coating the Fe_3O_4 nanoparticles by silica.[19] The Fe_3O_4 nanoparticles (0.5 g) were dispersed in a mixture of 95% EtOH (40 mL) and H_2O (10 mL). Then $Si(OEt)_4$ (0.5 mL) and a 25% solution of $NH_3 \cdot H_2O$ (1 mL) were added. The mixture was refluxed with stirring for 10 h. The unreacted $Si(OEt)_4$ was removed by three-times washing with EtOH with magnetic separation of the nanocomposite. Then the silica-coated nanoparticles were again dispersed in 95% EtOH (50 mL) and sonicated for 1 min. (3-Aminopropyl)-triethoxysilane (1 mL) was added and the mixture was refluxed for 10 h with stirring (see Scheme 1). The remaining (3-aminopropyl)triethoxysilane was removed by three-times washing with EtOH with magnetic separation of the nanocomposite using a permanent magnet with a $37.40 \cdot 10^4$ A m^{-1} field.

Grafting of the secondary antibodies to the silica coating of the nanoparticles (see Scheme 2) was performed by a known procedure.[20] The nanocomposite was dispersed in H_2O (50 mL), and 10 mL of the suspension was taken with a volumetric pipette and sonicated for 1 min. Then 25% glutaraldehyde (1 mL) and then secondary anti-measles virus antibodies (0.2 mg) were successively added. The mixture was stirred for 8 h at ~10 °C. The antibody—nanocomposite particles conjugate was purified from the unreacted components by means of a permanent magnet. The working suspension of the conjugate was prepared by dispersing a weighed portion (0.005 g) in a sterile saline solution (10 mL). Then the suspension was sonicated for 30 s.

Immunoassay procedure. Anti-measles virus IgG (10 μL, 1 mg mL^{-1}) was applied on the TGE working area and dried until the dispersion medium completely evaporated (stage 1 in Fig. 1). Measles virus antigen (NovO/96) solutions in the sterile physiological saline solution were prepared (concentrations of $2.33 \cdot 10^{-1}$, $2.33 \cdot 10^{-2}$, $2.33 \cdot 10^{-3}$, and $2.33 \cdot 10^{-4}$ mg mL^{-1}). Then 200 μL of solutions of each concentration were placed into the tubes, TGE with pre-immobilized anti-measles virus IgG were immersed, and the mixtures were incubated for 20 min at 37±0.1 °C (stage 2 in Fig. 1). After that, a suspension of the secondary anti-measles virus antibody conjugate with the nanocomposite particles (200 μL) was added into each tube. The conjugates were formed using a magnetic stand arranged behind the TGE bearing the immune complex at 37±0.1 °C for 30 min (stage 3 in Fig. 1). After washing, the TGE with the sandwich immune complex "secondary IgG antibodies—viral antigen—IgG/nanocomposite conjugate" was placed into a thermally stable glass beaker and the immune complex was destroyed by acid treatment.

A 1 *M* solution of CH_3COONa (1 mL) was placed into an electrochemical cell, and a $5 \cdot 10^{-3}$ *M* aqueous solution of pyrocatechol (0.1 mL) (for the formation of electrically active iron(III) complex) and the sample (0.2—2.0 mL) were added. The total volume of the solution was brought to 10 mL by adding deionized water. Electrodes were immersed into the cell and iron concentration was determined by inversion voltammetry using the TMGE.[21] Measurement conditions: preconcentration of the Fe^{3+} ions on the TMGE surface for 60—120 s (with stirring at $E = 0.1$ V), the solution conditioning time was 10 s at 0.1 V, the potential sweep rate (in the differential mode) was 0.5 V s^{-1} in the potential range $E = 0.10$—(−1.20) V, search area of the Fe^{3+} analytical signal was (−0.80)±0.05 V. The TMGE regeneration comprised 10 sweeps at a 0.5 V s^{-1} rate in the range $E =$ = 0.1—(−1.20) V. The maximum cathodic current for the reduction of the pyrocatechol complex with iron(III) was directly proportional to the Fe^{3+} content in the solution.

In the blank experiment, the immunoassay was performed by the above protocol except that in stage 2 (see Fig. 1), the TGE modified by antibodies was incubated in a solution containing no viral antigen.

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