

## VALIDATION OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR SEMIQUANTITATIVE DETERMINATION OF IgM ANTIBODIES AGAINST *CHLAMYDIA TRACHOMATIS*

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### ABSTRACT

The object of the following study is representation of the scientific and methodical foundation of the procedure of validation of means of serum *in vitro* diagnosis basing on the sample of ELISA for semiquantification of specific *Chlamydia trachomatis* IgM-antibodies. Validation characteristics (precision, diagnostic and analytic specificity, diagnostic sensitivity, relative linearity) were defined both at the beginning of the release of diagnostic set and the expiration date (as the element of stability study). The values of diagnostic sensitivity and specificity, defined via the in-process set of serums (20 positive and 50 negative) comprised 100%. The presence of other classes of Chlamydia antibodies in samples didn't influence the results of ELISA for specific IgM antibodies. Linearity of the method was sufficient. The intra-assay variation of results is between 3.1% to 6.4%, and intra-assay precision was from 1.5% to 8.4%, staying acceptable ( $\leq 10\%$ ) both at the moment of release and during expiration date. The ELISA method is validated, and the diagnostic kit is regarded as stable during 1 year.

**Keywords:** ELISA, validation, antibodies, *Chlamydia trachomatis*

### INTRODUCTION

Chlamydia is regarded as one of the most widespread sexually transmitted diseases in the world. According to data of World Health Organization, about 90 million people in the world are infected via sexual intercourse with *Chlamydia trachomatis* pathogen (Woodhall *et al.*, 2018). In Ukraine the infection rate reaches 80 people per 100 thousand citizens. Almost 16% of pregnant women are infected with *Ch. trachomatis*. The causation of tubal infertility by Chlamydia is proven in 50-60% of cases. 25% of cases of ophthalmic pathologies and respiratory diseases of newborns and infants are connected to Chlamydia infection (van Ess *et al.*, 2018).

One of the key components of dealing with the Chlamydia spreading is the effective diagnostics of this disease, which is realized via direct (detection of antigens, nucleic acids, microscopic examination and cultivation of agent) and indirect (detection of specific antibodies) methods. One of the methods, which has proven to be quite effective in detecting Chlamydia, is enzyme-linked immunosorbent assay (ELISA), the application of which provides the possibility to conduct differential diagnostics i.e. to define the stage and the character of the disease progression, which is especially difficult in case of chronic diseases. In this case the study of blood serums (plasma) are conducted with the view of detecting the presence of IgM, IgA and IgG antibodies, which are specific to agent's antigens (Winstanley *et al.*, 2017; Klestova *et al.*, 2019). During previous stages of our work we have acquired a set of monoclonal antibodies to human IgM and using them as a foundation have constructed an immunoenzyme set for quality (semiquantifying) detection of IgM antibodies and principal outer membrane protein of *Ch. trachomatis* (Nikolaenko *et al.*, 2005).

The estimation of validity of analytical methods is one of the key elements of provision of quality of pharmaceutical and biotechnology industries. Validation of analytical methods is the procedure of experimental confirmation that the method used is applicable for solving the set tasks (Lutsenko *et al.*, 2017). It is worth mentioning that means of *in vitro* serum diagnosis have certain peculiarities and dissimilarities from pharmaceutical products, resulting in difference in approaches to their bioanalytical unification, which may differ from similar methods applied to pharmaceutical products (Golembiovskaya *et al.*, 2019). In our previous studies we have conducted the analysis of requirements of national and international regulations on quality and safety of medical products for *in vitro* diagnosis and discussed the possibility of partial application of E.P. for this type of products (Golembiovskaya *et al.*, 2019). We have defined that the validation characteristics and parameters for bioanalytical unification for

qualitative (semiquantitative) test-kits for serum diagnosis might be intra-assay variation, intra-assay precision and replicability, diagnostic and analytical specificity, and for quantity means these are additional accuracy, linearity, analytical sensitivity and range of application. Taking into account the absence of obligatory recommendations concerning the conducting of bionalytical methods' validation used in *in vitro* serum diagnostics, we presume the urgency of creation of scientific-methodical guidelines for validation of immunoenzyme kits for qualifying and quantifying bioanalyses' conducting.

The object of the article is to validate the procedure and conducting of validation of ELISA designed for semiquantification of defining the specific *Ch. trachomatis* IgM antibodies.

### MATERIALS AND METHODS

#### ELISA kits

In our work we have used 3 scientific-production batches, which were studied right after the release and in 1 year, during expiration period (as the element of studying of kit stability).

#### Serum set

In order to perform validation an in-process serum set (PSS) was used. Positive samples were formed from the blood serum of patients, whose blood contained *Ch. trachomatis* IgM antibodies and had positive results to PCR during detection of agent's DNA in urogenital smear. Such serums were studied with the help of diagnostic kits "ChlamyBest-C.trachomatis IgM", Vector-Best, Russia, and "ELISA-VIDITEST anti-Chlamydia trachomatis IgM", VIDIA Ltd., Czech Republic. Only those smears which had positive results for all types of studies were chosen for inclusion into PSS. Negative samples didn't include specific *Ch. trachomatis* IgM-antibodies. Among IgM-negative samples we have chosen 5 serums, which simultaneously included both IgG and IgA antibodies to Chlamydia agent, and 15 samples which were only IgG antibodies positive (data of the serum were used for estimation of analytical sensitivity of analytes).

#### Indirect ELISA

Fusion proteins Pgp3 and MOMP of *Ch. trachomatis* were sorbed on 0,02 M carbon-bicarbonate buffer on 96-well culture plates for heterogeneous ELISA.

The plate was incubated for 12 hours at 4 °C, the phosphate-buffer saline (PBS) was washed thrice with adding of 0.05% tween-20 (PBST), pH 7.2-7.4 and held in BSA solution (10 mg/ml in PBS) for 1 hour with the temperature of 37 °C. The plates were dried in vacuum-flow closet and hermetically sealed in plastic bags. In such a way the plates were stored up to the point of the experiment conduction. Before the performing of the analysis the wells of the plate were filled with 90 µl of buffer solution for serum dilution (0.05 M tris-HCl buffer, pH 8.0, 0.15 M NaCl, 5 mM edetic acid, 0.5 mg/ml BSA, 0.2 % Tween-20) and 30 µl of the serum's studied sample (rate of dilution: 1:4); the mixture has been incubated for 60 minutes at 37 °C. Upon the incubation the plate wells were washed thrice with 300 µl of PBST and then the solution of the conjugate of monoclonal antibodies to human IgM with horseradish peroxidase; the mixture has been incubated for 30 minutes at 37 °C. After the incubation the plate wells were washed 4 times with 300 µl of PBST and then substrate-chromogenic solution (3,3',5,5'-tetramethylbenzidine, 0.003 % H<sub>2</sub>O<sub>2</sub>, 0.15 M citric buffer, pH 5.0). After termination of enzymatic reaction, the optical density (OD) was measured in wells with the length of wave being 450 nm.

**Mathematical (statistical) methods**

Statistical processing of experimental data was conducted with the help of corresponding guidelines (Galkin et al., 2017a; Galkin et al., 2018), and also Microsoft Excel software.

Cut-off value (CoV) was calculated via formula (1)

$$CoV = OD_{av-} + 3\sigma, \tag{1}$$

where OD<sub>av-</sub> – values of average OD for negative samples, σ – mean-square deviation (dispersion) of OD values of negative serums.

Percentage of positiveness (PP) was calculated via formula (2)

$$PP = CoV / TD_{st.K+}, \tag{2}$$

where CoV – cut-off value, OD<sub>st.K+</sub> – OD of a certain positive standard.

Coefficient of variation (CV) of the results of analysis were calculated via formula (3)

$$CV = \frac{SD}{X_{av}} \times 100\% \tag{3}$$

where SD – standard deviation of certain value, X<sub>av</sub> – arithmetical average of certain value.

**RESULTS**

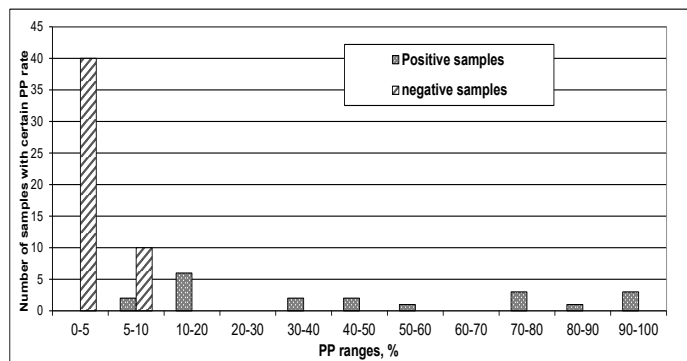
**Estimation of method's sensitivity and specificity**

As you know diagnostic specificity (DSp) characterizes the ability of method to define the particular component, for definition of which it was designed i.e. it characterizes the capacity of a certain method to register the minimum number of false-positive results. At the same time diagnostic sensitivity (DSe) is the parameter, characterizing the capacity of method to register the maximum number of real positive samples. In case of analysis for the presence of analytes of infection origins DSe reflects the percentage of infected people, who can be identified via application of this method (Sverstiuk, 2019). Estimation of DSe and DSp of immunoenzymic kits is conducted via usage of multiple serum sets: negative, positive low-titrated and serum-conversion ones. Such sets are manufactured as a commercial product and are used by many manufacturers of ELISA kits (e.g., SeraCare Life Sciences/Boston Biomedical Inc., USA, Scientific center of evaluation of medical products, Russia, Medico-Biological Union, Russia) or created by manufacturers independently for personal purposes (so called in-process serum sets). Taking into account that serum sets for evaluation of diagnostic quality of ELISA-kits for Chlamydia diagnostics are not widely used, we had to create our own PSS (20 positive and 50 negative samples).

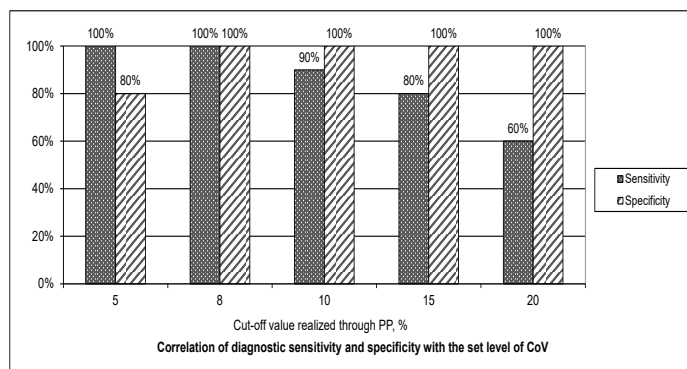
The key point in defining the DSe and DSp of quality (semiquantifying) analysis is the definition of the cut-off value. There are various methodical approaches to cut-off value's calculation. The most universal approach is addressed to application of formula (1), while at the same time other researchers define this parameter as the part (10...20%) of the ultimate result, acquired during testing of high-titrated serums (Sverstiuk et al., 2019; Parreno et al., 2010; Ederveen, 2010), using this parameter as the degree of positiveness (PP). Quantitative distribution of studied serums according to PP ranges is represented on Figure No.1 (the studies were conducted with the scientific-industrial kit 0016 during the 1<sup>st</sup> months after release). Cut-off value was calculated via formula (1) and was 0.146 absorbance units (a.u.), which corresponds to 8% of PP. The result of PSS serums' testing was evaluated depending on various cut-off values and thus calculating corresponding values of DSe and DSp (fig. 2). The data acquired prove that setting the cut-off value at 8% of PP provides the best values of both parameters of DSe and DSp (100%).

Analytical specificity (AS) was estimated through usage of 15 samples of PSS, which didn't contain IgA antibodies to Chlamydia agent: 5 serums, containing 2 classes of specific antibodies (IgA and IgG) and 15 samples, which were IgG-positive only. Those IgM-negative samples were used for diluting of positive serums (as the alternative to dilution solution) and investigated the coefficient of variation in scopes of one iteration of analysis (CV) during 4 repetitions for

different samples. The CV parameter was between 3% to 8%, which is quite sufficient and provides the grounds to state that the presence of antibodies of other classes in the studied samples doesn't effect the DSP (AS is acceptable).



**Figure 1** Distribution of studied serums of PSS according to ranges of positivity percentage

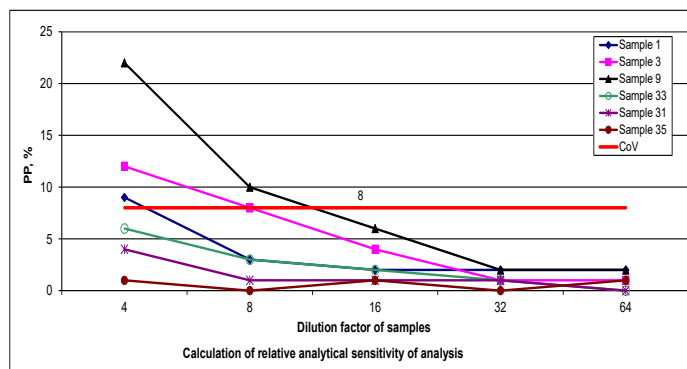


**Figure 2** Correlation of diagnostic sensitivity and specificity with the set level of CoV

Similar studies devoted to calculation of DSe, DSp and AS were conducted a year later with the set 0016 (during the expiration period) in order to confirm the stability. The following results were obtained: CoV was 0.146 optical units (8% of PP), DSe and DSp were 100%, during estimation of AS the CV didn't exceed 10%. Thus, validation characteristics during the expiration period remained on acceptable level.

**Estimation of detection value and linearity**

It's quite clear that it is impossible to set the absolute detection value (analytical sensitivity) for quality (semiquantifying) analysis, but we suggest to estimate this parameter through titrating of low-titrated positive serums. On Fig. 3 we can observe the results of titrating of 3 positive samples from PSS in comparison with similar study of 3 negative ones. One of three positive serums (sample 1) proved positive result only in dilution 1:4, the other (sample 3) proved the so called "grey zone" result in dilution 1:8 (PP±10%) and the 3 serum (sample 9) gave positive result in dilution 1:8.



**Figure 3** Calculation of relative analytical sensitive of analysis

Linearity of analysis was estimated through titrating of high-titrated serums of PSS, using the data acquired for obtaining of the equation of linear regression (fig. 4-5). Corresponding studies were conducted with the scientific-industrial set 0016 upon the 1<sup>st</sup> month of release. Slope factor of linear regression diagram for samples 15, 18 and 25 were the corresponding 1.592, 1.611 and 1.559, which is

acceptable for qualite analysis (Parreno et al., 2010; Ederveen, 2010; Schultheiss et al., 2009). Correlation ratio (r) between the theoretical and experimental values of OD was compared with the critical values for different levels of plausibility (Kannan et al., 2002). Obtained results (Table. 1) prove the acceptable level of correspondence of experimental data and OD results, calculated through the equation of linear regression for 3 studies samples of serum. The results of titrating of high-titrated serums and estimation of linearity during the expiration period of ELISA kit are presented in Table No.2 and confirm the preservation of acceptable level of studied characteristics after a year of ELISA kit's storage. We should mention that the correlation ratios of serums' OD values, obtained after release and in a year were in range of 0.999-1.000, which is the additional evidence of sufficient level of ELISA kit's stability.

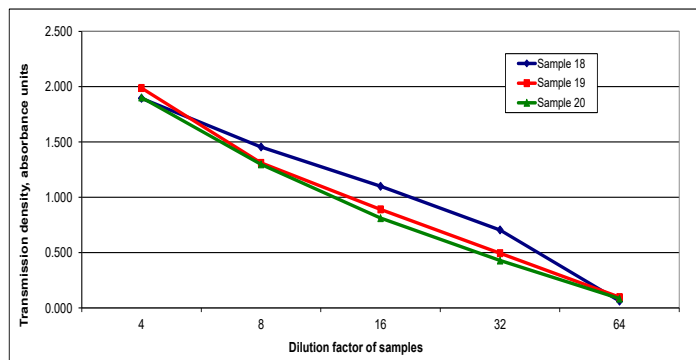


Figure 4 Calculation of analysis linearity through usage of high-titrated serums during the release period of ELISA kit

Table 1 Results of titrating of high-titrated serums and the estimation of linearity of ELISA kit (at the release)

Sample No.	Values of OD* for different dilutions of serums' samples					Equation of linear regression	Correlation ratio r and level of its plausibility
	1:4	1:8	1:16	1:32	1:64		
15	1.758/ 1.490	1.348/ 1.388	1.010/ 1.184	0.602/ 0.775	0.077/ -0.041	y = 1.592x-0.026	0.956 (p≤0.01)
19	1.977/ 1.502	1.303/ 1.392	0.855/ 1.173	0.475/ 0.734	0.047/ -0.143	y = 1.611x-0.027	0.896 (p≤0.05)
25	1.914/ 1.454	1.299/ 1.348	0.818/ 1.138	0.420/ 0.717	0.081/ -0.125	y = 1.559x-0.026	0.887 (p≤0.05)

Legend: The value of OD is presented in form experimental/theoretical. Arithmetical average values of experimental values of OD according to studies with 5 iterations are presented.

Table 2 Results of titrating of high-titrated serums and the estimation of linearity of ELISA kit (at the expiration period)

Sample No.	Values of OD* for various dilutions of serums' samples					Equation of linear regression	Correlation ratio r and it's plausibility rate
	1:4	1:8	1:16	1:32	1:64		
15	1.855/ 1.553	1.362/ 1.444	1.044/ 1.228	0.647/ 0.794	0.039/ -0.072	y = 1.683x-0.026	0.956 (p≤0.02)
19	1.970/ 1.474	1.288/ 1.368	0.804/ 1.155	0.465/ 0.728	0.074/ -0.124	y = 1.629x-0.027	0.882 (p≤0.05)
25	1.887/ 1.434	1.274/ 1.331	0.825/ 1.126	0.417/ 0.715	0.095/ -0.108	y = 1.507x-0.025	0.886 (p≤0.05)

Legend: The value of OD is presented in form experimental/theoretical. Arithmetical average values of experimental values of OD according to studies with 5 iterations are presented.

Determination of analysis' precision

As you know the precision may be considered on different levels i.e. intra-assay variation characterized the variations during making of several procedures of the analysis in the same conditions during a short period of time; inter assay precision takes into account the in-process variations; replicability characterizes the level of similarity of results in case of inter-laboratory experiments. In our study we estimated: intra-assay variation during studying of serums of positive and negative control in scopes of one analysis during 4 iterations on 4 ELISA-plates, realized through coefficient of variation CV<sub>intra</sub>; inter-assay variation during the studying of serums of positive and negative control in scopes of 3 analyses on different days by various operators and on different kits of the set, realized through the coefficient of variation CV<sub>inter</sub>. Results of corresponding

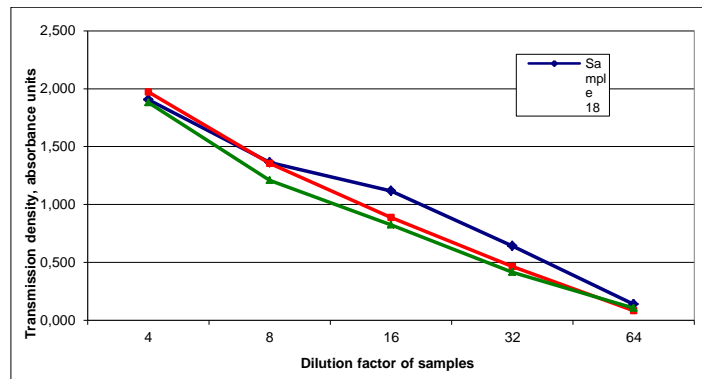


Figure 5 Calculation of analysis linearity through usage of high-titrated serums during the expiration period of ELISA kit

experiments are represented in tables No.3 and 4. The average intra-assay variation CV<sub>intra</sub> for experiments during release period was 4.7%, and during the expiration date – 4.75%. Intra-assay precision of analysis in case of application of different kits of the set varied from 1.5% to 8.4% (average CV<sub>inter</sub> =5.5%), and during the expiration period - from 2.4% to 7.8% (average CV<sub>inter</sub> = 4.6%). According to various guidelines different values of CV<sub>intra</sub> and CV<sub>inter</sub> are acceptable: certain authors suggest to define the rating of such parameters as CV ≤ 10% (Schultheiss et al., 2009), the others say that results are acceptable if these parameters don't exceed the value of 20% (Ederveen, 2010). The results of precision that we obtained confirm their acceptability during the release period and during the expiration period of ELISA kit.

Table 3 Results of ELISA's intra-assay variation study

Plate	OD in iterations	Average OD for a plate	Average OD for all plates	Standard deviation	CV <sub>intra</sub>
For positive control					
At the release					
1	1.214; 1.115; 1.114; 1.144	1.147	1.163	0.055	4.7%
2	1.220; 1.235; 1.114; 1.117	1.172			
3	1.132; 1.147; 1.158; 1.122	1.140			
4	1.288; 1.114; 1.157; 1.224	1.196			
At the expiration period					
1	1.125; 1.128; 1.205; 1.123	1.145	1.175	0.036	3.1%
2	1.205; 1.211; 1.118; 1.200	1.186			
3	1.204; 1.188; 1.158; 1.144	1.126			

4	1.220; 1.194; 1.174; 1.201	1.197			
For negative control					
At the release					
1	0.038; 0.034; 0.033; 0.035	0.035			
2	0.036; 0.034; 0.035; 0.037	0.036	0.036	0.002	4.7%
3	0.035; 0.038; 0.034; 0.035	0.036			
4	0.038; 0.035; 0.038; 0.037	0.037			
At the expiration period					
1	0.036; 0.037; 0.034; 0.032	0.035			
2	0.037; 0.034; 0.032; 0.036	0.035	0.035	0.002	6.4%
3	0.032; 0.035; 0.038; 0.034	0.035			
4	0.032; 0.037; 0.032; 0.037	0.035			

**Table 4** Results of ELISA intra-assay precision study

Analysis/ set series	OD in iterations	Average OD for series	Standard deviation	CV <sub>inter</sub>	Average value of CV <sub>inter</sub>
For positive control					
At the release					
1 / 0116	1.121; 1.119; 1.118; 1.222	1.145	0.051	4.5%	4.4%
2 / 0216	1.135; 1.299; 1.252; 1.121	1.202	0.087	7.3%	
3 / 0316	1.252; 1.291; 1.292; 1.288	1.281	0.019	1.5%	
At the expiration period					
1 / 0116	1.124; 1.122; 1.129; 1.221	1.149	0.048	4.2%	3.7%
2 / 0216	1.171; 1.204; 1.254; 1.124	1.188	0.055	4.6%	
3 / 0316	1.284; 1.222; 1.220; 1.235	1.240	0.030	2.4%	
For negative control					
At the release					
1 / 0116	0.038; 0.036; 0.031; 0.035	0.035	0.003	8.4%	6.6%
2 / 0216	0.036; 0.032; 0.037; 0.032	0.034	0.003	7.7%	
3 / 0316	0.039; 0.040; 0.042; 0.042	0.041	0.002	3.7%	
At the expiration period					
1 / 0116	0.037; 0.039; 0.040; 0.037	0.038	0.002	3.9%	5.5%
2 / 0216	0.037; 0.035; 0.034; 0.033	0.035	0.002	4.9%	
3 / 0316	0.040; 0.039; 0.040; 0.046	0.041	0.003	7.8%	

**DISCUSSION**

Validation of ELISA for quality (semiquantifying) detection of IgG antibodies *Ch. trachomatis* began with the estimation of sensitivity and specificity of analysis. The following study was preceded by formation of in-process panel of serums, the samples which contained and didn't contain specific IgM antibodies to Chlamydia agent. Literature data (Parreno et al., 2010; Schares et al., 2011; Galkin et al., 2013) confirm the possibility of application of two completely different approaches to calculation of OD cut-off value (Cut-off), which is actually a distinguishing point during calculation of result for studied samples. The first envisages the direct calculation basing on the average OD value of negative samples and their dispersion, and the second is in calculating this parameter as a certain part of the maximum result, obtained from high-titrated serums (with the application of such parameter as positivity percentage). The evaluation of plausibility of the methods may depend on additional information concerning the correlation of average OD values for positive and negative serums, and also results of testing of serums from standard serum panels in the designed ELISA kit. In our opinion, the realization of cut-off value though the positivity percentage (i.e. basing on the maximal OD value) is less adequate, but in conditions of lack of standard (characterized) control materials it may be acceptable; such approach is quite widespread (Parreno et al., 2010; Nizeyimana et al., 2013; Souza et al., 2012; Galkin et al., 2017b, Komar et al., 2019). In our study in case taking the cut-off value at 8% from OD of high-titrated serum, the diagnostic sensitivity and specificity were 100%. This result is sufficient, while usually this parameter is from 10% to 20% for ELISA kits (Parreno et al., 2010). The study of analytical specificity was conducted via estimation of CV levels during testing of samples, which didn't contain specific IgM antibodies, but contained other classed of Ig to *Ch. trachomatis*. The values of coefficient of variation didn't exceed 8%, which is acceptable (Parreno et al., 2010; Marynchenko et al., 2019; Umanets et al., 2018) and confirms that absence of influence of other classes of antibodies on detection in the designed ELISA.

Values of detection and linearity for quality (semiquantifying) analysis are impossible to calculate, but they can be estimated in comparison during analysis via titrating of low-titrated positive serums. The results of serums' titrating (1:4 – 1:64) allowed to calculate the slope factor of linear regression diagram: the obtained data during release period of ELISA kit and after 1 year of storage at 4 °C (1.473-1.698), which were quite acceptable for quality analysis (Golembiovska et al., 2019; Parreno et al., 2010; Ederveen, 2010; Nikolaenko et al., 2007). For estimation of analysis precision, the calculations of intra-assay variation (CV<sub>intra</sub>) and inter-assay variation (CV<sub>inter</sub>) were performed. These parameters varied in range from 2.5% to 9.6%, which is acceptable, as according to various guidelines (Parreno et al., 2010; Schultheiss et al., 2009; Kannan et al., 2002) CV cannot exceed 10% or 20%. Thus, the experiments performed

proved high quality of the designed ELISA-kit for quality (semiquantifying) detection of IgG antibodies to *Ch. trachomatis*. It should be mentioned that the suggested method of validation of quantity and quality (semiquantifying) ELISA may be used in ELISA kit for other purposes.

**CONCLUSIONS**

A scientific and methodical verification of validation procedure for *in vitro* serum diagnostics was conducted on the basis of ELISA kit for quality (semiquantifying) definition of specific *Ch. trachomatis* IgM antibodies. Validation characteristics were defined both at the beginning of the release of diagnostic set and the expiration date (as the element of defining of stability). The values of diagnostic sensitivity and specificity, defined via the intra-manufacturing set of serums (20 positive and 50 negative) comprised 100%. The presence of other classes of Chlamydia antibodies, IgG and IgA, in samples didn't influence the results of ELISA for specific IgM antibodies. Linearity of method is sufficient of qualitative assay. The intra-assay variation of results is between 3.1% and 6.4%, and intra-assay precision was from 1.5% to 8.4%, staying acceptable (≤ 10%) both at the moment of release and during expiration date. The ELISA method is validated with sufficient results and the diagnostic set is regarded as stable during 1 year.

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