A PROTOCOL OF DEVELOPMENT OF A SCREENING ASSAY FOR EVALUATING IMMUNOLOGICAL MEMORY TO VACCINE-PREVENTABLE INFECTIONS: SIMULTANEOUS DETECTION OF ANTIBODIES TO MEASLES, MUMPS, RUBELLA AND HEPATITIS B

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Multiplex screening assays for measuring antibodies to vaccine-preventable infections are routinely used in large-scale seroepidemiological studies, but not commercially available, because such studies are too specific and normally employ a particular type of the assay only once. This prompts researchers to develop their own solutions for exploring herd immunity. In this work we discuss theoretical principles and practical approaches to developing multiplex screening assays and give examples of protocols and recommendations based on our own experience in the field.

Keywords: immunological memory, humoral immunity, vaccine-preventable infections, measles, mumps, rubella, hepatitis B, serologic diagnosis, multiplex immunoassay

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ПРОТОКОЛ РАЗРАБОТКИ СКРИНИНГОВОЙ ТЕСТ-СИСТЕМЫ ДЛЯ ОЦЕНКИ ИММУНОЛОГИЧЕСКОЙ ПАМЯТИ К ВАКЦИНОУПРАВЛЯЕМЫМ ИНФЕКЦИЯМ: ОДНОВРЕМЕННОЕ ОПРЕДЕЛЕНИЕ ТИТРА АНТИТЕЛ К КОРИ, ПАРОТИТУ, КРАСНУХЕ И ГЕПАТИТУ В

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Скрининговые мультиплексные тест-системы для оценки уровня содержания антител к вакциноуправляемым инфекциям активно используются в масштабных сероэпидемиологических исследованиях. Между тем, они отсутствуют в виде коммерчески доступных продуктов, т. к. задачи подобных исследований достаточно узкоспецифичны и ограничены разовым применением таких наборов. В результате исследователи должны самостоятельно разрабатывать и внедрять мультиплексные тест-системы при изучении иммунологической памяти населения. В работе обсуждаются теоретические и практические основы разработки мультиплексных скрининговых тест-систем, приводятся протоколы и рекомендации, основанные на практическом опыте авторов.

Ключевые слова: иммунологическая память, гуморальный иммунитет, вакциноуправляемые инфекции, корь, паротит, краснуха, гепатит В, серодиагностика, мультиплексный иммунный анализ

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Excellent performance characteristics are crucial for screening assays used in large-scale seroepidemiologic studies. Such assays are designed to screen for antibodies against various vaccine-preventable infections and customized for equipment employed for multiplex suspension analysis. They are cost-effective and time-saving and require smaller sample volumes. However, there are no commercially available multiplex screening assays on the market, ready for use in large-scale seroepidemiologic research normally conducted by laboratories and involving dozens of thousands of samples. There are no

standard lists of antibodies to be measured by such assays, which disrupts their commercial potential, driving labs to develop their own in-house multiplex kits. In turn, this task requires a high level of expertise: lab personnel are expected to master special techniques of screening assay development and validation.

In this work we describe a protocol for the development of a multiplex immunoassay (MIA) to measure antibodies against a few vaccine-preventable infections, namely measles, mumps, rubella and hepatitis B (Fig. 1). We discuss possible problems associated with each stage of the process and offer solutions. The proposed protocol is used in the Laboratory of Translational Biomedicine of Gamaleya National Research Center for Epidemiology and Microbiology, Moscow. We provide detailed description for each step and use the actual data. We hope that this work will be a good guide for our colleagues from other laboratories in the development of multiplex solution-phase multiplex assays, aiding research on herd immunity to vaccine-preventable infections in Russia.

I. Design of the experiment

Protocols for designing and performing the indirect serological bead-based immunoassay were developed based on the recommendations of Luminex (USA), the manufacturer of MagPlex™-C microspheres and the MAGPIX analyzer [1], literature analysis [2–10], including a number of immunoassay manuals [11–13] and validation guides [11, 14–16], and our own experience.

Our method for antibody detection in blood samples relies on the use of capture antigens (AGs) covalently bound to the surface of magnetic beads and detection goat anti-human IgG antibodies conjugated to phycoerythrin. This method has a broader dynamic range, compared to ELISA, and allows simultaneous detection of up to 50 different analytes, if signal detection is performed on MAGPIX. The diagram of the method is shown in Fig. 1.

The schematic of the experiment is shown in Fig. 2.

Our multiplex immunoassay is intended for simultaneous detection of IgG against measles, rubella, mumps and hepatitis B (anti-HBs) in human blood serum. Sample preparation takes 1 hour, including incubations; another 10 minutes are allocated to reagents preparation and washing. With a 96-well plate the analysis takes about 40 min. The whole procedure lasts for less than 2 hours.

II. Preparation of monoplexes

1. Materials

The following components are needed to prepare a monoplex:

– antigens of those pathogens the samples will be tested for (our assay will be used to detect IgG antibodies to measles, mumps, rubella and hepatitis B);

– 4 different regions of magnetic carboxylated microspheres (beads) by MagPlexTM-C;

– serum panels previously characterized using any assay certified in Russia;

– detection antibodies, i.e. PE-conjugated goat anti-human IgG (One Lambda, Thermo Fisher Scientific, USA).

The antigens were selected based on the purpose underlying multiplex assay development. Ultimately, we aimed to estimate population immunity to vaccine-preventable infections. Vaccines against measles, rubella and mumps, registered in Russia, are live vaccines, therefore the priority was given to native antigens. We also used recombinant antigens. Vaccines against hepatitis B contain recombinant surface antigen virus (HBsAg) of the hepatitis B virus of ayw or adw genotypes, therefore, the latter were selected as the 4th component of the multiplex (Table 1).

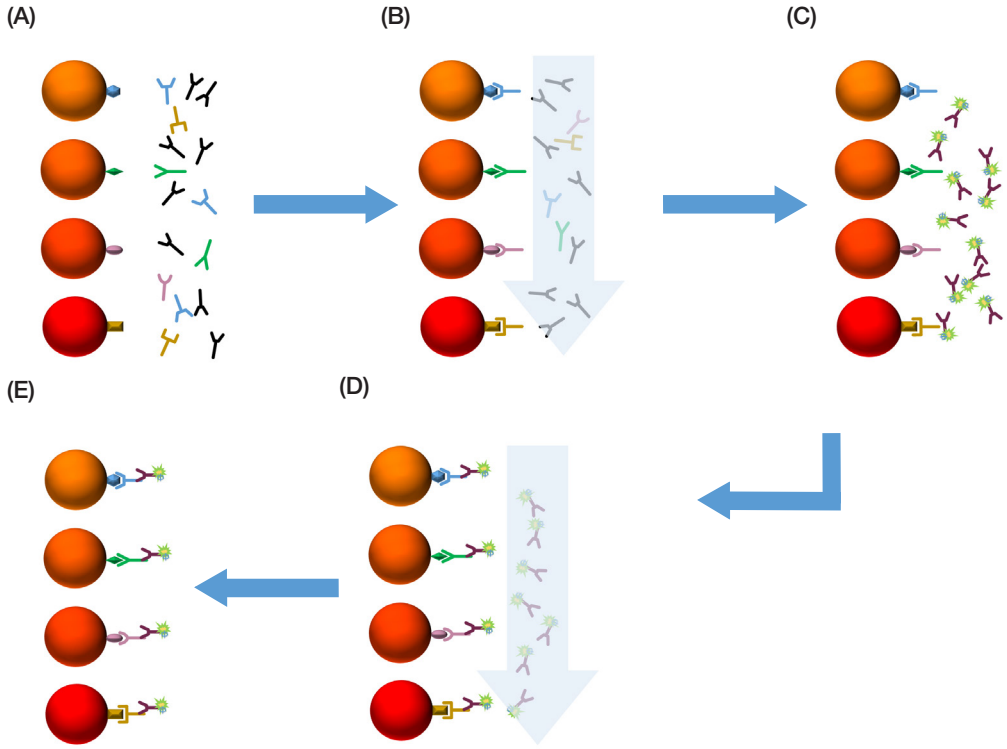

Fig. 1. Schematic representation of indirect serologic multiplex immunoassay, showing 4 bead regions, each bead coupled to a capture antigen. **(A)** Serum is added to the bead suspension; the first incubation is carried out. **(B)** Wash 1: unbound serum components are removed. **(C)** Phycoerythrin-conjugated anti-human detection antibodies are added to the coupled beads and the second incubation takes place. **(D)** Wash 2: unbound components (conjugate) are removed. **(E)** Beads are analyzed on the MAGPIX workstation

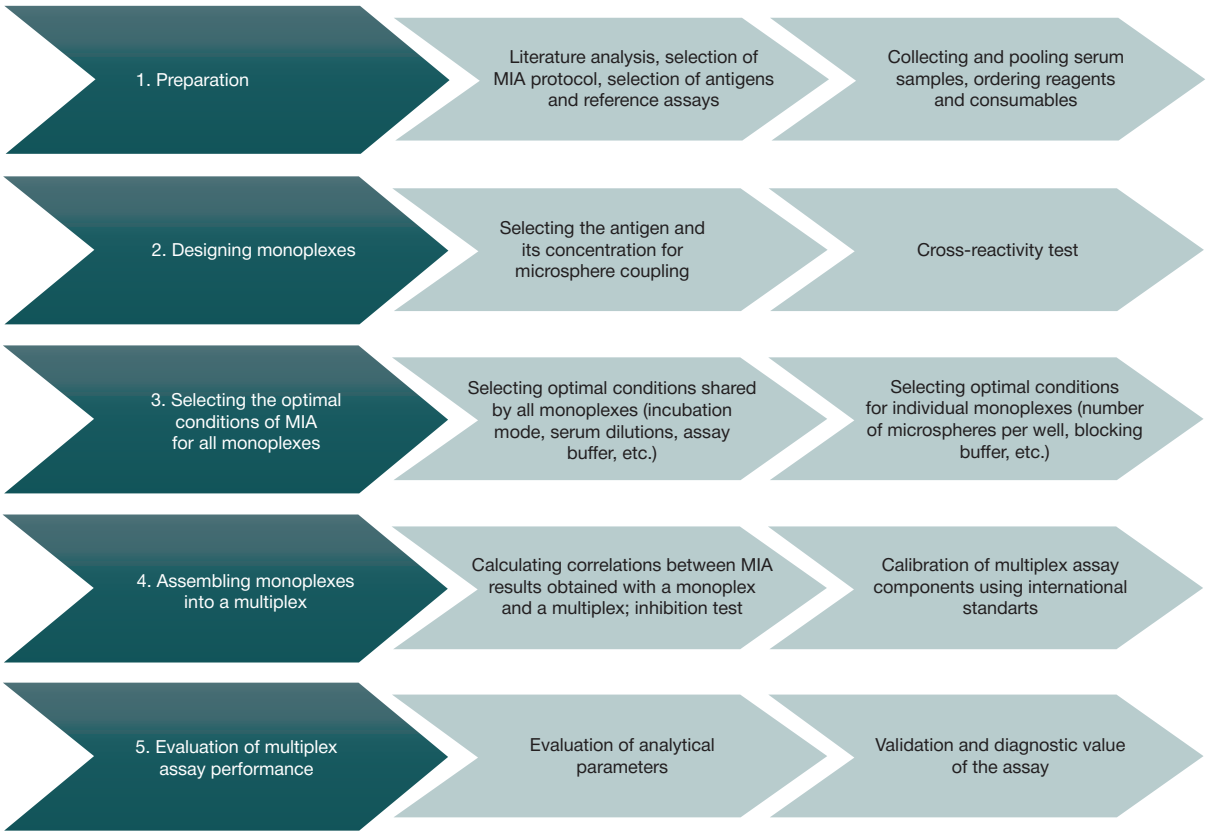

Fig. 2. Diagram showing steps of assay development

For simultaneous detection of IgG against 4 pathogens in a single reaction, we needed a set of 4 corresponding bead regions (MagPlex™-C microspheres). These microscopic beads (6.5 μm in diameter) are the basis of xMAP technology. They are color-coded with 2 to 3 fluorescent dyes, which allows generating up to 500 different regions and, therefore, designing multiplexes for measuring up to 500 analytes in one sample. Magnetic properties of the beads make the protocol simpler: using a magnetic rack for a polystyrene 96-well plate, one can separate the beads from the suspension contained in a well by mere shaking; the microspheres with the captured analyte will stay in the well pelleted by the magnet.

In the course of our experiment we prepared monoplex suspensions of beads conjugated with antigens and also evaluated the resulting multiplex using test samples of human serum. The serum panel must include the samples characterized by a well-established certified method. We used 98 serum samples run through ELISA-based assays (Vector-Best, Russia) and analyzed on automated workstations, including BioPlex (for rubella and measles; Bio-Rad, USA), Architect (for rubella and hepatitis B, Abbott, USA), Liason (for mumps; DiaSorin, Italy) and Immulite (for rubella; Siemens, Germany). To prevent damage to serum samples and to avoid multiple freeze-thaw cycles, all samples were diluted 1 : 2 in preautoclaved 100 % glycerol and stored at –20 ºC.

Microsphere/antigen/IgG complexes were fluorescently visualized using PE-conjugated goat anti-human IgG (detection antibodies), which selectively interacts with human IgG heavy chains.

To measure IgG concentrations in the studied serum samples, expressed in IU/ml, we constructed a calibration curve. Supply of international standards (IS) containing known quantities of immunoglobulins against a particular pathogen is limited; therefore, we used IS for calibration of serum-based secondary standards (SS). For our experiment we ordered WHO International Standards from the collection of the National Institute for Biological Standards and Control (NIBSC, UK) (Table 2). SS were used as an internal standard for the multiplex and as a reference calibration standard for each assay [11]. Because there are no international standards for mumps, we used calibration samples from a commercial ELISA kit. Immunoassay kits should contain human serum for quality control. We used controls from the NIBSC collection (Table 2).

2. Microsphere coupling – obtaining monoplexes

We used two different protocols to couple microspheres to the selected antigens. Both are based on carbodiimide chemistry, but one is a modification of the other. A two-step covalent coupling protocol was used in the case of the native rubella and mumps antigens and the recombinant measles antigen. This method is recommended if the activation molecule is more than 10 kDa in size. Otherwise, as is the case with recombinant HBsAg, the microspheres are modified with a chemical spacer (adipic acid dihydrazide, ADH) in the course of a single-step carmodiimide reaction. In our experiment, following microsphere modification, the capture antigens were coupled by a carbodiimide reaction to the spacer seated on the microsphere surface. It was necessary to “lift” the small peptide over the rough surface of the bead and to facilitate the reaction between the antigen and the analyte.

2.1 Coupling of 10^6 microspheres by a 2-step carbodiimide reaction

All solutions were prepared in distilled water (dH_2O) using the following reagents:

• NaH_2PO_4•H_2O (0.1 M NaH_2PO_4, pH 6.2) for the activation buffer;
• MES hydrate (50 mM MES, pH 5.0) for the binding buffer;
• phosphate buffered saline with 0.02 % Tween-20, 0.1 % BSA, 0.05 % NaN_3, pH 7.4 (below referred to as PBS-TBN) — the wash buffer;
• 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulfosuccinimide (s-NHS), 50 mg/ml of each reagent. Prepared immediately before use.

Equipment:
• magnetic rack for 1.5 ml Eppendorf tubes;
• ultrasonic bath;
• centrifuge/vortex mixer;
• lab rotator;
• automated cell counter or Goryaev chamber and microscope;
• low-bind 1.5 ml Eppendorf tubes.

Protocol for a two-step carbodiimide conjugation:
– preparation of microspheres
1. mix the flask with the microspheres by rotation for 2 min at 20 rpm;
2. transfer the required amount of microspheres to a clean tube; bring the volume up to 250 µL with dH_2O;
3. vortex/sonicate for 20 s, put the tube in the magnetic rack (below referred to as the magnet). Leave it to sit for 1 min, remove the supernatant;
– microspheres activation:
4. add 80 µL of the activation buffer to the tube, vortex for 20 s;
5. add 10 µL of EDC and s-NHS each, vortex for 20 s;
6. leave it to seat for 20 min, mix by vortexing for 20 s every 10 min;
– wash 1:
7. leave the tube on the magnet for 1 min, remove the supernatant;
8. add 250 µL of the binding buffer to the tube;
9. vortex/sonicate for 20 s, leave on the magnet for 1 min, remove supernatant;
10. repeat steps 8 and 9;
– bead coupling:
11. add to the tube 100 µL of the coupling buffer; vortex/sonicate for 20 s;
12. add the antigen (Table 3);
13. bring the solution volume up to 500 µL by adding the binding buffer;
14. place the tube on the rotator for 120 min at 20 rpm, protected from the light;
– wash 2:
15. leave the tube on the magnet for 1 min; remove the supernatant;
16. add to the tube 1000 µL of the wash buffer;
17. vortex/sonicate for 20 s; magnet — 1 min; remove the supernatant;
18. repeat steps 16 and 17 twice;
– finishing the procedure:
19. resuspend the microspheres in 1000 µL of the storage buffer (PBS-TBN or any other blocking buffer, selected experimentally);

Table 1. Antigen candidates for the assay

Infection	Type	Trade name	Manufacturer
Hepatitis B	Recombinant	Hepatitis B virus, subtype ayw	Bialexa, Russia
		Hepatitis B virus, subtype adw	
Rubella	Recombinant	Rubella virus E1 protein	Bialexa, Russia
		Rubella virus E2 protein	
		Rubella virus C protein	
	Native	Rubella K1S grade antigen	Microbix Biosystems, Canada
		Rubella K2S grade antigen	
		Rubella virus grade 2 antigen	Jena Bioscience, Germany
Measles	Recombinant	Measles virus nucleocapsid protein NCP	Kapel Biotech Company, Russia
	Native	Measles grade 2 antigen	Microbix Biosystems, Canada
		Measles virus antigen (Premium)	Jena Bioscience, Germany
		Measles virus antigen	
Mumps	Recombinant	Mumps virus nucleocapsid protein NCP	Kapel Biotech Company, Russia
	Native	Mumps grade 2 antigen	Microbix Biosystems, Canada
		Mumps/Parotitis virus antigen	Jena Bioscience, Germany

Table 2. Standards and serum controls used in the development of the multiplex assay

Infection	Standard/control name	Concentration or number of antibodies	Source
Hepatitis B	WHO International Standard Second International Standard for anti-hepatitis B surface antigen (anti-HBs) immunoglobulin, human	100 IU in a vial	NIBSC
	Anti-Hepatitis B Surface Antigen Quality Control Serum 1	219.0±16 IU/ml	NIBSC
Rubella	WHO International Standard Anti Rubella Immunoglobulin, Human	1600 IU in a vial	NIBSC
	Anti-Rubella Quality Control Reagent Sample 1	25.5±2,9 IU/ml	NIBSC
Measles	WHO International Standard 3rd International Standard for Anti-Measles	3 IU in a vial	NIBSC
	Anti-Measles Quality Control Reagent Sample 1	754.6 mIU/ml	NIBSC
Mumps	Anti-Mumps Quality Control Reagent Sample 1	728.6 EU/ml	NIBSC

20. measure bead concentrations in the cell counter or Goryaev chamber. Calculate the required amount of beads for MIA, accordingly;

21. store the coupled beads in the dark at +4 ºC for 16 h until further analysis (performed on the next day).

*2.2 Coupling of 1.5*10^6 microspheres by two one-step carbodiimide reactions using a spacer*

All necessary solutions were prepared in dH_2O using the following reagents:

- MES hydrate for the binding buffer (0.1 M MES, pH 6.0);
- MES hydrate for the wash buffer 1 (0.1 M MES, pH 4.5);
- phosphate buffered saline with 0.02 % Tween-20, 0.1 % BSA, 0.05 % NaN_3, pH 7.4 (below referred to as PBS-TBN) — the wash buffer 2;
- 100 mg/ml ADH (spacer) solution in the binding buffer;
- 100 mg/ml EDC solution in dH_2O. For (7) and (19) the solutions need to be prepared separately.

Equipment:

- magnetic rack for 1.5 ml Eppendorf tubes;
- ultrasonic bath;
- centrifuge/vortex mixer;
- lab rotator;
- automated cell counter of Goryaev chamber and microscope;
- low-bind 1.5 ml Eppendorf tubes.

Protocol for two one-step carbodiimide conjugations:

– preparation of microspheres:

1. mix the flask with microspheres by rotation for 2 min at 20 rpm;

2. transfer the required amount of microspheres to a clean tube, bring the volume up to 250 µL with dH_2O;

3. vortex/sonicate for 20 s, put the plate on the magnet; leave it to sit for 1 min, remove the supernatant.

4. add 500 µL of the binding buffer to the tube;

5. vortex/sonicate for 20 s; leave on the magnet for 1 min; remove the supernatant;

– coupling a spacer to beads:

6. add to the tube 30 µL of ADH (1mg per 500,000 beads);

7. add to the tube 30 µL of EDC (1mg per 500,000 beads);

8. bring the volume up to 500 µL with the binding buffer;

9. place the tube on the rotator for 60 min at 20 rpm, protected from the light;

– wash 1:

10. magnet – 1 min; remove the supernatant;

11. add 500 µL of the wash buffer 1 to the tube;

12. magnet – 1 min; remove the supernatant;

13. repeat steps 11 and 12 two more times;

– finishing spacer coupling:

14. resuspend microspheres in 1,000 µL of the wash buffer 1;

15. measure bead concentrations in the cell counter or Goryaev chamber. Calculate the required amount of beads for MIA, accordingly;

– coupling antigens with spacer on beads:

16. vortex/sonicate for 20 s; place on the magnet for 1 min; remove the supernatant;

17. add 100 µL of the binding buffer; vortex/sonicate for 20 s;

18. add the required amount of the peptide (Table 3);

19. add 30 µL of EDC (1 mg per 500,000 microbeads);

20. bring the volume up to 500 µL with the binding buffer;

21. mix by rotation for 120 min at 20 rpm, protected from the light;

– wash 2:

22. place the tube on the magnet. Leave for 1 min; remove the supernatant;

23. add 1,000 µL of the wash buffer 2;

24. vortex/sonicate for 20 s; magnet — 1 min; remove the supernatant;

25. repeat steps 23 and 24 two more times;

– finishing the procedure:

26. resuspend the microspheres in 1,000 µL of the storage buffer (PBS-TBN or any other blocking buffer, selected experimentally). For the hepatitis B monoplex we used PBS-TBN;

27. measure bead concentrations in the cell counter or Goryaev chamber. Calculate the required amount of beads for MIA, accordingly;

28. store the coupled beads in the dark at +4 ºC for 16 h for further analysis (performed on the next day).

3. Protocol for multiplexed indirect (serological) immunoassay

Before validating the performance of the multiplex, it is important to evaluate each monoplex component separately and optimize conditions for all monoplexes. This procedure is referred to as *Selecting the optimal conditions of MIA for all monoplexes* in Fig. 2. Evaluation is based on the analysis of serum samples using the following protocol.

Materials:

- a 96-well polystyrene plate (below referred to as the plate);
- 0.5 ml, 1.5 ml and 2 ml test tubes;
- a set of serum samples previously tested for the presence of target IgG;
- suspensions of ready-for-use microspheres conjugated with antigens;
- a 100X stock solution of phycoerythrin-conjugated detection antibodies;
- PBS-TBN.

Equipment:

- plate magnet (Magnetic Plate Separator, Luminex);
- centrifuge/vortex mixer;
- thermoshaker;
- ultrasonic bath;
- MAGPIX analyzer (Luminex).

The protocol:

1. preheat the thermoshaker to +37 ºC and switch on MAGPIX;

2. in microtubes, prepare 1 : 50 dilutions of serum samples using PBS-TBN;

3. prepare a 50 µL suspension of microspheres (2,500 beads per well/reaction):

- vortex/sonicate for 20 s,
- collect the required volume of the suspension to a new tube; adjust the transferred volume with PBS-TBN;

4. add 50 µL of the suspension and 50 µL of the diluted serum to each plate well. The final serum dilution will be 1 : 100;

5. place the plate in the thermoshaker and incubate for 30 min at +37 ºC by mixing at 800 rpm;

6. while the samples are incubating, prepare a solution of detection antibodies. Take the required volume of the stock antibody solution and dilute it 1 : 100 with PBS-TBN (you will need 50 µL of the final solution per well; final antibodies concentration should be 2.5 µg/ml per well);

7. remove the plate from the thermoshaker and leave it on the magnet for 2 min; then separate the liquid without removing the plate from the magnet;

8. wash the samples with PBS-TBN: add 100 µL PBS-TBN to each well using the multichannel pipette, mix in the

thermoshaker for 30 s, place the plate on the magnet for 2 min, remove the liquid, wash again;

9. resuspend the microspheres in 50 µL PBS-TBN per well;

10. add 50 µL of the prepared solution of detection antibodies to each well (see step 6);

11. second incubation: incubate for 30 min at +37 ºC: place the plate in the thermoshaker and mix at 800 rpm for 30 min;

12. wash with PBS-TBN (see step 8);

13. after washing, add 100 µL PBS-TBN to each well, mix in the thermoshaker for 30 s;

14. load the plate into MAGPIX and run the analysis.

Importantly, such parameters as temperature, incubation time, serum dilution, or a concentration of detection antibodies are variable, and these variations can affect fluorescence intensity (expressed in MFI units). Adjusting these parameters so that they would be equally beneficial for the performance of each monoplex is what optimization is about.

4. Optimization of monoplexes

The importance of the optimization step in the development of a multiplex assay cannot be overrated. Optimization is a search for the best conditions for each monoplex ensuring that the following requirements are met:

- low MFI values for the negative control (for example, rabbit serum or human serum free from immunoglobulins);
- the widest possible range of signal intensities in the serum samples with and without the studied antibodies;
- the results obtained using monoplexes are expected to be consistent with the results obtained using reference ELISA.

Optimization covers a wide range of parameters, including temperature and incubation time, serum dilution and a solution for serum dilution, or concentrations of detection antibodies, affecting the performance of all 4 monoplex components. But a few other factors also need to be optimized specific for each monoplex, such as a blocking buffer, antigen concentrations for coupling, and the number of beads in the monoplex per well. These parameters were accounted for, and the optimal conditions were selected for all monoplex components of the assay (Table 3).

Because some of the selected antigens can be recombinant (obtained through expression in *Escherichia coli*), target antigen preparations may be contaminated with the antigens of this bacterium. Antibodies to *E. coli* that are likely to be present in the studied serum sample may produce a false positive signal affecting the total MFI. To avoid this effect, we added the *E. coli* lysate (3 %) to the serum dilution buffer to bind *E. coli*-specific immunoglobulins [5].

Optimization also includes evaluation of cross-reactivity for each monoplex; the earlier it is performed, the better. Cross-reactivity tests show how specific is, for example, the monoplex with the measles virus antigen for IgG antibodies to the measles virus and whether it interacts with antibodies to other infections.

The cross-reactivity test is performed according to the protocol described above under the conditions presented in Table 3. Thus, the monoplex component of the assay containing beads conjugated to the measles antigen is tested using serum without IgG antibodies to measles but with IgG antibodies to other pathogens. Other monoplexes are tested in a similar manner. If MFI value is at the limit of detection (LOD), the monoplex is not cross-reactive; otherwise the capture antigen needs to be replaced.

At this stage of our experiment, we tested the optimized monoplexes designed to detect IgG antibodies to measles, rubella and mumps using a panel of 70 serum samples. Conditions are shown in Table 3. The results for MIA are presented in Fig. 3–5, expressed in MFI, because calibration was yet to be implemented, and the assay could not yet be used for quantification. ELISA kits (Vecto-Rubella-IgG, Vecto-Measles-IgG, Vecto-Mumps-IgG by Vector-Best) were used as reference.

The rubella monoplex successfully differentiated between serum samples with and without IgG antibodies to rubella; the results were consistent with those obtained by ELISA (Fig. 3). However, measles and mumps monoplexes were found to require further optimization, because they generated high MFI for serum samples that did not contain IgG antibodies (the values were close to or above the grey zone values, see Fig. 4, 5).

Clearly, without calibration we cannot compare our monoplexes and ELISA, but we still can estimate the performance of each monoplex and decide whether further optimization is required and the monoplex is ready for calibration.

Table 3. Optimal conditions for the developed immunoassay

Parameter		Monoplex			
Virus		Measles	Mumps	Rubella	Hepatitis B
Antigen	Name	Measles virus nucleocapsid protein NCP	Mumps/Parotitis virus antigen (native)	Rubella K2S grade antigen (native)	Recombinant HbsAg, subtypes ayw and adw
	Concentration, µg per million beads	5	10	10	20 (10 HbsAg ayw + 10 HbsAg adw)
PBS-TBN		PBS-TBN			
Serum dilution, dilution buffer		1 : 100, PBS-TBN + *E. coli* lysate			
Concentration of detection antibodies		2.5 µg /ml			
Incubation		• first incubation — 30 min, second incubation — 30 min • incubation temperature — +37 °C • mixing by rotation at 800 rpm			
Number of beads per well for each monoplex		2 500			

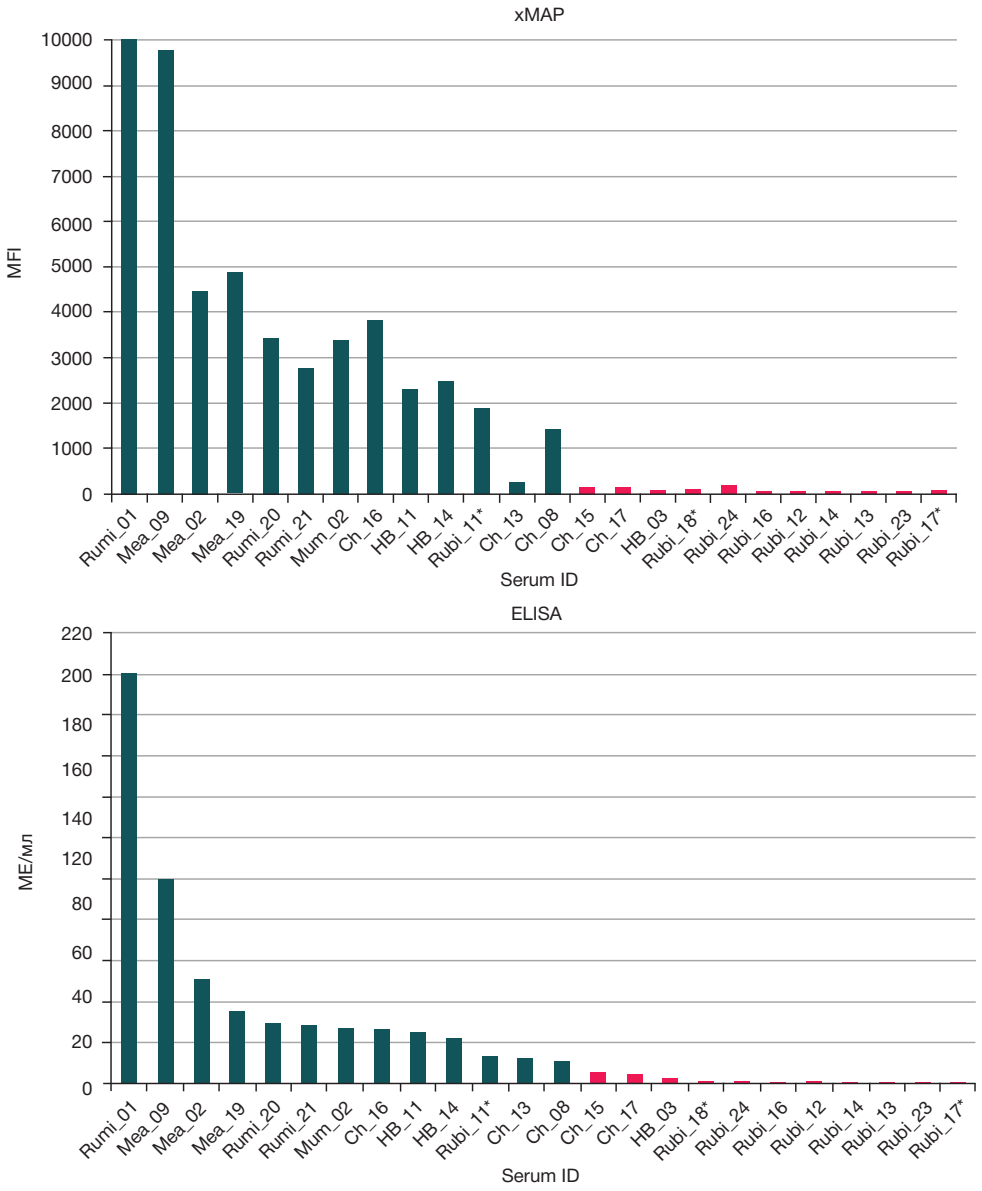

Fig. 3. Levels of IgG to rubella in human serum measured by xMAP and ELISA

Pooled serum samples were pretested in the reactions with monoplexes in a series of fourfold dilutions from 1 : 100 to 1 : 409,600. The resulting calibration curves were used to estimate whether serum pools could be used as secondary standards (Fig. 6).

III. From monoplex to multiplex

After each monoplex has been optimized, pretested using the serum panel and results have been compared to ELISA, the protocol is selected; in the next step monoplexes are combined into a multiplex and the results obtained using the multiplex and its monoplex components are compared. These tests are run in parallel using the serum panel [4]. Based on the results, correlations are established. At the moment, we are comparing qualitative characteristics of the multiplex for the detection of IgG antibodies to measles, rubella, mumps and hepatitis B and its monoplex components. Below we briefly describe the validation procedure for the multiplex.

When talking about optimization, we mentioned specificity of the assay. It should be evaluated in the optimization step (see 3 in Fig. 2). However, at this stage specificity and cross-reactivity should not be confused. Specificity is evaluated using homologous and heterologous inhibition of serum samples by antigens [3, 7–11]. For the inhibition test, a serum sample is needed with a known high concentration of IgG antibodies to all 4 pathogens. The sample is divided into 4 aliquots; each aliquot is incubated with one of 4 antigens. After preincubation, the multiplex is added to the serum.

The control (non-preincubated serum) is also included in the reaction. Homologous inhibition demonstrates specificity of a monoplex component of the multiplex assay for the homologous antigen, while heterologous inhibition indicates a nonspecific reaction between a monoplex and the heterologous antigen. Thresholds for homologous and heterologous inhibitions are 80–120 % and < 30 %, respectively. If the assay has passed the specificity test, standardization should be performed next, i.e. calibration using international standards (Table 2). First, serum samples should be selected with high concentrations of IgG antibodies against each pathogen; the samples are then pooled into a positive pool (which could be later used as the secondary standard). Then titration is performed of the international standard and the serum pool in a single run using a fourfold series of dilutions starting from 1 : 100 [11],

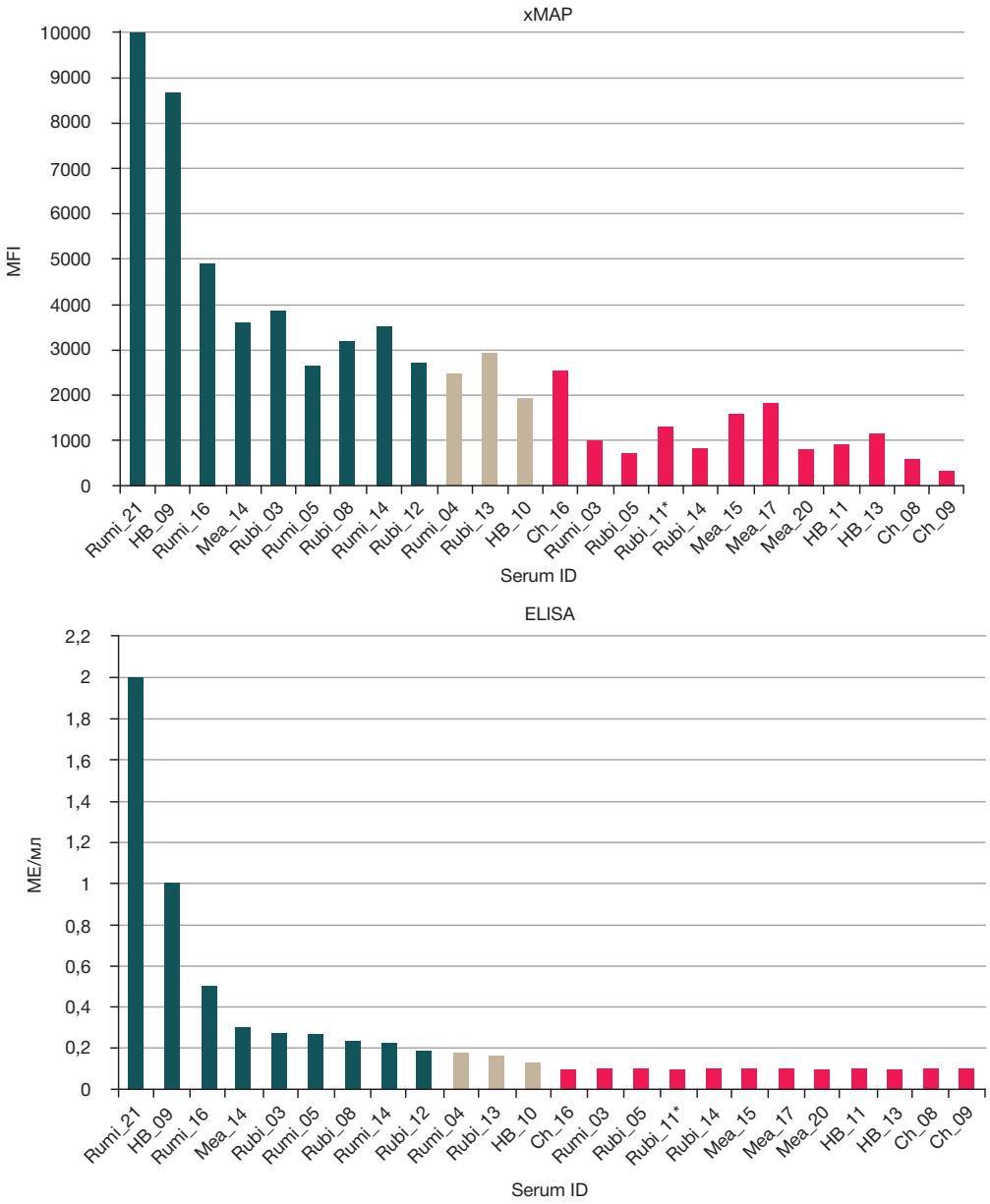

Fig. 4. Levels of IgG to measles in human serum measured by xMAP and ELISA

in each of 4 monoplexes according to the protocol, under the selected conditions. Based on the results, calibration curves are constructed using the predetermined values of international standards (expressed in IU/ml) and the values of the same standards obtained in the course of the experiment expressed in MFI for each monoplex. Calibration curves are used to determine concentrations of each analyte in the positive serum pool; then titration curves are constructed and conclusions are made whether this pool can be used as the secondary standard or it should be diluted.

Standardization of the multiplex is carried out following the same procedure (IS and SS titration in parallel, in the multiplex suspension). Multiplex calibration curves are compared with those for monoplexes, obtained in the previous step, and correlations are drawn. Then IS and SS titration curves are compared, and the decision is made whether the SS titration curve can be used as a calibration curve for the multiplex.

Then the optimal mathematical model is selected to accurately describe the obtained calibration curve and to calculate antibodies concentrations (MIA) expressed in international units.

IV. Analytical parameters, validation and diagnostic value of a screening assay

Validation is a series of experiments performed to reliably assess analytical parameters of the assay using a certified analytical method. Validation aims to test the feasibility of the developed assay and determine its limitations that may be critical for routine use. The assay has a lot of analytical parameters; some of them are analyzed and improved at the start of the experiment (for example, cross-reactivity). Any experiment involving serum samples with known analyte concentrations (detected by a certified technique) is, in its essence, a part of the validation process. It is impossible to say when the development of the assay ends and validation begins.

Below we describe basic analytical characteristics of the assay and propose a validation plan.

Sensitivity is in the broad sense of this word an ability to detect an analyte. It can be expressed as a minimal concentration of this analyte reliably detected by the assay. Analytical sensitivity is evaluated by running the zero sample without the analyte through the assay; the mean value of the results plus 2 to 3

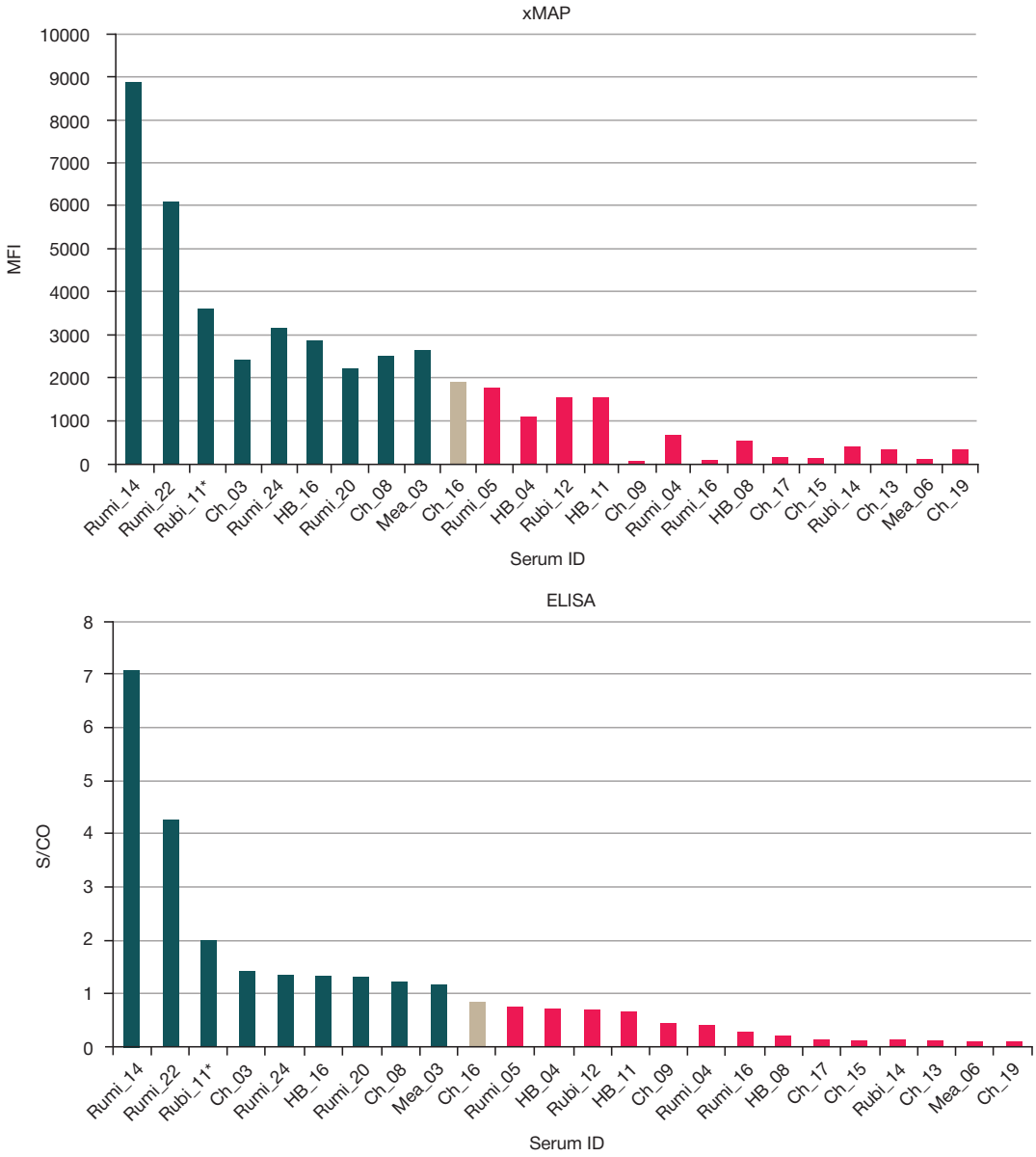

Fig. 5. Levels of IgG to mumps in human serum measured by xMAP and ELISA

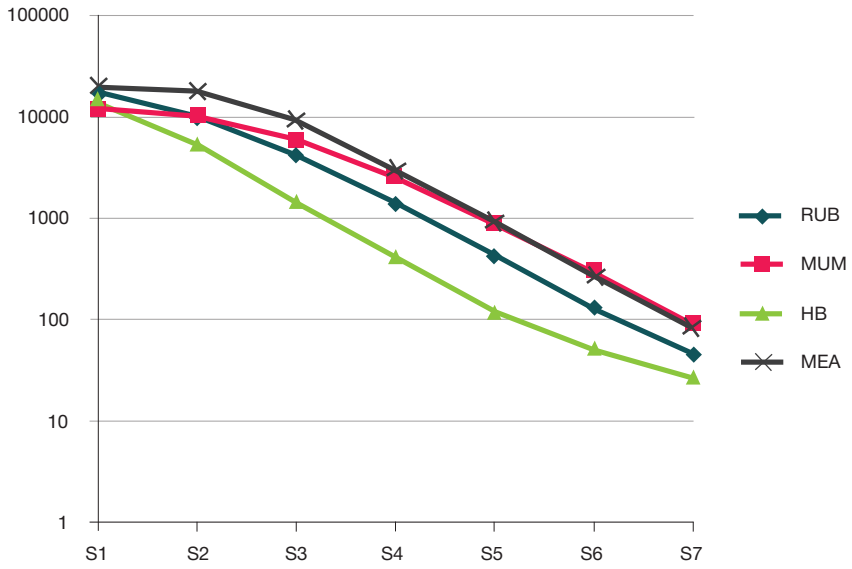

Fig. 6. Curves for serum pools dilutions with high antibody titers to rubella (RUB), measles (MEA), mumps (MUM) and hepatitis B (HB)

standard deviations are the analytical sensitivity. Another term for it is limit of detection (LOD). In other words, it is a minimal concentration of the analyte reliably detected (but no necessarily quantitatively) by the studied assay. ULOQ and LLOQ are upper and lower limits of quantification, the maximal and minimal analyte quantities that can be detected at certain accuracy and reproducibility values. Another type of sensitivity is functional sensitivity- a minimal analyte concentration detected in a series of runs with the coefficient of variance below a certain threshold (10–20 %).

Precision is the variability of the results obtained in a series of experiments with the same sample run through the assay under identical conditions; it is expressed as a coefficient of variance (CV, %). There are different types of precision. Within-run precision (intra-assay variation) should be < 10 %. Between-run precision (inter-assay variation) is expected to be < 20 %. Other types of precision include variations between reagents, equipment and even researchers. Reproducibility refers to the precision of the results obtained with the same technique but it different laboratories.

Specificity is an ability of the antibody to react with only one particular antigen (one of the antigen/antibody pair is the studied analyte). Cross-reactivity describes the extent of antibody ability to react with other substances besides the analyte/antigen.

Accuracy describes how consistent is the result obtained using the studied assay with the actual value of the measured parameter. Accuracy is crucial for all quantitative assays. It is determined by running a sample with a known quantity of the analyte through the assay. The ratio of the anticipated value to the measured one shows the degree of recovery.

Linearity is an ability of the assay to produce results directly proportional to the analyte concentration in the sample. To estimate linearity, a series of standard dilutions of the analyte is prepared, at concentrations ranging from 50 % to 130 % by diluting the original solution. The concentrations are then measured using the studied method. Based on the results the calibration curve is constructed representing the linear dependence between the calculated concentrations and the original concentrations (in normalized coordinates). To estimate linearity, r^2 correlation coefficient, the slops and the y-intercept are calculated.

The assay range is an interval between the maximal (ULOQ) and the minimal (LLoQ) analyte concentrations in the analyzed sample, for which the studied method has an acceptable level of precision, accuracy and linearity.

Apart from analytical characteristics, there are a few diagnostic parameters used to estimate the diagnostic value of an assay. They are analyzed in the course of assay development and compared to the results obtained using the reference method. Those include diagnostic sensitivity (frequency of analyte detection in the samples in comparison with the reference method; expressed in %); diagnostic specificity (frequency of the negative result, i.e. the absence of the analyte in the sample previously tested by a reference method; expressed in %), and some other.

All the parameters mentioned above can be assessed during the validation step. Fig. 7 shows a diagram representing a single assay run that can yield data on almost all assay parameters. Rows 1–3 and 10–12 of the plate contain calibrators in the standard dilution buffer (PBS-TBN). Eight dilutions of calibration samples are obtained in 6 replicates. These samples will provide information about the calibration curve, intra-assay variation, and the upper and lower limits of detection. Wells marked by B (blank wells) contain only the diluent (PBS-TBN) without the analyte. The mean value for these 12 wells will be used to determine the background signal, the limit of detection. Rows 4–6 C–H contain samples with the analyte diluted in the standard dilution buffer (PBS-TBN) in 3 replicates (S+B = sample+buffer). Rows 7–9 C-H contain samples with the analyte diluted in the original matrix (rabbit serum or human serum free of antibodies) (S+M = sample+matrix). The obtained data can be used to estimate accuracy, recovery from the diluent, linearity and intra-assay variation. To evaluate inter-assay variation, a few similar runs should be performed. Specificity is assessed by running cross-reactivity and inhibition tests described above.

Upon validation we plan to determine the diagnostic value of the assay and compare it to that of a commercial ELISA. We plan to analyze at least 400 serum samples: 100 per pathogen.

CONCLUSION

The xMAP technology has been recognized as a rapid, sensitive and accurate method for indirect serologic immunoassay. Multiplex immunoassays have demonstrated their efficiency in serological studies of herd immunity against vaccine-

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	B	B	B	B	B	B	S1	S1	S1
B	S2	S2	S2	B	B	B	B	B	B	S2	S2	S2
C	S3	S3	S3	S+B (1)	S+B (1)	S+B (1)	S+M (1)	S+M (1)	S+M (1)	S3	S3	S3
D	S4	S4	S4	S+B (2)	S+B (2)	S+B (2)	S+M (2)	S+M (2)	S+M (2)	S4	S4	S4
E	S5	S5	S5	S+B (3)	S+B (3)	S+B (3)	S+M (3)	S+M (3)	S+M (3)	S5	S5	S5
F	S6	S6	S6	S+B (4)	S+B (4)	S+B (4)	S+M (4)	S+M (4)	S+M (4)	S6	S6	S6
G	S7	S7	S7	S+B (5)	S+B (5)	S+B (5)	S+M (5)	S+M (5)	S+M (5)	S7	S7	S7
H	S8	S8	S8	S+B (6)	S+B (6)	S+B (6)	S+M (6)	S+M (6)	S+M (6)	S8	S8	S8

Fig. 7. Diagram illustrating the validation process of the studied assay. S1–8 — wells used to analyze calibrators and construct the calibration curve. B — wells containing a standard diluent; S+B — samples with the analyte diluted in a standard dilution buffer; S+M — samples with the analyte diluted in the matrix (rabbit serum or human serum free of antibodies)

preventable infections. They can simultaneously detect a number of different analytes, are fast and have excellent analytical characteristics. We hope that our tetraplex will aid large-scale seroepidemiological studies of herd immunity against vaccine-preventable measles, rubella, mumps and hepatitis B. The proposed protocol can be used for the development of other assays aimed to measure antibody titers against other vaccine-preventable and significant pathogens.

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