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Annals of Pediatrics & Child Health

Research Article

Performance of Multiplex Detection Method of IgM Class Antibodies against *Toxoplasma gondii*, Rubella and Human Cytomegalovirus

Rainara Moreno Sanches de Almeida[1]*, Guilherme Maerschner Ogawa[2], Paulo Rogério Siqueira Custódio[1], Maria Alcantara Socorro Sampaio[2], Newton Soares da Silva[3], Cristina Pacheco-Soares[1]

[1]*Instituto de Pesquisa e Desenvolvimento (IP&D), Universidade do Vale do Paraíba (UNIVAP), Brasil.*
[2]*Intercientífica, Parque Tecnológico UNIVAP, Brasil*
[3]*Instituto de Ciência e Tecnologia, Universidade Estadual Paulista (UNESP), Brasil*

***Corresponding author**

Rainara Moreno Sanches de Almeida, Instituto de Pesquisa e Desenvolvimento (IP&D), Universidade do Vale do Paraíba, Av. Shishima Hifumi 2911, São José dos Campos, São Paulo, Brasil, Tel: 55(12)988036744

Submitted: 24 November 2022

Accepted: 19 December 2022

Published: 23 December 2022

ISSN: 2373-9312

Keywords

- Neonatal screening
- Multiplex assay
- Magnetic beads
- Analytical validation

Abstract

Serological diagnosis during neonatal screening is crucial in disease prevention. Among the infectious diseases, the most common are toxoplasmosis, rubella, and cytomegalovirus. Traditional diagnostic methods are used to detect a single infectious agent per test. The use of multiplex detection methods increases productivity and reduces the amount of material used, resulting in a more efficient test from a technical, environmental, and economic point of view. The study's objective was to evaluate the performance of a new diagnostic method aimed at neonatal screening using the multiplex platform of magnetic microspheres from the company Luminex Corporation. For this, tests were carried out for analytical validation of the diagnostic product developed following the rules of the National Health Surveillance Agency (ANVISA) of Brazil. The parameters evaluated were repeatability, reproducibility, linearity, robustness, high dose, minimum detection limit, and analytical specificity. All data obtained met the acceptance criteria of RDC 166/17 of 2017 for the use of the diagnostic product in the national territory. Repeatability and reproducibility tests showed a CV of less than 15% between replicates of the same operator and different operators. The kit showed linearity throughout the operating range with R2 above 0.990, and no effect of high-efficiency dose was observed in the chosen working dilution. In addition, the kit did not show interference from the matrix with the results, and it was observed that small and deliberate changes in the incubation time of each reagent did not have a significant effect on the data obtained.

ABBREVIATIONS

ANVISA: National Health Surveillance Agency; RDC: Resolution of the collegiate board; CV: Variation Constance; TOX: Toxoplasmosis; RUB: Rubella; CMV: Cytomegalovirus.

INTRODUCTION

The detection of antibodies against infectious diseases is an activity widely used in several situations. Among the available laboratory detection techniques, the widely used method is the Enzyme-Linked Immunosorbent Assay (ELISA) which detects a single infectious agent by an assay based on antigen-antibody binding [1].

Although commonly used, traditional methods of serological diagnoses, such as ELISA, have the disadvantage of requiring more significant amounts of samples and time for analysis since each analyte of interest requires a specific assay.

New technologies were developed to optimize diagnostic methods, among which stands out is the xMAP® technology from Luminex Corporation, which allows the simultaneous analysis of several parameters in the same biological sample using microspheres [2]. Neonatal screening is necessary to prevent severe infections from affecting the fetus and neonate.

Several infectious agents could be vertically transmitted from mother to fetus via the transplacental route or direct contact during childbirth. TORCH's acronym represents the most common infections, including toxoplasmosis, rubella, and cytomegalovirus [3,4].

A multiplex kit was developed for detecting antibodies against infectious diseases using xMAP® technology of magnetic microspheres, aiming to meet the need to develop effective diagnostic methods for neonatal screening. The kit is intended to detect multiple and simultaneous anti-toxoplasmosis, anti-

rubella, and anti-human cytomegalovirus IgM antibodies in newborn samples.

Developing a new diagnostic product using microspheres demands studies on the performance and other parameters that an in vitro diagnostic kit must present to be used in Brazil. The National Health Surveillance Agency (ANVISA) created and evaluated these performance parameters.

For the validation and registration of the in vitro diagnostic kit and its entry into the national market, different studies are required following the rules of the Resolution of the Collegiate Board (RDC) No. 166 of July 24, 2017, published by ANVISA, which establishes the criteria for validation of analytical methods.

The objective of the present study was to evaluate the performance following the analytical validation criteria of a new diagnostic method for multiple and simultaneous detections of human antibodies of the IgM class against *Toxoplasma gondii* (TOX), rubella (RUB) and cytomegalovirus (CMV) in the field of newborn screening.

MATERIALS AND METHODS

Performance analysis

For the use of new diagnostic methods developed in Brazil, the product must meet ANVISA's analytical methods validation criteria. RDC No. 166 of July 24, 2017, applies to analytical methods used in pharmaceutical supplies, medicines, and biological products in all their production stages. Some validation parameters involve precision (repeatability and reproducibility), Analytical Specificity, High Dose, Linearity, Limit of Detection (LOD), and Robustness [5]. All tests carried out in the present study were based on the acceptance criteria for registering a new diagnostic product in Brazil and its use in the national territory.

Characterization and validation of clinical samples used.

Reference samples purchased from Seracare Life Science were used for the three parameters. These samples are Seracare's antibodies panel: antibody anti-*T. gondii* antibody panel (AccuSet™ Toxoplasmosis Performance Panel, catalog number 0820- 0321, lot 10344475), antibody IgM carrier sample Rubella (Seracare Rubella IgM positive plasma, catalog number DS-674 -M, lot 9254456), and antibodies carrying IgM anti-CMV (Seracare CMV IgM Positive plasma, catalog number DS-626-M, lot BM217341), all samples were supplied with a certificate of analysis and reference results from other methodologies.

Coupling of antigens to magnetic microspheres

Couplings were performed following the protocol provided by Luminex Corporation. (xMAP® Cookbook 5th Edition). First, the xMAP® magnetic microsphere solutions were added in different low-adhesion USA tubes (catalog 1415-2600). Microspheres 12 (MC10012), 45 (MC10045), and 72 (MC10072) were used for *Toxoplasma gondii*, Rubella, and Cytomegalovirus antigens, respectively. The microspheres were washed with NaH2PO4 (activation buffer) and incubated with EDC and Sulfo NHS for 20 minutes, protected from light, and on rotation in a tube shaker at 700 rpm. Then the microspheres were washed with PBS-TBN and incubated with the respective antigens for 2h. After incubation, the microspheres were washed and kept in rotation for 20 minutes with block buffer (PBS/1%BSA). Then the microspheres were centrifuged, resuspended in PBS-TBN buffer, and stored under refrigeration.

For reasons of commercial secrecy, the antigens and concentrations used cannot be made available. All couplings make up reagent 2 of the NeoMAP® 3plex IgM kit from Intercientífica, developed in this study, with registration number 80173700020 at ANVISA.

Assay protocol

First, the samples were eluted in 1.5 ml tubes with 200 µL of Elution Reagent (PBS, Tween20, 20% Azide, BSA, and *E. coli* extract) at a dilution of 1:200 for 60 minutes under agitation at 700rpm. After incubation, the assay plate was prepared by adding 25 µL of the pool of magnetic microspheres coupled to Toxoplasmosis, Rubella, and Cytomegalovirus antigens in each well. Then a washing step was performed by adding 50 µL of Washing Solution (PBS, Tween20, and 20% Azide). Next, 50 µL of the eluate was added to the wells and incubated for 90 minutes under agitation at 700 rpm, protected from light. After incubation, two washing steps were performed, and 50 µL of Anti-human IgM solution labeled with Biotin was added for 30 minutes under agitation on the plate shaker and protected from light. After the incubation, a new washing step was performed, and 50 µL of the Phycoerythrin solution conjugated with Streptavidin was added and maintained under agitation for 30 minutes in the plate shaker at 700 rpm. Then the solution was removed, 50 µL of Washing Solution was added and kept for 1 minute in the plate shaker, taken for reading in the Magpix equipment with analysis in the xPONENT® Software. For reasons of commercial secrecy, the antigens and concentrations used cannot be made available. As well as the Anti-Human IgM and Phycoerythrin conjugated with Streptavidin catalogs. All reagents make up the NeoMAP® 3plex IgM kit developed in this study and with registration 80173700020 at ANVISA.

Analysis of results

In all tests, the constant of variation (CV) between replicates of each sample was verified. For this, the formula below was used (**Figure 1).** In addition, GraphPad Prisma and Action Stat software were used for the statistical analysis of the data.

Measurement accuracy

The repeatability tests were carried out in the Research and Development laboratory of the company Intercientifica, and the reproducibility tests were carried out in the Quality Control laboratory in another sector of the same company, where all the equipment and the operator were different. The assays were carried out with nine high, medium, and low reactivity samples in triplicate and on other days.

$$CV = \left(\frac{Standard\ deviation}{Average}\right) X\ 100$$

Figure 1 Formula for constant of variation (CV).

Minimum Limit of Detection (LOD)

The LOD Limit of Detection must be demonstrated by obtaining the lowest amount of the analyte present in a sample that can be detected but not necessarily quantified under established experimental conditions. For this, 73 measurements of the blank were performed, with the blank consisting of all reagents, except for the sample. Statistical analysis was performed using the Action Stat software, which presented the minimum detection values for each parameter in the multiplex assay.

Analytical Specificity

Specificity was assessed by the method's ability to unambiguously identify or quantify the analyte of interest in the presence of components that may be present in the sample, such as matrix components. The test protocol was carried out using the blood components after separating the plasma in triplicate to verify if matrix interference occurs because it is in the plasma that the antibodies are found. Data analysis was performed using the formula below (figure 2), which considers the results in Mean Fluorescence Intensity (MFI) and the blank.

Linearity

The linearity of the method was verified through tests with highly positive samples for each parameter in serial dilution 1:2 with 16 drops. After the trial, linear regression analysis was performed on each sample, evaluating the regression coefficient (R2). The defined performance target was R2 greater than 0.99 according to ANVISA's acceptance criteria.

High Dose

The high dose prozone effect was verified using highly reactive samples for the three antigens. The test was carried out with samples in serial dilution 1:2 with eight drops starting with the dilution of the samples in 1:10.

Robustness

To verify whether small, deliberate changes in some steps cause changes in the reactivity of the samples. Tests were carried out with 10 minutes more and 10 minutes less than the standard incubation time of each step of the test protocol: sample elution, incubation with the microspheres, incubation with Anti-human IgM, and incubation with phycoerythrin. The acceptance criterion was a CV of less than 20% between samples with different incubation times compared to the reference (original time of each stage).

RESULTS

Measurement Accuracy

The repeatability test (Table 1), showed a CV of less than 15% in all samples, demonstrating no significant difference between triplicates for all analyzed parameters.

Although the acceptance criterion used was 15%, it is common for low reactivity samples to present a relative standard deviation above the recommended one due to the intrinsic variability of the method resulting from the high dynamic range (0-100,000MFI).

$$Analytical\ Specificity = MFI - Background$$

Figure 2 Formula used for analytical sensitivity analysis.

In Table 2, it is possible to observe CV less than 15% in all parameters when comparing the tests performed by two operators in two different laboratories.

Limit of Detection (LOD)

In all parameters, there was low reactivity in the blank, indicating no undesired reactivity of the elution and washing buffer with the microspheres coupled to the different antigens (Table 3).

Analytical specificity

Table 4 demonstrates that the reactivity of blood components in the absence of antibodies is below the detection limits of all parameters, indicating no significant reactivity of these components.

Table 1: Repeatability analysis.

TOX						
Sample	**Well 1**	**Well 2**	**Well 3**	**M**	**SD**	**CV%**
1	25737	27455	27450	27450	809	3
2	22508	25824	26491	25824	1742	7
3	8076	8626	8785	8626	304	3
4	3331	3929	4326	3929	409	10
5	2737	3514	3644	3513	400	11
6	3476	2967	3163	3163	210	7
7	370	376	319	370	26	7
8	346	336	324	336	9	3
9	287	203	254	254	34	13
RUB						
Sample	**Well 1**	**Well 2**	**Well 3**	**M**	**SD**	**CV%**
1	28094	30167	30621	30167	1100	4
2	26494	26115	25568	26115	380	1
3	20084	22046	21360	21360	813	5
4	15956	14871	15745	15745	470	3
5	10074	10350	9518	10074	346	3
6	6011	5815	5762	5815	107	2
7	3171	3113	2856	3113	137	4
8	1584	1542	1122	1542	209	13
9	773	732	668	732	43	6
CMV						
Sample	**Well 1**	**Well 2**	**Well 3**	**M**	**SD**	**CV%**
1	14934	15109	14604	14934	210	1
2	12386	12066	12469	12386	174	1
3	11111	12947	12580	12580	793	6
4	7422	7575	7471	7471	64	1
5	7546	8394	8094	8094	351	4
6	4402	4812	4066	4402	305	7
7	1236	1156	1151	1156	39	3
8	896	892	869	891	11	1
9	579	569	580	579	5	1

Abbreviations: M: medium; SD: Standard deviation; CV: Constance of variation.

Table 2: Reproducibility Analysis.

TOX										
Sample	**Well 1**	**Well 2**	**Well 3**	**M lab 2**	**SD**	**CV (%)**	**M lab 1**	**M Lab 1 vs. Lab 2**	**SD**	**CV (%)**
1	22495	23908	22571	22571	649	3	27450	25010	2440	10
2	22222	23608	24019	23608	769	3	25824	24716	1108	4
3	8021	7750	7951	7951	115	1	8626	8288	338	4
4	4158	4277	4107	4158	71	2	3929	4043	114	3
5	2761	2775	2446	2761	152	5	3514	3137	377	12
6	2616	2536	2326	2536	122	5	3163	2849	314	11
7	639	646	672	647	14	2	724	685	39	6
8	323	302	325	323	11	3	370	347	24	7
9	352	380	308	352	30	8	336	344	8	2
RUB										
Sample	**Well 1**	**Well 2**	**Well 3**	**M lab 2**	**SD**	**CV (%)**	**M lab 1**	**M Lab 1 vs. Lab 2**	**SD**	**CV (%)**
1	33277	36123	32433	33277	1579	5	30167	31722	1555	5
2	28718	30542	29543	29543	746	3	26115	27829	1714	6
3	24138	24113	23756	24113	174	1	21360	22736	1376	6
4	18332	16409	18236	18236	885	5	15745	16990	1245	7
5	12423	12187	12073	12187	146	1	10074	11130	1056	9
6	7573	6998	7270	7270	235	3	5815	6543	727	11
7	3844	3891	3921	3891	32	1	3113	3502	389	11
8	1868	1852	1545	1852	148	8	1542	1697	155	9
9	934	868	828	868	44	5	732	800	68	8
	CMV									
Sample	**Well 1**	**Well 2**	**Well 3**	**M lab 2**	**SD**	**CV (%)**	**M lab 1**	**M Lab 1 vs. Lab 2**	**SD**	**CV (%)**
1	15154	14815	14775	14815	170	1	12386	13601	1214	9
2	13316	16048	16051	16048	1289	8	12580	14314	1734	12
3	12101	12565	12182	12182	203	2	11259	11720	462	4
4	6373	6591	6306	6373	122	2	7471	6922	549	8
5	9202	8711	8484	8711	300	3	8094	8402	308	4
6	5041	4784	4372	4784	275	6	4402	4593	191	4
7	3385	3243	3306	3306	58	2	2771	3038	268	9
8	1188	1071	1115	1115	48	4	1156	1135	20	2
9	510	526	479	510	19	4	579	544	34	6

Abbreviations: Results from labs 1 and 2 with median, standard deviation, and CV of nine samples analyzed with the NeoMAP® 3plex IgM kit (M- Median, SD- standard CV – Constant Variation, Op- operator, Lab- laboratory).

Table 3: Limit of Detection in MFI of the NeoMAP® 3plex IgM Kit.

Limit of Detection (LOD)			
	TOX	**RUB**	**CMV**
Average	36	49	41
Standard deviation	5	9	4
Degrees of liberty	71	71	71
Detection limit	44	65	47

High Dose

In the figure below (Figure 3), it is possible to see that in the TOX parameter, a high dose effect occurs from the dilution of 1/40, causing a decrease in reactivity even with a higher concentration of antibodies. The RUB parameter occurs from the 1/160 dilution, and the CMV parameter from the 1/20 dilution.

Linearity

The response range is linear and occurs between 15 - 10,000 MFI with R2 and 0.99 correlation for TOX, 170 - 3,000 MFI with R2, 0.99 correlation for RUB, and 10 - 6,000 MFI with R2 and 0 correlation, 99 for CMV. At MFI values above or below these ranges, the test cannot correlate MFI with the concentration unit of the samples.

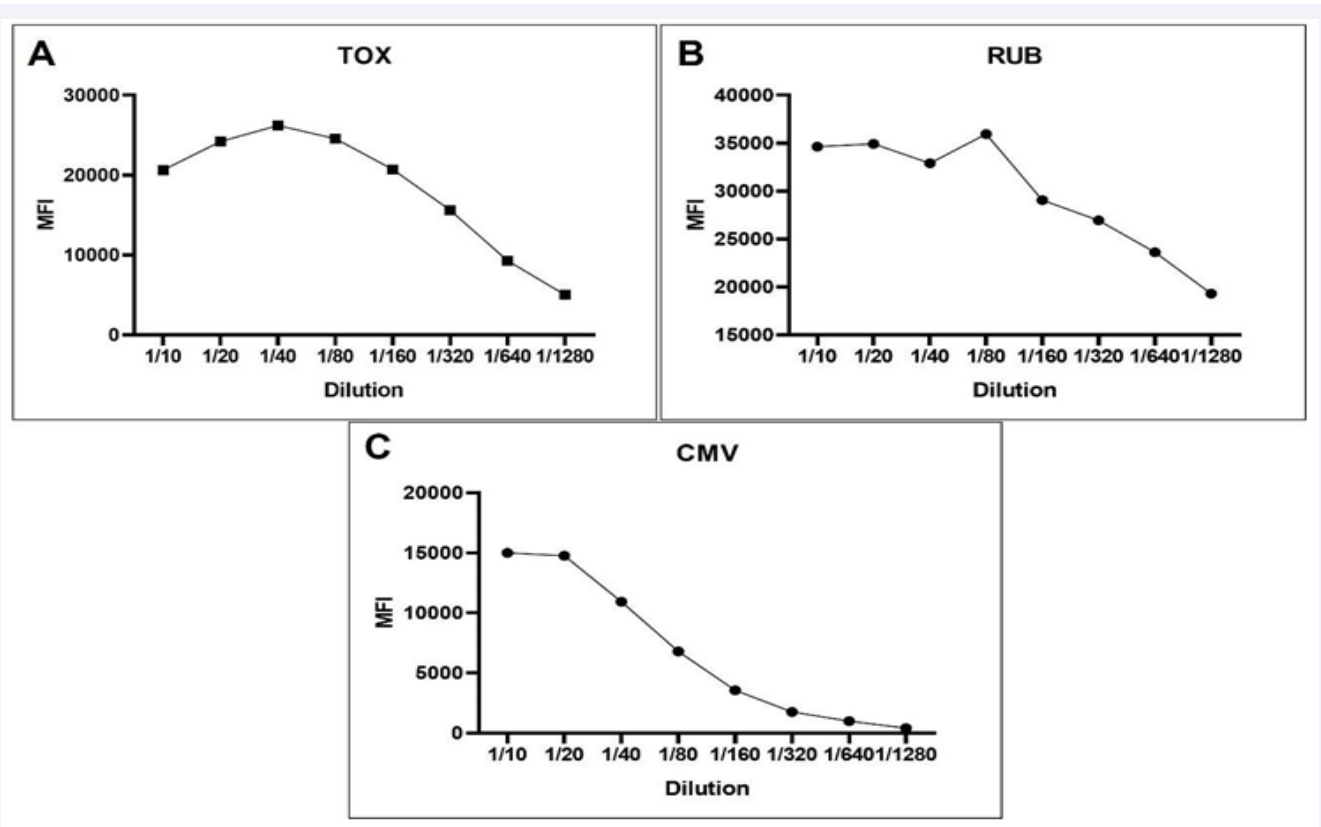

Figure 3 Reactivity curve of the samples in relation to the dilution factor in the parameters of TOX, RUB, and CMV. (A) TOX, (B) RUB and (C) CMV.

Robustness

Robustness studies indicate that variations of ten minutes plus or minus in incubations do not result in significant differences in the reactivity of samples with an overall CV of less than 15%. The results are in the tables below (Tables 5, 6, and 7).

DISCUSSION

Since the introduction of neonatal screening, several technological advances have been used for the early detection of congenital conditions. Implementing new methodologies allows for a significant increase in the number of diseases screened, with the capacity to include up to fifty disorders [5].

Recent technological advances have developed platforms capable of multiplexing multiple molecular and immunological assays for high-throughput screening. It is a modern platform whose main advantages are saving time and reagents [5].

Among the new technologies, the multiplex platform of magnetic microspheres from the company Luminex Corporation allows the development of assay methods with up to 100 different analytes in the same well of the assay plate [6].

Analytes must be coupled to these microspheres to perform target detection. The coupling is carried out by the chemical interaction of the carboxyl groups on the microspheres' surfaces with the proteins' primary amines. These bonds are covalent and most often performed with antibodies or specific antigens [8,9].

In general, these microspheres are used in scientific research,

Table 4: Analytical specificity.

	TOX	*RUB*	*CMV*
Replica 1	2	0.5	0
Replica 2	0	-4.5	-5
Replica 3	1	1.5	-4

Table 5: Reactivity of samples incubated 80, 90 and 100 minutes in the elution step.

TOX	**80min**	**90min**	**100min**	**M.**	**SD.**	**CV (%)**
1	615	623	615	615	4	1
2	21279	22669	21397	21396	629	3
3	1610	1629	2080	1629	217	13
4	507	518	572	518	28	5
RUB	**80min**	**90min**	**100min**	**M.**	**SD.**	**CV (%)**
1	27770	25990	26267	26267	782	3
2	181	249	222	222	28	13
3	329.5	389	423	389	38	10
4	53	65	60	60	5	8
CMV	**80min**	**90min**	**100min**	**M.**	**SD.**	**CV (%)**
1	453	552	457	457	46	10
2	3033	4540	3222	3222	670	21
3	9208	9778	12205	9778	1299	13
4	300	411	382	382	47	12

Abbreviations: M: Medium; SD: Standard deviation; CV: Constant of variation.

Table 6: Reactivity of samples incubated 20, 30 and 40 m minutes in the secondary antibody step.

TOX	20min	30min	40min	M.	SD.	CV (%)
1	640	623	698	640	32	5
2	18606	22669	22793	22669	1945	9
3	1717	1629	1907	1717	116	7
4	536	518	599	536	35	6
RUB	**20min**	**30min**	**40min**	**M.**	**SD.**	**CV (%)**
1	25301	25990	27931	25989	1113	4
2	188	249	230	230	25	11
3	393	389	423	393	15	4
4	60	68	68	65	3	5
CMV	**20min**	**30min**	**40min**	**M.**	**SD.**	**CV (%)**
1	493	552	508	507	25	5
2	2840	4540	4099	4098	720	18
3	10352	9778	10966	10351	485	5
4	386	411	419	411	14	3

Abbreviations: M: Medium; SD: Standard deviation; CV: Constant of variation.

and there needs to be more information about them in neonatal screening. Screening has the potential to prevent serious health problems, including death. Worldwide, neonatal screening programs have evolved from simple tests to comprehensive and complex systems capable of detecting more than 50 different conditions in different countries [9].

The screening program is used to track diseases and mainly to seek treatment and provide the family with resources so that those affected become healthy. In Brazil, more and more diseases are being implemented in the Unified Health System (SUS) screening programs, including the addition of infectious diseases such as Toxoplasmosis, Rubella, and Cytomegalovirus correspond to a high number of severe congenital infections [10].

With the expansion of screened diseases, developing new technologies that optimize and implement analyses in all regions, including the country's most remote regions, is essential. For the use of these new diagnostic methods developed in Brazil, the product must meet ANVISA's acceptance criteria for validation of analytical methods.

The precision of an analytical procedure expresses the proximity of agreement (degree of dispersion) between a series of measurements obtained from the same sample [11]. In this study, the repeatability and reproducibility assays performed for precision analysis showed a CV of less than 15% in all analyzes, demonstrating agreement between different tests with the same sample. The kit under development is considered semi-quantitative. This definition occurs because the kit qualitatively delivers the results, that is, positive or negative. However, occasionally quantitative values can be used, mainly in the case of recollection of the same patient.

The MFI values of a result can be compared with results from a later collection (from the same individual) to verify whether there has been a significant increase or decrease in reactivity. Thus, it is necessary to demonstrate that the MFI response of the product is proportional to the concentration of antibodies in the samples.

The linearity of the method is the ability to obtain results directly proportional to the concentration of the specific target antibody against the analyte of interest in the sample [12].

Our study verified linearity using samples with data on reactivity units. As these samples have unit values, it is possible to verify how the response occurs about the dose: MFI and AU/mL or s/co ratio. The tests indicate that all tested parameters can produce a response (in MFI) corresponding to the dosage of IgM antibodies in the reference samples.

The high dose prozone effect was verified using highly reactive samples for the three antigens. In the assay, it was possible to observe an effect of a high dose at the highest concentrations with a decrease in reactivity. However, this decrease did not occur significantly with CV less than 15% when comparing the MFI values. Furthermore, although there is inhibition of antigen-antibody binding, it is far from the dilution used in the study.

All parameters showed low LOD values, with 44, 65, and 47 MFI, respectively, for TOXO, RUB, and CMV. LOD corresponds to the lowest concentration of the analyte that can be detected, but not necessarily quantified, under established experimental conditions; this is because, usually, an assay cannot measure analyte concentrations down to zero [12].

Analytical specificity is the ability to unambiguously evaluate the analyte in the presence of other components that may be present in the samples, such as the matrix. The method under development must be able to produce a response only for the specific target [13].

In the study, the NeoMAP® 3plex IgM kit proved specific in all parameters, with no matrix reactivity in the assay performed only

Table 7: Reactivity of samples with incubation of 20, 30 and 40 minutes in the phycoerythrin step.

TOX	20min	30min	40min	M.	SD.	CV (%)
1	727	623	566	623	67	11
2	19032	22669	22974	22669	1791	8
3	1803	1629	1816	1803	85	5
4	567	518	623	567	43	8
RUB	**20min**	**30min**	**40min**	**M.**	**SD.**	**CV (%)**
1	25175	25990	28076	25990	1222	5
2	203	249	221	221	19	9
3	362	389	376	376	11	3
4	68	65	73	68	3	5
CMV	**20min**	**30min**	**40min**	**M.**	**SD.**	**CV (%)**
1	519	552	456	518	40	8
2	3392	4540	4200	4200	482	12
3	9879	9778	12273	9879	1153	12
4	379	411	415	411	16	4

Abbreviations: M: Medium; SD: Standard deviation; CV: Constant of variation.

with the red blood cell concentrate. That is, the kit could generate interference-free signals on the multiplex platform. Validation is fundamental for the efficient operation of new analytical methods and must be carried out at all stages, from the raw materials used to the finished product. The phrase validation implies a feasibility demonstration activity and aims to demonstrate that the developed product meets the recommended acceptance criteria and is suitable for the proposed objectives [10,11]. In the present study, the kit developed followed all the criteria proposed for product registration in Brazil and its use in the national territory.

CONCLUSION

The repeatability and reproducibility tests showed a CV of less than 15% in all samples, demonstrating the reliability of the data obtained in all tests. High-dose assays showed that at higher concentrations, there is a tendency to inhibit antigen-antibody binding, requiring a defined dilution of 1:200. The obtained data presented performance compatible with the validation criteria of analytical methods of ANVISA, being approved for use in the country. The data generated in this study are contained in the product registration (Registration number 80173700020).

ACKNOWLEDGMENTS

Acknowledgments to the Coordination for the Improvement of Higher Education Personnel (CAPES) for the master's scholarship that enabled the development of the study. Acknowledgments to the company Intercientifica for sharing the infrastructure of the Research and Development laboratory and for making the xMAP technology of magnetic microspheres available.

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