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HEALTH SCIENCES

Development of a validation protocol method for nucleic acid testing to detect human immunodeficiency virus, hepatitis C virus, and hepatitis B virus

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Abstract: The concern about the risks of viral infections transmission through blood transfusion has led into a search for improvements on screening tests used for the selection of blood donors. Molecular biology techniques applied in researches of viral genomes, known as Nucleic Acid-amplification-Test (NAT), represent a technology capable of increasing transfusion safety by shortening the diagnostic window period. In Brazil, the implementation of this technology for the detection of HIV, HCV and HBV occurred due to the implantation of the NAT Kit – produced by Immunobiological Technology Institute (Biomanguinhos-FIOCRUZ), in the Brazilian blood centers. The National Health Surveillance Agency attaches great importance to validation, since it standardizes, disciplines and regulates criteria for the registration of health products. This work aims to establish a protocol of performance validation by real-time PCR method, taking as the object of study the Bio-Manguinhos NAT Kit, in order to update the product registration or to meet any future needs to ensure all regulatory requirements for the performance validation of the real-time PCR diagnostic kit. The protocol developed followed the ICH recommendations. The results revealed that the adopted methodology contemplates the necessary requirements for compliance with the Brazilian legislation, as well as the established validation parameters.

Key words: validation studies, methods, real-time polymerase chain reaction, modification of products registration.

INTRODUCTION

Technological advances in research and development (R&D) in last decades have provided improvements in the quality of health of the population (Capucho et al. 2012). Over the years, concern about the risks of transmission of viral infections through blood transfusion has led to a search for improvements in screening tests in the selection of blood donors (Goodman 2004). To improve the quality of products derived from human blood and obtain greater transfusion safety, serological tests for several pathogens, including: the human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) are currently performed in Brazilian blood centers (Goodman 2004, Stramer et al. 2004).

Although these tests are performed in order to reduce or even eliminate the transmission of these viruses through blood transfusion, post-transfusion risks still persist (Stramer et al. 2004). This is basically due to the period of immunological window during which serological techniques are less effective because they do not have the capacity to detect antibodies at

this stage of the infection. In order to minimize this problem, the development of molecular diagnostic tests, based on polymerase chain reaction (PCR) in real time, allows the reduction of the immunological window period, since it is a much more sensitive technique (Jackson et al. 2003, Stramer et al. 2004).

Real-time PCR applied in the nucleic acid amplification test (NAT) is used for the detection of HIV, HBV and HCV in the screening of all blood donors in Brazil, which has allowed significant advances in detection of blood transfusion-transmitted infections during the immunological window period. Today, in a great extent of the world, the risk of transfusion transmission of an infectious agent has been drastically reduced by the introduction of state-of-the-art tests for antibodies and antigens, and by the introduction of the nucleic acid amplification test (FDA/Center for Biologics Evaluation and Research 2004, Stramer et al. 2004). NAT combines the advantages of direct and highly specific sequence detection of viral genomes (DNA or RNA) with an analytical sensitivity that is several orders of magnitude greater than antigen detection or virus isolation methods (Candotti & Allain 2013, Jackson et al. 2003)

In Brazil, the NAT HIV/HCV/HBV Kit used in the public blood network is a product of Bio-Manguinhos - Fiocruz, complementary to the serological tests offered in blood centers in the country (Rocha et al. 2018). This kit consists of the preparation of a pool of six samples together with the addition of a biosafe calibrator particle (internal control), followed by the automated extraction of nucleic acids (Petry 2013), amplification and detection of the targets. Regarding the targets of the kit in question, in general, these infections behave as follows: for HIV, serological tests are positive from around 15th to the 19th day. The detection of viral RNA by NAT, on the other hand, becomes significant from the 5th day of the beginning of infection, when the exponential replication phase of the virus begins (Fiebig et al. 2003, Kleinman et al. 2009). Regarding HCV, there is a significant gain in reducing the diagnostic window, because when NAT is performed around the 10th day after infection, the RNA can already be detected, while antibody research tests detect it after the 65th day (Kleinman et al. 2009, Petry 2013). For HBV, this window period can vary from 30 to 60 days, when serological tests are able to detect (Ministério da Saúde 2005), while NAT tests can detect HBV around the 10th day (BioManguinhos Fiocruz 2017). If it is necessary to perform any process of alteration in the NAT test, it is necessary to carry out tests regarding the validation of the product method after its alteration (ANVISA 2019). Considering the growing concern with the reliability of diagnostic tests and aiming to establish a method validation protocol for a real-time PCR diagnostic kit, this work aims to carry out the preparation and execution of performance validation tests of the new kit format NAT HIV/HCV/HBV manufactured by Bio-Manguinhos – Fiocruz, considering the change in the test calibration particles.

MATERIALS AND METHODS

Place of study execution

All tests were carried out at the Reactive Quality Control Laboratory (LACORE), which has a complete NAT platform and complies with the biosafety criteria required for molecular activities, including manipulation of derivatives of Genetically Modified Organisms (GMOs). The Brazilian NAT platform consists of automated sample pooling of blood donations using an automated workstation (Janus, PerkinElmer), automated nucleic acid isolation using a molecular biology workstation (BioRobot MDx, Qiagen), and an automated amplification on a

realtime PCR system (Applied Biosystems 7500, Thermo Scientific) (Rocha et al. 2018).

Samples used

The panel containing positive samples for HIV, HCV, HBV and HTLV (human T-lymphotropic virus) was provided by the Serological Panel Production Division (DIPPS) of Bio-Manguinhos, as well as the negative diluent. Its production comprises a series of procedures ranging from the raw material to the selection of the chosen samples. The analyzed samples are compatible with the country's epidemiological scenario. Therefore, all genotypes circulating in Brazil for these viruses were evaluated in product development. Positive samples were identified and characterized before use, being submitted to a viral quantification protocol and linearity tests with the viral load concentrations of interest. Viral quantification is performed using the COBAS® TaqMan® 48 methodology (viral quantification kit from Roche Molecular Diagnostics). After the viral load has been identified, serial dilutions are carried out in negative diluent up to the concentrations that are intended to be used in the routines of the quality control laboratory. These dilutions are analyzed with a NAT HIV/HCV/HBV Kit in order to evaluate the performance of the panel.

The HIV/HCV/HBV NAT Kit uses a mimetic viral particle (VLP), biosafe, called particle calibrator or internal control (IC), which controls all stages of the process. The modification performed in this IC consisted in using the Scramble Site Directed Mutagenesis technique to modify the target region in the IC plasmid in order to generate a new binding site for IC primers and prevent competition with HIV/WT primers. If the sample does not obtain a linear performance in its dilution, a new sample must be characterized. Thus, quality control defines an internal panel composed of standardized samples for use in tests for product release, stability tests, validations, and so on.

After all sample characterization steps are completed, the internal panel used in the validation tests was composed of the samples shown in Table I.

The positive sample for HTLV was not quantified, as it is not a target sample of the kit in question, being used only in the specificity analysis.

Batches used in validation

Three consecutive batches were produced for the validation and stability testing purposes, namely: 185NV001Z, 185NV002Z and 185NV003Z. Before starting the validation tests, the batches were approved by quality control according to the protocol for analysis of finished products already established for the kit format currently commercialized.

Experimental drawing

The tests were defined based on the recommendations described in the European Pharmacopoeia (European Pharmacopoeia 2020) aiming to meet the following specifications:

Detection limit assessment (LOD)

Three batches tested on different days were analyzed, according to pharmacopoeial recommendation. For these analyses, serial dilutions were performed in five concentrations

Table I. Description of the positive samples components of the inner panel.

Target	Identification	Viral load
HIV	359/18	5,14E+03
HCV	48/10	4,30E+04
HBV	1333/14	4,58E+06
Negative diluent	PN003/18	Not detectable

and 8 replicates of each were used, computing 24 results by concentration. Positive samples were diluted and stored at -80°C until their use. The total volume obtained from the dilution of the five concentrations of interest was enough to analyze all batches in order to ensure that the dilutions used were from the same processing. The analyzed concentrations were chosen in order to guarantee the Kit's detection limit, where, according to the manual, it is established in: 100 copies/ml for HIV, 100 IU/ml for HCV and 50 IU/ml for HBV. Serial dilutions followed the schemes described in Table II.

The analyses followed all the processes for using the Kit. The dilutions were manually pipetted into dedicated tubes and proceeded to the step of adding a calibrator particle in the automated sample pooling, subsequently followed by the steps of extracting the genetic material in the automated nucleic acid isolation and finally, amplification and detection in the 7500*Real-Time PCR System*.

The concentrations were determined in order to observe the performance of the Kit against concentrations close to the detection limit determined by the Kit manual, in order to demonstrate that the product remains within its specification.

Specificity assessment

Specificity was evaluated with 8 replicates of each positive sample from the internal panel - HIV, HCV and HBV, contaminated with an HTLV positive sample. The HTLV sample was used as a tool to represent a component of unexpected presence, and could be replaced by another one from another virus. The fact that the test unequivocally detects the nucleic acid only from the target sample, despite the presence of an unexpected component in the reaction, represents the specificity of the method.

The mixture carried out individually between the samples of HIV/HCV/HBV and HTLV was 1:1, with a final volume of 18 mL, in order to ensure that the mixtures used come from the same processing. For the HBV sample, a prior dilution in negative diluent was necessary, as its concentration is considerably high when compared to the HIV and HCV samples, so it was diluted to a concentration of 10^4 IU/mL and subsequently mixed with sample for HTLV.

In order to analyze the total of at least 100 negative samples as recommended in the pharmacopoeia, and also to ensure the specificity of the Kit, wells with negative sample added with calibrator particle were distributed on the analysis plates of each batch.

The analyses followed all the processes for using the Kit, the 600 µL representing the pool was composed of sample mixtures with HTLV. The mixtures were manually pipetted into the tubes dedicated to the Kit and went on to the step of adding a calibrator particle in the automated sample pooling, subsequently followed by the steps of extracting the genetic material in the automated nucleic acid isolation and finally, amplification and detection in the 7500*Real-Time PCR System*.

Robustness assessment

To demonstrate the robustness of the method, with regard to cross contamination, 20 wells with a high viral concentration sample were evaluated. In this test, positive samples were not diluted and were alternated with negative samples. Also to prove the robustness, 20 wells with samples with viral load levels at the threshold of the positive response were evaluated and the dilutions performed to obtain these concentrations are described in Tables III, IV and V. Both tests were performed for the 3 batches. As well as the LOD and specificity tests, all the processes for using the Kit were followed.

Table II. Serial dilution for HIV, HCV and HBV positive samples used in the detection limit evaluation test.

HIV Viral load (copies/mL)	150	125	100	75	50
HCV Viral load (IU/mL)	150	125	100	75	50
HBV Viral load (IU/mL)	75	50	25	15	7.5

Table III. Serial dilution for HIV-positive sample, highlighting the concentration used for the robustness test regarding the positive response threshold.

HIV 359/18						
Viral Load (Copies/ml)	**5.14E+03**	**1.60E+03**	**8.00E+02**	**4.00+E02**	**2.00E+02**	**1.00E+02**
Sample (µl)		1556	4000	6000	11000	20000
Diluent (µl)		3444	4000	6000	11000	20000
Final volume (µl)		5000	8000	1200	22000	40000

Table IV. Serial dilution for a HCV positive sample, highlighting the concentration used for the robustness test regarding the positive response threshold.

HCV 48/10							
Viral load (Copies/ml)	**4.30E+04**	**4.30E+03**	**2.40E+03**	**8.00E+02**	**4.00E+02**	**2.00E+02**	**1.00E+02**
Sample (µl)		1000	2791	3000	6000	11000	20000
Diluent (µl)		9000	2209	6000	6000	11000	20000
Final volume (µl)		10000	5000	9000	12000	22000	40000

Table V. Serial dilution for a HBV positive sample, highlighting the concentration used for the robustness test regarding the threshold of positive response.

	HBV 133/14							
Viral load (IU/ml)	**4.58E+06**	**4.58E+05**	**4.58E+04**	**4.58E+03**	**4.58E+02**	**2.29E+02**	**1.15E+02**	**5.73E+01**
Sample (µl)		400	500	500	800	6000	11000	20000
Diluent (µl)		3600	4500	4500	7200	6000	11000	20000
Final volume (µl)		4000	5000	5000	8000	12000	22000	40000

Statistical analysis

The evaluation of the dispersions of the Ct values obtained for the positive controls was performed with the Box-plot statistical tool, using the R software version 3.5.3. All replicas of positive controls (CR control) were compared by this tool in order to identify significant variations within the total set of tests performed for method validation.

RESULTS AND DISCUSSION

The first format of the Bio-Manguinhos NAT Kit contemplated the viral detection of HIV and HCV. With the insertion of the third target, HBV, it became necessary to change the positive control of the Kit, that is, the inclusion of a specific sequence for HBV in its composition. When performing such alteration of the product, it was necessary to revalidate the modified product, in order to, in addition to meeting institutional requirements, update the registration with the regulatory agency.

In the amplification and detection using the inputs of the amplification module for HIV/HCV, it is possible to detect the targets HIV, HCV and PC (calibrator particle), while when using the module for HBV it is possible to detect the targets HBV and PC. About AB controls (negative control) and CR (Positive control), it is expected that in the wells where AB controls are, the result will be the amplification only of the internal calibrator that is inserted in its composition, this control should not amplify the HIV, HCV or HBV targets. For CR controls, the result is expected to be amplification of HIV, HCV and HBV targets. These amplifications occur due to the target sequences inserted in its composition. With regard to the calibrator particle, it is an HIV-mimetic viral particle, with no replicative capacity in vivo, and for its amplification the reverse transcription step is necessary. Its amplification is expected to occur in all wells, ensuring the efficiency of the reaction in each well. Due to its nature, in the case of samples with a high viral load, competition for reagents may occur and their amplification may be impaired. This fact does not disregard the result, given the identification of the presence of the virus in the sample.

The tests carried out to validate the performance of the kit under study meet the requirements proposed by international regulations. The tests were defined according to the characteristics of the method, proposing that it will be considered validated if its characteristics comply with the criteria and limits established according to the particularity of the test and the disease under study (ANVISA 2017) The detection limit/analytical sensitivity was evaluated considering 03 batches of the product, 1 kit of each batch. The results obtained are shown in Table VI.

The detection limit of this technique is the smallest amount of nucleic acid that can be detected, but not necessarily quantified, with an exact value in the sample(ICH 1995). For practical reasons, the threshold of positive response for nucleic acid amplification techniques is determined, this being the minimum number of target sequences by unit of volume that can be detected in 95% of tests (ANVISA 2019, European Pharmacopoeia 2020).

The analysis of these results allows us to confirm the detection limit established by the Kit's manual, as 100% of the replicates were detected at concentrations of 100 copies/mL for HIV, 100 IU/mL for HCV and 50 IU/mL for HBV. In addition, these data suggest that the detection limit for the HCV and HBV targets could be determined at 75 IU/mL and 25 IU/mL respectively, a concentration lower than that described, as both had more than 95% detection of replicates in these concentrations. It is noteworthy that The European Medicine Evaluation Agency (EMEA) and the European Pharmacopoeia (EP) intend to prescribe HCV RNA testing of plasma pools, with the required test detection limit of 100 IU/ml (Lelie et al. 1998) The experimental drawing for detection limit evaluation, developed in the present study, presented satisfactory results and within what is recommended in Pharmacopoeias (ANVISA 2019, European Pharmacopoeia 2020). For evaluation of the analytical specificity of the methodology,

Table VI. Results obtained in the evaluation of the detection limit/analytical sensitivity.

Target	Concentration	Samples	Samples detected	Sensitivity (%)
HIV	150 (Copies/ml)	24	24	100
	125 (Copies/ml)		24	100
	100(Copies/ml)		24	100
	75 (Copies/ml)		22	91.67
	50 (Copies/ml)		20	83.33
HCV	150 (IU/ml)	24	24	100
	125 (IU/ml)		24	100
	100 (IU/ml)		24	100
	75 (IU/ml)		23	95.83
	50 (IU/ml)		20	83.33
HBV	75 (IU/ml)	24	24	100
	50 (IU/ml)		24	100
	25 (IU/ml)		24	100
	15 (IU/ml)		22	91.67
	7.5 (IU/ml)		14	58.33

8 sample replicates of each target were tested, contaminated with a positive sample for HTLV. This test was performed with 1 kit from each batch. The evaluation of the results showed unequivocal reactivity for the three targets, as shown below in Table VII.

These tests were able to demonstrate the specificity of the method, as the Kit's ability to distinguish between the target sample and the one originated from other components that could cause falsely reduced responses was observed, negatively affecting the test, with 100% specificity being obtained in the evaluation performed. We can also conclude that PCR inputs are highly specific, with no cross-response with another virus (HTLV). Furthermore, the 196 negative samples evaluated in the present study did not present a characteristic amplification profile for any target except for the calibrator particle (Figures 1, 2 and 3).

In graphs e of Figure 1, a of Figure 2 and c of Figure 3, unexpected signals for HCV were observed. Analyzing the profile of the amplified signal, it can be seen that they do not have a typical profile of the amplification curve, that is, their phases are not well defined (Figure 4). According to the product manual, sometimes an unspecific background signal may appear. These occurrences are due to a possible formation of probe dimers grouped by non-consumption in the reaction, unspecific degradation of the probe due to light incidence, thawing or other factors. Up to 5% of the presence of these background signal can be considered acceptable within a routine.

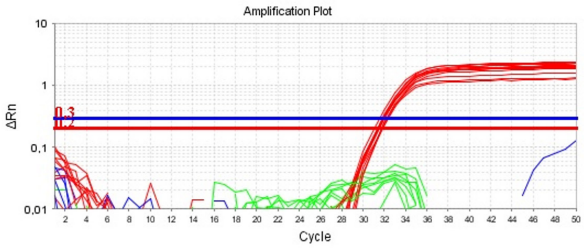
The results demonstrated the methodology's ability to distinguish the detection of targets of interest in a mixture contaminated with another similar virus species (HTLV), as well as the ability to only detect the reaction's internal control

Table VII. Results obtained in replicates contaminated with HTLV. Ct values (threshold cycle - cycle in which the sample's fluorescence exceeds the threshold line).

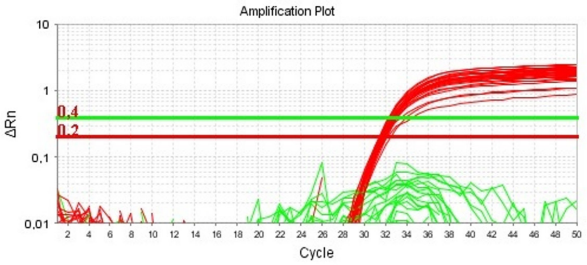
	185NV001Z		185NV002Z		185NV00Z	
Target	Ct	Samples detected	Ct	Samples detected	Ct	Samples detected
HIV	36.71	8/8	34.46	8/8	36.00	8/8
	35.46		36.73		36.69	
	38.30		34.87		34.92	
	34.09		34.77		34.72	
	36.47		36.04		37.12	
	33.84		35.13		34.75	
	40.67		34.23		34.05	
	34.01		35.23		34.41	
HCV	31.61	8/8	31.24	8/8	30.72	8/8
	30.55		30.77		30.52	
	31.22		30.86		31.02	
	30.88		30.91		31.09	
	30.63		31.07		30.93	
	30.84		30.97		30.87	
	30.65		30.82		31.08	
	31.46		30.91		31.26	
HBV	29.98	8/8	29.98	8/8	29.05	8/8
	29.94		30.00		30.08	
	29.94		29.94		29.65	
	30.12		29.74		29.61	
	30.02		29.78		29.73	
	29.73		29.98		29.81	
	29.91		30.16		29.66	
	30.04		30.16		29.96	

(calibrator particle) in the absence of the specific targets, confirming that the method meets the specificity criteria defined in pharmacopoeia, which establishes that it is convenient to test at least 100 negative samples and all results obtained are negative (ANVISA 2019).

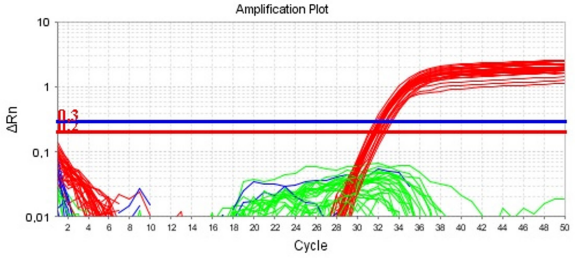
The experimental drawing chosen provided us with results that corroborate the concept that analytical specificity can also be confirmed by obtaining positive results obtained from samples that contain what is it wanted to detect, together with negative results from samples that do not contain what it is wanted to detect (Diniz 2013).



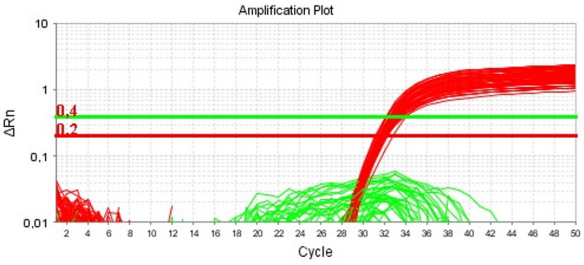
a) Amplification plot: LOD HIV and HCV



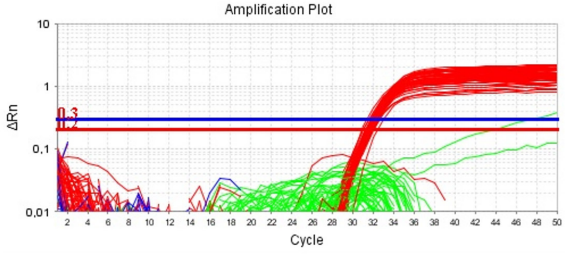
b) Amplification plot: LOD HBV + HTLV amplification with HBV dilution



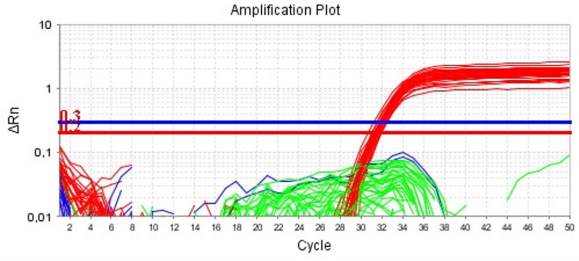
c) Amplification plot: LOD HBV+HTLV amplification



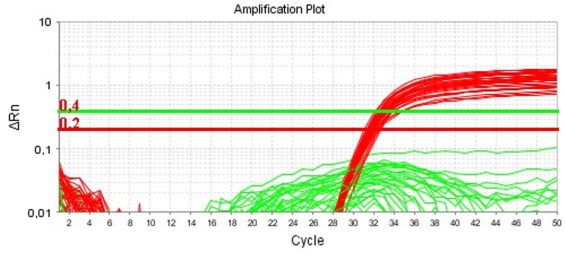
d) Amplification plot: Robustness with HBV high viral load



e) Amplification plot: Robustness with HIV/HCV high viral load

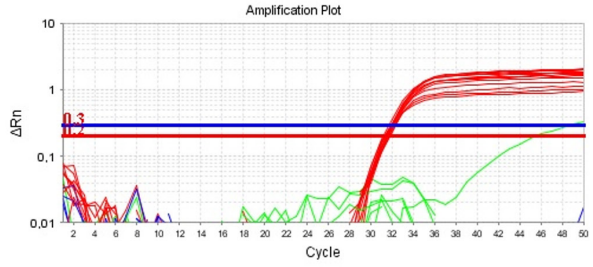


f) Amplification plot: Robustness with HIV/HCV low viral load

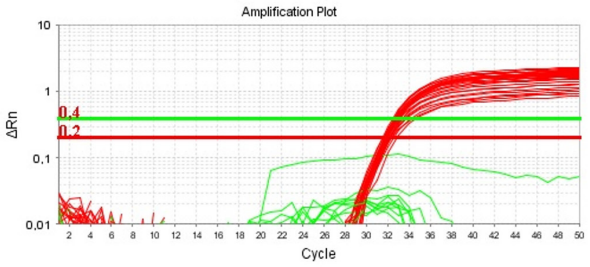


g) Amplification plot: Robustness with HBV low viral load

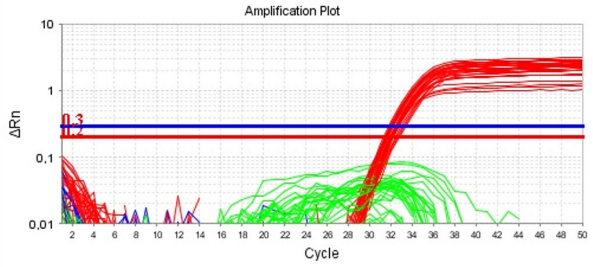
Figure 1. Graphs of results referring to negative samples arranged on plates belonging to batch referent to 185NV001Z. The red line refers to the threshold for internal control; the blue line refers to the threshold for HIV and HCV; the green line refers to the threshold for HBV. The amplification curves (in red) above the threshold lines demonstrate the detection of the internal control - calibrator particle. a) LOD HIV and HCV, b) LOD HBV+HTLV amplification with HBV dilution, c) LOD HBV+HTLV amplification with HIV/HCV dilution, d) Robustness with HBV high viral load, e) Robustness with HIV/HCV high viral load, f) Robustness with HIV/HCV low viral load, g) Robustness with HBV low viral load.



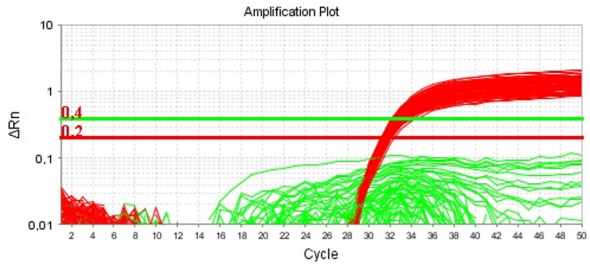
a) Amplification plot: LOD HIV and HCV



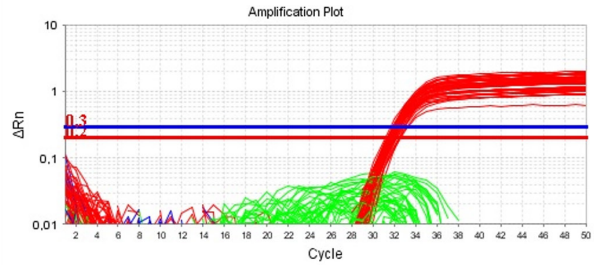
b) Amplification plot: LOD HBV + HTLV amplification with HBV dilution



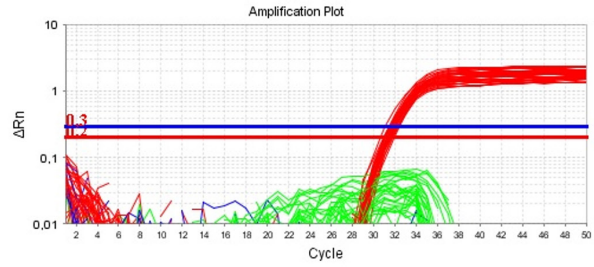
c) Amplification plot: LOD HBV+HTLV amplification with HIV/HCV dilution



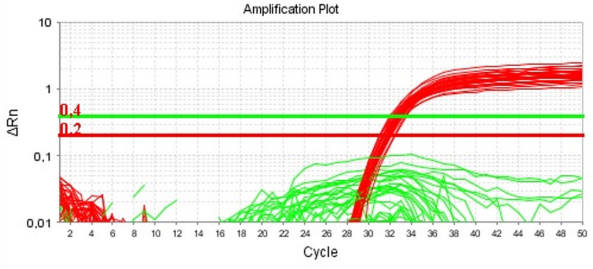
d) Amplification plot: Robustness with HBV high viral load



e) Amplification plot: Robustness with HIV/HCV high viral load

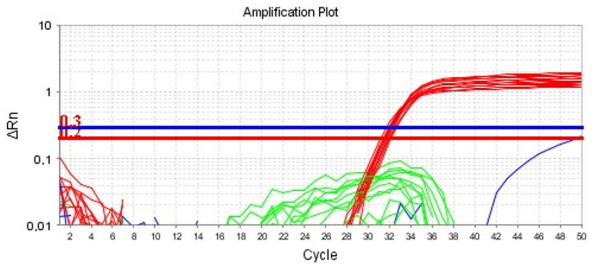


f) Amplification plot: Robustness with HIV/HCV low viral load

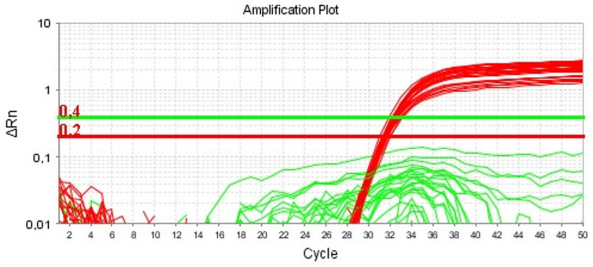


g) Amplification plot: Robustness with HBV low viral load

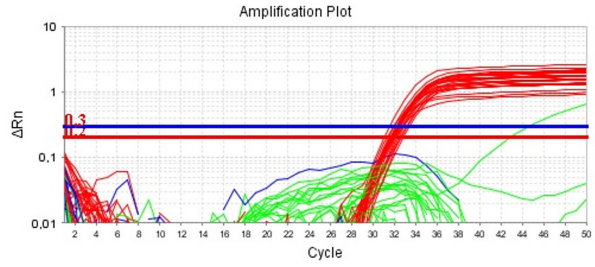
Figure 2. Graphs of the results referring to the negative samples arranged on the plates belonging to batch 185NV002Z. The red line refers to the threshold for internal control; the blue line refers to the threshold for HIV and HCV; the green line refers to the threshold for HBV. The amplification curves (in red) above the threshold lines demonstrate the detection of the internal control - calibrator particle. a) LOD HIV and HCV, b) LOD HBV+HTLV amplification with HBV dilution, c) LOD HBV+HTLV amplification with HIV/HCV dilution, d) Robustness with HBV high viral load, e) Robustness with HIV/HCV high viral load, f) Robustness with HIV/HCV low viral load, g) Robustness with HBV low viral load.



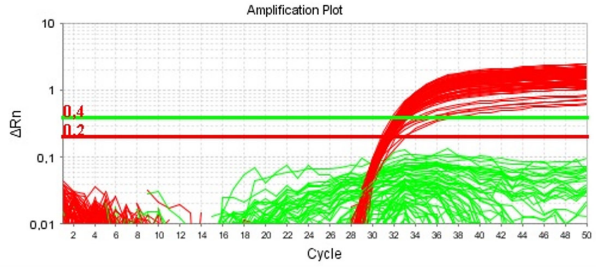
a) Amplification plot: LOD HIV and HCV



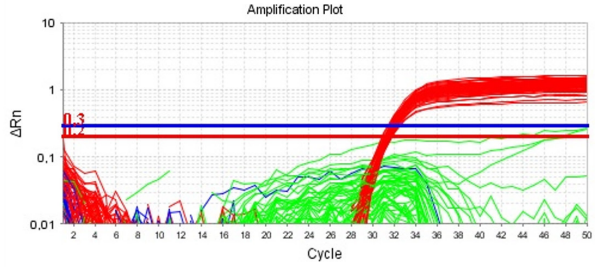
b) Amplification plot: LOD HBV + HTLV amplification with HBV dilution



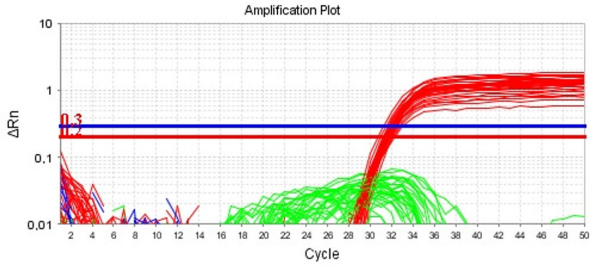
c) Amplification plot: LOD HBV+HTLV amplification with HIV/HCV dilution



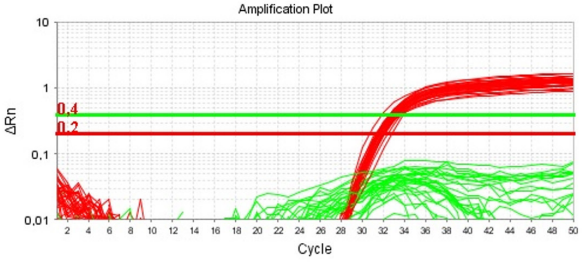
d) Amplification plot: Robustness with HBV high viral load



e) Amplification plot: Robustness with HIV/HCV high viral load



f) Amplification plot: Robustness with HIV/HCV low viral load



g) Amplification plot: Robustness with HBV low viral load

Figure 3. Graphs of results referring to negative samples arranged on plates belonging to batch 185NV003Z. The red line refers to the threshold for internal control; the blue line refers to the threshold for HIV and HCV; the green line refers to the threshold for HBV. The amplification curves (in red) above the threshold lines demonstrate the detection of the internal control - calibrator particle. a) LOD HIV and HCV, b) LOD HBV+HTLV amplification with HBV dilution, c) LOD HBV+HTLV amplification with HIV/HCV dilution, d) Robustness with HBV high viral load, e) Robustness with HIV/HCV high viral load, f) Robustness with HIV/HCV low viral load, g) Robustness with HBV low viral load.

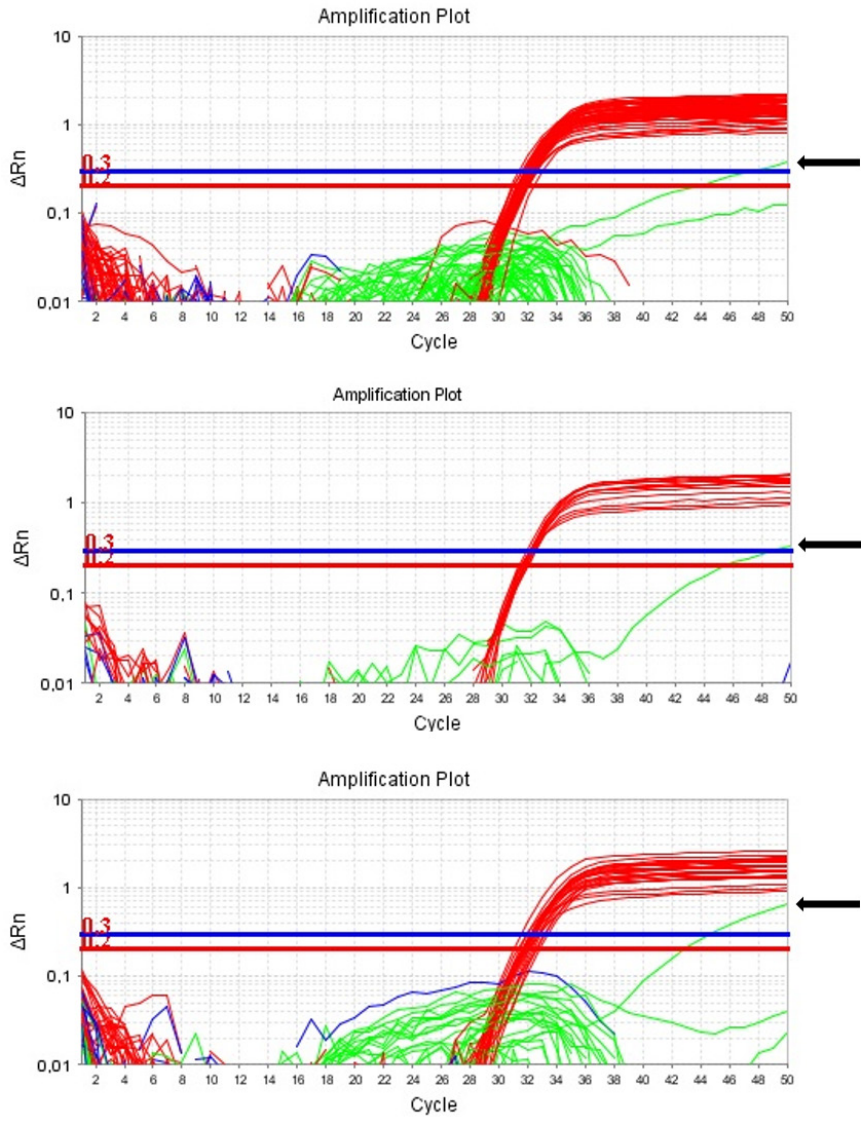


Figure 4. Graphs of results where unexpected signs for HCV were observed.

Robustness

The robustness of the method was analyzed using 20 wells containing positive samples at high viral concentration alternated with wells containing negative samples, in order to evaluate the possibility of cross-contamination. In all the experiments carried out, with the 3 batches, there was no evidence of cross contamination. We also analyzed 20 wells with samples containing viral load levels at the threshold of positive response (established by the kit's development team), where the HCV and HBV targets obtained all positive results. For HIV, however, it was observed that only one replicate did not show an amplification profile, however this loss is within the expected range for the detection limit of the product, as the total number of replicates detected represents 95% of positive results, as shown in the Table VIII.

Given the results obtained, it is concluded that the method is robust in relation to cross contamination and in relation to small variations, such as analysts and different days of performing testing (ICH 1995). The tests were considered sufficient to determine the robustness of the

Table VIII. Results obtained in 20 wells containing viral load levels at the Kit's detection threshold. Ct values (threshold cycle - cycle in which the sample's fluorescence exceeds the threshold line).

HIV			HCV			HBV		
185NV001Z	185NV002Z	185NV003Z	185NV001Z	185NV002Z	185NV003Z	185NV001Z	185NV002Z	185NV003Z
Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct
37.18	35.21	36.56	36.6	35.30	38.08	35.23	35.63	34.71
41.61	36.10	35.20	34.95	35.22	37.70	34.93	34.52	35.06
36.28	34.96	36.75	34.38	34.06	33.59	35.72	34.50	36.10
38.05	35.33	35.67	32.95	33.80	34.89	34.41	35.41	35.61
33.60	35.00	34.22	34.11	35.07	36.70	34.27	34.39	36.40
45.38	34.62	37.68	34.98	34.67	33.78	34.19	34.78	38.09
41.17	49.81	35.16	34.97	36.83	37.83	35.56	34.17	35.15
35.43	36.70	39.39	34.94	33.41	38.15	34.99	34.92	35.60
35.04	36.15	37.17	38.94	34.79	37.03	34.82	34.97	35.15
33.78	35.50	36.05	35.38	33.63	34.62	34.86	35.39	37.07
38.05	34.48	36.01	34.73	34.14	34.50	34.47	34.16	34.27
34.94	35.01	36.48	33.74	36.28	34.60	35.60	34.61	38.27
46.69	36.61	42.24	33.25	35.03	36.01	33.97	34.23	35.53
34.15	33.83	36.61	33.80	34.30	34.90	35.16	35.63	35.17
35.24	34.16	39.85	36.72	35.39	36.45	36.75	34.05	35.47
34.84	35.37	35.42	41.91	34.94	34.77	34.08	35.78	34.74
35.37	35.08	41.68	37.95	40.67	38.51	36.13	35.11	34.49
33.43	33.86	39.58	35.53	34.87	35.29	34.86	34.18	34.91
35.18	35.70	34.18	35.30	34.33	41.69	37.41	34.90	37.04
33.97	>50	34.84	35.51	33.73	34.92	35.26	34.81	34.93

method, as they followed what is recommended for regulatory purposes and are in accordance with the concept of robustness of an analytical method, where it is understood that it is about its ability to remain unchanged when subjected to small, but deliberate, variations in operating parameters and provides an indication of the feasibility of the technique under normal conditions of use (ANVISA 2019).

Positive controls

The positive control, called CR Control, was the altered input that led to the need for performance validation of the Bio-Manguinhos NAT HIV/HCV/HBV Kit. Thus, it is convenient to show the amplification performance of the new controls, which were evaluated in all tests in the present work. Furthermore, as it is a parameterized result, analyzing them makes it possible to observe the performance of the tests within the variations of the method.

As a test validation parameter, the NAT software used in this study uses a range of Ct values (threshold cycle - cycle in which the sample's fluorescence exceeds the threshold line) of 28 + 2.47, for each target, being any value below considered valid. All controls obtained Ct values within the recommended parameters (Table IX), and their dispersion was statistically evaluated.

Table IX. Results obtained in positive controls - CR.

HIV			HCV			HBV		
185NV001Z	185NV002Z	185NV003Z	185NV001Z	185NV002Z	185NV003Z	185NV001Z	185NV002Z	185NV003Z
Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct	Ct
26.35	26.46	26.74	27.23	27.23	27.25	27.05	27.01	26.85
26.80	26.55	26.02	27.06	27.09	26.77	26.95	26.94	26.91
26.22	26.50	26.62	27.14	27.41	27.07	27.26	27.45	26.88
26.41	26.28	26.34	27.14	27.22	27.08	27.08	27.21	26.99
26.18	26.67	26.22	26.95	27.09	27.05	27.31	27.30	27.15
26.27	26.73	26.52	26.90	27.01	27.16	36.81	27.00	27.05
26.85	26.53	26.59	27.41	27.45	27.27	27.32	26.97	27.01
26.13	26.61	26.59	27.32	27.22	27.35	27.06	26.70	26.93
26.86	26.35	26.89	27.28	26.84	37.08	27.19	27.01	26.96
26.78	26.46	26.28	27.03	29.99	26.86	26.95	27.05	27.00

It can be observed that the controls obtained results with Ct variations within the expected range, that is, all values below 30.47, as no outlier point was identified. We also observed through the box graphs that the dispersion of Cts for each target remained similar when compared between batches. Such results confirm the validity of the tests performed and guarantee the robustness of the new format of this input. It is important to highlight that, the tests employed different operators at all stages and these results represent excellent reproducibility and repeatability. This means that, despite the variation in the days the kits were analyzed and the individual operators, the tests obtained homogeneous results.

CONCLUSIONS

The protocol developed contemplates the necessary requirements to comply with Brazilian regulations and can be used to validate the performance of the Bio-Manguinhos NAT HIV/HCV/HBV Kit. The detection limit analysis confirmed the previously established limit for this Kit, as 100% of the replicates were detected at concentrations of 100 copies/ml for HIV, 100 IU/ml for HCV and 50 IU/ml for HBV. Tests for specificity were able to demonstrate the Kit's ability to distinguish the target sample as well as showing no characteristic amplification profile for any target except for the calibrator particle when negative samples were evaluated. The robustness analysis showed that the method did not show cross contamination and maintained the expected result even in the face of small variations, such as analysts and different days of testing. The tests were considered sufficient to determine the robustness of the method, as they followed what was recommended for regulatory purposes. All CR controls obtained Ct values within the recommended parameters. In the opinion issued by SEVAN/Bio-Manguinhos, the results were considered in compliance to meet the parameters established for validation.

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