Summary

The purpose of this study is to validate the method for detection of antibody to Human Immunodeficiency virus types 1 and 2 (HIV 1/2) in plasma pools. This method is described in the procedure "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools". It is an *in vitro* enzyme immunoassay (EIA) for the qualitative detection of antibodies to HIV-1 and HIV-2 in pools of human plasma.

Introduction

The purpose of this document is to describe the characteristics evaluated in order to validate the method "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools" and to report the results of the validation study in order to determine the suitability of the method for the determination of HIV-1 and HIV-2 in plasma pools.

Principle

In this method, the Abbott HIVAB® HIV-1/HIV-2 EIA 2.0 uses recombinant DNA-derived antigens corresponding to HIV-1 core and envelope and HIV-2 envelope proteins. These antigens will react with either or both antibody specificities present in human plasma. According to the package insert, Abbott HIVAB EIA was developed to allow the simultaneous detection of IgG and IgM antibodies to HIV-1 and HIV-2 to identify potentially reactive individual plasma units. All units comprising plasma pools made in Clayton have already been screened by this method. This procedure is designed for use with pooled plasma, and as such is not modified from the original FDA-licensed assay.

Validation Testing Facility

Bayer Corporation
Quality Assurance
8368 U.S. 70 West
Clayton, North Carolina 27520
(919) 553-5011

Description of Products Being Tested

Plasma pools were created using 50 or more individual units.

VIROTROL SCREENING Panel 1 is a testing panel made by Blackhawk BioSystems, Inc. It is composed of 3 samples that have been found to reactive or nonreactive using this test. The samples are: High Reactive Viral Marker, positive for HIV-1; Low Reactive Viral

Table 7: Detection Limit: Raw Data for Sensitivity Panels

	Operator 1			Operator 2		
Sensitivity Panel Sample	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
Cutoff Value[a]	0.029	0.030	0.027	0.034	0.033	0.029
H (0.00 ng/mL)	0.003 0.005	0.005 0.006	-0.006 0.004	0.008 0.005	0.004 0.004	0.001 0.004
F (0.065 ng/mL)	0.010 0.017	0.011 0.009	0.010 0.016	0.002 0.002	0.007 0.001	0.008 0.012
G (0.068 ng/mL)	0.008 0.013	0.010 0.013	0.004 0.005	0.235 (+)[b] 0.014	0.005 0.032	0.008 0.071 (+)
M (0.124 ng/mL)	0.012 0.010	0.012 0.010	-0.006 0.013	0.026 0.045 (+)	0.006 0.009	0.010 0.015
Q (0.131 ng/mL)	0.013 0.011	0.014 0.019	0.009 -0.002	0.019 0.029	0.016 0.020	0.011 0.153 (+)
L (0.230 ng/mL)	0.022 0.021	0.027 0.033 (+)	0.005 -0.014	0.029 0.026	0.021 0.018	0.024 0.030 (+)
D (0.274 ng/mL)	0.014 0.009	0.021 0.014	0.013 0.014	0.021 0.002	0.009 0.019	0.014 0.022
S (0.485 ng/mL)	0.043 (+) 0.044 (+)	0.081 (+) 0.050 (+)	0.043 (+) 0.028 (+)	0.042 (+) 0.043 (+)	0.040 (+) 0.053 (+)	0.410 (+) 0.281 (+)
P (0.525 ng/mL)	0.029 (+) 0.029 (+)	0.030 (+) 0.030 (+)	0.024 0.033 (+)	0.050 (+) 0.044 (+)	0.020 0.037 (+)	0.032 (+) 0.028
R (0.730 ng/mL)	0.065 (+) 0.038 (+)	0.043 (+) 0.046 (+)	0.044 (+) 0.074 (+)	0.104 (+) 0.029	0.119 (+) 0.042 (+)	0.149 (+) 0.070 (+)
B (0.784 ng/mL)	0.058 (+) 0.062 (+)	0.055 (+) 0.068 (+)	0.052 (+) 0.052 (+)	0.062 (+) 0.109 (+)	0.073 (+) 0.060 (+)	0.050 (+) 0.061 (+)
C (0.917 ng/mL)	0.052 (+) 0.042 (+)	0.050 (+) 0.053 (+)	0.047 (+) 0.051 (+)	0.054 (+) 0.060 (+)	0.034 (+) 0.041 (+)	0.044 (+) 0.053 (+)
N (1.002 ng/mL)	0.082 (+) 0.069 (+)	0.069 (+) 0.072 (+)	0.070 (+) 0.069 (+)	0.091 (+) 0.149 (+)	0.072 (+) 0.056 (+)	0.074 (+) 0.076 (+)
K (1.904 ng/mL)	0.145 (+) 0.132 (+)	0.140 (+) 0.144 (+)	0.134 (+) 0.106 (+)	0.151 (+) 0.143 (+)	0.143 (+) 0.535 (+)	0.147 (+) 0.157 (+)
J (2.040 ng/mL)	0.092 (+) 0.092 (+)	0.098 (+) 0.088 (+)	0.089 (+) 0.087 (+)	0.116 (+) 0.180 (+)	0.092 (+) 0.078 (+)	0.091 (+) 0.109 (+)
A (3.674 ng/mL)	0.170 (+) 0.160 (+)	0.175 (+) 0.172 (+)	0.168 (+) 0.139 (+)	0.182 (+) 0.186 (+)	0.158 (+) 0.152 (+)	0.164 (+) 0.169 (+)
E (3.999 ng/mL)	0.335 (+) 0.280 (+)	0.335 (+) 0.311 (+)	0.295 (+) 0.286 (+)	0.538 (+) 0.336 (+)	0.331 (+) 0.295 (+)	0.337 (+) 0.314 (+)

[a] Cutoff Value = Negative Control Mean + 0.025

[b] + indicates that value is ≥ the cutoff value and sample is positive for HBsAG

Table 8. Comparison of EIA and RIA Methods

Sensitivity Panel Sample	EIA	RIA
H (0.00 ng/mL)	100 % Nonreactive	100 % Nonreactive
F (0.065 ng/mL)	0% Reactive	0% Reactive
G (0.068 ng/mL)	17% Reactive	0% Reactive
M (0.124 ng/mL)	8% Reactive	0% Reactive
Q (0.131 ng/mL)	8% Reactive	0% Reactive
L (0.230 ng/mL)	8% Reactive	8% Reactive
D (0.274 ng/mL)	0% Reactive	0% Reactive
S (0.485 ng/mL)	100% Reactive	8% Reactive
P (0.525 ng/mL)	75% Reactive	0% Reactive
R (0.730 ng/mL)	92% Reactive	17% Reactive
B (0.784 ng/mL)	100% Reactive	8% Reactive
C (0.917 ng/mL)	100% Reactive	0% Reactive
N (1.002 ng/mL)	100% Reactive	33% Reactive
K (1.904 ng/mL)	100% Reactive	50% Reactive
J (2.040 ng/mL)	100% Reactive	83% Reactive
A (3.674 ng/mL)	100% Reactive	100% Reactive
E (3.999 ng/mL)	100% Reactive	100% Reactive

Appendix 1

Pool Data for Retrospective Study

HEPATITIS ANTIGEN POOLS 1998

			PRODUCT				NEGATIVE		POSITIVE
LOT#	DATE	TECH	1	2	3	Mean Absorbance	CONTROL	CUT-OFF	CONTROL
EE0064	1/1/99	SCB	0.008	0.006	0.036	0.017	-0.001	0.024	1.098
EE0065	1/4/99	JDD	0.013	0.003	0.003	0.006	0.004	0.029	1.026
EE0066	1/5/99	DRYK	0.005	0.001	0.01	0.005	0.002	0.027	0.874
EE0067	1/6/99	JAL	0.015	0.002	0.015	0.011	0.014	0.039	0.948
EE0068	1/8/99	JM	0.01	0.009	0.006	0.008	0.007	0.032	0.829
EE0069	1/8/99	JDD	-0.001	0.004	0.001	0.001	0.001	0.026	0.852
EE0070	1/9/99	JCR	0.023	0.001	0.016	0.013	0.002	0.027	0.983
EE0071	1/9/99	JCR	0.005	0.003	0.003	0.004	0.007	0.032	0.964
EE0072	1/12/99	DRYK	0.007	0.006	0.014	0.009	0.008	0.033	0.839
EE0073	1/13/99	SCB	0	0.002	0.007	0.003	0.001	0.026	0.896
EE0075	1/15/99	JAL	0.002	-0.003	0.006	0.002	0	0.025	0.903
EE0074	1/14/99	DRYK	0.007	0.007	0.015	0.010	0.01	0.035	0.856
EE0076	1/16/99	JCR	0	0.007	0.006	0.004	0.006	0.031	0.955
EE0077	1/20/99	JDD	0.012	0.016	0.023	0.017	0.008	0.033	0.983
EE0078	1/21/99	JDD	-0.002	-0.004	-0.001	-0.002	0.003	0.028	0.964
EE0079	1/24/99	JDD	0	-0.003	0.007	0.001	0.007	0.032	0.921
EE0080	1/27/99	MKM	0.007	0	0.008	0.005	0.002	0.027	0.834
EE0081	1/27/99	JDD	0.002	0.004	0.004	0.003	0.001	0.026	0.905
EE0082	1/28/99	JM	0.001	0.001	0.009	0.004	0.004	0.029	0.861
EE0084	1/29/99	JLK	0.012	0.011	0.01	0.011	0.009	0.034	0.854
EE0083	1/28/99	TAS	0.016	0.014	0.008	0.013	0.01	0.035	1
EE0085	1/29/99	TAS	0	0.005	0.007	0.004	0.01	0.0354	1.096
EE0086	2/2/99	JLK	0.003	-0.001	0.006	0.003	0.003	0.028	0.828
EE0087	2/3/99	MKM	0.005	0.007	0.009	0.007	0.005	0.03	0.805
EE0088	2/3/99	TAS	0.008	0.005	0.005	0.006	0.003	0.028	0.874
EE0089	2/4/99	THC	0.003	0.002	0.002	0.002	0	0.025	0.824
EE0090	2/4/99	JLK	0.002	0.004	0.008	0.005	0.004	0.029	0.978
EE0091	2/5/99	JAL	0.033	0.041	0.023	0.032	0.006	0.031	0.954
EE0092	2/10/99	JLK	0.01	0.004	0.008	0.007	0.012	0.037	0.879
EE0093	2/11/99	JDD	0.007	0.009	0.013	0.010	0.005	0.03	0.817
EE0094	2/12/99	KTM	0.011	0.01	0.002	0.008	0.005	0.03	0.916
EE0095	2/12/99	JLK	0.03	0.03	0.037	0.032	0.005	0.03	0.828
EE0096	2/13/99	JLK	0.002	-0.001	0.015	0.005	-0.003	0.022	0.912
EE0097	2/14/99	JAL	0.011	0.009	0.004	0.008	0.008	0.033	0.916
EE0098	2/15/99	JLK	-0.001	0.002	0.008	0.003	0.015	0.04	0.902
EE0099	2/16/99	MKM	0.007	0.01	0.003	0.007	0.007	0.032	0.696
EE0100	2/16/99	JLK	0.022	0.017	0	0.013	0.016	0.041	0.909
EE0101	2/17/99	EAP	0.006	0.011	0.006	0.008	0.007	0.032	0.862
EE0102	2/17/99	DRYK	0.014	0.01	0.003	0.009	0.004	0.029	0.85
EE0103	2/19/99	THC	0.007	0.001	0.033	0.014	0.007	0.032	0.99
EE0104	2/18/99	DRYK	0.003	0.001	0.014	0.006	0.006	0.031	0.856
EE0105	2/19/99	MKM	-0.002	0.004	0.01	0.004	0.003	0.028	0.829
EE0106	2/22/99	TAS	-0.003	-0.003	-0.001	-0.002	0.01	0.035	0.983
EE0108	2/23/99	TAS	0.014	0.008	0.026	0.016	0.009	0.034	0.836
EE0109	2/24/99	SCB	0.001	-0.002	0.01	0.003	0.008	0.033	0.918
EE0110	2/24/99	TAS	0.008	0.012	0.013	0.011	0.011	0.036	0.836
EE0111	2/25/99	JAL	0.002	0.004	0.003	0.003	0	0.025	0.85
EE0112	2/25/99	BT	0	0.001	0.007	0.003	0.006	0.031	0.964
EE0113	2/26/99	JDD	0.01	0.009	0.002	0.007	0.002	0.027	0.842
EE0121	3/9/99	SCB	0.014	0.01	0.004	0.009	0.004	0.029	0.751
EE0122	3/9/99	BT	0.004	0.012	0.015	0.010	0.002	0.027	0.737
EE0125	3/11/99	JLK	0.017	0.013	0.003	0.011	0.016	0.041	0.969
EE0126	3/16/99	BT	0.005	0	0.004	0.003	0.003	0.028	0.962
EE0127	3/17/99	JM	0.005	0.012	0.017	0.011	0.01	0.035	0.817
EE0128	3/17/99	JLK	0.014	0.013	0.005	0.011	0.008	0.033	0.945
EE0129	3/18/99	KTM	0	-0.001	0.002	0.000	0.003	0.028	0.869

			PRODUCT				NEGATIVE		POSITIVE
LOT#	DATE	TECH	1	2	3	Mean Absorbance	CONTROL	CUT-OFF	CONTROL
EE0131	3/19/99	MKM	0.02	-0.003	0.016	0.011	0.006	0.031	1.001
EE0132	4/23/99	BT	0.004	-0.005	0.007	0.002	-0.001	0.024	0.713
EE0133	4/24/99	TAS	0.001	0.002	0.001	0.001	0.002	0.027	0.781
EE0134	4/25/99	JLK	0.002	0.001	0.006	0.003	-0.001	0.024	0.778
EE0135	4/27/99	NM	0.003	0.005	0.012	0.007	0.004	0.029	0.751
EE0136	4/29/99	JLK	0.011	0.019	0.023	0.018	0.007	0.032	0.564
EE0138	4/30/99	BT	0.007	0.008	0.009	0.008	0	0.025	0.568
EE0139	5/1/99	TAS	0.001	0.002	0.005	0.003	0.007	0.032	0.709
EE0140	5/3/99	DRYK	0.003	0.003	0.01	0.005	0.001	0.026	0.651
EE0141	5/4/99	JM	0.001	0.007	0.005	0.004	0.003	0.028	0.751
EE0142	5/5/99	DRYK	0	0.008	0.001	0.003	0.001	0.026	0.655
EE0143	5/6/99	JM	0.011	-0.002	-0.004	0.002	-0.004	0.021	0.749
EE0144	5/11/99	JLK	0.009	0.003	0.009	0.007	0.002	0.027	0.668
EE0145	5/12/99	JM	0.008	0.008	0.009	0.008	0.01	0.035	0.51
EE0146	5/13/99	DRYK	0.003	0.015	0.002	0.007	0.004	0.029	0.584
EE0147	5/14/99	KTM	0.016	0.003	0.004	0.008	0.008	0.033	0.9
EE0148	5/15/99	TAS	0.006	0.006	0.012	0.008	0.004	0.029	0.827
EE0149	5/18/99	DRYK	-0.003	-0.001	0.005	0.000	0.004	0.029	0.751
EE0150	5/19/99	MKM	0.003	0.003	0.005	0.004	0.005	0.03	0.733
EE0151	5/20/99	SGK	0.007	0.008	0.007	0.007	0.005	0.03	0.73
EE0152	5/21/99	KTM	-0.003	0.009	0.001	0.002	0.002	0.027	0.757
EE0153	5/22/99	JDD	0.002	0.002	0.005	0.003	0.002	0.027	0.781
EE0154	5/25/99	TAS	0.011	0.006	0.002	0.006	0.002	0.027	0.771
EE0155	5/26/99	JM	0.006	0.005	0.009	0.007	0.005	0.03	0.561
EE0156	5/27/99	JDD	0.001	0.004	0.006	0.004	0.004	0.029	0.816
EE0157	5/28/99	MKM	0.007	0.005	0.001	0.004	0.007	0.032	0.562
EE0158	5/29/99	TAS	0.002	0.007	0.011	0.007	0.005	0.03	0.826
EE0159	6/1/99	BTR	0.016	0.013	0.019	0.016	0.008	0.033	0.731
EE0160	6/2/99	JM	-0.001	0.007	0.002	0.003	0.001	0.026	0.774
EE0161	6/3/99	BTR	0.002	-0.002	0.002	0.001	0.001	0.025	0.537
EE0162	6/4/99	JM	0.01	0.001	0.007	0.006	0.009	0.034	0.665
EE0163	6/5/99	TAS	0	-0.001	0.002	0.000	0.002	0.027	0.757
EE0164	6/8/99	BTR	0.001	-0.003	0	-0.001	-0.002	0.023	0.65
EE0165	6/9/99	JM	0.005	-0.003	0.007	0.003	0.004	0.029	0.671
EE0167	6/12/99	JLK	0	-0.001	0.004	0.001	0	0.025	0.933
EE0168	6/15/99	MKM	0.006	0.01	0.005	0.008	0.007	0.032	0.979
EE0170	6/16/99	KPG	0.006	0.001	0.007	0.005	0.006	0.031	0.959
EE0171	6/17/99	TS	0.006	0.003	0.002	0.004	0.006	0.033	0.983
EE0172	6/18/99	JM	0.004	0.006	0.08	0.030	0.005	0.03	0.941
EE0173	6/22/99	TS	0.006	0.001	0.008	0.005	0.005	0.03	0.883
EE0174	6/23/99	CJ	-0.003	-0.003	-0.006	-0.004	0.003	0.028	0.891
EE0175	6/24/99	JDD	0.005	0.007	0.005	0.006	0.007	0.032	0.898
EE0176	6/25/99	KTM	0.008	0.025	0.002	0.012	0.003	0.028	0.858
EE0177	6/28/99	BTR	0.001	0.005	0.001	0.002	0.002	0.027	0.944
100					Mean	0.007	0.005	0.030	0.835
					S.D.	0.006	0.004	0.004	0.124
					% c.v	91.093	80.309	13.133	14.890

Bayer Corporation
Pharmaceutical Division
Biological Products
Clayton, NC

T.2.53-01R

Validation Report of Test Methods/Other Methods Revision 1

Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools

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Table of Contents

Table 1: Summary of Assay Characteristics and Acceptance Criteria for Validation of the procedure "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools"

Characteristic	Validation Design	Statistics to Report	Actual Results
Precision (Repeatability)	Absorbance determined for 3 plasma pools, kit controls and HIV control panel	Mean, Standard Deviation, % CV	Operator 1 Kit Negative Control: Mean 0.009, SD 0.004, % CV 44.4 Kit HIV-1 Positive Control: 1.208, 0.134, 11.3 Kit HIV-2 Positive Control: 0.788, 0.050, 6.3 Plasma Pool 1: 0.009, 0.004, 44.1 Plasma Pool 2: 0.009, 0.004, 49.3 Plasma Pool 3: 0.006, 0.005, 71.2 HIV-1 High: 1.464, 0.070, 4.8 HIV-1 Low: 0.454, 0.020, 4.4 HIV-2 High: 0.978, 0.045, 4.6 HIV-2 Low: 0.311, 0.014, 4.4 HIV Negative: 0.012, 0.004, 33.3 Operator 2 Kit Negative Control: Mean 0.012, SD 0.005, % CV 41.7 Kit HIV -1 Positive Control: 1.084, 0.122, 11.3 Kit HIV -2 Positive Control: 0.772, 0.038, 4.9 Plasma Pool 1: 0.012, 0.004, 41.4 Plasma Pool 2: 0.010, 0.004, 44.9 Plasma Pool 3: 0.010, 0.005, 50.2 HIV-1 High: 1.762, 0.305, 17.3 HIV-1 Low: 0.594, 0.154, 25.9 HIV-2 High: 0.900, 0.057, 6.3 HIV-2 Low: 0.285, 0.022, 7.9 HIV Negative: 0.012, 0.006, 50
Precision (Intermediate)	Retrospective Data from 100 negative pools, kit negative control, and kit positive control	Mean, Standard Deviation, % CV	Mean, SD, % CV Pool: 0.011, 0.004, 37.2 Negative Control: 0.013, 0.005, 37 HIV-1 Positive Control: 0.985, 0.160, 16.2 HIV-2 Positive Control: 0.920, 0.135, 14.7
Specificity	Comparison of kit positive control to kit negative control	Fold-Difference	HIV-1: Operator 1 134-fold, Operator 2 90-fold Retrospective Pool Data: 76-fold (multiple operators) HIV-2: Operator 1 88-fold, Operator 2 64-fold Retrospective Pool Data: 71-fold (multiple operators)
Detection Limit	Comparison of kit negative control to cutoff	Fold-Difference	Operator 1 12-fold, Operator 2 9-fold Retrospective Pool Data: 1.8-fold (multiple operators)

Summary

The purpose of this study is to validate the method for detection of antibody to Human Immunodeficiency virus types 1 and 2 (HIV 1/2) in plasma pools. This method is described in the procedure "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools". It is an *in vitro* enzyme immunoassay (EIA) for the qualitative detection of antibodies to HIV-1 and HIV-2 in pools of human plasma.

Introduction

The purpose of this document is to describe the characteristics evaluated in order to validate the method "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools" and to report the results of the validation study in order to determine the suitability of the method for the determination of HIV-1 and HIV-2 in plasma pools.

Principle

In this method, the Abbott HIVAB® HIV-1/HIV-2 EIA 2.0 uses recombinant DNA-derived antigens corresponding to HIV-1 core and envelope and HIV-2 envelope proteins. These antigens will react with either or both antibody specificities present in human plasma. According to the package insert, Abbott HIVAB EIA was developed to allow the simultaneous detection of IgG and IgM antibodies to HIV-1 and HIV-2 to identify potentially reactive individual plasma units. All units comprising plasma pools made in Clayton have already been screened by this method. This procedure is designed for use with pooled plasma, and as such is not modified from the original FDA-licensed assay.

Validation Testing Facility

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Quality Assurance
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Description of Products Being Tested

Plasma pools were created using 50 or more individual units.

VIROTROL SCREENING Panel 1 is a testing panel made by Blackhawk BioSystems, Inc. It is composed of 3 samples that have been found to reactive or nonreactive using this test. The samples are: High Reactive Viral Marker, positive for HIV-1; Low Reactive Viral

Marker, positive for HIV-1 to a lesser extent; HIV-2 Level II, positive for HIV-2; HIV-2 Level I, positive to HIV-2 to a lesser extent; and, VIROCLEAR, negative for HIV types 1 and 2.

Validation Design

Precision (Repeatability): Three plasma pool samples and two control panels (one for each of the two virus types) consisting of a high reactive sample, a low reactive sample, and a negative sample were tested by each of two operators in triplicate on six separate days.

Precision (Intermediate): One-hundred negative plasma pools and their respective kit controls (positive and negative) were tested by multiple operators over a period of approximately 6 months.

Specificity: Specificity was addressed by comparing the kit negative control to the kit positive controls. In addition, kit negative and positive controls were compared from the retrospective data from the testing of 100 negative pools.

Detection Limit: The lower limit of detection is set by the cutoff value, which is defined by the kit manufacturer (Abbott). Since this is not a quantitative test, a numerical value cannot be placed on this response level. However, as part of the commercial antibody panel, samples designated as weakly positive were include in the study, to assess the sensitivity of the assay. In addition, a comparison was made between the cutoff value and the kit negative control absorbances from both the original validation and the retrospective pool data.

Discussion of Experimental Results

Precision (Repeatability) was determined by analyzing three plasma pool samples and one control panel consisting of an HIV-1 high reactive sample, HIV-2 high reactive sample, an HIV-1 low reactive sample, HIV-2 low reactive sample, and a negative (nonreactive) sample. All samples for each lot were analyzed by each of two operators on six separate days (Tables 2-5). The data is summarized in Table 6. All positive and negative kit controls were within kit specifications. Samples with low absorbance values (negative kit control, plasma pools, and nonreactive control panel sample) yielded % c.v.'s (coefficient of variation) that were greater than 30% (33.3% to 71.2%). For the kit controls, the % c.v.'s ranged from 4.4% to 25.9%.

Precision (Intermediate) was determined by evaluating retrospective data from 100 pools that tested negative for antibody to HIV type 1 and HIV type 2 and the kit controls (negative and positive) that were included with each determination. The % c.v.'s were approximately 16% for the HIV-1 positive control and 15% for the HIV-2 positive control (Table 7). For both the negative control and the 100 plasma pool samples, the % c.v. was 37%. This higher %c.v. is reflective of the low absorbance values for the negative control and the pool samples.

Specificity of the assay was demonstrated by the ratio of the mean of the positive control values to the mean of the negative control values, as summarized in Table 8. The HIV-1 positive control was approximately 134-fold higher than the negative control for Operator 1 and 90-fold higher for Operator 2. For the HIV-2 positive control, the ratios were 88-fold and 64-fold for Operators 1 and 2, respectively. In addition, the positive and negative control samples were compared from the 100 retrospective pool tests (Table 7). In the retrospective study, the HIV-1 positive control was 76-fold higher than the negative control, and the HIV-2 positive control was 71-fold higher than the negative control. Both sets of data show that there is ample spread between the positive and negative samples to yield confidence in assigning a sample as either reactive or nonreactive.

Detection Limit was assessed by comparing the absorbance values from the HIV control panel low reactive samples to the cutoff value and by comparing the cutoff value to the kit negative control. None of the 24 replicates of the HIV-1 low reactive samples and none of the 24 replicates of the HIV-2 low reactive samples fell below the cutoff value. The data from the original validation show that the cutoff value is approximately 9- to 12-fold higher than the negative control (Table 8).

To increase the sensitivity of the assay QA Change B0070 was initiated in May 1996. This QA change proposed modifying the calculation of the cutoff value. The cutoff value was originally calculated by: negative control mean + 0.100 (based on the kit's package insert). QA change B0070 changed the calculation of the cutoff value to: negative control mean + 0.010. The experimental data from the 300 pools evaluated for QA change B0070 supported tightening of the specification. QA change B0070 was adopted in July 1996. Therefore, the cutoff value used for the retrospective 100 negative pool study was calculated as follows: negative control mean + 0.010. For the retrospective 100 negative pools, the cutoff value is 1.8-fold higher than the negative control (Table 7).

Conclusions

Validation of the method CQAB 08-056 "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools" was accomplished by running a series of experiments that assessed the following characteristics: Precision, Specificity and Detection Limit. "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools", CQAB 08-056, is considered validated and suitable for its intended purposes.

References

1. CQAP 202 "Validation of Analytical Methods", Revision 3.
2. ICH Harmonized Tripartite Guideline on Validation of Analytical Procedures", May 1997

Attachments

Table 1:	Summary of Assay Characteristics and Acceptance Criteria for Validation of the procedure "Qualitative Determination of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools"
Table 2:	Operator 1: Absorbance Values for Samples Assayed Day1, Day 2, and Day 3
Table 3	Operator 1: Absorbance Values for Samples Assayed Day 4, Day 5, and Day 6
Table 4	Operator 2: Absorbance Values for Samples Assayed Day1, Day 2, and Day 3
Table 5	Operator 2: Absorbance Values for Samples Assayed Day1, Day 2, and Day 3
Table 6	Precision (Repeatability): Statistical Summary of Original Validation Data
Table 7	Precision (Intermediate), Specificity and Detection Limit: Statistical Summary of Retrospective Data and Comparison of Controls
Table 8	Specificity and Detection Limit: Comparison of Positive and Negative Controls
Appendix 1	Pool Data for Retrospective Study

Table 2: Operator 1: Absorbance Values for Samples Assayed Day1, Day 2, and Day 3

Sample		Day 1	Day 2	Day 3
Kit Negative Control:	Well 1	0.008	0.016	0.012
	Well 2	0.009	0.001	0.006
	Well 3	0.010	0.007	0.002
Mean		0.009	0.008	0.007
Kit Positive Control, HIV-1:	Well 1	1.155	1.071	1.217
	Well 2	1.114	1.089	1.176
Mean		1.135	1.080	1.197
Kit Positive Control, HIV-2:	Well 1	0.791	0.718	0.787
	Well 2	0.814	0.736	0.806
Mean		0.803	0.727	0.797
Plasma Pool 1:	Well 1	0.016	0.011	0.004
	Well 2	0.003	0.014	0.010
	Well 3	0.009	0.007	0.013
Mean		0.009	0.011	0.009
Plasma Pool 2:	Well 1	0.002	0.009	0.004
	Well 2	0.006	0.004	0.004
	Well 3	0.016	0.010	0.005
Mean		0.008	0.008	0.004
Plasma Pool 3:	Well 1	0.006	0.006	0.008
	Well 2	-0.006	0.009	0.006
	Well 3	0.008	0.016	0.002
Mean		0.003	0.010	0.005
Commercial Panel: HIV-1 High Reactive	Well 1	1.463	1.361	1.470
	Well 2	1.495	1.347	1.432
	Well 3	1.480	1.446	1.505
Mean		1.479	1.385	1.469
Commercial Panel: HIV-1 Low Reactive	Well 1	0.444	0.441	0.468
	Well 2	0.461	0.456	0.460
	Well 3	0.420	0.455	0.444
Mean		0.442	0.451	0.457
Commercial Panel: HIV-2 High Reactive	Well 1	0.970	0.958	0.942
	Well 2	1.008	1.009	0.965
	Well 3	0.943	1.019	0.977
Mean		0.974	0.995	0.961
Commercial Panel: HIV-2 Low Reactive	Well 1	0.334	0.307	0.311
	Well 2	0.294	0.313	0.297
	Well 3	0.291	0.318	0.317
Mean		0.306	0.313	0.308
Commercial Panel: Negative (Nonreactive)	Well 1	0.008	0.012	0.007
	Well 2	0.010	0.011	0.010
	Well 3	0.011	0.012	0.007
Mean		0.010	0.012	0.008
Cutoff Value[a]		0.109	0.108	0.107

[a] Cutoff Value = Negative Control Mean + 0.100

Table 3: Operator 1: Absorbance Values for Samples Assayed Day 4, Day 5, and Day 6

Sample		Day 4	Day 5	Day 6
Kit Negative Control:	Well 1	0.004	0.012	0.010
	Well 2	0.014	0.009	0.014
	Well 3	0.007	0.011	0.009
Mean		0.008	0.011	0.011
Kit Positive Control, HIV-1:	Well 1	1.121	1.271	1.258
	Well 2	1.115	1.309	1.204
Mean		1.118	1.290	1.231
Kit Positive Control, HIV-2:	Well 1	0.807	0.893	0.707
	Well 2	0.814	0.805	0.774
Mean		0.811	0.849	0.741
Plasma Pool 1:	Well 1	0.010	0.009	0.008
	Well 2	0.009	0.011	0.004
	Well 3	0.015	0.004	0.005
Mean		0.011	0.008	0.006
Plasma Pool 2:	Well 1	0.007	0.012	0.010
	Well 2	0.013	0.016	0.007
	Well 3	0.012	0.012	0.006
Mean		0.011	0.013	0.008
Plasma Pool 3:	Well 1	0.009	0.006	0.006
	Well 2	0.008	0.009	0.003
	Well 3	0.002	0.010	0.004
Mean		0.006	0.009	0.004
Commercial Panel: HIV-1 High Reactive	Well 1	1.400	1.536	1.393
	Well 2	1.420	1.532	1.527
	Well 3	1.421	1.627	1.496
Mean		1.414	1.566	1.472
Commercial Panel: HIV-1 Low Reactive	Well 1	0.424	0.486	0.458
	Well 2	0.433	0.491	0.463
	Well 3	0.447	0.483	0.444
Mean		0.435	0.487	0.455
Commercial Panel: HIV-2 High Reactive	Well 1	0.915	1.001	0.941
	Well 2	0.888	1.078	0.997
	Well 3	0.964	1.025	1.001
Mean		0.922	1.035	0.980
Commercial Panel: HIV-2 Low Reactive	Well 1	0.308	0.338	0.297
	Well 2	0.298	0.323	0.302
	Well 3	0.309	0.330	0.313
Mean		0.305	0.330	0.304
Commercial Panel: Negative (Nonreactive)	Well 1	0.012	0.017	0.017
	Well 2	0.015	0.015	0.008
	Well 3	0.010	0.019	0.009
Mean		0.012	0.017	0.011
Cutoff Value[a]		0.108	0.111	0.111

[a] Cutoff Value = Negative Control Mean + 0.100

Table 4: Operator 2: Absorbance Values for Samples Assayed Day1, Day 2, and Day 3

Sample		Day 1	Day 2	Day 3
Kit Negative Control:	Well 1	0.017	0.009	0.016
	Well 2	0.013	0.011	0.012
	Well 3	0.014	0.007	0.013
Mean		0.015	0.009	0.014
Kit Positive Control, HIV-1:	Well 1	1.094	0.961	0.997
	Well 2	0.974	0.880	1.019
Mean		1.034	0.921	1.008
Kit Positive Control, HIV-2:	Well 1	0.776	0.750	0.712
	Well 2	0.791	0.755	0.832
Mean		0.784	0.753	0.772
Plasma Pool 1:	Well 1	0.017	0.014	0.016
	Well 2	0.015	0.018	0.010
	Well 3	0.013	0.012	0.016
Mean		0.015	0.015	0.014
Plasma Pool 2:	Well 1	0.012	0.008	0.010
	Well 2	0.013	0.008	0.015
	Well 3	0.015	0.015	0.009
Mean		0.013	0.010	0.012
Plasma Pool 3:	Well 1	0.019	0.007	0.010
	Well 2	0.012	0.008	0.008
	Well 3	0.020	0.002	0.014
Mean		0.017	0.006	0.011
Commercial Panel: HIV-1 High Reactive	Well 1	2.077	2.008	2.090
	Well 2	2.200	1.956	2.021
	Well 3	2.052	2.012	2.019
Mean		2.110	1.992	2.043
Commercial Panel: HIV-1 Low Reactive	Well 1	0.795	0.707	0.715
	Well 2	0.776	0.712	0.727
	Well 3	0.783	0.708	0.745
Mean		0.785	0.709	0.729
Commercial Panel: HIV-2 High Reactive	Well 1	0.899	0.844	0.883
	Well 2	0.849	0.907	0.892
	Well 3	0.887	0.856	0.839
Mean		0.878	0.869	0.871
Commercial Panel: HIV-2 Low Reactive	Well 1	0.303	0.254	0.265
	Well 2	0.293	0.286	0.261
	Well 3	0.271	0.300	0.288
Mean		0.289	0.280	0.271
Commercial Panel: Negative (Nonreactive)				
Well 1		0.016	0.015	0.012
Well 2		0.018	0.014	0.005
Well 3		0.013	0.018	0.014
Mean		0.016	0.016	0.010
Cutoff Value[a]		0.115	0.109	0.114

[a] Cutoff Value = Negative Control Mean + 0.100

Table 5: Operator 2: Absorbance Values for Samples Assayed Day 4, Day 5, and Day 6

Sample		Day 4	Day 5	Day 6
Kit Negative Control:	Well 1	0.014	0.003	0.016
	Well 2	0.015	0.009	0.014
	Well 3	0.016	0.002	0.017
Mean		0.015	0.005	0.016
Kit Positive Control, HIV-1:	Well 1	1.240	1.140	1.132
	Well 2	1.249	1.081	1.235
Mean		1.245	1.111	1.184
Kit Positive Control, HIV-2:	Well 1	0.789	0.723	0.803
	Well 2	0.824	0.743	0.764
Mean		0.807	0.733	0.784
Plasma Pool 1:	Well 1	0.010	0.003	0.009
	Well 2	0.008	0.000	0.011
	Well 3	0.014	0.008	0.015
Mean		0.011	0.004	0.012
Plasma Pool 2:	Well 1	0.013	-0.003	0.010
	Well 2	0.012	0.009	0.010
	Well 3	0.010	0.004	0.007
Mean		0.012	0.003	0.009
Plasma Pool 3:	Well 1	0.009	0.008	0.004
	Well 2	0.009	0.005	0.008
	Well 3	0.015	0.012	0.005
Mean		0.011	0.008	0.005
Commercial Panel: HIV-1 High Reactive	Well 1	1.534	1.410	1.481
	Well 2	1.535	1.346	1.468
	Well 3	1.644	1.373	1.496
Mean		1.571	1.376	1.482
Commercial Panel: HIV-1 Low Reactive	Well 1	0.476	0.409	0.442
	Well 2	0.492	0.417	0.435
	Well 3	0.486	0.427	0.448
Mean		0.485	0.418	0.442
Commercial Panel: HIV-2 High Reactive	Well 1	1.047	0.900	0.934
	Well 2	1.002	0.846	0.907
	Well 3	0.971	0.868	0.874
Mean		1.007	0.871	0.905
Commercial Panel: HIV-2 Low Reactive	Well 1	0.310	0.264	0.269
	Well 2	0.312	0.278	0.290
	Well 3	0.342	0.264	0.284
Mean		0.321	0.269	0.281
Commercial Panel: Negative (Nonreactive)	Well 1	0.018	0.005	0.003
	Well 2	0.019	0.008	0.019
	Well 3	0.016	0.003	0.007
Mean		0.018	0.005	0.010
Cutoff Value[a]		0.115	0.105	0.116

[a] Cutoff Value = Negative Control Mean + 0.100

Table 6. Precision (Repeatability): Statistical Summary of Original Validation Data [a]

	Operator 1			Operator 2		
Sample	**Mean**	**S.D.**	**% c.v.**	**Mean**	**S.D**	**% c.v.**
Kit Negative Control	0.009	0.004	44.4	0.012	0.005	41.7
Kit HIV-1 Positive Control	1.208	0.134	11.1	1.084	0.122	11.3
Kit HIV-2 Positive Control	0.788	0.050	6.3	0.772	0.038	4.9
Plasma Pool 1	0.009	0.004	44.1	0.012	0.005	41.4
Plasma Pool 2	0.009	0.004	49.3	0.010	0.004	44.9
Plasma Pool 3	0.006	0.005	71.2	0.010	0.005	50.2
HIV-1 High Reactive	1.464	0.070	4.8	1.762	0.305	17.3
HIV-1 Low Reactive	0.454	0.020	4.4	0.594	0.154	25.9
HIV-2 High Reactive	0.978	0.045	4.6	0.900	0.057	6.3
HIV-2 Low Reactive	0.311	0.014	4.4	0.285	0.022	7.9
Panel Negative (Nonreactive)	0.012	0.004	33.3	0.012	0.006	50
Cutoff Value[b]	0.109	0.002	1.8	0.112	0.004	3.6

[a] Summary of data from Tables 2-5

[b] Cutoff Value = Negative Control Mean + 0.100

Table 7. Precision (Intermediate), Specificity and Detection Limit: Statistical Summary of Retrospective Data and Comparison of Controls[a]

Sample	Mean	S.D	% c.v.
Kit Negative Control	0.013	0.005	37
Kit HIV-1 Positive Control	0.985	0.160	16.2
Kit HIV-2 Positive Control	0.920	0.135	14.7
Plasma Pool	0.011	0.004	37.2
Cutoff Value[b]	0.023	0.005	21.2

Ratio: HIV-1 Negative/Positive	1.3%
Ratio: HIV-2 Negative/Positive	1.4%
Fold Difference: HIV-1 Positive/ Negative	76-fold
Fold Difference: HIV-2 Positive/ Negative	71-fold
Fold Difference: HIV-1 Positive/Cutoff	43-fold
Fold Difference: HIV-2 Positive/Cutoff	40-fold
Fold Difference: Cutoff/Negative	1.8-fold

[a] Individual pool and control data is in Appendix A
[b] Cutoff Value = Negative Control Mean + 0.010

Table 8. Specificity and Detection Limit: Comparison of Positive and Negative Controls

Control Sample	Operator 1	Operator 2
Negative Control Mean	0.009	0.012
Positive Control, HIV-1	1.208	1.084
Positive Control, HIV-2	0.788	0.772
Ratio: HIV-1 Negative/Positive	0.7%	1.1%
Ratio: HIV-2 Negative/Positive	1.1%	1.6%
Fold Difference: HIV-1 Positive/Negative	134-fold	90-fold
Fold Difference: HIV-2 Positive/Negative	88-fold	64-fold
Cutoff Value[a]	0.109	0.112
Fold Difference: HIV-1 Positive/Cutoff	11-fold	10-fold
Fold Difference: HIV-2 Positive/Cutoff	7-fold	7-fold
Cutoff/Negative	12-fold	9-fold

[a] Cutoff Value = Negative Control Mean + 0.100

Appendix 1

Pool Data for Retrospective Study

HIV 1/2 ANTIBODY

PRODUCT **NEGATIVE** **POSITIVE** **POSITIVE**

LOT #	DATE	TECH	1	2	3	Mean Absorbance	CONTROL	CUT-OFF	CONTROL 1	CONTROL 2
EE0064	1/1/99	KTM	0.014	0.014	0.02	0.016	0.018	0.028	1.141	0.988
EE0065	1/4/99	DRYK	0.011	0.002	0.008	0.007	0.015	0.025	1.082	0.839
EE0066	1/5/99	JDD	0.02	0.015	0.019	0.018	0.016	0.026	1.152	0.978
EE0067	1/6/99	JAL	0.024	0.014	0.014	0.017	0.018	0.028	1.057	0.988
EE0068	1/8/99	EAP	0.008	0.017	0.009	0.011	0.013	0.023	1.031	0.956
EE0069	1/8/99	JDD	0.013	0.008	0.015	0.012	0.021	0.031	1.022	0.907
EE0070	1/9/99	JDD	0.025	0.016	0.013	0.018	0.017	0.027	0.755	0.658
EE0071	1/9/99	JDD	0.013	0.01	0.027	0.017	0.012	0.022	0.805	0.82
EE0072	1/12/99	JDD	0.017	0.019	0.015	0.017	0.019	0.029	1.287	1.229
EE0073	1/13/98	JM	0.01	0.01	0.008	0.009	0.013	0.023	1.313	1.196
EE0074	1/14/99	JDD	0.016	0.009	0.008	0.011	0.017	0.027	1.319	1.091
EE0075	1/15/99	JM	0.009	0.007	0.015	0.010	0.017	0.027	1.333	1.092
EE0076	1/16/99	SCB	0.011	0.01	0.015	0.012	0.007	0.017	1.365	1.136
EE0077	1/20/99	BT	0.02	0.008	0.011	0.013	0.013	0.023	1.144	1.401
EE0078	1/21/99	BT	0.003	0.012	0.009	0.008	0.004	0.014	1.25	1.063
EE0079	1/24/99	BT	0.015	0.008	0.014	0.012	0.006	0.016	0.625	0.666
EE0080	1/27/99	JM	0.018	0.018	0.015	0.017	0.019	0.029	1.023	0.811
EE0081	1/27/99	BT	0.017	0.01	0.005	0.011	0.014	0.024	0.981	0.925
EE0082	1/28/99	KTM	0	0.004	-0.001	0.001	0.004	0.014	0.92	0.869
EE0083	1/28/99	JDD	0.009	0.005	0.01	0.008	0.02	0.03	1.058	0.96
EE0084	1/29/99	KTM	0.01	0.017	0.0105	0.013	0.023	0.033	0.894	0.976
EE0085	1/29/99	JDD	0.016	0.014	0.016	0.015	0.019	0.029	1.057	0.991
EE0086	2/2/99	BT	0.01	0	0.004	0.005	0.005	0.015	0.802	0.872
EE0087	2/3/99	JM	0.017	0.018	0.008	0.014	0.023	0.033	1.024	0.943
EE0088	2/3/99	JLK	0.006	0.006	0.015	0.009	0.018	0.028	0.886	0.833
EE0089	2/4/99	EAP	0.012	0.008	0.013	0.011	0.016	0.026	0.71	0.797
EE0090	2/4/99	BT	0.001	0.02	0.013	0.011	0.02	0.03	0.805	0.794
EE0091	2/5/99	JM	0.014	0.001	0.014	0.010	0.014	0.024	0.839	0.76
EE0092	2/10/99	JDD	0.017	0.013	0.008	0.013	0.014	0.024	0.933	0.894
EE0093	2/11/99	JLK	0.028	0.014	0.02	0.021	0.026	0.036	0.767	0.746
EE0094	2/12/99	JAL	0.022	0.008	0.015	0.015	0.013	0.023	0.883	0.818
EE0095	2/12/99	JDD	0.008	0.004	0.01	0.007	0.014	0.024	0.949	0.903
EE0096	2/13/99	JAL	0.024	0.018	0.022	0.021	0.016	0.026	0.784	0.736
EE0097	2/14/99	JLK	0.008	0.008	0.005	0.007	0.015	0.025	1.088	0.933
EE0098	2/15/99	BT	0.006	0.009	0.01	0.008	0.01	0.02	0.922	0.698
EE0099	2/16/99	EAP	0.005	0.01	0.019	0.011	0.004	0.014	0.829	0.689
EE0100	2/16/99	BT	0.012	0.008	0.018	0.013	0.012	0.022	1.042	0.865
EE0101	2/17/99	CRZ	0.008	0.005	0.005	0.006	0.008	0.018	0.831	0.705
EE0102	2/17/99	BT	0.008	0.007	0.009	0.008	0.007	0.017	0.974	0.774
EE0103	2/18/99	EAP	0.008	0.009	0.01	0.009	0.012	0.022	1.06	0.863
EE0104	2/18/99	JLK	0.016	0.019	0.01	0.015	0.015	0.025	1.088	0.867
EE0105	2/19/99	EAP	0.009	0.01	0.008	0.009	0.01	0.02	1.135	0.83
EE0106	2/22/99	DRYK	0.007	0.007	0.009	0.008	0.008	0.018	0.878	0.814
EE107	2/23/99	JM	0.015	0.013	0.011	0.013	0.021	0.031	1.034	0.772
EE0108	2/23/99	JDD	0.013	0.009	0.008	0.010	0.012	0.022	0.885	0.758
EE0109	2/24/99	EAP	0.012	0.01	0.015	0.012	0.013	0.023	0.91	0.85
EE0110	2/24/99	JDD	0.01	0.009	0.009	0.009	0.012	0.022	1.08	0.884
EE0111	2/25/99	SCB	0.012	0.012	0.014	0.013	0.021	0.031	1.114	0.812
EE0112	2/25/99	JLK	0.006	0.01	0.008	0.008	0.013	0.023	1.066	0.786
EE0113	2/26/99	JLK	0.012	0.011	0.01	0.011	0.013	0.023	0.94	0.711
EE0114	3/3/99	CRZ	0.009	0.008	0.009	0.009	0.008	0.018	0.962	0.758

			PRODUCT				NEGATIVE		POSITIVE	POSITIVE
LOT #	DATE	TECH	1	2	3	Mean Absorbance	CONTROL	CUT-OFF	CONTROL 1	CONTROL 2
EE0115	3/3/99	JLK	0.017	0.015	0.012	0.015	0.016	0.026	1.052	0.76
EE0116	3/4/99	THC	0.01	0.008	0.013	0.010	0.011	0.021	0.919	0.629
EE0117	3/4/99	JLK	0.013	0.013	0.016	0.014	0.017	0.027	1.129	0.936
EE0118	3/5/99	JAL	0.002	0.003	0	0.002	0.003	0.013	0.738	0.742
EE0119	3/6/99	EAP	0.012	0.013	0.011	0.012	0.009	0.019	0.808	0.794
EE0120	3/8/99	JLK	0.007	0.017	0.01	0.011	0.011	0.021	0.955	0.895
EE0121	3/9/99	EAP	0.019	0.011	0.015	0.015	0.01	0.02	0.681	0.85
EE0122	3/9/99	JLK	0.008	0.022	0.006	0.012	0.01	0.02	0.989	0.835
EE0123	3/10/99	BT	0.006	0.006	0.007	0.006	0.004	0.014	0.904	0.851
EE0124	3/11/99	EAP	0.009	0.011	0.013	0.011	0.01	0.02	0.936	0.988
EE0125	3/11/99	BT	0.013	0	0.02	0.011	0.012	0.022	1.162	0.981
EE0126	3/16/99	JDD	0.008	0.01	0.018	0.012	0.009	0.019	1.224	1.046
EE0127	3/17/99	SCB	0.012	0.002	0.013	0.009	0.01	0.02	0.904	0.943
EE0128	3/17/99	JDD	0.001	0.008	0.007	0.005	0.006	0.016	1.002	0.927
EE0129	3/18/99	EAP	0.024	0.013	0.017	0.018	0.021	0.031	1.239	0.921
EE0130	3/18/99	JLK	0.011	0.002	0.008	0.007	0.011	0.021	1.213	1.071
EE0131	3/19/99	EAP	0.006	0.007	0.005	0.006	0.009	0.019	0.84	0.99
EE0132	4/23/99	JLK	0.004	0.003	0.017	0.008	0.012	0.022	1.124	0.868
EE0133	4/24/99	JLK	0.016	0.012	0.01	0.013	0.011	0.021	1.124	0.853
EE0134	4/25/99	JAL	0.005	-0.002	0.003	0.002	0.002	0.012	1.121	0.831
EE0135	4/27/99	KPG	0.01	0.009	0.016	0.012	0.017	0.027	1.153	0.999
EE0136	4/29/99	DRYK	0.012	0.014	0.019	0.015	0.015	0.025	0.797	0.811
EE0137	4/30/99	JAL	0.008	-0.003	0.017	0.007	0.013	0.023	0.882	0.846
EE0138	4/30/99	JDD	0.018	0.02	0.02	0.019	0.021	0.031	1.075	0.939
EE0139	5/1/99	JAL	0.005	0.012	0.007	0.008	0.014	0.024	0.979	1.081
EE0140	5/3/99	BT	0.005	0.012	0.01	0.009	0.015	0.025	1.085	0.956
EE0141	5/4/99	TWB	0.014	0.006	0.017	0.012	0.021	0.031	1.127	1.103
EE0143	5/6/99	TWB	0.011	0.01	0.017	0.013	0.015	0.025	0.968	1.068
EE0144	5/11/99	DRYK	-0.003	0.013	0.006	0.005	0.01	0.02	0.709	0.875
EE0145	5/12/99	KB	0.017	0.01	0.008	0.012	0.018	0.028	1.063	1.067
EE0146	5/13/99	JLK	0.007	0.006	0.006	0.006	0.007	0.017	0.638	0.887
EE0147	5/14/99	KLB	0.015	0.016	0.015	0.015	0.018	0.028	1.013	0.957
EE0148	5/15/99	TAS	0.016	0.009	0.001	0.009	0.012	0.022	1.028	0.996
EE0149	5/18/99	JLK	0.009	0.011	0.013	0.011	0.012	0.022	0.605	1.026
EE0150	5/19/99	JM	0.005	0.014	0.01	0.010	0.015	0.025	1.015	1.092
EE0151	5/20/99	JLK	0.009	0.002	0.016	0.009	0.02	0.03	0.962	0.981
EE0152	5/21/99	NM	0.017	0.011	0.013	0.014	0.017	0.027	1.053	1.105
EE0153	5/22/99	JLK	0.029	0.019	0.017	0.022	0.016	0.026	0.996	0.952
EE0154	5/25/99	JLK	0.011	0.012	0.001	0.008	0.014	0.024	0.93	1.063
EE0155	5/26/99	KPG	0.01	0.011	0.014	0.012	0.011	0.021	0.69	0.773
EE0157	5/28/99	TWB	0.005	0.009	0.014	0.010	0.011	0.021	0.897	1.066
EE0158	5/29/99	JDD	0.0132	0.013	0.007	0.011	0.015	0.025	0.871	1.161
EE0159	6/1/99	JLK	0.014	0.017	0.015	0.016	0.012	0.022	0.942	1.135
EE0160	6/2/99	NM	0.01	0.01	0.017	0.012	0.018	0.028	1.024	1.096
EE0161	6/3/99	JLK	0.006	0.009	0.012	0.009	0.009	0.019	0.979	0.967
EE0162	6/4/99	TWB	0.004	0.004	0.004	0.004	0.014	0.024	0.959	1.031
EE0163	6/5/99	JLK	0.011	0.005	0.008	0.008	0.009	0.019	0.948	1.021
EE0164	6/8/99	JLK	0.012	0.011	0.023	0.015	0.015	0.025	1.217	0.812
EE0165	6/9/99	JM	0.015	0.005	-0.004	0.005	0.008	0.018	1.207	0.739
100					Mean	0.011	0.013	0.023	0.985	0.920
					SD	0.004	0.005	0.005	0.160	0.135
					%cv	37.210	36.977	21.160	16.231	14.652

Bayer Corporation
Pharmaceutical Division
Biological Products
Clayton, NC

T.2.53-02R

Validation Report of Test Methods/Other Methods

Addendum 1

Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools

Number of Pages
(including cover page)

5

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Valid From: JUL 15 1999

Introduction

This report is an addendum to Revision 1 of the validation report of the method "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools". Revision 1 included a discussion of the cutoff value as defined by the kit (cutoff = negative control mean + 0.100) and as modified through QA Change B0070 (cutoff = negative control mean + 0.01). This addendum addresses changing the cutoff to negative control mean + 0.025.

Discussion of Cutoff Value

To increase the sensitivity of the assay QA Change B0070 was initiated in May 1996. This QA change proposed modifying the calculation of the cutoff value. The cutoff value was originally calculated by: negative control mean + 0.100 (based on the kit's package insert). QA change B0070 changed the calculation of the cutoff value to: negative control mean + 0.010. The experimental data from the 300 pools evaluated for QA change B0070 supported tightening of the specification. QA change B0070 was adopted in July 1996.

Evaluation of the accuracy of the method showed that 30% of known negative samples tested positive when the cutoff value = negative control mean + 0.01 (Data not shown). Statistical analysis of 98 absorbance values from 44 test samples known to be negative for HIV-1(see Appendix 1 for raw data of CDC negative samples) yielded a mean absorbance of 0.017 with a standard deviation of 0.008 (Table 1). The 100 pools (with 300 absorbance values) evaluated in Revision 1 of the validation report yielded a mean absorbance of 0.011 and a standard deviation of 0.006. When both sets of data were combined, the overall mean was 0.012 with a standard deviation of 0.007. For these sets of data, the factor of 0.010 is 1.3, 1.7, and 1.4 times the standard deviation of the data, respectively. Increasing the factor to 0.025, would result in an increase of approximately 3 times the standard deviation for these samples.

Conclusions

The recommended cutoff value for this method "Qualitative Detection of Antibody to Human Immunodeficiency Virus Types 1 and 2 in Plasma Pools" is negative control mean + 0.025. This value is well below the cutoff value as defined by the kit manufacturer (Abbott Laboratories); therefore, the sensitivity of the assay would not be compromised and the number of false positives would be reduced.

Attachments

Table 1: Comparison of Standard Deviation of Absorbance Values to 0.01 Cutoff Factor

Appendix 1 Absorbance Values of CDC Negative Samples and 100 Pool Samples (Used in Revision 1 of the Validation Report)

Table 1. Comparison of Standard Deviation of Absorbance Values to 0.01 Cutoff Factor

Sample	**Mean**	**S.D**	**% c.v.**	Fold Difference: 0.01 Factor/S.D.
CDC Negative Samples	0.017	0.008	48.7	1.3
100 Pools	0.011	0.006	50.3	1.7
CDC Negative Samples + 100 Pools	0.012	0.007	54.2	1.4

Appendix 1

Absorbance Values of CDC Negative Samples and 100 Pool Samples
(Used in Revision 1 of the Validation Report)

Absorbance Values

CDC Negative Samples	100 Pools (Retrospective Data from Revision 1 of Validation Report	CDC Negative Samples + 100 Pools
0.012	0.014	0.014
0.011	0.011	0.011
0.01	0.02	0.02
0.007	0.024	0.024
0.013	0.008	0.008
0.005	0.013	0.013
0.014	0.025	0.025
0.006	0.013	0.013
0.003	0.017	0.017
0.013	0.01	0.01
0.018	0.016	0.016
0.014	0.009	0.009
0.011	0.011	0.011
0.013	0.02	0.02
0.021	0.003	0.003
0.014	0.015	0.015
0.02	0.018	0.018
0.016	0.017	0.017
0.024	0	0
0.013	0.009	0.009
0.024	0.01	0.01
0.016	0.016	0.016
0.014	0.01	0.01
0.019	0.017	0.017
0.031	0.006	0.006
0.014	0.012	0.012
0.026	0.001	0.001
0.021	0.014	0.014
0.02	0.017	0.017
0.019	0.028	0.028
0.019	0.022	0.022
0.02	0.008	0.008
0.014	0.024	0.024
0.029	0.008	0.008
0.025	0.005	0.005
0.011	0.005	0.005
0.006	0.012	0.012
0.028	0.008	0.008
0.031	0.008	0.008
0.023	0.008	0.008
0.023	0.016	0.016
0.027	0.009	0.009
0.03	0.007	0.007
0.021	0.015	0.015
0.043	0.013	0.013
0.025	0.012	0.012
0.033	0.01	0.01
0.048	0.012	0.012
0.011	0.006	0.006
0.017	0.012	0.012
0.022	0.009	0.009
0.02	0.017	0.017
0.017	0.01	0.01
0.026	0.013	0.013
0.012	0.002	0.002
0.009	0.012	0.012
0.013	0.007	0.007
0.009	0.013	0.013
0.01	0.008	0.008
0.015	0.006	0.006
0.02	0.009	0.009
0.011	0.013	0.013
0.01	0.008	0.008
0.008	0.012	0.012
0.015	0.001	0.001
0.031	0.024	0.024
0.009	0.011	0.011

Absorbance Values

CDC Negative Samples	100 Pools (Retrospective Data from Revision 1 of Validation Report	CDC Negative Samples + 100 Pools
0.018	0.005	0.006
0.007	0.004	0.004
0.01	0.015	0.015
0.007	0.005	0.005
0.021	0.01	0.01
0.014	0.012	0.012
0.015	0.008	0.008
0.012	0.018	0.018
0.015	0.005	0.005
0.008	0.005	0.005
0.011	0.014	0.014
0.01	0.011	0.011
0.011	-0.003	-0.003
0.014	0.017	0.017
0.018	0.007	0.007
0.015	0.015	0.015
0.012	0.016	0.016
0.027	0.009	0.009
0.013	0.005	0.005
0.02	0.009	0.009
0.011	0.017	0.017
0.01	0.029	0.029
0.016	0.011	0.011
0.016	0.01	0.01
0.011	0.006	0.006
0.016	0.0132	0.0132
0.01	0.014	0.014
0.018	0.01	0.01
0.008	0.006	0.006
0.011	0.004	0.004
0.003	0.011	0.011
	0.012	0.012
	0.015	0.015
	0.014	0.014
	0.002	0.002
	0.015	0.015
	0.014	0.014
	0.017	0.017
	0.026	0.026
	0.016	0.016
	0.01	0.01
	0.019	0.019
	0.01	0.01
	0.009	0.009
	0.007	0.007
	0.01	0.01
	0.008	0.008
	0.012	0.012
	0.006	0.006
	0.018	0.018
	0.01	0.01
	0.004	0.004
	0.005	0.005
	0.017	0.017
	0.014	0.014
	0	0
	0.018	0.018
	0.006	0.006
	0.008	0.008
	0.02	0.02
	0.001	0.001
	0.013	0.013
	0.014	0.014
	0.008	0.008
	0.004	0.004
	0.018	0.018
	0.008	0.008

Mean	0.0167778
SD	0.008161
% cv	48.65084

Absorbance Values

CDC Negative Samples	100 Pools (Retrospective Data from Revision 1 of Validation Report	CDC Negative Samples + 100 Pools
	0.009	0.009
	0.01	0.01
	0.008	0.008
	0.005	0.005
	0.007	0.007
	0.009	0.009
	0.018	0.018
	0.01	0.01
	0.007	0.007
	0.013	0.013
	0.009	0.009
	0.01	0.01
	0.009	0.009
	0.012	0.012
	0.01	0.01
	0.011	0.011
	0.006	0.006
	0.015	0.015
	0.006	0.006
	0.013	0.013
	0.003	0.003
	0.013	0.013
	0.017	0.017
	0.011	0.011
	0.022	0.022
	0.008	0.008
	0.011	0.011
	0	0
	0.01	0.01
	0.002	0.002
	0.008	0.008
	0.013	0.013
	0.002	0.002
	0.007	0.007
	0.003	0.003
	0.012	0.012
	-0.002	-0.002
	0.009	0.009
	0.014	0.014
	-0.003	-0.003
	0.02	0.02
	0.012	0.012
	0.012	0.012
	0.006	0.006
	0.01	0.01
	0.013	0.013
	0.01	0.01
	0.009	0.009
	0.016	0.016
	0.009	0.009
	0.011	0.011
	0.014	0.014
	0.002	0.002
	0.011	0.011
	0.019	0.019
	0.012	0.012
	0.011	0.011
	0.009	0.009
	0.013	0.013
	0.017	0.017
	0.01	0.01
	0.009	0.009
	0.004	0.004
	0.005	0.005
	0.011	0.011
	0.005	0.005
	0.02	0.02

Absorbance Values

CDC Negative Samples	180 Pools (Retrospective Data from Revision 1 of Validation Report	CDC Negative Samples + 180 Pools
	0.008	0.008
	0.019	0.019
	0.014	0.014
	0.009	0.009
	0.015	0.015
	0.013	0.013
	0.027	0.027
	0.015	0.015
	0.008	0.008
	0.008	0.008
	0.015	0.015
	0.015	0.015
	0.011	0.011
	0.009	0.009
	0.014	0.014
	0.015	0.015
	0.005	0.005
	-0.001	-0.001
	0.01	0.01
	0.0105	0.0105
	0.016	0.016
	0.004	0.004
	0.008	0.008
	0.015	0.015
	0.013	0.013
	0.013	0.013
	0.014	0.014
	0.006	0.006
	0.02	0.02
	0.015	0.015
	0.01	0.01
	0.022	0.022
	0.005	0.005
	0.01	0.01
	0.019	0.019
	0.018	0.018
	0.005	0.005
	0.009	0.009
	0.01	0.01
	0.01	0.01
	0.006	0.006
	0.009	0.009
	0.011	0.011
	0.008	0.008
	0.015	0.015
	0.009	0.009
	0.014	0.014
	0.008	0.008
	0.01	0.01
	0.009	0.009
	0.012	0.012
	0.013	0.013
	0.016	0.016
	0	0
	0.011	0.011
	0.01	0.01
	0.015	0.015
	0.006	0.006
	0.007	0.007
	0.013	0.013
	0.02	0.02
	0.018	0.018
	0.013	0.013
	0.007	0.007
	0.017	0.017
	0.009	0.009
	0.005	0.005

Absorbance Values

CDC Negative Samples	100 Pools (Retrospective Data from Revision 1 of Validation Report	CDC Negative Samples + 100 Pools
	0.017	0.017
	0.01	0.01
	0.003	0.003
	0.018	0.018
	0.019	0.019
	0.017	0.017
	0.02	0.02
	0.007	0.007
	0.01	0.01
	0.017	0.017
	0.017	0.017
	0.006	0.006
	0.008	0.008
	0.006	0.006
	0.015	0.015
	0.001	0.001
	0.013	0.013
	0.01	0.01
	0.016	0.016
	0.013	0.013
	0.017	0.017
	0.001	0.001
	0.014	0.014
	0.014	0.014
	0.007	0.007
	0.016	0.016
	0.017	0.017
	0.012	0.012
	0.004	0.004
	0.008	0.008
	0.023	0.023
	-0.004	-0.004
	0.011089	0.012
	0.005507082	0.011
	50.25417315	0.01
		0.007
		0.013
		0.005
		0.014
		0.008
		0.005
		0.013
		0.018
		0.014
		0.011
		0.013
		0.021
		0.014
		0.02
		0.018
		0.024
		0.013
		0.024
		0.018
		0.014
		0.019
		0.031
		0.014
		0.026
		0.021
		0.02
		0.019
		0.019
		0.02
		0.014
		0.029
		0.025

Absorbance Values

CDC Negative Samples	100 Pools (Retrospective Data from Revision 1 of Validation Report	CDC Negative Samples + 100 Pools
		0.011
		0.006
		0.028
		0.001
		0.023
		0.023
		0.027
		0.03
		0.021
		0.043
		0.026
		0.033
		0.048
		0.011
		0.017
		0.022
		0.02
		0.017
		0.025
		0.012
		0.009
		0.013
		0.009
		0.01
		0.015
		0.02
		0.011
		0.01
		0.008
		0.015
		0.031
		0.009
		0.018
		0.007
		0.01
		0.007
		0.021
		0.014
		0.015
		0.012
		0.015
		0.009
		0.011
		0.01
		0.011
		0.014
		0.016
		0.016
		0.012
		0.027
		0.012
		0.02
		0.011
		0.01
		0.016
		0.016
		0.011
		0.016
		0.01
		0.018
		0.009
		0.011
		0.003
		0.012474
		0.006758
		54.17917

Table of Contents

Validation of Test Methods

Validation of the Detection of HCV RNA in Plasma Pools using the Roche AmpliScreen™ HCV Test Kit, Version 2.0, on the COBAS AMPLICOR™ Analyzer

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Original is signed

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Name and Rank Date

_____ _____

Name and Rank Date

Valid from: 13 Jul 2004

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1. Introduction

Hepatitis C virus (HCV) is considered to be the principal etiological agent responsible for 90 to 95% of post-transfusion non-A, non-B hepatitis cases. HCV is a positive sense single-stranded RNA virus with a genome of approximately 10,000 nucleotides coding for 3,000 amino acids. As a blood-borne virus, it may be transmitted by blood and blood products. The prevalence of HCV infection is high in patients receiving organ transplants, blood transfusions or commercial clotting factors, in patients with percutaneous exposure through intravenous drug abuse and in patients undergoing renal dialysis. The global prevalence of HCV infection, as determined by immunoserology, ranges from 0.6% in Canada to 1.5% in Japan.

The COBAS AmpliScreen™ HCV Test, v2.0, an in vitro nucleic acid amplification test for the qualitative detection of HCV RNA in plasma, is designed to screen pooled samples of donated human plasma. The screening of pooled plasma and the subsequent culling of positive material ensures a reduction of the potential viral load in production pools. The reduction of viral load is significant to improving the efficacy of viral inactivation steps that occur during the production process.

Guidelines that indirectly regulate the viral load for HCV in plasma derived products have been in place since July 01, 1999 (CPMP/BWP/390/97). Nucleic acid amplification technology (NAT) must be used to screen plasma manufacturing pools for the presence of HCV RNA prior to release of final product within the European Union. Manufacturing-scale plasma pools are created from individual source plasma donations that have been screened by NAT for one or more blood borne viruses. These manufacturing pools may then be retested for these viruses, to ensure that negative manufacturing pools are processed.

2. Objective

The aim of this study was to demonstrate the robustness of the Roche COBAS AmpliScreen™ HCV kit, v2.0 and to prove equivalency with the previous method (Roche™ HCV microwell plate method, v2.0) when testing pool matrices of source plasma donations and recovered plasma donations. The validation protocol was conducted according to the ICH Harmonized Tripartite Guideline on the Validation of Analytical Procedures and the EDQM document PA/PH/OMCL (98) 22, DEF *Validation Of Nucleic Acid Amplification Technology (NAT) For The Detection Of Hepatitis C Virus (HCV) RNA In Plasma Pools* in accordance with the E.P. general chapter 2.6.21, *Nucleic Acid Amplification Techniques*.

3. Validation Testing Facility

Talecris Biotherapeutics, Inc.
Raleigh Test Lab
1200 New Hope Road
Raleigh, North Carolina 27610

4. Persons Involved in the Validation Study

Study Director:
Michael Gray, NAT Technical Operations Supervisor

Study Personnel:
Matt Selley, NAT Laboratory Technician, Operator 1
Rochelle Taylor, NAT Senior General Supervisor, Operator 2
Eugenia Vallido, NAT Chief Laboratory Technician, Operator 3

5. Description of Materials Being Tested

5.1 HCV Positive Material

The HCV in-house standard (QAV-749A) was used in these studies. This positive control consists of an HCV-positive plasma donation, calibrated against the WHO International Standard, at a titer of 57.5×10^6 IU/mL.

5.2 Robustness Study (Verification of assay sensitivity in varied plasma matrices)

Recovered plasma pool samples obtained from archive at the RTL have been tested previously and found negative for HCV. These previously blinded samples, representing pools of approximately 12,000 donations each, were combined to create sufficient volume of a homogeneous matrix to perform this validation.

Source plasma pools were prepared from RTL minipool samples that have been previously tested and found negative for HCV. A sufficient number of these samples were combined to represent no less than the minimum number of donors in a manufacturing pool (3,840) and to provide adequate sample volume to perform this validation.

5.3 Cross-Contamination Study

A multiple-source plasma pool (PCR-012), non-reactive for HCV, was used as a diluent in the preparation of HCV dilution for the cross-contamination study. This normal human plasma pool was also utilized as the negative control (NHP) tested along with the HCV in-house standard QAV-749A in the same study.

5.4 Specificity Study

A panel of 100 source plasma pools (3,840 donations each) served as the matrices for all of the final test samples used in the specificity study (Listed in Table 3). The 100 pools were created from plasma donation minipools that were previously tested and found negative for HCV.

The dilution scheme and composition of the samples tested during this validation study is outlined in Table 1.

Table 1 - Composition and Dilution Scheme of Sample Panels Tested During Validation

Stock	Dilution	Dilution Procedure	Final Sample
HCV In-house Standard (QAV-749A) at 57.5 x 10^6 IU/mL	1:100	0.3 mL QAV-749A + 29.7 mL Recovered Plasma Pool	HCV Intermediate at 57.5 x 10^4 IU/mL
HCV Intermediate at 57.5 x 10^4 IU/mL	1:100	0.3 mL of HCV Intermediate at 57.5 x 10^4 IU/mL + 29.7 mL Recovered Plasma Pool	HCV Intermediate at 57.5 x 10^2 IU/mL
HCV Intermediate at 57.5 x 10^2 IU/mL	1:57.5	2 mL of HCV Intermediate at 57.5 x 10^2 IU/mL + 113 mL Recovered Plasma Pool	HCV-positive (100 IU/mL) Recovered Plasma Pool panel.
HCV In-house Standard (QAV-749A) at 57.5 x 10^6 IU/mL	1:100	0.3 mL QAV-749A + 29.7 mL Source Plasma Pool	HCV Intermediate at 57.5 x 10^4 IU/mL
HCV Intermediate at 57.5 x 10^4 IU/mL	1:100	0.3 mL of HCV Intermediate at 57.5 x 10^4 IU/mL + 29.7 mL Source Plasma Pool	HCV Intermediate at 57.5 x 10^2 IU/mL
HCV Intermediate at 57.5 x 10^2 IU/mL	1:57.5	2 mL of HCV Intermediate at 57.5 x 10^2 IU/mL + 113 mL Source Plasma Pool	HCV-positive (100 IU/mL) Source Plasma Pool panel.
HCV In-house Standard (QAV-749A) at 57.5 x 10^6 IU/mL	1:100	0.5 mL QAV-749A + 49.5 mL PCR-012	HCV-positive (57.5 x 10^4 IU/mL) Cross-contamination panel

6. Analytical Test Method

The COBAS AmpliScreen™ HCV Test, v2.0, is based on five major processes: 1) specimen preparation, 2) reverse transcription of target RNA to generate cDNA copies, 3)

PCR amplification of target cDNA using HCV specific complementary primers, 4) hybridization of the amplified products to oligonucleotide probes specific to the target and 5) detection of the probe-bound amplified products by colorimetric determination.

6.1 Extraction of HCV Nucleic Acid from Human Plasma

HCV RNA is isolated from plasma. After an initial high-speed centrifugation step to concentrate virus particles, the HCV RNA is isolated by lysis of virus particles with a chaotropic agent followed by precipitation of the RNA with alcohol. The HCV Internal Control RNA is introduced into each specimen with the Lysis Reagent and serves as an extraction and amplification control for each individually processed specimen.

6.2 Internal Control

Reverse transcription and amplification of HCV Internal Control (IC) RNA occur simultaneously. The Master Mix reagent contains a biotinylated primer pair specific to HCV and IC target nucleic acid. The IC is a non-infectious RNA transcript that contains the identical primer binding sites as the HCV target and a unique probe binding region that allows IC amplicons to be distinguished from HCV amplicons. The IC is incorporated into each individual sample and is carried through the specimen preparation, reverse transcription, PCR amplification, hybridization and detection steps along with the HCV target. The detection of amplified HCV Internal control DNA is performed to ensure the integrity of the extraction and amplification process.

6.3 Detection of HCV in Plasma Pools Using Polymerase Chain Reaction Methodology

The COBAS AMPLICOR™ Analyzer chemically denatures the HCV and IC amplicons into single-stranded DNA immediately after PCR amplification. During the detection the amplicons are hybridized to target-specific oligonucleotide probes bound to magnetic particles, which increases the overall specificity of the assay. A colored complex is formed when particle-bound horseradish peroxidase catalyzes the oxidation of tetramethylbenzidine (TMB). The COBAS AMPLICOR™ Analyzer at a wavelength of 660 nm measures the absorbance.

6.4 Detection Controls

The Roche negative control (-) is designed to confirm acceptable performance of the assay. The absorbance for the Roche (-) control must be less than 0.100 at 660 nm. If the absorbance value is greater than or equal to 0.100, the entire run is invalid and must be repeated (sample preparation, amplification and detection).

The Roche positive control (+) is designed to confirm acceptable performance of the assay. The absorbance for the MP (+) control must be greater than or equal to 1.000 at

660 nm. If the absorbance value is less than 1.000 nm, the entire run is invalid and must be repeated (sample preparation, amplification and detection).

The Roche internal control absorbance (IC) for samples and controls must be greater than or equal to 0.200 at 660 nm for a valid test. A sample IC of less than 0.200 invalidates the result for that sample, unless the test result is positive. If the absorbance of the sample is greater than or equal to the cut-off value of 0.200 at 660 nm, the internal control result is disregarded and the sample is interpreted as positive. If the test result for the sample is invalid, the entire test procedure (sample preparation, amplification and detection) must be repeated.

The in-house positive control, at 100 IU/mL (in accordance with CPMP/BWP/390/97), must be greater than or equal to 0.200 at 660 nm for a valid test. If above conditions are not met, the entire run is discarded and all non-reactive samples are repeated. Reactive results obtained from runs with acceptable kit controls but invalid in-house controls results are treated as positive.

6.5 Test Disposition

A test sample is interpreted as negative for HCV RNA if the absorbance value (660 nm) for the sample is less than 0.200 and the A_{660} value for the internal control is greater than or equal to 0.200. If the A_{660} value for the test sample is less than 0.200 and the A_{405} value for the internal control is less than 0.200, the test for that sample is invalid and the sample must be retested (these samples would not be counted for purposes of this validation). If the absorbance value (660 nm) for the test sample is greater than or equal to 0.200, then the test sample is interpreted as positive for HCV RNA. Test samples with an A_{660} greater than or equal to 0.200 are interpreted as positive for HCV RNA regardless of the A_{660} value for the HCV IC.

7. Design of the Validation Study

The qualitative PCR assay described here functions as a limit test for impurities. Test results are interpreted as either positive or negative for a detectable analyte. This limit test was validated for assay robustness, repeatability, intermediate precision and specificity.

7.1 Sensitivity (Detection Limit)

The detection limit of the type of qualitative assay must not only account for the quantity of the nucleic acid units per unit volume, but it must also be expressed as a function of the positive-test rate for a specific nucleic acid load. The positive cut-off point is the minimum number of target sequences per volume sample, which can be detected in 95% of test runs. Roche has determined that the COBAS AmpliScreen™ HCV test, v2.0, can

detect HCV RNA at levels as low as 50 IU/mL with a 95% positivity rate for donor screening (COBAS AmpliScreen™ HCV Test, version 2.0 package insert).

7.2 Specificity

Specificity is primarily a function of primer selection in PCR assays. One hundred source pools of non-reactive plasma were tested to establish that the assay does not generate false-positive reactions from its integral components or pool constituents.

7.3 Repeatability and Intermediate Precision

Repeatability and intermediate precision were addressed as a function of the detection limit. As a component of assay execution, each test consisted of separate extraction, amplification and detection sessions, and the operator accounted for each phase of the assay. Operators used different COBAS instruments for the amplification and detection phase of the procedure . Source plasma pools and recovered plasma pools spiked with HCV at 100 IU/mL were tested in panels consisting of 8 samples each over a three day period for both recovered and source plasma pools. Operators were required to achieve individual (repeatability) and collective (intermediate precision) positive-tests at a rate of 95 to 100% in order to pass the validation.

7.4 Robustness

This validation addressed the issue of robustness through detection of HCV at the targeted cutoff in varied plasma backgrounds (recovered and source plasma) and the absence of cross-contamination between negative and high-titer HCV samples.

Test sensitivity in varied pool constituents was addressed by testing 24 individual plasma matrices consisting of both recovered (3 operators x 24 matrices) and source plasma (3 operators x 24 matrices) pools non-reactive for HCV containing 1,000 to 12,000 different donations. To meet the requirement set forth by the CPMP guideline (CPMP/BWP/390/97) and to compare with the previous method used at the RTL (Roche AmpliScreen™ HCV microwell test kit, v2.0, positive-test rate at 95% or better at a titer of 100 IU/mL in plasma pools), sample pools were spiked with HCV at 100 IU/mL. The robustness of assay performance under these conditions (varied pool constituents) was assessed by the ability to attain a positive-test rate of 95%.

Cross-contamination concerns were addressed by testing a panel of alternating non-reactive pools and non-reactive pools spiked with HCV to a titer of 57.5×10^4 IU/mL. One operator tested a panel consisting of 10 negative samples alternating with 10 high-titer HCV samples to detect the presence of HCV RNA. Robustness of assay performance was assessed by the ability of the test to accurately detect negative (no positives) and

high-titer HCV positive samples (100% positive) arranged in an alternating pattern (Figure 1).

8. Discussion of Experimental Results

This study of the COBAS AmpliScreen™ HCV Test, v2.0 for the detection of HCV RNA in plasma pools using polymerase chain reaction methodology provides a validation of the assay robustness.

Test specificity was addressed by testing 100 source plasma pools consisting of plasma samples found non-reactive during donor screening for HCV. These tests were carried out to establish that the assay does not generate false-positive reactions from its integral components or pool constituents. The validation requirement that no positive results be obtained was successfully achieved (Table 3).

The accuracy of the targeted 100 IU/mL value was assessed by multiple-operator testing of source plasma pools and recovered plasma pools spiked with the in-house standard to a genome titer of 100 IU/mL. The sensitivity requirement that the assay must detect positive samples within each test panel at a rate of 95 to 100% was met (Table 4 and 5). In addition, the overall positive test rates for the source plasma pools and recovered plasma pools were 100% and 99%, meeting the validation criterion. Test robustness was demonstrated with these test panels by the ability to detect HCV RNA at 100 IU/mL in complex plasma matrices representative of plasma pools at a rate of 95 to 100%. The performance of COBAS analyzers was deemed acceptable.

Cross-contamination concerns were addressed by extracting, amplifying and detecting a panel of alternating non-reactive pools and non-reactive pools spiked with HCV at a titer of 57.5×10^4 IU/mL. Robustness of assay performance was demonstrated by the ability of the test to accurately detect negative (no positives) and high-titer HCV positive samples (100% positive) arranged in a alternating pattern (Table 6, Figure 1).

Results shown were obtained in test runs that had successful positive, negative, and internal controls.

The results of this validation study are summarized in Table 2.

Table 2 - Results of the Tests in the Study to Validate the Process for the Detection of HCV RNA in Plasma Pools using Polymerase Chain Reaction Methodology

Validation level of analyte strength (sample type)	Type of Validation Testing	Acceptance Criteria	Results	Outcome
Negative diluent controls: Plasma pools	Specificity	No positives	0% positive	VALID
In-house test panel: Recovered plasma pools at 100 IU/mL	Robustness: (sensitivity), repeatability and intermediate precision	95 to 100% positive	Operator 1 100% Operator 2 100% Operator 3 96% Total 99%	VALID
In-house test panel: Source plasma pools at 100 IU/mL	Robustness: (sensitivity), repeatability, and intermediate precision	95 to 100% positive	Operator 1 100% Operator 2 100% Operator 3 100% Total 100%	VALID
Negative diluent controls: plasma pools	Robustness (cross-contamination)	No positives	0% positive	VALID
In-house test panel: Plasma pools spiked with HCV at 57.5×10^4 IU/mL	Robustness (cross-contamination)	100% positive	100% positive	

9. Conclusions

The COBAS AmpliScreen™ HCV Test, v2.0, met or exceeded the performance of the previous method used at the Raleigh Test Lab (Roche AmpliScreen™ HCV microwell plate method, v2.0) when testing plasma pools. In addition, the test meets the performance requirement recommended by the CPMP that states nucleic acid amplification technology (NAT) must be used to screen plasma manufacturing pools for the presence of HCV RNA prior to release of final product within the European Union. The COBAS AmpliScreen™ HCV Test, v2.0, can detect HCV RNA that is present in source plasma pools and recovered plasma pools at a titer of 100 IU/mL with a positive-test rate at or above 95%.

Table 3 - Absorbance Values (660 nm) and Detection Frequency for the Test Panel for the Confirmation of Test Specificity in Plasma Pools

TEST PANEL: HCV-negative plasma pools			
Sample	**Operator**	**A_{660} HCV**	**A_{660} Internal Control**
035A	Operator 1	0.006	3.846
035D	Operator 1	0.006	3.846
045F	Operator 1	0.004	3.671
045G	Operator 1	0.004	3.670
045H	Operator 1	0.006	3.546
045I	Operator 1	0.005	3.847
045J	Operator 1	0.004	3.847
047A	Operator 1	0.005	≥4.000
047B	Operator 1	0.005	3.847
047C	Operator 1	0.005	≥4.000
047D	Operator 1	0.007	3.847
047E	Operator 1	0.006	3.671
047F	Operator 1	0.005	3.847
047G	Operator 1	0.007	3.847
048A	Operator 1	0.004	3.847
048B	Operator 1	0.005	3.847
048C	Operator 1	0.005	3.847
048D	Operator 1	0.007	≥4.000
048E	Operator 1	0.003	3.734
048F	Operator 1	0.003	≥4.000
048G	Operator 1	0.004	3.734
048H	Operator 1	0.003	≥4.000
048I	Operator 1	0.003	≥4.000
048J	Operator 1	0.003	≥4.000
048K	Operator 1	0.004	3.734
053O	Operator 1	0.003	3.734
053P	Operator 1	0.004	≥4.000
063H	Operator 1	0.005	3.849
063I	Operator 1	0.004	3.849
064A	Operator 1	0.003	≥4.000
064B	Operator 1	0.004	3.673
064D	Operator 1	0.004	3.849
064E	Operator 1	0.004	3.673
064F	Operator 1	0.005	3.849
064G	Operator 1	0.004	3.849

Table 3 - Continued

TEST PANEL: HCV-negative plasma pools			
Sample	**Operator**	**A_{660} HCV**	**A_{660} Internal Control**
064H	Operator 1	0.003	3.673
064I	Operator 1	0.004	3.849
037G	Operator 1	0.005	3.673
040C	Operator 1	0.003	3.849
037D	Operator 1	0.003	3.548
040B	Operator 1	0.004	3.849
034B	Operator 1	0.005	3.849
033D	Operator 1	0.004	3.850
037B	Operator 1	0.004	3.850
038C	Operator 1	0.004	3.850
039F	Operator 1	0.003	3.563
054B	Operator 3	0.005	≥4.000
054C	Operator 3	0.009	≥4.000
054D	Operator 3	0.005	3.737
054F	Operator 3	0.006	3.737
054G	Operator 3	0.007	3.737
060A	Operator 3	0.006	3.737
060D	Operator 3	0.005	3.737
060E	Operator 3	0.005	≥4.000
060F	Operator 3	0.005	≥4.000
060G	Operator 3	0.006	≥4.000
060H	Operator 3	0.005	3.194
060I	Operator 3	0.005	3.261
060J	Operator 3	0.006	3.738
060K	Operator 3	0.006	3.562
060L	Operator 3	0.005	3.261
060M	Operator 3	0.006	≥4.000
061A	Operator 3	0.005	≥4.000
061B	Operator 3	0.006	≥4.000
061C	Operator 3	0.007	3.853
061D	Operator 3	0.006	3.853
062B	Operator 3	0.007	3.853
048L	Operator 3	0.009	3.853
048M	Operator 3	0.008	3.853
048N	Operator 3	0.008	≥4.000
048O	Operator 3	0.006	3.853
048P	Operator 3	0.008	≥4.000

Table 3 - Continued

TEST PANEL: HCV-negative plasma pools			
Sample	**Operator**	**A_{660} HCV**	**A_{660} Internal Control**
048Q	Operator 3	0.007	≥4.000
048R	Operator 3	0.006	≥4.000
048S	Operator 3	0.007	≥4.000
050B	Operator 3	0.005	3.847
050E	Operator 3	0.005	≥4.000
050F	Operator 3	0.006	3.847
050G	Operator 3	0.006	≥4.000
050H	Operator 3	0.005	≥4.000
053A	Operator 3	0.006	3.671
053B	Operator 3	0.005	≥4.000
053C	Operator 3	0.006	≥4.000
053D	Operator 3	0.006	3.847
053E	Operator 3	0.006	≥4.000
053F	Operator 3	0.006	≥4.000
053J	Operator 3	0.007	≥4.000
053K	Operator 3	0.008	3.847
053L	Operator 3	0.006	≥4.000
053M	Operator 3	0.007	3.847
053N	Operator 3	0.007	≥4.000
062C	Operator 3	0.006	≥4.000
062D	Operator 3	0.006	≥4.000
062F	Operator 3	0.006	≥4.000
062G	Operator 3	0.005	≥4.000
062I	Operator 3	0.004	≥4.000
062N	Operator 3	0.004	≥4.000
063C	Operator 3	0.003	≥4.000
063D	Operator 3	0.004	≥4.000
063G	Operator 3	0.005	≥4.000
Positive Rate		0/100	All positive
Detection Frequency		0 %	

Table 4 - Absorbance Values (660 nm) and Detection Frequency for Plasma Pools

TEST PANEL: Recovered plasma pools spiked with HCV RNA to 100 IU/mL								
	Operator 1			Operator 2			Operator 3	
Sample	A_{660} HCV	A_{660} Internal Control	Sample	A_{660} HCV	A_{660} Internal Control	Sample	A_{660} HCV	A_{660} Internal Control
RHVCN	0.006	≥4.000	RHVCN	0.006	3.851	RHVCN	0.002	3.848
R1A	3.735	3.737	R1B	≥4.000	3.851	R1C	≥4.000	3.847
R2A	3.735	≥4.000	R2B	3.453	3.851	R2C	3.669	≥4.000
R3A	≥4.000	3.737	R3B	3.851	3.851	R3C	3.669	3.847
R4A	3.735	3.737	R4B	≥4.000	3.675	R4C	≥4.000	3.671
R5A	3.735	≥4.000	R5B	3.851	3.851	R5C	3.845	3.672
R6A	≥4.000	3.737	R6B	3.851	3.851	R6C	3.846	3.848
R7A	≥4.000	≥4.000	R7B	3.851	≥4.000	R7C	3.846	3.672
R8A	3.736	≥4.000	R8B	3.851	≥4.000	R8C	≥4.000	3.848
R9A	≥4.000	3.739	R9B	3.851	3.852	R9C	3.557	≥4.000
R10A	3.261	3.739	R10B	3.674	3.676	R10C	≥4.000	≥4.000
R11A	≥4.000	≥4.000	R11B	3.550	3.852	R11C	3.733	≥4.000
R12A	≥4.000	≥4.000	R12B	3.675	3.676	R12C	3.733	≥4.000
R13A	3.738	3.739	R13B	3.675	3.852	R13C	≥4.000	≥4.000
R14A	3.738	≥4.000	R14B	3.851	3.676	R14C	≥4.000	≥4.000
R15A	≥4.000	≥4.000	R15B	3.675	3.852	R15C	3.335	≥4.000
R16A	≥4.000	3.739	R16B	3.851	3.852	R16C	≥4.000	≥4.000
RHCVN	0.005	3.739	RHCVN	0.005	3.676	RHCVN	0.004	≥4.000
R17A	≥4.000	≥4.000	R25B[a]	≥4.000	3.734	R17C	2.613	3.667
R18A	3.556	≥4.000	R26B[a]	≥4.000	3.734	R18C	1.826	3.667
R19A	≥4.000	≥4.000	R27B[a]	≥4.000	3.558	R19C	2.247	3.668
R20A	≥4.000	≥4.000	R28B[a]	≥4.000	3.035	R20C	0.006	3.668
R21A	≥4.000	≥4.000	R29B[a]	≥4.000	≥4.000	R21C	3.366	3.668
R22A	≥4.000	≥4.000	R30B[a]	≥4.000	3.734	R22C	1.307	0.937
R23A	≥4.000	≥4.000	R31B[a]	≥4.000	3.558	R23C	3.366	2.844
R24A	≥4.000	≥4.000	R32B[a]	3.734	3.734	R24C	2.215	2.374
RHCVN	0.003	≥4.000	RHCVN[a]	0.005	≥4.000	RHCVN	0.005	3.543
Positive Rate	24/24	All positive		24/24	All positive		23/24	All positive
Total Positive Rate	71/72							
Detection Frequency	100%			100%			96%	
Total Detection Frequency	99%							

[a] Sample set R17B-R24B was repeated due to a Roche positive control failure. Sample set R25B-R32B was used for repeat test.

Table 5 - Absorbance Values (660 nm) and Detection Frequency for Source Plasma Pools

TEST PANEL: Source plasma pools spiked with HCV RNA to 100 IU/mL								
	Operator 1			Operator 2			Operator 3	
Sample	A_{660} HCV	A_{660} Internal Control	Sample	A_{660} HCV	A_{660} Internal Control	Sample	A_{660} HCV	A_{660} Internal Control
SHVCN	0.004	3.738	SHVCN	0.006	≥4.000	SHVCN	0.002	3.672
S1A	≥4.000	≥4.000	S1B	3.550	3.851	S1C	3.846	3.672
S2A	≥4.000	3.738	S2B	≥4.000	3.851	S2C	3.670	3.673
S3A	≥4.000	≥4.000	S3B	3.851	3.851	S3C	3.846	3.673
S4A	≥4.000	3.738	S4B	3.851	3.851	S4C	3.846	3.849
S5A	3.736	≥4.000	S5B	≥4.000	3.675	S5C	3.846	3.673
S6A	3.737	≥4.000	S6B	3.851	≥4.000	S6C	3.846	3.673
S7A	3.737	≥4.000	S7B	≥4.000	3.851	S7C	3.846	3.673
S8A	≥4.000	3.562	S8B	3.851	≥4.000	S8C	3.847	3.673
S9A	3.738	3.739	S9B	3.675	3.853	S9C	≥4.000	≥4.000
S10A	≥4.000	3.739	S10B	3.851	3.853	S10C	≥4.000	≥4.000
S11A	≥4.000	3.739	S11B	3.851	3.853	S11C	≥4.000	≥4.000
S12A	≥4.000	≥4.000	S12B	3.851	3.677	S12C	3.190	≥4.000
S13A	≥4.000	3.739	S13B	3.675	3.677	S13C	2.013	≥4.000
S14A	3.739	3.739	S14B	3.675	3.853	S14C	≥4.000	≥4.000
S15A	≥4.000	3.563	S15B	2.255	≥4.000	S15C	≥4.000	≥4.000
S16A	≥4.000	3.739	S16B	3.851	3.853	S16C	3.734	≥4.000
SHCVN	0.005	≥4.000	SHCVN	0.006	3.853	SHCVN	0.005	≥4.000
S17A	≥4.000	≥4.000	S17B	3.735	≥4.000	S17C	2.553	2.891
S18A	≥4.000	≥4.000	S18B	≥4.000	≥4.000	S18C	3.445	3.845
S19A	≥4.000	≥4.000	S19B	≥4.000	≥4.000	S19C	1.031	3.846
S20A	≥4.000	≥4.000	S20B	≥4.000	≥4.000	S20C	2.968	2.628
S21A	3.733	≥4.000	S21B	≥4.000	≥4.000	S21C	1.673	3.545
S22A	≥4.000	≥4.000	S22B	3.735	≥4.000	S22C	3.542	≥4.000
S23A	≥4.000	≥4.000	S23B	≥4.000	≥4.000	S23C	2.600	3.147
S24A	3.733	≥4.000	S24B	≥4.000	≥4.000	S24C	3.843	3.369
SHCVN	0.003	≥4.000	SHCVN	0.004	3.736	SHCVN	0.005	≥4.000
Positive Rate	24/24	All positive		24/24	All positive		24/24	All positive
Total Positive Rate	72/72							
Detection Frequency	100%			100%			100%	
Total Detection Frequency	100%							

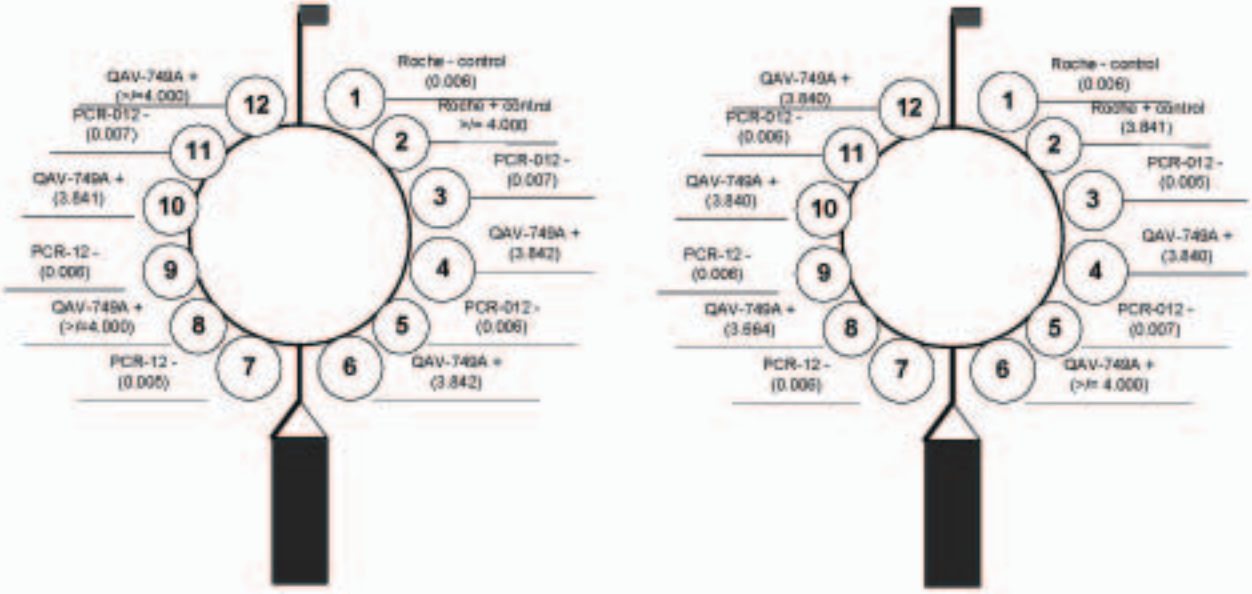
Table 6 - Cross-Contamination Study

Sample	Operator	A_{660} HCV	A_{660} Internal Control
PCR-012	Operator 3	0.007 (-)	≥4.000
QAV-749A	Operator 3	3.842 (+)	2.779
PCR-012	Operator 3	0.006 (-)	≥4.000
QAV-749A	Operator 3	3.842 (+)	3.100
PCR-012	Operator 3	0.005 (-)	≥4.000
QAV-749A	Operator 3	≥4.000 (+)	≥4.000
PCR-012	Operator 3	0.006 (-)	≥4.000
QAV-749A	Operator 3	3.841 (+)	≥4.000
PCR-012	Operator 3	0.007 (-)	≥4.000
QAV-749A	Operator 3	≥4.000 (+)	3.664
PCR-012	Operator 3	0.005 (-)	3.840
QAV-749A	Operator 3	3.840 (+)	3.538
PCR-012	Operator 3	0.007 (-)	≥4.000
QAV-749A	Operator 3	≥4.000 (+)	≥4.000
PCR-012	Operator 3	0.006 (-)	≥4.000
QAV-749A	Operator 3	3.664 (+)	≥4.000
PCR-012	Operator 3	0.006 (-)	3.839
QAV-749A	Operator 3	3.840 (+)	3.538
PCR-012	Operator 3	0.006 (-)	≥4.000
QAV-749A	Operator 3	3.840 (+)	≥4.000

Assay detection array with individual assay results are listed in Table 6.

For HCV-specific reactions a (+) or (-) is included to define the determined result. Sample identity and coordinates are defined according to their placement on the amplification and detection A-rings defined in Figure 1.

Figure 1



Talecris Biotherapeutics, Inc.
Clayton, NC

T.18.47-02

Validation of Test Methods

Validation of the Detection of HIV-1 RNA in Plasma Pools using the Roche AmpliScreen™ HIV-1 Test Kit, Version 1.5, on the COBAS AMPLICOR™ Analyzer

Number of Pages
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17

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Valid from: 13 Jul 2004

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1. Introduction

Human Immunodeficiency Virus (HIV-1) is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). HIV-1 infection can be transmitted by sexual contact, exposure to infected blood or blood products, or by an infected mother to the fetus. Within three to six weeks of exposure to HIV-1, infected individuals generally develop a brief, acute syndrome characterized by flu-like symptoms and associated with high levels of viremia in peripheral blood. In most infected individuals this is followed by an HIV-1 specific immune response and a decline of plasma viremia, usually within four to six weeks of the onset of symptoms. The prevalence of HIV-1 infection is 1.1% overall in the world, while 0.56% in North America and 0.25% in West Europe.

Serological screening assays have greatly reduced, but not completely eliminated the risk of transmitting viral infections by transfusion and blood products. HIV p24 antigen is the principle core protein of HIV-1 and is found in serum either free or bound by anti-p24 antibody. Free p24 antigen can be measured with commercially available enzyme immunoassays (EIA), which reduce the seroconversion window period (the time between infection and the rise of antibodies to the virus) by approximately five to six days. In theory, testing for viral nucleic acids should further reduce the residual transmission risk by detecting HIV-1 RNA in donations made during the seroconversion window period. Nucleic acid-based tests should also detect viremic units donated by carriers who do not seroconvert or who lack antibodies to epitopes detected by the immunological assays.

The COBAS AmpliScreen™ HIV-1 Test, v1.5, an in vitro nucleic acid amplification test for the qualitative detection of HIV-1 RNA in plasma, is designed to screen pooled samples of donated human plasma. The screening of pooled plasma and the subsequent culling of positive material ensure a reduction of the potential viral load in production pools. The reduction of viral load is significant to improving the efficacy of viral inactivation steps that occur during the production process.

Manufacturing-scale plasma pools are created from individual source plasma donations that have been screened by NAT for one or more blood borne viruses. These manufacturing pools may then be retested for these viruses, to ensure that negative manufacturing pools are processed.

2. Objective

The aim of this study was to demonstrate the robustness of the Roche COBAS AmpliScreen™ HIV-1 kit, v1.5 and to prove equivalency with the previous method (Roche™ HIV-1 microwell plate method, v1.5) when testing pool matrices of source plasma donations and recovered plasma donations. The validation protocol was conducted according to the ICH Harmonized Tripartite Guideline on the Validation of Analytical Procedures and the EDQM document PA/PH/OMCL (98) 22, DEF *Validation Of Nucleic Acid Amplification Technology (NAT) For The Detection Of Hepatitis C Virus*

(HCV) RNA In Plasma Pools in accordance with the E.P. general chapter 2.6.21, *Nucleic Acid Amplification Techniques.*

3. Validation Testing Facility

Talecris Biotherapeutics, Inc.
Raleigh Test Lab
1200 New Hope Road
Raleigh, North Carolina 27610

4. Persons Involved in the Validation Study

Study Director:
Michael Gray, NAT Technical Operations Supervisor

Study Personnel:
Farzaneh Sina, NAT, Chief Laboratory Technician, Operator 1
Matt Selley, NAT Laboratory Technician, Operator 2
Eugenia Vallido, NAT Chief Laboratory Technician, Operator 3

5. Description of Materials Being Tested

5.1 HIV-1 Positive Material

The HIV In-house Standard (NATHIV.990211) was used in these studies. This positive control consists of an HIV-1 positive plasma donation at a titer of 1.02×10^6 gEq/mL. The standard was calibrated against the WHO International Standard, and can be considered to have a potency of 3.55×10^6 IU/mL.

5.2 Robustness Study (Verification of assay sensitivity in varied plasma matrices)

Recovered plasma pool samples obtained from archive at the Raleigh Test Lab (RTL) have been tested previously and found negative for HIV-1. These previously blinded samples, representing pools of approximately 12,000 donations each, were combined to create sufficient volume of a homogeneous matrix to perform this validation.

Source plasma pools were prepared from RTL minipool samples that have been previously tested and found negative for HIV-1. A sufficient number of these samples were combined to represent the number of donations in a typical manufacturing pool (3,840) and to provide adequate sample volume to perform this validation.

5.3 Cross-Contamination Study

A multiple-source plasma pool (PCR-012), non-reactive for HIV-1, was used as a diluent in the preparation of HIV-1 dilution for the cross-contamination study. This normal human plasma pool was also utilized as the negative control (NHP) tested along with the HIV-1 In-house Standard NATHIV.990211 in the same study.

6. Specificity Study

A panel of 100 source plasma pools (3,840 donations each) served as the matrices for all of the final test samples used in the specificity study (Listed in Table 3). The 100 pools were created from plasma minipools that were previously tested and found negative for HIV-1.

The dilution scheme and composition of the samples tested during this validation study is outlined in Table 1.

Table 1 - Composition and Dilution Scheme of Sample Panels Tested During Validation

Stock	Dilution	Dilution Procedure	Final Sample
HIV-1 In-house Standard (NATHIV.990211)at 1.02×10^8 gEq/mL	1:100	0.3 mL NATHIV.990211 + 29.7 mL Recovered Plasma	HIV-1 intermediate at 1.02×10^6 gEq/mL
HIV-1 intermediate at 1.02×10^4 gEq/mL	1:510	0.2 mL HIV-1 intermediate at 1.02×10^4 gEq/mL + 101.8 mL Recovered Plasma	HIV-1 positive (20 gEq/mL) Recovered Plasma Pool panel
HIV-1 In-house Standard (NATHIV.990211)at 1.02×10^8 gEq/mL	1:100	0.3 mL NATHIV.990211 + 29.7 mL Source Plasma Pool	HIV-1 intermediate at 1.02×10^6 gEq/mL
HIV-1 intermediate at 1.02×10^4 gEq/mL	1:510	0.2 mL HIV-1 intermediate at 1.02×10^4 gEq/mL + 101.8 mL Source Plasma Pool	HIV-1 positive (20 gEq/mL) Source Plasma Pool
HIV-1 In-house Standard (NATHIV.990211)at 1.02×10^8 gEq/mL	1:100	0.5 mL NATHIV.990211 + 49.5 mL PCR-012	1.02×10^6 gEq/mL (Total Volume 50 mL)

7. Analytical Test Method

The COBAS AmpliScreen™ HIV-1 Test, v1.5 is based on five major processes. 1) Specimen preparation, 2) Reverse transcription of target RNA to generate cDNA copies, 3) PCR amplification of target cDNA using HIV-1 specific complementary primers, 4) Hybridization of the amplified products to oligonucleotide probes specific to the target, and 5) Detection of the probe-bound amplified products by colorimetric determination.

7.1 Extraction of HIV-1 Nucleic Acid from Human Plasma

HIV-1 RNA is isolated from plasma. After an initial high-speed centrifugation step to concentrate virus particles, the HIV-1 RNA is isolated by lysis of virus particles with a chaotropic agent followed by precipitation of the RNA with alcohol. The HIV-1 Internal Control RNA is introduced into each specimen with the Lysis Reagent and serves as an extraction and amplification control for each individually processed specimen.

7.2 Internal Control

Reverse transcription and amplification of HIV-1 Internal Control (IC) RNA occur simultaneously. The Master Mix reagent contains a biotinylated primer pair specific to HIV-1 and IC target nucleic acid. The IC is a non-infectious RNA transcript that contains the identical primer binding sites as the HIV-1 target and a unique probe binding region that allows IC amplicons to be distinguished from HIV-1 amplicons. The IC is incorporated into each individual sample and is carried through the specimen preparation, reverse transcription, PCR amplification, hybridization and detection steps along with the HIV-1 target. The detection of amplified HIV-1 Internal control DNA is performed to ensure the integrity of the extraction and amplification process.

7.3 Detection of HIV-1 in plasma pools using polymerase chain reaction methodology.

The COBAS AMPLICOR™ Analyzer chemically denatures the HIV-1 and IC amplicons into single-stranded DNA immediately after PCR amplification. During detection the amplicons are hybridized to target-specific oligonucleotide probes bound to magnetic particles, which increases the overall specificity of the assay. A colored complex is formed when particle-bound horseradish peroxidase catalyzes the oxidation of tetramethylbenzidine (TMB). The COBAS AMPLICOR™ Analyzer at a wavelength of 660 nm measures the absorbance.

7.3.1 Detection controls

The Roche Negative control (-) is designed to confirm acceptable performance of the assay. The absorbance for the Roche (-) Control must be less than 0.200 at 660 nm. If the absorbance value is greater than or equal to 0.200, the entire run is invalid and must be repeated (sample preparation, amplification and detection).

The Roche Positive control (+) is designed to confirm acceptable performance of the assay. The absorbance for the MP (+) Control must be greater than or equal to 1.000 at 660 nm. If the absorbance value is less than 1.000 nm, the entire run is invalid and must be repeated (sample preparation, amplification and detection).

The Roche Internal Control absorbance (IC) for samples and controls must be greater than or equal to 0.200 at 660 nm for a valid test. A sample IC of less than 0.200 invalidates the result for that sample, unless the test result is positive. If the absorbance of the sample is greater than or equal to the cut-off value of 0.200 at 660 nm, the Internal Control result is disregarded and the sample is interpreted as positive. If the test result for the sample is invalid, the entire test procedure (sample preparation, amplification and detection) must be repeated.

The In-house Positive Control must be greater than or equal to 0.200 at 660 nm for a valid test. If above conditions are not met, the entire run is discarded and all non-reactive

samples are repeated. Reactive results obtained from runs with acceptable kit controls but invalid in-house controls results are treated as positive.

7.3.2 Test disposition

A test sample is interpreted as negative for HIV-1 RNA if the absorbance value (660 nm) for the sample is less than 0.200 and the A_{660} value for the internal control is greater than or equal to 0.200. If the A_{660} value for the test sample is less than 0.200 and the A_{660} value for the internal control is less than 0.200, the test for that sample is invalid and the sample must be retested (these samples would not be counted for purposes of this validation). If the absorbance value (660 nm) for the test sample is greater than or equal to 0.200, then the test sample is interpreted as positive for HIV-1 RNA. Test samples with an A_{660} greater than or equal to 0.200 are interpreted as positive for HIV-1 RNA regardless of the A_{660} value for the HIV-1 IC.

8. Design of the Validation Study

The qualitative PCR assay described here functions as a limit test for impurities. Test results are interpreted as either positive or negative for a detectable analyte. This limit test was validated for assay robustness, repeatability, intermediate precision and specificity.

8.1 Sensitivity (Detection Limit)

The detection limit of the type of qualitative assay must not only account for the quantity of the nucleic acid units per unit volume, but it must also be expressed as a function of the positive-test rate for a specific nucleic acid load. The positive cut-off point is the minimum number of target sequences per volume sample, which can be detected in 95% of test runs. The Roche AmpliScreen™ microwell HIV-1 test, v1.5, was shown to detect HIV-1 RNA at levels as low as 20 gEq/mL with a 95% positivity rate.

8.2 Specificity

Specificity is primarily a function of primer selection in PCR assays. One hundred source plasma pools of non-reactive plasma were tested to establish that the assay does not generate false-positive reactions from its integral components or pool constituents.

8.3 Repeatability and intermediate precision

Repeatability and intermediate precision were addressed as a function of the detection limit. As a component of assay execution, each test consisted of separate extraction, amplification and detection sessions, and the operator accounted for each phase of the assay. Source plasma pools and recovered plasma pools spiked with HIV-1 at 20 gEq/mL were tested in panels consisting of 8 samples each over a three day period for both recovered and source plasma pools. Operators were required to achieve individual

(repeatability) and collective (intermediate precision) positive-tests at a rate of 95 to 100% in order to pass the validation.

8.4 Robustness

This validation addressed the issue of robustness through detection of HIV-1 at the targeted cutoff in varied plasma backgrounds (recovered and source plasma) and the absence of cross-contamination between negative and high-titer HIV-1 samples.

Test sensitivity in varied pool constituents was addressed by testing 24 individual plasma matrices consisting of both recovered (3 operators x 24 matrices) and source plasma (3 operators x 24 matrices) pools non-reactive for HIV-1 containing 1,000 to 12,000 different donations. To compare with the previous method used at the RTL (Roche AmpliScreen™ HIV-1 microwell test kit, v1.5, positive-test rate at 95% or better at a titer of 20 gEq/mL in plasma pools), sample pools were spiked with HIV-1 at 20 gEq/mL. The robustness of assay performance under these conditions (varied pool constituents) was assessed by the ability to attain a positive-test rate of 95%.

Cross-contamination concerns were addressed by testing a panel of alternating non-reactive pools and non-reactive pools spiked with HIV-1 to a titer of 1.02×10^4 gEq/mL. One operator tested a panel consisting of 10 negative samples alternating with 10 high-titer HIV-1 samples to detect the presence of HIV-1 RNA. Robustness of assay performance was assessed by the ability of the test to accurately detect negative (no positives) and high-titer HIV-1 positive samples (100% positive) arranged in an alternating pattern (Figure 1).

9. Discussion of Experimental Results

This study of the COBAS AmpliScreen™ HIV-1 Test, v1.5 for the detection of HIV-1 RNA in plasma pools using polymerase chain reaction methodology provides a validation of the assay robustness.

Test specificity was addressed by testing 100 source plasma pools consisting of plasma samples found non-reactive during donor screening for HIV-1. These tests were carried out to establish that the assay does not generate false-positive reactions from its integral components or pool constituents. The validation requirement that no positive results be obtained was successfully achieved (Table 3).

The accuracy of the targeted 20 gEq/mL value was assessed by multiple-operator testing of source plasma pools and recovered plasma pools spiked with the in-house standard to a genome titer of 20 gEq/mL. The sensitivity requirement that the assay must detect positive samples within each test panel at a rate of 95 to 100% was met (Tables 4 and 5). In addition, the overall positive test rates for the source plasma pools and recovered plasma pools were 100% and 99%, meeting the validation criterion. Test robustness was

demonstrated with these test panels by the ability to detect HIV-1 RNA at 20 gEq/mL in complex plasma matrices representative of plasma pools at a rate of 95 to 100%.

Cross-contamination concerns were addressed by extracting, amplifying, and detecting a panel of alternating non-reactive pools and non-reactive pools spiked with HIV-1 at a titer of 1.02×10^4 gEq/mL. Robustness of assay performance was demonstrated by the ability of the test to accurately detect negative (no positives) and high-titer HIV-1 positive samples (100% positive) arranged in an alternating pattern (Table 6, Fig. 1.)

Results shown were obtained in test runs that had successful positive, negative, and internal controls. Samples that were repeated are as follows:

- Operator 2 had a Roche Negative failure for sample set 062N-064D during the specificity study. Entire sample set was repeated.
- Operator 2 had an IC failure for sample 064I during the specificity study. Sample was repeated.

The results of this validation study are summarized in Table 2.

Table 2 - Results of the Tests in the Study to Validate the Process for the Detection of HIV-1 RNA in Plasma Pools using Polymerase Chain Reaction Methodology

Validation Level of Analyte Strength (Sample Type)	Type of Validation Testing	Acceptance Criteria	Results	Outcome
Negative diluent controls: plasma pools	Specificity	No positives	0% positive	VALID
In-house Test Panel: Recovered plasma pools at 20 gEq/mL	Robustness: (sensitivity), repeatability, and intermediate precision	95 to 100% positive	Op 1 100% Op 2 96% Op 3 100% Total 99%	VALID
In-house Test Panel: Source plasma pools at 20 gEq/mL	Robustness: (sensitivity), repeatability, and intermediate precision	95 to 100% positive	Op 1 100% Op 2 100% Op 3 100% Total 100%	VALID
Negative diluent controls: plasma pools	Robustness (cross-contamination)	No positives	0% positive	VALID
In-house Test Panel: plasma pools spiked with HIV-1 at 1.02×10^4 gEq/mL	Robustness (cross-contamination)	100% positive	100% positive	

10. Conclusions

The COBAS AmpliScreen™ HIV Test, v1.5, met or exceeded the performance of the previous method used at the Raleigh Test Lab (Roche AmpliScreen™ HIV-1 microwell plate method, v1.5) when testing plasma pools. The COBAS AmpliScreen™ HIV-1 Test, v1.5, can detect HIV-1 RNA in source plasma pools and recovered plasma pools at a titer of 20 gEq/mL with a positive-test rate at or above 95%.

Table 3 - Absorbance Values (660 nm) and Detection Frequency for the Test Panel for the Confirmation of Test Specificity in Plasma Pools

TEST PANEL: HIV-1 negative plasma pools			
Sample	**Operator**	**A_{660} HIV**	**A_{660} Internal Control**
035A	Operator 1	0.001	≥4.000
035D	Operator 1	0.001	≥4.000
045F	Operator 1	0.000	2.996
045G	Operator 1	0.003	≥4.000
045H	Operator 1	0.012	≥4.000
045I	Operator 1	0.002	≥4.000
045J	Operator 1	0.000	≥4.000
047A	Operator 1	0.002	≥4.000
047B	Operator 1	0.001	≥4.000
047C	Operator 1	0.001	≥4.000
047D	Operator 1	0.000	3.737
047E	Operator 1	0.001	≥4.000
047F	Operator 1	0.001	≥4.000
047G	Operator 1	0.001	3.738
048A	Operator 1	0.001	3.562
048B	Operator 1	0.001	≥4.000
048C	Operator 1	0.002	3.562
048D	Operator 1	0.001	3.738
048E	Operator 1	0.003	3.848
048F	Operator 1	0.004	3.848
048G	Operator 1	0.002	3.848
048H	Operator 1	0.005	3.848
048I	Operator 1	0.006	3.848
048J	Operator 1	0.003	≥4.000
048K	Operator 1	0.003	≥4.000
053O	Operator 1	0.003	3.849
053P	Operator 1	0.002	≥4.000
054B	Operator 1	0.002	≥4.000
054C	Operator 1	0.004	3.849
054D	Operator 1	0.002	3.849
054F	Operator 1	0.005	≥4.000
054G	Operator 1	0.005	3.849
060A	Operator 1	0.002	≥4.000
060D	Operator 1	0.005	≥4.000
040C	Operator 2	0.003	≥4.000
064E	Operator 2	0.004	≥4.000
064F	Operator 2	0.005	3.853
064G	Operator 2	0.003	≥4.000

Table 3 - Continued

TEST PANEL: HIV-1 negative plasma pools			
Sample	**Operator**	**A_{660} HIV**	**A_{660} Internal Control**
064H	Operator 2	0.004	3.853
064I	Operator 2	0.003	≥4.000
033D	Operator 2	0.005	≥4.000
034D	Operator 2	0.003	≥4.000
037B	Operator 2	0.003	≥4.000
037D	Operator 2	0.004	≥4.000
038C	Operator 2	0.005	3.852
037G	Operator 2	0.004	≥4.000
039F	Operator 2	0.004	≥4.000
040A	Operator 2	0.004	≥4.000
040B	Operator 2	0.005	3.852
053K	Operator 2	0.001	3.439
053L	Operator 2	0.001	≥4.000
053M	Operator 2	0.000	≥4.000
053N	Operator 2	0.000	≥4.000
062C	Operator 2	0.001	3.740
062D	Operator 2	0.001	≥4.000
062F	Operator 2	0.000	≥4.000
062G	Operator 2	0.000	≥4.000
062I	Operator 2	0.000	≥4.000
062N	Operator 2	0.004	3.847
063C	Operator 2	0.003	3.847
063D	Operator 2	0.004	3.847
063G	Operator 2	0.003	3.671
063H	Operator 2	0.004	3.847
063I	Operator 2	0.002	3.847
064A	Operator 2	0.003	3.847
064B	Operator 2	0.003	3.847
064D	Operator 2	0.007	≥4.000
060E	Operator 3	0.001	2.892
060G	Operator 3	0.001	3.737
060H	Operator 3	0.002	3.737
060I	Operator 3	0.001	≥4.000
060J	Operator 3	0.011	≥4.000
060K	Operator 3	0.001	3.737
060L	Operator 3	0.002	≥4.000
060M	Operator 3	0.001	3.737
061A	Operator 3	0.001	≥4.000
061B	Operator 3	0.000	≥4.000
061C	Operator 3	0.000	≥4.000
061D	Operator 3	0.000	≥4.000

Table 3 - Continued

TEST PANEL: HIV-1 negative plasma pools			
Sample	**Operator**	**A_{600} HIV**	**A_{600} Internal Control**
062B	Operator 3	0.002	≥4.000
048L	Operator 3	0.010	≥4.000
048M	Operator 3	0.003	≥4.000
048N	Operator 3	0.002	≥4.000
048O	Operator 3	0.002	≥4.000
048P	Operator 3	0.004	≥4.000
048Q	Operator 3	0.005	3.849
048R	Operator 3	0.004	3.849
048S	Operator 3	0.004	3.849
050B	Operator 3	0.005	≥4.000
050E	Operator 3	0.006	≥4.000
050F	Operator 3	0.005	≥4.000
050G	Operator 3	0.005	3.849
050H	Operator 3	0.006	≥4.000
053A	Operator 3	0.004	3.849
053B	Operator 3	0.004	3.849
053C	Operator 3	0.010	≥4.000
053D	Operator 3	0.008	3.849
053E	Operator 3	0.005	3.849
053F	Operator 3	0.006	≥4.000
053J	Operator 3	0.038	≥4.000
Positive Rate		0/100	All positive
Detection Frequency		0 %	

Table 4 - Absorbance Values (660 nm) and Detection Frequency for Recovered Plasma Pools

TEST PANEL: Recovered plasma pools spiked with HIV-1 RNA at 20 gEq/mL								
	Operator 1			Operator 2			Operator 3	
Sample	A_{660} HIV-1	A_{660} Internal Control	Sample	A_{660} HIV-1	A_{660} Internal Control	Sample	A_{660} HIV-1	A_{660} Internal Control
RHIVN	0.004	3.437	RHIVN	0.003	≥4.000	RHIVN	0.001	≥4.000
R1A	≥4.000	3.561	R1B	≥4.000	≥4.000	R1C	≥4.000	3.736
R2A	≥4.000	3.561	R2B	3.738	3.740	R2C	3.736	2.957
R3A	≥4.000	3.738	R3B	≥4.000	≥4.000	R3C	≥4.000	≥4.000
R4A	≥4.000	≥4.000	R4B	≥4.000	≥4.000	R4C	3.736	≥4.000
R5A	≥4.000	3.738	R5B	≥4.000	≥4.000	R5C	≥4.000	≥4.000
R6A	≥4.000	≥4.000	R6B	0.003	≥4.000	R6C	3.736	≥4.000
R7A	≥4.000	3.738	R7B	≥4.000	3.740	R7C	3.736	3.259
R8A	≥4.000	3.738	R8B	≥4.000	≥4.000	R8C	≥4.000	3.736
R9A	≥4.000	3.737	R9B	3.675	≥4.000	R9C	≥4.000	≥4.000
R10A	≥4.000	3.560	R10B	3.550	≥4.000	R10C	≥4.000	≥4.000
R11A	3.736	3.560	R11B	3.851	3.851	R11C	≥4.000	≥4.000
R12A	2.102	≥4.000	R12B	≥4.000	≥4.000	R12C	≥4.000	≥4.000
R13A	≥4.000	≥4.000	R13B	≥4.000	3.851	R13C	≥4.000	≥4.000
R14A	3.736	≥4.000	R14B	3.675	3.675	R14C	≥4.000	≥4.000
R15A	≥4.000	3.436	R15B	≥4.000	3.851	R15C	≥4.000	≥4.000
R16A	3.560	3.737	R16B	≥4.000	3.851	R16C	≥4.000	≥4.000
RHIVN	0.001	3.561	RHIVN	0.006	≥4.000	RHIVN	0.001	≥4.000
R17A	3.561	3.737	R17B	3.549	3.850	R17C	3.437	≥4.000
R18A	≥4.000	≥4.000	R18B	3.850	≥4.000	R18C	3.562	≥4.000
R19A	≥4.000	≥4.000	R19B	3.549	≥4.000	R19C	3.738	≥4.000
R20A	≥4.000	3.560	R20B	3.674	≥4.000	R20C	3.340	3.564
R21A	1.179	3.738	R21B	3.850	≥4.000	R21C	3.738	≥4.000
R22A	3.339	3.193	R22B	3.850	3.850	R22C	3.261	3.439
R23A	≥4.000	3.561	R23B	3.850	3.850	R23C	3.437	≥4.000
R24A	≥4.000	3.737	R24B	≥4.000	≥4.000	R24C	3.341	≥4.000
RHIVN	0.001	3.737	RHIVN	0.005	≥4.000	RHIVN	0.001	≥4.000
Positive Rate	24/24	All positive		23/24	All positive		24/24	All positive
Total Positive Rate	71/72							
Detection Frequency	100%			96%			100%	
Total Detection Frequency	99%							

Table 5 - Absorbance Values (660 nm) and Detection Frequency for Source Plasma Pools

TEST PANEL: Source plasma pools spiked with HIV-1 RNA at 20 gEq/mL								
	Operator 1		Operator 2			Operator 3		
Sample	A_{660} HIV-1	A_{660} Internal Control	Sample	A_{660} HIV-1	A_{660} Internal Control	Sample	A_{660} HIV-1	A_{660} Internal Control
SHIVN	0.002	3.562	SHIVN	0.001	3.343	SHIVN	0.001	≥4.000
S1A	≥4.000	≥4.000	S1B	3.563	3.564	S1C	≥4.000	≥4.000
S2A	3.737	≥4.000	S2B	≥4.000	≥4.000	S2C	≥4.000	3.259
S3A	3.737	≥4.000	S3B	≥4.000	3.741	S3C	≥4.000	2.957
S4A	3.561	≥4.000	S4B	≥4.000	3.741	S4C	≥4.000	≥4.000
S5A	≥4.000	3.562	S5B	≥4.000	2.896	S5C	≥4.000	≥4.000
S6A	3.737	3.738	S6B	≥4.000	3.000	S6C	3.736	3.736
S7A	≥4.000	3.562	S7B	3.740	3.565	S7C	≥4.000	≥4.000
S8A	2.716	3.738	S8B	3.740	≥4.000	S8C	≥4.000	≥4.000
S9A	≥4.000	≥4.000	S9B	≥4.000	3.852	S9C	3.738	≥4.000
S10A	3.736	3.737	S10B	3.850	3.852	S10C	≥4.000	≥4.000
S11A	3.736	3.737	S11B	3.851	3.853	S11C	≥4.000	≥4.000
S12A	≥4.000	3.737	S12B	≥4.000	3.853	S12C	3.738	≥4.000
S13A	3.037	3.737	S13B	≥4.000	3.853	S13C	≥4.000	≥4.000
S14A	≥4.000	3.737	S14B	≥4.000	3.853	S14C	≥4.000	3.738
S15A	3.736	3.738	S15B	≥4.000	3.853	S15C	≥4.000	≥4.000
S16A	3.736	≥4.000	S16B	≥4.000	≥4.000	S16C	≥4.000	≥4.000
SHIVN	0.002	≥4.000	SHIVN	0.004	3.853	SHIVN	0.001	≥4.000
S17A	≥4.000	3.260	S17B	3.850	≥4.000	S17C	3.340	≥4.000
S18A	≥4.000	3.261	S18B	3.850	3.851	S18C	3.562	≥4.000
S19A	≥4.000	3.340	S19B	3.674	≥4.000	S19C	3.437	≥4.000
S20A	≥4.000	3.261	S20B	3.850	≥4.000	S20C	3.562	3.739
S21A	3.737	≥4.000	S21B	3.850	3.852	S21C	3.562	≥4.000
S22A	3.737	3.437	S22B	3.850	≥4.000	S22C	3.738	≥4.000
S23A	3.737	≥4.000	S23B	3.850	≥4.000	S23C	3.562	≥4.000
S24A	3.339	3.562	S24B	3.673	3.852	S24C	3.738	≥4.000
SHIVN	0.003	≥4.000	SHIVN	0.004	3.851	SHIVN	0.001	3.195
Positive Rate	24 / 24	All positive		24/24	All positive		24/24	All positive
Total Positive Rate	72 / 72							
Detection Frequency	100%			100%			100%	
Total Detection Frequency	100%							

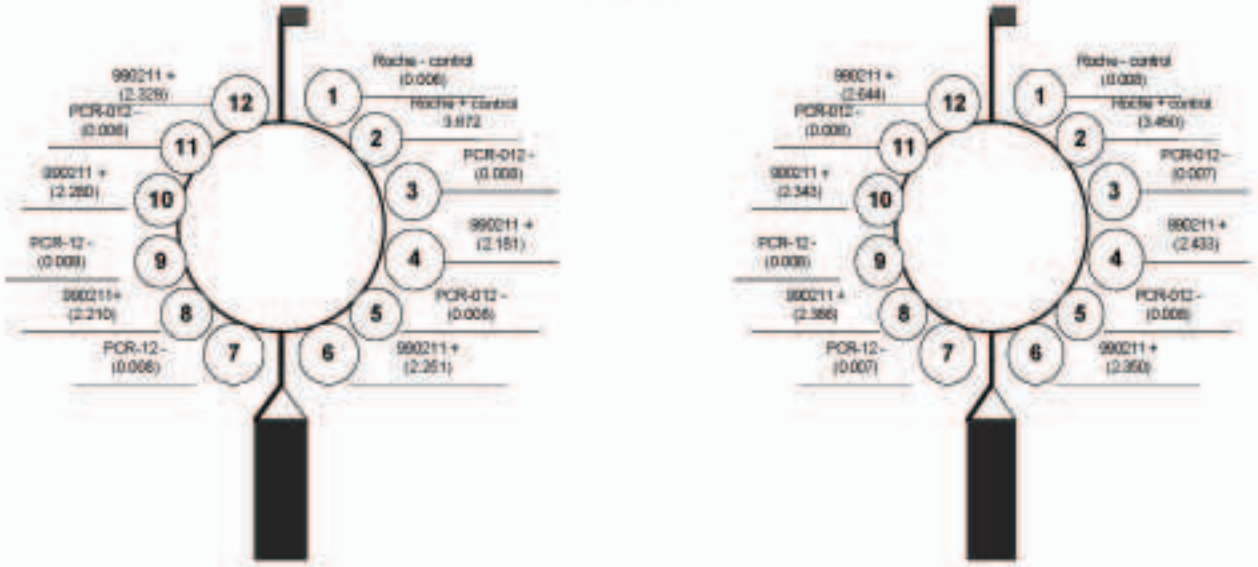
Table 6 - Cross-Contamination Study

Sample	Operator	A_{650} HIV-1	A_{650} Internal Control
PCR-012	Operator 2	0.008 (-)	≥4.000
NATHIV.990211	Operator 2	2.181 (+)	≥4.000
PCR-012	Operator 2	0.008 (-)	3.673
NATHIV.990211	Operator 2	2.251 (+)	≥4.000
PCR-012	Operator 2	0.008 (-)	≥4.000
NATHIV.990211	Operator 2	2.210 (+)	≥4.000
PCR-012	Operator 2	0.009 (-)	3.849
NATHIV.990211	Operator 2	2.280 (+)	≥4.000
PCR-012	Operator 2	0.006 (-)	3.849
NATHIV.990211	Operator 2	2.329 (+)	≥4.000
PCR-012	Operator 2	0.007 (-)	3.849
NATHIV.990211	Operator 2	2.433 (+)	≥4.000
PCR-012	Operator 2	0.008 (-)	3.849
NATHIV.990211	Operator 2	2.350 (+)	≥4.000
PCR-012	Operator 2	0.007 (-)	≥4.000
NATHIV.990211	Operator 2	2.386 (+)	≥4.000
PCR-012	Operator 2	0.008 (-)	≥4.000
NATHIV.990211	Operator 2	2.343 (+)	3.850
PCR-012	Operator 2	0.006 (-)	3.850
NATHIV.990211	Operator 2	2.644 (+)	3.674

Assay detection array with individual assay results are listed in Table 6.

For HIV-1 specific reactions a (+) or (-) is included to define the determined result. Sample identity and coordinates are defined according to their placement on the amplification and detection A-rings defined below in Figure 1.

Figure 1



Validation of Test Methods

Validation of the Detection of HBV DNA in Plasma Pools using the Roche AmpliScreen™ HBV Test Kit on the COBAS AMPLICOR™ Analyzer

Number of Pages
(including cover page)

17

Original is signed

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Valid from: 13 Jul 2004

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1. Introduction

Hepatitis B Virus (HBV) is considered to be one of the major etiologic agents that cause chronic and acute hepatitis, cirrhosis and hepatocellular carcinoma. HBV is a partially double-stranded circular DNA virus with a genome of approximately 3,200 bases that contains four overlapping open reading frames encoding for all viral proteins. As a blood-borne virus, HBV can be transmitted with a higher risk than HCV and HIV by blood and blood products. HBV is one of the most infectious diseases with about 350 million chronic hepatitis B carriers worldwide. The yearly new infections are about 200 to 300 thousand in the United States and approximately 1 million in Europe. The global prevalence of chronic HBV infection, as determined by immunoserology, ranges from <2% in western countries to ≥8% in Asian and African countries.

The presence of HBV antigens or antibodies in-patients infected with HBV has led to the development of immunoserological tests that are specific for these antigens or antibodies. Implementation of these tests has reduced, but not completely eliminated, the incidence of post-transfusion hepatitis. Currently, the most used common marker of HBV infection is the presence of HBV surface antigen (HBsAg). However, it has been reported that blood units from HBsAg negative donors caused post-transfusion hepatitis B in recipients and HBV DNA was prospectively detected in the donor's blood units by PCR. In theory, screening of blood donations for HBV DNA should further reduce the residual transmission risk. PCR tests should also detect viremic units donated by carriers who are in the window period, early acute infection, or late resolving infection that may not be detectable by the existing immunological assays.

The COBAS AmpliScreen™ HBV Test, an in vitro nucleic acid amplification test for the qualitative detection of HBV DNA in plasma, is designed to screen pooled samples of donated human plasma. The screening of pooled plasma and the subsequent culling of positive material ensures a reduction of the potential viral load in production pools. The reduction of viral load is significant to improving the efficacy of viral inactivation steps that occur during the production process.

Manufacturing-scale plasma pools are created from individual source plasma donations that have been screened by NAT for one or more blood borne viruses. These manufacturing pools may then be retested for these viruses, to ensure that negative manufacturing pools are processed.

2. Objective

The aim of this study was to demonstrate the robustness of the Roche COBAS AmpliScreen™ HBV kit when testing pool matrices of source plasma donations and recovered plasma donations. The validation protocol was conducted according to the ICH Harmonized Tripartite Guideline on the Validation of Analytical Procedures and the EDQM document PA/PH/OMCL (98) 22, DEF *Validation Of Nucleic Acid Amplification Technology (NAT) For The Detection Of Hepatitis C Virus (HCV) RNA In Plasma Pools*

in accordance with the E.P. general chapter 2.6.21, *Nucleic Acid Amplification Techniques*.

3. Validation Testing Facility

Talecris Biotherapeutics, Inc.
Raleigh Test Lab
1200 New Hope Road
Raleigh, North Carolina 27610

4. Persons Involved in the Validation Study

Study Director:
Michael Gray, NAT Technical Operations Supervisor

Study Personnel:
Matt Selley, NAT Laboratory Technician, Operator 1
Farzaneh Sina, NAT Chief Laboratory Technician, Operator 2
Jarrett Brown, NAT Chief Laboratory Technician, Operator 3
Eugenia Vallido, NAT Chief Laboratory Technician, Operator 4

5. Description of Materials Being Tested

5.1 HBV Positive Material

The HBV In-house Standard (NAT-098) was used in these studies. The standard consists of an HBV positive plasma donation, calibrated against the WHO international standard at a titer of 7.64×10^8 IU/mL.

5.2 Robustness Study (Verification of assay sensitivity in varied plasma matrices)

Recovered plasma pool samples obtained from archive at the Raleigh Test Lab (RTL) have been tested previously and found negative for HBV. These previously blinded samples, representing pools of approximately 12,000 donations each, were combined to create sufficient volume of a homogeneous matrix to perform this validation.

Source plasma pools were prepared from RTL minipool samples that have been previously tested and found negative for HBV. A sufficient number of these samples were combined to represent the number of donations in a typical manufacturing pool (3,840) and to provide adequate sample volume to perform this validation.

5.3 Cross-Contamination Study

A multiple-source plasma pool (PCR-012), non-reactive for HBV, was used as a diluent in the preparation of HBV dilution for the cross-contamination study. This normal human plasma pool was also utilized as the negative control (NHP) tested along with the HBV In-house Standard NAT-098 in the same study.

5.4 Specificity Study

A panel of 100 source plasma pools (3,840 donations each) served as the matrices for all of the final test samples used in the specificity study (Listed in Table 3). The 100 pools were created from plasma minipools that were previously tested and found negative for HBV.

The dilution scheme and composition of the samples tested during this validation study is outlined in Table 1.

Table 1 - Composition and Dilution Scheme of Sample Panels Tested During Validation

Stock	Dilution	Dilution Procedure	Final Sample
HBV In-house Standard (NAT-098) at 7.64×10^8 IU/mL	1:100	0.1 mL NAT-098 + 9.9 mL Recovered Plasma	HBV intermediate at 7.64×10^6 IU/mL
HBV intermediate at 7.64×10^6 IU/mL	1:100	0.1 mL HBV intermediate at 7.64×10^6 IU/mL + 9.9 mL Recovered Plasma	HBV intermediate at 7.64×10^4 IU/mL
HBV intermediate at 7.64×10^4 IU/mL	1:20	0.25 mL HBV intermediate at 7.64×10^4 IU/mL + 9.75 mL Recovered Plasma	HBV intermediate at 1.91×10^3 IU/mL
HBV intermediate at 1.91×10^3 IU/mL	1:106	0.9 mL HBV intermediate at 1.91×10^3 IU/mL + 94.6 mL Recovered Plasma	HBV positive (18 IU/mL) Recovered Plasma Pool Panel
HBV In-house Standard (NAT-098) at 7.64×10^8 IU/mL	1:100	0.1 mL NAT-098 + 9.9 mL Source Plasma	HBV intermediate at 7.64×10^6 IU/mL
HBV intermediate at 7.64×10^6 IU/mL	1:100	0.1 mL HBV intermediate at 7.64×10^6 IU/mL + 9.9 mL Source Plasma	HBV intermediate at 7.64×10^4 IU/mL
HBV intermediate at 7.64×10^4 IU/mL	1:20	0.25 mL HBV intermediate at 7.64×10^4 IU/mL + 9.75 mL Source Plasma	HBV intermediate at 1.91×10^3 IU/mL
HBV intermediate at 1.91×10^3 IU/mL	1:106	0.9 mL HBV intermediate at 1.91×10^3 IU/mL + 94.6 mL Source Plasma	HBV positive (18 IU/mL) Source Plasma Pool Panel
HBV In-house Standard (NAT-098) at 7.64×10^8 IU/mL	1:100	0.5 mL NAT-098 + 49.5 mL PCR-012	HBV positive 7.64×10^6 IU/mL Cross-Contamination Panel (Total Volume 50 mL)

6. Analytical Test Method

The COBAS AmpliScreen™ HBV test is based on four major processes: sample and control preparation; PCR amplification of target DNA using HBV specific complementary primers; hybridization of the amplified products to oligonucleotide

probes specific to the target(s); and detection of the probe-bound amplified product by colorimetric determination.

6.1 Extraction of HBV Nucleic Acid from Human Plasma

HBV DNA is isolated from plasma. After an initial high-speed centrifugation step to concentrate virus particles, the HBV DNA is isolated by lysis of virus particles with a chaotropic agent followed by precipitation of the DNA with alcohol. The HBV Internal Control DNA is introduced into each specimen with the Lysis Reagent and serves as an extraction and amplification control for each individually processed specimen.

6.2 Internal Control

In enzyme-based amplification processes such as PCR, efficiency can be reduced by inhibitors that may be present in the sample. The HBV Internal Control has been added to the COBAS AmpliScreen™ HBV Test to permit identification of processed samples containing substances that may interfere with PCR amplification. The HBV Internal Control is a DNA plasmid with primer binding regions identical to those of the HBV target sequence, a randomized internal sequence of similar length and base composition as the HBV target sequence, and a unique probe binding region that differentiates the HBV Internal Control amplicon from target amplicon. These features were selected to ensure equivalent amplification of the HBV Internal Control and the HBV target DNA.

6.3 Detection of HBV in Plasma Pools Using Polymerase Chain Reaction Methodology

The COBAS AMPLICOR Analyzer chemically denatures the HBV and IC amplicons into single-stranded DNA immediately after PCR amplification. During the detection the amplicons are hybridized to target-specific oligonucleotide probes bound to magnetic particles, which increases the overall specificity of the test. A colored complex is formed when particle-bound horseradish peroxidase catalyzes the oxidation of tetramethylbenzidine (TMB). The absorbance is measured by the COBAS AMPLICOR Analyzer at a wavelength of 660 nm.

6.3.1 Detection Controls

The Roche Negative control (-) is designed to confirm acceptable performance of the assay. The absorbance for the Roche (-) Control must be less than 0.200 at 660 nm. If the absorbance value is greater than or equal to 0.200, the entire run is invalid and must be repeated (sample preparation, amplification and detection).

The Roche Positive control (+) is designed to confirm acceptable performance of the assay. The absorbance for the MP (+) Control must be greater than or equal to 1.000 at 660 nm. If the absorbance value is less than 1.000 nm, the entire run is invalid and must be repeated (sample preparation, amplification and detection).

The Roche Internal Control absorbance (IC) for samples and controls must be greater than or equal to 0.200 at 660 nm for a valid test. A sample IC of less than 0.200 invalidates the result for that sample, unless the test result is positive. If the absorbance of the sample is greater than or equal to the cut-off value of 0.200 at 660 nm, the Internal Control result is disregarded and the sample is interpreted as positive. If the test result for the sample is invalid, the entire test procedure (sample preparation, amplification and detection) must be repeated.

The In-house Positive Control must be greater than or equal to 0.200 at 660 nm for a valid test. If above conditions are not met, the entire run is discarded and all non-reactive samples are repeated. Reactive results obtained from runs with acceptable kit controls but invalid in-house controls results are treated as positive.

6.3.2 Test Disposition

A test sample is interpreted as negative for HBV DNA if the absorbance value (660 nm) for the sample is less than 0.200 and the A_{660} value for the internal control is greater than or equal to 0.200. If the A_{660} value for the test sample is less than 0.200 and the A_{660} value for the internal control is less than 0.200, the test for that sample is invalid and the sample must be retested (these samples would not be counted for purposes of this validation). If the absorbance value (660 nm) for the test sample is greater than or equal to 0.200, then the test sample is interpreted as positive for HBV DNA. Test samples with an A_{660} greater than or equal to 0.200 are interpreted as positive for HBV DNA regardless of the A_{660} value for the HBV IC.

7. Design of the Validation Study

The qualitative PCR assay described here functions as a limit test for impurities. Test results are interpreted as either positive or negative for a detectable analyte. This limit test was validated for assay robustness, repeatability, intermediate precision, and specificity.

7.1 Sensitivity (Detection Limit)

The detection limit of the type of qualitative assay must not only account for the quantity of the nucleic acid units per unit volume, but it must also be expressed as a function of the positive-test rate for a specific nucleic acid load. The positive cut-off point is the minimum number of target sequences per volume sample, which can be detected in 95% of test runs. The COBAS AmpliScreen™ HBV test can detect HBV DNA at levels as low as 6 IU/mL with a 95% positivity rate.

7.2 Specificity

Specificity is primarily a function of primer selection in PCR assays. One hundred source pools of non-reactive plasma were tested to establish that the assay does not generate false-positive reactions from its integral components or pool constituents.

7.3 Repeatability and Intermediate Precision

Repeatability and intermediate precision were addressed as a function of the detection limit. As a component of assay execution, each test consisted of separate extraction, amplification and detection sessions, and the operator accounted for each phase of the assay. Source plasma pools and recovered plasma pools spiked with HBV at 18 IU/mL were tested in panels consisting of eight samples each over a three day period for both recovered and source plasma pools. Operators were required to achieve individual (repeatability) and collective (intermediate precision) positive-tests at a rate of 95 to 100% in order to pass the validation.

7.4 Robustness

This validation addressed the issue of robustness through detection of HBV at the targeted cutoff in varied plasma backgrounds (recovered and source plasma) and the absence of cross-contamination between negative and high-titer HBV samples.

Test sensitivity in varied pool constituents was addressed by testing 24 individual plasma matrices consisting of both recovered (3 operators x 24 matrices) and source plasma (3 operators x 24 matrices) pools non-reactive for HBV containing 1,000 to 12,000 different donations. To demonstrate robustness, sample pools were spiked with HBV DNA at 18 IU/mL (3 times the detection limit). The robustness of assay performance under these conditions (varied pool constituents) was assessed by the ability to attain a positive-test rate of 95%.

Cross-contamination concerns were addressed by testing a panel of alternating non-reactive pools and non-reactive pools spiked with HBV to a titer of 7.64×10^6 IU/mL. One operator tested a panel consisting of 10 negative samples alternating with 10 high-titer HBV samples to detect the presence of HBV DNA. Robustness of assay performance was assessed by the ability of the test to accurately detect negative (no positives) and high-titer HBV positive samples (100% positive) arranged in an alternating pattern (Figure 1).

8. Discussion of Experimental Results

This study of the COBAS AmpliScreen™ HBV Test for the detection of HBV DNA in plasma pools using polymerase chain reaction methodology provides a validation of the assay robustness.

Test specificity was addressed by testing 100 source plasma pools consisting of plasma samples found non-reactive during donor screening for HBV. These tests were carried out to establish that the assay does not generate false-positive reactions from its integral components or pool constituents. The validation requirement that no positive results be obtained was successfully achieved (Table 3).

The accuracy of the targeted 18 IU/mL value was assessed by multiple-operator testing of source plasma pools and recovered plasma pools spiked with the in-house standard to a genome titer of 18 IU/mL. The sensitivity requirement that the assay must detect positive samples within each test panel at a rate of 95 to 100% was met (Tables 4 and 5). In addition, the overall positive test rates for the source plasma pools and recovered plasma pools were 100% and 99%, meeting the validation criterion. Test robustness was demonstrated with these test panels by the ability to detect HBV DNA at 18 IU/mL in complex plasma matrices representative of plasma pools at a rate of 95 to 100%.

Cross-contamination concerns were addressed by extracting, amplifying, and detecting a panel of alternating non-reactive pools and non-reactive pools spiked with HBV at a titer of 7.64×10^6 IU/mL. Robustness of assay performance was demonstrated by the ability of the test to accurately detect negative (no positives) and high-titer HBV positive samples (100% positive) arranged in a alternating pattern (Table 6 and Figure 1).

Results shown were obtained in test runs that had successful positive, negative, and internal controls. Samples that were repeated are as follows:

- Operator 4 had a Roche Negative failure for sample set 050B-050D during the specificity study. Entire sample set was repeated.
- Operator 1 had an IC failure for samples 048R and 048P during the specificity study. Samples were repeated.
- Operator 2 had an IC failure for samples S2B and S7B during the robustness study. Samples were repeated.

The results of this validation study are summarized in Table 2.

Table 2 - Results of the Tests in the Study to Validate the Process for the Detection of HBV DNA in Plasma Pools Using Polymerase Chain Reaction Methodology

Validation Level Of Analyte Strength (Sample Type)	Type Of Validation Testing	Acceptance Criteria	Results	Outcome
Negative diluent controls: Plasma pools	Specificity	No positives	0% positive	**VALID**
In-house Test Panel: Recovered plasma pools at 18 IU/mL	Robustness: (sensitivity), repeatability, and intermediate precision	95 to 100% positive	Op 1 100% Op 2 96% Op 3 100% Total 99%	**VALID**
In-house Test Panel: Source plasma pools at 18 IU/mL	Robustness: (sensitivity), repeatability, and intermediate precision	95 to 100% positive	Op 1 100% Op 2 100% Op 3 100% Total 100%	**VALID**
Negative diluent controls: Plasma pools	Robustness (cross-contamination)	No positives	0% positive	**VALID**
In-house Test Panel: Plasma pools spiked with HBV at 7.64 x 10^6 IU/mL	Robustness (cross-contamination)	100% positive	100% positive	

9. Conclusions

The COBAS AmpliScreen™ HBV Test meets or exceeds the performance requirements recommended by the EDQM and adopted by the Raleigh Test Lab. The COBAS AmpliScreen™ HBV Test can detect HBV DNA in source plasma pools and recovered plasma pools at a titer of 18 IU/mL with a positive-test rate at or above 95%.

Table 3 - Absorbance Values (660 nm) and Detection Frequency for the Test Panel for the Confirmation of Test Specificity in Plasma Pools

TEST PANEL: HBV negative plasma pools			
Sample	**Operator**	**A_{660} HBV**	**A_{660} Internal Control**
081D	Operator 4	0.004	3.851
062N	Operator 4	0.004	≥4.000
064I	Operator 4	0.004	≥4.000
064F	Operator 4	0.004	3.851
053B	Operator 4	0.005	≥4.000
047F	Operator 4	0.004	3.850
060K	Operator 4	0.004	≥4.000
047E	Operator 4	0.011	3.850
064E	Operator 4	0.004	≥4.000
081C	Operator 4	0.005	≥4.000
081E	Operator 4	0.004	3.851
081G	Operator 4	0.004	3.851
081I	Operator 4	0.006	3.851
081M	Operator 4	0.004	≥4.000
081O	Operator 4	0.004	≥4.000
081J	Operator 4	0.004	3.851
081H	Operator 4	0.004	≥4.000
081F	Operator 4	0.009	≥4.000
062C	Operator 4	0.003	2.999
062D	Operator 4	0.002	3.739
062E	Operator 4	0.003	≥4.000
062G	Operator 4	0.003	3.739
062I	Operator 4	0.003	≥4.000
062J	Operator 4	0.003	3.563
062M	Operator 4	0.003	3.739
048A	Operator 4	0.003	3.438
063C	Operator 4	0.003	≥4.000
050B	Operator 4	0.003	3.854
047A	Operator 4	0.003	≥4.000
060M	Operator 4	0.004	≥4.000
061C	Operator 4	0.003	3.678
062F	Operator 4	0.003	3.854
063A	Operator 4	0.002	3.854
048D	Operator 4	0.003	≥4.000
062B	Operator 4	0.003	3.854
050D	Operator 4	0.003	3.854
047C	Operator 4	0.002	3.854
045I	Operator 4	0.003	≥4.000

Table 3 - Continued

TEST PANEL: HBV negative plasma pools			
Sample	**Operator**	**A_{660} HBV**	**A_{660} Internal Control**
048J	Operator 4	0.003	≥4.000
048B	Operator 4	0.003	3.854
064G	Operator 4	0.003	≥4.000
048C	Operator 4	0.003	≥4.000
048Q	Operator 4	0.004	≥4.000
064H	Operator 4	0.004	3.854
048E	Operator 4	0.004	3.854
063B	Operator 4	0.003	3.852
063D	Operator 4	0.003	3.852
063G	Operator 4	0.003	≥4.000
063H	Operator 4	0.004	≥4.000
063I	Operator 4	0.004	≥4.000
064A	Operator 4	0.004	3.852
064B	Operator 4	0.004	3.852
064C	Operator 4	0.004	≥4.000
064D	Operator 4	0.003	2.132
035A	Operator 4	0.003	3.852
035D	Operator 4	0.003	3.853
045F	Operator 4	0.003	3.853
045G	Operator 4	0.003	3.853
047B	Operator 4	0.004	3.853
045J	Operator 4	0.004	≥4.000
045H	Operator 4	0.005	≥4.000
053M	Operator 4	0.008	≥4.000
053N	Operator 4	0.003	≥4.000
048S	Operator 4	0.002	3.736
050E	Operator 4	0.002	≥4.000
050F	Operator 4	0.002	≥4.000
050G	Operator 4	0.002	≥4.000
060D	Operator 4	0.002	≥4.000
060E	Operator 4	0.002	3.338
060F	Operator 4	0.002	3.338
060H	Operator 4	0.003	≥4.000
060G	Operator 4	0.002	3.736
060I	Operator 4	0.002	3.561
048R	Operator 4	0.001	3.737
048P	Operator 4	0.002	≥4.000

Table 3 - Continued

TEST PANEL: HBV negative plasma pools			
Sample	**Operator**	**A_{660} HBV**	**A_{660} Internal Control**
053K	Operator 2	0.003	3.258
050H	Operator 2	0.002	3.736
053A	Operator 2	0.001	3.192
061E	Operator 2	0.001	≥4.000
061D	Operator 2	0.001	3.134
053F	Operator 2	0.001	3.435
053E	Operator 2	0.001	3.560
053D	Operator 2	0.002	3.736
053C	Operator 2	0.004	≥4.000
048K	Operator 1	0.004	3.844
048L	Operator 1	0.004	≥4.000
048N	Operator 1	0.003	3.844
048O	Operator 1	0.002	≥4.000
053P	Operator 1	0.003	≥4.000
054B	Operator 1	0.003	≥4.000
054C	Operator 1	0.003	3.844
054D	Operator 1	0.004	≥4.000
054F	Operator 1	0.004	≥4.000
054G	Operator 1	0.004	≥4.000
060A	Operator 1	0.003	≥4.000
060J	Operator 1	0.004	≥4.000
061L	Operator 1	0.004	3.845
061A	Operator 1	0.004	≥4.000
061B	Operator 1	0.003	3.845
061F	Operator 1	0.003	≥4.000
Positive Rate		0 / 100	All positive
Detection Frequency		0 %	

Table 4 - Absorbance values (660 nm) and Detection Frequency for Recovered Plasma Pools

TEST PANELs: Recovered plasma pools spiked with HBV DNA at 18 IU/mL								
	Operator 1			Operator 2			Operator 3	
Sample	A_{660} HBV	A_{660} Internal Control	Sample	A_{660} HBV	A_{660} Internal Control	Sample	A_{660} HBV	A_{660} Internal Control
RHBVN	0.003	≥4.000	RHBVN	0.002	≥4.000	RHBVN	0.002	3.546
R1A	3.676	3.852	R1B	0.392	3.193	R1C	3.546	3.546
R2A	3.551	≥4.000	R2B	3.192	3.038	R2C	3.847	3.671
R3A	3.852	3.852	R3B	0.174	0.935	R3C	3.546	3.671
R4A	3.852	≥4.000	R4B	3.560	3.260	R4C	3.671	3.546
R5A	3.852	3.852	R5B	2.297	3.436	R5C	3.671	3.671
R6A	≥4.000	3.852	R6B	3.736	3.339	R6C	3.847	3.671
R7A	3.852	3.852	R7B	3.435	3.338	R7C	3.671	3.546
R8A	3.676	3.853	R8B	≥4.000	≥4.000	R8C	3.848	3.671
R9A	≥4.000	3.737	R9B	3.338	3.260	R9C	≥4.000	≥4.000
R10A	≥4.000	≥4.000	R10B	3.736	≥4.000	R10C	3.739	3.564
R11A	3.561	3.737	R11B	3.435	3.260	R11C	3.739	3.439
R12A	≥4.000	≥4.000	R12B	3.435	≥4.000	R12C	≥4.000	3.564
R13A	≥4.000	≥4.000	R13B	3.435	≥4.000	R13C	3.739	≥4.000
R14A	≥4.000	≥4.000	R14B	3.736	3.260	R14C	3.563	≥4.000
R15A	≥4.000	≥4.000	R15B	≥4.000	≥4.000	R15C	≥4.000	≥4.000
R16A	≥4.000	≥4.000	R16B	3.338	3.737	R16C	3.341	3.440
RHBVN	0.002	≥4.000	RHBVN	0.002	3.561	RHBVN	0.003	3.564
R17A	3.258	≥4.000	R17B	3.737	3.437	R17C	3.451	3.372
R18A	≥4.000	≥4.000	R18B	≥4.000	3.561	R18C	3.451	3.673
R19A	≥4.000	≥4.000	R19B	≥4.000	≥4.000	R19C	3.548	3.451
R20A	≥4.000	3.560	R20B	≥4.000	≥4.000	R20C	3.372	3.451
R21A	3.133	3.736	R21B	3.737	≥4.000	R21C	3.451	3.372
R22A	3.736	3.736	R22B	3.436	≥4.000	R22C	3.451	3.548
R23A	3.082	≥4.000	R23B	3.737	≥4.000	R23C	3.548	3.673
R24A	3.134	≥4.000	R24B	3.339	≥4.000	R24C	3.548	3.548
RHBVN	0.004	≥4.000	RHBVN	0.003	3.136	RHBVN	0.003	3.451
Positive Rate	24/24	All positive		23/24	All positive		24/24	All positive
Total Positive Rate	71/72							
Detection Frequency	100%			96%			100%	
Total Detection Frequency	99%							

Table 5 - Absorbance Values (660 nm) and Detection Frequency for Source Plasma Pools

TEST PANEL: Source plasma pools spiked with HBV DNA at 18 IU/mL								
	Operator 1			Operator 2			Operator 3	
Sample	A_{660} HBV	A_{660} Internal Control	Sample	A_{660} HBV	A_{660} Internal Control	Sample	A_{660} HBV	A_{660} Internal Control
SHBVN	0.003	≥4.000	SHBVN	0.002	≥4.000	SHBVN	0.001	3.672
S1A	3.853	≥4.000	S1B	≥4.000	≥4.000	S1C	≥4.000	3.547
S2A	3.853	3.852	S2B	3.336	≥4.000	S2C	3.547	3.547
S3A	3.853	3.852	S3B	3.737	3.436	S3C	3.848	3.848
S4A	3.853	3.852	S4B	0.486	2.065	S4C	3.848	3.672
S5A	3.853	≥4.000	S5B	≥4.000	≥4.000	S5C	3.848	3.672
S6A	3.852	≥4.000	S6B	≥4.000	3.561	S6C	3.547	3.672
S7A	3.852	≥4.000	S7B	2.994	3.133	S7C	3.672	3.848
S8A	3.852	≥4.000	S8B	≥4.000	3.437	S8C	≥4.000	3.849
S9A	3.737	≥4.000	S9B	≥4.000	3.561	S9C	≥4.000	3.739
S10A	≥4.000	≥4.000	S10B	≥4.000	2.925	S10C	≥4.000	≥4.000
S11A	≥4.000	≥4.000	S11B	≥4.000	3.340	S11C	3.739	≥4.000
S12A	≥4.000	≥4.000	S12B	3.083	3.136	S12C	≥4.000	3.563
S13A	≥4.000	≥4.000	S13B	≥4.000	≥4.000	S13C	≥4.000	3.438
S14A	≥4.000	≥4.000	S14B	≥4.000	3.437	S14C	≥4.000	≥4.000
S15A	≥4.000	3.738	S15B	3.560	≥4.000	S15C	3.563	3.341
S16A	≥4.000	≥4.000	S16B	≥4.000	≥4.000	S16C	3.739	3.740
SHBVN	0.002	≥4.000	SHBVN	0.003	3.038	SHBVN	0.003	3.438
S17A	3.736	≥4.000	S17B	3.561	3.194	S17C	3.849	3.849
S18A	3.338	≥4.000	S18B	3.436	2.863	S18C	3.673	3.673
S19A	3.736	3.737	S19B	3.260	3.562	S19C	3.673	3.673
S20A	3.560	≥4.000	S20B	3.737	≥4.000	S20C	3.849	3.673
S21A	≥4.000	≥4.000	S21B	≥4.000	≥4.000	S21C	3.673	3.674
S22A	3.338	≥4.000	S22B	3.561	3.738	S22C	3.548	3.850
S23A	3.338	≥4.000	S23B	3.260	≥4.000	S23C	3.673	3.549
S24A	3.435	3.737	S24B	3.737	≥4.000	S24C	3.548	3.674
SHBVN	0.003	≥4.000	SHBVN	0.004	≥4.000	SHBVN	0.005	3.548
Positive Rate	24/24	All positive		24/24	All positive		24/24	All positive
Total Positive Rate	72/72							
Detection Frequency	100%			100%			100%	
Total Detection Frequency	100%							

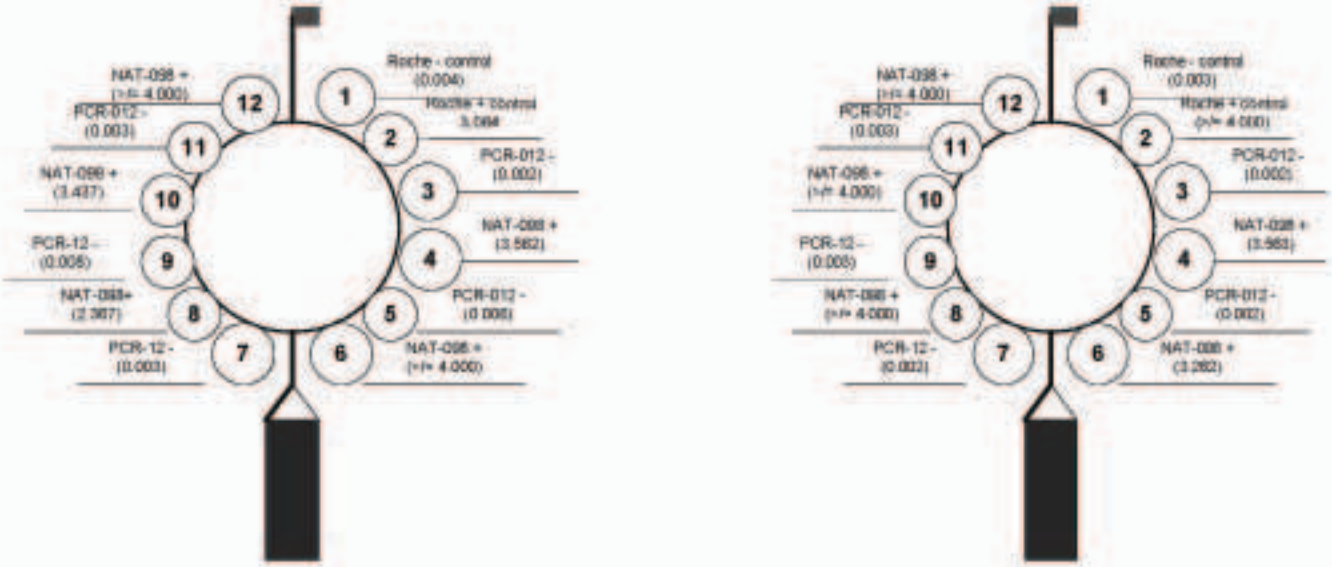
Table 6 - Cross-Contamination Study

Sample	Operator	A_{660} HBV	A_{660} Internal Control
PCR-012	Operator 4	0.002 (-)	≥4.000
NAT-098	Operator 4	3.562 (+)	≥4.000
PCR-012	Operator 4	0.006 (-)	≥4.000
NAT-098	Operator 4	≥4.000 (+)	≥4.000
PCR-012	Operator 4	0.003 (-)	≥4.000
NAT-098	Operator 4	2.367 (+)	2.387
PCR-012	Operator 4	0.008 (-)	3.341
NAT-098	Operator 4	3.437 (+)	≥4.000
PCR-012	Operator 4	0.003 (-)	≥4.000
NAT-098	Operator 4	≥4.000 (+)	≥4.000
PCR-012	Operator 4	0.002 (-)	≥4.000
NAT-098	Operator 4	3.563 (+)	3.563
PCR-012	Operator 4	0.002 (-)	≥4.000
NAT-098	Operator 4	3.262 (+)	≥4.000
PCR-012	Operator 4	0.002 (-)	3.262
NAT-098	Operator 4	≥4.000 (+)	3.739
PCR-012	Operator 4	0.003 (-)	≥4.000
NAT-098	Operator 4	≥4.000 (+)	3.739
PCR-012	Operator 4	0.003 (-)	3.739
NAT-098	Operator 4	≥4.000 (+)	≥4.000

Assay detection array with individual assay results are listed in Table 6.

For HBV specific reactions a (+) or (-) is included to define the determined result. Sample identity and coordinates are defined according to their placement on the amplification and detection A-rings defined below in Figure 1.

Figure 1



Talecris Biotherapeutics, Inc.
Clayton, NC

T.18.47-04

Validation of Test Methods

Method for the Detection of Parvovirus B19 DNA in Plasma Donation Mini-Pools using Polymerase Chain Reaction Methodology, Version 2: The Parvo B19 Test

Number of Pages
(including cover page)

46

Original is signed

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Valid from: 27 Jun 2005

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1. Introduction

Human Parvovirus B19 is the single member of the genus *Erythrovirus* in the *Parvoviridae* family. It is a non-enveloped, single stranded DNA virus that can cause symptoms with clinical relevance. Parvovirus B19 is the etiologic agent of the childhood illness erythema infectiosum (a.k.a. Fifth Disease), acute anemia resulting from transient aplastic crisis, and hydrops fetalis during early pregnancy. The two groups of people most susceptible to Parvovirus B19 infection are immune compromised individuals and pregnant women. Parvovirus B19 infection is highly contagious, primarily through the respiratory route. However, infection may be transmitted parenterally through the infusion of blood or blood-derived products. Its thermal stability and lack of envelope make it difficult to inactivate using conventional technologies, such as pasteurization and treatment with detergent or solvent.

The *Method for the Detection of Parvovirus B19 DNA in Donation Mini-Plasma Pools using Polymerase Chain Reaction Methodology, Version 2,* differs from the previous version of the test in several aspects. In the previous sample preparation method, plasma samples were centrifuged for one hour at high speed to pellet viral particles. In the alternate method for sample preparation, the chaotropic detergent cetyltrimethylammonium bromide (CTAB) is added to the plasma samples to aid in the disruption of viral particles and the recovery of nucleic acids. Parvo B19 Internal Control (IC) DNA is added immediately after the CTAB. Following a 15-minute incubation at room temperature, CTAB/nucleic acid complexes are pelleted during a 15-minute low-speed spin. The CTAB/nucleic acid complexes are processed in the same manner as the pelleted viral particles in the previous sample extraction method. In the previous detection method, only 2.5 µL of extracted sample are added to the amplification reaction. In the current method, 6.5 µL of sample extract are added to the amplification reaction. Because the internal control DNA is added during sample extraction, it serves as a control for nucleic acid recovery, sample transfer, amplification, and detection. Both tests employ a 50 µL amplification reaction and the amplification parameters are the same.

In the Parvovirus B19 Plasma Donation Test, Version 2, cetyltrimethylammonium bromide and Roche AmpliScreen™ Multiprep Sample Prep reagents are used for the recovery of Parvovirus B19 viral DNA from human plasma. The recovered DNA is amplified using oligonucleotide primers provided in the Digene *SHARP Signal*™ System Parvo B19 Probe/Primer set. Parvo B19 Internal Control DNA is added during sample preparation and is a control for nucleic acid recovery, sample transfer, amplification, and detection. Samples and controls are detected using the Digene *SHARP Signal*™ System Assay for PCR Products, a sandwich capture molecular hybridization assay that utilizes colorimetric detection.

2. Objective

The aim of the validation protocol was to validate the *Method for the Detection of Parvovirus B19 DNA in Donation Mini-Pools using Polymerase Chain Reaction Methodology, Version 2*. The validation protocol was performed according to the ICH guidelines for *Validation of Analytical Procedures*.[1] The methods described in the attendant procedures provide the basis for testing donor plasma mini-pools, representing 96 to 480 plasma donations, for the presence of Parvovirus B19. This method is also applicable to the resolution and confirmatory phases of testing for Parvovirus B19-elevated pools, where plasma pools representing either 8 or 12 plasma donations or individual samples are tested.

Specifically, the validation protocol was designed to address the application of the 1 mL CTAB/GITC nucleic acid extraction procedure coupled with target amplification using the Parvo B19 Probe/Primer set[2] and target detection using the Digene *SHARP Signal*™ System Assay for PCR Products.[3] The Parvo B19 Internal Control DNA, which is part of the Digene *SHARP Signal*™ System Parvo B19 IC Probe/Primer set,[4] is extracted and amplified in the same reaction as the Parvovirus B19 target and is detected in parallel. Parvo B19 Primers PVA and PV2B are contained in both the Parvo B19 Probe/Primer and the Parvo B19 IC Probe/Primer sets.

The PPTA Voluntary Standard for Parvovirus B19 states that each PPTA member company will develop an in-process testing program for plasma to prevent highly viremic units from entering manufacturing pools.[5] The cut-off limit, based on the testing protocol, will be set so that viremic plasma that would result in a manufacturing pool exceeding 1×10^5 IU/mL will be removed. Therefore, based on pooling practices, it would be sufficient to test for individual plasma donations with a Parvovirus B19 titer of 1×10^8 IU/mL. The test method to be validated here has been developed with a $\geq$2-$\log_{10}$ margin of safety. The targeted threshold for validation of the test is 5×10^3 IU/mL, the sensitivity required to detect a single donation at 5×10^5 IU/mL in a pool of 96. This targeted threshold supports testing plasma donation mini-pools in the range of 96 to 480 donations.

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[1] ICH Topic Q 2 B. *Validation of Analytical Procedures: Methodology. Step 4, Consensus Guideline. Note for Guidance on Validation of Analytical Procedures: Methodology*. (CPMP/ICH/281/95) (60 FR 27464) Effective 6 November 1996.
[2] Digene *SHARP Signal* System Parvo B19 Probe/Primer Set package insert, Digene Corporation, Gaithersburg, MD.
[3] Digene *SHARP Signal* System Assay for PCR Products package insert, Digene Corporation, Gaithersburg, MD.
[4] Digene *SHARP Signal* System Parvo B19 IC Probe/Primer Set package insert, Digene Corporation, Gaithersburg, MD.
[5] Parvovirus B19 Voluntary Standard (014369), Plasma Protein Therapeutics Association. Effective 4 April 2001.

3. Testing Facility

Talecris Biotherapeutics, Inc.
Pathogen Safety and Research
Nucleic Acid Technology Development Laboratory
1200 New Hope Road
Raleigh, North Carolina 27610
(919) 250-5059

4. Persons Involved in the Study

Study Director:
Todd Gierman, Ph.D., Staff Scientist

Study Personnel:
Martesa Williams, Associate Research Scientist, Operator 1
Kevin Sullivan, Associate Research Scientist, Operator 2
Anne Keen, M.S., Principal Associate Research Scientist, Operator 3

5. Dates of Study

Initiated: August 21, 2002
Completed: August 29, 2002

6. Description of Materials Tested

The Parvovirus B19 In-house Standard, NAT-056, was used. This standard consists of a Parvovirus B19-positive plasma donation, non-reactive for HIV-1, HCV and HBV using PCR methodologies. The Parvovirus B19 In-house Standard was calibrated against the 1st WHO International Standard for Parvovirus B19 DNA NAT Assays (99/800)[6] and the titer was determined to be 7.6×10^{11} IU/mL.[7] The B19 In-house Positive Control (IHP) tested with each panel was a sample spiked with Parvovirus B19 to 5×10^{3} IU/mL (Appendix A). The limit of detection for the Parvovirus B19 Plasma Donation Test, Version 2, has been strategically targeted to viral titers $\leq 1.0 \times 10^{4}$ IU/mL.

The HBV Eurohep Standard *adw*/Genotype A has a genome titer of 2.7×10^{9} Eurohep Units (EU)/mL.[8,9] In these studies, the HBV standard was utilized at 5×10^{5} EU /mL, a

[6] Saldanha, J., Lelie, N., Yu, M.W. and Heath, A. Establishment of the first World Health Organization International Standard for human Parvovirus B19 DNA nucleic acid amplification techniques. *Vox Sanguinis*, Vol. 82, pp. 24-31.
[7] Study to Establish an In-house Standard for B19 DNA NAT Assays: Calibration of a Parvovirus B19 DNA Control Against the 1st WHO International Standard for Human Parvovirus B19 DNA NAT Assays (99/800), NAT Development Lab, Department of Pathogen Safety
[8] Gerlich, W.H., Heermann, K.H., Thomssen, R., and the Eurohep Group. (1995) Quantitative assays for Hepatitis B virus DNA: Standardization and quality control. *Viral Hepatitis Reviews*, Vol. 1, No. 1, pp. 53-57.
[9] Heermann, K.H., Gerlich, W.H., Chudy, M., Schaefer, S., Thomssen, R., and the Eurohep Group. (1999) Quantitative detection of Hepatitis B virus DNA in two international reference plasma preparations. *J Clin Microbiol*, Vol. 37, No. 1, pp. 68-73.

1.85×10^{-4} dilution of the stock, in order to establish that the assay did not generate false-positive reactions from pool constituents, including non-targeted DNA.

A multiple-source plasma pool (NAT-094), non-reactive for HIV-1, HCV, HBV and Parvovirus B19 using PCR methodologies, was used as diluent for the preparation of Parvovirus B19 and as diluent for test panels. This normal human plasma pool was also utilized as the negative control (NHP) tested with each panel (Appendix A).

A panel of 120 initial combined samples (96 donations each) served as the matrices for the test samples for the validation of test specificity (Appendix B). The 120 pools were initial combined samples that were tested in the RTL Operations Laboratory for the presence of HIV-1, HCV, HBV and Parvovirus B19 using PCR methodologies. The 100-member panel prepared for testing during the confirmation of test specificity in Parvovirus B19 DNA-negative pools included the first 100 donor plasma mini-pools (NAT-099A1 to NAT-099A100).

The dilution scheme for and the composition of the samples tested during this validation study are outlined in Appendix B.

7. Analytical Test Method

The method for the detection of Parvovirus B19 is comprised of four major processes: extraction and recovery of DNA from human plasma, PCR amplification of a 265-base pair region of the gene that codes for the Non-Structural protein (NS-1), hybridization of the PCR product to a specific single-stranded RNA probe and detection of the PCR product by color formation.

Cetyltrimethylammonium bromide and Roche AmpliScreen™ Multiprep Sample Prep reagents[10] are used for the recovery of Parvovirus B19 viral DNA from human plasma. The recovered DNA is amplified using oligonucleotide primers provided in the Digene *SHARP Signal*™ System Parvo B19 Probe/Primer set. The Digene *SHARP Signal*™ System Parvo B19 Internal Control DNA is added during the extraction step and is a control for nucleic acid recovery, sample transfer, amplification, and detection.

The purified viral DNA and internal control (IC) DNA are co-amplified using common oligonucleotide primers from the Parvo B19 Probe/Primer or Parvo B19 IC Probe/Primer Sets[2,4] supplied by Digene. The target and internal control DNAs are then detected using specific RNA probes and the Digene *SHARP Signal* System Assay for PCR Products, a sandwich capture molecular hybridization assay that utilizes colorimetric detection.[3]

[10] AmpliScreen HIV-1Test, v 1.5 draft procedure, Roche Molecular Systems, Inc., Somerville, NJ.

The test for the detection of parvovirus DNA in donor plasma pools is a qualitative test for the presence of Parvovirus B19 DNA. Because the detection stage of the assay occurs following the post-linear generation of DNA amplicons, spectrophotometric readings based on enzyme-dependent color formation represent a non-quantitative indicator for the presence, or absence, of detectable Parvovirus B19 DNA. The determination of a positive versus a negative sample is defined by a minimum absorbance reading at 405 nanometers (nM) as defined by the specification of the individual assay. Such qualitative tests function as limit tests for the control of impurities.[11]

Detection of Parvovirus B19 DNA in Donor Plasma Pools using Polymerase Chain Reaction Methodology, Version 2

The purified viral and IC DNAs are co-amplified using oligonucleotide primers from the Parvo B19 Probe/Primer or Parvo B19 IC Probe/Primer Sets supplied by Digene.[2,4] Parvo B19 Primers PVA and PV2B are contained in both the Parvo B19 Probe/Primer and the Parvo B19 IC Probe/Primer sets. Immediately following amplification, aliquots of each PCR reaction are transferred to two wells of a microwell plate containing a denaturation reagent and are denatured into single-stranded DNA. An RNA probe complementary to the biotinylated strand of the Parvovirus B19 target amplicon is added to one well and an RNA probe complementary to the biotinylated strand of the internal control amplicon is added to the other well. RNA:DNA hybrids are captured through biotin onto the surface of streptavidin-coated microwells. The captured RNA:DNA hybrids are then bound by anti-hybrid antibody conjugated to alkaline phosphatase. The hybrid:antibody complexes are detected by measuring the alkaline phosphatase activity using *para*-nitrophenylphenol (PNPP) as the colorimetric substrate.

The Parvo B19 IC DNA consists of plasmid DNA containing the Parvovirus B19 internal control DNA sequence flanked by the Parvo B19 primer sequences.[4] The Parvovirus B19 target DNA and IC DNA are co-amplified using the same primer pair. The Parvovirus B19 target and Parvo B19 IC are detected in parallel reactions. An absorbance value greater than or equal to 0.500 at 405 nM indicates a positive internal control.

The positive cut-off value for the assay is calculated using the absorbance value (405 nM) for the amplified NHP control detected with the Parvovirus B19 RNA probe. The positive cut-off value is equal to the A_{405} of the NHP control + 0.100 A_{405} unit.

[11] ICH Topic Q 2 A. *Guideline for Industry: Text on Validation of Analytical Procedures: Definitions and Terminology: Availability* (60 FR 11260) Effective 1 March 1995.

Detection Controls

Target-specific Positive Assay Controls (PAC) supplied with the Digene *SHARP Signal* System Probe/Primer sets are designed to confirm the performance of the detection assay.[3] During Quality Control (QC) testing of the validation study reagents, the Parvo B19 PAC is tested in duplicate and the mean absorbance value (405 nM) with the Parvovirus B19 probe must be greater than or equal to the positive cut-off value. The Parvo B19 IC PAC is tested in duplicate and the average absorbance value (405 nM) with the Parvo B19 IC probe must be greater than or equal to the positive cut-off value. If any of these conditions is not met, the entire assay is considered invalid and amplification and detection must be repeated.

The Negative Assay Control (NAC) supplied with the Digene *SHARP Signal™ System* Assay for PCR Products is designed to confirm the performance of the detection assay.[3] During QC of the test reagents, the NAC is tested in duplicate with both the Parvovirus B19 probe and the Parvo B19 IC probe. The mean NAC absorbance value (405 nM) with each of the RNA probes must be less than or equal to 0.250. If any of these conditions is not met, the entire assay is considered invalid and amplification and detection must be repeated.

Assay Validation Controls

Valid tests are indicated by the appropriate performance of negative, positive, and internal controls. One negative control (NHP) is extracted, amplified and detected to ensure that the amplification reagents are not contaminated with target and to assist in determination of the positive cut-off value.[3] The absorbance value for the NHP sample must be less than 0.250 A_{405} units with the Parvovirus B19 detection probe and greater than or equal to 0.500 A_{405} units with the Parvo B19 IC detection probe. If either of these conditions is not met, the entire assay is invalid and extraction, amplification, and detection must be repeated.

One positive control sample, the in-house positive (IHP), is extracted, amplified and detected with each test run. The positive control is tested as a measure of success of each process within the assay.[3] Positive control samples are plasma samples spiked with Parvovirus B19 to 5.0×10^3 IU/mL. The performance of the positive control is assessed during the colorimetric assay. The average absorbance value (405 nM) for the IHP positive control sample must be greater than or equal to the positive cut-off value with the Parvovirus B19 probe. If this condition is not met, the entire assay is invalid and extraction, amplification, and detection must be repeated.

Test Disposition

A test sample is interpreted as negative for Parvovirus B19 DNA if the absorbance value (405 nM) for the sample is less than the positive cut-off value (NHP A_{405} + 0.100) and the A_{405} value for the internal control is greater than or equal to 0.500. If the A_{405} value for the test sample is less than the positive cut-off value and the A_{405} value for the internal control is less than 0.500, the test for that sample is invalid and the sample must be retested (these samples would not be counted for validation purposes). If the absorbance value (405 nM) for the test sample is greater than or equal to the positive cut-off value, then the test sample is interpreted as positive for Parvovirus B19 DNA. Test samples with an A_{405} greater than or equal to the positive cut-off value are interpreted as positive for Parvovirus B19 DNA regardless of the A_{405} value for the Parvo B19 IC DNA.

8. Design of the Validation Study

The qualitative PCR assay described here functions as a limit test for impurities.[12] Test results are interpreted as either positive or negative for a detectable analyte. Limit tests are validated for sensitivity and specificity. Additionally, this validation study was designed to address intermediate precision and aspects of assay robustness that could affect sensitivity and specificity. The 99% positive-test rate (limit of detection) of this assay was determined to be 1.7×10^3 IU/mL through testing of dilution panels derived from the Parvovirus B19 In-house Standard (NAT-056) (Appendix C).

This study was designed to examine the specificity of the Parvovirus B19 Plasma Donation Test, Version 2 for pooled donor plasma samples, in which nucleic acid is isolated using the 1 mL CTAB/GITC nucleic acid extraction procedure. Sample extracts were amplified and detected using the Digene *SHARP Signal* System Parvo B19 Probe/Primer set and the Digene *SHARP Signal* System Assay for PCR Products. EDQM guidelines for the *Validation of Nucleic Acid Technologies for the Detection of Nucleic Acid in Plasma Pools* indicate that assay specificity should be addressed by testing at least 100 negative plasma pools.[13] Specificity was to be demonstrated in this validation study by testing 100 donor plasma mini-pools (comprised of 96 plasma donations) found non-reactive for Parvovirus B19. Twenty donor plasma mini-pools found non-reactive for Parvovirus B19 and subsequently spiked with HBV at 5×10^5 Eurohep Units (EU)/mL were to be tested to demonstrate specificity in the presence of non-targeted DNA.

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[12] ICH Topic Q 2 A. *Guideline for Industry: Text on Validation of Analytical Procedures: Definitions and Terminology; Availability.* (60 FR 11260) Effective 1 March 1995

[13] Validation of Nucleic Acid Amplification Technology (NAT) for the Detection of Hepatitis C Virus (HCV) RNA in Plasma Pools, European Directorate for the Quality of Medicines (EDQM), Control Authority Batch Release of Blood Products, 2001.

The validation study also was designed to verify the sensitivity of the Parvovirus B19 Plasma Donation Test, Version 2. EDQM guidelines indicate that robustness should be demonstrated in at least 20 negative plasma pools spiked at three times the previously determined 95% cut-off value for the assay.[12] The targeted cut-off for the Parvovirus B19 Plasma Donation Test, Version 2, with a 1 mL sample volume is 5.0×10^3 IU/mL (approximately three times the previously determined 99% positive cut-off value of 1.7×10^3 IU/mL). The repeatability and robustness of the assay relative to the targeted cut-off value was assessed through multiple-operator testing of Negative Human Plasma (NHP) spiked with the Parvovirus B19 In-house Standard at a genomic titer of 5.0×10^3 IU/mL. Robustness was addressed further through the controlled variation of reagents or assay parameters. Lot-to-lot variation of hexadecyltrimethylammonium bromide (CTAB) was included as a robustness component. Another robustness component involved holding the PCR amplification at 91°C for 60 minutes before aborting the amplification and proceeding with the detection. In addition, matrix variation was addressed as a robustness component through the testing of NHP spiked with the Parvovirus B19 In-house Standard at a genomic titer of 5.0×10^3 IU/mL in the presence of the HBV Eurohep Standard adw/Genotype A at a titer of 5.0×10^5 EU/mL. Control of cross-contamination was to be demonstrated by testing alternating negative and Parvovirus B19 samples at a titer 100-fold greater than the assay cutoff (5.0×10^5 IU/mL) to demonstrate the absence of cross-contamination in the Parvovirus B19 Plasma Donation Test, Version 2.

Repeatability and intermediate precision were addressed as a function of the detection limit. As a component of assay execution, each test consisted of separate extraction, amplification, and detection sessions, and the operator accounted for each phase of the assay. Operators used different GeneAmp PCR System 9600 thermal-cyclers for the amplification phase of the procedure (Serial Number: P8383, S01627, N19606, S01497x and S01622).

For the validation study, Parvovirus B19 Amplification Master Mix was prepared and stored at -20°C. Quality control testing of the Master Mix and other reagents required for the validation studies was then performed. The Parvovirus B19 Amplification Master Mix was stored at -20°C for nine days before being utilized in the validation study.

This study was designed to address these issues in order to confirm that the Parvovirus B19 Plasma Donation Test, Version 2, with the 1 mL CTAB/GITC nucleic acid extraction method, PCR amplification and colorimetric detection is suitable for the testing of donor plasma pools. Individual studies and their acceptance criteria are further defined in the following sub-sections.

Sensitivity (Detection Limit)

Assay sensitivity relative to the 99% positive cut-off value was previously determined to be 1.7×10^3 IU/mL (Appendix C). The targeted cut-off of the assay is 5.0×10^3 IU/mL. The ability to achieve the targeted cut-off value was assessed through multiple-operator testing of NHP spiked with the Parvovirus B19 In-house Standard to a genome titer of 5.0×10^3 IU/mL. This titer represents a 6.58×10^{-9} dilution of the Parvovirus B19 In-house Standard. The assay must detect positive samples within each test panel at a rate of 95 to 100% in order to pass the validation.

Specificity

Specificity is primarily a function of primer selection in PCR assays. One hundred donor plasma mini-pools were tested to establish that the assay does not generate false-positive reactions from its integral components or pool constituents. Although the plasma mini-pools were tested for the presence of Parvovirus B19 nucleic acid, there is a possibility that a donor plasma mini-pool may contain low titers of Parvovirus B19. Due to the nature of the qualitative test and its inherent variability, there is a possibility that a pool may test positive for Parvovirus B19 DNA during these studies. Any positive result must be investigated as a discrepant result, and must be addressed in the context of assay performance. Such investigations include testing with a more sensitive method and may also include testing by an independent laboratory.

Repeatability and Intermediate Precision

Repeatability and intermediate precision were addressed simultaneously during the validation of test sensitivity. Each operator prepared a panel of NHP spiked with Parvovirus B19 at 5.0×10^3 IU/mL. The three panels, consisting of 20 samples each, were mixed so that each operator tested samples from all three dilution series. Operators were required to achieve individual (repeatability) and collective (intermediate precision) positive-tests at a rate of 95 to 100% in order to pass the validation.

Robustness

The validation addressed the issue of robustness by extracting NHP spiked with the Parvovirus B19 In-house Standard to 5.0×10^3 IU/mL with 5% (w/v) CTAB prepared with a different lot of Hexadecyltrimethylammonium Bromide (FisherChemical). In addition, robustness was addressed by testing NHP spiked with the Parvovirus B19 In-house Standard to 5.0×10^3 IU/mL in the presence of the HBV Eurohep Standard adw/Genotype A at a titer of 5×10^5 EU/mL. Another robustness component involved holding the PCR amplification at 91°C for 60 minutes before aborting the amplification and proceeding with the detection. Robustness was also demonstrated by testing

alternating negative (NHP) and Parvovirus B19 samples at a titer 100-fold greater than the assay cutoff (5.0×10^5 IU/mL) to demonstrate the absence of cross-contamination in the Parvovirus B19 Plasma Donation Test, Version 2. The acceptance criteria for the cross-contamination study required the test to accurately detect negative (no positives) and B19 positive samples (100% positive) arranged in a checkerboard pattern (Figure 1).

The elements of the validation study design and acceptance criteria are summarized in Table 1.

Figure 1 - Sample detection array for the cross-contamination study. Sample identity and plate coordinates are defined according to their placement in the detection microwell plate. The hybridization targets are indicated in the column headings; "B19" indicates that the samples in these columns are detected with the Parvovirus B19-specific RNA probe, whereas "IC" indicates that the samples in these columns are detected with the Parvo B19 internal control-specific RNA probe. B19 and IC columns are paired to match target and internal control as follows: 1 and 4, 2 and 5, 3 and 6.

	1 (B19)	2 (B19)	3 (B19)	4 (IC)	5 (IC)	6 (IC)
A	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**
B	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL
C	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**
D	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL
E	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**
F	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL
G	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	**Negative**
H	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL	Parvovirus B19 at 5×10^5 IU/mL	**Negative**	Parvovirus B19 at 5×10^5 IU/mL

Table 1 - Design and Acceptance Criteria for the Tests in the Study to Validate the Process for the Detection of Parvovirus B19 DNA in Donor Plasma Pools using Polymerase Chain Reaction Methodology, Version 2

Validation Level of Analyte Strength (Sample Type)	Type of Validation Testing	Number of Operators	Total Number of Samples per Operator	Acceptance Criteria
Negative diluent controls: donor plasma mini-pools	Specificity	3	2 at 33, 1 at 34	No positives
Negative diluent controls: donor plasma mini-pools spiked with HBV Eurohep standard *adw*/A at 5×10^5 EU/mL	Specificity	1	20	No positives
In-house Test Panel: NHP spiked with Parvovirus B19 at 5.0×10^3 IU/mL	Sensitivity, robustness, repeatability and intermediate precision	3	20	95 to 100% positive
In-house Test Panel: NHP spiked with Parvovirus B19 at 5.0×10^3 IU/mL and HBV Eurohep Standard *adw*/A at 5×10^5 EU/mL	Robustness	1	20	95 to 100% positive
In-house Test Panel: NHP spiked with Parvovirus B19 at 5.0×10^3 IU/mL - Samples extracted with 5% (w/v) CTAB prepared from a different lot of Hexadecyltrimethylammonium Bromide (FisherChemical)	Robustness	1	20	95 to 100% positive
In-house Test Panel: NHP spiked with Parvovirus B19 at 5.0×10^3 IU/mL - Amplification HOLD for 60 minutes at 91°C prior to aborting the program	Robustness	1	20	95 to 100% positive
Negative diluent control: NHP	Robustness (cross-contamination)	1	12	No positives
In-house test panel: NHP spiked with Parvovirus B19 at 5×10^5 IU/mL	Robustness (cross-contamination)	1	12	100% positive

9. Discussion of Experimental Results

This study of the Parvovirus B19 Plasma Donation Test, Version 2, was designed to provide a validation of the assay sensitivity and specificity and also to address some aspects of test robustness. The validation study results are summarized in Table 2. All results shown were obtained in test runs that had successful positive, negative and internal controls.

Test specificity was addressed through the testing of 100 donor plasma mini-pools consisting of plasma samples found non-reactive for Parvovirus B19. These tests were performed to demonstrate that the assay does not generate false-positive reactions from its integral components or pool constituents. The validation acceptance criteria for this panel required that there be no positive results. Ninety-nine of the 100 specificity panel members tested met the acceptance criteria (Table 3).

The possibility of a positive result in the specificity panel was previously addressed in the Validation Study Design section. In this validation, specificity panel member NAT-099A10 generated an A_{405} of 0.273 with a positive assay cut-off of 0.237 A_{405}. An in-house investigation was conducted to determine whether the positive result for the mini-pool could be due to the presence of low levels of Parvovirus B19 DNA. The extract from NAT-099A10 was amplified again and detected in quadruplicate using the Parvovirus B19 Plasma Donation Test, Version 2. All four replicates were negative (Appendix D). An additional 1 mL aliquot of NAT-099A10 was extracted using an alternate and more sensitive investigational method and tested in quadruplicate on the ABI PRISM® 7700 Sequence Detection System using fluorogenic PCR technology. Two of the four replicates exhibited low levels of amplification (Appendix D). The investigation indicated that the positive result for specificity panel member NAT-099A10 in the validation was most likely due to low-titer Parvovirus B19 and was not a false-positive reaction. NAT-099A10 was excluded from the specificity panel. The 99 remaining specificity panel results met the acceptance criteria of no positive results.

Test specificity was also addressed through the testing of 20 donor plasma mini-pools consisting of plasma samples found non-reactive for Parvovirus B19 subsequently spiked with HBV to 5.0×10^5 EU/mL. This test was performed to establish that the assay does not generate false-positive reactions from its integral components or pool constituents, including non-targeted DNA. The validation requirement that no positive results be obtained was achieved (Table 4).

Assay sensitivity relative to the 99% positive cut-off value was previously determined to be 1.7×10^3 IU/mL (Appendix C). The targeted assay cut-off for the Parvovirus B19 Plasma Donation Test, Version 2, is 5.0×10^3 IU/mL. The ability to achieve the targeted cut-off value was verified through multiple-operator testing of a multi-source plasma