EFS

#1.15

PROTOCOL FOR SAMPLE PRESERVATION

PLASMA BANKS VERSUS SERUM BANKS

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BACKGROUND

1.- The viral load tests registered with the AFSSAPS (French Agency for the Safety of Health Products) cannot be used currently on plasma samples.

2.- According to current practice in the laboratories involved in the biological qualification of donations, all viral serology analyses are carried out on serum. In fact, as part of the technical inspections carried out for registering virology kits, the tests are carried out on serum. It is the case, however, that suppliers suggest the use of serum or plasma in technical protocols.

3.- The laboratories involved in the biological qualification of donations may be obliged for health safety reasons (withdrawal of batch of reagents, equipment warning) to reassess the packed red blood cells within a period of up to 42 days (preservation time for red blood cells). To achieve this they must keep the samples in validated conditions so that they can have a molecular biology and/or serology reassessment carried out in the same traceability and safety conditions as with the initial assessment. In fact, the FAME study in 1998 was able to highlight a number of problems associated with current serum banks (sample size, sample transfer, labelling, etc.), which must be taken into consideration.

4.- According to recent data from the literature (J. Coste, unpublished, lectures from the AABB Congress in San Francisco), viral RNAs would be stable for at least 4 days at +4°C . Furthermore, according to good practice for biological qualification of donations, biological analyses must be carried out within a maximum period of 96 hours.

However, technical directive no. 3 of 21 December 1998 from the French National Blood Service (then known as AFS) on DNA banks stipulates that samples must be centrifuged within the 12 hours following collection, a timeframe based on the state of the art and knowledge at the time. In view of the laboratories involved in the biological qualification of donations being reorganised into single regional technical centres, this legal obligation with regard to centrifugation would seem difficult to fulfil given the restrictions resulting from the time required to transport the donations from the collection points to the centres and the general logistical requirements associated with the various collection centres being located so far away.

OBJECTIVES

The objectives of this study are as follows:

- Simplifying the current restrictions imposed in the AFS technical directive relating to DNA banks, by validating a centrifugation time of 96 hours.

- Defining the steps in a secure, automated national procedure in the event of a compulsory retest, in the case of both serology and viral load tests.

Consequently, the authors have evaluated the samples' stability with a view to validating:

- the screening for slight markers of viraemia (RNA), whatever the type of sample
- a centrifugation time of 96 hours
- the preservation of samples for 45 days at – 30°C, as well as their thawing method
- the use of plasma samples in serology, if donations have to be qualified again.

METHODOLOGY

The methodology selected is identical for both the serology study and the viral load test study and comprises two parts:

- The first part involves analysing designs of experiment using a small representative sample, making it possible to evaluate the impact of various factors on analytical performance.
- As a result of analysing the designs of experiment, we were able to validate in the second part of the methodology a larger sample, taking into account the different supplier kits used routinely by the technical centres.

1.- DESIGN OF EXPERIMENT

1.1.- FACTORS SELECTED AND JUSTIFICATION

1.1.1.- *Types of tubes*

Several types of tubes are intended to be used:

- For **plasma**:
  - **Conventional EDTA tube** (Becton Dickinson Vacutainer® ref. 367655): glass tube decanted into a secondary tube after centrifugation for freezing.
  - **BD PPT™ tube** (Becton Dickinson Vacutainer® ref. 362795): plastic tube with a plasma separator gel, providing a better extraction yield and allowing freezing immediately after centrifugation.
- For **serum**, same principle:
  - **Dry tube with gel** (Becton Dickinson Vacutainer® ref. 367784): glass tube decanted into a secondary tube after centrifugation for freezing.
  - **Tube BD SST™** (Becton Dickinson Vacutainer® ref. 368986) : plastic tube with serum separator gel already used for the DNA bank

The secondary tubes used for decanting are 5ml polypropylene 13*75 mm tubes, which can be immediately adjusted to the automated equipment used for qualifying donations.

For financial and organisational reasons particular to these technical centres involved in the biological qualification of donations, these four types of tube were selected for use in the survey:

TUBES USED IN TEST	Design of experiment CODE
• Conventional EDTA tube	1
• Dry tube with gel	2
• BD PPT™ tube	3
• BD SST™ tube	4

1.1.2.- *Times before centrifugation*

In a preliminary study we evaluated 4 periods of time before centrifugation: 12, 24, 48 and 96 hours. For the remainder of the study we compared the 96-hour period with the 12-hour period. The tubes were kept at ambient T° for 12 hours, then stored at 4°C according to the period of time being analysed and were centrifuged for 30 minutes at 3000g.

TIMES BEFORE CENTRIFUGATION	Design of experiment CODE
• 12h	1
• 24h	2
• 48h	3
• 96h	4

1.1.3.- *Sample preservation*

The minimum preservation time selected is 45 days, given that the maximum time limit packed red blood cells can be used for is 42 days. The purpose of preserving samples at the technical centres is in order to be able to retest labile blood products in stock in conditions where safety and optimum traceability are guaranteed, with the donor samples being preserved in the DNA bank. Only packed red blood cells and platelets are affected (5-day maximum time of use).

In fact, in the case of plasma samples sent to the Laboratoire Français de Fractionnement (French Fractionation Laboratory) analyses carried out are automatically rechecked, which also applies to plasma samples sent to the Bordeaux centre, whereby they undergo viral attenuation by detergent solvent treatment (PVA). In the case of plasma intended for therapeutic use, it is held in quarantine for 4 months while waiting for donors to be retested.

Furthermore, taking into account the current data from the literature, the preservation time and the financial constraints associated with keeping cold storage facilities at the technical centres, only the preservation temperature of –30°C will be tested.

In order to be able to compare results analyses were carried out before freezing (D0) and after freezing (D45).

PRESERVATION	Design of experiment CODE
• DO	1
• D45 at – 30°C	2

1.1.4.- *Thawing conditions*

In the case of an emergency, the samples would probably be thawed under emergency conditions. For this reason, two thawing temperatures were provided: 37°C in an oven and ambient temperature (22°C +/- 2°C). In fact, there is no data available indicating that gels are inert at 37°C.

After thawing all the tubes were homogenised by inverting them, then centrifuged at 3000g for 30 minutes in order to remove any fibrine, which could cause anomalies during the automated collection process.

THAWING METHOD	Design of experiment CODE
• Ambient T°	1
• 37°C in oven	2

1.2.- SAMPLING:

16 BD Vacutainer tubes (4 EDTA, 4 DRY, 4 PPT, 4 SST) were used to collect samples from healthy volunteers. Positive samples were obtained by in vitro contamination of negative samples collected, based on patient samples known to be positive. The HIV and HCV viral loads were quantified using the Amplicor Roche assay. These positive samples were samples taken from patients under the care of the CHRU (University Regional Hospital Centre) in Brest. In order to avoid any risk of outliers, each sample type was analysed on the basis of three different volunteers.

The samples analysed in the first part of the study produced the following results:

VIRAL LOAD TEST		SEROLOGY	
1	NEGATIVE	1	NEGATIVE
2	HCV RNA slightly positive ≈ 150 copies/ ml	2	HCV Ac slightly positive
3	HCV RNA strongly positive ≈ 10^4 copies/ ml	3	HCV Ac strongly positive
4	HCV RNA slightly positive ≈ 150 copies/ ml	4	HCV Ac slightly positive
5	HCV RNA strongly positive ≈ 10^4 copies/ ml	5	HCV Ac strongly positive
		6	HTLV Ac and HBs Ag POSITIVE

The evaluation of the genomes' stability for this first part of the study was carried out using the Chiron TMA assay, allowing simultaneous detection of both the HIV and HCV viruses.

The serological study involved screening for the five compulsory markers: HBs Ag, HBc Ac, HCV Ac, HIV Ac and HTLV Ac, using the Abbott PRISM assay.

The results were expressed in ratios (E/S).

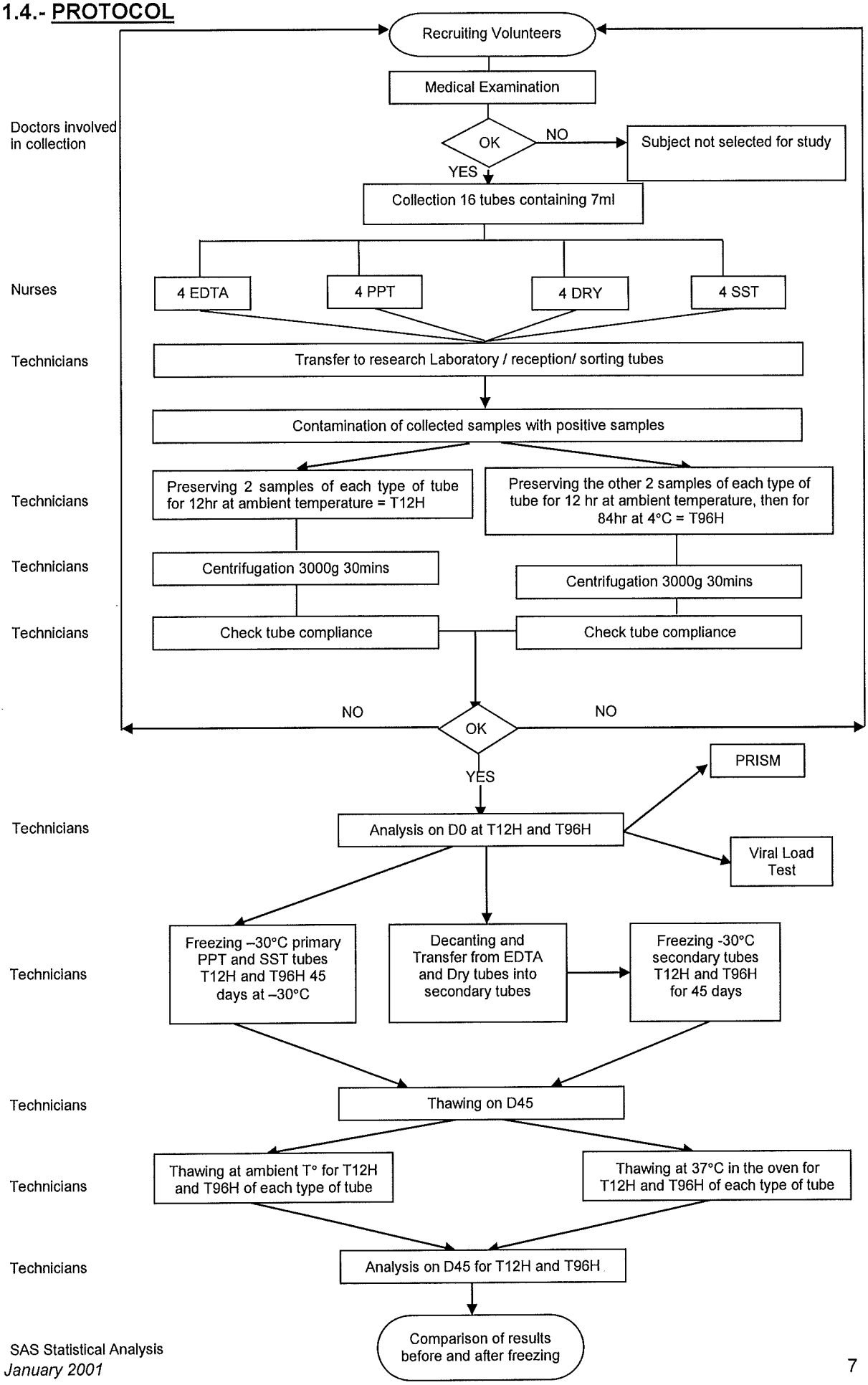
1.3.- INPUTTING DESIGNS OF EXPERIMENT

The various factors being analysed are coded so that the results obtained can be entered. The designs of experiment, with one for each type of sample, are analysed both at D0 and D45.

Example of design of experiment for negative samples:

Individual	Type of tube	Time before centrifugation	Thawing method	Analysis date D0/D45	VIRAL LOAD TESTING RESULTS
1	1 (EDTA)	1(12H)	1 (ambient T°)	1 (D0)	RESU 1
1	1	1	1	2 (D45)	RESU 2
1	1	1	2 (37°C)	1	RESU 3
1	1	1	2	2	RESU 4
1	1	2 (96H)	1	1	RESU 5
1	1	2	1	2	RESU 6
1	1	2	2	1	RESU 7
1	1	2	2	2	RESU 8
1	2 (PPT)	1	1	1	RESU 9
/..	/..	/..	/...	/...	/...

1.4.- PROTOCOL


Recruiting Volunteers
Medical Examination
Doctors involved in collection
OK
NO
Subject not selected for study
YES
Collection 16 tubes containing 7ml
Nurses
4 EDTA
4 PPT
4 DRY
4 SST
Technicians
Transfer to research Laboratory / reception/ sorting tubes
Contamination of collected samples with positive samples
Technicians
Preserving 2 samples of each type of tube for 12hr at ambient temperature = T12H
Preserving the other 2 samples of each type of tube for 12 hr at ambient temperature, then for 84hr at 4°C = T96H
Technicians
Centrifugation 3000g 30mins
Centrifugation 3000g 30mins
Technicians
Check tube compliance
Check tube compliance
NO
OK
NO
YES
PRISM
Technicians
Analysis on D0 at T12H and T96H
Viral Load Test
Freezing –30°C primary PPT and SST tubes T12H and T96H 45 days at –30°C
Decanting and Transfer from EDTA and Dry tubes into secondary tubes
Freezing -30°C secondary tubes T12H and T96H for 45 days
Technicians
Thawing on D45
Technicians
Thawing at ambient T° for T12H and T96H of each type of tube
Thawing at 37°C in the oven for T12H and T96H of each type of tube
Technicians
Analysis on D45 for T12H and T96H
Comparison of results before and after freezing

1.5.- STATISTICAL ANALYSIS OF RESULTS

The analysis of the results is carried out using SAS version 6.12, SAS/STAT module. The GLM (General Linear Model) procedure used for interpreting the results is based on analysis of variance (ANOVA), which allows the comparison of two or more mean values (T-test, Bonferroni test and Tukey test) after checking the goodness of fit (Kolmogorov-Smirnov test) and equality of variances (Levene test). This procedure can be used to analyse the impact of a factor on the "result" variable and to compare the interactions between the different factors by analysing the variations in experimental error on residual variance.

2- VALIDATION

The purpose of this second part of the study was to validate the protocol for preserving the samples collected under the conditions defined above for a sample of 300 blood donors before and after thawing using the technologies routinely used in the qualification laboratories.

Two EFS centres took part in this second part of the study:

- **For the viral load test:**
  - EFS Bretagne – Brest centre, B. MERCIER/I. DUPONT
    using the CHIRON TMA assay.
  - EFS Aquitaine – Bordeaux centre, F. HAU
    using the Roche Extractor- Cobas assay.
- **For the serology test**:
  - EFS Bretagne – Brest centre, manager of the study, I. DUPONT
    using the Abbott PRISM assay.
  - EFS Bretagne – Rennes centre, F. DURAND
    using a microplaque assay.

RESULTS

1.- DESIGN OF EXPERIMENT

1.1.- VIRAL LOAD TEST STUDY (TMA assay)

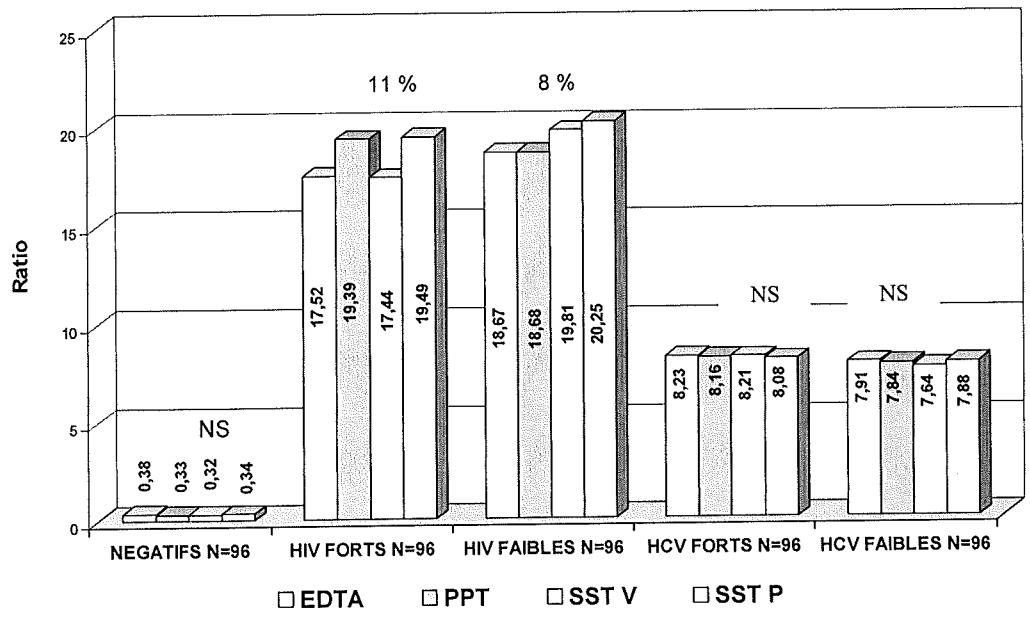
The mean values and variations observed (expressed in %) are given for each type of sample.

The table below provides an overview of the variation coefficients recorded by the various centres using the TMA assay during the feasibility study.

	VARIATION COEFFICIENTS (%)			
	BREST	DIJON	GRENOBLE	MONTPELLIER
Negative Calibrator (Mean RLU)	17.35	18.3	23.7	21.1
HIV Calibrator (Mean RLU)	7.33	8.7	11.4	6.2
HCV Calibrator (Mean RLU)	6.68	8.7	6.1	5.6
Negative Samples (RLU)	29.55	15.8	21.5	28.5

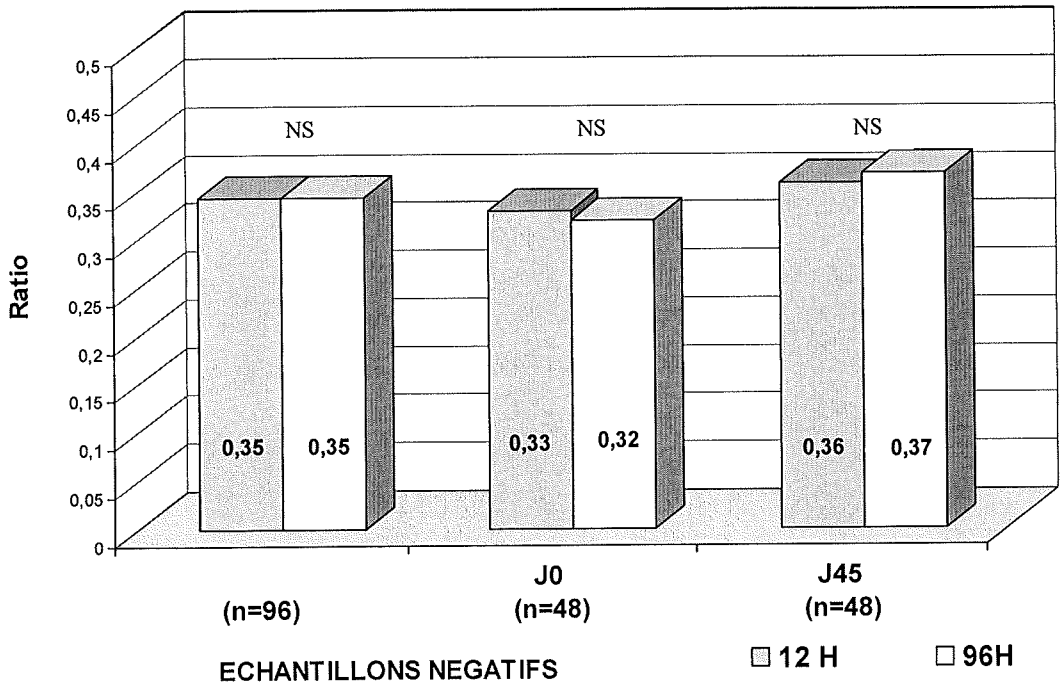
1.1.1.- The diagram below shows the results and variations in the mean values obtained with the different types of collection tubes used.

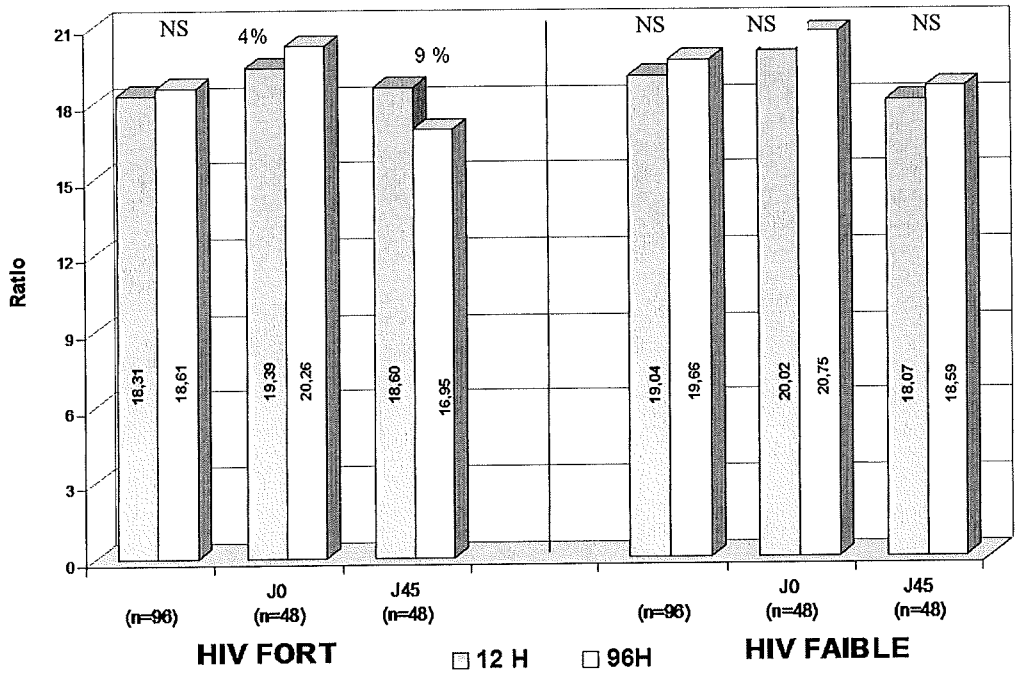
TYPE OF SAMPLES

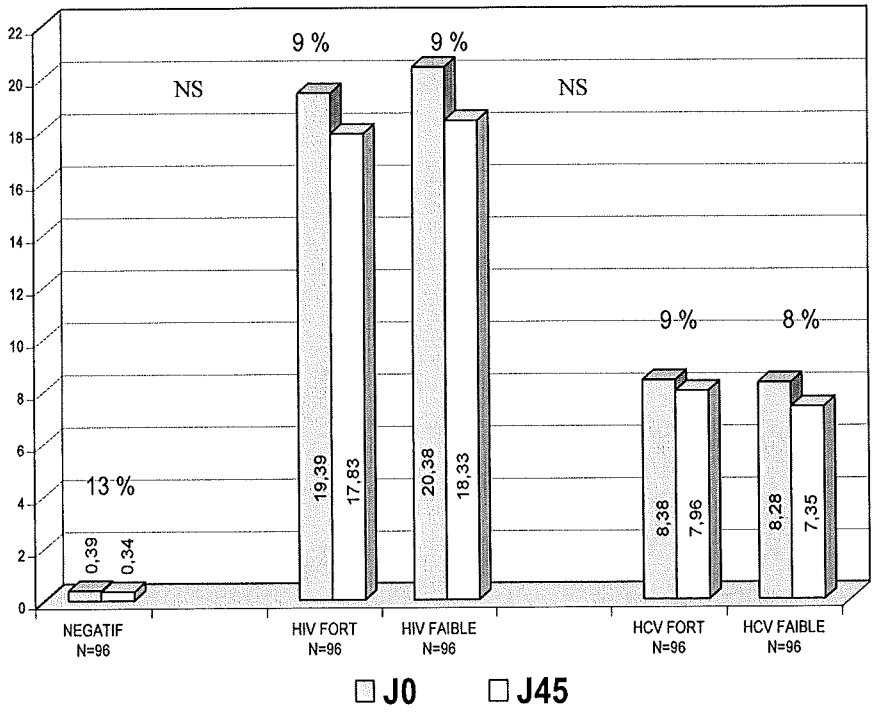


1.1.2.- The 3 diagrams which follow show the results and variations in the ratios obtained according to the time before centrifugation (12 h versus 96 h).

TIME BEFORE CENTRIFUGATION
12 h versus 96 h

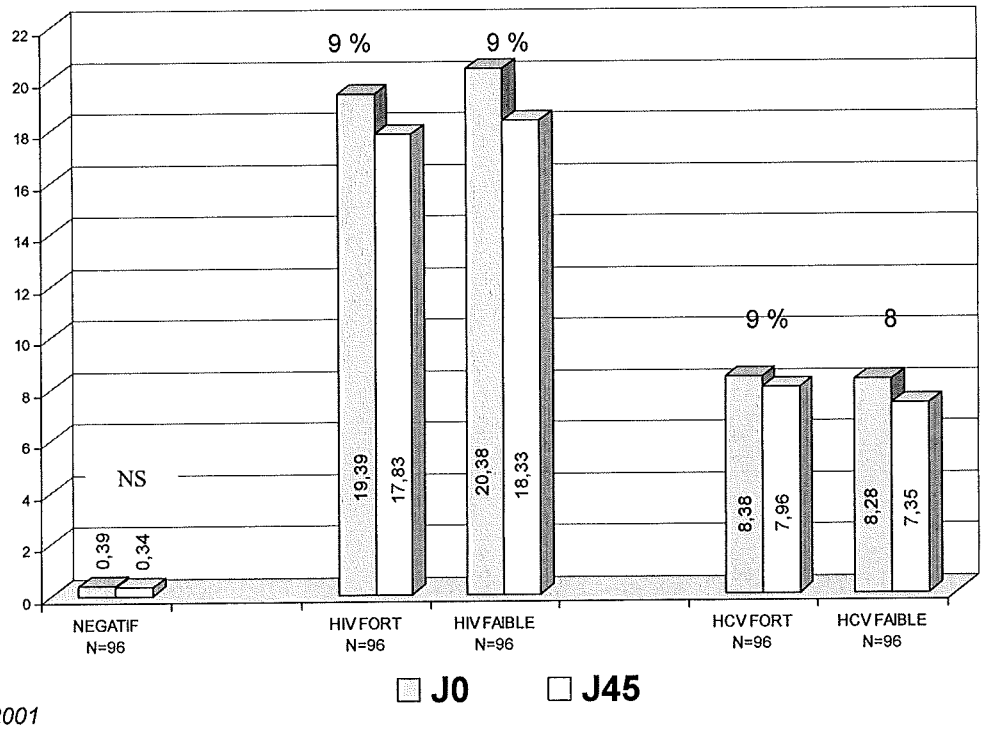






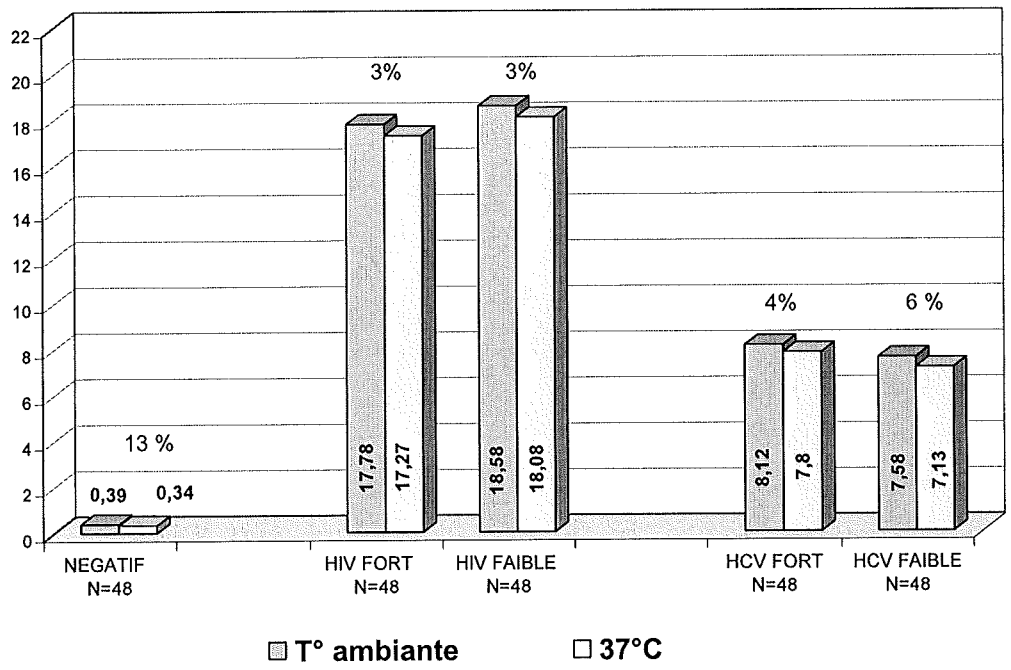
1.1.3.- The diagram below shows the results and the variations obtained after freezing the samples for 45 days at –30°C.

PRESERVING SAMPLES AT - 30° C
D0 versus D45



1.1.4.- The diagram below shows the results and the variations obtained with regard to the thawing conditions after preserving the samples for 45 days at -30° C (ambient temperature versus 37° C).

THAWING SAMPLES
Ambient temperature versus 37° C



Conclusion on Viral Load Testing

This preliminary study based on the designs of experiment has demonstrated that the time before centrifugation of the samples does not affect the analysis results in Viral Load Testing.

As this is a non-quantitative detection test, slight markers of viraemia showed up as positive after 96 hours of being preserved in whole blood, whatever the type of sample.

The signal observed for the slightly viraemic samples (<150 copies/ml) is also at saturation (19.83 vs. 20.33 for HIV and 8.38 vs. 7.83 for HCV).

The results were all validated after preserving samples for 45 days at -30°C, without even the thawing method at 37°C affecting the results.

The variations observed (in the order of 10%), including the interactions between the different factors analysed, match the variation coefficients obtained during the reproducibility study for the system, given the experimental error associated with the in vitro contamination of the tubes.

1.2.- SEROLOGICAL STUDY (PRISM assay)

The results for the HCV Ac and HIV Ac markers are shown below. The results for the HBs Ag and HTLV Ac are shown in the appendix.

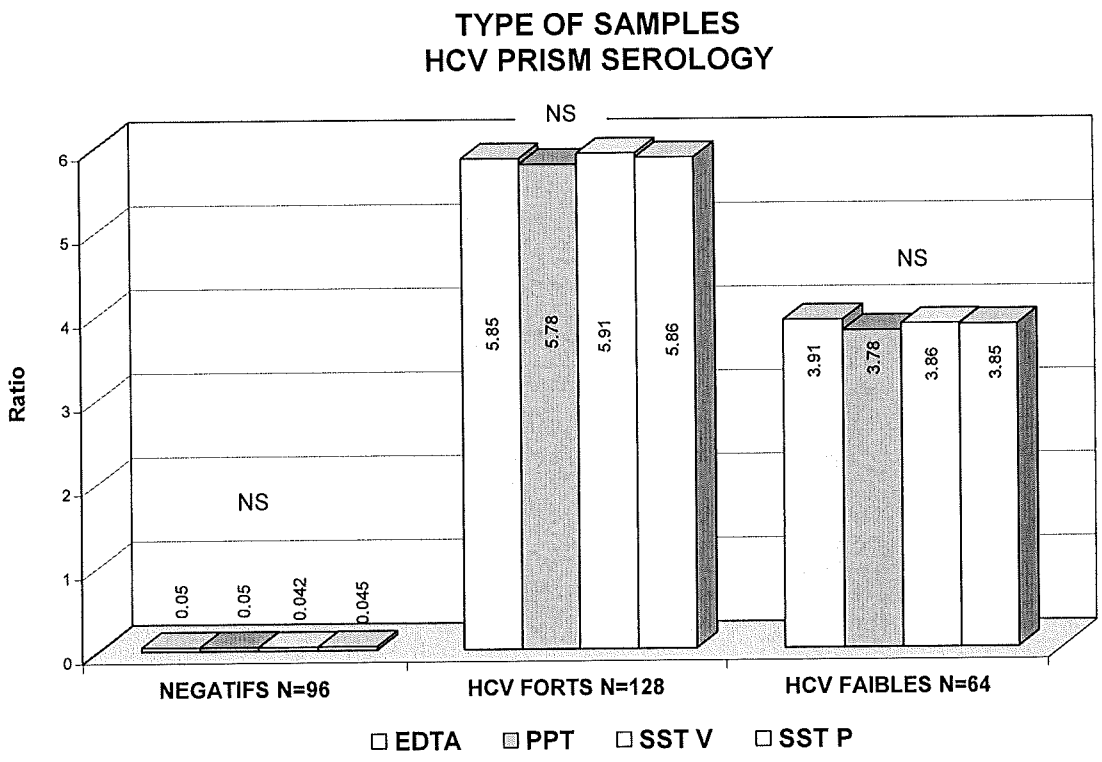
The mean values for the ratios and variations observed expressed in % are shown for each sample type.

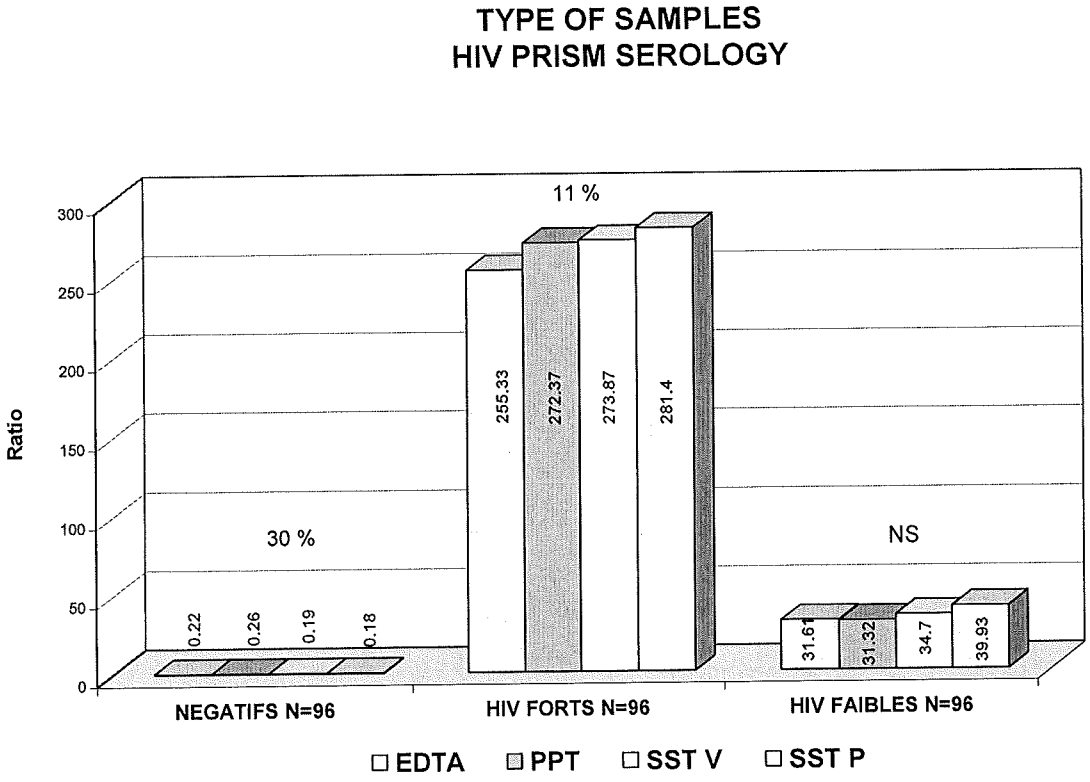
The table below provides an overview of the variation coefficients routinely observed by the Brest centre using the PRISM assay with the batches of reagents used for the study.

	HCV Ac	HIV Ac	HBs Ag	HTLV Ac
ABBOTT CO6-Positive control (1/2)	11.02	6.26	5.63	5.45
VIROTROL	7.64	6.79	12.14	14.09
Mean of Negative Samples	13.79	10.36	11.39	4.95

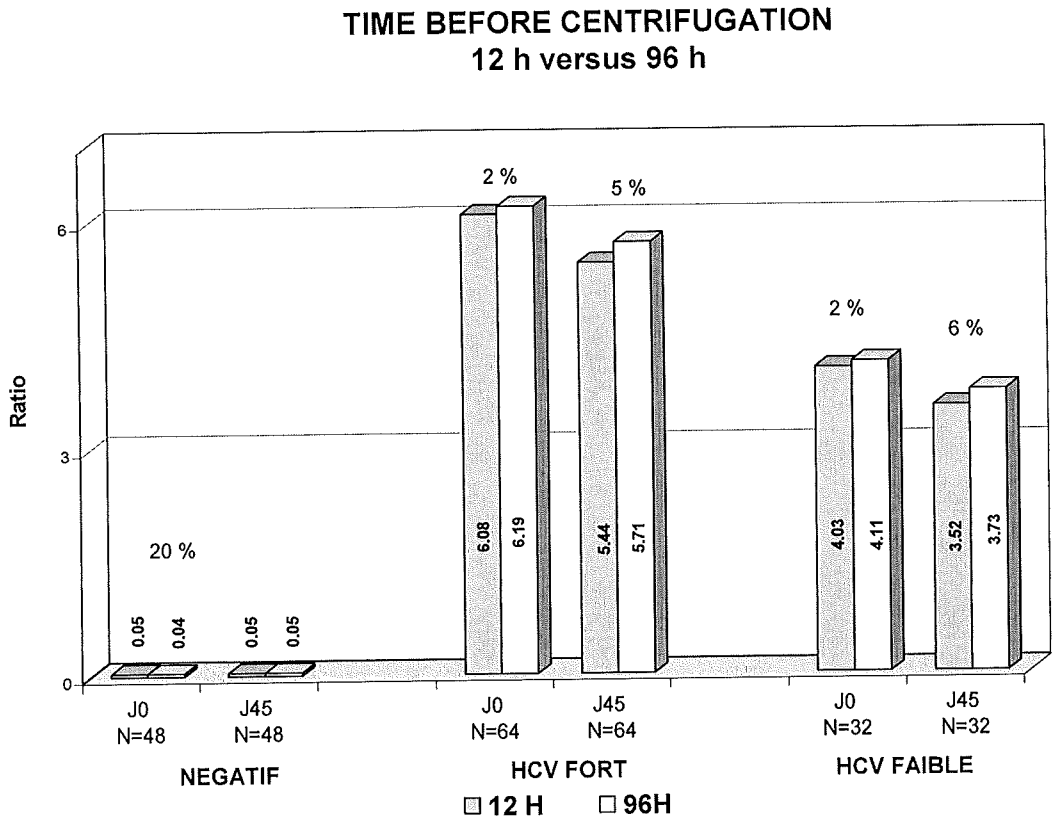
(Virotrol: BMD multiparameter control)

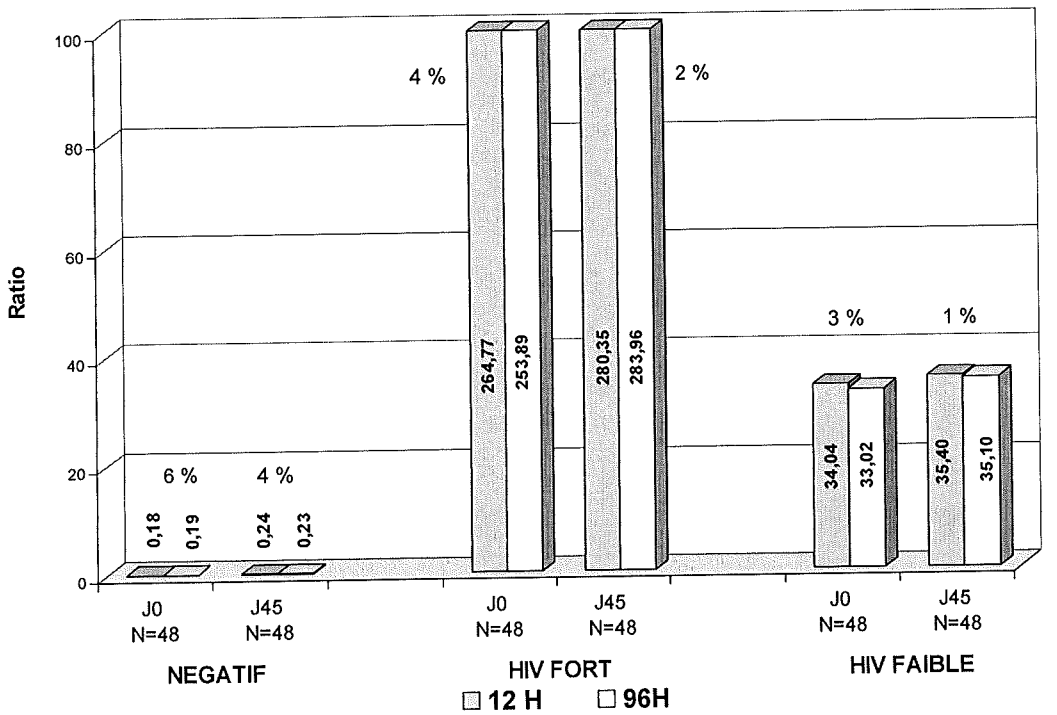
1.2.1.- The two diagrams below show the results and variations obtained using different types of collection tube.





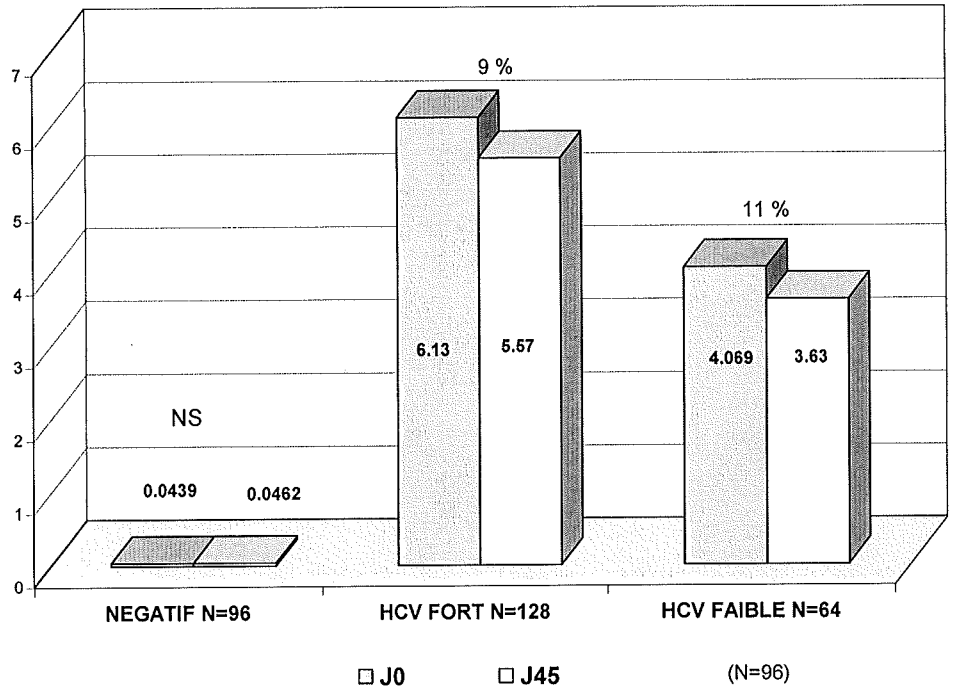
1.2.2.- The two diagrams below show the results and variations obtained with regard to the impact of the time before centrifugation.

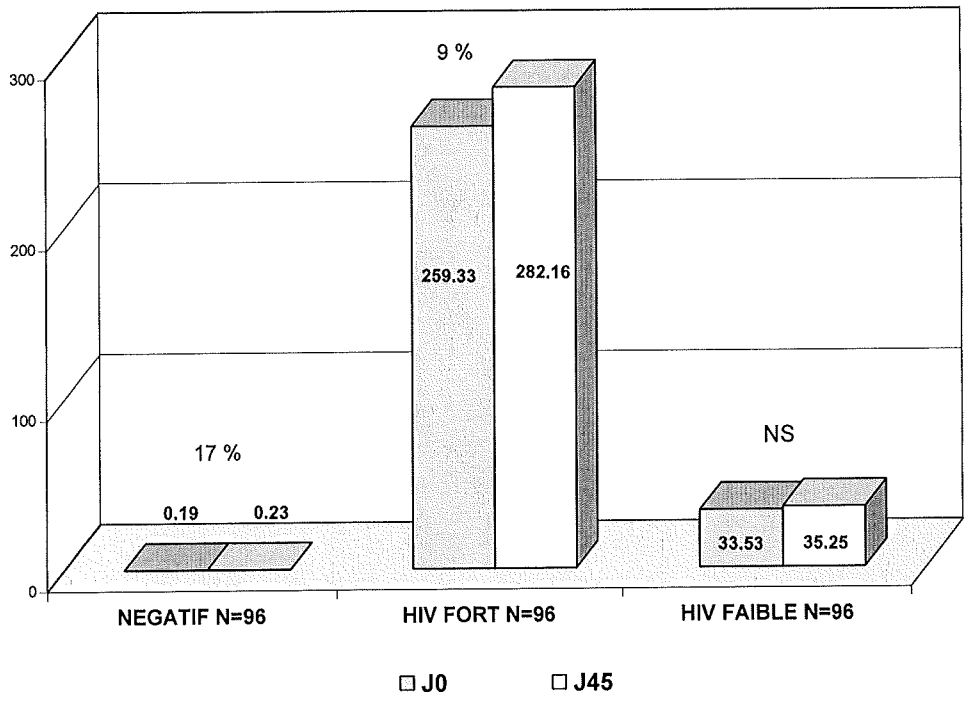




1.2.3.-The two diagrams below show the results and variations obtained after freezing the samples for 45 days at - 30° C.

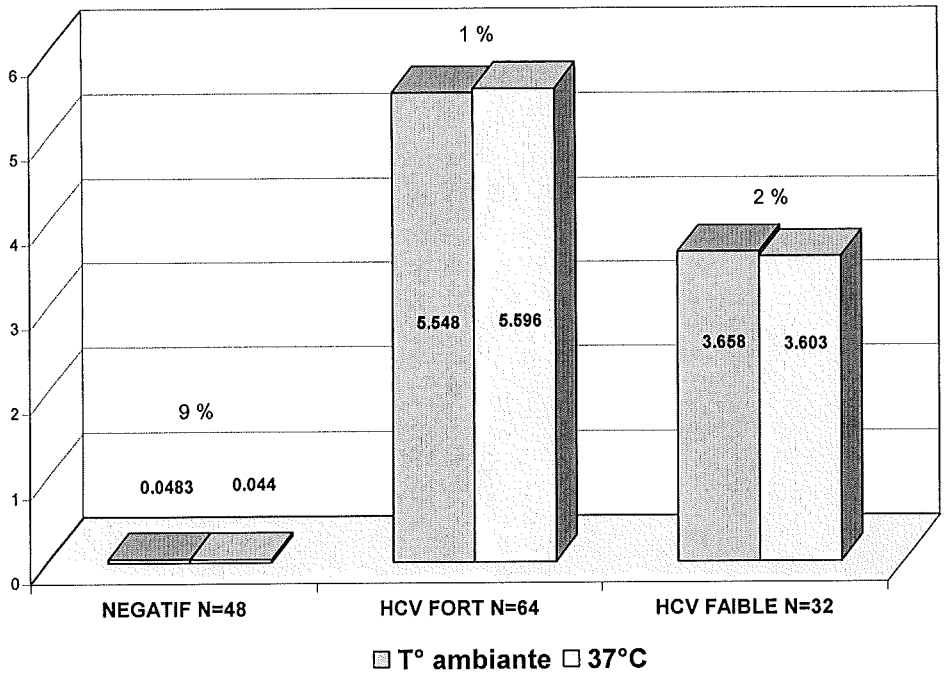
PRESERVING SAMPLES AT -30° C
D0 versus D45

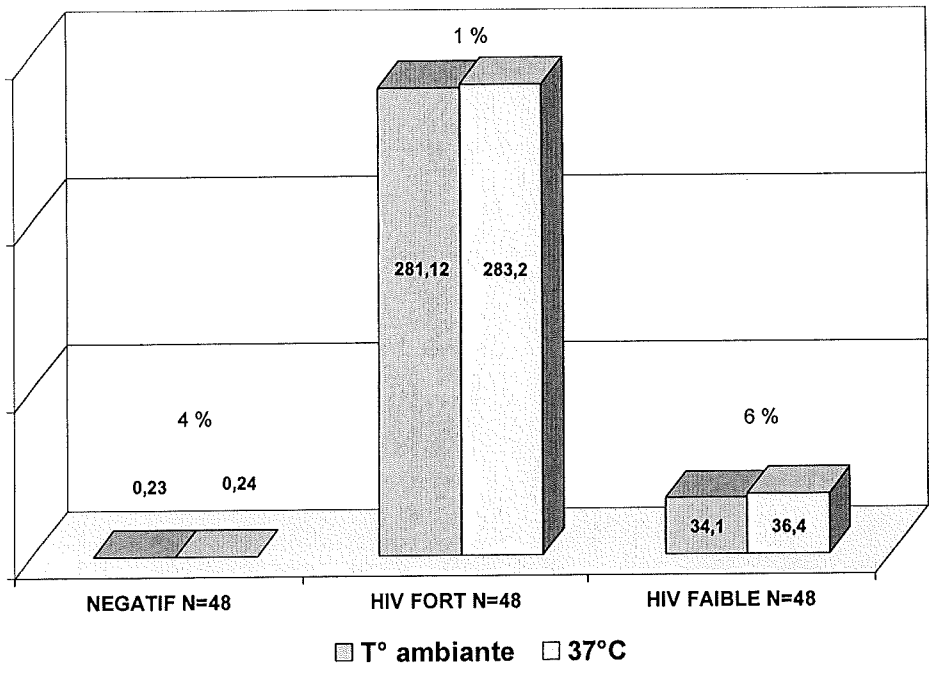




1.2.4.- The two diagrams below show the results and variations obtained with regard to the thawing conditions.

THAWING SAMPLES
Ambient temperature versus 37° C





Conclusion on serology

This preliminary study based on the designs of experiment has demonstrated that whether the sample type is plasma or serum does not affect the results of the serology analyses when carried out in accordance with the recommendations of the reagent kit suppliers.

The maximum waiting time of 96 hours for centrifugation before carrying out biological analyses, dictated by good practice in the biological qualification of donations, was validated during this preliminary study.

Samples may be thawed at 37°C and analysed after being preserved for 45 days at - 30°C.

General conclusion on the design of experiment

This first part of the study allowed us to demonstrate that:

- The sensitivity in serum and plasma is identical.
- Samples may be preserved at ambient temperature for 12 hours, then at 4°C for 4 days before centrifugation and analysed using viral load or serology testing.
- In these conditions, samples can be re-analysed, if necessary, after being frozen at - 30°C for 45 days.

2.- VALIDATION OF RESULTS

2.1.- VALIDATION OF RESULTS FOR VIRAL LOAD TESTING

In order to confirm the results obtained above, a series of 300 negative samples was analysed based on viral load testing using the Chiron assay (Brest) and the Organon-Roche assay (Bordeaux) These samples were collected using PPT tubes, kept at ambient temperature for 12 h, then for up to 96 h at 4°C before centrifugation. After analysis they were preserved for 45 days at –30°C, thawed at 37°C, centrifuged and re-analysed.

The table below shows the results obtained.

BORDEAUX Amplicor Roche Pools (DO)	
HIV	
D96H plasma PPT N=176 (3*48+32)	0.00625 +/- 0.0015
D45 plasma PPT N=297 (6*48+9)	0.00785+/- 0.00227
HCV	
D96H plasma PPT N=176 (3*48+32)	0.004 +/- 0.002
D45 plasma PPT N=297 (6*48+9)	0.00433 +/- 0.00152
BREST Unitary Chiron TMA (E/S)	
Simultaneous detection of HIV/HCV	
D96H plasma PPT N=309	0.360 +/- 0.0992 2 invalid (0.6%)
D45 plasma PPT N=309	0.392 +/- 0.102 6 invalid (1.9%)

It is worth noting the figure of 1.9% for invalid results during the analysis after freezing. These 6 samples turned out to be negative when the procedure was repeated manually

Conclusion

The conditions chosen during the preliminary phase have been validated.

2.2.- VALIDATION OF SEROLOGY RESULTS

In order to confirm the results obtained above, a series of 300 negative samples was analysed based on a serology test using the PRISM assay (Brest) and microplaque assay (Rennes). These samples were collected using PPT tubes, kept at ambient temperature for 12 h, then for up to 96 h at 4°C before centrifugation. After analysis they were preserved for 45 days at –30°C, thawed at 37°C, centrifuged and re-analysed.

The table below shows the results obtained.

SEROLOGY (E/S)	RENNES microplaque	BREST Prism	SEROLOGY (E/S)	RENNES microplaque	BREST Prism
HIV	**N=236**	**N=307**	**HBs Ag**	**N=224**	**N=307**
D0 serum SST V	0.02 +/- 0.057	0.250 +/- 0.048	**D0 serum SST V**	0.056 +/- 0.044	0.211 +/- 0.060
D96H plasma PPT	0.03 +/- 0.070	0.303 +/- 0.081	**D96H plasma PPT**	0.358 +/- 0.132	0.255 +/- 0.035
D45 plasma PPT	0.02 +/- 0.061	0.316 +/- 0.042	**D45 plasma PPT**	0.289 +/- 0.177 6 repeatable positives	0.234 +/- 0.038
HCV	**N=229**	**N=307**	**HBc Ac**	**N=228**	**N=307**
D0 serum SST V	0.02 +/- 0.059	0.065 +/- 0.032	**D0 serum SST V**	0.09 +/- 0.062	1.96 +/- 0.26
D96H plasma PPT	0.02 +/- 0.034	0.065 +/- 0.0316	**D96H plasma PPT**	0.15 +/- 0.090	1.96 +/- 0.28
D45 plasma PPT	0.289 +/- 0.177	0.074 +/- 0.395	**D45 plasma PPT**	0.09 +/- 0.07	1.99 +/- 0.29 1 repeatable positive

It should be noted that 6 repeatable false positive HBs Ag results were recorded with the microplaque assay. However, this rate of 3% is relatively low, taking into account the extreme conditions it was recorded in. We should only come across these conditions in very exceptional circumstances.

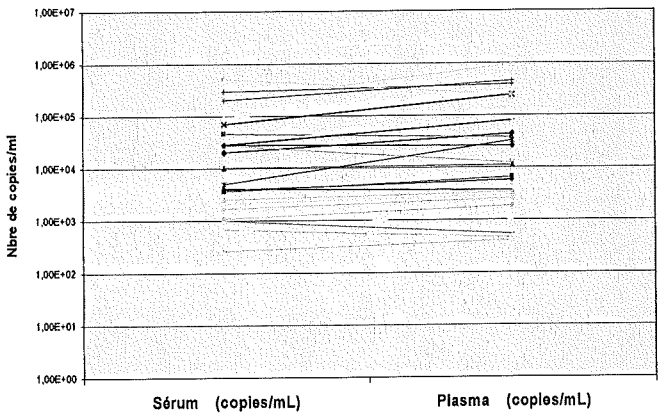
Conclusion

The conditions selected during the preliminary phase have been validated.

2.3.- QUANTIFYING VIRAL LOADS IN SERUM AND PLASMA

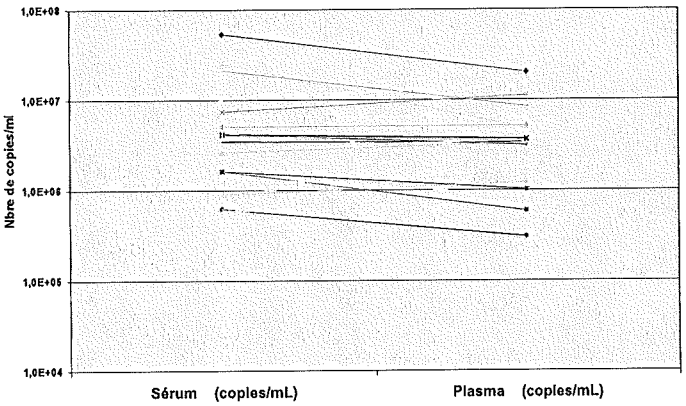
We also used the Amplicor Roche assay to quantify the viral load in native patients with either HCV or HIV by comparing the results obtained from the serum and plasma samples collected. The results are shown below.

VIRAL LOAD TESTING



N=30 N=30

HCV VIRAL LOAD TESTING



N=22 N=22

Conclusion from viral load comparison

No significant difference is observed when a comparison is made between the viral loads obtained in serum or plasma in the case of either HCV or HIV. The variations observed are lower than a log, which corresponds to the reproducibility limit achieved in our laboratory using the Amplicor Roche assay. The slight markers of viraemia (10^2-10^3 copies/ml for HIV and 10^5-10^6 copies/ml for HCV) corresponding to the viral loads observed in the serological window according to the physiopathological data (M. Bush, Workshop NAT, Dec. 99) were quantified in both serum and plasma.

GENERAL CONCLUSION

- The stability of viral RNAs is better than initially supposed
- The viral load is comparable in serum and plasma
- The following conditions have been validated both for viral load testing and the serology tests:
  - Collection using an EDTA tube
  - Storing samples at ambient temperature for a maximum of 12 hours
  - Storing samples between +2 and +8°C for more than 12 hours
  - Centrifugation within a maximum of 96 hours after collection
  - Analysis within a maximum of 96 hours after collection

- With regard to any repeat qualification as a result of a reagent being withdrawn, for instance, the following conditions have been validated both for viral load testing and the serology tests:
  - Freezing the primary tube (PPT) at - 30°C after centrifugation
  - Thawing at either ambient temperature or at + 37°C
  - Centrifugation time of 30 min. at 3000 g
  - Analysis carried out in normal routine conditions

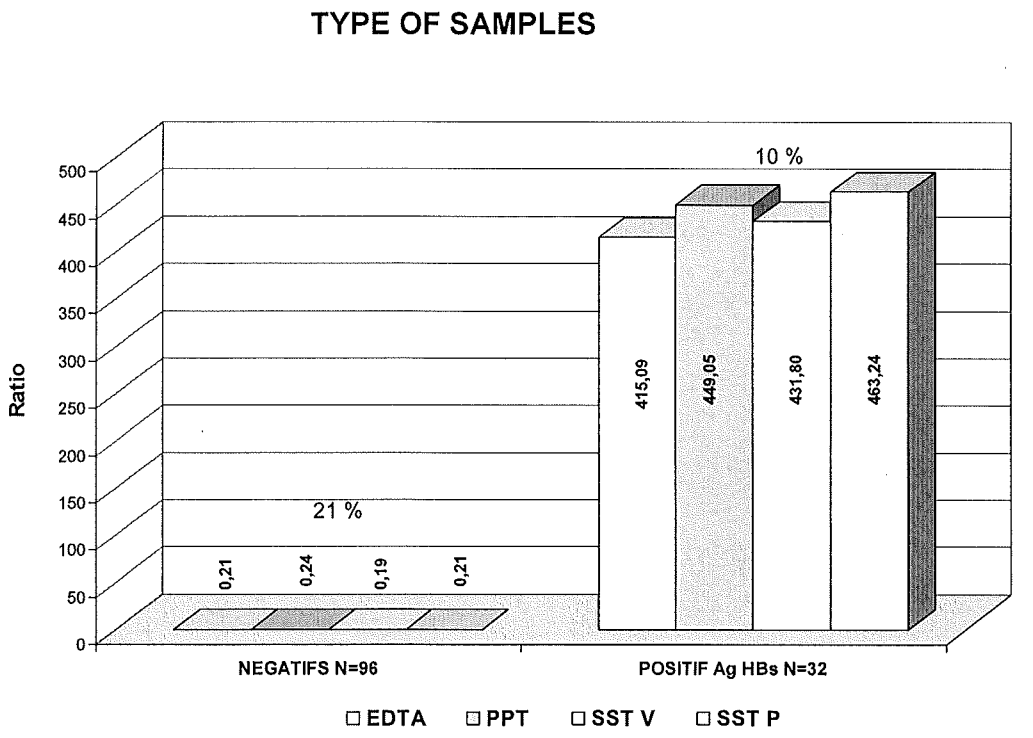
In these conditions, it would seem that the 12-hour time limit imposed by DNA banks can be extended to a limit of 96 hours in order to facilitate the logistics involved in the delivery of the collected samples.

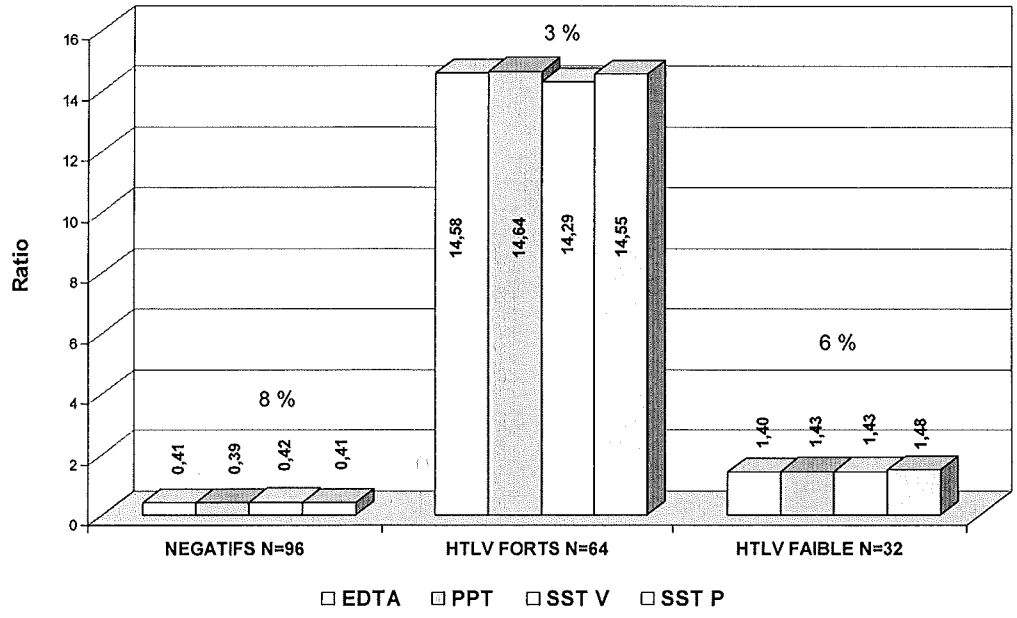
We propose preserving a primary gel PPT tube after centrifugation for 45 days at - 30°C, which allows any repeat qualification to be carried out for viral load testing and serology tests. The procedures for handling these tubes are still to be drawn up by the working group formed by Cécile CORNILLOT.

APPENDIX

Results obtained using the Abbott PRISM assay for HBs Ag and HTLV Ac markers, according to sample type

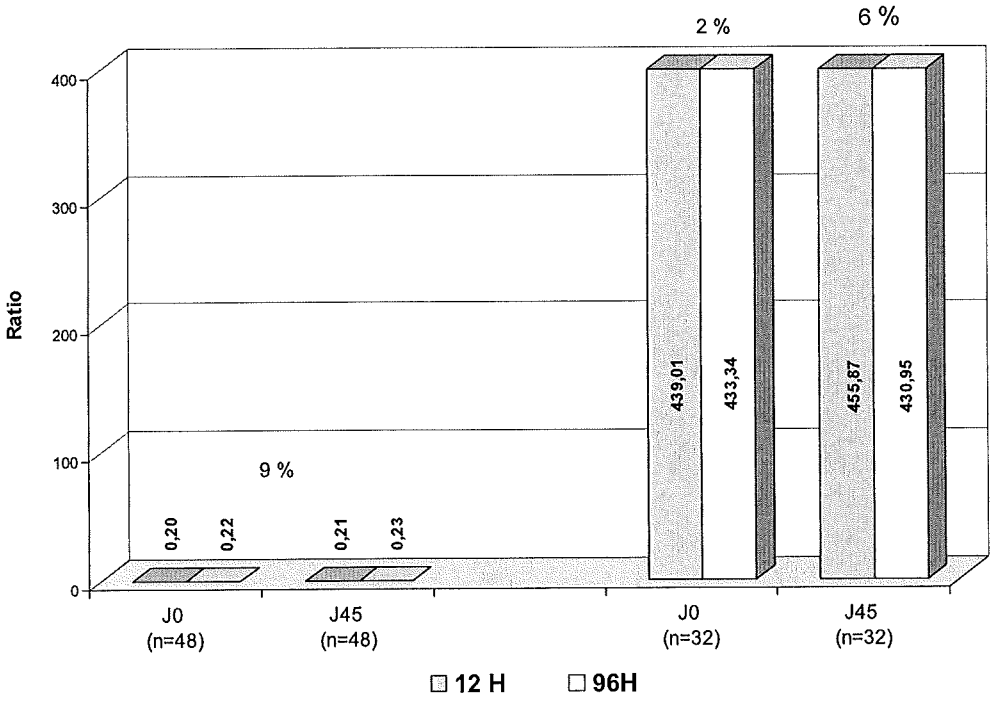
- The 2 diagrams below shows the results and variations obtained with regard to the type of collection tube.

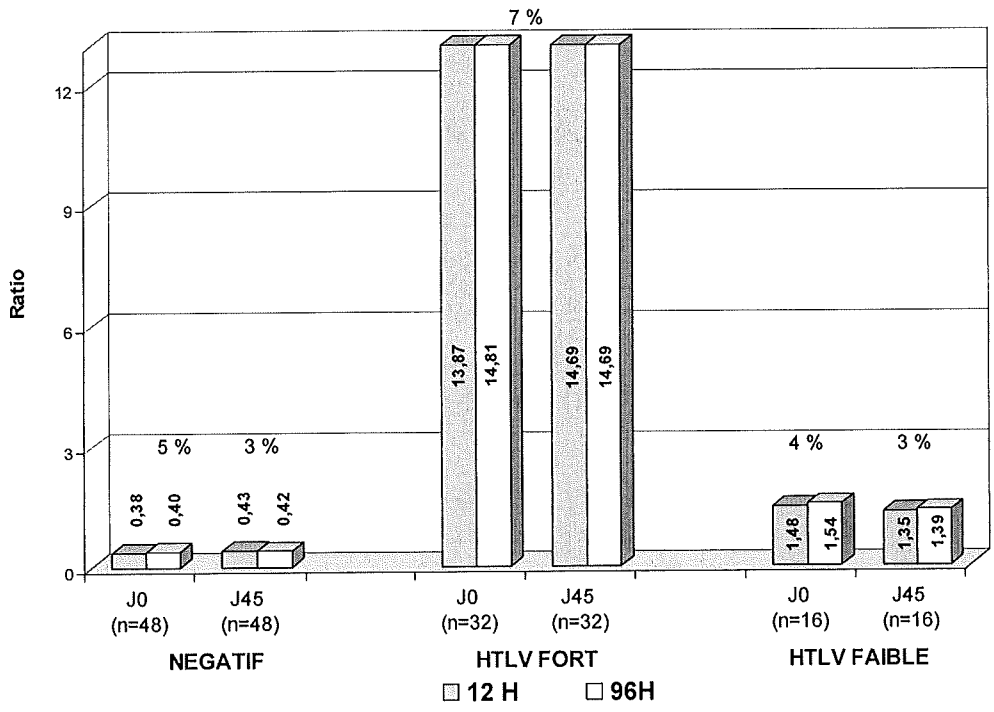




- The 2 diagrams below show the results and variations obtained with regard to the impact of the time before centrifugation.

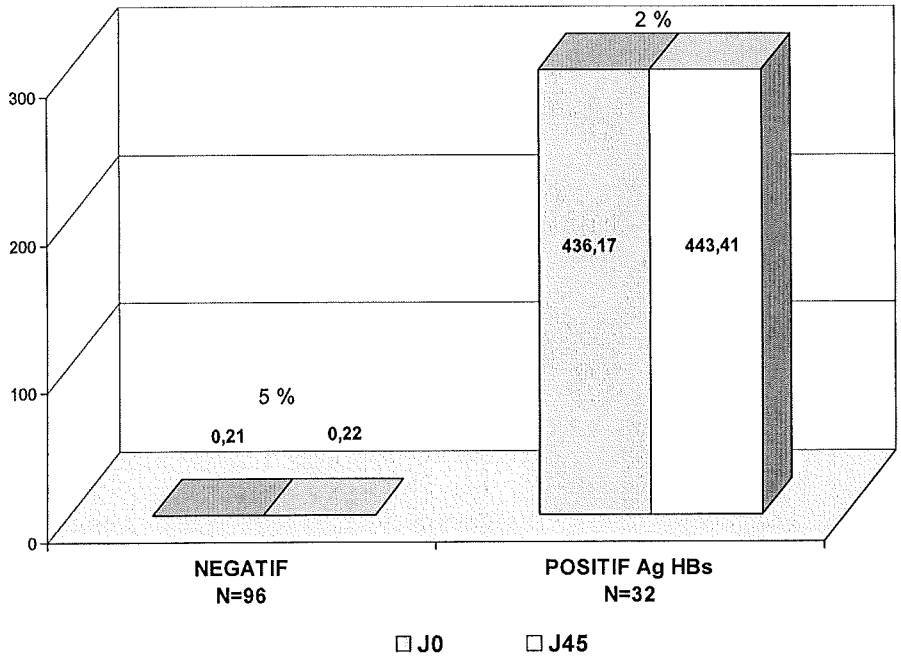
TIME BEFORE CENTRIFUGATION
12 h versus 96 h

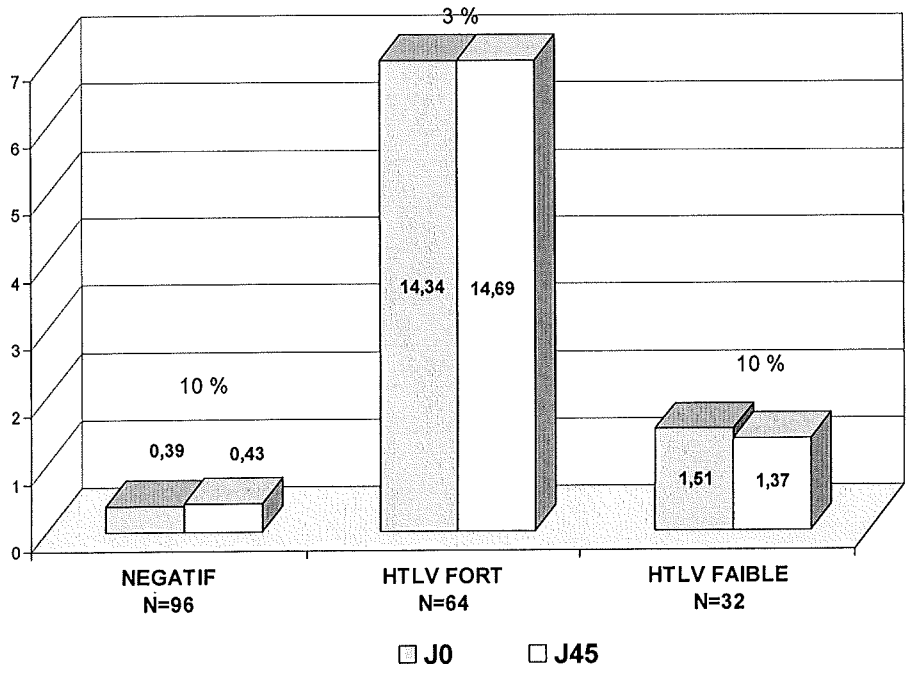




- The 2 diagrams below show the results and variations obtained with regard to preservation conditions (freezing for 45 days).

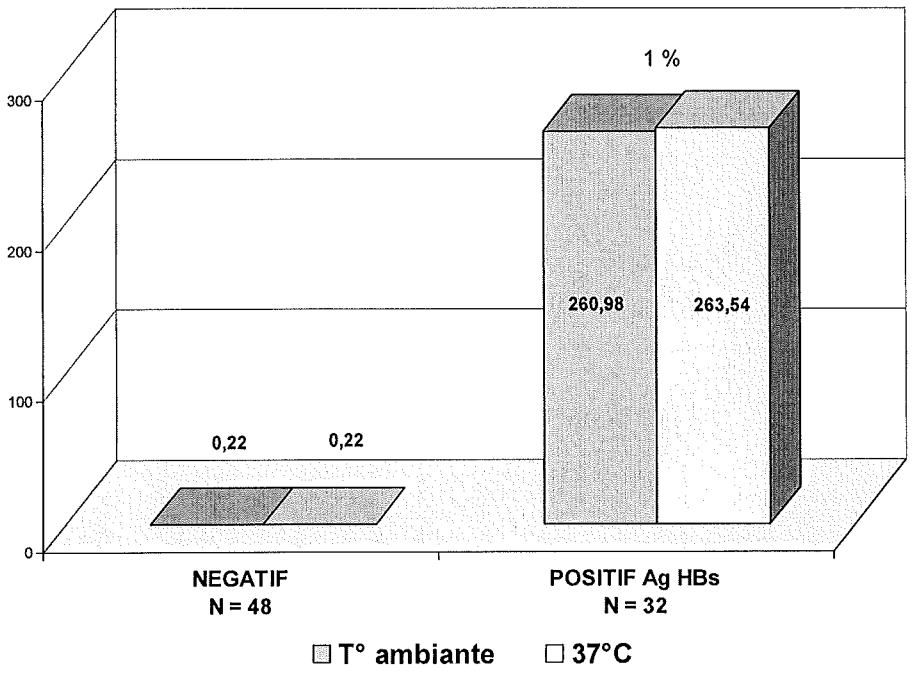
SAMPLE PRESERVATION AT - 30° C
D0 versus D45

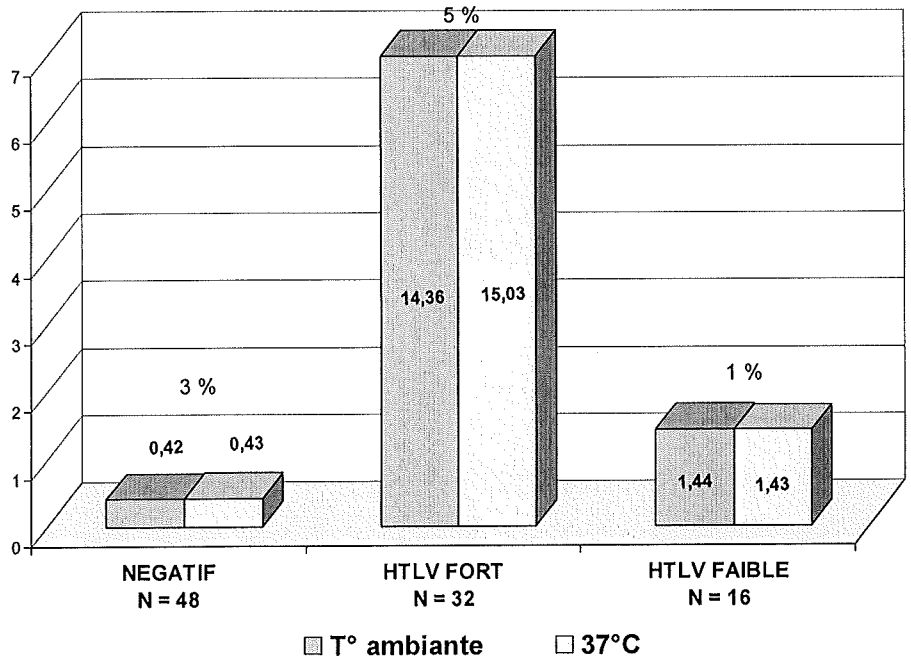




- The 2 diagrams below show the results and variations obtained with regard to thawing conditions.

THAWING SAMPLES
Ambient temperature versus 37° C





Results obtained from quantifying HCV and HIV RNA
Plasma/Serum comparison

HCV RNA (Amplicor ROCHE)		
Genotype	Serum (copies/ml)	Plasma (copies/ml)
1b	5.3E+07	2.0E+07
3	1.6E+06	5.8E+05
NF	1.4E+06	8.6E+05
1a	2.5E+07	4.7E+06
NF	1.6E+06	1.0E+06
1	6.1E+05	3.0E+05
NF	4.1E+06	3.1E+06
NF	3.4E+06	3.3E+06
1a	2.1E+07	8.3E+06
3	5.2E+06	4.4E+06
NF	1.0E+07	1.4E+07
NF	6.4E+06	6.2E+06
3	4.1E+06	3.6E+06
NF	4.2E+06	2.5E+06
NF	8.9E+05	1.2E+06
NF	3.1E+06	3.4E+06
1a	4.0E+06	3.4E+06
2	5.0E+06	5.1E+06
1	9.0E+05	1.6E+06
4	2.6E+06	3.3E+06
NF	7.3E+06	1.1E+07
NF	6.0E+05	3.9E+05

HIV RNA (Amplicor ROCHE)		
Genotype	Serum (copies/ml)	Plasma (copies/ml)
B	19606	42949
A	44670	36579
G	90983	252183
B	198031	915436
B	4871	31685
B	3769	5582
B	283504	375957
B	27515	77314
B	665	451
B	30147	83933
B	1911165	2551242
B	116250	252050
B	68786	236494
B	2079	2921
B	502413	233390
B	1315	1394
B	594	1493
B	256	<400
B	1647	2490
B	962	<400
B	2474	3279
B	26762	11822
B	290	982
B	194673	440582
B	977	527
NF	3974	3575
B	27447	25137
B	3549	6404
NF	9955	107630

#1.16

Performance evaluation study of the PAXgene™ Blood RNA System with regulatory compliance

PreAnalytiX
A QIAGEN / BD Company

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1. Introduction

Gene expression analysis in peripheral blood is an important tool in molecular research, pharmacogenomics, and diagnostics. Reliable analysis is challenged by *ex vivo* changes of expression patterns starting immediately at the time of blood collection. In 2001, PreAnalytiX successfully introduced the PAXgene Blood RNA System intended for the collection, storage, and transport of blood, stabilization of RNA and subsequent isolation and purification of intracellular total RNA from whole blood for research use only. The system consists of a blood collection tube (PAXgene Blood RNA Tube) and nucleic acid purification kit (PAXgene Blood RNA Kit).

This system is widely used for clinical research applications and drug trials. In addition, as RNA-based detection of minimal residual disease in leukemias and monitoring diseases on the molecular level become more important in managing patients, the need increases for an FDA-cleared (US) and CE-marked (EU) preanalytical RNA system for in vitro diagnostic (IVD) applications.

The aim of this study was to evaluate and verify the performance characteristics of a new, optimized PAXgene Blood RNA System (IVD) to support product claims specified in the submission to regulatory authorities. The product was submitted to the FDA using the de novo 510(k) process.

Here we describe a small subset of design verification experiments of the optimized new PAXgene Blood RNA System (IVD) to verify the system's performance, reliability, repeatability, and reproducibility claims.

2. Materials and methods

According to its intended use, system performance was verified with human whole blood with white blood cell counts in the range of $4.8 \times 10^6 - 1.1 \times 10^7$ cells/ml, collected from different donors and stored in PAXgene Blood RNA Tubes (BD, cat. no. 762162). RNA was subsequently extracted using the PAXgene Blood RNA Kit (762174 in Europe; 762164 in the US and Canada) according to the kit handbook.

For stability experiments, a reference method was used, combining standard blood collection tubes with EDTA as an anticoagulant and total RNA purification using a standard acid-phenol organic-extraction method (QIAzol Lysis Reagent) with silica-membrane–based RNA cleanup (QIAamp® RNA Blood Mini Kit). One-step, real-time, duplex RT-PCR assays were developed and used to analyze transcript levels by normalizing C_T (threshold cycle) values of analytes (IL1B) to those of 18S rRNA using the comparative $\Delta\Delta C_T$ method.

RNA was quantified and purity determined using UV spectrophotometric analysis. Genomic DNA contamination was assayed by real-time PCR assays of β-actin sequences and comparison with a standard curve. Inhibition of RT-PCR was analyzed by one-step real-time RT-PCR assays of β-actin sequences using a constant amount of template RNA.

All assays were validated according to method validation guidelines given in USP 27 <1225> "Validation of compendial methods" and ICHQ2A and ICHQ2B "Validation of analytical procedures". This study was conducted in compliance with regulatory guidelines to support product performance claims, and results were submitted to authorities in a 510(k) clearance process, followed by approval of the complete system by The Center for Devices and Radiological Health (CDRH) of the Food and Drug Administration (FDA) to be used as an IVD cleared product in April, 2005 (Regulation Number 21 CFR 866.4070).

3. Results — stabilization at 18–25°C

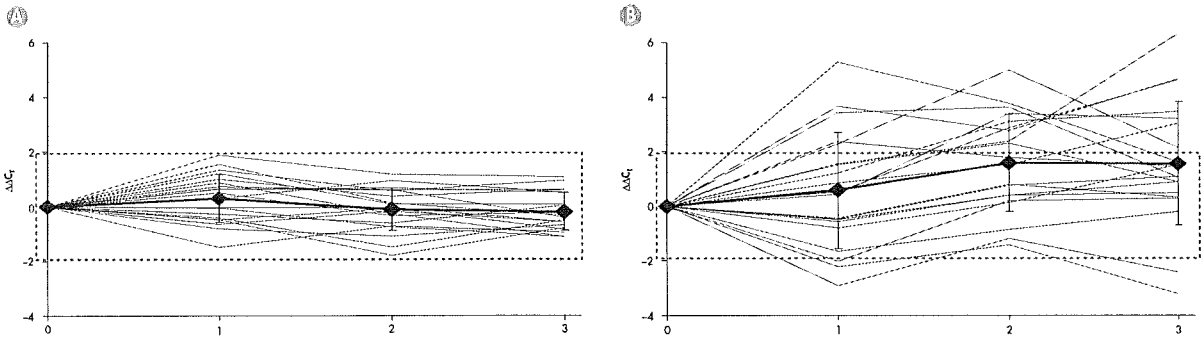


Figure 1 Duplicate blood samples were drawn from 10 donors and stored at 18–25°C for the indicated number of days, followed by total RNA purification. (A) Blood was collected and stored in PAXgene Blood RNA Tubes, and total RNA was purified using the PAXgene Blood RNA Kit. (B) Blood was collected and stored in standard blood collection tubes with EDTA as an anticoagulant, and total RNA was purified using a standard organic-extraction method with silica-membrane-based RNA cleanup. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 C_t).

- Stability: Transcripts remained stable for 3 days at 18–25°C using the PAXgene Blood RNA System, in contrast to significant changes using the reference method.
- Yield: 100% of samples purified using the PAXgene Blood RNA System had RNA yields ≥3 µg (n = 80).
- Purity: 100% of samples purified using the PAXgene Blood RNA System had A_{260}/A_{280} values in the range 1.8–2.2 (n = 80).

4. Results — stabilization at 2–8°C

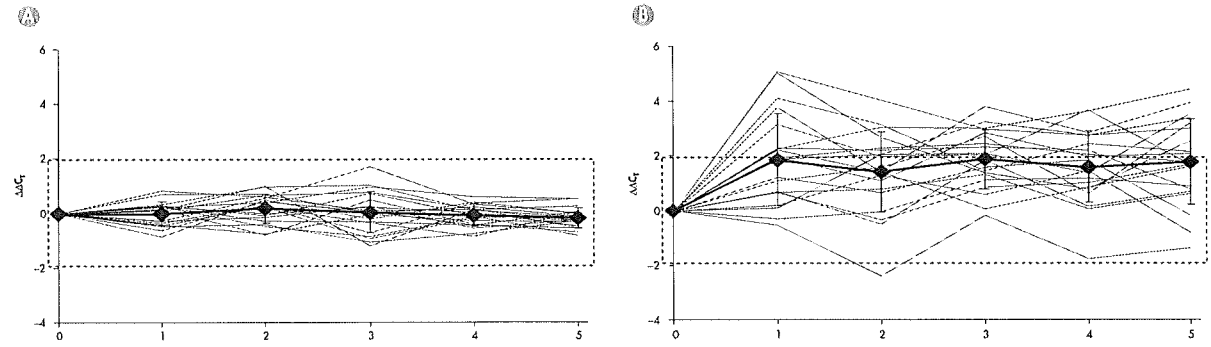


Figure 2 Duplicate blood samples were drawn from 10 donors and stored at 2–8°C for the indicated number of days, followed by total RNA purification. (A) Blood was collected and stored in PAXgene Blood RNA Tubes, and total RNA was purified using the PAXgene Blood RNA Kit. (B) Blood was collected and stored in standard blood collection tubes with EDTA as an anticoagulant, and total RNA was purified using a standard organic-extraction method with silica-membrane-based RNA cleanup. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 C_t).

- Stability: Transcripts remained stable for 5 days at 2–8°C using the PAXgene Blood RNA System, in contrast to significant changes using the reference method.
- Yield: 100% of samples purified using the PAXgene Blood RNA System had RNA yields ≥3 µg (n = 120).
- Purity: 100% of samples purified using the PAXgene Blood RNA System had A_{260}/A_{280} values in the range 1.8–2.2 (n = 120).

5. Results — stabilization at –20°C and –70°C

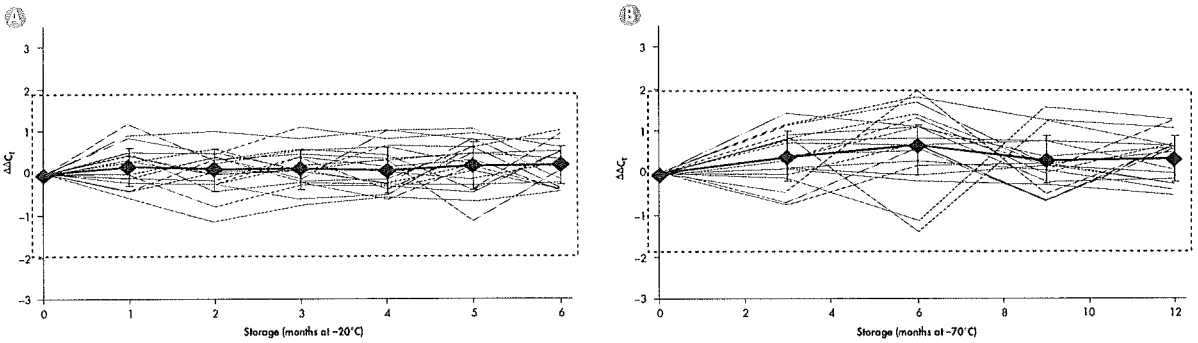


Figure 3 Duplicate blood samples were drawn from 10 donors into PAXgene Blood RNA Tubes and stored at –20°C or –70°C for the indicated number of months, followed by total RNA purification using the PAXgene Blood RNA Kit. Ⓐ PAXgene Blood RNA Tubes were stored at –20°C. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. Ⓑ PAXgene Blood RNA Tubes were stored at –70°C. Relative transcript levels of IL1B were determined by real-time, duplex RT-PCR, using 18S rRNA as an internal standard. The values for all samples are plotted, with means and standard deviations of all samples shown. The dashed lines indicate the ±3x total precision of the assay (1.93 C_T).

- Stability: Transcripts remained stable for 6 months at –20°C and 12 months at –70°C using the PAXgene Blood RNA System. (The reference method was not tested since freeze–thawing of unpreserved blood is known to result in heavy degradation or total loss of RNA.)
- Yield: 100% of samples stored at –20°C (n = 140) and 99% of samples stored at –70°C (n = 100) had RNA yields ≥3 µg.
- Purity: 100% of samples stored at –20°C (n = 140) and 99% of samples stored at –70°C (n = 100) had A_{260}/A_{280} values in the range 1.8–2.2.

6. Results — minimal genomic DNA and no RT-PCR inhibition

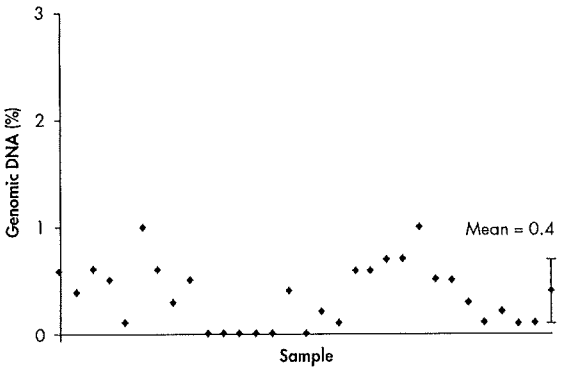


Figure 4 Duplicate blood samples were drawn from 15 donors into PAXgene Blood RNA Tubes and stored at 18–25°C for 1 day, followed by total RNA purification using the PAXgene Blood RNA Kit. Genomic DNA was detected by real-time PCR of the β-actin gene and quantified by comparison with a standard curve. Genomic DNA is reported as the percentage (w/w) of the total nucleic acids.

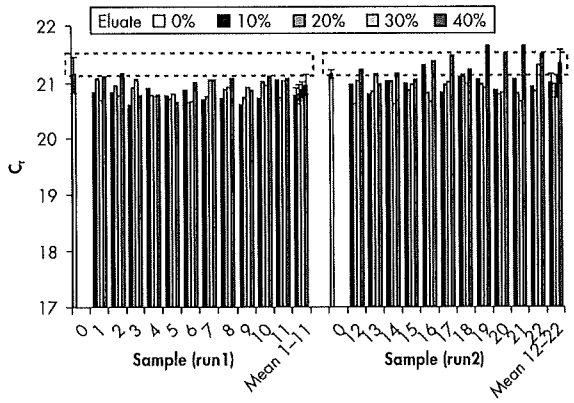


Figure 5 The PAXgene Blood RNA purification procedure was carried out 22 times using distilled water as the starting material instead of the pellet resulting from centrifugation of PAXgene Blood RNA Tubes. Each of the resulting "blank" eluates was used in real-time RT-PCR of the β-actin transcript with a constant amount of template RNA. The eluate comprised the indicated percentage of the reaction volume.

- Genomic DNA: All samples (100%, n = 30) fulfilled the design input specification with ≤1.0% genomic DNA (mean = 0.4%).
- RT-PCR inhibition: All samples (100%, n = 22) showed minimal effects on RT-PCR (<0.5 C_T), with eluates comprising up to 30% of the total reaction volume.

7. Results — lot-to-lot and user-to-user reproducibility

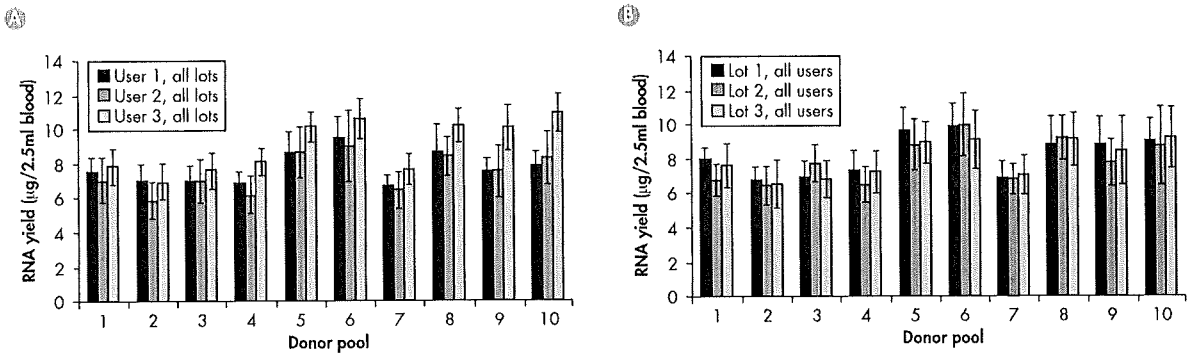


Figure 6 Blood samples from 30 different donors were collected in PAXgene Blood RNA Tubes (12 tubes per donor, 360 tubes in total). The contents of the tubes from 3 donors were pooled and subsequently realiquoted into 36 samples. These 36 samples per 3-donor-pool were processed by 3 different operators. Each operator used 3 different PAXgene Blood RNA Kit lots for the extraction and processed quadruplicate samples from each of the 10 donor pools. Ⓐ RNA yield and standard deviation are given for each operator (**User 1, User 2, User 3**) using all 3 lots. Ⓑ RNA yield and standard deviation are given for each lot (**Lot 1, Lot 2, Lot 3**) used by all 3 operators.

- Lot-to-lot reproducibility (within each user and between all lots calculated as CV of RNA yields per 12 replicates): CVs were ≤30% for 100% of all CVs.
- User-to-user reproducibility (within each lot and between all users per 12 replicates): CVs were ≤30% for 100% of all CVs.

8. Results — repeatability and reproducibility

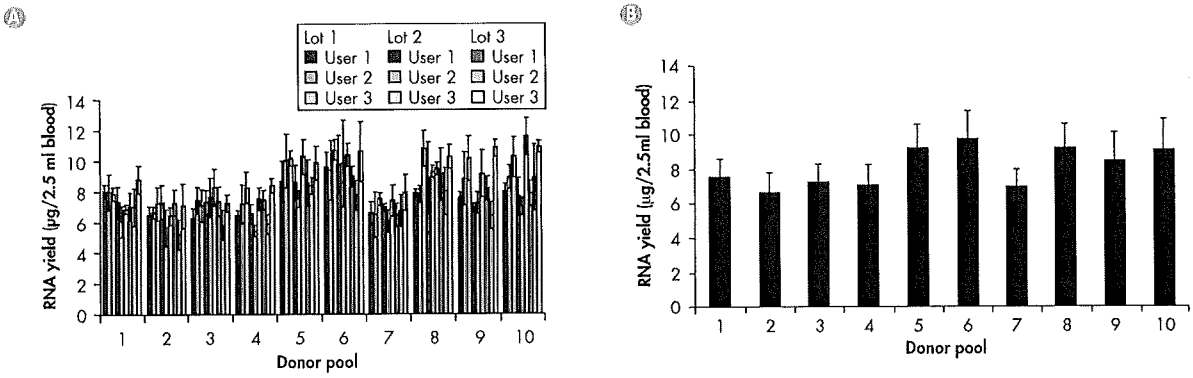


Figure 7 Individual and overall results from Figure 6. Ⓐ Repeatability within each lot and within each user. Ⓑ Reproducibility between all users and all lots.

- Repeatability (within each lot and within each user calculated as CV of RNA yield per quadruplicate sample preps): CVs were ≤25% for 98% (88/90) of all CVs.
- System reproducibility (between all users and all lots per 36 replicates calculated as CV of RNA yield per quadruplicate sample preps): CVs were ≤30% for 100% of all CVs.
- Yield and purity: All samples (n = 360) had RNA yields ≥3 µg (8.0 ± 1.7 µg per 2.5 ml blood sample [mean ± SD, range 4.5–13.7]) and A_{260}/A_{280} values of 2.0 ± 0.1 (mean ± SD, range 1.8–2.2), indicating the high reliability of the complete system.

9. Conclusions

This study demonstrates the high performance of the PAXgene Blood RNA System for the collection, storage, and transport of blood and stabilization of intracellular RNA in a closed tube and subsequent isolation and purification of intracellular RNA from whole blood for RT-PCR used in molecular diagnostic testing. Among the parameters tested were the following.

- In situ RNA stability: 3 days at 18–25°C, 5 days at 2–8°C, 6 months at –20°C, and 12 months at –70°C
- RNA purity: A_{260}/A_{280} values of 1.8–2.2 for ≥95% of samples
- RNA yield: ≥3 µg for ≥95% of samples (per 2.5 ml blood)
- Genomic DNA contamination: ≤1.0% for ≥95% of samples
- No significant RT-PCR inhibition when the eluate contributes up to 30% of reaction volume
- Repeatability of RNA yield: ≥95% of all CVs of donor-dependent yield are ≤25%
- Reproducibility of RNA yield: ≥95% of all CVs of donor-dependent yield are ≤30%

11.33

Evaluation of 16 x 100 mm BD Vacutainer Plus Conical Tubes with a Urinalysis Preservative for Urine Chemistry and Microscopic Analysis on the IRIS® 900 UDx

Evaluation of 16 x 100 mm BD Vacutainer Plus Conical Tubes with a Urinalysis Preservative for Urine Chemistry and Microscopic Analysis on the IRIS® 900 UDx

INTRODUCTION

BD Vacutainer Systems has developed a Urinalysis preservative in a 16x100 mm Plus (plastic) Conical Tube. This unique preservative provides maintenance of urine dipstick chemistries and microscopic components over 72 hours without the need for refrigeration. The preservative does not interfere with urine dipstick chemistries and microscopic analyses on various automated urine analyzers. The two primary urine analyzers on the market are the IRIS 900 UDx manufactured by Roche-BMC and International Remote Imaging Systems (IRIS) and the Clinitek Atlas manufactured by Bayer Diagnostics.

Clinical performance of the preservative was documented over time. Testing included evaluation of urine dipstick chemistries and microscopic analysis at initial time, 24 hours, 48 hours, and 72 hours after tube fill. In this study, urine dipstick chemistry testing and microscopic analysis was performed on the IRIS 900 UDx. The IRIS 900 UDx is comprised of the Roche Boehringer-Mannheim Super UA, a urine dipstick analyzer, and the IRIS, a flow cytometer. The IRIS is unique in that it does not require centrifugation of urine in order to classify cells, casts, and other microscopic urine elements.

The clinical trial was conducted at The University of North Carolina Hospitals in Chapel Hill, NC.

OBJECTIVE

Comparisons were made between the clinical performance of 16x100 mm Vacutainer(TM) Plus Conical Tubes with a Urinalysis preservative stored at room temperature (Evaluation) with 16x100 mm Vacutainer(TM) Plus Conical Tubes stored at 4C (Control) for urine dipstick chemistries using the IRIS 900 UDx at initial time, 24 hours (hr), 48 hr, and 72 hr after tube fill.

CLINICAL PROTOCOL AND PROCEDURE

Urine samples collected in this trial were excess specimens from those submitted to the laboratory for patient testing. The approximate amount of urine obtained from each specimen was 32 mL. In the initial phase of the study, one hundred and forty-one urine samples were collected. Four tubes were filled from each specimen for a total of 800 tubes.

All tubes tested in the study were 16x100 mm Plus Conical Tubes with an 8 mL draw. The Control Tube, Reorder# 364980, does not contain any preservative. The Evaluation Tube, Reorder# 364992, contains a urinalysis preservative comprised of chlorhexidine, ethyl paraben, and sodium propionate.

Urine dipstick chemistry analytes were assayed on the Super UA using Iristrips for color, pH, protein, glucose, ketone, bilirubin, blood, urobilinogen, leukocytes, and nitrite. Appearance was analyzed by transmission and light scattering.

Microscopic analysis was performed on each tube by flow microscopy on the IRIS 900 UDx. Identification of cells, crystals, casts, bacteria, and other articles were made. Specific gravity was also tested on the IRIS 900 UDx using a mass gravity meter.

The test methods/principles employed by this analyzer are:

Analytes and Methods		
Analyte	**Instrument**	**Principle**
Specific gravity	IRIS 900 UDx	Flow microscopic mass gravity meter
PH	Boehringer Mannheim Super UA	Double indicator of methyl red and brothymol blue
Protein	Boehringer Mannheim Super UA	Protein error of indicators
Glucose	Boehringer Mannheim Super UA	Double sequential enzyme reaction – glucose oxidase
Ketone	Boehringer Mannheim Super UA	Legal's test – sodium nitroferricyanide
Bilirubin	Boehringer Mannheim Super UA	Diazo reaction
Blood	Boehringer Mannheim Super UA	Pseudoperoxidase activity of hemoglobin
Urobilinogen	Boehringer Mannheim Super UA	Modified Erlich's reaction
Leukocytes	Boehringer Mannheim Super UA	Granulocytic Esterase
Nitrite	Boehringer Mannheim Super UA	Griess's reaction
Color	Boehringer Mannheim Super UA	Reagent pad color change
Appearance	Boehringer Mannheim Super UA	Transmission and scattering of light
Microscopic Constituents	IRIS 900 UDx	Flow Microscopy

A randomization schedule was employed to account for draw order of the tubes. All tubes, collected in duplicate, were filled and mixed by 10 inversions or until dissolution of the preservative. Tubes were stored at the designated conditions, either RT or refrigerated (approximately 4 C), for the duration of the 72 hour time period. Any tubes that were refrigerated were allowed to equilibrate to RT prior to assay. At each time interval, tubes were mixed before analysis by inverting or using a transfer pipet. An aliquot of 4 mL of urine from each tube type was pipetted into pediatric tubes at each time interval. Urine specimens were analyzed for urine dipstick chemistries and microscopic flow cytometry at initial time (within 2 hr of collection), 24 hr, 48 hr, and 72 hr following tube fill. A () 2 hour window was allowed at each time interval.

A sample size of 200 was designated for the initial phase of the study. After completion of 141 urine specimens, the data review showed there were several analytes; protein, ketones, bilirubin, urobilinogen, RBC and WBC, that required further investigation. In order to determine the root cause of the differences observed, the study design was revised to better understand the effect of the preservative on these analytes. In the second phase of the trial, each analyte was evaluated individually. Specimens that were positive for each of these specific analytes were selected.

Sample sizes vary for each analyte due to availability of specimens demonstrating positive responses. Sample analysis over time (initial, 24h, 48h, and 72h) was conducted for protein, ketones, bilirubin, urobilinogen, RBC and WBC. Over the 72-hour period, analyzed specimens were not obtained from a single collection tube due to volume restrictions. As with the initial phase of the trial, testing time intervals were 2 hours of any stated interval.

RESULTS AND DISCUSSION

Phase 1 Results

The Ordinal Logistic Regression method was used to model the data and draw inference. The Main Effects Plot examines the data by tube and by time. By tube, all data points across all time intervals are averaged for each tube type in order to evaluate the effect of the tube type. By time, at each time interval all data points for both the control and the preservative tube are averaged together to evaluate the effect of time on the tubes. The Interaction Plot compares the performance of the control tube with the preservative tube at each time interval. This plot presents the data best for a clinical evaluation of tube performance over time.

When reviewing the data, any p value that is <.05 is considered statistically significant. There were *no statistically significant differences* in the normal range demonstrated when comparing the performance of 16x100 mm Urinalysis Preservative Tubes, at room temperature, with 16x100 mm Plus Conical Tubes, at 4C, over 72 hours with specimen analysis on the IRIS 900 UDx using the above defined statistical model for the Interaction Plots.

The following observations demonstrate the percentage of results (per 141 donors) outside of the normal reference range for the respective analytes:

• Specific gravity	0%	• Nitrite	3%
• pH	18%	• Color	6%
• Protein	33%	• Appearance	6%
• Glucose	17%	• Blood	23%
• Ketone	9%	• Bilirubin	6%
• Urobilinogen	14%	• Leukocytes	15%
• WBC	7%	• RBC	4%

Phase 2 Results

Only positive data collected during phase one of the study for the selected outstanding analytes was analyzed. In addition, positive data, for the same select analytes that were collected during phase two, were merged and analyzed with phase one data. It is important to note that all of the positive data analyzed for this phase is not considered clinically abnormal. There are various degrees of abnormal results present; however, they are all deemed "positive" responses.

The protein data shows that Preservative Tubes shift protein results by approximately one grade from the initial Control Tube, however, over time the performance for both tube types remains unchanged. The observed one grade change is not considered clinically significant in a majority of cases.

Ketone data demonstrates no statistically significant difference between the two tube types. A statistically significant difference was observed with the urobilinogen data. Clinically, these differences can be attributed to time as well as the effect of the environment (i.e., light vs. dark). Statistical analysis determined no significant differences between Preservative Tubes and Control Tubes for bilirubin. Due to the similar nature of bilirubin and urobilinogen, it is anticipated that differences may be observed with bilirubin due to time and/or environmental factors.

WBC and RBC statistical analyses indicate that there are no significant differences between tube types, but there is a significant interaction between tube types and time. The graphs reveal a decrease in values for both WBC and RBC over time. Clinically, this can be attributed to handling differences in phase one and two of the study. Inadequate mixing in phase one of the study produced lower values over time. Phase two shows that providing proper attention to mixing prior to assay maintains the values over time.

Based on conclusions from the statistical analysis, *clinical equivalency is established* between the 16x100 mm Urinalysis Preservative Tubes when compared with the 16x100 mm Plus Conical Tubes with analysis on the IRIS 900 UDx over 72 hours. In addition, the presence of clinically abnormal results ensures the Urinalysis Preservative Tubes work effectively at room temperature over 72 hours in conditions beyond normal reference ranges except for urobilinogen and bilirubin. There is a minor shift in the protein determination. This shift is only 1 grade change in the <30 mg/dL range and may not be clinically significant

CONCLUSIONS

The findings of this study support both statistical and clinical equivalency between 16x100 mm Vacutainer™ Plus Urinalysis Preservative Tubes, stored at room temperature, when compared with 16x100 mm Plus Conical Tubes, stored at 4C, for urine dipstick chemistry and microscopic analysis on the IRIS 900 UDx over 72 hours.

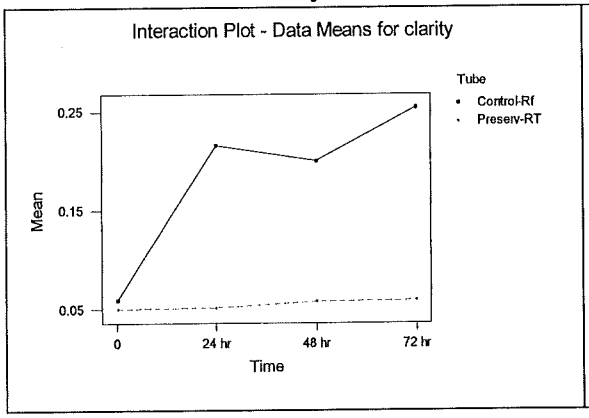
It should be noted that urobilinogen and bilirubin assays should be performed as soon as possible to reduce the effects of photosensitivity or stored in darkness.

Whenever changing any manufacturer's collection tube type or size for a particular laboratory assay, the laboratory director should review the tube manufacturer's data and/or previous data generated to establish/verify reference range data for your specific instrument and reagent system. Based on such information, the laboratory can then decide if changes are indicated.

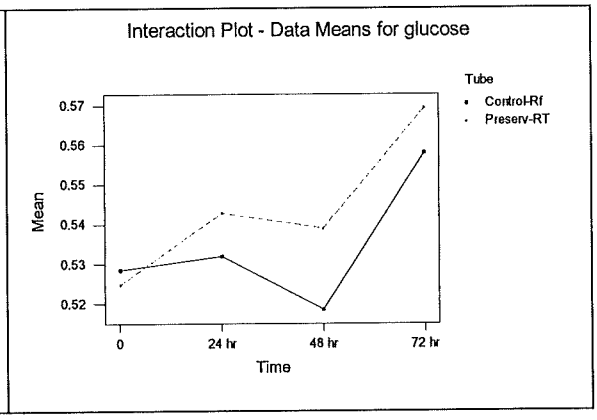
Phase One Graphs

Graph Legend	
Control - Rf	Solid Line ———
Preservative - RT	Dashed Line --------

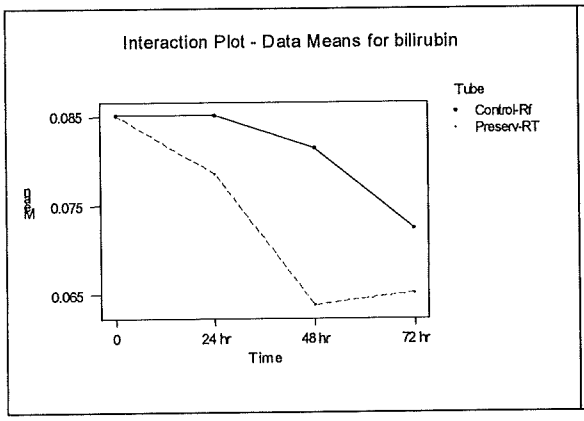
Clarity



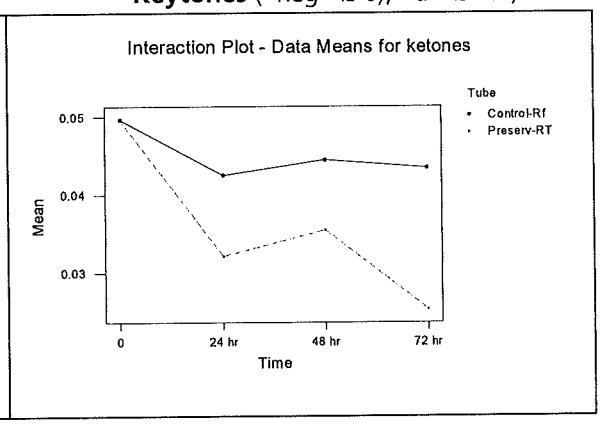
Glucose



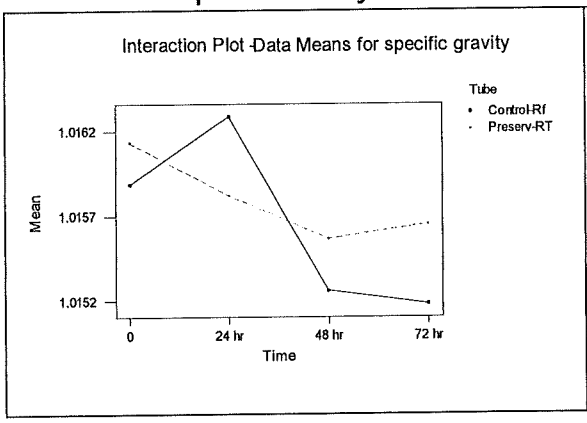
Bilirubin



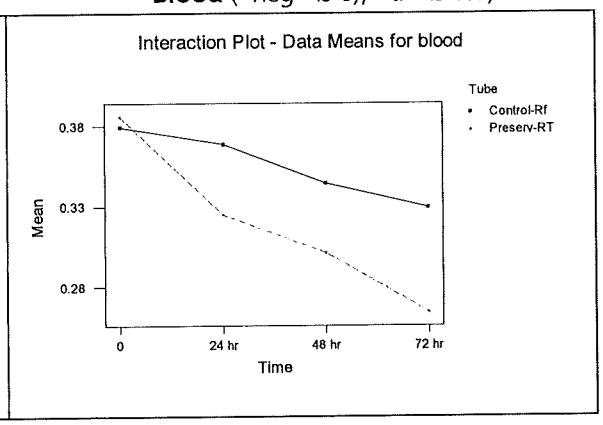
Keytones ("neg" is 0), "tr" is 0.5)



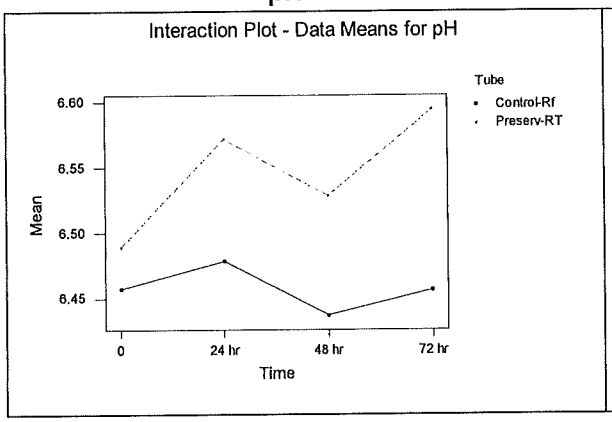
Specific Gravity



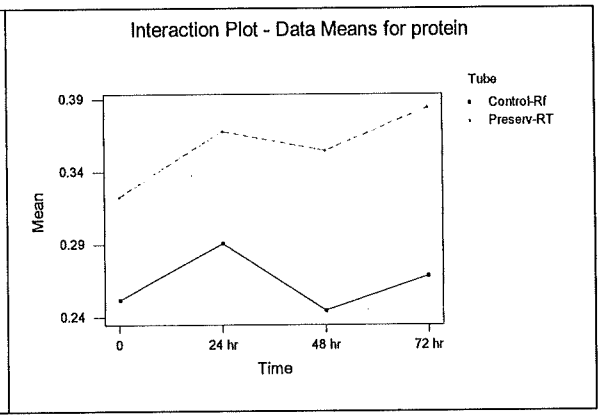
Blood ("neg" is 0), "tr" is 0.5)



pH



Protein

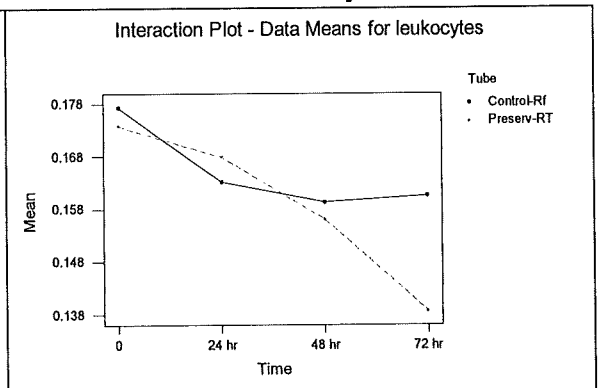


Nitrate

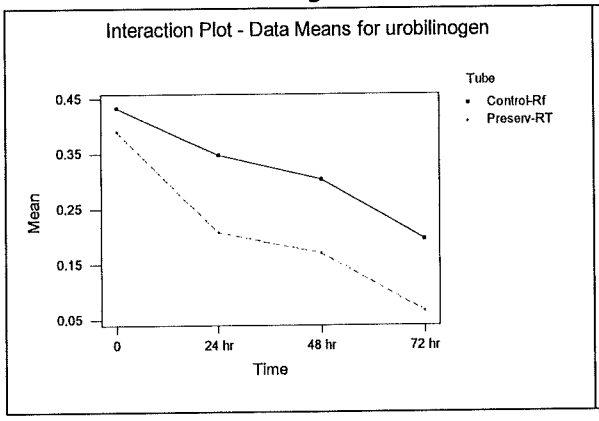
Binary Logistic Regression

Tube*	Time
Preserv-RT*24 hr	0.786
Preserv-RT*48 hr	0.816
Preserv-RT*72 hr	0.789

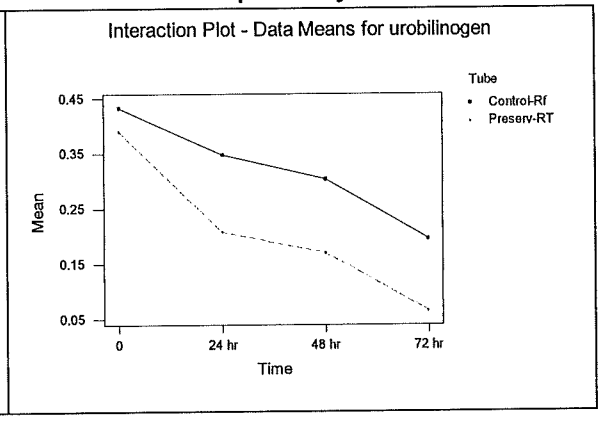
Leukocytes



Urobilingen

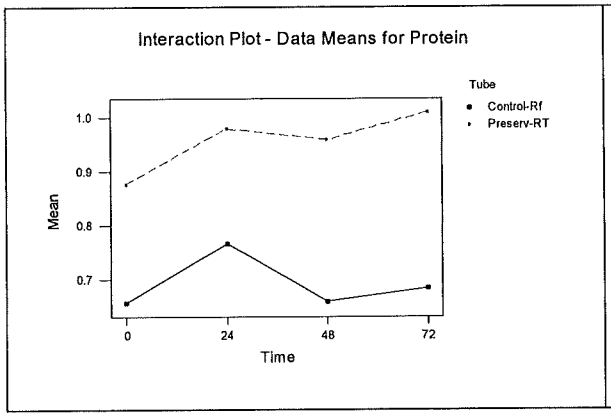


Microscopic Analysis: WBC/RBC

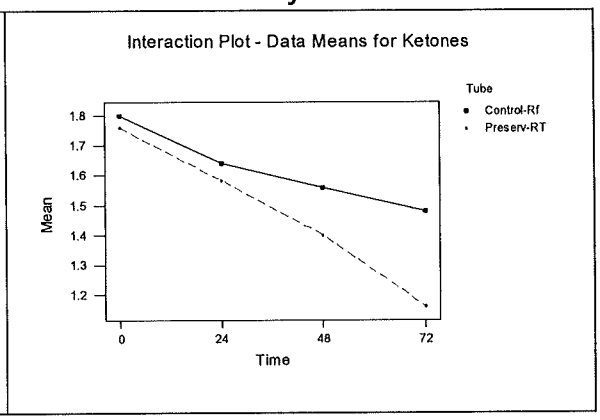


Phase Two Graphs

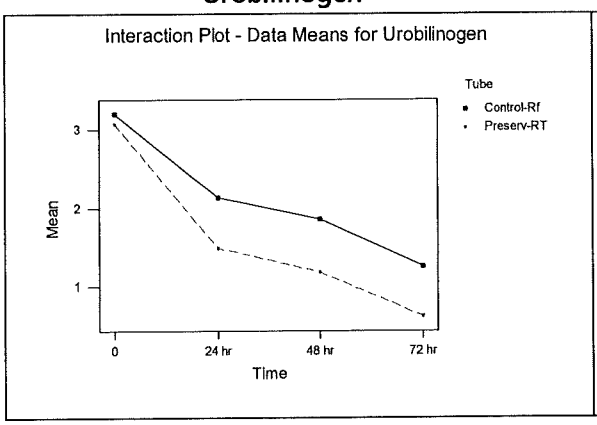
Protein



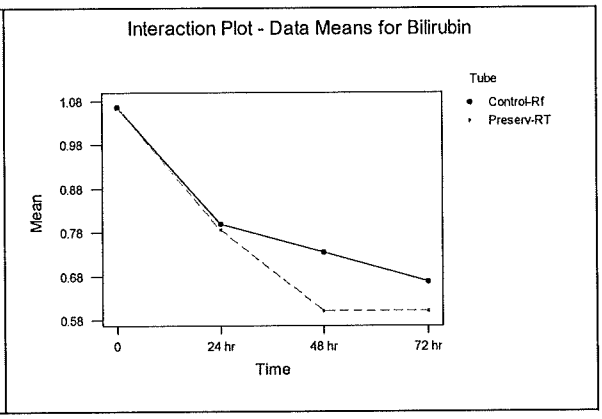
Keytones



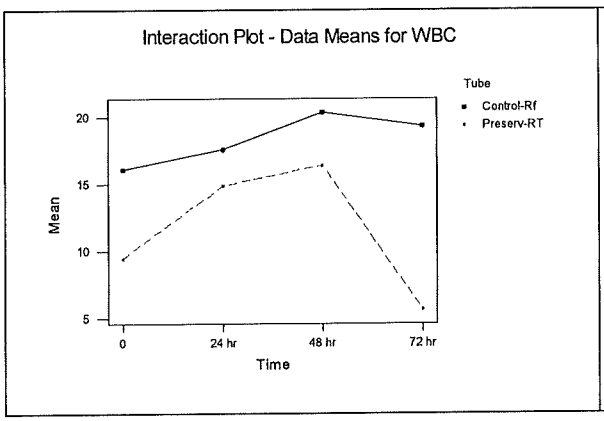
Urobilinogen



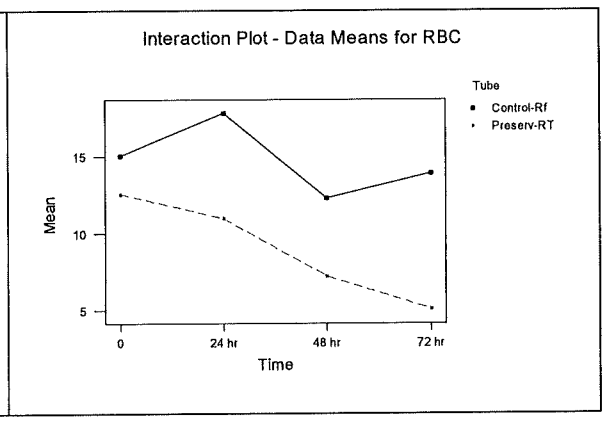
Bilirubin



WBC



RBC



Technical assistance

For more specific information on BD Vacutainer™ products, please call Technical Services at (800) 631-0174.

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