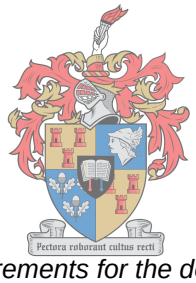
DRIED PLASMA SPOT TESTING – THE ANSWER FOR MAKING BLOOD TRANSFUSION TESTING SAFER IN AFRICA?

by

Charlotte Pistorius



Thesis presented in fulfilment of the requirements for the degree Master of Medical Science (Medical Virology) at the University of Stellenbosch

Supervisor: Prof. Wolfgang Preiser

Co-supervisor: Dr Arthur Bird

Faculty of Health Sciences

Division of Medical Virology, Department of Pathology

December 2018

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:

_____

Charlotte Pistorius

Date: December 2018

SUMMARY

Sub-Saharan Africa (SSA) has a unique set of challenges pertaining to blood transfusion. Two of the largest contributing factors are: (1) the most common disease states in SSA require large amounts of blood as a lifesaving intervention e.g. malaria, and (2) the highest burden of infectious diseases transmissible through transfusion (Tapko, Toure, & Sambo, 2014) is found in SSA. This has often led to the dichotomous donor base that exists in SSA, consisting of Voluntary Non-remunerated Blood donors (VNBD) and family or replacement donors (FRD), since transfusion centres are unable to supply the demand when relying only on VNBD.

VNBD are the safest blood donors as they have no monetary incentives and are under no direct social pressure to donate. Monetary incentives have been shown to entice individuals that know or suspect themselves to be infected with a blood borne agent to donate blood.

Nucleic Acid Testing (NAT) in conjunction with serological testing is the gold standard for testing, however the vast distances and high temperatures of SSA makes transport of traditional plasma samples a logistical nightmare. Many publications evaluating the stability, suitability and ease of use of dried blood spot (DBS) and dried plasma spot (DPS) for NAT have been published. Generally results have been shown to be comparable to traditional plasma samples. DBS are being used successfully in the early infant diagnosis (EID) programs for HIV by means of PCR testing especially in Africa.

Ethical approval was obtained to conduct a study to determine whether DBS and/or DPS testing would be suitable for use in a resource limited setting for blood screening.

Two cohorts were included. Cohort A consisted of 900 de-identified negative new donor samples. Cohort B consisted of 100 de-identified confirmed positive donor samples, 9 procured reactive samples, and a contamination panel. After routine donor testing was completed at Western Province Blood Transfusion Service, one DBS sample and one DPS sample for each blood donor was prepared and analysed with the Ultrio Elite Assay on the Panther analyser (Hologic Inc., USA).

Logistically DBS/DPS is well suited for the resource-poor countries as samples are:

a. Easy to obtain (fingerpick samples could be used).
b. Transport is simplified as samples will not leak or haemolyse due to high temperatures.
c. Samples can be stored at room temperature.

DBS/DPS samples demonstrated superb specificity. DPS samples would be suited for screening blood and reduced cost involved in NAT testing provided that the HBV sensitivity is increased. Further detailed economic viability and large-scale studies need to be performed to determine sensitivity and specificity within a specific population.

OPSOMMING

Bloedoortapping in Sub-Sahara Afrika(SSA) word geteister deur unieke uitdagings. Die twee grootste bydraende faktore is:

1) die mees algemene infeksies in SSA vereis menige bloedoortappings as ingrypende lewensredende en lewensverbeterende terapie bv. Malaria,

2) die grootse las van aansteeklike siektes, wat deur bloedoortapping oorgedra word, word in SSA aangetref (Tapko, Toure and Sambo, 2014).

Hierdie twee faktore het bygedra tot die ontstaan van die tweeledigeskenskersbasis wat in SSA gevind word. Die tweeledigeskenkersbais bestaan uit vrywillige onvergoede bloedskenkers(VOBS) en familie lede en/of vervangings skenkers(VS). Daar word dikwels staat gemaak op VS as gevolg van die feit dat bloedoortappingssentrums nie in staat is om die aanvraag te voorsien wanneer daar slegs op VOBS staat gemaak word nie.

VOBS is die veiligste bloedskenkers omdat hulle geen monetêre motiewe het nie en onder geen direkte sosiale druk verkeer om te skenk nie. Monetêre motiewe kan individue, wie kennis dra of vermoed dat hulle bloed besmet is met oordraagbare agente, aanspoor om te skenk.

Die kombinasie van nukleïensuurtoetsing (NAT) en serologiese toetsing is die goue standaard vir bloedoortappings-siftingstoetse, maar die lang afstande en hoë temperature van SSA maak die vervoer van tradisionele plasma monsters 'n logistieke nagmerrie. Menige publikasies oor die stabiliteit, geskiktheid en gemak van die gebruik van gedroogde bloed kol (DBK) en gedroogde plasma kol (DPK) vir NAT is reeds gepubliseer. Oor die algemeen het die resultate vergelykbaar met tradisionele plasma monsters vertoon. DBK word veral in Afrika suksesvol gebruik in die vroeë babadiagnose-programme (EID) vir die toetsing van menslike immuniteits virus deur middel van polimerase kettingreaksie.

Etiese goedkeuring was verkry vir die studie wat beoog om te bepaal of DBK/DPK geskik sal wees om te gebruik vir siftings-bloedoortappingstoetse in 'n hulpbron beperkte omgewing.

Twee kohorte was ingesluit by die studie. Kohort A het bestaan uit 900 gedeïdentifiseerde negatiewe nuwe skenkermonsters. Kohort B het bestaan uit 100 gedeïdentifiseerde bevestigde positiewe skenkersmonsters, nege aangekoopte reaktiewe monsters en 'n besmettings paneel. Nadat roetine siftings-bloedoortappingstoetse deur die Westelike Provinsie Bloedoortappingsdiens voltooi was, is een DBK monster en een DPK monster vir elke bloedskenker voorberei en getoets deur die Ultrio Elite reagens op die Panther instrument (Hologic Inc., VSA).

DBK/DPK is logisties geskik vir hulpbronbeperkte lande want die monsters:

a. Is maklik om te verkry (Vingerprikmonsters kan gebruik word).
b. Vervoer van monsters kan vereenvoudig word omdat monsters nie lek of hemoliseer weens hoë temperature nie.
c. Kan teen kamertempuratuur geberg word.

DBK/DPK het uitstekende spesifisiteit gedemonstreer (100%). DPK sal geskik wees vir die toeting van bloed en vermindering in koste verbonde aan NAT toetsing, slegs as die HBV sensitiwiteit verhoog word. Verdere ekonomiese, lewensvatbare en grootskaalse studies moet uitgevoer word om die sensitiwiteit en spesifisiteit binne 'n spesifieke bevolking te bepaal.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and organisations:

God

"Thank you! Everything in me says "Thank you!" Angels listen as I sing my thanks. I kneel in worship facing your holy temple and say it again: "Thank you!" Thank you for your love, thank you for your faithfulness." *Psalm 138:1-3*

Professor Preiser, Dr Bird, Russell and Kosma

"Thank you for thinking of me and then wondering how you could help. Thank you for doing what you did, instead of being too busy, or just forgetting about it. Thank you for inking me on your priority list, when you have so many other things to do: I am honoured; it meant a lot to me. Thank you"
By Joanna Fuchs

Tinus, Chanah en Mikah

Dankie vir al die koffie, geduld, koffie, verstaan, koffie, liefde, koffie, wasgoed, koffie, aandetes en ondersteuning (in daardie volgorde). Ek sal dit altyd onthou en waardeer.

Adri, Morné and the rest of the family

Thank you for your consistent and unfailing support it means the world to me.

Ilex Medical

Thank you for providing reagents, consumables and technical support. Without your support, I would not have been able to do this research

WPBTS

Bev thank you for pushing me. Amanda, Carmen and Lizzie thank you for your words of encouragement and support along the way. Greg Bellairs and Nicky du Toit, thank you for affording me the opportunity to study further and to use the Virology laboratory as my research base.

Dankie, Thank you, Vielen Dank, Enkosi

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND UNITS

ACD - Acid citrate dextrose

anti-HCV - Antibodies to Hepatitis C

anti-HIV - Antibodies to Human Immunodeficiency Virus

CDC - Centre for disease control

CKC - Cohen's Kappa Coeficient

CLIA - Chemiluminescence immuno assay

DBS - Dried blood spot

DNA - Deoxynulceic acid

DPS - Dried plasma spot

EID - Early infant diagnosis

FDA - Food and Drug Administration

FFP - Fresh frozen plasma

FRD -Family/Replacement donor

HBsAg - Hepatitis B surface antigen

HBV - Hepatitis B virus

HCV - Hepatitis C virus

HDN - Haemolytic disease of the newborn

HIV - Human immunodeficiency Virus

IC - Internal Control

ID_{50} - Infectious dose where 50% of the population would acquire the infection

LOA - limits of agreement

LOD - lowest level of detection

mℓ - Millileter

NAT - Nucleic acid test

PD - Paid donor

PEPFAR - U.S. President's Emergency Plan for AIDS Relief

RCC - Red cell concentrate

RDT - Rapid diagnostic test

RNA - Ribonucleic acid

SSA - Subsaharan Africa

TTD - Transfusion transmitted disease

TTI - Transfusion transmitted infection

UNAIDS - Joint United Nations Programme on HIV and AIDS

UNICEF - United Nations Children's Fund

VNBD - Voluntary non-renumerated blood donors

WHO - World Health organization

WHO SSA - World Health Organization Subsaharan Africa

WP - Window period

µℓ - Microlitre

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

1. INTRODUCTION

1.1. BLOOD TRANSFUSION

1.1.1. Historical development

The first recorded attempt at administration of blood took place in 1490 when an ill Pope Innocent VIII was given the blood of three young boys to “rejuvenate” him. However, the administration was not a success given that all 3 donors and the patient died. It is still debated whether the blood was given to the Pope orally or intravenously (Wiener, 1933; Gear, 1938; Learoyd, 2006; ‘Highlights of Transfusion Medicine History’, 2018).

After the unsuccessful attempt to save the Pope in the 15th century the transfusion scene was quiet until the early 17th century when various strides in the field where made. One of the most profound advances was made by William Harvey when he described the circulatory system and this enabled a quick succession of experiments. Richard Lower, an English physician, is credited with the first transfusion to animals. He did ground-breaking experiments with dogs, first giving them transfusions of various liquors, and then graduating to giving the blood of one dog to another. His contemporary Denys read about Lower’s work and started to perform his own experiments transfusing blood from dog to dog. Denys submitted his report on his first lamb to human transfusion in July 1667, but due to publishing delays it was only printed after Richard Lower’s report of the same experiment, hence he is often not credited with the first blood transfusion to humans. (Learoyd, 2006) The transfusion of animal blood to humans had dire consequences to the humans and was thus soon prohibited by law in England and France. After this law was passed we entered another lull of almost a hundred years in which no significant advances were made.

James Blundell is credited with the first human to human transfusion, in 1818, after transfusing a patient for the treatment of post-partum haemorrhage. However, there is a record of an earlier transfusion, which was performed by an American physician Philip Syng Physick, in 1795. Unfortunately Physick never published and so is seldom recognised (or even mentioned) as a pioneer in this field. (‘Highlights of Transfusion Medicine History’, 2018)

In the 20th century blood transfusion was revolutionised. In 1900 Karl Landsteiner, often referred to as the father of blood transfusion, and his team discovered the first three of the ABO blood groups, namely A, B, and O. This reduced transfusion fatalities significantly. Group AB was discovered by the same team in 1902. In 1914 the key to storing blood in glass bottles prior to infusion was found when the optimum dose of citrate for anti-coagulation was described. The first blood transfusion institute opened its doors in Moscow in 1926 led the Russian physician and scientist Alexander Bogdanov (Huestis, 2007).

The World Wars necessitated a large number of advances in blood transfusion techniques, apparatuses, and biomedical consumables. Most notably the use of acid-citrate-dextrose (ACD) and the freeze drying of blood plasma were introduced. In South Africa the Cape Peninsula Blood Transfusion Service was founded in 1938 (now known as the Western Province Blood Transfusion Service) at Groote Schuur Hospital. In 1940 Dr Phillip Levine and his colleagues describe what we now know to be the 'Rh' blood group system (Giblett, 1994) which was described as the main cause of haemolytic disease of the new born (HDN). In 1950 another remarkable change took place. This was the change from fragile, re-usable glass bottles to disposable, breakage-resistant plastic bags for the collection of blood. This change furthermore facilitated the introduction of component therapy as opposed to whole blood transfusions.

In 1947 the first routine testing on units was introduced. Initially each unit was only tested for ABO and syphilis. In 1971 Hepatitis B surface antigen (HBsAg) testing was introduced, followed by the introduction of HIV testing in 1985, and Hepatitis C antibody (Anti-HCV) testing in 1990 (Aoki *et al.*, 1993; Van der Poel, 1999; Busch and Kleinman, 2009). This completed the current testing menu of recommended serological testing assays (World Health Organization, 2010). For the next 10 years serological assays were constantly reengineered to be more specific and sensitive without major reductions to the window periods to detect infections. In 2002 the first FDA licensed Nucleic acid amplification test (NAT) became available from Grifols, making accessible a test that by reducing the window periods for detection mitigates the risk related to acquiring a transfusion transmitted infection significantly (Cable, Lelie and Bird, 2013).

1.1.2 Importance of blood transfusion

Blood transfusion is seen world-wide as a life-saving, life improving medical intervention and treatment option. Blood transfusions are indicated in various diagnosis.

Red cell concentrates are predominantly used to increase oxygen carrying capacity when the patient's ability to carry oxygen is impaired, e.g. following haemorrhage, bone diseases and certain deficiency disorders.

Different plasma products are used to increase circulatory volume and to correct clotting factor deficiencies as result of hereditary factor deficiency disorders, warfarin overdose or the effect of massive transfusions. Plasma products are extensively used in burns patients.

When a patient's platelet count is low, these patients are at risk of excessive bleeding (World Health Organization, 2002). A low platelet count could result from chemotherapy, cancer and some infections in which case platelet products may be required.

1.2. RISKS ASSOCIATED WITH BLOOD TRANSFUSION

1.2.1. General overview

Although life-saving, blood transfusion does carry the risk of complications which include transfusion transmitted infections and adverse transfusion reactions.

The main objective of blood transfusion policies are to ensure a safe and adequate blood supply appropriate to the needs of a country. Since less than half of the blood transfusion needs are being met in Sub Saharan Africa (SSA), in certain instances family replacement donors (FRD), paid donors (PD) and untested units are being transfused. To address this problem the WHO established the Blood Safety Unit. This unit has been tasked with assisting member states by providing basic guidelines to ensure all individuals have access to:

- an adequate blood supply
- at affordable prices
- as safe as possible for transfusion (within economic reason restraints)
- transfused only when needed (clinically indicated transfusion)
- within a sustainable blood transfusion program (Fleming, 1997)

The WHO has recommended the development of national blood policies. This requires that each country develop a strategy for testing, recruiting and maintaining adequate blood supplies. Although formal policies have been developed in 40 reporting countries only 22 countries have successfully implemented them.

1.2.2. Infectious risks

Infectious risks vary from transfusion service to transfusion service. A large percentage of the risk is mitigated by the screening assays used in the transfusion service. However, diseases that are not routinely screened for in blood transfusion testing do pose an additional infection risk.

Table 1.1: Infectious agents are known or present a theoretical risk to be transmitted through transfusion:(Kaur and Basu, 2005; Bihl *et al.*, 2007; CDC, 2013; Fung *et al.*, 2014)

Viral agents	*Parasitic diseases/ Bacterial agents*	*Prion diseases*
Hepatitis A	Chagas Disease	Creutzfeldt-Jakob disease
Hepatitis B (HBV)	Treponema Pallidum	Human variant Creutzfeldt-Jakob disease
Hepatitis C (HCV)	Plasmodium species	Chronic wasting disease
Hepatitis D	Trypanosoma cruzi	
Hepatitis E	Barbesia secies	
Human Immunodeficiency virus (HIV)	Leishmania species	
Human T Lymphocytic virus	*Borrelia burgdorferi*	
Epstein Barr Virus	*Toxoplasma gondii*	
Cytomegalo virus	Filarial infections	
Dengue Virus	*Anaplasma phagocytophilum*	
Chikungunya virus	*Rickettsia rickettsii*	
St Louis encephalitis virus		
Human herpesvirus 8		
Human Herpesvirus 6		
Human parvovirus B19		
Simian foamy virus		
West Nile Virus		
Transfusion transmitted Virus		
SEN Virus		

There are a number of reasons why blood donors are not screened for all transfusion transmitted diseases (TTD):

1. no screening assay exists for the TTD,
2. the incidence of the TTD is so low that screening would not be feasible,
3. the prevalence of the TTD is so high that most donors would be excluded (e.g. Malaria),
4. the costing of the screening assays is prohibitive,
5. and lack of expertise, to name a few.

In situations where there is no available test or it is not feasible to screen for a TTD marker, screening is often performed during donor interviews. This would then initiate deferrals for certain aspects, i.e. travel.

For an infectious agent to be transmissible through blood transfusion the agent must:

- be present in the plasma or cellular component of the donated blood.
- be able to use the blood stream as an entry point to the host.
- have an asymptomatic stage of the disease i.e. exist where the infected donor is free from signs or symptoms of the infection. (Government of Maharashtra Blood transfusion Council, 2005)

1.3. BLOOD BORNE VIRUSES

1.3.1 HIV

Human immunodeficiency virus is a member of the Retroviridae family. The virus infects certain human immune system cells which impair and destroy the functionality of these cells. As cell-mediated immunity is progressively lost susceptibility to infections increases. The virus is transmitted through contact with the blood or other body fluids of an infected person.

The infectious dose, where 50% of the population (ID_{50}) would acquire HIV, from a red blood cell concentrate (RCC) is calculated at 316 copies (Weusten *et al.*, 2011; Vermeulen *et al.*, 2013, 2014). Different viral loads could result in an infection because the plasma content of varies between products. The ID_{50} is calculated based on 20 mℓ of plasma within a RCC.

High viral replication takes place during the initial acute phase of the HIV infection. This results in a peak of HIV-RNA within the first few weeks of being infected. This peak is seen during or prior to the development of the p24 antigen and HIV antibodies as illustrated in Figure 1. *1*.

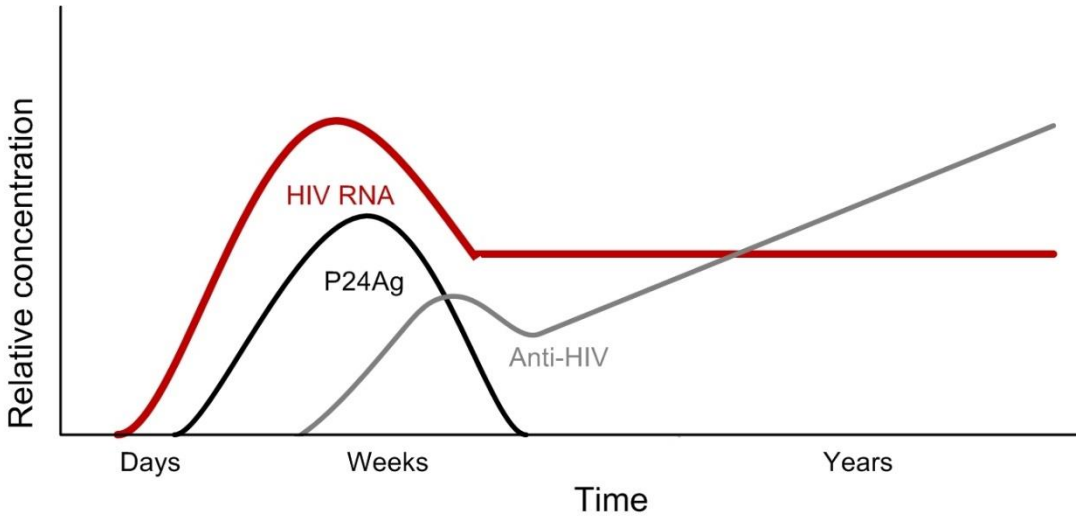


Figure 1. 1. The sequence of appearance of laboratory markers of HIV infection. This figure based on (Grant and Smith, 2015)

1.3.2 Hepatitis B

Hepatitis B virus is a member of the Hepadnaviridae family. The virus infects the liver and causes both chronic and acute infection. Chronic infection puts people at a high risk for liver cirrhosis and carcinoma. The virus is transmitted through contact with the blood or other body fluids of an infected person. Within the SSA Hepatitis B is predominantly transmitted through horizontal infection in childhood, sexual transmission, and transfusion transmitted transmission (Maponga, 2012).

With reference to blood transfusion, Hepatitis B has 2 different ID_{50}'s dependent on stage of the disease in the donor. The first is attained during the initial window period (WP) in the pre-acute phase where 3.16 copies are estimated to be the ID_{50} (Vermeulen *et al.*, 2012). The second is attained during the occult WP (Anti-HBc positive) where the estimated ID_{50} is 100-fold higher at approximately 316 copies (Vermeulen *et al.*, 2012). The lowest ID_{50} of 3.16 copies is used to simplify calculations of risk (Weusten *et al.*, 2011). Plasma content varies for different blood products, consequently, different viral loads could result in an infection. Transfusion with fresh frozen plasma (FFP) increases the risk of infection by approximately 9 times (Busch and Kleinman, 2009; Allain *et al.*, 2013).

In *Figure 1. 2* the two WP are shown with corresponding viral markers.

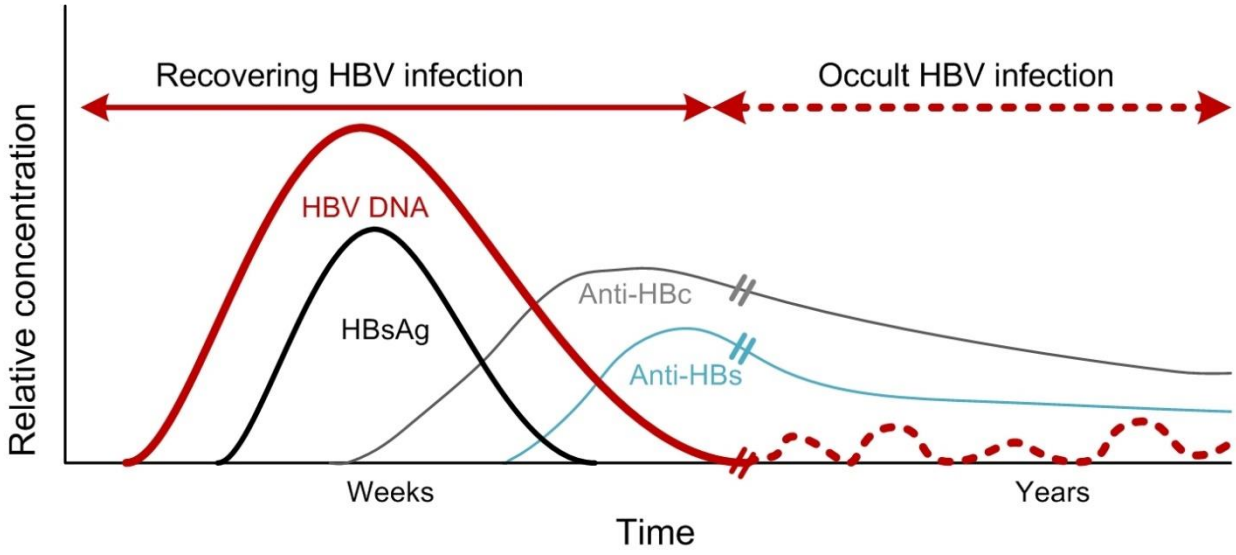


Figure 1. 2. Recovered and occult hepatitis B infection base on (candotti and laperche, 2018)

1.3.3 Hepatitis C

Hepatitis C virus is a member of the Flaviviridae family. Similarly to Hepatitis B, the virus causes liver disease that can be both chronic and acute. Chronic infections often develop into cirrhosis or liver cancer. The virus is transmitted through contact with the blood of an infected person. The infection is often associated with intravenous drug use, and transfusion of untested units. The risk of infection due to sexual transmission is low. Within SSA it is estimated that the overall prevalence of Hepatitis C is 3 % (Madhava, Burgess and Drucker, 2002).

In reference to blood transfusion, Hepatitis C, like Hepatitis B, has 2 different ID_{50}'s. The first ID_{50} is attained during the initial WP in the pre-acute phase where 3.16 (1-10) copies is estimated, this would be the worst case scenario. The second ID_{50} is calculated to be 316 (100-1000) copies during the anti-HCV positive stage since the anti-HCV provides some protection for the recipient (El Ekiaby *et al.*, 2015).

In *Figure 1.3* recovering and chronic infection periods are shown with the relevant viral markers.

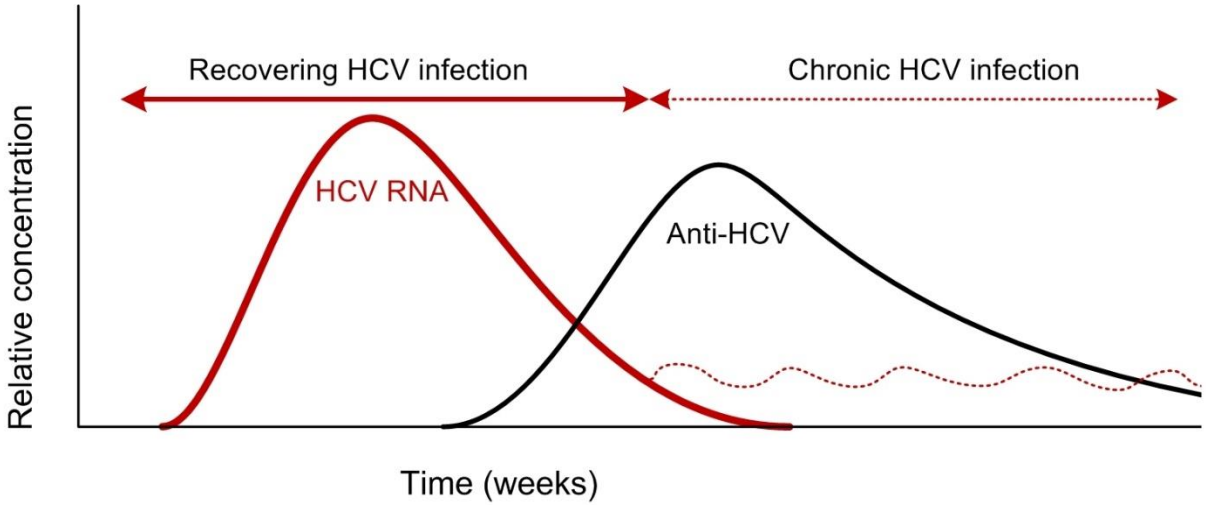


Figure 1.3. Graph depicting of recovering and chronic infection periods of Hepatitis C

1.4. RESEARCH RATIONALE

1.4.1 Blood transfusion in Africa

1.4.1.1. History

Blood transfusion has been practised in Africa since the early 1920s (Schneider, 2013). Organised blood services existed in SSA before World War II. These transfusion services were able to call on donors when patients required blood transfusions. The availability of doctors and facilities were the only limiting factor to performing transfusions during this time.

After independence, various SSA countries moved away from centralised testing. Regional services were established in a response to high costs, large transport distances and delays, limited blood supplies, and political unrest. Larger facilities where able to ensure limited blood transfusion services, however smaller hospitals could not meet demand. The smaller, often rural, transfusion services were forced to turn to family/replacement donors (FRD) and even paid donors (PD) to supplement their blood stocks. (Schneider, 2013)

A turning point was reached in transfusion testing when it became evident that HIV could be transmitted via blood transfusion, this led to international bodies such as the World Health Organisation (WHO) recognising that SSA needed help in order to ensure safe blood supplies. In 1994 it was noted that only ten out of 46 WHO SSA reporting countries could guarantee that blood had been screened for HIV (Tapko, Toure and Sambo, 2014). In response, the WHO has led various initiatives to increase blood safety in SSA. With the help of organisations such as PEPFAR, CDC and Safe Blood for Africa, some SSA countries have been able to improve their blood testing centres and staff education. In contrast, some SSA countries are still unable to meet even the most basic recommendations regarding blood transfusion and consequently do not have a sufficient, safe or sustainable blood supply. It has been estimated that less than 50% of blood transfusion needs are currently met in SSA (Tapko, Toure and Sambo, 2014).

1.4.1.2. Current status

As a rule low-income countries have a higher disease prevalence than their higher income counterparts. Conversely, higher income countries often have a lower prevalence of TTDs than low-income countries. First world countries (with low TTD prevalence) are able to screen donated blood with the most sophisticated screening tests available. In comparison, countries within the SSA (with a high TTD prevalence) are often unable to employ even the minimum screening recommendations.

A myriad factors contribute to the insufficient, or lack of, screening. These include economic instability, civil wars, lack of infrastructure, manmade- and natural disasters. SSA carries 10% of the world disease burden, as well as the highest disease burden for TTDs (Tapko, Toure and Sambo, 2014). In 2010 approximately 68% of people living with HIV worldwide lived in SSA, in an area with only 12% of the global population (WHO, UNICEF and UNAIDS, 2011). Within the blood donor population, which is often seen as a safer/lower risk population, the prevalence of TTDs is higher in low-income countries than in high-income countries as illustrated in *TABLE 1.2*.

Table 1.2. Prevalence of transfusion-transmissible infections in blood donations (Median, Interquartile range (IQR), by income groups(WHO, 2016)

	HIV	**HBV**	**HCV**
High-Income countries	0.003% (0.001% – 0.04%)	0.03% (0.008% – 0.18%)	0.02% (0.003% – 0.16%)
Upper middle-income countries	0.08% (0.006% – 0.2%)	0.39% (0.16% – 0.69%)	0.21% (0.05% – 0.42%)
Lower middle-income countries	0.20% (0.05% – 0.44%)	1.60% (0.94% – 4.13%)	0.40% (0.19% –1.5%)
Low-income countries	1.08% (0.56% – 2.69%)	3.70% (3.34% – 8.47%)	1.03% (0.67% – 1.80%)

With TTD prevalence this high in low-income countries, even within the blood donor base, it can be assumed that incidence cases will be high. Theoretically the implementation of NAT testing would provide greater benefit to low-income countries since NAT is able to detect infection during serological window periods which would be seen more frequently in high TTD prevalence countries (El Ekiaby, Lelie and Allain, 2010).

A large number of countries within SSA are unable to screen blood for transfusion in a quality assured manner. However, there are exceptions, some SSA countries have been able to increase their voluntary donations and improve their testing strategies. These countries have the potential to perform sophisticated centralised testing, but are still resolving terrain and infrastructure challenges, so small regional testing centres remain the norm. Some of these small testing centres have the capacity and ability to perform serological testing, but not yet NAT testing due to the infrastructure, expertise and cost involved.

The difficulty of transporting traditional plasma samples across vast distances between rural areas and cities on poor condition roads makes this one of the biggest deterrents for centralised testing. An additional constraint is that traditional plasma samples require cold chain management increasing cost and transport difficulty.

1.4.2. Blood safety in Africa

Africa has a unique set of challenges pertaining to blood transfusion. The two of largest contributing factors are:

1. the most common disease states in SSA require large amounts of blood as lifesaving intervention e.g. malaria,
2. the highest burden of infectious diseases transmissible through transfusion (Tapko, Toure and Sambo, 2014)is found in SSA.

This has led to the dichotomous donor base that exists in SSA. The donor base consists of voluntary non-remunerated blood donors (VNBD) and family/replacement donors (FRD). Transfusion centres are unable to meet the transfusion demand when relying only on VNBD.

VNBD are the safest blood donors as they have no monetary incentive, or direct social pressure to donate. Monetary incentives have been shown to entice individuals that know or suspect themselves to be infected with a blood-borne agent to donate blood. While VNBD are the safest donors, the effort and cost involved in testing, transport, donor retention and processing are often prohibitive in severely economically restrained countries. In contrast, the cost of testing, processing and delivery of a unit from a replacement donor is about 4 times less than the cost of a voluntarily donated unit (Bates et al., 2008).

The cost of a voluntarily donated unit is attributed to: the cost of running a testing facility utilising enzymatic or chemiluminescent immunoassays for screening for transfusion-transmitted infections (TTI); staff training and staffing costs; transport; donor management and donor recruitment. The lower cost of replacement donation is attributed to it being done in a hospital setting where only limited testing (often using lateral flow immunochromatographic/rapid point-of-care tests) is performed by hospital staff. Subsequently, only 42.3% of countries reporting to World Health Organisation (WHO) collect more than 90% of their blood supply from VNBD and 41% of countries collect more than 50% of their blood supply from FRD (World Health Organization, 2016).

Blood transfusion testing in SSA can be roughly broken down into 2 categories: Hospital-Based and National blood transfusion services. See **Error! Reference source not found.** for comparison of the above mentioned categories.

Table 1.3. Comparison of the two different transfusion testing categories

	Hospital- Based	**National blood transfusion services**
Donor Base	These testing centres focus on FRD. FRD are donors who donate when someone known to them requires a transfusion. FRD may unfortunately also include hidden paid donation as the donor may receive monetary contributions from the patient's family to present as a family FRD when no other suitable donor can be found by the patient's family (McCullough and McCullough, 2013; Asamoah-Akuoko *et al.*, 2017)	Rely on both VNBD and FRD Due to the cost involved with especially recruitment of VNBD less than 50 % of total blood donors on the African continent are VNBD. In contrast countries like Ivory Coast, Malawi and Burkina Faso who receive external funding (Tagny *et al.*, 2008) and other well established transfusion services like Uganda and South Africa are mostly or completely dependent on VNBD (Lund *et al.*, 2013)
Organisational structure	Situated in hospitals both regional and remote.	The testing centres are both regional and centralised.
Blood stock	They have limited blood stock and have limited ability to provide component therapy.	Larger blood stocks are available in the larger academic or university affiliated hospitals. However the amount of stored blood in SSA countries remains below the WHO minimum target for adequate blood supply (10 units per 1000 population per annum) with the exception of Botswana, Guyana, Namibia and South Africa (Chevalier *et al.*, 2016)
Testing	Limited TTI testing is performed as it is not sustainable to test relatively small quantities of blood with automated analysers utilizing chemiluminescence (CLIA). Rapid lateral flow tests (RDT) are used more frequently as these tests are readily available and require no equipment or electricity. However such rapid tests are usually less sensitive than laboratory-based and ensuring proficiency in conducting such tests is notoriously challenging.	Larger testing centres use automated CLIA analysers and the more remote settings use enzyme-linked immunosorbent assays or RDT tests. NAT is performed in only a handful of countries in SSA as part of their blood screening strategy. This is in contrast with the European blood transfusion services. However it has been reported that NAT would identify comparatively more HBV yield and occult cases in SSA and thus be more cost effective than in Europe (El Ekiaby, Lelie and Allain, 2010)

1.4.3. Dried blood and plasma spots for nucleic acid testing

In the last century applying biological fluids and/or blood to filter paper has changed a number of fields of study. This technique became widespread after Dr Robert Guthrie, in November 1961, found a novel way of screening for Phenylketonuria (PKU) (Robert Guthrie and Ada Susi, 1963). Guthrie was reportedly inspired by a Sherlock Holmes story where a desk blotter was used to solve a murder (Paul and Brosco, 2013). Dried blood spot (DBS) and dried plasma spot (DPS) sampling techniques have had a significant impact on, to name a few, forensic pathology, new-born metabolic screening, epidemiology, and drug analysis (Li *et al.*, 2014).

DNA extraction from dried blood spots on filter paper was first described in 1987 (McCabe *et al.*, 1987). Many studies evaluating the stability, suitability, and ease of use of DBS for NAT have since been published. Results have generally shown to be comparable to traditional plasma samples testing. DBS's success is attributed to the fact that it provides a robust sample that can be transported over large distance at temperatures at and above room temperature. This is essential in SSA where cold chain management of sample shipment and storage are challenging, and poor infrastructure results in adverse logistical issues. Agitation of the sample due to poor road conditions is not an issue with DBS.

Table 1.4 describes the advantages and disadvantages of DBS/DPS as compared to traditional liquid plasma samples.

Table 1.4.Advantages and disadvantages of DBS/DPS

Advantages	**Disadvantages**
Robust sample DBS/DPS samples can be stored at ambient temperatures for up to 45 days without risking nucleic acid integrity	Additional steps prior to testing Nucleic acids must first be eluted from DBS/DPS prior to testing
Lower Infection risk The viral envelope is damaged during the drying process (although HBV may remain infectious for a week) (Bond *et al.*, 1981). Thus, once dried, the biohazard is very low.	Small sample volume A typical DPS contains 70 µl of plasma and a typical DBS 35 µl of plasma volume. Several DBS/DPS can be eluted together to increase the sample volume for testing.
Sample No risk of sample leaking or breaking. The sample can be transported by normal mail as the dried blood matrix stabilises many analytes including DNA, infection risk is very low and the sample will not break and leak	Possible lower sensitivity Due to the small sample volume and elution process, the sample might show lower sensitivity when compared to traditional liquid plasma samples.

DBS are being used successfully in the early infant diagnosis (EID) programs for HIV by means of polymerase chain reaction (PCR) testing. As such DBS has revolutionised the EID program for HIV in

low-resource countries by reducing the cost and training involved in sample collection and transport. This reduction in cost has resulted in DBS being used extensively within limited resource settings allowing access to early diagnosis for infants and the required treatment, thus reducing morbidity and mortality in this population (Ciaranello *et al.*, 2011). DBS testing in the EID program within SSA has predominantly been used in conjunction with PCR (Chibwesha *et al.*, 2016) with manual or semi-automated extraction processes (Stevens *et al.*, 2009).

The Procleix Ultrio assay menu includes two versions for the Panther instrument. The Procleix Ultrio blood screening assay for blood donors simultaneously detects the presence of HIV-1/2, HCV RNA and HBV DNA, using the multiplex TMA technology. And the second is the Aptima assays (Aptima HIV-1, Aptima HCV and Aptima HBV) which are used during confirmation and identification of the virus of reactive Procleix Blood screening assay samples. These have individual probes specific for each virus and helps discriminates between them. Evaluations of DBS/DPS have been done using Aptima HIV-1 RNA qualitative assay (Sahoo *et al.*, 2016).

1.5. AIMS AND OBJECTIVE

1.5.1. Aims

The aim of this study is to validate a screening method using a novel sample type. We present the advantages of using this new method for screening donated blood for viral nucleic acid in resource constrained settings.

1.5.2. Objective

2. To demonstrate that dried blood spot (DBS) and / or dried plasma spot (DPS) testing is suitable for blood donor screening and can make good quality testing more widely available in SSA due to lowering costs.
3. To determine the diagnostic sensitivity and specificity of testing DPS and DBS samples, in comparison to testing of plasma samples as per routine protocol.
4. To determine the stability of DPS and DBS after being stored for three weeks at room temperature.

1.5.3. Hypothesis

1. DPS and DBS testing is compatible with the nucleic acid testing (NAT) platform used at WPBTS (Ultrio Elite assay on the Panther analyser).
2. Interpreted test results obtained using DPS and / or DBS are comparable to conventional plasma sample testing.
3. Sample stability is high over a limited period of time allowing DBS / DPS to be transported to a centralised blood testing centre.

1.6. ETHICAL CONSIDERATIONS

1.6.1. Routine informed consent

Each donor routinely completes and signs a Donor Questionnaire Addenda A which covers health and lifestyle questions prior to donating blood. In Point 1 of the consent section the donor gives consent for their blood to be tested for syphilis, Hepatitis B, Hepatitis C and HIV. In point 3 the donor gives consent to having their samples/blood products used for research purposes.

The tests being performed with the DBS /DPS are for the same three viruses (HIV, HBV and HCV) that are routinely screened each time a donor donates blood, i.e. no additional non-routine markers will be tested for.

1.6.2. Confidentiality

Samples will be de-identified prior to testing the DBS and DPS samples. At no time will a donor's identification be made available to any third party involved or not involved in this study.

CHAPTER TWO

2. RESEARCH DESIGN AND METHODOLOGY

2.1. MATERIALS

2.1.1. Ethical considerations

2.1.1.1. Routine Informed consent

At WPBTS blood donors are voluntary non-remunerated donors (VNRD) from within the borders of the greater Western Cape. This area is bordered by Plettenberg Bay, Springbok, and Beaufort West. Blood donors are recruited by blood drives, recruitment calls, recruitment SMS' and at blood donor clinics. Within the Western Cape, approximately 1.5% of the population donates or has donated blood. In 2017 70 140 donors donated, 20869 of which were first-time donors (Rix, no date). The donor base for 2016-2017 comprised of 49% females and 51% males, and included Asian (1%), Black (4%), Coloured (31%) and White (64%) donors. During this time WPBTS donors donated, on average, 2.17 donations per year.

Each donor routinely completes and signs a Donor Questionnaire which covers health and lifestyle questions prior to donating blood (Addendum A). In Point 1 of the consent section the donor gives consent for their blood to be screened routinely for syphilis, Hepatitis B, Hepatitis C and HIV. In point 3 the donor gives consent to having their samples/blood products used for research purposes.

This study will establish whether the Ultrio Elite Assay can be used for blood screening using DBS/DPS. The tests performed with the DBS/DPS are for the same three viruses (HIV, HBV and HCV) that are routinely screened for each time a donor donates blood, i.e. no additional non-routine markers were tested for. The only difference between routine testing and this study was the sample type. Published studies have shown that DBS and DPS are equally or slightly less sensitive than plasma testing (Stevens *et al.*, 2008).

2.1.1.2. Approval

Ethical approval was obtained from the University of Stellenbosch Health Research Ethics Committee (HREC 1) via Minimal Risk Review procedures on 15 September 2017 (Addendum B).
(Institutional Review Board (IRB) Number: IRB0005239. Protocol Reference number 0806.)

2.1.2. Study Population

The study population consisted of WPBTS blood donors that donated blood at the various blood donation clinics within the WPBTS donation area. These donors included both first-time donors (ND) and repeat donors (RD) who donated from November 2017 until January 2018.

All samples were drawn as per routine donation procedure. No additional blood samples were drawn from donors. The blood donor samples were first subjected to routine sample preparation which comprised of centrifugation. This was followed by routine testing which included NAT, serological, syphilis and blood group testing. Samples were stored in a refrigerated room after completion of testing, as per routine protocol. Only once all routine testing was completed and confirmed, were the samples used for this study.

At WPBTS all routine samples are interpreted using two separately completed algorithms. The two algorithms are the serological algorithm and the NAT algorithm. Only if the sample interpretation for both algorithms are the same, i.e. HIV reactive or non-reactive, is the sample regarded as a concordant reactive sample or concordant negative sample. All samples used in this study were categorised based on the WPBTS routine serological and NAT algorithms which are illustrated in Figure 2.1 and Figure 2.2. The Ultrio Elite is a multiplex assay which simultaneously detects RNA for HIV-1/2 and HCV, as well as HBV DNA, therefore discriminatory testing is performed as part of the NAT algorithm. The discriminatory probes discriminate which of the three viruses are responsible for the Ultrio Elite reactive result. The Discriminatory assays (dHXV) consist of 3 different discriminatory probes namely; the discriminatory probe for HBV (dHBV), the discriminatory probe for HCV (dHCV), and the discriminatory probe for HIV (dHCV).

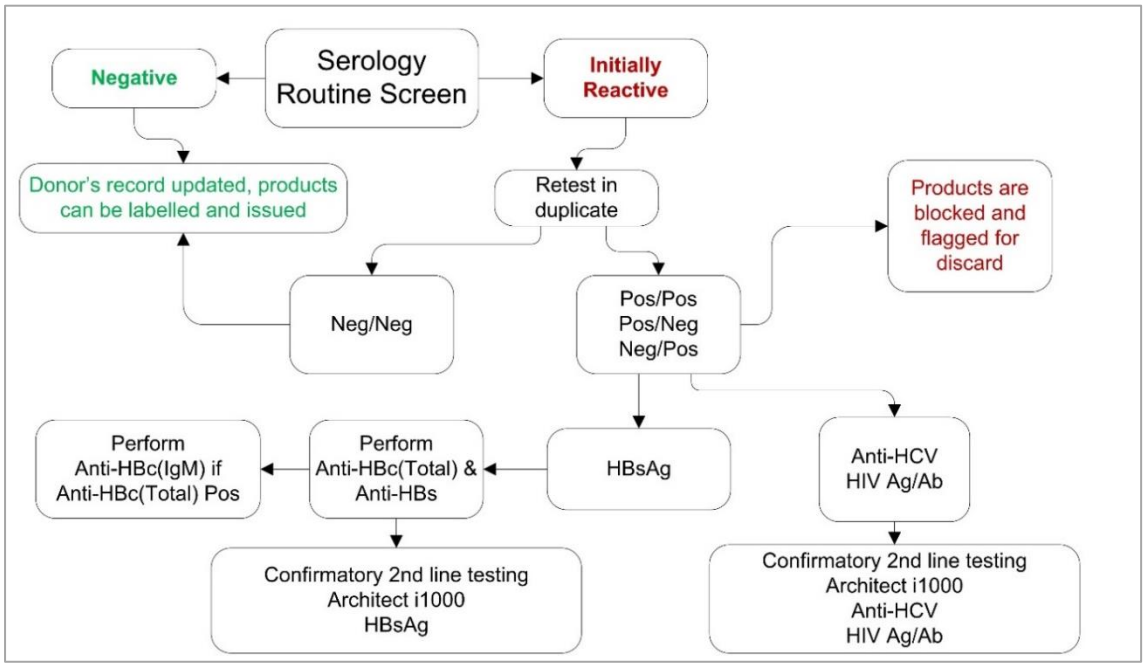


Figure 2.1. Simplified routine WPBTS serological algorithm

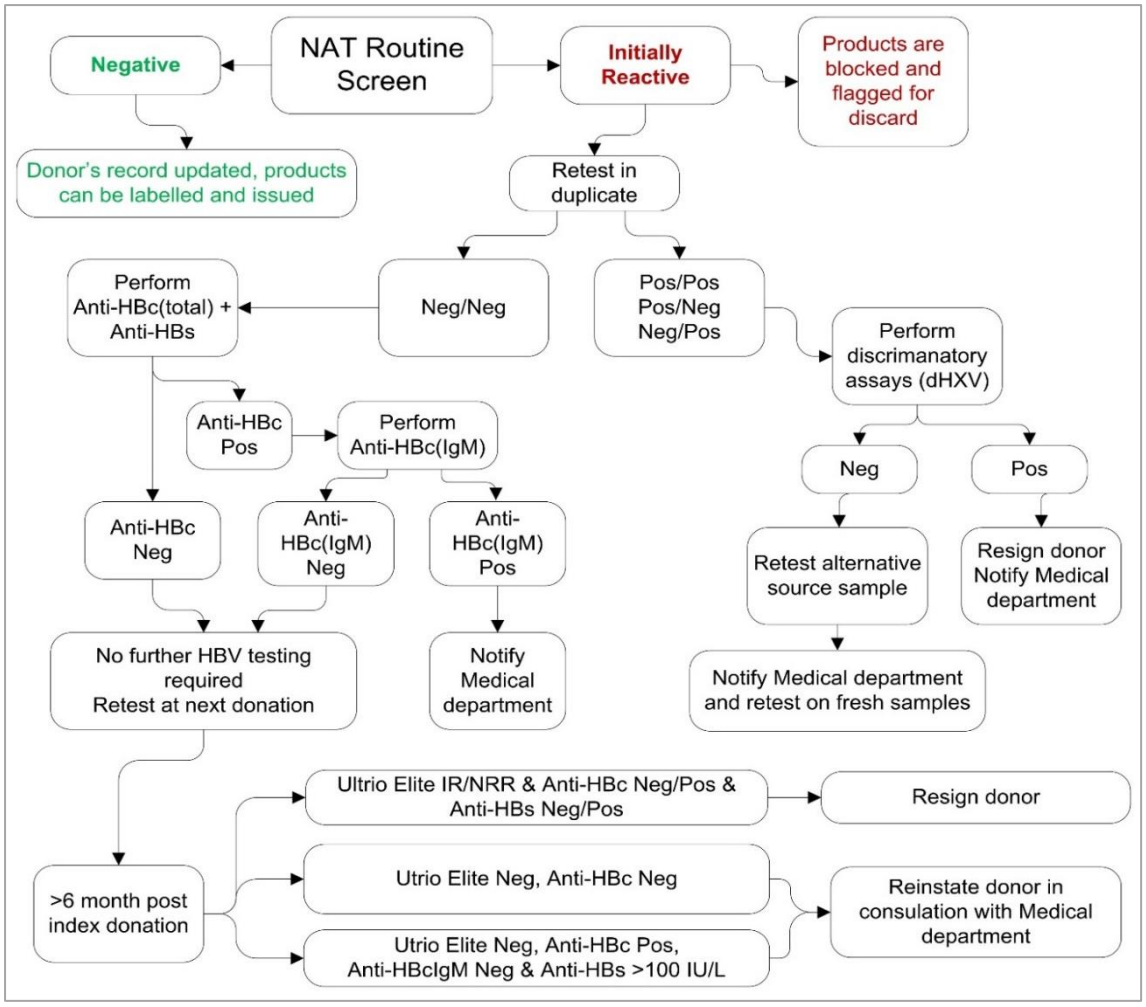


Figure 2.2. Simplified routine WPBTS NAT algorithm

2.1.2.1. Cohort A

This cohort consisted of 900 WPBTS routine first-time/new donors (ND) that were found to be negative on routine screening on the Panther analyser (Hologic Inc., USA).

The rationale behind selecting new donors is that they are not prone to a preselection bias that would otherwise have occurred due to repeat 'cross-reactive' donors. A 'cross-reactive' donor is a donor whose blood results appear to be reactive at first but after repeat testing or confirmatory testing are shown to be non-reactive. According to international standards 'cross-reactive' units are not to be transfused and as such must be discarded. At WPBTS these repeat 'cross-reactive' donors are requested not to donate or refrain from donating for a specified time due operational guidelines and requirements, not due to a potential infection. As a result, these repeat donors (RD) with cross-reactive results are removed from the donor base which could then result in preselection bias if we used RD for this cohort.

2.1.2.2. Cohort B

Cohort B consisted of reactive samples. Within this cohort 3 different sample populations were used, namely:

1. Contamination panel
2. WPBTS reactive donor samples
3. Procured samples

2.1.2.2.1. Contamination panel

A "contamination panel" consisting of 28 known positive plasma and twelve known negative plasma samples was run blind to control for possible carry-over from positive to negative samples. The panel contained samples positive for at least one of the viruses as determined by routine screening followed by confirmatory testing. The panel samples were numbered from A001 to A040 and were arranged in such a way that reactive samples would be processed directly after non-reactive samples and vice versa. The operator was blinded to the expected results until testing had been completed. The samples were handled using universal precautions. The samples were prepared, which included the making of DPS on cards, elutions and processed in numerical order from A001 to A040. Because only plasma samples were included in this panel only DPS was prepared. Reactive and non-reactive samples were prepared and processed on the same bench within one batch. In a real-world scenario results would not be available on donor samples prior to testing, therefore during this analysis reactive and non-reactive samples were handled in the same batch.

2.1.2.2.2. WPBTS donors found to be positive for viral markers on routine screening on the Panther analyser

This sample population consisted of 100 WPBTS donors found to be positive for viral markers on routine screening on the Panther analyser.

Due to the low prevalence of HIV, HBV and HCV among the donor population of WPBTS (799 donors out of 777 112 donations tested were found confirmed positive for any of the three viruses from 2013 until 2017), it is realistic to assume that most WPBTS donors will be negative for the routine viral markers. During the calendar year of 2017, 37 ND were found to be HIV positive (3 serology yields and 2 NAT yields, 32 concordant positive) 2 ND were found to be concordant HCV positive, and 72 ND were found to be HBV positive(5 serology yields, 2 NAT yields and 65 concordant positive). The average prevalence for all three markers for ND in 2017 is 0.032. In order to calculate diagnostic sensitivity, samples confirmed positive for one or more of the three viruses were included in the study. All confirmed concordant positive samples were confirmed by both serological screening on the cobas e602 system and the NAT testing on the Panther system. All confirmed NAT only positive samples were confirmed on the Panther system using samples of a different origin to exclude contamination.

2.1.2.2.3. Procured samples

Due to the low prevalence of the three viral markers in the donor population, three reactive validation plasma samples, with known viral loads, were procured for HIV-1, HBV, and HCV respectively from the *National Serology Reference laboratory (*Fitzroy, Australia*).* The objective of including these samples was to perform dilutions to determine the lowest level of detection/diagnostic sensitivity based on linear regression, as well as testing for stability after three weeks of storage.

2.2. METHODS

2.2.1. Study design

Nine hundred negative ND samples and one hundred confirmed positive donor samples, as defined by routine blood safety screening done at WPBTS, were additionally screened for viral nucleic acid using a dried blood spot kit. The confirmed positive samples had a variety of sample to cut-off (S/CO) values on both serology and NAT. After routine testing had been completed, one DBS sample and one DPS sample for each blood donor was prepared and analysed with the Ultrio Elite Assay on the Panther. Specificity was calculated based on Cohort A. Sensitivity was determined from the results of the reactive samples as well as the results of serial dilutions. The serial dilutions of procured samples with quantitative viral load values were used to calculate linearity and determine analytical assay sensitivity. Serial dilutions were made from at least one sample per analyte with known viral loads. Figure 2.3 illustrates the study design, algorithms used as well as the interpretation criteria.

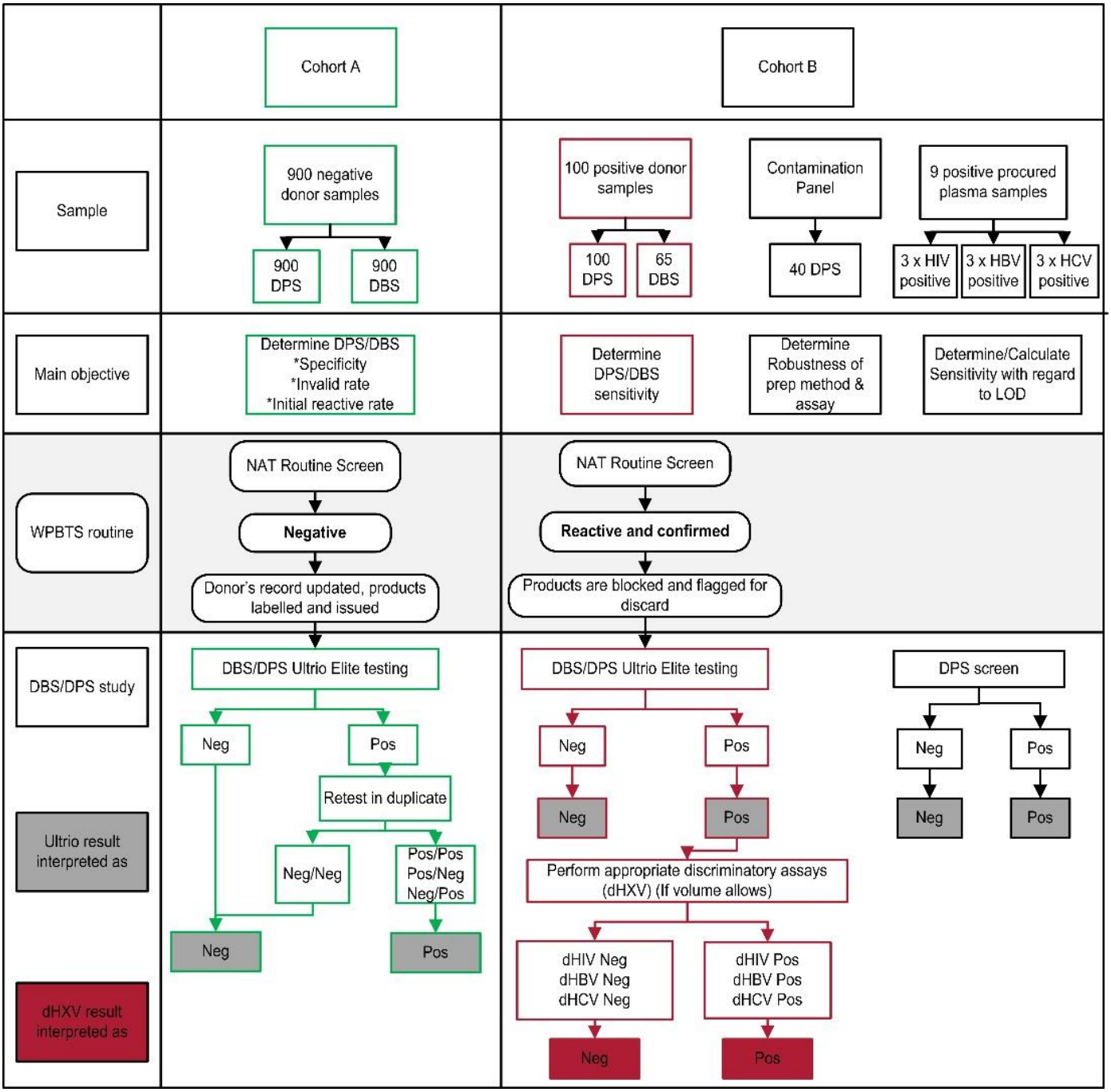


Figure 2.3. Flowchart of the study design, algorithms used and interpretation criteria

2.2.1.1. Specimen collection and preparation

Donors that present at a blood donation clinic are asked to complete the WPBTS Health and Lifestyle Questionnaire. Thereafter the WPBTS clinic staff evaluate the answers on the questionnaires, if the staff are satisfied with the questionnaire the donor is registered. Serial numbers are then placed on the relevant documentation and empty sample tubes. However, if the donor does not satisfy the donation questionnaire standards the donor will be deferred and no samples will be drawn. Point-of-care haemoglobin screening is performed on each registered donor by using the HemoCue machine (HemoCue AB, Ängelholm, Sweden). Only if the donor has a haemoglobin level of not less than 12.5 g/dℓ for females and not less than 13.5 g/dℓ for males, will the donor be allowed to donate (*Standards of Practive for Blood Transfusion in South Africa*, 2017).

Once a donor has been identified as being able to donate, serial numbers on the sample tubes are checked against the serial numbers on the Teruflex® blood pack (Terumo Penpol Private Limited, Thiruvananthapuram, India). The donor is then prepared for the donation event. The donor is identified by name and birthdate which is compared to the label on the questionnaire. The venepuncture site is cleaned with alcohol swabs and the needle is inserted into the donor's arm at an approximate angle of 25^{o} using an aseptic technique.

The pre-donation sampling pouch is filled first and clamped once it is appropriately filled. Three samples are drawn from the pre-donation sampling pouch of the Teruflex®. These samples are collected in ethylenediaminetetraacetic acid (K2EDTA) blood tubes manufactured by BD (Franklin Lakes, NJ USA). The Virology Department at WPBTS receives two of the three samples. The two Virology samples are identical 10 mℓ samples with the only difference being that the cap colour is different for these two samples: purple and gold. The purple-capped tube (K2EDTA) is used for the serological viral marker screens on the Cobas c8000 system (Roche Diagnostics, Mannheim, Germany). The gold-capped tube (K2EDTA) is used for the NAT on the Panther system (Grifols, CA, USA). After routine NAT testing the remainder of the sample was used for this study. Prior to routine NAT testing, samples are centrifuged for 15 minutes at between 4134 and 4231 gs depending on the make of the centrifuge which is consistent with the Ultrio Elite recommendations for sample preparation (Gen-Probe, 2015). Samples are then processed for routine testing, after which samples are kept upright in a cold room at 2-8 ^{o}C for up to 7 days. Within that period DBS/DPS were prepared for this study. The details and testing procedure flow chart is illustrated in Figure 2.4.

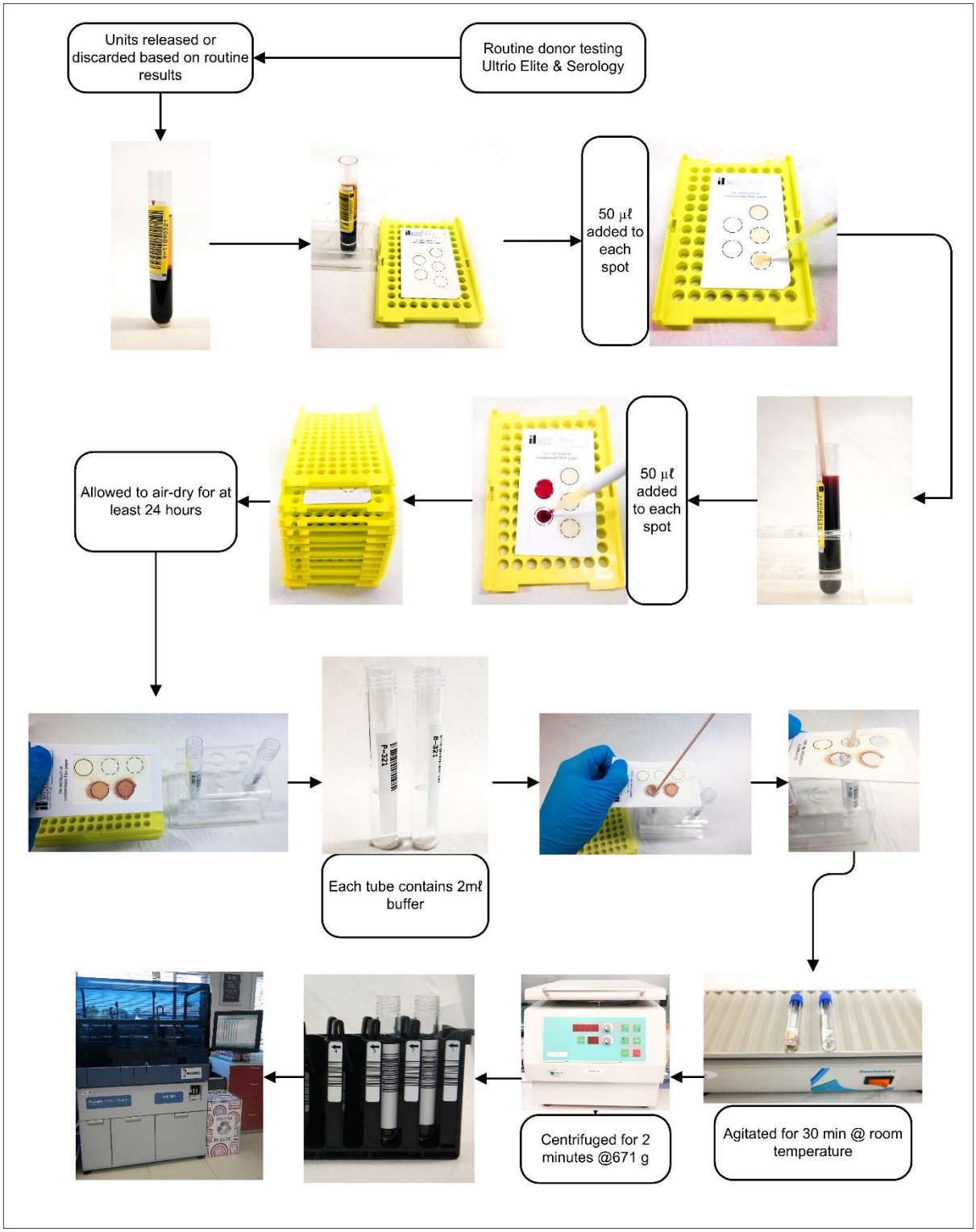


Figure 2.4. DBS/DPS sample preparation and testing flow chart.

2.2.2. DBS/DPS

2.2.2.1. Work area preparation

The laboratory bench areas where reagents were prepared were decontaminated daily with 0.5% sodium hypochlorite solution which was left on the surface for 15 minutes and then wiped down with water. A separate sample preparation area was established and decontaminated daily in the same manner as the reagent area.

2.2.2.2. Preparation

A 50 µℓ donor sample was added to each of the five spots present on the Ilex DBS spot collection card. The card is made of Munktell TFN filter paper (Ahlstrom-Munksjö, Stockholm, Sweden) and has four perforated 12 mm circles for the addition of samples. The fifth circle is not perforated and was kept in case of a discrepant result. Exactly 50 µℓ of plasma from the donor EDTA tube was pipetted onto each of the three of the five spots. Subsequently, the sample was mixed with wooden applicator stick and 50 µℓ whole blood was pipetted onto each of the other two spots. A calibrated precision pipette with disposable tips was used. The cards were placed on empty Panther disposable pipette tip trays as these trays could be stacked in a manner which enabled proper ventilation. The tray stacks were stable ensuring that the cards stacks did not fall over and contaminate each other. Samples were air dried for at least 24 hours prior to being eluted. During the drying process, the biological material binds to the card but the water molecules evaporate allowing proteins and nucleid acids, *inter alia*, to stabilise.

2.2.2.3. Elution of DBS/DPS

A 2000 µℓ of sample transport buffer (Gen-Probe, San Diego, CA, USA) was pipetted into a 5000 µℓ screw top plastic tube. The sample transport buffer was a 110 mM lithium lauryl sulphate (LLS) Buffered Solution ('GEN-PROBE ® APTIMA ® Adapter Kit', 2011). A calibrated precision pipette using disposable tips was used.

Serial numbers that correspond to the serial numbers of the samples on the cards were printed with either a B- or P- prefix denoting whether the sample was a dried blood or a dried plasma spot sample. Each card had both a P- and B- serial number. The corresponding set of serial numbers were then applied to the two plastic tubes containing the measured buffer solution. The corresponding spots were punched out straight into the tube containing the solution, using disposable wooden applicator sticks. The tube was capped using the screw cap and placed on a tube rocker, after which it was agitated for a minimum of 30 minutes at room temperature. Room temperature was controlled by a central air-conditioner and temperatures were digitally monitored to ensure a temperature between 15 ^{o}C and 25 ^{o}C. The tube rocker had a tilt angle range of 150 ^{o} to 210 ^{o} with a fixed speed of 24 rpm.

After incubation on the rocker, samples were centrifuged for two minutes at 671 g. Sample caps were removed by placing a piece of paper towel over the cap and then gently releasing the cap. The use of

a paper towel to open the sample cap prevents the formation of a possible aerosol and subsequent contamination of samples. Once the sample was uncapped, any bubbles present were removed by using a disposable plastic pipette. Thereafter samples were placed in the Panther sample racks with the barcode facing outward in order for the analyser to scan and record the serial number.

2.2.3. Panther

2.2.3.1. Analyser

The Panther analyser is a fully automated nucleic acid testing platform used for the NAT screening of individual donations at WPBTS.

Barcoded primary samples are loaded onto the Panther after which operator intervention is only required when the samples are removed. Wash Solution, Auto Detect 1, Auto Detect 2, Oil and Buffer for Deactivation (Universal Fluids) used by the analyser are labelled with RFID tags. Each component of the reagent and calibrator sets have unique barcodes that are read and recorded by the analyser providing complete traceability from sample to end result.

2.2.3.2. Procleix Ultrio Elite Assay

The Procleix Ultrio Elite (Grifols Diagnostic Solutions Inc, Spain) assay is a commercial nucleic acid test assay. It is a multiplex assay based on the transcription-mediated amplification principle. It has been designed to detect Human Immunodeficiency virus type -1 and -2 viral RNA, Hepatitis B viral DNA and Hepatitis C viral RNA in serum and plasma specimens from humans. The assay is a qualitative *in vitro* assay that is intended for blood screening of blood donors, of both live and cadaveric organ/tissue donors. Each result is validated by 1) calibration of the reagent set and 2) an internal control for which a result is generated simultaneously. Only if the internal control has the expected result will the sample test result be deemed as valid. The internal control on each individual specimen confirms that no inhibition took place during the testing process. The calibrator set consists of a negative calibrator (run in triplicate), HIV Positive calibrator (run in duplicate), Hepatitis C Positive calibrator (run in duplicate), and a Hepatitis B calibrator (run in duplicate). Calibration for each reagent set is only valid for 24 hours, after which a new calibration has to be performed if test reagents are still available on the reagent set.

Each test kit of 250 tests consists of:

1.) Internal control reagent (IC)

    A hydroxyethyl-piperazineethane-sulfonic acid (HEPES) buffered solution containing detergent and an RNA transcript

2.) Target capture reagent (TCR)

    A HEPES buffered solution containing detergent, capture oligonucleotides and magnetic microparticles

3.) Amplification reagents (AMP)

Primers, Deoxynucleotides (dNTPs), Nucleoside triphosphates (NTPs) and co-factors in trisaminomethane (TRIS) buffered solution containing ProClin 300 as a preservative

4.) Enzyme reagent

MMLV reverse transcriptase and T7 RNA polymerase in HEPES/TRIS buffered solution containing 0.05% sodium azide as preservative

5.) Probe reagent

Chemiluminescent oligonucleotide probes in succinate buffered solution containing detergent

6.) Selection Reagent

Borate buffered solution containing a surfactant

7.) Target enhancer reagent (TER)

A concentrated solution of lithium hydroxide

(Gen-Probe, 2015)

2.2.3.3. Principles

The Procleix Ultrio Elite assay process can be described in three main steps.

1) Sample preparation by means of target capture resulting in isolation and purification of the viral nucleic acid within the sample.
2) Amplification by means of transcription-mediated amplification resulting in the amplification of target nucleic acid sequences to produce multiple copies of RNA.
3) Detection by means of hybridization protection assay whereby the amplified nucleic acid signal is detected using light-emitting nucleic acid probes.

2.2.3.3.1.1. Sample preparation/Target Capture

The Panther analyser takes a multi-tube unit (MTU) from the input queue and puts it in the sample dispense station. These MTU's are 5 individual tubes that are moulded to form a single unit. In the sample dispense station, 400 µℓ working Target capture reagent (wTCR), which is the TCR and the IC reagent combined, containing the capture oligonucleotides, is added to each individual tube of the MTU. The capture oligonucleotides are short strands of ssDNA of specific base-pair sequence, i.e. the base pairs are *complementary* to the target viral sequence which targets a conserved region within each of the 3 viruses. Only one disposable pipette tip is used during the dispensing of wTCR into the five tubes of the MTU. 500 µℓ of the cooled sample is then added to an individual MTU tube using a disposable pipette tip for the addition of each sample. Thereafter wTCR and sample volume verification is performed to ensure that the exact amount of each was added to the tube. The sample is moved to the mixing station to ensure adequate mixing of the constituent fluids. The MTU is then moved to the high-temperature incubator. The temperature within this incubator is 64 ^{o}C. Detergent, within the TCR, lyses viral particles releasing nucleic acid which is then available for hybridization. At this stage, both target

sequences and the internal control RNA transcript are hybridized with matching oligonucleotides. Each of the oligonucleotides has a Poly-A tail, which allows for magnetic microparticles, with a poly-T tail, to bind to the oligo-target nucleic acid complexes when the sample is moved to the transition incubator.

The MTU is then cooled in a chiller ramp where magnets pre-capture the magnetic beads to the side of the MTU. Once the magnetic bead has captured the targets the MTU move to the magnetic wash station. In the Mag wash station, a magnetic force is applied and residual fluids are aspirated. The force is then removed and the wash solution is added, the sample is then mixed and the process repeated, resulting in purified target nucleic acid. After completion of the wash, 200 µℓ of oil is dispensed to minimize evaporation during the rest of the assay process.

2.2.3.3.1.2. Amplification

After the completion of the mag wash and the addition of oil, the MTU is moved to the AMP load station where 75µℓ of amplification reagent is pipetted into each tube.

The samples are then mixed and moved to the High-temperature incubator where the secondary structure of the RNA/DNA is disrupted and the primers anneal. The high temperature, 64^{o}C, promotes binding the primers to the target nucleic acid in this assay as the oligonucleotides contained within this assay is designed to optimally bind at 64 ^{o}C. Thereafter the MTU is moved for cooling to the transition incubator prior to enzyme reagent being added. The MTU is then moved to the AMP load station where 25 µℓ Enzyme reagent is pipetted into the sample. The MTU is mixed by means of an orbital mixer and transferred to the AMP/RT incubator for amplification at 42.7^{o}C. The amplification process uses the transcription mediated amplification (TMA) principle. The process initially differs for RNA and DNA target sequences but then follows the same loop after a dsDNA template which includes the T7 promoter has been created.

For RNA (HIV, HCV and IC): Primer 1, which includes a T7- promoter sequence, binds to the target allowing Reverse Transcriptase (RT) to create the first strand of cDNA, resulting in an RNA: DNA complex. RT degrades the target RNA by means of its RNase H activity which results in single-stranded cDNA. The second primer then binds to the cDNA allowing RT to create a second strand of cDNA. A dsDNA template including the T7-promoter sequence is produced.

For DNA (HBV): The T7 primer and displacer primer bind to the target. Thereafter RT extends the displacer primer and T7 primer. The Displacer primer elongation displaces the T7 DNA primer product, resulting in a T7 ssDNA. Primer 2 binds to the T7 DNA product and primes the second strand DNA. A dsDNA template including the T7-promoter sequence is now available for amplification.

Both RNA and DNA processes result in ds DNA templates including the T7 promoter sequence, RNA polymerase initiates transcription of RNA from a DNA template resulting in 100 to 1000's of RNA transcript. Primer 2 binds to RNA amplicon and creates the first strand of the new cDNA resulting in the completion of the RNA:DNA complex, after which the RT degrades the target RNA by means of its RNase H activity which results in a single-stranded cDNA. Primer 1 including the T7-promoter binds

and RT creates new double-stranded cDNA, the amplicon. The amplicon then becomes the new template and the process repeats itself until the reagent supply is exhausted. The amplified product is called the amplicon which can now be hybridized with the labelled nucleic acid probe in the hybridization protection assay.

2.2.3.3.1.3. Hybridization Protection Assay (HPA)

During the HPA the single-stranded amplicon from TMA is hybridised with a single-stranded nucleic acid probe that is labelled with an acridinium ester molecule. The sequence of the labelled nucleic acid probe complements the target sequence. The analyser moves the MTU to the HPA load station. 100 µℓ of probe reagent is dispensed into the tube and the tube content is mixed. Since the labelled probe complements the target sequence, it will bind if the target sequence is present. The MTU is then moved to the high-temperature incubator for hybridization. Once hybridization is complete the MTU is moved to the HPA load station where 250 µℓ Selection reagent is added, and then to the high-temperature incubator for the selection process. During the selection process, the un-hybridized AE is hydrolysed from any unbound probe as these AE are not "protected" within a double-stranded helix. As soon as the selection process has been completed the system dispenses Auto detect 1 and Auto detect 2 which react with the AE, emitting photons. The light-signal is read by the luminometer if a light signal is present. The sample results are validated by the dual kinetic assay. This technology allows the analyser to differentiate between kinetic profiles of the labelled probes. The IC, for example, is labelled with very rapid kinetics producing a rapid emission of light called a "flasher" whereas the sample kinetic profile is much slower thus producing relatively slower emission of light called a glower. A negative sample result will have a "glower" reading, while a reactive sample will have both a "flasher" and a "glower" reading.

2.2.3.4. Interpretation of results

Panther results are reported as sample to cut-off (S/CO) values and all calculations are performed by the software on the Panther analyser. For each analyte two cut-offs are determined during calibration, one for the analyte (produced by the glower) and one for the internal control of the sample (flasher). The analyte value is reported in relative light units (RLU) which are then converted to S/CO based on the cut-offs determined by calibration. An S/CO value equal to or greater than 1 is considered as reactive.

2.2.3.5. Confirmation of reactive results

2.2.3.5.1. Cohort A

Once a DBS sample result was found to be initially reactive, the DBS sample was retested in duplicate. If only 1 of the 3 results were found to be reactive the sample was considered as Initial-Reactive/Non-Repeat-Reactive (IR/NRR). No further testing was performed, however, for statistical calculations, these results were interpreted as negative. However, if two or all three results were found to be reactive, the sample was considered reactive and discriminatory (dHXV) testing was performed to identify the

virus present in the donor's blood sample. This testing algorithm is similar to the testing algorithm used routinely in WPBTS virology laboratory, a simplified version of which is illustrated in Figure 2.2. If the results obtained on the DPS/DBS and the original sample results were discrepant a fresh spot would have to be made, however, if the test result of the fresh spot correlates with the original sample's results no further testing would have been required. The interpretation of Cohort A results is also illustrated in Figure 2.3.

2.2.3.5.2. Cohort B

Plasma samples were confirmed reactive by routine NAT testing and re-testing of the plasma bag.

DBS/DPS samples were considered reactive if the sample was initially reactive. Discriminatory (dHXV) testing was performed on these samples, where volume allowed, to determine the virus present. The interpretation of Cohort B results is also illustrated in Figure 2.3.

.

2.2.3.6. Data processing and storage

De-identified DPS and DBS sample results from the Panthers were transmitted to eL@bs (PANTHER middleware) which served as a database for all DBS/DPS and NAT test results. Results could be distinguished based on the prefixes. The original plasma sample was denoted with an N- prefix, the DBS sample with a B-, and the DPS sample with a P-.

2.2.4. Statistical analysis

Statistical analysis was performed to compare the results from the original plasma sample versus the DPS/DBS sample.

Analysis of the 3 sets of results included:

- Sensitivity/Specificity
- Positive and negative predictive values (false positive/false negative)
- Statistical significance of any differences measured using concordance analysis which includes:
  - Cohen's Kappa coefficient (Stata version 15)
  - Or Bland Altman where relevant (Stata version 15)
- Calculating the lower level of detection for each virus

CHAPTER THREE

3. RESULTS

In this study, two different cohorts were used to evaluate the suitability of DBS and DPS as samples for routine screening of blood. Cohort A consisted of New Donors (ND) who tested negative for HIV, HBV, and HCV genome on the Panther Ultrio Elite during routine screening performed on a plasma sample. Cohort B consisted of samples that had been confirmed positive for at least one of the HIV, HBV, or HCV viruses.

3.1. COHORT A

Nine hundred screening-negative DBS and DPS samples were tested. The results of these tests are summarized in

Table *3.1*.

Table 3.1. Ultrio Elite results for DBS/DPS samples prepared from 900 screening negative samples.

	DBS	**DPS**
Number of samples found non-reactive	898/900	900/900
Number of samples found initially reactive	2/900	0/900
Number of samples found repeat reactive	0/2	N/A
Number of samples found initially invalid	5/900	0/900
Number of initially invalid samples found non-reactive on repeat testing.	5/5	N/A

Of the 900 known non-reactive DBS samples tested on Ultrio Elite, two tested initially reactive. When retested, as per algorithm, these two samples tested non-reactive. These two samples were therefore re-classified as non-reactive in accordance with the algorithm used in this study. The sample to cut-off (S/CO) values of the repeat tests were used for further calculations. In addition, with the inclusion of the repeat tests, all Cohort A DBS samples were regarded as non-reactive resulting in a 100% specificity (95% confidence limit (CL): 99.59% to 100.00%) for the Ultrio Elite.

Five of the 900 DBS samples were found initially invalid due to the internal control (IC) failing. On repeat testing valid non-reactive results (i.e. the internal controls did not fail) were obtained on these five samples.

All 900 known non-reactive DPS samples tested on Ultrio Elite were found to be non-reactive, resulting in a 100% specificity (95% CL: 99.59% to 100.00%).

3.1.1. S/CO values

On average, DBS samples had a higher and a wider distribution of S/CO values than both DPS and plasma samples. Comparing the mean S/CO values for the plasma and DBS samples a bias (-0.117) existed and results were not all within the 95% limits of agreement. On the other hand, when comparing the mean S/CO values for the plasma and DPS samples no bias (0.00) existed and most results were within the 95% limits of agreement. In addition, the difference between the mean S/CO values of DPS and DBS samples was significant. These results are illustrated in *Figure 3.1*, Figure 3.2, and Figure 3.3

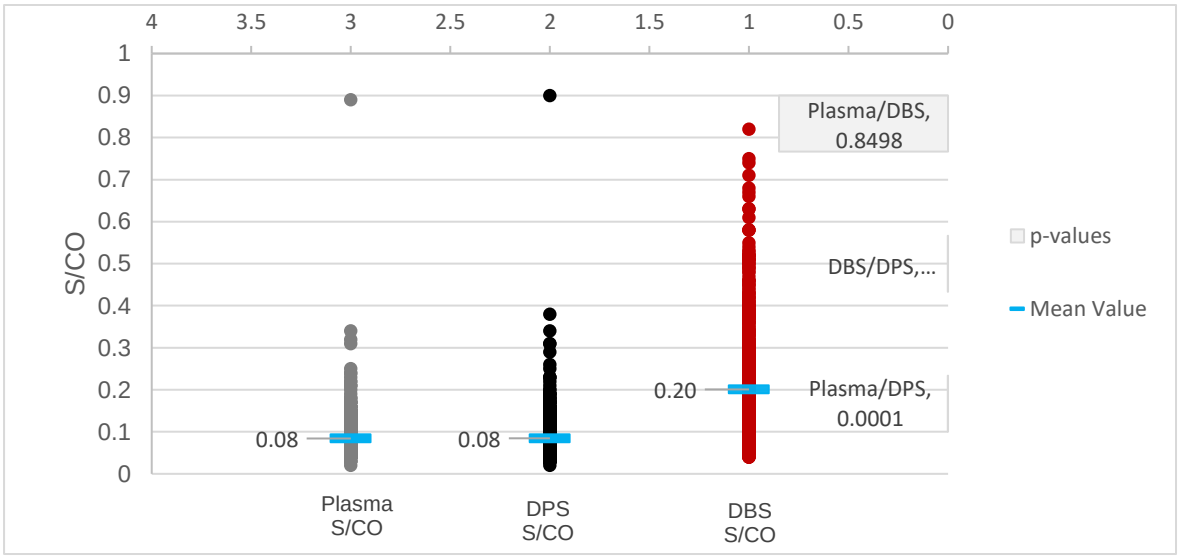


Figure 3.1. Distribution of S/CO values of negative plasma, DBS, and DPS samples tested. The mean and p-values are included.

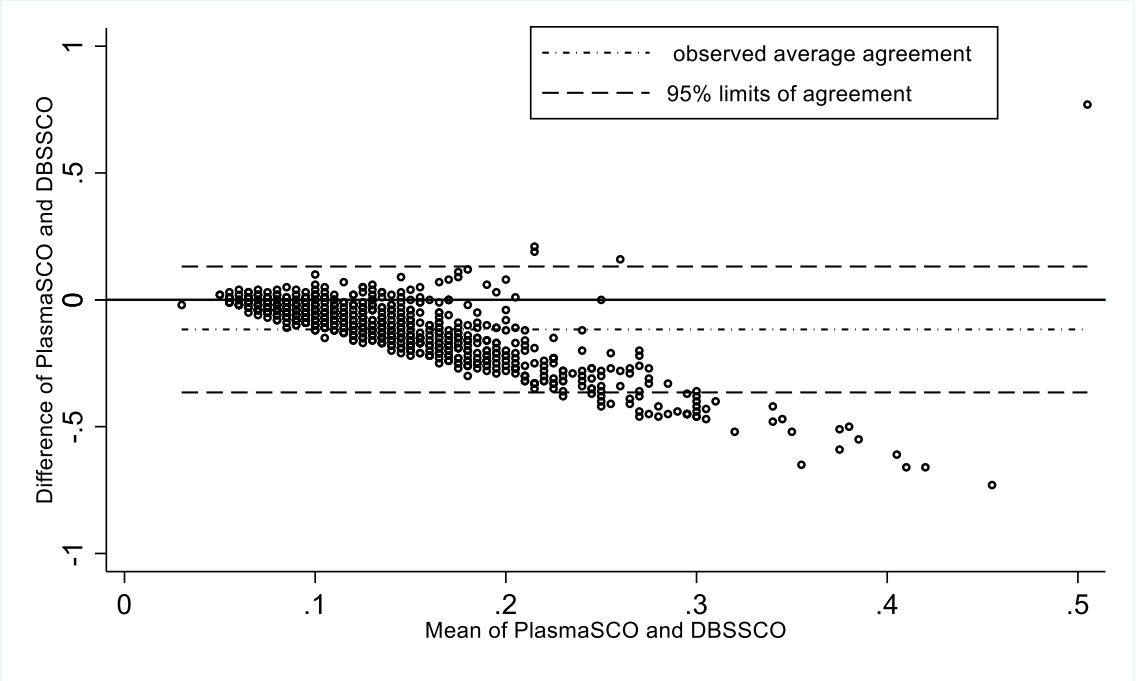


Figure 3.2. The Bland Altman chart plotting the difference between the plasma S/CO values and DBS S/CO values versus the mean of the plasma S/CO AND DBS S/CO values.

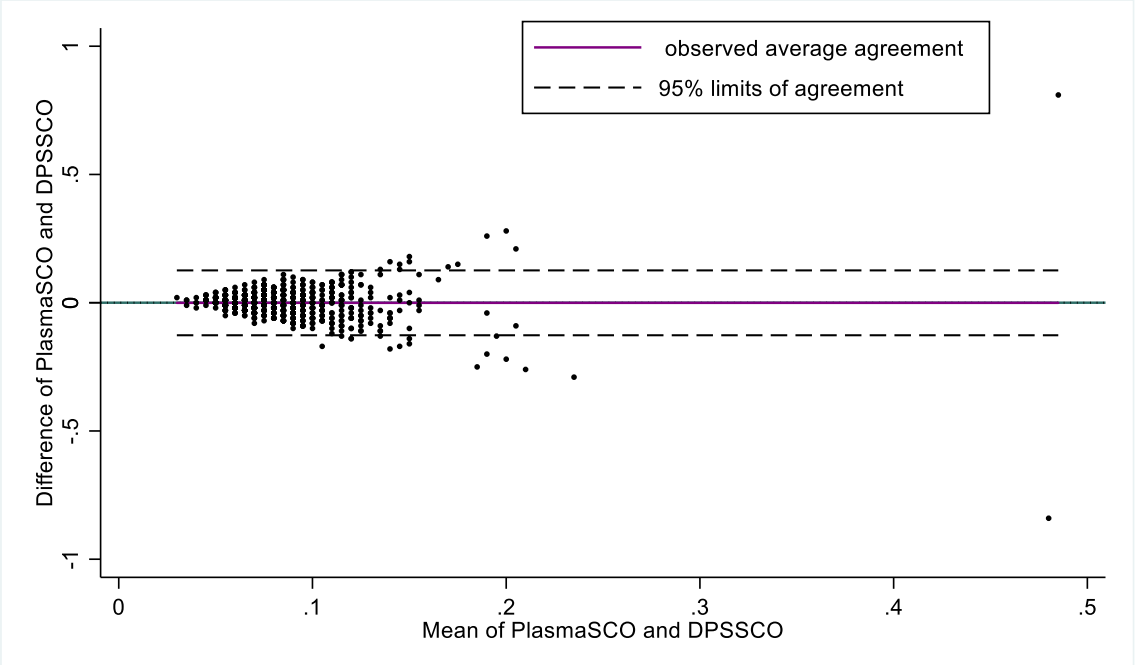


Figure 3.3. The Bland Altman chart plotting the difference between the plasma S/CO values and DPS S/CO values versus the mean of the plasma S/CO and DPS S/CO values.

3.1.2. Negative Predictive Values

Negative predictive values (NPV) for both DBS and DPS samples were 100% when calculated based on Cohort A. However, to be meaningful they needed to be re-calculated taking real-life donor prevalences in our region into account. The average prevalence of Hepatitis B, Hepatitis C, and HIV combined within the WPBTS blood donor base has been 0.038 for the past 5 years (2013 - 2017). This value is based on viral infection defined as a donor presenting

1. both NAT and serologically reactive for the appropriate virus (concordant),
2. NAT and discriminatory test reactive for the appropriate virus (NAT yield),
3. and serologically reactive (serological yield), with confirmatory serological test reactive and Western blot or immunoblot reactive.

In the last 5 years most reactive cases results were concordant (90%), followed by NAT yields (7%), and lastly serological yields (3%). The NAT yield consisted of window period (WP) samples (HIV and HBV) and occult HBV infections. Serological yields consisted of HIV elite controllers/donors on antiretroviral therapy as well as chronic HBV infections.

Using the NPV formula based on Bayes' theorem,

$$NPV = \frac{Specificity \times (1 - Prevalence)}{(1 - sensitivity) \times prevalence + specificity \times (1 - prevalence)}$$

and using the WPBTS donor average prevalence, the NPV would be 99.99 % for both DBS and DPS.

3.2. COHORT B

3.2.1. Contamination Panel

The contamination panel used consisted of 40 well characterized donor samples, 28 of which were known positive plasma samples and twelve that were known negative plasma samples. Contamination panel results are summarized in Addendum B. No contamination was observed. All negative samples tested negative as expected; however, three positive samples had false negative test results.

3.2.2. Donor samples

A hundred DBS and DPS samples were prepared from confirmed reactive donor samples from WPBTS.

3.2.2.1. HIV

Forty confirmed HIV-1 reactive samples were available for testing as DBS and DPS samples. Ten of these were available as plasma samples only and 30 as whole blood samples. Hence only 30 DBS samples could be prepared and tested while 40 DPS samples were available. The HIV test results of these samples are shown in Table 3.2 Note that the DBS false non-reactive sample was not the same sample as the DPS sample found to be false non-reactive. The HIV-1 sensitivity on plasma samples claimed for the Ultrio Elite assay is 100% (95% CL:98.3% to 100%) (Gen-Probe, 2015).

Table 3.2. : Summary of the 30 DBS and 40 DPS test results for the HIV reactive donor samples tested

Sample type	**Matched samples**		**Additional samples**
	DBS	**DPS**	**DPS**
Number of samples that tested reactive on Ultrio Elite	29/30	30/30	9/10
Number of samples that tested reactive on discriminatory assay for HIV-1/2 (dHIV)	29/29	29/29	9/10

Of the known HIV reactive samples 30 were tested as DBS. Twenty nine DBS samples were found to be reactive and one non-reactive, resulting in a HIV sensitivity for the Ultrio Elite using DBS samples of 96.67% (95% CL 82.87% to 99.92%). All 30 samples were also tested with dHIV, including the one that tested negative on the Ultrio Elite assay (outside of this study setting this sample would not have been included for the dHIV testing). All 30 samples tested reactive on dHIV.

Of the known HIV reactive samples 40 were tested as DPS. Thirty nine DPS samples were found to be reactive and one non-reactive, resulting in a HIV sensitivity for the Ultrio Elite using DPS samples of 97.50% (95% CL: 86.84% to 99.94%).

One sample, which was prepared as both DBS and DPS, was a window period sample. The window period sample had an HIV viral load of 45356 cps/mℓ but no detectable antibodies on the index sample. This sample was found to be reactive on both DBS and DPS. Approximately one month later, a follow-up sample from the donor showed they had developed Anti-HIV.

3.2.2.2. HBV

Fifty confirmed HBV reactive samples were available for testing as DBS and DPS. All 50 of these were available as plasma samples, but only 33 were available as whole blood samples. Hence, only 33 DBS samples could be prepared and tested, compared to the 50 DPS samples which could be prepared and tested. The HBV test results of these samples are shown in Table 3.3

The HBV sensitivity on plasma samples claimed for the Ultrio Elite assay is 100% (95% CL: 98.3% to 100%) (Gen-Probe, 2015).

Table 3.3. Summary of the 33 DBS and 50 DPS test results of HBV reactive donor samples.

Sample type	**Matched samples**		**Additional samples**
	DBS	**DPS**	**DPS**
Number of samples that tested reactive on Ultrio Elite	19/33	19/33	10/17
Number of samples that tested reactive on discriminatory Assay for HBV (dHBV)	18/19	19/19	6/10

Thirty-three known HBV reactive DBS samples were tested on Ultrio Elite, 19 of which were found to be reactive and 14 non-reactive. These results indicate a HBV sensitivity for the Ultrio Elite using DBS samples of 57.58% (95% CL: 39.22% to 74.52%). The 19 reactive DBS samples were further tested on the dHBV and 18 tested reactive for dHBV.

Fifty known HBV reactive DPS samples were tested on Ultrio Elite, 29 of which tested reactive and 21 non-reactive. These results indicate a HBV sensitivity for the Ultrio Elite using DPS samples of 58.00% (95% CL: 43.21% to 71.81%). The 29 reactive DPS samples were further tested on the dHBV. Of the samples tested on the dHBV, 25 were found to be reactive and 4 non-reactive.

Both the DBS and DPS samples tested on Ultrio Elite showed a statistically significant reduction in sensitivity compared to the plasma samples. Below is a table (Table 3.4) showing the S/CO values for each of the tests from the same sample source, with reference to any additional confirmatory tests that were performed.

Table 3.4. S/CO values for DBS and DPS samples that tested non-reactive for well characterised HBV reactive samples.

Serial Number	Plasma S/CO	DBS S/CO	DPS S/CO	HBsAg Serology S/CO	Supplementary test results
2	7.14	0.06	0.05	9.28	Confirmed Reactive
3	13.23	0.14	0.04	42.2	Confirmed Reactive
4	14.81	0.17	0.2	0.773	Confirmed Reactive
8	13.64	0.12	0.05	3.7	Confirmed Reactive
9	14.09	0.07	0.12	3856	Viral Load Detected LDL <116 cps/mL
18	14.97	0.07	0.12	30.58	Confirmed Reactive
20	13.06	0.08	0.04	6834	Confirmed Reactive
21	13.45	0.13	0.02	7427	Confirmed Reactive
23	13.20	0.26	0.1	1587	Viral Load Detected LDL <116 cps/mL
25	9.36	0.1	0.06	0.572	Confirmed Reactive
28	13.29	0.12	0.34	0.574	Confirmed Reactive
29	14.78	0.09	0.11	2355	Confirmed Reactive
32	14.15	0.07	0.05	3608	Confirmed Reactive
33	13.60	0.12	0.04	4879	Confirmed Reactive
11	14.22	13.24	0.05	2236	Confirmed Reactive
36	13.66	NT	0.12	7014	Viral Load Detected LDL <116 cps/mL
45	13.74	NT	0.1	0.593	Confirmed Reactive
46	13.38	NT	0.13	1255	Viral load 361 cps/mL
48	14.11	NT	0.13	0.538	Confirmed Reactive
49	14.65	NT	0.1	0.545	Confirmed Reactive
50	14.16	NT	0.1	0.593	Confirmed Reactive

3.2.2.3. HCV

Ten known HCV reactive samples were available for testing. All ten samples were reactive for both anti-HCV and NAT. No window period samples were available since the WPBTS donor population has a very low HCV prevalence (0.03%) and has not seen a donor during the HCV window period since the start of NAT testing in 2005. The HCV test results of these samples are shown in *Table 3.5*. The HCV sensitivity on plasma samples claimed for the Ultrio Elite assay is 100% (95% CL: 98.2% to 100%).

Table 3.5. *Summary of the two DBS and ten DPS test results of HCV reactive donor samples*

Sample type	**Matched samples**		**Additional samples**
	DBS	**DPS**	**DPS**
Number of samples that tested reactive on Ultrio Elite	2/2	2/2	8/8
Number of samples that tested reactive on Discriminatory Assay for HCV (dHCV)	2/2	2/2	7/7

Two known HCV reactive DBS samples were tested on Ultrio Elite and both were found to be reactive. This small sample size resulted in an HCV sensitivity for the Ultrio Elite using DBS samples of 100% (95% CL: 15.81% to 100.00%). Both DBS samples were as per algorithm further tested on dHCV and found to be positive.

Ten known HCV reactive DPS samples were tested on Ultrio Elite were all found to be reactive. The resulting HCV sensitivity for the Ultrio Elite using DPS samples was calculated to be 100% (95% CL: 69.15% to 100.00%). Of the 10 DPS reactive samples, 9 were further tested on the dHCV (the tenth DPS sample was excluded due to insufficient sample volume) and found to be reactive.

3.2.3. Procured samples

Three reactive validation plasma samples, with known viral loads, were procured for HIV-1, HBV, and HCV respectively from the *National Serology Reference laboratory (*Fitzroy, Australia*).* The samples were serially diluted with saline until non-reactive values were obtained. The objective of the dilutions were to determine analytical sensitivity and to calculate the lowest level of detection (LOD) for each virus using Ultrio Elite and DPS as sample type. A further objective was to test for stability of the DPS samples after three weeks of storage.

3.2.3.1. Results of samples tested within a week of preparing the sample on the DPS cards.

3.2.3.1.1. HIV

The reference HIV viral load results supplied by the National Serological Reference Laboratory (NRL) were 101 000, 193 000, 326 000 cps/mℓ for the three procured samples respectively. Addendum D and E give an overview of the S/CO results obtained for each dilution. The dilution factor was calculated by using the buffer volume divided by the amount of plasma that was added to the DPS. This factor can be used to calculate the viral load present in the DPS eluted sample.

When using the Ultrio Elite assay the lowest S/CO that would be interpreted by Panther as reactive is 1.00. Lowest level of detection (LOD) calculations were based on the linear regression formula derived from the scatter plot of the serial dilutions. The best fit linear equation was determined to be,

$$y = 4.3725x - 9.745$$

where x represents the log of the viral load and y represents the S/CO value. Therefore, when the S/CO value is 1.00 the linear relationship determines that the log of the viral load will be 2.09 cps/mℓ. We thus conclude that theoretically for a DPS sample the assay's LOD is 124.11 cps/mℓ present in the plasma sample. This means that the LOD for viral load within the eluted DPS sample is theoretically 6.21 cps/mℓ.

The lowest viral load that tested reactive using a DPS sample was 376.95 cps/mℓ in the plasma sample with only 18.85 cps/mℓ present in the elution that was tested. The levels of detection calculated in this study must be used with caution since these calculations were based on 30 data points derived from dilutions from only three unique samples, rather than from a comprehensive study involving probit analysis of multiple repeats at various viral loads.

3.2.3.1.2. HBV

The reference HBV viral load results supplied by the National Serological Reference Laboratory (NRL) were 135 520 000, 110 320 000, 868 000 000 cps/mℓ for the three procured samples respectively. Addendum F and G give an overview of the S/CO results obtained for each dilution. Results of the doubling serial dilutions are stipulated in Addendum F, however, due to the fact that no non-reactive S/CO was reached, new serial dilutions were made, using ten-fold dilutions. Results for the ten-fold serial dilutions are listed in Addendum G. Samples were tested in duplicate. Addendum H illustrates the results of both the HBV positive two-fold dilutions as well as the ten-fold dilutions.

Lowest level of detection (LOD) calculations were based on the linear regression formula derived from the scatter plot of the serial dilutions. The best fit linear equation was determined to be,

$$y = 2.5429x - 3.6656$$

where x represents the log of the viral load and y represents the S/CO value. Therefore, when the S/CO value is 1.00 the linear relationship determines that the log of the viral load will be 1.83 cps/mℓ. We thus conclude that theoretically for a DPS sample the assay's LOD is 68.32 cps/mℓ present in the plasma sample. This means that the LOD for viral load within the eluted DPS sample is theoretically 3.42cps/mℓ. These LOD were calculated using linear regression and are based on 48 data points derived from dilutions of 3 different samples.

3.2.3.1.3. HCV

The reference HIV viral load results supplied by the National Serological Reference Laboratory (NRL) were 1067800, 4598000, 2128000 cps/mℓ for the three procured samples respectively. Addendum I and J give an overview of the S/CO results obtained for each dilution.

LOD calculations were based on the linear regression formula derived from the scatter plot of the serial dilutions. The best fit linear equation was determined to be,

$$\mathrm{y} = 3.663\mathrm{x} - 12.63$$

where x represents the log of the viral load and y represents the S/CO value. Therefore, when the S/CO value is 1.00 the linear relationship determines that the log of the viral load will be 3.04 cps/mℓ. We thus conclude that theoretically for a DPS sample the assay's LOD is 1096.48 cps/mℓ present in the plasma sample. This means that the LOD for viral load within the eluted DPS sample is theoretically 54.82 cps/mℓ.

The Ultrio Elite package insert claims a detection limit (95% confidence limit) of 11.4 cps/mℓ. If detection limit is applied to the elution (using a dilution factor of 20) this can be converted into an HCV viral load of 228 cps/mℓ in the plasma sample prior to the preparation of the DBS/DPS. These LOD were calculated using linear regression that is based on 30 data points derived from dilutions of 3 different samples.

3.2.3.2. Result samples tested 3 weeks after spotting the sample on the DPS card.

As previously stated, one of the objectives of this study was to determine stability of DPS samples after three weeks of storage. In aid of this, a second undiluted HIV, HBV & HCV DPS card was prepared simultaneously as the first, but only tested 3 weeks later. The DPS card was kept at room temperature on the bench during these weeks.

*Table 3.6*Table 3.6 below the S/CO values obtained from the first test, done within one week after the DPS sample preparation and referred to as Test 1, and the second test, done after three weeks of

storage and referred to as Test 2, are listed.

Table 3.6. Comparison of S/CO values obtained for the DPS samples within one week (test 1) and three weeks (test 2) after preparation

	S/CO of Test 1	**S/CO of Test 2**
HIV	9.88	10.37
HBV	14.78	14.56
HCV	9.69	9.58

Comparing the S/CO values of Test 1 and Test 2, a 99.3% correlation was calculated, with an average S/CO difference between the two test sample sets of 0.02. Furthermore, the S/CO values for Test 1 and Test 2 were found not to be statistically significant (Student's t-test, $p > 0.05$).

The stability results within this study must be used with caution since these were based on three sample sets.

3.3. SUMMARY

In *Table 3.7* below, the specificity, sensitivity, NPV, PPV, and accuracy results obtained in this study for each cohort, and the combined cohorts where applicable, are tabulated to provide a summarised overview.

Table 3.7. Summary of specificity, sensitivity, NPV, PPV, and accuracy results for cohorts A and B

	DBS		DPS	
	n=	%	n=	%
Specificity				
Cohort A	900	100%	900	100%
Sensitivity				
Cohort B				
HIV	30	96.67%	40	97.50%
HBV	33	57.58 %	50	58 %
HCV	2	100 %	10	100 %
NPV				
Cohort A	900	100 %	900	100 %
HIV	30	99.99 %	40	99.99 %
HBV	33	98.46 %	50	97.72 %
HCV	2	100 %	10	100 %
Total	65	98.36 %	100	97.61 %
Overall NPV *Taking WPBTS donor prevalence into account*	965	99.99%	1000	99.99%
PPV				
Cohort B				
HIV *WPBTS donor prevalence 0.041%*	30	96.67%	40	97.50%
HBV *WPBTS donor prevalence 0.060%*	33	57.58 %	50	58 %
HCV *WPBTS donor prevalence 0.03%*	2	100 %	10	100 %
Total	65	76.92%	100	78.00%
Overall PPV *Taking WPBTS donor prevalence into account*	965	99.99 %	1000	99.99 %
Overall				
Accuracy	965	98.45%	1000	97.80%
Cohen's Kappa coefficient	965	0.8615	1000	0.8645

CHAPTER FOUR

4. DISCUSSION

The collection of blood donations varies tremendously from high-income to low-income countries. Low-income countries are often unable to meet the transfusion demand (WHO, UNICEF and UNAIDS, 2011). Of the blood donations collected in SSA only 85.5% (Tapko, Toure and Sambo, 2014) of these are screened, by means of serological, NAT, and/or rapid diagnostic testing (RDT), for transfusion transmitted diseases (TTDs).

4.1 BLOOD SCREENING

Ideally all blood donations should be screened for TTD. At a minimum, these tests should include Anti-HIV, HBsAg, Anti-HCV and syphilis testing. Where feasible, additional markers should be added, tailored to the country and their donor population's epidemiological situation. In particular, the nucleic acid test (NAT) for HIV, HBV and/or HCV should be included where possible. (World Health Organization, 2010)

4.2 DBS/DPS

DNA extraction from blood spots dried on filter paper was first described in 1987 (McCabe *et al.*, 1987). The first HIV-RNA viral extraction from a DPS sample was described in 2003 (Brambilla *et al.*, 2003). Since these initial descriptions many publications evaluating the stability, suitability, reduction in cost, and ease of use of DBS/DPS samples for NAT have been published. Results from these publications indicate that using DBS/DPS samples for screening and testing is comparable to using traditional plasma samples.

DBS samples are being used successfully in the early infant diagnosis (EID) programs for HIV by means of PCR testing. This advance in EID is especially relevant SSA where cold chain management of sample shipment and sample storage are challenging, and has enabled millions of children to be screened for HIV-RNA(Essajee, 2013). This example of a successful, cost effective intervention using DBS is a motivator to do more studies for the feasibility of applying DBS/DPS in order to improve access to medical services with SSA.

4.3 FINDINGS

During this study, 900 non-reactive and 100 reactive plasma samples were prepared as DBS and DPS samples. These samples were tested on the Panther analyser using the Ultrio Elite assay. The results were compared to the standard routine test results to determine sensitivity, specificity, and utility of DBS- and DPS-testing in a resource constraint blood transfusion setting.

Sensitivity is defined as the "*diagnostic sensitivity; the conditional probability that a person having a disease will be correctly identified by a clinical test, i.e., the number of true positive results divided by the total with the disease (which is the sum of the numbers of true positive plus false negative results). See also specificity."* (*Miller-Keane Encyclopaedia and Dictionary of Medicine, Nursing, and Allied Health*. 7th edn, 2003).

Specificity is defined as "*the probability that a person not having a disease will be correctly identified by a clinical test, i.e., the number of true negative results divided by the total number of all those without the disease (which is the sum of the number of true negative plus false positive results)."* (*Miller-Keane Encyclopaedia and Dictionary of Medicine, Nursing, and Allied Health*. 7th edn, 2003)

Overall screening sensitivity was found to be reduced when using both DBS and DPS samples. Screening specificity was comparable to routine screening but additional result interference was observed with the DBS samples.

4.3.1 Sensitivity

The sensitivity of a screening assay is one of the most important considerations in a blood transfusion setting. Acquiring a TTD could result in disease or even death of the transfusion recipient. Furthermore, it is important to identify a donor as being infected with a virus. The WHO Regional office for Africa compiled a strategy in 2001 to improve blood safety by improving both the quality and adequacy of blood products (Tapko, Toure and Sambo, 2014). One of the main objectives was a reduction of transfusion-acquired infections. Based on an estimated 2 million donations given annually Jayaraman *et al*. estimate that 15 600 TTD infections (13% HIV, 55% HBV, and 32% HCV) are acquired in SSA every year. If the WHO target of collecting 6.65 million donations annually in SSA was met, 51 580 TTD infections would be acquired annually. These would include, 28 595 cases of HBV, 16 625 cases of HCV and 6 650 cases of HIV (Jayaraman *et al.*, 2010).

The WHO recommends that all blood screening assays, whether serological or NAT, should have a minimum sensitivity and specificity of 99.5 %, and that test sensitivity and specificity should be as high as possible (World Health Organization, 2010). The reduction of the risk of an infectious unit entering the blood supply is largely determined by the sensitivity of the screening assays used. If assays with a 99.5% sensitivity were used, the 51 580 TTD infections annually acquired could be reduced to 33 250, therefore if the 6.65 million donation target were met, 6.62 million of the donations would be free of the TTD's tested for.

4.3.1.1 HIV

HIV is a leading cause of death and disease in SSA (Wang *et al.*, 2016). The burden of disease in SSA is disproportional, with only 14% of the world population residing in SSA it carries more than 65% (Tapko, Toure and Sambo, 2014; Kharsany and Karim, 2016) of the global HIV burden. The risk of acquiring an HIV infection from a transfusion in SSA is calculated to be 1 000 for every 1 000 000 donations collected (Jayaraman *et al.*, 2010). At WPBTS 100% of donated blood is screened for TTDs,

including testing for HIV serologically and by NAT. At WPBTS the overall TTD residual risk is calculated to be 1.31 per 1 000 000 donations, which is significantly lower than the SSA risk.

An HIV free generation can only be realised with the prevention of HIV transmission. Unsafe blood contributes to the transmission of HIV. Unsafe blood, due either to not being screened or being screened with low sensitivity antibody-only assays, is projected to contribute to 2 000 infections within SSA annually (Jayaraman *et al.*, 2010).

In counties where the prevalence of HIV is high, the number of incident infections is high. Therefore the risk of blood donations taking place during the window period is higher in high that in low HIV prevalence setting. El Ekaiby *et al.* concluded that the introduction of NAT would be of greater relative value in higher TTD prevalence settings (El Ekiaby, Lelie and Allain, 2010).

Within this study, one window period sample was tested using both DBS and DPS, and in both cases the samples had reactive results. This indicated that NAT using DBS or DPS samples would have identified the donor as being HIV positive, even though serological tests were negative.

The sensitivity of Ultrio Elite for plasma is claimed to be 100% (95% CL: 98.3% - 100%) (Gen-Probe, 2015). The HIV-1 sensitivity of Ultrio Elite using DBS and DPS was found to be 96.67 % and 97.50 %, respectively. DPS has a slightly higher sensitivity than DBS, this is most likely due to the increased plasma content in the DPS sample as compared to the DBS sample.

For HIV testing using DPS, linear regression suggested a lowest level of detection (LOD) of 24.11 cps/mℓ. When comparing our LOD with the LOD found in Mössner *et al*'s study, our LOD is lower than their reported LOD of 500 cps/mℓ. Mössner *et al*'s study also used the Ultrio Elite assay, however they used DBS as their sample type (Mössner *et al.*, 2016).

The infectious dose where 50% of the population (ID_{50}) would acquire HIV in a blood transfusion setting is calculated to be 632 copies (Weusten *et al.*, 2011; Vermeulen *et al.*, 2013) contained within the 20 mℓ plasma found in a red blood cell concentrate (RCC) unit. This translates to the blood donor having a viral load of c.31.6 cps/mℓ to be infectious. Each type of blood product contains different volumes of plasma. The higher the plasma content of the product the higher the potential absolute viral count in the product. Conversely, if the plasma content is very low the potential absolute viral count will be very low. Therefore as the plasma content differs in the products, different viral loads within the donor could result in an infection.

Within this study, the lowest viral load that tested reactive on a DPS sample was 380.18 cps/mℓ (2.58 log) which is much higher than the calculated detectable viral load of 24.11 cps/mℓ. The discrepancy could have been due to our small sample size and the possibility of a non-linear detection at low viral loads. If the calculated LOD were verified, using DPS samples for testing would prevent HIV transmission as it is lower than the ID_{50} of HIV which is 31.6 cps/mℓ. Comparing the viral load detection sensitivity for DPS and plasma samples, we found a reduction in sensitivity for HIV using DPS samples of approximately 1 $\log_{10}$ in viral load compared to the assay claims on plasma samples.

4.3.1.2 HBV

NAT was originally introduced as an additional safeguard in addition to serological screening. Originally it was thought that NAT testing would be most beneficial in the fight against HIV, since then it has been proven most useful in identifying occult Hepatitis B infection (OBI) cases (El Ekiaby, Lelie and Allain, 2010). To illustrate this, from 2011 up to and including 2017, 34 HIV NAT yield (i.e. HIV antibody negative) donations were received out of 1 113 916 donations, in comparison, a staggering 79 HBV NAT yield (i.e. Hepatitis B surface antigen negative) donations were received. Bearing in mind that an average of three different blood products is made from each donation, 102 potential HIV transmissions have been prevented compared to an astounding 237 potential HBV transmissions.

The use of DBS/DPS samples affected performance of HBV NAT adversely. The HBV reactive portion of Cohort B re-tested using DBS/DPS in this study had the lowest sensitivity. The 1 $\log_{10}$ reduction or more in sensitivity when using DBS/DPS instead of plasma samples with regard to HBV has been documented in a number of other studies (Jardi *et al.*, 2004; Lira *et al.*, 2009; Stene-Johansen *et al.*, 2016)The HBV sensitivity for Ultrio Elite using DBS and DPS was 57.58 % and 58.00 % respectively, much lower than the 100% HBV sensitivity for plasma samples stated in the package insert (Gen-Probe, 2015). The determined sensitivities were also lower than the universal recommended sensitivity of 99.5% for all TTD assays (World Health Organization, 2010). DPS had a slightly higher sensitivity to HBV than DBS, this was most likely due to the higher plasma content in the DPS sample as compared to the DBS sample.

The failure of detection in almost half of the DBS/DPS samples that should have been positive for HBV could be due to two reasons.

1. The viral loads in the samples tested were so low that they were undetectable in the eluate. The eluate comprised of 100µℓ of dried DBS/DPS sample which was eluted into a 2mℓ of buffer, thus the volume of plasma in the eluate was at least 20 fold lower than a traditional plasma sample. When the viral load is very low in the donor sample, the discrepancy between the HBV sensitivities of plasma and DBS/DPS are enhanced. To test this hypothesis four plasma samples from the original reactive Cohort B were sent for viral load testing, the viral load for three of the samples were found to be less than 116 cps/mℓ (unquantifiable) and the fourth sample 361 cps/mℓ. This observation is probably due to the nature of the HBV infection in the donors, there is a strong possibility the donors had occult HBV since most of the NAT yield donors within WPBTS were Anti-HBc positive.

2. The Ultrio Elite assay may not be as effective at denaturing the double stranded DNA as required, thus there was a reduction in the release of viral genomic DNA (Grabarczyk *et al.*, 2015). The prominently reduced sensitivity was only observed for HBV which supports the ineffective DNA denaturing hypothesis since HBV is the only DNA virus of the three viral genomes tested.

The ID_{50} has been calculated to be 3.16 virions (Weusten *et al.*, 2011) within a RCC for HBV by mathematical modelling. HBV does, however, have 2 different ID_{50} levels which are determined by the stage of the infection in the donor. The first ID_{50} is 3.2 virions during the initial window period (WP) of the HBV infection where the viral ramp-up is high (Vermeulen *et al.*, 2014). The second ID_{50} is during the Anti-HBc positive WP where the viral replication is reduced, and the ID_{50} is 100 fold lower at 316 virions.

Within this study the lowest HBV viral load that tested reactive using a DPS sample was 11032 cps/mℓ in the plasma sample (552 cps/mℓ present in the eluate). Only one sample with a higher viral load (13552 cps/mℓ) tested non-reactive. Mathematical modelling (linear regression) does, however, suggest that the Ultrio Elite should theoretically be able to detect HBV at levels as low as 68.32 cps/mℓ which is below the ID_{50} for the Anti-HBc positive window period phase of the HBV. The lowest viral loads that were found reactive were far above the infectious dose level for HBV therefore transmission would not be prevented based on DPS NAT only.

4.3.1.3 HCV

The prevalence of HCV within the WPBTS is very low, an average of 0.0030% of donations presented with concordant HCV in the last 5 years. For this reason only ten HCV reactive samples were available for testing on DPS and only two on DBS. WPBTS has not had an HCV window period donor since the implementation of NAT testing in October 2005.

The sensitivity for Ultrio Elite using DBS/DPS was 100% which matched the sensitivity claimed for Ultrio Elite on plasma samples (Gen-Probe, 2015). These levels are above the recommended 99.5% sensitivity levels for all TTD assays (World Health Organization, 2010). These result needs to be considered in the context of a very small sample size.

As with HBV, HCV has two ID_{50} levels for different infection stages. The first ID_{50} is calculated to be 3.16 cps/mℓ in RNA-positive anti-HCV negative donors, and the second ID_{50} is calculated to be 316 cps/mℓ when anti-HCV are present in the donor (Bruhn *et al.*, 2015; El Ekiaby *et al.*, 2015). The neutralizing effect of the HCV antibodies afford some degree of protection to the recipients.

In this study, the lowest viral load that tested reactive using a DPS sample /mℓ in the plasma sample with only 208 cps/mℓ present in the elution that was tested. This is lower than the calculated LOD for HCV based on linear regression. Not all low viral load samples tested reactive, and some samples with viral loads higher than 792 cps/mℓ tested non-reactive. This could be due to the fact to the very low viral load present in the dilution as a result of the large dilution factor (20) which results from adding two 50 µℓ samples to 2 000 µℓ of buffer.

Within this study the lowest HCV viral load that tested reactive using a DPS sample was 4176 cps/mℓ in the plasma sample (208 cps/mℓ present in the eluate). Mathematical modelling (linear regression) does, however, suggest that the Ultrio Elite should theoretically be able to detect HCV at 5252.7 cps/mℓ

which far above both ID_{50}s for HCV. Not all low viral load samples tested reactive; four samples with viral loads higher than 4176 cps/mℓ tested non-reactive.

The lowest viral loads that were found reactive were far above the infectious dose level for HCV therefore transmission would not be prevented based on DPS NAT only. The study of a bigger sample size could reduce the uncertainty of this conclusion.

4.3.1.4 Sample stability after three weeks

The S/CO change observed for all three reactive samples tested three weeks after preparation are within the intra-run standard deviation for each virus. Therefore, the change in the S/CO is not significant and the results could imply that DPS is stable for at least three weeks at room temperature. This finding is supported by a number of articles where stability using DBS/DPS is only affected at higher temperatures and longer storage periods. (Cassol *et al.*, 1991; Abe and Konomi, 1998; Bennett *et al.*, 2012; Stene-Johansen *et al.*, 2016). These findings should not be used in isolation as the sample size for stability testing was very limited and the viral loads of the samples tested were relatively high. Higher viral loads are generally more stable than lower viral loads (Stene-Johansen *et al.*, 2016).

4.3.1.5 Discriminatory probe (dHXV)

dHXV is designed as a follow-up probe for discriminating reactive samples after confirmed reactive Ultrio Elite test results. In this study all samples with sufficient volume that tested non-reactive on Ultrio Elite where nevertheless tested with dHXV, and some were subsequently found to be reactive. Thee of the nine samples for DBS and three of the 17 samples for DPS that tested non-reactive with Ultrio Elite tested reactive on dHXV. This is most likely due to low viral loads that are seen and not due to increased specificity of the discriminatory probe as the only assay where the dHXV probe has a higher sensitivity is dHIV (Gen-Probe, 2012).

4.3.1.6 Negative and Positive Predictive Value

The negative and positive predictive values of DBS/DPS, taking prevalence into account, are equal to those currently observed using plasma samples. PPV, without reference to prevalence is lower for DBS than for DPS within Cohort B. NPV and PPV, using the claimed plasma sensitivities and WPBTS donor population, were calculated to be 100%, which is higher than those calculated for both DBS and DPS.

NPV is predominantly based on specificity and prevalence while PPV is predominantly based on sensitivity and prevalence. Therefore when evaluating an assay for routine use, it important to ensure that the NPV/PPV is calculated using the parameters that are relevant to the testing facility to ensure that the NPV/PPV is acceptable.

4.3.2 Specificity

Specificity of a screening assay in a blood transfusion setting is not only important because it correctly identifies a donor as not being infected with a virus, but an assay with poor specificity also results in additional expenses.

Additional expenses arise from the chain of events after an initial reactive result is determined. Once a sample tests initially reactive will have to be repeat-tested. If the repeat test results are reactive (or on initial results depending on the algorithm) the associated blood products will have to be discarded. So evidently the additional expenses would consist of repeat testing, confirmatory testing, and the donation set.

The largest cost to a blood transfusion service is often overlooked - this cost results from the loss of potential income from the sale of the blood products and the potential loss of a donor. When a blood unit tests reactive, whether it be a true infection or a cross-reactive result, it is mandatory to discard such a unit. Therefore the unit of blood cannot generate an income. Furthermore, the donor is blocked/deferred for a specific length of time from donating blood which results in a loss of donated blood in addition to the cost for the confirmatory testing needed to reintroduce the donor back into the blood donor pool, on a subsequent donation. If inconclusive results (or true reactive results) are obtained it is also possible that a previous donated unit will be investigated and that a look-back procedure could be initiated. In most cases, a look-back procedure would entail contacting all recipients of the previous unit in question and performing a specific blood test on these donors to determine whether they have contracted the transfusion transmissible infection. The additional level of safety gained by performing a look-back procedure addresses concerns about donors being in the window period of an infection at the previous donation.

The Ultrio Elite assay claims 100% specificity (95% CL: 98.2% to 100%) on plasma samples (Gen-Probe, 2015). In real-life use, the WPBTS has observed an average specificity of 99.97% (95% CL: 99.96% to 99.97%) on 465 234 samples tested during the last 3 years (2015 - 2017).

The algorithm used in this study stipulates that if a sample was found initially reactive but non-reactive on repeat testing, it is regarded as non-reactive. In contrast, the WPBTS algorithm deems these donors as IR-NRR and counts them as false reactive because units are discarded based on the initial result. Should we have employed the WPBTS algorithm on this study the two initially reactive DBS samples would have been considered as false reactive, and the specificity for Ultrio Elite would have been calculated to be 99.78% for DBS (95% CL: 99.20% to 99.97%) and 100% for DPS (95% CL: 99.59% to 100.00%). However, if the specificity is calculated based on the algorithm used during this study, the resulting specificity for both DBS and DPS is 100% (95% CL: 99.59% to 100.00%).

NAT specificity is not only impacted by the prevalence of the diseases that are tested for, but also by the overall prevalence of diseases within a population. The rule of thumb is the higher the overall prevalence of viruses the higher the risk of a donor having circulating viral DNA or RNA from viruses

other than HIV, HBV or HCV which could result in cross-reactive results.

The Ultrio Elite assay states that an albumin level of 60 g/ℓ or higher could result in cross-reactive results (Gen-Probe, 2015). The normal average serum-albumin level is 40 g/ℓ (Margarson MP and Soni N., 1998) with a reference range of 3.4 to 5.4 g/dℓ. Fifty to sixty percent of the total protein in plasma is Albumin (Margarson MP and Soni N., 1998; Devaraj and Wheeler, 2015; Greco, Frank and Walton-Ziegler, Olivia, 2018). Therefore the normal reference range falls below the interference level.

However, a study performed in Ghana to establish population-based reference ranges suggested that the reference range for serum-Albumin in Ghana should be 46 – 68 g/ℓ (Koram *et al.*, 2007), thus placing the Ghanaian normal reference range above the interference level. Koram et al. suggest that the differences seen between the stated reference ranges and the normal values obtained could be due to the fact that the standard published reference ranges are based mostly on Caucasian sample populations, in addition to which genetical and environmental factors might play a role. This illustrates how important it is for each laboratory to establish their own data-sets using samples from the general population that the laboratory intends to screen.

4.3.2.1 S/CO value comparisons

The lack of red blood cells and the similarity of sample composition results in DPS and traditional plasma samples sharing a number of characteristics. Both DPS and plasma sample S/CO values are lower, with smaller standard deviations (see *FIGURE 3.1*). Each data set has a right-tailed S/CO distribution indicative of a higher frequency of low S/CO values than high S/CO values. The first quartile for the DBS dataset is higher than the third quartile of both the plasma and DPS samples. A lower and tighter S/CO distribution has the advantage of ensuring ease of interpretation of sample results and reducing the number of borderline results.

The difference between DPS and plasma samples, and DBS samples is most likely due to the presence of haemoglobin in the DBS samples. When the red blood cells dry, on the filter paper, the red cells lyse. The free haemoglobin is then eluted from the paper during the elution process. This is the only variable that is present in DBS, but not in DPS or plasma, since neither the plasma nor the DPS samples contain significant amounts of free-haemoglobin. Haemoglobin is primarily stored in red blood cells within the blood. The Ultrio Elite package insert notes that a haemoglobin content of 5000 mg/ℓ or more may cause interference of cross-reactivity (Gen-Probe, 2012). A minimum donation criteria is that each donor has a minimum haemoglobin level of 12.5 g/dℓ. This would result in 12.5 mg of haemoglobin present in the 100 µℓ whole blood used for making the DBS. This 12.5 mg of haemoglobin would then be placed in a 2 mℓ buffer solution for elution. This would then equates to 6250 mg/ℓ of haemoglobin. This minimum haemoglobin level (donors often have higher haemoglobin levels) is more than the level at which haemoglobin may cause interference or cross-reactivity.

4.3.2.2 Invalid results

On the Panther analyser, an invalid result can be obtained for numerous reasons, from instrument failure, inadequate sample volume to internal control failure (IC invalid). Internal Control (IC) is added to the reagents to control nucleic acid capture, amplification, and detection. For example, if the sample contains a substance that inhibits either one of these processes, the signal of the internal control will be below the calculated IC cut-off for samples, and therefore the result will be regarded as invalid. The type of invalid results observed with the DBS samples during this study were IC failures. During 2017, 87 IC invalid results were recorded at WPBTS during the routine screening of 153 947 donors. This amounts to an IC invalid rate of 0.06% for routine screening of plasma compared to the 0.50% invalid rate seen using DBS. If DBS was used for routine screening throughout 2017, there would have been a projected 770 invalid results. The reason for the high number of IC invalid results seen using DBS samples is most likely due to interference from haemoglobin.

4.3.3 Contamination Panel

Within SSA, facilities are usually not purpose-designed for transfusion testing. Instead existing infrastructure is adapted in a more or less suitable way. Under these conditions tests and processes have to be as robust as possible, and ideally should not require additional contamination precautions.

The ‘contamination panel’ consisting of forty samples was run blind, i.e. expected results were made available only after the testing was completed and results were compiled. The samples were handled as routine samples following the protocol set out in the methods section, with no additional precautions to limit contamination. All the samples were run by the same technologist.

Lack of contamination indicates that both sample preparation protocol and tests were robust. A confounding factor could be that all samples were run and prepared by the same technologist who has 15 years of good laboratory practice (GLP) experience. This human resource may not be available in most SSA laboratories.

4.3.4 Statistical Analysis

4.3.4.1 Accuracy

Although no official recommendations are available for the accuracy of blood transfusion screening tests, accuracy does provide an overall view of how well an assay performs. DBS samples had a slightly higher accuracy (98.45 %) than DPS samples (97.80%). This could be due to a larger number of HBV NAT yield samples included in the DPS samples than the DBS samples. This is the only metric where DBS samples apparently outperformed the DPS samples.

4.3.4.2 Bland Altman

When comparing the two different dried spot samples against the plasma “gold standard” there was no interpretational difference in Cohort A for both DBS and DPS testing when following the design of this study.

Using the Bland Altman analysis a bias was observed between plasma S/CO and DBS S/CO values. The bias increases as the S/CO values increase. A large number of S/CO values fall outside the 95% limits of agreement (LOA), this indicates that a correlation between the plasma S/CO values and DBS S/CO values does not exist.

Using the Bland Altman analysis no bias was observed between the plasma S/CO and DPS S/CO values. Almost all of the S/CO values fall inside the 95% LOA indicating that there is a correlation between the plasma S/CO and DPS S/CO values.

4.3.4.3 Cohen’s Kappa coefficient

Cohen’s Kappa is a statistical measure that is used to measure the agreement between two categorical datasets with an expected dichotomous outcome.

Within this study the agreement was found to be 98.45% between plasma S/CO and DBS S/CO values. The Cohen’s Kappa coefficient (CKC) was equal to 0.8615. Below in Table 4.1 Landis and Koch suggests the following values for interpretation. Which means that the agreement is almost perfect.

Table 4.1. Cohen’s kappa coefficient interpretations as described by Landis and Koch (Landis and Koch, 1977)

COHEN’S KAPPA COEFFICIENT	Agreement
0.00	Less than chance
0.00 – 0.20	Slight
0.21 – 0.40	Fair
0.41 – 0.60	Moderate
0.61 – 0.80	Substantial
0.81 – 1.00	Almost perfect

Within this study the agreement was found to be 97. 80% between plasma S/CO and DPS S/CO values. The CKC was equal to 0.8645. Which means that the agreement is almost perfect.

The plasma-DBS S/CO values comparison had a lower CKC than that of the plasma-DPS S/CO values comparison, showing that the plasma S/CO and DPS S/CO values had a greater agreement. We

choose to interpret this as an indicator that DPS samples are better suited for testing than the DBS samples.

4.4 PRACTICAL APPLICATION

One of the main questions this study set out to answer is whether DBS/DPS would be suitable for blood donor screening using Ultrio Elite as ID-NAT screening assay on the Panther systems. This questions will now be discussed from several practical perspectives.

To make NAT feasible, centralised NAT testing would have to be performed. The two main advantages of centralised testing are that

1. better quality assurance (QA) can be established and maintained (procedures, policies and testing can be better controlled.)
2. and that testing would be cheaper, due to economies of scale.

4.4.1 Quality Assurance

The success of NAT within SSA would be heavily dependent on the QA. QA should not only be applied to testing laboratories but to all blood transfusion activities. QA measures should include but will not necessarily be limited to:

- Adequate training of staff regarding:
    - Donor interviewing
    - Collecting DBS/DPS
    - Processing the DBS/DPS samples for testing
    - Testing samples on the Panther analyser
- Accurate recording of results and other information
- Adequate documentation of processes and procedures
- Adequate retention and retrieval of documents
- Safe working conditions
- Adequate personal protective equipment
- Adequate documentation with regard to change management

4.4.2 Collection and processing of DBS and DPS

4.4.2.1 Sample preparation

When looking at sample collection, DBS samples are the easiest to obtain. Capillary blood can be applied directly to the DBS card after a copper sulphate test or other appropriate test for haemoglobin has been performed by means of a finger prick. A foreseen risk is that the incorrect DBS sample application could render the sample unsuitable for testing, thus making the donation unusable.

In our study we found that DPS samples provide better results than DBS samples. DPS samples require a venous sample to be taken and centrifuged prior to DPS sample preparation. The addition of collecting plasma in a tube for DPS would add to overall costs. Additional infrastructure and supplies would also be needed. The following additional items would be needed to prepare a DPS sample (that are not required when preparing a DBS sample):

1. Hypodermic needles
2. Specimen tubes with caps
3. Centrifuge (Which either require a battery or electricity)

The additional step of using a venous plasma sample could result in incorrect sample labelling and sample mix-up in the pre-analytic area. Plasma samples are lighter in colour than whole blood, and thus visual confirmation that the sample was applied to the DPS card is not as prominent as whole blood applied to a DBS card.

Roche diagnostics (Mannheim, Germany) has designed a novel DBS card that produces a DPS, this present a possible solution to many of the challenges faced preparing DPS in rural areas. It is called the Cobas® Plasma Separation Card (Roche Diagnostics, Mannheim, Germany). This card claims to have detected a viral load of 91.5 cps/mℓ when using it in combination with the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test (Roche, 2017).

Once the DBS/DPS cards are prepared there is no difference in the preparation of the sample.

During this study, samples were allowed to air dry on the bench making use of empty Panther tip trays. The holes for the tips in these trays ensured that the cards dried adequately due to good ventilation. The good ventilation also made it possible to stack the trays, thus conserving space. Repurposing these trays was a cheap, durable option as the Panther tip trays are generally discarded once the tips have been used. The reuse of Panther tip trays is a scalable solution since trays could be sent from the central testing laboratory to the rural areas with the sample collection transport. During this study the laboratory temperature was controlled, but in a number of studies DBS has been shown to be stable at temperatures above room temperature. The effect of humidity in transport without climate control could be reduced by placing desiccants in the shipping container/bag.

4.4.2.2 Laboratory results

Overall, our study found DPS to be a better sample than DBS with regard to laboratory results.

DPS samples had greater sensitivities and specificities, had fewer invalid results, less initial-reactive-non-repeat-reactive results, lower S/CO values for negative samples, and their S/CO values were more comparable to plasma S/CO values than DBS. The increased sensitivity of DPS is probably due to the larger plasma input volume.

The Invalid result rate of the DBS would not result in an efficient testing management as it is approximately ten times more than the average invalid rate seen within WPBTS. Should DBS be used an invalid result could mean that insufficient volume would be available for discriminatory testing if an initial invalid sample was found to be subsequently reactive.

4.4.3 Testing strategies

Blood screening services usually test their complete TTD test panel simultaneously using samples taken at the same time as the blood unit is collected (World Health Organization, 2010). Novel strategies suggest a number of various combinations which include sequential testing of TTD markers and using rapid testing prior to donation. Both of these strategies are aimed to reduce costs. When sequential testing strategies are employed cost is saved by not testing all the deemed markers if a unit is reactive, in other words only negative units would have been tested against the full testing panel. Rapid testing pre-donation is not recommended by the WHO (World Health Organization, 2010; Bloch, Vermeulen and Murphy, 2012), but this strategy does reduce costs as a blood collection set is not "wasted" as a reactive donor is deferred prior to donating. It also has the marginal benefit of allowing donors to be counselled immediately if they test reactive for a TTD marker. Additional safety can be built into the rapid test screening strategy by adding additional post-donation testing. Some studies have looked at the viability of combining rapid tests and NAT testing (van Hulst M, Owusu-Ofori S, Sarkodie F, Nsiah-Asare A, Candotti D,Postma MJ, 2008; El Ekiaby, Lelie and Allain, 2010) for blood screening. Both these studies concluded that this testing strategy would not compromise safety and would reduce testing costs. The combination of rapid tests and NAT testing has not been limited to blood transfusion but has also been documented in the diagnostic field (Kania *et al.*, 2013).

The true benefit of NAT is the identification of blood donations during the window period or occult phase of viral infection thus reducing the risk of transmission. By investigating the use of DBS and DPS for NAT testing, this study was able to demonstrate that the Ultrio Elite was able to detect an HIV-1 window period sample. HIV-2 was not included in this study as the donor demographics of the WPBTS does not include HIV-2 donors. If this sample type (DBS/DPS) is to be used in areas where HIV-2 is endemic further analysis and studies need to be performed. Window period HBV infections are not often seen at WPBTS as seroprevalence for anti-HBc is high in the general population base. Therefore, a combination of window period and occult HBV samples was used for the study. Ultrio Elite, was not

reliably able to detect the NAT only HBV infections using DBS or DPS samples. No HCV window period samples were tested, both DBS and DPS samples had a 100% sensitivity for concordant HCV.

Individual testing strategies and testing algorithms must be evidence-based and cognisant of the national and regional epidemiology of TTD. The epidemiology studies should be conducted on both first-time and repeat donors.

4.4.4 Transport

Transportation within SSA, where distances are long and roads are not always well maintained, poses one of the greatest hindrances to centralised testing. The cost of transporting plasma samples from rural areas to centralized testing centres would require specialized vehicles that could maintain the cold chain of the samples, and also be equipped for biological safety precautions as liquid samples (which could be infectious) would be transported. This would result in added costs and could potentially delay the availability of blood. If DBS/DPS samples were transported no or minimal cold chain management would be required, and samples would carry a low infectious risk (Bond *et al.*, 1981). Samples could even be mailed, although this strategy could introduce delays to testing to such an extent that test results might not be available when the unit is required.

One of the developments with regard to transportation of blood products is the utilisation of unmanned aerial vehicles (also referred to as drones) as currently used in Rwanda and Tanzania. In Rwanda drones routinely deliver units of blood. These drones can fly within an average radius 80 km, and reach their destinations within in 30 minutes.

Taking into account the carrying capacity of such a drone (1.8 kg) and the weight of a DBS/DPS sample card (+/- 2g), approximately 900 DBS/DPS cards (with no packaging added) could be transported (Zipline, no date; Rosen, 2017) in a single flight. DBS/DPS cards could then be flown to centralised laboratories and tested. Results could then be sent back to the peripheral collection centres. Drones in Tanzania are also used to deliver urgent medical supplies, and thus present the case that such drones could also be employed to deliver blood from peripheral centres to rural hospitals thus reducing delivery time (Zipline, no date; Amukele *et al.*, 2017; Rosen, 2017; Becker, 2018). Currently the drones used by Zipline are deployed for a delivery form a centralised base after a sms is sent from the requesting hospital.

As illustrated, the use of DBS/DPS for testing opens up various transportation solutions much better suited for SSA.

4. CONCLUSION

Innovative screening strategies such as pre-donation rapid testing followed by NAT testing at a centralized site (van Hulst M, Owusu-Ofori S, Sarkodie F, Nsiah-Asare A, Candotti D,Postma MJ, 2008) could revolutionise the safety of blood screening even in resource-constrained settings. One of the largest hindrances, apart from training, in the above strategy would be transportation from donation site to the centralised laboratory.

DBS/DPS samples can be transported with no or minimal cold chain management, and carry a low infectious risk (Bond *et al.*, 1981). Should DBS/DPS samples yield comparable test results to plasma samples, they would pose a viable, transportable specimen that could be processed and tested in a centralised laboratory. Using DBS/DPS samples would reduce some of the costs associated with NAT testing.

This study found that the diagnostic specificities of both DBS and DPS samples were equal to that of plasma samples, thus indicating that either would be a reasonable tool for screening of donated blood. However, the sensitivities for both HCV and HIV were lower using DBS/DPS samples than for plasma samples. This was expected for samples with such small input volumes and large dilutions. The HBV reactive DBS/DPS samples showed very erratic results which render them unsuitable for testing; further development of the assay to increase the sensitivity to HBV would be recommended.

DPS performed marginally better than DBS, and would be suited for screening blood, especially if the HBV sensitivity of the assay were to be increased. Further detailed economic viability and large-scale studies need to be performed to determine sensitivity and specificity within a specific population.

With regard to sample integrity, both DBS and DPS displayed reliable results even after a three week period of storage at room temperature. An additional advantage of collecting dried blood or plasma specimens on cards is that collection costs and complexity would be reduced.

REFERENCES

Allain, J. P. *et al.* (2013) 'Infectivity of blood products from donors with occult hepatitis B virus infection', *Transfusion*, 53(7), pp. 1405–1415. doi: 10.1111/trf.12096.

Aoki, S. K. *et al.* (1993) 'Evidence of hepatitis in patients receiving transfusions of blood components containing antibody to hepatitis C.', *Blood*, 82(3), pp. 1000–1005.

Asamoah-Akuoko, L. *et al.* (2017) 'Blood donors' perceptions, motivators and deterrents in Sub-Saharan Africa - a scoping review of evidence', *British Journal of Haematology*, (May), pp. 864–877. doi: 10.1111/bjh.14588.

Bates, I. *et al.* (2008) 'Maternal mortality in sub-Saharan Africa: The contribution of ineffective blood transfusion services', *BJOG: An International Journal of Obstetrics and Gynaecology*. doi: 10.1111/j.1471-0528.2008.01866.x.

Bihl, F. *et al.* (2007) 'Transfusion-transmitted infections', *Journal of Translational Medicine*, 11(Table 1), pp. 0–35. doi: 10.1186/1479-5876-5-25.

Bond, W. W. *et al.* (1981) 'Survival of hepatitis B virus after drying and storage for one week.', *Lancet (London, England)*. England, pp. 550–551.

Brambilla, D. *et al.* (2003) 'Multicenter Evaluation of Use of Dried Blood and Plasma Spot Specimens in Quantitative Assays for Human Immunodeficiency Virus RNA: Measurement, Precision, and RNA Stability', *JOURNAL OF CLINICAL MICROBIOLOGY*, 41(5), pp. 1888–1893. doi: 10.1128/JCM.41.5.1888–1893.2003.

Busch, P. and Kleinman, S. H. (2009) 'Hepatitis C infection: Recent insights relevant to transfusion safety', *Vox sanguinis*, 96, pp. 22–23. doi: 10.1111/j.1751-2824.2009.01214.x.

Cable, R., Lelie, N. and Bird, A. (2013) 'Reduction of the risk of transfusion-transmitted viral infection by nucleic acid amplification testing in the Western Cape of South Africa: A 5-year review', *Vox Sanguinis*, 104(2), pp. 93–99. doi: 10.1111/j.1423-0410.2012.01640.x.

Candotti, D. and Laperche, S. (2018) 'Hepatitis B Virus Blood Screening: Need for Reappraisal of Blood Safety Measures?', *Frontiers in Medicine*, 5(February), pp. 1–10. doi: 10.3389/fmed.2018.00029.

CDC (2013) 'CDC - Diseases and Organisms - Blood Safety'. Available at: http://www.cdc.gov/bloodsafety/bbp/diseases_organisms.html.

Chevalier, M. S. *et al.* (2016) 'Morbidity and Mortality Weekly Report', 12(5), pp. 2011–2014. doi: 10.15585/mmwr.mm6505a4.

Chibwesha, C. J. *et al.* (2016) 'Point-of-care virologic testing to improve outcomes of HIV-infected children in Zambia: A clinical trial protoco', *Journal of Acquired Immune Deficiency Syndromes*, 72, pp.

S197–S201. doi: 10.1097/QAI.0000000000001050.

Ciaranello, A. L. *et al.* (2011) 'Early infant HIV-1 diagnosis programs in resource-limited settings: Opportunities for improved outcomes and more cost-effective interventions', *BMC Medicine*. doi: 10.1186/1741-7015-9-59.

El Ekiaby, M. *et al.* (2015) 'Viremia levels in hepatitis C infection among Egyptian blood donors and implications for transmission risk with different screening scenarios', *Transfusion*, 55(6), pp. 1186–1194. doi: 10.1111/trf.13061.

El Ekiaby, M., Lelie, N. and Allain, J. P. (2010) 'Nucleic acid testing (NAT) in high prevalence-low resource settings', *Biologicals*, 38(1), pp. 59–64. doi: 10.1016/j.biologicals.2009.10.015.

Essajee, S. (2013) 'Update on EID testing : Global Progress & Emerging Challenges'. Clinton Health Access initiative.

Fleming, A. F. (1997) 'HIV and Blood Transfusion in Sub-Saharan Africa', *Transfus. Sci*, 18(2), pp. 167–179.

Fung, M. *et al.* (eds) (2014) *Technical Manual*. 18th edn. Maryland: AABB.

Gear, J. . (1938) 'the History of Blood Transfusion', *the Leech*, April, pp. 61–64.

Gen-Probe, I. (2015) 'Procleix Ultrio Elite Assay', pp. 1–44.

'GEN-PROBE ® APTIMA ® Adapter Kit' (2011). San Diego, CA, pp. 1–2.

Giblett, E. R. (1994) 'Philip Levine 1900—1987 A Biographical Memoir'. Washingto D.c.: National Academy of sciences.

Government of Maharashtra Blood transfusion Council (2005) *Blood Transfusion Transmitted Diseases*, *Blood Transfusion Transmitted Diseases*. Available at: http://www.mahasbtc.com/blood-transfusion-transmitted-diseases.

Grabarczyk, P. *et al.* (2015) 'Inclusion of human immunodeficiency virus Type 2 (HIV-2) in a multiplex transcription-mediated amplification assay does not affect detection of HIV-1 and hepatitis B and C virus genotypes: A multicenter performance evaluation study', *Transfusion*, 55(9), pp. 2246–2255. doi: 10.1111/trf.13193.

Grant, R. M. and Smith, D. K. (2015) 'Integrating Antiretroviral Strategies for Human Immunodeficiency Virus Prevention: Post- and Pre-Exposure Prophylaxis and Early Treatment', *Ofid*, 2(Suppl 1), pp. 1–8. doi: 10.1093/o.

'Highlights of Transfusion Medicine History' (2018) *American Association of Blood Banks*. Available at: http://www.aabb.org/tm/Pages/highlights.aspx.

Huestis, D. W. (2007) 'Alexander Bogdanov: The Forgotten Pioneer of Blood Transfusion', *Transfusion*

Medicine Reviews, 21(4), pp. 337–340. doi: 10.1016/j.tmrv.2007.05.008.

van Hulst M, Owusu-Ofori S, Sarkodie F, Nsiah-Asare A, Candotti D,Postma MJ, A. J. (2008) 'Cost-effectiveness of pre-donation screening blood donors with rapid tests and subsequent nucleic acid amplification testing of blood donations in a resource-poor setting', *Vox Sanguinis*, 95(S1), p. 261.

Jardi, R. *et al.* (2004) 'Usefulness of dried samples for quantification and molecular characterization of HBV-DNA', *Hepatology*, 40(1), pp. 133–139. doi: 10.1002/hep.20275.

Kaur, P. and Basu, S. (2005) 'Transfusion-transmitted infections: existing and emerging pathogens.', *Journal of postgraduate medicine*, 51(2), pp. 146–151.

Landis, J. R. and Koch, G. G. (1977) 'The Measurement of Observer Agreement for Categorical Data Published by : International Biometric Society Stable URL : http://www.jstor.org/stable/2529310', *Biometrics*, 33(1), pp. 159–174. Available at: /www.jstor.org/stable/2529310.

Learoyd, P. (SCIENTIFIC & T. T. M. – L. D. L. B. C. (2006) 'A Short History of Blood Transfusion', in *National Blood Service - Scientific & Technical Training*. Leeds Blood Centre, p. 18.

Li, W. *et al.* (2014) *Dried Blood Spots: Applications and Techniques*, *Dried Blood Spots: Applications and Techniques*. doi: 10.1002/9781118890837.

Lira, R. *et al.* (2009) 'Use of dried blood samples for monitoring hepatitis B virus infection', *Virology Journal*, 6, pp. 1–6. doi: 10.1186/1743-422X-6-153.

Lund, T. C. *et al.* (2013) 'The blood supply in Sub-Saharan Africa: Needs, challenges, and solutions', *Transfusion and Apheresis Science*. Elsevier Ltd, 49(3), pp. 416–421. doi: 10.1016/j.transci.2013.06.014.

Madhava, V., Burgess, C. and Drucker, E. (2002) 'Epidemiology of chronic hepatitis C virus infection in sub-Saharan Africa.', *The Lancet infectious diseases*, 2(May), pp. 293–302. doi: 10.1016/S1473-3099(02)00264-5.

Maponga, T. (2012) 'An investigation of hepatitis B virus in antenatal women tested for human immunodeficiency virus, in the Western Cape Province of South Africa', (March). Available at: https://scholar.sun.ac.za/handle/10019.1/20416.

McCabe, E. R. *et al.* (1987) 'DNA microextraction from dried blood spots on filter paper blotters: potential applications to newborn screening.', *Human genetics*. Germany, 75(3), pp. 213–216.

McCullough, T. K. and McCullough, J. (2013) 'Strengthening blood programs in developing countries', *Transfusion and Apheresis Science*. Elsevier Ltd, 49(3), pp. 408–415. doi: 10.1016/j.transci.2013.07.006.

Paul, D. and Brosco, J. (2013) *The PKU Paradox: A Short History of a Genetic Disease By*. Baltimore: John Hopkins University Press.

Van der Poel, C. L. (1999) 'Hepatitis C virus and blood transfusion: past and present risks.', *Journal of hepatology*, 31 Suppl 1, pp. 101–6.

Rix, J. (no date) *WPBTS Integrated report*. Available at: http://oro.open.ac.uk/6497/.

Robert Guthrie and Ada Susi (1963) 'A simple Phenylalanine method for detecting phenylketonuria in large popluations of newborn infants', (September).

Roche (2017) 'Cobas Plasma separation card'. Pleasonton: Roche Molecular Systems.

Rosen, J. W. (2017) *Zipline Expands Drone Delivery of Medical Supplies*, *MIT Technology Review*. Available at: https://www.technologyreview.com/s/608696/zipline-expands-drone-delivery-of-medical-supplies/.

Sahoo, M. K. *et al.* (2016) 'Evaluation of the aptima HIV-1 Quant Dx assay using plasma and dried blood spots', *Journal of Clinical Microbiology*, 54(10), pp. 2597–2601. doi: 10.1128/JCM.01569-16.

Schneider, W. H. (2013) 'History of blood transfusion in sub-saharan africa', *Transfusion Medicine Reviews*. Elsevier Inc., 27(1), pp. 21–28. doi: 10.1016/j.tmrv.2012.08.001.

Standards of Practive for Blood Transfusion in South Africa (2017).

Stene-Johansen, K. *et al.* (2016) 'Dry blood spots a reliable method for measurement of Hepatitis B viral load in resource-limited settings', *PLoS ONE*, 11(11), pp. 1–9. doi: 10.1371/journal.pone.0166201.

Stevens, W. *et al.* (2008) 'Performance of a novel human immunodeficiency virus (HIV) type 1 total nucleic acid-based real-time PCR assay using whole blood and dried blood spots for diagnosis of HIV in infants', *Journal of Clinical Microbiology*. doi: 10.1128/JCM.00754-08.

Stevens, W. S. *et al.* (2009) 'Ultra-high-throughput, automated nucleic acid detection of human immunodeficiency virus (HIV) for infant infection diagnosis using the Gen-Probe Aptima HIV-1 screening assay', *Journal of Clinical Microbiology*, 47(8), pp. 2465–2469. doi: 10.1128/JCM.00317-09.

Tagny, C. T. *et al.* (2008) 'Blood safety in Sub-Saharan Africa: A multi-factorial problem', *Transfusion*, 48(6), pp. 1256–1261. doi: 10.1111/j.1537-2995.2008.01697.x.

Tapko, J. B., Toure, B. and Sambo, L. G. (2014) *Status of Blood Safety in the WHO African Region: Report of the 2010 Survey*.

Vermeulen, M. *et al.* (2012) 'Hepatitis B virus transmission by blood transfusion during 4 years of individual-donation nucleic acid testing in South Africa: Estimated and observed window period risk', *Transfusion*, 52(4), pp. 880–892. doi: 10.1111/j.1537-2995.2011.03355.x.

Vermeulen, M. *et al.* (2013) 'Comparison of human immunodeficiency virus assays in window phase and elite controller samples: viral load distribution and implications for transmission risk', *Transfusion*, 53(10), pp. 2384–2398. doi: 10.1111/trf.12117.Comparison.

Vermeulen, M. *et al.* (2014) 'A mathematical approach to estimate the efficacy of individual-donation and minipool nucleic acid amplification test options in preventing transmission risk by window period and occult hepatitis B virus infections', *Transfusion*, 54(10), pp. 2496–2504. doi: 10.1111/trf.12657.

Weusten, J. *et al.* (2011) 'Refinement of a viral transmission risk model for blood donations in seroconversion window phase screened by nucleic acid testing in different pool sizes and repeat test algorithms', *Transfusion*, 51(1), pp. 203–215. doi: 10.1111/j.1537-2995.2010.02804.x.

WHO (2016) *Blood safety and availability*, *World Health Organisation*. Available at: http://www.who.int/en/news-room/fact-sheets/detail/blood-safety-and-availability.

WHO, UNICEF and UNAIDS (2011) 'Global HIV/AIDS Response Progress Report 2011', *Vasa*, pp. 1–233. doi: ISBN 978 92 4 150298 6.

Wiener, A. S. (1933) *History of blood transfusion*, *Journal of the American Medical Association*. doi: 10.1001/jama.1933.02740030056029.

World Health Organization (2002) *The Clinical Use of Blood*. Genieva.

World Health Organization (2010) *Screening Donated Blood for Transfusion- Transmissible Infections Recommendations*. Geneva: WHO Press.

World Health Organization (2016) 'Blood safety and availability', *World Health Organization*. Available at: http://www.who.int/mediacentre/factsheets/fs279/en/.

Zipline (no date) *Zipline — Service — Zipline provides a seamless delivery service, rain or shine.* Available at: http://www.flyzipline.com/service/ (Accessed: 27 July 2018).

ADDENDA

ADDENDUM A. WPBTS CONFIDENTIAL DONOR QUESTIONNAIRE PAGE 2

DONOR LABEL

BAR CODE

Section 1 | LIFESTYLE QUESTIONNAIRE

Please circle your answers.

Please read all questions carefully and answer honestly. Your answers will be treated confidentially.

1	Do you consider your blood safe to be transfused to a patient?	No	Yes	STAFF SECTION
2	Have you or your sexual partner **ever** used recreational/street drugs by nose, mouth or injection needle?	No	Yes	
3	**In the past 6 months have you:** Had a tattoo, body piercing, ear piercing or permanent make-up applied?	No	Yes	
	Had Raatib, ritual scarring, ritual piercing, ritual circumcision, blood sharing or been stabbed?	No	Yes	
	Taken antiretroviral (ARV's) medication, including Truvada?	No	Yes	
4	**For Health Care Workers and their partners only:** **In the past 6 months:** Have you or your sexual partner had a needle stick or skin penetrating injury; or had skin, eye or mouth contact with another person's blood?	No	Yes	
The following questions are of a sexual nature. We ask these questions as sexual contact may cause infectious diseases like HIV/AIDS. "Sexual contact" refers to vaginal sex (contact between penis and vagina); oral sex (mouth or tongue contact with vagina, penis or anus) and anal sex (contact between penis and anus). Where applicable, please answer "Yes" to the following questions even if a condom was used:				
5	Do you have AIDS or are you HIV positive?	No	Yes	
	Have you ever had sexual contact with anyone who has AIDS or is HIV positive?	No	Yes	
	Are you only giving blood for an HIV test?	No	Yes	
6	**In the past 6 months (with or without a condom):** - Have you started having sexual contact with a new sexual partner?	No	Yes	
	- Have you had sexual contact with more than one person?	No	Yes	
	- To the best of your knowledge has your sexual partner had sexual contact with more than one person?	No	Yes	
	- Have you had sexual contact with someone whose sexual history you do not know?	No	Yes	
	- Have you had sexual contact with anyone who takes money, drugs or other favours for sex?	No	Yes	
	- Have you received money, drugs or other payment for sex?	No	Yes	
	- Are you a sex worker?	No	Yes	
	- Have you been sexually assaulted?	No	Yes	
7	**In the past 6 months:** Have you or your sexual partner had any sexually transmitted disease (STD) including genital herpes, syphilis, gonorrhoea (drop) or human papilloma virus?	No	Yes	

DECLARATION — Please read and sign the declaration and consent before donating blood. — **CONSENT**

1. I have read and understood the pamphlet "Important Information for Blood Donors".
2. To the best of my knowledge all the information supplied is the truth.
3. I understand that if I have not answered these questions truthfully this could endanger the patient and lead to legal proceedings against me. I undertake that should I for any reason deem my blood not safe for use, I will immediately inform WPBTS.
4. I confirm that I am 16 years of age or older.
5. I understand that the information on this form will be kept in a secure facility indefinitely under my donor code, not my name.
6. I understand the donation process and the possible risks involved as explained.

1. I consent to my blood being tested for Syphilis, Hepatitis B, Hepatitis C and HIV.
2. I understand that I will be informed of any test results that are important to my health or affect my ability to donate blood.
3. I consent that samples of my blood and/or donation data may be used on occasion for scientific research, the objective of which is to improve the safety of the blood supply and donor health and well-being. Blood products or samples may be used in limited circumstances for research and/or the preparation of diagnostic reagents utilised by blood banks and related laboratories. On occasion the Service may permit researchers to request additional samples from me with my consent.
4. I consent to the administration of such fluids and medications as deemed necessary in the management of an untoward donor reaction.
5. I consent to the infusion of fluids, medications and re-infusion of my own blood components during apheresis collection procedures.
6. I consent to being offered information on the Service's Iron Replacement Programme and that any decision to take the iron replacement tablets rests with me.

Please do not sign until you have answered all the questions and read the declaration and consent.

Cell number: Tel number:	
Name and surname:	
Date of birth:	
Donor's signature:	

FOR OFFICE USE:				
Interview done	No		Yes	
Signature: Phlebotomist				
Signature: Interviewer (only if interview was done)				

CLN 12 (06 Jun 18)

Addendum B. Health Research Ethics approval

UNIVERSITEIT STELLENBOSCH UNIVERSITY

Approved

New Application

Ethics Reference #: 0806

Title: DRIED PLASMA SPOT TESTING – THE ANSWER FOR MAKING BLOOD TRANSFUSION TESTING SAFER IN AFRICA?

Dear Charlotte Pistorius

The **New Application** received on 14/08/2017 16:26 , was reviewed by members of the **Health Research Ethics Committee** via Minimal Risk Review procedures on 15 September 2017 and was approved.

Please note the following information about your approved research protocol:

Protocol approval period: This project has been approved for a period of one year from the date of this approval letter.

Please remember to use your **protocol number** (0806) on any documents or correspondence with the HREC concerning your research protocol.

Please note that this decision will be ratified at the next HREC full committee meeting. HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:

Please note a template of the progress report is obtainable on https://applyethics.sun.ac.za/Project/Index/875 and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2015 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel:+27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and documents please visit: https://applyethics.sun.ac.za/Project/Index/875

If you have any questions or need further assistance, please contact the HREC office at 0219389207.

Sincerely,

Franklin Weber

Coordinator

Health Research Ethics Committee 1

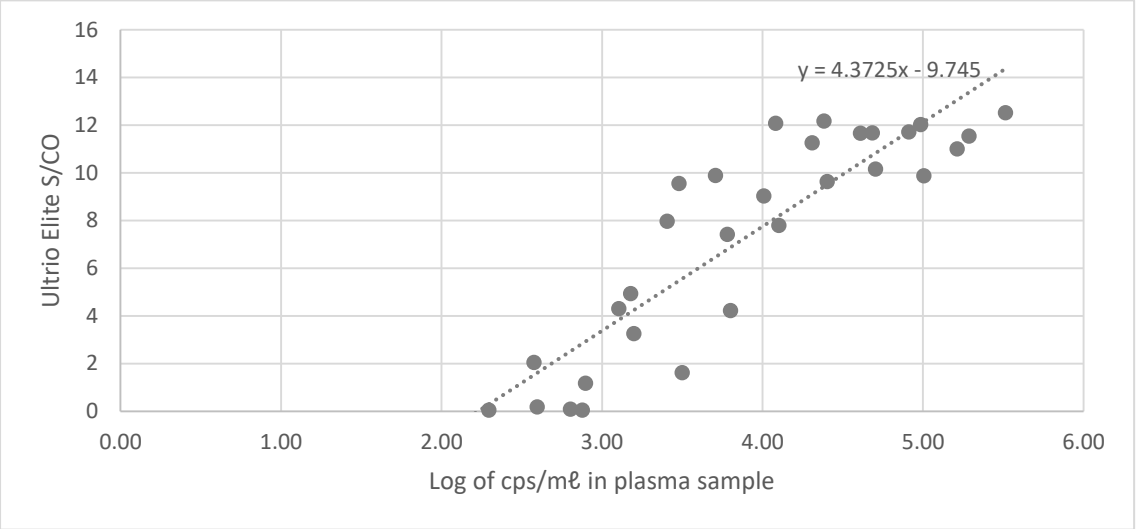
ADDENDUM C. CONTAMINATION PANEL RESULTS

	Ultrio Elite original sample interpretation	Virus	DPS Interpretation
A001	Nonreactive	N/A	Nonreactive
A002	Reactive	HIV	Reactive
A003	Nonreactive	N/A	Nonreactive
A004	Reactive	HIV	Reactive
A005	Reactive	HCV	Reactive
A006	Nonreactive	N/A	Nonreactive
A007	Nonreactive	N/A	Nonreactive
A008	Reactive	HBV	Reactive
A009	Nonreactive	N/A	Nonreactive
A010	Reactive	HBV	Reactive
A011	Reactive	HIV	Reactive
A012	Reactive	HCV	Reactive
A013	Reactive	HBV	Reactive
A014	Reactive	HCV	Reactive
A015	Nonreactive	N/A	Nonreactive
A016	Reactive	HIV	Reactive
A017	Reactive	HIV	Reactive
A018	Reactive	HIV	Reactive
A019	Reactive	HBV	Reactive
A020	Nonreactive	N/A	Nonreactive
A021	Reactive	HIV	Reactive
A022	Reactive	HBV	**Nonreactive**
A023	Reactive	*HIV*	**Nonreactive**
A024	Nonreactive	N/A	Nonreactive
A025	Reactive	HCV	Reactive
A026	Reactive	HBV	Reactive
A027	Reactive	HBV	**Nonreactive**
A028	Nonreactive	N/A	Nonreactive
A029	Reactive	HIV	Reactive
A030	Nonreactive	N/A	Nonreactive
A031	Reactive	HCV	Reactive
A032	Nonreactive	N/A	Nonreactive
A033	Reactive	HIV	Reactive
A035	Reactive	HBV	Reactive
A036	Reactive	HIV	Reactive
A037	Reactive	HBV	Reactive
A038	Reactive	HIV	Reactive
A039	Reactive	HCV	Reactive
A040	Nonreactive	N/A	Nonreactive

ADDENDUM D. RESULTS OF PROCURED HIV-SAMPLE DILUTIONS

Serial Number	Viral Load (cps/mℓ)	Dilution	Dilution factor	Calculated viral load present in DPS eluted sample (log units)	DPS Result S/CO
HIV1	101000	1	20	3.70	9.88
	50500	2	20	3.40	10.16
	25250	4	20	3.10	9.64
	12625	8	20	2.80	7.8
	6312.5	16	20	2.50	4.22
	3156.25	32	20	2.20	1.62
	1578.12	64	20	1.90	3.25
	789.06	128	20	1.60	1.18
	394.53	256	20	1.30	0.18
	197.27	512	20	0.99	0.05
HIV2	193000	1	20	3.98	11.54
	96500	2	20	3.68	12.03
	48250	4	20	3.38	11.68
	24125	8	20	3.08	12.17
	12062.5	16	20	2.78	12.08
	6031.25	32	20	2.48	7.42
	3015.625	64	20	2.18	9.55
	1507.8125	128	20	1.88	4.94
	753.90625	256	20	1.58	0.05
	376.953125	512	20	1.28	2.05
HIV3	326000	1	20	4.21	12.52
	163000	2	20	3.91	11.01
	81500	4	20	3.61	11.72
	40750	8	20	3.31	11.66
	20375	16	20	3.01	11.26
	10187.5	32	20	2.71	9.03
	5093.75	64	20	2.41	9.89
	2546.875	128	20	2.10	7.97
	1273.44	256	20	1.80	4.31
	636.72	512	20	1.50	0.09

ADDENDUM E. COMBINED S/CO FOR HIV PROCURED SAMPLES (N=30)



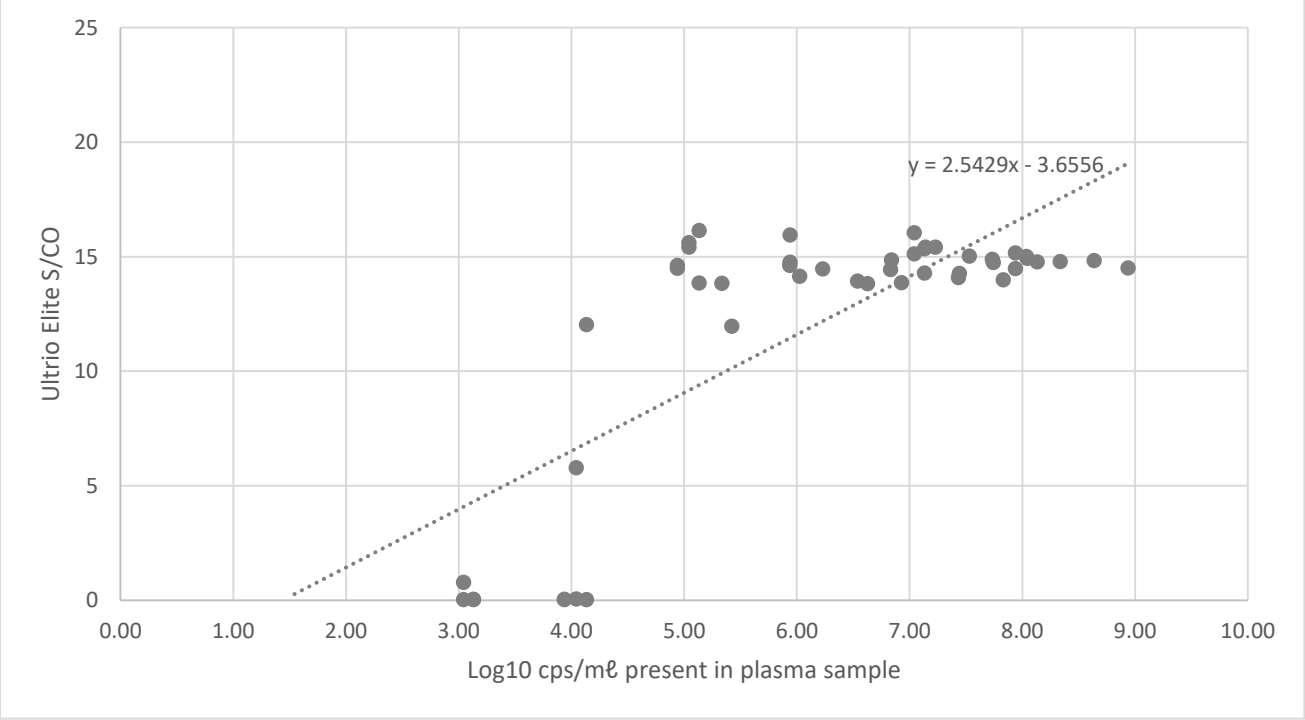
ADDENDUM F. RESULTS OF DOUBLING SERIAL DILUTIONS OF PROCURED HBV REACTIVE SAMPLES

Serial number	Dilution	Calculated cps/mℓ	Log of Calculated cps/mℓ	Dilutional factor	DPS Result S/CO
HBV1	1	135520000	8.13	20	14.78
	2	67760000	7.83	20	13.99
	4	33880000	7.53	20	15.03
	8	16940000	7.23	20	15.41
	16	8470000	6.93	20	13.87
	32	4235000	6.63	20	13.82
	128	1058750	6.02	20	14.14
	512	264687.53	5.42	20	11.96
HBV2	1	110320000	8.04	20	14.92
	2	55160000	7.74	20	14.75
	4	27580000	7.44	20	14.27
	8	13790000	7.14	20	15.41
	16	6895000	6.84	20	14.86
	32	3447500	6.54	20	13.93
	128	861875	5.94	20	14.62
	512	215468.74	5.33	20	13.84
HBV3	1	868000000	8.94	20	14.51
	2	434000000	8.64	20	14.83
	4	217000000	8.34	20	14.79
	8	108500000	8.04	20	15.01
	16	54250000	7.73	20	14.89
	32	27125000	7.43	20	14.09
	128	6781250	6.83	20	14.44
	512	1695312.5	6.23	20	14.46

ADDENDUM G. RESULTS OF TEN-FOLD SERIAL DILUTIONS MADE FROM PROCURED HBV REACTIVE SAMPLES

Serial Number	Dilution	Calculated cps/mℓ	Calculated cps/mℓ	Dilutional factor	DPS Result S/CO #1	DPS Result S/CO #2
HBV01	10	13552000	7.13	20	15.34	14.28
	1000	135520	5.13	20	16.15	13.85
	10000	13552	4.13	20	0.03	12.04
	100000	1355.2	3.13	20	0.04	0.03
HBV02	10	11032000	7.04	20	16.05	15.12
	1000	110320	5.04	20	15.41	15.61
	10000	11032	4.04	20	0.05	5.79
	100000	1103.2	3.04	20	0.03	0.78
HBV03	10	86800000	7.94	20	15.16	14.48
	1000	868000	5.94	20	15.95	14.76
	10000	86800	4.94	20	14.5	14.62
	100000	8680	3.94	20	0.03	0.04

ADDENDUM H. SCATTER PLOT OF ALL RESULTS OF PROCURED HBV-SAMPLE DILUTIONS (N=48)



ADDENDUM I. RESULTS OF PROCURED HCV-SAMPLE DILUTIONS

Serial Number	Dilution	Calculated cps/mℓ	Log of Calculated cps/mℓ	Dilutional factor	DPS Result S/CO
HCV1	1	1067800	6.03	20	9.69
	2	533900	5.73	20	9.56
	4	266950	5.43	20	9.46
	8	133475	5.13	20	9.84
	16	66737.50	4.82	20	9.41
	32	33368.75	4.52	20	0.29
	64	16684.38	4.22	20	0.14
	128	8342.19	3.92	20	0.51
	256	4171.09	3.62	20	0.71
	512	2085.55	3.32	20	0.3
HCV2	1	4598000	6.66	20	9.98
	2	2299000	6.36	20	9.42
	4	1149500	6.06	20	10.04
	8	574750	5.76	20	9.36
	16	287375	5.46	20	9.82
	32	143687.50	5.16	20	0.84
	64	71843.75	4.86	20	1.55
	128	35921.88	4.56s	20	3.17
	256	17960.94	4.25	20	3.9
	512	8980.47	3.95	20	4.17
HCV3	1	2128000	6.33	20	9.84
	2	1064000	6.03	20	9.84
	4	532000	5.73	20	9.51
	8	266000	5.42	20	9.86
	16	133000	5.12	20	7.06
	32	66500	4.82	20	1.44
	64	33250	4.52	20	1.72
	128	16625	4.22	20	2.48
	256	8312.50	3.92	20	2.51
	512	4156.25	3.62	20	2.58

ADDENDUM J. SCATTER PLOT OF RESULTS OF PROCURED HCV-SAMPLE DILUTIONS (N=30)