Dissertation

entitled

Improvement in the antibody detection sensitivity of the Murex Ab/Ag Combination HCV assay.

Submitted

by

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DECLARATION

I, Nityanandi Diaksha Sukhdeo, declare that this dissertation (Improvement in antibody detection sensitivity of the Murex Ab/Ag Combination HCV assay) is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

It is being submitted for the degree of MSc in Life Sciences at the University of South Africa.

I further declare that I have not previously submitted this work, or part of it, for examination at UNISA for another qualification or at any other higher education institution.

27th day of May 2024

DEDICATION AND ACKNOWLEDGEMENTS

This dissertation is dedicated to my parents.

Thank you for your unwavering support and encouragement through this process.

Thank you to UNISA and Professor John Dewar, for allowing me this Recognition of Prior Learning opportunity.

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ABSTRACT

Globally, the hepatitis C virus is one of the leading causes of chronic liver diseases, with a high incidence of these diseases developing liver cancer. Hepatitis C infection may be cured, but its effects may only be realised many years after infection. While much has been done to prevent and mitigate the risk of infection, populations in underdeveloped countries remain vulnerable. Thus, in many countries within sub-Saharan Africa, access to high quality healthcare for many transmissible diseases remains limited. It is estimated that only 5% of those infected with hepatitis C virus are diagnosed, while almost none have treatment options. These statistics have alarming implications for the availability and accessibility of safe blood and blood products. In resource-constrained countries, access to traditional screening methods is limited and expensive, requiring burdensome specialised equipment and training. It is, therefore, imperative to find high quality tests that are accurate, simple to use, and require minimal resources. The Murex HCV Ag/Ab Combination assay is a high-quality serological assay that may be used to screen blood, as it is able to simultaneously detect both HCV antigen and anti-HCV antibodies. A positive result may then be confirmed using more expensive molecular techniques.

The Murex HCV Ag/Ab Combination assay whilst being able to simultaneously detect both antigen and antibodies to HCV, is more sensitive in its detection of the hepatitis C viral particles. The antibody detection aspect of the assay, although robust in its detection of the antibodies to core region 1, 2, and 3 as well as non-structural proteins, has been difficult to prepare. Specifically, detection of antibodies to core region 3 required the addition of large amounts of the detection molecule during preparation to achieve the required assay sensitivity. This not only makes the assay more expensive but also impacts the specificity of the assay by increasing the risk of non-specific binding of assay reagents.

The aim of this study was to improve the ability of the of the Murex HCV Ag/Ab Combination assay to detect antibodies generated to core region 3 of the HCV genome in blood serum by: optimizing the preparation of the enzyme-peptide conjugate; developing an improved method for the removal of excess/unwanted reagents and optimizing the sensitivity of the core capture molecule coated on the microtitre wells.

This experimental study identified variables within the current preparation process for both the capture and detection molecules and evaluated them to determine their effect on the Murex HCV Ab/Ag Combination Assay. A separate capture molecule specific to each region of the

core represented in the Murex HCV Ab/Ag Combination Assay was prepared by the linkage of the each of the peptides to a single bovine serum albumin (BSA) molecule via sulfhydryl bonds. This molecule was then coated onto microtitre wells at a concentration of a 0.2 µg/ml. The detection molecule was prepared by: i) increasing the concentration of sulfo-SMCC modified horseradish peroxidase to 1250 µM; ii) providing sulfo-SMCC in 2.5-fold excess to horseradish peroxidase; iii) providing a 500 mM peptide solution for linkage to the modified horseradish peroxidase; iv) cross-linking the core peptide and modified horseradish peroxidase in pH 6.0 buffer and, v) removing excess and unbound reagents using a Sephadex G25 column and eluting with pH 6.0 buffer. The sensitivity, specificity, and stability of the optimised core and detection molecules were evaluated using a quality control containing antibodies specific to core region 3 only, the antibody positive control contained within the Murex HCV Ab/Ag Combination Assay, as well as 90 normal human serum samples known to be negative for hepatitis C antibodies.

The incorporation of these steps improved the sensitivity of detection of antibodies to core region 3 by 36%, the specificity of the assay by 13%, and the limit of detection was lowered by 50.9%. In addition, the optimised method did not adversely affect the stability of the Murex HCV Ab/Ag Combination Assay.

Incorporation of the suggested steps and reagents may optimize the sensitivity and specificity of the Murex HCV Ab/Ag Combination Assay for the screening of donor blood and blood products. This will provide a more reliable, less burdensome, and inexpensive alternative for the screening of blood in countries with resource constraints. Thus, this will allow more people to access treatment options and positively contribute to the World Health Organizations (WHO) target of decreasing mortality by 65% by 2030.

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LIST OF ABBREVIATIONS

2ME	β-mercaptoethanol
Ab	Antibody
Ag	Antigen
AP	Alkaline phosphatase
DTT	Dithiothreitol
DMSO	Anhydrous dimethyl sulfoxide
DSS	Dried serum spot
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
HCV	Hepatitis C Virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IFU	Instructions for use
KN	Kit Negative Control
l	Litre
ml	millilitre
MWCO	Molecular weight cut off
NAT	Nucleic Acid Testing
NaCl	Sodium chloride
NaOH	Sodium hydoxide
NEM	N-Ethylmaleimide
NHS	Normal human serum
NHS ester	N-hydroxy succinimide
nm	Nanometer
NAT	Nucleic acid testing
OD	Optical density
PBS	Phosphate buffered saline.
PVP	Polyvinylpyrrolidone
QC	Quality control
RNA	Ribonucleic acid
SAMSA	S-Acetylmercaptosuccinic anhydride

SATA	*N*-Succinimidyl S-acetylthioacetate
S/CO	Sample to cut-off ratio
SMCC	Succinimidyl-4-(N -maleimidomethyl) cyclohexane-1-carboxylate
Sulfo-EMCS	*N*-ε-maleimidocaproyl-oxysulfosuccinimide ester
Sulfo-SMCC	Succinimidyl-4-(N -maleimidomethyl) cyclohexane-1-carboxylate (water soluble)

LIST OF TERMS

Conformational stability of a protein: Degree of folded versus unfolded states. The maintenance of a specific conformation is important in ELISA, whereby a specific reaction may proceed.

Inert protein: Protein containing minimal reactive ability.

Non-specific binding: Attachment of unwanted molecules to the solid phase, thus impacting the specificity of the ELISA assay.

Specificity: The specificity of an ELISA assay is its ability to separate and identify the target analyte from all other analytes present in a sample or that which is applied to the assay. It is a crucial requirement for an assay to be specific for the target analyte as this contributes to the accuracy of the test.

Sensitivity: The sensitivity of an ELISA assay is the lowest value of an analyte in the assay buffer at which the analyte can be statistically distinguished from the background. This is a calculated value determined by comparing the numbers of multiple samples of the low standard concentration and the blank.

Efficacy: The efficacy of an ELISA assay is the ability of the test to produce an accurate result.

Coating buffer: The coating buffer used to coat the surface of microtitre wells for ELISA is a solution containing the pre-selected analytes required for the capture of constituents contained within a sample. The pre-selected analytes are then able to passively adsorb to the surface of the microtitre well.

Blocking buffer: The blocking buffer used in the preparation of the coated microtitre wells for ELISA is a solution containing additives that are capable to binding to unoccupied sites on the microtitre wells' surface. This buffer is applied after the coating buffer to "block" sites that are not occupied by the capture molecule(s).

Fixing buffer: The fixing buffer used in the preparation of the coated microtitre wells for ELISA is a solution containing additives that are capable of binding to unoccupied sites on the microtitre wells' surface. It is an additional blocking step.

CHAPTER 1: BACKGROUND

Infection with hepatitis C virus (HCV) causes an acute or chronic inflammation of the liver (Thomas & Lemon, as cited by World Health Organization (WHO), 2002). The hepatitis C virus is a 30-60 nm (Zuckerman, 1996) blood-borne, enveloped, single stranded, positive sense RNA virus, sharing protein sequences with both pestiviruses and flaviviruses (Miller & Purcell, 1990). In 1989, scientists at the Chiron Corporation identified and designated it as hepatitis C virus (Houghton, 2009) and is now known to cause non-A, non-B hepatitis in 70–95% of parenterally transmitted cases (WHO, 2002).

1.1 HCV infection

This virus is transmitted primarily following exposure to infected blood and blood products (Jacyna & Thomas, 1990; WHO, 2012) leading to HCV infection of between 60 and 180 million people with an additional 1-2 million people infected annually, contributing to around 300 00 deaths annually (Lanini et al., 2016; Petruzziello et al., 2016; WHO, 2021a). Infection with HCV according to country indicates a high prevalence in Egypt (4,4% - 15%) (Anwar et al., 2021), as well as Pakistan (6.8%) and Italy (1,5%) (Lanini et al., 2016). Symptoms include fatigue, nausea and jaundice (WHO, 2012). Chronic infection may progress to cirrhosis of the liver, hepatocellular carcinoma and possible death and may be cured using antiviral therapy. However, there is no vaccine available (WHO, 2012) and so early diagnosis of infection is critical to enable successful treatment, to improve quality of life and to help prevent the spread of infection (WHO, 2002).

In 2022, the WHO estimates that 91 million Africans are infected with hepatitis B or C, with 1% of these individuals being infected with hepatitis C virus. Thus, 26% of the global hepatitis B and C burden is in Africa, with 125 000 related deaths in 2020. A review of the literature estimated the HCV seroprevalence to be 3.0% in sub-Saharan Africa. Southern and Eastern Africa were estimated at 1.6%, West Africa at 2.4%, and Central Africa at 6% (Madhava et al., 2002) with the highest HCV seroprevalence being recorded in Egypt, followed by countries in Central Africa (Gabon, Angola and Cameroon) and West Africa (Burkina Faso and Benin) (Riou et al., 2016).

The effects of the COVID-19 pandemic on viral hepatitis diagnosis, monitoring and surveillance are also of concern in sub-Saharan Africa. The currently strained healthcare systems in the region require a systemic and pre-determined approach to the elimination of viral hepatitis (Kazmi et al., 2021). In addition, the frequency of diagnosis of hepatitis C infection is low, with only 5% of the infected being diagnosed with very few patients receiving treatment. These

statistics have severe implications for the safety of blood and blood products, particularly as only 80% of blood donations are subject to quality assurance screening (WHO, 2022).

1.2 Detection of HCV infection

Diagnosis of infection with the hepatitis C virus may be facilitated by the use of a variety of techniques - serological assays for detection of HCV antibodies (enzyme immunoassays), nucleic acid testing (polymerase chain reaction) and genotyping and each technique may be used to detect infection at different stages of the disease (Richter, 2002). The polymerase chain reaction (PCR) is the most sensitive technique for early detection of genes that express HCV viral proteins, however sample contamination and cost implications may be associated with its use as a confirmatory test following a positive EIA result (Lok & Gunaratnam, 1997). While PCR remains the gold standard for the clinical diagnosis of HCV infection, ELISA assays are considered the benchmark for immunoassays particularly for its application in the screening of blood and blood products.

Combination ELISA assays for the simultaneous detection of HCV core antigen and HCV antibodies are a beneficial alternative to anti-HCV and HCV RNA assays for blood screening (Laperche et al., 2005), and for transfusion purposes where reducing the window period is imperative (Lambert, 2007). In countries where the cost of the use of the gold standard for the diagnosis and screening of infectious diseases is prohibitive, alternative techniques are recommended (Haselbeck et al., 2022). The WHO also recognizes that HCV combination assays are an appropriate alternative to HCV RNA assays when NAT cannot b implemented (WHO, 2014).

African countries, where resources are often limited, require guidelines for HCV screening of patients already infected with HIV as antibody-only assays have indicated both false negative and false positive results (Rouet et al., 2015). These authors conducted a study evaluating a fourth generation ELISA in the central African country of Gabon, where 762 HIV positive samples were tested on the G4 Monolisa™ HCV Ag-Ab ULTRA ELISA assay (Bio-Rad, Marnes-la-Coquette, France). This assay shows a specificity for hospitalized patients of 99.5%, and for those patients where interfering substances such as HIV may be present, a specificity of 100%. The assay has a sensitivity of 100%, respectively (Bio-Rad, 2009a). Of the samples tested, 67 samples had a sample-to-cut off (S/CO) greater than 1 (tested positive), of which 70.1% (n=47) were positive for HCV RNA. The most appropriate S/CO for the detection of active infection of HCV for the G4 Monolisa™ HCV Ag-Ab ULTRA ELISA assay was 1.7, indicating a specificity of 91.3% and sensitivity of 97.9%. This was confirmed using a recombinant

immunoblot assay, therefore recommending this combination of diagnostic tests in resource-limited areas (Rouet et al., 2015).

A study conducted in northwestern Nigeria compared the results of ELISA and rapid screening techniques in the detection of the hepatitis B surface antigen (HBsAg). Nigeria is a developing country, located in sub-Saharan Africa where quality healthcare remains a concern (Schoch & Lakner, 2020). Nigeria relies solely on rapid screening tests for the serological screening of blood donors to prevent transfusion-transmission HBV, despite the knowledge that ELISA and nucleic acid testing (NAT) are known to be more sensitive and primarily used in the developed world. Another Nigerian study showed that 100 donor blood samples tested negative using rapid screening techniques but 9 out of these samples (9%) tested positive for the HBsAg when re-tested on the Monolisa™ HBsAg ULTRA ELISA test (Bio-Rad, Marnes-la-Coquette, France). This ELISA test detects hepatitis B surface antigen in human serum and plasma (Bio-Rad France, 2009b). The Monolisa™ HBsAg ULTRA is a single step sandwich ELISA with a specificity and sensitivity of 99.94% and 100% respectively (Bio-Rad France 2009b). The high false negative failure rate (9%) indicated in this study has important implications for the availability of safe blood in Nigeria (Erharbor et al., 2014)

A further Nigerian study on the safety of blood transfusions reviewed HIV serological screening (Orkuma et al., 2014). The analytical sensitivity of assay 1 (the gold standard rapid testing assay used in that study) was compared to ELISA techniques. A total of 440 random donor samples were serially tested using ELISA and a rapid test. The rapid test determined 16 out of 440 (3.6%) were HIV-seropositive whereas the ELISA test indicated 41 out of 440 (9.3%) were seropositive. The rapid test returned a negative result in 25 samples whereas the ELISA returned a positive result for the same samples, thus indicating a 61% false negative rate compared to the ELISA technology used. Orkuma et al. (2014) concluded that rapid testing techniques for the screening of donor blood is insufficient in the prevention of transfusion transmissible HIV. This study also recommended the use of combined HIV antigen-antibody ELISA kits for serological screening in Nigerian hospital-based blood banks.

1.3 HCV enzyme assays

Enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) are predicated on the specific nature of antibodies for their antigenic stimulus, whereby a recognition molecule (antigen or antibody) is reacted with an enzyme-labeled antibody for detection of a molecule of diagnostic interest (Berg et al., 2002). Quantification of this reaction is possible via the reaction of the bound enzyme labeled complex with an appropriate substrate leading

to a relative change in colour of the reactants within the microtiter plate well (Berg et al., 2002). These tests provide an excellent tool for the diagnosis of infectious diseases (Voller et al., 1978). Enzyme immunoassays for HCV are particularly useful for large scale donor blood screening (WHO, 2012). It is a simple, convenient and inexpensive technique (Berg et al., 2002) that is extensively employed for detecting specific HCV molecules present in a sample.

The EIA was the earliest test developed for HCV diagnosis (Kuo et al., 1989) and was especially suitable in detecting the presence of anti-HCV antibodies in serum (Zuckerman, 1996). Following the first EIA for HCV detection, four generations of EIAs have since been developed, initially to detect antibodies produced to one of various epitopes of the HCV proteins and later to detect viral core proteins (Richter, 2002).

The first generation of indirect enzyme immunoassay for HCV diagnosis was based on the detection of anti-HCV IgG but this test displayed low sensitivity in high prevalence populations and a high incidence of false positives in low prevalence populations (Richter, 2002).

In 1992, the Food and Drug Administration (FDA) approved a second generation of indirect HCV EIA (Richter, 2002). This EIA incorporated additional recombinant structural (C22-3) regions from the nucleocapsid and non-structural (NS3, NS4, C100 and C200) regions expressed from the HCV genome (Vrielink et al., 1997). These second-generation tests were able to detect 20% and 10% more antibodies in patients with acute hepatitis and chronic hepatitis, respectively (Richter, 2002).

In 1996, the FDA approved a third generation of indirect EIAs, which incorporated the NS5 antigen in its format and allowed antibody detection on average, 26 days earlier than the second generation EIAs, thus improving the sensitivity of these tests (Richter, 2002).

Fourth generation EIAs employ recombinant antigens from the structural (C-22) and nonstructural (NS3, NS4 and NS5) regions that have been treated to maintain optimum protein conformation to further increase the sensitivity of this technology (Berry et al., 2005).

The Murex Ab/Ag HCV combination assay is a fourth generation, sandwich immunoassay (Dean & Perry, 2007) for the simultaneous detection of HCV core antigen and anti-HCV antibodies in human plasma or serum. This enables increased diagnostic sensitivity by the significant reduction of the window period of HCV detection compared to conventional serological assays (Rodgers & Burch, 2008). The Murex HCV Ab/Ag Combination assay has a demonstrated diagnostic sensitivity of 100%, when tested on 509 specimens from patients with es-

tablished HCV infections. In the early phase of HCV infection, diagnostic sensitivity was established by testing 265 commercially available specimens, in which the Murex HCV Ab/Ag Combination Assay detected 196 as compared to less than half of these (n=94) detected by a competitor's antibody only assay. During the early phase of infection, the Murex HCV Ab/Ag Combination Assay can detect HCV infection 20.57 days earlier than an antibody only assay. When tested on seroconversion panels containing samples in the early phase of infection known to be negative by serological assays and nucleic acid detection, the Murex HCV Ab/Ag Combination assay was able to detect infection one day after NAT, and an of average 33.2 days earlier than an antibody only assay (DiaSorin South Africa, 2014). The Murex HCV Ab/Ag Combination Assay has a diagnostic specificity of 99.82% when tested on 8292 routine donor samples from two different blood donor centers. The initial and repeat reactive was 0.18% (DiaSorin South Africa, 2014). Specificity of the Murex HCV Ab/Ag Combination assay was also evaluated on 376 samples from patients with health conditions other than HCV, such samples from pregnant women, patients with autoimmune diseases, and other viral infectious diseases. Thes specimens had the potential to cross react with the assay. In this sample population, the Murex HCV Ab/Ag Combination was found to have a diagnostic specificity of 99.73%, as 1 (1/376) sample was reactive (DiaSorin South Africa, 2014).

An independent study by Dean and Perry (2007) showed that the Murex HCV Ab/Ag Combination Assay detected infection 3.5 days earlier than a competitor Ag-only assay and 5.5 days after PCR, recommending it as alternative to PCR for early detection of infection.

The Murex HCV Ab/Ag Combination assay (DiaSorin, South Africa) was evaluated against a third generation anti-HCV assay, where 403 serum samples were collected from four different patient groups. These were a control group of healthy patients, a group containing patients infected with HCV, a group containing patients with HCV/HIV co-infections, and a group of patients with uremia. It was found that the Murex HCV Ab/Ag Combination assay (DiaSorin, South Africa) demonstrated a specificity of 95.4% compared to the anti-HCV assay. Thus, the study recommended the Murex HCV Ab/Ag Combination assay (DiaSorin, South Africa) as a possible alternative diagnostic measure particularly in immunocompromised scenarios (Yang et al., 2011)

The sensitivity of the Murex combination assay is based on the ability of the Ab/Ag conjugate to detect complexes bound to the wells of microtitre plates (Rodgers & Burch, 2008). Currently, the wells of polymeric microtitre plates are pre-coated with a carbonate buffer containing anti-core monoclonal antibody (MAb), recombinant antigen and peptides; for binding of viral antigen to the MAb and anti-HCV antibodies to the immunodominant regions of structural and

non-structural viral epitopes (Rodgers & Burch, 2008). These proteins and the surface of the well are blocked with a phosphate buffered saline (PBS) solution containing an inert protein to prevent non-specific binding. Thereafter conformational stability is achieved through the application of, and incubation with, a fixing buffer containing sucrose and polyvinylpyrrolidone (PVP). The functionality of the coated plates is confirmed by the performance of appropriate testing and analysis (Rodgers & Burch, 2008). Thereafter, the HCV Ab/Ag conjugate is produced to suit the functionality of the coated plates.

The Ab/Ag conjugate is prepared through a matching process to a particular batch of microtitre plates, using internal controls that are indicative of sensitivity and specificity. A solution appropriate for lyophilisation is spiked at pre-determined concentrations with five separate detection molecules and then freeze-dried for storage and transportation purposes. Prior to manufacture of the Ab/Ag conjugate, these detection molecules are engineered sequentially through the specific linkage of each separate peptide, recombinant protein and antibody to HRP. Thus, important ELISA kit reagents consist of a monoclonal antibody and a recombinant protein that enable the binding to viral core antigens and non-structural antibodies present in serum, respectively. Three separate peptides representing different regions of the HCV core protein enable the binding to the different structural antibodies present in serum (Rodgers & Burch 2008).

This process typically involves an antibody with a specific binding affinity for an antigen being linked to an enzyme to enable visualization of the antigen of interest, following the degradation of an appropriate substrate (Berg et al., 2002). There are various enzymes available for use, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase, and glucose oxidase (Voller et al., 1978). The HRP is the most popular choice (Hermanson, 2013) and is a heme-containing glycoprotein (Veitch, 2004) with a molecular weight of 40 000 that is found in the roots of horseradish. It is advantageous in diagnostic assays due to its reactivity with hydrogen peroxide which results in the formation of an intermediate that dissociates in the presence of an electron donor. This dissociation elicits a quantifiable colour change. Other advantages of HRP include its small size and stability, particularly under the conditions required for conjugation, and retention of activity after conjugation to another molecule (Hermanson, 2013).

In order to effectively conjugate molecules, conditions under which conjugation occur must be optimized. Conjugation should occur in a buffer that is appropriate for the reactivity of the molecules e.g., phosphate buffers are optimal for reaction of thiols at physiological pH. The pH and ionic strength of the reaction buffers are of utmost importance as this will determine

whether the correct reactions occur. Dissolution of the linking reagent must be ascertained as many are reactive or insoluble in aqueous solutions. Organic solvents such as methanol and dimethylformamide (DMF) may be used. Temperature and length of reaction time are also of importance to promote optimal cross-linking (Brinkley, 1992). It is often necessary to determine the extent to which linkage has occurred. This may be achieved by measuring the concentration of free residues by, among others, colorimetric and spectroscopic methods (Brinkley, 1992).

Of particular importance to this project is the detection molecule representing core region 3 of the HCV genome. The freeze-dried Murex HCV Ab/Ag combination conjugate contains this specific molecule in addition to four other molecules at different concentrations. The detection molecule specific to core region 3 is an amino acid sequence that is covalently linked via the formation of thioether bonds to horseradish peroxidase to form an enzyme-peptide conjugate (Rodgers & Burch, 2008). It is of paramount importance that contaminants such as unreacted label molecules and excess cross-linking reagents be removed from the modified molecule (Brinkley, 1992).

There are several established techniques available for protein purification, based on size, ionic properties and binding capabilities. These may be used singly or in combination to achieve the required purity (Berg et al., 2002). These techniques include gel filtration or size exclusion chromatography, ion exchange chromatography, and other adsorptive techniques such as affinity binding of a molecule with a specific ligand (Berg et al., 2002). Protein concentration and fractionation achieved by protein precipitation (Burgess & Deutscher, 2009) may also be used for protein purification. Dialysis is another separation technique that can be used, whereby molecules move by diffusion across a semi-permeable membrane (Berg et al., 2002).

This study will focus on the capture and detection molecules of the Murex HCV Ab/Ag Combination assay that are able to bind to antibodies produced to the immunogenic region of the core (structural) region of the HCV capsid protein. Whilst this pair of molecules continue to perform at DiaSorin South Africa as per the claims listed in the assays instructions for use (IFU); it is good practice to periodically review diagnostics assays already on the market due to the high amount of genetic sequence diversity and variation, i.e., genetic heterogeneity. HCV has a global distribution of the more 30 genotypes, in addition to its ability to present quasispecies distribution in infected individuals (Kato, 2003). HCV diagnosis and pathogenesis may be hindered by this fact as well as the virus's ability to change the genetic sequences circulating in an individual during the course of an infection. (Kato, 2003). These changing sequences allows the infection to remain active as the antibodies produced by the host earlier

in the infection are unable to provide protection (Bukh et al., 1995). It was noted; after analysis of the core, envelope, and nonstructural regions of HCV isolates, that there was variation in the genetic sequences for these regions within a genotype. The distinction of these regions between genotypes were also not clear. This has profound implications for ELISA assays, as its performance relies on the detection of the antigen and antibodies related to specific genetic regions (Bukh et al., 1995).

In addition, an evaluation of the Monolisa Ag/Ag Ultra V2 (Bio-Rad, Marnes la Coquette, France) and the Murex HCV Ab/Ag Combination assay (DiaSorin, South Africa) was conducted on 1102 recently diagnosed HIV positive clinical dried serum spots (DSS) eluates. These samples were evaluated for possible co-infection with hepatitis C. The study found that both assays performed similarly when detecting HCV antigen and antibodies in undiluted clinical serum/plasma samples. However, the Monolisa Ag/Ag Ultra V2 (Bio-Rad, Marnes la Coquette, France) was more sensitive in its detection of hepatitis C antibody eluates and their subsequent dilutions than the Murex HCV Ab/Ag Combination assay (DiaSorin, South Africa). Dried serum spot or plasma spot samples eluates of HCV seroconversion panels and longitudinal antibody serum samples were used to evaluate sensitivity. During the evaluation of the HCV seroconversion plasma samples; the Murex assay was reactive earlier than the Bio-Rad assay for antigen positive bleeds. The evaluation indicated that the Bio-Rad assay had a false positive rate of 1.9% (2/105) versus the Murex assay, which had a false positive rate of 22.8% (28/123) (Eshetu et al., 2020). As a higher false positive rate will affect the overall specificity of an assay, this study indicates that the specificity of the Murex HCV Ab/Ag Combination assay for samples with HIV co-infection is 77.8% (95/123). This contrasts with the diagnostic specificity of 99.73% for specimens with potential cross reactivity as listed in the Murex HCV Ab/Ag Combination assay IFU. Thus, this study further supported the need to review the Murex HCV Ab/Ag Combination assay, in order to improve its sensitivity and specificity for the detection of antibodies.

The capture molecule is a peptide conjugate containing peptides representing three regions of the core of HCV capsid protein, that has been immobilized onto the surface of a microtitre well. However, this study will only focus on core region 3.

The detection molecule is an enzyme-peptide conjugate employed in the binding and visualization of antibodies specific to core region 3 that have been captured onto the microtitre well. These peptide reagents are particular to the Murex HCV Ab/Ag Combination Assay and this study intends to improve the overall sensitivity and specificity of this reagent and so of the kit as a whole.

1.4 Problem statement

The Murex HCV Ab/Ag Combination Assay has been commercially available since 2008 and the method for preparation of its components have not been updated since then. Since inception of the assay, during the bulk preparation and internal processing of the detection molecule that binds to antibodies specific to core region 3;it was noted that the efficacy of the internal quality assurance measures that detect and assay this region of the core, perform significantly lower as compared to the other core-detecting quality assurance measures. Therefore, a large amount of this enzyme-peptide conjugate is required during bulk preparation to ensure that the resultant freeze-dried conjugate can titrate the test antibodies at acceptable predetermined standards. On average, more than 20 times the amount of the detection molecule for core region 3 is required for preparation of the freeze-dried conjugate, compared to the detection molecules for core region 1 and 2. This suggests that the preparation of this enzyme-peptide conjugate may be improved, or there may be impurities present that have an inhibitory effect on its performance, or a combination of these, or other factors. In addition, the use of large amounts of enzyme-peptide conjugate containing horseradish peroxidase affect the specificity of the overall assay, by increasing the possibility for non-specific binding. Improvement in this process will enable the preparation of a higher quality, final freeze-dried conjugate, allowing the Murex HCV Ab/Ag Combination assay to continue to meet its product claims in terms of sensitivity and specificity. Unfortunately, no literature is available documenting this problem, as the Murex HCV Ab/Ag Combination Assay is commercially available, and the techniques used in preparation of its components are proprietary information. Such detail and history of the preparations are only available as batch records and may not be disclosed. In terms of specificity and sensitivity, the Murex Ab/Ag Combination assays continues to perform as per the claims listed in its Instructions for Use (IFU), and this problem is currently limited to the performance of the quality assurance measures used in the preparation of the freeze-dried conjugate.

It is crucial to maintain the sensitivity and specificity of the Murex HCV Ab/Ag Combination assay, as it may provide an inexpensive, convenient and sensitive alternative to PCR (Dean & Perry, 2007). Additionally, the continuous improvement of EIAs to date indicates that this technique is robust and that further improvement to increase sensitivity is possible.

1.5 Study aims and objectives.

Thus, the aim of this study was to maintain the sensitivity and specificity of the Murex HCV Ab/Ag Combination assay by improving the assays' ability to detect antibodies in blood serum that were generated to the protein expressed by the core region 3 of the HCV genome.

In achieving this aim, the following study objectives were addressed:

1. to improve the conjugation and activity of an enzyme-peptide detection molecule used to detect antibodies specific for the structural (core) region 3 of the hepatitis C virus.
2. to develop an improved method for the removal of excess unbound reagents from the detection molecule.
3. to improve the sensitivity of the assay by improving the ability of the capture molecule coated onto the microtitre wells to bind to antibodies generated to core region 3.

CHAPTER 2: INTRODUCTION

The liver is the second largest organ in the human body, weighing approximately 1.4 kg, and is situated in the upper, right section of the abdomen. The main functions of the liver include: (1) secretion of bile for digestion, (2) metabolism of carbohydrates, proteins and lipids, (3) the processing of hormones, toxins and drugs found in the bloodstream, (4) storage of vitamins, (5) activation of vitamin D and (6) phagocytosis of aged red blood cells and certain types of bacteria (Tortora & Derrickson, 2008). As such, the liver is critically important to maintaining health but is vulnerable to viral and bacterial infection, is affected by adverse reactions to drugs and toxins, is damaged by metabolic diseases and various causes of ischaemia – all conditions requiring immediate diagnosis or risk liver disease and/or acute liver failure (Sherlock & Dooley, 2008).

2.1 Hepatitis

Hepatitis is an inflammatory condition of the liver, most commonly as a result of infection by a hepatitis virus. However, the condition can also be caused by other infection, abuse of drugs and alcohol, or by certain autoimmune conditions (WHO, 2019). Hepatitis can be self-limiting or have no clinical presentation, making diagnosis and follow up treatment exceptionally difficult (Thomas & Lemon cited in WHO, 2002). However, hepatitis can manifest as acute or chronic liver inflammation beginning with fibrosis, progressing to cirrhosis and eventually to hepatocellular carcinoma, the most prevalent type of cancer (American Cancer Society 2018) while infection with hepatitis A virus (HAV), hepatitis B virus (HBV) and hepatitis C virus (HCV) may clinically present as jaundice, abdominal pain, influenza-like symptoms and fatigue (Gupta, 2018).

2.1.1 Viral hepatitis

In 2017, the Global Hepatitis Report issued by the World Health Organization reported 1.34 million fatalities resulting from viral hepatitis infection in 2015, a figure surpassing death associated with HIV and similar to that of tuberculosis. Although mortality rates for both HIV and tuberculosis showed a decline in 2015, mortality associated with viral hepatitis showed a 22% increase compared to the figure for 2000 (WHO, 2017). Both the American Cancer Society as well as the WHO lists chronic infection with the hepatitis B and hepatitis C viruses as the highest contributors towards liver cancer (Liver Cancer Risk Factors, 2019; WHO, 2017).

There are five known types of hepatitis viruses - HAV, HBV, HCV, HDV and HEV and infection with any of these viruses may cause acute or chronic liver disease. It is impossible to distinguish the type of hepatitis virus from its clinical manifestation, but each type of hepatitis virus is unique regarding its transmission, epidemiology, diagnosis and treatment management (WHO, 2019).

2.1.1.1 Hepatitis A virus (HAV)

Hepatitis A virus (HAV) belongs to the *Picornaviridae* family and is a single stranded, positive sense RNA virus without an envelope (Thomas et al., 2013). The virus is found in human stool and is transmitted via direct contact or following ingestion of contaminated water and food. The spread of HAV is exacerbated in areas with limited access to clean drinking water and proper ablution facilities. Infection with HAV is often mild and does not cause chronic liver disease - as reflected in WHO estimates from 2016 that infection with HAV accounted for 7134 fatalities. Clinically, a patient infected with HAV recovers, after which he/she becomes immune to repeat infections, but severe/fatal infections may result from extensive liver necrosis and atrophy. A safe and effective vaccine for the HAV is available (WHO, 2018).

2.1.1.2 Hepatitis B virus (HBV)

Hepatitis B virus (HBV) is a small, enveloped, partially double stranded DNA virus, belonging to the family *Hepadnaviridae* (Ozaras & Arends, 2019). The virus is transmitted by contact with infected blood, blood products, semen and other bodily secretions. Infection with HBV may cause chronic and acute liver disease that can eventually lead to liver cancer and death. In 2019, the WHO estimated that 296 million people were chronically infected with HBV, with 1.5 million new infections reported each year. A safe and effective vaccine conferring nearly 100% immunity is available for HBV (WHO, 2023).

2.1.1.3 Hepatitis D virus (HDV)

Hepatitis D virus (HDV) belongs to the *Deltavirus* family. It is a small, spherical virus surrounded by a layer of hepatitis B envelope proteins (Gupta, 2018). The HDV is only able to infect persons who are already infected with HBV, as it requires HBV for viral replication in the host cell. The virus is transmitted in the same way as HBV and HDV can also present as a superinfection when the virus infects a person who has already contracted HBV. This dual infection characteristic results in an exacerbation of the clinical symptoms causing expeditious progression to hepatocellular carcinoma and death. Globally, the WHO estimates that 5% of people infected with HBV are also infected with HDV, with these people mainly located in Mongolia, the Republic of Moldova, and western and central Africa. Therefore, vaccination against the HBV is of vital importance as it simultaneously confers immunity to the HDV (WHO, 2023).

2.1.1.4 Hepatitis E (HEV)

Hepatitis E virus (HEV) is a non-enveloped, single stranded RNA virus, belonging to the *Hepeviridae* family (Meng, 2008). The virus is found in infected stools and is primarily waterborne. Transmission occurs through contact with contaminated food and water (Ozaras & Arends, 2019). The HEV is only enveloped in host cell membranes providing it with a unique mechanism for resistance to neutralising antibodies (Yin et al., 2016; Das et al., 2023). Infection with Hepatitis E is endemic in low-income countries, where access to clean drinking water can be limited. The WHO estimates that annually there are 20 million new Hepatitis E infections. In 2015, 44 000 fatalities resulted from infection involving the HEV. The infection is usually self-limiting; however, it has a high rate of morbidity and mortality in pregnant women and new-borns. A vaccine was developed in 2011 but is only available in China (WHO, 2018).

2.1.1.5 Hepatitis C virus (HCV)

Hepatitis C viruses are single stranded, enveloped, positive sense RNA viruses classified in the *Flaviviridae* family of viruses (Bukowski, 2009), and is the exclusive member of the genus *Hepacivirus* (Bartenschlager & Lohmann, 2000). The virus was first observed in 1975 (WHO, 2002). It was designated non-A, non-B and is now known to cause non-A, non-B hepatitis in 70–95% of transfusion-associated hepatitis cases (Jacyna & Thomas, 1990). In 1989, scientists at the Chiron Corporation identified and designated it as HCV (Houghton, 2009). This virus is a bloodborne, parentally transmitted virus, spread primarily through exposure to infected blood and blood products (WHO, 2012). The virus causes both acute and chronic liver disease although infection can be asymptomatic.

Antiviral treatment for infection with HCV is available and can have a cure rate of 95%. This treatment will be described below. However, despite extensive research, a vaccine against HCV infection is not yet available (WHO, 2019). These alarming statistics, coupled with the asymptomatic feature of hepatitis C infection, highlight the need for reliable and accurate diagnostics.

2.2 Epidemiology of hepatitis C virus infection

Infection with HCV is globally prevalent and WHO statistics from 2015 indicate that HCV infection is highest in Europe and the Eastern Mediterranean. Globally, 71 million people live with hepatitis C-induced chronic liver inflammation and in 2016 there were an estimated 399 000 fatalities due to HCV-associated liver cancer and cirrhosis. From a global perspective, the prevalence of chronic hepatitis C virus infection is high in Egypt at 22%, Pakistan at 4.8% and China at 3.2% (WHO, 2012). In these countries, the most prevalent mode of transmission of the virus is considered to be contaminated or inadequately sterilised needles used by healthcare professionals during the provision of health care services (Wasley & Alter, 2000).

Whilst statistics reflect that Europe and the Mediterranean region have a high prevalence of HCV infection, the socio-economic context of African must be taken into account particularly as around 8 million Africans are chronically infected (WHO, 2024) and 20% of the global HCV-related viraemic burden is in sub-Saharan Africa (Mohd Hanafiah et al., 2013). In sub-Saharan Africa, the death toll from liver cirrhosis has nearly doubled from 53 000 in 1980 to 103 000 in 2010, second only to HBV infection as the leading cause of mortality related to hepatocellular carcinoma (Mokdad et al., 2014; Sonderup et al., 2017). Thus, infection with HCV is increasingly becoming a major public health concern in Africa and it is estimated that 0.8% of the population in South Africa in 2015 was infected with HCV (Wedemeyer et al., 2015).

2.3 Treatment of hepatitis C infection

As mentioned, infection with HCV is amongst the leading causes of cirrhosis and fibrosis of the liver and hepatocellular carcinoma and so it is the most common requisite for liver transplantations (Shahid, 2018). Most cases (80%) of HCV infection remain asymptomatic for decades allowing for liver damage and so diagnosis is often late following the onset of symptoms. The action of the immune system can eliminate recent HCV infection but when this is unsuccessful, chronic hepatitis C infection sets in, and treatment becomes imperative. The WHO guidelines recommend pan-genotypic direct-acting antivirals (DAAs) for all cases of chronic infection in people over the age of 18 (WHO, 2018). In treating infection in adolescents between the ages of 12 and 17 years, the WHO recommends the use of sofosbuvir/ledipasvir for

HCV genotypes 1, 4, 5 and 6, and sofosbuvir/ribavirin for HCV genotype 2 and 3. Until recently, infections in children under the age of 12 involved deferment of treatment until 12 years of age but in October 2022 the WHO recommended the use of DAA treatment for children with chronic HCV infection who were over the age of 3 years (WHO, 2022). Such DAAs are effective in curing almost all such infections within 12 to 24 weeks but such treatment is expensive, even in developed countries. However, generic medicines are becoming increasingly available and affordable, particularly in developing countries (WHO Hepatitis C, 2019). A complicating factor to both diagnosis and treatment of hepatitis C infections are hepatitis C virus plus human immunodeficiency virus (HIV) co-infections.

Current global estimates of co-infection indicate that 5.5 million people are currently living with HCV and HIV, of which 2.5 million are in South East Asia and Africa (Rouet et al., 2015; Mohd Hanafiah et al., 2013). This portends accelerated disease progression as a result of the compromised immune system, as well as the current availability of treatment for HIV infection but not for HCV infection.

2.4 Mechanism of infection of hepatitis C virus

The hepatitis C virus infection strategy is represented in Figure 1. This figure indicates that exposure to infective bodily fluids may introduce HCV virions into the body allowing them to circulate in the bloodstream, or they may be surrounded by host lipoproteins (Dustin et al., 2016).

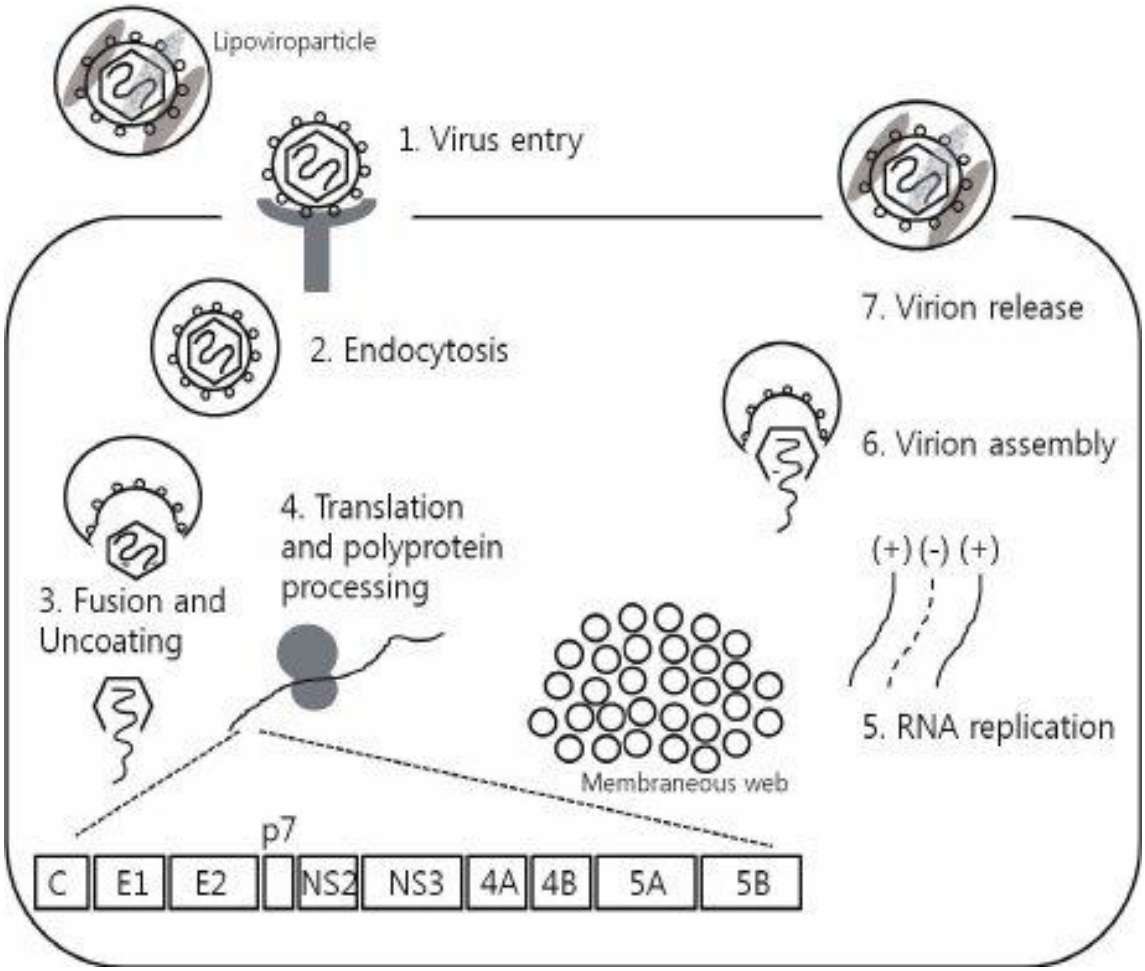


Figure 2.1: A schematic representation of hepatitis C virus replication. Adopted from Kim and Chang (2013).

The mechanism of cellular infection involves the attachment of the viral particle onto the host cell, followed by penetration of the host cell membrane (Bartenschlager & Lohmann, 2000). Once the virus has entered the hepatocyte cytoplasm, the viral capsid disintegrates to release the HCV RNA allowing its translation into a polyprotein in the rough endoplasmic reticulum. This polyprotein is cleaved into smaller proteins, each of which have different viral replication, structural and assembly functions allowing the assembly of nascent viral particles. Once viral assembly is complete, the intact virions are released from the infected cell by exocytosis.

The hepatitis C viruses closely associate with the hosts' endogenous lipoproteins to facilitate evasion of immune detection and response (Dustin et al., 2016), a process closely linked with lipid metabolism (Kim and Chang, 2013). During active infection, the hepatitis C virus is able to produce 10 trillion virion particles per day (Neumann et al., as cited in Kamili et al., 2012).

Once the virus has replicated in the liver cell, it is present and able to cause infection in bodily

fluids facilitated by endogenous immunomodulation capabilities, where it is able to alter both the adaptive and innate host immune responses to its advantage. An example of this is that the hepatitis C core antigen is able to alter cytotoxic T cell differentiation, which results in extended replication cycles with little or no seroconversion (Francavilla et al., 2004).

2.5 Hepatitis C virus genome

2.5.1 Hepatitis C protein components

The core component of the HCV capsid structure is a dimeric protein containing two domains, one hydrophilic and the other hydrophobic. The charged hydrophilic domain (D1) aids in nucleocapsid formation. The hydrophobic domain (D2) enables attachment and binding to the endoplasmic reticulum. The envelope proteins, E1 and E2, are located at the N-terminus of the polyprotein and form a non-covalent heterodimer that is postulated to be the basic functional unit of the envelope facilitating the entry of the viral particle into the host cell (Popescu et al., 2011).

The non-structural (NS) proteins, i.e., NS3, NS4 and NS5, are situated at the C-terminus of the polyprotein and constitute the viral replication complex. Protein NS3 is responsible for the processing of a helicase and a serine protease that binds to NS4A, which processes the cleavage sites of the polyprotein. Protein NS3 is associated with viral assembly as is protein NS4B, a hydrophobic protein containing four transmembrane regions that is also responsible for modification of membrane architecture. Protein NS5A is an RNA-dependent RNA polymerase and is the most important polyprotein component in viral replication where it is involved in viral assembly as well as regulation of replication (Popescu et al., 2011).

Protein p7 and the NS2 portions of the polyprotein are also non-structural in nature in that p7 is a small viroporin associated with assembly and subsequent release of the intact viral particle into the host blood stream while NS2 is a multi-functional transmembrane protein: it catalyses the processing of the C-terminus as well as assisting in the viral assembly process by connecting the structural and non-structural proteins (Popescu et al., 2011).

Serological responses in the host cell are directed to these polyprotein component antigens allowing their use in diagnostic assays for detection of HCV infection (Zein, 2000). This is discussed further under diagnosis of hepatitis C below.

2.5.2 Structure of the hepatitis C virus genome

The hepatitis C virion has a diameter of 30-60 nm (Zuckerman, 1996) and is a blood-borne, enveloped, single stranded, positive sense RNA virus, sharing protein sequences with both

pestiviruses and flaviviruses (Miller and Purcell, 1990). The virus genome is approximately 9600 nucleotides in length and has an untranslated region (UTR) at both the 5' and 3' ends as well as an open reading frame (ORF) encoding a polyprotein consisting of around 3 000 amino acids. Peptidases and proteases in the structural and non-structural regions, respectively, are able to cleave the larger polyprotein into 10 single proteins (Kamili et al., 2012).

The hepatitis C virus is distinctive in its ability to evade host immune detection and associated responses due to the mutability of its genome. These mutations are caused by errors that occur during the rapid replication and poor proofreading by viral RNA polymerase (Dustin et al., 2016). The resulting genetic diversity is categorized into several genotypes, within which are at least 100 geographically distinct subtypes (Simmonds et al. cited in Budkowska, 2009).

2.5.3 Hepatitis C genotypes

The heterogeneity of hepatitis C viruses has allowed their division into genotypes 1 to 7 that can be further categorised into 86 subtypes (Smith et al., 2014; Palladino et al., 2018). Genotypes 1, 2, and 3 are prevalent worldwide, whereas 4, 5, and 6 are specific to certain geographical locations. Genotypes 4 and 5a are concentrated in the Middle East and the northern regions of South Africa while genotype 6 is concentrated in China and Southeast Asia (Ju et al., 2015). Genotype 1 is most widely spread and occurs mainly in high to middle income regions and is responsible for causing 44% of global infections. The second most prevalent is genotype 3 responsible for 25% of all global infections. Genotype 3 occurs in lower middle-income countries in Southern Asia. As many as 15% of all global infections are caused by genotype 4, particularly in low-income countries in Africa and the Middle East (Blanch et al. cited in Palladino et al., 2018). The rarest genotype, genotype 5, is found mainly in Southern Africa where 40% of the South African population infected with hepatitis C are infected with this genotype. Within South Africa, the frequency of infection of genotype 5 is followed by genotype 1b with 30% of the population infected (Wedemeyer et al., 2015). As almost half of the infected South African population is infected with the rarest genotype, this further substantiates the critical need for quality diagnostic tools to determine HCV infection. As HCV shows genetic heterogeneity (Kato, 2009), this study also substantiates the need to independently validate HCV assays that, over time, still effectively detect evolving populations of hepatitis C viruses with acquired mutations and altered phenotypes.

2.6 Diagnosis of hepatitis C virus

It is of critical importance that early diagnostic screening processes and tests are in place, in order to meet the WHO, target of decreasing mortality from liver disease by 65% and the reduction of new infections by 90% by 2030 (WHO, 2021a). Therefore, early diagnosis of infection will allow for treatment regimens to be instituted to achieve this target.

Diagnosis of infection with the hepatitis C virus makes use of a variety of techniques: serological assays for detection of specific HCV antibodies (anti-HCV), nucleic acid testing (polymerase chain reaction) as well as genotyping. Each technique may be used to screen different stages of infection (Richter, 2002).

The polymerase chain reaction (PCR) is considered the most sensitive technique for early detection of gene sequences that express HCV viral proteins, however sample contamination and cost implications may force its use as a confirmatory test following a positive ELISA result (Lok & Gunaratnam, 1997). Typically, blood screening for hepatitis C infection is initiated by screening for HCV antibodies using an anti-HCV serological test. A positive serological test is followed by a nucleic acid test required to test for the presence of HCV RNA, in order to determine if there is chronic infection. This is important for treatment plans, as the immune response of approximately a third of those infected (i.e. who test anti-HCV positive) is sufficient to eliminate the virus without treatment. In addition, early diagnosis is key not only to the development of treatment plans, but also in the prevention of further transmission. The WHO advises proper diagnostic options, and thereafter treatment, care, and education be made available in regions with a high antibody seroprevalence (WHO, 2021b). High quality rapid tests at point-of-care (POC) are becoming increasingly popular in sub-Saharan Africa, as it offers simpler, cheaper, and more decentralised methods of delivery to populations that have access constraints. However, laboratory-based testing is still the preference for accurate diagnosis of infection (WHO, 2021a).

Enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) provide an excellent tool for the diagnosis of infectious diseases (Voller et al., 1978). The acronyms EIA and ELISA may be used interchangeably. More recently, ELISA techniques have been shown to have particular application in the screening of blood donors for infectious diseases, such as HIV (Ayuba et al., 2021).

Anti-HCV tests are indirect in nature and rely on the use of enzyme-linked immunosorbent assays to detect specific anti-HCV antibodies in the blood sample of seroconverted individuals.

These assays are discussed in detail below. The serological window period is on average 6 to 8 weeks from infection (Gupta et al., 2014) or may be up to twelve months in patients who are immune deficient (Van der Poel et al., 1994; Ré et al., 2005). This introduces an additional layer of complexity in detecting hepatitis C, particularly in the blood bank market, where the availability of suitable blood and/or blood products is of critical importance. In addition, co-infection with HIV can result in an inhibition of the immune response, where HCV seroconversion is undetectable by indirect methods. This can result in false negative results, and in turn, the possibility of a patient receiving infected blood (Mullis et al., 2013; Fox et al., 2015).

There are many advantages to the use of ELISA assays: it is simple to use and relatively easy to execute. The technique requires small volumes of the samples and reagents. Adsorption to a plastic surface is uncomplicated. Washing of the microplate surface with a detergent removes excess unbound reagents. The ELISA assays are also sensitive, exhibiting detection abilities of 0.01 – 1 µg per ml and are also inexpensive compared to other techniques, with both start-up and reagent costs being relatively low. Equipment required by this technique is easily available. The technique is versatile, meaning that it can be executed under conditions that do not meet state of the art (Crowther, 2001).

These simple, convenient, versatile, and inexpensive assays are predicated on (1) the specific interaction between an antibody and the antigen that stimulated their synthesis, allowing (2) a recognition molecule (antigen or antibody) to react with an enzyme-labelled antibody for (3) a colour change that indicates detection of a molecule of interest (Berg et al., 2002). These authors also described quantification of this reaction involving serial dilution of sample followed by the reaction of the bound enzyme-labelled complex with an appropriate substrate leading to a relative change in colour of the reactants within adjacent wells.

Enzyme immunoassays for HCV may be performed manually with comparatively few instrument requirements but may also be automated on platforms for high throughput screening and are of particular use for large scale donor blood screening (WHO, 2012). There are four different types of ELISAs available for diagnosis: direct, indirect, sandwich and competitive (Crowther, 2001).

2.6.1 Direct ELISA

The basis of the direct ELISA involves a direct reaction of the enzyme-labelled antibody with the antigen (Crowther, 2001). This method of ELISA is the simplest and fastest to perform.

2.6.2 Indirect ELISA

Indirect ELISAs are the most popular and are used to detect antibodies in serum samples, following the binding of antigens coated on a plate surface with antibodies present in a serum sample (Berg et al., 2002). Recognition of this bound complex is facilitated by the addition of an enzyme-libelled antibody conjugate followed by enzyme substrate (Voller et al., 1976).

2.6.3 Competitive ELISA

Competitive ELISAs are somewhat different from other types of ELISAs, in that they involve the competitive binding of labelled and unlabelled antigens for binding sites on an antibody (Voller et al., 1976). Unlike other forms of ELISAs, reaction colorimetric intensity is inversely proportional to the concentration of antigen present in the sample (Crowther, 2001).

2.6.4 Sandwich ELISA

Of particular relevance to this study are sandwich ELISAs. These are based on the premise of a capture antibody as well as a labelled detection antibody, whereby the antigen is captured between these two antibody moieties (Crowther, 2001). In this application, two antibodies that bind to different antigenic sites are used. The primary or capture antibody, which has a high specificity for the antigen of interest, is adsorbed onto a solid surface; in this instance, the inner wall of a polymeric microtitre plate. The antigen is then applied. The secondary or detection antibody, which binds to a different antigenic site from the capture antibody is then applied (Crowther, 2001). The detection antibody is usually enzyme-labelled, in order to elicit a colour change upon addition of an appropriate substrate solution (Voller et al., 1976). This form of ELISA is advantageous in that it both detects and quantifies the molecule of interest (Berg et al., 2002).

The ELISA was the earliest test developed for HCV diagnosis (Kuo et al., 1989) and was especially suitable in detecting the presence of anti-HCV antibodies in serum (Zuckerman, 1996). Following the first ELISA for HCV detection, four generations of ELISAs have since been developed, initially to detect antibodies produced to one of various epitopes of the HCV proteins and later to detect viral core proteins (Richter, 2002).

- The first generation of indirect enzyme immunoassay for HCV diagnosis was based on the detection of anti-HCV IgG making use of the C100-3 section of the NS4 region of the HCV polyprotein (Richter, 2002). However, this test displayed low sensitivity in high prevalence populations and a high incidence of false positives in low preva-

lence populations (Richter, 2002). Antibodies in response to this section of the genome were also found to be generated at a much later stage of infection, in chronically infected patients (Zuckerman, 1996), reducing the sensitivity and usefulness of this test.

- In 1992, the Food and Drug Administration (FDA) approved a second generation of indirect HCV ELISA (Richter, 2002). This ELISA incorporated additional recombinant structural (C22-3) regions from the nucleocapsid and non-structural (NS3, NS4, C100 and C200) regions from the HCV polyprotein (Vrielink et al., 1997). In comparison to the first-generation assays, these second-generation tests were able to detect 20% and 10% more antibodies in patients with acute hepatitis and chronic hepatitis, respectively (Richter, 2002). Antibodies to the C22 structural region are detectable at an early stage of infection (Zuckerman, 1996), facilitating diagnosis of infection 6 weeks earlier than that of the first-generation tests (Vrielink et al., 1997). Sensitivity, as well as specificity, was improved by this generation of ELISAs (Zuckerman, 1996).
- In 1996; the FDA approved a third generation of indirect ELISAs, which incorporated the NS5 antigen into its format and allowed antibody detection on average, 26 days earlier than the second generation ELISAs, thereby improving the sensitivity of these tests (Richter, 2002). Third generation ELISAs exhibited improved sensitivity for a sub-group that generated antibodies to the NS5 region (Vrielink et al., 1997).
- Fourth generation ELISAs employ recombinant antigens from the structural (C-22) and non-structural (NS3, NS4 and NS5) regions that have been treated to maintain optimum protein conformation to further increase the sensitivity of this technology (Berry et al., 2005).

2.7 The Murex HCV Ab/Ag Combination Assay

The patent application for the development of this assay was submitted by Rodgers and Burch (2007) and the kit was evaluated by Dean and Perry (2007) prior to the approval of the application.

The Murex Ab/Ag HCV combination assay is a fourth generation, qualitative, direct sandwich enzyme-linked immunoassay (Dean & Perry, 2007) for the simultaneous *in vitro* detection of the hepatitis C core antigen and anti-HCV antibodies in human plasma or serum (Rodgers and Burch, 2008). These authors further described that this assay is unique in its ability to reduce the window period between infection and detection. In addition, the combination format is of particular use as it can be eliminate the complication of anti-HCV false-negative

results in cases where immunosuppression due to HIV co-infection is present (Thio et al., 2000). The contents of this diagnostic kit is indicated in Figure 2 and includes (1) pre-coated microtitre wells, (2) sample diluent, (3) Tween saline wash buffer concentrate, (4) negative and positive controls, (5) freeze-dried conjugate, (6) conjugate diluent, (7) substrate concentrate and (8) substrate diluent (Rodgers & Burch, 2008).

Briefly, sample is incubated in wells of microtitre plates at 36-38°C for 60 minutes, before Tween saline wash buffer is used to remove excess unbound proteins present in each sample well. Antigens and antibodies, if present in the sample, will form bound complexes on the surface of each microtitre well, by binding to the coated capture molecules. Reconstituted conjugate is added to the wells and incubated at ambient temperature (approximately 15-28°C) for 60 minutes, allowing specific interaction between the conjugate and the bound complexes. Washing with Tween saline after the incubation period removes unbound conjugate before detection of the bound complex is achieved. This involves specific catalytic cleavage of free substrate, 3,3', 5,5'-tetramethylbenzidine (TMB), by horseradish peroxidase (HRP) when added to each well and incubated at 36-38°C for 30 minutes. Following termination of the reaction with sulphuric acid, the cleaved substrate daughter compound is detected colorimetrically at 448 nm (reference wavelength 620/690 nm). A positive result is indicated by a blue-green colour that changes to orange upon addition of sulphuric acid; whilst a negative result can be identified by the change to between yellow and pink (Rodgers & Burch, 2008). The test can be used on automated platforms, as well as manually and this allows for a high degree of versatility, which is especially beneficial in resource-limited countries.

Figure 2.2: The Murex HCV Ab/Ag Combination 450 Test Kit (Photo by DiaSorin, South Africa)

2.7.1 The basis of the Murex HCV Ab/Ag Combination Assay

The sensitivity of the Murex HCV combination assay is based on the ability of the Ab/Ag conjugate to detect antigen-antibody complexes bound to the wells of microtitre plates (Rodgers and Burch, 2008). Currently, the wells of polymeric microtitre plates are pre-coated with an anti-core monoclonal antibody (MAb), recombinant antigen, as well as peptides representing selected epitopes of HCV core region, with the aim of binding the viral antigen to the monoclonal antibody (MAb) and the anti-HCV antibodies to the immunodominant regions of the structural (core) and non-structural viral epitopes (Rodgers & Burch, 2008). These proteins are blocked with an appropriate inert protein to prevent non-specific binding, thereafter conformational stability is achieved through the application and incubation of a fixing solution. The functionality of the coated plates is confirmed by the performance of appropriate testing and analysis (Rodgers & Burch, 2008).

Thereafter, the HCV Ab/Ag conjugate is prepared to suit the functionality of the coated plates. Figure 2.3 below illustrates the functionality of the Murex Ab/Ag-coated microtitre plates. The

Murex Ab/Ag conjugate is prepared to include levels of individual enzyme peptides conjugates to each capture complex coated on the microtitre plate. This is performed to ensure that the sensitivity of all antibodies and the hepatitis C core viral antigen meet the claims of the Murex Ab/Ag assay.

The Ab/Ag conjugate is prepared through a matching process to a particular batch of plates, using internal controls that indicate sensitivity and specificity. A solution appropriate for lyophilisation is spiked at pre-determined concentrations with five separate detection molecules and then freeze-dried for storage and transportation purposes. Prior to manufacture of the Ab/Ag conjugate, these five detection molecules are engineered sequentially through specific linkage of each separate peptide, recombinant protein, and antibody, to horseradish peroxidase. A monoclonal antibody and a recombinant protein enable the binding to viral core antigens and to non-structural antibodies present in serum, respectively. Three separate peptides representing different regions of the HCV core protein enable the binding to the different structural antibodies present in serum (Rodgers & Burch 2008). Figure 3 is a schematic representation of the components of the Murex HCV Ab/Ag Combination assay.

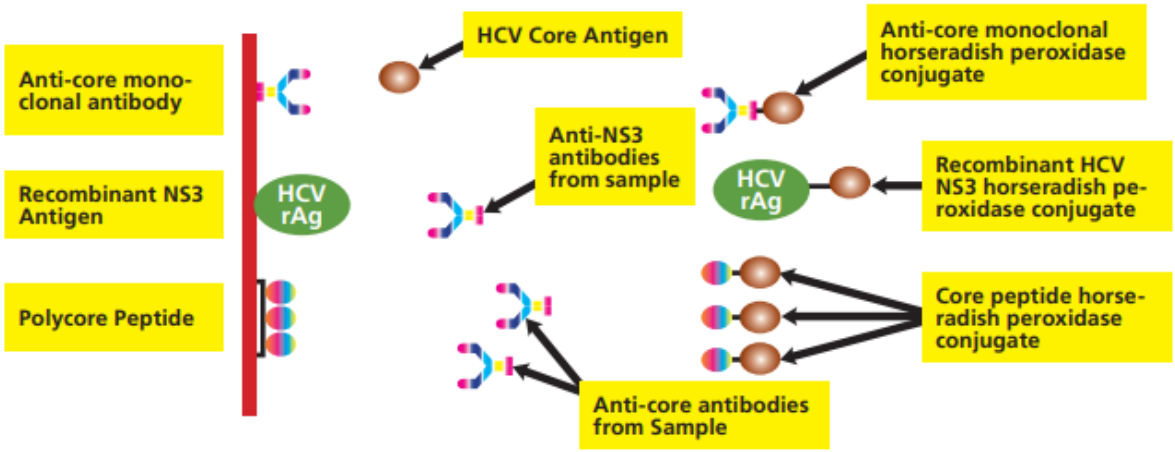


Figure 2.3: Example of a fourth generation ELISA: Sandwich format of the Murex HCV Ab/Ag Combination Assay. Adopted from 07-Murex Ab/Ag Combination Assay Technical Flyer: M0870004234 12281 0810).

2.7.2 Sensitivity and specificity of the Murex HCV Ab/Ag Combination Assay

The Murex HCV Ab/Ag Combination Assay facilitates increased diagnostic sensitivity by significantly reducing the window period of HCV detection compared to conventional serological assays (Rodgers & Burch, 2008).

A study reported by El-Emshaty et al. (2011) in Egypt compared the results of the Murex HCV Ab/Ag Combination Assay against reverse transcription polymerase chain reaction (RT-PCR). The study recommended the Murex HCV Ab/Ag Combination Assay as a suitable alternative when nucleic acid testing could not be performed. The study also cited the test's increased sensitivity, specificity and accuracy, as compared to the currently available anti-HCV tests. In addition, the researchers cited reduced incubation periods, ease of use, cost efficiency and reproducibility of results as additional benefits of the Murex HCV Ab/Ag Combination Assay. These recommendations were based on samples taken at various stages of infection, i.e., pre-seroconversion, window period and post the immune response. A total of 39 patients with chronic renal failure were used in this study. Of these, 20 patients were known to be anti-HCV positive. The Murex HCV Ab/Ag Combination Assay and the RT-PCR performed comparatively, identifying the same 18 samples as positive, and a single sample as negative. The Murex HCV Ab/Ag Combination Assay identified one sample as positive when the RT-PCR test returned a negative result. Of the samples, 19 were anti-HCV negative. Again, both tests performed comparatively, identifying 15 samples as negative, 3 samples were in the window period and were detected as positive, and one sample was positive on RT-PCR and negative on the Murex HCV Ab/Ag Combination Assay (El-Emshaty et al., 2011).

Another independent study by Dean and Perry (2007) indicated that the Murex HCV Ab/Ag combination assay detected infection 3.5 days earlier than a competitor Ag-only assay and 5.5 days after PCR, thus recommending it as alternative to PCR for early detection of infection particularly in resource-limited developing countries. The study evaluated donor HCV negative and positive donor samples, as well as an extensive commercial seroconversion panel specific to hepatitis C. Results of the study showed that of the donor samples, all 200 positive samples were detected correctly, whilst 4 out the 200 negative samples were initially, but not repeat reactive. A total of 211 seroconversion panels were evaluated, with 157 out of 197 panels correctly detected (Dean & Perry, 2007).

2.7.3 Core antibody detection by the Murex HCV Ab/Ag Combination Assay

Whilst the selling point of the Murex HCV Ab/Ag Combination Assay is the ability of detect the presence of viral molecules in blood specimens, antibody detection is as important as it aims

to detect the patients' immune response following seroconversion, once the viral load has diminished, or when the infection has been spontaneously cleared by the immune system. In addition, the antibody detection ability is important in the blood bank market, when spontaneously cleared infected blood is donated for use. An HCV infection clears spontaneously in about 25% of those infected. Generally, post-infection the viral load remaining detectable for 2 -14 days, with antibodies detectable in the blood 20 – 150 days after exposure (Grebely et al., 2012). Thus, the Murex HCV Ab/Ag Combination Assay provides a complete solution for the detection of HCV infection as it is able to detect antibodies generated in the patient to both the structural (core) and the non-structural regions of the hepatitis C core antigen.

This ability is due to the fact that the coated microplate contains capture molecules that are specific to these regions, and the lyophilized conjugate contain detection molecules that bind the molecules that have attached to the capture molecule (Rodgers & Burch, 2008). Such core detection is three-fold, in that the assay is able to detect three different types of antibodies generated following infection. As described below, this detection involves an initial coating of the inside of the microtitre wells with a core capture complex, which will bind to antibodies generated to the core region of the virus present a blood sample (Rodgers & Burch, 2008).

2.7.4 Polycore capture complex.

Three different peptides, each corresponding to a different immunodominant region of the core protein of the hepatitis C virus, are conjugated to a carrier molecule, forming a core capture-peptide complex.

One of these three core peptides is of particular interest to this project. This peptide is a 15 amino acid peptide sequence, that has been labelled with a S-acetylmercaptosuccinic anhydride (SAMSA) group at one end, and a hydroxyl (-OH) group on the other end. This peptide is representative of core region 3 of the hepatitis C virus. The addition of the SAMSA group onto the peptide allows the addition of a sulfhydryl (-SH) functional group that is protected by an acetyl group (Rodgers & Burch, 2008). During conjugation, the sulfhydryl group is accessed chemically by a de-masking agent so as to enable linkage to the cross-linker.

The core capture peptide complex, including all three immunodominant regions, is then titrated to determine the optimal concentration for use in the manufacture of the coated microtitre wells. These concentrations range from 40-120 ng/ml (Rodgers & Burch, 2008).

2.7.5 Solid phase immobilisation of antigens and antibodies

Immobilisation onto the 96 well Nunc MaxiSorp™ microtitre well walls is achieved by spiking a 0.05 M carbonate/bicarbonate buffer with the appropriate amounts of the polycore complex, as well as the capture antibody and N3 protein and allowing passive adherence to the polystyrene of the microtitre surface during incubation (Rodgers & Burch, 2008).

Thereafter, a blocking buffer containing 0.2% casein is applied to prevent non-specific binding of proteins to the detection complex or free sites on exposed surface of the well (Rodgers and Burch, 2008). As the basis of the ELISA technology is the antigen-antibody interaction, it is of paramount importance that such non-specific interactions are minimised. Unoccupied space on the surface of the microtitre wells can be blocked using either detergent or protein blockers, or a combination of these (Crowther, 2001). The microtitre wells containing the blocking buffer are incubated overnight at ambient temperature to facilitate passive adsorption of the blocking component onto the free microplate well surfaces.

Once the incubation period has elapsed and the surplus blocking buffer removed, the empty wells are treated with a fixing buffer. This fixing buffer is a solution containing polyvinyl pyrrolidone (PVP) and sucrose and is incubated similarly to the blocking buffer. This step is then followed by an incomplete aspiration of the fixing buffer which results in a small residual volume remaining in the wells. This volume is allowed to dry and forms a small cloudy pellet at the base of the microtitre well (Rodgers & Burch, 2008). The pellet is an immobilisation technique to “fix” the capture molecules onto the plate, as well as ensuring that the molecules remain in the appropriate conformations require for linkage to molecules in the sample.

2.8 Factors affecting coating of microtitre plates.

2.8.1 Microtitre plate composition

Immunoassays are dependent on the passive adsorption of molecules onto the surface of microtitre wells. Microplate surfaces are generally made up of polystyrene, a type of plastic with a hydrophobic structure. Polypropylene, polycarbonate and nylon are also used. In addition, the chemical structure of these surfaces can be modified (Corning Incorporated Life Sciences, 2015).

The surface chemical composition is pivotal to the reaction as it anchors the capture molecule. Immobilisation occurs through molecular, hydrophobic interactions between the polystyrene and the protein. This is effected by the availability of contact points on the binding surface in order to capture a medium-to-large molecules onto its surface, whilst retaining the activity of

the molecule (Crowther, 2001). The passive binding surfaces are selected based on these capture points and the type of molecule requiring immobilisation. The hydrophilicity, or affinity for binding water molecules, differentiates these binding surfaces into four categories, i.e., hydrophobic, slightly hydrophilic, hydrophilic, and very hydrophilic. Hydrophilic surfaces are used for binding immunoglobulins, as well as, other hydrophobic or hydrophilic biomolecules, and are well suited for sandwich ELISA formats (Crowther, 2001).

Passive binding has several disadvantages which must be considered during assay development. These include desorption of proteins and other complexes during assay processing. Non-specific binding of unwanted proteins and biomolecules may also occur (Esser, 2010a). In addition, the hydrophobic adsorption process is arbitrary, often resulting in the bound molecules being inaccessible to the target molecules (Shmanai et al., 2001). This development may be mitigated by modification of the microplate surface (Esser, 2010a). Polystyrene exposed to gamma-irradiation is one such method of modification. The gamma rays create a positive charge, resulting in a combination of the hydrophobic and hydrophilic regions on the microplate surface. This combination surface regulates the arrangement of adsorbed molecules, thereby creating more accessible capture molecules.

Two commercially available gamma-irradiated polystyrene surface microtitre plates may be used. The Greiner MICROLON® plates (Greiner Bio-One, Austria) are polystyrene plates modified to include hydrophilic contact points within their array of 600 µl wells. These plates have a binding capacity for mouse IgG of 600 ng/cm² (Greiner Bio-One, 2014). Alternatively, the Nunc MaxiSorp™ microtitre plates (Thermo Fisher Scientific, Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL, USA) are also polystyrene but are modified by gamma irradiation to produce a more hydrophilic surface that can bind molecules with both hydrophobic and polarised hydrophilic surfaces with a maximum binding capacity for IgG of 650 ng/cm^2 (Thermo Fisher Scientific, 2016).

2.8.2 Binding capacity

Determining binding capacity is subject to two factors: microtitre well surfaces have a limited number of binding sites, and the characteristics of the protein being bound. In general terms, saturation levels are between 50 – 500 ng/well contained in 50 µL of coating buffer (Crowther, 2001).

2.8.3 Blocking buffers

With the basis of ELISAs being the coating of reagents onto the surface of microtitre wells, care must be taken to ensure that only the reagent of choice be coated onto the well surface so that only the required reaction is able to proceed. Blocking of these spaces with inert non-reactive proteins can prevent non-specific binding to the wells. This will allow the assay to produce a more accurate reaction signal, reduce background noise and improve sensitivity (Gibbs, 2001). The most suitable blocking buffer for any particular application will effectively deter non-specific binding, confer a stabilizing effect to the target molecules, and will not interfere or cross-react with assay components (Duhamel & Johnson, 1985).

Blocking buffers may use a range of blocking agents to achieve this goal and these substances may be divided into two broad categories, i.e., detergents blockers and protein blockers (Crowther, 2001). Therefore, the selection of these agents requires careful consideration as detergent and protein blockers are used at various stages in an assay including during plate coating, sample addition, conjugate addition and washing (Gibbs, 2001). Proteins, sera derived from animals, casein, skimmed milk powder, gelatine or detergents such as Tween-20, Triton X-100 and sodium dodecyl sulphate can also be used as potential blocking agents.

2.8.3.1 Protein blockers

Protein blockers such as skimmed milk or BSA function to occupy vacant sites on the microplate surfaces, inhibits denaturation and provide stability to bound molecules (Crowther, 2001).

Bovine serum albumin

Bovine serum albumin (BSA) is a large protein derived from serum and is a ubiquitous blocking agent. It is used as an additive to blocking buffers at concentrations between 1% and 3% (Gibbs, 2001). Advantages of using BSA include it's solubility, large molecular weight, and compatibility with other proteins. In addition, it is easily sourced, has storage flexibility, and is cost effective.

Bovine serum albumin has a molecular weight of 67 000 which is particularly useful in blocking non-specific, protein-surface interactions (Gibbs, 2001). However, the large molecular weight can be disadvantageous should there be inefficient adsorption to the surface of the microtitre as this will leave large surfaces remaining open to allow nonspecific binding to occur. This disadvantage can be mitigated by using a second blocking agent such as casein in the buffer, in addition to BSA (Pratt and Roser, 2010). Another disadvantage is that BSA batches may

display significant batch-to-batch variability and may contain contaminants such as other proteins (Crowther, 2001). In addition, BSA may be unable to block certain surfaces and has been shown to cross react with antibodies where the antigenic stimulus was a BSA-conjugate (Duhamel & Johnson, 1985).

Non-fat dairy milk

Non-fat dairy milk (NFDM) is another widely used blocking agent, used at concentrations between 0.1% and 3% depending on the assay requirements. It is inexpensive, displays a high degree of molecular diversity and is the blocking agent of choice for covalent surfaces (Duhamel & Johnson, 1985). However, there are several disadvantages associated with NFDM. It is imperative to prepare and store NFDM appropriately as it is known to be become unstable. In addition, NFDM also exhibits significant batch-to-batch variability (Gibbs, 2001).

2.8.3.2 Detergent blockers

Detergent blockers are temporary, symmetrical, low molecular weight molecules with both a hydrophilic and a hydrophobic end (Butler, 1996). Detergent blockers can be either non-ionic or ionic, however only non-ionic detergents may be used for blocking of polystyrene.

Detergent blockers are often used together with proteins blockers, where the detergent is removed during the washing steps of the assay. Detergents may be used in other assay reagents, such as washing solutions. In this application, the detergent is used to remove any non-specific molecules that may have bound to the plate during the assay steps (Esser 2010b). It will also serve to block spaces created as a result of any unwanted desorption of molecules during the assay (Gibbs, 2001).

2.8.3.3 Non-protein blockers

A disadvantage of the use of protein blockers is its potential to cross react, by binding non-specifically when used in the plate preparation. There are commercially available non-protein blockers that may be used in lieu of proteins, particularly in sensitive assays like those that use avidin-biotin systems. The Protein-Free blocking buffer available from Thermo Scientific (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL, USA) is a ready to use buffer that can applied directly to the microtitre wells. Polyvinylpyrrolidone (PVP) is a non-protein reagent that modifies hydrophobic sites into hydrophilic sites, thus prohibiting binding and may be used by itself or in combination with other blockers (Gibbs, 2001).

2.8.4 Capture and Detection Molecule pairing

2.8.4.1 Concentration of the substance being adsorbed

Crowther (2001) determined that the ideal concentration of the capture molecule in approximately 50 µl of coating buffer, lies within the range of 1 – 10 µg/ml. Coating with the capture molecule(s) in this concentration range is expected to saturate all available binding sites on the plastic surface of the microtitre plate. Saturation is dependent, however, on the purity of the capture molecule. A higher purity capture molecule will allow for greater adsorption of the protein of choice to the plastic matrix. It is important to find the optimal concentration for a specific application as very large or small quantities can affect its binding ability to the surface.

The application of highly concentrated capture molecules onto the microplate surface will result in the formation of layers or stacks, which is not ideal for accessibility by the detection molecule. In such a case, an assay's binding efficiency is reduced as the stacks can have detrimental effects on molecular orientation, causing the functional groups required for binding to become inaccessible. In addition to a reduction in binding efficiency, stacking has other disadvantages. Layers of capture molecules are unstable, as they have not adsorbed to the microplate surface, but rather have been stacked onto other capture molecules. The application of subsequent reagents is impacted as it may not be in contact with all subsequently required sites for binding. The removal of unbound reagents is also impacted because the wash step may be inadequate to remove the reagents caught between the layers of the capture molecule. This allows for contamination and increases the potential for the occurrence of unwanted side reactions (Crowther, 2001).

The application of large amounts of proteins above the particle saturation point of the hydrophobic surfaces can lead to the formation of loosely bound multilayers. These layers can also leach from the adsorption surfaces. This can result in open, unblocked sites on the microtitre surface, which are able to now bind arbitrarily to unwanted to molecules contained within each of the reagents applied to the plate throughout the assay (Hermanson, 2013).

2.8.5 Stereochemistry

Stereochemistry ai the study of the orientation of bonds in 3-dimensional space. Single bonds or sigma (σ) bonds are able to rotate within the molecule, depending on the types of atoms contained. This rotational ability allows for varying arrangements of the atoms resulting in different conformational structures of a particular type of molecule, called conformational isomers or conformers. Conformers can result in changes to the properties of a molecule (Merriam-Webster Dictionary, 2021).

Therefore, accessing the target molecules is an important factor to consider. A chemical reaction is facilitated by one substance acting as a nucleophile. For binding to occur, the nucleophile must be in close physical proximity to the target molecule. Bulky reactive groups can impede the accessibility to the target molecule, a phenomenon called steric hindrance (Wade & Simek, 2016).

2.8.5.1 Steric hindrance and its effect on ELISA

The Merriam Webster dictionary defines steric hindrance as the hindrance of chemical reactions as a result of the arrangement of atoms in a molecule (Meriam-Webster, 2021). The physical proximity of the chemical groups surrounding a particular group can inhibit access to the target group, thereby resulting in slower or even outright prevention of the reaction (Gallegos et al., 2022). The phenomenon of steric hindrance has an effect on the signal efficiency in ELISA applications (Crowther, 2001). This can be caused when increasing the concentration of the capture molecule during the coating of the microtitre wells in order to improve the sensitivity of the assay. A higher concentration of the capture molecule in the microtitre well may cause crowding of the coated molecules. The higher concentration may also cause molecular conformational changes, as well as orientational changes. This is particularly problematical in ELISA assays, as their performance is based on the processing of specific reactions, which require the availability of specific reactive groups. Conformational and orientation changes in the capture molecule may cause it to become inaccessible to the target molecule, therefore inhibiting the detection molecule. Thus, steric hindrance must be considered when attempting to improve assay performance, by increasing the concentration of the capture molecule. In practice, the optimal concentration of the capture molecule may be investigated by the coating of the microtitre wells with a range of concentration. The coated microtitre wells are tested using the applicable ELISA protocol, and the results reviewed to determine the optimal concentration (Hermanson, 2013).

2.9 Manufacture of the detection molecule

As mentioned previously, the detection molecule is comprised of five (5) sequentially engineered enzyme-conjugates, which are titrated to the coated microtitre plates. Of particular importance to this project is the detection molecule for core region 3, one of the three detection molecules contained in the Murex HCV Ab/Ag conjugate. The peptide corresponds to region 3 of the hepatitis C core region. The peptide is modified with a SAMSA group at one end to allow for linkage to sulfo-SMCC-modified horseradish peroxidase (Rodgers and Burch, 2008).

2.10 Chemistry of conjugation

Conjugation or cross-linking refers to the linking of biomolecules via their functional groups. In this context, the biomolecules that will be modified are proteins. Only four (4) functional groups can be used for conjugate, i.e., primary amines, carboxyls, sulfhydryls, and carbonyls. Primary amines (-NH2) occur at the N-terminus and in lysine residue side chains and are present on the outer surface of proteins. This external feature is beneficial in that conjugation can occur whilst maintaining protein structural integrity. Carboxyls (-COOH) are present at the C-terminus, as well as in the side chains of glutamic acid and aspartic acid. These functional groups also occur on protein surfaces. Sulfhydryl (-SH) groups are present in cysteine side chains, and form part of a proteins' secondary or tertiary structure. Reduction of the disulphide bonds between cysteine residues produce free sulfhydryls, which can be conjugated to a range of other reactive groups. Sulfhydryl conjugation requires an extra step (reduction of the disulphide bond) and requires some modification of protein structure. Carbonyl (-CHO) groups such as ketones and aldehydes can be modified onto glycoproteins by oxidation (Pierce Biotechnologies, 2020).

Unlike amines or carboxylates, sulfhydryls are not usually present on proteins. Thus, the use of heterobifunctional crosslinkers with a sulfhydryl can be used to specifically target a region. For this reason, sulfhydryl modification is common in the development of conjugation and modification strategies. Sulfhydryls can be generated by reducing disulfides already present in a molecule or by using a variety of thiolation reagents (Hermanson, 2013).

2.11 Modification of enzymes

Enzymes may be modified by the introduction of reactive groups which facilitate their cross-linkage to other biomolecules (Hermanson, 2013). Cross-linking is of particular importance as the basis of most ELISAs is the exploitation of the catalytic ability of enzymes to convert substrate molecules into quantifiable and detectable (chromogenic, fluorescent or chemiluminescent) products. This process typically involves an antibody with a specific binding affinity for an antigen being linked to an enzyme to enable visualization of the antigen of interest, following the degradation of an appropriate substrate (Berg et al., 2002). There are various enzymes available for use, such as horseradish peroxidase, alkaline phosphatase, β-galactosidase and glucose oxidase (Voller et al., 1978).

2.11.1 Horseradish peroxidase

Horseradish peroxidise (HRP) is a heme-containing plant glycoprotein extracted from the roots of horseradish. The enzyme has a relatively small molecular weight of 40 000 (Hermanson,

2013). This small size and its chemical stability under conjugation conditions makes it the most popular choice for labelling antibodies and antigens in immunoassays (Avrameas and Guilbert, 1972). The secretory glycoprotein has three lysine residues (amine reactive groups) that may be used for linkage to other biomolecules (Navapour et al., 2014). The optimal functional pH range for horseradish peroxidase is 7.0 (Hermanson, 2013). The heme structure provides the reddish-brown colour and can be spectrophotometrically measured at a wavelength of 403 nm. The Reinheitzahl ratio is often used to measure the purity of the HRP and is calculated by using the absorption value at 403 nm versus absorption at 275 nm. Furthermore, it is advantageous in diagnostic assays due to its reactivity with a substrate solution to form an easily quantifiable end product. Studies indicate that 80% of antibody conjugates are prepared for application in diagnostic assays. The reactivity exploited in these assays occurs between HRP and its substrate, hydrogen peroxide. A stable intermediate is created, which dissociates in the presence of an appropriate electron donor. As a result, the electron donor is oxidized. This chemical reaction, in a suitable solution, can elicit a quantifiable colour change (Hermanson, 2013).

Lyophilised HRP can be stored for long periods of time without deterioration. In addition, at 4°C, the purified liquid HRP can be stored for months without significant deterioration. The HRP molecule has the ability to build conjugates with a high or low ratio of antibody to enzyme, depending on the requirement, whilst still being highly specific. As a result of the relatively small size of HRP, small antibody-enzyme conjugates can be produced which are better able to access antigens. Disadvantages of HRP include the relatively low number of primary amines that are available for linkage, as well as sensitivity to anti-bacterial agents such as sulfide, cyanide and azide. In addition, the practical substrate development time of HRP is limited to approximately 60 minutes, and often even shorter times (Hermanson, 2013). As such, assays developed using this enzyme must ensure their protocols are set up to account for this limitation.

2.12 Cross-linking strategies

There are various cross-linking strategies that can be employed for conjugation of molecules. These strategies are dependent on the application of the final conjugated molecule. Factors such as chemical specificity and reactivity, the presence and nature of spacer arms, cell membrane permeability, cleavability of the cross-linker and the nature of the moieties present must all be considered when selecting a cross linker as well as the strategy for cross-linking (Narain, 2013).

Of these factors, the chemical reactivity and specificity are especially important. Reactive groups such as amines (lysine residues), thiols (cysteine residues), phenols (tyrosine residues) and carboxylic acids (aspartic acid) are sites commonly used for linkage of one molecule to another (Brinkley, 1992). These reactive groups determine the chemical specificity of a cross linker and, thus, the cross-linkage strategy (Narain, 2013).

The requirement for other functional groups, must also be assessed. These groups may be used for labelling, for downstream application of the final conjugate. Labels may be radioactive, fluorescent, etc. The ability of the final conjugate to permeate cells and/or membranes, must also be assessed. In addition, a determination of whether the bond may be reversed, is also necessary when selecting a strategy (Narain, 2013).

Homobifunctional and heterobifunctional cross-linkers such as glutaraldehyde and succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), respectively, may be used (Brinkley, 1992).

2.12.1 Homobifunctional cross linkers

Homobifunctional crosslinkers have identical reactive groups on either end of their spacer arms and can be conjugated to the molecule of choice in a single-step reaction. They are widely used in industry in a range of applications, depending on the reactive groups required (Narain, 2013).

An active intermediate can be formed by reacting the homobifuctional crosslinkers with one protein. Thereafter, the intermediate is able to crosslink with other proteins. Intramolecular cross-linking with its polypeptide chain is possible, leading to polymerization and a subsequent precipitation of the protein from solution. Single step conjugation using homobifunctional crosslinkers exacerbate the issue of polymerization, as there is a lack of control over the cross-linking process. This can be mitigated by employing a multi-step cross-linking strategy, whereby the cross linker is reacted with one protein. Thereafter, excess reagents are removed from the solution. The second protein is then added to this solution, which completes the conjugation process. However, multi-step conjugation strategies do not eliminate all of the advantages associated with homobifunctional crosslinkers, as conjugation with other modified molecules is still a possibility (Hermanson, 2013).

Despite its simplicity and widespread use, homobifunctional cross-linkers have several disadvantages. These include the possibility of self-conjugation, polymerization and intracellular

cross-linking (Narain, 2013). In addition, the lack of distinction between different types of proteins can result in false positives and/or high noise levels that are difficult to interpret accurately.

2.12.1.1 Glutaraldehyde

Glutaraldehyde is a widely used homobifunctional cross linker. Cross linkage using glutaraldehyde is able to occur through two different pathways. One pathway makes use of sodium cyanoborohydride by reacting amines with aldehydes, enabling amine linkages with an aldehyde group for conjugation to another molecule. The second pathway consists of double bond formation by the reaction of glutaraldehyde polymers with amines. The end product contains aldehydes and double bonds that are capable of linkage with other amines. (Hermanson, 2013).

2.12.2 Heterobifunctional cross linkers

Heterobifunctional cross-linkers have different reactive groups at either end of their spacer arms (Hermanson, 2013).

Heterobifunctional cross-linkers have specific application as they allow linkage of each reactive group sequentially, providing site-specific linkage that effectively controls conjugation (Hermanson, 2013). This is affected by initially reacting the most unstable group whilst the other reactive group remains unreacted. The two-step sequential reaction of the reactive groups significantly reduces polymerization and intramolecular cross-linking (Narain, 2013). Three-step reaction strategies may also be employed, when a protein does not contain the specific functionality required for coupling. A specific functionality can be introduced onto the target protein by activation of the first protein with the heterobifunctional crosslinker, followed by a purification step to remove any unreacted reagent. Thereafter, modification of the second protein introduces the specific functionality for coupling to the activated first protein. The modified protein, and the activated protein are then mixed together, to allow the final conjugation process to occur (Hermanson, 2013).

Specific functionalities may be introduced into the structure of a macromolecule by reacting existing functionalities with a modification reagent that must contain the functionality required for coupling. Thus, an amine can be converted to a sulfhydryl group using 2-iminothiolane (Traut's reagent). Alternatively, N-succinimidyl-S-acetylthioacetate (SATA) can use its active NHS ester (N-hydroxy succinimide) end to react with amino acids or other molecules, thereby introducing a protected sulfhydryl group. This strategy can also be used to modify low reactive

functionalities into highly reactive groups. The selective reduction of a protein's cysteine residues can also be used to create active sulfhydryls, which may be used for cross-linking. An alternative modification of molecules for conjugation purposes is the use of spacer arms that are engineered into the molecule. These spacer arms are useful in extending functional groups away from a molecules surface as they allow for the reduction of the effects of steric hindrance as cross-linking proceeds. In addition, the use of spacer arms is advantageous, in that they create more chemically active complexes (Hermanson, 2013).

Cross-linkers with a spacer arm between an amine-reactive succinimidyl ester and a thiol-reactive group are most extensively used in conjugation reactions. Maleimides, haloacetylyls, and pyridyl disulfides are thiol reactive groups ubiquitously used for cross-linkage (Narain, 2013).

Whilst modification reagents have immense value in their ability to modify molecules with specific functional target moieties, there are several caveats. Alteration of the chemical structure of a molecule affects the structure which, in turn, can affect molecular activity. Selection of a modification reagent, and a cross-linking strategy requires an in depth understanding of the native conformation, the effects of modification of critical groups, and the possibility of blocking active sites during modification.

2.12.2.1 N-Succinimidyl S-Acetylthioacetate

N-Succinimidyl S-Acetylthioacetate (SATA) is an N-hydroxysuccinimide (NHS) ester of S-acetylthioacetic acid (Pierce Biotechnologies, Rockford, USA). The heterobifunctional cross-linker has a 2.8 Å spacer arm, allowing for the addition of a protected sulfhydryl group onto a primary amine of the molecule requiring linkage by covalent bonds. In this protected state, the SATA-modified molecule may be stored for extended periods of time, providing an advantage in commercial applications. During the conjugation process, the protected sulfhydryl group can be exposed by addition of hydroxylamine-HCl.

An advantage of SATA is that it is particularly useful in reactions when the molecules requiring linkage do not contain sulfhydryl groups, or when these groups are not physically available (Pierce Biotechnology 2011). Another advantage of SATA is the ability to introduce a sulfhydryl without the need for a reducing agent as this allows for modification without disruption of the native conformation of the protein, thereby preserving protein integrity and activity.

Antibodies are a good example of proteins that do not contain sulfhydryl groups for linkage. Thus, SATA is beneficial is this regard, as it is known to introduce up to six (6) sites for binding per polyclonal antibody, without a decrease in antigen binding capacity. There is also the

possibility of including more SATA molecules per antibody, without a loss of antigen binding capacity.

An enzyme modified to include maleimide groups can be immediately reacted with the modified antibody, after deacetylation with hydroxylamine-HCl. After deprotection, the solution must be used immediately and not stored or risk the formation of disulphide bonds and a subsequent loss of binding ability as there will be fewer sulfhydryl groups available for reaction with maleimide groups. The extent to which sulfhydryl modification has occurred can be assessed by using the Ellman's assay. This strategy is ubiquitous in the conjugation methods for immunoassays. In addition, SATA can be dissolved in organic solvents, such as dimethyl sulphoxide (DMSO), dimethyl formamide (DMF), and methylene chloride. A range of buffer compositions can be used, at a pH range between 7.0 and 7.6. Phosphate buffers are a common choice as buffers that contain extraneous amines, or those that facilitate hydrolysis of the NHS (N-hydroxy succinimide) ester must be avoided (Hermanson, 2013).

2.12.2.2 S-Acetylmercaptosuccinic anhydride

S-Acetylmercaptosuccinic anhydride (SAMSA) is reactive towards amines as this molecule has a protected sulfhydryl group which is made available for conjugation by treatment with hydroxylamine-HCl. An amide linkage is formed by nucleophilic attack of the amine toward the anhydride. A side effect of the reaction is a free carboxyl group which can change the overall charge of the molecule, resulting in a change of protein conformation. S-Acetylmercaptosuccinic anhydride may be dissolved in an organic solvent such as DMF (Hermanson, 2013).

2.12.2.3 SMCC and Sulfo-SMCC (Succinimidyl-4-(N -maleimidomethyl) cyclohexane-1-carboxylate)

Succinimidyl-4-(N -maleimidomethyl) cyclohexane-1-carboxylate (SMCC), and its water-soluble equivalent, sulfosuccinimidyl-4--(N-maleimidomethyl) cyclohexane-1-carboxylate, are N-hydroxysuccinimide (NHS) esters (Pierce Biotechnologies, Rockford, USA). The SMCC and sulfo-SMCC compounds are particularly useful heterobifunctional cross-linkers and are commonly used for linkage of biomolecules (Hermanson, 2013).

Sulfo-SMCC and SMCC contains an 8.3 Å spacer arm with a sulfo-NHS-ester and flanking maleimide reactive groups. The NHS-ester is able to link with a protein's primary amines by forming stable amide bonds (Pierce Biotechnology, 2020). The maleimide group reacts with both sulfhydryl and amine groups, depending on the pH of the reaction conditions. Reaction with sulfhydryls is selected for at the pH range of 6.5 to 7.5, over which the reaction is specific for amines. A pH of 7 has been shown to be optimal for linkage of maleimide with sulfhydryls,

as the rate of reaction is 1000 times greater than that maleimide reaction with amines (Hermanson, 2013).

Conjugation of antibodies or peptides to enzymes is achieved by the activation of the enzyme with SMCC. This introduces maleimide groups onto horseradish peroxidase, by reaction of its NHS ester with amine groups to form amide bonds. Any unbound cross linker is then removed from the solution. The purified activated enzyme, with its newly introduced maleimide groups are then able to react with sulfhydryl groups on the protein of choice (Hermanson, 2013).

Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate is not soluble in water and requires an organic solvent such as DMF or DMSO to facilitate dissolution. However, organic solvents are known to inhibit protein activity. Sulfo-SMCC is the water-soluble derivative of SMCC and confers the ability to add the crosslinker directly to reaction buffers. Sulfo-SMCC enables solubility of 10 mg/ml at room temperature. The water solubility is effected by an NHS ring with a negatively charged sulfonate group. A 10 mM concentration of sulfo-SMCC is possible in a 50 mM sodium acetate, at pH 5.0. The same concentration is possible in 50 mM sodium borate, at pH 7.6. A 0.1 M sodium phosphate solution, in the pH range of 6 to 7.5, can be used to dissolve sulfo-SMCC to yield a concentration of 10 mM. Sulfo-SMCC can also be dissolved in an organic solvent such as DMF or DMSO, so as to reduce hydrolysis-associated activity loss. The maleimide group remains stable for 64 hours at 4°C, at a pH of 7, when conjugated in a 0.1 M sodium phosphate buffer (Pierce Biotechnology, 2020).

2.13 Maleimide chemistry

Maleic acid imides or maleimides result from the reaction between maleic acid and amine derivatives and are most commonly contained within the structures of heterobifunctional cross linkers. Alkylation of the maleimide double bond by the sulfhydryl group allows for the formation of a stable thioether bond. This process is facilitated by nucleophilic attack by the thiolate anion on a carbon atom adjacent to the maleimide group. This reaction is exclusively selected within a pH range of 6.5 to 7.5. Hermanson, in Bioconjugate Techniques, describes that, at pH 7.0, this reaction is 1000 times greater than the reaction of maleimide groups with amines. At pH values higher than 7.5, amines and sulfhydryl may compete for the maleimide groups present in a solution. This can result in mixed and unwanted reactions, i.e. maleimides reacting with both sulfhydryls and amines. Hydrolysis of maleimide groups can cause the formation of an open maleamic acid, which is not reactive toward sulfhydryl groups.

Hydrolysis is also a problem after the sulfhydryl maleimide reaction has occurred. The probability of forming maleamic acid with an open structure increases at high pH. In addition, the functional group near the maleimide group also plays in a role in the probability of hydrolysis. Steric hindrance also effects hydrolysis of maleimide groups, as it can be exploited to prevent unwanted side reactions (Hermanson, 2013).

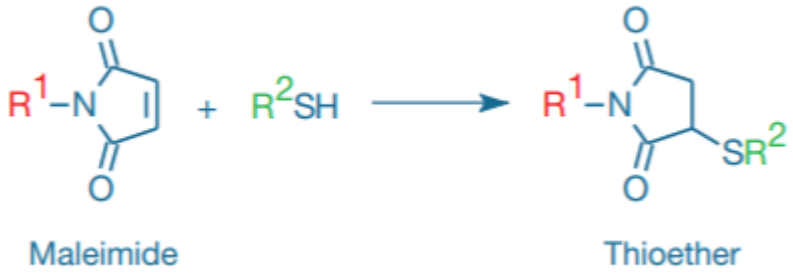


Figure 2.4: A maleimide group reacting with a sulfhydryl resulting in the formation of a thioether bond. Adopted from Hermanson (2013).

2.13.1 Carrier molecules

Carrier molecules are often used in the preparation of hapten-carrier conjugates, and are extensively used in the production of antibodies, vaccine development, and the research into immune responses. However, carrier molecules can also be used in the development of conjugates for immunoassays, particularly when the capture molecules are of low molecular weight (Hermanson, 2013).

Bovine serum albumin (BSA) with its large size has numerous functional groups that can be exploited for cross-linking purposes. A single molecule of BSA has 59 lysine groups, however only 30 to 35 of these are available for modification. In addition, a single BSA molecule has a free cysteine sulfhydryl, 19 tyrosine phenolate residues, 17 histidine imidazole groups, and a number of carboxylate groups. Thus, the BSA molecule has a net negative charge (p.I .1) (Hermanson, 2013).

The BSA can remain in solution despite significant hapten modification. However, there is the risk of precipitation from solution when BSA is used to cross-link hydrophobic peptides and sparingly soluble molecules. This can be mitigated by tailoring the BSA modification process to the specific properties of the target molecules (Hermanson, 2013).

2.14 Conjugation conditions

In order to effectively conjugate molecules, conditions under which conjugation occur must be optimized. Conjugation should occur in a buffer that is appropriate for the reactivity of the molecules e.g., phosphate buffers are optimal for reaction of thiols at physiological pH. The pH and ionic strength of the reaction buffers are of utmost importance as this will determine whether the correct reactions occur. Dissolution of the linking reagent must be ascertained as many are reactive or insoluble in aqueous solutions. Organic solvents such as methanol and dimethylformamide (DMF) may be used (Hermanson, 2013).

Sulfhydryls, however, are sensitive to oxidation, during which disulphide bonds are formed. Therefore, to prevent oxidation, all oxygen within the conjugation buffers must be removed. This can be achieved by degassing or the use of 0.01 – 0.1 M EDTA. The EDTA is used to prevent chelation of metal ions (Hermanson, 2013).

A further point to note is that lyophilized enzymatic activity in organic solvents is dependent on the pH of the solvent used for dissolution of the enzyme, during the preparatory phase (Kwon and Ito, 2013).

Temperature and length of reaction time are also of importance to promote optimal cross-linking (Brinkley, 1992). It is often necessary to determine the extent to which linkage has occurred and this may be achieved by measuring the concentration of free residues by, among other means, colourimetric and spectroscopic methods (Brinkley, 1992).

Hermanson recommends maintaining high concentrations of proteins as the target molecule in the reaction medium favours modification of amines. The reaction can be controlled to achieve the optimal product by calibration of the molar ratio of the cross linker to the target molecule (Hermanson, 2013).

The pH of the reaction buffer is critical in order to allow the correct reactions to be selected. The maleimide group present on SMCC and sulfo-SMCC react exclusively with sulfhydryls when the pH of the reaction buffer is in the range of 6.7 to 7.5. The optimal pH for linkage of maleimides to sulfhydryl moieties is pH 7.0. The reaction rate at pH 7.0 is 1000 greater with maleimides than with amines. At pH greater than 7.5, the maleimide group favours linkage with amine groups. There is an increased risk of hydrolysis of the maleimide group as pH increases, resulting in the formation of an open maleamic acid ring. This opened ring is now unreactive to sulfhydryls, thus eliminating the possibility of being used for linkage. In addition, the structural change from maleimide to maleamic acid ring is exacerbated as pH increases. Post conjugation, the maleimide-sulfhydryl linkage is also vulnerable to hydrolysis, if the pH

conditions are increased during further processing. Up to pH 7.5, the maleimide group attached to SMCC remains stable. The cyclohexane ring present on SMCC, as well as the absence of an aromatic structure provides an advantage to this crosslinker, by conferring stability to the maleimide group. Maintenance of neutral pH for two hours at a temperature of 30° C, has been shown to cause a 4% degradation of the maleimide groups of SMCC. This characteristic is particularly beneficial when conjugating proteins, as it allows for the formation of stable maleimide activated intermediates.

N, N'-o-phenylenedimaleimide, N, N'-oxydimethylenedimaleimide are examples of cross-linkers that are exhibit lower stability as pH increases (Hermanson, 2013).

The effects of the pH of the reaction buffer below pH 6.5 on the ability to maleimide groups to react with sulfhydryls has not been described in the literature.

2.15 Purification of cross-linked biomolecules

It is of paramount importance that contaminants, unreacted label molecules and excess cross-linking reagents be removed from the modified molecule (Brinkley, 1992). There are several established techniques available for protein purification that are based on molecular size, ionic properties, and binding capabilities. These may be used singly or in combination and the most popular methods are described below as they provide high resolution with an acceptable yield of the protein of interest (Berg et al., 2002).

2.15.1 Dialysis

Dialysis is a separation technique whereby molecules move by selective diffusion across a semi-permeable membrane (Berg et al., 2002). This tried and trusted technique offers a safe means of buffer exchange and desalting in physiological conditions, without the risk of damage to the protein being purified (Burgess and Deutscher, 2009). However, the disadvantages of the method are relatively low speed of purification as well as the need to change the dialysis buffer during processing (Scopes, 1993).

A high concentration of a particular analyte is placed within a dialysis membrane that is then placed within a relatively large volume, low concentration buffer solution. Dialysis membrane contains pores of varying molecular weight cut-offs (MWCO) that facilitate the movement of molecules smaller than the pore size into the dialysis buffer (Burgess and Deutscher, 2009). Thus, molecules larger than the pores will remain inside the membrane (Nath, 2008) and so dialysis separates larger molecules from small molecules such as small contaminants (Berg et al., 2002).

A variety of membrane pore sizes are available, with MWCO ranging from 1000 to 50 000, allowing the separation of molecules in solution from contaminants that are tailored specifically to the sample. Further to this, dialysis membranes are also available in a wide range of formats, from tubing of varying thickness to specialized dialysis cassettes and devices (Scopes, 1993).

As dialysis is accomplished by the process of diffusion, maintenance of a concentration gradient is important in achieving effective separation of molecules. Therefore, the following factors influence the effectiveness of the technique:

- **Temperature:** Higher temperatures speed the rate of diffusion, which would theoretically speed up the rate of the movement across the dialysis membrane. However, thermal stability must be evaluated, particularly with proteins, in order to maintain integrity of the sample being purified (Pierce Biotechnology, 2016).
- **Sample Concentration:** The rate of dialysis is faster in samples of high concentrations, due to the steeper concentration gradient between the inside of the dialysis membrane and the external dialysate buffer. Higher concentrations cause molecules to move through the pores of the membrane at a higher speed in an attempt to reach equilibrium, thus enabling faster separation. Therefore, separation in dialysis buffers volumes that are several orders of magnitude larger than the sample of interest is one way to exploit this characteristic (Pierce Biotechnology, 2016).
- **Molecular weight of the molecule:** There is an inverse proportional relationship between the molecular weight of a substance and the rate at which it is able to move through the dialysis membrane. Lower molecular weight substances tend to move faster than those of a larger molecular weight. Therefore, a thorough understanding of the sample being separated is essential when designing dialysis protocols (Pierce Biotechnology, 2016).
- **Membrane Surface area:** The speed at which impurities are able to move through the dialysis membrane is directly proportional to the surface area of the membrane exposed to the dialysate. This characteristic can be exploited by selecting longer, narrower tubing. In addition, ensuring that the dialysate is in constant motion by stirring during the dialysis period further increases exposure of the sample to the dialysate (Pierce Biotechnology, 2016).
- **Membrane thickness:** An inversely proportional relationship exists between membrane thickness and the rate of diffusion (Pierce Biotechnology, 2016).

2.15.2 Size exclusion chromatography

Gel filtration, gel permeation or size exclusion chromatography is an adsorptive chromatographic technique that separates molecules on the basis of size (Berg et al., 2002) and molecular shape (Walsh, 2002). According to Walsh (2002), fractionation in size exclusion chromatography causes separation according to the molecular mass of the proteins rather than the molecular shape. Thus, gel filtration can be applied to many of the same processes as dialysis.

The sample containing the molecules of interest is allowed to transude through a column of porous beads such as Sepharose or Sephadex. These are insoluble, hydrated polymeric beads made from agarose or dextran that allow small molecules to enter the pores of the beads in the porous matrix, but excludes larger molecules that remain in solution outside the beads. The result is that larger molecules move through the column faster and are eluted first, whereas the smaller molecules follow a more meandering path within and through the matrix. Thus, they are eluted after the larger molecules (Stellwagen, 2009). There are two forms of this technique - group separation and fractionation (Hagel, 2001).

Group separation involves the separation of high and low molecular weight molecules into two distinct groups. Sample elution is isocratic, allowing samples to be loaded and collected in the buffer of choice while the need for sample adjustment after separation is negated. This technique allows the solution to be desalted and/or buffer exchanged (Aguilar, 2003).

Fractionation separates molecules that are very similar in size (Aguilar, 2003). As the sample volume is restricted in this separation technique, it only allows the sample volume to be between 0.5% – 5.0% of the column bed volume, whereas group separation allows a sample volume of 30% of the column bed volume (Aguilar, 2003).

Gel filtration has some advantages over dialysis (Rolet-Menet et al., 2000). Gel filtration is able to separate molecules of different sizes faster and more efficiently. A column is pretreated with the buffer of choice before application of the sample requiring separation. The purified sample is then collected in the buffer with which the column was treated. This process is faster and requires a smaller volume of buffer than does dialysis. This more efficient method reduces processing time and is particularly useful when working with time sensitive processes.

Gel filtration, as described above, provides a longer path for smaller molecules within a column. Thus, larger molecules are separated much earlier. This is useful when separating samples

containing contaminants smaller than the molecules of interest. The smaller contaminants also remain on the column and can be disposed of easily (Berg et al., 2002).

Gel filtration products have more robust packaging than do dialysis membranes, thus the integrity of the resin is not as easily compromised as dialysis membranes. Gel filtration products are able to separate samples containing organic solvents, such as DMSO, whereas dialysis membranes may become compromised both physically and chemically (Pierce Protein Methods, 2006).

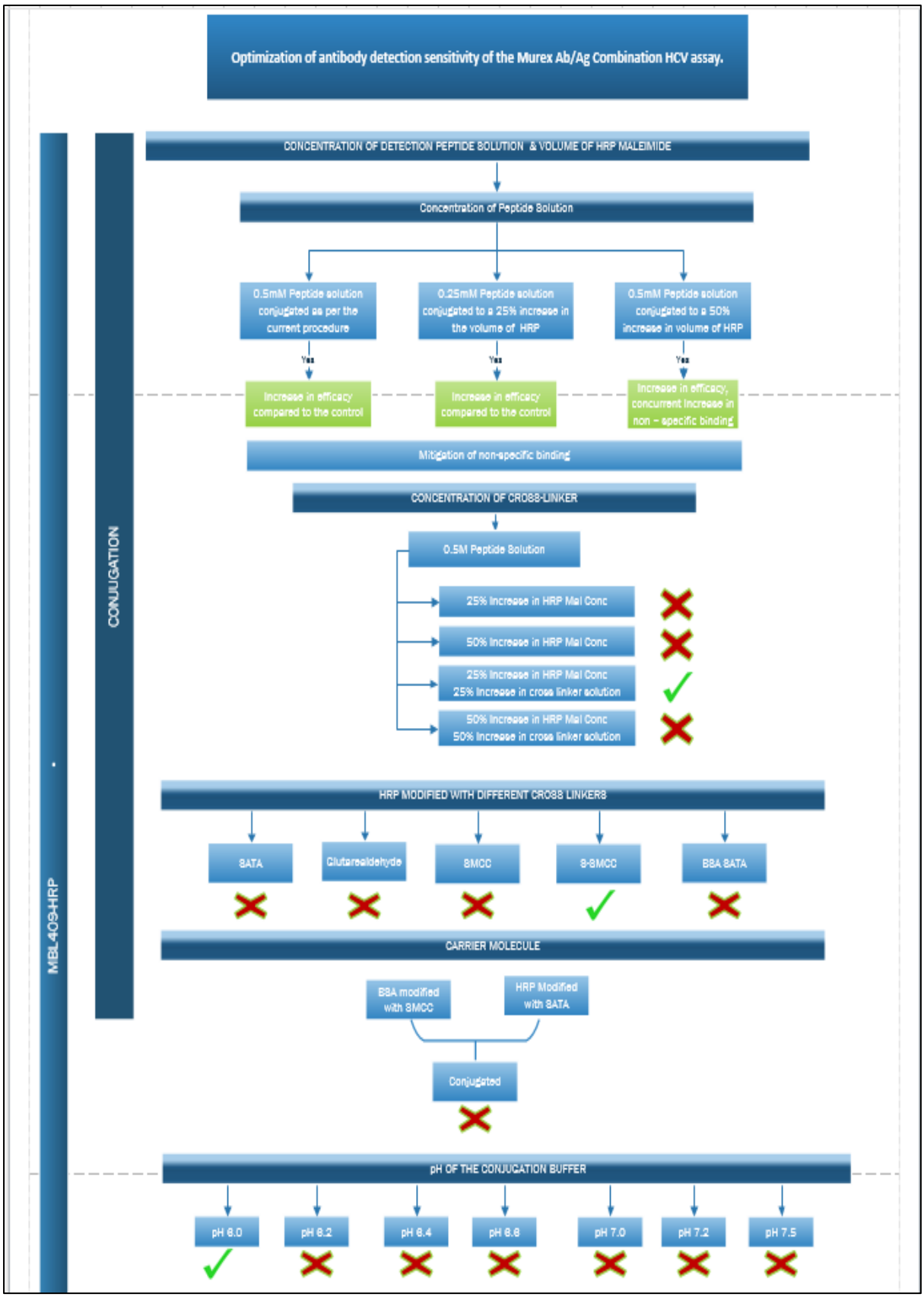
There are a variety of media options available, such as those made of dextrans (Sephadex) and agaroses (Sepharose). This form of separation is advantageous, in that it is inexpensive and easily available (Aguilar, 2003). Another advantage of size exclusion chromatography is its relatively simple execution, as it does not necessarily require instrumentation. The technique is versatile, in that, any soluble substance can be separated (Mori and Barth, 1999).

CHAPTER 3: MATERIALS AND METHODS

This chapter describes the variables tested in the synthesis for both the core capture and detection molecules. The standard methods of synthesis for both core detection and capture molecules used at DiaSorin South Africa are described in Appendix B3.1 and B3.2, respectively.

Figure 3.1 below provides a representation of the flow of the research from the start of this study through to its conclusion. The green ticks indicate the variables that improved the synthesis process, whereas the red crosses indicate the variables that did not affect or negatively affected the preparation of these reagents. The methods presented below were limited to the variables that indicated an improved result. The methods that did not affect or negatively affected the reagent preparation can be found in Appendix B5.

Each variable was tested sequentially, as detailed in the sections below, and following analysis of the results, the best result was selected and added to the experimental protocol in the following section, unless otherwise stated. This approach was employed in order to optimize material usage and mitigate the introduction of additional requirements during each experiment. The outcome of each section is detailed in the Results and Discussion chapter.


Optimization of antibody detection sensitivity of the Murex Ab/Ag Combination HCV assay.
MBL409HRP
CONJUGATION
CONCENTRATION OF DETECTION PEPTIDE SOLUTION & VOLUME OF HRP MALEIMIDE
Concentration of Peptide Solution
0.5mM Peptide solution conjugated as per the current procedure
0.25mM Peptide solution conjugated to a 25% increase in the volume of HRP
0.5mM Peptide solution conjugated to a 50% increase in volume of HRP
Yes
Yes
Yes
Increase in efficacy compared to the control
Increase in efficacy compared to the control
Increase in efficacy, concurrent increase in non – specific binding
Mitigation of non-specific binding
CONCENTRATION OF CROSS-LINKER
0.5M Peptide Solution
25% Increase in HRP Mal Conc
50% Increase in HRP Mal Conc
25% Increase in HRP Mal Conc
25% Increase in cross linker solution
50% Increase in HRP Mal Conc
50% Increase in cross linker solution
HRP MODIFIED WITH DIFFERENT CROSS LINKERS
SATA
Glutaraldehyde
SMCC
S-SMCC
BSA SATA
CARRIER MOLECULE
BSA modified with SMCC
HRP Modified with SATA
Conjugated
pH OF THE CONJUGATION BUFFER
pH 6.0
pH 6.2
pH 6.4
pH 6.8
pH 7.0
pH 7.2
pH 7.5

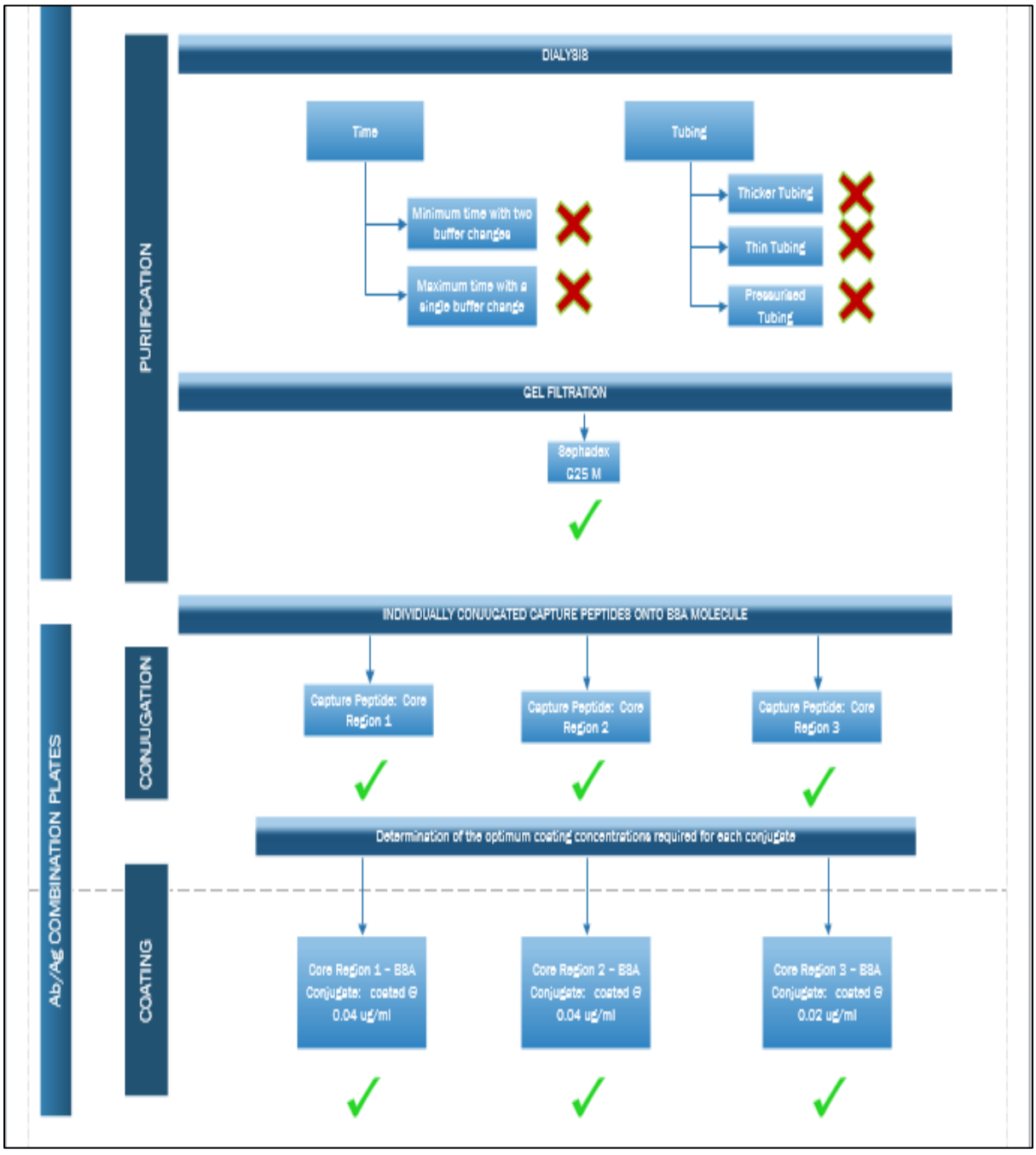


Figure 3.1: Flowchart indicating the variables tested in this study.

3.1 Research to improve the detection molecule.

3.1.1 Objective 1: Concentration of sulfo-SMCC modified horseradish peroxidase and the detection peptide solution.

3.1.1.1 Concentration of sulfo-SMCC modified horseradish peroxidase.

A 100 mg/ml solution of horseradish peroxidase was prepared by dissolving a total of 2 g of horseradish peroxidase (BBI Solutions, Gwent, UK) in 20 ml of 25 mM HEPES, 1 mM EDTA (pH 7.8), as described in Appendix B3.1.1. The HRP-maleimide concentration of 2.08 mM was determined by A4 assay.

A 1 mM solution of sulfo-SMCC modified horseradish peroxidase (control) was prepared as per the standard method described in Appendix B3.1.3.

A 1.25 mM solution was prepared by adding 2.67 ml of 25 mM HEPES/1 mM EDTA (pH 6.8) to 4 ml of sulfo-SMCC modified horseradish peroxidase.

3.1.1.2 Concentration of detection peptide

The method used at DiaSorin South Africa makes use of a 0.1 mM detection peptide solution for conjugation to sulfo-SMCC modified horseradish peroxidase.

A 10 mM core-peptide solution was prepared by dissolving 6 mg of the peptide representing core region 3 (Auspep, Victoria, Australia) in 382 µl of DMSO (Sigma Aldrich, Missouri, USA), as detailed in Appendix B3.1.3.

Thereafter, a series of dilutions of the 10 mM solution was prepared in a 25 mM HEPES/1 mM EDTA (pH 6.8) solution. Peptide concentrations: i.e., 0.25 mM, 0.5 mM and 0.75 mM were prepared by adding 90 µl of the 10 mM core-peptide solution, to 3.5 ml, 1.8 ml and 1.1 ml of 25 mM HEPES/1 mM EDTA (pH 6.8) solution, respectively.

Linkage to sulfo-SMCC modified horseradish peroxidase, and removal of excess/unbound reagents were executed as per the standard procedure in use at DiaSorin South Africa as described in Appendix B3.1.3 and B3.1.4.

3.1.1.3 Evaluation of the detection molecule with increased concentration of sulfo-SMCC-modified horseradish peroxidase and the detection peptide solution

a. Test Method:

The detection molecules were prepared at peptide concentrations of 0.25 mM, 0.5 mM, and 0.75 mM, respectively. A range of dilutions were prepared for each concentration and for the

control. These dilutions were as follows: 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/1500, and 1/2000. The detection molecule prepared at each scenario and the control were then tested as per the standard method for qualification, at DiaSorin South Africa. Testing was conducted using the Murex HCV Ag/Ab Combination assay and a commercially available coated micro-plate.

The following samples were tested as per the layout detailed in Table C1.1 of Appendix C.1.

- The negative control (KN) contained within the kit.
- Quality control reference standard containing antibodies specific to core region 3 of the hepatitis C genome was used to assess the detection molecule's performance.

b. Evaluation of the data and statistical analysis

The mean OD at 448 nm/690 nm was determined spectrophotometrically; thereafter the S/CO was calculated to compare the different concentrations to the control. This was calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality control for core region 3 was divided by its corresponding cut-off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample was considered positive if the S/CO value is greater than 1.0.

The difference in the performance of each test scenario compared to the control was calculated and expressed as a percentage. A colour scale was applied to highlight the increasing deviation from the control.

One-way ANOVA as described by evolutionary biologist and statistician Ronald Fisher was conducted in Minitab (R) 19 software to determine if any variation in the performance of the experimental detection molecules were statistically significant (Fisher, 1918). The dependent variable was the S/CO values achieved for each of the scenarios tested, whereas the independent variable was the peptide concentrations (Laerd Statistics, 2018). The following were applied:

A α-level of 0.05

N_0: The means are equal.

N_a: The means are not equal.

A Tukey pairwise comparison, as described by John Tukey was used after ANOVA to compare all possible pairs of means with each other (Benjamini & Braun, 2002). As such this test was conducted in Minitab (R) 19 software to determine which concentrations performed statistically

significantly differently from one another with the aim of identifying the optimal peptide concentration for preparation of the detection molecule.

3.1.2 Objective 1: Mitigation of non-specific binding by increasing the concentration of cross-linking reagent used in the modification of horseradish peroxidase.

For each experiment, 500 mg of horseradish peroxidase was dissolved in 5 ml of 25 mM HEPES/1 mM EDTA (pH 6.8).

A 100 mg/ml solution of sulfo-SMCC (Pierce Biotechnologies, Rockford, USA) was prepared as described in Appendix B2.11.

The sulfo–SMCC modified horseradish peroxidase was diluted to a final concentration of 1250 µM, despite the concentration of sulfo-SMCC offered.

A 10 mM core-peptide solution was prepared by dissolving 5 mg of the peptide representing core region 3 (Auspep, Victoria, Australia) in 319 µl of DMSO (Sigma Aldrich, Missouri, USA), as per the standard method for preparation. Thereafter, a 0.1 mM solution was prepared by adding 64 µl of the 10 mM peptide solution to 1.28 ml of 25 mM HEPES/1 mM EDTA (pH 6.8).

3.1.2.1 Cross linker offered to horseradish peroxidase as a 2.25-fold excess and conjugated to 500 mM peptide solution.

A volume of 138 µl of 100 mg/ml sulfo-SMCC solution was added to the horseradish peroxidase. Excess unbound reagents were removed from the modified horseradish peroxidase solution as detailed in Appendix B3.1.2.

The absorbance value at 280 nm was 0.610. The extinction coefficient of 2.988 was applied to the absorbance value to determine the protein concentration in mg/ml. The protein concentration was determined as 83.370 mg/ml. The absorbance at 324 nm was 0.752. The extinction coefficient of 2.475 was used to determine the maleimide concentration, of 2351.5 µM. The sulfo-SMCC solution was diluted to 1250 µM by the addition of 3.337 ml of 25 mM HEPES/1 mM EDTA (pH 6.8).

Conjugation was achieved by the drop-wise addition of 1.344 ml of peptide solution to 0.269 ml of 1250 µM sulfo-SMCC modified horseradish peroxidase, whilst continuously swirling. Then, 54 µl of 500 mM hydroxylamine was the added to the solution and incubated for 20 hours at 2-8°C. The conjugation reaction was stopped as per the standard method for preparation of the detection molecule. Excess/unbound reagents were also removed by dialysis as per the standard method.

3.1.2.2 Cross linker offered to horseradish peroxidase as a 2.5-fold excess and conjugated to 500 mM peptide solution.

A volume of 163 µl of 100 mg/ml sulfo-SMCC solution was added to the horseradish peroxidase. Excess unbound reagents were removed from the modified horseradish peroxidase solution as detailed in Appendix B3.1.2.

The absorbance value at 280 nm was 0.610. The extinction co-efficient of 2.988 was applied to the absorbance value to determine the protein concentration in mg/ml. The protein concentration was determined as 83.37 mg/ml. The absorbance at 324 nm was 0.706. The extinction co-efficient of 2.475 was used to determine the maleimide concentration of 2537.37 µM. The sulfo-SMCC solution was diluted to 1250 µM by the addition of 3.337 ml of 25 mM HEPES/1 mM EDTA (pH 6.8).

Conjugation was achieved by the drop-wise addition of 1.344 ml of peptide solution to 0.269 ml of 1250 µM sulfo-SMCC modified horseradish peroxidase, whilst continuously swirling. A volume of 54 µl of 500 mM hydroxylamine was then added to the solution, and this was incubated for 20 hours at 2 - 8°C. The conjugation reaction was stopped as per the standard method for preparation of the detection molecule. Excess/unbound reagents were also removed by dialysis as per the standard method.

3.1.2.3 Cross-linker applied to horseradish peroxidase as a 2.75-fold excess and conjugated to 500 mM peptide solution.

A volume of 192 µl of 100 mg/ml sulfo-SMCC solution was added to the horseradish peroxidase. Excess unbound reagents were removed from the modified horseradish peroxidase solution as detailed in Appendix B3.1.2.

The absorbance value at 280 nm was 0.610. The extinction co-efficient of 2.988 was applied to the absorbance value to determine the protein concentration in mg/ml. The protein concentration was determined as 83.37 mg/ml. The absorbance at 324 nm was 0.690. The extinction co-efficient of 2.475 was used to determine the maleimide concentration, of 2602.02 µM. The sulfo-SMCC solution was diluted to 1250 µM by the addition of 3.337 ml of 25 mM HEPES/1 mM EDTA (pH 6.8).

Conjugation was achieved by the drop-wise addition of 1.344 ml of peptide solution to 0.269 ml of 1250 µM sulfo-SMCC modified horseradish peroxidase, whilst continuously swirling. Then, 54 µl of 500 mM hydroxylamine was the added to the solution, and incubated for 20 hours at 2 - 8° C. The conjugation reaction was stopped as per the standard method for

preparation of the detection molecule. Excess/unbound reagents were also removed by dialysis as per the standard method.

3.1.2.4 Evaluation of the detection molecule when the concentration of the cross-linker was increased.

a. Test Method:

The detection molecules prepared using sulfo-SMCC were provided at 2.25-fold excess, 2.5-fold excess and 2.75-fold excess, were then tested alongside a detection molecule prepared as per the standard method of preparation of the core region 3 detection molecule. A range of dilutions were prepared for each concentration, i.e., i.e., 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/1500, and 1/2000. The detection molecule prepared at each scenario was then tested as per the standard method for qualification, at DiaSorin South Africa.

The testing was conducted as per the Murex HCV Ag/Ab Combination protocol detailed in the Instructions for Use (IFU) (DiaSorin South Africa, 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4.

The following samples were tested as per the layout detailed in Table C1.1 of Appendix C.1.

- The Negative Control (KN) contained within the kit.
- Quality control reference standard containing antibodies specific to core region 3 of the hepatitis C genome was used to assess the detection molecule's performance.

b. Evaluation of the data and statistical analysis

The mean OD at 448 nm/690 nm was determined spectrophotometrically. Thereafter the S/CO was calculated to compare the detection molecules prepared using the different concentrations of the sulfo-SMCC to the control. This was calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality control for core region 3 was then divided by its corresponding cut off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample is considered positive if the S/CO value is greater than 1.0.

The one-way ANOVA test (Fisher, 1918) was conducted in Minitab (R) 19 software to determine if any variation in the performance of the experimental detection molecules was statistically significant. The dependent variable was the S/CO values achieved for each of the scenarios tested, whereas the independent variable was the concentration of the sulfo-SMCC (Laerd Statistics, 2018). The following were applied:

A α-level of 0.05

N_0: The means are equal.

N_a: The means are not equal.

A Tukey pairwise comparison was used after ANOVA to compare all possible pairs of means with each other (Benjamini & Braun, 2002). As such, this test was conducted in Minitab (R) 19 software to determine which concentrations performed statistically significantly differently from one another with the aim of identifying the optimal concentration of sulfo-SMCC for preparation of the detection molecule.

3.1.3 Objective 1: pH of the conjugation buffer

The standard method for preparation of the core region 3 detection molecule makes use of 25 mM HEPES, 1 mM EDTA (pH 6.8) solution. This experiment investigated the effect of pH and conductivity on the efficiency of the cross-linking process, and the removal of excess un-wanted reagents.

3.1.3.1 Preparation of 25 mM HEPES, 1 mM EDTA

A volume of 1 L of 25 mM HEPES/1 mM EDTA conjugation buffer was prepared as described in section B2.2. The volume was divided into 100 ml aliquots, and the pH was adjusted to 6.0, 6.2, 6.4, 6.6, 7.0, 7.2, and 7.5, either by addition of 5M HCl or 5M NaOH and an Orion Star A211 Benchtop pH meter (Thermo Scientific, Pittsburgh PA, USA). The conductivity of the conjugation buffers, with the adjusted pH was measured to determine if there were any changes to the electrical conductivity of the solution. This was measured using an Orion Star A222 portable conductivity meter (Thermo Scientific, Pittsburgh PA, USA).

3.1.3.2 Preparation of the detection molecule

The core region 3 detection molecule for each pH solution was then prepared as described in sections B3.1.2 – B3.1.3, except for the pH of the 25mM HEPES/1mM EDTA conjugation buffer. In addition, removal of excess and unwanted reagents was performed as described in section B3.1.4 but in the same solution as the conjugation process.

3.1.3.3 Evaluation of the detection molecule at various conjugation buffer pH

a. Test Method

The detection molecules prepared when varying the pH of the conjugation buffer were tested, alongside a detection molecule prepared as per the standard method of preparation of the

core region 3 detection molecule (pH 6.8). A range of dilutions were prepared for each pH, i.e., 1/350, 1/500, 1/750, and 1/1000. The detection molecule prepared at each scenario was then tested as per the standard method for qualification, at DiaSorin South Africa.

The testing was conducted as per the Murex HCV Ag/Ab Combination protocol detailed the Instructions for Use (IFU) (DiaSorin South Africa 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4.

The following samples were tested as per the layout detailed in Table C1.1 of Appendix C.1.

- The negative control (KN) contained within the kit.
- Quality control reference standard containing antibodies specific to core region 3 of the hepatitis C genome was used to assess the detection molecule's performance.

b. Evaluation of the data and statistical analysis

The mean OD at 448 nm/690 nm was determined spectrophotometrically; thereafter the S/CO was calculated to compare the detection molecules prepared with buffers of differing pH to the control. This is calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality control for core region 3 was then divided by its corresponding cut-off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample is considered positive if the S/CO value is greater than 1.0.

The performance data for each pH at each dilution was reviewed and the scenario showing the highest S/CO value was compared to the control (pH 6.8).

A two sample T-test (Wadhwa & Marappa-Ganeshan, 2003) was conducted on the selected data using in Minitab ® 19 software to determine if any variation in the performance of the experimental detection molecules were statistically significant.

3.1.4 Objective 2: Separation of excess unbound reagents from the detection molecule

Core region 3 detection molecules were prepared in 10 ml volumes as per the standard method described in Appendix B3.1.2 and B3.1.3.

3.1.4.1 Removal of excess/unwanted reagents from the core detection conjugate by dialysis.

As dialysis is the standard method for removal of unconjugated or excess reagents from the enzyme conjugate, variation in the membrane format was tested. The contact material of all

dialysis membranes tested was regenerated cellulose acetate, with a MWCO of 10 000 Daltons. The first 10 ml enzyme conjugate was pipetted into dialysis tubing with a diameter of 6.3 mm and a wall thickness of 0.050 mm. The second 10 ml enzyme conjugate was pipetted into dialysis tubing with a diameter of 16 mm and a membrane thickness of 0.020 mm. The third test enzyme conjugate was pipetted into a 15 ml Slide-A-Lyzer G2TM dialysis cassette (Thermo Scientific, Massachusetts, USA). Each of the filled membranes were then placed in 1000 ml of 25 mM HEPES/1 mM EDTA (pH 6.8) solution at 2-8°C, and agitated with a magnetic flea plea, as is standard practice at DiaSorin South Africa. The enzyme conjugate was allowed to dialyze for 6 hours, thereafter the dialysis was refreshed, and dialysis was allowed to occur for a subsequent 18 hours.

3.1.4.2 Removal of excess/unwanted reagents from the core detection conjugate by gel filtration.

Enzyme peptide conjugates were prepared in 10 ml volumes. Removal of excess and unwanted reagents was achieved by gel filtration. To this end, four (4) PD-10 columns (GE Healthcare, Buckinghamshire, UK) containing Sephadex G25 Medium resin were pre-treated with five (5) column volumes of 25 mM HEPES/1 mM EDTA, pH 6.8 solution. Thereafter, 2.5 ml of the enzyme conjugate was then applied to each prepared PD-column and allowed to completely enter the gel bed. Once the solution had entered the gel bed, 3.2 ml of 25 mM HEPES 1mM EDTA, pH 6.8 solution was applied to the top of each column. The eluate containing the enzyme conjugate, determined by visual inspection, was then collected in glass bottles, and pooled together.

3.1.4.3 Evaluation of the detection molecule after removal of excess unbound reagents.

a. Test Method:

The detection molecules prepared when varying the method for removal of excess/unbound reagents were tested alongside a detection molecule prepared as per the standard method of preparation of the core region 3 detection molecule. A range of dilutions were prepared for each pH, i.e., 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/1500, and 1/2000. The detection molecule prepared at each scenario was then tested as per the standard method for qualification at DiaSorin South Africa.

The testing was conducted as per the Murex HCV Ag/Ab Combination protocol detailed the Instructions for Use (IFU) (DiaSorin South Africa 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4.

The following samples were tested as per the layout detailed in Table C1.1 of Appendix C.1.

- The negative control (KN) contained within the kit.
- Quality control reference standard containing antibodies specific to core region 3 of the hepatitis C genome was used to assess the detection molecule's performance.

b. Evaluation of the data and statistical analysis:

The mean OD at 448 nm/690 nm was determined spectrophotometrically. Thereafter the S/CO was calculated for each detection molecule. This was calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality control for core region 3 was then divided by its corresponding cut off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample is considered positive if the S/CO value is greater than 1.0.

One-way ANOVA (Fisher, 1918) was conducted in Minitab (R) 19 software to determine if any variation in the performance of the experimental detection molecules were statistically significant. The dependent variable was the S/CO values achieved for each of the scenarios tested, whereas the independent variable was the pH of the buffer (Laerd Statistics, 2018). The following were applied:

A α-level of 0.05

N_0: The means are equal.

N_a: The means are not equal.

The detection molecule with the highest S/CO were selected and a two sample T-test was conducted on the selected data as described by Wadhwa & Marappa-Ganeshan (2003) in Minitab ® 19 software to determine if any variation in the performance of the experimental detection molecules were statistically significant. The standard deviations were assessed to determine variability within the data set.

3.2 Improvement of the conjugation of the capture molecule.

3.2.1 Objective 3: Linkage of individual core peptides to Bovine Serum Albumin (BSA)

3.2.1.1 Preparation of sulfo-SMCC modified Bovine Serum Albumin (BSA)

A volume of 7.5 ml of sulfo-SMCC modified BSA was used to prepare each of the conjugates. Based on this volume, and the maleimide concentration, the total maleimide content was calculated to be 6840.9 nM. A 100 mg/ml solution of each peptide was prepared for conjugation.

An amount of 18 mg of the capture peptide for core region 1 was dissolved in 180 µl of DMSO. Then 13 mg of the capture peptide for core region 2 was dissolved in 133 µl of DMSO. An amount of 13 mg of the capture peptide for core region 3 was dissolved in 130 µl of DMSO. The entire contents of each peptide solution were added to 7.5 ml of the sulfo-SMCC modified bovine serum albumin. Thereafter, 375 µl of 500 mM hydroxylamine was incubated with each of the peptide mixtures. All three conjugates were incubated concurrently, at ambient temperature (18°C to 25°C), away from direct light, on a mechanical roller, for 18.5 hours.

At the end of the incubation period, each of the conjugation reactions were terminated by the addition of 958 µl of a 1/201 dilution of β-mercaptoethanol (0.14 µl per nM of modified BSA) and incubated for 20 minutes at ambient temperature. Thereafter, 1368 µl of 100 mM NEM solution (0.2 µl per nmol of modified BSA) was added to conjugate and allowed to incubate for 15 minutes, at ambient temperature, away from direct light.

3.2.1.2 Removal of excess/unbound reagents from the individual capture complexes

The excess unbound reagents contained within the three individual capture complexes were then removed by dialysis. The capture peptide complexes were purified by dialysis in a cold pH 6.8 25 mM HEPES 1 mM EDTA solution, at 200 x the total conjugate volume. This meant that each of the core region 1 capture peptide complex, the core region 2 capture peptide complex, and the core region 3 capture peptide complex was purified in 2.066 L of dialysis buffer. Dialysis was performed at 2-8°C, in size 1 dialysis tubing MWCO 12000 – 14000 DA (MediCell Membranes Ltd, London, UK). The conjugates were allowed to dialyse for an initial 8 hours, after which the buffer was refreshed. Thereafter, dialysis was allowed to progress for a subsequent 14 hours.

3.2.1.3 Determination of the concentration of each core capture complex

The concentration of the capture peptide complexes for each of the regions was determined by dividing the weight of the peptide used by the final volume of the conjugate after removal

from dialysis. The concentration of the capture peptide complex for core region 1 was 1.764 mg/ml, for core region 2 was 1.300 mg/ml, and the core region 3 was 1.287 mg/ml.

3.2.1.4 Determination of the optimum concentration of the each of the individual core-capture complexes for coating on the microtitre wells

The optimum coating concentration for each core capture complex was determined by coating the conjugates onto microtitre wells, at a range of concentrations as detailed below. This method only deviated from the standard method for determination of concentration by the use of three separate core capture complexes coated at different concentrations, rather than one polycore capture complex.

a. Coating stage

Each of the individually prepared core capture complexes were coated onto microtitre plates (Nunc MaxiSorp, Thermo Fisher Scientific, Roskilde, Denmark) at concentrations of 0.02 µg/ml, 0.04 µg/ml, 0.06 µg/ml, and 0.08 µg/ml. The polycore capture complex (control) was coated onto the wells at a concentration of 0.04 µg/ml. One coated microtitre plate (96 wells) was prepared per scenario tested.

The core antigens at each concentration tested, as well as the antigen capture antibody and recombinant NS3 antigen, were added to the standard pH 9.6 50 mM carbonate/bicarbonate coating buffer, containing 20 mM DTT. 115 µl of the coating buffer containing the antigens and antibody were dispensed in a 96 well microtitre plate (Nunc MaxiSorp, Thermo Fisher Scientific, Roskilde, Denmark). The microplates were incubated at ambient temperature overnight.

b. Blocking stage

After the incubation with the core antigens and the capture antibody at room temperature, 115 µl of 0.2% casein in PBS blocking buffer (pH 7.4) was added into each carbonate/bicarbonate-containing well and incubated at room temperature overnight.

c. Fixing stage

After incubation, the coating buffer/blocking buffer mixture was completely aspirated from each well of the microtitre plate. Immobilisation onto the solid phase was achieved by dispensing a fixing buffer that contains 40 mM of DTT. A volume of 135 µl fixing buffer (pH 6.0), was pipetted into each well of the microtitre plate and incubated at room temperature overnight. The partial aspiration of the fixing buffer ensured a residual volume of fixing buffer remained in each well. The remaining buffer was then dried onto the microtitre well, by incubation at 37°C for at least 12 hours.

3.2.1.5 Evaluation of the individually prepared core capture molecules

a. Test Method:

The individually prepared capture molecules to core region 1, core region 2, and core region 3, were tested alongside a commercially available coated Murex HCV Ag/Ab Combination microtitre plate, as per the standard Murex HCV Ag/Ab Combination protocol detailed in the Instructions for Use (IFU) (DiaSorin South Africa, 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4.

The capture molecules were coated at concentrations of 0.02 µg/ml, 0.04 µg/ml, 0.06 µg/ml, and 0.08 µg/ml and the performance of each was compared to the microtitre wells coated using the polycore complex at a concentration of 0.04 µg/ml.

The following samples were tested as per the layout detailed in Table C2.1 of Appendix C.2.
The Negative Control (KN) contained within the kit.
Quality control reference standards, each containing antibodies specific to the core region 1, core region 2, and core region 3 of the hepatitis C genome was used to detect the efficacy of the test plates.

b. Evaluation of the data and statistical analysis:

The mean OD at 448 nm/690 nm was determined spectrophotometrically. Thereafter, the S/CO was calculated to compare the individually prepared core capture complexes to the polycore capture complex. This was calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality controls for each core region was divided by its corresponding cut-off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample is considered positive if the S/CO value is greater than 1.0.

3.3 Improved preparation of the core detection and capture molecules

3.3.1 Improved method for the preparation of the core capture molecule

The individual core capture complexes prepared in section 3.2,1, were coated on microtitre wells (Nunc MaxiSorp, Thermo Fisher Scientific, Roskilde, Denmark).

The concentrations of the core-capture complex for core region 1 and core region 2 were coated at a final concentration of 0.4 µg/ml, and core capture complex was coated at a final concentration of 0.02 µg/ml.

The NS3 and the antigen capture antibody were coated as per the standard procedure at DiaSorin South Africa. Blocking and immobilisation onto the solid phase proceeded as per the standard method at DiaSorin South Africa.

3.3.2 Improved method of preparation of the core detection molecule

3.3.2.1 Preparation of the sulfo-SMCC modified horseradish peroxidase

A total of 2 g of horseradish peroxidase (BBI Solutions, Gwent, UK) was dissolved in 20 ml of 25 mM HEPES/1 mM EDTA, pH 7.8 solution at ambient temperature, as is the procedure used at DiaSorin South Africa.

Then, 62.5 mg of S-SMCC (Pierce Biotechnologies, Rockford) was dissolved in 0.625 ml of DMSO (Sigma Aldrich, Missouri, USA), at ambient temperature, resulting in a 100 mg/ml solution of the cross-linking reagent.

The cross-linking agent was added at 2.5-fold excess to horseradish peroxidase. A volume of 548 µl of 100 mg/ml of S-SMCC solution was then slowly added dropwise to the 20 ml of HRP solution, whilst continuously swirling. The resulting HRP-maleimide solution was then incubated for approximately 45 minutes, away from direct light.

The unbound S-SMCC was removed from the solution by gel filtration using PD-10 columns (GE Healthcare, Buckinghamshire, UK) containing Sephadex G25 Medium resin. These columns were prepared by allowing the ethanol in which it was stored to run through, then equilibrating the columns with 25 ml of 25 mM HEPES/1 mM EDTA, pH 7.8 solution (five (5) column volumes). A volume of 2.5 ml of HRP-maleimide solution was then applied to each prepared PD-column and allowed to completely enter the gel bed. Once the solution had entered the gel bed, 3.2 ml of 25 mM HEPES/1 mM EDTA, pH 7.8 solution was then applied to the top

of the column, with the aim of elution of the purified HRP-maleimide. The eluate containing HRP, determined by visual inspection, was then collected in glass bottles and pooled.

The absorbance value at 280 nm was 0.539. The extinction co-efficient of 2.988 was applied to the absorbance value to determine the protein concentration in mg/ml. The protein concentration was determined as 72.17 mg/ml or 1804.2 µM. The absorbance at 324 nm was 0.844. The extinction co-efficient of 2.475 was used to determine the maleimide concentration, of 1620.2 µM.

The sulfo-SMCC modified horseradish peroxide solution was diluted to 1250 µM by the addition of 0.867 ml of 25 mM HEPES/1 mM EDTA (pH 7.8).

3.3.2.2 Preparation of the core region 3 detection molecule

A 10 mM peptide solution was prepared by dissolving 25 mg of the peptide representing core region 3 (Auspep, Victoria, Australia) in 1.59 ml DMSO. The solution was gently mixed by swirling for 20 to 30 minutes at ambient temperature to ensure complete dissolution. A 500 µM peptide solution was prepared by the addition of 30.248 ml 25 mM HEPES/1mM EDTA, pH 6.0 solution HEPED buffer.

A volume of 5.773 ml of 500 µM peptide solution was added to 28.862 ml of 1250 µM HRP-maleimide solution, and incubated with 5.773 ml 500 mM hydroxylamine, at 2 - 8° C for 20 hours. The reaction was terminated by the sequential addition of 1.443 ml 2-ME (incubated at ambient temperature for 20 minutes), thereafter 2.887 ml NEM ((incubated at ambient temperature for 12 minutes).

3.3.2.3 Purification of detection peptide conjugated to horseradish peroxidase

A volume of 2.5 ml of HRP-maleimide solution was applied to each PD-column. The solution was allowed to enter the gel bed completely. Once the solution had entered the bed of the column, 3.2 ml of 25mM HEPES/1mM EDTA, pH 6.0 was applied to the top of each column. The purified HRP-maleimide, identified by the reddish-brown colour, was then collected in glass bottles and pooled.

3.3.3 Preparation of the Murex HCV Ab/Ag freeze-dried combination conjugate

Freeze dried conjugates, as provided in the Murex HCV Ab/Ag Combination kit, were prepared using the improved detection conjugate, and along with the four other enzyme linked detection conjugates. This was done to assess the improved core detection conjugate, in relation to the other core, NS3, and antigen detection conjugates, to ascertain if there are any adverse interactions between them.

3.3.3.1 Preparation of lyophilisation solution

The lyophilization solution's formulation is proprietary, confidential information that cannot be provided in detail. However, in order to contextualise the makeup of the freeze-dried conjugate; the solution is a liquid containing sucrose and casein. The function of this buffered solution is to keep the antigen and antibodies stable during the lyophilisation process, by maintaining the detection molecules in the proper conformations. The lyophilisation solution may be stored at 2ºC to 8°C for up to 2 weeks.

3.3.3.2 Preparation of the Murex HCV Ab/Ag freeze-dried conjugate

The improved conjugate was spiked at 1/2000, and the other detection conjugates were added to the lyophilisation as per the standard method at DiaSorin South Africa. Therefore, a volume of 1.0 ml of the improved core region 3 detection was added in order to prepare a total volume of 2000 ml of lyophilisation solution, as per the standard method in use at DiaSorin South Africa. A control combination conjugate was prepared by spiking a standard core region 3 detection molecule at 1/350, i.e., 5.714 ml in 2000 ml of lyophilisation solution. The detection molecule spiked lyophilisation solution was freeze dried in LyoPro Freeze Dryer (Hull Corporation, Hatboro, USA) as per the Murex HCV Ag/Ab Combination conjugate freeze-drying protocol currently in use at DiaSorin South Africa.

3.3.4 Evaluation of the optimised Murex HCV Ab/Ag freeze-dried combination conjugate on the optimised Murex HCV Ab/Ag microtitre wells

a. Test method:

The conjugates prepared in section 3.3.3 were assessed on the improved coated microtitre wells, prepared in section 3.3.1. A working strength solution of the freeze-dried conjugates was prepared by pouring the entire contents of a bottle of the Murex HCV Ab/Ag conjugate diluent onto the freeze-dried conjugate pellet, as directed by the Murex HCV Ab/Ag Combination Assay IFU (DiaSorin South Africa 2014). Sample diluent, wash buffer, TMB substrate concentrate and diluent, and conjugate diluent were used from a standard Murex HCV Ag/Ab Combination kit. The optimised coated microplate and conjugate were tested alongside a control. This was the Murex HCV Ag/Ab Combination microplate and conjugate, that had been prepared as per standard method of synthesis.

The sensitivity (efficacy and limits of detection), specificity, and stability, of the optimised coated microplate and conjugate combination were assessed, using the samples listed below, as per the layouts detailed in Appendix C3.1, C3.2, C3.3, and C3.4:

- **Sensitivity - Efficacy:** The negative control provided in the Murex HCV Ab/Ag Combination Assay was selected to assess the non-specific binding capacity of the conjugate (Appendix C3.1, Table C3.1).
- **Sensitivity - Efficacy:** A serological panel specifically used to detect antibodies to core region 3 of the HCV genome was used to assess efficacy of the optimised core and detection molecules. This serological panel is an internal control manufactured specifically for the Murex HCV Ab/Ag Combination Assay and is considered proprietary information. Thus, the details of these cannot be disclosed and are not commercially available. The quality control was tested as per:
- **Sensitivity - Limits of Detection:** A dilution series of antibody positive control was prepared to assess the detection limit of the optimised method as per Appendix C3.3, Table C3.7.
- **Specificity:** 90 normal human serum samples as per the layout described in Appendix C3.2 (Table C3.4) were used to assess specificity. Human serum samples known to be negative for the presence of the HCV antigen and antibodies were procured from the South African Blood Services. These samples were provided routinely to DiaSorin South Africa as per a commercial agreement between the two entities, for the specificity testing and batch release of all ELISA products prepared at the site. These samples were used for evaluation of the specificity of the improved microtitre plate and conjugate.
- **Accelerated stability:** Testing was conducted on the optimised microtitre wells and freeze-dried conjugate combination using the negative control and internal quality control specifically used to detect antibodies generated to core region 3. Seven (7) samples of the microtitre plate and conjugate were stored at 37°C for 7 weeks. The seven (7) samples of one batch of microtitre plates and matched conjugated manufactured according to the standard method were also stored at the same temperature, to serve as the control. A fresh microtitre plate and conjugate (both test and control) were tested once per week over seven (7) weeks, as per layout described in Appendix C3.4 (Table C3.11). At time zero (T=0) and week seven (7), five (5) replicates were tested, whereas during weeks one (1) to six (6), a single replicate was tested per week.

b. Evaluation of the optimised core capture and detection molecules and statistical methods

The mean OD at 448 nm/690 nm was determined spectrophotometrically. Thereafter the S/CO was calculated to compare the improved molecules with those of the standard method.

The following statistical methods were used:

- Sensitivity - Efficacy testing: A two sample T-test (Wadhwa & Marappa-Ganeshan, 2003) was conducted on the selected data in Minitab ® 19 software to determine statistically significance between the reagents prepared using the improved and standard methods . The standard deviations were assessed to determine variability within the data set.
- Specificity: Descriptive statistics was conducted in Microsoft Excel before a histogram was plotted to determine the distribution of the samples in the data set (Yannis, 2004). A two sample T-test (Wadhwa & Marappa-Ganeshan, 2003) on the selected data was conducted in Minitab ® 19 software to determine statistically significance between the reagents prepared using the improved and standard methods. The standard deviations were assessed to determine variability within the data set.
- Sensitivity – Limit of Detection: Regression analysis was conducted in Microsoft excel to theoretically determine the dilution at which the assay would return a positive result (S/CO = 1.0).

CHAPTER 4: RESULTS AND DISCUSSION

The variables assessed and the associated methods used to prepare the respective reagents were executed as described in Chapter 3.

The study set out to use various combinations of ELISA kit components to test which optimised its efficacy and sensitivity. However, it may be appropriate to mention a limitation to this study. The reagents and compounds described in this study were prepared according to standard methods used at DiaSorin South Africa. The quality and efficacy of these compounds were then assessed against data associated from a commercially available Murex HCV Ab/Ag Combination test kit. Thus, much of the data and research surrounding this kit remain proprietary and confidential to DiaSorin South Africa. As a consequence, the data generated for the variables tested cannot be compared to current literature for similar assays and are discussed against generally accepted ELISA principles, standards and methods. In addition, the results presented and discussed below are limited to those variables that indicated an improved result.

4.1 Improvement of the detection molecule

4.1.1 Varying the concentrations of sulfo-SMCC horseradish peroxidase and peptide concentration.

This section of the study assessed the effects of peptide concentration on the sensitivity of the conjugated core region 3 detection molecule. In optimising the preparation of this detection molecule, a variable was the concentration of the peptide component that was increased from 0.25 mM, 0.5 mM to 0.75 mM before being linked to 1250 µM sulfo-SMCC modified horseradish peroxidase. The prepared detection molecules were then compared to a detection molecule prepared as per the standard method of synthesis, i.e., a peptide concentration of 0.10 mM and 1000 µM sulfo-SMCC modified horseradish peroxidase.

The OD values of the data generated for each test scenario and the control were converted to S/CO and plotted on a bar graph in Microsoft Excel, to indicate and compare the performance of the detection molecules. This data are presented below:

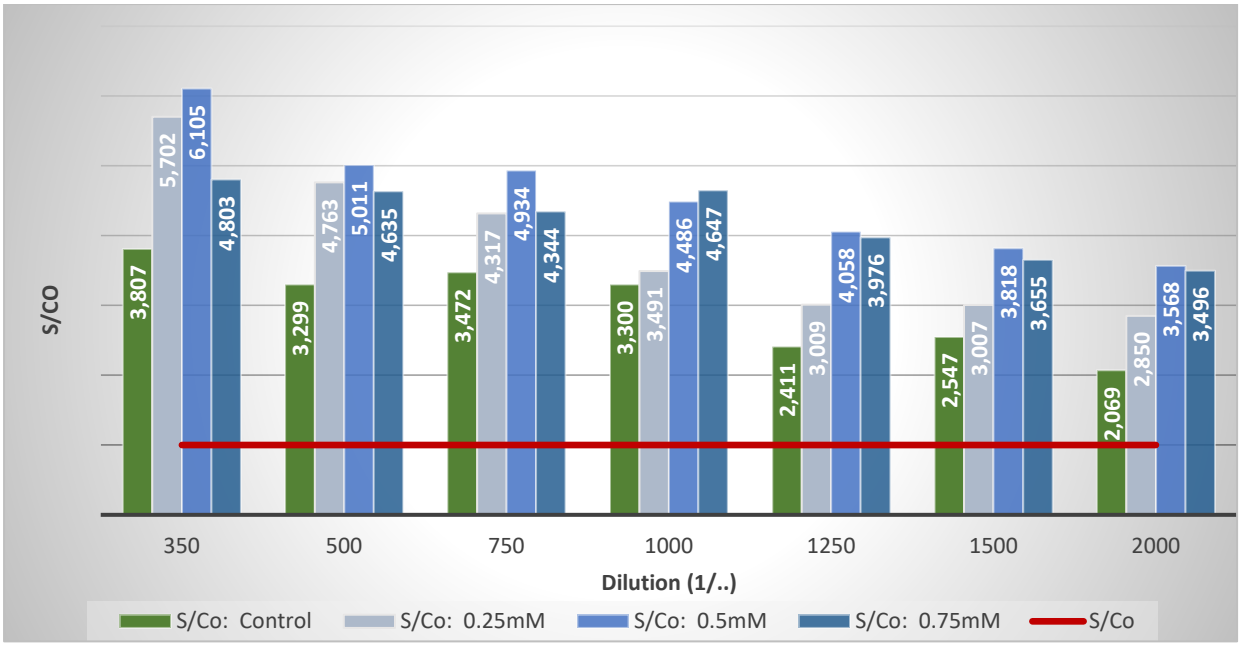


Figure 4.1: S/CO results for a dilution series of the synthesised detection molecule, when the peptide concentration was incrementally increased.

Note 1: Testing was conducted using the Murex HCV Ag/Ab Combination assay, with a commercially available microplate. In addition, a conjugate used as a control was synthesised using a peptide concentration of 0.1 mM.

Note 2: S/CO = Sample to cut-off; mM = Millimolar

In evaluating the detection molecules, a sample to cut off ratio of 1 (S/CO = 1) indicated a positive result.

Figure 4.1 shows a comparison between the resulting S/CO ratios and, thus, the efficacy of the detection conjugate prepared at various concentration of peptides. Trends noted in figure indicated that the control ratio is relatively low when compared to the synthesised detection complexes; there was a gradual reduction in efficacy of the detection molecule across the dilution range; the detection molecule was still effective at a dilution of 1/2000 and, in general, the 0.5 mM peptide solution returned the highest positive result, followed closely by the 0.75 mM peptide solution - the only exception to this was at the 1/1000 dilution, where the 0.75 mM molecule performed better than the 0.5 mM molecule.

The table below details the individual OD units and its conversion to S/CO for the 1/2000 dilution for each of the scenarios. The 1/2000 dilution was selected for analysis as it has the lowest concentration of the detection molecule that is still able to provide a positive result, as indicated in Figure 4.2. The difference in the performance of each test scenario compared to the control was calculated and expressed as a percentage. A colour scale was applied to

highlight the increasing deviation from the control. The difference in the performance of the test scenarios and the control is indicative of improvement to the sensitivity of the detection complex.

Table 4.1: Individual OD values, as well as corresponding S/CO values for the 1/2000 dilution of the detection molecule, when various peptide concentrations were tested.

Note 1: The difference for the OD and the S/CO values were expressed as percentage of the control.
Note 2: OD = Optical density; S/CO = Sample to cut-off; mM = Millimolar

	OD values obtained when using a 1/2000 dilution of the detection molecule						
	Control	0.25 mM		0.5 mM		0.75 mM	
		Value	% Diff.	Value	% Diff.	Value	% Diff.
Replicate 1	0.611	0.771	26.19%	0.858	40.43%	0.85	39%
Replicate 2	0.567	0.771	35.98%	0.885	56.08%	0.801	41%
Replicate 3	0.605	0.731	20.83%	0.898	48.43%	0.804	33%
Replicate 4	0.569	0.700	23.02%	0.827	45.34%	0.899	58%
Cut-off	0.284	0.261		0.243		0.239	
S/CO: Rep 1	2.150	2.957	37.56%	3.531	64.26%	3.560	66%
S/CO: Rep 2	1.995	2.957	48.23%	3.642	82.58%	3.355	68%
S/CO: Rep 3	2.128	2.803	31.72%	3.695	73.63%	3.368	58%
S/CO: Rep 4	2.002	2.685	34.11%	3.403	70.02%	3.765	88%

Figure 4.2 below indicates that the 0.5 mM detection peptide solution performs consistently better than the other scenarios tested, when analysing both the OD values as well as the S/CO values. When assessed against OD units and S/CO, the 0.25 mM peptide solution performed 26% and 38% better respectively, relative to the control. The 0.5 mM detection peptide solution performed 48% and 73% better than the control, whereas the 0.75 mM detection peptide solution performed 43% and 70% better than the control.

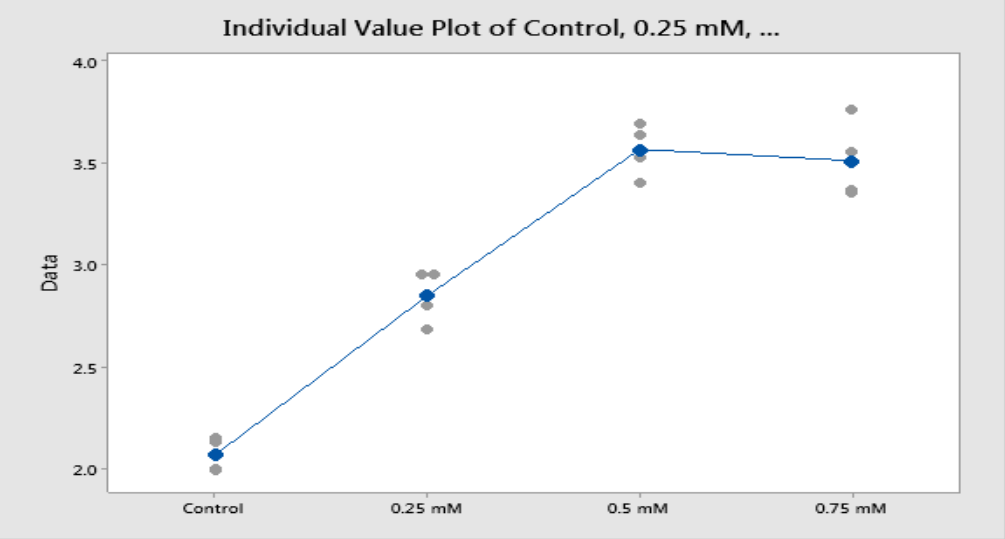


Figure 4.2: Individual value plot of S/CO values of the peptide concentrations at 1/2000 dilution.

Note 1: The individual value plot shows the S/CO value for each replicate at the 1/2000 dilution as shown in Table 4.1. All testing was conducted using the Murex HCV Ab/Ag Combination Assay.

Note 2: S/CO = Sample to cut-off; mM = Millimolar

One-way ANOVA and a Tukey Pairwise Comparison were conducted on the 1/2000 dilution data sub-set, for all peptide concentration scenarios, using Minitab (R) 19 software. This analysis was performed to determine if the improvements indicated in Table 4.1 are statistically significant. An α-level of 0.05 was applied, and the analysis are indicated on Table C1.8 in Appendix C1. The p-value is 0.000, which is less than the α-level value of 0.05, indicating that there is a significant difference in the S/CO values of the four different peptide concentrations used in the manufacture of the detection molecule, at the 1/2000 dilution.

The S/CO values of all peptide concentrations tested are higher than the control, indicating that increasing the detection peptide concentration significantly improves the sensitivity of the detection molecule. The standard deviation of each individual value for the control molecule is 0.0818, the lowest of the scenarios tested. Conversely, the 0.75 mM peptide solution has the highest standard deviation, at 0.1933, indicating greater variability in the data set. The individual value box plot (Figure 4.2) further supports this by providing a graphical representation of the distribution of the S/CO values for each peptide concentration. The S/CO values for the control molecule are clustered tightly around the mean value, whereas those of the 0.75 mM scenario indicates a larger range in the values that contribute to the mean.

The S/CO values for the 0.5 mM and 0.75 mM peptide solutions are distinctly higher than that of the 0.25 mM peptide solution. However, there is not a significant difference between the

S/CO values of the 0.5 mM and the 0.75 mM peptide solution. The Tukey Pairwise comparison confirms this observation (Figure C1.1 in Appendix C). The mean of the control compared to the means of the 0.25 mM, the 0.5 mM and, the 0.75 mM peptide solutions respectively, are significantly different as the 95% confidence interval do not contain a zero. Therefore, increasing the detection peptide concentration significantly affects the S/CO values of the quality control specific for core region 3 antibodies, up to a molarity of 0.5 mM. In addition, increasing the concentration beyond 0.5 mM is an unnecessary step. Furthermore, increasing the dilution of the detection molecule to 1/2000 while still maintaining efficacy increases cost effectiveness of the overall Murex HCV Ab/Ag Combination assay. The concentration of the peptide used for conjugation is prepared from a 10 mM solution stock solution (as per B3.1.3), where the peptide is dissolved into the organic solvent DMSO. Thereafter, the 0.1 mM solution is prepared by dilution in 25 mM HEPES/1 mM EDTA (pH 6.8) solution. Similarly, the preparation of a 0.5 mM peptide solution for conjugation will not utilise additional quantities of the peptide representing core region 3.

The results of increasing the peptide concentration in further excess than is used in the standard method of synthesis demonstrates that the sensitivity of the core region 3 detection molecule is improved up to a concentration of 0.5 mM. The S/CO values for each concentration tested are higher than the control, with the 0.5 mM and the 0.75 mM concentrations performing similarly. The peptide concentration of 0.5 mM was selected for use in the improved manufacturing method as its data demonstrated the largest S/CO value at the lowest dilution tested (1/2000), with the least variability in each individual value. Increasing the sensitivity and decreasing variability in a data set for ELISA is important as it ensures each test performs consistently, thus improving the robustness of the assay.

Hermanson (2013) recommended maintaining high concentrations of proteins or the target molecule within each reaction. The reaction can be controlled to achieve the optimal product by calibration of the molar ratio of the cross linker to the target molecule. The increase in sensitivity when increasing the peptide concentration, as compared to the control core region 3-HRP could be attributed to the availability of “free” binding sites on the activated horseradish peroxidase enzyme. Cross-linking did not proceed on these “free” sites as the peptide offered in the control conjugate was not adequate to saturate all available binding sites. These available sites were now able to link to the additional peptide offered, but only up to a concentration of 0.5 mm. The decrease in sensitivity demonstrated at 0.75 mM, as well as the increase in variability, could be due to the saturation of the binding sites on the activated

horseradish peroxidase. Thus, increasing the peptide concentration beyond 0.5 mM does not result in an increase in the sensitivity of the core region 3 detection molecule.

4.1.2 Varying the concentration of the cross-linking agent.

An increase in the excess sulfo-SMCC offered to horse radish peroxidase was tested in order to determine if more of the cross linker would bind to the enzyme, thereby creating more sites to which core region 3 peptide can bind.

Sulfo-SMCC was added to the 0.5 mM peptide solution at 2-fold, 2.25-fold, 2.5-fold, and 2.75-fold excess. The rationale behind testing the effect of increasing the concentration of the cross-linking agent was the inclusion of additional reactive sites on the enzyme to compensate for the higher peptide concentration offered for conjugation. All other aspects of the preparation process remained as per the standard method for preparation. The control used as a reference was synthesised using the 0.1 mM peptide solution and 2-fold excess of sulfo-SMCC.

The OD values of the data generated on each test were converted to S/CO and plotted on a bar graph in Microsoft Excel, to indicate and compare the performance of the detection molecules. This data are presented in Figure 4.3 below:

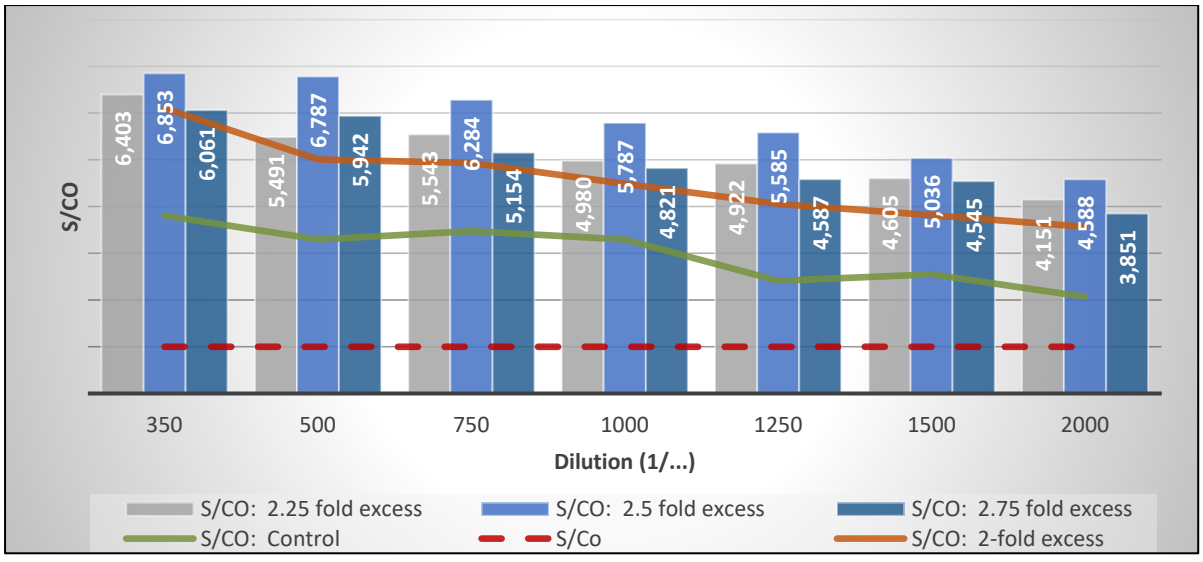


Figure 4.3: S/CO results for a dilution series of the synthesised detection molecule, when the S-SMCC concentration was incrementally increased.

Note 1: Testing was conducted using the Murex HCV Ag/Ab Combination assay, with a commercially available microplate and conjugate used as a control.

Note 2: S/CO = Sample to cut-off; mM = Millimolar

The S/CO values for all test scenarios were higher than the control but as the peptide concentrations of the experiment detection molecules were higher than the control, this difference is expected. The 2.5-fold excess S-SMCC test scenario showed the highest S/CO value of all the test scenarios at each dilution tested, followed by the 2.25-fold excess, the 2.75-fold excess, and the 2-fold excess scenario.

One-way ANOVA (Table C1.13 in Appendix C1), and a Tukey Pairwise Comparison (Table C1.14 and Figure C1.2 in Appendix C1) was conducted on the 1/2000 dilution data sub-set for all test scenarios (excluding the control), on Minitab (R) 19 software. This was conducted in order to determine if the differences indicated in Figure 4.3 above are statistically significant. In addition, the data were presented as an individual value plot and a box plot.

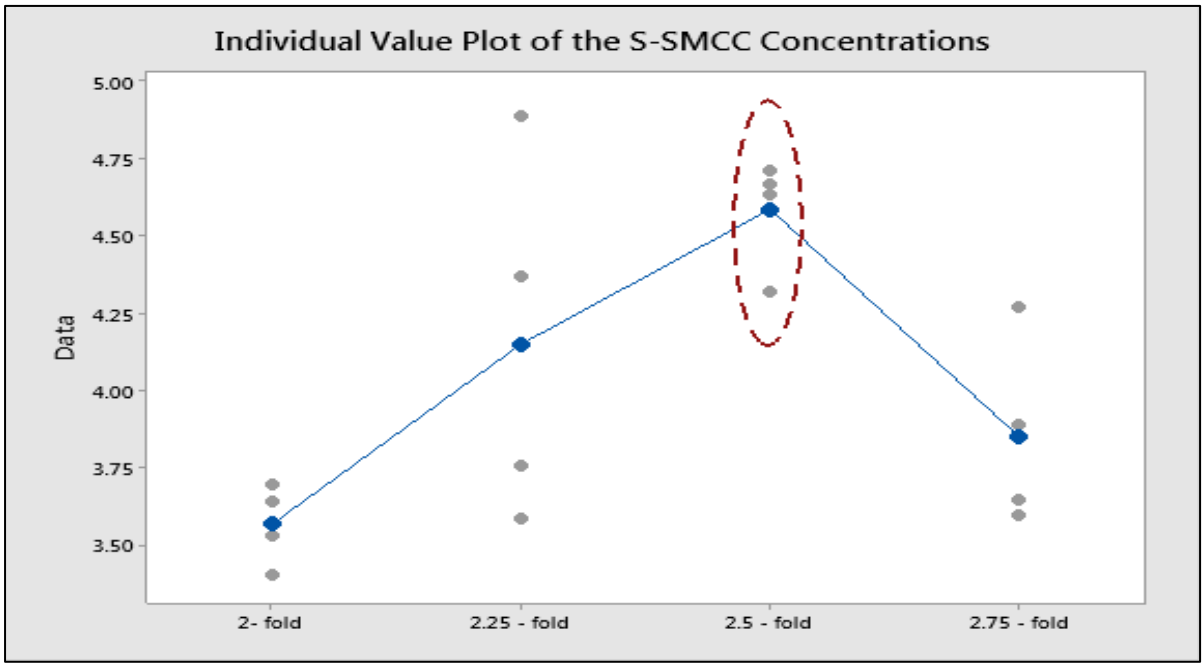


Figure 4.4: Individual value plot of S/CO values of the S-SMCC concentrations.

Note 1: The individual value plot shows the S/CO value for each replicate at the 1/2000 dilution.

Note 2: Refer to Appendix C, Table C1.13 and C1.14 for the one-way ANOVA and Tukey pairwise analyses, respectively.

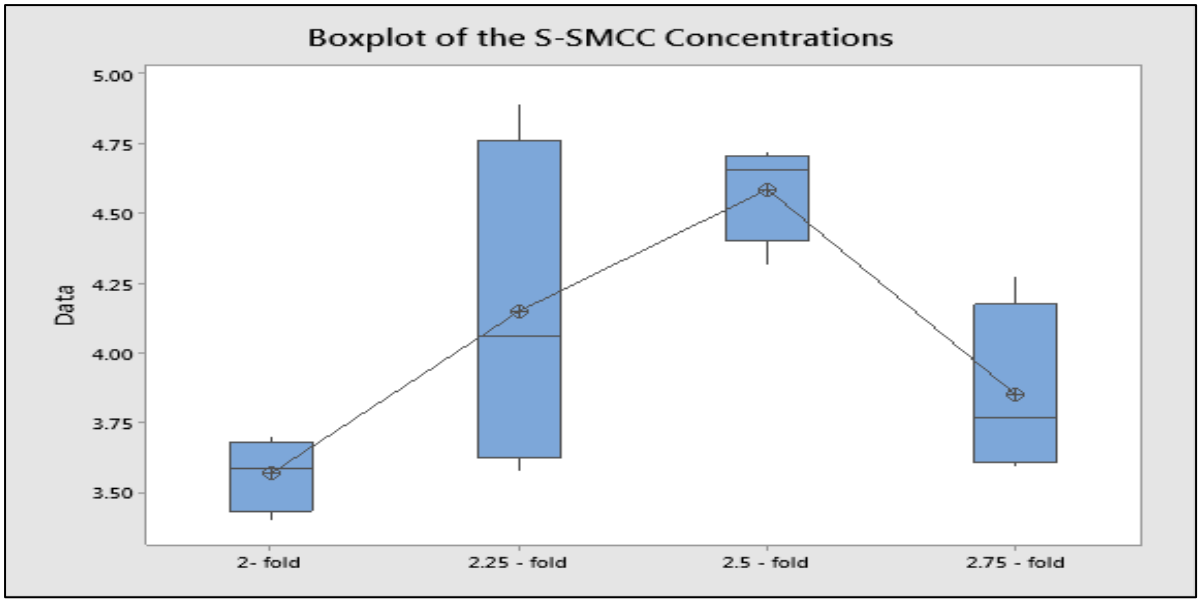


Figure 4.5: Boxplot of S/CO values of the S-SMCC concentrations.

Note: The box plot shows the S/CO value for each replicate at a 1/2000 dilution.

The p-value is 0.009 of the one-way ANOVA is less than the α-level of 0.05, indicating that the means of the different S-SMCC concentrations are significantly different. Evaluation of the means of each of the scenarios as well as the individual value plot (Figure 4.4) indicated that the 2.5-fold excess S-SMCC had the highest mean S/CO value (4.587), and that the 2-fold excess S-SMCC scenario showed the lowest mean S/CO value (3.5679). This was confirmed by the pairwise comparison of the mean S/CO values of the test scenarios, where the 2.5-fold excess S-SMCC scenario and the 2-fold excess scenario were the only two scenarios that do not share a group (Figure C1.2 in Appendix C). The Tukey pairwise comparisons compare every mean to every other mean in the data set. When two means do not share a group designated by a letter, then those two means are significantly different from one another. Therefore, the detection molecule prepared using the 2.5-fold excess S-SMCC scenario and the 2-fold excess scenario were statistically significantly different from each other.

As indicated in the individual value plot, the highest individual S/CO value was obtained for the 2.25-fold excess scenario, with a mean of 4.151. This mean is lower than that of the 2.5-fold excess scenario. The 2.25-fold excess S-SMCC scenario data set indicates a standard deviation of 0.598, the highest of all scenarios analysed, reflecting a high degree of variability. This is confirmed by the box plot representation of the data (Figure 4.5).

Providing a further excess of sulfo-SMCC than is used in the standard method of synthesis to a 1250 µM solution of horseradish peroxidase, then followed by linking the modified enzyme to 0.5 mM peptide solution, demonstrated an increase in the sensitivity of the core region 3

detection molecule. The mean S/CO value for 2.5-fold excess scenario was highest at the 1/2000 dilution, indicating that this scenario had the largest effect on the efficacy of the core region 3 detection molecule.

The concentration of horseradish peroxidase was increased from 1000 µM to 1250 µM to accommodate the increase in the cross-linker used in the preparation of the enzyme conjugate, i.e., a 250 µM increase in the concentration of horse radish peroxidase for a 2.5-fold increase in sulfo-SMCC. The increase in enzyme concentration allowed for an increased number of sites available on the enzyme for linkage. Pierce Biotechnologies (2016) recommends a 10 to 50 molar excess of sulfo-SMCC be added to the protein containing the amine, in this case, horseradish peroxidase. As such, a 2.5-fold excess is still less than the recommended amount for saturation of the available amine groups. However, it is more than the amount offered by the standard method. The results show that a 2.5-fold molar excess of sulfo-SMCC to horseradish peroxidase is the optimal proportion to ensure saturation of the amine reactive groups on the enzyme. Thus, increasing the number of maleimide groups available for linkage to the peptide representing core region 3 was shown to increase the efficacy of the detection molecule.

4.1.3 pH of the conjugation and storage buffer

This section of the study tested the effects of the conjugation buffer on the reactivity of the detection molecule. The pH is significant in cross-linkage processes, as it has the ability to expose or protect the functional groups available for conjugation. Thus, by varying the pH of the conjugation buffer, a functional group may be specifically selected.

The study prepared 25 mM HEPES/1 mM EDTA, as per the standard method of synthesis but varied the pH as follows: 6.0, 6.2, 6.4, 6.6, 6.8 (control), 7.0 and 7.5. Enzyme-peptide conjugates were then prepared as per the standard method, each using a particular buffer to achieve conjugation.

The prepared detection molecules were tested as per the standard method for qualification at DiaSorin South Africa.

The OD values of the data generated on each test were converted to S/CO and plotted on a multi-Variate chart in Minitab (R) 19 software, to indicate and compare the performance of the detection molecules.

The chart in Figure 4.6 depicts the S/CO results for the performance of the internal quality control that detects core region 3, when the pH of the conjugation buffer was varied for the preparation of the detection molecule.

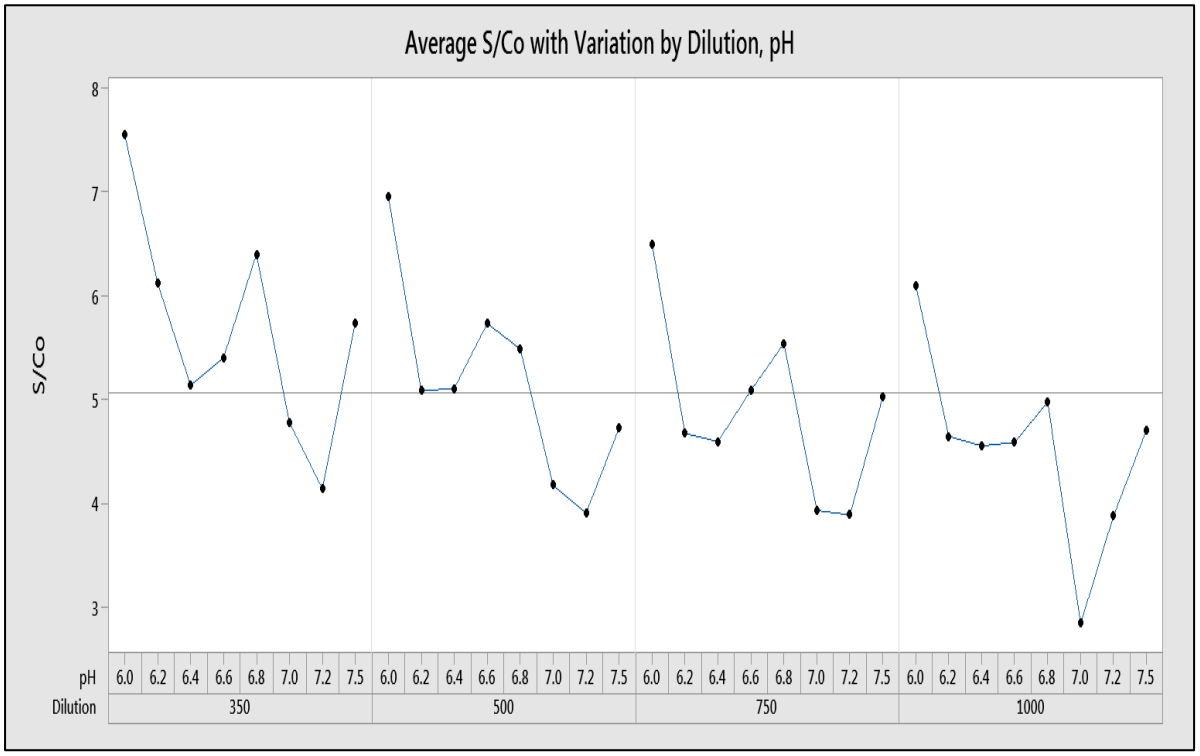


Figure 4.6: Multivariate chart of activity of the synthesised detection molecule according to pH

Note: Dilutions of 1/350, 1/500, 1/750, and 1/1000 were used to test the synthesised detection molecule.

The S/CO values for pH 6.0, and 6.8 were consistently higher than the S/CO values for the other tested pH values. pH 6.8 served as the control, as this is the pH of conjugation buffer used during the standard method of synthesis. The mean S/CO for conjugate prepared using the pH 6.0 buffer was 6.103, as compared to 4.980 of the conjugate prepared using the pH 6.8 buffer. This is a 23% increase in the efficacy of the core region 3 detection molecule.

The two-sample t-test was performed on the individual values of the 1/1000 dilution to determine if the difference between the S/CO values are statistically significant. The analysis can be found in Table C1.18 in Appendix C1. The p-value is 0.002, which is less than the α-level value of 0.05, indicating that there was a significant difference in the S/CO values for pH 6.0 and pH 6.8 conjugation buffer used in the manufacture of the detection molecule, at a 1/1000 dilution. The use of pH 6.0 conjugation buffer when preparing the detection molecule improved the S/CO values obtained when tested on the Murex HCV Ab/Ag Combination Assay.

The results of changing the pH of the conjugation buffer demonstrated that pH 6.0 rather than pH 6.8 had the largest effect on the efficacy of the core region 3-HRP. Hermanson (2013) described that alkylation of the maleimide double bond by the sulfhydryl group forms a stable and non-reversal thioether bond. This reaction is exclusively selected for pH range of 6.5 to 7.5, as the process is facilitated by nucleophilic attack by the thiolate anion on a carbon atom adjacent to the maleimide group. Above pH 7.5, the amine and sulfhydryls compete for the maleimide groups, resulting in mixed and unwanted reactions. There is no available literature on the low pH and its effect on the stability of the thioether bond. Therefore, this result is unexpected and must be investigated further to determine the root cause of the increase in efficacy of the detection molecule when pH 6.0 buffer was used for cross-linking and storage.

4.1.4 Removal of excess/unbound reagents from the detection molecule

This section of the study focused on comparing the results of two methods – dialysis and exclusion chromatography – that were used to remove excess unbound reagents contained within the detection molecule. Adequate removal of these unbound reagents is important, as it has the potential to impact the assay's overall performance. The presence of the unbound reagents could result in unwanted side reactions, or reactions that are not able to be visualised or quantified, thus diminishing the sensitivity of the assay.

Important factors to consider include molecular size of the detection molecule and the molecules that require removal, purification format, temperature, and the time taken to remove the unwanted molecules. This study tested these factors, however only performance data for the purification format is presented below:

Dialysis Membrane:

The detection molecule was prepared as per the standard method of preparation at DiaSorin South Africa. However, the removal of excess reagents was achieved by varying the dialysis membranes, i.e., size 1 tubing is used in the standard method as the control, and the use of size 2 tubing and a dialysis cassette were the experiment methods used to remove unlinked molecules from the detection molecule.

The OD values of the data generated on each test was converted to S/CO and plotted on a bar graph in Microsoft Excel, to indicate and compare the performance of the detection molecule purified using these techniques. Figure 4.7 below indicates these results.

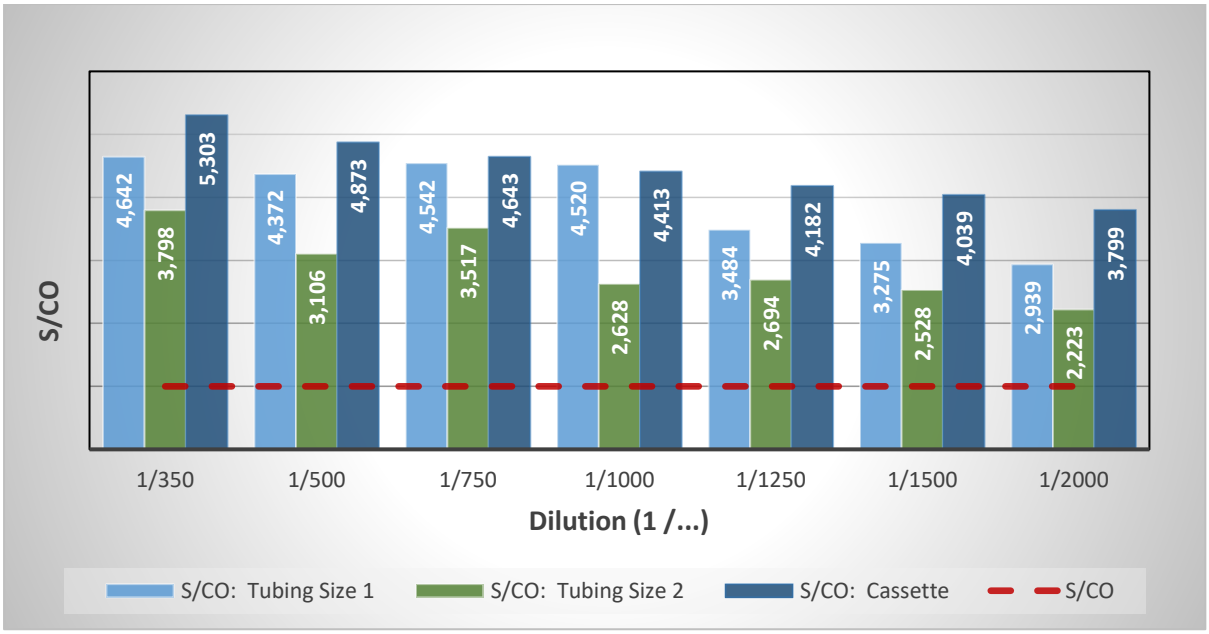


Figure 4.7: S/CO results for a dilution series of the synthesised detection molecule, when the dialysis membrane format was varied.

Note: The dilution range of 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/500, and 1/2000 were used for the testing of the synthesised detection molecule. Testing was conducted using the Murex HCV Ag/Ab Combination assay.

The S/CO values of the size 2 tubing are consistently lower than the other scenarios, across all dilutions tested. One-way ANOVA analysis was conducted on the 1/2000 data set, to determine if there are statistical difference between the scenarios tested. This analysis is presented on Table C1.22 in Appendix C1.

The individual value plot for the performance data for all scenarios in shown in Figure 4.8 below.

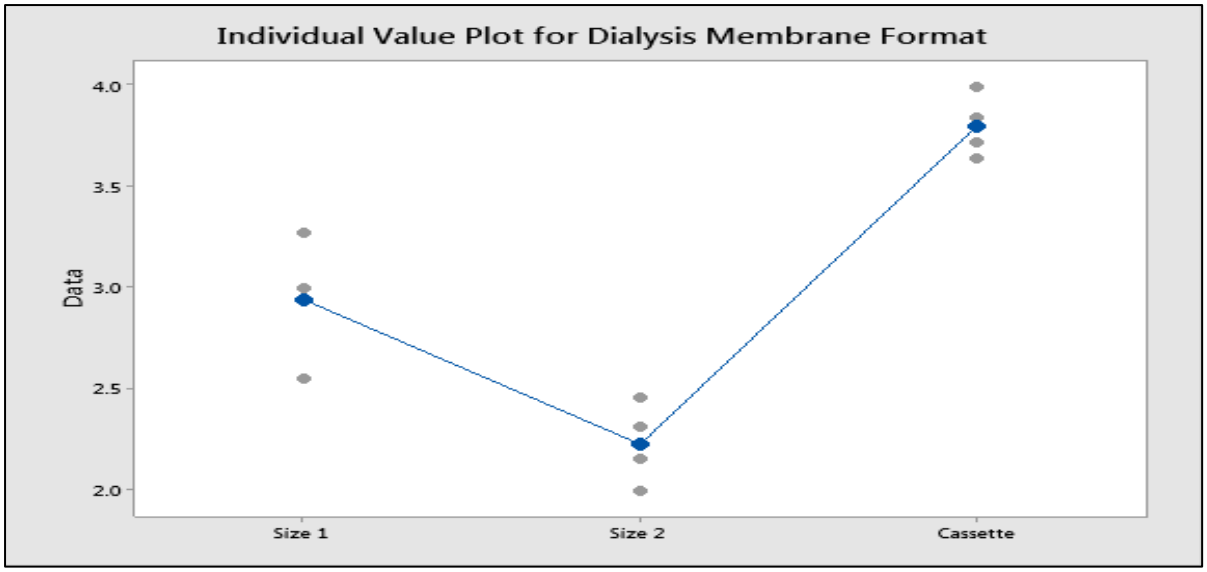


Figure 4.8: Individual value plot of S/CO values of the synthesised detection molecule when the dialysis format was varied.

Note: The individual value plot shows the S/CO value for each replicate at the 1/2000 dilution.

The p value is 0.000, which is less than the α-level of 0.05, indicating that the S/CO values for each of the scenarios are statistically different from each other. The individual value plot indicates that the cassette scenario has the largest mean S/CO value, whereas the size 2 dialysis tubing has the lowest mean S/CO. Therefore, these analyses, indicates that dialysis through the cassette membrane indicates the largest improvement to the S/CO.

Gel Filtration:

PD-10 columns containing Sephadex G25 resin was used to remove excess and unwanted reagents from the detection complex, in this experiment. The resultant detection molecule was tested as per the standard method for qualification, at DiaSorin South Africa. This scenario was tested compared to the detection molecule purified using a dialysis cassette.

The OD values of the data generated on each test was converted to S/CO and plotted on a bar graph in Microsoft excel, to indicate and compare the performance of the detection molecule purified using these techniques. A summary of the performance of both scenarios is presented in Figure 4.9 below.

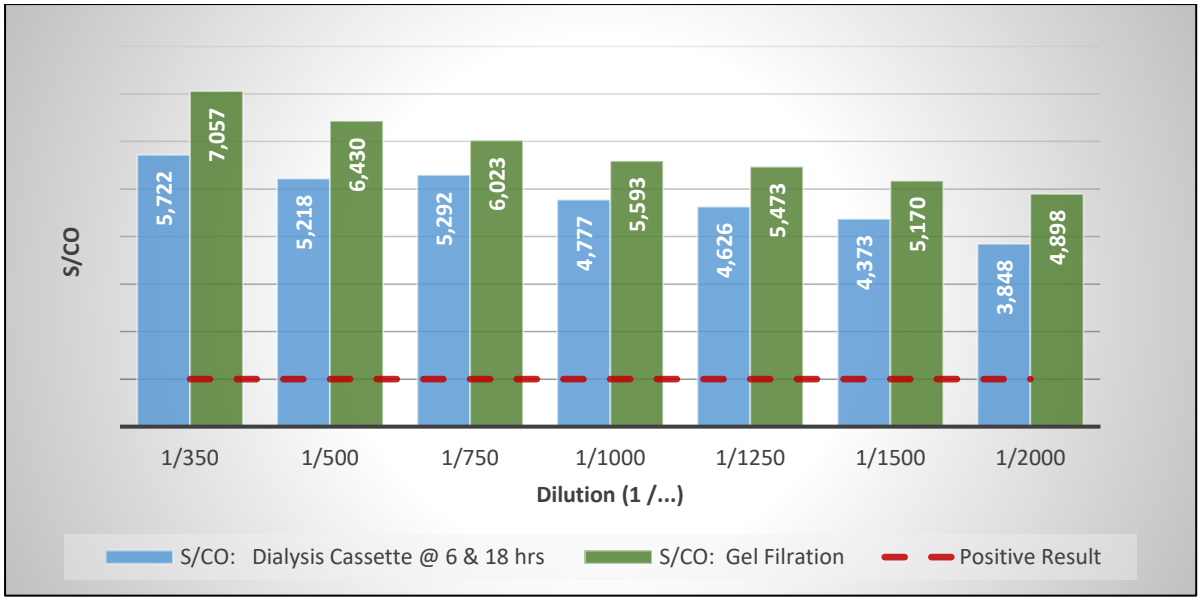


Figure 4.9: S/CO results for a dilution series of the synthesised detection molecule prepared using gel filtration and a dialysis cassette.

Note: The dilution range of 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/500, and 1/2000 was used for the testing of the synthesised detection molecule. Testing was conducted using the Murex HCV Ag/Ab Combination assay.

Comparison of the S/CO values of both test scenarios indicate that the gel filtration consistently has a larger S/CO value than the dialysis cassette. At the lowest dilution tested, 1/2000, the mean of the S/CO value for the gel filtration scenario is 27% higher than that of the dialysis cassette. A two-sample t-test was conducted on Minitab (R) 19 software, on the individual data points, to determine if the difference is statistically significant.

This analysis is presented on Table C1.23 in Appendix C1. A box plot of this data is shown in Figure 4.10 below:

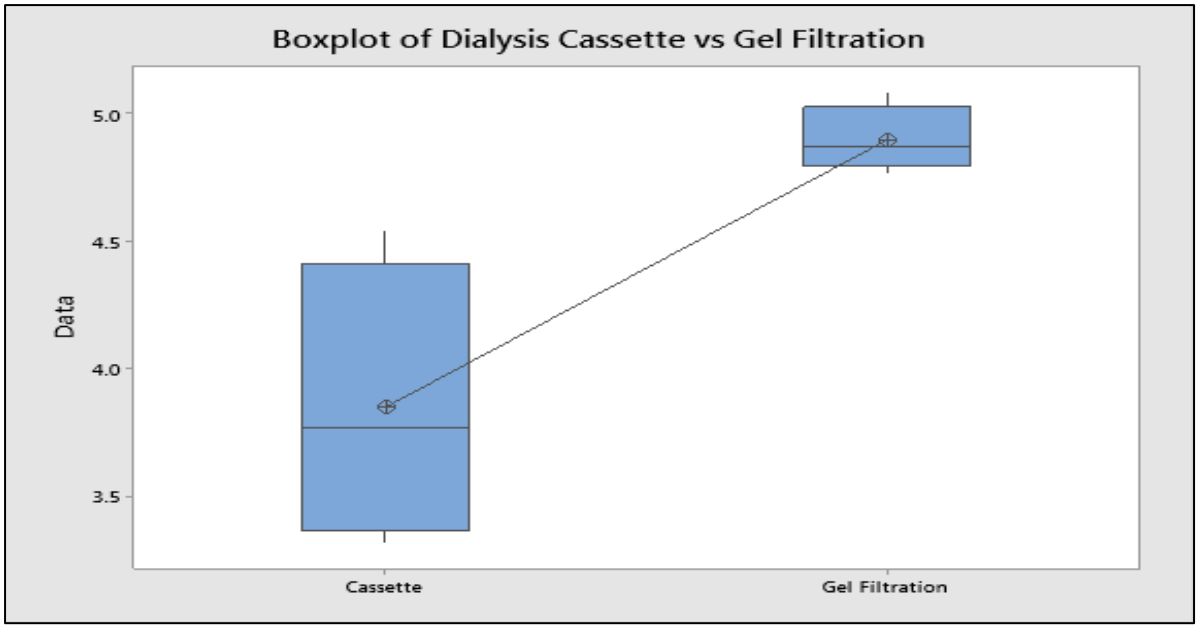


Figure 4.10: Boxplot of S/CO values of the synthesised detection molecule when prepared using gel filtration and a dialysis cassette.

Note: The Boxplot shows the S/CO value for each replicate at the 1/2000 dilution.

The p value is 0.035 which, although higher, is still lower than the α-level of 0.05 applied to test. This indicates there is a statistical difference between the S/CO values obtained for scenarios. However, it is interesting to note that the standard deviation (0.554) of the dialysis cassette scenario is considerably higher than that of the gel filtration scenario (0.129), indicating that there is large degree of variability within the data set. This is confirmed by the box plot chart (Figure 4.10). Therefore, gel filtration has the most beneficial and consistent effect on the S/CO values.

These results indicate that the use of gel filtration produces conjugates that generate a higher S/CO value, as well as more consistent OD values of the replicates tested.

The results indicate that the use of G25 Sephadex resin improved the S/CO values in comparison to the dialysis cassette. At the highest dilution tested, 1/2000, the mean of the S/CO value for the gel filtration scenario is 27% higher than that of the dialysis cassette.

Whilst dialysis and gel filtration are used to remove substances based on size, dialysis is predicated on the retention of target molecules within the dialysis membrane (Nath 2008), whilst allowing the molecules of small molecular weights to diffuse through the dialysis membrane - this separates large from small molecules such as contaminants (Berg et al., 2002).

On the other hand, Sephadex G25 is a gel filtration resin that separates unbound or excess products based on size of the molecular size (Berg et al., 2002) and molecular shape (Walsh 2002). Thus, it retains substances of small molecular weight in the resin, whilst allowing the larger (target) molecules such as peptides to quickly exit the resin.

In addition to both dialysis and gel filtration allowing separation of molecules according to their molecular weight, albeit differently, dialysis is also affected by temperature, membrane thickness and surface area, and sample concentration (Pierce Biotechnology 2016). However, gel filtration does not have these latter constraints.

Sephadex G 25 Medium has a dry particle diameter of 50 to 150 µm and a hydrated particle diameter of between 84 µm and 262 µm. The fraction range (Mr) is between 1000 – 5000 for globular proteins. This fractionation range facilitates group separation of molecules larger than 5000 Daltons from molecules smaller than 1000 Daltons. The core region 3 – HRP has an approximate molecular weight of 46222, with the peptide representing core region 3 having a molecular weight of approximately 1680 dissolved in DMSO which has a molecular weight of 78,14 Daltons. Therefore, group separation of the core region 3 – HRP from the DMSO would have occurred, because the low molecular weight of DMSO would allow it to enter the pores of the beads of Sephadex G25 resin and remains in the column longer than the core region 3 – HRP. The unbound core region 3 peptide is also able to enter the pores of the beads but will follow a shorter path than the DMSO molecules as it is larger. As a result of its large size, the core region 3 - HRP will be first molecule to exit the column and can be collected immediately. Thus, because of the large difference in molecular weights, the use of gel filtration can more effectively and efficiently separate the conjugate from excess and unwanted reagents.

4.1.5 Assessment of the detection molecule: Improved method vs standard method

The detection molecule was prepared by including all the improved variables into the preparation methods, i.e., the increased peptide concentration, increased concentration of the cross-linker, the conjugation buffer at pH 6.0, and purified using gel filtration. The resultant conjugate was diluted in the Murex Ab/Ag conjugate diluent, i.e., 1/1350, 1/1500, 1/1750, and 1/2000, as is the standard method for qualification of the detection molecule at DiaSorin South Africa. A control batch was prepared as per the standard method of synthesis and qualified alongside the test batch.

The OD values of the data generated were converted to S/CO and plotted on a bar graph in Microsoft excel, to indicate and compare the performance of the detection molecule for both scenarios. A summary of the performance of both scenarios is presented in Figure 4.11 below.

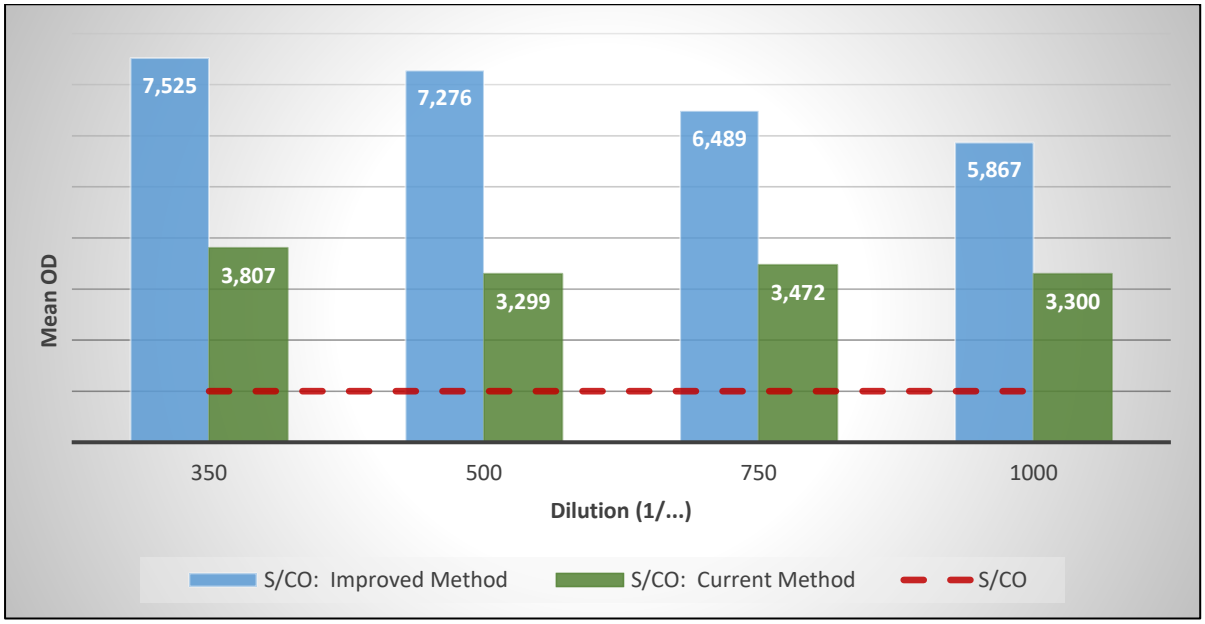


Figure 4.11: S/CO results for a dilution series of the synthesised detection molecule prepared using the improved method and the standard method.

Note: The dilution range of 1/350, 1/500, 1/750, and 1/1000 were used for the testing of the synthesised detection molecule. Testing was conducted using the Murex HCV Ag/Ab Combination assay.

The S/CO values of the improved method are considerably higher than those obtained for the standard method, across all the dilutions tested. At the lowest dilution, 1/1000, the improved method shows an improvement of 78% over the standard method.

4.2 Improvement to the capture molecule

This section of the study assessed the effect of separately cross-linking each of the three core detection peptides to a different BSA molecule, thus creating individual core peptide conjugates for coating on the microtitre wells. The core region – BSA was then titrated by coating microtitre wells with a range of concentrations (0.02 µg/ml, 0.04 µg/ml, 0.06 µg/ml or 0.08 µg/ml.) to assess and determine the optimal concentration.

4.2.1 Conjugation of the individual core capture peptides to bovine serum albumin

The individual enzyme-peptide conjugates prepared for each region of the core was coated at 0.04 µg/ml onto the microtitre wells, and subsequently tested as per the standard method for qualification of the polycore capture complex. A polycore capture complex was coated onto microtitre wells at the same concentration (0.04 µg/ml) to serve as a control.

The OD values of the data generated were converted to S/CO and plotted on a bar graph in Microsoft excel, to visualise the core performance. A summary of the performance of both scenarios is presented in Figure 4.12 below.

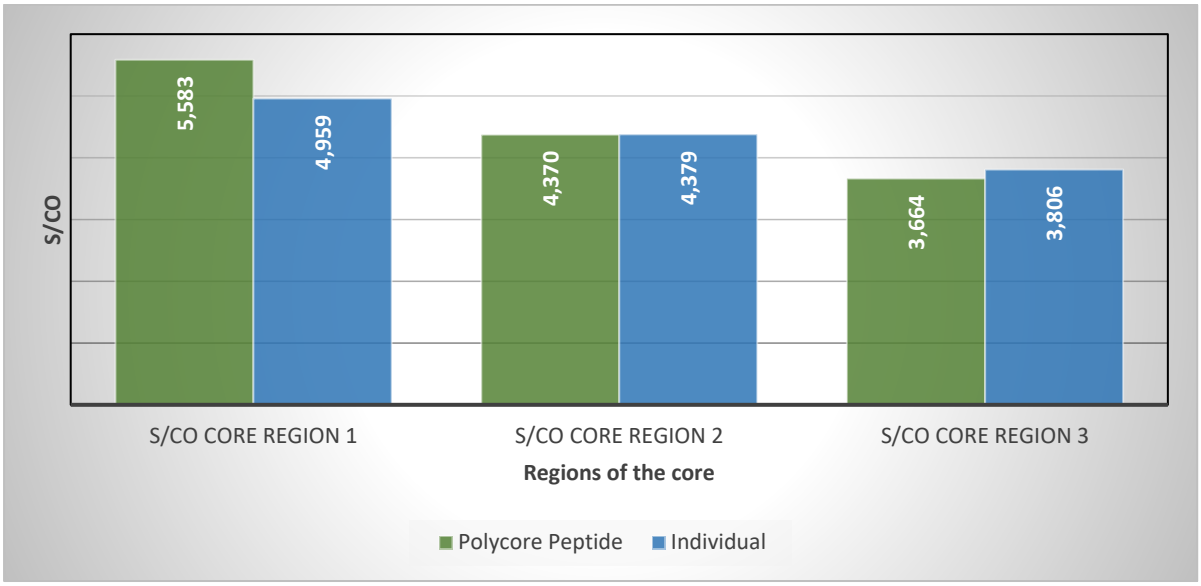


Figure 4.12: S/CO results for the polycore capture molecule and the individual core capture molecules.

Note: The polycore capture molecule and the individual core capture molecules were coated at 0.4 µg/ml*. Testing was conducted using the Murex HCV Ag/Ab Combination assay.*

The S/CO obtained from the three individually prepared core detection peptide conjugates when compared to the standard polycore preparation show very little difference in their values. The S/CO returned a marginally higher (4%) result for core region 3 – BSA compared to its polycore equivalent. Conversely for core region 1; the polycore molecule performed 13% better than the core region 1 – BSA. The performance of core region 2 were comparatively the same, with a negligible difference (0,009) in the S/CO values obtained for each scenario.

4.2.2 Titration of the core region 3 – BSA

The enzyme-peptide conjugate prepared using only the core region 3 peptide linked to BSA was coated at a range of concentrations onto the microtitre wells, and subsequently tested as per the standard method for qualification of the polycore capture complex.

The OD values of the data generated were converted to S/CO and plotted on a line graph in Microsoft Excel to visualise performance at each concentration. A summary of the performance of both scenarios is presented in Figure 4.13 below.

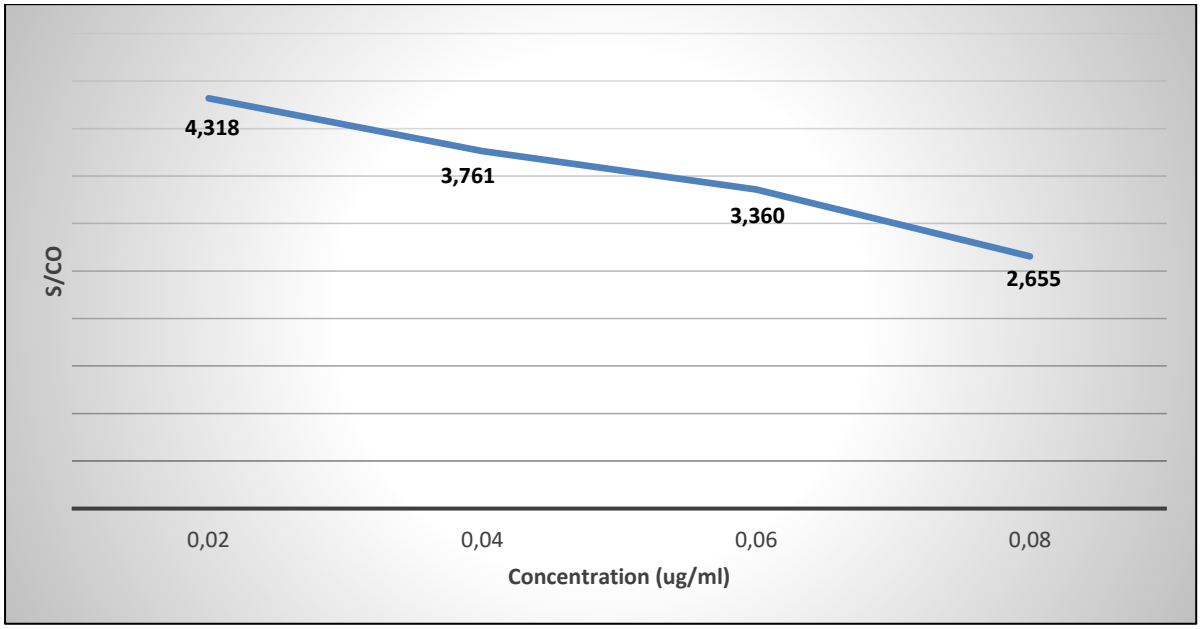


Figure 4.13: S/CO values of a concentration series for core region 3 – BSA coated on microtitre plates.

Note: Core regions 1 - BSA and Core regions 2 - BSA were coated at 0.04 µg/ml. Core region 3 – BSA was coated at 0.02 µg/ml, 0.04 µg/ml, 0.06 µg/ml, and 0.08 µg/ml. Testing was conducted using the Murex HCV Ag/Ab Combination assay.

The highest S/CO ratio obtained for core-region 3-BSA was obtained when it was coated at 0.02 µg/ml. As the concentration was increased, so a steady decrease in the S/CO values was noted. The standard concentration for coating of the polycore capture molecule is 0.04 µg/ml. However, there is a 15% increase in the S/CO value when the core region 3 – BSA is coated at 0.02 µg/ml, compared to coating at a concentration of 0.04 µg/ml. Therefore, 0.02 µg/ml is the optimal concentration for improvement of sensitivity to core region-3 core antibodies.

The preparation of the core region 3 – BSA, and subsequent coating of this molecule at a concentration of 0.02 µg/m has been shown to improve the sensitivity of the Murex HCV Ab/Ag Combination Assay to antibodies generated to core region 3 of the hepatitis C virus. This translated to a 15% increase in sensitivity as compared to the standard concentration for coating along with a 50% reduction in the use of polycore capture molecule.

The BSA molecule is large, with a molecular weight of 67 000 (Gibbs, 2001), and has a number of amine groups available for linkage per molecule (Hermanson, 2013). The standard method for preparation of the polycore complex requires the sequential addition of the three different peptides representing the amino acid sequences of three areas of the HCV core, to sulfo-SMCC-activated BSA. These three peptides may bind at different efficiencies, thus resulting in a polycore that better represents one or more regions of the core. The preparation of the individual core region-BSA conjugates mitigates this possibility, as all maleimide groups on the BSA molecule are available only to a single region of the core.

The results of the testing of the different concentrations of the core region-BSA conjugates allows for the improvement in the detection of each region. The efficacy of core region 3 was most improved when coated at 0.02 µg/ml, which is half the concentration of core region 3 used in the standard practice. This result may be due to the effects of steric hindrance. Crowther (2001) described the effects of steric hindrance on the signal generated by the ELISA assay, indicating that high concentrations of the capture molecule during coating can suppress the signal of the assay. The higher concentrations of the polycore capture complex may cause crowding on the microtitre well surface, thus causing molecular orientation changes. These changes may block the specific reactive groups on the core region 3 capture molecule coated on the microtitre well surface, thereby causing them to be inaccessible to the target molecule contained within the patient sample and so resulting in a decrease in the signal of the Murex Ab/Ag Combination ELISA assay for the detection of core region 3 of the hepatitis C virus.

4.3 Assessment of the final freeze-dried conjugate of the detection molecule on the improved coated microtitre plates

This section of the study assessed the effect of the improved methods of preparation for both the capture and detection complexes as it is used to prepare the coated microtitre well and the freeze-dried kit conjugate.

The improved reagents were tested as per the standard procedure at DiaSorin South Africa, alongside a commercially available product. Sensitivity, specificity, and stability of the improved reagents were compared to the commercially available product. These results are presented below:

4.3.1 Sensitivity - Efficacy

The efficacy of an ELISA assay is the ability of the test to produce an accurate result.

Efficacy testing of the reagents prepared was performed using a serological panel with the specifically ability to detect core region 3. The OD values were converted to S/CO values, and plotted on a bar graph, for the reagents prepared using the improved and standard methods. This was done in order to compare the performance of the reagents prepared using both methods. These data are represented in Figure 4.14 below.

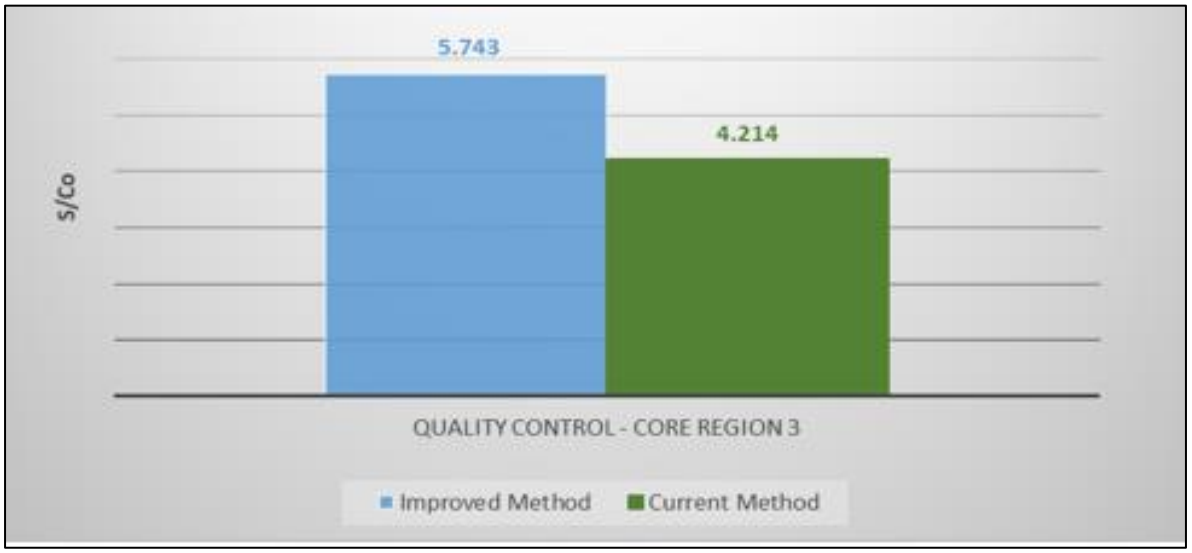


Figure 4.14: S/CO values depicting the microtitre plate and conjugate prepared using the improved method in comparison to the commercially available product prepared using the standard methods of synthesis.

Note: Testing was conducted using the Murex HCV Ag/Ab Combination assay.

The S/CO value for the improved conjugate (1/2000) was 36% higher than the S/CO value obtained for the conjugate manufactured (1/350) using the standard method.

A two-sample t-test was conducted on Minitab (R) 19 software on the individual data points, to determine if the difference was statistically significant.

This analysis is presented in Table 4.2 below:

Table 4.2: Two-sample T-test of the S/CO of microtitre plate and conjugate prepared using the improved method and compared to the commercially available product.

Two-Sample T-Test and CI: 1/350, 1/2000

Method

μ_1: mean of 1/350
μ_2: mean of 1/2000
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
1/350	6	4.2142	0.0789	0.032
1/2000	6	5.7430	0.0910	0.037

Estimation for Difference

Difference	95% CI for Difference
-1.5287	(-1.6399, -1.4175)

Test

Null hypothesis $H_0: \mu_1 - \mu_2 = 0$
Alternative hypothesis $H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-31.10	9	0.000

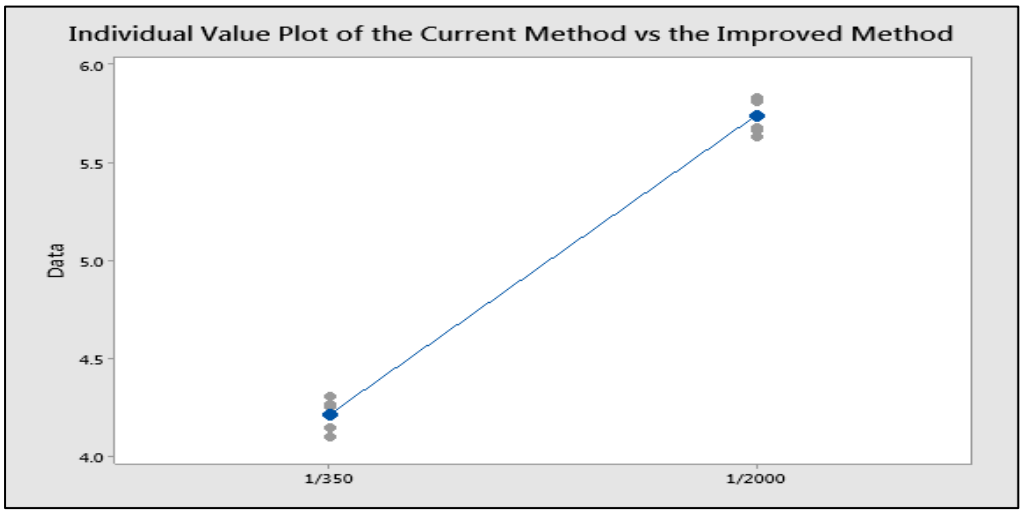


Figure 4.15: Individual value plot of the S/CO of microtitre plate and conjugate prepared using the improved method and compared to the commercially available product.

Note: The Individual value plot shows the S/CO value for each replicate of the capture and detection molecules prepared using both methods. The freeze-dried conjugate using the improved method was spiked at a 1/2000 dilution, whereas the control was spiked at 1/350.

The OD values for the replicates for both scenarios show a high level of consistency, as demonstrated by the individual value plot, where all replicates are clustered tightly around the mean value. In addition, the two-sample T-test has a p-value of 0 indicating that there is a statistically significant difference between the OD values of the two scenarios. This indicates that the molecules prepared using the improved method show a significant increase in sensitivity, without compromising the repeatability of the individual replicates.

4.3.2. Specificity: 90 Normal Human Serum Samples

The specificity of an ELISA assay is its ability to separate and identify the target analyte from all other analytes present in a sample or that which is applied to the assay. It is a crucial requirement for an assay to be specific for the target analyte as this contributes to the accuracy of the test. Specificity testing of the prepared reagents was performed using 90 normal human serum (NHS) samples that were routinely procured from the South Africa National Blood Services. These samples were used to assess the specificity of ELISA reagents and products at DiaSorin South Africa and were not procured exclusively for this study.

The OD values for each of the 90 NHS samples tested, for each scenario were compared in to determine if there were differences. These data are presented below for the Improved method (Table 4.3) and the standard method (Table 4.4).

Table 4.3: OD 448 nm/690 nm data for the specificity testing of the microtitre plate and conjugate prepared using the improved method.

Note: Testing was conducted using the Murex HCV Ag/Ab Combination assay.

Raw Data	1	2	3	4	5	6	7	8	9	10	11	12
A	0.166	0.149	0.221	0.236	0.192	0.132	0.150	0.148	0.227	0.152	0.172	0.160
B	0.170	0.150	0.145	0.165	0.144	0.141	0.149	0.134	0.127	0.136	0.158	0.159
C	2.061	0.146	0.129	0.140	0.135	0.136	0.121	0.125	0.132	0.123	0.148	0.205
D	2.046	0.170	0.159	0.132	0.132	0.132	0.131	0.226	0.171	0.126	0.157	0.128
E	2.015	0.153	0.143	0.189	0.143	0.147	0.160	0.125	0.125	0.128	0.150	0.162
F	2.087	0.160	0.141	0.150	0.154	0.131	0.121	0.131	0.121	0.129	0.147	0.142
G	0.169	0.145	0.141	0.128	0.128	0.144	0.146	0.257	0.125	0.144	0.221	0.214
H	0.179	0.160	0.144	0.142	0.147	0.150	0.158	0.143	0.152	0.155	0.319	0.301
	Improved Method											

Table 4.4: OD 448 nm/690 nm data for the specificity testing of the commercially available micro-titre plate and conjugate prepared using the standard method.

Note: Testing was conducted using the Murex HCV Ag/Ab Combination assay.

Raw Data	1	2	3	4	5	6	7	8	9	10	11	12
A	0.189	0.230	0.272	0.222	0.207	0.166	0.170	0.192	0.249	0.175	0.176	0.168
B	0.187	0.502	0.316	0.226	0.198	0.169	0.173	0.171	0.185	0.175	0.159	0.171
C	2.022	0.170	0.259	0.217	0.209	0.162	0.165	0.166	0.160	0.149	0.162	0.218
D	2.011	0.202	0.170	0.151	0.157	0.158	0.168	0.164	0.199	0.152	0.172	0.153
E	2.034	0.205	0.156	0.192	0.178	0.175	0.165	0.170	0.158	0.162	0.154	0.163
F	2.019	0.175	0.171	0.167	0.178	0.148	0.143	0.169	0.148	0.149	0.154	0.156
G	0.183	0.164	0.184	0.151	0.141	0.142	0.167	0.192	0.148	0.155	0.177	0.169
H	0.200	0.166	0.168	0.164	0.163	0.147	0.179	0.165	0.138	0.165	0.145	0.195
	Current Method											

Descriptive statistics in Microsoft Excel were applied to the OD values of the data sets for both scenarios and a histogram was generated. This was undertaken to analyse the performance of the 90 NHS samples, thereafter, to compare the specificity of both scenarios. These results are presented in Table 4.5 below:

Table 4.5: Descriptive statistics comparing the data sets of the microtitre plate and conjugate prepared using the improved and standard method of synthesis.

Descriptive Statistics				
Improved Method			**Current Method**	
Column1			*Column1*	
Mean	0.155664778		Mean	0.179493333
Standard Error	0.003830365		Standard Error	0.004783531
Median	0.1463		Median	0.16845
Mode	0.1485		Mode	0.1683
Standard Deviation	0.036338031		Standard Deviation	0.045380555
Sample Variance	0.001320453		Sample Variance	0.002059395
Kurtosis	6.823570763		Kurtosis	29.04234689
Skewness	2.416977146		Skewness	4.610003661
Range	0.1983		Range	0.3636
Minimum	0.121		Minimum	0.1384
Maximum	0.3193		Maximum	0.502
Sum	14.00983		Sum	16.1544
Count	90		Count	90
Confidence Level(95.0%)	0.007610853		Confidence Level(95.0%)	0.009504773

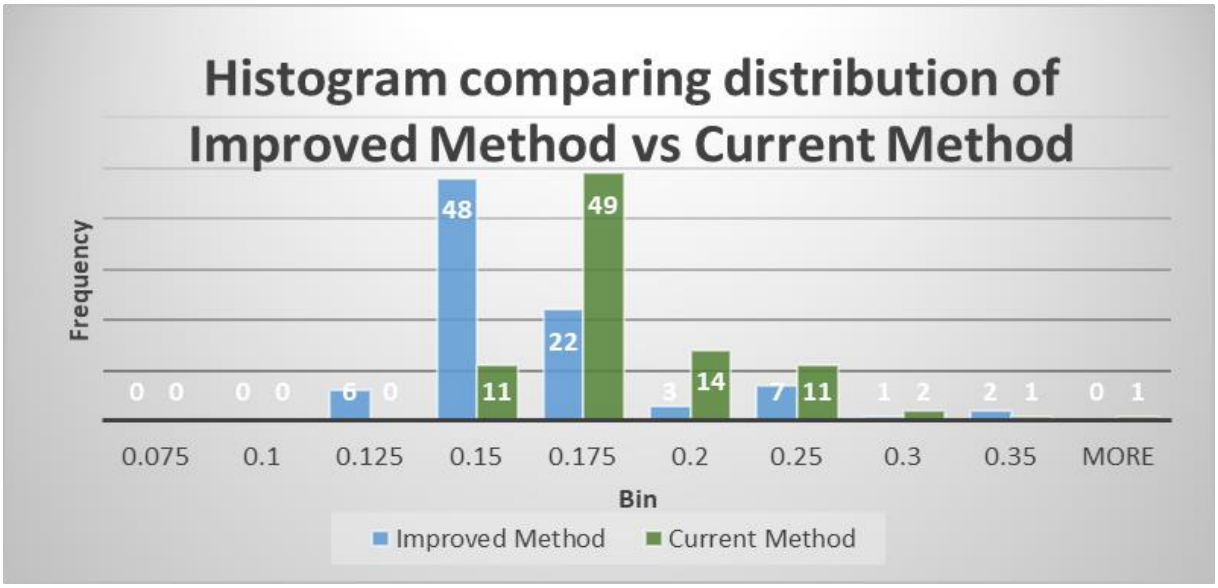


Figure 4.16: Histogram comparing the data sets of the microtitre plate and conjugate prepared using the improved and standard method of synthesis.

As indicated in Figure 4.16, the histogram of both the improved and standards methods are bell shaped, with a positive skewness. This means that the tails on the right are longer than those on the left, with the improved method displaying a “shorter tail” (2.417) than the standard method (4.610).

The mean of the data sets are higher than their respective median values indicating that the mean values are affected by outliers in the data set, i.e., the improved method has a mean and median value of value of 0.156, and 0.146, respectively. This compares with the standard method that showed a mean and median value of 0.179, and 0.169, respectively. The most frequent value (mode) of both data sets is also lower than the mean values, further supporting the presence of the outliers that affected the mean OD values of the normal human serum sampled.

The improved method has 84% of the individual OD values below 0.175, and 16% of the values above the 0.175. The standard method has 67% of its values below 0.175, and 33% of its values above 0.175. The standard deviation of the improved method (0.0363) is lower than that of standard method (0.454), indicating more variability in the data for the reagents prepared using the standard method. This implies that the reagents prepared using the improved method are more accurate, therefore more reliable in ensuring the assay returns the correct result.

A two-sample t-test was conducted on the data sets to determine if the difference between the two mean values is statistically significant. An individual value plot of the two data sets was also conducted, to graphically represent the OD values in relation to each other. This is presented below:

Table 4.6: Two-sample T-test the comparing the OD values of the 90 normal human serum samples for both the standard and improved methods.

Two-Sample T-Test and CI: Current Method, Improved Method

Method

μ_1: mean of Current Method
μ_2: mean of Improved Method
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
Current Method	90	0.1795	0.0454	0.0048
Improved Method	90	0.1557	0.0363	0.0038

Estimation for Difference

Difference	95% CI for Difference
0.02383	(0.01173, 0.03593)

Test

Null hypothesis $H_0: \mu_1 - \mu_2 = 0$
Alternative hypothesis $H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
3.89	169	0.000

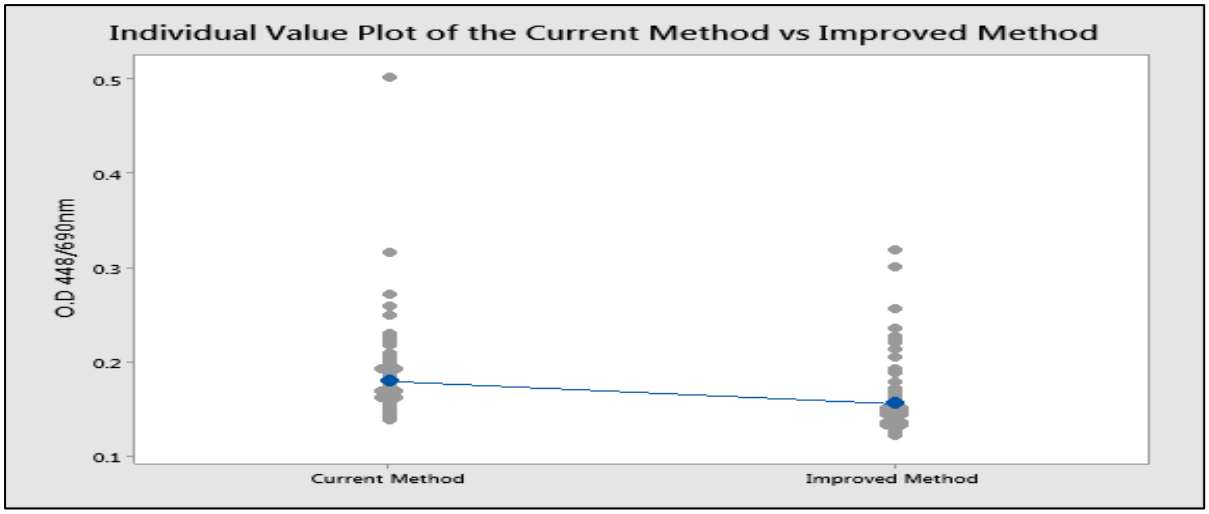


Figure 4.17: Individual value plot of the OD values of the 90 normal human serum samples for both the standard and improved methods.

The p-value is 0 indicating that there is a statistically significant difference between the OD values. This may be ascribed to the mean OD of the improved method being 13% lower than that of the standard method and the improved method indicating less variability in the OD values that are used to calculate the mean, as demonstrated in the individual value plot. In addition, the mean has also shifted closer to 0.150 than the standard method.

4.3.3 Sensitivity - Limits of Detection (LoD)

Limits of detection (LoD) is the lowest concentration of an analyte concentration that can be reliably distinguished from the "analytical noise" of the assay. This is especially significant for the purposes of this study, as the objective of this study was to improve the detection of the core region of 3 of the HCV genome. A dilution series of the antibody positive control was prepared and tested. The S/COO ratio was calculated from the mean OD of each of the dilutions. These calculations may be found in Appendix C3.3.

The OD values of the data generated on the LoD study for both scenarios were converted to S/CO and plotted on a line graph in Microsoft Excel. A trend line was also added, to indicate and compare the performance for both scenarios. A summary of the performance of both scenarios is presented in Figure 4.18 below.

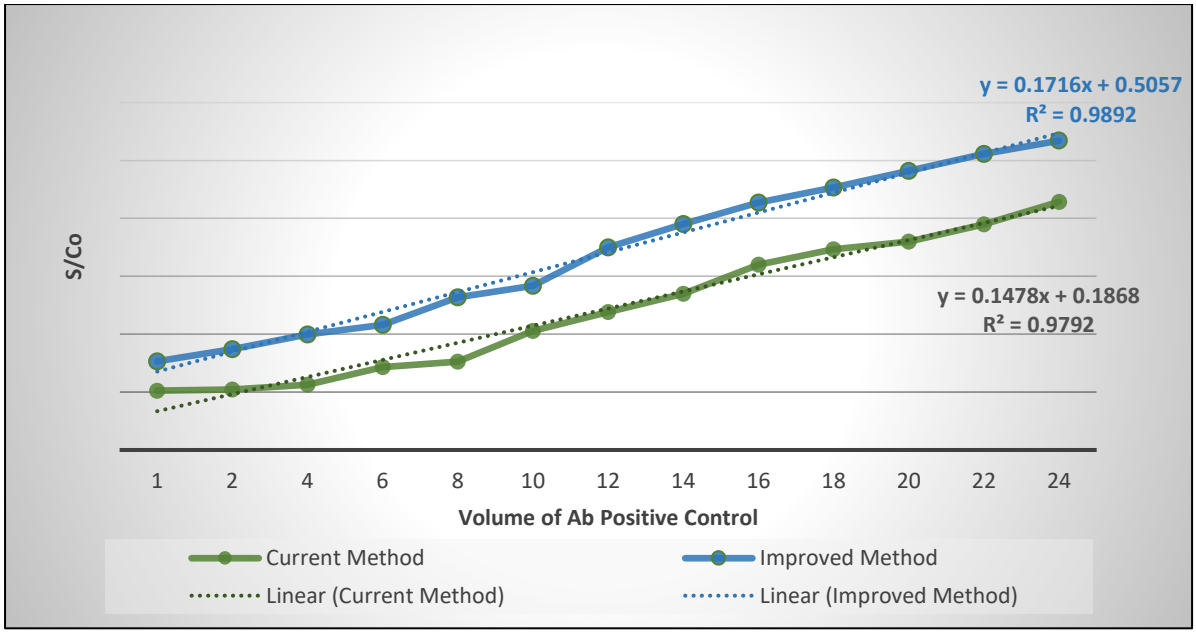


Figure 4.18: S/CO results for a dilution series of the antibody (Ab) positive control for the microtitre plate and conjugate prepared using the improved method and the standard methods.

Note: Testing was conducted using the Murex HCV Ag/Ab Combination assay.

The equation of the trend lines as shown in Figure 4.18 was used to calculate the minimum volume (µl) of antibody positive control required to return a positive result (S/CO = 1). This was performed for both the improved and standard methods of synthesis. This calculation is shown in Table 4.7 below.

Table 4.7: Calculation of the volume (µl) of antibody (Ab) positive control required to return a positive result (S/CO = 1)

Scenario	R-Squared	Trend line Equation	Volume (µl) of Ab positive control required for achieving a positive S/CO value of 1.0
Standard Method	0.98	y = 0.1478x + 0.1868	5.5
Improved Method	0.99	y = 0.1716x + 0.5057	2.8

The calculations shown in Table 4.7 indicate that for the improved microtitre plate and conjugate, 2.8 µl of antibody positive control is sufficient sample to return a S/CO = 1, which is a

positive result. The microplate and conjugate synthesised using the standard method required 5.5 µl of antibody positive control to return a sample to cut off value of 1.0. Thus, the microplate and conjugate matched manufactured using the improved method requires 50.9% less sample to return a positive result.

4.3.4 Stability

Accelerated stability, where the reagents are subject to exaggerated, environmental conditions are designed to generate performance data of the reagent. Typically, an increase in storage temperature is used to achieve the desired degradation.

Accelerated stability testing was conducted to assess the rate of degradation of the improved microtitre plate and conjugate. A control was tested alongside the improved batch - this was a commercially available microtitre plate and conjugate, prepared according to the standard method. The reagents were stored at 37°C over seven (7) weeks (acceleration factor =8). This was done to review the reagent performance over the shelf life of both the microtitre plate and conjugate. The data generated from this study provided an understanding of the impact of the change to both the microtitre plate and conjugate.

The OD values of the data generated on each test occasion over the 8-week study was converted to S/CO and plotted on a bar graph in Microsoft Excel. A regression line was also added, to indicate and compare the rate of degradation for both scenarios. A summary of the performance of both scenarios is presented in Figures 4.19 and 4.20 below.

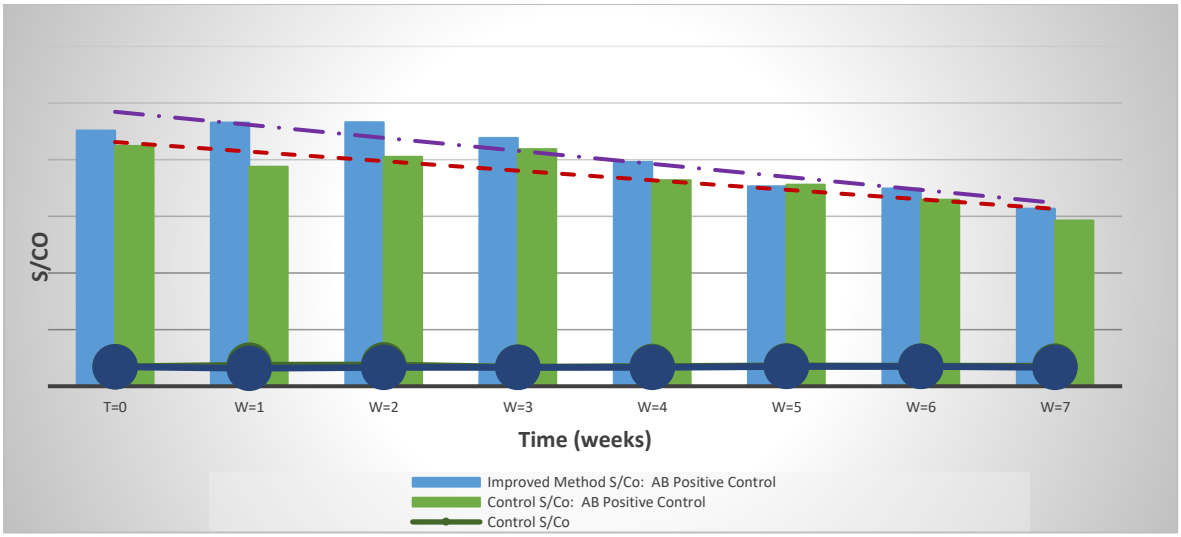


Figure 4.19: S/CO values of the antibody (Ab) positive control for the accelerated stability testing of the microtitre plate and conjugate prepared using the improved and standard methods of preparation.

Note: Testing was conducted using the Murex HCV Ag/Ab Combination assay.

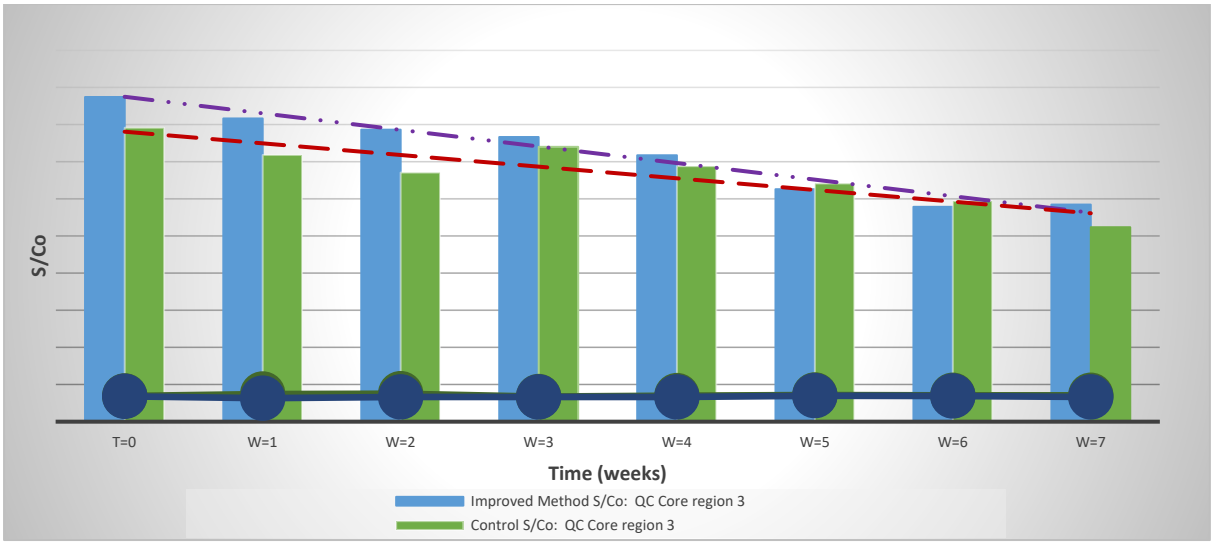


Figure 4.20: S/CO values of the core region 3 quality control for the accelerated stability testing of the microtitre plate and conjugate prepared using the improved and standard methods of preparation.

Note: Testing was conducted using the Murex HCV Ag/Ab Combination assay.

The percentage decline was calculated based on each scenario's performance at the beginning of the study versus performance at the end. A summary of the performance of both scenarios is presented in Table 4.8 below.

Table 4.8: Calculation of the percentage decline of coated microtitre well and conjugate prepared using the improved method of synthesis and a commercially available coated microtitre well and conjugate.

Improved Method % Decline: Ab Positive Control	-30.503
Control % Decline: Ab Positive Control	-31.1014
Improved Method % Decline: QC Core Region 3	-33.174
Control Method % Decline: Ab Positive Control	-33.864

The stability of the improved method for manufacturing was comparable to that of the standard method. The rates of decline for both the antibody positive control and the quality control representing core region 3 for the improved method is 30.503% and 33. 174%, respectively. The standard method indicates a 31.101% and 33.864% respectively, for the antibody positive control and quality control representing core region 3.

4.4 Improved preparation methods for the detection of antibodies generated to core region 3

4.4.1 Sensitivity, Specificity, and Stability

The sensitivity of the Murex HCV Ab/Ag Combination Assay for detection of antibodies to core region 3 of the hepatitis C viral genome was improved by 36% when prepared using the improved methods for synthesis, as compared to the conjugate prepared using the standard method. The sensitivity of the assay for core region 3 was improved despite the use of a smaller volume of the core region 3 detection molecule. The standard method requires an 83% larger volume of the core region 3 detection molecule as compared to the conjugate prepared using the improved method. This is since the conjugate prepared using the standard method was spiked at 1/350, whereas the conjugate prepared using the improved method was spiked at 1/2000.

In addition, a smaller volume of antibody positive sample was required when the improved method was used for preparation of both the Murex HCV Ab/Ag combination conjugate and coated microtitre wells. A sample volume of 2.8 µl vs 5.5 µl for the improved and the standard methods, translating into a 50.9% decrease in the sample volume required for a positive result.

This result demonstrates that it is possible to spike the conjugate at a lower titre and still improve the detection of antibodies to core region 3. In addition, a smaller volume of sample is needed to return a positive result allowing for more tests to be conducted on a single prepared sample.

These results have important implications for the study comparing the Murex HCV Ab/Ag Combination (DiaSorin, South Africa) assay and the Monolisa Ag/Ab Ultra V2 (Bio-Rad, Marnes-la-Coquette, France) in samples with HIV co-infection, which found that the Bio-Rad assay was more sensitive than the Murex assay (Eshetu et al., 2020). As sensitivity has now been improved by amending the preparation of the core capture and detection molecules, the Murex Ab/Ag Combination assay may now be better able to detect HCV antibodies to core region 3 in eluates and their subsequent dilutions. In addition, the assay may be able to perform more reliably when detecting subtypes of HCV in dilutions of dried serum or plasma spots, as it now able to detect smaller volumes in a sample.

With regard to specificity, the same study comparing the Murex and Bio-Rad assays indicated that 22.8% (28/123) dried serum spot samples were initially reactive on the Murex assay, but confirmatory testing showed them to negative. This is compared the 1.9% (2/105) false positive rate indicated by the Monolisa Ag/Ab Ultra assay (Eshetu et al., 2020). As the Murex assay has a higher false positive rate, thus a lower diagnostic specificity of 77.2% compared to Bio-Rad's 1.9% and 98.1%, respectively; the improvement of 13% in specificity demonstrated in this study may result in a lower false positive rate in potentially cross-reactive specimens.

The improved preparation methods do not impact the overall stability of the Murex HCV Ab/Ag Combination Assay. The Murex Ab/Ag Combination coated microtitre wells and conjugate prepared using the improved and the standard methods both show a decline a similar rate, i.e., 30 to 34%. Thus, indicating that the improved methods for preparation does not affect the long-term stability of the Murex HCV Ab/Ag Combination Assay.

In a 2016 review of the current practices of HCV diagnosis, it was noted that there was a difference in diagnostic approaches when screening blood for suitability and that used for the development of a treatment regime (Cloherty et al., 2016). The Murex HCV Ab/Ag Combination Assay may now be more reliable, and thus perform more competitively with other HCV combination assays, when used for blood screening in low-income countries.

CHAPTER 5: CONCLUSIONS

The aim of this study was to improve the sensitivity of the Murex HCV Ab/Ag Combination assay for the detection of antibodies generated to core region 3 of the hepatitis C genome, as well as improve the overall assay specificity. The value of this study is related to the periodic review of the ELISA assays in order for them to remain relevant with regard to the heterogeneity of HCV. In addition, it serves to review reagent preparation to ensure that kits are produced as efficiently and cost effectively as possible.

The sensitivity of the Murex HCV Ab/Ag Combination assay for the detection of antibodies generated to core region 3 of the hepatitis C genome was improved by changing the methods for preparing both the capture and detection molecule.

Efficacy of the core detection was most significantly improved when conjugation occurred at pH 6.0. The concentration of the enzyme was increased to 1250 µM and modified by offering a 2.5-fold increase in sulfo-SMCC. The detection molecule was prepared using a 0.5 mM

peptide solution. Finally, the detection molecule was passed through G25 Sephadex resin in 10 ml PD -10 disposable columns. The sensitivity of the core capture molecule for antibodies generated to core region 3 coated on the microtitre wells was improved by the preparation of individual core capture molecules and the subsequent ascertainment of the most appropriate microtitre plate coating level for each. Core region 3 – BSA should be coated at 0.02 µg/ml, whilst core region 1 – BSA and core region 2 – BSA should be coated at 0.04 µg/ml. This was not included in the original objectives submitted during the research proposal stage of this study. However, as the study progressed, it was discerned that there were limitations to the extent to which the sensitivity could be improved without reviewing both the capture and detection molecules.

This study was also limited by the fact that the difficulties noted during the bulk preparation processes are historical and have only been identified internally at DiaSorin South Africa. There is no record of the issue in the public domain. Further to this, there is little relevant research available to which to compare this study's findings. As such, the study was limited to comparing the performance of the Murex HCV Ab/Ag Combination assay as it is currently prepared to the performance when the improvements were made. Finally, the study did not use HCV serum positive clinical samples for evaluation of the different variables as these are difficult to obtain in South Africa. The antibody positive control used in the Murex HCV Ab/Ag combination assay is prepared using serum positive for antibodies generated to the hepatitis C virus. This serum is chemically inactivated with an added preservative. HCV negative (normal human serum) clinical samples were used to assess the specificity of the improved assay.

The incorporation of the improvements determined in this study into the bulk preparation processes for both the capture and detection molecules, would result in the Murex HCV Ab/Ag Combination assay being able to maintain and improve its claims for sensitivity and specificity. Thus, continuing to provide a robust, less expensive alternative for blood screening, and enabling access to safer blood and blood products particularly in sub-Saharan Africa.

CHAPTER 6: FUTURE STUDIES

Purification of the core region 3 detection molecule can be conducted on a purification system such as an AKTA Purifier on Sephadex G25 to further optimize purity. Online monitoring of absorbance values at 280 nm can be used to determine when the enzyme cross-linked to the peptide elutes off the column. This can be monitored to remove unconjugated horseradish

peroxidase from the final product, thereby further improving purity, and by extension specificity of the detection molecule.

In addition, a study using clinical samples with potentially cross-reactive specimens to evaluate the Murex HCV Ab/Ag Combination assay for detection of HCV antigen and antibodies in serum and plasma should be conducted to determine if the sensitivity and specificity of the assay has been improved. The evaluation should be conducted on reagents prepared using the improved methods determined in this study.

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APPENDIX A: APPROVAL

Ethical clearance obtained from UNISA

UNISA university of south africa

2014-04-14

Ref. Nr.: 2014/CAES/082

To:
Student: ND Sukhdeo **Student nr: 45691037**
Supervisor: Prof J Dewar
Department of Life and Consumer Sciences
College of Agriculture and Environmental Sciences

Dear Prof Dewar and Ms Sukhdeo

Request for Ethical approval for the following research project:

Optimisation of antibody detection sensitivity of the Murex Ab/Ag Combination HCV assay

The application for ethical clearance in respect of the above mentioned research has been reviewed by the Research Ethics Review Committee of the College of Agriculture and Environmental Sciences, Unisa. Ethics clearance for the above mentioned project (Ref. Nr.: 2014/CAES/082) **is given** for the duration of the study.

The applicant is reminded that the permission letter from SANBTS must be submitted as soon as it is available.

Please be advised that should any part of the research methodology change in any way as outlined in the Ethics application (Ref. Nr.: 2014/CAES/082), it is the responsibility of the researcher to inform the CAES Ethics committee. In this instance a memo should be submitted to the Ethics Committee in which the changes are identified and fully explained.

The Ethics Committee wishes you all the best with this research undertaking.

Kind regards,

Prof E Kempen,
CAES Ethics Review Committee Chair

Prof MJ Linington
Executive Dean: College of Agriculture and Environmental Sciences

University of South Africa
Preller Street, Muckleneuk Ridge, City of Tshwane
PO Box 392 UNISA 0003 South Africa
Telephone: +27 12 429 3111 Facsimile: +27 429 12 429 4150
www.unisa.ac.za

Permission obtained from DiaSorin South Africa

UNISA university of south africa

Request for permission to conduct research at DiaSorin South Africa

Optimization of the antibody detection sensitivity of the Murex Ab/Ag Combination HCV assay.

Tanja Postma
22 Kyalami Boulevard
Managing Director
tanja.postma@diasorin.co.za

Dear Tanja,

I, Nityanandi Diaksha Sukhdeo, am doing research Professor John Dewar, a professor, etc in the Department of Life Sciences towards a Master of Science degree, at the University of South Africa. We have funding from DiaSorin South Africa for the purposes of improvement of the Murex Ab/Ag Combination product already on the market. We are inviting you to participate in a study entitled Optimization of the antibody detection sensitivity of the Murex Ab/Ag Combination HCV assay.

The aim of the study is to improve the detection of antibodies generated to a particular region of the core of the freeze-dried conjugate of the Murex Anti-HCV combination kit.

Your company has been selected because DiaSorin in the owner of the Murex Brand, and thus the owner of the intellectual property related to the Murex Ab/Ag Combination Assay.

The study will entail a review of the current manufacturing processes, and test variables sequentially to determine which is the most appropriate to improve the process and the performance of the product.

This study is beneficial as it will lead to both process, and, financial efficiencies.

There are no potential risks identified.

Feedback procedures will entail regular sessions to discuss the progress of the study, challenges, and next steps.

Yours sincerely

Nityanandi Diaksha Sukhdeo
Quality Control Team Leader

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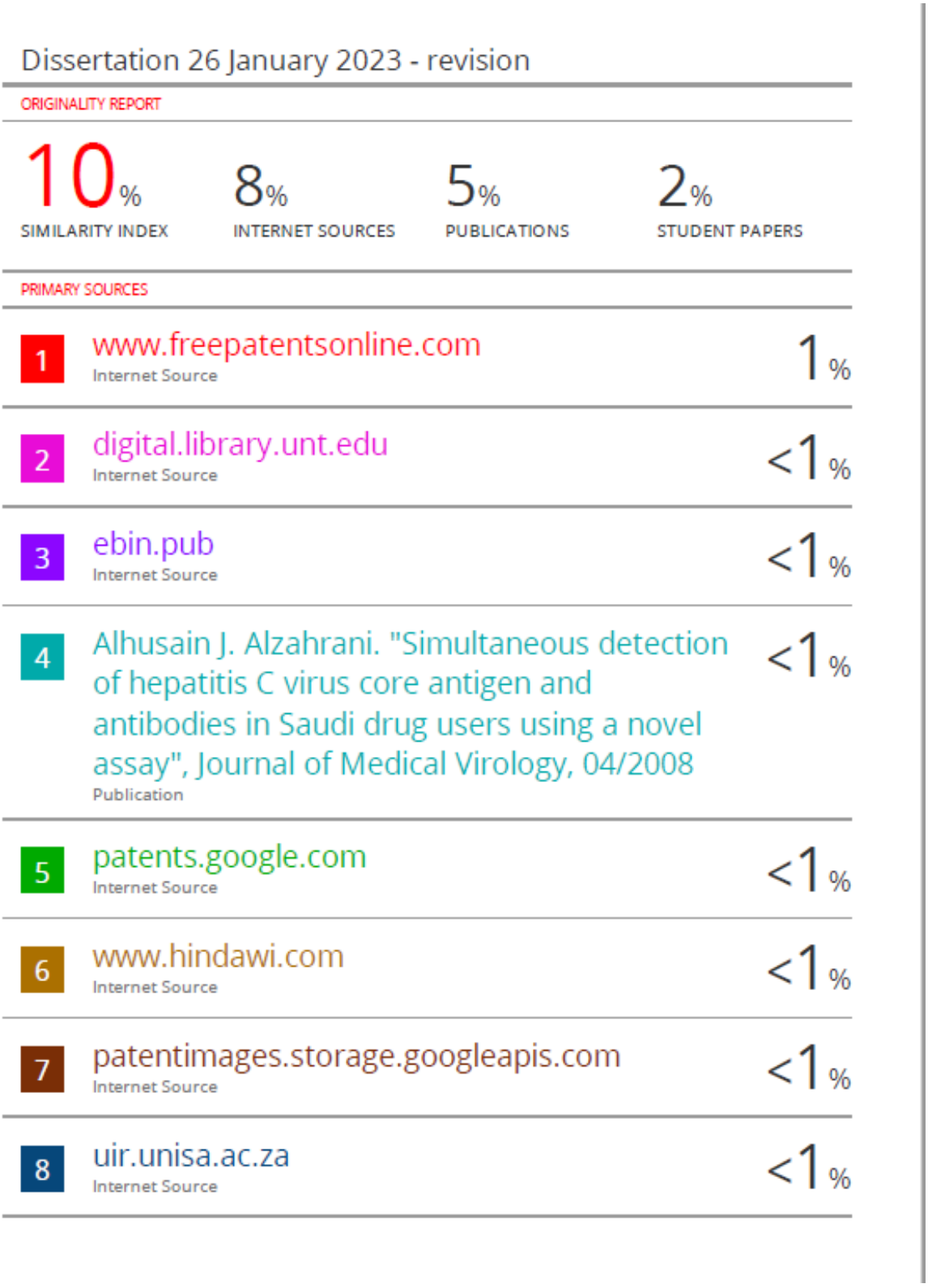

Dissertation 26 January 2023 - revision

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APPENDIX B: CHEMICALS AND SUPPLIERS, REAGENT/BUFFER PREPARATION, and, STANDARD METHODS OF PREPARATION

Appendix B1: Chemicals and suppliers

Table B1.1: List chemicals and suppliers

MATERIALS	MANUFACTURER
HEPES	Sigma Aldrich, St. Louis, MO, USA
Hydroxylamine	VWR International, Bridgeport, NJ, USA
Triethanolamine hydrochloride	Sigma Aldrich, St. Louis, MO, USA
Aldrithiol 4	Sigma Aldrich, St. Louis, MO, USA
95% Ethanol:	Merck KGaA, Darmstadt, Germany
Molecular sieve 4A, 1/16-inch pellets:	VWR International, Bridgeport, NJ, USA
β-mercaptoethanol	Merck KGaA, Darmstadt, Germany
Dimethyl sulfoxide (from which water is removed)	Sigma Aldrich, St. Louis, MO, USA

N-ethylmaleimide (NEM)	Sigma Aldrich, St. Louis, MO, USA
Bovine serum albumin (BSA)	Sigma Aldrich, St. Louis, MO, USA
Size 1 dialysis membrane with a MWCO of between 12000 – 14000 (diameter of 6.3 mm and a wall thickness of 0.050 mm.)	MediCell Membranes Ltd, London, UK
Size 2 dialysis membrane with a MWCO of between 12000 – 14000 (diameter of 16 mm and a membrane thickness of 0.020 mm)	MediCell Membranes Ltd, London, UK
sulfo-SMCC	Pierce Biotechnologies, Rockford, USA
SATA	Pierce Biotechnologies, Rockford, USA
Sodium carbonate	VWR International, Bridgeport, NJ, USA
Sodium hydrogen carbonate	Merck KGaA, Darmstadt, Germany
Sodium chloride	Merck KGaA, Darmstadt, Germany
di-Sodium hydrogen orthophos-phate 2-hydrate	VWR International, Bridgeport, NJ, USA
Potassium di-hydrogen ortho-phosphate	VWR International, Bridgeport, NJ, USA
10% casein (Hammerstein grade)	BD GmbH, Heidelberg, Germany
Sodium chloride	Merck KGaA, Darmstadt, Germany

Potassium chloride	VWR International, Bridgeport, NJ, USA
semi-instant skimmed milk powder	Dairy Crest, Telford, UK
30% Bovine serum albumin	Merck Millipore, Billerica, MA, USA
PVP	VWR International, Bridgeport, NJ, USA
Sucrose	VWR International, Bridgeport, NJ, USA
Nunc MaxiSorp™ microtiter wells	Thermo Scientific, Massachusetts, USA)
Nunc MediSorp™ microtiter wells	Thermo Scientific, Massachusetts, USA)
Greiner MICROLON® 200 microtitre wells	Greiner Bio-One, Austria
Greiner MICROLON® 600 microtitre wells	Greiner Bio-One, Austria
Horseradish peroxidase	BBI Solutions, Gwent, UK
5 ml Slide-A-Lyzer G2TM dialysis cassette	Thermo Scientific, Massachusetts, USA)
PD-10 columns containing Sephadex G25 Medium resin	(GE Healthcare, Buckinghamshire, UK)
0.5 M Sulphuric Acid	Merck KGaA, Darmstadt, Germany

Appendix B2: Preparation of buffers and reagents

B2.1: 25 mM HEPES 1 mM EDTA (pH 7.8): A quantity of 6 g of HEPES (Sigma Aldrich, St. Louis, MO, USA), was dissolved in 800 ml of distilled water. Thereafter, 2 ml of 500 mM EDTA solution at pH 8.0, was added and allowed to stir for 10 minutes. The pH of this solution was adjusted to 7.8 by addition of 5 M NaOH. Thereafter, the distilled water was added to a final volume of 1000 ml. This buffer can be stored at 2 to 8°C for up to 1 month.

B2.2: 25 mM HEPES 1 mM EDTA (pH 6.8): A quantity of 6 g of HEPES (Sigma Aldrich, St. Louis, MO, USA), was dissolved in 800 ml of distilled water. Thereafter, 2 ml of 500 mM EDTA solution at pH 8.0, was added and allowed to stir for 10 minutes. The pH of this solution was adjusted to 6.8 by addition of 5 M NaOH. Thereafter, the distilled water was added to a final volume of 1000 ml. This buffer can be stored at 2 to 8°C for up to 1 month.

B2.3: 500 mM Hydroxylamine (pH 7.0): A quantity of 3.45 g of Hydroxylammonium chloride (VWR International, Bridgeport, NJ, USA), was dissolved in 80 ml of distilled water. Thereafter, 18. 56 g of Triethanolamine hydrochloride (Sigma Aldrich, St. Louis, MO, USA) was added, and allowed to dissolve fully. An amount of 0.2 ml of 500 mM EDTA solution at pH 8.0, was added and allowed to stir for 10 minutes. The pH of the resulting solution was adjusted to 7.0 by addition of 5 M NaOH. Lastly, the distilled water was added to a final volume of 100 ml. This buffer can be stored at 2 to 8°C for up to 1 month.

B2.4: 500 mM A4 Solution: A quantity of 11 mg Aldrithiol 4 (98%) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 1 ml of 95% Ethanol (Merck KGaA, Darmstadt, Germany). This buffer can be stored at 2 to 8°C for up to 1 month.

B2.5: Anhydrous dimethyl sulfoxide: A quantity of 5 g molecular sieve 4A, 1/16-inch pellets (VWR International, Bridgeport, NJ, USA) was added to a volume of 100 ml of dimethyl sulfoxide (Sigma Aldrich, St. Louis, MO, USA). This solution can be stored at room temperature for up to 1 year.

B2.6: 1/500 β-mercaptoethanol (2-ME) solution: A volume of 20 µl β-mercaptoethanol (Merck KGaA, Darmstadt, Germany) was prepared in 9.98 ml 25 mM HEPES 1 mM EDTA (pH 6.8). This solution must be used immediately to prepare a 1/48 000 dilution β-mercaptoethanol solution.

B2.7: 1/48 000 β-mercaptoethanol (2-ME) solution: A volume of 50 µl of 1/500 β-mercaptoethanol solution was added to 4.75 ml of 25 mM HEPES 1 mM EDTA (pH 6.8). This solution must be used immediately.

B2.8: 1/201 β-mercaptoethanol (2-ME) solution: A volume of 50 µl of β-mercaptoethanol solution was added to 10 ml of 25 mM HEPES 1 mM EDTA (pH 6.8). This solution must be used immediately.

B2.9: 100 mM NEM solution**:** A quantity of 125 mg N-ethylmaleimide (NEM) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 10 ml distilled water. This solution must be used immediately.

B2.10: Preparation of 5mg/ml bovine serum albumin (BSA): A 30% solution of bovine serum albumin (Merck Millipore, Illinios, USA) was purified by dialysis at 2°C to 8°C in a size 1 dialysis membrane with a MWCO of between 12000 – 14000 (MediCell Membranes Ltd, London, UK) using a pH 7.8, 25 mM HEPES, 1 mM EDTA solution provided at 200 x the volume of the BSA solution. Two changes of the HEPES-EDTA solution were used, each for a period between 4 and 24 hours. The concentration of the BSA solution was determined spectrophotometrically using a T80 UV-VIS Spectrophotometer (PG Instrument Ltd, London, UK) set at a wavelength of 280 nm in order to determine the concentration of the 30% BSA stock solution.

A 5 mg/ml solution was prepared from the 30% BSA stock solution by adding 3.45 ml of the BSA solution to 18.08 ml of a 25 mM HEPES, 1 mM EDTA (pH 7.8) solution.

B2.11: Preparation of 100 mg/ml sulfo-SMCC in DMSO: A quantity of 61.6 mg of sulfo-SMCC (Pierce Biotechnologies, Rockford, USA) was dissolved in 1616 µl of DMSO.

B2.12: 0.05 M Carbonate/Bicarbonate coating buffer (pH 9.6): An amount of 1.6 g of sodium carbonate (VWR International Bl, Bridgeport, NJ, USA), and 2.94 g sodium hydrogen carbonate (Merck KGaA, Darmstadt, Germany), was dissolved in 750 ml of distilled water. The pH was adjusted to 9.6 with 5 M sodium hydroxide or 5 M hydrochloric acid, as applicable. Thereafter, the distilled water was added to a final volume of 1000 ml. This buffer can be stored at 2 to 8 °C for up to 2 weeks.

B2.13: 0.2% casein in PBS blocking buffer (pH 7.4): An amount of 16 g sodium chloride (Merck KGaA, Darmstadt, Germany), 0.4 g potassium chloride (VWR International, Bridgeport, NJ, USA), 2.88 g di-sodium hydrogen orthophosphate 2-hydrate (VWR International, Bridgeport, NJ, USA), and 0.4 g potassium di-hydrogen orthophosphate (VWR International, Bridgeport, NJ, USA), were dissolved in 900 ml distilled water. The pH of the solution was adjusted to 7.4 using 5 M sodium hydroxide or 5 M hydrochloric acid. Thereafter, 20 ml of 10% casein (Hammerstein grade) (BD GmbH, Heidelberg, Germany) was added and allowed to stir for 5 minutes. Distilled water was added to a final volume of 1000 ml. This buffer can be stored at 2 to 8°C for up to 2 weeks.

B2.14: 0.2% Non-fat dairy milk in PBS blocking buffer (pH 7.4 (pH 7.4): An amount of 16 g sodium chloride (Merck KGaA, Darmstadt, Germany), 0.4 g potassium chloride (VWR International, Bridgeport, NJ, USA), 2.88 g di-sodium hydrogen orthophosphate 2-hydrate (VWR International, Bridgeport, NJ, USA), and 0.4 g potassium di-hydrogen orthophosphate (VWR International, Bridgeport, NJ, USA), was dissolved in 900 ml distilled water. The pH of the solution was adjusted to 7.4 using 5 M sodium hydroxide or 5 M hydrochloric acid. Thereafter, 2 g of semi-instant skimmed milk powder (Dairy Crest, Telford, UK) was added and allowed to stir for 5 minutes. Distilled water was added to a final volume of 1000 ml. This buffer can be stored at 2 to 8°C for up to 2 weeks.

B2.15: 0.1% BSA, 0.1% Casein in PBS blocking buffer (pH 7.4): An amount of 16 g sodium chloride (Merck KGaA, Darmstadt, Germany), 0.4 g potassium chloride (VWR International, Bridgeport, NJ, USA), 2.88 g di-sodium hydrogen orthophosphate 2-hydrate (VWR International, Bridgeport, NJ, USA), and 0.4 g potassium di-hydrogen orthophosphate (VWR International, Bridgeport, NJ, USA), was dissolved in 900 ml distilled water. The pH of the solution was adjusted to 7.4 using 5 M sodium hydroxide or 5 M hydrochloric acid. Thereafter, 10 ml 10% Casein (Hammarsten grade) (BD GmbH, Heidelberg, Germany) and 1 ml 30% Bovine serum albumin (Merck Millipore, Billerica, MA, USA) was added and allowed to stir for 5 minutes. Distilled water was added to a final volume of 1000 ml. This buffer can be stored at 2 to 8°C for up to 2 weeks.

B2.16: Fixing Buffer (pH 6.0): The formulation of the fixing buffer is proprietary information and cannot be disclosed. Briefly this buffer contains PVP and Sucrose to preserve the coated antigens and antibodies onto the microplate surface.

Appendix B3: Standard preparation of the core and detection molecules

B3.1 Standard preparation of the core detection molecule at DiaSorin South Africa:

B3.1.1 Preparation of sulfo-SMCC modified horseradish peroxidase.

A 100 mg/ml solution of horseradish peroxidase (BBI Solutions, Gwent, UK) was prepared in a pH 7.8 solution of 25 mM HEPES, 1 mM EDTA, by adding 6.01 g of horseradish peroxidase to 60.1 ml of pH 7.8 25 mM HEPES, 1 mM EDTA. The horseradish peroxidase was dissolved by gentle swirling.

B3.1.2 Inclusion of Maleimide groups to Horseradish peroxidase (HRP) using Sulfo-SMCC

A 100 mg/ml sulfo-SMCC solution was prepared as described in B2.11.
A 100 mg/ml solution of horseradish peroxidase (BBI Solutions, Gwent, UK) was prepared in a pH 7.8 solution of 25 mM HEPES, 1 mM EDTA, by adding 6.01 g of horseradish peroxidase to 60.1 ml of pH 7.8 25 mM HEPES, 1 mM EDTA. The horseradish peroxidase was dissolved by gentle swirling. A two-fold excess of the 100 mg/ml sulfo-SMCC solution was added to the horseradish peroxidase solution (BBI Solutions, Gwent, UK), i.e., a volume of 1.316 ml of 100 mg/ml sulfo-SMCC was added dropwise to the horseradish peroxidase solution. The resultant solution was allowed to react by incubation at 20° C, away from direct light, for 45 minutes.

At the conclusion of the incubation, the excess cross-linking reagents was removed by gel filtration. The solution was passed through PD-10 desalting columns containing Sephadex G25 resin (GE Healthcare, Uppsala, Sweden), equilibrated with 25 ml of 25 mM HEPES/1 mM EDTA (pH 6.8) buffer solution. The reddish-brown eluate collected in glass bottles and pooled together.

The protein and the maleimide concentration of the HRP-maleimide solution, was determined by spectrophotometry. A T80 UV-VIS Spectrophotometer (PG Instrument Ltd, London, UK) was set at a wavelength of 280 nm. A solution of β-mercaptoethanol was reacted with horse-radish peroxidase/sulfo-SMCC solution, and the absorbance read at a wavelength of 324 nm on a T80 UV-VIS Spectrophotometer (PG Instrument Ltd, London, UK). The extinction co-efficient of 2.475 (Pierce Biotechnology, 2008) is applied to the absorbance value of 0.752 to determine the maleimide concentration of 2351.513 µM.

B3.1.3 Linkage of the detection-peptide to the horseradish peroxidase.

The HRP-maleimide solution prepared in section 3.2.3 was diluted to 1000 µM by adding a volume of 70.476 ml 25 mM HEPES/1 mM EDTA (pH 6.8) solution.

A 10 mM solution of the core detection peptide was prepared by dissolving 106.349 mg of the detection peptide in 6.773 ml DMSO (Sigma Aldrich, Missouri, USA). Thereafter, the 10 mM core peptide solution was further diluted to 0.1 mM by adding 693.594 ml of 25 mM HEPES/1 mM EDTA (pH 6.8) solution.

Modified horseradish peroxidase was reacted with peptide solution one in a ratio of 2:1 to optimise conjugation. In practice, 677.38 ml of the core detection peptide solution was added dropwise to 135.476 ml modified horseradish peroxidase solution, whilst continuously swirling. Thereafter, 500 mM hydroxylamine was added to the solution at a ratio of 1:5, with the aim of exposing the sulfhydryl groups on s-SMCC-modified HRP (Hermanson 1996). As such, 27.095 ml of 500 mM hydroxylamine was added to the solution and incubated at 2°C to 8°C for between 16 and 24 hours. At the conclusion of the incubation, the conjugation reaction was stopped by the addition 50 µl of 1/201 β-mercaptoethanol (2ME) per ml of 1000 µM HRP-maleimide to block free maleimide groups. Thus, 6.774 ml of 1/201 β-mercaptoethanol (2ME) was added and incubated at 18ºC to 25ºC for 20 minutes. Free sulfhydryl's are then blocked by adding 100 µl of 100 µM N-ethylmaleimide per ml of 1000 µM HRP-maleimide. Thus, 13.458 ml was added and incubated at 18ºC to 25ºC for 20 minutes.

B3.1.4 Removal of excess/unwanted reagents from the core detection conjugate

The excess reagents, i.e., unbound peptide, unbound stop solutions etc. contained in the detection peptide conjugate solution was removed by dialysis.

Size 1 dialysis tubing of a MWCO between 12000 – 14000 DA (MediCell Membranes Ltd, London, UK) was pre-treated by soaking in 25 mM HEPES/1 mM EDTA (pH 6.8) solution for approximately 20 minutes. The core-peptide conjugate was pipetted into the pre-treated dialysis tubing, and submerged into 25 mM HEPES/1 mM EDTA (pH 6.8) solution at 100 x the volume of the conjugate for a maximum of 24 hours at 2°C to 8°C. The buffer was changed twice during the 24-hour period.

B3.2 Standard preparation of the core capture molecule at DiaSorin South Africa:

B3.2.1 Inclusion of maleimide groups onto bovine serum albumin (BSA)

A 60 mg/ml solution of the cross-linking reagent was made by dissolving 60 mg of sulfo-SMCC (Pierce Biotechnologies, Rockford, USA) in 1 ml of DMSO (Sigma Aldrich, St Louis, MO, USA). Then, a 600 µl aliquot of this 60 mg/ml sulfo-SMCC solution was added to 20 ml of the 5 mg/ml BSA solution, whilst gently swirling for 5 minutes. The resulting solution was then incubated at 18°C to 25°C, away from direct light, for 90 minutes. Any unbound cross-linking reagent

was removed from the solution by dialysis in 25 mM HEPES 1 mM EDTA (pH 6.8). The volume of buffer needed for dialysis was 200 x the volume of the BSA-maleimide solution, i.e., 4.12 l. Two changes of buffer solutions were used, each for a period of 4 hours and 19 hours, respectively. BSA-maleimide was stored at 2 to 8°C until use. The total maleimide content of sulfo-SMCC modified BSA was determined by A4 assay. A 1/20 dilution was prepared, i.e., 60 µl of the sulfo-SMCC modified-BSA in 0.9 ml 25 mM HEPES, 1 mM EDTA (pH 6.8), and 0.240 ml of 1/48 000 β-mercaptoethanol. This sample was pipetted into a cuvette to completely fill it, ensuring that there were no air bubbles present, or drops of sample on the outside of the cuvette. The cuvette was then placed into a T80 UV-VIS Spectrophotometer (PG Instrument Ltd, London, UK) set at a wavelength of 280 nm. The spectrometer then generated an absorbance value of 0.154, when read at 280 nm. The wavelength of the spectrophotometer was changed to a wavelength of 324 nm. The same sample was removed from the spectrophotometer, and a volume of 4 µl A4 solution was added and mixed vigorously. Thereafter, the sample was read at 324 nm, producing an absorbance value of 0.445. A control was prepared by diluting 0.240 ml 1/48 000 β-mercaptoethanol in 0.960 ml 25 mM HEPES, 1 mM EDTA (pH 6.8), after which an absorbance value of 1.463 at a wavelength of 324 nm was generated, after the addition of 4 µl of A4 solution. The maleimide concentration was determined indirectly by subtracting the sample absorbance value from the control absorbance value at 324 nm. The maleimide concentration was determined to 1028.28 µM. This is based on the absorbance value of 1 µM-SH equalling 0.0198 at 324 nm, 1 mg/ml BSA equalling 0.595 at 280 nm, the molecular weight of BSA at 66 000 Da, and the 1/20 dilution of the sample.

B3.2.2 Preparation of the polycore capture complex.

Three peptides, each representing core region 1, 2, and 3 of the hepatitis C genome, were used in the manufacture of the polycore capture complex. These peptides were separately dissolved in DMSO. The peptide solutions were added sequentially to sulfo-SMCC modified BSA, the volumes were calculated based on the maleimide concentration and a fixed ratio of each peptide representing the core. This ratio cannot be disclosed, as it is proprietary information. However, the ratio is inversely proportional to the reactivity of the peptide for antibodies in a sample. The maleimide concentration was calculated as described in B3.2.1 above, i.e., 1028.28 µM. As such, a volume of 7 µl, 3 µl, and 5 µl of the peptides representing core region 1, 2, and 3, respectively were added sequentially whilst gently stirring. In order to maintain the correct sulfhydryl configuration throughout the conjugation process, a volume of 250 µl of 500 mM hydroxylamine was added the BSA-peptide solution. The resultant solution was then incubated away from direct light, at room temperature, on a roller, for between 16 and 24 hours.

The conjugation reaction was stopped by the sequential addition of 143 µl 1/201 β-mercaptoethanol, thereafter 206 µl N-ethylmaleimide to block any unconjugated maleimide and sulfhydryl groups, respectively.

The excess unbound reagents in the polycore capture complex was then removed by dialysis. This was done by soaking size 1 dialysis tubing of a MWCO between 12000 – 14000 DA in 25 mM HEPES/1 mM EDTA (pH 6.8) solution for approximately 15 minutes. The polycore capture complex was pipetted into the pre-treated tubing and submerged into 25 mM HEPES/1 mM EDTA (pH 6.8) solution at 200 x the volume of the solution to be purified. The buffer was changed twice over a maximum of 48 hours, each change was at a minimum of 4 hours and maximum of 24 hours, at 2°C to 8°C.

Appendix B4: The Murex HCV Ab/Ag Combination Assay Test Method

1. A volume of 50 µl of sample diluent from the kit was added to each microtitre well using a 10 -100 µl 8-channel Finnpipette (Thermo Fisher Scientific, Vantaa, Finland).
2. A volume of 50 µl of each sample (as per the layout described) was added into the sample diluent in each microtitre well.
3. The sample/sample diluent-filled microtitre wells were incubated at 37°C +/-1 °C for 60 minutes in a microplate incubator (Stuart SI505 Microplate Incubator, Staffordshire, UK).
4. A working strength solution of Tween/Saline was prepared by diluting the 20 x concentrate in the Murex HCV Ab/Ag Combination kit with 2500 ml of distilled water. The sample/sample diluent-filled microplate was then washed five times, on a Thermo Wellwash Versa Microplate washer (Thermo Fisher Scientific, Vantaa, Finland), by filling each well with 500 µl of the working strength Tween/Saline washer solution.
5. At the end of five wash cycles, the wash solution remaining in the wells were aspirated leaving the wells empty. Excess wash buffer was removed from the wells by inverting the microplate and tapping gently on a lint free wipe.
6. The freeze-dried conjugate was reconstituted using the entire contents of the conjugate diluent, both of which were found in the Murex HCV Ab/Ag Combination kit.
7. A volume of 120 µl of reconstituted kit conjugate was then added to each microtitre well, and incubated away from direct light, at ambient temperature (~15° C to 28° C) for 60 minutes.
8. The conjugate-filled microtitre wells were then washed as described above.
9. A working strength solution of TMB was prepared by adding an equal volume of TMB Diluent to an equal volume of Orange TMB Substrate Concentrate (both of which are

found the Murex HCV Ab/Ag Combination kit). A volume of 80 µl of working strength TMB solution was then added to each microtitre well.

10. The TMB-filled microtitre wells were then incubated in a microplate incubator (Stuart SI505 Microplate Incubator, Staffordshire, UK) at 37° C +/-1 °C for 30 minutes.
11. The reaction was stopped by the addition of 50 µl 0.5 M Sulphuric Acid (Merck KGaA, Darmstadt, Germany).
12. Absorbance was determined by reading the OD values at 448 nm and a reference wavelength of 690 nm on a Tecan Infinite F50 Absorbance Microplate Reader (Tecan Trading AG, Switzerland).

Appendix B5: Preparation methods for the capture and detection molecule where an improvement was not indicated:

B5.1 Methods for testing variables on the core detection molecule:

B5.1.1 HRP Modified with different crosslinkers.

The peptide representing core region 3 has a protected but chemically accessible sulfhydryl group available for linkage. Horseradish peroxidase has an amine group available for conjugation. As such, the cross-linkers and associated cross-linking strategies that were assessed are detailed below:

Glutaraldehyde

Glutaraldehyde was assessed as a cross linker of interest to this project. However, a decision was taken to exclude this cross-linking reagent from the experimental testing. The standard method in use for conjugation at DiaSorin South Africa employs the use of a heterobifunctional cross-linking reagents in order to maintain stability of the conjugates in the long term. In addition, core region 3-HRP is one of three enzyme-linked core peptide-conjugates, and one of five enzyme-linked conjugates that make up the final Murex Ab/Ag Combination conjugate. Therefore, in order to maintain the stability of the final freeze-dried conjugate, glutaraldehyde was excluded as a possible cross-linking reagent.

Sulfo-EMCS

A 100 mg/ml solution of sulfo-EMCS was prepared in DMSO, by the addition of 61.6 mg of sulfo-EMCS (Pierce Biotechnologies, Rockford, USA) was dissolved in 1.616 ml of DMSO.

A mass of 500 mg of horseradish peroxidase was dissolved in 5 ml of 25 mM HEPES/1 mM EDTA (pH 6.8). A 100 mg/ml solution of sulfo-EMCS (Pierce Biotechnologies, Rockford, USA) was prepared as described in section 3.2.3.2. The sulfo–EMCS modified horseradish peroxidase was diluted to a final concentration of 1000 µM, by the addition of 1.251 ml 25 mM HEPES/1 mM EDTA (pH 6.8).

Linkage to the detection peptide, and removal of excess reagents was conducted as described in Appendix B3.1.3 and B3.1.4.

Evaluation of the detection molecule when the cross-linker was changed:

Test Method:

The detection molecules prepared using the different cross-linkers were tested, alongside a detection molecule prepared as per the standard method of preparation of the core region 3 detection molecule. A range of dilutions were prepared for each concentration, i.e., i.e., 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/1500, and 1/2000. The detection molecule prepared at each scenario was then tested as per the standard method for qualification, at DiaSorin South Africa.

The testing was conducted as per the Murex HCV Ag/Ab Combination protocol detailed the Instructions for Use (IFU) (DiaSorin South Africa 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4.

The following samples were tested as per the layout detailed in Table C1.1 of Appendix C.1.

- The Negative Control (KN) contained within the kit.
- Quality control reference standard containing antibodies specific to core region 3 of the hepatitis C genome was used to assess the detection molecule's performance.

Evaluation of the data and statistical analysis:

The mean OD at 448/690 nm was determined spectrophotometrically; thereafter the S/CO was calculated. This is calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality control for core region 3 was then divided by its corresponding cut-off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample is considered positive if the S/CO value is greater than 1.0.

B5.1.2 Linkage of the core region 3 peptide and HRP to a carrier molecule

The standard method for introducing sulfhydryl groups onto horseradish peroxidase uses sulfo-SMCC to target cysteine residues on the detection peptide. This experiment made use of SATA for the introduction of sulfhydryls onto horseradish peroxidase, and sulfo-SMCC to modify BSA. These activated molecules were then reacted with the detection peptide to pre-pare the core region 3 conjugate.

The detection peptide of core region 3 was cross-linked to a carrier molecule. In this instance, bovine serum albumin (Merck Millipore, Illinios, USA) was used.

B5.1.2.1 Preparation of SATA modified horseradish peroxidase.

The 30% bovine serum albumin (BSA) was prepared and diluted to 5 mg/l, and thereafter modified with sulfo-SMCC as described in Appendix B2.10. The maleimide concentration of the modified-BSA was calculated to be 1028.28 µM.

An amount of 0.2 g of Horseradish peroxidase (BBI Solutions, Gwent, UK) was dissolved in 2 ml of 50 mM Phosphate/1 mM EDTA (pH 7.8) solution. A 40 mg/ml solution was made up by dissolving 16 mg of SATA (Pierce Biotechnologies, Rockford, USA) in 400 µl of DMSO (Sigma Aldrich, Missouri, USA). The cross-linker was added as two-fold excess over horse-radish peroxidase, therefore 58 µl of the SATA solution was added to the 2 ml horseradish peroxidase solution, in a dropwise fashion whilst continuously stirring. The resultant conju-gate solution was allowed to incubate for 45 minutes, at room temperature, away from direct light.

The excess cross-linking reagent was removed from the conjugate by gel filtration.

During the incubation of the conjugate, a PD 10 column containing Sephadex G-25 M resin (GE Healthcare, Buckinghamshire, UK) was equilibrated with 5 column volumes of 50 mM Phosphate, 1 mM EDTA (pH 6.8) solution.

At the end of the conjugate incubation period, 2 ml of SATA-modified horseradish peroxidase solution was applied to the column. Once the entire quantity of the solution had entered the gel bed of the PD 10 column, 3.7ml of 50 mM Phosphate/1 mM EDTA (pH 6.8) solution was applied in order to elute the conjugate.

A 1/80 dilution of the SATA modified horseradish peroxidase was prepared in the 50 mM Phosphate, 1 mM EDTA (pH 6.8) solution. Thereafter, the protein concentration was deter-mined spectrophotometrically on T80 UV-VIS Spectrophotometer (PG Instrument Ltd, London, UK) set at a wavelength of 280 nm. The optical density reading at 280 nm was 0.455, to which

an extinction co-efficient of 5.975 (Pierce Biotechnology 2008) was applied to deter-mine the concentration in mg/ml. The concentration of the STA modified horseradish peroxidase was 60.92 mg/ml. Thereafter, the 4.445 ml of 25 mM HEPES/1 mM EDTA (pH 6.8) was added to 1.9 ml of the SATA-modified horseradish peroxidase, to a final concentration of 50 mg/ml.

B5.1.2.1 Linkage of sulfo-SMCC modified Bovine Serum Albumin and SATA modified horseradish peroxidase to the core detection peptide:

A volume of 6.345 ml of the 50 mg/ml SATA-modified horseradish peroxidase was added dropwise to an equal amount of sulfo-SMCC modified BSA. Thereafter, 1269 µl of 500 mM hydroxylamine was added to the conjugate solution. The resultant solution was incubated at ambient temperature, away from direct light, for 65 minutes.

During the incubation of the BSA and HRP, 4.540 mg of core capture peptide was dissolved in 45 µl of DMSO (Sigma Aldrich, Missouri, USA), and made up a 100 mg/ml solution. The entire amount of dissolved peptide was added dropwise to the HRP/BSA solution, whilst continuously swirling. The conjugate was incubated for 19 hours at 2ºC to 8°C away from di-rect light.

At the end of the incubation period, the conjugation reaction was stopped by addition of 128 µl 1/201 β-mercaptoethanol solution (0.2 µl per mg of BSA). The resultant solution was then incubated at room temperature in the dark for 15 minutes. Thereafter, 182 µl of 12.5 mg/ml NEM solution (5 µl per mg of HRP) was added and the conjugate solution was incubated at room temperature in the dark for 10 minutes.

Removal of excess/unwanted reagents from the core detection conjugate:

Separation of excess unbound reagents was achieved by dialysis in 2609 ml (200 x the conjugate volume) of cold 25 mM HEPES, 1 mM EDTA (pH 6.8) buffer. The conjugate was pipetted into size 1 dialysis tubing, and immersed in cold buffer, along with a magnetic flea. This was placed atop a magnetic stirrer platform. Dialysis was allowed to progress at 2 - 8°C for an initial 3 hours after which, the buffer was refreshed. Thereafter, dialysis progressed for a subsequent 19 hours.

Evaluation of the detection molecule when core region 3 peptide and HRP was linked to a carrier molecule:

Test Method

The detection molecules prepared using the carrier molecule was tested, alongside a detection molecule prepared as per the standard method of preparation of the core region 3 detection molecule. A range of dilutions were prepared for each conjugate, i.e., 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/1500, and 1/2000. The detection molecule prepared at each scenario was then tested as per the standard method for qualification, at DiaSorin South Africa.

The testing was conducted as per the Murex HCV Ag/Ab Combination protocol detailed the Instructions for Use (IFU) (DiaSorin South Africa 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4.

The following samples were tested as per the layout detailed in Table C1.1 of Appendix C.1.

- The Negative Control (KN) contained within the kit.
- Quality control reference standard containing antibodies specific to core region 3 of the hepatitis C genome was used to assess the detection molecule's performance.

Evaluation of the data and statistical analysis:

The mean OD at 448/690 nm was determined spectrophotometrically; thereafter the S/CO was calculated. This is calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality control for core region 3 was then divided by its corresponding cut-off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample is considered positive if the S/CO value is greater than 1.0.

B5.1.3 Preparation of core region 3 detection molecule when testing variables related to removal of excess/unbound reagents by dialysis.

The detection molecule was prepared as per the standard method described in B3.1.1 to B3.1.3. As dialysis is the standard method for removal of unconjugated or excess reagents from the enzyme conjugate; variation in the time, temperature, and membrane format was tested. Separation of the unbound and excess reagents was done by the emersion of filled dialysis membranes in a cold 25 mM HEPES/1 mM EDTA (pH 6.8) solution that was agitated by inclusion of a magnetic flea and placement on a magnetic stirrer platform. The contact material of all dialysis membranes tested was regenerated cellulose acetate, with a MWCO of 10 000 Daltons.

Temperature

Dialysis tubing with a diameter of 6.3 mm, a width of 10 mm, and a membrane thickness of 0.050 mm (MediCell Membranes Ltd, London, UK) was used for these experiments. The tubing was pre-treated at room temperature, by emersion in 2 5 mM HEPES/1 mM EDTA (pH 6.8).

Enzymes peptide conjugates prepared in duplicate and in 10 ml volumes, were then placed in the dialysis tubing and secured with clips. One enzyme conjugate was placed on a magnetic stirrer at 2 - 8°C, as whilst the other was placed at room temperature (average temperature of 21.2°C during the course of the dialysis). The conjugates were allowed to dialyze for an initial 6 hours, the buffer refreshed and dialyzed for a subsequent 18 hours.

Time:

Dialysis tubing with a diameter of 6.3 mm, a width of 10 mm, and a wall thickness of 0.050 mm (MediCell Membranes Ltd, London, UK) was used for these experiments. The tubing was pre-treated with at room temperature, by emersion in 2 5mM HEPES/1 mM EDTA (pH 6.8).

Enzymes peptide conjugates prepared in triplicate and in 10 ml volumes were placed in the dialysis tubing and secured with clips. The tubing was then placed in a 25 mM HEPES, 1 mM EDTA (pH 6.8) solution, at 2 - 8°C, as is standard practice at DiaSorin South Africa.

The first enzyme conjugate was allowed to dialyze for 3 hours at a time, with two changes of the dialysis buffer. The second was allowed to dialyze for 6 hours, and a subsequent 18 hours. The third enzyme conjugate was allowed to dialyze for 24 hours at a time, with two changes of the dialysis buffer.

Evaluation of the detection molecule when testing variables related to removal of excess/unbound reagents by dialysis.

Test Method:

The detection molecules prepared when varying the method for removal of excess/ unbound reagents, were tested alongside a detection molecule prepared as per the standard method of preparation of the core region 3 detection molecule. A range of dilutions were prepared for each pH, i.e., 1/350, 1/500, 1/750, 1/1000, 1/1250, 1/1500, and 1/2000. The detection molecule prepared at each scenario was then tested as per the standard method for qualification, at DiaSorin South Africa.

The testing was conducted as per the Murex HCV Ag/Ab Combination protocol detailed the Instructions for Use (IFU) (DiaSorin South Africa 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4.

The following samples were tested as per the layout detailed in Table C1.1 of Appendix C1.

- The Negative Control (KN) contained within the kit.
- Quality control reference standard containing antibodies specific to core region 3 of the hepatitis C genome was used to assess the detection molecule's performance.

Evaluation of the data and statistical analysis:

The mean OD at 448/690 nm was determined spectrophotometrically; thereafter the S/CO was calculated. This is calculated by firstly adding 0.2 to the mean OD of the negative control value, to determine the cut-off value. Thereafter, the average of the quality control for core region 3 was then divided by its corresponding cut-off for that test. As per the Murex HCV Ab/Ag Combination Assay Instruction for Use (IFU), a sample is considered positive if the S/CO value is greater than 1.0.

B5.2 Methods for testing variables on the core capture molecule:

Immobilisation of the core capture complex onto the solid phase

A composite experiment was designed that tested various microplate surfaces, various concentrations of core capture complex onto the microplate surface, as well, as a range of blocking buffers.

The Solid Phase

Polystyrene microtitre plates were evaluated for improvement in the detection of antibodies to the hepatitis C virus. As such, the currently used Nunc MaxiSorp™ microtitre surface was tested alongside the Nunc MediSorp™ microtitre surface, the Greiner MICROLON® 600 microtitre surface and the Greiner MICROLON® 200 microtitre surface.

Coating concentration of the core-capture complex

The polycore complex and the core peptides individually conjugated to BSA were coated at 0.02 µg/ml, 0.04 µg/ml, 0.06 µg/ml, and 0.08 µg/ml. This level was determined by coating microtitre wells with a range of concentrations of the polycore capture-complex. The range concentrations used was between 20 ng/ml to 80 ng/ml, i.e., 20 ng/ml, 40 ng/ml, 60 ng/ml, and 80 ng/ml.

Active ingredient in the blocking buffer (PBS/Casein)

A non-protein blocker, a mix of casein and BSA, non-fat dairy milk, as well as the current 0.2% casein solution were manufactured and tested. All blocking buffers were prepared as per the standard method in use at DiaSorin South Africa. See sections B2.13, B2.14 and B2.15 for details of the preparation of the buffers.

Coating stage

A volume of 115 µl of the coating buffer, containing the different concentrations of individually conjugated BSA-core peptide, as well as, the standard concentrations of the antibody and antigens excluded from this study, were applied to four (4) different microtitre surfaces, i.e., Greiner MICROLON® 200, Greiner MICROLON® 600 (Greiner Bio-One GmbH, Frickenhausen, Germany), Nunc MaxiSorp™ and Nunc MediSorp™ (Thermo Fisher Scientific, Roskilde, Denmark). The wells were then incubated overnight at room temperature.

Blocking stage

Three different types of blocking buffers were applied on top of the coating buffer after the overnight room temperature incubation. These buffers were:

pH 7.4 PBS containing 0.1% BSA and 0.1% casein,

pH 7.4 PBS containing 0.2% non-fat dairy milk,

protein-free blocking buffer (Pierce Biotechnology, Thermo Fisher Scientific, Rockford, IL, USA)

The resultant mixture was incubated as per the standard protocol and aspirated from the wells after 12 hours.

Fixing stage

This additional step for immobilisation of the molecules to the microtitre plate well surface was performed as per section B2.16.

Evaluation of the core capture molecules immobilised onto the solid phase.

The coated microplate was tested using the standard Murex HCV Ag/Ab Combination Testing protocol as per the Instructions for Use (IFU) (DiaSorin South Africa 2014) using components from the Murex HCV Ag/Ab Combination kit.

Test Method:

The microtitre wells were tested, alongside a commercially available coated Murex HCV Ag/Ab Combination microtitre plate, as per the standard Murex HCV Ag/Ab Combination protocol detailed the Instructions for Use (IFU) (DiaSorin South Africa 2014). The test method for the Murex HCV Ab/Ag Combination assay is described in Appendix B4. Concentrations of 0.02 µg/ml, 0.04 µg/ml, 0.06 µg/ml, and 0.08 µg/ml for each core capture complex were tested on each of the buffers and microplate surfaces as per the layout detailed in Table C2.1 of Appendix C.2.1.

The following samples were tested:

The Negative Control (KN) contained within the kit.

Quality control reference standards, each containing antibodies specific to the core region 1, core region 2, and core region 3 of the hepatitis C virus was used to detect the efficacy of the test plates.

Evaluation of the data:

The mean OD at 448/690 nm was determined spectrophotometrically; thereafter the S/CO was calculated for each test and compared to the commercially available microplate prepared as per the standard method at DiaSorin South Africa.

APPENDIX C: SUPPORTING DATA

Appendix C1: Testing of variables for improvement of the detection molecule

C1.1: Concentration of sulfo-SMCC modified horseradish peroxidase and the detection peptide solution.

Table C1.1: Plate Layout for the testing variables related to improvement of the detection molecule.

Well Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3
B	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3
C	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3
D	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3	QC Core region 3
E	KN	KN	KN	KN	KN	KN	KN	KN
F	KN	KN	KN	KN	KN	KN	KN	KN
G	KN	KN	KN	KN	KN	KN	KN	KN
H	KN	KN	KN	KN	KN	KN	KN	KN
Dilution:	**1/…**	**1/…**	**1/…**	**1/…**	**1/…**	**1/…**	**1/…**	**REF**

Table C1.2: OD 448 nm/690 nm results for the testing of 0.10 mM peptide concentrations for the detection molecule in the ELISA format (Control).

Well Position	1	2	3	4	5	6	7	8
A	1.112	0.790	0.914	0.747	0.701	0.812	0.611	2.030
B	1.094	1.014	1.041	4.082	0.565	0.641	0.567	1.245
C	1.091	0.786	1.165	1.145	3.618	0.596	0.605	1.301
D	0.978	0.749	0.137	0.657	4.027	0.480	0.569	1.267
E	0.059	0.041	0.040	0.018	0.046	0.033	0.029	0.093
F	0.071	0.048	0.020	0.053	0.072	0.038	0.119	0.150
G	0.072	0.049	0.038	0.076	0.058	0.051	0.154	0.131
H	0.121	0.074	0.040	0.083	0.074	0.071	0.035	0.176
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Note: Values highlighted in red were excluded from the data analysis, as these were considered contamination due to insufficient washing.

Table C1.3: OD 448 nm/690 nm results for the testing of 0.25 mM peptide concentrations for the detection molecule in the ELISA format.

Well Position	1	2	3	4	5	6	7	8
A	1.652	1.246	1.095	0.875	0.842	0.793	0.771	1.147
B	1.555	1.283	1.177	0.895	0.827	0.765	0.771	1.042
C	1.557	1.245	1.183	0.922	0.736	0.760	0.731	1.206
D	1.463	1.227	1.125	0.960	0.800	0.833	0.700	1.202
E	0.068	0.058	0.058	0.085	0.052	0.057	0.053	0.156
F	0.069	0.058	0.070	0.050	0.059	0.061	0.060	0.170
G	0.067	0.056	0.060	0.052	0.086	0.050	0.073	0.126
H	0.088	0.069	0.073	0.059	0.068	0.080	0.057	0.111
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.4: OD 448 nm/690 nm results for the testing of 0.50 mM peptide concentrations for the detection molecule in the ELISA format.

Well Position	1	2	3	4	5	6	7	8
A	1.689	1.344	1.201	1.211	0.993	0.990	0.858	1.287
B	1.690	1.319	1.249	1.236	0.952	0.885	0.885	1.158
C	1.610	1.328	1.254	1.112	0.996	0.892	0.898	1.030
D	1.623	1.346	1.269	1.084	0.999	0.898	0.827	1.315
E	0.054	0.065	0.041	0.052	0.038	0.039	0.035	0.118
F	0.052	0.067	0.047	0.054	0.048	0.043	0.036	0.116
G	0.076	0.064	0.082	0.052	0.038	0.041	0.036	0.182
H	0.101	0.069	0.038	0.062	0.047	0.037	0.043	0.143
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.5: OD 448 nm/690 nm results for the testing of 0.75 mM peptide concentrations for the detection molecule in the ELISA format.

Well Position	1	2	3	4	5	6	7	8
A	1.299	1.140	1.098	1.136	1.0043	0.876	0.85	1.200
B	1.223	1.451	1.103	1.196	0.968	0.887	0.801	1.239
C	1.300	1.149	1.110	1.208	0.967	0.882	0.804	1.245
D	1.269	1.136	1.116	1.033	0.999	0.871	0.899	1.267
E	0.063	0.081	0.086	0.040	0.042	0.039	0.040	0.121
F	0.074	0.061	0.051	0.038	0.043	0.034	0.036	0.115
G	0.061	0.047	0.042	0.061	0.049	0.039	0.036	0.120
H	0.071	0.052	0.040	0.045	0.046	0.050	0.043	0.117
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.6 Peptide Concentration Data Summary.

Negative Control							
	350	**500**	**750**	**1000**	**1250**	**1500**	**2000**
Negative Control: Control	0.081	0.053	0.035	0.058	0.063	0.048	0.084
Cut-off: Control	**0.281**	**0.253**	**0.235**	**0.258**	**0.263**	**0.248**	**0.284**
Negative Control: 0.25 mM	0.073	0.063	0.065	0.062	0.066	0.062	0.061
Cut-off: 0.25 mM	**0.273**	**0.263**	**0.265**	**0.262**	**0.266**	**0.262**	**0.261**
Negative Control: 0.5 mM	0.071	0.066	0.052	0.055	0.043	0.040	0.043
Cut-off: 0.5 mM	**0.271**	**0.266**	**0.252**	**0.255**	**0.243**	**0.240**	**0.243**
Negative Control: 0.75 mM	0.065	0.063	0.055	0.046	0.046	0.041	0.039
Cut-off: 0.75 mM	**0.265**	**0.263**	**0.255**	**0.246**	**0.246**	**0.241**	**0.239**
Negative Control Upper limit	**0.200**	**0.200**	**0.200**	**0.200**	**0.200**	**0.200**	**0.200**

QC Core Region 3: Mean OD @ 448 nm/690 nm and S/CO							
	350	**500**	**750**	**1000**	**1250**	**1500**	**2000**
QC Core Region 3: Control	1.069	0.835	0.814	0.850	0.633	0.632	0.588
S/CO: Control	**3.807**	**3.299**	**3.472**	**3.300**	**2.411**	**2.547**	**2.069**
QC Core Region 3: 0.25 mM	1.557	1.250	1.145	0.913	0.801	0.788	0.743
S/CO: 0.25 mM	**5.702**	**4.763**	**4.317**	**3.491**	**3.009**	**3.007**	**2.850**
QC Core Region 3: 0.5 mM	1.653	1.334	1.243	1.144	0.985	0.916	0.867
S/CO: 0.5 mM	**6.105**	**5.011**	**4.934**	**4.486**	**4.058**	**3.818**	**3.568**
QC Core Region 3: 0.75 mM	1.273	1.219	1.107	1.143	0.978	0.879	0.835
S/CO: 0.75 mM	**4.803**	**4.635**	**4.344**	**4.647**	**3.976**	**3.655**	**3.496**
QC Core Region 3: Lower Limit	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**
QC Core Region 3: Upper Limit	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**
S/CO	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**

QC Core Region 3 - neg							
	350	**500**	**750**	**1000**	**1250**	**1500**	**2000**
QC Core Region 3-neg: Control	0.988	0.782	0.780	0.792	0.571	0.584	0.504
QC Core Region 3-neg: 0.25 mM	1.484	1.188	1.080	0.852	0.735	0.726	0.683
QC Core Region 3-neg: 0.5 mM	1.582	1.268	1.191	1.089	0.942	0.876	0.824
QC Core Region 3-neg: 0.75 mM	1.208	1.156	1.052	1.097	0.932	0.839	0.796
QC Core Region 3: Lower Limit	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**
QC Core Region 3: Upper Limit	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**

Table C1.7 One-way ANOVA of the detection molecules prepared using the different peptide concentrations.

One-way ANOVA: Control, 0.25 mM, 0.5 mM, 0.75 mM

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	Control, 0.25 mM, 0.5 mM, 0.75 mM

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	5.8983	1.96610	100.51	0.000
Error	12	0.2347	0.01956		
Total	15	6.1330			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.139862	96.17%	95.22%	93.20%

Means

Factor	N	Mean	StDev	95% CI
Control	4	2.0686	0.0818	(1.9162, 2.2210)
0.25 mM	4	2.8504	0.1321	(2.6981, 3.0028)
0.5 mM	4	3.5679	0.1294	(3.4155, 3.7203)
0.75 mM	4	3.5120	0.1933	(3.3597, 3.6644)

Pooled StDev = 0.139862

Table C1.8 Tukey Pairwise comparison of the detection molecules prepared using different peptide concentrations.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping		
0.5 mM	4	3.5679	A		
0.75 mM	4	3.5120	A		
0.25 mM	4	2.8504		B	
Control	4	2.0686			C

Means that do not share a letter are significantly different.

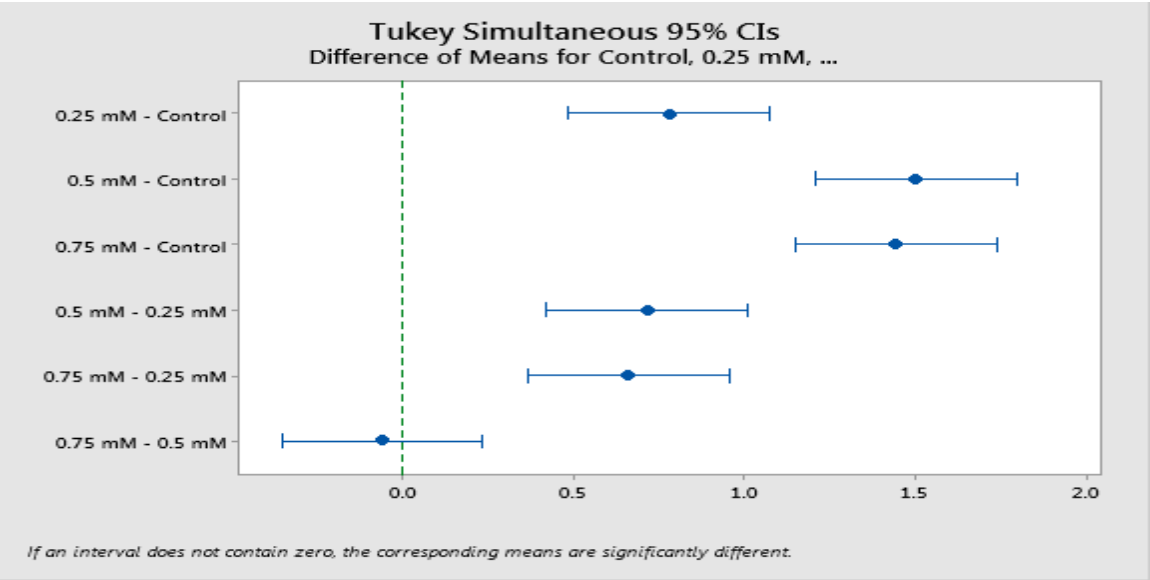

Figure C1.1. Tukey Pairwise Comparison on Minitab (R) 19 software of peptide concentration.

Note: The 1/2000 dilution of the detection molecule was assessed for each test scenario.

C1.2: Concentration of sulfo-SMCC

Table C1.9: OD 448 nm/690 nm results for the testing of 2.25-fold increase in S-SMCC concentrations offered to 0.5 mM peptide solution for the detection molecule on the ELISA format.

Well Position	1	2	3	4	5	6	7	8
A	1.421	1.312	1.365	1.243	1.069	1.022	0.844	1.288
B	1.557	1.304	1.454	1.173	1.132	0.979	0.885	1.275
C	1.748	1.375	1.288	1.154	1.194	1.140	1.029	1.411
D	1.664	1.335	1.220	1.146	1.256	1.215	1.152	1.359
E	0.046	0.041	0.038	0.039	0.037	0.036	0.036	0.112
F	0.048	0.042	0.037	0.036	0.035	0.036	0.035	0.111
G	0.053	0.043	0.039	0.035	0.036	0.037	0.035	0.109
H	0.051	0.044	0.047	0.037	0.037	0.037	0.036	0.104
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.10: OD 448 nm/690 nm results for the testing of 2.5-fold increase in S-SMCC concentrations offered to 0.5 mM peptide solution for the detection molecule on the ELISA format.

Values highlighted in red were excluded from the data analysis, as these were considered contamination due to insufficient washing.

Well Position	1	2	3	4	5	6	7	8
A	1.732	2.004	3.367	1.561	1.361	1.207	1.133	1.413
B	1.777	1.678	1.629	1.452	1.440	1.240	1.152	1.334
C	1.778	1.704	1.565	1.434	1.377	1.209	1.056	1.317
D	1.758	1.663	1.500	1.288	1.267	1.274	1.141	1.447
E	0.050	0.050	0.047	0.045	0.043	0.048	0.047	0.151
F	0.065	0.050	0.059	0.056	0.043	0.042	0.044	0.162
G	0.050	0.042	0.044	0.042	0.044	0.044	0.044	0.134
H	0.063	0.049	0.046	0.048	0.045	0.045	0.042	0.147
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.11: OD 448 nm/690 nm results for the testing of 2.75-fold increase in S-SMCC concentrations offered to 0.5 mM peptide solution for the detection molecule on the ELISA format.

Well Position	1	2	3	4	5	6	7	8
A	1.658	1.533	1.393	1.141	1.294	1.151	0.945	1.250
B	1.644	1.582	1.522	1.385	1.319	1.157	1.122	1.313
C	1.624	1.574	1.439	1.246	1.219	1.235	1.022	1.354
D	1.693	1.526	1.346	1.126	1.191	1.120	0.958	1.151
E	0.076	0.067	0.065	0.068	0.079	0.085	0.116	0.225
F	0.068	0.059	0.051	0.049	0.104	0.048	0.045	0.167
G	0.090	0.066	0.142	0.051	0.063	0.047	0.045	0.143
H	0.058	0.054	0.048	0.048	0.049	0.046	0.045	0.151
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.12: S-SMCC Concentration Data Summary.

Negative Control							
Dilution	**350**	**500**	**750**	**1000**	**1250**	**1500**	**2000**
Negative Control: Control	0.081	0.053	0.035	0.058	0.063	0.048	0.084
Negative Control: 2.25-fold excess	0.050	0.043	0.040	0.037	0.036	0.037	0.036
Negative Control: 2.5-fold excess	0.057	0.048	0.049	0.048	0.044	0.045	0.044
Negative Control: 2.75-fold excess	0.073	0.062	0.077	0.054	0.074	0.057	0.063
QC Core region 3: Mean OD @ 448 nm/690 nm							
QC Core region 3: Control	1.069	0.835	0.814	0.850	0.633	0.632	0.588
QC Core region 3: 2.25-fold excess	1.598	1.332	1.332	1.179	1.163	1.089	0.978
QC1728: 2.5-fold excess	1.761	1.682	1.565	1.434	1.361	1.233	1.121
QC1728: 2.75-fold excess	1.655	1.554	1.425	1.225	1.256	1.166	1.012
QC Core region 3 - neg							
QC Core region 3-neg: Control	0.988	0.782	0.780	0.792	0.571	0.584	0.504
QC Core region 3-neg: 2.25-fold excess	1.548	1.289	1.292	1.142	1.127	1.053	0.942
QC Core region 3-neg: 2.5-fold excess	1.704	1.634	1.516	1.386	1.318	1.188	1.076
QC Core region 3-neg: 2.75-fold excess	1.582	1.492	1.349	1.171	1.182	1.109	0.949
Cut-off (KN + 0.2)							
Cut-off: Control	0.281	0.253	0.235	0.258	0.263	0.248	0.284
Cut-off: 2.25-fold excess	0.250	0.243	0.240	0.237	0.236	0.237	0.236
Cut-off: 2.5-fold excess	0.257	0.248	0.249	0.248	0.244	0.245	0.244
Cut-off: 2.75-fold excess	0.273	0.262	0.277	0.254	0.274	0.257	0.263
QC Core region 3: Lower Limit	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**	**0.712**
QC Core region 3: Upper Limit	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**	**2.009**
S/CO							
S/CO	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**
S/CO: Control	3.807	3.299	3.472	3.300	2.411	2.547	2.069
S/CO: 2.25-fold excess	6.403	5.491	5.543	4.980	4.922	4.605	4.151
S/CO: 2.5-fold excess	6.853	6.787	6.284	5.787	5.585	5.036	4.588
S/CO: 2.75-fold excess	6.061	5.942	5.154	4.821	4.587	4.545	3.851

Table C1.13: One-way ANOVA of the detection molecules prepared using the different sulfo-SMCC concentrations.

One-way ANOVA: 2- fold, 2.25 - fold, 2.5 - fold, 2.75 - fold

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0.05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	4	2- fold, 2.25 - fold, 2.5 - fold, 2.75 - fold

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	2.283	0.7610	6.08	0.009
Error	12	1.503	0.1252		
Total	15	3.786			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.353875	60.31%	50.38%	29.43%

Means

Factor	N	Mean	StDev	95% CI
2- fold	4	3.5679	0.1294	(3.1824, 3.9534)
2.25 - fold	4	4.151	0.598	(3.765, 4.536)
2.5 - fold	4	4.5875	0.1789	(4.2020, 4.9730)
2.75 - fold	4	3.851	0.308	(3.465, 4.236)

Pooled StDev = 0.353875

Table C1.14: Tukey Pairwise comparison of the detection molecules prepared using the different sulfo-SMCC concentrations.

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

Factor	N	Mean	Grouping	
2.5 - fold	4	4.5875	A	
2.25 - fold	4	4.151	A	B
2.75 - fold	4	3.851	A	B
2- fold	4	3.5679		B

Means that do not share a letter are significantly different.

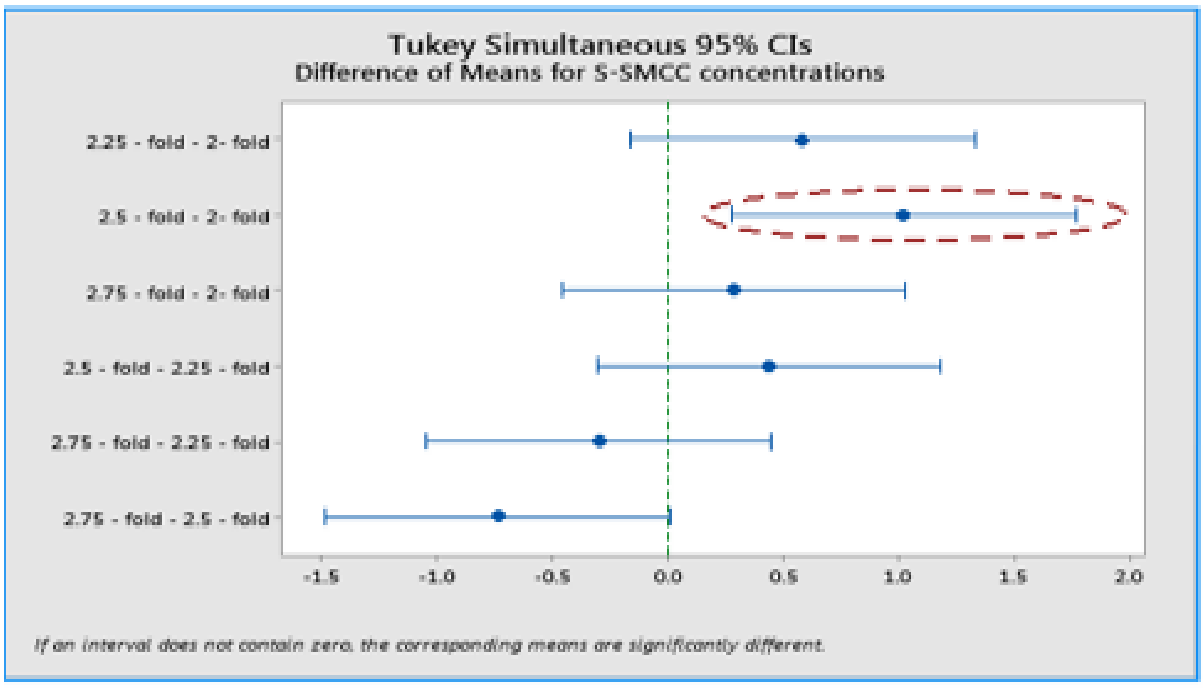

Figure C1.2. Tukey Pairwise Comparison on Minitab (R) 19 software of S/CO values of the S-SMCC concentrations.

Note: A 1/2000 dilution was assessed for each test scenario.

C1.3: pH

Table C1.15: OD 448 nm/690 nm results for the preparation of the core region 3 detection complex when the pH of the buffer is pH 6.8.

	Control (pH 6.8)				
	1	2	3	4	5
A	1.421	1.312	1.365	1.243	1.288
B	1.557	1.304	1.454	1.173	1.275
C	1.748	1.375	1.288	1.154	1.411
D	1.664	1.335	1.220	1.146	1.359
E	0.046	0.041	0.038	0.039	0.112
F	0.048	0.042	0.037	0.036	0.111
G	0.053	0.043	0.039	0.035	0.109
H	0.051	0.044	0.047	0.037	0.104
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**REF**

Table C1.16: OD 448 nm/690 nm results for the preparation of the core region 3 detection complex when the pH of the buffer is pH 6.0.

	pH 6.0				
	1	2	3	4	5
A	1.871	1.654	1.460	1.395	0.788
B	2.007	1.854	1.758	1.596	0.637
C	2.139	1.759	1.652	1.558	0.587
D	1.792	1.729	1.546	1.598	0.601
E	0.064	0.055	0.048	0.064	0.257
F	0.069	0.049	0.051	0.052	0.183
G	0.051	0.045	0.044	0.044	0.158
H	0.050	0.057	0.045	0.048	0.150
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**REF**

Table C1.17: pH of Preparation Buffer Data Summary.

Negative Control				
Dilution	350	500	750	1000
Control (6.8)	0.050	0.043	0.040	0.037
6	0.058	0.051	0.047	0.052
6.2	0.057	0.067	0.050	0.044
6.4	0.083	0.072	0.058	0.053
6.6	0.081	0.067	0.057	0.054
7	0.075	0.064	0.059	0.051
7.2	0.110	0.096	0.075	0.063
7.5	0.050	0.087	0.057	0.056
QC-Core region 3: Mean O.D @ 448 nm/690 nm				
Control (6.8)	1.598	1.332	1.332	1.179
6	1.952	1.749	1.604	1.537
6.2	1.576	1.358	1.168	1.136
6.4	1.454	1.392	1.187	1.155
6.6	1.517	1.531	1.308	1.165
7	1.316	1.101	1.020	0.715
7.2	1.283	1.158	1.069	1.020
7.5	1.437	1.355	1.290	1.204
QC-Core region 3 -neg				
Control (6.8)	1.548	1.289	1.292	1.142
6	1.894	1.698	1.557	1.485
6.2	1.519	1.291	1.118	1.092
6.4	1.371	1.320	1.129	1.102
6.6	1.436	1.464	1.251	1.112
7	1.241	1.038	0.961	0.664
7.2	1.173	1.062	0.995	0.958
7.5	1.387	1.269	1.234	1.149
QC1728: Lower Limit	0.712	0.712	0.712	0.712
QC1728: Upper Limit	2.009	2.009	2.009	2.009
Cut-off (KN + 0.2)				
Control (6.8)	0.250	0.243	0.240	0.237
6	0.258	0.251	0.247	0.252
6.2	0.257	0.267	0.250	0.244
6.4	0.283	0.272	0.258	0.253
6.6	0.281	0.267	0.257	0.254
7	0.275	0.264	0.259	0.251
7.2	0.310	0.296	0.275	0.263
7.5	0.250	0.287	0.257	0.256
S/Co				
Control (6.8)	6.403	5.491	5.543	4.980
6	7.557	6.958	6.500	6.103
6.2	6.126	5.088	4.678	4.650
6.4	5.143	5.110	4.599	4.557
6.6	5.404	5.736	5.092	4.592
7	4.784	4.178	3.936	2.847
7.2	4.144	3.912	3.894	3.886
7.5	5.746	4.729	5.029	4.712
S/Co	1.000	1.000	1.000	1.000

Table C1.18: Two sample T-Test of S/CO values of the synthesised detection molecule when pH 6.0 and 6.8 were used for preparation.

Two-Sample T-Test and CI: Control, pH 6.0

Method

μ_1: mean of Control
μ_2: mean of pH 6.0
Difference: $\mu_1 - \mu_2$

Equal variances are assumed for this analysis.

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
Control	4	4.980	0.186	0.093
pH 6.0	4	6.103	0.383	0.19

Estimation for Difference

Difference	Pooled StDev	95% CI for Difference
-1.123	0.301	(-1.644, -0.601)

Test

Null hypothesis $H_0: \mu_1 - \mu_2 = 0$
Alternative hypothesis $H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-5.27	6	0.002

C1.4: Removal of excess/unbound reagents

Table C1.19: OD 448 nm/690 nm results for the preparation of the core region 3 detection complex when excess reagents are removed from the solution using size 1 dialysis tubing (control).

	Dialysis Size 1 Tubing @ 6 & 18 hrs (Control)							
	1	2	3	4	5	6	7	8
A	1.845	1.670	1.590	1.451	1.395	1.320	1.209	1.544
B	1.863	1.663	1.511	1.412	1.409	1.270	1.201	1.312
C	1.854	1.610	1.509	1.412	1.364	1.273	1.197	1.442
D	1.814	1.622	1.515	1.407	1.366	1.348	1.188	1.453
E	0.059	0.052	0.054	0.051	0.046	0.051	0.041	0.143
F	0.065	0.053	0.055	0.052	0.055	0.048	0.045	0.144
G	0.060	0.057	0.052	0.058	0.051	0.056	0.042	0.138
H	0.061	0.059	0.056	0.055	0.059	0.053	0.051	0.135
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.20: OD 448 nm/690 nm results for the preparation of the core region 3 detection complex when excess reagents are removed from the solution using the dialysis cassette.

	Dialysis cassette @ 6 & 18 hrs							
	1	2	3	4	5	6	7	8
A	1.540	1.320	1.351	1.225	1.122	1.154	1.010	1.582
B	1.591	1.319	1.410	1.231	1.130	1.121	0.848	1.329
C	1.520	1.317	1.373	1.243	1.267	1.120	0.805	1.384
D	1.414	1.398	1.402	1.245	1.256	1.136	1.112	1.482
E	0.065	0.051	0.051	0.053	0.048	0.051	0.051	0.137
F	0.067	0.052	0.067	0.061	0.057	0.067	0.047	0.134
G	0.063	0.056	0.063	0.062	0.066	0.063	0.042	0.141
H	0.065	0.067	0.065	0.059	0.061	0.055	0.041	0.133
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.21: OD 448 nm/690 nm results for the preparation of the core region 3 detection complex when excess reagents are removed from the solution using Sephadex G25 resin (gel filtration).

	Gel Filtration							
	1	2	3	4	5	6	7	8
A	1.186	1.103	1.156	1.202	0.903	0.847	0.820	1.509
B	1.174	1.010	1.152	1.178	0.887	0.831	0.727	1.512
C	1.178	1.115	1.166	1.124	0.892	0.820	0.743	1.517
D	1.169	1.114	1.165	1.127	0.875	0.817	0.711	1.505
E	0.056	0.052	0.052	0.055	0.055	0.052	0.051	0.127
F	0.051	0.048	0.054	0.056	0.052	0.051	0.055	0.101
G	0.055	0.042	0.052	0.058	0.051	0.055	0.054	0.143
H	0.052	0.051	0.060	0.056	0.063	0.054	0.061	0.156
Dilution:	**1/350**	**1/500**	**1/750**	**1/1000**	**1/1250**	**1/1500**	**1/2000**	**REF**

Table C1.22: One-way ANOVA of S/CO values of the synthesised detection molecule at the 1/2000 dilution when the dialysis membrane format was varied.

One-way ANOVA: Size 1, Size 2, Cassette

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	3	Size 1, Size 2, Cassette

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	4.9845	2.49223	48.69	0.000
Error	9	0.4606	0.05118		
Total	11	5.4451			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.226233	91.54%	89.66%	84.96%

Means

Factor	N	Mean	StDev	95% CI
Size 1	4	2.939	0.298	(2.683, 3.195)
Size 2	4	2.223	0.200	(1.967, 2.479)
Cassette	4	3.7992	0.1564	(3.5433, 4.0551)

Pooled StDev = 0.226233

Table C1.23: Two sample T-Test of S/CO values of the synthesised detection molecule at the 1/2000 dilution when excess unbound reagents were removed by dialysis cassette and gel filtration.

Two-Sample T-Test and CI: Cassette, Gel Filtration

Method

μ_1: mean of Cassette
μ_2: mean of Gel Filtration
Difference: $\mu_1 - \mu_2$

Equal variances are not assumed for this analysis.

Descriptive Statistics

Sample	N	Mean	StDev	SE Mean
Cassette	4	3.848	0.554	0.28
Gel Filtration	4	4.898	0.129	0.064

Estimation for Difference

Difference	95% CI for Difference
-1.050	(-1.955, -0.144)

Test

Null hypothesis	$H_0: \mu_1 - \mu_2 = 0$
Alternative hypothesis	$H_1: \mu_1 - \mu_2 \neq 0$

T-Value	DF	P-Value
-3.69	3	0.035

Appendix C2: Improvements to the capture molecule

Table C.2.1: Plate layout used for the efficacy testing of the improved microtitre plate and conjugate and the microtitre plate and conjugate produced using the standard method.

Well Position	1	2	3	4
A	KN	KN	KN	KN
B	KN	KN	KN	KN
C	Core region 1	Core region 1	Core region 1	Core region 1
D	Core region 1	Core region 1	Core region 1	Core region 1
E	Core region 2	Core region 2	Core region 2	Core region 2
F	Core region 2	Core region 2	Core region 2	Core region 2
G	Core region 3	Core region 3	Core region 3	Core region 3
H	Core region 3	Core region 3	Core region 3	Core region 3
Capture Complex	Individual		Polycore	

C.2.1. Efficacy of the individually prepared capture complexes vs polycore complex when coated at 0.04 µg/ml.

Table C2.2: OD 448 nm/690 nm results for the comparative testing of individually prepared capture molecules vs the polycore complex. The individually prepared and coated complexes, as well, as the polycore complex was coated at a final concentration of 0.04 µg/ml.

Well Position	1	2	3	4
KN	0.157	0.152	0.154	0.150
KN	0.152	0.159	0.124	0.142
Core region 1	1.770	1.772	1.902	1.911
Core region 1	1.735	1.765	1.914	1.922
Core region 2	1.504	1.494	1.459	1.455
Core region 2	1.659	1.561	1.583	1.490
Core region 3	1.391	1.385	1.295	1.224
Core region 3	1.355	1.274	1.230	1.271
Capture Complex	Individual		Polycore	

Table C2.3: Individual vs Polycore capture complexes Data Summary.

Mean O.D (448/690 nm)	Individual	Polycore
KN	0.155	0.143
Core region 1	1.761	1.912
Core region 2	1.555	1.497
Core region 3	1.351	1.255
S/Co	Individual	Polycore
Cut-off	0.355	0.343
Core Region 1	4.959	5.583
Core Region 2	4.379	4.370
Core Region 3	3.806	3.664

C.2.2. Titration of the individually prepared capture molecule.

Table C2.4: OD 448 nm/690 nm results for the titration of the individually prepared capture molecules. The individually prepared and coated complexes were coated at concentrations of 0.02 µg/ml, 0.04 µg/ml, 0.06 µg/m, and 0.08 µg/ml.

	Well Position	1	2	3	4
	KN	0.193	0.145	0.157	0.167
	KN	0.124	0.156	0.143	0.152
	Core region 1	0.123	0.129	0.131	0.142
	Core region 1	0.183	0.174	0.161	0.117
	Core region 2	1.571	1.333	1.115	0.871
	Core region 2	1.501	1.390	1.126	0.879
	Core region 3	1.498	1.241	1.151	0.899
	Core region 3	1.575	1.317	1.285	1.009
Capture Complex	Core Region 1	0.04 µg/ml	0.04 µg/ml	0.04 µg/ml	0.04 µg/ml
	Core Region 2	0.04 µg/ml	0.04 µg/ml	0.04 µg/ml	0.04 µg/ml
	Core Region 3	0.02 µg/ml	0.04 µg/ml	0.06 µg/ml	0.08 µg/ml

Table C.25: Data Summary of the titration of individually prepared core region 3 capture complexes.

	Mean O.D @ 448/690 nm			
	0.02 µg/ml	0.04 µg/ml	0.06 µg/ml	0.08 µg/ml
KN	0.156	0.151	0.148	0.145
Core Region 3	1.536	1.320	1.169	0.915
Cut Off	0.356	0.351	0.348	0.345
S/Co	4.318	3.761	3.360	2.655

Appendix C3: Testing of the improved capture and detection molecules

C3.1. Efficacy Testing:

Table C3.1: Plate layout used for the efficacy testing of the improved microtitre plate and conjugate and the microtitre plate and conjugate produced using the standard method of preparation.

Layout:												
	1	2	3	4	5	6	7	8	9	10	11	12
A	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN
B	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN
C	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN	KN
D	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture	QC: Antigen capture
E	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1	QC: Core Region 1
F	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2	QC: Core Region 2
G	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3	QC: Core Region 3
H	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein	QC: Non structural protein
	Current Method						Improved Method					
	Efficacy											

Table C.3.2: OD 448 nm/690 nm results for the efficacy testing of both the improved and control microtitre plate and conjugate.

Raw Data												
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.233	0.225	0.199	0.201	0.221	0.216	0.148	0.137	0.149	0.137	0.129	0.142
B	0.210	0.242	0.187	0.211	0.213	0.209	0.113	0.132	0.114	0.112	0.119	0.115
C	0.186	0.213	0.189	0.186	0.218	0.202	0.118	0.129	0.124	0.128	0.111	0.109
D	1.276	1.252	1.287	1.307	1.271	1.199	1.205	1.299	1.276	1.289	1.222	1.264
E	1.562	1.692	1.581	1.565	1.521	1.542	1.684	1.641	1.665	1.672	1.681	1.701
F	1.412	1.423	1.444	1.451	1.414	1.419	1.454	1.431	1.473	1.438	1.469	1.442
G	1.677	1.745	1.739	1.724	1.694	1.761	1.836	1.846	1.902	1.897	1.852	1.895
H	0.907	0.937	0.911	0.922	0.899	0.971	0.996	0.903	0.917	0.930	0.919	0.951
	Current Method						Improved Method					
	Efficacy											

Table C3.3: Summary of efficacy performance on both the improved and control microtitre plate and conjugate.

Efficacy				
	Mean O.D 448/690 nm		S/Co	
	Current Method	Improved Method	Current Method	Improved Method
Cut off	0.409	0.326		
Negative Control	*0.209*	*0.126*		
Quality Control - Antigen Capture	1.265	1.259	3.094	3.864
Quality Control - Core Region 1	1.577	1.674	3.857	5.137
Quality Control - Core Region 2	1.427	1.451	3.490	4.453
Quality Control - Core Region 3	*1.723*	*1.871*	*4.214*	*5.743*
Quality Control - Non-Structural	0.925	0.936	2.261	2.873

C3.2. Specificity: Testing of normal human serum (HCV negative) samples

A collection of 90 normal human serum samples was tested on the control (standard method) and test microtitre plate and conjugate (improved method of preparation).

Table C3.4: Plate layout used for testing of normal human serum samples on the standard control and test microplate and test conjugate containing detection antibody-horseradish peroxidase conjugate at a dilution of 1/500.

	1	2	3	4	5	6	7	8	9	10	11	12
A	KN	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
B	KN	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
C	KP Ag	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
D	KP Ag	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
E	KP Ab	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
F	KP Ab	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
G	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
H	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS	NHS
	Matched Plate and Conjugate											

Key:	
KN	Kit Negative Control
KP Ag	Antigen Positive Control
KP Ab	Antibody Positive Control
NHS	Normal Human Serum

Table C3.5: Side by side comparison of OD 448 nm/690 nm of the 90 normal human serum samples for both the standard and improved methods of synthesis.

	Improved	Current
1	0.169	0.183
2	0.179	0.200
3	0.149	0.230
4	0.150	0.502
5	0.146	0.170
6	0.170	0.202
7	0.153	0.205
8	0.160	0.175
9	0.145	0.164
10	0.160	0.166
11	0.221	0.272
12	0.145	0.316
13	0.129	0.259
14	0.159	0.170
15	0.143	0.156
16	0.141	0.171
17	0.141	0.184
18	0.144	0.168
19	0.236	0.222
20	0.165	0.226
21	0.140	0.217
22	0.132	0.151
23	0.189	0.192
24	0.150	0.167
25	0.128	0.151
26	0.142	0.164
27	0.192	0.207
28	0.144	0.198
29	0.135	0.209
30	0.132	0.157
31	0.143	0.178
32	0.154	0.178
33	0.128	0.141
34	0.147	0.163
35	0.132	0.166
36	0.141	0.169
37	0.136	0.162
38	0.132	0.158
39	0.147	0.175
40	0.131	0.148
41	0.144	0.142
42	0.150	0.147
43	0.150	0.170
44	0.149	0.173
45	0.121	0.165
46	0.131	0.168
47	0.160	0.165
48	0.121	0.143
49	0.146	0.167
50	0.158	0.179
51	0.148	0.192
52	0.134	0.171
53	0.125	0.166
54	0.226	0.164
55	0.125	0.170
56	0.131	0.169
57	0.257	0.192
58	0.143	0.165
59	0.227	0.249
60	0.127	0.185
61	0.132	0.160
62	0.171	0.199
63	0.125	0.158
64	0.121	0.148
65	0.125	0.148
66	0.152	0.138
67	0.152	0.175
68	0.136	0.175
69	0.123	0.149
70	0.126	0.152
71	0.128	0.162
72	0.129	0.149
73	0.144	0.155
74	0.155	0.165
75	0.172	0.176
76	0.158	0.159
77	0.148	0.162
78	0.157	0.172
79	0.150	0.154
80	0.147	0.154
81	0.221	0.177
82	0.319	0.145
83	0.160	0.168
84	0.159	0.171
85	0.205	0.218
86	0.128	0.153
87	0.162	0.163
88	0.142	0.156
89	0.2139	0.169
90	0.3014	0.195

C3.3. Limit of detection (LoD):

A dilution series of the antibody positive control was prepared using the Murex Ab/Ag Sample diluent and the antibody positive control. The dilution series was prepared as per the table below:

Table C3.6: Preparation of the dilutions of the antibody positive control, in the Murex Ab/Ag sample, for the testing of the freeze-dried detection antibody-horseradish peroxidase conjugates.

Volume of antibody positive control / well	Volume of sample diluent / well
1 µl	99 µl
2 µl	98 µl
4 µl	96 µl
6 µl	94 µl
8 µl	92 µl
10 µl	90 µl
12 µl	88 µl
14 µl	86 µl
16 µl	84 µl
18 µl	82 µl
20 µl	80 µl
22 µl	78 µl
24 µl	76 µl

The dilution series was tested as per the layout below:

Table C3.7: Plate layout used for the testing of the improved core region 3 detection molecule.

Plate Layout:								
	1	2	3	4	5	6	7	8
A	KN	Ab: 2 µl	Ab: 10 µl	Ab: 18 µl	KN	Ab: 2 µl	Ab: 10 µl	Ab: 18 µl
B	KN	Ab: 2 µl	Ab: 10 µl	Ab: 18 µl	KN	Ab: 2 µl	Ab: 10 µl	Ab: 18 µl
C	KN	Ab: 4 µl	Ab: 12 µl	Ab: 20 µl	KN	Ab: 4 µl	Ab: 12 µl	Ab: 20 µl
D	KN	Ab: 4 µl	Ab: 12 µl	Ab: 20 µl	KN	Ab: 4 µl	Ab: 12 µl	Ab: 20 µl
E	KN	Ab: 6 µl	Ab: 14 µl	Ab: 22 µl	KN	Ab: 6 µl	Ab: 14 µl	Ab: 22 µl
F	KN	Ab: 6 µl	Ab: 14 µl	Ab: 22 µl	KN	Ab: 6 µl	Ab: 14 µl	Ab: 22 µl
G	Ab: 1 µl	Ab: 8 µl	Ab: 16 µl	Ab: 24 µl	Ab: 1 µl	Ab: 8 µl	Ab: 16 µl	Ab: 24 µl
H	Ab: 1 µl	Ab: 8 µl	Ab: 16 µl	Ab: 24 µl	Ab: 1 µl	Ab: 8 µl	Ab: 16 µl	Ab: 24 µl
	Current Method				Improved Method			

Table C3.8: OD 448 nm/690 nm results for testing of the antibody positive control dilution series on the both the improved and control microtitre plate and conjugate.

Raw Data:								
	1	2	3	4	5	6	7	8
A	0.214	0.213	0.405	0.697	0.132	0.302	0.471	0.774
B	0.187	0.204	0.415	0.683	0.134	0.291	0.493	0.769
C	0.212	0.219	0.478	0.714	0.139	0.341	0.593	0.812
D	0.185	0.231	0.471	0.720	0.149	0.337	0.597	0.827
E	0.196	0.259	0.547	0.769	0.140	0.374	0.648	0.873
F	0.195	0.312	0.527	0.782	0.147	0.362	0.679	0.867
G	0.209	0.305	0.629	0.844	0.267	0.458	0.728	0.902
H	0.199	0.302	0.646	0.863	0.254	0.439	0.725	0.915
	Current Method				Improved Method			

Table C3.9: Summary of results showing the mean OD 448 nm/690 nm and S/CO values for both the improved and control microtitre plate and conjugate.

Limits of Detection (LoD)				
	Current Method		Improved Method	
	Mean O.D	S/Co	Mean O.D	S/Co
KN	0.198		0.140	
Cut off	**0.398**		**0.340**	
Ab: 1 µl	0.204	0.512	0.261	0.766
Ab: 2 µl	0.209	0.524	0.297	0.872
Ab: 4 µl	0.225	0.565	0.339	0.997
Ab: 6 µl	0.286	0.717	0.368	1.082
Ab: 8 µl	0.304	0.762	0.449	1.318
Ab: 10 µl	0.410	1.029	0.482	1.417
Ab: 12 µl	0.475	1.192	0.595	1.749
Ab: 14 µl	0.537	1.349	0.664	1.951
Ab: 16 µl	0.638	1.602	0.727	2.136
Ab: 18 µl	0.690	1.733	0.772	2.268
Ab: 20 µl	0.717	1.801	0.820	2.409
Ab: 22 µl	0.776	1.948	0.870	2.558
Ab: 24 µl	0.854	2.144	0.909	2.671

C3.4. Accelerated Stability testing of the improved manufacturing method:

Stability of the improved method was assessed by conducting efficacy testing over a period of seven weeks. A control (standard method of preparation) was tested alongside this.

Table C3.10: Accelerated stability testing schedule.

Stability testing occasion at 37 ^{o} C (Acceleration factor = 8)		Replicates
T=0	Time zero	x 5
Week 1	represents 8 weeks at 2-8^{o}C	x1
Week 2	represents 16 weeks at 2-8^{o}C	x1
Week 3	represents 24 weeks at 2-8^{o}C	x1
Week 4	represents 32weeks at 2-8^{o}C	x1
Week 5	represents 40 weeks at 2-8^{o}C	x1
Week 6	represents 48 weeks at 2-8^{o}C	x1
Week 7	represents 56 weeks at 2-8^{o}C	x 5

Table C3.11: Plate layout used for stability testing.

	1	2
A	KN	KN
B	KN	KN
C	KP Ab	KP Ab
D	KP Ab	KP Ab
E	QC Core Region3	QC Core Region3
F	QC Core Region3	QC Core Region3
G	NHS	NHS
H	NHS	NHS
	Test Combination	**Control Combination**

Key:	
KN	Kit Negative Control
KP Ab	Antibody Positive Control
QC Core Region 3	QC Core Region 3
NHS	Normal Human Serum

Table C3.12: OD 448 nm/690 nm results for stability testing of the control (standard method) and improved microtitre plate/conjugate throughout the stability programme.

	Accelerated Stability Data: Improved method Vs Current Manufacturing Method																			
T=0																				
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control										
KN	0.129	0.131	0.111	0.098	0.158	0.170	0.189	0.187	0.114	0.155										
KN	0.119	0.123	0.200	0.143	0.139	0.171	0.124	0.130	0.130	0.148										
AB Positive Control	1.681	1.520	1.449	1.238	1.681	1.520	1.591	1.497	1.430	1.428										
AB Positive Control	1.611	1.579	1.283	1.337	1.611	1.579	1.471	1.436	1.605	1.544										
QC Core Region3	1.302	1.401	1.125	1.367	1.302	1.401	1.239	1.319	1.361	1.373										
QC Core Region3	1.503	1.498	1.571	1.354	1.503	1.498	1.305	1.256	1.401	1.222										
NHS	0.139	0.129	0.139	0.240	0.141	0.168	0.183	0.187	0.214	0.191										
NHS	0.177	0.180	0.177	0.168	0.181	0.161	0.164	0.197	0.201	0.151										
	T=0, Replicate 1		T=0, Replicate 2		T=0, Replicate 3		T=0, Replicate 4		T=0, Replicate 5											
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control								
KN	0.106	0.209	0.153	0.152	0.122	0.135	0.121	0.155	0.139	0.169	0.148	0.148								
KN	0.126	0.134	0.114	0.201	0.140	0.141	0.148	0.148	0.158	0.148	0.146	0.163								
AB Positive Control	1.511	1.451	1.509	1.492	1.459	1.401	1.304	1.267	1.209	1.281	1.214	1.149								
AB Positive Control	1.433	1.428	1.599	1.557	1.444	1.427	1.345	1.289	1.252	1.273	1.209	1.193								
QC Core Region3	1.196	1.369	1.258	1.239	1.249	1.203	1.199	1.296	1.111	1.100	1.009	1.000								
QC Core Region3	1.386	1.300	1.364	1.286	1.291	1.298	1.201	1.123	1.071	1.202	1.002	1.113								
NHS	0.129	0.122	0.187	0.208	0.206	0.214	0.229	0.194	0.203	0.221	0.196	0.201								
NHS	0.207	0.114	0.238	0.193	0.223	0.221	0.217	0.229	0.215	0.212	0.229	0.247								
	W=1		W=2		W=3		W=4		W=5		W=6									
WK=7																				
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control										
KN	0.128	0.157	0.186	0.157	0.137	0.163	0.145	0.137	0.121	0.161										
KN	0.121	0.151	0.159	0.122	0.136	0.187	0.117	0.123	0.097	0.178										
AB Positive Control	1.002	1.028	1.025	1.036	1.030	1.012	1.108	1.098	1.086	1.024										
AB Positive Control	1.067	1.034	1.110	1.014	1.019	1.014	1.023	1.093	1.035	0.985										
QC Core Region3	1.001	0.992	1.008	0.890	0.982	0.876	0.982	0.873	0.945	0.966										
QC Core Region3	0.987	0.989	0.969	0.987	0.971	1.003	0.985	0.863	0.953	0.816										
NHS	0.236	0.235	0.195	0.217	0.203	0.321	0.188	0.225	0.201	0.207										
NHS	0.164	0.213	0.193	0.170	0.189	0.127	0.188	0.209	0.201	0.207										
	W=7, Replicate 1		W=7, Replicate 2		W=7, Replicate 3		W=7, Replicate 4		W=7, Replicate 5											

Table C3.13: Summary of results showing the mean OD @ 448 nm/690 nm and sample to cut off (S/CO) was calculated per sample on each of the occasions.

Summarised Accelerated Stability Data								
	Improved Method							
	T=0	W=1	W=2	W=3	W=4	W=5	W=6	W=7
Improved Method: KN	0.141	0.116	0.133	0.131	0.135	0.149	0.147	0.135
Improved Method: AB Positive Control	1.541	1.472	1.554	1.452	1.325	1.231	1.212	1.051
Improved Method: QC Core Region3	1.493	1.291	1.311	1.270	1.200	1.091	1.006	0.978
Improved Method: NHS	0.171	0.168	0.213	0.215	0.223	0.209	0.212	0.196
Improved Method S/Co	0.341	0.316	0.333	0.331	0.335	0.349	0.347	0.335
Improved Method S/Co: AB Positive Control	4.516	4.655	4.661	4.382	3.958	3.530	3.489	3.138
Improved Method S/Co: QC Core region 3	4.373	4.083	3.933	3.834	3.586	3.130	2.896	2.922
	Control							
	T=0	W=1	W=2	W=3	W=4	W=5	W=6	W=7
Control: KN	0.146	0.171	0.177	0.138	0.151	0.159	0.155	0.153
Control:AB Positive Control	1.468	1.440	1.525	1.414	1.278	1.277	1.171	1.034
Control:QC Core Region3	1.369	1.335	1.263	1.251	1.210	1.151	1.057	0.926
Control:NHS	0.177	0.118	0.200	0.217	0.212	0.217	0.224	0.213
Control S/Co	0.346	0.371	0.377	0.338	0.351	0.359	0.355	0.353
Control S/Co: AB Positive Control	4.246	3.875	4.049	4.185	3.638	3.558	3.294	2.925
Control S/Co: QC Core region 3	3.960	3.593	3.353	3.701	3.443	3.207	2.972	2.619
Improved Method % Decline: Ab Positive Control	-30.503							
Control % Decline: Ab Positive Control	-31.1014							
Improved Method % Decline: QC Core Region 3	-33.174							
Control Method % Decline: QC Core Region 3	-33.864							