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**Development of molecular and serological assays  
for diagnosis and surveillance of Crimean-Congo  
haemorrhagic fever virus**

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Danelle Pieters

May 2015

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# **Development of molecular and serological assays for diagnosis and surveillance of Crimean-Congo haemorrhagic fever virus**

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B. Med. Sc. Hons.

**Submitted in fulfilment of the requirements in respect of the M. Med. Sc. Degree qualification Medical Virology in the Department of Medical Microbiology and Virology in the Faculty of Health Sciences at the University of the Free State**

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## **Declaration**

I, Danelle Pieters, certify that the dissertation hereby submitted for the M.Med.Sc Medical Virology qualification at the University of the Free State is my independent effort and has not previously been submitted for a qualification at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.



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Danelle Pieters

## Abstract

Crimean-Congo haemorrhagic fever virus (CCHFV) an arthropod-borne virus associated with haemorrhagic disease in humans. The global distribution of CCHFV correlates with that of ticks from the *Hyalomma* genus. CCHFV infection is diagnosed by detection of viral nucleic acid using reverse-transcription polymerase-chain-reaction (RT-PCR) or other molecular assays, by virus isolation from infected cell culture or suckling mouse brain or by detection of anti-CCHFV antibodies using enzyme-linked immunosorbent assay (ELISA) or immunofluorescence assay (IFA). High biocontainment facilities are required for virus isolation and preparation of whole virus native antigen for use in serological assays. Currently, treatment is limited to supportive therapy. CCHFV is currently emerging and re-emerging in many regions, which emphasize the requirement for safe, reliable and inexpensive assays to increase diagnostic capacity and monitor emergence of the virus.

A nucleic acid sequence-based amplification (NASBA) molecular assay for detection of CCHFV ribonucleic acid (RNA) was developed. The assay can be performed without the requirement for sophisticated laboratory equipment. A commercially available enzyme mixture and buffer were compared with a more cost effective and easier to obtain in-house enzyme mixture and amplification buffer. Specificity of the NASBA assays were determined by testing viral RNA extracted from Vero cell culture infected with genetically diverse southern African CCHFV strains. A total of 41/48 samples tested were positive. Sensitivity of the NASBA assays was determined using dilutions of viral RNA and transcribed RNA to detect minimal copy number that could be amplified. The NASBA assay was able to detect at least 3.7 RNA copies. Diagnostic application of the NASBA assays was investigated by amplifying RNA extracted from clinical samples and the results compared with two commercial real-time RT-PCR assays. A total of 20/22 samples tested positive using the NASBA whereas the commercially available assays were able to amplify 22/22 samples. Subsequently, the inhibitory effect of sera on the amplification of CCHFV RNA using the NASBA assay was investigated using sera spiked with transcribed RNA.

Two expression systems were investigated for the expression of recombinant CCHFV nucleocapsid protein (NP) for use in serological assays. The baculovirus expression system was initially investigated. The open reading frame of the S segment of a CCHFV strain was codon optimized for expression in insect cells. A pFastBac HT B transfer vector containing the



optimized CCHFV NP gene was prepared and used to transform DH10Bac™ *Escherichia coli* cells to transpose the optimized CCHFV NP gene to a bacmid. The recombinant bacmid was utilized to transfect *Spodoptera frugiperda* 9 cells. The cell lysates were analysed, however, no expression of the CCHFV NP could be confirmed. A mammalian expression system was subsequently investigated. A pcDNA™ 3.1D/V5-His-TOPO.CCHFV.NP construct was used to transfect baby hamster kidney-21 cells. Expression of CCHFV NP was detected in transiently transfected cells using IFA and serum collected from a convalescent CCHFV patient.

To profile the immune response against CCHF viral proteins, 15 sera collected from convalescent patients at various times after onset of illness were tested for antibody against CCHFV NP and glycoproteins (GP) using commercially available slides. The antigen slides were prepared from transfected cells expressing recombinant CCHFV NP and GP. Antibody against CCHFV GP and NP were detected in all samples. End point titers of anti-CCHFV NP and GP were determined for two serum samples. Commercially available slides are expensive and therefore have limited application for testing large numbers. Application of in-house antigen slides prepared from transfected cells expressing CCHFV NP were tested using IFA and 14 sera collected from convalescent CCHFV patients. All sera tested positive, suggesting that preparation of a stable cell line expressing CCHFV NP is warranted for application in detection of antibody against CCHFV.

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## List of Abbreviations

× g – times gravity

°C – degrees Celsius

ℓ – litre

μg – microgram

μℓ – microlitre

μM – micromolar

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AcNPV – *Autographa californica* nuclear polyhedrosis virus

AMV RT – avian myeloblastosis virus reverse transcriptase

BHK – baby hamster kidney

BLAST – Basic Local Alignment Search Tool

bp – base pair

BSA – bovine serum albumin

BSL-4 – biosafety level 4

CCHF – Crimean-Congo haemorrhagic fever

CCHFV – Crimean-Congo haemorrhagic fever virus

cDNA – complementary DNA

CTP – cytidine triphosphate

DIG – digoxigenin

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleoside triphosphate

DTT – dithiothreitol

*E. coli* – *Escherichia coli*

ELISA – enzyme-linked immunosorbent assay

EOC – enzyme-linked oligonucleotide capture

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

G<sub>C</sub> – glycoprotein matured when cleaved from C-terminal of GPC

G/C – GTP and CTP

GFP – green fluorescent protein

G<sub>N</sub> – glycoprotein matured when cleaved from N-terminal of GPC

GP – glycoproteins

GPC – glycoprotein precursor

GTP – guanosine triphosphate

h – hour

HAZV – Hazara virus

His – histidine

HRPO – horse-radish peroxidase

IC – internal control

IFA – immunofluorescence assay

IgG – immunoglobulin G

IgM – immunoglobulin M

IL – interleukin

IPTG – isopropyl-thiogalactoside

kDa – kiloDaltons

L – large segment

LB – Luria Bertani	RE – restriction endonuclease
LB/amp – LB media with ampicillin	RNA – ribonucleic acid
M – molar	RNase H – ribonuclease H
Me – medium segment	rpm – revolutions per minute
mg – milligram	RT-LAMP – reverse transcription loop-mediated isothermal amplification
MgCl <sub>2</sub> – magnesium chloride	RT-PCR – reverse transcriptionPCR
min – minutes	RVFV – Rift Valley fever virus
mℓ – millilitre	s – seconds
mM – millimolar	S – small segment
MOI – multiplicity of infection	SA – South Africa
mRNA – messenger RNA	SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Na <sub>2</sub> EDTA – disodium ethylenediaminetetraacetate	<i>Sf</i> – <i>Spodoptera frugiperda</i>
NASBA – nucleic acid sequence-based amplification	<i>Sf9</i> – <i>Spodoptera frugiperda</i> 9
NEAA – nonessential amino acids	SKI-1 – subtilisin/kexin-isozyme-1
Neg - negative	SNF – supernatant fluid
NFW – nuclease free water	<i>spp.</i> – species
ng – nanogram	SPU – Special Pathogens Unit
NICD – National Institute for Communicable Diseases	STAT-1 – signal transducer and activator of transcription-1
nm – nanometer	TAE – tris-acetate-disodiummethylenediaminetetraacetate
NP – nucleocapsid protein	TBS – tris-buffered saline
ORF – open reading frame	TBS-T – 1× TBS containing 1% Tween® 20
p/s – penicillin and streptomycin mixture	TEMED – tetramethylethylenediamine
PBS – phosphate buffered saline	U/μℓ – units/microliter
PBS-T – 1× PBS containing 1% Tween® 20	USA – United States of America
PCR – polymerase chain reaction	V – Volts
pmol – picomole	VBD – Vector-Borne Disease
Pos – positive	YFV – yellow fever virus
PVDF – Polyvinylidene fluoride	

# Chapter 1 – Introduction and literature review

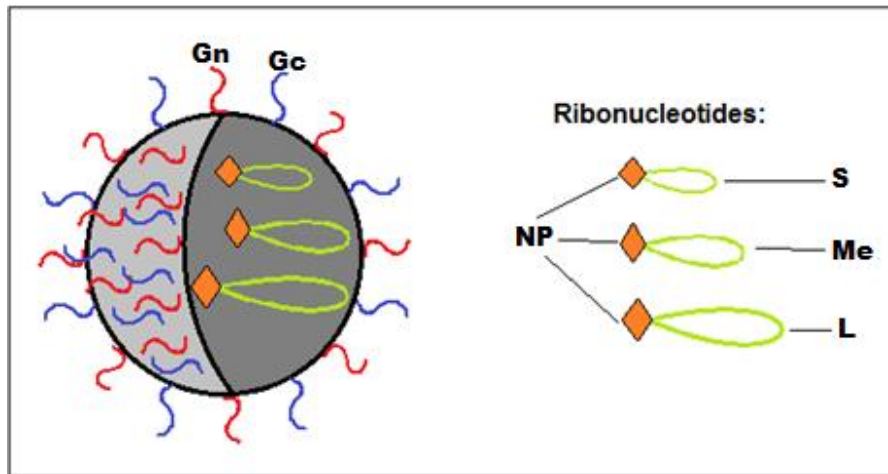
## 1.1. History of the virus and introduction

The first evidence of Crimean-Congo haemorrhagic fever (CCHF) dates back to Tajikistan in the 12<sup>th</sup> century, where a disease accompanied by severe haemorrhage and an arthropod associated with this disease was described (Hoogstraal, 1979). However, in modern times Crimean haemorrhagic fever was first described in Crimea when  $\pm$  200 military personnel became infected during 1944 at the end of World War II. The causative virus was first isolated in 1967. It became evident that this virus was indistinguishable from the virus isolated in 1956 in Belgian Congo, current Democratic Republic of the Congo, and the combined name Crimean-Congo haemorrhagic fever virus was adopted (Casals, 1969; Hoogstraal, 1979).

The first case of CCHFV was identified in South Africa in 1981, when a schoolboy contracted the disease following a tick bite while attending “veldschool” in Bloemhof. Serological surveys were subsequently performed to determine the prevalence of the virus in the country and it was confirmed that the virus was widely distributed in South Africa and had been present for many years prior to 1981 (Swanepoel et al, 1983; Swanepoel et al, 1985). There have been 219 laboratory confirmed cases in southern Africa up to December 2014, which includes 197 from South Africa and 22 from Namibia (Msimang et al, 2013; personal communication J Paweska and J Weyer). The case fatality rate in southern Africa is 24%. The majority of cases (45.2%) resulted from tick bite or squashing ticks and 38.4% occurred in patients who had contact with fresh blood or other tissues of livestock and/or ticks. Only 3.6% of cases have resulted from nosocomial infections and 12.2% had no direct evidence of contact but patients lived in or visited a rural area unknown (information provided by JT Paweska and J Weyer). The majority of cases occurs in males and is frequently associated with occupational exposure, particularly farmers and farm workers. Three species of *Hyalomma* ticks are found in South Africa, namely *H. marginatum rufipes*, *H. glabrum*, and *H. truncatum* and serve as vectors of the virus in nature. These are two host ticks with immature ticks feeding on hares and ground feeding birds, with adult ticks feeding on a variety of larger wild and domestic herbivores (Shepherd et al, 1987). The ticks are distributed in the central and western areas of South Africa with two of the three species absent in the eastern and southern coastal areas. The tick distribution correlates with the distribution of human cases and antibody determined in cattle herds from serological surveillance (Burt et al, 1993).

## 1.2. Virus classification and characteristics

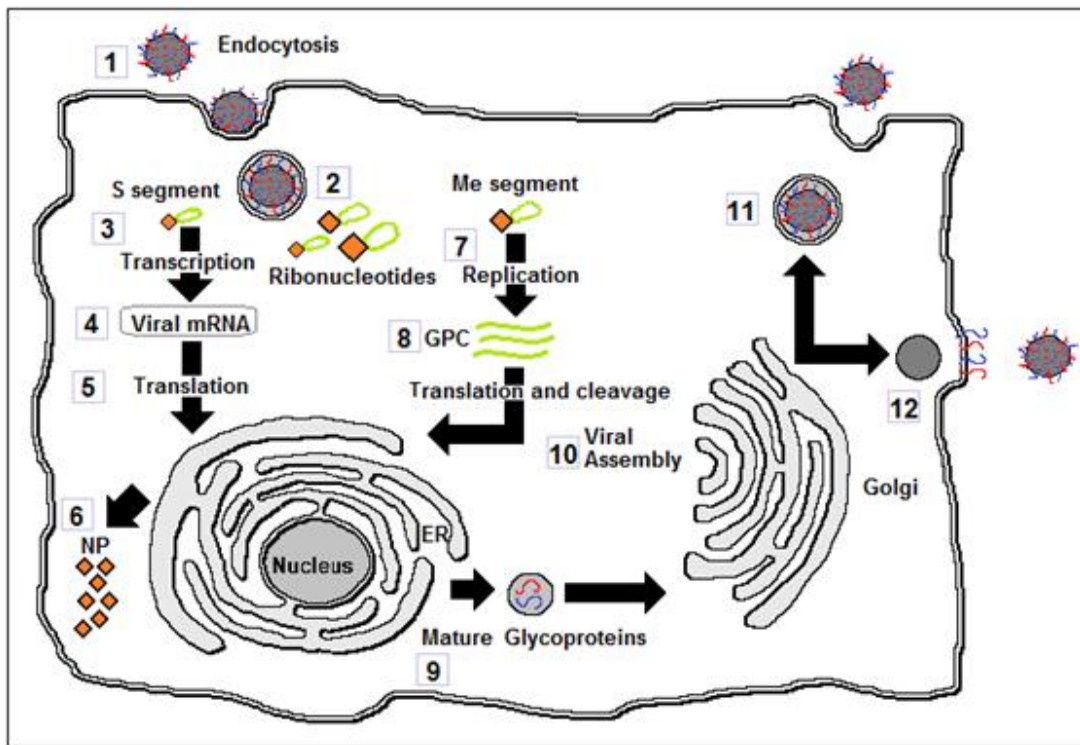
CCHFV is a member of the *Bunyaviridae* family which is comprised of enveloped viruses with single-stranded, negative sense, tripartite RNA genomes (Casals, 1969; Hoogstraal, 1979). The *Bunyaviridae* family is divided into five genera; *Orthobunyavirus*, *Hantavirus*, *Phlebovirus*, *Nairovirus* and *Tospovirus* (Elliot, 1997). CCHFV is classified within the *Nairovirus* genus, which is divided into seven groups. The classification was originally based on the antigenic relationships and supported more recently with the genetic relationships. The seven groups consist of 34 described viruses of which only three are known to be human pathogens (Schmaljohn and Nichol, 2007), CCHFV, Dugbe virus, that causes mild febrile disease and thrombocytopaenia, and Nairobi sheep disease virus, which causes fever and haemorrhagic gastroenteritis (Burt et al, 1996; Schmaljohn and Nichol, 2007). CCHF virions are spherical, with diameters of  $\pm 90$  nanometer (nm), and surrounded by a lipid envelope, derived from the host cell, that contains the viral encoded glycoproteins (GP) (Marriott and Nuttal, 1996). The GP are spike-like structures which attach to host cell receptors and likely induce neutralizing antibodies (Flick and Whitehouse, 2005; Schmaljohn and Nichol, 2007). The tripartite genome consists of a small (S), medium (Me) and large (L) segment and is illustrated in Figure 1.1. These segments encode the NP, glycoprotein precursor (GPC) and RNA-dependent RNA polymerase, respectively (Schmaljohn and Nichol, 2007). The segments all contain complementary nucleotide regions at the ends, which are highly conserved within the *Bunyaviridae* family. These complementary regions lead to the formation of closed circular RNAs due to non-covalent intra-strand interaction. The circular RNA segments provide functional promoter regions for the viral encoded polymerase and are complexed with the viral encoded NP to form ribonucleocapsids (Schmaljohn and Nichol, 2007).



**Figure 1.1. Schematic representation of virion and genome ribonucleocapsids.**

$G_N$  &  $G_C$  – mature GP, NP – nucleocapsid protein, S – small segment ribonucleotide, Me – medium segment ribonucleotide, L – large segment ribonucleotide

In order to replicate, the virus gains entry to the host cell by endocytosis when the mature GP ( $G_N$  and  $G_C$ ) on the surface of the spherical virus particle recognize and attach to receptor sites on the host cell (Schmaljohn and Nichol, 2007). Replication of the viral genome occurs in the cytoplasm of the host cell (Schmaljohn and Nichol, 2007) and is illustrated in Figure 1.2. The replication strategies of members of the *Bunyaviridae* family are similar (Schmaljohn and Nichol, 2007). Fusion of the viral membrane with the cytoplasmic vesicle membrane releases the ribonucleocapsid segments into the cytoplasm, where transcription of the S segment is initiated. The resultant viral mRNAs are capped and translated to NP. When sufficient NP is present, replication of the other viral segments commences. This process is mediated by the virally encoded RNA-dependent RNA polymerase (Schmaljohn and Nichol, 2007). The large GPC polyprotein is processed to yield the mature  $G_N$  and  $G_C$  GP. The precursor protein is co-translationally cleaved by signalase in the endoplasmic reticulum to yield pre- $G_N$  and pre- $G_C$  molecules. The cellular protease subtilisin/kexin-isozyme-1 (SKI-1) is responsible for the proteolytic cleavage that yield the N-terminus of the mature  $G_N$  glycoprotein. The specific protease involved in the cleavage of the pre- $G_C$  glycoprotein to yield the mature  $G_C$  glycoprotein is yet to be identified.



**Figure 1.2. Schematic representation of viral replication in the host cell.**

1- Endocytosis; 2- Release of viral ribonucleotides after fusion of viral membrane with cytoplasmic vesicle; 3- Initiation of S segment transcription; 4- Resulting mRNAs are capped; 5- Translation of capped mRNAs in endoplasmic reticulum; 6- Sufficient amount of NP to initiate viral replication via viral polymerase; 7- Replication of Me segment is highlighted in the figure; 8- GPC (mRNA of Me segment) is translated and cleaved within the ER; 9- Mature GP are localised to Golgi apparatus; 10- Viral assembly commences in the Golgi apparatus; 11- Mature virions bud from Golgi vesicles through plasma membrane; 12- Alternative budding of virions through plasma membrane, where mature GP are anchored in the host cell plasma membrane.

The mature GP are localized to the Golgi apparatus, where viral assembly occurs (Schmaljohn and Nichol, 2007). The virions possibly mature by budding into cytoplasmic vesicles in the Golgi region and bud from the Golgi vesicles out of the host cell when these vesicles fuse with the plasma membrane of the host cell. Alternatively, the budding site is possibly defined by the retention of the GP at a particular site in the plasma membrane of the host cell (Schmaljohn and Nichol, 2007).

RNA viruses usually have relatively high rates of mutation because their polymerases are extremely prone to error (Holland et al, 1998). However, arthropod-borne RNA viruses usually

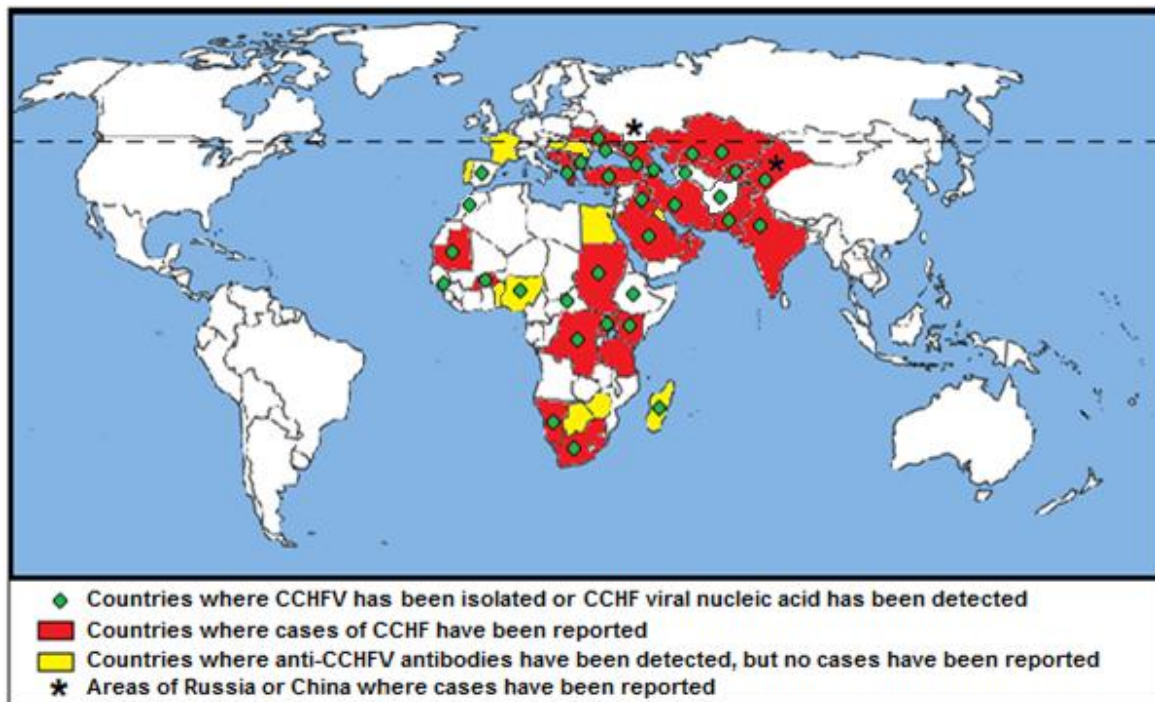
have lower rates as these viruses have to obtain fitness in order to survive within the arthropod host and the amplifying vertebrate host. In the case of CCHFV, a high level of genome plasticity has been observed, with 20%, 31% and 22% difference within the nucleotide sequences of the S, Me and L segments of different strains, respectively. Difference within the amino acid sequences of the NP, GP and RNA-dependent RNA polymerase of different strains has been observed as 8%, 27% and 10%, respectively (Deyde et al, 2006). The important motifs and the lengths of both the RNA segments and the open reading frames (ORFs) remain highly conserved, suggesting that CCHFV can tolerate moderate diversity within the genome and proteins whilst maintaining high fitness in both arthropod and vertebrate hosts (Deyde et al, 2006). The Me segment has been shown to have the highest rate of mutation accumulation. As the Me segment encodes the components by which the virus gains entry into host cells, it can be suspected that this region undergoes various mutations in order to adjust the GP so that it would be able to attach to both specific arthropod and specific vertebrate cells. Commonly, the L segment encoding the viral polymerases of RNA viruses are the most highly conserved regions, but in the case of CCHFV the L segment has higher rates of mutation than the S segment. Therefore, the S segment is the most conserved genome segment and the encoded NP the most conserved protein of CCHFV (Hewson et al, 2004; Deyde et al, 2006). The global diversity of CCHFV strains is apparent through the eight groups into which the strains are categorized on the basis of the phylogenetic relationships of the segments' sequences. Seven groups were initially proposed as Africa 1, Africa 2, Africa 3, Asia 1, Asia 2, Europe 1 and Europe 2 (Chamberlain et al, 2005) and was adapted to group I – West Africa, group II – Democratic Republic of the Congo, group III – South Africa and West Africa, group IV – Asia and the Middle East, group V – Europe and Turkey, group VI – Greece and group VII – Mauritania by Deyde et al (2006). More recent isolates from China do not cluster within the existing groups and appear to represent a new group (Zhou et al, 2013). These groupings demonstrate that certain CCHFV strains have travelled over great distances, as indicated by similarities between geographically distinct isolates in groups III and IV for example. These similarities could be due to the movement of CCHFV-infected ticks carried on livestock or CCHFV-infected livestock or migration of birds carrying CCHFV-infected ticks. The different segments of a CCHFV strain can be classified in different groups, which could be due to RNA segment reassortment or recombination (Deyde et al, 2006). RNA segment reassortment has been observed in the *Bunyaviridae* in the *Orthobunyavirus* genus where Bunyamwera virus and Batai virus reassorted resulting in Ngari virus, which is associated

with outbreaks of haemorrhagic fever in regions of Africa (Gerrard et al, 2004). Reassortment has been observed for CCHFV with a higher frequency of Me segment reassortment than with the S and L segments. This could merely indicate that reassortment of the Me segment results in high fitness viable virus more frequently than S or L segment reassortment (Hewson et al, 2004; Deyde et al, 2006; Burt et al, 2009; Kondiah et al, 2010). It was proposed that the S and L segments of one virus tend to end up together in the same novel particle during reassortment between S segments or between L segments as there apparently is a strong interrelationship between the encoded NP and RNA-dependent RNA polymerase (Chamberlain et al, 2005). Reassortment probably occurs during co-infection of ticks as viraemia of vertebrate hosts is short-lived in comparison to the long-term infection observed in ticks. Ticks feed on more than one host during their life cycle which could contribute to reassortment (Hewson et al, 2004; Deyde et al, 2006). Homologous recombination has been observed in RNA viruses. In CCHFV this has only been observed within the S segment and there is currently insufficient evidence to support recombination in the Me or L segment. The observed recombination events of the S segment involve only short genome regions (Deyde et al, 2006).

### **1.3. Global distribution**

The worldwide distribution of CCHFV correlates with that of *Hyalomma spp.* ticks (Hoogstraal, 1979). CCHFV has the greatest distribution of all tick-borne viruses currently known and is still emerging in previously naïve regions of the world (Ergönül, 2006). The virus circulates between ticks and various small and large vertebrate animals which act as hosts to the ticks (Hoogstraal, 1979). Various strains of CCHFV have been identified which seem to be restricted to the specific geographical locations they were originally isolated from (Burt and Swanepoel, 2005; Ergönül, 2006). Figure 1.3 is a schematic presentation of the worldwide distribution of CCHFV, based on the references that follow in this section. Countries where the presence of CCHFV was evident in the form of virus isolation from ticks or livestock, or detection of anti-CCHFV antibodies in surveillance studies of humans or livestock were included even though the disease has not been diagnosed in that country.





**Figure 1.3. Schematic representation of the global distribution of CCHFV.**

Map indicates areas where CCHFV was isolated from a tick, animal or human patient and detection of antibodies during surveillance of humans and/or animals. The stippled line indicates the 50° parallel north, which is the northern distribution limit of *Hyalomma spp.* ticks.

Since the initial identification of CCHFV, human cases have been reported from South Africa, Namibia, Democratic Republic of the Congo, Tanzania, Kenya, Sudan, Uganda, Burkina Faso, Mauritania, Albania, Kosovo (formerly part of Yugoslavia), Yugoslavia, Bulgaria, the former Soviet Union, Kazakhstan, Uzbekistan, Turkmenistan, Tajikistan, Armenia, Azerbaijan, China, Pakistan, Iran, Iraq, Oman, United Arab Emirates and Saudi Arabia (Simpson et al, 1967; Woodall et al, 1967; Hoogstraal 1979; Burney et al, 1980; Suleiman et al, 1980; Tantawi et al, 1980; Al-Tikriti et al, 1981; Gear et al, 1982; Saluzzo et al, 1984; Saluzzo et al, 1985; Yu-Chen et al, 1985; Swanepoel et al, 1987; Watts et al, 1989; Schwarz et al, 1995; El Azazy et al, 1997; Hassanein et al, 1997; Dunster et al, 2002; Papa et al, 2002a; Papa et al, 2002b; Papa et al, 2002c; Nabeth et al, 2004a; Nabeth et al, 2004b). In addition to the previous mentioned countries the presence of CCHFV was evident through virus isolation or detection of viral nucleic acid from ticks or animals in Madagascar, Ethiopia, Central African Republic, Nigeria, Senegal, Morocco, Greece and Afghanistan (Causey et al, 1970; Wood et al, 1978; Hoogstraal, 1979; Mathiot et al,

1988; Watts et al, 1989; Palomar et al, 2013) and through serological evidence in Botswana, Zimbabwe, Egypt, Portugal, France, Hungary, Benin and Kuwait (Hoogstraal, 1979; Blackburn et al, 1982; Al-Nakib et al, 1984; Filipe et al, 1985; Swanepoel et al, 1987; Watts et al, 1989).

Since 2002 CCHFV has emerged in Turkey with more than 7 000 CCHF cases reported thus far (Maltezou et al, 2010a), in Greece the first case of CCHF was reported in 2008 (Papa et al, 2008), in 2011 the first CCHF case in India was reported (Mishra et al, 2011; Patel et al, 2011) and serological evidence of CCHFV was recently detected in Romania (Ceianu et al, 2012). CCHFV was isolated from adult *Hyalomma lusitanicum* ticks that were collected from red deer in Spain and was shown to be genetically similar to strains circulating in Africa through phylogenetic analysis (Estrada-Pena et al, 2010). Subsequently, a CCHFV isolate from ticks that were collected from migratory birds in Morocco was found to be identical to isolates from Sudan and Mauritania and showed 98.9% identity with the isolate from Spain (Palomar et al, 2013). Even though no CCHF cases have been reported to date, CCHFV could emerge in regions of south-western Europe that are currently non-endemic where competent vector species are present. The emergence of CCHFV in several Balkan countries raises concerns that the virus could expand its current distribution and establish new endemic foci (Maltezou et al, 2010b; Maltezou and Papa, 2010). CCHFV has re-emerged in south-western regions of the Russian Federation in 1999 after an absence of 27 years. Re-emergence and emergence could be influenced by global warming resulting in changed weather patterns which in turn influences the tick populations. Additionally, increased livestock trading, changes in farming practices and land development could influence CCHFV re-emergence and emergence (Randolph and Rogers, 2007; Maltezou et al, 2010b; Maltezou and Papa, 2010).

#### **1.4. Vectors and hosts**

CCHFV has been isolated from several ixodid ticks, but as the distribution of CCHFV correlates with the spread of *Hyalomma spp.* ticks, shown in Figure 1.4, these ticks are considered to be the main vector of this virus (Hoogstraal, 1979). The virus has been isolated from more than 30 different tick species, but it is important to note that isolating virus from a tick does not necessarily indicate that the specific species is a vector, but could be due to having a recent blood meal on a CCHFV-infected animal. In cases like these further experiments are necessary to determine whether or not the tick species could indeed be a vector (Hoogstraal, 1979).



**Figure 1.4.**Image of a *Hyalomma spp.* tick.

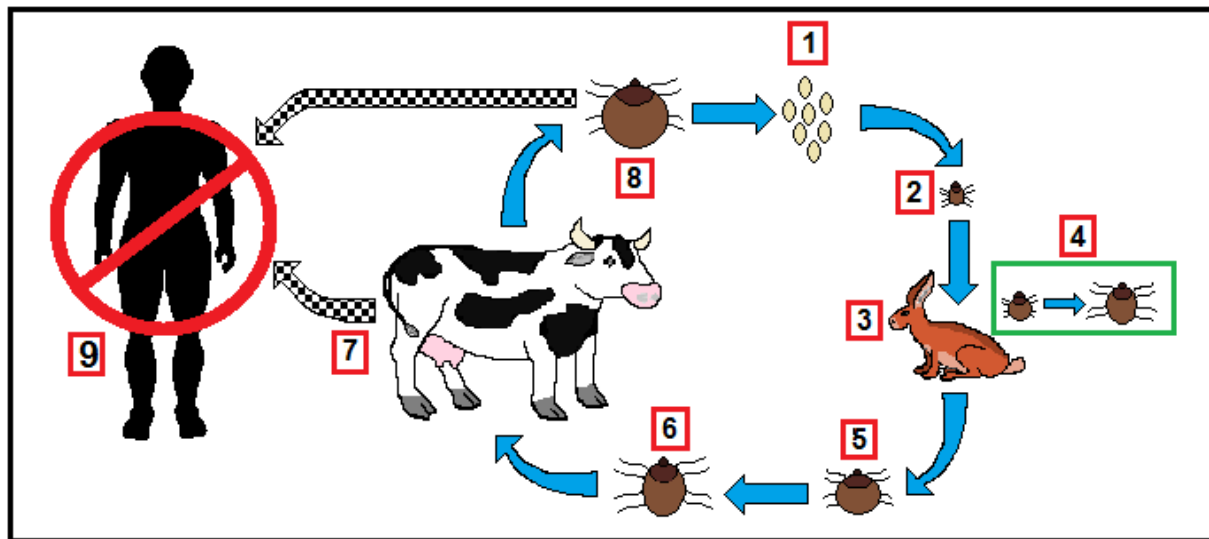
*Hyalomma spp.* ticks have typical white and brown bands on their legs.

Ticks become infected whilst feeding on viraemic hosts and can subsequently transmit the virus to a second host (Hoogstraal, 1979). Transovarial transmission (transmission from infected female to eggs) of CCHFV is not considered to occur sufficiently to maintain the viral life cycle without an amplification mechanism. Therefore the infection of immature ticks is a more important amplification mechanism than the subsequent infection of the adult ticks on the second hosts (Hoogstraal, 1979). The preferred hosts of the larvae and nymphs are small mammals; like hares, hedgehogs or ground dwelling birds (Hoogstraal, 1979; Shepherd et al, 1987b; Van Niekerk et al, 2006); while adult ticks prefer to feed on large vertebrates; like cattle, zebras, African buffaloes, eland antelope, rhinoceros, ostriches and giraffes (Hoogstraal, 1979; Burt et al, 1993). CCHFV causes viraemia that can last for about a week in animals with no evidence of resulting disease from the infection. Clinical disease as a result of natural CCHFV infection has only been reported in humans, while laboratory infection of suckling mice is fatal (Hoogstraal, 1979).

The role of birds in the viral life cycle is not yet clear. CCHFV positive ticks have been recovered from birds proven to be negative for virus, antigen or antibodies (Hoogstraal, 1979; Shepherd et al, 1987a; Jameson et al, 2012). With the exception of ostriches, experimental infection of birds has proven unsuccessful which might indicate that many birds are refractory to CCHFV infection (Hoogstraal, 1979). However, the phenomenon of non-viraemic transmission of CCHFV to *Hyalomma marginatum rufipes* while attached to ground feeding birds has been demonstrated, which might indicate that some bird species may play a role in amplifying the virus (Jones et al, 1987; Zeller et al, 1994). Ostriches, which are readily infected with CCHFV by ticks and can become highly viraemic for up to a week, may act as amplifying hosts (Swanepoel et al, 1998).

### **1.5. Lifecycle of Crimean-Congo haemorrhagic fever virus in nature**

Once a tick is infected by CCHFV, it remains infected for the remainder of its life (Mardani and Keshtkar-Jahromi, 2007). There are three stages in the life cycle of ixodid ticks; larvae, nymphs and adult ticks. Most *Hyalomma* species behave as two-host ticks, where the larvae and nymph stages attach to and feed on the same host after which the satiated nymphs detach to molt into the adult ticks that feed on a second host. Transovarial and transstadial transmission (transmission from one infected instar to the next instar in the tick life cycle) are included in the tick-vertebrate-tick cycle which involves quite a variety of wild and domestic animals that act as hosts (Hoogstraal, 1979). The full life cycle of *Hyalomma spp.* ticks is shown in Figure 1.5. The female adult tick drops off from the host to lay eggs, subsequently initiating new tick life cycles. These vectors can be infected by CCHFV at different stages; as hatched larvae on the first host, as nymphs on the first host, as adults on the second host or the eggs can be infected transovarially by the infected female. The second hosts are usually large vertebrates, either domestic or wild livestock, which act as amplifying hosts (Hoogstraal, 1979; Burt et al, 2007). It is important to note that ticks not only act as vectors in the life cycle of CCHFV, but also as hosts (Whitehouse, 2004).



**Figure 1.5. The lifecycle of CCHFV in nature.**

1 – Eggs laid by a female tick, can be infected transovarially; 2 – Hatched larvae attach to and feed on small animals, like; 3 – hares, hedgehogs or ground dwelling birds; 4 – Engorged larvae molt into nymphs on the initial host, where the nymphs continue to feed (if the larva was infected whilst feeding on the initial host, the nymph will be infected transstadially); 5 – Engorged nymphs drop off the initial host to molt into adult ticks (6) (if the nymph was infected whilst feeding on the initial host, the adult tick will be infected transstadially); 6 – Adult ticks attach to and feed on large vertebrate, like; 7 – cattle, zebra, buffalo, etc. The second host is also the location where male and female ticks mate; 8 – Engorged female ticks drop off to lay their eggs, thus initiating new life cycles; 9 – Humans can be infected through tick bite or contact with infected blood or tissue from infected livestock, as they do amplify the virus they are seen as dead-end hosts

Humans are infected accidentally. They do not act as amplifying hosts and are considered as dead-end hosts. Humans acquire CCHFV through a bite from CCHFV-infected tick, squashing a CCHFV-infected tick between the fingers or from direct contact with infected blood or tissue from animals or human patients to broken skin or mucus membranes (Hoogstraal, 1979). The incubation period range from 1 – 3 days after infection by tick bite and 5 – 9 days after infection by infected blood or tissue (Hoogstraal, 1979).

Climate has an influence on the emergence of CCHFV as it influences various aspects of the life cycle of the vectors and hosts (Ergönül, 2006; Estrada-Peña et al, 2012; Jameson et al, 2012). Animal movement and trade have been shown to influence the global distribution of the virus (El-Azazy and Scrimgeour, 1997; Khan et al, 1997; Rodriguez et al, 1997; Whitehouse, 2004;

Ergönül, 2006). Migratory birds, especially ground-feeding birds, may play a role in the distribution of *Hyalomma spp.* ticks and even in the introduction of these ticks to non-endemic regions (Hoogstraal et al, 1961; Hoogstraal et al, 1963; Hoogstraal, 1979; Palomar et al, 2013). The potential for ticks to survive in the new surroundings is mostly dependent on the climate conditions and availability of appropriate hosts (Estrada-Peña et al, 2012; Jameson et al, 2012).

## **1.6. Prevention and control**

People who are at risk are those with occupations exposing them to large domestic animals such as farmers and farm workers in endemic areas, abattoir workers, veterinarians and hospital and laboratory staff (Hoogstraal 1979; Ozkurt et al, 2006; Burt et al, 2007). No definite gender- or age-specific association has been made. Precautions that can be taken by veterinarians, farmers, farm workers and abattoir workers include wearing gloves and other protective clothing and/or tucking trousers into boots or socks to decrease the risk of exposing naked skin to ticks or infected blood or tissue (Hoogstraal, 1979). Human clothing can be treated by pyrethroid preparations to kill ticks or just be impregnated in permethrin to reduce the risk of tick bites (Swanepoel et al, 1998). These precautions, unfortunately, are not always practical. People travelling near areas that are endemic for *Hyalomma spp.* ticks associated with CCHFV should regularly examine their clothes and skin for ticks, try not to squash the ticks whilst removing and make use of insect repellents. People living in endemic areas should be aware of any possible exposure and try to take the necessary precautions (Flick and Whitehouse, 2005; Ergönül, 2006). Livestock treated with acaricide have been shown to effectively reduce the number of ticks on the animals (Flick and Whitehouse, 2005). Surveillance, by IgG detection, of CCHFV infection in livestock or wildlife can be done to monitor further spread of the virus to non-endemic areas as a result of trade or other type of movement of the animals or migratory birds (Burt et al, 1993, Burt et al, 1994; Maltezou et al, 2010b). Barrier-nursing techniques can be exercised by medical staff involved in treatment of CCHFV infected patients. Patients can be isolated in a room with negative pressure (Hoogstraal, 1979; Ergönül, 2006) and can be given general supportive therapy (Ergönül, 2006; World Health Organization). Ribavirin, a guanosine analog shown to be promising especially when administered at day four of illness, is the only current form of treatment (Tignor and Hanham, 1993; Ergönül et al, 2004; Keshtkar-Jahromi et al; 2011). The efficacy of this drug is not completely clear as contradicting evidence has been found in the few studies conducted on this matter (Fisher-Hoch et al, 1995; Mardani et al, 2003; Ergönül et al,

2004; Smego et al, 2004; Izadi and Salehi, 2009; Keshtkar-Jahromi et al; 2011; Ceylan et al, 2013). Duygu et al (2011) have shown that diverse CCHFV strains respond differently to ribavirin *in vivo*. In certain instances, patients have received intramuscular or intravenous anti-CCHF immunoglobulin, but this form of treatment has not been fully evaluated with respect to administration of a standardized product with known neutralizing antibody titers and should be further evaluated to determine the efficacy (Keshtkar-Jahromi et al; 2011; Kubar et al, 2011). Currently there are no approved vaccines available for treatment. There is, however, a vaccine derived from inactivated mouse brain currently in use in Bulgaria, but it is not available outside the country due to the method of preparation and the efficacy is not well quantified (Hoogstraal, 1979; Papa et al, 2004; Keshtkar-Jahromi et al; 2011).

### **1.7. Infection course and clinical presentation**

CCHF usually presents as febrile illness with haemorrhage from multiple sites, however the course of illness likely depends on the immune response of the host, which differs from one person to the next (Hoogstraal, 1979; Swanepoel et al, 1987; Swanepoel et al, 1989). CCHF usually occurs in four stages: incubation, pre-haemorrhagic, haemorrhagic and convalescence (Hoogstraal, 1979). The incubation period is usually short, about 3 – 7 days, depending on viral dose and route of exposure (Hoogstraal, 1979, Swanepoel et al, 1989). Sudden onset of illness occurs during the pre-haemorrhagic period, with symptoms like headache; high fever (39°C - 41°C) lasting 4 – 5 days; chills; back-, joint- and stomach pain; dizziness; sore eyes and photophobia. Malaise, myalgia, nausea, vomiting, sore throat and loss of appetite occur early during illness in many cases (Hoogstraal, 1979; Swanepoel et al, 1987; Swanepoel et al, 1989). In some cases mood changes occur over the first 2 days of illness, when patients experience confusion and aggression outbreaks (Swanepoel et al, 1987). The haemorrhagic period is commonly short, about 2 – 3 days, and develops very quickly (Hoogstraal, 1979; Mardani and Keshtkar-Jahromi, 2007). By day 3 – 6 of illness a petechial rash may appear on the trunk and limbs, usually followed by large bruises, Figure 1.6.



**Figure 1.6. Petechial rash on the arms of a patient infected with CCHFV.**

Picture courtesy of Prof. Burt.

In severe cases patients are extremely prone to bleeding from the nose, gastrointestinal tract, uterus and urinary tract and the respiratory tract (Hoogstraal, 1979; Swanepoel et al, 1989). Hepatorenal and pulmonary failure can be expected from day 5 of illness and onward (Hoogstraal, 1979; Swanepoel et al, 1989). Atypical bleeding has also been observed (Ergönül, 2006). The mortality rate is 3 – 30%, with deaths occurring between days 5 – 14 of illness (Hoogstraal, 1979; Ergönül, 2006; Burt et al, 2007; Vorou et al, 2007; Yilmaz et al, 2009). The mortality rates coupled to nosocomial infections are usually higher (Whitehouse, 2004; Ergönül, 2006). Patients that do recover start to improve from day 9 – 15 of illness, when the infection is in the convalescent stage. However, some symptoms like slight confusion, asthenia, conjunctivitis, amnesia, tachycardia, temporary complete hair loss and loss of hearing may continue for a month or even longer (Hoogstraal, 1979; Swanepoel et al, 1989; Schwarz et al, 1997; Bray, 2007; Burt et al, 2007).

### **1.8. Pathogenesis**

CCHFV has the ability to disable the host immune response by attacking and manipulating the cells that initiate the antiviral response, a pathogenic feature shared with other haemorrhagic fever viruses. Infection of host endothelium cells plays a key role in the pathogenesis of CCHFV. Damage to the endothelium contributes to haemostatic failure when degranulation and

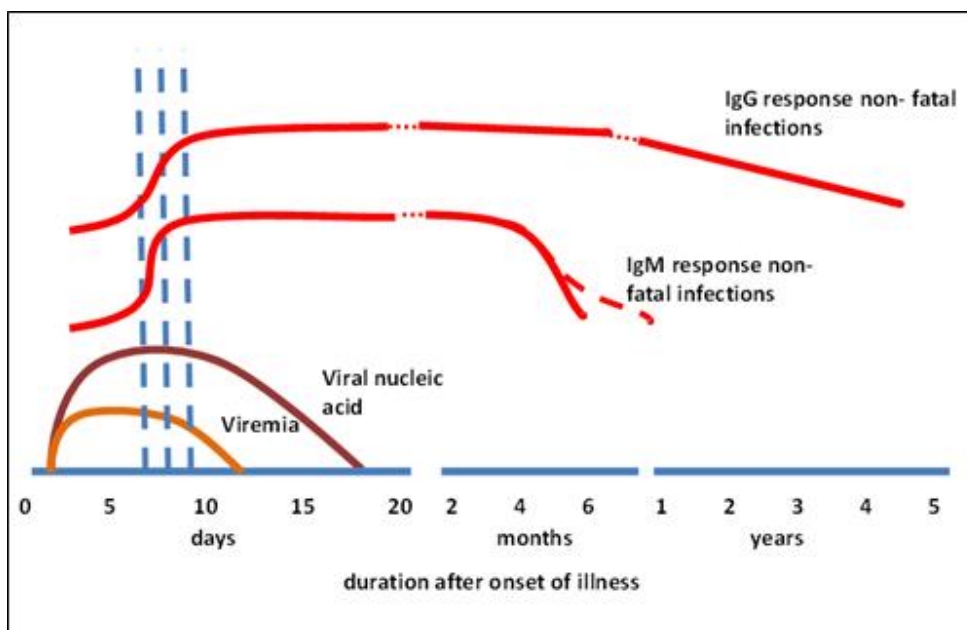


platelet aggregation are stimulated, accounting for the characteristic rash and having the consequence of activating an intrinsic coagulation cascade. Certain cytokines that are secreted by T<sub>helper</sub>1 cells may participate in other pathogenetic aspects of this virus (Ergönül, 2006; Schmaljohn and Nichol, 2007). The viral GP play an important role in the pathogenesis of the virus as they facilitate viral adherence to host cells (Schmaljohn and Nichol, 2007). Following viral entry, the virus replicates in dendritic cells and other local tissues. This is followed by migration to the local lymph nodes and dissemination through the lymph and blood monocytes to the liver, spleen and other tissues and/or organs. The infected macrophages from the different tissues and/or organs result in the secondary infection of the permissive parenchymal cells (Chen and Cosgriff, 2000; Geisbert and Jarhling, 2004). Even though lymphocytes may not be infected during the course of illness, they can still be killed in vast numbers by apoptosis – as seen in other forms of septic shock (Chen and Cosgriff, 2000; Geisbert and Jarhling, 2004). Impaired haemostasis necessitates endothelial cell, platelet and/or coagulation factor dysfunction (Burt et al, 1997; Chen and Cosgriff, 2000; Geisbert and Jarhling, 2004). These events may largely be due to infected monocytes and macrophages releasing cytokines, chemokines and other pro-inflammatory mediators, like interleukin (IL)-1 and IL-6, tumour necrosis factor-alpha, etc., (Ergönül, 2006). CCHFV infection seems to mostly affect the hepatocytes (Burt et al, 1997). Very little is known otherwise about the pathogenesis of this virus as it requires biosafety level 4 (BSL-4) facilities to study the virus, most cases occur in regions where there are limited clinical pathology facilities and suitable small mammalian models, a signal transducer and activator of transcription-1 (STAT-1) knockout mouse model and a type 1 interferon receptor-knockout mouse model, have only recently been identified (Bente et al, 2010; Bereczky et al, 2010). Hazara virus (HAZV), the closest relative to CCHFV in the *Nairovirus* genus, has been proposed as an alternative model to study CCHFV pathogenesis, as handling of HAZV does not require BSL-4 facilities (Flusin et al, 2011). Further studies are required to clearly understand the pathogenesis of this virus. Cerebral haemorrhage, severe dehydration, severe anaemia, shock associated with diarrhoea, lung oedema, myocardial infarction and pleural effusion are additional factors that contribute to the high fatality risk. Multiple organ failure is frequently the cause of death (Ergönül et al, 2004).

## 1.9. Diagnosis

Rapid diagnosis is essential to prevent nosocomial outbreaks and transmission of the virus in the community (Ergönül, 2006). Therefore clinicians require an accurate history (Whitehouse, 2004; Mardani and Keshtkar-Jahromi, 2007). Handling of samples that might contain viable virus requires high biological containment laboratories, usually BSL-4 (Flick and Whitehouse, 2005; Ergönül, 2006). CCHF should be distinguished from other tick-borne infections like borreliosis, leptospirosis and rickettsiosis, and other viral haemorrhagic fevers that are prevalent in the same suspected area (Hoogstraal, 1979; Burt, 2011).

Sudden onset of febrile illness (usually within a week after possible exposure) can be indicative of CCHF. The occurrence of leucopenia or leucocytosis, thrombocytopenia and elevated levels of alanine transaminase and aspartate transaminase early in the course of disease can be supportive evidence of CCHFV infection (Ergönül, 2006; Burt et al, 2007). Figure 1.7. illustrates the various stages in the course of CCHFV infection and application of tests will depend on the stage of illness.



**Figure 1.7. Diagnosis through course of infection (Burt, 2011).**

*Figure 1, Laboratory diagnosis of Crimean-Congo haemorrhagic fever virus infections, Future Virology, Vol. 6, No. 7, Pages 831-841*

Due to the biohazardous nature of the virus it is classified as a class four pathogen and culturing and handling the virus is performed within the confines of a BSL-4 facility. In South Africa there is one such facility within the Center for Emerging and Zoonotic Pathogens at the National Institute for Communicable Diseases. Tests performed at this laboratory for confirmation of infection during the acute phase of include detection of viral nucleic acid in serum samples using molecular techniques, or by isolation of the virus in cell culture or from inoculation of suckling mice (Burt, 2011). There are several molecular assays currently available for detection of CCHF viral nucleic acid (Burt, 2011) and most target the gene encoding the nucleocapsid, the most conserved gene. During the acute phase of illness viral nucleic acid can be detected for up to 16 days after the onset of illness via conventional or real-time RT-PCR. These methods are highly specific and sensitive rapid tests and can be used to determine the viral load (Burt et al, 1998; Burt et al, 2011). The first CCHFV real-time RT-PCR was based on SYBRGreen detection of the amplicons (Drosten et al, 2002), but as SYBRGreen is an interchelating detection reagent it would detect any double-stranded deoxyribonucleic acid (DNA). Therefore, more specific methods were designed to detect the amplicons in real-time RT-PCR in the form of detection probes. A TaqMan-probe-based one step real-time RT-PCR was developed for the detection and quantification of CCHFV RNA (Yapar et al, 2005) and, similarly, a FRET-probe-based assay was developed for the diagnosis of CCHFV strains circulating within the Balkan region only, evading the problems associated with high strain variation (Duh et al, 2006). With real-time RT-PCR one can quantify the viral load, which can subsequently be used as a predictor of infection, where a viral load greater than  $1 \times 10^8$  RNA copies per millilitre (mL) plasma can be considered to predict a fatal outcome (Cevik et al, 2007; Duh et al, 2007; Mustafa et al, 2007; Papa et al, 2007; Wölfel et al, 2007). A TaqMan-minor groove binding assay was developed to accommodate genetic diversity by using smaller probes (Garrison et al, 2007). A Simple-Probe® real-time polymerase chain reaction (PCR) assay was designed in order to distinguish between reassorted and non-reassorted CCHFV isolates in southern Africa (Kondiah et al, 2010). These methods are of great importance for diagnosing CCHFV infection in fatal cases, as these patients usually fail to develop adequate antibody responses against the virus, rendering serological tests useless because of undetectable antibody levels (Burt et al, 1994; Burt et al, 1998). The amplicons can be used to determine the partial sequence of the viral genome and the genetic relationship to other strains of the virus. Molecular methods have the advantages that nucleic acid can be detected directly from field-collected ticks (Whitehouse, 2004) and are safer to use as

there is no need to culture live virus. There are other molecular methods available like the low density macro-array designed to detect a broad range of CCHFV strains using multiple probes. This technique implements a biotinylated primer mix and visualization of the hybridized probes are done through a colourimetric assay. This method has the advantages that it can be used by laboratories with less sophisticated equipment and it allows basic genotyping of the isolates (Wölfel et al, 2009). A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for detection of the CCHFV S segment RNA was recently developed (Osman et al, 2013). A combination of six primers is used to amplify the target region at 63°C and can be visualized with the naked eye, thereby evading the use of expensive and sophisticated equipment. The sensitivity of the RT-LAMP assay was the same as for a nested RT-PCR, making this assay a helpful tool for rapid diagnosis of acute infection. Another method is a nucleic acid sequence-based amplification (NASBA) assay which is based on the amplification of conserved regions of the viral genome using complementary oligonucleotide primers, probes and three enzymes, RNase H, a reverse transcriptase and T7 RNA-dependent RNA polymerase, at a constant temperature (Compton, 1991). This method has the advantage that it can be implemented by laboratories which lack thermocyclers, however, there has yet to be a NASBA developed for the diagnosis of CCHFV.

Viral antigen can be detected in serum samples taken during the acute phase by direct enzyme-linked immunosorbent assay (ELISA) (Jacobson, 1998; Burt et al, 2007) (Figure 1.7). Detection of viral antigen is not as sensitive as virus isolation or RT-PCR and is infrequently used. Virus can be isolated during the acute phase from either inoculated susceptible cell cultures or intracerebral inoculated day-old mice in BSL-4 facilities (Ergönül, 2006; Burt et al, 2007; Burt, 2011). Virus can be detected and identified, after proliferation in cell cultures, by the use of IFA with specific monoclonal or polyclonal antibodies. Isolation can be accomplished within 1 – 5 days. CCHFV causes little to no cytopathic effects, thereby necessitating the use of IFA to identify the virus. Even though isolation of virus from infected mice takes 5 – 9 days, the mouse inoculation method is more sensitive for the isolation of virus present at low amounts. Inoculated mice sicken or die and the virus can be identified from the brain of the mice by indirect IFA (Ergönül, 2006; Burt et al, 2007; Burt, 2011).

In convalescent samples, collected from non-fatal cases, the diagnosis is confirmed by demonstration of an immune response using ELISA or indirect IF tests. Patients with fatal infections frequently do not develop a demonstrable antibody response. In most non-fatal cases

immunoglobulin M (IgM) antibody can be detected as early as day 5 (Tang et al, 2003) of illness and IgG antibody can be detected from days 7 – 9 of illness by IFA and ELISA (Burt et al, 1994; Jacobson, 1998). In some instances antibodies have been detected as early as day 2 after the onset of illness (Personal communication, Burt). Most currently used ELISA methods, especially for IgM detection, make use of a capture method which often necessitates the use of polyclonal antibodies raised in animals (Shepherd et al, 1989; Burt et al, 1993; Tang et al, 2003; Saijo et al, 2005; Garcia et al, 2006). ELISA (direct or indirect) can make use of recombinant NP to detect antibodies against CCHFV (Marriott et al, 1994; Saijo et al, 2002b, Tang et al, 2003; Saijo et al, 2005; Garcia et al, 2006; Samudzi et al, 2012), which evades the problem of culturing live virus in BSL-4 facilities. Both IgM and IgG are present in the sera of all survivors by day 9 of illness. In non-fatal cases the IgM activity declines to undetectable levels in  $\pm$  4 months, but it has been detected for up to 12 months (Burt et al, 2011). IgG activity declines gradually and can still be detected up to 12 years after infections (Samudzi et al, 2012). Recent or current infection can be indicated by IgM activity within a single sample;  $\geq$  fourfold increase in IgG activity or seroconversion within paired samples (Burt et al, 2007; Burt, 2011). It should be noted that false-positives are often detected with indirect ELISA due to the presence of rheumatoid factor, an anti-IgG antibody of the IgM family, and pre-treatment of samples are necessary in instances like pregnancy, rheumatoid-arthritis and during acute phase of some viral infections where rheumatoid factor might be circulating (Dowall et al, 2012). In patients with a fatal outcome, CCHF can be confirmed by virus isolation, detection of viral nucleic acid in serum samples or liver samples taken post mortem by PCR, or by demonstrating CCHFV antigen by immunohistochemical techniques on paraffin-embedded liver sections (Burt et al, 1997).

#### **1.10. Novel assays and non-infectious reagents for diagnosis and detection**

Due to the emergence of CCHFV and the threat of spread in eastern Europe, diagnostic tools requiring less sophisticated equipment and high containment facilities for preparation may play an important role in increasing diagnostic and surveillance capacity.

The NASBA assay was developed in 1991 (Compton, 1991) and has since been used in the design of rapid diagnostic tests for various medically significant pathogens (Kievits et al, 1991; Lanciotti and Kerst, 2001; Keightley et al, 2005; Lau et al, 2006; Lau et al, 2008; Loens et al, 2008; Mugasa et al, 2009; Boulet et al, 2010; Lau et al, 2010), but there has yet to be a NASBA assay designed to detect CCHFV nucleic acid. Briefly in a NASBA, the initial primer attaches to

the 3' end of the target region on the RNA template. The RNA template is reverse transcribed by a reverse transcriptase, after which ribonuclease H (RNase H) degrades the RNA in the newly formed RNA/complementary DNA (cDNA) hybrid. The second primer (modified to include sequence coding for a T7 promoter) binds to the 5' end of the cDNA and T7 RNA-dependent RNA polymerase produces RNA strands complementary to the cDNA. These RNA strands initiate new cycles of the amplification, rendering the amplification reaction cyclic. The amplification product can be detected using a real-time detection probe, electrochemiluminescence or with optical detection using enzyme-linked oligonucleotide capture (EOC) (Lau et al, 2006). As NASBA does not require a thermocycler, it can be seen as a rather inexpensive, rapid and safe method for detecting viral nucleic acid with possible field use.

Serological assays are essential tools to detect anti-CCHFV antibodies during the convalescent phase for diagnosis or surveillance purposes. These techniques utilize native or recombinant antigens to detect antibodies against the specific virus in serum samples. Native antigens are prepared from culturing live virus, which requires BSL-4 facilities. Recombinant antigens are produced by manipulation of a viral gene and expression in susceptible cell lines. Recombinant antigens have advantages and disadvantages compared to native antigens. Recombinant proteins can be manufactured in laboratories without high biocontainment facilities as this process does not predispose the laboratory worker to possible infection, however, factors like incorrect folding of the protein and/or decreasing stability of the expressed protein over a period of time could lead to the recombinant proteins being non-functional and impractical.

There are various expression systems available that can be used to express a recombinant antigen and the desired system is selected depending on the desired outcome. Bacterial expression systems produce extremely high yields, but because of their prokaryotic properties they cannot perform post-translation modifications. These modifications are necessary for the correct folding of the protein and the absence thereof might cause the epitopes to be positioned incorrectly. Without the correct positioning of the epitopes the recombinant protein may not function as the native antigen functions in serological tests and hence requires biological testing. In our laboratory recombinant CCHFV NP have been expressed using bacterial expression systems and utilized to detect anti-CCHFV antibodies in human sera either via indirect ELISA and western blot (Samudzi et al, 2012; Rangunwala et al, 2014). However, the bacterially expressed recombinant CCHFV NP was unstable and high level background signals were observed in

ELISA and western blot likely due to antibodies directed against *Escherichia coli* (*E. coli*) present in human sera.

Mammalian systems possess the necessary post-translation abilities and the recombinant protein will thus be folded correctly, but the product yield is very low. Therefore, the large scale production of the recombinant protein can be expensive and laborious. However, mammalian cells can be transfected with an expression vector containing the target gene and a resistance to an antibiotic like neomycin, which allow the selection of transfected cells. Subsequently, a stable cell line can be prepared that will continuously express the desired recombinant protein, which can be used to prepare antigen slides for IFA. Recombinant CCHFV NP has been expressed continuously in a HeLa cell line for preparation of antigen slides utilized in indirect IFA assays and expressed from a recombinant Semliki Forest alphavirus replicon in BSR cells, a clone of baby hamster kidney (BHK)-21 cells for detecting anti-CCHFV antibodies in ELISA (Saijo et al, 2002a; Garcia et al, 2006).

Baculovirus systems can be placed as an intermediate between bacterial and mammalian expression systems as they produce average yield and possess the ability to perform some post-translational modifications on the expressed protein. This system utilizes insect cells to express the recombinant protein which is significant as the vectors of CCHFV are insects (Marino, 1989; Hodgson, 1993). Recombinant CCHFV NP has been expressed from *Spodoptera frugiperda* (*Sf*) 21 insect cells and from Tn5 insect cells using a baculovirus expression system and used to detect anti-CCHFV antibodies via ELISA in a limited number of sera (Marriott et al, 1994; Saijo et al, 2002b; Tang et al, 2003; Saijo et al, 2005).

### **1.11. Problem identification**

CCHFV is an emerging threat in non-endemic regions of south-eastern Europe and southern Asia (Papa et al, 2008; Maltezou et al, 2010a; Patel et al, 2011), and a re-emerging threat in endemic areas, as in south-western regions of the Russian Federation (Maltezou et al, 2010a; Maltezou and Papa, 2010). Rapid diagnosis is important for isolation of the patient and for protection of healthcare and laboratory workers. Due to the high fatality rate accompanied by the propensity to cause human-to-human and laboratory infections and the absence of any specific vaccine or treatment other than administration of ribavirin there are only a limited number of laboratories with adequate biocontainment facilities for culturing live virus and preparing laboratory reagents. For these reasons the virus can be seen as a potential bioterrorism agent (Borio et al, 2002; Deyde

et al, 2006). In order to increase the diagnostic and surveillance capacity it is important to produce safe, reliable and inexpensive methods that can be used in less sophisticated laboratories. Molecular assays play a key role in rapid detection of CCHFV during the acute phase of illness and hence the development of novel molecular assays provides additional options for diagnostic laboratories. Serological assays are important for detection of antibody responses during the convalescent phase and for surveillance purposes. Recombinant antigens that do not require high biocontainment facilities for preparation could play an important role in development of serological tests.

## **Aim**

The aim of this study was to develop a novel simple molecular assay for detecting CCHF viral nucleic acid and to produce recombinant CCHFV NP for development of safe assays for detecting anti-CCHFV antibodies.

## **Objectives**

The objectives of this study were to

1. design and optimize a NASBA assay for CCHFV,
2. evaluate the NASBA for viral nucleic acid detection of South African isolates of CCHFV,
3. express recombinant CCHFV NP in insect cells or mammalian cell lines and
4. evaluate the recombinant CCHFV NP as an antigen for detection of antibody against CCHFV.

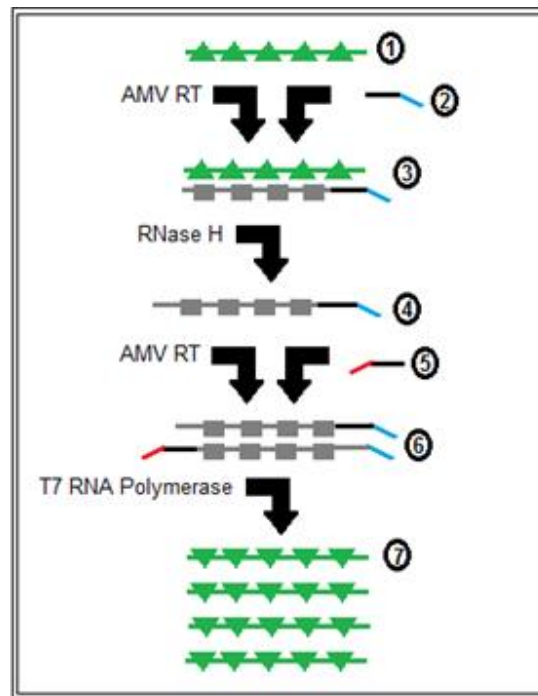


## **Chapter 2 – Development and validation of a nucleic acid sequence-based amplification assay for detection of Crimean-Congo haemorrhagic fever viral RNA**

### **2.1. Introduction**

Early diagnosis of CCHF is essential in order to isolate the patient, avoid subsequent infection of healthcare workers and for early administration of supportive therapy and/or ribavirin. Currently confirmation of acute CCHFV infection is diagnosed through isolation of virus, detection of viral RNA by RT-PCR or other molecular techniques, or detection of antigen by ELISA. Virus isolation can only be performed in BSL-4 facilities, whereas detection of viral antigen or RNA can be done with minimal BSL facilities after the serum or plasma samples have been inactivated at 56°C for 30 minutes (min) or following the lysis step of nucleic acid extraction. Detection of antigen is dependent on the time after onset that the sample was collected as viraemia occurs for less than 10 days, depending on the specific individual. However, this technique is seldom used as it lacks sensitivity (Burt, 2011). Viral RNA has been detected from day 1 to day 18 but more frequently during the acute phase up to days 7 – 9 after onset (Burt, 2011). Molecular techniques employ consensus primers to amplify a conserved region of the target genome. The S segment of the CCHFV is the most conserved region of the genome (Hewson et al, 2004; Deyde et al, 2006) and is the target of most molecular assays. Most of the molecular techniques available for detection of CCHF viral RNA require expensive thermocyclers, however there are few techniques that can be employed by laboratories with less sophisticated equipment, like a water bath or heating block. A NASBA assay is an isothermal amplification that employs three enzymes simultaneously and can be done using a water bath or heating block. As a NASBA assay does not require a thermocycler, it can be seen as a rather inexpensive, rapid, sensitive and safe alternative method for detecting viral nucleic acid. The NASBA assays that have been fully validated for viral nucleic acid detection of avian influenza virus, foot-and-mouth disease virus, Newcastle disease virus, classical swine fever virus and porcine reproductive and respiratory syndrome virus have shown sensitivity equal to or higher than that of real-time RT-PCR (Lau et al, 2006). To date there has yet to be a NASBA assay designed to amplify and detect CCHF viral RNA. A NASBA assay utilizes a reverse transcriptase, RNase H and T7 RNA polymerase.

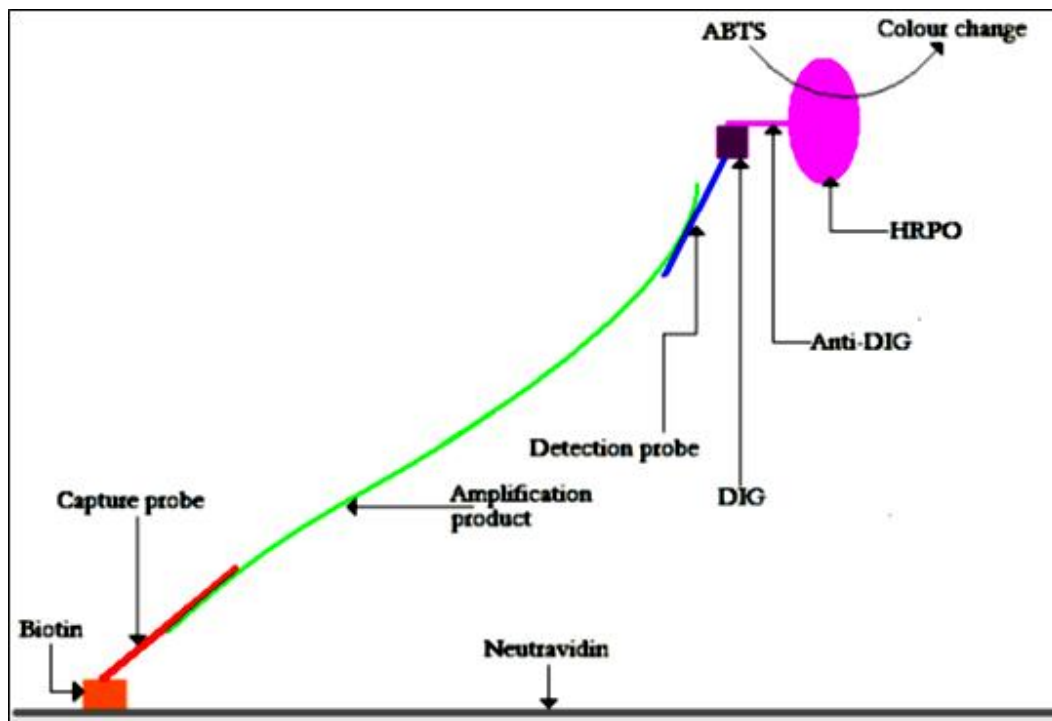
The sequences of the primers are modified to include specific sequences. The T7 promoter sequence has to be included in one of the primers as it is required in the amplification process. It is important to note that the T7 promoter sequence is active in double-stranded form. The various detection methods available for a NASBA assay require specific sequences to be included in the other primer. Figure 2.1. illustrates the concept of a NASBA assay incorporating the T7 promoter sequence in the reverse primer and the EOC capture probe sequence in the forward primer and explains the need for the three enzymes. The EOC detection method is the



**Figure 2.1. A schematic illustration of a NASBA assay.**

Target RNA (1) is reverse transcribed by reverse transcriptase after attachment of the reverse primer coupled with T7 promoter site (2), resulting in a cDNA/RNA hybrid (3). RNase H hydrolyses the RNA from the cDNA (4). The forward primer coupled with the capture sequence (5) attaches to the cDNA and is elongated by reverse transcriptase, resulting in double-stranded cDNA (6). As the T7 promoter site is in active form T7 RNA polymerase amplifies antisense RNA (7). Note that any of these antisense RNA molecules could initiate new amplification cycles, rendering this process cyclic.

least expensive of the various detection methods. Figure 2.2. illustrates the EOC detection method. This method is based on a colourimetric visualization process and requires modified probes. The capture probe is usually biotinylated and the detection probe is usually conjugated to digoxigenin (DIG). A mixture of the amplification product, the capture probe, detection probe and hybridization buffer is added to wells in which neutravidin has been coated to the wells. The biotin on one end of the capture probe binds to the neutravidin, while the other end of the capture probe binds to the amplification product. The detection probe, designed to be



**Figure 2.2. Illustration of optical detection through EOC for a NASBA assay.**

Wells is coated with neutravidin prior to the detection. The biotin on the 5' end of the capture probe attaches to the neutravidin, while the 3' end attaches to the amplification product. The DIG-labelled detection probe is added and attaches to the bound amplification product. Anti-DIG conjugated to HRPO is added and attaches to the 3' end of the detection probe. ABTS is added and confers a colour change from colourless to green in the presence of CCHFV nucleic acid, which can be measured by a spectrophotometer (absorbance at 405nm).

complementary to a region within the amplification product, binds to the bound amplification product. Anti-DIG conjugated to horse-radish peroxidase (HRPO) is added. Lastly, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) confers a colour change from transparent to green in the presence of CCHF viral nucleic acid.

Primers were specifically designed for the development of a NASBA using an enzyme mixture and buffers prepared in-house. This assay was designated the CCHFV NASBA assay and was designed to detect CCHFV RNA from southern African isolates. The enzyme mixture and buffers of the CCHFV NASBA assay were compared with a commercially available enzyme mixture and buffers provided in the NucliSENS® EasyQ Basic Kit v2 (bioMérieux, Marcy-l'Etoile, France). The assay utilizing the NucliSENS® EasyQ Basic kit was designated CCHFV NucliNASBA assay.

## **2.2. Methods and materials**

### **2.2.1. Samples**

All RNA samples were extracted prior to this study at the Centre for Emerging and Zoonotic Diseases (CEZD) at the National Institute for Communicable Diseases (NICD), Johannesburg.

#### **2.2.1.1. RNA extracted from infected Vero cell culture**

The nucleotide sequences of S segments of different CCHFV strains have shown 20% genome plasticity (Deyde et al, 2006). To determine the specificity of the primers for detection of genetically diverse CCHFV isolates, RNA was extracted from Vero cell culture infected with 48 southern African CCHFV isolates. These isolates were obtained from patients and sequence data from previous phylogenetic studies indicated that the genetic diversity among southern African isolates ranged up to 18% (Burt and Swanepoel; 2005, Burt et al, 2009). RNA was extracted using the guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). In addition RNA extracted from Vero cell culture infected with Rift Valley fever virus (RVFV) and yellow fever virus (YFV), respectively, was kindly supplied by Dr Paweska at the NICD and from our laboratory using the vaccine strain 17D of YFV, respectively. RNA extracted from Vero cell culture infected with SPU 4/81 using the QIAamp® Viral RNA Mini kit (QIAGEN, Hilden, Germany) was selected as control and was serially diluted to determine

the minimum detection limit of the NASBA assays. It should be noted that the concentration of the RNA extracted from SPU 4/81 was not determined.

#### **2.2.1.2. RNA extracted from patient serum samples**

Clinical samples contain low levels of CCHFV RNA and might contain substances that could inhibit the NASBA assay, hence the assays were validated using clinical samples to investigate potential for diagnostic application. RNA was extracted, using the QIAamp® Viral RNA Mini Kit according to the manufacturer's instructions, from 22 serum samples collected from patients with confirmed CCHFV infections and from 16 serum samples collected from patients with suspected viral haemorrhagic fevers but confirmed as non-CCHFV infected cases.

#### **2.2.2. Nested RT-PCR**

The detection of CCHF viral RNA using a conventional nested RT-PCR (Rodriquez et al, 1997) (Table 2.1) was performed to confirm the presence of CCHF viral RNA in the samples extracted from infected Vero cell culture. The first round was performed with the Titan™ One-Tube RT-PCR system (Roche, Mannheim, Germany) according to the manufacturer's protocol. The Titan system is based on the functions of avian myeloblastosis virus reverse transcriptase (AMV-RT) and Expand High Fidelity enzymes in one reaction tube. Briefly, the reaction mixture was prepared in a 50.0 microliter (μℓ) volume consisting of 15.0 picomole (pmol) of the F2 primer, 15.0 pmol of the R3 primer, 10.0 units/microliter (U/μℓ) Protector RNase Inhibitor, 5.0 millimolar (mM) dithiothreitol (DTT), 1× RT-PCR buffer (1.5 mM MgCl<sub>2</sub> and dimethyl sulfoxide (DMSO), 0.4 mM deoxyribonucleoside triphosphate (dNTP) (Sigma-Aldrich, Missouri, United States of America (USA)), 1.0 μℓ Titan enzyme mix, 1.0 μℓ RNA template and nuclease free water (NFW) (Ambion Inc., Texas, USA) to 50.0 μℓ. The reaction mixture was subjected to the following cycling conditions using the GeneAmp 9700 (Applied Biosystems, Life Technologies, Oregon, USA): 50 degrees Celsius (°C) for 30 min, 94°C for 2 min, 30 cycles of 94°C for 10 seconds (s), 44°C for 30 s, 68°C for 45 s and a final extension time of 7 min at 68°C.

The second round was performed using *Taq* DNA Polymerase (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa (SA)) according to manufacturer's instructions.

Briefly, a reaction mixture consisted of 1× *Taq* buffer with magnesium chloride (MgCl<sub>2</sub>), 0.4 pmol of the F3 primer and 0.4 pmol of the R2 primer, 0.4 mM dNTP, 0.025 U/μl *Taq* DNA Polymerase, 10.0 μl template from the first round and NFW up to 50.0 μl. It is important to note that only samples that were not detected during the first round of the nested RT-PCR were used as template for the second round. The reaction mixture was subjected to the following cycling conditions: 95°C for 1 min; 30 cycles of 95°C for 30 s, 43°C for 30 s, 72°C for 30 s; and a final extension time of 5 min at 72°C.

The PCR products were separated by electrophoresis using 1% SeaKEM® LE agarose (Lonza, Maine, USA) gel in 1× tris-acetate-disodiummethylenediaminetetraacetate (TAE) buffer (Appendix D) with 1% ethidium bromide (0.6 microgram (μg)/ml) (Sigma-Aldrich, St. Louis, Missouri, USA) and visualized using a UV Transilluminator (UviPro Gold, UVItec, Cambridge, United Kingdom). The O'GeneRuler DNA ladder mix SM 1173 (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA) was used as size marker. A 6× loading dye (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA) was mixed with 5.0 μl of the DNA samples and loaded onto the gel. Gel electrophoresis was performed utilizing a Bio-Rad PowerPac Basic system (Bio-Rad, California, USA) at 80 Volts (V) for 40 min.

**Table 2.1. Primers used for the nested PCR**

Primer name	Sequence (5' - 3')	Position on CCHFV strain 10200*	Melting temperature (°C)
F2	TGG ACA CCT TCA CAA ACT C	135 – 153	44
F3	GAA TGT GCA TGG GTT AGC TC	290 – 309	47
R2	GAC ATC ACA ATT TCA CCA GG	549 – 530	45
R3	GAC AAA TTC CCT GCA CCA	670 – 653	43

\* Genbank Accession number U88410

### 2.2.3. NASBA

The NucliSENS® EasyQ Basic Kit v2 is a commercial kit that supplies an enzyme mixture, comprising of lyophilized AMV-RT, RNase H, T7 RNA polymerase and bovine serum albumin

(BSA), and buffer for use in NASBA assays. It is important to note that the primers and/or probes are not supplied with this kit. The NucliSENS® EasyQ Basic Kit v2 is not readily available and is more costly than the enzyme mixture and buffer prepared for use in the CCHFV NASBA assay.

#### **2.2.3.1. Primer and probe design**

Sequences from 33 southern African CCHFV isolates, consisting of a 450 base pair (bp) region of the S segment, were retrieved from GenBank ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)) (Accession numbers: AY905625; AY905626; AY905627; AY905628; AY905629; AY905630; AY905631, AY905632, AY905633, AY905634, AY905635, AY905636, AY905637, AY905638, AY905639, AY905640, AY905641, AY905642, AY905643, AY905644, AY905645, AY905646, AY905647, AY905648, AY905649, AY905650, AY905651, AY905652, U84635, U84636, U84637, U84638, U84639) and aligned using ClustalX (<http://www.clustalx.software.informer.com/2.1/>) (Appendix A). Primers were designed based on the alignment in order to flank a conserved 250 bp region and a detection probe was designed to bind within the 250 bp region. The primers and probes were modified as required for optical detection by EOC (Table 2.2). Briefly, the reverse primer included the T7 promoter sequence on the 3' end, the forward primer included the sequence identical to the capture probe on the 5' end, the capture probe was biotinylated on the 5' end and the detection probe was labelled with DIG on the 3' end. The specificity of the primers was tested using Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Prior to optimizing the CCHFV NASBA, the primers were tested for their ability to detect and amplify CCHF viral RNA extracted from Vero cell culture infected with SPU 103/87 using the Titan™ One-Tube RT-PCR system according to the manufacturer's instructions as described in 2.2.2. with the following modifications, the NASBA F primer and the NASBA R primer were used as primer pair with an annealing temperature of 64°C. The amplicons were visualised by agarose gel electrophoresis as described in 2.2.2.

**Table 2.2. NASBA primers and probes**

<b>Primer/Probe (Position on sequence)*</b>	<b>Sequence (5' – 3')</b>	<b>Additional sequence (5' – 3')</b>	<b>Function of additional sequence</b>	<b>Other modifications</b>
NASBA F (bp 299-321)*	TGG GT/CT AGC TCC ACT/C GGC ATT GT	GAT GCA AGG TCG CAT ATG AG	Identical to Capture probe sequence	None
NASBA R (bp 553-530)*	GAC ATT/C ACA ATT/C TCG CCA GG	AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG G	T7 promoter sequence	None
Capture probe (N/A)	ATG CAA GGT CGC ATA TGA GT	None	n/a	Biotinylated (5' end)
Detection probe (bp 470-492)*	TTC CGT GTC AAT GCA AAC ACA GC	None	n/a	Labelled with DIG (3' end)

\* SPU 415/85 (Genbank Accession number U88415) was used as reference strain

n/a = not applicable

### 2.2.3.2. CCHFV NASBA assay

The in-house developed enzyme mixture and buffer were optimized using CCHF viral RNA extracted from Vero cell culture infected with SPU 103/87. A reaction mixture was prepared in a 15.0 µl volume consisting of CCHFV NASBA amplification buffer (Appendix D), 0.9 mM of each dNTP, 1.8 mM of each ribonucleoside triphosphate (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA), 0.45 pmol of NASBA F primer, 0.45 pmol of NASBA R primer, 2.0 µl of RNA template and NFW up to 15.0 µl. The optimal concentrations of tris(hydroxymethyl)aminomethane, magnesium chloride (MgCl<sub>2</sub>), potassium chloride (KCl), DTT and DMSO used in the CCHFV NASBA amplification buffer were determined by titrations. The tested concentrations ranged as follows, 30 – 100 mM for tris(hydroxymethyl)-aminomethane, 18 – 180 mM for MgCl<sub>2</sub>, 60 – 100 mM for KCl, 75 – 100 mM for DTT and 10 – 30% for DMSO. For the negative control, the RNA template was replaced with NFW. The reaction mixture was incubated at 65°C for 5 min and cooled to 41°C for 5 min using a thermocycler. A 5.0 µl aliquot of the in-house enzyme mixture, consisting of 2.0 U/µl of T7 RNA polymerase (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria,SA), 0.005 U/µl



of RNase H (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA), 0.41 U/ $\mu\text{l}$  of AMV-RT (Roche, Mannheim, Germany) and 0.09  $\mu\text{g}/\mu\text{l}$  of BSA (Roche, Mannheim, Germany) (Oehlenschläger et al, 1996), was added to each reaction mixture and the reaction mixtures were incubated at 41°C for 90 min. The EOC detection process followed, briefly eight-well strips (F8 Polysorp UNFRA Nunc-Immuno module, Nunc A/S, Denmark) were coated with 100.0  $\mu\text{l}$  neutravidin (5 mg/ml) (Life Technologies, Oregon, USA), diluted 1:100 in tris-buffered saline (TBS) (Appendix D), overnight. The detection mixture was prepared in a 100.0  $\mu\text{l}$  volume/well consisting of 5.0  $\mu\text{l}$  of amplicon and 95.0  $\mu\text{l}$  of hybridization mixture, which consisted of 0.26 micromolar ( $\mu\text{M}$ ) of capture probe, 0.26  $\mu\text{M}$  of detection probe, 10.0  $\mu\text{l}$  of NASBA-EOC hybridization buffer (Appendix D) and NFW up to 100.0  $\mu\text{l}$ . The wells were incubated at 41°C for 1 hour (h). The supernatant was discarded and the wells washed three times with 200.0  $\mu\text{l}$  1 $\times$  TBS. A 100.0  $\mu\text{l}$  aliquot of anti-DIG conjugated to HRPO (50 U) (Roche, Mannheim, Germany), diluted 1:500 in TBS, was added to each well and the wells incubated at 37°C for 30 min. The supernatant was discarded and the strips washed three times with 200.0  $\mu\text{l}$  1 $\times$  TBS, where after a 100.0  $\mu\text{l}$  aliquot of ABTS (KPL, Maryland, USA) was added to each well. The absorbance values were read at 405 nm using a plate reader (Sunrise<sup>TM</sup>, Tecan, Männedorf, Switzerland) at 5 min intervals for 40 min. The optimal incubation period with ABTS was determined as 40 min. The cut-off value was calculated for the CCHFV NASBA assay as average absorbance values of known negative samples + 2 standard deviations.

Three CCHFV RNA samples were selected randomly and used to compare the CCHFV NASBA assay performance using a water bath to performance using a heating block.

### **2.2.3.3. CCHFV NucliNASBA assay**

The NucliSENS® EasyQ Basic Kit v2 was used according to the manufacturer's protocol. The NucliSENS enzyme mixture was prepared by reconstituting the lyophilized enzyme sphere in 55.0  $\mu\text{l}$  enzyme diluent, consisting of an aqueous sorbitol solution containing biocide, and left at room temperature for less than 1 h. The reaction mixture was prepared in a 15.0  $\mu\text{l}$  volume consisting of 8.0  $\mu\text{l}$  reagent premix (consisting of lyophilized nucleotides, DTT and  $\text{MgCl}_2$ ) (reagent sphere reconstituted in 80.0  $\mu\text{l}$  reagent diluent, consisting of Tris/HCl and 45% DMSO), 3.0  $\mu\text{l}$  KCl/NFW solution (80 mM), 5.0 pmol of each of the NASBA F primer and NASBA R primer, 2.0  $\mu\text{l}$  of RNA template and NFW up to 15.0  $\mu\text{l}$ . For the negative control,

the RNA template was replaced with NFW. The reaction mixture was incubated at 65°C for 5 min and cooled to 41°C for 5 min using a thermocycler. A 5.0 µl aliquot of the NucliSENS enzyme mixture was added to each reaction mixture and the reaction mixtures were incubated at 41°C for 90 min. The EOC detection process was performed and the cut-off value was calculated as described in 2.2.3.2.

#### **2.2.4. Control RNA transcript**

An RNA control, for use in optimizing the NASBA assays and determining minimum detection levels, was prepared by transcription.

##### **2.2.4.1. Preparation of control RNA transcript construct**

RNA extracted from tissue culture infected with SPU 103/87 was amplified using the Titan<sup>TM</sup> One-Tube RT-PCR system according to the manufacturer's protocol, using the NASBA primer pair designed in this study and the cycling conditions described in section 2.2.2.1. The amplicon was visualized by agarose gel electrophoresis as described in 2.2.2.1. The PCR product was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) according to the manufacturer's instructions. Briefly, the remainder of the PCR product was added to an equal volume of Membrane Binding Solution and the tube was vortexed briefly. The mixture was transferred to an SV minicolumn in a collection tube and incubated at room temperature for 1 min. The SV minicolumn assembly was clarified at 16 000× g for 1 min. The flowthrough was discarded from the collection tube. The SV minicolumn was washed with a 700.0 µl aliquot of Membrane Wash Solution, with added ethanol, at 16 000× g for 1 min. The flowthrough was discarded from the collection tube. The wash was repeated with a 500.0 µl aliquot of Membrane Wash Solution at 16 000× g for 5 min and the flowthrough was discarded from the collection tube. The empty SV minicolumn was centrifuged 16 000× g for 1 min with the inner lid of the microcentrifuge off and placed in a sterile 1.5 ml tube. A 30.0 µl aliquot of NFW was added to the SV minicolumn and incubated at room temperature for 1 min. The assembly was centrifuged 16 000× g for 1 min and the SV minicolumn was discarded. The DNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA).

The PCR amplicon acquired a 3' adenine overhang during amplification which facilitated ligation into the pGEM®-T Easy vector (Promega, Wisconsin, USA), which is linearized at base 60 and has a single thymidine added to the 3' end. The pGEM®-T Easy vector also contains a *lacZ* gene which encodes  $\beta$ -galactosidase, an enzyme that normally breaks down galactose into lactose and glucose, but can convert X-gal (a colourless substrate) to a blue coloured product. The *lacZ* gene contains multiple cloning sites where the PCR fragment is inserted, thus interrupting the *lacZ* gene. This disruption leads to production of a non-functional  $\beta$ -galactosidase enzyme and X-gal is not converted into a blue coloured product. Thus, colonies consisting of positively transformed cells appear white and colonies consisting of untransformed cells appear blue. It is important to note that a DNA insert that consists of a multiple of three bases and lacks in-frame stop codons does not disrupt the *lacZ* gene and in this case the colonies consisting of positively transformed cells may also appear blue.

Briefly, the ligation reaction consisted of 0.3 Weiss units/ $\mu\text{l}$  T4 DNA ligase, 1 $\times$  Rapid ligation buffer, 5.0 nanogram (ng)/ $\mu\text{l}$  pGEM®-T Easy vector, 60.3 ng of the PCR product and NFW up to 10.0  $\mu\text{l}$ . The reaction was incubated at 4°C for 16 h. The ligation product was used to transform OneShot® Top10 chemically competent cells (Invitrogen, California, USA) according to the manufacturer's instructions. Briefly, the 10.0  $\mu\text{l}$  ligation product was added to a 100.0  $\mu\text{l}$  aliquot of OneShot® Top10 chemically competent cells that was completely thawed on ice. The cells were gently mixed and placed on ice for 20 min. The tube was transferred to a water bath at 42°C for 50 s and returned to ice for 2 min. A 900.0  $\mu\text{l}$  aliquot of SOC media (Appendix D) was added to the cells and the cells were incubated at 37°C for 90 min whilst shaking at 150 revolutions per minute (rpm). A 100.0  $\mu\text{l}$  aliquot of the cells was spread out on agar plates containing 100.0  $\mu\text{g}/\text{ml}$  ampicillin (Roche, Mannheim, Germany), 0.1 mM isopropyl-thiogalactoside (IPTG) (Separations, Randburg, SA) and 80.0  $\mu\text{g}/\text{ml}$  X-gal (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA). The plates were incubated at 37°C for 16 h. The transformation efficiency was calculated as described in the manufacturer's instructions as  $1.6 \times 10^7$  colony forming units/ $\mu\text{g}$ .

#### **2.2.4.2. Confirmation of positively transformed cells**

White colonies were selected and divided into two aliquots. One aliquot of each colony was used in a PCR reaction to confirm positively transformed cells using the GoTaq® Flexi DNA

Polymerase system (Promega, Wisconsin, USA). The DNA template was prepared by incubating one aliquot of each white colony in a 10.0  $\mu\text{l}$  aliquot of NFW at 95°C for 2 min. Briefly, a reaction mixture consisted of 1 $\times$  Green GoTaq® Flexi buffer, 1.5 mM MgCl<sub>2</sub> solution, 0.4 mM dNTP, 0.4 pmol T7 forward primer (5' TAA TAC GAC TCA CTA TAG GG 3'), 0.4 pmol NASBA R primer, 0.025 U/ $\mu\text{l}$  GoTaq® DNA Polymerase, 10.0  $\mu\text{l}$  DNA template and NFW up to 50.0  $\mu\text{l}$ . Cycling commenced with a single step at 95°C for 2 min; 30 cycles of 95°C for 30 s, 43°C for 30 s, 72°C for 30 s; and a final extension time of 5 min at 72°C. Genes that were inserted in the incorrect orientation would not have been amplified with this primer pair. The amplicons were separated and visualised by agarose gel electrophoresis as described in 2.2.2.1. The remaining aliquot of a colony consisting of positively transformed cells was grown in 5.0 ml of Luria Bertani (LB) media containing 100.0  $\mu\text{g/ml}$  ampicillin (LB/Amp) at 37°C whilst shaking at 150 rpm for 16 h. The pGEM-T Easy plasmid containing the insert was purified with the PureYield Plasmid Miniprep system (Promega, Wisconsin, USA) according to the manufacturer's instructions and was designated pGEMTE.CCHFV.S. Briefly, the LB/Amp overnight culture was collected by centrifugation at 16 000 $\times$  g for 30 s, the supernatant was discarded and the pellet reconstituted in a 600.0  $\mu\text{l}$  aliquot of distilled water. A 100.0  $\mu\text{l}$  aliquot of Cell Lysis buffer was added and mixed by inverting. A 350.0  $\mu\text{l}$  aliquot of Neutralization solution was added, mixed by inverting and centrifuged at 16 000 $\times$  g for 3 min. The supernatant was transferred to a minicolumn and centrifuged at 16 000 $\times$  g for 15 s. A 200.0  $\mu\text{l}$  aliquot of Endotoxin Removal wash was added and followed by centrifugation at 16 000 $\times$  g for 15 s. A 400.0  $\mu\text{l}$  aliquot of Column Wash solution was added and followed by centrifugation at 16 000 $\times$  g for 30 s. The purified plasmid was eluted in a 30.0  $\mu\text{l}$  aliquot of NFW by centrifugation at 16 000 $\times$  g for 15 s. The DNA concentration and purity were measured as described in 2.2.4.1. The purified pGEMTE.CCHFV.S construct was linearized using a selected restriction endonuclease (RE), SphI, that recognizes a specific short sequence, 5' GCATGC 3', and cleaves between specific bases within the short sequence, 5' GCATG`C 3'. Briefly, a digestion reaction consisted of 2.0  $\mu\text{l}$  purified pGEMTE.CCHFV.S, 1.0  $\mu\text{l}$  SphI (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA), 2.0  $\mu\text{l}$  buffer K (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA) and NFW up to 20.0  $\mu\text{l}$ . The digestion reaction was incubated at 37°C for 2 h. The digested product was visualised by agarose gel electrophoresis as described in 2.2.2.1. along with the undigested plasmid.

#### **2.2.4.3. RNA transcription and purification**

Template for the preparation of the control RNA transcript was prepared by amplification of the linearized pGEMTE.CCHFV.S construct using GoTaq® Flexi DNA Polymerase as described in 2.2.4.2. with the following modifications, the NASBA F primer and Sp6 reverse primer (5' ATT TAG GTG ACA CTA TAG 3') were used as the primer pair and the annealing temperature was adjusted to 41°C. A DNA template including the Sp6 site, which is required for the Sp6-dependent transcription, was produced and visualised by agarose gel electrophoresis as described in 2.2.2.1. The PCR product was purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions and the DNA concentration and purity were measured as described in 2.2.4.1.

The control RNA transcript was prepared using the Sp6 MEGAscript® Kit (Ambion Inc., Texas, USA) according to the manufacturer's protocol. Briefly, a reaction mixture consisted of 5.0 mM adenosine triphosphate solution, 5.0 mM cytidine triphosphate (CTP) solution, 5.0 mM guanosine triphosphate (GTP) solution, 5.0 mM uridine triphosphate solution, 1× Reaction buffer, 2.0 µl Sp6 enzyme mix, 3.0 µl CCHFV.S.Sp6 control fragment DNA and NFW up to 20.0 µl. The reaction mixture was incubated at 37°C for 16 h.

The SV Total RNA Isolation system (Promega, Wisconsin, USA) combines the nucleoprotein disruption properties of guanidine thiocyanate with the RNase inactivation properties of β-mercaptoethanol. Dilution of the sample in the presence of high guanidine thiocyanate causes selective precipitation of cellular proteins, while the RNA remains in solution. The lysate is cleared of precipitated proteins and cellular debris through centrifugation. The RNA is selectively precipitated with ethanol and bound to the silica surface of the glass fibres in the spin column. The binding reaction occurs rapidly due to the disruption of water molecules by chaotropic salts, favouring the adsorption of nucleic acids to the silica. RNase-free DNase I is applied directly to the silica surface to digest contaminating genomic DNA and contaminating salts, proteins and cellular impurities is washed away in two steps with one wash buffer. The essentially pure fraction of total RNA is eluted in NFW.

Total RNA was purified from the transcription reaction using the SV Total RNA Isolation system according to the manufacturer's instructions with the following modifications, a 175.0 µl aliquot of the Lysis buffer was added directly to the 20.0 µl transcription reaction and the RNA eluted in a 50.0 µl aliquot of NFW. The RNA concentration and purity was measured using a

Nanodrop 2000 spectrophotometer and the number of copies of RNA was estimated. The estimate was determined as:

$$\frac{\text{amount of ssRNA (ng)} \times \text{Avogadro's number (mol}^{-1}\text{)}}{\text{length of ssRNA (bp)} \times 10^9 \times \text{average weight of RNA base (g/mol)}}$$

Ten-fold dilutions of the NASBA RNA control transcript were prepared and used to determine the sensitivity and the minimum detection levels of the NASBA assays.

### **2.2.5. Commercial RT-PCR assays**

RNA extracted from clinical samples were tested using two commercially available real-time RT-PCR assays. The tests were performed by Dr Weyer at the CEZD, NICD, Johannesburg.

#### **2.2.5.1. TaqMan OneStep RT-PCR Kit**

The TaqMan OneStep RT-PCR Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions as described previously (Wölfel et al, 2007). The primer and probe sets used were designed based on an alignment of S segment sequences of sixty one CCHFV isolates of seven globally distinct regions. Thus, the CCforSE01 primer (5' CAA GGG GTA CCA AGA AAA TGA AGA AGG C 3') and the CCrevSE02 (5' GCC ACA GGG ATT GTT CCA AAG CAG AC 3') were designed to flank a 181 bp region near the 5' end of the CCHFV S segment and used as primer pair with an annealing temperature of 59°C. The following probes were designed, CCprobeSE01 (5' FAM-TGT CAA CAC AGC AGG GTG CAT GTA GAT-TAMRA+p 3'), CCprobeSE03 (5' FAM-TGT AAG CAC GGC AGG GTG CAT GTA AAT-TAMRA+p 3') and CCprobeSE0A (5' FAM-ACT CCA ATG AAG TGG GGG AAG AAG CT-TAMRA+p 3'), as design of a single probe compatible with CCHFV isolates from all the distinct regions was not possible. Briefly, the reaction mixture consisted of 1× OneStep RT-PCR buffer, 0.4 mM dNTP, 0.6 µM of primer CCforSE01, 0.6 µM of primer CCrevSE02, 1.0 µM of probe CCprobeSE01, 1.0 µM of probe CCprobeSE03, 1.0 µM of probe CCprobeSE0A, 32.0 µg BSA, 2.0 µl OneStep RT-PCR enzyme, 5.0 µl RNA template and NFW up to 25.0 µl. The cycling parameters were as follows, reverse transcriptase at 50°C for 30 min, denaturation at 95°C for 15 min, 45 cycles of 94°C for 15 s and 59°C for 30 s and a final cooling at 30°C for 30 s.

#### **2.2.5.2. RealStar® CCHFV RT-PCR Kit 1.2**

The RealStar® CCHFV RT-PCR Kit 1.2 (Altona Diagnostics GmbH, Hamburg, Germany) utilizes an internal control (IC) to determine possible inhibition of the RT-PCR reaction and to confirm the integrity of the reagents and is supplied with target-specific primers and probes. The current set of primers and probes supplied with the kit were designed to compensate for accumulation of mutations, but as circulating CCHFV strains evolve by accumulating multiple mutations and RNA recombination, the primer and probe sets may have to be updated to avoid obtaining false negative results. The functioning of the RealStar® CCHFV RT-PCR Kit 1.2 is based on a reverse transcription process to produce cDNA from the target RNA template, PCR process to amplify the target cDNA and real-time probes labelled with fluorescent reporter and quencher dyes to detect the target cDNA. The probe specific to the CCHF viral RNA is labelled with the fluorophore FAM and the probe specific to the IC is labelled with a fluorophore exhibiting similar reactions as Cy3. The use of distinguishable dyes allows parallel detection of CCHF viral RNA and the IC. The IC was added to the specimen/lysis buffer mixture during the RNA extraction process at a volume of 10% of the final elution volume. The reaction mixture consisted of 15.0 µl of the mastermix and 10.0 µl of the RNA sample or positive or negative control. The following cycling conditions were used, reverse transcription at 45°C for 10 min, denaturation at 95°C for 10 min, 45 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 30 s, and a final cooling at 40°C for 30 s.

#### **2.2.6. Inhibitors of the CCHFV NASBA**

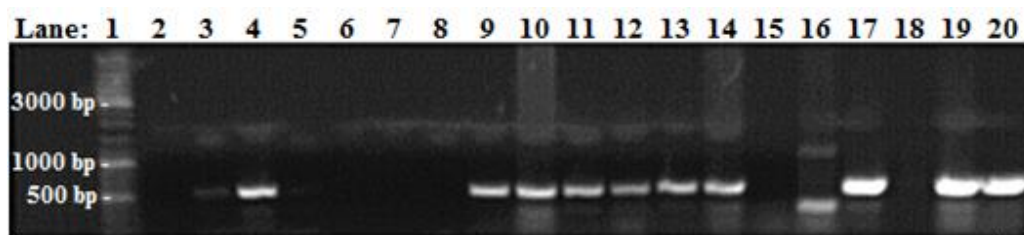
Serum samples were collected from four volunteers and designated Vector-Borne Disease (VBD) 16/14, VBD 19/14, VBD 20/14 and VBD 21/14. Sera were spiked with the RNA control transcript prior to RNA extraction and use in CCHFV NASBA assay to determine whether there are substances in the human sera that could inhibit the CCHFV NASBA assay. Seven aliquots of 130.0 µl were taken from each serum sample, six of the aliquots spiked with 10.0 µl of the RNA control transcript at copy numbers ranging from  $5 \times 10^5$  –  $5 \times 10^6$ . The seventh aliquot had 10.0 µl NFW added to it and served as a negative control. All twenty-eight spiked serum aliquots were subjected to RNA extraction using the SV Total RNA Isolation System (Spin Protocol) (Promega, Wisconsin, USA) according to the manufacturer's instructions with the following modifications, a 560.0 µl aliquot of the lysis buffer was added directly to the 140.0 µl spiked

serum and the RNA eluted in a 50.0  $\mu\text{l}$  aliquot of NFW. The RNA samples were tested using the CCHFV NASBA assay as described in 2.2.2.3. and compared with RNA control transcript at similar copy numbers used as template in the CCHFV NASBA assay.

## 2.3. Results

### 2.3.1. Nested RT-PCR on CCHFV RNA samples extracted from infected Vero cell culture

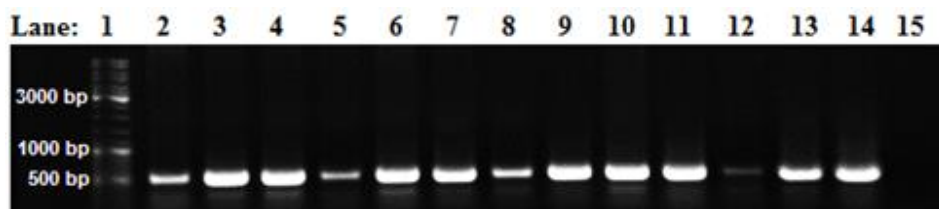
RNA extracted from Vero cell culture infected with 48 genetically diverse southern African isolates were tested with a nested PCR to confirm the presence of CCHFV RNA. Figure 2.3.a. – 2.3.d. show the agarose gel electrophoretic analysis of the amplicons of the first round of the nested RT-PCR. Primers F2 and R3 amplify a PCR product of predicted size 536 bp. In summary RNA extracted from Vero cell culture infected with SPU 259/84, SPU 381/85, SPU 396/85, SPU 408/85, SPU 415/85, SPU 582/86, SPU 593/86, SPU 444/89 and SPU 61/90 were not detected during the first round of the nested RT-PCR.



**Figure 2.3.a. Agarose gel electrophoretic analysis of 18 of 48 amplicons of the first round of the nested PCR to confirm the presence of CCHFV RNA extracted from infected tissue culture.**

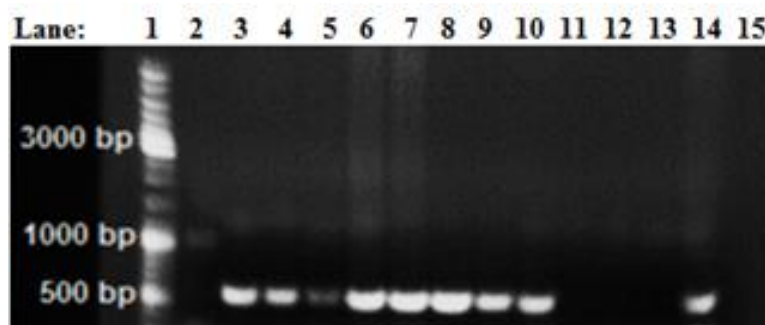
Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; the following lanes present amplicons with different RNA samples as template: 2. SPU 259/84; 3. SPU 94/85; 4. SPU 97/85; 5. SPU 381/85; 6. SPU 396/85; 7. SPU 408/85; 8. SPU 415/85; 9. SPU 23/86; 10. SPU 196/86; 11.SPU 361/86; 12.SPU 536/86; SPU 560/86; 14.SPU 566/86; 15.SPU 582/86; 16.SPU 593/86; 17.SPU 177/87; 18.Negative control; 19.SPU 244/87; 20. SPU 383/87





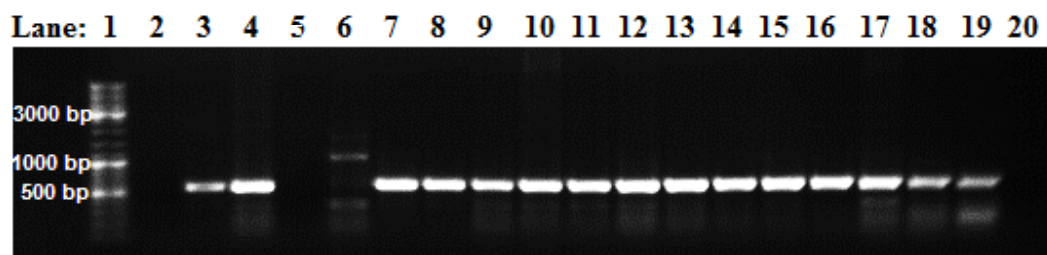
**Figure 2.3.b. Agarose gel electrophoretic analysis of 14 of 48 amplicons of the first round of the nested PCR to confirm the presence of CCHFV RNA extracted from infected tissue culture.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; the following lanes present amplicons with different RNA samples as template: 2. SPU 486/87; 3. SPU 556/87; 4. SPU 18/88; 5. SPU 45/88; 6. SPU 71/88; 7. SPU 203/88; 8. SPU 273/88; 9. SPU 497/88; 10. SPU 498/88; 11.SPU 130/89; 12.SPU 281/89; 13.SPU 337/89; 14.SPU 372/89; 15. SPU 444/89



**Figure 2.3.c. Agarose gel electrophoretic analysis of 10 of 48 amplicons of the first round of the nested PCR to confirm the presence of CCHFV RNA extracted from infected tissue culture.**

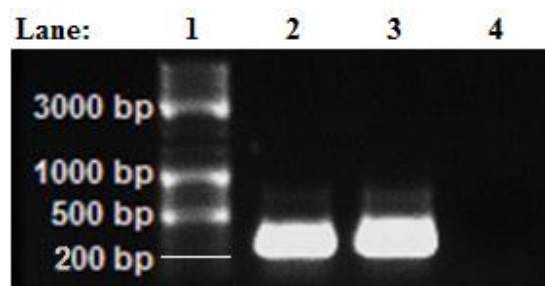
Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; the following lanes present amplicons with different RNA samples as template: 2. SPU 61/90; 3. SPU 380/90; 4. SPU 50/93; 5. SPU 247/85; 6. SPU 215/90; 7. SPU 172/93; 8. SPU 15/92; 9. SPU 24/01; 10. SPU 51/01; 11.Negative sample in the form of NFW instead of RNA; 12.Yellow fever viral RNA; 13. Rift Valley fever viral RNA; 14. SPU 409/87; 15. Empty



**Figure 2.3.d. Agarose gel electrophoretic analysis of 19 of 48 amplicons of the first round of the nested PCR to confirm the presence of CCHFV RNA extracted from infected tissue culture.**

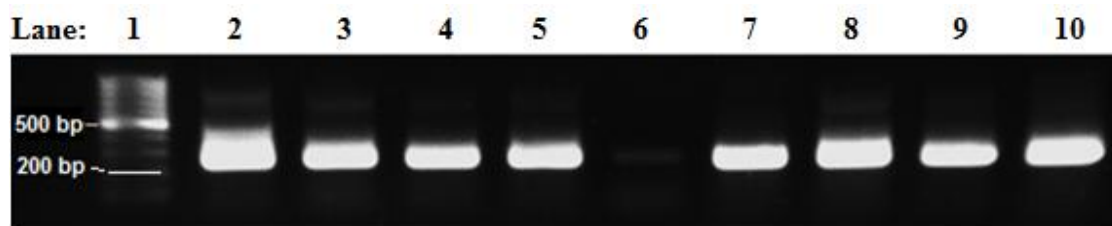
Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; the following lanes present amplicons with different RNA samples as template: 2. SPU 259/84; 3. SPU 94/85; 4. SPU 372/89; 5. SPU 444/89; 6. SPU 61/90; 7. SPU 380/90; 8. SPU 50/93; 9. SPU 247/85; 10. SPU 215/90; 11.SPU 19/88; 12.SPU 378/90; 13.SPU 381/90; 14.SPU 382/90; 15.SPU 388/90; 16.SPU 172/93; 17.SPU 15/92; 18.SPU 24/01; 19.SPU 51/01; 20. Negative control

Negative samples were subjected to a nested reaction. Figure 2.3.e. and Figure 2.3.f. show the Agarose gel electrophoretic analysis of the amplicons of the second round of the nested PCR. Primers F3 and R2 amplify a PCR product of predicted size 260 bp. In summary all of the RNA samples were positive using the nested RT-PCR. The results obtained with the nested RT-PCR are summarized in Table 2.4.



**Figure 2.3.e. Agarose gel electrophoretic analysis of 2 of 11 amplicons of the second round of the nested PCR to confirm the presence of CCHFV RNA extracted from infected tissue culture.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; the following lanes present amplicons with different RNA samples as template: 2. SPU 259/84; 3. SPU 94/85; 4. Empty



**Figure 2.3.f. Agarose gel electrophoretic analysis of 9 of 11 amplicons of the second round of the nested PCR to confirm the presence of CCHFV RNA extracted from infected tissue culture.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1143; the following lanes present amplicons with different RNA samples as template: 2. SPU 381/85; 3. SPU 396/85; 4. SPU 408/85; 5. SPU 415/85; 6. SPU 582/86; 7. SPU 593/86; 8. SPU 281/89; 9. SPU 444/89; 10. SPU 61/90

### 2.3.2. Diluted RNA extracted from infected Vero cell culture

The cut-off was calculated using data from sixteen known negative samples tested in duplicate (Appendix B) and was determined as a value of 0.156 and 0.166 for the CCHFV NASBA and the CCHFV NucliNASBA, respectively. RNA extracted from Vero cell culture infected with SPU 4/81 was diluted -0.5 logs from 0 to -5.5 and used as template to test the sensitivity of the NASBA assays. Table 2.3. shows the results obtained. The CCHFV NASBA assay detected RNA diluted  $\leq 10^{-4.5}$ . The CCHFV NucliNASBA assay detected RNA diluted  $\leq 10^{-5.5}$ .

**Table 2.3. Detection of serially diluted RNA extracted from infected Vero cell culture**

Dilution	CCHFV NASBA	CCHFV NucliNASBA
$10^{-0.0}$	Positive	Positive
$10^{-0.5}$	Positive	Positive
$10^{-1.0}$	Positive	Positive
$10^{-1.5}$	Positive	Positive
$10^{-2.0}$	Positive	Positive
$10^{-2.5}$	Positive	Positive
$10^{-3.0}$	Positive	Positive
$10^{-3.5}$	Positive	Positive
$10^{-4.0}$	Positive	Positive

**Table 2.3. (Continued)**

$10^{-4.5}$	Positive	Positive
$10^{-5.0}$	Negative	Positive
$10^{-5.5}$	Negative	Positive

### 2.3.3. Validation of CCHFV NASBA assay using RNA extracted from infected Vero cell culture

The analytical specificity of the CCHFV NASBA assay was determined using RNA extracted from Vero cell cultures infected with 48 genetically diverse southern African CCHFV isolates. RNA extracted from Vero cell culture infected with SPU 103/87 was used as template to determine the optimal concentration of the reagents used in the CCHFV NASBA assay. Figure 2.4. shows the visual presentation of the results obtained using different concentrations of DMSO.



**Figure 2.4. Visual presentation of the CCHFV NASBA results using RNA extracted from SPU 103/87 as template and different DMSO concentrations.**

Wells: 1 - 3. Amplicon amplified in presence of a final concentration of 10% volume/volume (v/v) DMSO; 4. NFW instead of RNA during the amplification; 5 - 7. Amplicon amplified in presence of a final concentration of 15% v/v DMSO; 8. NFW.

Table 2.4. shows the results of the CCHFV NASBA assay compared with the results of the nested RT-PCR. Two samples were negative using the NASBA and negative in the first round of the nested PCR, but positive using the second round of the nested RT-PCR. The results suggest either a lower level of viral RNA and that the nested RT-PCR may be able to detect

lower levels or alternatively inhibition of the CCHFV NASBA. Sequence data were available for two of the seven RNA samples that were not detected with the CCHFV NASBA assay. Further inspection revealed that there were some mismatches within the NASBA primer and probe binding regions of SPU 582/86 (Appendix A). A single mismatch within the binding region of the NASBA Reverse primer was observed with SPU 415/85 (Appendix A). The CCHFV NASBA assay was able to detect RNA in 41/48 (85.4%) of the samples. No amplification was detected from RNA extracted from Vero cell cultures infected with RVFV and YFV. A detailed list of the results can be found in Appendix C.

**Table 2.4. Detection of RNA extracted from infected Vero cell culture**

	CCHFV NASBA	Nested RT-PCR	
		First round	Second round
	Number of samples		
<b>Positive</b>	41	39	9
<b>Negative</b>	7	9	0

#### **2.3.4. Amplification using water bath or heating block**

RNA extracted from Vero cell culture infected with SPU 408/85, SPU 103/87 and SPU 498/88 were selected and tested by the CCHFV NASBA assay using a water bath and a heating block. Table 2.5. shows the results obtained. RNA extracted from Vero cell culture infected with SPU 103/87 and SPU 498/88 was amplified with both instruments, respectively, but RNA extracted from Vero cell culture infected with SPU 408/85 was negative, in concordance with the results obtained with the CCHFV NASBA assay using a thermocycler.

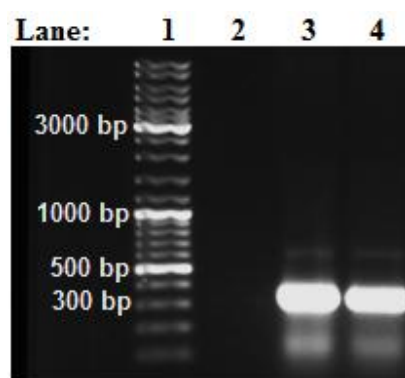
**Table 2.5. Comparison of efficiency of water bath with the efficiency of a heating block for performing the CCHFV NASBA assay**

SPU number	Water bath	Heating block	Thermocycler
408/85	Negative	Negative	Negative
103/87	Positive	Positive	Positive
498/88	Positive	Positive	Positive
NFW instead of RNA	Negative	Negative	Negative

### 2.3.5. Preparation of a control RNA transcript

A control RNA transcript was prepared for use as a positive control, to determine the lower detection limit of the NASBA assays and to determine the inhibitory effects of serum on the CCHFV NASBA assay.

A PCR fragment was amplified using RNA extracted from Vero cell culture infected with SPU 103/87 as template. The expected size band was 305 bp and, as shown in Figure 2.5.a., the bands visualized aligned approximately with the 300 bp band of the DNA size marker.

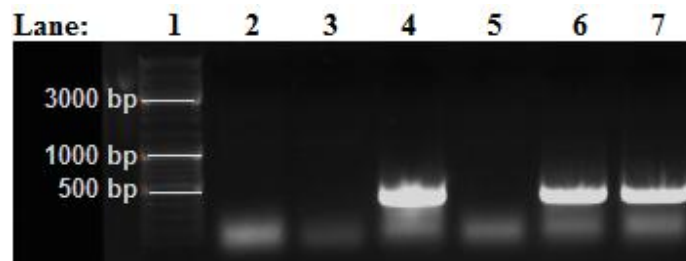


**Figure 2.5.a. Agarose gel electrophoretic analysis of the amplicon from a Titan™ One tube RT-PCR to prepare the NASBA RNA control construct.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. Empty; 3 & 4. Amplicon with RNA extracted from SPU 103/87 as template

The expected size of the amplification product to confirm correct orientation of the insert in pGEM T-Easy was 328 bp. As shown in Figure 2.5.b., the bands in lanes 4, 6 and 7 are observed

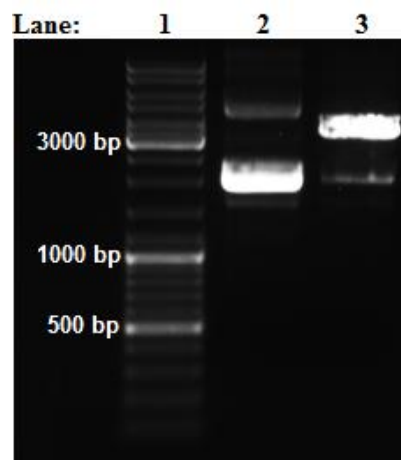
just above the 300 bp band of the DNA marker.



**Figure 2.5.b. Agarose gel electrophoretic analysis of the amplicons from a PCR on one aliquot of selected white colonies to confirm the correct orientation of the NASBA control transcript insert in pGEM.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2 – 7. Amplicons from colonies 1 – 6 with one aliquot of six different white colonies as template.

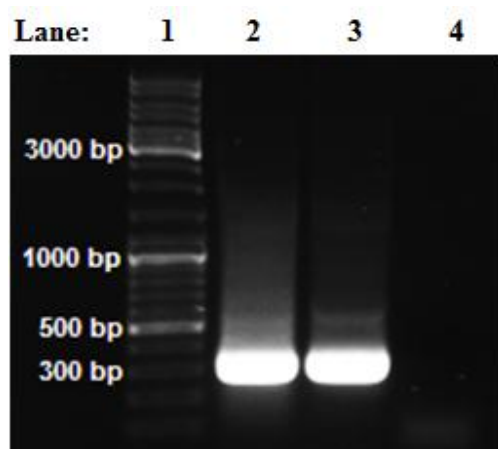
The expected size of digested plasmid was 3 266 bp. Figure 2.5.c. shows that the band visualized in Lane 3 was just above the 3 000 bp band of the DNA marker. The lower bands observed in Lanes 2 and 3 which migrated further down the gel likely represent supercoiled DNA plasmid.



**Figure 2.5.c. Agarose gel electrophoretic analysis of the RE digestion of the purified pGEMTE.CCHFV.S construct.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. Undigested pGEMTE.CCHFV.S construct; 3. Digested pGEMTE.CCHFV.S construct

The predicted size of the amplicon tested with the NASBA F and NASBA R primers was 305 bp and, as shown in Figure 2.5.d., the bands visualized aligned approximately with the 300 bp band of the DNA marker.



**Figure 2.5.d. Agarose gel electrophoretic analysis of the amplicons of a Titan™ One tube RT-PCR to test the NASBA RNA control transcript.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2 & 3. Amplicon with the RNA control transcript as template; 4. Negative control.

### 2.3.6. Determination of the lower detection limits of the NASBA assays

The copy number of the control RNA transcript was calculated as  $3.7 \times 10^{12}$  copies/ $\mu\text{l}$ . Fourteen ten-fold dilutions were prepared from the control RNA transcript and used to determine the sensitivity of the CCHFV NASBA assay and the CCHFV NucliNASBA assay. Table 2.6. shows the results obtained, where the CCHFV NASBA assay detected  $\geq 3.7$  copies of RNA and the CCHFV NucliNABSA assay detected  $\geq 37$  copies of RNA.

**Table 2.6. Detection of serially diluted control RNA transcript specimens**

RNA copy number	CCHFV NASBA	CCHFV NucliNASBA
$3.7 \times 10^{12}$	Positive	Positive
$3.7 \times 10^{11}$	Positive	Positive
$3.7 \times 10^{10}$	Positive	Positive



**Table 2.6. Continued**

RNA copy number	CCHFV NASBA	CCHFV NucliNASBA
$3.7 \times 10^9$	Positive	Positive
$3.7 \times 10^8$	Positive	Positive
$3.7 \times 10^7$	Positive	Positive
$3.7 \times 10^5$	Positive	Positive
$3.7 \times 10^4$	Positive	Positive
$3.7 \times 10^3$	Positive	Positive
$3.7 \times 10^2$	Positive	Positive
$3.7 \times 10^1$	Positive	Positive
$3.7 \times 10^0$	Positive	Negative
$3.7 \times 10^{-1}$	Negative	Negative
$3.7 \times 10^{-2}$	Negative	Negative

### 2.3.7. Application of the NASBA assay using clinical samples

RNA extracted from clinical samples collected from patients with suspected viral haemorrhagic fever were tested for CCHF viral nucleic acid using two commercially available real-time RT-PCR assays, the CCHFV NASBA assay and the CCHFV NucliNASBA assay. A total of 16/16 samples were confirmed to be negative using all four assays. The clinical cut-off was calculated using data from 16 known negative samples tested in triplicate (Appendix B) and was determined as a value of 0.122 and 0.117 for the CCHFV NASBA and the CCHFV NucliNASBA, respectively. A total of 22/22 samples tested were positive using the commercial real-time RT-PCR assays. In comparison 14/22 and 20/22 were positive using the CCHFV NASBA assay and the CCHFV NucliNASBA assay, respectively. Although the CCHFV NASBA assay detected lower copies of RNA in the control, it detected fewer clinical samples. This is likely a result of inhibition of the amplification reaction. It is likely that a larger sample number is required to clarify this point. A detailed list of the results can be found in Appendix C.

**Table 2.7. Detection of RNA samples extracted from patient sera**

	<b>CCHFV NASBA</b>	<b>CCHFV NucliNASBA</b>	<b>TaqMan OneStep RT-PCR kit</b>	<b>RealStar® CCHFV RT-PCR kit 1.2</b>
	<b>Number of samples</b>			
<b>Positive</b>	14	20	22	22
<b>Negative</b>	24	18	16	16

**2.3.8. Factors inhibiting the CCHFV NASBA assay**

Sera were collected from four volunteers and spiked with the control RNA transcript to evaluate the effect of potential inhibitors on the amplification of CCHF viral RNA using the CCHFV NASBA assay. Table 2.8. shows the results obtained. The CCHFV NASBA assay was able to detect at least 2000 RNA copies extracted from VBD 16/14, at least 200 RNA copies extracted from VBD 20/14 and at least 20 RNA copies extracted from VBD 21/14, but was unable to detect RNA extracted from VBD 19/14.

**Table 2.8. Detection of serial dilutions of RNA extracted from spiked sera**

<b>RNA copy number</b>	<b>VBD 16/14</b>	<b>VBD 19/14</b>	<b>VBD 20/14</b>	<b>VBD 21/14</b>
0	Negative	Negative	Negative	Negative
2	Negative	Negative	Negative	Negative
20	Negative	Negative	Negative	Positive
200	Negative	Negative	Positive	Positive
2000	Positive	Negative	Positive	Positive
20000	Positive	Negative	Positive	Positive
200000	Positive	Negative	Positive	Positive

## 2.4. Summary

Molecular assays are frequently used to diagnose acute CCHFV infections. Molecular assays provide a rapid result and are useful for isolation of the patient to protect healthcare workers and for administration of supportive therapy at an early stage of infection. In an attempt to prepare a rapid, safe and inexpensive molecular assay to increase the diagnostic capacity of CCHFV, a NASBA assay for detection of CCHF viral RNA was developed and optimized. The in-house enzyme mixture and buffer were compared with a commercially available enzyme mixture and buffer provided in the NucliSENS® EasyQ Basic Kit v2. RNA samples extracted from CCHFV-infected Vero cell culture and from clinical samples were supplied by the CEZD, NICD, Johannesburg. Additionally, RNA extracted from RVFV- and YFV-infected cell culture were tested for specificity. The RNA extracted from infected cell culture were initially tested using a nested RT-PCR, which confirmed CCHF viral RNA in a total of 48/48 samples. To distinguish positive from negative results using the NASBA assays, a cut-off value was determined using a panel of negative samples as values of 0.156 and 0.166, respectively. RNA extracted from cell culture infected with SPU 4/81 was selected as control, serially diluted and tested to determine the lower detection limit of the NASBA assays. The CCHFV NASBA assay was able to detect RNA diluted  $\leq 10^{-4.5}$  and the CCHFV NucliNASBA assay was able to detect RNA diluted  $\leq 10^{-5.5}$ . The results suggest that the CCHFV NASBA was less sensitive for detection of RNA from isolate SPU 4/81 than the CCHFV NucliNASBA assay. In the absence of absolute concentration of RNA used to prepare the dilutions a relative sensitivity can be determined and not absolute sensitivity. The ability of the NASBA primers to detect genetically diverse strains of CCHFV from southern Africa was evaluated using RNA extracted from Vero cell culture infected with 48 southern Africa CCHFV isolates. The sequence data available for these isolates suggests that the diversity ranged p to 18% (Burt and Swanepoel, 2005; Burt et al, 2009). A total of 41/48 samples tested positive using the CCHFV NASBA assay. RNA extracted from RVFV- and YFV-infected cell culture tested negative. To facilitate the use of a NASBA assay in low resource laboratories or in field stations the use of a water bath and heating block was tested to replace the thermocycler. Three RNA samples extracted from CCHFV-infected cell culture were randomly selected and tested using the CCHFV NASBA with a water bath and a heating block. The results obtained with the water bath and the heating block were in concordance with the results obtained with a thermocycler. A control RNA transcript was prepared from PCR

template and was used to assist with validation of the CCHFV NASBA assay, to determine the lower detection limit of the NASBA assays and to investigate the inhibitory effects of sera. The lower detection limits were determined as 3.7 copies RNA and 37 copies RNA for the CCHFV NASBA assay and the CCHFV NucliNASBA assay, respectively. The application of the CCHFV NASBA assay as a diagnostic test was evaluated using RNA extracted from clinical samples. RNA from serum samples collected from 22 CCHFV-infected patients and 16 patients with suspected viral haemorrhagic fever were tested with two commercial real-time RT-PCR assays and a total of 22/22 were positive with 16/16 suspected cases testing negative. A clinical cut-off value was determined using the panel of clinical samples known to be negative for CCHF viral RNA as a value of 0.122 for the CCHFV NASBA and 0.117 for the CCHFV NucliNASBA. The application of the CCHFV NASBA assay as a diagnostic test was evaluated using the RNA extracted from clinical samples. In comparison with the commercial assays, 14/22 and 20/22 tested positive using the CCHFV NASBA assay and the CCHFV NucliNASBA assay, respectively. From the suspected cases, 16/16 samples tested negative using all four assays. The ability of the CCHFV NASBA assay to detect RNA extracted from clinical samples was disappointing. A possible explanation could be the presence of inhibitors in the sera which was briefly investigated using spiked sera. Serum samples were collected from four volunteers and spiked with a range of 0 – 200 000 copies of the control RNA transcript. The RNA were extracted and tested with the CCHFV NASBA assay, which was able to detect at least 2000 copies RNA, 200 copies RNA and at least 20 copies RNA extracted, respectively, from three volunteers. However, no RNA could be detected from the sera of the fourth volunteer despite repeated extractions and amplifications.

In summary, the results suggest that the CCHFV NASBA assay had lower sensitivity than the commercial assays and there may be inhibitors of the reaction in human serum. The assay could have diagnostic application if the limitations are considered, with the inclusion of an internal control to determine if a negative result is possibly due to inhibition of the reaction. In the absence of sophisticated laboratory equipment the assay could have application in building diagnostic capacity however, in addition to including an internal control for detecting inhibition of amplification reaction, the lower sensitivity suggests that in the event of a negative result a confirmatory assay would be required.

## **Chapter 3 – Development of serological assays for detection of antibodies against Crimean-Congo haemorrhagic fever virus**

### **3.1. Introduction**

Serological assays are useful tools to confirm CCHFV infection and for surveillance studies. In most non-fatal CCHF cases IgM and IgG antibodies can be detected as early as day 5 (Tang et al, 2003) of illness using IFA and in some instances as early as day 3 using ELISA. In general most survivors have detectable IgG and IgM antibody by day 5 and onwards of illness (Burt et al, 1994; Jacobson, 1998, Tang et al, 2003; Saijo et al, 2005). The IgM activity declines to undetectable levels in  $\pm$  four months, but it has been detected for up to 12 months (Burt et al, 2011). The IgG activity declines gradually but is likely lifelong and has been detected 12 years after illness (Samudzi et al, 2012). Recent or current infection can be indicated by IgM activity within a single sample;  $\geq$  fourfold increase in IgG titer or seroconversion within paired samples (Burt et al, 2007; Burt, 2011). False-positive results can occur due to the presence of rheumatoid factor, an anti-IgG antibody of the IgM family, and pre-treatment of samples when testing for IgM may be indicated (Dowall et al, 2012). Current serological assays frequently require BSL-4 facilities to prepare reagents, for instance indirect IFA usually utilize antigen slides prepared from CCHFV-infected cell culture and ELISA techniques frequently utilize sucrose-acetone-extracted antigen prepared from infected mouse brain suspension and inactivated with 0.1%  $\beta$ -propiolactone (Burt, 2011). Most ELISA methods used currently are based on a capture method, especially for IgM antibody detection, which often necessitates the use of polyclonal antibodies raised in animals (Shepherd et al, 1989; Burt et al, 1993; Tang et al, 2003; Saijo et al, 2005; Garcia et al, 2006). Direct or indirect ELISA and IFA can make use of recombinant proteins to detect anti-CCHFV antibodies (Marriott et al, 1994; Saijo et al, 2002a, Saijo et al, 2002b, Tang et al, 2003; Saijo et al, 2005; Garcia et al, 2006; Samudzi et al, 2012), without the necessity to culture live virus in BSL-4 facilities.

The CCHFV S segment encoding the NP is the most conserved region of the genome (Deyde et al, 2006). The NP has been shown to be highly immunogenic (Schmaljohn and Nichol, 2007) and is frequently targeted for preparation of recombinant antigens for diagnostic assays. Recombinant NP have been successfully expressed via baculovirus expression systems (Marriott

et al, 1994; Saijo et al, 2002b) and tested in ELISA with sera from infected patients (Marriott et al, 1994; Saijo et al, 2002b; Saijo et al, 2005; Garcia et al, 2006).

In an attempt to prepare recombinant antigen for use in ELISA or transfected cells for preparation of antigen slides for IFA, two expression systems were investigated. A recombinant baculovirus expressing the CCHFV NP was prepared using the Bac-to-Bac® system for transfection of *Spodoptera frugiperda* 9 (*Sf9*) insect cells. Additionally, the pcDNA™ 3.1 Directional TOPO® Expression kit was used to prepare a recombinant pcDNA™ 3.1D/V5-His-TOPO® plasmid for expression of the CCHFV NP for transfection of mammalian cells.

### **3.2. Methods and materials**

#### **3.2.1. Serum samples**

Serum samples were collected from a convalescent CCHFV-infected patient and from a volunteer known to be negative for anti-CCHFV antibodies. Each sample was allocated a laboratory number, designated VBD on accession. These sera were previously tested in our laboratory using ELISA with recombinant CCHFV NP in an unrelated study (Samudzi et al, 2012; Rangunwala et al, 2014). Titer of the sera was > 1:100 and hence should be diluted 1:10 for use in IFA.

#### **3.2.2. Bac-to-Bac system for baculovirus expression of recombinant CCHFV NP**

The Bac-to-Bac® expression system utilizes a baculovirus shuttle vector, or bacmid. The system relies on the generation of recombinant bacmid through site-specific transposition in *E. coli* cells instead of homologous recombination in insect cells. As with all baculovirus expression systems the expression of the foreign gene is driven by the polyhedron promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV), which is highly transcribed during the late stages of baculovirus infection (Luckow et al, 1993; Anderson et al, 1996). AcNPV has a large double-stranded circular DNA genome, 130 kb, with multiple recognition sites for various RE. The foreign gene is inserted into a plasmid transfer vector downstream of the baculovirus promoter through directed cloning and the recombinant plasmid transfer vector is used to generate recombinant bacmid through transposition. The recombinant bacmid is used to transfect insect cells.

In this study the ORF of the CCHFV S segment was inserted into a plasmid transfer vector, pFastBac, through directed cloning in the insertion site flanked by Tn7. This led to the formation of a mini-Tn7 element. The recombinant pFastBac plasmid was used to transpose DH10Bac<sup>TM</sup> *E. coli* cells that contain the bacmid. The mini-Tn7 element was transposed to the bacmid through the functioning of a helper plasmid contained in the DH10Bac<sup>TM</sup> *E. coli* cells. The recombinant bacmid was used to transfect insect cells, where the expression of the recombinant protein was controlled by the baculovirus promoter (Luckow et al, 1993; Anderson et al, 1996).

#### **3.2.2.1. Optimizing and synthesizing the ORF of the CCHFV S segment for baculovirus expression**

The ORF of the S segment of the CCHFV strain SPU 415/85 was codon optimized for expression in insect cells by GenScript (GenScript USA inc., New Jersey, USA) and supplied in the pUC57 transfer plasmid. The optimized gene was 1458 bp in length, with BamHI sites included at the 5' and 3' ends of the gene (Appendix E) to facilitate RE digestion from pUC57 and insertion into the pFastBac HT B plasmid. The vector map and features of the pUC57.CCHFV.NP.opt construct are shown in Appendix E.

#### **3.2.2.2. Primer design**

Primers were designed to flank the codon optimized CCHFV NP gene. Additionally, primers were designed to flank the cloning site of the pFastBac HT B plasmid. The primer sequences and positions are shown in Table 3.1.

**Table 3.1. Primers for analysis of baculovirus expression construct**

Primer name	Sequence (5' – 3')	Tm (°C)	Position on gene/plasmid
CCHFV NP bac opt F	GGA TCC GAA AAC AAG ATC GAG GTC AAC AAC AAG GAC	61	1 – 36*
CCHFV NP bac opt R	GGA TCC GGG ATG ATG TTG ACT GAT GTA GCG TTG	61	1448 – 1425*
pFastBac HT B F	CCG GAT TAT TCA CGT CCC ACC ATC GGG	61	3999 – 4018**
pFastBac HT B R	GTA CTT CTC GAC AAG CTT GGT ACC GCA TGC	59	4235 – 4206**

\* These positions are relative to the codon optimized CCHFV NP gene received from GenScript.

\*\* These positions are relative to the pFastBac HT B plasmid

### 3.2.2.3. Preparation of the recombinant CCHFV NP construct

The pFastBac HT vector contains a polyhedrin promoter for high-level protein expression, a multiple cloning site, an N-terminal 6× Histidine (His)-tag, for downstream protein purification, and a Tobacco Etch Virus protease cleavage site, for removal of the His-tag (Polayes et al, 1996). The pFastBac HT vector is available in three different reading frames (Polayes et al, 1996) and for the purpose of this project the pFastBac HT B vector (Appendix E) was selected in order to clone the CCHFV NP gene in frame with the stop codon of the plasmid.

One Shot® Top10 chemically competent *E. coli* cells were transformed as described in 2.2.4.1. with the following modifications, 40.0 µg of pUC57.CCHFV.NP.opt and 25.0 µg pFastBac HT B plasmid were used to transform the *E. coli* cells, respectively. White colonies from each plate were selected and grown in 5.0 ml aliquots of LB/Amp at 37°C for 16 h. Plasmid was purified from the cultures using the PureYield Plasmid Miniprep system (Promega, Wisconsin, USA) according to the manufacturer's protocol. Briefly, a 1.5 ml aliquot of each overnight culture was added to separate, sterile 2.0 ml tubes and centrifuged for 30 s at 16 000× g, after which the supernatant fluid (SNF) was discarded. This step was repeated. Each pellet was completely resuspended in a 600.0 µl aliquot of distilled water. A 100.0 µl aliquot of Cell Lysis buffer with



added RNase A supplied in the kit was added to each of the tubes and the tubes were inverted until the content turned blue. A 350.0  $\mu\text{l}$  aliquot of Neutralization solution supplied in the kit was added to each tube and the tubes were inverted until the content turned completely yellow. The tubes were centrifuged 16 000 $\times$  g for 3 min. The SNF of each tube was transferred to separate minicolumns in collection tubes and centrifuged at 16 000 $\times$  g for 15 s. The DNA was washed with a 200.0  $\mu\text{l}$  aliquot of Endotoxin removal and a 400.0  $\mu\text{l}$  aliquot of Column wash with added ethanol. The DNA was eluted in a 30.0  $\mu\text{l}$  aliquot of NFW.

The CCHFV.NP.opt gene was rescued from pUC57 through RE digestion using BamHI. Briefly, a digestion reaction consisted of 1.0  $\mu\text{l}$  BamHI (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA), 2.0  $\mu\text{l}$  10 $\times$  BamHI buffer (Fermentas, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA), 5.0  $\mu\text{l}$  pUC57.CCHFV.NP.opt plasmid DNA and NFW up to 20.0  $\mu\text{l}$ . The digestion reaction was incubated at 37°C for 2 h. The 20.0  $\mu\text{l}$  aliquot of the digested product and a 5.0  $\mu\text{l}$  aliquot of the undigested plasmid were visualized by agarose gel electrophoresis as described in 2.2.2.1. The DNA representing the CCHFV.NP.opt gene was excised from the agarose gel and purified using Wizard® SV Gel and PCR Clean up system as described in 2.2.4.1. with the following modifications: the agarose gel slice was weighed, Membrane Binding Solution was added at a ratio of 10.0  $\mu\text{l}$  of solution per 10.0 milligram (mg) of agarose gel slice and the agarose gel slice was dissolved at 65°C for 10 min. The tube was briefly vortexed and centrifuge. The dissolved agarose gel mixture was added to an SV minicolumn and the remainder of the procedure was performed as described in 2.2.4.1.

The pFastBac HT B plasmid (Invitrogen, California, USA) was prepared for cloning by digestion of the plasmid using 5.0  $\mu\text{l}$  pFastBac HT B plasmid DNA, 1.0  $\mu\text{l}$  BamHI, 2.0  $\mu\text{l}$  10 $\times$  BamHI buffer and NFW up to a total volume of 20.0  $\mu\text{l}$ . The digestion reaction was incubated at 37°C for 2 h. Digested and undigested plasmids were visualized by agarose gel electrophoresis and the DNA concentration was measured as described in 2.2.2.1. The linearized pFastBac HT B plasmid was dephosphorylated using Antarctic Phosphatase (New England Biolabs, Massachusetts, USA). Briefly, the reaction consisted of 120.0 ng linearized pFastBac HT B, 5.0 U Antarctic Phosphatase, 1 $\times$  Antarctic Phosphatase Reaction buffer (New England Biolabs, Massachusetts, USA) and NFW up to 20.0  $\mu\text{l}$ . The reaction was incubated at 37°C for 60 min and the enzyme deactivated at 65°C for 5 min.

The CCHFV.NP.opt was ligated into the linearized and dephosphorylated pFastBac HT B plasmid. Briefly, the ligation reaction consisted of 50.0 ng linearized pFastBac HT B plasmid, 15.0 ng CCHFV.NP.opt., 0.3 Weiss units/ $\mu\text{l}$  T4 DNA ligase, 1 $\times$  Rapid ligation buffer and NFW up to 10.0  $\mu\text{l}$ . The reaction was incubated at 4°C for 16 h.

The ligation product was used to transform OneShot® Top10 chemically competent *E. coli* as described in 2.2.4.1. The transformation efficiency was calculated as described in the manufacturer's instructions as  $3.7 \times 10^8$  colony forming units/ $\mu\text{g}$ .

#### **3.2.2.4. Confirmation of positively transformed cells**

White colonies were selected and divided into two aliquots. One aliquot of each colony was digested with BamHI as described in 3.2.2.3. The digested products were separated and visualised by agarose gel electrophoresis as described in 2.2.2.1.

The remaining aliquot of each colony that was shown to be a positive transformant was grown in 5.0 ml of LB/Amp at 37°C whilst shaking at 150 rpm for 16 h. The 5.0 ml overnight culture was used to inoculate 20.0 ml LB/Amp and was incubated at 37°C whilst shaking at 150 rpm for 3 h. The pFastBac HT B.CCHFV.NP.opt.plasmid was purified using the QIAGEN Plasmid *Plus* Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, the 25.0 ml LB/Amp overnight culture was collected by centrifugation at 6 000 $\times$  g for 15 min in a 50.0 ml Falcon tube (Thermo Scientific, Illinois, USA), the SNF was discarded and the pellet resuspended in a 4.0 ml aliquot of Buffer P1 with added RNase A. A 4.0 ml aliquot of Buffer P2 was added, the content mixed by inverting and incubated at room temperature for 3 min. A 4.0 ml aliquot of Buffer S3 was added, mixed by inverting and the lysate transferred to the QIAfilter cartridge in a sterile 50.0 ml Falcon tube. The lysate was incubated at room temperature for 10 min. A plunger was inserted into the QIAfilter cartridge and gently pressed down, forcing the lysate through the filter. A 2.0 ml aliquot of Buffer BB was added to the filtered lysate and mixed by inverting. The lysate mixture was transferred to a QIAGEN Plasmid *Plus* Midi spin column with a tube extender attached to a vacuum manifold. The solution was drawn through the QIAGEN Plasmid *Plus* Midi spin column through use of the vacuum pump. The QIAGEN Plasmid *Plus* Midi spin column was washed with a 700.0  $\mu\text{l}$  aliquot of Buffer ETR and the liquid was drawn through using the vacuum pump. The wash was repeated with a 700.0  $\mu\text{l}$  aliquot of Buffer PE and the liquid was drawn through using the vacuum pump. The

QIAGEN Plasmid *Plus* Midi spin column was removed from the vacuum manifold and placed in a collection tube. The assembly was centrifuged at  $10\,000\times g$  for 1 min. The QIAGEN Plasmid *Plus* Midi spin column was placed in a sterile 1.5 ml tube and a 100.0  $\mu\text{l}$  aliquot of NFW was added to the centre of the QIAGEN Plasmid *Plus* Midi spin column. The assembly was incubated at room temperature for 1 min and clarified at  $16\,000\times g$  for 1 min. The DNA concentration and purity were measured as described in 2.2.4.1.

The correct orientation of the CCHFV.NP.opt was confirmed by PCR using GoTaq® Flexi DNA Polymerase system as described in 2.2.4.2. with the following modifications: the CCHFV NP bac opt F primer (Table 3.1.) and pFastBac HT B reverse primer (Table 3.1.) were used and the reaction was performed with an annealing temperature of 61°C. Genes that were inserted in the incorrect orientation would not have been amplified with this primer pair.

### **3.2.2.5. Preparation and purification of the recombinant bacmid**

The site-specific transposition of the recombinant plasmid transfer vector prior to transfection of insect cells distinguishes the Bac-to-Bac® system from other baculovirus expression systems. The bacmid contains a mini-F replicon and an *attTn7* site in the polyhedrin locus of AcNPV (Appendix E). The mini-F replicon allows autonomous replication, a selectable kanamycin marker and stable segregation of plasmids at low copy number. The *attTn7* is a target site for the bacterial transposon Tn7. The Tn7 transposon inserts at a high frequency into the *attTn7* site located on the *E. coli* chromosome and into DNA segments of plasmids that contain the *attTn7* site (Luckow et al, 1993; Anderson et al, 1996).

The recombinant pFastBac HT B.CCHFV.NP.opt construct was diluted 1:10 000 and used to transpose MAX Efficiency® DH10Bac™ competent cells (Invitrogen, California, USA) containing both the bacmid and helper plasmid according to the manufacturer's instructions. Briefly, a 100.0  $\mu\text{l}$  aliquot of Max Efficiency® DH10Bac™ competent cells was thawed completely on ice and 36.6 pg pFastBac HT B.CCHFV.NP.opt plasmid DNA was added. The cells were gently mixed and incubated on ice for 30 min. Cells were heat shocked for 45 s at 42°C and returned to the ice for 2 min. A 900.0  $\mu\text{l}$  aliquot of SOC media was added to the cells and the cells were incubated at 37°C whilst shaking at 150 rpm for 4 h. A 100.0  $\mu\text{l}$  aliquot of the cells were spread on LB plates containing 50.0  $\mu\text{g/ml}$  kanamycin sulfate (Sigma-Aldrich, Missouri, USA), 7.0  $\mu\text{g/ml}$  gentamicin (Sigma-Aldrich, Missouri, USA), 10.0  $\mu\text{g/ml}$

tetracycline (Sigma-Aldrich, Missouri, USA), 0.1 mM IPTG and 80.0 µg/ml X-gal at 37°C for 24 h. The transformation efficiency was calculated as  $2.4 \times 10^5$ . White colonies were selected and divided into two aliquots. One aliquot was used to confirm the correct insertion of the CCHFV.NP.opt gene into the bacmid by PCR using GoTaq® Flexi DNA Polymerase system according to the manufacturer's protocol as described in 2.2.4.2. with the following modifications, the M13 Forward primer (5' CCC AGT CAC GAC GTT GTA AAA CG 3') and M13 Reverse primer (5' AGC GGA TAA CAA TTT CAC ACA GG 3') were used with a annealing temperature of 55°C. The positions of the M13 Forward and M13 Reverse primers are shown in Appendix E. The amplicons were separated and visualised by agarose gel electrophoresis as described in 2.2.2.1.

The remaining aliquot of a colony consisting of positively transposed cells was grown in 5.0 ml of LB media containing 50 µg/ml kanamycin sulfate, 7 µg/ml gentamicin and 10 µg/ml tetracycline at 37°C for 16 h whilst shaking at 150 rpm. The overnight culture was used to inoculate 20.0 ml of LB media containing 50 µg/ml kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline at 37°C for 3 h whilst shaking at 150 rpm. The composite bacmid was purified using QIAGEN Plasmid *Plus* Midi kit, as described in 3.2.2.4., and the purified composite bacmid was sequenced to confirm the insert of the optimized CCHFV NP gene using the ABI PRISM® BigDye™ Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, California, USA). Briefly, the 10.0 µl sequencing reaction consisted of 3.2 pmol primer, 2.0 µl Terminator Ready Reaction mix, 1× sequencing buffer and 752.5 ng composite bacmid. The composite bacmid was sequenced in duplicate using the CCHFV M13 Forward primer and the M13 Reverse primer. The following cycling conditions were used: 96°C for 1 min, 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min and cooling at 4°C for unlimited time. The reaction was purified according to the manufacturer's instructions. Briefly, the reaction was added to a 10.0 µl aliquot of NFW, gently mixed and transferred to a 1.5 ml tube containing 5.0 µl 125 mM disodium ethylenediaminetetraacetate (Na<sub>2</sub>EDTA) and 60.0 µl 100% ethanol. The tube was vortexed for 5 s and incubated at room temperature for 15 min. The tube was centrifuged at 14 000× g for 20 min at 4°C. The SNF was discarded and a 500.0 µl aliquot of ice cold 70% ethanol was added. The tube was centrifuged at 14 000× g for 10 min at 4°C and the SNF was discarded. The tube was incubated with an open lid at 37°C for 16 h. The sequencing reactions were submitted to The Department of Microbiology, Biochemistry and

Food Biotechnology, Faculty of Natural and Agricultural Sciences, University of the Free State, Bloemfontein, SA for electrophoresis. The nucleotide sequence data was edited using BioEdit version 7.1. (<http://bioedit.software.informer.com/7.1/>) and ChromasPro version 2.1.1. ([www.technelysium.com.au/ChromasLite211Setup.exe](http://www.technelysium.com.au/ChromasLite211Setup.exe)), aligned using ClustalX with nucleotide sequence data the CCHFV.NP.opt gene received from GenScript and analyzed using BLAST to confirm the identity of the nucleotide sequence.

#### **3.2.2.6. Transient transfection of *Sf9* insect cells**

Transfection of *Sf9* insect cells with the recombinant bacmid.CCHFV.NP construct was done according to the manufacturer's instructions in the Bac-to-Bac® baculovirus expression system manual. A fresh dilution of exponentially growing *Sf9* cells, kindly supplied by Dr CM Knox from the Department of Microbiology at Rhodes University, was prepared in TC-100 medium (Invitrogen, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (FBS) (Delta Bioproducts, Kempton Park, SA) and 1% penicillin and streptomycin mixture (p/s) (Lonza, Verviers, Belgium) in a T-25 flask (Corning® Life Sciences, New York, USA) incubated at 28°C for 16 h. Wells in a six-well plate (Corning® Life Sciences, New York, USA) were seeded with  $8 \times 10^5$  cells and incubated at 28°C for 90 min. The transfection complexes were prepared as follows: a 8.0 µl aliquot of Cellfectin® II reagent (Invitrogen, California, USA) was diluted in a 100.0 µl aliquot of TC-100 media supplemented with 10% FBS for each well, 1.0 ng, 2.0 ng and 5.0 ng purified composite bacmid DNA was diluted in a 100.0 µl aliquot of TC-100 media supplemented with 10% FBS and the diluted Cellfectin® reagent was added drop wise to the diluted DNA, gently mixed and allowed to incubate for 30 min at room temperature. The transfection complexes were added drop wise to each of three seeded wells, respectively, and allowed to incubate for 5 h at 28°C, after which the media was replaced with fresh TC-100 media supplemented with 10% FBS and 1% p/s. The remaining three seeded wells of the plate functioned as the negative controls and did not receive any transfection complexes. The plate was incubated at 28°C for 72 h.

Table 3.2.indicates various conditions used to optimize transfections including different ratios of DNA to transfection reagent and earlier passages of insect cells. Recombinant bacmid was freshly prepared for transfection attempts 5 – 8. All transfection experiments were subsequently analysed for expression of the recombinant CCHFV NP.

**Table 3.2. Variations in the *Sf9* transfection attempts**

	Transfection attempt number							
	1	2	3	4	5	6	7	8
Passage number of <i>Sf9</i> cells stock	12	12	12	12	8	8	8	8
DNA concentration (ng/ $\mu\text{l}$ )	1	2	5	2	2	2	2	2
Volume of Cellfectin® II transfection reagent ( $\mu\text{l}$ )	8	8	8	10	8	10	6	8
Incubation time of <i>Sf9</i> cells with transfection complexes (h)	5	5	5	5	5	5	5	16

### 3.2.2.7. Analysis of the recombinant CCHFV NP

The cell culture media from transfected cells and non-transfected control cells were harvested. The transfected and non-transfected cells were harvested and stored. A 250.0  $\mu\text{l}$  aliquot of cell culture media from each transfection was added to a T-25 flask seeded with  $2.2 \times 10^6$  *Sf9* cells in 6.0 ml TC-100 media supplemented with 10% FBS and 1% p/s. The cells were incubated at 28°C for 72 h. The cell culture media was collected and stored as passage one SNF (P1 SNF). The attached cells were passaged by gently knocking the side of each flask and resuspended the detached cells in 2.0 ml supplemented TC-100 media. These cells were passage one (P1) of transfected cells. A 1.0 ml aliquot of the P1 cells was used to seed a T-25 flask containing 5.0 ml supplemented TC-100 media and the flask was incubated at 28°C for 72 h. Cells were passaged a further four times and from each passage aliquots of resuspended cells, designated P2 to P5, and harvested cell culture media, designated P2 SNF to P5 SNF, were stored for further analysis. A 250.0  $\mu\text{l}$  aliquot of each cell fraction was lysed using 100.0  $\mu\text{l}$  NET/BSA (Appendix D) on ice for 30 min. The tubes were centrifuged at  $2\,000\times g$  at 4°C for 10 min and SNF was removed and stored as post lysis SNF fractions. The pellet was resuspended in a 100.0  $\mu\text{l}$  aliquot of phosphate buffered saline solution (PBS) pH 7.4 (Appendix D). The resuspended cell pellets (P1 to P5), the post lysis SNF1 to SNF5 fractions and the SNF (P1SNF to P5SNF) were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot.

### **3.2.2.7.i. SDS-PAGE**

Proteins were analysed using SDS-PAGE. Briefly, a 10% separation polyacrylamide gel consisted of 2.667 ml of acryl/bisacrylamide (Sigma-Aldrich, Missouri, USA), 1.065 ml of 3 M (pH 8.8) tris(hydroxymethyl)aminomethane (Merck, Gauteng, SA), 4.267 ml of distilled water, 200.0 µl of 10% SDS (BDH Chemicals Ltd, Poole, England), 54.0 µl of 10% ammonium persulfate (Promega, Wisconsin, USA) and 10.0 µl of tetramethylethylenediamine (TEMED) (Merck, Darmstadt, Germany) was prepared. The gel was poured into the BG-verMINI glass plate assembly (BayGene Biotech Company Ltd, Beijing, China) to ~85% of the capacity. A layer of amyl alcohol was added and the gel was allowed to set at room temperature. The 4% stacking gel consisted of 400.0 µl of acryl/bisacrylamide, 210.0 µl of 0.5 M (pH 6.8) tris(hydroxymethyl)aminomethane, 2.37 ml of distilled water, 30.0 µl of 10% SDS, 60.0 µl of 10% ammonium persulfate and 6.0 µl of TEMED. The layer of amyl alcohol was removed and the stacking gel was added to the BG-verMINI glass plate assembly, the comb was inserted into the stacking gel and the gel allowed to set at room temperature. The protein samples were prepared using the 5× Lane Marker Reducing Sample Buffer (Thermo Scientific, Illinois, USA). Briefly, 5.0 µl of 5× Lane Marker Reducing Sample Buffer was mixed with 20.0 µl of each sample, the sample mixture was boiled at 95°C for 5 min and cooled to room temperature. A 10.0 µl aliquot of PageRuler™ Unstained Protein Ladder (Thermo Scientific, Illinois, USA) and the samples were loaded into the empty wells of the polyacrylamide gel. The 1× tris/glycine/SDS running buffer was prepared by diluting 10× premixed tris/glycine/SDS running buffer (Bio-Rad, California, USA) 1:10 in distilled water and was used to fill the bath of the cell. Gel electrophoresis was performed using the BG-Power 300 system (BayGene Biotech Company Ltd, Beijing, China) at 60 V for 30 min, followed by 140 V for 90 min. The gel was stained and destained using the Fairbanks staining method (Appendix D).

### **3.2.2.7.ii. Western blot**

For western blot analysis polyacrylamide gels were prepared as described above using a 10.0 µl aliquot of PageRuler™ Prestained Protein Ladder (Thermo Scientific, Illinois, USA) instead of the PageRuler™ Unstained Protein Ladder and the protein gel was transferred to a nitrocellulose membrane. Briefly, the BioTrace™ Polyvinylidene fluoride (PVDF) transfer membrane (Pall

Corporation, Florida, USA) was activated in methanol (Merck, Gauteng, SA) for 3 min whilst shaking at 100 rpm, rinsed in distilled water for 2 min and soaked in transfer buffer (Appendix D) for 3 min whilst shaking at 100 rpm. The protein gel was soaked in transfer buffer for 15 min. Additionally, two pieces of Protean blotting paper (Bio-Rad, California, USA) were soaked in transfer buffer whilst shaking at 100 rpm. The gel was transferred to the membrane using Trans-blot® SD Semi-Dry Transfer Cell (Bio-Rad, California, USA). The PVDF membrane was washed with TBS containing 1% Tween® 20 (Promega, Wisconsin, USA) (1% TBS-T) for 10 min. The primary antibody was prepared as a 1:50 dilution of mouse anti-His antibody (Roche, Mannheim, Germany) in 10% fat free milk/TBS (Appendix D). The PVDF membrane was incubated with the primary antibody for 16 h at 4°C. The PVDF membrane was washed three times with TBS-T whilst shaking at 100 rpm. The secondary antibody was prepared as a 1:10 dilution of Fast Western Mouse Optimized HRP reagent from the Pierce® Fast Western blot kit, SuperSignal West Pico Substrate, Mouse (Thermo Scientific, Illinois, USA) in 10% fat free milk/TBS. The PVDF membrane was incubated with the secondary antibody for 1 h at room temperature whilst shaking at 100 rpm. The wash step was repeated as described above with an additional 10 min wash with TBS. The SuperSignal West Pico Solution was prepared according to the manufacturer's instructions. Briefly, a 5.0 ml aliquot of SuperSignal West Pico Luminol/Enhancer Solution, provided in Pierce® Fast Western blot kit, SuperSignal West Pico Substrate, Mouse (Thermo Scientific, Illinois, USA), was mixed with a 5.0 ml aliquot of SuperSignal Stable Peroxide Solution, provided in Pierce® Fast Western blot kit, SuperSignal West Pico Substrate, Mouse (Thermo Scientific, Illinois, USA). The PVDF membrane was incubated in the SuperSignal West Pico solution for 1 min at room temperature, clear plastic wrap was folded around the PVDF membrane and the PVDF membrane was moved to a dark room. The wrapped PVDF membrane was exposed to CL-XPosure™ Film (Thermo Scientific, Illinois, USA) for 1 min. The film was incubated in X-Ray Developer (Axim Africa X-Ray Industrial and Medical (Pty) Ltd, Midrand, SA) for 3 min and placed in X-Ray Fixer (Axim Africa X-Ray Industrial and Medical (Pty) Ltd, Midrand, SA) for 2 min. Finally, the film was rinsed with water to remove excess fixer and visualized.



### **3.2.2.7.iii. IFA**

Transfected cells were used to prepare antigen slides to determine if CCHFV NP had been expressed using IFA. A 250.0  $\mu\text{l}$  aliquot of P2 – P5 cells was centrifuged at  $2\,000\times g$  for 2 min, the SNF was discarded and each pellet resuspended in 50.0  $\mu\text{l}$   $1\times$  PBS supplemented with 10% FBS to assist with adherence of cells to the glass slides. A 5.0  $\mu\text{l}$  aliquot of the cell suspension was applied to each well of an eight-well glass slide (Flow Laboratories, United Kingdom). The cells were dried on the glass slides at  $37^{\circ}\text{C}$  and the dried cell suspensions were fixed in paraformaldehyde (Merck, Darmstadt, Germany) at  $-20^{\circ}\text{C}$  for 16 h. The glass slides were equilibrated to room temperature and a 5.0  $\mu\text{l}$  aliquot of permeabilization buffer ( $1\times$  PBS with 10% sucrose (Merck, Gauteng, SA) and 1% Tween® 20) was applied to each well. The slides were incubated at  $37^{\circ}\text{C}$  for 20 min at room temperature and washed in  $1\times$  PBS. The primary antibody solutions were prepared by diluting sera from a convalescent CCHF patient and sera from a volunteer known to be negative for anti-CCHFV antibodies 1:10 in permeabilization buffer. The cells were reacted with serum samples designated VBD 30/10 and VBD 7/12 by incubating in a humidified container at  $37^{\circ}\text{C}$  for 90 min. VBD 30/10 was collected from a survivor of CCHF infection and was anti-CCHFV IgG antibody positive. VBD 7/12 was collected from a volunteer with no history of CCHFV infection and was negative for anti-CCHFV IgG antibody. The cells were washed three times in  $1\times$  PBS. The secondary antibody solution was prepared by diluting affinity purified fluorescein isothiocyanate (FITC) labeled goat anti-human IgG (H+L) (KPL, Maryland, USA) 1:40 in Evans Blue (BDH Chemicals Ltd, Poole, England) and the solution was vortexed for 10 s. A 5.0  $\mu\text{l}$  aliquot of the secondary antibody solution was applied to the cells and incubated in a humidified container at  $37^{\circ}\text{C}$  for 30 min. Finally, the cells were washed as described previously, dried, mounted with a coverslip and visualized under the Nikon Optiphot fluorescent microscope (Nikon, Tokyo, Japan) using a 495 nm wavelength filter and  $40\times$  magnification lens.

### **3.2.3. The use of the pcDNA<sup>TM</sup> 3.1 Directional TOPO® Expression kit for mammalian expression of a recombinant CCHFV NP**

The pcDNA<sup>TM</sup> 3.1 Directional TOPO® Expression kit (Invitrogen, California, USA) provides the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO® reagents and One Shot® TOP10 chemically competent *E. coli* cells. The pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO® vector features a human cytomegalovirus

immediate early promoter which facilitates high-level expression of the desired gene in various mammalian cell lines (Boshart et al, 1985; Nelson et al, 1987), a TOPO® cloning site which facilitates rapid directional cloning of blunt-end PCR products into the vector, a carboxyl terminal peptide containing the V5 epitope and a polyhistidine tag which facilitates detection and purification of the recombinant protein and a neomycin resistance gene which facilitates selection for stable cell lines using geneticin. The pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO® vector facilitates efficient directional cloning of the desired gene to produce a recombinant vector that can be used to transfect mammalian cells to express the desired recombinant protein. The vector map and features are shown in Appendix E. For the purpose of this project a recombinant pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct, that was prepared in a previous study in our laboratory (Rangunwala, 2013) (Appendix E), was used to transfect baby hamster kidney (BHK)-21 cells.

### **3.2.3.1. Preparation of the recombinant vector for transfection of mammalian cells**

A 100.0 ng aliquot of the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct was used to transform One Shot® TOP10 chemically competent *E. coli* cells as described in 2.2.4.1. White colonies were selected and divided into two aliquots. One aliquot was used in a PCR reaction to confirm positively transformed cells using the GoTaq® Flexi DNA Polymerase system as described in 2.2.4.2. with the following modifications: the T7 forward primer and the R3 reverse primer were used as primer pair with an annealing temperature of 43°C. Genes that were inserted in the incorrect orientation would not have been amplified with this primer pair. The amplicons were separated and visualised by agarose gel electrophoresis as described in 2.2.2.1. The remaining aliquot of each colony consisting of positively transformed cells was grown in 5.0 ml LB/Amp at 37°C whilst shaking at 150 rpm for 16 h. The 5.0 ml overnight culture was used to inoculate 20.0 ml LB/Amp and was incubated at 37°C whilst shaking at 150 rpm for 3 h. The pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP.plasmid was purified using the QIAGEN Plasmid *Plus* Midi kit according to the manufacturer's instructions as described in 3.2.2.4. The DNA concentration and purity were measured as described in 2.2.4.1.

Additionally, a Sindbis virus-based DNA launch replicon prepared and supplied by Prof. Mark Heise (Carolina Vaccine Institute, University of North Carolina, North Carolina, USA) was used as a positive transfection control. The replicon, designated pSIN-DLR-GFP contained a gene

encoding green fluorescent protein (GFP) and genes conferring resistance to kanamycin and geneticin (vector map and features are shown in Appendix E). Briefly, a 100.0 ng aliquot of the pSIN-DLR-GFP construct was used to transform One Shot® TOP10 chemically competent *E. coli* cells, as described in 2.2.4.1 with the following modifications, 2× TY/kan plates (Appendix D) were used instead of LB/Amp plates. White colonies were selected and divided into two aliquots. One aliquot was used in a PCR reaction to confirm positively transformed cells by PCR using the GoTaq® Flexi DNA Polymerase system as described in 2.2.4.2. with the following modifications: the pSIN forward primer (5' AAA TAG TCA GCA TAG TAC ATT 3') and the pSIN reverse primer (5' TGC AAT TTC CTC ATT TTA TTA 3') were used as primer pair with an annealing temperature of 54°C. Genes that were inserted in the incorrect orientation would not have been amplified with this primer pair. The amplicons were separated and visualised by agarose gel electrophoresis as described in 2.2.2.1. The remaining aliquot of a colony consisting of positively transformed cells was grown in 5.0 ml 2× TY/kan media (Appendix D) at 37°C whilst shaking at 150 rpm for 16 h. The 5.0 ml overnight culture was used to inoculate a 20.0 ml aliquot of 2× TY/kan media and was incubated at 37°C whilst shaking at 150 rpm for 3 h. The pSIN-DLR-GFP plasmid was purified using the QIAGEN Plasmid *Plus* Midi kit according to the manufacturer's instructions as described in 3.2.2.4. The DNA concentration and purity were measured as described in 2.2.4.1.

### **3.2.3.2. Transfection of BHK-21 mammalian cells**

Exponentially growing BHK-21 cells, kindly supplied by Dr CM Knox from the Department of Microbiology at Rhodes University, were prepared in BHK growth media (Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Verviers, Belgium) supplemented with 5% FBS, 1% p/s, 1% *Levo*-Glutamine (Lonza, Verviers, Belgium) and 1% nonessential amino acids (Lonza, Verviers, Belgium)) in a T-25 flask incubated at 37°C for 16 h. A round glass coverslip (12 millimetre in diameter) was added to each of the wells in a 24-well plate (Corning® Life Sciences, New York, USA), followed by a 1.0 ml aliquot of BHK growth media, each well was seeded with  $7.5 \times 10^5$  cells and the plate incubated at 37°C for 16h. The transfection complexes were prepared according to the Lipofectamine® 2000 DNA Transfection reagent (Invitrogen, California, USA) manufacturer's instructions. Briefly, a 2.0 µl aliquot of Lipofectamine® 2000 reagent was diluted in a 50.0 µl aliquot of Opti-MEM® I Reduced Serum media (Life Technologies, Oregon,

USA) for each transfection and incubated at room temperature for 25 min. Meanwhile, 2.0 ng of the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP plasmid was diluted in a 50.0 µl aliquot of Opti-MEM® I Reduced Serum media for each transfection and incubated at room temperature for 5 min. Additionally, 2.0 ng of the pSIN-DLR-GFP plasmid was diluted in a 50.0 µl aliquot of Opti-MEM® I Reduced Serum media for use as a positive transfection control and incubated at room temperature for 5 min. The ratios of DNA to Lipofectamine® 2000 reagent used were determined previously in the laboratory. Each of the Lipofectamine® 2000 reagent dilutions was added to the corresponding DNA dilution, gently mixed and incubated at room temperature for 20 min. The growth media was removed from each well in the seeded 24-well plate and replaced with a 1.0 ml aliquot of transfection media (DMEM supplemented with 5% FBS). The transfection complexes were added drop wise to each well and the plate was incubated in a humidified container at 37°C with 5% CO<sub>2</sub> for 24 h. Two of the seeded wells were used as negative controls and did not receive any transfection complexes.

### **3.2.3.3. Confirmation of expressed recombinant proteins**

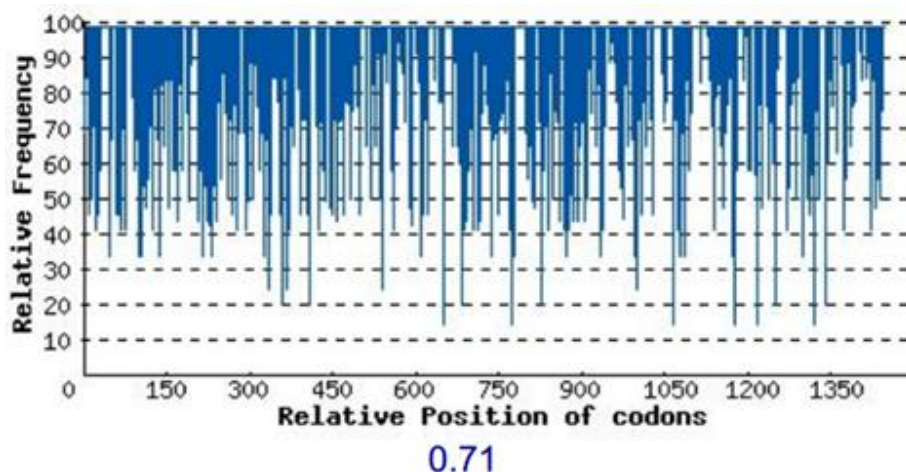
Expression of recombinant NP was confirmed using IFA. Briefly, the transfection media was removed from each well and replaced with an ice cold 1:1 methanol (Merck, Gauteng, SA) and acetone (Merck, Gauteng, SA) solution and the plate was incubated at -20°C for 1 h. The round glass coverslips were removed from each well and placed on clean microscope slides. The coverslips on which the cells were transfected with pSIN-DLR-GFP were visualized directly under fluorescent microscope, as described in 3.2.2.7.iii. IFA was performed on cells transfected with the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP plasmid to detect expressed CCHFV NP. A 100.0 µl aliquot of blocking solution (Appendix D) was applied to each coverslip and incubated for 20 min at room temperature. The primary antibody solution was prepared by diluting VDB 30/10 and VBD 7/12 1:10 in blocking solution. The excess blocking solution was removed and a 50.0 µl aliquot of each serum sample was added to transfected cells on a coverslip. The microscope slides were placed in a humidified container and incubated at 37°C for 90 min. The coverslips were washed three times in 1× PBS containing 1% Tween® 20 (PBS-T). The secondary antibody solution was prepared as described in 3.2.2.7.iii. A 25.0 µl aliquot of the secondary antibody solution was applied to each coverslip, the microscope slides were placed in a humidified container and incubated at 37°C for 30 min. The coverslips were washed three

times in PBS-T and allowed to dry completely. The coverslips were mounted using fluorescence mounting medium (Dako, Honeydew, SA) and visualized under fluorescent microscope as described in 3.2.2.7.iii.

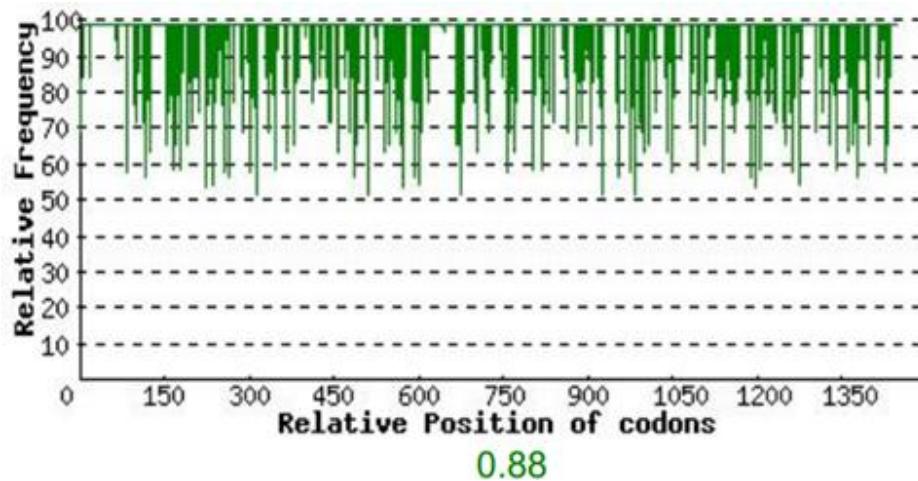
### 3.3. Results

#### 3.3.1. Preparation of the pFastBac HT B.CCHFV.NP.opt construct

The ORF of the CCHFV S segment was codon optimized for expression in insect cells. The codon bias can be evaluated by various indices, the Codon Adaptation Index (CAI) (Sharp and Li, 1987), and effective number of codons usage of a reference group of genes (Sharp and Li, 1987). Its values range from 0, when the codon usage of a sequence and that of the reference set are very different, and 1, when both codon usages are the same. This index is the most effective and most commonly used of all codon bias measures for predicting gene expression levels (Lithwick and Margalit, 2003, Hanry and Sharp, 2007) and is the index that was used in this study (Samudzi et al, 2012). Figure 3.1.a. & 3.1.b. show the codon adaptation index of the native and optimized CCHFV.NP gene, respectively. A codon adaptation index of 1.0 is considered to be perfect in the desired expression organism and a codon adaptation index of  $> 0.8$  is regarded as good, in terms of high gene expression levels. The codon adaptation index value was improved from 0.71 to 0.88.

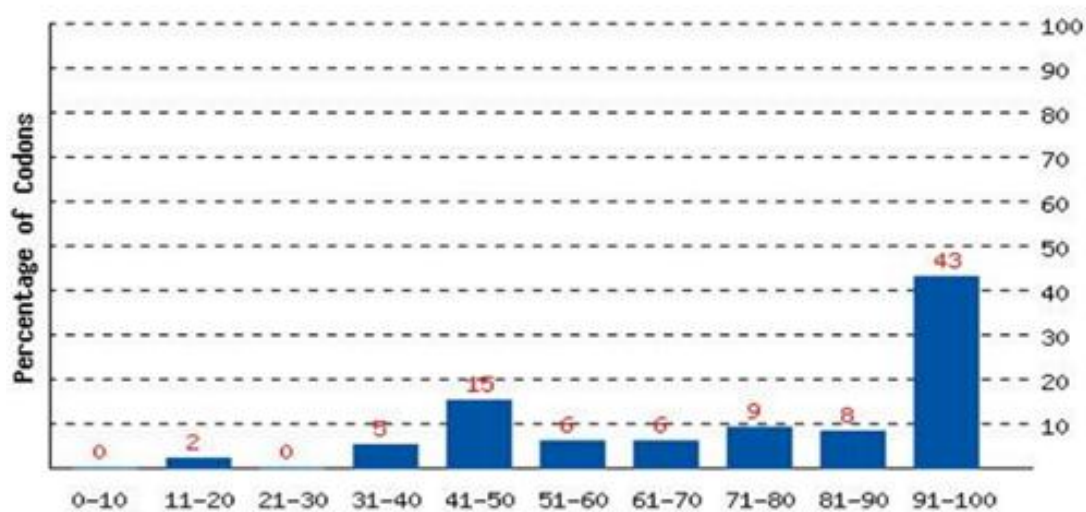


**Figure 3.1.a. The distribution of codon usage along the length of the native CCHFV.NP gene sequence.**

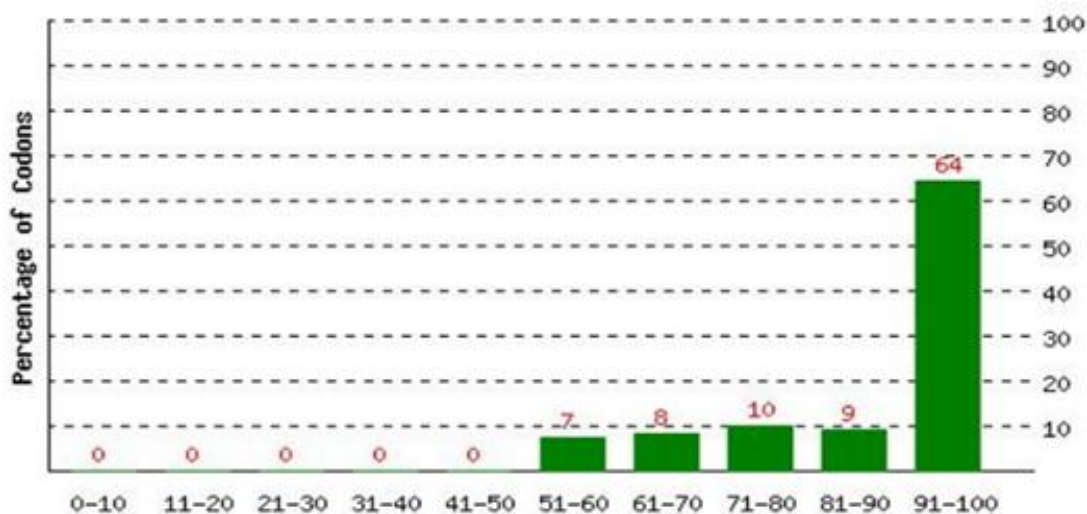


**Figure 3.1.b.**The distribution of codon usage along the length of the optimized CCHFV.NP gene sequence.

Figure 3.1.c. & 3.1.d. show the frequency of optimal codons of the native and optimized CCHFV.NP gene, respectively. The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism. The unfavourable peaks were optimized to prolong the half-life of the messenger RNA (mRNA).

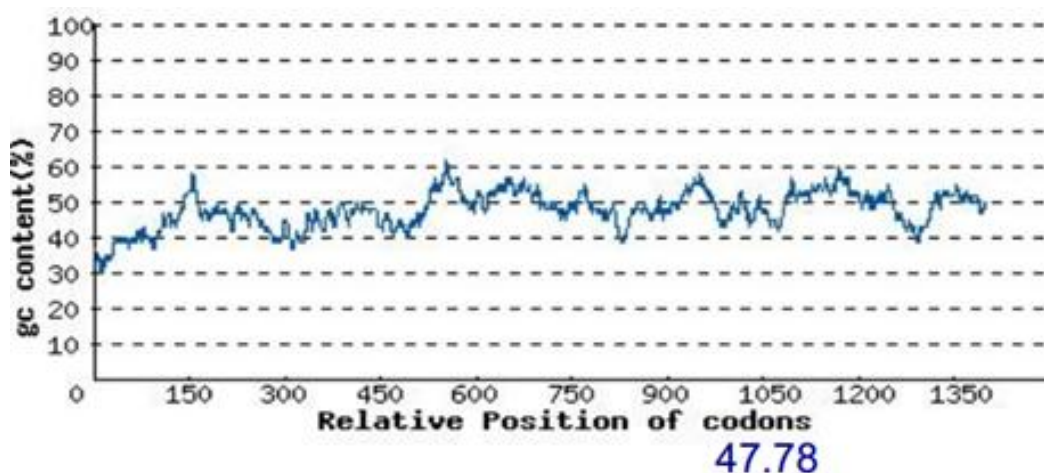


**Figure 3.1.c.** The percentage distribution of codons in computed codon quality groups of the native CCHFV.NP gene sequence.

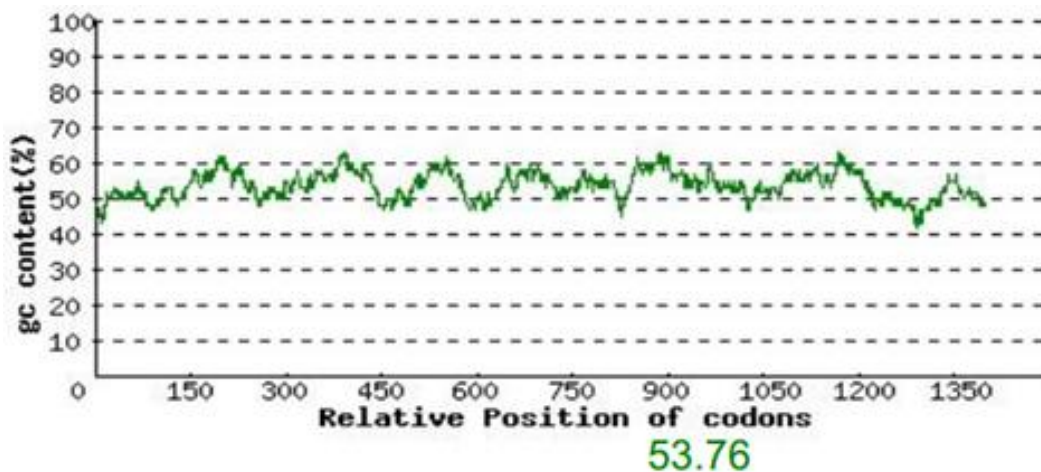


**Figure 3.1.d. The percentage distribution of codons in computed codon quality groups of the optimized CCHFV.NP gene sequence.**

Figure 3.1.e. & 3.1.f. show the percentage GTP and CTP (G/C) content of the native and optimized CCHFV.NP gene, respectively. The ideal percentage range of G/C content is between 30 – 70%. The G/C content was improved from 47.78% to 53.76% to prolong the half-life of the mRNA.

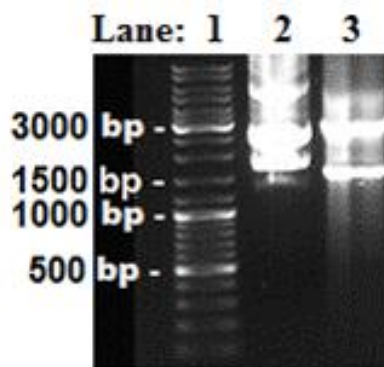


**Figure 3.1.e. The average G/C content of the native CCHFV.NP gene sequence.**



**Figure 3.1.f. The average G/C content of the optimized CCHFV.NP gene sequence.**

Figure 3.2.a. shows the undigested and digested pUC57.CCHFV.NP.opt. The size of the CCHFV.NP.opt gene is 1 458 bp. The lower band in lane 3 aligned approximately with the 1 500 bp band of the DNA size marker.

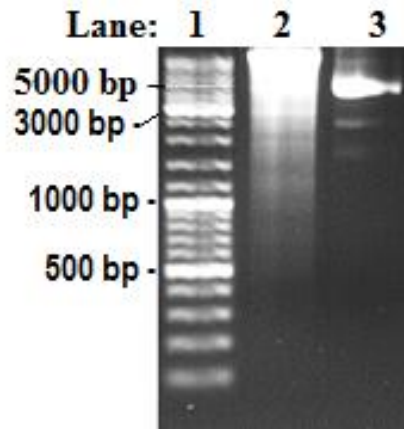


**Figure 3.2.a. Agarose gel electrophoretic analysis of the digestion of the pUC57.CCHFV.NP.opt construct.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. Undigested pUC57.CCHFV.NP.opt plasmid; 3. Digested pUC57.CCHFV.NP.opt plasmid



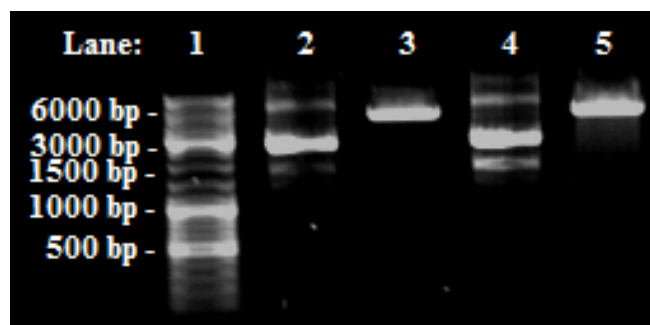
Figure 3.2.b. shows the undigested and digested pFastBac HT B plasmid. The size of the pFastBac HT B plasmid was 4 856 bp. Lane 3 shows a band approximately aligned with the 5 000 bp band of the DNA size marker.



**Figure 3.2.b. Agarose gel electrophoretic analysis of the digestion of the pFastBac HT B plasmid.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. Undigested pFastBac HT B plasmid; 3. Digested pFastBac HT B plasmid

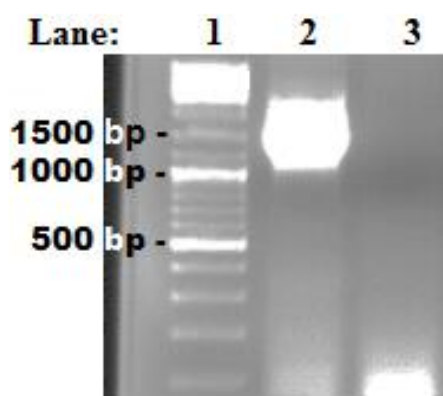
Figure 3.2.c. shows the undigested and digested plasmid. The size of the pFastBac HT B.CCHFV.NP.opt construct was 6 314 bp. Lane 3 and 5 show bands approximately aligned with the 6 000 bp band of the DNA size marker. Lane 2 and 4 show lower bands approximately aligned with the 1 500 bp band of the DNA size marker.



**Figure 3.2.c. Agarose gel electrophoretic analysis of the digestion of the pFastBac HT B.CCHFV.NP.opt construct.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. Digested pFastBac HT B.CCHFV.NP.opt construct; 3. Undigested pFastBac HT B.CCHFV.NP.opt construct; 4. Digested pFastBac HT B.CCHFV.NP.opt construct; 5. Undigested pFastBac HT B.CCHFV.NP.opt construct.

The correct orientation of the inserted CCHFV.NP.opt gene was confirmed using PCR with the CCHFV NP bac opt Forward and pFastBac HT B Reverse primers. The expected size of the amplification product was 1 630 bp. As shown in Figure 3.2.d. the band in lane 2 approximately aligned with the 1 500 bp band of the DNA marker. The band in lane 3 is likely due to primer-dimer.

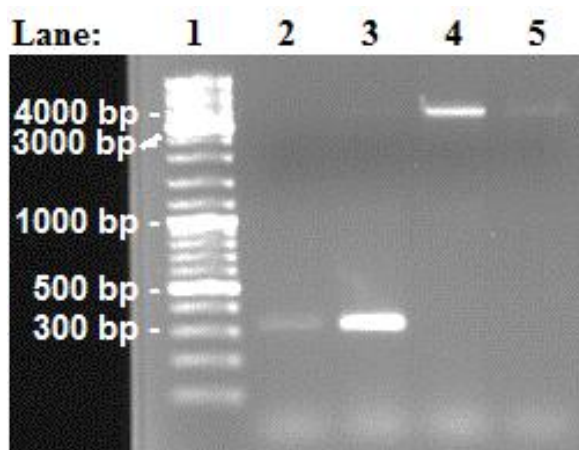


**Figure 3.2.d. Agarose gel electrophoretic analysis of the amplicons from a PCR on one aliquot of selected white colonies to confirm the correct orientation of the CCHFV.NP.opt insert in pFastBac HT B.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. Amplicon from colony 1; 3. Amplicon from colony 2.

### 3.3.2. Preparation of the recombinant bacmid

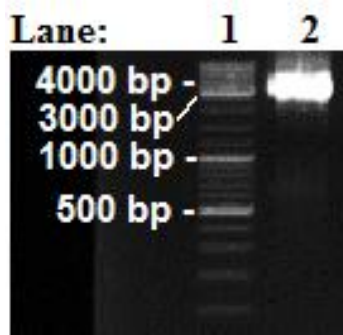
The expected size of the amplicons consisting of the bacmid with the insert was 3 885 bp and the expected size of the amplicons consisting of empty bacmid was 300 bp. Figure 3.3.a. shows a band in lane 4 approximately aligned with the 4 000 bp band of the DNA size marker.



**Figure 3.3.a. Agarose gel electrophoretic analysis of the amplicons from a PCR on one aliquot of selected white colonies to confirm the presence of the CCHFV.NP.opt insert in the bacmid.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2 – 5. Amplicons from colonies 1 – 4.

Figure 3.3.b. shows a band in lane 2 approximately aligned with the 4 000 bp band of the DNA size marker.



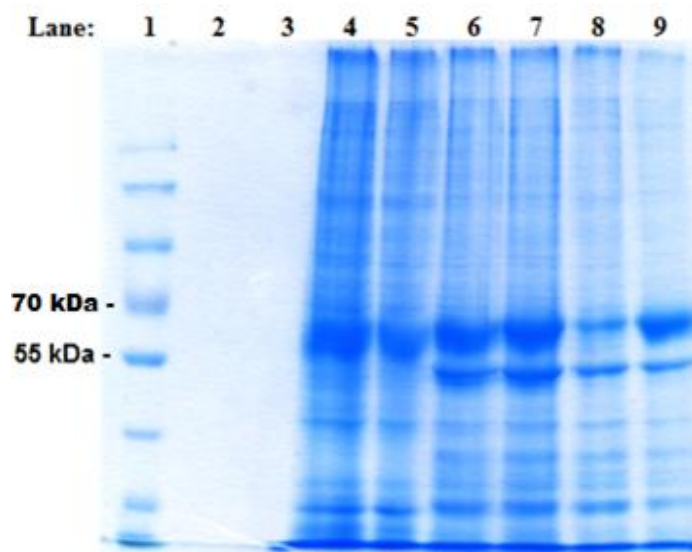
**Figure 3.3.b. Agarose gel electrophoretic analysis of the purified recombinant bacmid.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. An aliquot of the purified recombinant bacmid.

The purified recombinant bacmid was sequenced to confirm the presence of the CCHFV.NP.opt gene. The sequencing reactions were performed in duplicate using the M13 Forward primer and the M13 Reverse primer. The sequencing results were analysed and the edited sequence was aligned with the nucleotide sequence of the CCHFV.NP.opt gene that was received from GenScript. The alignment is shown in Appendix A.

### 3.3.3. Analysis of the transient *Sf9* transfections

Figure 3.4. shows the SDS-PAGE analysis of the resuspended cell pellets, post lysis SNF and cell culture media of P5 and control of the second transfection attempt. The expected size of the recombinant CCHFV NP was ~ 54 kiloDaltons (kDa).



**Figure 3.4. SDS-PAGE analysis of the *Sf9* insect cells transfected with the recombinant bacmid to confirm the presence of the recombinant CCHFV NP.**

Lanes: 1. Protein size marker; 2 & 3. Empty; 4.P5 resuspended cell pellet; 5.Control resuspended cell pellet; 6.P5 post lysis SNF; 7. Control post lysis SNF; 8. P5SNF; 9. Control SNF.

There were no bands indicating expression of the recombinant CCHFV NP observed in lanes 4, 6 and 8, which contained the transfected cell lysates when compared with lanes 5, 7 and 9, which

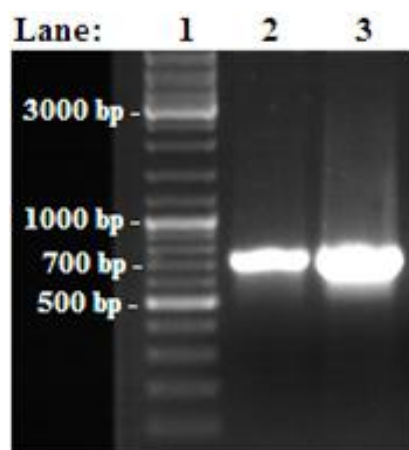
contained the control cell lysates. Similar gels were visualized for the other passages and the other transfection attempts.

Additionally, the resuspended cell pellets, post lysis SNF and cell culture media of P2 – P5 of each transfection attempt were analysed using western blot. No bands indicating expression of recombinant CCHFV NP, about 54 kDa, were observed on any of the films, results not shown.

Lastly, the transfected and non-transfected cells were analysed using IFA. Cells of P2 – P5 were tested in duplicate using a serum sample collected from a convalescent CCHFV-infected patient and a serum sample known to be negative for anti-CCHFV antibodies, collected from a volunteer. No fluorescence was observed with any of the transfected cells.

### 3.3.4. Preparation of the constructs for mammalian transfection

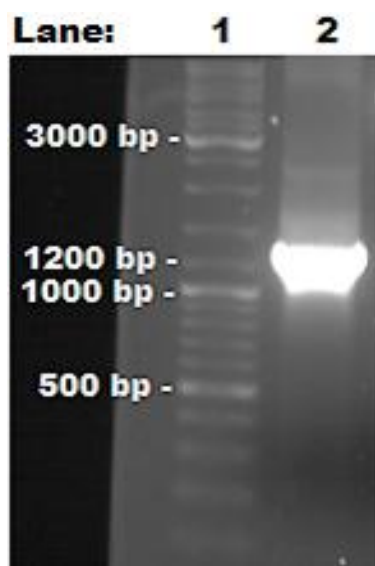
Figure 3.5.a. shows bands in lanes 2 and 3 just above the 700 bp band of the DNA size marker. The expected size of the amplicons analysed by PCR with the T7 Forward and R3 Reverse primers was 762 bp.



**Figure 3.5.a. Agarose gel electrophoretic analysis of the amplicons from a PCR on one aliquot of selected white colonies to confirm the presence of the CCHFV.NP. insert in the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2 & 3. Amplicons from colonies 1 & 2.

Figure 3.5.b. shows a band in lane 2 above the 1 200 bp band of the DNA size marker. The expected size of the amplicons analysed by PCR with the pSIN Forward and pSIN Reverse primers was 1 301 bp.



**Figure 3.5.b. Agarose gel electrophoretic analysis of the amplicons from a PCR on one aliquot of a selected white colony to confirm the presence of the GFP insert in the pSIN-DLR-GFP construct.**

Lanes: 1. O'GeneRuler DNA ladder mix SM 1173; 2. Amplicon from colony 1.

### **3.3.5. Analysis of the transient BHK-21 transfections**

BHK-21 cells were transfected with the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct and the pSIN-DLR-GFP construct. The pSIN-DLR-GFP vector was used as transfection control. Expression of recombinant CCHFV NP was demonstrated by IFA. The transfection efficiency of  $\pm 90\%$  for BHK-21 cells expressing GFP was estimated by observation of number of green fluorescent cells and total number of cells visualized in at least four fields under the fluorescent microscope. A transfection efficiency of  $\pm 15\%$  was estimated similarly for the BHK-21 cells expressing CCHFV NP.

### 3.4. Summary

Serological assays are frequently used to detect anti-CCHFV antibodies for diagnostic and surveillance purposes. In an attempt to prepare antigen that did not require high biosafety facilities, two recombinant expression systems were investigated. Each system has different advantages and applications. Baculovirus expressed recombinant proteins could have application in ELISA, whereas most mammalian system express yields of protein too low for application in ELISA. However, expression of recombinant antigen from stably transfected mammalian cells could likely have application in preparation of antigen slides for IFA. To investigate the baculovirus expression system, the S segment ORF of the CCHFV strain SPU 415/85 was codon optimized for protein expression in insect cells. The codon adjustment index, frequency of optimal codons and G/C content was improved to reduce the use of tandem rare codons, prolong the half-life of mRNA, improve ribosomal binding, increase stability of mRNA and modify negative cis-acting sites. The optimized gene was synthesized to include BamHI restriction sites on the 5' and 3' ends and supplied in pUC57. In order to rescue the optimized gene for the pUC57 vector, the pUC57.CCHFV.NP.opt was digested with BamHI. To prepare the pFastBac HT B plasmid for cloning, the plasmid was linearized by digesting with BamHI and dephosphorylated. The multiple cloning site of the pFastBac HT B plasmid is situated within a Tn7 transposon. The optimized gene was ligated into the prepared pFastBac HT B plasmid and the ligation reaction used to transform *E. coli* cells. Positive transformants were identified by digestion with BamHI and the correct orientation of the optimized gene was confirmed using PCR. To facilitate high frequency of cloning, the bacmid contains an *attTn7* site. In order to prepare the recombinant bacmid, the pFastBac HT B.CCHFV.NP.opt construct was used to transform DH10Bac™ *E. coli* cells which contain the helper plasmid. Through the assistance of the helper plasmid the Tn7 transposon element containing the optimized gene transposed into the bacmid. Positive transformants were identified and the correct orientation of the optimized gene confirmed as mentioned previously. Additionally, the recombinant bacmid was sequenced to confirm the presence of the optimized gene. The recombinant bacmid was used in to transfect Sf9 insect cells using various transfection conditions. The cell lysates of each transfection attempt were analysed using SDS-PAGE, western blot and IFA, however, no expression of CCHFV NP could be detected.

To investigate the mammalian expression system, a pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct, prepared in a previous study in our laboratory, and a pSIN-DLR-GFP construct for use as a transfection control was used to transform *E. coli* cells. Although the control was prepared from a different plasmid it was included as an indication that the BHK-21 cells were able to be transfected using the reagents and conditions applied. Plasmid for transfection was purified from bacterial cultures and the presence of the genes of interest confirmed by PCR. In-house antigen slides were prepared using cells transfected with the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct. Expression of CCHFV NP was determined by IFA using an anti-CCHFV IgG positive serum sample. The transfection efficiency of the BHK-21 cells expressing GFP was calculated as  $\pm 90\%$ . In comparison the transfection efficiency of the cells expressing CCHFV NP was lower, approximately 15%.

In summary, the results suggest that attempts to express CCHFV NP using baculovirus system were not successful. However, recombinant CCHFV NP was expressed from transiently transfected mammalian cells and recombinant antigen was detected by IFA using human anti-CCHFV IgG. The application of transfected cells warrants further investigation to determine if generation of a stable cell line has application in preparation of antigen slides for IFA.



## **Chapter 4 – Profiling the antibody response against Crimean-Congo haemorrhagic fever viral proteins**

### **4.1. Introduction**

Selection of recombinant antigens for inclusion in development of assays for diagnosis or surveillance requires a knowledge of which viral proteins are immunodominant and the kinetics of antibody responses. Currently, little is known regarding the antibody responses against different CCHF viral proteins. The most extensive evaluation of antibody responses was performed on serum samples collected from southern African CCHFV-infected patients using ELISA and IFA (Shepherd et al, 1989; Burt et al, 1994). These assays were based on native antigen and confirmed that a humoral antibody response is frequently not detectable during the first few days after onset of illness. In nonfatal CCHF cases, ELISA can detect anti-CCHFV IgM and IgG antibodies as early as day 3, but more frequently from day 5, and IFA can detect anti-CCHFV IgM and IgG antibodies from day 5 (Burt et al, 1994; Jacobson, 1998, Tang et al, 2003; Saijo et al, 2005). More recently, recombinant CCHFV NP antigens have been used to detect IgG antibody using ELISA. Samudzi et al (2012) demonstrated that bacterially expressed recombinant CCHFV NP could detect antibody in human convalescent sera at least 12 years after infection. Rangunwala et al (2014) prepared recombinant CCHFV NP from genetically diverse strains of CCHFV, a South African isolate and a Greek isolate. Recombinant NP from either strain was able to detect antibody in sera collected from South African convalescent patients indicating significant antigenic similarity among the diverse strains.

In this chapter, the application of cells transiently transfected in-house with a plasmid expressing CCHF NP was evaluated for detection of antibody against CCHFV from human sera. The results were compared with commercially available slides prepared using cells expressing either CCHFV NP or GP. The implication was that if the transiently transfected cells could be used to detect anti-CCHF IgG, it warranted preparation of a stable cell line that would be significantly more cost effective than the commercial slides. In addition, although we have previously shown that CCHFV-infected patients develop antibody against CCHFV NP that is detectable at least 12 years after illness, the detection and duration of antibody specifically against CCHFV GP has not yet been described. In this study the commercial slides were used to determine the presence and duration of IgG antibody against CCHFV GP.

## **4.2. Methods and materials**

### **4.2.1. Serum samples**

Serum samples were collected from 15 survivors of CCHFV in southern Africa at different time periods after onset of illness and from eight volunteers known to be negative for anti-CCHFV antibodies. Informed consent was obtained from the survivors and the volunteers. Each sample was allocated a laboratory number, designated VBD. Two of the 15 sera were selected, diluted two-fold from 1:2, and used to compare anti-CCHFV NP and anti-CCHFV GP titers.

### **4.2.2. Transfected cells**

The pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct was used to transfect BHK-21 mammalian cells. A 24-well plate was prepared for transfection, as described in 3.2.3.2. Cells were transfected with the pSIN-DLR-GFP plasmid as positive transfection control. Non-transfected cells were included as a negative control. Transfected cells and controls were used to prepare antigen slides, as described in 3.2.3.2, and detection of anti-CCHFV IgG in 22 human sera (14 positive and eight negative) was confirmed using IFA, as described in 3.2.3.3.

### **4.2.3. Commercial IFA slides**

The Crimean-Congo fever virus Mosaic 2 (IgG) kit (EUROIMMUN AG, Lübeck, Germany) contains slides for use in indirect IFA. Each slide contains ten fields and each field contains three BIOCHIPS. The first BIOCHIP is coated with cells expressing the CCHFV NP, the second BIOCHIP is coated with transfected cells expressing the CCHFV GP and the third BIOCHIP is coated with untransfected cells. A total of 15 sera from survivors were tested for anti-CCHFV IgG using the Crimean-Congo fever virus Mosaic 2 (IgG) kit. Briefly, the slides were equilibrated to room temperature and the serum samples, as described in 3.2.1, were diluted 1:10 in the sample buffer provided with the kit. A 30.0 µl aliquot of each diluted serum sample was applied to an empty reaction field on the reagent tray provided with the kit, slides were placed upon the reagent tray and incubated at room temperature for 30 min. The slides were rinsed with PBS-Tween provided with the kit, and immersed in PBS-Tween for 5 min. Meanwhile, a 25.0 µl aliquot of the fluorescein labelled anti-human IgG with Evans Blue, provided with the kit, was applied to an empty reaction field on the reagent tray. The slides were removed from the

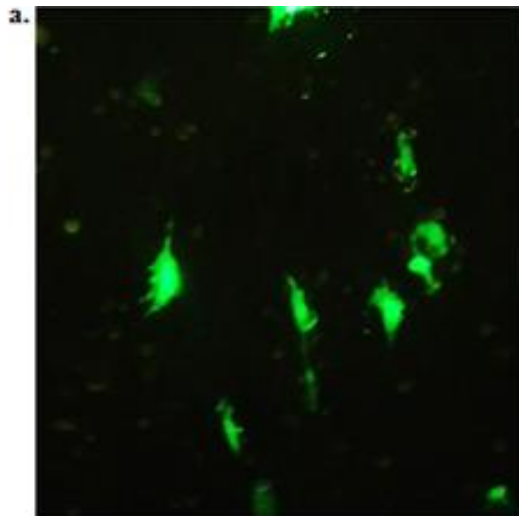
PBS-Tween, dried and placed upon the reagent tray with detection antibody and incubated at room temperature for 30 min. The slides were washed as previously and mounted with cover slip for visualization under fluorescent microscope, as described in 3.2.2.7.iii.

Two serum samples were selected, diluted two-fold (1:2 – 1:128 000) and tested to determine end point titers of the IgG antibody against CCHFV NP and CCHFV GP as described above.

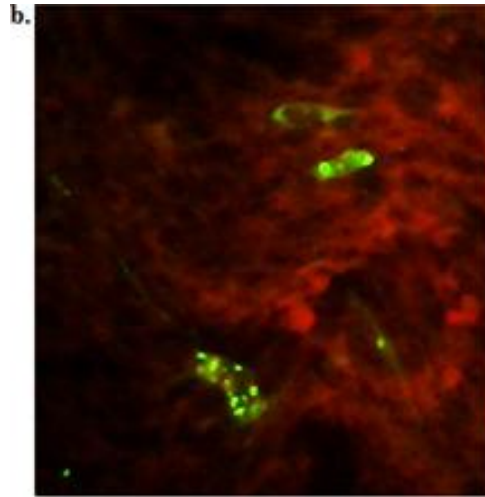
### **4.3. Results**

#### **4.3.1. Mammalian expression of recombinant CCHFV NP**

Figure 4.1.a. and Figure 4.1.b. show pictures of transfected BHK-21 cells expressing GFP and recombinant CCHFV NP, respectively. Anti-CCHFV antibodies were detected in a total of 14/14 serum samples from CCHFV survivors. Note that one of the 15 serum samples collected from convalescent patients was not tested using the in-house antigen slides due to limited volume. No antibody was detected in 8/8 serum samples collected from volunteers with no history of CCHFV infection.



**Figure 4.1.a. Transiently transfected BHK-21 cells expressing GFP visualized using the Nikon Optiphot fluorescent microscope at a magnification of 400 $\times$ .**

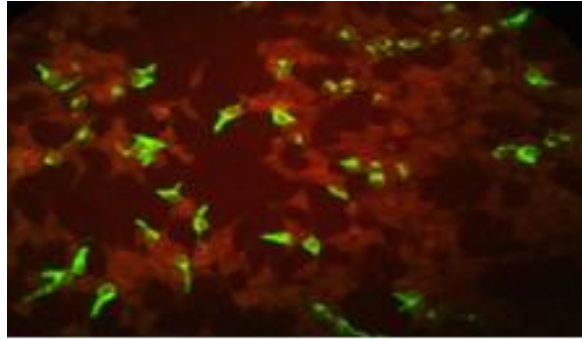


**Figure 4.1.b. Transiently transfected BHK-21 cells expressing the recombinant CCHFV NP reacted against anti-CCHFV IgG in convalescent patient sera visualized using the Nikon Optiphot fluorescent microscope at a magnification of 400×.**

The expressed recombinant CCHFV NP is visible appearing as inclusion bodies within the BHK-21 cells.

#### **4.3.2. Commercial IFA**

A total of 23 sera were tested for antibody against CCHFV using the antigen slides provided in the Crimean-Congo fever virus Mosaic 2 (IgG) kit. Anti-CCHFV IgG antibody against CCHFV NP and GP was detected in 15/15 serum samples from CCHFV survivors. No antibody was detected in 8/8 serum samples collected from volunteers with no history of CCHFV infection. Figure 4.2. shows anti-CCHFV sera reacting with transfected cells expressing recombinant CCHFV NP on commercial slides.



**Figure 4.2.**The BIOCHIP coated with cells expressing CCHFV NP on the CCHF antigen slides visualized using the Nikon Optiphot fluorescent microscope at a magnification of 200×.

Untransfected cells were counterstained with Evans Blue, which stains the cell walls red.

Table 4.1. shows the results of the Crimean-Congo fever virus Mosaic 2 (IgG) kit compared with the results of the transient BHK-21 transfection IFA tests and the time after onset of illness that the samples were collected.

**Table 4.1. Detection of anti-CCHFV IgG using commercial antigen slides and in-house prepared antigen slides.**

VBD number	Time after onset collected	BHK-21 transfection IFA	EUROIMMUN NP	EUROIMMUN GP
66/08	9 months	Positive	Positive	Positive
67/08	7 years	Positive	Positive	Positive
69/08	4 years	Positive	Positive	Positive
6/09	3 months	Positive	Positive	Positive
29/10	19 days	Positive	Positive	Positive
30/10	10 years	Positive	Positive	Positive
51/10	2 years	Positive	Positive	Positive
52/10	2 years	Positive	Positive	Positive

**Table 4.1. Continued**

<b>VBD number</b>	<b>Time after onset collected</b>	<b>BHK-21 transfection IFA</b>	<b>EUROIMMUN NP</b>	<b>EUROIMMUN GP</b>
53/10	10 years	Positive	Positive	Positive
6/11	11 months	Positive	Positive	Positive
29/11	10 months	Positive	Positive	Positive
31/11	5 months	Positive	Positive	Positive
51/11	2.5 years	Positive	Positive	Positive
54/11	2 years	Positive	Positive	Positive
01/13	16 days	<b>Not done</b>	Positive	Positive
23/07	n/a	Negative	Negative	Negative
07/13	n/a	Negative	Negative	Negative
48/13	n/a	Negative	Negative	Negative
49/13	n/a	Negative	Negative	Negative
50/13	n/a	Negative	Negative	Negative
51/13	n/a	Negative	Negative	Negative
52/13	n/a	Negative	Negative	Negative
53/13	n/a	Negative	Negative	Negative

n/a – not applicable

Table 4.2. shows the results from diluted sera to determine end point titers for IgG directed against CCHFV NP and GP using the commercial antigen slides.

**Table 4.2. Comparison of end point titers of IgG antibody directed against CCHFV NP and CCHFV GP.**

VBD number	Time after onset collected	End point titer	
		EUROIMMUN NP	EUROIMMUN GP
29/10	19 days	1:64 000	1:16 000
01/13	16 days	1:64 000	1:32 000

#### 4.4. Summary

Understanding the kinetics of antibody responses and which viral proteins induce humoral immune responses provide useful information for preparation of recombinant antigens for use as diagnostic and surveillance tools. In-house antigen slides were prepared using cells transfected with pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct. The pSIN-DLR-GFP plasmid was used as a positive transfection control. Anti-CCHFV NP IgG was detected in 14 samples collected from convalescent patients and eight samples collected from volunteers with no history of CCHFV infection were negative. Although assumed to be present antibodies directed specifically against the CCHFV GP and the duration of IgG has not previously been reported in publications. To profile the immune response directed against the CCHF viral proteins, transfected cells expressing recombinant CCHFV NP and GP were analysed using IFA. The antigen slides provided in the kit consisted of 10 reaction fields and each field contained of three BIOCHIPS consisting of non-transfected mammalian cells, transfected mammalian cells expressing CCHFV NP and transfected mammalian cells expressing CCHFV GP, respectively. All samples from survivors tested positive for antibody against both CCHFV NP and GP. Two samples collected early after onset of illness were selected and diluted two-fold to determine end point titers. The IgG antibody titer against CCHFV NP was 64 000 in both the serum samples and IgG antibody titer against CCHFV GP was 16 000 and 32 000. Earlier sera were not available for testing due to the risk of viraemia.

In summary, the results obtained with the antigen slides prepared in-house were in concordance with that of the commercial antigen slides. Anti-CCHFV GP IgG antibody was identified using

the commercial antigen slides. Comparison of the end point titers for IgG antibody against CCHFV NP and GP using two serum samples collected early after onset of illness suggests that anti-CCHFV NP IgG titers were higher, possibly due to more abundant CCHFV NP. The CCHFV NP is known to be a more abundant protein during infections. The results suggest that antibodies are directed against CCHFV NP and GP, indicating that these proteins are both candidates for recombinant diagnostic and surveillance tools. Preparation of a stable cell line expressing CCHFV NP for preparation of antigen slides would be a more cost effective option that warrants further investigation.



## Chapter 5. Discussion

CCHFV is an RNA virus of the *Nairovirus* genus within the *Bunyaviridae* family with a global distribution correlating with that of *Hyalomma spp.* ticks (Hoogstraal, 1979). Crimean haemorrhagic fever was first described during 1944 at the end of World War II and the causative virus was first isolated in 1967. The combined name Crimean-Congo haemorrhagic fever virus was adopted when it became evident that the Crimean haemorrhagic fever virus was indistinguishable from the Congo virus isolated in 1956 in Belgian Congo, current Democratic Republic of the Congo (Casals, 1969; Hoogstraal, 1979).

The virus has a single-stranded, tripartite, negative sense RNA genome consisting of S, Me and L segments encoding the NP, GPC and the RNA-dependent RNA polymerase, respectively. CCHFV is an arthropod-borne zoonosis associated with severe haemorrhagic disease in humans. CCHF cases are characterized by sudden onset of fever accompanied by headache, myalgia and/or joint pain and progresses quickly to haemorrhage in the form of nose bleeds, bleeding from the gums, petechia and/or internal haemorrhage (Hoogstraal, 1979). Multiple organ failure is usually the cause of death (Swanepoel et al, 1989). Very little is known about the pathogenesis of CCHFV, as high biocontainment facilities required to study the virus are expensive to maintain and therefore the number of laboratories is limited worldwide.

CCHFV poses a health threat to people living in endemic areas whose work places them at high risk of possible exposure to CCHFV and a concerning threat against healthcare workers where human-to-human contact plays a role in viral transmission. Therefore precautions, such as isolation of the patient and barrier nursing techniques, should be in place where possible cases of CCHF can be considered likely to occur to limit possible exposure to CCHFV. Treatment is mostly supportive therapy. Ribavirin is administered in some cases, but the efficacy of this antiviral drug is yet to be firmly established (Fisher-Hoch et al, 1995; Mardani et al, 2003; Ergönül et al, 2004; Smego et al, 2004; Izadi and Salehi, 2009; Duygu et al, 2011; Keshtkar-Jahromi et al; 2011; Ceylan et al, 2013).

Diagnosis of CCHFV is usually done through detection of viral nucleic acid using RT-PCR, virus isolation from infected cell culture or suckling mice, or detection of anti-CCHFV antibodies by ELISA or IFA (Burt, 2011). However, RT-PCR requires the use of expensive equipment, virus isolation can only be done in high biocontainment facilities and humans only develop antibody responses 3 – 5 days after the onset of illness in nonfatal cases and frequently

no antibodies are produced in fatal infections, rendering detection of antibodies of little use during an acute infection. Viral haemorrhagic fever reference laboratories frequently make use of a battery of tests targeting different analytes to account for the different window periods in which those specific analytes can be detected. Diagnosis of a single patient often requires conducting assays for detection of antigen or genetic material, IgM and IgG antibodies separately, and virus isolation.

The emergence in non endemic regions and re-emergence in endemic areas that experienced long periods without CCHF cases emphasize the requirement for safe, rapid and inexpensive assays to increase diagnostic and surveillance capacity of CCHFV.

NASBA is an isothermal molecular assay developed in 1991 (Compton, 1991). This assay has since been applied for detection of various medically significant pathogens (Kievits et al, 1991; Lanciotti and Kerst, 2001; Keightley et al, 2005; Lau et al, 2006; Lau et al, 2008; Loens et al, 2008; Mugasa et al, 2009; Boulet et al, 2010; Lau et al, 2010). The principle of the assay is based on simultaneous functioning of reverse transcriptase, RNase H and T7 RNA polymerase under isothermal conditions and using primers designed to include specific sequences additional to the target sequences. Advantages of NASBA assays are that the isothermal amplification does not require a thermocycler, each amplification step can initiate the moment that an intermediate component becomes available and the single-stranded products can be detected using various probe hybridization methods (Loens et al, 2002). In contrast, RNA integrity, limited length of target sequence, RNA amplification and amplification inhibitors are main disadvantages of NASBA assays (Loens et al, 2002). The integrity of RNA can be affected during sample collection, processing or storage prior to extraction and addition of RNase inhibitors, such as guanidine isothiocyanate, is usually required to preserve integrity (Loens et al, 2002). The length of the target sequence ranges between 120 – 250 bp and lengths outside this range might not be amplified efficiently (Loens et al, 2002). The enzymes utilized in NASBA assays are not thermostable and an increase in temperature beyond 42°C might affect the functionality of the enzymes (Loens et al, 2002). Various components with inhibitory effects on NASBA are present in human sera and false-negative results might be observed (Loens et al, 2002). The RNA extraction method should be carefully considered to have the ability to reduce the effects of the inhibitory components (Fanson et al, 2000; De Paula et al, 2003). Pre-treatment of samples using proteinases might be necessary (Al-Soud et al, 2001, Goldschmidt et al, 2014). The use of

an internal control is required to evaluate false-negative results due to amplification inhibitors (Sooknanan and Malek, 1995). There has yet to be a NASBA developed for the detection of CCHF viral nucleic acid. A NASBA assay has the potential to increase diagnostic capacity of CCHFV as it can be implemented in field situations or by laboratories with limited resources.

High biocontainment facilities (BSL-4) are required for culturing CCHFV and production of native antigen. Recombinant proteins are a useful and safe alternative to native antigens, but correct protein folding and processing are required for recombinant proteins to retain functionality as native antigens. Bacterial, mammalian and baculovirus expression systems have been implemented for the expression of recombinant CCHFV NP (Marriott et al, 1994; Saijo et al, 2002a; Saijo et al, 2002b; Tang et al, 2003; Saijo et al, 2005; Garcia et al, 2006; Samudzi et al, 2012; Rangunwala et al, 2014) for detection of anti-CCHFV antibodies using ELISA and preparation of antigen slides for use in IFA. Utilization of recombinant CCHFV proteins by serological assays can increase diagnostic and surveillance capacity of CCHFV, as preparation of the proteins do not require BSL-4 facilities.

The aims of this study were to develop a novel simple molecular assay for detecting CCHF viral nucleic acid and to produce recombinant CCHFV NP for development of safe assays for detecting anti-CCHFV antibodies.

An in-house NASBA was designed as a molecular assay with diagnostic application in laboratories with limited resources. RNA were extracted from Vero cell culture infected with 48 genetically diverse CCHFV strains, from serum samples collected from 22 confirmed CCHFV-infected patients and from serum samples collected from 16 patients with suspected viral haemorrhagic fever but confirmed as non-CCHFV cases. Nested RT-PCR was used to test RNA extracted from infected Vero cell culture and a total of 48/48 samples tested positive. The NASBA primers were designed to target a 250 bp region based on a multiple alignment of a 450 bp region within the ORF of the S segment of genetically diverse CCHFV isolates. The recommended size to target for NASBA is 150 bp to 250 bp however, some isolates have more than 20% heterogeneity between the gene encoding CCHFV NP and therefore it was difficult to target a region smaller than 250bp using available sequence data. A probe for detection of the amplicon was designed to bind in a region flanked by the primer pair. An enzyme mixture and amplification buffer was developed in-house, designated CCHFV NASBA assay, and compared with an enzyme mixture and buffer provided in a commercial NucliSENS® EasyQ Basic kit.

The lower detection limits of the CCHFV NASBA assay and the CCHFV NucliNASBA assay were determined using ten-fold dilutions of RNA extracted from Vero cell culture infected with SPU 4/81. The CCHFV NASBA assay detected RNA diluted  $\leq 10^{-4.5}$  and the CCHFV NucliNASBA assay detected RNA diluted  $\leq 10^{-5.5}$ . To determine the ability of the NASBA primers to detect genetically diverse CCHFV strains, RNA extracted from Vero cell culture infected with 48 different isolates of CCHFV were tested using the CCHFV NASBA assay. A total of 41/48 samples tested positive. To determine why seven samples were not amplified using the CCHFV NASBA assay the available sequence data was analysed (Appendix A). Nucleotide sequence data were available for two (SPU 415/85 and SPU 582/86) of the seven samples that tested negative. Further analysis revealed that SPU 415/85 had a single nucleotide mismatch in the priming area of NASBA R, indicating that the nucleotide sequence was conserved within the priming and binding areas. SPU 582/86 had three nucleotide mismatches in the priming area of NASBA F, four nucleotide mismatches in the binding area of the detection probe and a single nucleotide mismatch in the priming area of NASBA R. This could indicate that SPU 582/86 tested negative likely due to the number of nucleotide mismatches within the priming area of NASBA F and the binding area of the detection probe. However, five (SPU 259/84, SPU 396/85, SPU 408/85, SPU 415/85 and SPU 582/86) of the seven samples that tested negative using the CCHFV NASBA also tested negative during the first round of the nested RT-PCR, likely indicating that the RNA levels within these samples was lower than other samples. The RNA samples had been subjected to multiple freeze-thaw cycles which might affect the RNA integrity. For application in laboratories with few resources and lack of sophisticated equipment, the use of a water bath and a heating block was briefly compared using three RNA samples extracted from infected Vero cell culture and tested using the CCHFV NASBA assay. The results correlated with the results obtained using a thermocycler, indicating that the NASBA could be performed using less sophisticated equipment. Control RNA was transcribed from PCR template and used as positive control, to determine the lower detection limits of the NASBA assays and to determine if there are inhibitors of the CCHFV NASBA assays in human sera. The lower detection limits were determined as  $\geq 3.7$  copies of RNA and  $\geq 37$  copies of RNA for the CCHFV NASBA assay and the CCHFV NucliNASBA assay, respectively. The diagnostic application of the NASBA assays was determined using RNA extracted from patient sera and the results were compared with that of two commercial real-time RT-PCR assays.

The commercial assays detected 100% of the samples, the CCHFV NASBA detected 63.7% and the CCHFV NucliNASBA detected 90.9%. All suspected VHF samples shown to be negative for CCHF viral RNA using commercial RT-PCRs tested negative using the NASBA assays. To investigate why the CCHFV NASBA assay lacked sensitivity, and assuming that inhibitory factors may play a role, sera spiked with transcribed RNA were analysed. Sera were collected from four volunteers and spiked with the control RNA transcript. The CCHFV NASBA assay detected RNA from three serum samples, but was unable to detect RNA extracted from VBD 19/14 despite repeated attempts. This finding likely indicated that components inhibiting the CCHFV NASBA may be present in certain clinical samples and this may have influenced the amplification of RNA from clinical samples. Human sera contain various components and contaminants, such as IgG, heparin, lactoferrin, bile salt, hemoglobin, calcium chloride ( $\text{CaCl}_2$ ), iron chloride ( $\text{FeCl}_3$ ) and EDTA, which could inhibit polymerase enzymes (Al-Soud et al, 2000; Al-Soud et al, 2001). Further investigation is required to identify the inhibitory components, followed by selection of an RNA extraction method or pre-treatment process that might reduce or eliminate the inhibitory components prior to amplification. The CCHFV NASBA assay could have application as a rapid diagnostic assay especially in laboratories with limited resources, however, an internal control should be incorporated and a negative result should be confirmed by another independent assay.

Two expression systems were investigated for preparation of recombinant antigens which can be utilized in serological assays. An insect cell-baculovirus expression system has significance as the main vectors of CCHFV are insects. Additional advantages of the baculovirus expression system are that expressed proteins are processed post-translationally and the protein yield is sufficient for application in ELISA. A mammalian expression system has significance as expressed proteins will have correct post-translational modifications, however, in general the yield of mammalian expression systems is insufficient for application in ELISA, but the transfected cells can be utilized to prepare antigen slides for use in IFA. Transiently transfected cells are useful to determine if the cells have application in IFA prior to preparation of a stable cell line. In order to investigate the baculovirus expression system, the ORF of the S segment of a CCHFV isolate was codon optimized for expression in insect cells. Optimization assists with increasing the half-life and stability of mRNA, reduces the use of tandem rare codons, improves ribosomal binding and modified negative cis-acting sites. The optimized gene was synthesized

to include BamHI restriction sites on the 5' and 3' ends, which were used to rescue the optimized gene for downstream application. The gene was ligated into the pFastBac HT B plasmid and used to transform *E. coli* cells. Positive transformants were identified using digestion with BamHI and the correct orientation of the optimized gene was confirmed using PCR. Plasmid DNA was purified and used to transform DH10Bac™ cells containing the bacmid and helper plasmid. The Tn7 element containing the optimized gene was transposed into the *attTn7* region within the bacmid. Positive transformants and correct orientation of the optimized gene were confirmed and the recombinant bacmid was sequenced. The recombinant bacmid was used to transfect Sf9 insect cells. Various transfection conditions were attempted and the transfected cells and cell culture media were analysed using SDS-PAGE, western blot and IFA. Expression of CCHFV NP was not detected in any of the cultures. Various factors could have contributed to lack of expression including physiological and biochemical factors. Factors, such as multiplicity of infection (MOI), age of culture, growth phase of the culture, correct processing and assembly of the expressed protein, culturing time post infection, cytotoxins and DNA concentration should be considered (Kioukia et al, 1995; Wong et al, 1996; Martinez-Torrecuadrada et al, 2000; Zhang et al, 2005; Lin et al, 2014). Low MOI has advantages and disadvantages when compared with high MOI. Theoretically, all cells are infected immediately when infecting at high MOI, resulting in synchronous infection and cell growth ceases completely. In contrast, infecting at low MOI requires low viral titers and primarily infected cells subsequently infect neighbouring cells. Eventually all cells become infected or lose their affinity for virus attachment (Wong et al, 1996). Infecting at high MOI requires high viral titers and the problem with passage effect is bound to appear. Virus attachment rates depend on virus concentration, cell concentration and virus-binding capacity, which all vary along the cell growth curve (Zhang et al, 2005). Earlier passages of Sf9 cells were applied to address the issue of cell culture age. The increased DNA synthesis during the synthesis phase of the cell cycle assists virus in replication of its genome at an increased rate, due to the availability of precursors and enzymes (Kioukia et al, 1995). The Sf9 cells were seeded 30 min prior to transfection to allow just enough time for the cells to attach before introducing the transfection complexes, so that cells would be in an early phase of the growth cycle. In order to determine the processing of recombinant proteins and identifying factors affecting the correct assembly of recombinant proteins, Martinez-Torrecuadrada et al (2000) compared two baculovirus transfer plasmids, pFastBac and pAcYM1. The expression of

the recombinant pFastBac transfer vector produced rigid tubular structures, where expression of the recombinant pAcYM1 transfer vector led to the production of virus-like particles. The results suggest that different promoters within the transfer vectors led to the production of different proteins, even though the same gene was used (Martinez-Torrecuadrada et al, 2000). This factor could not be addressed in this study as expression of recombinant CCHFV NP was not confirmed. Cell culture might lose affinity for virus infection with age (Zhang et al, 2005). Cells and cell culture media were harvested at different times post transfection in our study. Different concentrations of the transfection reagent were used to investigate the cytotoxic effect on the *Sf9* cells. Different DNA concentrations were used to optimize the likelihood that the DNA would be introduced in the *Sf9* cells and to investigate the cytotoxic effect of the foreign DNA on the *Sf9* cells. Further investigation is required to determine optimal conditions for baculovirus expression of recombinant CCHFV NP and to identify inhibitory components that affect the transfection process or virus replication process.

CCHFV NP was subsequently expressed using a mammalian expression system. The pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct, prepared previously in our laboratory, was used to transfect BHK-21 mammalian cells. The pSIN-DLR-GFP plasmid was used as positive transfection control. Although the plasmids differed and the pSIN-DLR-GFP plasmid was not an optimal control to determine DNA and lipofectamine ratios, it did provide an indicator that the cells were transfected and the techniques were working. The pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct and the pSIN-DLR-GFP plasmid were used to transform *E. coli* cells and positive transformants were identified using PCR. Purified plasmids were used to transfect BHK-21 mammalian cells. Transfected BHK-21 cells expressing GFP were visualized directly and showed a high transfection efficacy, approximately 90%. Expression of recombinant CCHFV NP was confirmed by reacting the transfected BHK-21 cells with a serum sample collected from a convalescent CCHFV-infected patient. Transfection efficacies were much lower for the plasmid expressing CCHFV NP but this could be due to use of different plasmids, different promoters influencing the levels of protein expressed and difference in size of the proteins (27 kDa GFP versus 54 kDa CCHFV NP).

Immune profiling is important to determine which viral proteins induce strong humoral antibody responses and which can subsequently be used to prepare recombinant antigens for application as diagnostic and surveillance tools. Antigen slides prepared from transfected mammalian cells

expressing recombinant CCHFV NP and GP, provided in a commercial kit, were tested using IFA and sera collected from 15 convalescent CCHFV-infected patients and from eight volunteers with no history of CCHFV infection. All sera tested positive against the CCHFV NP and GP and the known negative sera tested negative. Two of the 15 sera collected early after onset of illness from convalescent patients were selected, diluted two-fold and used to determine the end point titers of anti-CCHFV IgG. End point titers for anti-CCHFV NP IgG were determined as 64 000 for both samples and 16 000 and 32 000 for anti-CCHFV GP IgG. In-house antigen slides were prepared from the transfected BHK-21 cells expressing recombinant CCHFV NP and tested using IFA. A total of 14/14 sera from CCHFV-infected patients tested positive and 8/8 known negative sera tested negative. A stable cell line expressing recombinant CCHFV NP, for preparation of in-house antigen slides, could have application in IFA for diagnostic and surveillance purposes and will be more cost effective than the commercial antigen slides for testing large number of samples.

In conclusion, CCHFV has the propensity to spread in Europe where the *Hyalomma spp.* tick is present and climate changes favouring tick populations. Since 2002 Turkey has had over 8 000 CCHFV cases per year. In contrast, South Africa has about one to ten cases per year. However, the current Ebola virus outbreak in Africa emphasizes the dire need to continually investigate options for the development of safe reagents for detection of dangerous pathogens and to investigate viral proteins that induce detectable antibody responses. Eventhough an internal control should be included and negative results should be confirmed using another assay, the CCHFV NASBA assay is more cost effective, is easier to obtain and has diagnostic application in low resource countries. Investigation of immunodominant antigens are important to consider when developing serological assays. Factors such as antigenic cross reactivity and duration of antibody contribute to understanding the kinetics of antibody responses, which in turn contributes to correct interpretation of results. Identifying antibodies against CCHFV NP and GP in sera collected years after onset of illness has application in surveillance studies. Preparation of antigen slides from stable cell lines expressing CCHFV NP and GP is more cost effective and could increase diagnostic and surveillance capacity of CCHFV.



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## Appendix A – CLUSTALX 2.1 Alignments

### 1. CCHFV partial S segment sequence alignment for NASBA primer design

The 450 bp region position within the S segment ORF is between bp 131 – 580 relative to the SPU 415/85 isolate, bp 1 of the ORF is the first base of the ATG codon.

The sequence titles are the SPU number followed by the Genbank Accession number.

**Key:**

-  - Forward primer site
-  - Detection probe site
-  - Reverse primer site

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		135	145	155	165	175	185	195	205
SPU107/00	AY905646	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU106/00	AY905645	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGACGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU536/86	AY905632	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU422/86	U84638	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTAGAGGCA
SPU234/01	AY905651	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU45/88	U84637	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGAAGCA
SPU187/86	AY905628	ACAAGTTTGT	GTTCCAAATG	GCCAGCGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTATG	CGTCTGCTCT	GGTGGAGGCA
SPU381/86	AY905630	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTATG	CGTCTGCTCT	GGTGGAGGCA
SPU24/01	AY905648	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAGAAAGGAT	TCCATCTACG	CGTCTGCTCT	AGTGGAGGCA
SPU170/00	AY905647	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAGAAAGGAT	TCCATCTACG	CGGTTGCTCT	AGTGGAGGCA
SPU509/86	AY905631	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CCGATGATGC	ACAGAAAGGAT	TCCATCTACG	CGTCTGCTCT	AGTGGAGGCA
SPU40/00	AY905644	ACAGATTTGT	GTTCCAAATG	GCCGGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTATG	CATCTGCTCT	GGTGGAGGCA
SPU245/87	U84639	ACAGATTTGT	GTTCCAAATG	GCCGGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTATG	CATCTGCTCT	GGTGGAGGCA
SPU566/86	AY905633	ACAGATTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTATG	CATCTGCTCT	GGTGGAGGCA
SPU198/98	AY905640	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU234/99	AY905641	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGTT	TCCATCWACG	CGTCTGCTCT	GGTGGAGGCA
SPU70/01	AY905650	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	GCAAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU399/01	AY905652	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCTATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU196/86	AY905629	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCTATCTACG	CGTCCGCTCT	GGTGGAGGCA
SPU378/90	AY905638	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCTATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU355/99	AY905642	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCTATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU51/01	AY905649	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCTATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU259/84	AY905625	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCTATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU18/88	AY905635	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCTATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU103/87	AY905634	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU27/00	AY905643	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTATG	CGTCTGCTCT	GGTGGAGGCA
SPU415/85	AY905627	ACAAGTTTGT	GTTCCAAATG	GCCAGTGCCA	CTGATGATGC	ACAAAAGGAT	TCCATCTACG	CGTCTGCTCT	GGTGGAGGCA
SPU582/86	U84635	ATAGGTTTGT	ATTCCAGATG	GCCAGTGCAA	CCGATGATGC	ACAAAAGGAC	TCCATCTATG	CATCTGCTCT	AGTGGAGGCA
SPU247/85	U84636	ATAGGTTTGT	ATTCCAGATG	GCCAGTGCAA	CCGATGATGC	ACAAAAGGAT	TCCATCTATG	CATCTGCTCT	AGTGGAGGCA
SPU94/85	AY905626	ATAGGTTTGT	ATTCCAGATG	GCCAGTGCAA	CCGATGATGC	ACAAAAGGAT	TCCATCTATG	CATCTGCTCT	AGTGGAGGCA
SPU281/89	AY905637	ACAAGTTTGT	ATTCCAGATG	GCCAGTGCAA	CCGATGATGC	ACAAAAGGAT	TCCATCTATG	CATCTGCTCT	AGTGGAGGCA
SPU60/89	AY905636	ACAGGTTTGT	ATTCCAGATG	GCTAGTGCAA	CCGATGATGC	ACAAAAAGAT	TCCATCTATG	CATCTGCCTT	GGTTGAGGCT
SPU68/98	AY905639	ATAGGTTTGT	ATTCCAGATG	GCTAGTGCAA	CCGAAGATGC	ACAAAAAGAT	TCCATCTATG	CATCTGCTCT	GGTTGAGGCT
Clustal Consensus		* * * * *	* * * * *	** * * *	* * * * *	** * *	** * * *	* * *	* * * *

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		215	225	235	245	255	265	275	285	
SPU107/00	AY905646	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTCG	AATGGTTCGA
SPU106/00	AY905645	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTCA	GCTCCACTGG	CATTGTGAAG	AAGGGACTTG	AATGGTTCGA
SPU536/86	AY905632	ACAAAATTTT	GTGCACCTGT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAA	AGGGGACTTG	AATGGTTCGA
SPU422/86	U84638	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU234/01	AY905651	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU45/88	U84637	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU187/86	AY905628	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU381/86	AY905630	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU24/01	AY905648	ACAAAGTTTT	GTGCACCTAT	ATACGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AAAGGACTTG	AATGGTTCGA
SPU170/00	AY905647	ACAAAGTTTT	GTGCACCTAT	ATACGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AAAGGACTTG	AATGGTTCGA
SPU509/86	AY905631	ACAAAGTTCT	GTGCACCTAT	ATACGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AAAGGACTTG	AATGGTTCGA
SPU40/00	AY905644	ACAAAATTCT	GTGCACCTAT	ATACGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AAGGGACTTG	AATGGTTCGA
SPU245/87	U84639	ACAAAATTTT	GTGCACCTAT	ATACGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AAGGGACTTG	AATGGTTCGA
SPU566/86	AY905633	ACAAAGTTTT	GTGCACCTAT	ATACGAGTGT	GCA	TGGGTTA	GCTCCACCGG	CATTGTGAAG	AAGGGACTTG	AATGGTTCGA
SPU198/98	AY905640	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU234/99	AY905641	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU70/01	AY905650	ACAAAATTTT	GTGCACCTAT	ATATGAGTGC	GCG	TGGGTTA	GCTCCACTGG	CATTGTAAAG	AGGGGACTTG	AATGGTTCGA
SPU399/01	AY905652	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACCGG	CATTGTGAGG	AGGGGACTTG	AATGGTTCGA
SPU196/86	AY905629	ACAAAATTCT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU378/90	AY905638	ACAAAGTTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU355/99	AY905642	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU51/01	AY905649	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU259/84	AY905625	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGAGGCTTTG	AATGGTTCGA
SPU18/88	AY905635	ACAAAATTTT	GTGCACCTAT	ATATGAGTAT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AGGGGACTTG	AATGGTTCGA
SPU103/87	AY905634	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCG	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AAGGGACTTG	AATGGTTCGA
SPU27/00	AY905643	ACAAAATTTT	GTGCACCTAT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAG	AAGGGACTTG	AATGGTTCGA
SPU415/85	AY905627	ACAAAATTTT	GTGCACCTGT	ATATGAGTGT	GCA	TGGGTTA	GCTCCACTGG	CATTGTGAAA	AGGGGCCTTG	AATGGTTCGA
SPU582/86	U84635	ACTAAGTTCT	GTGCACCTAT	ATACGAGTGT	GCA	TGGGTCA	GCTCCACCGG	TATAGTCAAG	AAGGGCCTTG	AGTGGTTCGA
SPU247/85	U84636	ACTAAGTTCT	GTGCACCTAT	ATACGAGTGT	GCA	TGGGTCA	GCTCCACCGG	CATAGTCAAG	AAGGGCCTTG	AATGGTTCGA
SPU94/85	AY905626	ACTAAGTTCT	GTGCACCTAT	ATACGAGTGT	GCA	TGGGTCA	GCTCCACCGG	CATAGTCAAG	AAGGGCCTTG	AATGGTTCGA
SPU281/89	AY905637	ACTAAGTTCT	GTGCACCTAT	ATACGAGTGT	GCA	TGGGTCA	GCTCCACCGG	CATAGTCAAG	AAGGGCCTTG	AATGGTTCGA
SPU60/89	AY905636	ACCAAGTATT	GTGCTCCCAT	ATATGAATGT	GCC	TGGGTTA	GCTCCACAGG	CATAGTGAAA	AGGGGCCTTG	AGTGGTTTGA
SPU68/98	AY905639	ACCAAGTATT	GTGCTCCCAT	ATATGAATGT	GCC	TGGGTTA	GCTCCACAGG	CATAGTGAAA	AGAGGCCTTG	AGTGGTTTGA
Clustal Consensus		** * *	***** *	*** ** *	** ***** *	***** **	** ** *	* * ** ** *	* ***** **	

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		295	305	315	325	335	345	355	365
SPU107/00	AY905646	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAGCTTG
SPU106/00	AY905645	AAAAAATGCA	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAGCTTG
SPU536/86	AY905632	AAAAAATGCG	GGCACCATTA	AGTCTGGGGA	TGAAAGTTAT	ACGGAGCTAA	AAGTCGACGT	CCCAAAAATA	GAACAGCTAG
SPU422/86	U84638	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCAAAAATA	GAACAGCTTG
SPU234/01	AY905651	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAGCTTG
SPU45/88	U84637	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAGCTTG
SPU187/86	AY905628	AAAAAATGCG	GGCACCATCA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU381/86	AY905630	AAAAAATGCG	GGCACCATCA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU24/01	AY905648	AAAAAATGCA	GGCACTATTA	AGTCCTGGGA	TGAAAGTTAC	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU170/00	AY905647	AAAAAATGCA	GGCACCATTA	AGTCCTGGGA	TGAAAGTTAC	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU509/86	AY905631	AAAAAATGCA	GGCACTATTA	AGTCCTGGGA	TGAAAGTTAC	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU40/00	AY905644	AAAAAATGCA	GGCACCATTA	AGTCCTGGGA	TGAAAGTTAC	ACTGAACTAA	AAGTCGACGT	CCCGAAAATA	GAACAACTTG
SPU245/87	U84639	AAAAAATGCA	GGCACCATTA	AGTCCTGGGA	TGAAAGTTAC	ACTGAACTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU566/86	AY905633	AAAAAATGCA	GGCACCATTA	AGTCCTGGGA	TGAAAGTTAC	ACTGAACTAA	AAGTCGACGT	CCCGAAAATA	GAACAACTTG
SPU198/98	AY905640	AAAAAATGCG	GGCACCATTA	AGTCATGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU234/99	AY905641	AAAAAATGCG	GGCACCATTA	AGTCATGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU70/01	AY905650	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU399/01	AY905652	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU196/86	AY905629	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU378/90	AY905638	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU355/99	AY905642	AAAAAATGCG	GGCACCATCA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGATGT	CCCGAAAATA	GAACAACTTG
SPU51/01	AY905649	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAC	ACCGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU259/84	AY905625	AAAAAATGCG	GGTACCATTA	AGTCTTGGGA	TGAAAGTTAC	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU18/88	AY905635	AAAAAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCAAAAATA	GAACAACTTG
SPU103/87	AY905634	AAAGAATGCG	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAGCTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU27/00	AY905643	AAAAAATGCA	GGCACCATTA	AGTCTTGGGA	TGAAAGTTAT	ACTGAACTAA	AAGTTGACGT	CCCGAAAATA	GAACAACTTG
SPU415/85	AY905627	AAAGAATGCA	GGAACATATCA	AGTCTTGGGA	TGAGAGCTAT	ACTGAGCTTA	AAGTTGGTGT	CCCCAAAATA	GAGCAGCTTG
SPU582/86	U84635	AAAGAATGCA	GGAACATATCA	AGTCTTGGGA	TGAGAGCTAT	ACTGAGCTTA	AAGTTGGTGT	CCCCAAAATA	GAGCAGCTTG
SPU247/85	U84636	AAAGAATGCA	GGAACATATCA	AGTCTTGGGA	TGAGAGCTAT	ACTGAGCTTA	AAGTTGGTGT	CCCCAAAATA	GAGCAGCTTC
SPU94/85	AY905626	AAAGAATGCA	GGAACATATTA	AGTCTTGGGA	TGAGAGCTAT	ACTGAGCTTA	AAGTTGGTGT	CCCCAAAATA	GAGCAGCTTG
SPU281/89	AY905637	AAAGAATGCA	GGAACCATCA	AGTCTTGGGA	TGAGAGCTAT	ACTGAGCTTA	AAGTTGGTGT	CCCCAAAATA	GAGCAGCTTG
SPU60/89	AY905636	AAAAAACACA	GGAACCATCA	AGTCTTGGGA	TGAGAGCTAC	ACTGAACTGA	AAGTGGATGT	GCCCCAAAATT	GAACAGCTTG
SPU68/98	AY905639	AAAAAACACA	GGAACCATCA	AGTCTTGGGA	TGAGAGCTAC	ACTGAACTGA	AAGTGGATGT	GCCCCAAAATT	GAACAACTTG
Clustal Consensus		*** ** *	** ** ** *	***** *****	*** ** **	** ** ** *	***** * **	** *****	** ** **



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		375	385	395	405	415	425	435	445	
SPU107/00	AY905646	CCAATTACCA	ACAGGCTGCC	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU106/00	AY905645	CCAATTACCA	ACAGGCTGCT	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU536/86	AY905632	CCAATTACCA	ACAGGCTGCC	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGTAAC
SPU422/86	U84638	CCAATTACCA	ACAGGCTGCC	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU234/01	AY905651	CCAATTACCA	ACAGGCTGCC	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCAGC	TCTGAGCAAC
SPU45/88	U84637	CTAATTACCA	ACAAGCTGCC	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU187/86	AY905628	CTAATTACCA	ACAGGCTGCC	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU381/86	AY905630	CTAATTACCA	ACAGGCTGCC	TTGAAATGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU24/01	AY905648	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU170/00	AY905647	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU509/86	AY905631	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGCAAC
SPU40/00	AY905644	CCAATTACCA	ACAAGCTGCC	TTGAAGTGGA	GAAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU245/87	U84639	CCAATTACCA	ACAAGCTGCC	TTGAAGTGGA	GAAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU566/86	AY905633	CCAATTACCA	ACAAGCTGCC	TTGAAGTGGA	GGAAAGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU198/98	AY905640	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU234/99	AY905641	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU70/01	AY905650	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGC	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAT
SPU399/01	AY905652	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU196/86	AY905629	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU378/90	AY905638	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU355/99	AY905642	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU51/01	AY905649	CCAATTACCA	ACAGGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU259/84	AY905625	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGAAAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU18/88	AY905635	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU103/87	AY905634	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU27/00	AY905643	CCAATTACCA	ACAAGCTGCC	TTGAAATGGA	GAAAGGACAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TTTGAGCAAC
SPU415/85	AY905627	CAAACCTACCA	ACAGGCTGCT	CTAAAGTGGA	GGAAGGATAT	AGGT	TTCCGT	GTCAATGCAA	ACACAGCGGC	TCTGAGTAAC
SPU582/86	U84635	CAAACCTACCA	ACAGGCTGCT	CTAAAGTGGA	GGAAGGATAT	AGGT	TTCCGT	GTCAACGCCA	ATACGGCAGC	CTTGAGCAAC
SPU247/85	U84636	CAAACCTACCA	ACAGGCTGCT	CTAAAGTGGA	GGAAGGATAT	AGGT	TTCCGT	GTCAACGCCA	ATACGGCAGC	CTTAAGCAAC
SPU94/85	AY905626	CAAACCTACCA	ACAGGCTGCT	CTAAAGTGGA	GGAAGGATAT	AGGT	TTCCGT	GTCAACGCCA	ATACGGCAGC	CTTGAGCAAC
SPU281/89	AY905637	CAAACCTACCA	ACAGGCTGCT	CTAAAGTGGA	GGAAGGATAT	AGGT				

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		455	465	475	485	495	505	515	525	
SPU107/00	AY905646	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGA	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU106/00	AY905645	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGA	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU536/86	AY905632	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU422/86	U84638	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAAATGCTG	TCAGACATGA
SPU234/01	AY905651	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU45/88	U84637	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU187/86	AY905628	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU381/86	AY905630	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU24/01	AY905648	AAAGTCCTCG	CAGAGTACAA	GGTT	CCTGGC	GAGATTGTGA	TGTC	CGTCAA	AGAGATGCTG	TCGGACATGA
SPU170/00	AY905647	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	CGTCAA	AGAGATGCTG	TCGGACATGA
SPU509/86	AY905631	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	CGTCAA	AGAGATGCTG	TCAGACATGA
SPU40/00	AY905644	AAAGTCCTTG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	CGTCAA	AGAGATGCTG	TCTGACATGA
SPU245/87	U84639	AAAGTCCTTG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	CGTCAA	AGAAATGCTG	TCTGACATGA
SPU566/86	AY905633	AAAGTCCTTG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	CGTCAA	AGAGATGCTG	TCTGACATGA
SPU198/98	AY905640	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU234/99	AY905641	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU70/01	AY905650	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU399/01	AY905652	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU196/86	AY905629	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU378/90	AY905638	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU355/99	AY905642	AAAGTCCTAG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU51/01	AY905649	AAAGTCCTCG	CAGAGTACAA	AGTC	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU259/84	AY905625	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GATATTCTGA	TGTC	TGTCAA	AGAGATGCTG	TCTGACATGA
SPU18/88	AY905635	AAAGTCCTCG	CAGAGTACAA	AGTA	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU103/87	AY905634	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU27/00	AY905643	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGC	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU415/85	AY905627	AAAGTCCTCG	CAGAGTACAA	AGTT	CCTGGA	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTG	TCAGACATGA
SPU582/86	U84635	AAAGTCCTTG	CAGAATACAA	AGTC	CCTGGT	GAAATTGTGA	TGTC	TGTCAA	AGAAATGTTA	TCAGACATGA
SPU247/85	U84636	AAAGTCCTTG	CAGAATACAA	AGTC	CCTGGT	GAAATTGTGA	TGTC	TGTCAA	AGAAATGTTA	TCAGACATGA
SPU94/85	AY905626	AAAGTCCTTG	CAGAATACAA	AGTC	CCTGGT	GAAATTGTGA	TGTC	TGTCAA	AGAAATGTTA	TCAGACATGA
SPU281/89	AY905637	AAAGTCCTTG	CAGAATACAA	AGTC	CCTGGT	GAAATTGTGA	TGTC	TGTCAA	AGAAATGTTA	TCAGACATGA
SPU60/89	AY905636	AAAGTCCTCT	CAGAGTACAA	GGTT	CCTGGT	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTT	TCAGACATGA
SPU68/98	AY905639	AAAGTTCTCT	CAGAGTACAA	GGTT	CCTGGT	GAGATTGTGA	TGTC	TGTCAA	AGAGATGCTT	TCAGACATGA
Clustal Consensus		***** **	**** *****	**	***** **	*** **	****	*****	*** **	** ***** *

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		535	545	555	565	575
SPU107/00	AY905646	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU106/00	AY905645	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU536/86	AY905632	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU422/86	U84638	GAACCAGATT	CTAAACAGAG	GTGGCGATGA	GAATCCACGT	GGCCCTGTGA
SPU234/01	AY905651	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU45/88	U84637	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATGGACGT	GGCCCTGTGA
SPU187/86	AY905628	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU381/86	AY905630	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU24/01	AY905648	AAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU170/00	AY905647	AAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU509/86	AY905631	AAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU40/00	AY905644	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCAGTGA
SPU245/87	U84639	GAACCAAATA	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCAGTGA
SPU566/86	AY905633	AAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU198/98	AY905640	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU234/99	AY905641	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU70/01	AY905650	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTAA
SPU399/01	AY905652	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU196/86	AY905629	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU378/90	AY905638	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU355/99	AY905642	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU51/01	AY905649	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCCGTGA
SPU259/84	AY905625	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU18/88	AY905635	GAACCAAATT	CTAAACAGAG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU103/87	AY905634	GAACCAAATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU27/00	AY905643	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU415/85	AY905627	GAACCAGATT	CTAAACAGGG	GTGGTGATGA	GAATCCACGT	GGCCCTGTGA
SPU582/86	U84635	GAACCTGATC	CTCAACAGAG	GTGGCGATGA	GAACCCACGC	GGTCCAGTGA
SPU247/85	U84636	GAACCTGATC	CTCAACAGAG	GTGGCGATGA	GAACCCACGC	GGTCCAGTGA
SPU94/85	AY905626	GAACCTGATC	CTCAACAGAG	GTGGCGATGA	GAACCCACGA	GGTCCAGTAA
SPU281/89	AY905637	GAACCTGATC	CTCAACAGAG	GTGGCGATGA	GAACCCACGC	GGTCCTGTGA
SPU60/89	AY905636	AAACCTGATC	CTTAACAGAG	GGGGTGATGA	GAACCCAAGA	GGCCCAGTAA
SPU68/98	AY905639	GAACCTGATC	CTTAACAGAG	GGGGTGATGA	GAACCCAAGA	GGCCCAGTAA
Clustal Consensus		*** **	** ***** *	* ** *****	*** * *	** ** * *

## 2. CCHFV isolates SPU 415/85 and SPU 582/86 aligned with the primers and detection probe

The sequence titles are the SPU numbers followed by the Genbank Accession numbers.

### 2.1. Alignment with NASBA Forward primer:

```

                ....|....| ....|....| ...
                248       258
SPU415/85 | AY905627 TGGGT TAGCT CCACTGGCAT TGT
SPU582/86 | U84635  TGGGT TAGCT CCACCGGTAT AGT
NASBA_F_1   TGGG C TAGCT CCAC GGCAT TGT
NASBA_F_2   TGGG C TAGCT CCACTGGCAT TGT
NASBA_F_3   TGGG T TAGCT CCACCGGCAT TGT
NASBA_F_4   TGGG T TAGCT CCACTGGCAT TGT
Clustal Consensus ***** ** ** **

```

### 2.3. Alignment with the NASBA Reverse primer:

```

                ....|....| ....|....|
                5       15
SPU415/85 | AY905627 CCTGG AGAGA TTGTGATGTC
SPU582/86 | U84635  CCTGG TGAAA TTGTGATGTC
NASBA_R_1   CCTGGCGAGA TTGT GATGTC
NASBA_R_2   CCTGGCGAAA TTGT GATGTC
NASBA_R_3   CCTGGCGAGA TTGT AATGTC
NASBA_R_4   CCTGGCGAAA TTGT AATGTC
Clustal Consensus ***** ** * *****

```

### 2.2. Alignment with the NASBA Detection probe:

```

                ....|....| ....|....| ...
                419       429
SPU415/85 | AY905627 TTCCGTGTCA ATGCAAACAC AGC
SPU582/86 | U84635  TTCCGTGTCA AGCCAATAC GGC
DET_PROBE  TTCCGTGTCA ATGCAAACAC AGC
Clustal Consensus ***** * ** ** **

```

#### Key:

- X – Degenerate bases within NASBA Forward primer
- X – Degenerate bases within NASBA Reverse primer
- X – Single base mismatches between SPU 415/85 and NASBA R primer
- X – Single base mismatches between SPU 582/86 and NASBA Forward / Reverse primer / Detection probe

### 3. Sequencing results of the recombinant bacmid:

The recombinant bacmid was sequenced using the M13 F and M13 R primer pair and aligned with the CCHFV.NP.opt gene nucleotide sequence. The BamHI restriction sites are positioned at bp 1 – 6 and bp 1453 – 1458. The second codon of the CCHFV.NP.opt ORF starts at bp 7 and ends at bp 1452.

```

      10      20      30      40      50      60      70      80      90     100
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  GGATCCGAAAAACAAGATCGAGGTCAACAACAAGGACGAGATGAACAAGTGGTTCGAGGAGTTCAAGAAGGGCAACGGTCTGGTGGACACATTACCAACT
   *****

      110     120     130     140     150     160     170     180     190     200
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  CTTACAGCTTCTGCGAGAGCGTTCCAACTTGGACAAGTTCGTGTTCCAGATGGCTTCAGCCACTGACGATGCCCAAAGGATAGCATCTACGCTTCAGC
   *****

      210     220     230     240     250     260     270     280     290     300
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  CCTCGTCGAGGCCACTAAGTTCTGCGCTCCGGTGTACGAATGTGCCTGGGTCTCCTCTACAGGCATCGTTAAGCGTGGACTGGAGTGGTTTCGAAAAGAAC
   *****

      310     320     330     340     350     360     370     380     390     400
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  GCTGGAACAATCAAGTCGTGGGACGAGTCCTACACCGAACTCAAGGTGGATGTCCCCAAGATCGAGCAGTTGGCTAACTACCAGCAAGCTGCCCTGAAGT
   *****

```

```

          410          420          430          440          450          460          470          480          490          500
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  GGCGCAAGGACATCGGATTCAGGGTGAACGCCAACACCGCTGCCCTCAGCAACAAGGTGTTGGCTGAGTACAAGGTCCCTGGTGAAATCGTTATGTCAGT
   GGCGCAAGGACATCGGATTCAGGGTGAACGCCAACACCGCTGCCCTCAGCAACAAGGTGTTGGCTGAGTACAAGGTCCCTGGTGAAATCGTTATGTCAGT
   *****

          510          520          530          540          550          560          570          580          590          600
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  GAAGGAGATGCTCTCGGACATGATCCGTCGCAGGAACCAGATCTTGAACAGAGGTGGCGACGAAAACCCAAGAGGCCCGGTCTCCCGTGAGCACGTTGAT
   GAAGGAGATGCTCTCGGACATGATCCGTCGCAGGAACCAGATCTTGAACAGAGGTGGCGACGAAAACCCAAGAGGCCCGGTCTCCCGTGAGCACGTTGAT
   *****

          610          620          630          640          650          660          670          680          690          700
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  TGGTGTCGTGAATTCGTCAAGGGAAAGTACATCATGGCTTTCAACCCCCCTTGGGGTGACATCAACAAGTCAGGTAGATCGGGAATCGCTCTGGTGGCTA
   TGGTGTCGTGAATTCGTCAAGGGAAAGTACATCATGGCTTTCAACCCCCCTTGGGGTGACATCAACAAGTCAGGTAGATCGGGAATCGCTCTGGTGGCTA
   *****

          710          720          730          740          750          760          770          780          790          800
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  CCGGATTGGCTAAGCTGGCCGAGACTGAAGGAAAGGGTGTGTTTCGACGAGGCCAAGAAGACAGTCGAAGCTTTGAACGGATACCTGGACAAGCACAAAGGA
   CCGGATTGGCTAAGCTGGCCGAGACTGAAGGAAAGGGTGTGTTTCGACGAGGCCAAGAAGACAGTCGAAGCTTTGAACGGATACCTGGACAAGCACAAAGGA
   *****

          810          820          830          840          850          860          870          880          890          900
1  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
2  CGAGGTGGATCGCGCTTCCGCCGATTCTATGATCACTAACCTGCTCAAGCACATCGCTAAGGCCCAGGAAGTGTACAAGAACAGCTCAGCTCTCAGGGCC
   CGAGGTGGATCGCGCTTCCGCCGATTCTATGATCACTAACCTGCTCAAGCACATCGCTAAGGCCCAGGAAGTGTACAAGAACAGCTCAGCTCTCAGGGCC
   *****

```

```

          910          920          930          940          950          960          970          980          990          1000
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1  CAGGGCGCTCAAATCGACACCGCCTTCTCGTCCTACTACTGGCTGTACAAGGCTGGAGTTACCCCCGAGACTTTCCCTACAGTGTGCGAGTTCCTCTTCG
2  CAGGGCGCTCAAATCGACACCGCCTTCTCGTCCTACTACTGGCTGTACAAGGCTGGAGTTACCCCCGAGACTTTCCCTACAGTGTGCGAGTTCCTCTTCG
      *****

          1010         1020         1030         1040         1050         1060         1070         1080         1090         1100
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1  AATTGGGCAAGCAACCCCGCGGCACTAAGAAGATGAAGAAGGCCTTGCTGTCTACACCTATGAAGTGGGGCAAGAAGCTGTACGAGCTCTTCGCTGACGA
2  AATTGGGCAAGCAACCCCGCGGCACTAAGAAGATGAAGAAGGCCTTGCTGTCTACACCTATGAAGTGGGGCAAGAAGCTGTACGAGCTCTTCGCTGACGA
      *****

          1110         1120         1130         1140         1150         1160         1170         1180         1190         1200
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1  TACCTTCCAGCAAAACCGCATCTACATGCACCCAGCCGTCCTGACAGCTGGCAGGATCTCTGAAATGGGAGTCTGCTTCGGTACCATCCCAGTTGCTAAC
2  TACCTTCCAGCAAAACCGCATCTACATGCACCCAGCCGTCCTGACAGCTGGCAGGATCTCTGAAATGGGAGTCTGCTTCGGTACCATCCCAGTTGCTAAC
      *****

          1210         1220         1230         1240         1250         1260         1270         1280         1290         1300
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1  CCGGACGATGCTGCCCAGGGCTCTGGACACACTAAGAGCATCCTGAACCTCAGAACCAACACTGAGACAAACAACCCTTG TGCCCGTACCATCGTGAAGC
2  CCGGACGATGCTGCCCAGGGCTCTGGACACACTAAGAGCATCCTGAACCTCAGAACCAACACTGAGACAAACAACCCTTG TGCCCGTACCATCGTGAAGC
      *****

          1310         1320         1330         1340         1350         1360         1370         1380         1390         1400
      ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
1  TGTTGGAATCCAGAAGACTGGTTTCAACATCCAAGACATGGATATCGTCGCTTCCGAGCACCTCTTGACCAATCTCTCGTTGGCAAGCAGTCCCCATT
2  TGTTGGAATCCAGAAGACTGGTTTCAACATCCAAGACATGGATATCGTCGCTTCCGAGCACCTCTTGACCAATCTCTCGTTGGCAAGCAGTCCCCATT
      *****

```

```

          1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
1  CAAAAACGCTTACAACGTCAAGGGCAACGCTACATCAGTCAACATCATCTAAGGATCC
2  CAAAAACGCTTACAACGTCAAGGGCAACGCTACATCAGTCAACATCATCTAAGGATCC
   *****

```

**Key:**

1 – Sequence obtained from recombinant bacmid using M13 F and R primer pair

2 – Nucleotide sequence of the CCHFV.NP.opt gene

GGATCC – BamHI restriction sites



## Appendix B – Raw absorbance data obtained with the NASBA assays

All absorbance values were measured at a wavelength of 405 nm.

### 1. Calculation of the cut-off value determined using results obtained from panel of known negative samples

Samples containing NFW instead of RNA and samples containing NFW instead of amplicon were tested in duplicate using the CCHFV NASBA and the CCHFV NucliNASBA and used to determine the cut-off values. Alternatively, RNA extracted from tissue culture infected with a non-CCHF virus could be used to determine the cut-off value.

**Table B.1. Absorbance data of known negative reactions tested with the NASBA assays**

	Absorbance values							
NFW instead of RNA during amplification	0.043	0.065	0.054	0.160	0.060	0.061	0.029	0.025
	0.037	0.027	0.056	0.168	0.146	0.033	0.032	0.032
NFW instead of amplicon during detection	0.040	0.031	0.052	0.077	0.198	0.024	0.027	0.034
	0.084	0.048	0.120	0.057	0.138	0.031	0.024	0.066

CCHFV NASBA Cut-off

= Average + 2 × (Standard deviation)

= 0.069938 + 2 × (0.043198)

= 0.156333

= 0.156

CCHFV NucliNASBA Cut-off

= Average + 2 × (Standard deviation)

= 0.06 + 2 × (0.053067)

= 0.166135

= 0.166

### 2. Absorbance data obtained from serial dilutions of RNA extracted from infected tissue culture and tested using the CCHFV NASBA and CCHFV NucliNASBA

RNA extracted from Vero cell culture infected with SPU 4/81 was diluted and tested in duplicate using the CCHFV NASBA and the CCHFV NucliNASBA.

**Table B.2. Absorbance data from the diluted CCHFV RNA tested with the NASBA assays**

Dilution	CCHFV NASBA				CCHFV NucliNASBA			
	Absorbance values		Mean	Result	Absorbance values		Mean	Result
$10^0$	2.273	2.697	2.485	Positive	2.621	2.322	2.472	Positive
$10^{-0.5}$	2.581	2.413	2.497	Positive	2.493	2.014	2.254	Positive
$10^{-1.0}$	2.509	2.377	2.443	Positive	2.149	2.269	2.209	Positive
$10^{-1.5}$	0.918	1.148	1.033	Positive	1.994	2.061	2.028	Positive
$10^{-2.0}$	2.261	2.421	2.341	Positive	2.107	2.125	2.116	Positive
$10^{-2.5}$	2.639	2.459	2.549	Positive	2.154	2.356	2.255	Positive
$10^{-3.0}$	0.888	0.792	0.840	Positive	2.492	2.512	2.502	Positive
$10^{-3.5}$	2.858	2.902	2.880	Positive	2.671	2.565	2.618	Positive
$10^{-4.0}$	2.170	2.371	2.271	Positive	2.639	2.607	2.623	Positive
$10^{-4.5}$	1.384	1.775	1.580	Positive	2.152	2.240	2.196	Positive
$10^{-5.0}$	0.128	0.132	0.130	Negative	0.529	0.651	0.590	Positive
$10^{-5.5}$	0.105	0.116	0.111	Negative	0.492	0.341	0.417	Positive

### 3. Specificity of NASBA.

Absorbance data from RNA extracted from infected tissue culture tested with the CCHFV NASBA. Each RNA sample was tested in triplicate.

**Table B.3. Absorbance data from RNA samples extracted from CCHFV-infected tissue culture tested with the CCHFV NASBA assay**

SPU number	Absorbance values			Mean	Result
259/84	0.051	0.187	0.197	0.145	Negative
94/85	0.400	0.482	0.417	0.433	Positive
97/85	0.396	0.468	0.405	0.423	Positive
247/85	0.700	1.029	1.385	1.038	Positive
381/85	0.256	0.262	0.268	0.262	Positive
396/85	0.051	0.057	0.038	0.049	Negative
408/85	0.092	0.183	0.085	0.120	Negative
415/85	0.103	0.171	0.167	0.147	Negative
23/86	0.371	0.512	0.374	0.419	Positive
196/86	1.653	1.107	1.014	1.258	Positive
361/86	0.380	0.458	0.494	0.444	Positive
536/86	0.263	0.518	0.479	0.420	Positive
560/86	0.541	0.572	0.201	0.438	Positive

**Table B.3. Continued**

<b>SPU number</b>	<b>Absorbance values</b>			<b>Mean</b>	<b>Result</b>
566/86	0.129	0.865	0.272	0.422	Positive
582/86	0.043	0.104	0.135	0.094	Negative
593/86	0.148	0.301	0.220	0.223	Positive
103/87	1.077	0.639	1.890	1.202	Positive
177/87	0.404	0.418	0.354	0.392	Positive
244/87	0.488	0.619	0.234	0.447	Positive
383/87	0.476	0.453	0.325	0.418	Positive
409/87	0.063	0.047	0.055	0.055	Negative
486/87	0.503	0.452	0.287	0.414	Positive
556/87	0.528	0.497	0.262	0.429	Positive
18/88 <sup>a</sup>	0.614	0.283	0.399	0.432	Positive
19/88 <sup>a</sup>	1.239	1.115	0.784	1.046	Positive
45/88	0.361	0.501	0.419	0.427	Positive
71/88	0.594	0.248	0.436	0.426	Positive
203/88	0.284	0.419	0.485	0.396	Positive
273/88	0.373	0.219	0.053	0.215	Positive
497/88	0.263	0.617	0.287	0.389	Positive
498/88	0.402	0.384	0.345	0.377	Positive
130/89	0.319	0.427	0.403	0.383	Positive
281/89	0.052	0.063	0.041	0.052	Negative
337/89	0.521	0.237	0.388	0.382	Positive
372/89	0.332	0.359	0.344	0.345	Positive
444/89	0.229	0.228	0.245	0.234	Positive
61/90	0.480	0.926	0.520	0.642	Positive
215/90	1.266	0.997	1.238	1.167	Positive
378/90 <sup>b</sup>	1.576	1.249	1.183	1.336	Positive
380/90 <sup>b</sup>	1.143	1.169	1.132	1.148	Positive
381/90 <sup>b</sup>	0.635	1.023	1.267	0.975	Positive
382/90 <sup>b</sup>	0.917	1.038	1.405	1.120	Positive
388/90 <sup>b</sup>	1.218	1.116	1.668	1.334	Positive
15/92	1.009	0.794	0.987	0.930	Positive
50/93	0.872	1.003	0.915	0.930	Positive
172/93	0.989	1.217	0.857	1.021	Positive
24/01	1.419	0.823	0.530	0.924	Positive
51/01	0.805	0.621	0.164	0.530	Positive
RVFV	0.028	0.032	0.032	0.031	Negative
YFV	0.037	0.059	0.074	0.057	Negative

<sup>a</sup> & <sup>b</sup> - samples taken from the same patient

#### 4. Comparison of results using a water bath and a heating block

Each RNA sample was tested in duplicate.

**Table B.4. Absorbance data from three RNA samples extracted from infected tissue culture tested with the CCHFV NASBA assay using a water bath or heating block**

SPU numbers and negative sample	408/85	103/87	498/88	NFW
	Absorbance values			
Water bath	0.036	0.645	0.131	0.017
	0.036	0.218	0.264	0.032
Heating block	0.031	1.189	1.184	0.021
	0.021	0.556	0.587	0.069

#### 5. Sensitivity of the NASBA assays

Absorbance data obtained from serial dilutions of RNA extracted from tissue culture infected with SPU 4/81 tested with the CCHFV NASBA and CCHFV NucliNASBA.

**Table B.5. Absorbance data from the serially diluted control RNA transcript tested with the NASBA assays**

Copy number	CCHFV NASBA				CCHFV NucliNASBA			
	Absorbance values		Mean	Result	Absorbance values		Mean	Result
$3.7 \times 10^{12}$	1.596	1.492	1.544	Positive	1.347	1.482	1.415	Positive
$3.7 \times 10^{11}$	1.353	1.289	1.321	Positive	1.286	1.321	1.304	Positive
$3.7 \times 10^{10}$	1.355	1.492	1.424	Positive	1.311	1.305	1.308	Positive
$3.7 \times 10^9$	1.379	1.293	1.336	Positive	1.197	1.29	1.244	Positive
$3.7 \times 10^8$	1.392	1.408	1.400	Positive	1.237	1.186	1.212	Positive
$3.7 \times 10^7$	1.395	1.402	1.399	Positive	1.256	1.181	1.219	Positive
$3.7 \times 10^6$	1.63	1.432	1.531	Positive	1.309	1.226	1.268	Positive
$3.7 \times 10^5$	1.501	1.382	1.442	Positive	1.184	1.085	1.135	Positive
$3.7 \times 10^4$	1.441	1.408	1.425	Positive	1.336	1.293	1.315	Positive
$3.7 \times 10^3$	1.222	1.238	1.230	Positive	1.337	1.359	1.348	Positive

**Table B.5. Continued**

CCHFV NASBA					CCHFV NucliNASBA			
Copy number	Absorbance values		Mean	Result	Absorbance values		Mean	Result
$3.7 \times 10^2$	1.171	1.125	1.148	Positive	0.984	1.148	1.066	Positive
$3.7 \times 10^1$	1.472	1.351	1.412	Positive	0.581	0.648	0.615	Positive
$3.7 \times 10^0$	0.847	0.926	0.887	Positive	0.129	0.135	0.132	Negative
$3.7 \times 10^{-1}$	0.156	0.143	0.150	Negative	0.072	0.075	0.074	Negative
$3.7 \times 10^{-2}$	0.075	0.083	0.079	Negative	0.038	0.042	0.040	Negative

## 6. Clinical application of NASBA assays

6.1. Calculation of the clinical cut-off value determined using results obtained from panel of known negative clinical samples

**Table B.6. Absorbance data from the RNA samples extracted from known negative patient sera tested with the NASBA assays**

SPU number	CCHFV NASBA					CCHFV NucliNASBA				
	Absorbance values		Mean	Results		Absorbance values		Mean	Results	
09/12	0.027	0.038	0.019	0.028	Negative	0.091	0.047	0.093	0.077	Negative
20/12	0.048	0.087	0.093	0.076	Negative	0.082	0.081	0.093	0.085	Negative
24/12	0.099	0.105	0.092	0.099	Negative	0.037	0.084	0.036	0.052	Negative
29/12	0.095	0.029	0.035	0.053	Negative	0.032	0.071	0.038	0.047	Negative
48/12	0.091	0.062	0.077	0.077	Negative	0.089	0.060	0.072	0.074	Negative
185/12	0.028	0.085	0.040	0.051	Negative	0.106	0.111	0.144	0.120	Negative
189/12	0.107	0.099	0.084	0.097	Negative	0.128	0.083	0.095	0.102	Negative
04/13	0.018	0.026	0.034	0.026	Negative	0.012	0.083	0.037	0.044	Negative
11/13	0.106	0.011	0.095	0.071	Negative	0.086	0.095	0.024	0.068	Negative
13/13	0.048	0.073	0.052	0.058	Negative	0.032	0.046	0.038	0.039	Negative
14/13	0.038	0.039	0.042	0.040	Negative	0.037	0.035	0.029	0.034	Negative
23/13	0.014	0.037	0.028	0.026	Negative	0.061	0.052	0.047	0.053	Negative
70/13	0.129	0.084	0.106	0.106	Negative	0.085	0.092	0.074	0.084	Negative
78/13	0.094	0.107	0.098	0.100	Negative	0.079	0.080	0.083	0.081	Negative
81/13	0.093	0.091	0.102	0.095	Negative	0.083	0.088	0.070	0.080	Negative
84/13	0.059	0.028	0.041	0.043	Negative	0.049	0.033	0.043	0.042	Negative

$$\begin{aligned}
&\text{CCHFV NASBA Clinical Cut-off} \\
&= \text{Average} + 2 \times (\text{Standard deviation}) \\
&= 0.06538 + 2 \times (0.02851) \\
&= 0.1224 \\
&= 0.122
\end{aligned}$$

$$\begin{aligned}
&\text{CCHFV NucliNASBA Clinical Cut-off} \\
&= \text{Average} + 2 \times (\text{Standard deviation}) \\
&= 0.06763 + 2 \times (0.0245) \\
&= 0.11663 \\
&= 0.117
\end{aligned}$$

6.2. Absorbance data obtained testing RNA extracted from clinical specimens tested with the CCHFV NASBA and the CCHFV NucliNASBA. Each RNA sample tested in triplicate.

**Table B.7. Absorbance data from the RNA samples extracted from CCHFV-infected patient sera tested with the NASBA assays**

SPU number	CCHFV NASBA					CCHFV NucliNASBA				
	Absorbance values			Mean	Result	Absorbance values			Mean	Result
44/08	0.936	0.995	1.042	0.991	Positive	2.930	2.830	2.479	2.746	Positive
51/08	0.105	0.118	0.095	0.106	Negative	1.383	1.502	1.423	1.436	Positive
96/08	2.185	2.841	2.985	2.670	Positive	2.900	2.783	2.481	2.721	Positive
167/08	0.127	0.114	0.148	0.130	Positive	2.957	2.487	2.914	2.786	Positive
168/08	0.129	0.096	0.118	0.114	Negative	2.987	2.839	2.885	2.904	Positive
189/08	0.272	0.283	0.295	0.283	Positive	0.174	0.130	0.163	0.156	Positive
264/08	0.103	0.085	0.094	0.094	Negative	2.488	2.385	2.592	2.488	Positive
313/08	1.078	0.972	1.304	1.118	Positive	2.409	2.749	2.904	2.687	Positive
324/08	0.069	0.070	0.081	0.073	Negative	0.083	0.093	0.082	0.086	Negative
155/09	0.163	0.184	0.179	0.175	Positive	1.184	1.382	1.157	1.241	Positive
334/09	0.182	0.174	0.166	0.174	Positive	0.482	0.474	0.377	0.444	Positive
74/10	2.349	2.584	2.148	2.360	Positive	2.204	2.185	2.391	2.260	Positive
131/10	0.396	0.249	0.258	0.301	Positive	2.195	2.006	2.142	2.114	Positive
132/10	0.087	0.076	0.060	0.074	Negative	0.124	0.165	0.128	0.139	Positive
385/10	1.338	1.286	1.371	1.332	Positive	0.894	0.726	0.943	0.854	Positive
399/10	0.767	0.824	0.295	0.629	Positive	1.283	0.927	1.392	1.201	Positive
401/10	0.159	0.069	0.134	0.121	Positive	0.295	0.618	0.421	0.445	Positive
01/13	0.421	0.587	0.248	0.419	Positive	0.538	0.683	0.893	0.705	Positive
12/13	1.209	1.184	1.234	1.209	Positive	1.239	1.934	1.607	1.593	Positive
39/13	0.126	0.148	0.093	0.122	Negative	1.830	1.382	1.748	1.653	Positive
44/13	0.094	0.102	0.098	0.098	Negative	0.092	0.105	0.112	0.103	Negative
192/13	0.108	0.138	0.110	0.119	Negative	0.111	0.120	0.138	0.123	Positive

## 7. Inhibition of CCHFV NASBA

Serum samples collected from four volunteers were spiked with different dilutions of the control RNA transcript and tested with the CCHFV NASBA in duplicate.

**Table B.8. Absorbance data from the RNA extracted from serum samples spiked with dilutions of the control RNA transcript tested with the CCHFV NASBA assay**

Number of copies used to spike sera		0 (NFW)	$2 \times 10^0$	$2 \times 10^1$	$2 \times 10^2$	$2 \times 10^3$	$2 \times 10^4$	$2 \times 10^5$
VBD 16/14	Absorbance values	0.031	0.047	0.139	0.058	0.827	1.292	1.313
		0.040	0.081	0.065	0.065	0.698	1.324	1.339
	Mean	0.036	0.064	0.102	0.062	0.763	1.308	1.326
	Result	Neg	Neg	Neg	Neg	Pos	Pos	Pos
VBD 19/14	Absorbance values	0.063	0.078	0.074	0.060	0.076	0.086	0.103
		0.061	0.084	0.091	0.061	0.077	0.092	0.104
	Mean	0.062	0.081	0.083	0.061	0.077	0.089	0.104
	Result	Neg	Neg	Neg	Neg	Neg	Neg	Neg
VBD 20/14	Absorbance values	0.059	0.035	0.047	0.170	0.838	1.362	0.831
		0.035	0.038	0.044	0.131	0.771	1.339	0.935
	Mean	0.047	0.037	0.046	0.151	0.805	1.351	0.883
	Result	Neg	Neg	Neg	Pos	Pos	Pos	Pos
VBD 21/14	Absorbance values	0.073	0.065	0.815	1.225	1.344	1.300	1.147
		0.067	0.064	0.767	1.147	1.390	1.366	1.218
	Mean	0.070	0.065	0.791	1.186	1.367	1.333	1.183
	Result	Neg	Neg	Pos	Pos	Pos	Pos	Pos

Neg – Negative, Pos – Positive

## Appendix C – Detailed results of the NASBA assays

### 1. Results obtained testing RNA extracted from infected cell culture with the CCHFV NASBA assay and the nested PCR

**Table C.1. Detection of RNA extracted from CCHFV-infected Vero cell culture**

SPU number	CCHFV NASBA	Nested PCR	
		First round	Second round
259/84	Negative	Negative	Positive
94/85	Positive	Negative	Positive
97/85	Positive	Positive	Not done
247/85	Positive	Positive	Not done
381/85	Positive	Positive	Not done
396/85	Negative	Negative	Positive
408/85	Negative	Negative	Positive
415/85	Negative	Negative	Positive
23/86	Positive	Positive	Not done
196/86	Positive	Positive	Not done
361/86	Positive	Positive	Not done
536/86	Positive	Positive	Not done
560/86	Positive	Positive	Not done
566/86	Positive	Positive	Not done
582/86	Negative	Negative	Positive
593/86	Positive	Negative	Positive
103/87	Positive	Positive	Not done
177/87	Positive	Positive	Not done
244/87	Positive	Positive	Not done
383/87	Positive	Positive	Not done
409/87	Negative	Positive	Not done
486/87	Positive	Positive	Not done



**Table C.1. Continued**

SPU number	CCHFV NASBA	Nested RT-PCR	
		First round	Second round
556/87	Positive	Positive	Not done
18/88 <sup>a</sup>	Positive	Positive	Not done
19/88 <sup>a</sup>	Positive	Positive	Not done
45/88	Positive	Positive	Not done
71/88	Positive	Positive	Not done
203/88	Positive	Positive	Not done
273/88	Positive	Positive	Not done
497/88	Positive	Positive	Not done
498/88	Positive	Positive	Not done
130/89	Positive	Positive	Not done
281/89	Negative	Positive	Not done
337/89	Positive	Positive	Not done
372/89	Positive	Positive	Not done
444/89	Positive	Negative	Positive
61/90	Positive	Negative	Positive
215/90	Positive	Positive	Not done
378/90 <sup>b</sup>	Positive	Positive	Not done
380/90 <sup>b</sup>	Positive	Positive	Not done
381/90 <sup>b</sup>	Positive	Positive	Not done
382/90 <sup>b</sup>	Positive	Positive	Not done
388/90 <sup>b</sup>	Positive	Positive	Not done
15/92	Positive	Positive	Not done
50/93	Positive	Positive	Not done
172/93	Positive	Positive	Not done
24/01	Positive	Positive	Not done
51/01	Positive	Positive	Not done

<sup>a</sup> & <sup>b</sup> indicates samples taken from the same patient

**2. Results obtained from RNA extracted from clinical samples and tested with the NASBA assays and the commercial RT-PCR assays**

**Table C.2. Detection of RNA samples extracted from patient sera**

	<b>SPU number</b>	<b>CCHFV NASBA</b>	<b>CCHFV NucliNASBA</b>	<b>TaqMan OneStep RT-PCR kit</b>	<b>RealStar CCHFV RT-PCR kit 1.2</b>
<b>Positive panel</b>	44/08	Positive	Positive	Positive	Positive
	51/08	Negative	Positive	Positive	Positive
	96/08	Positive	Positive	Positive	Positive
	167/08	Positive	Positive	Positive	Positive
	168/08	Negative	Positive	Positive	Positive
	189/08	Positive	Positive	Positive	Positive
	264/08	Negative	Positive	Positive	Positive
	313/08	Positive	Positive	Positive	Positive
	324/08	Negative	Negative	Positive	Positive
	155/09	Positive	Positive	Positive	Positive
	334/09	Positive	Positive	Positive	Positive
	74/10	Positive	Positive	Positive	Positive
	131/10	Positive	Positive	Positive	Positive
	132/10	Negative	Positive	Positive	Positive
	385/10	Positive	Positive	Positive	Positive
	399/10	Positive	Positive	Positive	Positive
	401/10	Positive	Positive	Positive	Positive
	01/13	Positive	Positive	Positive	Positive
	12/13	Positive	Positive	Positive	Positive
	39/13	Positive	Positive	Positive	Positive
	44/13	Negative	Negative	Positive	Positive
	192/13	Negative	Positive	Positive	Positive

**Table C.2. Continued**

	<b>SPU number</b>	<b>CCHFV NASBA</b>	<b>CCHFV NucliNASBA</b>	<b>TaqMan OneStep RT-PCR kit</b>	<b>RealStar CCHFV RT-PCR kit 1.2</b>
<b>Negative panel</b>	09/12	Negative	Negative	Negative	Negative
	20/12	Negative	Negative	Negative	Negative
	24/12	Negative	Negative	Negative	Negative
	29/12	Negative	Negative	Negative	Negative
	48/12	Negative	Negative	Negative	Negative
	185/12	Negative	Negative	Negative	Negative
	189/12	Negative	Negative	Negative	Negative
	04/13	Negative	Negative	Negative	Negative
	11/13	Negative	Negative	Negative	Negative
	13/13	Negative	Negative	Negative	Negative
	14/13	Negative	Negative	Negative	Negative
	23/13	Negative	Negative	Negative	Negative
	70/13	Negative	Negative	Negative	Negative
	78/13	Negative	Negative	Negative	Negative
	81/13	Negative	Negative	Negative	Negative
	84/13	Negative	Negative	Negative	Negative

## **Appendix D – Composition of buffers, media and solutions**

### **TAE buffer**

Prepare the following:

– 0.5 M disodium ethylenediaminetetraacetate ( $\text{Na}_2\text{EDTA}$ ) solution

84.05 g  $\text{Na}_2\text{EDTA}$  (SAARChem, Krugersdorp, SA)

adjust pH to 8.0 using HCl (Merck, Gauteng, SA)

in 500.0 ml distilled water

– 50× TAE buffer

100.0 ml aliquot of 0.5 M  $\text{Na}_2\text{EDTA}$  solution

242.0 g tris(hydroxymethyl)aminomethane (Merck, Gauteng, SA)

57.1 ml aliquot of glacial acetic acid (Merck, Gauteng, SA)

in 1.0 litre (ℓ) distilled water

– 1× TAE buffer

20.0 ml aliquot of the 50× TAE buffer in 2.0 ℓ distilled water

### **CCHFV NASBA Amplification buffer**

Prepare the following:

– 3.6 M  $\text{MgCl}_2$  stock

14.36 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Merck, Gauteng, SA) in 20.0 ml NFW

– 2.52 M KCl stock

1.9 g KCl (Merck, Gauteng, SA) in 10.0 ml NFW

– 0.29 M tris stock

1.05 g tris(hydroxymethyl)aminomethane

adjust pH to 8.3 using HCl

in 30.0 ml NFW

– 25× CCHFV NASBA Amplification buffer  
68.75 µl aliquot of 0.29 M tris stock  
15.0 µl aliquot of 2.52 M KCl stock  
1.75 µl aliquot of 3.6 M MgCl<sub>2</sub> stock  
22.5 µl aliquot of 100 mM DTT (Roche, Mannheim, Germany)  
50.0 µl aliquot of DMSO (Merck, Darmstadt, Germany)

A 6.32 µl aliquot of the CCHFV NASBA Amplification buffer was used per CCHFV NASBA reaction.

### **10× TBS stock solution**

60.6 g tris(hydroxymethyl)aminomethane  
87.6 g sodium chloride (NaCl) (Merck, Gauteng, SA)  
adjust pH to 7.5 using HCl  
in 1.0 l distilled water.

### **1× TBS working solution**

50.0 ml aliquot of the 10× TBS stock solution in 500.0 ml distilled water.

### **NASBA-EOC hybridization buffer**

Prepare the following:

– 50 mM tris-HCl stock  
0.303 g tris(hydroxymethyl)-aminomethane  
adjust the pH to 8.8 using HCl  
in 50.0 ml distilled water

– NASBA-EOC hybridization buffer  
0.25 g BSA (Roche, Mannheim, Germany)  
5.0 ml aliquot of the 50 mM tris-HCl stock  
in 25.0 ml 20× SSPE (Gibco, Life technologies, New York, USA)

## **SOC media**

Prepare the following:

– 1 M NaCl stock

2.9 g NaCl in 50.0 ml distilled water

– 1 M KCl stock

3.73 g KCl in 50.0 ml distilled water

– 2 M  $\text{Mg}^{2+}$  stock

10.17 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  and 12.33 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in 50.0 ml distilled water, filter sterilize the solution using a 0.2  $\mu\text{m}$  filter.

– 2 M glucose stock

18.02 g glucose (Merck, Gauteng, SA) in 50.0 ml distilled water, filter sterilize the solution using a 0.2  $\mu\text{m}$  filter

– 50.0 ml volume of SOC media

1.0 g Tryptone (Oxoid, Hampshire, England)

0.25 g Yeast extract (Oxoid, Hampshire, England)

0.5 ml 1 M NaCl stock

0.125 ml 1 M KCl stock

in 45.0 ml distilled water.

The solution was autoclaved and allowed to cool to room temperature.

A 0.5 ml aliquot of 2 M  $\text{Mg}^{2+}$  stock and a 0.5 ml aliquot of 2 M glucose stock were added to the solution.

Final volume adjusted to 50.0 ml using distilled water. The SOC media was aliquoted in 1.0 ml volumes and stored at 4°C.

## **NET/BSA**

4.3 g NaCl

1.04 g  $\text{Na}_2\text{EDTA}$

3.02 g tris(hydroxymethyl)aminomethane

0.5 g BSA

2.5 ml aliquot of Nonidet P40 (BDH Laboratory Supplies, Poole, England)

in 500.0 ml distilled water

### **10× PBS stock solution**

160.0 g NaCl

4.0 g KCl

35.6 g disodiumhydrogenphosphatedihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) (Merck, Gauteng, SA)

4.8 g potassiumdihydrogenphosphate ( $\text{KH}_2\text{PO}_4$ ) (Merck, Gauteng, SA)

in 2.0 l distilled water.

### **1× PBS working solution**

A 100.0 ml aliquot of the 10× PBS stock solution

in 1.0 l distilled water

### **Fairbanks staining/destaining method**

Prepare the following:

– Fairbanks A buffer

0.5 g Coomassie Blue (Fluka AG, Germany)

250.0 ml aliquot of isopropanol (Merck, Gauteng, SA)

100.0 ml aliquot of glacial acetic acid (Merck, Gauteng SA)

in 1.0 l distilled water

– Fairbanks B buffer

0.05 g Coomassie Blue

100.0 ml aliquot of isopropanol

100.0 ml aliquot of glacial acetic acid

in 1.0 l distilled water

– Fairbanks C buffer

0.02 g Coomassie Blue

100.0 ml aliquot of glacial acetic acid

in 1.0 l distilled water

– Fairbanks D buffer

100.0 ml aliquot of glacial acetic acid

in 1.0 l distilled water

The SDS-PAGE gels were stained in sufficient Fairbanks A buffer to cover the gels in a closed container overnight whilst shaking at 100 rpm. The container was heated in a microwave until the buffer started to boil. The container was removed and the SDS-PAGE gels were incubated at room temperature for 10 min whilst shaking at 100 rpm. The Fairbanks A buffer was discarded from the container and the SDS-PAGE gels were rinsed with distilled water. The distilled water was discarded and sufficient Fairbanks B buffer was added to cover the gels. The above process was repeated for the Fairbanks B, C and D buffers and the SDS-PAGE gels were finally incubated in distilled water overnight.

#### **Transfer buffer for western blot**

3.03 g tris(hydroxymethyl)aminomethane

14.4 g glycine (Promega, Wisconsin, USA)

200.0 ml aliquot of methanol

in 1.0 l distilled water

#### **10% fat free milk/TBS**

1.0 g Elite fat free milk powder (Clover SA (Pty) Ltd, Roodepoort, SA) in 10.0 ml 1× TBS

#### **2× TY/kan plates**

7.5 g Agar bacteriological (Agar no. 1) (Oxoid, Hampshire, England)

2.5 g NaCl

8.0 g Tryptone

5.0 g Yeast extract

in 500.0 ml distilled water.

The mixture was autoclaved and allowed to cool to ~60°C before adding a 300.0 µl aliquot of 50mg/ml kanamycin. The solution was mixed and used to pour into empty petri dishes.



**2× TY/kan media**

5.0 g NaCl

16.0 g Tryptone

10.0 g Yeast extract

in 1.0 ml distilled water

The mixture was autoclaved and allowed to cool to room temperature before adding a 300.0 µl aliquot of 50mg/ml kanamycin, final concentration 30 mg/ml.

**IFA Blocking solution**

5.0 g saccharose (Merck, Gauteng, SA)

0.25 ml aliquot of Triton X-100 (Promega, Wisconsin, USA)

in 50.0 ml 1× PBS

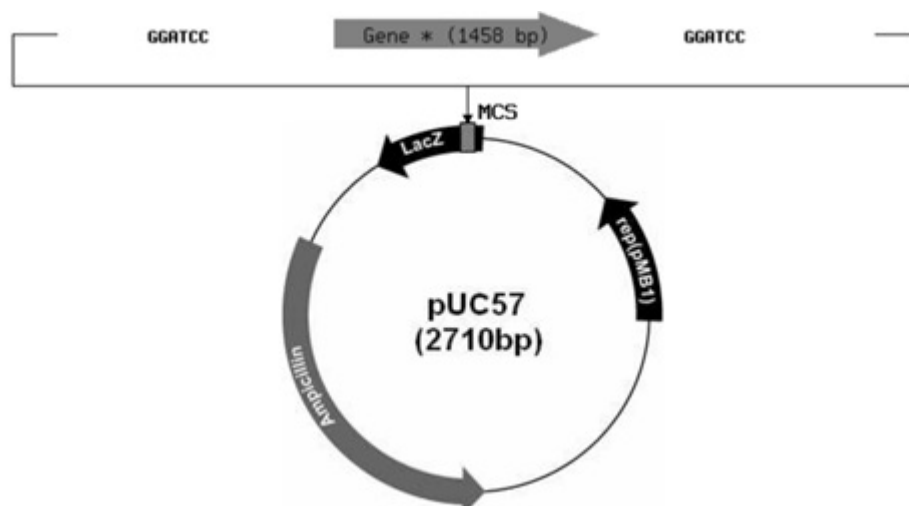
## Appendix E – Gene sequences and vector maps

### 1. The nucleotide sequence of the CCHFV.NP.opt gene (1458 bp) and the vector map of pUC.CCHFV.NP.opt construct

Position	Sequence
1 – 50	GGATCCGAAAACAAGATCGAGGTCAACAACAAGGACGAGATGAACAAGTG
51 – 100	GTTTCGAGGAGTTCAAGAAGGGCAACGGTCTGGTGGACACATTACCAACT
101 – 150	CTTACAGCTTCTGCGAGAGCGTTCCAAACTTGGACAAGTTCGTGTTCCAG
151 – 200	ATGGCTTCAGCCACTGACGATGCCCAAAAGGATAGCATCTACGCTTCAGC
201 – 250	CCTCGTCGAGGCCACTAAGTTCTGCGCTCCGGTGACGAATGTGCCTGGG
251 – 300	TCTCCTCTACAGGCATCGTTAAGCGTGGACTGGAGTGGTTCGAAAAGAAC
301 – 350	GCTGGAACAATCAAGTCGTGGGACGAGTCCTACACCGAACTCAAGGTGGA
351 – 400	TGTCCCAAGATCGAGCAGTTGGCTAACTACCAGCAAGCTGCCCTGAAGT
401 – 450	GGCGCAAGGACATCGGATTCAAGGTGAACGCCAACACCGCTGCCCTCAGC
451 – 500	AACAAGGTGTTGGCTGAGTACAAGGTCCCTGGTGAAATCGTTATGTCAGT
501 – 550	GAAGGAGATGCTCTCGGACATGATCCGTCGCAGGAACCAGATCTTGAACA
551 – 600	GAGGTGGCGACGAAAACCCAAGAGGCCCGGTCTCCCGTGAGCACGTTGAT
601 – 650	TGGTGTCTGTAATTCGTCAAGGGAAAGTACATCATGGCTTTCAACCCCC
651 – 700	TTGGGGTGACATCAACAAGTCAGGTAGATCGGGAATCGCTCTGGTGGCTA
701 – 750	CCGGATTGGCTAAGCTGGCCGAGACTGAAGGAAAGGTGTGTTTCGACGAG
751 – 800	GCCAAGAAGACAGTCGAAGCTTTGAACGGATACCTGGACAAGCACAAAGGA
801 – 850	CGAGGTGGATCGCGCTTCCGCCGATTCTATGATCACTAACCTGCTCAAGC
851 – 900	ACATCGCTAAGGCCCAGGAAGTGTACAAGAACAGCTCAGCTCTCAGGGCC
901 – 950	CAGGGCGCTCAAATCGACACCGCCTTCTCGTCCTACTACTGGCTGTACAA
951 – 1000	GGCTGGAGTTACCCCCGAGACTTTCCTACAGTGTGCGAGTTCCCTCTTCG
1001 – 1050	AATTGGGCAAGCAACCCCGCGGCACTAAGAAGATGAAGAAGGCCTTGCTG
1051 – 1100	TCTACACCTATGAAGTGGGGCAAGAAGCTGTACGAGCTCTTCGCTGACGA
1101 – 1150	TACCTTCCAGCAAAACCGCATCTACATGCACCCAGCCGTCCTGACAGCTG
1151 – 1200	GCAGGATCTCTGAAATGGGAGTCTGCTTCGGTACCATCCCAGTTGCTAAC
1201 – 1250	CCGGACGATGCTGCCCAGGGCTCTGGACACACTAAGAGCATCCTGAACCT
1251 – 1300	CAGAACCAACACTGAGACAAACAACCCCTTGTGCCCGTACCATCGTGAAGC
1301 – 1350	TGTTTCGAAATCCAGAAGACTGGTTTCAACATCCAAGACATGGATATCGTC
1351 – 1400	GCTTCCGAGCACCTCTTGACCAATCTCTCGTTGGCAAGCAGTCCCCATT
1400 – 1450	CCAAAACGCTTACAACGTCAAGGGCAACGCTACATCAGTCAACATCATCT
1451 – 1458	AAAGATCC

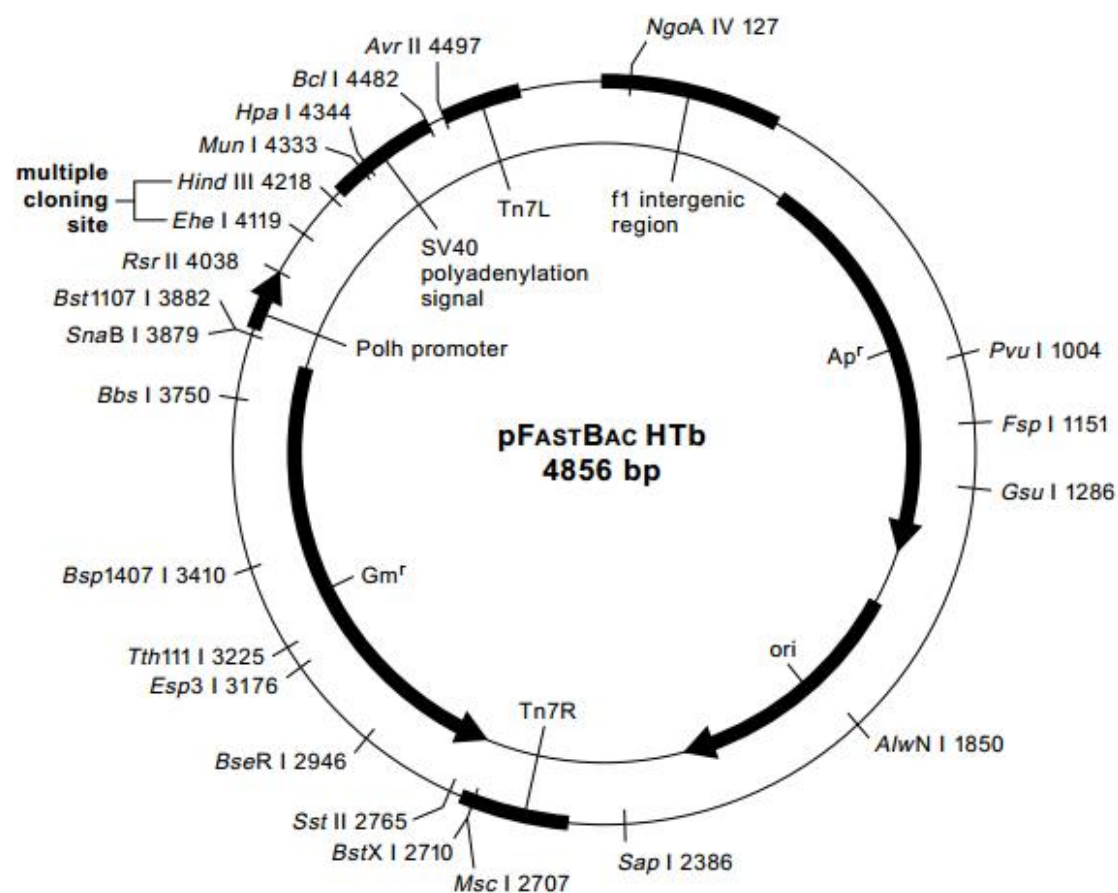
#### Key:

AGATCC – BamHI restriction sites



**Figure E.1. Schematic illustration of the vector map of the pUC.CCHFV.NP.opt construct.** As supplied by GenScript

## 2. A vector map of pFastBacHT B



**Figure E.2. Schematic illustration of the vector map of the pFastBacHT B expression vector.** Available from Bac-to-Bac® manual

### 3. The nucleotide sequence of the CCHFV NP gene of isolate SPU 415/85 cloned in frame in the pFastBacHT B vector

AACC**ATG**TCGTACTAC**CATCACCATCACCATCAC****GATTACGATATCCCAACGACC****GAAAACCTGTATTTTCAGGG**  
**G**CCATG**GGATCC**GAAAACAAGATCGAGGTCAACAACAAGGACGAGATGAACAAGTGGTTCGAGGAGTTCAAGAA  
 GGGCAACGGTCTGGTGGACACATTCACCAACTCTTACAGCTTCTGCGAGAGCGTTCCAAACTTGGACAAGTTCGT  
 GTTCCAGATGGCTTCAGCCACTGACGATGCCCCAAAAGGATAGCATCTACGCTTCAGCCCTCGTCGAGGCCACTAA  
 GTTCTGCGCTCCGGTGTACGAATGTGCCTGGGTCTCCTCTACAGGCATCGTTAAGCGTGGACTGGAGTGGTTCGA  
 AAAGAACGCTGGAACAATCAAGTCGTGGGACGAGTCCTACACCGAACTCAAGGTGGATGTCCCCAAGATCGAGCA  
 GTTGGCTAACTACCAGCAAGCTGCCCTGAAGTGGCGCAAGGACATCGGATTCAGGGTGAACGCCAACACCGCTGC  
 CCTCAGCAACAAGGTGTTGGCTGAGTACAAGGTCCCTGGTGAAATCGTTATGTCTAGTGAAGgAGATGCTCTCGgA  
 CATGATCCGTGCGAGGAACCAGATCTTGAACAGAGGTGGCGACGAAAACCCAAGAGGCCCGGTCTCCCGTGAGCA  
 CGTTGATTGGTGTCTGTAATTCGTCAAGGGAAAGTACATCATGGCTTTCAACCCCCCTTGGGGTGACATCAACAA  
 GTCAGGTAGATCGGGAATCGCTCTGGTGGCTACCGGATTGGCTAAGCTGGCCGAGACTGAAGGAAAGGTGTGTT  
 CGACGAGGCCAAGAAGACAGTCGAAGCTTTGAACGGATACCTGGACAAGCACAAGGACGAGGTGGATCGCGCTTC  
 CGCCGATTCTATGATCACTAACCTGCTCAAGCACATCGCTAAGgCCCAGGAACTGTACAaGAaCAGCTCAGCTCT  
 CAGGgCCcAGGgCGCTCAAaTCGACACcGCcTTCTCGTCcTACTACTGgCTGTACAaGgCTGgAGTTACCCcGA  
 GACTTtCCCTACAGTGTGCGAGTTCCTCTTCGAaTtGGgCAaGCAaCCCCGCGGCACTAAGAAGATGAAGAAGGC  
 CTTGCTGTCTACACCTATGAAGTGGGGCAAGAAGCTGTACGAGCTCTTCGCTGACGATACCTTCCAGCAAAACCG  
 CATCTACATGCACCCAGCCGTCCTGACAGCTGGCAGGATCTCTGAAATGGGAGTCTGCTTCGGTACCATCCCAGT  
 TGCTAACCCGGACGATGCTGCCCAGGGCTCTGGACACACTAAGAGCATCCTGAACCTCAGAACCAACACTGAGAC  
 AAACAACCCTTGTGCCCGTACCATCGTGAAGCTGTTGCAAATCCAGAAGACTGGTTTCAACATCCAAGACATGGA  
 TATCGTCGCTTCCGAGCACCTCTTGACCAATCTCTCGTTGGCAAGCAGTCCCCATTCCAAAACGCTTACAACGT  
 CAAGGGCAACGCTACATCAGTCAACATCATC**TAA****GGATCC**GGAATTCAAAGGCCTACGTCGACGAGCTCACTAGT  
 CGCGGCCGCTTTTCGAATCTAGAGCCTGCAGTCTCGAGGCATGCGGTACCAAGCTTGTCGAGAAGTAC**TAG**AGGAT  
 CATAATC

#### Key:

**ATG**– Start codon

**CATCACCATCACCATCAC** – 6× His affinity tag

**GATTACGATATCCCAACGACC** – Spacer region

**GAAAACCTGTATTTTCAGGGC** – rTEV protease cleavage site

**GGATCC** – BamHI restriction sites

GAA ... TAA – ORF of SPU 415/85 S segment

**TAA/TAG** – Stop codons

#### 4. The predicted amino acid sequence of the CCHFV NP gene of isolate SPU 415/85 cloned in frame in the pFastBacHT B vector

The predicted amino acid sequence of the pcDNA<sup>TM</sup>3.1D/V5-His-TOPO.CCHFV.NP construct was obtained using ExPASy: SIB Bioinformatics Resource Portal Translate tool (<http://web.expasy.org/translate/>).

M S Y Y H H H H H H D Y D I P T T E N L Y F Q G A M G S E N K I E V N N K D  
E M N K W F E E F K K G N G L V D T F T N S Y S F C E S V P N L D K F V F Q  
M A A T D D A Q K D S I Y A S A L V E A T K F C A P V Y E C A W V S S T G I  
V K R G L E W F E K N A G T I K S W D E S Y T E L K V D V P K I E Q L A N Y  
Q Q A A L K W R K D I G F R V N A N T A A L S N K V L A E Y K V P G E I V M  
S V K E M L S D M I R R R N Q I L N R G G D E N P R G P V S R E H V D W C R  
E F V K G K Y I M A F N P P W G D I N K S G R S G I A L V A T G L A K L A E  
T E G K G V F D E A K K T V E A L N G Y L D K H K D E V D R A S A D S M I T  
N L L K H I A K A Q E L Y K N S S A L R A Q G A Q I D T A F S S Y W L Y K A  
G V T P E T F P T V S Q F L F E L G K Q P R G T K K M K K A L L S T P M K W  
G K K L Y E L F A D D T F Q Q N R I Y M H P A V L T A G R I S E M G V C F G  
T I P V A N P D D A A Q G S G H T K S I L N L R T N T E T N N P C A R T I V  
K L F E I Q K T G F N I Q D M D I V A S E H L L H Q S L V G K Q S P F Q N A  
Y N V K G N A T S V N I I **Stop** G S G I Q R P T S T S S L V A A A F E S R A  
C S L E A C G T K L V E K Y **Stop**

##### Key:

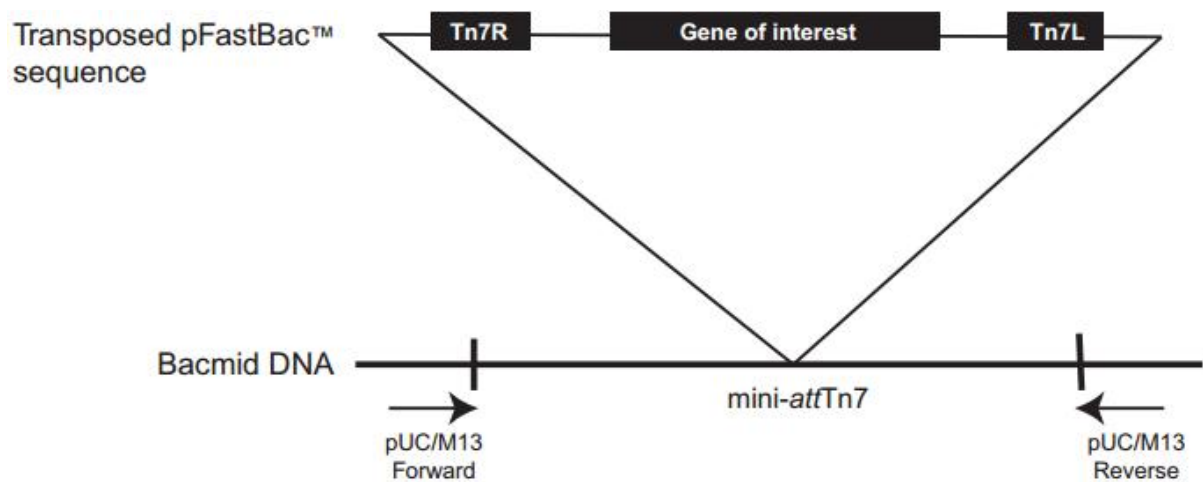
H H H H H H - 6× His affinity tag

E N L Y F Q G - rTEV protease cleavage site

E N K ... N I I - predicted amino acid sequence of CCHFV NP of SPU 415/85

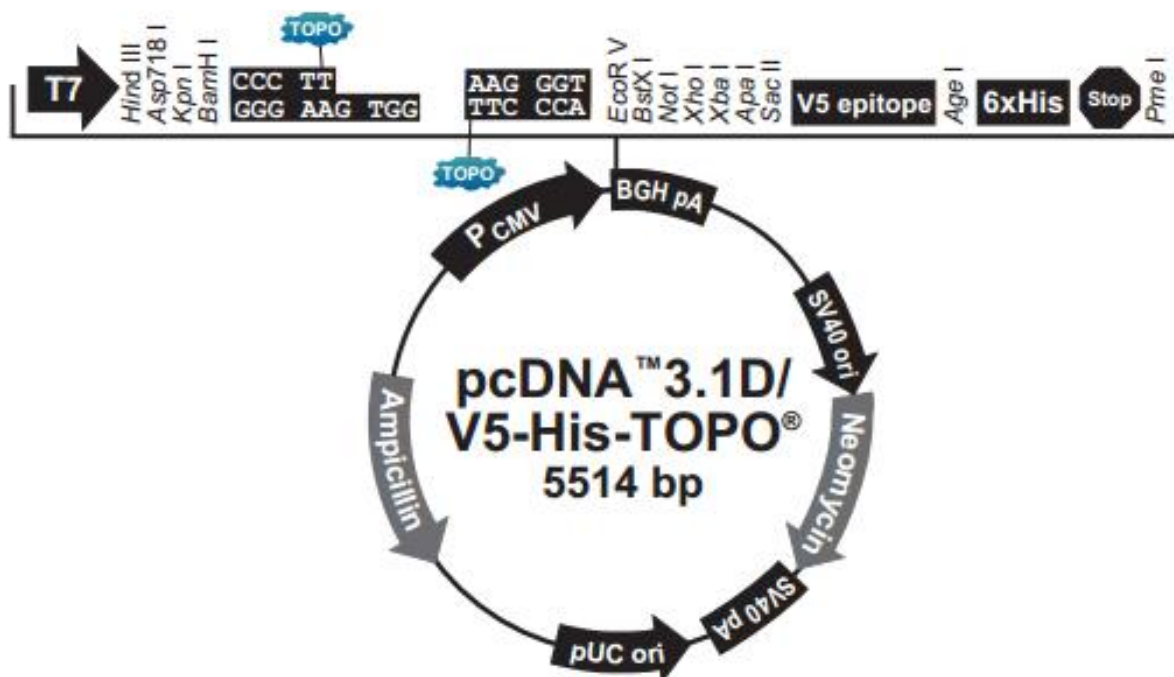
**Stop** - Stop codons

## 5. Position of the Tn7 transposon and the M13 primers



**Figure E.3.** Schematic illustration of the position of the Tn7 transposon on the recombinant bacmid and the M13 primers on the bacmid. Available from Bac-to-Bac® manual

## 6. A vector map of pcDNA™3.1D/V5-His-TOPO® vector



<b>Comments for pcDNA™3.1D/V5-His-TOPO®</b> <b>5514 nucleotides</b> CMV promoter: bases 232-819 T7 promoter/priming site: bases 863-882 TOPO® recognition site 1: bases 930-934 Overhang sequence (complementary strand): bases 935-938 TOPO® recognition site 2: bases 939-943 V5 epitope: bases 1011-1052 Polyhistidine (6xHis) tag: bases 1062-1079 BGH reverse priming site: bases 1102-1119 BGH polyadenylation signal: bases 1108-1332 SV40 early promoter and origin: bases 1833-2142 Neomycin resistance gene: bases 2217-3011 SV40 early polyadenylation signal: bases 3189-3319 pUC origin: bases 3700-4373 (complementary strand) Ampicillin ( <i>bla</i> ) resistance gene: bases 4518-5378 (complementary strand) <i>bla</i> promoter: bases 5379-5477 (complementary strand)
--

**Figure E.4. Schematic illustration of the vector map and features of the pcDNA™ 3.1D/V5-His-TOPO® expression vector.** Available from pcDNA™ 3.1 Directional TOPO® Expression Kit manual

## 7. The nucleotide sequence of the CCHFV NP gene of isolate SPU 92/01 cloned in frame in the pcDNA™ 3.1D/V5-His-TOPO® vector

TAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGTTAAGCTTGGTACCGAGCTCGGATCCAGTACCCTT**CAC**  
**CATG**GAAAACAAAATAGAGGTGAATAACAAAGATGAGATGAACAAGTGGTTCGAAGAGTTCAAAAAGGGAATGG  
 ACTTGTGGACACCTTCACAACTCCTATTCTTTTGTGAGAGTGTTCCAAATTTGGACAAGTTTGTGTTCCAAAT  
 GGCCAGTGCCACTGATGATGCACAAAAGGATTCTATCTACGCGTCTGCTCTGGTGGAGGCAACAAAGTTTGTGTC  
 ACCTATATATGAGTGTGCGTGGGTAGCTCCACTGGCATTGTGAAGAGGGGACTTGAATGGTTCGAAAAAATGC  
 GGGCACCATTAAAGTCTGGGATGAAAGTTATACTGAGCTAAAAGTTGACGTCCCGAAAAATAGAACAACCTTGCCAA  
 TTACCAACAAGCTGCCTTAAATGGAGAAAGGACATAGGTTTCCGTGTCAATGCAACACAGCTGCTTTGAGCAA  
 CAAAGTCCTTGCAGAGTACAAAGTTCCTGGCGAGATTGTGATGTCTGTCAAAGAGATGCTGTCAGACATGATTCTG  
 GAGAAGGAACCAATTCTAAACAGGGGTGGTGATGAGAATCCACGTGGCCCTGTGAGCCGTGAGCATGTGGACTG  
 GTGCAGGGAGTTTGTCAAAGGCAAATACATCATGGCCTTCAACCCACCATGGGGGGACATCAACAAGTCAGGCCG  
 TTCAGGAATAGCACTTGTGCGCAACAGGCCTTGCCAAGCTTGCAGAGACTGAAGGAAAGGGAGTTTTTGACGAAGC  
 CAAAAGACCGTGGAGGCCCTCAATGGGTATCTGGACAAGCACAAAGGACGAAGTTGACAGGGCGAGTGCTGACAG  
 CATGATAACAAACCTTCTCAAGCACATTGCCAAGGCACAGGAGCTTTATAAGAATTCGTCTGCACTCCGTGCACA  
 AGGTGCACAGATTGACACTGCTTTAGCTCATACTATTGGCTTTACAAAGCTGGCGTGACCCAGAAACCTTCCC  
 GACGGTGTGCGAGTTCCTCTTCGAGCTAGGGAAGCAGCCAAGAGGTACCAAGAAAATGAAGAAGGCTCTGCTGAG  
 CACCCCAATGAAGTGGGGGAAGAACTTTATGAGCTCTTTGCCGACGATTCTTTCCAGCAGAACAGGATCTACAT  
 GCACCTGCCGTGCTTACAGCTGGCAGAATCAGTGAAATGGGAGTCTGCTTTGGGACAATCCCCGTGGCCAATCC

TGATGATgCTGCCCCAAGGATCTGGACATACCAAGTCCATTCTCAACCTCCAGACTAACACCGAGACCAACAATCC  
 ATGTGCCAGGACCATTGTCAAGCTGTTTGAATTCAGAAAACAGGGTTCAACATTCAGGACATGGACATAGTGGC  
 CTCTGAGCACTTGCTACACCAGTCTCTTGTGGCAAGCAATCTCCATTCCAGAATGCCTACAACGTCAAGGGCAA  
 TGCCACCAGTGTTAACATTATCAAGGGTCAAGACAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCT  
 AGAGGGCCCGCGGTTCGAAAGGTAAGCCTATCCCTAACCCCTCTCCTCGGTCTCGATTCTACGCGTACCGGT  
 TCACCATCACCATTGAGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTT

### Key:

TAATACGACTCACTATAGGG - T7 priming site

GGATCC - BamHI restriction site

CACCATG - Kozak sequence

GAA ... ATC - ORF of SPU 92/01 S segment

CGGCCG - NotI restriction site

GGT ... ACG - V5 epitope

CATCATCACCATCACCAT - 6× HIS tag

TGA - Stop codon

## 8. The predicted amino acid sequence of the CCHFV NP gene of isolate SPU 92/01 cloned in frame in the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO® vector

The predicted amino acid sequence of the pcDNA<sup>TM</sup> 3.1D/V5-His-TOPO.CCHFV.NP construct was obtained using ExpAsy: SIB Bioinformatics Resource Portal Translate tool (<http://web.expasy.org/translate/>).

S D P V P F T M E N K I E V N N K D E M N K W F E E F K K G N G K V D T F T  
 N S Y S F C E S V P N L D K F V F Q M A S A T D D A Q K D S I Y A S A L V E  
 A T K F C A P I Y E C A W V S S T G I V K R G L E W F E K N A G T I K S W D  
 E S Y T E L K V D V P K I E Q L A N Y Q Q A A L K W R K D I G F R V N A N T  
 A A L S N K V L A E Y K V P G E I V M S V K E M L S D M I R R R N Q I L N R  
 G G D E N P R G P V S R E H V D W C R E F V K G K Y I M A F N P P W G D I N  
 K S G R S G I A L V A T G L A K L A E T E G K G V F D E A K K T V E A L N G  
 Y L D K H K D E V D R A S A D S M I T N L L K H I A K A Q E L Y K N S S A L  
 R A Q G A Q I D T A F S S Y Y W L Y K A G V T P E T F P T V S Q F L F E L G  
 K Q P R G T K K M K K A L L S T P M K W G K K L Y E L F A D D S F Q Q N R I  
 Y M H P A V L T A G R I S E M G V C F G T I P V A N P D D A A Q G S G H T K  
 S I L N L Q T N T E T N N P C A R T I V K L F E I Q K T G F N I Q D M D I V  
 A S E H L L H Q S L V G K Q S P F Q N A Y N V K G N A T S V N I I K G Q D N



S A D I Q H S G G R S S L E G P R F E G K P I P N P L L G L D S T R T G H H  
H H H H Stop

**Key:**

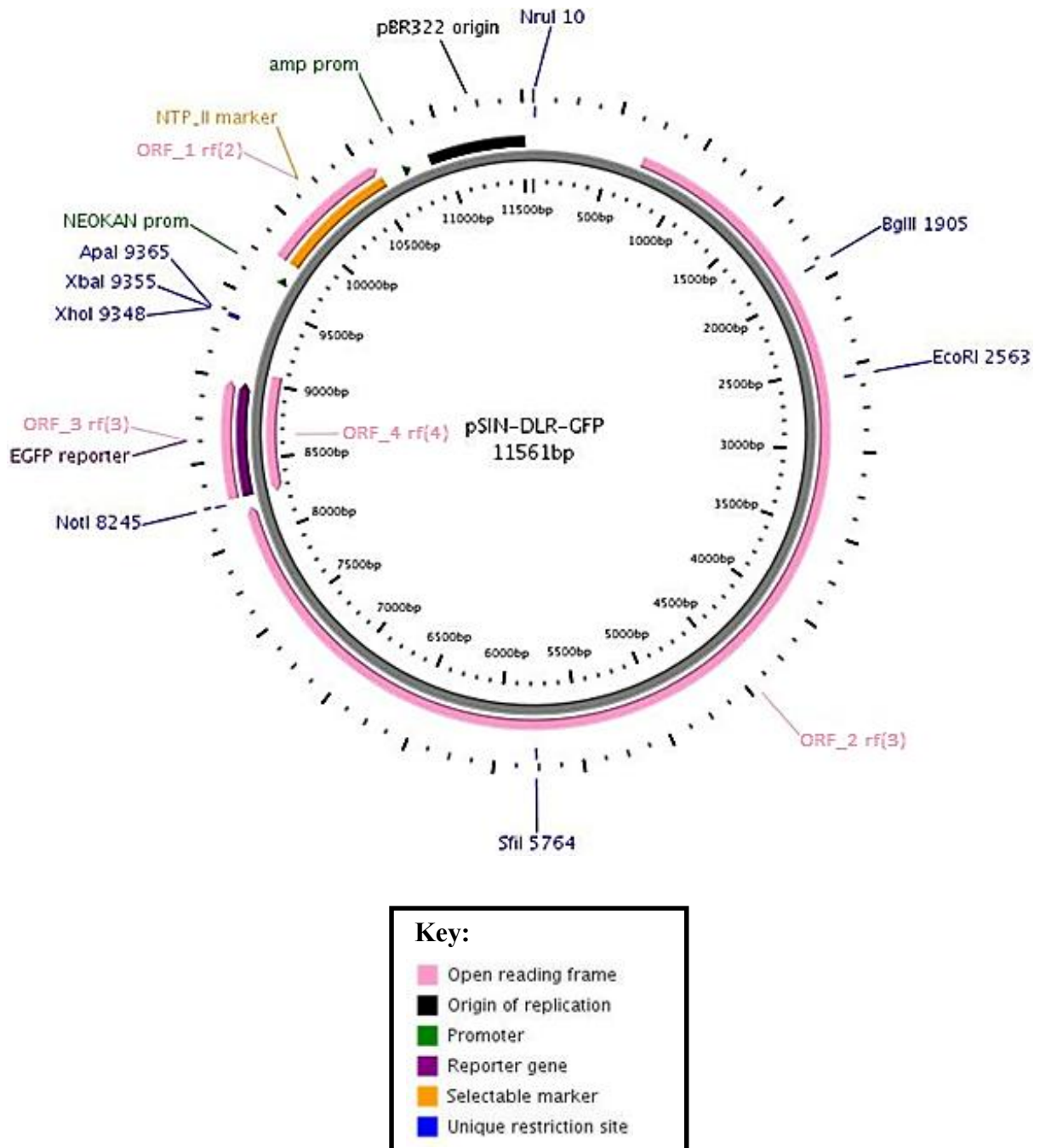
M E N ... N I I - predicted amino acid sequence of CCHFV NP of SPU 92/01

K P I ... D S T - V5 epitope

H H H H H H - 6× HIS tag

Stop - Stop codon

## 9. A vector map of the pSIN-DLR-GFP vector



**Figure E.5. Schematic illustration of the vector map and features of the pSIN-DLR-GFP expression vector.** As supplied by Prof. Mark Heise (Carolina Vaccine Institute, University of North Carolina, North Carolina, USA)

## **Appendix F – Presentations and publications**

### **Oral presentation:**

Title: Development of a nucleic acid sequence-based amplification (NASBA) assay for detection of Crimean-Congo haemorrhagic fever viral RNA

Forum: Faculty of Health Sciences Research Forum, University of the Free State, Bloemfontein, South Africa

Date: 27 – 29 August 2014

### **Publications:**

Title: Development and evaluation of a nucleic acid sequence-based amplification assay for detection of Crimean-Congo haemorrhagic fever viral nucleic acid

Authors: D Pieters<sup>1</sup>, P Jansen van Vuren<sup>2</sup>, J Weyer<sup>2</sup>, JT Paweska<sup>2</sup> and FJ Burt<sup>1</sup>

Departments: Medical Microbiology and Virology<sup>1</sup>, University of the Free State, Bloemfontein;  
Centre for Emerging and Zoonotic Diseases<sup>2</sup>, National Institute for Communicable Diseases,  
Johannesburg

Status: In progress

## **Appendix G – Letters of ethics approval and permission**

This section contains the letters of ethics approval and a letter of permission to use Figure 1.6. Please note that the remaining figures and pictures were self produced.

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Internal Post Box G40  
☎ (051) 4052812  
Fax (051) 4444359

E-mail address: StraussHS@ufs.ac.za

Ms H Strauss/hv

2012-03-07

REC Reference nr 230408-011  
IRB nr 00006240

PROF F BURT  
DEPARTMENT OF MEDICAL VIROLOGY (G23)  
NHLS  
FACULTY OF HEALTH SCIENCES  
UFS

Dear Prof Burt

ETOVS NR 152/06

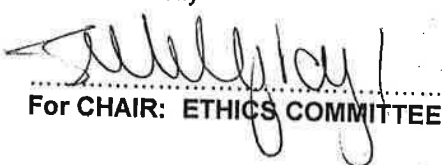
PROF F BURT

DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY

PROJECT TITLE: IMMUNE RESPONSES IN SURVIVORS OF CRIMEAN-CONGO HAEMORRHAGIC  
FEVER AND EVALUATION OF CANDIDATE VACCINES.

- You are hereby kindly informed that the Ethics Committee approved the following at the meeting held on 6 March 2012:
  - **Addition of postgraduate student: D Pieters student no 2007002104, M.Med.Sc., Department of Medical Microbiology and Virology**
- Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.
- Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.
- The Committee must be informed of any serious adverse event and/or termination of the study.
- A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the ETOVS reference number in correspondence to the Ethics Committee secretariat.

Yours faithfully

  
For CHAIR: ETHICS COMMITTEE



Research Division  
Internal Post Box G40  
☎ (051) 4052812  
Fax (051) 4444359

E-mail address: StraussHS@ufs.ac.za

Ms H Strauss/hv

2012-04-13

REC Reference nr 230408-011  
IRB nr 00006240

PROF F BURT  
DEPT OF MEDICAL MICROBIOLOGY AND  
VIROLOGY  
FACULTY OF SCIENCES  
UFS

Dear Prof Burt

ETOVS NR 152/06

PROF F BURT

DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY

PROJECT TITLE: IMMUNE RESPONSES IN SURVIVORS OF CRIMEAN-CONGO HAEMORRHAGIC FEVER  
AND EVALUATION OF CANDIDATE VACCINES

- You are hereby kindly informed that the Ethics Committee approved the following at the meeting held on 10 April 2012:
  - ***Amendment to protocol***
  - ***Extension to timeline until December 2013***
- Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.
- Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.
- The Committee must be informed of any serious adverse event and/or termination of the study.
- A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the ETOVS reference number in correspondence to the Ethics Committee secretariat.

Yours faithfully

  
CHAIR: ETHICS COMMITTEE



Research Division  
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☎ (051) 4052812  
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E-mail address: StraussHS@ufs.ac.za

Ms H Strauss/hv

2013-11-06

REC Reference nr 230408-011  
IRB nr 00006240

PROF F BURT  
DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY  
FACULTY OF HEALTH SCIENCES  
UFS

Dear Prof Burt

ETOVS NR 152/06

PROF F BURT

DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY

PROJECT TITLE: IMMUNE RESPONSES IN SURVIVORS OF CRIMEAN-CONGO HAEMORRHAGIC  
FEVER AND EVALUATION OF CANDIDATE VACCINES.

- You are hereby kindly informed that the Ethics Committee approved the following and it will be condoned at the meeting scheduled for 26 November 2013:
  - *Amendment to the protocol*
  - *Extension of the study period to December 2014*
  - *Two additional collaborators: Dr A Meyers and Mr R Atkinson from the University of Cape Town*
- Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.
- Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.
- The Committee must be informed of any serious adverse event and/or termination of the study.
- All relevant documents e.g. signed permission letters from the authorities, institutions, changes to the protocol, questionnaires etc. have to be submitted to the Ethics Committee before the study may be conducted (if applicable).
- A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the ECUFS reference number in correspondence to the Ethics Committee secretariat.





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Ms H Strauss/hv

2014-04-14

REC Reference nr 230408-011  
IRB nr 00006240

PROF F BURT  
DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY  
FACULTY OF HEALTH SCIENCES  
UFS

Dear Prof Burt

**ETOVS NR 152/06A**

**PROF F BURT  
VIROLOGY**

**DEPT OF MEDICAL MICROBIOLOGY AND**


**PROJECT TITLE: IMMUNE RESPONSES IN SURVIVORS OF CRIMEAN-CONGO  
HAEMORRHAGIC FEVER AND EVALUATION OF CANDIDATE VACCINES.**

1. You are hereby kindly informed that the Ethics Committee approved the following at the meeting on 8 April 2014:
  - Extension Study:
    - Ecufs nr 152/06B  
Researcher: Student Miss D Pieters (M.Med.Sc.)  
Project title: Development of molecular and serological assays for diagnosis and surveillance of Crimean-Congo haemorrhagic fever virus.
2. Committee guidance documents: Declaration of Helsinki, ICH, GCP and MRC Guidelines on Bio Medical Research. Clinical Trial Guidelines 2000 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition (2006); the Constitution of the Ethics Committee of the Faculty of Health Sciences and the Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines.
3. Any amendment, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.
4. The Committee must be informed of any serious adverse event and/or termination of the study.
5. All relevant documents e.g. signed permission letters from the authorities, institutions, changes to the protocol, questionnaires etc. have to be submitted to the Ethics Committee before the study may be conducted (if applicable).
6. A progress report should be submitted within one year of approval of long term studies and a final report at completion of both short term and long term studies.



7. Kindly refer to the ETOVS/ECUFS reference number in correspondence to the Ethics Committee secretariat.

Yours faithfully



.....  
**PROF WH KRUGER**  
**CHAIR: ETHICS COMMITTEE**

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## Opsomming

Krimiaanse-Kongo hemorrhagiese koors virus (KKHKV) is 'n lid van die *Bunyaviridae* familie in die genus *Nairovirus*. Die virus word geassosiëer met hemorrhagiese koors in mense. KKHKV word oorgedra deur bosluise en die wêreldwye verspreiding stem ooreen met dié van bosluise in die *Hyalomma* genus. KKHKV-infeksie word gediagnoseer deur opsporing van virale nukleïensuur deur gebruik te maak van tru-transkripsie polimerase ketting reaksie (TT-PKR) of ander molekulêre toetse, virus isolasie vanuit geïnfekteerde sel kulture of suigeling muisbreine of deur opsporing van teen-KKHKV teenliggame deur gebruik van ensiem-gekoppelde immunoabsorberende toets of immunofluoreserende toets. Hoë bio-veiligheid fasiliteite word benodig vir virus-isolasie en vir voorbereiding van natuurlike antigene wat gebruik kan word in serologiese toetse. Tans is behandeling beperk tot ondersteunende terapie en vroeë toediening van ribavirin. Die verskyning van KKHKV in nie-endemiese gebiede en heropduiking van die virus in endemiese gebiede na lang periodes van afwesigheid, tesame met die bogenoemde feite, beklemtoon die aanvraag na vinnige, veilige, betroubare en goedkoop toetse vir diagnostiese en observering doeleindes om sodoende diagnostiese kapasiteit vir KKHKV te verhoog en om die verskyning van die virus te monitor.

'n Nuwe nukleïensuur volgorde-gebaseerde amplifikasie (NVGA) toets is ontwikkel vir opsporing van KKHK virale nukleïensuur. Hierdie toets kan uitgevoer word sonder gesofistikeerde toerusting. 'n Ensiem mengsel en amplifiseringsbuffer wat voorsien is in die kommersiële NucliSENS® EasyQ Basic kit is vergelyk met die ensiem mengsel en amplifisering buffer wat in ons laboratorium ontwikkel is, om uitvoering te vergemaklik. Ribonukleïensuur (RNS) wat ekstraheer is vanuit Vero sel kulture, geïnfekteer met geneties diverse KKHKV isolate van suidelike Afrika, is gebruik om die spesifisiteit van die KKHKV NVGA toets te bepaal en om die KKHKV NVGA met TT-PKR te vergelyk. 'n Totaal van 41/48 monsters het 'n positiewe resultaat gelever nadat dit met die KKHKV NVGA getoets is. Die sensitiwiteit van die KKHKV NVGA en die kommersiële NVGA is bepaal deur verdunde RNS, wat ekstraheer is vanuit 'n KKHKV-geïnfekteerde Vero sel kultuur, en verdunnings van 'n sintetiese RNS kontrole te toets. Die KKHKV NVGA was in staat daartoe om 3.7 RNS kopieë te amplifiseer terwyl die kommersiële KKHKV NVGA ten minste 37 kopieë kon amplifiseer. Die diagnostiese toepassing van die NVGA toetse is ondersoek deur RNS, wat ekstraheer is vanuit kliniese monsters, te toets en te vergelyk met kommersiële ware-tyd TT-PKR toetse. Die kommersiële

ware-tyd TT-PKR en NVGA toetse het RNS in 22 of 17 van die 22 bekende positiewe monsters onderskeidelik opgespoor, terwyl die NVGA wat in ons laboratorium ontwikkel is dit slegs in 10 kon opspoor.

Hierna is die inhiberende effek van serum op die amplifisering van KKHK virale RNS deur middel van KKHKV NVGA ondersoek. Serum monsters is versamel van vier vrywilligers en met verdunnings van die sintetiese RNS kontrole verryk. Die RNS is ekstraheer en getoets met die KKHKV NVGA. Die resultate dui op die moontlike teenwoordigheid van inhiberende faktore in sekere van die kliniese monsters wat getoets is.

Twee proteïen-uitdrukking sisteme, 'n baculovirus uitdrukking sisteem en 'n soogdier uitdrukking sisteem, is ondersoek vir die uitdrukking van rekombinante KKHKV kapsied proteïen (KP). Die nukleïensuur volgorde van die geen wat verantwoordelik is vir die uitdrukking van die KKHK virale KP is geoptimaliseer vir uitdrukking in insek selle. 'n pFastBac HT B oordrag plasmied, wat die geoptimaliseerde geen bevat, is voorberei en gebruik om DH10Bac™ *Escherichia coli* selle te transformer om sodoende die geoptimaliseerde geen na die bakmied oor te plaas. Die rekombinante bakmied is gebruik om *Spodoptera frugiperda* 9 insek selle te transfekteer. Menige transfeksie pogings is gemaak met verskeie kondisies. Die selle en die sel kultuur media is geanaliseer, maar geen bewys van suksesvolle uitdrukking van die rekombinante KKHK virale kapsied kon gevind word nie. 'n pcDNA™ 3.1D/V5-His-TOPO oordrag plasmied wat die KKHK virale kapsied geen bevat, is voorheen voorberei in ons laboratorium, en is gebruik om baba hamsternier (BHN)-21 selle te transfekteer. 'n pSIN-DLR-GFP plasmied, wat groen-fluoresenserende-proteïen uitdruk, is gebruik as positiewe transfeksie kontrole. Die getransfekteerde BHN-21 selle is geanaliseer deur immunofluoreserende toets (IFT) en daar is bevind dat die selle positief gereageer het teenoor 'n serum monster geneem van 'n herstellende KKHKV pasiënt.

Die profiel van die immuuniteitsreaksie teenoor die KKHK virale proteïene is bepaal deur kommersiële antigeen IFT kassette te gebruik om vir teenliggame teen KKHKV KP en glikoproteïene te toets in serum monsters wat versamel is op verskillende tydstippe na aanvang van siekte in 15 herstellende KKHKV pasiënte. Die antigeen kassette is voorberei deur gebruik te maak van getransfekteerde soogdier selle wat rekombinante KKHK virale KP en glikoproteïene onderskeidelik uitdruk. Twee van die 15 serum monsters is gekies, tweevoudig verdun en gebruik om die eindpunt konsentrasies van teenliggame teen die KKHKV KP en

glikoproteïene te bepaal deur middel van IFT. Die toepassing van die antigeen kassette wat in die laboratorium voorberei is met getransfekteerde BHN-21 selle wat die rekombinante KKHK virale kapsied uitdruk is getoets deur middel van IFT en is gereageer teen serum monsters wat geneem is van herstellende KKHKV-geïnfekteerde pasiënte en ook van vrywilligers met geen geskiedenis van KKHKV infeksie nie. Al die serum monsters van herstellende KKHK pasiënte het 'n positiewe toets resultaat gelewer, wat daarop dui dat voorbereiding van 'n stabiele sel lyn wat die KKHKV KP uitdruk geregverdig is vir toepassing in die opsporing van teenliggame teen KKHKV.