DEVELOPMENT OF A RAPID DIAGNOSTIC PLATFORM FOR NUCLEIC ACID TESTING OF INFECTIOUS PATHOGENS

Vorgelegt von

Diplom-Ingenieur

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Von der Fakultät III – Prozesswissenschaft

der Technischen Universität Berlin

zur Erlangung des akademischen Grades

Doktor der Ingenieurwissenschaften

-Dr.-Ing.-

genehmigte Dissertation

Promotionsauschuss:

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Berichter:	Prof. Dr. Roland Lauster
Berichter:	Prof. Dr. Jens Kurreck

Tag der wissenschaftliche Aussprache: 16. August 2012

Berlin 2012
D 83

The presented Ph.D. thesis was conducted from June 2009 to July 2012 at Robert Koch Institute, Berlin, Germany under supervision of Prof. Dr. Matthias Niedrig.

DECLARATION OF AUTHORSHIP

I certify that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigations, except as acknowledged. The present work has not been submitted, either in part or completely, for a degree at this or any other University.

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ABSTRACT

Multiple factors, including climate change, economic development, human demographics and behavior, international tourism and global trade, have contributed to the emergence and spread of infectious diseases in many countries worldwide thus becoming a major public health problem. Besides the natural incidents, the potential use of infectious pathogens for bioterror attack is an additional risk to the human population. Anthrax, severe acute respiratory syndrome (SARS), dengue (DEN), malaria, yellow fever (YF), west nile (WN) fever, bird flu (H5N1), swine flu (H1N1 2009), and hemolytic uremic syndrome and bloody diarrhea (e.g. EHEC O104:H4) are some of the recent emerging and re-emerging infectious diseases that have caused outbreaks of human diseases. The burden of morbidity and mortality associated with these infectious diseases affects the human population globally and causes widespread social panic.

The recent advances in biochemical methods and microfluidic technologies open a new perspective for point-of-care diagnostics of infectious pathogens. This can eliminate the high complexity associated with molecular testing currently performed in large hospitals, reference- and research-laboratories. However, cost-effectiveness, commercial availability, automation, and integrated diagnostic platforms are still the essential targets to develop affordable diagnostic tests.

This thesis focuses on the establishment of novel biochemical methods and the development of diagnostic platforms for the rapid detection of infectious diseases. All the important methods of nucleic acid testing including sample pre-treatment, sample preparation, nucleic acid amplification, and detection have been developed to overcome the major existing problems for the development of molecular diagnostics and tested for the rapid detection of recent emerging and re-emerging infectious pathogens.

This work presents the achievements made towards establishing molecular diagnostic platforms ranging from high-tech to a simple platform using the existing and newly developed nucleic acid detection techniques. On one hand, an automated microfluidic diagnostic platform was developed utilizing a novel sample preparation and isothermal amplification technologies for biodefense applications. On the other hand, the same techniques were combined with existing simple biosensor devices such as fluorescence readers and lateral flow strips to develop molecular diagnostics of emerging and re-emerging pathogens such as influenza viruses and flaviviruses. Some of these techniques were successfully applied for the molecular diagnostics of infectious diseases in reference laboratories and low-resource settings, while other techniques are going to be tested under real conditions for the detection of infectious pathogens.

In conclusion, simple and accurate diagnostic tests were developed to combat the infectious diseases, and alternative approaches were explored in order to make these techniques available and accessible to wider range of people. Furthermore, some of these diagnostic methods could be an useful tool for the detection of emerging and re-emerging diseases. They would have a beneficial impact on public health care permitting the important assistance for patient management, disease surveillance, epidemiological studies, and rapid identification of infectious diseases in emergencies.

ZUSAMMENFASSUNG

Mehrere Faktoren wie z.B. die Klimaveränderung, die wirtschaftliche und demographische Entwicklung, menschliches Verhalten sowie internationaler Tourismus und Handel, haben weltweit zum Auftreten und zur Ausbreitung von Infektionskrankheiten geführt. Dies hat in vielen Ländern zu einem großen Problem der öffentlichen Gesundheit beigetragen. Die potenzielle Nutzung von Infektionserregern für bioterroristische Angriffe ist neben dem natürlichen Auftreten der Erreger ein zusätzliches Risiko für die Bevölkerung. Anthrax, schweres akutes Atemnotsyndrom (SARS), Dengue (DEN), Malaria, Gelbfieber (YF), West-Nil (WN), Vogelgrippe (H5N1), Schweinegrippe (H1N1 2009), hämolytisch-urämisches Syndrom und blutiger Durchfall (z.B. EHEC O104: H4) sind einige der kürzlich aufgetretenen „Emerging“ und „Re-emerging“ Infektionskrankheiten, die Krankheitsausbrüche beim Menschen verursacht haben. Die mit diesen Infektionskrankheiten verbundene Belastung durch Morbidität und Mortalität betrifft die menschliche Population weltweit und verursacht oft soziale Ängste.

Die aktuellen Fortschritte von biochemischen Methoden und Mikrofluidik-Technologien öffnen eine völlige neue Perspektive bei der Point-of-Care-Diagnostik für Infektionserreger. Die momentan angwendeten molekularen Teste sind sehr Komplex und daher können sie derzeit nur in großen Krankenhäusern, Referenz- und Forschungs-Laboren durchgeführt werden. Die wesentlichen Aufgaben zur Entwicklung günstiger diagnostischer Teste wären daher Wirtschaftlichkeit, kommerzielle Verfügbarkeit, Automatisierung und integrierte Diagnostik-Plattformen.

In dieser Arbeit geht es um die Etablierung neuer biochemischer Methoden und um die Entwicklung von diagnostischen Plattformen für die schnelle Erkennung von Infektionskrankheiten. Hierzu wurden wichtige Methoden der Nukleinsäure-Testung einschließlich Probenaufreinigung, Probenvorbereitung, Nukleinsäureamplifikation und Detektion weiterentwickelt, um die bestehenden Probleme der molekularen Diagnostik zu überwinden. Mit dieser neuen Methode wurde dann die schnelle Erkennung einiger kürzlich aufgetretener „Emerging“ und „Re-emerging“ Infektionserreger getestet.

Diese Arbeit präsentiert die erfolgreichen Schritte zur Etablierung von molekulardiagnostischen Plattformen (von „High-Tech“ bis zu einfacheren Plattformen) durch Nutzung von bestehenden und neu entwickelten Nukleinsäure-Nachweis-Techniken. Zum einen wurde eine automatisierte diagnostische Plattform basierend auf Mikrofluidik entwickelt. Dabei wurde eine neuartige Probenvorbereitung und isothermale Amplifikationstechnologie zwecks biologischer Sicherheit eingesetzt. Zum anderen wurden die gleichen Techniken mit vorhandenen einfachen Biosensoren wie Fluoreszenzdetektoren und Lateral-Flow-Streifen kombiniert, um die molekulare Diagnostik von „Emerging“ und „Re-emerging“ Infektionserregern wie Flavi-Viren und Influenza-Viren zu entwickeln. Einige dieser Techniken wurden erfolgreich zur molekularen Diagnostik von Infektionskrankheiten sowohl in Referenzlaboratorien als auch in einfach ausgestatteten Einrichtungen angewendet, wo auch zukünftig andere Techniken unter realen Bedingungen zur Erkennung von Infektionserregern getestet werden sollen.

Zusammengefasst sind einfache und präzise diagnostische Teste zur Bekämpfung von Infektionskrankheiten entwickelt worden. Dazu wurden auch alternative Ansätze entwickelt, um Verfügbarkeit und Zugang zu dieser Technik auch für wenig entwickelte Gebiete mit Bedarf zu erleichtern. Darüber hinaus eignen sich einige dieser diagnostischen Verfahren als nützliches Werkzeug zum Nachweis von „Emerging“ und „Re-emerging“ Krankheiten und können für die öffentliche Gesundheitsversorgung

vorteilhaft sein. Sie könnten eine wichtige Hilfe für Patienten-Management, Überwachung von Krankheiten und epidemiologische Studien sein, sowie die schnelle Identifizierung von Infektionskrankheiten in Notfällen ermöglichen.

ABBREVIATIONS

µTAS	Micro-total-analytical systems
BA	*Bacillus anthracis*
BHQ	Black hole quenchor
CDC	Centre of Disease Control and Prevention
ChikV	Chikungunya virus
CMC	Carboxy methyl cellulose
CP 19	Camelpox virus 19
CPE	Cytopathic effects
Ct	Threshold cycle
ddNTP	Dideoxynucleotide
DENV	Dengue virus
ELISA	Enzyme-linked immunosorbent assay
FAM	6-carboxy-fluorescine
FCS	Fetal calf serum
Ftu	*Francisella tularensis*
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Geq	Genome equivalents
HA	Haemagglutanin
HDA	Helicase dependent amplification
IFA	Immunofluorescence assay
IVD	*in-vitro* diagnostics
ivRNA	in vitro transcribed RNA
JEV	Japanese Encephalitis virus
LAMP	Loop-mediated amplification
LFS	Lateral flow stripe
LNA	Locked nucleotide
LOC	Lab-on-chip
MAGs	Magnetic beads
MARV, MbgV	Marburg virus
MDx	Molecular disgnostics
MGB	Minor groove binder
NALF	Nucleic acid based lateral flow assay
NASBA	Nucleic acid sequence based amplification
NAT	Nucleic acid testing

Pan-Flavi assay	Pan-Flavivirus real-time RT-qPCR
PCR	Polymerase chain reaction
PFU	Plaque forming units
PK	Proteinase K
PRNT	Plaque reduction neutralization tests
RAH	Rapid amplification/hybridization
real-time qPCR	Quantitative real-time PCR
real-time RT-qPCR	Quantitative real-time RT-PCR
RPA	Recombinase polymerase amplification
RSSEV	Russian Spring Summer Encephalitis virus
RT-PCR	reverse transcriptase-polymerase chain reaction
RVFV	Rift valley fever virus
rxn	Reactions
SAEs	Severe adverse events
SARS	Severe acute respiratory syndrome
SDA	Strand displacement amplification
SigV	Sigma virus
SLEV	Saint Louis encephalitis virus
S-OIV A	Swine-origin influenza A
SONDE	Szenario-orientierte Notfall Diagnostik für den Feld Einsatz
TBEV	Tick-borne encephalitis virus
TCID 50	Tissue Culture Infection Dose 50
THF	Tetrahydrofuran
TMA	Transcription-mediated amplification
USUV	Usutu virus
VACV	Vaccinia virus
WHO	World health organisation
WNV	West nile virus
YFV	Yellow fever virus
Ype	*Yersinia pestis*
ZEBO	Ebola virus Zaire

PUBLICATIONS

Parts of this work have been published at the following journals or conferences

Publications related to this Work

1. **RapidSTRIPE H1N1 test for detection of the pandemic swine origin influenza A (H1N1) virus.**
   Patel P, Graser E, Robst S, Hillert R, Meye A, Hillebrand T, Niedrig M. J Clin Microbiol. 2011 Apr; 49(4):1591-3. Epub 2011 Jan 19.

2. **Molecular diagnosis of flaviviruses**.
   Domingo C, Patel P, Linke S, Achazi K, Niedrig M.
   Future Virology 6 (9): 1059-1074.DOI 10.2217/fvl.11.77 (2011).

3. **Development of one-step quantitative reverse transcription PCR for the rapid detection of flavivirus**s.
   Patel P, Landt O, Kaiser M, Faye O, Koppe T, Lass U, Sall AA, Niedrig M
   Virology Journal in progress

Other Publications

4. **RNA interference inhibits replication of tick-borne encephalitis virus in vitro.**
   Achazi K, Patel P, Paliwal R, Radonić A, Niedrig M, Donoso-Mantke O.
   Antiviral Res. 2012 Jan; 93(1):94-100. Epub 2011 Nov 9.

5. **Advanced yellow fever virus genome detection in point-of-care facilities and reference laboratories.**
   Domingo C, Patel P, Yillah J, Weidmann M, Méndez JA, Nakouné ER, Niedrig M.
   Clinical Microbiology in accepted

Conferences

1. **Enrichment of viruses by AJ enrichment kit**
   Pranav Patel, Timo Hillebrand & Niedrig M
   Presented as a talk at Life Science Conference, 05.05.-06.05.2010, Jena

2. **Development Of A Rapid Diagnostic Platform For Detection Of Category A Biothreat Pathogens In The Field**
   Pranav Patel, Manfred Weidmann, Katharina Achazi, Sonja Linke, Oliver Strohmeier, Daniel Mark, Thomas van Oordt, Josef Drexler, Michael Eberhard, Felix von Stetten and Matthias Niedrig
   Presented as a poster at 10th ASM Biodefence and Emerging Diseases conference, Washington DC, USA, 2012 & BMBF-Innovationsforum "Zivile Sicherheit" Berlin, Germany

CONTENTS

ACKNOWLEDMENT

First, I would like to thank Prof. Dr. Matthias Niedrig for giving me the opportunity to do my Ph.D. thesis in his lab and his guidance throughout my Ph.D. I am very thankful to my supervisor Sonja Linke and Katharina Achazi for their excellent guidance during my Ph.D. I would also like to thank our colleagues from SONDE project Manfred Weidmann & Frank Hufert from Dept. of Virology, Göttingen; Oliver Strohmeier & Thomas Van Oordt from HSG-IMIT, Freiburg; Josef Drexler & Michael Eberhard from Qiagen Lake Constance; Timo Hillebrand & Elmara Graser from AJ Innuscreen and Olfert Landt from TIb MolBiol for their kind support, guidance, and valuable discussions.

Furthermore, I would like to thank Cristina Domingo, Ellen Zimowski, Hewad Laraway, Nina Stock, Nadine Litzba, Patricia Bußmann, Anette Teichmann, and Marco Kaiser for technical assistance concerning lab, advice, and encouragement during my work.

I am feeling glad to be a part of **AG Niedrig, ZBS1** and thankful to all the members of our department at Robert Koch Institute. I am never going to forget the extraordinary atmosphere of the group, which made me feel like home. All the celebrations, sport activities, and outgoing activities of the group help me a lot to make hard work easy-going.

Once I am very thankful to Regina Schaedler, Katharina Achazi, Vipul Patel, and Gregory Stevens for the critical reading and correction of this manuscript. I am grateful to my wife for sharing the good moments with me and supporting me throughout my Ph.D.

This PhD was supported by the German Ministry of Education and Research (BMBF) joint research project S.O.N.D.E. In this context, I am also thankful to BMBF.

BACKGROUND

INFECTIOUS PATHOGENS

Infectious pathogens are disease-causing organisms such as viruses, bacteria, fungi, or parasites. They have threatened humankind throughout the history and today pose a considerable threat to global health. There have been a number of epidemic diseases caused by infectious pathogens recorded in last centuries, such as a plague, cholera, influenza, measles, smallpox, malaria and typhoid, which claimed the life of millions (Fauci 2001; Feldmann, Czub et al. 2002; Morens, Folkers et al. 2004). Through vaccination and the use of antibiotics, humans have tried to combat infectious diseases, but the rapid evolution of microorganisms has allowed them to escape from these defenses. In addition, some of the zoonotic pathogens have evolved as major human pathogens through the adaptation to new hosts and new environments. Multiple factors, including economic development and land use, human demographics and behavior, international travel and commerce as well as climate changes are contributing to the emergence and spread of infectious diseases worldwide, which represent today a major public health problem (Morens, Folkers et al. 2004).

In recent years, infectious diseases have been classified into the following categories: emerging diseases (newly appeared diseases), re-emerging diseases (old diseases which occur again) and deliberately emerging diseases (diseases which are caused by the deliberate release of infectious pathogens through bioterrorism, or use of bioweapons) (Fauci 2006). In the past 30 years, more than 50 emerging pathogens have been identified (Fauci 2001; Olano and Walker 2011). Some important examples of the recent emerging diseases are acquired immunodeficiency syndrome (Human immunodeficiency virus (HIV)), hemorrhagic diseases (Ebola virus, Marburg virus, Sabia virus, Hantaan virus), hepatitis (Hepatitis A, B, C, D and E virus), encephalitic diseases (Hendra virus, Nipah virus), severe acute respiratory syndrome (SARS), respiratory diseases (H5N1 influenza virus and H1N1 influenza virus), drugs-resistant *staphylococcus aureus* and hemolytic uremic syndrome and bloody diarrhea (*Escherichia coli* O157:H7, EHEC O104:H4).

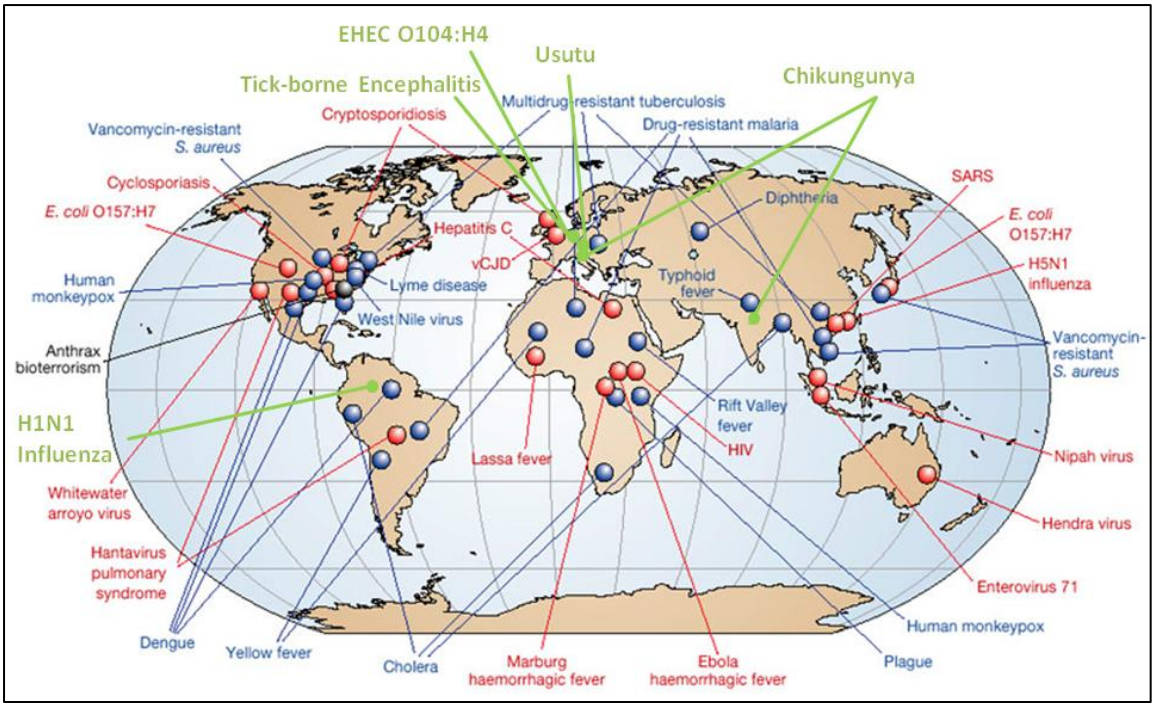


Figure 1. Examples of emerging and re-emerging infectious diseases worldwide. In red: newly emerging diseases; In blue: re-emerging/resurging diseases; In black: a 'deliberately emerging' disease, green, recent emerging & re-emerging disease (Morens, Folkers et al. 2004). (Source: http://www.nature.com/nature/journal/v430/n6996/fig_tab/nature 02759 _F1 .html)

Besides new emerging diseases, some re-emerging diseases have also occurred in new or extended geographic regions. Flaviviruses like West Nile virus (WNV), Yellow fever virus (YFV), Dengue virus (DENV) and Usutu virus (USUV) as well as Enterovirus 71, Mumps virus, Chikungunya virus (ChikV), *Clostridium difficile, Streptococcus Group A, Staphylococcus aureus* and *Mycobacterium tuberculosis* are some important pathogens causing re-emerging diseases in the last decades. WNV was first introduced though migratory birds in the United States (US) in 2001 and had never been reported in the US previously (Calisher CH, 2000). Recently, outbreaks of re-emerging pathogens including among the others WNV, USUV, ChikV, Tuberculosis, DENV, YFV and H5N1 are continuously reported in different parts of the world. Additionally, some infectious pathogens like *Mycobacterium tuberculosis, Staphylococcus aureus* and *Plasmodium falciparum* have developed resistance to drugs through evolution over the past years (Fauci 2001). Many of above-mentioned pathogens can be misused for bioterrorism purposes, which represent a very sensitive issue for civil security around the world. One known example of a deliberated release of infectious pathogen is the anthrax attacks of 2001 in the United States (Jernigan, Stephens et al. 2003).

All these emerging and re-emerging diseases pose constant threat to the humans and are among the top 10 death causes according to WHO (WHO 2011). Therefore, research on infectious diseases, reporting of outbreaks and surveillance studies are important measures of preparedness to fight infectious diseases and improve the public health globally.

MOLECULAR DIAGNOSTICS OF INFECTIOUS DISEASES

Advances in microbiology, molecular biology, immunology, and bioinformatics have made it possible to develop accurate and sensitive diagnostic methods for the detection of microbes. These diagnostic methods can be classified into the following categories: 1) Direct detection of pathogens by classical microbiology and virology cultivation methods; 2) Detection by the physical properties of the pathogens by microscopy and molecular analytic methods; 3) Biochemical techniques based on the protein-protein interaction (e.g. enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA)); 4) Molecular techniques based on the detection of nucleic acids from the pathogens (e.g. Polymerase chain reaction, Hybridization, DNA arrays). Many of these methods are applied routinely in clinical diagnostics of infectious diseases on the basis of availability of the methods, laboratory setup, and technical skills.

Classical cultivation methods are the gold standard for detection of pathogens and allow direct detection of viable pathogens. Nevertheless, these methods are very laborious and time-consuming. Other direct detection methods based on microscopy and molecular analytics may also allow an easy detection of pathogens in time. However, these methods require expensive instruments and complex sample preparations, which limit their application in routine clinical diagnosis. Biochemical methods utilizing antibodies are also widely used in the diagnostics of infectious pathogens. These methods require pathogen-specific antibodies, which are mostly produced by immunizing animals or by *in- vitro* hybridoma technologies. However, the production of antibodies is also laborious and time-consuming. Many tests like, ELISA, IFA and lateral flow assays have been successfully employed using antibodies for the detection of infectious pathogens. However, they have several limitations such as restricted multiplex detection and high-throughput screening, low specificity, and variable sensitivity. In contrast to all the above-mentioned methods, molecular methods offer many advantages like high sensitivity and specificity, rapidity, high-throughput capability, multiplex detection, and reproducibility. The use of molecular techniques is not restricted to confirm a clinical

diagnosis, but it has also led to the discovery of new pathogens, the detection of outbreaks, and the identification of geographical distributions of pathogens.

Since the development of Polymerase Chain Reaction (PCR) in the early 80´s, molecular testing based on nucleic acid amplification has revolutionized the field of molecular biology, and this technology has emerged as an ideal healthcare tool. PCR based methods are the dominant technology for nucleic acid amplification, and have become a new gold standard for diagnostic testing in microbiological laboratories (Cirino, Musser et al. 2004). PCR amplifies DNA through a repeated cycle of heating and cooling using a thermal cycler. There are many diagnostic markers available for the PCR technology utilizing genomic sequences and genes of pathogens. The availability of an online genome database allows the easy development of the required assay components like primers and probes. Furthermore, many well-established protocols and commercial kits are available to perform PCR, which allows also rapid development of sensitive and specific assay with high reproducibility. There are many different formats of PCR available such as conventional PCR, reverse transcriptase PCR (RT-PCR), nested PCR, multiplex PCR and real-time PCR. The major benefits of real-time PCR over other PCR methods are rapidly in time, low risk of a contamination, high specificity, and high sensitivity. Real-time PCR is now replacing the conventional cell culture and traditional PCR methods for the molecular diagnostics of infectious diseases (Ratcliff, Chang et al. 2007).

Real-time PCR is based on real-time monitoring of increase of a fluorescence signal during DNA amplification. In molecular diagnostics, the main formats of real-time PCR are SybrGreen, hydrolysis probes (Taqman), molecular beacons probes or hybridization probes (FRET) (Holland, Abramson et al. 1991; Tyagi and Kramer 1996; Mangasser-Stephan, Tag et al. 1999; Ponchel, Toomes et al. 2003). The SybrGreen is a dsDNA binding dye and emits fluorescence upon excitation, which can be monitored during DNA amplification in a SybrGreen based real-time PCR. In contrast, the hydrolysis probes containing a reporter fluorescent dye on the 5' end, and a quencher dye on the 3' end, were used in a Taqman real-time PCR. During DNA amplification, the polymerase with 5´exonuclease activity cleaves the probe and separates the reporter dye from the quencher dye. This allows the detection of fluorescence signal in real-time (Fig. 2). The Nowadays Taqman real-time PCR is the most widely used method in molecular diagnostics and basic research.

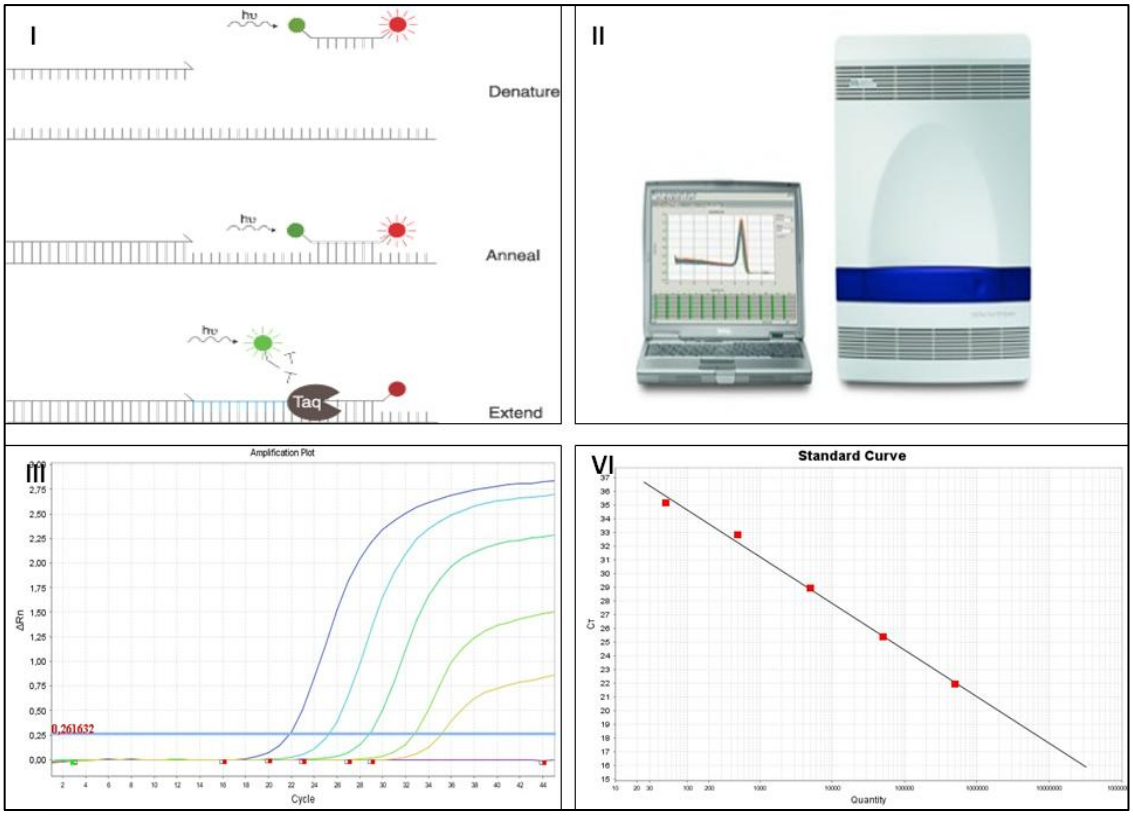


Figure 2. Overview of Taqman probe based real-time PCR. I) The TaqMan probe is dual labeled oligonucleotide with a reporter dye at the 5' end and acceptor dye at 3' end. The polymerases with 5'-nuclease activity used in the polymerase chain reaction (PCR) cleave hydrolysis probes during the DNA extension step, which separates the detectable reporter fluorophore (in green) from a quencher (in red). Fluorescence emitted when excited by an external light source (hν) at each PCR cycle is proportional to the amount of product formed. II) An example of real-time PCR instrument (ABI 7500 by Applied Biosystems) III) A typical amplification plot of real-time PCR is shown. The ΔRn in logarithmic scale is shown on the y-axis, where ΔRn represents the normalized reporter signal minus the baseline signal. The cycle number is shown on the x-axis. IV) A typical standard curve derived from the amplification of serial dilution series of the plasmid standards.

As an alternative to PCR, varieties of isothermal methods have been developed in the past 10 years. Some of the most important isothermal methods include Strand Displacement Amplification (SDA), Nucleic Acid Sequence Based Amplification (NASBA), Loop-mediated Amplification (LAMP), Helicase Dependent Amplification (HDA) and Recombinase Polymerase Amplification (RPA) as reviewed previously (Niemz A 2011). These isothermal amplification methods just require a single constant temperature for DNA amplification and provide sensitive amplification of DNA within an hour. In the recent years, many of these techniques are being used to develop the point-of-care diagnostics, and are becoming a true competitor to PCR based methods.

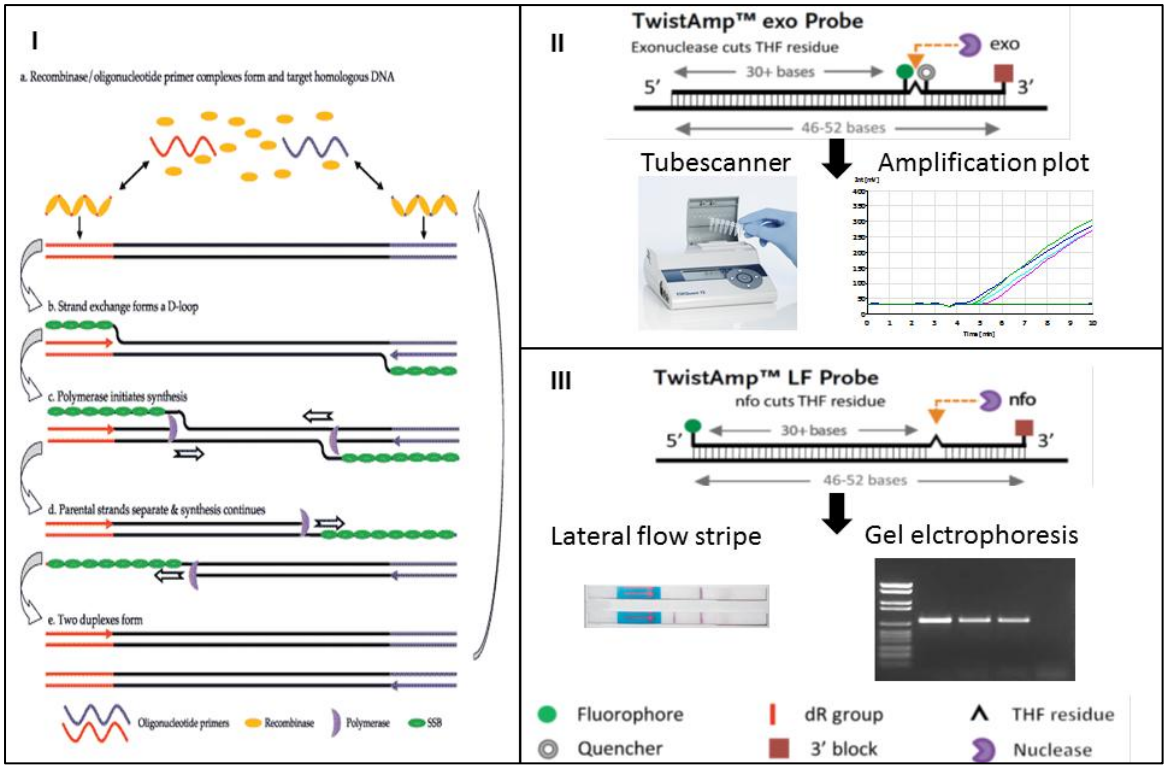


Figure 3. Overview of recombinase polymerase amplification (RPA) techniques. I) Scheme of RPA reaction. A recombinase-primer complex scans a DNA helix and initiates primer binding. Single strand DNA binding proteins stabilize the separated strain; then DNA amplified analogue to PCR, but only constant 37 °C is needed. II) RPA exo probe contains dT(FAM) - THF - dT(BHQ) site. Exonuclease III cuts THF and small probe section is released which seperates quencher from fluorophore and allows the emission of fluorescence. Fluorescence can be monitored in real-time by using the ESE Tubescanner (Qiagen Lake Constant). III) RPA nfo probe contains THF site and is labeled with fluorophore at 5´-end and block at 3´-end. Nfo cuts THF and small probe is released. Polymerase elongates the DNA strand at site of cleavage. The amplification products can be detected by lateral flow strip or gel electrophoresis as endpoint analysis. (Source: www.twistDx.com, (Piepenburg, Williams et al. 2006))

RPA was first described in 2006 (Piepenburg, Williams et al. 2006) and is one of the most promising next generation molecular diagnostic method. RPA employs prokaryotic recombinase and other proteins including UvsX, UvsY, Gp32 and *Bacillus subtilis* (Bsu) Pol I. The UvsX recombinase protein forms a complex with primers and guides the primer to find homologous sequences in DNA without the need of a heating step. Once the homologous sequence is found, primers bind to the target sequence through a D-loop formation and displace the parental strand. This reaction is assisted by UvsY and Gp32 proteins. Primers are then extended by Bsu Pol I, which lead to DNA amplification (Fig. 3 I). The RPA reaction has some analogy to PCR such as oligonucleotide-primed

DNA synthesis and exponential amplification of DNA. However, RPA works at ambient temperatures of from 24 °C to 45 °C, obviating the need for sophisticated heating devices. This is a major advantage over PCR and other isothermal methods. Furthermore, different detection formats are available for RPA. Utilizing oligonucleotide-probes, the RPA reaction can be monitored by the fluorescence detection in real-time, or can be detected as endpoint analysis by using gel electrophoresis or lateral flow strip (LFS) (Fig. 3 II and III).

Nowadays molecular testing is used in various fields such as basic research, medical diagnostics, agriculture, biodefense, and food testing. In the future, molecular testing may become a component of routine laboratory work for detecting infectious pathogens, to monitor the outbreaks, and to provide important data for surveillance and epidemiological studies, thus improving the public health system.

OBJECTIVES OF THIS PH.D. THESIS

Recent advances in nucleic acid amplification technology have revolutionized the field of molecular diagnostics and, *In-Vitro* Diagnostics (IVD) has become a fast-growing industry worldwide. The application of molecular diagnostic tools opens a new perspective of diagnostics for infectious pathogens, even in less developed clinical settings. However, only a small number of the potentially capable laboratories are using the molecular diagnostics due to the high cost of these techniques and the shortage of commercial kits, well-equipped laboratories, and trained personnel. In contrast, point-of-care testing would eliminate the high complexity associated with molecular testing currently performed in large hospitals, reference and research laboratories. However, cost-effectiveness, commercial availability, automation, and integrated diagnostic platforms are still the essential targets to make this approach a success.

The aim of this Ph.D. work is to develop and establish methods for a complete workflow of nucleic acid diagnostics including sample pre-treatment, sample preparation, nucleic acid amplification, and detection. These methods should be sensitive and specific but also accessible, rapid and affordable, so they can be widely implemented in diagnostic laboratories. Furthermore, these methods would be used in combination with microfluidic systems to develop integrated molecular diagnostic platforms or nucleic acid testing (NAT) for low-resource settings, in order to overcome the challenges of developing a “sample to answer” platform.

This thesis is divided into four chapters.

In the first chapter, novel biochemical methods including all steps of NAT have been established for the detection of biothreat pathogens. These methods were then implemented on the centrifugal microfluidic platform to obtain a “sample to answer” platform, which should allow fully automated and rapid identification of biothreat pathogens in emergencies.

The second chapter describes a molecular diagnostic method based on real-time PCR for the identification of important flaviviruses, which can be used in well-equipped reference laboratories to confirm flavivirus infections.

The third chapter is reserved for an alternative molecular diagnostic system combining rapid PCR with a lateral flow strip as an endpoint analysis, which was specially developed for the diagnosis of pandemic H1N1 2009 influenza virus in low-resource settings.

In the last chapter, an instrument-free molecular diagnostic method utilizing an isothermal amplification was developed for the detection of yellow fever virus in low-resource and in-field settings.

Finally, these molecular diagnostic platforms were compared with each other in a general conclusion.

Chapter 1. INTEGRATED MICROFLUIDIC DIAGNOSTIC PLATFORM

DEVELOPMENT OF A RAPID DIAGNOSTIC PLATFORM FOR DETECTION OF CATEGORY A BIOTHREAT PATHOGENS IN THE FIELD

A INTRODUCTION

A.1 BIOLOGICAL THREAT AGENTS

Biological threat agents are highly contagious microorganisms (bacteria, viruses and fungi), or toxins produced by these microorganisms. They are present in nature but could also be genetically engineered. The natural or deliberate release of such agents can cause the death in humans, animals or plants, lead to panic and enormous economic loss. The Centre of Disease Control and Prevention (CDC), USA, has categorized biothreat agents into categories “A”, “B”, or “C” according to potential threat, efficiency of transmission, and severity of illness caused (Table A-1). Category “A” threats include Botulinum toxin and important infectious agents such as Smallpox viruses, *Bacillus anthracis, Yersinia pestis, Francisella tularensis* and hemorrhagic fever disease-causing RNA viruses. The pathogens from Category “A” pose the highest risk in term of public health, as they can be transmitted easily from person to person and cause high mortality rates among the population causing widespread panic. Category “B” threats include agents such as *Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei,* many foodborne & waterborne pathogens and Ricin toxin. In contrast to category “A” pathogens, category “B” pathogens spread at lower speed and cause moderate or low death rates. Category “C” threats include emerging pathogens, which could be engineered for mass dissemination in the future because of their availability and ease of production as well as their potential threat (CDC Factsheet Bioterrorism Agents).

In the last three decades, progress in biotechnology and genetic engineering has revolutionized the field of molecular biology. On one hand, these technologies are used in biological and medical research for peaceful purposes, on the other hand, they can be misused to develop and produce biological weapons. A well-known example of bioterrorism attack is the use of Anthrax spores after 9th Sep. 2001 in the US. Such biological weapons pose a significant danger and were being produced mainly for military purposes. Nevertheless, bioweapons are not the only option for bioterrorism, as the use of naturally occurring foodborne and waterborne pathogens can cause the similar damage in terms of death and social disruption. Therefore, monitoring, rapid screening and surveillance studies of biological threat agents are important for identifying, controlling their spread, taking appropriate quarantine measures, and providing better patient treatment in a timely manner.

Table A-1. List of biological threat agents classified into different categories by Centre for Disease Control and Prevention, USA. Some of the main agents are listed in each classified category "A", category "B" and category "C" based on the potential threat of the pathogens.

CDC Category A agents	CDC Category B agents	CDC Category C agents
Bacillus anthracis (anthrax) *Clostridium botulinum* (botulism) *Yersinia pestis* (plague) *Variola major* (smallpox) and other pox viruses *Francisella tularensis* (tularemia) Viral hemorrhagic fevers • Arenaviruses • Bunyaviruses • Flaviviruses • Filoviruses	*Burkholderia pseudomallei* *Coxiellaburnetii (Q fever)* *Brucella species (brucellosis)* *Burkholderia mallei (glanders)* *Chlamydia psittaci (Psittacosis)* Ricin toxin (from Ricinus communis) Epsilon toxin *of Clostridium perfringens* *Staphylococcus enterotoxin B* *Typhus fever (Rickettsia prowazekii)* *Food- and water-borne Pathogens*	Emerging infectious disease threats such as Nipah virus and additional hantaviruses Tickborne hemorrhagic fever viruses Tickborne encephalitis viruses Prions Antimicrobial resistance, excluding research on sexually transmitted organisms

Table A-2. List of category A biothreat pathogens included as a target in the SONDE project for nucleic acid testing.

RNA agents	DNA agents
Ebola Zaire Virus (-)-stranded-RNA-Virus	*Bacillus anthracis* gram-positive bacterium (DNA)
Ebola Sudan Virus (-)-stranded-RNA-Virus	*Francisella tularensis* gram-negative bacterium (DNA)
Marburg Virus (-)-stranded-RNA-Virus	*Yersinia pestis* gram-negative bacterium (DNA)
Rift Valley Fever Virus (-)-stranded-RNA-Virus	Variola Virus double stranded-DNA Virus

In the past ten years, many countries have initiated Chemical, Biological, Radiological, and Nuclear (CBRN) security projects and they have contributed in various ways to progress in improving the detection, identification and monitoring of biological threat agents. In addition, the strategies for preparedness, prevention, and response to

biological threat agents have improved due to various funding research programs related to Biosecurity. In the “Szenario-orientierte Notfall Diagnostik für den Feld Einsatz” (SONDE) project, some of the important bacterial and viral biothreat agents from CDC category A list has been included in nucleic acid testing (NAT) by microfluidic molecular diagnostic platform (Table A-2).

A.2 INTEGRATED MOLECULAR DIAGNOSTIC PLATFORM

Nucleic acid testing (NAT) of infectious microorganism is an important method in molecular diagnostics (MDx), and applied in fields ranging from basic research and clinical diagnostics to monitoring of food-borne pathogens and biodefense applications. NAT is typically done in centralized laboratories using complex and expensive instruments and requires highly trained personnel. Therefore, these time-consuming NAT methods are restricted to stationary facilities and cannot be applied for mobile and point-of-need diagnostics. In contrast, an integrated MDx platform offers advantages such as rapid diagnostics, easy operation, and on-site testing under low-resource settings.

A common integrated MDx platform for NAT includes three main steps: sample preparation, amplification, and detection. Sample preparation methods are based on chemical and mechanical lysis, which release the nucleic acids (RNA/DNA) from the microorganism and serve as a template in NAT. After isolation of nucleic acids, the target region is amplified using polymerase chain reaction (PCR) or isothermal amplification methods. The successive amplification of the target is detected either during amplification in real-time, or at the end of the reaction as an endpoint analysis. Most of these techniques (real-time quantitative PCR, loop mediated amplification etc.) use detection of a fluorescence signal or turbidity in real-time.

In the past ten years, many efforts have been made to develop an integrated MDx platform, which allows an automated analysis of samples including all three steps of NAT. More recently, several fully integrated large and small MDx platforms for NAT are commercially available and are presented in Table A-2. Examples of large MDx platforms are Gen-probe Tigris, Roche Cobas, Siemens Versant and Becton Dickenson Viper systems, which are floor-standing automated devices employing complex robotics. Major benefits of these large platforms include the ability to process large numbers of samples and cost-efficiency. However, they are mainly used in centralized laboratories due to their high complexity. In contrast, small MDx platforms utilize Lab-on-chip or microfluidic technologies to integrate all the important steps of NAT in disposable cartridges, which can be used in portable devices. These have been successfully demonstrated by small

MDx platforms like Cepheid GeneXpert, Becton Dickenson BD Max, Enigma ML, Idaho technology Filmarray, Nanoshere Varigene and Iquum Liat analyzer. These systems allow the rapid detection of samples within one hour and can be applied in low-resource, in-field, and point-of-need settings.

Table A-3. A List of large and small molecular diagnostic (MDx) platforms for nucleic acid testing (NAT). Large MDx platforms are indicated by black text and small MDx platform are presented in red text.

Company	Platform	Amplificaion	Run time (min)	Multiplex capability	Throughput/run	Website
Abbott	Abbott m2000	PCR	-	-	24-96 samples	www.abborrmolecular.com
Alere	iNAT	Isothermal	-	-	-	www.alere.com
Becton Dickinson	BD Max Viper®	Isothramal (SDA)	90	1	24 samples	www.bd.com
Biocartis	MDx	PCR	-	-	1 sample	www.biocartis.com
Cepheid	GeneXpert	PCR	40	5	1 sample	www.cepheid.com
Enigma	Enigma ML	PCR	30	6-12	1 sample	www.enigmadiagnostic.com
Gen-Probe	Tigris	Isothemal (TMA)	210	-	20 sample	www.gen-probe.com
Idaho technologies	FilmArray	PCR	60	20+	1 sample	www.idahotech.com
Iquum	Liat Analyzer	PCR	<60	6	1 sample	www.iquum.com
Nanosphere	Verigene	PCR	-	-	1 sample	www.nanoshere.us
Siemens	Versant	PCR	-		96 Samples	www.siemens.com
Roche	Cobas	PCR	-	-	96 samples	www.roche.de

A.3 LAB-ON-CHIP PLATFORM FOR NUCLEIC ACID TESTING

Lab-on-chip (LOC) devices, also known as micro-total-analytical systems (µTAS) are miniaturized analytical devices, which integrate the workflow of laboratory methods in single small devices. The size of LOC devices range from a fingertip to a credit card. They are fabricated in glass, polymer, or silicon using processes such as lithography, chemical etching, and laser machining. Major advantages of these devices are the handling of µL to nL volumes, low consumption of reagents, short response time and low production costs. Advances in LOC technology have revolutionized the field of biotechnology and medicine in recent decades, where LOC based devices were used in NAT, protein analysis, proteomics and genomics. More recently, the use of LOC has dramatically increased in the field of point-of-care (POC) diagnostics, as reviewed previously (Yager, Edwards et al. 2006; Mark, Haeberle et al. 2010; Gulliksen, Keegan et al. 2012).

As a part of the consortium of the BMBF funded SONDE project, the Institute of Microsystems Engineering (IMTEK) from the University of Freiburg has developed a state-of-the-art LOC based integrated MDx platform. The idea was to integrate the steps of molecular diagnostics: enrichment of pathogens, sample preparation, target amplification, and detection on one or more chips. These chips are fabricated using glass wafers and platinum electrodes, as described previously (Vulto, Dame et al. 2010; Podszun, Vulto et al. 2012). The microfluidic structure and phaseguides were formed within dry film resist by lamination and lithographic techniques. The concept of this LOC and fabricated chip are presented in Figure A-1. The whole workflow of diagnostic methods can be performed using one or more chip modules, which can be later fabricated as a single module.

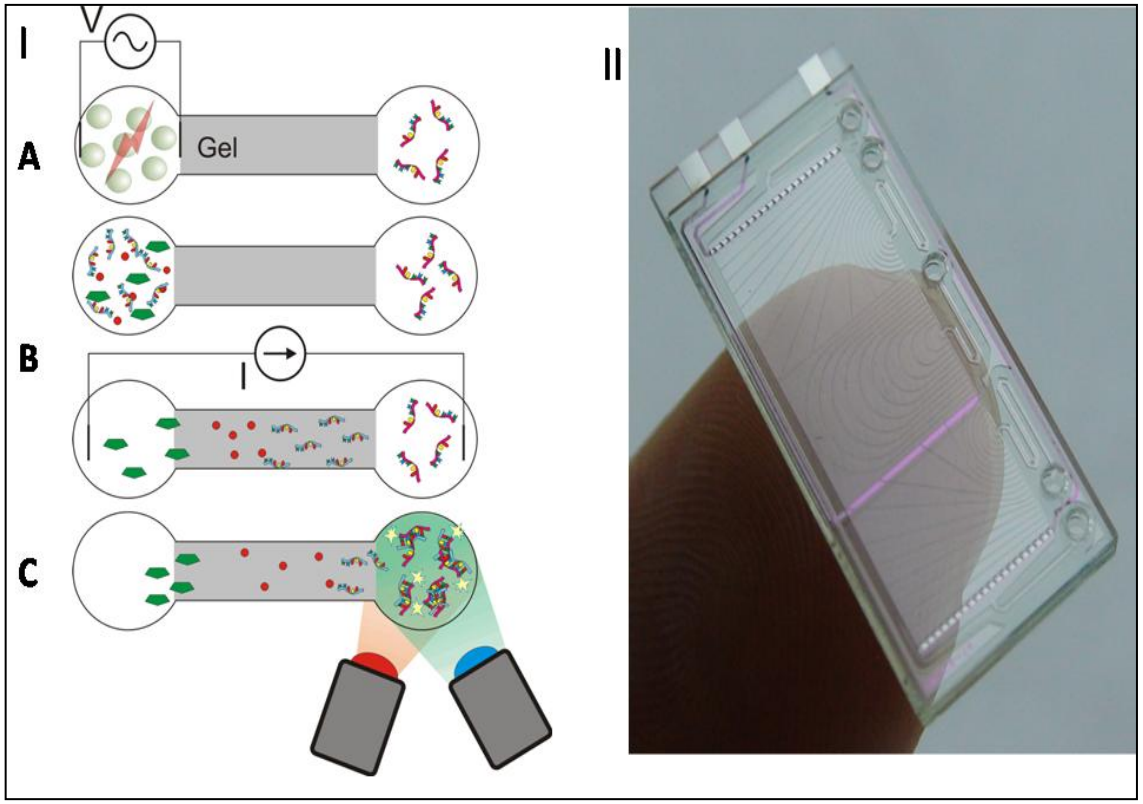


Figure A-1. The Lab-on-chip platform developed by IMTEK, University of Freiburg. I) The concept of LOC is presented in three diagnostic steps; A: Sample preparation by thermo-electrolysis of pathogens after enrichment and purification of nucleic acids on a small poly-acrylamide gel by on-chip gel electrophoresis, B: Amplification of the target region by Polymerase Chain Reaction (PCR) or isothermal amplification methods, C: Detection of amplified products by fluorescence measurement. II) The photograph of the microfluidic chip including phaseguide structures, different square chambers, platinum electrodes, and inlet and outlet connections (Source: IMTEK, University of Freiburg; (Vulto, Dame et al. 2010)).

The LOC technology by IMTEK is a hybrid device, which integrates fluidic and electronic components on the same chip, allowing precise liquid handling and sample testing (Fig. A-1). Physical properties of bacteria and viruses are used to trap them to one electrode according to their surface charges. Subsequently the trapped agents are lysed by thermo-electrolysis without need of any chemical reagents or enzymes. After lysis, nucleic acids from the samples are fragmented and purified by on-chip gel electrophoresis within 10 min. Finally, the purified nucleic acids are analyzed using PCR or isothermal amplification methods by on- or off-chip testing. The whole process of LOC testing can be done using a disposable chip and a portable LOC device.

A.4 CENTRIFUGAL LABDISC PLATFORM FOR NUCLEIC ACID TESTING

Another type of LOC system, which uses centrifugal forces for liquid handling, mixing, metering, and initiating reactions, is referred as a lab-on-disc (LabDisc) platform. In the past 10 years, the centrifugal LabDisc platform has been intensively developed for biomedical applications, and progress in this technology has made it one of the most promising biological analysis platforms in molecular diagnostics (Gorkin, Park et al. 2010; Siegrist, Amasia et al. 2010).

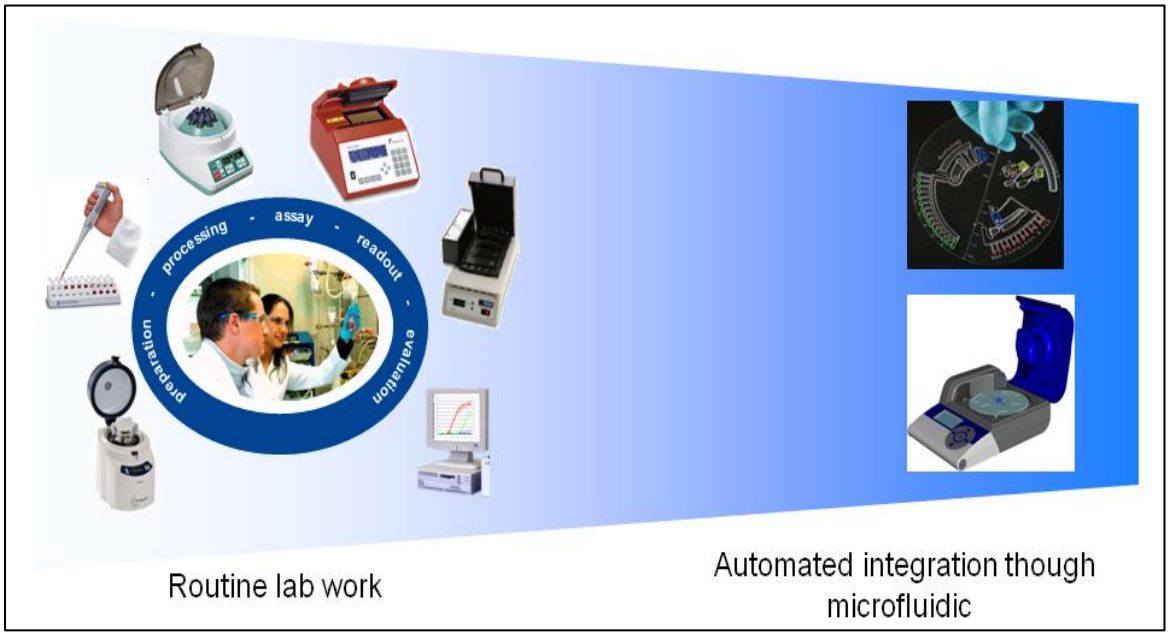


Figure A-2. Concept and vision of the LabDisc platform. NAT and protein testing protocols can be adapted and integrated on a LabDisc cartridge. By use of a LabDisc cartridge with the portable SONDE player device, the routine laboratory workflow molecular diagnostics can be performed automatically under low-resource settings. (Source: HSG-IMIT, Freiburg and Qiagen Lake Constance, original picture is modified and adapted to this work).

In contrast to common state-of-the-art LOC systems, the centrifugal LabDisc platform offers many advantages such as minimal instrumentation, simple liquid handling due to passive components and centrifugal forces, compact microfluidic design and automation and integration of different processes. Piccolo system by Abaxis, Gyrolab workstation of Gyros and GeneDisc system by Pall are some of the examples of commercial diagnostic devices based on a centrifugal microfluidic platform.

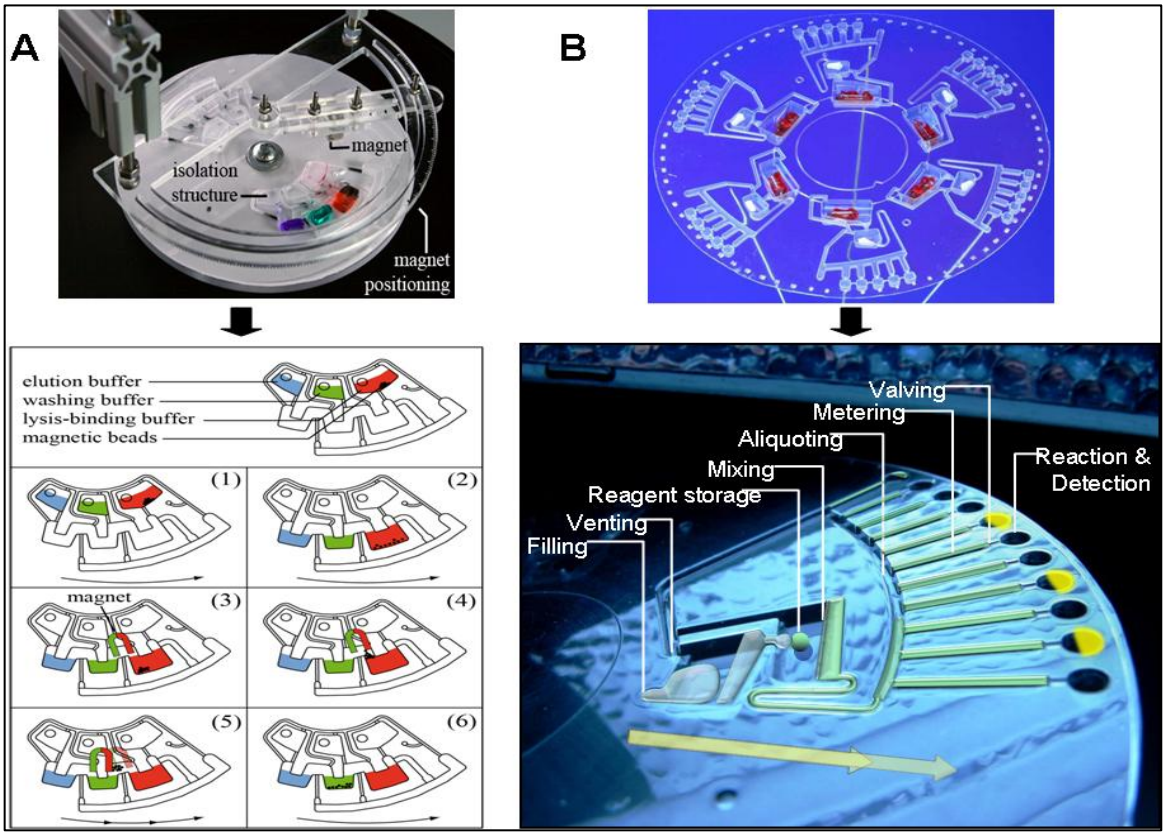


Figure A-3. An Overview of Centrifugal LabDisc platform A) A foil cartridge mounted to centrifugal test set up and schematic depiction of magnetic beads (MAGs) transport sequence. MAGs were transported from one chamber to another by using a permanent magnet attached to fix position by spinning the disk. B) A foil cartridge assembled with liquid reagent containers and lyophilized reagents featuring 6 fluidic structures and scheme of the fluidic processing steps in the foil cartridge. (Source: HSG-IMIT, Freiburg; (Focke, Stumpf et al. 2010; Lutz, Weber et al. 2010)).

As part of the SONDE project, a centrifugal LabDisc platform was developed by HSG-IMIT, Freiburg, as an integrated MDx platform for rapid detection of infectious diseases. This platform consists of a disposable LabDisc cartridge, in which the integrated biochemical analysis such as NAT and protein analysis can be performed automatically

(Figure A-2). The LabDisc is adapted from the common pharmaceutical blister packaging technology and fabricated using a biocompatible Cyclo Olefin polymer (COP) foil by micro-thermoforming as described previously (Focke, Stumpf et al. 2010; Focke, Stumpf et al. 2010). The fabricated LabDisc cartridge contains microfluidic channels and reaction chambers. There are no active components such as pumps and valves as the fluid flow is controlled by centrifugal forces. All microfluidic operations like liquid transport, metering, aliquoting and mixing of reaction and sample separation are simply controlled by the spinning frequency of the LabDisc (Fig. A-3). For cartridge processing, a portable device the “SONDE player” was developed by the company Qiagen Lake Constance (a partner in the SONDE consortium), which includes a disc motor, magnets for bead transport, heating coil and fan for biochemical assays, detectors for a fluorescence and luminescence detection, and the software . This device is small and portable, making it a suitable device in combination with the LabDisc platform for point-of-care diagnostics.

A.5 AIM OF THIS STUDY

The increased threat of infectious diseases and bioterrorist incidents pose danger to public. In response to such a threat, a system for rapid detection and identification of biothreat agents is needed. Therefore, the SONDE project intends to develop a suitcase-size platform for the detection of category A biothreat pathogens by nucleic acid and antigen detection. The consortium of the SONDE project unifies leading competences in the microsystems technology and the molecular diagnostics. There are seven project partners from research institutes (Centre for biological security (ZBS), Robert Koch Institute (RKI); Institute of Virology at University of Gottingen (UMG); Department of Microsystem technology (IMTEK & HSG-IMIT), Freiburg; Centre for material research Freiburg (FMF) and Institute of Molecular Medicine and Cell research (IMMZ) and industry (Qiagen Lake Constance). The microfluidic platforms offer a great solution for adapting a NAT onto an automated platform by eliminating the need of laboratories with a highly complex MDx instrument. Therefore, the SONDE consortium focuses on the research, design, and development of microfluidic platforms for the rapid detection of biothreat pathogens.

The aim of this work was to develop and establish the following molecular methods, which can be later integrated into the bio-analytical systems (the Lab-on-chip platform and the centrifugal LabDisc platform).

- Development of a virus model system for on-chip enrichment analysis on the Lab-on-chip platform

- Development of an enrichment method for viral pathogens from large volume samples
- Development of a sample preparation method for the biothreat agents
- Establishment and optimization of nucleic acid detection protocols for the biothreat agents (real-time PCR and real-time RPA assays)
- Testing of the final “sample to answer” MDx platform

These methods should also fulfil the following important criteria: easy to perform, minimal use of instruments, ambient condition for reagent storage, scalable reagent volume and time effectiveness. Furthermore, the developed integrated MDx platform should be optimized to obtain a “sample to answer” diagnostic system for biothreat pathogens and should be compared to the manual laboratory and commercially available systems.

B MATERIALS AND METHODS

B.1 CELL CULTURE

B.1.1 CULTIVATION OF ADHERENT CELLS

The most common method of cell culture is cultivation in a cell culture flask. Depending on the cell line, the cultivation conditions and medium compositions, different types of cell culture flask are used. Eukaryotic cell lines and one insect cell line were used for the experiments. Vero E6 and Vero B4 cells were cultivated at 37 °C and 5 % CO_2 using D-MEM media with 5 % fetal calf serum (FCS) and 1 % glutamine. PS cells were cultivated at 37 °C using L-15 media with 5 % FCS and 1 % glutamine. In contrast to eukaryotic cell lines, C6/36 cell line was cultivated at 28 °C using L-15 media with 5 % FCS.

Frozen cells were thawed in a water bath at 50 °C, and cells were resuspended in 9 ml fresh cell culture media. Cells were centrifuged at 1000 x g, and cell pellet was resuspended in fresh 20 ml of cell culture media. Cells were then cultivated in a cell culture flask (175 cm^2) and placed in an appropriate incubator according to cell culture media, and cultivated for 3 to 5 days. Cell sub culturing must be done at a particular frequency to maintain cell lines. At a density of 90 %-100 %, medium was removed from the cell culture flask, and cells were washed with PBS. Trypsin was applied to the cell for the detachment of cells from their matrix and each other. Excessive trypsin was removed from the cells and the flask was incubated at 37 °C until the cells dissociated from the flask surface. Fresh medium was then added to the cells and the cells were resuspended by pipetting. Cells were sub-cultured in a new flask at normal inoculation density, and incubated at 37 °C with or without 5 % CO_2, depending on the medium.

The cultivation of C6/36 cell line differed from usual adherent cell culture. At a density of 90 %-100 % cells, the media were removed from the cells, and fresh media was added. C6/36 cells were removed from the cell flask surface using a cell scraper. Cells were diluted in order to maintain log phase growth. After splitting, cells were incubated at 28 °C. Cell culture were regularly tested for mycoplasma contamination by mycoplasma PCR.

B.1.2 ESTIMATION OF CELL NUMBER

The cell number was estimated by direct microscopic measurement. 10-20 µl cell suspension was applied to a Neubauer counting chamber, and four outer cell chambers were counted directly by microscope. The counted cell number was multiplied by factor

of ¼* 10^4 to calculate the initial cell number per milliliter. If the cell suspension was diluted, a dilution factor was taken into account.

B.2 CLASSIC VIROLOGY METHODS

B.2.1 VIRUS CULTIVATION AND INACTIVATION

Most of the viral pathogens used in this study were obtained as inactivated material. Yellow fever virus 17D (YFV 17D), Camelpox virus (CP 19), Rift valley fever virus (RVFV) and Sigma virus (SigV) were cultivated under BSL-2 or BSL-3 conditions according to the relevant biosafety requirements. YFV 17D and RVFV were cultivated in Vero E6 cells, whereas CP 19 was cultivated in HEp-2 cells. Sigma virus was cultivated in C6/36 cells.

For virus cultivation, 50-100 µl of the virus stock solution was added to a monolayer of cells. Cells were incubated for up to 6 days at optimal growth conditions and observed over time for the cytopathic effects (CPE). The cell culture was harvested when CPE were clearly visible and 60-80 % of the cells were dying. Cells with cell culture supernatant were centrifuged at 4000 x g, and the supernatant was stored at minus 80 °C.

For virus inactivation, virus containing cell culture supernatant was heated to 56 °C for 1 h and subsequently gamma-irradiated at 40 kilo Grey.

B.2.2 PLAQUE ASSAY FOR TITRATION OF VIRUS SUSPENSION

The plaque assay is a method to determine the total number of infectious virus particles in any given virus suspension. The infectious units are presented as plaque forming units per ml (PFU/ml).

For determination of the virus titer, serial dilution of virus suspension was prepared and seeded to a defined quantity of cells. The virus spreads from infected cells to adjacent cells. After attachment of cells to the flask surface and viral infection, the suspension was overlaid by viscous carboxymethyl cellulose (CMC) solution preventing further diffusion of viruses. After a certain time duration, so-called plaques are formed on the cell monolayer because of the cytopathic effect of the virus on cells. Each plaque is formed from a single infectious virus particle thus allowing an accurate calculation of the virus titer. These plaques can be detected visually. The infectivity titer is expressed in PFU/ml and obtained by the following equation:

$$Virus\ titer = Plaque\frac{number}{Volume*Dilution}\ PFU/mL.$$

For a YFV plaque assay, accurate 10 fold serial dilution of virus suspension was prepared in L-15 cell culture media. A suspension of 200 µl PS cells at a concentration of $6*10^5$ cells/ml was seeded in each well of 24 well plate. The same amount of virus dilution was added to each well; then the plate was incubated at 37 °C for 4 h. After 4 h, 400 µl of CMC overlay media (1.6 % CMC in L-15 cell culture media) was added to each well. For negative controls, the same amount of media was added to the well instead of virus dilution. The well plate was incubated for 4 days at 37 °C. After 4 days incubation, the media was removed from the well plate and cells were fixed with 3.7 % formaldehyde for 15 min. The plaques were stained with a naphthalene black solution for 20 min. Plates were dried; then plaques counted directly.

In contrast to the plaque assay of YFV, the plaque assay for RVFV and CP 19 virus was performed using 200 µl of Vero E6 cells at a concentration of $1.6*10^6$ cells/ml. The virus dilutions and CMC media were prepared in D-MEM cell culture media. All other procedures were as described above.

B.2.3 $TCID_{50}$ ASSAY FOR TITRATION OF VIRUS SUSPENSION

Many viruses do not cause plaques in the plaque assay. In this case, Tissue Culture Infection Dose 50 ($TCID_{50}$) method is used to determine virus titer based on detection of cytopathic effects caused by viruses in cell culture. $TCID_{50}$ is also a simple method to quantify virus titer. It is defined as the dilution of virus that is needed to infect the 50 % of inoculated cells. Usually the $TCID_{50}$ assay is performed in a 96 well plate. In case of pure cytopathic effect (CPE), plaques can be stained by the naphthalene black solution. Calculation of $TCID_{50}$ is based on a counting of positive infected wells. These data can be used to determine $TCID_{50}$ of the virus suspension by following equation of the Spearman-Kärber method:

$$TCID50 = Highest\ dilution\ giving\ 100\%\ \boldsymbol{CPE} + \frac{1}{2} - \frac{Total\ number\ of\ test\ results\ showing\ CPE}{numberof\ test\ units\ per\ dilution}.$$

Virus titration of Ljungan virus was performed on Vero B4 cells in a 96-well plate by using 10-fold serial dilutions of the sample from 10^{-1} to 10^{-8} according to standard procedures. 100 µl of $1*10^4$ vero B4 cells/ml were plated into a 96-well plate and 100 µl of virus dilutions were added to each well in quadruplet. Plates were inoculated at 37 °C and 5 % CO_2 for 10 days. After 10 days, the media were removed and cells were fixed with 3.7 % formaldehyde per well for 15 min. Formaldehyde was then removed, and cells

were stained with naphthalene black. The virus titer in $TCID_{50}$ was estimated by determining the virus dilution and counting the number of wells showing cytopathic effects by the Spearman-Kärber method.

B.2.4 VIRUS PURIFICATION BY ULTRACENTRIFUGATION

The virus supernatant of YFV 17D and CP 19 were purified by ultracentrifugation to obtain concentrated and purified virus particles. YFV 17D virus supernatant (180 ml) was concentrated by ultracentrifugation through 45 ml of 25 % sucrose, prepared on phosphate-buffered saline (PBS), in a Beckman type 19 rotor using a 250 ml vessel at 19,000 rpm and 4 °C for 14 h. CP 19 virus supernatant (180 ml) was concentrated by ultracentrifugation through 45 ml of 30 % sucrose, prepared on PBS, in Beckman type 19 rotor using a 250 ml vessel at 18,000 rpm and 4 °C for 3 h. In both cases, the supernatant was removed from the vessels and the virus pellet was dissolved in 1 ml of Tris pH9 buffer overnight at 4 °C. then virus suspensions were stored at -80 °C.

B.2.5 LABELING OF VIRUSES

The enveloped particles of YF 17D and CP 19 particles primarily contain proteins, lipids and viral nucleic acids. Lipid membranes and structural proteins of the viruses can be labeled with lipophilic carbocyanine dyes $DiOC_6(3)$. To stain the viral particles, suspensions containing virus particles were incubated with a $DiOC_6(3)$, 10 µM final concentration and incubated at room temperature for 20 min, after which they were subjected to 18,000x g centrifugation for 10 min (CP 19) and 45 min (YFV 17D). The supernatant was removed, and the virus pellet was rinsed in distilled water and centrifuged again at 18,000x g for 10 or 45 min. The virus pellet was then resuspended in distilled water and kept at 4 °C until further use.

B.3 SAMPLE PRE-TREATMENT

B.3.1 VIRUS ENRICHMENT BY USING LAB-ON-CHIP TECHNIQUES

Macro- to micro-volume concentration of viable bacteria was successfully performed in a microfluidic chip (Podszun, Vulto et al. 2012). The enrichment principle is based on free flow electrophoresis and is demonstrated for Gram positive bacteria. Bacteria from a suspension flow were trapped on a gel interface that separates the trapping location from integrated actuation electrodes in order to enable non-destructive trapping (Fig B-1). This method was tested to concentrate viruses on the chips. The chips were fabricated using the full wafer fabrication process, as described previously (Vulto, Podszun et al. 2011).

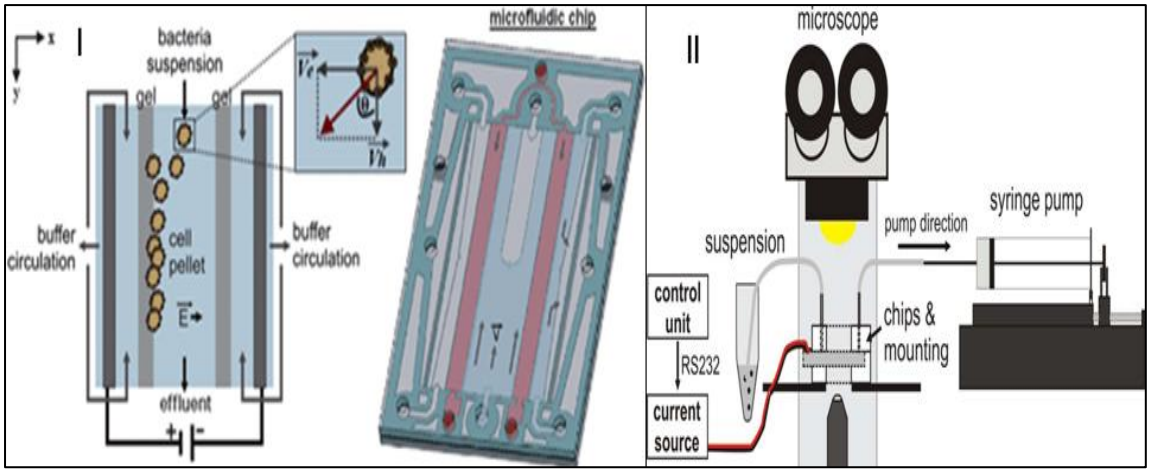


Figure B-1. Principle of electrophoretic trapping of biological agents on Lab-on-chip and experimental setup: I) A bacterial/virus suspension was pumped vertically through the chip and an electric field E applied laterally. Bacteria and viruses exhibit a negative surface charge and thus can be electrophoretically captured from a continuous flow II) Chips were placed in amounting on an inverted fluorescence microscope. The inlets and outlets were connected via silicone tubes to Eppendorf tubes and a syringe pump. (Source: IMTEK, Freiburg; (Podszun, Vulto et al. 2012)).

To concentrate viruses on the chip, the chips were first filled with polyacrylamide gel and placed in a holder on an inverted fluorescence microscope. The experimental setup is schematically illustrated in Figure B-1. Purified suspension of YFV 17D and CP 19 were labeled with $DiOC_6(3)$ dye as described above. Labeled virus suspension (100 µl) was injected continuously using a syringe pump at a flow rate of 3 µl/min over 30 min. The chip was provided with a constant current of 123 µA. Accumulation of viruses at the electrode was observed by fluorescence microscopy. After 30 min, the accumulated viruses were resuspended in the buffer by inverting the current for 1 min. Viruses were recovered from the chip with a pipette and further analyzed by molecular techniques to determine the enrichment factor.

B.3.2 VIRUS ENRICHMENT BY USING POLYMER

Enrichment of pathogens were performed using InRICHMENT Virus Reagents from InRICHMENT Virus DNA/RNA kit (Analytik Jena, Jena) according to the manufacturer's instructions. To enrich viruses from cell culture media or patient samples like plasma, serum or urine, 1 ml of sample volume was treated with InRICHMENT virus reagents. Briefly, 20 µl of reagent 1(containing polymers) was added to the sample and the tube was inverted to bind the polymer to the viruses. Reagent 2 (precipitation reagent) was added and the sample was vortexed, leading to polymerization of the polymer. The polymer-virus complex was collected by centrifugation at 12,000 rpm for 2 min. The

supernatant was discarded and the pellet of polymer-virus complex was resuspended in 50-100 µl of reagent 3. The proteinase K was not added, so samples were not lysed after enrichment.

B.4 PURIFICATION OF NUCLEIC ACIDS

B.4.1 PURIFICATION OF TOTAL NUCLEIC ACIDS FROM BACTERIAL AND VIRAL PATHOGENS

For conventional extraction of DNA/RNA from 200 µl bacteria or virus samples, Chemagic NA body fluid kit, innuPREP virus DNA/RNA kit or Instant MP basic kit were used according to the manufacturer's instructions. The DNA/RNA was eluted in 60-100 µl of RNase free sterile water.

B.4.2 PURIFICATION OF NUCLEIC ACIDS FROM LARGE VOLUME SAMPLE UP TO 1 ML

For extraction of viral RNA from 1 ml samples the following kits were used according to the manufacturer's instructions: QIAamp UltraSens Virus kit (Qiagen, Hilden, Germany), High Pure Viral Nucleic Acid Large Volume Kit (Roche Diagnostics, Mannheim, Germany) and InRICHMENT Virus DNA/ RNA Kit (Analytik Jena AG, Jena, Germany). RNA was eluted in 60 µl RNase free sterile water.

B.4.3 PREPARATION OF PLASMID DNA ON SMALL SCALE

Alkaline lysis is a basic method for any kind of DNA preparations. Qiagen Miniprep kit is used for plasmid DNA preparation. Mini scale preparation is very fast and can yield large quantities of DNA. Briefly, a pellet was recovered from a 2 ml overnight bacterial culture and resuspended in 250 µl solution A and solution B subsequently. This preparation was mixed up thoroughly and incubated for 30 seconds to lyse bacteria. Solution C was added and mixed up. This solution is centrifuged for 5 min, and clear supernatant was applied to a spin column. Plasmid DNA binds to the spin filter during incubation of 1 minute. After centrifugation of the spin column, 750 µl wash buffer was added to remove other contaminants. Finally, the plasmid DNA was eluted with 50-100 µl elution buffer (TE/ DNase-free RNase) and stored at -20 °C.

B.4.4 DETERMINING THE CONCENTRATION OF NUCLEIC ACIDS SOLUTION

A measurement of the optical density at 260 nm is a simple and fast method to determine the concentration of nucleic acids in a solution. This measurement requires a photometer with an ultraviolet (UV) lamp. The concentration of nucleic acids can be

calculated from the absorbance measured at 260 nm, the dilution factor, and a specific multiplication factor. The multiplication factor is specific to RNA or DNA.

$$Concentration\ \frac{\mu g}{ml} = OD260 * Dilution\ factor * Multiplication\ factor$$

Information about protein contaminants can be obtained from the ratio of OD_{260} to OD_{280}, and exact calculation of nucleic acid is possible. A nucleic acid measurement was carried out by biophotometer with the use of an ultra-micro cuvettes or nanodrop. It is not possible to measure concentration below 100 ngusing very low volume.

B.5 NUCLEIC ACID AMPLIFICATION AND DETECTION

B.5.1 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

The reverse transcription of RNA into cDNA was performed using the reverse transcriptase innuScript RT enzyme. In brief, 10 µl RNA was mixed with 5 µl of RT-master mix containing 1.5 µl of 10 x RT-buffer, 4 µM of dNTPs, 1.4 mM of DTT, 1 µM of random primer and RT-enzyme. The temperature parameters were set for 30 min at 55 °C and 10 min at 95 °C on a thermal cycler (Analytik Jena AG/Biometra, Jena, Germany).

For cDNA synthesis using the SuperScript™ II RT enzyme, 11.6 µl purified RNA was added in a 20 µl reaction volume with 4 µl 5× First-Strand Buffer (Invitrogen, Karlsruhe, Germany), 4 µl of 0.1 M DTT (Invitrogen), 0.4 µl of dNTPs (25 mM each) and 1 µM of random primer. The template RNA and primer were heated up to 65 °C for 5 min and rapidly cooled to 4 °C. After addition of 1 µl of SuperScript™ II RT (200 U/µl) (Invitrogen), samples were incubated for 60 min at 42 °C for reverse transcription. The reaction was stopped by enzyme inactivation at 75 °C for 15 min and cooled to 4 °C. The cDNA were stored at −20 °C until further use.

B.5.2 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a simple method for the amplification of a target DNA fragment. PCR is widely used in clinical laboratories for molecular detection of pathogens. For amplification of DNA or cDNA, PCR was performed using the Platinum Taq polymerase in a total volume of 25 µl containing PCR buffer, primer pair, magnesium chloride, primers and 2.5 µl of template DNA (Table B-2). Thermal cycling was done on a PCR cycler under the following conditions: 15 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C; 10 min at 72 °C. After PCR reaction, the products were analyzed by gel electrophoresis. The used primers are listed in Table B-1.

Table B-1. List of oligonucleotides used for PCR

PCR system & name of oligonucleotide		Primer & Probe	Sequence in orientation 5´-> 3´	Tm (°C)
Cloning, sequencing	M13 F	Forward	gTAAAACgACggCCAgT	50
	M13 R	Reverse	AACAgCTATgACCATg	50
Promoter primer	SP6	Forward	ATTTAggTgACACTATAg	60
Promoter primer	T7	Reverse	TAATACgACTCACTATAggg	60
Mycoplasma testing	GPO-3	Forward	gggAgCAAACaggAtTagATACCCT	60
	MGSO	Reverse	TgCACCATCTgTCACTCTgTTAACCTC	60
Internal control	GAPDH F	Forward	CCATggAgAAggCTggggCT	60
	GAPDH R	Reverse	ggTggTgCAggAggCATTgCT	60

Table B-2. PCR reaction mixture and cycler conditions

Reagents	Volume [µL]	Time	T [°C]	cycle
dest. Water	13.4	10 min	94	
10 x Taq-Buffer	2.5	30 sec	94	40 cycles
dNTPs (10 mM)	0.5	120 sec	50-60	
$MgCl_2$ (50 mM)	0.75	30 sec	72	
Forward Primer (10 µM)	0.5	10 min	72	
Reverse Primer (10 µM)	0.5	Hold	4	
Platinum®*Taq* (5 U/µL)	0.1			
DNA	2.5			

B.5.3 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is the simplest and most common method for separating and identifying DNA fragments of different sizes (0.5 to 25 kb fragments). Depending on the size of the fragments, an agarose gel of specific concentration (1 %- or 2 %- agarose gel) was prepared to separate DNA fragments effectively. Agarose was heated with electrophoresis buffer, until it dissolved completely and then gel was spread in a gel tank with a comb. After the gel becomes harder, it was placed into a flow migration chamber. The gel was then covered with electrophoresis buffer. The DNA solution was pipetted into sample wells and a voltage was applied. The DNA migrates through the gel under application of the electrical field. Ethidium bromide was added as a DNA staining dye directly in the agarose gel before pouring it into the gel tank. After sufficient migration, the gel was observed under UV light.

B.5.4 COLONY PCR

Colony PCR is a method to determine the correct insertion of a cloning fragment into a plasmid in a bacterial clone. The clones were selected with tips and directly dipped into

PCR master mix (Table B-3). The same clones were also plated onto an agar plate as a backup. Primers were chosen for colony PCR depending on the plasmid. Cloning of the insert into the plasmid was confirmed by observation of the expected fragment size of the insert by the gel electrophoresis.

Table B-3. Colony PCR reaction mixture and cycler condition

Reagents	Volume [µL]	Time	T [°C]	cycle
dest. Water	20.0	10 min	94	
10 x Taq-Buffer	2.5	30 sec	94	35 cycles
dNTPs (10 mM)	0.5	30 sec	60	
$MgCl_2$ (50 mM)	0.75	1 min	72	
Forward Primer (10 µM)	0.5	10 min	72	
Reverse Primer (10 µM)	0.5			
Platinum®*Taq* (5 U/µL)	0.2			
DNA	-			

B.6 QUANTITATIVE TAQMAN REAL-TIME PCR

Quantitative real-time PCR (real-time qPCR) is a modern method to quantify nucleic acids. In a TaqMan PCR, a fluorescence labeled probe is digested by the 5´ to 3´exonuclease activity of the DNA polymerase to produce a fluorescence signal. In order to digest the probe, it must hybridize entirely to the amplified target DNA. Primer and probe sequences used in this project are listed in Table B-4. Real-time qPCR was performed using the Platinum Taq polymerase in a total volume of 25 µl containing 5 µl of cDNA or DNA, 2.5 µl of 10 x Reaction Mix, 4 mM $MgCl_2$, 400 nM of each primer, 100 nM of probe and 2.5 µM of dNTPs. Thermal cycling was done on a Stratagene Mx3000 or ABI 7500 cycler instrument under the following conditions: 15 min at 95 °C; 45 cycles of 15 s at 95 °C and 30 - 60 s at 60 °C (Table B-5). To quantify genomic copies, a ten-fold serial dilution of the standard plasmid (10–10^6 copies/µl) was tested within the same sample run. Data analysis was performed by the software provided with the instrument.

For quantification of the genomic RNA of pathogens, real-time qPCR can be used in either a two-step or a one-step format. In the two-step format, cDNA is first synthesized from RNA; then cDNA is used as a template for real-time RT-qPCR. In the one-step format, RNA is directly used as the template. In this case the reagents for real-time PCR contain an RT-enzyme, so cDNA synthesis and real-time PCR are performed in a single reaction.

Table B-4. List of oligonucleotides and probes used in this study

Pathogen	Target gene	Primer & Probe	Sequenz 5´->3´	Tm
Orthopox virus	IL3 gene	OPV 12/13 F	GCCAATTGTCTTTCTCTTTTACTGA	60° C
		OPV 12/13 R	GAAAACATTTAAGGATGAATCCATCT	
		OPV TMGB	FAM-CCTTCTATAgATCTgAgAAT NQF MGB	
Rift valley virus	S segment	RVF FP	TGCCACGAGTYAGAGCCA	60° C
		RVF RP	TTGAACAGTGGGTCCGAGA	
		RVF P	FAM-TCCTTCTCCCAGTCAGCCCCAC-BHQ	
Ebola virus zaire	NP gene	ENZ FP	ATGATGGAAGCTACGGCG	60° C
		ENZ RP	AGGACCAAGTCATCTGGTGC	
		ENZ P	FAM-CCAGAGTTACTCGGAAAACGGCATG-BHQ1	
Ebola virus sudan	NP gene	ENS FP	TTGACCCGTATGATGATGAGAGTA	60° C
		ENS RP	CAAATTGAAGAGATCAAGATCTCCT	
		ENS P	FAM-CCTGACTACGAGGATTCGGCTGAAGG-BHQ1	
Marburg virus	NP gene	MN FP	CAATTCCACCTTCAGAAAACTG	60° C
		MN RP	GCTAATTTTTCTCGTTTCTGGCT	
		MN P	FAM-CACACACAGTCAGACACTAGCCGTCCT-BHQ1	
Sigma virus	G gene	SIGV FP	gTgACATTCCAAgTAACTgATT	56° C
		SIGV RP	CAACggCAgTTTggATA	
		SIGV P	Cy5-CCCTCCgTgTCCTCCCggTACC-BBQ	
Bacillus antracis	PagA (pXO1)	Pag-F	CggATCAAgTATATgggAATATAgCAA	60° C
		Pag-R	CCggTTTAgTCgTTTCTAATggAT	
		Pag-TM	FAM-CTCgAACTggAgTgAAgTgTTACCgCAAAT-BBQ	
Bacillus antracis	CapC (pXO2)	Cap-F	ACgTATggTgTTTCAAgATTCATg	60° C
		Cap-R	ATTTTCgTCTCATTCTACCTCACC	
		Cap-TM	FAM-CCACggAATTCAAAAATCTCAAATggCAT-BBQ	
Francisella tularensis	FopA	Ft 821 F	TTgggCAAATCTAgCAggTCA	60° C
		Ft 921 R	ATCTgTAgTCAACACTTgCTTgAACA	
		Ft 851 TM	FAM-AAgACCACCACCAACATCCCAAgCA XT-p	
Yersinia pestis	Pla	Yp Pla F	gCTTTATgACgCAgAAACAgg	60° C
		Yp Pla R	CTgTAgCTgTCCAACTgAAACg	
		YP Pla TM	FAM-ATATgACCTCAATgTgAAAggCTggTTACTCCXTp	
Yellow fever virus 17D	NS3	YFV NS3 F	GAGCGACAGCCCCGATTTCT	60° C
		YFV NS3 R	AGGTCCAGTTGATCGCGGC	
		YFV NS3 TM	FAM-TggTCAACgTCCAgACAAAACCgAgCxTTg-PH	
Yellow fever virus	5´-UTR	YFV all F	gCTAATTgAggTgYATTggTCTgC	60° C
		YFV all R	CTgCTAATCgCTCAAMgAACg	
		YFV all TM	FAM-ATCgAgTTgCTAggCAATAAACAC-TAMARA	
Ljungan virus	5´-UTR	LV F	gCggTCCCACTCTTCACAg	60° C
		LV R	gCCCAgAggCTAgTgTTACCA	
		LV TMGB	FAM-TgTCCAgAggTgAAAgC-MGB-NFQ	

FP: forward primer, RP: reverse primer, MGB: minor groove binding, BBQ, BHQ1: Black hole quencher, Ph or p: phosphate, Tm: melting temperature, FAM/TAMARA/Cy5: fluorescence dye

For quantification of the RNA of pathogens, QuantiTect virus kit, Transcriptor One-Step RT-PCR Kit or AgPath-ID One-Step RT-PCR Kit were used according to the manufacturer´s instructions. When commercial kits were used for one-step real-time RT-qPCR, the primer and probe concentrations and protocol were optimized and adapted to the reagents. Either published assays from project partners or in-house real-time qPCR were used in this study (Table B-4) (Ellerbrok, Nattermann et al. 2002; Weidmann, Muhlberger et al. 2004; Weidmann, Sanchez-Seco et al. 2008; Kramski, Drozd et al. 2011).

Table B-5. Real-time qPCR reaction mixture and cycler conditions

Reagents	Volume [µL]	Time	T [°C]	cycle
Dest. Water	14.05	10 min	95	
10 x Taq-Buffer	2.5	15 sec	95	45 cycles
dNTPs (10 mM)	0.5	30 sec	60	
$MgCl_2$ (50 mM)	0.75	10 min	72	
Forward Primer (10 µM)	0.75			
Reverse Primer (10 µM)	0.75			
Probes (10 µM) 0.25 each	0.25			
Platinum®*Taq* (5 U/µL)	0.2			
DNA	5.0			

B.7 RECOMBINASE POLYMERASE AMPLIFICATION FOR NUCLEIC ACID AMPLIFICATION

Recombinase Polymerase Amplification (RPA) was used as an isothermal amplification method and alternative approach to PCR for nucleic acid amplification. There are different commercial RPA kits (TwistDx, Cambridge, UK) available with options for electrophoresis, fluorescent and lateral flow detection methods and for DNA and RNA targets. In contrast to this, the real-time RPA assay was used to detect the DNA amplification from target DNA or RNA by real-time fluorescence measurements using a fluorescence reader.

B.7.1 REAL-TIME RPA ASSAY

The real-time RPA assay was performed using either the TwistAmp exo kit or the TwistAmp exo RT kit according to manufacturer's instruction. The TwistAmp exo RT kit contains in addition to the RPA reagents, an RT-enzyme which provides the DNA amplification from RNA targets. In final experiments, only the TwistAmp exo RT kit were used for DNA amplification from both DNA and RNA targets. Briefly, 37.7 µl of rehydration solution was mixed with 2.1 µl of each primer (10 µM) and 0.6 µl of target

specific RPA exo probe (10 µM). Master mix (42.5 µl) was then added to the dry reagent pellet and mixed by pipetting the reaction mixture. Template genomic DNA or RNA from the pathogen (5 µl) was added to this mixture. Finally, 3.5 µl of $Mg(OAC)_2$ (280 mM) was added to the solution to start the reaction. Tubes containing reactions were vortexed and centrifuged shortly, and placed in the ESE Quant Tubescanner for real-time monitoring of fluorescence. The reaction was performed at 39 °C for 15-20 min. Data were analyzed by the Tubescanner studio software Version 1.0. All the real-time RPA assays were developed by our project partner from the Department of Virology Göttingen, UMG (Euler, Wang et al. 2012; Euler, Wang et al. 2012; Podszun, Vulto et al. 2012).

B.7.2 Optimization of real-time RPA assays for the LabDisc platform

A real-time RPA assay for the LabDisc was optimized for 10 µl volumes. RPA reactions of 50 µl, 20 µl and 10 µl were prepared as shown in table B-6. To prepare 20 µl and 10 µl of RPA reaction, a master mix for 50 µl of reaction was prepared normally and distributed in 18 µl and 9 µl to tubes. Subsequently, template (DNA/RAN) and water were added to tubes for 20 µl and 10 µl of RPA reactions (Table B-6).

Table B-6. RPA reaction mixture for 50 µl, 20 µl and 10 µl of reaction volumes.

Reagents	50 µl volume	20µl volume	10µl volume
RPA Buffer	36.7	14.68	7.34
Probe TM [10 µM]	0.6	0.24	0.12
Forward Primer [10 µM]*	2.1	0.84	0.42
Reverse Primer [10 µM]*	2.1	0.84	0.42
Magnesium acetate	3.5	1.4	0.7
Master mix	45	18	9
Template DNA/RNA	1	1	1
Dry reagent*	1x	1x	1x
PCR Water	4	1	0
Total	50µl	20µl	10µl

***These parameters were changed for testing 20 µl and 10 µl of RPA reactions.**

Two following parameters were changed and tested for optimization of 20 µl and 10 µl of RPA reaction: 1) Use of double amount of primers and probe 2) Use of 1.5 X dry RPA reagents. All RPA reactions were carried out at 41 °C for 15-20 min using the ESE Quant tubescanner, and data was analyzed by IsoAmp software.

B.8 CENTRIFUGAL LABDISC PLATFORM

B.8.1 TESTING OF REAL-TIME RPA ASSAY ON THE GENESLICE

Optimized RPA assay in a 10 µl reaction volume was tested using the GeneSlice cartridge and the LabDisc platform. The GeneSlice cartridge contains the microfluidic structure for aliquoting, which has eight reaction chambers for nucleic acid testing. Briefly, 73 µl of rehydration solution, 4.2 µl of forward/reverse primer (10 µM each), 1.2 µl of probe (10 µM), 7 µl of $Mg(OAC)_2$ (280 mM) and 10 µl of the DNA/RNA template were mixed to a final volume of 100 µl. Master mix (100 µl) were then added to the 3 X dry reagent pellet and mixed by pipetting the reaction mixture. After this, 90 µl of reaction mixture was pipetted in the GeneSlice and placed in a GeneSlice holder (Fig. B-2).

Figure B-2. The GeneSlice cartridge and holder for the LabDisc platform. The GeneSlice contains the microfluidic structure for aliquoting of the reaction in a 10 µl of reaction in each of the eight chambers. (Source: HSG-IMIT, Freiburg)

The prepared GeneSlice holder was then placed in the SONDE player and the RPA reaction mixture aliquoted in the GeneSlice at a final reaction volume of 10 µl. Eight RPA reactions of 10 µl were simultaneously measured in the SONDE player at a constant temperature of 41 °C for 20-25 min. The Data was finally analyzed by IsoAmp Software.

B.8.2 SAMPLE TO ANSWER TESTING OF LABDISC PLATFORM USING RPA LABDISC CARTRIDGE

The RPA LAbDisc was designed and fabricated as described previously (Focke, Stumpf et al. 2010). Primers and probe of the RPA assays were dried at room temperature on the RPA LabDisc as shown in Figure B-3. Three RPA reagent pellets were then placed in the mixing chamber on the RPA LabDisc and the RPA LabDisc was sealed with a

transparent adhesive foil. The RPA LabDisc was fixed in a LabDisc holder and placed on the SONDE player.

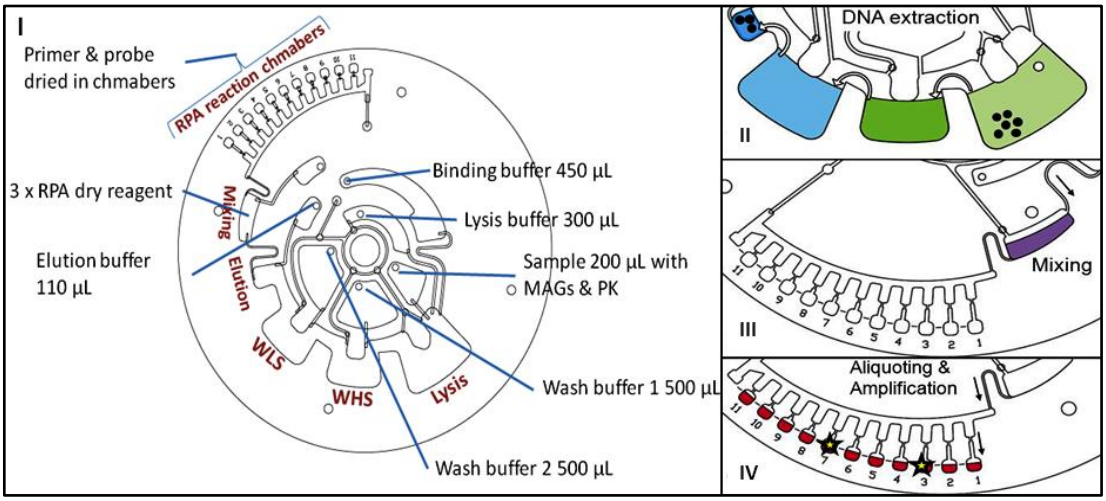


Figure B-3. Microfluidic process flow of the RPA LabDisc. I) Sample with magnetic beads (MAGs), lysis-, binding- and two washing buffers (WHS and WLS) were pipetted into inlets indicated by blue lines. Through spinning of this LabDisc, samples were gated radially outwards, and reagents are transported into appropriate chambers shown in red. II) After lysis, the MAGs were transported through a washing chamber to the elution chamber by magnetic actuation. III) The eluted sample is gated into chambers containing pre-stored dry RPA reagents and mixed. IV) The master mix is aliquted in 10 µl volumes in RPA reaction chambers, where primers and probes for RPA assays are pre-stored.

A test sample of 200 µl was mixed directly with 20 µl of magnetic beads (MAGs) and 20 µl of proteinase K (PK) before processing, and then pipetted into the sample chamber. Lysis buffer, binding buffer, and wash buffers (Instant MP basic kit) were then loaded into the corresponding inlets and automatic processing of the sample was started as described in Figure B-3 I. After the lysis step, nucleic acids were captured by MAGs, which were transported via a bead transport mechanism using fixed magnets. During one minute pause in the RPA LAbDisc protocol, 110 µl of elution buffer comprising PCR grade water, RPA rehydration buffer and $MgO(AC)_2$ were added manually to the elution chamber. After the elution of nucleic acids, the elute was mixed with the dry RPA reagent (Fig B-3 III). Finally, this mixture was aliquoted into 10 µl volumes in chambers containing the pre-stored primers and probes (Fig. B-3 IV). The RPA reaction in these chambers was monitored in the SONDE player using a fluorescence detector at 41 °C for 20-30 min.

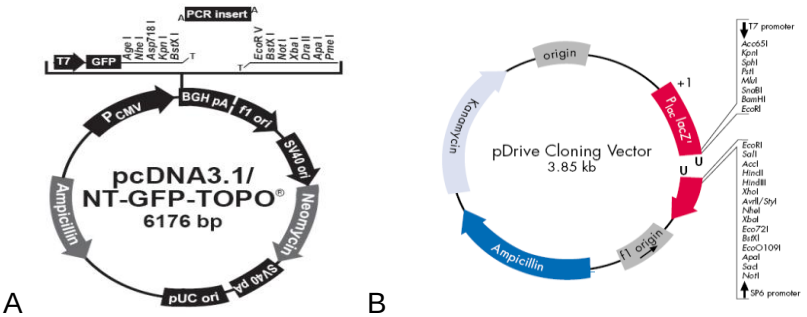
B.9 CLONING OF DNA FRAGMENTS AND PREPARATION OF STANDARD PLASMID FOR PCR

B.9.1 CLONING OF DNA FRAGMENTS INTO PLASMID

DNA fragments prepared by PCR are cloned into an appropriate vector on the base of fragment sizes. In the classical procedure, restriction cleavage sites are introduced at the 5´end of both primers. The PCR product is cut and ligated into a suitable vector. There are many other commercial methods available for cloning of fragments, like TA cloning and TOPO TA cloning, which are reliable and rapid. Both methods are based on Taq Polymerase, which generally produces nonspecific overhangs of one base, in most case adenosine. A vector with a Thymine/Uridin overhang can be constructed to simplify the cloning of DNA fragments with adenosine overhangs. The TOPO TA cloning kit is commercially available from Invitrogen. In this kit, a topoisomerase-activated TA vector is used instead of a ligase. The ligase reaction takes 5 min and is much quicker than conventional T4 ligase reaction. The plasmid constructs are then transformed into competent *Escherichia coli* bacteria with the help of calcium chloride method, as described under B. 10. The bacterial culture is then plated on selective medium. The clones are selected by blue-white screening, or an antibiotic resistance marker depending on the vector constructs.

Table B-7. List of cloning vectors

Name	Type	Sorce
pcDNA3.1/NT-GFP-TOPO-Vector	Eucaryotic vector	Invitrogen, Karlsruhe
pDrive Cloning-Vector	Eucaryotic vector	Qiagen, Hilden



A B

Figure B-4. The map of cloning vectors. A) Cloning vector pcDNA3.1 from Invitrogen. B) Cloning vector pDrive from Qiagen.

The TOPO TA cloning kit from Invitrogen or the PCR cloning kit from Qiagen was used for preparation of the standard plasmids (Table B-7 & Fig. B-4). The fresh PCR products

were cloned into the pDrive cloning vector of the Qiagen PCR cloning kit or Invitrogen TOPO TA cloning kit as described above. An ampicillin resistance marker selected the clones and overnight cultures of clones were prepared for plasmid isolation according to manufacturer´s instruction. Plasmids with inserts were isolated from overnight culture using the Qiagen plasmid mini kit, according to the manufacturer´s instructions.

B.10 TRANSFORMATION WITH THE CALCIUM CHLORIDE METHOD

The calcium chloride method is a classic method of making bacteria competent for transformation. Preserved competent bacteria One Shot Top 10 *E. coli* were used for transformation. 50 µl of competent bacteria One Shot Top 10 *E. coli* were thawed on ice. 450 µl of chilled $CaCl_2$ was then added to the bacteria and the solution was carefully mixed. 5 µl of Ligation mix was added to the 100 µl mix of bacteria, mixed well and incubated for 20 min on ice. After that, the bacteria suspension was heated up to 43.5 °C for 45 s and kept once on ice for 5 min. Super optimal broth (SOC) medium (200 µl) was added to this suspension and incubated at 37°C for 30 min on a shaker. The suspension (50 µl and 200 µl) was cultured on selective LB plates (100 µg/ml Ampicillin, for blue-white screening: 10 µl 1 M IPTG and 40 µl of X-Gal) and incubated overnight at 37 °C.

B.11 DNA SEQUENCING

ABI PRISM Big Dye Terminator Cycle sequencing Ready reaction kit was used for the sequencing of plasmids and PCR products. This sequencing method is known as dideoxy sequencing (also called the chain terminator method or Sanger method). It is based on the use of dideoxynucleotides (ddNTP´s) along with the normal nucleotides. The 3´dideoxynucleotides lack a 3´ hydroxyl group and when it is incorporated into an extending DNA strand, DNA replication cannot continue, as the 3´ hydroxyl group is not available for the addition of further nucleotides. After addition of the dideoxynucleotide, the growing DNA chain is terminated randomly. Many DNA molecules are produced at the same time and this process results in the formation of the DNA molecules, which have the common 5´end, but varying in a specific base at the 3´end. The DNA fragments are separated by polyacrylamide gel electrophoresis and the sequence of newly synthesized DNA can be read.

An automated sequencing method was used to sequence the DNA strands and plasmids. The sequencing reaction was performed with dideoxynucleotides, each labeled with a different receptor dye (Table B-8). Therefore, the reaction was performed in a single tube, and the DNA fragments were separated by a capillary tube containing the gel matrix.

Table B-8. Reaction mixture for sequencing and cycler conditions

Reagents	Volume [µL]	Time	T [°C]	cycle
dest. Water	5.0	1-2 min	96	
6 x BigDye buffer	1.5	10 sec	96	25 cycles
BigDye 3.1 mix	1.0	5 sec	45-60	
Primer (10 µM)	0.5	4 min	60	
Mix	8.0	Hold	4	
DNA template[10-20 ng]	2.0			
Total volume	10			

C RESULTS

On behalf of the SONDE project, our collaborative project partners developed two microfluidic platforms in parallel: the lab-on-chip, and the LabDisc. The main aim of this study was to develop or establish basic methods for the nucleic acid testing workflow for the detection of category "A" biothreat pathogens, and to optimize these methods for their integration into the microfluidic MDx platform. Finally, the developed MDx platform would tested for detection of the biothreat agents and the results compared with the manual laboratory methods.

C.1 DEVELOPMENT OF METHODS FOR THE LAB-ON-CHIP PLATFORM

The Lab-on-chip (LOC) method combines three diagnostic steps: target enrichment, nucleic acid purification, and nucleic acid detection. In this work, target enrichment on lab-on-chip is presented only. The small amount of sample volume (10 µl to 100 µl) can be applied to a miniaturized molecular diagnostic system like the LOC. This factor limits the sensitivity of molecular biological assays. Therefore, an approach to concentrate the pathogen has been tested on a LOC in order to enhance the sensitivity of the diagnostic system.

C.1.1 DEVELOPMENT OF METHOD FOR LABELING OF VIRUSES WITH A FLUORESCENCE DYE

The ability to concentrate of bacteria on an LOC system was successfully demonstrated previously (Puchberger-Enengl, Podszun et al. 2011). To demonstrate the concentration of viruses on this LOC system, two viruses were used as model organisms: 1) YFV 17D as a model for small RNA viruses, and 2) CP 19 as a model for DNA viruses. Both of these viruses were cultivated on cell culture in large amounts, and purified using ultracentrifugation as described in Materials and Methods. The purified viruses were then labeled with a $DiOC_6(3)$ fluorescence dye as described above in the methods. To check the quality of the labeled viruses, 10 µl of fluorescently labeled CP 19 and YFV 17D in 10-fold dilutions were dried directly on a glass slide and fixed with 4 % of formaldehyde for 20 min. Then, the slides were analyzed under a fluorescence microscope. The fluorescently labeled CP 19 and their aggregates were clearly visible as green spots under the fluorescence microscope (Fig. C-1). Similarly, fluorescently labeled YFV 17D aggregates were also visible as green spots (data not shown). These labeled viruses are used in the next steps to investigate the microfluidic capture of viruses on the LOC system.

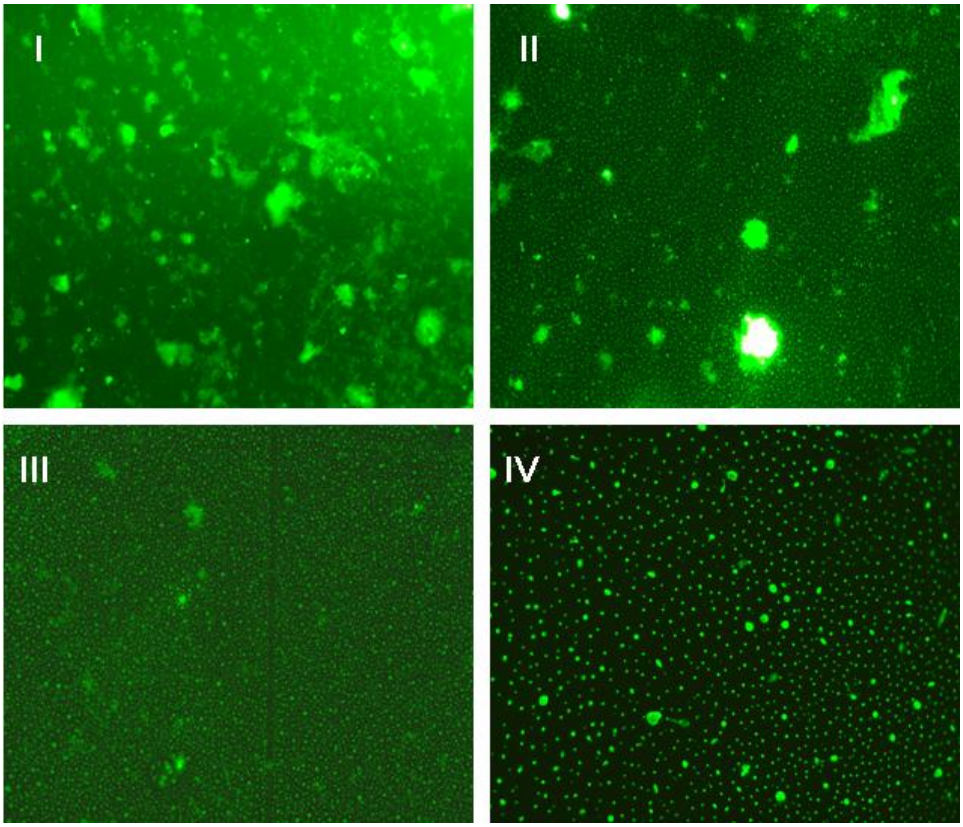


Figure C-1. Visualization of the fluorescently labeled CP 19 with $DiOC_6$(3). 10 µl of fluorescently labeled CP 19 in 10-fold serial dilution was fixed on a glass slide and visualized by fluorescence microscopy. In higher dilutions, fluorescently labeled CP 19 and their aggregates were clearly visible as green spots. I) Dilution 1:10, II-III) Dilution 1:100, and IV) Dilution 1:1000.

C.1.2 ELECTROPHORETIC TRAPPING OF LABELED VIRUSES ON THE LAB-ON-CHIP SYSTEM

For the electrophoretic trapping of viruses, 100 µl of CP 19 and YF 17D virus stock diluted in 0.5 X TBE buffer was injected continuously for 30 min as described in methods. The electrophoretic trapping and release processes for CP 19 are shown in Fig. C-2. The accumulation of CP 19 on the gel barrier is shown in Fig. C-2 II. Viruses were deflected to one electrode by the resulting force (F_{res}) of two main forces: F_E (electrophoretic force) and F_v (viscous force). The release of CP 19 from the electrodes was achieved by changing the direction of voltage for 1 min and is shown in Fig. C-2 III and IV. CP 19 were detached from the electrodes and moved to the central part of the chip, when the direction of voltage was changed for 1 min. It was clearly visible that the viruses were not completely recovered from the electrode and a small amount of viruses remained in the gel barrier (Fig C-2 IV). In a next step, these viruses were recovered from the chip and analyzed by real-time PCR.

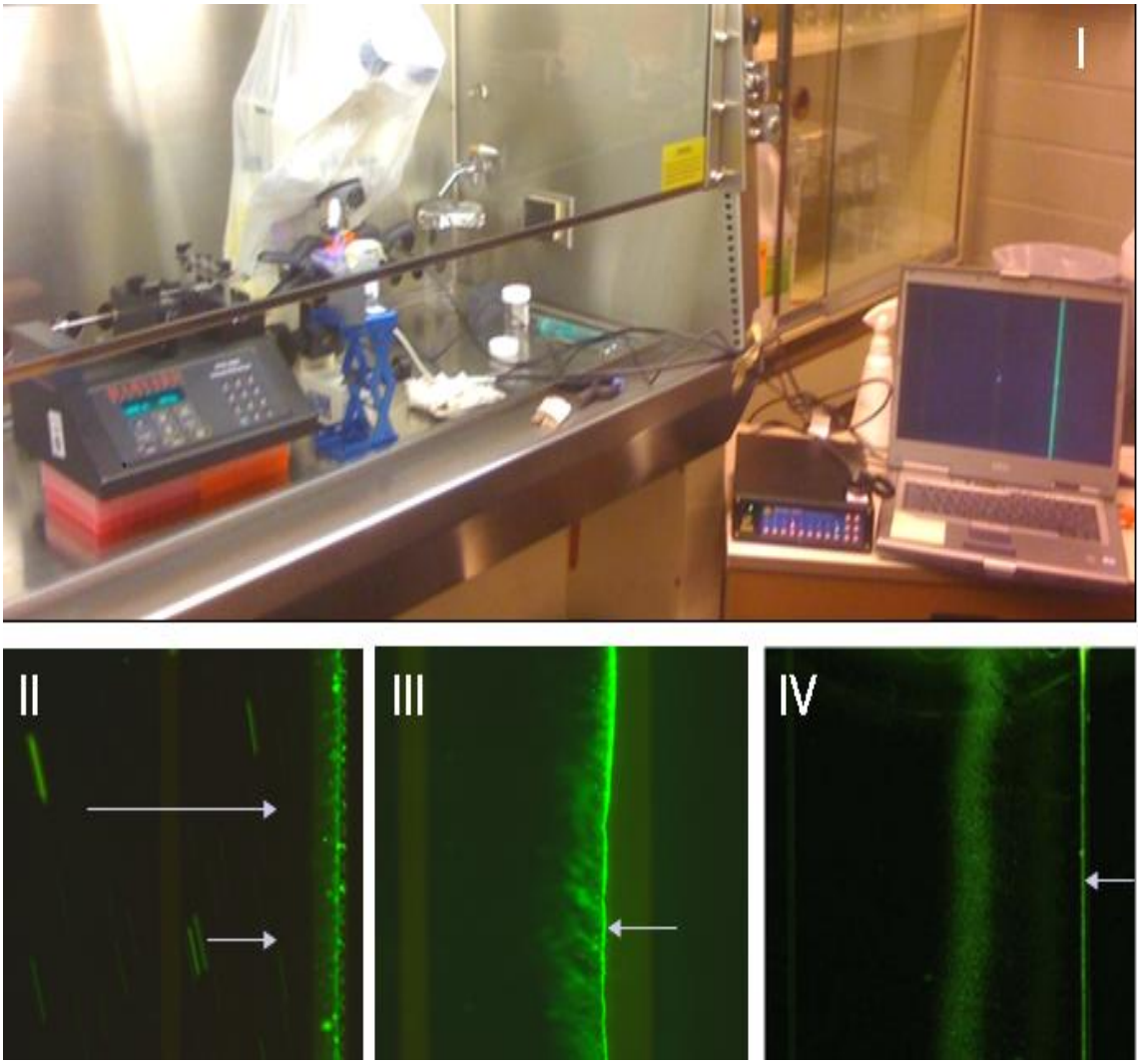


Figure C-2. Experimental setup and electrophoretic enrichment of the fluorescently labeled CP 19: I) The chip is placed under an inverse fluorescence microscope in a Biosafety hood for observation during the electrophoretic trapping of viruses, II) Fluorescently labeled CP 19 were deflected to the positive electrode due to resulting force and accumulated at this electrode indicated by white arrow. III) The viruses were released of from the electrodes by changing the direction of voltage for 1 min. IV) CP 19 were detached from the electrodes and moved to the central part of the chip. A small amount of viruses remained in the gel barrier indicated by white arrow.

Through repeated experiments, using labeled YF 17D viruses, the same results were obtained as those from CP 19 viruses in previous experiments, and electrophoretic trapping was here successfully demonstrated. However, there were still problems to completely recover the viruses from the gel barrier (data not shown).

C.2 DEVELOPMENT AND OPTIMIZING NAT METHODS FOR THE CENTRIFUGAL LABDISC PLATFORM

This work focuses on developing, establishing and optimizing molecular methods for nucleic acid testing such as pathogen enrichment, nucleic acid purification, and nucleic acid detection for the centrifugal LabDisc platform.

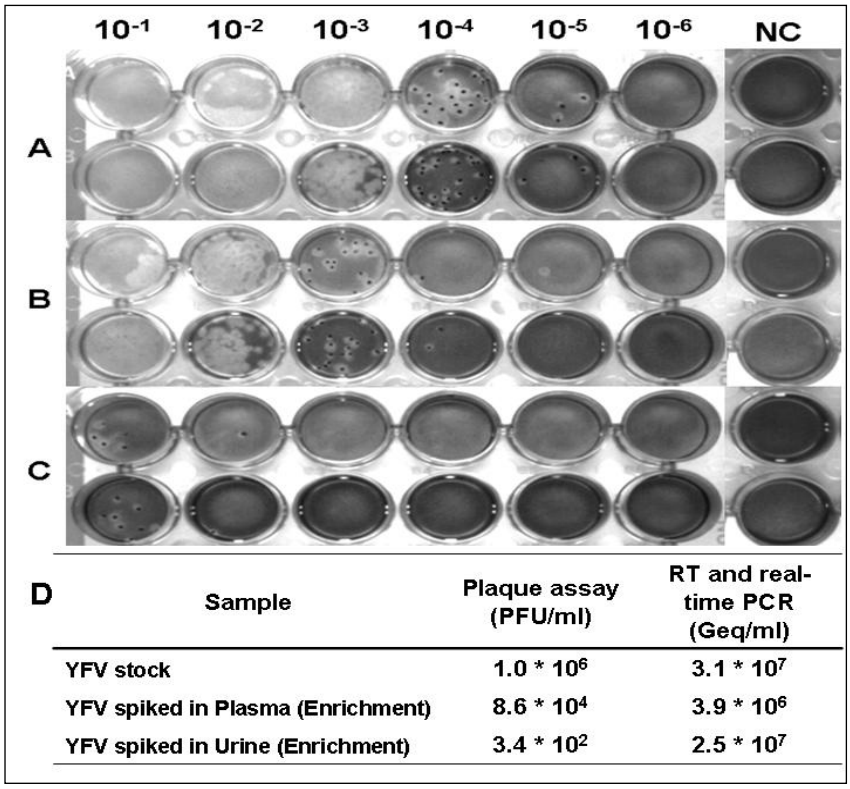
C.2.1 TESTING OF POLYMER BASED ENRICHMENT METHOD FOR VIRUSES

In the present study, a novel method to concentrate viruses and purify nucleic acids from large volumes of biological fluids (up to 1 ml) was tested and optimized. This polymer based InRichment kit was provided by AJ Innuscreen for testing and further development purposes. This method utilizes a polymer, which binds to viruses nonspecifically and forms a polymer-virus complex. The complex can be collected by centrifugation, and viruses can be resuspended into small volumes and used further for molecular applications.

C.2.1.1 Virus capturing by use of the polymer based InRICHMNET system

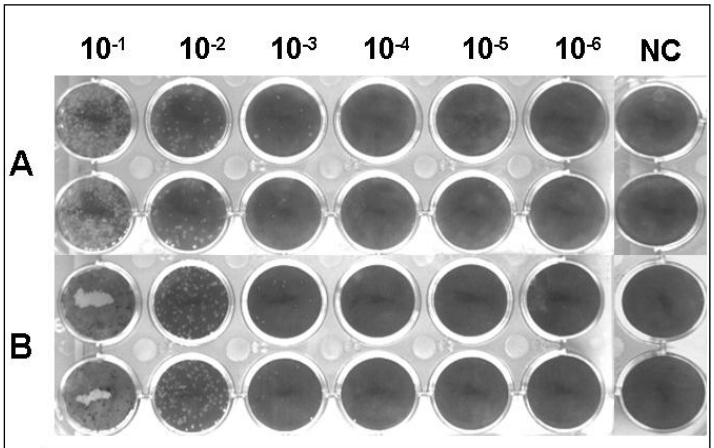
The InRICHMENT system is able to enrich virus particles using InRICHMENT virus reagents. In order to investigate if the viruses are still infectious before lysis, 200 µl viruses were spiked in 800 µl of plasma, urine, or PBS and subsequently recovered by InRICHMENT virus reagents. The volume of recovered samples was again set up to 200 µl. To determine virus titer and genome RNA copies, half of these samples were applied to the plaque assay, and the other half of the samples to cDNA synthesis, followed by the real-time PCR as described above in Materials and Methods. The same amount of virus stock without enrichment treatment was used as a control to determine the virus titer and the virus genome copies per ml.

The results for the determination of YFV titer and viral genome copies are presented in Fig. C-3. Virus titer and viral genome copies found in YFV stock were $1*10^6$ PFU/ml and $3.1*10^7$ Geq/ml, respectively. If plasma was spiked with viruses and recovered by the InRICHMENT virus reagent, approximately 1.5 log loss in virus titer and viral genome copies was found after testing. This could be due to loss of virus particles and free viral RNA during the enrichment followed by nucleic acid isolation. Similar concentrations of viral genome copies were detected by real-time PCR in the virus enrichment from urine samples spiked with virus, as compared it to the control, but the virus titers were 5 logs lower than the control.



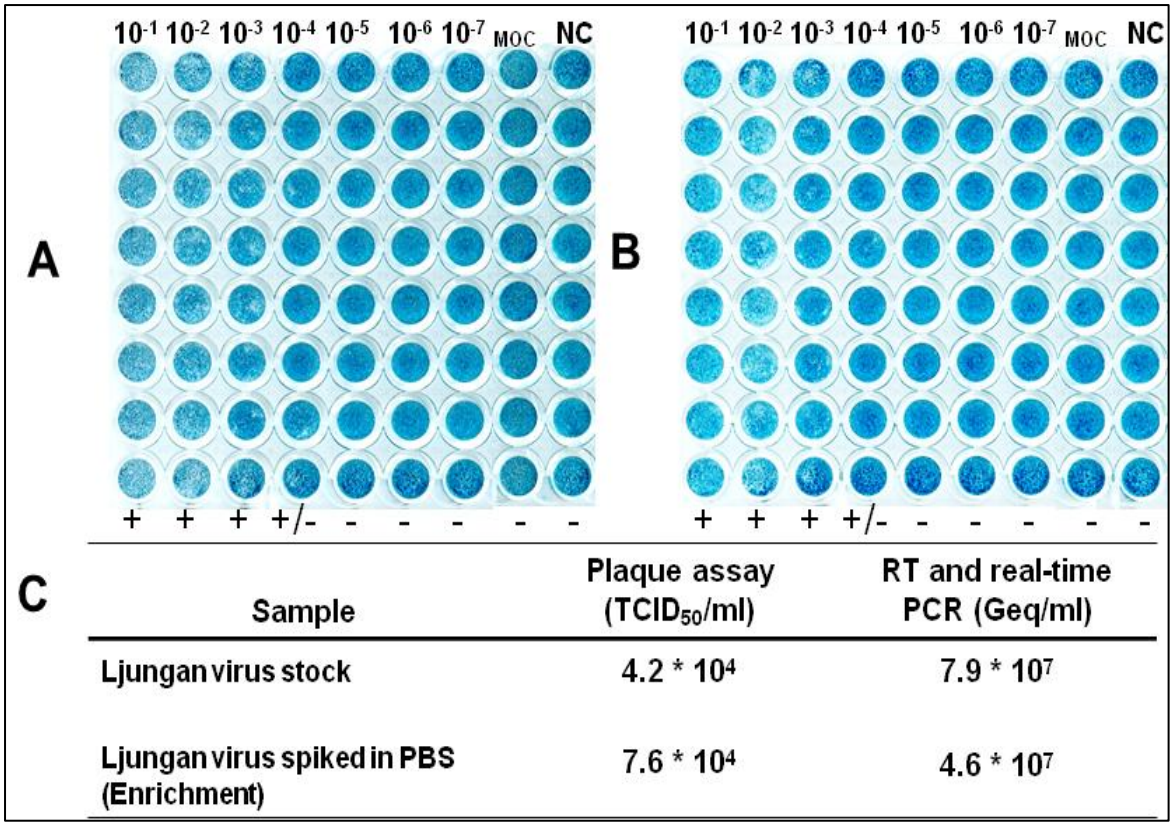
Sample	Plaque assay (PFU/ml)	RT and real-time PCR (Geq/ml)
YFV stock	$1.0 * 10^6$	$3.1 * 10^7$
YFV spiked in Plasma (Enrichment)	$8.6 * 10^4$	$3.9 * 10^6$
YFV spiked in Urine (Enrichment)	$3.4 * 10^2$	$2.5 * 10^7$

Figure C-3. Recovery of YFV from the sample matrix by the virus enrichment and analysis by virus titration and real-time PCR. A) Virus titration of the YFV stock solution by plaque assay. B) Virus titration of YFV recovered from the plasma samples by plaque assay. C) Virus titration of YFV recovered from the urine sample by plaque assay. D) Results achieved by virus titration and real-time PCR analysis. PFU: plaque forming units, Geq: genome equivalents.



Sample	Plaque assay (PFU/ml)	RT and real-time PCR (Geq/ml)
CP 19 virus stock	$3.04 * 10^4$	$3.0 * 10^7$
CP 19 virus spiked in Urine (Enrichment)	$7.14 * 10^4$	$1.8 * 10^7$

Figure C-4. Camelpox viruses recovered from the sample matrix by virus enrichment and the analysis by virus titration and real-time PCR. A) Virus titration of CP 19 stock solution by plaque assay. B) Virus titration of CP19 recovered from urine by the plaque assay. C) Results by virus titration and real-time PCR analysis. PFU: plaque forming units, Geq: Genome equivalents



C

Sample	Plaque assay ($TCID_{50}$/ml)	RT and real-time PCR (Geq/ml)
Ljungan virus stock	$4.2 * 10^4$	$7.9 * 10^7$
Ljungan virus spiked in PBS (Enrichment)	$7.6 * 10^4$	$4.6 * 10^7$

Figure C-5. Recovery of Ljungan virus from sample matrix by virus enrichment and analysis by virus titration and real-time PCR. A) Virus titration of the Ljungan virus stock solution by TCID50. B) Virus titration of Ljungan virus recovered from urine by TCID50 assay. C) Results achieved by virus titration and real-time PCR analysis. TCID50: Tissue Culture Infection Dose 50, Geq: Genome equivalents, +: cytopathic effect present in whole column, -: cytopathic effect not present in whole column, +/-: cytopathic effects only some wells in one column.

To demonstrate proof-of-principle of the enrichment method for other viruses, CP 19 (a model for enveloped DNA virus) and Ljungan virus (a model for non-enveloped RNA virus) were spiked in urine and PBS, respectively. Intact viruses from these samples were recovered by InRICHMENT virus reagent and processed as described above to determine viral genome copies and virus titer. The results presented in Figure C-4, show that CP 19 was well recovered from urine after enrichment. In fact, CP 19 spiked in urine and recovered by InRICHMENT virus reagent, resulted in similar amounts of viral genome copies and virus titer as in the control (Fig. C-4). Samples spiked with Ljungan virus and recovered by InRICHMENT virus reagent, also showed similar amounts of viral genome copies and virus titers as in the control (Fig. C-5).

C.2.1.2 Analysis of polymer and concentrated viruses in polymer by Electron microscopy

To investigate the functionality of the InRICHMENT reagents, the virus samples were analyzed for polymer-virus complexes by electron microscopy after enrichment. Orthopox virus particles have a size of 200x300 nm and a typical morphology. Therefore, they are easy to detect by electron microscopy. The Camelpox virus was chosen with the virus titer of $1.5*10^9$ PFU/ml for this study. 50 µl of virus supernatant was added to PBS to a final volume of 1 ml and recovered by InRICHMENT reagents into 50 µl of reagent 3. This should completely dissolve the polymer complex. This sample was analyzed by electron microscopy after appropriate inactivation of the virus.

Polymer from InRICHMENT kit forms complexes when the precipitation reagent was added to the polymer. The structure of polymer complexes was revealed under the electron microscope. The polymer forms threadlike structure with a thickness of about 5 - 10 nm, which are again complexes in dense networks (Fig. C-6 A-B). The typical morphology of orthopox viruses was visible by electron microscopy in the control with Camelpox virus (Fig.C-6 C). In the sample, where Camelpox viruses were recovered by InRICHMENT reagents, virus aggregates were clearly visible and the polymer structure was completely dissolved, as expected. The Camelpox viruses were surrounded by an excess of membrane debris of cell origin from the virus supernatant (Fig. C-6 D-F). The results here suggest that the viruses and cell membrane debris were captured by the dense and complex network of the polymer and could be collected in a pellet through centrifugation.

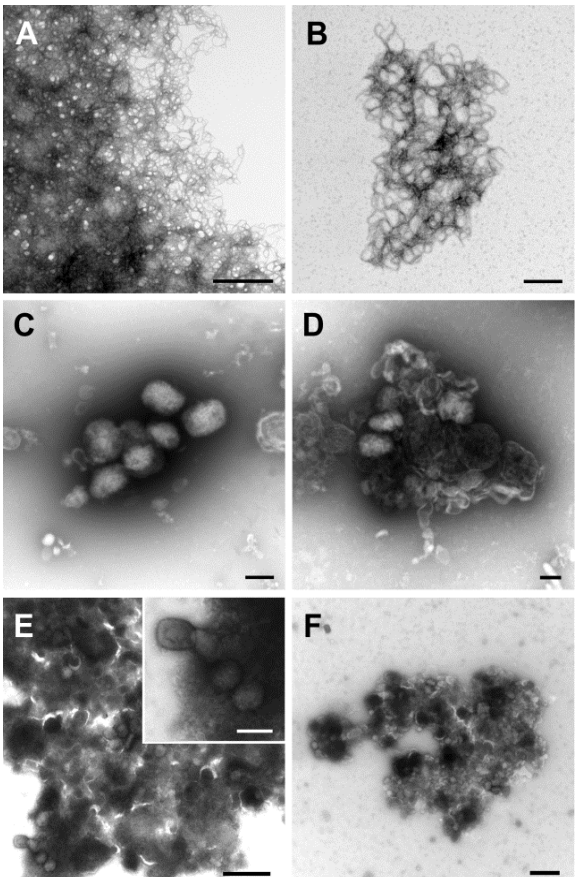


Figure C-6. Electron micrographs of polymer and Camelpox virus. A-B) Electron micrograph of polymer complex. C) Electron micrograph of Camelpox virus. D-F) Electron micrograph of polymer-virus complex after enrichment. Bars: A = 500 nm, B = 200 nm, C = 200 nm, D = 200 nm, E = 1 µm, F = 1 µm.

C.2.1.3 Comparison of three high-volume viral RNA extraction methods

To test and compare the extraction efficiency for YFV from large volumes, 10-fold serial dilutions of YFV 17D were prepared in PBS, plasma and urine. A sample volume of 1 ml of each dilution was tested in duplicate using all three extraction methods as described previously. After nucleic acid purification and cDNA synthesis, these samples were subjected to real-time PCR for genome detection of YFV.

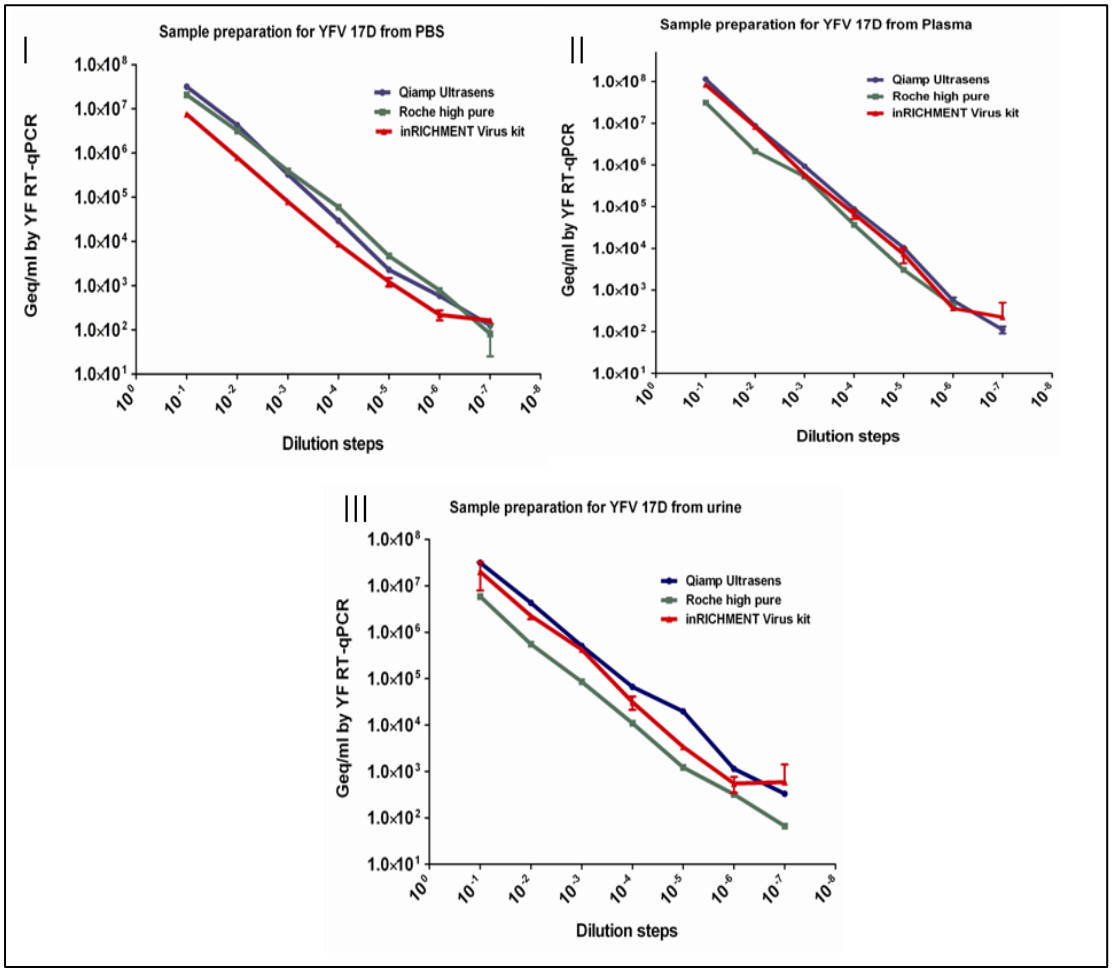


Figure C-7. Comparison of the different large volume nucleic acid extraction kits from 1 ml of YFV 17D spiked samples. I) Virus spiked in PBS. II) Virus spiked in plasma. III) Virus spiked in urine.

The response of real-time PCR used in this study was linear for 10^6 to 10^1 plasmids/reaction linearly. The quantification of cDNA copies was performed using standard plasmids (10^1 - 10^6 copies/µl). The YFV genome copies detected by real-time PCR for samples processed by all three methods showed good linearity and the results were comparable for all three extraction methods (Fig.C-7). YFV genome copies were detected in all spiked samples and diluted in PBS by real-time PCR after three extraction

methods. The results showed that the number of YFV genome copies obtained by High Pure Viral nucleic acid in the large volume system was higher than the two other methods for samples diluted in PBS (Fig. C-7 I). In samples diluted in plasma and urine, higher YFV genome copies were found by real-time PCR for extraction after QIamp Ultrasens system, when compared to the other two systems. Thus, the higher dilutions containing the lowest viral load were detected with greater sensitivity using the QIamp Ultrasens system and InRICHMENT DNA/RNA system than with the High Pure Viral nucleic acid large volume system (Fig. C-7 II-III). For instance, the InRichment RNA/DNA system detected the highest amount of genome copies in the most dilute of all three spiked samples. This was observed for all the experiments.

C.2.2 DEVELOPMENT OF A SAMPLE PREPARATION METHOD FOR VARIOUS BIOTHREAT AGENTS

While many protocols and commercial kits exist for purification of DNA or RNA, most are optimized for a single sample type or organism type. My goal was to develop a single kit and protocol that can process a variety of samples and organism types, including bacterial spores from biothreat agents such as *Bacillus anthracis*. In commercial kits, samples are usually incubated with a silica-gel membrane to bind in order to extract the nucleic acids. This procedure is labor-intensive and is not suitable for automation. In contrast, magnetic particles based kits are easily adaptable to automated nucleic acid purification. Therefore, the aim was to develop and optimize a nucleic acid extraction kit based on magnetic particles. Development of this kit was done in collaboration with AJ Innuscreen Company using reagents from the existing kits in combination with paramagnetic beads.

C.2.2.1 Pre-testing of the Instant MP basic extraction method on different matrices

The Instant MP basic extraction kit using paramagnetic particles (MAGs) was tested on 4 selected model pathogens: 1) YFV/ RVFV as a model for RNA viruses 2) Camelpox virus/ Vaccinia virus as a model for DNA viruses 3) *Yersinia pestis* as a model for gram negative bacteria 4) *Bacillus anthracis* as a model for gram positive bacteria. Whole blood, plasma, and serum were chosen as matrices due to the importance of these samples in molecular diagnostics. The same amount of these four model organisms was spiked in PBS, plasma, serum and whole blood, and then RNA/DNA was extracted using the Instant MP basic extraction method. These nucleic acid extracts were analyzed by real-time PCR as described above in Materials and Methods.

The results obtained for nucleic acid detection in the extracts of the four models from different matrices are presented as Ct value in Table C-1. A lower Ct value means higher amounts of detected nucleic acids after extraction, and indicates better extraction efficiency, whereas a higher Ct value means a lower amount of detected nucleic acids in the extracts. The used extraction method was able to purify nucleic acids efficiently from the spiked PBS sample for the four model pathogens. Therefore, Ct values obtained from the spiked PBS samples were used as reference values to compare the extraction efficiency of other matrices. In contrast to spiked PBS samples, the extraction efficiency decreased in whole blood, plasma and serum spiked samples for all model organisms excepting *Y. pestis* in the following order: plasma < serum < whole blood (Table C-1). Overall, the extraction method showed better extraction efficiency for plasma spiked samples than for serum and whole blood. These results support that plasma is a better matrix to prepare spiked samples for testing extraction methods.

Table C-1. Data of the pre-testing of extraction methods for different pathogens using various matrices.

Model	Model pathogen	Sample spiked in			
		Blood	Serum	Plasma	PBS
		Mean Ct	Mean Ct	Mean Ct	Mean Ct
RNA virus	**YFV 17D**	29.55	28.05	24.67	19.36
DNA virus	**CP 19**	24.64	24.96	24.33	20.72
Gram + bacteria (Plasmid pXO2)	***B. anthracis***	30.99	29.27	28.5	25.27
Gram + bacteria (Plasmid pXO1)	***B. anthracis***	30.01	29.29	27.62	24.7
Gram - bacteia (tmRNA)	***Y. pestis***	20.78	21.32	21.32	18.91

Ct: threshold cycle, YFV: yellow fever virus, CP 19: camelpox virus 19

C.2.2.2 Testing the efficiency of the Instant MP basic kit

A defined amount of model pathogen was spiked in plasma, and a 10-fold serial dilution was prepared in order to determine the efficiency of the chosen extraction method. After extraction, the amount of nucleic acids in the extracts was determined and quantified by real-time qPCR or RT-qPCR. For quantification of nucleic acids, either a plasmid, or in vitro transcribed RNA (ivRNA) standards, were used in the same real-time PCR runs along the samples.

The results from testing of the extraction efficiency by the Instant MP basic extraction method is illustrated exemplarily for RVFV (a model RNA virus) in Fig. C-8. The theoretical reference values of RVFV RNA copies are plotted as a blue line in the graph.

Under optimal extraction conditions, the determined RNA copies should be in the range of these reference values. The results show that all dilutions of RVFV in plasma were correctly detected down to the detection limit of 100 Geq/ml. However, the determined RNA copies of RVFV in plasma were not linear in relation to the reference values (Fig. C-8 I). If RVFV was diluted in PBS and plasma: PBS (1:1), RNA copies after extraction were detected close to the reference volume (Fig. C-8 II). In addition, plotted RNA copies in serial dilutions showed linear values. Similar results were obtained for the other three model pathogens (Vaccina virus, *B. anthracis* and *Y. pestis*) spiked in plasma (data not shown). Overall, the Instant MP basic extraction method was able to purify total nucleic acid from the four model pathogens from plasma and detection limits were down to 100 Geq/ml.

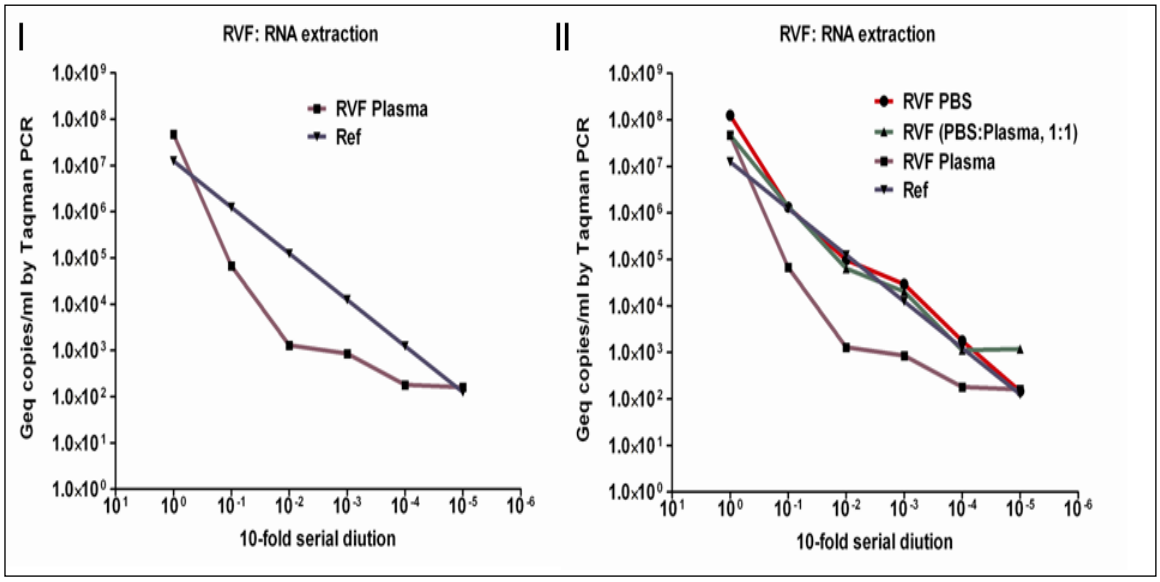


Figure C-8. Determination of the extraction efficiency of the Instant MP basic extraction method: I) A 10-fold serial dilution of rift valley fever virus (RVFV) in plasma samples was tested in real-time RT-qPCR after extraction. II) The 10-fold serial dilution of RVF in plasma, PBS and plasma:PBS (1:1). The samples were tested in real-time RT-qPCR after extraction. RVF: rift valley fever, Geq: Genome equivalents.

C.2.2.3 Comparison of Instant MP basic extraction method to a commercial kit

The Chemagic NA Body Fluid Kit allows isolation of pathogenic nucleic acids from different kinds of sample materials in one run. This kit was used to compare with the Instant MP basic extraction results for four model organisms. Additionally, proteinase K (PK) was included in the Instant MP basic method and tested in parallel. Pathogens were

spiked in plasma, and the same amount of spiked plasma was used to test three different extraction methods.

The determined Ct values of nucleic acid extracts from four model pathogens are presented in Table C-2. The results show that the Chemagen kit performed better than the Instant MP basic method for purifying nucleic acids from bacteria and DNA virus model pathogens, but the purification of RNA from viruses was less efficient. When PK was included in the Instant MP basic kit, the efficiency of this kit to purify total nucleic acid was increased by 2-3 Ct values, and the results were comparatively good as obtained by the Chemagen extraction method (Table C-2). Furthermore, the nucleic acid purification of viruses was considerably improved in comparison to the previous two methods. In further experiments, PK was included in the Instant MP basic kit as a component.

Table C-2. Comparison of different extraction methods for purifying nucleic acids from different pathogens in plasma.

Model	Model pathogen	Spiked plasma extracted by		
		Chemagen kit	Instant MP basic kit	Instant MP basic kit + PK
		Mean Ct	Mean Ct	Mean Ct
RNA virus	**RVFV**	25.66	24.08	22.81
DNA virus	**VACV**	22.74	25.40	21.44
Gram + bacteria (Plasmid pXO1)	***B. anthracis***	28.01	30.52	29.37
Gram - bacteria (tmRNA)	***Y. pestis***	15.96	16.62	16.37

Ct: threshold cycle; PK: proteinase K

C.2.2.4 Testing extraction efficiency of final Instant MP basic kit on different biothreat pathogens

The SONDE microfluidic platform includes the following pathogens in the target panel to be tested: RVFV, VACV, MARV, ZEBO, *B. anthracis*, *Y. pestis*, *F. tularensis* and Sigma virus (Internal control). These pathogens were 10-fold diluted and spiked in plasma to achieve a final concentration of 10^1 to 10^4 genomic copies in the samples. Additionally, 2 µl of Sigma virus at a concentration of 10^5 copies was added to prepare a spiked plasma dilution for monitoring the performance of the extraction procedure. These samples were then extracted in duplicate using the optimized Instant MP basic kit, and analyzed by real-time PCR methods to determine the efficiency of the extraction method.

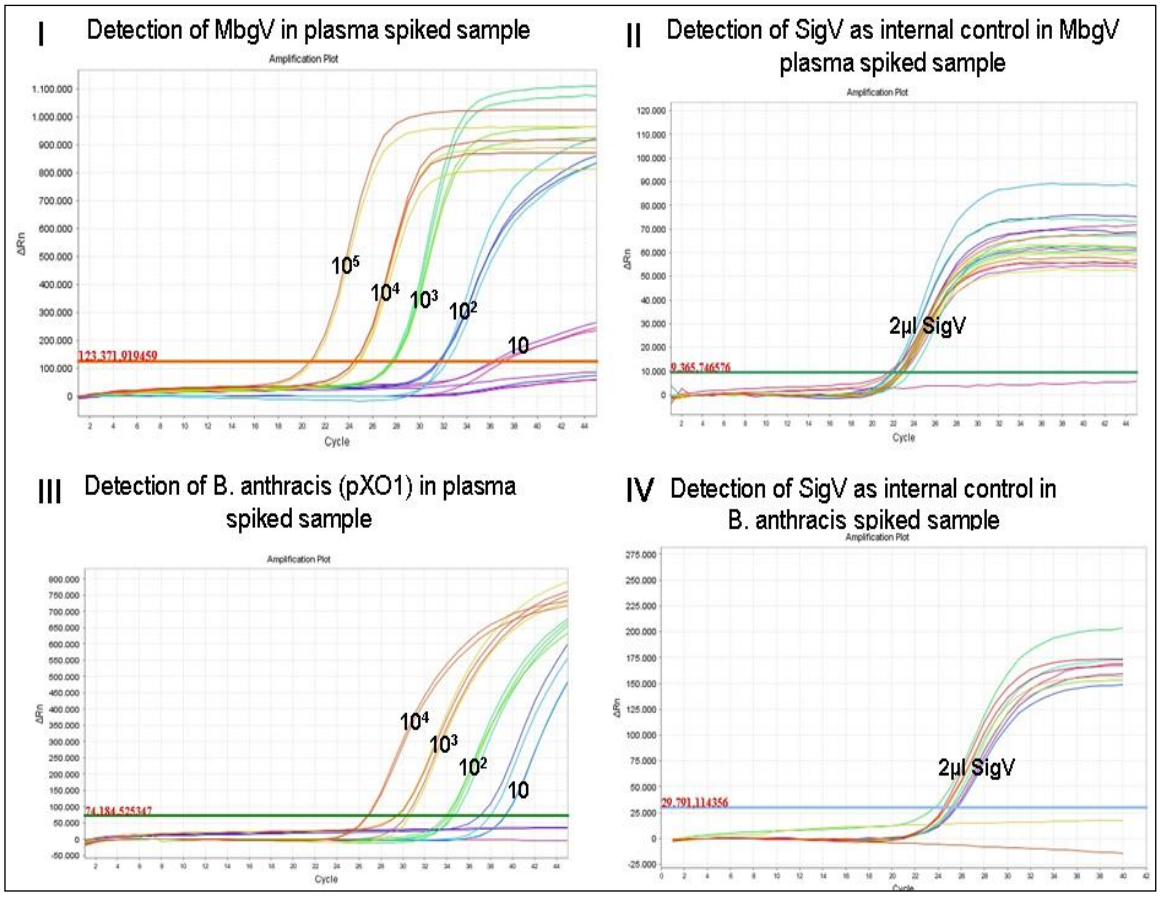


Figure C-9. Representative data for the performance of the Instant MP basic kit as an example from one bacterial and viral agent. I, III) Instant MP basic kit data shown for Marburg virus and *B. anthracis*. II, IV) Instant MP basic kit data shown for sigma virus as an internal control in extracts of Marburg virus and *B. anthracis*.

The results of the performance of the Instant MP basic kit obtained for one bacterial (*B. anthracis)* and one viral pathogen (Marburg virus) are illustrated in Fig. C-9. The data are presented as amplification plots, where the measured fluorescence in the sample is plotted against the number of PCR cycles. Fig. C-9 shows that the samples of pathogen spiked in plasma for a range of 10^1 to 10^4 genomic copies were efficiently recovered by the Instant MP basic kit, and well detected well by real-time PCR methods. In addition, the internal control was successfully detected in all extracts of Marburg virus and *B. anthracis* showing low variations in the detected number of genomic copies of sigma virus (Fig. C-9). These results indicate good reproducibility and robustness of the extraction method. Similar results were also obtained for the other five pathogens spiked in plasma (data not shown). Overall, the Instant MP basic kit was able to purify all eight pathogens efficiently from plasma with detection limits down to 10 copies of nucleic acid.

C.2.3 ESTABLISHMENT AND OPTIMIZATION OF NUCLEIC ACID DETECTION FOR THE CENTRIFUGAL LABDISC PLATFORM

While PCR methods require several temperature cycles to amplify the target nucleic acid, isothermal amplification is performed at a single temperature. This advantage of isothermal techniques allows to simplify the process of the diagnostic platform. Therefore, the SONDE partner from UMG developed a specific and sensitive isothermal amplification method using the Recombinase Polymerase Amplification (RPA) for detection of the biothreat agents. In this work, RPA assays were established as a manual reference method and optimized for the centrifugal LabDisc platform.

C.2.3.1 Performance of recombinase polymerase amplification

To detect category A Biothreat pathogens included in the SONDE target panel, nine specific and sensitive RPA assays were developed by our project partner (Euler, Wang et al. 2012; Euler, Wang et al. 2012). To determine the performance of RPA, a 10-fold serial dilution of the nucleic acids from target pathogens were prepared and tested with RPA. The same samples were measured by real-time PCR and results were compared to those from RPA.

The amplification plots of real-time PCR and RPA are illustrated in Fig. C-10 taking an example of one DNA and one RNA target. In real-time PCR, the measured fluorescence of the samples was plotted against the threshold cycles. For RPA amplification plots, the measured fluorescence of the samples was plotted against time (min). A comparison of the results from RPA and real-time PCR showed that both of these methods were able to detect presented *B. anthracis* pXO1 plasmid as DNA target down to the detection limit of 10 copies, and Marburg virus genomic RNA as an RNA target down to the detection limit of 100 copies. These results clearly show that the performance of RPA is as good as real-time PCR. Additionally, an RPA run requires less than 15 min for amplification of DNA and RNA target, whereas real-time PCR requires more than 90 min.

For more insight into RPA performance, it was tested with nucleic acid extracts of target pathogens, previously used to determine the extraction efficiency. The results of RPA performance are shown in table C-3 for each model pathogen. These data indicate excellent sensitivity of RPA compared to the results from real-time PCR with a detection limit of 10-100 Geq/rxn. Furthermore, RPA assays were also tested on nucleic acid extracts of other pathogens and they performed similarly as real-time PCR assays (data not shown).

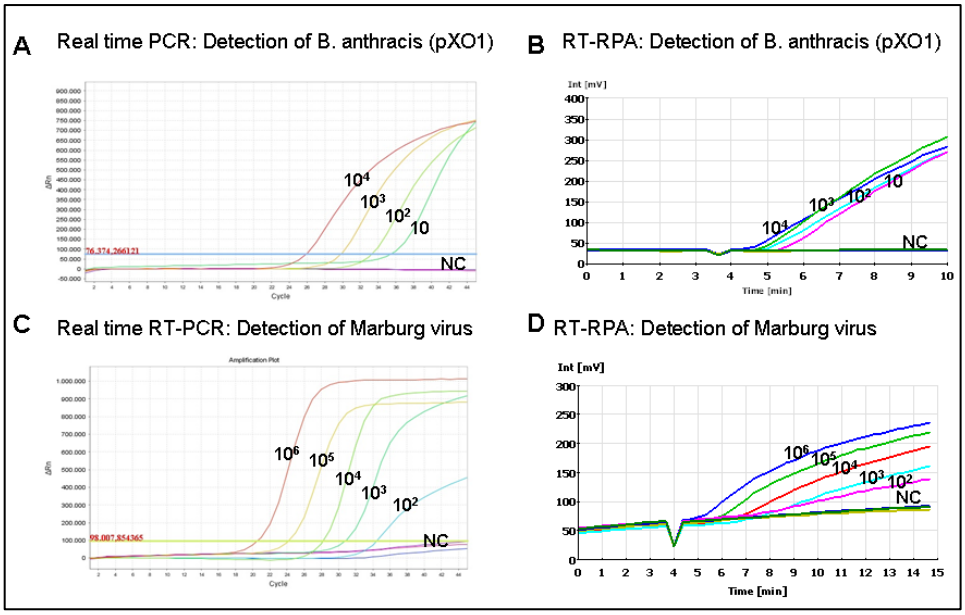


Figure C-10. The comparison of real time PCR and RT-RPA. Exemplarily results for the detection of viral genome by real time-PCR and RT-RPA are shown for one DNA target and one RNA target. The RT-RPA kit was used for both viral RNA and DNA detection. A, C) Detection of *B. anthracis* (pXO1 plasmid) and Marburg virus in plasma extracts by real-time PCR. B, D) Detection of *B. anthracis* (pXO1 plasmid) and Marburg virus in plasma extracts by RT-RPA.

Table C-3. The performance of RPA in the nucleic acid extracts. Inactivated whole organisms were spiked into plasma, and nucleic acid was extracted. One model pathogen of each category (gram positive, gram negative bacteria, DNA- and RNA virus) was tested. SIGV represents the internal positive control.

Organism	Sensitivity of real time PCR in extracts GE/rxn	Sensitivity of RPA in extracts GE/rxn
B. anthracis (pagA)	10	10
Y. pestis	10	10
Vaccinia virus	10	10
Marburg virus	10-100	10-100
Sigma virus	1	10

Geq/rxn: genome equivalent per reaction. *Instead of Rift valley fever virus, Marburg virus extracts were used as a model pathogen for RNA viruses

C.2.3.2 Optimizing RPA reaction for the LabDisc platform

RPA is normally carried out in 50 µl of the reaction volume in tubes. However, in the cartridge of the LabDisc platform, RPA was done in 10 µl volume. For this approach, the RPA reaction were first optimized in a tube. In a first step, RPA reactions were carried out in 50 µl, 20 µl and 10 µl volume and the results compared. In a second step two other parameters, primers & probe concentration, and amount of dry reagent per 10 µl reaction were varied, and the results obtained were compared.

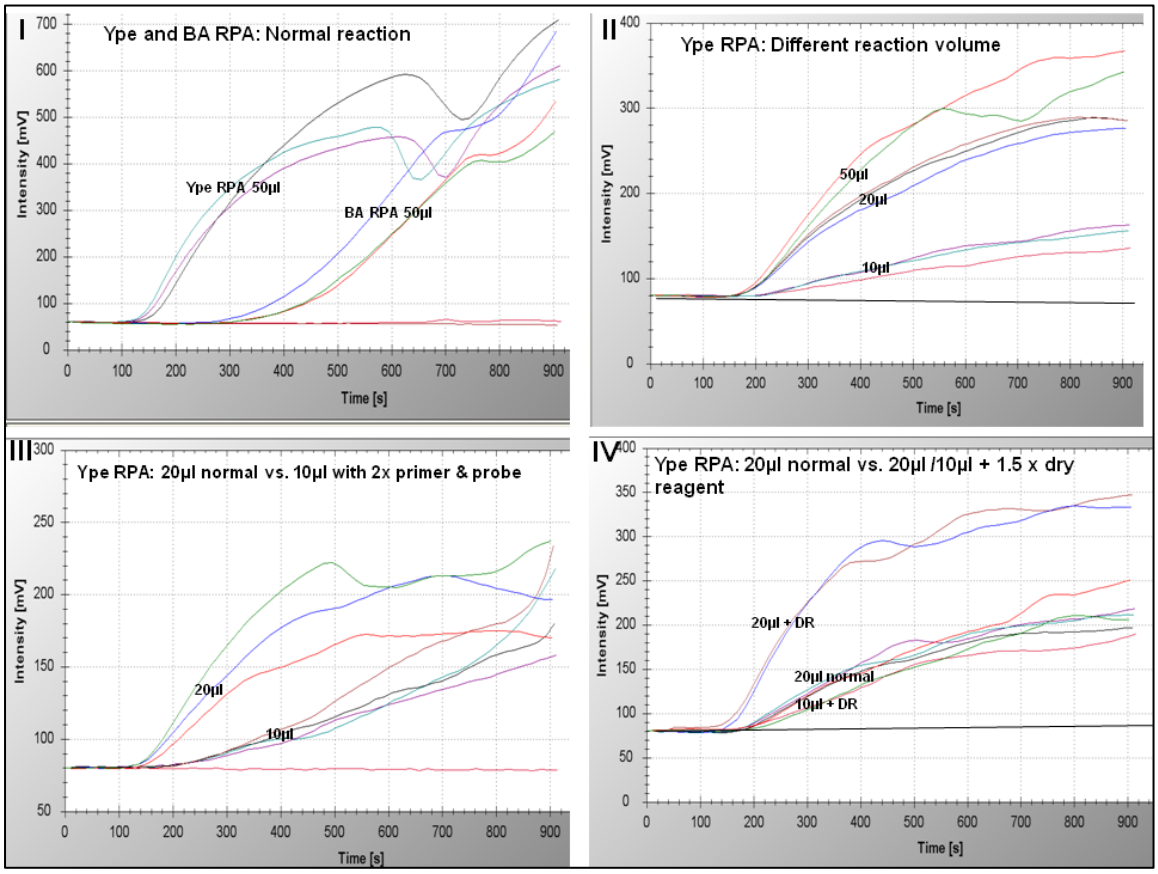


Figure C-11. RPA amplification plots of RPA optimization in 10 µl reaction volumes. I) Amplification plot for 50µl of RPA reaction of *Y. Pestis* (Ype) and *B. anthracis* (BA). II) Amplification plot for Ype RPA reaction in different volume. III) Testing of 10 µl Ype RPA reaction using 2 x primers & probe concentration. IV) Testing of 10 µl Ype reaction using 1.5 x dry RPA reagents.

For optimization of the RPA reaction, Ype and BA (pXO1) RPA assays were first tested on nucleic acid extracts of these pathogens in spiked plasma samples. Comparing the amplification plots of Ype and BA RPA assay in Fig C-11 I, it can be seen that the Ype amplification curves show a typical sigmoid shape and amplification is much faster than

the BA assays. For this reason, Ype assay was chosen as a model assay and optimized for 10 µl of reaction volume. For RPA assays performed in 50 µl, 20 µl and 10 µl of the reaction volumes with the same amount of template DNA, comparisons of the amplification plots showed the 20 µl RPA reaction gave a similar amplification curve as the 50 µl of RPA reaction. In contrast to this, the amplification curves for 10 µl of RPA reactions were flater than those of 20 µl and 50 µl, indicating a lower signal. (Fig C-11 II).

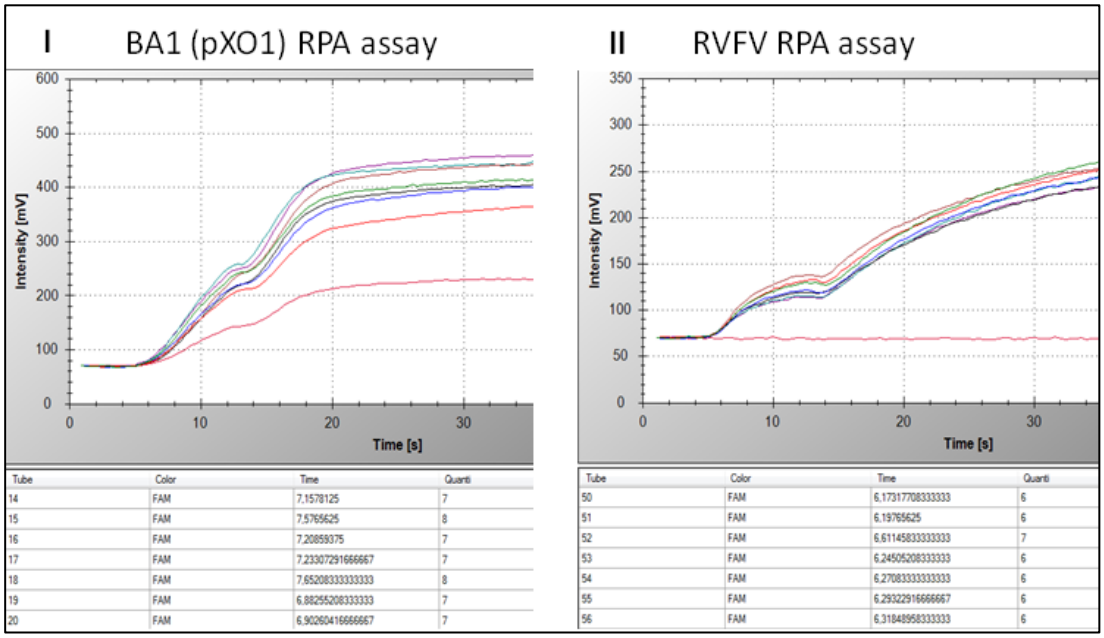
To optimize the RPA reaction in 10 µl, double amount of primers & probe concentration compared to the normal RPA reactions were tested and the results were compared with those of the 20 µl standard reaction. Fig C-11 III illustrates the results of these experiments, and show that the primers & probe concentration has no effect on the sensitivity of the 10 µl of RPA reaction. However, the use of 1.5 x RPA dry reagents instead of 1 x RPA dry reagent increased the sensitivity of the 10 µl RPA reaction and the amplification curves were identical to the normal 20 µl of RPA reaction (Fig. C-11 IV). Additionally, it can be observed in Fig. C-11 IV that the 20 µl reaction with 1.5 x dry reagents showed the highest sensitivity among all the normal and optimized reaction. The results suggest that the RPA reaction can be carried out in a reaction volume of 10 µl and the sensitivity can clearly be increased by increasing the amount of RPA dry reagents.

C.2.3.3 Testing of the optimized RPA reaction using the GeneSlice cartridges

After optimization of RPA reaction in a 10 µl volume, all the RPA assays were tested with the optimized protocol using the GeneSlice cartridge, which is mainly used for validation of the assay by HSG-IMIT.

An RPA assay for each pathogen was prepared in a 100 µl reaction volume as described in the Material and Methods. The complete RPA reaction including target nucleic acids were pipetted into the GeneSlice and aliquoted automatically to 10 µl volumes into the amplification chamber on the GeneSlice by the SONDE player. The RPA reactions were then monitored by fluorescence measurement. The amplification plots of these RPA reactions are plotted over time as shown in Figure C-12, as exemples for one DNA and one RNA target. For BA1 RPA assay, all eight chambers of the GeneSlice gave good and reproducible fluorescence signals, which were detected after of 7-8 min (Fig. C-12 I). For RVFV assay, 7 of the 8 chambers gave good and reproducible fluorescence signals after 6-7 min. During aliquoting of the RVFV assay, the last chamber was empty, and therefore no signal was obtained in this case. All other RPA assays (Ype, Ftu, ZEboV, MARV) performed using the Geneslice cartridges provided good results and a

reproducible signal was obtained for each amplification chamber of the GeneSlice cartridge (data not shown).



Tube	Color	Time	Quanti
14	FAM	7,1578125	7
15	FAM	7,5765625	8
16	FAM	7,20859375	7
17	FAM	7,23307291666667	7
18	FAM	7,65208333333333	8
19	FAM	6,88255208333333	7
20	FAM	6,90260416666667	7

Tube	Color	Time	Quanti
50	FAM	6,17317708333333	6
51	FAM	6,19765625	6
52	FAM	6,61145833333333	7
53	FAM	6,24505208333333	6
54	FAM	6,27083333333333	6
55	FAM	6,29322916666667	6
56	FAM	6,31848958333333	6

Figure C-12. Results from testing of 10 µl RPA reaction using the GeneSlice cartridges in the LabDisc platform. I) An example of RPA assay in 10 µl for *B. anthracis* as a DNA target. II) An example of RPA assay in 10 µl for the Rift Valley fever virus (RVFV) as an RNA target. The amplification curves are plotted against time and threshold time is used as a quantitative measurement of the RPA assay.

C.2.4 "SAMPLE TO ANSWER" TESTING OF BIOTHREAT PATHOGENS USING RPA LABDISC

For the "sample to answer" testing of biothreat pathogens, three bacterial pathogens (*B. anthracis (BA), Y. pestis (Ype)* and *F. tularensis (Ftu))* were chosen as targets. Testing samples were prepared by spiking 20 µl of bacterial suspension in 180 µl plasma, whereas samples with three pathogens were prepared by spiking 20 µl of each bacterial suspension in 140 µl plasma. 200 µl of these samples was applied to the RPA LabDisc for "sample to answer" testing as described under B 7.2. The same 200 µl of samples were manually extracted by Instant MP basic kit, and measured in triplicate by real-time qPCR and real-time RPA as described above. Results obtained from manual testing were compared to those obtained from the RPA LabDisc testing as a "sample to answer" method.

Results for the testing of three bacterial biothreat pathogens by the RPA LabDisc and manual methods are presented in Table C-4. Testing of the single and multiple

pathogens spiked plasma by real-time qPCR revealed that the genome equivalent (Geq) in 200 µl spiked samples of BA, Ype and Ftu were $2.6 * 10^4$, $5 * 10^7$ and $4.8 * 10^8$, respectively. The same concentration was found in spiked plasma samples with single or multiple pathogens (Table C-4 I and II). Manual testing of these samples by real-time RPA also shows that the measured amounts of detected nucleic acids expressed by detection time [min] were almost the same for spiked samples with single or multiple pathogens. In manual testing of real-time RPA, the detection times of the RPA reactions for BA, Ype and Ftu were 4.8, 2.2 and 3.6 min, respectively. Manual RPA testing was done in less than 50 min. In contrast to this, real-time qPCR testing required about 2 h. In "sample to answer" testing by the RPA LabDisc, similar results were obtained to those from manual testing. Comparing Table C-4 I to II shows that the detection of RPA reaction in time [min] for spiked samples with single or multiple pathogens were almost the same as previous results. The RPA LabDisc requires less than 50 min to detect different pathogens.

Table C-4. Testing of spiked plasma samples with single or multiple bacterial biothreat agents. The applied concentration of each pathogen in the sample was quantified by real-time qPCR. The results from real-time RPA and RPA LabDisc are measured in time [min].

I

				In Tube	In RPA LabDisc
Single pathogen spiked in plasma	Given Geq/mL	Measured by real-time PCR Geq/mL	Applied concentration in 20 µl sample	Measured by RPA time [min]	Measured by RPA time [min]
Bacillus anthracis spores	3.00E+08	1.30E+07	2.60E+04	4.8	36.0
Francisella tularensis	3.00E+10	2.50E+10	5.00E+07	2.2	42.0
Yersinia pestis	4.00E+10	2.40E+11	4.80E+08	3.6	38.0

II

				In Tube	In RPA LabDisc
Mix pathogen spiked in plasma	Given Geq/mL	Measured by real-time PCR Geq/mL	Applied concentration in 20 µl sample	Measured by RPA time [min]	Measured by RPA time [min]
Bacillus anthracis spores in BFY	3.00E+08	1.30E+07	2.60E+04	4.5	36.0
Francisella tularensis in BFY	3.00E+10	2.50E+10	5.00E+07	2.2	41.0
Yersinia pestis in BFY	4.00E+10	2.40E+11	4.80E+08	3.5	38.0

BFY: Mixture of *B. anthracis*. *Y. pestis*. *F. tularensis*

The “sample to answer” testing of three bacterial biothreat pathogens was demonstrated by using the RPA LabDisc. Nucleic acid extraction was successfully performed on RPA LabDisc and required less than 35 min. RPA reaction on the LabDisc at 10 µl was done within 20 min. The drying of primers and probes in the amplification chamber was successful in testing the three biothreat pathogens. The obtained signal intensity was higher for the BA assay than the Ype and the Ftu assays with the RPA LabDisc testing (Fig. C-13 I). For Ftu and Ype, the amplification signal varied in all three chambers. In contrast, the amplification curves for BA were in the same range for RPA LabDisc testing (Fig. C-13 II).

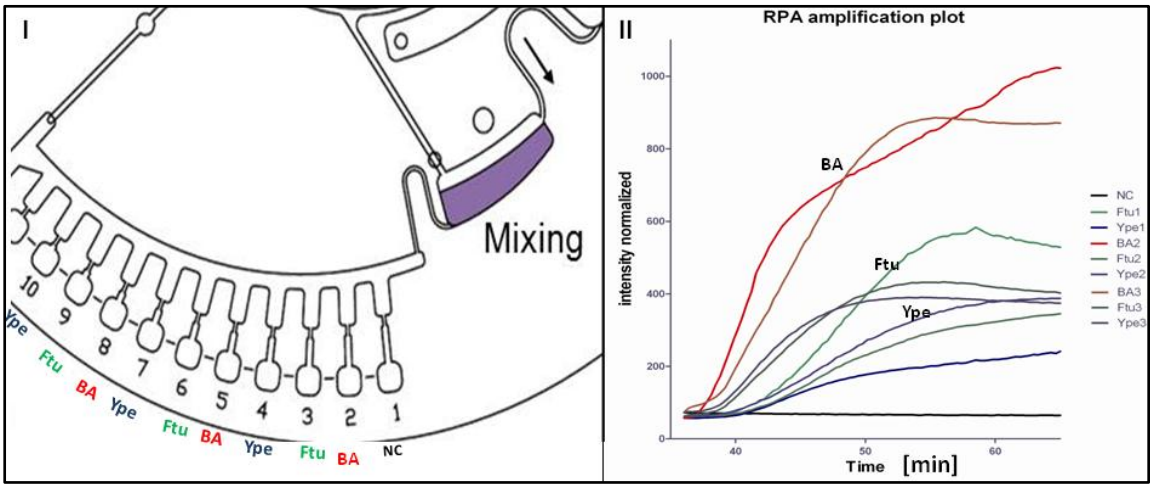


Figure C-13. RPA LabDisc testing for “sample to answer” detection exemplarily performed on plasma sample spiked with multiple bacterial biothreat agents. I) Ten amplification chambers on the LabDisc were used to pre-store RPA primers and probes for different assays. II) The amplification plots show the successful amplification of *B. anthracis* in chambers 5 & 8; *F. tularensis* in chambers 3, 6, & 9; and *Y. Pestis* in chambers 4, 7 & 10. There was no signal obtained in chambers 1 & 11, as no primers and probes were pre-stored in these chambers.

D DISCUSSION

D.1 DEVELOPMENT OF METHODS FOR THE LAB-ON-CHIP PLATFORM

The LOC system by IMTEK for concentrating viruses in small volumes on a chip was tested and could be later used for NAT by integrated LOC devices. The two following model pathogens for testing of this approach were chosen: YFV 17D as a model for RNA viruses and CP 19 virus as a model for DNA virus. As these pathogens can be handled under Biosafety level-2 conditions easily, these pathogens were chosen for proof-of-principle testing instead of biothreat pathogens. The LOC system was already tested for the electrophoretic capture of bacteria on the chip previously (Puchberger-Enengl, Podszun et al. 2011; Podszun, Vulto et al. 2012). Visual analysis of bacterial pathogen on microfluidic devices are particularly done using a simple light microscope. In contrast to these, viral pathogens cannot be analyzed by use of the light microscope, as the size of viruses ranges from 10 to 300 nm. Electron microscopes (EM) are generally used to visualize the viruses, but this method required complex sample preparation including fixation and negative staining of the sample for visualization of viruses. Therefore, EM was not used to analyze the interaction of viruses in microfluidic chips.

Labeling of external membrane components of a virus such as the capsid or envelope can be easily done with fluorescent dyes; the labeled viruses can then be visualized using fluorescence microscopy techniques. This approach was already described for the analysis of Vaccinia virus in a microfluidic device previously (Demir Akin 2004). This method labels virus membranes and structural proteins nonspecifically. Therefore, it is important to use the purified virus suspension to exclude cell debris and other cell culture contaminants. Using this described approach, the purified YFV 17D and CP 19 viruses were successfully labeled by lipophilic carbocyanine dyes $DiOC_6(3)$. Additionally, visualized aggregates of labeled viruses were visualized under a fluorescence microscope. This method has opened new possibilities for our testing of viruses on LOC devices.

LOC devices for NAT process small sample volume for the analysis. Therefore, the concentration of pathogens in the applied sample is crucially important for such a test. Many attempts have been made for concentrating viral pathogens on-chip using the functionalized particles or electrokinetic methods (Lui, Cady et al. 2009). Dielectrophoresis was also used for concentration of viral pathogens previously (Bhattacharya, Salamat et al. 2008). None of the described methods uses electrophoretic capturing for viruses for microfluidic devices. Surfaces of viruses are

made of membranes and proteins containing carboxylate and phosphoryl groups. Due to these ionized groups, viruses exhibit negative surface charge. Therefore, viruses can be deflected to the electrode by applying an electrical field. This phenomenon was observed by on-chip electrophoresis using labeled virus particles. In these experiments, both YFV 17D and CP 19 viruses were attracted to anodes when an electrical field was applied to the viruses on the chip. By pumping the sample containing viruses through chip over 30 min, viruses were captured by electrophoresis from the sample, and the viruses were successfully resuspended in a 5 µl volume on the chip by changing the direction of the electrical field. Using this approach, the sample volume was reduced from 100 µl to 5 µl, concentrating viruses, which is important for subsequent NAT analysis of viruses on the chip. However, the viruses were not completely recovered after the electrophoretic capture. To avoid direct lysis of viruses on the electrode, a polyacrylamide gel barrier was placed before the electrodes on the chip. Some amount of virus particles was irreversibly captured in the pores of the polyacrylamide gel. Therefore, it was difficult to quantify the amount of concentrated viruses by molecular methods. To solve this problem, the complete concept of the chip needed to be designed without use of the polyacrylamide gel on the chip.

By working with this LOC system at IMTEK, many disadvantages like small processing volume, handling of devices, and limitations for integration of components in the chip fabrication process were found. Furthermore, clinical samples like plasma, serum or blood could not be used, as on-chip electrophoresis depends on the conductivity of liquids. Because of this limitation, the use of LOC devices from IMTEK were only restricted to analysis of swab samples. Considering this experiment, the complete LOC is re-designed by our project partner and this chip for concentrating the viruses is directly combined with nucleic acid isolation chip, as described previously (Vulto, Dame et al. 2010). This LOC system is now being tested for its functionality, and will be combined with other chip modules in future for amplification and detection of nucleic acids to obtain an integrated MDx device.

D.2 Development of molecular methods for the Centrifugal LabDisc platform

Sample preparation, amplification, and detection are the three basic methods in NAT, which are important to develop the integrated MDx platforms. Many different methods and protocols are available for all three steps of NAT, but these methods must be adapted in order to implement them on integrated MDx platforms. In this study, basic methods for NAT were developed and/or established to make them suitable for the

centrifugal LabDisc platform and allow a sensitive detection of the biothreat agents. Finally, these methods were integrated onto the LAbDisc platform, and the LabDisc tested as a “sample to answer” platform for rapid detection of biothreat agents.

D.2.1 ENRICHMENT OF VIRUSES BY A POLYMER BASED METHOD

Sample preparation and target enrichment are an often neglected, but critical area in molecular diagnostics for infectious diseases (Dineva, MahiLum-Tapay et al. 2007). Unfortunately, only a small volume of sample of around 200 µl can be processed for nucleic acid extraction using conventional sample preparation methods or commercial kits, which also limits the sensitivity of molecular biological assays. Therefore, sample pre-treatment such as enrichment of infectious pathogens is crucially important in sample preparation for the molecular testing of biological samples. In this study, a novel method was described, which is applicable to various biological samples for capturing nonspecifically different viruses from 1 ml sample volumes. These captured viruses can be easily used for either the infectivity assay, or nucleic acid purification. Major advantages of this method over traditional methods like PEG precipitation, ultracentrifugation, or filtration are speed, simplicity and cost effectiveness.

In the field of molecular diagnostics, a number of methods have been reported recently that describe enhancement of PCR sensitivity by virus concentration. These include: 1) The use of polyethyleneimine conjugated magnetic beads; 2) the use of cationic magnetic beads; 3) the use of poly tentacles containing magnetic particles; and 4) the use of Apolipoprotein H (Iwata, Satoh et al. 2003; Satoh, Iwata et al. 2003; Arkhis, Elaissari et al. 2010; Adlhoch, Kaiser et al. 2011). However, the capture of non-enveloped virus was reported to be problematic (Satoh, Iwata et al. 2003; Arkhis, Elaissari et al. 2010). It has been demonstrated here that the InRICHMENT kit using a polymer was able to capture enveloped model viruses such as YFV 17D & CP 19, and non-enveloped model virus such as Ljungan virus efficiently from various matrices. This method allowed the use of large sample volume (1 ml to 10 ml) in comparison to conventional commercial methods, and concentrated the samples in a 50 µl of volume after enrichment. These concentrated samples were successfully used for quantitative analysis of the virus titer by virus infectivity assays, and the genome copies by real-time PCR (Fig. C-3, C-4 & C-5). The results obtained show clearly that the combination of the enrichment method with the sample preparation method has enhanced the sensitivity for detecting virus particles and viral genomes. Furthermore, the mechanism of the enrichment process was revealed by electron microscopy using CP 19 as a model organism. The results suggest that the viruses and cell membrane rests were captured

clearly by formation of a dense and complex network of polymer, which were then collected mechanically through centrifugation (Fig. C-6). In addition, the polymer complex was dissolved by the reagents after capturing the viruses as described by the manufactures. In a similar mechanism, YFV and Ljungan virus seems to have been captured and concentrated by InRICHMENT reagents. This supports the results from in vitro infectivity assays that the polymers did not inhibit the binding of viruses to the cells. Additionally, it was found that this enrichment method needs no elaborate equipment and adapts well to scale up. Nearly physiological conditions can be maintained during the enrichment process, and at the same time, this system has provided higher recovery yields for viruses with fewer steps in the procedure.

Recently, some commercial kits have become available, which allow the nucleic acid extraction from up to 1 or even 2 ml of a sample. These test kits were developed mainly to examine blood, plasma, or serum samples for the detection of viral pathogens tolerating the extraction of high sample volumes, concentrating viral nucleic acids in samples, and allowing the downstream detection of very low viral titers. As a simple and feasible method for virus concentration, the InRichment method was tested for extraction of RNA from large volumes and compared with recent available two commercial kits. These kits also allow manual viral nucleic extraction from 1 ml sample volumes. All three methods use different technologies to enable large sample volume extraction. One method scales up the standard extraction via silica gel membranes, whereas the other two methods use an enrichment step before or after lysis to reduce the working volume (Dovc-Drnovsek, Emersic et al. 2008; Domingo, Yactayo et al. 2011). These kits have been tested with emphasis on practicability and sensitivity for sample preparation using yellow fever virus 17D (YFV 17D) as an RNA model virus. The results suggest here that the enrichment method using the InRichment kit efficiently concentrates viruses and purifies RNA from various matrices like plasma, urine, or PBS. The results obtained were as good as the other two commercial methods for purification of viral RNA (Fig. C-7). The InRICHMENT method was more sensitive to the samples with the lower virus concentrations. It could be that the inhibitory substances may be removed from the sample during the enrichment procedure, as the InRichment method captures the viruses mechanically through polymer complexes and excludes small inhibitors from the samples.

In summary, a novel method for enrichment of viral pathogens from large volume samples was established to enhance the sensitivity of NAT. This method is perfectly suitable for the centrifugal LabDisc platform, as the viruses-polymer complexes are

captured by centrifugation. Furthermore, this method can be easily combined with the sample preparation method to isolate nucleic acids from large volume samples to improve the sensitivity of NAT.

D.2.2 Development of a Sample preparation method for the LabDisc platform

The extraction of nucleic acids is the first and most important step in molecular diagnostics. Nucleic acids such as a DNA and RNA can be isolated from cells, tissues, microorganisms and viruses by sample preparation methods for downstream analytical processes in NAT. A large number of commercial and in-house methods are available for nucleic acid extraction. Most of the commercial extraction kits are based on solid-phase nucleic acid extraction utilizing silica matrices, glass particles, or magnetic particles; which allows simple, rapid, and efficient purification of nucleic acids. These methods comprise four major steps: Lysis of pathogens, binding of nucleic acids to a solid phase, washing, and elution.

Nucleic acid extraction methods based on magnetic beads has become a new trend in manual and automated nucleic acid purification. These kits do not require buffers containing organic solvents, the repeated use of centrifugation, or vacuum filtration and separation of columns, thus reducing the time and complexity of the extraction method (Tan and Yiap 2009). Therefore, the magnetic beads based nucleic acid extraction methods are easy to implement in automated devices and are used widely in commercial automated devices nowadays (e.g. Promega Maxwell 16 system, Anaytik Jena Innupure system, Thermo Scientific KingFisher Flex system). After an extensive testing and comparing the different commercial kits, it was found that the Instant MP basic kit utilizing magnetic beads was as good as a silica column based commercial kits. Therefore, this kit was chosen for the centrifugal LabDisc platform. Additionally, this kit allows the extraction of the nucleic acids from the various clinical samples (whole blood, plasma, serum, or urine).

For detection of biothreat agents, sample preparation methods are applied that are commonly used for detection of infectious pathogens in samples such as blood or blood components, powder and air (Lim, Simpson et al. 2005). Many of these bacterial and viral biothreat pathogens are found in detectable amount in blood and blood components such as serum and plasma of infected patients (Lim, Simpson et al. 2005; HEALTH 2011). Clinical diagnostic would in most of cases, be the early warning system for the infection with biothreat pathogens; therefore, the sample preparation for biothreat agents was adapted for testing clinical samples. One agent for each agent category (gram-

positive, gram-negative bacteria, DNA- and RNA virus) was tested in spiked human blood, plasma, and serum to determine the extraction efficiency using the Instant MP basic kit. The results from this testing suggest that all four pathogens can be detected in plasma, as well as in whole blood and serum. Blood contains many substances like heme, erythrocytes and other components that can inhibit the downstream process of NAT (Al-Soud and Radstrom 2001). Therefore, the detected amount of pathogens was lower in spiked blood samples with pathogens. According to this testing, plasma was chosen as a sample for the NAT analysis with the Instant MP basic kit.

To evaluate the efficiency of the Instant MP basic kit, 10-fold serial dilution of each of four model pathogens was prepared in human plasma and subjected to real-time PCR after nucleic acid isolation. The efficiency analysis shows that this method was able to purify the total nucleic acids from all four pathogens efficiently with detection limits down to 100 Geq/ml. When samples were prepared by the 10-fold serial dilution in plasma, the detected amount of genome copies was not linear to the reference value. This phenomenon was seen in the dilution of all four pathogens (Fig. C-8). As the samples were prepared by a 10-fold serial dilution in plasma : PBS (1:1) or PBS only, the detected amounts of genome copies were linear to the reference values. These results indicated that the direct serial dilution of pathogens in plasma lead to this failure, which could be clearly avoided by diluting the plasma 1:1 with PBS. On the other hand, the dilution of plasma samples could also beneficially avoid inhibition of the following process like nucleic acid detection, as the plasma contains many PCR-inhibitory substances (Drosten, Panning et al. 2002). Therefore, the extraction efficiency can be clearly increased by diluting the plasma samples before sample preparation. Other approaches to increase the extraction efficiency are to use of the proteinase K (PK). PK cleaves glycoproteins and inactivates RNase and DNase in lysis solution with SDS. Use of the PK in nucleic acid extraction has shown an increased sensitivity in the downstream process such as nucleic acid detection by PCR (Gobbers, Oosterlaken et al. 2001). The use of PK in the Instant MP basic kit also increases extraction efficiency for samples spiked with viral and bacterial pathogens. Due to the beneficial effect of the PK in extraction, it was included as a standard reagent for the Instant MP basic kit.

After extensive testing and optimizing the extraction method, finally the Instant MP basic kit was tested on eight biothreat agents (BA, Ype, Ftu, RVFV, MARV, ZEBO, SigV and VACV) included in the SONDE project. A defined amount of these biothreat pathogens in the range of 10^4 to 10^1 genome copies were spiked in plasma and detected by the real-time PCR after isolating the total nucleic acids. The results show that the optimized

Instant MP basic kit efficiently isolates nucleic acids from spiked plasma samples with all eight pathogens, and the detection limit was down to the 10 copies/assay. Furthermore, all the components of this kit including PK can be stored at a room temperature. Lysis of the samples can be performed at ambient conditions and handling of extraction procedure is easy due to use of the MAGs. As this Instant MP basic kit fulfilled the all the important criterion for point-of-care settings, this method was integrated into the centrifugal LabDisc platform as an extraction method.

D.3 ESTABLISHMENT AND OPTIMIZATION OF NUCLEIC ACID DETECTION

PCR is the most widely used technique for DNA amplification and detection in molecular biology. This method requires extensive thermal cycling for *in vitro* amplification of the DNA. In contrast, isothermal amplification method offers many advantages over the traditional PCR based methods. One of the main advantages is that a single constant temperature is needed for amplification of DNA and the isothermal method can be performed using a simple heating device. In addition, isothermal amplification is easy-to-perform, cost effective as well as robust and provides good sensitivity and specificity for molecular diagnostics (Vincent, Xu et al. 2004; Piepenburg, Williams et al. 2006; Kaneko, Kawana et al. 2007; Chow, McCloskey et al. 2008; Mori and Notomi 2009). Therefore, an isothermal amplification method so called Recombinase Polymerase Amplification (RPA) was chosen as the nucleic acid detection method by the SONDE consortium for the centrifugal LabDisc platform. The main advantages of the RPA are: 1) the reaction can be performed at 37 °C to 42 °C; 2) the reaction requires only 15-20 min; 3) the reaction allows different detection format including real-time monitoring of fluorescence; and 4) all the RPA reagents are provided as lyophilized reagents, which are suitable for storage at ambient conditions. Using commercial RPA kits, sensitive and specific RPA assays have been developed by our project partner from UMG, Göttingen for the detection of biothreat pathogens, as described previously (Euler, Wang et al. 2012; Euler, Wang et al. 2012).

RPA assays for the detection of biothreat agents have been established and optimized for the centrifugal LabDisc platform. The testing of this assay shows that the sensitivity of RPA is as good as the sensitivity of real-time PCR, as described in literature (Piepenburg, Williams et al. 2006). Furthermore, DNA amplification of RNA and DNA templates was done using a single RPA kit (Twistamp exo RT kit) within 15-20 min. The RPA assays for detection of biothreat agents were highly sensitive and 4-5 times faster than real-time PCR (Fig. C-10). However, all these RPA assays needed to be optimized in a 10 µl reaction in order to integrate them into the LabDisc platform. When the RPA

assay was performed in 50 µl, 20 µl and 10 µl reaction volumes, the observed signal intensity in 10 µl was very low in comparison to the 50 µl and 20 µl reactions. This result indicates that the RPA assay in 10 µl is less sensitive. A possible reason for this could be the reduced amount of reagents in the smaller volumes. Miniaturization of RPA reaction is at a certain amount of reaction volume is possible, as shown for 20 µl of reaction. However, the reduced reaction volume in this case of 10 µl contains fewer reagents to amplify target nucleic acid, which could lead to the reduced signal intensity.

There was no improvement in the signal intensity of the RPA reaction using double amounts of primer and probes in 20 µl and 10 µl reactions. As the amount of RPA reagents was increased 1.5 times in 20 µl and 10 µl reactions, the signal intensity of these RPA assays was significantly increased (Fig. C-11). These results indicate that the RPA reaction can be performed in smaller volumes, if the RPA reagent concentration is increased 1.5 times. Additionally, the RPA reaction with increased reagent concentration for 20 µl reactions was more sensitive than 10 µl reactions. However, the designed LabDisc cartridge can process only 10 µl of reaction mixture. Furthermore, this approach was successfully proven for all eight RPA assays performed in 10 µl reactions using the GeneSlice. By optimizing the RPA reaction, the reaction volume was successfully reduced to 10 µl for the final integration of the RPA assays onto the LabDisc platform. This approach also offers advantages like low-cost, high-throughput, low reagent consumption, easy liquid handling, and portability of the microfluidic cartridge.

D.4 "SAMPLE TO ANSWER" TESTING OF RPA LABDISC FOR THE DETECTION OF BIOTHREAT AGENTS

This study demonstrates the utility of the centrifugal LabDisc platform using the RPA LabDisc cartridge and protocols by HSG-IMIT and the SONDE player by QLC for the "sample to answer" testing of biothreat pathogens. The developed extraction method and the optimized RPA assays for biothreat agents from this work have been successfully integrated on the RPA LabDisc cartridge according to need. The processing of the RPA LabDisc was also done completely automatically using the SONDE player.

In a proof-of-principle testing, all three bacterial biothreat pathogens (BA, Ftu and Ype) were tested on the centrifugal LabDisc platform. In this study, plasma spiked with one bacterial pathogen, or all three bacterial pathogens were processed automatically using the RPA LabDisc cartridge. The same samples were analyzed by two following manual laboratory methods: Nucleic acid extraction followed by real-time PCR, and nucleic acid extraction followed by real-time RPA assay. The results revealed that all these samples

were automatically extracted, and nucleic acids of the biothreat pathogens were correctly detected by the centrifugal LabDisc platform utilizing the RPA LabDisc. The “sample to answer” testing required the total time of less than 1 h. In the first 30 min, the samples were extracted on the RPA LabDisc, and the nucleic acids were isolated. Subsequently, the nucleic acids were mixed with dry RPA reagents and detected by fluorescence measurement in real-time within 20-30 min. Samples with single and multiple pathogens were detected in the same range as the “sample to answer” testing, which indicates that there were no inhibitory effects or cross reactions of the detection methods for different pathogens.

The result of manual testing by real-time PCR after nucleic acid isolation shows that all three pathogens were also correctly identified and the amount of the nucleic acids were quantified. Similarly, manual testing by real-time RPA after nucleic acid isolation detected all the samples correctly within 1 h. In both cases, the manual extraction was done in 40 min. The real-time PCR has required more than 2 h, whereas the manual real-time RPA was done in less than 10 min. Comparing these results, it is evident that the RPA assays using the manual methods, or the automated LabDisc platform could be performed in less than 1 h for NAT. Until now, there is only one commercially available method: GeneXpert by Cepheid, which can perform the “sample to answer” testing in this turnaround time. However, GeneXpert allows detection of a maximum of three targets using the multiplex reaction on a single cartridge. Depending on the pathogens, different cartridges are needed for testing by GeneXpert, which limits the simultaneous detection of different pathogens. In contrast to this, the RPA LabDisc has 11 detection chambers, which can be used for detection of 10 pathogens and 1 internal control simultaneously. The RPA LabDisc offers geometric multiplex reactions, which means that 11 single reactions can be performed simultaneously instead of the multiple reactions in one chamber. This approach using the RPA LabDisc also simplifies fluorescence monitoring devices. Only a single fluorescence labeled probe and detector are needed to perform 11 assays simultaneously using the RPA LabDisc.

In this study, manual laboratory methods were successfully integrated onto the centrifugal LabDisc platform, and whole NAT was performed automatically under 1 h. The utilization of MAGs based extraction method and RPA on the RPA LabDisc cartridge simplified the whole NAT testing workflow and enabled detection of biothreat pathogens simultaneously as a “sample to answer” testing. In future, all eight viral and bacterial pathogens included in the SONDE project can be tested using a single RPA LabDisc cartridge. Further development will focus also on the optimization of the RPA LabDisc

design, storage of reagents and reduction in time for sample preparation. This will allow the robust and reproducible automated NAT testing of the biothreat pathogens. Due to the portable design of the centrifugal LabDisc platform, it is perfectly suitable for point-of-care testing, low-resource, and in-field settings.

Chapter 2. REAL-TIME PCR PLATFORM

DEVELOPMENT OF ONE-STEP REAL-TIME REVERSE TRANSCRIPTION PCR FOR THE RAPID DETECTION OF FLAVIVIRUSES

A INTRODUCTION

A.1 FLAVIVIRUSES

The genus Flavivirus of the family *Flaviviridae* consists of more than 70 virus species including many arthropods-borne viruses. It contains highly pathogenic agents such as a Yellow Fever virus (YFV), West Nile virus (WNV), Japanese Encephalitis virus (JEV), Tick-borne Encephalitis virus (TBEV) and Dengue virus (DENV) which cause encephalitis or hemorrhagic fever and induce some of the most important emerging diseases in humans (Mackenzie, Gubler et al. 2004).

YFV is the prototype and name-giving member of the genus and family. Flaviviruses comprise three epidemiologically distinct groups: the mosquito-borne group, the tick-borne group and the unknown vector viruses (Porterfield 1980). They are enveloped positive-stranded RNA viruses with a genome of approximately 11 kb. The viral genome encodes a large single polyprotein from which three structural proteins, Capsid (C), Envelope (E) and Membrane (M) and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, are produced (Heinz and Allison 2000).

Flaviviruses pose a serious threat to public health around the world, especially in developing countries. In recent years, there was a dramatic increase in cases of DENV infection globally. This disease is endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the Western Pacific. About 50–100 million cases of DENV infection are reported worldwide every year, resulting in 500,000 cases of Dengue hemorrhagic fever requiring hospitalization and about 25,000 fatalities (WHO Dengue fever factsheet, 2009). Yellow Fever is endemic in 45 countries in Africa and Latin America, and annually YFV causes approximately 200,000 cases of human infections worldwide, resulting in 30,000 deaths (WHO YFV factsheet 2011). WNV is one of the most widespread flaviviruses and is distributed worldwide. WNV lineage 1 is endemic in the Americas, India and Australia; lineages 1 and 2 are known in Africa and Europe (Bakonyi, Hubalek et al. 2005; Bakonyi, Ivanics et al. 2006; Venter, Human et al. 2009). The recent WNV outbreak reported in August 2010 in Greece has been the first large outbreak in humans in Europe since the Romanian outbreak in 1996–1997 (Papa, Danis et al. 2010). JEV is the leading cause of viral encephalitis in Asia with 30,000–50,000 clinical cases reported annually and 10,000 deaths (CDC JEV factsheet). In addition, the increased spread of TBEV virus, which caused an infection of central nervous system, has been recognized in endemic area of Europe. The reported human cases of TEBV infections have dramatically increased by 400 % in these areas in last 30

years according to the ECDC (ECDC Factsheet for health professionals 2010) and pose danger to public health in European countries.

A.2 DIAGNOSIS OF FLAVIVIRUSES

The species in the family *Flaviviridae* include many human pathogenic viral agents causing a mild flu like to severe encephalitis or haemorrhagic illnesses. Therefore, clinical syndrome based diagnosis is not reliable to distinguish flavivirus infection from other infections and laboratory diagnosis needed to confirm the causative agent of infections. Serological tests based on the detection of virus-specific antibodies are generally performed for conventional flavivirus diagnosis. However, these tests have several limitations: immunological cross reactivity among Family *Flaviviridae* as well as differentiation of group- and serotype-specific flavivirus in endemic areas, where more than one flavivirus are co-circulating.

At present, several diagnostic techniques including molecular techniques and serological methods are used by most laboratories to detect a dengue infection. After the onset of illness, the virus can be detected in serum, plasma, circulating blood cells and other tissues for 2-7 days, and the duration of this viremic phase and viral load detected vary depending on the infecting virus as presented in Table A-1 (Domingo, Patel et al. 2011). Virus isolation, detection of viral nucleic acids and detection of viral antigens are common techniques used to diagnose flavivirus infection during the early phase. Seven days after onset of infection, the serological methods based on detection of virus-specific antibodies can be used. The serological methods comprise the immunofluorescence assay (IFA), the immunoglobulin M capture enzyme linked immunosorbent assay (IgM capture ELISA), the IgG ELISA and the virus-specific antigen capture ELISA. One limitation of serological methods is inability to differentiate flaviviruses due to the serological cross-reactions among the different flaviviruses (Hunsperger, Yoksan et al. 2009).

In recent years, molecular approaches based on detection of genomic viral RNA by the reverse transcription-Polymerase chain reaction (RT-PCR), modified RT-PCR methods, real-time PCR and isothermal amplification methods are playing an important role in diagnosis of flavivirus infections during the acute phase. In comparison to virus isolation and serological methods, molecular techniques are rapid, highly specific and sensitive, and allow the differentiation of flaviviruses. Since the early 1990s, several group-specific and generic molecular approaches have been developed for the detection of flaviviruses as reviewed previously (Lanciotti 2003; Sekaran and Artsob 2007; Domingo, Patel et al.

2011). A number of attempts have been made to detect simultaneously several flaviviruses in a single assay, using RT-PCR (Johnson, Wakeley et al. 2010). All of these assays vary in targeted region of the genome, assay format and final detection method.

Table A-1. Diagnostic algorithms of flaviviruses (Domingo, Patel et al. 2011).

	Acute phase	Convalescent phase	Preferred sample*	Viral load expected
YFV	RT-PCR, RT-qPCR, **IgM**, virus isolation	**IgM, IgG**	**Serum**, plasma, tissue	High
DENV	**RT-PCR**, RT-qPCR, **NS1 Ag**, **IgM**, virus isolation	**IgM, IgG**	**Serum**, plasma, CSF, PBMCs	up to 10^6 virions/ml
WNV	RT-PCR, IgM, IgG	**IgM, IgG**	**CSF**, serum	Low
JEV	RT-PCR, **IgM,** IgG	**IgM, IgG**	**CSF**, Sera, Blood, PBMCs	Low
TBEV	RT-PCR, **IgM, IgG**	**IgM, IgG**	**CSF**, Serum	Low

*** For molecular diagnosis**
The most commonly used methods in diagnostic laboratories are shown in bold
Ag: antigen; CSF: cerebrospinal fluid; PBMCs: Peripheral Blood Mononuclear Cells

YFV: Yellow fever virus; DENV: Dengue viruses; WNV: West Nile virus; JEV: Japanese encephalitis virus; TBEV: Tick-borne encephalitis virus; IgM: Immunoglobulin M; IgG: Immunoglobulin G; RT-PCR: reverse transcriptase polymerase chain reaction

For the identification of flaviviruses, molecular amplification methods targeting regions of highly conserved NS3 and NS5 genes have been described widely, predominantly in the format of nested, hemi-nested RT-PCR or SYBR green real-time PCR. Nested and hemi-nested techniques present the lower sensitivity than specific flavivirus approaches, and they are more prone to cross contamination. In contrast to these methods, real-time RT-qPCR utilizing a specific internal fluorogenic probe (Taqman probe) offers excellent sensitivity and minimizes the risk of cross contamination as the whole reaction occurs in one-step format in closed tubes. In addition, the use of real-time RT-qPCR allows the quantification of genome copies when a standard curve is included in the assay. Besides conventional Taqman probes also DNA probes with the conjugated minor groove binder (MGB) group or locked nucleotides (LNA) were used in real-time PCR, which forms extremely stable duplexes with single-stranded DNA targets, allowing shorter probes with the high melting temperature (Kutyavin, Afonina et al. 2000; Goldenberg, Landt et al. 2005).

A.3 AIM OF THIS STUDY

The aim of this study was to develop a rapid, sensitive and reliable real-time RT-qPCR assay for the simultaneous detection of several important flaviviruses. To realize such an assay, conserved NS5 region of flavivirus genome was used as a target for designing the LNA probe. The introduction of locked nucleic acids (LNA) probe in real-time RT-qPCR increases the assay's robustness, specificity and sensitivity. Furthermore, the method can be standardized to quantify the viral load using this specific LNA probe based real-time PCR assay.

B MATERIALS AND METHODS

B.1 VIRUSES AND ISOLATION OF VIRAL RNA

All flavivirus and non-flavivirus strains used were derived from cell culture and provided by the Robert Koch Institute, Berlin, Germany. The following inactivated and stable virus preparations were used in this study: DENV-1 VR344 (Thai 1958); DENV-2 VR345 (TH-36 strain); DENV-3 VR216 (H87 strain); DENV-4 VR217 (H241 strain); WNV Uganda strain (AY532665); WNV Israel (H. Bin, Sheba Medical Centre, Israel), Usutu virus (AY453411); JEV (ATCC SA14-14-2); Saint Louis Encephalitis virus (SLEV) (ATCC VR-1265); TBEV strain K23 (AF091010); YFV strain 17D (X03700); YFV strain ASIBI (AY640589); YFV strain Brazil; YFV strain Ivory Coast; Russian Spring Summer Encephalitis virus (RSSEV); Chikungunya virus (LR 2006); Chikungunya virus African isolate; Sindbis virus; Rift Valley Fever virus and Influenza A virus subtype H5N1 (A/dk/Germany R603/06 H5N1). Two different standard preparations of H1N1 Influenza viruses (A/California/04/2009 and A/Hamburg/04/2009) used in this study were provided by the European Network for Diagnostics of Imported Viral Diseases (ENIVD). Viral RNA was isolated from 140 µl aliquots of cell culture supernatants, using the QIAamp Viral Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 100 µl of elution buffer and stored at -80 °C until further use.

B.2 TAQMAN PRIMERS AND PROBE

Available full-length flavivirus sequences, partial sequences covering the NS5 region from the database (NCBI) and previously published primers (Scaramozzino, Crance et al. 2001; Chien, Liao et al. 2006) were aligned using MEGA5 software. Based on the alignment, degenerate generic primers (Table 1) and corresponding primers representing all known sequence variants were designed for generic amplification of flaviviruses. In addition, 22 sequence variant-specific fluorescent-labelled flavivirus probes and two degenerate probes containing LNA nucleotides were designed. LNA nucleotide analogues contain a 2'–5' bridge, keeping the ribose moiety in an RNA-like configuration. They bind tighter to the complement sequence and thus allow the use of shorter probes. This is particularly important when only short conserved regions in the target sequence are available or in order to link the binding power of the probe to a more specific or conserved sequence motif.

Table B-1. Oligonucleotide sequence of primers and probes used in Pan-Flavi real-time RT-PCR assay.

Primers & Probe	Sequence (5´→ 3´)	Concentration (pmol)	Orientation[a]	T_m (°C)
Flavi all sense	TACAACATgATggggAARAgAgARAA	10	S	54.7
DEN4 F	AACATgATgggRAAACgTgAGAA	10	S	60.18
Flavi all antisense	gTgTCCCAgCCNgCKgTgTCATCWgC	10	AS	69.6
Flavi all probe 3 mix	[b]FAM-Tg**+g**TWYATgT**+g**gYTNg**+g**RgC-BBQ	5	S	69.5
	[c]FAM-CCgTgCCATATggTATATgTggCTgggAgC-BBQ	0.5	S	74.7
	[d]FAM-TTTCTggAATTTgAAgCCCTgggTTT-BBQ	0.5	S	68

Tm: temperature, Locked nucleic acid bases are written as '+_', e.g., +A
Degenerated bases: R = (A/G), W = (A/T), K = (T/G), Y = (C/T), N = (A/G/T/C)
[a] S: sense orientation, AS: antisense orientation.
[c] Flavi all Sonde
[d] Flavi Sonde YFV
[e] Flavi Sonde DEN4

B.3 PAN-FLAVIVIRUS REAL-TIME RT-PCR ASSAY

The pan-flavivirus real-time RT-PCR assay (Pan-Flavi assay) was performed using the Transcriptor One-Step RT-PCR Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. 5 µl of RNA, 10 pmol of primer Flavi all sense, 10 pmol of primer Flavi all antisense, 10 pmol of primer DEN4 F and 1 µl of probe mixture Flavi probe 3 (Table 1) was added to the Transcriptor One-Step RT-PCR master mix. Optimized cycling conditions were as following: reverse transcription at 55 °C for 2 min, denaturation at 95 °C for 30 s and 45 cycles of 95 °C for 10 s and 60 °C for 25 s. The amplification was performed on a LightCycler 480 instrument, software version 1.5 (Roche, Basel, Switzerland).

To quantify the Pan-Flavi assay, the serial dilution of standard plasmids (10–10^6 copies/µl) were tested in duplicate within the same sample run and compared. Standard plasmid was prepared by cloning the PCR product of YFV using a TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions and quantified by spectrophotometric method.

B.4 SPECIFIC REAL-TIME RT-PCR FOR DIFFERENT FLAVIVIRUSES

For the specific detection, already published group-specific assays were used. Except for DENV and JEV, where in-house assays were used.

TBEV-specific primers and probe directed to the NS1 gene were used to detect and quantify genomic RNA of TBEV as described previously (Achazi, Nitsche et al. 2011). Real-time PCR was carried out in one step on an ABI 7500 cycler (Applied Biosystems, California, USA), using the Superscript III Platinum One-Step RT-qPCR Kit (Invitrogen, Karlsruhe, Germany).

A WNV-specific diagnostic real-time PCR assay for the detection of lineages 1 and 2 was used to detect and quantify genome equivalent copies (Geq) of WNV as described previously (Linke, Ellerbrok et al. 2007). Real-time PCR was performed in a two-step format on the Stratagene MX 3000 cycler (Agilent Technologies, Santa Clara, USA).

YFV-specific primer combination YFV FP/RP and probe YFV LNA2 were used to detect and to quantify the genomic RNA of YFV as described previously (Weidmann, Faye et al. 2010). The assay was performed in an one-step format on the LightCycler 480 instrument using the QuantiTect Virus Kit (Qiagen, Hilden, Germany).

DENV-genomic RNA was tested and quantified by an in-house real-time quantitative RT-PCR as described previously (Domingo, Niedrig et al. 2010). The assay is a DENV group-specific assay and was carried out in one-step format on an ABI 7500 real-time PCR system using the AgPath-ID One-Step RT-PCR Kit. Plasmid standards were used for the quantification of the DENV Geq.

A JEV-specific in-house real-time PCR assay was used for detection and quantification of JEV geq. Real-time PCR was performed in a two-step format on the Stratagene MX 3000 cycler. cDNA was synthesized by reverse transcription of 5 µl purified RNA in a 20 µl reaction volume with 4 µl of 5× First-Strand Buffer, 4 µl of 0.1 M DTT, 0.4 µl of 25 mM dNTPs and 1 µl of SuperScript RT (200 U/µl) (Invitrogen, Karlsruhe, Germany). The template RNA was heated to 65 °C for 5 min and rapidly cooled to 4 °C. After addition of the master mix, samples were incubated for 60 min at 37 °C for reverse transcription. The reaction was stopped by enzyme inactivation at 93 °C for 5 min, and then cooled to 4 °C. cDNA was stored at −20 °C until further use. After cDNA synthesis, RT-qPCR was performed using the Platinum Taq polymerase (Invitrogen, Karlsruhe, Germany) in a total volume of 25 µl containing 5 µl of cDNA, 2 µl of 10x Reaction Mix, 4 mM $MgCl_2$, 300 nM of each primer, 100 nM of probe and 2.5 µM of dNTPs. Thermal cycling was done on a

Stratagene Mx3000 cycler instrument under the following conditions: 15 min at 95 °C; 45 cycles of 15 s at 95 °C and 30 s at 60 °C. To quantify JEV genomic copies, the ten-fold serial dilution of the standard plasmids (10–10^6 copies/μl) were tested within the same sample run.

C RESULTS

C.1 DESIGN AND OPTIMIZATION OF PAN-FLAVI ASSAY

Primers already published (Scaramozzino, Crance et al. 2001; Chien, Liao et al. 2006) were aligned against genomic sequences of all flaviviruses available in the NCBI database to verify flavivirus-generic amplification capacity. Based on the published primers, degenerate primers were designed with some modifications to increase broad-spectrum flavivirus amplification. In addition, flavivirus species-specific primers were selected in order to replace the degenerate primers with a mixture of individual primers and reduce the overall degeneration. Altogether 17 different forward and 15 reverse non-degenerate primers with 4 reverse primers at a different location have been used. Testing of the primer mix with viral RNA revealed that the performance of this primer mix was not better than that using degenerate primers containing wobble positions, with the exception of DEN4 (data not shown). A specific F-primer for DEN4 improved the primer performance significantly (Table 1). Also for the probes, 22 specific sequences covering all known sequence variants and established mixtures optimized to yield comparable threshold cycle (Ct) values have been used and similar signal levels have been found for all tested viruses. The results were compared with two single probes containing degenerate positions and LNA bases. One LNA probe gave reliable results (Fig. C-1).

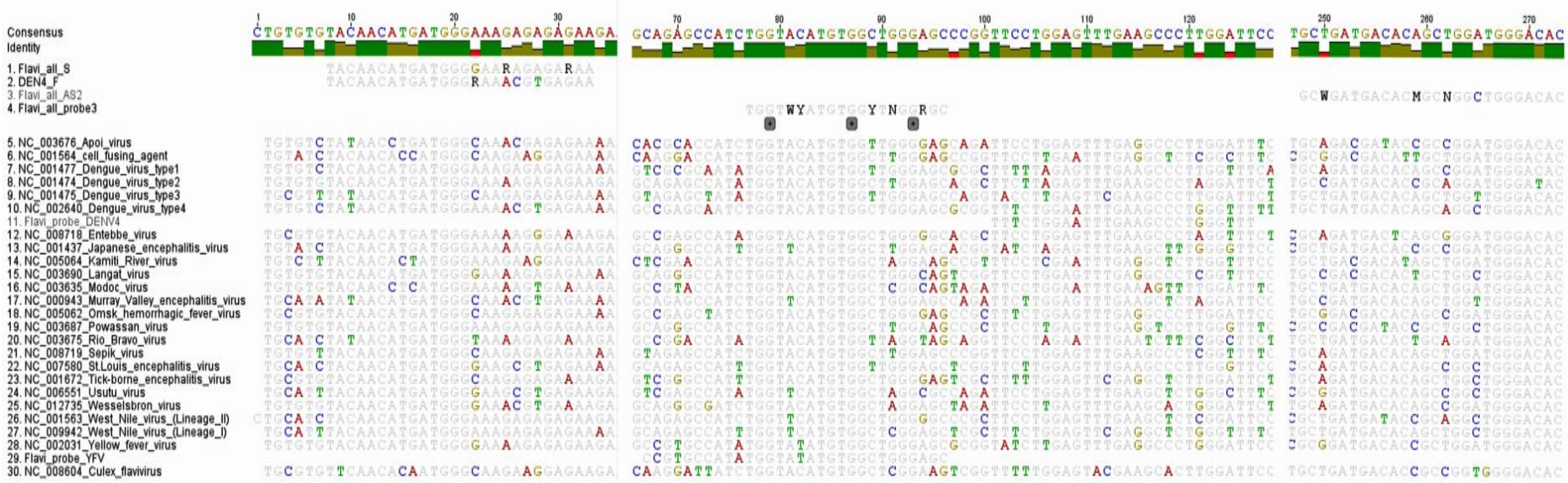


Figure C-1. Sequence alignment of primers and probe with 26 flaviviruses conserved region of NS5 gene. + Symbol under oligonucleotides indicates the position of a locked nucleotide.

C.2 EVALUATION OF SPECIFICITY OF THE PAN-FLAVI ASSAY

The specificity of the Pan-Flavi assay was tested against a panel of 16 flaviviruses, one virus of the *Bunyaviridae* family, three viruses of the Togaviridae family and three viruses of the *Orthomyxoviridae* family. RNA isolates from chosen panels were amplified using the Pan-Flavi assay. All 16 flaviviruses were amplified by this assay, showing 100 % amplification of the tested flaviviruses. Neither the non-flaviviruses nor the negative controls were amplified, demonstrating the absence of cross-reactivity of the Pan-Flavi assay with non-flaviviruses (Table C-2).

C.3 EVALUATION OF SENSITIVITY OF THE PAN-FLAVI ASSAY

To determine the linearity of the assay, the 10-fold serial dilution of the standard plasmids were tested on the Pan-Flavi assay. The assay was shown to be linear for the standard plasmid tested over a range of five log10 dilutions. The lowest number of standard plasmid detected by the Pan-Flavi assay was 10 copies/reaction.

The sensitivity of the Pan-Flavi assay to detect the viral RNA copies was determined by using the 10-fold serial dilution of the RNA of DENV-1, YFV strain 17D and TBEV strain K23. These RNA stocks were quantified by virus species-specific real-time RT-PCR as described previously (Domingo, Niedrig et al. 2010; Weidmann, Faye et al. 2010; Achazi, Nitsche et al. 2011). The Pan-Flavi assay's sensitivities were 100 copies for YFV 17D, 10 copies for DENV-1 and 10 copies for TBEV K23 (Table C-1).

Table C-1. Test of sensitivity of Pan-Flavi assay with viral RNA dilution.

Quantity(Geq/rxn)	Ct values for DENV-1	Ct values for TBEV strain K23	Ct values for YFV strain 17D
1×10^7	16.8	16.6	-
1×10^6	20.5	19.5	20.8
1×10^5	23.4	23.0	24.2
1×10^4	27.4	26.2	27.6
1×10^3	30.2	29.4	30.1
1×10^2	37.1	31.7	36.4
1×10^1	39.7	33.0	No Ct

Geq: genome equivalent copies, Ct: threshold cycle, YFV: Yellow fever virus, TBE: Tick-borne encephalitis virus, DENV: Dengue virus

C.4 COMPARISON OF THE PAN-FLAVI ASSAY WITH VIRUS SPECIES-SPECIFIC REAL-TIME RT-PCR

RNA isolates of DENVs, WNVs, YFVs, TBEVs and JEVs were detected quantitatively by using a virus species-specific assay, and the results were compared with those obtained by the Pan-Flavi assay for these viruses (Table C-2).

Table C-2. Performance of the Pan-Flavi assay to detect different flaviviruses and its comparison with flavivirus-specific assays.

Virus	Strain/ Acc. No.	Pan-flavi assay		Virus-specific assay	
		Mean of Ct Value	Mean of Geq/ml	Mean of Ct Value	Mean of Geq/ml
Flaviviridae					
DENV-1	ATCC VR-344	15.6	2.0×10^9	15.9	4.6×10^9
DENV-2	ATCC VR-345	20.8	6.6×10^7	18.8	6.7×10^8
DENV-3	ATCC VR-1256	19.4	1.7×10^8	20.3	2.4×10^8
DENV-4	ATCC VR-1257	16.3	1.3×10^9	16.2	3.9×10^9
YFV 17D	X03700	19.2	1.9×10^8	20.5	5.6×10^8
YFV ASIBI	RKI reference strain	23.7	9.1×10^6	23.7	4.7×10^7
YFV Brazil	RKI reference strain	21.2	5.0×10^7	22.5	1.2×10^8
YFV Ivory Coast	RKI reference strain	21.4	4.2×10^7	21.3	3.1×10^8
WNV Uganda	AY532665, lineage 1	28.7	3.2×10^5	26.8	3.4×10^6
WNV Israel	H. Bin, SMC, Israel lineage 2	19.8	1.2×10^8	19.9	4.0×10^8
TBEV K23	AF091010	16.3	1.3×10^9	16.8	2.1×10^8
RSSEV	RKI reference strain	24.2	6.7×10^6	19.4	4.1×10^6
TBEV Louping ill	RKI reference strain	20.6	7.4×10^7	20.4	2.0×10^7
JEV	ATCC SA14-14-2	19.4	2.4×10^8	16.7	7.7×10^8
SLEV	ATCC VR-1265	22.2	2.6×10^7	N. d.	N. d.
Usutu virus	AY453411	22.5	2.1×10^7	N. d.	N. d.
Bunya viridae					
Rift valley fever virus		No Ct	Negative	N. d.	N. d.
Togaviridae					
Chikungunya virus	LR 2006	No Ct	Negative	N. d.	N. d.
Chikungunya virus	ST05.African isolate	No Ct	Negative	N. d.	N. d.
Sindbis virus	RKI reference strain	No Ct	Negative	N. d.	N. d.
Influneza virus					
H1N1 Hamburg	A/Hamburg/04/2009 H1N1	No Ct	Negative	N. d.	N. d.
H1N1 California	A/California/04/2009 H1N1	No Ct	Negative	N. d.	N. d.
H5N1	A/dk/Germany R603/06 H5N1	No Ct	Negative	N. d.	N. d.
Negative control		No Ct	Negative	No Ct	Negative

Geq: genome equivalent copies, Ct: threshold cycle, N.d.: not done

DENV: Dengue virus, JEV: Japanese encephalitis virus, RSSEV: Russian spring-summer encephalitis virus, WNV: West Nile virus, TBEV: Tick-borne encephalitis virus, SLEV: Saint Louis encephalitis virus, RVFV: Rift valley fever virus, YFV: Yellow fever virus

All four serotypes of DENVs were detected by the Pan-Flavi assay and a Dengue-specific assay. The Ct values and geq/ml determined by both methods were almost identical except for DENV-2. Lower values by one log for DENV-2 were detected by the Pan-Flavi assay when compared with the Dengue-specific assay. For all YFVs, the results obtained with the Pan-Flavi assay and YFV-specific assay were completely comparable with each other. Both WNV lineages were detected well by the Pan-Flavi and a WNV-specific assay. However, lower values by one log for WNV lineage 1 were detected by the Pan-Flavi assay when compared with a WNV-specific assay. All three subtypes of TBEV were also detected by the Pan-Flavi assay and a TBEV-specific assay well. The Ct values and geq/ml determined by both methods were identical except for RSSEV. For the detection of JEV, the Pan-Flavi assay had half a log lower sensitivity than a JEV-specific assay. As WNV- and JEV-specific assays were performed in two-step formats, this being the possible reason for their higher sensitivity when compared to the Pan-Flavi assay. Overall, the performance of the Pan-Flavi assay was comparable to virus species-specific assays except for minor differences for some strains of flaviviruses.

D DISCUSSION

The continuous emergence of flaviviruses is a reality and poses major public health concerns around the world. Techniques used for molecular diagnosis and surveillance of flaviviruses should be able to detect rapidly and identify a wide range of flavivirus species with a high level of specificity and sensitivity. For this purpose, a LNA probe-based real-time RT-PCR (Pan-Flavi assay) for the detection of flavivirus RNA by one single method was successfully developed. Demonstrating the ability of the Pan-Flavi assay to detect the flavivirus genus, most of the important human-pathogenic flaviviruses have been tested. The Pan-Flavi assay was also tested with other viruses like phleboviruses, alphaviruses and influenza viruses to determine the specificity.

In recent years, generic approaches targeting highly conserved NS3 and NS5 genes have been described for the diagnosis of flaviviruses (Domingo, Niedrig et al. 2010; Johnson, Wakeley et al. 2010; Weidmann, Faye et al. 2010; Achazi, Nitsche et al. 2011; Domingo, Patel et al. 2011). These methods were predominantly in the format of nested RT-PCR or SYBR green real-time RT-PCR and in particular required post-amplification methods such as sequencing or melting temperature analysis. These methods have generally shown variable or lower sensitivity than the species-specific approaches when evaluated during the EQA exercises for molecular diagnosis of different flaviviruses (Domingo, Niedrig et al. 2010). However, in some cases their performance was comparable to those from the species-specific methods (Scaramozzino, Crance et al. 2001; Domingo, Niedrig et al. 2010). Therefore, the use of generic methods for the molecular diagnosis of flaviviruses is only advisable when post-amplification techniques (sequencing, melting temperature) are performed afterwards to identify the specific detection of flaviviruses.

The primer sequences with some degenerate nucleotides (Fig. C-1) were chosen so that it would hybridize to a target sequence that is conserved among most flavivirus species. These degenerate primers showed a better efficiency in detecting the broad range of flaviviruses, when compared to the previously published primers. Designing a single probe for the universal detection of flaviviruses is challenging because of the genetic diversity of flaviviruses and the restricted length of a conserved region in the alignment of flavivirus sequences. To overcome this problem, LNA probe was chosen for the Pan-Flavi assay. Major advantages of LNA probes in comparison to other real-time PCR formats are the shorter probe length, biological stability, increased sensitivity, specificity and quenching efficiency which has been demonstrated by many studies (Frieden,

Hansen et al. 2003; Letertre, Perelle et al. 2003; Johnson, Haupt et al. 2004; Ugozzoli, Latorra et al. 2004; Weidmann, Faye et al. 2010). Also in this study, a LNA-based probe has enabled a real-time PCR assay to detect different flaviviruses in one reaction.

At present, RT-PCR in nested or hemi-nested format is used most frequently. It requires sequencing for the identification of viruses and needs approximately one day for experimentation. In addition, this format carries a high risk of contamination caused by open handling of highly concentrated PCR products, increasing the risk of false positives. In contrast, the Pan-Flavi assay presented here requires about 50 minutes for specific and sensitive detection of several flaviviruses in one reaction. The detection sensitivity of YFV, DENV, TBEV, JEV and WNV, using the Pan-Flavi assay described here was close to or as good as that of flavivirus species-specific real-time RT-PCR assays. Three possible explanations for these minor differences can be considered. First, the variation in nucleotide sequences of the flaviviruses and the degenerate oligonucleotides have led to variable results in the real-time PCR assay. Secondly, different kits were used for the Pan-Flavi assay and flavivirus species-specific assays. In addition, WNV and JEV-specific assays were performed in a two-step PCR compared to the one-step Pan-Flavi assay. One-step PCR assays are also reportedly less sensitive than two-step PCR assays (Wacker and Godard 2005).

The sensitivity of the Pan-Flavi assay to detect RNA was demonstrated by using a 10-fold serial dilution of viral RNA from DENV-1, YFV and TBEV. This assay was able to detect 10–100 viral RNA copies per reaction depending on the flavivirus. As a wide range of human-pathogenic flaviviruses has been detected rapidly with a high level of sensitivity by the Pan-Flavi assay, this method could be a very useful tool for the detection of important flaviviruses in the livestock samples. Additionally, this method allows quantification of flavivirus RNA by use of standard plasmids or standardized in-vitro RNA, providing information regarding viral load and can be easily established to other laboratories. Furthermore, the length of the amplicon in the Pan-Flavi assay is about 260 bp and sufficient to distinguish different flaviviruses based on the sequence variability. If sequencing or pyrosequencing is combined with this assay, it would be even possible to differentiate flavivirus species rapidly. Therefore, the Pan-Flavi assay could be an useful tool to confirm the presence of flaviviruses in sample without any post amplification method, and it will complement the species-specific flavivirus assays. In future, this Flavi-all assay is tested on other flaviviruses to check the broad range of this assay moreover validated by screening livestock and clinical samples for detection of flaviviruses.

In summary, a single-tube Pan-Flavi assay was successfully developed and evaluated by comparing it with the flavivirus species-specific assays. The Pan-Flavi assay would be an useful tool for the detection of flaviviruses in clinical and livestock samples, and would aid in the molecular diagnosis and surveillance studies of various vectors and non-human hosts.

Chapter 3. RAPIDSTRIPE DETECTION SYSTEM

RAPIDSTRIPE H1N1 TEST FOR DIAGNOSIS OF THE PANDEMIC SWINE-ORIGIN INFLUENZA A (H1N1)

A INTRODUCTION

A.1 SWINE ORIGIN INFLUENZA A (H1N1) VIRUS

In April 2009, a novel swine-origin influenza A (H1N1) virus (S-OIV A) was detected in specimens of several patients in the United States and Mexico. This virus spreads person-to-person, probably in the same way that seasonal influenza viruses spread (WHO 2009). On June 11 2009, the World Health Organization declared influenza pandemic, caused by novel S-OIV A (H1N1) and raised the pandemic alert level to phase 6. Through rapid and frequent international travel, this virus spread worldwide to more than 214 countries and overseas territories or communities reporting laboratory confirmed cases of pandemic influenza A H1N1, including at least 18,337 deaths by 16 July 2010 (WHO H1N1 2009 update 2010).

In the 20th century, there have been three pandemics of influenza origin, A/H1N1 from 1918 to 1919 (“Spanish influenza”), A/H2N2 from 1957 to 1963 (“Asian influenza”) and A/H3N2 from 1968 to 1970 (“Hong Kong influenza”). In 1918, an H1N1 virus, the Spanish flu closely related to avian viruses adapted to replicate efficiently in humans and was the most devastating, causing about 20 million human deaths. The pandemic that began in April 2009 was caused by a novel S-OIV A (H1N1) that represents a quadruple re-assortment of two swine strains, one human strain, and one avian strain of influenza; the largest proportions of genes coming from swine influenza viruses. The sequence analysis of the new S-OIV (H1N1) (A/California/04/2009) showed that the virus has an unique genome composition of human, swine and avian influenza A viruses that has not been previously identified (Dawood, Jain et al. 2009).

A.2 DIAGNOSIS OF S-OIV A (H1N1)

The signs and symptoms caused by novel S-OIV A (H1N1) have been characterized by self-limited, uncomplicated febrile respiratory illness and similar to those of seasonal influenza like fever, sore throat, cough, headache, rhinorrhea and myalgia. Vomiting and diarrhea have been also common, neither of which is typical for seasonal influenza (Cao, Li et al. 2009; Dawood, Jain et al. 2009; Louie, Acosta et al. 2009). Compared to the previous influenza pandemic (H1N1) of 1918 and 1919, a significant minority of cases has been severe (Fraser, Donnelly et al. 2009).

Rapid diagnosis of influenza is important to the introduction of antiviral therapy and quarantine measures, since antiviral therapy should preferably be initiated within 24 h

after the appearance of the patient's first clinical symptoms (van der Vries and Schutten 2010). According to the guideline for diagnosis of pandemic S-OIV A (H1N1) by the Centers for Disease Control and Prevention (CDC, USA), the probable clinical case must be confirmed by either positive RT-PCR or virus culture (CDC). Real time RT-PCR is the recommended test for the diagnosis of pandemic S-OIV A (H1N1) (Ellis, Iturriza et al. 2009). Isolation of S-OIV A (H1N1) by virus culture is also a diagnostic conformation of infection, but may not yield timely results for clinical management. Several rapid antigen tests and immunofluorescence tests are available for the diagnosis of pandemic S-OIV A (H1N1). However, the sensitivity of these tests varies widely (Vasoo, Stevens et al. 2009; Ganzenmueller, Kluba et al. 2010; Karre, Maguire et al. 2010) and they are not able to distinguish between the seasonal and new pandemic S-OIV A (H1N1) strains (CDC). Thus, the differentiation needs to be achieved by RT-PCR or by virus culture, followed by hemagglutinin inhibition tests. In RT-PCR based methods, a detection of amplified PCR products requires either time-consuming gel electrophoresis separation or expensive real time PCR instruments. The rapid detection of PCR products on lateral flow strip (LFS) as endpoint analysis would be a simple and cost-effective alternative to these laborious, expensive, and instrument-dependent methods.

A.3 AIM OF THIS STUDY

Lateral flow tests based on the principle of immunochromatography are currently used for qualitative analysis of several types of analytes (Posthuma-Trumpie, Korf et al. 2009). A new advanced method of lateral flow test, so called nucleic acid lateral flow (NALF) assay allows the detection of genetic material such as nucleic acids. This article describes a NALF assay called rapidSTRIPE assay used as a molecular-genetic rapid test for the diagnosis of the pandemic S-OIV A (H1N1). This assay is based on rapid amplification/hybridization (RAH) technology (Analytik Jena AG, Jena, Germany). The aim of this study was to evaluate the rapidSTRIPE assay based on rapid amplification/hybridization reaction coupled to instrument independent detection of the amplification products by user friendly dipsticks. Furthermore, the diagnostic sensitivity and specificity for rapidSTRIPE assay were determined and compared to the real-time RT-PCR method (Schulze, Nitsche et al. 2010), which is considered widely as the gold standard (Ellis, Iturriza et al. 2009).

B MATERIALS AND METHODS

B.1 VIRUS STRAINS AND CLINICAL SPECIMENS

Two different standard preparations of H1N1 Influenza viruses (A/California/04/2009 and A/Hamburg/04/2009) were provided by the European Network for Diagnostics of imported Viral Diseases Collaborative Laboratory Response Network (ENVID-CLRN). Different representative influenza A subtype virus strains (A/Brisbane/59/07 H1N1, A/Caledonia/20/99 H1N1, A/Brisbane/10/07 H3N2, A/Wellington/1/04 H3N2, A/dk/Germany R603/06 H5N1, A/dk/Vietnam TG24-01/05/H5N1, A/Italy/472/99/ H7N1, A/Germany/R11/01/H7N1); influenza virus B (B/Malasiya/250604 (Victoria lineage), B/Iangsu/10/03 (Yamagata lineage)) for specificity test were provided for the specificity test by the National Reference Center for Influenza, Robert Koch Institute (RKI), Berlin, Germany.

B.2 RNA EXTRACTION AND cDNA SYNTHESIS

The specimens were collected in different patient centers in Saxony, Germany as pharyngeal or nasal swabs and stored at 4 °C up to further use. Total RNA from swabs and the reference virus material was extracted by module 1 of rapidSTRIPE H1N1 Assay KingFischer system (KF) (Analytik Jena AG, Jena, Germany) according to the manufacturer's instructions, whereas viral RNA were extracted from collected swabs as well as swabs spiked with virus standards using Innuprep RNA virus KFFLX kit (Analytik Jena AG, Jena, Germany) and Kingfisher FLX automated purification system (Thermo Scientific, Finnalnd). Samples were eluted in 120 µl of elution buffer. Nucleic acid extraction can be performed either manually or automatically using rapidSTRIPE H1N1 assay KF system. In addition, RNA samples from clinical specimens included in this study were tested with in-house glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RT-PCR to check the quality of the extracted RNA samples using AffinityScript One-Step RT-PCR Kit (Agilent Technologies, Santa Clara, USA) according to manufacturer's instruction. The primer set was designed to span introns and restricted only to PCR amplification of cDNA templates. RNA was subjected to PCR with Primer GAPDHF: 5´-CCATGGAGAAGGCTGGGGCT-3´ and Primer GAPDHR: 5´-GGTGGTGCAGGAGGCA-TTGCT-3´. Subsequently, the amplification products were analysed by 2% ethidium bromide-stained agarose gel and visualized under UV light. Subsequently, the amplification products were analysed by 2% ethidium bromide-stained agarose gel and visualized under UV light.

The cDNA synthesis was performed by module 2 of the rapidSTRIPE H1N1 Assay KF system, where 10 µl of RNA were mixed with 5 µl of an RT-master mix containing 10x RT-buffer, 4 µM of dNTPs, 1.4 mM of DTT, 1.25 µM of primer HN1 and RT-enzyme at a final volume of 15 µl. The temperature parameters were set to 30 min at 55 °C and 10 min at 95 °C on a speed cycler or Alpha SC cycler (Analytik Jena AG, Jena, Germany). Samples of cDNA were used for real time RT-PCR and rapidSTRIPE H1N1 assay. Additionally, the cDNA synthesis was performed using 1 µM random hexamer primer under the temperature parameter of 37 °C at 45 min and 10 min at 95 °C for further specificity tests and stored at -80 °C up to further use.

B.3 REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR (RT-QPCR)

As the method of comparison, an RT-qPCR targeting the Haemagglutanin (HA) gene developed at Robert Koch Institute was chosen (Schulze, Nitsche et al. 2010). After cDNA synthesis, RT-qPCR was performed using the Platinum Taq polymerase (Invitrogen) in a total volume of 25 µl containing 2 µl of cDNA, 2 µl of 10x Reaction Mix, 4 mM $MgCl_2$, 300 nM of each primer FluSW H1 F236 and FluSW H1 R318, 100 nM FluSW H1 TM 292 probe and 2.5 µM of dNTPs. All primers and probes were synthesized by Metabion, Martinsried, Germany. The thermal cycling was done on Stratagene Mx3000 cycler instrument (Agilent Technologies, Inc., Santa Clara, USA) under the following conditions: 15 min at 95 °C; 45 cycles of 15 s at 95 °C and 30 s at 58 °C. To quantify the RT-qPCR, the 10-fold serial dilution of the standard plasmids (10 - 10^6 copies/µl) were tested in duplicate within a same sample run and compared. Standard plasmid was prepared by cloning fresh PCR products using a TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) according to the kit instruction and quantified by spectrometric method.

B.4 OLIGONUCLEOTIDES FOR LATERAL FLOW STRIP TEST

Multiple-sequence alignments of the HA gene of S-OIV A (H1N1) was performed by analyzing HA gene available from public gene database by Geneious pro 4.0.3 software. Primers and probe used in this assay were designed by modifying published RT-qPCR assay for S-OIV A (H1N1) from RKI (Schulze, Nitsche et al. 2010) to customize LFS assay and compared to the consensus sequence achieved from the alignment for its specificity. Furthermore, predicted specificity of the primers and probe were analyzed with BLSTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer HN2 was labeled with biotin

at its 5´end and probe HN was labeled with fluorescein isothiocyanate (FITC) at its 5´end (Table B-1). All primers and probe were synthesized by Metabion, Martinsried, Germany.

Table B-1. Oligonucleotides used for rapidSTRIPE H1N1 assay

Primer/probe	Target gene of S-OIV A	Oligonucleotide sequence (5´-3´)	Tm (°C)
Primer HN1	HA	TGGGAAATCCAGAGTGTGAATCACTCTC	65
Primer HN2	HA	Biotin-CGTTCCATTGTCTGAACTAGRTGTTTCC	65
Probe HN	HA	FITC-AGCAAGCTCATGGTCCTACATT	55

B.5 LATERAL FLOW STRIP (LFS) ASSAY BASED ON NUCLEIC ACID AMPLIFICATION

After cDNA synthesis, amplification/hybridization was performed by module 2 of rapidSTRIPE H1N1 Assay KF system according to the manufacturer's instructions. In brief, 3 µl of the cDNA were subjected to PCR in 25 µl of final volume reaction containing 2.5 µl of 10xSpeedAmp PCR buffer, 150 nm of primer HN1 and 300 nm of primer HN2, 300 nm of probe HN, 2.5 µM of dNTPs using innuTaq Hot DNA Polymerase. The thermal cycling was performed using Speed cycler or Alpha SC cycler under following conditions: 2 min at 95 °C; followed by 42 cycles of 4 s at 95 °C, 4 s at 62 °C and 20 s at 72 °C; and final cycle for hybridization 5 min at 95 °C, 10 min at 52 °C. Final detection was carried out using module 3 of rapidSTRIPE H1N1 Assay KF system according to the kit instructions. Briefly, 15 µl of amplification/hybridization product was added to the sample pad on lateral flow strip and placed into the tube containing 150 µl of running buffer at room temperature. As the lateral flow strip comes into contact with the running buffer, the biotin labeled amplification/hybridization products flows into the strip to react first with the streptavidin gold particles and attract them to form a conjugate for the visualization. When the amplification/hybridization product-gold conjugate reaches the detection line, this complex is captured by anti FITC antibodies resulting in a visible purple line. The excess of colloidal gold conjugate is captured at the control line to monitor the flow along the strip (Fig. B-1).

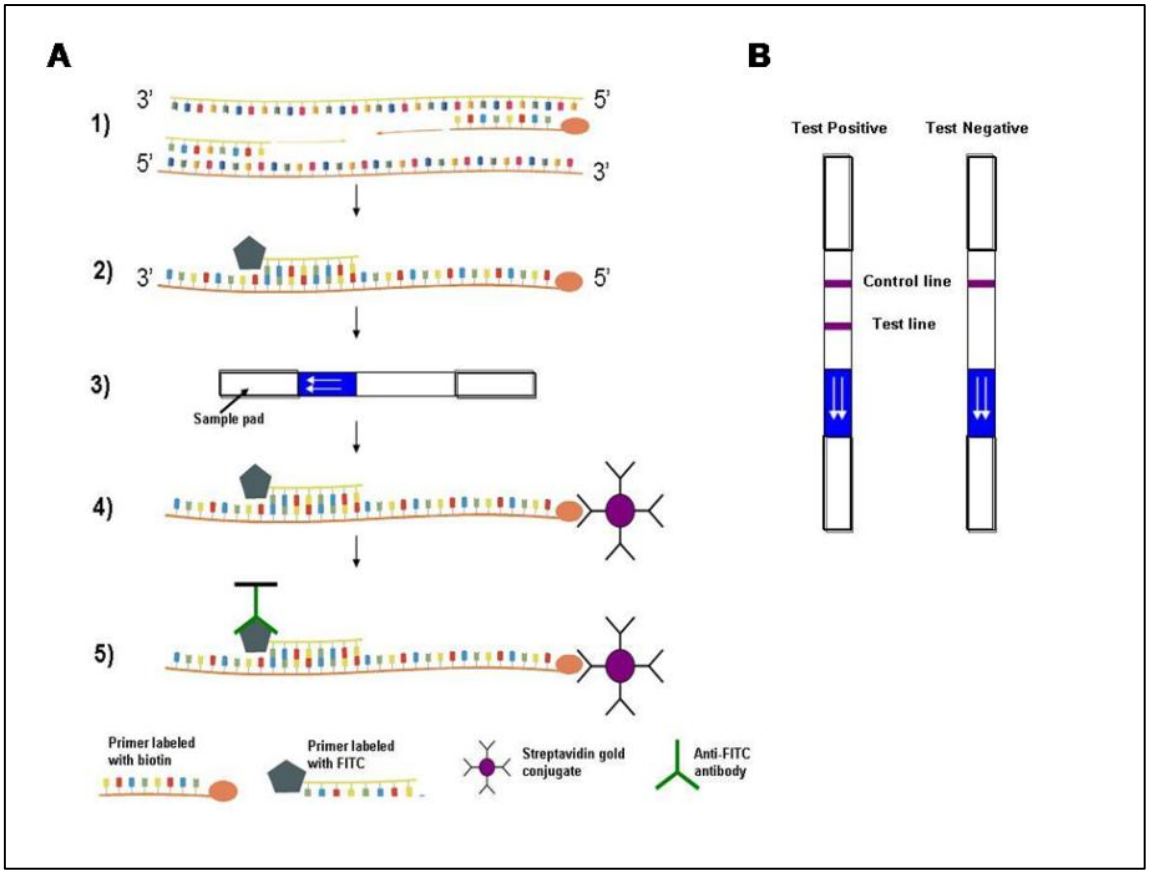


Figure B-1. Schematic diagram of rapidSTRIPE assay. A: A basic principle of rapidSTRIPE assay. 1) PCR amplification of the synthesized cDNA is performed with a forward primer and a biotin-tagged reverse primer. 2) PCR fragment is denatured, and hybridization of sequence specific probe with FITC-label takes places. 3) Then amplification/hybridization product is applied to the lateral flow strip on sample pad and placed in a tube containing the running buffer. 4) On the lateral flow strip (LFS), amplification/hybridization product conjugates with streptavidin gold particles and flows further. 5) The conjugated amplification/hybridization product with streptavidin gold particles is captured by anti-FITC antibodies at the detection line on the LFS. Thus, the gold particles accumulate in this part of LFS and the purple detection line becomes visible. B: Detection of amplification/hybridization on LFS. Control line is a conjugate control and gives answers about the quality of LFS. A test is positive when the detection line and the control line are visible. A test is negative when only the control line is visible.

The result was read visually after 10 min of incubation. A test was considered positive when detection line as well as control line was visible. A test was considered negative when only the control line was visible (Fig. B-1). In case of no visible control line or only visible detection line, a test result was counted as invalid.

C RESULTS

C.1 SENSITIVITY OF THE LFS ASSAY AND COMPARISON WITH RT-QPCR

In order to determine the sensitivity of the rapidSTRIPE H1N1 Assay KF system, serial diluted concentrations of the viral strain A/Hamburg/04/2009 were applied on LFS assay as well as on reference RT-qPCR after cDNA synthesis. Tenfold serial dilution of S-OIV A (H1N1) RT-qPCR specific standard plasmids were used to determine the genome equivalent copies (Geq). Down to 8 Geq copies/assay of S-OIV A (H1N1) were detected per assay by the reference method RT-qPCR (Fig C-1 & C-2), where down to 84 Geq copies/assay of S-OIV A (H1N1) were detected clearly per LFS assay (Fig. C-2). In this case, LFS assay showed 10-fold lower sensitivity than the reference method RT-qPCR. RT-qPCR was performed in duplicate while LFS assay was performed as a single sample (Fig. 2).

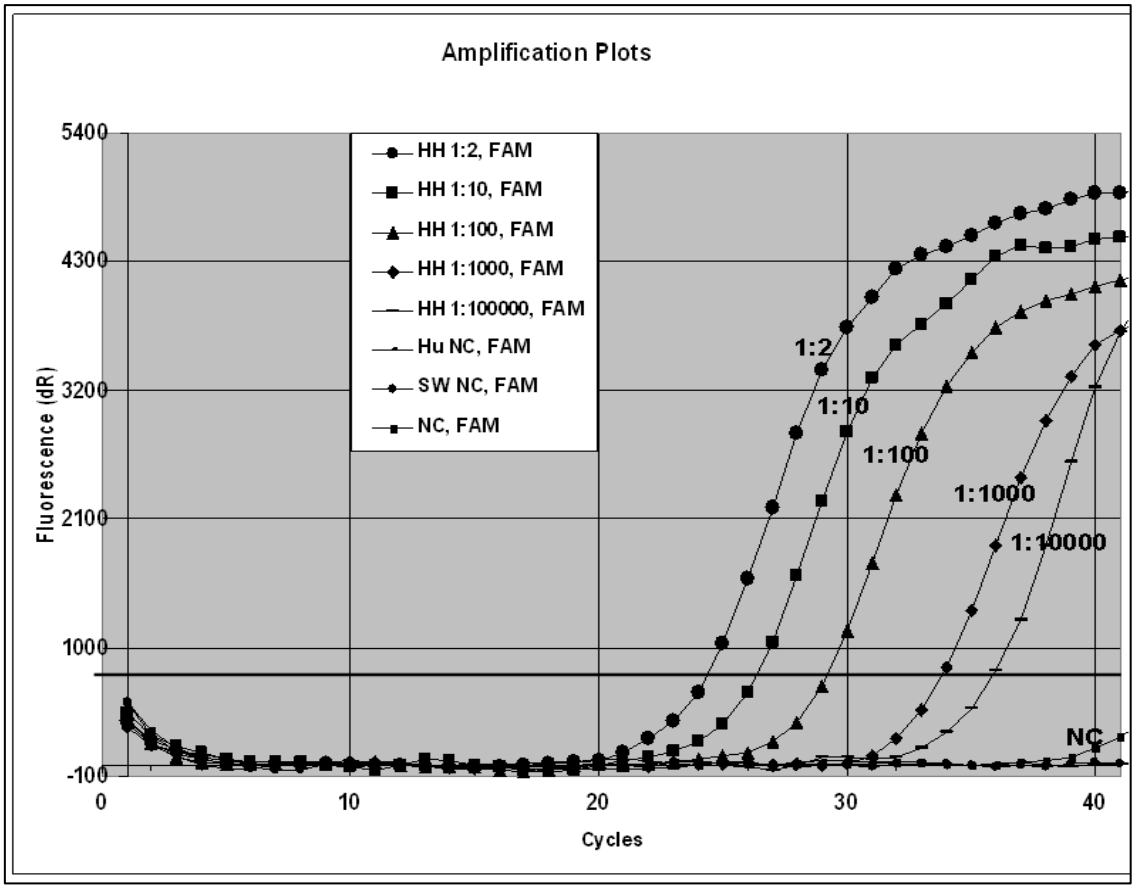


Figure C-1. Sensitivity of RT-qPCR. Serial dilution of the viral strain A/Hamburg/04/2009 were used to determine the Geq copies of S-OIV as a reference method.

Sample dilution	1:2	1:10	1:100	1:1000	1:10000	Human negative control	Swab control
Ct Value (RT-qPCR)*	24.46	26.45	29.28	33.89	35.95	No ct	No ct
Geq per assay	4.20E+04	8.40E+03	8.40E+02	8.40E+01	8.40E+00	-	-
Lateral flow stripe (LFS)**	Positive	Positive	Positive	Positive	Negative	Negative	Negative

Figure C-2. Sensitivity of LFS assay. Serial dilutions of the viral strain A/Hamburg/04/2009 were used to evaluate the sensitivity of LFA assay and compared with the reference method RT-qPCR. Test strip control lines were visible for all the samples. Detection lines were visible below the test line for the first four dilutions of viral samples.

The same experiments have been repeated 3 times independently for rapidSTRIPE H1N1 assay and compared for reproducibility. All three times, up to 84 Geq copies/assay of S-OIV A (H1N1) were detected positive on LFS by rapidSTRIPE H1N1 assay (Data not shown here).

C.2 SPECIFICITY OF THE LFS ASSAY

The specificity of the LSF assay was assessed by testing 10 different strains of subtypes of influenza virus A and influenza virus B and human control negative swab material. All influenza viruses, but S-OIV A (H1N1), yield a negative result demonstrating the high specificity of the LFS assay. The reference RT-qPCR was also negative for all reference influenza A and influenza B strains, excepting two strains of influenza A (A/Brisbane/59/07 H1N1 and A/dk/Germany R603/06 H5N1) (Table C-1). Both of influenza A viruses were detected at the threshold of detection with Ct values of 38.01 and 38.8, respectively.

To control the quality of the cDNA of reference influenza viruses, in house RT-qPCR assays for influenza A and B viruses (Analytik Jena AG, Jena, Germany) was performed on all samples. All the influenza viruses were positive on RT-qPCR flu A+B assay. A

swab from human negative controls and swab controls were negative for all the three assays (Table C-1).

Table C-1. Specificity test of two references RT-qPCR and rapidSTRIPE test with representative influenza virus subtypes.

Influenza Viruses	RKI RT-qPCR assay*	In-house flu A+B assay*	LFS assay**
	Ct value	Ct value	
A/Hamburg/04/2009 H1N1	24.46	23.69	Positive
A/Brisbane/59/07 H1N1	38.02	29.3	Negative
A/Caledonia/20/99 H1N1	No ct	23.5	Negative
A/Brisbane/10/07 H3N2	No ct	28	Negative
A/Wellington/1/04 H3N2	No ct	25.5	Negative
A/dk/Germany R603/06 H5N1	38.8	24.4	Negative
A/dk/Vietnam TG24-01/05/H5N1	No ct	21.3	Negative
A/Italy/472/99/H7N1	No ct	20.3	Negative
A/Germany/R11/01/H7N1	No ct	31.5	Negative
B/Malasiya/2506/04 (Victoria lineage)	No ct	21.2	Negative
B/Iangsu/10/03 (Yamagata lineage)	No ct	24.5	Negative
Human virus-negative swab	No ct	No ct	Negative
Swab negative control	No ct	No ct	Negative
Influenza A + B negative control	-	No ct	Negative

***Samples were measured in duplicate in RT-qPCR assay and flu A+B assay**
**** Samples were measured once in LFS assay**
Ct: threshold cycle

C.3 DIAGNOSTIC PERFORMANCE OF THE RAPIDSTRIPE H1N1 ASSAY KF SYSTEM

174 viral RNA samples obtained from patient nasal swabs included in this study were positive for in-house GAPDH RT-PCR. The same RNA samples were also tested with the rapidSTRIPE H1N1 assay and RT-qPCR. The Ct values obtained by RT-qPCR for positive samples ranged from 22.58 to 38.9. 105 samples out of 174 samples (60.3%) resulted positive and 69 (39.7%) resulted negative by RT-qPCR. Of the 105 samples that were detected positive in RT-qPCR, 92 were tested positive by rapidSTRIPE H1N1 assay, providing a sensitivity of 88% and a positive predictive value of 96%. Of the 69 samples that were tested negative in RT-qPCR, 65 were tested negative by rapidSTRIPE H1N1 assay, providing a specificity of 94% and a negative predictive value

of 84% (Table C-2). The overall agreement between the two assays was 90.2% (157/174).

Table C-2. Performance of the LFS assay compared to the reference methods for detecting S-OIV H1N1

Lateral flow strip assay	Reference method (RT-qPCR)		Performance characteristics (%)			
	Positive	Negative	Sensitivity	Specificity	PPV	NPV
Positive	92	4	88 (92/105)		96	
Negative	13	65		94 (65/69)		83
Total (n=174)	n=105	n=69				

PPV: positive predictive value; NPV: negative predictive value
This study includes 174 patient samples. The sensitivity and specificity of assay were calculated 88% and 94%, respectively. The PPV and NPV were calculated 96% and 83%, respectively.
For the RT-qPCR, all samples with a Ct value > 39 were counted as a negative.

C.4 INSTRUMENT, COST AND WORK LOAD

The rapidSTRIPE H1N1 assay KF system in combination with the KingFischer automated extraction system was used as an alternative method to the conventional PCR methods during the 2009 H1N1 pandemic at MLO MVZ GbR, Dresden and more than 5,000 samples have been tested by this system.

Table C-3. Cost of assay, instrument and duration of test for rapidSTRIPE assay system.

Assay/Instrument	Cost	Samples tested per run	Time for test
RapidSTRIPE H1N1 assay (NA isolation to detection LFS)	10 €/sample	-	2.5 h
Nucleic acid automated extraction system (KingFischer Flex 96 instrument)	ca. 35000 €	96	1 h
Thermal cycler (Alpha SC or Speed cycler, Analytik Jena)	8000 €	96	-

The use of this system revealed some interesting properties regarding time duration and cost effectiveness of the test. The time duration of the test from extraction up to final detection was 3 hours for 96 samples. The cost of the rapidSTRIPE H1N1 assay including manual nucleic acid extraction was 10 € per sample and the costs of instruments to perform rapidSTRIPE test was estimated at 43,000 € (Table C-3). The

maximal capacity of MLO MVZ GbR, Dresden for the detection of the S-OIV A (H1N1) was estimated 250 samples per day by one person using automated extraction system.

D DISCUSSION

This study demonstrates the usefulness of the rapidSTRIPE H1N1 assay for the rapid detection of novel S-OIV H1N1 as point-of-care diagnostic setting. This assay showed cross-reactivity neither with other influenza A and B viruses nor with human negative control material providing a good specificity profile required for diagnostic accuracy. At least 84 geq copies/assay could be detected by the rapidSTRIPE H1N1 assay, corresponding to 8,400 viral RNA copies in 100 µl RNA or 8400 virus particles in the initial sample (exp. swab sample). The rapidSTRIPE test showed an overall sensitivity of 88% and specificity of 94% in comparison to RT-qPCR, the widely preferred method for diagnosis of S-OIV A (H1N1) (Ellis, Iturriza et al. 2009). The total cost of the rapidSTRIPE H1N1 test including a manual nucleic acid extraction is about 10 € per sample.

Several PCR-based assays for the detection of the S-OIV A (H1N1) have been developed and published soon after the emergence of the pandemic 2009 H1N1 virus (Carr, Gunson et al. 2009; Panning, Eickmann et al. 2009; Poon, Chan et al. 2009; Wang, Gao et al. 2009; Whiley, Bialasiewicz et al. 2009). Also new rapid assays like real-time nucleic acid sequenced based amplification and multi-fluorescent real-time RT-PCR have been developed to detect novel S-OIV A (H1N1) (Dong, Zhang et al. 2009; Ge, Cui et al. 2009). All these molecular detection methods as real-time RT-qPCR have been broadly used in medical diagnostic laboratories, because of their high sensitivity and specificity. RT-qPCR is regarded widely as a gold standard for diagnosis of influenza viruses (Ellis, Iturriza et al. 2009). However, RT-qPCR is relatively expensive and requires trained laboratory expertise and extensive evaluation, which limit the broad use of in house assays (Vasoo, Stevens et al. 2009).

Rapid antigen-antibody based influenza tests as point-of-care tests have been used since they require only 10-15 min and minimal expertise for testing. They also provide a source of data for clinical management of the patients. However, high virus concentration is required to yield a positive rapid test (Drexler, Helmer et al. 2009). Depending on the virus load of the respiratory sample, an overall sensitivity of 40% to 69% has been reported for rapid antigen-antibody based influenza tests, among different commercial tests (Ganzenmueller, Kluba et al. 2010; Herzum, Lutz et al. 2010; Karre, Maguire et al. 2010). A negative test obtained with rapid antigen-antibody based tests still requires a need of confirmation by the PCR based methods or virus culture, followed by hemagglutination inhibition test.

Nucleic acid based lateral flow assay is a similar method as a rapid antigen-based test, but a dual labeled amplicon is detected here after amplification instead of antigen. Such a NALF test using primers with two different tags has been described for the detection of pathogenic bacteria from food (Blazkova M, Koets M et al. 2009) and for rapid detection of malaria (Mens, van Amerongen et al. 2008) previously. There is a high risk of false positive results caused by primer dimers for this NALF test using tagged primers. The rapidSTRIPE H1N1 assay is a NALF test using RAH technology, which generates amplification/hybridization products in a single reaction without any post amplification modification. Higher specificity in comparison to rapid antigen-antibody based systems is achieved here by use of the target specific probe and rapid PCR. The use of a target sequence specific tagged probe for subsequent hybridization to single stranded amplification product helps here to eliminate nonspecific reaction due to the primer dimers.

Recently, rapid oligochromatographic assay (Perez-Ruiz, Navarro-Mari et al. 2010) and NALF test combining the isothermal PCR (Wu, Curran et al. 2010) methods have been developed for detection of the pandemic 2009 H1N1 virus. In the rapid oligochromatographic assay, amplification products were first denatured; then carried out on LFS, where hybridization took place under thermal conditions. This whole procedure needs extra manual steps in comparison to the rapidSTRIPE assay. In the rapidSTRIPE assay, amplification/hybridization products were generated in a single reaction and carried out directly on LFS at room temperatures without any modification. The major advantage of isothermal methods in comparison to rapidSTRIPE assay is that, there is no need of thermal cycling hardware. However, a complex assay design, an extensive optimization, and other reagents are required for such an isothermal PCR, where the rapidSTRIPE assay can be easily adapted from any conventional real time PCR assay and all reagents are commercially available. Furthermore, the rapidSTRIPE H1N1 assay KF system provides all reagents needed from the nucleic acid isolation to the final detection on LFS in one single system.

The rapidSTRIPE H1N1 assay described here is commercially available under the CE mark of in vitro diagnostic products and fulfills the CE requirement of European Guidelines for quality management ISO 9001:2000 and in-vitro diagnostic EN ISO 13485:2003. Furthermore, over 500 repetitions of the same samples with different batches of the kits demonstrated a 100% reproducibility of the results by the manufacturer.

In summary, the rapidSTRIPE H1N1 assay offers a powerful tool for specific detection of S-OIV A (H1N1) in about 3 hours starting from swab sampling to nucleic acid isolation, RT-PCR and rapid amplification/hybridization until final detection of the PCR products on a LFS. This rapid assay allows the semi-quantitative detection of S-OIV A (H1N1) with several advantages as quickness, cost effectiveness and long-term stability. The readout of the test is performed optically, which makes independent of an instrument specific analysis system. The results clearly demonstrate that the rapidSTRIPE assay in combination with the KingFischer automated nucleic acid extraction system allows the investigation of 250 patient samples per day by one person. This system can easily be used as a high throughput screening system for laboratories not equipped with real time PCR instruments and resource-poor diagnostic settings during the epidemical situation.

Chapter 4. Recombinase Polymerase Amplification Assay for Low-Resource Settings

Recombinase Polymerase amplification assay for the detection of Yellow fever virus in low-resource settings

A INTRODUCTION

A.1 YELLOW FEVER VIRUS

Yellow fever (YF) is one of the most lethal and acute viral hemorrhagic disease transmitted by infected mosquitoes. YF is naturally distributed throughout the tropical South-Americas and sub-Saharan Africa, but not reported in Asian countries; and remains one of the major public health problems in these areas of the world. According to World Health Organization, there are globally 200,000 estimated cases of Yellow fever annually, causing 30,000 deaths with case fatality rates of 20 to 50 %. YF is endemic in tropical areas of Africa and South-America and poses risk to a total combined population of over 900 million people (WHO YFV fact sheet 2011). In the last two decades, a reemergence of YF was recognized in these endemic regions and cases of YF infections have increased due to the following main reasons: declining population immunity, deforestation, urbanization, climate change and frequent travels from endemic countries (Staples, Gershman et al. 2010).

YF is caused by Yellow fever virus (YFV), a prototype member of the genus *Flavivirus*, a family *Flaviviridae*. YFV contains a positive-sense, single-stranded RNA genome of approximately 11 kb. The viral genome has only a single open reading frame encoding single large polypeptide. This polypeptide is co- and post-translationally proceeded by host and virus-encoded proteases into at least ten proteins: three structural proteins (capsid, pre-membrane and envelope) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers, Hahn et al. 1990).

Several different species of *Aedes* and *Haemogogus* mosquitos are known to transmit the YFV. The Transmission of YFV occurs between mosquito and human in urban cycle or between mosquito and other primates in sylvatic cycle. YFV can be also transmitted transovarially between the mosquitos. An intermediate cycle occurs in humid and semi-humid parts of Africa, where YFV is transmitted between human and non-human primates through *Aedes* mosquitos (Monath, Guirakhoo et al. 2003; WHO YFV fact sheet 2011).

The disease symptoms caused by YFV infection ranges from nonspecific flu-like illness to classic YF, which is characterized by severe liver and renal dysfunction, circulatory shock, and hemorrhage. Most of the patients recover 3-4 days after onset of infection. However, about 15 %-25 % patients develop the severe disease, with a case fatality rate

of up to 50 %. There is no specific therapy treating the YF; patients can be only treated symptomatically. Vaccination is the only and most effective measure for preventing YF.

A.2 VACCINATION AND ADVERSE EVENTS AFTER VACCINATION

In 1927, YFV (Asibi strain) was first isolated from a human patient in West Africa and the YF-17D live-attenuated vaccine was developed in 1936 by the empirical method of sequential passage of the YFV Asibi in a mouse and chicken embryo tissue. Two sub-strains of YFV (17D - 204 and 17DD) derived from a common ancestor (wild type Asibi strain) are used for manufacturing of live attenuated YF vaccines (Domingo and Niedrig 2009).

The YF vaccine is delivered as a single subcutaneous dose. It is safe and provides an effective immunity against YFV within 10 days for 90 % of the recipients. The YF vaccine is not allowed for children under 6 years and immunocompromised people. The YF vaccine is well tolerated, with a low rate of severe adverse events (SAEs). Although SAEs to YFV are rare, an increased number of SAEs after the YF vaccination was recently reported (Barrett and Teuwen 2009; Domingo and Niedrig 2009). The reasons for SAEs are still poorly understood and it needs more investigation of YFV vaccine strains (Stock, Boschetti et al. 2012). However, the risk of death from YF is far greater than the risk of SAEs related to the vaccine. Therefore, vaccination remains an important measure for preventing YF in endemic areas. At least 60 %- 80 % population in affected areas must be vaccinated to prevent outbreaks. WHO had initiated preventive vaccination campaigns in the 12 West African countries at risk of the YF disease. Between 2007 and 2010, ten African countries have completed mass vaccination campaigns. Despite these efforts, vaccination coverage in African countries is very low in comparison to South-American countries (Domingo and Niedrig 2009; WHO YFV fact sheet 2011). Therefore diagnostic tools to detect YFV and rapid response through emergency vaccination campaigns are essential to control outbreaks.

A.3 DIAGNOSIS OF YFV

The diagnosis of YFV infection is particularly difficult because the symptoms are similar to severe malaria, dengue hemorrhagic fever, leptospirosis, viral hepatitis and other hemorrhagic fevers. Many laboratory techniques like virus isolation, immunoassay and RT-PCR assays, have been applied routinely for diagnosis of YFV. Virus isolation can be performed during the febrile period of the YF disease by inoculation of patient´s blood sample on cell culture. Immunofluorescence assays and RT-PCR can be used to determine the successive virus isolation. Other alternatives besides virus isolation for

diagnosis during the febrile period of YF disease are molecular techniques like conventional RT-PCR, Taqman real-time RT-PCR and YF antigen detection. Seven days after onset of symptoms, several blood tests can detect yellow fever specific antibodies produced in response to the infection. Different serological tests like IgM capture and IgG ELISA, plaque reduction neutralization tests (PRNT) and rapid tests can be performed to detect these YF-specific antibodies after viremic phase. PRNT is a standard serological method in virology to detect virus neutralizing antibodies. Virus and antibody mixture is inoculated on virus susceptible cells, and the effect of antibodies on virus infectivity is measured to determine the titer. Different read out systems are developed for PRNT. The PRNT is a very reliable, mainly serological test, but in contrast to other serological tests, it is a lab and time intensive assay.

Molecular methods based on the detection of viral genomes have replaced the gold standard method “virus isolation” and play an important role for diagnosis in the acute phase of YFV infection. Molecular assays are rapid, sensitive, and highly specific in comparison to other methods. Many of quantitative RT-PCR assays have been described for the detection and quantification of YFV. Most of these methods are based on Taqman probe chemistry and provide good sensitivity and specificity. Although RT-qPCR provides many advantages, it is restricted to well establish laboratories and cannot be used for in-field diagnosis. In contrast to this, new molecular methods based on isothermal amplification like Loop mediated amplification (LAMP), Helicase dependent amplification (HAD), Recombinase polymerase amplification (RPA) and Transcription-mediated amplification (TMA) offer simple, rapid, specific and cost-effective alternatives to RT-qPCR and can be easily used for the in-field applications using simple laboratory settings.

A.4 AIM OF THIS STUDY

The aim of this study was to develop an YFV nucleic acid testing system for resource-poor and field settings, which can be used to monitor YFV infection and SAEs caused by the YF vaccine during mass vaccination campaigns in endemic African countries. Using Recombinase polymerase amplification (RPA), an isothermal approach, a molecular diagnostic assay was developed for rapid detection of YFV, which could be performed either instrument-free or with a small portable instrument for in-field or resource-poor settings. Additionally, all the reagents needed for testing can be stored at ambient conditions.

B MATERIALS AND METHODS

B.1 VIRUSES AND ISOLATION OF VIRAL RNA

All flavivirus and non-flavivirus strains used were derived from cell culture and provided by the Robert Koch Institute, Berlin, Germany. The following inactivated and stable virus preparations were used in this study: DENV-1 VR344 (Thai 1958); DENV-2 VR345 (TH-36 strain); DENV-3 VR216 (H87 strain); DENV-4 VR217 (H241 strain); WNV Uganda strain (AY532665); WNV Israel (H. Bin, Sheba Medical Centre, Israel), Usutu virus (AY453411); JEV (ATCC SA14-14-2); Saint Louis Encephalitis virus (SLEV) (ATCC VR-1265); TBEV strain K23 (AF091010); YFV strain 17D (X03700); YFV strain ASIBI (AY640589); YFV strain Brazil; YFV strain Ivory Coast and Russian Spring Summer Encephalitis virus (RSSEV). The external quality assurance (EQA) panel of YFV used in this study was provided by the European Network for Diagnostics of Imported Viral Diseases (ENIVD).

Viral RNA was isolated from 140 µl aliquots of cell culture supernatants, using the QIAamp Viral Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted in 100 µl of elution buffer and stored at -80 °C until further use. For in-field application, Instant MP basic kit can be used for RNA extraction from clinical samples according to the manufacturer´s instructions.

B.2 PRIMER AND PROBE FOR RPA

Available full-length YFV sequences and partial sequences covering the 5´-UTR region from the database (NCBI) were aligned using Geneious 5.0 software. Based on the alignment, degenerate generic primers and corresponding primers representing all known sequence variants were designed for generic amplification of different YFV strains. In contrast to PCR, RPA requires primer consisting of up to 30 nt. According to Piepenburg and colleagues, primers in length of 30 to 35 nt are recommended for RPA (Piepenburg, Williams et al. 2006). Although, primers in length of 27 to 29 nt have used for YFV RPA nfo assay. Whereas, primers in length of 30 nt were used for YFV RPA exo assay. RPA nfo probe and RPA exo probes, two restriction probes were designed according to the guidelines provided by TwistDx manual.

B.3 REAL-TIME PCR AND PLASMID STANDARD

The YFV-specific primers combination YFV all F/R and probe YFV all TM were used to detect and quantify genomic RNA of YFV as described previously (Domingo, Patel, 2012

in progress). The assay was performed in one-step format on the ABI 7500 instrument using the QuantiTect Virus Kit (Qiagen, Hilden, Germany).

To quantify the RT-qPCR, the 10-fold serial dilution of the standard plasmids (10 - 10^6 copies/µl) were tested in duplicate within the same sample run and compared. Standard plasmid was prepared by cloning fresh PCR products of YFV all assay using a TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) according to the kit instruction and quantified by spectrometric method. The YFV all assay and plasmids were kindly provided by Dr. C. Domingo.

The YFV LNA probe based real-time RT-qPCR assay was used as described previously (Weidmann, Faye et al. 2010).

B.4 LATERAL-FLOW STRIPE RPA ASSAY

Lateral-flow stripe RPA (LFS-RPA) assay was performed using either TwistAmp nfo kit or Twist nfo RT kit according to manufacturer's instruction. For detection of RNA target using TwistAmpnfo kit, 1 µl of RT-Enzyme was additionally added to the RPA reaction. As the TwistAmpnfo RT kit was commercially available, only this kit was used for LFS-RPA assay. Briefly, 29.5 µl of rehydration solution were mixed with 2.1 µl of each primer (10 µM), 0.6 µl of target specific RPA nfo probe (10 µM) and 8.2 µl of PCR grade water. Master mix of 42.5 µl was then added to the dry reagent pellet and mixed by pipetting up and down the reaction mixture. 5 µl of template (genomic DNA or RNA from the pathogens) was added to this mixture. Finally, 2.5 µl of $Mg(OAC)_2$ (280 mM) was added to the 47.5 µl of the solution and the reaction was started through this step. The tubes containing reactions were vortexed and centrifuged shortly, and then placed into a thermo block or a PCR cycler at 39 °C for 20 min. After the amplification step, one µl of amplification product was diluted in 9 µl of PBST buffer and carried onto the sample pad of lateral flow strip (LFS). The strips were then placed in tubes containing 100 µl of PBST buffer. The result was read visually after 10 min of incubation. A test was considered positive when the detection line as well as the control line was visible. A test was considered negative when only the control line was visible.

Amplification products of the LFS-RPA assay were also analyzed by gel electrophoresis as endpoint detection. 25 µl of amplification product was loaded on a 1.5 % agarose gel and run for 40 min at 110 V. Gel was stained with ethidium bromide and visualized under UV light.

Table B-1. List of oligonucleotides and probe for the LFS-RPA assay

Pathogen	Name of oligos	Sequences 5´-> 3´
YFV	**YF RF**	AAATCCTGTGTGCTAATTGAGGTG**Y**ATTG
	YF RR	Biotin-TTCTGGTCARTTCTCTGCTAATCGCTC
	YF Rprobe nfo	**FAM-**CTGCAAATCGAGTTGCTAGGCAATAAACAC**[THF]**TTTGGATTAATTTT**R**ATCGTT**-P**

YFV: Yellow fever virus, FAM: 6-Carboxyfluorescein, TFH: tetrahydrofuran, P: 3' phosphate to block elongation

B.5 REAL-TIME RPA ASSAY

The real-time RPA assay was performed using the TwistAmp exo RT kit according to the manufacturer's instruction. The TwistAmp exo RT kit contains additionally to RPA reagent an RT-enzyme, which allows the DNA amplification of RNA targets. Briefly, 37.7 µl of rehydration solution were mixed with 2.1 µl of each primer (10 µM) and 0.6 µl of target specific RPA exo probe (10 µM). Master mix of 42.5 µl was then added to the dry reagent pellet and mixed by pipetting up and down the reaction mixture. 5 µl of template (viral RNA) was added to this mixture. Finally, 3.5 µl of $Mg(OAC)_2$ (280 mM) was added to the 47.5 µl of solution and reaction was started through this step. The tubes containing reactions were vortexed and centrifuged shortly; then placed in the ESE Quant Tubescanner for real-time monitoring of fluorescence. Reaction was performed at 39 °C for 15-20 min, and data were analyzed by Tubescanner studio software Version 1.0.

Table B-2. List of oligonucleotides and probe for the real-time RPA assay

Pathogen	Name of oligos	Sequences 5´-> 3´
Yellow fever virus	**YFV RF2**	AAATCCTGKGTGCTAATTGAGGTGYATTGG
	YFV RR2	ACATDWTCTGGTCARTTCTCTGCTAATCGC
	YFV Rprobe exo	gCAAATCgAgTTgCTAggCAATAAACACATT(BHQd**T)g(THF)A(FamdT)**TAATTTTRATCgTTC--**SpacerC3**

FAM: 6-Carboxyfluorescein, TFH: tetrahydrofuran, BHQ: black hole quencher

C RESULTS

C.1 DEVELOPMENT OF LFS-RPA ASSAY FOR YFV

The LFS-RPA assay was first developed using the TwistAmp nfo kit, which was able to detect only DNA template. Therefore, cDNA was synthesized from the 10-fold serial dilutions of YFV RNA; then applied to the LFS-RPA assay. The successive amplification was detected as an endpoint measurement either by lateral flow strip or gel electrophoresis. The results of two-step LFS-RPA assay are represented in Fig. C-1. The two-step LFS-RPA has successfully detected the 10-fold serial dilution of YFV RNA by gel electrophoresis and LFS. Comparing Fig. C-1 I and II show that the detection of YFV RNA by LFS was more sensitive than by gel electrophoresis. Endpoint detection by LFS was able to detect YFV RNA down to a dilution of $1:10^6$, while only down to a dilution of $1:10^4$ of YFV RNA was detected by gel electrophoresis.

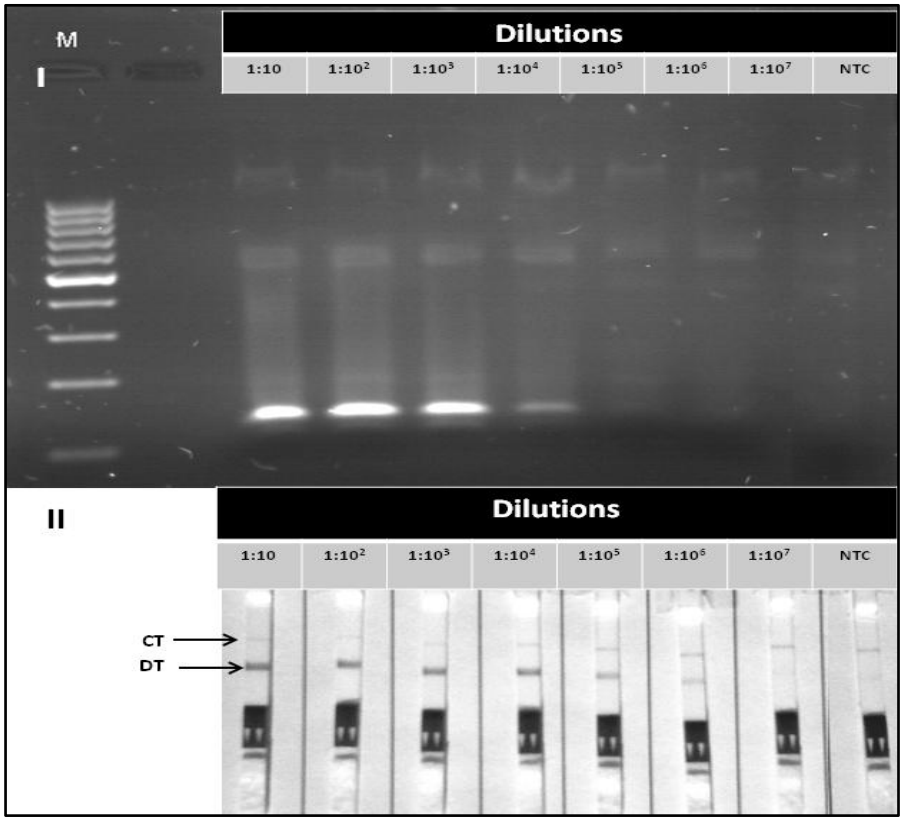


Figure C-1. Sensitivity of two-step lateral flow strip-RPA assay (LFS-RPA assay) for the detection of the 10-fold serial dilution of Yellow Fever virus RNA. I) Sensitivity of LFS-RPA assay as observed by 115 bp amplicon on 1.5 % gel by gel electrophoresis. II) Sensitivity of LFS-RPA assay as observed by detection line on LFS. CT: control line, DT: detection line.

To perform LFS-RPA assay in one-step format, reverse transcriptase (RT) enzyme was added to master mix of TwistAmp nfo kit and YFV RNA dilution were directly used as a template. The results of one-step LFS-RPA assay is shown in Fig. C-2 and reveals that the LFS-RPA assay can be successfully done in one-step format using RT-enzyme with a detection limit of down to a dilution of $1:10^5$ of YFV RNA. However, the sensitivity of this assay was one log less in comparison to two-step LFS-RPA assay.

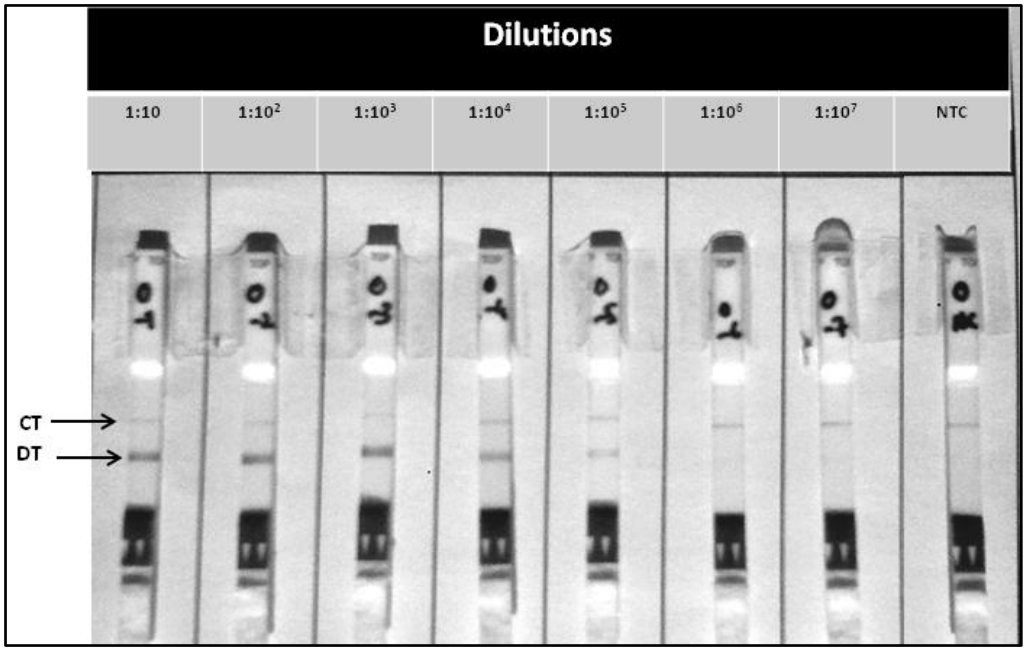


Figure C-2. Sensitivity of one-step LFS-RPA assay for detection of the 10-fold serial dilution of YFV RNA. CT: control line, DT: detection line.

C.2 PERFORMANCE OF THE LFS-RPA ASSAY AND COMPARISON WITH RT-QPCR

As it´s known from the previous experiments, the LFS-RPA assay can be performed by adding RT-enzyme in 1-step format. Therefore, TwistDx Company has provided on request TwistAmp nfo RT kits for one-step LFS-RPA assay. To determine the sensitivity of the assay employing the TwistAmp nfo RT kit, 10-fold serial dilution of YFV RNA were tested by this method. The same RNA dilutions were tested by RT-qPCR to quantify the amount of RNA copies and the results were compared with those from one-step LFS-RPA assay.

The real-time RT-qPCR used as a reference assay was able to detect down to 1-10 genome equivalent copies/reaction (Geq/rxn) with linearity over a wide dynamic range from 10^0 to 10^6 Geq/rxn. When serial dilution of YFV RNA were tested by this assay and genome copies were determined by use of the standard plasmids, down to 2 Geq/rxn

were detected by this method with excellent sensitivity. The same 10-fold serial diluted RNA were also tested by the LFS-RPA assay to compare the sensitivity. The data show that the LFS-RPA assay was able to detect the virus down to 23 Geq/rxn with linearity over a range of 10 to 10^6 Geq/rxn (Fig C-3). The LFS-RPA assay for YFV was one log less sensitive than real-time RT-qPCR.

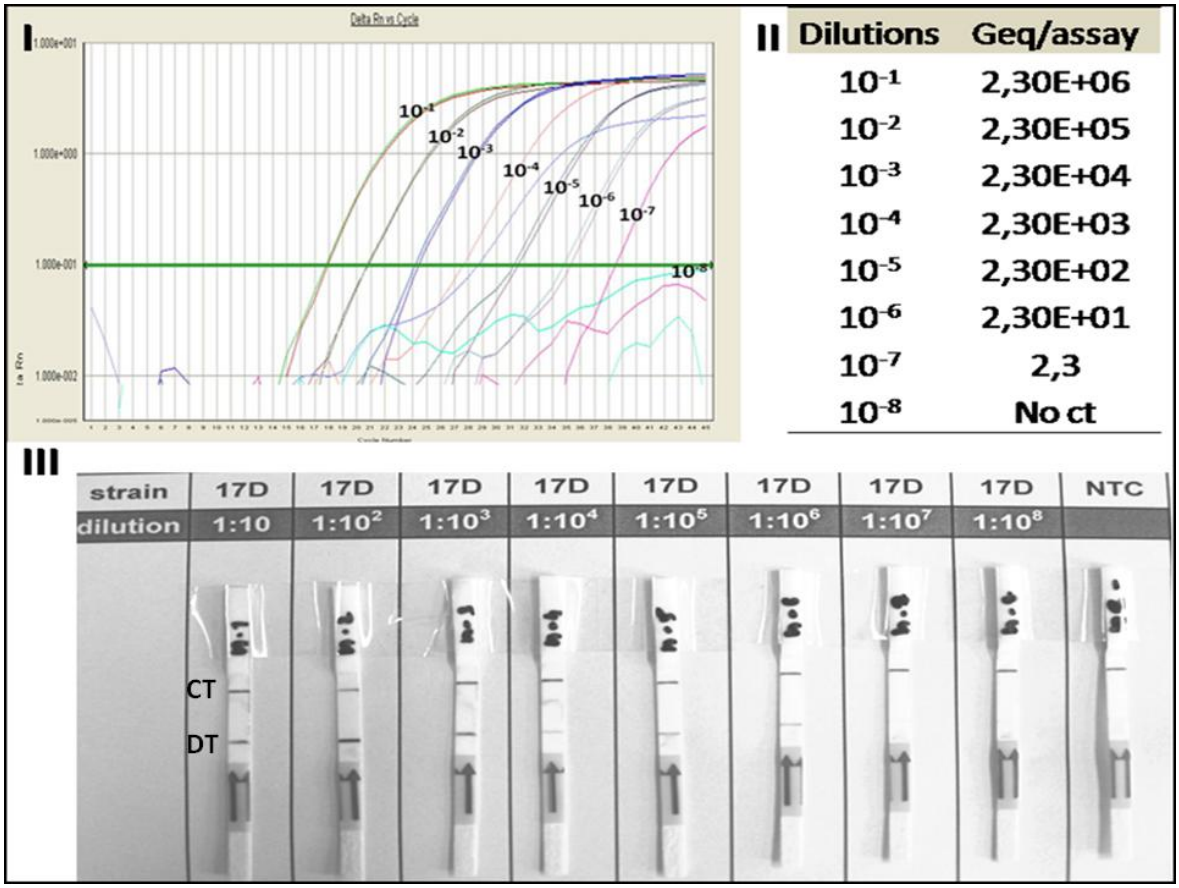


Figure C-3. Sensitivity of LFS-RPA assay vs. sensitivity of real-time RT-qPCR. I) The amplification plot of the real-time RT-qPCR. II) The genome copies/rxn determined in 10-fold serial dilutions by the real-time RT-qPCR. III) The sensitivity of LFS-RPA assay by detection on the LFS as an endpoint analysis. CT: control line, DT: detection line.

C.3 SPECIFICITY OF THE LFS ASSAY

The specificity of the LFS-RPA assay was assessed by testing one YFV vaccine stain 17D, three YFV wild type strains and other eleven closely related flaviviruses. This assay was highly specific to YFV and able to detect all the wild type strains efficiently. Furthermore, this assay showed no cross-reaction with the other eleven closely related flaviviruses, when tested on the LFS-RPA assay using RNA of these flaviviruses in higher concentration.

Sample No	Pathogen (1:10 diluted RNA Ct = ~24 to 30)	RPA
1	YF 17D	
2	YF Asibi	
3	YF Brazil	
4	YF Ivory Coast	
5	DEN1	
6	DEN2	
7	DEN3	
8	DEN4	
9	FSME K603	
10	FSME (Louping ill)	
11	JE	
12	RSSE	
13	SLE	
14	West Nile (Uganda)	
15	West Nile (Israel)	
	NC	

Figure C-4. Specificity of YFV LFS-RPA assay

An YFV EQA panel was tested to check the performance of the YFV LFS-RPA assay. Testing of this YFV EQA panel reveals that the LFS-RPA assay has successfully detected 8 of 10 YFV positive samples containing dilution of different YFV strains as detailed in Fig. C-5. Two YFV positive samples (#14 and #6), which were a false negative in the LFS-RPA assay showed a faint detection band on LFS as one of the negative controls and therefore they were suggested as negative.

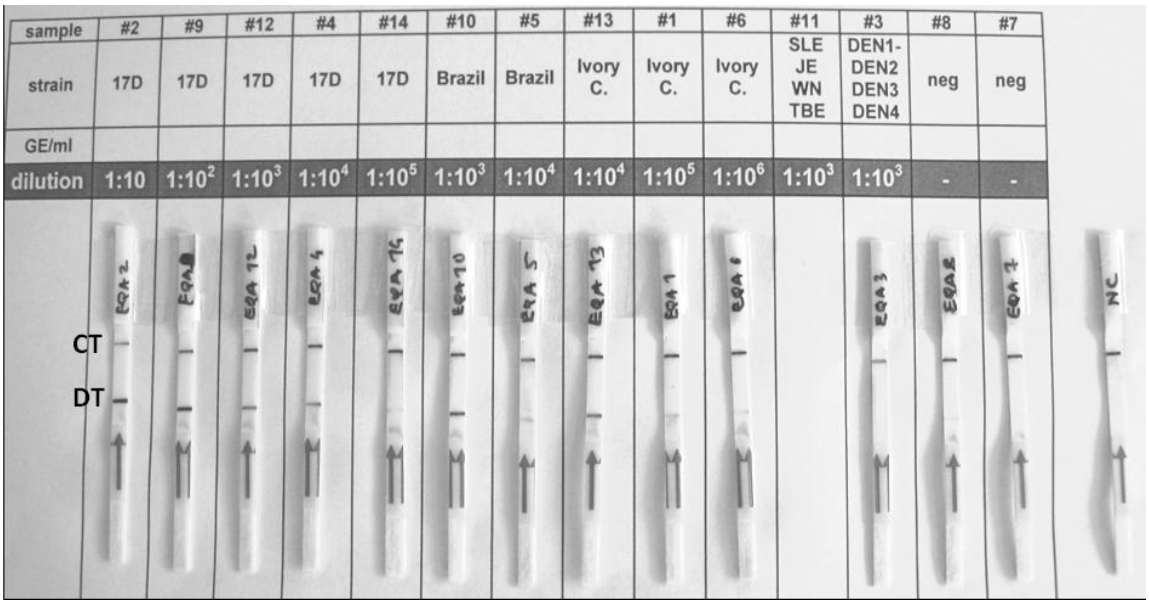
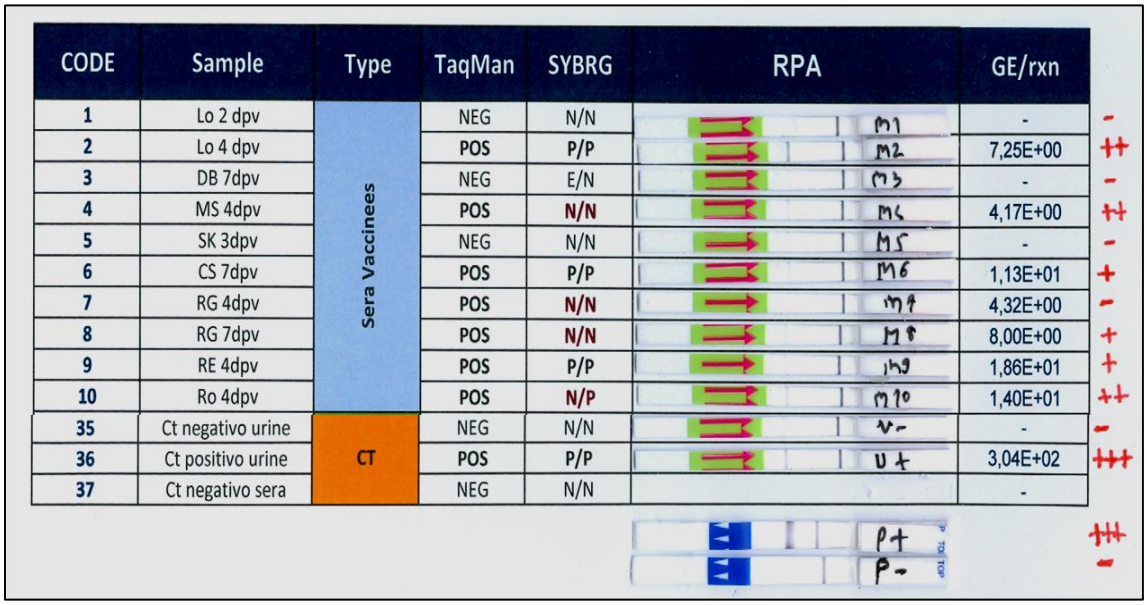


Figure C-5. Performance of LFS-RPA assay for detection of the YFV EQA panel. CT: control line, DT: detection line.

One of three negative samples (#8) of the EQA panel has also shown faint detection band as observed in Fig. C-5. In LFS-RPA assay, the tubes with amplification products were opened after reaction and diluted with PBST. This open handling of amplification product might lead to contamination.

C.4 TESTING CLINICAL SAMPLES

Ten sera samples of YFV vaccines were tested with LFS-RPA assay, and results were compared to those results obtained by real-time RT-qPCR. Seven of ten samples resulted positive, and three resulted negative by reference real-time RT-qPCR method. Of these seven positive samples, 6 samples were tested positive by the YFV LFS-RPA assay. All three negative samples were correctly detected by real-time RT-qPCR and LFS-RPA assay. The overall agreement between the two assays was 90 % (9/10).



CODE	Sample	Type	TaqMan	SYBRG	RPA	GE/rxn	
1	Lo 2 dpv	Sera Vaccinees	NEG	N/N		-	-
2	Lo 4 dpv		POS	P/P		7,25E+00	++
3	DB 7dpv		NEG	E/N		-	-
4	MS 4dpv		POS	N/N		4,17E+00	++
5	SK 3dpv		NEG	N/N		-	-
6	CS 7dpv		POS	P/P		1,13E+01	+
7	RG 4dpv		POS	N/N		4,32E+00	-
8	RG 7dpv		POS	N/N		8,00E+00	+
9	RE 4dpv		POS	P/P		1,86E+01	+
10	Ro 4dpv		POS	N/P		1,40E+01	++
35	Ct negativo urine	CT	NEG	N/N		-	-
36	Ct positivo urine		POS	P/P		3,04E+02	+++
37	Ct negativo sera		NEG	N/N		-	
							+++
							-

Figure C-6. Testing of clinical samples by the LFS-RPA assay and it´s comparison with real-time RT-qPCR.

C.5 PERFORMANCE OF REAL-TIME RPA ASSAY FOR YFV

As an alternative to the LFS-RPA assay, a real-time RPA assay was developed using the TwistAmp exo RT kit. This assay can be done in a closed tube system to avoid contamination. According to RPA primer design guidelines, longer primers lead to faster reaction and provide the better sensitivity. Therefore, the real-time RPA assay was performed using the 30 nt long primers YFV RF2/ YFV RR2 and YFV Rprobe exo in contrast to LFS-RPA assay.

Using a 10-fold serial dilution of YFV RNA, the real-time RT-qPCR showed linearity over a wide range of log 7 to 1 Geq/rxn and the detection limit was down to 10 Geq/rxn as published before (Weidmann M, 2010). Comparing Fig C-7 I and II show that the minimum detection limit of real-time RPA and real-time RT-qPCR was 100 Geq/rxn and 10 Geq/rxn, respectively. Real-time RPA was here one log less sensitive than real-time RT-qPCR. Furthermore, real-time RPA was also able to detect different YFV wild type strains as shown in Fig C-7 III.

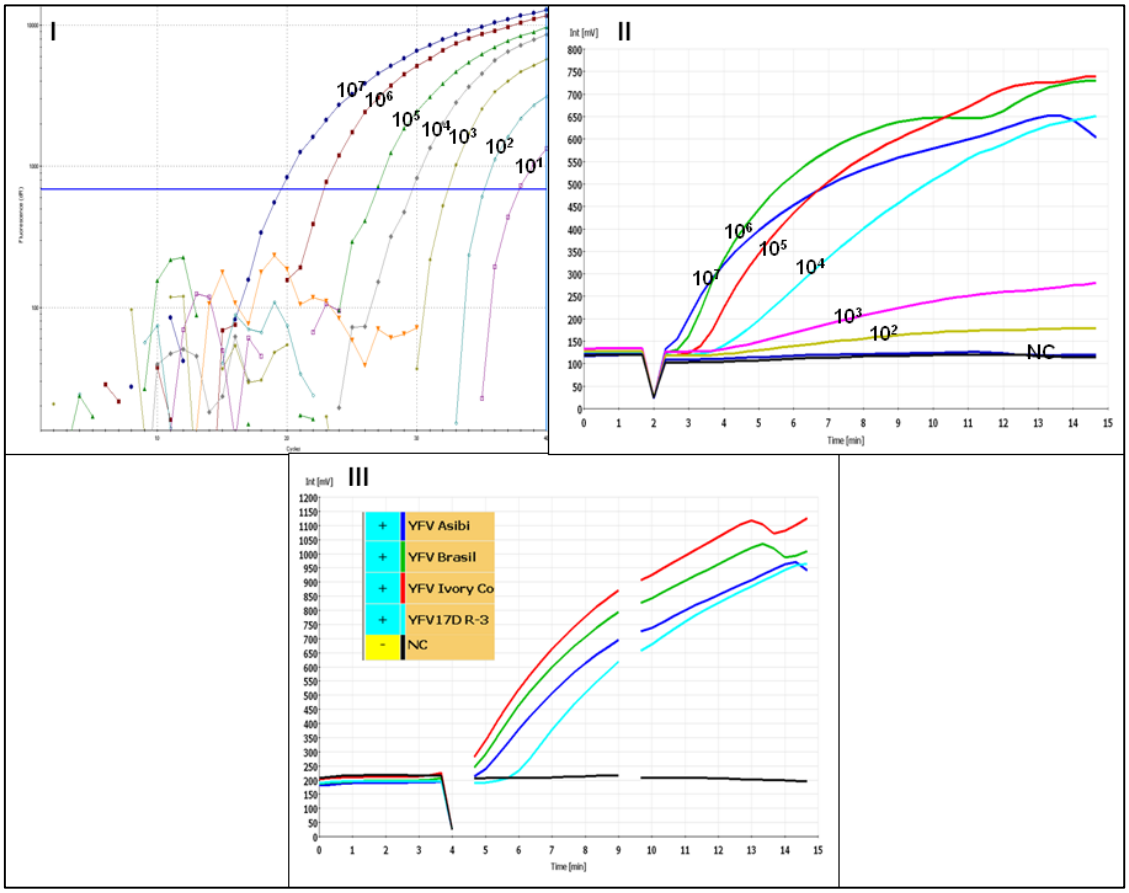


Figure C-7. Comparative sensitivity of real-time RPA vs. real-time RT-qPCR assay I) The amplification plot of real-time RT-qPCR for detection of 10-fold serial dilutions of YFV RNA II) The amplification plot of real-time RPA assay for detection of the 10-fold serial dilution of YFV RNA III) Detection of different YFV wild type strains by the real-time RPA.

D DISCUSSION

Yellow fever remains a medically neglected disease and spreads continuously around Africa and South America (Monath 1997, Barnett 2006). Despite preventive WHO vaccination campaigns in African countries, there is still a high risk of YF in endemic areas. The main reason for this is low vaccination coverage. Therefore, reporting of YFV cases in endemic areas is very important to take appropriate emergency vaccination measures. On the other hand, it is also important to monitor SAEs during mass vaccination campaigns to differentiate them from real infections. If SAEs caused by the YF vaccine is false-positive diagnosed, it may lead to wrong concerns about safety of YF vaccine. For all these reasons, there is a need for specific and sensitive detection methods of Yellow fever virus, which can be applied in low-resource settings or in field conditions.

In the last decade, many molecular assays based on RT-PCR have been developed for the detection of YFV in the acute phase of infection as summarized previously (Sekaran and Artsob 2007; Domingo and Niedrig 2009). Now these molecular assays are widely considered as a diagnostic tool due to high specificity and sensitivity in contrast to other methods. While PCR based methods require extensive thermal cycling and expensive equipment, other nucleic acid amplification methods like isothermal amplification need single constant temperature and therefore it can be performed using the simple equipment like a water bath or a heating block. Several isothermal techniques like SDA, NASBA, LAMP, RCA, HDA, RPA and many others have been developed as an alternative to PCR as summarized previously (Gill and Ghaemi 2008; Kim and Easley 2011; Niemz, Ferguson et al. 2011).

Isothermal amplification methods like SDA, LAMP, RCA, TMA and NASBA are based on strand displacement and utilize DNA or RNA polymerase lacking of 5′-3′ exonuclease activity. Furthermore, the reaction schemes are more complicated in isothermal techniques than in PCR. All these factors limit the use of the exonuclease Taqman probe in the isothermal amplification techniques excepting HDA. In HDA reaction, the exonuclease Taqman probe was successfully utilized as in PCR based methods (Tong, Tang et al. 2008). Nevertheless, some isothermal amplification methods have used a variety of alternative probe-based detection methods such as molecular beacons with NASBA (Leone, van Schijndel et al. 1998), fluorescence transfer probes with SDA (Little, Andrews et al. 1999) and restriction probe with RPA (Piepenburg, Williams et al. 2006). However, these formats of isothermal amplification require the same fluorescence

detector as real-time PCR. In contrast to fluorescence detection, lateral flow strip (LFS) as an endpoint analysis offers an economical alternative for isothermal amplification methods.

RPA amplifies DNA from DNA/RNA template at a constant temperature in the range of 25°C to 42°C and offers the different detection formats by use of the restriction probes like endpoint analysis by gel electrophoresis or LFS and real-time monitoring. All RPA reagents are commercially available as dried reagents, which can be stored at ambient conditions. Furthermore, RPA allows sensitive and specific detection within 15 min. Due to all these advantages; RPA was chosen for developing a diagnostic platform for low-resource settings.

In this study, LFS-RPA assay for rapid detection of YFV was developed. This assay can be easily adapted to different laboratories. Two different real-time RT-qPCR assays have been established targeting a conserved 5´-non coding region (NCR) of the YFV genome. Both of real-time RT-qPCR assays were able to detect down to 10 Geq/rxn of YFV. In this study, primers and probe for the LFS-RPA assay were designed in the same conserved 5´-NCR of the YFV genome. The developed LFS-RPA assay using these primers and probe has detected all the different YFV strains and showed no cross-reactivity to other closely related flaviviruses (Fig. C-4 and C-5). This LFS-RPA assay showed a detection limit down to 23 copies of YFV genome comparisons with real-time RT-qPCR (Fig. C-3). In wild type yellow fever infection and SAEs, higher viremia level up to 8 log 10 PFU/ml is reported. Whereas low viremia was reported in YFV vaccinees and does not exceed 2.3 log 10 PFU/ml or 2.23 log 10 Geq/ml (Barrett and Teuwen 2009; Domingo and Niedrig 2009; Domingo, Yactayo et al. 2011). As 10 serum samples of vaccinees have been tested by established real-time RT-qPCR, seven YFV samples were detected positive and viral load was at the same level as described in literature. The LFS-RPA assay for YFV has detected six out of seven YFV positive samples. All these results support that the sensitivity of developed LFS-RPA assay is in the acceptable range for diagnostic of YFV not only in wild type infection, but also in the sera of vaccines.

Besides the analytical performance, the RPA amplifies target RNA/DNA within 15-20 min. Therefore, the speed of RPA reaction is five times higher than real-time RT-qPCR. As the LFS-RPA assay only needs a heating block or water bath, it can be easily performed in low-resource setting laboratories without the need of expensive instruments. Furthermore, the use of the lateral flow strip as a final readout for results eliminates need of complex analytical systems including computer. Because of all these

advantages, RPA is perfectly suitable for low-resource settings, in field conditions and point-of-need diagnostic settings.

LFS-RPA assay allows simple, rapid, sensitive, and qualitative detection of YFV, but open handling of RPA amplification products has led to the contamination. Therfore, the rules for the diagnostic settings have to be followed strictly for working with the LFS-RPA assay. As an alternative to LFS-RPA, a real-time RPA assay was developed by modifying the primer and probe from LFS-RPA assay for the RPA exo system. The major benefit of real-time RPA assay is that the whole reaction can be performed by using the TwistAmp exo system in a closed system to avoid contamination problems. According to TwistDx manual guidelines, the amplification product of real-time RPA is also completely digested, once the reaction is accomplished. This could also help to eliminate contamination problems, while working with molecular diagnostic methods. Nevertheless, a portable tubescanner device is needed in order to perform real-time RPA reaction. The testing of real-time RPA assay for YFV showed that this assay detected all different strains of YFV. The detection limit of the real-time RPA assay was 100 Geq/rxn, when compared to the real-time RT-qPCR. The real-time RPA was one log less sensitive than real-time RT-qPCR, but the sensitivity of real-time RPA can be optimized further by changing reaction parameters. The results suggest that the real-time RPA assay can be also used for low-resource; in-field and point-of-care settings as LFS-RPA assay and eliminates on the other hand need of lateral flow strips. However, the need portable power supply and computer for the tubescanner device makes it a little instrument dependent.

In summary, isothermal nucleic acid detection methods were successfully developed employing the LFS-RPA assay or real-time RPA assay. Both of these assays can be easily applied in low-resource settings as an alternative to traditional laboratory based molecular diagnostic assays. According to need and scenario, one of these RPA assays can be used for rapid detection of YFV. The results suggest that the RPA has a great potential to develop a simple point-of-care diagnostic platform. In future, these methods and their functionality have to be tested under real conditions in Africa during mass vaccination campaigns.

GENERAL CONCLUSION AND OUTLOOK

This Ph.D. describes the laboratory based and point-of-care molecular diagnostic platforms, which can be used according to need, scenario, and availability of technology for the detection of infectious pathogens. The major advantages and disadvantages of these developed systems are summarized in Table 3.

Table 3. Advantages and disadvantages of different molecular diagnostic platforms.

Type of MDx platform	Advantages	Disadvantages
LabDisc platform	Automated “sample to answer” testing Simple to perform Suitable for in-field and low-resource setting High geometric multiplex capability	Technically complex Difficult for mass production yet Not suitable for high throughput system
Real-time PCR platform	Multiple detection format Multiplex capability High sensitivity and specificity Rapid in time (1 - 2 hour) Viability of software and tools to design assay High throughput system	Expensive Instruments Technically complex Restricted to trained laboratories Cost-intensive
rapidSTRIPE detection system	Easy detection by use of the lateral flow strips Easy to perform High sensitivity and specificity High throughput system Good alternative to real-time PCR format Simple and cost-effective	Limited multiplex capability Semi-quantitative analysis
Recombinase polymerase amplification	Multiple detection format Simple and easy method Little or no hardware required Stable lyophilized reagents High sensitivity and specificity Perfect suitable for low-resource settings High sensitivity and specificity	Limited multiplex capability Semi-quantitative analysis Critical probe design

In the first chapter, the developed/established methods and the microfluidic platform provide a valuable insight about an integrated MDx platform and offer great solutions to overcome problems of NAT platforms regarding automation, reagent storage, sample preparation, multiplex detection, sensitivity, cost, and portability. The described LabDisc platform is one of few successful “sample to answer” platforms for the rapid detection of infectious pathogens. Many advantages such as fully automated testing, rapid in timely, low reagent consumption, minimal handling and need of disposables makes the LabDisc platform a potential “sample to answer” platform for rapid nucleic acid testing in the field of medical, biodefense and research. In future, further developments in reagent storage, mass production, and cost-effective manufacturing of such a LabDisc system will fulfill the demand of existing and emerging health care needs.

The second chapter describes a novel real-time PCR method utilizing LNA-probes for the detection of important re-emerging flaviviruses to complement reference methods. This method would be an useful tool for the detection of many flaviviruses simultaneously in livestock samples, and would aid in the molecular diagnosis and surveillance studies of various vectors and non-human hosts. In future, this method can be used to monitor the activity of flaviviruses in endemic regions providing important data for the surveillance studies.

In the third chapter, the RapidSTRIPE H1N1 test based on nucleic acid lateral flow assay was developed for diagnosis of a swine-origin influenza A (H1N1) virus during the H1N1 2009 pandemic. This test is simple and cost-effective and allows the specific detection of the S-OIV A (H1N1) from a swab sampling to the final detection within 2-3 hours. This method was successfully used as an alternative to classical real-time PCR methods, and has proven its functionality as a cost-effective, simple, and high-throughput system.

The final chapter describes reliable, sensitive, and specific diagnostic methods utilizing an isothermal amplification and a minimal instrument setup. These methods will provide a useful alternative to current MDx technologies at an affordable level and allow molecular diagnostics in low-resource and field settings. This type of decentralized routine diagnostics would also improve monitoring of YFV outbreaks, local disease surveillance, and response to epidemics in YFV endemic region of the world.

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SUPPLEMENTARY DATA

LAB EQUIPMENT AND MATERIALS

Table 0-1. List of the lab equipment used in this study

Equipment	Source
Autoclave	
▪ Automat 21/2	Webeco, Bad Schwartau
▪ Varioklav	H+P Labortechnik, Oberschleissheim
Clean bench	
▪ Clean bench small	The Baker company
▪ Clean bench	Thermo scientific, Finnland
Centrifuge	
▪ Sigma 3K30C	B.Braun, Melsungen
▪ Sigma 1-13	B.Braun, Melsungen
▪ Sigma 1-40	B.Braun, Melsungen
▪ Sigma 1-15K	Thermo scientific, Finnland
Freezer	
▪ -20 °c	Libeherr, Biberach a.d. Riss
▪ -80 °c	Libeherr, Biberach a.d. Riss
Incubator	
▪ Bacterial culture(with shaker)	New Brunswick scientific, USA
▪ Cell culture (with 5 % co_2)	Heraeus, Hanau, Germany
Gel electrophoresis System	Bio-RAD, München
ESE Quant Tubescanner	Qiagen Lake Constance, Germany
Glassware (flasks, pipette ect.)	Schott Glas, Mainz
JEOL JEM-2100 electron microscope	Massachusetts, USA
Kingfisher FLX system	Thermo scientific, Finnalnd
Microscope	
▪ Fluorescent microscope (Axioskop 20)	Zeiss, Jena
▪ Optical light microscope (Axiophot)	Zeiss, Jena
▪ Eurostar Mikroskope	Euroimmun, Lübeck
Nanodrope spectrometer	Thermo scientific, Finnalnd
Neubauer -Cell counting chamber	Roth, Karlsruhe
Photometer (Biophotometer)	Eppendorf, Hamburg
Pipettball (Accujet®)	Brand, Wertheim
Pipette (10 µl, 100 µl, 1000 µl)	Eppendorf, Hamburg Gilson, USA
Real-time PCR cycler	
• ABI 7500 Real-time PCR system	California, USA

• Lightcycler 480	Roche, Switzerland
• MX 3000P™ QPCR sytem	Stratagene, USA
Scales	Sartotius, Göttingen
Sequencer (ABI PRISM® 3100 genetic analyser)	Applied Biosystems, Foster city, USA
SONDE Player	Qiagen Lake Constance, Stockach
SorvallMikro Ultracentrifuge	Thermo scientific, Finnland
Thermoblock	Eppendorf, Hamburg
Thermal cycler	
▪ Biometra T gradient cycler	Biometra, Göttingen
▪ Biometra TRIO thermoblock	Biometra, Göttingen
Transilluminator TC-312A	Spectroline, Westburry, USA
Thermal cycler	
▪ Biometra T gradient cycler	Biometra, Göttingen
▪ Biometra TRIO thermoblock	Biometra, Göttingen
Voltmeter	Biometra, Göttingen
Vortex®	Roth, Karlsruhe

LIST OF CHEMICALS AND ENZYMES

Table 0-2. List of used lab materials

Type	Source
ABgene PCR plattes (Thermo fast 96)	Abgene, Epsom, UK
Cell culture flask (25 cm^3, 75 cm^3, 175 cm^3)	Nunc, Wiesbaden
Cuvette	Eppendorf, Hamburg
Disposableseropipette (2 ml, 5 ml, 10 ml, 25 ml)	B.Braun, Melsungen
Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml)	Eppendorf, Hamburg
Falcon tubes (15 ml, 50 ml)	Nunc, Wiesbaden
Filter paper	Schleicher&Schuell, Dassel
MagnaRackmagentic rack	Invitrogen, Karlsruhe
PCR 8 strip tubes	Eppendorf, Hamburg
Pipette tips with filter	Biozym, Oldenburg
Pipette tips without filter	Eppendorf, Hamburg
Titerplane	Euroimmune AG, Lübeck
Ultra clear cap strips	Abgene, Epsom, UK
Well-Platte(6-well, 24-well, 96-well)	Nunc, Wiesbaden

Table 0-3. List of used chemicals and enzymes

Chemical	Source
Aceton	Roth, Karlsruhe
Agar	Invitrogen, Karlsruhe
Agarose	Biozym, Oldenburg
Ampicillin	Sigma-Aldrich, München
Bovine Serum Albumin (BSA)	NewenglandBiolabs, Frankfurt a. Main
Calcium Chloride	Roth, Karlsruhe
Chloroform	Roth, Karlsruhe
Dimethylsulfoxid	Merck, Darmstadt
DiOC6(3) (3,3´-dihexyloxacarbocyanine iodide)	Sigma-Aldrich, München
Dithiothreitol	Invitrogen, Karlsruhe
DNAse	Invitrogen, Karlsruhe
dNTP mix	Eppendorf, Hamburg
Ethanol	Roth, Karlsruhe
Ethidiumbromid	Roth, Karlsruhe
Evan´s blue	Sigma-Aldrich, München
Fetal bovine serum (FCS)	PAA Laboratories, Linz
Formaldehyde	Roth, Karlsruhe
Magnesiumchlorid	Invitrogen, Karlsruhe
Molecular bi dest. Water	Eppendorf, Hamburg
Penicillin/Streptomycin	PAA Laboratories, Linz
SOC media	Invitrogen, Karlsruhe
Superscript II	Invitrogen, Karlsruhe
Taq-Polymerase	Invitrogen, Karlsruhe
Trypsin	PAA Laboratories, Linz

MEDIA FOR CELL CULTURE

Mostly, the cell culture media in routine use is chemically defined. In addition 5 – 10 % FCS and 1 % of 2 mM L-Glutamine are added the media for the good growth of cell culture. Phenol red is added to the media as a pH indicator. Generally, cells grow at pH 7 to 7.4. Phenol red is red at pH 7.4 and orange at pH 7.0. In acidic condition it becomes yellow at pH 6.5. Antibiotics also can be added to the media to prevent contamination of bacteria and fungus in cell culture.

Table 0-4. List of media and additives

Media	Additional	Source
DMEM (Dulbecco's Modified Eagle Media)	10 % FCS, 1 % Penicillin-Streptomycin, 1 % L-Glutamin	GIBCO Invitrogen, Karlsruhe
L15 (Leibovitz-Media)	5 % FCS, 1 % Penicillin-Streptomycin, 1 % L-Glutamin	GIBCO Invitrogen, Karlsruhe

CELL LINES

Table 0-5. List of cell lines used in this study

Cell line	Origin	Media for cultivation	Source
C6/36 cells	Asian tiger mosquito, larva tissue	L-15 + 5 % FCS	RKI
Hep-2 cells	Human, epidermoid carcinoma	D-MEM + 5 % FCS + 1 % Glutamin	RKI
PS cells	Pig, kidney cells	L-15 + 5 % FCS + 1 % Glutamin	RKI
Vero B4 cells	African green monkey, kidney epithelial cells	D-MEM + 5 % FCS + 1 % Glutamin	RKI
Vero E6 cells	African green monkey, kidney epithelial cells	D-MEM + 5 % FCS + 1 % Glutamin	RKI