Aus dem Max von Pettenkofer-Institut für Hygiene und Medizinische Mikrobiologie

Institut der Ludwig-Maximilians-Universität München

Lehrstuhl: Virologie

Leitung: Prof. Dr. Oliver T. Keppler

Next Generation Sequencing as screening method for resistance associated variants in Hepatitis C Virus before treatment with direct acting antivirals

Dissertation

zum Erwerb des Doktorgrades der Medizin

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

Vorgelegt von:

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aus Freising

2019

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Tag der mündlichen Prüfung: 11.07.2019

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Danksagung:

Mein besonderer Dank gilt Matthias Götte unter dessen Aufsicht und in dessen Labor ich die Experiment für diese Arbeit durchführen durfte, Christopher Ablenas für die großartige Betreuung, sowie allen anderen Mitarbeitern des Dept. of Microbiology and Immunology an der McGill University für die Hilfe und Unterstützung.

Ich danke vor allem meinem Doktorvater Prof. Dr. Josef Eberle für die wertvolle Kritik, Unterstützung und durchgehende Begleitung meiner Arbeit.

Danke auch meiner Familie, meinen Freunden und meinem Freund Merlin für Ermutigung, Unterstützung, Ansporn und viel Geduld.

Veröffentlichung:

Christopher Ablenas, Natan Bensoussan, Laura Mayrthaler, and Matthias Götte:

Comparing Genetic Variation within the Three Domains of the Hepatitis C Virus NS3 Helicase by Deep Sequencing

Poster präsentiert auf dem Canadian HCV Symposium in Banff 2015.

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List of abbreviations:

Amp	amplicon
bp	base pairs
cDNA	complementary deoxyribonucleic acid
ddH2O	double-distilled water
DAA	direct acting antiviral
DNA	deoxyribonucleic acid
E.coli	Escherichia coli
f	femto = 10^{-15}
fw	forward
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IFN	interferon
IFN-alpha	interferon-alpha
kb	kilobases
m	milli = 10^{-3}
MPC	Magnetic Particle Collector
µ	micro = 10^{-6}
n	nano = 10^{-9}
NS2/3	non-structural protein 2/3
NS5b	non-structural protein 5b
p	pico = 10^{-12}
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PTP	Pico Titer Plate

pIFN-alpha	pegylated interferon alpha
qPCR	quantitative polymerase chain reaction
R^2	square of the correlation coefficient R
RAV	resistance associated variant
RBV	ribavirin
rev	reverse
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcription
SVR	sustained virological response
TAE	tris acetate-EDTA
WT	wildtype

I Introduction

I.1 Hepatitis C

The hepatitis C virus (HCV) is a 9.6 kb positive-sense, single-stranded RNA virus of the *Flaviviridae* family. Its genome "encodes a long polyprotein of more than 3000 amino acids that is proteolytically processed to generate 10 mature viral proteins" [1]. Viral structural proteins (core, E1, and E2) are encoded by the N-terminal part of the polyprotein. Viroporin p7 and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) encode for enzymes like a serine protease, RNA helicase and the RNA dependent RNA polymerase as well as assembly factors for the replication complex [2, 3].

6 HCV-genotypes (*1* to *6*) and approximately 100 subtypes (*a* to *n*) have been identified. Genotypes differ in at least 30% of their sequence and subtypes in about 20–25% [4]. Globally, genotype 1 is the most common one with 46%, followed by genotypes 3 (22%) and 2 (13%). The most common subtype is genotype 1b, responsible for 22% of all infections. In North America, Latin America and Europe, genotype 1 is dominant (62–71%), while in Asia genotype 3 is most prevalent (39%). Egypt excluded, where genotype 4 accounts for 93% of all infections, genotype 1 is also predominant in North Africa and the Middle East (46%). Australia is dominated by genotype 1 (53%), followed by genotype 3 (39%) [5].

With approximately 2–3% of the world's population (more than 170 million people) chronically infected [1, 3], hepatitis C virus represents a worldwide health burden. While Germany belongs to one of the countries with a lower HCV-prevalence, the Robert-Koch-Institut (RKI) indicates on its website that, according to studies from 2008 to 2011, HCV-RNA could be identified in at least 0.3% of the German population. However, the real prevalence might be higher because people with a higher risk for HCV infection (prisoners, drug users, immigrants) were underrepresented in the study [6]. About 75% of HCV-infections in adults go on to become chronic infections [4], leading to severe liver disease, including cirrhosis and hepatocellular carcinoma amongst other complications [1]. Compared to HCV monoinfected patients, HIV/HCV co-infected individuals show an accelerated disease progression and poor treatment response to pIFN-alpha and RBV [7, 8].

HIV and HCV share similar risk factors like the use of injection drugs, contact with infected blood as through needles or blood transplants and sex with an infected partner. But because the possibility of HCV transmission is much higher when exposed to infectious blood then during sexual contact, HIV-positive patients have a 8–35-fold higher chance (79–95%) of being co-infected with HCV when they acquired their HIV infection from drug use and not from sexual exposures [9].

The Swiss HIV Cohort Study, investigating 6534 HIV-infected individuals from 1998 until 2011, found 33.3% of HIV infected patients who used injection drugs to be co-infected with hepatitis C. For the non-drug user, homo- and heterosexual groups the incidence rates were much lower (3.0% and 0.8%) [10].

I.2 Therapy

Early therapy of chronic hepatitis C used to only consist of interferon-alpha (IFN-alpha), before ribavirin (RBV) was added to the treatment. The sustained virological response (SVR), defined as undetectable HCV RNA levels (<50 IU/ml) 24 weeks after the cessation of treatment, was thereby increased to about 50%. In 2000 the introduction of pegylated interferon-alpha (pIFN-alpha), with a longer half-life, provided the advantage of weekly administration [11].

In 2011, the first two direct acting antivirals (DAAs), telaprevir and boceprevir were introduced into clinical practice. When combined with pIFN-alpha and RBV, these two protease-inhibitors helped to increase SVR rates by about 30% in treatment-naïve, and 26–60% in treatment-experienced patients infected with genotype 1 [11].

Simeprevir and other second or third generation NS3/4A inhibitors show less side effects and a higher barrier to resistance [11]. Thanks to its 40h half-life, simeprevir needs to be administered only once a day. It is well tolerated, but typically causes asymptomatic high bilirubin levels [11].

Sofosbuvir, a NS5b polymerase inhibitor promises high SVR rates after 24 weeks of treatment for patients infected with genotype 1, 2 and 3, as monotherapy over 12 weeks or when combined with RBV and pIFN-alpha. Common adverse drug events are headache, fatigue, rash, and anemia amongst others [12].

The development of DAAs has “opened a new era to the possibility of IFN-free therapy, lower pill-burden, increased treatment success rate, as well as reduced duration of therapy” [13].

Severe IFN side-effects such as flu-like symptoms and anemia can even be avoided by an IFN-free regimen like the combination of only the two DAAs simeprevir and sofosbuvir, as investigated by the COSMOS-study [14], where SVR rates after 12 weeks were found to be 92% in all patients. Minor adverse events found were fatigue, headache and nausea. Serious adverse events had only been seen in 2% of the patients, all of them treated with simeprevir and sofosbuvir only or in combination with RBV over 24 weeks.

Even without any prophylactic vaccine available [1], some clinicians optimistically “expect eradication of HCV on the earth within a few decades” [13]. On the other hand DAAs are very expensive, which limits the options on an interferon-free therapy for wide-spread use in all infected individuals, not only, but especially in developing countries [13]. Poor response rates in patients with liver cirrhosis and the emergence of resistance variants require a search for better therapies [1].

Pre-existing resistance-associated variants (RAV) and polymorphisms can be critical for treatment outcome, but usually are below viral sequence resolution limits [8]. However, with next generation sequencing like Roche’s 454 deep sequencing platform, extremely low percentages of variants in the genome can be detected, as low as 0.5–1%.

Typical examples are polymorphisms at positions R155 and D168 in NS3A, associated with resistance against boceprevir, telaprevir, simeprevir and vaniprevir. S122A, S122R and S122G provide a certain resistance against simeprevir. Q80K and Q80R were found in less than 10% of treatment failure patients but provide 420-times and 305-times respectively the normal resistance compared to genotype 1b wild type replicon RNA when combined with R155K [15]. The highly variable polymorphism C316Y/N is located in NS5b and therefore associated with resistance against polymerase-inhibitors like sofosbuvir [16].

I.3 Aim of the experiments

I.3.1 Mutant Mix Experiment

In preparation for the second experiment, deep sequencing of a mix of *in vitro* transcribed mutants S122R, R155K, D168V and a wild-type was conducted. To investigate the accuracy of deep sequencing especially at low percentages and low RNA input, these mutants were mixed together and the results were then compared back to the original percentages. Considering that, during treatment, viral loads can sometimes get very low in plasma from patients infected with HCV, the aim was to find a threshold for concentration of RNA at which deep sequencing results are still reliable. To mimic how cellular RNA might interfere with RNA extraction and reverse transcription of viral RNA in practice, RNA from Huh-7 Lunet cells was added as "background-RNA".

I.3.2 Plasma Samples Experiment

For the second experiment, 16 plasma samples from treatment-naïve, HCV/HIV co-infected patients (11 genotype 1a and 5 genotype 1b samples) were screened for pre-existing variants in the NS3 and NS5b regions. Especially the two resistance-associated polymorphisms Q80K in NS3 and C316N in NS5b were of interest. If commonly present in treatment-naïve patients, screening for these mutations before starting a highly expensive and maybe ineffective treatment with DAAs might be worth considering.

II Materials and methods

II.1 Materials

II.1.1 Consumables, chemicals, cell line

Product	Provider	Specification
96 well plate	Greiner	black
Agarose	BioShop Canada Inc.	Cat. No: AGA 002.100
Agencourt AMPure XP - PCR Purification	Beckman Coulter	A63880 Lot: 14632800
Ammonium acetate	BioShop Canada Inc.	3M pH 7
Ampicillin	BioShop Canada Inc.	
Bacteria		E.coli, strain: XL-1 Genotype: *rec*A1 *end*A1 *gyr*A96 *thi*1 *hsd*R17 *sup*E44 *rel*A1 *lac*
Cell line	Dr. Ralf Bartenschlager	Huh-7 Lunets
Chloroform	BioShop Canada Inc.	
Conical tube	Falcon	
Diatec 8-strip PCR tube	Diamed	Product Code: 420-1375
DNA dye	Denville Scientific Inc.	GreenGlo DNA dye Cat. No: CA3600
DNA ladder	GeneDirex	100 bp H3 RTU (ready to use) With orange G tracking dye
dNTP-Mix	Thermo Scientific	10 mM each, 0,2 ml Cat. No: R0191 Lot: 00196662
Ethanol	Commercial alcohols	Cat. No: P016EAAN Lot 16404
Flasks	Sarstedt	
Gloves	Kimberly-Clark	REF 50706 KC 300 Sterling Nitrile Powder-free
Glycogen		
Isopropyl alcohol	BioShop Canada Inc.	Cat. No: ISO920.4 Lot: 4A32510
Kimwipes disposable wipers	Kimberly-Clark	Kimtech Science
Lens cleaning wipes	Zeiss	Pre-moistened
LB media	BioShop Canada Inc.	

Media	Wisent	Modified Eagles Media (DMEM) 10% Fetal Bovine Serum (FBS) 5% Penicillin 5% Streptomycin 5% Non-essential amino acids 0.5 mg/ml G418 (Geneticin)
Nucleoside Solutions	Biobasic	ATP, GTP, CTP, UTP
PBS	Wisent	
PCR tubes	Eppendorf	
Phenochloroform	BioShop Canada Inc.	
Phusion HF Buffer	Thermo Scientific	Contains 7,5 mM MgCl2 F-518Sx Lot: 00154037
RNA ladder	RiboRuler	High Range RNA Ladder Cat. No: SM1821
RNA loading dye	Thermo Scientific	Fermentas 2x RNA Loading dye Cat. No: R0641
SOC Medium Solution	BioShop Canada Inc.	Cat. No: SOC 001.101 10 ml
Sparkleen solution	Fisher Scientific	Cat. No: 04-320-4
Sterile disposable cell spreader	Life Science Products Inc.	Optimum
Sodium Acetate	BioShop Canada Inc.	3M pH 5.2
TAE buffer	BioShop Canada Inc.	1L TAE 50x = 242g Tris 57,1 ml Glacid acetic acid 100 ml of 0,5M ECTA pH 8.0
Trypan Blue Solution	Mediatech Inc.	Cat. No. 25-900Cl
Trypsin	Wisent	
Tween-20 Surfact-Amps Detergent Solution	Thermo Scientific	
Water	Thermo Scientific	HyClone, HyPure Molecular Biology Grade Water Cat. No: SH30538.03
Water	EMD Millipore	HPLC Grade Cat. No: WX0008-1

Table 1: Consumables, chemicals, cell line.

II.1.2 Enzymes

Enzyme	Provider	Specification
DNA Polymerase And Buffer	Thermo Scientific	PhusionTM High- Fidelity DNA Polymerase Cat. No: F-530S
DNA Polymerase 10x Pfu Buffer	Agilent	Cat. No: 600135
Fast digest restriction enzymes Dpn1 ScaI XhoI And 10x FD Buffer	Thermo Scientific	 Cat. No: FD1703 Cat. No: FD0434 Cat. No: FD0694
Green-2-Go-qPCR Mastermix	Bio Basic Inc.	Cat No: QPCR004-S
Reverse Transcriptase and 10x RT reaction buffer	Agilent	AccuScriptTM High Fidelity RT- PCR Kit Cat. No: 600180
TURBO DNase 10x DNase Buffer	Life Technologies	TURBO DNA-free kit Cat. No: AM1907

Table 2: Enzymes.

II.1.3 Kits

Kit	Provider	Specification
Compact Prep Plasmid Midi Kit	Qiagen	Plasmid Midi Kit (25) Cat. No: 12843
GS Junior emPCR kit (Lib-A)	Roche	Bead recovery reagents, emPCR reagents (Lib-A), Oil and Breaking Kit Cat. No: 05996520001
GS Junior Sequencing Kit	Roche	Reagents and Enzymes, Buffers, Packing Beads and Supplement CB Cat. No: 05996554001
GS Junior sipper maintenance kit	Roche	Cat. No: 07138857001
GS Junior Titanium Control Bead Kit	Roche	Cat. No: 05996643001
GS Junior Titanium PicoTiterPlate Kit	Roche	Cat. No: 05996619001
PicoGreen Quant-iT dsDNA Assay Kit	Invitrogen	Cat. No: P7589
RNA-extraction Kit (from plasma)	Qiagen	QIAmp Viral RNA Mini Kit Cat. No: 52904
RNA-extraction Kit (from cells)	Qiagen	RNeasy Mini Kit 50 Cat. No: 74104

Table 3: Kits.

II.1.4 Laboratory equipment

Device	Provider	Model
Centrifuge	Desaga Sarstedt-Gruppe M2	Cat. No. 185101 (Year 2004)
Centrifuge	Sigma Laboratory centrifuges	Model 6K 15
Centrifuge	Thermo Scientific	Sorvall Legend RT Plus Cat. No: 75004377
Gelbox	Liberty2 Biokey American Instruments Inc.	High Speed/ bufferless/ conventional gel system Model: LBT2, Cat No: SYS-LBT2 Serial No: 10444
GS Junior 454 Bead counter	Roche	Cat. No: 06594662001
GS Junior 454 BDD counterweight and adaptor	Roche	Cat. No: 05889103001
GS Junior 454 Bead deposition device	Roche	Cat. No: 05996473001
GS Junior 454 Sequencer	Roche	Cat. No: 05922160001
Heat block (digital)	VWR	Cat. No: 12621-108
Hemocytometer	Assistent Germany	Improved Neubauer, bright-line 0,1mm, Order No: 422/12
Hood	Nuaire	Biological Safety Cabinet Model No: NU-425-400
Hood	Nuaire	Biological Safety Cabinet Model No: NU-425-300
Hood	Ottawa Cabinet Company Ltd.	Model number: CC475C
Incubator	Thermo Scientific	Cat. No: 3110 FormaTM Series II 3110 Water-Jacketed CO2 Incubator
Labquake shaker	Lab Industries Inc.	Cat No: 400-110
Magnetic Particle Collector (MPC)	Life Technologies Invitrogen	DynaMagTM 2 Magnet Cat. No: 12321D
Microscope	Zeiss	
Nanodrop	Thermo Scientific	Nanodrop2000 Spectrophotometer
Power supply	Thermo Electron Corporation	Cat. No: 105ECA-LVD
PCR machine	Eppendorf	Mastercycler gradient
Real Time/qPCR machine	Biotech Inc. CR Corbett Research	Model Rotor Gene 3000
Spectra Max Microplate Reader	Molecular Devices	M5
Tube disperser	Roche	IKA Ultra Turrax Cat. No: 05943353001
Vacuum pump (oil free)	Fisher Scientific	Maxima Dry Type PU1308-N82.0-9.01
Vortex Genie2	Scientific Industries	Model G-560 Cat. No: SI-0236
Water bath	Fisher Scientific	IsoTemp Digital Control Water bath Model 210
Molecular Imager	Biorad	Gel Doc XR

Table 4: Laboratory equipment.

II.1.5 Pipets and tips

Device	Provider	Specification
Barrier Tips	MultiGuard	10, 100, 1000 µl
Electronic Pipet	Brand	Handy Step
Falcon serological pipet tips	Fisher Scientific	2 ml Cat. No: 357507
Low Binding Tips Next Generation Pipet Tip Refill System	VWR	100, 200, 250 µl Cat. No: 89079-476
Nunc Serological Pipet	Thermo Scientific Inc.	50 ml disposable plastic pipet Cat. No: 170358
PD-Tips	Brand	
Progene Universal Fit Tips	Ultident Scientific	1000 µl Cat. No: 87A1000
Manual Pipets	Eppendorf Research	10, 100, 1000 µl
Serological pipet	VWR	10 ml Cat No: 89130-898
Tips Quick Rack Transfer System	BioScience Inc.	0.1-10 µl graduated Cat. No: 30470

Table 5: Pipets and tips.

II.1.6 Plasma samples

The plasma samples were drawn between 2008 and 2013, with the exception of one sample from 2003. 11 of the patient samples were genotype 1a and 5 were genotype 1b. All patients were treatment-naïve and HIV/HCV-co-infected. The viral load ranged between 573,382 and 17,900,000 copies/ml. They were generously provided by a collaboration with Dr Marina Klein from McGill University Health Centre, Division of Infectious Diseases and Chronic Viral Illness Service.

All plasma samples were shipped on dry ice and stored at -80°C.

Unique ID	Inclusion date	HCV genotype	HCV load	Date drawn
A24	26.02.2013		10,995,129	26.02.2013
A42	26.02.2013		2,697,194	26.02.2013
A57	03.04.2013		5,510,698	03.04.2013
A85	09.08.2012		2,262,668	09.08.2012
A93	16.10.2013		573,382	16.10.2013
A94	15.08.2012	1a	3,730,617	15.08.2012
B5	30.05.2011		2,406,201	30.05.2011
B49	19.08.2009		11,467,682	25.08.2009
B51	23.06.2008		16,129,846	23.06.2008
B84	21.08.2009		1,165,396	28.08.2009
C1	25.05.2010		4,786,301	25.05.2010
A76	07.03.2013		9,958,944	07.03.2013
B35	25.01.2010		7,721,399	25.01.2010
B37	25.08.2009	1b	1,109,882	25.08.2009
B57	25.08.2008		17,900,000	25.08.2008
C6	13.05.2003		2,130,000	03.07.2003

Table 6: List of plasma samples.

II.1.7 Plasmids

1a.US.77.H77

HCV 1b 5.1 Replicon

HCV NS3 in pET 21b

II.1.8 Primers

All deep sequencing primers used were full length HPLC purified. Chemical synthesis of primers starts at their 3'-end which may result in a truncated 5'-end in some cases. However, the 5'-end contains the important adapter sequence, key (TCAG) and Multiplex Identifier sequence (MID), which are important for binding to capture beads during emPCR and sequencing primers as well as identification of the amplicon during analysis. With an incomplete 5'-end, amplicons might not be detectable by deep sequencing.

Deep sequencing primers are assembled:

5' – Adaptor sequence (fw/rev) – *key* – MID – template specific sequence – 3'

Primer name	Primer sequence
Adaptor sequence fw (with *key*)	5'-CGTATCGCCTCCCTCGCGCCA*TCAG*-3'
Adaptor sequence rev (with *key*)	5'-CTATGCGCCTTGCCAGCCCGC*TCAG*-3'
MID 1	5'-ACGAGTGCGT-3'
MID 2	5'-ACGCTCGACA-3'
MID 3	5'-AGACGCACTC-3'
MID 4	5'-AGCACTGTAG-3'
MID 5	5'-ATCAGACACG-3'
MID 6	5'-ATATCGCGAG-3'
MID 7	5'-CGTGTCTCTA-3'
MID 8	5'-CTCGCGTGTC-3'
MID 9	5'-TAGTATCAGC-3'
MID 10	5'-TCTCTATGCG-3'
Template specific sequences:	
NS3p Amp7 fw	5'-TGTAGACCAAGACCTCGTCG-3'
NS3p Amp7 rev	5'-GTGGGCCACCTGGAAT-3'
NS3p Amp9 fw	5'-GTGACATCATCAACGGCTTG-3'
NS3p Amp9 rev	5'-TGCCTCGTGACCAGGTAA-3'
NS5b C316 Amp fw	5'-ACCCGCTGTTTTGACTCAAC-3'
NS5b C316 Amp rev	5'-GATGTTATCAGCTCCAAGTCGT-3'
S122R fw	5'-GCCGGCGGGGCGACCGCAGGGGGAGCCTACTCTCC-3'
S122R rev	5'-GGAGAGTAGGCTCCCCCTGCGGTCGCCCCGCCGGC-3'

Random Hexamer Primer	Thermo Scientific	120 µl, 100 µM Cat. No: S0142

Table 7: Primer assembly.

II.1.9 Software

Software	Provider
GS Junior Amplicon Variant Analyzer 3.0	Roche
GS Junior Run Browser 3.0	Roche
GS Junior Sequencer 454	Roche
SoftMaxPro 5.2	Molecular Devices
Rotorgene analysis software 6.1.93	Corbett Research

Table 8: Software.

II.2 Methods

II.2.1 Deep sequencing work flow

For both experiments, 454 GS Junior deep sequencing system was used.

Deep sequencing work flow consists of three major steps: Amplicon library preparation, emulsion based clonal amplification (emPCR) and sequencing.

RNA has to be converted into single stranded cDNA. Using PCR, double stranded DNA amplicons are amplified using primers specifically designed for the deep sequencing protocol. Amplicons then have to be cleared from any short by-products like primer-dimers and any remaining primer by using AMPure magnetic beads and a Magnetic Particle Collector (MPC). After purification, only highly pure PCR product eluted in TE buffer is left. The presence of by-products in a library can be tested for by carrying out a quality control PCR (QCPCR). Amplified short products show up as secondary bands when run on an electrophoresis gel. Additionally, purity can be tested with the help of the Agilent 2001 Bioanalyzer. The library needs to be quantified fluorometrically using Quant-it Picogreen dsDNA Assay Kit. Based on the results, the concentration of each amplicon can be calculated and amplicons can then be diluted to a uniform concentration and pooled so that for the emPCR a certain number of molecules of library DNA per capture bead are added.

For the emPCR, DNA template and special capture beads are mixed in an emulsion of water and oil, every water droplet ideally containing only one capture bead and one DNA template. During the following PCR, the DNA amplicon gets amplified while being attached to the adaptor on the bead until every capture bead contains millions of clonal copies. Afterwards, the emulsion has to be broken and treated with “melt solution” to leave PCR products single stranded. In the end as not all water droplets with a DNA capture bead will contain a DNA template, only DNA-containing capture beads are to be kept. This step is called “enrichment”.

After adding special sequencing primers, between 500,000 and 2 million enriched beads are loaded on the Pico Titer Plate (PTP). With the help of GS Junior bead counter, the percentage of enrichment can be estimated. The PTP device consists of hundreds of thousands of microscopic

wells. Every well contains ideally one Capture Bead with DNA. Additionally, three other layers of reagents (enzymes, packing beads, PPiase beads) are loaded onto the plate. During sequencing nucleotides (TACG) flow across the surface of the PTP one at a time for 200 cycles. With every nucleotide binding to DNA on the Capture Beads a chemiluminescent signal is generated, registered by a 6 megapixel CCD camera. With more than 50,000 reads obtained in parallel, deep sequencing gives detailed information on nature and quantity of sequence variants in the amplicon library.

II.2.2 Mutant Mix Experiment

II.2.2.1 Mutagenesis

The in vitro transcribed mutants D168V and R155K were already present at the lab from previous experiments. For the Mutant Mix Experiment the mutant S122R was created and then transcribed into RNA. A HCV 1b 5.1 Replicon plasmid, 10x pfu Buffer and DNA Polymerase by Agilent were used.

On ice, the following reagents were mixed together three times:

Volume (µl)	Reagent	Concentration
18,2	**ddH_2O**	
2,5	**Buffer (pfu)**	10x
0,625	**dNTP Mix**	10 mM
1,25	**Primer Forward** S122R	10 µM
1,25	**Primer Reverse** S122R	10 µM
0,58	**5.1 Replicon Plasmid**	17.3 µg/µl
0,6	**DNA Polymerase (pfu)**	25 units/µl
total: 25		

Table 9: Master Mix Mutagenesis.

A PCR was then conducted according to the following protocol, using a different annealing temperature for each of the three tubes (51.0°C, 52.5°C, 56.5°C):

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	95°C	2 min	35 cycles
Step 2	Denaturation	95°C	30 sec	
Step 3	Annealing	51.0°C	30 sec	
		52.5°C		
		56.5°C		
Step 4	Extension	72°C	5.5 min	
Step 5	Hold	4°C		

Table 10: Amplicon PCR protocol Mutagenesis.

0.6 µl of Dpn1 was added to the 3 PCR products. Tubes were put in a heat block at 37°C and left 1h to digest. 90 µl of E.coli from a glycerol stock at -80°C were slowly thawed on ice. By the flame, 3 µl of PCR product and 30 µl of bacteria were pipetted into sterile 1.7 ml tubes. Tubes were stored on ice for 30 minutes, then heat shocked at 42°C for 45 seconds. 200 µl of SOC medium solution was added using sterile pipet tips (autoclaved). Tubes were incubated in a lab shaker for 20 minutes at 37°C. By the flame of a Bunsen burner, liquid was poured from the three tubes into three plates with ampicillin and distributed with a sterile spreader. Plates were incubated upside down at 37°C overnight.

Working by the flame: The next day, a 15 ml conical tube was filled with 4.5 ml LB media and 10 µl Ampicillin. One single colony per plate was picked with a sterile pipet and transferred into the 15 ml tube, which was then incubated in a lab shaker at 37°C overnight.

The next day, bacteria were mini-prepped using Qiagen Compact Prep Plasmid Midi kit, following its manual.

Mini-prep products were nanodropped and then stored at -20°C until further proceeding.

II.2.2.2 Digestion

4 µl of FD Buffer, 1 µl of each the restriction enzymes XhoI and ScaI were added to 26 µl of DNA S122R (at a concentration of 115 ng/µl) and mixed with 8 µl of RNase-free water. The contents (40 µl total) were incubated for 2 hours at 37°C. With RNase-free water, the mix was brought up to a volume of 200 µl before 300 µl of Phenochloroform were added. After mixing carefully, the tube was centrifuged for 5 minutes at approximately 13,000 rpm. The upper layer (150 µl) was extracted and added to 300 µl of chloroform. After mixing, the tube was spun for 5 minutes in a bench centrifuge. The upper layer was extracted and added to 600 µl of 100% ethanol, 20 µl of 3 M Sodium Acetate at a pH of 5.2 and 0.5 µl of Glycogen at a concentration of 20 mg/ml. The mixture was stored at -20°C overnight.

On the next day, the tube was centrifuged at 4°C for 1 hour at 13000 rpm. Supernatant was removed and 300 µl of 70% ethanol were added. After spinning it again at 4°C for 1 hour for 13000 rpm, supernatant was again removed and 300 µl of 100% ethanol were added. After spinning it for a third time at 4°C for 1 hour at 13000 rpm, supernatant was removed and DNA was left to air dry for 10 minutes. DNA was resuspended in 42 µl of RNase-free water. 2 µl were then used to determine its concentration with the help of nanodrop.

II.2.2.3 In vitro transcription

In a 1.7 ml Eppendorf tube, 8 µl of linear DNA (0.8 µg), 2 µl of each ATP, CTP, GTP and UTP solution and 2 µl of enzyme mix and 2 µl of 10x reaction buffer were mixed by pipetting up and down several times. The tube was incubated at 37°C for 4 hours. Afterwards, 1 µl of TURBO DNase was added, contents were mixed and then incubated at 37°C for 15 minutes. 115 µl of RNase-free water and 15 µl of Ammonium acetate (3M, pH7) were added, along with 300µl of Phenol Chloroform. After spinning at 4°C for 10 minutes, 300 µl of chloroform were added. The tube was again centrifuged at 4°C for 10 minutes. After that, the top layer was transferred to a new 1.7ml tube with 400 µl of isopropanol. The new tube was stored at -20°C overnight.

On the next day, the mix was centrifuged at 4°C for 1 hour. Supernatant was removed. 300 µl of 70% ethanol were added. After spinning for a second time at 4°C for 1 hour, supernatant was removed and 300 µl of 100% ethanol were added. The tube was spun a third time at 4°C for 1 hour and the supernatant was again taken out and discarded. After letting it air dry for 10 minutes, RNA was resuspended in 50 µl of RNase-free water and nanodropped to find its concentration.

II.2.2.4 Start of new cell line

A new cell line of Huh-7 Lunets was started (cells from stock frozen with liquid nitrogen).

Huh-7 Lunet cells from a liquid nitrogen stock were slowly thawed on ice, and then defrosted in a 37°C water bath. The tube was flicked several times. The cells were then put in 10 ml warm media in a 15 ml falcon tube and mixed by pipetting up and down 2-3 times. The tube was centrifuged for 5 minutes at 3600 rpm at room temperature. The media was removed and replaced by 10 ml fresh media. Cells were mixed by pipetting up and down several times. The whole content was added to a 20 ml flask. 5 ml of media were added and the flask was kept at 37°C overnight. On the next day, old media was removed and replaced by 15 ml of fresh media. 3 days later cells appeared to be about 75-80% confluent, so they were split: Old media was removed from the flask and discarded. 10 ml sterile PBS were used to wash the bottom of the flask, then removed and discarded. 3 ml of trypsin were added and the flask was moved around so that trypsin covered the entire bottom of the flask. Trypsin was then removed and the flask was incubated at 37°C for 5 min. Only 1/6 of the cells was kept, by adding 6ml of media to the cells, pipetting up and down and then adding the 6 ml media plus cells to a 15 ml falcon tube. 1 ml of this was put back in the flask, together with 14 ml of fresh media.

II.2.2.5 Cell harvesting and cell counting

7 days after starting the new cell line, supernatant was harvested. Old media was removed from the flask, cells were washed with sterile PBS, then treated with trypsin and incubated at 37°C for 5min. 6 ml of media was added to the flask, cells and media were mixed well by pipetting up and down and then transferred to a 15 ml falcon tube.

10 µl of cells were added to an Eppendorf tube with 90 µl of trypan blue and mixed. 10 µl of this mixture was placed on the grid of a hemocytometer and cells were counted under a microscope. Cells in 4 squares (0.2 x 0.2mm) of the counting chamber, each consisting of 16 small squares (0.05 x 0.05mm) were counted and the mean value was calculated. The calculated number of cells times 10,000 equals the number of cells per µl in the falcon tube. As 6 ml of media had been transferred into the falcon tube, the number of cells per µl times 6000 equals the total number of cells. For the RNA extraction, 10^5 to 10^6 cells were needed.

In the meantime, the remaining 6 ml of cells and media were spun at 1000 rpm. Supernatant was removed and cells were resuspended in 10 ml sterile PBS and again spun at 1000 rpm. Supernatant was removed and the remaining cells were used for a RNA extraction.

II.2.2.6 RNA extraction

RNA was extracted with RNeasy Mini Kit from Qiagen, following its manual RNeasy Mini Handbook June (2012) [17].

RNA was then nanodropped to determine its concentration and stored at -80°C until further proceeding.

II.2.2.7 Mix at RNA level

A mix, containing 85% of WT RNA (plasmid pET in 21b), 12%, 2.7% and 0.3% of the in vitro transcribed mutants S122R, R155K and D168V was created (see table 11).

RNA	Mix
WT	85%
S122R	12%
R155K	2.7%
D168V	0.3%

Table 11: Original concentrations of the RNA mutants.

In order to have a RNA mix with a final concentration of 5ng/µl, 4 samples at 100ng/µl each were used. 4.25 µl of the WT RNA, 0.6 µl of the S122R mutant, 1.35 µl of a 1/10 dilution of the R155K mutant and 1.5 µl of a 1/100 dilution of the D168V mutant were mixed together. These first 7.7 µl contained 500 ng of RNA mix. 92.3 µl of RNase-free water was added in order to have 100 µl of a final concentration of 5ng/µl (see figure 1).

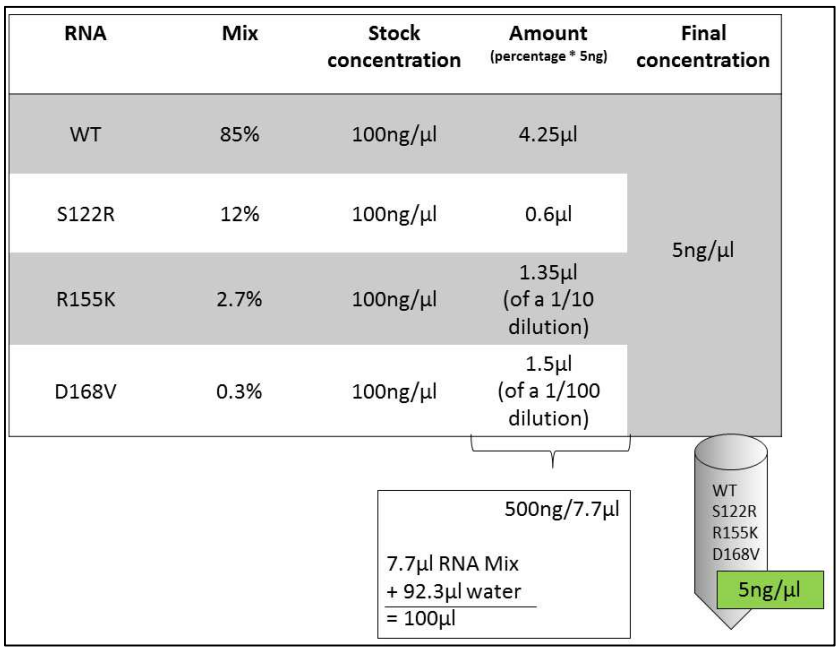


Figure 1: Calculation of the amounts of mutant RNA to be added to the Mutant Mix.

This RNA mix at a concentration of 5 ng/µl was then further diluted 1/10, 1/100, 1/1000, 1/10,000 and 1/100,000 to get final concentrations of 0.5 ng/µl, 50 pg/µl, 5 pg/µl, 0.5 pg/µl and 50 fg/µl (see figure 2).

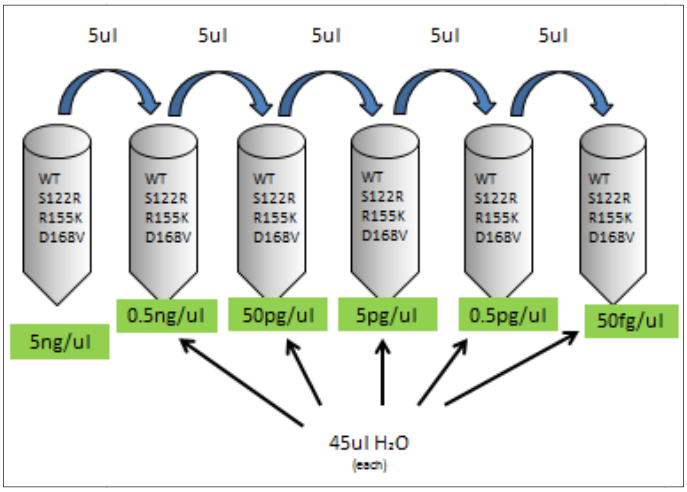


Figure 2: Serial dilution of the RNA Mutant Mix.

II.2.2.8 Reverse Transcription

A reverse transcription of each dilution of the RNA mix (5 ng/µl, 0.5 ng/µl, 50 pg/µl, 5 pg/µl, 0.5 pg/µl and 50 fg/µl) was conducted according to the following protocol (see tables 12, 13).

Additionally, a reverse transcription of the WT plasmid pET in 21b (78 ng/µl) was conducted following the same protocol.

In order to simulate the RNA background coming from cellular RNA that might interfere with the extraction and reverse transcription of viral RNA from human plasma, a total of 998.3 ng of harvested RNA from Huh-7 Lunet cells was added to the reaction.

Volume (µl)	Reagent	Concentration
8.51	**ddH2O**	
2	**AccuScript RT Buffer**	
1	**Random Primer**	
2	**dNTP**	10mM
1.49	**RNA background from cells**	670 ng/µl
1	**Mutant Mix RNA** 5ng, 0.5ng, 50pg, 5pg, 0,5pg, 50fg / **WT plasmid pET in 21**	
total: 16.5		

Table 12: Master Mix Reverse transcription Mutant Mix Experiment.

The mix was incubated at 65°C for 5 minutes, then briefly cooled at room temperature for 5 minutes. The following components were added to the reaction mix:

Volume (µL)	Reagents	Concentration
2	**DTT**	100 mM
1	**Accuscript RT**	40 U/µl
0.5	**Rnase Inhibitor**	

Table 13: Master Mix 2 Reverse transcription Mutant Mix Experiment.

The following program was run:

	Temperature	Time
Preheat	25°C	10 min
	42°C	60 min
	70°C	10 min
Hold	4°C	

Table 14: Reverse transcription protocol Mutant Mix Experiment.

II.2.2.9 Primer optimization (primer tests) NS3p Amp7 MID1

Mutants S122R, R155K and D168V are located in the protease region of NS3. The amplicon covering all three mutants was named NS3p Amp7.

The primers NS3p Amp7 MIDx forward and reverse were already at the lab from previous experiments. Primers generating amplicons for deep sequencing have to be very exact, leaving no short by-products such as free adaptors and adaptor dimers, as those can later interfere with the emPCR by binding to capture beads amongst other reasons. Therefore, they were tested on a HCV 1b 5.1 Replicon plasmid. First a concentration gradient, then in a second reaction a temperature gradient was tested and the products were run on a 1.5% agarose electrophoresis gel (as described in section II.2.2.12), in order to look for secondary bands showing short products. Temperature and concentration showing the best conditions (clear band, no faint secondary bands) were used for further experiments.

A concentration gradient of the primer was created by making a serial diluting of each forward and reverse primer, starting from 10 µM and 5 times diluting one in two (see figure 3).

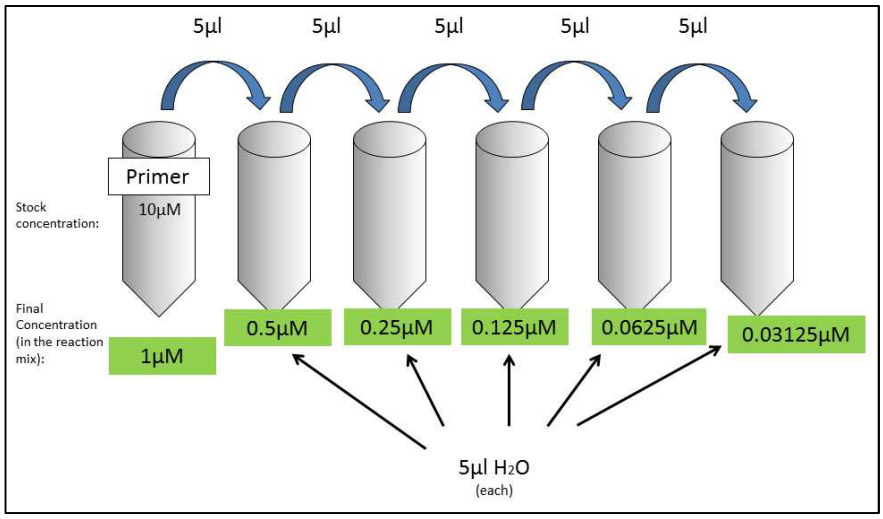


Figure 3: Concentration gradient for primer tests.

For the temperature gradient, annealing temperatures 51.0°C, 52.5°C, 54.2°C, 56.5°C, 59.1°C, 61.8°C and 64.5°C were used.

Tables 15 and 16 show the protocol for the concentration gradient reaction, tables 17 and 18 for the temperature gradient reaction.

Volume (µl)	Reagent	Concentration
13.8	**ddH2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
2.5	**Primer Forward**	10 µM
	NS3p Amp7 MID1	
2.5	**Primer Reverse**	10 µM
	NS3p Amp7 MID1	
0.15	**HCV 5.1 1b Replicon plasmid**	33.2 ng/µl
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 15: Master Mix primer test NS3p Amp7 MID1 concentration gradient.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	64.5°C	30	
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 16: PCR program primer test NS3p Amp7 MID 1 concentration gradient.

Volume (µl)	Reagent	Concentration
17.6	**ddH2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
0.625	**Primer Forward**	10 µM
	NS3p Amp7 MID1	
0.625	**Primer Reverse**	10 µM
	NS3p Amp7 MID1	
0.15	**HCV 5.1 1b Replicon plasmid**	33.2 ng/µl
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 17: Master Mix primer test NS3p Amp7 MID1 temperature gradient.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	51.0°C	30 sec	
		52.5°C		
		54.2°C		
		56.5°C		
		59.1°C		
		61.8°C		
		64.5°C		
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 18: PCR program primer test NS3p Amp7 MID 1 temperature gradient.

II.2.2.10 qPCR

To quantify the cDNA after the reverse transcription, a Real Time quantitative PCR (qPCR) was conducted, according to the following protocol (see table 19). The reaction consisted of 40 cycles. NS3p Amp7 (without MID) was used as primer.

Reagent	Volume (µl)
Green-2-go Mastermix	10
Forward Primer 10uM	1
Reverse Primer 10 µM	1
PCR Water	7
Template	1
Total	20

qPCR Program

	Temperature (ºC)	Time
Hold	95	10 min
Cycling	95	30 sec
	59	60 sec
	72	90 sec
Melt	72 - 95	

Rising 1 degree each step
45 second wait after 1st step
Then 5 second wait after each step afterwards

Place #	Description	Conc.	unit
1	**Standard** HCV1b Neo WT	1.5625	ng/µl
2		0.78125	ng/µl
3		0.390625	ng/µl
4		0.195313	ng/µl
5		0.097656	ng/µl
6		0.048828	ng/µl
7		0.024414	ng/µl
8		0.012207	ng/µl
9		0.006104	ng/µl
10		0.003052	ng/µl
11		0.001526	ng/µl
12		0.000763	ng/µl
13		0.000381	ng/µl
14		0.000191	ng/µl
15	**Blank**		
16	Mutant Mix 5 ng/µl		
17	Mutant Mix 0.5 ng/µl		
18	Mutant Mix 50 pg/µl		
19	Mutant Mix 5 pg/µl		
20	Mutant Mix 0.5 pg/µl		
21	Mutant Mix 50 fg/µl		
22	HCV 1b WT control		

Table 19: qPCR protocol Mutant Mix Experiment.

II.2.2.11 Amplicon PCR

An amplicon PCR was conducted on the 6 reverse transcription products according to the following protocol (see tables 21, 22). 0.2 ml tubes were used. The annealing temperature was 52.5°C.

NS3p Amp7 MIDx was used as primer. For each dilution of the mutant mix, a different MID was used (see table 20). The final concentration of primer in the reaction mix was 0.25 µM.

As a positive control served the plasmid WT NS3 in pET 21b from the reverse transcription, and one PCR containing only RNase-free water instead of cDNA served as negative control.

cDNA	MID
5 ng/µl	MID 1
0.5 ng/µl	MID 2
50 pg/µl	MID 3
5 pg/µl	MID 4
0.5 pg/µl	MID 5
50 fg/µl	MID 6
WT control	MID 6
Negative control	MID 1

Table 20: Assignment of MIDs to Mutant Mix dilutions.

Volume (µl)	Reagent	Concentration
34	**ddH_2O**	
10	**Buffer**	
1	**dNTP Mix**	10 mM
1.25	**Primer Forward**	10 µM
	NS3p Amp7 MIDx	
1.25	**Primer Reverse**	10 µM
	NS3p Amp7 MIDx	
2	**cDNA**	
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 50		

Table 21: Master Mix Mutant Mix cDNA.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	52.2°C	30 sec	
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 22: PCR protocol Mutant Mix cDNA.

II.2.2.12 Run on 1.5% agarose gel

Amplicons of the 6 dilutions of the Mutant Mix (5 ng, 0.5 ng, 50 pg, 5 pg, 0.5 pg and 50 fg), the positive control (WT from pET in 21b plasmid) and the negative control were run on a 1.5% agarose gel for approximately 30–40 minutes at 130V and 60–80A, along with a 100bp DNA ladder as follows:

1.5 g of agarose were melted in 100 ml TAE buffer by heating it up in a microwave for 2 minutes. When cooled down to approximately 55°C, 10 µl of Greenglo DNA dye were added. Afterwards the liquid was poured into a gelbox with a comb and left to dry. Once dried, the comb was removed. At the cathode and anode ends of the box the chambers were filled with TAE buffer. The middle chamber containing the gel was filled up with RNase-free water. Before loading 5 µl the samples into the wells of the dried gel, they were mixed with 1 µl of orange G tracking dye. 5 µl of the 100 bp DNA ladder were loaded next to the samples. A picture of the gel was taken with gel doc system by biorad.

II.2.2.13 Library Purification

Purification was conducted according to GS Junior Amplicon Library Preparation Method Manual (March 2012) [18] section 3.2.2 for PCR tubes:

At first, 22.5 µl of molecular biology grade water were pipetted into 1.5 ml tubes (one per amplicon). Then, 22.5 µl of each PCR product was added to each 1.5 ml tube. Into each tube, 72.0 µl of AMPure beads were transferred and then mixed by vortexing for 5 seconds. Tubes

were incubated for 10 minutes at room temperature and then placed in a Magnetic Particle Collector (MPC) where they were again incubated for 5 minutes at room temperature. With the tubes still in the MPC, the supernatant was removed and discarded without disturbing the beads. Then, 200 µl of 70% ethanol (freshly prepared) were added to each tube and mixed by vortexing for 5 seconds. Tubes were put back in the MPC and incubated for 1 minute at room temperature. With the tubes still in the MPC, supernatant was again removed and discarded without disturbing the beads. The wash with 70% ethanol was repeated. Open tubes were then placed on a heatblock at 37°C until the pellet had dried sufficiently (not longer than 5 minutes to avoid overdrying). 10 µl of 1xTE was added to each tube and mixed by vortexing. Tubes were placed in the MPC and incubated for 2 minutes at room temperature. With the tubes still in the MPC, supernatant was transferred to new screw cap o-ring 1.5 ml tubes. Purified amplicons were stored at -20°C until further proceeding.

II.2.2.14 Library quality control PCR (QCPCR)

A library quality control PCR was conducted according to section 3.3 of the GS Junior Amplicon Library Preparation Method Manual (March 2012) [18]. For each amplicon to be tested, the following mix was prepared (see table 23):

Reagent	**Volume**
Molecular Biology Grade Water	39 µl
PCR Buffer 10x	5 µl
dNTP mix (10mM each)	2 µl
Forward Primer (10µM)	1 µl
Reverse Primer (10µM)	1 µl
Amplicon Library (2x10^8 molecules/µl, estimated)	1 µl
Polymerase	1 µl
Total	50 µl

Table 23: QCPCR mix.

The PCR program was run according to the following table (see table 24):

1x	**94°C**	**11 min**
20x	94°C	1 min
	60°C	1 min
	72°C	1 min
1x	72°C	10 min
	At 4°C on hold	

Table 24: QCPCR program.

Samples were then run on a 1.5% agarose gel to amplify and detect any contaminating small PCR products (as described in section II.2.2.12).

II.2.2.15 Bioanalyzer

Additionally, quality control PCR products as well as purified amplicons were later sent to another lab to be run on Agilent 2100 Bioanalyzer system which is very sensitive in detecting contaminating short fragments.

II.2.2.16 PicoGreen Quantification (Library quantitation)

To quantify the library fluorometrically, Quant-it Picogreen dsDNA Assay Kit was used. As recommended in the GS Junior Amplicon Library Preparation Method Manual (March 2012) [18], the assay was carried out in duplicates (both samples and standard curve).

Eight 1.5 ml tubes were labelled 1-8 and 1xTE was transferred into them as follows:

Tube 1 594 µl

Tubes 2-8 300 µl

6 µl of DNA standard provided with the kit was transferred to tube 1 (1/100 dilution) and the tube was vortexed for 10 seconds. 300 µl were then transferred from tube 1 to tube 2, from

tube 2 to tube 3 and so on until tube 7, each time the tubes were vortexed for 10 seconds. Tube 8 remains without DNA standard. 100 µl of each DNA standard dilution were transferred to the wells of a black fluorometer plate as follows (see table 25):

row	1	2	3	4
Line	Standard (amount of DNA)		Amplicon DNA to measure	
A	100 ng	100 ng		
B	50 ng	50 ng		
C	25 ng	25 ng		
D	12.5 ng	12.5 ng		
E	6.25 ng	6.25 ng		
F	3.13 ng	3.13 ng		
G	1.56 ng	1.56 ng		
H	0 ng	0 ng		

Table 25: Black fluorometer plate.

99 µl of 1x TE Buffer were transferred to the remaining wells (2 per amplicon). 1.0 µl of each purified amplicon DNA sample was transferred to the appropriate wells of the fluorometer plate. Samples were mixed by pipetting up and down 4 times with a multichannel pipet. 100 µl of a 1:200 dilution of PicoGreen reagent were added to each well. Samples and standards were mixed by pipetting up and down 4 times. The fluorescence was then measured with Spectra Max Microplate reader.

Wavelengths: excitation ~ 480 nm, emission ~ 520 nm

As all assays had been carried out in duplicates, each mean value was calculated by adding both values and dividing them by two. Then, the fluorescence value of the reagent blank was subtracted from that of each of the samples. A standard curve was created by plotting the fluorescence emission intensity versus DNA concentration.

II.2.2.17 Amplicon dilution and pooling

Based on the PicoGreen results, the concentration of each amplicon was calculated as follows, according to GS Junior Amplicon Library Preparation Method Manual (March 2012) [18]:

Molecules/µl = [sample conc (*ng/µl*) * 6.022×10^{23}] / [656.6×10^{9} * amplicon length (*bp*)].

Amplicons were then separately diluted down to 10^9 molecules per µl in 1x TE buffer by adding 1 µl in the volume of TE buffer calculated using the following formula:

{[Molecules/µl (from Step 1) / 10^9] -1} µl.

The amplicons were then pooled by mixing together 10 µL of each of the dilutions. The pooled samples were further diluted to 10^7 molecules per µl by adding 2 µl of the amplicon pool to 198 µL of Molecular Biology Grade Water.

The GS Junior Amplicon Library Preparation Method Manual (March 2012) [18] recommends an input of 2 molecules of Library DNA per capture bead. The volume of DNA library needed can be calculated using the following formula:

µl of DNA library per tube = (Desired molecules per bead * 10 million beads) / (library concentration in molecules/µl).

A volume between 5 µL and 30 µL has to be added during emPCR in the end. Depending on these numbers and the concentrations, all samples had to be diluted further down to 2×10^5 molecules per µl.

Pooled amplicons were stored at -20°C.

II.2.2.18 emPCR

The emPCR was conducted according to GS Junior emPCR Amplification Method Manual Lib-A (May 2010) [19]:

All kit components except for the Enzyme Mix and PPiase tubes which were kept at -20°C, were thawed at room temperature and then mixed by vortexing. The tube of Additive was thawed for 5 minutes in a heat block at 55°C and then vortexed.

The tube of emulsion oil was mixed for 10 seconds and then poured into the Turrax stirring tube. 1x Mock Mix was prepared by mixing 430 µl of Mock Mix with 1.72 ml of Molecular Biology Grade Water. Then, 2.0 ml of 1x Mock Mix was added to the emulsion oil in the Turrax stirring tube. The Ultra Turrax Tube Drive was set to 4000 rpm for 5 minutes and started.

Two reaction mixes (Live Amp A and Live Amp B) were prepared according to tables 26 and 27 and then stored on ice.

Reagent	Volume (µl)
Molecular Biology Grade Water	205
Additive	260
Amp Mix	135
Amp Primer B	40
Enzyme Mix	35
PPiase	1
Total:	676

Table 26: Live Amp A.

Reagent	Volume (µl)
Molecular Biology Grade Water	205
Additive	260
Amp Mix	135
Amp Primer A	40
Enzyme Mix	35
PPiase	1
Total:	676

Table 27: Live Amp B.

1 ml of 1x Wash Buffer was added to both tubes of Capture Beads and vortexed. The Capture Beads were pelleted in a bench centrifuge by spinning, rotating, spinning. Supernatants were removed. Beads were once more washed with Wash Buffer. The volume of DNA library needed was calculated using the following formula:

µl of DNA library per tube = (Desired molecules per bead * 5 million beads) / (library concentration in molecules/µl).

According to the manual, approximately 2 molecules of library DNA per Capture bead yields a bead enrichment between 5% and 20% (equals 500,000 to 2 million enriched beads).

As the library DNA had been diluted to a concentration of $2x10^5$ molecules/µl and the desired number of molecules per bead was 0.7 (due to previous experiences with similar experiments), 17.5 µl of DNA library were added to each washed Capture Beads A and B. Both tubes were vortexed. To the tube of Capture Beads B, 600 µl of Live Amp Mix B were added. After vortexing, the entire content was added to the Turrax stirring tube. The Ultra Turrax Tube Drive was set to 2000rpm for 5 minutes and started to mix the emulsion.

Afterwards, Live Amp Mix A was equally handled.

100 µl of emulsion were aliquoted into nine 8-strip cap tubes, using HandyStep electronic pipet. A PCR was conducted according to the following program (see table 28):

1x	4 minutes at 94°C
50x	30 seconds at 94°C 4.5 minutes at 58°C 30 seconds at 68°C
End	10°C on hold

Table 28: emPCR program.

II.2.2.19 Bead recovery and enrichment

Bead recovery and enrichment were conducted according to GS Junior emPCR Amplification Method Manual Lib-A (May 2010) [19]:

In an externally ventilated hood, the emulsion was aspirated from all wells of the strip tubes into a 50 ml conical tube, using a bench top vacuum system. Tubes were rinsed twice with 100

µl isopropanol per well. The rinse, as well as an additional approximately 5 ml isopropanol were added to the conical tube.

For the following steps the conical tube was taken out of the ventilated hood. It was vortexed and 35 ml isopropanol were added before vortexing again. Beads were pelleted in a centrifuge at 930 x *g* for 5 minutes. Supernatant was discarded carefully. 10 ml of Enhancing Buffer were added and the tube was vortexed to resuspend the pellet. Isopropanol was added to a final volume of 40 ml and the tube was vortexed. Beads were pelleted in a centrifuge at 930 x *g* for 5 minutes and again supernatant was poured out and discarded. For another wash, isopropanol was added to a final volume of 35 ml, the tube was vortexed and the beads then pelleted in a centrifuge at 930 x g for 5 minutes. Supernatant was removed. Finally, Enhancing Buffer was added up to a final volume of 35 ml, the tube was vortexed and spun in a centrifuge at 930 x *g* for 5 minutes. Supernatant was taken out, leaving approximately 2 ml of Enhancing Buffer in the tube. The DNA bead suspension was transferred into a 1.7 ml tube. After spinning, rotating, spinning the small tube, supernatant was discarded. The 50 ml tube was rinsed with 1 ml of Enhancing Buffer which was then added to the small tube. The tube was again spun, rotated, spun and supernatant was taken out. The bead pellet was rinsed twice with 1 ml of Enhancing Buffer, spun, rotated, spun both times and supernatant was discarded.

1 ml of Melt Solution (125 µl of 10M NaOH in 9.875 ml of Molecular Biology Grade Water) was added to the 1.7 ml tube with DNA bead suspension. After incubating for 2 minutes at room temperature, the tube was vortexed, spun, rotated, spun and supernatant was discarded. The melt step was repeated once. 1 ml of Annealing Buffer was added. After vortexing, spinning, rotating, spinning the supernatant was taken out. The annealing step was repeated twice. 45 µl of Annealing Buffer and 25 µl of Enrichment Primer were added to the beads. The tube was vortexed and placed in a heat block at 65°C for 5 minutes and then promptly cooled on ice for 2 minutes. 1 ml of Enhancing Buffer was added, the tube was vortexed, spun, rotated, spun and supernatant was discarded. The enhancing step was repeated twice. 1 ml of Enhancing Buffer was added, the tube was vortexed and set aside at room temperature until further proceeding.

A tube of brown Enrichment Beads from the kit was vortexed one minute to resuspend its contents completely. The tube was then placed for 3 minutes in a Magnetic Particle Collector (MPC) to collect the beads. After discarding the supernatant, 500 µl of Enhancing Buffer were added and the tube was vortexed. Beads were again pelleted using the MPC. Supernatant was taken out. The wash with 500 µl of Enhancing Buffer was repeated. After discarding the supernatant, 80 µl of Enhancing Buffer were added and the tube was vortexed.

The 80 µl of brown Enrichment Beads in Enhancing Buffer were added to the 1.7 ml tube with DNA Bead Suspension. The mix was vortexed and then incubated for 5 minutes at room temperature on a lab rotator. Afterwards, the tube was placed for 4 minutes on the MPC. The MPC was inverted several times. The supernatant was carefully taken out, without touching the magnetic brown beads. The pellet was then washed several times (approximately 6 to 10 times) until no more white DNA beads were aspirated (supernatant was collected in extra tubes and spun in a centrifuge in order to make sure that no more white beads were left).The washes were conducted as follows: After adding 1 ml of Enhancing Buffer, the tube was vortexed and then placed in the MPC. Approximately 3 minutes later, supernatant was carefully taken out.

To the tube of enriched beads, 700 µl of Melt Solution were added. After vortexing, the tube was placed in the MPC until the beads had pelleted. With the tube still on the MPC, the supernatant containing the enriched DNA beads was transferred to a new 1.7 ml tube. The brown beads were again treated with 700 µl Melt Solution and supernatant was added to the new tube. To make sure that no brown beads had been aspirated with all the white beads, the new 1.7 ml tube was spun, rotated, spun in a bench top centrifuge. Supernatant was discarded. 1 ml of Annealing Buffer was added and the tube was vortexed. After spinning, rotating, spinning the supernatant was taken out. The wash with Annealing Buffer was repeated twice. Finally, 100 µl of Annealing Buffer were added and the tube was vortexed.

Then, 25 µl of Seq Primer from the kit was added, the tube was vortexed and then placed in a heat block at 65°C. After 5 minutes, the tube was promptly cooled on ice for 2 minutes. Afterwards, 1 ml of Annealing Buffer was added. After spinning, rotating, spinning the

supernatant was discarded. The wash with Annealing Buffer was repeated three times, but after spinning, rotating, spinning for the last time, supernatant was left in the tube.

II.2.2.20 Bead counting

A 1.7 ml tube provided with the kit was used. The tube was put in the GS Junior Bead Counter to estimate the number of enriched beads. The upper line of the window corresponds to 20% (equals 2 million beads), the lower line represents 5% (equals 500,000 beads).

Beads were stored at +4°C until further proceeding.

II.2.2.21 Deep sequencing

The actual sequencing run was conducted following GS Junior Sequencing Method Manual (March 2012) [20]:

First, kit components of the Reagents and Enzyme box were thawed: All 2.0 ml tubes were stored on ice to thaw. The empty container was filled with tab water. The 50 ml conical tube and 10-tubes Reagents-cassette were placed back in the container for thawing. Packing Beads and Supplement CB were kept on ice together with the 2.0 ml tubes.

A pre-wash was conducted: Therefore all Sipper tubes in the 454 machine were replaced by those from the new kit. The pre-wash cassette was installed in the instrument after rinsing it twice with nanopure water and then filling its tubes with Pre-wash Buffer till approximately 1cm from the top of the tubes. The bottle of Pre-wash Buffer was placed next to an empty waste bottle. A used PTP device was still there in the machine.

To the Buffer CB bottle, 6.6 ml of Supplement CB were added and contents were mixed by inverting the bottle 10 times. Then, 40 ml of the Buffer CB were transferred into a clean 50 ml conical tube and placed on ice. The Apyrase tube was spun in a centrifuge for 5 seconds at

10,000 rpm. 6.5 µl of Apyrase were added to the 50 ml conical tube containing CB Buffer. Contents were mixed by inverting the conical tube 10 times and then stored on ice.

The bead loading gasket and cartridge seal from the PTP device tray were washed together with the Bead Deposition Device (BDD) in a Sparkleen solution, cleaned with a toothbrush, diligently rinsed with nanopure water and thoroughly dried with Kimwipes. Altogether, they were assembled with the PTP device.

Four different layers were loaded onto the PTP device as follows (see table 29). Every time the BDD was centrifuged before loading the next layer. To keep liquid inside the BDD during spinning, both loading port and air vent were covered with tape.

Bead Layer	Bead Type
Layer 1	Enzyme Beads Pre-layer
Layer 2	DNA and Packing Beads
Layer 3	Enzyme Beads Post-layer
Layer 4	PPiase Beads

Table 29: Layers of enzymes and beads to be loaded onto the PTP device.

Through the loading port, 350 µl of Buffer CB from the 50 ml conical tube were pipetted onto the PTP device. The BDD was spun for 5 minutes at 4,013 rpm.

As 400,000 DNA beads were attempted to be sequenced because this amount of beads had shown to yield the best results in previous experiments, 0.32% of the 1,250,000 enriched beads were transferred into a new 1.7 ml tube. This was done by adjusting the volume of enriched beads to exactly 1 ml and then taking out 320 µl of bead suspension. Beads were then pelleted in a centrifuge and enough supernatant was removed to leave only 100 µl in the tube.

Control Beads XLTF were centrifuged for 5 seconds at 10,000 rpm. After pipetting up and down 5 times to resuspend the beads, 6 µl of them were added to the new 1.7 ml tube of enriched DNA beads (not more than 100 µl). 500 µl of CB Buffer from the 50 ml conical tube were added to the DNA Beads. The tube was vortexed and incubated for 20 minutes at room temperature on a lab rotator.

Packing Beads were three times washed with 1 ml of CB buffer from the 50 ml conical tube as follows: CB Buffer was added, beads were centrifuged at 10,000 rpm for 5 minutes and

supernatant was carefully removed. After the third wash, 200 µl of CB Buffer from the 50 ml conical tube was added, the contents were mixed by vortexing and then stored on ice.

1 ml of CB Buffer from the 50 ml conical tube was added to each PPiase Beads and Enzyme tube. Both tubes were vortexed and then put in a Magnetig Particle Collector (MPC) to pellet the beads. The MPC was inverted several times before letting the beads pellet completely. Supernatants were removed. Both bead types were washed three times with 1ml CB Buffer from the 50 ml conical tube, as explained before. After the third wash, 400 µl of CB Buffer from the 50 ml conical tube was added to the Enzyme Beads and 410 µl were added to the PPiase Beads. Both tubes were vortexed. Two new 1.7 ml tubes were labelled Pre-layer and Post-layer and prepared as follows (see table 30):

Reagents	CB Buffer (50 ml tube)	Enzyme Beads	Total volume
Pre-layer	300 µl	110 µl	410 µl
Post-layer	180 µl	230 µl	410 µl

Table 30: Pre-layer and Post-layer mixes.

Both tubes were vortexed and stored on ice.

The tube of DNA Beads was retrieved from the lab rotator and spun in a centrifuge for 10 seconds at 10,000 rpm. Enough supernatant was removed to leave 50 µl in the tube. The Polymerase and Polymerase Cofactor were spun for 5 seconds at 10,000 rpm. Then, 40µl of Polymerase, 20 µl of Polymerase Cofactor and 65 µl of CB Buffer from the 50 ml conical tube were added to the DNA Beads. The mixture was vortexed and incubated on the lab rotator at room temperature for 10 minutes.

Afterwards, the tube of Packing Beads was vortexed and 175 µl of them were transferred into the tube of DNA Beads. The DNA Beads were again incubated on the lab rotator at room temperature for 5 minutes.

From the BDD device, as much supernatant as possible was carefully pipetted out through the port hole. After vortexing the tube of Pre-layer, 350 µl of it were promptly loaded onto the PTP device. The BDD was centrifuged for 5 minutes at 4,013 rpm.

In the meantime, 1 ml of DTT was added to the bottle of Buffer CB, after vortexing. After inverting the tube of Substrate TW 10 times, 44 ml of it were transferred into the bottle of Buffer CB. The bottle was then inverted 10 times.

From the BDD, as much supernatant as possible was carefully pipetted out through the port hole. After mixing it thoroughly, 350 µl of DNA and Packing Beads suspension were loaded onto the PTP device. The BDD was spun again for 10 minutes at 4,013 rpm.

In the meantime, the Pre-wash cassette and Pre-wash bottle were removed from the 454 machine and discarded. The waste bottle was emptied and place back in the instrument. After changing gloves to avoid contamination, the Reagents cassette was inverted 20 times to mix. All caps were carefully removed, avoiding contamination from one tube to another. The Reagents cassette was placed in the instrument. The bottle of Buffer CB was placed next to the waste bottle. By clicking Proceed on the computer, the prime of the instrument was started.

After changing gloves, as much supernatant as possible was carefully pipetted out through the port hole from the BDD. After vortexing, 350 µl of Post-layer were promptly loaded through the loading port. The BDD was spun for 10 minutes at 4,013 rpm.

In the meantime, the used PTP device in the 454 machine was removed. After changing the gloves, the surface of the cartridge was wiped with a Kimwipe and 50% ethanol. The surface of the PTP cartridge was cleaned with a Kimwipe and a 10% solution of Tween-20 Surfact-Amps Detergent Solution. The camera was wiped with a Zeiss pre-moistened cleaning tissue.

From the BDD, as much supernatant as possible was carefully pipetted out through the port hole. After vortexing, 350 µl of PPiase Beads were loaded onto the PTP device. The BDD was centrifuged for 5 minutes at 4,013 rpm.

After spinning, as much supernatant as possible was carefully pipetted out through the port hole from the BDD. The PTP device was removed from the BDD and inserted onto the cartridge seal in the 454 instrument. The back of the PTP device was cleaned with a Kimwipe, the camera door was closed and the sequencing run was started.

II.2.2.22 Analysis

Data from the sequencing run was analyzed with the help of GS Junior 454 Sequencing System Software Manual, v 2.5p1 (August 2010) [21]. As reference sequence, against which the sequencing reads are aligned and compared so variations can be detected, served a HCV 1b NS3 sequence. Multiplex Identifiers (MIDs) helped identifying the single amplicons.

II.2.3 Plasma Samples Experiment

II.2.3.1 Primer generating / Fusion primer design

Amplicon Primers were designed with the help of IDT-Integrated DNA Technologies, using a HCV 1a, 1b, 1c consensus sequence as reference sequence. According to 454 Sequencing System Guidelines for Amplicon Experimental Design (July 2011), deep sequencing requires amplicons between 200 and 600 bp in length, including adapter sequences [22]. The selected amplicons for this project are located in NS2/NS3p (NS3p Amp9) and in NS5b. The first one starts in NS2 at position 554 and covers the first part of NS3p until position 329. It is 427 bp long (including primers).

Deep sequencing primers contain a certain adapter sequence, key (TCAG) and Multiplex Identifier sequence (MID) which are important for binding to capture beads during emPCR and sequencing primers as well as identification of the amplicon during analysis.

The amplicon in NS5b contains 434 bp (including primers). It starts at position 661 of NS5b and ends at position 1094.

II.2.3.2 Primer optimization (primer tests) NS3p Amp9, NS5b C316

Primers generating amplicons for deep sequencing have to be very exact, leaving no short secondary products (as described in section II.2.2.9)

With a standard polymerase chain reaction, the primer pairs NS3p Amp9 MID1 fw, rev and NS5b C316 MID1 fw, rev were tested (see tables 31–40). Products were run on a 1.5% agarose electrophoresis gel to look for secondary bands showing short products (as described in section II.2.2.12). Temperature and concentration showing the best conditions (clear band, no secondary faint bands) were used for further experiments.

The fw primer of NS3p Amp9 is located in NS2. Therefore the primer pair was tested on a 1a.US.77.H77 plasmid, which covers both NS2 and NS3. A concentration gradient for NS5b fw and rev was tested on a HCV 1b 5.1 Replicon plasmid. For the temperature gradient, both the HCV 1b 5.1 Replicon plasmid and a 1a.US.77.H77 plasmid were used. The concentration gradient was created as explained in section II.2.2.9. Phusion Buffer and DNA Polymerase were used.

Volume (µl)	Reagent	Concentration
16.8	**ddH_2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
0.625	**Primer Forward**	10 µM
	NS3p Amp9 MID1	
0.625	**Primer Reverse**	10 µM
	NS3p Amp9 MID1	
1	**pH77/deltaE1-p7**	5 ng/µl
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 31: Master Mix primer test NS3p Amp9 MID1 temperature gradient.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	52.5°C	30 sec	
		56.5°C		
		61.8°C		
		64.5°C		
		69.1°C		
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 32: PCR program primer test NS3p Amp9 MID1 temperature gradient.

Volume (µl)	Reagent	Concentration
13	**ddH2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
2.5	**Primer Forward**	10 µM
	NS3p Amp9 MID1	
2.5	**Primer Reverse**	10 µM
	NS3p Amp9 MID1	
1	**pH77/deltaE1-p7**	5 ng/µl
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 33: Master Mix primer test NS3p Amp9 MID1 concentration gradient.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	56.5°C	30 sec	
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 34: PCR program primer test NS3p Amp9 MID1 concentration gradient.

Volume (µl)	Reagent	Concentration
13.7	**ddH2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
2.5	**Primer Forward**	10 µM
	NS5b C316 MID1	
2.5	**Primer Reverse**	10 µM
	NS5b C316 MID1	
0.29	**5.1 Replicon plasmid**	17.3 ng/µl
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 35: Master Mix primer test NS5b C316 MID1 temperature gradient with 5.1 replicon plasmid.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	51.0°C	30 sec	
		52.5°C		
		56.5°C		
		61.8°C		
		64.5°C		
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 36: PCR program primer test NS5b C316 MID1 temperature gradient with 5.1 replicon plasmid.

Volume (µl)	Reagent	Concentration
13.7	**ddH_2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
2.5	**Primer Forward**	10 µM
	NS5b C316 MID1	
2.5	**Primer Reverse**	10 µM
	NS5b C316 MID1	
1	**pH77/deltaE1-p7**	5 ng/µl
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 37: Master Mix primer test NS5b C316 temperature gradient with pH77/deltaE1-p7.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	51.0°C	30 sec	
		52.5°C		
		56.5°C		
		61.8°C		
		64.5°C		
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 38: PCR program primer test NS5b C316 temperature gradient with pH77/deltaE1-p.

Volume (µl)	Reagent	Concentration
13.7	**ddH2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
2.5	**Primer Forward**	10 µM
	NS5b C316 MID1	
2.5	**Primer Reverse**	10 µM
	NS5b C316 MID1	
0.29	**5.1 Replicon plasmid**	17.3 ng/µl
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 39: Master Mix primer test NS5b C316 MID1 concentration gradient with 5.1 replicon plasmid.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	56.5°C	30 sec	
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 40: PCR program primer test NS5b C316 MID1 concentration gradient with 5.1 replicon plasmid.

II.2.3.3 RNA extraction

RNA was extracted from the 28 plasma samples on 2 different days (first 8 samples on July 30th, then 20 more samples on August 6th 2014), following instructions of Qiagen RNeasy Mini Kit (June 2012) [17]. In order not to contaminate the samples with DNA from plasmids and to get some safety instructions on how to handle infectious blood plasma, the extraction was conducted at the Lady Davis Institute (LDI) Montreal. Plasma and extracted RNA where shipped on dry ice. From the 28 samples, 16 were used for deep sequencing in the end.

At the first RNA extraction, RNA was eluted in 60 µL of Buffer AVE, as recommended in the Qiamp Viral RNA Mini Handbook (June 2012) [23]. The second time, to increase the final concentration, RNA was eluted in only 30 µL.

As a negative control, RNase-free water instead of plasma was added to one reaction per RNA extraction.

II.2.3.4 Reverse transcription

The negative controls from the RNA extractions were further used to the reverse transcription. As positive control served RNA WT NS3 in pET 21b and 1a.US.77.H77

For targets bigger than 2kb, Agilent AccuScript High Fidelity 1st Strand cDNA Synthesis Kit Instruction Manual recommends an input of 200-5000ng of RNA to the reaction [24]. Up to 11 µl of RNA can be added. As the main part of eluted RNA from the RNA extraction is carrier RNA, to simplify calculations only carrier RNA was taken into calculating the quantity of RNA added to the reaction.

5600ng of carrier RNA in 60 µL of Buffer AVE equals 93.3 ng/µL. When multiplied by 11, because up to 11 µl of RNA can be added, this equals 1026.3 ng, which is within the recommended quantity for a target bigger than 2 kb.

5600 ng/30 µL = 186.7 ng/µL. When multiplied by 11 this equals 2057 ng.

For all reverse transcriptions, the maximum volume (=11 µl) was added.

The reverse transcription was conducted following this protocol (tables 41, 42, 43):

Volume (µl)	Reagent	Concentration
0	**ddH2O**	
2	**AccuScript RT Buffer**	
1	**Random Primer**	
2	**dNTP**	10mM
11	**RNA**	
total: 16.5		

Table 41: Master Mix Reverse transcription Plasma Samples Experiment.

The mix was incubated at 65°C for 5 minutes, then briefly cooled at room temperature for 5 minutes. The following components were added:

Volume (µL)	Reagents	Concentration
2	DTT	100 mM
1	Accuscript RT	40 U/µl
0.5	Rnase Inhibitor	

Table 42: Master Mix 2 Reverse transcription Plasma Samples Experiment.

The following program was run:

	Temperature	Time
Preheat	25°C	10 min
	42°C	60 min
	70°C	10 min
Hold	4°C	

Table 43: Reverse transcription program Plasma Samples Experiment.

cDNA was stored at -20°C until further proceeding.

II.2.3.5 qPCR

19 extracted cDNA samples as well as two WT controls (one from a 1a.US.77.H77 plasmid and one from RNA) were compared to 14 standards and one blank, containing RNase-free water. The 14 standards are dilutions from a HCV 1b plasmid.

Real Time PCR was conducted following this protocol (see tables 44 and 45):

Reagents	Volume (µl)
Green-2-go Mastermix	10
Forward Primer 10uM	1
Reverse Primer 10 uM	1
PCR Water	7
Template	1

Table 44 qPCR Mastermix.

qPCR Program

	Temperature (ºC)	Time	
Hold	95	10	minutes
Cycling	95	30	seconds
	59	60	seconds
	72	90	seconds
Melt	72 - 95		

Rising 1 degree each step

45 second wait after 1st step

Then 5 second wait after each step afterwards

Place #	Description	Conc.	unit
1	**Standard** HCV1b Neo WT	1.5625	ng/µl
2		0.78125	ng/µl
3		0.390625	ng/µl
4		0.195313	ng/µl
5		0.097656	ng/µl
6		0.048828	ng/µl
7		0.024414	ng/µl
8		0.012207	ng/µl
9		0.006104	ng/µl
10		0.003052	ng/µl
11		0.001526	ng/µl
12		0.000763	ng/µl
13		0.000381	ng/µl
14		0.000191	ng/µl
15	**Blank**		
16	A12-02		
17	A24-10		
18	A42-10		
19	A57-10		
20	A61-11		
21	A85-03		
22	A93-03		
23	A94-01		
24	B05-08		
25	B49-03		
26	B51-01		
27	B84-01		
28	C01-15		
29	A76-05		
30	B33-05		
31	B35-04		
32	B37-03		
33	B57-01		
34	C06-01		
35	WT control from plasmid		
36	WT control from RNA		

Table 45: qPCR protocol Plasma Samples Experiment.

II.2.3.6 Amplicon PCR

Following the protocols as shown in tables 50, 51, 52 and 53, four different PCRs were conducted in order to get enough samples working for deep sequencing. For some, amplification was not or not sufficiently possible, due to either damage at shipping or a failure of the reverse transcription or amplicon PCR. The negative control used for the RNA extraction was included in the reactions (-Ve), as well as several other negative controls, containing RNase-free water instead of a sample (-Ve (H2O)).

Despite the fact that the NS3p fw primer is located in NS2-region, a NS3-NS5b replicon plasmid was used as a positive control for the first two reactions, showing obviously no band on the gel. For the third reaction a standard material (strain 1a.US.77.H77) was used, giving a strong band. The fourth reaction was for NS5b samples and therefore HCV 1b 5.1 Replicon plasmid served as positive control. Phusion Buffer and DNA Polymerase were used.

Primers NS3p Amp9 MIDx fw and rev were used for the first 3 reactions, NS5b MIDx fw and rev for the last PCR. Samples and MIDs were assembled as follows (see tables 46, 47, 48, 49):

cDNA	MID
A12-02	MID 2
A24-10	MID 3
A42-10	MID 4
A57-10	MID 5
A61-11	MID 6
A85-03	MID 7
A93-03	MID 8
A94-01	MID 9
WT control	MID 10
-Ve	MID 2
-Ve (H2O)	MID 3
-Ve (H2O)	MID 4
-Ve (H2O)	MID 5

Table 46: Assignment of MIDs to plasma samples first amplicon PCR.

cDNA	MID
B05-08	MID 2
B51-01	MID 3
B49-03	MID 4
B57-01	MID 5
A76-05	MID 6
B33-05	MID 7
B35-04	MID 8
B37-03	MID 9
WT control	MID 10
-Ve	MID 2
-Ve (H2O)	MID 3
-Ve (H2O)	MID 5
-Ve (H2O)	MID 7

Table 47: Assignment of MIDs to plasma samples second amplicon PCR.

cDNA	MID
C01-15	MID 6
B84-01	MID 7
WT control	MID 10
-Ve (H2O)	MID 6

Table 48: Assignment of MIDs to plasma samples third amplicon PCR.

cDNA	MID
A76-05	MID 6
B33-05	MID 7
B35-04	MID 8
B37-03	MID 9
B57-01	MID 5
C06-01	MID 4
A24-10	MID 4
A42-10	MID 3
A57-10	MID 2
A85-03	MID 7
A93-03	MID 6
WT control	MID 10
-Ve	MID 9
-Ve (H2O)	MID 8
-Ve (H2O)	MID 7
-Ve (H2O)	MID 6

Table 49: Assignment of MIDs to plasma samples fourth amplicon PCR.

Volume (µl)	Reagent	Concentration
14.5	**ddH_2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
1.25	**Primer Forward**	10 µM
	NS3p Amp9 MIDx	
1.25	**Primer Reverse**	10 µM
	NS3p Amp9 MIDx	
2	**cDNA from plasma**	
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 50: Master Mix plasma samples from first and second RNA extraction using NS3p Amp9.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	56.5°C	30 sec	
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 51: PCR program plasma samples from first and second RNA extraction using NS3p Amp9.

Volume (µl)	Reagent	Concentration
14.5	**ddH2O**	
5	**Buffer**	
0.5	**dNTP Mix**	10 mM
1.25	**Primer Forward**	10 µM
	NS5b C316 MIDx	
1.25	**Primer Reverse**	10 µM
	NS5b C316 MIDx	
2	**cDNA from plasma**	
0.5	**DNA Polymerase (phusion)**	2 units/µl
total: 25		

Table 52: Master Mix plasma samples from first and second RNA extraction NS5b.

PCR Program		Temperature	Time	
Step 1	Initial Denaturation	98°C	30 sec	30 cycles
Step 2	Denaturation	98°C	10 sec	
Step 3	Annealing	54.2°C	30 sec	
Step 4	Extension	72°C	15 sec	
Step 5	Extension	72°C	7 min	
Step 6	Hold	4°C		

Table 53: PCR program plasma samples from first and second RNA extraction NS5b.

II.2.3.7 Library purification

Library purification was conducted according to the GS Junior Library Preparation Method Manual (March 2012) [18] as described for the Mutant Mix Experiment (section II.2.2.13).

II.2.3.8 Library Quality Control PCR (QCPCR)

Library Quality Control PCR was conducted according to the GS Junior Library Preparation Method Manual (March 2012) [18] as described for the Mutant Mix Experiment (section II.2.2.14).

II.2.3.9 Bioanalyzer

Purified samples and amplicons were sent to another lab to be run on Agilent 2100 Bioanalyzer system as described for the Mutant Mix Experiment (section II.2.2.15).

II.2.3.10 PicoGreen Quantification (Library Quantitation)

PicoGreen Quantification was conducted according to the GS Junior Library Preparation Method Manual (March 2012) [18] as described for the Mutant Mix Experiment (section II.2.2.16).

For NS5b the concentration of the WT control was higher than the highest standard curve value. Therefore this sample was diluted 1/2 and re-measured immediately. For the calculation, the dilution factor 2 was taken into account.

II.2.3.11 Amplicon dilution and pooling

Amplicon dilution and pooling was conducted according to the GS Junior Library Preparation Method Manual (March 2012) [18] as described for the Mutant Mix Experiment (section II.2.2.17).

All three amplicon libraries were pooled and the mix was brought to a final concentration of $2x10^5$ molecules/µl.

II.2.3.12 emPCR

The emPCR was conducted according to the GS Junior emPCR Amplification Method Manual Lib-A (May 2010) [19] as described for the Mutant Mix Experiment (section II.2.2.18).

Previous experiments had shown that the enrichment can be too high when using the 2 molecules per µl recommended by the manual. That is why for the samples from the first RNA

extraction, the desired number of molecules per bead was 1. Therefore 25 µl of the samples pool were used.

As the enrichment from the emPCR of the samples from the first RNA extraction was 5% and therefore at the lower end of the window, the attempted number of molecules per bead for samples from the second RNA extraction was 1.2, and 30 µl of the pooled samples were used.

For NS5b, 1.1 molecules per bead were desired and therefore 27.5 µl of the pool were used.

II.2.3.13 Bead recovery and enrichment

Bead recovery and enrichment were conducted according to the GS Junior emPCR Amplification Method Manual Lib-A (May 2010) [19] as described for the Mutant Mix Experiment (section II.2.2.19).

II.2.3.14 Bead counting

Beads were counted according to the GS Junior emPCR Amplification Method Manual Lib-A (May 2010) [19] as described for the Mutant Mix Experiment (section II.2.2.20).

II.2.3.15 Deep sequencing

Deep sequencing was conducted according to the GS Junior Sequencing Method Manual (March 2012) [20] as described for the Mutant Mix Experiment (section II.2.2.21).

As described in section II.2.2.21, for all three deep sequencing runs, a little less than the recommended amount of enriched beads was loaded onto the PTP.

II.2.3.16 Analysis

Data from the sequencing run was analyzed with the help of GS Junior 454 Sequencing System Software Manual, v 2.5p1 (August 2010) [21], as explained in section II.2.2.22. For both NS5b and the protease region a 1a.US.77.H77 plasmid was used as a reference sequence.

Multiplex Identifiers (MIDs) helped identifying the single amplicons.

III Results

III.1 Mutant Mix Experiment

III.1.1 Primer optimization (primer tests) NS3p Amp7

The first gel picture (figure 4) shows the concentration gradient for the primer pair NS3p Amp7 MID1 forward and reverse. The amplicon created with the help of those primers (NS3p Amp7) contains all three mutants of the first experiment: S122R, R155K and D168V. As explained in chapter II.2.2.9, an HCV 1b 5.1 Replicon plasmid served as template. Due to the fact that a higher annealing temperature is associated with a higher specificity of the primers —a low annealing temperature promotes false binding sites— the concentration gradient was conducted using a high annealing temperature (64.5°C). Subsequently, in a second run, different temperatures were tested to find the best working annealing temperature, as shown in the second gel picture (figure 5). In further experiments, 0.25 µM were used as final concentration of the primers in the reaction mix while 52.5°C were used as annealing temperature.

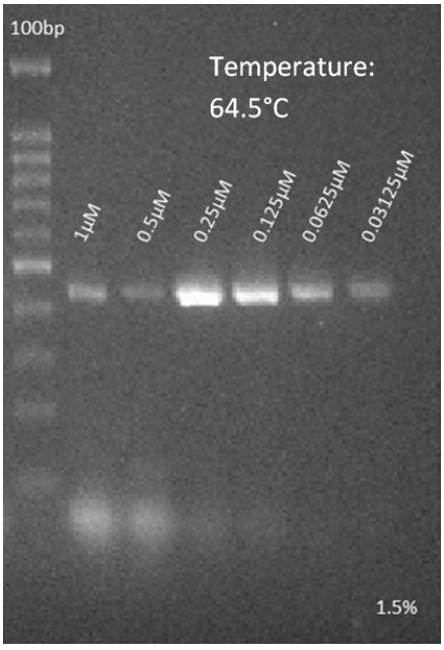


Figure 4: Primer test NS3p Amp7 MID1 concentration gradient with six different concentrations from 1 µM to 0.03125 µM.

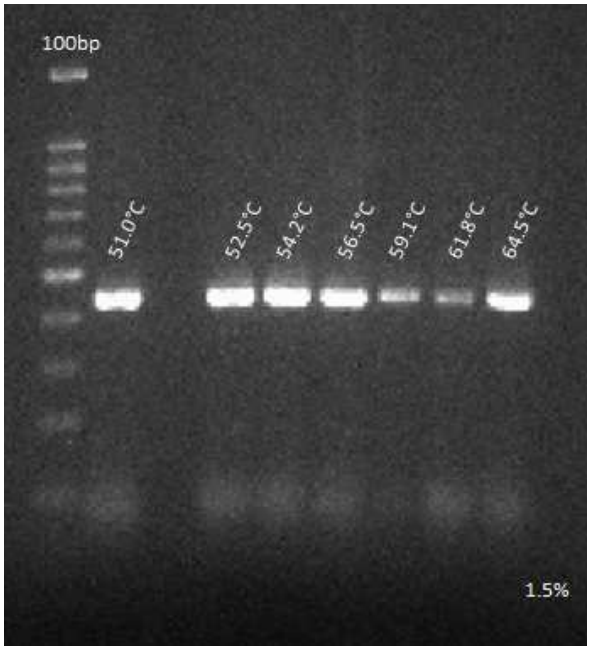


Figure 5: Primer test NS3p Amp7 MID1 temperature gradient using seven different annealing temperatures from 51.0°C to 64.5°C at a concentration of 0.25 µM.

III.1.2 qPCR

A Real Time quantitative PCR (qPCR) was conducted to quantify the six products of the reverse transcription. Those measurements were compared to 14 reference measurements with known concentrations. Results are shown in table 54. Standard number 14 was left out of the analysis because its tube opened during the reaction. Although the measured concentrations of the six cDNA samples do not decouple each time going from the lowest to the highest concentration, one might conclude that the measured amount of cDNA multiplies in each process. However, the concentrations of the cDNA samples now do not resemble exactly the original concentrations at RNA level, which can mean that the reverse transcription had worked better on some of the samples than others. But it is certainly enough to conclude that the reverse transcription had worked for at least five of the six samples. As for the sample with originally the lowest concentration (50 fg/µl), the reverse transcription might not have worked properly which is why it then does not show on the gel from the amplicon PCR in chapter III.1.3.

Place #	description	measured conc.	unit
15	Blank	0.000063	ng/µl
16	Mutant Mix 5 ng/µl cDNA	0.421567	ng/µl
17	Mutant Mix 0.5 ng/µl cDNA	0.050026	ng/µl
18	Mutant Mix 50 pg/µl cDNA	0.012178	ng/µl
19	Mutant Mix 5 pg/µl cDNA	0.002353	ng/µl
20	Mutant Mix 0.5 pg/µl cDNA	0.000462	ng/µl
21	Mutant Mix 50 fg/µl cDNA	0.000111	ng/µl
22	pET in 21b WT control	2.6821	ng/µl

Table 54: qPCR results without standards Mutant Mix Experiment.

Figure 6 shows the standard curve of the reaction. The Ct value (Cycle Threshold) of each sample and standard except standard 14 is shown against their concentrations. R^2 is 0.96172, indicating an acceptable alignment of the data points and therefore reliable results of the qPCR. The efficiency is 0.65, indicating that the reaction itself did not run ideally. This observation is in accordance to the fact that the graphs in figure 7 sometimes do not reach a plateau.

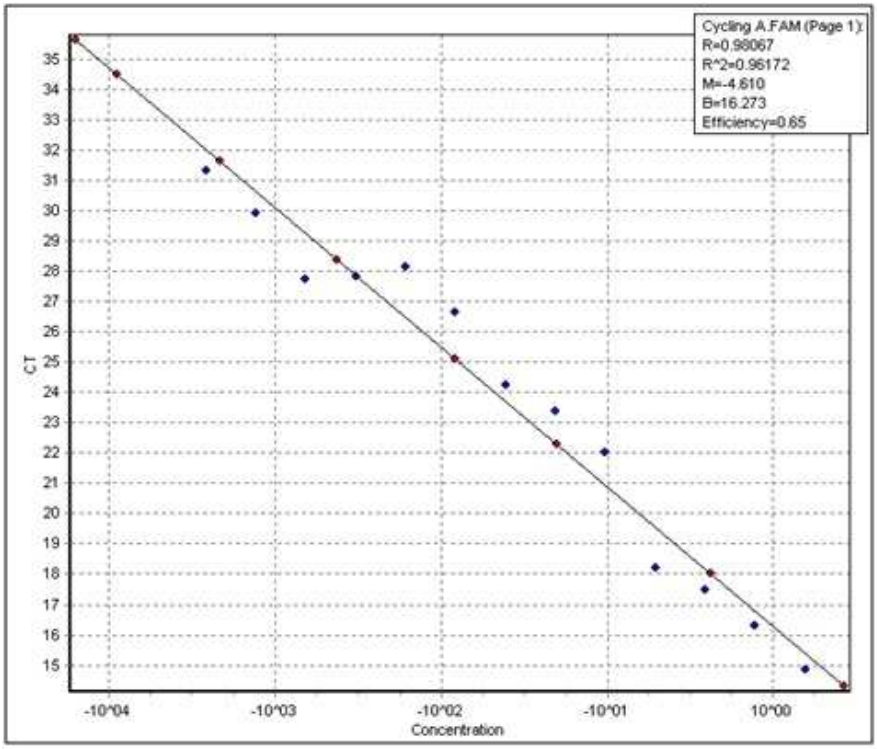


Figure 6: qPCR standard curve Mutant Mix Experiment showing the Ct value of each standard (blue) and sample (red) against their concentrations.

Figure 7 shows the intensity of a fluorescence signal as an indicator for amplification of all standards and samples (except standard number 14) during 40 cycles. The WT control gives the strongest signal, even stronger than the highest standard. The baseline for all curves is flat and their linear phases are long. However some samples do not reach a plateau due to the fact that their initial concentration was low, i.e. more cycles would have been necessary to get the samples to reach a steady state. The samples rise in order from highest to lowest concentration. As mentioned before, these results indicate that the reverse transcription had worked which allowed a continuation of further experiments.

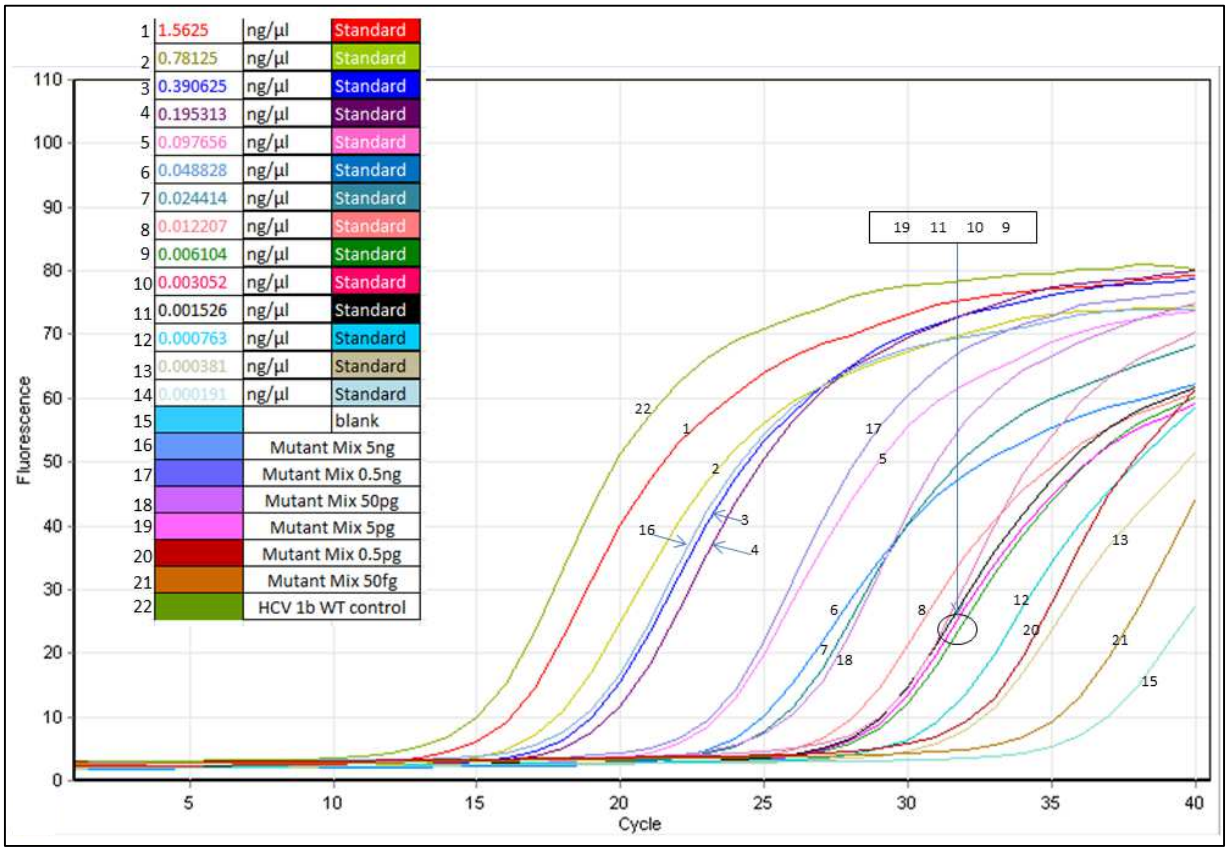


Figure 7: qPCR Mutant Mix Experiment showing amplification of the WT control, six samples and 14 standards during 40 cycles.

III.1.3 Amplicon PCR

An amplicon PCR was conducted on the six reverse transcription products “Mutant Mix” at original concentrations of 5 ng/µl, 0.5 ng/µl, 50 pg/µl, 5 pg/µl, 0.5 pg/µl and 50 fg/µl using the primer pair NS3p Amp7 MIDx forward and reverse. The plasmid WT NS3 in pET 21b served as positive control. The samples show clear, single bands on the agarose gel. The perceptibility of the bands decreases from 5 ng/µl to 0.5 pg/µl, with no visible band left of the 50 fg/µl dilution. This was already suggested by the low concentration of this sample after the qPCR. The 50fg/µl dilution was therefore not used for further experiments. Positive and negative controls are both as expected (see figure 8).

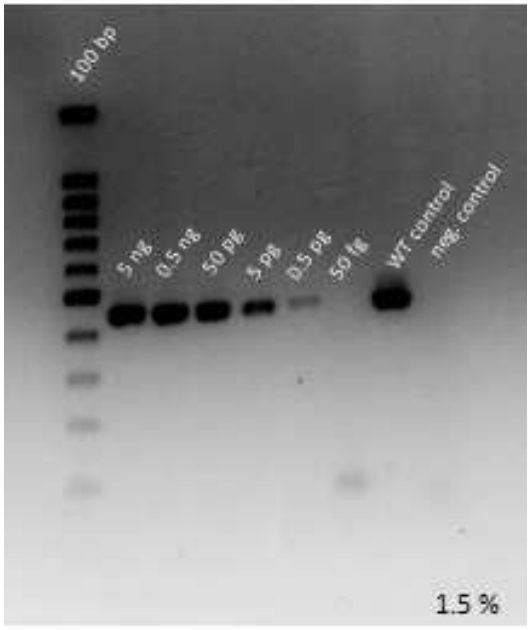


Figure 8: Amplicon PCR Mutant Mix Experiment showing six samples of double stranded DNA at original concentrations ranging from 5 ng/µl to 50 fg/µl on the agarose gel.

III.1.4 Quality Control PCR (QCPCR)

After Library Purification as described in chapter II.2.2.13, a Quality Control PCR was conducted on the five remaining samples from the amplicon PCR at original concentrations of 5 ng/µl, 0.5 ng/µl, 50 pg/µl, 5 pg/µl, and 0.5 pg/µl. All of them, as well as the WT control show strong single, clear bands. The negative control shows no band (see figure 9). Primers for the reaction were provided by GS Junior.

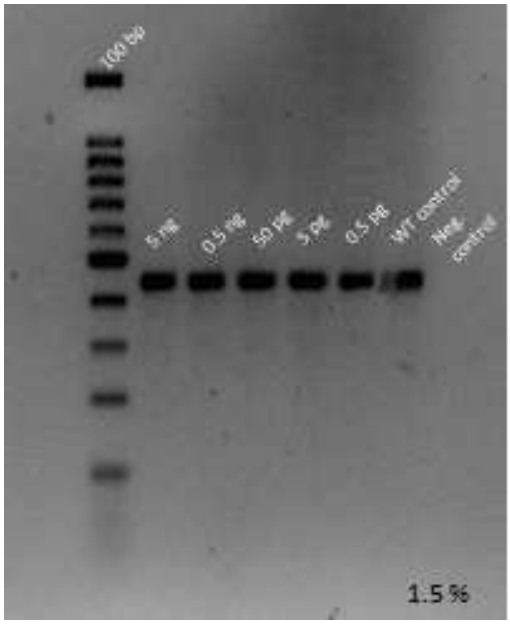


Figure 9: QCPCR Mutant Mix Experiment with five samples as well as the positive control showing distinct, strong bands on the gel.

III.1.5 Bioanalyzer

In order to look for smaller by-products of the reactions which can later interfere with deep sequencing, QCPCR products as well as the purified amplicons were sent to another lab to be run on the Agilent 2100 Bioanalyzer system. The QCPCR samples were pooled together and sequenced in pairs, for efficiency reasons. One good Bioanalyzer result is shown in figure 10. The two peaks which are labelled in green and purple represent the ladder. One single, distinct

peak in the middle between 80 and 85 bp shows the sample. There are no additional smaller peaks and the baseline is flat, without any undefined extra peaks.

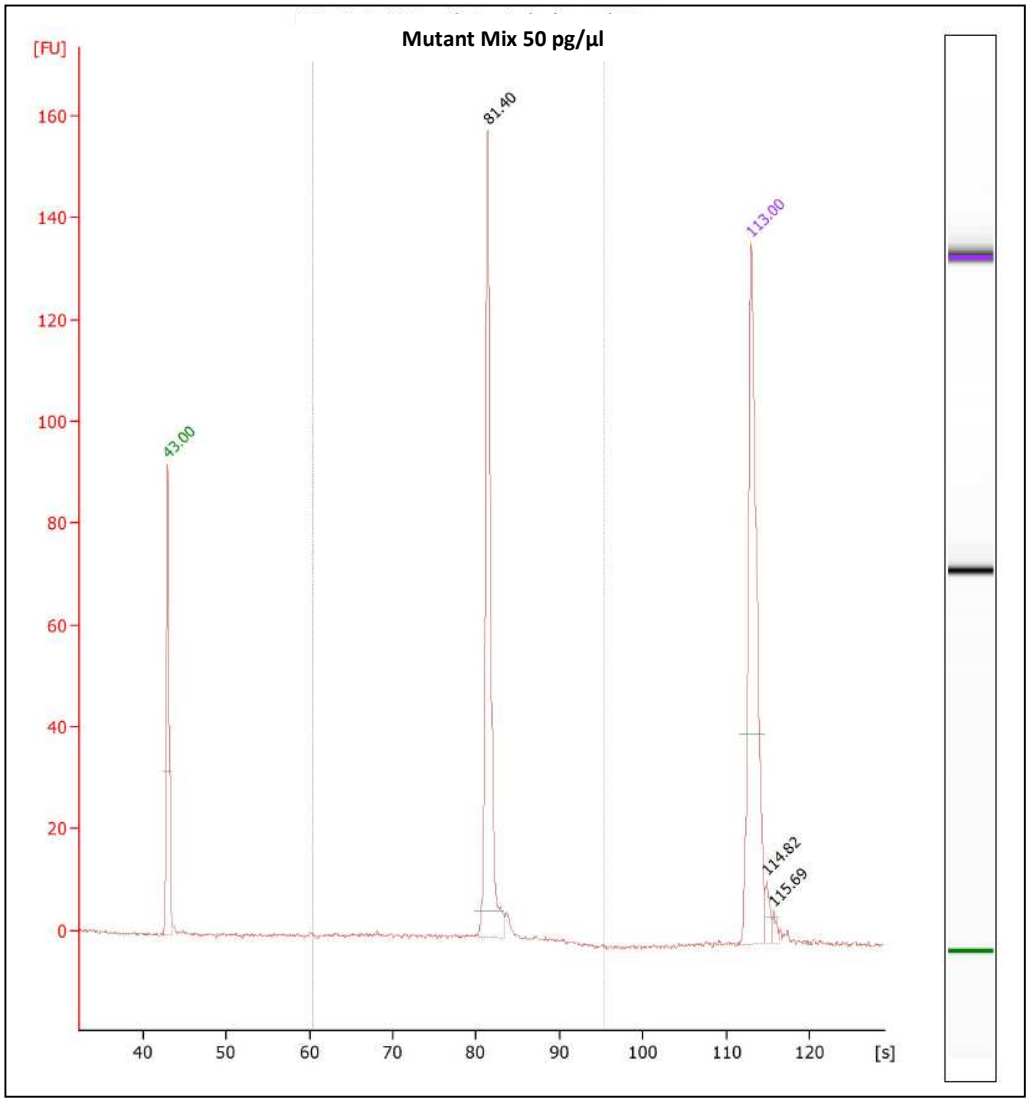


Figure 10: Bioanalyzer result 50 pg/µl Mutant Mix Experiment showing a fluorescent signal representing the quantity of DNA in a reaction between 40 to 120 seconds as well as two ladders at 43.00 and 113.00 seconds.

In comparison, the scan of the bioanalyzer at this time with the most additional peaks is shown in figure 11. Because it is mainly the short fragments which can interfere with deep sequencing, even the extra peaks in this picture at 92 – 95.64 seconds were not deemed important as they are larger than the sample and also are in their intensity about 0.04% of the amount of the sample. Peak heights are related to quantity. The lower the peak, the less DNA is present. The

single peaks at 79.12 and 83.83 seconds are that close to the sample, that they represent most likely denaturated DNA belonging to the sample, and not contamination.

For the remaining electropherograms, see the appendix.

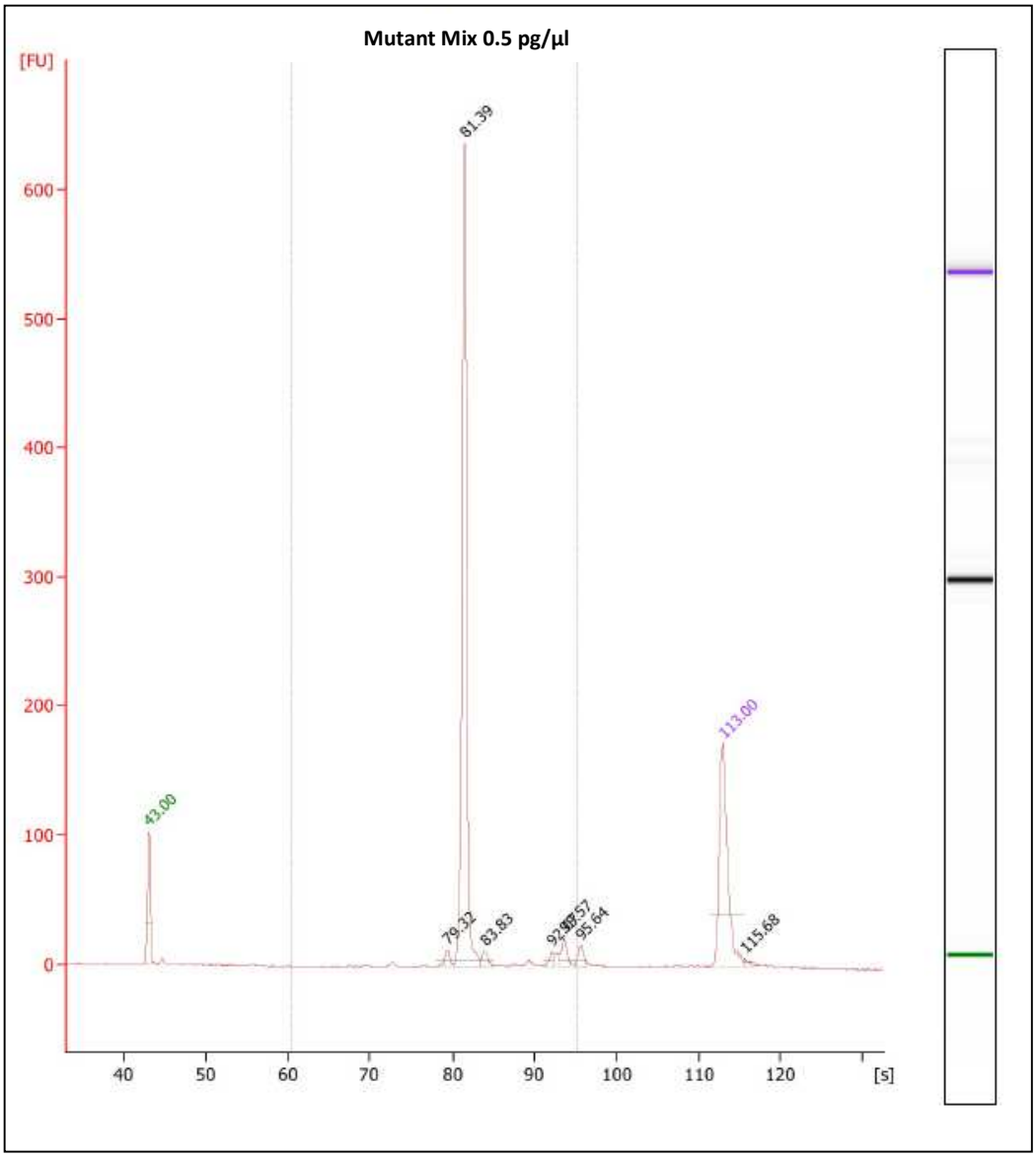
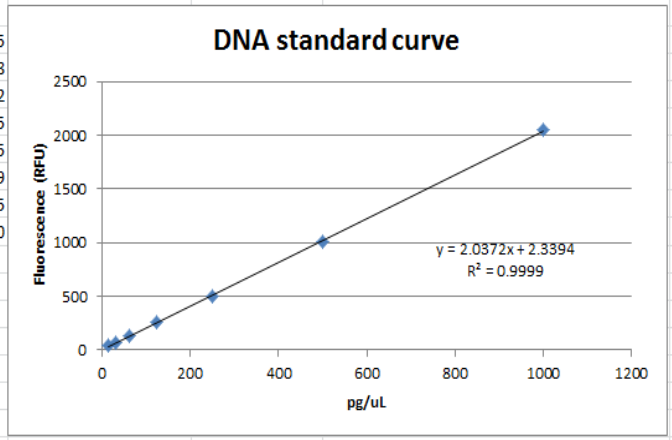


Figure 11: Bioanalyzer result 0.5 pg/µl Mutant Mix Experiment with several small additional peaks.

III.1.6 PicoGreen Quantification (Library Quantitation)

The library was quantified using PicoGreen Quantification. All values of the samples fall within the range of the standards. The R^2 value is higher than 0.98, as recommended in GS Junior Library Preparation Method Manual (March 2012) [18], suggesting that the results of the quantification are reliable. As expected, the WT control shows the highest concentration while the amount of library DNA of the samples decreases from the originally 5 ng/µl sample to the 0.5 pg/µl sample (see figure 12).

DNA (pg/uL)	F (rfu)		F_{avg} (rfu)	F_{avg} Corrected
1000	2059.431	2042.691	2051.061	2048.465
500	998.195	1019.757	1008.976	1006.38
250	502.222	509.694	505.958	503.362
125	257.808	260.513	259.1605	256.5645
62.5	132.416	139.211	135.8135	133.2175
31.25	73.97	74.74	74.355	71.759
15.625	41.611	41.986	41.7985	39.2025
0	2.458	2.734	2.596	0



unknowns	F (rfu)		F_{avg} Corrected	pg/uL	dilution factor	conc. Corrected for dilution factor ng/uL
Mutant Mix 5 ng	1262.414	1145.373	1201.2975	588.53	1	58.85
Mutant Mix 0.5 ng	1286.536	945.97	1113.657	545.51	1	54.55
Mutant Mix 50 pg	754.031	773.109	760.974	372.39	1	37.24
Mutant Mix 5 pg	398.506	530.906	462.11	225.69	1	22.57
Mutant Mix 0.5 pg	90.67	104.334	94.906	45.44	1	4.54
WT control	1354.715	1366.116	1357.8195	665.36	1	66.54

Figure 12: PicoGreen results from Mutant Mix Experiment showing the amount of DNA in the five samples plus the wild type control.

III.1.7 Enrichment

The enriched capture beads were counted using GS Junior Bead Counter. White enriched beads show exactly in the middle between upper (20%) and lower (5%) line of the window, resulting in an enrichment of 12.5% (see figure 13). Loading to little DNA onto the plate for the deep sequencing run results in good quality but low data output, while too much amount of DNA leads to poor image resolution and problems with the analysis. Also, experience from previous experiments had shown that an amount of approximately 400,000 beads for the deep sequencing run leads to satisfying results. That is why 0.32% of those enriched beads were used for the run.

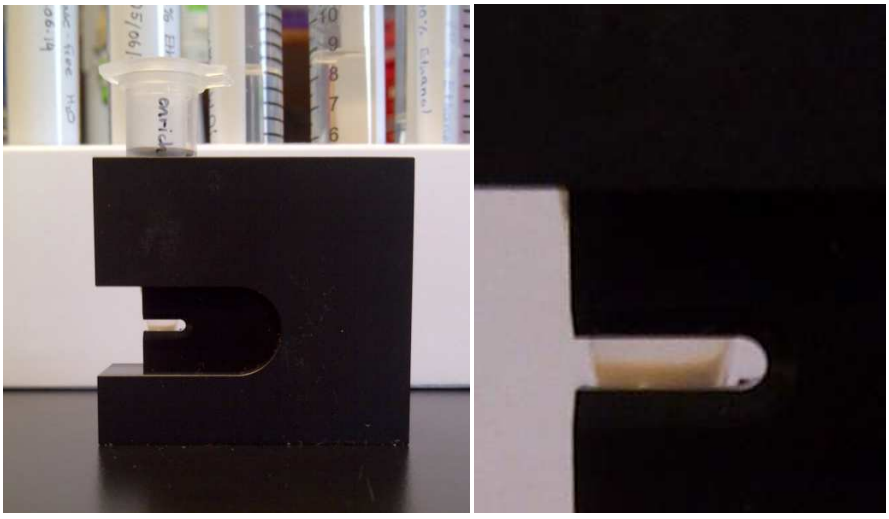


Figure 13: Enrichment Mutant Mix Experiment showing white capture beads filling out exactly the lower half of the window, resulting in an enrichment of about 12.5% or 1,250,000 beads.

III.1.8 Numbers from the run

There are some criteria to determine whether a deep sequencing run was successful. For instance, GS Junior recommends the ratio of Passed Filter Wells / Key Pass Wells to be higher than 25–50% and Key Pass Wells / Total Raw Wells to be higher than 90%. In this case, Raw Wells means the number of all detected signals on the plate during the run, while Key Pass Wells equals all the Raw Wells starting with a valid key sequence. Passed Filter Wells are the

total of reads which pass all of the program's filters. In this experiment, Passed Filter Wells/ Key Pass Wells was 0.55 or 55% while Key Pass Wells / Total Raw Wells was 0.97 or 97%, and therefore within the recommended range by GS Junior Data Analysis Terminology and Benchmarks [25] (see table 55).

TCAG (Library)	Region	Total
Raw Wells	187,291	187,291
Key Pass Wells	180,795	180,795
Passed Filter Wells	99,807	99,807
Total bases	39,030,823	39,030,823
Average Read Length	391,06	391,06
Length Std Deviation	30,09	
Longest Read Length	720	720
Shortest Read Length	40	40
Median Read Length	393.0	393.0
Modal Read Length	393.0	393.0

Table 55: Numbers from the deep sequencing run of the Mutant Mix Experiment.

III.1.9 Deep sequencing results

Deep sequencing results from the region of interest are shown in table 56. Variants corresponding to the codon changes for S122R, D168V and R155K are highlighted. As the mutant codon R155K differs in two bases from its wild-type (positions 463 and 464), both variants were combined by the software and the common percentage was defined.

Variant	Mix 5ng MID1	Mix 0.5ng MID2	Mix 50pg MID3	Mix 5pg MID4	Mix 0.5pg MID5	WT control MID6
267:C/-	0,16	0,34	0,48	0,32	0,36	0,98
310.5:-/G	0	0,56	0,73	0,54	0,52	0,34
364:A/C	8,49	9,16	9,38	10,67	13,58	0
462.5:-/A	2,03	1,7	1,83	1,43	0	0
463:C/A	3,02	2,67	2,77	3,1	2,7	0
464:G/A	2,19	1,87	2,17	2,38	2,7	0
466:G/-	0,83	0,79	0,6	0,72	0	0
503:A/T	0,31	0,35	0,28	0,34	0,37	0
R155K	2,19	1,87	2,17	2,38	2,7	0
WT	89.02	88.62	88.17	86.61	83.35	

Original percentages	
WT	85%
S122R	12%
R155K	2.7%
D168V	0.3%

Mutant	WT Codon	Mutant Codon
S122R	AGC	CGC
R155K	CGG	AAG
D168V	GAC	GTC

Table 56: Deep sequencing results, original percentages and codon changes Mutant Mix Experiment.

In order to be able to compare the deep sequencing results back to the original percentages, the error for each result was calculated (see table 57). Numbers from the WT differ the least from their originals. But even for sample with the lowest concentration, the results are valid.

In %	Error = ((measured value - original value)/original value) *100%				
	5 ng/µl	0.5 ng/µl	50 pg/µl	5 pg/µl	0.5 pg/µl
WT	4.52	4.26	3.6	1.86	-1.98
S122R	-29.25	-23.67	-21.83	-11.08	13.17
R155K	-18.89	-30.74	-19.63	-11.85	0
D168V	3.33	16.67	-6.67	13.33	23.33

Table 57: Error calculation for deep sequencing results Mutant Mix Experiment.

III.2 Plasma Samples Experiment

III.2.1 Primer optimization (primer tests) NS3p Amp9, NS5b C316

The primer pairs NS3p Amp9 MID1 forward and reverse and NS5b C316 MID1 forward and reverse were tested in order to find the best concentrations and annealing temperatures. Because the forward primer of the amplicon NS3p Amp9 is located in the NS2 region, as explained in chapter II.2.3.2 the primer pair was tested on a 1a.US.77.H77 plasmid covering both NS2 and NS3.

As for NS3p Amp9, first a temperature gradient was conducted using five different annealing temperatures (see figure 14). Because a high primer concentration is associated with the building of primer-dimers and other short by-products, a comparatively low primer concentration of 0.25 µM was used for the temperature test. Then the primer pair was tested at six different concentrations (1, 0.5, 0.25, 0.125, 0.06, 0.03 µM) at an annealing temperature of 56.5°C (see figure 15). This temperature was also used as annealing temperature for the amplicon PCR, while 0.5 µM were further used as final primer concentration in the reaction mix.

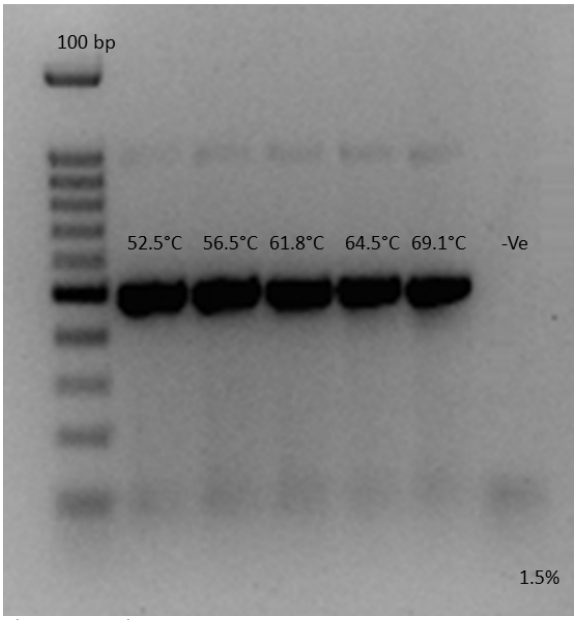


Figure 14: Primer test NS3p Amp9 MID1 temperature gradient using five different annealing temperatures at a primer concentration of 0.25 µM.

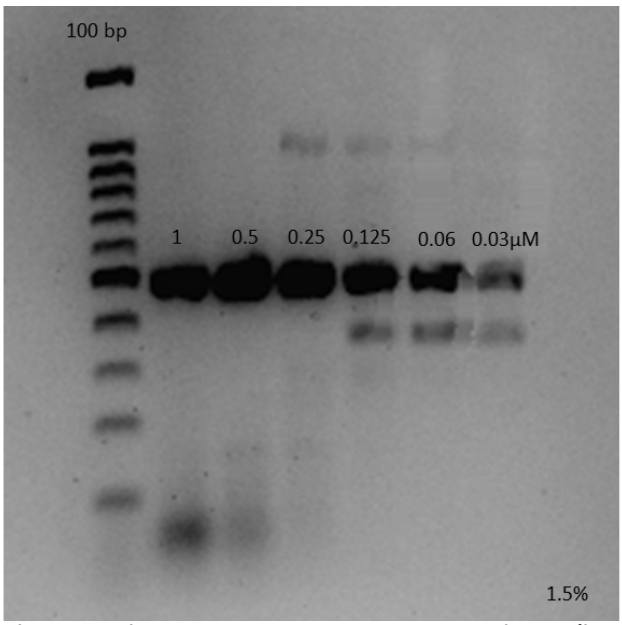


Figure 15: Primer test NS3p Amp9 MID1 concentration gradient with six different concentrations from 1 to 0.03 µM.

The primer pair NS5b C316 forward and reverse was tested on both a HCV 1b 5.1 Replicon plasmid and a 1a.US.77.H77 plasmid for the temperature gradient, whereas for the concentration gradient only the HCV 1b 5.1 Replicon plasmid was used (see figures 16 and 17).

The temperature gradient was conducted at a primer concentration of 1 µM. Previous temperature gradients at a primer concentration of 0.5 µM had not worked, showing no bands on the gel for any of the different annealing temperatures, which is why the PCR was repeated at a higher primer concentration and with two different plasmids. As the concentration gradient shows, the primer pair should actually work at a lower concentration as well. Why the temperature gradient hadn't worked previously could not be determined.

For the primer pair NS5b C316 fw and rev, 54.2°C, which is the program's next temperature step between the two best working temperatures 52.5°C and 56.5°C was further used as annealing temperature. As final primer concentration in the reaction mix 0.5 µM was used.

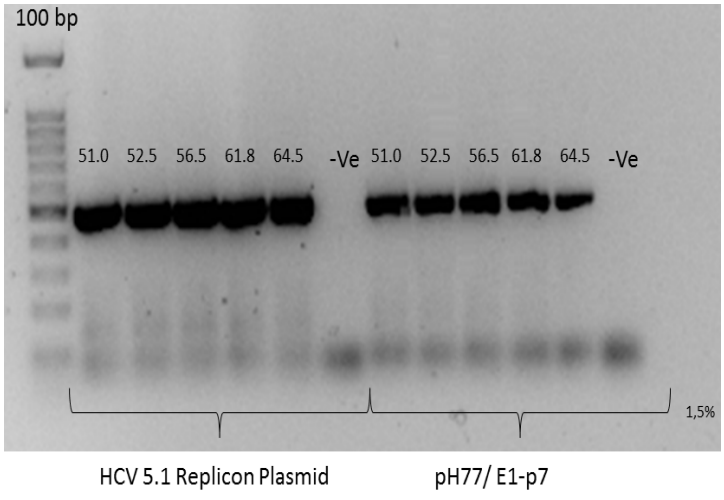


Figure 16: Primer test NS5b C316 temperature gradient using five different annealing temperatures at a primer concentration of 1 µM on two different plasmids.

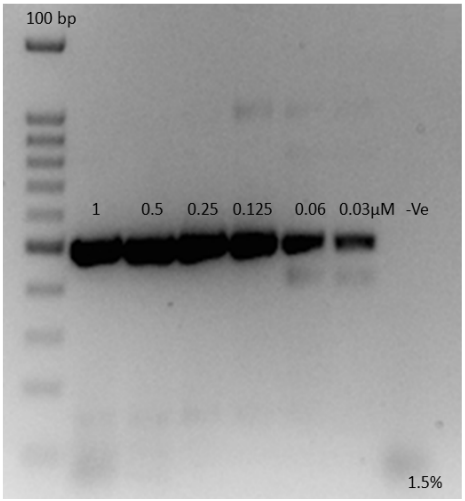


Figure 17: Primer test NS5b C316 concentration gradient using the 5.1 Replicon plasmid as template with six different concentrations at an annealing temperature of 56.5°C.

III.2.2 qPCR

As described in chapter II.2.3.5, a Real Time quantitative PCR (qPCR) was conducted to quantify the 19 products of the reverse transcription in comparison with 14 standards with known concentrations. Results are shown in table 58, along with the blank and two wild-type controls.

Place #	description	measured conc.	unit
15	Blank	0.0000381	ng/µl
16	A12-02	0.0001747	ng/µl
17	A24-10	0.0003161	ng/µl
18	A42-10	0.0001978	ng/µl
19	A57-10	0.0002128	ng/µl
20	A61-11	0.0006115	ng/µl
21	A85-03	0.0005432	ng/µl
22	A93-03	0.0003022	ng/µl
23	A94-01	0.0012086	ng/µl
24	B05-08	0.0001502	ng/µl
25	B49-03	0.0005899	ng/µl
26	B51-01	0.0003343	ng/µl
27	B84-01	0.0002768	ng/µl
28	C01-15	0.0038123	ng/µl
29	A76-05	0.0035481	ng/µl
30	B33-05	0.0003057	ng/µl
31	B35-04	0.0007062	ng/µl
32	B37-03	0.0004122	ng/µl
33	B57-01	0.0003937	ng/µl
34	C06-01	0.0002227	ng/µl
35	WT control from plasmid	3.2789139	ng/µl
36	WT control from RNA	0.0109424	ng/µl

Table 58: qPCR results without standards Plasma Samples Experiment.

Figure 18 shows the standard curve of the reaction. As explained in chapter III.1.2, the Ct value (Cycle Threshold) of each sample and standard is shown against their concentrations. R^2 is 0.96441, indicating a good alignment of the data points and therefore reliable results of the qPCR. The efficiency is 0.44, indicating that the reaction itself did not run ideally.

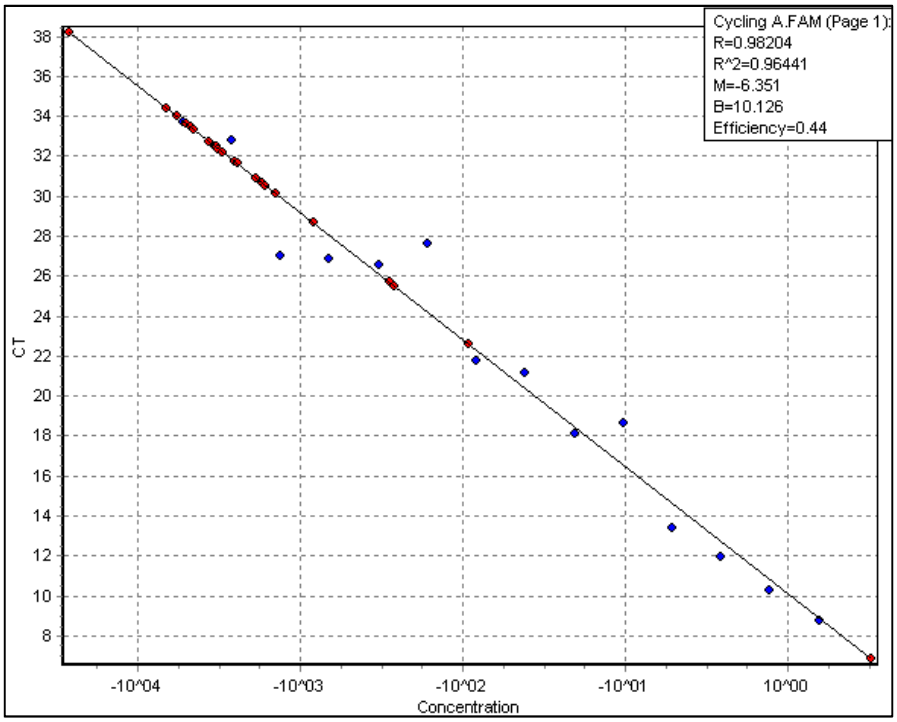


Figure 18: qPCR standard curve Plasma Samples Experiment showing the Ct value of each standard (blue) and sample (red) against their concentrations.

Figure 19 shows the intensity of fluorescence of all standards and samples during 40 cycles. The WT control from plasmid gives the strongest signal, even stronger than the highest standard. The baseline for all curves is flat, linear phases are long, but some samples do not reach a plateau, indicating that their concentration was low at the start of the reaction. But since they all enter the linear phase and differ clearly from the blank, the reverse transcription must have worked sufficiently, so they were all used for the following experiments.

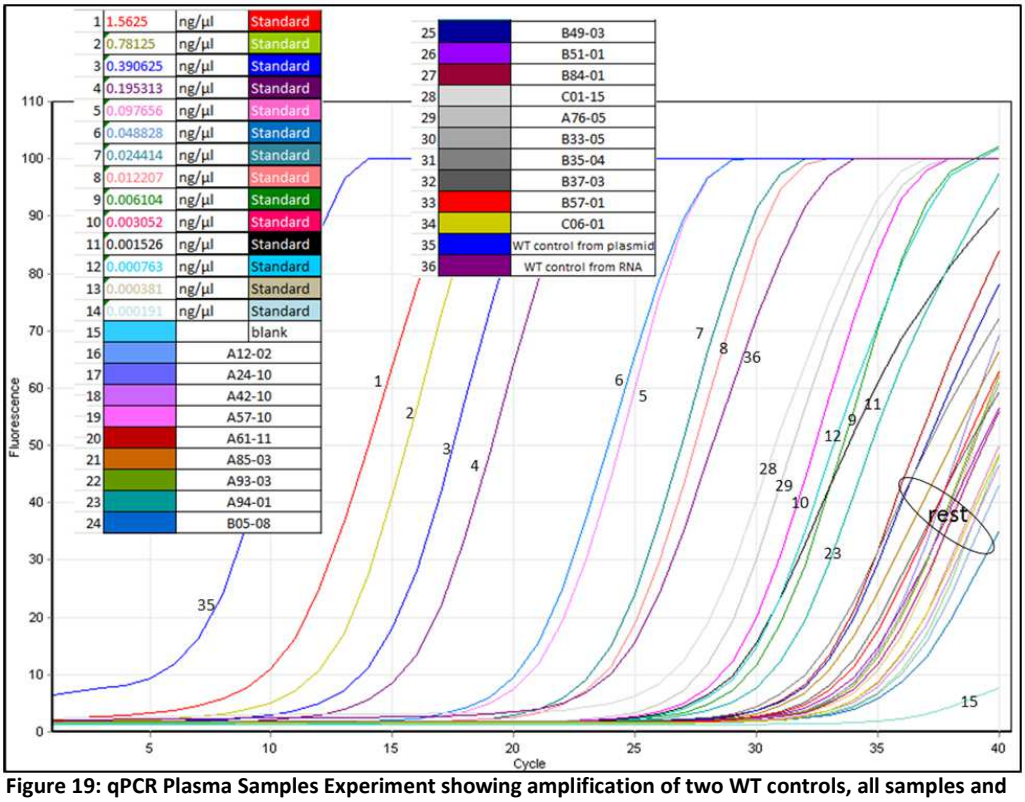


Figure 19: qPCR Plasma Samples Experiment showing amplification of two WT controls, all samples and 14 standards during 40 cycles.

III.2.3 Amplicon PCR

The following gel pictures (figures 20–23) show the results of four different polymerase chain reactions. As some samples show no or only faint bands on the gels (A12-02, A61-11, B33-05), amplification of them was unfortunately not successful. This could be either due to damage at shipping and storing or a failure of the reverse transcription or amplicon PCR. The sample B33-05 was immediately left out, samples A12-02 and A61-11 were purified, but then left out and not used for the actual deep sequencing run, because the quality control PCR was not successful. In figures 20 and 21, the positive controls are not visible, due to the fact that a NS3 plasmid had been used even though the forward primer is located in the NS2 region. Positive controls serve to show that the mix and amplification conditions were correct. As most of the samples were successfully amplified, the reaction conditions and the mix had obviously worked.

So the experiment was accepted without repetition. The positive controls in figures 22 and 23 however show strong bands. More importantly, the negative controls in all four PCR gels are as expected.

For the three deep sequencing runs, from genotype 1a samples A24-10, A42-10, A57-10, A85-03, A93-03, A94-01, B05-08, B49-03, B51-01, B84-01, C01-15, and from the genotype 1b A76-05, B35-04, B37-03, B57-01 and C06-01 were used.

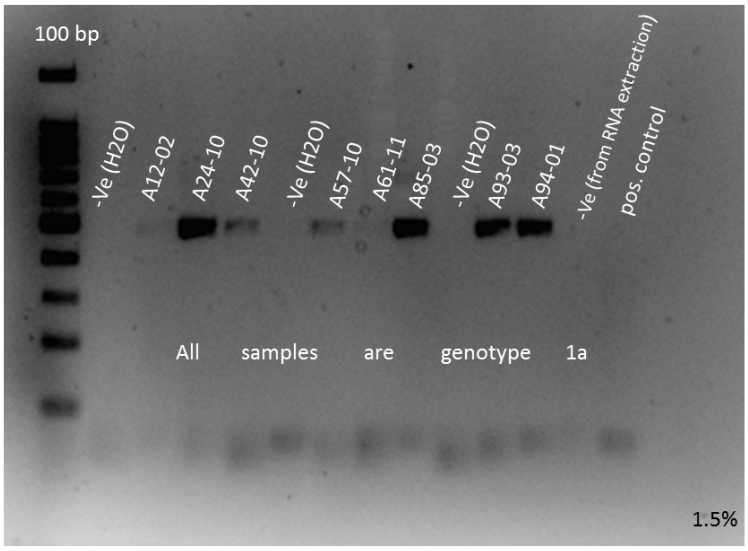


Figure 20: Amplicon PCR plasma samples from 1st RNA extraction, NS3.

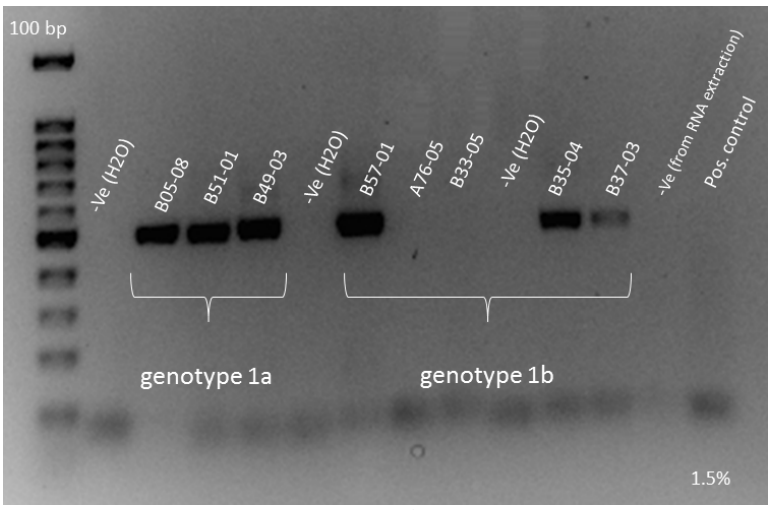


Figure 21: Amplicon PCR plasma samples from 2nd RNA extraction, NS3.

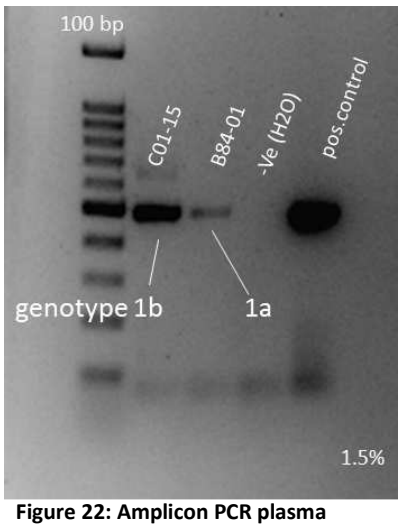


Figure 22: Amplicon PCR plasma samples 2nd RNA extraction, NS3 only 2 samples.

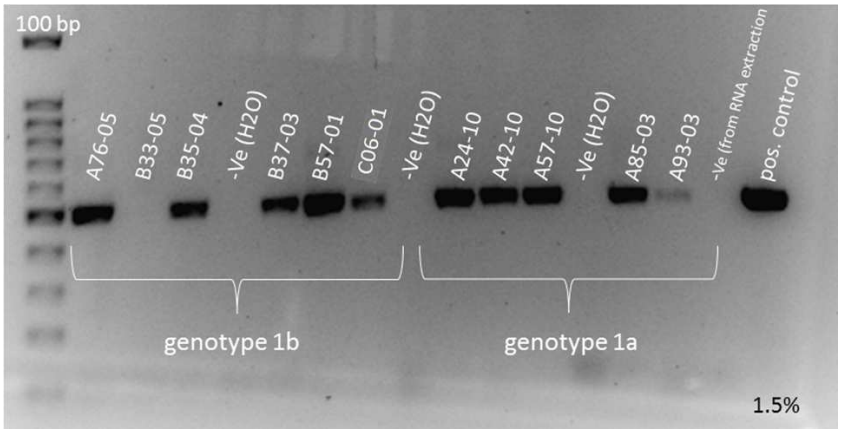


Figure 23: Amplicon PCR plasma samples NS5b.

III.2.4 Library quality control PCR (QCPCR)

In all the gels (figures 24, 25), the positive control is positive, negative controls are negative. Samples show single, clear bands and no secondary bands. Samples A12-02 and A61-11 in figure 24 show only very faint bands and were therefore left out from further experiments.

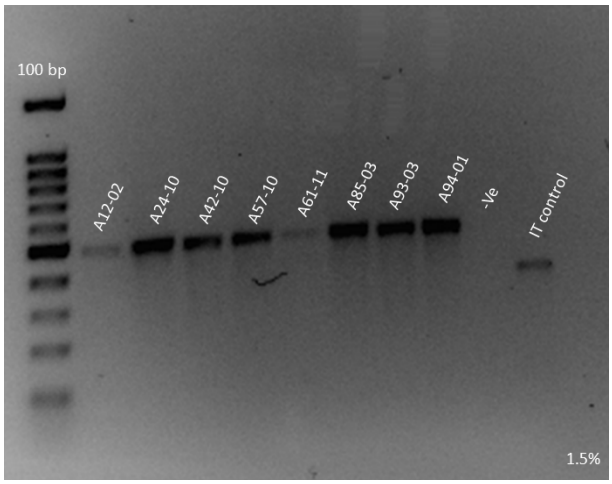


Figure 24: QCPCR Plasma Samples from 1st RNA extraction.

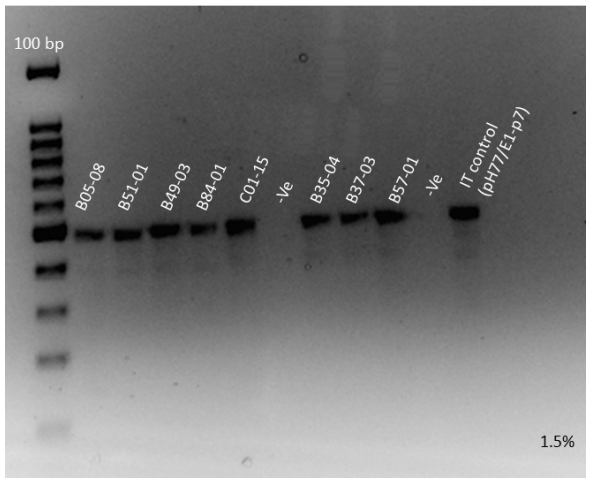


Figure 25: QCPCR Plasma Samples from 2nd RNA extraction.

III.2.5 Bioanalyzer

Figure 26 shows the Bioanalyzer result of a pure sample. One distinct, single peak represents the amplicon, no additional peaks other than the two ladders are visible. In comparison a less pure sample is shown (figure 27). As explained in chapter III.1.5 it is mostly short by-products which will interfere with deep sequencing, therefore the additional peaks at 499 bp and 560 bp were not seen as relevant. The broad base of the sample or any smaller peaks very close to the sample are more likely the result of denaturation of the sample than contamination. Also, the QCPCR samples had been pooled together in order to save costs and time (as explained in chapter III.1.5), so a broader and less distinct peak in this case is due to the fact that two samples were run together. For the rest of the Bioanalyzer results see the appendix.

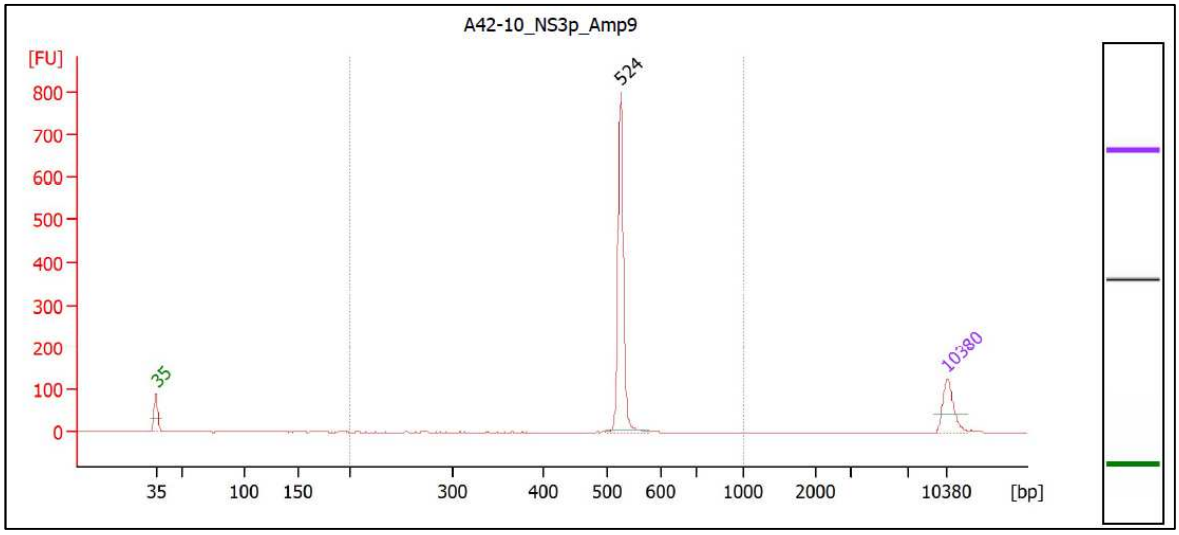


Figure 26: Bioanalyzer example plasma sample A42-10.

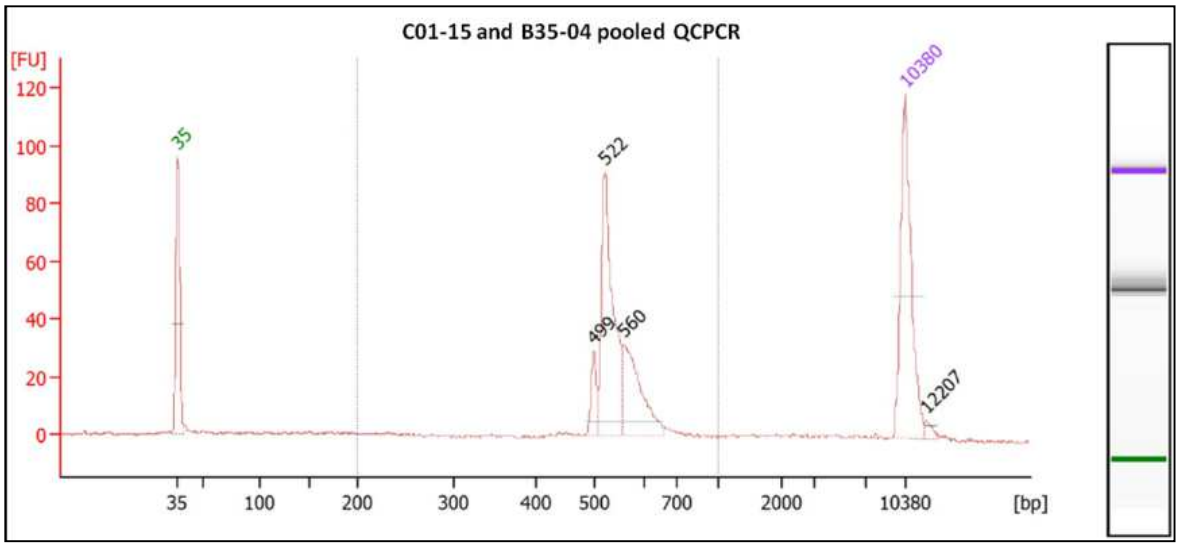


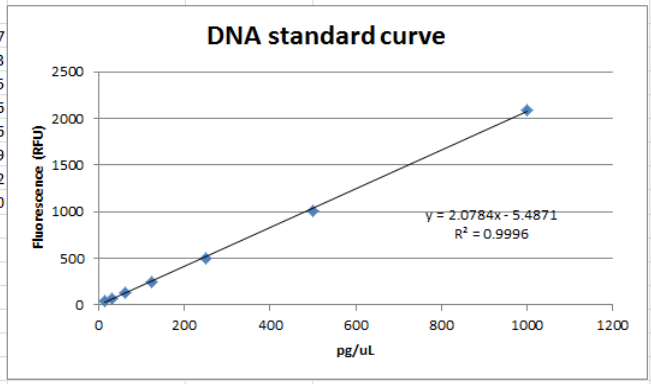
Figure 27: Bioanalyzer example plasma samples C01-15 and B35-04 QCPCR pooled.

III.2.6 PicoGreen Quantification (Library Quantitation)

The library was quantified using PicoGreen Quantification (see figures 28–30). After re-measuring NS5b (see chapter II.2.3.10), all values of the samples fall within the range of the standard curve. As recommended in GS Junior Library Preparation Method Manual [18], the square of the correlation coefficient, R^2, is higher than 0.98, suggesting that the results of the quantification are reliable. Six samples with amplicon NS3 which were originally from the first RNA extraction were arranged together, eight NS3 samples from the second RNA extraction and eight NS5b samples. This grouping was later continued at the three actual deep sequencing runs.

III.2.6.1 Results from 1st RNA extraction NS3

DNA (pg/uL)	F (rfu)		F_{avg} (rfu)	F_{avg} Corrected
1000	2055.179	2129.751	2092.465	2089.727
500	1000.545	1012.757	1006.651	1003.913
250	511.933	502.53	507.2315	504.4935
125	253.106	257.062	255.084	252.346
62.5	129.947	134.581	132.264	129.526
31.25	69.457	71.177	70.317	67.579
15.625	40.039	42.141	41.09	38.352
0	2.916	2.56	2.738	0

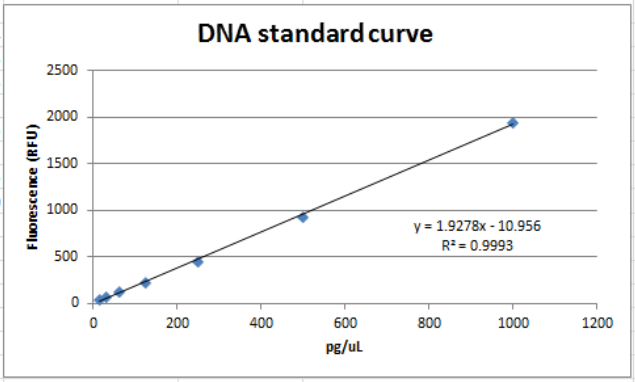


unknowns	F (rfu)		F_{avg} Corrected	pg/uL	dilution factor	conc. Corrected for dilution factor ng/uL
A24-10	476.5	460.596	465.81	226.76	1	22.68
A42-10	57.29	52.772	52.293	27.80	1	2.78
A57-10	61.983	54.863	55.685	29.43	1	2.94
A85-03	421.583	398.66	407.3835	198.65	1	19.86
A93-03	264.997	286.111	272.816	133.90	1	13.39
A94-01	258.346	235.584	244.227	120.15	1	12.01

Figure 28: PicoGreen results from first RNA extraction NS3 showing the amount of DNA of six samples from the first RNA extraction.

III.2.6.2 Results from 2nd RNA extraction NS3

DNA (pg/uL)	F (rfu)		F_{avg} (rfu)	F_{avg} Corrected
1000	1940.05	1935.96	1938.007	1935.154
500	918.374	939.037	928.7055	925.8525
250	453.236	457.46	455.348	452.495
125	217.689	226.461	222.075	219.222
62.5	120.009	122.087	121.048	118.195
31.25	65.123	66.567	65.845	62.992
15.625	37.812	37.659	37.7355	34.8825
0	2.486	3.22	2.853	0

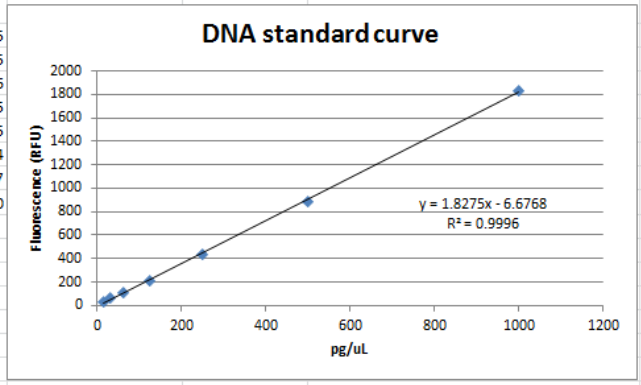


unknowns	F (rfu)		F_{avg} Corrected	pg/uL	dilution factor	conc. Corrected for dilution factor ng/uL
B05-08	101.926	83.292	89.756	52.24	1	5.22
B51-01	201.323	203.249	199.433	109.13	1	10.91
B49-03	261.641	247.392	251.6635	136.23	1	13.62
B84-01	52.665	54.396	50.6775	31.97	1	3.20
C01-15	411.254	456.484	431.016	229.26	1	22.93
B35-04	184.121	177.091	177.753	97.89	1	9.79
B37-03	54.066	52.481	50.4205	31.84	1	3.18
B57-01	411.566	388.918	397.389	211.82	1	21.18
pos. Control pH77/E1-p7	1071.66	1027.08	1046.519	548.54	1	54.85

Figure 29: PicoGreen results from second RNA extraction NS3 showing the amount of DNA of eight NS3 samples from the second RNA extraction.

III.2.6.3 Results from NS5b samples

DNA (pg/uL)	F (rfu)		F_{avg} (rfu)	F_{avg} Corrected
1000	1806.83	1863.22	1835.021	1832.6645
500	878.315	907.857	893.086	890.7295
250	436.728	441.897	439.3125	436.956
125	211.5	218.322	214.911	212.5545
62.5	114.785	116.341	115.563	113.2065
31.25	63.904	63.057	63.4805	61.124
15.625	34.998	34.849	34.9235	32.567
0	2.353	2.36	2.3565	0



unknowns	F (rfu)		F_{avg} Corrected	pg/uL	dilution factor	conc. Corrected for dilution factor ng/uL
A76-05	747.605	724.669	733.7805	405.17	1	40.52
B35-04	428.426	420.741	422.227	234.69	1	23.47
B37-03	302.371	441.136	369.397	205.79	1	20.58
A85-03	475.839	450.257	460.6915	255.74	1	25.57
C06-01	209.575	209.108	206.985	116.91	1	11.69
WT control pH77..	1136.62	1097.24	1114.572	613.54	2	122.71
A42-10	434.723	425.572	427.791	237.74	1	23.77
B57-01	902.974	959.764	929.0125	512.01	1	51.20
A57-10	661.641	648.184	652.556	360.73	1	36.07

Figure 30: PicoGreen results showing the amount of DNA of eight NS5b samples.

III.2.7 Bead counting

As explained in chapter III.1.7, loading to little amount of DNA onto the plate for the deep sequencing run leads to good quality but low data output while too much amount of DNA equals poor image resolution and problems with the analysis, which is why the best amount of beads for the deep sequencing run was determined for each of the three runs individually. According the GS Junior emPCR Amplification Method Manual, an enrichment between 5% and 20% will generate satisfactory sequencing results [19], but previous experiments had shown that an amount of DNA beads at the lower end of the window yields the best results.

III.2.7.1 Results from 1st RNA extraction NS3

The white enriched capture beads match up with the lower line of the window on the bead counter, resulting in an enrichment of 5% (see figure 31). 90% of the beads, i.e. 450,000 beads were used for the deep sequencing run.

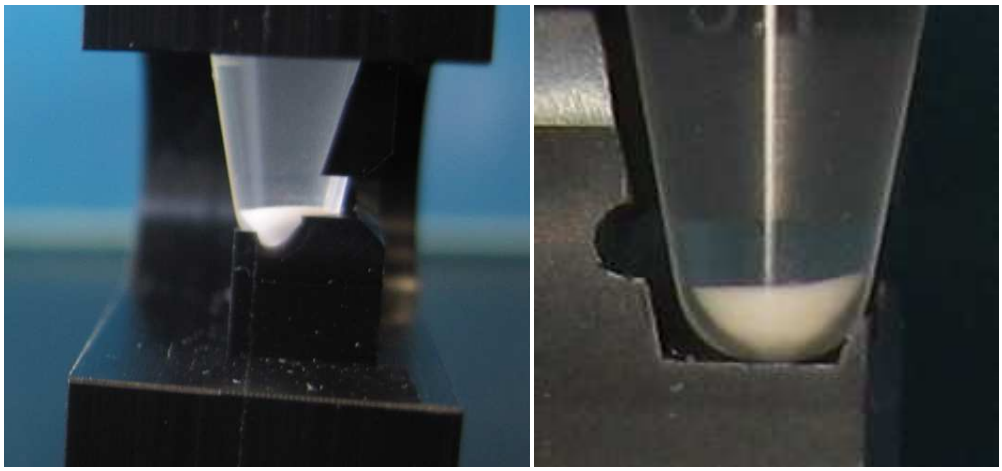


Figure 31: Enrichment NS3 samples from first RNA extraction showing the white beads on the bottom of the 1.7ml tube matching up with the lower line of the window of the bead counter, corresponding to about 5% enrichment or 500,000 beads.

III.2.7.2 Results from 2nd RNA extraction NS3

White enriched beads show in the lower half of the window, resulting in an enrichment of 8–10% (see figure 32). 50% of the beads were used for the run.

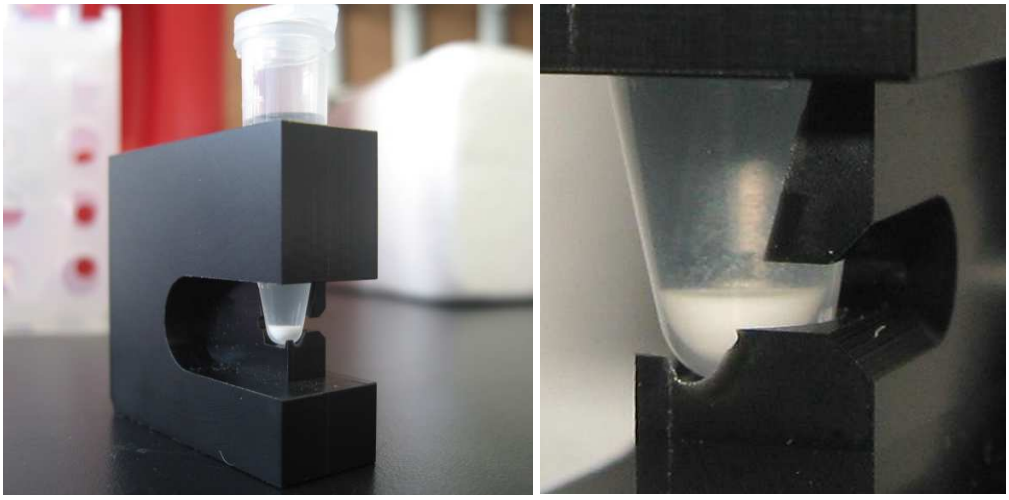


Figure 32: Enrichment NS3 samples from second RNA extraction showing enriched beads approximately covering the lower half of the window, representing an enrichment of 8–10% or 800,000 to 1,000,000 beads.

III.2.7.3 Results from NS5b samples

This time, white enriched beads show slightly under the lower line of the window, resulting in an enrichment of 4% (see figure 33). All of the beads were used for the deep sequencing run.

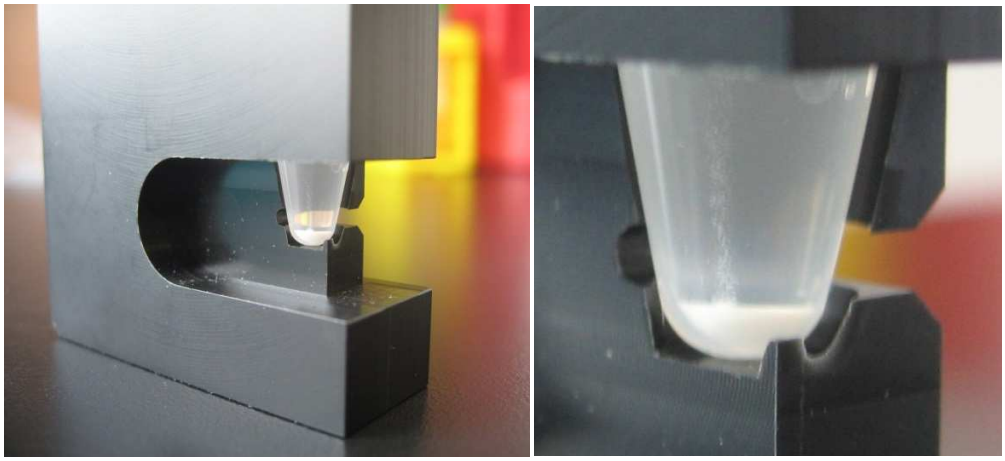


Figure 33: Enrichment NS5b samples with beads showing slightly under the lower line of the window, meaning about 4% or 400,000 of enriched capture beads were collected.

III.2.8 Numbers from the run

As explained in chapter III.1.8, Raw Wells means the number of all detected signals on the plate during the run, while Key Pass Wells equals all the Raw Wells starting with a valid key sequence. Passed Filter Wells are the total of reads which pass all of the program's filters. As mentioned, GS Junior recommends the ratio of Passed Filter Wells / Key Pass Wells to be higher than 25–50% and Key Pass Wells / Total Raw Wells to be higher than 90%. Also, the higher the number of reads per amplicon, the more likely a minor variant at a very low percentage actually represents a real mutation and not just sequencing error.

III.2.8.1 Results for the 1st deep sequencing run

The number of reads per amplicon ranged between 10,080 and 19,708. Passed Filter Wells/ Key Pass Wells = 0.55 or 55%. KeyPassWells/totalRawWells is 0.96 or 96%, as recommended by GS Junior Data Analysis Terminology and Benchmarks [25] (see table 59).

TCAG (Library)	Region	Total
Raw Wells	185,623	185,623
Key Pass Wells	178,408	178,408
Passed Filter Wells	97,812	97,812
Total bases	43,501,586	43,501,586
Average Read Length	444.75	444.75
Length Std Deviation	28.02	
Longest Read Length	688	688
Shortest Read Length	40	40
Median Read Length	447.0	447.0
Modal Read Length	447.0	447.0

Table 59: Numbers from first deep sequencing run.

III.2.8.2 Results for the 2nd deep sequencing run

For the second run, the number of reads per amplicon ranged between 5,001 and 13,019. Passed Filter Wells/ Key Pass Wells = 0.45 or 45%, as recommended. KeyPassWells/totalRawWells is 0.95 or 95%, as recommended (see table 60).

TCAG (Library)	Region	Total
Raw Wells	160,878	160,878
Key Pass Wells	153,492	153,492
Passed Filter Wells	69,033	69,033
Total bases	30,676,853	30,686,853
Average Read Length	444.38	444.38
Length Std Deviation	27.29	
Longest Read Length	511	511
Shortest Read Length	47	47
Median Read Length	447.0	447.0
Modal Read Length	447.0	447.0

Table 60: Numbers from second deep sequencing run.

III.2.8.3 Results for the 3rd deep sequencing run

The number of reads per amplicon ranged between 3,848 and 7,477. Unfortunately, for the amplicon with the lowest number of reads this does not allow any conclusions on variants lower than 1.3%. Passed Filter Wells/ Key Pass Wells = 0.66, or 66%. A little low is KeyPassWells/totalRawWells: 0.89 or 89% (see table 61). But with a very high number of total filter passed reads, it is very close to the recommended percentage.

TCAG (Library)	Region	Total
Raw Wells	81,710	81,710
Key Pass Wells	72,463	72,463
Passed Filter Wells	47,951	47,951
Total bases	21,658,263	21,658,263
Average Read Length	451.67	451.67
Length Std Deviation	20.36	
Longest Read Length	819	819
Shortest Read Length	45	45
Median Read Length	453.0	453.0
Modal Read Length	453.0	453.0

Table 61: Numbers from third deep sequencing run.

III.2.9 Deep sequencing results

In three deep sequencing runs, a total of 16 samples was analyzed, there from eleven with genotype 1a and five with genotype 1b. 14 samples (11x 1a and 3x 1b) were sequenced for NS2/NS3p, looking for Q80K amongst other variants. Eight samples were sequenced for NS5b (three 1a and five 1b) looking for C316 and other variants. Three 1a samples and three 1b samples were sequenced for variants both in the NS2/NS3p and NS5b region (see table 62).

	samples	1st run	2nd run	3rd run
Genotype 1a	A24-10	NS2/NS3p		
	A42-10			NS5b
	A57-10			
	A85-03			
	A93-03			
	A94-01			
	B05-08		NS2/NS3p	
	B49-03			
	B51-01			
	B84-01			
	C01-15			
Genotype 1b	A76-05			
	B35-04			
	B37-03			
	B57-01			
	C06-01			

Table 62: Plasma samples sequenced for NS2/3 and NS5b.

Ten out of 14 samples sequenced for the NS3 region showed the RAV Q80K. At nine of them, the percentage of viral copies with Q80K within one patient's plasma was very high (between 97.86% and 100%). One of them was genotype 1b. One sample showed both variants Q80K (at 84%) and Q80R (16%) (see appendix).

One of the 1a samples positive for Q80K showed also the RAV V36L at 48%.

Only one of the eight samples sequenced for NS5b showed the mutation C316N at a very low percentage (1.2%). But with a read number of 3848 for this amplicon this equals 46 reads for the mutation, which according to 454 GS Junior Sequencing System Guidelines is probably coincidental, as the threshold for a mutation to be considered real is 50 reads [22].

34 variants were found in NS2, whereas 58 showed up in NS3. However, neither NS2 nor NS3 were sequenced completely.

Q80K was among the three most prevalent variants, together with Q199R in the NS2 region and T40A in NS3.

Numerous other variants were found at each run in NS2, NS3 and NS5b without resulting in any aminoacid changes and therefore representing only silent mutations or polymorphisms. Some of them, however, are located at positions which typically show clinically relevant mutations like positions 36, 43, 54 and 55 in NS3 (see appendices 2, 3 and 4).

IV. Discussion

IV.1 Mutant Mix

IV.1.1 Analysis of the run

According to 454 GS Junior Sequencing System Guidelines [22], a mutation should appear 50 times as a minimum cut-off in order to decide that it is real and not just coincidental, due to sequencing error. For instance, for an amplicon with 5000 reads, 1% and higher can be considered a real mutation. For this thesis, all numbers below this threshold were left out of the analysis.

According to GS Junior Data Analysis Terminology and Benchmarks [25], totalPassFiltering/totalKeyPass should be above 25–50% and the fraction of KeyPassWells/totalRawWells is recommended to be higher than 90%. The numbers from the Mutant Mix deep sequencing run match the criteria (see section III.1.8).

IV.1.2 Deep sequencing accuracy

The aim of the Mutant Mix Experiment was to find a threshold for RNA input at which deep sequencing results are still reliable. The numbers show that mutants can still be picked up from RNA added at a quantity of only 0.5 pg/µl even for the mutant added at the lowest percentage (0.3%).

In summary, we found that as long as a sample shows up on a PCR-gel after amplification, even as a faint band only, deep sequencing numbers still match the percentages from the input, within error, and can therefore be considered accurate.

IV.2 Plasma samples

IV.2.1 Analysis of the run

The recommendations for amplicon run results are described in section III.2.8. The numbers from the plasma samples deep sequencing run match the criteria, except for the 3rd deep sequencing run where KeyPassWells/totalRawWells are only 0.89 or 89%. However, this number is right below the threshold, and is therefore considered to be sufficiently close to the recommended percentage, as ultimately we still obtained a very high number of total filter passed reads. The raw wells from the 3rd run are low compared to the two other runs (only half of the raw wells from the 2nd run). As a result, the number of reads is also low for some of the amplicons in the 3rd run. As described in section IV.1.1, all variants below the threshold for reads to be considerable are hence excluded from the analysis and also do not show up in the table in the appendix.

Major cross-contamination between the samples can be ruled out by simply aligning deep sequencing results from each patient and comparing variants in each sample to the others (see lists of variants in the appendix).

Insertions which show up in the deep sequencing results are most likely due to sequencing error, as the 454 has a tendency to show insertions on homo-polymeric stretches and are therefore left out of the analysis.

At some positions, the plasmid control sequence differs from the reference sequence. This is simply explained with the high genetic variability of HCV where at those positions the plasmid sequence does not match the reference sequence used.

IV.2.2 Discussing the method

Errors can occur throughout the whole experimental setup, from the RNA extraction to the actual sequencing. Cross-contamination can be ruled out as described previously. Deep sequencing is a rather expensive and time-consuming method, limiting the number of samples that can be processed. However, with a small number of samples like in this thesis, a selection bias is prone to happen.

But in view of the results from the Mutant Mix Experiment, deep sequencing proves to qualify as a method for investigating plasma samples for RAVs, even for patients with low viral loads. Unlike for the paper from Jabara et al. [8] (see IV.2.3), for this thesis a Lib-A emPCR Amplification Kit was used. The advantages of bidirectional sequencing with the use of two primers, compared to the unidirectional sequencing with the Lib-L Kit are that it provides higher accuracy. Determining whether a variant is valid or not can be easier when it shows up in both forward and reverse reads independently. However, unidirectional sequencing can cover a wider part of the genome and therefore screen for more variants at once [22].

A systematic review from 2017 compares several currently available HCV sequencing methods for RAVs, in order to find the ideal protocol, describing strengths and limitations of current methods. The ideal method is described as a full length, pan-genotypic, cost effective method with a low limit of detection. However, no method matching all of those features was found [26].

In order for the protocol in this thesis to be generally applicable, multi- or pan-genotypic primers would have needed to be used. In this case, the primers were matching genotype 1a and 1b samples. Other genotypes, however, were not included. But, with the extensive genetic variability of the Hepatitis C virus, it is difficult to find the ideal method of sequencing. Only a small number of primers generate amplicons which cover all regions of interest but at the same time are still applicable to more than one genotype. Template-independent amplification methods which can provide multi-genotypic and full length sequencing at the same time “have a high limit of detection” [26].

IV.2.3 Resistance-associated variants (RAVs) in NS2/3 and NS5b

The surprisingly high prevalence of Q80K in plasma samples included in this thesis (10 out of 14 samples which had been sequenced for NS3 show the polymorphism) raises several questions: Is this high prevalence and high frequency similar to findings from other experiments or studies? Is it due to the fact that the Hepatitis C Virus in HCV/HIV-co-infected patients is more likely to mutate? How significant is the impact of the polymorphism Q80K on a treatment that includes simeprevir? And in consequence of this, should patients be screened for Q80K and other RAVs prior to treatment with DAAs?

In the paper "Hepatits C virus NS3 sequence diversity and antiviral RAV frequency in HCV/HIV co-infection", published on 4 August 2014, around the same time as the experiments for this thesis were conducted, 20 monoinfected HCV-positive patients were compared to 20 HIV/HCV-co-infected patients [8]. All 40 of them had been deep sequenced for Q80K as well as other resistance-associated variants located in NS3. A total number of 40 subjects might not be completely sufficient for a sound statistical analysis. However, the results were similar to the findings in this thesis: 17 out of the 40 subjects showed the variant Q80K. In 11 of the monoinfected and in 6 of the co-infected patients, Q80K was present with a frequency of at least 99% each.

Unlike the study by Jabara *et al.*, for this thesis patients infected with genotype 1a and 1b were included, but only HCV/HIV co-infected subjects.

With only 3 samples of genotype 1b screened for Q80K there is unfortunately not enough data to make a statement on subtype predisposition for the aminoacid change. When consulting the literature, however, it seems that this polymorphism is much more prevalent in subtype 1a than it is in 1b. The article on a meta-analysis of Q80K prevalence in 3349 HCV genotype 1-infected patients "Prevalence of the hepatitis C virus NS3 polymorphism Q80K in genotype 1 patients in the European region" comes to the conclusion that Q80K is present at approximately 7.5% overall. The percentages vary, depending on subtype and region. Subtype 1a shows the variant Q80K at 19.8%, 1b only at 0.5% [27]. However, if HCV/HIV-co-infection has an impact on RAV-rates was not investigated here.

A clinical study published by Enass A. Abdel-Hameed et al. in 2017 finds RAVs prevalent in 73.3% of HCV/HIV co-infected subjects. Among them, Q80K showed the highest prevalence: 46.7%, which might suggest that a co-infection with HIV leads to a higher mutation rate of the hepatitis C virus. After a 12 week course with PegIFN/ribavirin, RAV rates went up to 83.3%, with Q80K at 56.7%, indicating that non DAA treatments also pressure the virus to develop mutations [28].

Another possible explanation for the very high prevalence of RAVs, especially Q80K in this study could be that the authors had investigated only a local group of infected patients. Interaction between the individuals, i.e. the virus being transmitted from one subject to others within the study, could lead to a founder effect. Although the authors do not explicitly tell how close the 30 subjects were, all of them had been previously enrolled in a clinical trial with broad geographic representation [29].

An article from 2016 investigating the prevalence of RAVs in the NS3 region in patients infected with HCV genotype 1a found the prevalence of Q80K in HCV/HIV co-infected individuals to be 12.9% while it was 9.6% in HCV mono-infected subjects. This supports the theory that HCV/HIV co-infection could be one reason for the high prevalence of Q80K in samples from this thesis [30].

According to another article, the RAVs Q80K and Q80R show up in less than 10% of treatment failure patients but provide 420-fold respectively 305-fold the normal resistance to simeprevir when combined with R155K [15]. However, patients with the RAV Q80K still have high SVR rates of 92% when treated with a combination of simeprevir plus sofosbuvir with or without ribavirin. Only when other negative predictive factors are present at the same time (previous treatment, liver cirrhosis) Q80K leads to (significantly) worse treatment results [31, 32].

Normally, pre-existing resistance-causing mutations in the genome of bacteria or viruses exist at low levels until the beginning of treatment, when they become dominant due to their selective advantage [8]. The only way to predict the outcome of a therapy with DAAs would be to screen for those mutations using a method which can detect mutants at very low percentages, such as deep sequencing.

More rarely, resistance-associated variants represent not just a minor fraction of the genome but are in fact already dominant in treatment-naïve patients, in which case they could easily be picked up by population sequencing as well.

IV.2.4 Implication

With the introduction of the first DAAs emerged the hope for a cure of HCV-infected patients with “short treatment duration and acceptable adverse events” [13]. During the following years research dealt with reducing adverse side effects by omitting IFN from the treatment. The COSMOS randomised study investigated in 2014 a completely IFN-free regimen by treating 167 patients with chronic HCV with either a combination of simeprevir and sofosbuvir only, or RBV plus the two DAAs. As SVR of 92% were achieved after 12 weeks in 154 patients while serious adverse events were only seen in 2% of the patients, this IFN-free treatment seems to be both “efficacious and well tolerated” [14]. Guidelines of the DGVS (Deutsche Gesellschaft für Gastroenterologie, Verdauungs- und Stoffwechselkrankheiten), member of the AWMF (Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachangestellten) from 2015 already do not approve IFN-based regimes as standard therapy anymore [33]. The latest guidelines from 2018 recommend several IFN-free, DAA-based regimes. As for an HCV/HIV co-infection, treatment is recommended to be the same as for monoinfected patients [31].

Resistance-associated variants like Q80K and C316N can theoretically cause regimens to fail, especially when DAAs play a main part in this treatment. At the beginning of DAAs it hence looked like important tasks for future research were to further determine the prevalence and frequency of RAVs as well as their total impact on treatments with DAAs.

In comparison: with HIV, general screening for RAVs before starting treatment is more common. The German AIDS Society (Deutsche AIDS Gesellschaft e.V.) for instance recommends in its guidelines from 2017 to do a genotypic test on every HIV infected patient for resistance causing mutations before starting treatment [34].

However, new drugs for HCV are admitted every year and today regimens which are independent of the genotype and of a pre-treatment analysis exist.

The latest AWMF guidelines for treatment of Hepatitis C Virus infection from 2018 list several different combinations of DAAs as effective IFN-free regimens, amongst them the combination of simeprevir and sofosbuvir plus ribavirin for genotypes 1 and 4, but not as first line therapy. Some of those regimens like the 12-week-course of sofosbuvir and velpatasvir even show SVR rates of at least 95% overall (with the exceptions of 91% SVR in genotype 3-infected patients with compensated liver cirrhosis and 90% SVR in genotype 3-infected patients with prior treatment) for all genotypes 1 to 4, in patients with or without cirrhosis, and without prior screening for RAVs and are therefore recommended for first and re-therapy for all genotypes mostly independent of RAVs [31].

As for the combination of simeprevir plus sofosbuvir: SVR rates drop from 92% in patients with liver cirrhosis without Q80K to only 74% in patients with both liver cirrhosis and Q80K according to the OPTIMIST-2 study [32].

The AWMF guidelines recommend pre-treatment viral resistance analysis for three scenarios only:

- a NS5A resistance analysis should be conducted beforehand when treating with the second generation NS3 protease inhibitor grazoprevir and NS5A inhibitor elbasvir for 12 weeks for patients infected with HCV genotype 1a
- Patients infected with HCV genotype 3 can undergo NS5A resistance analysis before starting treatment with daclatasvir and velpatasvir in combination with sofosbuvir
- After treatment with DAAs failed, patients should have viral resistance analysis in order to choose the right regimen for re-therapy [31].

Although it is not determined whether this pre-treatment analysis should be conducted via next generation sequencing or population sequencing, the authors postulate that only RAVs at a percentage of 15% or higher (a level which can be detected by population sequencing) have significant influence on SVRs [31, 35].

One major problem of DAAs remains their expensiveness. According to the free educational website from the University of Washington “Hepatitis C Online”, a 12-week course with simeprevir costs $66,000, while an interferon-free regimen with simeprevir (Olysio) plus sofosbuvir (Sovaldi) for 12 weeks, as recommended in the COSMOS-study costs approximately $150,000 (depending on exchange rates about 150,000€). A 12 week course of sofosbuvir plus velpatasvir as recommended by the AWMF guidelines costs about $75,000. However, that is about 300 times higher than the estimated production costs [36].

As for Germany: in 2015, sofosbuvir provider Gilead and german health insurance companies (GKV) agreed on a price for a 12 week course of sofosbuvir of 43,562€, which put the real costs as sold by pharmacies (Apothekenverkaufspreis) in the end at around 53,000€ [37, 38]. Since then, not much has changed. In 2017 the association “Ärzte ohne Grenzen” together with organisations from 17 other countries objected in front of the European Patent Office to Gilead’s patent for sofosbuvir, arguing that the costs for the drug are unreasonably high. In September 2018 the European Patent Office decided against them. In consequence, Gilead keeps its patent for sofosbuvir [39].

Prices for other DAAs can sometimes be difficult to find out, since they are determined in negotiations between insurance companies and providers.

Presuming that pre-treatment analysis is conducted via population sequencing, costs are about 260€, corresponding to the costs for genotype resistance sequencing of HIV according to the website of “Kassenärztliche Bundesvereinigung” [40].

As a very rough estimation, the screening of 203 patients for the variant Q80K would cost as much as treating one patient with a 12 week course of sofosbuvir.

The critical question in this matter is therefore, how cheap new therapies (for example simeprevir/sofosbuvir with or without RBV) would have to be so that screening for RAVs does not pay off.

If 1000 patients were treated, without previous screening, with a combination of DAAs like for example simeprevir plus sofosbuvir with or without RBV, costs for this regime would be 1000 times x.

If a pre-treatment screening with population sequencing was taken into account, costs would rise up to 260€ + x, 260€ being the costs for the pre-treatment analysis of one patient. Assuming that the prevalence of Q80K is only 7.5% [27], 75 out of 1000 patients would benefit from another regime, not containing simeprevir. Meanwhile, the rest of the 925 patients without Q80K still would have been screened and then treated. Their costs would hence be 260€ + x.

$$
\begin{aligned}
1000x &< 75 \cdot 260€ + 925 \cdot (260€ + x) \\
\Leftrightarrow 1000x &< 19500\ € + 240500\ € + 925x \\
\Leftrightarrow 75x &< 260000\ € \\
\Leftrightarrow x &< 3467\ €
\end{aligned}
$$

With a higher prevalence of Q80K, the result for x would be even lower.

Only if the treatment costs for simeprevir plus sofosbuvir with or without RBV were lower than 3,467€, screening for Q80K with population sequencing would not pay off. Of course, the current costs are about 10 to 50 times higher and it does not look like as if they are going to drop any time soon.

However, as previously mentioned, literature shows that the prevalence of Q80K only seems to result in treatment failure when other negative predictive factors such as liver cirrhosis are present [31, 32, 35].

Meanwhile, the costs for sequencing are in free fall. According to an article from 2009 on Spiegel Online, sequencing the whole human genome at that time cost $48,000 [41]. The Assman-Stiftung describes on its website costs for whole genome sequencing of only 1000€ in the year 2012 [42]. And in 2017 the American company Illumina presented the new sequencer “NovaSeq” and promised on its website the $100 genome in the future [43].

As for this study, Q80K shows up at either close to 0% or almost 100% (all or nothing). As such it could be easily detected by population sequencing as well [31, 35]. Furthermore, any variant

with a frequency lower than 15% within the viral population does not seem to significantly impact treatment success and should therefore not influence a treatment decision [35, 26] During the time of the experiments for this study, it looked like population sequencing was maybe a cheaper, but definitely a more practical method since most labs are provided with PCR machines while next generation sequencing machines were still highly expensive in purchase and maintenance. Today, next generation sequencing has become much more accessible and its costs have decreased. But even if screening is performed with population sequencing and Q80K does not show up, deep sequencing for its presence as a minor variant still remains an option.

NGS also allows for a closer look on how exactly the virus reacts to a treatment with DAAs especially in patients with treatment failure or relapse, as demonstrated by Bonsall et al. in 2018, where the authors hypothesise that “the selective pressures exerted by modern treatments, coupled with the ability of HCV to mutate may have a significant role in shaping the future HCV pandemic” [44].

In conclusion, while population sequencing seems to be the preferable method for genotypic resistance testing, the significance of next generation sequencing lies mostly in the analysis of patient samples during treatment failure, in order to investigate the exact order of selection within certain viral populations.

Abstract

Hepatitis C Virus infection affects more than 170 Million people worldwide. With the introduction of direct acting antivirals (DAAs) into therapy in 2011, treatment of chronic hepatitis C infection has experienced major improvement showing less side-effects and higher treatment success rates compared to early hepatitis C therapy consisting of a combination of only pegylated interferon-alpha (pIFN-alpha) and ribavirin (RBV). However, as with every new antiviral or antibiotic therapy, resistance-associated variants emerge with treatment. Some resistance-associated polymorphisms are even found to be pre-existing in treatment-naïve patients. Unfortunately, DAAs also remain very expensive, which limits the options on a world-wide use and interferon-free regimens.

Because of similar pathways of infection, HCV/HIV co-infection is not uncommon but complicates treatment, leads to higher complication rates and possibly a higher prevalence of resistance-associated variants (RAVs).

For this thesis, the main goals were to establish a screening method for RAVs in plasma from HCV/HIV co-infected patients with deep sequencing and to investigate deep sequencing accuracy especially at low RNA input (low viral load) and low prevalence of RAVs within one patient.

A serial dilution of a mix of *in vitro* transcribed mutants S122R, R155K, D168V and a wild-type was sequenced. It was found that mutants can still be picked up when RNA is added at a quantity of only 0.5 pg, even for the mutant added at the lowest percentage (0.3%). In conclusion, deep sequencing proves to qualify as a method for investigating plasma samples for resistance-associated variants, even for patients with low viral loads.

Afterwards, it was shown in an experiment with few samples that screening for RAVs using deep sequencing or population sequencing prior to treatment with DAAs would make sense, especially given the high costs for DAAs. Therefore, 16 plasma samples from HCV/HIV co-infected, treatment-naïve patients were screened for pre-existing variants in the NS3 and NS5b regions of the Hepatitis C Virus using deep sequencing. Patients were infected with HCV

genotypes 1a and 1b. Especially the two resistance-associated polymorphisms Q80K in NS3 and C316N in NS5b were of interest. Q80K provides a certain resistance against simeprevir, while C316N is associated with resistance against polymerase-inhibitors like sofosbuvir.

A number of genetic variations of the original consensus sequence were found. As for Q80K, the polymorphism was found in 10 out of 14 samples. Each time, the percentage of viral copies with Q80K within one patient's plasma was very high (> 97% in 9 samples, 84% in 1 sample). C316N however, was only present at a very low percentage (1.2%) in one out of 8 samples.

With such high percentages of variants, screening with population sequencing would be effective enough. The significance of deep sequencing then probably lies in the analysis of patient samples during treatment failure, in order to investigate the exact order of selection within certain viral populations.

However, the number of samples in this study is too low to make a valid statistical statement. More studies with higher numbers of samples would be necessary to support this theory.

Zusammenfassung

Weltweit sind mehr als 170 Millionen Menschen mit dem Hepatitis C Virus infiziert. Dank der 2011 auf den Markt gekommenen direct acting antivirals (DAAs) hat sich die Therapie von Patienten mit chronischer Hepatitis C deutlich verbessert. Geringere Nebenwirkungen und höhere Erfolgsraten zeigen sich im Vergleich mit der herkömmlichen Therapie bestehend aus der Kombination von pegyliertem Interferon-alpha (pIFN-alpha) und Ribavirin (RBV). Wie bei jeder neuen antiviralen oder antibiotischen Therapie entwickeln sich allerdings auch im Hepatitis C Virus Resistenzen vermittelnde genetische Varianten oder Mutationen, sogenannte resistance-associated variants (RAVs). Manche resistenz-vermittelnde Polymorphismen sind sogar bereits vor Therapiebeginn existent. Leider ist die Therapie mit DAAs nach wie vor sehr teuer, wodurch die Möglichkeiten einer weltweiten Nutzung sowie die Aussicht auf Interferon-freie Therapien begrenzt werden.

HCV und HIV haben ähnliche Übertragungswege, weswegen Koinfektionen keine Seltenheit sind. Allerdings kompliziert eine HCV/HIV-Koinfektion die Behandlung, führt zu höheren Komplikationsraten und möglicherweise auch zu einer höheren Prävalenz an RAVs.

Die beiden Hauptziele dieser Arbeit waren zum einen, die technische Durchführung eines Screenings auf RAVs mittels Deep Sequencing zu etablieren, und zum anderen die Genauigkeit von „Next-Generation-Sequencing“ (NGS) besonders bei niedriger Viruslast und niedriger Prävalenz von RAVs im Patienten zu untersuchen.

Hierfür wurde eine Serienverdünnung von einem Mix aus *in vitro* transkribierten Mutanten S122R, R155K, D168V und dem Wildtyp sequenziert. Es wurde festgestellt, dass Mutanten auch dann noch detektiert werden, wenn nur 0,5 pg RNA zugegeben wurden. Selbst der Mutant mit dem geringsten Anteil am Mix (0,3%) zeigte sich in den Ergebnissen. Zusammenfassend gesagt beweist sich NGS als eine gute Methode um Plasmaproben auf RAVs zu untersuchen, selbst bei Patienten mit niedriger Viruslast.

Anschließend wurde an einer geringen Anzahl an Proben gezeigt, dass ein routinemäßiges Screening auf RAVs mittels NGS oder Population Sequencing sinnvoll wäre, besonders aufgrund

der hohen Kosten für DAAs. Es wurden 16 Plasmaproben von HCV/HIV-koinfizierten, bisher nicht behandelten Patienten auf präexistente RAVs in den Regionen NS3 und NS5b gescreent. Die Patienten waren mit HCV-Genotyp 1a und 1b infiziert. Hauptaugenmerk lag auf den beiden Polymorphismen Q80K in NS3 und C316N in NS5b. Durch Q80K erlangt das Virus eine gewisse Resistenz gegen Simeprevir, wohingegen C316N mit einer Resistenz gegen Sofosbuvir assoziiert ist.

Es wurden eine Reihe von genetischen Abweichungen von der jeweiligen Konsensussequenz gefunden. In 10 von 14 Proben fand sich Q80K, davon neunmal zu über 97% und einmal zu 84%. C316N hingegen fand sich nur in einer von acht Proben, zu nur 1,2%.

Bei solch hohen Anteilen von Virusvarianten wäre ein Sequenzieren mit Population Sequencing völlig ausreichen, der Stellenwert von NGS läge dann vermutlich in einer Feinanalyse von Patientenproben, die während eines Therapieversagens gewonnen werden, um die genaue Abfolge der Selektion bestimmter Viruspopulationen darzustellen.

Für eine statistisch belastbare Aussage ist die Probenzahl allerdings deutlich zu gering und weitere Studien mit größeren Patientenzahlen sind vonnöten um diese Aussage zu unterstützen.

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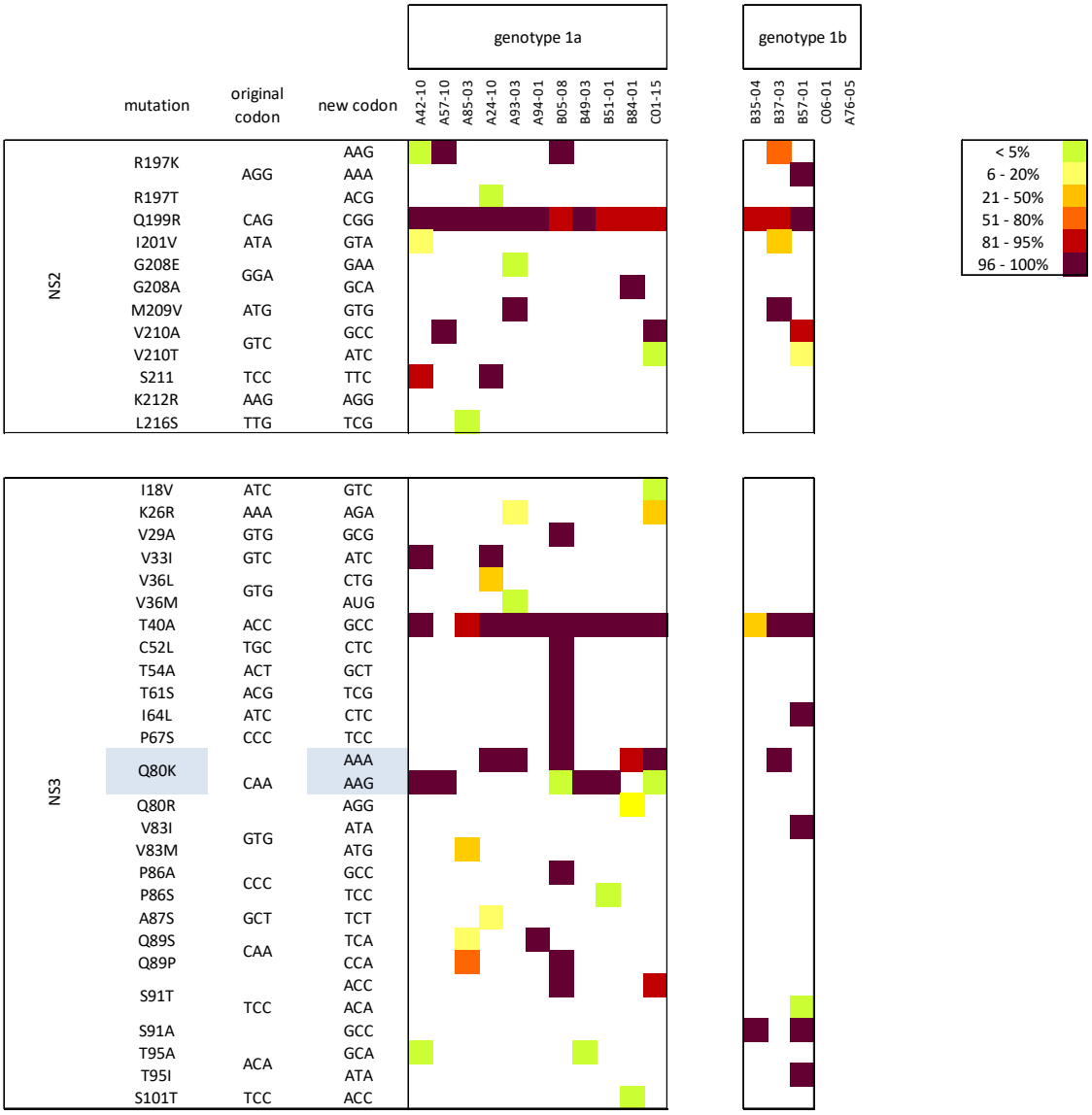
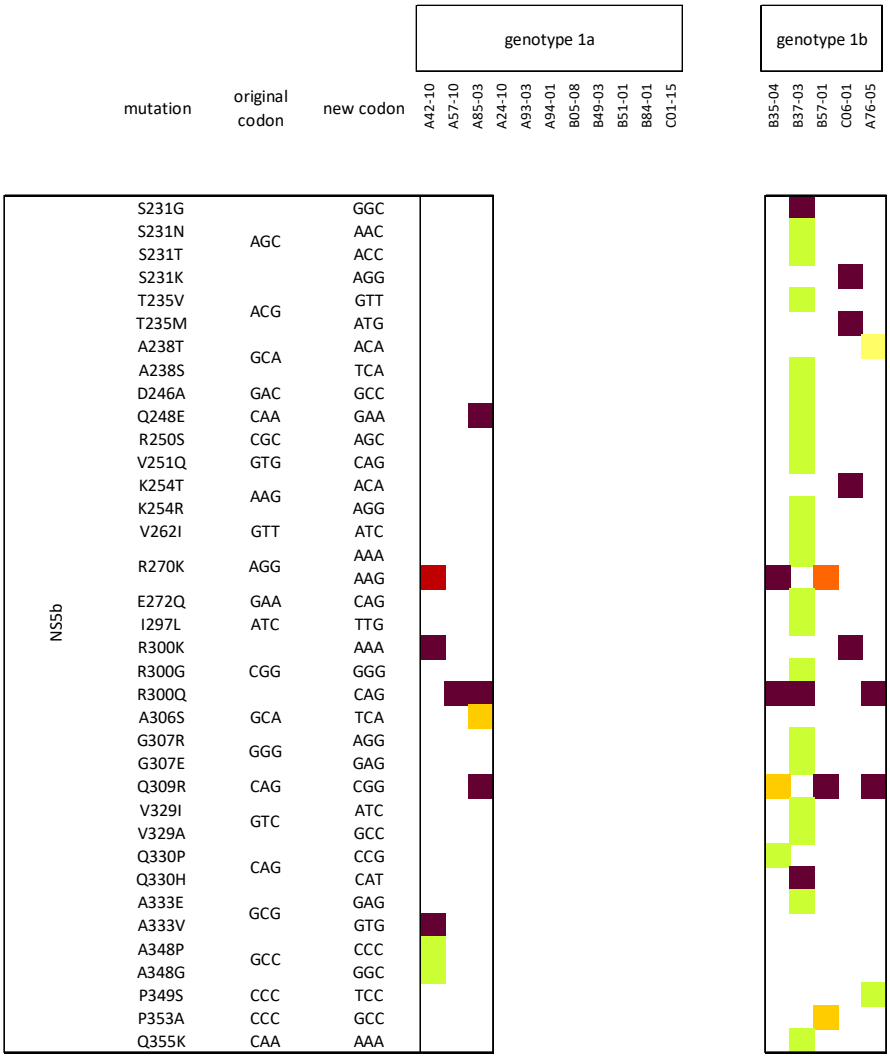
44. Bonsall D, Black S, Howe AY, et al. (2018): Characterization of hepatitis C virus resistance to grazoprevir reveals complex patterns of mutations following on-treatment breakthrough that are not observed at relapse. *Infection and Drug Resist*ance 2018;11:1119-1135. Published 2018 Aug 8. DOI:10.2147/IDR.S156581

Appendix:

Heat map: presence and relative frequency of RAVs



Heat map part 2



List of variants first deep sequencing run

reference sequence		Re.1a.US.77.H77											
genotype		all samples genotype 1a											
Number of reads		13713	19708	12900	10653	14097	10080	15518					
Variant	**aminoacid position in NS2/3**	**A24-10 %**	**A42-10 %**	**A57-10 %**	**A85-03 %**	**A93-03 %**	**A94-01 %**	**WT control (H77 plasmid) %**	**variant**	**original codon**	**new codon**		
576:C/T	192	0	0,67	0	0,15	0	0	0	P192 silent	CCC	CCT		576
582:T/C	194	99,88	99,12	0,29	4,57	100	0	0	S194 silent	TCT	TCC		582
585:C/T	195	0	1,06	0	0	0	0	0	A195 silent	GCC	GCT		585
588.5:-/G	196,1666667	1,64	0	0	0	0	0	0					588,5
588:T/C	196	45,9	0	99,59	100	0	0	0	R196 silent	CGT	CGC		588
589.5:-/C	196,5	0,98	0	0	0	0	0	0					589,5
589:A/C	196,3333333	0,14	0	0	7,59	0	0	0	R197 silent		CGG		589
590:G/A	196,6666667	0	1,52	99,67	0	0	0	0	R197K	AGG	AAG		590
590:G/C	196,6666667	0,77	0	0	0	0	0	0	R197T		ACG		590
592:G/C	197,3333333	0,42	0	0	0	0	0	0	G198R		CGC		592
594:C/G	198	0,42	0	0	0	0	0	0		GGC	GGG		594
594:C/T	198	0	1	0	0	0,56	0	0	G198 silent		GGT		594
595-596:CA/--		1	0	0	0	0	0	0					595-596
595:C/A	198,3333333	0	0	0	0	0,72	0	0	Q199K		AAG		595
595:C/G	198,3333333	0,61	0	0	0	0	0	0	Q199E		GAG		595
596:A/-	198,6666667	1,53	1,2	0	0,79	0	0	0	Q199 deletion	CAG	C-A		596
596:A/G	198,6666667	97,63	98,51	100	99,21	99,67	100	0	Q199R		CGG		596
597:G/A	199	0	0	0	21,18	0	99,6	0	Q199 silent		CAA		597
597:G/C	199	0,42	0	0	0	0	0	0	Q199H		CAC		597
599.5:-/C	199,8333333	0,5	0	0	0	0	0	0					599,5
599:A/G	199,6666667	0,42	0	0	0	0	0	0	E200G	GAG	GGG		599
600.5:-/G	200,1666667	0,69	0	0	0	0	0	0					600,5
600:G/-	200	1,31	0	0	0	0	0	0	E200 deletion	GAG	GA-		600
600:G/A	200	44,56	0,71	0	0	0	0	0	E200 silent		GAA		600
601.5:-/A	200,5	0,69	0	0	0	0	0	0					601,5
601:A/G	200,3333333	0	7,08	0	0	0	0	0	I201V		GTA		601
602:T/C	200,6666667	0	0,33	0	0	0	0	0	I201T	ATA	ACA		602
604:C/T	201,3333333	99,68	1,37	0,67	1,09	0	0	0	L202 silent	CTG	TTG		604
609:T/C	203	100	2,56	100	99,7	99,58	100	0	L203 silent	CTT	CTC		609
612:G/A	204	0	98,95	99,81	95,03	100	99,86	0			GGA		612
612:G/C	204	0	0,39	0	0	0	0	0	G204 silent	GGG	GGC		612
612:G/T	204	100	0	0	0	0	0	0			GGT		612
615:A/C	205	0,47	0,92	0	0	0	0	0			CCC		615
615:A/G	205	98,98	0	0	100	100	100	0	P205 silent	CCA	CCG		615
615:A/T	205	0	99,08	0	0	0	0	0			CCT		615
618:C/T	206	0	4,76	0	0	0	0	0	A206 silent	GCC	GCT		618
621:C/T	207	0	99,84	98,66	100	19,66	100	0	D207 silent	GAC	GAT		621
623:G/A	207,6666667	0	0,45	0	0	3,91	0	0	G208 E		GAA		623
624:A/C	208	0	0	11,23	0	0	0	0	G208 silent	GGA	GGC		624
624:A/G	208	0	0,72	0,36	10,44	1,11	0	0			GGG	GAG -> E (1 and 3)	624
625:A/G	208,3333333	0	0	0	0,19	100	0	0	M209V	ATG	GTG		625
626:T/C	208,6666667	0	0	0	0	0,49	0	0	M209T		ACG	GCG -> A (1 and 2)	626
629:T/A	209,6666667	0	0	0,46	0	0	0	0	V				629
629:T/C	209,6666667	0	0,61	99,54	0	0	0	0	V210A	GTC	GCC		629
630:C/T	210	0	0,7	0	0	0	0	0	V				630
633:C/T	211	100	90,4	0	0	0,46	0	0	S211	TCC	TTC		633
634:A/C	211,3333333	0	0,44	0	0	0	0	0	K212				634
635:A/G	211,6666667	0,36	0,83	0	0	0,42	0	0	K212	AAG			635
636:G/A	212	0	1,77	0	0	0	0	0	K212silent		AAA		636
639:G/A	213	0	0,14	0	0,39	0	0	0	G213	GGG			639
643:A/C	214,3333333	0	0	0	0,38	0	0	0	R215	AGG			643
646:T/C	215,3333333	0	0,53	0,93	2,2	0	0	0	L216S	TTG	TCG		646
649:C/T	216,3333333	0	0	0	0	77,11	0	0	L217 silent		TTG		649
651:G/A	217	0	0	0	0,97	0	0,58	0	L	CTG			651
651:G/C	217	99,37	0	0	0	0	0	0	L217 silent		CTC		651
651:G/T	217	0,63	0	0	0	0	0	0	L				651
654:G/A	1	0	0,56	0	34,32	0	0	0	A1 silent	GCG	GCA		654
654:G/T	1	0	0	0	0	0,67	0	0			GCT		654
657:C/T	2	0	55,19	0,14	0	0,34	53,41	0	P2 silent	CCC	CCT		657
660:C/T	3	0	5,53	0	0,42	0,12	56,67	0	I3 silent	ATC	ATT		660
663:G/A	4	0	0	0	0,61	0	0	0	T4 silent	ACG	ACA		663
666:G/A	5	0	0,92	0	0	0,42	0	0	A5 silent	GCG	GCA		666
669:C/T	6	0	0,36	0,48	99,28	0,65	99,86	0	Y6 silent	TAC	TAT		669
672:C/T	7	0	96,61	0,6	0	0	0	0	A7 silent	GCC	GCT		672
681:G/A	10	92,42	0	0	99,86	0	99,64	0	T10 silent	ACG	ACA		681
684:A/G	11	96,85	98,83	100	99,69	100	100	0	R11 silent	AGA	AGG		684
688:C/A	12,33	0	0	0	0	0,28	0	0	L13I	CTC	ATC		688
690:C/T	13	17,92	0	0	34,73	0	0	0	L13 silent	CTC	CTT		690
691-693:CTA/TNG		0	0	0	98,79	0	99,05	0			TTG		
691:C/T	13,33	0	0,65	0	98,79	0,2	99,19	0	L14 silent	CTA	TTA		691
693:A/G	14	0	0	0	99,48	0	99,86	0			CTG	TTG -> L silent	693
696:G/A	15	0	0,3	0	48,74	0	99,33	0	G15 silent	GGG	GGA		696
699:T/C	16	0	9,33	100	99,86	0,14	100	0	C16 silent	TGT	TGC		699
700:A/G	16,33	0	0,52	0	0	0	0	0	I17V	ATA	GTA		700
705:C/A	18	0	0	0	0,95	0	0	0	I18 silent	ATC	ATA		705
705:C/T	18	0	0,35	0	98,18	20,08	99,47	0			ATT		705
711:C/T	20	0	0,5	0	0	1,21	0	0	S20 silent	AGC	AGT		711
712:C/T	20,33	0	1,88	0	99,86	0	100	0	L21 silent	CTG	TTG		712
717:T/C	22	0	1,61	99,71	94,73	100	100	0	T22 silent	ACT	ACC		717
720:C/T	23	0	0,49	0	0	0	0	0	G23 silent	GGC	GGT		720
723:G/A	24	0	99,24	98,67	2,55	0	0	0	R24 silent	CGG	CGA		723
726:C/T	25	0	1,64	0	0	0,14	0	0	D25 silent	GAC	GAT		726
728:A/G	25,67	0	0	0	0	6,32	0	0	K26R	AAA	AGA		728
729:A/G	26	100	6,21	0	0,54	0	0,3	0	K26 silent		AAG		729
732:C/T	27	0	0,58	0	27,14	0	0	0	N27 silent	AAC	AAT		732
735:A/G	28	0	1,77	0	99,48	100	99,85	0	Q28 silent	CAA	CAG		735
744:T/C	31	0	0,9	0	0,35	0,86	0	0	G31 silent	GGT	GGC		744
747:G/A	32	0	0,48	0	0	0	0	0	E32 silent	GAG	GAA		747
748:G/A	32,33	95,62	98,95	0	0	0	0	0	V33I	GTC	ATC		748
750:C/T	33	0	0,92	0	88,83	0	99,47	0	V33 silent		GTT		750
753:G/A	34	0	0,34	0	0	0	0	0	Q34 silent	CAG	CAA		753

754:A/G	34,33	0	0	0,32	0	0	0	0	I35V	ATC	GTC	754
756:C/T	35	100	99,53	100	97,81	99,06	100	0	I35 silent		ATT	756
757:G/A	35,33	0	0	0	0	0,38	0	0	V36M		ATG	757
757:G/C	35,33	48,16	0	0	0	0	0	0	V36L	GTG	CTG	757
759:G/C	36	0	0	0	0,33	0	0	0	V36 silent		GTC	759
762:A/G	37	0	0,79	0	0	0	0	0	S37 silent	TCA	TCG	762
765:T/C	38	100	1,22	0	0	80,64	0	0	T38 silent	ACT	ACC	765
768:T/C	39	0	1,27	0,79	99,43	0	0	0	A39 silent	GCT	GCC	768
769:A/G	39,33	100	100	0	94,29	100	99,65	0	T40A	ACC	GCC	769
771:C/T	40	0	0,49	0	0,42	0	0	0	T40 silent		ACT	771
773:A/G	40,67	0	0	0	0	0	0	100				773
774:A/G	41	0	0	1,07	99,86	0	99,87	0	Q41 silent	CAA	CAG	774
777:C/A	42	0	0,32	0,14	0	0	99,67	0			ACA	777
777:C/G	42	0	0	0	0	0	0,33	0	T42 silent	ACC	ACG	777
777:C/T	42	0	99,53	99,65	0,42	98,76	0	0			ACT	777
780:C/T	43	0	1,78	0,71	0,03	1,13	0	0	F43 silent	TTC	TTT	780
781-783:CTG/TNA		0	0	0	0	0	0	0				
781:C/T	43,33	0	1,77	0,59	1,16	37,97	0	0	L44 silent	CTG	TTG	781
783:G/A	44	0	0,62	0	0,44	0	0	0			CTA	TTA -> L silent	783
786:A/G	45	0	0,43	0	0	0,16	0	0	A45 silent	GCA	GCG	786
789:G/A	46	0	1,7	0	0	4	0	0	T46 silent	ACG	ACA	789
789:G/C	46	0	0,55	0	100	0	100	0			ACC	789
792:C/T	47	0	0,73	0	0	0	0	0	C47 silent	TGC	TGT	792
795:C/T	48	0	0,64	0	0,78	0	0	0	I48 silent	ATC	ATT	795
798:T/C	49	0	0,77	0	99,97	0	100	0	N49 silent	AAT	AAC	798
804:A/G	51	100	100	0	99,97	0	100	0	V51 silent	GTA	GTG	804
807:C/T	52	0	0	0,39	0	0	0	0	C52 silent	TGC	TGT	807
813:T/C	54	0	0,37	0	0,83	0,58	0	0	T54 silent	ACT	ACC	813
816:C/T	55	0	1,29	0	0	0,14	0	0	V55 silent	GTC	GTT	816
819:C/T	56	0,5	0	0	0	0	0	0	Y56 silent	TAC	TAT	819
822:C/T	57	100	0	0	0,35	0	0	0	H57 silent	CAC	CAT	822
825:G/A	58	0	0,35	0,13	0	100	0	0	G58 silent	GGG	GGA	825
828:C/G	59	0	0	0	0,37	0	0	0	A59 silent	GCC	GCG	828
828:C/T	59	99,37	98,92	99,65	0	100	0	0			GCT	828
831:A/G	60	0	0	0	0	0	4,22	0	G60 silent	GGA	GGG	831
834:G/A	61	0	0	0	100	0	100	0	T61 silent	ACG	ACA	834
835:A/C	61,33	0	99,64	0	0	0	0	0	R62 silent	AGG	CGG	835
840:C/T	63	100	0,51	0,27	1,07	0	0	0	T63 silent	ACC	ACT	840
843:C/T	64	100	8,62	0,33	0,82	2,4	0	0	I64 silent	ATC	ATT	843
846:A/G	65	0	0	0,29	99,88	77,99	100	0	A65 silent	GCA	GCG	846
849:A/G	66	0	0,76	0	0,23	0	0	0	S66 silent	TCA	TCG	849
851:C/T	66,67	0	0,3	0	0	0	0	0	P67L		CTC	851
852:C/A	67	0	0	99,23	0	0	0	0	P67 silent	CCC	CCA	852
852:C/T	67	0	100	0	0	0	18,69	0			CCT	852
855:G/A	68	0	0,14	0,51	12,99	8,15	0	0	K68 silent	AAG	AAA	855
858:T/C	69	0	1,84	0,42	0	0	0	0	G69 silent	GGT	GGC	858
861:T/C	70	0	95,31	0	0	0	0	0	P70 silent	CCT	CCC	861
862-864:GTC/ANG		0	0	0	0	0	0	0				
862-864:GTC/ANT		0,62	0	0	0	0	0	0	V71I		ATT	
862:G/A	70,33	0,62	0	0	0	0	0	0		GTC	ATC	862
864:C/G	71	1,96	0	0	0	0	0	0	V71 silent		GTG	864
864:C/T	71	94,35	99,65	88,15	63,21	100	99,3	0			GTT	ATT -> I	864
867:C/T	72	0	7,57	0	0	0	0	0	I72 silent	ATC	ATT	867
876:T/C	75	28,43	91,76	97,84	28,04	100	100	100	Y75 silent	TAT	TAC	876
879:C/T	76	0	0,61	0	0	2,38	0	0	T76 silent	ACC	ACT	879
882:T/C	77	0	54,85	13,25	1,85	0,16	0	0	N77 silent	AAT	AAC	882
885:G/A	78	0	0	0	72,55	0	99,21	0	V78 silent	GTG	GTA	885
886:G/C	78,33	0	0,5	0	0	0	0	0	D79H	GAC	CAC	886
888:C/T	79	0	0,55	0	99,38	0	100	0	D79 silent		GAT	888
889-891:CAA/ANG		0	99,6	97,86	0	0	0	0	Q80K		AAG	
889:C/A	79,33	100	100	99,74	0	100	0	0	Q80K	CAA	AAA	889
891:A/G	80	0	99,6	97,86	0,42	0	0	0	Q80 silent		CAG	891
894:C/T	81	100	0	0,46	0,23	0	0	0	D81 silent	GAC	GAT	894
897:T/C	82	99,67	1,64	0	100	0,13	99,81	0	L82 silent	CTT	CTC	897
898-900:GTG/ANA		0	0	0	20,92	0	0	0				
898:G/A	82,33	0	0	0	20,92	0	0	0	V83M	GTG	ATG	898
900:G/A	83	0	0,14	0	99,68	0	99,67	0	V83 silent		GTA	ATA -> V83I (1 and 3)	900
903:C/G	84	0	1,13	0	0	0	0	0	G84 silent	GGC	GGG	903
909:C/T	86	0	2,54	0,19	0	0,54	42,58	0	P86 silent	CCC	CCT	909
910:G/A	86,33	0,45	0	0	0	0	0	0	A87T	GCT	ACT	910
910:G/T	86,33	5,8	0	0	0	0	0	0	A87S		TCT	910
912:T/-	87	0	0	0	2,84	0	0,18	0			GC-	912
912:T/C	87	0	0,4	0,16	97,16	0	99,82	0	A87 silent		GCC	912
915:T/C	88	0	1,61	0,67	0,36	0,21	0	0	P88 silent	CCT	CCC	915
915:T/G	88	0	0,36	0	0	0	0	0			CCG	915
916-917:CA/TC		0	0	0	20,28	0	97,26	0	Q89S		TCA	
916:C/T	88,33	0	0	0	20,28	0	100	0	Q89 STOP		TAA	TCG -> Q89S (1,2 and 3)	916
917:A/C	88,67	0	0	0	98,93	0	97,26	0	Q89P	CAA	CCA	917
918:A/-	89	0	0	0	1,07	0	0	0			CA-	918
918:A/G	89	0	99,63	0	0	64,62	100	0	Q89 silent		CAG	918
921:T/A	90	0	0	100	0	0	0	0			GGA	921
921:T/C	90	0	99,34	0	1,03	0	0	0	G90 silent	GGT	GGC	921
921:T/G	90	0	0	0	0	19,66	0	0			GGG	921
924:C/T	91	0	0	0	0	100	0	0	S91 silent	TCC	TCT	924
927:C/T	92	0	99,49	0	0	0	0	0	R92 silent	CGC	CGT	927
930:A/G	93	0	1,22	0	0,16	0	0	0	S93 silent	TCA	TCG	930
931:T/C	93,33	0	10,65	0	1,68	0	0	0	L94 silent	TTG	CTG	931
933:G/A	94	99,85	0	0	92,07	0,35	99,71	0	L94 silent		TTA	933
934:A/G	94,33	0	0,78	0	0	0,14	0	0	T95A	ACA	GCA	934
936:A/G	95	100	0,93	0	0	0	0	0	T95 silent		ACG	936
939:C/T	96	0	0	0	0,2	0,28	0	0	P96 silent	CCC	CCT	939
942:C/T	97	0	0	0	0,37	0	0	100	C97 silent	TGC	TGT	942
945:C/T	98	0	2,65	0	97,98	0,55	99,82	0	T98 silent	ACC	ACT	945
948:C/T	99	0	99,3	0,32	0	1,23	0	0	C99 silent	TGC	TGT	948
951:C/T	100	0	0,28	0	0	0	0	0	G100 silent	GGC	GGT	951
954:C/T	101	0	0,54	0,47	0	0	0	0	S101 silent	TCC	TCT	954
957:G/A	102	9,34	0,67	0	7,76	0	0,38	0	S102 silent	TCG	TCA	957
960:C/T	103	0	0,35	0,4	0	0	0	0	D103 silent	GAC	GAT	960

all samples genotype 1a

NS2

NS3

Q80

plasmid sequence different from reference sequence

clincally relevant mutations

silent mutation

mutation

actual mutation

List of variants second deep sequencing run

reference sequence		Re.1a.US.77.H77												
genotype		genotype 1a					genotype 1b							
number of reads		9926	7804	13019	5001	7921	6690	6498	5904	5264				
Variant	aminoacid position in NS2/3	B05-08 %	B49-03 %	B51-01 %	B84-01 %	C01-15 %	B35-04 %	B37-03 %	B57-01 %	WT control (H77 plasmid DNA from Selena) %	variant	original codon	new codon		Variant
576:C/T	192	98,23	0,78	0	0,55	0	0	98,19	0	0	P192 silent	CCC	CCT		576
579:C/T	193	0	0	0	0	0	0,1	0	98,46	0	V193 silent	GTC	GTT		579
582:T/A	194	56,98	0	0	0	0	0	0	0	0	S194 silent	TCT	TCA		582
582:T/C	194	37,79	18,98	99,77	99,11	98,97	67,41	96,46	4,74	0			TCC		582
588:T/C	196	100	7,16	0	0,63	0	0	99,56	100	0	R196 silent	CGT	CGC		588
589:A/C	196,3333333	0	0	0	2,15	0	0	0	0	0	R197 silent		CGG		589
590:G/A	196,6666667	100	0	0	0	0	0	52,82	0	0	R197K	AGG	AAG		590
591:G/A	197	0	0	0	0	0	0	0	99,52	0			AAA		591
594:C/T	198	0	0	72,79	0	0	0,65	0	0	0	G silent	GGC	GGT		594
596:A/G	198,6666667	90,56	97,58	92,78	89,97	93,83	87,63	94,51	100	0	Q199 R	CAG	CGG		596
597:G/A	199	0	0	0	0	3,3	0	0	99,63	0	Q199 silent		CAA		597
601:A/G	200,3333333	0	0	0	0	0	0	37,02	0	0	I201 V	ATA	GTA		601
604:C/T	201,3333333	0,18	1	0	100	0	0	3,2	0	0	L 202 silent	CTG	TTG		604
606:G/A	202	0	6,8	0	0	0	0	0	0	0			CTA		606
609:T/C	203	14,47	95,04	99,26	99,49	100	99,78	99,88	95,77	0	L 203 silent	CTT	CTC		609
612:G/A	204	0	94,82	95,29	99,14	99,44	67,48	0	100	0	G 204 silent	GGG	GGA		612
612:G/C	204	0	0	0	0,49	0	0	0	0	0			GGC		612
615:A/G	205	95,85	0,9	0	0,18	0	98,99	0,99	99,6	0	P 205 silent	CCA	CCG		615
618:C/T	206	0	0	0	0,52	0	0	0	0	0	A 206 silent	GCC	GCT		618
621:C/T	207	99,56	23,84	100	100	34,1	97,69	100	97,28	0	D207 silent	GAC	GAT		621
623:G/C	207,6666667	0	0	0	100	0	0	0	0	0	G208 A		GCA		623
624:A/C	208	0	0	0	0,62	0	0	0	0	0		GGA	GGC		624
624:A/G	208	0	3,68	0	0	0	0	0	0	0	G 208 silent		GGG		624
624:A/T	208	0	0	0	92,09	0	0	0	0	0			GGT		624
625:A/G	208,3333333	0	0	0	0	0	0	99,55	0	0	M209 V	ATG	GTG		625
627:G/A	209	0	0	0	0	0	0	0,67	0	0	M209 I		ATA		627
628:G/A	209,3333333	0	0	0	0	2,41	0	0	8,97	0	V210 I	GTC	ATC	V210T =ACC	628
629:T/C	209,6666667	0	0	0	0	100	0	0	100	0	V210 A		GCC		629
633:C/T	211	0	6,06	0	0	0	0	0	0	0	S211 silent	TCC	TCT		633
635:A/G	211,6666667	0	0	0	4,28	0	0	0	0	0	K212 R	AAG	AGG		635
639:G/A	213	0	0	2,21	0	0,2	0,27	0	0	0	G 213 silent	GGG	GGA		639
645:G/A	215	0	0	61,23	0	99,83	0	0	0	0	R215 silent	AGG	AGA		645
646:T/C	215,3333333	0	0	100	85,23	0	0	0	70,51	0	L216 silent	TTG	CTG		646
649:C/T	216,3333333	0	0	97,99	0	0	0	0	0	0			TTG		649
651:G/A	217	0	0	0	0,4	0	2,54	0	0	0	L217 silent	CTG	CTA		651
651:G/T	217	0	0	0	0	0	3,02	0	0	0			CTT		651
654:G/A	1	100	0	0	0	0	30,8	0	0	0	A1 silent	GCG	GCA		654
657:C/T	2	0	0	0	12,45	0	0,26	0,62	0	0	P2 silent	CCC	CCT		657
660:C/T	3	0	0	0	0,17	0	0	41,31	0,49	0	I3 silent	ATC	ATT		660
663:G/A	4	0	0	0	0	0	0	0	97,58	0	T4 silent	ACG	ACA		663
663:G/C	4	0	0	0	0	100	0	0	0	0			ACC		663
666:G/A	5	0	0	0	85,21	0	0	0	0	0	A5 silent	GCG	GCA		666
669:C/T	6	0	0	0	1,23	0	99,82	0,17	0	0	Y6 silent	TAC	TAT		669
672:C/T	7	0	0	0	0	0	0	0	31,03	0	A7 silent	GCC	GCT		672
675:G/A	8	0	0	0	0	0	3,29	0	0	0	Q8 silent	CAG	CAA		675
681:G/A	10	100	0	99,71	5,83	99,59	100	0	97,21	0	T10 silent	ACG	ACA		681
682:A/C	10,33333333	0	0	0	0	67,5	0	0	0	0	R11 silent	AGA	CGA		682
684:A/G	11	100	100	99,85	99,72	99,9	99,64	99,45	100	0			AGG		684
690:C/T	13	0	0	0	0	0	0	0	47,17	0	L13 silent	CTC	CTT		690
691:C/T	13,33333333	0	0	0,35	8,43	0,34	98,46	0	85,12	0	L14 silent	CTA	TTA		691
693:A/G	14	0,16	0	0	100	0,34	99,59	0,86	100	0			CTG		693
696:G/A	15	3,27	0	0	6,48	3,13	99,77	0,64	98,69	0	G15 silent	GGG	GGA		696
699:T/C	16	99,86	99,78	100	99,75	0,27	100	100	99,58	0	C16 silent	TGT	TGC		699
703:A/G	17,33333333	0	0	0	0	3,13	0	0	0,34	0	I18V	ATC	GTC		703
705:C/T	18	0	0	0	0,34	0	46,25	0	99,58	0	I18 silent		ATT		705
708:C/G	19	0	0	0	0	0	0	0,99	0	0	T19 silent	ACC	ACG		708
711:C/T	20	0	0	99,62	0	0	0	0	0	0	S20 silent	AGC	AGT		711
712:C/T	20,33333333	0	0	1,81	0,45	0	0	0	0	0			TTG		712
714:G/A	21	93,21	0	0	0	35,01	0	99,76	0	0	L21 silent	CTG	CTA		714
714:G/T	21	0	0	0	0	0	98,95	0	0	0			CTT		714
717:T/A	22	0	0	0	0	0	0	0	99,76	0	T22 silent	ACT	ACA		717
717:T/C	22	0	8,24	0	0,17	0	99,71	100	0	0			ACC		717
720:C/T	23	0	0	32,26	0	0	0	0	0,04	0	G23 silent	GGC	GGT		720
721:C/A	23,33333333	0	0	64,13	0	0	0	0	0	0			AGG		721
723:G/A	24	0	92,02	99,6	84,15	0	0	0	0	0	R24 silent	CGG	CGA		723
723:G/C	24	0	0	0	15,27	0	0	0	0	0			CGC		723
726:C/T	25	0	0	0	0	0	0	13,33	0	0	D25 silent	GAC	GAT		726
728:A/G	25,66666667	0	0	0,13	0	22,51	0	0	0	0	K26R	AAA	AGA		728
729:A/G	26	0,47	100	32,57	0,94	0	0	99,72	99,66	0	K26 silent		AAG		729
732:C/T	27	0	0,44	0	0	0	0	0	0	0	N27 silent	AAC	AAT		732
735:A/G	28	0	0	0	0	99,83	0	0	99,48	0	Q28 silent	CAA	CAG		735
737:T/C	28,66666667	100	0	0	0	0	0	0	0	0	V29A	GTG	GCG		737
738:G/A	29	0	0	65,16	0	0	0	0	0	0	V29 silent		GTA		738
739:G/-	29,33333333	0	0	0,57	0	0,17	0	0	0,34	0	E30 delete	GAG	AG		739
744:T/A	31	0	0	0	0	2,12	0	0	0	0	G31 silent	GGT	GGA		744
744:T/C	31	100	100	0	0,37	0	0	0	1,18	0			GGC		744
747:G/A	32	0	0	0	0	0,42	0	0	0	0	E32 silent	GAG	GAA		747
750:C/A	33	0	1,24	0	0	0	0	16,01	0,04	0	V33 silent	GTC	GTA		750
750:C/T	33	0	4,2	0	1,03	0	0	0,14	99,52	0			GTT		750
753:G/A	34	0	30,45	0	0	0	0	0	0,04	0	Q34 silent	CAG	CAA		753
756:C/T	35	100	80,8	100	99,83	77,44	99,81	99,88	99,76	0	I35 silent	ATC	ATT		756
759:G/A	36	99,01	0	0	0	0	0	0	98,64	0	V36 silent	GTG	GTA		759
765:T/C	38	0	0	0	0	96,34	0,29	0	29,84	0	T38 silent	ACT	ACC		765
768:T/C	39	0	0	3,4	0,86	0	67,43	0	0	0	A39 silent	GCT	GCC		768
769:A/G	39,33333333	100	100	100	100	100	38,48	100	100	0	T40A	ACC	GCC		769
773:A/G	40,66666667	0	0	0	0	0	0	0	0,04	100	Q41R	CAA	CGA		773
774:A/G	41	94,15	0	0	0	0	99,6	99,83	100	0	Q41 silent		CAG		774
777:C/T	42	0	2,28	98,25	98,35	97,39	0	0	0	0	T42 silent	ACC	ACT		777
780:C/T	43	0	2,98	0	0,15	0	0	0	0,15	0	F43 silent	TTC	TTT		780
781:C/T	43,33333333	99,66	1,18	0	0	0	0,51	0	0	0	L44 silent	CTG	TTG		781
786:A/C	45	1,15	0	0	0	0	0	6,59	0	0			GCC		786
786:A/G	45	0	0	97,54	0	0,61	0	0	0	0	A45 silent	GCA	GCG		786
786:A/T	45	98,85	0	0	0	0	0	93,41	0	0			GCT		786
789:G/A	46	1,4	0,28	0	0,6	0	0	0	0	0			ACA		789
789:G/C	46	0	0	0	0	0	99,45	0	96,95	0	T46 silent	ACG	ACC		789
789:G/T	46	0	0	0	0	0	0,55	0	2,91	0			ACT		789
792:C/T	47	0	0	0	0	0,34	0	0	0	0	C47 silent	TGC	TGT		792
795:C/T	48	0	61,33	0	0	0	0	0	0	0	I48 silent	ATC	ATT		795
798:T/C	49	0	99,9	0	0	0	3,97	0	98,91	0	N49 silent	AAT	AAC		798
801:G/A	50	0	0	0	0,18	0	0	62,45	96,73	0	G50 silent	GGG	GGA		801
804:A/G	51	100	0	0	100	0	97,58	99,84	100	0	V51 silent	GTA	GTG		804
805:T/C	51,33333333	100	0	0	0	0	0	0	0	0	C52L		CTC		805

806:G/T	51,66666667	100	0	0	0	0	0	0	0	0	C52L	TGC	CTC		806
807:C/T	52	0	0	0	0	0,92	0,51	0	0	0	C52 silent		TGT		807
811:A/G	53,33333333	99,97	0	0	0	0	0	0	0	0	T54A	ACT	GCT		811
813:T/C	54	0	0	99,73	0	99,08	26,67	5,77	0,63	0	T54 silent		ACC		813
816:C/T	55	5,2	0	0	0	0,93	0,4	0	0	0	V55 silent	GTC	GTT		816
822:C/T	57	99,63	14,78	3,51	0	0,73	97,35	0,13	67,41	0	H57 silent	CAC	CAT		822
825:G/A	58	0,13	0	0	100	0	0	0	0	0	G58 silent	GGG	GGA		825
828:C/T	59	0	97,08	99,12	100	0,75	66,49	0	2,84	0	A59 silent	GCC	GCT		828
831:A/G	60	0	0	0	0	0	0	1,68	0	0	G60 silent	GGA	GGG		831
832:A/T	60,33333333	100	0	0	0	0	0	0	0	0	T61S	ACG	TCG		832
834:G/A	61	0	0	0	0	0	0	0	99,67	0	T61 silent		ACA		834
835:A/C	61,33333333	0	1,4	30,7	0	0	0	0	0	0	R62 silent	AGG	CGG		835
840:C/T	63	0	0	0	0,31	0	0	99,87	0	0	T63 silent	ACC	ACT		840
841:A/C	63,33333333	99,77	0	0	0	0	0	0	100	0	I64L		CTC		841
843:C/A	64	100	0	0	0	0	0	0	0	0	I64 silent	ATC	ATA		843
843:C/T	64	0	2,54	0,3	0	0	1,05	0,87	0	0			ATT		843
846:A/G	65	0	3,86	0	0	100	0	99,63	100	0	A65 silent	GCA	GCG		846
849:A/G	66	0	1,24	0	0,31	0	0	0	0	0	S66 silent	TCA	TCG		849
850:C/T	66,33333333	100	0	0	0	0	0	0	0	0	P67S	CCC	TCC		850
852:C/T	67	0	0	1,41	3,28	0,29	0,9	0	0	0	P67 silent		CCT		852
855:G/A	68	0	100	2,01	0	0	0	0	0	0	K68 silent	AAG	AAA		855
858:T/C	69	0	0	100	0,38	0,17	0	0,94	0	0	G69 silent	GGT	GGC		858
861:T/C	70	0	94,82	0	0	0	99,04	0	0	0	P70 silent	CCT	CCC		861
864:C/T	71	98,41	1,24	0	99,65	98,39	98,82	83,74	93,13	0	V71 silent	GTC	GTT		864
870:G/A	73	0	0	100	0	0	0	0	0	0	Q73 silent	CAG	CAA		870
876:T/C	75	0,32	9,32	1,05	99,74	100	0	36,45	2,28	100	Y75 silent	TAT	TAC		876
882:T/C	77	0	0	0	0	94,77	2,97	0	0,39	0	N77 silent	AAT	AAC		882
885:G/A	78	99,31	0	0	0	0	99,85	99,47	100	0	V78 silent	GTG	GTA		885
889:C/A	79,33333333	100	100	100	100	100	0	100	0	0	Q80K		AAA	AGG -> R (all 3)	889
890:A/G	79,66666667	0	0	0	15,56	0	0	0,46	0	0	Q80R	CAA	CGA	AAG -> K (1 and 3)	890
891:A/G	80	3,78	100	100	100	1,1	0	0,66	0	0	Q80 silent		CAG		891
894:C/T	81	0	0	0	0	0	0	1,43	0	0	D81 silent	GAC	GAT		894
897:T/C	82	0	0	0	100	4,38	100	0,12	95,7	0	L82 silent	CTT	CTC		897
898:G/A	82,33333333	0	0	0	0	0	0	0	100	0	V83M	GTG	ATG	ATA -> I (V83I)	898
900:G/A	83	0	0	0	0	65,1	68,8	0,61	100	0	V83 silent		GTA		900
903:C/T	84	0	1,06	30,6	0	6,14	0	62,57	0	0	G84 silent	GGC	GGT		903
907:C/G	85,33333333	100	0	0	0	0	0	0	0	0	P86A		GCC		907
907:C/T	85,33333333	0	0	1,05	0	0	0	0	0	0	P86S	CCC	TCC		907
909:C/T	86	0	0	0	0,17	34,63	0	0	0	0	P86 silent		CCT		909
911:C/-	86,66666667	0	0	0	0	0	0	0	0,33	0		GCT	G-C		911
912:T/C	87	0	0	0	0,35	0	0	0	0	0	A87 silent		GCC		912
913:C/T	87,33333333	0	0	0	0	0	0	0	0,52	0	P88S	CCT	TCT		913
915:T/C	88	0,16	17,22	0	0	0	100	0,18	98,95	0	P88 silent		CCC		915
917-920:AAGG/---	#WERT!	0	0	0	0	0	0	0	0,52	0					917-920:AAGG/---
917:A/C	88,66666667	100	0	0	0	0	0	0	0	0	Q89P	CAA	CCA		917
918:A/G	89	0	99,38	0	0	0	0	99,56	69,86	0	Q89 silent		CAG		918
921:T/A	90	0	0	0	2,59	2,34	0	0	0	0	G90 silent	GGT	GGA		921
921:T/C	90	100	0	0	0,68	6,58	0,4	0	0	0			GGC		921
922:T/A	90,33333333	100	0	0	0	99,69	0	0	0	0	S91T		ACC		922
922:T/G	90,33333333	0	0	0	0	0,1	100	0	100	0	S91A		GCC		922
923:C/G	90,66666667	0	0	0	0	5,79	0	0	0	0	S91C	TCC	TGC		923
924:C/A	91	0	0	0	0	0	0	0	2,36	0	S91 silent		TCA	AGC -> S (1 and 2)	924
924:C/T	91	0	16,94	0	0,14	0	0	3,09	61,51	0			TCT		924
927:C/T	92	0	0	0	99,52	0	0	0	0	0	R92 silent	CGC	CGT		927
930:A/G	93	0,18	0	0	99,86	0	0	0,83	0	0	S93 silent	TCA	TCG		930
931:T/C	93,33333333	55,93	0	0	2,91	3,65	3,01	37,59	94,55	0	L94 silent	TTG	CTG		931
933:G/A	94	0	0	98,72	0	0	0	0	100	0	L94 silent		TTA		933
934:A/G	94,33333333	0	1,04	0	0	0	0	0,53	0	0	T95A		GCA		934
935:C/T	94,66666667	0	0	0	0	0	0	0	96,15	0	T95I	ACA	ATA		935
936:A/G	95	100	0	0	0	0	0	0,46	100	0	T95 silent		ACG	ATG -> M (2 and 3)	936
939:C/A	96	0	0	0	0	99,58	0	0	0	0			CCA		939
939:C/G	96	0	0	0	0	0,42	0	0	0	0	P96 silent	CCC	CCG		939
939:C/T	96	0	0	0	0	0	0	0,55	1,99	0			CCT		939
942:C/T	97	0	0	0	0	0	0	0	0	100	C97 silent	TGC	TGT		942
945:C/G	98	0	0	0	0	0	0,4	0	0	0	T98 silent	ACC	ACG		945
945:C/T	98	0	0	2,92	100	0	66,98	0	0	0			ACT		945
948:C/T	99	97,53	99,74	0	7,39	0	0	0	0	0	C99 silent	TGC	TGT		948
951:C/T	100	0	75,96	0	0	0	0	0	0	0	G100 silent	GGC	GGT		951
952:T/A	100,3333333	0	0	0	1,68	0	0	0	0	0	S101T	TCC	ACC		952
954:C/T	101	0	0	2,52	0	0	0	0	50,56	0	S101 silent		TCT		954
957:G/A	102	99,72	0	0	0	0	99,6	0	0,61	0			TCA		957
957:G/C	102	0	0	0	97,12	0	0	0	0	0	S102 silent	TCG	TCC		957
957:G/T	102	0	0	0	0,28	0	0	0	0	0			TCT		957

NS2
NS3

genotype 1a | genotype 1b

Q80

plasma sequence different from reference sequence

clinically relevant mutation

mutation

silent mutation

actual mutation

List of variants third deep sequencing run

reference sequence		Re.1a.US.77.H77												
genotype		genotype 1a			genotype 1b									
Number of reads		6385	7477	5359	4300	5750	3848	4455	3759	5514				
Variant	aminoacid position in NS5b	A42-10 %	A57-10 %	A85-03 %	A76-05 %	B35-04 %	B37-03 %	B57-01 %	C06-01 %	WT control (IT H77 RNA from Selena) combined %	variant	original codon	new codon	Variant
678.5:-/T		0	0	0	0	0	0	0	100	0				678.5:-/T
691-693:AGC/GAG		0	0	0	0	0	0	0	0	0	S231E		GAG	691-693:AGC/GAG
691-693:AGC/GAT		0	0	0	0	0	0	0	0	0	S231D		GAT	691-693:AGC/GAT
691-693:AGC/GCG		0	0	0	0	0	0	0	0	0	S231A		GCG	691-693:AGC/GCG
691-693:AGC/GCT		0	0	0	0	0	0	0	0	0			GCT	691-693:AGC/GCT
691:A/G	230,333333	0	0	0	0	0	95,71	0	0	0	S231G	AGC	GGC	691
692:G/A	230,666667	0,55	0	0	0,33	0	2,88	0,29	99,57	0	S231N		AAC	692
692:G/C	230,666667	0	0	0	0	0	5,38	0	0	0	S231T		ACC	692
693:C/G	231	0	0	0	0	0	0	0	99,63	0	S231R		AGG	693
693:C/T	231	0	0	14,54	0,63	0	2,88	0,29	0	0	S231 silent		AGT	693
696:C/T	232	1,03	2,65	0	0,58	99,81	96,91	99,71	1,17	0	D232 silent	GAC	GAT	696
703-705:ACG/GTT		0	0	0	0	0	2,88	0,16	0	0	T235V		GTT	703-705:ACG/GTT
703:A/G	234,333333	0	0	0	0	0	2,88	0,16	0	0	T235A	ACG	GCG	703
704:C/T	234,666667	0	0	0	0	0	2,88	0,29	100	0	T235M		ATG	704
705:G/T	235	0	0	0	0	0	2,88	0,29	0	0	T235 silent		ACT	705
712:G/A	237,333333	0	0	0	8,65	0	0	0	0	0	A238T	GCA	ACA	712
712:G/T	237,333333	0	0	0	0	0	2,88	0,29	0	0	A238S		TCA	712
717:T/C	239	99,22	97,87	100	100	99,84	100	0,29	97,69	0	I239 silent	ATT	ATC	717
723:A/G	241	0	0	98,25	100	38,71	0,21	99,19	0	0	Q241 silent	CAA	CAG	723
733:C/T	244,333333	0,7	0,47	100	0,4	0	2,88	0,29	0	0	L245 silent	CTG	TTG	733
737:A/C	245,666667	0	0	0	0	0	2,88	0,16	0	0	D246A	GAC	GCC	737
741:C/T	247	0	99,09	0	0	0	0	0	99,52	0	P247 silent	CCC	CCT	741
742-744:CAA/GNG		0	0	0	0	0	0	0	0	0			GAG	742-744:CAA/GNG
742:C/G	247,333333	0	0	100	0	0	2,88	0,29	0	0	Q248E	CAA	GAA	742
744:A/G	248	99,08	99,77	0	0,12	0,19	97,12	0	100	0	Q248 silent		CAG	744
745.5:-/G		0,49	0	0	0	0	0	0	0	0				745.5:-/G
748-750:CGC/ANA		0	0	0	0	0	2,88	0,29	0	0	R250 silent		AGA	748-750:CGC/ANA
748-750:CGC/ANT		0	0	0	0	0	0	0	0	0			AGT	748-750:CGC/ANT
748:C/A	249,333333	0	0	0	0	0	2,88	0,29	0	0	R250S	CGC	AGC	748
750:C/A	250	0	0	0	0,12	0,19	3,3	0,29	0	0			CGA	750
750:C/T	250	1,05	8,88	0	0,72	0,75	0,6	2,9	0,85	0	R250 silent		CGT	750
751-752:GT/CA		0	0	0	0	0	2,88	0,16	0	0	V251Q		CAG	751-752:GT/CA
751:G/C	250,333333	0	0	0	0	0	2,88	0,16	0	0	V251L	GTG	CTG	751
752:T/A	250,666667	0	0	0	0	0	2,88	0,29	0	0	V251E		GAG	752
755-756:CC/TT		0	0	0	0	0	0	0	0	0			GTT	755-756:CC/TT
755:C/T	251,666667	0	0	0	0,93	0	0	0	0	0	A252V	GCC	GTC	755
756:C/T	252	0	0	0,11	0	0	0	27,03	0	0	A252 silent		GCT	756
759:C/A	253	0	0	0	0	0	2,88	0,29	0	0			ATA	759
759:C/T	253	0,56	0	0	0,12	0,5	0,42	0,72	0,4	0	I253 silent	ATC	ATT	759
761-762:AG/CA		0	0	0	0	0	0	0	100	0	K254T		ACA	761-762:AG/CA
761-762:AG/GA		0	0	0	0	0	0	0	0	0	K254R		AGA	761-762:AG/GA
761:A/C	253,666667	0	0	0	0	0	0	0	100	0	K254Q	AAG	CAG	761
761:A/G	253,666667	0	0	0	0	0	2,88	0,16	0	0	K254R		AGG	761
762:G/A	254	0	0	0	0	0	0	0	100	0	K254 silent		AAA	762
765:C/A	255	0	0	0	0	60,05	0	0	0	0			TCA	765
765:C/G	255	0	0	0	0	0,24	2,88	0,29	0	0	S255 silent	TCC	TCG	765
765:C/T	255	0	1,2	100	99,88	0	0	0	0	0			TCG	765
768:C/T	256	0	0	0	70,19	0	0	0	0	0	L256 silent	CTC	CTT	768
771:T/A	257	0	0	0	0,35	0	2,88	0,29	0	0	T257 silent	ACT	ACA	771
771:T/C	257	0,41	0	99,76	98,28	99,5	96,96	98,97	0,51	0			ACC	771
775:A/C	258,333333	0	0	0	0	0	2,88	0,16	0	0	R259 silent	AGG	CGG	775
780:T/G	260	0	1,06	0	0	0	0	0	0	0	L260 silent	CTT	CTG	780
783:T/C	261	0	0,47	0,9	0	0	2,88	0,16	0	0	Y261 silent	TAT	TAC	783
784-786:GTT/ANC		0	0	0	0	0	2,88	0,29	0	0	V262I	GTT	ATC	784-786:GTT/ANC
784:G/A	261,333333	0	0	0	0	0	2,88	0,29	0	0			ATT	784
786:T/C	262	0	0	90,71	98,65	98,9	4,08	92,62	0,21	0	V261 silent		GTC	786
792:C/T	264	0	0	0	0	0,42	0	0	100	0	G264 silent	GGC	GGT	792
795:T/C	265	0,53	0	0	0	0	2,88	0,16	1,04	0	P265 silent	CCT	CCC	795
798:T/C	266	0,78	0	0	0,28	0	0	99,42	0	0	L266 silent	CTT	CTC	798
798:T/G	266	0	0	0	0	0	2,88	0,16	0	0			CTG	798
801:C/T	267	0	0	0	0,16	0	2,88	0,29	0	0	T267 silent	ACC	ACT	801
804:T/C	268	0,47	0	6,1	0	0	0	68,91	0	0	N268 silent	AAT	AAC	804
807:A/T	269	0	0	0	0	0	2,88	0,16	0	0	S269 silent	TCA	TCT	807
808.5:-/A		1,61	0	0	0	0	0,21	0,85	0	0				808.5:-/A
809-810:GG/AA		0,56	0	0	0	0	2,88	0,29	0	0	R270K	AGG	AAA	809-810:GG/AA
809:G/A	269,666667	92,34	0	0	0,12	96,07	3,3	57,62	0	0			AAG	809
810:G/A	270	1,11	0	0	0	0	2,88	0,29	0,69	0	R270 silent		AGA	810
814-816:GAA/CNG		0	0	0	0	0	2,88	0,16	0	0	E272Q		CAG	814-816:GAA/CNG
814:G/C	271,333333	0	0	0	0	0	2,88	0,16	0	0		GAA	CAA	814
816:A/G	272	99,3	87,87	0	0,67	3,9	100	0,29	99,49	0	E272 silent		GAG	816
822:C/T	274	0,27	0	0	99,77	2,1	0	0	0	0	C274 silent	TGC	TGT	822
828:C/T	276	1,19	100	99,89	100	67,86	3,3	99,12	75,92	0	Y276 silent	TAC	TAT	828
832-834:AGG/CNA		0	0	0	0	0	0	0	0	0			CGA	832-834:AGG/CNA
832:A/C	277,333333	0	0	0	0	0	2,88	0,16	0	0	R278 silent	AGG	CGG	832
834:G/A	278	0,42	0	4,72	0	0,78	96,73	99,57	1,46	0			AGA	834
840:C/T	280	0	99,63	0	0	0	0	0	0	0	R280 silent	CGC	CGT	840
843:G/A	281	0,33	0,41	0,9	99,12	0	0	0,38	82,42	0	A281 silent	GCG	GCA	843
849:C/T	283	0	0	0	0	0	2,88	0,29	0	0	G283 silent	GGC	GGT	849
852:A/G	284	0	0	0	0,42	0,78	0	0,13	0,29	0	V284 silent	GTA	GTG	852
858:A/G	286	0,39	87,19	0,65	0,26	100	3,09	100	0	0	T286 silent	ACA	ACG	858
861:T/C	287	0	0	13,25	0	0	3,01	0,16	0	0	T287 silent	ACT	ACC	861
867:T/C	289	0	0	100	99,88	0	2,88	0,61	0,51	0	C289 silent	TGT	TGC	867
870:T/A	290	0	0	98,77	0	0	0	0	0	0			GGA	870
870:T/C	290	0	0,55	0	99,56	0,35	0,36	0	0	0	G290 silent	GGT	GGC	870
870:T/G	290	0	0	1,23	0	0	0	0	0	0			GGG	870
873:C/T	291	0,89	0	0,26	0,49	0	3,04	0,29	0	0	N291 silent	AAC	AAT	873
882:T/A	294	0	0	0	0	0	2,88	0,29	0,13	0	T294 silent	ACT	ACA	882
882:T/C	294	0	0	0	0,28	0	0	0	99,6	0			ACC	882
885:C/T	295	0	0	0	0	0	3,17	0,29	0	0	C295 silent	TGC	TGT	885
888:C/T	296	0	0	0	0	0,78	0	0	0	0	Y296 silent	TAC	TAT	888
889-891:ATC/TNG		0	0	0	0	0	2,88	0,16	0	0	I297L		TTG	889-891:ATC/TNG
889-891:ATC/TNT		0	0	0	0	0	0	0	0	0	I297F		TTT	889-891:ATC/TNT
889:A/T	296,333333	0	0	0	0	0	2,88	0,16	0	0		ATC	TTC	889
891:C/G	297	0	0	0	0	0	2,88	0,29	0	0	I297M		ATG	891
891:C/T	297	0	0	94,51	0,44	0	0,34	0	0	0	I297 silent		ATT	891
894:G/A	298	95,58	0	0	0	0	0	0	0	0	K298 silent	AAG	AAA	894
897:C/T	299	0,31	0	0	0	0	0	0	0	0	A299 silent	GCC	GCT	897
898-900:CGG/AAA		99,66	0	0	0	0	0	0	99,63	0	R300K		AAA	898-900:CGG/AAA
898-900:CGG/AAC		0	0	0	0	0	0	0	0	0	R300N		AAC	898-900:CGG/AAC
898-900:CGG/ACC		0	0	0	0	0	0	0	0	0	R300T		ACC	898-900:CGG/ACC
898-900:CGG/GAA		0	0	0	0	0	0	0	0	0	R300E		GAA	898-900:CGG/GAA
898-900:CGG/GCA		0	0	0	0	0	0	0	0	0	R300A		GCA	898-900:CGG/GCA
898-900:CGG/GCC		0	0	0	0	0	0	0	0	0			GCC	898-900:CGG/GCC
898:C/A	299,333333	100	0	0	0	0	0	0	100	0	R300 silent	CGG	AGG	898
898:C/G	299,333333	0	0	0	0	0	2,88	0,29	0	0	R300G		GGG	898
899:G/A	299,666667	100	99,13	100	99,88	99,6	96,91	0	100	0	R300Q		CAG	899

899:G/C	299,666667	0	0	0	0	0	2,88	0,16	0	0	R300P		CCG		899
900:G/A	300	99,66	100	100	100	99,79	90,93	97,28	99,63	0			CGA		900
900:G/C	300	0	0	0	0	0	0	2,56	0	0	R300 silent		CGC		900
900:G/T	300	0	0	0	0	0	2,88	0,16	0	0			CGT		900
903:A/G	301	0,64	0	0	0	0	3,01	0,16	0	0	A301 silent	GCA	GCG		903
915:C/T	305	0	0	0	0	0	2,88	0,29	0	0	A305 silent	GCC	GCT		915
916-918:GCA/TNG		0	0	0	0	0	0	0	0	0	A306S		TCG	916-918:GCA/TNG	
916:G/T	305,333333	0	0	31,01	0	0	0	0	0	0		GCA	TCA		916
918:A/G	306	97,7	0	0	0,35	0	2,88	0,16	23,33	0	A306 silent		GCG		918
919-921:GGG/AAA		0	0	0	0	0	0	0	0	0	G307K		AAA	919-921:GGG/AAA	
919:G/A	306,333333	0	0	0	0	0	2,88	0,29	0	0	G307R	GGG	AGG		919
920:G/A	306,666667	0	0	0	0	0	2,88	0,29	0	0	G307E		GAG		920
921:G/A	307	0	0	0	17,16	0	0	0	0	0	G307 silent		GGA		921
926:A/G	308,666667	0	0	100	99,88	39,39	0	98,27	0	0	Q309R	CAG	CGG		926
936:C/G	312	0	0	0	0	0	2,88	0,29	0	0			ACG		936
936:C/T	312	0,36	0	0	0	0	0	0	0	0	T312 silent	ACC	ACT		936
944:T/C	314,666667	0	0	0	0	0	0	0	0,29	0	V315A		GCG		944
945:G/A	315	0	0	0	0	0	2,88	0,29	0	0	V315 silent	GTG	GTA		945
946-948:TGT/AAC		0	0	0	0	0	1,2	0	0	0	C316N		AAC	946-948:TGT/AAC	
946:T/A	315,333333	0	0	0	0	0	1,2	0	0	0	C316S		AGT		946
947:G/A	315,666667	0	0	0	0	0	1,2	0	0	0	C316Y	TGT	TAT		947
948:T/C	316	0,66	0	0	0	0	2,88	0,16	0,88	0	C316 silent		TGC		948
951:C/A	317	0	0	0	0	0	2,88	0,29	0	0	G317 silent	GGC	GGA		951
954:C/T	318	1,06	0	0	0	0	0	0	1,68	0	D318 silent	GAC	GAT		954
958-960:TTA/CNG		0	0	0	0	0	0	0	0	0	L320 silent		CTG	958-960:TTA/CNG	
958-960:TTA/CNT		0	0	0	0	0	2,88	0,16	0	0	L320 silent		CTT	958-960:TTA/CNT	
958:T/C	319,333333	98,89	100	0	0	0	2,88	0,76	0	0	L320 silent	TTA	CTA		958
960:A/G	320	0	0	0	0,84	3,37	6,21	0	0	0	L320 silent		TTG		960
960:A/T	320	0	0	0	0	0	2,88	0,16	0	0	L320F		TTT		960
967-969:ATC/GNT		0	0	0	0	0	0	0	0	0	I323V		GTT	967-969:ATC/GNT	
967:A/G	322,333333	0	0	0	0,49	0	0	0	0	0		ATC	GTC		967
969:C/T	323	0	0,68	0	0	0	0	0,18	0	0	I323 silent		ATT		969
972:T/C	324	0,34	0	0	0	0	0	0	0	0	C324 silent	TGT	TGC		972
975:A/G	325	99,45	100	0	1,21	0,52	0,21	0	99,41	0	E325 silent	GAA	GAG		975
978-980:TGC/CCA		0	0	0,58	0,93	40,8	0	0	0	0				978-980:TGC/CCA	
978:T/C	326	0,89	0,4	0,58	0,93	41,22	3,25	0,16	97,87	0					978
979-980:GC/CA		0	0	100	98,77	99,39	0	99,71	0	0				979-980:GC/CA	
979:G/C	326,333333	0	0	100	98,77	99,39	0	99,71	0	0					979
980:C/A	326,666667	0	0	100	99,88	99,81	0	99,71	0	0					980
981:G/A	327	0	0	1,06	0	0	0	0	0	0					981
984:G/A	328	0	0	99,46	0,12	99,81	0,42	96,18	0	0	G328 silent	GGG	GGA		984
985-987:GTC/ACT		0	0	0	0	0	0	0	0	0	V329T		ACT	985-987:GTC/ACT	
985:G/A	328,333333	0	0	0	0	0	2,88	0,29	0	0	V329I	GTC	ATC		985
986:T/C	328,666667	0	0	0	0	0	2,88	0,16	0	0	V329A		GCC		986
987:C/T	329	0	0	0	0	0	95,53	0	0	0	V329 silent		GTT		987
989-990:AG/CA		0	0	0	0	0	0	0	0	0			CCA	989-990:AG/CA	
989-990:AG/CC		0	0	0	0	0	0	0	0	0	Q330P		CCC	989-990:AG/CC	
989-990:AG/CT		0	0	0	0	0	0	0	0	0			CCT	989-990:AG/CT	
989:A/C	329,666667	0	0	0	0	1,86	0	0	0	0		CAG	CCG		989
990:G/A	330	0	0	0	0	0	2,88	0,29	0	0	Q330 silent		CAA		990
990:G/C	330	0	0	0	0	0	0,57	0	0	0	Q330H		CAC		990
990:G/T	330	0	0	0	0	0	96,34	0	0	0			CAT		990
993:G/A	331	0	0	0,54	0	0	0	0	0	0	E331 silent	GAG	GAA		993
996:C/T	332	0,33	0	0,41	0,81	90,19	0	98,95	0	0	D332 silent	GAC	GAT		996
998-999:CG/AA		0	0	0	0	0	0	0	0	0	A333E		GAA	998-999:CG/AA	
998-999:CG/TA		0	0	0	0	0	0	0	0	0	A333V		GTA	998-999:CG/TA	
998:C/A	332,666667	0	0	0	0	0	2,88	0,29	0	0	A333E	GCG	GAG		998
998:C/T	332,666667	98,65	0	0	0	0	0	0	0	0	A333V		GTG		998
999:G/A	333	0	0	0	98,79	93,74	0	99,21	0	0	A333 silent		GCA		999
1002:G/A	334	0	0	0	0,28	0	0	0	0	0	A334 silent	GCG	GCA		1002
1003:A/G	334,333333	0	0	0	0,28	0	0	0	0	0	S335G	AGC	GGC		1003
1004:G/A	334,666667	0	0	0	0	0	0	0,52	0	0	S335N		AAC		1004
1008:G/A	336	0	0	0	0	0	2,88	0,29	0	0	L336 silent	CTG	CTA		1008
1009-1011:AGA/CNG		0	0	0	0	0	2,88	0,16	0	0			CGG	1009-1011:AGA/CNG	
1009:A/C	336,333333	100	100	100	99,88	0	2,88	99,87	100	0	R337 silent	AGA	CGA		1009
1011:A/G	337	0	0	0	0	0	2,88	0,16	0	0			AGG		1011
1014:C/T	338	0	0	63,33	0	0	0	0	0	0	A338 silent	GCC	GCT		1014
1020:G/A	340	0,33	0	0	0	0	0	0	0	0	T340 silent	ACG	ACA		1020
1026:T/C	342	0,36	0	0	0	0	0	0	0	0	A342 silent	GCT	GCC		1026
1032:C/T	344	0	0	0	0	0	2,88	0,16	0	0	T344 silent	ACC	ACT		1032
1035:G/A	345	0	0	0	0	0,78	3,12	0,16	0	0	R345 silent	AGG	AGA		1035
1038:C/T	346	87,39	0	84,61	0,49	0,31	52,62	98,97	0	0	Y346 silent	TAC	TAT		1038
1041.5:-/C		0,69	0	0,17	0,21	0	0	2,07	0,13	0				1041.5:-/C	
1041:C/T	347	0	0	0	1,4	0	3,04	0,16	0,19	0	S347 silent	TCC	TCT		1041
1042:G/C	347,333333	2,85	0	0	0	0	0	0	0	0	A348P		CCC		1042
1043.5:-/T		0	0	3,41	1,86	0	0	7	0	0				1043.5:-/T	
1043:C/G	347,666667	2,85	0	0	0	0	0	0	0	0	A348G	GCC	GGC		1043
1044.5:-/T		0	0	1,72	2,81	0,5	0	0,4	0	0				1044.5:-/T	
1044:C/T	348	0	0	88,39	92,81	99,17	0	90,98	0	0	A348 silent		GCT		1044
1045:C/T	348,333333	0	0	0,6	2,09	0	0	0,58	0	0	P349S	CCC	TCC		1045
1050.5:-/T		0	2,09	0	0	8,47	0	0,22	0	0				1050.5:-/T	
1050:C/-		87,3	12,48	49,73	41,95	0,24	96,36	1,23	97,63	80,58				1050:C/-	
1050:C/G	350	0	0	0	0	0	0,13	1,28	0	0	P350 silent	CCC	CCG		1050
1050:C/T	350	0,42	82,41	0	0,12	89,51	3,51	12,95	0,45	0			CCT		1050
1056.5:-/G	352,166667	0	0	0	0	0	0	1,55	0	0					1056,5
1056:C/T	352	0	0	12,99	0	0	0	0	0	0	D352 silent	GAC	GAT		1056
1057-1059:CCC/GNG		0	0	0	0	0	0	0	0	0	P353A		GCG	1057-1059:CCC/GNG	
1057-1059:CCC/GNT		0	0	0	0	0	0	0	0	0			GCT	1057-1059:CCC/GNT	
1057:C/G	352,333333	0	0	0	0	0	0	32,35	0	0		CCC	GCC		1057
1059:C/G	353	0	0	0	0	0	2,75	0,16	0	0	P353 silent		CCG		1059
1059:C/T	353	0	0	3,28	0	0	0	0	0,43	0			CCT		1059
1061:C/-		57,04	7,38	0,26	0	0	26,33	0,18	56,66	57,44				1061:C/-	
1062-1063:AC/--		0	0	29,02	34,49	0,12	0	10,71	0	0				1062-1063:AC/--	
1062:A/-	354	0	0	62,38	67,84	56,19	0	46,87	0	0					1062
1062:A/C	354	0	0	30,29	24,07	38,21	5,35	52,3	0	0			CCC		1062
1062:A/G	354	0	0	0	0,33	0,19	0,21	0,13	0	0	P354 silent	CCA	CCG		1062
1062:A/T	354	0	0	0,13	0,23	5,41	0	0,54	0	0			CCT		1062
1063-1064:CA/--		0	0	6,74	6,3	0	0	0	0	0				1063-1064:CA/--	
1063-1065:CAA/ANG		0	0	0	0	0	0	0	0	0	Q355K		AAG	1063-1065:CAA/ANG	
1063:C/-	354,333333	0	0	35,96	41	0,12	1,51	10,86	0	0		CAA			1063
1063:C/A	354,333333	0	0	0	0	0	1,38	0	0	0	Q355K		AAA		1063
1065:A/G	355	0	0	31,39	17,51	0	0	0,36	0	0	Q355 silent		CAG		1065
1068:A/G	356	0	0	0	0,51	0	0	0	0	0	P356 silent	CCA	CCG		1068
1071:A/G	357	99,79	98,65	1,18	0,74	0	0	0,4	90,02	0	E357 silent	GAA	GAG		1071
Var_175		0	0	0	0	0	0	0	0	0				Var_175	
Var_176		0	0	0	0	0	0	0	0	0				Var_176	

genotype 1a | genotype 1b

C316

plasmid sequence different from reference sequence

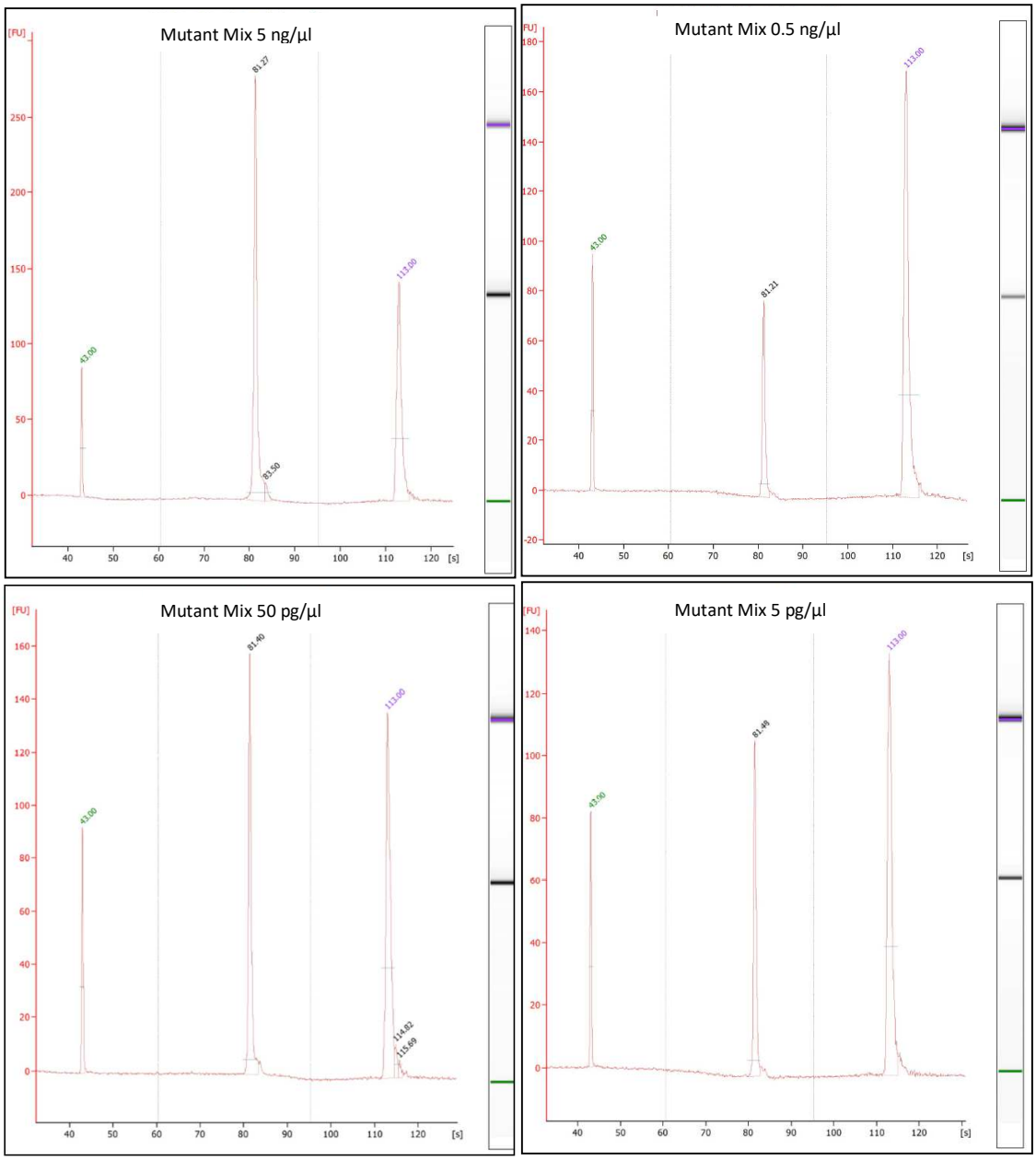
clincally relevant mutations

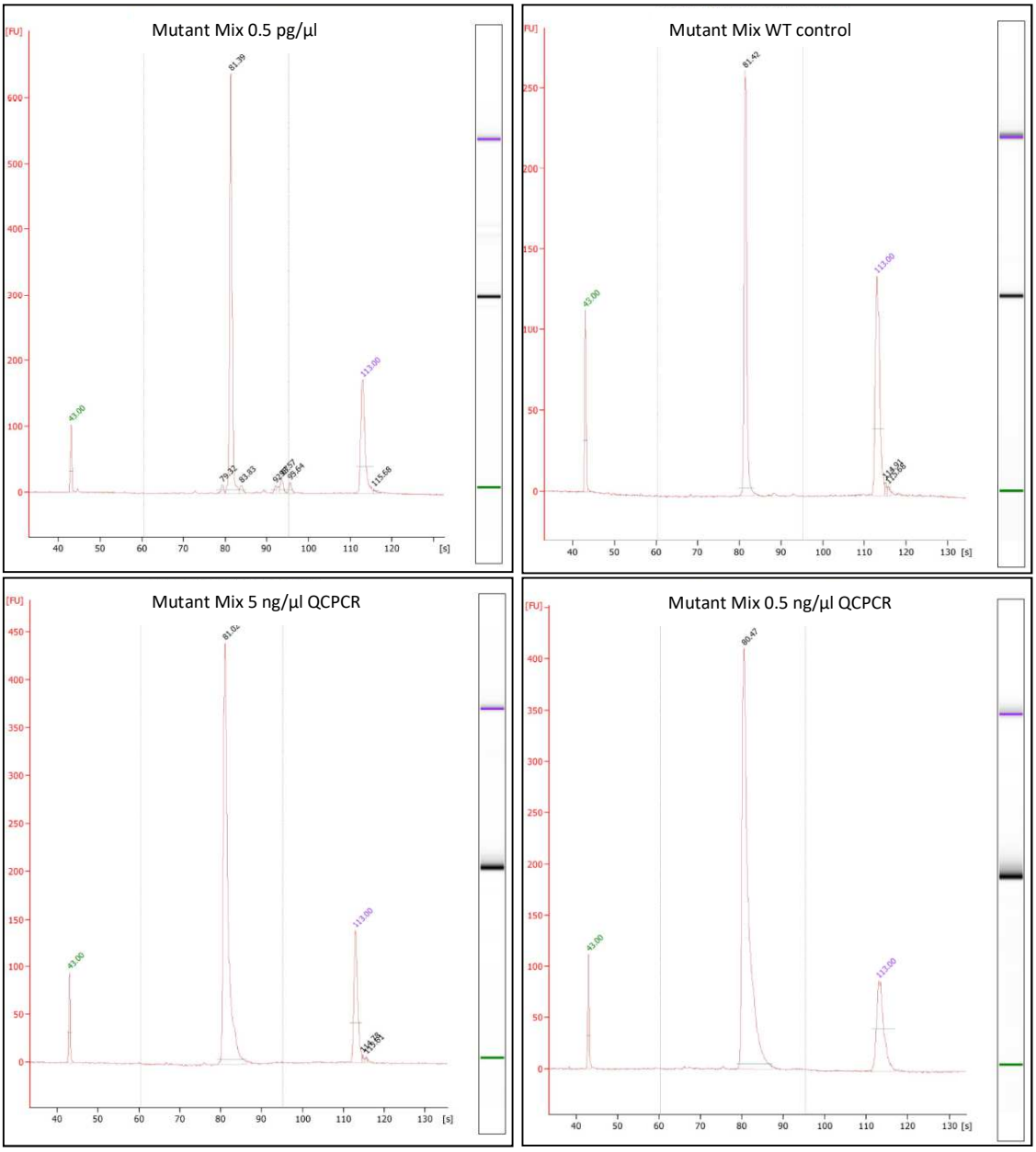
mutation

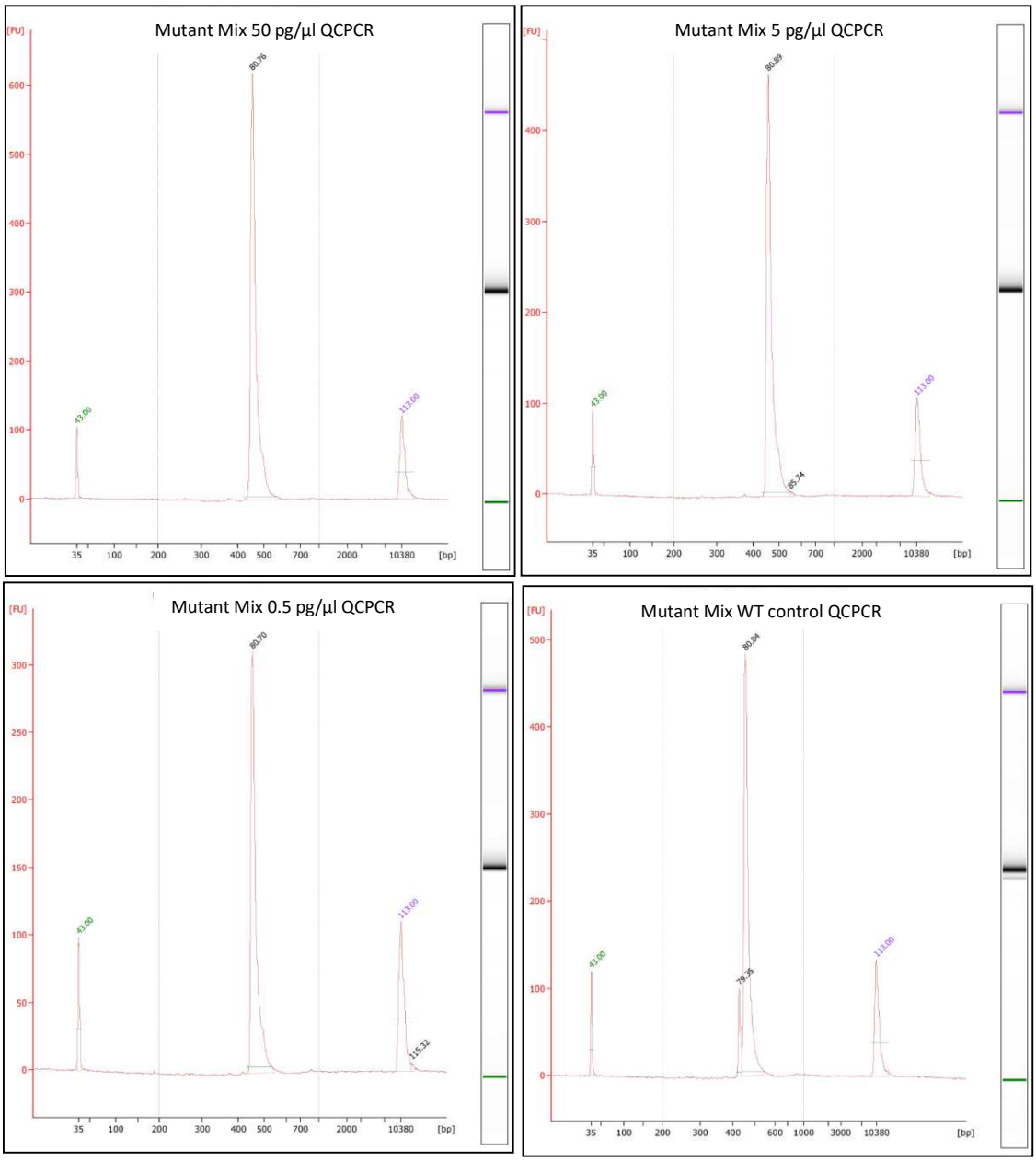
silent mutation

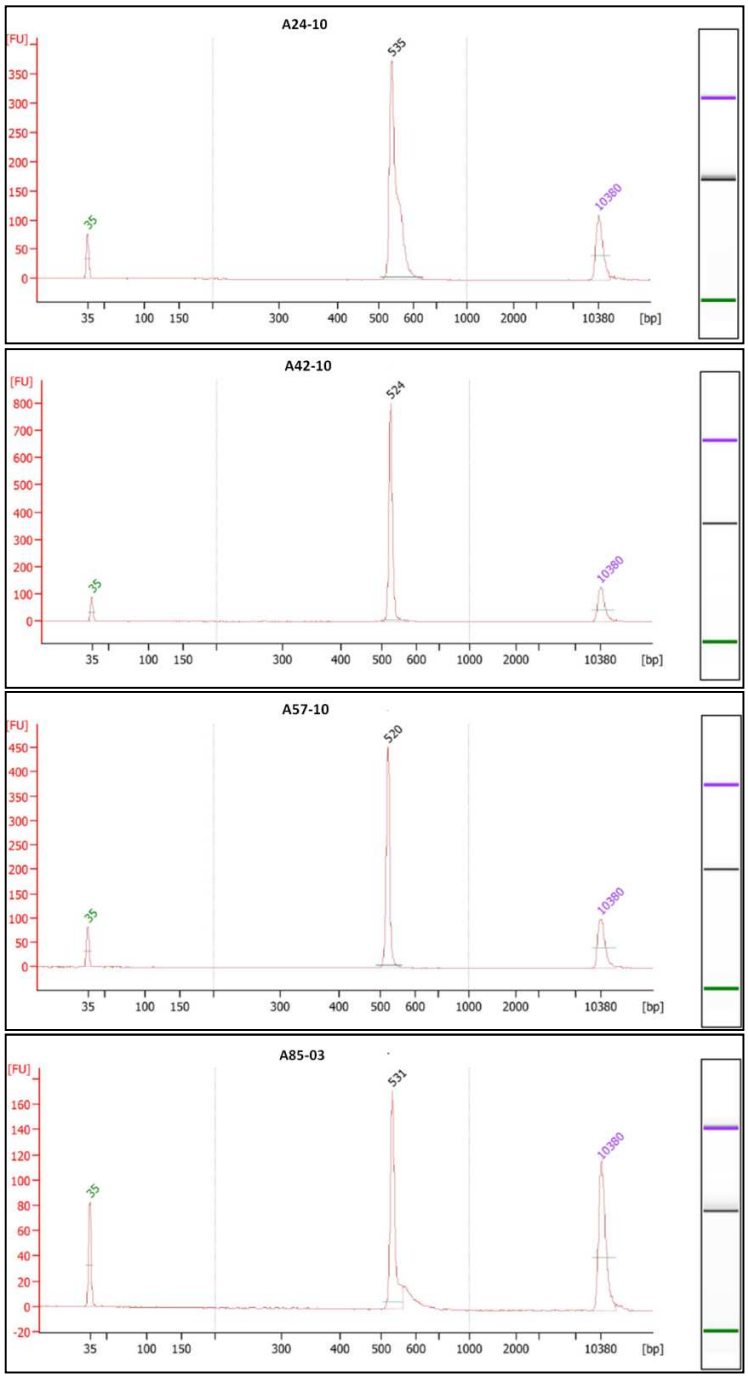
actual mutation

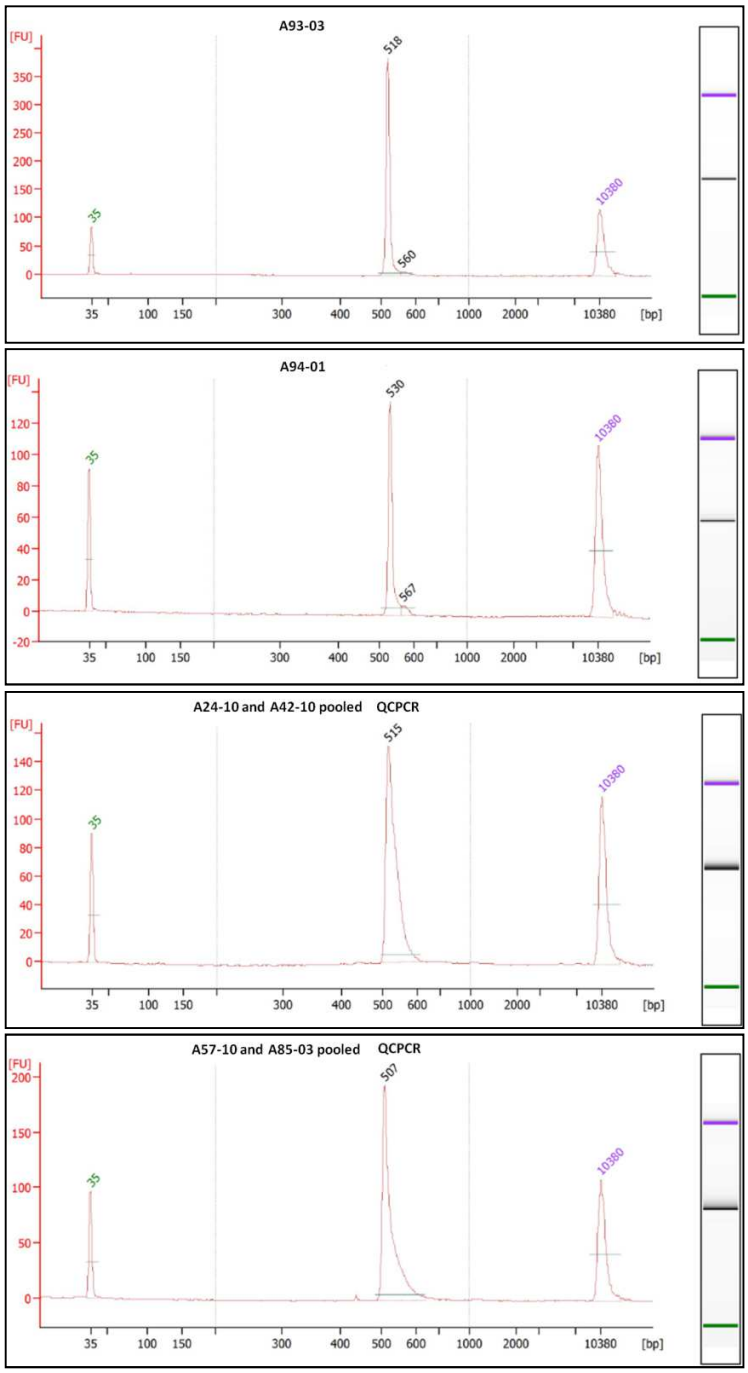
Bioanalyzer electropherograms

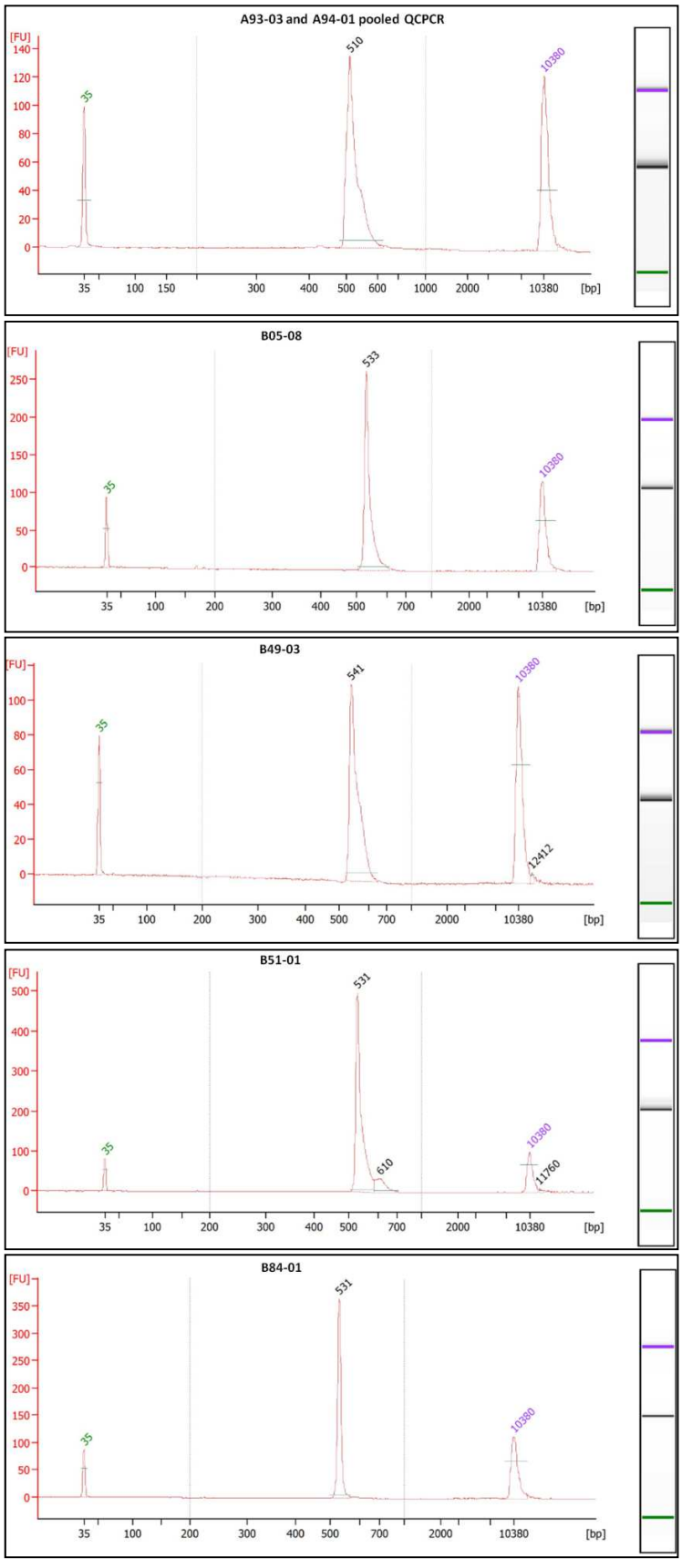


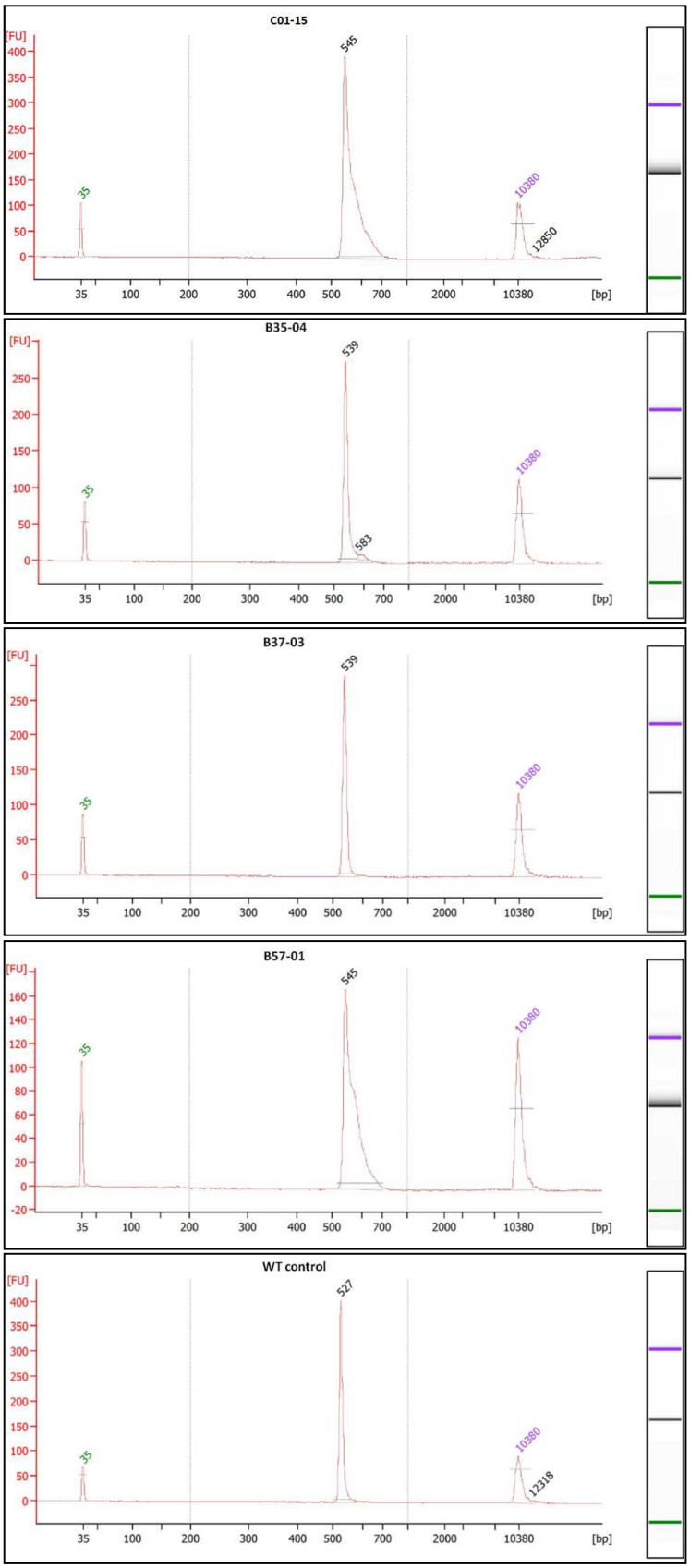

Mutant Mix 0.5 pg/µl
Mutant Mix WT control
Mutant Mix 5 ng/µl QCPCR
Mutant Mix 0.5 ng/µl QCPCR
[FU]
[s]

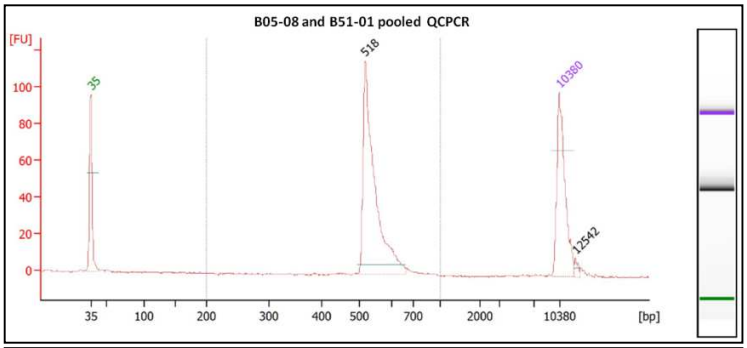

Mutant Mix 50 pg/µl QCPCR
Mutant Mix 5 pg/µl QCPCR
Mutant Mix 0.5 pg/µl QCPCR
Mutant Mix WT control QCPCR
[FU]
[bp]
35 100 200 300 400 500 700 2000 10380
35 100 200 300 400 600 1000 3000 10380

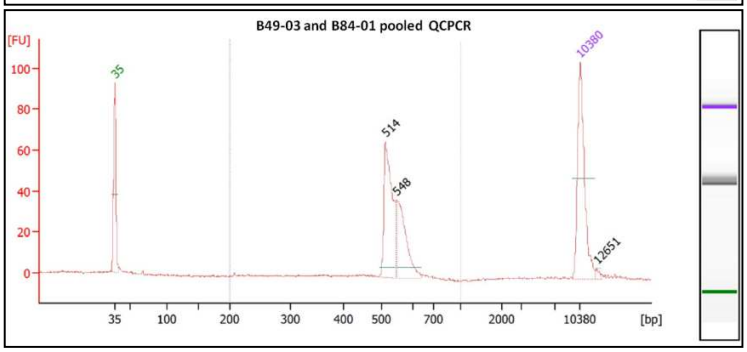

A24-10
[FU]
350
300
250
200
150
100
50
0
535
35
10380
35 100 150 300 400 500 600 1000 2000 10380 [bp]
A42-10
[FU]
800
700
600
500
400
300
200
100
0
524
35
10380
35 100 150 300 400 500 600 1000 2000 10380 [bp]
A57-10
[FU]
450
400
350
300
250
200
150
100
50
0
520
35
10380
35 100 150 300 400 500 600 1000 2000 10380 [bp]
A85-03
[FU]
160
140
120
100
80
60
40
20
0
-20
531
35
10380
35 100 150 300 400 500 600 1000 2000 10380 [bp]

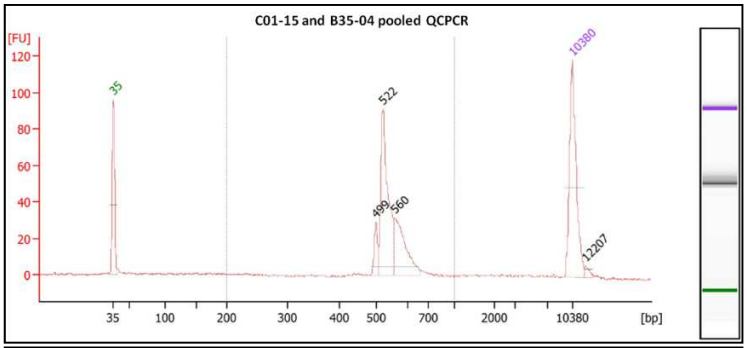

A93-03
[FU]
518
10360
35
560
35 100 150 300 400 500 600 1000 2000 10380 [bp]
A94-01
[FU]
530
10360
35
567
35 100 150 300 400 500 600 1000 2000 10380 [bp]
A24-10 and A42-10 pooled QCPCR
[FU]
515
10360
35
35 100 150 300 400 500 600 1000 2000 10380 [bp]
A57-10 and A85-03 pooled QCPCR
[FU]
507
10360
35
35 100 150 300 400 500 600 1000 2000 10380 [bp]

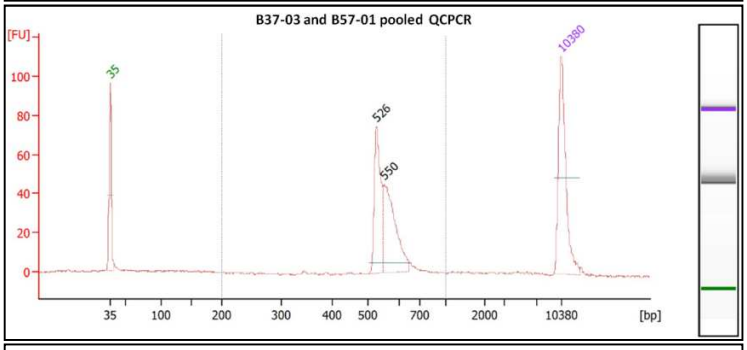

A93-03 and A94-01 pooled QCPCR
[FU]
510
10380
35
[bp]
B05-08
533
B49-03
541
12412
B51-01
531
610
11760
B84-01

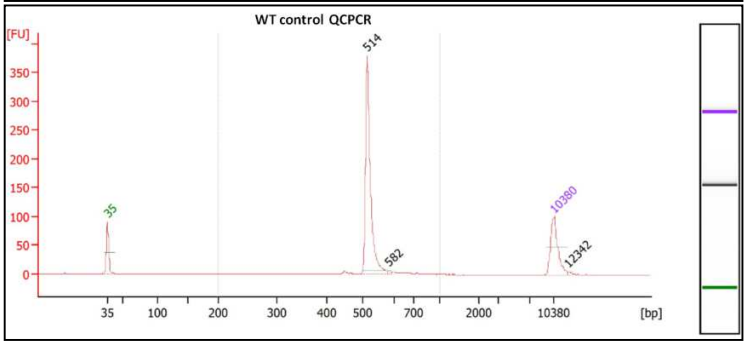

C01-15
[FU]
545
35
10380
12850
[bp]
B35-04
539
583
B37-03
B57-01
WT control
527
12318

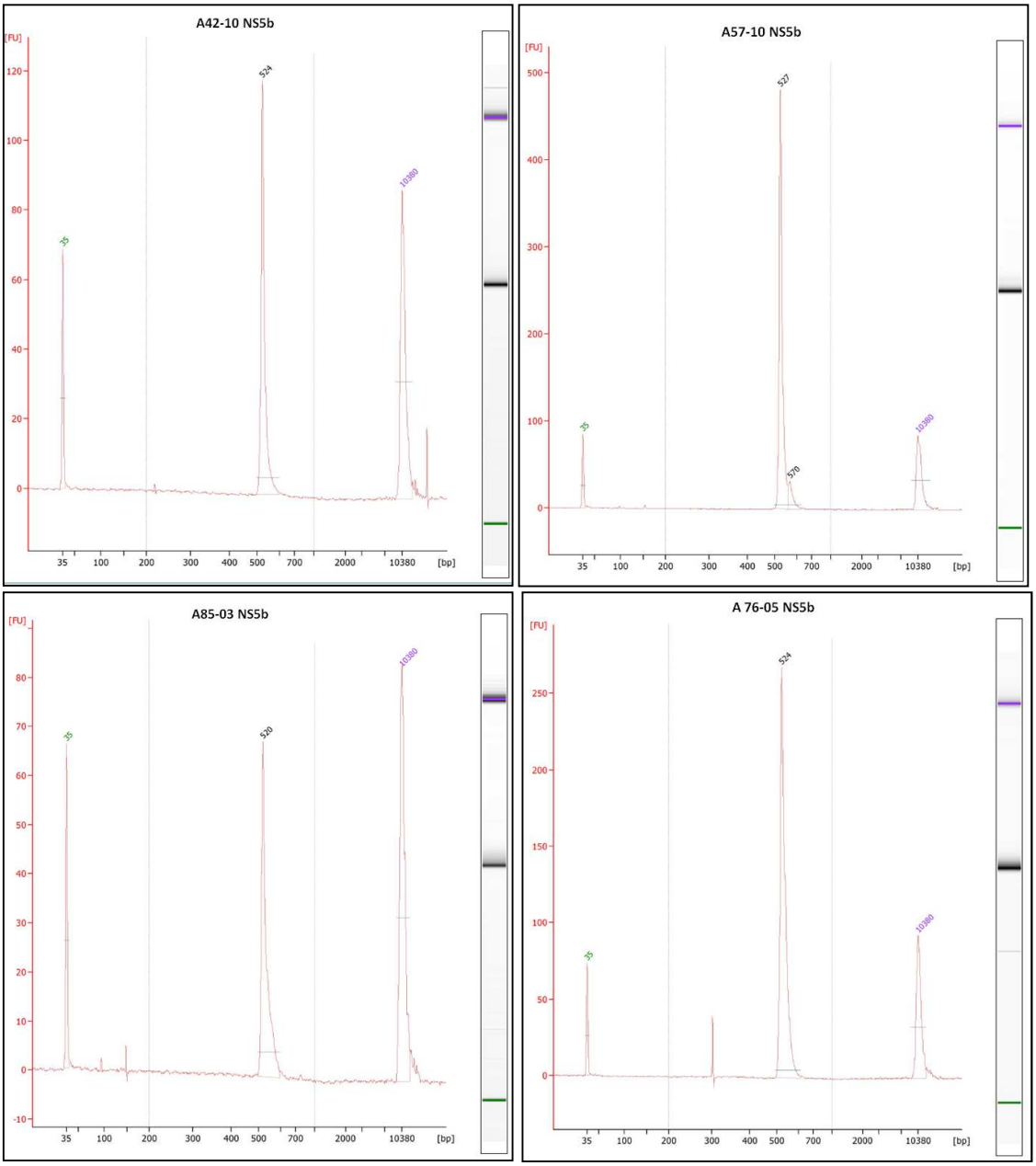

B05-08 and B51-01 pooled QCPCR
[FU]
35
518
10380
12542
[bp]


B49-03 and B84-01 pooled QCPCR
[FU]
35
514
548
10380
12651
[bp]


C01-15 and B35-04 pooled QCPCR
[FU]
35
499
522
560
10380
12207
[bp]


B37-03 and B57-01 pooled QCPCR
[FU]
35
526
550
10380
[bp]


WT control QCPCR
[FU]
35
514
582
10380
12342
[bp]


A42-10 NS5b
[FU]
524
10380
35
35 100 200 300 400 500 700 2000 10380 [bp]
A57-10 NS5b
[FU]
527
570
10380
35
35 100 200 300 400 500 700 2000 10380 [bp]
A85-03 NS5b
[FU]
520
10380
35
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A 76-05 NS5b
[FU]
524
10380
35
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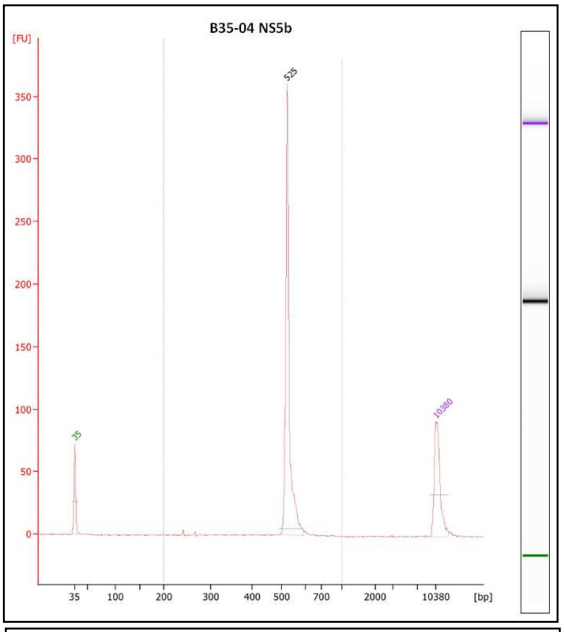
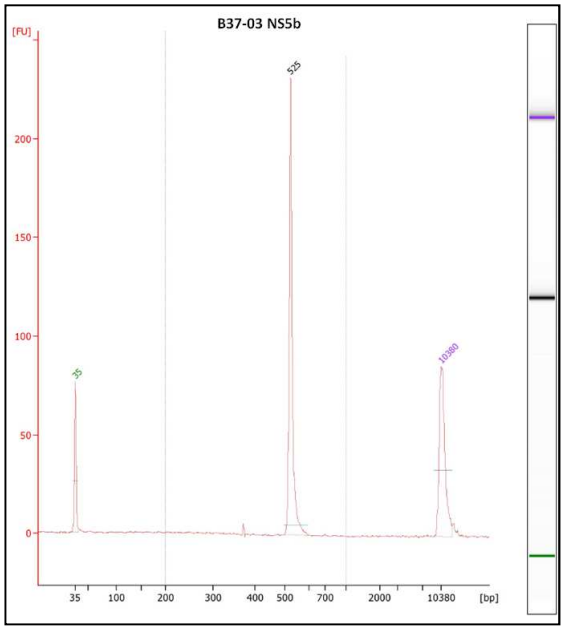
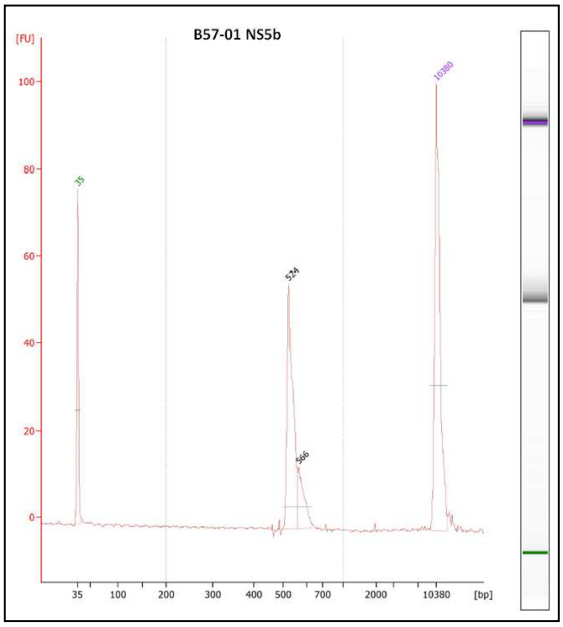
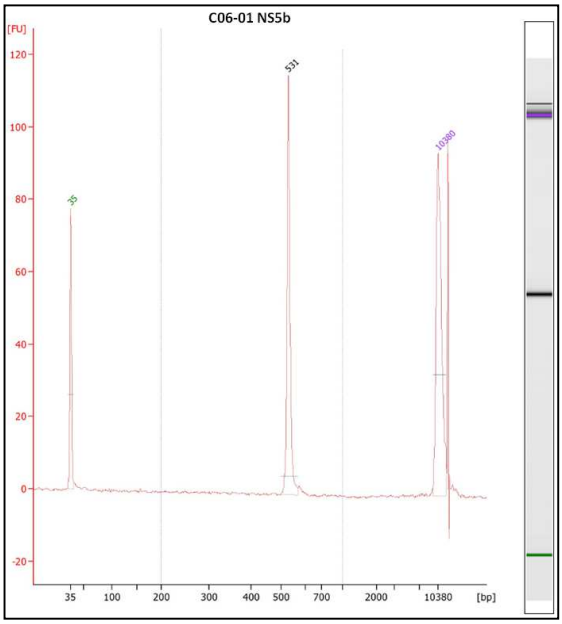
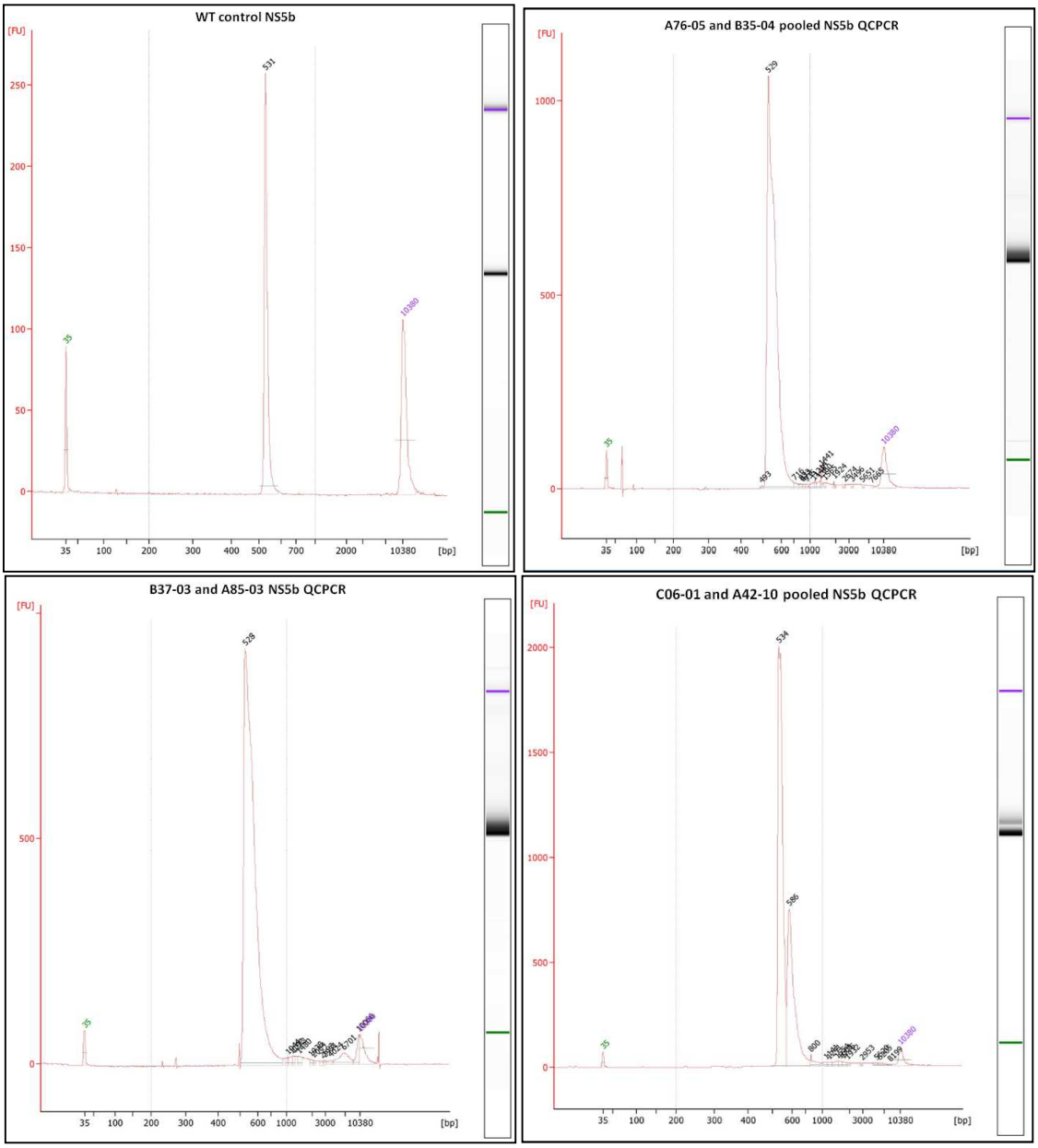
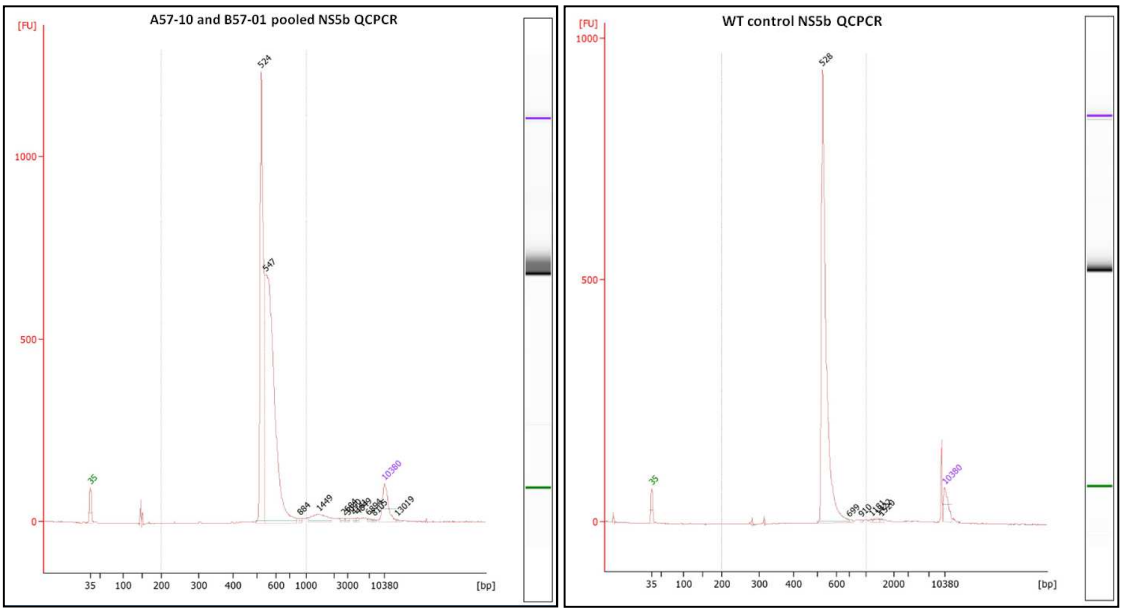

B35-04 NS5b
[FU]
350
300
250
200
150
100
50
0
525
35
10380
35 100 200 300 400 500 700 2000 10380 [bp]


B37-03 NS5b
[FU]
200
150
100
50
0
525
35
10380
35 100 200 300 400 500 700 2000 10380 [bp]


B57-01 NS5b
[FU]
100
80
60
40
20
0
534
566
35
10380
35 100 200 300 400 500 700 2000 10380 [bp]


C06-01 NS5b
[FU]
120
100
80
60
40
20
0
-20
531
35
10380
35 100 200 300 400 500 700 2000 10380 [bp]


WT control NS5b
[FU]
531
35
10380
[bp]
A76-05 and B35-04 pooled NS5b QCPCR
[FU]
529
35
10380
[bp]
B37-03 and A85-03 NS5b QCPCR
[FU]
528
35
[bp]
C06-01 and A42-10 pooled NS5b QCPCR
[FU]
534
586
35
10380
[bp]


A57-10 and B57-01 pooled NS5b QCPCR
[FU]
1000
500
0
524
547
35
10380
13019
35 100 200 300 400 600 1000 3000 10380 [bp]
WT control NS5b QCPCR
[FU]
1000
500
0
528
35
10380
35 100 200 300 400 600 2000 10380 [bp]