



universität
wien

MASTERARBEIT

Titel der Masterarbeit

New methods for fluorescence labelling of
Human Rhinovirus in live cell imaging

Verfasser

Matthias Pilecky, BSc.

angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2013

Studienkennzahl lt. Studienblatt:

A 066 863

Studienrichtung lt. Studienblatt:

Master Biologische Chemie

Betreuerin / Betreuer:

Dr. Heinrich Kowalski

ACKNOWLEDGEMENT

Completing this work would not have been possible without the help of many people, which more than deserve to be mentioned here.

In particular I would like to thank my supervisor Dr. Heinrich Kowalski for giving the possibility to join his group. During the development of this project together with him I learnt a lot and got a great insight into this fascinating topic.

Furthermore I want to thank Prof. Dieter Blaas, who always supported me with his knowledge and experience upon the field of rhinoviruses, for his ideas and a great time with him.

My special thanks belong to Irene Gösler for her advices concerning viral growth and tissue culture, as well as for providing material and her patience while explaining new techniques.

I would like to express my gratitude to my colleagues Irena Corbic, for her introduction to the lab and the picture she contributed to this thesis, Shushan Harutyunyan, for her time spending with me together at the FCS and Mohit Kumar and Karin Waclawek, for their constant motivation and moral support.

Sincere thanks are also given to Josef Gotzmann for his introduction into the world of laser microscopy and his efforts to teach me how to be a good user of microscopes.

In addition I would like to thank my parents for their support during my whole studies. And finally I want to appreciate the constant motivation and support by all my friends.

TABLE OF CONTENTS

Acknowledgement	3
1. Introduction	8
1.1. Picornaviruses and the Human Rhinovirus	8
1.1.1. The diversity of picornaviruses	8
1.1.2. The viral lifecycle and genome.....	9
1.2. The Human Rhinovirus (HRV).....	14
1.2.1. Diversity and pathology of Human Rhinovirus	14
1.2.2. Virus capsid structure	15
1.2.3. Uncoating/Penetration by Rhino- and Poliovirus	17
1.2.4. The Genome of HRV (14)	18
1.3. Live cell imaging	19
1.4. Tetra-Cystein detection tags	21
1.5. GFP like RNA-aptamers	23
1.5.1. The chemistry of GFP	23
1.5.2. Spinach and spinachlike aptamers.....	24
1.6. Aim of the work.....	26
2. Materials and Methods.....	27
2.1. DNA/RNA materials, production and modification	27
2.1.1. HRV14 plasmids.....	27
2.2. Secondary structure prediction of 24-2/HRV14 hybrids	27
2.3. Protein sequence modification and insertion mutagenesis by overlap extension PCR.....	28
2.4. Polymerase Chain Reactions	29
2.4.1. 5' and 3' PCR products	29
2.4.2. Overlap extension of 3' and 5' constructs	29
2.5. Routine (plasmid) cloning	30
2.5.1. Preparation of electrocompetent cells	30
2.5.2. Electroporation	30
2.5.3. Plasmid isolation from bacteria	31

2.6. DNA/RNA standard methods.....	31
2.6.1. DNA/RNA separation	31
2.6.2. DNA GEL extration	31
2.6.3. DNA/RNA phenolextraction and precipitation.....	31
2.6.4. Restriction digests and ligation	32
2.6.5. RNA transcription	32
2.6.6. DNA/RNA concentration measurement.....	32
2.6.7. Sequencing and sequencing primers.....	32
2.7. Tissue culture methods	33
2.7.1. Cells, culture materials and basic techniques	33
2.7.2. Transfection of HeLa cells with viral RNA & harvesting virus	33
2.7.3. Plaque assay	34
2.7.4. Virus production	34
2.8. Fluorescence methods	35
2.8.1. RNA fluorescence assay.....	35
2.8.2. Fluorescence microscopy	35
3. Results.....	36
3.1. The Spinach Projekt	36
3.1.1. Structur prediction and sequence of Spinach/HRV14 hybrids.....	36
3.1.2. Plasmid construction	39
3.1.3. Fluorescence spectrum of RNA-DFHBI complexes.....	40
3.1.4. Viral growth	41
3.1.5. Standard fluorescence images of transfection.....	42
3.1.6. Standard fluorescence images of infection.....	43
3.1.7. Sequence analysis of isolated virus	44
3.1.8. A further 3'-UTR construct (IC).....	45
3.2. The FIAsh-Tag Projekt.....	46
3.2.1. Target site selection and modelling	46
3.2.2. PCR.....	51
3.2.3. Hybridization and amplification	52
3.2.4. Sequencing of PCR products.....	53
3.2.3. Plasmid construction	54

4. Discussion.....	55
4.1. Spinach project.....	55
4.2. FIAsh Project	58
4.3. Conclusion	58
5. References.....	59
6. Appendices.....	74
Abstract	74
Zusammenfassung	75
6.1. Abbreviations	76
6.2. Sequence of the plasmid HRV14-as3f-wt	77

1. INTRODUCTION

1.1. PICORNAVIRUSES AND THE HUMAN RHINOVIRUS

1.1.1. THE DIVERSITY OF PICORNAVIRUSES

The family of picornaviruses (Pico = small) includes many infamous disease causing viruses like poliovirus, foot-and-mouth disease virus (FMDV), hepatitis A, or encephalomyelitis virus. (See figure 2) Among the genus “enterovirus” there are three species of human rhinovirus (HRV) termed A, B and C – the origin of most common colds. (For more information about the classification of virus visit the homepage of international committee on taxonomy of virus [1]) Generally speaking picornaviruses are about 30nm in diameter with an icosahedral shaped capsid (built by 60 units of VP1, VP2, VP3 and VP4 each; see figure 1), contain one single strand (+)-RNA of about 6.5-9.5 kb encoding one polypeptide of around 2-2.5 amino acids. [2]

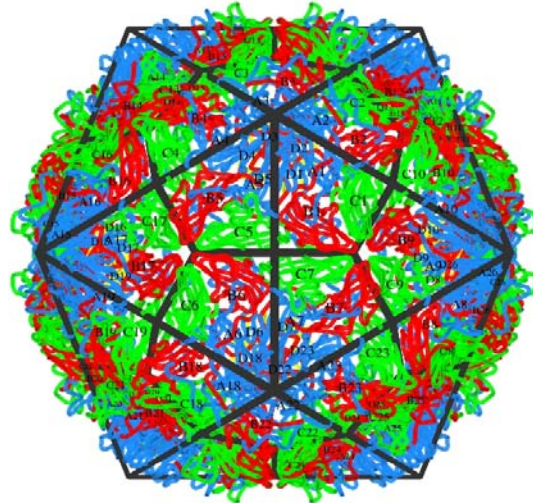


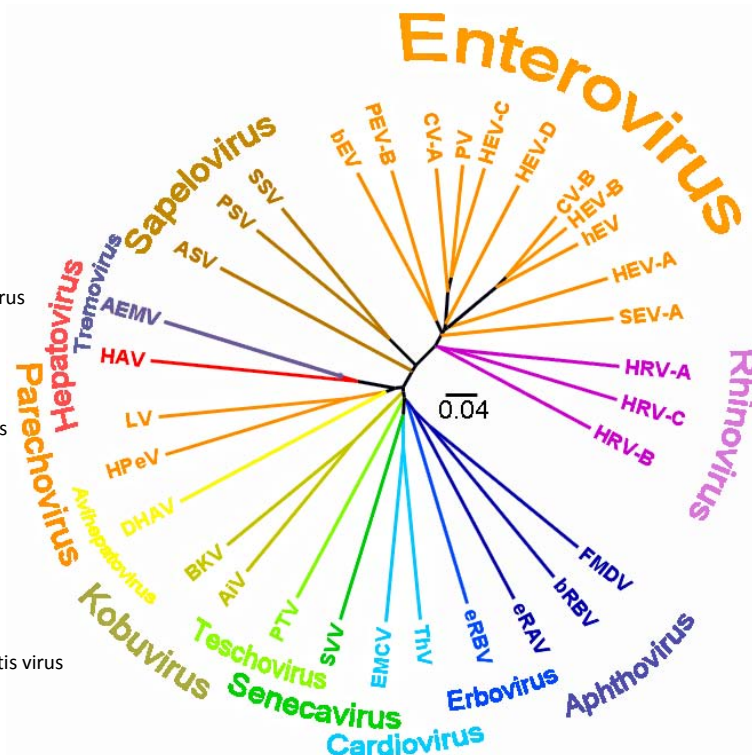
Figure 1 Modified X-Ray image of HRV2. Picture taken from Virus Particle Explorer [VIPERdb] <http://viperb.scripps.edu/>

Figure 2 Phylogenetic tree of picornaviridae. RNA sequences were aligned with ClustalW and data was processed with FigTree and Adobe Photoshop.

Enterovirus:

bEV	bovine Enterovirus
PEV	porcine Enterovirus B
CV	coxsackievirus
PV	Poliovirus
HEV	human Enterovirus
SEV	simian Enterovirus
HRV	human Rhinovirus
hEV	human Echovirus

FMDV	Foot-and-mouth-diseas virus
bRBV	bovine rhinitis B virus
eRAV	equine rhinitis A virus
eRBV	equine rhinitis B virus
ThV	Theilers virus
EMCV	Encephalomyocarditisvirus
SVV	Seneca Valley Virus
PTV	porcine Teschovirus
AV	Aichi Virus
BKV	bovine Kobuvirus
DHAV	duck Hepatitis A virus
HPeV	human Parechovirus
LV	Ljungan virus
HAV	Hepatitis A virus
AEMV	avian encephalomyocarditis virus
ASV	avian Sapelovirus
PSV	porcine Sapelovirus
SSV	simian Sapelovirus



1.1.2. THE VIRAL LIFECYCLE AND GENOME

A virus is designed to infect a host cell and use cellular proteins to replicate. Therefore it first has to approach a suitable target cell. The process of infection begins by attaching to specific receptors, which restricts the infection spectrum to certain species and cell types. Concerning picornaviruses receptors are mainly members of the immunoglobulin and integrin receptor family, but also scavenger receptor and others are used. [3] [4] [5] [6] In case of picornavirus binding to a receptor and/or exposure to low pH alters their conformation irreversibly, so that they become – termed by their sedimentation properties – 135S particles in case of rhino- and poliovirus. [7] [8] The mechanism of this transformation is not fully understood. [9] An important feature seems to be the exposure of the amphipathic N-terminal region of VP1. [10]

	<i>Receptor</i>	<i>Accessory factors</i>	<i>Major syndromes</i>
<u>Enterovirus</u>			
Coxsackievirus A 2,3,5,7,8,10,12,14,16	N/A		Herpangina, hand-foot-and-mouth disease, respiratory disease, meningitis, poliomyelitis
Enterovirus 71	N/A		Hand-foot-and-mouth disease, meningitis, paralysis
Coxsackievirus A9	$\alpha\text{v}\beta 3$	MAP-70, $\beta 2$ -m	(See CVAs)
Coxsackievirus B1-6	HCAR		Myocarditis, pleurodynia
Coxsackieviruses B1,3,5	DAF (=CD55)	$\alpha\text{v}\beta 6$	Meningitis, respiratory disease, neonatal infections
Echoviruses 1,8	$\alpha 2\beta 1$		Meningitis, encephalitis
Echovirus 3,6,7,11,12,13,19,21,24,25,29,30,33	DAF	$\beta 2$ -m, CD59	Pleurodynia, exanthema
Polioviruses 1-3	CD155		Poliomyelitis, (meningitis)
Coxsackieviruses A13,17,18,20,21,24	ICAM-1		Common cold, infantile diarrhea
Coxsackievirus A21, 24	ICAM-1		Acute hemorrhagic conjunctivitis
Enterovirus 70 (68)	DAF		Acute hemorrhagic conjunctivitis
<u>Rhinovirus</u>			
Major group (~90 types)	ICAM-1		Common cold
Minor group (~20 types)	LDL		Common cold
Third group (HRV-C)	N/A		Common cold
<u>Hepatovirus</u>			
Hepatitis A virus	HAVcr-1		Type A hepatitis
<u>Aphthovirus</u>			
FMDV A12	$\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 6$		Foot-and-mouth disease (cloven-footed livestock)
<u>Cardiovirus</u>			
Encephalomyocarditis virus, mengovirus	VCAM-1		Encephalitis, myocarditis

Table 1 Human pathogens of picornaviridae with their receptor and the disease they cause. Adopted from [2] (2002); $\alpha\text{v}\beta 3$, $\alpha\text{v}\beta 6$, $\alpha 2\beta 1$ = integrins; DAF = Decay Acceleration Factor (CD55); CD155 = Poliovirus receptor (Pvr); ICAM-1 = intercellular adhesion molecule 1; HCAR = human coxsackievirus B and adenovirus 2 receptor; VCAM = vascular cell adhesion molecule; HAVcr-1 = receptor for hepatitis A virus; $\beta 2$ -m = $\beta 2$ microglobulin (part of MHC I); MAP-70 (part of MHC I); N/A = Not available;

After binding, virus is internalized via endosomes. [11] There either by pore formation or membrane disruption RNA gets into the cytoplasm, leaving an empty capsid (80S particle) behind. [12]

The (+)-RNA serves as template for both translation and (-)-strand production. Since the RNA-dependent RNA-polymerase is not carried with the virus particle first the polyprotein has to be translated. [13] The picornaviral polyprotein (see Figure 3) consists of 3 domains which are the initial products of proteolytic cleavage by two virus encoded proteases, and are then further cut into the mature proteins.

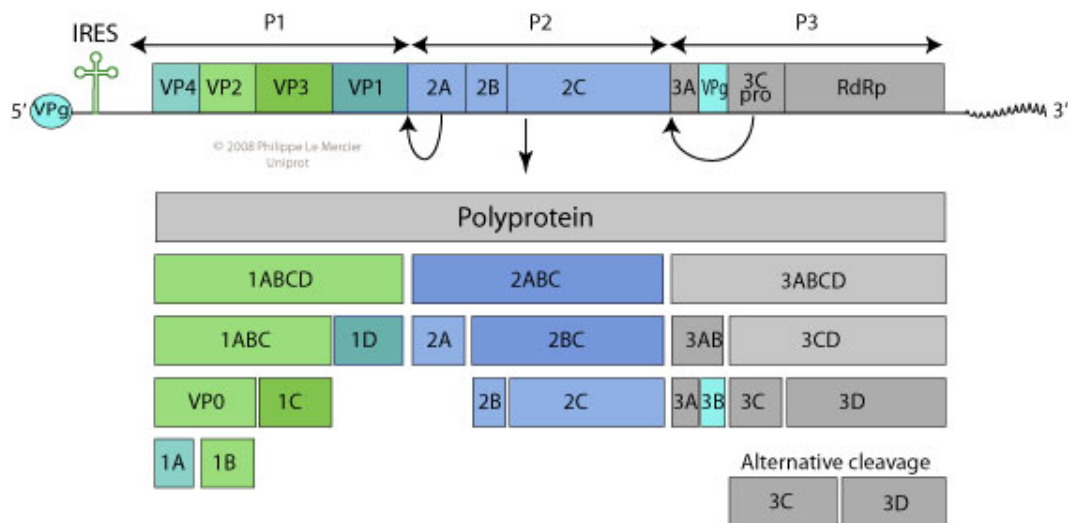


Figure 3 Protease processing of picornaviral polyprotein. The figure shows the typical proteolytic processing of picornaviral polyprotein in case of enterovirus. In some cases a leader protease is encoded in front of VP4. Picture taken from Viralzone, ExPASy (viralzone.expasy.org)

In some cases (e.g. cardiovirus) a leader protease is encoded in front, but in most picornaviral genomes the first domain (P1) only contains all structural proteins (VP1-VP4). The second part (P2) comprises 2A (in case of polio- and rhinovirus a cysteine protease, which separates P2 from P1 and degrades eukaryotic initiation factor eIF-4G to shut down host cell protein synthesis and therefore recruit all resources for virus production [14]), 2B (Ca²⁺-Channel for suppression of host cell early apoptosis [15] [16]) and 2C (assembles to hexamers, formation of replication vesicles by membrane recruitment, shows ATPase activity [17] [18]). The third domain (P3) consists of 3A (inhibition of protein secretion, resulting in reduced MHC I expression on the cell surface for antigen presentation [19]) and 3B (VPg, a small peptide primer for virus RNA synthesis [20]), a second chemotrypsin-like serine proteinase (3C) as well as the RNA-dependent RNA-Polymerase (3D). [21] Still not all biochemical activities of the picornaviral proteins are explored yet.

Replication and translation of viral polyprotein and RNA critically depends on cis-acting RNA elements which act like an origin of replication at both ends of the RNA and one within the open reading frame. (see figure 4) (not shown for all picornaviruses yet) [22]

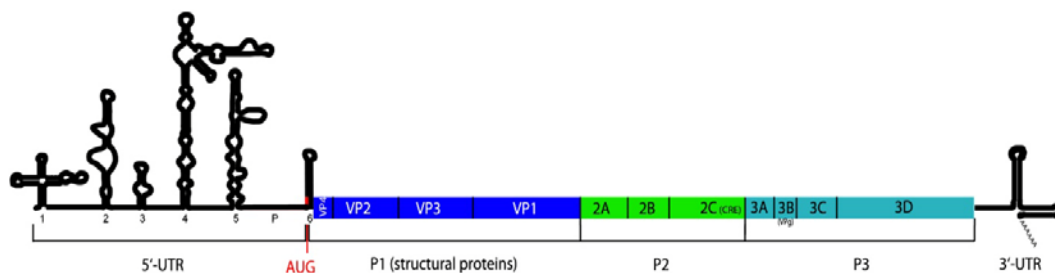


Figure 4 Schematic representation of a picornaviral genome. In case of aphtho- and cardioviruses also a Leader Protease (L) is encoded in front of VP4. 5'-UTR: 1 (cloverleaf), 2-5 (IRES) [23]; P1 (structural proteins); P2 (non-structural proteins; necessary for adapting host cell to efficient virus replication); P3 (non-structural proteins; Proteins necessary for virus replication); 3'-UTR is different for each type. In case of poliovirus the CRE is located within the coding region of 2C. Picture drawn in Adobe Photoshop.

The several hundred nucleotides long 5'-UTR, which is also linked covalently to the hydroxyl group of tyrosine-3 in VPg (See figure 5) [24] [25], is highly structured featuring domain I (which forms a cloverleaf in polio- [26], entero- and rhinoviruses [27] – not such a defined structure in others) and the internal ribosome entry site (IRES), which is absolutely necessary for the translation of the RNA by eukaryotic ribosomes. The cloverleaf interacts with both viral protease 3CD and polycytosine binding proteins [28], forming a complex which is important for switching from translation to replication. [29] For the 3'-UTR several structures in different types of picornavirus have been identified. The distal 3'-NCR

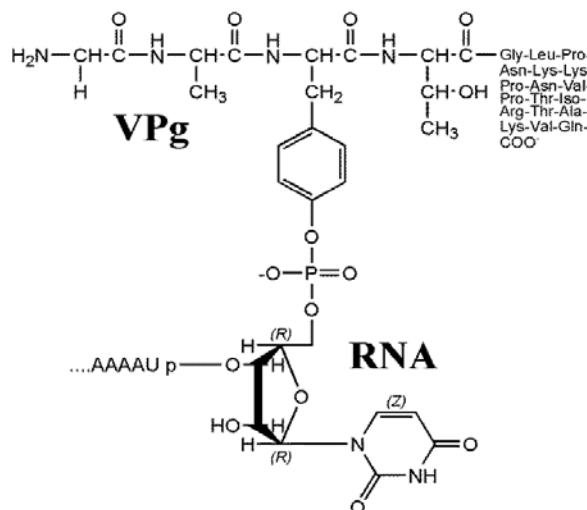


Figure 5 Chemical structure of VPg covalently linked to the viral RNA. By the complex of 3CD and VPg the first nucleotides using CRE as template are synthesized. Picture drawn with ChemDraw.

also bears a genetically encoded poly-A stretch of variable length (20-150nt [30]), however at least 12nt are necessary for the binding of human poly-adenosine binding protein (PABP). [31] Polio- and rhinovirus fail to replicate efficiently when most of the noncoding 3'-End is deleted, but are still viable, yielding about 10-100 fold less infectious progenies than wild type virus. [32] [33] PABP is partly

cleaved by 3C to suppress host cell protein production [34] while intact PABP binds in a looping interaction to the viral poly-A and 3CD attached to the cloverleaf, resulting in a nearly circular plus sense RNA molecule. [35]

In that way poly-A at the 3'-end and VPg-Linked poly-U can serve as templates for RNA replication. [36] To obtain an uridylated VPg, an internal cis-acting responsive element (CRE or Oril) interacts with 3CD and serves as template for synthesising the first nucleotides being attached to the small peptide primer. [20] CRE is a structure within the ORF and can be found at different positions, depending on the type of Virus. [37]

It is assumed that at first several polyproteins are produced from the input virus RNA following hydrolysis of the VPg until the protease/polymerase protein 3CD reaches a critical concentration, binds to the cloverleaf to direct (-)-RNA-strand synthesis, which proceeds in opposite direction to the translating ribosomes. Since both processes cannot run simultaneously on the same RNA molecule this constitutes an important regulatory mechanism determining the fate of individual RNA templates. [38] [39] The synthesized negative strand RNA is then used as template for the production of genomic positive sense RNA.

3CD displays increased proteolytic activity compared to 3C alone and is required for efficient cleavage of the enteroviral polyproteins P1, P2 and P3. [40] Proteins are not processed at once but intermediate proteins (e.g. 2BC, 3AB) are generated (see figure 3). Cleavage of the structural proteins also advances particle formation and therefore directs towards encapsidation. [41]

Proteases 2A and 3C also shut down host cell protein production by various methods like cleaving transcription factors [42] [43] or translation factors [44] [45] and consequently using all cellular resources just for virus production.

Genome replication occurs before and independently of encapsidation [46] in close association with virus-induced membrane vesicles and sheets giving rise to unique organelles termed replication complexes [47]. The newly generated vesicles are 50-350nm in diameter and recruited by 2BC and 2C probably from the ER. [48] [49] Replicated RNA is released from these vesicles for translation, until enough structural proteins (VP1, VP3 and VP0, which is uncleaved VP4 & VP2) are produced. [50] This processing of the P1 region by 3CD results in the self assembly of 5 copies each of VP1, VP3 and VP0 to a 14S pentameric particle. [51] Twelve 14S particles then assemble together to an 80S procapsid (80S is also the sedimentation coefficient of an empty capsid (B-particle) after cell infection) and with one (+)-strand RNA molecule, which eventually results in the 150S provirion. Finally VP0 is possibly autocatalytically cleaved into VP2 & VP4 resulting in the mature virion. [50]

The encapsidation process is not yet fully understood. Especially the mechanism switching from replication to virion formation has not been identified yet. 5-(3,4-dichlorophenyl) methylhydantoin is an inhibitor of particle formation and inhibits posttranslational cleavage, [52] maybe by inhibition of 2C. [53] Further studies also revealed that 14S particles maybe have to be activated promoting the encapsidation. [54] It is also assumed, that replication and virus packaging do not occur independently. However it was shown, that poliovirus capsid proteins can encapsidate different picornaviral RNAs, but it seems that the particular viral capsids are optimized for their specific RNA as shown in trans-encapsidation studies. [55]

It is assumed, that cell lysis occurs independently from virus production, as just more and more membrane is recruited for virus replication [56] until the cell membrane is permeable for small proteins (approximately 1000 Da) in the mid of the infection. [57] [58] Ongoing alterations of the cytoplasmic concentrations especially cations lead to membrane depolarisation and disruption setting the synthesised virus particles free. [59]

1.2. THE HUMAN RHINOVIRUS (HRV)

1.2.1. DIVERSITY AND PATHOLOGY OF HUMAN RHINOVIRUS

While the Human Rhinovirus (HRV) has been a genus itself for a long period the International Committee of Taxonomy of Virus (ICTV) since 2009 classifies it as member of the genus enterovirus and distinguishing three different types of rhinoviruses: HRV-A, -B and -C [1]

In parallel HRVs are divided into major and minor group based on their cognate host cell receptor. While the major group of HRV uses ICAM-1 [60] and the minor group LDL-receptor family (e.g. LDL, LRP or VLDL receptor) [61] the target molecules for recently discovered HRV-C [62] is still unknown. [63] Some major group HRV also use heparan sulphate as additional receptor either naturally or when selected for growth in an ICAM-1 deficient environment. [64] [65] Initial binding to proteoglycans might be advantageous, since ICAM-1 is very weakly expressed in unstimulated nasal epithelial cells and nasal mucosa of healthy individuals and only unregulated during the course of inflammation as triggered by rhinoviral infection, which in turn facilitates spreading of major group viruses within the respiratory tract. [66]

HRV is the major cause of the common cold [67], but only few, scattered infected airway epithelial cells are usually detected. The main symptoms are therefore unrelated to cell death but rather precipitated by the patient's innate and adaptive immune response. [68] HRV infections are usually limited to the upper respiratory tract, but may spread to the lower airways leading to more severe symptoms especially in children and also exacerbate asthma and chronic obstructive pulmonary diseases (COPD). [69] [70] [71] [72] HRV-A and -C are more frequently associated with lower respiratory tract infections than HRV-B with some reports proposing the highest pathogenic potential for species C. [73] [74] Though normally not fatal, rhinoviral infections are associated with huge financial expenses mostly due to the appreciable loss of working days. [75] Notably, despite considerable knowledge on the picornavirus life cycle, no drug is presently available to specifically combat rhinovirus infections. This altogether warrants further examination of the infection cycle, ranging from the early phase of rhinovirus attachment and penetration of the host cell to the ultimate release of infectious particles, using new approaches such as described in this thesis, in order to facilitate identification of new drug targets.

1.2.2. VIRUS CAPSID STRUCTURE

The X-Ray structure of several human rhinoviruses has been obtained, representing members of the major and minor group. [76] [77] [78] The capsid consists of 60 so called protomers containing one copy of each structural protein. While VP1, VP2 and VP3 form the outer surface of the virion, VP4 lies entirely interior at the capsid/RNA interface. VP4 is cleaved from VP2 as the last step of virus maturation which may be catalyzed by the entry of RNA into the capsid. [79] The capsid displays three different types of symmetrical centres: 5 copies of

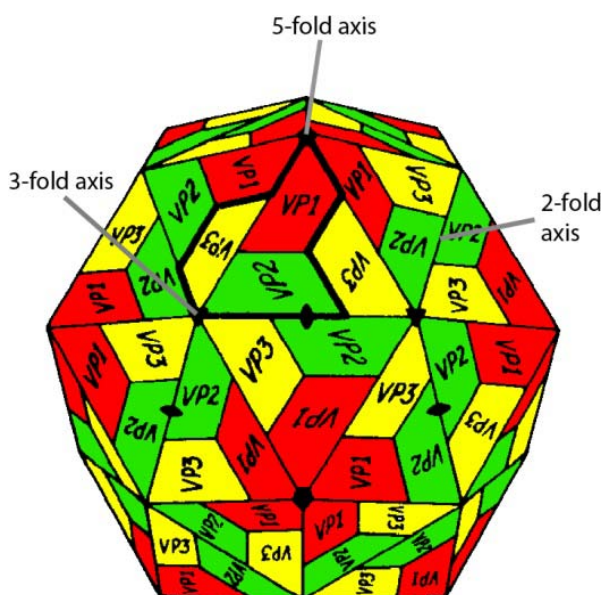


Figure 6 Schematic image of an icosahedral picornaviral capsid built by 60 units of protomers (bold circled). The three different symmetrical axis are shown. Picture adapted from [80]

VP1 surround a 5-fold axis, 3 copies of VP3 and VP2 each enclose a 3-fold axis and two VP2 subunits are symmetrically arranged at a 2-fold axis. [80] The VP1 forms a star-shaped plateau at the 5-fold axis and is surrounded by a circular depression, 2nm deep and about 2-3nm wide, which has been termed canyon. [81] The walls and floor of the canyon are formed by VP1 and VP3 [82] with different residues contributed by HRV-A and HRV-B. [83] Beneath the canyon a hydrophobic pocket was found [78], occupied by fatty acids in case of most HRVs [77], but not of HRV-14 [84]. New findings, however, suggest that these so called pocket factors are unlikely to play a crucial role in the rhinoviral infection cycle. [85] Additionally the surface contains host cell binding sites, which attach to the aforementioned receptors. Concerning major group viruses like HRV-14 the binding site for ICAM-1 is hidden from the host's antibodies within the canyon. [86] In contrast minor group viruses like HRV-2 bind with exposed loops decorating the plateau at the 5-fold axis to various LDL-receptor family members. [87] In both cases the canyon has to be flexible for viral uncoating. [88] Stabilizing the canyon by hydrophobic interaction (e.g. pleconaril [89]) prevents uncoating of the virus, [90] while destabilizing the RNA/capsid interaction (e.g. dansylaziridine) also increases the flexibility of the capsid and makes it more vulnerable to tryptic digestion. [88] Although this seems to be a good target for antiviral drugs, none of these compounds have entered phase III studies yet, mainly due to severe side effects. Furthermore a quick adaptation of rhinoviruses has been observed in case of pleconaril resulting in a mutant which is not affected at all. [88] It is also hard to address all known rhinovirus species at once. (see figure 7) For a review about the current antirhinoviral drug development see [91].

Crystallographic studies also revealed the presence of bivalent cations in the 5-fold axis. For HRV-14, -1A and 3 it seems to be a Ca^{2+} , whereas for HRV-16 a Zn^{2+} ion. In this context the cations might have a stabilizing effect on the capsid, although extraction by EDTA did not trigger uncoating-associated changes. [92] HRVs show high sensitivity to acidic conditions and are completely unstable below pH 3. [93] Nevertheless HRV14 mutants resisting and even requiring low pH could be generated by artificial evolutionary pressure. [94]

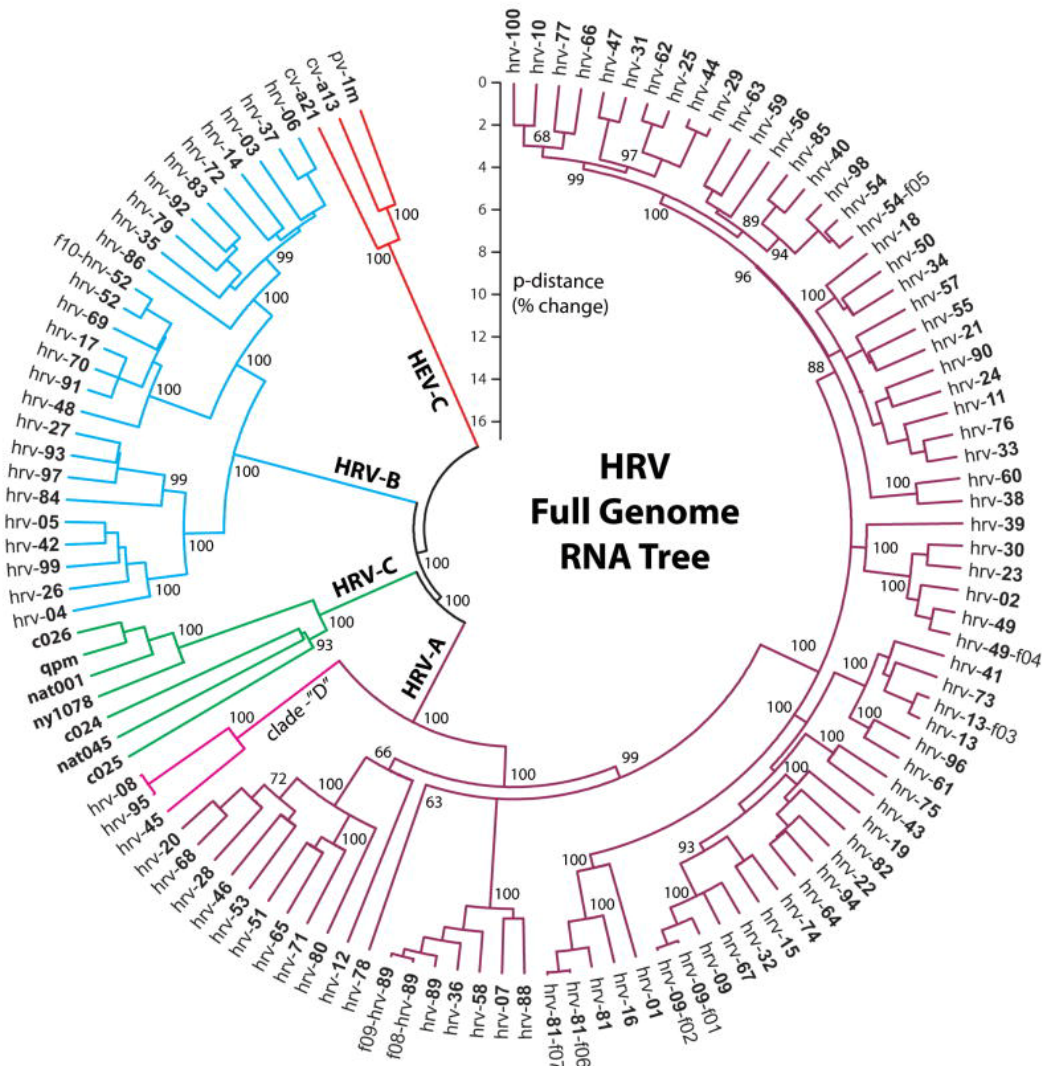


Figure 7 Phylogenetic tree of known rhinoviruses. Picture taken from [95]

1.2.3. UNCOATING/PENETRATION BY RHINO- AND POLIOVIRUS

To investigate the uncoating of picornaviruses many studies have been done on human rhinoviruses and on the closely related poliovirus, but still some features, like the exact exit site of RNA and the main driving force, remain unknown. [8] Upon binding of poliovirus (and major group rhinoviruses) to its receptor the energy needed to undergo several conformational changes and convert to an 135S particle is reduced by about 50 kcal/mol. [96] This includes the loss of VP4 [97], moving 31 amino acids of the N-terminus of VP1 from inside the capsid onto the viral surface, which does not allow to bind new receptors anymore, but enables to bind to liposomal membranes. [10] The internalisation of viral particles is dependent on dynamin and in most cases on clathrin [98] (an exception would be e.g. heparin sulphate-binding HRV8 [99]) taking the virus up into endosomes. [100] By using FISH it has been determined that the uptake of HRV into early endosomes happens quite fast (5 min), thereafter virus is transferred to endosomal carrier vehicles, where low pH assist uncoating. [101] The more sensitive a virus is to low pH the more successful the virus uncoats. [102] Both VP4 and VP1 seem to play an important role in penetrating the endosomal lipid bilayer and delivering the RNA into the cytosol. [103] There is some evidence, that a conformational change along the 2-fold axis induced by VP2 allows VP4 to exit, as well as detaches the RNA. [104] [105] VP4 might be of importance for enabling the RNA to penetrate the endosomal membrane. [106] In case of HRV14 the current believe is that RNA is released into the cytoplasm concomitant with endosomal rupture, in contrary to HRV-2, which seems to transfer the genomic material through *de novo* formed endosomal pores. [107] Regarding the mechanism of RNA exit from the capsid very little is known so far. Experiments with atomic force microscopes indicate that RNA forms local structures [108] [109], which opens up the question how such structures fit through the small pores formed in the conformationally altered virus shell, as in case of HRV2 and what is the source providing the energy to unfold. [110]

1.2.4. THE GENOME OF HRV (14)

The genome of HRV14 has the following features [111]:

Name	nucleotide	
	from	to
VPg-TT	1	2
Cloverleaf	3	81
IRES	116	628
ORF-AUG	629	631
VP4	629	835
VP2	836	1621
VP3	1622	2329
VP1	2330	3196
2A	3197	3634
2B	3635	3925
2C	3926	4915
3A	4916	5170
3B	5171	5239
3C	5240	5785
3D	5786	7165
ORF-UAG	7166	7168
Stem-Loop	7177	7212
Poly-A	7213	

As for all picornaviruses discussed before, the RNA is covalently linked to the Tyr³-Residue of the small VPg (3B) protein. [112] The cloverleaf [29] is followed by a short cytosine-rich spacer region and an internal ribosome entry site type I. (see figure 9) [23] [113] [114] The open reading frame encodes for the typical picornaviral structural (VP1-VP4) and non-structural proteins, as discussed before and reviewed in [115]. HRV14 has only one stem-loop at the 3'-UTR of its genome in proximity the poly-adenosine tail. [116] (see figure 8)

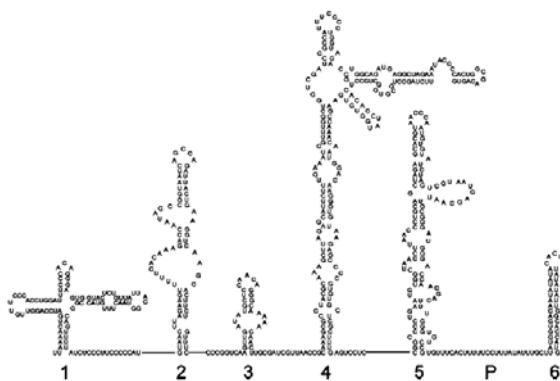


Figure 9 Structure of the 5'-UTR of HRV-2, which is closely related to that of HRV14, 1=cloverleaf; 2-5=IRES; 6=Start of ORF [23]

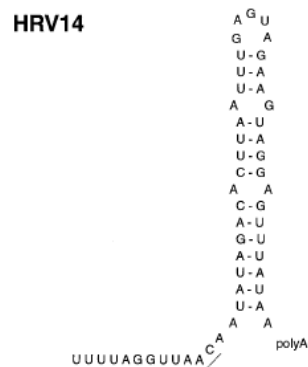


Figure 8 Structure of the 3'-UTR of HRV-14; the first 6 nt are part of the ORF [116]

1.3. LIVE CELL IMAGING

For a long time standard techniques involved fixation of cells and thereafter staining with e.g. antibodies, or in newer times short nucleic acid probes as in fluorescence in situ hybridization (FISH). Fixation not only affects the cell architecture but also does not allow obtaining dynamic information anymore. With advancing technology and increasing use of GFP localisation trafficking studies of proteins as well as gene expression assays became popular. [117] However, on the one hand GFP is quite big and might influence the natural behaviour of a molecule and on the other hand tracking of other biomolecules like nucleic acids was still not possible satisfyingly without several modifications. The first live cell imaging of RNA experiment was done by Ainger et al. and involved microinjection of fluorescently labelled full-length mRNA. [118] Based on this idea also live FISH experiments were designed, which however, lacking the possibility of washing of unhybridised probes, caused high background signals and required microinjection of single cells. [119] Nowadays several more sophisticated approaches are used to observe the natural behaviour of nucleic acids as viral RNA. Especially Förster resonance energy transfer (FRET)-based systems, in which a donor and acceptor molecule have to be in close proximity to obtain a fluorescence signal improved the noise-to-background ration tremendously. [120] Today the best established options for nucleic acid tracking are molecular beacons (MB). [121] [122] MBs are modified oligonucleotids which form a stem-loop and carry a fluorophore on one and a quencher on the other end. In that way the probes theoretically fluoresce only upon binding to their target sequence. Usually, an increase in intensity between 10- and 200-fold is observed, depending on the sample and the efficiency of the MB in terms of hybridisation. [123] It is also possible to discriminate between perfectly matched and mismatched MBs, which is due to their thermodynamic behaviour. (See figure 10) [124] Concerning the delivery of MBs into cells better methods were developed than microinjection. Linkage to the tat-peptide resulted in efficient uptake of MBs by different cell types and did not interfere with the system. [125] [126]

Finally proteins which bind to specific RNA sequences like MS2 RNA-binding protein of MS2 bacteriophage were expressed fused to GFP and a nuclear localisation signal to reduce background fluorescence. However, this system only shows modified RNA and not native. [127]

The mentioned techniques have already been used to detect viral RNA *in vivo*. [128] [129] However, none of those were able to visualize the cell entry phase of viral RNA *in vivo*.

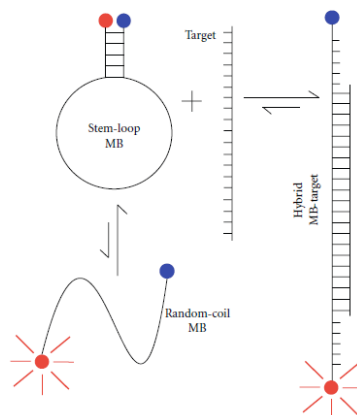


Figure 10 The three states of a molecular beacon: Stem-loop – where the fluorophore is quenched; Random-coil or hybridized to target. Taken from [121]

In contrast to RNA, fluorescent labelling of proteins is easier, since more different functional groups can be targeted. Reactive dyes for example are commercially available for reactions with amines, ketones, carboxylic acids, aldehydes and thiols (invitrogen.com) and cover the whole spectrum used in fluorescence microscopy. Mostly N-hydroxy-succinimide-activated carboxyesters (NHS-activated) dyes are used for primary amines, since at least one primary amine is available in every protein.

Recently “Quantum Dots”, fluorescent nanometer-scale semiconductor crystals with high level of brightness and extreme photostability are increasingly used to label biomolecules. However, they are also bigger and in contrast to GST or TC-tag do not allow the tagging of newly synthesised proteins, albeit there are several applications in the field of biology, where they have been successfully established. [130]

Brandenberg et al. used Cy5 to react with the polioviral capsid and simultaneous labelled the viral RNA with Syto 82, which binds to certain RNA secondary structures and which was incorporated into the viral capsid. Studies with this system in HeLa cells showed, that the Syto 82 signal decreased after 10 minutes of infection in about 60% of labelled PV1, which was due to RNA release. [131] This system however has two weaknesses. First, only previously labelled virus is visible, thus it is only useful for infection studies, and second, both dyes are sensitive to photo bleaching. We chose two different new labelling methods, which are able to bind to specific RNA/protein sequences and can be used to label newly synthesized viral proteins/RNA. Both techniques involve small membrane permeable molecules and can be used for live *in vivo* imaging. This could allow tracking of both viral uncoating, similar as it has been done in fixed cells before [101], and replication and assembly. FAsH – one of the labelling techniques we chose – has been recently used for super resolution imaging (photoactivated localization microscopy; PALM) of the assembly of the HIV-1 capsid [132], which might also become an attractive possibility for rhinovirus.

1.4. TETRA-CYSTEINE DETECTION TAGS

Derivatives of fluorophors which are able to bind covalently to a tetracysteine motif (TC-Tag: cys-cys-pro-gly-cys-cys) in any protein and allow detecting and quantifying the amount of this peptide *in vivo* are commercially available. [133] They were first described by Griffin, Tsien et al., who used 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein which is membrane-permeant and does not fluoresce until binding to the tetra-cysteine sequence.

Compared to GFP the TC-tag has much less influence on the target protein's structure and mass. [134] The Fluorescein Arsenical Helix Binder (FIAsh), or the as well

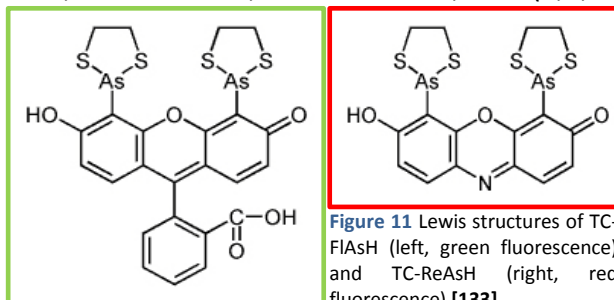


Figure 11 Lewis structures of TC-FIAsh (left, green fluorescence) and TC-ReAsH (right, red fluorescence) [133]

commercially available Red Arsenical Helix Binder (ReAsH) are aromatic fluorophores with two trivalent arsenics. (see figure 11) Originally they were developed to bind to a peptide known to form an α -helix (AREACCRECCARA). (Reviewed in [135]) Further studies however revealed that other tags bind the fluorophore much stronger [136] and notably do not have a helical conformation (but still the system is called "Helix Binder"). [137] The higher affinity of this optimized sequence known as TC-tag also resulted in reduced background signal. [138] These properties have been even improved with the design of flanking amino acids which further stabilize the optimal structure (see figure 12) for the

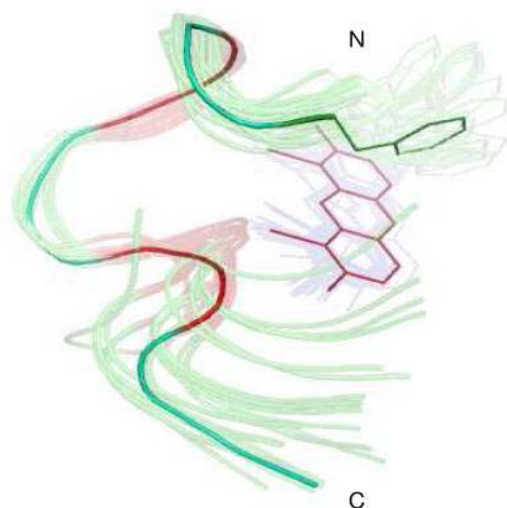


Figure 12 NMR model of the optimized FIAsh-Tag sequence FLNCCPGCCMEP. The peptide folds into a hairpin structure. Picture taken from [137]

binding of Arsenical Helix Binder: FLN*MEP and HRW*KTF. [139] It is also assumed, that the improved fluorescent properties of those two variants is due to the increased electronic density provided by aromatic side-chains, that is, especially phenylalanine, but to some extent also by tryptophan in the latter one. [136]

Arsenic helical binder with different excitation and emission wavelengths have been developed (e.g. CHOxAsH [388nm|433nm]), which also have the advantage of stability even at lower pH (depending on the peptide used), however the background fluorescence was too high for commercial applications. [140] [138]

Arsenic is known to inhibit proteins due to their high affinity to sulphur, which causes the high toxicity of arsenic. [141] To avoid cellular damage in this case, the arsenics are modified with 1,2-ethanedithiol which binds tighter to the

organometallic arsenic than any cellular proteins. [142] A short TC-Tag has an even higher affinity to the arsenical compound which avoids toxic effects when used in cell culture. [134] Successful establishment of this method for various cell biological issues has been reported [143] [144] and also FRET-compatible probes were developed. [145] Disadvantages of this tagging are high background signals if the sample is not extensively washed with e.g. British Anti-Lewisite (BAL) or similar thiol group containing molecules. [146] Arsenic has been used as well to purify proteins with vicinal dithiols via affinity chromatography before, which is an additional advantage of the TC-Tag. [147] Naturally occurring pseudo-tetrahcyteine sequences have been investigated regarding their affinity to FIAsH and ReAsH. Proteins, which naturally showed fluorescence upon treating with the dye were found in bacteria and might be responsible for background fluorescence, despite not being bright enough to be used on a regular base. [148] Rhinoviruses can only incorporate little extra genetic material due to size limitations in effective packaging of its RNA genome. Indeed, only about 5% of the 7,100 nucleotides can be inserted in select positions of the open reading frame. [128] This severely restricts the type and size of Tag, which can be used for real-time fluorescent imaging of cognate protein products. For example, the popular but relatively large enhanced green fluorescent protein (eGFP) and discosoma sp. red fluorescent protein (DsRed), respectively have been used to determine more easily the replication of coxsackievirus [149] [150] and the oncolytic picornavirus seneca valley virus [151] in infected cells and tissue. In these examples, the cDNA coding for eGFP was always flanked by viral protease cleavage sites to enable its authentic isolation from the polyprotein during the cleavage cascade described previously. To the best of our knowledge there is only one report, where hybrids of picornaviral proteins with (auto)fluorescent tags were created. In this work, the protease 2A of poliovirus was fused to DsRed with variable impact on viability of the recombinant virus. [152] However, this approach is not possible with structural proteins. Also the maturation time of eGFP and other fluorescent proteins limits their application for real-time studies involving rapid processes following protein synthesis such as encapsidation at sites of RNA replication.

Previous experiments by Arnold and co-workers have shown that the surface loops of HRV14 can accommodate foreign sequences, albeit only in the range of oligopeptides. Specifically, a surface-localized loop of VP2 has already been modified successfully to present an antigenic epitope of HIV-1. [153] Another likely innocuous insert site has been chosen in this thesis project based on an alignment of the amino acid sequences of the capsid proteins of all known rhinoviruses. This led to the discovery that VP1 shows high variability at sequences in proximity to the C-Terminus, which are hence most likely not subject to selection for a particular structure or function. This stretch was also recently discovered as novel antigenic site. [154] Both regions in VP2 and VP1, respectively, are at the viral surface and small insertion likely will not affect the ability to bind to the viral receptor in case of major group virus (e.g. HRV14) and were therefore chosen to being used as target site for a TC-Tag insert.

1.5. GFP LIKE RNA-APTAMERS

1.5.1. THE CHEMISTRY OF GFP

GFP was first described by Shimomura et al. in 1962 and obtained from the jellyfish *Aequorea Victoria*. [155] Over the last 20 years Green Fluorescent Protein (GFP) was widely used for tagging proteins, screening protein expression, investigating cell signalling and many other possible applications. [156] [157] [158] The structure of the chromophore was first discovered by Shimomura in 1979. [159] Three amino acids (Ser65 – Tyr66 – Gly67) undergo an autocatalytic reaction resulting in 4-hydroxybenzylidene imidazolinone (HBI) [160], albeit the last step (α,β -double bond of Y66) requiring molecular oxygen and thus being the time limiting step with 2h for WT. [161] The reaction is promoted by the formation of a β -barrel consisting of 11 β -sheets. [162] Tyr66 is not necessary for the cyclization and may be replaced by tryptophan for cyan FP, histidine for blue FP [156], or other residues obtaining non-fluorescent variants. [163] Nevertheless Tyr66 is conserved in all natural fluorescent proteins. [164] Undoubtedly it has an important role in the excited-state proton transfer. [165] Studies with GFP revealed a network of hydrogen bonds and the solution to why GFP has two different absorption/emission bands: State B (phenolate - excitation 470-480nm) shows usual photo physics; state A (phenol - excitation 390-400nm) however, upon excitation changes its pK_a to near zero, becoming strongly acidic and transferring its proton via the hydrogen bond network. (see figure 13) [166] In modified FPs this mechanism is partially blocked resulting in dual emission GFPs (deGFPs) which are able to act as pH-indicator. [167] [168] [169]

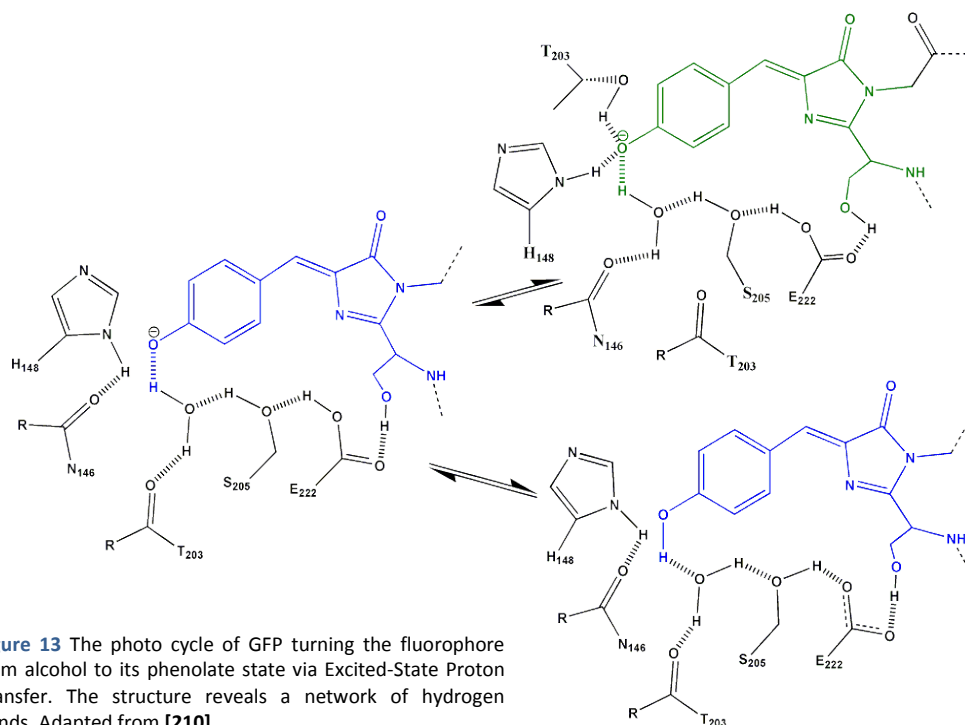


Figure 13 The photo cycle of GFP turning the fluorophore from alcohol to its phenolate state via Excited-State Proton Transfer. The structure reveals a network of hydrogen bonds. Adapted from [210]

An important aspect concerning the advantage of embedding the chromophore into a protein might be the instability of anionic HBI derivatives in water and E/Z-isomerisation at the α,β -double bond due to its low energy barrier. [170] GFP was improved by mutational experiments resulting in a 35 times brighter signal by enhanced GFP (eGFP). [171] In addition the whole rainbow of colours is available today. [164] [172] Designing multiple FPs with different absorption and emission wavelengths is interesting for two reasons: First multilabeling is possible and second corresponding pairs of FPs can be used as FRET donor and acceptor respectively as has been shown simultaneously by two different groups for intracellular Ca^{2+} sensors. [173] [174]

It should be mentioned that in any case the dehydration of the Y66 α,β -bond remains the time limiting step taking still 30 minutes in the improved S65T (eGFP) variant. [175] This has to be considered when using fluorescent proteins for cell signalling and other time dependent applications requiring newly translated fusion proteins.

For *in vivo* experiments cytotoxicity also has to be considered. In case of FPs the critical parameter is the production of reactive oxygen species (ROS) during fluorescence [176], especially $^1\text{O}_2$, also responsible for photobleaching damage. Molecules and proteins intercepting ROS can prolong the viability of cells. [177] Last-mentioned there is also a protein termed KillerRed which is purposeful used to inactivate fused proteins or killing cells within minutes upon excitation. [178]

1.5.2. SPINACH AND SPINACHLIKE APTAMERS

Synthetic HBI is not fluorescent.

[179] The same holds true for unfolded, denatured GFP [180]

Using SELEX-experiments [181]

Paige, Jaffrey and co-workers accomplished the selection of RNA-

aptamers binding to HBI and derivatives. Depending on both the

small molecule and the RNA-

Aptamer different colours are accessible, resulting in a broad

spectrum similar to known FPs and in one instance, with a quantum

yield of almost 50% of the landmark eGFP. This aptamer termed “Spinach” (24-2, with minimum structure

24-2-min) binds specifically the phenolate anion of 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) resulting in enhanced emission of fluorescence. [182] The fluorophore is bound in the major loop of spinach, which is surrounded by three stem-loops. Especially the second one is essential for the stability of the aptamer. This property has very recently been used to detect the levels of cellular metabolites by replacing the latter stem by another aptameric



Figure 14 The 2D structure prediction of the Spinachaptamers, which preferable bind to the phenolate anion of a HBI derivate. [182]

sequence specifically binding a metabolite of interest and therefore stabilizes the whole construct enabling spinach to bind to DFHBI and become a fluorescent metabolite-reporter. [183]

We decided to use the 24-2min sequence, since it shows almost the same properties as 24-2, however, as discussed before, viruses show limited capacity concerning inserts and less nucleotides reduce the chance of unwanted base pairing. Furthermore we determined four possible sites for the insertion of the 24-2min sequence in non-coding regions, since the ORF was not an option due to the sequence containing several stop codons. The first uracils could be separated from the cloverleaf resulting in an insert before (*CL*). The spacer region after the cloverleaf (*SP*) and before the IRES was found to be highly variable and was therefore the region of choice. In poliovirus PCBP binds to these nucleotides, however no evidence was found for that in rhinovirus. Still this region might affect the pathogenic potential of an individual HRV. [184] The same variability was found for the 3'-UTR where an insert site could be before the terminal stem-loop (*SM*) or after the stem-loop before the poly-A (*PA*). No function for the 3'-UTR terminal sequence was determined so far, despite the conservation of the structure in HRV. [184]

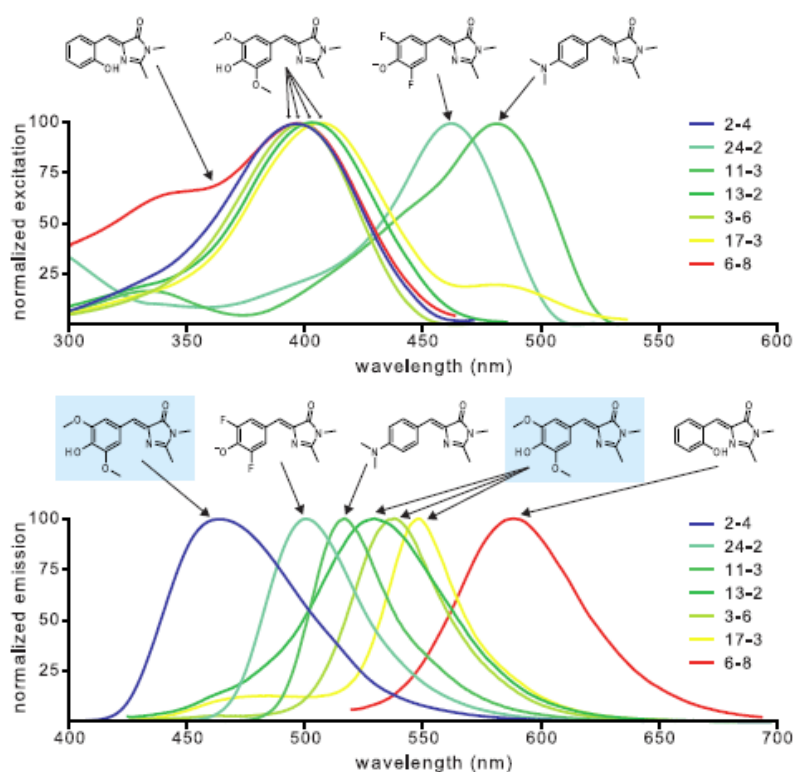


Figure 15 Taken from [182]: Available RNA-Fluorophore constructs resulting in the corresponding excitation (above) and emission (below) spectra. In case of DMHBI (Dimethoxy-HBI; blue shading) the used aptamer determines the physical properties.

1.6. AIM OF THE WORK

The main project termed “Spinach” dealt with the development of spinach-viral-RNA hybrids. This included the identification of proper target insertion sites in the genome of the HRV-14, used as a model throughout these studies, with especially avoiding unwanted base pairing, which could destroy secondary structures of either the RNA aptamer or the virus. Additionally unique restriction sites were added, which will facilitate future cloning of new spinach-like aptamer generations into the same positions as chosen in this project. These positions were limited to non coding regions since the currently available aptamers contain several stop codons and therefore a cloning into the open reading frame was not an option.

The side project termed “Flash” was developed later, when we thought it would be beneficial to also be able to track down virus capsid proteins of newly produced virus and their fate in real-time by live cell imaging. Therefore it was searched for not conserved loops (in terms of known rhinovirus sequences) in different capsid proteins and the recombinant sequences were designed. The modification was done with PCR and the PCR product religated into the original plasmids.

If a system like recombinant capsid shell with recombinant RNA (a TC-Spinach-HRV14 hybrid) can be fully developed it would theoretically allow visualizing the entry pathway of a rhinovirus, its replication and assemble. This would allow for example co localisation studies of FIAsh-tagged capsid protein with Spinach-tagged viral RNA. Proof of principle would gain a new possibility to reveal unexplored facts of viruses in general. Furthermore in contrast to other systems both the TC-tag and the 24-2 Spinach aptamer are encoded within the viral genome and therefore it should be possible to visualize replicated virus as well. That means that a whole infection cycle, including infection, replication and assembly could be detected in live cell imaging by using just one system, which, to our knowledge, has not been done yet before.

2. MATERIALS AND METHODS

2.1. DNA/RNA MATERIALS, PRODUCTION AND MODIFICATION

2.1.1. HRV14 PLASMIDS

For all experiments with HRV14 a pSP72 plasmid was used, which contains a full length HRV14 cDNA (Gen Bank Accession Number L05355) with a single silent mutation 3472 G->A originally constructed by Tim Skern [94] and kindly provided by David Neubauer. For the complete sequence see appendix.

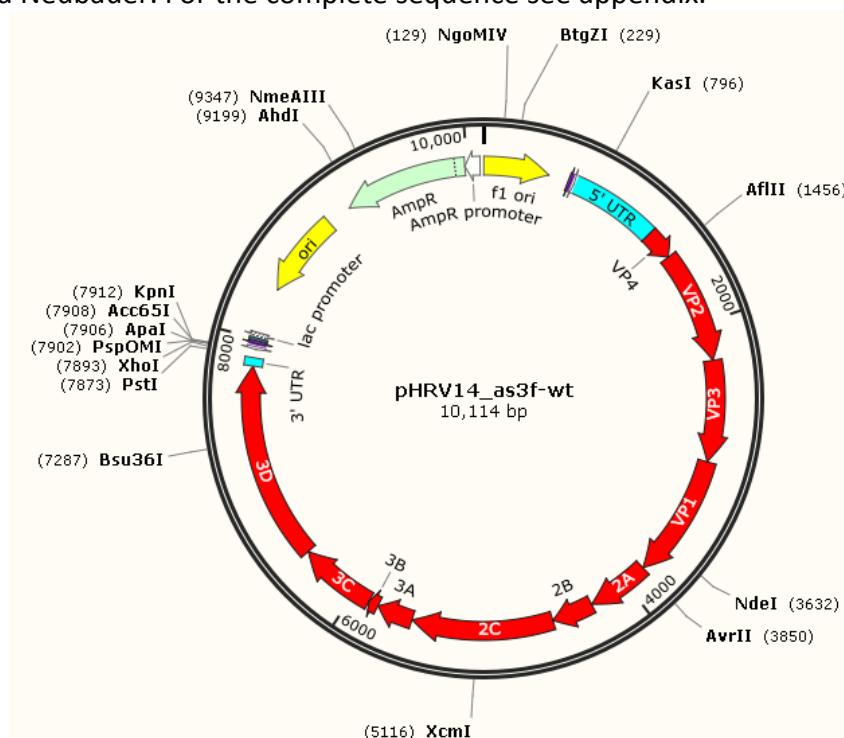


Figure 16 Schematic representation of pHRV14-as3f-WT as it was produced by Skern et al.; [94] Image made with SnapGene

2.2. SECONDARY STRUCTURE PREDICTION OF 24-2/HRV14 HYBRIDS

All secondary structure predictions were done with the “Vienna RNA Websuite” [185] [186]. The exact insertion positions were optimized in terms of probability of incorrect base pairing using the parameters by Turner [187] [188] and Andronescu [189] allowing GU-base pairs at the end of helices and avoiding single base pairs. The simulation temperature was set at 37°C.

The corresponding cDNA sequences were synthesised by Invitrogen using their GeneArt® gene synthesis service. They were delivered within different plasmids containing an ampicilin resistance gene.

2.3. PROTEIN SEQUENCE MODIFICATION AND INSERTION MUTAGENESIS BY OVERLAP EXTENSION PCR

The following oligonucleotide primers for overlap extension PCR-mediated synthesis of the various TC-Tag cDNA sequences were ordered from Sigma-Aldrich (*Steinheim, Germany*). The red letters are the tag-related sequences.

Primer	Sequence	nt
VP1-TC 5' fw	CCAAAAGAGTGGGCGATTACACATGGCAAAGTGCTTC	37
VP1-TC 5' rv	GCAGCATCCCGGGCAACAATTCTGTATTCTAGGATAATTTGTGCGCCCTATTGATGTGTAGGGT	64
VP1-TC 3' fw	TGTTGCCCGGGATGCTGCTAATTAAGAAGAGGAAAGGTGACAT	44
VP1-TC 3' rv	ATCCAACCTACCTCCAGCTGTTA	21
VP1-FLN 5' fw	CGAAATCCAAAAGAGTGGGCGATTAC	26
VP1-FLN 5' rv	CATGCAGCATCCCGGGCAACAGTTGAGGAAATTCTTAGGATAATTTGTGCGCCCTATTGA	60
VP1-FLN 3' fw	TTCTCAACTGTTGCCCGGGATGCTGCATGGAACCAAGTAATTAAGAAGAGGAAAG	55
VP1-FLN 3' rv	ATGTCCTCCTGTTGAGACTA	20
VP1-HRW 5' fw	GGCGATTACACATGGCAAAGTGCTTC	26
VP1-HRW 5' rv	AAACGTCTTGAGCATGGGCCACACAGCTGTGATTCCTTAGGATAATTTGTGCGCCCTATTGA	63
VP1-HRW 3' fw	AATCACAGCTGGTGTGGCCCATGCTGCAAGACGTTTATTAAGAAGAGGAAAGGTGACATTAAATC	65
VP1-HRW 3' rv	ACCATGTCCTCCTGTTGAGACTA	23
VP2-Var1 5' fw	CATCATCAGCTGGTCAATCACTGTC	25
VP2-Var1 5' rv	GCAGCATCCCGGGCAGCATGCAGATGATAAATCTATACCACGTTAC	48
VP2-Var1 3' fw	TGCTGCCCGGGATGCTGCAATGAAGTGGGAGGCCTGTCAAGGATGTC	47
VP2-Var1 3' rv	GCGCCCTATTGATGTGTAGGGTAGTGCTCTG	31
VP2-Var2 5' fw	CATCAGCTGGTCAATCACTGTC	22
VP2-Var2 5' rv	GGGCTCCATGCAGCACCCGGGGCAGCAGTTCAGAAAATGCAGATGATAAATCTATACCACGTTAC	65
VP2-Var2 3' fw	TTTCTGAAGTGTGCCCCGGGTGCTGCATGGAGCCCAATGAAGTGGGAGGCCTGTCAAGGATGTC	66
VP2-Var2 3' rv	GCGCCCTATTGATGTGTAGGGTAGTGCTCTG	31
VP2-Var3 5' fw	GAATGCGGCTAACCTTAACCTAGAG	26
VP2-Var3 5' rv	AAACGTCTTGAGCACCCCGGACAAACACAGCTGTGTGCAGATGATAAATCTATACCACGTTAC	66
VP2-Var3 3' fw	CACAGCTGGTGTGTCCGGGTGCTGCAAGACGTTTATGAAGTGGGAGGCCTGTCAAGGATGTCATA	69
VP2-Var3 3' rv	TGTAGGGTAGTGCTCTGGGTGCTCTTGAA	30

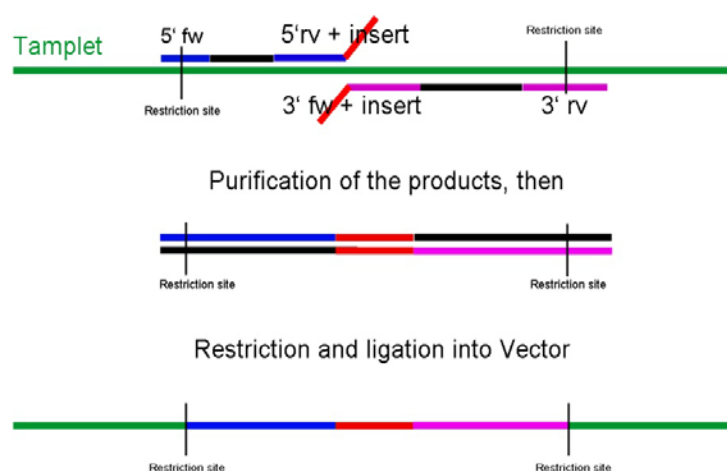


Figure 17 Graphical representation of the overlap PCR. In Step 1 two PCRs are done amplifying the strand between the insert position and the next unique restriction site. The 5' reverse and the 3' forward primer contain the insert (complementary) sequence. The product is cleaned from primers and put together in a next PCR step where the insert serves as a primer for the complementary strand. Then the 5' forward and 3' reverse primer are added to obtain more products. Finally it is cloned into the vector via the unique restriction sites.

2.4. POLYMERASE CHAIN REACTIONS

All PCR reactions were done – if not stated otherwise - with 1U *Pfu* DNA polymerase from Promega (Madison, USA) in Pfu-Buffer with dNTP-Mix (Promega) in a final concentration of 200µM for dATP, dCTP, dGTP and dTTP each, 0.5µM of each primer and 10ng-50ng of DNA template.

2.4.1. 5' AND 3' PCR PRODUCTS

PCR program for TC-tags designed for insertion into VP1:

1.	95°C	10 min
2.	95°C	30 sec
3.	65°C	30 sec
4.	72°C	3 min
5.	Go to 2.	30 cycles

VP2 primers needed 10% DMSO (Sigma-Aldrich, Madison, USA) additionally and the setup of a touchdown PCR, which were used to obtain better PCR results for difficult reactions before. [190] [191]

PCR program for TC-tags designed for insertion into VP2:

1.	95°C	10 min
2.	95°C	30 sec
3.	68°C [-0.3°C/cycle]	30 sec
4.	72°C	5 min
5.	Go to 2.	30 cycles

Removal of excess primers, important for the subsequent hybridization step, was done with E.Z.N.A. Cycle Pure Kit (*OmegaBiotek, Norcross, USA*) according to the manufactures protocol.

2.4.2. OVERLAP EXTENSION OF 3' AND 5' CONSTRUCTS

Several different procedures for the initial hybridization of 3' and 5' PCR products have been tried. However the one described here in the end gave the best results. 0.1 nmol of both 3' and 5' PCR product were mixed with buffer, 200µM dNTP and 1U *Pfu* DNA polymerase. Before step 10 the same amount of dNTP and DNA polymerase were added to the reaction.

1.	95°C	10 min	8.	72°C	5 min
2.	95°C	30 sec	9.	Go to 6.	15 cycles
3.	68°C	2.5 min	10.	95°C	30 sec
4.	72°C	5 min	11.	68°C [-0.3°C/cycle]	30 sec
5.	Go to 2.	15 cycles	12.	72°C	6 min
6.	95°C	1 min	13.	Go to 10.	35 cycles
7.	55°C	2.5 min			

The desired PCR product obtained in above hybridization/amplification reaction was re-amplified as follows: 0.5µl of this reaction was transferred into a new PCR reaction with 5' forward and 3' reverse primers or additionally designed helper primers.

VP1 fw	CATGGCAAAGTGCTTCAAAC	for amplification of VP1 PCR hybrids
VP1 rv	GATGTATAAATCCCACCGTACC	
VP2 fw	CATCAGCTGGTCAATCACTGTC	for amplification of VP2 PCR hybrids
VP2 rv	CGTGCTTTGCCCTGTGATAAAC	

1. 95°C 10 min
2. 95°C 30 sec
3. 65°C 30 sec
4. 72°C 7 min
5. Go to 2. 30 cycles

The final constructs were purified via gel electrophoresis. (see 2.6.2.)

2.5. ROUTINE (PLASMID) CLONING

2.5.1. PREPARATION OF ELECTROCOMPETENT CELLS

An overnight culture of transformed E. coli was transferred into 1 volume of fresh LB-medium and grown to an OD600 of 0.6-0.8. The culture was put on ice for 45 minutes and centrifuged for 3 minutes with 4000g at 4°C. The supernatant was discarded, the pellets resuspended in cold autoclaved Millipore water and centrifuged again under the same conditions. The resulting pellet was resuspended in 0.05 volume of cold 10% glycerol and centrifuged for 10 minutes with 4000g at 4°C. The pellet was finally resuspended in 0.003 volumes of cold 10% glycerol, aliquots were shock frozen in liquid nitrogen and stored at -80°C. [192]

2.5.2. ELECTROPORATION

Electrocompetent cells prepared as described above were slowly thawed on ice. A 50µl aliquot was gently mixed with 50-100ng of plasmid DNA. Electroporation was done using 2mm cuvettes (PeqLab, Erlangen, Germany). The pulse was set at 2.5 kV and 25µF resulting in a time constant of 3 to 5 msec. LB-Medium was then added immediately to the cells followed by 45 min incubation at 37°C before spreading the transformed bacteria on an appropriate antibiotic-containing LB-plate.

2.5.3. PLASMID ISOLATION FROM BACTERIA

Plasmids were isolated from transformed Bacteria grown overnight in LB-Medium containing 100µg/ml ampicilin. For isolation OmegaBiotek's E.Z.N.A. Plasmid Mini Kit II was used. Bacteria were pelleted and treated according to the manufactures protocol.

Both inserts contain an unique restriction site (Spinach: SacI, TC-Tag: XmaI) not present in pHRV14-as3f-WT, which was used to quickly verify by restriction digest (see 2.6.4), whether the purified plasmid was correct. It was then sent for sequencing (see 2.6.) for final confirmation of the inserts authenticity.

2.6. DNA/RNA STANDARD METHODS

2.6.1. DNA/RNA SEPARATION

Agarose was obtained from GenXpress (Vienna, Austria). Depending on the size of fragment(s) to be separated, a 1%, 2% or 3,5% gel was prepared by dissolving the respective amount of agarose in hot 1xTAE (4,84 g/l Tris, 1,20 g/l acetic acid, 1mM EDTA), which was also used as running buffer (at room temperature). Markers were from Biozym (Quantitas DNA Marker 200bp-10000bp), Promega (100bp DNA ladder) and NEB (100bp DNA ladder). Loading dye was from Invitrogen. DNA/RNA separation was done at 90V until the front reached half of the gel front. Gels were put into 1% ethidiumbromid solution for 20 minutes. Gels were imaged with GelDocIt-Imaging System (UVP, Upland, USA)

2.6.2. DNA GEL EXTRATION

Gel Extraction was done from an 0,75% gel dissolved in hot TAE Buffer containing 1M Guanosine-HCl which was dissolved in DMSO (final concentration of DMSO: 5%) to prevent damage by UV-light. The DNA was extracted with E.Z.N.A. Gel extraction Kit (*OmegaBiotek, Norcross, USA*) according to manufactures protocol.

2.6.3. DNA/RNA PHENOLEXTRACTION AND PRECIPITATION

Contaminating proteins were removed from DNA/RNA preparations by extraction samples twice with phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10mM Tris, pH 8.0, 1mM EDTA (Sigma-Aldrich). DNA/RNA were precipitated by adding 0.1 volumes of 3M NaAc and 1 volume of 2-propanol. Samples were placed at -80°C for 10 minutes and then centrifuged at 13000 g, 4°C for 10 minutes. The pellet was washed with cold 70% ethanol and centrifuged again as before. The pellet was air dried and resuspended in nuclease free water (QIAgen)

2.6.4. RESTRICTION DIGESTS AND LIGATION

Restriction digests were done with all enzymes obtained from New England Biolabs (NEB, Ipswich, USA) within their recommended buffer system. For double digest their online tool “Double Digest Finder” (www.neb.com) was consulted to find the optimal conditions. If not otherwise mentioned digest was done at 37°C for 2 hours followed an inactivation period of 20 minutes at 80°C using either NEBuffer 3 or 4 and if recommended 1% BSA (NEB).

Bsu36I, PstI (NEBuffer 3 + BSA) Kasi, NgoMIV, AflII, NdeI, AvrII (NEBuffer 4 + BSA) To check for the inserts in case of Spinach SacI in case of TC-Tag XmaI (both NEBuffer 4) were used.

Ligation was done using T4-Ligase (Promega) in a 10µl reaction with 100ng of vector and a 1:3 excess of insert at 16°C overnight.

2.6.5. RNA TRANSCRIPTION

For *in vitro* transcription of viral RNA from cDNA under the control of a T7 promoter, the recombinant plasmid was initially cut with PstI located immediately after the insert’s poly-A sequence. The 3’ overhang was blunted by incubation with the “Large Klenow Fragment” (NEB, Ipswich, USA) in the presence of 100µM dNTPs at 25°C for 1h. Enzymes were removed by phenolextraction as described before. DNA was diluted in a final concentration of 125ng/µl.

RNA was *in vitro* transcribed from linearized template DNA using the RNA T7 RIBOMAX™ Express large Scale RNA Production System (Promega) according to manufacturer’s protocol. This includes also a treatment with DNase to digest the DNA template.

2.6.6. DNA/RNA CONCENTRATION MEASUREMENT

DNA/RNA concentrations were measured with Nanodrop ND-1000 (PeqLab)

2.6.7. SEQUENCING AND SEQUENCING PRIMERS

Every DNA modification was controlled by sequencing (LGC genomics, Teddington, Middlessex, UK) using the following primers:

primer	sequence	remarks
3' seq	CCCAGAATACAGCGTACTTAGG	+/-strand sequencing of 3'-UTR
5' seq	ATACCTCGCTCCTGGGAAAC	specific RT-Primer for 5' end (instead of oligo dT)
SP fw	GGATGGGTATCCACCAT	sequence primer for SP constructs
VP1 seq	CACCCAGAGCACTACCCTAC	for sequencing of VP1 insert region
VP2 seq	AGAACACCACTGGCTTAC	for sequencing of VP2 insert region

2.7. TISSUE CULTURE METHODS

2.7.1. CELLS, CULTURE MATERIALS AND BASIC TECHNIQUES

For all experiments Hela Ohio cells (European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK) were used grown in Culture Medium [DMEM with phenolred (Gibco, Paisley, UK) supplied with 10% FCS (Sigma-Aldrich, Taufkirchen, Germany) and 1% PenStrep (Gibco, Paisley, UK)] at 37°C with 5% CO₂-Level.

Almost confluent cells were washed with PBS and harvested with 1ml/25cm² Trypsin (PAA, Pasching, Austria) for 5 minutes at 37°C. Thereafter 3 volumes of Culture Medium were added and thoroughly mixed by pipetting several times up and down to dissociate cell clumps. If not otherwise mentioned cells were counted in a Thoma-chamber and diluted to obtain the following cell number in the respective volume per containment:

96-well plate	1000 cells/well	100 µl
12-well plate	10 ⁵ cells/well	1 ml
6-well plate	3x10 ⁵ cells/well	2.5 ml
T-25 flask	7x10 ⁵ cells	5 ml
T-75 flask	1,5x10 ⁶ cells	12 ml
T-162 flask	3x10 ⁶ cells	25 ml
T-225 flask	5x10 ⁶ cells	50 ml

All culture plastics were obtained from Corning (Amsterdam, The Netherlands).

2.7.2. TRANSFECTION OF HELA CELLS WITH VIRAL RNA & HARVESTING VIRUS

RNA-Transfection was done with Lipofectamin 2000 (Invitrogen, Paisley, UK) in a 12-well plate. Cells were seeded in Culture medium the day before. On the day of transfection medium was replaced by Opti-MEM (Gibco, Paisley, UK). 1.5 µg *in vitro* transcribed virus RNA and 3 µl of Lipofectamin were each diluted in 100 µl Opti-MEM, followed by gently shaking the tubes for 5 minutes. The tube contents were combined, mixed and incubated for half an hour at room temperature. Finally, the mixture was added to each well containing cells and media (Opti-MEM) and the plates were incubated for 24 hours at 37°C and 5% CO₂. (Recombinant) HRV14 produced by the transfected HeLa cells was harvested by freeze-thawing 3 times followed by centrifuging at 2,000g for 5 minutes to remove the cell debris.

2.7.3. PLAQUE ASSAY

HeLa cells were seeded in a 12-well plate the day before. On the day of infection, adherent cells were washed with PBS and incubated for 4h with virus-containing supernatant diluted 1:1 with infection medium [DMEM without Phenol red, 2% FCS, 30mM MgCl₂ (Sigma-Aldrich, Steinheim, Germany)]. The medium was aspirated and replaced by a 1:1 mixture of 2x infection medium and melted 2% low-melting agarose (Sigma-Aldrich, Steinheim, Germany) cooled down to 37°C. Plates were incubated for 2 days at 34°C and subsequently stained with 0.05% Neutral red (Sigma, St. Louis, USA). Plaques were picked with a sterile Pasteur pipette and resuspended in infection medium. To visualize the cytopathic effect (CPE) and plaques, respectively, for documentation by photography, plates were stained with crystal violet (Merck, Darmstadt, Germany).

Essentially the same procedure was used to show virus production immediately after transfection. Transfection was done as described before, but only for 4 hours. Cells were washed with PBS, overlaid with low-melting agarose and plaques identified by staining with crystal violet.

2.7.4. VIRUS PRODUCTION

(Recombinant) virus picked from plaques was used to infect a new 12-well plate of HeLa Ohio cells. Plates were incubated at 34°C until complete lysis of cells, but not more than 5 days. Plates were freeze-thawed 3 times, the lysates of each well combined and centrifuged at 2,000g for 5 minutes; the supernatant was mixed 1:1 with infection medium and used to inoculate a 6-well plate. After complete lysis, the cycle of freeze-thawing and centrifugal clearance was repeated. Collected virus was then amplified by infection of cells seeded in a T-25, a T-75 flask and finally a T-162 flask. After each scaling-up step, virus was released and crudely purified essentially as described for the tissue culture plate format. Virus obtained from the T-162 flask was eventually pelleted in an Optima L-80 XP Ultracentrifuge (Beckman, Fullerton, USA) with a Type 45 Ti rotor at 186,000g for 2 hours at 4°C. The (recombinant) HRV14 was resuspended in a minimum amount of PBS (about 0.5ml) and stored at -80°C (= working stock).

Determination of virus concentration was done by a TCID₅₀ assay. A 96-well plate was infected with different dilutions of one virus preparation (12 wells for each dilution). Cells were incubated for 5 days at 34°C, stained with crystal violet and the number of wells with completely lysed cells evaluated for each virus dilution. The number of PFU/ml was then obtained from Poisson statistics.

$$(\text{PFU/ml} = 0.7 \times \text{TCID}_{50}/\text{ml})$$

2.8. FLUORESCENCE METHODS

2.8.1. RNA FLUORESCENCE ASSAY

For recording emission fluorescence spectra of RNA-DFHBI complexes a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Sunnyvale, USA) was used. RNA was dissolved in PBS with 30mM MgCl (final concentration of RNA: 1µg/100µl) and 20µM 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI, Lucerna Inc., New York, USA) [stock solution: 20mM in DMSO]. For each RNA species three wells were excited at 465nm and the mean spectrum was plotted from 485nm to 550nm.

2.8.2. FLUORESCENCE MICROSCOPY

Standard fluorescence microscopy was done on an Axiovert 200M Inverted Fluorescent Microscope, confocal microscopy was done on an LSM710 (both Zeiss, Oberkochen, Germany)

Cells (3,000/well) were seeded on Lab-Tek Chamber Slides (Nunc, Roskilde, Denmark) the day before imaging. Cells were either transfected with RNA (according to the previous protocol) or infected with virus. Imaging was done in infection medium or PBS with 30mM MgCl₂ in the presence of 20µM DFHBI. Counterstaining of nuclei was done with Hoechst 33342 (Invitrogen, Paisley, UK). For imaging spinach the standard GFP filterset was used. In case of LSM710 for Hoechst 33342 a 405nm laser, for the excitation of DFHBI a 458nm laser was used.

Images were recorded in autoexposure mode and processed with Axiovert 4.1.

3. RESULTS

3.1. THE SPINACH PROJEKT

3.1.1. STRUCTUR PREDICTION AND SEQUENCE OF SPINACH/HRV14 HYBRIDS

Fusion of the SPINACH aptamer sequence to the 3' end of the small noncoding 5S RNA resulted in its fluorescent detection after transfection of cells and staining with DFHBI, with a distribution as observed for the corresponding endogenous RNA. [182] We therefore reasoned that engineering human rhinovirus RNA with SPINACH might similarly allow its direct and facile fluorescent live-cell imaging in infected cells.

As model for these experiments we have chosen the well-characterized major group serotype HRV14, and the minimal, fully functional, Spinach aptamer sequence 24-2 min for tagging of the genomic RNA. [182] Its size of ca. 80 nucleotides is well within the approx. 5-8% increase of the ~7,000 to 8,500 nucleotide long genome tolerated for productive packaging into the picornavirus capsid. [193] High virus infectivity requires intact 5' and 3' ends [194] [31], which excludes direct fusion of SPINACH with the termini of the genomic RNA as the obviously simplest approach. Translation of the aptameric sequence indicated the presence of at least one stop codon in each of the three possible reading frames, limiting the choice of insertion sites to the 5'- and 3'-UTR of the viral RNA. A further restriction was avoidance of primary sequences known to be involved in the regulation of translation and/or plus and minus strand replication. These cis-elements are mostly distributed in conserved secondary structures such as the cloverleaf and IRES sequence in the 5'-UTR and the stem-loop in the 3'-UTR.

By taking into account these additional constraints, this resulted in the identification of four distinct contiguous small stretches of non-coding virus RNA for insertion of the Spinach aptamer (see figure 18), with either unknown or possibly only minor impact (inferred from the literature and/or the considerable nucleotide polymorphism of these sites within rhinovirus serotypes) on the viability of HRV14 [116] [195] [196]. An exception is the region between the 3' stem-loop and poly(A) tail, which did not tolerate insertion of a small oligonucleotide [116]. Since the reason was not further investigated, this stretch was nonetheless chosen as potentially suitable target site for Spinach.

To prevent perturbation of vital secondary structure elements, the folding pattern of each in silico aptamer-tagged HRV14 sequence was predicted using RNAfold [185]. Only such combinations were selected, which maintained (almost) correct folding of the HRV14 body and the incorporated Spinach (24-2 min) sequence. Evidently, disruption of crucial tertiary interactions and/or a possible steric blockage of factors binding to any of the cis-regulatory elements by the nearby aptamer sequence cannot be excluded by this still relatively undeveloped analysis method.

Each possible combination of SPINACH aptamer flanked by the respective proximal HRV14 sequences (including distal restriction sites required for engineering the viral cDNA) inclusively its predicted secondary structure is shown in Figures 19 to 22.

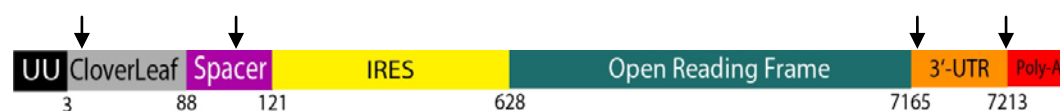


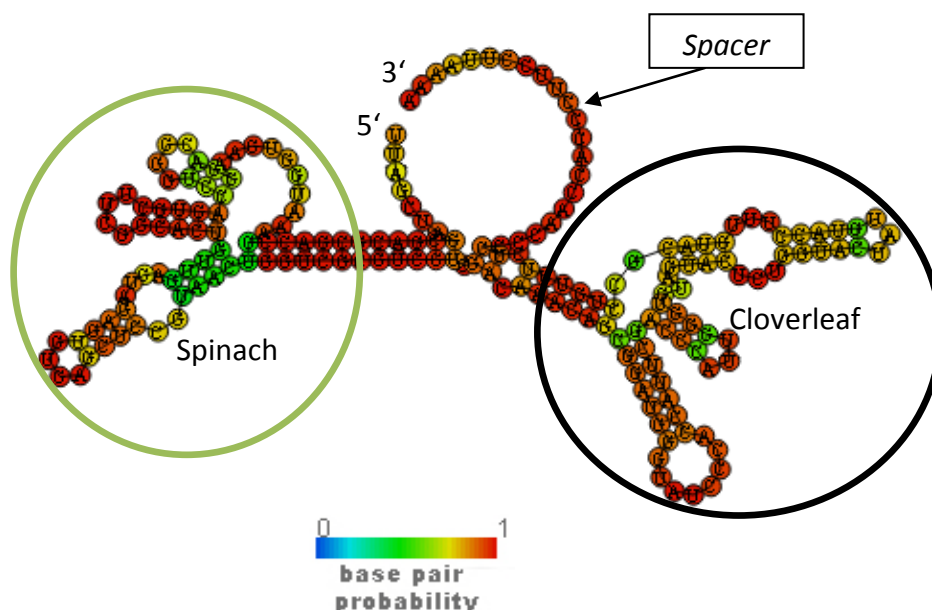
Figure 18 Schematic representation of the HRV Genome. possible insertion sites were: After the start uracils at position 3; within the spacer or in the 3'-UTR. Picture drawn with Adobe Photoshop

3.1.1.1. 5'-UTR PRECLOVERLEAF REGION

GCCGGCTTT...(for full sequence see attachment)**...GCGTAATACGACTCACT**
ATAGGTTAGCTAGCGACGCGACCGAAATGGTGAAGGACGGGTCCAGTGCTTCGGCACTGTTGAGTA
GAGTGTGAGCTCCGTAACTGGTCGCGTC**GTCGAC**AAACAGCGGATGGGTATCCCACCATTTCGACCC
ATTGGGTGTAGTACTCTGGTACTATGTACCTTTGTACGCCTGTTTCTCCCAACCACCCTTCCTTA
AAATTCCCACCCATGAAACGTTAGAAGCTTGACATTAAAGTACAATAGGT**GGCGCC** (765 nt)

Green: spinach (24-2 min); Gray: cloverleaf; Pink: T7-promoter; light gray: viral start nucleotides
Bold and Underlined: Sites for restriction enzymes in series: NgoMIV, NheI, Sall, KsaI

Figure 19
 Prediction I with RNAfold of the sequence above. According to the simulation both the cloverleaf and the spinach aptamer stay intact and do not interact with bases of the spacer region



3.1.1.2. 5'-UTR SPACER REGION

GCCGGCTTT...(for full sequence see attachment)...GCGTAATACGACTCACT
 ATAGGTAAAAACAGCGGATGGGTATCCCACCATTGACCCATTGGGTGTAGTACTCTGGTACTATG
 TACCTTTGTACGCCTGTTTCTCCCCAACCA**CGGTCCG**GACGCGACCGAAATGGTGAAGGACGGGTC
 CAGTGCTTCGGCACTGTTGAGTAGAGTGTGAGCTCCGTAACGGTCGCGTC**CCCGGGTTCTTAAA**
ATTCCCACCCATGAAACGTTAGAAGCTTGACATTAAAGTACAATAGGT**GGCGCC** (763 nt)

Gray: UU + CloverLeaf; Pink: Spacer; Green: 24-2 Aptamere; Yellow: IRES
Bold and Underlined: Sites for restriction enzymes in series: NgoMIV, RsrII, XmaI, KasI

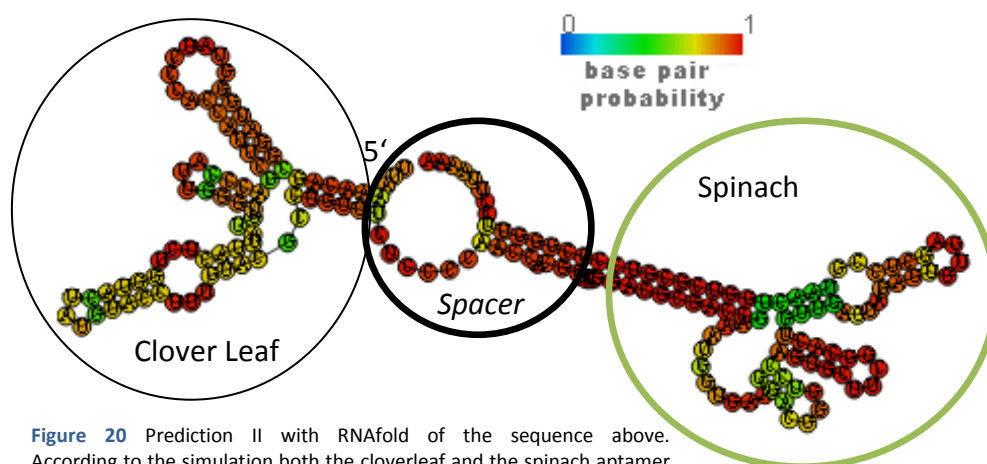


Figure 20 Prediction II with RNAfold of the sequence above. According to the simulation both the cloverleaf and the spinach aptamer stay intact and do not interact with bases of the spacer region.

3.1.1.3. 3'-UTR FOLLOWING ORF

CCTCAGGGTG...(for complete sequence see attachment)...TAGGTTAACA
GCTAGCGACGCGACCGAAATGGTGAAGGACGGGTCCAGTGCTTCGGCACTGTTGAGTAGAGTGTGA
GCTCCGTAACTGGTCGCGTC**GTCGACA**TATAGACACTTAATTTGAGTAGAAGTAGGA**TTTAT**AAA
 AAAAAAAG**CCTGCAG** (681 nt)

Blue: ORF (Polymerase); Green: (24-2 Spinach Aptamere); Yellow: 3'-Structure; Violet: For replication necessary sequence
Bold and Underlined: Sites for restriction enzymes in series: Bsu36I, NheII, SalI, PstI

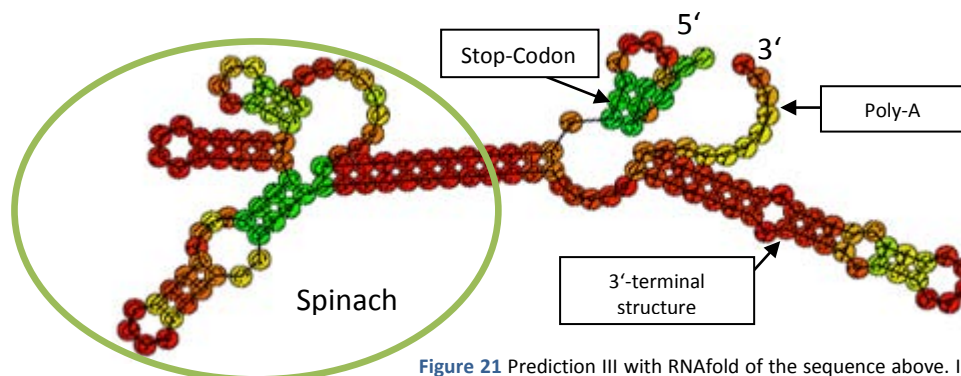


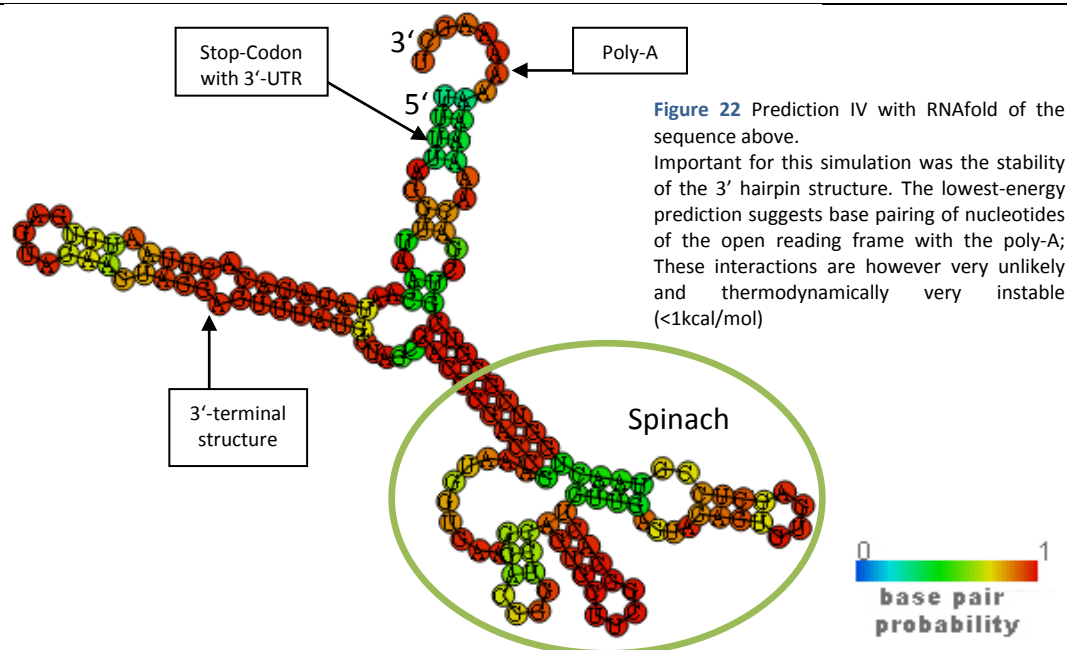
Figure 21 Prediction III with RNAfold of the sequence above. Important for this simulation was the stability of the 3' hairpin structure

3.1.1.4. 3'-UTR PRE-POLY-A POSITION

CCTCAGGGTG...(for complete sequence see attachment)...**TAG**GTTAACA
ATATAGACACTTAATTTGAGTAGAAGTAGGAGTTTATGCTAGCGACGCGACCGAAATGGTGAAGGA
CGGGTCCAGTGCTTCGGCACTGTTGAGTAGAGTGTGAGCTCCGTAACCTGGTCGCGTCGTCGACAAA
 AAAAAAAG**CTGCAG** (681 nt)

Blue: ORF (Polymerase); Green: (24-2 Spinach Aptamere); Yellow: 3'-Structure; Violet: For replication necessary sequence

Bold and Underlined: Sites for restriction enzymes in series: Bsu36I, NheII, SalI, PstI



3.1.2. PLASMID CONSTRUCTION

All 4 ordered sequences could be successfully relegated into a cut vector resulting in the modified plasmids: pHRV14-as3f-CL (aptamer before the cloverleaf), pHRV14-as3f-SP (aptamer within the spacer region), pHRV14-as3f-SM (aptamer before the spacer) and pHRV14-as3f-PA (aptamer before the poly-adenosine-tail). The correct sequence for all non-coding regions was confirmed by LGC genomics (Teddington, Middlesex, UK) using the following sequencing primers:

pHRV14 RNA fw	CCCAGTCACGACGTTGTAAA	5'-UTR sequencing
pHRV14 RNA rv	CGCGCAATTAACCCTCACTA	3'-UTR sequencing

All four plasmids were transcribed into RNA successfully as confirmed by electrophoresis.

3.1.3. FLUORESCENCE SPECTRUM OF RNA-DFHBI COMPLEXES

The examination of RNA-DFHBI complexes revealed, that 3 out of 4 constructs showed promising fluorescence spectra. The maximum for all constructs was in the range from 501nm (HRV PA) to 503nm (HRV SM) which coincides with spinach-RNA hybrid spectra published by Paige et al. Interestingly the different constructs show different fluorescence intensity at the same concentrations (1 μ M RNA). The Pre-3'-StemLoop (SM) construct is about 1.5 times brighter than the Spacer (SP) variant.

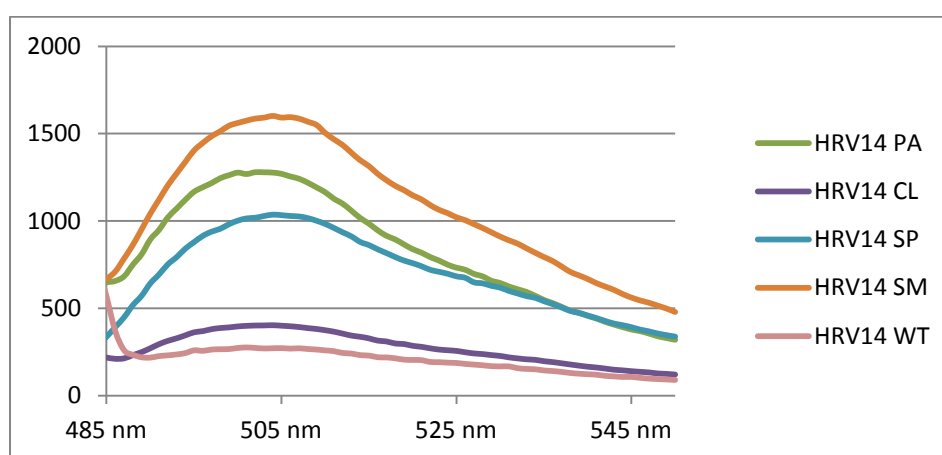


Figure 23 Spectra of RNA-DFHBI complexes. The aptamere was inserted at different positions: PA=pre poly-A; CL=pre cloverleaf; SP=Spacer region; SM=pre 3' stem-loop; WT=wild type. PA,SP and SM showed promising fluorescence. Y-Axis in RFU (relative fluorescence units). Wavelength step: 1nm, each wavelength measured 8 times.

Later we also received a plasmid from Samie Jeffrey, the developer of Spinach, which contained the spinach 24-2 min sequence fused to a T7 promotor and has been used for *in vitro* transcription. The fluorescence of the Spinach RNA was about twice as intense compared to that of the SM construct. However, no signal could be seen in transfection experiments. (see 3.1.5.)

3.1.4. VIRAL GROWTH

In order to determine whether the genome modifications affect the viral growth rate the same amount of each *in vitro* transcribed RNA was transfected with Lipofectamin 2000 into HeLa cells. After 24h the supernatant was used for plaque purification. While 5' constructs (CL, SP) grew nearly as fast as WT virus, it could clearly be seen that 3' modifications (PA, SM) hardly produced any plaques. This was even more precise when virus was produced in constantly larger amounts, where HRV14-SP took on average 2 days, HRV14-PA more than 5 days for a complete lysis. (see 2.3.4.)

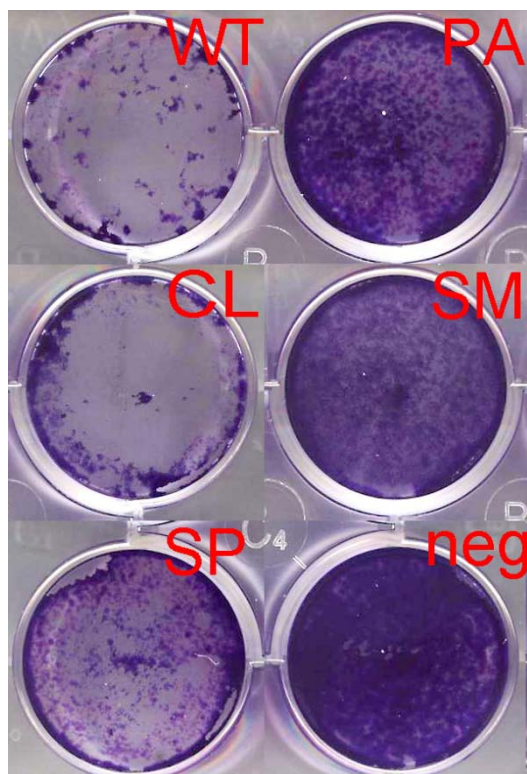


Figure 24 HeLa-H1 were transfected with HRV14 chimeric (CL, SP, SM, PA) and wild type (WT) RNA and stained with crystal violet at 24 hours post transfection (p.t.). Negative control: transfection reagent without RNA. Note the considerable differences in the extent of CPE. WT RNA and the CL construct behaved almost identical and showed the by far largest degree of cell lysis.

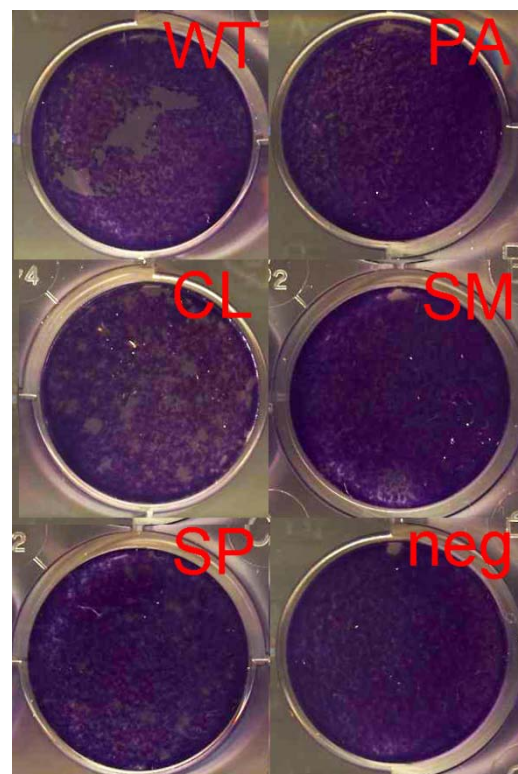


Figure 25 Plaque assay of viable recombinant HRV14 recovered from HeLa-H1 transfected with CL, SP, SM, PA and WT HRV14 RNA 24 hours post transfection. Crystal violet staining was done at 2 days post infection. While the CL construct produced a plaque size comparable to wild type, the plaques of HRV14-SP smaller. Both 3'-UTR constructs gave rise to only tiny plaques

The amount of plaque forming units per transfection was also determined by a statistical TCID₅₀ assays according to Reed & Muench with a WT virus as control. Compared to that virus gained from the supernatant of CL-transfected cells had about the same amount, while SP showed about 85%, and SM and PA below 2% of wild-type TCID₅₀.

3.1.5. STANDARD FLUORESCENCE IMAGES OF TRANSFECTION

Since virus replication comes along with a high rate of mutations, it could not be precluded, that the aptamer sequence changes due to selective pressure. Therefore imaging was done after 5 hours of RNA transfection. At early timepoints (30 Minutes, 1 h and 2h) no fluorescence could be observed.

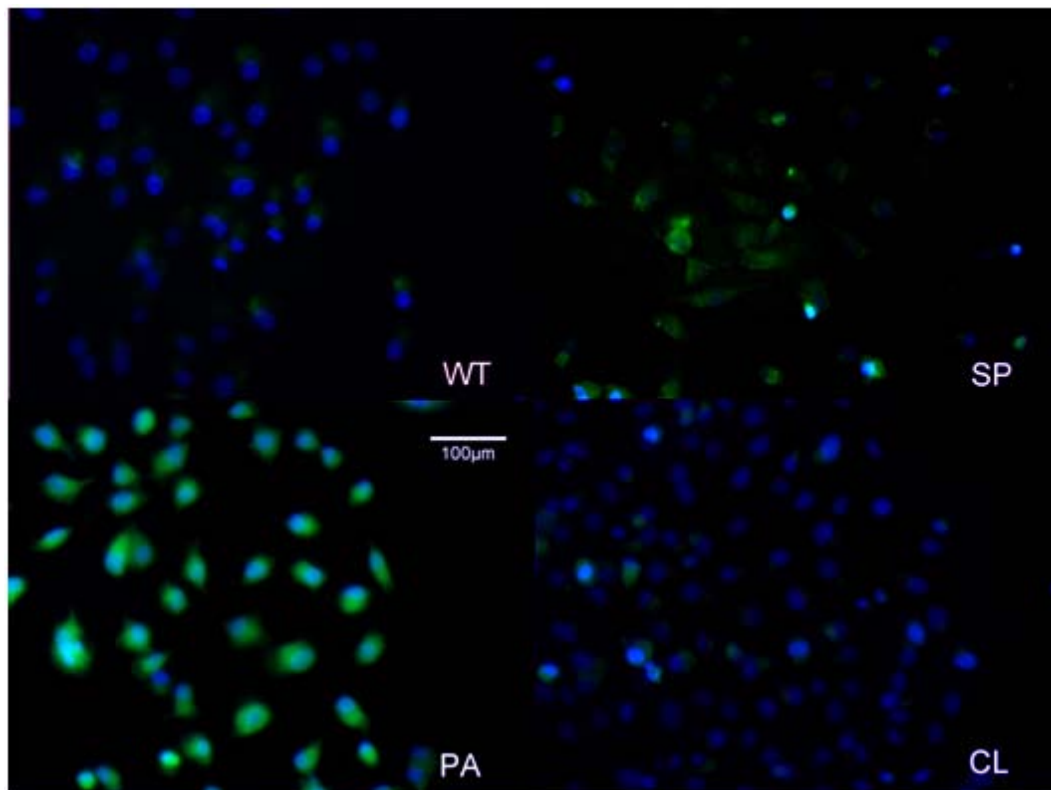


Figure 26 Fluorescence microscopy images (20x) of HeLa Ohio cells transfected with Lipofectamin 2000 and viral RNA for 5 hours. Counterstaining = Hoechst 33342; Imaged in PBS in presence of 20µM DFHBI. Image of SM construct was similar to that of PA.

Both 3' constructs (PA and SM [not shown] type) showed fluorescence upon excitation with standard GFP filter set, which could be clearly distinguished from background fluorescence as seen in wild type. This was not true for CL constructs which was not unexpected after previous experiments. The other 5' construct (SP) showed some fluorescence but not as bright as seen by the 3' modifications. At this point no further experiments with CL modified virus were done.

The required exposure time was quite long (about 5 seconds) which was not unexpected, but still much longer than used by Paige et al. for their experiments. As a positive control *in vitro* transcribed RNA of a Spinach plasmid kindly provided by Paige et al. was used, which unfortunately did not show any signal in this experiment.

A further problem was high background when imaged in infection media. As assumed by Paige and Jeffrey (personal communication) various flavins within the medium could be the problem. Therefore imaging was done in PBS.

3.1.6. STANDARD FLUORESCENCE IMAGES OF INFECTION

Plaque-isolated recombinant virus (as described in the methods) were used for infection studies. Since the system should be used for *in vivo* imaging it was important, that the background of flavins in the medium does not disturb too much. The fluorescence could be seen clearly for the PA and the SM construct as in the previous experiment. Also the brightness of the SP modification did not change. In contrast to the transfection experiment the fluorescence had a granular pattern, which has already been observed by counter double RNA-strand immune staining before (figure 28) [197] and have been described as organelle-like replication vesicles for viruses before. [198] [17]

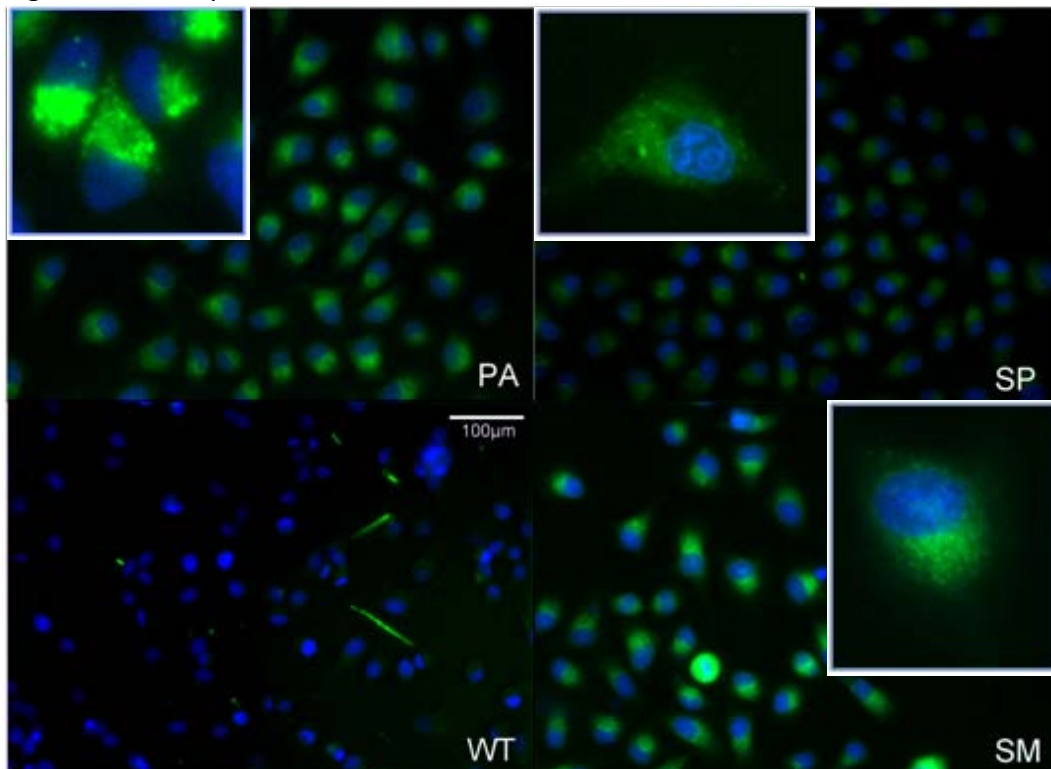
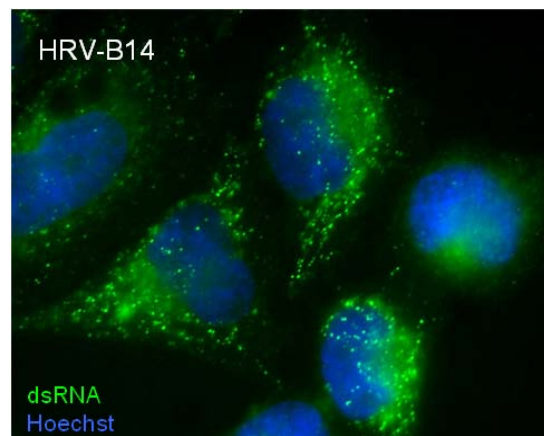


Figure 27 Fluorescence microscopy images (20x) of HeLa Ohio cells infected with the respective virus over night. (Isolated by plaque purification) Counterstaining = Hoechst 33342; Imaged in infection medium containing 20µM DFHBI; The brightness of each constructs corresponds to the findings of the previous experiment. In contrast to the transfection a granular structure can be seen which corresponds to images done with immune staining. (Figure 28) [199]

Only a small region was fluorescent on the slide. The experiment was very dependent on the isolation and could not be repeated with every plaque purified virus. For any further passage no fluorescence was observed. It seems that the viral RNA loses its ability to bind to DFHBI quite quickly.

Figure 28 Confocal microscopy image (63x) 24h after transfection with WT RNA, stained with a-double-strand-RNA antibody and Hoechst 33342. Thanks to Irena Corbic for providing this image.



Furthermore no fluorescence could be observed at earlier timepoints. This might be due to the low intensity and the low number of fluorescent molecules. Therefore infection experiments with confocal microscopy experiments have been tried. However, despite multiple attempts with different isolation no fluorescence could be detected by confocal microscopy.

3.1.7. SEQUENCE ANALYSIS OF ISOLATED VIRUS

Viral RNA was isolated from concentrated virus (about 10^7 pfu/ml) of a T225 flask ([SP]: passage 4; [PA]: passage 5) and reversed transcribed, as described in the methods, for PA and SP constructs. Sequencing was done with the specific 3' and 5' primers. The results in both cases were blasted in the NCBI nucleotide database. This revealed that the virus sequence was identical with the original HRV-14wt sequence.

```
>gb|L05355.1|HRV14POL Human rhinovirus type 14 polyprotein gene
Length=7212

Score = 320 bits (173), Expect = 2e-84
Identities = 176/177 (99%), Gaps = 1/177 (1%)
Strand=Plus/Minus

Query 1 TATACCTCGCTCCGGGG-AACAGAAAGTGCTTGTACATAGACACCATTGGATATGGCGCCA 59
      |||
Sbjct 210 TATACCTCGCTCCGGGGAAACAGAAAGTGCTTGTACATAGACACCATTGGATATGGCGCCA 151

Query 60 CCTATTGTACTTTAATGTCAAGCTTCTAACGTTTCATGGGTGGGAATTTTAAGGAAGGGT 119
      |||
Sbjct 150 CCTATTGTACTTTAATGTCAAGCTTCTAACGTTTCATGGGTGGGAATTTTAAGGAAGGGT 91

Query 120 GGTGTTGGGGAGAAAACAGGCGTACAAAAGGTACATAGTACCAGAGTACTACACCCAATGG 176
      |||
Sbjct 90 GGTGTTGGGGAGAAAACAGGCGTACAAAAGGTACATAGTACCAGAGTACTACACCCAATGG 34

>gb|K02121.1|HRV G Human rhinovirus type 14 (HRV14), complete genome
Length=7212

GENE ID: 1461213 HRVBcpl | polyprotein [Human rhinovirus 14]
(10 or fewer PubMed links)

Score = 73.8 bits (37), Expect = 7e-11
Identities = 37/37 (100%), Gaps = 0/37 (0%)
Strand=Plus/Plus

Query 14 ATATAGACACTTAATTTGAGTAGAAGTAGGAGTTTAT 50
      |||
Sbjct 7176 ATATAGACACTTAATTTGAGTAGAAGTAGGAGTTTAT 7212
```

Table 2 Screenshots of the NCBI BLASTs of 5'-UTR SP (above) and 3'-UTR PA (below); In both cases the sequences were identically with the native HRV14-WT sequence. Query = sample sequence; Sbjct = compared blasted sequence. The adenosine on position 18 of the Query in the above SP sequence is missing because the exact number of adenosine repeats at this position could not be determined.

The experiment (growth of the virus and isolation of RNA) was repeated to definitively rule out any possible cross-contamination by wild-type HRV14, with the same results. We conclude from these data that the RNA aptamer has been deleted after serial passage of these two recombinant viruses. Unexpectedly, the original wild type sequence was fully restored in each instance.

3.1.8. A FURTHER 3'-UTR CONSTRUCT (IC)

While the SPINACH tag of recombinant HRV14 PA was precisely deleted at an early stage of virus replication, the SM recombinant was hardly infective. However, since both recombinants initially showed appreciable fluorescence intensity, we screened the small 3'-UTR for additional insertion site(s) with possibly lesser impact on virus viability. The five nucleotides forming the loop in the conserved 3' stem-loop structure are highly variable within rhinovirus strains. We therefore fused 24-2 min with the 3' stem-loop of HRV-14 by insertion into the polymorphic pentameric loop as shown in the inset of figure 29. The characterization of this construct will be part of future investigations.

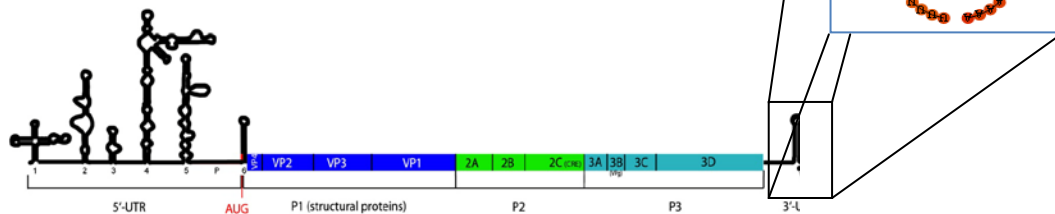


Figure 29 Scheme of an HRV-genome (actually poliovirus genome, or?). The inset shows the secondary structure of the 3' UTR with the spinach aptamer inserted into the polymorphic nucleotides forming the top of the conserved 3' stem-loop present in all rhinoviruses.

3.2. THE FLASH-TAG PROJECT

3.2.1. TARGET SITE SELECTION AND MODELLING

To insert the TC-Tag into the viral capsid two strategies were prosecuted. First an antigenic region at the C-terminus of VP-1 was recently discovered, which showed high variability in a sequence alignment of known HRVs-A and HRVs-B. [154] [83] Second Arnold et al. modified a turn of VP-2 to insert HIV-1 antigens, which was also used as target for inserting the tag. [153] [200] [201]

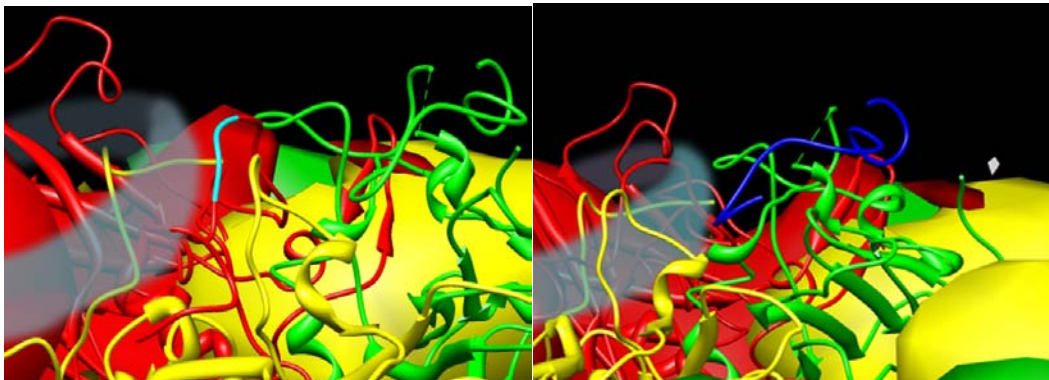


Figure 30 3D Presentation of [211] with Chimera. The left image shows the residues where the insert was cloned into VP1 (blue), the right image shows HIV-1 looped which was inserted into VP2 at the same position the TC-Tag was inserted. Red: VP1, Green: VP2, Yellow: VP3. The blue circle represents the canyon. Both positions are surface loops which show high variability indifferent serotypes of human rhinovirus.

At both positions we wanted to try the standard TC-Tag (CCPGCC), the FLN-Tag (FLNCCPGCCMEP) and the HRW-Tag (HRWCCPGCCKTF). Therefore we used overlap extension PCR. Concerning VP1 the 3 modifications named VP1-TC (standard Tetra-Cystein-Tag), VP1-FLN (FLN-Tag) and VP1-HRW (HRW-Tag) were edited in a way that known sequences from other viruses or HRV-14 itself were preserved. For example the amino acid sequence EP occurs at position 275 and 276 in HRV-14wt at an ideal position for the FLN-Tag. Also the KTF motif of the HRW-Tag can be found at a close position in some other human rhinoviruses. Although no modifications at the flexible c-terminus of VP1 are known so far, this seemed to be a promising approach. Models were calculated using Swiss-Model. In case of VP2 the constructs were planned strictly according to Arnold et al. as an insertion between A158 and N159.

3.2.1.1. VP2 TETRA-CYSTEIN TAGS

Arnold et al. were working on a modified HRV14 which expresses HIV-1 antigens (the ELDKWA epitope) on its surface. Therefore they did a lot of in silico calculations and modifications of HRV14-VP2. The important thing was that the epitope still had to have the right conformation and presents a target site for α -HIV-1 antibodies. [153]

By using Swiss-Model we calculated three-dimensional models for 3 different FIAsh-Tags (TC, FLN and HRW) when inserted into VP2.

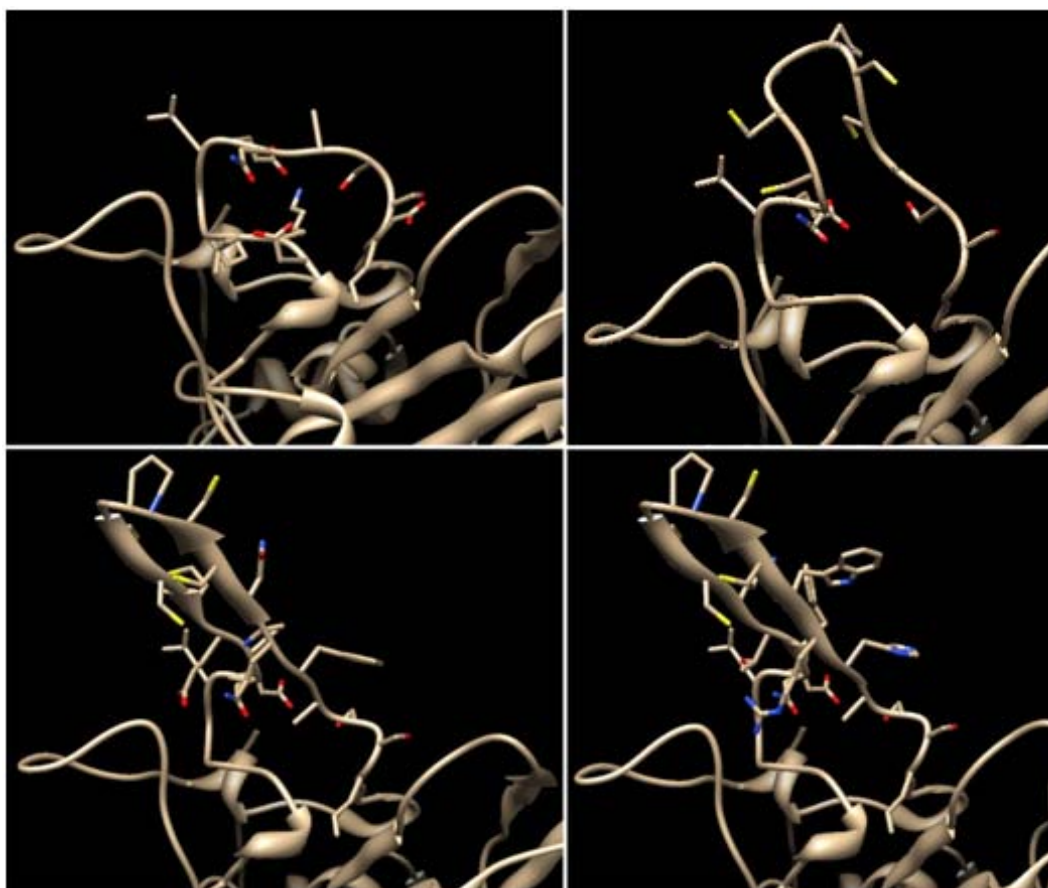


Figure 31 Predicted models (by Swiss-Model) for 3 different inserts of FIAsh-Tags in a loop of VP2 of HRV-14. Upper left: Wild-Type; Upper right: Standard TC-Tag (CCPGCC); Lower left: FLNCCPGCCMEP; and Lower right: HRWCCPGCCCKTF; The program predicted a β -turn for FLN & HRW tag, which deviates from the NMR model described before. [137]

The important thing was that all cysteine residues still allow the binding of the arsenic compound. The prediction forecasted a β -sheet-like structure for FLN- and HRW-tag – in contrast to TC-tag – in which the residues are directed into opposite position.

In this case the fluorophore would not be able to bind to the tag efficiently, however, the prediction is based on structure alignments and only unknown sequences are calculated *de novo*. Therefore no adaptation was done.

3.2.1.2. VP1 TETRA-CYSTEIN TAGS

Concerning VP1 the 3 modifications called VP1-TC (standard Tetra-Cystein-Tag), VP1-FLN (FLN-Tag) and VP1-HRW (HRW-Tag) were obtained by insertion of each tag into slightly different positions of NIm-IV to optimally exploit the natural amino acid context of HRV-14. For example the amino acid sequence EP, which occurs at position 275 and 276 in Nim-IV of HRV-14-wt became the distal part of the FLN-tag after the latter's insertion between Asn274 and V277 of HRV-14. Also the KTF motif of the HRW-Tag inserted between Asn274 and Ile278 can be found immediately upstream of the residue corresponding to Ile278 in some human rhinoviruses. The standard TC-tag was inserted between Asp275 and Pro276.

Since the last amino acids (DIKSY) of VP1 are important for protease cleavage of the P1 precursor from the rest of the polyprotein, the tag had to be inserted into the high variable region localized immediately before the exposed C-terminus instead of attachment to the terminal tyrosine. (See alignment on the next page) Here the cysteines are modeled in close spatial proximity to each other, which is a premise for the binding of the dye.

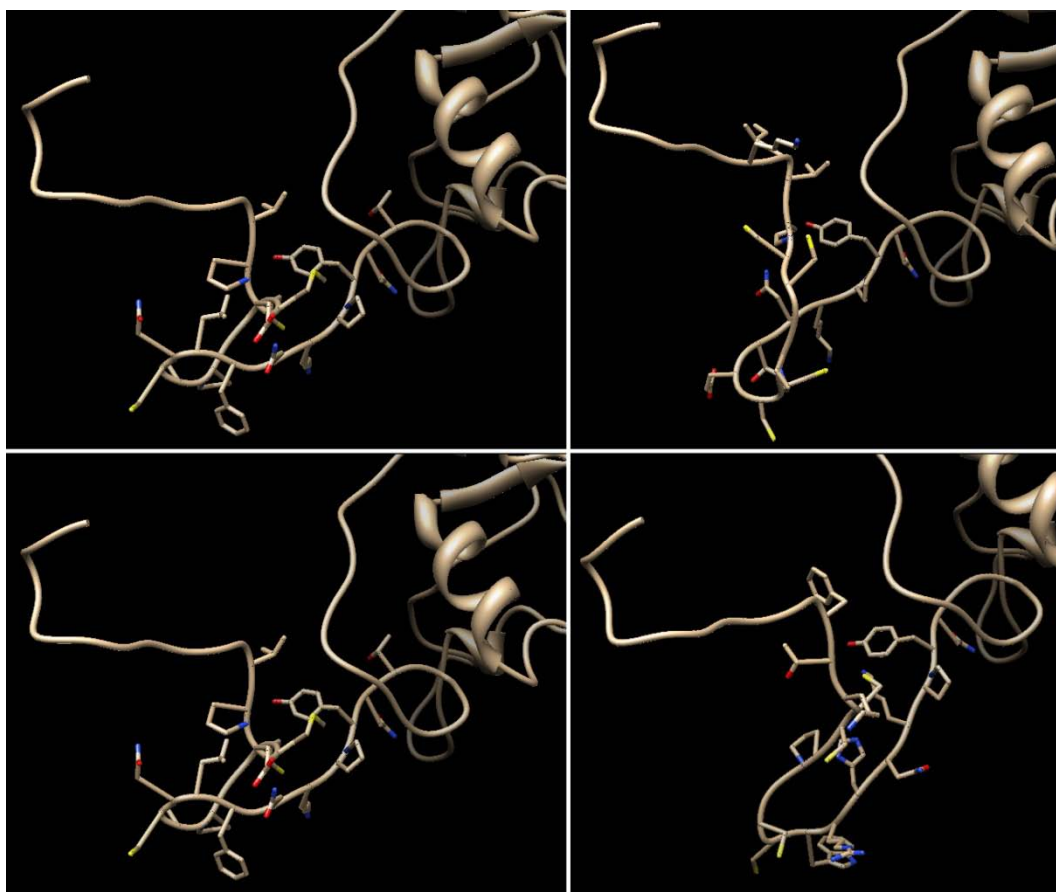


Figure 32 Predicted models (by Swiss-Model) for 3 different inserts of FIaSH-Tags. Upper left: Wild-Type; Upper right: Standard TC-Tag (CCPGCC); Lower left: FLNCCPGCCMEP; and Lower right: HRWCCPGCKTF; It is assumed, that the improved fluorescent properties of the latter two is due to the increased electronic density by aromatic side-chains. In this case the structure agrees more with the NMR experiments of the peptides only. [137]

HRV-A1 KAKHTKAWCPRPPRAVPYTHSHVTNYPETG--DVTTAIVRRNT-----ITTA
 HRV-A1B KAKHTKAWCPRPPRAVPYTHSRVTNYPKGTG--DVTTAIVPRAS-----MKTV
 HRV-A16 KAKHTKAWCPRPPRAVQYSHHTTNYKLSTEV--HNDVAIRPRTN-----LTTV
 HRV-A81 KAKHTKAWCPRPPRAVQYTHTHVTNYPKLETDV--HTSVAIRPRTS-----LTNV
 HRV-A55 KAKHIKAWCPRPPRAVEYTHTHVTNYPKKTGDG--TEKTAIKYRRD-----IKTV
 HRV-57 KAKHVKAWCPRPPRAIEYTHTHVTNYPKVGK--QEETAIKYRRD-----IKVV
 HRV-A21 KAKHVKAWCPRPPRAVEYTHTHVTNYPKIANH--EVTSAVESRRT-----IVTV
 HRV-A33 KAKHVKVWCPRPPRAVEYTHTHVTNYPKSTTR--EVKTAIRPRAT-----IKTA
 HRV-A76 KAKHVKVWCPRPPRAVEYTYTHVTNYPKPHSG--DVQTAIRPRAT-----IKTA
 HRV-A11 KAKHVKVWCPRPPRAVEYTHTHVTNYPKQEG--QVKTAVKARKT-----IKTA
 HRV-A24 KAKHIKVWCPRPPRAVEYTHTHVTNYPKHQTR--EVKTAIEPRRG-----IKTV
 HRV-A90 KAKHIKVWCPRPPRAVEYTHTHVTNYPKHATR--ELKTAIRPRKT-----ITTA
 HRV-A34 KAKHVKTWCPRPRAVEYTHTHVTNYPKVRGE--TEKTAIKPRAK-----ITMA
 HRV-A50 KAKHIKVWCPRPPRAVEYTHTHVTNYPKSDA--TEKTAIATRPK-----ITVA
 HRV-A18 KAKHVKAWCPRPPRAVEYTHTHVTNYPKREG--REKTAIVPRAR-----ITMA
 HRV-A10 KAKHVKAWCPRPPRAVPYTHAVSTNYPKPHGK--ELQIFIRSRDDP-----KVVTA
 HRV-A100 KAKHVKAWCPRPPRAVPYTHSHSTNYPKHEG--DVQIFIRPRDDP-----KFVTA
 HRV-A66 KAKHVKAWCPRPLRAVPYTTVNSTNYPMPHTG--DLQIFIKPRTD-----KVVNV
 HRV-A77 KAKHIKAWCPRPPRAVPYTAVDSTNYPKMRG--DVQIFIKERASP-----KVVTF
 HRV-A25 KAKHVKAWCPRPPRAVPYKYVDFNNYAASDN--VDIFIQPRNS-----LTKTA
 HRV-A62 KAKHVKAWCPRPPRAVPYKYVDFNNYAASDS--VDIFIKSRQN-----LQTA
 HRV-A29 KAKHVKAWCPRPPRVVPYKYVGLTNYTLKE---EDTVVESRPS-----LMTA
 HRV-A44 KAKHVKAWCPRPPRVVPYKYVGLTNYTLKE---TDTVVEPRHS-----IMTA
 HRV-A31 KAKHVKAWCPRPPRAVPYRYVGSTNYPKPDEN--EVTIFVKHRDNP-----KIITA
 HRV-A47 KAKHVKAWCPRPPRAVPYRYVGSTNYPKPDQG--EVAIFIEHREN-----KFITA
 HRV-A40 KAKHIKAWCPRAPRAVPYTHTHSTNYPKQEG--EVQIFLKERAS-----LTTV
 HRV-A85 KAKHVKAWCPRAPRAVPYTHTRSTNYPVQDG--EVKIFLKERAS-----LTTA
 HRV-A54 KAKHIRAWCPRAPRAVPYTHTRSTNYPREG--DPTIFLKHRTN-----LVTA
 HRV-A98 KAKHIRAWCPRAPRAVPYTHTRSTNYPQDG--EPTIFLKHRRD-----LVTA
 HRV-A59 KAKHVKAWCPRAPRAVPYTHSYVTNYPKIAGK--EPEIFLKPRMN-----ITTA
 HRV-A63 KAKHIRAWCPRAPRAVPYTHSYVTNYPKITGQ--ETEIFLKPRAT-----IKTA
 HRV-A56 KAKHIRAWCPRAPRAVPYTHAVSTNYPKPDG--DVQIFLKPRES-----LTTF
 HRV-A38 KAKHVKAWCPRPPRAVEYRHTHVNNYPKPDQG--EVTMIPTRTNIR-----TIVNV
 HRV-A60 KAKHVKAWCPRPPRAVEYRHTHVNNYRPDDG--EAAITIPIRTDIR-----AIRTV
 HRV-A2 KAKHVKAWCPRPPRALEYTRAHRTNFKIEDR--SIQTAIVTRPI-----ITTA
 HRV-A49 KAKHVKAWCPRPPRALEYTRAHRTNFKIQNR--DIKTGIKSRPI-----ITTA
 HRV-A23 KAKHVKAWCPRPPRALEYTRAHRTNFKIEGE--NVKSRVAHRPA-----VITA
 HRV-A30 KAKHVKAWCPRPPRALEYTRAHRTNFKIDGR--EVKSRVEHRAR-----VTTA
 HRV-A39 KAKHVKAWCPRAPRAVPYTHSNVTNYPKVRDG--EPTLFIKPREN-----LTTA
 HRV-A9 KAKHVKAWCPRPPRAVEYIHTHVNNYPKSEG--EYSTVIPVRDNVR-----AVRNV
 HRV-A32 KAKHVKAWCPRPPRAVEYTHTHVTNYPKSTG--DYTTAIQTRHVR-----AVKNV
 HRV-A67 KAKHVKAWCPRPPRAVEYIHTHVNNYRPETG--EAQTVIPVRADVR-----TIRNV
 HRV-A15 KAKHVKAWCPRPPRAVEYTHTHVTNYPKPDG--DVTTVIPTRENVR-----AIVNV
 HRV-A74 KAKHVKAWCPRPPRAVEYTHTHVTNYPKQEG--DVTTVIPTRR-----SIVNV
 HRV-A64 KAKHVKAWCPRPPRAVGYTHTNVTNYPKPSKG--EYTPPVPSRNSPR-----DIVTV
 HRV-A94 KAKHVKAWCPRPPRAVGYTHTHVTNYPKSEG--EYKPPVVRNSPR-----DIVTV
 HRV-A22 KAKHVKAWCPRPPRAVGYTHTHVTNYPKPSVG--DYTLPIPTRANPR-----QILNV
 HRV-A82 KAKHVKAWCPRPPRAVGYTHTHVTNYPKSQG--DYSVVIPVRESPR-----QILNV
 HRV-A19 KAKHIKAWCPRPPRAVEYTHTHVTNYPKQTG--EVTLPPIERDNP-----HIKNV
 HRV-A43 KAKHIKAWCPRPPRAVEYTHTHVTNYPKREGK--EVETAIVSRRD--I-----KIVNA
 HRV-A75 KAKHIKAWCPRPPRAVEYTFRRVTNYPKRDGQ--QVEIAIEPRRD--V-----KFVNA
 HRV-A13 KAKHVKAWCPRPPRAVEYTNVHVNNYPKGTG--DVAVSIVPRANVR-----EIRNF
 HRV-A73 KAKHVKAWCPRPPRAVEYTNNAHVNTNYPKTDG--EVTTAIRHRDNP-----AIQNF
 HRV-A41 KAKHVKAWCPRPPRAVEYTNVHVNTNYPKAGA--EIVASVRPRDNVR-----QVRNY
 HRV-A61 KAKHVKVWCPRPPRAVEYTNVHLTNYPKDKSEKQVTTFIKPRANLR-----EIRTF
 HRV-A96 KAKHVKVWCPRPPRAVEYTNVHLTNYPKPNN--EVTTFIKPRENLR-----DIRNF
 HRV-A7 KAKHIKAWCPRPPRAVPYQHTHSTNYPVQNG--EVATQIKTRANLF-----TLKSA
 HRV-A88 KAKHIKAWCPRPPRAVPYQHTHSTNYPKDG--EVATQIKTRRDVY-----TVTTA
 HRV-A36 KAKHIKAWCPRPPRAVPYQHTHSTNYPKSG--EITTQIKTRPNVF-----TVG--
 HRV-A89 KAKHIKAWCPRPPRAVAYQHTHSTNYPKSN--EATTQIKTRPDVF-----TVTNV
 HRV-A58 KAKHIKAWCPRPPRAVPYQFTHSTNYPIDSG--EVTTQIKPRTNVF-----TITSA
 HRV-A20 KAKHISAWCPRAPRAVPYQHTKSTNLVPRTGE--ITTHIRFRNTVRDLAYPTMTNLG----
 HRV-A68 KAKHISAWCPRAPRAVPYQHTKSTNLVPKEGD--IKTHIKFRNTVKDLAYPPELANLG----
 HRV-A28 KAKHVSAWCPRPPRAVAYQHTYSPNFVPQEGD--VETHIKFRTDVK--QITTVG--
 HRV-A53 KAKHISAWCPRPPRAVAYQHTYSPNFVPQTGT--VETHIKFRPDVKDVT--SVMTAG--
 HRV-A46 KAKHIKAWCPRAPRAVPYQHIYNPNFKTTQPETIPDTHIKIRRDIK-----YIKTAG--
 HRV-A80 KAKHIKAWCPRAPRAVPYQHTYSPNFKNDES--IPDTQIKIRDNIR-----QVRTVG--
 HRV-A51 KAKHVSAWCPRPPRAVAYQHTYSPNFVPEGEGLKTKIKTRADIKLVN-----FG--
 HRV-A65 KAKHITAWCPRPPRAVAYQHTYSTNFPVSGGLTNLRTQIKTRDNKIVN-----FG--
 HRV-A71 KAKHVRWAWCPRPPRAVAYQSTYTTFNFPVQDGINSIKTIKIRRDIKEVSN-----YG--
 HRV-A101 KAKHISAWCPRPPRAVAYQVHNTNFVPETG--QVEIQIPTRTDLTTVG-----TVG--
 HRV-A12 KAKHISAWGPRPPRAVPYQHIHNPKNYKTSNGV--PDNRVKLRETLT-----TVG--
 HRV-A78 KAKHISAWGPRPPRAVPYQHIYNPNYKTEEGT--PDTKVAIRANIK-----TVG--
 HRV-A8 KAKHIKAWCPRPPRAVTNHIYNPNYVREGVTPETKVKYRAEVT-----TI

```

HRV - A95      KAKHIKAWCPPPRAVTYNHIYNPNYVREGVTPETKVKYRAEVT-----TI
HRV - A45      KAKHIKAWCPPPRAVTYNHTYNPNYVRADET-ATKVQTRANVT-----TV
HRV - C6       KPKHTAAWIPRPPRATQYLHKFSTNYNPKPT--KGSNELEPKHFLKYRDD----ITNITTL
HRV - C26      KPKHTAAWIPRPPRATQYLHKFSTNYNPKPT--KGSNELEPKHFLKYRDD----ITSITTL
HRV - C3       KPKHTIAWIPRPPRATQYLHKFSTNYNPKPT--SGSTELEPKHFFKYRQD----ITSITNL
HRV - C11      KPKHTIAWIPRPPRATGYTHRYSTNYNPKVS--DDSTLTRDHYIETR-----ASVKTA
HRV - C25      KPKHTIAWIPRPPRATGYTHKYSTNYNPKVS--DDSTLTRDHYIETR-----ATVTTA
HRV - C24      KPKHTIAWIPRPPRATQYLHKFSTNYHVKKP-DDTTGLLIQKHFINHR-----TDIKTA
HRV - C49      KPKHTIAWIPRPPRATQYLHKFSTNYHVKKP-GDDTGLLIQKHFIKPR-----GDIKTA
HRV - C15      KPKHTIAWIPRPPRATQYLHKYSTNYHYK-P-NADEHILEDHFIKTR-----PLISSA
HRV - B6       RAKHVEAWIPRAPRALPYETIGRTNYPKN--KIVPVIKKRE-----NITTY
HRV - B37      RAKHVEAWIPRAPRALPYETIGRTNYPK--MITPVIKKRD-----NITTY
HRV - B3       RAKHVEAWIPRAPRALPYVSGRTNYPK--DSKTIKKRT-----NIKTY
HRV - B83      RAKHVQWVPRAPRALPYVSGRTNYPK--NIKPVIEKRT-----SIKQY
HRV - B92      RAKHVEAWIPRAPRALPYVSGRTNYPK--AIVPVIKKRS-----LITNY
HRV - B79      RAKHVEAWIPRAPRALPYVSGRTNYPK--DAKPIIAKRE-----NIMKY
HRV - B35      RAKHVQAWIPRPPRALPYVIGRTNYPK--PIKPIIKRD-----QITQY
HRV - B14      RAKLVEAWIPRAPRALPYTSIGRTNYPK--NTEPVIKKRKG-----DIKSY
HRV - B72      RAKHIEAWIPRAPRALPYTSIGRTNYPK--NPQPVIIKKRG-----DIKTY
HRV - B86      RAKHIQTWIPRAPRALPYETIGRTNYPK--NPPKIIKKRD-----TINTY
HRV - B17      RAKHIRAWVPRPPRALPYTSIGRTNYPK--TRNKVIARRD-----SITTY
HRV - B70      RAKHIRAWVPRPPRALPYTSIGRTNYPK--THSKVIATRD-----SITTY
HRV - B91      RPKHIRAWIPRPPRALPYTSIGRTNYPK--RHSKVIATRD-----SITTY
HRV - B52      RAKHIRAWVPRPPRALPYTSIGRTNYPK--NHGKIIAKRD-----KITTY
HRV - B5       RAKHIRAWVPRAPRALPYLHIGRTNYPK--SPQNPIKTRK-----TISTY
HRV - B42      RAKHIRAWIPRAPRALPYLHIGRTNYPK--NPKNPIKTRD-----SISTY
HRV - B99      RAKHIRAWIPRAPRALPYLHIGRTNYPK--NSTNPIKTRD-----SISTY
HRV - B26      RAKHVRAWVPRAPRALPYLHIGRTNYPK--QSKNPIKTRX-----TISTY
HRV - B4       RAKHIRAWIPRAPRALPYLHIGRTNYPK--NPDSPKIPRE-----TINTY
HRV - B27      RAKHVLAWIPRPPRAKPYLSIGRTNYPK--KTGNIIPKRN-----NISTY
HRV - B93      RAKHVMAWIPRPPRAKPYLIGRTNYPK--KSGHVIPKRD-----GISTY
HRV - B97      RAKHVMAWIPRPPRAKPYLIGRTNYPK--GSDNIIPKRN-----GISTY
HRV - B84      RAKHVMAWIPRPPRAKPYLHIGRTNYPK--GSQSIIPKRD-----TISTY

```

Table 3 Alignment of C-terminus of VP1 of known HRV sequences (data obtained from NCBI protein database) using ClustalW. The last five amino acids are quite conserved, which might be important for protease cleavage. Before those however a variable region (NimIV) could be available for manipulation.

Based on the alignment we developed three different sequences for VP1

```

HRV14-VP1-WT   RAKLVEAWIPRAPRALPYTSIGRTNYPKNTEPVIKKRKGDIKSY
HRV14-VP1-TC   RAKLVEAWIPRAPRALPYTSIGRTNYPKNTECCPGCCPVIKKRKGDIKSY
HRV14-VP1-FLN  RAKLVEAWIPRAPRALPYTSIGRTNYPKNFLNCCPGCCMEPVIKKRKGDIKSY
HRV14-VP1-HRW  RAKLVEAWIPRAPRALPYTSIGRTNYPKNHRWCCPGKTFIKKRKGDIKSY

```

The model further was used to determine the distance between two bound fluorophores when simultaneously bound to the viral capsid. Using known crystal structure data the calculated distance is about 67 angstroms between two VP1 along the 5-fold axis bound arsenic helical binder molecules, which is enough to avoid possible self-quenching.

3.2.2. PCR

We used overlap extension PCR to engineer each of these TC-tag sequences into VP1 and VP2 of the infectious HRV-14 wt cDNA already employed for the SPINACH project. The alternative approach, using synthetic cDNA (as was chosen for insertion of the 24-2 aptamer into the virus genome) was abandoned due to its considerable costs for a larger number of constructs.

The PCR primers were designed separately for each construct to avoid undesired primer dimers and improve the yield. The expected length of each PCR-product is shown in table 4

V1 (VP2-TC)	5'	568 nt	3'	1820 nt
V2 (VP2-FLN)	5'	565 nt	3'	2192 nt
V3 (VP2-HRW)	5'	846 nt	3'	1806 nt
VT (VP1-TC)	5'	371 nt	3'	423 nt
VF (VP1-FLN)	5'	349 nt	3'	182 nt
VH (VP1-HRW)	5'	332 nt	3'	182 nt

Table 4 Expected length of PCR Products

At first no product was obtained from VP2 3' PCR reaction. Different supplements were tested known to improve amplification by acting on inhibitory secondary structures of GC rich sequences (combination of 0.5-2 M betaine and/or 2-10% DMSO). [190] The biggest yield was seen in PCR reactions with additionally added 10% DMSO. Products were purified and primers were removed as described in the methods and used for the following hybridization reaction.

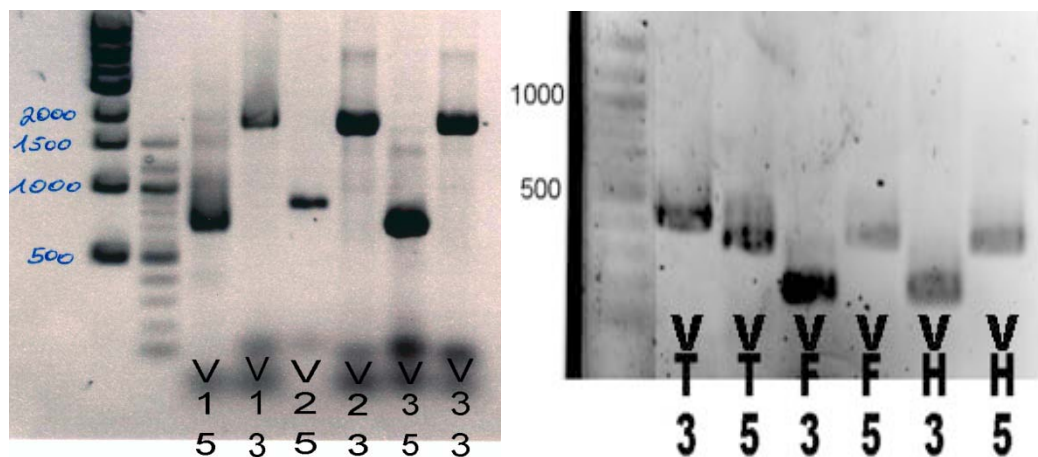


Figure 33 3' and 5' products of VP1 and VP2 protein modifications. In case of VP2 (left) additionally 10% DMSO had to be used to gain products. (V15 = Var 1 5', etc.)

3.2.3. HYBRIDIZATION AND AMPLIFICATION

Hybridization was done as described in the methods without the addition of DMSO. Subsequent PCR amplification was carried out with VP1 and VP2 helper primers to avoid primer dimers and increase the efficiency of the PCR. In case of VP1 the expected products are about 530 bp, while the products of VP2 are about 2350 bp long. As shown in figure 34, a main product of correct length was produced in every instance and was then purified as described in the methods.

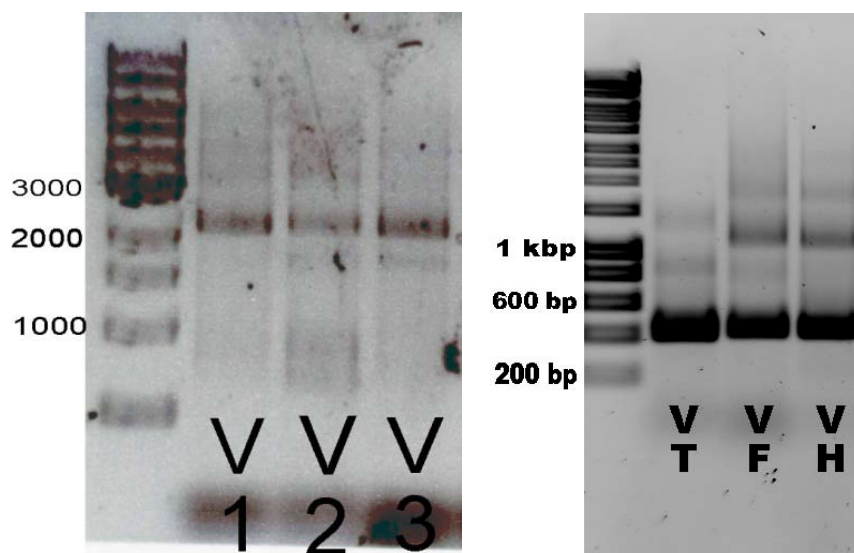


Figure 34 Gel electrophoresis of PCR hybridization products. VP2 products (left) should contain in average 2350 bp, VP1 products (right) about 530 bp.

Due to occurring problems in the later ligation step the products were cut at this stage with AflIII/NdeI or NdeI/AvrII respectively and the fragments of the correct length purified by gel electrophoresis and extraction.

3.2.4. SEQUENCING OF PCR PRODUCTS

To ensure that the correct products were produced by extension overlap PCR, the cDNA was gelpurified and submitted to custom sequencing analysis with primers listed in 2.6.7. All PCR products except VP2-Var3 (HRW) yielded interpretable data (Figure 34 and 35); in each instance the DNA sequence corresponded fully to the expected hybrid cDNA.

3.2.4.1. VP2 PCR PRODUCTS

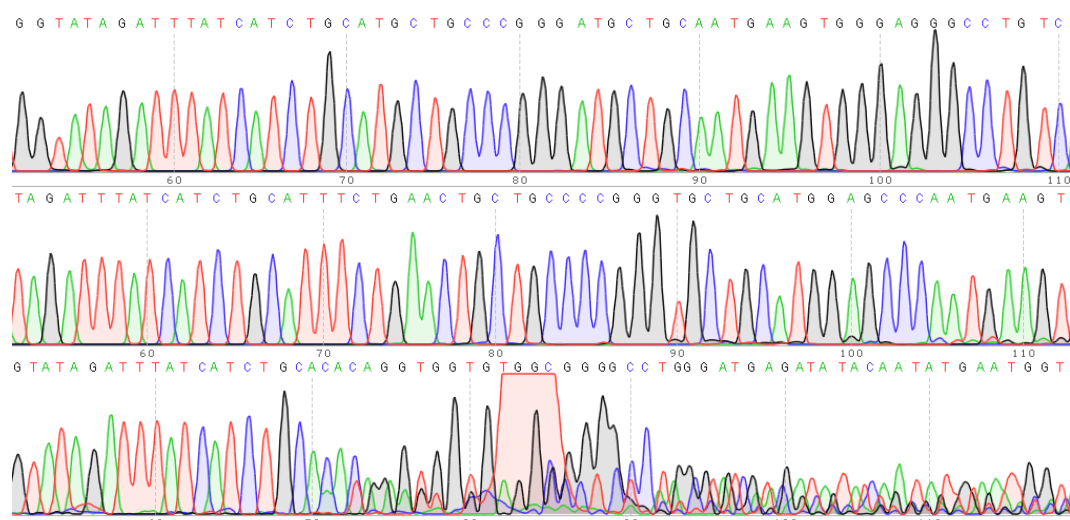


Figure 35 Chromatograms of VP2 PCR products. TC (top) and FLN (middle) –tag resulted in satisfying sequences. Obviously the hybridization of HRW (bottom) was facing some difficulties and could not be achieved.

3.2.4.2. VP1 PCR PRODUCTS

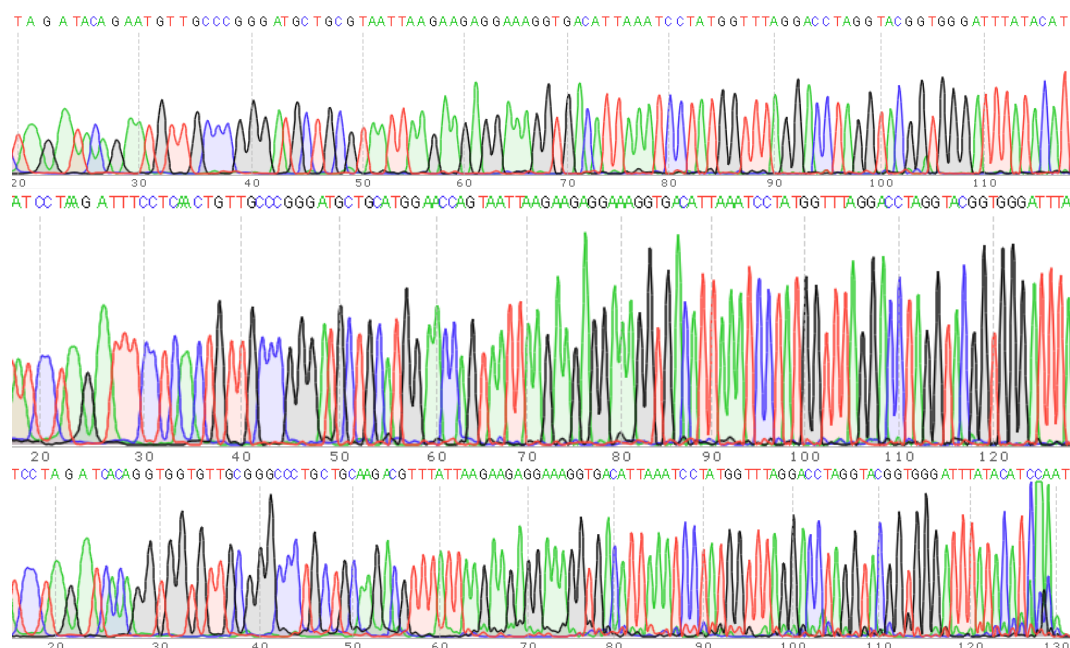


Figure 36 Chromatograms of VP1 PCR products. All three (top: TC; middle: FLN; bottom: HRW) sequences agreed with the designed.

3.2.3. PLASMID CONSTRUCTION

Due to several problems at the time of this thesis no plasmid with a correct sequence could be obtained. Although ligation resulted in colonies and isolated plasmids could be cut with XmaI (unique restriction site within the TC-tag) sequencing resulted in not interpretable sequences. Within the time frame it was not possible to solve this problem.

4. DISCUSSION

4.1. SPINACH PROJECT

The aim of this master thesis was to produce a transgenic virus, which contains the short DFHBI binding Spinach RNA aptamer within its genome and which can be used for infection, replication and assembly studies. Although a fluorescence signal of Spinach/viral RNA hybrids was detectable under the fluorescence microscope, the constructs are still facing some limitations. The fluorescence intensity was lower compared to GFP, to similar fluorescent tags which have been used before and even to the original RNA aptamer developed by Paige. This might be due to the much larger size of viral genome compared to the short 5S RNA which they were using for the first Spinach experiments. Because only one fluorescence molecule is available per RNA, it is important to determine the detection limit of this method, if single molecule imaging is possible and whether the advantages justify the effort, especially considering the development of molecular beacons (MB) linked with tat-peptides. This system allows the delivery of MBs into the cells and therefore real time imaging of RNA replication, however not of processes of the infection (e.g. endocytosis). [202] This might be possible with Spinach-tagged RNA, if a signal is still emitted despite the low pH of endosomes. Furthermore the high resistance of Spinach to photo bleaching could make this system superior for live cell imaging.

3 of 4 recombinant viruses replicated significantly slower than wild type HRV14. The fourth construct was not fluorescent *in vitro* upon incubation with DFHBI, had the same growth properties as wild type HRV-14 and was therefore dismissed from further analysis. Although not further investigated its aptamer sequence likely became misfolded when inserted between the terminal two uridine residues and the cloverleaf of HRV-14, which would explain the lack of appreciable association with DFHBI. The observed replication defect of most chimeric viruses was not entirely unanticipated: Although all aptamer insertion sites in the noncoding regions have been chosen as to maximally avoid disruption of important cis regulatory elements and their secondary structure, other aspects may play a role which cannot be predicted by such simple approach. For example it must be considered, that insertion of aptamers can cause steric blockage for RNA binding proteins, which has been described before. [203] This might be the reason for the lower replication rates as experiments of Meredith et al. indicate that the 3D polymerase interacts with the 3'-UTR. [204]

Addition of the fluorophore may disrupt tertiary interactions and/or generate new inappropriate e.g. pseudoknot interactions in the RNA hybrids not addressed by the RNAfold algorithm. Finally as later discussed in more detail, melting of the unusually stable spinach aptamer stem-loop structure as required during replication could constitute a severe problem. It is however rather unlikely that the underlying causes of the compromised replication are the same for each construct, as the HRV14-SP (tagged in the 5'-UTR) developed a cytopathic effect considerably faster than the 3'-UTR inserts (HRV14-SM and

HRV14-PA). This was also reflected in the amount of virus collected from transfected HeLa cells and the different plaque size. That the 3'-UTR tagged viruses are more severely affected than the 5'-UTR-tagged HRV-14 is quite intriguing with respect to the relative unimportance of the 3'-UTR in picornavirus replication.

As shown here, between the second (when virus was still fluorescent in the microscope) and the fourth to fifth passage (when aiming at isolation of a greater amount of virus) the RNA aptamer was precisely deleted from the HRV14 backbone of the two recombinants (SP, PA) which grew fast enough to be subject for further analysis. In some cases there was even no fluorescence when using virus directly from plaque purification. We assume that this is due to selective pressure, since wild-type virus is able to replicate far more efficiently. An increased viral growth rate has been observed during passage four and five for both 3' and 5' modifications.

An interesting finding is that the aptamer was deleted from the virus genome thereby altering the sequence to the original wild type. We suggest that this high tendency of aptamer-deletion is due to high unfolding energy which is required for denaturing the spinach stem and therefore cannot readily be amplified by rhinoviral 3D RNA polymerase. This agrees with experiments by Rohll et al. who observed a lack of replication in a recombinant poliovirus, where an extremely stable stem was created. (about -35 kcal/mol, which is about the same value as calculated for the 24-2min Spinach aptamer – the value for the native stem of HRV14 is about -5 kcal/mol) [116]

Requiring higher unfolding energy the polymerase could be forced to jump to the next easy accessible (in this case native) nucleotide of the plus-strand template during negative-strand synthesis. Also recombination events could have happened, resulting in removing the aptamer. Such phenomena have already been observed in poliovirus when a second VPg was inserted. [205] Pilipenko et al. proposed a three step model for recombination events, which they discovered in recombinant Theiler's murine encephalomyelitis virus (TMEV). First the synthesis of the nascent strand is paused by misincorporations, then the RNA polymerase dissociates together with the 3' end of the nascent strand, which finally reanneals with another template strand. [206]

As a final point it must not be neglected that for cDNA synthesis, which then was used for sequencing UTRs, another polymerase (Superscript III by Invitrogen) was used, which might also have troubles concerning sequences with strong secondary structures. Nevertheless due to the higher temperature during this reaction (55°C) it is more unlikely. It should also be mentioned, that some experiments showed that modifications tend to be more stable when virus is grown on lower temperatures. [207] However, if our theory concerning the high unfolding energy of the spinach is true this might be contra productive in this case.

In this analysis it was also shown *in vitro* and *in vivo*, that both 3' constructs had significantly higher fluorescence than the 5' spacer modification. The 5' cloverleaf construct did not fluoresce at all.

However the 3' pre-poly-adenosine (PA) variant had a quite slow replication rate which increased rapidly with every passage. As we found out later on, this was due to the deletion of the aptamer and therefore returning to wild-typ. It seems that for efficient replication the 3' sequence GTTTAT has to be in context with the poly-A terminus as mentioned before. [32]

The 3' stem-loop (SM) variant grew extremely slow that uninfected cells soon predominated in infected cultures and it was therefore not possible to gain enough amounts for further experiments. This was quite surprising, since it has been reported, that 3' modifications at this position do not massively affect viral replication that much. [208] Additional it has been proved that the HRV14 minus-strand can be synthesized successfully even if a major part of the 3'-UTR is deleted. [209] It seems that no rescue mutation or even the deletion of the whole aptamer, as it was the case for the PA construct, is possible for an insert at this position. More investigations beyond the scope of this thesis are required to determine if the aptamer insert compromises the replication of (-) or (+)-strand synthesis or even influences processes like packaging or the infection of a new host cell which could reveal new insights into the function of the 3'-UTR.

The 5'-UTR spacer (SP) construct showed already less fluorescence intensity in *in vitro* experiments. This might be either due to wrong base pairing and therefore weakening the affinity of the aptamer for DFHBI, or other influences, which shift the equilibrium from complex to aptamer and unbound dye.

The new construct (IC) might deal with some of the mentioned troubles. The conserved 3'-UTR stem-loop, on top of which the aptamer has been grafted, is basically preserved, however the folding free energy is again high which may be prohibitive for proper replication of this chimeric RNA as discussed before, despite the different context. Rohll et al. suggested that the structure itself, rather than the sequence of the 3'-UTR is important for viral replication, since their PV3/HRV14-3'-UTR chimera was able to replicate successfully. Especially the stem seems to play an important role, since modification of the loop, though reduced, resulted in replication of the virus. [116]

Still single molecule observation of infection could be possible using virus harvested immediately after transfection, however, further studies have to be done first. For example data of the properties of the aptamere-fluorophore complex under conditions like in endosomes has to be collected, since Paige et al. observed lower intensity at pH 6 or lower. [182]

4.2. FLASH PROJECT

In contrast to the recently developed RNA aptamers arsenic helical binder have been used for a longer period and more experiments about applications in biology have been published. That means that once virus is produced the fluorescent methods are comparable with the literature, which is hardly possible for the Spinach.

Three approaches have been developed for each VP1 and VP2 using the currently most common tags for arsenic helical binder (CCPGCC; FLNCCPGCCMEP; HRWCCPGCCKTF)

Unfortunately within the frame of time of this thesis it was not possible to get beyond sequence confirmation of the PCR products. Once the correct sequence of the plasmid is confirmed, viral RNA production by *in vitro* transcription and later transfection into HeLa cells will be carried out for production of recombinant virus. If such virus is viable, staining with either FIAsh or ReAsH can be done. The use of ReAsH in particular would allow double labeling with Spinach.

4.3. CONCLUSION

Facing several difficulties so far it is hard to tell if the “Spinach” aptamer is useful for the intended purpose. At least one out of four constructs has worked quite well, when used immediately after transfection, demonstrating the feasibility of the approach. However, after few passages a deletion of the aptamer occurs. A further developed 3' construct might combine high fluorescence intensity with a wild-type like growth rate and might be resistant to any deletion and therefore allow a more certain conclusion. Additionally studies concerning the detection limit and the conditions required for fluorescence have to be done. Maybe virus harvested from an up scaled transfection, not yet mutated, can be used for infection and replication studies. The FIAsh-Tag constructs could not be developed completely due to limits of time.

5. REFERENCES

- [1] ICTV, "International committee on taxonomy of viruses," 2009. [Online]. Available: www.ictvdb.org. [Accessed April 2012].
- [2] B. Semler and E. Wimmer, *Molecular Biology of Picornaviruses*, Washington, D.C.: ASM Press, 2002.
- [3] B. Sherry, A. Mosser, R. Colonno and R. Rueckert, "Use of monoclonal antibodies to identify four neutralization immunogens on a common cold picornavirus, human rhinovirus 14," *J. Virol.*, no. 57, pp. 246-257, 1986.
- [4] P. Freimuth, L. Philipson and S. Carson, "The Coxsackievirus and Adenovirus Receptor," *Curr. Top Microbiol. Immunol.*, vol. 323, pp. 67-87, 2008.
- [5] V. Racaniello, "One Hundred Years of Poliovirus Pathogenesis," *Virology*, vol. 1, pp. 9-16, 2006.
- [6] J. Ruiz-Sáenz, Y. Goez, W. Tabares and L.-H. A., "Cellular Receptors for Foot-and-Mouth-Disease Virus," *Intervirology*, vol. 4, pp. 201-212, 2009.
- [7] D. Bubeck, D. Filman, N. Cheng, A. Steven, J. Hogle and D. Belnap, "The structure of the Poliovirus 135s cell entry intermediate at 10-Angstrom Resolution Reveals the Location of an externalized Polypeptide that binds to Membranes," *J. Virol.*, vol. 79, no. 12, pp. 7745-7755, Jun 2005.
- [8] R. Fuchs and B. D., "Uncoating of human rhino viruses," *Rev. Med. Virol.*, vol. 20, pp. 281-297, 2010.
- [9] O. Flore, C. Fricks, D. Filman and J. Hogle, "Conformation changes in poliovirus assembly and cell entry," *Semin. Virol.*, no. 1, pp. 429-438, 1990.
- [10] C. Fricks and J. Hogle, "The cell-induced conformational change of poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding.," *J. Virol.*, no. 64, pp. 1934-1945, 1990.
- [11] N. Bayer, D. Schober, E. Prchla, F. Murphy, D. Blaas and R. Fuchs, "Effect of bafilomycin A1 and nocodazole on endocytic transport in HeLa cells: implications for viral uncoating and infection," *J. Virol.*, no. 72, pp. 9645-9655, 1998.
- [12] B. Tsai, "Penetration of nonenveloped viruses into the cytoplasm.," *Annu Rev Cell Dev Biol*, no. 23, pp. 23-43, 2007.
- [13] E. Wimmer, C. Hellen and X. Cao, "Genetics of poliovirus," *Annu. Rev. Genet.*, no. 27, pp. 353-436, 1993.
- [14] J. Seipelt, A. Guarné, E. Bergmann, M. James, W. Sommergruber, I. Fita and T. Skern, "The Structures of Picornaviral Proteinases.," *Virus Res.*, vol. 62, no. 2, pp. 159-68, 1999.
- [15] F. van Kuppeveld, A. de Jong, W. Melchers and P. Willems, "Enterovirus protein 2B po(u)res out the calcium: a viral strategy to survive," *Trends in Microbiology*, vol. 13, no. 2, pp. 41-44, 2005.
- [16] L. Martinez-Gil, M. Bano-Polo, N. Redondo, S. Sanchez-Martinez, J. Nieva, L. Carrasco and I. Mingarro, "Membrane Integration of Poliovirus 2B Viroporin," *J Virology*, vol. 85, no. 21, pp. 11315-11324, 2011.

- [17] T. Sweeney, V. B. D. Cisnetto, M. Bailey, J. Wilson, X. Zhang, G. Belsham and S. Curry, "Foot-and-Mouth Disease Virus 2C is a Hexameric AAA+ Protein with a Coordinated ATP Hydrolysis Mechanism," *J. Biol. Chem.*, vol. 285, no. 32, pp. 24347-24359, 2010.
- [18] P. Adams, E. Kandiah, G. Effantin, A. Steven and E. Ehrenfeld, "Poliovirus 2C protein forms homo-oligomeric structures required for ATPase activity," *J Biol Chem*, vol. 284, no. 33, pp. 22012-22021, 2009.
- [19] N. Teterina, Y. Pinto, J. Weaver, K. Jensen and E. Ehrenfeld, "Analysis of Poliovirus Protein 3A Interactions with Viral and Cellular Proteins in Infected Cells," *J. Virol.*, vol. 85, no. 9, pp. 4284-4296, 2011.
- [20] B. Steil and D. Barton, "Cis-active RNA elements (CREs) and picornavirus RNA replication," *Virus Res.*, vol. 139, pp. 240-252, 2009.
- [21] A. Totsuka and Y. Moritsugu, "Hepatitis A Virus Proteins," *Intervirology*, vol. 42, no. 2-3, pp. 63-68, 1999.
- [22] V. Agol, A. Paul and E. Wimmer, "Paradoxes of the replication of the picornaviral genome," *Virus Research*, vol. 62, no. 2, pp. 129-147, 1999.
- [23] R. Jackson and A. Kaminski, "Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond," *RNA*, vol. 1, pp. 985-1000, 1995.
- [24] H. Pathak, J. Arnold, P. Wiegand, M. Hargittai and C. Cameron, "Picornavirus Genome Replication assembly and organization of the VPg Uridylylation ribonucleoprotein (initiation) complex," *J. Biol. Chem.*, vol. 282, pp. 16202-16213, 2007.
- [25] A. Paul, J. van Boom, D. Filippov and E. Wimmer, "Protein-primed RNA synthesis by purified poliovirus RNA polymerase," *Nature*, vol. 393, pp. 280-284, 1998.
- [26] R. Andino, G. Rieckhof, P. Achacoso and D. Baltimore, "Poliovirus RNA synthesis utilizes an RNP complex formed around the 5'-end of viral RNA," *The EMBO Journal*, vol. 12, no. 9, pp. 3587-3598, 1993.
- [27] W. Xiang, K. S. Harris, L. Alexander and E. Wimmer, "Interaction between the 5'-terminal cloverleaf and 3AB/3CDpro of poliovirus is essential for RNA replication," *J. Virol.*, vol. 69, no. 6, pp. 3658-3667, 1995.
- [28] B. Kempf and D. Barton, "Poly(rC) Binding Proteins and the 5' Cloverleaf of Uncapped Poliovirus mRNA Function during De Novo Assembly of Polysomes," *J Virology*, vol. 82, no. 12, pp. 5835-5846, 2008.
- [29] H. Huang, A. Alexandrov, X. Chen, T. Barnes, H. Zhang, K. Dutta and S. Pascal, "Structure of an RNA hairpin from HRV-14," *Biochemistry*, pp. 8055-64, 2001.
- [30] P. Ahlquist and P. Kaesberg, "Determination of the length distribution of poly(A) at the 3' terminus of the virion RNAs of EMC virus, poliovirus, rhinovirus, RAV-61 and CPMV and of mouse globin mRNA," *Nucleic Acids Res.*, vol. 7, pp. 1195-1204, 1979.
- [31] P. Sarnow, "Role of 3'-End sequences in Infectivity of Poliovirus transcripts made in vitro," *J. Virol.*, vol. 63, no. 1, pp. 467-470, 1989.
- [32] S. Todd and B. Semler, "Structure-infectivity analysis of the human rhinovirus genomic RNA 3' non-coding region," *Nucleic Acids Research*, vol. 24, no. 11, pp. 2133-2142, 1996.
- [33] D. Brown, C. Cornell, G. Tran and J. S. B. Nguyen, "An authentic 3' noncoding region is necessary for efficient poliovirus replication," *J. Virol.*, vol. 79, pp. 11962-11973, 2005.

- [34] C. Rivera and R. Lloyd, "Modulation of enteroviral Proteinase cleavage of poly(A)-binding Protein (PABP) by conformation and PABP-Associated Factors.," *Virology*, vol. 375, no. 1, pp. 59-72, 2008.
- [35] J. Herold and R. Andino, "Poliovirus RNA replication requires genome circularization through a protein-protein bridge," *Mol. Cell*, vol. 74, pp. 581-591, 2001.
- [36] B. Steil, B. Kempf and D. Barton, "Poly(A) at the 3' End of Positive-Strand RNA and VPg-Linked Poly(U) at the 5' End of Negative-Strand RNA Are Reciprocal Templates during Replication of Poliovirus RNA," *J. Virol.*, vol. 84, no. 6, pp. 2843-2858, 2010.
- [37] S. Cordey, D. Gerlach and T. Junier, "The cis-acting replication elements define human enterovirus and rhinovirus species," *RNA*, vol. 14, pp. 1568-1578, 2008.
- [38] A. Gamarnik and R. Andino, "Switch from translation to RNA replication in a positive-stranded RNA Virus," *Genes Dev.*, vol. 12, no. 15, pp. 2293-2304, 1998.
- [39] R. Perera, S. Dajiogo, B. Walter, J. Nguyen and B. Semler, "Cellular Protein Modification by Poliovirus: The two Faces of Poly(RC)-Binding Protein," *J. Virol.*, vol. 81, no. 17, pp. 8919-8932, 2007.
- [40] T. Schultheiss, Y. Kusov and V. Gauss-Müller, "Proteinase 3C of Hepatitis A Virus (HAV) cleaves the HAV Polyprotein P2-P3 at all sites including VP1/2A and 2A/2B," *Virology*, vol. 198, no. 1, pp. 275-281, 1994.
- [41] A. Paul, "Possible unifying mechanism of Picornavirus genome replication," in *Molecular Biology of Picornaviruses*, Washington D.C., ASM Press, 2002, pp. 227-246.
- [42] R. Sharma, S. Raychaudhuri and A. Dasgupta, "Nuclear Entry of Poliovirus Protease-Polymerase Precursor 3CD: Implications for Host Cell Transcription shut-off".
- [43] S. Amineva, A. Aminev, A. Palmenberg and J. Gern, "Rhinovirus 3C Protease Precursors 3CD and 3CD' localize to the Nuclei of infected cells," *J. Gen. Virol.*, vol. 85, no. 10, pp. 2969-2979, 2004.
- [44] A. Castelló, E. Alvarez and L. Carrasco, "The multifaceted Poliovirus 2A Protease: Regulation of gene expression by Picornavirus Proteases," *J. Biomed Biotechnol.*, p. doi:10.1155/2011/369648, 2011.
- [45] A. Haghighat, Y. Svitkin, I. Novoa, E. Kuechler, T. Skern and N. Sonenberg, "The eIF4G-eIF4E Complex is the Target for direct cleavage by the rhinovirus 2A Proteinase," *J. Virol.*, vol. 70, no. 12, pp. 8444-8450, 1996.
- [46] H. Oh, H. Pathak, I. Goodfellow, J. Arnold and C. Cameron, "Insight into Poliovirus Genome Replication and Encapsidation Obtained from Studies of 3B-3C Cleavage Site Mutants," *J. Virol.*, vol. 83, no. 18, pp. 9370-9387, 2009.
- [47] D. Egger, R. Gosert and K. Bienz, "Role of Cellular Structures in Viral RNA Replication," in *Molecular Biology of Picornaviruses*, Washington D.C., ASM Press, 2002, pp. 247-253.
- [48] M. Cho, N. Teterina, D. Egger, K. Bienz. and E. Ehrenfeld, "Membrane Rearrangement and Vesicle Induction by Recombinant Poliovirus 2C and 2BC in Human Cells," *Virology*, vol. 2002, no. 1, pp. 129-145, 1994.
- [49] D. Suhy, T. Giddings and K. Kirkegaard, "Remodeling the Endoplasmic Reticulum by Poliovirus Infection and by Individual Viral Proteins: an Autophagy-Like Origin for Virus-Induced Vesicles," *J. Virol.*, vol. 74, no. 19, pp. 8953-8965, 2000.

- [50] L. Leong, C. Cornell and B. Semler, "Processing Determinants and Functions of Cleavage Products of Picornavirus Polyproteins," in *Molecular Biology of Picornaviruses*, Washington D.C., ASM Press, 2002, pp. 187-197.
- [51] Y. Verlinden, A. Cuconati, W. Wimmer and B. Rombaut, "Cell-Free Synthesis of Poliovirus: 14S Subunits are the Key Intermediates in the Encapsidation of Poliovirus RNA," *J Gen. Virol.*, vol. 81, no. 11, pp. 2751-2754, 2000.
- [52] Y. Verlinden, A. Cuconati, E. Wimmer and B. Rombaut, "The antiviral compound 5-(3,4-dichlorophenyl) methylhydantoin inhibits the post-synthetic cleavages and the assembly of poliovirus in a cell-free system," *Antivir. Res.*, vol. 48, pp. 61-69, 2000.
- [53] L. Vance, N. Moscufo, M. Chow and B. Heinz, "Poliovirus 2C Region Functions during Encapsidation of Viral RNA," *J Virology*, vol. 71, no. 11, pp. 8759-8765, 1997.
- [54] W. Lee and W. Wang, "Human Rhinovirus Type 16: Mutant V1210A requires Capsid-Binding Drug for Assembly of Pentamers to form Virions during Morphogenesis," *J. Virol.*, vol. 77, no. 11, pp. 6235-6244, 2003.
- [55] X.-Y. Jia, M. Van Eden, M. Busch, E. Ehrenfeld and D. Summers, "trans-Encapsidation of a Poliovirus Replicon by Different Picornavirus Capsid Proteins," *J Virol.*, vol. 72, no. 10, pp. 7972-7977, 1998.
- [56] J. Nieva, A. Agirre, S. Nir and L. Carrasco, "Mechanisms of Membrane Permeabilizations by Picornavirus 2B Viroporin," *FEBS Lett.*, vol. 552, no. 1, pp. 68-73, 2003.
- [57] L. Carrasco, "Membrane Leakiness after Viral Infection and a new Approach to the Development of Antiviral Agents," *Nature*, vol. 272, no. 5655, pp. 694-699, 1978.
- [58] J. Lacal and L. Carrasco, "Relationship between Membrane Integrity and the Inhibition of Host Translation in Virus-Infected Mammalian Cells. Comparative Studies between Encephalomyocarditis Virus and Poliovirus," *Eur. J. Biochem.*, vol. 127, no. 2, pp. 359-366, 1982.
- [59] A. Schaefer, J. Kühne, R. Zibirre and G. Koch, "Poliovirus-Induced Alterations in Hela Cell Membrane Functions," *J. Virol.*, vol. 44, no. 2, pp. 445-449, 1982.
- [60] J. Greve, G. Davis, A. Meyer, C. Forte, S. Yost, C. Marlor, M. Kamarck and A. McClelland, "The major human rhinovirus receptor is ICAM-1," *Cell Press*, vol. 56, no. 5, pp. 839-847, 1989.
- [61] N. Verdaguer, I. Fita, M. Reithmayer, R. Moser and D. Blaas, "X-Ray Structure of a Minor Group Human Rhinovirus bound to a Fragment of its Cellular Receptor Protein," *Nature Structural & Molecular Biology*, vol. 11, pp. 429-434, 2004.
- [62] D. Lamson, N. Renwick, V. Kapoor, Z. Liu, G. Palacios, J. Ju, A. Dean, K. St George, T. Briesse and W. Lipkin, "MassTag polymerase-chain-reaction detection of respiratory pathogens, including a new rhinovirus genotype, that caused influenza-like illness in New York State during 2004-2005," *Journal of infectious diseases*, vol. 194, pp. 1398-1402, 2006.
- [63] Y. Bochkov, A. Palmenberg, W. Lee, J. Rathe, S. Amineva, X. Sun, T. Pasic, N. Jariour, S. Liggett and J. Gern, "Molecular modeling, organ culture and reverse genetics for a newly identified human rhinovirus C," *Nature Medicine*, vol. 17, pp. 627-632, 2011.

- [64] A. Reischl, M. Reithmayer, G. Winsauer, R. Moser, I. Gosler and D. Blaas, "Viral evolution toward change in receptor usage: adaptation of a major group human rhinovirus to grow in ICAM-1-negative cells.," *J. Virol.*, vol. 75, pp. 9312-9319, 2001.
- [65] A. Khan, J. Pichler, A. Rosemann and D. Blaas, "Human rhinovirus type 54 infection via heparan sulfate is less efficient and strictly dependent on low endosomal pH," *J. Virol.*, vol. 81, pp. 4625-4632, 2007.
- [66] B. Winter, E. Arruda, T. Witek, S. Marlin, M. Tsianco, D. Innes and F. Hayden, "Expression of ICAM-1 in nasal epithelium and levels of soluble ICAM-1 in nasal lavage fluid during human experimental rhinovirus infection.," *Arch. Otolaryngol Head Neck Surg*, vol. 128, pp. 131-136, 2002.
- [67] H. Rotbart and F. Hayden, "Picornavirus infections: a primer for the practitioner," *Arch. Fam. Med.*, vol. 9, pp. 913-920, 2000.
- [68] J. Hendley, "The host response, not the virus, causes the symptoms of the common cold," *Clin. Infect. Dis.*, vol. 26, pp. 847-848, 1998.
- [69] S. Johnston, "Overview of virus-induced airway diseases.," *Proc. Am. Thorac. Soc.*, vol. 2, pp. 150-156, 2005.
- [70] D. Proud, "Role of Rhinovirus Infections in Asthma," *Asian Pac. J. Allergy Immunol.*, vol. 29, no. 3, pp. 201-208, 2011.
- [71] M. Gavalá, P. Bertics and J. Gern, "Rhinoviruses, allergic inflammation and Asthma," *Immunol. Rev.*, vol. 242, no. 1, pp. 69-90, 2011.
- [72] B. Winther, "Rhinovirus Infections in the upper Airway," *Proc. Am. Thorac. Soc.*, vol. 8, no. 1, pp. 89-89, 2011.
- [73] N. Fuji, A. Suzuki, S. Lupisan, L. Sombrero, H. Galang, T. Kamigaki, R. Tamaki, M. Saito, R. Aniceto, R. Olveda and H. Oshitani, "Detection of Human Rhinovirus C Viral Genome in Blood among Children with Severe Respiratory Infections in the Philippines," *PLoS One*, vol. 6, no. 11, p. e27247, 2011.
- [74] E. Broberg, J. Niemelä, E. Lahti, T. Hyypia, O. Ruuskanen and M. Waris, "Human Rhinovirus C-Associated severe Pneumonia in a Neonate," *J. Clin. Virol.*, vol. 51, no. 1, pp. 79-82, 2011.
- [75] A. Fendrik, A. Monto, B. Nightengale and M. Sarnes, "The economic burden of non-influenza-related viral respiratory tract infection in the United States," *Arch. Intern. Med.*, vol. 163, pp. 486-494, 2003.
- [76] A. Hadfield, W. Lee, R. Zhao, M. Oliveira, I. Minor, R. Rueckert and M. Rossmann, "The Refined Structure of Human Rhinovirus 16 at 2.15 Å Resolution: Implications for the Viral Life Cycle.," *Structure*, vol. 5, no. 3, pp. 427-441, 1997.
- [77] N. Verdager, D. Blaas and I. Fita, "Structure of Human Rhinovirus Serotype 2 (HRV2)," *J. Mol. Biol.*, vol. 300, no. 5, pp. 1179-1194, 2000.
- [78] M. Oliveira, R. Zhao, W. Lee, M. M. I. Kremer, R. Rueckert, G. Diana, D. Pevear, F. Dutko and M. McKinlay, "The Structure of Human Rhinovirus 16.," *Structure* 1993, vol. 1, no. 1, pp. 51-68.
- [79] R. Basavappa, R. Sved, O. Flore, J. Icenogle, D. Filman and J. Hogle, "Role and Mechanism of the Maturation Cleavage of VP0 in Poliovirus Assembly: Structure of the Empty Capsid Assembly Intermediate at 2.9 Å Resolution," *Protein Sci.*, vol. 3, no. 10, pp. 1651-1669, 1994.

- [80] M. Rossmann and J. Johnson, "Icosahedral RNA Virus Structures," *Annu. Rev. Biochem.*, vol. 58, pp. 533-573, 1989.
- [81] M. Rossmann, "The Canyon Hypothesis. Hiding the host cell Receptor Attachment Site on a viral Surface from immune surveillance.," *J. Biol. Chem*, vol. 264, no. 25, pp. 14587-14590, 1989.
- [82] N. Olson, P. Kolatkar, M. Oliveira, R. Cheng, J. Greve, A. McClelland, T. Baker and M. Rossmann, "Structure of a Human Rhinovirus Complexed with its Receptor Molecule.," *Proc. Natl. Acad. Sci. USA*, vol. 90, no. 2, pp. 507-511, 1993.
- [83] P. Laine, S. Blomqvist, C. Savolainen, K. Andries and T. Hovi, "Alignment of Capsid Protein VP1 Sequences of all Human Rhinovirus Prototype strains: conserved motifs and functional domains," *J. Gen. Virol.*, vol. 87, no. 1, pp. 129-138, 2006.
- [84] R. Zhao, D. Pevear, M. Kremer, V. Giranda, J. Kofron, R. Kuhn and M. Rossmann, "Human Rhinovirus 3 at 3.0 Å Resolution.," *Structure*, vol. 4, no. 10, pp. 1205-1220, 1996.
- [85] U. Katpally and T. Smith, "Pocket Factors are Unlikely to play a major role in the Life Cycle of Human Rhinovirus," *J. Virol.*, vol. 81, no. 12, pp. 6307-6315, 2007.
- [86] M. Rossmann, N. Olson, P. Kolatkar, M. Oliveira, R. Cheng, J. Greve, A. McClelland and T. Baker, "Crystallographic and Cryo EM Analysis of Virion-Receptor Interactions.," *Arch. Virol. Suppl.*, vol. 9, pp. 531-541, 1994.
- [87] E. Hewat, E. Neumann, J. Conway, R. Moser, B. Ronacher, T. Marlovits and B. D., "The cellular Receptor to Human Rhinovirus 2 Binds Around the 5-Fold Axis and not in the Canyon: a Structural View," *EMBO J.*, vol. 19, no. 23, pp. 6317-6325, 2000.
- [88] N. Reisdorph, J. Thomas, U. Katpally, E. Chase, K. Harris, G. Siuzdak and T. Smith, "Human Rhinovirus Capsid Dynamics is Controlled by Canyon Flexibility," *Virology*, vol. 314, no. 1, pp. 34-44, 2003.
- [89] N. Florea, D. Maglio and D. Nicolau, "Pleconaril, a Novel Antipicornaviral Agent," *Pharmacotherapy*, vol. 23, no. 3, pp. 339-348, 2003.
- [90] J. Lewis, B. Bothner, T. Smith and G. Siuzdak, "Antiviral agent blocks breathing of the common cold virus.," *Proc. Natl. Acad. Sci. USA*, no. 95, pp. 6774-6778, 1998.
- [91] J. Rollinger and M. Schmidtke, "The Human Rhinovirus: Human-Pathological Impact, Mechanisms of Antirhinoviral Agents, and Strategies for their Discovery.," *Med. Res. Rev.*, vol. 31, no. 1, pp. 42-92, 2011.
- [92] R. Zhao, A. Hadfield, M. Kremer and M. Rossmann, "Cations in Human Rhinoviruses," *Virology*, vol. 6, no. 227, pp. 13-23, 1997.
- [93] J. Hughes, D. Thomas, V. Hamparian and H. Cramblett, "Acid liability of rhinovirus type 14: effect of pH, time, and temperature," *Proc. Soc. Exp. Biol. Med.*, vol. 144, pp. 555-560, 1973.
- [94] T. Skern, H. Torgersen, H. Auer, E. Kuechler and D. Blaas, "Human Rhinovirus Mutant resistant to low pH," *Virology*, vol. 183, pp. 757-763, 1991.
- [95] A. Palmenberg, J. Rathe and S. Liggett, "Analysis of the complete genome sequences of human rhinovirus," *J. Allergy Clin. Immunol.*, vol. 125, no. 6, pp. 1190-1199, 2010.
- [96] S. Tsang, B. McDermott, V. Racaniello and J. Hogle, "Kinetic Analysis of the Effect of Poliovirus Receptor on Viral Uncoating: The Receptor as a Catalyst.," *J. Virol.*, vol. 75, no. 11, pp. 4984-4989, 2001.

- [97] K. Lonberg-Holm and B. Korant, "Early Interaction of Rhinoviruses with Host Cells," *J. Virol.*, vol. 9, no. 1, pp. 29-40, 1972.
- [98] H. Grunert, K. Wolf, K. Langner, D. Sawitzky, K. Habermehr and H. Zeichhardt, "Internalization of human rhinovirus 14 into HeLa and ICAM-1-transfected BHK cells," *Med. Microbiol. Immunol. Berl.*, vol. 186, pp. 1-9, 1997.
- [99] A. Khan, A. Pickl-Herk, L. Gjadzik, T. Marlovits, R. Fuchs and D. Blaas, "Entry of a heparan sulphate-binding HRV8 variant strictly depends on dynamin but not on clathrin, caveolin, and flotillin," *Virology*, vol. 412, no. 1, pp. 55-67, 2011.
- [100] V. Giranda, B. Heinz and M. Oliveira, "Acid-induced structural changes in human rhinovirus 14: possible role in uncoating.," *Proc. Natl. Acad. Sci USA*, vol. 89, pp. 10213-10217, 1992.
- [101] M. Brabec-Zaruba, B. Pfanzagl, D. Blaas and R. Fuchs, "Site of Human Rhinovirus RNA Uncoating Revealed by Fluorescent In Situ Hybridization," *J Virology*, vol. 83, no. 8, pp. 3770-3777, 2009.
- [102] N. Bayer, E. Prchla, M. Schwab, D. Blaas and R. Fuchs, "Human Rhinovirus HRV14 uncoats from early endosomes in the presence of Bafilomycin," *FEBS Letters*, vol. 463, no. 1-2, pp. 175-178, 1999.
- [103] P. Danthi, M. Tostea, Q. Li and M. Chow, "Genome Delivery and Ion Channel Properties are Altered in VP4 Mutants of Poliovirus," *J. Virol*, vol. 77, no. 9, pp. 5266-5274, 2003.
- [104] E. Hewat, E. Neumann and D. Blaas, "The Concerted Conformational Changes during Human Rhinovirus 2 Uncoating.," *Mol. Cell.*, vol. 10, no. 2, pp. 317-326, 2002.
- [105] L. Xing, J. Casasnovas and R. Cheng, "Structural Analysis of Human Rhinovirus Complexed with ICAM-1 reveals the Dynamics of Receptor-Mediated Virus Uncoating.," *J. Virol.*, vol. 77, no. 11, pp. 6101-6107, 2003.
- [106] W. Lee, S. Monroe and R. Rueckert, "Role of Maturation Cleavage in Infectivity of Picornaviruses: Activation of an Infectosome," *J. Virol.*, vol. 67, no. 4, pp. 2110-2122, 1993.
- [107] D. Schober, P. Kronenberger, E. Prchla, D. Blaas and R. Fuchs, "Major and minor receptor group human rhinoviruses penetrate from endosomes by different mechanisms.," *J. Virol*, vol. 72, pp. 1354-1364, 1998.
- [108] Y. Kuznetsov, S. Daijogo, J. Zhou, B. Semler and A. McPherson, "Atomic Force Microscopy Analysis of Icosahedral Virus RNA," *J. Mol. Biol.*, vol. 347, no. 1, pp. 41-52, 2005.
- [109] F. Kienberger, R. Zhu, R. Moser, D. Blaas and P. Hinterdorfer, "Monitoring RNA Release from Human Rhinovirus by Dynamic Force Microscopy.," *J. Virol.*, vol. 78, no. 7, pp. 3203-3209, 2004.
- [110] M. Brabec, D. Schober, E. Wagner, N. Bayer, R. Murphy, D. Blaas and R. Fuchs, "Opening of Size-Selective Pores in Endosomes during Human Rhinovirus serotype 2 in vivo uncoating monitored by single-organelle flow analysis," *J. Virol.*, vol. 79, no. 2, pp. 1008-1016, 2005.
- [111] P. Callahan, S. Mizutani and R. Colonno, "Molecular cloning and complete sequence determination of RNA genome of human rhinovirus type 14," *NCBI Reference Sequence: NC_001490.1*, 1985.

- [112] Y. Lee, A. Nomoto, B. Detien and E. Wimmer, "A Protein Covalently linked to Poliovirus Genome RNA.," *Proc. Natl. Acad. Sci. USA*, vol. 74, no. 1, pp. 59-63, 1977.
- [113] K. Bedard and B. Semler, "Regulation of picornavirus gene expression," *Microbes and Infection*, vol. 6, pp. 702-713, 2004.
- [114] O. Fernández-Miragall, S. López de Quinto and E. Martínez-Salas, "Relevance of RNA Structure for the Activity of Picornavirus IRES Elements," *Virus. Res*, vol. 139, no. 2, pp. 172-182, 2009.
- [115] J. Lin, T. Chen, K. Weng, S. Chang, L. Chen and S. Shih, "Viral and Host Proteins Involved in Picornavirus Life Cycle," *J. Biomed. Sci.*, vol. 16, no. 1, pp. 103; doi:10.1186/1423-0127-16-103, 2009.
- [116] J. Rohll, D. Moon, D. Evans and J. Almond, "The 3' Untranslated Region of Picornavirus RNA: Features Required for Efficient Genome Replication," *Journal of Virology*, vol. 12, pp. 7835-7844, 1995.
- [117] M. Chalfie, Y. Tu, G. Euskirchen, W. Ward and D. Prasher, "Green fluorescent protein as a marker for gene expression.," *Science*, vol. 263, pp. 802-805, 1994.
- [118] K. Ainger, D. Avossa, F. Morgan, S. Hill, C. Barry, E. Barbarese and J. Carson, "Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes," *J Cell Biol*, vol. 123, pp. 431-441, 1993.
- [119] C. Molenaar, S. Marras, J. Slats, J. Truffert, M. Lemaitre, A. Raap, R. Dirks and H. Tanke, "Linear 2' O-Methyl RNA probes for the visualization of RNA in living cells.," *Nucleic Acids Res.*, vol. 29, p. E89, 2001.
- [120] A. Tsuji, H. Koshimoto, Y. Sato, M. Hirano, Y. Sei-Iida, S. Kondo and K. Ishibashi, "Direct observation of specific messenger RNA in a single live cell under a fluorescence microscope," *Biophys. J.*, vol. 78, pp. 3260-3274, 2000.
- [121] R. Monroy-Contreras and L. Vaca, "Molecular Beacons: Powerful Tools for Imaging RNA in Living Cells," *J Nucleic Acids*, vol. 2011, p. 741723, 2011.
- [122] Z.-Q. Cui, Z.-P. Zhang, X.-E. Zhang, J.-K. Wen, Y.-F. Zhou and W.-H. Xie, "Visualizing the dynamic behavior of poliovirus plus-strand RNA in living host cells," *Nucleic Acids Res.*, vol. 33, no. 10, pp. 3245-3252, 2005.
- [123] S. Tyagi and F. Kramer, "Molecular beacons: probes tht fluoresce upon hybridization," *Nature Biotechnology*, vol. 14, no. 3, pp. 303-308, 1996.
- [124] G. Bonnet, S. Tyagi, A. Libchaber and F. Kramer, "Thermodynamic basis of the enhanced specificity of structured DNA probes," *Proc Natl Acad Sci USA*, vol. 96, no. 11, pp. 6171-6176, 1999.
- [125] N. Nitin, P. Santangelo, G. Kim, S. Nie and G. Bao, "Peptide-linked molecular beacons for efficient delivery and rapid mRNA detection in living cells," *Nucleic Acids Res*, vol. 32, no. 6, p. e58, 2004.
- [126] A. Chen, M. Behlke and A. Tsourkas, "Efficient cytosolic delivery of molecular beacon conjugates and flow cytometric analysis of target RNA," *Nucleic Acids Res.*, vol. 36, no. 12, p. e69, 2008.
- [127] E. Bertrand, P. Chartrand, M. Schaefer, S. Shenoy, R. Singer and R. Long, "Localization of ASH1 mRNA particles in living yeast," *Mol Cell*, vol. 2, pp. 437-445, 1998.

- [128] N. Teterina, C. J. K. Lauber, E. Levenson, A. Gorbalenya and E. Ehrenfeld, "Identification of tolerated insertion sites in poliovirus non-structural proteins," *Virology*, vol. 409, no. 1, pp. 1-11, 2011.
- [129] P. Ganguli, W. Chen and M. Yates, "Detection of musine norovirus-1 by using TAT peptide-delivered molecular beacons," *Appl Environ Microbiol*, vol. 77, no. 15, pp. 5517-5520, 2011.
- [130] I. Medinitz and H. Mattoussi, "Quantum dot-based resonance energy transfer and its growing application in biology," *Physical Chemistry Chemical Physics*, no. 11, pp. 17-45, 2009.
- [131] B. Brandenburg, L. Lee, M. Lakadamyali, M. Rust, X. Zhuang and J. Hogle, "Imaging Poliovirus Entry in Live Cells," *PLoS Biology*, vol. 5, no. 7, p. e183, 2007.
- [132] M. Lelek, F. Di Nunzio, R. Henriques, P. Charneau, N. Arhel and C. Zimmer, "Superresolution imaging of HIV in infected cells with FIAsh-PALM," *PNAS*, vol. 109, no. 22, pp. 8564-8569, 2012.
- [133] MolecularProbes, "TC-FIAsh/TC-ReAsH In-Cell Tetracysteine Tag Detection Kits," *Product description by Invitrogen*, 2011.
- [134] B. Griffen, S. Adams and R. Tsien, "Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells," *Science*, vol. 281, pp. 269-272, 1998.
- [135] A. Pomorski and A. Krezel, "Exploration of Biarsenical Chemistry - Challenges in Protein Research," *ChemBioChem*, no. 12, pp. 1152-1167, 2011.
- [136] C. Hoffmann, G. Gaietta, A. Zürn, S. Adams, S. Terrillon, M. Ellisman, R. Tsien and M. Lohse, "Fluorescent labeling of tetracysteine-tagged proteins in intact cells," *Nature Protocols*, vol. 5, no. 10, pp. 1666-1677, 2010.
- [137] F. Madani, J. Lind, P. Damberg, S. Adams, R. Tsien and A. Gräslund, "Hairpin Structure of a Biarsenical - Tetracysteine Motif Determined by NMR Spectroscopy," *J Am. Chem. Soc.*, vol. 131, pp. 4613-4615, 2009.
- [138] S. Adams, R. Campbell, L. Gross, B. Martin, G. Walkup, Y. Yao, J. Llopis and R. Tsien, "New biarsenical ligands and tetra cysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications.," *J Am Chem Soc*, vol. 124, no. 21, pp. 6063-6076, 2002.
- [139] B. Martin, B. Giepmans, S. Adams and R. Tsien, "Mammalian cell-based optimization of the biarsenical binding tetracysteine motif for improved fluorescence and affinity," *Nature Biotechnology*, no. 23, pp. 1308-1314, 2005.
- [140] J. Zhang, R. Campbell, A. Ting and R. Tsien, "Creating new fluorescent probes for cell biology," *Nature Reviews Molecular Cell Biology*, no. 3, pp. 906-918, 2002.
- [141] J. Webb, *Enzyme and Metabolic Inhibitors*, New York: Academic Press, 1963.
- [142] E. Webb and R. Van Heyningen, "The Action of British Anti-Lewisite (BAL) on Enzyme Systems.," *Biochem J.*, vol. 41, no. 1, pp. 74-78, 1947.
- [143] G. Gaietta, T. Deerinck, S. Adams, J. Bouwer, O. Tour, D. Laird, G. Sosinsky, R. Tsien and M. Ellisman, "Multicolor and Electron Microscopic Imaging of Connexin Trafficking," *Science*, vol. 296, pp. 503-507, 2002.
- [144] J. Estévez and C. Somerville, "FIAsh-Based Live-Cell Fluorescent Imaging of Synthetic Peptides Expressed in Arabidopsis and Tobacco.," *Biotechniques*, vol. 41, no. 5, pp. 569-574, 2006.

- [145] C. Spagnuolo, R. Vermeij and E. Jares-Erijman, "Improved Photostable FRET-Competent Biarsenical-Tetracysteine Probes Based on Fluorinated Fluoresceins.," *J. Am. Chem. Soc.*, vol. 128, no. 37, pp. 12040-12041, 2006.
- [146] K. Stroffekova, C. Proenza and K. Beam, "The protein-labeling reagent FLASH-EDT2 binds not only to CCXXCC motifs but also non-specifically to endogenous cysteine-rich proteins," *Eur J Physiol*, no. 442, pp. 859-866, 2001.
- [147] E. Kalef and C. Gitler, "Purification of Vicinal Dithiol-Containing Proteins by Arsenical-Based Affinity Chromatography.," *Methods Enzymol.*, vol. 233, pp. 395-403, 1994.
- [148] T. Wang, P. Yan, T. Squier and M. Mayer, "Prospecting the Proteome: Identification of Naturally Occurring Binding Motifs for Biarsenical Probes," *Chem Bio Chem*, vol. 8, no. 16, pp. 1937-1940, 2007.
- [149] R. Feuer, I. Mena, R. Pagarigan, M. Slifka and J. Whitton, "Cell cycle status affects coxsackie virus replication, persistence, and reactivation in vitro," *J Virology*, vol. 76, no. 9, pp. 4430-4440, 2002.
- [150] J. Tabor-Godwin, C. Ruller, N. Bagalso, N. An, R. Pagarigan, S. Harkins, P. Gilbert, W. Kiosses, N. Gude, C. Cornell, K. Doran, M. Sussman, J. Whitton and R. Feuer, "A novel population of myeloid cells responding to coxsackievirus infection assists in the dissemination of virus within the neonatal CNS.," *J Neurosci.*, vol. 30, no. 25, pp. 8676-8691, 2010.
- [151] J. Poirier, P. Reddy, n. Idamakanti, S. Li, K. Stump, K. Burroughs, P. Hallenbeck and C. Rudin, "Characterization of a full-length infectious cDNA clone and a GFP reporter derivative of the oncolytic picornavirus SVV-001," *J Gen Virol.*, vol. 93, no. 12, pp. 2606-2613, 2012.
- [152] N. Teterina, E. Levenson and E. Ehrenfeld, "Viable polioviruses that encode 2A proteins with fluorescent protein tags.," *J Virology*, vol. 84, no. 3, pp. 1477-1488, 2010.
- [153] G. Arnold, P. Velasco, A. Holms, T. Wrin, S. Geisler, P. Phung, Y. Tian, D. Resnik, X. Ma, T. Mariano, C. Petropoulos, J. Taylor, H. Katinger and E. Arnold, "Broad Neutralization of Human Immunodeficiency Virus Type 1 (HIV-1) Elicited from Human Rhinoviruses That Display the HIV-1 gp41 ELDKWA Epitope," *J Virology*, vol. 83, no. 10, pp. 5087-5100, 2009.
- [154] K. Kalnin, Y. Yan and H. Kleanthous, "Novel Neutralizing Immunogen (NIMIV) of Rhinovirus and its Use for Vaccine Applications". USA Patent 12/442988, 10 1 2007.
- [155] O. Shimomura, F. Johnson and Y. Saiga, "Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the luminous hydromedusan Aequorea," *Journal of Cellular and Comparative Physiology*, vol. 59, no. 3, pp. 223-239, 1962.
- [156] R. Tsien, "The green fluorescent protein," *Annu. Rev. Biochem.*, vol. 67, pp. 509-544, 1998.
- [157] J. Tavaré, L. Fletcher and G. Welsh, "Using green fluorescent protein to study intracellular signalling," *Journal of Endocrinology*, vol. 170, pp. 297-306, 2001.
- [158] T. Mistrelli and D. Pector, "Applications of Green Fluorescent Protein in cell biology and biotechnology," *Nature Biotechnology*, vol. 15, pp. 961-964, 1997.
- [159] O. Shimomura, "Structure of the chromophore of Aequorea green fluorescent protein," *FEBS Letters*, pp. 220-222, 1979.

- [160] A. Cubitt, R. Heim, S. Adams, A. Boyd, L. Gross and R. Tsien, "Understanding, improving and using green fluorescent proteins," *Trends Biochemical Sci.*, vol. 20, pp. 448-455, 1995.
- [161] B. Reid and G. Flynn, "Chromophore Formation in Green Fluorescent Protein," *Biochemistry*, vol. 36, no. 22, pp. 6786-6791, 1997.
- [162] F. Yang, L. Moss and G. Phillips, "The molecular structure of green fluorescent protein," *Nature Biotechnology*, vol. 14, pp. 1246-1251, 1996.
- [163] D. Barondeau, C. Kassmann, J. Tainer and E. Getzoff, "Understanding GFP posttranslational chemistry: structures of designed variants that achieve backbone fragmentation, hydrolysis, and decarboxylation.," *J. Am. Chem. Soc.*, vol. 128, pp. 4685-4693, 2006.
- [164] S. Remington, "Fluorescent proteins: maturation, photochemistry and photophysics," *Current Opinion in Structural Biology*, vol. 16, pp. 714-721, 2006.
- [165] M. Chatteraj, B. King, G. Bublitz and S. Boxer, "Ultra-fast excited state dynamics in green fluorescent protein: multiple states and proton transfer," *Proc. Natl. Acad. Sci. USA*, vol. 93, pp. 8362-8367, 1996.
- [166] K. Brejic, T. Sixma, P. Kitts, S. Kain, R. Tsien, M. Ormo and S. Remington, "Structural basis for dual excitation and photoisomerization of the *Aequorea victoria* green fluorescent protein.," *Proc. Natl. Acad. Sci. USA*, vol. 94, pp. 2306-2311, 1997.
- [167] G. Hanson, T. McAnaney, E. Park, M. Rendell, D. Yarbrough, S. Chu, L. Xi, S. Boxer, M. Montrose and S. Remington, "Green fluorescent protein variants as ratiometric dual emission pH sensors. 1. Structural characterization and preliminary application.," *Biochemistry*, vol. 41, no. 52, pp. 15477-15488, 2002.
- [168] T. McAnaney, E. Park, G. Hanson, S. Remington and S. Boxer, "Green fluorescent protein variants as ratiometric dual emission pH sensors. 2. Excited-state dynamics," *Biochemistry*, vol. 41, no. 52, pp. 15489-15494, 2002.
- [169] T. McAnaney, X. Shi, P. Abbvad, H. Jung, S. Remington and S. Boxer, "Green fluorescent protein variants as ratiometric dual emission pH sensors. 3. Temperature dependence of proton transfer.," *Biochemistry*, vol. 44, no. 24, pp. 8701-11, 2005.
- [170] S. Olsen and S. Smith, "Bond Selection in the Photoisomerization Reaction of Anionic Green Fluorescent Protein and Kindling Fluorescent Protein Chromophore Models.," *J. Am. Chem. Soc.*, vol. 130, no. 1, pp. 8677-8689, 2007.
- [171] G. Zhang, V. Gurtu and S. Kain, "An Enhanced Green Fluorescent Protein Allows Detection of Gene Transfer in Mammalian Cells," *Biochemical and Biophysical Research Communications*, vol. 227, no. 3, pp. 707-711, 1996.
- [172] J. Hasegawa, T. Ise, K. Fujimoto, A. Kikuchi, E. Fukumora, A. Miyawaki and Y. Shiro, "Excited States of Fluorescent Proteins, mKO and DsRed: Chromophore-Protein Electrostatic Interaction Behind the Color Variations," *J. Phys Chem*, vol. 114, pp. 2171-2179, 2010.
- [173] A. Miyawaki, J. Llopis, R. Heim, J. McCaffery, J. Adams, M. Ikura and R. Tsien, "Fluorescent Indicators for Ca²⁺ Based on Green Fluorescent Proteins and Calmodulin," *Nature*, vol. 388, no. 6645, pp. 882-887, 1997.

- [174] V. Romoser, P. Hinkle and A. Persechini, "Detection in Living Cells of Ca²⁺ Dependant Changes in the Fluorescence emission of an Indicator Composed of two Green Fluorescent Protein Variants linked by a Calmodulin-binding sequence. A New Class of Fluorescent Indicators.," *J. Biol. Chem.*, vol. 272, no. 20, pp. 13270-13274, 1997.
- [175] R. Heim, A. Cubitt and R. Tsien, "Improved Green Fluorescence," *Nature*, vol. 373, no. 6516, pp. 663-664, 1995.
- [176] R. Dixit and R. Cyr, "Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guideline for non-invasive fluorescence microscopy," *Plant. J.*, vol. 36, no. 2, pp. 280-290, 2003.
- [177] L. Greenbaum, C. Rothmann, R. Lavi and Z. Malik, "Green fluorescent protein photobleaching: a model for protein damage by endogenous and exogenous singlet oxygen.," *Biol. Chem.*, vol. 381, pp. 1251-1258, 2000.
- [178] M. Bulina, D. Chudakov, O. Britanova, Y. S. D. Yanushevich, T. Chepurnykh, E. Merzlyak, M. Shkrob, S. Lukyanov and K. Lukyanov, "A genetically encoded photosensitizer," *Nat. Biotechnol.*, vol. 24, no. 1, pp. 95-99, 2006.
- [179] H. Niwa, S. Inouye, T. Hirano, T. Matsuno, S. Kojima, M. Kubota, M. Ohashi and F. Tsuji, "Chemical nature of the light emitter of the Aequorea green fluorescent protein," *Proc. Natl. Acad. Sci. USA*, vol. 93, p. 13617, 1996.
- [180] W. B. S. Ward, "Reversible denaturation of Aequorea green-fluorescent protein: physical separation and characterization of the renatured protein," *Biochemistry*, vol. 21, p. 4535, 1982.
- [181] G. Aquino-Jarquín and J. Toscano-Garibay, "RNA Aptamer Evolution: Two decades of Selection," *Int. J. Mol. Sci.*, vol. 12, no. 12, pp. 9155-9171, 2011.
- [182] J. Paige, K. Wu and S. Jaffrey, "RNA Mimics of Green Fluorescent Protein," *Science*, vol. 333, pp. 642-646, 2011.
- [183] J. Paige, T. Nguyen-Duc, W. Song and S. Jaffrey, "Fluorescence Imaging of Cellular Metabolites with RNA," *Science*, vol. 335, p. 1194, 2012.
- [184] A. Palmenberg, D. Spiro, R. Kuzmickas, S. Wang, A. Djikeng, J. Rathe, C. Fraser-Liggett and S. Liggett, "Sequencing and Analyses of All Known Human Rhinovirus Genomes Reveal Structure and Evolution," *Science*, vol. 324, pp. 55-59, 2009.
- [185] A. Gruber, R. Lorenz, S. Bernhart, R. Neuböck and I. Hofacker, "The Vienna RNA Website," *Nucleic Acid Res.*, vol. 36, pp. 70-74, 2008.
- [186] I. Hofacker, W. Fontana, P. Stadler, S. Bonhoeffer, M. Tacker and P. Schuster, "Fast Folding and Comparison of RNA Secondary Structures," *Monatshefte f. Chemie*, vol. 125, pp. 167-188, 1994.
- [187] D. Mathew, J. Sabina and M. T. H. Zucker, "Expanded Sequence Dependence of Thermodynamic Parameters Provides Robust Prediction of RNA Secondary Structure.," *Journal of Molecular Biology*, vol. 288, no. 5, pp. 911-940, 1999.
- [188] D. Mathews, M. Disney, J. Childs, S. Schroeder, M. Zuker and D. Turner, "Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure.," *Proc Natl Acad Sci USA*, vol. 101, no. 19, pp. 7287-7292, 2004.
- [189] M. Andronescu, A. Condon, H. Hoos, D. Mathews and K. Murphy, "Efficient parameter estimation for RNA secondary structure prediction," *Bioinformatics*, vol. 23, no. 13, pp. i19-i28, 2007.

- [190] S. Frackman, G. Kobs, D. Simpson and D. Storts, "Betaine and DMSO: Enhancing Agents for PCR," *Promega Notes*, no. 65, pp. 27-31, 1998.
- [191] R. Don, P. Cox, B. Wainwright, K. Baker and J. Mattick, "'Touchdown' PCR to circumvent spurious priming during gene amplification," *Nucleic Acid Research*, vol. 19, no. 14, p. 4008, 1991.
- [192] H. Withers, "Direct Plasmid Transfer between Bacterial Species and Electrocuting," *Methods Mol Biol.*, vol. 47, pp. 47-54, 1995.
- [193] K. Konduru, S. Nakamura and G. Kaplan, "Hepatitis A virus (HAV) packaging size limit," *BioMed Central*, vol. 6, 2009.
- [194] S. Mizutani and R. Colonno, "In vitro synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14," *J Virology*, vol. 56, no. 2, pp. 628-632, 1985.
- [195] J. Rohll, N. Percy, R. Ley, D. Evans, J. Almond and W. Barclay, "The 5'-untranslated region in Picornavirus RNAs contain Independent Functional Domains Essential for RNA Replication and Translation," *Journal of Virology*, vol. 68, no. 7, pp. 4384-4391, 1994.
- [196] C. Tapparel, T. Junier, D. Gerlach, S. Cordey, S. Van Belle, L. Perrin, E. Zdobnov and L. Kaiser, "New complete genome sequences of human rhinoviruses shed light on their phylogeny and genomic features," *BMC Genomics*, vol. 8, p. 224, 2007.
- [197] F. Weber, W. V., S. Rasmussen, R. Hartmann and S. Paludan, "Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses," *J Virology*, vol. 80, no. 10, pp. 5059-5064, 2006.
- [198] J. den Boon, A. Diaz and P. Ahlquist, "Cytoplasmic Viral Replication Complexes," *Cell Host Microbe*, vol. 8, no. 1, pp. 77-85, 2011.
- [199] A. Jurgeit, S. Moese, P. Roulin, A. Dorsch, M. Lötzerich, W. M. Lee and U. F. Greber, "An RNA replication-center assay for high content image-based quantifications of human rhinovirus and coxsackievirus infections," *Virology Journal*, vol. 7, no. 264, pp. 1-13, 2010.
- [200] D. Resnick, A. Smith, S. Gesiler, A. Zhang, E. Arnold and G. Arnold, "Chimeras from a human rhinovirus 14-human immunodeficiency virus type 1 (HIV-1) V3 loop seroprevalence library induce neutralizing responses against HIV-1," *J. Virology*, vol. 69, no. 4, pp. 2406-2411, 1995.
- [201] A. Smith, S. Geisler, A. Chen, D. Resnick, B. Roy, P. Lewi, E. Arnold and G. Arnold, "Human Rhinovirus Type 14:Human Immunodeficiency Virus Type 1 (HIV-1) V3 Loop Chimeras from a Combinatorial Library Induce Potent Neutralizing Antibody Responses against HIV-1," *J. Virology*, vol. 72, no. 1, pp. 651-659, 1998.
- [202] H. Yeh, M. Yates, A. Mulchandani and W. Chen, "Visualizing the dynamics of viral replication in living cells via Tat peptide delivery of nuclease-resistant molecular beacons," *PNAS*, vol. 105, no. 45, pp. 17522-17525, 2008.
- [203] C. Srisawat and D. Engelke, "RNA affinity tags for purification of RNAs and ribonucleoprotein complexes," *RNA*, vol. 7, no. 4, pp. 632-641, 2001.
- [204] J. Meredith, J. Rohll, J. Almond and D. Evans, "Similar Interactions of the Poliovirus and Rhinovirus 3D Polymerases with the 3' Untranslated Region of Rhinovirus 14," *J Virology*, vol. 73, no. 12, pp. 9952-9958, 1999.

- [205] X. Cao, R. Kuhn and E. Wimmer, "Replication of Poliovirus RNA Containing Two VPg Coding Sequences Leads to a Specific Deletion Event," *J Virology*, vol. 67, no. 9, pp. 5572-5578, 1993.
- [206] E. Pilipenko, A. Gmyl and V. Agol, "A model for rearrangements in RNA genomes," *Nucleic Acids Research*, vol. 23, no. 11, pp. 1870-1875, 1995.
- [207] P. Li, X. Bai, Y. Cao, C. Han, Z. Lu, P. Sun, H. Yin and Z. Liu, "Expression and Stability of Foreign Epitopes Introduced into 3A Nonstructural Protein of Foot-and-Mouth-Disease Virus," *PLoS one*, vol. 7, no. 7, p. e41486, 2012.
- [208] A. Pierangeli, M. Bucci, P. Pagnotti, A. Degener and R. Bercoff, "Mutational analysis of the 3'-terminal extra-cistronic region of poliovirus RNA: secondary structure is not the only requirement for minus strand RNA replication," *FEBS Letters*, vol. 374, no. 3, pp. 327-332, 1995.
- [209] S. Todd, J. Towner, D. Brown and B. Semler, "Replication-Competent Picornaviruses with Complete Genomic RNA 3' Noncoding Region Deletions," *J Virology*, vol. 71, no. 11, pp. 8868-8874, 1997.
- [210] D. Stoner-Ma, A. Jaye, P. Matousek, M. Towrie, S. Meech and P. Tonge, "Observation of Excited-State Proton Transfer in Green Fluorescent Protein using Ultrafast Vibrational Spectroscopy," *J. Am. Chem. Soc.*, vol. 127, pp. 2864-2865, 2005.
- [211] J. Ding, A. Smith, S. Geisler, X. Ma, G. Arnold and E. Arnold, "Crystal Structure of a Human Rhinovirus that Displays Part of the HIV-1 V4 Loop and Induces Neutralizing Antibodies against HIV-1," *Structure*, vol. 10, pp. 999-1011, 2002.

Figure 1 Modified X-Ray image of HRV2.....	10
Figure 2 Phylogenetic tree of Picornaviridae.....	8
Figure 3 Protease processing of picornaviral polyprotein.....	10
Figure 4 Schematic representation of a picornaviral genome.	11
Figure 5 Chemical structure of VPg covalently linked to the viral RNA.....	11
Figure 6 Schematic image of an icosahedral picornaviral capsid	15
Figure 7 Phylogenetic tree of known rhinoviruses.....	16
Figure 8 Structure of the 3'-UTR of HRV-14.....	18
Figure 9 Structure of the 5'-UTR of HRV-2.....	18
Figure 10 The three states of a molecular beacon.	19
Figure 11 Lewis structures of TC-FIAsH and TC-ReAsH.....	21
Figure 12 NMR model of FLNCCPGCCMEP	21
Figure 13 The photo cycle of GFP turning the fluorophore from alcohol to its phenolate state via Excited-State Proton Transfer.....	23
Figure 14 The 2D structure prediction of the Spinachaptamers.	24
Figure 15 Available RNA-Fluorophore constructs resulting in the corresponding excitation (above) and emission (below) spectra.....	25
Figure 16 Schematic representation of pHRV14-as3f-WT.....	27
Figure 17 Graphical representation of the overlap PCR.....	28
Figure 18 Schematic representation of the HRV Genome.	37
Figure 19 Prediction I (CL).....	37
Figure 20 Prediction II (SP).....	38
Figure 21 Prediction III (SM)	38
Figure 22 Prediction IV (PA)	39
Figure 23 Spectra of RNA-DFHBI complexes.....	40
Figure 24 Transfection of RNA with Lipofectamin 2000.....	41
Figure 25 Plaque assay 2 days after infection.	41
Figure 26 Fluorescence microscopy images (20x) of HeLa Ohio cells transfected with Lipofectamin 2000 and viral RNA for 5 hours.....	42
Figure 27 Fluorescence microscopy images (20x) of HeLa Ohio cells infected with the respective virus over night.	43
Figure 28 Confocal microscopy image (63x) 24h after transfection with WT RNA, stained with a-double-strand-RNA antibody and Hoechst 33342.....	42
Figure 29 Prediction V (IC)	45
Figure 30 3D Presentation of [209] with Chimera.	46
Figure 31 Predicted models (by Swiss-Model) for 3 different inserts of FIAsH-Tags in a loop of VP2.	47
Figure 32 Predicted models (by Swiss-Model) for 3 different inserts of FIAsH-Tags in VP1	48
Figure 33 3' and 5' products of VP1 and VP2 protein modifications.....	51
Figure 34 Gel electrophoresis of PCR hybridization products..	52
Figure 35 Chromatograms of VP2 PCR products.	53
Figure 36 Chromatograms of VP1 PCR products.	53

6. APPENDICES

ABSTRACT

We inserted an RNA aptamer called spinach which form a fluorescent complex with the small compound DFHBI into the genome of human rhinovirus 14 (HRV-14) aiming at facile dynamic fluorescent monitoring of its fate in order to gain new insight into the life-cycle of the viruses most frequently associated with the common cold. The sequence of the aptamer required insertion into non-coding regions of HRV-14, where the known key cis-regulatory elements forming conserved secondary structures like the cloverleaf or the IRES had to be kept intact. These constraints reduced the spectrum of available options to only 4 target sites: At the 5'-UTR after the two start Uracils – before the cloverleaf; at the 5'-UTR after the cloverleaf – before the IRES within the short polymorphic spacer region; or within the 3'-UTR between the end of the open reading frame and before the conserved stem-loop and between the stem-loop and poly A tract. The Vienna RNA Websuite was used to analyze for and rule out misfolding of the chimeric viral RNA as a result of aptamer tagging. We found that the insertion of spinach resulted in replication defective viruses in three instances, with severity dependent on the insertion site. The fourth construct was comparable to wild type but likely carried a misfolded inactive aptamer. Two recombinant viruses could be passaged on HeLa cells but [the virus is] proofed genetically unstable and deleted the aptamer, surprisingly without leaving behind any truncated sequences. Three chimeric RNA species became fluorescent in the presence of DFHBI in vitro and also in vivo when transfected into HeLa cells. First-generation plaque isolated recombinant viruses carrying the equivalent spinach-tagged genomic RNA gave rise to fluorescent signals in HeLa cells but only at the peak of infection (5 h post infection) which presently precludes dynamic analysis of early events such as endosomal RNA release. As a further project we also planned to insert a tetra-cystein-tag into a capsid protein, either VP1 or VP2. However, due to limitations in time this task could not be completed within this master thesis.

ZUSAMMENFASSUNG

Diese Thesis behandelt die Insertion eines RNA-Aptamers, genannt Spinach, welches mit dem kleinen Molekül DFHBI einen fluoreszierenden Komplex bildet, in das Genom des humanen Rhinovirus 14 (HRV-14). Damit soll es ermöglicht werden die virale RNA während eines gesamten Infektionszykluses in der Zelle zu beobachten um neue Erkenntnisse über die gesamte Replikation des Virus zu erhalten, welcher am häufigsten mit der gemeinen Erkältung assoziiert ist. Die Sequenz des Aptamers musste in nicht kodierende Regionen des Genoms insertiert werden, wo auch bereits bekannte Schlüsselstrukturen wie „Cloverleaf“ und „IRES“ intakt bleiben mussten. Das reduzierte die Anzahl der möglichen Stellen auf 4 Positionen: In der 5'-UTR jeweils vor und nach dem Cloverleaf und zwei in der 3'-UTR. Um die Stabilität der Konstruktionen und um ungewünschte Strukturbildung vorherzusagen wurde die Vienna RNA Websuite verwendet.

Die Einfügung des Aptamers resultierte in drei Fällen in einem defekten Virus, wobei das Ausmaß von der Position des Aptamers abhing. Das vierte Konstrukt zeigte Wachstumsraten vergleichbar mit dem nativen Virus, trug jedoch ein nicht fluoreszierendes Aptamer mit sich. Zwei der drei leuchtenden rekombinanten Viren konnten in HeLa-Zellen gezüchtet werden, zeigten sich jedoch genetisch instabil und deletierten das Aptamer interessanterweise punktgenau.

Drei Konstrukte fluoreszierten in Gegenwart von DFHBI *in vitro* und *in vivo* transifiziert in HeLa Zellen. Die erste Passage von aus Plaques isolierten rekombinanten Viren zeigten ebenfalls Fluoreszenz 5 Stunden nach der Infektion. Als ein weiteres Projekt wurde die Einführung eines Tetra-Cystein-Tags in ein Kapsidprotein (entweder VP1 oder VP2) geplant. Wegen des beschränkten Zeitrahmens konnte dies jedoch nicht während der Masterarbeit fertig gestellt werden.

6.1. ABBREVIATIONS

aa	amino acid
AMP	Adenosinemonophosphate
bp	base pair
cDNA	copy DNA
COPD	Chronic obstructive pulmonary disease
CRE	cis-acting responsive element
CV	Coxsackievirus
DAF	Decay Acceleration Factor
DFHBI	3,5-difluoro-4-hydroxybenzylidene imidazolinone
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
eGFP	enhanced GFP
ER	endoplasmatic reticulum
FCS	Fluorescence Correlation Spectroscopy
FISH	Flurescence In Situ Hybridisation
FIAsH	Flurescein Arsenical Helix Binder
FMDV	Foot-and-mouth Disease Virus
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
HAV	Hepatitis A Virus
HBI	4-hydroxybenzylidene imidazolinone
HCAR	human coxackievirus B and adenovirus 2 receptor
HeLa	Henriette Lang cells
HEV	Human Enterovirus
HRV	Human rhinovirus
ICAM	Intercellular adhesion molecule
IRES	Internal ribosome entry site
L(V)LDL	(Very) Low Density Lipoprotein
LB	Lysogeny broth medium
LRP	
MB	Molecular Beacon
MHC	Majorhistocompatibility complex
mRNA	messenger RNA
nt	nucleotids
NTP	Nucleotidtriphosphate
ORF	open reading frame
PABP	Polyadenosine binding protein
PAGE	Polyacrylamid gel electrophoresis
PBS	Phosphate buffered solution
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
Pvr	Poliovirus receptor

TCATCCCTATTGCCCTCTTACAGTACCAACTGGAGCAACTCCCTCACTCCCTATAACAGTCACA
 ATAGCACCTATGTGCACTGAGTTCTCTGGGATAAGGTCCAAGTCAATTGTGCCACAA GGTTCG
 CAACTACAACCTTTGCCGGGCTCAGGACAATTCTTGACACAGATGACAGGCAATCCCTCAAGTGC
 ACTGCCAAATTATGAGCCAACTCCAAGAATACACATACTAGGGGAAAGTTCATAACTTGCTAGAA
 ATTATACAGGTAGATACACTCATTCCTATGAACAACACGGCATACAAAAGATGAGGTTAACAGTT
 ACCTCATACCACTAAATGCAAAACAGGCCAAATGAGCAGGTTTGTGGACAAACCTGTTTATTGG
 TGATGGGGTCTTCAAACTACTCTTCTGGGTGAAATTTGTCAGTACTATACACATTGGTCTGGAT
 CACTTAGATTCTCTTGATGTATACTGGTCTGCTTGTCCAGTGCTAACTCACTCTAGCATACA
 CCCCSCCTGGTGGTGGTCCACAGGACAGGAGAGAAGCAATGCTAGTACTCATGTTGTCT
 GGGATATTGGTCTGCAATCCACCATAGTAATGACAATACCATGGACATCAGGGGTGCASTTTA
 GATATACTGATCCAGATACATACACCAAGTGTGGCTTCTATCATGTTGGTATCAAACCTTCTCT
 ATACTTCCCCAGAAACGACCGGCCAGGTCTACTTATTATCATTCATAAGTGCATGTCCAGATT
 TAAGCTTAGGCTGATGAAAGATACTCAAACCTATCTCAGAGACTGTTGCACTCACTGAAGGCTTA
 GGTGATGAATTAGAAGAAGTCATCGTTGAGAAAACGAAACAGACGGTGGCCTCAATCTCATCT
 GGTCAAAACACACACAAAAAGTCCCCATACTAACTGCAAACGAAACAGGGGCCACAATGCCT
 GTTCTCCATCAGACAGCATAGAAACAGAACTACCTACATGCACITTAATGGTTCAGAACTG
 ATGTAGAATGCTTTTTGGGTCGTGCAGCTTGTGTGCATGTAACGAAATACAAAACAAAGATGC
 TACTGGAATAGATAATCAGAGAGAAGCAAAATTGTTCAATGATTGGAAAAATCAACCTGTCAGC
 CTTGTCCAACCTAGAAAGAACTGGAACCTTCACTTATGTTAGGTTTGATTCTGAGTATACCAT
 ACTGGCCATGCTGTCAACCTGATTGACAACTATTCAAGCAATTTGGTGGTCCAAGCCATG
 TATGTTCCACATGCTGCCCGAAATCCAAAGAGTGGGGCATTACACATGGCAAAGTCTTCA
 AACCCAGTGATTCTCAAGGTGGGGGATACATCAAGGTTTAGTGTGCCTTATGTAGGATTGG
 CATCAGCATATAATTGTTTTATGATGGTTACTCACATGATGATGCAGAACTCAGTATGGCATA
 ACTGTTCTAAACCATAGGGTAGTATGGCATTGAGAATAGTAAATGAACATGATGAACATAAAA
 CTCTGTCAAGATCAGAGTTTATCAGGGGCAAAGCACGTTGAAGCATGGATTCCAAGAGCAC
 CCAGAGCACTACCTACACATCAATAGGGCGCACAAATATCTAAGAATACAGAACCAGTAAT
 TAAGAAGAGGAAAGGTGACATTAATCCTATGGTTTAGGACCTAGGTACGGTGGGATTATAC
 ATCAAATGTTAAATTAATGACAAATCTCAACAAAGTCAACAGAACTACAAGAAGTGGCGAAAGAT
 CCCTATCCAAATAGAGATTTAGCAATAGTCTCAACAGGAGGACATGGTGCAGAAACAATACCA
 CACTGTAACCTGTACATCAGGTGTTTACTATTCCACATATTACAGAAAGTATTACCCCATATTG
 TGAAGGCCACCAACATCTGGATTGAAGGAAACCTTATTACCAAGTAGATTTCAAGCAGGA
 GTGATGAAAGGGGTGGGCCAGCAGAACCAGGAGACTGCCGTGGGATTTTGAGATGCATACA
 TGGTCCCATTGGATTGTTAACAGCTGGAGGTAGTGGATATGTTTGTGCTGACATACGACAG
 TTGGAGTGATCGCAGAGGAACAGGGGCTGAGTGATTACATCACAGGTTTGGGTAGAGCTTTT
 GGTGTCGGGTTCACTGACCAATCTCAACAAAGTCAACAGAACTACAAGAAGTGGCGAAAGAT
 TTCTCACCAAAAAGTTTGTCCAAAGTGGTCAAAATGTTTTAGCTTTAGTGATCATTGTCAG
 AAATCATGATGACTTGGTCACTGTTACGGCCACTCTAGCACTACTTGGATGTGATGGATCTCCCT
 GGAGATTTCTGAAGATGTACATTTCAAACACTTTCAGGTGCCTTACATTGAAAGACAAGCAAA
 TGATGGATGGTTGAGAAAGTTAATGATGCATGTAATGCTGCAAGGGATTGGAATGGATTGC
 TAATAAGATTTCCAACTGATTGAATGGATAAAAAACAAAGTACTTCCCCAAGCCAAAGAAAAA
 CTAGAATTTGTAGTAACTCAAACTTGTATATACTAGAGAGACAAATAACCCATGCATA
 TCTCGAATCCAACAGGAAAAACGAGAGCAGTTGTTCAACAAGTATTGTTGGTTGGAACAAA
 TGTGCAAAAGTTTGCCCACTTATGCCGTTGAATCAAAAAGAACTAGGGAACCTCAAGAACAA
 AATGGTAAATTATATGAATTTAAAGTAAACAAAGAACTGAACCAAGTGTGTGTTAATCCAT
 GGTACACCCGGTTCTGGTAAATCATTAAACATCCATTGTGGGACGTGCAATTGCAGAACT
 TCAATTACGAGTATATTCACTTCCACAGATCCCAAGCACTTTGATGGTTATCAGCAACAGGAA
 GTTGTGATTATGGATGATCTGAACCAAAATCCAGATGGACAGGATATAAGCATGTTTTGTCAA
 TGGTTTCTTCAGTGGATTCTTGCTCCAATGGCTAGTTAGATAACAAGGGCATGTTATTCACC
 AGTAATTTGTCTAGGCTCCACAAATCTAACACACTAAGCCCCAACAACTTGAATCCTGA
 AGCTTTATGTAGGATTTGGTTTACCTAGATATATGTTTGCATACTACCTACACAAAGAATG
 GAAAACCTCAATGCAGGCATGTCAACCAAGACATGCAAAGATTGCCATCAACCATCTAATTTCAA
 GAAATGTTGCCCTAGTCTGTGAAAAGCTATTAGCTTGGTAGACAGAACTACCAACGTTAGG
 TATAGTGTGGATCAACTGGTCACGGCTATTATAAGTGATTTCAAGAGCAAAATGCAAATTACAG
 ATTCCTAGAAAACACTGTTTCAAGGACAGTGATAAAGATTAGAGATTGATGTTTGCAACAC
 ACCACCTTCAGAAATGTATCAACGATTACTGAAATCTGTAGATTGAGAAGAGATTAGGGAATAT
 TGTAAGAAGAAGAAATGGATTATACCTGAAATTCCTACCAACATAGAAAGGGCTATGAATCAA
 GCCAGCATGATTATTAATCACTTCTGATGTTTGCAGTACATTAGGTATGTTTATGTCATTTAT
 AAATTGTTTGTCAAACCTCAAGGACCATATTCTGGTAACCCGCCTCACAATAAACTAAAAGCCCC
 AACTTTACGCCCAGTTGTTGTGCAAGGACCAACACAGAATTTGCACTATCCCTGTTAAGGAAA
 AACATAATGACTATAACAACCTCAAAGGGAGAGTTACAGGGTTAGGCATACATGATCGTGTCT
 GTGTGATACCCACACACGACAGCCTGGTGATGATGTACTAGTGAATGGTCAGAAAATTAGAG
 TTAAGGATAAGTACAAATAGTAGATCCAGAGAACATTAATCTAGAGCTTACAGTGTGACTTT
 AGATAGAAATGAAAAATTGAGAGATATCAGGGGATTATATCAGAAGATCTAGAAGGTGTGGA
 TGCCACTTTGGTAGTACATTCAAATAACTTTACCAACACTATCTAGAAGTTGGCCCTGTAACAA

VP3

VP1

NdeI

AvrII

2A^{pro}

2B

2C

3A

3B

3C^{pro}

TGGCAGGACTTATTAATTTGAGTAGCACCCCCACTAACAGAATGATTCTGTTATGATTATGCAAC
 AAAAAGTGGGAGTGTGGAGGTGTGCTGTGCTACTGGTAAGATCTTTGGTATTCATGTTGG
 CGGTAAATGGAAGACAGGATTTTCAGCTCAACTTAAAAACAATATTTGTAGAGAAACAAGG
 CCAAGTAATAGCTAGACATAAGGTTAGGGAGTTTAAACATAAATCCAGTCAACACGGCAACTAA
 GTCAAAATTACATCCCAGTGTATTTATGATGTTTTCCAGGTGACAAGGAACCTGCTGTATTGA
 GTGACAATGATCCAGACTGGAAGTTAAATTGACTGAATCATTATTCTCTAAGTACAAGGGGAA
 TGTAAATACGGAACCCACTGAAAATATGCTTGTGGCTGTAGACCATTATGCAGGGCAACTATTA
 TCACTAGATATCCCCACTTCTGAACCTTACACTAAAAGAAGCATTATATGGAGTAGATGGACTAG
 AACCTATAGATATTACAACAGTGCAGGATTTCCCTATGTGAGTCTTGGGATCAAAAAGAGAGA
 CATTCTGAATAAAGAGACCCAGGACACAGAAAAGATGAAGTTTTATCTAGACAAGTATGGCAT
 TGACTTGCTCTAGTTACATATATTAAGGATGAATTAAGAAGTGTGACAAAAGTCCGATTAGGG
 AAAAGTAGATTAATTGAAGCCTCCAGTTTGAATGATTCTGTTAACATGAGAATGAACTAGGCA
 ACCTTTACAAAGTCCATCAAAATCCCGGTGTTCTGACTGGATCAGCAGTGGGTGTGATCC
 TGATGTGTTTTGGTCTGTCATCCCTTGCTTAATGGATGGGCACCTGATGGCATTGATTACTCTA
 ATTTTGATGCCTCTTTGTCACCAGTTTGGTTTGTCTGTCTAGAGAAGTTTTGACCAAGTAGGC
 TTTGACGGCTCTTCATTAATTCAATCAATTTGTAATACCCATCATATCTTAGGGATGAAATATAT
 GTGGTTGAAGGTGGCATGCCCTCAGGGGTGTCAGGAACACAGCATATTCATTCCATGATCAACA
 ACATAATCATTAGGACTTTGATATTAGATGCATATAAAGGAATAGATTTAGACAAACTTAAAAAT
 CTTAGCTTACGGTGATGATTTGATTGTTTCTTATCCTTATGAAGTGGATCCACAAGTGTGGCAA
 CTCTTGGTAAAAATTATGGACTAACCATCACACCCAGACAAATCTGAACTTTTACAAAAATG
 ACATGGGAAAACTTGACATTTTTAAAGAGATACTTCAAGCCTGATCAACAATTTCCCTTTTGGT
 TCACCCAGTTATGCCATGAAAGATATACATGAGTCAATCAGATGGACAAAGGATCCTAAAAAC
 ACACAGGATCACGTCGGATCATTATGCATGTTAGCATGGCACTCAGGAGAAAAAGAGTACAAT
 GAATTCATTGAGAAGATCAGAACTACTGACATTGGAAAAATGTCTAATCTCCAGAAATACAGCG
 TACTTAGGAGGCGTGGTTGGACCTCTTTAGGTTAAACAATATAGACACTTAATTTGAGTAGAA
 GTAGGAGTTTATAAAAAAGAGTgcagcatgaagcttcagctgctcagggggggcccggtaccagctt
 ttgtcccttagtgagggttaattgcgccttgccgtaatcatggcatagctgttctgtgaaattgtatccgctcacaat
 tccacacaacatacagcggaagcataaagttaaagcctgggtgctaatgagtgagctaaactcacattaattgcgttg
 cgctcactccccgtttccagtcgggaacacctgtcgtgccagctgcattaatgaatcgccacgcgcgggagagcggtt
 gcgtattgggcctctccgcttctcgtcactgactgcgtcgctcggtcttcggctgcggcgagcggtatcagctcactca
 aaggcggttaatacgttatccacagaatcaggggataacgcaggaagaacatgtgagcaaaaggccagcaaaaggcca
 ggaaccgttaaaaggccgctgtcgtgcgtttttccataggtccgccccctgacgagcatcacaaaaatcgacgtcaagt
 cagaggtggcgaaccgcagagactataagataccaggcgtttccccctggaagctccctcgtgcgtctcctgttccgac
 cctccgcttaccggatacctgtccgcttttcccttcgggaagcgtggcgctttctcatagctcacgcttaggtatctcagtt
 cggtgtaggtcggtcctcaagctgggtgctgtgcacgaacccccgttcagcccgaccgctgcgcttatccggttaactat
 gtcttgagtcgaaccggtaagacacgacttatcccactggcagcagccactggttaacaggattagcagagcgaggtatgt
 aggcggtgctacagagttcttgaagtggggcctaactacggctacactagaaggacagttatttggtatctgcgtctgctga
 agccagttaccttcggaagaaagttggtgagctttgatccggaacaaaccaccgctggtagcggtggtttttttgttgca
 agcagcagattacgcgcagaaaaaaggatctcaagaagatccttgatctttctacggggtctgacgctcagtggaacga
 aaactcacgttaagggttttggcatgagattatcaaaaaggatcttcacctagatccttttaaataaaaatgaagtttaa
 atcaatctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaacagtgaggcacctatctcagcgatctgtct
 atttcgttcacatagttgctgactccccgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgca
 atgataccgcagagaccacgctcacggctccagatttatcagcaataaaccagccagccggaaggccgagcgcagaagt
 ggtcctgcaactttatccgctccatccagcttattaattgttgcgggaagctagagtaagtgttgcagttaatagtttgc
 gcaacgttgttgccattgctacagcagctgtggtgtcacgctgtcgtttggtatggcttcattcagctccggttcccaacgatc
 aaggcgagttacatgatccccatgttgcgcaaaaagcggttagctccttcggtcctccgatcgttgcagaagtaagttggc
 cgagtggtatcactcatggttatggcagcactgcataattcttactgtcatgcatccgttaagatgcttttctgtgactggtg
 agtactcaaccaagtcattctgagaaatagtgatcggcgacagggtgtccttcccggcgtaatacggggataataccgcg
 ccacatagcagaactttaaaagtgtcatcattggaacacgttcttcggggcgaaactctcaaggatcttaccgctgttgag
 atccagttcgaatgaaccactcgtgcacccaactgatctcagcatctttactttcaccagcgttttctgggtgagcaaaaaca
 ggaaggcaaaatgccgcaaaaagggaataaggcgacacggaaatgtgaatactcatacttctcttttcaatattattg
 aagcatttatcagggttattgtctcatgagcgatacatattgaatgtatttagaaaaataacaatagggttccgcgcac
 atttccccgaaaagtgccac

3D^{pol}

Bsu36I

Stem-Loop
 Poly-A

PstI

Curriculum Vitae

Personal Data

Name: Matthias Pilecky
Date of birth: September 18th, 1988 in Melk
Address: Prandtauerstr. 8/2, A-3390 Melk
Phone: +43/664/88543969
E-Mail: matthias.pilecky@univie.ac.at

School Education

1998-2006 Stiftsgymnasium Melk with linguistic focus;
Term paper in biology (Cystic Fibrosis) in context of the school leaving exams

Studies

2007-2010 Bachelor studies „Molecular Biotechnology“ at the university for applied science (FH Campus Wien)
2010-2013 Master studies „Biological Chemistry“ with focus on food chemistry at the university of vienna

Internships / Jobs

january 2012 - january 2013	Masterthesis at the Department for medical biochemistry of the Max F. Perutz Laboratories
january 2010 - june 2010	Bachelorthesis at the institute for biomedical research of the medical university vienna
september 2006	Internship at the institute for biophysics at the JKU Linz

Further Training

since 2007 Voluntary work with the Austrian red cross as paramedic and group leader; frequent training on the (emergency) medical field