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

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Wien, 2013
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.
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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 polyprotein of around 2-2.5 amino acids. [2]
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. 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. The mechanism of this transformation is not fully understood. An important feature seems to be the exposure of the amphipathic N-terminal region of VP1.

### Table 1
Human pathogens of picornaviridae with their receptor and the disease they cause. Adopted from [2] (2002); αvβ3, αvβ6, αvβ1 = 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; β2-m = β2 microglobulin (part of MHC I); MAP-70 (part of MHC I); N/A = Not available;

<table>
<thead>
<tr>
<th>Enterovirus</th>
<th>Receptor</th>
<th>Accessory factors</th>
<th>Major syndromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coxsackievirus A9</td>
<td>αvβ3</td>
<td>MAP-70, β2-m</td>
<td>Herpangina, hand-foot-and-mouth disease, respiratory disease, meningitis, poliomyelitis</td>
</tr>
<tr>
<td>Coxsackievirus B1,3,5,7,8,10,12,14,16</td>
<td>HCAR</td>
<td>αvβ6</td>
<td>Myocarditis, pleurodynia, Meningitis, respiratory disease, neonatal infections</td>
</tr>
<tr>
<td>Coxsackievirus 1,8</td>
<td>α2β1</td>
<td>DAF</td>
<td>Meningitis, encephalitis</td>
</tr>
<tr>
<td>Echovirus 3,6,7,11,12,13,19,21,24,25,29,30,33</td>
<td>β2-m, CD59</td>
<td>DAF</td>
<td>Pleurodynia, exanthema</td>
</tr>
<tr>
<td>Polioviruses 1-3</td>
<td>CD155</td>
<td>N/A</td>
<td>Poliomyelitis, (meningitis)</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>N/A</td>
<td>N/A</td>
<td>Common cold, infantile diarrhea</td>
</tr>
<tr>
<td>Coxsackievirus A21, 24</td>
<td>ICAM-1</td>
<td>N/A</td>
<td>Acute hemorrhagic conjunctivitis</td>
</tr>
<tr>
<td>Coxsackievirus 70 (68)</td>
<td>DAF</td>
<td>N/A</td>
<td>Acute hemorrhagic conjunctivitis</td>
</tr>
</tbody>
</table>

| Hepatovirus | HAVcr-1 | N/A | Type A hepatitis |
| Aphthovirus | N/A | N/A | Foot-and-mouth disease (cloven-footed livestock) |
| Cardiovirus | VCAM-1 | N/A | Encephalitis, myocarditis |
After binding, virus is internalized via endosomes. There either by pore formation or membrane disruption RNA gets into the cytoplasm, leaving an empty capsid (80S particle) behind. 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. 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.

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 chymotrypsin-like serine proteinase (3C) as well as the RNA-dependent RNA-Polymerase (3D). Still not all biochemical activities of the picornaviral proteins are explored yet.

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)
Replication and translation of viral polypeptide 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 typ. 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 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 uridyalted VPg, an internal cis-acting responsive element (CRE or OriI) 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 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]:

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide from</th>
<th>Nucleotide to</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPg-TT</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cloverleaf</td>
<td>3</td>
<td>81</td>
</tr>
<tr>
<td>IRES</td>
<td>116</td>
<td>628</td>
</tr>
<tr>
<td>ORF-AUG</td>
<td>629</td>
<td>631</td>
</tr>
<tr>
<td>VP4</td>
<td>629</td>
<td>835</td>
</tr>
<tr>
<td>VP2</td>
<td>836</td>
<td>1621</td>
</tr>
<tr>
<td>VP3</td>
<td>1622</td>
<td>2329</td>
</tr>
<tr>
<td>VP1</td>
<td>2330</td>
<td>3196</td>
</tr>
<tr>
<td>2A</td>
<td>3197</td>
<td>3634</td>
</tr>
<tr>
<td>2B</td>
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</tr>
<tr>
<td>2C</td>
<td>3926</td>
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<tr>
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<td>5239</td>
</tr>
<tr>
<td>3C</td>
<td>5240</td>
<td>5785</td>
</tr>
<tr>
<td>3D</td>
<td>5786</td>
<td>7165</td>
</tr>
<tr>
<td>ORF-UAG</td>
<td>7166</td>
<td>7168</td>
</tr>
<tr>
<td>Stem-Loop</td>
<td>7177</td>
<td>7212</td>
</tr>
<tr>
<td>Poly-A</td>
<td>7213</td>
<td></td>
</tr>
</tbody>
</table>

As for all picornaviruses discussed before, the RNA is covalently linked to the Tyr₁-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 8** Structure of the 3’-UTR of HRV-14; the first 6 nt are part of the ORF [116]

**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]
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 where 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. FlAsH – 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-CYSTEIN 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 teta-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 (FlAsH), or the as well 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 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-tetra-cysteine sequences have been investigated regarding their affinity to FlAsH 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*. 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. The structure of the chromophore was first discovered by Shimomura in 1979. Three amino acids (Ser65 – Tyr66 – Gly67) undergo an autocatalytic reaction resulting in 4-hydroxybenzylidene imidazolinone (HBI), albeit the last step (α,β-double bound of Y66) requiring molecular oxygen and thus being the time limiting step with 2h for WT. The reaction is promoted by the formation of a β-barrel consisting of 11 β-sheets. Tyr66 is not necessary for the cyclization and may be replaced by tryptophan for cyan FP, histidine for blue FP, or other residues obtaining non-fluorescent variants. Nevertheless Tyr66 is conserved in all natural fluorescent proteins. Undoubtedly it has an important role in the excited-state proton transfer. 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 pKₐ to near zero, becoming strongly acidic and transferring its proton via the hydrogen bond network. (see figure 13) In modified FPs this mechanism is partially blocked resulting in dual emission GFPs (deGFPs) which are able to act as pH-indicator.

![Figure 13](image)

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 $\alpha,\beta$-double bound 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 $\alpha,\beta$-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 $^1$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
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 assembly. This would allow for example co localisation studies of FlAsH-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.

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

The following oligonucleotid 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>nt</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1-TC 5’ fw</td>
<td>CCAAAAGACTGGGGAGACATTACATGGCAAAGTGCTTC</td>
<td>37</td>
</tr>
<tr>
<td>VP1-TC 5’ rv</td>
<td>GCAGCATCCCCGGCAACATTTGATTTTCGTTAATTGTTGCTGCAGATGGTGGTGTG</td>
<td>64</td>
</tr>
<tr>
<td>VP1-TC 3’ fw</td>
<td>GTTTCCCGGAGATGCTGCAGATTAAGAAGAGGAAAGGACAT</td>
<td>44</td>
</tr>
<tr>
<td>VP1-TC 3’ rv</td>
<td>ATCCACTACCTCCACGTGTA</td>
<td>21</td>
</tr>
<tr>
<td>VP1-FLN 5’ fw</td>
<td>CGAAAATCCAAAGGAGGAGGGGATTAC</td>
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</tr>
<tr>
<td>VP1-FLN 5’ rv</td>
<td>CATGACAGATCCCCGGCAACAGTGGAGGAAATTCCTAGGATAATTGTTGCTGCGATGGTCT</td>
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<tr>
<td>VP1-FLN 3’ fw</td>
<td>TTCTCCACTGGCTTGCAAGTGCATGGAACAGTAAATAGAAGAGGAAAG</td>
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</tr>
<tr>
<td>VP1-FLN 3’ rv</td>
<td>ATGCTCTCCTGTGGAGACT</td>
<td>20</td>
</tr>
<tr>
<td>VP1-HRW 5’ fw</td>
<td>GGGGATGACATTACATGGCAAAGGAGGACAT</td>
<td>26</td>
</tr>
<tr>
<td>VP1-HRW 5’ rv</td>
<td>AAAACGTAGACATTACAGTGGCAGCATTTGCAATTGACAGCTGTCATGAGTTGCTGCAAGTGAAGGAGGAGGAGGACAT</td>
<td>63</td>
</tr>
<tr>
<td>VP1-HRW 3’ fw</td>
<td>ACCATGCTCCTCCTGATGGAGGACAT</td>
<td>23</td>
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<tr>
<td>VP1-HRW 3’ rv</td>
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<td>25</td>
</tr>
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<td>VP2-Var1 5’ fw</td>
<td>GCAGCATCCCCGGCAACATTTGATTTTCGTTAATTGTTGCTGCAGATGGTGGTGTG</td>
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</tr>
<tr>
<td>VP2-Var1 5’ rv</td>
<td>TGCTCCACCTCCACGTGTA</td>
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</tr>
<tr>
<td>VP2-Var1 3’ fw</td>
<td>GGCAAAATCCAAAGGAGGAGGGGATTAC</td>
<td>31</td>
</tr>
<tr>
<td>VP2-Var1 3’ rv</td>
<td>CATGACAGATCCCCGGCAACAGTGGAGGAAATTCCTAGGATAATTGTTGCTGCGATGGTCT</td>
<td>22</td>
</tr>
<tr>
<td>VP2-Var2 5’ fw</td>
<td>GGGCCTGATGACATTACAGTGGCAGCATTTGCAATTGACAGCTGTCATGAGTTGCTGCAAGTGAAGGAGGAGGACAT</td>
<td>65</td>
</tr>
<tr>
<td>VP2-Var2 5’ rv</td>
<td>TTCTCCACTGGCTTGCAAGTGCATGGAACAGTAAATAGAAGAGGAAAG</td>
<td>66</td>
</tr>
<tr>
<td>VP2-Var2 3’ fw</td>
<td>GGCAAAATCCAAAGGAGGAGGGGATTAC</td>
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<td>VP2-Var2 3’ rv</td>
<td>GAATGCGGCTCACCTGCAATGAGCTGCAATGGAAGGAGGAGGAGGACAT</td>
<td>26</td>
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<td>VP2-Var3 5’ fw</td>
<td>AAACGTAGACATTACAGTGGCAGCATTTGCAATTGACAGCTGTCATGAGTTGCTGCAAGTGAAGGAGGAGGACAT</td>
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<td>CACAGCTGATGACATTACAGTGGCAGCATTTGCAATTGACAGCTGTCATGAGTTGCTGCAAGTGAAGGAGGAGGACAT</td>
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<tr>
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<td>TGTAGGCTGATGACATTACAGTGGCAGCATTTGCAATTGACAGCTGTCATGAGTTGCTGCAAGTGAAGGAGGAGGACAT</td>
<td>30</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1 fw</td>
<td>CATGGCAAAGTGCTTCAAAC</td>
<td>for amplification of VP1 PCR hybrids</td>
</tr>
<tr>
<td>VP1 rv</td>
<td>GATGTATAAATCCCACCGTACC</td>
<td></td>
</tr>
<tr>
<td>VP2 fw</td>
<td>CATCAGCTGGTCAATCACTGTC</td>
<td>for amplification of VP2 PCR hybrids</td>
</tr>
<tr>
<td>VP2 rv</td>
<td>CGTGCTTGGCCCTGTAACAC</td>
<td></td>
</tr>
</tbody>
</table>

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 pHV14-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 EXTRACTION

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 and 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](http://www.neb.com)) was consulted to find the optimal conditions. If not otherwise mentioned digest was done at 37°C for 2 hours followed by 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, Middelsex, UK) using the following primers:

<table>
<thead>
<tr>
<th>primer</th>
<th>sequence</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’ seq</td>
<td>CCCAGAATACACGGTACTTAGG</td>
<td>+-strand sequencing of 3’-UTR</td>
</tr>
<tr>
<td>5’ seq</td>
<td>ATACCTCGCTCTGGGAAC</td>
<td>specific RT-Primer for 5’ end (instead of oligo dT)</td>
</tr>
<tr>
<td>SP fw</td>
<td>GGTGGAATCCGCTGTAACTT</td>
<td>sequence primer for SP constructs</td>
</tr>
<tr>
<td>VP1 seq</td>
<td>CACCCAGAGCACTACCTAC</td>
<td>for sequencing of VP1 insert region</td>
</tr>
<tr>
<td>VP2 seq</td>
<td>AGAACACCAACTGCTTCAC</td>
<td>for sequencing of VP2 insert region</td>
</tr>
</tbody>
</table>
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:

<table>
<thead>
<tr>
<th>Container Type</th>
<th>Cells/well</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>1000</td>
<td>100 µl</td>
</tr>
<tr>
<td>12-well plate</td>
<td>10⁵</td>
<td>1 ml</td>
</tr>
<tr>
<td>6-well plate</td>
<td>3x10⁵</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>T-25 flask</td>
<td>7x10⁵</td>
<td>5 ml</td>
</tr>
<tr>
<td>T-75 flask</td>
<td>1.5x10⁶</td>
<td>12 ml</td>
</tr>
<tr>
<td>T-162 flask</td>
<td>3x10⁶</td>
<td>25 ml</td>
</tr>
<tr>
<td>T-225 flask</td>
<td>5x10⁶</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

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, overlayed 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 MgCl₂ (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. STRUKTUR 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.

**3.1.1.1. 5′-UTR PRECLOVERLEAF REGION**

\[
\text{GCCGGCTTT... (for full sequence see attachment)...GCCAACATCTCTCACTCTAGCTACCACGACCAGAAATGCTCAAGACGCGGTCAGTCGTCGCTGCTCAGACGACCGACCGACCGAAATGGTGAAGGACGGGTCCAGTGCTTCGGCACTGTTGAGTGGAGTGTGAGCTCCGTAACTGGTCGCGTCGACAAACAGCGGATGGGTATCCCACCATTCACCCCTTTTTACCGCC (765 nt)}
\]

*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, SalI, Kasi

---

**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

GCCGCC... (for full sequence see attachment) ...GGTATACGACTCACC

ATACGTTAACACCGGATGGGATCGACGACCTCCACATCCGAGCCGATGCTTGAGCTCCGACCT

CTCTTTCAGCGCTGTTGTCGGCCAGCCACGAGAAATGCTGAGAGGACCCCGAC

GGTCCGAGACGCGACG

GCAGTGTGTCTGTCGAC

CCGGG

TTCCTTAAA

ATTCCCACCCATGAAACGTTAGAAGCTTGACATTAAAGTACAATAGGTG

GGCGCC (763 nt)

Gray: UU + CloverLeaf; Pink: Spacer; Green: 24-2 Aptamere; Yellow: IRES

**Bold and Underlined:** Sites for restriction enzymes in series: NgoMIV, Rsrl, 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

CCTCAGG... (for complete sequence see attachment) ...TAGTTAAC

GCTAGCAGCGACCCAGAGTGTGAGGACCGGGTCCAGTTCGACTCTCCAGTGGATGACTAGCT

GTCTCACTGTTGCGGTGTGCTCGACAATAGCAGTTTTGAGTAGAAGTAGGAAC

AAAAAGCTGCAG (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

CCTCAGGTG... (for complete sequence see attachment)...TAGGTTAACAGATTAGACACTTAATTTGAGTAGAGAAGTAGGAAGGTTATCTCTGCTAGCAGCGCACGAAATGGAAGGACCATCGCATCGTGACGTTGATCGCTGACAAA

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

Stop-Codon with 3′-UTR

Poly-A

3′-terminal structure

Figure 22 Prediction IV with RNAfold of the sequence above. Important for this simulation was the stability of the 3′ hairpin structure. The lowest-energy prediction suggests base pairing of nucleotides of the open reading frame with the poly-A; These interactions are however very unlikely and thermodynamically very instable (<1 kcal/mol)

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 polyadenosine-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 CCCAGTCACGAGTTGTAAA 5′-UTR sequencing
pHRV14 RNA rv CGCGCAATTAACCCTCACTA 3′-UTR sequencing

All four plasmids were transcribed into RNA successfully as confirmed by electrophoresis.
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 µM RNA). The Pre-3’-StemLoop (SM) construct is about 1.5 times brighter than the Spacer (SP) variant.

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 \textit{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.)

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.

![Fluorescence microscopy images](image)

**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](image)

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](image)

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.

**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 HRV 14-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.
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]

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 inserttion between A158 and N159.
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 FlAsH-Tags (TC, FLN and HRW) when inserted into VP2.

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.
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 Nlm-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 Nlm-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 FlAsH-Tags. Upper left: Wild-Type; Upper right: Standard TC-Tag (CCPGCC); Lower left: FLNCCPGCCMEP; and Lower right: HRWCCPGCCKTF; 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]
<table>
<thead>
<tr>
<th>HRV-A95</th>
<th>KAKHIKAWCPRPPRATYHNYYVEEGVTPETHVKYAEVT</th>
<th>TITTY</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRV-A45</td>
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<td>TV</td>
</tr>
<tr>
<td>HRV-C6</td>
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</tr>
<tr>
<td>HRV-C26</td>
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</tr>
<tr>
<td>HRV-C11</td>
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<tr>
<td>HRV-A95</td>
<td>KAKHIKAWCPRPPRATYHNYYVEEGVTPETHVKYAEVT</td>
<td>TITTY</td>
</tr>
</tbody>
</table>

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  RAKLVEAWIPRAPRALPTYSGRTNYPKNTEPVKKRKGDIKSY
HRV14-VP1-TC  RAKLVEAWIPRAPRALPTYSGRTNYPKNTECCPGCCPKVIKKRKGDIKSY
HRV14-VP1-FLN  RAKLVEAWIPRAPRALPTYSGRTNYPKNTEFLNCCPGCMEPVKIKRKKGDIKSY
HRV14-VP1-HRW  RAKLVEAWIPRAPRALPTYSGRTNYPKNHEWCCPGKTFIKIKRKKGDIKSY

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

<table>
<thead>
<tr>
<th>Construct</th>
<th>5’ Length</th>
<th>3’ Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1 (VP2-TC)</td>
<td>568 nt</td>
<td>1820 nt</td>
</tr>
<tr>
<td>V2 (VP2-FLN)</td>
<td>565 nt</td>
<td>2192 nt</td>
</tr>
<tr>
<td>V3 (VP2-HRW)</td>
<td>846 nt</td>
<td>1806 nt</td>
</tr>
<tr>
<td>VT (VP1-TC)</td>
<td>371 nt</td>
<td>423 nt</td>
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<tr>
<td>VF (VP1-FLN)</td>
<td>349 nt</td>
<td>182 nt</td>
</tr>
<tr>
<td>VH (VP1-HRW)</td>
<td>332 nt</td>
<td>182 nt</td>
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</table>

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.)](image)
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.](image)

Due to occurring problems in the later ligation step the products were cut at this stage with AflII/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 gel purified 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 stand is paused by misincorpoations, 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 successfull. 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 aptamer-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 FlAsH 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 FlAsH-Tag constructs could not be developed completely due to limits of time.
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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


6.1. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosinemonophosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>CRE</td>
<td>cis-acting responsive element</td>
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<td>CV</td>
<td>Coxsackievirus</td>
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<tr>
<td>DAF</td>
<td>Decay Acceleration Factor</td>
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<tr>
<td>DFHBI</td>
<td>3,5-difluoro-4-hydroxybenzylidene imidazolinone</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>DNA</td>
<td>Desoxyribonucleic acid</td>
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<td>eGFP</td>
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<td>ER</td>
<td>endoplasmatic reticulum</td>
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<td>FCS</td>
<td>Fluorescence Correlation Spectroscopy</td>
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<td>FISH</td>
<td>Fluorescence In Situ Hybridisation</td>
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<td>FIAsh</td>
<td>Flurescein Arsenical Helix Binder</td>
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<td>FMDV</td>
<td>Foot-and-mouth Disease Virus</td>
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<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>HAV</td>
<td>Hepatitis A Virus</td>
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<tr>
<td>HBI</td>
<td>4-hydroxybenzylidene imidazolinone</td>
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<td>HCAR</td>
<td>human coxackievirus B and adenovirus 2 receptor</td>
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<td>Human Enterovirus</td>
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<td>Human rhinovirus</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
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<tr>
<td>L(V)LDL</td>
<td>(Very) Low Density Lipoprotein</td>
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<td>Plaque Forming Unit</td>
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6.2. SEQUENC E OF THE P LASMID HRV14-AS3F-WT

The complete sequence of pHRV14-as3f-wt as sequenced by Tim Skern et al. [94]
The HRV14 genome contained in the sequence is written in capital letters. Letters with green background indicated insertion position for Spinach, red background indicates insertion position chosen for FlashTag. Underlined sequence parts mark used unique restriction sites.
Curriculum Vitae

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