Frog and Toad herpesvirus detection 2018-2019

Project co-financed by FOEN (contract n. R212-1468) and Vontobel Foundation (Zurich).

Summary as November 2018

This research project aims to develop two distinct, but complementary diagnostic tests to reveal the presence of *Ranid herpesvirus* 3 (RHV3), a recently discovered herpesvirus associated with severe skin proliferative disease, in common frogs (*Rana temporaria*) in Switzerland (Origgi et al., 2017). As many herpesviruses, RHV3 is expected to be present in its "lytic" form in acutely infected frogs and in its "latent" form in chronically infected individuals. Once is present in its lytic form, the virus is normally present in large amounts and shed in the environment making its detection possible by tests such as real-time PCR (RT-PCR). Differently, in its latent form, no virus is normally shed, and the presence of the virus cannot be detected by RT-PCR. However, antibodies developed against the virus are circulating in the infected individuals during latency. Consequently, a serological test (ELISA) would allow the detection of the exposure to the virus. The development of an RT-PCR and of an ELISA test would then allow the detection of the virus and of the exposure to it in common frogs independently of the clinical stage of the disease. These two complementary tests will allow an estimation of the prevalence of RHV3 in the common frog populations in Switzerland, helping to clarify the role of this virus in amphibian disease ecology and preservation of biodiversity.

This research project comprises multiple steps including:

1) Real time PCR development
    a. Target selection
    b. Primer and probe design
    c. Positive control development
    d. Sensitivity assessment
2) ELISA development
    a. Antigen development
    b. Monoclonal antibody development
    c. Assay development
3) Real time PCR and ELISA test validation.

Current status of our research as November 2018

RT-PCR development

We have selected the DNA polymerase gene, a very conserved gene among herpesviruses, as an ideal target for the RT-PCR. The high conservation of the gene makes very unlikely the occurrence of frequent mutations in this gene, allowing the detection of the virus even in slightly divergent strains. We have designed three pairs of primers and associated probes. Following testing with a *sybr green* format, we have selected the primer pair showing the best sensitivity and with the best dissociation curve. Following, we have cloned the target DNA amplicon (105nt) into a cloning vector (pGEM-T-easy), determined its molecular weight and prepared ten-fold dilution suspensions of the target plasmid to set up a standard curve to determine the limit of detection of the system. The selected protocol allowed the detection of up to a single copy of the target plasmid corresponding to 1 viral genome equivalent.

Ongoing future steps:

1) Validation of the set-up RT-PCR system for the detection of RHV3 genomic DNA in samples derived from a transmission study carried out in common frogs.
2) Development of a qualitative PCR protocol for the combined detection of RHV3 genomic DNA and of that of *Bufonid herpesvirus* 1 (BfHV1) a novel herpesvirus that we discovered in common toads (Bufo bufo) (Origgi et al., 2018).
3) If time and resources will allow, development of a RT-PCR system for the detection of BfHV1 genomic DNA will also be developed similarly to what has been done for RHV3.

ELISA development

Antigen development

The first step for the ELISA development is the production of the antigen, which is the core of the test itself. Given the unsuccessful attempts to grow RHV3 in cell culture, so far, we have decided to produce recombinant antigen. The selected targets include the Major capsid protein (MCP), a large viral protein, well conserved among herpesviruses know to be antigenic and consequently ideal as a surrogate antigen for the whole virus. Together with the recombinant RHV3 MCP we have also selected a second protein, which is named ORF118, which has been determined to be the most expressed of the RHV3 protein in infected individuals by transcriptomic analysis, recently carried out by our group. The selection of a "core" antigen as MCP, together with a highly expressed and consequently highly exposed antigen to the frog immune system is considered an appropriate strategy to achieve a proper antigenic substrate for the ELISA development. The genes expressing the MCP and ORF118 protein have been consequently amplified by high fidelity PCR protocols and cloned into expression vectors (pET30C+). Following full sequencing of the cloned genes in order to assess their integrity and proper reading frame, the expression vectors and consequently used to transform competent bacteria for protein expression (BL21 DE3). Each of the cloned gene had a "histidine-tag" located 5' for detection. Additionally, site-directed mutagenesis was carried out in order to place an additional histidine-tag 3' to the cloned genes. The mutagenesis protocol was successful with the ORF118 gene, whereas is still to be carried out with the MCP gene. Following the expression in competent bacteria, western blot analysis revealed the presence of strong bands both in the bacterial extracts from ORF118 carrying either one or two histidine tags. The predicted size of the full length fusion protein including RHV3-ORF118 is approximately 100KDa. The bands observed in the western blot corresponds to protein of approximately 45, 50 and 80 KDa possibly suggesting the expression of a truncated ORF118 (N'portion) protein. Additional analyses are currently ongoing to determine meaning of the observed result. The expression of MCP has not be detected by western blot analysis, however additional analyses are being currently carried out to determine the reasons for this lack of detection, despite sequencing confirming the presence of full length gene into the expression vector in the correct reading frame.

Ongoing future steps:

Protein expression

We are planning to achieve full expression of both ORF118 and MCP. Re-sequencing of the expression vectors, together with cloning of portions of the MCP gene, instead of its full length are being considered.

Surrogate viral proteins

We have recently purchased a strain of *Ranid herpesvirus* 2 (RHV2), from ATCC. RHV2 is the only viral strain among the *Batrachoviruses*, the genus which comprises all the frog herpesviruses known to date, which is known to grow in culture. Despite the differences existing between RHV3 and RHV2, the two viruses share great similarities in several of their constituent proteins. Accordingly, it is highly probably that RHV2 might serve as not specific, but cross-reacting antigen for RHV3. The virus is currently being grown and we aim to evaluate its cross reactivity with RHV3 and if it is possible to actually use it as replacement of RHV3. The use of the whole virus would provide great advantages vs the use of single proteins in term of sensitivity, although specificity might not be as high. However, at the current development stage, we consider this a viable option. Should it be successful, we also plan to admix the recombinant RHV3 MCP and 118 to the whole RHV2 proteins.

Monoclonal antibody development

A monoclonal antibody directed against the common frog immunoglobulins is currently being developed by one of our collaborators at the Istituto Zooprofilattico in Brescia, Italy. Common frog immunoglobulins will be purified and then used as antigen in BALBc mice to develop an hybridoma that will be then screened to select specific clones directed, ideally, against distinct groups of common frog immunoglobulins.

<u>Ongoing future steps</u>

Additionally to the development of specific monoclonal antibodies against common frog immunoglobulin we intend to test a monoclonal antibody developed against *Xenopus* sp. IgY. An aliquot of this antibody has been provided to us by Professor Jacques Robert, from the University of Rochester, USA and will be evaluated soon.

ELISA setting

Once the antigen and the monoclonal antibody will be available, it will be possible to start the setup of the ELISA test together with the already available positive and negative control sera obtained from RHV3-naturally infected frogs and from naïve individuals, respectively. Positive and negative sera are also available from RHV3-experimentally infected and uninfected frogs, respectively as well. We expect to have the reagents available by the spring/summer 2019.

ELISA and Real Time PCR development for RHV3 detection in Free-Ranging Common frogs (*Rana temporaria*)

This research project aims to develop two distinct, but complementary diagnostic tests to reveal the presence of *Ranid herpesvirus* 3 (RHV3), a recently discovered herpesvirus associated with severe skin proliferative disease, in common frogs (*Rana temporaria*) in Switzerland (Origgi et al., 2017). As many herpesviruses, RHV3 is expected to be present in its "lytic" form in acutely infected frogs and in its "latent" form in chronically infected individuals. Once is present in its lytic form, the virus is normally present in large amounts and shed in the environment making its detection possible by tests such as real-time PCR (RT-PCR). Differently, in its latent form, no virus is normally shed, and the presence of the virus cannot be detected by RT-PCR. However, antibodies developed against the virus are circulating in the infected individuals during latency. Consequently, a serological test (ELISA) would allow the detection of the exposure to the virus. The development of an RT-PCR and of an ELISA test would then allow the detection of the virus and of the exposure to it in common frogs independently of the clinical stage of the disease. These two complementary tests will allow an estimation of the prevalence of RHV3 in the common frog populations in Switzerland, helping to clarify the role of this virus in amphibian disease ecology and preservation of biodiversity.

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Below are listed the different steps that are being carried out to accomplish this project.

<u>Real Time PCR</u>

Target selection

The aim of the development of a real time PCR test for RHV3 detection was to make available a diagnostic test that would be effective in revealing acutely infected viral-shedding frogs. The test is supposed to detected the broadest number of potential distinct strains of RHV3 circulating in Switzerland and potentially in Europe. Accordingly, a very well conserved gene among herpesviruses, the DNA polymerase gene was selected. Given the high degree of conservation of the DNA polymerase gene, which is under a (evolutionary) negative selective pressure, it would be unlikely that RHV3 strains would diverge strongly enough to accumulate nucleotide mutations to inhibit primers and probe annealing on the selected target.

Primer and probe design

The nucleotide sequence of the RHV3 type strain was previously made available by our group (Origgi et al., 2017). The nucleotide gene of the DNA polymerase gene was selected, and a series of primer pairs and associated probes were obtained using the software Genious 10.0 using standard settings. A total of three primers pairs and associated probes were then obtained. The list of primers and probes are listed below:

Primer pair and probe set 1:

RHV3_RT_1_FW

5'-GCA GGA CAC AAT TCA AGC GG-3'

RHV3_RT_1_RV

Primer pair and probe set 2:

5'-TTT ACT GCG GTA TAG CGC CC-3'

RHV3_RT_2_FW

5'-TTA TCA TGC GAT CCA CCG GG-3'

RHV3_RT_2_RV

5'-CTT GTG ATA GTC GGG GTG GC-3'

Primer pair and probe set 3:

RHV3_RT_3_FW

5'-TCG TGG ATG TGT CGA GTT CC-3'

RHV3_RT_3_RV

5'-AGT TCA GCC ACG TAA TCC CC-3'

Primer and probe selection

The primer pairs were tested in a syber green format carried out according standard settings. Of the three primers pairs assessed, pair # 1 was selected as the best pair on the basis of the sensitivity and dissociation curve profile.

Positive control cloning

Once the best performing primers were selected (RHV3_RT_1 set), the positive control consisting of the DNA sequence amplified by the chosen primers was obtained by qualitative PCR (Taq Pol, Red cap). The amplicon was then cloned into the cloning vector pGEM-T-Easy (Promega, Madison, WI, USA) according to the manufacturer's instructions. Positive white colonies (Blue/White selection) were amplified and the plasmid was isolated and sequenced. The sequenced amplicon matched with the expected amplified RHV3 DNA polymerase gene, partial sequence and was 105 nt long, as expected.

Positive control molecular weight and titration procedure

The molecular weight of the positive control DNA amplicon cloned into the expression vector and the correspondent number of molecules contained in 1ng of DNA was determined according to the following formula (https://bitesizebio.com/20669/how-to-calculate-the-number-of-molecules-in-any-piece-of-dna/) based on the Avogadro number:

Number of copies=(ng*6.022x10^{23})/(length*1x10^{9}*650)

Accordingly:

Number of copies=(ng*6.022x10^{23})/(105+3015*1x10^{9}*650)

Number of copies=(ng*6.022x10^{23})/(3120x10^{9}*650)

Number of copies per nanogram=(6.022x10^{23})/(3.12x10^{14}*6.5)

Number of copies per nanogram=(6.022x10^{9})/(20.28)

Number of copies per nanogram=(6.022x10^{8})/(2.028)=600,022,000/2.28=2.641228x10^{8}

This means that 100 million copies of the target are present in 0.3786ng

Real time sensitivity test

Following the calculation showed above, we determined that 10^{9} copies of the target would have been present in 3.786 ng of plasmid. Accordingly, this amount of plasmid DNA was added to 1ml of DD H2O. Two serial dilutions (10 folds) named A and B, ranging from 10^{6} to 10^{-2} were prepared to be tested in real time format. Samples were tested in triplicates.

Real time PCR standard curve and limit of detection

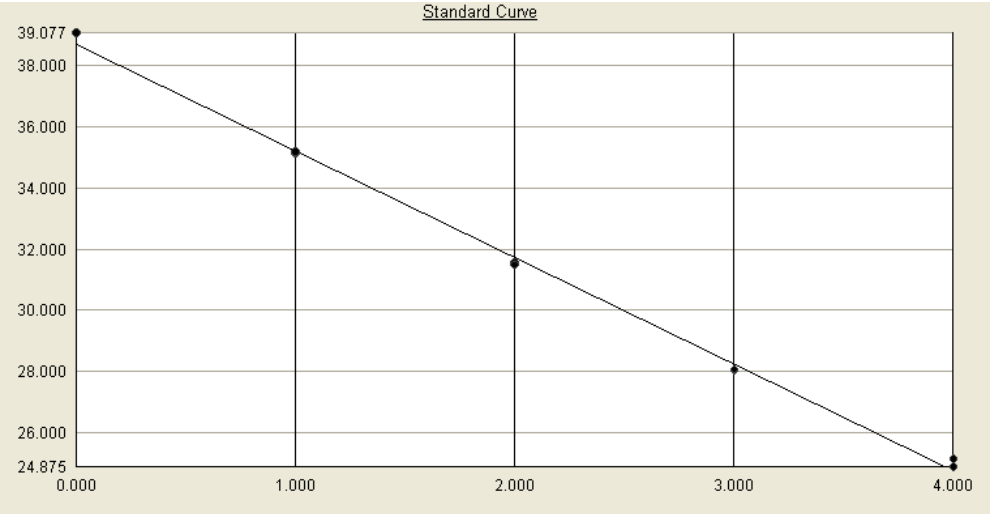


Figure 1. Standard curve. All the points of the standard are closely aligned (R2= 0.99)

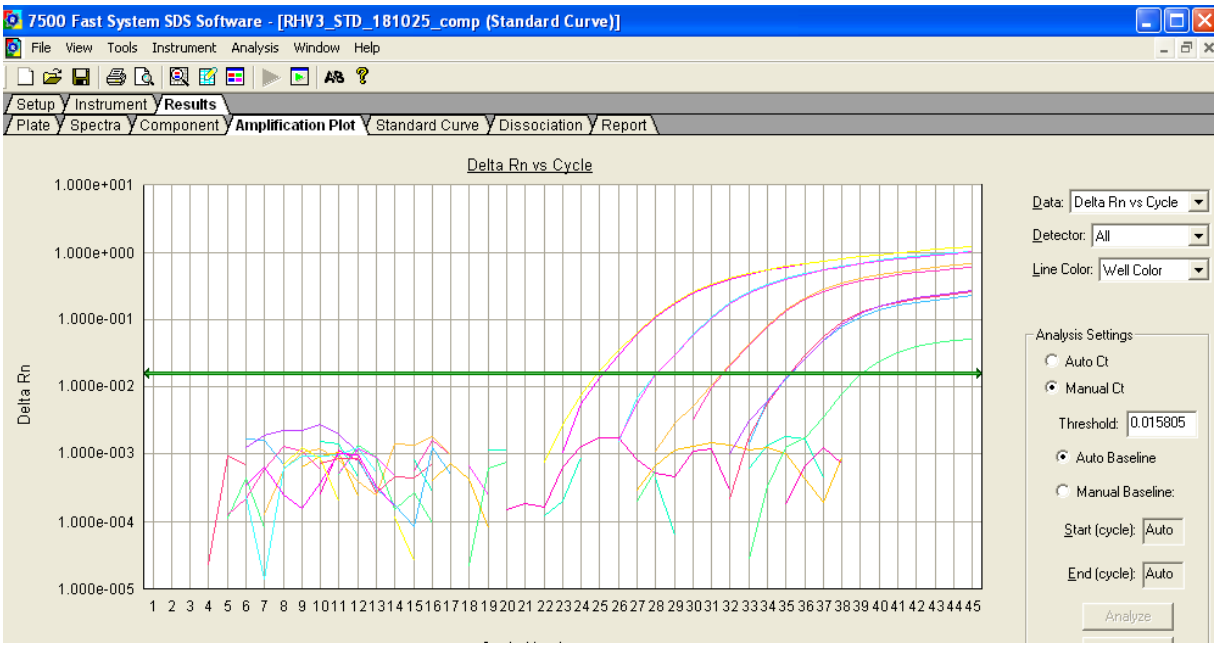
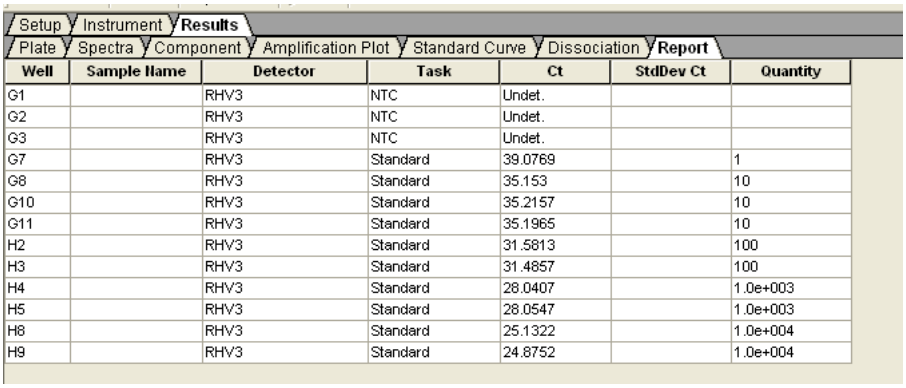


Figure 2. The amplification plots of the replicates show consistent reproducibility.



Well	Sample Name	Detector	Task	Ct	StdDev Ct	Quantity
G1		RHV3	NTC	Undet.		
G2		RHV3	NTC	Undet.		
G3		RHV3	NTC	Undet.		
G7		RHV3	Standard	39.0769		1
G8		RHV3	Standard	35.153		10
G10		RHV3	Standard	35.2157		10
G11		RHV3	Standard	35.1965		10
H2		RHV3	Standard	31.5813		100
H3		RHV3	Standard	31.4857		100
H4		RHV3	Standard	28.0407		1.0e+003
H5		RHV3	Standard	28.0547		1.0e+003
H8		RHV3	Standard	25.1322		1.0e+004
H9		RHV3	Standard	24.8752		1.0e+004

Figure 3. The amplification report shows the range of target detection of the RHV3 genomic DNA.

The Real time PCR developed for the detection of RHV3 genomic DNA is able to reveal the presence of as few as 1 copy of RHV3 genome equivalent (CT 39) in ideal conditions, using a purified plasmid as target.

The final conditions determined for the RT-PCR reaction are:

1st step: Denaturation at 95C for 2 minutes

2nd step: Annealing and extension at 60C for 1 minute (for 45 cycles)

The PCR mix include: 10 microliters of MM (AB, Taqman mix), 900 nM of each primer, 250nM of the RGB primer probe (AB), target DNA, DDH_20 up to 20 microliters.

Qualitative PCR for detection of RHV3 and BfHV1

A qualitative PCR aiming to detect both the presence of the genomic DNA of RHV3 and of the newly discovered Bufonid herpesvirus 1 (Origgi et al., 2018) is currently under way (Master project)

ELISA (Enzyme-linked Immunosorbent Assay)

The ELISA test aims to detect the presence of anti-RHV3 antibodies circulating in the blood of chronically infected common frogs. This test would complement the RT-PCR, able to detect the presence of RHV3 DNA during the acute stage of the infection.

Antigen development

ELISA development requires that the specific antigen (RHV3 viral proteins) would be available. Despite multiple isolation attempts carried out on commercial cells lines (ICR-2A, IZS Lombardia ed Emilia, Italy; TH-1 (ATCC, B1) and primary cell lines (Toad kidney cells-TEKC-1), RHV3 has never been isolated. Accordingly, in order to obtain an antigen to be used in the ELISA format, we considered the development of recombinant antigens.

Gene/protein targets

Ideal protein targets are considered those with high antigenic properties and that are highly expressed by the virus. Among *Herpesvirales*, the major capsid protein is a large protein, which compose the inner shell (capsid) of the virion structure and it is known to be highly antigenic. Accordingly, this was selected as a first protein target. Additionally, thanks to a transcriptomic investigation, we have shown that ORF118 is the most expressed gene within the RHV3 genome during the acute stage of the disease. Accordingly, ORF118 was also selected as an ideal target for recombinant antigen production.

Amino acid and nucleotide sequences of the target RHV3 antigenic proteins/genes and antigenic maps

RHV3_Major Capsid Protein (MCP)

```
  1 ATGACTAACGTTTTCATGTATAGTAATTCTAGGGGTAGGAAGGTG
     M  T  N  V  F  M  Y  S  N  S  R  G  R  K  V

 46 GACACGCGTGGGTTACAAAATACCAACCCTCCGGGTAGGAATATA
     D  T  R  G  L  Q  N  T  N  P  P  G  R  N  I

 91 GACTGGAACGTTTTAAACGATGCAACCTTTCTTGATCAGTTTCGC
     D  W  N  V  L  N  D  A  T  F  L  D  Q  F  R

136 AATATCGTGGCCGTGAACCGACTTTCCACATCCGATTTCTACGAC
     N  I  V  A  V  N  R  L  S  T  S  D  F  Y  D

181 TACTTTGCCGGACCCAACAGACGTGGGGACAATTATGTAGACAAT
     Y  F  A  G  P  N  R  R  G  D  N  Y  V  D  N

226 AGAAATGTACTGAGGATGGTCCGACCCGACGGTTTGAACATAAAC
     R  N  V  L  R  M  V  R  P  D  G  L  N  I  N
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 271 GATTTTCAAACAGATGTGGTCGTTCCGATGATGATGGTGCGTTCT
      D  F  Q  T  D  V  V  V  P  M  M  M  V  R  S

 316 CTGGCCGTCATGACGGCGGCCATGCAGAGCAATTACAACGCATGT
      L  A  V  M  T  A  A  M  Q  S  N  Y  N  A  C

 361 TTTCTGCTGTCTAATCTTTTGTTGCCCAGGAAAACACCCGGCCCA
      F  L  L  S  N  L  L  L  P  R  K  T  P  G  P

 406 AGACCGGTCTTCAAGTACACGGCTTCAAAAAGAGAAATCCGACCC
      R  P  V  F  K  Y  T  A  S  K  R  E  I  R  P

 451 GCTCACGCCGGTACGTTAAGAGAAATACCAGACCGCAGCAGCGTC
      A  H  A  G  T  L  R  E  I  P  D  R  S  S  V

 496 ACAAACACAGAAATGACCGTTGCGCTAAAATCATACACCAATGGT
      T  N  T  E  M  T  V  A  L  K  S  Y  T  N  G

 541 ATTGCCGTTCCGGATTCCTCAATCTTTGCGAAAATAAGCAGAGAA
      I  A  V  P  D  S  S  I  F  A  K  I  S  R  E

 586 CAAGCAATGATGGCGTTGGAAGCAGAAATGGTTAACTTCAGTGAG
      Q  A  M  M  A  L  E  A  E  M  V  N  F  S  E

 631 GGTTGGAAAGCTTGTATCGCCAAATTACATTATGAGTATTGTGCC
      G  W  K  A  C  I  A  K  L  H  Y  E  Y  C  A

 676 GGCCAACCAAAGTTTAGCGAGCTCTATTTGGCGAACAACCGTAGG
      G  Q  P  K  F  S  E  L  Y  L  A  N  N  R  R

 721 AATTTCACAGAGAGGGGCCGACTCGGGTGTCTTATGGAACTTATC
      N  F  T  E  R  G  R  L  G  C  L  M  E  L  I

 766 CACTTAGAGAATACCATTGAAGGCTGTATCAATAAGGACCCGTCC
      H  L  E  N  T  I  E  G  C  I  N  K  D  P  S

 811 AATATTTCACCCGTCATGCTTGCCGCCTGTCACATGATTCACGAG
      N  I  S  P  V  M  L  A  A  C  H  M  I  H  E

 856 CAAGGCAACGACCACATTATTGTTTGCCCGAAAAGAATAGTTCCT
      Q  G  N  D  H  I  I  V  C  P  K  R  I  V  P

 901 CCCGAAGCCGGTGGTTTGATCATAACCAACATTCCGTACGAGGAA
      P  E  A  G  G  L  I  I  T  N  I  P  Y  E  E

 946 AAAGTTTATAATTATTCAACGGCCGGCGCCGATTCAAACGACATG
      K  V  Y  N  Y  S  T  A  G  A  D  S  N  D  M

 991 GTAATGGACGTTGATTGGTCGACGGAAGTTGTAGCCAACGCAATG
      V  M  D  V  D  W  S  T  E  V  V  A  N  A  M

1036 AAGGACCCACGCATCAATAAAATTGAACCAGCACCATCACAGCCT
      K  D  P  R  I  N  K  I  E  P  A  P  S  Q  P
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1081 GCCCTTGACGATCGCCTATACGCCACGCTTTTGCAAGAAAATGGA
      A  L  D  D  R  L  Y  A  T  L  L  Q  E  N  G

1126 GAAACATTCATGCGAATGCCAGCCATTCGAACCGGATACACAGAA
      E  T  F  M  R  M  P  A  I  R  T  G  Y  T  E

1171 CCAATTCCAATGATTTTACTTGACAACGTACCACTCATGAAAAAC
      P  I  P  M  I  L  L  D  N  V  P  L  M  K  N

1216 GGTCCGTGTATGGCCCAACCCCTCATGAGAAATTGCACAAAACGC
      G  P  C  M  A  Q  P  L  M  R  N  C  T  K  R

1261 CTCTTTTACACAGTGGGTGCAGTTCCCAACTCTTACACGCAGTAC
      L  F  Y  T  V  G  A  V  P  N  S  Y  T  Q  Y

1306 AAAAACAAATCCTTTTACTCGAACAATAGCGTGTTGAAATACAGT
      K  N  K  S  F  Y  S  N  N  S  V  L  K  Y  S

1351 GACGTCGCAAGTAAATGCCCAAGTTCAACATTCATCGTTAACTAC
      D  V  A  S  K  C  P  S  S  T  F  I  V  N  Y

1396 GATGAAAAGGGAACGCCAAAAGAAGTCACGCTTTCCATGCTTCAC
      D  E  K  G  T  P  K  E  V  T  L  S  M  L  H

1441 AAGTCCGCAAACACTGAGCGGCTTTTTTTGGTTTCAGACTTCGAA
      K  S  A  N  T  E  R  L  F  L  V  S  D  F  E

1486 ACACGAATGCGGCGGATATTTGACGACGATTTTATGAAAAACCTC
      T  R  M  R  R  I  F  D  D  D  F  M  K  N  L

1531 GGGGGGAACCACACCGACGAAGTATCGTACATTAAGAATGCTTTA
      G  G  N  H  T  D  E  V  S  Y  I  K  N  A  L

1576 ATTTTATATCACGAACCAGATTCAGCGCTTAAACTTGAGCACCCG
      I  L  Y  H  E  P  D  S  A  L  K  L  E  H  P

1621 TATTGCGTATTGGAAAGTAGTCCAAGGAACATGATGTTCCCCATT
      Y  C  V  L  E  S  S  P  R  N  M  M  F  P  I

1666 CGTCCGTTTCCACACAACCACGCTCAGACACATAGCGTTATCAAA
      R  P  F  P  H  N  H  A  Q  T  H  S  V  I  K

1711 CCTAGGTTCTGTATGTGGAACTACCCCCTAATGTCAGCACAGCAC
      P  R  F  C  M  W  N  Y  P  L  M  S  A  Q  H

1756 GCAGAATACGTTGGTGTTCTTTCTAGTATAAAATGTTCTGTTGGA
      A  E  Y  V  G  V  L  S  S  I  K  C  S  V  G

1801 GAGGATGCAGATTTAATCAAGTTTCTGGAAATGTGTTACGCGGGT
      E  D  A  D  L  I  K  F  L  E  M  C  Y  A  G

1846 TTCGGAAACGTAAGCACAAACAGCGTATTCAACCTGGTGGTCGCA
      F  G  N  V  S  T  N  S  V  F  N  L  V  V  A
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1891 AAGGCAATTTCATTTATCGGAATGACAGCTCCTGACATGAACCGA
      K  A  I  S  F  I  G  M  T  A  P  D  M  N  R

1936 TTAATCGACCAAGCGGGGAATGGCTTCAAACGGGACGCTCAATTA
      L  I  D  Q  A  G  N  G  F  K  R  D  A  Q  L

1981 CTTTTGATAAACGAAAACAAATTACAAGGGCAAATTTTTAACACT
      L  L  I  N  E  N  K  L  Q  G  Q  I  F  N  T

2026 AGCTATGCAACTGGTACAGTGCCCATTACCCCTGCTGTACCTGCA
      S  Y  A  T  G  T  V  P  I  T  P  A  V  P  A

2071 GCTGGCGCATTCTTGTACGATGATAGGTTGGTGCAAACCGTTACC
      A  G  A  F  L  Y  D  D  R  L  V  Q  T  V  T

2116 CCGGATGAAGATAAATGTACTCAATTACTAAAATATATTTTACAG
      P  D  E  D  K  C  T  Q  L  L  K  Y  I  L  Q

2161 ATGGTAGATCACGTTGTTGAAACAAACGGTAAAACGCTCTTGGTG
      M  V  D  H  V  V  E  T  N  G  K  T  L  L  V

2206 TCAGACGCTTACTGCCAAGTGGTGTACTGTACAGTACTGGGTTTG
      S  D  A  Y  C  Q  V  V  Y  C  T  V  L  G  L

2251 CTTCGTGAAAATTTAAGTATGTTAGATTCACCAATCATTTGTTCC
      L  R  E  N  L  S  M  L  D  S  P  I  I  C  S

2296 TATTTTGACTTTAAAAACAAATTAGATAAAGCCATAGCGATTGCG
      Y  F  D  F  K  N  K  L  D  K  A  I  A  I  A

2341 GATTTAATCACGCAAGGTCAGCTGGCTTTTATGCGGGCGGTGGAA
      D  L  I  T  Q  G  Q  L  A  F  M  R  A  V  E

2386 AGTACCATGGTGGACCCACTCAACATGCTGTTATTGCCACATACA
      S  T  M  V  D  P  L  N  M  L  L  L  P  H  T

2431 ATCCCCAGCTACATCCACAAGACCTACGACTTCAATAAAAAGTAC
      I  P  S  Y  I  H  K  T  Y  D  F  N  K  K  Y

2476 ACCTCCTATTATCAAAACATGTTCGCCGCCGTTTGCTGTAATCAG
      T  S  Y  Y  Q  N  M  F  A  A  V  C  C  N  Q

2521 GAATATGCTTTGCGGACAAGCGACAACAATGCGGTGTTCAAGTCA
      E  Y  A  L  R  T  S  D  N  N  A  V  F  K  S

2566 GACGCGTGCGGCGTCATGCGCCCAAGCACAAATGTGTTGACGAGA
      D  A  C  G  V  M  R  P  S  T  N  V  L  T  R

2611 ACATTTGGAAGCATTTATAAAGACGGGAGTAAAGCATATGATTTT
      T  F  G  S  I  Y  K  D  G  S  K  A  Y  D  F

2656 TCAACGGTTGATGGGCAGTGTAAAGCAAGGCAATTGAACAATACT
      S  T  V  D  G  Q  C  K  A  R  Q  L  N  N  T
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2701 TATTCGGCAGTGGTGGAATCTTACACCGATAGCTTTTATTGTAGA
      Y  S  A  V  V  E  S  Y  T  D  S  F  Y  C  R

2746 GCTTCAACATCCATCGCAGAACGCGTAAACGAAATGCTCTATAGA
      A  S  T  S  I  A  E  R  V  N  E  M  L  Y  R

2791 ATGCCAAATGCGTTAAAGCTACGCGAGGTTGTTGAGGGCGCGGCA
      M  P  N  A  L  K  L  R  E  V  V  E  G  A  A

2836 AGGCACGTTGAAAATTTTACATGCGCGGCAGCCACAGGTACTAGA
      R  H  V  E  N  F  T  C  A  A  A  T  G  T  R

2881 ACGCCTACTGCAATCTTATTCCCATTTCATTCCGCCAGCGCTCTT
      T  P  T  A  I  L  F  P  F  H  S  A  S  A  L

2926 GCTGGACAACACGATAACGTTTATTTATTTAACGTTTCAAACTTC
      A  G  Q  H  D  N  V  Y  L  F  N  V  S  N  F

2971 CGCTTGAATATGACACCTGAGAACATACACGAGACATTTAAATAC
      R  L  N  M  T  P  E  N  I  H  E  T  F  K  Y

3016 TATTACGATGTATGGAACAGAGACGTTAACAATAATAACCCACTT
      Y  Y  D  V  W  N  R  D  V  N  N  N  N  P  L

3061 CAATTTCACTCATGTAGTGGGCCAAAACAGTTCAGAGCGTGTTCC
      Q  F  H  S  C  S  G  P  K  Q  F  R  A  C  S

3106 GATAATCCTTGGAACGGTATATTAAGCCCGACTTTTGTTGCTAGA
      D  N  P  W  N  G  I  L  S  P  T  F  V  A  R

3151 CGTCATCTGCTCCAAAATGAAATGGGTGGGCTACCATTTAACATG
      R  H  L  L  Q  N  E  M  G  G  L  P  F  N  M

3196 TTTTTAATGGGTTTACACTATTACTCCGGTATCAATTATCAAAAC
      F  L  M  G  L  H  Y  Y  S  G  I  N  Y  Q  N

3241 GTCACCCAAAAATTCGATAAAAAATATTATTCGGGCTGGGCTTAT
      V  T  Q  K  F  D  K  K  Y  Y  S  G  W  A  Y

3286 GCTTGTGTGCGGACAATGCGTTATATTGGCCACAGTGCCGTTGCA
      A  C  V  R  T  M  R  Y  I  G  H  S  A  V  A

3331 ATGCCTCGAAAAAAAGCACTTCAGGTGCACGGCATGAAAGCAATC
      M  P  R  K  K  A  L  Q  V  H  G  M  K  A  I

3376 AAAGATCACAATCCTGGTGACGAAGCGCGCTTTGTTAGTTCTCAG
      K  D  H  N  P  G  D  E  A  R  F  V  S  S  Q

3421 ATCGACATGGCAATTATACCCGATCAGATGGGAACGCATGGGTGT
      I  D  M  A  I  I  P  D  Q  M  G  T  H  G  C

3466 GTGGTCCCTAACGTATTTATTTCCGACATAGAAGGCGTGAACAGT
      V  V  P  N  V  F  I  S  D  I  E  G  V  N  S
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3511 TTTTTTTTAAATCCTAAAAAATTACCACCAATGAACAATTCCGCC
      F  F  L  N  P  K  K  L  P  P  M  N  N  S  A

3556 CTCGAGGGAACACTATATCGAAAATCGATCACCAGCGACGTTGAG
      L  E  G  T  L  Y  R  K  S  I  T  S  D  V  E

3601 AACTTTACCCTGGTTTTAGACTGGTTTAACGGTATGGTGCCAGAA
      N  F  T  L  V  L  D  W  F  N  G  M  V  P  E

3646 ACTATGTCTGGCAGCAGTAAAACCTGCGAAAGTATCATGCCTTTA
      T  M  S  G  S  S  K  T  C  E  S  I  M  P  L

3691 AATGGACGATACACCCAAATGTACATGGGTGCTGATCCAAACACT
      N  G  R  Y  T  Q  M  Y  M  G  A  D  P  N  T

3736 AATATCTTCAGCGAAAAAGATCCGATGAGTGGACGATATTGCGAA
      N  I  F  S  E  K  D  P  M  S  G  R  Y  C  E

3781 TCTCCGACTTTGCTCAACCATTCCGATACAGCACTTTCGATCGCT
      S  P  T  L  L  N  H  S  D  T  A  L  S  I  A

3826 GAAACAAGTTCTTATCTGTCGAATCTCATGTACACGAGTAATCCC
      E  T  S  S  Y  L  S  N  L  M  Y  T  S  N  P

3871 GCGTCCAGTTTCTTAAGCGCCGCGTCTTATAATAATATTGCGGAA
      A  S  S  F  L  S  A  A  S  Y  N  N  I  A  E

3916 ACAATGGCCGATCTCACATTTGGGCGATTCCAATTCATGCCGCAA
      T  M  A  D  L  T  F  G  R  F  Q  F  M  P  Q

3961 AACGGACAAGAACTGCTAAACACTCTGTTTCAACGAGCCCTTGCT
      N  G  Q  E  L  L  N  T  L  F  Q  R  A  L  A

4006 ACTAACTCGTCGGTAGAAACACCCTTTACGAATCGGCTGGGCATG
      T  N  S  S  V  E  T  P  F  T  N  R  L  G  M

4051 ATGTTTGCTGATTCTTACGTCATTGGGAAGGGGGGTGGACTTTAT
      M  F  A  D  S  Y  V  I  G  K  G  G  G  L  Y

4096 TTAATTAAAATTCAAAATAACAATTACGATAATCAACAATTTGAA
      L  I  K  I  Q  N  N  N  Y  D  N  Q  Q  F  E

4141 CTGAACCCGAGAGTTGTGGGAGAGGGAATAAGCCCAACCTATCTT
      L  N  P  R  V  V  G  E  G  I  S  P  T  Y  L

4186 TTCCATCCAACAAGCAATGAACCCTTCCTTAGAAATCTAGGAAAA
      F  H  P  T  S  N  E  P  F  L  R  N  L  G  K

4231 ATGTCTTGCTTACCGTTTATCTCTAGAGATCTAGTGAACGTGCGA
      M  S  C  L  P  F  I  S  R  D  L  V  N  V  R

4276 AGTGCGGTCGACAGTGGAGCGCTCGCACAGAAAGGTGAAAAATGA
      S  A  V  D  S  G  A  L  A  Q  K  G  E  K  *
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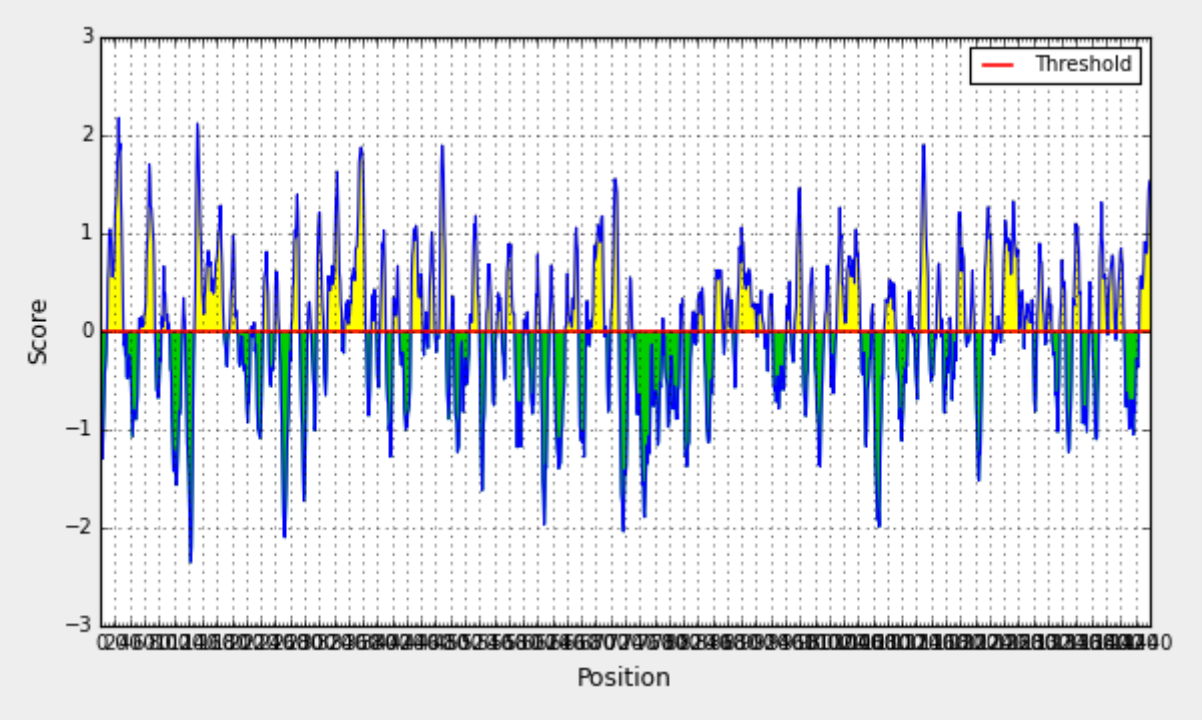

Figure 4. Predicted antigenic peptides in RHV3-MCP

No.	Start	End	Peptide	
68	1286	1295	YTSNPASSFL	10
6	177	188	YTNGIAVPDSSI	12
66	1238	1267	YMGADPNTNIFSEKDPMSGRYCESPTLLNH	30
22	465	474	YDEKGTPKEV	10
36	701	711	VQTVTPDEDKC	11
7	206	207	VN	2
20	446	447	VL	2
72	1357	1361	VIGKG	5
37	726	730	VETNG	5
55	1109	1114	VAMPRK	6
47	918	922	TSIAE	5
69	1299	1305	SYNNIAE	7
39	796	799	STMV	4
45	874	900	SIYKDGSKAYDFSTVDGQCKARQLNNT	27
62	1194	1199	SITSDV	6
46	902	910	SAVVESYTD	9
23	482	484	SAN	3
50	973	980	SALAGQHD	8
74	1384	1393	RVVGEGISPT	10
10	240	245	RNFTER	6

No.	Start	End	Peptide	
13	297	306	RIVPPEAGGL	10
16	372	378	QENGETF	7
14	312	331	PYEEKVYNYSTAGADSNDMV	20
40	812	814	PSY	3
5	129	169	PRKTPGPRPVFKYTASKREIRPAHAGTLREIPDRSSVTNTE	41
27	557	568	PFPHNHAQTHSV	12
3	84	94	PDGLNINDFQT	11
64	1229	1229	P	1
1	9	31	NSRGRKVDTRGLQNTNPPGRNID	23
52	1011	1043	NRDVNNNNPLQFHSCSGPKQFRACSDNPWNGIL	33
73	1371	1381	NNNYDNQQFEL	11
63	1212	1225	MVPETMSGSSKTCE	14
51	994	1001	MTPENIHE	8
32	638	656	MTAPDMNRLIDQAGNGFKR	19
4	113	116	MQSN	4
24	507	520	MKNLGGNHTDEVSY	14
53	1058	1060	MGG	3
28	581	581	M	1
2	53	74	LSTSDFYDYFAGPNRRGDNYVD	22
18	402	410	LMKNGPCMA	9
75	1395	1404	LFHPTSNEPF	10
26	544	550	LESSPRN	7
33	667	668	KL	2
61	1176	1187	KKLPPMNNSALE	12
38	772	772	K	1
17	384	391	IRTGYTEP	8
54	1076	1091	INYQNVTQKFDKKYYS	16
41	816	816	H	1
65	1232	1234	GRY	3
31	617	622	GNVSTN	6
19	426	442	GAVPNSYTQYKNKSFYS	17
8	211	211	G	1
34	673	691	FNTSYATGTVPITPAVPAA	19
70	1317	1324	FMPQNGQE	8
12	285	289	EQGND	5
25	530	536	EPDSALK	7
11	262	273	EGCINKDPSNIS	12

No.	Start	End	Peptide	
48	942	947	EGAARH	6
60	1166	1167	EG	2
15	335	364	DWSTEVVANAMKDPRINKIEPAPSQPALDD	30
21	451	459	DVASKCPSS	9
67	1269	1279	DTALSIAETSS	11
58	1148	1153	DQMGTH	6
42	820	828	DFNKKYTSY	9
30	597	603	CSVGEDA	7
44	858	868	CGVMRPSTNVL	11
9	224	230	CAGQPKF	7
49	953	963	CAAATGTRTPT	11
59	1155	1155	C	1
71	1335	1345	ATNSSVETPFT	11
29	583	586	AQHA	4
43	843	854	ALRTSDNNAVFK	12
57	1124	1136	AIKDHNPGDEARF	13
35	693	694	AF	2
56	1116	1116	A	1

Table 1. Predicted antigenic peptides in RHV3_MCP

RHV3_ORF118

```
  1 ATGTTCCTTACGTTCACTATAAGCGCGATCTTATTTGCCAAAGTA
     M  F  L  T  F  T  I  S  A  I  L  F  A  K  V

 46 CAGAGCAACCCAGAAGAAATTTGCATTTCTCCGCTTGCCTCTACA
     Q  S  N  P  E  E  I  C  I  S  P  L  A  S  T

 91 ACCGACTTAATAAAGAACACCAGTAATAATAATGTGATTGTGGGT
     T  D  L  I  K  N  T  S  N  N  N  V  I  V  G

136 CCACGGCAAACACTCGGTTTAGTTGCAGATGACCCCAACGATCTT
     P  R  Q  T  L  G  L  V  A  D  D  P  N  D  L

181 ATTGCAAACATAACGAATGACTGGATCGGCTTTGGAGACGGGAGA
     I  A  N  I  T  N  D  W  I  G  F  G  D  G  R

226 ACAAATTCTAACGATTCAAATTGGCCCGCCGTTCGAGATATTCCC
     T  N  S  N  D  S  N  W  P  A  V  R  D  I  P

271 TTGGAAGCAGCTTCCAAATTCGTTGGTGTTCGCGGGTCCTCCCTC
     L  E  A  A  S  K  F  V  G  V  R  G  S  S  L
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 316 ATTTACTCAATGTTTGGATTTCCAAATAAAACGGACTATGTATAC
      I  Y  S  M  F  G  F  P  N  K  T  D  Y  V  Y

 361 CAGGGGTGTGGAGCAGAGAAGATTTTTTATGAGGGTGCGTCTTCT
      Q  G  C  G  A  E  K  I  F  Y  E  G  A  S  S

 406 ATAAACACAGGAGAAAGCTGCAACTTTGTCAGTTGGGCGACCGTG
      I  N  T  G  E  S  C  N  F  V  S  W  A  T  V

 451 CGGAAAGCGTTTTACAATTTGTTCTATGCCGCGGCATCAAAGTAT
      R  K  A  F  Y  N  L  F  Y  A  A  A  S  K  Y

 496 AGGTGCTTATCTTTAGCAAACGTCACTGTCGCATACGACAAAGAT
      R  C  L  S  L  A  N  V  T  V  A  Y  D  K  D

 541 TATAAGGTTCAATTTCCAACTGGTGGATACGCTGGAACTGGAAAC
      Y  K  V  Q  F  P  T  G  G  Y  A  G  T  G  N

 586 TTTAAGATGGCTTCCGACAACTGTAGCGGCACTTGGCTACAGAAC
      F  K  M  A  S  D  N  C  S  G  T  W  L  Q  N

 631 CCACTTTATTATGTAAACATAACTTTGGCTTACTATAGCGTCTGC
      P  L  Y  Y  V  N  I  T  L  A  Y  Y  S  V  C

 676 GGTTCTATCAATGATAACCATCGTAAAACTCTATTTACCTTACCC
      G  S  I  N  D  N  H  R  K  T  L  F  T  L  P

 721 AGTACTAGCAACGGGCAGACGTGTGACGAGCATTTGGTTGAGCTG
      S  T  S  N  G  Q  T  C  D  E  H  L  V  E  L

 766 TGTTATTGGACCTTGCCTGCATCTTTTAGCTATAGTATTGACTGC
      C  Y  W  T  L  P  A  S  F  S  Y  S  I  D  C

 811 GGAGGTTATTATCAATATTACACCTATAGTAACGGTACTAACATA
      G  G  Y  Y  Q  Y  Y  T  Y  S  N  G  T  N  I

 856 TCAGTACCAATCATTGATTACAGACAAGGTACACCCTTTAGTAAC
      S  V  P  I  I  D  Y  R  Q  G  T  P  F  S  N

 901 GCTGCCCAACCTATGATAGTATGTAACCCTATTCTTTTAAAACCA
      A  A  Q  P  M  I  V  C  N  P  I  L  L  K  P

 946 GGAAATTATTATGCACTCAGTAATGGATACCGAGTAATGGCGCCT
      G  N  Y  Y  A  L  S  N  G  Y  R  V  M  A  P

 991 TCTAGAAGCTATTGTTTTGGAGGAGGAGAGGCTTCTCATATTCAC
      S  R  S  Y  C  F  G  G  G  E  A  S  H  I  H

1036 GTAGTTCAGAGTGTAAATGCAACCCTCGACCTTCTGAATGACGAC
      V  V  Q  S  V  N  A  T  L  D  L  L  N  D  D

1081 CTAGAGTTAGCCTGCAAAGCTAACCCGAAGTGTATTTATCATAAG
      L  E  L  A  C  K  A  N  P  K  C  I  Y  H  K
```

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1126 AAAAAAAGCGCATACGTCGCCACGGCCCCGGATGGCGTAAATGGG
      K  K  S  A  Y  V  A  T  A  P  D  G  V  N  G

1171 GACTTGGAAATGCGTAGAAGATTTGAGGGTTTATTACAACCTAAC
      D  L  E  M  R  R  R  F  E  G  L  L  Q  P  N

1216 ACCACGTGCACATTTTCGGGTGGTTCCTTTCAAGGTTCAAGCGTA
      T  T  C  T  F  S  G  G  S  F  Q  G  S  S  V

1261 AGTTCCAGCAATTATGCTACTCCCTCAATAGGTAAATGCCCCGTC
      S  S  S  N  Y  A  T  P  S  I  G  K  C  P  V

1306 ATTGCACCTATAACGATAGAATATCCAAATGAAAGATCGGTTTTT
      I  A  P  I  T  I  E  Y  P  N  E  R  S  V  F

1351 GGGAAAAAATCAATGATCATGAATTACTCTAATGCGACTTTCATT
      G  K  K  S  M  I  M  N  Y  S  N  A  T  F  I

1396 AATATATCGAATTCAGATTTGTACACACAGAATGTATTGCTGTTA
      N  I  S  N  S  D  L  Y  T  Q  N  V  L  L  L

1441 GGTGCCGTAGAAACTGGTTATAAAGTCATCAATGTGCTACAAACG
      G  A  V  E  T  G  Y  K  V  I  N  V  L  Q  T

1486 GGAGAAAATAATATTAAACTATCCAATACAGCAATGTTCACACAT
      G  E  N  N  I  K  L  S  N  T  A  M  F  T  H

1531 GCGGTTATTGCGACAAACCTCACCATAGCATCCGTTATTAAATCG
      A  V  I  A  T  N  L  T  I  A  S  V  I  K  S

1576 ATTGGAAAATCATCGGGTTTAGAATTCATACCTTTTGCATTAAGT
      I  G  K  S  S  G  L  E  F  I  P  F  A  L  S

1621 AGTACAAATCAGACACAAACAGCTTTGATTTCAAAAACATCAGAC
      S  T  N  Q  T  Q  T  A  L  I  S  K  T  S  D

1666 GATATACGTGGTAATTTGAGTTTGCGATTCGTTGATGCAATAGAA
      D  I  R  G  N  L  S  L  R  F  V  D  A  I  E

1711 AATGTACAAAGTATGTACGCACGATGGCGGACCAGTAATATATCA
      N  V  Q  S  M  Y  A  R  W  R  T  S  N  I  S

1756 AGTAACGGTGTTTACGTTTTAACACCTAACATTTTTACAAAAGAT
      S  N  G  V  Y  V  L  T  P  N  I  F  T  K  D

1801 TGCTGGGATTTTAATTCAGATGTATGCAACGATAATTGTTTTAAG
      C  W  D  F  N  S  D  V  C  N  D  N  C  F  K

1846 CAAATTTTTGAAAACGAGATTGTATTCACTACCCCAAAGCCCACA
      Q  I  F  E  N  E  I  V  F  T  T  P  K  P  T

1891 ACAACGCTAAATACCACCAGTTCTTCAATTACACAAAGCACTACT
      T  T  L  N  T  T  S  S  S  I  T  Q  S  T  T
```

```
1936 ACACTTAAATCAACAATTTCCACTTTAAAGTCTATTATATCCACA
      T  L  K  S  T  I  S  T  L  K  S  I  I  S  T

1981 ACCAAAACTTCCAATGAAGCAAACAAAAACTCACGTTACTCATTT
      T  K  T  S  N  E  A  N  K  N  S  R  Y  S  F

2026 ATTCTATTAAGTGTTTTGTGTTTTGTTTTGTTATGCAATAATATT
      I  L  L  S  V  L  C  F  V  L  L  C  N  N  I

2071 TAA
      *
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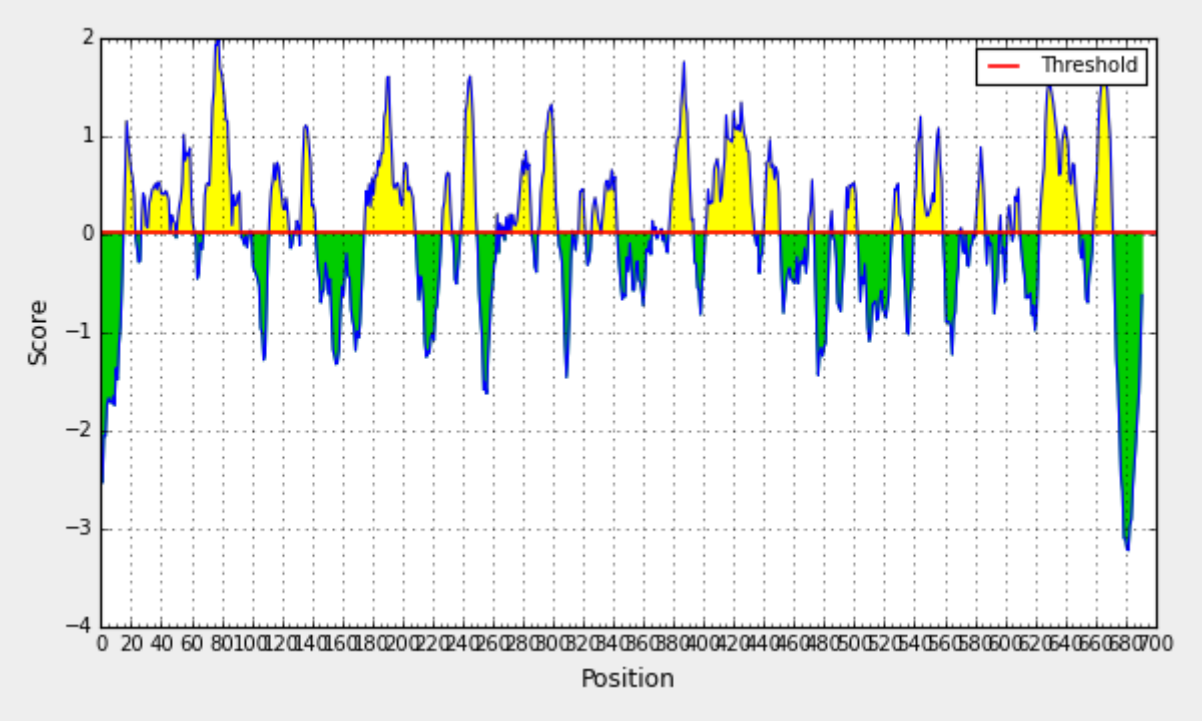

Figure 5. Predicted antigenic peptide in RHV3_ORF118

No.	Start	End	Peptide	
1	16	22	QSNPEEI	7
2	27	45	LASTTDLIKNTSNNNVIVG	19
3	47	49	RQT	3
4	51	62	GLVADDPNDLIA	12
5	69	92	IGFGDGRTNSNDSNWPAVRDIPLE	24
6	99	99	G	1
7	112	124	FPNKTDYVYQGCG	13
8	128	128	I	1
9	130	131	YE	2
10	133	142	ASSINTGESC	10

No.	Start	End	Peptide	
11	175	208	VAYDKDYKVQFPTGGYAGTGNFKMASDNCSGTWL	34
12	227	233	SINDNHR	7
13	239	249	LPSTSNGQTCD	11
14	263	263	S	1
15	265	265	S	1
16	267	267	S	1
17	269	271	DCG	3
18	273	285	YYQYYTYSNGTNI	13
19	291	303	DYRQGTPFSNAAQ	13
20	313	313	L	1
21	317	321	NYYAL	5
22	326	331	RVMAPS	6
23	333	342	SYCFGGGEAS	10
24	366	368	KAN	3
25	372	372	I	1
26	377	393	KSAYVATAPDGVNGDLE	17
27	402	433	LQPNTTCTFSGGSFQGSSVSSSNYATPSIGKC	32
28	440	450	TIEYPNERSVF	11
29	470	473	SDLY	4
30	484	485	ET	2
31	494	502	QTGENNIKL	9
32	525	531	SIGKSSG	7
33	540	558	SSTNQTQTALISKTSDDIR	19
34	570	571	EN	2
35	581	587	TSNISSN	7
36	597	597	F	1
37	602	604	WDF	3
38	607	609	DVC	3
39	623	649	VFTTPKPTTTLNTTSSSITQSTTTLKS	27
40	659	671	STTKTSNEANKNS	13

Table 2. Predicted antigenic peptides in RHV3_ORF118

(Selected) Gene amplification

The primers were designed to amplify the entire gene sequence with the exception of the very few 5' codons and to eliminate the stop codon located 3'. This strategy was necessary to 1) clone the gene in frame with the 5' fusion protein sequence present in the selected expression vector (pET30C+,

Novagen, USA), 2) avoid the stop of the translation at the original stop codon of the selected genes that would have determined the termination of the recombinant protein prior the histidine tag located 3' the end of the gene sequence on the expression vector. The selected primer design allowed to obtain a fusion protein with a 5' histidine tag in frame with the selected RHV3 protein targets. Furthermore, the primers were designed to contain 15 nucleotides 5', which matched with the insertion sequence of the selected expression vector (pET30C+) and containing an EcoRV site that would allow to excide the whole RHV3 selected and cloned genes. Amplification was carried out with proofreading DNA polymerase (Phusion II, Agilent) according to the manufacturer's instructions.

The selected genes, RHV3_MCP and ORF118 were amplified from RHV3 genomic DNA. Direct amplification of the MCP gene was obtained in two steps. First, the MCP gene sequence was amplified with a "short" pair of primers:

FW: 5'-AGC GCT CCA CTG TCG ACC G-3'

RV: 5'-GTT TTC ATG TAT AGT AAT TCT AGG-3'

Followed by a "long" set of primers carrying the 5' 15 "signature" nucleotides identical to those present on pET30C+:

MCP_FW: 5'-ACA AGG CCA TGG GAT ATC GTA ATT CTA GGG GTA GGA AGG T-3'

MCP_RV: 5'-TTC GGA TCC ACA GAT ATC CTC CAC TGT CGA CCG CAC TT-3'

Differently, ORF118 was amplified using a single set of primer carrying the pET30C+ signature 15 nucleotides.

ORF118_FW: 5'-ACA AGG CCA TGG GAT ATC GTA CGT TCA CTA TAA GCG CGA T-3'

ORF_118_RV: 5'-TTC GGA TCC ACA GAT ATC CAA AAC ACA AAA CAC TTA ATA GAA TAA ATG AGT AAC-3'

Detailed RHV3_MCP amplified gene sequence

Infusion primer FW

ACAAGGCCATGGGAT **ATC** GTAATTCTAGGGGTAGGAAGGT

Red: pET 15 nucleotide signal (infusion)along with two extra pET nucleotide and a third one to get in frame; green: specific gene sequence

Infusion primer RV

TTCGGATCCACAGAT ATC**C** TCCACTGTCGACCGCACTT

Major capsid protein

5-CGACAAGGCCATGG**GATATC** ATG ACT AAC GTT TTCATGTATAGTAATTCTAGG-3'

(5' gene end deleted ATG ACT AAC GTT TTC ATG TAT A)

(FW primer-CAA GGC CAT GGG ATA TCG TAA TTC TAG GGG TAG GAA GGT GG-)

From here

FW

5′-ACAAGGCCATG ggatatc **GT AATTCTAGGGGTAGGAAGGTGG-3′**

ACACGCGTGGGTTACAAAATACCAACCCTCCGGGTAGGAATATAGACTGGAACGTTTTAAACGATGCAACCTTTC
TTGATCAGTTTCGCAATATCGTGGCCGTGAACCGACTTTCCACATCCGATTTCTACGACTACTTTGCCGGACCCA
ACAGACGTGGGGACAATTATGTAGACAATAGAAATGTACTGAGGATGGTCCGACCCGACGGTTTGAACATAAACG
ATTTTCAAACAGATGTGGTCGTTCCGATGATGATGGTGCGTTCTCTGGCCGTCATGACGGCGGCCATGCAGAGCA
ATTACAACGCATGTTTTCTGCTGTCTAATCTTTTGTTGCCCAGGAAAACACCCGGCCCAAGACCGGTCTTCAAGT
ACACGGCTTCAAAAAGAGAAATCCGACCCGCTCACGCCGGTACGTTAAGAGAAATACCAGACCGCAGCAGCGTCA
CAAACACAGAAATGACCGTTGCGCTAAAATCATACACCAATGGTATTGCCGTTCCGGATTCCTCAATCTTTGCGA
AAATAAGCAGAGAACAAGCAATGATGGCGTTGGAAGCAGAAATGGTTAACTTCAGTGAGGGTTGGAAAGCTTGTA
TCGCCAAATTACATTATGAGTATTGTGCCGGCCAACCAAAGTTTAGCGAGCTCTATTTGGCGAACAACCGTAGGA
ATTTCACAGAGAGGGGCCGACTCGGGTGTCTTATGGAACTTATCCACTTAGAGAATACCATTGAAGGCTGTATCA
ATAAGGACCCGTCCAATATTTCACCCGTCATGCTTGCCGCCTGTCACATGATTCACGAGCAAGGCAACGACCACA
TTATTGTTTGCCCGAAAAGAATAGTTCCTCCCGAAGCCGGTGGTTTGATCATAACCAACATTCCGTACGAGGAAA
AAGTTTATAATTATTCAACGGCCGGCGCCGATTCAAACGACATGGTAATGGACGTTGATTGGTCGACGGAAGTTG
TAGCCAACGCAATGAAGGACCCACGCATCAATAAAATTGAACCAGCACCATCACAGCCTGCCCTTGACGATCGCC
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TTATCTCTAGAGATCTAGTGAACGTGCGAAGTG**CGGTCGACAGTGGAGCGCTCGCACAG** AAA GG A TAT
CTGTGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGG

RV primer

5′-TTC GGA TCC ACA GAT ATC CTT TCT GTG CGA GCG CTC CAC TGT CGA CCG-3′

(RV primer GGATATCTGTGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCG)

Detailed amplification of the RHV3_ORF118 gene

ORF118

In fusion primers

FW

ACAAGGCCATGGGAT ATC GTACGTTCACTATAAGCGCGAT

RV

TTCGGATCCACAGAT ATCC AAAACACAAAACACTTAATAGAATAAATGAGTAAC

(5′ gene end deleted ATG TTC CT)

From here

FW

5′-CAA GGC CAT GGG ATA TCG TAC GTT CAC TAT AAG CGC GAT CTT ATT TGC C-3′

CAAGGCCATG

Gga Tat **CG T ACG TTC ACT ATA AGC GCG ATC TTA TTT GCC**
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TCCAATGAAGCAAACAAAAACT**CAC GTTACTCATTTATTCTATTAAGTGTTTTGTGTTTT G**

RV

5′-CGG ATC CAA CGA TAT CCA AAA CAC AAA ACA CTT AAT AGA ATA AAT GAG TAA CGT G-3′

(3′ gene end deleted TAA)

Cloning by InFusion

The selected genes (RHV3 MCP and ORF188 were cloned into Novagen pET30C+ expression vector using the infusion technology according to the manufacturer's instructions. Briefly, a "signal" stretch of 15 nucleotides with the same sequence present at the plasmid insertion site were added 5' to both the FW and RV primers used to amplify the selected genes (see above). Ligation through recombination would then occur.

The selected plasmid was pET30C+ carrying a Kanamicin-resistence cassette. Following ligation by recombinantion, the recombinant product was transformed in Agilent Top 10 competent cells according to the manufacturer's instructions. Bacterial colonies were picked and plasmid extracted by conventional protocols. Presence of the cloned genes was assessed by restriction digestion (EcoRV) which consisted of bands of the expected size (approx.. 2Kb for ORF118 and 4Kb for MCP).

Following are the map and sequence of the plasmids.

Map and sequence of the MCP gene (MCP 1His**) cloned into pET30C+**

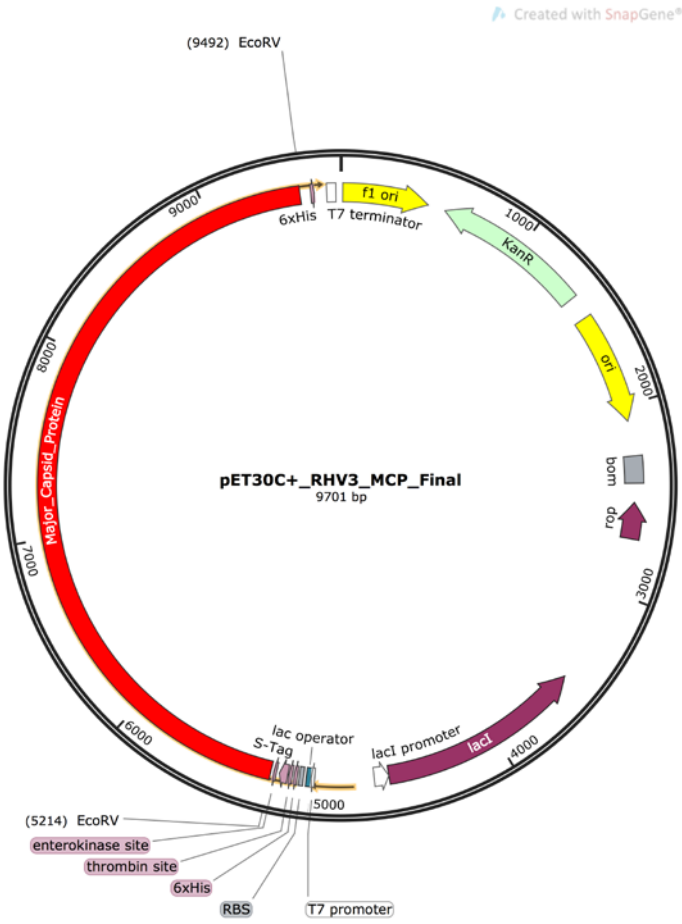

Figure 6. RHV3_MCP map.

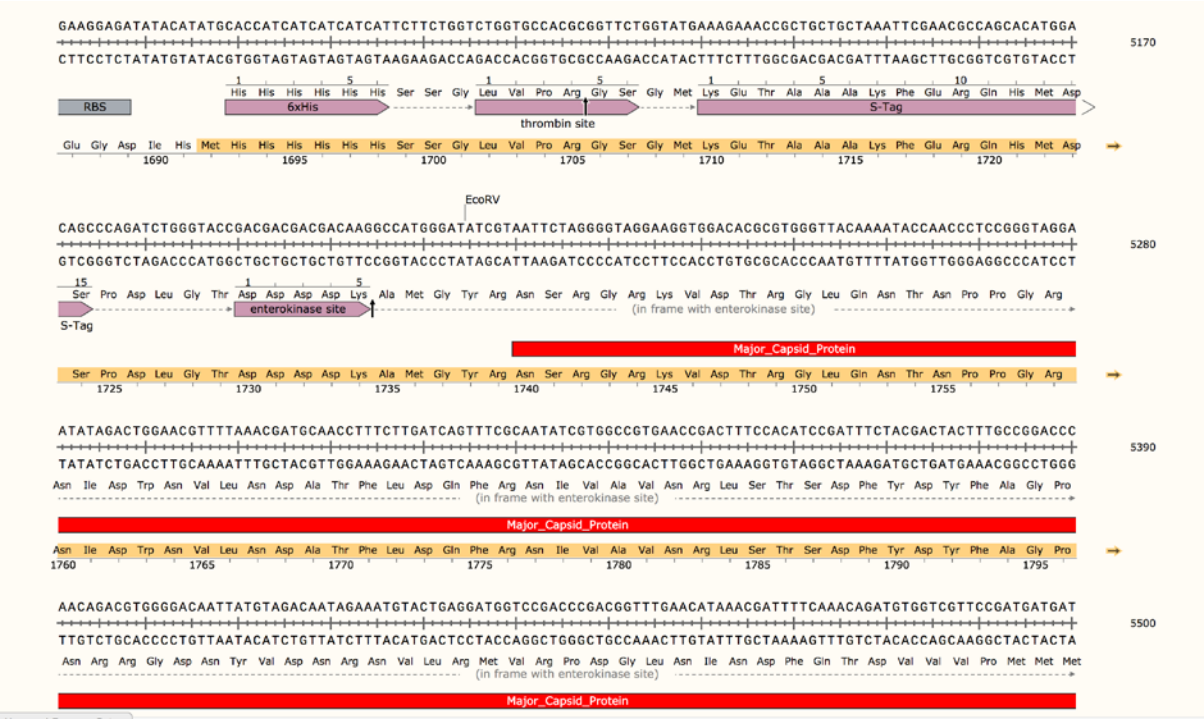

Figure 7. N' region of the recombinant protein

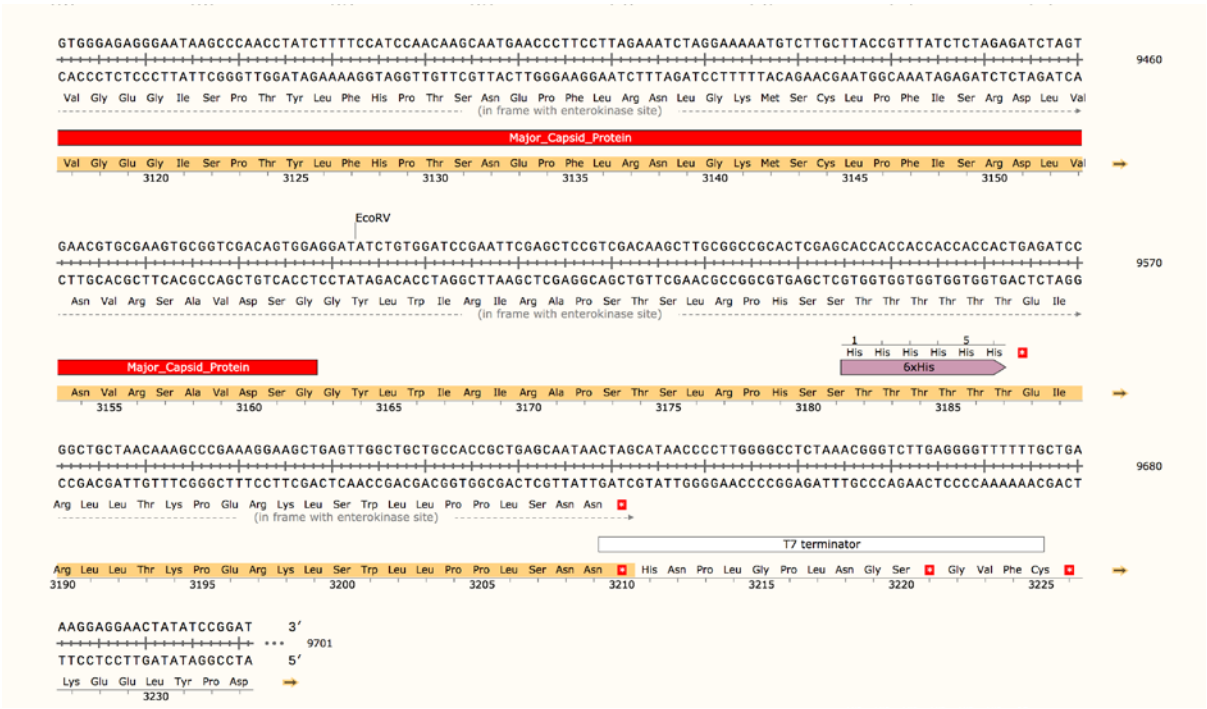

Figure 8. C'region of the recombinant protein

MCP sequence (cloned gene with N' histidin tag)

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GACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCG
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Map and sequence of the ORF118 gene (ORF118 1His**) cloned into pET30C+**

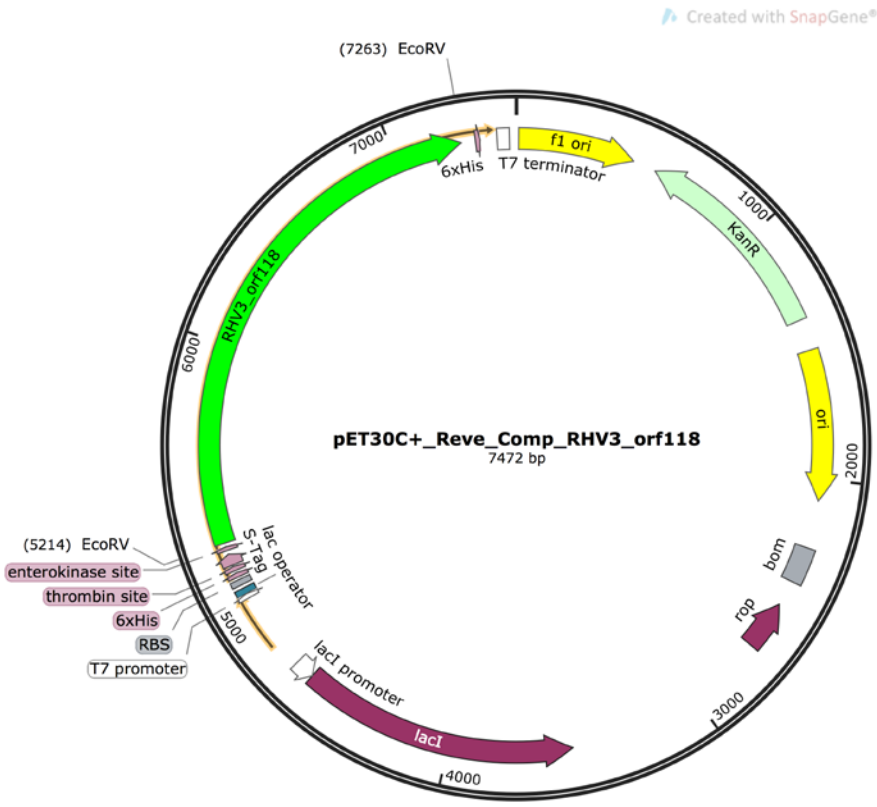

Figure 9. RHV3_ORF118_His1 map

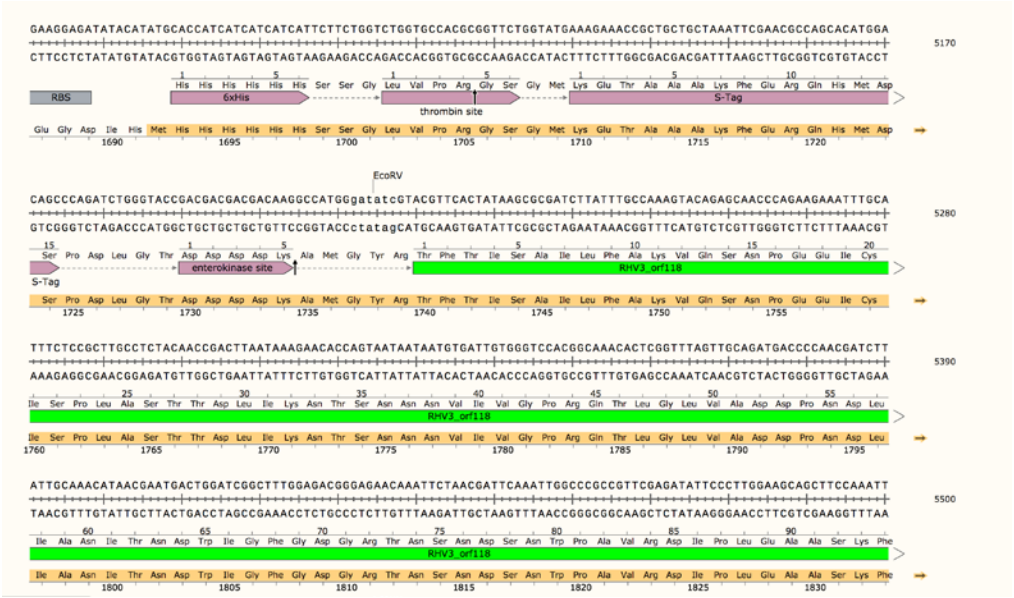

Figure 10. N' region of the recombinant protein

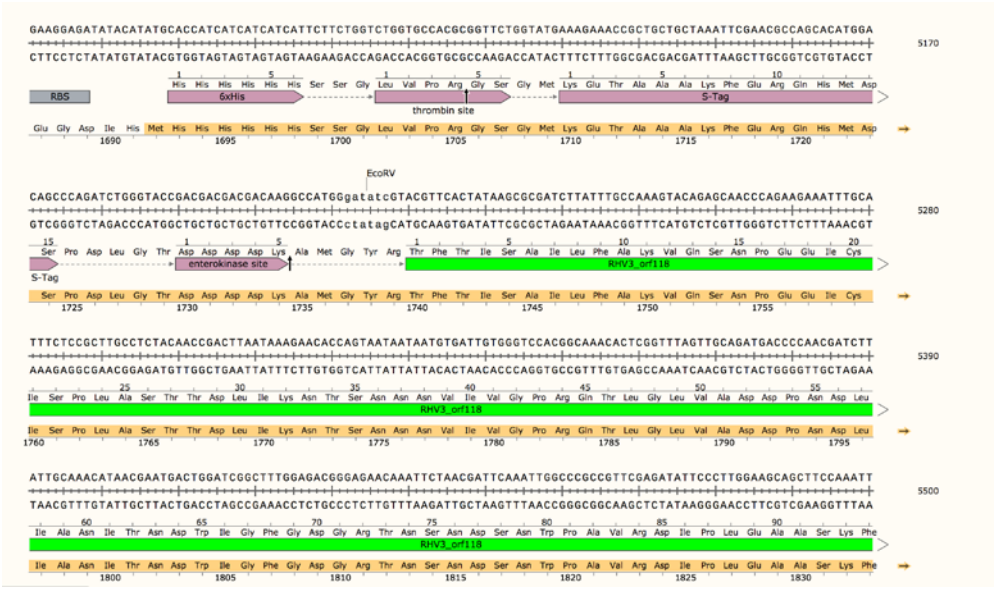

Figure 11. C' region of the recombinant protein

118_1His seq sequence (cloned gene)

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Site directed mutagenesis to obtain a double hisitidin tag N' and C'

The cloned genes were in frame with the 5' Histidin tag present in the expression vector but not with that located 3'. In order to place also the 3' cassette in frame, site directed mutagenesis was carried out using Phusion II DNA polymerase and a primer set designed according to the specifications of the manufacturer's (Agilent). More specifically a nucleotide (in red) was inserted at position 7316 of the 118 construct and at position 9560 of the MCP construct.

Name: Mut_InFus_RHV3_FW

Sequence (5'-3'): GCC GCA CTC GAG CCA CCA CCA CCA C

Name: Mut-InFus_RV3

Sequence (5'-3'): GTG GTG GTG GTG GCT CGA GTG CGG C

Map and sequence of the ORF118 gene (ORF118 2His**) cloned into pET30C+**

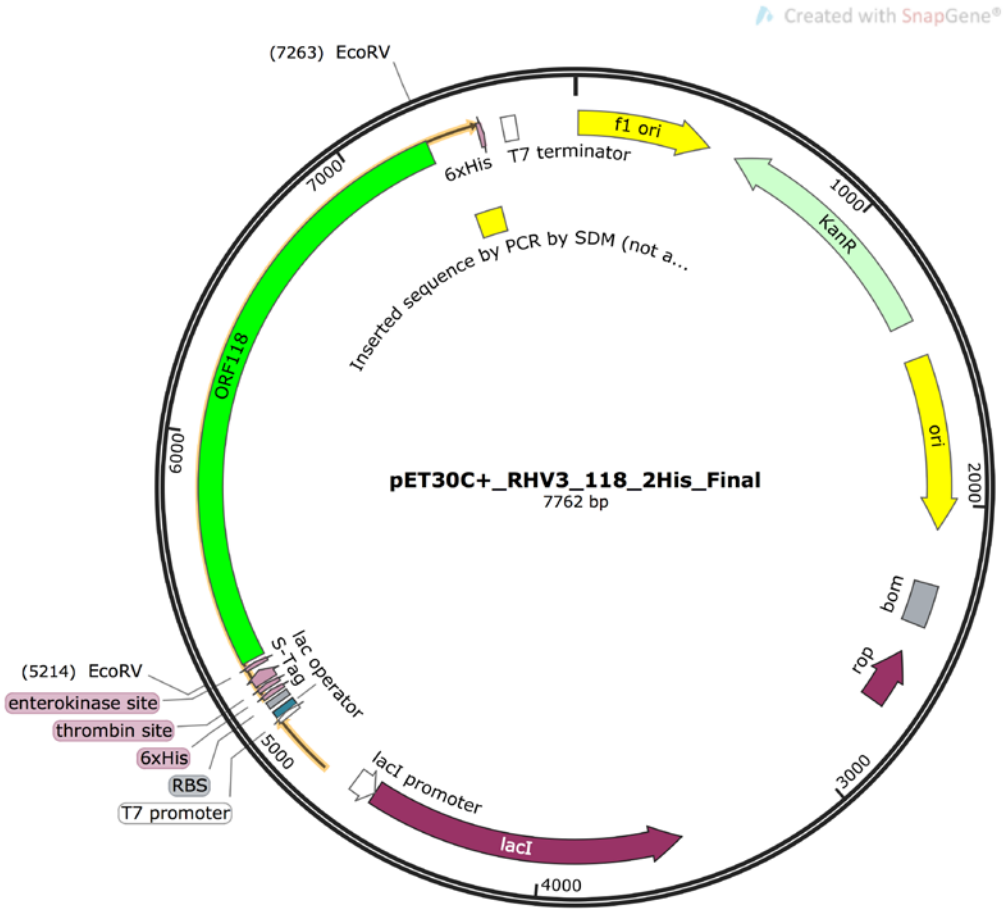

Figure 12. RHV3_ORF118_His2

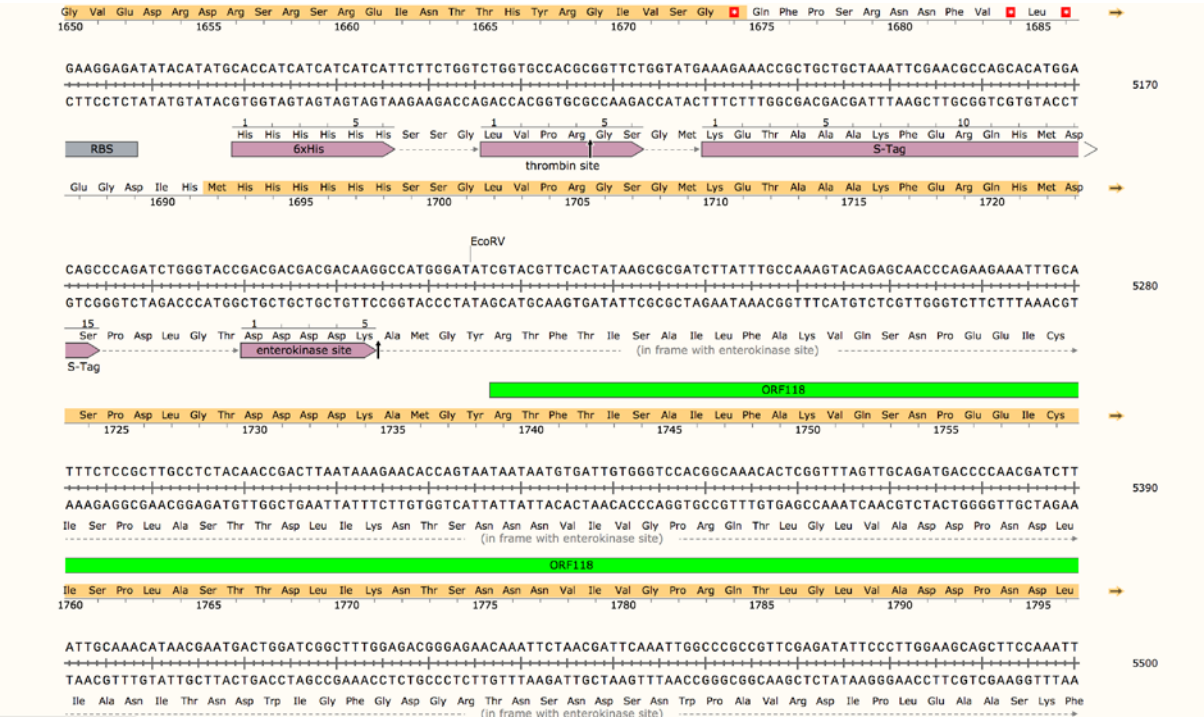

Figure 13. N' region of the recombinant protein

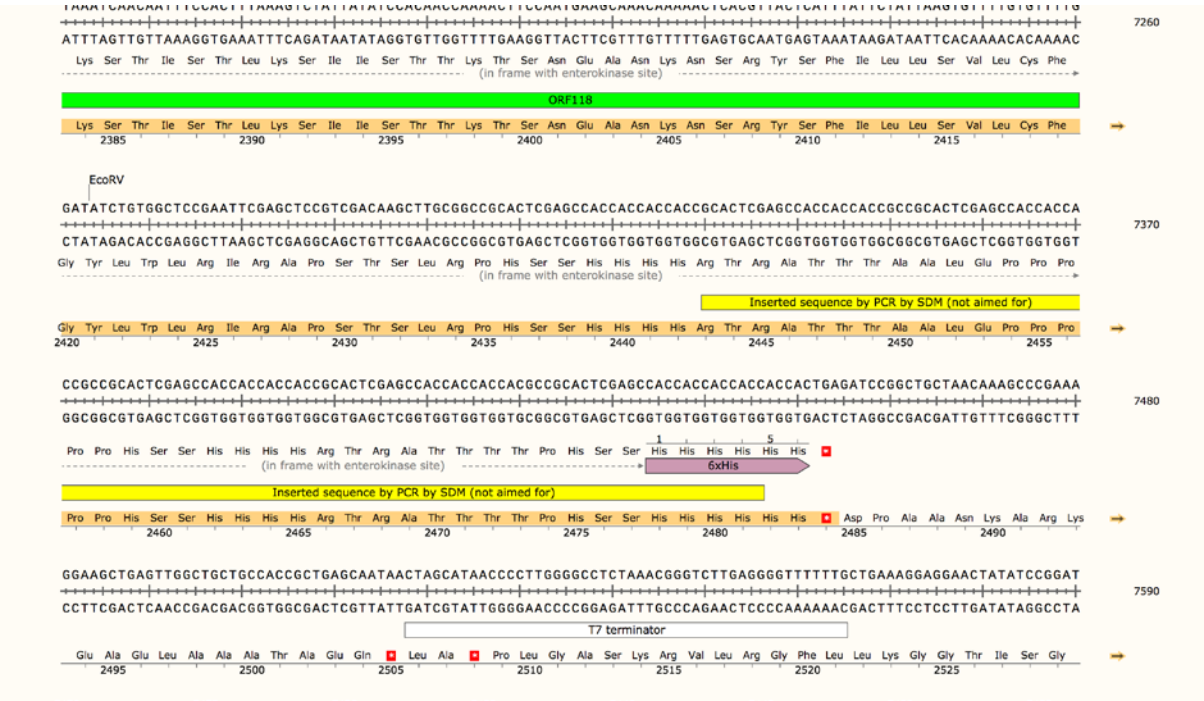

Figure 14. C' region of the recombinant protein. The ORF118_2his map shows several nucleotides inserted at the 3' end of the cloned gene, which are considered to have been addeded erroneously during the Infusion ligation reaction.

118 2His map sequence (gene cloned)

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TTCAAGCGTAAGTTCCAGCAATTATGCTACTCCCTCAATAGGTAAATGCCCCGTCATTGCACCTATAA
CGATAGAATATCCAAATGAAAGATCGGTTTTTGGGAAAAAATCAATGATCATGAATTACTCTAATGCG

ACTTTCATTAATATATCGAATTCAGATTTGTACACACAGAATGTATTGCTGTTAGGTGCCGTAGAAAC
TGGTTATAAAGTCATCAATGTGCTACAAACGGGAGAAAATAATATTAAACTATCCAATACAGCAATGT
TCACACATGCGGTTATTGCGACAAACCTCACCATAGCATCCGTTATTAAATCGATTGGAAAATCATCG
GGTTTAGAATTCATACCTTTTGCATTAAGTAGTACAAATCAGACACAAACAGCTTTGATTTCAAAAAC
ATCAGACGATATACGTGGTAATTTGAGTTTGCGATTCGTTGATGCAATAGAAAATGTACAAAGTATGT
ACGCACGATGGCGGACCAGTAATATATCAAGTAACGGTGTTTACGTTTTAACACCTAACATTTTTACA
AAAGATTGCTGGGATTTTAATTCAGATGTATGCAACGATAATTGTTTTAAGCAAATTTTTGAAAACGA
GATTGTATTCACTACCCCAAAGCCCACAACAACGCTAAATACCACCAGTTCTTCAATTACACAAAGCA
CTACTACACTTAAATCAACAATTTCCACTTTAAAGTCTATTATATCCACAACCAAAACTTCCAATGAA
GCAAACAAAAACTCACGTTACTCATTTATTCTATTAAGTGTTTTGTGTTTTGGATATCTGTGGCTCCG
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CCACCGCCGCACTCGAGCCACCACCACCGCCGCACTCGAGCCACCACCACCACCGCACTCGAGCCACC
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GGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGG
TCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGATTGGCGAATGGGACGCGCCCTGTAGCG
GCGCATTAAGCGCGGCGGGTGTGGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGC
GCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAA
ATCGGGGGCT

Clone sequencing

Full sequencing of the cloned gene product was also carried out. Please see maps and obtained sequences above.

Protein expression

BL21 DE3 bacteria were transformed with the expression vectors obtained (ORF118_His1, ORF118_His2, MCP_His1) according standard protocols. The bacteria were then pelleted and lysed and resolved on an acrylamide gel (10%), transferred on a nitrocellulose membrane and stained with an anti_Histidin tag monoclonal antibody. Below are shown the blotting results:

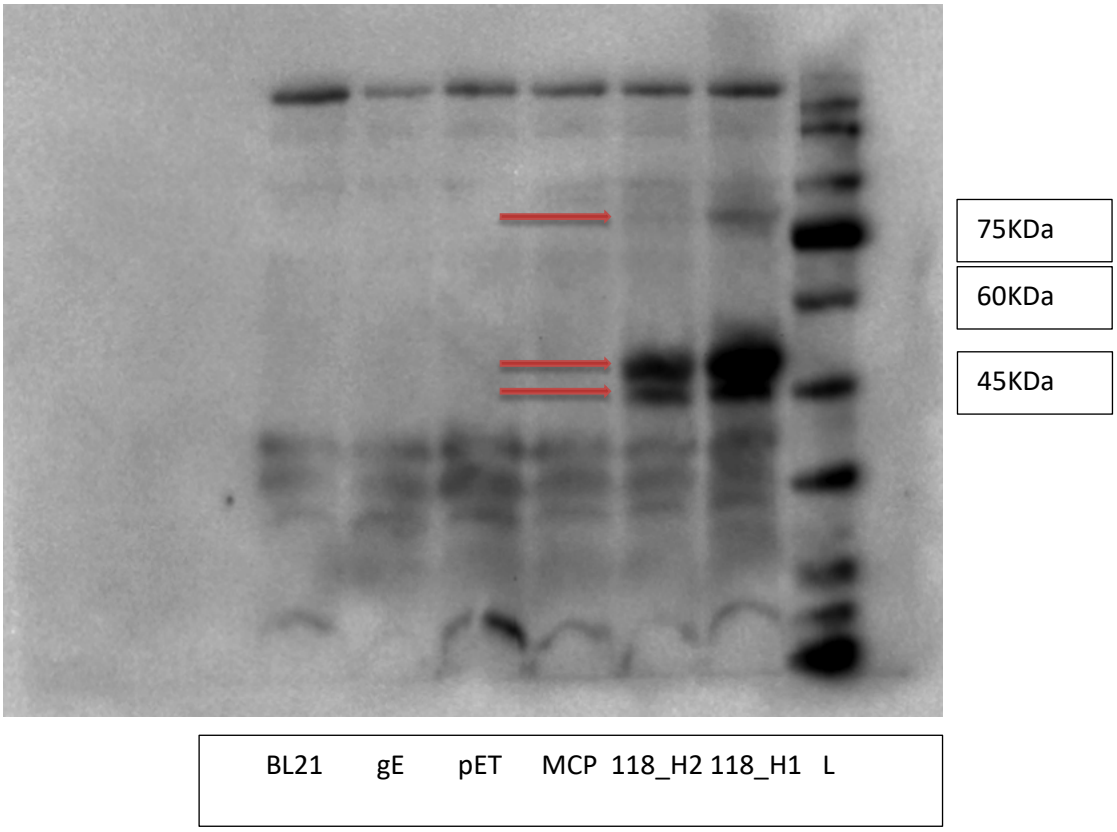

Figure 15. The western blot reveals the presence of three bands which are detectable only in the lanes where the proteins expressed by the bacteria transformed with the ORF118 plasmids. No unique bands are detected in any other lane, including that containing the proteins expressed by the MCP plasmid. The expected size of the full length cloned ORF118 is expected to be approximately 100 KDa. The detected bands are of approximately 45, 50 and 80 KDa suggesting the expression of a slightly shortened or truncated protein. (At the bottom of the gels are the loaded antigens: L=ladder; 118_H1= Orf118 with N'-His tag; 118_H2= Orf118 with N' and C' His tag; MCP=Major capsid protein; pET=empty plasmid; gE=irrelevant recombinant protein; BL21=Bacteria carrying no expression vector)

Protein Purification

To be carried out

Antibody production

Whole serum collected from RHV3 negative common frogs has been delivered to the Istituto Zooprofilattico della Lombardia e dell'Emilia (IZS), in Brescia, Italy where the common frog immunoglobulins will be purified and a specific monoclonal antibody will be obtained according to standard protocols. The purification of the common frog immunoglobulin is currently ongoing.

Summary as November 2019

Real Time PCR

During the past months we have worked on developing a new Real time PCR to detect BfHV1. This quantitative PCR is expected to complement the already developed quantitative PCR for RaHV3 detection. The BfHV1 quantitative PCR is able to detect as few as 10 copies of the target, in ideal conditions.

Consensus PCR

A consensus, qualitative PCR is also been developed. This PCR protocol is intended as a first line diagnostic PCR that is supposed to amplify all the known amphibian herpesviruses (Batrachoviruses) and ideally all those yet to be discovered. This PCR is based on degenerate primers annealing to a very conserved region of the Terminase gene of Batrachoviruses. A number of primer sets has been developed and currently being tested on the Batrachovirus targets

ELISA

Antigen

Additionally to the orf118, we have been able to clone the partially deleted sequence of the Major capsid protein (MCP). The gene has been cloned in the same expression vector used to express orf 118 (pET30C+). Both the putative expressed orf118 and deleted MCP are being purified to then be tested both in western and ELISA format.

Concurrently, we have run in a western blot format the protein extract obtained from RaHV3 infected frogs and we have used the primary sera from both naturally infected and not infected frog as primary serum. A number of bands have been highlighted, however, several of these bands were also observed in the membrane stained with sera from (putatively) uninfected frogs. Further investigations are undergoing to understand the nature of these bands.

Monoclonal antibody

The development of a specific monoclonal antibody against common frog immunoglobulin was critically impaired by the amount of total serum that could be used for the extraction of the antigen (frog Ig) that would have been used as the antigen for the mice immunization. The amount of immunoglobulin that could be obtained from 1ml of serum was not enough to obtain the required amount of antigen by the Istituto Zooprofilattico della Lombardia a dell'Emilia of Brecia, Italy. Accordingly, we have decided to rely on a monoclonal antibody that was developed for *Xenopus laevis* and that was shown to cross-react with the common frog immunoglobulin.

References

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