# PRODUCTION OF RECOMBINANT IRIDOVIRUS AND NERVOUS NECROSIS VIRUS CAPSID PROTEINS FOR APPLICATION TO AQUACULTURE

# METHODOLOGY

## 3.1 Introduction

This research was divided into five major parts. The first part was the synthesis of synthetic genes; the second was the construction of expression vector using pGS21a and pET22b; the third was the protein expression studies in *E. coli* BL21 (DE3); the fourth part was recombinant protein purification using HisTrap FF (GE Healthcare, England) and the last part was the induction of polyclonal antibodies against recombinant NNV major capsid protein in rat model (Sprague Dawley).

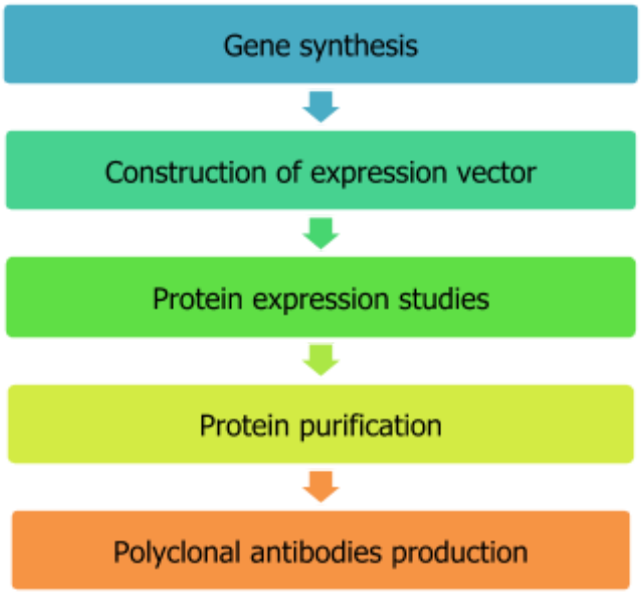



**Figure 3.1: Flowchart of five major parts of research methodology.**

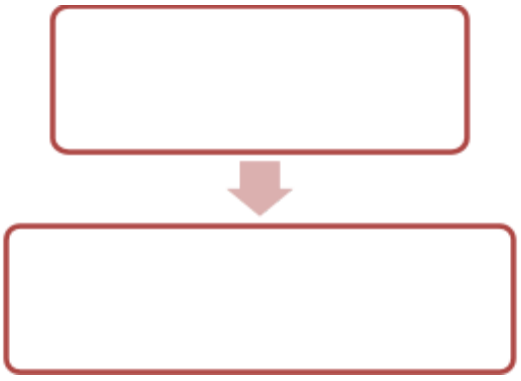
### 3.2 Source of DNA Sequences

Five nucleotide sequences coding for the target mature genes were retrieved from NCBI database. Table 3.1 shows the target genes for this study. Two genes are coding for major capsid protein of iridovirus and nervous necrosis virus. Another three genes are coding for growth hormone, Type I interferon and prolactin of groupers. The nucleotide sequences were modified where rare codons were removed and substituted with preferred codons of *E. coli* according to *E. coli* codon usage data from the Kazusa DNA Research Institute. The modification was applied without disrupting amino acid sequences of the target genes. Synthetic genes were synthesized by GENEWIZ (USA) with the inclusion of 5′- BamHI and 3′- EcoRI restriction enzyme sites. The synthetic genes were cloned into the pUC57 vector by GENEWIZ and delivered in the form of lyophilized plasmids. Native and modified sequences of target genes were analysed by Countcodon program (http://www.kazusa.or.jp/codon/countcodon.html) where the codons were clustered into codon groups according to the amino acids formed.

**Table 3.1: Nucleotide sequences of target genes retrieved from NCBI database.**

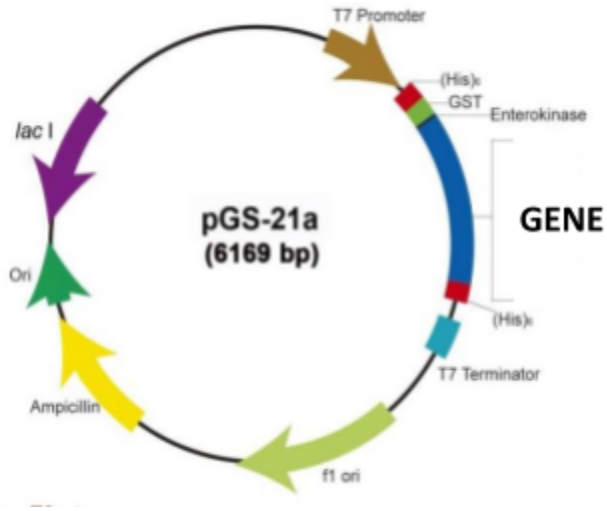
| Gene | Organism | NCBI Accession No. | Size (bp) |
|---|---|---|---|
| **Iridovirus major capsid protein (IRI)** | Grouper Iridovirus (GIV) | AY666015 | 1383 |
| **Nervous necrosis major capsid protein (NNV)** | Red-spotted grouper NNV (RGNNV) | EF558369 | 1011 |

### 3.3 General Methods in Construction of Expression Vector in *E. coli* Top 10



In order to clone the target genes to the expression vectors (pGS21a and pET22b), the above flow was followed. Plasmids pUC57 harboring the synthetic genes first digested using BamHI and EcoRI. Then, the genes were cloned into the vectors. The overview of construction of expression vector is as follow: First, both target genes and expression vectors were digested using respective enzyme combination. After that, digested fragments were separated in agarose gel and extracted. Then, the digested

fragments were ligated to respective vectors and transformed into E. coli. Positive transformants were screened.



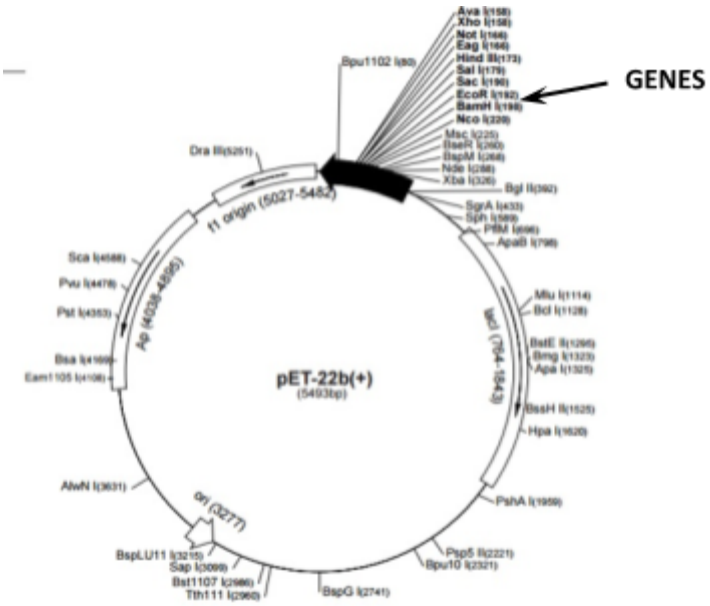



**Figure 3.2: Insertion of genes into expression vectors after digestion with BamHI and EcoRI.**

### 3.3.1 Restriction Enzyme Digestion

DNA restriction was performed with restriction enzyme BamHI, EcoRI and NcoI (ThermoScientific, USA). The reaction mixture was prepared in 20 µl in PCR tubes. The reaction mixtures were prepared as in Table 3.2 below. pUC57 from GENEWIZ harbouring the synthetic genes and expression vector pGS21a and pET22b were digested using restriction enzymes BamHI and EcoRI. The digestion reaction produced sticky ends on both synthetic genes and expression vector.

**Table 3.2: The components for restriction enzyme digestion**

| Components | 1X (µl) | Final Concentration |
|---|---|---|
| **10X Tango buffer** | 4.0 | 2X |
| **EcoRI** | 0.5 | 5 u |
| **BamHI** | 0.5 | 5 u |
| **Vector** | 5.0 | |
| **Sterilized water** | 10 | |
| **Final volume** | **20** | |

The restriction reactions were incubated at 37°C for 3 hours (hr) followed by 80°C heat deactivation to terminate enzyme digestion reaction.

### 3.3.2 Agarose Gel Electrophoresis

Digested products were centrifuged briefly before mixed with 2 µl of loading dye for each 20 µl reaction. The mixture was then loaded onto wells on 2% agarose gel and separated based on size with 100 volt for 45 minutes (min). 1X TBE buffer was used for electrophoresis. The agarose gel was stained with ethidium bromide solution for 5 min and visualized using AlphaImager UV transiluminator (ProteinSimple, USA). The approximate sizes of synthetic genes (GH, IFN, IRI, NNV and PRL) were estimated by comparison to 1kb DNA ladder (ThermoScientific, USA).

### 3.3.3 DNA Extraction from Agarose Gel

Target DNA fragments (GH, IFN, IRI, NNV, PRL and pGS-21a) were purified using Gel Extraction Kit (Qiagen, Netherlands). Target fragments were identified based on size and excised from the agarose gel with sterile scalpels. The gel slices placed in 1.5 ml centrifuge tubes and 500 µl of buffer QG was added. The tubes were incubated at 50˚C until the agarose gel dissolved completely. Then, 200 µl of isopropanol was added to the samples and mixed by inverting for 10 times. The samples were then transferred to QIAquick columns and centrifuged at 15 294 x g for 1 min. The flow-through was discarded and columns were placed back in the same collection tubes. Buffer PE (700 µl) was added to remove salt residue then centrifuged again at 15 294 x g for 1 min. The flow-through was discarded. After that, empty QIAquick columns were centrifuged for an additional 1 min at 15 294 x g in order to discard ethanol residue. The QIAquick columns were placed into clean 1.5 ml centrifuge tubes before 15µl Buffer EB was added to the center of the QIAquick membrane and left for 2 min at room temperature. The digested fragments were eluted by centrifuging at 15 294 x g for 2 min. The purified fragments (GH, IFN, IRI, NNV, PRL, pGS21a and pET22b) stored at -20˚C until further use.

### 3.3.4 Ligation

DNA ligation was done using T4 DNA ligase obtained from Thermo Scientific (USA). Vector and insert DNA were mixed. Ligation mixture was prepared based on Table 3.3.

**Table 3.3: The components of ligation reaction**

| Components | 1X | Final concentration |
|---|---|---|
| Linear vector (pGS21a/pET22b) (20-100 ng) | 2 µl / 50 ng | ~50ng |
| Purified synthetic DNA | 6 µl | 1 : 3 molar ratio |
| 10x DNA Ligase | 2 µl | 1x |
| T4 DNA Ligase (5u/µl) | 2 µl | 10 units |
| Water, nuclease free | 8 µl | - |
| **Total volume** | 20 µl | - |

All procedures were done on ice and prepared in 200 µl PCR tubes. The tubes were briefly centrifuged and the ligation components were mixed gently by pipetting up and down. The ligation process was carried out by incubation at 4°C for overnight. The ligation mixtures were then used directly for bacterial transformation.

**Table 3.4: Expression constructs after genes were inserted.**

| Gene | Constructs with pGS21a | Constructs with pET22b |
|---|---|---|
| **IRI** | pGS21a-IRI | pET22b-IRI |
| **NNV** | pGS21a-NNV | pET22b-NNV |

### 3.3.5 Competent Cells Preparation

A single *E. coli* Top10 bacterial colony was grown in 10 ml of Lysogeny broth (LB) without ampicillin. The culture was incubated for overnight at 37°C (180 rpm).

Then, 250 µl of overnight culture was transferred into a 50 ml falcon tube to inoculate 25 ml fresh LB broth. The culture was incubated at 37°C until $OD_{600}$ nm reached ~0.4 – 0.7 (approximately in 2 hr). The culture was transferred into ice and incubated for 15 min. Cells were recovered by centrifugation at 1 699 x g for 10 min

(4˚C). LB medium was decanted and cells were resuspended in 10 ml of 100mM cold calcium chloride ($CaCl_2$). After that, it was left on ice for 30 min before centrifuged as above. Pelleted cells were then resuspended in 1.5 ml of 100 mM cold $CaCl_2$ + 15% glycerol. Competent cells were kept in -80˚ until further use.

### 3.3.6 Transformation of gene constructs into *E. coli* Top10 (Heat Shock)

The transformation was done by using previously prepared chemically competent cells (Section 3.4.5). Competent cell aliquots (100 µl each) were thawed on ice and added with 10 µL of respective ligation products (Section 3.4.4). The competent cells and ligation products were mixed by pipetting gently. The mixtures were then incubated on ice for 30 min following heat shock at 42˚C for 40 sec to allow DNA transfer into cells. After heat shock, the tubes were quickly transferred on ice for 5 min. Then, 50 µl of cells were spread on LB agar supplemented with 100 µg/ml ampicillin. Transformed cells acquired ampicillin resistant, thus allowing them to grow on LB ampicillin (LB-amp) plates. Non-transformants will not be able to break ampicillin therefore unable to survive on LB-amp plates. LB-amp plates were incubated at 37˚C for 12 hr and single colonies were picked and transferred onto new LB-amp agar plates.

### 3.3.7 Colony Polymerase Chain Reaction (PCR) for Clone Selection

Colony PCR is a simple method to screen for positive transformants which no prior plasmid DNA extraction is required. High temperature will lyse the membrane cell thus exposing DNA fragment of interest for PCR amplification. Single colonies were picked into 0.2 ml PCR tubes containing 20 µl of PCR reaction mixture (Table 3.4). The sequences of the primers are indicated in Table 3.5. The primers bind to both vectors' sequence that flank outside the target genes.

**Table 3.5: The components of polymerase chain reaction (PCR)**

| Components | 1x (µl) | Final Concentration |
|---|---|---|
| 5x Colorless GoTaq Flexi Buffer | 4.0 | 1x |
| $MgCl^2$ (25mM) | 1.2 | 1.5 mM |
| Taq DNA Polymerase (5u/µl) | 0.2 | 1 unit |
| dNTP Mix (10mM) | 0.2 | 0.15 mM |
| Forward primer (10µM/ 1ml) | 0.1 | 0.1 µM/ ml |
| Reverse primer (10µM/1ml) | 0.1 | 0.1 µM/ ml |
| Water | 14.2 | |
| **Total** | **20** | |

**Table 3.6: Primer sequences**

| Primer | 5′ to 3′ sequence | Length | Tm (˚C) |
|---|---|---|---|
| **T7 promoter** | 5' TAATACGACTCACTATAGGG 3' | 20 | 47.7 |
| **T7 terminator** | 5' GCTAGTTATTGCTCAGCGG 3' | 19 | 51.1 |

PCR was carried out in a PCR thermocycler (Bio-Rad, USA) following cycle conditions stated in Table 3.6. PCR products were analyzed by gel electrophoresis and positive transformants were identified based on respective band sizes of each synthetic gene.

**Table 3.7: PCR cycle conditions**

| Event | Temperature (°C) | Duration |
|---|---|---|
| **Initial melting** | 95 | 3 min |
| **Melting** | 95 | 20 sec |
| **Annealing** | 58 | 40 sec |
| **Elongation** | 72 | 10 sec |
| **Final elongation** | 72 | 7 min |

### 3.3.8 Plasmid Miniprep from *E. coli*

Small scale plasmid isolation was carried out with GeneJET Plasmid Miniprep Kit (ThermoScientific, USA) and was done according to the manufacturer's manual. 5 ml overnight cultures were prepared in LB-amp (50 µg/ml) broth from single colonies picked from LB-amp plates. Cells were harvested by centrifugation at 15 294 x g for 1 min. Then it was used for plasmid extraction as per the mentioned protocol.

After plasmid extraction, second verification was performed to check whether an insert has successfully cloned into a vector, enzyme digestion was carried out as in 3.3.1. Digested products separated in agarose gel and identified based on respective sizes.

### 3.4 Sequencing

Plasmids with inserts were sequenced in both directions to confirm the authenticity of the nucleotide sequences using T7 promoter and T7 terminator primers. Purified plasmids (15 µl) were sent for DNA sequencing service (1stBASE, Singapore). Target sequences were assembled from forward and reverse sequencing data. Sequence alignment was performed using the NCBI blast algorithm compared with the synthetic DNA sequences.

### 3.5 Cloning Vector Constructs into Expression Host *E. coli* BL21 (DE3)

After sequence confirmation, the resulting expression constructs were respectively transformed into expression host *E. coli* strain BL21 (DE3) as described in 3.4.6 – 3.4.9.

### 3.6 Glycerol Stock (15%) Preparation of *E. coli* Top10 and BL21 (DE3)

Each positive transformants were used to inoculate 5 ml LB-amp broth for overnight incubation at 37˚C. Then, 1 ml of overnight culture was mixed with 225 µl of 80 % glycerol. Glycerol stocks were stored in -80˚C with minimum thawing step.

### 3.7 Expression of Gene Constructs in Expression Host *E. coli* BL21 (DE3)

Expression studies were conducted to determine the level of expression of recombinant growth hormone (GH), Type I Interferon (IFN), iridovirus major capsid protein (IRI), NNV major capsid protein (NNV) and prolactin (PRL) in *E. coli* BL21 (DE3).

#### 3.7.1 *E. coli* BL21 (DE3) Culture and Induction

Individual fresh 5 ml of LB broth containing 50 µg/ml of ampicillin (LB-amp) was inoculated and cultured at 37˚C (180 rpm) for overnight. Then, all 5 ml of culture was collected by centrifugation at 10 621 x g (10 min, 4˚C) before resuspended to cell density of 1.0 of $OD_{600}$ using LB-amp broth as diluent. After adjustment, 1 ml of uninduced culture was harvested by centrifugation at 15 294 x g for 1 min. The medium was discarded and samples were stored in -20˚C.

Fresh LB-amp broth (5 ml) was inoculated with 1% (v/v) or 50 µl of overnight culture. When the cell density reached $OD_{600}$=0.4 – 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) (Amresco, USA) was added to a final concentration of 0.1 mM to induce recombinant protein expression at 12˚C (180 rpm). After 24 hr of incubation, the cells were collected and resuspended with LB-amp broth to obtain cell density of 1.0 at $OD_{600}$ nm. Then, 1 ml of induced culture was transferred into a new 1.5 ml centrifuge tube and cells were collected at 15 294 x g for 1 min. All samples were stored in -20˚C until further use.

After that, incubation temperature for induction was optimized. Three different temperatures were used (12˚C, 20˚C and 37˚C) after addition of IPTG. Recombinant expression was also performed for target genes cloned in pGS-21a. Qualitative comparison was done.

### 3.7.2 Protein Extraction (Soluble and Insoluble)

Protein was extracted according to modified method of Kong and Guo (2014) and Shimmoto *et al*. (2010). Pelleted cells were thawed on ice and resuspended with 100 µl (10% of volume harvested) PBS buffer. The cells were sonicated (30%, 20 sec, 1 on, 1 off) on ice. Sonicated samples centrifuged at 15 294 x g for 1 min to separate insoluble and soluble fractions. After that, the supernatant was collected as soluble fraction and insoluble fraction added with 100 µl PBS buffer for SDS-PAGE.

### 3.7.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All components for resolving gel listed in Table 3.7 were mixed together except for 10% ammonium persulfat (APS) (Sigma, USA) and tetramethyethylenediamine (TEMED) (Sigma, USA). After addition of APS and TEMED, resolving gel solution was immediately transferred into the gel cast to three-quarters of the height of the. The resolving gel was overlaid with isopropanol. Isopropanol prevented dehydration and maintained an anaerobic environment for polymerization. The gel was left to be polymerized for 15 min.

**Table 3.8: Components for 10% Resolving Gel**

| Components | Volume |
|---|---|
| **Distilled water (dH2O)** | 2.765 ml |
| **30% Acrylamide** | 2.333 ml |
| **1.5 M Tris HCl (pH 8.8)** | 1.75 ml |
| **10% SDS** | 70 µl |
| **10% APS (100 mg/ml)** | 70 µl |
| **TEMED** | 11.55 µl |
| **Total Volume** | **~7.00 ml** |

After 15 min, isopropanol was removed with distilled water. All components of the stacking gel listed in Table 3.8 were mixed and the mixture was quickly pipetted on top of resolving gel. A 0.75 mm comb was placed to create wells in stacking gel. The

comb was removed gently after stacking gel was fully polymerized. Samples (10 µl) were mixed with 2.5 µl of Laemlli's sample buffer and then heated at 95˚C for 5 min. The proteins were separated by SDS-PAGE on 10% acrylamide gel and visualized by CBB staining.

**Table 3.9: Components for Stacking Gel**

| STACKING GEL | Volume |
|---|---|
| **Distilled water (dH2O)** | 1.406 ml |
| **30% Acrylamide** | 0.300 ml |
| **500 mM Tris HCl (pH 6.8)** | 0.250 ml |
| **10% SDS** | 20 µl |
| **10% APS (100 mg/ml)** | 20 µl |
| **TEMED** | 4.0 µl |
| **Total Volume** | **~2.00 ml** |

### 3.8 Peptide Identification

Targeted recombinant proteins' bands were dissected out and sent for peptide identification (Proteomics International Pty Ltd, Australia). The protein samples were trypsin digested and peptides extracted according to standard techniques (Bringans et al. Proteomics 2008). Peptides were analysed by matrix-assisted laser desorption ionization time-of-flight (MALDITOF-TOF) mass spectrometer using a 4800 Proteomics Analyzer (AB Sciex, USA). Spectra were analysed to identify protein of interest using Mascot sequence matching software (Matrix Science, UK) with Ludwig NR Database.

### 3.9 rNNV Expression for Purification

#### 3.9.1 Culture of *E. coli* BL21 (DE3) Harboring rNNV

rNNV recombinant protein was produced in medium scale for purification. Fresh 10 ml LB-amp broth was inoculated with *E. coli* BL21 (DE3) carrying rNNV construct and incubated at 20˚C (180 rpm). Then, 1 ml of overnight culture was harvested and stored in -20˚C (uninduced sample). The remaining cells were harvested (10 621 x g, 10 min) and resuspended to OD600 nm 1.0 with PBS buffer.

Fresh LB-amp broth (100 ml) in 500 ml conical flask inoculated with 1% of medium volume (1 ml inoculation) of overnight culture. When cell density reached OD600 nm=0.4 – 0.6, IPTG was added to final concentration of 0.1 mM. The cultures were incubated at 20˚C (180 rpm) for 12 hr. After 12 hr incubation, final OD600 nm value was observed (100 µl of cell diluted with 900 µl of LB-amp broth). Another 1 ml was harvested and stored in -20˚C (unsonicated induced sample).

The remaining cells were collected and weighed before resuspended in 10 ml PBS buffer to wash off the remaining medium. After discarding the PBS buffer, the cells were sonicated in 10 ml ice-cold His binding buffer (PBS, 20 mM imidazole) (30%, 1 min) twice. After soluble fraction collected (10 621 x g centrifugation, 5 min), cell lysate was resuspended in His binding buffer.

#### 3.9.2 Affinity Chromatography Purification of rNNV Protein

His binding buffer and elution buffer (PBS, 500 mM imidazole) were chilled prior to run. The soluble fraction was loaded onto 5 ml Nickel column (GE Healthcare, England), pre-equilibrated with His binding buffer. The column was washed with five column volumes of His binding buffer, and bound proteins were eluted with His elution buffer. The fraction eluted by 500 mM imidazole was collected and stored in -20˚C until further use. The purified protein was used as the immunogen to generate polyclonal antibodies in rat (Sprague Dawley).

### 3.9.3 Bradford Assay

Protein concentrations were determined using Bradford method (Bradford, 1976) (Sigma, USA) with bovine serum albumin (Sigma, USA) as standard.

## 3.10 Immunization of Rats (Sprague Dawley)

Twelve-week-old male rats (Sprague Dawley) were injected 3 times at 2-week intervals with purified soluble recombinant major capsid protein of NNV. The rats received subcutaneous injections of 50 µg/kg of purified protein that was emulsified with equal volume of complete Freund's adjuvant (Santa Cruz Biotechnology, USA) for the first injection and in combination with PBS for the subsequent injections. Control rats received PBS containing complete Freund's adjuvant. Sera were collected every week via retro-orbital under diethyl ether anaesthesia and stored at -20˚C for subsequent assay.

## 3.11 Enzyme-Linked Immuno Assay (ELISA)

Indirect ELISA was performed as described in Abcam protocol book with slight modification to determine the antibody titers of anti-rNNV sera (total immunoglobulin).

In brief, 50 µl diluted antigen (final concentration of 50 ng/well) coated the wells of 96-well plate. The microplate incubated for 2 hr at 4˚C overnight. The coating solution discarded and washed by filling the wells with 100 µl washing solution (PBS, 0.05% Tween 20). The wash solution removed by flicking the microplate gently, then patted on paper towel to remove remaining drops. Blocking solution (50 µl) (PBS, 5% BSA) added to block the remaining binding sites in the coated wells for 2 hr. After that, washing step repeated twice. In-house produced rats' anti-rNNV sera were diluted (1:100) with primary dilution buffer (PBS, 0.5% BSA, 0.05% Tween 20) and alkaline phosphatase (AP) -conjugated anti-rat Ig was diluted (1:1000) with secondary dilution

buffer (PBS, 1% BSA). Diluted sera (100 µl) added and incubated for 2 hr. After washing step, diluted AP-conjugated anti-rat Ig (50 µl) added and incubated for another 2 hr. The substrate was prepared by dissolving AP tablet in diethanolamine substrate buffer (DSB) (Tris 0.1 M, NaCl 0.1 M, $MgCl_2$ 0.00498 M, 10% diethanolamine, pH 9.5). Washing step repeated again and 50 µl of substrate (AP, 16.67 µg/ well) was added to each well. After 50 min incubation, 100 µl of stop solution added into the wells and absorbance was read at 405 nm with Tecan microplate reader.

## SEQUENCE DATA FOR IRIDOVIRUS CAPSID PROTEIN

```
1     acatgtaccacaggcgcaggtgtgaccagcggtttcatcgacttagccacctacgataac  60
      T  C  T  T  G  A  G  V  T  S  G  F  I  D  L  A  T  Y  D  N
61    ctggaccgcgcactgtatggtggtaaggacgccaccacctactttatcaaagagcactac  120
      L  D  R  A  L  Y  G  G  K  D  A  T  T  Y  F  I  K  E  H  Y
121   ccggttggttggtttaccaagctgccgacaatggccacccgcgtgagtggcaaccctgca  180
      P  V  G  W  F  T  K  L  P  T  M  A  T  R  V  S  G  N  P  A
181   ttcggccaggaatttagtgtgggcgttccgcgtagcggcgactatgtgctgaatgcctgg  240
      F  G  Q  E  F  S  V  G  V  P  R  S  G  D  Y  V  L  N  A  W
241   ctgaccctgaagacaccggagattaagctgctggacaccaatcgcctgggcgcaaatggc  300
      L  T  L  K  T  P  E  I  K  L  L  D  T  N  R  L  G  A  N  G
301   acagtgcgttggaccaagaacctgatgcataacgccgtggaacacgcaagcctgaccttc  360
      T  V  R  W  T  K  N  L  M  H  N  A  V  E  H  A  S  L  T  F
361   aatgacatctgtgcccagcagttcaacacagcctacctggacgcctggacacagttcaac  420
      N  D  I  C  A  Q  Q  F  N  T  A  Y  L  D  A  W  T  Q  F  N
421   atgtgcgagggcaagcgtatcggctatgacaatatgatcggcaacacaagcgacatgaca  480
      M  C  E  G  K  R  I  G  Y  D  N  M  I  G  N  T  S  D  M  T
481   aacccgacaccggcacagggtcaggatggtgcacgcaccttacctagcaaaaacctggtg  540
      N  P  T  P  A  Q  G  Q  D  G  A  R  T  L  P  S  K  N  L  V
541   ctgccgctgccgttttttttagtcgcgactgcggcctggcattaccgacagtggtgctg  600
      L  P  L  P  F  F  F  S  R  D  C  G  L  A  L  P  T  V  V  L
601   ccgtacaacgaaattcgcattaacattaaactgcgcagcctgcaggagttactggtgttc  660
      P  Y  N  E  I  R  I  N  I  K  L  R  S  L  Q  E  L  L  V  F
661   cagaataaggataccggcaatgtgattcctatcagcgccaccgacatcgccggcggttta  720
      Q  N  K  D  T  G  N  V  I  P  I  S  A  T  D  I  A  G  G  L
721   gccgataccgtggaggcctacgtgtacatgaccgtgggcctggtgagcaacgttgaacgc  780
      A  D  T  V  E  A  Y  V  Y  M  T  V  G  L  V  S  N  V  E  R
781   tgtgcaatggccggtaccgtgcgtgacatggttgtggagcagatgcaagcagcaccgacc  840
      C  A  M  A  G  T  V  R  D  M  V  V  E  Q  M  Q  A  A  P  T
841   catatcgtgaatccgcagaacaccaacaacgtgcacgtggacatgcgcttcagccacgcc  900
      H  I  V  N  P  Q  N  T  N  N  V  H  V  D  M  R  F  S  H  A
901   gtgaaggccctgtttttcatggtgcagaacgttacctacaagagcgtgggcagcaactac  960
      V  K  A  L  F  F  M  V  Q  N  V  T  Y  K  S  V  G  S  N  Y
961   acctgtgtgacaccggttaacggtccgggcaatacagtgatggaaccggccatgagtgtt  1020
      T  C  V  T  P  V  N  G  P  G  N  T  V  M  E  P  A  M  S  V
1021  gatcctatcaagagtgccagcctgacctacgagaacacaacccgtctggcaaacatgggc  1080
      D  P  I  K  S  A  S  L  T  Y  E  N  T  T  R  L  A  N  M  G
1081  gtggagtactacagcctggttcagccttggtacttcagcgccagtatccctgtgtacaca  1140
      V  E  Y  Y  S  L  V  Q  P  W  Y  F  S  A  S  I  P  V  Y  T
1141  ggttaccacatgtacagttacgccctgaacgtgggcagtgtgcaccctagcggtagcaca  1200
      G  Y  H  M  Y  S  Y  A  L  N  V  G  S  V  H  P  S  G  S  T
1201  aattacggccgcctgacaaacgccagcatcacagtgaccatgagtcctgaaagcgttgtg  1260
      N  Y  G  R  L  T  N  A  S  I  T  V  T  M  S  P  E  S  V  V
1261  gcagcagccggcggcggtaacaacaacagcggttacaatgagccgcagcgctttgccctg  1320
      A  A  A  G  G  G  N  N  N  S  G  Y  N  E  P  Q  R  F  A  L
1321  gtggttattgccgttaaccataacgttatccgcatcatgaacggtagtatgggcttcccg  1380
      V  V  I  A  V  N  H  N  V  I  R  I  M  N  G  S  M  G  F  P
1381  att  1383
      I
```

**IRIDOVIRUS : The nucleotide sequence of codon modified IRI and its deduced amino acid sequence. The amino acid sequence is in bold capital letters.**

```
1     gtacgtaaaggtgaaaaaaaattagcaaaaccagcgacgaccaaggcggctaatccacag  60
      V  R  K  G  E  K  K  L  A  K  P  A  T  T  K  A  A  N  P  Q
61    ccgcgccgtcgcgccaataaccgccgccgttccaatcgcaccgatgccccggtgagtaag  120
      P  R  R  R  A  N  N  R  R  R  S  N  R  T  D  A  P  V  S  K
121   gctagcaccgtaacgggttttggccgtggcactaacgatgtccacctgtccggcatgagc  180
      A  S  T  V  T  G  F  G  R  G  T  N  D  V  H  L  S  G  M  S
181   cgcattagccaagcggtacttccggccggtaccgggacggatggttatgtcgtcgtcgat  240
      R  I  S  Q  A  V  L  P  A  G  T  G  T  D  G  Y  V  V  V  D
241   gcaaccatcgtaccagatcttttgcctcgccttggccacgcagcacgtatttttcaacgt  300
      A  T  I  V  P  D  L  L  P  R  L  G  H  A  A  R  I  F  Q  R
301   tatgcagtagagacactggaatttgagatccagccaatgtgtcccgctaatactggaggc  360
      Y  A  V  E  T  L  E  F  E  I  Q  P  M  C  P  A  N  T  G  G
361   ggatacgtagccggctttctgccggaccccactgacaacgaccatacctttgatgcctta  420
      G  Y  V  A  G  F  L  P  D  P  T  D  N  D  H  T  F  D  A  L
421   caggctacccgtggagctgttgtggcaaaatggtgggaatcccgcactgttcgtccgcag  480
      Q  A  T  R  G  A  V  V  A  K  W  W  E  S  R  T  V  R  P  Q
481   tatactcgtactctgctgtggacgagctccggcaaagagcaacgcctgacgagtccaggc  540
      Y  T  R  T  L  L  W  T  S  S  G  K  E  Q  R  L  T  S  P  G
541   cgtctgattctgctttgcgtcggcaataatacggatgtggttaacgtctcagtattatgc  600
      R  L  I  L  L  C  V  G  N  N  T  D  V  V  N  V  S  V  L  C
601   cgctggagcgttcgtttaagcgtacccagcttggaaaccccggaggaaaccactgcacct  660
      R  W  S  V  R  L  S  V  P  S  L  E  T  P  E  E  T  T  A  P
661   attatgactcagggctccctgtataacgatagcctgagtaccacggatttcaaatctatc  720
      I  M  T  Q  G  S  L  Y  N  D  S  L  S  T  T  D  F  K  S  I
721   ctgctgggctcgaccccgctggatattgcccccgatggtgcagtcttccagctggaccgt  780
      L  L  G  S  T  P  L  D  I  A  P  D  G  A  V  F  Q  L  D  R
781   ccgctgagcatcgattattcactgggtactggtgatgttgatcgcgcggtttattggcat  840
      P  L  S  I  D  Y  S  L  G  T  G  D  V  D  R  A  V  Y  W  H
841   atcaaaaaatttgccggaaatgcaggtactccggcaggttggtttcgttgggggatctgg  900
      I  K  K  F  A  G  N  A  G  T  P  A  G  W  F  R  W  G  I  W
901   gacaacttcaacaagaccttcacagatggtgtcgcgtattatagcgatgagcaaccgcgt  960
      D  N  F  N  K  T  F  T  D  G  V  A  Y  Y  S  D  E  Q  P  R
961   cagatccttctgccggttggtactgtttgtacccgcgttgactctggcaat          1011
      Q  I  L  L  P  V  G  T  V  C  T  R  V  D  S  G  N
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**NNV MAJOR CAPSID: The nucleotide sequence of codon modified NNV and its deduced amino acid sequence. The amino acid sequence is in bold capital letters.**