

# Preparation of divalent antigen-displaying enveloped virus-like particles using a single recombinant Bombyx mori nucleopolyhedrovirus bacmid in silkworms

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1 **Preparation of divalent antigen-displaying enveloped virus-like**  
2 **particles using a single recombinant *Bombyx mori* nucleopolyhedrovirus**  
3 **bacmid in silkworms**

4

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20

21 **Abstract**

22 Silkworms have been used as a host for the production of recombinant proteins in a  
23 baculovirus expression system using *Bombyx mori* nucleopolyhedrovirus (BmNPV). To  
24 coexpress several recombinant proteins, a silkworm must be coinfecting with several  
25 recombinant BmNPVs, which requires a difficult DNA manipulation procedure. In this  
26 study, we constructed recombinant BmNPVs containing three expression cassettes, Rous  
27 sarcoma virus (RSV) Gag protein, surface antigen 1 of *Neospora caninum* (NcSAG1) and  
28 SAG1-related sequence 2 of *N. caninum* (NcSRS2), by Gibson assembly and the Bac-to-  
29 Bac system, designated BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS.  
30 BmNPV/SAG-SRS-Gag was expressed in silkworms and characterized. NcSAG1 and  
31 NcSRS2 were purified with RSV Gag proteins using sucrose density gradient  
32 centrifugation and affinity chromatography. RSV Gag formed virus-like particles (RSV-  
33 LPs) at a diameter of 20–30 nm based on transmission electron microscopy (TEM).  
34 Immuno-TEM analysis showed that both NcSAG1 and NcSRS2 were displayed on the  
35 surface of the RSV-LPs. These results indicate that RSV-LPs displaying two different  
36 kinds of proteins were produced in the hemolymph of silkworm larvae by the single  
37 polycistronic strategy. This expression platform is efficient for generating multiantigen-  
38 displaying VLPs and facilitates the development of vaccines against infectious diseases.

39

40 Keywords: BmNPV, coexpression, polycistronic, virus-like particle, *Neospora caninum*

41

## 42 **1. Introduction**

43

44 Viral structural proteins form virus-like particles (VLPs) without any assembly steps  
45 during the protein expression process regardless of the presence of the viral envelope  
46 (Zeltins, 2013). VLPs mimic viruses in terms of their structure. However, since they do  
47 not have any viral genetic materials, VLPs have been applied to vaccine platforms and  
48 drug delivery strategies as antigens and carriers (Charlton Hume et al., 2019).

49 To form intact VLPs, several structural proteins that resemble virions  
50 morphologically must be simultaneously expressed. For example, triple-layered  
51 rotavirus-like particles can be generated by coexpression of VP2, VP6 and VP7 in insect  
52 cells (Shoja et al., 2015). Thus, to mimic virus particles, several proteins can be expressed  
53 by a coexpression strategy, such as matrix 1 protein, hemagglutinin and neuraminidase in  
54 influenza VLPs (Kang et al., 2009). Additionally, the coexpression of structural and other  
55 proteins can generate hybrid VLPs containing full-length proteins from distinct viruses  
56 and recombinant protein-displaying VLPs (Choi et al., 2018; Ludwig and Wagner, 2007).

57 Insect cells can produce many kinds of VLPs by the baculovirus expression system  
58 and stably transformed expression system (Lee et al., 2011; Shoja et al., 2015; Sokolenko  
59 et al., 2012). The baculovirus expression system is the first choice to produce recombinant  
60 proteins in insect cells because of its ease of use and rapidity. To coexpress several  
61 recombinant proteins in the baculovirus expression system, normally, several kinds of  
62 recombinant baculoviruses have to infect insect cells simultaneously (coinfection  
63 strategy). This method causes an imbalanced expression of the recombinant proteins due  
64 to incomplete coinfection, which leads to the formation of incomplete VLPs (Sokolenko  
65 et al., 2012).

66           Alternatively, to achieve the simultaneous coexpression of recombinant proteins in  
67 the baculovirus expression system, a recombinant baculovirus containing several protein  
68 expression cassettes (a single polycistronic strategy) has been used (Latham and Galarza,  
69 2001; Pushko et al., 2005). In this case, only one recombinant baculovirus is used to infect  
70 insect cells, which leads to a more homogeneous expression of recombinant proteins than  
71 in the case of the coinfection strategy (Shanks and Lomonossoff et al., 2000). However,  
72 the construction of this type of recombinant baculovirus is very tedious and time-  
73 consuming. Recently, several gene cloning techniques have been developed to replace the  
74 conventional DNA ligase-based cloning method. A MultiBac system that is based on  
75 recombination technology using different recombinases facilitates the expression of many  
76 kinds of protein complexes, including VLPs (Pelosse et al., 2017). The uracil-specific  
77 excision reagent (USER) method with the MultiBac system has also been applied for the  
78 expression of protein complexes (Zhang et al., 2016). The biGBac system, based on the  
79 Gibson assembly principle, achieved the simultaneous expression of 17 subunits of  
80 human anaphase promoting complex/cyclosome using a single recombinant baculovirus  
81 (Gibson et al., 2009; Weissmann et al., 2016). This single polycistronic strategy is also  
82 very useful for the expression of protein complexes in insect cells.

83           Previous studies have employed *Autographa californica* multiple  
84 nucleopolyhedrovirus (AcMNPV) as a vector in insect cells, while *Bombyx mori*  
85 nucleopolyhedrovirus (BmNPV) has been used in silkworm. Silkworm larvae and pupae  
86 are favorable hosts for the large-scale production of recombinant proteins due to their  
87 high protein production capacity (Kato et al., 2010; Motohashi et al., 2005; Usami et al.,  
88 2010), including producing VLPs (Masuda et al., 2018; Minkner et al., 2018; Watanabe  
89 et al., 2017; Xu et al., 2019). In this study, antigen-displaying enveloped VLPs were

90 expressed in silkworm larvae using a single recombinant BmNPV containing three  
91 protein expression cassettes. A single polycistronic strategy was also suitable for the  
92 expression of intact VLPs displaying these antigens in silkworm larvae compared to the  
93 coinfection strategy.

94

95

## 96 **2. Materials and methods**

97

### 98 *2.1. Cell culture and silkworm rearing*

99 Bm5 cells were maintained in Sf-900 II medium (Thermo Fisher Scientific K. K.,  
100 Tokyo, Japan) supplemented with antibiotic-antimycotic solution (Thermo Fisher  
101 Scientific K. K.) and 10% FBS (Thermo Fisher Scientific K. K.) at 27°C. The fourth-  
102 instar silkworm larvae were purchased from Ehime Sansyu (Ehime, Japan). Silkworm  
103 larvae were reared on an artificial diet, Silkmate 2S (Nosan, Yokohama, Japan), at 25°C.

104

### 105 *2.2. Construction of recombinant baculoviruses*

106 The recombinant BmNPV containing the Rous sarcoma virus Gag protein expression  
107 cassette (BmNPV/Gag) was previously constructed (Deo et al., 2011). To express surface  
108 antigen 1 of *Neospora caninum* (NcSAG1) fused with the C-terminal domain of GP64  
109 from BmNPV (BmGP64), BmNPV/SAG1-GP64TM constructed previously was used  
110 (Kato et al., 2015). To express SAG1-related sequence 2 of *N. caninum* (NcSRS2) fused  
111 with the C-terminal domain of GP64 from BmNPV (BmGP64), BmNPV/SRS2-GP64TM,  
112 which was constructed previously, was also used (Kato et al., 2015).

113 To construct a single polycistronic BmNPV containing Gag, NcSAG1 and NcSRS2

114 expression cassettes, the expression cassettes were first amplified by PCR using three  
115 primer sets {set 1 ( $\alpha$ -1-F and  $\beta$ -1-R), set 2 ( $\beta$ -2-F and  $\gamma$ -2-R), set 3 ( $\gamma$ -3-F and  $\omega$ -4-R)}  
116 (Table 1). Additionally, a linear pFastbac1 vector (Thermo Fisher Scientific K. K.) was  
117 prepared by PCR using primer set 4 ( $\omega$ -pFB-F and  $\alpha$ -pFB-R) (Table 1). These DNA  
118 fragments (100 ng each) were assembled by Gibson assembly (Gibson et al., 2009;  
119 Gibson 2011). Then, pFB/SAG-SRS-Gag and pFB/SAG-Gag-SRS were constructed (Fig.  
120 1). These plasmids were individually transformed into *Escherichia coli* BmDH10Bac  
121 (Motohashi et al., 2005), and BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS  
122 bacmids were extracted from white colonies.

123 These recombinant BmNPV bacmids (10  $\mu$ g DNA containing the helper plasmid)  
124 were injected into silkworm larvae using chitosan (Kato et al., 2016). After 6-7 days,  
125 hemolymph was collected from recombinant BmNPV bacmid-injected silkworm larvae.  
126 Virus titers in the hemolymph were determined according to a protocol reported  
127 previously (Kato et al., 2009). Briefly, the genomic DNA was extracted from hemolymph  
128 by High Pure Viral Nucleic Acid Kit (Roche Diagnostics K. K., Tokyo, Japan) according  
129 to the manufacturer's protocol. Quantitative PCR (qPCR) was carried out using  
130 THUNDERBIRD SYBR qPCR Mix (TOYOBO, Otsu, Japan) with Bm-ie1 F and Bm-  
131 ie1 R primers (Table 1). Mx3000P system (Stratagene, La Jolla, CA, USA) and MxPro  
132 software (Stratagene) was used for the analysis.

133

### 134 2.3. Antigen-displaying VLPs in silkworm larvae

135 Using the coinfection strategy, three recombinant BmNPVs (BmNPV/Gag,  
136 BmNPV/SAG1 and BmNPV/SRS2) with the same titer ( $1 \times 10^8$  pfu) were injected into  
137 silkworm larvae. Using the single polycistronic strategy, BmNPV/SAG-SRS-Gag or

138 BmNPV/SAG-Gag-SRS was injected into silkworm larvae. After 4 days, the hemolymph  
139 and fat body were collected. 1-Phenyl-2-thiourea (200 mM) was added to the hemolymph  
140 at 2 mM, and 0.1 g of the fat body was suspended in 1 mL of Tris-buffered saline (pH  
141 7.6) containing 0.1% Triton X-100. The fat body was disrupted by sonication, and the  
142 homogenate was centrifuged at  $10,000 \times g$ . The supernatant was used as an extract of the  
143 fat body.

144 For the purification of VLPs, the collected hemolymph was centrifuged at  $10,000 \times$   
145  $g$ . The resulting supernatant (4 mL) was centrifuged on a 20% sucrose cushion at  $122,000$   
146  $\times g$ . The pellet was suspended in phosphate-buffered saline (PBS, pH 7.4) by sonication.  
147 The suspension was subjected to 20–60% sucrose density gradient centrifugation at  
148  $122,000 \times g$  for 3 h. The fractions containing VLPs were subjected to anti-DDDDK tag  
149 affinity chromatography. Approximately 100  $\mu$ L of anti-DDDDK tag purification gel  
150 (Medical & Biological Laboratories, Nagoya, Japan), which was equilibrated with PBS,  
151 was added into the fractions and incubated at  $4^{\circ}\text{C}$  with gentle stirring for 1 h. The gel was  
152 washed with PBS, and proteins were eluted with 0.1 M glycine-HCl (pH 3.0) in each  
153 fraction (100  $\mu$ L). Immediate neutralization was carried out by the addition of 1 M Tris-  
154 HCl (pH 9.0).

155 Genomes of recombinant BmNPVs were extracted by a High Pure Viral Nucleic Acid  
156 Kit (Roche Diagnostics K. K., Tokyo, Japan) according to the manufacturer's protocol.  
157 To detect the genomes of recombinant BmNPVs, a part of the *ie-1* gene of BmNPV was  
158 amplified by PCR. PCR was performed with the *ie-1* primer set Bm-*ie1* F and Bm-*ie1* R  
159 (Table 1) using the extracted recombinant BmNPV DNA as a template.

160

161 2.4. *SDS-PAGE and western blotting*



162 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was  
163 carried out to separate proteins using 12% acrylamide. After SDS-PAGE, the proteins  
164 were transferred to a polyvinylidene fluoride (PVDF) membrane using the Mini Trans-  
165 Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The membrane was  
166 blocked in 5% skim milk in TBST (pH 7.6), followed by incubation with 10000-fold-  
167 diluted mouse anti-DDDDK tag monoclonal antibody (Medical & Biological  
168 Laboratories) for NcSAG1 and NcSRS2 detection or 1000-fold-diluted mouse anti-Gag  
169 serum for RSV Gag detection. As a secondary antibody, 10000-fold-diluted horseradish  
170 peroxidase (HRP)-linked goat anti-mouse IgG (Medical & Biological Laboratories) was  
171 used. Detection of proteins was performed using Immobilon Western Chemiluminescent  
172 HRP Substrate (Merck Millipore, Billerica, MA, USA). Protein bands were detected on a  
173 Fluor-S MAX Multi-Imager (Bio-Rad).

174

#### 175 2.5. *Transmission electron microscopy (TEM)*

176 Purified samples were loaded on a TEM grid (649, Nisshin EM, Tokyo, Japan) and  
177 stained with 2% phosphotungstic acid. Particles were observed by JEM-2100F (JEOL,  
178 Tokyo, Japan) operated at 100 kV. For immuno-TEM, TEM grid-loaded samples were  
179 blocked with 1% BSA, followed by washing with PBS 3 times. Then, the grid was  
180 incubated with 100-fold-diluted mouse anti-DDDDK tag monoclonal antibody  
181 (MEDICAL & BIOLOGICAL LABORATORIES). As a secondary antibody, 100-fold-  
182 diluted anti-IgG + IgM (H+L), Mouse, Goat-Poly, Gold 10 nm, EM (EMGAF10, BBI,  
183 Solutions, Crumlin, UK) was used. Staining with phosphotungstic acid was carried out  
184 before observation by TEM.

185

186

### 187 **3. Results and discussion**

188

#### 189 *3.1. Construction of recombinant BmNPV*

190 Recombinant BmNPVs BmNPV/Gag, BmNPV/NcSAG1 and BmNPV/NcSRS2 (Fig.  
191 1A–C) was previously constructed for the expression of RSV Gag protein, NcSAG1, and  
192 NcSRS2 in silkworms, respectively (Deo et al., 2011; Kato et al., 2015). The signal  
193 sequence of NcSAG1 or NcSRS2 at the N-terminus was deleted, and the signal sequence  
194 of bombyxin from *B. mori* was fused at the N-terminus instead. Additionally, the  
195 transmembrane and cytoplasmic domains of GP64 from BmNPV were also fused with  
196 NcSAG1 and NcSRS2 at the C-terminus for the display of NcSAG1 and NcSRS2 on the  
197 RSV-LPs. Using these recombinant BmNPVs, NcSAG1 and NcSRS2-displaying RSV-  
198 LPs were prepared by coinfection strategy (Xu et al., 2019). In this study, we attempted  
199 to construct a single recombinant BmNPV containing three expression cassettes by  
200 Gibson assembly (Gibson et al., 2009). We constructed two recombinant BmNPVs  
201 (BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS2) containing the three expression  
202 cassettes described in Fig. 1D and E.

203

#### 204 *3.2. Expression of RSV-LPs*

205 To prepare NcSAG1 and NcSRS2-displaying RSV-LPs, these three proteins were  
206 coexpressed in silkworm larvae. Three different recombinant BmNPVs (BmNPV/Gag,  
207 BmNPV/NcSAG1, and BmNPV/NcSRS2) were coinjected to express the three proteins.  
208 RSV Gag (61 kDa) was observed in both the hemolymph and fat body, but NcSAG1 and  
209 NcSRS2 were observed only in the fat body (Fig. 2A). This result suggested that most

210 NcSAG1 and NcSRS2 proteins were expressed in the fat body and that a small amount  
211 may be displayed on the RSV-LPs. Estimated molecular weight of NcSAG1 and NcSRS2  
212 was 36 and 40 kDa, respectively (Kato et al., 2015). However, NcSAG1 was detected at  
213 between 40 and 50 kDa in the fat body by western blot as several bands and NcSRS2 was  
214 also detected at between 50 and 60 kDa as a single band. Now this discrepancy of each  
215 molecular weight is unknown, but some post-translational modification including *N*-  
216 glycosylation might be carried out. Several bands of NcSAG1 detected in the fat body  
217 might be caused by some post-translational modification.

218 In addition to coinfection of three recombinant BmNPVs, the three proteins were  
219 coexpressed in silkworm larvae using single recombinant BmNPV, BmNPV/SAG-SRS-  
220 Gag or BmNPV/SAG-Gag-SRS2 (Fig. 2B). The expression pattern of each protein by  
221 BmNPV/SAG-SRS-Gag or BmNPV/SAG-Gag-SRS2 was almost the same as that in the  
222 coinfection of the three recombinant BmNPVs. However, the expression level of each  
223 protein using BmNPV/SAG-SRS-Gag (lane 2 of Fig. 2A and B) was higher than that  
224 obtained using the coinfection strategy with the three recombinant BmNPVs. This result  
225 suggests that the reduction in the burden of multiple recombinant BmNPV infections by  
226 the use of the single polycistronic strategy may lead to the improvement of the expression  
227 level of recombinant proteins. BmNPV/SAG-Gag-SRS2 also produced a larger amount  
228 of NcSAG1 compared to the coinfection strategy, but only a faint band of NcSRS2 was  
229 detected compared to that observed with BmNPV/SAG-SRS-Gag. This result suggests  
230 that the order of the three expression cassettes may have affected their expression levels.  
231 Using an anti-Gag antibody, several proteins were detected below the full-length Gag  
232 protein (Fig. 2A). These protein bands may be produced by the processing of expressed  
233 Gag protein because Gag protein is processed into a protein matrix consisting of p2, p10

234 and capsid domains in insect cells (Deo et al., 2011).

235 RSV-LPs collected from the hemolymph expressed using the single polycistronic  
236 strategy were concentrated and purified using sucrose density gradient centrifugation (Fig.  
237 3). Using BmNPV/SAG-SRS-Gag, NcSAG1 and NcSRS2 were detected with Gag  
238 protein in the same fractions (fractions 4–7), even though these two proteins were not  
239 detected in the hemolymph (Fig. 2B). These results suggested that NcSAG1 and NcSRS2  
240 were displayed on the RSV-LPs. However, NcSRS2 was hardly observed for  
241 BmNPV/SAG-Gag-SRS. This result corresponded to the expression level of each protein  
242 in Fig. 2B. To confirm the display on the RSV-LPs, RSV-LPs in fractions 4–7 were  
243 purified by affinity chromatography. The three proteins were detected in the same elution  
244 fractions using BmNPV/SAG-SRS-Gag (Fig. 4A). However, more NcSAG1 was detected  
245 than NcSRS2, which was observed as only a faint band. This result indicates that  
246 NcSAG1 and NcSRS2 are displayed on the RSV-LPs but not at the same amounts. This  
247 result corresponded to the expression level of each protein in Figs. 2 and 3. In our previous  
248 study, NcSAG1 and NcSRS2 were displayed on BmNPV particles (Kato et al., 2015). To  
249 investigate the possibility of NcSAG1 and NcSRS2 being displayed on the BmNPV  
250 particles, detection of BmNPV genomic DNA was carried out in the elution fractions by  
251 PCR to amplify a part of the *ie-1* gene. Amplified DNA fragments were detected in  
252 fraction 1, but NcSAG1 and NcSRS2 were not detected in fraction 1. On the other hand,  
253 in fractions 2–4, no amplified DNA fragments were detected, and the three recombinant  
254 proteins were detected using BmNPV/SAG-SRS-Gag. This result indicates that NcSAG1  
255 and NcSRS2 were displayed on the RSV-LPs. From 1 mL of hemolymph, 3.0 and 2.7  $\mu$ g  
256 of RSV-LPs were prepared using BmNPV/SAG-SRS-Gag and BmNPV/SAG-Gag-SRS,  
257 respectively.

258 The morphology of RSV-LPs purified by affinity chromatography was investigated  
259 by TEM. Particles were 20–30 nm in diameter (Fig. 5A), and this size is smaller than that  
260 in previous reports (Deo et al., 2011; Kato et al., 2015). In this study, RSV-LPs were  
261 sonicated to be suspended after precipitation from the hemolymph by ultracentrifugation.  
262 In addition, acidic buffer was used to elute RSV-LPs from an anti-DDDDK tag  
263 purification gel. These steps may cause the difference in the particle size between this  
264 study and previous studies. To confirm the display of NcSAG1 and NcSRS2 on the  
265 surface of RSV-LPs, immuno-TEM was carried out. Gold particles were observed on the  
266 surface of the RSV particles (Fig. 5B and C), indicating that NcSAG1 and NcSRS2 were  
267 displayed on the surface of RSV-LPs because both NcSAG1 and NcSRS2 were fused  
268 with the FLAG tag.

269 We previously reported that NcSAG1 and NcSRS2-displaying RSV-LPs, which were  
270 prepared in silkworms by the coinfection strategy using three different recombinant  
271 BmNPVs, were not sufficient to prevent *N. caninum* infection in mice (Xu et al., 2019).  
272 Optimization of each recombinant baculovirus, which is often difficult, has to be carried  
273 out to balance the expression of each protein when multiple proteins are coexpressed by  
274 the use of multiple recombinant baculoviruses (Mena et al., 2007). In silkworm larvae  
275 and pupae, the single polycistronic strategy using a single recombinant baculovirus is  
276 better for the coexpression of recombinant proteins to produce protein complexes (Liu et  
277 al., 2020; Yao et al., 2012; Zhou et al., 2011). In this study, we achieved the coexpression  
278 of RSV Gag protein, NcSAG1 and NcSRS2 using the single polycistronic strategy in  
279 silkworm larvae to prepare divalent antigen (NcSAG1 and NcSRS2)-displaying RSV-LPs.  
280 Compared to the coinfection strategy, the single polycistronic strategy facilitated  
281 improved expression of multiantigen-displaying RSV-LPs in silkworm larvae. This

282 platform can be utilized for the preparation of antigen-displaying VLPs, regardless of the  
283 kind of VLPs (enveloped and nonenveloped) and will be helpful for the development of  
284 vaccines against infectious diseases.

285

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289

## 290 **References**

291

292 Charlton Hume, H.K., Vidigal, J., Carrondo, M.J.T., Middelberg, A.P.J., Roldão., Lua,  
293 L.H.L., 2019. Synthetic biology for bioengineering virus-like particle vaccines.  
294 *Biotechnol. Bioeng.* 116, 919–935.

295 Choi, J.M., Bourassa, V., Hong, K., Shoga, M., Lim, E.Y., Park, A., Apaydin, K., Udit,  
296 A.K. 2018. Polyvalent Hybrid Virus-Like Nanoparticles with Displayed Heparin  
297 Antagonist Peptides. *Mol. Pharm.* 15, 2997–3004.

298 Deo, V.K., Tsuji. Y., Yasuda. T., Kato. T., Sakamoto. N., Suzuki. H., Park. E.Y. Expression  
299 of an RSV-gag virus-like particle in insect cell lines and silkworm larvae. *J. Virol.*  
300 *Methods* 177, 147–152.

301 Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A.3rd., Smith, H.O.  
302 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat.*  
303 *Methods* 6, 343–345.

304 Gibson, D.G. 2011. Enzymatic Assembly of Overlapping DNA Fragments. *Methods*  
305 *Enzymol.* 498, 349–361, 2011.

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306 Kang, S.M., Yoo, D.G., Lipatov, A.S., Song, J.M., Davis, C.T., Quan, F.S., Chen, L.M.,  
307 Donis, R.O., Compans, R.W. 2009. Induction of long-term protective immune  
308 responses by influenza H5N1 virus-like particles. PLoS One 4, e4667.

309 Kato, T., Manoha, S.L., Tanaka, S., Park, E.Y. High-titer preparation of *Bombyx mori*  
310 nucleopolyhedrovirus (BmNPV) displaying recombinant protein in silkworm larvae  
311 by size exclusion chromatography and its characterization. BMC Biotechnol. 9, 55.

312 Kato, T., Kajikawa, M., Maenaka, K., Park, E.Y. 2010. Silkworm expression system as a  
313 platform technology in life science. Appl. Microbiol. Biotechnol. 85, 459–470.

314 Kato, T., Otsuki, T., Yoshimoto, M., Itagaki, K., Kohsaka, T., Matsumoto, Y., Ike, K., Park,  
315 E.Y. 2015. *Bombyx mori* nucleopolyhedrovirus displaying *Neospora caninum*  
316 antigens as a vaccine candidate against *N. caninum* infection in mice. Mol. Biotechnol.  
317 57, 145–54.

318 Kato, T., Yui, M., Deo, V.K., Park, E.Y. 2015. Development of Rous sarcoma virus-like  
319 particles displaying hCC49 scFv for specific targeted drug delivery to human colon  
320 carcinoma cells. Pharm. Res. 32, 3699–3707.

321 Kato, T., Arai, S., Ichikawa, H., Park, E.Y. 2016. Versatility of chitosan/BmNPV bacmid  
322 DNA nanocomplex as transfection reagent of recombinant protein expression in  
323 silkworm larvae. Biotechnol. Lett. 38, 1449–1457.

324 Latham, T., Galarza, J.M. 2001 Formation of wild-type and chimeric influenza virus-like  
325 particles following simultaneous expression of only four structural proteins. J. Virol.  
326 75, 6154–6165.

327 Lee, J.M., Chung, H.Y., Kim, K.I., Yoo, K.H., Hwang-Bo, J., Chung, I.S., Park, J.H. 2011.  
328 Synthesis of double-layered rotavirus-like particles using internal ribosome entry site  
329 vector system in stably-transformed *Drosophila melanogaster*. Biotechnol. Lett. 33,

330 41-46.

331 Liu, X., Yang, X., Mehboob, A., Hu, X., Yi, Y., Li, Y., Zhang, Z. A construction strategy  
332 for a baculovirus-silkworm multigene expression system and its application for  
333 coexpression of type I and type II interferons. *Microbiologyopen* 9, e979.

334 Ludwig, C., Wagner, R. 2007. Virus-like particles-universal molecular toolboxes. *Curr.*  
335 *Opin. Biotechnol.* 18, 537–45.

336 Masuda, A., Lee, J.M., Miyata, T., Sato, T., Hayashi, S., Hino, M., Morokuma, D.,  
337 Karasaki, N., Mon, H., Kusakabe, T. Purification and characterization of  
338 immunogenic recombinant virus-like particles of porcine circovirus type 2 expressed  
339 in silkworm pupae. *J. Gen. Virol.* 99, 917–926.

340 Mena, J.A., Ramírez, O.T., Palomares, L.A. 2007. Population kinetics during  
341 simultaneous infection of insect cells with two different recombinant baculoviruses  
342 for the production of rotavirus-like particles. *BMC Biotechnol.* 7, 39.

343 Minkner, R., Baba, R., Kurosawa, Y., Suzuki, S., Kato, T., Kobayashi, S., Park, E.Y. 2018.  
344 Purification of human papillomavirus-like particles expressed in silkworm using a  
345 *Bombyx mori* nucleopolyhedrovirus bacmid expression system. *J. Chromatogr. B*  
346 *Analyt. Technol. Biomed. Life Sci.* 1096, 39–47.

347 Motohashi, T., Shimojima, T., Fukagawa, T., Maenaka, K., Park, E.Y. 2005. Efficient  
348 large-scale protein production of larvae and pupae of silkworm nuclear polyhedrosis  
349 virus (BmNPV) bacmid system, *Biochem. Biophys. Res. Commun.*, 326, 564–569.

350 Pelosse, M., Crocker, H., Gorda, B., Lemaire, P., Rauch, J., Berger, I. 2017. MultiBac:  
351 from protein complex structures to synthetic viral nanosystems. *BMC Biol.* 15, 99.

352 Pushko, P., Tumpey, T.M., Bu, F., Knell, J., Robinson, R., Smith, G. 2005. Influenza virus-  
353 like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus



354 induce protective immune responses in BALB/c mice. *Vaccine* 23, 5751–5759.

355 Shanks, M., Lomonosoff, G.P. 2000. Co-expression of the capsid proteins of Cowpea  
356 mosaic virus in insect cells leads to the formation of virus-like particles. *J. Gen. Virol.*  
357 81, 3093–3097.

358 Shoja, Z., Tagliamonte, M., Jalilvand, S., Mollaei-Kandelous, Y., De Stradis, A.,  
359 Tornesello, M.L., Buonaguro, F.M., Buonaguro, L. 2015. Formation of self-assembled  
360 triple-layered rotavirus-like particles (tRLPs) by constitutive co-expression of VP2,  
361 VP6, and VP7 in stably transfected high-five insect cell lines. *J. Med. Virol.* 87, 102–  
362 111.

363 Sokolenko, S., George, S., Wagner, A., Tuladhar, A., Andrich, J.M., Aucoin, M.G. 2012.  
364 Co-expression vs. co-infection using baculovirus expression vectors in insect cell  
365 culture: Benefits and drawbacks. *Biotechnol. Adv.* 30, 766–781.

366 Usami, A., Suzuki, T., Nagaya, H., Kaki, H., Ishiyama, S. 2010. Silkworm as a host of  
367 baculovirus expression. *Curr. Pharm. Biotechnol.* 11, 246–250.

368 Watanabe, S., Iizuka, T., Hatama, S., Kanno, T., Mase, M., Shibahara, T. Production of  
369 highly immunogenic virus-like particles of bovine papillomavirus type 6 in silkworm  
370 pupae. *Vaccine* 35, 5878–5882.

371 Weissmann, F., Petzold, G., VanderLinden, R., Huis In 't Veld, P.J., Brown, N.G., Lampert,  
372 F., Westermann, S., Stark, H., Schulman, B.A., Peters, J.M. 2016. biGBac enables  
373 rapid gene assembly for the expression of large multisubunit protein complexes. *Proc.*  
374 *Natl. Acad. Sci. USA.* 113, E2564–2569.

375 Xu J., Hiramatsu R., Suhaimi H., Kato T., Fujimoto A., Tokiwa T., Ike K., Park EY. 2019.  
376 *Neospora caninum* antigens displaying virus-like particles as a bivalent vaccine  
377 candidate against neosporosis. *Vaccine* 37:6426–6434.

378 Yao, L., Wang, S., Su, S., Yao, N., He, J., Peng, L., Sun, J. Construction of a baculovirus-  
379 silkworm multigene expression system and its application on producing virus-like  
380 particles. PLoS One 7, e32510.

381 Zhang, Z., Yang, J., Barford, D. 2016. Recombinant expression and reconstitution of  
382 multiprotein complexes by the USER cloning method in the insect cell-baculovirus  
383 expression system. Methods 95:13–25.

384 Zeltins A. 2013. Construction and characterization of virus-like particles: a review. Mol.  
385 Biotechnol. 53, 92–107.

386 Zhou, Y., Chen, H., Li, X., Wang, Y., Chen, K., Zhang, S., Meng, X., Lee, E.Y., Lee, M.Y.  
387 2011. Production of recombinant human DNA polymerase delta in a Bombyx mori  
388 bioreactor. PLoS One 6, e22224.

389

390 **Table 1.** Used primers

Name	5'-3'
$\alpha$ -1-F	AACGCTCTATGGTCTAAAGATTTACTCCGGAATATTAATAG
$\beta$ -1-R	AAACGTGCAATAGTATCCAGTTTTAGATTTCACTTATCTGG
$\beta$ -2-F	AAACTGGATACTATTGCACGTTTACTCCGGAATATTAATAG
$\gamma$ -2-R	AAACATCAGGCATCATTAGGTTTTAGATTTCACTTATCTGG
$\gamma$ -3-F	AAACCTAATGATGCCTGATGTTTACTCCGGAATATTAATAG
$\omega$ -3-R	AAACTAAGCTATGTGAACCGTTTTAGATTTCACTTATCTGG
$\omega$ -pFB-F	AAACACTGACATTGACTTGGTTTCCCGGTCCGAAGCGCGCG
$\alpha$ -pFB-R	AAATCTTTAGACCATAGAGCGTTCTATTAATATTCCGGAGT
Bm-ie1 F	CCCGTAACGGACCTTGTGCTT
Bm-ie1 R	TTATCGAGATTTATTTACATACAACAAG

391

392

393 **Figure legends**

394

395 **Fig. 1.** Constructs of recombinant BmNPVs used in this study. P<sub>PH</sub> and SV40 pA indicate  
396 the polyhedrin promoter and simian virus 40 polyadenylation sequence, respectively.  $\alpha$ ,  
397  $\beta$ ,  $\gamma$ ,  $\omega$  indicate linker sequences for Gibson assembly (Weissmann et al., 2016). A,  
398 BmNPV/Gag; B, BmNPV/SAG1; C, BmNPV/SRS2; D, BmNPV/SAG-SRS-Gag; E,  
399 BmNPV/SAG-Gag-SRS.

400

401 **Fig. 2.** Western blot of expressed RSV Gag protein, NcSAG1, and NcSRS2 in silkworm  
402 larvae by the coinfection and single polycistronic strategies. In the case of the coinfection  
403 strategy, BmNPV/Gag, BmNPV/NcSAG1 and BmNPV/NcSRS2 were coinjected into  
404 silkworm larvae at 1:1:1. In the case of the single polycistronic strategy, BmNPV/SAG-  
405 SRS-Gag or BmNPV/SAG-Gag-SRS2 was injected into silkworm larvae. Hemolymph  
406 and fat bodies were collected at 4 days post injection, and each sample was detected with  
407 anti-Gag antibody (A) and anti-DDDDK (B). 1: Mock, 2: BmNPV/SAG-SRS-Gag, 3:  
408 BmNPV/SAG-Gag-SRS, 4, 5: the coinfection strategy (the three recombinant BmNPVs  
409 were mixed). Asterisks show protein bands detected by each antibody and # shows non-  
410 specific bands.

411

412 **Fig. 3.** Sucrose density gradient centrifugation of hemolymph from silkworm larvae  
413 injected with BmNPV/SAG-SRS-Gag (A) or BmNPV/SAG-Gag-SRS2 (B). The  
414 hemolymph was centrifuged on a 25% sucrose cushion at  $122,000 \times g$  and resuspended  
415 in PBS by sonication. The suspension was subjected to 25-60% sucrose density gradient  
416 centrifugation. Each fraction was collected, and each protein was detected by western blot.

417 Asterisks show the bands detected by each antibody in fraction 4 to 7. RSV-LPs were  
418 purified from collected fractions (fraction 4 to 7).

419

420 **Fig. 4.** Purification of NcSAG1 and NcSRS2-displaying RSV-LPs from the fractions of  
421 sucrose density gradient centrifugation. The hemolymph was centrifuged on a 25%  
422 sucrose cushion at  $122,000 \times g$  and suspended in PBS by sonication. The suspension was  
423 subjected to 25–60% sucrose density gradient centrifugation. Fractions containing RSV  
424 Gag protein, NcSAG1 and NcSRS2 were collected, and NcSAG1 and NcSRS2-  
425 displaying RSV-LPs were purified by anti-DDDDK tag purification gel. The elution was  
426 carried out with 0.1 M glycine-HCl buffer (pH 3.0). BmNPV/SAG-SRS-Gag (A) and  
427 BmNPV/SAG-Gag-SRS2 (B) were used for the coexpression of the three recombinant  
428 proteins, and the same volume of hemolymph was used for purification using both  
429 recombinant BmNPV. Asterisks show the bands detected by each antibody in elution  
430 fractions.

431

432 **Fig. 5.** Morphology of NcSAG1 and NcSRS2-displaying RSV-LPs purified by affinity  
433 chromatography. A TEM image (A) and Immuno-TEM (B) of NcSAG1 and NcSRS2-  
434 displaying RSV-LPs. (C) Magnified immuno-TEM image. Arrows indicate secondary  
435 antibody-conjugated gold nanoparticles.











