Plasmid vectors for the construction of recombinant fowlpox viruses carrying multiple vaccine antigens and immunomodulatory molecules

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Construction of Plasmid Vectors for the Construction of Recombinant Fowlpox Viruses (rFPVs)

Plasmid transfer vectors for the insertion of foreign genes into three locations of the fowlpox virus (FPV) genome were constructed using standard techniques of DNA manipulation including PCR mutagenesis, restriction endonuclease digestion, and subcloning (Figure S1). Plasmids are based upon the pBlue® plasmid (Stratagene, La Jolla, CA, USA) for the pAF and pKG series or pUC19 for the pCH34 series.

pAF09. The dominant selection vector pAF09 has been previously described (13). A gene can be inserted immediately downstream of the F5 promoter; an early transcription termination signal, T, should be added immediately downstream of the coding sequence of the gene. In addition, the BglII site can be used for the insertion of a FPV promoter-gene construct into this plasmid. Both sites can be used to allow the simultaneous insertion of two genes of interest into this plasmid and thus into the FPV genome. Amplification and selection of recombinants are based on the coexpression of Escherichia coli xanthine guanine phosphoribosyl transferase gene (Ecogpt) and β-galactosidase (β-gal) gene, respectively.

pAFtd. The transient dominant selection vector pAFtd was constructed from pAF09. The β-gal and Ecogpt markers were removed from pAF09 by BamHI/BglII digestion and religation of the 5365-bp fragment (Figure S1). The β-gal and Ecogpt markers were then reinserted into the resulting plasmid (pAF09del) outside the FPV sequences and within the plasmid backbone. This was achieved by the digestion of pAF09del with Nhel, which has a unique site immediately adjacent to the left-hand FPV genome flanking sequence. Synthetic oligonucleotide linkers Aflink1 and 2 (Table S1) were ligated into the Nhel site to insert BglII and NcoI sites, yielding the plasmid pAFdellink. A 4159-bp fragment containing the BglII and EcoRTIIiII markers was recovered from pAF09 by BamHI/BglII digestion. This fragment was then ligated into the BglII site created by the insertion of the synthetic linker in pAFdellink. The resulting plasmid, pAFtd, allows the insertion of promoter-gene constructs in the intragenic region between thymidine kinase (TK) and the adjacent uncharacterized open reading frame ORF X using transient dominant selection with the unique restriction endonuclease sites of HindIII, PstI, SalI and SmaI available (Figure S1).

pKG10a. The transient dominant selection vector pKG10a was constructed by PCR amplification and cloning of the FPV genome region, F6, F7, and F9 (Table S2; Reference 14) through a series of intermediate plasmid constructs. pKG02, a 863-bp PCR fragment amplified using primer 1 and primer 4 from FPV genomic DNA spanning the 3′ end of F5 and the full lengths of the F6 and F7 genes was cloned into pBlueScript M13+ digested with EcoRI and SalI. During the PCR amplification, 5′ EcoRI and 3′ BglII and SalI restriction endonuclease sites were added. pKG03, a 686-bp PCR fragment amplified using primer 2 and primer 3 from FPV genomic DNA spanning the promoter and the full length of the F9 gene was cloned into pBlueScript M13+ digested with SalI and HindIII. During the PCR amplification, SalI/NcoI sites were added 5′ of the promoter and a HindIII site 3′ of the F9 gene open reading frame for cloning and further manipulation. pKG04 was constructed by cloning the SalI/HindIII fragment encompassing the F9 gene recovered from pKG03 into pKG02, which had been digested with SalI and HindIII. This plasmid contains the FPV genomic regions of F6, F7, and F9 with the unique restriction endonuclease sites of BglII, SalI, and NcoI, separating the F7 open reading frame from the promoter immediately upstream of the F9 gene. To construct pKG05, the HindIII site in pKG04 was removed by HindIII digestion, the KlN° fragment of DNA polymerase I filled in, and religated. pKG08 was...
constructed to facilitate the cloning of the selection markers outside the FPV genome regions by digesting pKG05 with EcoRI and NdeI and religating with oligonucleotides (Table S1, 23259 and 23258) that inserts a BamHI site immediately adjacent to the F5 and F6 genes. To construct pKG09, the β-gal and EcoGpt markers were recovered from pAF09 as a 4159-bp fragment by BamHI/BglII digestion and ligated into pKG08 digested with BamHI. pKG10a, the remaining unique BamHI site in pKG09a, was deleted by BamHI digestion, Klenow fragment of DNA polymerase I filled in, and religated. The unique restriction endonuclease sites for BglII and SalI are available for insertion of promoter-gene constructs in the intragenic region between F7 and F9. The selection markers β-gal and EcoGpt are within the plasmid backbone, external to the FPV regions used for homologous recombination for transient dominant selection insertions into the FPV genome.

pCH34. The transient dominant selection vector pCH34 was constructed by PCR amplification and cloning of the FPV genome region, M-1, M-1 3′, and M-2 3′, which flanks the site of naturally occurring insertion of the reticuloendotheliosis provirus (REV) into

![Figure S1. Dominant and transient dominant insertion vectors.](image_url)
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Table S2. Genome Locations of Fowlpox Virus (FPV) Sequences Used in the Plasmid Vectors

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Flanking Sequences</th>
<th>Original References</th>
<th>FPV Genome Locations as per Full Genome Sequence</th>
<th>FPV Genes Flanking Insertion Sites</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>pAF09</td>
<td>I6, I5, TK, X, I3</td>
<td>Boyle et al. a, 13, Binns et al. b</td>
<td>86637–88823</td>
<td>FPV087–FPV088</td>
<td>21</td>
</tr>
<tr>
<td>pAFtd</td>
<td>I6, I5, TK, X, I3</td>
<td>Boyle et al. a, 13, Binns et al. b</td>
<td>86637–88823</td>
<td>FPV087–FPV088</td>
<td>21</td>
</tr>
<tr>
<td>pCH34</td>
<td>M-1, M-1 3′, M-2 3′</td>
<td>15</td>
<td>231691–234277</td>
<td>FPV201–FPV203</td>
<td>21</td>
</tr>
<tr>
<td>pKG10a</td>
<td>F6, F7, F9</td>
<td>14</td>
<td>164133–165651</td>
<td>FPV133–FPV134</td>
<td>21</td>
</tr>
</tbody>
</table>


cSequence numbers and gene locations are as per the fully sequenced pathogenic FPV challenge strain (GenBank® NC_002188). The full genome of FPV M3 has not been sequenced.

the FPV genome (Table S2; Reference 15). A series of intermediate plasmid constructs were used to manipulate the FPV sequences. pCH19 was generated by cloning EcoRI fragments of the FPV M3 genome into pUC19 digested with EcoRI. A plasmid containing an 8.5-kb FPV genome fragment flanking the REV long terminal repeat (LTR; Reference 15) was identified by hybridization with REV LTR sequences. pCH21 was generated by BamHI digestion of pCH19 and religation of the plasmid fragment carrying a 2.5-kb (EcoRI/BamHI) fragment of the FPV genome containing the REV LTR. pCH29 was derived from pCH21 by digestion with SacI, which is within the REV LTR sequence, and PCR amplification of the whole plasmid with primers 33103 and 33104 (Table S1), designed to delete the REV LTR sequences and to insert a unique BclI site at the location of the deleted REV LTR. The BclI-digested PCR product was self-ligated to generate pCH29. pCH30 was generated by BamHI digestion of pCH29 (the BamHI site within the multiple cloning site originating from pUC19) and ligation with the β-gal and Ecogpt markers from pAF09 by BamHI/BglII digestion as a 4159-bp fragment. pCH33 was generated from pCH30 by BamHI/HindIII digestion, Klenow fragment of DNA polymerase I filled in, and religated. This manipulation provided the ability to insert additional restriction endonuclease sites at the REV LTR site to improve the utility of the plasmid vector. pCH34 was generated by a three fragment ligation of (i) a NcoI/ScaI fragment encompassing the majority of the plasmid backbone and selection markers from pCH33, (ii) a NeoI/BclI fragment amplified by PCR, and (iii) a NeoI/ScaI fragment amplified by PCR. The two PCR products were generated using plasmid pCH33 as a template and two primer pairs (pCH33No2 and pCH32DBB1, and pCH33No1 and pCH32DB2) as per Table S1. Unique BamHI and XhoI restriction endonuclease sites were inserted in the intragenic region between M-1 and M-1 3′, and the REV genome LTR was deleted during the manipulation and cloning. The selection markers of β-galactosidase and Ecogpt are within the plasmid backbone external to the FPV regions used for homologous recombination for transient dominant selection insertions into the FPV genome. Full deduced nucleotide sequences are available on request for the final vectors pAF09, pAFtd, pKG10a, and pCH34.