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(57) Abrégé/Abstract:
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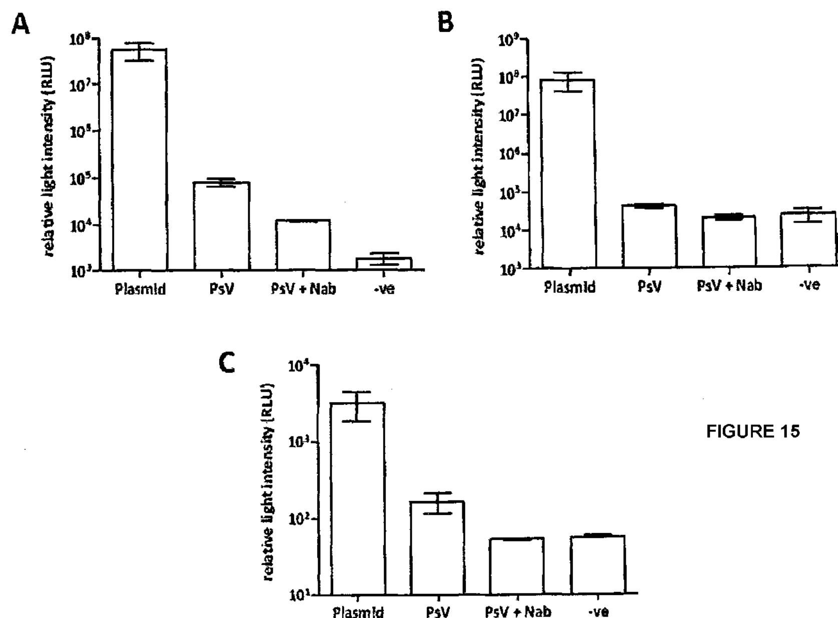

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PLANT PRODUCED HUMAN PAPILLOMAVIRUS PSEUDOVIRION

BACKGROUND OF THE INVENTION

The present invention relates to a method of producing human papillomavirus (HPV) pseudovirions in plant cells, the plant produced pseudovirions per se, a neutralisation assay using the plant produced pseudovirions and pharmaceutical compositions comprising the plant produced pseudovirions.

The applicants have use novel autonomously replicating vectors in conjunction with previously developed, non-replicating vectors to produce HPV-16 pseudovirions (PsVs) *in planta*. Preliminary expression trials established optimal conditions and timeframes for production of each individual element required for assembly of HPV PsVs. The structural elements required for PsV production are HPV L1 and L2 proteins, produced by non-replicating plant expression vectors pTRAc-hL1 and pTRAc-hL2, respectively; and circular double-stranded DNA from one of three replicons derived from pRIC3-mSEAP, pRIC3-mSEAP+ or pRIC3-mluc+. Putative PsV particles, as well as L1/L2 virus-like particles (VLPs) produced in absence of the replicons, were harvested from plants and purified by successive gradient ultracentrifugation steps. Gradient fractions containing L1 were pooled and dialysed against high-salt (0.5M) NaCl PBS, to obtain purified PsVs. These were confirmed by electron microscopy to be conformationally similar to VLPs and PsVs produced in other systems, and by PCR to contain the corresponding encapsidated replicon DNA. Purified PsVs were used to demonstrate their use in a neutralisation assay. Two of the three PsVs created, namely mSEAP and mluc+ PsVs, demonstrated successful pseudoinfection and neutralisation with a common HPV16 neutralising antibody, while mSEAP+ PsVs showed no reporter gene expression after pseudoinfection of mammalian cells. This is the first known report of the production and purification of HPV PsVs, as well as L1/L2 VLPs *in planta*, as well as the first demonstration of a pseudovirion-based neutralisation assay (PBNA) using plant-produced PsVs.

Cervical cancers caused by high-risk HPV are the second most prevalent form of cancer in women in developing countries. Africa in particular has been identified as a high risk region for the disease. Recently developed L1 VLP vaccines,

Cervarix® and Gardasil™, protect against HPV-16 and HPV-18, or HPV-6, HPV-11, HPV-16 and HPV-18 infection, respectively. Both currently available vaccines, Cervarix® and Gardasil™, elicit a strong and protracted neutralising antibody response, and have been shown to have sustained efficacy up to 5 years post-administration. While these vaccines have shown great promise in reducing the burden of disease, development and production of VLP vaccines remains prohibitively expensive, particularly in developing countries.

A key element of any HPV vaccine development initiative is the pseudovirion-based neutralisation assay (PBNA). Induction of neutralising antibodies is currently the best estimate of vaccine candidate efficacy for second generation HPV vaccine testing. Until recently, the identification of serum neutralising antibodies relied on the use of enzyme-linked immunosorbent assay (ELISA) or neutralisation assays using whole virus (Dessy et al., 2008). However, improvements in HPV PsV production efficiency in the last decade have allowed the development of the PBNA. Developed by John Schiller's group at the Center for Cancer Research, this assay uses mammalian cells for intracellular production of PsVs expressing a secreted alkaline phosphatase (SEAP) reporter gene (Buck et al., 2005a), and has since become the gold standard for testing neutralisation of candidate HPV vaccines, allowing rapid and un-biased screening of neutralising antibodies and epitopes (Stanley et al., 2008). While this production method has been shown to be extremely effective for production of PsVs, cell culture production is expensive, and SEAP assay kits are particularly expensive in comparison to other commonly used reporter assays such as luciferase or GFP. There is a need, therefore, to develop alternative PsV production methods to allow for affordable candidate vaccine development and, in particular, inexpensive testing of immune sera.

The production of neutralising IgG antibodies in response to vaccination has long been understood to be a key aspect of protective immunity (Robbins et al., 1995). It has been suggested that it may be possible to accurately estimate the required level of neutralising antibody required for protection, provided that the concentration, isotype and secondary biological activity of these antibodies could be accurately measured (Robbins et al., 1995). Neutralisation assays were developed as a method of accurately quantifying the neutralising capabilities of immune sera,

usually in response to a live viral or vaccine candidate challenge, as well as identify neutralising epitopes (Ochsenbauer and Kappes, 2009; Yeager et al., 2000).

The first demonstration of *in vitro* neutralisation of papillomavirus was by Dvoretzky et al. (1980), who demonstrated neutralisation with rabbit-produced Bovine papillomavirus type 1 (BPV-1) antisera to confirm the role of BPV-1 in focus formation in mouse cell lines. Early efforts to establish a robust, sensitive *in vitro* neutralisation assay for HPVs were hampered by difficulties in production of infectious virus. Production of infectious virions *in vitro* was first achieved by grafting HPV-11-infected material into athymic mice – grafts were left to develop into condylomatous cysts over a period of 3-5 months, before being harvested and purified for HPV virions (Kreider et al., 1987). This method was utilised to produce virions for use in the first *de facto* neutralisation assay. Neutralising monoclonal antibodies were identified and isolated from HPV-11 or BPV-1 antisera. These antibodies were then used to demonstrate neutralisation of intact virions by ELISA, as well as identifying several neutralising conformational epitopes (Christensen et al., 1990). The same group used this method to successfully identify neutralising HPV antibodies in human sera for the first time, and further demonstrated that ELISA was a good indicator of the presence of neutralising antibodies in human sera (Christensen et al., 1992). Another approach coupled the neutralisation of HPV-11 infection with RT-PCR detection of HPV mRNA transcripts to create a semi-quantitative neutralisation assay (Smith et al., 1995). While these approaches were nominally successful in identification of neutralising antibodies, detection remained limited at best, and the procedures used were time-consuming and expensive.

A major step forward in neutralisation assay technology came with the advent of PsV production, which abrogated the need for the expensive and time-consuming xenograft production method. Roden et al. (1996) used hamster BPHE-1 cells to generate BPV-1 or HPV-16 PsVs. These were used to demonstrate focus formation in C127 cells, using the technique demonstrated by Dvoretzky et al. (1980). These researchers further showed that neutralising antibodies in HPV-16 antisera prevented focus formation, demonstrating a quantitative neutralisation assay of a high-risk HPV type using PsVs for the first time (Roden et al., 1996). In this report, the authors noted that the focus transformation assay required 2-3 weeks, and that inclusion of a marker or reporter gene would greatly improve the speed of the assay. This was first

attempted by chemically linking a β-lactamase (BLAM) reporter plasmid to VLPs or infectious virions, and incubating these with PV antisera before infecting various mammalian cell lines. Early attempts demonstrated neutralisation, but resulted in <1% infection of cells with these PsVs (Muller et al., 1995). Yeager et al. (2000) and Bousarghin et al. (2002) demonstrated this approach more successfully, using a BLAM or luc reporter plasmids and an alternative method of attaching the plasmid to VLPs. More importantly, several groups generated PsVs with encapsidated reporter genes, and demonstrated their use for neutralisation assays (Buck et al., 2005a; Fleury et al., 2008; Kawana et al., 1998; Rossi et al., 2000; Stauffer et al., 1998; Touze and Coursaget, 1998; Unckell et al., 1997). While early attempts were inefficient due to poor PsV production levels, this was improved upon by intracellular generation of high yields of L1/L2 PsVs and incorporation of a SEAP reporter plasmid (Buck et al., 2004). These PsVs were used with a commercially available SEAP detection kit to demonstrate a pseudovirion-based neutralisation assay that was at least as sensitive as, and potentially more type-specific than, the standard ELISA-based neutralisation assay (Pastrana et al., 2004).

While the system developed by Pastrana et al. (2004) is considered the current 'best practice' neutralisation assay, there remains room for improvement. In particular, the costs of PsV production could be greatly decreased by the use of a less expensive production system (Brondyk, 2009). Recombinant protein expression in plants has been demonstrated to have a significantly lower cost of production when compared to production in mammalian cells (Tiwari et al., 2009). As such, plant expression may provide an attractive alternative for the production of PsVs for use in the PBNA.

Expression of recombinant proteins in plants has developed over the last twenty years from a curiosity in the late 1980s to a medically and industrially relevant production system today. Early efforts relied on transformation of plants to produce stable transgenic lines. This was achieved through biolistic delivery or, more recently, agroinfiltration (Daniell et al., 2009). While transgenic protein production remains a useful and viable system, advances in transient expression methods and technology have positioned transient expression as the preferred method for industrial-scale production in plants (Rybicki, 2010). Two key factors that have played a central role

in this transition are viral, or virus-derived, expression vectors, and the development of agroinfiltration technology.

Agroinfiltration was originally developed to as an alternative to biolistic bombardment for the stable transformation of plants (Kapila et al., 1997). This process relies on the DNA transfer capability of *A. tumefaciens* to introduce foreign DNA to plant cells. *A. tumefaciens* can be used to transfer a transgene located in the transfer DNA (T-DNA) segment of the Ti plasmid into plants infiltrated with a bacterial suspension of the transformed bacterium. The T-DNA is transported to the plant nucleus, and this allows for transformation of the plant through integration of the T-DNA into the plant genome (Zupan et al., 2000). Importantly, however, a transgene incorporated into the T-DNA may also be transiently expressed, from non-integrated or episomal T-DNA, resulting in systemic expression of a recombinant protein without the need for stable transformation (Kapila et al., 1997).

Viral vectors were the first transient expression method developed for plants. Early efforts simply inserted a recombinant gene or epitope into the genome of viruses such as TMV, cowpea mosaic virus (CPMV), or PVX, either fused to the viral coat protein or separately, under control of a duplicated subgenomic viral promoter (Durrani et al., 1998; Gleba et al., 2007; Turpen et al., 1995). While this application produced immunogenic protein, expression levels were lower than those found in transgenic plants. Other problems with these 'first-generation' viral vectors included a tendency to revert to the natural virus, constraints on insert size, difficulty of administration, and an inability to form VLPs (Kohl et al., 2006; Rybicki, 2010; Varsani et al., 2006).

These limitations prompted further work to develop 'second generation', or deconstructed, viral vectors. This approach used only the desirable viral elements, in particular the replicative machinery, to manufacture synthetic vectors capable of inducing transgene expression in plants. While these vectors are usually not infectious on their own, when coupled with agroinfiltration technology they can result in systemic transient expression of protein at levels comparable to that of transgenic plants (Tiwari et al., 2009). This approach has the advantages of short time frames (3-7 days) when compared to stable transformation (6-9 months), significant expression levels, and rapid and easy scale-up and purification. This makes

agroinfiltration-mediated transient expression via viral vectors an ideal approach for the production of medically relevant proteins and particles in plants. Of particular interest is the use of transient expression for the production of VLPs and PsVs in plants, as there is potential for a reduction in cost when compared to traditional systems (Santi et al., 2006).

Papillomavirus L1 VLPs have been produced by several groups in plants. Most have used transgenic plants (Biemelt et al., 2003; Warzecha et al., 2003) with resulting low yields. Early attempts at transient expression of L1 also yielded low levels of expression, as well as an apparent inability to form VLPs (Varsani et al., 2006). However, agroinfiltration of an *Agrobacterium* vector coding for a human codon-optimised L1 protein provided a much higher protein yield, and demonstrated that transient expression of HPV-16 VLPs at high levels is a feasible approach for the production of immunogenic HPV candidate vaccines (Maclean et al., 2007).

The vector used to produce L1 at such high expression levels – pTRAc – was developed at the Fraunhofer Institute for Molecular Biology and Applied Biology. This vector utilises a CaMV 35S promoter with duplicated transcriptional enhancer, chalcone synthase 5'-untranslated region, and CaMV 35S polyadenylation signal for foreign gene expression. This vector has also been used to express minor capsid protein L2 in plants (Pereira, 2008). However, coexpression of L1 and L2 has not previously been conclusively demonstrated to form VLPs *in planta*.

A further development in vector technology has been the use of single-stranded DNA plant geminiviruses in the genus *Mastrevirus*, family *Geminiviridae*, to create replicating vectors. These replicating vectors incorporate a viral Ori (origin of replication) sequence that is duplicated on either side of a gene expression cassette. The replicating vectors further may or may not include a viral replication-associated protein (*Rep*) gene. Agroinfiltration of a single *Rep*-containing replicon construct, or of a replicon construct plus a *Rep* construct expressed in trans by standard techniques, results in release of a plasmid-like "replicon" which multiplies under the control of Rep protein up to copy numbers of several thousand per cell (Regnard et al., 2010). This can result in significantly increased expression of genes of interest compared to non-replicating vector expression. While the expression of a geminivirus *Rep* gene and cognate (eg: Ori sequence from the same virus) replicon construct in a

plant cell leads to replication of the replicon, this is not known to occur in mammalian cells.

Encapsidation or covalent attachment of DNA by HPV VLPs to form PsVs has been demonstrated in yeast, insect, bacterial and mammalian cell systems (Buck et al., 2005a; Roden et al., 1996; Rossi et al., 2000; Unckell et al., 1997). Buck et al. (2005a) demonstrated that intracellular encapsidation of the pseudogenome is more efficient than *in vitro* disassembly-reassembly methods for the production of HPV PsVs, probably due to cellular factors that assist in correct assembly of the virions (Buck et al., 2008; Fleury et al., 2008; Peng et al., 2011). Currently, HPV pseudovirions have not been successfully expressed in plant expression systems. As discussed above, transient expression in plants offers several significant advantages for this application: protein expression in plants has been shown to be safe, cheaper than other expression systems, and potentially extremely rapid (Ma et al., 2005; Schillberg et al., 2005). A further significant advantage is that there is no need for downstream processing of proteins (e.g. glycosylation), as for bacterial recombinant protein expression systems (Giorgi et al., 2010). While it has been noted that N-glycosylation may differ in plants (specifically, plants cannot synthesise β-1,4-galactose and sialic acid), this problem can be overcome by recent advances in transgenic tobacco to provide 'humanised' glycosylation machinery (Bakker et al., 2006; Gleba et al., 2007). Further, it has been suggested that glycosylated L1 or L2 are not an important part of the assembled virion (Zhou et al., 1993).

In this application the inventors evaluated the feasibility of expressing HPV L1/L2 pseudovirions with an encapsidated mammalian reporter cassette, derived from a replicating geminivirus-derived vector, *in planta*. To achieve this, pTRAc plasmids expressing L1 and L2 proteins were co-infiltrated into plants with novel autonomously replicating plasmids, developed in this study, to create HPV L1/L2 PsVs. Further, we purified these particles by density-based centrifugation, for subsequent testing in a mammalian system.

This invention describes, for the first time, the successful production of HPV PsVs in plants, and testing of the PsVs in a standard PBNA. HPV L1/L2 VLPs, as well as PsVs containing a mammalian reporter cassette pseudogenome derived from the geminivirus Bean yellow dwarf virus (BeYDV), were produced in large quantities

in planta. The particles readily encapsidated the pseudogenome DNA provided by the replicating vectors. Further, they were easily purified, stable at high temperature, and were conformationally indistinguishable from PsVs produced in other systems. Most importantly, they were successfully used to perform a PBNA in mammalian cells. Transient plant-based production of HPV PsVs is a feasible strategy, and should be further investigated as a low-cost alternative to mammalian cell culture for PsV production.

SUMMARY OF THE INVENTION

The present invention provides pseudovirions produced in plant cells, methods for producing the pseudovirions in plants, a neutralisation assay using the plant produced pseudovirions and pharmaceutical compositions comprising the plant produced pseduovirions.

The present invention teaches that transient co-expression of HPV L1 and HPV L2 in a plant cell together with concurrent replication, to high copy number, of a ssDNA virus-derived replicon containing a gene encoding a heterologous polypeptide of interest, results in the HPV L1 and HPV L2 assembling into virus-like particles encapsidating the replicon to form pseudovirions. Further, the pseudovirions and method for producing them described in this invention provide a significant advancement for the potential production of vaccines and DNA delivery vehicles for use in gene therapy.

According to a first aspect of the invention there is provided for a method for producing a human papillomavirus (HPV) pseudovirion in a plant cell. The method comprises a step of introducing into the plant cell a first nucleic acid encoding a HPV L1 polypeptide and a second nucleic acid encoding a HPV L2 polypeptide, wherein the first and second nucleic acids are contained on at least one expression vector. The method further comprises a step of introducing a replicating vector comprising a third nucleic acid encoding a heterologous polypeptide into the plant cell. The HPV L1 polypeptide and HPV L2 polypeptide are consequently expressed in the plant cell, and the replicating vector is replicated, in order to produce a high copy number of the replicating vector in the plant cell. The expressed HPV L1 and HPV L2 polypeptides

subsequently assemble, together with a copy of the replicating vector, and encapsidate the replicating vector to produce a HPV pseudovirion.

It will be appreciated that the first and second nucleic acids are operably linked to regulatory sequences that allow for expression of the HPV L1 and HPV L2 polypeptides.

In one embodiment of the invention replication of the replicating vector is initiated by a regulatory protein. It will be appreciated that the regulatory protein will be encoded by a fourth nucleic acid which is operably linked to regulatory sequences, which allows for the expression of the regulatory protein. It will further be appreciated that the fourth nucleic acid may be expressed from either (i) a nucleic acid sequence contained on the replicating vector, (ii) a nucleic acid sequence contained on the at least one expression vector, (iii) a nucleic acid sequence contained on an independent vector, not being the vector of (i) or (ii) above; or (iv) a nucleic acid sequence integrated into the genomic DNA of the plant cell. Preferably, expression of the regulatory protein in the presence of the replicating vector initiates replication of the replicating vector.

In another embodiment of the invention it will be appreciated that the third nucleic acid sequence is operably linked to a regulatory sequence which allows for expression of the heterologous polypeptide in a mammalian cell.

In a preferred embodiment of the invention the third nucleic acid encoding the heterologous polypeptide, comprises a gene selected from the group consisting of a reporter gene, a therapeutic gene or a gene encoding an antigenic polypeptide, such as a gene encoding HPV E6 or E7 oncoprotein-derived constructs for treating cervical lesions or carcinomas caused by HPVs. Preferably, the gene encoding the heterologous polypeptide is a reporter gene selected from a luciferase gene or a secreted alkaline phosphatase gene.

In a further embodiment of the invention the polynucleotides encoding the HPV L1 and HPV L2 polypeptides are from HPV 16. It will however be appreciated by a person skilled in the art that the present invention will work just as effectively for a HPV type for which virus like particles can be produced, including but not limited to

HPV 6, HPV 11, HPV 18, HPV 31, HPV 33, HPV 45, HPV 48, HPV 52, and/or HPV 58, or combinations thereof.

In yet another embodiment of the invention the method comprises a step of recovering the HPV pseudovirion from the plant cell.

According to a second aspect of the invention, there is provided for an assay for detecting the presence of a neutralising antibody to HPV in a subject. The assay including the steps of producing a first sample by combining the HPV pseudovirion produced according to the method above, with a biological sample from the subject to form a biological sample composition, wherein the heterologous polypeptide is a reporter polypeptide. Producing a second sample by combining the HPV pseudovirion produced according to the method above, with a control sample, wherein the control sample does not contain a HPV neutralising antibody, in order to form a control sample composition, wherein the heterologous polypeptide is a reporter polypeptide. The assay further comprising the step of contacting and incubating a mammalian cell capable of being infected with HPV with the first (biological) sample composition or with the second (control) sample composition and thereafter assaying the expression of the reporter polypeptide, wherein a decreased expression of the reporter polypeptide in the mammalian cells contacted with the first sample composition, as compared to mammalian cells contacted with the second sample composition is indicative of the presence of a HPV neutralising antibody in the biological sample.

Preferably, the reporter polypeptide used in the assay is selected from either a luciferase (luc) or a secreted alkaline phosphatase (SEAP) polypeptide.

More preferably, the assay is performed on a biological sample from a human subject.

A third aspect of the invention provides for a HPV pseudovirion comprising a capsid, wherein the capsid comprises a HPV L1 and a HPV L2 polypeptide, wherein the capsid encapsidates a replicating vector that encodes a heterologous polypeptide. Wherein, the heterologous polypeptide is operably linked to a regulatory

sequence that allows for its expression in a mammalian cell, and further, wherein the HPV pseudovirion is produced in and recovered from a plant cell.

In a preferred embodiment of the invention replication of the replicating vector is initiated, in a mammalian cell infected by the HPV pseudovirion, in the presence of a regulatory protein. Preferably, the regulatory protein is encoded by a nucleic acid sequence operably linked to a regulatory sequence that allows for the expression of the regulatory protein in the mammalian cell. It will be appreciated by those skilled in the art that the regulatory protein may be expressed from a nucleic acid sequence contained on the replicating vector, a nucleic acid sequence contained on an independent vector; or from a nucleic acid sequence integrated into the genomic DNA of the mammalian cell. It will further be appreciated that expression of the regulatory protein in the mammalian cell in the presence of the replicating vector will result in the replication of the replicating vector.

Preferably, the heterologous polypeptide of this embodiment of the invention is selected from the group consisting of a reporter polypeptide, a therapeutic polypeptide or an antigenic polypeptide, such as a gene encoding HPV E6 or E7 oncoprotein-derived constructs for treating cervical lesions or carcinomas caused by HPVs.

According to a fourth aspect of the present invention there is provided for a pharmaceutical composition comprising a human papillomavirus pseudovirion produced by the method described above or containing the human papillomavirus pseudovirion described above and a pharmaceutically acceptable carrier or adjuvant. It will be appreciated that the pharmaceutical composition may be a vaccine composition or a DNA delivery vehicle.

BRIEF DESCRIPTION OF THE FIGURES

Non-limiting embodiments of the invention will now be described by way of example only and with reference to the following figures:

Figure 1: 2nd generation vector pRIC3 and replicon. ColE1 ori, origin of replication for *Escherichia coli*; RK2 ori, origin of replication for *Agrobacterium*

tumefaciens; bla, ampicillin/carbenicillin resistance bla gene; LB and RB, left and right borders for T-DNA integration; P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5'-untranslated region; MCS, multiple cloning site, pA35S, CaMV 35S polyadenylation signal; LIR, BeYDV long intergenic region; SIR, BeYDV short intergenic region; rep, BeYDV rep gene. The curved bar inside the plasmid maps indicates the T-DNA transferred into the plant cell during transfection.

Figure 2: Construction of pRIC3-mSEAP and replicon. Autonomously replicating plasmid pRIC3-mSEAP. (A) Final cloning steps to create pRIC3-mSEAP. (B) EF-1α, elongation factor 1 alpha promoter; SEAP, Secreted Alkaline Phosphatase gene; SV40 PolyA, simian virus 40 polyadenylation signal. The curved bar inside plasmid map indicates T-DNA transfected into plant cells. (C) Circularised replicon after release from T-DNA.

Figure 3: Construction of pRIC3-mSEAP+ and replicon. pRIC3-mSEAP+ autonomously replicating plasmid. (A) Final cloning steps in construction of pRIC3-mSEAP+. (B) EF-1α, elongation factor 1 alpha promoter; SEAP, Secreted Alkaline Phosphatase gene; SV40 PolyA, simian virus 40 polyadenylation signal; CaMV 35S, cauliflower mosaic virus promoter region, EGFP, enhanced green fluorescent protein gene; pA35SS, CaMV 35S polyadenylation signal. The curved bar inside plasmid map indicates T-DNA transfected into plant cells. (C) Circularised replicon after release from T-DNA.

Figure 4: Construction of pRIC3-mluc+ and replicon. Autonomously replicating plasmid pRIC3-mluc+. (A) Final cloning steps to create pRIC3-mluc+. (B)CMV I/E/P + pCapR, cytomegalovirus intron/enhancer/promoter region with pCapR enhancer; luc, firefly luciferase reporter gene; BGH polyA, bovine growth hormone polyadenylation signal. CaMV 35S, cauliflower mosaic virus promoter region, EGFP, enhanced green fluorescent protein; pA35SS, CaMV 35S polyadenylation signal. The curved bar inside plasmid map indicates T-DNA transfected into plant cells. (C) Circularised replicon after release from T-DNA.

Figure 5: *Agrobacterium* expression vector pTRAc. P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5' untranslated region; pA35S, CaMV 35S polyadenylation signal; SAR, scaffold attachment region of the tobacco Rb7 gene; LB and RB, the left and right borders for T-DNA integration; ColE1ori, origin of replication for *E. coli*; RK2ori, origin of replication for *Agrobacterium*; bla, ampicillin/carbenicillin-resistance gene.

Figure 6: Principle of Inverse PCR. Diagram illustrating the principle of inverse PCR. Primers (→) designed to amplify only recircularised replicon DNA, but not the parent vector. Vector recircularised at the duplicated LIR.

Figure 7: PCR amplification of (A) pRIC3-mSEAP and pRIC3-mSEAP+ and (B) pRIC3-mluc+ replicons shows replicational release of T-DNA. MW, molecular weight marker; mSEAP, pRIC3-mSEAP; mSEAP+, pRIC3-mSEAP+, mluc+, pRIC3-mluc+, plant, uninfiltrated plant DNA (negative control).

Figure 8: Optimisation of L1 and L2 expression. Time trial of (A) hL1 and (B) hL2 expression levels at various infiltration ODs, at 1-7 and 1-5 dpi, respectively. Protein is indicated (→) at 55kDa (hL1) and approximately 65kDa (hL2). MW, molecular weight marker, with sizes indicated in kDa; +, crude plant-produced hL1 (A) and bacterially-produced hL2 (B)

Figure 9: qPCR time trial of replicating vectors. qPCR analysis of DNA extracted from plants infiltrated with (A) pRIC3-mSEAP, (B) pRIC3-mSEAP+, and (C) pRIC3-mluc+, 1-7dpi. Copy number is shown as log10 scale. Error bars indicate standard error of the mean (N=3)

Figure 10: Expression of PsV structural elements. PCR was used to confirm presence of (A) pRIC3-mSEAP, pRIC3-mSEAP+, and (B) pRIC3-mluc+ replicons 3dpi, in plants co-infiltrated with pTRAc-hL1, pTRAc-hL2 and pRIC3-mSEAP, -mSEAP+ or -mluc+, respectively. A band at approximately 2.1 Kbp (→) indicates replicational release. MW, molecular weight marker, sizes shown on left; PsV, crude plant extract; +, DNA only (positive control); -, plant DNA (negative control). Western blotting for L1 (C) and L2 (D) confirm presence of both structural HPV proteins 4dpi in crude extract from plants co-infiltrated with pTRAc-hL1, pTRAc-hL2 and pRIC3-mSEAP, -mSEAP+ or -mluc+. VLP, pTRAc-hL1 and –hL2 only; mSEAP, pRIC3-mSEAP PsV, mSEAP+, pRIC3-mSEAP+ PsV; mluc+. pRIC3-mluc+ PsV; +, crude plant-produced hL1 (C) or bacterially produced L2 (D); MW, molecular weight marker, sizes indicated in kDa.

Figure 11: Caesium chloride gradient ultracentrifugation of VLPs and PsVs. Densitometry analysis of L1 dot blots shows distribution of L1 after Caesium chloride ultracentrifugation and fractionation. Dot Blots (left panels) of CsCl fractions 1-19 were probed with CamVir-1 anti-L1 antibody. * on dot blots, and grey panel on graphs, indicate fractions pooled for dialysis; +, crude plant extract (positive control). Density of CsCl fractions (♦) was compared to relative intensity (□, arbitrary units) of

L1 (right panels) to estimate density of purified particles. (A) L1/L2 VLPs, (B) pRIC3-mSEAP, (C) pRIC3-mSEAP+, (D) pRIC3-mluc+.

Figure 12: Purification of PsVs. Coomassie staining (A, B) and western blot for L1 (C, D) of various stages of purification of PsVs, separated by SDS-PAGE. Western blots were probed for L1 using the commercial anti-L1 antibody CamVir-1. MW, molecular weight marker, sizes shown on right; C, crude plant extract; CsCl, pooled caesium chloride gradient fractions; dial., dialysed pooled fractions, +, crude hL1 extract (positive control).

Figure 13: Electron micrographs of purified PsVs. (A) and (E) pRIC3-mSEAP, (B) and (F) pRIC3-mSEAP+, (C) and (G) pRIC3-mluc+ PsVs (previous page) and (D) and (H) L1/L2 VLPs were purified by CsCl gradient ultracentrifugation. Purified PsVs size varied from 30-120 nm in diameter. White arrows indicate small (30-40 nm) particles, Grey arrows indicate standard-sized HPV PsV particles (50-60 nm), and black arrows indicate large (100-120 nm) particles. (I) Crude plant extract serves as negative control. Scale bars are indicated (left panel, 0.5 µm; right panel and (D), 200 nm, (I), 100 nm).

Figure 14: Presence of DNA in purified PsVs. PCR amplification of (A) pRIC3-mSEAP, pRIC3-mSEAP+, and (B) pRIC3-mluc+ replicon (2.1 Kbp, →) indicates presence of DNA in purified PsV particles after digestion with proteinase K (PrK). MW, molecular weight marker; PrK +/-, PrK digestion; PsV +, pooled L1-containing fractions, PsV -, non-L1 containing fraction (fraction 18); DNA, replicon DNA extract (positive control), V, PrK-digested purified L1/L2 VLPs (negative control); P, plant DNA (negative control).

Figure 15: Reporter gene expression in PsV-pseudoinfected mammalian cells. (A) SEAP expression 72 hours post pRIC3-mSEAP transfection (Plasmid) or mSEAP-PsV pseudoinfection (PsV) in relative light units (RLU), expressed in logarithmic scale (log10). (B) SEAP expression for pRIC3-mSEAP+, as previously. (C) luc expression for pRIC3-mluc+, as previously. –ve, negative control (media only). All experiments performed in triplicate. Error bars show standard deviation between triplicates.

Figure 16: mSEAP mammalian expression cassette (SEQ ID NO: 8).

Figure 17: mSEAP+ cassette: expression cassette comprising a SEAP mammalian cassette and EGFP plant cassette (SEQ ID NO: 9).

Figure 18: mluc+ cassette: expression cassette comprising a mluc mammalian cassette and EGFP plant cassette (SEQ ID NO: 10).

Figure 19: Cloning strategy for insertion of the SV40 origin of replication (SV40ori) into the pRIC3-mSEAP and pRIC3-mSEAP+ vectors.

Figure 20: SEAP expression in mammalian cells infected with mSEAP+ or mSEAP+ SV40ori PsVs. Negative control is non-infected cells. SEAP values are in relative light units (RLU) and pseudovirion dilutions are given on X-axis.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the invention are shown.

The invention as described should not to be limited to the specific embodiments disclosed and modifications and other embodiments are intended to be included within the scope of the invention. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

As used throughout this specification and in the claims which follow, the singular forms "a", "an" and "the" include the plural form, unless the context clearly indicates otherwise.

The terminology and phraseology used herein is for the purpose of description and should not be regarded as limiting. The use of the terms "comprising", "containing", "having" and "including" and variations thereof used herein, are meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Provided herein is a method for producing a human papillomavirus (HPV) pseudovirion in a plant cell. "Papillomaviruses" are DNA viruses from the family *Papillomaviridae* that infect the skin and mucous membranes of animals, preferably mammals, and even more preferably humans. A "VLP" or "virus-like particle" refers to the capsid-like structure which results from the assembly of the HPV L1 protein alone, or with the HPV L2 capsid protein. These structures are antigenically and

morphologically similar to actual HPV virus particles or virions. Virus-like particles do not include viral genetic material; accordingly, these particles are not infectious.

The term "pseudovirion" or "PsV" refers to a papillomavirus virus-like particle including the papillomavirus capsid proteins in which a plasmid or vector containing a heterologous gene of interest has been encapsidated. The pseudovirions of the invention contain non-native genetic material which can be transferred by the virus to an animal cell, preferably a mammalian cell, and most preferably to a human cell. The non-native genetic material may include a plasmid encoding a therapeutic gene, reporter gene, a gene encoding an antigenic polypeptide, such as a gene encoding HPV E6 or E7 oncoprotein-derived constructs for treating cervical lesions or carcinomas caused by HPVs, and/or any other heterologous gene of interest under the control of a mammalian promoter, which can be delivered to a mammalian cell by the pseudovirion. In this specification "encapsidated" refers to the plasmid or vector being enclosed within the capsid of the virus-like particle.

The term "protein" should be read to include "peptide" and "polypeptide" and *vice versa.*

The method of the invention includes the steps of introducing a first polynucleotide encoding an HPV L1 polypeptide and a second polynucleotide encoding an HPV L2 polypeptide into a plant cell. It will be appreciated that the first and second polynucleotides may be contained on either a single or on two vectors.

The term "vector" refers to some means by which polynucleotides or gene sequences can be introduced into a cell. There are various types of vectors known in the art including plasmids, viruses, bacteriophages and cosmids. Generally polynucleotides or gene sequences are introduced into a vector by means of a cassette. The term "cassette" refers to a polynucleotide or gene sequence that is expressed from a vector, for example, the polynucleotide or gene sequences encoding the HPV L1 and HPV L2 proteins. A cassette generally comprises a gene sequence inserted into a vector, which in some embodiments, provides regulatory sequences for expressing the polynucleotide or gene sequences. In other embodiments, the polynucleotide or gene sequence provides the regulatory sequences for its expression. In further embodiments, the vector provides some

regulatory sequences and the nucleotide or gene sequence provides other regulatory sequences. "Regulatory sequences" include but are not limited to promoters, transcription termination sequences, enhancers, splice acceptors, donor sequences, introns, ribosome binding sequences, poly(A) addition sequences, and/or origins of replication.

The method further includes the step of introducing a third polynucleotide sequence into a plant cell. The third polynucleotide sequence is in the form of a replicon which is contained within a larger vector. The replicon itself contains a polynucleotide encoding a heterologous polypeptide of interest. In order for amplification of the replicon encoding the heterologous polypeptide of interest to proceed, replicational release of the replicon from the larger vector must occur in a plant cell. Once it is released from the larger vector the replicon, together with an expression cassette comprising a polynucleotide or gene sequence encoding the heterologous polypeptide of interest, is further replicated to high copy number in the plant cell. Due to structural elements in its polynucleotide make up the replicon that is released from the larger vector recircularises to form a smaller plasmid containing the polynucleotide or gene sequence encoding the heterologous polypeptide of interest.

The co-expression of the HPV L1 and HPV L2 polypeptides in the plant cell results in the L1 and L2 polypeptides self-assembling into virus-like particles in the cell. As a result of the high copy number of the replicon in the cell copies of the replicon are encapsidated directly into the virus-like particles during assembly in the cell to form pseudovirions. This is in contrast to indirect methods of incorporating a polynucleotide of interest into a pseudovirion by chemically or mechanically separating the virus-like particles and introducing a polynucleotide of interest into the virus-like particle to form a pseudovirion.

The replicating vector is held to be incapable of replicating in a mammalian cell, this is due to the fact that while the plant virus-derived Rep protein may be capable of initiating replication of the replicon in an animal cell, preferably a mammalian cell, and most preferably to a human cell, in the vector system described the Rep protein is expressed from a plant-specific promoter sequence which is highly unlikely to be recognised in a mammalian cell. On the other hand, the gene encoding the heterologous polypeptide is, however, capable of being expressed in a

mammalian cell because it is under the control of a well-characterised mammalian promoter as part of the expression cassette.

It will be appreciated by a person skilled in the art that the gene encoding the Rep protein may be contained on the replicating vector, on another vector (such as a vector containing a cassette encoding the HPV L1 and/or HPV L2 polypeptide), it may further be integrated into the DNA of the plant cell in which the pseudovirions are produced, or it may be integrated, together with a suitable promoter, into the DNA of a mammalian cell into which the carrier vector is introduced by the pseudovirion. The presence of the Rep protein in the plant or mammalian cell may result in the initiation of replication of the replicating vector and production of the replicon to high copy number.

The gene encoding the heterologous polypeptide contained on the replicon may include a gene selected from the group consisting of a reporter gene, a therapeutic gene and possibly other genes related to desirable human or animal vaccine proteins – such as HPV L1 or L2, HIV Gag or Env, and others.

It will be appreciated that a "reporter gene" may be selected from any nucleic acid encoding a polypeptide or protein whose transcription, translation and/or post-translation activity can be detected. Examples of reporter genes include genes for luciferase, secreted alkaline phosphatase, green fluorescent protein, beta-galactosidase, and the like. The expression of the reporter polypeptide is used in the present invention as an indicator of the presence of neutralising antibodies to HPV in a sample. The pseudovirions of the invention can thus be used in a neutralisation assay for detection of neutralising antibodies to HPV in a subject.

It will also be appreciated that the replicon may be derived from any single-stranded DNA virus of plants, including geminiviruses and nanoviruses, as well as from bird and mammalian circoviruses, or from parvoviruses, and/or bacterial ssDNA viruses or bacterial plasmids that replicate via a similar rolling circle DNA replication strategy. All that is required is a Rep protein or equivalent, expressed in the presence of a DNA construct carried in a larger plasmid, which incorporates at least one origin of replication sequence (Ori) recognised by the Rep so as to allow the initiation of rolling circle replication.

The pseudovirion neutralisation assay of the invention could be used for the development of a HPV pseudovirus neutralisation kit which could be used to test the effectiveness of potential HPV vaccine candidates.

The delivery of the replicon from the pseudovirion to a mammalian cell is a clear indicator that the pseudovirions of the invention are capable of being used as DNA delivery vehicles for the purposes of gene therapy.

Production of the pseudovirions of the invention in plants has certain benefits over the current mammalian cell production methods. Among others the cost of production of plant derived pseudovirions is substantially lower than the cost of production in mammalian cells. Currently, pseudovirions are only produced in mammalian cancer-derived cultured cells: this production method poses certain safety issues in that the pseudovirions could encapsidate oncogenes from the cell lines. This could result in a subject who is treated with these pseudovirions being "infected" with cancer-causing genes. Further, propagation of pseudovirions in mammalian cell lines could result in other viruses and/or contaminants being encapsidated in the capsid.

The method of production of the pseudovirions of the invention in plants is a simple process and removes the possibility of oncogene or mammalian virus contamination. The process is also highly scalable. Further, should plant virus-derived replicating DNA be encapsidated into the pseudovirions of the invention this plant virus-derived DNA will not be capable of replicating in mammalian cells or of combining with other mammalian viruses or transposon like sequences.

The following examples are now offered by way of illustration and not by way of limitation of the invention described herein.

EXAMPLE 1

Replicon production in plants

Plant expression vectors

To express pseudovirions (PsVs) in *N. benthamiana* plants, several plant expression vectors were utilised. Replicating vectors were constructed which would replicate *in planta* to form replicons or pseudogenomes for packaging by HPV L1 and L2 capsid proteins into PsVs. The replicating vectors were constructed by adapting the previously developed geminivirus-derived pRIC3 vector (Figure 1) to produce the replicating vectors. Two different mammalian expression cassettes encoding genes for the reporter gene products SEAP (mSEAP cassette) and luc (mluc cassette) were utilized to create the replicating vectors. Both of the mammalian cassettes were incorporated into pRIC3 with the extant EGFP plant cassette (+), hereinafter designated mSEAP+ (SEQ ID NO: 9; Figure 17) and mluc+ (SEQ ID NO: 10; Figure 18) serving to increase the overall replicon size (pRIC3-mSEAP+ and pRIC3-mluc+), while the SEAP cassette (SEQ ID NO: 8; Figure 16) was also incorporated in place of the plant cassette to create a smaller replicon (pRIC3-mSEAP). HPV VLPs have been reported to package pseudogenomes of approximately 5-8 Kbp in size, whereas larger or smaller pseudogenomes are not packaged at all (Buck et al., 2004; Touze and Coursaget, 1998). To accommodate these size constraints, three vectors were created with different reporter genes and resulting in replicons of different sizes, namely:

a) pRIC3-mSEAP – pRIC3 with a mammalian cassette encoding the SEAP reporter gene in place of the current plant cassette (4.8 Kbp replicon/pseudogenome) (Figure 2)

b) pRIC3-mSEAP+ – pRIC3 with the addition of a mammalian SEAP cassette, inserted upstream of the plant cassette (6.6 Kbp replicon/pseudogenome) (Figure 3)

c) pRIC3-mluc+ - pRIC3 with the addition of an alternative mammalian cassette encoding the luc reporter gene, inserted upstream of the plant cassette (7.6 Kbp replicon/pseudogenome) (Figure 4)

Further to these, the plant expression vector pTRAc (gifted by Prof. Dr. Rainer Fischer; Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany) expressing HPV-16 L1 (SEQ ID NO: 13) or HPV 16 L2 (SEQ ID NO: 14) human codon-optimised genes (pTRAc-hL1 and pTRAc-hL2, respectively) were used for production of L1 and L2 capsid proteins. This vector, shown in Figure 5, targets L1

and L2 expression to the cytoplasm, and pTRAc-hL1 has demonstrated high expression levels for L1 *in planta* (Maclean et al., 2007).

Transformation of *Agrobacterium tumefaciens*

Plasmids were isolated from *E. coli* using a QIAGEN® Plasmid Miniprep Kit. These were then introduced into *Agrobacterium tumefaciens* strain GV3101::pMP90RK via electroporation, as described by Maclean et al. (2007). *A. tumefaciens* cells were made electrocompetent by the method described by Shen and Forde (1989). 200 ng of plasmid DNA was added to a chilled electroporation cuvette (Molecular BioProducts, Inc.), along with 100 µl of electrocompetent cells. After 5 minutes of incubation on ice, cells were electroporated using a Bio-Rad GenePulser™ under the following conditions: 1.8 kV, 25 µF, 200 Ω. 900 µl of antibiotic-free Luria broth was added to the electroporated cells, which were incubated for 2 hours at 27°C. Recombinant clones were screened by antibiotic selection with rifampicin (50 µg/ml), carbenicillin (50 µg/ml), and kanamycin (30 µg/ml). Plates were incubated at 27°C for 48 hours to allow for colony formation, and screened for positive clones by colony PCR.

Agroinfiltration of *N. benthamiana*

Agroinfiltration of *N. benthamiana* plants was performed as described by Maclean et al. (2007). *Nicotiana benthamiana* plants were grown from seed in a controlled plant growth room. The plants were grown at 22°C, with 16 hours of light per day for 6 weeks. Plants were agroinfiltrated by syringe or by vacuum with a bacterial suspension of recombinant *A. tumefaciens* at an optical density (OD) of 0.25, 0.5, 0.75 or 1. Briefly, a syringe was used to force *A. tumefaciens* bacterial suspension into the abaxial air spaces in several leaves per plant. The plants were allowed to grow as normal, and leaf samples were harvested at 1-7 days post infiltration (dpi). For vacuum infiltration, whole plants were submerged in 500ml of bacterial suspension, and placed in a vacuum chamber. A vacuum of -90 kilopascal (kPa) was maintained for 5 seconds, then rapidly released (10-15 kPa.sec^{-1}). Plants were grown as normal, and harvested at 4 dpi.

Quantitative PCR

qPCR analysis was performed to determine whether replication of the replicon was occurring in plants. A single 0.5cm leaf disc was incubated at 95°C for

10 minutes with 100 µl Extraction Buffer from the Extract'n'Amp Plant PCR Kit (Sigma Aldrich). This was diluted with 100 µl Dilution buffer, and stored at -20°C until needed. qPCR was performed using the 2x SybrGREEN ReadyMix from the same kit. Primers lucQ-F (5'-CAA CTG CAT AAG GCT ATG AAG AGA-3' (SEQ ID NO:1)) and lucQ-R (5'-ATT TGT ATT CAG CCC ATA TCG TTT-3' (SEQ ID NO:2)) were used to amplify a 153 bp fragment of the luciferase gene, and primers SEAPQ-F (5'-CCT TGA CCC CGC ACA GGT A-3' (SEQ ID NO:3)) and SEAPQ-R (5'-GGC TCT GTC CAA GAC ATA CAA TGT A-3' (SEQ ID NO:4)) were used to amplify an 83 bp fragment of the SEAP gene. All primers were used at a final concentration of 0.4 mM. qPCR cycling was performed on a Corbett RotorGene 6000 (Corbett), using cycling parameters as follows: for the luciferase reaction 95°C for 2 minutes; 40 cycles of 95°C for 5 seconds, 57°C for 5 seconds, and 72°C for 5 seconds; and melt curve analysis from 72-95°C for 5 seconds per degree and for the SEAPQ reaction 95°C for 2 minutes; 40 cycles of 95°C for 5 seconds, 54°C for 5 seconds, and 72°C for 5 seconds; and melt curve analysis from 72-95°C for 5 seconds per degree. qPCR was performed with three technical repeats per sample, with a sample population size of three (N=3). Data was analysed using RotorGene Q Series 2.0.2 software (Corbett). Ct values were normalised to total DNA concentration for each sample.

Inverse PCR

The replicon construct of the present invention was derived from the genome of Bean yellow dwarf mastrevirus (BeYDV). This includes two copies of the Long Intergenic Region (LIR) of BeYDV, flanking a construct comprising a mammalian promoter to the 5' side of a reporter gene, a BeYDV-derived Short Intergenic Region (SIR), and the BeYDV Rep gene under the control of its native promoter in the LIR sequence. Introduction of the carrier plasmid into plant cells results in transcription from the BeYDV Rep promoter of Rep mRNA, and translation of the Rep protein. This protein binds to the BeYDV Ori within LIR sequences, and causes a single-strand nick in the sequence 5'-TAATATT/AC-3'; host repair polymerases extend the free 3'-end up to the second LIR TAATATTAC sequence. Release of a single-stranded unit-length replicon DNA allows recircularisation via a stem-loop sequence encoded in the LIR, with ligation to a circular molecule by the Rep protein circular ssDNA molecules are converted to dsDNA by host polymerases, and Rep can then be transcribed from these to amplify their presence as autonomous replicons, just as the native virus replicates. Alternatively, a replicon may be generated by the

expression in trans of a Rep protein from another co-agroinfiltrated construct, and replication would continue only as long as Rep was co-expressed.

This process occurs in plant cells because the native Rep promoter is recognised by plant transcription factors: this does not appear to happen in mammalian cells, meaning the replicon would be replicationally inert and would only be transcribed to allow expression of the transgene.

A variation of inverse PCR, as described by Regnard et al. (2010), was utilised to confirm recircularisation of the replicon (Figure 6) . Primers were designed to amplify a DNA fragment (approximately 2.1 Kbp) encompassing the site of recircularisation for each replicon. PCR reactions to confirm recircularisation of the pRIC3mluc+Replicon were performed with the GoTaq Kit (Promega), 2.5 mM Mg^{2+}, primers polyA35SS-F (5'-AGG GTT CTT ATA GGG TTT CGC TC-3' (SEQ ID NO:5)) and CMV-R (5'-CCC TGT AAC GTA TGT GAG A-3' (SEQ ID NO:6)), under the following conditions: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1-3 minutes; and 72°C for 5 minutes. PCR reactions to confirm recircularisation of the pRIC3mSEAP Replicon and pRIC3mSEAP+Replicon were performed with the GoTaq Kit (Promega), 2.5 mM Mg^{2+}, primers Rep-F (5'-TCC ATC GTG CGT CAG ATT TGC G-3' (SEQ ID NO:7)) and SEAPQ-R (SEQ ID NO:4), under the following conditions: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 1-3 minutes; and 72°C for 5 minutes.

Replicating vectors undergo replicational release in plants

The three novel vectors, pRIC3-mSEAP, pRIC3-mSEAP+ and pRIC3-mluc+ were designed and tested in *N. benthamiana*. All three vectors were cloned into *A. tumefaciens* GV3101::pMP90RK, and plants were infiltrated at an OD_{600} of 0.5. DNA was harvested from plants at 3 dpi, and tested for replicational release by PCR. Primers were designed to amplify a 2.1 Kbp fragment of the replicon, incorporating the LIR (see Figures 2C, 3C and 4C) using the Inverse PCR reactions described above for pRIC3mluc+, pRIC3-mSEAP and pRIC3-mSEAP+. According to the design of this experiment PCR amplification product would only be produced in the presence of recircularised replicon. PCR amplification (Figure 7) of an approximately 2.1 Kbp product confirmed that the replicon was formed in plants individually infiltrated with pRIC3-mSEAP and pRIC3-mSEAP+ (Figure 7A), and pRIC3-mluc+ (Figure 7B). This

confirms that these vectors form a recircularised replicon in plant cells, and are suitable vectors for pseudogenome production.

The pRIC3 vector backbone has been previously demonstrated to form replicons that replicate to high copy number within the plant cell, relative to non-replicating vector pTRAc (Regnard et al., 2010). Plants were infiltrated individually with each replicating vector at OD 0.5, and DNA was harvested at 1, 3, 5 and 7 dpi. qPCR was used to determine the increase in replicon copy number from 1 to 7 dpi with each of the replicating vectors using the reactions and reaction conditions described above for pRIC3mluc+, and pRIC3-mSEAP and pRIC3-mSEAP+, respectively. Analysis showed a 100-1000-fold increase in gene copy number for all three vectors at from 1 to 3 dpi, with maintenance at similar copy number up to 7 dpi (Figure 9). pRIC3-mSEAP (Figure 9A), pRIC3-mSEAP+ (Figure 9B) and pRIC3-mluc+ (Figure 9C) all show very similar increases in copy number. This is consistent with previous observations for pRIC and pRIC3 (Ogle, 2008; Regnard et al., 2010).

The three vectors were tested for their ability to replicate autonomously in plants. PCR analysis confirmed that the appropriate replicons were formed as expected. To elucidate whether the autonomously replicating vectors were, in fact, producing high copy numbers of the replicons *in planta*, qPCR analysis was employed. qPCR analysis of DNA samples harvested up to 7 dpi showed that replicon copy number for all plasmids was amplified between 100- and 1000-fold between 1 and 3 dpi, and that this high copy number was maintained up to 7 dpi. This is similar to the results obtained by Regnard et al. (2010), who showed a near-identical increase in the pRIC vectors used to generate replicons encoding the HIV p24 gene or EGFP. Replicating vectors developed by other groups have demonstrated similar increases in replicon copy number (Huang et al., 2009; Zhang and Mason, 2006). Our result demonstrates that the use of these replicating vectors for the generation of high quantities of replicon DNA in plants is a feasible strategy for producing sufficient pseudogenome DNA in plant host cells for PsV production.

EXAMPLE 2

PsVs production *in planta*

SDS-PAGE and Western Blotting

SDS-PAGE was performed to analyse HPV-16 hL1 and hL2 protein production in plants. Protein was extracted from plants agroinfiltrated with *A. tumefaciens* GV3101::pMP90RK pTRAc-hL1 and/or pTRAc-hL2. Briefly, three 0.5 cm leaf discs were harvested at 1, 3, 5, and 7 dpi, frozen in liquid N_2, and ground in a microcentrifuge tube using a plastic pestle. 100 µl of 0.5M NaCl PBS with 1x complete EDTA-free protease inhibitor cocktail (Roche) (hL1) or 8M urea in H2O (hL2) was added to the ground leaf material and mixed thoroughly. Samples were centrifuged at 13000 rpm for 5 minutes, and the supernatant was reserved. This centrifugation step was repeated, and the supernatant was stored at -20°C. For SDS-PAGE analysis, 8 µl of 5x loading dye containing β-mercaptoethanol was added to 32 µl of soluble protein, and samples were incubated at 95°C for 7 minutes. These were then loaded on 10% SDS-polyacrylamide gels using the Mini-PROTEAN® Tetra SDS-PAGE system (Bio-Rad), and electrophoresed at 130V for approximately 120 minutes. These gels, and nitrocellulose membranes, were equilibrated for 10 minutes in transfer buffer before being transferred to a nitrocellulose membrane at 15V for 90 minutes using a Bio-Rad Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell. Membranes were incubated with blocking buffer for 60 minutes, then probed for L1 overnight, using commercially available CamVir-1 primary monoclonal antibody (Abcam, ab69) diluted 1 in 10000 in blocking buffer. Membranes were washed for 4x10 minutes in blocking buffer, probed with goat anti-mouse AP-conjugated secondary antibody (Sigma, A3562) diluted 1 in 5000 in blocking buffer for two hours, washed 4x10 minutes in blocking buffer without skim milk powder, and visualised using BCIP/NBT Phosphatase substrate (KPL). For L2, a similar protocol was employed. Primary antibody was rabbit-produced anti-L2 primary polyclonal serum produced in our laboratory and used at 1 in 5000 dilution, and secondary antibody was goat anti-rabbit AP-conjugated antibody (Sigma, A3687).

Optimisation of protein expression in plants

Expression of hL1 and hL2 was optimised by a 1-7 dpi time trial. Plants were agroinfiltrated with a range of bacterial suspension OD_{600} values (0.25-1.0). Protein was harvested at 1, 3, 5, and 7 dpi, and separated by SDS-PAGE. hL1 and hL2 expression was analysed by western blotting using anti-L1 CamVir-1, and an anti-L2 polyclonal antibody raised in rabbits (Figure 8), respectively. Expression of recombinant protein was detected at all OD_{600} values tested, from 3 dpi, for both hL1 and hL2, at the expected size. While L2 is an approximately 50 kDa protein, it has

been widely observed to migrate at approximately 80 kDa (Muller et al., 1995). The highest expression detected for hL1 was at OD_{600} 0.25, from 3-7 dpi (Figure 8A). The highest expression of hL2 was also seen in those plants infiltrated at OD_{600} 0.25, at 3 dpi (Figure 8B). As such, agroinfiltration parameters of OD_{600} 0.25 at 4 dpi was chosen for optimal hL1 and hL2 expression in further experiments.

Production of PsVs in plants

To produce HPV PsVs, *N. benthamiana* plants were co-infiltrated with pTRAc-hL1, pTRAc-hL2, along with each of pRIC3-mSEAP, pRIC3-mSEAP+, and pRIC3-mluc+, separately. Based on data demonstrated above, agroinfiltration with pTRAc vectors was at an OD_{600} of 0.25, while replicating vectors were agroinfiltrated at OD_{600} of 0.5, and particles were harvested at 4 dpi. pTRAc-hL1 and pTRAc-hL2 were also co-infiltrated without a replicating vector, with the intention of producing HPV L1/L2 VLPs. This was performed by vacuum infiltration for the production of large volumes of biomass. DNA and crude protein were extracted at 4 dpi, in order to confirm the presence of all components necessary for PsV formation by PCR and western blotting (Figure 10). PCR amplification of a 2.1 Kbp fragment confirmed that replicon formation was occurring for all three replicating constructs at 4 dpi (Figure 10, panels A and B). Lanes demarcated 'PsV' indicate replicon formation in plants co-infiltrated with pTRAc-hL1 and -hL2, while those marked '+' are from plants infiltrated with replicating vector alone, and serve as a positive control. Western blotting analysis with CamVir-1 (hL1) and a rabbit polyclonal antibody (hL2) confirmed expression of both L1 (Figure 10C) and L2 (Figure 10D) at 4 dpi, in plants infiltrated with L1 and L2 alone ('VLP'), or L1 and L2 coinfiltrated with a replicating vector ('mSEAP', 'mSEAP+' and 'mluc+'). This was independently confirmed in at least three separate co-infiltration experiments. Notably, the intensity of the band corresponding to L2 (Figure 10D) showed marked variability between repeats in all constructs.

The production of HPV-16 PsVs in plants was successful for each of the three replicating vectors constructed. This work relied on the findings of several earlier papers, in particular that of Maclean et al. (2007). That study demonstrated that humanised L1 was expressed at high levels, and spontaneously assembled into VLPs *in planta* using the pTRAc vector. This, along with unpublished results for

pTRAc-hL2 from the same group, demonstrated the feasibility of these vectors for HPV particle production in plants.

It has been widely demonstrated that both HPV L1 and L2 are required for efficient packaging of DNA into the HPV virion, in both natural virions and PsVs (Ma et al., 2011; Okun et al., 2001; Stauffer et al., 1998). Further, it was recently established that the presence of L2 in the PsV capsid increases DNA packaging efficiency 10-fold (Holmgren et al., 2005). In this study, both L1 and L2 were co-expressed to allow for maximum potential DNA encapsidation. No investigations into differential packaging in the presence and absence of L2 were performed; however, little or no L1 signal was visible in fractions 14-16 (buoyant density 1.26-1.28 g/ml - corresponding to the density of VLPs with no encapsidated DNA). This lack of 'light' particles suggests that packaging of DNA by HPV particles in plants is very efficient indeed, resulting in few or no particles without encapsidated DNA. This is in contrast to other PsV production methods, all of which show a peak corresponding to 'light' particles, or VLPs. This is in particular true for the VLP disassembly-reassembly method (as demonstrated by Touze and Coursaget (1998)), which usually has a packaging efficiency well below 50% (Touze and Coursaget, 1998; Unckell et al., 1997).

This efficient packaging is a distinct advantage for the plant production approach, although this must be tempered with the observation that not all DNA packaged is necessarily pseudogenome DNA. This is clearly demonstrated by the L1/L2 VLPs produced here, which were seen to be mostly 'heavy' particles, indicating encapsidated DNA. As these were produced in the absence of a replicating vector, DNA packaged was either the pTRAc plasmids used to produce VLPs, or miscellaneous plant DNA. Both pTRAc-hL1 and pTRAc-hL2 (7.7 Kbp and 7.5 Kbp, respectively) fall below the maximum size of 7.9-8 Kbp for pseudogenomes that can be packaged effectively into HPV L1/L2 PsVs (Buck et al., 2005b; Touze and Coursaget, 1998). It is possible that these plasmids, or miscellaneous DNA, were packaged into assembling HPV particles instead of the intended replicon DNA – it has been suggested that VLPs produced in mammalian cells encapsidate miscellaneous cellular DNA (Roden et al., 1996). In any event, the extremely efficient replication observed in the three replicating vectors used here, as well as that observed when pRIC was compared to pTRAc in a previous study (Regnard et al.,

2010), suggest that the vast majority of plasmids present in the plant during PsV assembly would be the reporter pseudogenomes. As such, the potential for pTRAc plasmids to be packaged may not hold any relevance to the outcome of this study. Future work to elucidate all DNA species encapsidated into plant-produced PsVs is important for a full understanding of the PsV assembly process *in planta*, as well as for their use in neutralising assays.

Structural analyses of L1 and L2 suggest that DNA associates in a non-specific manner, based on the overall pH and charge of internal structural motifs (Fay et al., 2004; Garcea and Gissmann, 2004; Li et al., 1997; Pereira et al., 2009). Presumably, this allows for *in vitro* PsV assembly, demonstrated by several investigators, in the absence of mammalian cellular factors (Oh et al., 2004; Shi et al., 2001; Touze and Coursaget, 1998). In mammalian cells, there is evidence to suggest that chaperones (particularly karyopherins) play a role in the assembly and DNA packaging of natural HPV virions, and it seems likely that these are responsible for efficient intracellular PsV production (Bird et al., 2008; Chromy et al., 2006). Chaperones, in particular Heat shock protein 70 (Hsp70) and karyopherins, have been shown to play a role in the assembly of diverse viruses, including plant viruses (Kunik et al., 1999; Sullivan and Pipas, 2001). Interestingly, the ER associated chaperone Binding Protein (BiP) has been demonstrated to take part in folding and assembly of recombinant antibodies in transgenic plants (Nuttall et al., 2002). These data suggest that the molecular machinery required for papillomavirus assembly and DNA encapsidation is conserved across all eukaryote systems, and is responsible for the efficient PsV assembly observed here.

EXAMPLE 3

Purification of pseudovirions

Extraction and purification of particles

To produce particles, plants were vacuum-infiltrated with *A. tumefaciens* GMV3101::pMP90RK containing pTRAc-hL1, pTRAc-hL2 and either pRIC3luc, pRIC3mSEAP or pRIC3mSEAP+. Protein and DNA were harvested at 4 dpi, as described above. Western blotting, as described above, was used to confirm the presence of L1 and L2 protein, and inverse PCR, as described above, was used to confirm that replicational release had taken place. Whole plants were harvested 4

dpi. Particles were purified following a variation of the protocol described by Varsani et al. (2003), with some modifications. Whole leaves were weighed, and ground with liquid nitrogen in a pestle and mortar, or macerated thoroughly at room temperature. Cold 0.5M NaCl PBS was added to the leaf material at a ratio of 1:2 (w:v), and samples were homogenised in an T25 Ultra-Turrax high shear mixer (IICA®) at 13000 rpm for 10 minutes on ice. Homogenate was kept on ice for a further 2 hours before being centrifuged at 8000g for 20 minutes at 4°C in a Beckman Coulter Avanti J25i centrifuge with a Beckman JA-14 rotor. Supernatant was filtered through 4 layers of Miracloth (Calbiochem), and layered onto a 7 ml, 40% sucrose cushion (w/v). The samples were centrifuged at 100000g for 3 hours at 4°C in an Optima™ L-100 XP centrifuge (Beckman Coulter) with a Beckman Coulter SW32Ti rotor. The supernatant and sucrose cushion were removed, the pellet was resuspended in 1 ml 0.4 g/ml CsCl in PBS, and clarified on an Eppendorf 5424 tabletop centrifuge at 13000 rpm for 10 minutes. The supernatant was diluted in 5 ml of 0.4 g/ml CsCl in PBS, and subjected to centrifugation at 100000g for 24 hours in an L-100 XP ultracentrifuge with a Beckman SW55Ti rotor at 10°C.

Identification of VLPs and pseudovirions in CsCl gradient

After centrifugation, the CsCl gradient was fractionated manually or using a Foxy Jr. fractionator (ISCO). The density of each fraction was determined using a hand refractometer (ATAGO) to read the refractive index at 25°C, and International Critical Tables (Kellogg, 1927) were used to convert refractive index to buoyant density.

A dot blot was performed to confirm the presence of L1 in the CsCl fractions. Briefly, 1 µl of each fraction was dropped onto a nitrocellulose membrane. The membrane was blocked for 30 minutes in blocking buffer, then probed for L1 as described above. Membranes were scanned and analysed using GeneTools densitometry software (SynGene), and relative spot intensity was normalised to L1 presence in crude plant extract. L1-positive fractions were pooled and dialysed overnight against 0.5M NaCl in PBS to remove CsCl.

In order to confirm the presence of the DNA replicon in the PsVs, Proteinase K was added to the fractions, which were incubated at 55°C for 3 hours to allow full digestion of the PsV protein shell, before undergoing inactivation at 95°C for 10

minutes. Inverse PCR was used to amplify an approximately 2.1 Kbp DNA fragment from the samples, for the pRIC3mluc+Replicon reactions were performed with the GoTaq Kit (Promega), 2.5 mM Mg^{2+}, primers polyA35SS-F (5'-AGG GTT CTT ATA GGG TTT CGC TC-3' (SEQ ID NO:5)) and CMV-R (5'-CCC TGT AAC GTA TGT GAG A-3' (SEQ ID NO:6)), under the following conditions: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1-3 minutes; and 72°C for 5 minutes. PCR reactions to confirm recircularisation of the pRIC3mSEAP Replicon and pRIC3mSEAP+ Replicon were performed with the GoTaq Kit (Promega), 2.5 mM Mg^{2+}, primers Rep-F (5'-TCC ATC GTG CGT CAG ATT TGC G-3' (SEQ ID NO:7)) and SEAPQ-R (SEQ ID NO:4), under the following conditions: 95°C for 3 minutes; 25 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 1-3 minutes; and 72°C for 5 minutes.

Electron microscopy

To confirm the presence of VLPs and PsVs, transmission electron microscopy (TEM) was used. Copper grids were rendered hydrophilic by glow-discharge using a Model 900 SmartSet Cold Stage Controller (Electron Microscopy Sciences) at 25mA for 30 seconds. Grids were incubated for 1-30 minutes with VLP or PsV samples, washed three times with dH2O, and particles were stained with 2% uranyl acetate (w/v). Grids were viewed on a Tecnai F20 transmission electron microscope (FEI) or a LEO912 transmission electron microscope (Zeiss) at 14500X, 19000X or 50000X magnification. 10 fields of view were captured at 50000X magnification for all samples, and three fields of view were captured at 19000X magnification for L1/L2 VLP samples, and 14500X for PsV samples.

Purification and identification of plant-produced PsVs

Having confirmed the presence of all necessary elements comprising PsVs in infiltrated plants (L1 protein, L2 protein, as well as mSEAP, mSEAP+ and mluc+ replicons), VLPs and PsVs were isolated from crude plant extract using variations of the method described by Varsani et al. (2003). Briefly, homogenised PsV-containing plant material was subjected to ultracentrifugation on a 40% sucrose cushion. The resulting pellet was resuspended in 0.4 g/ml CsCl in PBS, and subjected to isopycnic ultracentrifugation to separate particles on the basis of buoyant density.

After centrifugation, samples were fractionated and analysed for the presence of L1 by dot blotting, using anti-L1 CamVir-1, as it was thought that this would indicate the presence of VLPs and PsVs as a result of its association with L1, a vital component of these particles. Densitometry analysis of L1 signal on the dot blots indicated the presence of putative HPV VLPs or PsVs, and compared to the buoyant density of each fraction of the gradient, calculated as a function of refractive index (Figure 11). Previous work has reported that HPV L1/L2 VLPs with encapsidated DNA (PsVs) have a buoyant density of 1.32-1.34 g/ml, while VLPs (without DNA) have a buoyant density of 1.26-1.28 g/ml (Rossi et al., 2000; Touze and Coursaget, 1998). L1 was seen to be present in all fractions, with a distinct peak in signal corresponding to a buoyant density of 1.33 g/ml, suggesting that these particles contain encapsidated DNA (Figure 11). Interestingly, L1/L2 VLPs demonstrated an L1 peak at a buoyant density of 1.30-1.33 g/ml, which corresponds to a 'heavy' particle (Figure 11A). This suggests that these particles encapsidated DNA with similar efficiency to those co-infiltrated with replicating vectors. A secondary peak was seen at a density of 1.25 g/ml in L1/L2 VLPs (Figure 11B) and at a density of 1.27 g/ml in particles purified from plants infiltrated with pRIC3-mSEAP (Figure 11B). This suggests that in these two samples, small quantities of particles were formed without encapsidated DNA. These results are each representative of at least three separate purification procedures. Fractions 8-11 were pooled and dialysed against high-salt PBS to obtain purified PsVs, and fractions 17-18 were pooled and dialysed as a non-PsV control. These were used for further analysis by electron microscopy, western blotting, and PCR.

Figure 12 shows several key stages in the purification process, separated on and SDS-PAGE gel. Coomassie staining reveals the removal of the majority of protein contaminants from the purified samples (Figure 12A and 12B). A protein band is present at approximately 55 kDa in purified samples in both Figure 12A and 12B, which is likely purified L1. L2, which migrates at approximately 90 kDa, is not visible in the Coomassie-stained gels. This is expected, as L2 is present in HPV VLPs and PsVs in much smaller quantities than L1 (a maximum ratio of L1:L2 is estimated at 5:1). Western blotting analysis with CamVir-1 shows a clear increase in concentration and purity of L1 in all samples (Figure 12C and 12D).

In order to further confirm the presence of L1/L2 VLPs and PsVs, dialysed samples were examined by transmission electron microscopy (Figure 13). All samples showed the presence abundant particles, of sizes ranging from 30-120 nm. 54% of mSEAP-PsVs (Figure 13A and 13E) were 40-70 nm in diameter, while 47% of mSEAP+-PsVs (Figure 13B and 13F) and 50% of mluc+-PsVs (Figure 13C and 13G) 73% of L1/L2 VLPs (Figure 13D and 13H) were of a similar size. Infectious HPV virions are usually between 50 and 60 nm in diameter. These particles showed a similar morphology to other examples of plant-produced HPV particles (Maclean et al., 2007; Warzecha et al., 2003).

To confirm that replicon DNA was encapsidated to form PsVs, pooled L1-containing fractions were digested with proteinase K to release the encapsidated pseudogenome, followed by inverse PCR with replicon-specific primers as described above to confirm the presence of replicon DNA (Figure 14). PCR amplification confirmed that mSEAP, mSEAP+ (Figure 14A) and mluc+ (Figure 14B) PsVs contained the expected DNA replicon. No replicon DNA was amplified in samples not treated with proteinase K, indicating that the DNA was encapsidated, and not found outside of the virion shell. Fractions 17 and 18 from each sample were pooled and dialysed. PCR amplification of these fractions, before or after proteinase K digestion, as well as amplification of proteinase K-treated L1/L2 VLPs, yielded no amplification products.

As a preliminary measure of quantity of PsVs in each sample, DNA concentration of proteinase K-treated samples was read using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Table 1 shows NanoDrop readings for all three PsV types. As a broad first estimate at particle concentration for each type, DNA concentration was used to calculate the number of pseudogenomes present per microliter, using the formula:

$$no.of\ molecules = \frac{total\ DNA\ (ng)}{660.bp.N_A / 10^{-9}}$$

where ng is nanograms of DNA in 1 µl, bp is pseudogenome size in base pairs, and N_A is Avogadro's constant. Results can be seen in Table 1. Concentration of molecules for all three PsV types was in the billions of particles per millilitre. This

data assumes that all DNA present was pseudogenome DNA, and that each PsV packaged exactly one copy of the pseudogenome. Taken together, these results indicate the successful production *in planta* of PsVs containing a reporter gene for the first time.

Table 1: Estimated DNA and particle concentration of purified PsVs

	mSEAP	mSEAP+	mluc+
DNA (ng/µl)	8.56	14.33	14.95
PsVs (pseudogenomes/µl)	1.63×10^9	2.04×10^9	1.84×10^9

Purification of plant-produced PsVs

The purification method developed for extracting HPV VLPs of Varsani et al., 2003 (with the modifications described above) proved to be successful, for the purification of PsVs. One concern was the use of liquid N_2 for preliminary grinding of plant material. While this step was not, in itself, a problem, cycles of freezing and thawing, as well as freezing plant material for long-term storage at -70°C, resulted in degradation of PsV particles (data not shown). As such, the protocol was modified slightly to replace grinding of frozen leaf material with finely chopping the leaf material in high-salt PBS before proceeding directly to the homogenisation step. This alteration noticeably decreased degradation of PsVs – PCR amplification of the replicon before purification of PsVs showed much more amplification product in fresh plant material when compared to frozen (data not shown).

Electron micrographs clearly demonstrate the successful assembly and isolation of HPV L1/L2 VLPs and PsVs in plants. The PsVs produced demonstrated an unusual variability in size, when compared to other VLP and PsV production methods (Buck et al., 2004; Maclean et al., 2007; Touze and Coursaget, 1998). The broad size range, from 30nm to 120 nm in diameter, may be due to pooling of fractions corresponding to CsCl density of 1.30-1.33 g/ml. HPV L1/L2 PsVs should be found at a density of 1.32-1.34 g/ml, and as such, some PsVs smaller and larger than the expected 50-60 nm size range may have been pooled, resulting in the variability shown. Other researchers have seen similar results in transgenic plants, and suggest that the smaller-sized particles may be assembly intermediates (Biemelt et al., 2003). It is also possible that the differential sizes seen here are due to an assembly process that differs substantially to that of HPV virions in mammalian cells.

Previous reports on the production of HPV PsVs has used benzonase treatment coupled with PCR to demonstrate that DNA is encapsidated within the virion shell, and not merely associated with the virion (Rossi et al., 2000; Unckell et al., 1997). PsVs produced in this study were not degraded by the 95°C PCR denaturation step, as demonstrated by no amplification of pseudogenome DNA in PsV samples not digested with proteinase K. As such the protein shell needed to be digested prior to PCR pseudogenome amplification in order to demonstrate the presence of pseudogenome DNA. The significance of this is twofold. Firstly, benzonase treatment was not required to demonstrate encapsidation of DNA, and was subsequently not used. Second, and possibly more importantly, this demonstrates that these PsVs are remarkably stable, even under mildly denaturing conditions. This is an important observation. VLPs are generally relatively unstable, and need to be treated with some care to avoid collapsing the particle (Mach et al., 2006). While PsVs are generally more stable, most that have been produced in other systems are not as stable as these plant-produced PsVs have been demonstrated to be. Accordingly, this suggests an important advantage over traditional PsV production systems.

The data presented here is the first clear evidence for successful production and purification of plant-produced L1/L2 VLPs. While this was not the primary aim of the project, production of L1/L2 VLPs was useful, in that it allowed a comparison of VLPs and PsVs produced in plants. Electron micrographs clearly show regular particles of 40-70nm in diameter. The low number of VLPs shown relative to the numbers of PsVs is a result of less starting material – VLPs were purified from approximately 25% (by fresh leaf weight) of the crude plant material used for PsV production. Western blots seen in (Figure 9C and 9D) clearly show the presence of both L1 and L2 in plants co-infiltrated with pTRAc-L1 and pTRAc-L2. Levels of L2 varied noticeably between different co-infiltration experiments, including co-infiltrations for the production of PsVs. This is not unsurprising, in that the ratio of L1:L2 has been shown to vary between 5:1 and 30:1 in HPV virions and L1/L2 VLPs. This first evidence of L1/L2 VLP production in plants is an encouraging new landmark in plant-based production of HPV VLP vaccines.

A first estimate of final concentration of PsVs yielded figures in the billions per microliter. This estimate is an inherently rough one: several assumptions are made, and the starting data – DNA concentration obtained by spectrophotometry, is far from accurate. These assumptions are 1) that all DNA present was encapsidated pseudogenome DNA, and 2) that each PsV packaged exactly one copy of the pseudogenome. However, it is safe to assume these estimates would not be out by more than two orders of magnitude. Several other researchers have tried to quantitate PsV concentration from various systems, usually using L1 quantitation by ELISA (Fleury et al., 2008), L1 ELISA in combination with PCR (Unckell et al., 1997) or estimating transducing units from reporter assay data (Buck et al., 2004). Future improvements on this system will require an accurate estimate of PsV concentration, such as that provided by ELISA quantitation.

Total yield and concentration factor were not determined. It is clear from electron micrographs and western blots that there was a marked concentration of particles. Western blotting of various stages of purification (Figure 12) clearly shows an increase in L1 signal, suggesting an approximate doubling in concentration from the crude plant sample to the dialysed PsVs. However, this is by no means a quantitative assay, and as such no firm conclusions can be reached on that basis. Further work to determine PsV concentration is an important next step in evaluating the efficiency of plant production of PsVs.

This study successfully demonstrated the feasibility of producing PsVs in plants. However, much work remains to fully elucidate the production method and efficiency of production for plant-produced PsVs to be a feasible alternative to current methods. Most importantly, quantitation of PsVs produced is a necessary next step. This could be achieved with relative simplicity, by L1 ELISA, as demonstrated by Touze and Coursaget (1998). Another important step is the investigation of all DNA species incorporated into virions, to avoid issues of contamination when using these PsVs. Lastly, an exciting possibility is a simplified purification protocol. The complexity of the current protocol was necessitated by the instability of VLPs. However, the demonstrated stability of these PsVs suggest that a much 'harsher' virus extraction protocol, such as those used for plant virus extraction (E.P. Rybicki, personal communication), could be equally successful in purifying PsVs at a fraction of the time and cost.

EXAMPLE 4

Neutralisation of pseudovirion infection

Pseudovirion neutralisation assay

To determine whether plant-produced PsVs were useful for PBNA, mammalian cells were pseudoinfected with plant-produced PsVs. HEK293TT cells were trypsinised and resuspended in neutralisation media (standard growth media, using DMEM lacking in phenol red) at a density of 0.3×10^6 cells/ml, and plated at 100 µl/well in a 96-well plate. Cells were grown at 37°C for 3-4 hours. 60 µl of each PsV was added per well, in triplicate, and grown for 72 hours. For the PsVs containing a SEAP replicon, cell culture medium was harvested. For those containing the luciferase replicon, media was removed, the cells were washed once with PBS, and an appropriate volume of Cell Culture Lysis Buffer (Promega, 20 µl for 96-well plates, 400 µl for 6-well plates) was added to the cells. Cells were rocked on an orbital shaker for 15 minutes, and stored at -20° C overnight.

To measure luciferase production in mammalian cells, the Luciferase Assay System kit (Promega) was used, as per kit instructions. 100 µl luciferase substrate luciferin was added to 20ul of cell lysate. Luminescence was read on a Modulus Microplate Reader (Turner BioSystems).

Western Blotting was used to confirm SEAP expression after transfection. 32 µl of cell culture media from cells transfected with the SEAP cassette was used for SDS-PAGE, as described above. Blots were probed with a sheep-produced polyclonal anti-calf intestinal alkaline phosphatase (anticiAP) primary antibody (Abcam, ab7330), and mouse anti-sheep alkaline phosphatase-conjugated secondary antibody (Sigma, A8062). SEAP activity was assayed using the Great EscAPe SEAP Chemiluminescence Kit (Clontech Laboratories, Inc.), at 0.6 volumes of those described in kit instructions. Briefly, 50 µl of cell culture media was harvested at 72 hours post-transfection. 15 µl was added to 45 µl dilution buffer, and incubated at 65°C for 30 minutes. Samples were placed on ice for 5 minutes, before 60 µl of SEAP Substrate Solution was added, and samples were incubated at room temperature for 30-60 minutes. Luminescence was detected on a Modulus

Microplate Reader (Turner BioSystems) for 10 seconds. All samples were assayed in triplicate, and standard deviation was calculated for all samples

To confirm that plant produced PsVs were able to be used for the PBNA, neutralisation of PsVs was assayed using a known HPV-16 neutralising antibody, following the protocol described by Buck et al. (2005a). HEK293TT cells were prepared as described by Pastrana et al. (2004). 60 µl of PsVs were incubated with 15 µl neutralising antibody HPV-16.V5 (developed by Christensen et al. (1996)) at a dilution of 1 in 4000 (for a final dilution 1 in 20000) on ice for 60 minutes. 75 µl of PsVs were added dropwise to cells in triplicate, and cells were incubated for 72 hours. Luciferase and SEAP activity were assayed as previously described. Standard deviation was calculated for all samples.

Pseudovirion-based neutralisation assay using plant-produced PsVs

In order to demonstrate that plant produced HPV-16 PsVs are an effective biological tool for use in the PBNA, PsVs were tested for reporter expression in mammalian cells, as well as for neutralisation with a commonly used HPV-16 neutralising antibody, HPV16.V5. HEK293TT cells were grown in 96 well plates, and pseudoinfected with 60 µl of purified, undiluted PsVs in 0.5M NaCl-PBS, with or without prior incubation with HPV16.V5 monoclonal antibodies diluted 1:20000 in neutralisation media. Successful infection of mammalian cells with PsVs, as well as neutralisation of PsVs, was demonstrated by luc or SEAP reporter gene expression in these cells. Figure 15 shows reporter gene expression for cells 72h post-infection with mSEAP (Figure 15A), mSEAP+ (Figure 15B) and mluc+ (3.12C) PsVs, with or without the presence of neutralising antibodies (PsV, or PsV+NAb, respectively). The negative control (-ve) for each experiment – mammalian cells with 60 µl of neutralisation media added - provides a baseline reading in RLU, while transfection with the corresponding endotoxin free plasmid DNA is used for the positive control. Pseudoinfection with mSEAP PsVs elicited a clear positive SEAP response (Figure 15A), although not as strong as that in cells transfected with plasmid DNA by lipofection. Incubation with neutralising antibody partially neutralised infection, as demonstrated by a decrease in SEAP signal. Pseudoinfection with mSEAP+ PsVs did not show a strong SEAP signal above the baseline level provided by the negative control (Figure 15B). As such, neutralisation of mSEAP+ PsVs was not observed. Pseudoinfection with mluc+ PsVs elicited a weak luciferase signal, although clearly

above that of the negative control. Incubation with HPV16.V5 NAb completely neutralised luciferase expression, resulting in expression identical to the negative control.

PsV testing and PBNA in mammalian cells

For plant-produced PsVs to be a useful tool for vaccine testing, it is vital to demonstrate their use in the pseudovirion-based neutralisation assay. PsVs were tested for pseudogenome reporter gene expression and PBNA activity using the Great EscAPe SEAP Chemoluminescence Kit (Clontech Laboratories, Inc.) or the Luciferase Assay Sytem (Sigma). The Great EscAPe kit is used for the widely accepted PBNA protocol developed by the Schiller laboratories, for its sensitivity and ease of use (Buck et al., 2005a). Luciferase has seen broad utility as an easy and sensitive reporter assay, and was chosen due to its low cost Great EscAPe system, as well as to test an alternative pseudogenome size and reporter system.

Of the three PsV types produced, two (mSEAP PsVs and mluc+ PsVs) showed low-level reporter activity after pseudoinfection of mammalian cells, while one (mSEAP+) showed little or no reporter activity. A preliminary neutralisation assay using a well-established mouse monoclonal HPV-16 neutralising antibody HPV16.V5 (Christensen et al., 1996), demonstrated partial neutralisation of mSEAP PsV infection, and complete neutralisation of mluc+ PsV infection. It is unclear why mSEAP+ PsVs failed to induce reporter gene expression in mammalian cells. The SEAP cassette is clearly functional, as demonstrated by successful reporter gene expression by mSEAP PsVs. The plant cassette incorporated into the pseudogenome is unlikely to be the cause – mluc+ PsVs also incorporated an identical plant expression cassette without affecting expression. It is possible that it was due to low concentration of particles in comparison to the other two PsV types – while estimates of concentration based on presence of DNA revealed no major differences, electron micrographs showed less particles in the mSEAP+ PsV samples compared to the other two PsV types. While preliminary, these data provide an initial proof-of-concept for the production of PsVs *in planta* for use in the PBNA.

Reporter gene expression after pseudoinfection was considerably lower than expected. Most previous PBNA studies have needed to dilute PsVs up to 1000000-fold in order to be within the linear range of the SEAP assay. Preliminary calculations

determined PsV particle concentration to be similar to that obtained by Buck et al. (2005a). Accordingly, it would be expected that infectivity would be similar. However, this was not the case: the PsVs tested here showed limited reporter gene expression, even though they were added undiluted to cells. Expression by pseudoinfection was lower than DNA transfected by lipofection. Total DNA added by FuGene transfection to each well of a 96-well plate was approximately 200 ng per well, while total DNA in a 60 µl PsV sample, as determined by NanoDrop spectrophotometry, was 500-900 ng, depending on the sample. Previous work has shown that infectivity of PsVs can be quite low - Roden et al. (1996) estimated an infectivity of 1 in 10000 cells, while Unckell et al. (1997) and Touze and Coursaget (1998) estimated ratios of 1:2000, 1:1000, respectively. However, this does not fully explain the poor expression following pseudoinfection. It is likely that the cause of this discrepancy is the buffer that the PsVs were dialysed into after purification, namely 0.5M NaCl-PBS. Changes in cell culture media osmolality (a measure of the concentration of particles in solution) have a marked effect on mammalian cells. Physiological osmolality is estimated at 290-320 mOsm/kg for mammalian tissues (Waymouth, 1970). Any major deviation from this, such as the introduction of large quantities of NaCl into cell culture media, is likely to seriously affect the growth of the cells, as well as their ability to produce recombinant protein. This is likely to be the case here, and a priority for future work is to repeat these experiments with PsVs in a buffer with less salt. This buffer was chosen because it has been shown to aid in stability of VLPs produced in plants (Varsani et al., 2003). However, these PsVs have demonstrated marked stability to denaturing conditions, and it is likely that they will be stable in PBS.

EXAMPLE 5

Improvement of reporter plasmid and PBNA using the improved plasmid

Introduction of the SV40 origin of replication

In order to assess whether reporter gene expression could be improved the SV40 origin of replication (SV40ori) was cloned into the pRIC3-mSEAP, pRIC3-mSEAP+ and pRIC3-mluc vectors (Figure 19). The inventors hypothesized that the inclusion of the SV40ori in the plasmid would increase the amplification of SEAP in HEK293TT cells and would improve reporter protein yields. The vectors that contained the SV40ori (pRIC3-mSEAP-SV40ori (SEQ ID NO:15), pRIC3-mSEAP+-

SV40ori (SEQ ID NO:16) and pRIC3-mLuc+-SV40ori (SEQ ID NO:17)) were tested in tissue culture by adding the DNA to HEK293TT cells and comparing expression levels with those obtained with the original DNA vectors.

Production of mSEAP-SV40ori PsVs

pRIC3-mSEAP-SV40ori, pTRAc-hL1 and pTRAc-hL2 were infiltrated in plants as described above. PSVs which contained a replicating vector encoding a polypeptide for SEAP were produced using both mSEAP+ and mSEAP+-SV40ori constructs. Crude plant extracts were added directly to either continuous (poured the day before) or discontinuous (freshly poured) Optiprep gradients (20%, 33%, 40%, 50%). The gradients were spun for 6 hours at 32,000 rpm. The fractions that produced the darkest blots on a dot blot from the discontinuous gradients for both mSEAP+ and mSEAP+-SV40ori were pooled and used to pseudo-infect HEK293TT cells. PSVs were diluted in DMEM media, 5 x 10^5 cells were added to 6 well-plates, incubated at 37 °C for 3 hours and 400 µl of the PSVs (diluted 1:1, 1:10. 1:100 and 1:1000 in DMEM) were dropped into the cells. The plates were incubated at 37 °C for 3 days. SEAP activity was assessed in the HEK293TT cells by assessing alkaline phosphate activity using dot blots. The inventors were able to detect AP in the supernatant of cells infected with mSEAP+ and mSEAP+-SV40+ori. A SEAP kit was then used to determine the amount of SEAP in supernatant (Table 2, Figure 20). No significant increase in activity was detected in the mSEAP+-SV40ori PSVs.

Table 2: Determination of SEAP levels in relative light units (RLU) per dilution of plant produced PsVs added to HEK293TTcells. Negative control is no DNA and positive control is mSEAP+DNA only.

mSEAP+		mSEAP+-SV40ori	
Dilution of PsVs	**RLU**	**Dilution of PsVs**	**RLU**
1	970,000	1	1,140,000
10	1,080,000	10	12,300
100	9,200	100	9,080
1000	8,500	1000	4,660
Neg	3,380		
DNA 1µg	33,900,000		

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CLAIMS

1. A method for producing a human papillomavirus (HPV) pseudovirion in a plant cell, the method comprising the steps of:

(i) introducing into the plant cell:

(a) a first nucleic acid encoding a HPV L1 polypeptide,

(b) a second nucleic acid encoding a HPV L2 polypeptide,

wherein the first and second nucleic acids are contained on at least one expression vector,

(c) a replicating vector derived from a geminivirus comprising an origin of replication (Ori) sequence recognised by a viral replication associated (Rep) protein, the replicating vector further comprising a third nucleic acid encoding a heterologous polypeptide,

wherein the replicating vector is capable of undergoing rolling circle replication, and

(d) a fourth nucleic acid encoding a Rep protein,

wherein replication of the replicating vector is initiated by the Rep protein, and

wherein the third nucleic acid sequence is operably linked to a regulatory sequence which allows for the expression of the heterologous polypeptide in a mammalian cell;

(ii) expressing the HPV L1 polypeptide, the HPV L2 polypeptide and the Rep protein in the plant cell, and

(iii) replicating the replicating vector from the Ori sequence recognised by the Rep protein in the plant cell, in order to produce a high copy number of a pseudogenome, wherein the pseudogenome is smaller than the replicating vector, is replicationally inert in mammalian cells in the absence of the Rep protein, and comprises the third nucleic acid,

wherein the expressed HPV L1 and HPV L2 polypeptides assemble with a copy of the pseudogenome and encapsidate the pseudogenome to produce a HPV pseudovirion.

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2. The method of claim 1, wherein the first and second nucleic acids are operably linked to regulatory sequences that allow for expression of the HPV L1 and HPV L2 polypeptides.

3. The method of claim 1 or 2, wherein the Rep protein is expressed from at least one of the group selected from:

(i) a nucleic acid sequence contained on the replicating vector;

(ii) a nucleic acid sequence contained on the at least one expression vector;

(iii) a nucleic acid sequence contained on an independent vector, not being the vector of (i) or (ii) above; and

(iv) a nucleic acid sequence integrated into the genomic DNA of the plant cell;

wherein expression of the Rep protein in the presence of the replicating vector results in replication of the replicating vector to produce a high copy number of the pseudogenome in the plant cell.

4. The method of any one of claims 1 to 3, wherein the third nucleic acid encoding the heterologous polypeptide comprises a gene selected from the group consisting of a reporter gene, a therapeutic gene and a gene encoding an antigenic polypeptide.

5. The method of claim 4, wherein the gene encoding the heterologous polypeptide is a reporter gene selected from a luciferase gene and a secreted alkaline phosphatase gene.

6. The method of any one of claims 1 to 5, further comprising a step of recovering the HPV pseudovirion from the plant cell.

7. A HPV pseudovirion produced according to the method of any one of claims 1 to 6, the HPV pseudovirion comprising a capsid, wherein the capsid comprises a HPV L1 and a HPV L2 polypeptides, wherein the capsid encapsidates the pseudogenome comprising the third nucleic acid encoding the heterologous polypeptide, wherein the third nucleic acid is operably linked to a regulatory sequence that allows for the

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expression of the heterologous polypeptide in a mammalian cell, wherein replication of the replicating vector is initiated from the Ori sequence recognised by the Rep protein, and wherein the HPV pseudovirion is produced in and recovered from the plant cell.

8. The HPV pseudovirion of claim 7, wherein replication of the pseudogenome is initiated in a mammalian cell infected by the HPV pseudovirion in the presence of a Rep protein, wherein the Rep protein is encoded by a nucleic acid sequence operably linked to a regulatory sequence that allows for the expression of the regulatory protein in the mammalian cell, wherein the Rep protein is expressed from any one of the group consisting of:

(i) a nucleic acid sequence contained on the replicating vector;
(ii) a nucleic acid sequence contained on an independent vector; and
(iii) a nucleic acid sequence integrated into the genomic DNA of the mammalian cell,

wherein expression of the Rep protein in the mammalian cell results in the replication of the pseudogenome.

9. The HPV pseudovirion of claim 7, wherein the heterologous polypeptide is selected from the group consisting of a reporter polypeptide, a therapeutic polypeptide and an antigenic polypeptide.

10. An assay for detecting the presence of a neutralising antibody to HPV in a subject, the assay including the steps of:

(i) combining the HPV pseudovirion of any one of claims 7 to 9, with a biological sample from the subject to form a biological sample composition, wherein the heterologous polypeptide is a reporter polypeptide;
(ii) combining the HPV pseudovirion of any one of claims 7 to 9, with a control biological sample, wherein the control biological sample does not contain a HPV neutralising antibody, to form a control sample composition, wherein the heterologous polypeptide is a reporter polypeptide;

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(iii) contacting and incubating a mammalian cell capable of being infected with HPV with the biological sample composition of (i) or the control sample composition of (ii); and

(iv) assaying the expression of the reporter polypeptide;

wherein decreased expression of the reporter polypeptide in the mammalian cells contacted with the biological sample composition, as compared to mammalian cells contacted with the control sample composition, is indicative of the presence of a HPV neutralising antibody in the biological sample.

11. The assay of claim 10, wherein the reporter polypeptide is selected from either a luciferase and a secreted alkaline phosphatase polypeptide.

12. The assay of claim 10 or 11 wherein the subject is a human.

13. A pharmaceutical composition comprising the HPV pseudovirion of any one of claims 7 to 9 and a pharmaceutically acceptable carrier or adjuvant.

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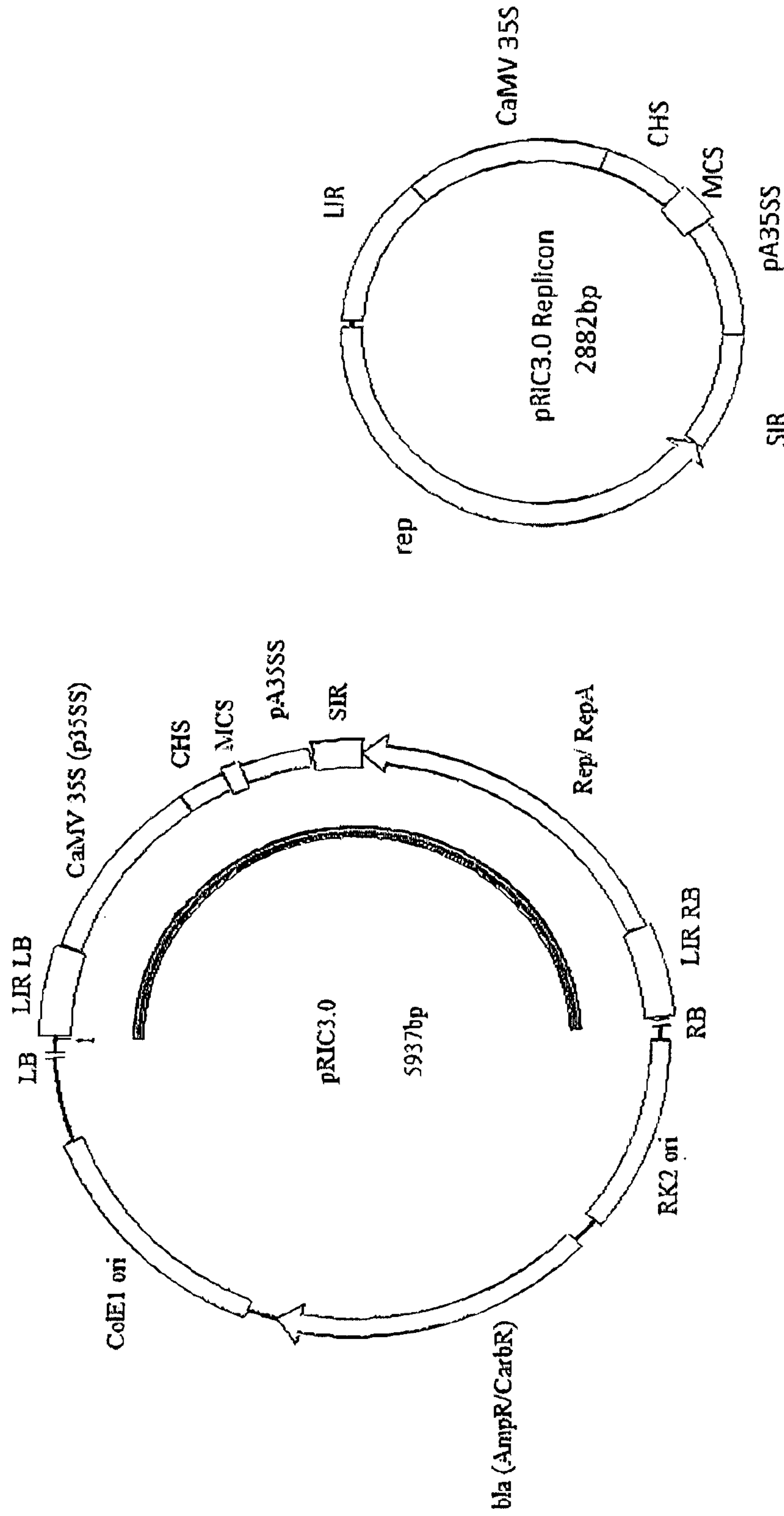


FIGURE 1

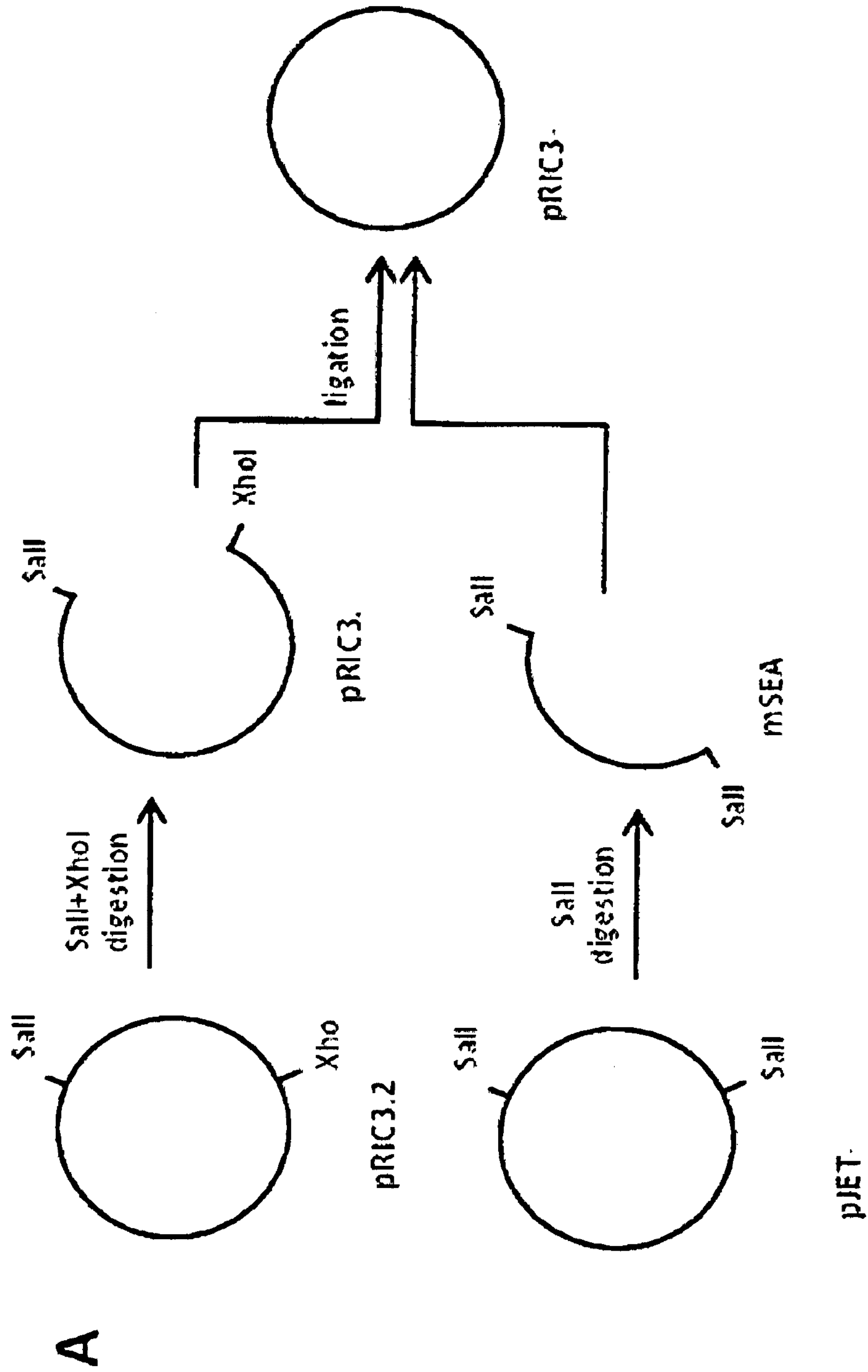


FIGURE 2

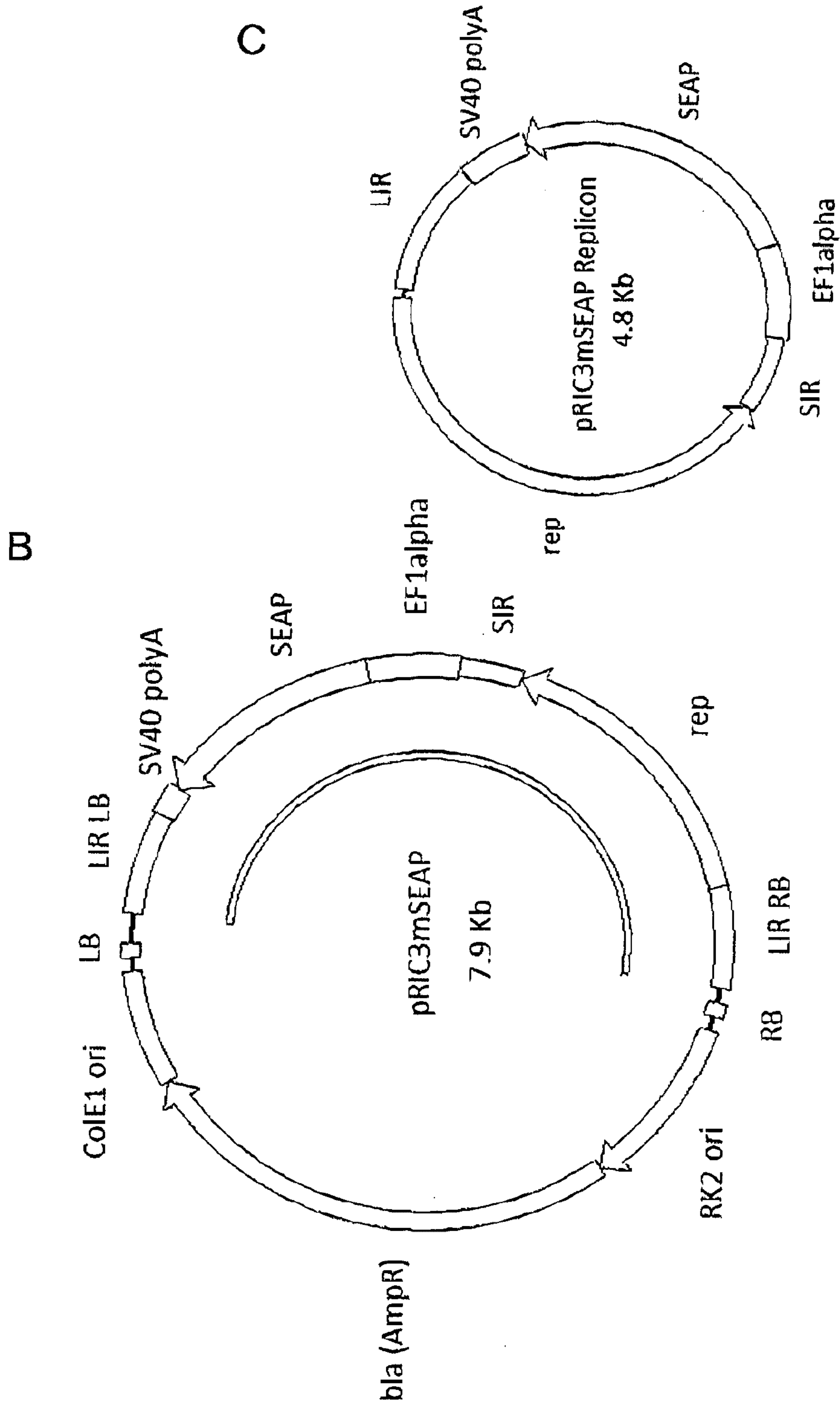
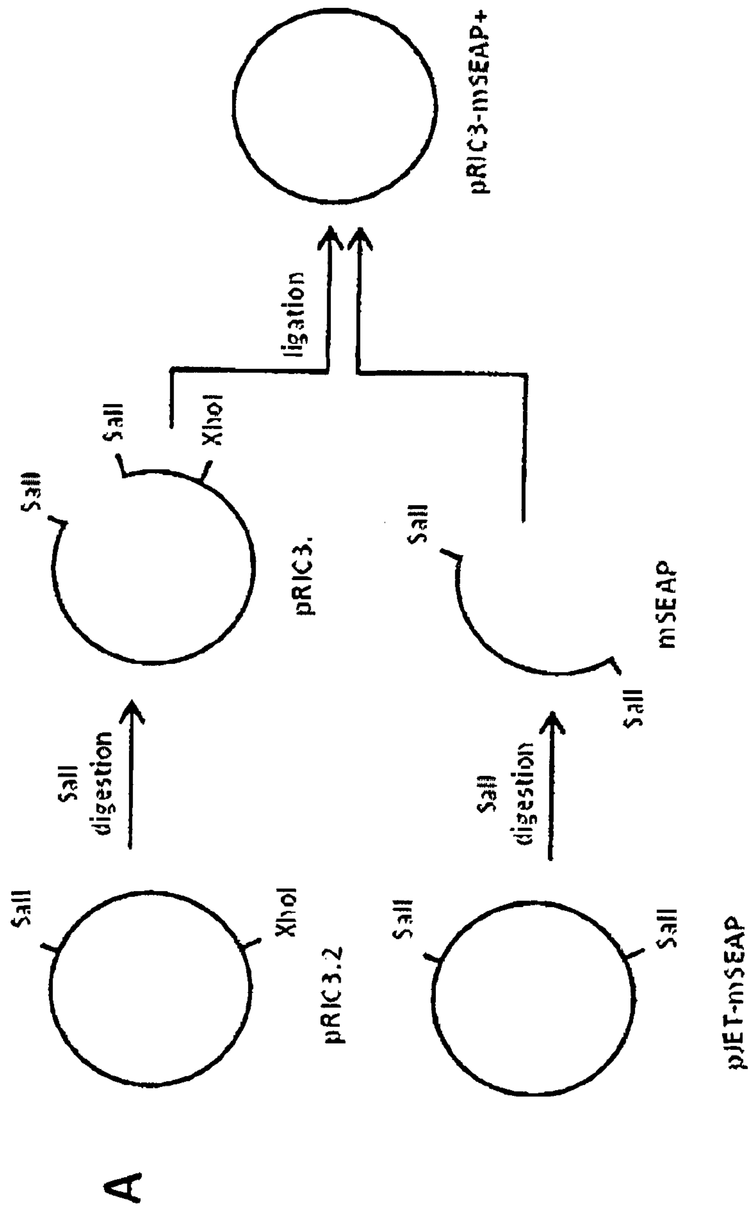


FIGURE 2 CONT



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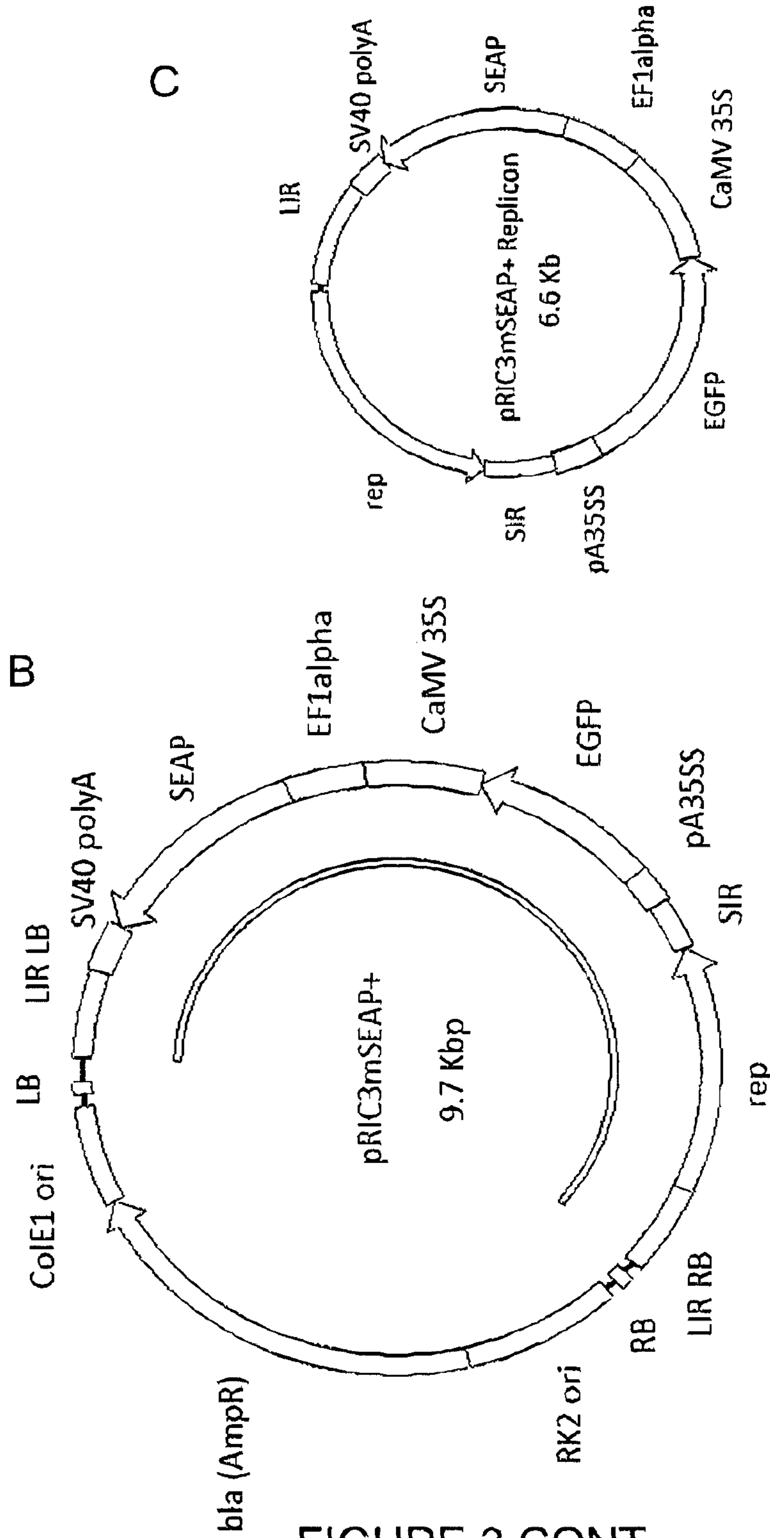
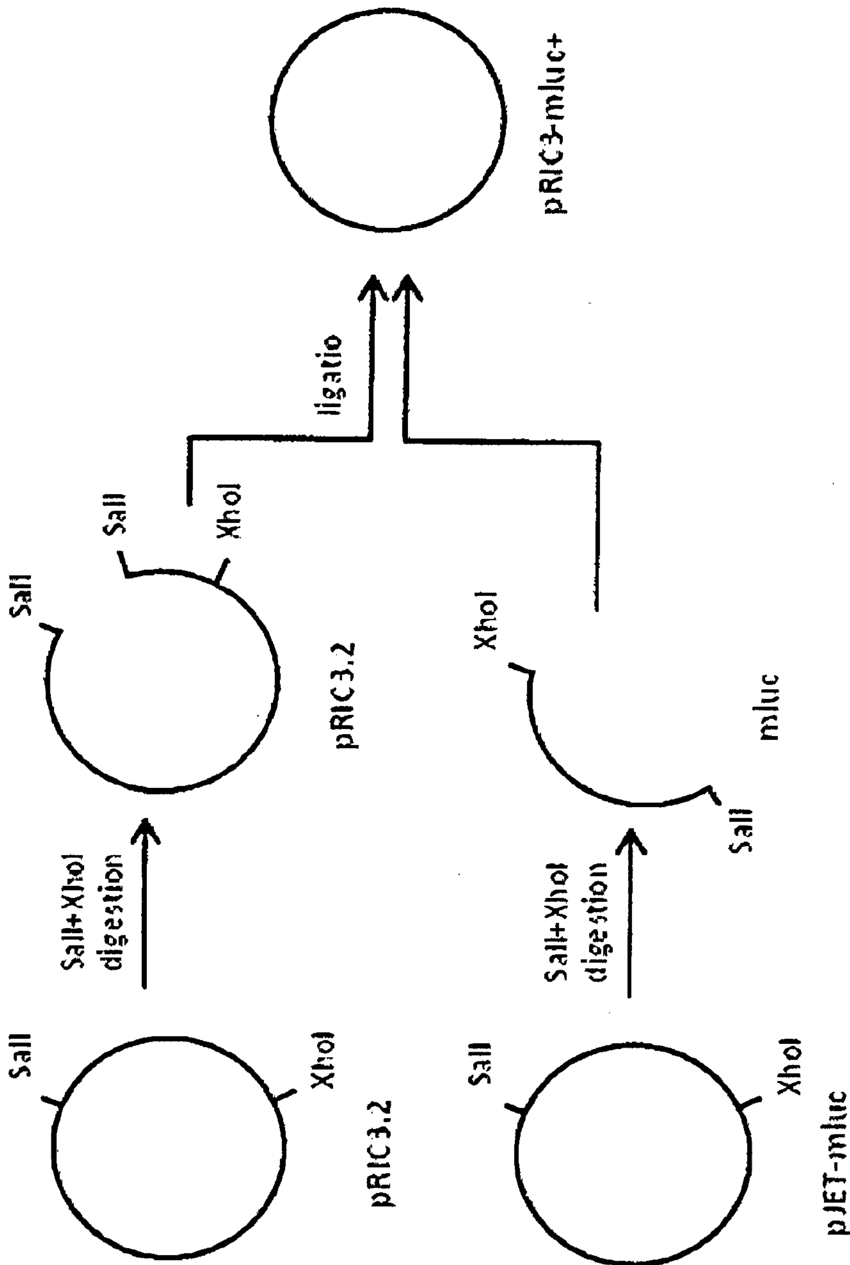


FIGURE 3 CONT



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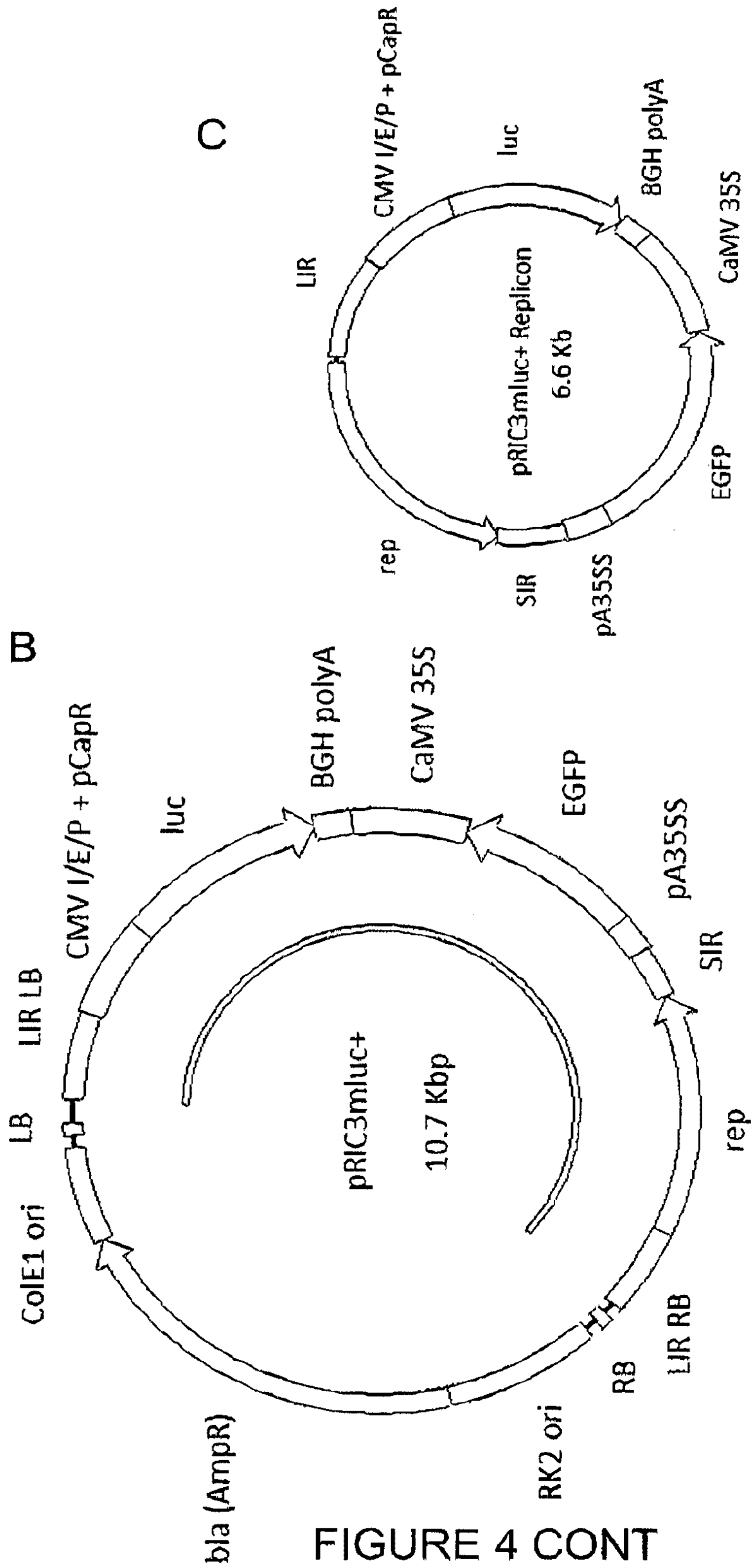
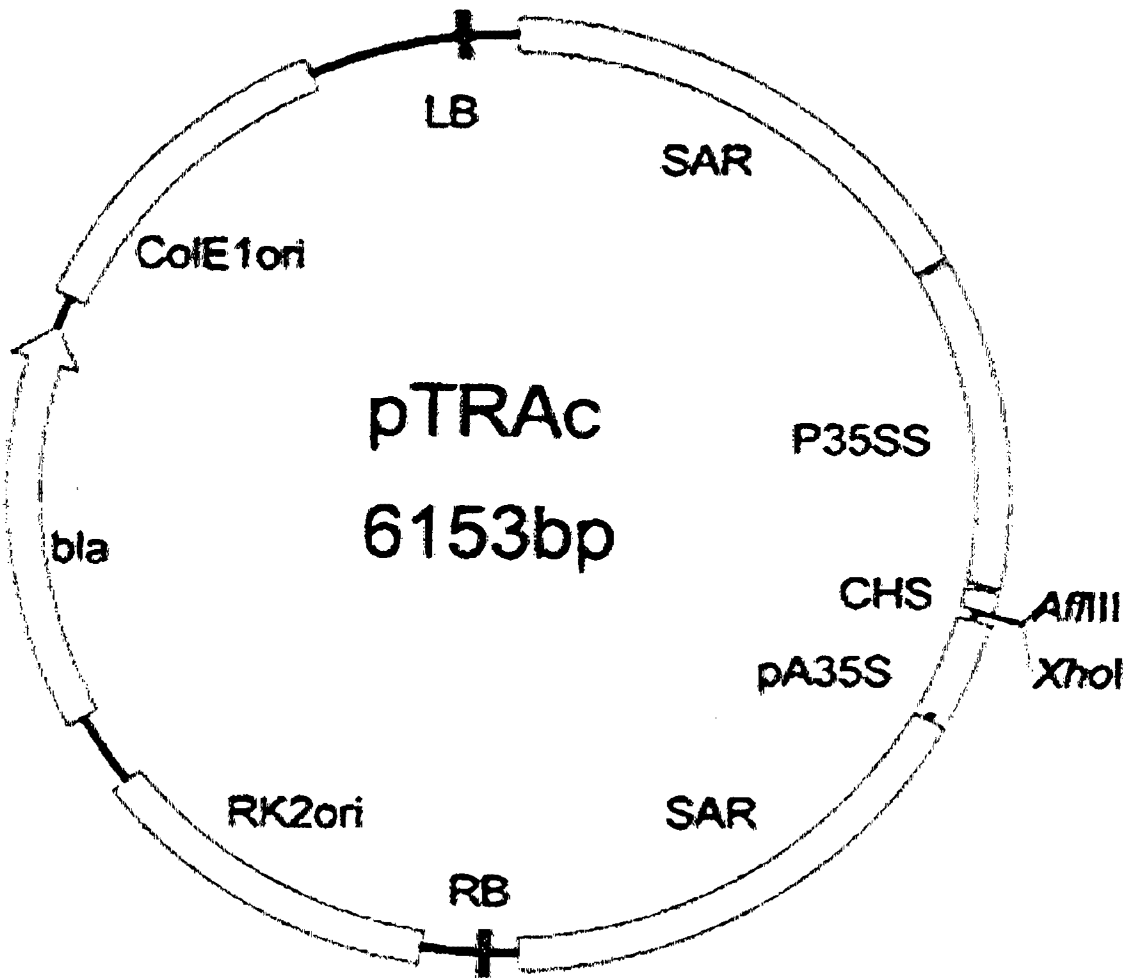


FIGURE 4 CONT



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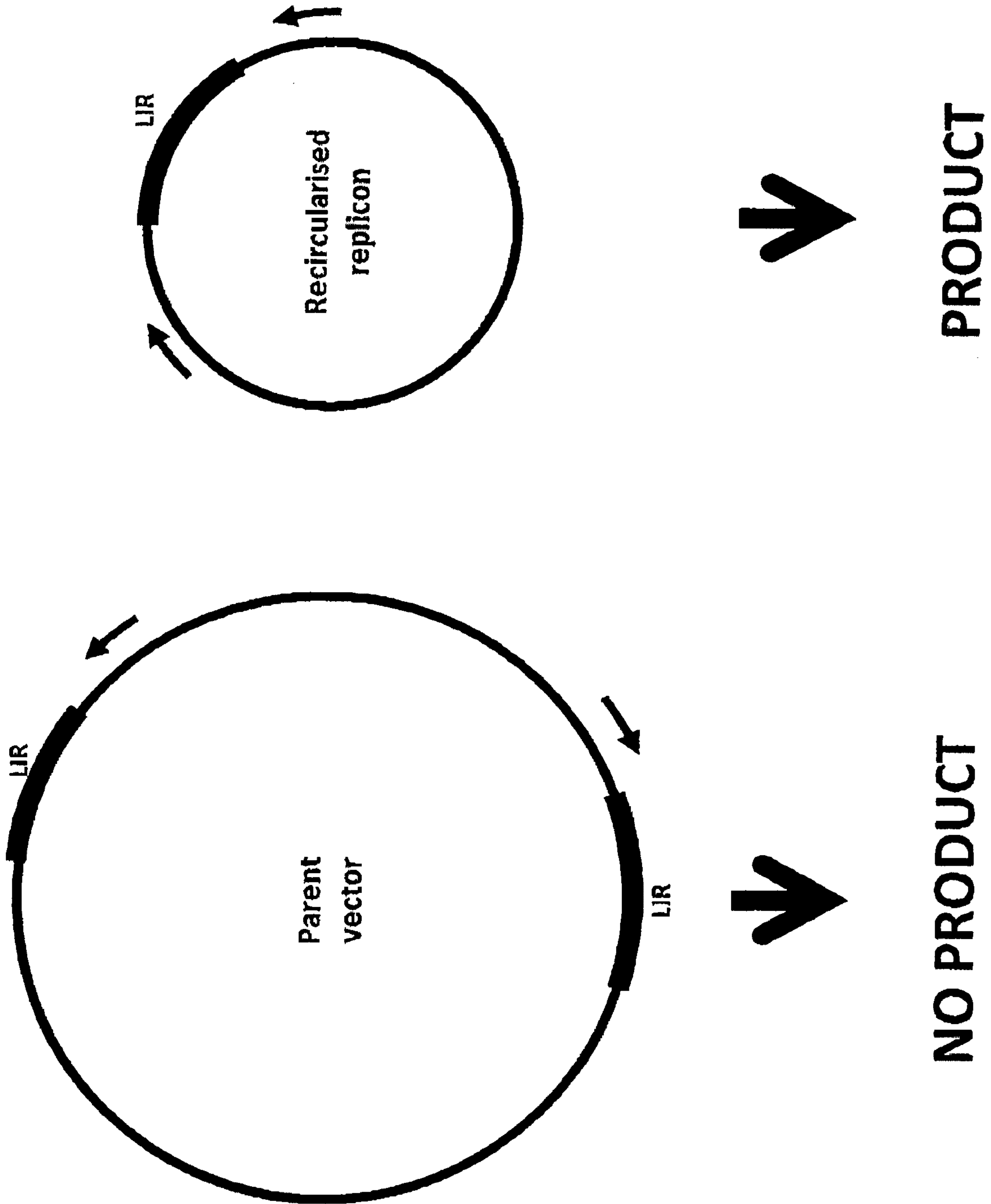

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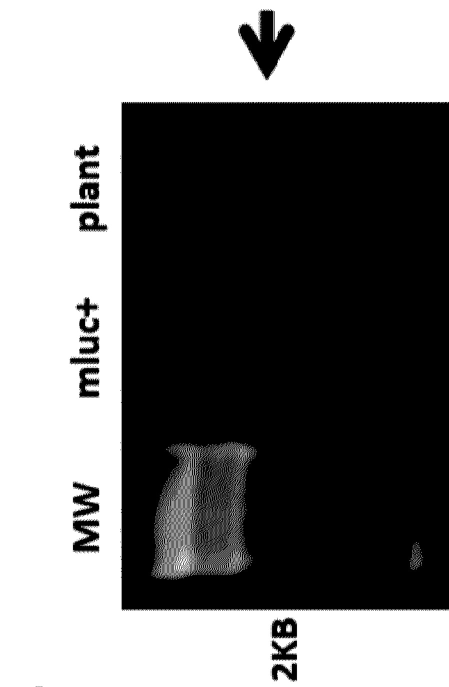

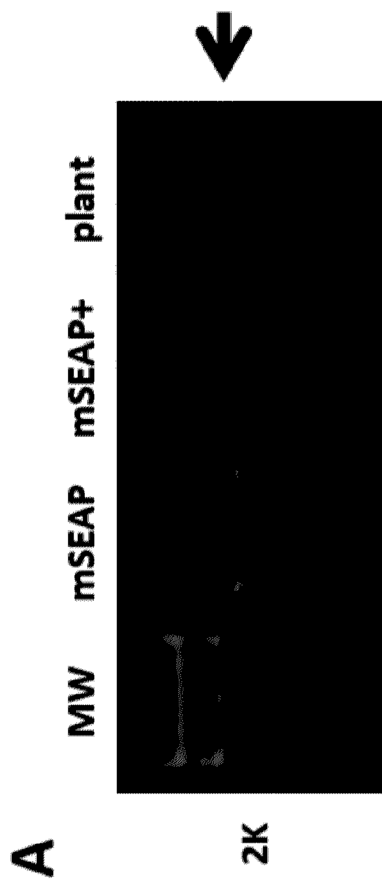

FIGURE 7

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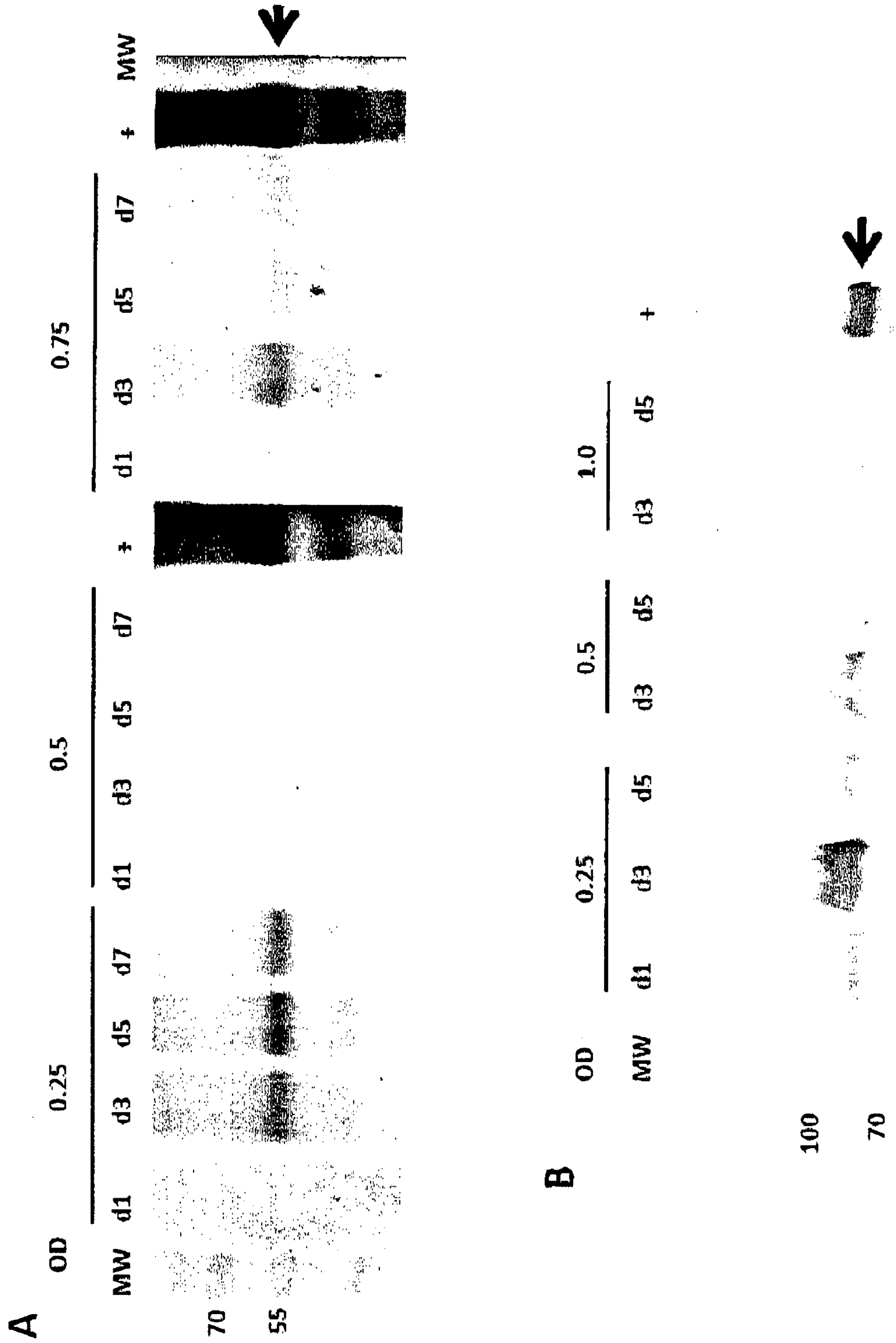


FIGURE 8

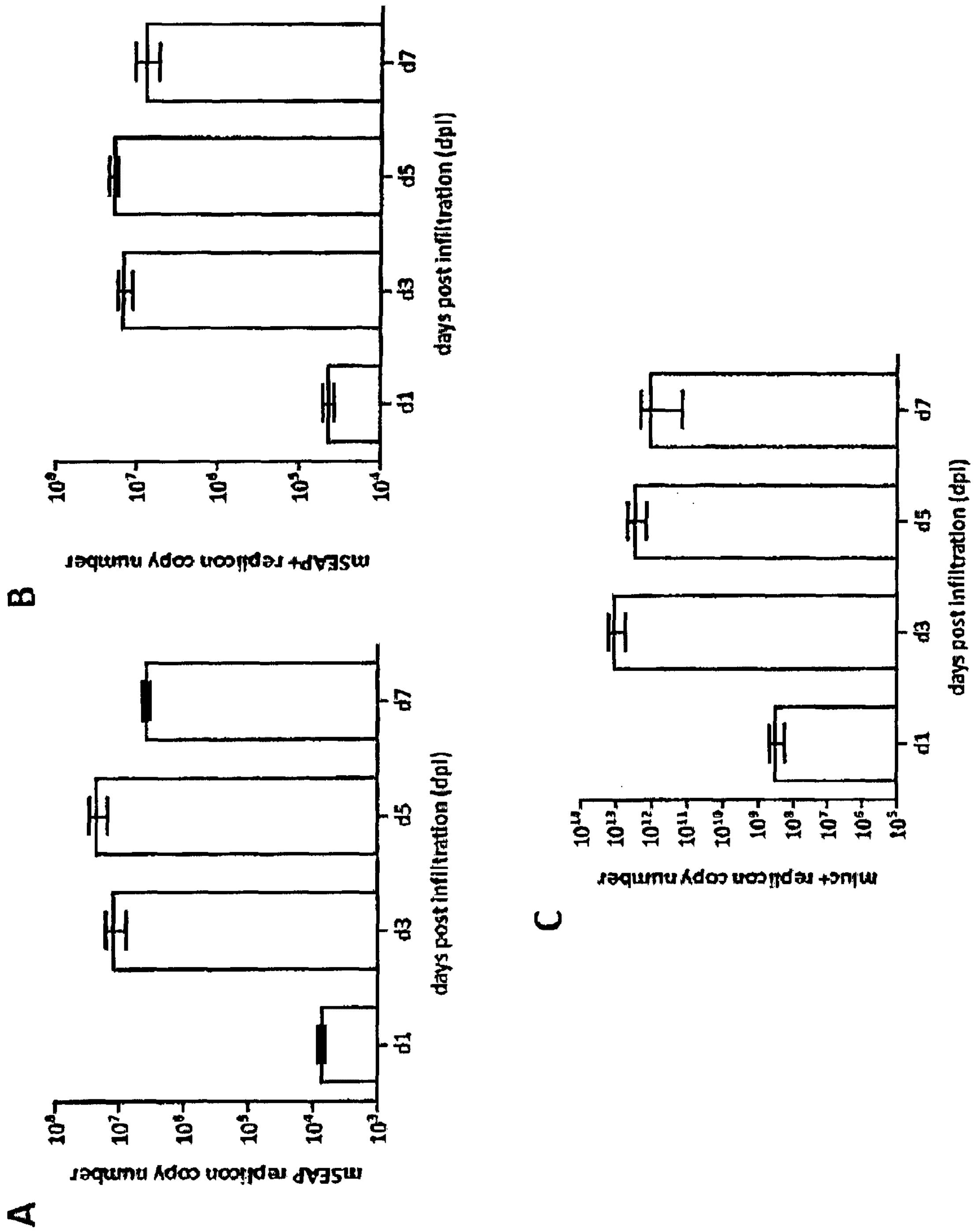


FIGURE 9

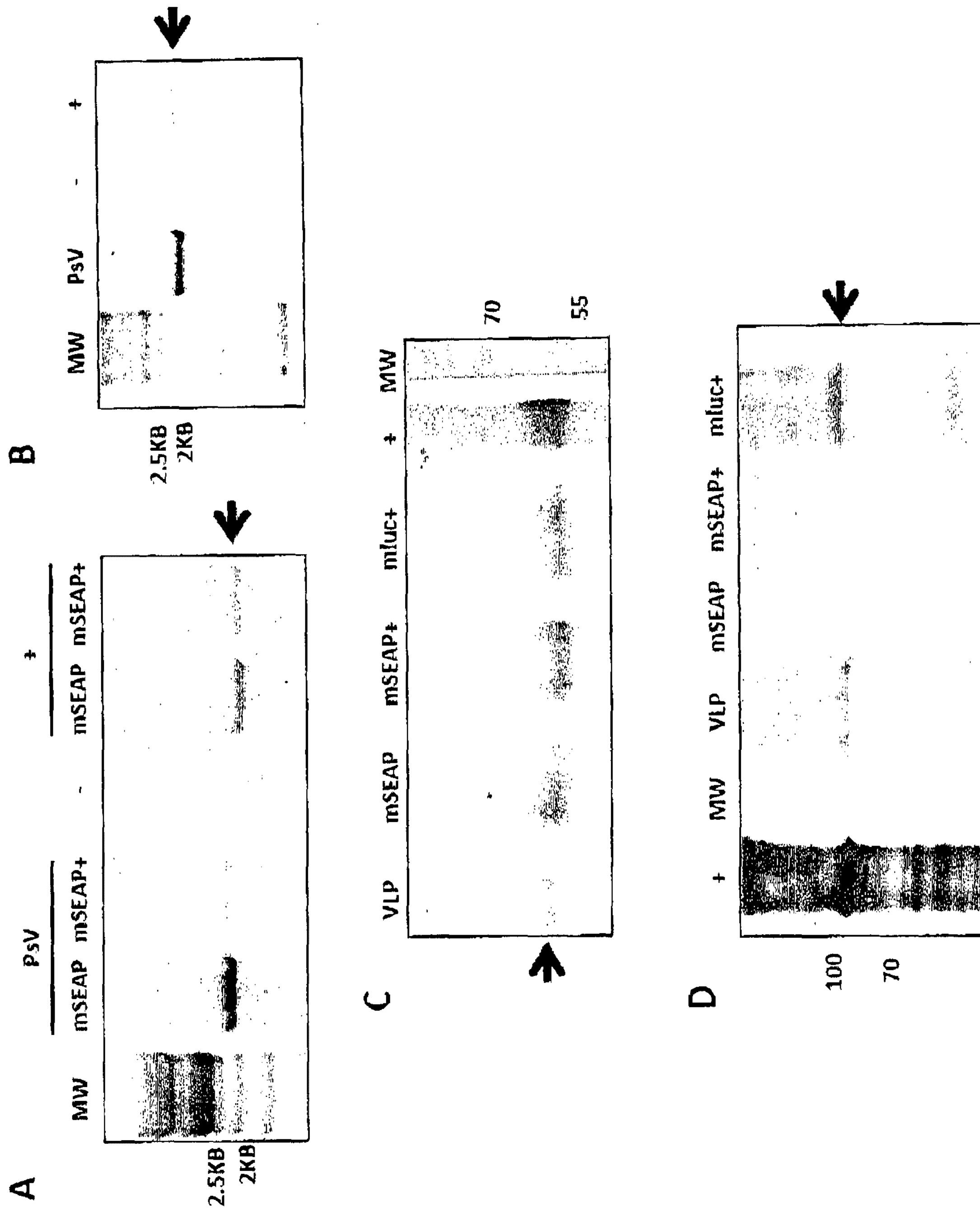
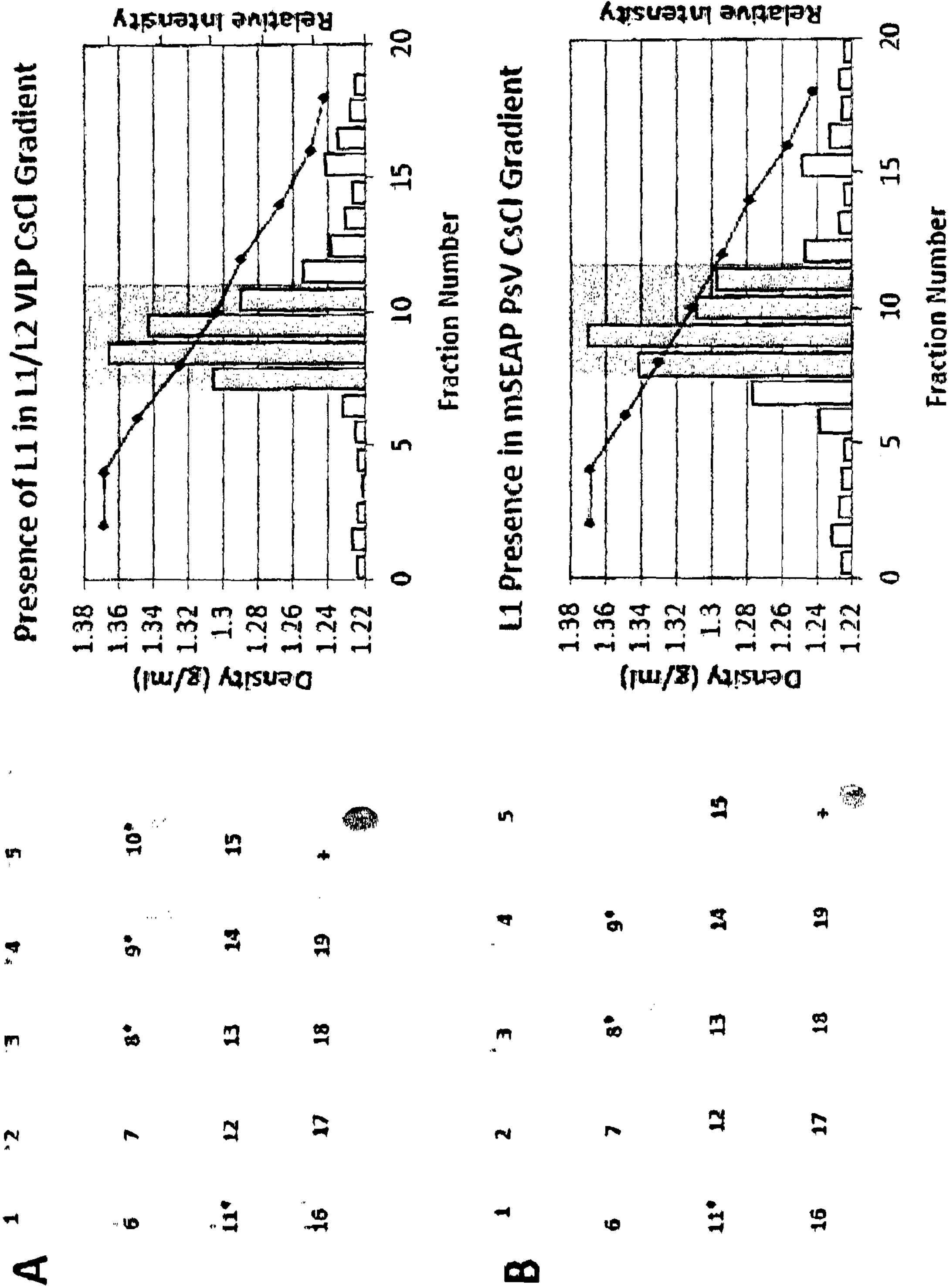


FIGURE 10



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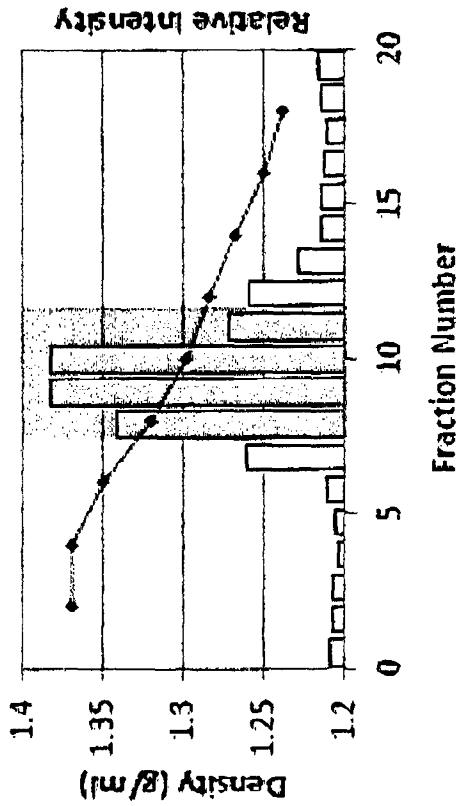

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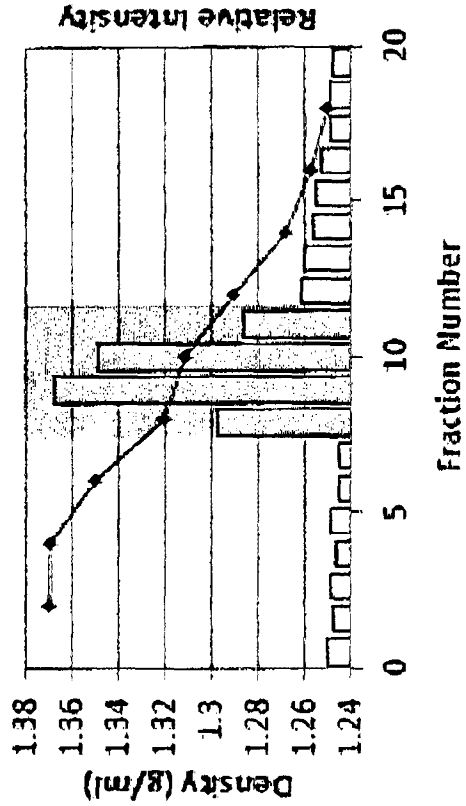

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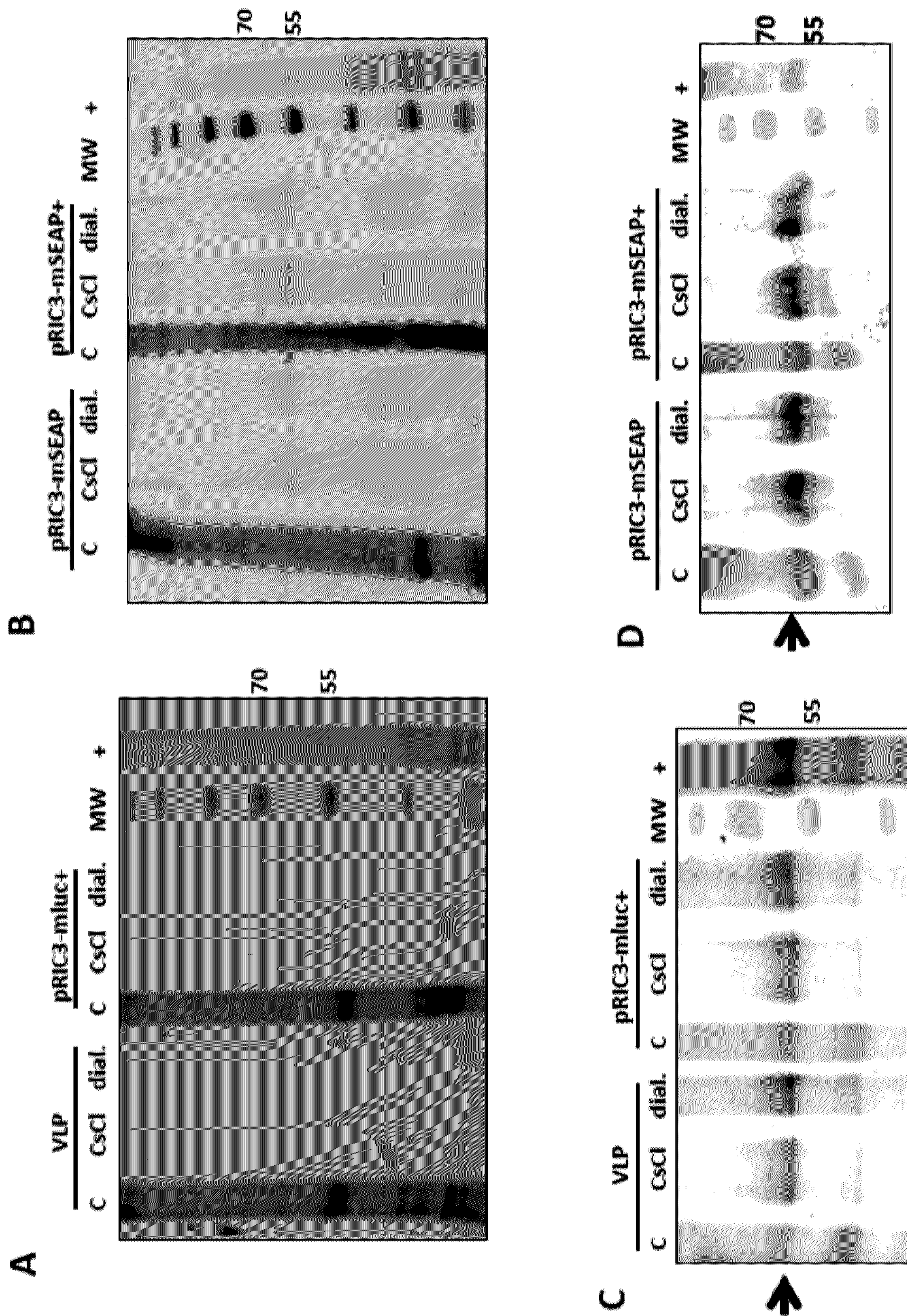
FIGURE 11 CONT



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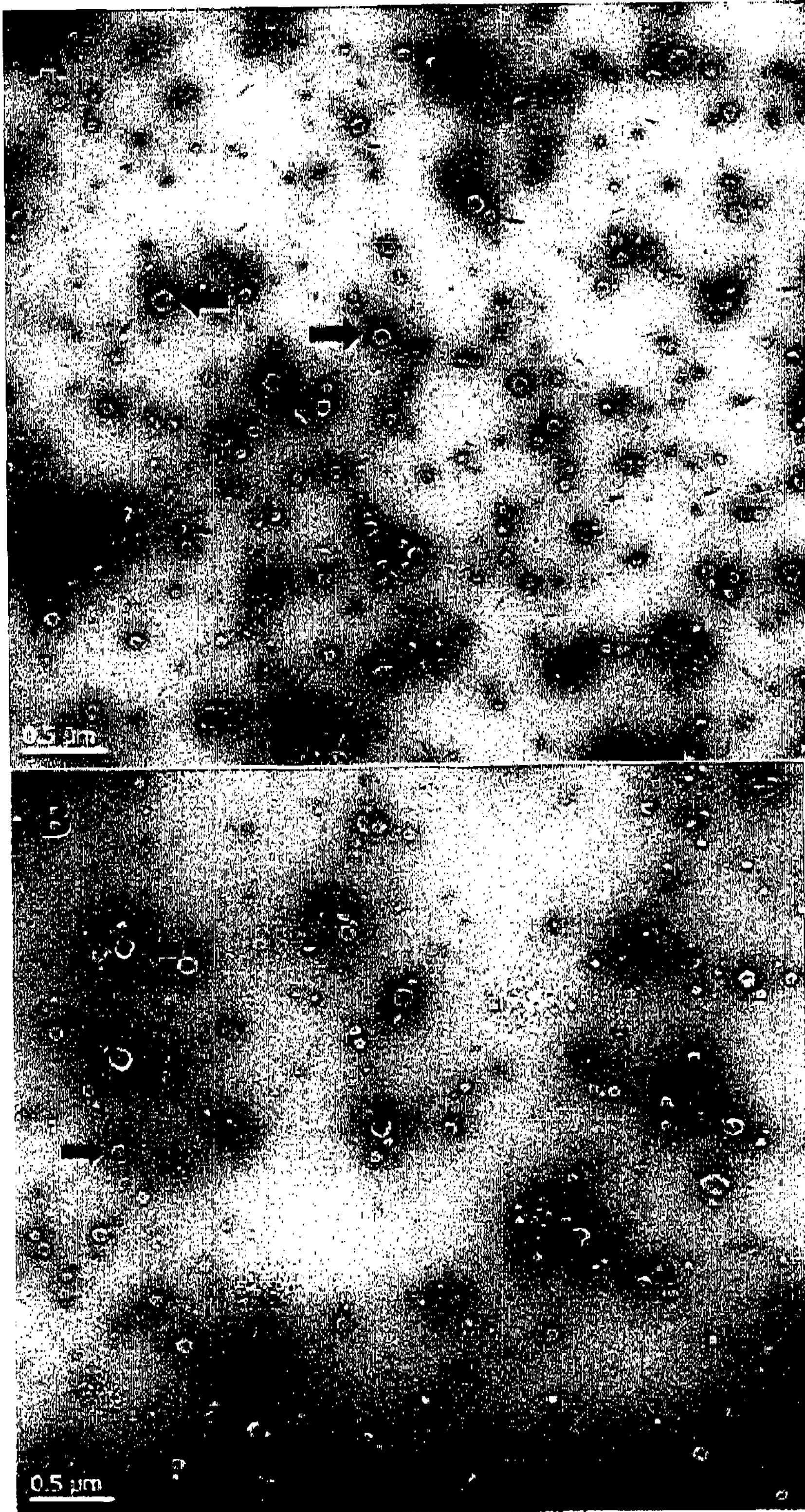

FIGURE 13

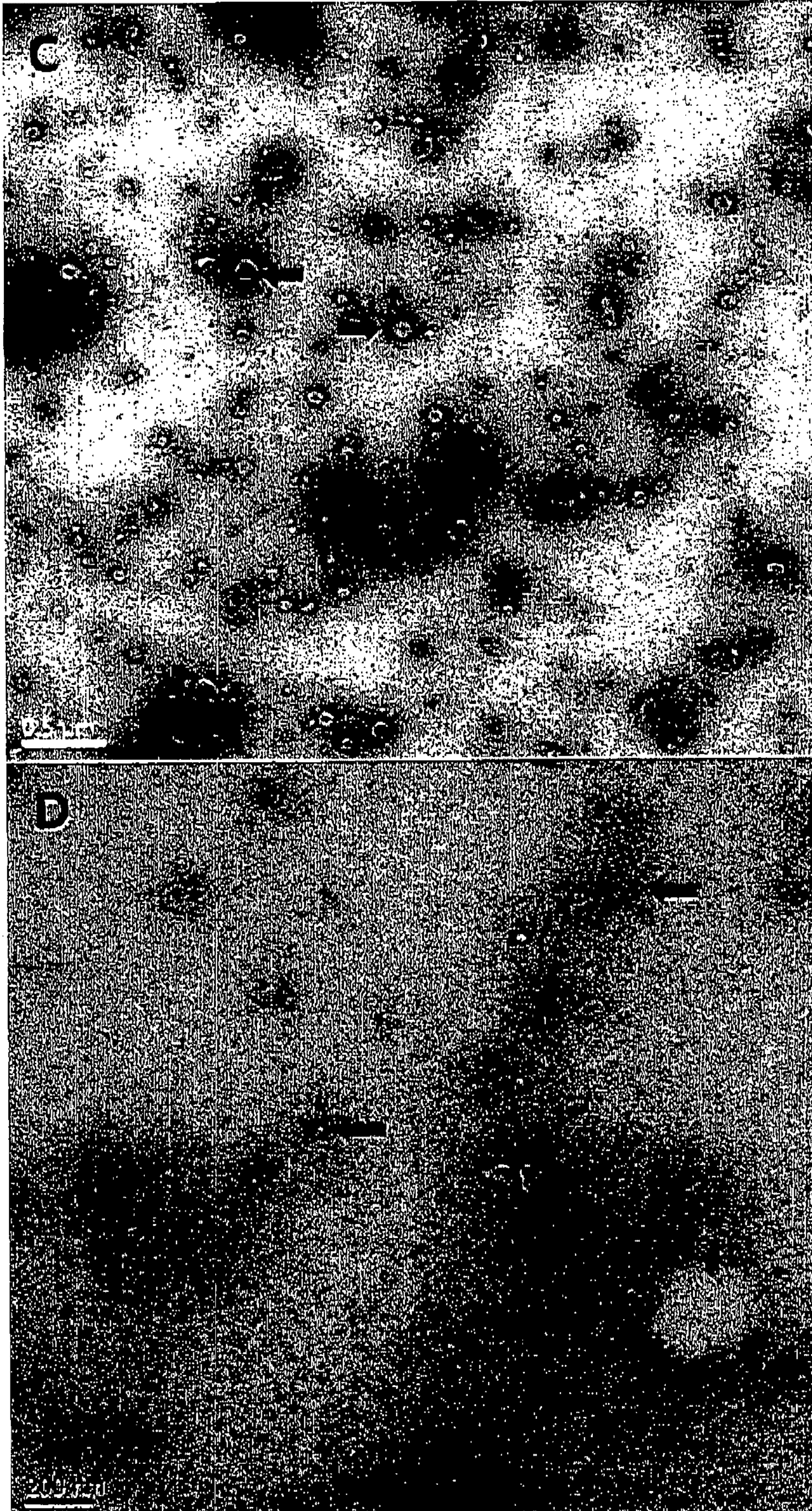

FIGURE 13 CONT

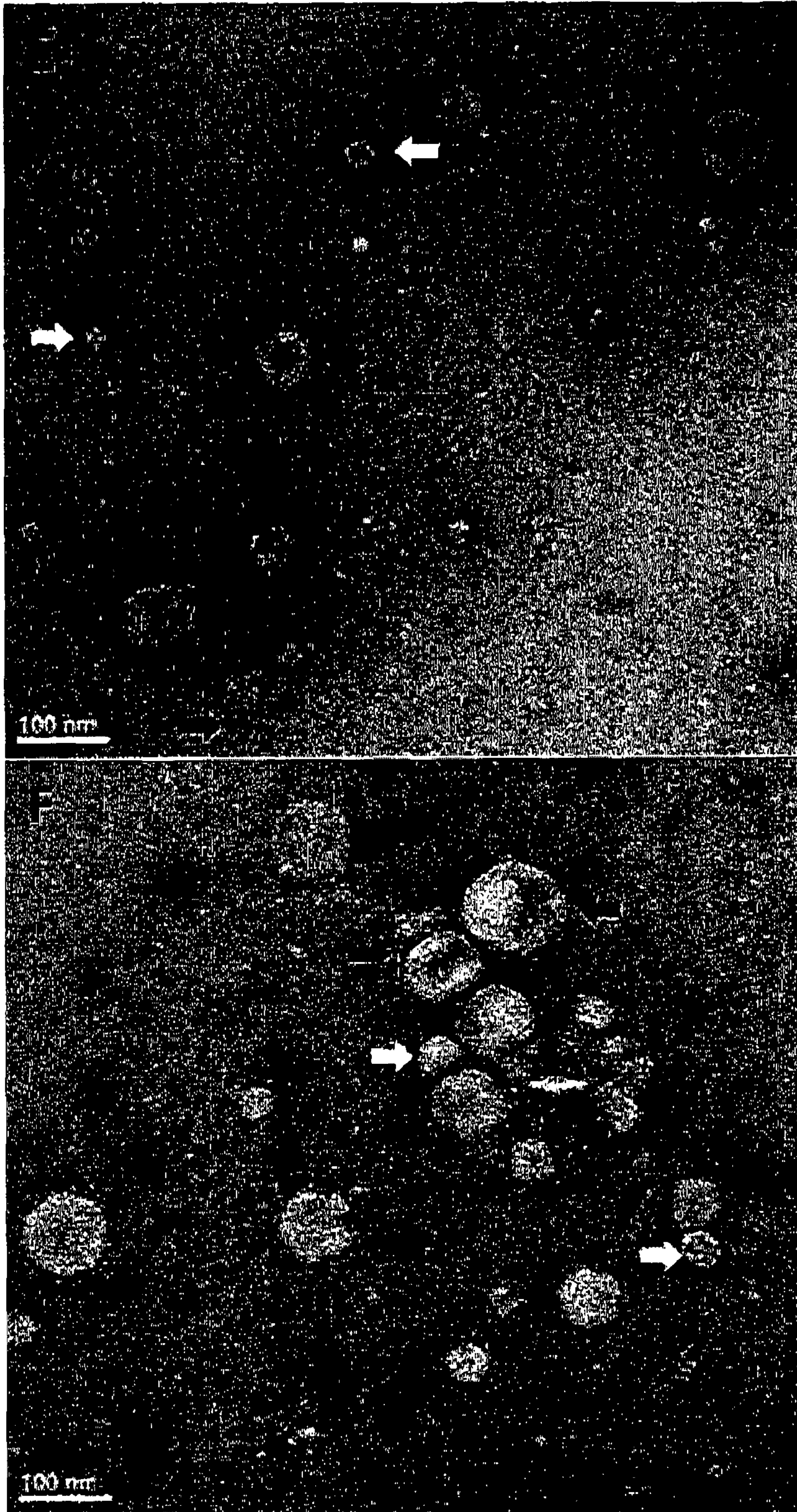

FIGURE 13 CONT

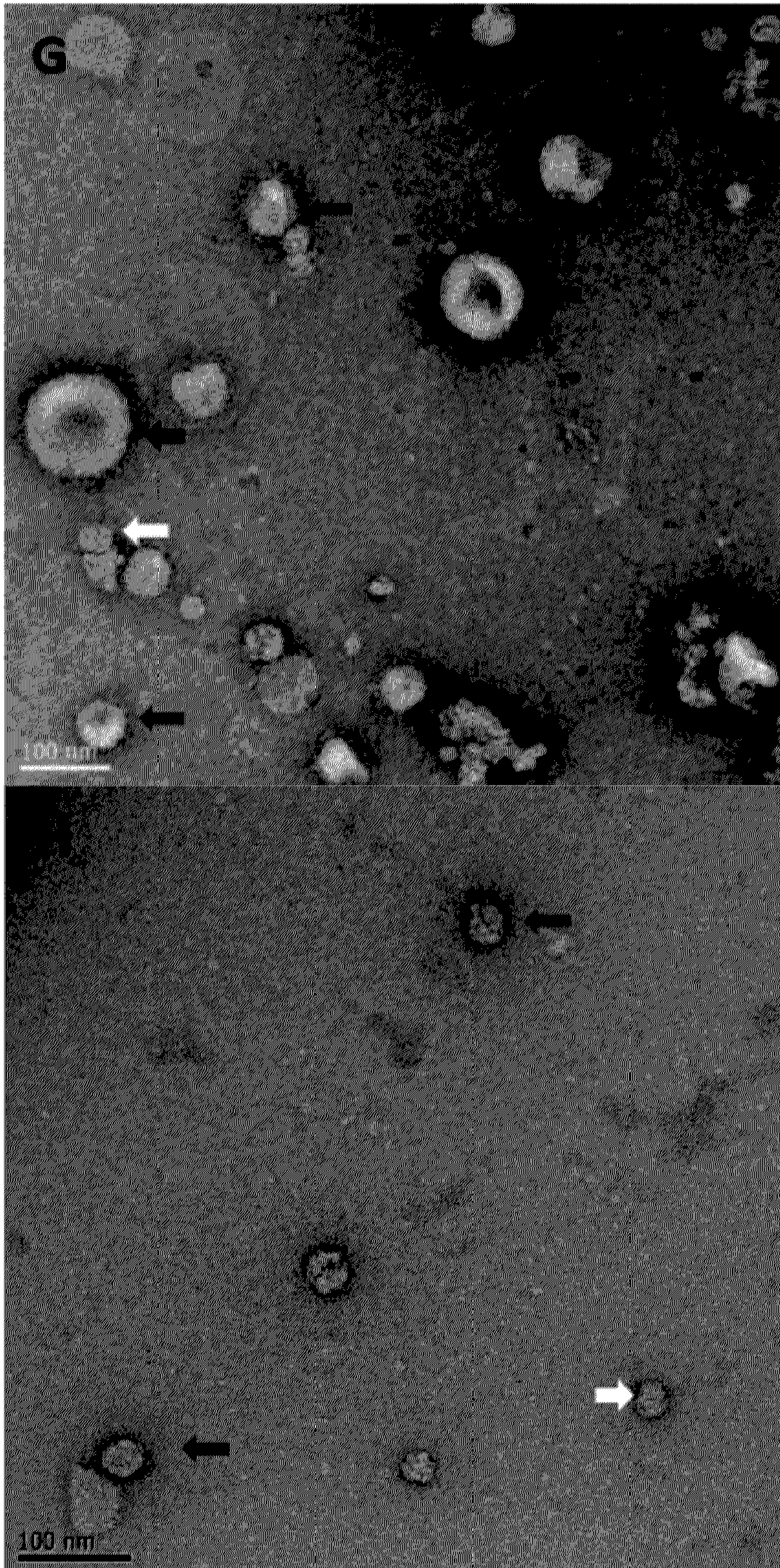

FIGURE 13 CONT

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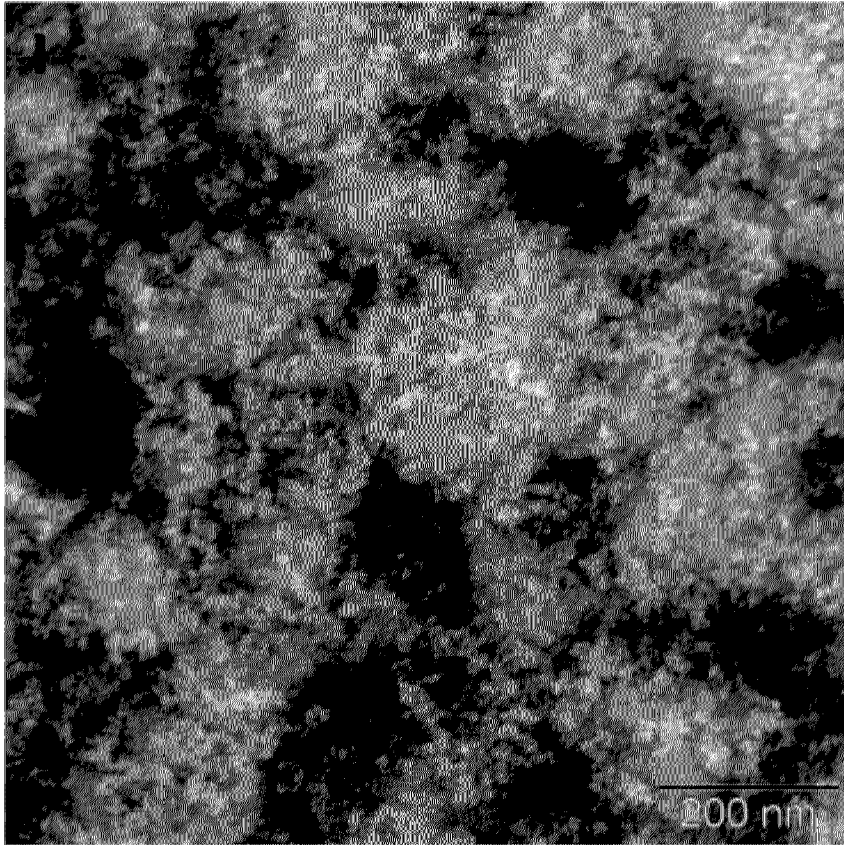

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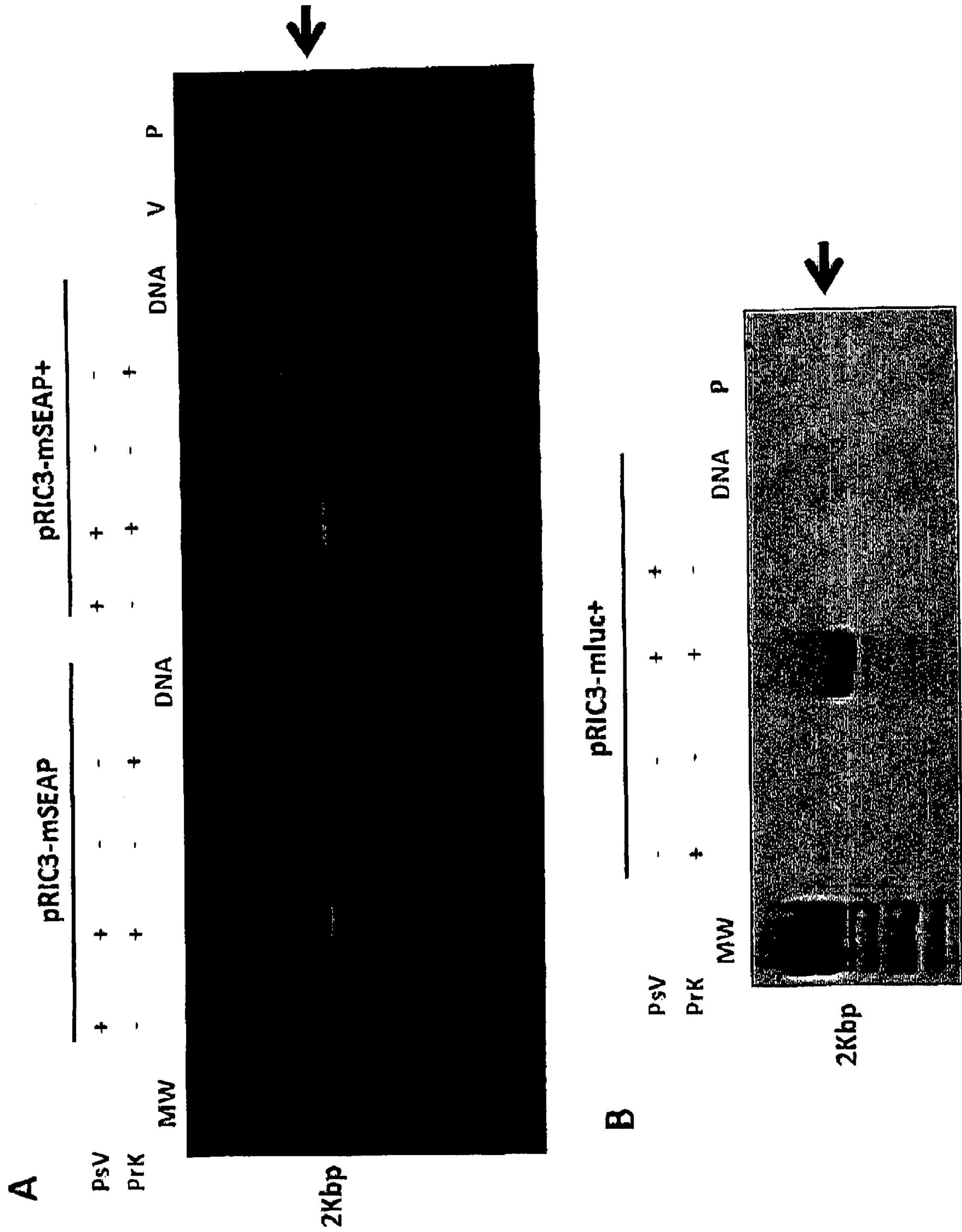

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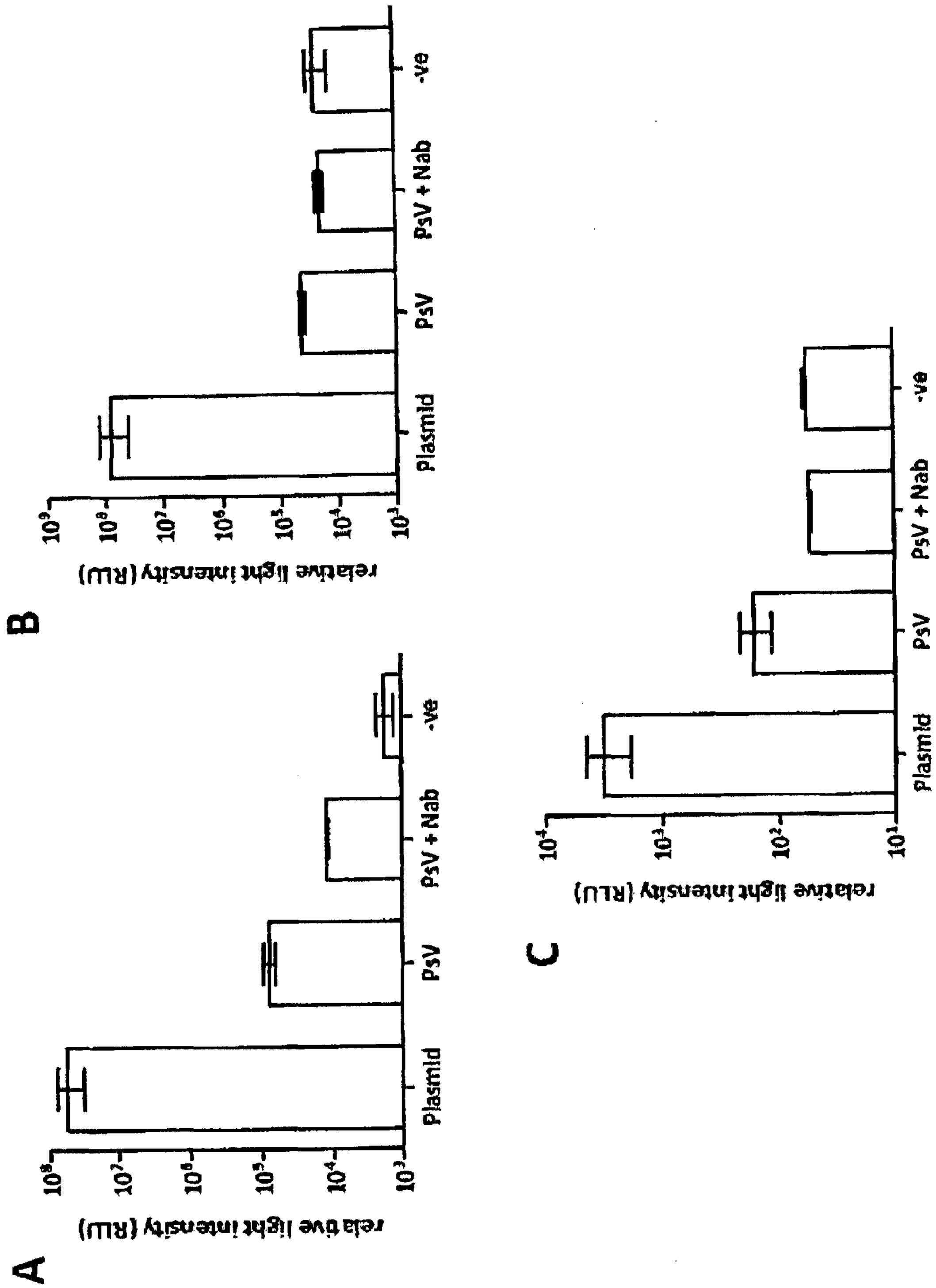

FIGURE 15

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FIGURE 16

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agtggattga tgtgatatct ccactgacgt aagggatgac gcacaatccc actatccttc    3720
```

FIGURE 17

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gttgtcgggc agcagcacgg ggccgtcgcc gatgggggtg ttctgctggt agtggtcggc     4080
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ccccaggatg ttgccgtcct ccttgaagtc gatgcccttc agctcgatgc ggttcaccag     4260
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FIGURE 17 CONT

SUBSTITUTE SHEET (RULE 26)

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FIGURE 18

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FIGURE 18 CONT

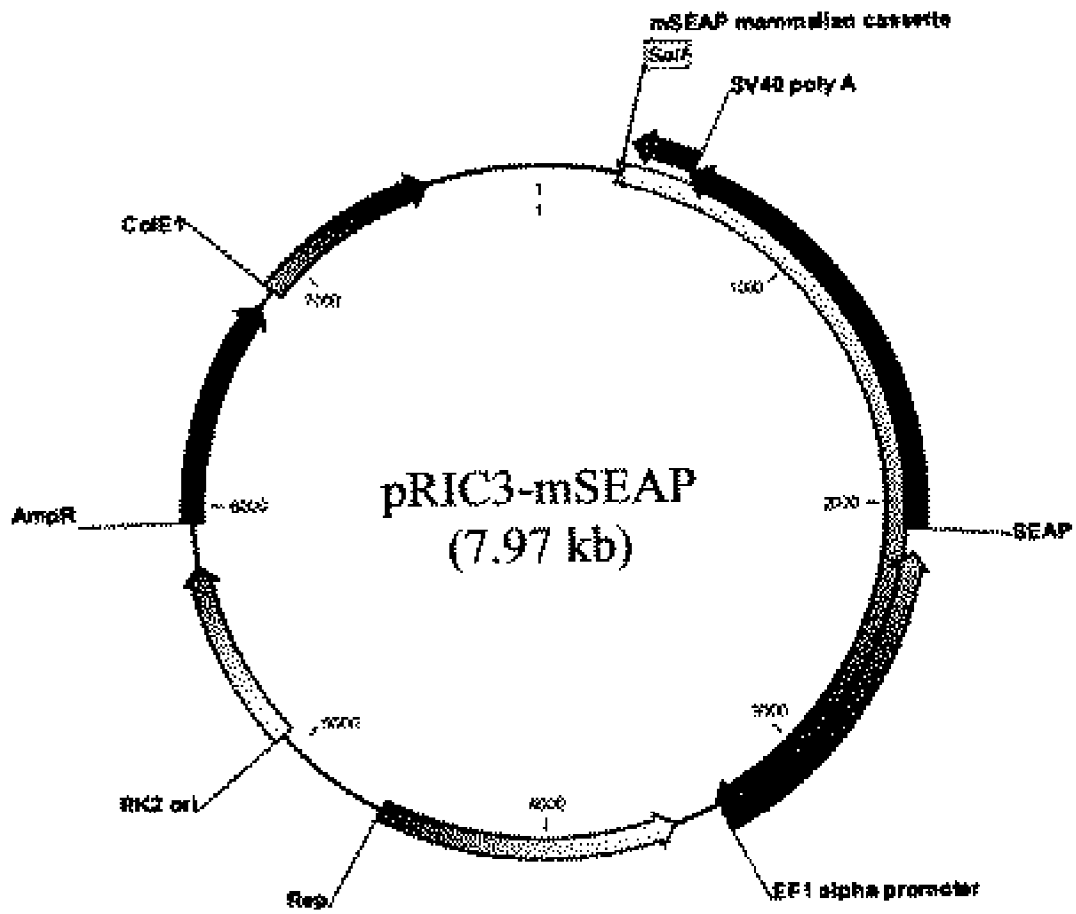
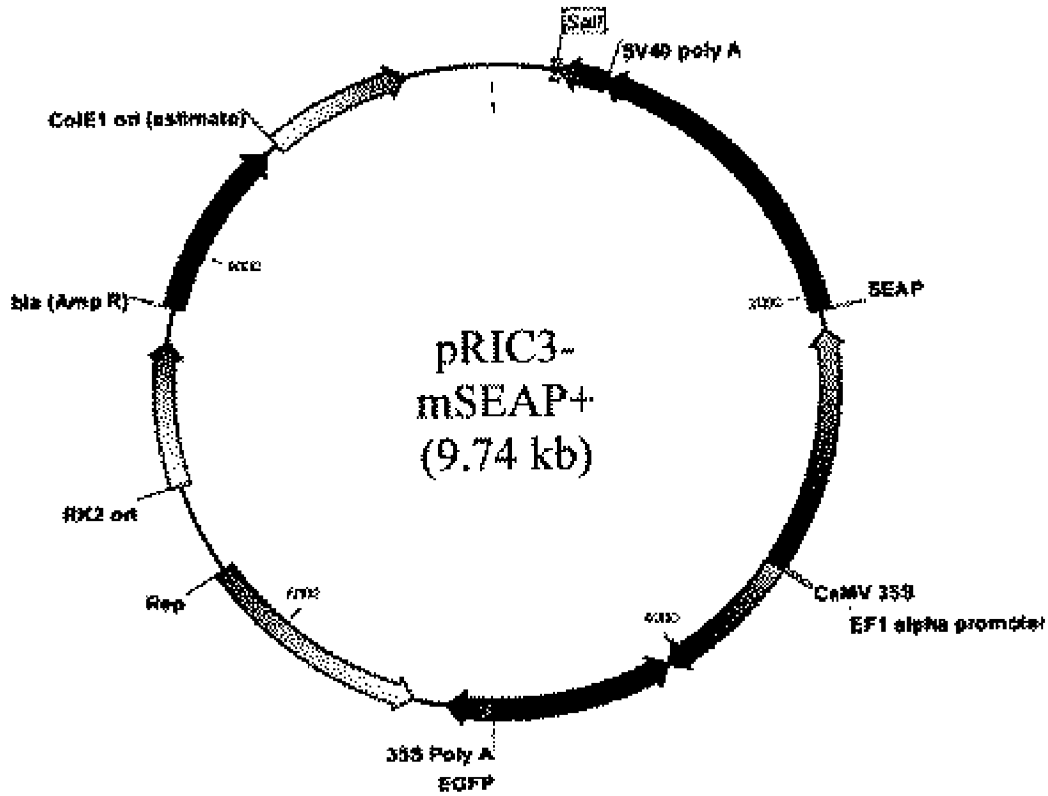
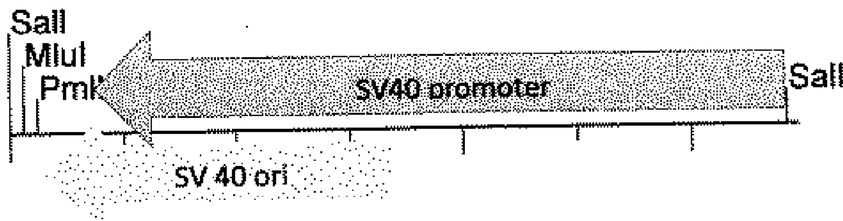
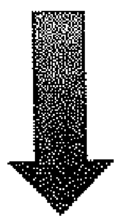


FIGURE 19

Date Reçue/Date Received 2020-12-16







Sal I digest of vector and SV40 ori+promoter gene and Ligation

FIGURE 19 CONT

Date Reçue/Date Received 2020-12-16

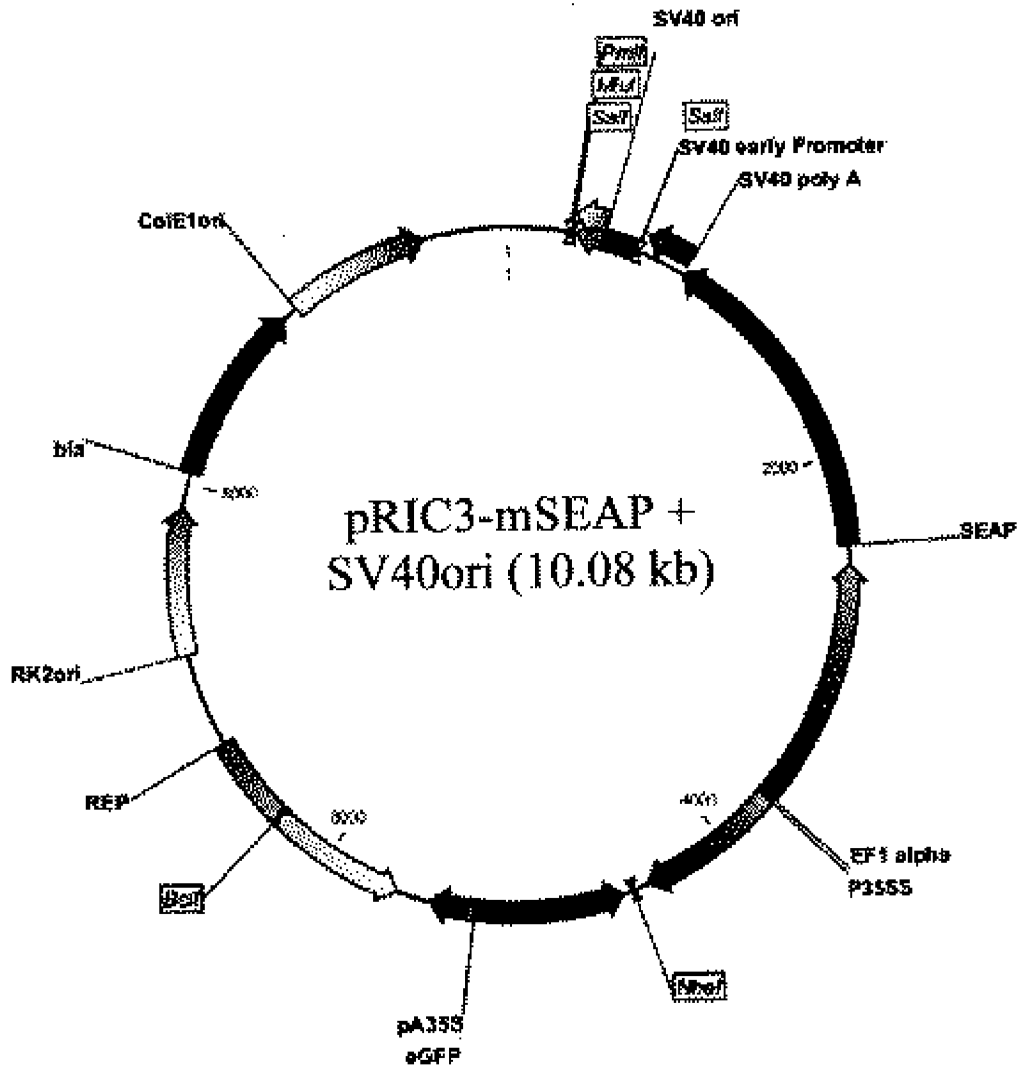


FIGURE 19 CONT

Date Reçue/Date Received 2020-12-16

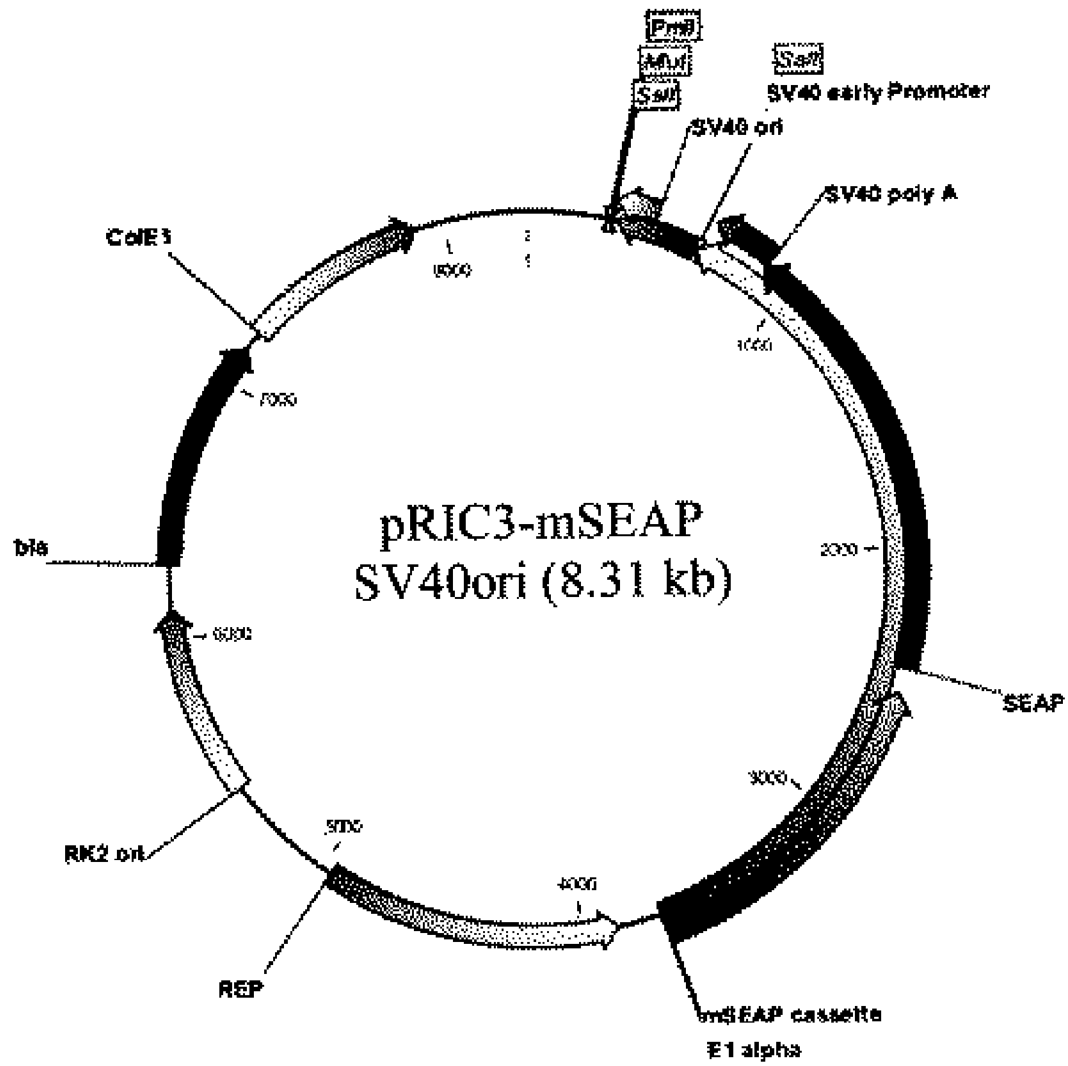


FIGURE 19 CONT

Date Reçue/Date Received 2020-12-16

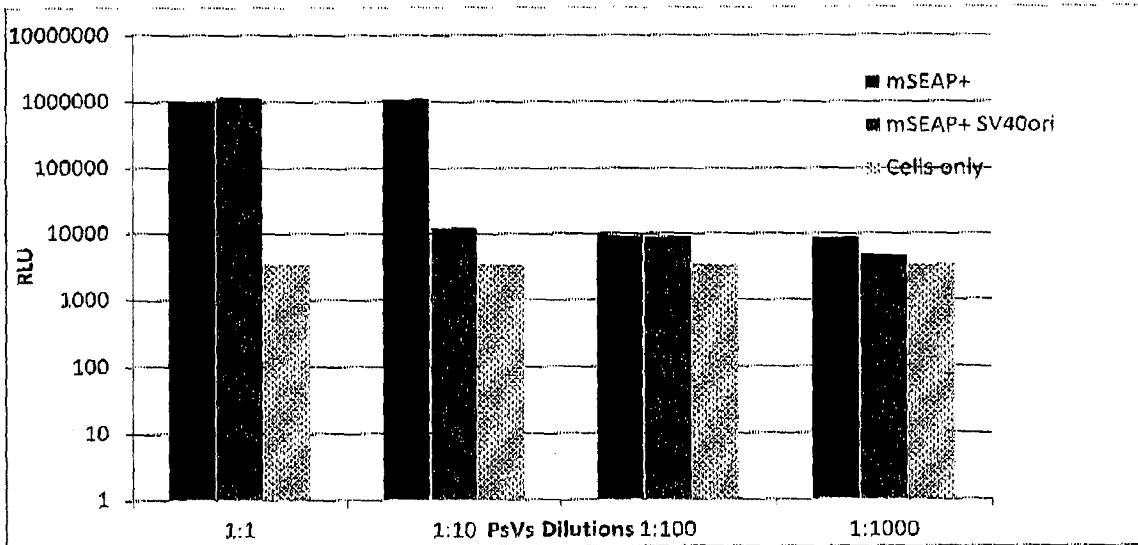


FIGURE 20