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<p>(21) International Application Number: PCT/NL98/00717</p> <p>(22) International Filing Date: 17 December 1998 (17.12.98)</p> <p>(30) Priority Data: 97203989.5 18 December 1997 (18.12.97) EP</p> <p>(71) Applicants (for all designated States except US): STICHTING INSTITUUT VOOR DIERHOUDERIJ EN DIERGEZONDHEID [NL/NL]; Edelhertweg 15, NL-8219 PH Lelystad (NL). BAYER AKTIENGESELLSCHAFT [DE/DE]; Konzernbereich RP, Patente und Lizenzen, D-51368 Leverkusen (DE).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): KRETZDORN, Dietmar [DE/DE]; Landrat-Trimborn-Strasse 61, D-42799 Leichlingen (DE). VAN DE WIEL, Dirk, Franciscus, Marinus [NL/NL]; Lijsterbeslaan 17, NL-3843 JJ Hardewijk (NL). DE SMIT, Abraham, Johannes [NL/NL]; De Patrijs 10, NL-8251 MB Dronten (NL). MOORMANN, Robertus, Jacobus, Maria [NL/NL]; De Telgang 12, NL-8252 EH Dronten (NL). HAMANN, Erik, Kees [NL/NL]; Gondel 1601, NL-8243 BT Lelystad (NL).</p>	<p>(74) Agent: OTTEVANGERS, S., U.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i></p>	
<p>(54) Title: PROTEIN EXPRESSION IN BACULOVIRUS VECTOR EXPRESSION SYSTEMS</p>		
<p>(57) Abstract</p> <p>The invention relates to a method to increase protein expression in baculo vector virus expression systems. The invention provides a method to produce a recombinant protein in insect-cell culture which comprises selecting a recombinant baculovirus expressing said protein, growing insect cells in growth medium in a culture vessel with a sufficient volume to contain at least 2 liters and infecting the cells with an inoculum of at least one baculovirus at a cell density of <math>1 \times 10^5</math> to <math>5 \times 10^6</math> cells/ml with an m.o.i of <math>&lt;0.01</math>. The invention furthermore provides a method to produce recombinant pestivirus E2 or E<sup>ms</sup> protein or fragments thereof in insect cell culture characterised by a final concentration of said protein (fragments) in the growth medium at harvest of at least 100 <math>\mu\text{g/ml}</math>. The invention furthermore provides a method to produce recombinant follicle stimulating hormone, <math>\alpha</math>-units and/or <math>\beta</math>-units and complexes and fragments thereof, at a concentration in the growth medium at harvest of at least 15 <math>\mu\text{g/ml}</math>.</p>		

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Title: Protein expression in baculovirus vector expression systems.

The invention relates to methods to increase protein or polypeptide expression in baculovirus vector expression systems. When recombinant-DNA techniques were developed, expectations were high regarding to large-scale protein production using genetically modified bacteria. The majority of commercially attractive proteins, however, necessarily undergo post-translational modifications before they can become biologically active proteins. Hence animal cells are now more frequently used to produce recombinant proteins.

Among animal cells, insect cells are of growing importance for the production of recombinant proteins. A convenient and versatile baculovirus vector system using insect cells has been developed. Information on the physiology of insect cells is rather scarce, however, vaccines produced via baculovirus recombinant techniques are generally well accepted. An example is the use of a baculovirus-expressed gp 160 envelope protein of human immunodeficiency virus type I as a possible AIDS vaccine in clinical trials.

Until now large-scale production of baculovirus-expressed proteins in insect cells is limited to bioreactors of up to about 10 liters. For scale up, suspension cultures offer the best possibility. In large scale production (see Tramper et al., Rec. Adv. Biotech., 1992, 263-284; Power and Nielsen, Cytotechnology 20: 209-219, 1996) special emphasis should be given to factors influencing cell growth and virus production. Variations in such factors greatly influence the final level of recombinant protein production.

Baculoviruses are characterised by rod-shaped virus particles which are generally occluded in occlusion bodies

Baculoviruses are characterised by rod-shaped virus particles which are generally occluded in occlusion bodies (also called polyhedra). The family Baculoviridae can be divided in two subfamilies: the Eubaculovirinae comprising two genera of occluded viruses: nuclear polyhedrosis virus (NPV) and granulosis virus (GV), and the subfamily Nudobaculovirinae comprising the non-occluded viruses. The cell and molecular biology of *Autographa californica* (Ac)NPV has been studied more in detail.

10 Many proteins have been expressed in insect cells infected with a recombinant baculovirus encoding that protein. Encoding means that such viruses are provided with a nucleic acid sequence encoding a heterologous protein, and often are further provided with regulating nucleic acid sequences, such as a promoter. Most often the polyhedrin promoter is used to express a foreign gene, but the p10 promoter is equally well suitable and used as well.

20 Several cell-lines are available for infection with recombinant baculovirus. The cell line SF-21 was derived from ovarian tissue of the fall armyworm (*Spodoptera frugiperda*). A clonal isolate, SF-9, available from the American Type Culture Collection (CRL 1711) is more or less a standard cell-line for *in vitro* production of recombinant virus and is said to be superior in producing recombinant virus. Other cell-lines are e.g. the Hi-Five cell-line and the Tn-368 and Tn-368A cell-lines obtained from the cabbage looper (*Trichoplusia ni*). The most widely used media in which insect cells grow include TNM-FH, BML-TC/10, AND IPL-41. These media are usually supplemented with more or less defined components, such as mammalian sera, in particular foetal calf serum. Serum replacements have also been applied to insect-cell culture, and serum-free media, such as Ex-cell 400™ and Sf900 are commercially available.

Insect cells in general grow on solid supports as well as in suspension, but are reported to give higher yields of virus when grown on solid supports. Infection is most efficient when cells are infected in the exponential growth phase but the amount of polyhedra and virus produced per cell, however, does not vary significantly between cells infected during different stages in the cell cycle. Cell density has great influence on virus production. Insect cells can show a form of contact inhibition resulting in reduced virus production at higher cell densities.

The initial multiplicity of infection (m.o.i.), which is the number of infectious viruses per cell, generally influences both the fraction of infected cells and the number of polyhedra per cell at the end of infection. Optimal m.o.i. for virus production is generally considered to be at around 20-30. In a study (Licari and Bailey, Biotech. Bioeng., 37: 238-246, 1991) with a recombinant baculovirus expressing  $\beta$ -galactosidase, Sf-9 cells were infected with m.o.i. values between 0 and 100. The  $\beta$ -galactosidase yield increased and cell density decreased with increasing m.o.i. It is generally thought that increasing or decreasing m.o.i. has only a limited effect on the maximum achievable yield of a recombinant protein per infected cell. Choosing low m.o.i. however, allows reduction of virus stock needed for infection and minimises the risk of the generation of defective interfering particles of baculovirus. If a batch culture of insect cells is infected at high m.o.i. (>5), the ensuing infection process will be essentially synchronous, i.e. all cells will go through the infection cycle simultaneously. When cells are infected at an m.o.i. 1-5 in a batch culture, the culture will no longer be synchronous. The culture will initially be composed of non-infected cells and cells at different points in their individual infection cycle, until all cells have been infected and the production of wanted

protein comes to an end. In general, in such cultures the production levels are much lower. The culture behaviour is the combined behaviour of the individual cells that are each in a different phase of production; explaining the sub-optimal production levels. In a continuous culture non-infected cells are added continuously and the culture will obviously be asynchronously infected.

Through designing mathematical models it is thought possible to predict complex behaviours such as those observed when infecting cells at low m.o.i. or when propagating virus in a continuous culture system. A purely empirical analysis of the same phenomena is considered very difficult if not impossible. At present, three models are known, the Licari & Bailey, the de Gooijer and the Power & Nielsen model. These are, despite their complexity and the effort that has gone into developing them, all first generation models, postulating about the behaviour of baculoviruses expressing a model recombinant protein ( $\beta$ -galactosidase) expressed under control of the polyhedrin promotor. They summarise, to a large extent, our present quantitative understanding of the interaction between baculovirus and insect cells, when looked upon as a black box system, with disregard to DNA and RNA accumulation and the infection cycle. The binding and initial infection processes are still quantitatively poorly understood and further work in this area is much needed.

The baculovirus expression system offers a powerful tool for recombinant protein production. Several cell-culture configurations can be used for large-scale production. These systems, however, need further optimisation to take full advantage of their potential. For commercial application large-scale and low-cost production is pivotal. Polyhedra-production systems reported in large-scale cell cultures should be dramatically improved to meet the commercial demands for a price-competitive product.

The invention provides a method for large-scale recombinant protein production using the baculovirus expression system allowing increased or improved yields of the wanted protein.

5 The invention provides a method to produce and improve yield of a recombinant protein in insect-cell culture which comprises selecting a recombinant baculovirus encoding said protein, growing insect cells in growth medium in a culture vessel, and infecting the cells with a multiplicity of  
10 infection of  $<0.01$ .

In a preferred embodiment, the invention provides a method to increase yield of a recombinant protein produced in insect-cell culture which comprises selecting a recombinant baculovirus encoding said protein, growing insect cells in  
15 growth medium in a culture vessel and infecting the cells with an inoculum of at least one baculovirus with a multiplicity of infection of  $<0.01$ . Increasing yield has been a topic of several research groups. For example, Chen et al, (Drug metabolism and Disposition: 24 p399-405, 1997) studied  
20 the possibility of optimising the MOI for a co-infection approach, whereby two different baculovirus expressed proteins were produced in insect cell culture. Contrary to the results described herein they find for their two proteins a best MOI of approximately 0.015 to 0.03. Reducing the MOI  
25 to  $<0.01$  reduced the yield of the co-infection system of Chen et al. Radford et al (Cytotechnology 24, 73-81, 1997), not being hindered by studying a co-infection system, clearly indicate that MOI's  $>1$  should always be used to maximise final process yields, again teaching against the findings of  
30 the present invention. They state that it is impossible to produce larger amounts of protein and virus per cell using low MOI, and suggest adjusting the time of infection (TOI) instead.

Others, such as Nguyen et al ( J.Biotech. 31, 205-217, 1993) do not find a solution in changing MOI, but aim at increasing yields by applying fed-batch cultures, or change the temperature under which the virus is grown (Wu et al, 5 Cytotechnology, 9, 141-147, 1992; King et al, Biotechnol. Bioeng, 38, 1091-1099, 1991) and avoid growing the virus under MOI <0.01.

These earlier results clearly differ from those provided in this description (see for example figure 1), 10 where cultures infected with an MOI <0.01 (such as 0.003, 0.001, or even 0.0001) reached higher yields than those infected with MOI 0.01 or 0.1.

A preferred embodiment of the invention provides a method to produce and improve yield of a recombinant protein 15 in insect-cell cultures not grown in monolayer cultures. Conventional laboratory methods to produce proteins in the baculovirus expression vector system in small amounts use monolayer cultures of insect cells in culture flasks. These static cultures are normally infected with a high MOI, to 20 ensure synchronous infection. It is pivotal that all cells are infected before the monolayer has become confluent, since contact inhibition will lead to metabolic changes in the cells, which may affect the final product yield. Since it is difficult to accurately establish the cell 25 density in monolayer cultures it is impossible to carry out MOI experiments. It is even more useless to use a low MOI (<0.01) to infect the cultures. Both over- and underestimation of the cell density at the time of infection will lead to a significantly suboptimal protein yield. 30 Routinely, one uses a high MOI, which guarantees a synchronous infection of the total cell population. This implies that large virus stocks are needed to infect the monolayer culture to achieve optimal yields.

Scale-up of protein production using monolayer cultures 35 simply means using more tissue culture flasks. The production

of large amounts of protein using monolayer cultures is very labour-intensive. Furthermore it is not possible to regulate and/or monitor important culture parameters such as dissolved oxygen concentration and pH.

5           The invention now provides a method suitable for all insect cell cultures the invention provides the insight that a low MOI is beneficial for optimising yield in insect cultures other than monolayer cultures.

10           Different types of culture vessels can be used for culturing insect cells other than in monolayer culture. The aim of every fermentor design is achieving sufficient aeration and high cell density, while keeping the shear forces as low as possible (Tramper et al. In: Recent advantages in biotechnology (eds. Vardar-Sukan and Sukan) 15 1992). In the majority of the cases described in the literature a stirred tank bioreactor equipped with a gas sparger is used. In this type of vessel, homogeneity is achieved by using an impeller. This type of fermentor can be operated in different methods. First of all, the batch 20 method. This is the most straightforward and simple method. Cells are cultured, virus is added and the product is harvested at the end of the infection. A more complicated method is fed-batch culture. A concentrated mixture of nutrients is added to the culture vessel to achieve higher 25 cell densities and higher volumetric product yields (Nguyen et al. 1993 Journal of Biotechnology vol. 31, p.205-217). This is more complicated since it is not always clear what the limiting nutrient is. Cancelling one nutrient limitation by adding this substrate may directly lead to another 30 substrate limitation and thus not necessarily to higher product yields.

          A different method of mixing is used in airlift bioreactors (Wu et al. 1992 Cytotechnology vol. 9 p. 141-147). This type of vessel consists of two cylinders. The 35 cylinder with the smallest diameter (draft tube) is placed inside the cylinder with the bigger diameter (downcommer). In

the center cylinder air or another gas is sparged, creating an upward flow. At the top of the fermenter the gas leaves the fluid and the fluid goes down outside the center cylinder. In this way, both aeration and homogeneity are achieved using sparging of gas only, due to the difference in density in the draft tube and the downcommer. This method may reduce shear stress. However, continuous aeration rates are needed to achieve proper mixing.

Another method to raise the living cell density in a fermentor is by including a spin filter or another cell-retaining device. This is called perfusion. This allows to remove waste medium from the fermentor and addition of fresh medium, while retaining the cells in the fermentor. This method results in a lot of extra equipment and more difficult fermentor operation (Caron et al. 1994 Biotechnology and Bioengineering vol. 43, p. 881-891). Furthermore, methods such as a macroporous-bed and immobilisation in a gel-matrix are reported. This type of methods relies on immobilisation of the cells on or inside a matrix, making it possible to remove waste medium and add fresh medium, without diluting the cell culture. If cell densities, contact inhibition and other related problems of monolayer cultures would be manageable, a method according to the invention could not only be applied in above various culture systems but in monolayer culture as well.

For example, a preferred embodiment of the invention provides a method to produce and improve yield of a recombinant protein in insect-cell cultures which comprises selecting a recombinant baculovirus encoding said protein, growing the insect cells in growth medium in a culture vessel with a sufficient volume to contain at least 2 liters and infecting the insect cells with an inoculum of at least one baculovirus with an m.o.i of  $<0.01$  PFU of said baculovirus/cell. The invention provides a method wherein multiplicities of infection are used that are considerably lower than for example the m.o.i. of 1-5 leading to an

asynchronously infected culture. By infecting insect cultures with a baculovirus using an m.o.i. as provided by the invention, an optimal balance is achieved between the speed of replication of the cells in relation to the speed of replication of the virus, thereby allowing optimal expression of the wanted protein. A method provided by the invention can be easily adjusted to higher or lower cell densities by adjusting the m.o.i. as well, wherein the relative ratio of virus particles available for infecting the cells in the various phases of replication remains according to the multiplicities and densities provided by the invention. A preferred embodiment of the method according to the invention comprises growing the cells in a culture vessel with a sufficient volume to contain at least 10, more preferably at least 20, more preferably at least 50 or 250 liters growth medium, thereby allowing scaling-up of baculovirus cultures expressing heterologous proteins. One can for example use a culture vessel with a volume that is larger than needed for the volume of growth medium that is present e.g. one can use 100L culture vessels to cultivate 20-70 liters cell-culture. A preferred embodiment of the method according to the invention comprises infecting the cells at a cell density of  $1 \times 10^5$  to  $5 \times 10^6$  cells/ml, more preferably at  $5 \times 10^5$  to  $1,5 \times 10^6$  cells/ml, thereby keeping the actual volume of the virus inoculum within easily manageable limits. Yet another embodiment of the method according to the invention comprises infecting the cells with an m.o.i.  $\leq 0.005$ , such as 0.003, 0.001, 0.0005 or 0.00025 whereby the inoculum is kept as small as possible. A preferred embodiment of the method according to the invention comprises selecting a recombinant baculovirus expressing the wanted protein under control of the p10 promotor. The p10 promotor is playing a role in cell lysis, absence of the p10 protein causes the infected cells to remain intact and prevents the release of polyhedra from

infected cells, thereby reducing reinfection rates but not infectivity per se. The whole process of virus infection can now be checked visually, due to the fact that the polyhedrin gene, and thus the polyhedra, are still present. Virus  
5 infection can be observed as dense protein particles that accumulate in the cell nucleus. Another embodiment of the method according to the invention comprises growing the insect cells in a batch culture system, thereby minimising the accumulation of defective interfering baculovirus  
10 particles, which can compromise infection and replication, of yet uninfected cells. Another embodiment of the method provided by the invention comprises growing the insect cells in suspension, preferably in a culture vessel, such as a fermentor, which can be (moderately) stirred. The use of  
15 (stirred) suspension cultures, especially when combined with using a recombinant baculovirus wherein the wanted protein is under control of the p10 promotor to visually check virus growth (but other methods of checking, e.g. in the case of using the polyhedrin promotor, such as observing CPE are also  
20 available) allows for a better control of the culture.

Moderate stirring of the suspension guarantees a homogenous culture in which no substrate gradients are built up and in which the cells are not subjected to too high shear forces. Furthermore, stirring results in an efficient  
25 transfer of viruses from infected to non-infected cells, giving a higher efficiency of virus infection of cells. Since initially only about 0.1-0.3% of the cells is infected, the remaining 99.7-99.9% of cells are allowed to grow and multiply.

30 The invention provides a method to express and produce recombinant protein of various origin. An example provided by the invention is the production of pestivirus derived protein to a concentration of said protein in the growth medium at harvest of at least 100, 120 or 150 µg/ml, more preferably at

least 200 or even 300 µg/ml. The wanted protein can also be used to prepare antigenic substances for veterinary or medical use, e.g. incorporated in a vaccine or in a diagnostic test. The wanted protein produced by a method according to the invention can for example be used to prepare a vaccine.

An example provided by the invention is the pestivirus E2 protein or fragments thereof, which can for example be used to prepare a vaccine against pestivirus infections, such as classical swine fever in pigs. In a preferred embodiment, the invention provides a vaccine comprising recombinant pestivirus E2 or E<sup>rns</sup> protein or fragments thereof characterised in that it is not being immunoaffinity purified and preferably confers protection against a pestivirus infection at the PD95 level after one single vaccination with one dose. This is particularly relevant for CSFV vaccination. When applied, CSFV vaccination generally is performed during a mass campaign in an area where an outbreak of CSFV has occurred. This calls for rapid vaccination of large numbers of animals in a relatively short period. In such a mass campaign it is of imminent importance that an adequate protection level (the number of pigs that are protected against the wild type virus infection) is achieved rapidly. Waiting for several weeks after a first vaccination for a second vaccination in order to achieve protection greatly hampers and delays the control of the disease. Differences between various methods to produce the recombinantly expressed E2 protein, even when comparing E2 (fragments) expressed in baculovirus, exist. In earlier reported E2 protein production cultures, the E2 protein (fragment) yield varied between 20-90 µg/ml (Hulst et al., J. Virol. 5435-5442, 1993; Hulst and Moormann, Cytotechnology 20:271-279, 1996), further necessitating immunoaffinity-purification with monoclonal antibodies to obtain the necessary and relevant E2

antigenic mass for single shot vaccination. Another method (using a fragment of E2 described in EP 0389034), which uses E2 harvested from the supernatant of insect cells without further immunoaffinity purification, results in an E2 based vaccine that is injected twice before a satisfying (protective) immune response is obtained. Although a vaccine (Porcilis®Pesti) comprising E2 antigen is currently registered, this vaccine needs to be applied twice, thereby seriously hampering the usefulness of vaccinating against classical swine fever infections with this vaccine since it takes at least two vaccinations, with a 4 weeks interval, to provide the wanted immune response.

These problems (which are solved by the present invention), among others, relate to a low concentration of the relevant antigenic substance, in this case the E2 protein (fragments), in the starting material, e.g. the cell culture supernatant, from which the vaccine is prepared. In theory, one can further accumulate antigenic mass by purification and condensation methods known in the art, however, this does not lead to a commercially attractive vaccine production but causes high costs per dose. Another example, is the pestivirus E<sup>ns</sup> protein, which can also be used in a vaccine and in diagnostic tests, or in other (therapeutic) substances.

For example, Ruggli et al (Virus Genes 10:115-126, 1995) grow a baculovirus expressing E2 in monolayer insect cell culture to a maximum yield of no more than 5-10µg/10<sup>6</sup> cells. Furthermore, Moser et al (Vet. Microbiol. 51:41-53) grow the E2 of Ruggli et al in monolayer insect cell culture using a MOI of 5 and cannot produce enough antigen in unconcentrated form for ELISA purposes. In their experience further purification by nickel-chelate affinity chromatography of the protein is a prerequisite to simplify

handling and improve ELISA quality. No vaccine preparation was contemplated with the thus prepared E2 protein.

When vaccination was the aim of the research, it was found that vaccination needed to occur twice, using an immunopurified E2 protein, to achieve a certain measure of protection. For example, in Hulst et al (J. Virol. 67:5435-5442, 1993), WO 95/35380, and van Rijn et al (J. Gen. Virol. 77:2737-2745, 1996), E2 was produced in monolayers and immunoaffinity purified to achieve a protective vaccine.

An example provided by the invention is a vaccine comprising recombinant CSFV E2 protein (fragments) which, now that sufficient large amounts can be produced, need not longer be immunoaffinity purified before it is incorporated in a vaccine that confers protection (at a protective dose level of 95% (PD95)) against a classical swine fever virus infection within two to three weeks after the animals received one single vaccination with one dose. A method provided by the invention provides a vaccine comprising recombinant pestivirus E2 or E<sup>rns</sup> protein or fragments thereof that confers protection against a pestivirus infection after one single vaccination with one dose, while the protein (fragment) has not been purified by immunoaffinity. The invention also provides a vaccine comprising a protein provided by the invention which additionally comprises an adjuvant. Suitable adjuvants are known to the average person skilled in the art, e.g. Freund adjuvants, or aluminum hydroxide, or emulsion, such as water-in-oil or double water-in-oil or oil-in-water emulsions. The wanted protein can also be used to prepare other substances for example veterinary or medical use. Yet another example provided by the invention is a hormone-like substance such as the follicle stimulating hormone (FSH,  $\alpha$ -units and/or  $\beta$ -units and complexes and fragments thereof), which can for example be produced by infecting an insect cell culture with one baculovirus

expressing the  $\alpha$ -unit and/or with another baculovirus  
expressing the  $\beta$ -unit in the culture. A method according to  
the invention can also be used for large-scale and low-cost  
production of (recombinant) baculoviruses as bio-  
5 insecticides. A preferred embodiment is the use of  
recombinant viruses utilising the p10 promotor for foreign  
gene expression in the production of bio-insecticides since  
insects generally get less well infected by baculovirus  
lacking the polyhedra gene. Culturing other recombinant  
10 baculoviruses expressing other recombinant proteins with a  
method according to the invention and production and/or use  
of such viruses proteins for incorporation in insecticidal,  
medical, therapeutic, and/or antigenic substances or products  
is within the skills of the artisan. The invention is further  
15 illustrated in the experimental part but is not limited  
thereto.

**Experimental part**

The genus *Pestivirus* of the family *Flaviviridae* conventionally consists of classical swine fever virus (CSFV), border disease virus (BDV), and bovine viral diarrhea virus (BVDV). Genomes of several BVDV and CSFV strains have been sequenced (Renard et al., 1987 EP application 0208672; Collett et al., 1988, *Virology* 165, 191-199; Deng and Brock, 1992, *Virology* 1991, 865-679; Meyers et al., 1989, *Virology* 171, 555-567; Moormann et al., 1990, *Virology* 177, 184-188). For BDV, only incomplete genomic nucleotide sequences are yet available. The pestivirus genome is a positive-stranded RNA molecule of about 12.5 kilobases containing one large open reading frame. The open reading frame is translated into a hypothetical polyprotein of approximately 4,000 amino acids, which is processed by virus- and cell-encoded proteases. The open reading frame is flanked by two highly conserved small nontranslated regions, which are probably involved in the replication of the genome. The 5'-noncoding region also plays a role in initiation of translation.

The polyprotein which is co- and posttranslationally processed by cellular and viral proteases contains all the viral structural and nonstructural proteins (for review see C.M. Rice: In *Fields Virology*, Third Edition, 1996 *Flaviviridae: The Viruses and their Replication: Chapter 30: pp. 931-959*). The viral structural proteins, among which the envelope proteins E<sup>ns</sup>, E1 and E2, are located in the N-terminal part of the polyprotein. The nonstructural proteins among, which the serine protease NS3 and RNA replicase complex NS5A and NS5B, are located in the C-terminal part of the polyprotein. Animals infected with a pestivirus develop antibodies against E<sup>ns</sup>, E2 and NS3. However, only antibodies directed against E2 are strongly virus neutralizing. whereas those directed

against E<sup>ns</sup> and NS3 have only a low virus neutralizing capacity or none at all. This knowledge prompted us to start evaluation the suitability of E2 as CSF subunit marker vaccine. In this setup, E<sup>ns</sup>, and/or NS3 could be used for  
5 development of the diagnostic test accompanying the E2 marker vaccine.

To date, BDV and BVDV have been isolated from different species, whereas CSFV seems to be restricted to swine. Pestiviruses are structurally and antigenically closely  
10 related. Envelope glycoprotein E2 is the most immunogenic and most variable protein of pestiviruses. We cloned E2 genes of many different pestivirus strains, including those from a deer and a giraffe. The E2 genes were transiently expressed, characterized with monoclonal antibodies, sequenced and  
15 compared, P.A. van Rijn et al., 1997, Virology, 237: 337-348. Based on these data, we can delineate six major groups within the pestivirus genus. Four groups correspond to defined genotypes, whereas the two other groups are new genotypes within the pestivirus genus. One group comprises CSFV strains  
20 isolated from swine. A second group consists of BDV strains Moredun, L83 and X818, which have been isolated from sheep, and strain F from swine. A third group contains strain BD78 from sheep, strain 5250 from swine and strain 178003 from cattle. On the basis of E2, these viruses are very similar to  
25 BVDV strains associated with acute severe outbreaks of bovine viral diarrhea, so called type 2 BVDV. The fourth group consists of BVDV strains predominantly originating from cattle. This BVDV-group can be divided into two subtypes or subgroups BVDV-1a and 1b: BVDV-1a contains viruses from the  
30 USA, like NADL and Oregon, and some others, like 150022 and 1138 from Europe. Subgroup BVDV-1b contains strain Osloss and several Dutch isolates. The fifth and sixth "group" could be proposed as two new genotypes and contain strains Deer and Giraffe, respectively.

The development of marker vaccines for veterinary use has led to new concepts for control and eradication of economically important viral diseases of livestock. The use of a marker vaccine allows serological discrimination between vaccinated and field-virus infected animals, and thereby a controlled elimination of the virus. For instance, in most member states of the EU vaccination against Aujeszky's disease (AD) is allowed with gE-negative vaccines only. Animals infected with an AD field virus do develop antibodies against gE. An ELISA test which detects these antibodies is used for the detection of infected animals, which subsequently may be removed from a herd, and for monitoring the status of field virus infections in a herd during (prolonged) vaccination campaigns. Eventually, the aim is to reach a field virus free status of a herd. Vaccination can then be discontinued and a serological surveillance program to guard this status should come into force. Thus, vaccination with a marker vaccine will reduce the costs of eradication campaigns which rely on stamping out of infected herds, instead of only the infected individual animal, significantly, and if performed consequently on a large enough scale, and during a long enough period, may even be a faster method to reach the field virus free status of the pig population than stamping out.

Now and in the future, when current endemic diseases like AD have been eradicated, there is still use for marker vaccines, e.g. to control outbreaks of diseases which have been eradicated from the population, and against which there is no routine vaccination anymore. In such cases, where an animal population is serologically naive, highly contagious diseases may spread explosively, and can then cause enormous economic losses.

Many examples have shown that the baculovirus system supports high level expression of heterologous proteins. High

level expression is highly advantageous because dead subunit vaccines are generally only capable of eliciting a protective immune response when a large amount of antigen is applied.

In our first approach two recombinant baculoviruses, one  
5 expressing E2 with a TMR (BacE2[+]) and the other expressing E2 without a TMR (BacE2[-]), were constructed (Hulst et al., 1993, J. Virol. 67:5435-5442). Note that in this publication, and other comparable publications, E2 is still designated with its old name E1. E2 without a TMR was secreted into the  
10 cell culture medium whereas E2 with a TMR was not.

Furthermore, both E2s reacted identically with monoclonal antibodies representing each of the four antigenic domains on E2. This suggested that the antigenic properties of cell-associated and secreted E2 were identical, and because  
15 secreted E2 was produced to much higher levels than cell-associated E2 (tenfold higher), it was decided to test secreted immunoaffinity purified E2 in a vaccination trial in pigs. Two vaccine formulations containing 20 µg E2/ml and 100 µg E2/ml in a double water-oil adjuvant were prepared. Of  
20 four groups of 2 SPF pigs, two were vaccinated IM with 20 µg E2, and two with 100 µg E2. After 28 days, 1 group vaccinated with 20 µg E2 and 1 group vaccinated with 100 µg E2, were revaccinated with the same dose E2. At day 42 all animals were challenged intranasally with virulent CSFV strain  
25 Brescia. Regardless of the vaccine dose applied all vaccinated pigs had mounted neutralizing antibodies at day 28, though to different levels. Up till day 42, the day of challenge, antibody titers kept rising to high levels in all animals whether they were boosted or not. Therefore, it was  
30 not surprising that even animals which were vaccinated only once with a dose of 20 µg immunoaffinity purified E2, were already completely protected against CSF (Hulst et al., 1993 J. Virol. 67:5435-5442).

Because animals infected with pestiviruses invariably develop antibodies against E<sup>rns</sup> (Kwang et al., 1992, Vet.Microbiol. 32: 281-292), a second viral envelope glycoprotein, it is one of the viral proteins most suitable for diagnostic test development in conjunction with E2. However, there is also one report which suggests that E<sup>rns</sup> could be an important antigen for the protection of pigs against CSF (König et al., 1995, J. Virol. 69:6479-6486). In these studies E<sup>rns</sup> was expressed with a live virus vaccinia vector. Animals vaccinated simultaneously by 3 different routes (intradermally, intravenously, and intraperitoneally) with 5 x 10<sup>7</sup> PFU of vaccinia-E<sup>rns</sup> recombinant virus per injection site, survived an IN challenge with a lethal dose of CSFV strain Alfort at 5 weeks post vaccination, without showing any signs of CSF.

To evaluate the suitability of E<sup>rns</sup> as a dead subunit vaccine, baculovirus expressed E<sup>rns</sup> of strain Brescia (conform Hulst et al., 1994, Virology, 200: 558-565) was tested in pigs. Groups of 6 SPF pigs were vaccinated once IM with 5.0 and 20 µg E<sup>rns</sup>, respectively (Moormann et al., 1996, Proc. 14<sup>th</sup> Intern. Pig. Vet. Society Congress, pp. 25-29, Bologna, Italy). A third group of 4 nonvaccinated SPF pigs served as control. Three weeks after vaccination all animals were challenged IM with 10<sup>5</sup> TCID<sub>50</sub> of strain Behring. Within 5 days after challenge all animals vaccinated with the lowest dose E<sup>rns</sup> developed severe signs of CSF. Within 14 days after challenge, 2 animals died, 3 were killed when moribund, and 1 apparently recovered. Five of these animals, including the one that recovered, were positive in the IFT. After challenge, all animals vaccinated with the highest dose E<sup>rns</sup> showed more or less severe signs of CSF and high fever for several days, but within 14 days, 4 of 6 animals recovered, 1 animal had died, and another was killed when moribund. Of the 4 surviving animals only 1 appeared positive in the IFT. In

contrast, all control animals showed severe signs of CSF within 5 days after challenge, died within 14 days after challenge, and were positive in the IFT (Moormann et al., 1996, Proc. 14<sup>th</sup> Intern. Pig. Vet. Society Congress, pp. 25-5 29, Bologna, Italy).

We concluded that baculovirus expressed E<sup>2ns</sup> can protect pigs against a heterologous CSFV challenge, albeit with a much lower efficacy than baculovirus expressed E2 does.

Follicle stimulating hormone (FSH) belongs to the 10 family of glycoprotein hormones, which are produced either in the pituitary (luteinizing hormone, LH; thyroid stimulating hormone, TSH) or in the placenta (human chorionic gonadotropin, hCG; pregnant mare serum gonadotropin, PMSG). Within a species, each of these hormones consists of a common 15 alpha subunit, which is non-covalently bound to a hormone specific beta subunit. Purified FSH, administered alone or in combination with LH, is widely used to induce a superovulatory response in many species, including cattle.

A problem in the cow is, that bovine FSH is difficult 20 to purify in substantial quantities from bovine pituitaries. For this reason, FSH of ovine (oFSH), porcine (pFSH) or equine (eFSH, PMSG) origin is commonly used for superovulation treatment of cows. However, application of brain tissue derived material is not free of risk, due to the 25 possible presence of prion like proteins, which can cause bovine spongiforme encephalopathy (BSE) in cows, and possibly a variant of Creutzfeldt-Jacob disease (CJD) in humans. Furthermore, the use of placenta derived material has the disadvantage of a very long biological half-life, which 30 necessitates its neutralisation by the injection of specific antibodies. Finally, all currently used FSH preparations do contain some LH activity which is considered responsible, at least in part, for the observed large variation in superovulation results.

For these reasons it seems likely, that superovulation treatment of cows can benefit from the application of recombinant bovine FSH (rbFSH) produced in non-mammalian cells, such as insect cells (baculovirus expression system).

5

## **MATERIALS AND METHODS**

### **1. An example of the preparation of a production cell culture (PCS) of SF21 cells**

10 A cryo vial with 1.5 ml of SF21 working cell seed (WCS) (total number of cells is  $4-10 \times 10^6$  cells/ml) is thawed to a temperature of 20-30°C. After thawing, the content of the vial is transferred to a 15 ml Falcon tube, containing 8.5 ml serum free medium SF900II, and suspended. After suspension, 15 the content of the Falcon tube is centrifuged at 100-200xg for 10 minutes to precipitate the cells. The medium is discarded and the pellet suspended in 4-6 ml of SF900II. The suspended cells are transferred to a 100 ml shake flask, containing 10 ml SF900II. The cells are cultured for 3-7 days 20 at 26-30°C by placing the flask on an orbital shaker platform at 40-80 rpm. Cell growth and cell viability are monitored by taking in process samples. When the cell density is  $1.0-6.5 \times 10^6$  cells/ml, the cells are passed to two 500 ml shake 25 flasks, containing 100 ml SF900II each. This corresponds to a 10-fold dilution of the cells. Again, the cells are cultured for 3-7 days at 26-30°C by placing the flask on an orbital shaker platform shaking at 40-80 rpm. Cell growth and cell viability are constantly monitored by in-process control. When the cell density is  $1.0-6.5 \times 10^6$  cells/ml, the cells are 30 passed for a second time to, in this case, six to eleven 500 ml shake flasks, containing 100 ml SF900II each. Excess cell material is discarded. Also in this case, a 10-fold dilution is achieved. The cells are cultured for 3-7 days at 26-30°C by placing the flask on an orbital shaker platform

shaking at 40-80 rpm. Cell growth and cell viability are constantly monitored by in-process control. When the cell density is  $1.0-6.5 \times 10^6$  cells/ml, 500 ml or 1000 ml of the suspension, containing the cells, is passed to a 5 liter or  
5 10 liter fermentor, containing approximately 4.5 liter or 9 liter of SF900II, respectively. This corresponds to a 10-fold dilution of the cells. The cells are cultured for 3-7 days at 26-30°C. The suspension is constantly stirred (50-100 rpm). Cell growth and cell viability are constantly  
10 monitored by in-process control. When the cell density is  $1.0-6.5 \times 10^6$  cells/ml, the content of the 5 liter fermentor is passed to a 50 l fermentor, containing approximately 40 liter of SF900II. The cells are cultured at 26-30°C until a density of  $\pm 5-15 \times 10^5$  cells/ml is reached. The suspension  
15 is constantly stirred (50-100 rpm). Samples are taken for in-process control.

## **2. An example of the production of E2 antigen**

To the above mentioned cell suspension, 1-2 ml of  
20 Working Seed Virus (WVS) (BacE2[-]), containing  $\pm 10^7$  TCID<sub>50</sub>/ml is added. The suspension is incubated at 28°C for 3-8 days until 70-100% of the cells show a cytopathic effect. During the incubation, samples are taken for in-process control. Next, the suspension is clarified by removal of the cells by  
25 microfiltration. The obtained filtrate (i.e. the antigen solution) is collected and stored at  $\leq -20^\circ\text{C}$  until the inactivation step (3.3) is started. Samples are taken for in-process control. To determine antigen content, samples are  
30 for example tested in an enzym-linked immuno assay, to determine antigenic mass, or in a protein assay to determine actual weight per volume of the water protein, or by a combination of such methods.

### 3. An example of the virus inactivation

The virus is inactivated by adding 2-Bromoethyl-ammoniumbromide (BEA) to a concentration of 10 mmol/l. By adjusting the pH to 8.2-8.7 and the temperature at 34-37°C, BEA is converted to 2-bromoethyl-imminebromide (BEI), which is the active component to inactivate the virus. Virus kill is checked by taking samples for in-process control. The inactivation takes 24-72 hours. After inactivation, < 1 infectious particle per 10000 liter may be present. After virus inactivation, BEI is neutralized by adding sodium-thiosulphate to a concentration of 5-25 mmol/l and adjusting the pH to 5.7-6.3. Samples are taken for in-process control. The inactivated and neutralized antigen solution is transferred into 1 and/or 5 liter bags and stored at  $\leq -20^{\circ}\text{C}$ .

15

### 4. An example of the formulation

The frozen antigen solution is thawed at 22-28°C and diluted with SF900II or PBS to an antigen solution of 50 µg/ml. Thiomersal is added as an anti-microbiological agent to 100 µg/ml. Samples are taken for in-process control. This solution (i.e. the first water phase) is stored at 2-8°C for < 3 days. Meanwhile, the oil phase has been prepared by mixing Marcol 52 with Montanide 80 (9:1). Also this solution is stored at 2-8°C for no more than 3 days. The oil phase is sterile filtered through a 0.22 µm-sterile filter. Samples are taken for in-process control. Finally, the second water phase is prepared by mixing phosphate buffered saline (PBS) or SF900II medium with Montanox 80 (98:2), and thiomersal is added to a final concentration of 100 µg/ml. This solution is stored at 2-8°C until use (< 3 days). Before use, the first water phase is sterile filtered. Samples are taken for in-process control. The first emulsion is prepared by mixing the first water phase with the oil phase (1:1.1). This emulsion may be stored at 2-8°C no more than 3 days. Samples are taken

20  
25  
30

for in-process control. The double water-in-oil emulsion is prepared by emulsifying the first emulsion with the second water phase (2.1:1). The double emulsion is stored at 2-8°C in a quarantine storage room until filling in vials. Samples  
5 are taken for in-process control.

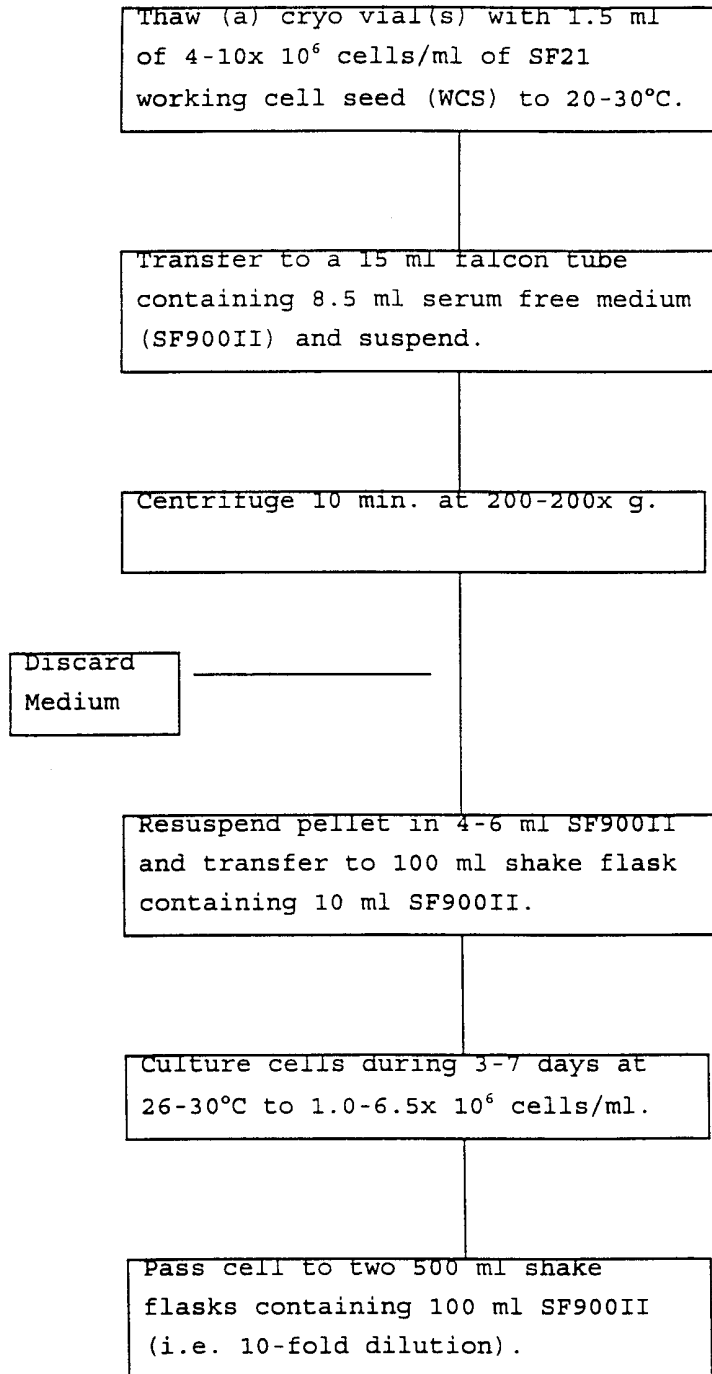
#### **5. An example of the filling and capping**

The double emulsion solution is filled aseptically in a class A zone in the clean room. The filling volume is 51, 102  
10 or 255 ml in 50, 100 or 250 ml vials, respectively. The filling volume is constantly monitored, by checking the weight of the filled volume. Immediately after filling, the vials are stoppered and capped. Finally, the vials are stored in a quarantine storage room at 2-8°C after which the  
15 quality control is initiated.

**Example of a scheme for 50 liter fermentor scale E2 subunit vaccine**

Preparation of an SF 21 Production cell culture (PCS)

5



Culture cells during 3-7 days at  
26-30°C to 1.0-6.5x 10<sup>6</sup> cells/ml.

Dilute cells 10-fold and pass  
cells to six to eleven 500 ml shake  
flasks containing 100 ml SF900II.

27

Culture cells during 3-7 days at 26-30°C to 1.0-6.5x 10<sup>6</sup> cells/ml.

Transfer 500 ml to a 5 l Fermentor, containing appr. 4.5 l SF900II or 1000 ml to a 10 l fermentor containing appr. 9 l SF900II.  
Culture cells during 3-7 days at 26-30°C to 1.0-6.5x 10<sup>6</sup> cells/ml.

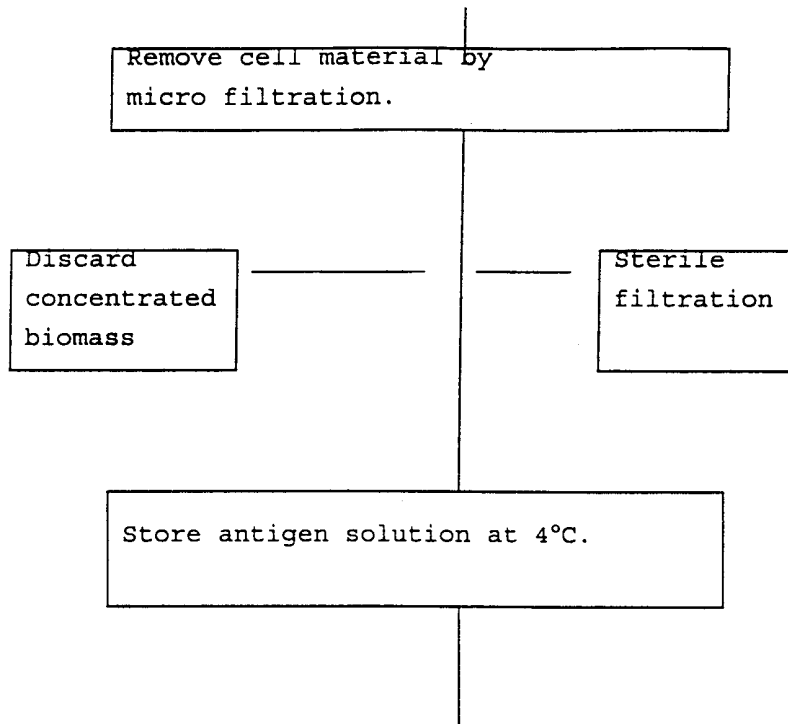
Transfer to a 50 Fermentor, containing appr. 40 l SF900II.  
Culture cells at 26-30°C to a cell density of 5-15x 10<sup>5</sup> cells/ml.

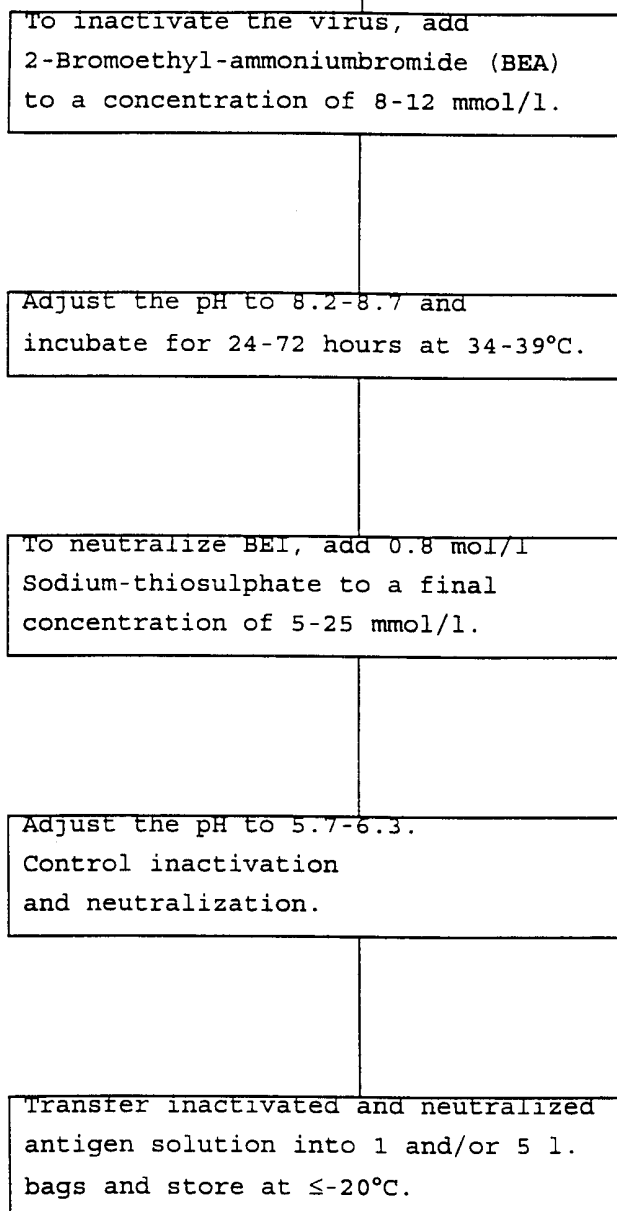
#### Production of E2 antigen

5

Add 1-10 ml of Working Seed Virus (WSV), containing ± 10<sup>7</sup> TCID<sub>50</sub>/ml.

Incubate at 28°C (3-8 days) until 50-100% of cells show Cytopathic effect (CPE).



Virus inactivation

Formulation

Prepare the oil phase by mixing Marcol 52 and Montanide 80 (9:1). The mix is sterile filtered (0,22  $\mu\text{m}$ ). Store at 2-8°C.

Thaw the frozen antigen solution at 22-28°C.

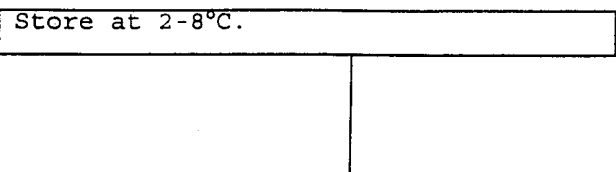
Prepare the first water phase by mixing the thawed antigen solution with SF900II to 50  $\mu\text{g}$  antigen/ml. Add thiomersal to 100  $\mu\text{g}/\text{ml}$ . Store at 2-8°C.

Prepare the second water phase by mixing PBS with Montanox 80 (0.98:0.02) and thiomersal to a final concentration of 100  $\mu\text{g}/\text{ml}$ . The phase is sterile filtered (0,22  $\mu\text{m}$ ). Store at 2-8°C.

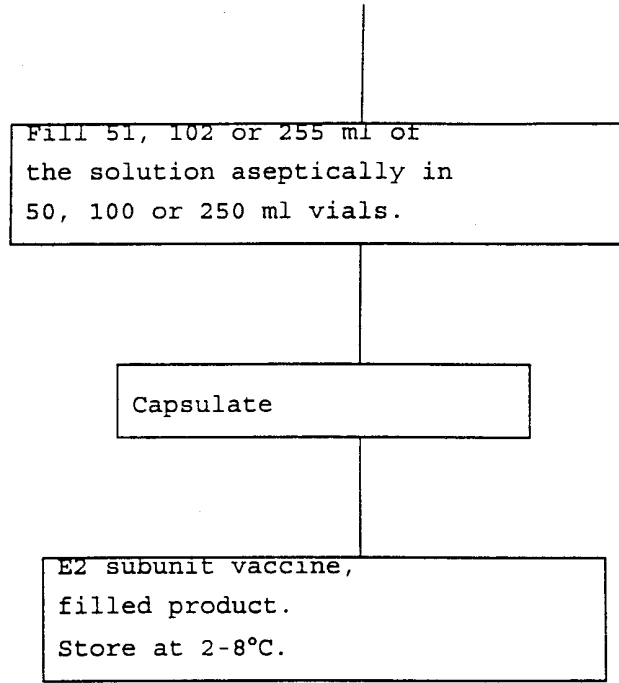
Prepare the first emulsion by mixing the oil phase and the first water phase (1.1:1). Store at 2-8°C.

Prepare the double emulsion by mixing the first emulsion with the second water phase (2.1:1).

Store at 2-8°C.



Filling and capping



## 6. An example of an E2 stability experiment

### Purpose

5 To investigate the stability of the E2 protein in an infected  
cell culture (MOI\*0.0001) after prolonged culturing under  
otherwise normal conditions. Due to the lytic nature of the  
infection process of the baculovirus in the cell culture,  
10 proteases might be released in the medium late in the  
infection cycle. Whether or not this results in degradation  
of the E2 protein and thus in a decrease of the volumetric E2  
protein levels, is the topic of this experiment.

### Materials and methods

15

When medium or SF900II is mentioned, SF900II medium with  
0.2% Pluronic F68 is meant.

A vial with SF21 cells is thawed and cultured in 10 ml  
of SF900II in a 100 ml shake flask in an incubator on an  
20 orbital shaker platform. When the cell culture reaches the  
required cell density, the cells are diluted tenfold to 100  
ml in a 500 ml shake flask. When this cell culture reached  
the required cell density, it was passaged by diluting  
tenfold to 100 ml again. Excess cell material was discarded.  
25 When this cell culture reached the required cell density it  
was diluted tenfold to three 500 ml shake flasks with 100 ml  
volume per flask. Excess material was discarded. The shake  
flasks are placed on an IKS orbital shaker platform, stirring  
at 70 rpm in an 28 °C incubator.

30 At a cell density of  $0.612 \cdot 10^5$  cells per ml, 63  $\mu$ l of a 100x  
diluted virus suspension is added to flasks nr. 2 and 3.  
Shake flask nr. 1 is kept as an uninfected blank. The MOI  
with which the cultures are infected is  
 $(0.063 \cdot 0.01 \cdot 0.8 \cdot 10^7) / (100 \cdot 0.612 \cdot 10^5) = 0.00008$

35 The 100x diluted virus suspension that was used, was  
prepared as outlined below. MSV vial 253 was thawed by QC and

diluted 100x in TC100 medium, supplemented with 10% FBS (lot nr. 97QC0139) and stored at -70°C in 0.5 ml portions.

Regularly, samples are taken from both infected shake flask cultures and the uninfected shake flask for  
5 determination of the condition of the cell culture. Sampling was performed as outlined below.

Approximately 1.1 ml of cell material is spun down in 15 ml Falcon tubes in IEC Centra GP8R centrifuge for 6 minutes at 1000 rpm. Then the supernatant is 0.45 µm filtered through  
10 a Gelman Acrodisc syringe filter to discard cells that might still be present. 0.4 ml samples are stored in Nalgene cryogenic vials at -70 °C for later analysis.

#### Results and conclusions

15

As can be seen in the data in figure 1 and the graphs in figure 2, the cell growth curves in flask 2 and 3 develop more or less similarly. After 191 hpi, no more cell counts were done, since virtually all cells were dead and infected.  
20 The 139 hpi sample of flask 2 is left out of the graph. The cell counts of this sample are much higher than both the previous and following sample. This indicates that probably some precipitation has occurred before or after sampling resulting in an inaccurate sample that was mixed with the  
25 trypan blue solution. This assumption is confirmed by the fact that both the viability and infection percentage are in accordance with expected results. This means that nothing unusual is happening in the infected culture itself. This inaccurate sampling will most likely have no effect on the E2  
30 concentration in the sample, since the E2 is present in the medium and not intracellular.

The E2 concentration in flask 2 rises quite rapidly to a maximum of >190 µg/ml around 139 hpi and then slowly decreases to 170 µg/ml at 215 hpi. Then the E2-content drops  
35 more quickly to a final level of <100 µg/ml at 306.5 hpi.

The profile in flask 3 is more or less the same. The maximum E2 protein content is also reached around 139 hpi, but it is slightly higher than in flask 2. The E2 concentration slowly decreases from 233  $\mu\text{g/ml}$  at 139 hpi to 212  $\mu\text{g/ml}$  at 191 hpi before the concentration drops more rapidly to 141  $\mu\text{g/ml}$  215 hpi. Flask 2 and 3 show a good correlation.

At  $t=44.25$  hpi shake flask nr.1, the blank, was also counted. Living cell density was  $2.435 \cdot 10^6$  cells/ml, dead cell density  $0.190 \cdot 10^6$  and total cell density  $2.625 \cdot 10^6$  cells/ml, giving a viability of 92.8%. The living cell density is significantly higher than in both flasks 2 and 3, indicating that infection is already slowing down cell growth. This blank culture was subsequently passaged.

The following table shows the results of the immunoblotting assay performed on a selection of the samples of flask 2.

Sample (hpi)	V3	Degraded V3	V8	Degraded V8
0	-	-	-	-
44	-	-	-	-
115	+	-	-	-
139	+	-	+	-
164,5	+	-	+	-
191	+	+	+	-
288	+	+	+	+
306.5	+	+	+	-

'-' indicates not detectable, '+' indicates detectable

As can be seen in this table, epitopes V3 and V8 are not yet detectable in the 0 and 44 hpi samples. This correlates well with the results of the E2-ELISA. In the samples from 115 hpi onwards, intact E2 is detected, since both V3 and V8 are detected in the E2 band.

From 191 hpi onwards, a second band, from a smaller protein, is visible on the gel. Immunoblotting with V3 and V8 in two separate blots shows that the degradation product does contain the V3 epitope, but not the V8 epitope. No V8 is  
5 detected anywhere else on the blot but in the intact E2 band. This assay clearly shows that degradation of the E2 protein does occur due to the presence of proteases. In fact, this may be held responsible for the drop in the E2 protein content which is observed during the microfiltration step of  
10 the bulk E2 antigen solution. In this downstream processing step, cells are removed before the virus inactivation starts.

Therefore it is most likely that the presence of a protease causes the degradation of the E2 protein.

#### 15 7. An example of a MOI experiment

##### Purpose

To further investigate the relationship between MOI  
20 (multiplicity of infection, number of viruses per cell), maximum cell density and volumetric E2-content. On the one hand, infection of the complete cell population must be completed before the medium is exhausted, but on the other hand, infection of the total population at low cell  
25 concentration results in suboptimal protein yields.

##### Materials and methods

When medium is mentioned, SF900II medium is meant.

30

76 ml of cell suspension was diluted in 444 ml SF900II in a 1000 ml bottle and gently mixed. The living cell density just before dilution of the culture was  $3.41 \cdot 10^6$  cells/ml, the dead cell density  $0.190 \cdot 10^6$ , giving a viability of 94.8%.  
35 This dilution should give a cell density of  $\pm 5 \cdot 10^5$  cells/ml.

Furthermore, gentamycin was added to a final concentration of 10  $\mu\text{g/ml}$ . The cell suspension was divided in nine 50 ml portions in 250 ml shake flasks and excess cell material was discarded. The first 50 ml was transferred to shake flask 1, the final 50 ml to shake flask 5.

5 nrs. 1 and 6 are used for MOI=0 (blank),  
nrs. 2 and 7 for MOI=0.000001,  
nrs. 3 and 8 for MOI=0.00001  
nrs. 4 and 9 for MOI=0.0001  
10 nr. 5 for MOI=0.001

The preparation of the virus stock is as follows. A dilution range is made of a vial with 100x diluted virus suspension (the  
15 vial contains  $0.8 \cdot 10^5$  plaque forming units (pfu) per ml). This 100x diluted virus stock solution was prepared as follows.

Master seed virus was thawed and diluted 100x in TC100 medium, supplemented with 10% FBS and stored at  $-70^\circ\text{C}$  in 0.5  
20 ml portions.

0.4 ml of this 100 x diluted virus solution is diluted with 3.6 ml of SF900II medium, resulting in a dilution factor of  $10^{-1}$ . 1 ml of this solution is added to 9 ml of medium,  
25 resulting in a dilution factor of  $10^{-2}$ , 1 ml of this solution is added to 9 ml of medium to give a dilution factor of  $10^{-3}$  and 1 ml of this solution is diluted tenfold to give a dilution factor of  $10^{-4}$ . 3.1 ml of medium is added to shake flasks 1 and 6 (blank flasks), containing the cell culture.  
30 3.1 ml of virus dilutions  $10^{-2}$ ,  $10^{-3}$  and  $10^{-4}$  are added to shake flasks 4 and 9, 3 and 8 and 2 and 7 respectively. These shake flasks already contain the cell culture.

In 3.1 ml of virus dilution  $10^{-2}$ , there are  $3.1 \cdot 10^{-2} \cdot 0.8 \cdot 10^5 = 2.5 \cdot 10^3$  pfu present. In a shake flask with 50  
35 ml of cell suspension  $0.5 \cdot 10^6 \cdot 50 = 2.5 \cdot 10^7$  cells are present. So, addition of 3.1 ml of virus dilution  $10^{-2}$  to 50 ml of the

cell suspension results in an MOI of  $2.5 \cdot 10^3 / 2.5 \cdot 10^7 = 0.0001$ .

#### Making

comparable calculations for the other virus dilutions, MOI counts of 0.00001 and 0.000001 are obtained for virus dilutions  $10^{-3}$  and  $10^{-4}$  added to 50 ml of cell culture. Finally, 2.85 ml of dilution  $10^{-1}$  is added to shake flask nr. 5, resulting in a MOI of 0.00092 instead of 0.001.

A 3.1 ml sample is taken of each shake flask immediately after virus addition. The cells in the samples are spun down in an IEC Centra GP8R centrifuge in 15 ml Falcon tubes (10 min 1000 rpm). Sampling is carried out going from low MOI to high MOI to prevent virus transmission from an infected cell suspension with high MOI to a cell suspension infected with low MOI. After centrifugation, samples are  $0.45 \mu\text{m}$  filtered (to remove cells that might still be present in the supernatant) and divided over 3 Nalgene cryovials and stored at  $-70^\circ\text{C}$  for testing later on.

The cell density of shake flasks 1 and 5 was determined immediately after virus addition. These were the first and the last flask to be inoculated with cells. Since the cell densities were almost identical, it was assumed that all flasks had a cell density equal to the average value of both cell counts. The initial living cell density was  $0.423 \cdot 10^6$  cells/ml, whereas  $0.012 \cdot 10^6$  cells were dead, giving a viability of 97.3%.

All flasks were placed on the Labotech 300 orbital shaker platform, stirring at approximately 75 rpm in the  $28^\circ\text{C}$  room.

At 23, 95, 125, 144, 173 and 194 hpi (hours post infection) all flasks were sampled. Samples of which the cell density was determined had a volume of 3.3 ml, of which 0.2 ml was used to determine the cell density. The remaining 3.1 ml is handled as mentioned above for the  $t=0$  sample. Samples of cultures that were not counted, had a volume of 3.1 ml. At 194 hpi the cell density was determined and the experiment was terminated. The remaining

cell suspension (approximately 30 ml) was stored in 3 portions of 1 ml in Nalgene cryovials and 4 portions of approximately 6 ml in 15 ml Falcon tubes.

## 5 Results and conclusions

The results show that there is a good correlation between MOI and optimum cell density and, more importantly, between MOI and E2 protein yield. This can be seen in graph where the maximum volumetric E2 protein yield of the cultures infected with MOI 0.001 is set at 100%. It shows that the volumetric E2 protein yield increases with decreasing MOI. Up to a MOI of 0.0001 the cell density is infected before the maximum achievable cell density is reached. Using an MOI of 0.00001 or less results in an infection where the medium is exhausted before the infection of the culture is completed, resulting in suboptimal protein yields.

The conclusion can therefore be drawn that, the lower the MOI used, the higher the E2 protein yield, provided that the medium is not exhausted before the infection process and E2 production is completed.

### **Recombinant baculoviruses expressing bFSHa and/or bHSH $\beta$**

Transfervectors pDW-Alpha-9.1 and pDW-Beta-3.1 were constructed. Sf21 cells were cotransfected with pDW-Alpha-9.1 or pDW-Beta-3.1 and wild-type (wt) AcNPV/M021 DNA isolated from extracellular virus particles (PCT application: Wo 96/25696). Polyhedrin-positive plaques expressing  $\beta$ -galactosidase were isolated and analyzed for expression of bFSHa or bHSH $\beta$  by ELISA of culture media. One plaque-purified bFSHa virus (AcNPVa3.4) and one plaque-purified bHSH $\beta$  virus (AcNPV $\beta$ 1.4) were used to prepare virusstocks with a TCID50 of approximately  $10^7$  and  $10^8$ , respectively. Production of FSH

occurred according to the methods described above and resulted in production levels varying from 17 to 33 µg/ml.

#### **PD<sub>95</sub> Trial**

5           Assessment of the dose (µg) of E2 needed after one vaccination to protect 95% (PD<sub>95</sub>) of the vaccinated pigs against a challenge with a 100 LD<sub>50</sub> of the virulent CSFV strain Brescia.

#### **10   Animals**

          Twenty six specific-pathogen-free (SPF) pigs, 6-7 weeks old, were randomly divided on arrival in three groups of eight (A-C) and one group of two (D). The animals were housed in stables B18, B19, B20 and B21 within the high containment facilities of the ID-DLO. The animals were left to  
15           acclimatise for three days. The animals were fed once a day, in a trough, with complete foodpellets (Hope Farms), and could drink water from a nipple ad libitum.

**Vaccination and challenge**

Three formulations of double-water-in-oil adjuvanted vaccine were prepared as described above, each with a different concentration of E2 antigen; 32.0, 8.0 and  
5 2.0 µg/dose. The pigs were inoculated once intramuscularly, each pig receiving one dose, 2 cm behind the left ear (A: 2 µg E2, B: 8 µg E2, C: 32 µg E2, D: 0 µg E2). The control group D was inoculated with DOE adjuvant only and the  
10 sentinel pigs were not inoculated at all. Three weeks after vaccination each animal, except the sentinels, was challenged intranasally with 100 50% lethal doses (= 100 Va LD<sub>50</sub>) of CSFV strain Brescia 456610. Just before challenge the  
15 sentinels were separated from their group and returned 24 hours later. Viral contents of the inoculum was determined by titration of a sample taken after return from the stable.

**Clinical observation**

The pigs were checked daily by the animal technicians, abnormal findings were recorded and if necessary the  
20 supervising veterinarian was called. Each group was observed at least 15 minutes per day before and during feeding time and cleansing of the stable.

A reduction in food uptake of the group or an individual animal was noted.

25 Bodytemperatures (rectal) were recorded during nine days after vaccination and for 20 days after challenge.

**Blood analysis after challenge**

EDTA-blood samples were collected on day 0, 2, 4, 7, 10  
30 and 14 after challenge to monitor changes in leucocyte and trombocyte numbers. A decrease in the number leucocytes (leucopenia) and thrombocytes (thrombocytopenia) in the blood is one of the typical signs of CSF. Normal cell counts for white blood cells and thrombocytes in conventional swine

range between respectively 11-23  $10^9/1$  and 320-720  $10^9/1$ . For SPF pigs these values are a bit lower; 6-12  $10^9/1$  and 300-700  $10^9/1$ . Both mentioned ranges vary in each pig. The blood cell analyses was performed with a Medonic® CA 570 coulter  
5 counter. Leucopenia and thrombocytopenia were defined as cell/platelets counts considerably lower than the minimum number mentioned above, preferably for more than one day.

#### **Virus spread/excretion and viral detection**

10 The temperature, leucocyte- and trombocyte counts and seroconversion of the sentinels were the parameters used to detect virus transmission from the inoculated animals to these animals. At post-mortem tissue samples were collected from the following organs: tonsil, spleen, kidney and ileum  
15 and were tested, by direct immunofluorescent technique for the presence of viral antigen. Cryostat sections (4  $\mu\text{m}$  thick, two per organ) from these tissue samples were fixed and incubated with a polyclonal swine anti-pestivirus FITC-conjugated serum. After washing the sections were read under  
20 a fluorescence microscope. Results were expressed as positive (=fluorescence) or negative (= no fluorescence).

#### **Serological response**

25 Serum of all pigs was collected at 0, 2 and 3 weeks post-vaccination and at death. Samples were stored at  $-20^{\circ}\text{C}$  and assayed in a virus neutralization test (VNT) and the Ceditest®, an ELISA for detecting CSF specific antibody. CSFV neutralizing antibody titres in serum were determined in a microtitre system.  
30 Serial twofold dilutions of serum were mixed with an equal volume of a CSFV (strain Brescia) suspension which contained 30-300 TCID<sub>50</sub>. After incubation for 1 hour at  $37^{\circ}\text{C}$  in a CO<sub>2</sub> incubator approximately 25.000 PK 15 cells per well were added. after four days the microtitre plates were treated as

mentioned above and read microscopically. The CSFV neutralizing titre was expressed as the reciprocal of the highest dilution that neutralized all virus.

#### 5 **Statistical evaluation**

Determination of the 95% protective dose was based on the assumption that a CSFV neutralizing Ab titer of  $\geq 50$  stands for full protection.

10

### **RESULTS**

For animal numbers throughout this section compare tables 1 or 2.

#### **Clinical observation after vaccination**

15 Vaccination did not have any adverse effect on the pigs, food uptake and body temperature remained normal. Among group A and C mild diarrhea, anorexia and depression was seen on day 3 after vaccination. One pig from group A (no.448) vomitted regularly during the whole period and stayed behind  
20 in growth. Pig no. 438 from group B vomitted once. The temperature of pig no. 434 (group C, sentinel) was slightly elevated, this animal was suffering from lameness of the right hind leg. Food uptake increased during the period between vaccination and challenge from 3 to 6 kg per  
25 group/day.

#### **Clinical observation after challenge**

Unvaccinated control animals developed signs of CSF on three (no. 59) and six (no. 60) days after challenge: fever,  
30 huddling, shivering, loss of appetite and depression were seen till death, 10 days after challenge. Furthermore, the animals developed cyanosis, paresis posterior, diarrhea and severe vomiting. Both animals were killed being moribund.

All the pigs vaccinated with 2 µg of E2 (group A) developed signs of disease 2-3 days after challenge, consisting mainly of fever, huddling, depression and anorexia. Fever lasted from 2-10 days and maximum  
5 temperatures ( $T_{max}$ ) varied from 40.7-42.2°C. From day seven onwards among pigs 443-446 fever disappeared and the food uptake increased towards the amount before challenge. But pig 448 was found dead on day nine and pig 447 developed acute CSF (convulsions, paresis posterior) and was killed when  
10 moribund on the same day. Both sentinels remained normal till day 7-9 after challenge after which they developed acute CSF and were killed when moribund on day 20.

In the animals of groups B and C clinical signs and duration of disease were milder as the payload of E2 antigen  
15 increased.

In group B fever (435-439) lasted to day 27 and  $T_{max}$  varied between 40.2 and 41.7°C. Pig no. 436 resisted the challenge with very mild clinical signs. One pig (no. 440) died from acute CSF after 18 days, killed moribund. The  
20 remaining five animals from group B recovered. Both sentinels remained normal until day 11-12 after challenge after which they developed acute CSF and were killed on day 20.

In group C mild fever was seen in two (430,431) out of six animals, from day 4-6 and  $T_{max}$  varied from 40.5-41.2°C. No  
25 clinical signs were noted except slight depression on day six after challenge. As before challenge pig no. 430 vomitted.

Both sentinels remained normal till the end of the experiment.

### 30 **Blood analysis after challenge**

From group A only one pig (450), the sentinel, developed clear leucopenia and thrombocytopenia. Pigs no. 445 and 446 were trombocytopenic on day 7 and 10 after challenge, pig 449 on day 10 and 14. One pig (440) from group B

developed leucopenia and thrombocytopenia the others remained normal. Both sentinels showed signs of a developing thrombocytopenia on day 14. Leucopenia and thrombocytopenia were not detected in group C, including the sentinels.

5 Both control animals (no. 59 and 60) became thrombocytopenic from day 7 onwards.

#### **Virus spread and viral antigen detection after challenge**

Back titration of the inoculum rendered a virus titre  
10 of 2.55 TCID50/ml after return from the stable. CSFV viral antigen (Table 1) was detected in all the selected tissue samples of the controls and sentinels from groups A and B. One out of six inoculated animals was positive in groups A and B. And non in group C including the sentinels.

15

#### **Serological response**

Seroconversion for CSFV was defined as a titre  $\geq$  25 in the VNT. The result of the backtitration, in order to determine the amount of Brescia virus used in the VNT, was 41  
20 TCID50/well.

None of the controls (D) or sentinel animals seroconverted during the experiment (table 2). Two out of six animals from group A had seroconverted after three weeks. But four out of six animals boosted after challenge (table 2).  
25 Groups B and C had seroconverted on day 21 after vaccination and all the animals boosted after challenge. The latter only indicating a successful replication of the challenge virus in the animal.

#### **30 Conclusion**

The PD<sub>95</sub> dose per animal was determined as 32  $\mu$ g of E2 in 2 ml of DOE adjuvant given once. With this dose, clinical signs of disease after a challenge with a highly virulent

CSFV strain remained minimal and no spread of virus to contact animals occurred. In summary, four groups (A-D) of six pigs were vaccinated by intramuscular route with respectively 32(A), 8(B), 2(C) and 0  $\mu\text{g}$  Ed (D) per 2 ml  
5 adjuvant (DOE). Two non-vaccinated pigs were kept within each group (A-C) to detect virus excretion causing contact infections (sentinels). After three weeks the animals were challenged by intranasal route with 100 LD<sub>50</sub> of the highly virulent CSFV strain Brescia. Clinical, virological and  
10 serological parameters were measured in order to assess the efficacy of this E2 sub-unit vaccine. As expected animals in group C resisted the challenge better than the animals from the other groups. No viral antigen was detected in tissue samples from the animals inoculated with the highest dose of  
15 E2 (32  $\mu\text{g}$ ) and disease was absent in this group. The PD<sub>95</sub> dose was calculated on the assumption that an NPLA titre of  $\geq 50$  (on 21 days after vaccination) gives full protection (no spread of virus). This resulted in a PD<sub>95</sub> dose of 32  $\mu\text{g}/\text{animal}$ .

20 To further assess the level of protection against a challenge with 100 LD<sub>50</sub> virulent CSFV at 2 and 3 weeks after vaccination, two groups (A-B) of six pigs were vaccinated by the intramuscular route with 32  $\mu\text{g}$  of E2. Two non-vaccinated pigs were kept within each group (A-B) to detect virus  
25 excretion from the vaccinated pigs causing contact infections. After two (A) and three (B) weeks the vaccinated animals and control animals were challenged by intranasal route with 100 LD<sub>50</sub> of the highly virulent CSFV strain Brescia. Clinical, virological and serological parameters  
30 were measured in order to assess the efficacy of the E2 sub-unit vaccine two and three weeks after vaccination. As expected, group B resisted the challenge with less clinical symptoms than group A. Transmission of virus to the sentinels occurred only in group A and in the leucofractions of all the

animals from both groups virus was detected. Only one animal in group B had seroconverted on day 14 after vaccination (no. 414). From the same group one animal had not seroconverted after 21 days but was protected, had fever for only two days and seroconverted within eleven days after challenge. Only the controls and one of the sentinels (group A) developed leucopenia and thrombocytopenia. No viral antigen was detected in any of the tissue samples from the animals of groups A and B. Concluding, all animals were protected against the challenge on two and three weeks after vaccination with a single dose.

To further assess the level of protection against a challenge with 100 LD<sub>50</sub> virulent CSFV at 3 and 6 months after vaccination, two groups (A-B) of six pigs were vaccinated by the intramuscular route with 32 µg of E2. Two non-vaccinated pigs were kept within each group (A-B) to detect virus excretion from the vaccinated pigs causing contact infections. After three (A) and six (B) months the vaccinated animals and control animals were challenged by intranasal route with 100 LD<sub>50</sub> of the highly virulent CSFV strain Brescia. Clinical, virological and serological parameters were measured in order to assess the efficacy of the E2 sub-unit vaccine after this period. All the animals from group A and B resisted the challenge with minor clinical symptoms. And only the controls developed leucopenia and thrombocytopenia. Transmission of virus to the sentinels did not occur. And virus was not detected in any of the leucofractions selected of both groups. Only one animal in group B had seroconverted on day 28 after vaccination (no. 1983). No viral antigen was detected in any of the tissue samples from the animals of groups A, B and the sentinels. In conclusion, all animals were protected against the challenge on three and six months after vaccination with a single dose, and virus transmission did not occur.

BVDV strains 4800, 150022 and 178003 were used to generate experimental E2 subunit vaccines. The E2 genes of these strains were expressed in the baculovirus expression system (Hulst et al., 1993, J. Virol. 67: 5435-5442) (P.A. van Rijn et al., 1996, I). Gen. Virol., 77: 2737-2745). The *Spodoptera frugiperda* cell line, SF21, was used for propagation of the recombinant baculoviruses and the production of E2 proteins. SF21 cells were grown at 28°C, in serum free SF900 medium (GIBCO BRL). Confluent monolayers of SF21 cells were infected with recombinant baculovirus at a multiplicity of infection of 5 TCID<sub>50</sub> (50% tissue culture infective dose) per cell. After 4-5 days the cultures showed a cytopathic effect of 80-90%. The cells were centrifuged for 10 minutes at 1500\*g and the supernatant, containing baculovirus and E2, was collected and stored at -20°C. To inactivate baculovirus, a 2-bromoethyl-imminebromide (BEI) treatment was performed according to standard procedures. Briefly, 600 µl BEI and 600 µl 1 M NaOH were mixed. After 30 minutes at 20°C, 150 ml supernatant was added and stirred for 24 hours at 20°C. Then 20 ml of 25% thiosulphate was added. Inactivation of the baculovirus was confirmed by titration of the supernatant on SF21 cells.

Experimental BVDV vaccines consisted of the supernatants, containing E2, in a double wateroil emulsion. The oil contained 90% Marcol 52 (Esso) and 10% Montanide 80 (Seppic). The water fraction (PBS+) contained 98% phosphate buffered saline, 2% Montanox 80 (Seppic) and 100 ppm thiomersal. Supernatant, oil and PBS+ were used in the proportion of 10:11:10. First supernatant and oil were emulsified and then PBS+ was added and emulsified. A control vaccine was similarly prepared of the supernatant of SF21 cells infected with wild-type baculovirus. After preparation the vaccines were found to be sterile.

Our preliminary assumption that the antigen amount in the 3 vaccines would be similar, because of the comparability of the expressed proteins, was incorrect. The differences in antigen amount are probably caused by differences in expression in the insect cells. Vaccine 150022 contained 55 µg of E2 per dose and was protective for the fetus after 2x vaccination (day 0, and day 21). (Brusche et al. 1997, Vaccine in press). Vaccine 4800 and vaccine 178003 contained 12 µg and 17 µg of E2 per dose, respectively, and were not protective for the fetus. This result suggests a correlation between the amount of E2 protein and foetal protection. However, a difference in immunogenicity of E2 glycoproteins and inability of the challenge strains to cross the placenta may also account for the different outcomes of the challenge. None of the vaccines protected against heterologous BVDV challenge.

The results of this study are promising for further development of BVDV subunit vaccines, since we have shown that foetal protection can be achieved by vaccination with envelope glycoprotein E2. Furthermore, it is a marker vaccine which allows discrimination between vaccinated animals and animals infected with a field virus strain. This is advantageous in the light of future BVDV eradication programs.

25

### **Discussion**

The developed production process is aimed at achieving optimal heterologous protein production encoded by genes inserted into baculovirus in insect cells. The Baculovirus Expression Vector System (BEVS) is very well suited for producing different recombinant proteins, since correct protein folding and accurate post translational processing

30

results in biologically active proteins for animal and human applications. The baculovirus contains 2 non-essential genes, which are transcribed from very powerful promoters, the p10 and the polyhedrin promoters. Deletion mutagenesis of the p10 gene (van Oers et al, J. Gen Virol. 74: 563-574; 1993) showed that it is not essential for virus replication in cell culture, p10, however, is playing a role in cell lysis, absence of the p10 protein causes the infected cells to remain intact and prevents the release of polyhedra from infected cells, thereby reducing reinfection rates but not infectivity per se. The products of the genes (P10 (or fibrillin) and polyhedrin) are expressed late during the infection phase. Replacing these genes by the gene of the required protein can (in case of the polyhedrin promoter) theoretically result in yields of up to 50% of total protein production of the insect cell culture, resulting in expression levels of over 1 gram polyhedrin protein per liter in culture (Maiorella et al., (1988) Large-scale insect cell culture for recombinant protein production. Bio/technology, 6, 1406-1410). The multiplicity of infection (MOI, ratio of virus density per ml and cell density per ml) is a key parameter for the optimisation of the protein production. An obvious reason for infection with a low multiplicity of infection is to keep the virus inoculum as small as possible. If the infection takes place at a density of 2 million cells per ml at an MOI of 0.1 in a 50L fermentor, then  $1 \times 10^{10}$  viruses are needed. This would require an inoculum of around 1 liter. Furthermore, an extra production step is needed to make such an inoculum. Two major drawbacks of attached cultures are the inefficient medium usage and oxygen limitation. Due to the fact that the solubility of oxygen in aqueous fluids is relatively poor, oxygen will be the limiting substrate for the cells in monolayer cultures. The determining factor for maximum cell density in static

cultures is the available surface. Once the surface is covered with a monolayer of cells, cell growth will come to a halt. This results in a cell density of approx.  $1 \cdot 10^6$  cells/ml medium. In suspension cultures no oxygen limitation is present and the availability of other substrates is the limiting factor for cell density. This limitation occurs at a higher cell density than oxygen limitation and therefore the cell density becomes higher than in static cultures. Oxygen limitation is prevented by using an oxygen electrode to monitor the dissolved oxygen concentration. Once it drops below a certain value, oxygen is automatically added to the suspension, either by sparging or by addition via the head space of the fermentor. Moderate stirring of the suspension guarantees a homogenous culture in which no substrate gradients are built up and in which the cells are not subjected to too high shear forces. Furthermore, stirring results in an efficient transfer of viruses from infected to non-infected cells, giving a higher efficiency of virus infection of cells. Since initially only about 0.1-0.3% of the cells is infected, the remaining 99.7-99.9% of cells are allowed to grow and multiply. Virus particles that are produced in the infected cells are released 12-20 hours post infection (Dee, K.U. and Shuler, M.L. 1997. Biotechnology Progress, 13, 14-24). The number of released virus particles per cell is 100-200, which can infect hitherto non-infected cells in a new cycle. It will take several cycles before enough virus particles are produced to infect all cells present in the culture. The aim is to complete the protein production of the final infection passage before the medium is exhausted and all metabolic processes come to a halt. It is also undesirable to achieve complete infection at a cell density that is suboptimal, for then the medium will not be used efficiently resulting in a lower volumetric yield of the heterologous protein. The whole process of virus infection

can be checked visually, due to the fact that the polyhedrin gene is still present. Virus infection can be observed as dense protein particles that accumulate in the cell nucleus. To prevent shear damage to the cells Pluronic F-68 is added to the medium to a final concentration of 0.2%. Furthermore, if necessary, antifoam A is added to prevent excessive foaming, which also results in cell death. The total amount of antifoam A added to a 50L culture is on average 20 ml. The optimal virus density to infect the cells can be calculated. This density depends on the growth rate of the cells, the time post infection at which the new virus particles are released, and the number of new virus particles produced per infected cell. Illustrating the method provided by the invention is the production of the pestivirus E2 protein. Although this protein was known as a potent antigen, E2(fragment) production for a vaccine or a diagnostic test is not always successful. Despite a PD50 as low as 2 µg (when using a particularly immunogenic E2 fragment), a problem is how to accrue sufficient amounts of vaccine doses with sufficient antigenic mass in a commercially attractive way to enable protective vaccination of large groups of animals by one single vaccination per animal. This is particularly relevant for CSFV vaccination. When applied, CSFV vaccination generally is performed during a mass campaign in an area where an outbreak of CSFV has occurred. This asks for rapid vaccination of large numbers of animals in a relatively short period. In such a mass campaign it is of imminent importance that an adequate protection level (the number of pigs that are protected against the wild type virus infection) is achieved rapidly. Waiting for several weeks after a first vaccination for a second vaccination in order to achieve protection greatly hampers and delays the control of the disease. Differences between various methods to produce the recombinantly expressed E2 protein, even when comparing

E2 (fragments) expressed in baculovirus, exist. In earlier reported E2 protein production cultures, the E2 protein (fragment) yield varied between 20-90  $\mu\text{g/ml}$  (Hulst et al., J. Vir. 5435-5442, 1993; Hulst and Moormann, Cytotechnology 20:271-279, 1996), further necessitating immunoaffinity-purification with monoclonal antibodies to obtain the necessary and relevant E2 antigenic mass for single shot vaccination. Another method (using a fragment of E2 described in EP 0389034), which uses E2 harvested from the supernatant of insect cells without further immunoaffinity purification, results in a E2 based vaccine that is injected twice before a satisfying (protective) immune response is obtained. These problems, among others, relate to a low concentration of the relevant antigenic substance, in this case the E2 protein (fragments), in the starting material, e.g. the cell culture supernatant, from which the vaccine is prepared. In theory, one can further accumulate antigenic mass by purification and condensation methods known in the art, however, this does not lead to a commercially attractive vaccine production but causes high costs per dose. Production runs using a method provided by the invention in our 50L fermentor routinely results in a yield of around 200-300  $\mu\text{g/ml}$  CSFV E2 protein fragments, enough to theoretically vaccinate 100.000 animals per culture. Cells are infected at a density of 0.5 to  $1.5 \cdot 10^6$  cells/ml with 1 to 10 ml virus inoculum containing approx.  $10^7$  TCID<sub>50</sub>/ml. The culture is preferably harvested when >50-80% of the cells show CPE. This is about 100-150 hours post infection. In earlier E2 protein production cultures, cells were (synchronously) infected with an MOI > 1, resulting in a threefold lower protein yield than as provided by the present invention. In a method provided by the invention a 50L fermentor is inoculated with 5L of cell suspension grown in a 5L fermentor or 10L of all suspension, grown in a 10L fermentor. The initial cell density is around

3\*10<sup>5</sup> cells/ml. Cells are grown to the calculated cell density before virus is added to the suspension. Downstream processing starts with the removal of the cells and polyhedra by microfiltration. A hollow fiber microfiltration device is connected to the fermentor and the material is pumped through the filtration module with a pore size of 0.22 µm. The retentate flow is recirculated over the fermentor and the permeate flow is collected in a 100L vessel. When filtration is completed, the antigen solution, which is now cell-free but still contains infectious baculoviruses, is inactivated in the 100L vessel. Generally, 2-bromoethyl-ammoniumbromide (BEA) is added to the suspension to a final concentration of 8-12mM. The pH is raised from about 5.8 to 8.2-8.7 by adding 2M NaOH. This pH-shifts converts BEA to BEI (2-bromoethyl-imminebromide). This is the DNA-inactivating agent. pH is carefully monitored and regulated at 6-10 and the temperature is kept at 34-39. After 6 hours of inactivation, the antigen solution is transferred to a second inactivation vessel. This ensures, that all material in the vessel has been in contact with BEI and thus will be inactivated. Drops of fluid, containing virus, but not containing BEI, could be present in the first vessel, but not in the second. Baculoviruses, present in a concentration of 10<sup>4</sup>-10<sup>7</sup> pfu/ml are degraded to a value <10<sup>-7</sup> pfu/ml (<1 virus particle per 10 m<sup>3</sup>). This is the same norm as used for viral vaccines that are based on viruses that are able to infect the host animal (like FMD). Pigs are no hosts for baculoviruses. The inactivated antigen bulk is stored at ≤-20°C until it is formulated into a water-oil-water emulsion. A single dose containing 32 µg E2 (dose volume 2 ml) is sufficient to give protection (>PD95) against classical swine fever, from 2 weeks up to at least 6 months after vaccination. The production process is designed in such a way, that scaling-up of the process is straightforward, and use of 250L or even larger fermentors is possible. Scale-up

of a static culture production is also straightforward; just use more tissue culture flasks. However, the total protein production routinely achieved in the 50L fermentor would take approximately 7000 T175 tissue culture flasks. Cell growth and infection can be monitored and regulated better in a fermentor, since an oxygen and pH-electrode are present. In tissue culture flasks flask-to-flask variation is probably present, but it cannot be quantified. Inactivation is monitored and regulated much more accurate in the vessels as was done in the static culture production. Volumetric production levels of other proteins expressed in the BEVS (in our institute) also improve considerably if cells are grown with a method provided by the invention. For example, the yield of bovine FSH increased by a factor of 3-4, using a 10L fermentor. Cells were co-infected at an MOI of 0.003 of each of 2 recombinant baculoviruses at a cell density of  $1.1 \cdot 10^6$  cells/ml. The yield of different E2 proteins of BVDV and of E<sup>2ns</sup> proteins or BVDV and/or CSFV in suspension cultures in shake flasks increased threefold. The method is also applicable to other recombinant proteins.

Table 1. Viral antigen detection by the Immunofluorescence technique on cryostat sections of different tissues.

Group	Animal nr.	Lab nr.	Tissue				Death d.p.c.
			Tonsil	Spleen	Kidney	Ileum	
A.	443	1	-	-	-	-	20
2 $\mu$ E2	444	2	-	-	-	-	20
	445	3	-	-	-	-	20
	446	4	-	-	-	-	20
	447	5	+	-	+	+	9
	448	6	-	-	-	-	9
	449 (S)	7	+	+	+	+	20
	450 (S)	8	+	+	+	+	20
B.	435	9	-	-	-	-	20
8 $\mu$ E2	436	10	-	-	-	-	20
	437	11	-	-	-	-	20
	438	12	-	-	-	-	20
	439	13	-	-	-	-	20
	440	14	+	+	+	---	18
	441 (S)	15	+	+	+	+	20
	442 (S)	16	+	+	+	+	20
C.	427	17	-	-	-	-	20
32 $\mu$ g E2	428	18	-	-	-	-	20
	329	19	-	-	-	-	20
	430	20	-	-	-	-	20
	431	21	-	-	-	-	20
	432	22	-	-	-	-	20
	433 (S)	23	-	-	-	-	20
	434 (S)	24	-	-	-	-	-
D.	59	25	+	+	+	+	10
	60	26	+	+	+	+	1

5 - = no fluorescence detected

+ = fluorescence detected

--- = no data

\* = animals were slaughtered at the end of the experiment 20 dpc.

Table 2. Results of the Virus Neutralization Test

Group	Animal nr.	Lab nr.	Days post vaccination			
			0	14	21	41 (at death)
A.	443	1	<12,5	19	75	>1600
2 $\mu$ E2	444	2	<12,	<12,	19	>1600
	445	3	<12,	<12,	<12,	>1600
	446	4	<12,5	<12,5	12,	>1600
	447	5	<12,5	<12,5	19	<12,5
	448	6	<12,5	19	37	---
	449 (S)	7	<12,5	<12,5	<12,5	<12,5
	450 (S)	8	<12,5	<12,5	<12,5	<12,5
B.	435	9	<12,5	<12,5	100	>1600
8 $\mu$ E2	436	10	<12,5	<12,5	400	>1600
	437	11	<12,5	75	600	>1600
	438	12	<12,5	37	400	>1600
	439	13	<12,5	37	75	>1600
	440	14	<12,5	<12,5	37	600
	441 (S)	15	<12,5	<12,5	<12,5	<12,5
	442 (S)	16	<12,5	<12,5	<12,5	<12,5
C.	427	17	<12,5	50	150	>1600
32 $\mu$ g E2	428	18	<12,5	37	600	>1600
	329	19	<12,5	>1600	1200	>1600
	430	20	<12,5	<12,5	50	>1600
	431	21	<12,5	<12,5	800	>1600
	432	22	<12,5	<12,5	800	>1600
	433 (S)	23	<12,5	<12,5	<12,5	<12,5
	434 (S)	24	<12,5	<12,5	<12,5	<12,5
D.	59	25	<12,5	<12,5+	<12,5	+
	60	26	<12,5	<12,5	<12,5	+

--- = no data

CLAIMS

1. A method to increase yield of a recombinant protein produced in insect-cell culture which comprises selecting a recombinant baculovirus encoding said protein, growing insect cells in growth medium in a culture vessel and infecting the  
5 cells with an inoculum of at least one baculovirus with a multiplicity of infection of  $<0.01$ .
2. A method according to claim 1 which comprises growing the cells in a culture vessel with a sufficient volume to contain  
10 2, more preferably 10, more preferably 50 liters growth medium, more preferably 250 liters growth medium.
3. A method according to claim 1 or 2 which comprises infecting the cells at a cell density of  $1 \times 10^5$  to  $5 \times 10^6$  cells/ml, more preferably  $5 \times 10^5$  to  $1.5 \times 10^6$  cells/ml.
4. A method according to claim 1, 2 or 3 which comprises  
15 infecting the cells with a multiplicity of infection of  $\leq 0.005$ .
5. A method according to claim 4 wherein the multiplicity of infection is  $\leq 0.003$ .
6. A method according to any one of claims 1-5 which  
20 comprises selecting a recombinant baculovirus expressing the wanted protein under control of the p10 promotor.
7. A method according to any one of claims 1-6 which comprises growing the insect cells in suspension, preferably in a culture vessel, such as a fermentor, which can be  
25 (moderately) stirred.
8. A method according to any of claims 1-7 to produce recombinant pestivirus E2 or E<sup>ns</sup> protein or fragments thereof.
9. A method to increase yield of a recombinant pestivirus E2  
30 or E<sup>ns</sup> protein or fragments thereof produced in insect cell culture characterised by a final concentration of said

protein(fragments) in the growth medium at harvest of at least 100 µg/ml.

10. A method according to claim 9 wherein the concentration is larger than 120 µg/ml, or at least 150 µg/ml, more preferably at least 200 µg/ml.
11. Use of a method according to any of claims 1-10 to produce an antigenic substance.
12. Use according to claim 11 wherein the antigenic substance is a pestivirus protein(fragment), more preferably a classical swine fever virus protein(fragment).
13. A vaccine comprising an antigenic substance which is obtainable by using a method according to any of claims 1-10.
14. A vaccine comprising recombinant pestivirus E2 or E<sup>rns</sup> protein or fragments thereof characterised in that it is not being immunoaffinity purified and preferably confers protection against a pestivirus infection at the PD95 level after one single vaccination with one dose.
15. A vaccine according to claim 14 wherein the pestivirus infection is a classical swine fever infection.
16. A vaccine according to one of claims 13-15 additionally comprising adjuvant, said adjuvant preferably comprising a double water-in-oil emulsion.
17. A method to produce recombinant follicle stimulating hormone, α-units and/or β-units and complexes and fragments thereof, at a concentration in the growth medium at harvest of at least 15 µg/ml.
18. A method according to claim 17 wherein the concentration is at least 20 µg/ml, more preferably at least 25 µg/ml.
19. A method according to claim 17 or 18 comprising infecting an insect cell culture with one baculovirus expressing the α-unit and with another baculovirus expressing the β-unit in the culture.
20. Use of a method according to any of claims 1-7 or 17-19 to produce a hormone-like substance.

21. Use according to claim 20 to produce a hormone-like substance for incorporation in a pharmaceutical composition to treat reproductive disorders.

Figure 1 Data of E2 stability in prolonged culture experiment

## Shake flask nr. 2

Time (hpi)	Living (#/ml)	Dead (#/ml)	Total (#/ml)	Viability (%)	Living inf (#/ml)	Dead inf (#/ml)	Inf (%)	E2-content ( $\mu\text{g/ml}$ )	
-1	$0.612 \cdot 10^6$	$0.018 \cdot 10^6$	$0.630 \cdot 10^6$	97.1	0	0	0.0	0.0	
20.75	$1.305 \cdot 10^6$	$0.048 \cdot 10^6$	$1.353 \cdot 10^6$	96.5	0	0	0.0	0.0	
44.25	$2.030 \cdot 10^6$	$0.110 \cdot 10^6$	$2.140 \cdot 10^6$	94.9	0	0	0.0	0.0	
94.75	$2.125 \cdot 10^6$	$0.415 \cdot 10^6$	$2.540 \cdot 10^6$	83.7	$0.715 \cdot 10^6$	$0.395 \cdot 10^6$	43.7	96.8	
115	$1.145 \cdot 10^6$	$1.610 \cdot 10^6$	$2.755 \cdot 10^6$	41.6	$0.585 \cdot 10^6$	$1.550 \cdot 10^6$	77.5	171.0	
139	$0.608 \cdot 10^6$	$2.168 \cdot 10^6$	$2.776 \cdot 10^6$	21.9	$0.364 \cdot 10^6$	$2.136 \cdot 10^6$	90.1	193.5	
164.5	Sampling only, no cell count								176.9
191	$0.072 \cdot 10^6$	$2.540 \cdot 10^6$	$2.612 \cdot 10^6$	2.8	$0.072 \cdot 10^6$	$2.540 \cdot 10^6$	100.0	176.4	
215	Sampling only, no cell count								136.3
288	Sampling only, no cell count								102.0
306.5	Sampling only, no cell count								96.6

## Shake flask nr. 3

Time (hpi)	Living (#/ml)	Dead (#/ml)	Total (#/ml)	Viability (%)	Living inf (#/ml)	Dead inf (#/ml)	Inf (%)	E2-content ( $\mu\text{g/ml}$ )	
-1 hpi	$0.736 \cdot 10^6$	$0.024 \cdot 10^6$	$0.760 \cdot 10^6$	96.8	0	0	0	0	
20.75	$1.179 \cdot 10^6$	$0.045 \cdot 10^6$	$1.224 \cdot 10^6$	96.3	0	0	0	0	
44.25	$2.075 \cdot 10^6$	$0.085 \cdot 10^6$	$2.160 \cdot 10^6$	96.1	0	0	0	0	
94.75	$1.815 \cdot 10^6$	$0.525 \cdot 10^6$	$2.340 \cdot 10^6$	77.6	$0.475 \cdot 10^6$	$0.485 \cdot 10^6$	41.0	101	
115	$1.285 \cdot 10^6$	$1.510 \cdot 10^6$	$2.795 \cdot 10^6$	46.0	$0.615 \cdot 10^6$	$1.425 \cdot 10^6$	73.0	196	
139	$1.060 \cdot 10^6$	$3.420 \cdot 10^6$	$4.480 \cdot 10^6$	23.7	$0.584 \cdot 10^6$	$3.380 \cdot 10^6$	88.5	233	
164.5	Sampling only, no cell count								204
191	$0.104 \cdot 10^6$	$2.692 \cdot 10^6$	$2.796 \cdot 10^6$	3.7	$0.104 \cdot 10^6$	$2.692 \cdot 10^6$	100.0	212	
215	Sampling only, no cell count								141
288	Sampling only, no cell count								N.D.
306.5	Sampling only, no cell count								N.D.

N.D. = not determined



Figure 2 (continued)

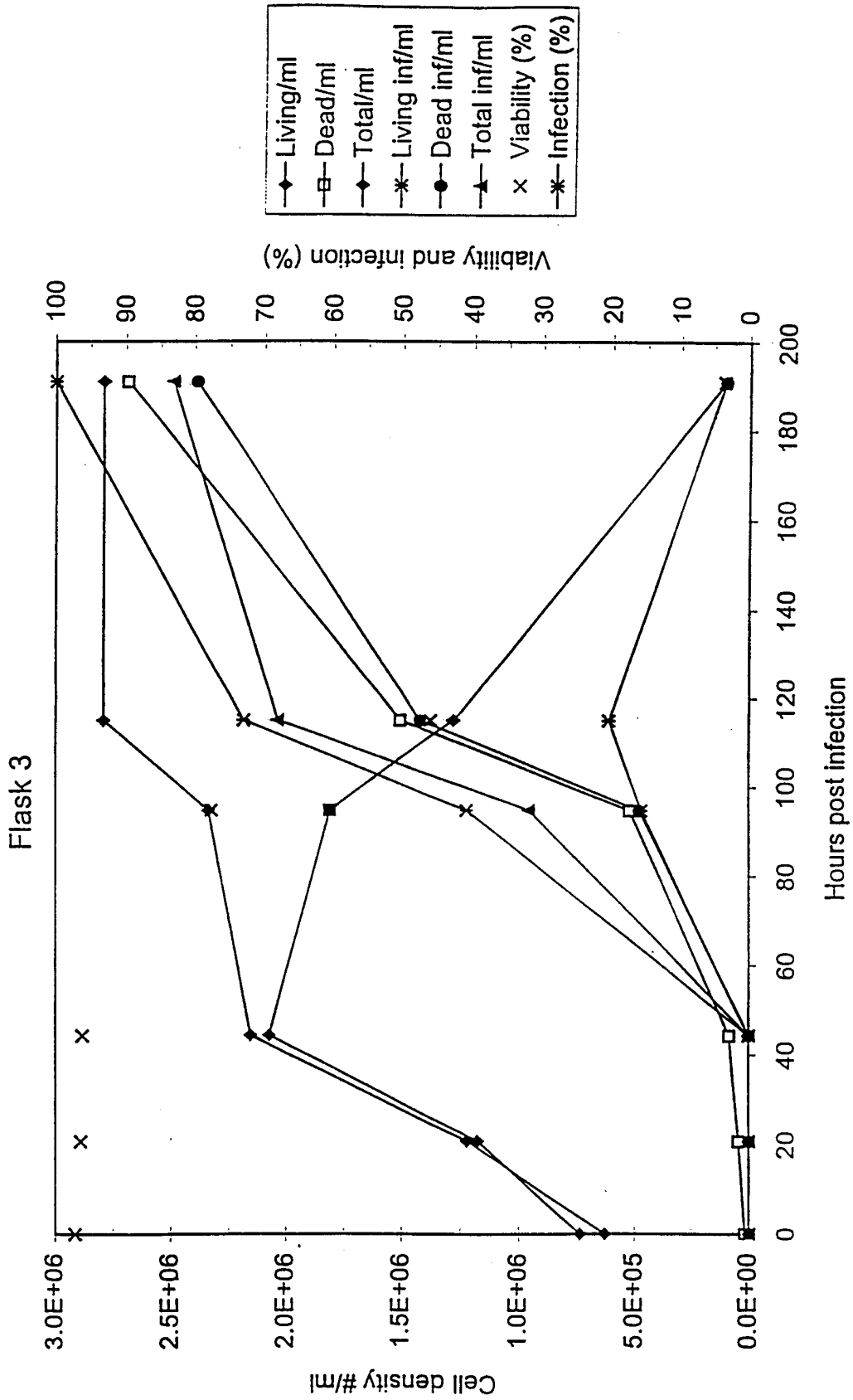


Figure 2 (continued)

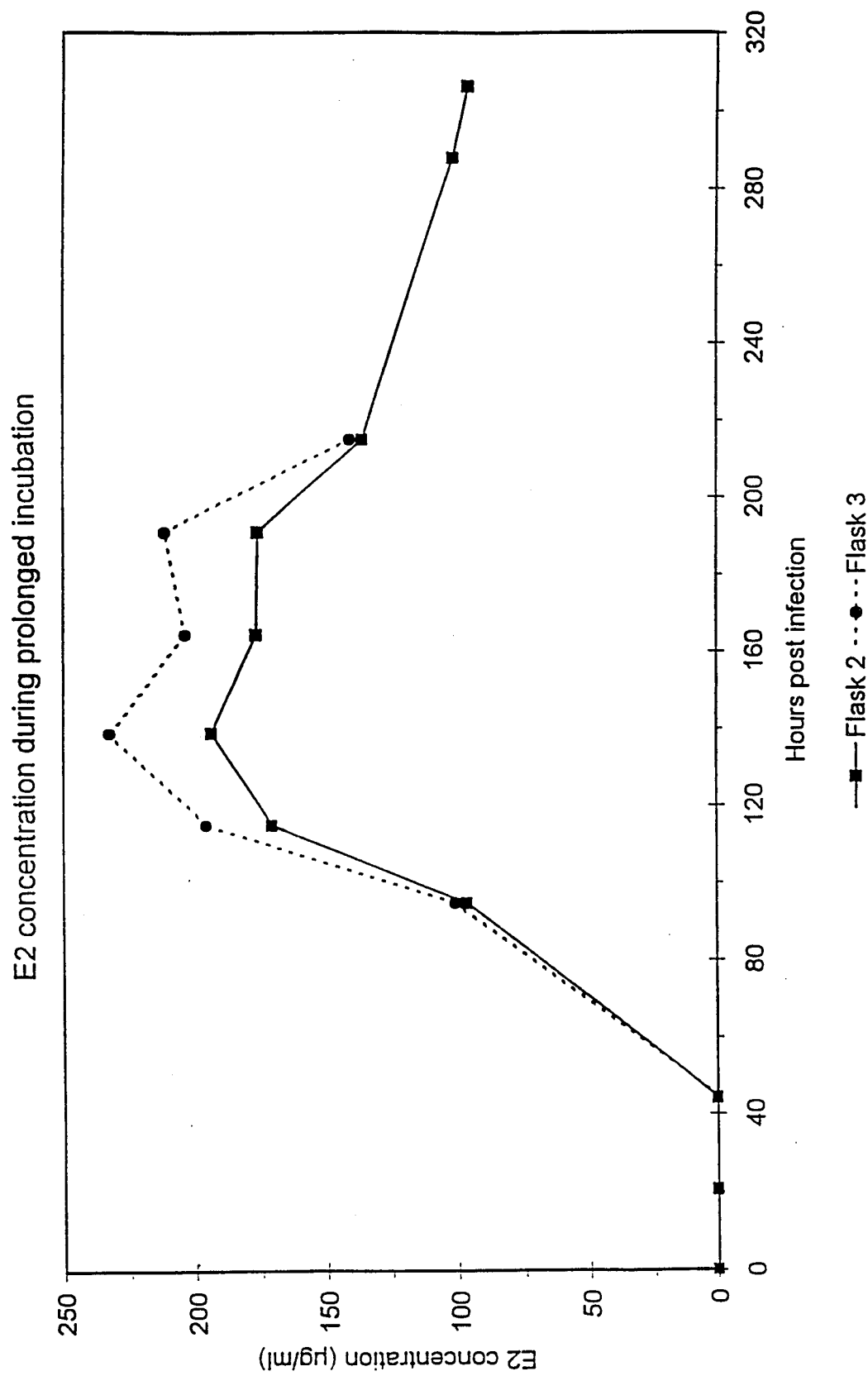


Figure 3 Data of MOI experiment

## Shake flask nr.1 MOI=0

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.12*10 <sup>5</sup>	8.91*10 <sup>5</sup>	2.36*10 <sup>6</sup>	2.57*10 <sup>6</sup>	2.43*10 <sup>6</sup>	2.51*10 <sup>6</sup>	2.01*10 <sup>6</sup>
Dead cells/ml	1.6*10 <sup>4</sup>	2.10*10 <sup>4</sup>	1.80*10 <sup>5</sup>	2.70*10 <sup>5</sup>	3.65*10 <sup>5</sup>	7.50*10 <sup>5</sup>	1.09*10 <sup>6</sup>
Total cells/ml	4.28*10 <sup>5</sup>	9.12*10 <sup>5</sup>	2.54*10 <sup>6</sup>	2.84*10 <sup>6</sup>	2.79*10 <sup>6</sup>	3.26*10 <sup>6</sup>	3.09*10 <sup>6</sup>
Viability (%)	96.3	97.7	92.9	90.5	86.9	77.0	64.9
Living inf cells/ml	0	0	0	0	0	0	0
Dead infected/ml	0	0	0	0	0	0	0
Total infected cells/ml	0	0	0	0	0	0	0
Infection (%)	0	0	0	0	0	0	0
E2-content (µg/ml)	N.D.	N.D.	N.De	N.D.	N.D.	N.D.	N.D.

N.D. = not determined

N.De = not detectable

## Shake flask nr.2 MOI=0.000001

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.23*10 <sup>5</sup>	9.75*10 <sup>5</sup>	2.67*10 <sup>6</sup>	2.34*10 <sup>6</sup>	1.91*10 <sup>6</sup>	8.60*10 <sup>5</sup>	5.65*10 <sup>5</sup>
Dead cells/ml	1.3*10 <sup>4</sup>	2.40*10 <sup>4</sup>	9.00*10 <sup>4</sup>	4.50*10 <sup>5</sup>	9.30*10 <sup>5</sup>	2.03*10 <sup>6</sup>	2.30*10 <sup>6</sup>
Total cells/ml	4.35*10 <sup>5</sup>	9.99*10 <sup>5</sup>	2.76*10 <sup>6</sup>	2.79*10 <sup>6</sup>	2.84*10 <sup>6</sup>	2.89*10 <sup>6</sup>	2.86*10 <sup>6</sup>
Viability (%)	97.3	97.6	96.7	83.9	67.2	29.8	19.8
Living inf cells/ml	0	0	0	9.00*10 <sup>4</sup>	4.95*10 <sup>5</sup>	3.85*10 <sup>5</sup>	3.45*10 <sup>5</sup>
Dead infected/ml	0	0	0	6.00*10 <sup>4</sup>	4.50*10 <sup>5</sup>	1.34*10 <sup>6</sup>	1.89*10 <sup>6</sup>
Total infected cells/ml	0	0	0	1.50*10 <sup>5</sup>	9.45*10 <sup>5</sup>	1.72*10 <sup>6</sup>	2.24*10 <sup>6</sup>
Infection (%)	0	0	0	5.4	33.3	59.5	78.1
E2-content (µg/ml)	N.D.	N.D.	1.4	19.4	49.8	107.5	144.8

## Shake flask nr.3 MOI=0.00001

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.23*10 <sup>5</sup>	7.95*10 <sup>5</sup>	2.31*10 <sup>6</sup>	1.67*10 <sup>6</sup>	1.31*10 <sup>6</sup>	6.40*10 <sup>5</sup>	3.00*10 <sup>5</sup>
Dead cells/ml	1.3*10 <sup>4</sup>	2.70*10 <sup>4</sup>	9.50*10 <sup>4</sup>	7.70*10 <sup>5</sup>	1.29*10 <sup>5</sup>	1.74*10 <sup>6</sup>	1.96*10 <sup>6</sup>
Total cells/ml	4.35*10 <sup>5</sup>	8.22*10 <sup>5</sup>	2.40*10 <sup>6</sup>	2.44*10 <sup>6</sup>	2.59*10 <sup>6</sup>	2.38*10 <sup>6</sup>	2.26*10 <sup>6</sup>
Viability (%)	97.3	96.7	96.0	68.4	50.4	26.9	13.3
Living inf cells/ml	0	0	1.35*10 <sup>5</sup>	4.80*10 <sup>5</sup>	4.85*10 <sup>5</sup>	3.80*10 <sup>5</sup>	2.50*10 <sup>5</sup>
Dead infected/ml	0	0	4.00*10 <sup>4</sup>	4.85*10 <sup>5</sup>	9.35*10 <sup>5</sup>	1.43*10 <sup>6</sup>	1.85*10 <sup>6</sup>
Total infected cells/ml	0	0	1.75*10 <sup>5</sup>	9.65*10 <sup>5</sup>	1.42*10 <sup>6</sup>	1.81*10 <sup>6</sup>	2.10*10 <sup>6</sup>
Infection (%)	0	0	7.3	39.5	54.8	76.2	92.9
E2-content (µg/ml)	N.D.	N.D.	24.6	100.7	135.1	177.5	195.7

## Shake flask nr.4 MOI=0.0001

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.23*10 <sup>5</sup>	7.50*10 <sup>5</sup>	1.35*10 <sup>6</sup>	6.65*10 <sup>5</sup>	2.40*10 <sup>5</sup>	N.D.	N.D.
Dead cells/ml	1.3*10 <sup>4</sup>	1.80*10 <sup>4</sup>	1.75*10 <sup>5</sup>	8.95*10 <sup>5</sup>	1.36*10 <sup>6</sup>	N.D.	N.D.
Total cells/ml	4.35*10 <sup>5</sup>	7.68*10 <sup>5</sup>	1.52*10 <sup>6</sup>	1.56*10 <sup>6</sup>	1.60*10 <sup>6</sup>	N.D.	N.D.
Viability (%)	97.3	97.7	88.5	42.6	15.0	N.D.	N.D.
Living inf cells/ml	0	0	1.01*10 <sup>6</sup>	6.15*10 <sup>5</sup>	2.20*10 <sup>5</sup>	N.D.	N.D.
Dead infected/ml	0	0	1.60*10 <sup>5</sup>	8.90*10 <sup>5</sup>	1.36*10 <sup>6</sup>	N.D.	N.D.
Total infected cells/ml	0	0	1.17*10 <sup>6</sup>	1.51*10 <sup>6</sup>	1.58*10 <sup>6</sup>	N.D.	N.D.
Infection (%)	0	0	76.6	96.5	98.4	N.D.	N.D.
E2-content (µg/ml)	N.D.	N.D.	140.5	200.4	216.6	215.7	212.2

Figure 3 (continued)

## Shake flask nr.5 MOI=0.001

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.34*10 <sup>5</sup>	4.89*10 <sup>5</sup>	7.60*10 <sup>5</sup>	4.90*10 <sup>5</sup>	1.25*10 <sup>5</sup>	N.D.	N.D.
Dead cells/ml	0.8*10 <sup>4</sup>	2.70*10 <sup>4</sup>	1.60*10 <sup>5</sup>	8.55*10 <sup>5</sup>	7.30*10 <sup>5</sup>	N.D.	N.D.
Total cells/ml	4.42*10 <sup>5</sup>	5.16*10 <sup>5</sup>	9.20*10 <sup>5</sup>	1.35*10 <sup>6</sup>	8.55*10 <sup>5</sup>	N.D.	N.D.
Viability (%)	98.2	94.8	82.6	36.4	14.6	N.D.	N.D.
Living inf cells/ml	0	0	7.05*10 <sup>5</sup>	4.60*10 <sup>5</sup>	1.25*10 <sup>5</sup>	N.D.	N.D.
Dead infected/ml	0	0	1.55*10 <sup>5</sup>	8.55*10 <sup>5</sup>	7.30*10 <sup>4</sup>	N.D.	N.D.
Total infected cells/ml	0	0	8.60*10 <sup>5</sup>	1.32*10 <sup>6</sup>	8.55*10 <sup>5</sup>	N.D.	N.D.
Infection (%)	0	0	93.5	97.8	100.0	N.D.	N.D.
E2-content (µg/ml)	N.D.	N.D.	170.6	185.7	187.9	184.5	133.3

## Shake flask nr.6 MOI=0

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.23*10 <sup>5</sup>	7.05*10 <sup>5</sup>	2.52*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Dead cells/ml	1.3*10 <sup>4</sup>	4.50*10 <sup>4</sup>	1.65*10 <sup>5</sup>	N.D.	N.D.	N.D.	N.D.
Total cells/ml	4.35*10 <sup>5</sup>	7.50*10 <sup>5</sup>	2.68*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Viability (%)	97.3	94	93.8	N.D.	N.D.	N.D.	N.D.
Living inf cells/ml	0	0	0	N.D.	N.D.	N.D.	N.D.
Dead infected/ml	0	0	0	N.D.	N.D.	N.D.	N.D.
Total infected cells/ml	0	0	0	N.D.	N.D.	N.D.	N.D.
Infection (%)	0	0	0	N.D.	N.D.	N.D.	N.D.
E2-content (µg/ml)	N.D.	N.D.	N.De	N.D.	N.D.	N.D.	N.D.

## Shake flask nr.7 MOI=0.000001

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.23*10 <sup>5</sup>	8.64*10 <sup>5</sup>	3.59*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Dead cells/ml	1.3*10 <sup>4</sup>	3.30*10 <sup>4</sup>	1.75*10 <sup>5</sup>	N.D.	N.D.	N.D.	N.D.
Total cells/ml	4.35*10 <sup>5</sup>	8.97*10 <sup>5</sup>	3.77*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Viability (%)	97.3	96.3	95.4	N.D.	N.D.	N.D.	N.D.
Living inf cells/ml	0	0	0	N.D.	N.D.	N.D.	N.D.
Dead infected/ml	0	0	0	N.D.	N.D.	N.D.	N.D.
Total infected cells/ml	0	0	0	N.D.	N.D.	N.D.	N.D.
Infection (%)	0	0	0	N.D.	N.D.	N.D.	N.D.
E2-content (µg/ml)	N.D.	N.D.	1.0	22.7	58.6	162.1	180.8

## Shake flask nr.8 MOI=0.00001

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.23*10 <sup>5</sup>	7.38*10 <sup>5</sup>	2.27*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Dead cells/ml	1.3*10 <sup>4</sup>	2.10*10 <sup>4</sup>	5.00*10 <sup>4</sup>	N.D.	N.D.	N.D.	N.D.
Total cells/ml	4.35*10 <sup>5</sup>	7.59*10 <sup>5</sup>	2.32*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Viability (%)	97.3	97.2	97.8	N.D.	N.D.	N.D.	N.D.
Living inf cells/ml	0	0	1.25*10 <sup>5</sup>	N.D.	N.D.	N.D.	N.D.
Dead infected/ml	0	0	5.00*10 <sup>3</sup>	N.D.	N.D.	N.D.	N.D.
Total infected cells/ml	0	0	1.30*10 <sup>5</sup>	N.D.	N.D.	N.D.	N.D.
Infection (%)	0	0	5.6	N.D.	N.D.	N.D.	N.D.
E2-content (µg/ml)	N.D.	N.D.	24.8	151.3	166.9	174.8	232.2

Figure 3 (continued)

Shake flask nr.9 MOI = 0.0001

Time	0 hpi	23 hpi	95 hpi	125 hpi	144 hpi	173 hpi	194 hpi
Living cells/ml	4.23*10 <sup>5</sup>	7.41*10 <sup>5</sup>	1.45*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Dead cells/ml	1.3*10 <sup>4</sup>	1.50*10 <sup>4</sup>	1.20*10 <sup>5</sup>	N.D.	N.D.	N.D.	N.D.
Total cells/ml	4.35*10 <sup>5</sup>	7.56*10 <sup>5</sup>	1.57*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Viability (%)	97.3	98	92.4	N.D.	N.D.	N.D.	N.D.
Living inf cells/ml	0	0	1.12*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Dead infected/ml	0	0	1.20*10 <sup>5</sup>	N.D.	N.D.	N.D.	N.D.
Total infected cells/ml	0	0	1.24*10 <sup>6</sup>	N.D.	N.D.	N.D.	N.D.
Infection (%)	0	0	78.7	N.D.	N.D.	N.D.	N.D.
E2-content (µg/ml)	N.D.	N.D.	113.9	281.3	264.4	298.3	208.6

Relative volumetric E2 protein yield

MOI	Maximum E2 yield (µg E2/ml)	Average of duplicates (µg E2/ml)	Relative E2 yield (%)
1*10 <sup>-1</sup> (V&S-E2-002)	57.3		
1*10 <sup>-1</sup> (V&S-E2-002)	59.6	58.5	48.1
1*10 <sup>-2</sup> (V&S-E2-002)	85.4		
1*10 <sup>-2</sup> (V&S-E2-002)	89.8	87.6	72.1
1*10 <sup>-3</sup> (V&S-E2-002)	120.6		
1*10 <sup>-3</sup> (V&S-E2-002)	122.3	121.5	100.0
1*10 <sup>-4</sup> (V&S-E2-002)	169.6		
1*10 <sup>-4</sup> (V&S-E2-002)	173.2	171.4	141.2
1*10 <sup>-3</sup> (V&S-E2-004)	187.9	187.9	100.0
1*10 <sup>-4</sup> (V&S-E2-004)	216.6	257.0	
1*10 <sup>-4</sup> (V&S-E2-004)	298.3		136.7
1*10 <sup>-5</sup> (V&S-E2-004)	195.7		
1*10 <sup>-5</sup> (V&S-E2-004)	232.2	214.0	113.8
1*10 <sup>-6</sup> (V&S-E2-004)	144.8		
1*10 <sup>-6</sup> (V&S-E2-004)	180.8	162.8	86.6





Figure 4 (continued)

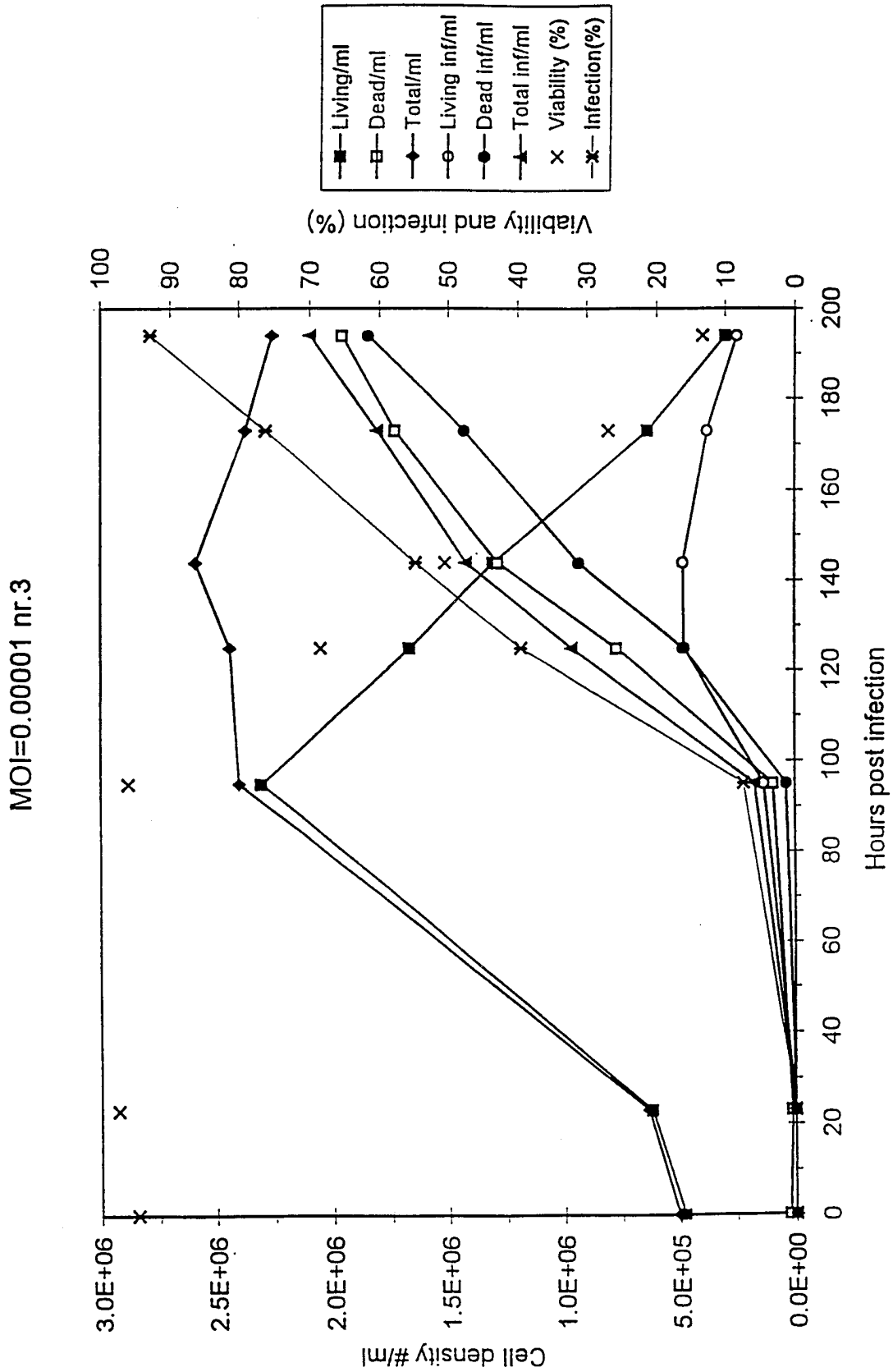




Figure 4 (continued)

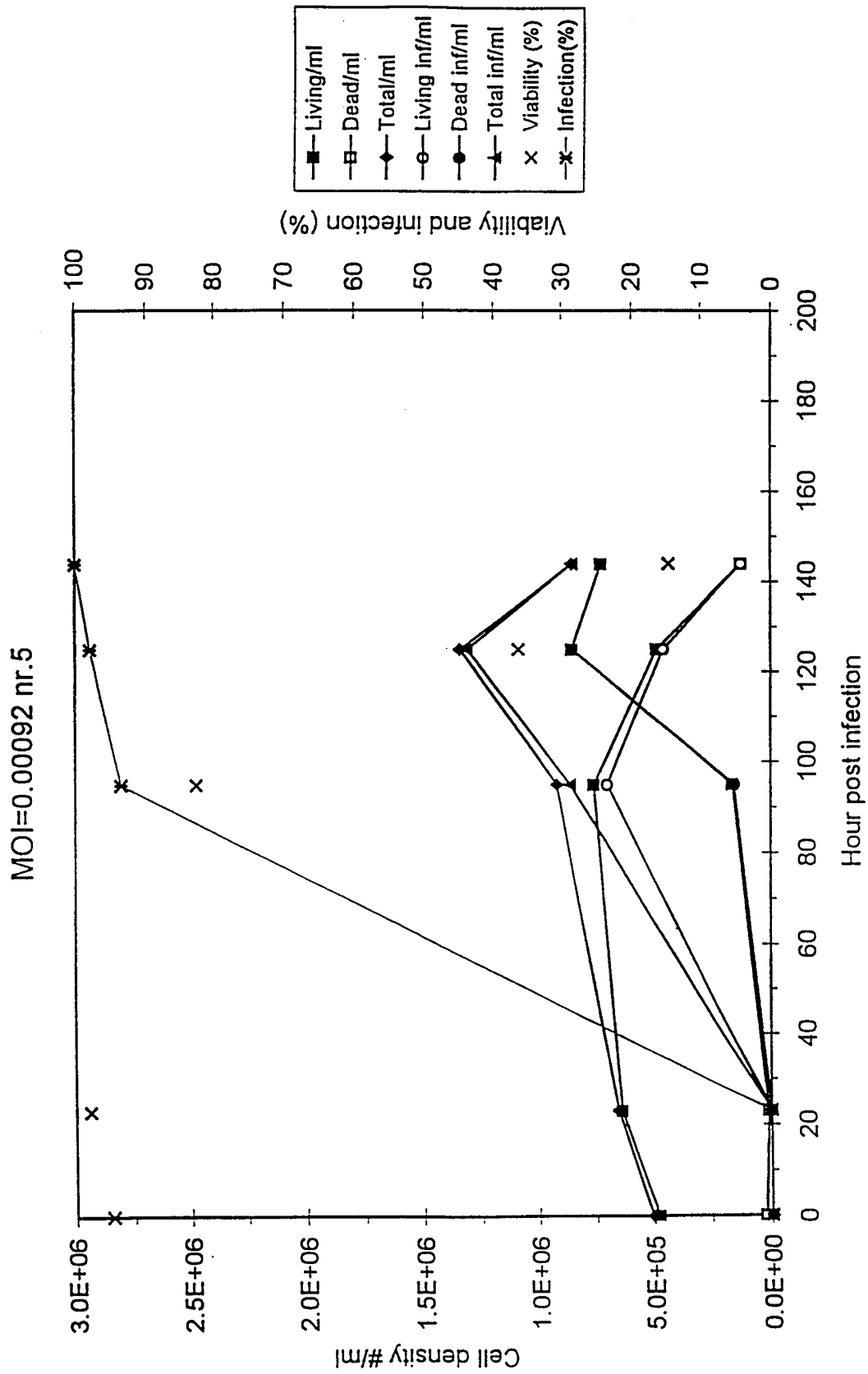


Figure 4 (continued)

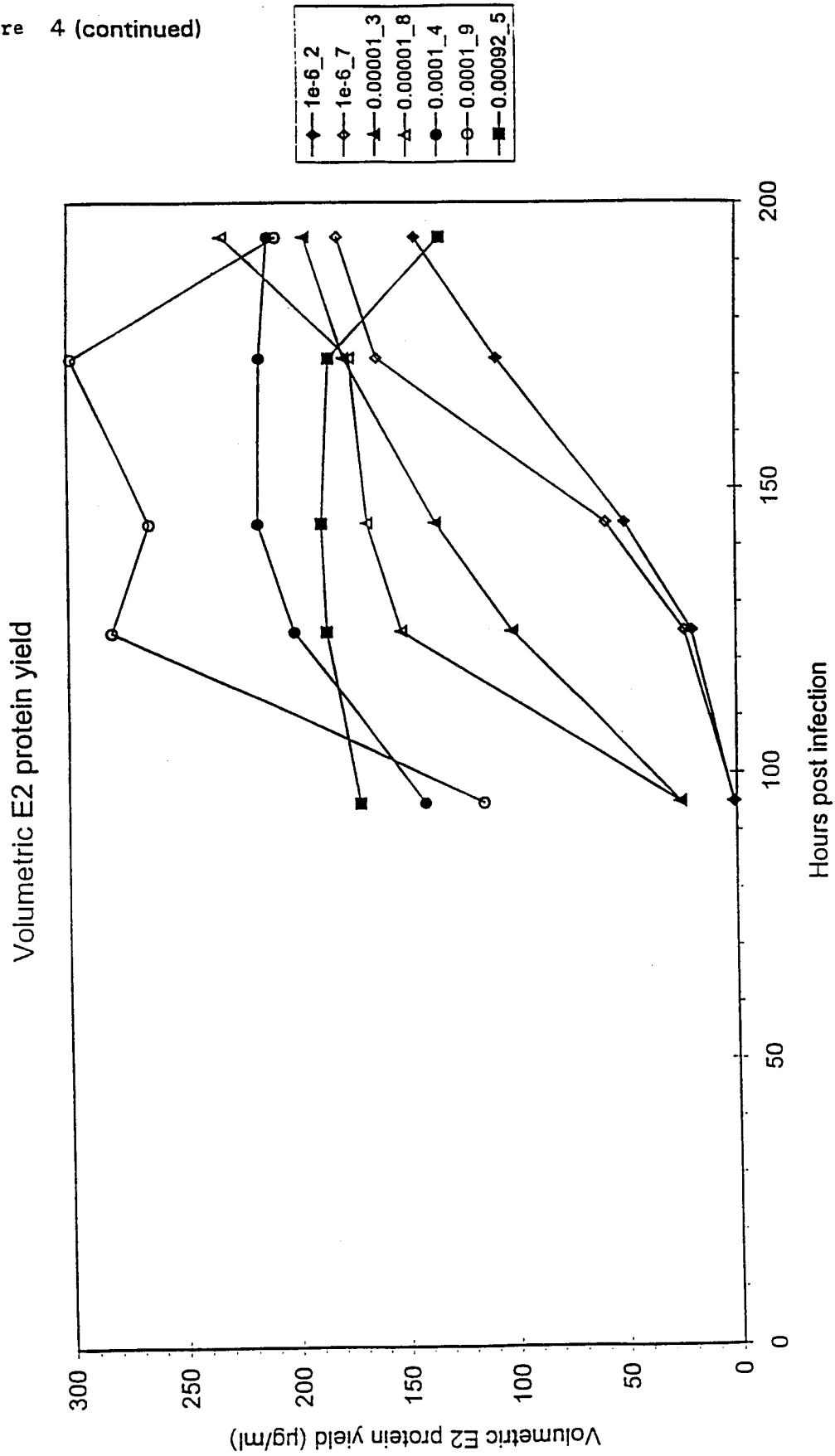


Figure 4 (continued)

