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(54) Title: PRODUCTION OF RECOMBINANT IGM IN PER.C6 CELLS

(57) Abstract: The invention provides an immortalized human retina cell, per.C6 TM expressing E1A and E1B proteins of an adenovirus, wherein said cell comprises recombinant nucleic acid encoding an IgM molecule. The invention also provides a method for recombinant production of an IgM molecule, said method comprising culturing a cell according to the invention and expressing said recombinant nucleic acid encoding an IgM.



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PRODUCTION OF RECOMBINANT IGM IN PER.C6 CELLS

FIELD OF THE INVENTION

5 The invention relates to the field of recombinant protein production. More in particular, the invention relates to the production of immunoglobulins of class M (IgM) in recombinant mammalian host cells.

BACKGROUND OF THE INVENTION

10 Immunoglobulin molecules may be one of five classes based on amino acid sequence of the constant region of the molecule. These classes are IgG, IgM, IgA, IgD and IgE. Each class has different biological roles based on class-specific properties
15 (Roitt, I., Brostoff, J., Make, D. (2001). Immunology. 6th edition, pub. Mosby).

IgM is the first immunoglobulin produced by B cells in response to stimulation by antigen, and is present at around 1.5 mg/ml in serum with a half-life of 5 days. One IgM monomer
20 comprises two heavy chains and two light chains. The heavy chains consist of an N-terminal variable region, followed by four constant regions. At the C-terminus there is a tail-piece which has a function in multimerisation of the molecule. Within the constant regions are cysteine residues that form
25 disulphide bonds with a second heavy chain, and each heavy chain is covalently linked by a disulphide bond to a light chain. Light chains may be of the kappa or lambda class. IgM is unique among immunoglobulins in that the monomeric unit exists mainly in a pentameric or hexameric structure, the five
30 or six monomeric units being all identical (Fig. 1). A J chain may also be present in the structure: this binds to the tail-piece at the C-terminus of the heavy chains and helps to

mediate multimerisation (Yoo et al., 1999). It is commonly observed that IgM containing a J chain exists as a pentamer, and IgM without a J chain exists as a hexamer. The quaternary structure may also impact on the biological activity of the complete molecule (Wiersma, et al., 1998; Johansen et al., 2000). A complete IgM has a mass of approximately 1000 to 1200 kDa.

Each IgM heavy chain has five or six potential N-glycosylation sites on each of the 10 to 12 heavy chains in one IgM molecule, and the glycans make up around 12% of the mass of the molecule. While little is known about the biological activity of IgM glycans, it is likely that they play a role in protein function as has been demonstrated for the glycans present on IgG. The J chain is also glycosylated. Studies on the glycosylation of human IgM have mostly relied on data from pathological IgM derived from patients with a macroglobulinaemia, but there are some studies from cell-lines producing IgM (Leibiger et al., 1998; Wang et al., 2003). A wide range of glycan structures have been observed on IgM molecules, including high mannose, bi- and tri-antennary structures. However IgMs produced in non-human cell-lines have been seen to contain Gal α (1,3)Gal structures, and N-glycolylneuraminic acid: these are not found in humans and are potentially immunogenic (Leibiger et al., 1998). This is an important factor in the production of IgM if it is to be administered therapeutically.

Natural IgM molecules often have a low affinity for antigen; however this is compensated by the high valency, which gives the IgM molecule a high avidity for its target. However if an antigen binding region with a high affinity, e.g. of an IgG, is converted to an IgM format, then the avidity is likely to be extremely high. Multivalency also

results in the ability to aggregate bacteria and other cells, making them easier to eliminate.

IgM binds complement with higher affinity than IgG (the hexameric form being more active in this respect than the pentameric form; Wiersma et al., 1998), providing a highly potent mechanism for complement dependent cytotoxicity. As a result, IgM is often the preferred class of immunoglobulin physiologically to combat bacterial infection.

Another mechanism by which antibodies are thought to eliminate target cells is by binding to, and often cross-linking, cell surface receptors (Ghetie et al., 1997; Tutt et al., 1998; Longo, 2002). This can lead to activation of signalling pathways resulting in arrest of cell growth or apoptosis. The multivalency of IgM makes this molecule potentially very potent at cross-linking surface receptors: IgM molecules have been seen to sit like a crab on the surface of a cell, the variable regions bent over to bind at the cell surface.

There is already evidence in the literature that IgMs may be of value as therapeutics. A natural IgM antibody has been implicated in regression of neuroblastoma cells in human patients, suggesting that it can function as a physiological tumour defence mechanism (Ollert et al, 1996). This has also been studied as a potential therapeutic against neuroblastoma (Engler et al., 2001). There are also data which show that an IgM specific for human gastrointestinal adenocarcinomas is more potent than the IgG format of the same antibody in lysing a colon carcinoma cell-line, possibly as a result of increased complement deposition on the cells (Fogler et al., 1989). The monoclonal IgG antibody OKT3 was the first monoclonal to be used in the clinic, and is used to treat renal allograft rejection; one drawback to this therapy is the release of

cytokines. To try to reduce this, the same antibody was tested in an IgM format in a mouse model where it was observed successfully to reduce inflammation (Choi et al., 2002). There is also much interest in anti-microbial activities of immunoglobulins, a function for which IgM is ideally suited for reasons mentioned above. Studies have also been performed which show that IgM molecules against bacterial lipopolysaccharides can reduce mortality in septic patients with Gram-negative bacteremia (Bogard et al., 1993; Krieger et al., 1993; Seifert et al., 1996).

While these potential advantages of IgM are clear, there is little data in the literature regarding production in cell lines (see Yoo et al, 2002; Knight et al, 1992). Wood et al (1990) reported that IgM could be produced in CHO cells with an initial production of 1 to 1.5 pg mu chain per cell per day. This rose to approximately 30 pg per cell per day after gene amplification with methotrexate and 2'-deoxycoformycin. However, amplification is often associated with instability of expression (Kim et al, 1998; Barnes et al, 2003). Moreover, IgMs produced in non-human cell-lines have been seen to contain Gal α (1,3)Gal structures, and N-glycolylneuraminic acid: these are not found in humans and are potentially immunogenic (Leibiger et al., 1998).

Therefore, a need remains for a good production platform for recombinant IgM production, without the drawbacks associated with the existing platforms.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Cartoon showing hexameric IgM (with no J chain).

Fig. 2. Vector for expression of intact IgM. IgM-encoding regions (light chain, heavy chain), CMV promoters and the neomycin resistance marker are indicated.

5 Fig. 3. Expression levels of IgM-expressing PER.C6TM cells. Cells from 25 different clones were seeded at 1×10^6 cells per well of a 6-well dish and allowed to grow for 4 days. Supernatant was then harvested and assayed for IgM by ELISA.

10 Fig. 4. Reducing SDS-PAGE (stained with Coomassie Blue) of crude cell culture supernatant from clones producing IgM, material after Q-sepharose chromatography, and final purified IgM.

15 Fig. 5. Gel filtration HPLC analysis of purified sample of IgM. A: PER.C6TM anti-EpCAM IgM; B: IgG standard; C: human IgM standard; D: molecular weight standards (proteins of 670, 158, 44, 17 and 1 kDa). Elution times of the main peaks are shown for the immunoglobulin samples; elution time of the 670kDa
20 molecular weight standard is also shown.

Fig. 6. Binding of IgM to LS174T cells. FACS-derived mean fluorescent intensity (MFI) is shown.

25 Fig. 7. Complement dependent cytotoxicity test of anti-EpCAM IgM on LS174T cells. Also present as controls are anti-EpCAM IgG, GBSIII IgG (an antibody which binds a bacterial surface antigen and hence acts as a negative control), as well as no antibody.

30

Fig. 8. MALDI-MS spectrum of (de-sialylated) glycans released from anti-EpCAM IgM produced in PER.C6TM. The proposed

structures of the two main glycan species are indicated. Key:

○ N-Acetylglucosamine, ● galactose, ○ mannose, △ fucose

DESCRIPTION OF THE INVENTION

The characteristics of a platform for production of IgM would preferably include high IgM productivity and human-type glycosylation of the molecule. It is demonstrated herein that PER.C6TM cells are capable of efficiently producing and secreting recombinant IgM molecules. High levels of functional IgM are expressed from recombinant cells without the need for amplification of the copy number of the recombinant nucleic acid encoding the IgM. The expressed IgM is in multimeric form and contains mainly biantennary N-linked glycans with a high galactose content. Glycan structures that are known to be immunogenic in man, such as Gal α (1,3)Gal structures and N-glycolylneuraminic acid, have not been found on the IgM produced according to the invention.

In one aspect, the invention provides an immortalized human retina cell expressing ElA and ElB proteins of an adenovirus, wherein said cell comprises recombinant nucleic acid encoding an IgM molecule in expressible format. In another aspect the invention provides a method for recombinant production of an IgM molecule, said method comprising: a) providing an immortalized human retina cell expressing ElA and ElB proteins of an adenovirus, wherein said cell further comprises recombinant nucleic acid encoding an IgM molecule in expressible format; and b) culturing said cell and expressing said recombinant nucleic acid encoding an IgM. In certain embodiments, the method further comprises the step of: c) isolating the recombinant IgM from said cells, from the

culture medium or from both said cells and the culture medium. In another aspect, the invention provides for the use of an immortalized human retina cell expressing E1A and E1B proteins of an adenovirus for recombinant expression of IgM molecules.

5 In preferred embodiments the cells of the invention are PER.C6TM cells or derived therefrom.

DETAILED DESCRIPTION OF THE INVENTION

10 The present invention teaches that cells derived from human retina cells, which have been immortalized by introduction of E1 sequences from an adenovirus, are a good production platform for recombinant IgM molecules. A method for immortalization of embryonic retina cells has been

15 described in US patent 5,994,128. Accordingly, an embryonic retina cell that has been immortalized with E1 sequences from an adenovirus can be obtained by that method. Such a cell expresses at least the E1A region of an adenovirus, and preferably also the E1B region. E1A protein has transforming

20 activity, while E1B protein has anti-apoptotic activities. The cells of the invention therefore preferably express E1A and E1B proteins of an adenovirus. In preferred embodiments, such cells are derived from PER.C6TM cells. A PER.C6TM cell as used herein, is a cell having essentially the characteristics as

25 the cells deposited at the ECACC on 29 February 1996, under number 96022940. Cells derived from a PER.C6TM cell according to the invention can be obtained by introduction of foreign genetic material encoding an IgM molecule into such PER.C6TM cells. Preferably said cells are from a stable clone that can

30 be selected and propagated according to standard procedures known to the person skilled in the art. A culture of such a clone is capable of producing recombinant IgM molecules. Cells

according to the invention preferably are able to grow in suspension culture in serum-free medium.

It has previously been shown that PER.C6TM cells can express intact human IgG (WO 00/63403), that such IgGs have human-type glycans and said cells can be grown at large scale (Jones et al, 2003; Nichols et al, 2002). However, no specific data have been provided for other immunoglobulin classes. The present invention teaches that these cells can efficiently produce an entirely different class of immunoglobulins that have very different characteristics from IgG, i.e. IgM molecules. It was unexpectedly found that IgM can be produced in PER.C6 cells at levels comparable to those for IgG. Moreover, the produced IgM was shown to be functional and to have a human-type glycosylation. These aspects could not be foreseen based upon data of the much smaller IgG molecules.

To obtain expression of nucleic acid sequences encoding IgM, it is well known to those skilled in the art that sequences capable of driving such expression can be functionally linked to the nucleic acid sequences encoding the IgM molecules, resulting in recombinant nucleic acid molecules encoding an IgM in expressible format. Functionally linked is meant to describe that the nucleic acid sequences encoding the IgM antibody fragments or precursors thereof are linked to the sequences capable of driving expression such that these sequences can drive expression of the antibodies or precursors thereof. Useful expression vectors are available in the art, e.g. the pcDNA vector series of Invitrogen. Where the sequence encoding the IgM polypeptide of interest is properly inserted with reference to sequences governing the transcription and translation of the encoded polypeptide, the resulting expression cassette is useful to produce the IgM of interest, referred to as expression. Sequences driving expression may

include promoters, enhancers and the like, and combinations thereof. These should be capable of functioning in the host cell, thereby driving expression of the nucleic acid sequences that are functionally linked to them. Promoters can be

5 constitutive or regulated, and can be obtained from various sources, including viruses, prokaryotic, or eukaryotic sources, or artificially designed. Expression of nucleic acids of interest may be from the natural promoter or derivative thereof or from an entirely heterologous promoter. Some well-

10 known and much used promoters for expression in eukaryotic cells comprise promoters derived from viruses, such as adenovirus, e.g. the E1A promoter, promoters derived from cytomegalovirus (CMV), such as the CMV immediate early (IE) promoter, promoters derived from Simian Virus 40 (SV40), and

15 the like. Suitable promoters can also be derived from eucaryotic cells, such as methallothionein (MT) promoters, elongation factor 1 α (EF-1 α) promoter, actin promoter, an immunoglobulin promoter, heat shock promoters, and the like. In one embodiment the sequence capable of driving expression

20 comprises a region from a CMV promoter, preferably the region comprising nucleotides -735 to +95 of the CMV immediate early gene enhancer/promoter. This gives particularly high expression levels in cells expressing E1A of an adenovirus.

Culturing a cell is done to enable it to metabolize,

25 and/or grow and/or divide and/or produce recombinant proteins of interest. This can be accomplished by methods well known to persons skilled in the art, and includes but is not limited to providing nutrients for the cell. The methods comprise growth adhering to surfaces, growth in suspension, or combinations

30 thereof. Several culturing conditions can be optimized by methods well known in the art to optimize protein production yields. Culturing can be done for instance in dishes, roller

bottles or in bioreactors, using batch, fed-batch, continuous systems, hollow fiber, and the like. In order to achieve large scale (continuous) production of recombinant proteins through cell culture it is preferred in the art to have cells capable of growing in suspension, and it is preferred to have cells capable of being cultured in the absence of animal- or human-derived serum or animal- or human-derived serum components. Thus purification is easier and safety is enhanced due to the absence of additional animal or human proteins derived from the culture medium, while the system is also very reliable as synthetic media are the best in reproducibility.

The conditions for growing or multiplying cells (see e.g. Tissue Culture, Academic Press, Kruse and Paterson, editors (1973)) and the conditions for expression of the recombinant product may differ somewhat, and optimization of the process is usually performed to increase the product yields and/or growth of the cells with respect to each other, according to methods generally known to the person skilled in the art. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach (M. Butler, ed., IRL Press, 1991).

The IgM is expressed in the cells according to the invention, and may be recovered from the cells or preferably from the cell culture medium, by methods generally known to persons skilled in the art. Such methods may include precipitation, centrifugation, filtration, size-exclusion chromatography, affinity chromatography, cation- and/or anion-exchange chromatography, hydrophobic interaction chromatography, and the like. In certain aspects of the invention, the isolation of the IgM comprises an anion exchange chromatography step and/or a gel filtration step.

It is demonstrated herein that IgM can be expressed at high levels without the necessity for first amplifying the nucleic acid sequences encoding the IgM within the host cells. This has the advantage that no large copy numbers are required
5 for efficient expression according to the invention, in contrast to previously described recombinant IgM production systems, where amplification was required to obtain levels of around 30 pg μ chain per cell per day. PER.C6 cells expressing IgG at high levels have been shown to contain
10 usually between 1 and 10 copies of the nucleic acid encoding the IgG per cell (Jones et al, 2003). Methods to determine copy numbers are known to the person skilled in the art of molecular biology, and include Southern blotting, quantitative PCR, fiber-FISH, and the like. Hence, the invention provides
15 for a method according to the invention wherein the cells comprise 1-20, usually between 1 and 10 copies per cell of the recombinant nucleic acid encoding the IgM molecule. This has the advantage of establishing a production system fast, as no labour-intensive and time consuming amplification step is
20 needed to obtain clones with sufficiently high expression levels for analysis. Moreover, such cells are expected to be more stable than cells containing high copy numbers of the transgene, that are reported to display instability upon propagation of the cells (Kim et al, 1998; Barnes et al,
25 2003). Therefore, also in view of regulatory requirements the cells and methods according to the invention are an improvement over those of the prior art. Preferably, cells in the method of the invention express at least 5 pg IgM per seeded cell per day, more preferably at least 20 pg per seeded
30 cell per day.

The IgM production system of the invention is not dependent upon co-expression of the J-chain for production of

functional IgM in the form of multimers. Optionally however, J-chain may be co-expressed.

An IgM molecule is an immunoglobulin wherein the heavy chains are mu chains. An IgM molecule can have a pentameric or
5 hexameric structure. An IgM molecule according to the invention may be of any origin, including human, rodent, chimeric, humanized, and the like, however human IgMs are preferred in the invention. Using a human cell line for the production provides these molecules with a human-type
10 glycosylation, resulting in production of IgM molecules that are not recognized as foreign by the human immune system, because both the polypeptide and the glycan portion are human. The person skilled in the art will be aware of the possibilities to obtain human IgM sequences. The sequences for
15 the constant regions are known, and are also provided herein. Sequences encoding human variable regions may e.g. be obtained by known methods such as phage display (methods e.g. described in CF Barbas III et al, Phage Display. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New
20 York, 2001) or by immunizing transgenic mice that comprise genetic material encoding a human immunoglobulin repertoire (Fishwild et al, 1996; Mendez et al, 1997).

Antibodies may be used as naked molecules, or they may be in the form of immunoconjugates or labelled molecules, and so
25 used as a magic bullet to deliver a cargo to a tumor or infection for therapy or imaging (Carter, 2001; Borrebaeck and Carlsson, 2001; Park and Smolen, 2001). Immunoconjugates comprise antigen binding domains and a non-antibody part such as a toxin, a radiolabel, an enzyme, and the like. IgM
30 molecules may be labelled in the same way as IgG molecules, but the high avidity would likely mean that they are less likely to dissociate from the target antigen once bound. This

advantageously could deliver a cargo to a target cell more permanently. Hence, the term IgM molecule as used herein includes naked IgM molecules, but may also refer to immunoconjugates comprising IgM molecules.

5 The IgM generated in this study is against the human tumour antigen EpCAM (epithelial cell adhesion molecule), a 40kDa glycoprotein expressed on the surface of colon carcinoma cells. The high expression of EpCAM on colon carcinomas makes it an attractive target for immunotherapy. The antibody was
10 isolated from a semi-synthetic phage library as a single chain Fv fragment named UBS54 (WO 01/48485; Huls et al, 1999). In one aspect the invention therefore provides a recombinant human IgM molecule that is capable of binding to EpCAM. In other embodiments said human IgM molecules are used for the
15 preparation of a medicament and/or for direct treatment of a disease such as cancer.

EXAMPLES

The invention will now be described by some examples, not to
20 be construed to limit the scope of the invention. The practice of this invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology, and recombinant DNA, which are within the skill of the art. See e.g. Sambrook, J. and
25 Russell, D.W. (2001). Molecular cloning: a laboratory manual. Pub. Cold Spring Harbor Laboratory Press; Current Protocols in Molecular Biology, Ausubel FM, et al, eds, 1987; the series Methods in Enzymology (Academic Press, Inc.); PCR2: A Practical Approach, MacPherson MJ, Hams BD, Taylor GR, eds,
30 1995; Antibodies: A Laboratory Manual, Harlow and Lane, eds, 1988.

Example 1. Construction of pEpcamIgM expression vector.

An expression plasmid was generated which encodes both the light and heavy chains of an IgM antibody that recognizes EpCAM. The DNA encoding the antigen-binding region of this antibody was first isolated from a scFv phage display library (Huls et al, 1999). A leader sequence and constant regions were added essentially as described in Boel et al, 2000. The genomic DNA encoding the light and heavy chains (genomic sequences of the antibody-encoding regions provided in SEQ. ID. NOs. 1 and 2, amino acid sequences of the encoded anti-EpCAM IgM provided in SEQ. ID. NOs. 3 and 4) were then amplified using PCR to append convenient restriction sites, and then cloned into the expression vector pcDNA3002 (Neo). The following primers were used for PCR of the light chain:

E001: CCTGGCGCGCCACCA**ATGG**CATGCCCTGGCTTCCTGTGG (SEQ. ID. NO. 5)
 E002: CCGGGTTAACTA**CTA**CACTCTCCCCTGTTGAAGC (SEQ. ID. NO. 6)

The following primers were used for PCR of the heavy chain:

E003: GGAGGATCCGCCACCA**ATGG**CATGCCCTGGCTTCCTGTGG (SEQ. ID. NO. 7)
 S02: GGAACGCTAGCT**TCAG**TAGCAGGTGCCAGCT (SEQ. ID. NO. 8)

The start codon (E001, E003) and stop codon (E002, S02) are in bold. Restriction sites AscI (E001), HpaI (E002), BamHI (E003) and NheI (S02) are underlined. Primers E001 and E003 also include a Kozak sequence (italics). The light chain fragment of approximately 0.9kb was digested with AscI-HpaI and inserted into pcDNA3002 (Neo) digested with the same enzymes. The heavy chain fragment of approximately 2.3kb was then digested with BamHI-NheI and inserted into pcDNA3002 (Neo) containing the light chain digested with the same enzymes. The resulting plasmid is pEpcamIgM (Fig. 2). The generated construct contains DNA encoding a kappa light chain and a mu heavy chain, both preceded by a CMV promoter. The expression vector pcDNA3002 (Neo), which has been described in

international patent application PCT/NL02/00841, was deposited on December 13, 2001 at the European Collection of Cell Cultures (ECACC) under number 01121318.

5 **Example 2.** Transfection of PER.C6TM cell line and production of IgM.

Cells were transfected with pEpcamIgM by a lipofectamine based method. In brief, PER.C6TM cells were seeded at 3.5×10^6 cells per 96mm tissue culture dish. For each dish, 2µg plasmid
10 DNA was mixed with 10µl lipofectamine (Gibco); this was added to the cells in serum free DMEM medium (total volume 7ml) and incubated for 4 hours. This was then replaced with DMEM medium (i.e. containing serum). The following day (and for the ensuing 3 weeks) cells were grown in DMEM medium in the
15 presence of 0.5mg/ml Geneticin (G418) to select for clones that were stably transfected with the plasmid. Stable clones were picked from the plate and twenty-five were selected for analysis of IgM productivity by ELISA analysis. In brief, cells were plated at 1×10^6 cells per well of a 6-well dish in
20 DMEM serum. These were incubated for 4 days, after which time supernatant was harvested and IgM concentration measured. For ELISA analysis, wells of a 96-well plate were coated with antibody raised against Ig kappa light chain. After blocking with a BSA solution, samples were added to wells at varying
25 dilutions and incubated for 1 hour. The standard used was human IgM (Accurate Chemical cat. YSRTPHP003). After washing, detection antibody (HRP-labelled anti-IgM) was applied for 30 minutes. After a further washing step, substrate O-phenylene diamine dihydrochloride was added. Antibody concentration was
30 determined by comparing optical density at 492nm with that of the known antibody standard. The results are shown in Fig. 3, and the highest-producing clones picked up so far produce

around 27 pg IgM/seeded cell/day. This is comparable to results seen when IgG-producing cell lines are measured with the same analysis. It is possible that further screening will allow picking up clones with even higher expression levels.

5 Production of antibody was performed in serum-free medium. Thus the adherent cells in tissue culture flasks were transferred to roller bottles in PER.C6TM suspension growth medium (under which conditions the cells grow in suspension). After one week, supernatant was harvested and electrophoresed

10 on reducing SDS-PAGE (Fig. 4). The heavy and light chains that comprise the intact, secreted antibody are the predominant protein species. The material was then purified using standard techniques over HiTrap Q (Amersham Biosciences) anion exchange chromatography (which primarily concentrates material) and

15 then gel filtration chromatography. Material was loaded on Q-sepharose FF (Amersham Biosciences) in 20mM sodium phosphate pH8.0 and eluted with a gradient of NaCl. Peak fractions were pooled and loaded on to a HiLoad 26/60 Superdex 200 (Amersham Biosciences) and eluted in PBS. Purity of IgM after each step

20 is shown in Fig. 4.

Example 3. HPLC gel filtration of IgM.

In order to test whether the IgM produced was monomeric or multimeric, purified IgM was electrophoresed over an HPLC

25 gel filtration column (Zorbax GF450 (Agilent) in 250mM sodium phosphate buffer pH6.8). Other control samples included recombinant human IgG, human IgM (Accurate Chemical cat. YSRTPHP003) and molecular weight standards. This is shown in Fig. 5. The small peak before the main IgM elution peak is

30 likely to be the void volume of the column. PER.C6TM IgM elutes at a similar position to human serum IgM, and is larger than the protein standard of 670kDa (the first peak in this

chromatogram). There is no evidence for monomeric or other low valency molecules.

Example 4. Binding to LS174T cells.

5 LS174T cells (ATCC number CL-188) express EpCAM antigens and were therefore used as targets for determination of anti-EpCAM binding and hence IgM integrity. Cells were harvested from DMEM medium and washed in PBS. Cells were transferred to Falcon FACS tubes (0.25×10^6 cells per FACS tube) and washed
10 with 2 ml PBS/0.5% BSA (wash and incubation buffer; further indicated as WB). After centrifugation at 300x g for 5 min, supernatant was removed and cells were resuspended in 100 μ l antibody dilutions.

Serial dilutions of 16, 4, 1, 0.25, 0.062, 0.016 and
15 0.004 μ g/ml IgM were prepared in WB. Three negative controls were used; 1) cells incubated without primary and secondary antibody, further referred to as "no antibody", 2) cells incubated with only secondary antibody (PE-control), and 3) GBSIII, a negative control which recognizes a bacterial
20 antigen, at a concentration of 40 μ g/ml.

After 30 min of incubation at 4°C, cells were washed with 2 ml WB. Samples were centrifuged for 5 min at 300x g and supernatant was removed. Cells were resuspended in 100 μ l goat anti-human kappa PE (diluted WB 1:100 or 1:250) and incubated
25 for 20 min at 4°C. Subsequently, cells were washed with 2 ml WB, and centrifuged for 5 min at 300x g. The cell pellet was resuspended in 250 μ l WB and samples were analyzed on a FACS Calibur in FL2 channel.

The IgM binds to the LS174T cells in a concentration dependent
30 manner (Fig. 6). This shows that the protein is correctly folded.

Example 5. CDC activity.

CDC activity of anti-EpCAM IgM was tested with LS174T colon carcinoma cells as target cells and human complement serum (Quidel).

Briefly, LS174T cells were harvested at 70% confluency from DMEM medium. At a concentration of 1×10^6 cells/ml, cells were labeled for 15 min at 37°C with 1:50000 calcein-AM (stock concentration 3.3 µg calcein/µl DMSO) in CDC medium. Cells were washed 2 times in CDC medium and diluted to 1×10^6 cells/ml in the same medium. Anti-EpCAM IgG, anti-GBSIII and anti-EpCAM IgM were diluted in CDC medium to different concentrations varying from 160 to 0.006 µg/ml. Samples included 50 µl antibody, 50 µl of labeled target cells, 50 µl serum and 50 µl medium. Two negative controls were used; 1) GBS III, an antibody directed against antigen III of Streptococcus group B, and 2) samples without antibody ("no antibody"). Fifty µl CDC medium replaced the antibody solution in the "no antibody" control sample. Anti-EpCAM IgG was used as a positive control. Samples were incubated for 4 hours at 37 °C in 10% CO₂ incubator and then analysed by FACS (FACSCalibur, Becton Dickinson). The percentage of lysis of target cells by the complement was determined after gating the calcein positive target cells in the FL1 channel. Propidium iodide (PI; 0.4 µg/ml) was added to determine the percentage of dead cells in the gated population. PI was detected in the FL2 channel. The percentage of dead cells was calculated by [the number of both PI positive and calcein positive cells], divided by the total number of calcein positive cells, multiplied by 100%. The presence of IgM and IgG caused complement-dependent cell lysis in a concentration-dependent manner (Fig. 7). The IgM was

approximately 10-fold more potent in this assay than the IgG:
1.5 μ g/ml IgG gave the same percentage cell lysis as 0.15 μ g/ml
IgM. Negative controls (no antibody and GBSIII) gave
background signals. In other CDC assays the IgM was at least
5 10 times more potent than IgG in this assay (data not shown).
This shows that the PER.C6TM produced IgM is functionally
active.

Example 6. MALDI-MS analysis of glycans present on PER.C6TM
10 IgM.

Purified IgM samples in 20 mM sodium phosphate (pH 7.2)
were digested with PNGase F which releases the N-linked
glycans. The glycan pools were desialylated using neuraminidase
and were analyzed in the reflector mode on an Applied
15 Biosystems Voyager DE Pro MALDI mass spectrometer. The matrix
was 2,5-dihydroxybenzoic acid (10 mg/ml) in 50/50/0.1
acetonitrile/water/trifluoroacetic acid. Spectra were obtained
in the positive ion mode and glycans were detected as sodium
adducts, $[M+Na]^+$.
20 Results are shown in Fig. 8. The main peak is a biantennary,
fully galactosylated structure with a core fucose. Therefore,
in one aspect of the invention at least 50% of the N-linked
sugar structures of the produced IgM are biantennary, fully
galactosylated (not having a terminal N-Acetylglucosamine)
25 structures with a core fucose. Mass data (combined with
knowledge of fully analyzed glycan structures found on
recombinant erythropoietin produced on PER.C6TM cells (...))
indicate that other minor structures include tri- and tetra-
antennary glycans, as well as some hybrid structures. Glycans
30 containing a Lewis X structure (structures with an additional
fucose attached to the N-acetylglucosamine in the antenna) are
also observed at low levels.

The examples above demonstrate that PER.C6™ cells may be transfected with a plasmid expressing IgM to give cells with a high IgM productivity. Moreover the IgM is structurally sound and functionally active, and glycans which may prove immunogenic to humans have not been observed.

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CLAIMS

1. A method for recombinant production of an IgM molecule,
said method comprising:
 - 5 a) providing an immortalized human retina cell expressing E1A and E1B proteins of an adenovirus, wherein said cell further comprises recombinant nucleic acid encoding an IgM molecule in expressible format;
 - b) culturing said cell and expressing said recombinant
10 nucleic acid encoding an IgM.
2. A method according to claim 1, wherein said cells
comprise 1-20 copies per cell of said recombinant nucleic
acid encoding the IgM molecule.
15
3. A method according to claim 1 or 2, wherein the cells in
the culture produce at least 5 pg IgM/seeded cell/day.
4. A method according to claim 3, wherein the cells in the
20 culture produce at least 20 pg IgM/seeded cell/day.
5. A method according to anyone of claims 1-4, wherein the
IgM that is produced is essentially devoid of
Gal α (1,3)Gal structures.
25
6. A method according to anyone of claims 1-5, wherein the
IgM that is produced is essentially devoid of N-
glycolylneuraminic acid.
- 30 7. A method according to anyone of claims 1-6, wherein at
least 50% of the N-linked sugar structures on the IgM
that is produced are biantennary, fully glycosylated

structures with a core fucose.

8. A method according to anyone of claims 1-7, further comprising the step of:
- 5 c) isolating the recombinant IgM from said cells, from the culture medium or from both said cells and the culture medium.
9. A method according to anyone of claims 1-8, wherein said
- 10 IgM molecule is a human IgM molecule.
10. A method according to anyone of claims 1-9, wherein said cells are in suspension in said culture.
- 15 11. A method according to anyone of claims 1-10, wherein said culturing is performed at least part of the time in a serum-free culture medium.
12. A method according to anyone of claims 1-11, wherein
- 20 said IgM molecule is capable of specifically binding to the EpCAM antigen.
13. A method according to anyone of claims 1-12, wherein said cell does not comprise recombinant nucleic acid
- 25 encoding a J-chain.
14. An immortalized human retina cell expressing E1A and E1B proteins of an adenovirus, wherein said cell comprises recombinant nucleic acid encoding an IgM molecule in
- 30 expressible format.

15. An immortalized human retina cell according to claim 14,
wherein said IgM molecule is a human IgM molecule.
- 5 16. Use of an immortalized human retina cell expressing E1A
and E1B proteins of an adenovirus for recombinant
expression of IgM molecules.
- 10 17. A method according to anyone of claims 1-13, or an
immortalized human retina cell according to claim 14 or
15, or use according to claim 16, wherein said
immortalized human retina cell is derived from a PER.C6TM
cell.

Fig. 1

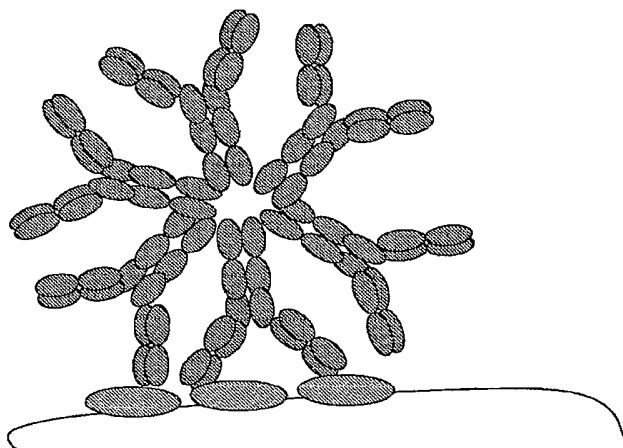


Fig. 2

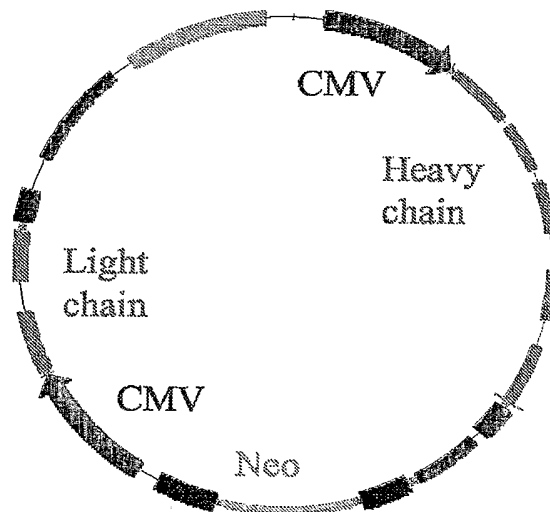


Fig. 3

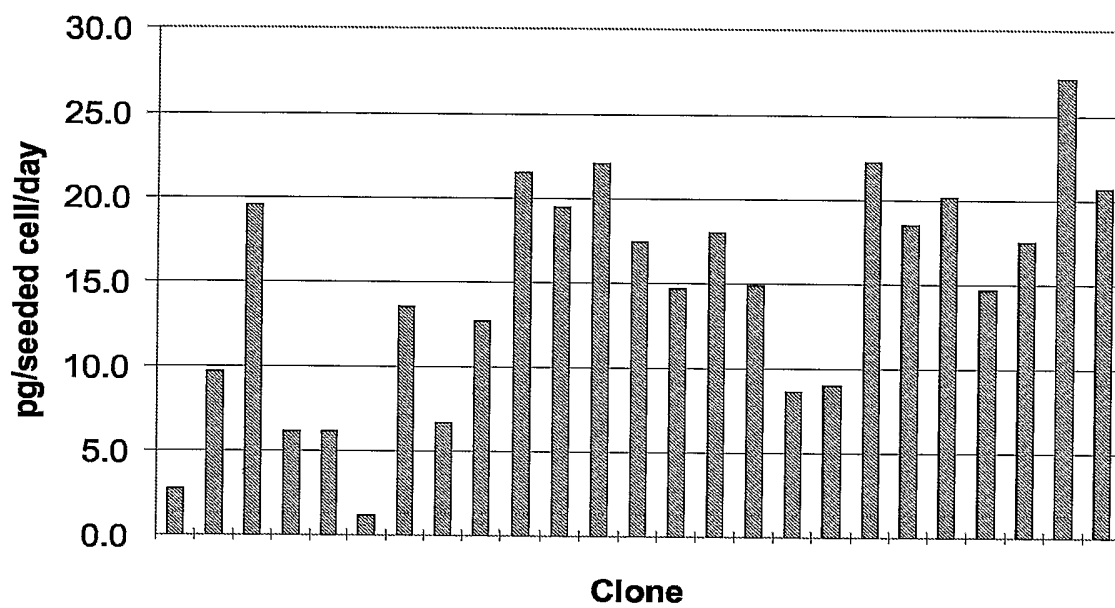


Fig. 4.

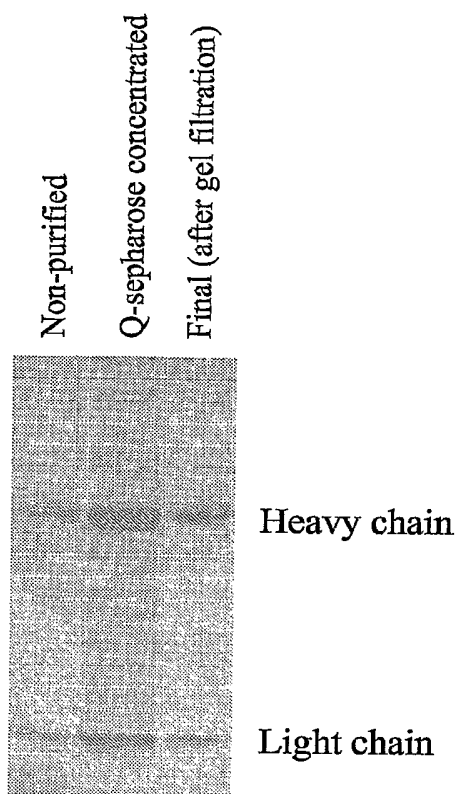


Fig. 5.

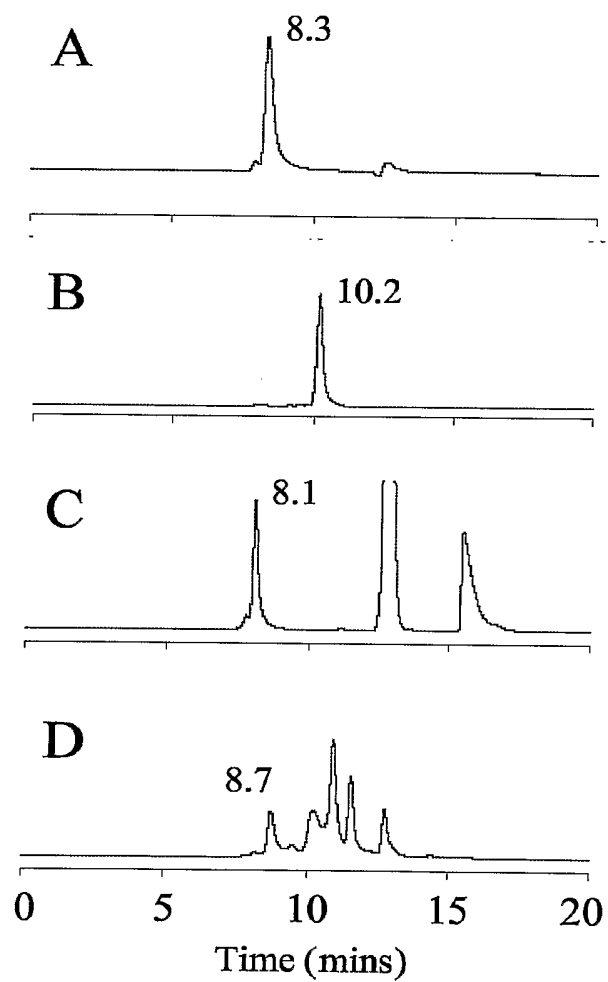


Fig. 6.

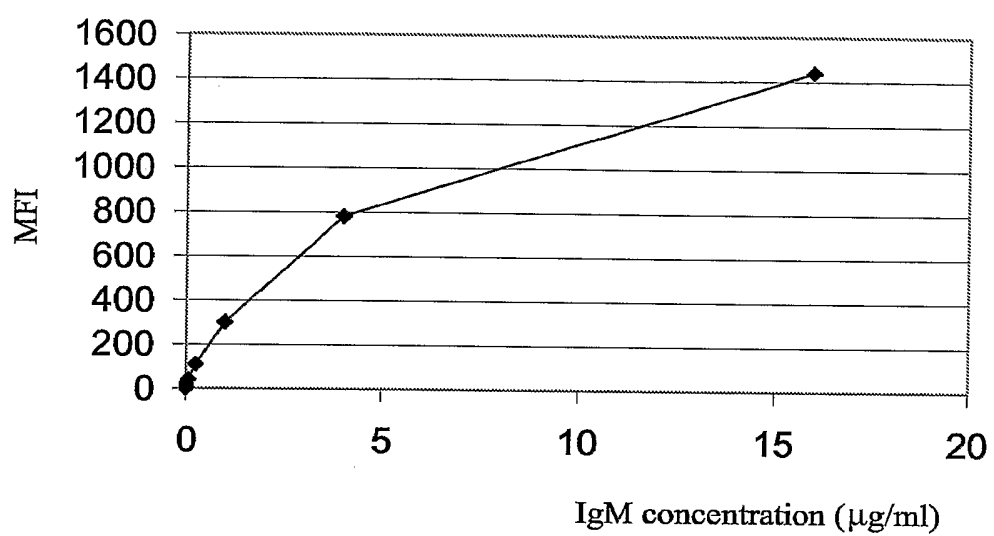


Fig. 7.

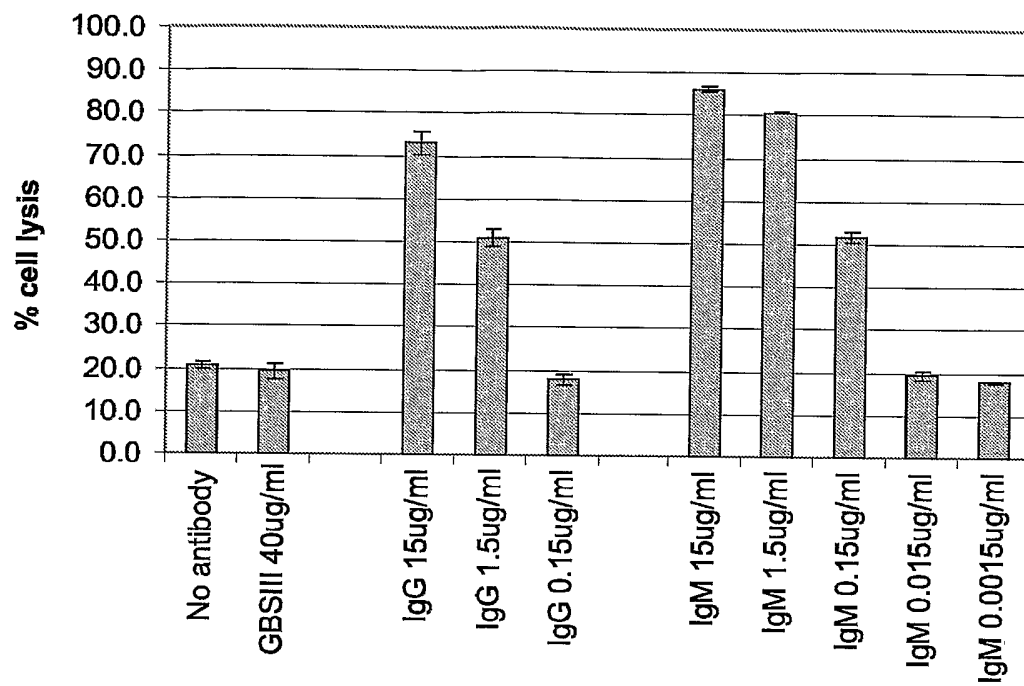
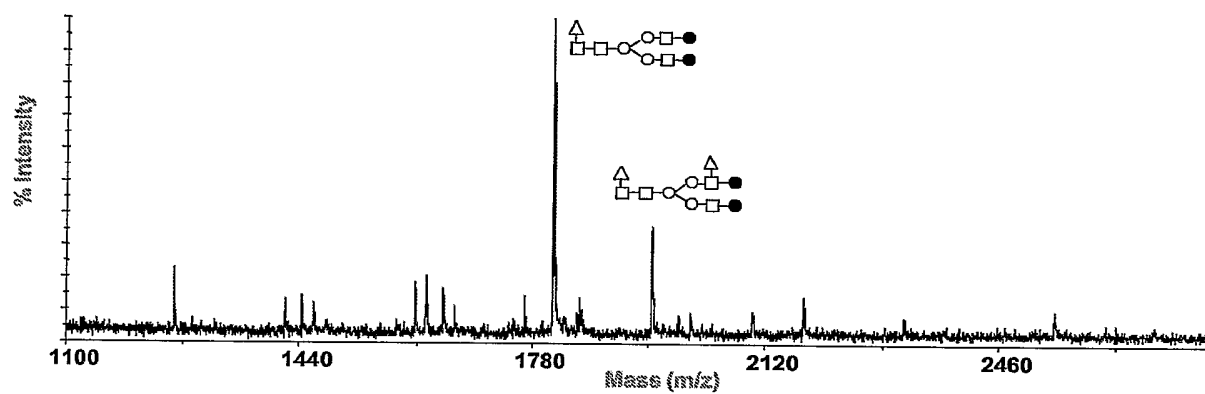


Fig. 8



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/050844

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K16/30 C07K16/00 C12N5/10 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 01/85797 A (PEASE LARRY R ;MAYO FOUNDATION (US); MILLER DAVID J (US); RODRIGUE) 15 November 2001 (2001-11-15) page 132, line 25 - page 137, line 8; example 5	1-17
Y	WO 00/63403 A (SCHOUTEN GOVERT JOHAN ;HATEBOER GUUS (NL); BOUT ABRAHAM (NL); INTR) 26 October 2000 (2000-10-26) page 57, line 7 - page 65, line 34	1-17
Y	WO 89/01975 A (CELLTECH LTD) 9 March 1989 (1989-03-09) page 9, line 8 - page 11, line 26 page 15, line 22 - page 23, line 20	1-17
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *G* document member of the same patent family

Date of the actual completion of the international search

11 October 2004

Date of mailing of the international search report

19/10/2004

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Fax: (+31-70) 340-3016

Authorized officer

Le Flao, K

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2004/050844

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. type of material
- ☒ a sequence listing
- ☐ table(s) related to the sequence listing
- b. format of material
- ☒ in written format
- ☒ in computer readable form
- c. time of filing/furnishing
- ☒ contained in the international application as filed
- ☒ filed together with the international application in computer readable form
- ☐ furnished subsequently to this Authority for the purpose of search
2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/050844

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JONES DAVID ET AL: "High-level expression of recombinant IgG in the human cell line PER.C6." BIOTECHNOLOGY PROGRESS, vol. 19, no. 1, February 2003 (2003-02), pages 163-168, XP002256988 ISSN: 8756-7938 cited in the application the whole document	1-17
A	SMITH R I ET AL: "Addition of a mu-tailpiece to IgG results in polymeric antibodies with enhanced effector functions including complement-mediated cytotoxicity by IgG4" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO. BALTIMORE, US, vol. 154, no. 5, 1 March 1995 (1995-03-01), pages 2226-2236, XP002111659 ISSN: 0022-1767 the whole document	1-17
A	FALLAUX F ET AL: "New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses" HUMAN GENE THERAPY, XX, XX, vol. 9, no. 9, 1 September 1998 (1998-09-01), pages 1909-1917, XP002111070 ISSN: 1043-0342 the whole document	1-17

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/050844

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