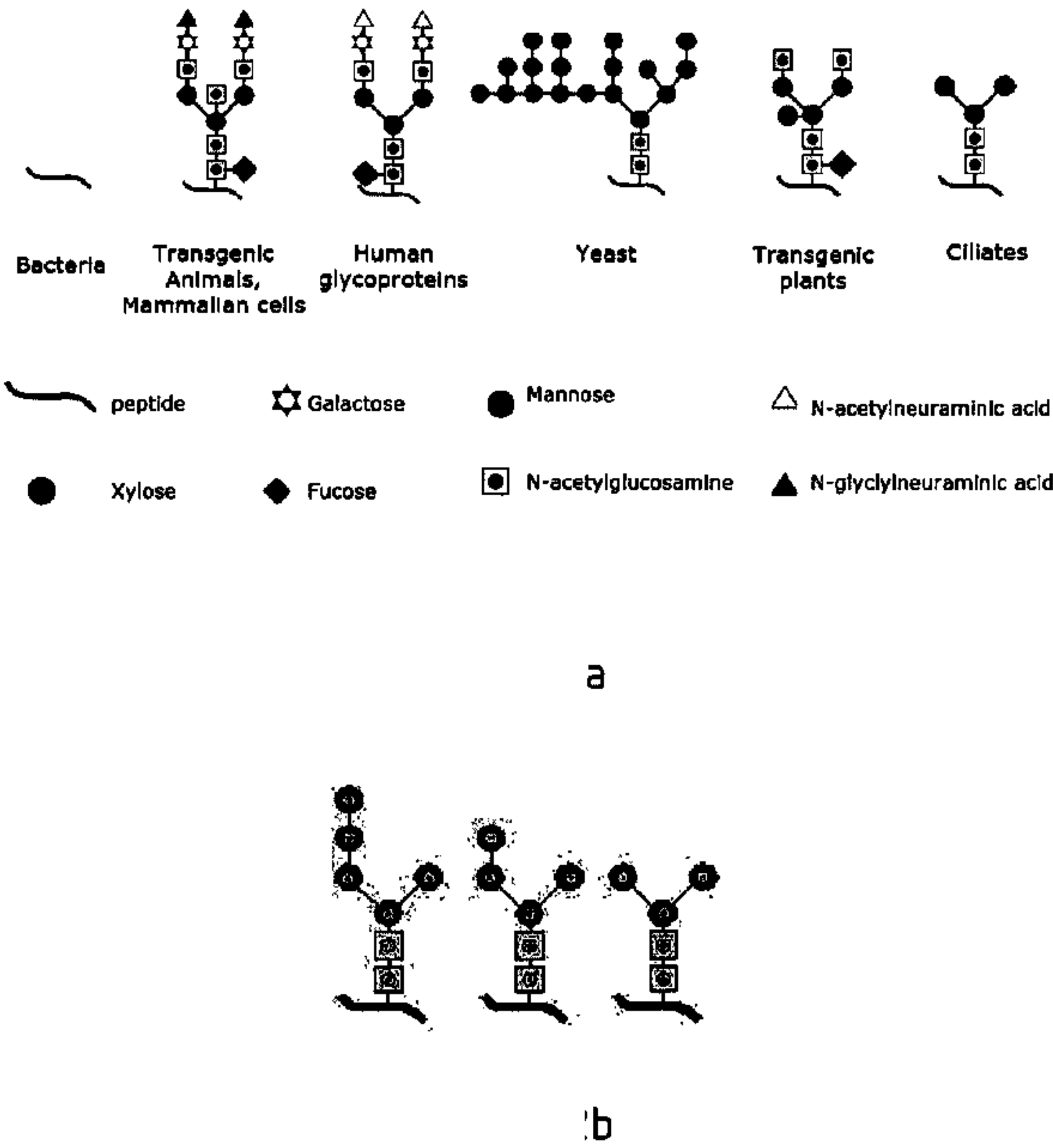




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(54) Titre : EXPRESSION D'ANTICORPS MONOCLONAUX DANS DES CELLULES HOTES CILIEES  
(54) Title: EXPRESSION OF MONOCLONAL ANTIBODIES IN CILIATE HOST CELLS



(57) Abrégé/Abstract:  
The present invention is related to a system for the heterologous expression of a monoclonal Antibody (mAb) or a fragment or derivative thereof, said system comprising at least one ciliate host cell, and incorporated, into said ciliate host cell, at least one heterologous nucleic acid molecule encoding for said monoclonal Antibody, or a fragment or derivative thereof.

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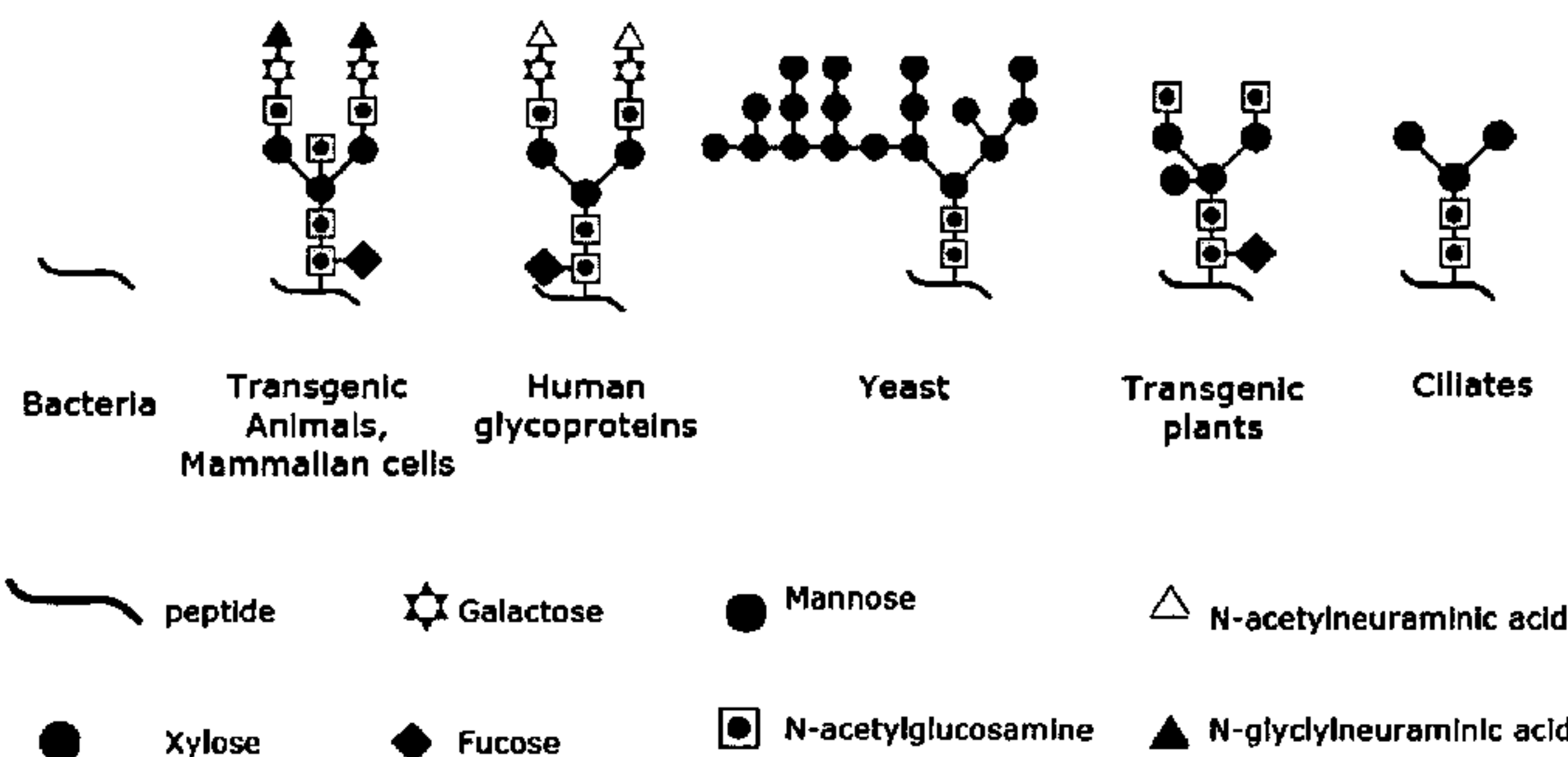


Fig. 2a

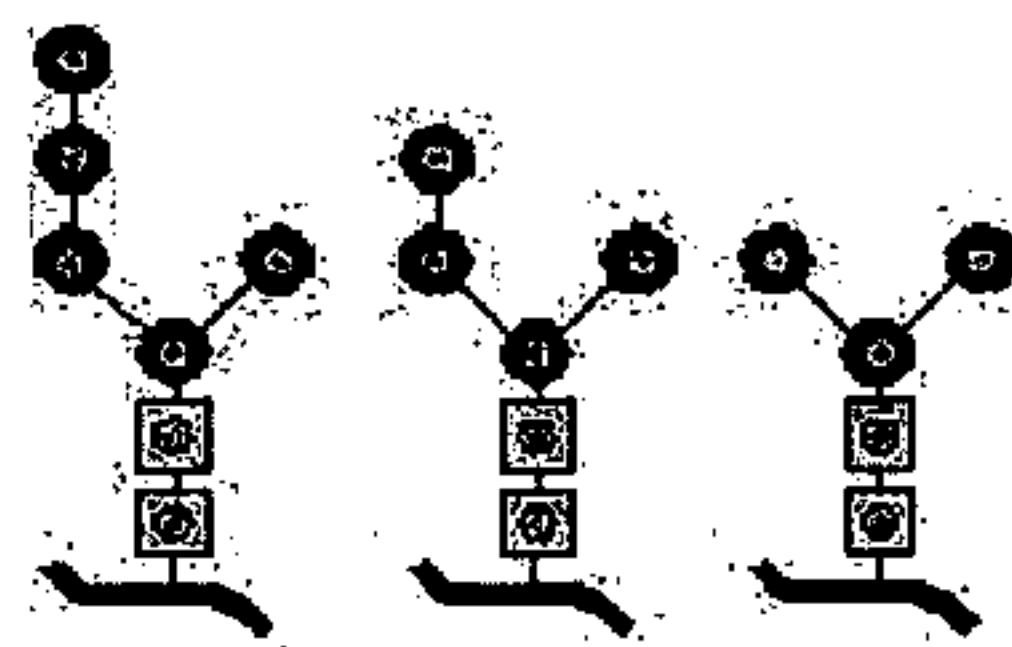


Fig. 2b

(57) Abstract: The present invention is related to a system for the heterologous expression of a monoclonal Antibody (mAb) or a fragment or derivative thereof, said system comprising at least one ciliate host cell, and incorporated, into said ciliate host cell, at least one heterologous nucleic acid molecule encoding for said monoclonal Antibody, or a fragment or derivative thereof.

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10           EXPRESSION OF MONOCLONAL ANTIBODIES IN CILIATE HOST CELLS

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The present invention relates to a system for the heterologous expression of a monoclonal Antibody (mAb) in a ciliate host cell.

20    Today, the main indications of monoclonal antibodies for human therapy are cancer, autoimmune diseases and infectious diseases.

One mechanism of action is to interrupt the signalling pathways of growth factors which promote tumor-associated angiogenesis, as for example done by Avastin<sup>TM</sup> (Bevacizumab),  
25    which targets Vascular Endothelial Growth Factor (VEGF), thus starving the tumor. Other targets are, for example, Placental growth factor (PLGF). While for these purposes a high target affinity is mandative, which is mediated by the Complementary Determining Region (CDR) regions located on the variable regions of antibody light and heavy chains (V<sub>L</sub> and V<sub>H</sub>), antibody effector functions as exerted by the Fc region are not crucial. For these purposes, antibody  
30    fragments devoid of an Fc region (like scFv, or Fabs) can be used

Other mechanisms of action are the binding of cytokines, like TNFalpha (Humira<sup>TM</sup>), or the blocking of growth factor receptors, like erbB-2 (Erbtux<sup>TM</sup>), of viral surface antigens necessary



for cell entry, like RSV F-Protein (Synagis<sup>TM</sup>), or of receptors responsible for blood clotting, like IIb/IIIa-receptors of RBC (ReoPro<sup>TM</sup>).

However, monoclonal antibodies can also be used for target cell-killing applications, e.g., for elimination of cancer cells or pathogens. While conjugated antibodies, i.e., artificial antibodies carrying a particular cytotoxin, have been developed for this purpose, unconjugated antibodies devoid of a particular cytotoxin can meet this goal as well by evoking respective immune responses. For these purposes, however, a functional Fc region is necessary, are provided in IgGs, particularly IgG1. Basically, four different mechanisms are known in this context:

- The Fc region of target-cell bound antibodies can bind to Fc gamma receptors (FcγRs, particularly FcγRI, FcγRIIa and/or FcγRIII) on the surface of immune effector cells, and trigger FcγR-mediated killing of the target cells by immune effectors (“Antibody-Dependent Cellular Cytotoxicity”, or ADCC);
- The Fc region of target-cell bound antibodies can bind to soluble proteins of the complement system found in blood (e.g., C1q), and trigger complement mediated lysis of target cells (“Complement-Dependent Cytotoxicity”, CDC);
- Direct binding of the antibody to the target molecules can trigger cell death-inducing mechanisms, such as apoptosis (Antibody-Dependent Apoptosis), or can block the action of cell survival factors, such as growth factors;
- Opsonisation of a target cell by antibody-mediated binding of macrophages or neutrophils, and subsequent phagocytosis.

ADCC is a mechanism of cell-mediated immunity whereby an effector cell of the immune system actively lyses a target cell that has been bound by specific antibodies. It is one of the mechanisms through which antibodies, as part of the humoral immune response, can act to limit and contain infection. Classical ADCC-mediating effector cells are natural killer (NK) cells; but monocytes and eosinophils can also mediate ADCC. ADCC is part of the adaptive immune response due to its dependence on a prior antibody response.

Therapeutic antibodies which are used to elicit an ADCC in target cells need an Fc region in order to be recognized by Fc gamma receptors of the said effector cells. Examples for such antibodies are Herceptin, which recognizes erbB-2 and binds preferably to tumor cells

overexpressing erbB-2, or Rituxan<sup>TM</sup>, which binds to the CD20 receptor in malignant B-Cells. One other potential mechanism is to bring two or more different entities into close proximity, namely by using a bi- or higher specific antibody construct. This is for example useful to re-direct T cells against tumor cells, in cases the tumor cells can escape from T cell attack, e.g., by mutation, or loss, of their MHC class I entities, or by secreting messenger substances that suppress T cell activation. One approach is to combine two scFv antibodies, out of which one is directed against a T-cell-receptor (e.g., CD 3), while the other one is directed against a tumor cell antigen (e.g. EGFR).

- Another approach is to connect (by means of a fusion molecule comprising two different complementary determining regions in both Fv chains, and a Fc-region), a tumor cell (e.g., by means of a Fv binding to EGFR or EpCAM), a T-cell (e.g., by means of another Fv binding to a T-cell receptor, like CD3) and an effector cell, such as a monocyte, a macrophage or a natural killer cell (by means of the Fc region which is detected by Fc gamma receptors on such effector cells). This approach brings together the anti-tumor effect of T killer cells, which induce tumor cell lysis and apoptosis, and of effector cells, which eliminate tumor cells by phagocytosis or apoptosis, while they release cytokines which further stimulate T cell activity.

- Yet another approach is to design an Antibody, in which two different antigens can be recognized by one antigen binding site, both with high affinity. Such antibodies may in future replace combination therapies with two different antibodies. Furthermore, such antibodies could also be used to combine different epitopes of the same antigen, especially of soluble antigens, to increase the binding avidity and *in vivo* potency.

- Currently, antibodies or fragments or derivatives thereof for therapeutical use are expressed either in *E. coli* or mammalian cell lines, like CHO (chinese hamster ovary) cells. These systems do not allow to enhance ADCC or provide antibodies with multiple specificity, and have some other disadvantages.

- Antibodies produced in *E. coli* come without a glycosylation or other post translational modifications, and have therefore limited capabilities related to ADCC. Furthermore, *E. coli* strains do not secrete proteins into the medium, so cells have to be lysed and antibodies need



thorough purification. Another well-known problem is the incorrect folding of proteins which can lead to the formation of insoluble inclusion bodies. As a consequence, *E. coli* is only suitable for the production of Fab and scFv fragments, which have poor serum half life.

- 5 Eukaryotic expression systems also suffer from a number of drawbacks. Yeast expression systems tend to produce hyperglycosylated proteins rich in mannose, which often lead to unwanted immune reactions when the therapeutic antibody is administered to a patient. Baculovirus transfected insect cell systems cause problems due to hypoglycosylation, which negatively affects the effector function of therapeutic antibodies. Furthermore, the major  
10 disadvantage are the catalytic properties of infectious baculovirus that narrows the window for full IgG production.

- Mammalian and human cell lines, like CHO and Per.C6 cells, are difficult to culture and grow, and expensive to upscale. Additionally, these cells have have high demands related to the  
15 culture medium. Moreover, mammalian and human cell lines bear the risk of infections with bacteria and viruses of human or animal origin.

### **Object of the invention**

- 20 It is one object of the present invention to provide a system for the expression of antibodies, or fragments or derivatives thereof, which does not have the disadvantages set forth above.

- It is one other object of the present invention to provide a system which allows the production of antibodies, or fragments or derivatives thereof, with increased ADCC, CDC, Antibody-  
25 Dependent Apoptosis or Antibody-Dependent Opsonisation.

It is one other object of the present invention to provide a system which allows the production of antibodies, or fragments or derivatives thereof, with multiple specificity.

- 30 It is one other object of the present invention to provide a system which allows the production of antibodies, or fragments or derivatives thereof, with an extended serum half life.

These objects are met with a system according to the independent claim. Dependent claims describe preferred embodiments, while other independent claims describe variants and/or alternatives.

## 5 Summary of the invention

According to the invention, a system for the heterologous expression of a monoclonal Antibody (mAb) or a fragment or derivative thereof, is provided, said system comprising

- 10 a) at least one ciliate host cell, and
- b) incorporated, into said ciliate host cell, at least one heterologous nucleic acid molecule encoding for said monoclonal antibody, or a fragment or derivative thereof.

15 The term “heterologous expression”, as used herein, shall refer to the protein expression of a gene, a nucleic acid or a cDNA, which is foreign to the organism in which the expression occurs.

As used herein, the term "nucleic acid molecule" is intended to indicate any single- or double stranded nucleic acid molecule comprising DNA (cDNA and/or genomic DNA), RNA (preferably mRNA), PNA, LNA and/or Morpholino. Preferably, said nucleic acid molecule  
20 comprises a cDNA encoding for a monoclonal antibody, or a fragment or derivative thereof.

The term “cDNA”, as used herein, shall refer to a DNA molecule which encodes for a protein to be expressed, and is devoid of any non-encoding parts, like introns. In many cases, a cDNA has been directly synthesized from an RNA template using reverse transcriptase, and an oligo dT-  
25 primer. However, the term shall as well comprise synthetic genes and encoding DNAs otherwise obtained.

Nucleic acid sequences encoding for given monoclonal antibodies against given targets can be taken from literature. In European Patent EP0590058B1, for example, the amino acid sequences  
30 of the V<sub>L</sub> domain and the V<sub>H</sub> domain of the humanized monoclonal anti-Her-2/neu Antibody Herceptin<sup>TM</sup> (Trastuzumab) are disclosed. Other references describe even the amino

acid sequence for full IgGs. With this information, the skilled person could design a cDNA encoding for such antibody, and use it for the purpose of the present invention.

Other resources are, for example, the public DrugBank database, which provides sequence  
5 information for most monoclonal antibodies, or fragments or derivatives thereof.

As used herein, the term “monoclonal Antibody (mAb)”, shall refer to an antibody composition having a homogenous antibody population, i.e., a homogeneous population consisting of a whole immunoglobulin, or a fragment or derivative thereof. Particularly preferred, such antibody is  
10 selected from the group consisting of IgG, IgD, IgE, IgA and/or IgM, or a fragment or derivative thereof.

As used herein, the term “fragment” shall refer to fragments of such antibody retaining, in some cases, target binding capacities, e.g.  
15

- a CDR (complementarity determining region)
- a hypervariable region,
- a variable domain (Fv)
- an IgG heavy chain (consisting of V<sub>H</sub>, C<sub>H</sub>1, hinge, C<sub>H</sub>2 and C<sub>H</sub>3 regions)
- 20 • an IgG light chain (consisting of V<sub>L</sub> and C<sub>L</sub> regions), and/or
- a Fab and/or F(ab)<sub>2</sub>.

As used herein, the term “derivative” shall refer to protein constructs being structurally different form, but still having some structural relationship to, the common antibody concept, e.g. scFv, as  
25 well as bi-, tri- or higher specific antibody constructs. All these items are explained below.

The term “host cell”, as used herein, has two different meanings which may be understood according to the respective context. In the context of heterologous protein expression, the term “host cell” refers to a transgenic cell which is used as expression host. Said cell, or its  
30 progenitor, has thus been transfected with a suitable vector comprising the cDNA of the protein to be expressed.



As used herein, the term “ciliate host cell” shall refer to a cell from the phylum Ciliophora (formerly: Ciliata), e.g., protozoans characterized by the presence of hair-like organelles called cilia and a nuclear dimorphism.

5

As used herein, the term “incorporated” shall refer to the fact that the said nucleic acid has entered the host cell in such way that it is ready for protein expression. Such incorporation can have different types in ciliates, e.g. “episomal incorporation” (e.g. the nucleic acid molecule, like a plasmid, has not entered the cellular nucleus, but replicates, and is translated, in the  
10 cytoplasm), and “integrative incorporation” (e.g. the nucleic acid molecule has integrated into the cellular genome).

Ciliates have some surprising properties which make them suitable for use as expression hosts for monoclonal antibodies, or fragments or derivatives thereof. In contrast to *E. coli*, they can  
15 not only produce scFv and Fab, but also full scale immunoglobulines (IgG). Furthermore, the produced antibody can be secreted into the medium, so cell lysis and extraction from the cell pellet is not necessary.

Compared to mammalian cell lines, antibody expression is very cheap, as ciliates have little  
20 demands related to the culture medium, and can be cultured in liquid cultures.

Furthermore, the inventors have realized that for ciliates, unlike as for bacteria or higher eukaryotes, no specific viruses are known so far. This might be due to the nuclear dimorphism which is common to ciliates. Another reason for this might be the unusual codon usage and  
25 AT-rich genome in Ciliates. The inventors do thus assume that pathogenic viruses of higher organisms cannot amplify in most ciliates. The fact that, as known so far, ciliates are not susceptible for viruses, arises as a surprising advantage. This means that in production processes based on Ciliates, amplification or growth of adventitious viruses does not occur. This means, furthermore, that in case a protein is produced for therapeutic use, costly virus  
30 depletion procedures as necessary in industrial processes with human and animal cell cultures can be skipped.

Ciliate systems have, however, some other advantages with respect to the expression of monoclonal antibodies. These will be discussed in the following.

5 Despite the said advantages, ciliate expression systems are still relatively unknown, and the person skilled in the art, when being asked about potential heterologous protein expression systems, would rather think of *E. coli*, yeast, insect cell systems (baculovirus) and mammalian cell lines.

10 Methods for the transformation of ciliates, which can be used in the context of the present invention, comprise, among others, microinjection, electroporation and particle bombardment, and are, for example, described in Tondravi & Yao (1986), Gaertig & Gorovsky (1992) and Cassidy-Hanley et al (1997).

15 Methods for transformation and heterologous protein expression have been described for a few protists (WO 00/58483 and WO 00/46381). The generation of mitotically stable transformants of the ciliate *Tetrahymena thermophila* can be achieved after transfection either of the somatic macronucleus or the generative micronucleus by microinjection, electroporation or by particle bombardment.

20 Selection of the transformants can be performed using different selection markers like the neomycin resistance (Weide et al. 2006, BMC) and the integration of the heterologous genes by homologous DNA recombination, which results in stable thymidin-auxotrophic *Tetrahymena* cells (Weide et al. 2006, BMC). In addition, the use of blasticidin S (Weide et al. 2007, BMC) or paclitaxel (WO 00/46381) resistance has also been considered.

25

Preferably, the encoding nucleic acid is codon optimized for a ciliate expression host. The term “codon optimized”, as used herein, shall refer to a process in which the cDNA encoding the heterologous protein to be expressed is adapted to a host specific codon usage which derives from the universal genetic code scheme. Ciliates have an AT-rich genome, with *Tetrahymena*  
30 DNA consisting of approximately 75% AT (see Fig. 9). The codon usage differs from that in other organisms particularly in how often a codon is used to encode a given amino acid (“codon bias”). If the non-optimized cDNA encoding a heterologous protein uses codons which are rarely used in ciliates this might strongly affect the protein expression efficiency.



This means, in turn, that heterologous protein expression can improve dramatically when the codon frequency of the gene under study is matched to that of the ciliate expression system. Moreover, many ciliates, among them *Tetrahymena*, utilize non-canonical nucleotide codes with UAA and UAG triplets encoding for glutamine, while in most other organisms these  
5 codons are used as stop codon which terminate translation. This may lead to the fact that foreign (non ciliate) genes carrying UAA and UAG triplets as stop codon are not correctly expressed. For this purpose, before transforming the ciliate host cell, the cDNA encoding a heterologous protein should be code optimized in such way that UAA and UAG triplets are amended into UAA. Code optimization can for example be accomplished by site directed  
10 mutagenesis, or by *de novo* cDNA synthesis.

In a preferred embodiment of the present invention, said monoclonal Antibody (mAb), or a fragment or derivative thereof, has an N-glycan structure which is essentially fucose-free. Proteins expressed in eukaryotic expression systems undergo a process of post-translational  
15 modification, which involves glycosylation. Eukaryotic expression systems which have been established today for the production of IgG and other monoclonal antibodies comprising an Fc region add N-glycans to the polypeptide chains. In IgG, the most important N-glycan is bound at Asn 297 of both C<sub>H</sub>2 chains (see Fig. 1), which comprises, among others, N-acetyl-neuraminic acid (sialic acid), N-acetyl-glucosamine, galactose, mannose, and fucose residues.  
20 This applies, basically, for transgenic plant expression systems as well as for mammalian cell lines (see Fig. 2), insect cell lines etc. In all these cases, the N-glycan comprises at least one fucose residue which is bound either  $\alpha$ -3-glycosidically or  $\alpha$ -6-glycosidically to the N-acetyl-glucosamine residue bound to the Asn residue of the polypeptide chain.

In contrast thereto, ciliates produce an N-glycan structure which is significantly different from  
25 the glycosylation patterns produced by the above mentioned expression systems in that it does not contain fucose.

As used herein, the term “essentially fucose-free” means that the share of monoclonal antibodies, or fragments or derivatives thereof, carrying one or more fucose residues in one or  
30 more N-glycans, preferably in Asn 297 N-Glycans, does not exceed 10 %, preferably 5 %, more preferably 1 %, and most preferably 0.1 % of the total of monoclonal antibodies, or fragments or derivatives thereof, produced with the system according to the invention.



Furthermore, production of recombinant antibodies in human cell lines (PerC6) as well as in common mammalian cell lines lead to glycosylation profiles that varies with culture conditions and over the course of the culture period. This reduced fidelity in antibody glycosylation pattern contributes to an diminished therapeutic efficiency and increases the risk of adverse effects (Jefferis 2005). Ciliates, in contrast, are able to secrete proteins with an highly reproducible biantennary oligomannose N- glycosylation structure (Banno et al. 1993). The like consistent glycosylation pattern lead to an uniform serum half life, reduced risk of adverse effects and likely enables an uniform and well manageable therapeutic effect.

10 In another preferred embodiment, said monoclonal Antibody (mAb), or a fragment or derivative thereof, has at least one effect selected from the group consisting of

- increased Antibody-Dependent Cellular Cytotoxicity (ADCC)
- increased Complement-Dependent Cytotoxicity (CDC),
- 15 • increased Antibody-Dependent Apoptosis, and/or
- increased Antibody-Dependent Opsonisation.

Recent studies have shown that monoclonal antibodies having a reduced amount of fucose in its glycosylation pattern exhibit much higher Antibody-Dependent Cellular Cytotoxicity (ADCC) activity as compared to fucosylated antibodies. Again, it is basically position Asn 297 where a lack of fucose residues leads to the increased ADCC. The mechanism behind the increased ADCC of a low/no-fucose Antibody seems to be mediated by an increased affinity of a so modified Fc region to Fc $\gamma$ R, for example Fc $\gamma$ IIIa (CD16), the major Fc receptor for ADCC in human immune effector cells (Shields et al, 2002).

25

Potential targets for therapeutic antibodies according to the present invention, eliciting an ADCC, are shown in the following table, which is not to be construed as limiting the scope of the present application (target abbreviations have been taken from standard literature):

target	potential indication	Antibody example
CD3	graft versus host disease, kidney transplantation	OKT3
CD4	T-cell-lymphoma	HuMAX CD4
CD5	B/T cell antigen: MCL, CLL, CTCL, autoimmune	

CD19	non hodgkin lymphoma, B cell malignancies and autoimmune diseases	AFM12, XmAb 5574; XmAb5871
CD20	non hodgkin lymphoma, rheumatoid arthritis, chronic lymphocytic leukemia, follicular non-Hodgkin's lymphoma, diffuse large B cell lymphoma	Rituxan, Bexxar <sup>TM</sup> , HuMAX <sup>TM</sup> CD20, Zevalin <sup>TM</sup>
CD22	Non-Hodgkin-Lymphome	LymphoCide
CD30	treatment of Hodgkins Lymphoma and anaplastic large cell lymphoma	XmAb2513; MDX060 (5F11)
CD33	acute myeloid leukemia	Mylotarg <sup>TM</sup>
CD38	multiple myeloma	HuMAX CD38
CD40	Alzheimer disease pathogenesis; B cell malignancies and autoimmune diseases	XmAb 5485
CD52	B-cell chronic lymphocytic leukaemia	Campath <sup>TM</sup>
CD70	hematological malignancies	SGN70 and SGN75
CEA	colorectal/lung/breast cancer	CEA-Scan
CTAA 16.88	colorectal/breast/prostatic/lung/ovarian/pancreatic cancer	HumaSpect-Tc
GD2	solid cancers	BIW-8137
VEGF-R/FLT-1	breast and colon cancer	BIW-8556
GM2	lung and brheumatoid arthritisin cancer	BIW-8962
IL-5 receptor	Asthma	BIW-8405
EGFR/Her2-neu	metastatic colorectal cancer and head and neck cancer, squamous cell carcinoma of the head and neck (SCCHN)	Erbixux, HuMAX EGFr
EpCAM	colon, breast and prostate cancer (solid tumors)	MT201, Panorex <sup>TM</sup>
ErbB2	metastatic breast cancer overexpressing ErbB2	Herceptin
FOLR1	ovarian cancer	
PLAC1	breast cancer, NSCL, ovarian cancers	GT468
CLDN18.2	gastric and pancreatic cancers	GC182
Histone H1	lung/uterine cancer gliomasarcoma	Cotara
CD317	multiple myeloma	anti-HM1.24
Muc1	colon carcinoma	PankoMab
PSMA	prostatic cancer	ProstaScint
VEGF	metastatic colorectal cancer	Avastin

Table 1



It is important to understand that the skilled person has full access to manufacturing protocols and amino acid sequences of the above mentioned antibodies, and will thus be able to apply the teaching of the present invention to all of the said antibodies, e.g., in order to enhance the ADCC evoked by the latter.

5

US company Xencor has developed a modular suite of antibody components by engineering antibody Fc regions for select amino acid changes. In some cases, these Fcs have reportedly been shown to increase the ADCC more than 100-fold, resulting, among others, in ADCC killing even against cell lines expressing low levels of antigen and the reduction of doses of mAb while maintaining the same cytotoxic effect. However, the authors draw no causal relationship between their modifications, which seem to be based on a random mutation/selection process, and the resulting effects, i.e. increased ADCC. For this reason, the concept is not fully reproducible, and it is unknown whether or not it can be generalized to other Antibodies.

15

Japanese company BIOVA has developed a CHO (Chinese Hamster Ovary) cell line for the expression of mAbs with increased ADCC. In this cell line, the gene encoding for the  $\alpha$ -1,6 Fucosyltransferase ("FUT8") enzyme has been knocked out. Thus, during post translational glycosylation, fucose residues cannot be added to the N-glycosylation site of the antibodies. It is claimed that mAbs thus produced show an enhanced ADCC activity. The method is described in EP1176195. A major drawback of this technique is that it does not ensure a 100 % fucose free product. Defucosylation is highly dependent on a potentially remaining enzyme activity of the  $\alpha$ -1,6 fucosyltransferase, and thus subject to significant variance, particularly on a batch-to-batch comparison. Furthermore, the system is only available in CHO cells (so-called FUT8 knock out CHO), which are suboptimal expression hosts for some mAb expression applications.

25

US company Glycart has developed cell lines for the production of mAbs which carry a heterologous gene for encoding the oligosaccharide-modifying enzyme beta (1,4)- N-acetylglucosaminyltransferase III (GnT III). When these cells are later on transfected with a DNA encoding for a mAb, they will produce mAbs which first undergo a normal glycosylation process, including the incorporation of fucose residues. In a second step, the fucose residues are then cleaved by means of the GnT III enzyme. The resulting proteins are thus more or less

30



unfucosylated and exhibit an increased ADCC. Again, this technique does not ensure a 100 %  
fucose free product. Defucosylation is highly dependent on the activity of said GnT III enzyme,  
and thus subject to significant variance, particularly on a batch-to-batch comparison.

- 5 US company Eureka Therapeutics is advertising that they have developed a method to enhance  
ADCC in therapeutic antibodies which they have named MAGE (“Magnified ADCC through  
Glycosylation Engineering “). However, technical details of the method have not been  
revealed.
- 10 Surprisingly the inventors of the present invention have found in their experiments, that ciliates  
produce antibodies that can induce ADCC, although the N-glycan structure is different to  
typical antibodies expressed in mammalian cells. Subsequently, the inventors of the present  
application found in their experiments that ciliates produce antibodies with an N-glycan  
structure in the Fc-region, which does not contain fucose. This can be an explanation for the  
15 increased ADCC effector function, compared to antibodies expressed in mammalian cells.

The system according to the invention thus provides an economical, simple and reliable method  
for the production of monoclonal antibodies, or fragments or derivatives thereof, which have a  
drastically increased ADCC and thus a highly enhanced therapeutic potential.

20

- It is important to mention that yeast-based expressions systems (e.g., *Saccharomyces sp.*, or  
*Pichia sp.*) also produce unfucosylated N-glycans (see Fig. 2) rich in mannose. While these  
expression systems are subject of intensive research, particularly for the production of  
antibodies, or fragments or derivatives thereof (Wei et al, 2008), it seems that the major focus  
25 of research is directed to modifying the glycosylation pattern of a yeast-based expression  
system in such way that it is similar to human glycosylation patterns (Gerngross, 2004). This,  
in turn, would not only be useful for antibodies, or fragments and derivatives thereof, but also  
for other biopharmaceuticals expressed.

- 30 Today, no reports are available which indicate that antibodies, or fragments or derivatives  
thereof, produced in yeast strains have an increased ADCC, or CDC, or Antibody-Dependent  
Apoptosis, nor that the lack of fucosylation has any other particular effect. This again indicates

that a lack of fucosylation alone does not automatically mean an increased ADCC – a fact well known by the skilled person.

Furthermore, it seems that therapeutical antibodies, or derivatives or fragments thereof,  
5 produced with the system according to the invention, also have an increased CDC.

Furthermore, it seems that therapeutical antibodies, or derivatives or fragments thereof,  
produced with the system according to the invention, also have increased  
Antibody-Dependent Apoptotic effects.

10

Furthermore, it seems that therapeutical antibodies, or derivatives or fragments thereof,  
produced with the system according to the invention, also have increased  
Antibody-Dependent Opsonization effects.

15 In a particularly preferred embodiment, it is provided that additional N-glycosylation sites are introduced into the antibody, or fragment or derivative thereof, which is to be expressed. This can be done by introducing, for example by site-directed mutagenesis, or by deliberate exchange of amino acid residues, additional N-glycosylation motifs, i.e., tripeptide sequences Asn-X-Ser or Asn-X-Thr, where X can be any amino acid, although Pro and Asp are rarely  
20 found. If for example the antibody, or fragment or derivative thereof, has, somewhere in its chain, the motif “Gly-X-Ser”, one could substitute “Gly” by “Asn”, in order to create an additional N-glycosylation site. It is of course necessary to make sure that the said substitution does not affect important properties of the protein, like target affinity, binding by Fc gamma receptors (FcγRs) or the like.

25

However, the exceptional N-glycosylation pattern, does, at first sight, rule out the use of *Tetrahymena thermophila* as expression system for antibodies for therapeutical use, as the skilled person would consider that such abnormal glycosylation pattern affects immunocompatibility of the antibodies thus produced. The inventors have, however, shown  
30 that these presumptions are not correct.

Furthermore, ciliate expression systems have other advantages in comparison to other protein expression systems, like mammalian cell lines, which are discussed below.



In yet another preferred embodiment, said monoclonal Antibody (mAb), or a fragment or derivative thereof, has an extended serum half life.

5 Serum half life is an important issue in monoclonal antibodies used for therapeutic purposes, as an extension of the former may help to reduce the dosage and/or the administration frequency. As monoclonal antibodies can not be administered orally, this would help to improve the patient compliance, while reducing costs because of lower doses and minimizing risks related to the way of administration.

10

A major pathway of removing dissolved proteins from the serum is the asialoglycoprotein receptor-mediated clearance in the liver. Usually, mammalian proteins are N-glycosylated with bifurcated N-glycans having two or more terminal sialic acid residues (N-acetyl-neuraminic acid), which are backed up by beta-galactose residues (see Fig. 2). This applies both for a  
15 subject's intrinsic proteins as well as for proteins heterologous expressed, e.g., in a mammalian cell line, and administered to said subject.

During the protein life span, the terminal sialic acid residues are gradually removed from the glycan chain because of ubiquitous neuraminidases, until the galactose residues are exposed.  
20 These are then recognized by asialoglycoprotein receptors, which are lectins abundant in the liver binding galactose residues of many desialylated plasma proteins. After being recognized, the said proteins are subject to endocytosis, and will then be degraded in the liver.

As shown above, proteins heterologous expressed in ciliates have neither terminal sialic acid  
25 residues, which could be removed by free floating neuraminidases, nor galactose residues, which could serve as a target for asialoglycoprotein receptors. For this reason, monoclonal antibodies, or fragments or derivatives thereof, which have been heterologous expressed in ciliates, are not subject to the asialoglycoprotein receptor-mediated clearance, and do therefore have an enhanced serum half life. The ciliate – expression approach has some significant  
30 advantages over other approaches to extend antibody serum half life, which all involve more or less dramatic modifications of the basic antibody concept, the consequences of which for immunogenicity and the like are difficult to predict. These approaches are discussed in the following.



US company Domantis tries to extend serum half life by using an anti-albumin domain bound to antibodies, while Genentech Inc. has developed an approach in which the galactose content in CH<sub>2</sub>-N-glycans is increased. PDL BioPharma Inc. developed an approach in which some amino acid residues in the Fc-region are substituted by others, thus leading to an extension of serum half life. Furthermore, the concept of PEGylation is well known to the skilled in order to extend serum half life of a protein.

In yet another preferred embodiment of the invention, the said system further comprises

- c) a promoter operably linked to said nucleic acid molecule, and/or
- d) a signal sequence operably linked to said nucleic acid molecule, which signal sequence accounts for the secretion of the monoclonal antibody, or the fragment thereof, encoded by the said nucleic acid molecule, into the extracellular medium.

The term “operably linked” as used herein, means that a nucleotide sequence, which can encode a gene product, is linked to a promoter and/or a signal sequence in such way that the promoter regulates expression of the gene product under appropriate conditions.

The term “promoter”, as used herein, shall refer to a regulatory region of DNA generally located upstream (towards the 5' region of the sense strand) of a gene or a cDNA, that contains essential genetic elements which allow or even enhance transcription of the gene, or the cDNA.

The term “signal sequence”, as used herein, shall refer to a nucleic acid sequence which encodes for an oligopeptide (“signal peptide” or “transit peptide”) which directs the transport of a protein to certain organelles such as the nucleus, mitochondrial matrix, endoplasmic reticulum, chloroplast, apoplast and peroxisome. Almost all proteins that are transported to the endoplasmic reticulum have a sequence consisting of 5-10 hydrophobic amino acids at the N-terminus. Those signal peptides are cleaved from the protein by a signal peptidase after the cotranslational insertion of the protein into the lumen of the ER. Most proteins are then transported via Golgi apparatus downstream on the secretory pathway.

Promoters suitable for antibody expression in ciliates are, for example, disclosed in WO2007006812A1 which is also registered for the applicant of the present invention. Therein, a heat-inducible promoter and a metallothionein-promoter are disclosed which can also be used for the purposes of the present invention.

Suitable signal sequences are, for example, disclosed in WO03078566A1 which is also registered for the applicant of the present invention. Therein, two signal peptides particularly preferred in the context of the present invention are disclosed, namely the endogenous signal peptide of the antibody heavy and light chain, and the ciliate lipase signal peptide.

Furthermore, a vector for the transfection of a ciliate host cell is provided, said vector comprising at least one nucleic acid molecule encoding for a monoclonal Antibody (mAb), or a fragment or derivative thereof.

The term "vector", as used herein, refers to a molecular vehicle used to transfer foreign genetic material into another cell. The vector itself is generally a DNA sequence that consists of an insert (sequence of interest) and a larger sequence that serves as the "backbone" of the vector. The purpose of a vector to transfer genetic information to another cell is typically to isolate, multiply, or express the insert in the target cell.

The term "plasmid", as used herein, refers to plasmid vectors, i.e., circular DNA sequences that are capable of autonomous replication within a suitable host due to an origin of replication ("ORI"). Furthermore, a plasmid may comprise a selectable marker to indicate the success of the transformation or other procedures meant to introduce foreign DNA into a cell and a multiple cloning site which includes multiple restriction enzyme consensus sites to enable the insertion of an insert. Plasmid vectors called cloning or donor vectors are used to ease the cloning and to amplify a sequence of interest. Plasmid vectors called expression or acceptor vectors are specifically for the expression of a gene of interest in a defined target cell. Those pplasmid vectors generally show an expression cassette, consisting of a promoter, the transgene and a terminator sequence. Expression plasmids can be shuttle plasmids containing elements that enable the propagation and selection in different host cells.



In yet another embodiment of the present invention, a system for the heterologous expression of a monoclonal Antibody (mAb) or a fragment or derivative thereof is provided, said system comprising a ciliate host cell which has been obtained by conjugation of at least two ciliate host  
5 cells according to the invention.

All ciliates exhibit a nuclear dimorphism with two structurally and functionally different types of nuclei. The large, somatic macronucleus (MAC) is actively expressed during vegetative multiplication. The MAC contains 45 chromosome copies and divides by amitosis. The small,  
10 diploid micronucleus (MIC) is the germline and contains 5 pairs of chromosomes. The MIC stores the genetic information for the sexual progeny. During the vegetative phase the MIC divides mitotically. The life cycle of ciliates consists of alternating haplophases and diplophases with reference to the germline. The cell reproduction is exclusively asexual and occurs only in the diplophase.

The above approach utilizes a unique feature of ciliate host cells, namely that they can exchange genetic matter by conjugation. Under certain conditions ciliates will enter a conjugation cycle, the sexual stage of the life cycle. In *Tetrahymena*, for example, cells can be induced to conjugate by mixing cells belonging to at least two out of seven different mating  
20 types, and moderately starving them. During this stage, two cells pair to exchange haploid gametic nuclei. The nuclear events of conjugation normally include meiosis, gamete nucleus formation, fertilization, and nuclear differentiation. Conjugation includes the only - and very brief - haploid stage of the ciliate life cycle; it follows meiosis and quickly ends at fertilization. This process is conserved among the majority of ciliates.

The claimed approach utilizes the unique feature of ciliate host cells, namely the exchange genetic material during conjugation. The main stations of the conjugation process are shown in Fig. 6.

30 At the start of conjugation, micronuclei in paired cells undergo meiosis, generating four haploid pronuclei. Three of these pronuclei are destroyed, while the remaining one divides to form two gametic nuclei: a "migratory" pronucleus and a "stationary" pronucleus. Migratory



pronuclei are exchanged through a temporary junction of the two cells; these then fuse with a stationary pronucleus to form a zygotic nucleus in each cell.

5 The zygotic nucleus divides twice to form four genetically identical nuclei, whereas the old macronucleus is degraded. Two of the four zygotic clones (anterior products) develop into new macronuclei, which undergo a wide array of genome rearrangements, including chromosome breakage, programmed DNA elimination, and telomere addition. In *Tetrahymena* these processes generate approximately 300 individual macronuclear chromosomes. Each chromosome is then amplified to 45 copies, completing development of the macronuclear  
10 genome.

One of the two remaining zygotic clones is degraded; the other, the new micronucleus, divides mitotically during the first asexual reproductive cycle. The daughter cells each receive one micronucleus and one macronucleus in this division, yielding the normal complement of nuclei  
15 found in vegetatively growing ciliate cells.

The principle of conjugation is absent in prokaryotic expression systems as well as in most other eukaryotic expression systems, like yeasts, insect cell systems (baculovirus), mammalian expression systems, like CHO cells, or transgenic plants or mammals. Ciliate host cell which  
20 are used for recombinant expression of bi- or higher specific antibodies using conjugating of two different antibody expressing host cells according to the above system need to meet some requirements:

- a) the at least two ciliate cells need to be from different mating types
- 25 b) the at least two ciliate cells need to have incorporated a nucleic acid molecule, encoding for a monoclonal Antibody (mAb), or a fragment or derivative thereof, into its micronucleus.

By conjugating the cells, a cell can be produced which carries a combination of both nucleic  
30 acid molecules, and can thus produce a new monoclonal antibody or antibody construct, or fragments or derivatives thereof, composed of the combination of the two parent antibodies, constituting e.g. both antigen specificities.

The following table gives an overview over some potential constellations which are within the scope of said invention. Indicated are the possible resulting combination antibodies, fragments or derivatives thereof after the conjugation of two host cells each carries an nucleic acid encoding for a monoclonal antibody. The list is not to be construed as limiting the scope of the present invention.

cell 1 prior to conjugation	cell 2 prior to conjugation	after conjugation
whole IgG A	whole IgG B	bispecific full IgG F(ab) <sub>2</sub> A + Fc B
heavy chain (V <sub>H</sub> ) + light chain (V <sub>L</sub> )	heavy chain (V <sub>H</sub> ) + light chain (V <sub>L</sub> )	whole IgG
heavy chain (V <sub>H</sub> ) + light chain (V <sub>L</sub> ) against target 1	heavy chain (V <sub>H</sub> ) + light chain (V <sub>L</sub> ) against target 2	quadroma (bispecific)
scFv specific against target 1 (e.g. EGFR)	scFv specific against target 2 (e.g. CD3)	diabody or tandem scFv
heavy chain (V <sub>H</sub> ) + light chain (V <sub>L</sub> ) against tumor cell target (e.g. EGFR)	heavy chain (V <sub>H</sub> ) + light chain (V <sub>L</sub> ) against T-cell receptor (e.g. CD3)	trispecific antibody binding tumor cell, T cell and effector cell
Fab from antibody A	Fab from antibody B	bispecific F(ab) <sub>2</sub>
Fab from antibody A	Fab from antibody B	F(ab) <sub>2</sub> with bispecific antigen binding site (V <sub>L</sub> (A) + V <sub>H</sub> (B))
bispecific whole IgG (A+B)	full IgG C	trispecific whole IgG: F(ab) <sub>2</sub> A+B + Fc from C

Table 2

- 10 In another embodiment of the invention a ciliate host cell transfected with at least one vector according to the invention, or obtained by conjugation of at least two ciliate host cells according to the invention, is provided.

Furthermore, according to another embodiment of the present invention, a library comprising  
 15 at least two ciliate host cells according to the invention, or at least two systems according to the invention, are provided wherein each host cell has incorporated at least one heterologous nucleic acid molecule encoding for an antibody, or fragment or derivative thereof, preferably in form of a vector, and wherein at least two ciliates are selected in such way that they can conjugate with one another.



Such library could, for example, comprise stable transfected ciliate host cells which each carry a nucleic acid molecule encoding for an antibody or fragment or derivate thereof (see below) specific against a given target (see, e.g. Tables 1 and 3). For each nucleic acid should encode for a given antibody or fragment or derivate thereof, host cells of at least two, preferably more, different mating types should be available. In case a bispecific antibody construct is to be built, two host cells can be selected from the library which carry the nucleic acid molecules for the two antibody or fragment or derivate thereof needed for the said construct. The said host cells should be from different mating types in order to conjugate them.

- 10 In a preferred embodiment of the system according to the invention, or a ciliate host cell according to the invention, the said ciliate is a member of the family *Tetrahymenidae*.

In a particularly preferred embodiment, the said transgenic ciliate is *Tetrahymena* sp. (particularly *Tetrahymena thermophila*). *Tetrahymena* is a nonpathogenic unicellular eukaryotic microorganism which has been established in a few laboratories as an expression host. It features a number of advantages which make it suitable for heterologous protein expression. *Tetrahymena* is a broadly examined model organism, and, in over 50 years of basic research, no viruses or endoparasites were observed. Examinations with indicator cell lines revealed no endogenous infectious agents like viruses or mycoplasma, which can infect higher animals.

First of all, the above considerations as related to codon usage in ciliates apply for *Tetrahymena* as well. Furthermore, high copy number plasmids are available for *Tetrahymena*, containing an origin of replication (ori) from a minichromosomal rDNA. This minichromosomal rDNA is present in up to 9.000 copies per cell. Beyond that stable integration can take place into the macronuclear DNA, in which all genes are present in 45-fold copy number. The high gene dose is the ideal precondition for an efficient protein biosynthesis and thus for a high productivity. In contrast to bacteria, ciliates of the genus *Tetrahymena* secrete biologically proteins very efficiently to the supernatant.

30

*Tetrahymena* is able to attach posttranslational modifications to proteins, like disulfid bridges, GPI anchor, phosphorylation, acetylation and glycosylation, which are more similar to those in mammalian cells than those detected in yeast or in other eukaryotic expression systems.

Unlike mammalian cells, *Tetrahymena* combines the ease of growth with short generation times (1,5 - 3 h), and cost reduction, as chemically defined media can be used and no need for peptides or serum components, like growth factors, exists.

5

Batch, fed-batch and continuous fermentation of *Tetrahymena* with cell densities up to  $2 \times 10^7$  cells/ml and dry weights of up to 80 g/L are established, and production enlargements (upscaling) up to 1000 L could be demonstrated without any problem. In feasibility studies with reporter proteins space-time yields of 50 - 90 pg/cell a day could already be achieved.

10

First experiments with homologous expression resulted in a yield of over 200 mg/L a day for secreted proteins. *Tetrahymena* can be fermented in conventional production facilities for microbiological expression systems (bacteria or yeasts). This means that no costly modifications in existing production plants or a new building of the production facilities are necessary.

15

In another preferred embodiment of the present invention, a monoclonal Antibody (mAb), or a fragment or derivative thereof is provided, said Antibody or fragment being produced with a system according to the invention, with a ciliate host cell according to the invention and/or with a process according to the invention.

20

Preferably, the monoclonal antibody, fragment or derivative according to the invention binds to at least one of the targets set forth in Table 1 (ADCC) or 3 (non ADCC)

Targets which are not involved in ADCC are listed in the following table, which is exemplary

25

only and not to be construed as limiting the scope of the present invention.

30

target	indication	Antibody example
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target	indication	Antibody example
TNF- $\alpha$	rheumatoid arthritis, psoriasis, Morbus Bechterew, Morbus Crohn	Adalimumab, Golimumab, Infliximab
CD25	prophylaxis of tissue rejection after kidney transplantation	Basiliximab, Daclizumab
CD3	treatment of tissue rejection after organ transplantation	Muromonab-CD3 (murine)
CD49d ( $\alpha_4$ -Integrin)	multiple sclerosis	Natalizumab
interleukin 6 receptor	rheumatoid arthritis	Tocilizumab
Interleukin 12/23	plaque-psoriasis	Ustekinumab
RSV surface antigen	prophylaxis of RSV in newborn	Motavizumab Palivizumab
VEGF-A	wet macular degeneration	Lucentis <sup>TM</sup>
CD11a-antigen	psoriasis	Efalizumab
Immunglobulin E	asthma bronchiale	Omalizumab

Table 3

Again, it is important to understand that the skilled person has full acces to manufacturing  
5 protocols and amino acid sequences of the above mentioned antibodies, and will thus be able to  
apply the teaching of the present invention to the said antibodies, e.g., in order to enhance the  
serum half-life of the latter.

Furthermore, the monoclonal antibody, fragment or derivative according to the invention, is  
10 selected from the group consisting of

- murine, chimeric, humanized and/or human mAb,
- IgG, scFv, Fab and/or F(ab)<sub>2</sub>,
- modified antibody format

15

Methods for the production and/or selection of chimeric, humanized and/or human mAbs are  
known in the art. For example, US6331415 by Genentech describes the production of chimeric  
antibodies, while US6548640 by Medical Research Council describes CDR grafting techniques

and US5859205 by Celltech describes the production of humanized antibodies. In vitro antibody libraries are, among others, disclosed in US6300064 by MorphoSys and US6248516 by MRC/Scripps/Stratagene. Phage Display techniques are for example disclosed in US5223409 by Dyax. Transgenic mammal platforms are for example described in  
 5 US200302048621 by TaconicArtemis.

IgG, scFv, Fab and/or F(ab)<sub>2</sub> are antibody formats well known to the skilled person. Related enabling techniques are available from the respective textbooks.

10 As used herein, the term “Fab” relates to an IgG fragment comprising the antigen binding region, said fragment being composed of one constant and one variable domain from each heavy and light chain of the antibody

As used herein, the term “F(ab)<sub>2</sub>” relates to an IgG fragment consisting of two Fab fragments  
 15 connected to one another by disulfide bonds.

As used herein, the term “scFv” relates to a single-chain variable fragment being a fusion of the variable regions of the heavy and light chains of immunoglobulins, linked together with a short linker, usually serine (S) or glycine (G). This chimeric molecule retains the specificity of the  
 20 original immunoglobulin, despite removal of the constant regions and the introduction of a linker peptide.

Modified antibody formats are for example bi- or trispecific antibody constructs, as for example given in Table 2, antibody-based fusion proteins, antibody-drug conjugates,  
 25 immunotoxins and the like. Some of these formats are listed in the following table, which is not to be construed as limiting the scope of the present invention.

30

company	format
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Affimed	scFv – Diabody - scFv
Unilever	Camelid Antibodies
Ablynx	camelid VHH
Domantis	variable regions of heavy (V <sub>H</sub> ) or light (V <sub>L</sub> ) chain
Scancell	tumor epitopes on a IgG structure with unchanged FC domain
Hybritech	trifunctional antibodies
Trion Pharma	trifunctional IgG
Affitech	antibodies with T-cell epitopes between $\beta$ -strands of constant domains, and new V-regions specific for antigen presenting cells
Affitech	antibody fragments that can cross link antigen and antibody effector molecules
Vaccibody AS	bivalent homodimers, each chain consisting of scFv targeting unit specific for antigen presenting cells
Planet Biotechnology	IgA (two IgG structures joined by a J chain and a secretory component), expressed in a plant host, secretory component replaced by a protection protein
Trubion	variable regions of heavy (V <sub>H</sub> ) and light (V <sub>L</sub> ) chain + Fc
Haptogen	homodimeric heavy chain complex found in immunized nurse sharks, lacking light chains
AdAlta	recombinant shark antibody domain library
Xencor	altered Fc region to enhance affinity for Fc gamma receptors, thus enhancing ADCC
Arana	new world primate framework + non-new world primate CDR
City of Hope	"minibody"
Seattle Genetics	antibody-drug conjugate technology with enzyme-cleavable linkers

**Table 4**

In other preferred embodiments, the monoclonal antibody, fragment or derivative according to  
5 the invention, has at least one feature selected from the group consisting of

- increased ADCC, CDC, and/or Antibody-Dependent Apoptosis,
- extended serum half life, and/or
- bi, tri- or multispecificity.

10

As used herein, the terms “increased ADCC”, “increased CDC”, “increased Antibody-Dependent Apoptosis”, “increased Antibody-Dependent Opsonization” and “extended serum

half life” relate to a comparison with antibodies that have been produced with conventional Antibody expression systems, e.g., mammalian cells or *E. coli*. ADCC, CDC, Antibody-Dependent Apoptosis and serum half life can be measured with assays commercially available.

- 5 The terms “bi-“, “tri-“ or “multispecificity” refer to antibodies, fragments or derivatives thereof which have at least two domains exhibiting affinity against at least two different epitopes, preferably of at least two different targets. Some examples for such antibodies, fragments or derivatives are given in Table 2 and Fig. 4.
- 10 The purpose of such antibodies, fragments or derivatives thereof is to bring two or more different entities into close contact, namely by using a bi- or higher specific antibody construct. This is for example useful to re-direct T cells against tumor cells, in cases the tumor cells can escape from T cell attack, e.g., by mutation, or loss, of their MHC class I entities, or by secreting messenger substances that suppress T cell activation. One approach is to combine
- 15 two scFv antibodies, out of which one is directed against a T-cell-receptor (e.g., CD 3), while the other one is directed against a tumor cell antigen (e.g. EGFR).

Another approach is to connect, by means of two different complementary determining regions in both Fv chains, and by the Fc-region, a tumor cell (e.g., by means of a Fv binding to EGFR),

20 a T-cell (e.g., by means of another Fv binding to a T-cell receptor, like CD3) and an effector cell, such as a monocyte, a macrophage or a natural killer cell (by means of the Fc region which is detected by Fc gamma receptors on such effector cells). This approach brings together the anti-tumor effect of T killer cells, which induce tumor cell lysis and apoptosis, and of effector cells, which eliminate tumor cells by phagocytosis or apoptosis, while they release

25 cytokines which further stimulate T cell activity.

The following table gives an overview of some exemplary targets in bispecific antibodies (first & second column) and trispecific antibodies (all three columns), but is not to be construed as limiting the scope of the present invention. Other suitable target epitopes are listed in Table 1.

30

Effector cell epitope	Target cell epitope	T cell activating epitope
FcγRI	EGFR	CD3
FcγRIIa	EpCAM	CD64



Fc $\gamma$ RIII	CD20	CD16
Fc $\gamma$ RIII	CEA	CD89
Fc $\gamma$ RIII	CD19	CD89

**Table 6**

Some features of bispecific antibodies, including potential formats as well as targets, are  
5 discussed in Kufer et. al (2004), while features of trispecific antibodies, including potential  
formats as well as targets, are for example discussed in Ruf and Lindhofer (2001).

Furthermore, a process for the production of at least one monoclonal Antibody (mAb), or a  
fragment or derivative thereof, in a ciliate host cell, is provided, said process comprising the  
10 steps of

- a) transfecting at least one ciliate host cell with at least one nucleic acid molecule encoding  
for said monoclonal antibody, or a fragment or derivative thereof, or, preferably, with at  
least one vector according to the invention, and
- 15 b) culturing the host cell under conditions which allow expression of a protein.

In another preferred embodiment of the present invention, a process for the production of at  
least one monoclonal Antibody (mAb), or a fragment or derivative thereof, in a ciliate host  
cell, is provided, said process comprising the steps of

- c) transfecting at least two different ciliate host cells with at least one nucleic acid molecule  
encoding for an antibody, or a fragment or derivative thereof, or, preferably, with at least  
one vector according to the invention,
- d) conjugating the said two ciliate host cells, or offspring thereof, in order to obtain at least  
25 one ciliate cell carrying at least two different nucleic acid molecules encoding for at least  
two different antibodies, or fragments or derivatives thereof, and
- e) culturing the ciliate cell thus produced under conditions which allow expression of a  
protein.

30 Furthermore, a process for the production of a pharmaceutical composition is provided, said  
process comprising the steps of

- a) expressing an antibody, or a fragment or derivative thereof protein according to the invention in a ciliate expression system according to the invention, and
- b) isolating and/or purifying the protein thus obtained.

5 Furthermore, a pharmaceutical composition is provided, said composition comprising an Antibody, or a fragment or derivative thereof, according to the invention, and/or produced with a method according to the invention.

## Disclaimer

10

The particular combinations of elements and features in the above detailed embodiments are exemplary only; the interchanging and substitution of these teachings with other teachings in this and the patents/applications referenced are also expressly contemplated. As those skilled in the art will recognize, variations, modifications, and other implementations of what is described herein can occur to those of ordinary skill in the art without departing from the spirit and the scope of the invention as claimed. Accordingly, the foregoing description is by way of example only and is not intended as limiting. The invention's scope is defined in the following claims and the equivalents thereto. Furthermore, reference signs used in the description and claims do not

15

20 limit the scope of the invention as claimed.

## Brief description of the examples and drawings

Additional details, features, characteristics and advantages of the object of the invention are disclosed in the subclaims, and the following description of the respective figures and examples, which, in an exemplary fashion, show preferred embodiments of the present invention. However, these drawings should by no means be understood as to limit the scope of the invention.

25

30

## Examples

### 1. Construction of expression vectors



The synthetic genes for the heavy and light chain of the antibody Gk1.5 (see SEQ ID NOs 1 and 2) were cloned into the donor vector. The expression cassettes from all donor vectors were transferred into the acceptor vector (see Fig. 5) using a Cre dependent recombinase system.

5

## 2. Cultivation of wildtype *Tetrahymena* and transformation of expression plasmids

Wildtype *Tetrahymena thermophila* strains (e.g. B 1868/4, B 1868/7 and B 2068/1) were cultivated in skimmed milk medium, in SPP or in chemically defined medium. The transformation of the *T. thermophila* cells was performed as previously described in Cassidy-Hanley et al. 1997.

## 3. Detection of antibody Gk1.5

15 Transformed *Tetrahymena* cells were cultivated in SPP medium under selection pressure at 30 °C in a shaker at 80 rpm. Target gene expression was induced by heat shock at 41 °C (HSP- P) or by addition of 20 nM Cd<sup>2+</sup> (MTT1- P) of logarithmically growing cultures.

Aliquots of the culture were harvested 24h after induction of target gene expression. Afterwards cells and cell free supernatant was gained, respectively. The cells were solubilized in ice cold RIPA-buffer (5000 cells/μl in 150mM NaCl, 10mM TrisHCl, 5mM EDTA, 0.1% SDS, 0.1% DOC, 1% Triton<sup>TM</sup> X100, 2,5μg/ml E64, pH 7.4) and incubated for 15 minutes in a sonicator. SDS-PAGE and Western blot analysis were done according to the art. Briefly, aliquots of either disrupted cells (i.e. 10 000 cells) or cell free supernatant were added to Laemmli sample buffer  
25 (125mM Tris HCl pH 6.8, 10% Glycerol, 4% SDS) and separated using 8-16 % SDS-PAGE. The proteins were transferred to nitrocellulose membranes and blocked in PBS containing 0.05 % Tween<sup>TM</sup> 20 and 5 % bovine serum albumin (PBS/TBSA). The expression of recombinant heavy and light chain of the antibody in transformed Ciliates was detected by an Hrp conjugated anti-rat-whole IgG-antibody. After washing the blots were

30

developed by chemoluminescence using Super Signal West Pico Chemoluminescent Substrate (Perbio, Fischer Scientific) in combination with conventional X-ray film development. Fig. 8 shows Western blots of cell lysates and supernatants of transformed cells at different time points after the induction of target gene expression.

5

#### 4. Production of antibody Gk1.5

For fermentations a Sixforce multifermenter (0.5 Litre) equipped with standard marine impellers were used. Stirrer speed was limited to 900 rpm; pO<sub>2</sub> was set to 25% and pH was set to 7.0, respectively. Fermentations were carried out in standard medium.

10

#### Figures

Fig. 1 shows a schematic representation of an immunoglobulin G (IgG). An IgG is composed of two identical light chains (each composed of two domains, V<sub>L</sub> and V<sub>H</sub>) and two identical heavy chains (each composed of four domains, V<sub>H</sub>, C<sub>H</sub>1, C<sub>H</sub>2 and C<sub>H</sub>3). Antigen binding surface is formed by the variable domains of heavy and light chains and the effector function, such as complement activation and binding of cytotoxic cells is mediated by the V<sub>C</sub> region of the antibody.

20

Fig. 2 shows an overview of N-glycan structures of different taxa. Generally, the term „N-glycosylation“ refers to glycosylation of the amino acid residue asparagine (N). Here, an oligosaccharide chain is attached by oligosaccharyltransferase to those asparagine residues which occur in the tripeptide sequences Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except Pro. It is obvious that, while prokaryotes have no N-glycosylation at all, ciliates feature N-glycan structures which are devoid of the fucose side chain and lack, furthermore, terminal sialic acid residues (n-acetyl-neuraminic acid) backed up by beta-galactose residues. Fig. 2b shows potential variations in the said pattern in some ciliate species.

25

Fig. 3 shows a schematic representation of an IgG and its fragments and derivatives. Fig. 3A represents an whole IgG antibody. In Fig. 3B an F(ab)<sub>2</sub> and in Fig. 3C an Fab fragment is shown (removal of the Fc-fragment). The inclusion of a genetically engineered leucine zipper enables dimer association. Using recombinant technology the generation of smaller antibody

30



fragments is possible. The single chain variable fragment (scFv, Fig. 3E) combines the V<sub>L</sub> and V<sub>H</sub> domains joined by a flexible synthetic linker sequence. The shortening of the linker sequence results in the formation of diabodies (Fig. 3F) and triabodies (Fig. 3G) or even tetrabodies (not shown). The scFv-fragment has been further modified to include constant  
 5 domains of the antibody like the C<sub>H</sub>3 domain resulting in the development of minibodies Fig. 3D.

Fig. 4 shows a schematic representation of possible combinations of antibodies and antibody fragments to generate bi- and trivalent specificities. The combination of two different  
 10 antibodies (e.g. antibody A and B) by mating stable transfected *Tetrahymena* cells results in different possible bispecific antibodies, shown in Fig. 4A. In Fig. 4B the possible combination of antibody fragments are shown resulting in different bi-and trispecific F(ab)<sub>2</sub> and dia- and minibodies. In Fig 4C the possible combination of an antibody (e.g. antibody C) or antibody fragment with an bispecific antibody or antibody fragment by mating stable transfected  
 15 *Tetrahymena* cells resulting in different possible tri-and multispecific antibodies and antibody fragments are shown.

Fig. 5A shows the expression plasmids for use in the ciliate *Tetrahymena thermophila* encoding the heavy and the light chain of the antibody is shown, representing the one plasmid  
 20 approach. The plasmid contains an ampicillin (AmpR) and chloramphenical (CmR) resistance gene for selection in *E. coli*, a *T. thermophila* specific origin (rDNA ori) for plasmid stability in *T. thermophila*, a neomycin based selection cassette (NeoR) for identification of transformed ciliates and the two open reading frames of the target gene (heavy and light chain) under the control of an inducible promoter and followed by *T. thermophila*'s [beta]-tubulin 2 terminator  
 25 sequence (BTU2).

In Fig. 5B and C expression plasmids are shown for the use in the ciliate *Tetrahymena thermophila* representing the two plasmid approach. In Fig. 5B the plasmid contains the 5' and 3' flanking regions of the *Tetrahymena* gene DHFR for directed integration of the heterologous  
 30 gene, an ampicillin (AmpR) and chloramphenical (CmR) resistance gene for selection in *E. coli*, and a blasticidin S selection cassette (BsdR) for identification of transformed ciliates and the open reading frame of either the heavy or the light chain of the desired antibody under the control of an inducible promoter and followed by *T. thermophila*'s [beta]-tubulin 2 terminator

sequence (BTU2). In Fig. 5C the expression plasmid encoding the corresponding heavy or light chain of the antibody and containing the same features as listed for Fig. 5A.

Fig. 6 shows a schematic overview of the different stages in *Tetrahymena* conjugation.

- 5 Conjugation process starts with pairing of cells homozygous for alternative alleles at one locus. The MIC (small circles) nested in but physically separate from the MAC (large circles). The MICs undergo meiosis and generate four haploid nuclei, only one of them remains functional (anterior meiotic product) and the other three disintegrate. In this stage the meiotic crossover occurs, followed by the reciprocal exchange of the migratory pronuclei, which fuse with the
- 10 stationary pronuclei of the recipient cell, forming the zygote nucleus. The zygote nucleus undergoes two mitotic divisions resulting in four different genetically identical diploid nuclei. At this stage the old MAC is degraded. Then the anterior products differentiate into new MACs and the posterior products remain diploid MICs. The cells separate (called now exconjugants) and undergo the first postzygotic cell division forming four karyonide cells.
- 15 Each karyonide receives an independently differentiated new MAC and a mitotic copy of a functional MIC. Karyonides then begin vegetative multiplication by binary fission.

- Fig. 7 shows a schematic overview of the transformation of *Tetrahymena* cells using one episomal and one integrative expression plasmid. This two plasmid approach leads to stable
- 20 transfected *Tetrahymena* cells producing whole IgG and exhibit an thymidin auxotrophy.

- Fig. 8 shows representative immunoblots of the anti-CD4 antibody Gk1.5 and its fragments expressed in *Tetrahymena thermophila* cells. In Fig. 8A, expression of Gk1.5 and its fragments in the cell pellet and in the supernatant of stable transformed cells is shown after different times
- 25 of induction of the recombinant protein expression (p.i.) which were cultivated in a multi-fermenter (0.5L lab-scale). The anti-CD4-antibody clone Gk1.5 from eBioscience served as a positive control. Staining took place using an Hrp-conjugated anti-rat-IgG. In Fig. 8B, a representative immunoblot of *Tetrahymena* expressed antibody Gk1.5 and its fragments after purification of the produced supernatant using a protein G column is shown.

30

Fig. 9 shows a comparison between codon usage in *Tetrahymena thermophila* and *Homo sapiens*. The latter is applicable for monoclonal antibodies, or fragments or derivatives thereof, being expressed in a mammalian cell line. See text for further explanations.



Fig. 10 shows the genetic code as used in ciliates, particularly in *Tetrahymena*. The non-canonical nucleotide codes UAA and UAG, which encode for glutamine, are printed in bold. According to the general genetic code, these triplets are, however, stop codons (see striked  
5 through triplets). “1LC” stands for “one letter code”, whereas “3LC” stands for “three letter code”.

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**What is claimed is**

1. A system for the heterologous expression of a monoclonal Antibody (mAb) or an antigen binding fragment or derivative thereof, said system comprising
- 5 a) at least one ciliate host cell, and
- b) incorporated, into said ciliate host cell, at least one heterologous nucleic acid molecule encoding for said monoclonal Antibody, or an antigen binding fragment or derivative thereof, and
- the system further comprising
- 10 c) a signal sequence operably linked to said nucleic acid molecule, which signal sequence accounts for the secretion of the monoclonal antibody, or the antigen binding fragment or derivative thereof, encoded by the said nucleic acid molecule, into the extracellular medium,
- wherein the ciliate is a member of the family Tetrahymenidae,
- 15 and wherein the antigen binding fragment or derivative is at least one selected from the group consisting of
- scFv, and
  - bi-, tri- or higher specific antibody constructs.
- 20 2. The system of claim 1, wherein said monoclonal Antibody (mAb), or an antigen binding fragment or derivative thereof, has an N-glycan structure which is essentially fucose-free.
3. The system of claim 1 or 2, wherein said monoclonal Antibody (mAb), or an antigen binding fragment or derivative thereof, has at least one effect selected from the group
- 25 consisting of
- increased Antibody-Dependent Cellular Cytotoxicity (ADCC),
  - increased Complement-Dependent Cytotoxicity (CDC),
  - increased Antibody-Dependent Apoptosis,
  - increased Antibody-Dependent Opsonisation and
  - 30 • an extended serum half-life,
- each increase being relative to at least one Antibody (mAb), or an antigen binding fragment or derivative thereof being produced in a mammalian cell.



4. A system for the heterologous expression of a monoclonal Antibody (mAb) or an antigen binding fragment or derivative thereof, said system comprising a ciliate host cell which has been obtained by conjugation of at least two ciliate host cells of the system of claim 1, wherein the ciliate is a member of the family Tetrahymenidae.
- 5
5. A ciliate host cell transfected with at least one vector comprising at least one nucleic acid molecule encoding for a monoclonal Antibody (mAb), or an antigen binding fragment or derivative thereof provided by the system of claim 4, or obtained by conjugation of at least two ciliate host cells of the system of claim 1, wherein the ciliate is a member of the family
- 10 Tetrahymenidae.
6. A library comprising at least two ciliate host cells of claim 5, or at least two systems of claim 4, wherein each host cell has incorporated at least one heterologous nucleic acid molecule encoding for an Antibody, or an antigen binding fragment or derivative thereof,
- 15 and wherein at least two ciliates are selected in such way that they can conjugate with one another, and the ciliate is a member of the family Tetrahymenidae.
7. The library of claim 6, wherein the ciliate host cell comprises a vector.
- 20 8. A monoclonal Antibody (mAb) being produced with the system of any one of claims 1 – 4, or with the ciliate host cell of claim 5, wherein the ciliate is a member of the family Tetrahymenidae which antibody or antigen binding fragment or derivative further binds to at least one of the targets selected from CD3, CD4, CD5, CD19, CD20, CD22, CD30, CD33, CD38, CD40, CD52, CD70, CEA, CTAA 16.88, GD2, VEGF-R/FLT-1, GM2, IL-5 receptor,
- 25 EGFR/Her2-neu, EpCAM, ErbB2, FOLR1, PLAC1, CLDN18.2, Histone H1, CD317, Muc1, PSMA, VEGF, TNF- $\alpha$ , CD25, CD3, CD49d ( $\alpha$ 4-integrin), interleukin 6 receptor, interleukin 12/23, RSV surface antigen, VEGF-A, CD11a-antigen and immunoglobulin E .
9. The monoclonal Antibody of claim 8, which has at least one feature selected from the
- 30 group consisting of
- increased ADCC, CDC, and/or Antibody-Dependent Apoptosis,
  - extended serum half-life, and
  - bi, tri- or multispecificity.

10. A process for the production of at least one monoclonal Antibody (mAb), or an antigen binding fragment or derivative thereof, in a ciliate host cell, said process comprising the steps of
- a) transfecting at least two different ciliate host cells with at least one nucleic acid molecule encoding for an antibody, or a fragment or derivative thereof,
  - b) conjugating the said two ciliate host cells, or offspring thereof, in order to obtain at least one ciliate cell carrying at least two different nucleic acid molecules encoding for at least two different antibodies, or fragments or derivatives thereof, and
  - c) culturing the ciliate cell thus produced under conditions which allow expression of a protein, wherein the ciliate is a member of the family Tetrahymenidae.
11. The process of claim 10, wherein the ciliate host cell comprises a vector comprising at least one nucleic acid molecule encoding for a monoclonal Antibody (mAb), or an antigen binding fragment or derivative thereof.
12. A monoclonal Antibody (mAb) or antigen binding fragment or derivative thereof, produced with the process of claim 10, wherein the ciliate is a member of the family Tetrahymenidae, which antibody or antigen binding fragment or derivative thereof further binds to at least one of the targets selected from CD3, CD4, CD5, CD19, CD20, CD22, CD30, CD33, CD38, CD40, CD52, CD70, CEA, CTAA 16.88, GD2, VEGF-R/FLT-1, GM2, IL-5 receptor, EGFR/Her2-neu, EpCAM, ErbB2, FOLR1, PLAC1, CLDN18.2, Histone H1, CD317, Muc1, PSMA, VEGF, TNF- $\alpha$ , CD25, CD3, CD49d ( $\alpha_4$ -integrin), interleukin 6 receptor, interleukin 12/23, RSV surface antigen, VEGF-A, CD11a-antigen and immunoglobulin E.



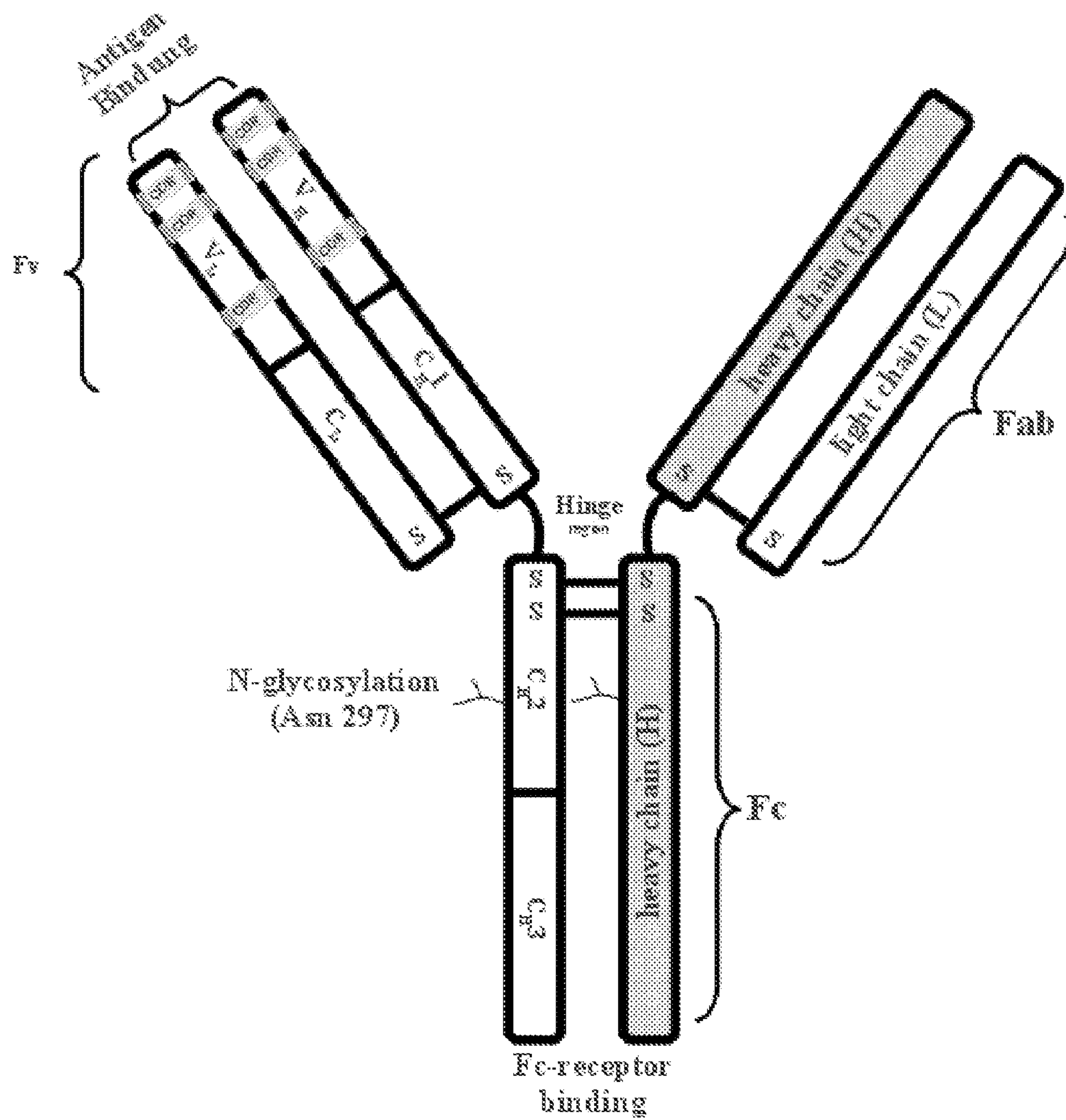
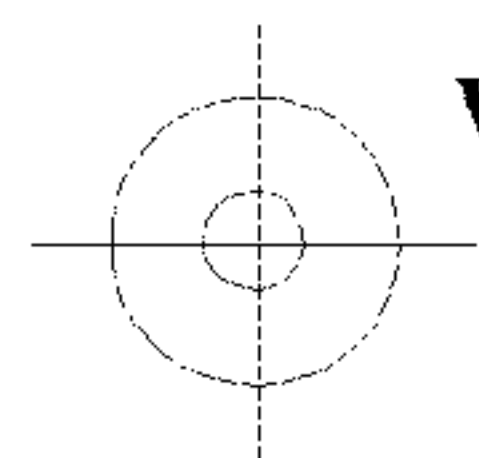
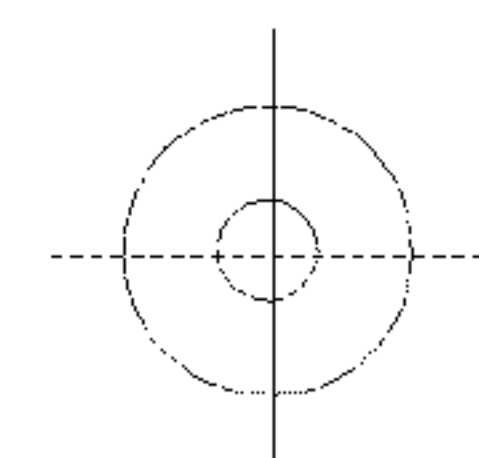


Fig. 1



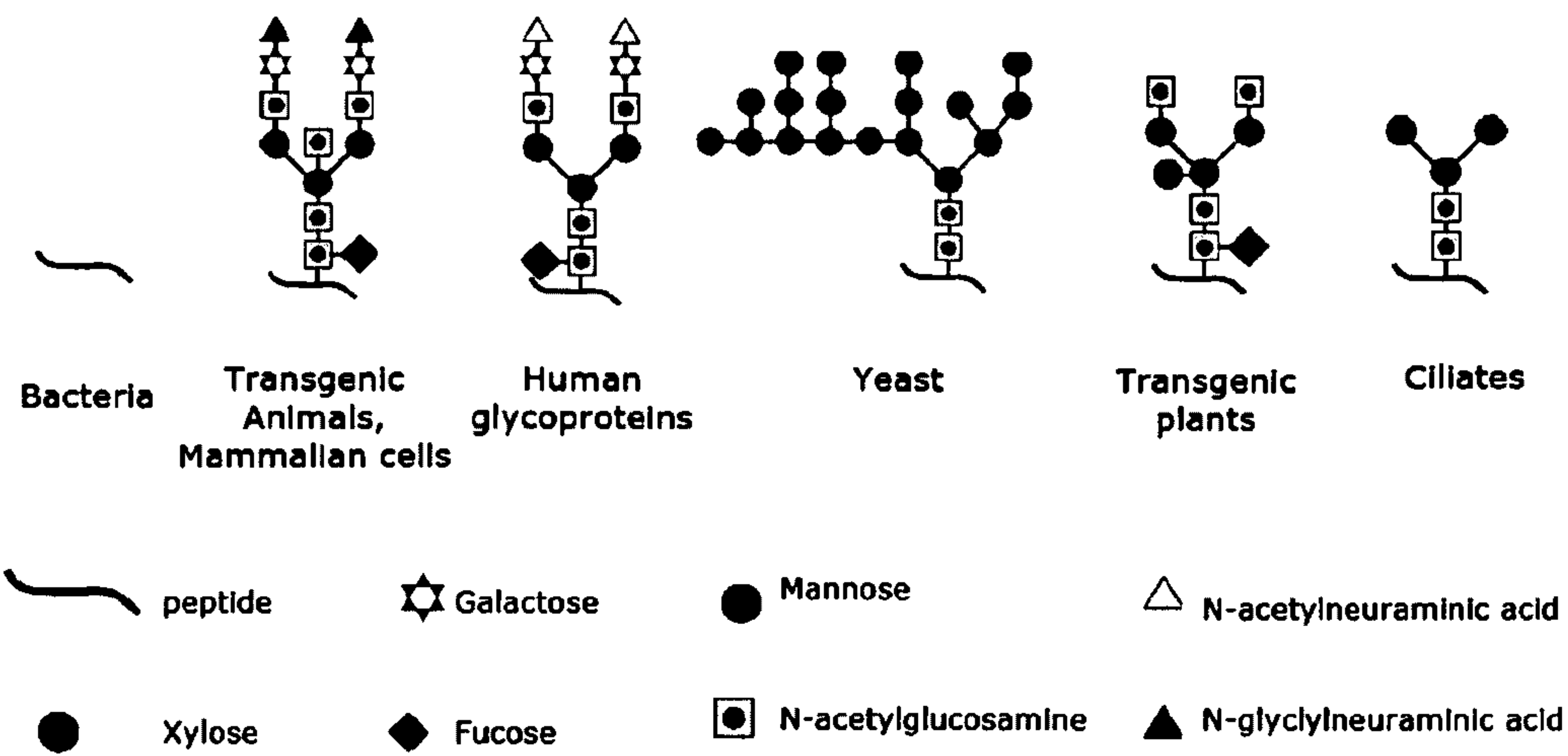
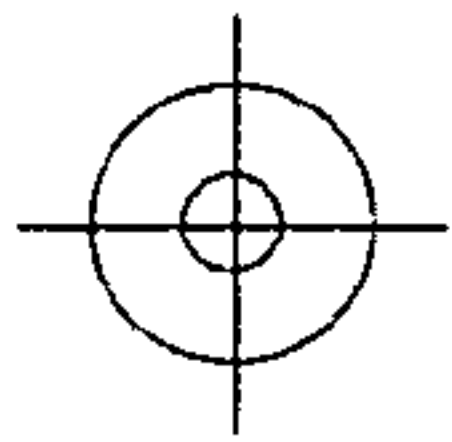


Fig. 2a

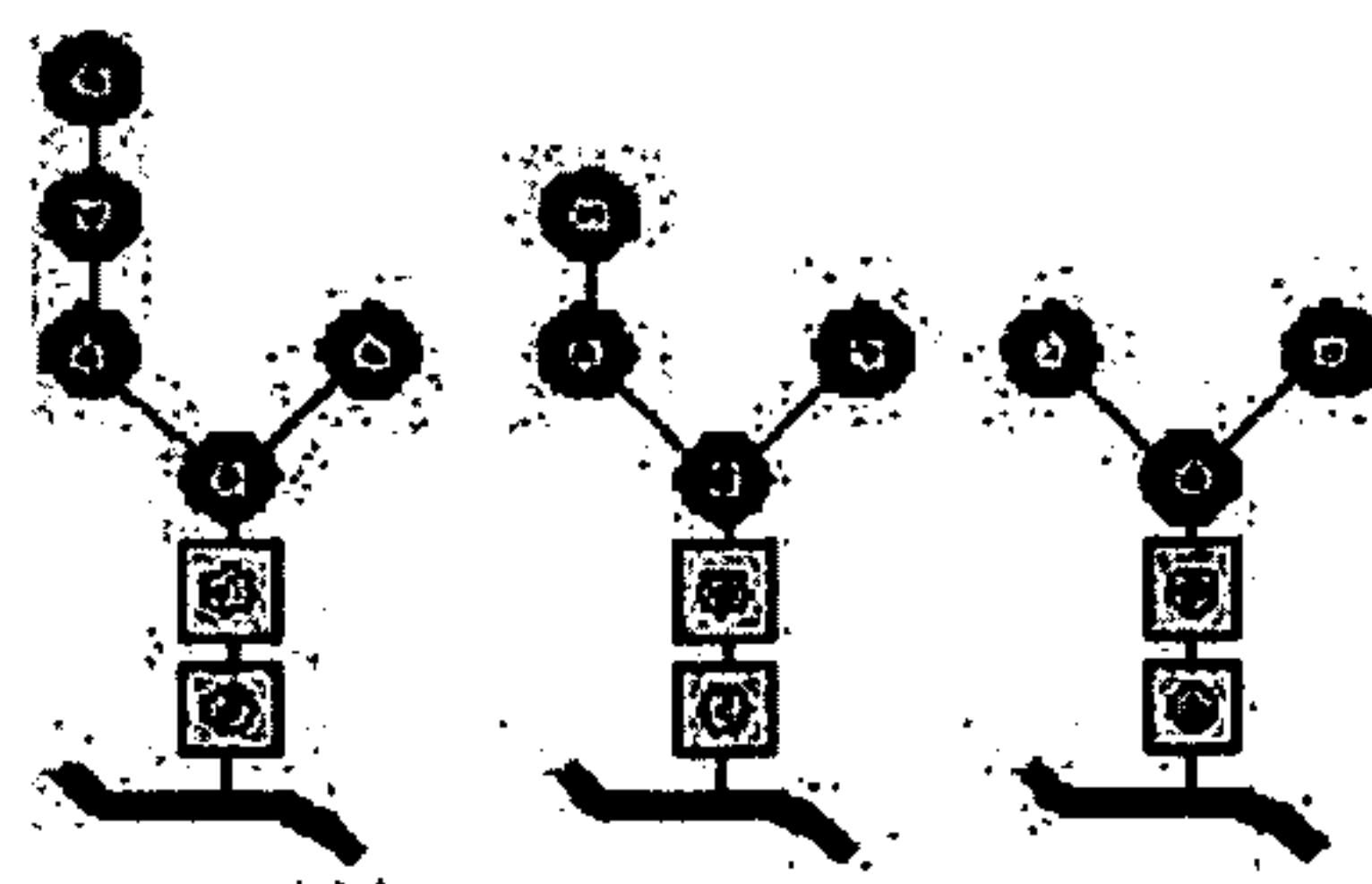
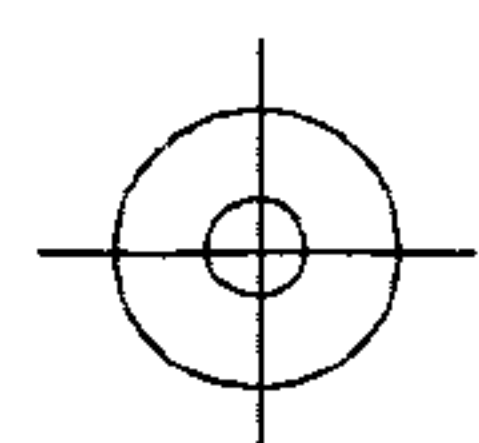


Fig. 2b





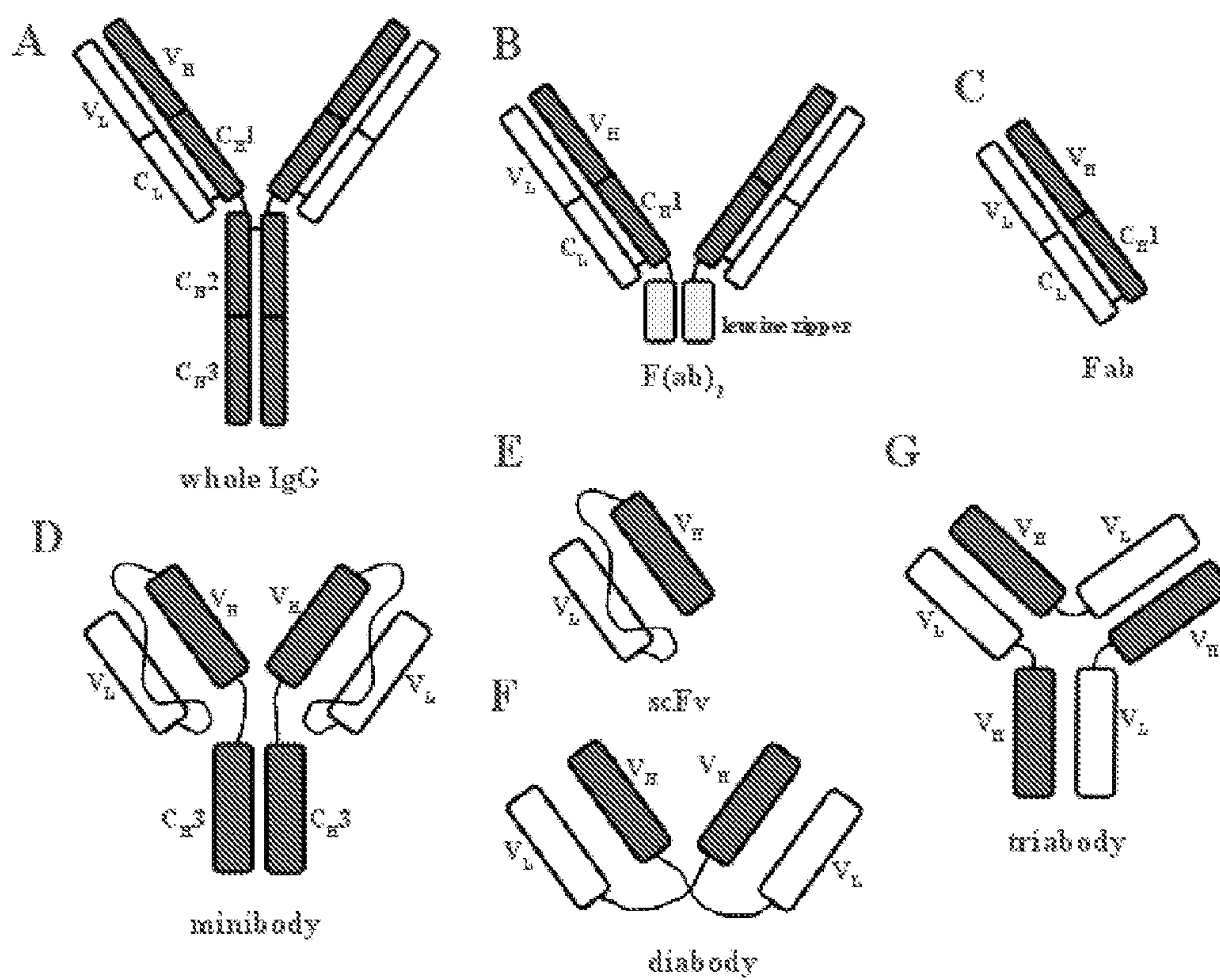
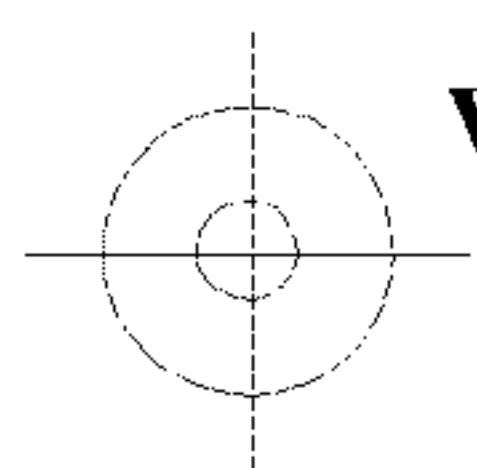
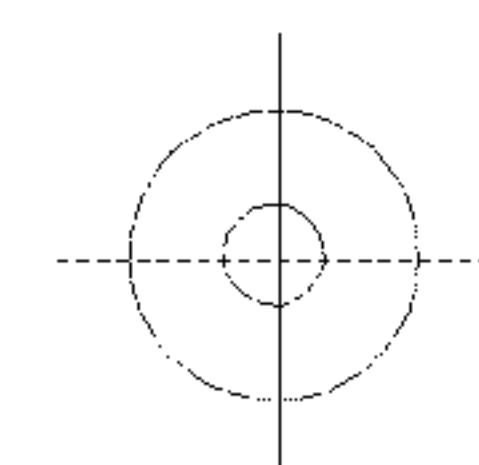


Fig. 3



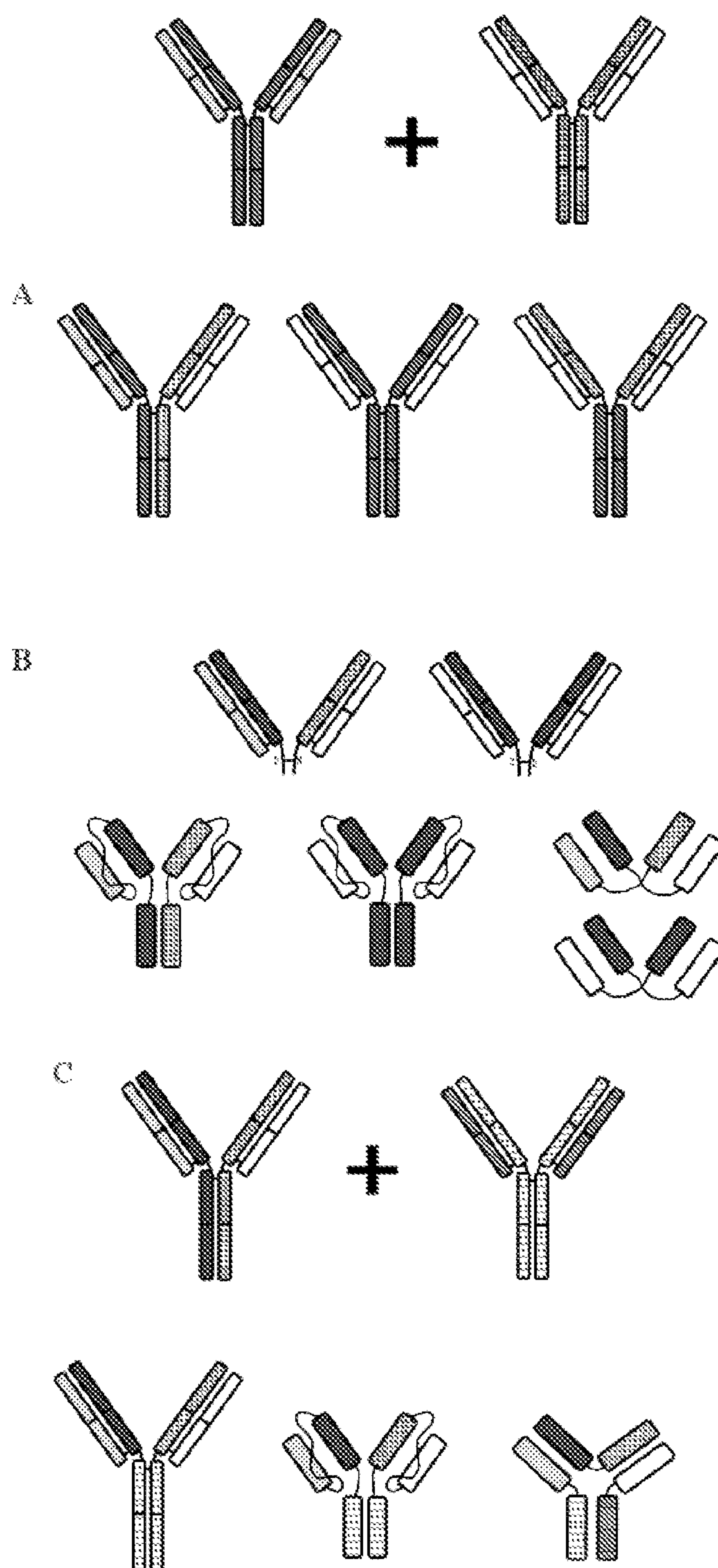


Fig. 4



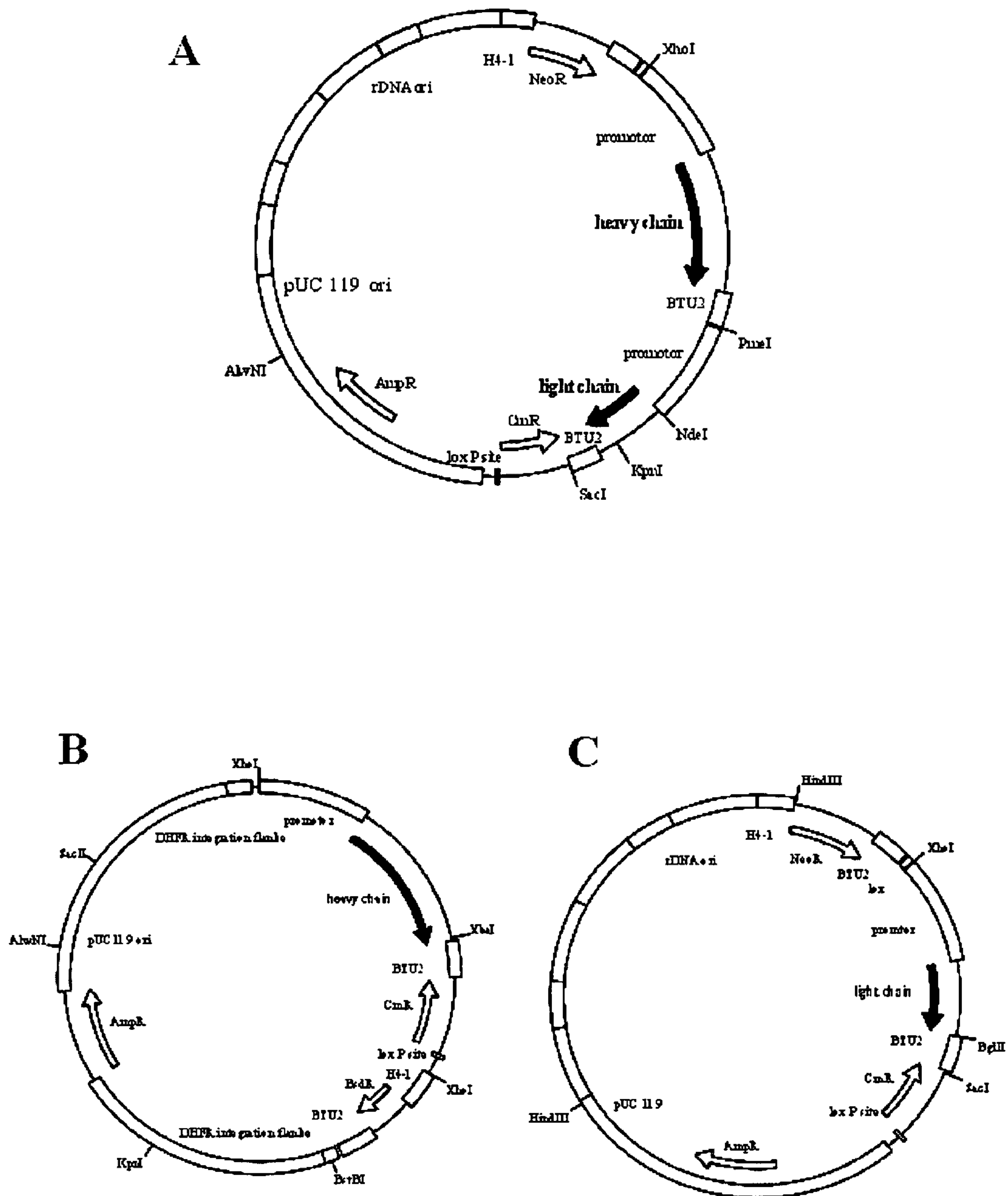
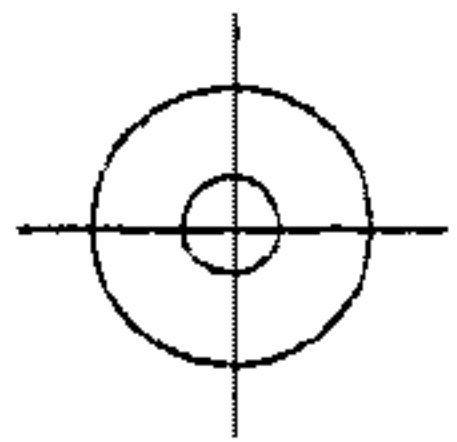
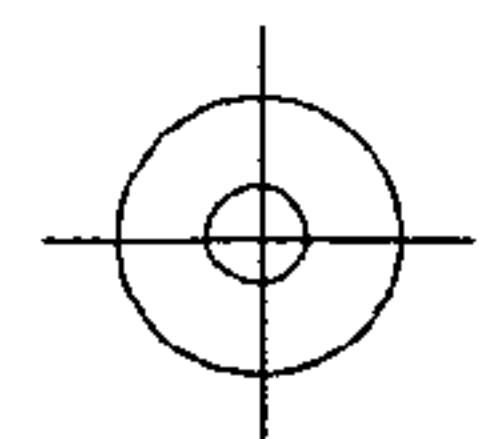


Fig. 5



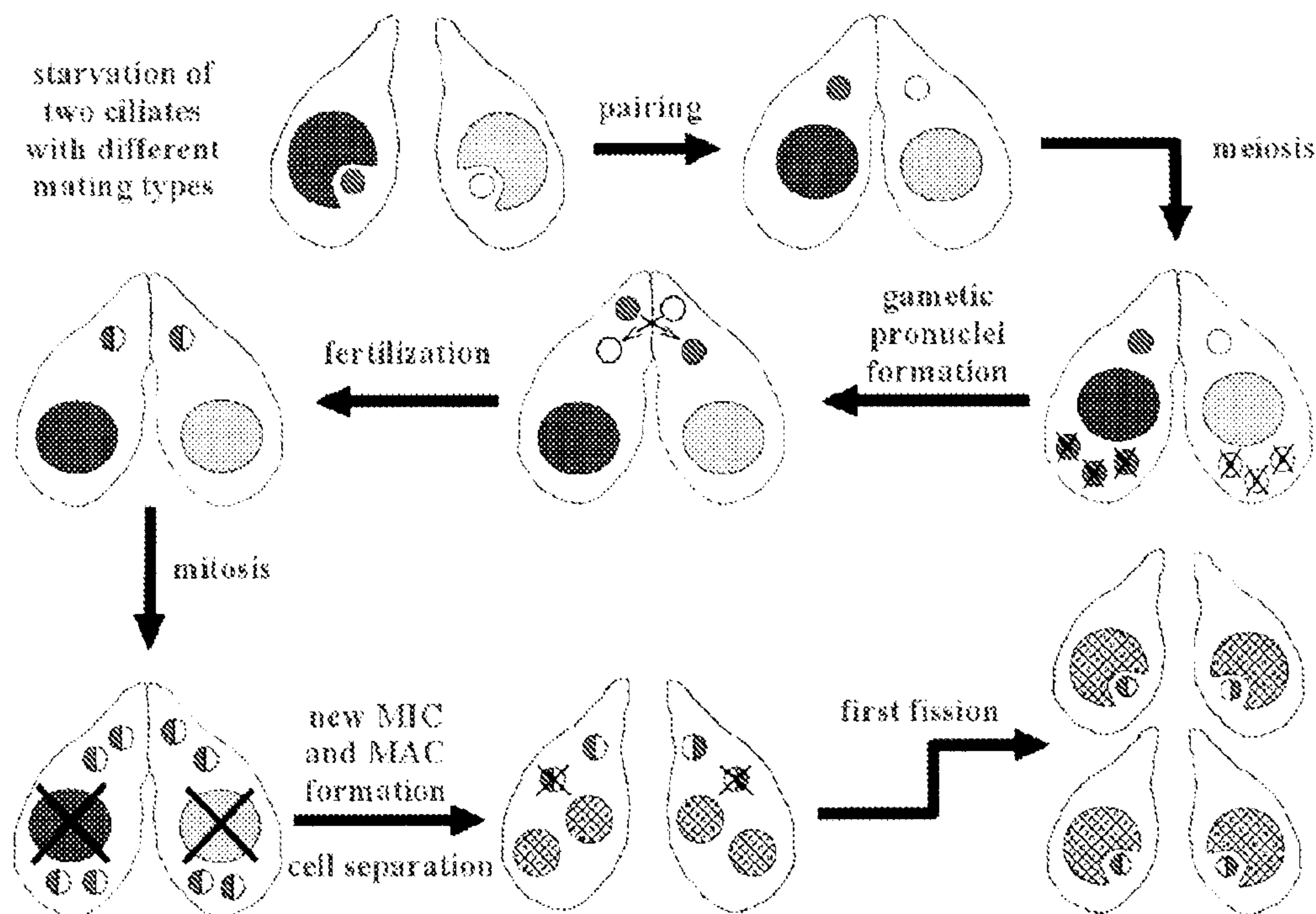


Fig. 6

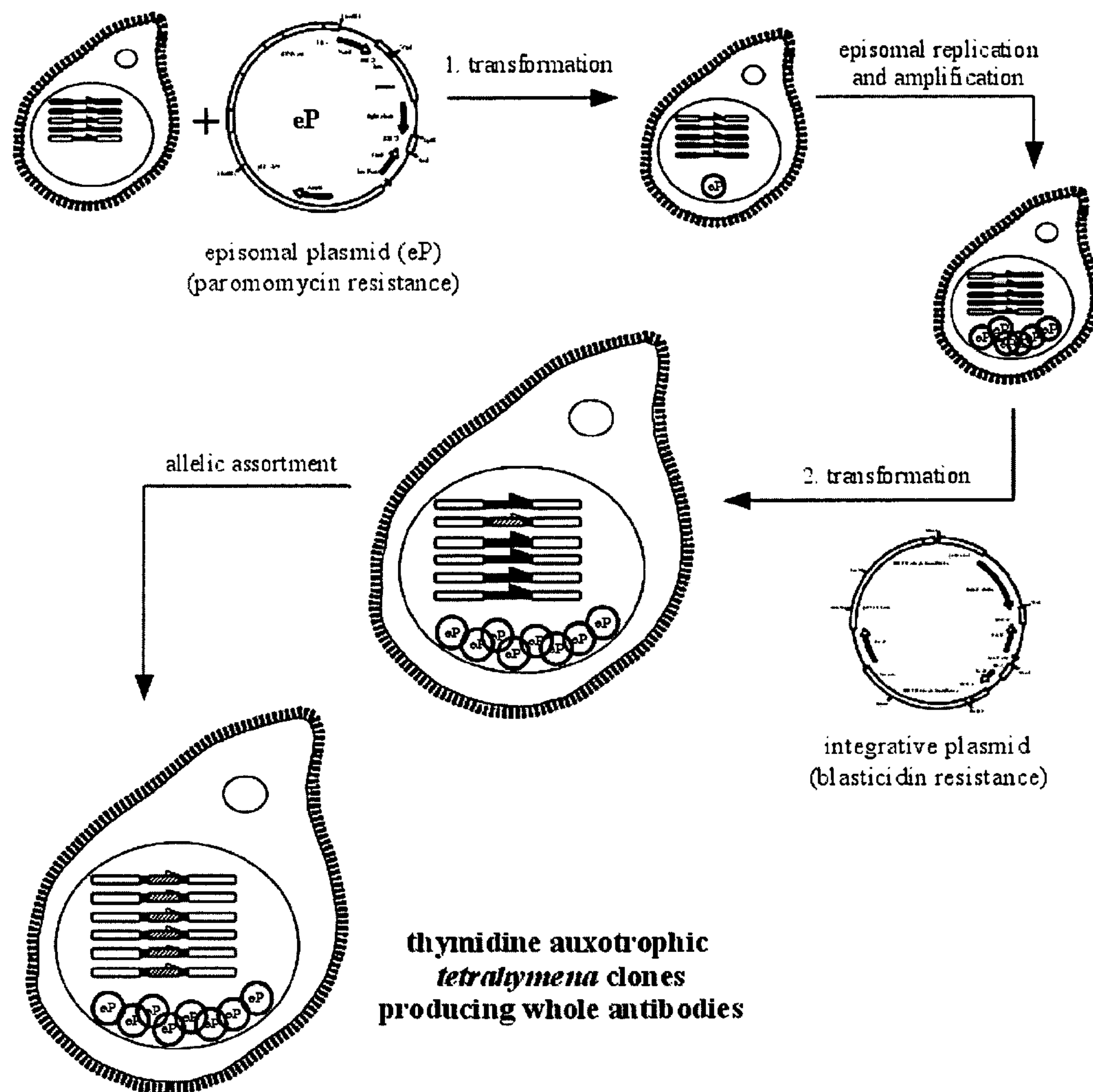
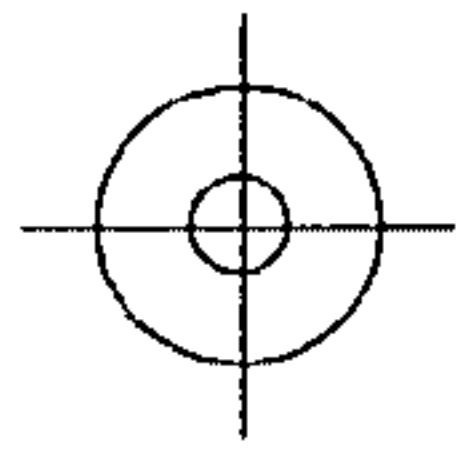
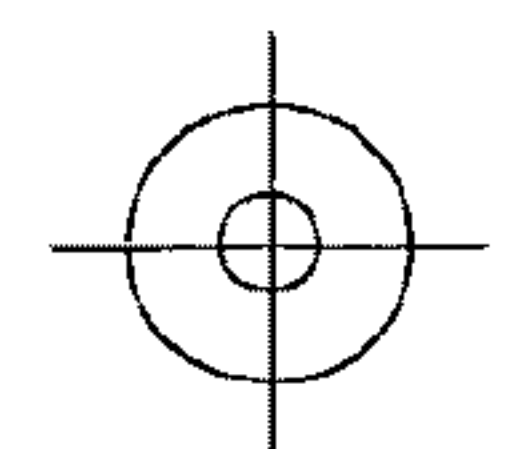


Fig. 7





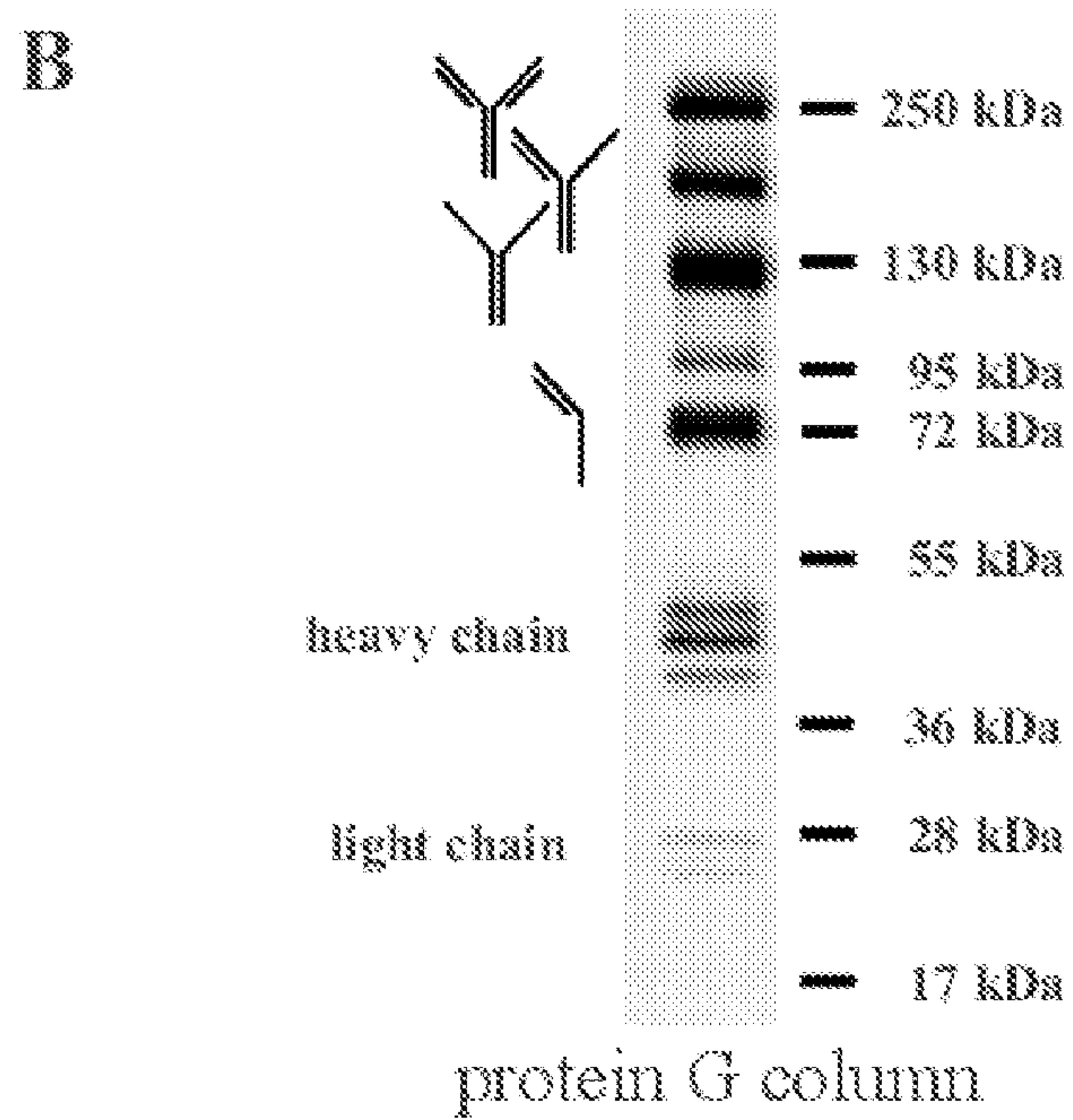
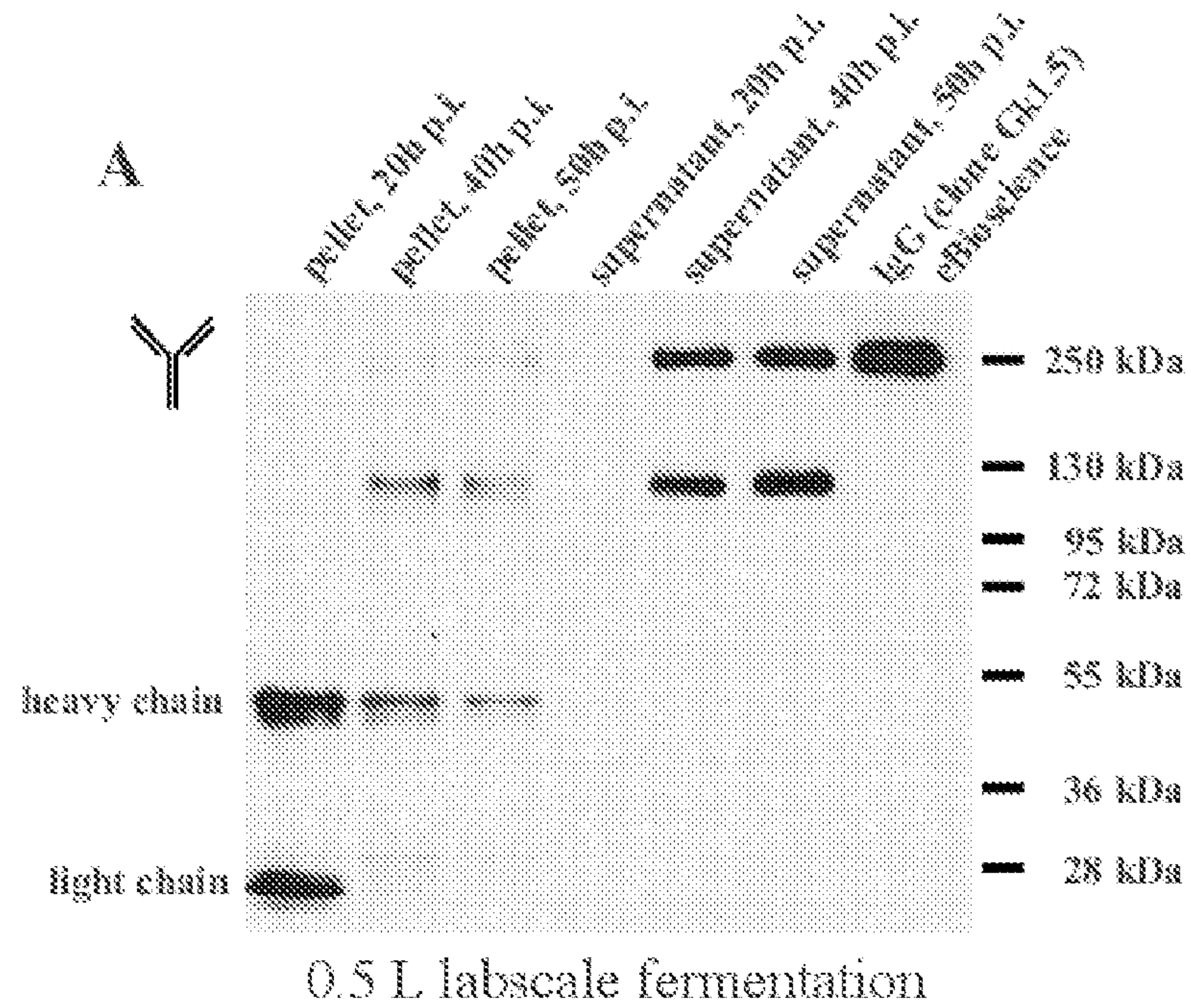
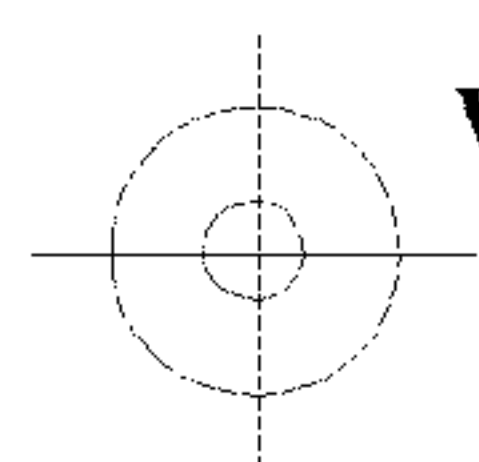


Fig. 8



***Tetrahymena thermophila* [gbinv]: 283 CDS's (145922 codons)**

**fields: [triplet] [frequency: per thousand] ([number])**

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UUC 19.4 ( 2827)	UCC 6.5 ( 948)	UAC 14.5 ( 2110)	UGC 8.8 ( 1282)
UUA 29.8 ( 4346)	UCA 16.8 ( 2453)	UAA 36.8 ( 5366)	UGA 2.0 ( 286)
UUG 14.1 ( 2054)	UCG 1.5 ( 222)	UAG 11.0 ( 1606)	UGG 7.4 ( 1080)
CUU 20.3 ( 2955)	CCU 17.6 ( 2574)	CAU 8.7 ( 1267)	CGU 4.6 ( 677)
CUC 10.3 ( 1497)	CCC 4.6 ( 676)	CAC 6.4 ( 930)	CGC 0.9 ( 136)
CUA 7.4 ( 1078)	CCA 8.2 ( 1202)	CAA 19.8 ( 2894)	CGA 0.5 ( 73)
CUG 2.6 ( 378)	CCG 0.5 ( 68)	CAG 3.3 ( 477)	CGG 0.1 ( 8)
AUU 39.3 ( 5733)	ACU 27.2 ( 3968)	AAU 48.0 ( 7002)	AGU 13.5 ( 1963)
AUC 16.2 ( 2367)	ACC 7.8 ( 1140)	AAC 24.2 ( 3530)	AGC 9.2 ( 1344)
AUA 19.1 ( 2783)	ACA 14.8 ( 2153)	AAA 58.7 ( 8562)	AGA 26.6 ( 3887)
AUG 19.3 ( 2811)	ACG 0.8 ( 111)	AAG 34.3 ( 5001)	AGG 2.8 ( 412)
GUU 25.8 ( 3763)	GCU 30.3 ( 4428)	GAU 42.5 ( 6208)	GGU 24.5 ( 3576)
GUC 10.1 ( 1469)	GCC 7.5 ( 1098)	GAC 12.4 ( 1815)	GGC 4.3 ( 629)
GUA 11.6 ( 1693)	GCA 11.8 ( 1726)	GAA 58.2 ( 8499)	GGA 15.1 ( 2205)
GUG 3.1 ( 451)	GCG 0.6 ( 88)	GAG 11.2 ( 1630)	GGG 1.5 ( 216)

Coding GC 32.53% 1st letter GC 38.64% 2nd letter GC 31.25% 3rd letter GC 27.69%

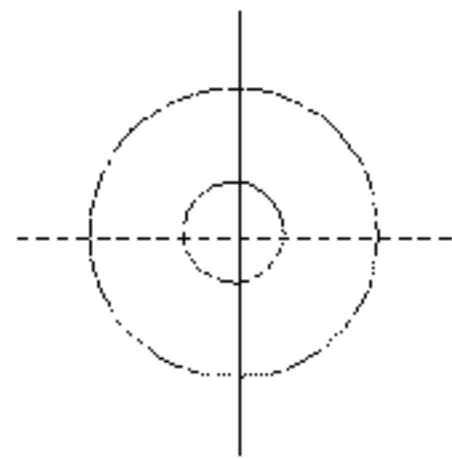
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UUG 12.9(525688)	UCG 4.4(179419)	UAG 0.8( 32109)	UGG 13.2(535595)
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GUG 28.1(1143534)	GCG 7.4(299495)	GAG 39.6(1609975)	GGG 16.5(669768)

Coding GC 52.27% 1st letter GC 55.72% 2nd letter GC 42.54% 3rd letter GC 58.55%

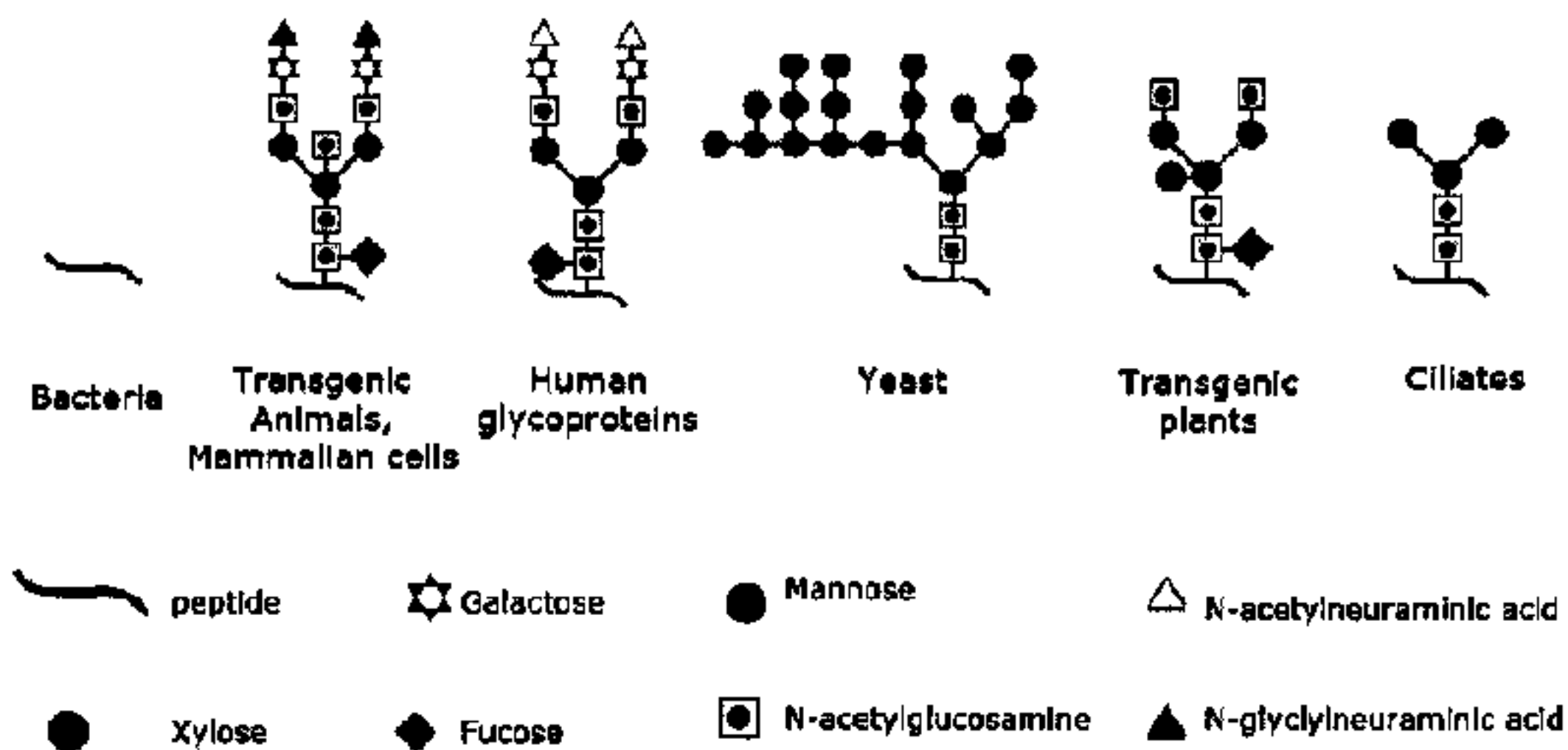
Fig. 9



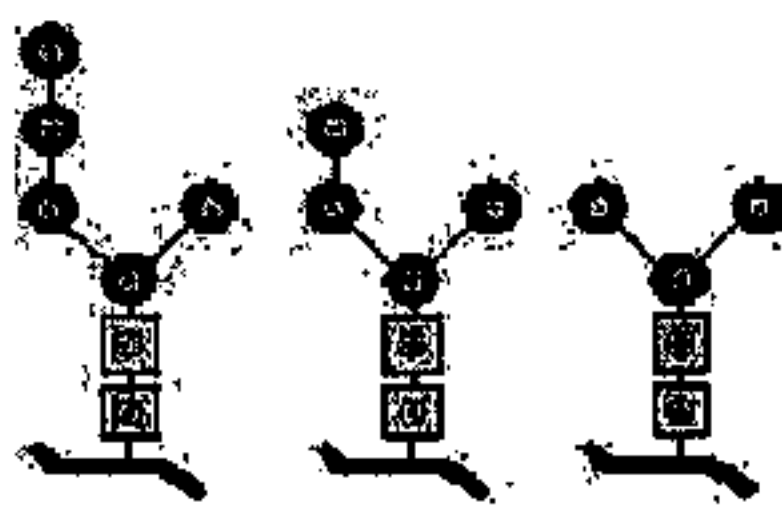
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A	Ala	Alanine	GCU	GCA	GCC	GCG		
C	Cys	Cysteine	UGU	UGC				
D	Asp	Aspartic acid	GAU	GAC				
E	Glu	Glutamic acid	GAA	GAG				
F	Phe	Phenylalanine	UUU	UUC				
G	Gly	Glycine	GGU	GGA	GGC	GGG		
H	His	Histidine	CAU	CAC				
I	Ile	Isoleucine	AUU	AUA	AUC			
K	Lys	Lysine	AAA	AAG				
L	Leu	Leucine	UUA	CUU	UUG	CUC	CUA	CUG
M	Met	Methionine /START	AUG					
N	Asn	Asparagine	AAU	AAC				
P	Pro	Proline	CCU	CCA	CCC	CCG		
Q	Gln	Glutamine	CAA	CAG	UAA	UAG		
R	Arg	Arginine	AGA	CGU	AGG	CGC	CGA	CGG
S	Ser	Serine	UCU	UCA	AGU	AGC	UCC	UCG
T	Thr	Threonine	ACU	ACA	ACC	ACG		
V	Val	Valine	GUU	GUA	GUC	GUG		
W	Trp	Tryptophan	UGG					
Y	Tyr	Tyrosine	UAU	UAC				
		STOP	UGA	UAA	UAG			

Fig. 10





a



b