



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(21) International Application Number: PCT/US99/14841</p> <p>(22) International Filing Date: 30 June 1999 (30.06.99)</p> <p>(30) Priority Data: 60/091,771                      6 July 1998 (06.07.98)                      US</p> <p>(71) Applicant (for all designated States except US): THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; 6011 Executive Boulevard, Rockville, MD 20852 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): STEWART, Colin, L. [US/US]; 280 N. Fullerton Avenue, Montclair, NJ 07042 (US). SHATZER, Teresa [US/US]; 206 Stadium Circle, Inwood, WV 25428 (US). SULLIVAN, Teresa [US/US]; 3903 Highland Avenue, Myersville, MD 21773 (US). CHEN, Jim-Ray [US/US]; Apartment 202, 342A Highview Terrace, Frederick, MD 21702 (US). HERNANDEZ, Lidia [US/US]; 20130 Darlington Drive, Montgomery Village, MD 20886 (US).</p>	<p>(74) Agents: CARROLL, Peter, G. et al.; Medlen &amp; Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> With international search report.</p>	
<p>(54) Title: COMPOSITIONS AND METHODS FOR <i>IN VITRO</i> FERTILIZATION</p>		
<p>(57) Abstract</p> <p>Compositions and methods are described to enhance embryo implantation. Cytokines capable of binding to a receptor complex (the complex comprising a member of the hematopoietic cytokine receptor family) are utilized to treat female mammals, and in particular, females receiving <i>in vitro</i> fertilized embryos.</p>		

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## COMPOSITIONS AND METHODS FOR *IN VITRO* FERTILIZATION

### FIELD OF THE INVENTION

5 The present invention relates to compositions and methods for *in vitro* fertilization (IVF), and in particular, enhancing implantation frequencies and blocking implantation in mammals (including but not limited to humans) using LIF and LIF antagonists, respectively.

### BACKGROUND

10 Infertility is a problem that afflicts approximately 9% of couples in the United States who wish to have children. The causes of infertility are many and varied. Whatever the cause in the particular case, treatment can be very expensive. Indeed, it is estimated that the annual cost of treating infertile couples in the United States is approximately 1 billion dollars.

15 Frequently, treatment involves the use of *in vitro* fertilization (IVF) in which eggs from the woman are recovered and fertilized with the male's sperm. The resulting embryo(s) are then surgically transferred back to the woman's uterus for development to continue. However, only about 25% (at best) of the transplanted embryos implant properly with even fewer resulting in live birth. Failure of transplanted embryos to  
20 implant is thus a major difficulty in treating the larger problem of infertility.

One obvious approach to improving the chance of success is to increase the number of embryos being implanted. While the use of multiple embryos (*e.g.* the transfer of up to four embryos to the uterus at the same time) increases the chances of one of them implanting, the recipient also runs the risk of multiple implants and consequently multiple  
25 births. *See* Elsner *et al.*, *Am J Obstet Gynecol* 177:350-355 (1997). This method is also unsatisfactory in that if the uterus is physically and chemically unprepared to receive embryos because the hormonal regulation is dysfunctional, simply transplanting more embryos will not solve the problem.

30 A number of hormones (*e.g.* estrogen, progesterone, etc.) have been identified as having a role in the preparation of the uterus to receive a transplanted embryo, and thus many attempts to improve implantation frequency have focused on the regulation of these hormones. For example, estrogen appears to play a significant role in regulating uterine

receptivity to embryo implantation, and thus one treatment of implantation failure is to regulate (*e.g.* increase) the estrogen level of the host. This approach to treatment frequently backfires, however, as it is thought that this can cause implantation failure due to improper uterine physiology and chemical receptivity induced by the estrogen treatment. Moreover, increased levels of estrogens employed during IVF treatment have been implicated in severe complications of the Ovarian Hyperstimulation Syndrome (OHS). *See e.g. MacDougall et al., Hum Reprod 7:597-600 (1992).*

What is needed is an effective substitute for the standard hormonal treatment currently used to increase uterine receptivity to embryo implantation. A one-time, non-invasive direct administration of an estrogen substitute would allow increased use and effectiveness of treatment of implantation failure, without the side effects of increased estrogen levels.

## DEFINITIONS

The term "hematopoietic cytokine receptor family" is the recognized family of receptors [*see e.g. T. Taga and T. Kishimoto, "Cytokine receptors and signal transduction," FASAB J. 6:3387 (1992)*] having structural similarity to IL-6-R, and more particularly, having structural similarity with respect to the approximately 200 amino acid-homologous regions of IL-6-R, said region characterized by four conserved cysteine residues in the amino-terminal half and a WSXWS motif at its carboxyl-terminal end (wherein W is Trp; S is Ser; and X is a nonconserved amino acid). Members of this family include IL-2-R, IL-3-R, IL-4-R, IL-5-R, IL-6-R, IL-7-R, IL-9-R, EPO-R, G-CSF-R, GM-CSF-R and LIF-R.

A subset of the above-named receptors typically exist as a "receptor complex" comprising the above-named molecule in association with another protein called gp130. Thus, the corresponding ligands that bind the above-named receptors typically bind the complex. Thus, the present invention contemplates "a cytokine capable of binding to a receptor complex comprising a member of the hematopoietic cytokine receptor family." Examples of such ligands are IL-6 and LIF. Other examples include Oncostatin M, Ciliary Neurotrophic Factor and Cardiotrophin-1.

The present invention also contemplates molecules that are simply capable of binding to gp130, such molecules useful for the treatment of females to enhance implantation frequencies.

5 An "estrogen-free" composition contains no detectable estrogen (e.g. detectable by immunological assay). A "substantially estrogen-free" composition contains only background amounts of estrogen. A female "subsequently" not treated with estrogen is a female who, subsequent to the claimed method step (e.g. the step of introducing an embryo into said female), does not receive estrogen. A female "that has not been treated with estrogen" is a female who has received no exogenous estrogen prior to the first step  
10 of the claimed method.

### SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for *in vitro* fertilization (IVF), and in particular, enhancing implantation frequencies and blocking implantation in  
15 mammals (including but not limited to humans) using LIF and LIF antagonists, respectively.

With respect to enhancing implantation frequencies, the present invention contemplates treatment of females with the cytokine Leukemia Inhibitory Factor (LIF). The present invention contemplates the direct injection of LIF, which leads to the  
20 embryo's normal development to birth and also, the use of LIF as a replacement to using estrogen for *in vitro* fertilization.

It is not intended that the present invention be limited to only one type of LIF. A variety of LIF proteins are contemplated, including but not limited to LIF from human (SEQ ID NO:1), murine (SEQ ID NO:2), rat (SEQ ID NO:3), porcine (SEQ ID NO:4)  
25 and ovine (SEQ ID NO:5) (see Figure 1). In a preferred embodiment, the LIF administered according to the present invention is recombinantly produced LIF.

It is also not intended that the present invention be limited to LIF. In one embodiment, the present invention contemplates a method, comprising: a) providing i) a female to be implanted with at least one embryo, and ii) an estrogen-free composition,  
30 comprising a cytokine capable of binding to a receptor complex comprising a member of the hematopoietic cytokine receptor family; and b) administering said estrogen-free composition to said female under conditions such that said embryo implants. In a

preferred embodiment, said cytokine is LIF and said embryo is an *in vitro* fertilized embryo.

In a preferred embodiment, the present invention contemplates a method, comprising: a) providing i) an embryo, ii) a female to be implanted with said embryo, and  
5 iii) an estrogen-free composition, comprising a cytokine capable of binding to a receptor complex comprising a member of the hematopoietic cytokine receptor family; b) introducing said embryo into said female, said female subsequently not treated with exogenous estrogen (*i.e.* estrogen given exogeneously) and c) administering said estrogen-free composition to said female under conditions such that said embryo implants. In a  
10 preferred embodiment, said cytokine is LIF and said embryo is an *in vitro* fertilized embryo.

It is also not intended that the present invention be limited to LIF. In one embodiment, the present invention contemplates a method, comprising: a) providing i) a  
15 female that has not been treated with exogenous estrogen, said female to be implanted with at least one embryo, and ii) an estrogen-free composition, comprising a cytokine capable of binding to a receptor complex comprising a member of the hematopoietic cytokine receptor family; and b) administering said estrogen-free composition to said female under conditions such that said embryo implants. In a preferred embodiment, said cytokine is LIF and said embryo is an *in vitro* fertilized embryo.

In another embodiment, the present invention contemplates a method, comprising:  
20 a) providing i) a female that has not been treated with exogenous estrogen, said female to be implanted with at least one embryo, and ii) an estrogen-free composition, comprising a ligand capable of binding to gp130; and b) administering said estrogen-free composition to said female under conditions such that said embryo implants. In a preferred embodiment,  
25 said embryo is an *in vitro* fertilized embryo.

## DESCRIPTION OF THE DRAWINGS

Figure 1 shows an alignment of the predicted amino acid sequence of human (SEQ ID NO:1), murine (SEQ ID NO:2), rat (SEQ ID NO:3), porcine (SEQ ID NO:4) and ovine  
30 (SEQ ID NO:5) versions of LIF (the predicted signal sequence is shown by a solid line above the sequence and potential N-linked glycosylation sites are indicated by #).

Figure 2 shows the nucleic acid sequence (SEQ ID NO:6) and amino acid sequence (SEQ ID NO:7) of gp130.

Figure 3 schematically shows the cDNA encoding a fusion protein of IL-6 and sIL-6R.

5

## DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for *in vitro* fertilization (IVF), and in particular, enhancing implantation frequencies and blocking implantation in mammals (including but not limited to humans) using LIF and LIF antagonists, respectively.

10

With respect to enhancing implantation frequencies, the present invention contemplates treatment of females with the cytokine Leukemia Inhibitory Factor (LIF).

LIF has been identified as having a role in the implantation process and attempts have been made to increase implantation frequency by either culturing the embryos in LIF-containing medium (*see e.g.* U.S. Patent No. 5,366,888 to Fry et al., hereby incorporated by reference) or continuously administering LIF to the host. *See Stewart et al., Nature 359:76-79 (1992)*. These techniques have met with some success, but are inferior in that surgery is involved in the implantation of a micro-pump and that they do not address the problem caused by the estrogen treatment.

20

### A. Leukemia Inhibitory Factor (LIF) And The LIF Receptor

LIF is a member of a family of proteins (the IL-6 family of cytokines) that show some structural similarities. They sometimes have similar effects on the same cell, because they can bind to each other's receptors and stimulate them. What all these factors have in common is that for their specific receptors to function properly, it appears that they have to associate with the gp130 protein. Without being limited to any particular mechanism, the present invention contemplates that other members of the IL-6 family of cytokines will have the same effects on embryo implantation as LIF, and in particular, those members that require the LIF receptor $\alpha$  are expected to be particularly effective.

25

The receptor complex that LIF binds to at the cell surface is comprised of two proteins: the LIF receptor $\alpha$  and another protein called gp130 (see Figure 2). When LIF binds to the LIF receptor $\alpha$  this complex then binds to gp130 which causes the receptor to

30

"fire" so activating a number of processes and responses in the cells to which LIF has bound. While the present invention is not limited to any particular mechanism, this association of LIF + LIF receptor $\alpha$  with gp130 appears to be essential for the whole receptor complex to function properly.

5           As noted above, the present invention contemplates the administration of ligands capable of binding gp130. Such compounds can be readily screened for and tested in a binding assay with gp130.

### **B. LIF Antagonists**

10           In one embodiment, the present invention relates to compositions and methods for blocking implantation in mammals (including but not limited to humans) using LIF antagonists. A variety of LIF antagonists are contemplated, including but not limited to mutant versions of LIF and antibodies to LIF. Mutant LIF can be LIF with amino acid modifications, substitutions and deletions.

15           In one embodiment, LIF is altered such that it cannot bind to the LIF receptor complex. Such altered LIF molecules are readily tested (usually by labeling them) for binding to the LIF receptor on cells. Such an altered LIF molecule can then be used to block implantation in normal female mammals (*e.g.* females having normal levels of functional LIF).

20

### **C. Formulations And Preparations**

          It is not intended that the present invention be limited by the particular nature of the therapeutic preparation used for administration of the cytokines useful for enhanced implantation. For example, such compositions can be provided together with  
25           physiologically tolerable liquid, gel or solid carriers, diluents, adjuvants and excipients.

          These therapeutic preparations can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will vary according to the type of use and mode of administration, as well as the particularized  
30           requirements of individual hosts.

          Such compositions are typically prepared as liquid solutions or suspensions, or in solid forms. Oral formulations for cancer usually will include such normally employed

additives such as binders, fillers, carriers, preservatives, stabilizing agents, emulsifiers, buffers and excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, 5 sustained release formulations, or powders, and typically contain 1%-95% of active ingredient, preferably 2%-70%.

The compositions are also prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared.

10 The antagonists of the present invention are often mixed with diluents or excipients which are physiological tolerable and compatible. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH buffering agents.

15 Additional formulations which are suitable for other modes of administration, such as topical administration, include salves, tinctures, creams, lotions, and, in some cases, suppositories. For salves and creams, traditional binders, carriers and excipients may include, for example, polyalkylene glycols or triglycerides.

## 20 **EXPERIMENTAL**

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

### **EXAMPLE 1**

25 In this example, LIF is shown to be useful as a replacement for estrogen. Mice were ovariectomized and allowed to recover for 18 days before starting experimental manipulation. Priming of the uterus by steroid hormones was started by the injection of 100ng of Estrogen on each of 3 consecutive days. On the 6th day the mice were given a single injection of Progesterone in the form of Depo-Provera. Three days later an average 30 of 6 blastocysts, either directly isolated from females or from cultures started the previous day when the embryos were at the 8-cell stage were surgically transferred to the right uterine horn. After the mice had recovered from surgery they were either given 1

injection of 40ng of estrogen or 3 injections, 1 hour apart, of 5 $\mu$ g LIF. Four days later (equivalent to Day 7-8 of pregnancy) the mice were sacrificed for analysis, and the implantation sites recovered. Examination of the deciduas revealed that they contained morphologically normal embryos at the right stage of development.

5 The results presented in Table 1 using this particular experimental protocol reveal that the injection of LIF can unequivocally replace the injection of estrogen for inducing embryo implantation. In turn this demonstrates that LIF could be used as an alternative to already established hormonal treatments for increasing the frequency of obtaining successful implantations and viable embryonic development in the treatment of human  
10 infertility following *In Vitro Fertilization* and transfer of the resulting embryo to the mother's uterus.

Table 1  
Injection of Recombinant LIF Substitutes for  
Nidatory (implantational) Estrogen at inducing Blastocyst Implantation

Blastocyst Type at time of Transfer	Implantation Agent	Nos. Blastocysts Transferred (Nos. of recipients)	Nos. Pregnant	Nos. Implanted
D4 direct	E2	36 (6)	3	11
D4 cultured	E2	36 (6)	4	16
20 D4 direct	LIF	36 (6)	6	30
D4 cultured	LIF	42 (7)	5	16
D4 direct	Saline (control)	30	0	0

#### EXAMPLE 2

25 In this example, a soluble IL-6 ligand/receptor fusion protein (sIL-6RFP) is tested. The sequences for IL-6 and IL-6R are known. See e.g. K. Yamasaki *et al.*, "Cloning and expression of the human Interleukin-6 (BSF-2/IFN  $\beta$ 2) receptor" *Science* 241:825-828 (1988). A cassette (Figure 2) consisting of human sIL-6R cDNA (corresponding to amino acid residues 113-323) and human IL-6 cDNA (corresponding to amino acid residues 29-

212) fused by a synthetic DNA linker coding for the amino acid sequence Arg-Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser-Val-Glu was constructed by the PCR technique and cloned into the *Pichia pastoris* expression vector pPIC9 (Invitrogen, San Diego, CA) using the restriction enzymes *Sna*BI and *Eco*RI. Cleavage of the signal peptide in transfected yeast cells leads to the secretion of the fusion protein with an NH<sub>2</sub>-terminal extension of 8 amino acid residues (Glu-Lys-Arg-Glu-Ala-Glu-Ala-Tyr). The fusion protein was purified from yeast supernatants by anion-exchange chromatography and gel filtration and visualized by SDS-PAGE and silver staining.

This fusion protein is essentially a hybrid protein comprised of IL-6 already bound to its specific receptor. This complex will then bind to any cell that has gp130 and activate the cell. To test the protein, pseudopregnant wild type mice were ovariectomized on Day 3 of pregnancy. Six females had ten 8-cell stage embryos surgically transferred to their right uterine horn and were injected with progesterone. Five days later 3 mice were each injected with 2μg of sIL-6RFP and the other 3 were injected with a saline control. Three days later the mice were examined for the presence of implanted embryos. The results are shown in Table 2.

Table 2

Treatment	Nos. of Mice Carrying 10 Embryos Each	Nos. with Implantation Sites	Nos. of Embryos Implanted
sIL-6RFP	3	2	8 and 10
Saline	3	0	Total of 15 <u>unimplanted</u> embryos recovered

The results show that this fusion protein is effective at inducing embryo implantation in the absence of estrogen, and thus can be used as an alternative to LIF.

SEQUENCE LISTING

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 Shatzer, Teresa  
 Sullivan, Teresa  
 Chen, Jim-Ray  
 Hernandez, Lidia

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## CLAIMS

We claim:

1. A method, comprising:
  - a) providing i) a female mammal that has not been treated with exogenous estrogen, said female to be implanted with at least one embryo, and ii) an estrogen-free composition, comprising a cytokine capable of binding to a receptor complex comprising a member of the hematopoietic cytokine receptor family; and
  - b) administering said estrogen-free composition to said female under conditions such that said embryo implants.
2. The method of Claim 1, wherein said female mammal is a human.
3. The method of Claim 1, wherein said embryo is an *in vitro* fertilized embryo.
4. The method of Claim 1, wherein said cytokine is LIF.
5. A method, comprising:
  - a) providing i) a female that has not been treated with exogenous estrogen, said female to be implanted with at least one embryo, and ii) an estrogen-free composition, comprising a ligand capable of binding to gp130; and
  - b) administering said estrogen-free composition to said female under conditions such that said embryo implants.
6. The method of Claim 1, wherein said female mammal is a human.
7. The method of Claim 1, wherein said embryo is an *in vitro* fertilized embryo.

8. The method of Claim 5, wherein said ligand comprises at least a portion of the receptor for IL-6.

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Figure 1



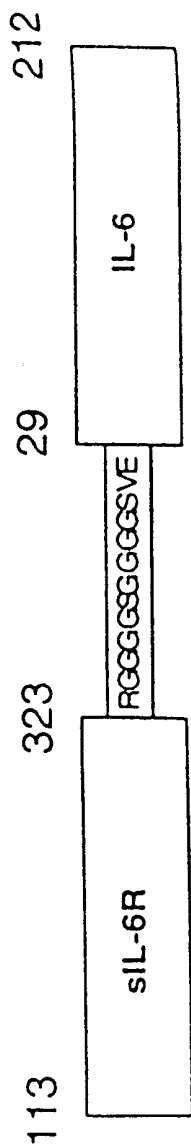


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/14841

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 38/16, 38/17, 38/19; G01N 33/53  
US CL :424/85.1; 512/2, 8, 12; 435/7.1, 7.2, 7.21  
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)

U.S. : 424/85.1; 512/2, 8, 12; 435/7.1, 7.2, 7.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

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

APS, CAS ONLINE, MEDLINE, EMBASE, BIOSIS, CAPLUS  
search terms: cytokine, leukemia inhibitory factor (LIF), in vitro fertilization, gp130, ligand, IL-6 receptor

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUNGLISON et al. Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultures in serum-free medium. Human Reproduction. 1996, Vol. 11, No. 1, pages 191-196.	1-8
A	JURISICOVA et al. Recombinant human leukemia inhibitory factor does not enhance in vitro human blastocyst formation. Fertility and Sterility. November 1995, Vol. 64, No. 5, pages 999-1002.	1-8
A	BORINI et al. Use of Recombinant Leukemia Inhibitory Factor in Embryo Implantation. Annals of the New York Academy of Sciences. 1997, Vol. 828, pages 157-161.	1-8

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	*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
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*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
*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)	* & * document member of the same patent family
*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	

Date of the actual completion of the international search

20 SEPTEMBER 1999

Date of mailing of the international search report

07 OCT 1999

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PREMA MERTZ

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/14841

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,366,888 A (FRY ET AL) 22 November 1994 (22/11/94), see entire document.	1-8