

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 July 2006 (27.07.2006)

PCT

(10) International Publication Number
WO 2006/078624 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2006/001543

(22) International Filing Date: 17 January 2006 (17.01.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/645,251 19 January 2005 (19.01.2005) US

(71) Applicant (for all designated States except US): **CHILDREN'S HOSPITAL MEDICAL CENTER** [US/US];
3333 Burnet Avenue, Cincinnati, Ohio 45229 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **GARDNER, Anne, M.** [US/US]; 8409 Barnesburg Road, Cincinnati, Ohio 45247 (US). **GARDNER, Paul, R.** [US/US]; 8409 Barnesburg Road, Cincinnati, Ohio 45247 (US).

(74) Agents: **LYMAN, Beverly, A.** et al.; Wood Herron & Evans, L.L.P., 2700 Carew Tower, Cincinnati, Ohio 45202 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

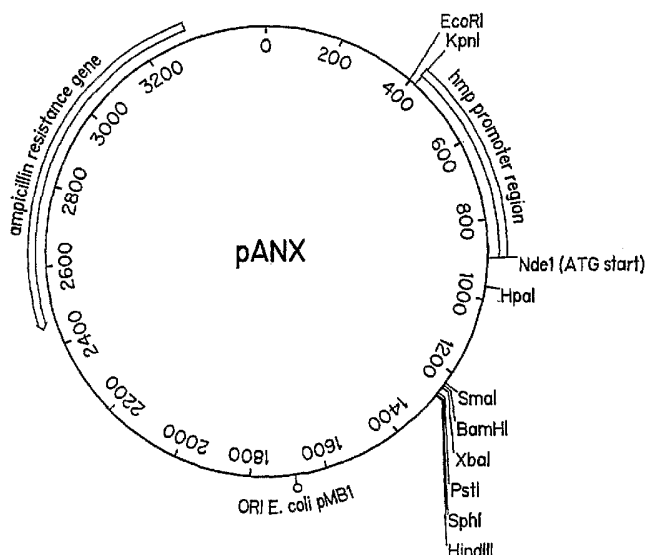
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: VECTOR TO INDUCE EXPRESSION OF RECOMBINANT PROTEINS UNDER ANOXIC OR MICROAEROBIC CONDITIONS



(57) Abstract: A protein expression vector that expresses large quantities of recombinant proteins under anoxic or microaerobic conditions by inducing expression with nitrate. The vector backbone is pUC19 and protein expression is driven by the E. coli flavo-hemoglobin promoter, which is inducible by nitrate, nitrite, or nitric oxide under conditions of low oxygen. The Nde1 site of pUC19 has been destroyed by filling in with Klenow fragment and religating the vector. An Nde1 site in the promoter provides an in-frame start methionine and a standard polylinker is available for ease of subcloning. The vector is named pANX for Plasmid Anaerobic eXpression.

-1-

VECTOR TO INDUCE EXPRESSION OF RECOMBINANT PROTEINS UNDER ANOXIC OR MICROAEROBIC CONDITIONS

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. GM65090 awarded by the National Institutes of Health.

Field of the Invention

The invention is directed to vectors used to express recombinant proteins.

Background

Many commercially important protein products are synthesized in *Escherichia coli* (*E. coli*). The two major goals targeted in cell-based recombinant protein production, in order to maximize protein production, are high cell density and high-level gene expression. Culture performance is optimized when these two goals are simultaneously met. High cell density can be obtained in culture using fed-batch cultivation, in which concentrated medium is gradually fed into a bioreactor, as described in Riesenber, D., and R. Guthke, 1999, *High-cell-density cultivation of microorganisms*. Appl. Microbiol. Biotechnol. 51:422-430, the disclosure of which is incorporated by reference herein in its entirety. High-level protein production in *E. coli* requires strong, inducible promoters that are capable of initiating rapid protein synthesis and that can quickly produce large amounts of protein after induction. Inducible expression vectors including *lac*, *trp*, *tac*, and phage T7 promoters have been successfully used as described in Makrides, S. C., 1996, *Strategies for achieving high-level expression of genes in Escherichia coli*. Microbiol Rev. 60:512-538, the disclosure of which is incorporated by reference herein in its entirety. These vectors rely on aeration throughout the growth and induction phases of culture. Inadequate oxygenation results in poor cell growth and reduced protein yields. Maintaining proper aeration once a culture is dense is difficult and expensive.

Aeration may lead to other problems as well. For example, overexpression of foreign proteins in *E. coli* frequently results in the formation of insoluble inclusion bodies composed of inactive protein as described in Villaverde, A., and M. M. Carrio, 2003, *Protein aggregation in recombinant bacteria: biological role of inclusion bodies*. Biotechnol. Lett. 25:1385-1395, the disclosure of which is incorporated by reference herein in its entirety. The mechanism of inclusion body formation is not completely understood. It is believed that the overexpressed gene products cannot be correctly folded and processed to achieve the native protein structure. Further, it is believed that aeration is a factor in inclusion body formation. For example, NorR is a transcription factor that regulates the expression of the anoxic NorVW nitric oxide reductase in *E. coli*. NorR is normally expressed in low levels. Attempts to overexpress the protein by conventional methods of induction (under conditions of aeration) were unsuccessful due to the fact that the protein contains an oxygen labile heme co-factor. Thus, overexpression of the protein led to formation of insoluble inclusion bodies, substoichiometric heme content, and no active, purifiable protein. Attempts to reduce the formation of inclusion bodies, including increasing the expression of chaparones, and processing proteins, have met with limited success. (See Goloubinoff, P., A. A. Gatenby, and G. H. Lorimer, 1989, GroE *heat-shock proteins promote assembly of foreign prokaryotic ribulose biphosphate carboxylase oligomers in Escherichia coli*. Nature. 337:44-47, and Ostermeier, M., K. DeSutter, and G. Georgiou, 1996, *Euraryatic protein disulfide isomerase complements Eschrichia coli dsbA mutants and increases the yield of a heterologous secreted protein with disulfide bonds*. J Biol Chem. 271:10616-10622, the disclosures of which are incorporated by reference herein in their entireties.)

As can be seen, one drawback to the use of vectors that rely on aeration is that many proteins react unfavorably with molecular oxygen, and thus cannot be expressed in a usable form under conditions of high aeration. As examples of oxygen-reactive proteins, nitric oxide dioxygenases (NODs) catalyze the reaction $\text{NO} + \text{O}_2 + \text{e}^- \rightarrow \text{NO}_3^-$, but can also release toxic superoxide radical and hydrogen peroxide as byproducts. NODs normally provide a free radical defense mechanism by detoxifying nitric oxide (NO). NO is a radical that builds up to toxic amounts when induced by responses to infections, foreign bodies, or tissue injury. At low levels, NO acts as a signal and controls diverse physiological processes including vasotension and O₂ delivery to tissues. NOD protects diverse cells and organisms from NO poisoning, growth inhibition and killing. NOD also modulates NO signaling pathways controlling vasorelaxation.

The structure and enzymatic function of NOD from *E. coli*, a flavohemoglobin-type NOD, has been reported (Gardner et al., Proc. Natl. Acad. Sci. USA 95, 13089 (1998) which is expressly incorporated by reference herein in its entirety). The reaction steps for flavohemoglobin-catalyzed NO dioxygenation incorporate the NADPH, FAD, and O₂ dependence, as well as other features, of the mammalian hemoglobin. Mammalian NOD is a microsomal cytochrome P450 oxidoreductase (EC 1.6.2.4)-driven heme-dependent enzyme (Hallstrom et al. Free Radic. Biol. Med. 37(2) (2004)), which is expressly incorporated by reference herein in its entirety). NODs are examples of proteins that react with oxygen to form toxic superoxide and hydrogen peroxide.

Uses for NOD and other proteins are thus desirable. However, the expression of NOD and other oxygen-sensitive or oxygen-reactive proteins is difficult under conditions of high aeration. Thus, a protein expression vector that expresses large quantities of recombinant proteins under anoxic or microaerobic conditions is desirable.

SUMMARY OF THE INVENTION

In one embodiment, the present invention includes an expression vector for the production of proteins, such as oxygen-sensitive, oxygen-radical-generating, or oxygen-reactive proteins, in *E. coli*. The vector is referred to herein as pANX (Plasmid ANaerobic eXpression). Use of the pANX vector for the expression of proteins occurs under anoxic or microaerobic conditions. Nitrate may be used as both the respiratory substrate to support a high level of growth under low aeration or anoxic conditions and as the inducer of expression from a nitrate inducible promoter from the *hmp* gene (the *E. coli* flavohemoglobin promoter). Protein expression may also be induced by nitrite or nitric oxide. Using the pANX vector, the induction and purification of protein, such as the heme-containing transcription factor NorR in substantial quantities, can be achieved. Of the soluble protein produced using the pANX vector of the present invention, about 20%-30% was heme-containing NorR. This results from the anoxic induction conditions used to induce protein expression from pANX. The pANX vector can also be used to overexpress other proteins, including, but not limited to, the mammalian protein heme oxygenase 1 (HOX-1), the oxygen-sensitive NorV/NorW nitric oxide reductase, *C. albicans* flavohemoglobin, and several site-directed mutants of *E. coli* NOD. Proteins such as flavohemoglobin, which can be produced in a soluble form by conventional expression plasmids, are reliably induced in the pANX vector as well.

Expression from the vector of the present invention can be induced by nitrate under anoxic or microaerobic conditions, because the *hmp* promoter used in the vector is nitrate inducible. (Poole et al., *Nitric Oxide, Nitrite, and Fnr Regulation of hmp (Flavo-hemoglobin) Gene Expression in Escherichia coli K-12*, Journal of Bacteriology, (Sept. 1996), pp. 5487-5492, which is expressly
5 incorporated by reference herein in its entirety). As a result, the present invention eliminates the need to maintain constant aeration, and thus is simpler and less expensive.

The pANX vector includes a modified pUC19 vector and a nitrate-inducible promoter. For example, protein expression is driven by the *E. coli* flavohemoglobin promoter, which is inducible by nitrate under anaerobic and microaerobic conditions. An Nde1 site provides an in-frame start
10 methionine and a polylinker is available for subcloning.

Another embodiment of the invention is a protein expression vector adapted to express recombinant proteins under anoxic conditions.

The protein expressed may include, but is not limited to, NOD, NorR, NorV, or HOX-1.

Another embodiment of the invention is a protein expression vector adapted to express
15 recombinant proteins under microaerobic conditions. The protein expressed may include, but is not limited to, NOD, NorR, NorV, or HOX-1.

Another embodiment of the invention is a method of expressing a recombinant protein under anoxic conditions. The method includes introducing a gene coding for a particular protein into a vector, and expressing the protein in a host cell by inducing expression with nitrate, nitrite, or nitric
20 oxide.

Another embodiment of the invention is a method of expressing a recombinant protein under microaerobic conditions. The method includes introducing a gene coding for a particular protein into a vector, and expressing the protein in a host cell by inducing expression with nitrate.

Another embodiment of the invention is a method of expressing a recombinant protein
25 under microaerobic conditions. The method includes introducing a gene coding for a particular protein into a vector, and expressing the protein in a host cell by inducing expression with nitrite or nitric oxide.

Another embodiment of the invention is a protein expression vector including a nitrate-inducible promoter for anoxic or microaerobic expression of proteins.

Another embodiment of the invention is a method for expressing proteins in a vector including the use of a nitrate-inducible promoter for anoxic or microaerobic expression of proteins. In one embodiment, the *hmp* promoter is used.

Another embodiment of the invention is a method for expressing oxygen-sensitive, oxygen-radical-generating, or oxygen-reactive proteins in a host by providing to a pUC vector a nitrite-inducible promoter with an insert of DNA sufficient to encode a functional oxygen-sensitive, oxygen-radical-generating, or oxygen-reactive protein, and providing nitrite to induce the promoter, thereby expressing the oxygen-sensitive, oxygen-radical-generating, or oxygen-reactive protein.

Another embodiment of the invention is a pUC plasmid containing an insert of a promoter inducible by at least one of nitrate, nitrite, or nitric oxide, thereby forming a modified pUC plasmid. This modified pUC plasmid induces expression of an inserted gene encoding oxygen-sensitive, oxygen-reactive, or oxygen-radical-generating proteins under at least microaerobic conditions.

Another embodiment of the invention is an isolated inducible promoter including a fragment of the 812 bp *E. coli* flavohemoglobin upstream region operably linked to a nitric oxide dioxygenase coding region. The promoter fragment is sufficient to induce expression of a gene encoded therein by at least one of nitrate, nitrite, or nitric oxide.

Another embodiment of the invention is a method of generating a nitrate inducible expression vector by inserting an expression-inducing fragment of an *E. coli* flavohemoglobin upstream region and coding region for nitric oxide dioxygenase into a pUC vector, thereby forming a modified pANX vector; ligating into the pANX vector a DNA sequence encoding at least one of an oxygen-sensitive, oxygen-radical-generating, or oxygen-reactive protein; transforming a bacterial host with the ligated pANX vector; and selecting colonies that contain the ligated pANX vector.

Another embodiment of the invention is an *E. coli* transformed with the pANX vector.

These and other advantages will be apparent in light of the following figures and detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram of an embodiment of the pANX vector;

Fig. 2A shows the induction of NOD expression using the pANX vector, with Lane 1 depicting molecular weight markers in kD, Lane 2 depicting pANX, and Lane 3 depicting pANXNOD;

Fig. 2B shows the induction of NorR expression using the pANX vector, with Lane 1 depicting pANX, Lane 2 depicting pANXNORR, and Lane 3 depicting molecular weight markers in kD;

Fig. 2C shows the induction of NorV expression using the pANX vector, with Lane 1 depicting molecular weight markers in kD, Lane 2 depicting pANX, and Lane 3 depicting pANXNORV;

5 and

Fig. 2D shows the induction of HOX-1 expression using the pANX vector, with Lane 1 depicting pANX, Lane 2 depicting pANXHOX-1, and Lane 3 depicting molecular weight markers in kD.

DETAILED DESCRIPTION

The present invention includes an expression vector for the production of oxygen-sensitive or reactive-oxygen (e.g., superoxide radical) generating proteins in *E. coli*. The vector is referred to as pANX herein (Plasmid ANaerobic eXpression). Use of the pANX vector for the expression of proteins occurs under anoxic (i.e., anaerobic) or microaerobic conditions. "Anoxic" and "anaerobic" refer to conditions in which oxygen is absent. "Microaerobic" refers to conditions with suboptimal oxygen. Nitrate can be used as both the respiratory substrate to support a high level of growth under low aeration (i.e., microaerobic) or anoxic conditions and as the inducer of expression from a nitrate inducible promoter. An example of such a promoter is from the *hmp* gene (the *E. coli* flavohemoglobin promoter).

The protein expression vector of the present invention is a plasmid. Plasmids are naturally occurring accessory chromosomes in a host, i.e., naturally occurring circular DNA molecules that carry genes for antibiotic inactivation, toxin production, and/or breakdown of natural products. These plasmids replicate independently of the host chromosome.

As is well known to those skilled in the art, particular plasmids to express a target protein may be created through modification of existing plasmids. In general, plasmids are modified by cleaving the circular DNA using endonucleases, and DNA fragments can then be inserted. A particular organism, such as *E. coli*, takes up the plasmid. Insertion of DNA from another source into the cloning site is confirmed by PCR (polymerase chain reaction) or DNA sequencing. *E. coli* provides the transcriptional and translational machinery to express the protein encoded in the insert of the plasmid.

The pANX protein expression vector described herein is a modified pUC19 vector. The base plasmid vector pUC19 contains a polylinker with unique cloning sites for multiple restriction nucleases and an ampicillin resistance gene to permit identification of transformed cells. More

specifically, the base pUC19 vector is a small, high-copy number *E. coli* plasmid that is 2,686 base pairs in length. The base pUC19 plasmid contains: (1) the pMB1 replicon *rep* responsible for the replication of plasmid (the high-copy number of pUC plasmids is a result of the lack of the *rop* gene and a single point mutation in *rep* of pMB1); (2) the *bla* gene, coding for β -lactamase that confers resistance to ampicillin (the source being plasmid pBR322, but differing from pBR322 by two point mutations); (3) a region of the *E. coli* operon *lac*-containing CAP protein binding site, a promoter P_{lac} , a *lac* repressor binding site, and a 5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of β -galactosidase.

To allow for the expression of protein under anoxic or microaerobic conditions, the pANX vector of the present invention includes a nitrate-inducible promoter in addition to the base pUC19 vector. Unless specifically differentiated, a nitrate-inducible promoter also encompasses nitrite and nitric oxide inducible promoters. In particular, the *hmp* promoter of the flavohemoglobin gene is used as the nitrate-inducible promoter. As described above, the *E. coli* flavohemoglobin gene (*hmp*) encodes a nitric oxide dioxygenase (NOD) that converts nitric oxide (NO) to nitrate, as described in Gardner, P. R., A. M. Gardner, L. A. Martin, and A. L. Salzman 1998 *Nitric oxide dioxygenase: an enzymic function for flavohemoglobin*. Proc Natl Acad Sci U S A. 95:10378-10383, the disclosure of which is incorporated by reference herein in its entirety. NOD functions to detoxify NO generated as an intermediate in nitrogen metabolism or from exposure to the immune system. NOD (flavohemoglobin) is rapidly and strongly induced under both aerobic and anoxic exposure to NO, as described in Poole, R. K., M. F. Anjum, J. Membrillo-Hernández, S. O. Kim, M. N. Hughes, and V. Stewart. 1996. *Nitric oxide, nitrite, and Fnr regulation of hmp (flavohemoglobin) gene expression in Escherichia coli K-12*. Journal of Bacteriology, 178:5487-5492, the disclosure of which is incorporated by reference herein in its entirety.

In vivo, the *hmp* promoter responds to anoxic nitrate and nitrite in addition to NO. (See Poole, R. K., et al.) Thus, the present invention includes a nitrate inducible expression plasmid using the upstream promoter region of *hmp*. Expression of oxygen-sensitive proteins would be facilitated by induction under conditions of low oxygen, or in the absence of oxygen. With reference to Fig. 1, in one embodiment the pANX vector of the present invention includes 812 bp of the *E. coli* flavohemoglobin upstream region and a portion of the NOD coding region cloned into pUC19. In alternate embodiments, the pANX vector may include less than 812 bp, such as a 244 bp sequence [SEQ. ID. NO. 6], and may

include coding regions for genes of interest other than the region coding for NOD. Functionally, the DNA for the promoter sequence must include a nitrate-, nitrite-, or nitric oxide-inducible sequence, -35 and -10 bp RNA polymerase binding sites, and a ribosome-binding site. For example, all of these elements are present in the 244 bp sequence of SEQ. ID. NO. 6. In the pANX vector, the Nde1 site of pUC19 has been destroyed by filling in with Klenow fragment and religating the vector. Genes of interest are directionally cloned into pANX using a 5' Nde1 site in the promoter region and any convenient 3' polylinker site. A start methionine is encoded in the Nde1 site. Plasmids are maintained in the culture by ampicillin selection.

The 812 bp region in the pANX vector of Fig. 1 includes the *hmp* promoter (480 bp) and a partial NOD coding region (335 bp). The vector, in this illustrated embodiment, is constructed generally by subcloning the promoter and part of the NOD coding region into pUC 19. There is a common ATG sequence coding for methionine that overlaps in the sequences of the promoter and the NOD coding region. In alternate embodiments, to express other proteins, the NOD coding region is replaced with a region coding for an alternate protein. The ATG sequence would be part of any other gene inserted in the vector. The ATG is part of the Nde1 restriction site, which is where other genes are inserted in-frame in the vector.

In other embodiments, the promoter region may include a smaller fragment than the 480 bp fragment discussed above. In general, any fragment that codes for inducibility via nitrate, nitrite, or nitric oxide would be sufficient. In order to produce such a fragment, one skilled in the art could first linearize the plasmid using Eco R1 restriction enzyme. Next, one would generate a nested series of deletions extending into the promoter, using, for example, a Bal31 exonuclease digestion. These shorter sequences would then be cloned into a reporter vector that lacks a promoter and assayed for inducibility of the reporter gene by nitrate, nitrite, or nitric oxide. Once the minimal length inducible sequence is determined, then nucleotides within this region would be randomly mutated by error-prone PCR ("EP-PCR") to identify which nucleotides are essential and which are not essential for such inducibility. Briefly, sense and antisense primers are generated to the 5' and 3' ends of the DNA promoter segment. The average number of mutations per DNA fragment is controlled as a function of the number of EP-PCR doublings performed. The PCR fragments are subcloned into the reporter vector and screened for inducibility by nitrate, nitrite, or nitric oxide. These techniques are described in

Current Protocols in Molecular Biology On Line (2004) FM Ausubel, ed. Wiley Intersciences, USA, which is incorporated by reference herein in its entirety.

Restriction endonuclease-digested DNA fragments are ligated into the pANX-cloning vector that has been cleaved with restriction endonucleases to create ends that will bind with the fragments containing the gene of interest. The ligation mixture is then used to transform an appropriate host, such as an *E. coli* strain. Colonies containing recombinant vectors are screened by PCR and confirmed with DNA sequencing.

In particular, an aerobic culture is used to start pANX expression cultures. Once the pANX plasmid containing the target gene is transformed into any common *E. coli* strain, the culture is grown aerobically to mid log phase in Luria Broth containing ampicillin. Luria Broth contains 10 g Bacto-Tryptone, 5 g yeast extract, 10 g NaCl prepared in 1 liter of deionized water, pH 7.2, and autoclaved for 60 minutes. Luria Broth is commercially available from Difco Laboratories of Detroit, Michigan. Aerobic growth represses expression of the target protein and allows for a rapid growth rate of healthy cells.

To induce proteins from pANX, the starter culture is inoculated into nitrate-containing modified TB medium (Terrific Broth) (8 g Bacto-Tryptone, 24 g yeast extract, 5.04 g glycerol prepared in 900 ml deionized water; it is autoclaved 60 min., cooled, and 100 ml of filter sterilized 23.1 g/L KH_2PO_4 (anhydrous), 125.4 g/L K_2HPO_4 (anhydrous) supplemented with 10mM nitrate, 150 $\mu\text{g}/\text{ml}$ ampicillin is added in 1L flasks (Pyrex # 4980) at an OD 0.01). Terrific Broth is commercially available from Difco Laboratories of Detroit, Michigan. Because nitrate is used as an anoxic respiratory substrate, high levels of glucose are not required to support culture growth. It has been determined that glucose is actually inhibitory to protein induction from the pANX vector and thus the growth medium has been modified to contain less glucose by decreasing the amount of Bacto-Tryptone. Growth on glucose can also result in the production of toxic levels of acetate and other acids once oxygen saturation is reduced. Anoxic respiratory growth on nitrate eliminates this problem and avoids the addition of costly induction chemicals such as IPTG.

In particular, the cultures were grown at 37°C with low shaking until saturation was reached. Growth and induction in static versus low aeration cultures was comparable (20-50% protein of interest). However, the final cell density was greater under microaerobic conditions (OD 2-4 for low aeration and OD 1.5-2 for static). Higher aeration (>200 RPM) resulted in little protein induction. The degree of aeration is directly proportional to the speed of shaking. Thus, increasing the speed of

shaking in revolutions per minute increases the aeration. Cultures were harvested with minimal oxygen exposure and frozen. Cell pellets were lysed and the target protein was purified from the soluble protein fraction by standard means known to one skilled in the art.

The pANX vector was used to produce recombinant proteins that were sensitive to oxygen, as will be described further below and in the Examples.

Because of its cytotoxicity, an organism will metabolize NO. In *E. coli*, NO exposure induces metabolism in two ways: (1) an aerobic mechanism which induces NOD (encoded by the *hmp* gene); (2) and an anoxic mechanism, which induces NO reductase (containing two iron atoms and encoded by two genes: NorV and NorW genes). The NorV and NorW genes are transcriptionally controlled by an adjacent σ^{54} dependent transcription regulator called NorR, which is a heme protein.

More specifically, the NorV/W genes encode a novel diiron-containing NO reductase. In *E. coli*, NO is detoxified by an O₂-independent NO reductase encoded by the NorV (flavorubredoxin) and NorW (NADH: flavorubredoxin oxidoreductase) genes arranged in a bicistronic operon and controlled by the adjacent σ^{54} -RNA polymerase-dependent regulator, NorR. *E. coli* NorR has been overexpressed and purified using the pANX vector. Based on its electronic absorption spectra, NorR is a heme protein. Ferrous NorR shows a Soret peak at 424nm and α and β maxima at 557nm and 531nm, respectively. Oxidized NorR has a Soret peak at 412nm and a broad band at 534nm. These spectra are consistent with low-spin, hexacoordinate heme. NO binds ferrous NorR to form a five-coordinate nitrosyl ferrous heme, similar to the NO sensing soluble guanylate cyclase of mammalian cells. Although CO binds heme, only NO is physiologically active. While not being bound by any theory, the spectrum of CO bound NorR is similar to the CO sensing transcription factor CooA of *Rhodospirillum rubrum*. NorR contains a His111-X-Cys113 site reminiscent of the Cys75-X-His77 heme iron ligand-switch motif in CooA. It is believed that NorR senses NO through its heme and transduces this signal through structural changes resulting from the switch from hexacoordinate to pentacoordinate heme. This alters the conformation of the σ^{54} interacting domain and activates transcription of the NorV/W genes. NorR orthologs are found adjacent to NorV/W in *S. typhimurium*, *K. pneumoniae*, and *S. flexneri*, and adjacent to *hmp* in *P. aeruginosa*, *V. cholera*, and *B. fungorum*, suggesting a central role for NorR in bacterial NO stress responses.

In summary, the present invention includes a protein expression vector (pANX) and an expression system that is nitrate-inducible under anoxic conditions or conditions of low oxygen. The low

oxygen growth conditions favor enhanced production and solubility of oxygen-sensitive proteins. Since the *hmp* promoter used in the pANX vector can be maximally induced under anoxic or microaerobic conditions, cells are grown rapidly under aerobic conditions, and then the promoter is induced by anoxic or microaerobic growth on nitrate. Thus, nitrate serves simultaneously as inducer, respiratory substrate, and energy source. Due to its low cost and lack of toxicity compared to IPTG, nitrate may provide an inexpensive alternative for the induction of *hmp* promoter mediated heterologous gene expression. In addition, induction under low oxygen eliminates the complications and expense of maintaining sufficient oxygenation in dense cultures of *E. coli*. Proteins, such as NOD, that constitutively produce damaging oxygen radicals, such as hydrogen peroxide or superoxide, can be expressed by this system. Proteins sensitive to oxygen damage are also successfully expressed from pANX. Although this procedure can be used for laboratory scale recombinant protein production, the production of other proteins of commercial interest will also be improved by this system and it can be adapted to industrial production using methods known to one skilled in the art. In particular, this is performed by an increase in the scale of production. At laboratory scale, 5 ml to 1 liter cultures may be used. In one embodiment, eight 1-liter batches may be used, from which a final yield of 50 mg to 1 g of greater than 90% pure protein may be expected.

As described above, pANX is a derivative of pUC19 in which a gene of interest, such as the NOD upstream region, has been inserted in the polylinker at the *Sma*I site. Protein expression is driven by the *E. coli* flavohemoglobin promoter. The sequence of one embodiment of this pANX vector (coding for NOD) is shown in SEQ. ID. NO. 1.

The sequence of the base pUC19 vector is shown in SEQ. ID. NO. 2.

The sequence of the NOD coding region is shown in SEQ. ID. NO. 3.

The sequence of the polylinker region is shown in SEQ. ID. NO. 4.

The sequence of the *hmp* promoter region is shown in SEQ. ID. NO. 5.

The sequence of a 244 bp fragment of the *hmp* promoter region is shown in SEQ. ID. NO. 6.

The principles of the present invention will be more apparent with reference to the following Examples.

Example 1: Protein Expression Using the pANX Vector

The pANX vector, in this embodiment, is a derivative of pUC19 in which the NOD upstream region (812 bp *PvuII* to *SmaI* fragment) was inserted in the polylinker at the *SmaI* site. The *NdeI* site of pUC19 was destroyed by filling in with Klenow fragment and religating. A start methionine was encoded in the *NdeI* site in the NOD promoter. The NOD promoter can be induced to high level of expression with 10 mM nitrate in low aeration or anaerobic cultures.

Cultures of *E. coli* AB1157 with various protein coding regions subcloned in pANX were grown aerobically to OD₅₅₀ 0.6 in LB (Luria Broth) with 50 µg/ml ampicillin. The cultures were used to inoculate 900 ml of modified Terrific Broth to an optical density of 0.01 at 550 nm. Beef liver catalase (Roche) 260 U/ml final was added for protection of proteins sensitive to H₂O₂ damage. The cultures were grown overnight at 37°C with slow shaking (~100 revolutions per minute) in a rotary shaker-water bath. Cells were harvested by centrifugation and washed in 50 mM Tris-HCl, pH 8.0 at 4°C, 1mM EDTA, with catalase 260 U/ml (Buffer A). The cell pellets were frozen on dry ice and stored at -70°C.

For analysis of soluble protein expression, cell pellets were thawed at 37°C and lysed by sonication in chilled Buffer A at a cell volume:buffer ratio of about 1:4. The extracts were clarified by centrifugation >10,000 X g for 30 min. Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

For gel analysis of protein induction, lysates were separated by SDS-PAGE on 8-16% gradient gels (Invitrogen) and proteins were visualized by Coomassie blue staining. Protein expression was quantitated by densitometric scanning of the gels.

Example 2: Induction of NOD

Referring to Fig. 2A, the coding region for NOD was subcloned in pANX, cultures were grown, and protein expression induced as described in Example 1. Induction and purification of NOD led to high yields, such as 50-200 mg of 90% pure protein from a 12-liter batch. NOD produced a low level of superoxide from bound oxygen and certain site-directed mutations in the NOD active site increased constitutive superoxide radical and hydrogen peroxide production to toxic levels. These proteins can only be efficiently produced under conditions of low aeration where superoxide radical and hydrogen peroxide production is limited.

Example 3: Induction of NorR

Referring to Fig. 2B, the coding region for NorR was subcloned in pANX, cultures were grown, and protein expression induced as described in Example 1. In the present invention, pANX was used to express the full-length NorR transcription factor in high yield, such as 50 mg of 90% pure protein from an 8-liter batch and in a soluble form (See Fig. 2B).

Example 4: Induction of NorV

The oxygen-sensitive diiron protein NorV was also expressed using pANX. The coding region for NorV was subcloned in pANX, cultures were grown, and protein expression induced as described in Example 1. Expression of NorV from pANX resulted in a high yield of soluble active protein (see Fig. 2C), such as 500 mg-1 g of 90% pure protein from an 8-liter batch. Loss of diiron resulted in inactivation of the NorV protein, when it was expressed under aerobic conditions. The NorV protein purified from nitrate induced low aeration cultures retained substantial iron and remained soluble.

Example 5: Induction of HOX-1

Referring to Fig. 2D, the coding region for human HOX-1 (heme oxygenase) was subcloned in pANX, cultures were grown, and protein expression induced as described in Example 1. Soluble HOX-1 was obtained in significant yield, such as 50-100 mg- of 90% pure protein from an 8-liter batch.

Other variations or embodiments of the invention will also be apparent to one of ordinary skill in the art from the above figures and descriptions. Thus, the forgoing embodiments are not to be construed as limiting the scope of this invention.

What is claimed is:

1. A vector to express recombinant proteins, comprising the nucleic acid sequence SEQ. ID. NO. 2, wherein said proteins are expressed under microaerobic or anoxic conditions.
2. The vector of claim 1, further comprising a promoter inducible by nitrate, nitrite, or nitric oxide.
3. The vector of claim 2, wherein said promoter comprises the nucleic acid sequence SEQ. ID. NO. 5.
4. The vector of claim 2, wherein said promoter comprises the nucleic acid sequence SEQ. ID. NO. 6.
5. The vector of claim 1, wherein said proteins are expressed in *E. coli*.
6. The vector of claim 5, wherein expression of said proteins is induced by a nitrate-, nitrite-, or nitric oxide-inducible *hmp* promoter of the flavohemoglobin gene of *E. coli*.
7. The vector of claim 1, wherein said proteins are oxygen-sensitive or oxygen-reactive proteins.
8. The vector of claim 7, wherein said oxygen-sensitive and oxygen-reactive proteins are selected from the group consisting of heme-oxygenase I, NorV/NorW nitric oxide reductase, *C. albicans* flavohemoglobin, a site-directed *E. coli* flavohemoglobin, and NorR transcription factor.
9. A vector to express recombinant proteins under microaerobic or anoxic conditions, said vector including a promoter that is inducible by nitrate, nitrite, or nitric oxide.
10. The vector of claim 9, wherein said promoter comprises the nucleic acid sequence SEQ. ID. NO. 5.
11. The vector of claim 9, wherein said promoter comprises the nucleic acid sequence SEQ. ID. NO. 6.

12. The vector of claim 9, wherein said proteins are expressed in *E. coli*.
13. The vector of claim 12, wherein said promoter is of the flavohemoglobin gene of *E. coli*.
14. The vector of claim 9, for use in expressing oxygen-sensitive or oxygen-reactive proteins.
15. The vector of claim 14, wherein said oxygen-sensitive and oxygen-reactive proteins are selected from the group consisting of heme-oxygenase I, NorV/NorW nitric oxide reductase, *C. albicans* flavohemoglobin, a site-directed *E. coli* flavohemoglobin mutant, flavohemoglobin, and NorR transcription factor.
16. A method of expressing a recombinant protein under anoxic or microaerobic conditions, comprising:
 - introducing a gene coding for a protein of interest into a vector capable of inducing expression of said protein of interest under anoxic or microaerobic conditions, wherein said vector comprises the nucleic acid sequence SEQ. ID. NO. 2; and
 - expressing said protein of interest in a host cell by inducing expression of said protein of interest with at least one of nitrate, nitrite, or nitric oxide.
17. The method of claim 16, said vector further comprising a promoter inducible by nitrate, nitrite, or nitric oxide, said promoter comprising the nucleic acid sequence SEQ. ID. NO. 5.
18. The method of claim 16, said vector further comprising a promoter inducible by nitrate, nitrite, or nitric oxide, said promoter comprising the nucleic acid sequence SEQ. ID. NO. 6.
19. A method of generating a nitrate inducible expression vector comprising:
 - inserting an expression-inducing fragment of an *E. coli* flavohemoglobin promoter into a pUC vector, thereby forming a pANX vector;
 - ligating into the pANX vector a DNA sequence encoding at least one of an oxygen-sensitive, oxygen-radical-generating, or oxygen-reactive protein;
 - transforming a bacterial host with the ligated pANX vector; and

selecting colonies that contain the ligated pANX vector.

20. The method of claim 19, further comprising reducing inclusion bodies in recombinant proteins by expressing recombinant proteins in said pANX vector under anoxic or microaerobic conditions, wherein said promoter is inducible by at least one of nitrate, nitrite, and nitric oxide.

1/3

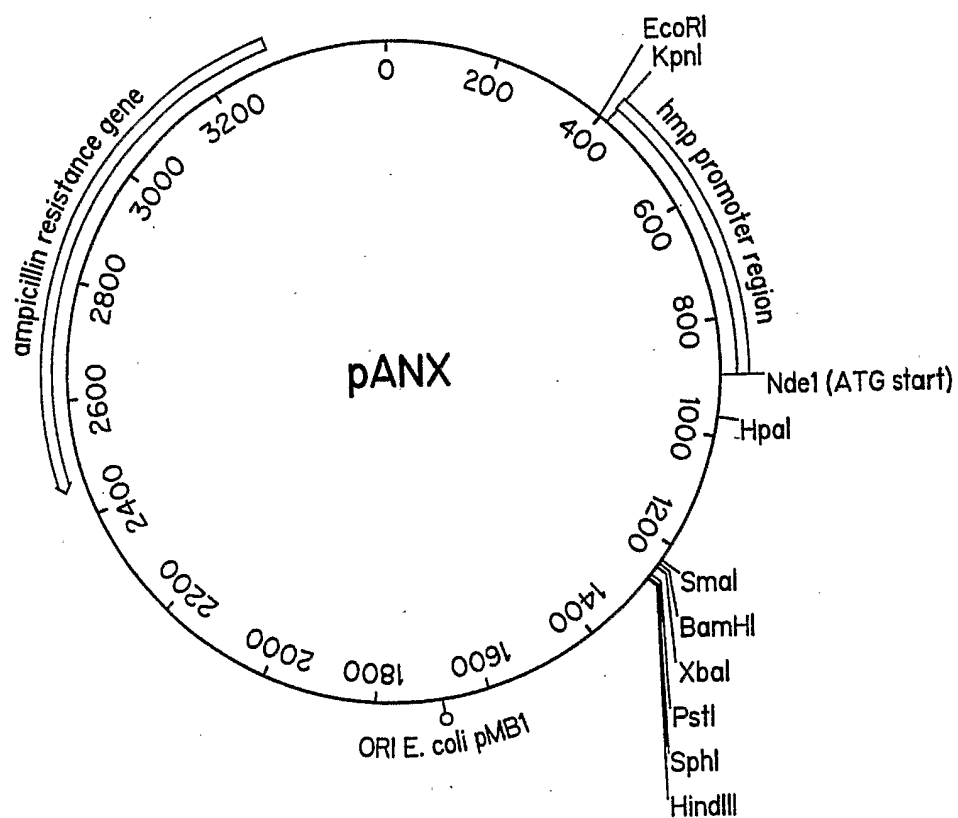


FIG. 1

2/3

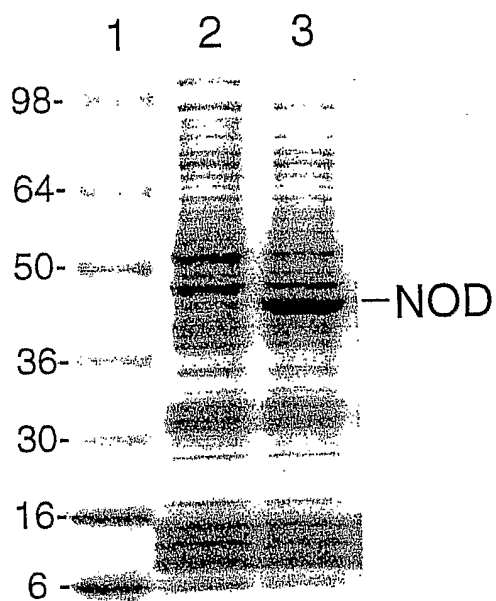


FIG. 2A

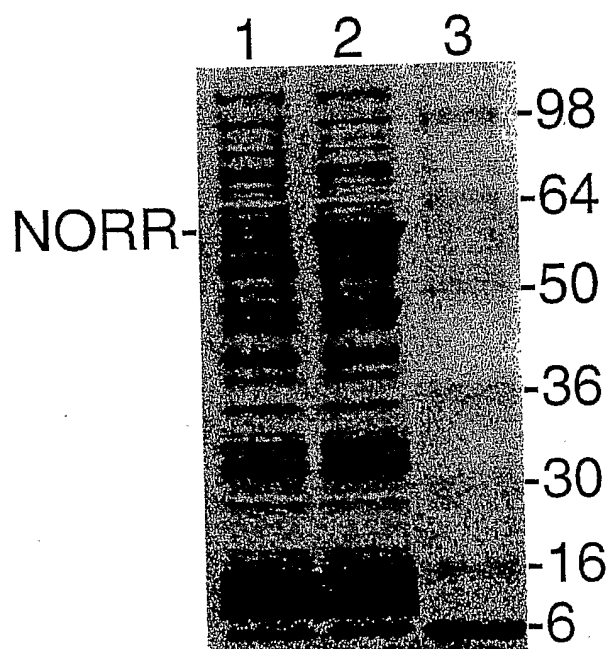


FIG. 2B

3/3

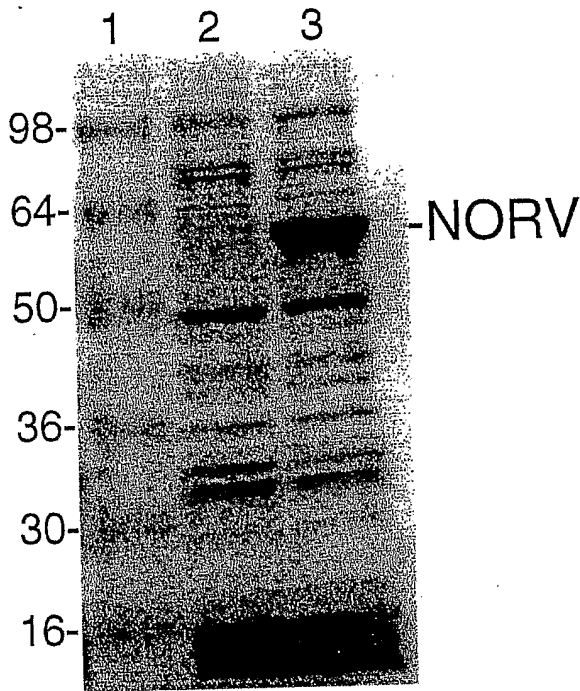


FIG. 2C

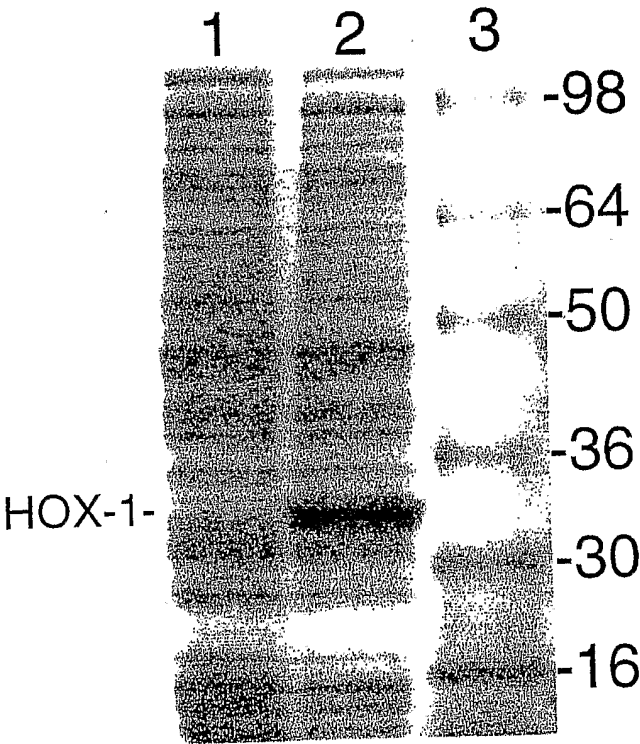


FIG. 2D

Sequence Listing
SEQUENCE LISTING

<110> Children's Hospital Medical Center

<120> Vector to Induce Expression of Recombinant Proteins Under Anoxic or Microaerobic Conditions

<130> CMC-168WO

<150> 60/645,251

<151> 2005-01-19

<160> 6

<170> PatentIn version 3.3

<210> 1

<211> 3503

<212> DNA

<213> Homo sapiens

<400> 1

tcgcgcgttt cggatgatgac ggtgaaaacc tctgacacat gcagctcccg gagacgggtca	60
cagcttgtct gtaagcggat gccgggagca gacaagcccg tcagggcgcg tcagcgggtg	120
ttggcgggtg tcggggctgg cttaactatg cggcatcaga gcagattgta ctgagagtgc	180
accatatatg cgggtgtgaaa taccgcacag atgcgtaagg agaaaatacc gcatcaggcg	240
ccattcgcca ttcaggctgc gcaactgttg ggaagggcga tcggtgcggg cctcttcgct	300
attacgccag ctggcgaaag ggggatgtgc tgcaaggcga ttaagttggg taacgccagg	360
gttttcccag tcacgacgtt gtaaaacgac ggccagtga ttcgagctcg gtaccctga	420
gaaccctgcg cctgcattac gcgcgggctg gtgtagtttt cggaggcgat cagttcgatg	480
tgctcttcct gacgtacttt ttcctgctcc atagcctgcc acagttcggc atcataatcg	540
gcaatgttca tttcacgctt taacatccgc atctcctgac tcagctaaca ataaaatatt	600
tggcctttat aggcggtcct gttggacaac ggcgaacagt ataaccgaat cattgtgcga	660
taacaggtct tgacaaagga atttacgcaa acgattacct tcaggctacg caaggctttg	720
gagaataaag agcttgcaac cggaacgga tttcttttca ggtttgtgat gcaaattttt	780
cacttcatca cattctttct gaaaaacacc aaagaacat ttacattgca gggctatttt	840
ttataagatg catttgagat acatcaatta agatgcaaaa aaaggaagac catatgcttg	900
acgtcctaac catcgctaca gtaaaagcca ccatcccttt actggtggaa acggggccaa	960
agttaaccgc ccatttctac gaccgtatgt ttactcataa cccagaactc aaagaaattt	1020
ttaacatgag taaccagcgt aatggcgatc aacgtgaagc cctgtttaac gctattgccg	1080
cctacgccag taatattgaa aacctgcctg cgctgctgcc agcggtagaa aaaatcgcgc	1140
agaagcacac cagcttccag atcaaaccgg aacagtacaa catcgtcggg gaacacctgt	1200
tggcaacgct ggacgaaatg ttcagcccgg gggggatcct ctagagtcga cctgcaggca	1260

Sequence Listing

tgcaagcttg gcgtaatcat ggtcatagct gtttcctgtg tgaaattggt atccgctcac	1320
aattccacac aacatacgag ccggaagcat aaagtgtaaa gcctgggggtg cctaattgagt	1380
gagctaactc acattaattg cgttgcgctc actgcccgtt ttccagtcgg gaaacctgtc	1440
gtgccagctg cattaatgaa tcggccaacg cgcggggaga ggcggtttgc gtattgggcg	1500
ctcttccgct tcctcgctca ctgactcgct gcgctcggtc gttcggctgc ggcgagcggt	1560
atcagctcac tcaaaggcgg taatacgggt atccacagaa tcaggggata acgcaggaaa	1620
gaacatgtga gcaaaaggcc agcaaaaggc caggaaccgt aaaaaggccg cgttgctggc	1680
gtttttccat aggctccgcc cccctgacga gcatcacaaa aatcgacgct caagtcagag	1740
gtggcgaaac ccgacaggac tataaagata ccaggcggtt cccctggaa gctccctcgt	1800
gcgctctcct gttccgaccc tgccgcttac cggatacctg tccgcctttc tcccttcggg	1860
aagcgtagcg ctttctcaat gctcacgctg taggtatctc agttcggtgt aggtcggtcg	1920
ctccaagctg ggctgtgtgc acgaaccccc cgttcagccc gaccgctgcg ccttatccgg	1980
taactatcgt cttgagtcca acccggtgaag acacgactta tcgccactgg cagcagccac	2040
tggtaacagg attagcagag cgaggtatgt aggcggtgct acagagttct tgaagtgggtg	2100
gcctaactac ggctacacta gaaggacagt atttggtatc tgcgctctgc tgaagccagt	2160
taccttcgga aaaagagttg gtagctcttg atccggcaaa caaaccaccg ctggtagcgg	2220
tggttttttt gtttgcaagc agcagattac gcgcagaaaa aaaggatctc aagaagatcc	2280
tttgcattct tctacggggt ctgacgctca gtggaacgaa aactcacggt aagggatttt	2340
ggtcatgaga ttatcaaaaa ggatcttcac ctatagctct ttaaattaaa aatgaagttt	2400
taaatcaatc taaagtatat atgagtaaac ttggtctgac agttaccaat gcttaatcag	2460
tgaggcacct atctcagcga tctgtctatt tcgttcatcc atagttgcct gactccccgt	2520
cgtgtagata actacgatac gggagggtct accatctggc ccagtgctg caatgatacc	2580
gcgagacca cgctcaccgg ctccagattt atcagcaata aaccagccag ccggaagggc	2640
cgagcgcaga agtggctctg caactttatc cgctccatc cagtctatta attgttgccg	2700
ggaagctaga gtaagtagtt cgccagttaa tagtttgctc aacgttggtt ccattgctac	2760
aggcatcgtg gtgtcacgct cgtcgtttgg tatggcttca ttcagctccg gttccaacg	2820
atcaaggcga gttacatgat ccccatgtt gtgcaaaaaa gcggttagct ccttcggtcc	2880
tccgatcgtt gtcagaagta agttggccgc agtggttatc ctcatggta tggcagcact	2940
gcataattct cttactgtca tgccatccgt aagatgcttt tctgtgactg gtgagtactc	3000
aaccaagtca ttctgagaat agtgtatgcg gcgaccgagt tgctcttgcc cggcgtcaat	3060
acgggataat accgcgccac atagcagaac tttaaaagtg ctcatcattg gaaaacgttc	3120

Sequence Listing

ttcggggcga aaactctcaa ggatcttacc gctgttgaga tccagttcga tgtaaccac	3180
tcgtgcaccc aactgatctt cagcatcttt tactttcacc agcgtttctg ggtgagcaaa	3240
aacaggaagg caaatgccg caaaaaaggg aataaggcg acacggaaat gttgaatact	3300
catactcttc ctttttcaat attattgaag catttatcag ggttattgtc tcatgagcgg	3360
atacatattht gaatgtattht agaaaaataa acaaataggg gttccgcgca catttccccg	3420
aaaagtgcc cctgacgtct aagaaacat tattatcatg acattaacct ataaaaatag	3480
gcgtatcacg aggccctttc gtc	3503

<210> 2
 <211> 2686
 <212> DNA
 <213> Homo sapiens

<400> 2	
gcgccaata cgcaaaccgc ctctccccgc gcgttgccg attcattaat gcagctggca	60
cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaatg tgagttagct	120
cactcattag gcacccagg ctttacactt tatgcttccg gctcgtatgt tgtgtggaat	180
tgtgagcgga taacaatttc acacaggaag cagctatgac catgattacg ccaagcttgc	240
atgcctgcag gtcgactcta gaggatcccc gggtagcgag ctgcaattca ctggccgctc	300
ttttacaacg tcgtgactgg gaaaaccctg gcgttaccca acttaatcgc cttgcagcac	360
atcccccttt cgccagctgg cgtaatagcg aagaggcccg caccgatcgc ccttcccaac	420
agttgcgag cctgaatggc gaatggcgcc tgatgcggta ttttctcctt acgcatctgt	480
gcggtatttc acaccgata tgggtgactc tcagtacaat ctgctctgat gccgcatagt	540
taagccagcc ccgacacccg ccaacacccg ctgacgcgcc ctgacgggct tgtctgtctc	600
cggcatccgc ttacagacaa gctgtgaccg tctccgggag ctgcatgtgt cagaggtttt	660
caccgtcatc accgaaacgc gcgagacgaa agggcctcgt gatacgcta tttttatagg	720
ttaatgtcat gataataatg gtttcttaga cgtcaggtgg cacttttcgg ggaaatgtgc	780
gcggaacccc tatttgttta tttttctaaa tacattcaaa tatgtatccg ctcatgagac	840
aataaccctg ataaatgctt caataatatt gaaaaaggaa gagtatgagt attcaacatt	900
tccgtgtcgc cttattccc ttttttgagg cattttgcct tcctgttttt gctcaccag	960
aaacgctggg gaaagtataa gatgctgaag atcagttggg tgcacgagt ggttacatcg	1020
aactggatct caacagcggg aagatccttg agagttttcg ccccgaagaa cgttttccaa	1080
tgatgagcac ttttaaagtt ctgctatgtg gcgcggtatt atcccgtatt gacgccgggc	1140
aagagcaact cggtcgccgc atacactatt ctcaaatga cttggttgag tactcaccag	1200
tcacagaaaa gcatcttacg gatggcatga cagtaagaga attatgcagt gctgccataa	1260

Sequence Listing

```

ccatgagtga taacactgcg gccaaacttac ttctgacaac gatcggagga ccgaaggagc 1320
taaccgcttt ttgacacaac atggggggatc atgtaactcg ccttgatcgt tgggaaccgg 1380
agctgaatga agccataacca aacgacgagc gtgacaccac gatgcctgta gcaatggcaa 1440
caacgttgcg caaactatta actggcgaac tacttactct agcttcccgg caacaattaa 1500
tagactggat ggaggcggat aaagttgcag gaccacttct gcgctcggcc cttccggctg 1560
gctgggtttat tgctgataaa tctggagccg gtgagcgtgg gtctcgcggg atcattgcag 1620
cactggggcc agatggtaag ccctcccgtc tcgtagttat ctacacgacg gggagtcagg 1680
caactatgga tgaacgaaat agacagatcg ctgagatagg tgccctactg attaagcatt 1740
ggtaactgtc agaccaagtt tactcatata tacttttagat tgatttaaaa cttcatTTTT 1800
aattttaaag gatctagggtg aagatccttt ttgataatct catgaccaa atcccttaac 1860
gtgagttttc gttccactga gcgtcagacc ccgtagaaaa gatcaaagga tcttcttgag 1920
atcctttttt tctgcgcgta atctgctgct tgcaaacaaa aaaaccaccg ctaccagcgg 1980
tggtttgttt gccggatcaa gagctaccaa ctctttttcc gaaggtaact ggcttcagca 2040
gagcgcagat accaaatact gttcttctag tgtagccgta gttaggccac cacttcaaga 2100
actctgtagc accgcctaca tacctcgctc tgctaatcct gttaccagtg gctgctgcca 2160
gtggcgataa gtcgtgtctt accgggttgg actcaagacg atagttaccg gataaggcgc 2220
agcggtcggg ctgaacgggg ggttcgtgca cacagcccag cttggagcga acgacctaca 2280
ccgaactgag atacctacag cgtgagctat gagaaagcgc cacgcttccc gaaggagaaa 2340
aggcggacag gtatccggta agcggcaggg tcggaacagg agagcgcacg agggagcttc 2400
cagggggaaa cgcctgggtat ctttatagtc ctgtcgggtt tcgccacctc tgacttgagc 2460
gtcgatTTTT gtgatgctcg tcaggggggc ggagcctatg gaaaaacgcc agcaacgcgg 2520
ccttttttac gttcctggcc ttttgctggc cttttgctca catgttcttt cctgcgttat 2580
cccctgattc tgtggataac cgtattaccg cctttgagtg agctgatacc gctcgccgca 2640
gccgaacgac cgagcgcagc gagtcagtga gcgaggaagc ggaaga 2686

```

<210> 3
 <211> 335
 <212> DNA
 <213> Homo sapiens

```

<400> 3
atgcttgacg ctcaaaccat cgctacagta aaagccacca tccctttact ggtggaaacg 60
gggccaaagt taaccgcca tttctacgac cgtatgttta ctcataacct agaactcaaa 120
gaaattttta acatgagtaa ccagcgtaat ggcgatcaac gtgaagccct gtttaacgct 180
attgccgcct acgccagtaa tattgaaaac ctgcctgcgc tgctgccagc ggtagaaaaa 240

```


Sequence Listing

atcgcgcaga agcacaccag cttccagatc aaaccggaac agtacaacat cgtcgggtgaa 300
 cacctgtttg caacgctgga cgaaatgttc agccc 335

<210> 4
 <211> 44
 <212> DNA
 <213> Homo sapiens

<400> 4
 cccggggggg atcctctaga gtcgacctgc aggcattgcaa gctt 44

<210> 5
 <211> 480
 <212> DNA
 <213> Homo sapiens

<400> 5
 ctgagaaccc tgcgcctgca ttacgcgcgg gctggtgtag ttttcggagg cgatcagttc 60
 gatgtgctct tcttgacgta ctttttcctg ctccatagcc tgccacagtt cggcatcata 120
 atcggcaatg ttcatttcac gctttaacat ccgcatctcc tgactcagct aacaataaaa 180
 tttttggcct ttataggcgg tctgtttgga caacggcgaa cagtataacc gaatcattgt 240
 gcgataacag gtcttgacaa aggaatttac gcaaacgatt accttcaggc tacgcaaggc 300
 tttggagaat aaagagcttg caaccggaaa cggatttctt ttcaggtttg tgatgcaaat 360
 ttttcacttc atcacattct ttctgaaaaa caccaaagaa ccatttacat tgcagggcta 420
 ttttttataa gatgcatttg agatacatca attaagatgc aaaaaaagga agaccatatg 480

<210> 6
 <211> 244
 <212> DNA
 <213> Homo sapiens

<400> 6
 ttgtgcgata acaggtcttg acaaaggaat ttacgcaaac gattaccttc aggctacgca 60
 aggctttgga gaataaagag cttgcaaccg gaaacggatt tcttttcagg tttgtgatgc 120
 aaatTTTTTca cttcatcaca ttctttctga aaaacaccaa agaaccattt acattgcagg 180
 gctattttttt ataagatgca tttgagatac atcaattaag atgcaaaaaa aggaagacca 240
 tatg 244