# IMPROVED HUMAN LONG PENTRAXIN 3 EXPRESSION SYSTEM AND USES THEREOF

## FIELD OF THE INVENTION

The present invention relates to human derived cellular system able to express high levels of the human pentraxin 3 (hPTX3) protein, methods and material used.

## BACKGROUND TO THE INVENTION

Human long pentraxin 3, PTX3 or hPTX3 (GeneBank Accession Number BD 131701) is a multimeric glycoprotein composed of eight subunits linked by disulphide bridges.

The authors of the present invention have already designed an expression system for the production of PTX3 in HEK293F human cell line. This system is based on a plasmid containing the neomycin resistance gene wherein the PTX3 gene is under the control of a human Ubiquitin C promoter sequence. Such system avoids the potential formation of a chimeric PTX3 derived from endogenous production of PTX3 by a cell line of non-human origin. The best producer isolated clone, named 2F12, was selected among those obtained. A production of about 20 mg/L of PTX3 was obtained, which was not sufficient for commercial production needs.

With the aim to increase PTX3 expression levels, the authors of the present invention have constructed a plasmid in which the PTX3 gene was under CMV promoter control. The plasmid was used to re-transfect the PTX3 expressing clone 2F12. The productivity level detected in the new isolated transfectomas was higher that expected, around 80 mg/L.

## DESCRIPTION OF THE INVENTION

A clone of human origin expressing high levels of human PTX3 was obtained using an experimental strategy , including the following steps:

a) construction of plasmid expression cassettes carrying the human PTX3 under the control of CMV promoter;

b) insertion of the hygromycin resistance cassette in said plasmid, in order to select stable transfectomas originating from a re-transfection of PTX3 expressing clone G418 resistant;

c) verifying the identity and the levels of expressed recombinant proteins;

d) biochemical characterization of the recombinant hPTX3.

The content of PCT/EP2009/050937 is hereby incorporated by reference.

It is an object of the invention an eukaryotic expression vector comprising a nucleotide sequence encoding for the human long pentraxin PTX3 protein under the control of an effective promoter and a nucleotide sequence encoding for a selectable marker, having essentially the sequence of SEQ ID 1.

The vector is preferentially use to transform host cell, preferably wherein the host cell is a recombinant human cell already able to express the human long pentraxin PTX3 protein, more preferably wherein the recombinant human cell is the recombinant 293F/PTX3/2F12 clone deposited at ECACC with no. 08011001. In a particular aspect the vector is linearized.

It is another object of the invention a recombinant cell able to express the human long pentraxin PTX3 protein encoded by the vector as above disclosed, preferably it is recombinant HEK293F cell line, more preferably it is the recombinant MS24PTX clone deposited at Health Protection Agency, Culture Collections Centre For Emergency Preparedness and Response Salisbury UK, with no. 09072902.

It is another aspect of the invention the use of the recombinant cell as above disclosed for the production of human long pentraxin PTX3 protein.

It is another object of the invention a process for the production of the recombinant human long pentraxin PTX3 protein comprising:

a) transfecting a recombinant human cell already expressing recombinant human long pentraxin PTX3 protein, with a selectable plasmid in which the human long pentraxin gene is under the control of the CMV promoter ;
b) selecting and growing transfected recombinant human cell;
c) purifying the human long pentraxin PTX3 protein from the culture medium of the transfected recombinant human cell.

Preferably the recombinant human cell expressing a recombinant human long pentraxin PTX3 protein is a recombinant HEK293F cell line, more preferably it is the recombinant 293F/PTX3/2F12 clone deposited at ECACC under number 08011001.

In a preferred embodiment the purification step includes at least one of the following step: anionic-exchange chromatography, hydroxyapatite chromatography or size exclusion chromatography.

It is another object of the invention a process for the production of the recombinant human long pentraxin PTX3 protein comprising:

a) co-transfecting contemporarily or sequentially human cells with a first vector having essentially the sequence of SEQ ID 1 and a second vector having essentially the sequence of SEQ ID 2;

b) selecting and growing the double transfected cells;

b) purifying the human long pentraxin PTX3 protein from the culture medium of double transfected cells.

It is another object of the invention a process for the production the recombinant human long pentraxin PTX3 protein comprising the step of growing the recombinant MS24PTX clone and purifying the human long pentraxin PTX3 protein from the culture medium.

The invention will be now illustrated by means of non limiting examples, referring in particular to the following figures:

**Figure 1**: pSASSI-hPTX3 map and main features

**Figure 2**: Growth, viability and productivity in Spinner Flask of (A) MS24PTX clone and (B) 293F/PTX3/2F12 clone.

**Figure 3**: characterization by SDS-PAGE gradient 4-15% (A) and Size Exclusion Chromatography (B) of recombinant human PTX3 purified from MS24PTX clone.

**Figure 4**: FGF2 binding capability of hPTX3 clone 293F/PTX3/2F12 and hPTX3 clone MS24PTX.

**Figure 5:** pSC1-hPTX3 map and main features.

EXAMPLES

**Example 1: clone 293F/PTX3/2F12**

Construction of the Plasmid pSC1-PTX3

1. Construction of pSG/Ub

1.1 Preparation of the Human Ubiquitin C Promoter sequence

The human ubiquitin C promoter is taken from pUB/Bsd plasmid (Invtrogen, Cat. n. V512-20), by amplification with PCR. As part of the cloning strategy, recognition sequences for restriction endonucleases are introduced at both ends. A BsaAI site is built in the upstream amplification primer and an EcoRI site in the downstream primer. The amplified region corresponds to nucleotides 1941 to 3161 in the sequence of pUB/Bsd.

The oligonucleotides are designed as follow:

5'p UbC: length: 26mer (SEQ ID 3) ATATCACGTG ATC TGG CCT CCG CGC C

3'p UbC: length: 23mer (SEQ ID 4) GGAATTC GGT CCG GTC TAA CAA A

The protocol for amplification is the following: 1 ng/µl of plasmid DNA, 2mM MgCl2, 0,2 mM dNTPs, 400nM of each primer, 1X supplied buffer and 0,04 u/ml of Taq DNA polymerase

(Sigma Genosys); temperature profile: 3 min 94°C, 30 times (30 sec. 94°C, 30 sec. 46°C, 2 min 72°C), 5 min 72°C, cooling at 4°C until further use.

The amplification product (1238 bp) is purified by silica membrane spin column ( NucleoSpin, Machery-Nagel GmbH & Co.), ligated in pGEM-T-Easy vector (Promega Cat. n. A1360) and transformed into E.coli host strain HB2151 (Pharmacia Biotech). Transformants are selected by growth on LB medium supplemented with 50 mg/l ampicillin

Plasmids DNA, isolated from ampicillin resistant colonies, are checked by restriction analysis with StuI plus SacI enzymes (expected ~ 3650 and 600 bp fragments)

Plasmids showing the correct restriction pattern are further checked by sequence analysis of the entire insert and subsequently digested with EcoRI (Sigma-Genosys) and BsaAI (New England Biolabs) restriction enzymes.

Human Ubiquitin C promoter is purified via agarose gel separation and elution on silica membrane spin column.

1.2 Preparation of the vector fragment pSG5

Plasmid pSG5 (4076 bp, Stratagene) was cut with the restriction enzymes EcoRI (Sigma-Genosys) and BsaAI (New England Biolabs); the resulting fragments are 1432 and 2644 bp long. The 2644 bp fragment, containing the backbone of pSG5, was prepared and purified via agarose gel electrophoresis plus silica membrane spin column.

1.3 Preparation of pSG/Ub

DNA fragments prepared in steps 1.1 and 1.2 were ligated using T4 DNA ligase (Promega) and transformed in HB2151 E. coli cells. Transformants were selected by growth on LB medium supplemented with 50 mg/l ampicillin.

Plasmid DNA, isolated from ampicillin resistant colonies, was checked by restriction analysis with EcoRI plus SacII enzymes (expected: 2670 and 1192 bp fragments). A plasmid DNA, with the expected restriction pattern, was designed as pSG/Ub.

2. Construction of pSC1

2.1 Preparation of the Neomycin Resistance Cassette (NeoR)

The Neomycin Resistance Cassette (NeoR) was taken from pcDNA3 plasmid (5446 bp, Invitrogen), amplifying it by PCR. As part of the cloning strategy, recognition sequences for restriction endonuclease AflIII were introduced at both ends. The amplified region corresponds to nucleotides 1788 to 3252 in the sequence of pcDNA3 and includes the SV40 promoter and origin of replication, the neomycin resistance ORF, and the SV40 poliA signal.

The oligonucleotides are designed as follows:

5'NeoR (SEQ ID 5) ATATACATG TCC CCA GGC AGG CAG AA

3'NeoR (SEQ ID 6) ATATACAT GTAT ACA GAC ATG ATA AG

Protocol for amplification was the following: 1 ng/µl of plasmid DNA, 2mM MgCl2, 0,2 mM dNTPs, 400nM of each primer, 1X supplied buffer and 0,04 u/µl of Taq DNA polymerase (Sigma Genosys); temperature profile: 3 min 94°C, 30 times (30 sec. 94°C, 30 sec. 46°C, 2 min 72°C), 5 min 72°C, cooling at 4°C until further use.

The amplification product (1484 bp) was purified by silica membrane spin column, ligated in pGEM-T-Easy vector (Promega Cat. n. A1360) and transformed into E.coli host strain HB2151. Transformants are selected by growth on LB medium, supplemented with 50 mg/l ampicillin

Plasmids DNA, isolated from ampicillin resistant colonies, are checked by restriction analysis with SmaI plus SacI enzymes (expected ~ 1200 and 3300 bp fragments).

Plasmids showing the correct restriction pattern were further checked by sequence analysis of the entire insert and subsequently digested with AflIII (New England Biolabs) restriction enzymes.

NeoR cassette (1471 bp) was purified via agarose gel separation and elution on silica membrane spin column.

2.2 Preparation of the vector fragment pSG/Ub

Plasmid pSG/Ub, prepared in step 1.3, was linearized by AflIII digestion and purified on silica membrane spin column.

2.3 Preparation of pSC1

DNA fragments prepared as in steps 2.1 and 2.2 were ligated using T4 DNA ligase (Promega) and transformed in JM109 E. coli strain (New England Biolabs). Transformants were selected by growth on LB medium, supplemented with 50 mg/l ampicillin.

Antibiotic resistant colonies were preliminarliy analyzed by PCR amplification with 5'NeoR and 3'NeoR oligonucleotides, as previously described, and subsequently, purified plasmids were checked by restriction analysis. For this purpose, SmaI (position 602, inside NeoR sequence) and SacII (position 4142, inside UbC sequence) enzymes were used. A plasmid DNA, with the expected restriction pattern (3540 and 1793 bp fragments), was designed as pSC1.

3. Construction of pSC1-PTX3

3.1 Preparation of the hPTX3 coding sequence

The hPTX3 (GeneBank Accession Number BD 131701) sequence was taken from pSG5-PTX3 (WO 99/32516 "Pharmaceutical compositions containing the long pentraxin PTX3 ) by BamHI (Roche Applied Science) digestion. Human PTX3 fragment (1463 bp) was purified by agarose gel electrophoresis and silica membrane spin column.

3.2 Preparation of the vector fragment pSC1

The pSC1 vector was linearized by BamHI digestion and purified on silica membrane spin column.

3.3 Construction and verification on pSC1-PTX3

DNA fragments prepared in steps 3.2 and 3.3 were ligated using T4 DNA ligase (Roche Applied Science) and transformed in JM109 E. coli strain. Transformants were selected by growth on LB medium, supplemented with 50 mg/l ampicillin and preliminarily screened by PCR with two oligonucleotides complementary to PTX3 sequence.

The oligonucleotides sequences are:

5'PTX (SEQ ID 7) GTGAGAACTCGGATGATTATGAT

3'PTX (SEQ ID 8) TGAAACATACTGAGCTCCTCCAT

In a final volume of 10 µl, reagents for amplification were: 1 µl of boiled colony (1 colony in 50ml of water), 2mM MgCl2, 0,2 mM dNTPs, 320nM of each primer, 0,06% Formamide, 1X supplied buffer and 0,08 u/µl of Taq DNA polymerase (Sigma Genosys); temperature profile: 3 min 96°C, 30 times (30 sec. 94°C, 30 sec. 58°C, 2 min 72°C), 5 min 72°C, cooling at 4°C until further use.

Plasmid purified from colonies positive to PCR screening, were digested with SalI restriction enzyme (Roche Applied Science) to check the orientation of hPTX3 insert. A plasmid with the expected restriction pattern (6619 and 177 bp) was sequenced in the regions coding for UbC promoter, NeoR cassette and hPTX3 and identified as pSC1-PTX3.

The new plasmid (pSC1-PTX3) was then constructed with PTX3 cDNA sequence located under ubiquitin promoter control and neomycin resistance gene under SV40 promoter control; all other features and plasmid map are represented in figure 1.

The complete sequence of pSC1-PTX3 is as follows (SEQ ID 2). The pSC1-hPTX3 sequence is represented starting from the first EcoRI site (figure 5). The sequence deriving from pSG5 containing PTX3 cDNA is underlined. The starting codon (ATG) and termination codon are in bold.

pSC1-PTX3 (SEQ ID 2)

AATTCGGATCCCCCGGGCTGCAGGAATTCCGGCTCAAACTCAGCTCACTTGAGAGTCTCCTCCCGCCAGCTGTGGAA
AGAACTTTGCGTCTCTCCAGCA**ATG**CATCTCCTTGCGATTCTGTTTTGTGCTCTCTGGTCTGCAGTGTTGGCCGAGA
ACTCGGATGATTATGATCTCATGTATGTGAATTTGGACAACGAAATAGACAATGGACTCCATCCCACTGAGGACCCC
ACGCCGTGCGACTGCGGTCAGGAGCACTCGGAATGGGACAAGCTCTTCATCATGCTGGAGAACTCGCAGATGAGAGA
GCGCATGCTGCTGCAAGCCACGGACGACGTCCTGCGGGGCGAGCTGCAGAGGCTGCGGGAGGAGCTGGGCCGGCTCG
CGGAAAGCCTGGCGAGGCCGTGCGCGCCGGGGGCTCCCGCAGAGGCCAGGCTGACCAGTGCTCTGGACGAGCTGCTG
CAGGCGACCCGCGACGCGGGCCGCAGGCTGGCGCGTATGGAGGGCGCGGAGGCGCAGCGCCCAGAGGAGGCGGGGCG
CGCCCTGGCCGCGGTGCTAGAGGAGCTGCGGCAGACGCGAGCCGACCTGCACGCGGTGCAGGGCTGGGCTGCCCGGA
GCTGGCTGCCGGCAGGTTGTGAAACAGCTATTTTATTCCCAATGCGTTCCAAGAAGATTTTTGGAAGCGTGCATCCA
GTGAGACCAATGAGGCTTGAGTCTTTTAGTGCCTGCATTTGGGTCAAAGCCACAGATGTATTAAACAAAACCATCCT

GTTTTCCTATGGCACAAAGAGGAATCCATATGAAATCCAGCTGTATCTCAGCTACCAATCCATAGTGTTTGTGGTGG
GTGGAGAGGAGAACAAACTGGTTGCTGAAGCCATGGTTTCCCTGGGAAGGTGGACCCACCTGTGCGGCACCTGGAAT
TCAGAGGAAGGGCTCACATCCTTGTGGGTAAATGGTGAACTGGCGGCTACCACTGTTGAGATGGCCACAGGTCACAT
TGTTCCTGAGGGAGGAATCCTGCAGATTGGCCAAGAAAAGAATGGCTGCTGTGTGGGTGGTGGCTTTGATGAAACAT
TAGCCTTCTCTGGGAGACTCACAGGCTTCAATATCTGGGATAGTGTTCTTAGCAATGAAGAGATAAGAGAGACCGGA
GGAGCAGAGTCTTGTCACATCCGGGGGAATATTGTTGGGTGGGGAGTCACAGAGATCCAGCCACATGGAGGAGCTCA
GTATGTTTCA**TAA**ATGTTGTGAAACTCCACTTGAAGCCAAAGAAAGAAACTCACACTTAAAACACATGCCAGTTGGG
AAGGTCTGAAAACTCAGTGCATAATAGGAACACTTGAGACTAATGAAAGAGAGAGTTGAGACCAATCTTTATTTGTA
CTGGCCAAATACTGAATAAACAGTTGAAGGAAAGACATTGGAAAAAGCTTATCGATACCGTCGACCTCGAGGGGGGG
CCCGGGGATCCAGATCTTATTAAAGCAGAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCA
CAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCAT
GTCTGGTCGACTCTAGACTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGT
ATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTCCCCAGGCA
GGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAG
AAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTC
CGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGCC
TCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTCCCGGGAGCTTGTAT
ATCCATTTTCGGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTT
CTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTG
TTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGA
CGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGG
GAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTA
TCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACA
TCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGC
TCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGAT
GCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGA
CCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCG
TGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGA
CTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTAT
GAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTT
CTTCGCCCACCCCAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATA
AAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGTATACATGTG
AGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTG
ACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCC
CCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGG
AAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTG
TGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACAC
GACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTT
GAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCG
GAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAG
ATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA
CTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTT
TTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCA
GCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACC
ATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAG
CCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCT
AGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTC
GTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG
CGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCA
CTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTG
AGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTT
TAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCG
ATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGG
AAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCTTTTTTCAATATT
ATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGG
GTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAA
TAGGCGTATCACGAGGCCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCG
GAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGG
GTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGC
ACAGATGCGTAAGGAGAAAATACCGCATCAGGAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTT
GTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATA

GGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAAC
CGTCTATCAGGGCGATGGCCCACTACGTGATCTGGCCTCCGCGCCGGGTTTTGGCGCCCCCCGCGGGCGCCCCCCTC
CTCACGGCGAGCGCTGCCACGTCAGACGAAGGGCGCAGCGAGCGTCCTGATCCTTCCGCCCGGACGCTCAGGACAGC
GGCCCGCTGCTCATAAGACTCGGCCTTAGAACCCCAGTATCAGCAGAAGGACATTTTAGGACGGGACTTGGGTGACT
CTAGGGCACTGGTTTTCTTTCCAGAGAGCGGAACAGGCGAGGAAAAGTAGTCCCTTCTCGGCGATTCTGCGGAGGGA
TCTCCGTGGGGCGGTGAACGCCGATGATTATATAAGGACGCGCCGGGTGTGGCACAGCTAGTTCCGTCGCAGCCGGG
ATTTGGGTCGCGGTTCTTGTTTGTGGATCGCTGTGATCGTCACTTGGTGAGTAGCGGGCTGCTGGGCTGGCCGGGGC
TTTCGTGGCCGCCGGGCCGCTCGGTGGGACGGAAGCGTGTGGAGAGACCGCCAAGGGCTGTAGTCTGGGTCCGCGAG
CAAGGTTGCCCTGAACTGGGGGTTGGGGGGAGCGCAGCAAAATGGCGGCTGTTCCCGAGTCTTGAATGGAAGACGCT
TGTGAGGCGGGCTGTGAGGTCGTTGAAACAAGGTGGGGGGCATGGTGGGCGGCAAGAACCCAAGGTCTTGAGGCCTT
CGCTAATGCGGGAAAGCTCTTATTCGGGTGAGATGGGCTGGGGCACCATCTGGGGACCCTGACGTGAAGTTTGTCAC
TGACTGGAGAACTCGGTTTGTCGTCTGTTGCGGGGGCGGCAGTTATGGCGGTGCCGTTGGGCAGTGCACCCGTACCT
TTGGGAGCGCGCGCCCTCGTCGTGTCGTGACGTCACCCGTTCTGTTGGCTTATAATGCAGGGTGGGGCCACCTGCCG
GTAGGTGTGCGGTAGGCTTTTCTCCGTCGCAGGACGCAGGGTTCGGGCCTAGGGTAGGCTCTCCTGAATCGACAGGC
GCCGGACCTCTGGTGAGGGGAGGGATAAGTGAGGCGTCAGTTTCTTTGGTCGGTTTTATGTACCTATCTTCTTAAGT
AGCTGAAGCTCCGGTTTTGAACTATGCGCTCGGGGTTGGCGAGTGTGTTTTTGTGAAGTTTTTTAGGCACCTTTTGAA
ATGTAATCATTTGGGTCAATATGTAATTTTCAGTGTTAGACTAGTAAATTGTCCGCTAAATTCTGGCCGTTTTTGGC
TTTTTTGTTAGACCGGACCG

A human cell line (HEK293F) has been chosen for its ability to grow in suspension and in a serum and protein free medium (Florian M Wurm "Production of recombinant protein therapeutics in cultivated mammalian cells" Nature Biotechnology 22(11):1393-1398, 2004, Yan SC et al. "Characterization and novel purification of recombinant human protein C from three mammalian cell lines" Biotechnology (N.Y.) 1990 Jul 8 (7): 655-61. "Use of cell lines for the production of influenza virus vaccines: an appraisal of technical, manufacturing, and regulatory considerations" Initiative for Vaccine Research, World Health Organization, Geneva, Switzerland (10 Aprile 2007). To transfect HEK293F, pSC1-PTX3 plasmid was used either in a linear (PvuI digested) or in a circular form. The best transfection yield was obtained with linearized plasmid; clones selection was done on a productivity base and growth capability. After several rounds of subcloning the 2F12 clone was selected.

The human clone 2F12, expressing hPTX3, has been deposited at ECACC (European Collection of Cell Cultures, Health Protection Agency, Porton Down, Wiltshire SP4 0JG, UK) on January 10, 2008, pursuant to Budapest Treaty condition under deposit number 08011001. The experimental details are described below.

3.4 Recombinant 293F-cells generated from pSC1-PTX3

*Transfection and subcloning*

$10^6$ cells/ml 293F (Invitrogen cat n° R790-07) were seeded in a 125 ml spinner flask in a final Freestyle medium volume of 28 ml the day of transfection. The pSC1/PTX3 plasmid was then allowed to adsorb to the 293fectin reagent (GIBCO/Invitrogen) according to the manufacturer's protocol.

In brief, in two separate tube, 30 µg of pSC1-PTX3 circular or PvuI linearized were diluted in 1 ml of Optimem (GIBCO/Invitrogen, Carlsbad, CA, USA) and 40 µl of 293fectin (Invitrogen)

diluted to 1 ml with Optimem. Both solution were incubated for 5 minutes at room temperature then mixed (final volume 2 ml) and incubated for 30 minutes in the same conditions. DNA/lipid cocktail was added to cells and incubated at 37°C, 5% CO2 with agitation (120 rpm). After cultivation for 36 hours, the medium was changed into selection medium (200 ml Freestyle medium+ 500 μg/ml of G418) and the transfected cells were plated in ten 96wells plates, 200μl/well. After 15 days highest producers cell-pools were determined by ELISA and amplified in 24wells, 6wells and T25flask.

Recombinant cell-pools obtained were subcloned with 1 cells per well in 96wells plates, in 50% fresh medium and 50% conditioned medium.

**Example 2: clone MS24PTX**

Construction of the plasmid pSASSI-hPTX3

1. Construction of pCEPlightΔ

pCEP4 plasmid (Invitrogen cat. n. V044-50), in which was previously cloned an antibody light chain, loosing a portion of the Multiple Cloning Site and the BamHI restriction site, was cut with the restriction enzymes EcoRV and ClaI (Roche Applied Science); the digestion allowed to obtain a plasmid without the Epstein-Barr-Virus replication origin (oriP) and the nuclear antigen (encoded by the EBNA-1 gene) that permit extrachromosomal replication. The resulting fragments were 6910 and 4281 bp long. The 6910 bp fragment, containing the backbone of pCEP, was purified via agarose gel electrophoresis plus silica membrane spin column. Since ClaI generates sticky end, the fragment was filled in, using T4 DNA polymerase (Roche Applied Science) with the follow protocol: 150 ng of ClaI/EcoRV purified fragment (38 μl), 5 μl of 10x T4 DNA polymerase buffer, 4 μl of dNTP mix 2.5mM, 3 μl of T4 DNA polymerase (1U/μl). After 15 minutes at 37°C, the reaction was stopped at 70°C for 5 minutes then on ice. The fragment was purified on a silica membrane spin column and ligated on itself over night at room temperature, by using T4 DNA ligase (Promega). TOP10 competent cells (Invitrogen) were transformed with the ligation mixture and transformants selected by growth on LB plates supplemented with 100 mg/L ampicillin.

Plasmid DNA, isolated from ampicillin resistant colonies, was designed as pCEPlightΔ.

2. Preparation of vector fragment containing Hygromycin resistance and CMV promoter.

The Hygromycin Resistance Cassette together with the cytomegalovirus (CMV) immediate early enhancer/promoter was taken from pCEPlightΔ amplifying it by PCR. As a part of the cloning

strategy, recognition sequence for restriction endonuclease BamHI was introduced in the oligonucleotide annealing to the 3' end of CMV promoter.

The amplified region about 5500 bp, included CMV promoter, Hygromycin gene under the control of TK promoter together with TK polyA signal.

The oligonucleotide are designed as follows:

oligo CMV (SEQ ID 9) 5'GAGAACTGTAACGTTGGATCCAGCTGG 3'

oligo H (SEQ ID 10) 5'GTGTACAAAGGATCCAGACATGATAAG 3'

Protocol for amplification was the following: 2 ng of pCEPlightΔ, 200nM of each primer, 0,2 mM dNTPs, 1X supplied buffer, 1.5 µl DMSO, 0.5 µl Taq DNA polymerase (Phusion), final volume 50 µl; temperature profile: 1 min 98°C, 35 times (10 sec. 98°C, 30 sec. 55°C, 3 min. 72°C), 10 min. 72°C, cooling at 4°C until further use.

The amplification product (~5500 bp) was purified via agarose gel electrophoresis plus silica membrane spin column. Purified fragment was ligated to itself and use to transform TOP10 competent cells (Invitrogen). Plasmid DNA, isolated from ampicillin resistant colonies, was checked by restriction and sequence analysis. and was designed as pCEPΔBam.

3. Preparation of hPTX3 gene

The hPTX3 (GeneBank Accession Number BD 131701) sequence was taken from pSC1-PTX3 as indicated above by BamHI (Roche Applied Science) digestion. Human PTX3 fragment (1463 bp) was purified by agarose gel electrophoresis and silica membrane spin column.

4. Preparation of pCEPΔBam-hPTX3

The pCEPΔBam vector was linearized by BamHI digestion and purified on silica membrane spin column. pCEPΔBam linearized and DNA fragment corresponding to hPTX3 gene prepared in step 3 were ligated using T4 DNA ligase (Roche Applied Science) and used to transform TOP10 E. coli strain. Transformants were selected by growth on LB medium, supplemented with 100 mg/L ampicillin and preliminarily screened restriction analisys to evaluate PTX3 fragment orientation.

5. Preparation of SV40 polyadenilation signal

The SV40 polyA signal was taken from pCEPΔlight plasmid amplifying it by PCR. As part of the cloning strategy, recognition sequences for restriction endonucleoases HindIII and XhoI were introduced at fragment ends respectively.

The oligonucleotides were designed as follows:

PCEPSVH (SEQ ID 11) 5'AAGCTTAGACATGATAAGATACATTG 3'

PCEPSVX (SEQ ID 12) 5'CTCGAGAGTCGACCGGTCATGGCTGC 3'

Protocol for amplification was the following: 1 ng of pCEPlightΔ, 200nM of each primer, 0,2 mM dNTPs, 1X supplied buffer, 2 µl MgCl2 50mM, 0.5 µl Taq DNA polymerase (Invitrogen), final volume 50 µl; temperature profile: 1 min 94°C, 30 times (30 sec. 94°C, 1 min. 55°C, 1 min. 72°C), 15 min. 72°C, cooling at 4°C until further use.

The amplification product (~420 bp) was purified via agarose gel electrophoresis plus silica membrane spin column.

6. Preparation of pSASSI-hPTX3

Purified fragment corresponding to SV40 polyA signal and pCEPΔBam-hPTX3 were digested with HindIII/XhoI restriction enzymes and ligated using T4 DNA ligase (Promega). Ligation mixture was used to transform TOP10 competent cells (Invitrogen) and transformants were selected by growth on LB plates containing 100 mg/L of ampicillin.

Plasmid DNA isolated from ampicillin resistant colonies, was checked by restriction and sequence analysis and the plasmid was designated pSASSI-hPTX3.

The complete sequence of pSASSI-HPTX3 is as follow (SEQ ID 1). The pSASSI-hPTX3 sequence is represented starting from the BamHI site (figure1). The sequence of hPTX3 is in small letters. The starting codon (atg) and the termination codon (taa) are in bold.

pSASSI-HPTX3 (SEQ ID 1).

```
GGATCCCCCG GGCTGCAGGA ATTCCGGCTC AAACTCAGCT CACTTGAGAG TCTCCTCCCG        60
CCAGCTGTGG AAAGAACTTT GCGTCTCTCC AGCAATGCAT CTCCTTGCGA TTCTGTTTTG       120
TGCTCTCTGG TCTGCAGTGT TGGCCGAGAA CTCGGATGAT TATGATCTCA TGTATGTGAA       180
TTTGGACAAC GAAATAGACA ATGGACTCCA TCCCACTGAG GACCCCACGC CGTGCGACTG       240
CGGTCAGGAG CACTCGGAAT GGGACAAGCT CTTCATCATG CTGGAGAACT CGCAGATGAG       300
AGAGCGCATG CTGCTGCAAG CCACGGACGA CGTCCTGCGG GGCGAGCTGC AGAGGCTGCG       360
GGAGGAGCTG GGCCGGCTCG CGGAAAGCCT GGCGAGGCCG TGCGCGCCGG GGGCTCCCGC       420
AGAGGCCAGG CTGACCAGTG CTCTGGACGA GCTGCTGCAG GCGACCCGCG ACGCGGGCCG       480
CAGGCTGGCG CGTATGGAGG GCGCGGAGGC GCAGCGCCCA GAGGAGGCGG GGCGCGCCCT       540
GGCCGCGGTG CTAGAGGAGC TGCGGCAGAC GCGAGCCGAC CTGCACGCGG TGCAGGGCTG       600
GGCTGCCCGG AGCTGGCTGC CGGCAGGTTG TGAAACAGCT ATTTTATTCC CAATGCGTTC       660
CAAGAAGATT TTTGGAAGCG TGCATCCAGT GAGACCAATG AGGCTTGAGT CTTTTAGTGC       720
CTGCATTTGG GTCAAAGCCA CAGATGTATT AAACAAAACC ATCCTGTTTT CCTATGGCAC       780
AAAGAGGAAT CCATATGAAA TCCAGCTGTA TCTCAGCTAC CAATCCATAG TGTTTGTGGT       840
GGGTGGAGAG GAGAACAAAC TGGTTGCTGA AGCCATGGTT TCCCTGGGAA GGTGGACCCA       900
CCTGTGCGGC ACCTGGAATT CAGAGGAAGG GCTCACATCC TTGTGGGTAA ATGGTGAACT       960
GGCGGCTACC ACTGTTGAGA TGGCCACAGG TCACATTGTT CCTGAGGGAG GAATCCTGCA      1020
GATTGGCCAA GAAAAGAATG GCTGCTGTGT GGGTGGTGGC TTTGATGAAA CATTAGCCTT      1080
CTCTGGGAGA CTCACAGGCT TCAATATCTG GGATAGTGTT CTTAGCAATG AAGAGATAAG      1140
AGAGACCGGA GGAGCAGAGT CTTGTCACAT CCGGGGGAAT ATTGTTGGGT GGGGAGTCAC      1200
```

AGAGATCCAG CCACATGGAG GAGCTCAGTA TGTTTCA**TAA** ATGTTGTGAA ACTCCACTTG 1260
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GGGTGTCGGG GCGCAGCCAT GACCGGTCGA CTCTCGAGGG GGGGCCCGGG GATCCAACGT 1860
TACAGTTCTC CAGTGCATGT AATCCCTTCA GTTGGTTGGT ACAACTTGCC AACTGGGCCC 1920
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GTCAGATCTC TAGAAGCTGG GTACCAGCT 7469

**Example 3**

Recombinant MS24PTX clone generated by pSASSI-hPTX3 transfection

1. Transfection and subcloning

$10^6$ cells/ml 293F/PTX3/2F12 were seeded in a 125 ml spinner flask in a final Freestyle medium volume of 28 ml the day of transfection. The pSASSI-hPTX3 plasmid was then allowed to adsorb to the 293fectin reagent (GIBCO/Invitrogen) according to the manufacturer's protocol.

In brief, 30 µg of pSASSI-hPTX3 NruI linearized was diluted in 1 ml of Optimem (GIBCO/Invitrogen, Carlsbad, CA, USA) and 40 µl of 293fectin (Invitrogen) diluted to 1 ml with Optimem. Both solution were incubated for 5 minutes at room temperature then mixed (final volume 2 ml) and incubated for 30 minutes in the same conditions. DNA/lipid cocktail was added to cells and incubated at 37°C, 5% $CO_2$ with agitation (120 rpm). After cultivation for 36 hours, the medium was changed into selection medium (200 ml Freestyle medium+ 200 µg/ml of Hygromycin) and the transfected cells were plated in ten 96wells plates, 200µl/well. After 15

days highest producers cell-pools were determined by ELISA and amplified in 24wells, 6wells and T25flask.

Recombinant cell-pools obtained were subcloned with 1 cells per well in 96wells plates, in 50% fresh medium and 50% conditioned medium.

2. ELISA detection of recombinant hPTX3

Purified PTX3 or PTX3 secreted in the culture supernatant were titrated using a sandwich ELISA. To detect PTX3, 96-well Nunc Maxisorb microtiter plates (Nunc, Roskilde, Denmark) were coated overnight, at 4°C, with 700 ng/ml of the rat monoclonal antibody MNB4 anti-human PTX3 (Alexis™ Biochemicals, Lausen, Switzerland) in 15mM sodium carbonate buffer, pH 9.6. Wells were washed with PBS plus 0.05% Tween-20 (PBS-Tw, washing solution) and blocked with 300µl of PBS-Tw containing 5% dry milk, for 2 hours at room temperature.

Cell supernatants or purified recombinant human PTX3 were added to the wells, diluted in washing solution plus 1% BSA. A standard curve, made with purified recombinant human PTX3 from CHO cells, ranging from 0 to 100 ng/ml, was done for quantification. After 1 hour of incubation at 37°C, bound PTX3 was detected using biotin-conjugated polyclonal rabbit anti-PTX3 antibody, followed by incubation with streptavidin conjugated to horseradish peroxidase (Sigma-Aldrich, USA). Finally 2.2'-azino-bis 3-ethylbenxthiazoline-6-sulfonic acid (Sigma Chemical Co. USA) was added for color development and optical density at 405 nm was assessed using a Microplate Reader Model 3550 EIA (Bio-Rad, Hercules, CA, USA).

**Example 4**

Comparison of growth, viability and productivity of clones 293F/PTX3/2F12 and MS24PTX

Cells deriving from the two clones were seeded at a density of 1.000.000 cells/ml (viability ≥90%) in a 500 ml of FreeStyle 293 medium in 1 liter Spinner flasks. The growing, the viability and the productivity were monitored for about 1 week until cells start die.

Figure 2 shows the growth, viability and productivity of the MS24PTX (panel A) and 293F/PTX3/2F12 (panel B) clones in the same seeding and growing conditions. As shown in the figure, with the re-transfection of the PTX3 expressing clone 293F/PTX3/2F12 with a new plasmid in which PTX3 is under the control of CMV promoter (MS24PTX clone) we were able to obtain about 4 fold increase in PTX3 productivity.

**Example 5**

Purification of recombinant human PTX3 from MS24PTX clone

Culture supernantant from MS24PTX clone, grown in spinner flask, was loaded onto a Q-SepharoseTM Fast Flow (GE Healthcare, UK) packed column. Retained material was eluted using a nonlinear gradient. The PTX3-containing fraction was directly applied to a ceramic Hydroxyapatite (BioRad, Hercules, CA, USA) packed column. The retained material was eluted by increasing phosphate concentration in a nonlinear fashion. The PTX3-containing fraction was concentrated and buffer changed on a ultrafiltration membrane (Pellicon-Biomax 100, Millipore) than characterized by Size Exclusion Chromatography on Biosep SEC S4000 (Phenomenex) and SDS-PAGE (Figure 3).

**Example 6**

Binding of h-PTX3 to FGF2

The binding of purified recombinant hPTX3 to FGF2 was assessed in an ELISA system. A 96-wells plate (Falcon 3912) was coated with 2 μg/ml of FGF2 (Calbiochem) in PBS and incubated overnight at 4°C. Wells were washed with PBS plus 0.1% Triton X-100 (PBS-Tr, washing solution) and blocked with 200 μl of PBS-Tr containing 3% BSA (PBS-B blocking and diluent solution) for 2 hours at room temperature. After washing, binding was performed adding 100 μl of samples, diluted in PBS-B at PTX3 concentrations ranging from 0 to 120 ng/ml, and incubating the plate at 37°C for 1 hr. After wash, plates were incubated with 100 μl/well of 100 ng/ml rabbit anti-PTX3 polyclonal antibody (1 hr at 37°C), washed again and incubated with 100 μl of horseradish peroxidase-labeled goat anti-rabbit IgG (1:1000 in PBS-B; 1 hr at 37°C). After washing, 100 μl of chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) (sigma-Aldrich) were added and after 10-15 min, the reaction was stopped adding 100 μl of HCl 1M and absorbance determined using a Microplate Reader Model 3550 EIA (Bio-Rad, Hercules, CA, USA). (Figure 4).

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| **1-3** | **Identification of deposit** | |
| 1-3-1 | Name of depositary institution | ECACC European Collection of Cell Cultures |
| 1-3-2 | Address of depositary institution | Health Protection Agency - Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom |
| 1-3-3 | Date of deposit | 10 January 2008 (10.01.2008) |
| 1-3-4 | Accession Number | ECACC 08011001 |
| **1-4** | **Additional Indications** | Cell Line 293F/PTX3/2F12 |
| **1-5** | **Designated States for Which Indications are Made** | All designations |
| **2** | **The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:** | |
| **2-1** | **page** | 2 |
| **2-2** | **line** | 8 |
| **2-3** | **Identification of deposit** | |
| 2-3-1 | Name of depositary institution | ECACC European Collection of Cell Cultures |
| 2-3-2 | Address of depositary institution | Health Protection Agency - Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom |
| 2-3-3 | Date of deposit | 29 July 2009 (29.07.2009) |
| 2-3-4 | Accession Number | ECACC 09072902 |
| **2-4** | **Additional Indications** | cell line MS24PTX |
| **2-5** | **Designated States for Which Indications are Made** | All designations |

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CLAIMS

1- An eukaryotic expression vector comprising a nucleotide sequence encoding for the human long pentraxin PTX3 protein under the control of an effective promoter and a nucleotide sequence encoding for a selectable marker, having essentially the sequence of SEQ ID 1.

2- Use of the vector according to claim 1 for transforming a host cell.

3- The use of the vector according to claim 2 wherein the host cell is a recombinant human cell already able to express the human long pentraxin PTX3 protein.

4- The use of the vector according to claim 3 wherein the recombinant human cell is the recombinant 293F/PTX3/2F12 clone deposited at ECACC with no. 08011001.

5- The use of the vector according to any of previous claims wherein the vector is linearized.

6- A recombinant cell able to express the human long pentraxin PTX3 protein encoded by the vector according to any of previous claim 1.

7- The recombinant cell according to claim 6 being a recombinant HEK293F cell line.

8- The recombinant cell according to claim 7 being the recombinant MS24PTX clone .

9- Use of the recombinant cell according to any one of claims 6-8 for the production of human long pentraxin PTX3 protein.

10- A process for the production of the human long pentraxin PTX3 protein comprising:

d) transfecting a recombinant human cell already expressing recombinant human long pentraxin PTX3 protein, with a plasmid in which the human long pentraxin gene is under the control of the CMV promoter;
e) selecting and growing transfected recombinant human cell;
f) purifying the human long pentraxin PTX3 protein from the culture medium of the transfected recombinant human cell.

11- The process according to claim 10 wherein the recombinant human cell expressing a recombinant human long pentraxin PTX3 protein is a recombinant HEK293F cell line.

12- The process according to claim 11 wherein the recombinant HEK293F cell line expressing a recombinant human long pentraxin PTX3 is the recombinant 293F/PTX3/2F12 clone deposited at ECACC under number 08011001.

13- The process according to claim 12 wherein the transfected recombinant human cell is MS24PTX clone.

14- The process of claims 10 to 13 wherein the purification step includes at least one of the following step: anionic-exchange chromatography, hydroxyapatite chromatography or size exclusion chromatography.

15- A process for the production of the recombinant human long pentraxin PTX3 protein comprising:

a) co-transfecting contemporarily or sequentially human cells with a first vector having essentially the sequence of SEQ ID 1 and a second vector having essentially the sequence of SEQ ID 2;

b) selecting and growing the double transfected cells;

b) purifying the human long pentraxin PTX3 protein from the culture medium of double transfected cells.

16- A process for the production the recombinant human long pentraxin PTX3 protein comprising the step of growing the recombinant MS24PTX clone and purifying the human long pentraxin PTX3 protein from the culture medium.