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CLONING OF IL2 IN pTZ19R AND ITS SITE DIRECTED MUTAGENESIS USING KUNKEL'S METHOD

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Abstract- Interleukin-2 (IL-2) is a lymphokine that exerts immunoregulatory effects on a variety of cells such as T cells, activated B cells and Natural Killer (NK) cells. Its biological effects are mediated through specific interactions with cell surface receptors present on target cells. Due to its role in generating normal immune responses, IL 2 is used in the treatment of a variety of tumors and infectious diseases. The downside of IL 2 is that the molecule is rather unstable. The aim of the current research is to generate IL-2 mutant with improved potency.This objective is accomplished by the use of cloning and Site directed Mutagenesis (SDM). For this purpose,first, wild type IL-2 mRNA was isolated from cultured T lymphocytes. This was converted into cDNA with the help of Reverse Transcription-PCR. Future work includes amplification of cDNA fragments and cloning into a phagemid vector pTZ19R. Transformation will be carried out in *E.coli RZ1032* and the ssDNA obtained as the end result of phagemid replication will be used as a template DNA to carry out modified Kunkel's method of site directed mutagenesis.

Key words: lymphokine, Reverse Transcription PCR, phagemid vector,Transformation

I .INTRODUCTION

Interleukin 2 (IL 2), originally defined as T-cell growth factor (Gillis et al., 1998) is a cytokine produced by T- helper cells when activated through interaction with an antigen presenting cell (APC). IL 2 molecule was first characterized as a variably glycosylated 15 kDa protein capable of supporting long term T lymphocyte proliferation (Robb *et al*., 1991). IL 2 exterts a variety of immunoregulatory effects including activation and expansion of lymphocyte subsets such as T-helper cells, cytotoxic and suppressor cells, B cells, Natural Killer (NK) cells and also cytotoxic macrophages. Aldesleukin, a recombinant form of native IL-2 (rIL-2), was the first cytokine of the interleukin class to be approved by the US FDA as a cancer therapy. The major application of aldesleukin is in the treatment of renal cell carcinoma (RCC), but it is also being studied in other cancers in phase I/II clinical trials. A disadvantage of IL 2 therapy is its stringent concentration range. Undesirable inflammatory responses are activated at IL 2 concentrations above 100 pM through stimulation of CD 56 dim NK cells (Fehniger *et al.*, 2002) while stimulation of T cells is not achieved below 1 pM, when administered intravenously IL 2 is rapidly cleared from the body. IL 2 serum concentrations are in the nanomolar range initially and fall rapidly with a double exponential clearance rate with half lives of 12.9 and 8.5 mins respectively (Konrad *et al*., 1990). Thus it is difficult to maintain the therapeutically effective serum concentration range (1 – 100 pM) over a sustained period of time. This narrow therapeutic window of effective concentration coupled with rapid systemic clearance adversely affects IL 2 therapy.

II. MATERIALS AND METHODS

A. BIOINFORMATICS TOOLS:

To begin with, the PDB ID of human IL2 and the receptor IL-2R was used. The protein data bank (PDB) is a repository for the 3D structural data of large biological molecules such as proteins. The RCSB(Research Collaboratory for Structural Bioinformatics)- PBD also provides a variety of tools and resources.

The PDB ID of Human IL 2 in RCSB is 1M47

The PDB ID of Human IL-2R in RCSB is 2B51.

Residues expected to play key roles in the stabilization of proteins (SRs) are selected by combining several methods based mainly on the interactions of a given residue with its spatial, rather than its sequential neighbourhood and by

considering the evolutionary conservation of the residues. The SRide server is located at http://SRide.enzim.hu.

Stability of a protein can be determined by finding its instability index value for which tools such as PROTPARAM are used. A value less than 40 indicates that the protein is stable whereas a value greater than 40 is indicative that the protein is unstable. The various physical and chemical parameters of the protein was assessed using Protparam. The computed parameters include molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydrophobicity. The primers were designed using an online bioinformatics tool called PrimerX. A text file containing DNA template sequence was uploaded or the sequence was pasted onto the text area.

The substitution to be introduced was entered with their change in nucleotide and position. From the various primes obtained a primer with suitable melting temperature, number of nucleotides and molecular weight was chosen.

B. *DEVELOPMENT OF T LYMPHOCYTE CULTURE:*

Buffy coats harvested from peripheral blood was cultured in a T flask containing RPMI 1640 medium supplemented with Penicillin ,Streptomycin and Fetal Bovine Serum. The mitogen Phytohaemagglutinin (PHA) was added to the culture. The cell culture flask was incubated in a CO_2 incubator at 5% humidity and 37ºC. On the 2^{nd} day cell count was carried out using Tryphan Blue dye and haemocytometer.

C. ISOLATION OF RNA AND cDNA SYNTHESIS

RNA isolation was carried out using TRIZOL reagent. To 1 ml of resuspended cells, 1 ml of TRIZOL and 250µl of chloroform were added and centrifuged at 10,000rpm for 5 mins. 3 layers were formed with the top aqueous layer containing the RNA. The RNA obtained was quantified using UV spectrophotometer at 260nm and 280 nm. OD_{260}/OD_{280} ratio was calculated.

TABLE 1: IL 2 PRIMER DATA SHEET

SNO	**Primer Seq 5'-3'**	**Mer**	**OD**	**MW**	**T_m**	**%GC**
1	5'-ATGTACAGGATGCAACTCCTGTC-3'	23	11.9	7023	64.1	46
2	5'-TCAAGTCAGTGTTGAGATGATGC-3'	23	16.2	7118	64.1	43

For cDNA synthesis, the first stand cDNA was synthesised from the RNA sample. The vial containing the RNA was placed at 65ºC for 10 minutes and then at room temperature for 10 minutes and spun briefly. The following reagents were added RNase Inhibitor, 0.1M DTT,RT Buffer (5X), 30mM dNTP mix and M-MuLV Reverse Transcriptase. The solution was mixed well and incubated at 37ºC for 1 hour. It was incubated at 95ºC for 2 minutes to denature RNA-cDNA hybrids, spun briefly, and then quickly placed on ice. The second strand was synthesised by RT-PCR using the first strand as template. Taq DNA polymaerase, dNTP mix and the IL2 specific primers were added to the reaction mixture and amplification was carried out for 30 cycles.

D. RESTRICTION DIGESTION AND LIGATION:

The phagemid vector pTZ19R was digested using Hind III and the IL2 cDNA was ligated to the digested vector using T4 DNA ligase.

E. TRANSFORMATION AND PLASMID DNA ISOLATION:

E.coli RZ1032 cells were cultured in LB medium and competent cells were prepared using Calcium Chloride method. Transformation was carried out by mixing these competent cells with the recombinant pTZ19R. This mixture was placed on ice for 30 mins and then immediately placed on water bath at 42˚C for 2 mins thus providing heat shock. The cells were then snap chilled on ice for 10 mins. Transformed cells were screened using blue white screening method. From the transformed colonies , the plasmid DNA was isolated using alkaline lysis method.

F. SITE DIRECTED MUTAGENESIS:

Escherichia coli strains used for the various steps were RZ1032 and TG1. The first step of mutagenesis involves

annealing the mutagenic primer to the single stranded plasmid DNA (template). The reaction mixture from the annealing reaction was extended using Sequenase (Amersham) and the newly synthesized strands were ligated using T4 DNA ligase. The extended heteroduplex mixture was then incubated with *E.coli UDG* (~100 ng) at 37°C for 30 minutes and this reaction mixture was transformed into TG1.

III. RESULTS

A. SRIDE TOOL TO DETERMINE VARIOUS STABILIZING RESIDUES IN IL-2 GENE

TABLE 2: Stabilizing residues in IL 2 Gene

SNO	RESIDUE	CONS. SCORE	Hp	LRO	SC
1.	Leu17	9	20.57	0.03279	1
2.	Leu21	9	26.40	0.03279	1
3.	Leu118	7	20.76	0.02459	1
4.	Cys125	9	23.41	0.03279	1

Cons. Score- Conservation score; Hp- Hydrophobicity; LRO-Long Range Order; SC- Stabilizing Centres.

B. *PROTPARAM RESULTS:*

INSTABILITY INDEX VALUE OF MUTANT IL -2

TABLE 3: *SINGLE POINT MUTATIONS AND THEIR INSTABILITY INDEX VALUE:*

MUTATION POSITION	INSTABILITY INDEX
46 (Met – Ile)	**43.40**
69 (Val- Ala)	47.71
74(Gln- Pro)	47.71
91(Val – Arg)	47.71
97(Lys- Gln)	50.91
113 thr- asn)	50.58
120(Arg- Gly)	43.79
128(Ile- Thr)	47.71

The above table shows that mutation at 46th position has least instability index value.

INSTABILITY INDEX VALUE OF MULTI POINT MUTATION

DOUBLE POINT MUTATION AND THEIR INSTABILITY INDEX VALUE

TABLE 4:At position 46

MUTATION POISTION	INSTABILITY INDEX
46 (Met- Ile) & 69 (Val-Ala)	43.22
46(Met- Ile) & 74 (Gln- Pro)	42.87
46(Met- Ile) & 91 (Val- Arg)	43.22
46(Met- Ile)&97 (Lys- Gln)	46.46
46 (Met-Ile)&113 (Thr- Asn)	46.09
46 (Met- Ile) & 120 (Arg-Gly)	**39.30**
46 (Met- Ile) & 128 (Ile-Thr)	43.22

The above table shows that mutation at position (46, 120) has the least instability index value.

TABLE 5: At position 69

MUTATION POSITION	INSTABILITY INDEX
69 (Val- Ala) & 74 (Gln- Pro)	47.35
69 (Val- Ala) &91 (Val- Arg)	47.71
69 (Val- Ala) &97 (Lys- Gln)	50.95
69 (Val- Ala) &113 (Thr- Asn)	50.58
69 (Val- Ala) &120 (Arg- Gly)	**43.79**
69 (Val- Ala) &128(Ile- Thr)	47.71

The above table shows that mutation at position (69,120) has the least instability index value.

TABLE 6 :At position 74

MUTATION POSITION	INSTABILITY INDEX
74 (Gln- Pro) & 91 (Val- Arg)	47.35
74 (Gln- Pro) &97(Lys- Gln)	50.59
74 (Gln- Pro)	50.22

&113 (Thr- Asn)	
74 (Gln- Pro) &120(Arg-Gly)	**43.43**
74 (Gln- Pro) &128(Ile- Thr)	47.35

The above table shows that mutation at position (74,120) has the least instability index value.

TABLE 7 :At position 91

MUTATION POSITION	INSTABILITY INDEX
91(Val- Arg) &97 (Lys- Gln)	50.95
91(Val- Arg) &113(Thr-Asn)	50.58
91(Val- Arg) &120(Arg-Gly)	**43.79**
91(Val- Arg) &128(Ile- Thr)	47.71

The above table shows that mutation at position (91,120) has the least instability index value.

TABLE 8: At position 113

MUTATION POSITION	INSTABILITY INDEX
113 (Thr- Asn)& 120(Arg- Gly)	46.66
113 (Thr-Asn)& 128(Ile- Thr)	50.58

The above table shows that mutation at position (113,120)has the least instability index value.

TABLE 9: At position 120

MUTATION POSITION	INSTABILITY INDEX
120(Arg-Gly)&128 (Ile-Thr)	43.79

TABLE 10 : TRIPLE POINT MUTATION AND THEIR INSTABILITY INDEX VALUE:

MUTATION POSITIONS	INSTABILITY INDEX
46(Met- Ile), 120(Arg- Gly) &69(Val-Ala)	39.30
46(Met- Ile), 120(Arg-Gly) & 74(Gln- Pro)	**38.95**
46(Met- Ile), 120(Arg- Gly) &91 (Val-Arg)	39.30
46(Met- Ile), 120(Arg- Gly) &97(Lys- Gln)	42.54
46(Met- Ile), 120(Arg- Gly) &113(Thr-Asn)	42.54
46(Met- Ile), 120(Arg- Gly) &128 (Ile- Thr)	39.30
69(Val- Ala), 74 (Gln-Pro)&128(Ile-Thr)	47.35
91(Val-Arg), 97 (Lys- Gln) &113 (Thr- Asn)	53.99

The above table shows that mutation at position (46,120 and 74) has the least instability index value

SYBYL TRIPOS RESULTS:

TABLE 11: LIGAND ENERGY VALUE:

MUTATION POSITION	ENERGY VALUE (kCals/mol)
Wild type	-356.439
Mutant IL-2 74 (Gln-Pro),46(Met-Leu) & 120 (Arg-Gly)	-358.345
Mutant IL-2 91(Val-Arg)	-356.448
Mutant IL-2 128(Ile-Thr)	-354.942
Mutant IL-2 74(Gln- Pro)	-353.864
Mutant IL-2 91(Val-Arg),97(Lys-Gln)&113(Thr-Asn)	-353.864
Mutant IL-2 69(Val-Ala)	-353.436
Mutant IL-2 91(Val-Arg),46(Met-Leu),120(Arg-Gly)	-351.207
Mutant IL-2 46(Met-Leu)&120 (Arg-Gly)	-348.461
Mutant IL-2 69(Val-Ala)& 74(Gln-Pro)	-348.215
Mutant IL-2 46 (Met-Leu),120(Arg-Gly)&128(Ile- Thr)	-347.655
Mutant IL-2 69(Val-Ala),74(Gln-Pro)&128(Ile-Thr)	-346.705
Mutant IL-2 69 (Val-Ala),46(Met-Leu)&120(Ile-Thr)	-346.153

The ligand energy values obtained from TRIPOS-SYBYL for various mutational positions.

TABLE 12 : COMPARISON OF THE MUTANT IL-2 WITH THE INSTABILITY INDEX AND ENERGY VALUE

MUTATIONAL POSITION	ENERGY VALUE (kCal/Mol)	INSTABILITY INDEX
Mutant IL-2 74 (Gln-Pro),46(Met-	-358.345	38.95

Leu)&120(Arg-Gly)		
Mutant Il-2 91(Val-Arg)	-356.448	47.71
Wild type	-356.439	47.71
Mutant IL-2 74(Gln-Pro)	-353.864	47.71
Mutant IL-2 69(Val-Ala)	-353.436	47.71
Mutant IL-2 91(Val-Arg),46(Met-Leu),120(Arg-Gly)	-351.207	39.30
Mutant IL-2 46(Met-Leu),120(Arg-Gly)&128(Ile-Thr)	-347.655	39.30
Mutant IL-2 69(Val-Ala),46(Met-Leu)&120(Ile-Thr)	-346.153	39.30

The instability index value and the energy value obtained from Protparam and Tripos SYBYL were compared.

Mutation position (74,46,120) showed the least instability index and has the energy value higher than the wild type. This is a better mutation position to create a recombinant IL-2 which is stable and has higher biological potency.

The primers required for creating a stable and potent mutanIL-2 are:

TABLE 13 : Site Directed Mutagenesis Primer 1 – Position 120

Seq name	Primer seq 5'-3'	Mer	T_m (^oC)	%GC	MW(Da)
Reverse (R)	GTTTTCCATTAGGTAGGCAAGTCTTTAAGATG	32	68.2	38	9884
Forward (F)	CATCTTAAAGACTTGCCTACCTAATGGAAAAC	32	68.2	38	9760

TABLE 14:Site Directed Mutagenesis Primer 2- Position 74

Seq name	Primer seq 5'-3'	Mer	T_m (^oC)	%GC	MW(Da)
Reverse (R)	GAAGTTTTTGCTCGGGCGCCAGGTTCAG	28	75.5	54	8635
Forward (F)	CTGAACCTGGCGCCCGAGCAAAAACTTC	28	75.5	54	8542

TABLE 15 : Site Directed Mutagenesis Primer 3- Position 46

Seq name	Primer seq 5'-3'	Mer	T_m (^oC)	%GC	MW (Da)
Reverse (R)	CCGGAAGAACCGTCCAGTCTTTAAGATG	30	75.2	50	8911
Forward (F)	CATCTTAAAGACTTGGACGGGTTCTTCGG	30	75.2	50	9213

C. LYMPHOCYTE CULTURE:

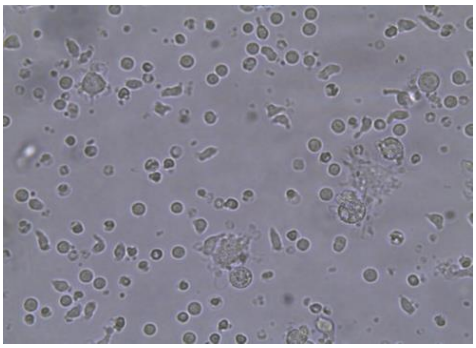

FIGURE 1: Lymphocytes in PHA$^-$ medium

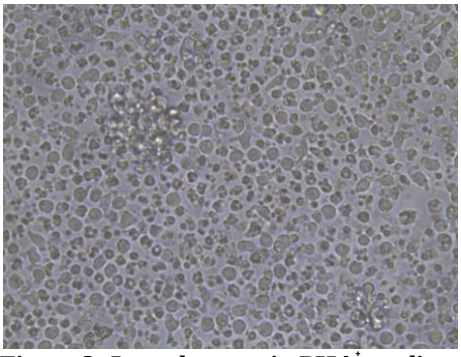

Figure 2 :Lymphocytes in PHA^{+} medium

The above figures clearly show that PHA increases the lymphocytes number and thus increasing the overall mRNA content of the T lymphocyte culture.

D. cDNA CONVERSION

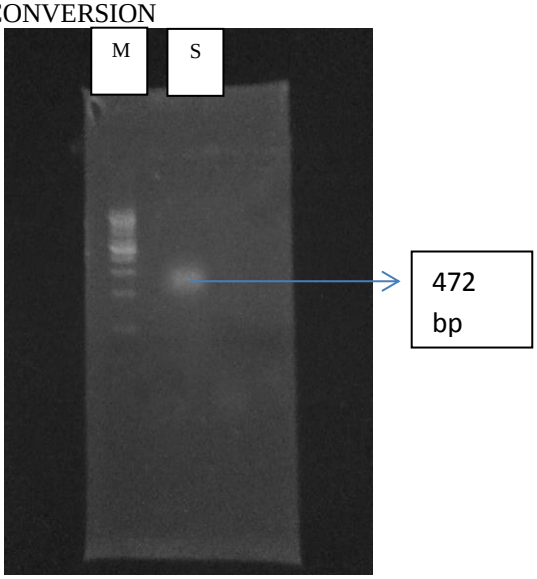

Figure 3:DNA on 1% agarose gel: The cDNA obtained from quantified mRNA was run on the agarose gel against a 1Kb ladder loaded in lane 1 marked as M and the cDNA sample was loaded in the second lane marked as S.

E. TRANSFORMATION AND SCREENINIG

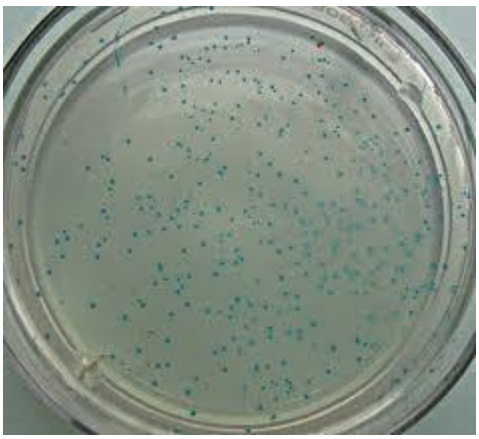

Figure 4: Transformed *E.coli* RZ1032: The white colonies here represent transformed E.coli RZ1032 containing pTZ19R cells with IL-2 cDNA insert, while blue colonies represent the untransformed E.coli RZ1032 with no insert cDNA on medium containing X-Gal.

To confirm the presence of IL-2 cDNA in the vector pTZ19R, plasmid DNA isolation was performed and the isolated DNA was run on 1% agarose gel along with marker. This has been represented in the figure given below.

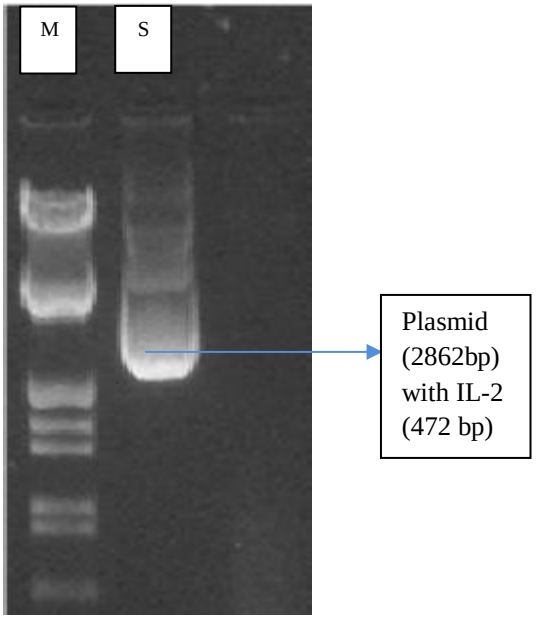

Figure 5 :Transformed vector in 1% agarose gel: The ligated plasmid containing IL-2 insert have been isolated and loaded in lane 2 labelled as S and compared with 20kb ladder loaded in lane 1 marked as M.

IV DISCUSSION

The results obtained from SRide using wild type human IL 2 showed that Leu17, Leu21, Leu118 and Cys125 are stabilising residues. Though the cysteine at 125th position is not involved in any disulphide bond, it cannot be mutated to serine or any other residue as it is a stabilizing centre.

The instability index for various single, double and triple point mutations were found using Protparam. It was found that 91,46,120/46,120,128/46,74,120 had the least instability index values. Hence these positionswere modelled using FUGUE and ORCHESTRAR and their energy values were calculated inTripos-SYBYL. The results obtained showed that the mutant IL-2 with positions 46,74, 120 has better stability than the other mutants. These positions were used to design the promers required for site directed mutagenesis. With the results obtained the invitro studies have been carried out.

PBMC procedure has been carried out and mononuclear cells were isolated for further analysis. Since T lymphocytes are needed for the study the culture was stimulated by the use of a mitogen phytohemogluttinin (PHA), is a glycoprotein found in uncooked red kidney beans. It has been isolated, purified and have been used for culturing of T lymphocytes. The mitogen causes T cells to divide by up regulating the expression of IL-2 and IL-2R. This causes autostimulation and increased transcription of IL-2 mRNA. After 8-16 hrs, when the stimulation of IL2 gene is at its maximum, the culture was used to isolate mRNA and then subsequently synthesize cDNA.

The IL-2 cDNA is then introduced inside pTZ19R phagemid vector using Hind III restriction enzyme and T4 DNA ligase. The vector is then transformed into *E.coli* RZ1032 and selected by blue white screening method. Plasmid DNA is then isolated from the transformed colonies and further work can be carried out to perform site directed mutagenesis.

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VI. REFERENCES

1. ACTG Laboratory Technologist Committee (2004). 'Preparation of PHA-stimulated uninfected donor peripheral blood mononuclear cells'pp.1-7.
2. Andrew J.H.Gearing., Alan P. Johnstone and Robin Thrope (1985). 'Production and Assay of the interleukins a review' Journal of Immuno Methods Vol. 83 pp.1-27.
3. Atkins MB., Lotze MT., Dutcher JP., Fisher RI, Weiss G., Margolin K., Abrams J., Sznol M., Parkinson D., Hawkins M., Paradise C., Kunkel L and Rosenberg SA (1999). 'High-dose recombinant interleukin-2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993'. J clin oncol Vol. 17 pp.2105-2116.
4. Berndt W.G., Chang D.Z., Smith K.A. & Ciardelli, T.L (1994). 'Mutagenic analysis of a receptor contact site on interleukin-2: preparation of an IL-2 analong with increased potency' Biochemistry Vol. 33 pp.6571-6577.
5. Cantrell D.A., Smith K. A. (1984). 'The interleukin-2 T-cell system: a new cell growth model' Science Vol. 224 pp.1312-1316.
6. Chang D.Z., Tasayco M.L & Ciardelli T.L. (1995). 'Structural analogs of interleukin-2: a point mutation that facilitates biological response' Mol. Pharm Vol. 47 pp. 206-211.
7. Fehniger TA., Cooper MA., Fuchs A., Caliguri M.,(2002),'NK cell and DC interactions', Trends in Immunology,Vol 1 pp 47-52
8. Gillis S., Baker P., Urdal D., (1998) 'Expression ,Purification and Characterization of Recombinant marine granulocyte macrophage colony stimulating factor and bovine interleukin 2 from yeast'Vol. 55 pp 287-293.
9. Grimm, E.A., Mazumder, A., Zhang, H.Z., Rosenberg, S.A. (1982). 'Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2- activated autologous human peripheral blood lymphocytes' J. Exp. Med. Vol. 155 pp.1823-1841
10. Hollander, G. A., Zuklys, S., Morel, C., Mizoguchi, E., Mobisson, K., Simpson, S., Terhorst, C., Wishar, W., Golan, D. E., Bhan, A. K., Burakoff, S. J. (1998). 'Monoallelic expression of the interleukin-2 locus' Science Vol. 279 pp.2118-2121.

11. Handa, P. and Varshney, U. (1998) Rapid and reliable site-directed mutagenesis using Kunkel's approach. *Indian J. Biochem. Biophys.* **35,** 63–66.
12. Konrad M., Hemstreet G., Hersh E .,Bradley E., (1990) 'Pharmacokinetics of Recombinant Interleukin 2 in Humans' J of Cancer Research
13. Kovaics, E.J., Becker, S.K., Longo, D.L., Versio, L. and Young, H.A., (1989). 'Cytokine gene expression during the generation of lymphokine activated killer cells: early induction of IL-1b by IL-2' Cancer Res Vol. 49 pp.940-944.
14. Liu, Y., Xun-Yan, Xiao, Min Sun, Ying-He, Hu, Ke-Qing, Ou-Yang, Shao-Xi, Cai and Zi-Chung, Hua. (2006). 'Expression and purification of a Mutant of Human Interleukin-2 in *Pichia pastoris'* Applied Biochemistry and biotechnology Vol. 133 pp.2273-2289.
15. Smith, K.A. (1988). 'Interleukin-2: inception, impact, and implications' Science Vol. 240 pp.1169-1176.
16. Smith, K.A., Lachman, L.B., Oppenheim, J.J., Favata, M.F. (1980). 'The functional relationship of the interleukins' J Exp Med. Vol. 6 pp.1551-1556.
17. Stewart, J.A., Belinson, J.L., Moore, A.L., Dorighi, J.A., Grant, B.W., Haugh, L.D., Roberts, J.D., Albertini, R.J., Branda, R.F. (1990). 'Phase I trial of intaperitoneal recombinant interleukin-2/ lymphokine-activated killer cells in patients with ovarian cancer' Cancer Res Vol. 50 pp.6302-6310.
18. Talmadge, J.E. (1985). 'Immunoregulation and immunostimulation of murine lymphocytes by recombinant human interleukin-2' J Biol Response Mod. Vol. 4 pp.18-34.
19. Wang, Z., Smith, D. G., and Mosbaugh, D. W. (1991) Overproduction and characterization of the uracil-DNA glycosylase inhibitor of bacteriophage PBS2. *Gene*
20. Xiaowei Wang and Brian Seed (2003). 'A PCR primer bank for quantitative gene expression analysis' Nucleic Acids Research Vol.31 (24) pp.1-8.