Cloning of the
B3 gene from
A20 Cells

Chapter II
2.1 INTRODUCTION:
B3 was initially detected on B cells that had been activated with LPS for 72 hrs. It was found, from partial characterization, to be a glycophosphoprotein of 39kD. Tryptic digestion and subsequent peptide sequencing indicated that it had 95% homology to Pyruvate Kinase M2 isoform (Dass S Vinay et al, 1995). The Pyruvate Kinase M2 (PK M2) isoform consists of a tetrameric structure. Each monomer is encoded by 530 amino acids (approximately 55kD). Moreover, PK is a cytosolic, glycolytic enzyme, with no obvious membrane spanning regions. This enzyme catalyzes the rate limiting step in glycolysis and no glycosylated forms of this enzyme have been reported till date.

During the initial characterization of B3, we obtained a peptide sequence "LNFDHETHEY" that lies 72 amino acids from the N terminal and this sequence has 90 % homology to the PK sequence "LNFSHGTHEY", with substitutions at two positions, Serine to Aspartic acid and Glycine to Glutamine. (Fig 2.1)

In a subsequent attempt to sequence B3, the following protein sequence "AEAAIYHLELFEE" was obtained, which once again exhibited homology to PK. The newly obtained sequence lies approximately 450 bp upstream of the C terminal. Since we knew two sequences that were present in B3 as well as in PK, pairs of primers were designed based on this data.

Using the published data on Pyruvate Kinase (Takenaka M et al.1989; Izumi S et al, 1995) two primers, one from the N- terminal (referred to as PK upper) and another from the extreme C- terminal end (referred to as PKL) were designed. The B3 specific primer (B3 sense) corresponding to the "AEAAIYHLELFEE" stretch was designed along with the antisense primer (B3 antisense) of the same region. In addition a middle primer, 800bp from the N terminal was also designed. The strategy focussed on the assumption that using a combination of these primers it would be possible to identify the entire gene, depending on the common sequences present in both PK as well as in B3 (Fig 2.1).

Further experiments in our laboratory demonstrated that the B3 molecule is also expressed constitutively on the A20 (ATCC TIB 208) cell membrane. A20 is a B cell lymphoma cell line derived from a spontaneous reticulum cell neoplasm found in an old (older than 15 months) Balb/c mouse. These cells are IgG+, Ia⁺ Fc⁺, Ig M⁻, IgA⁻ and complement receptor negative. (Kim KJ et al, 1979; Glimcher LH, et al, 1982). This cell line is capable of presenting both alloantigens and protein antigens to alloreactive and antigen reactive T lymphocytes in a MHC restricted fashion,
Fig:2.1 : MOUSE PYRUVATE KINASE M2 SEQUENCE INDICATING PEPTIDE SEQUENCE DAT PRIMERS FOR AMPLIFICATION.

+3 E V R P R T S G A T M P K P H S E A G T
5'1 GAA GTA CSG CSG AGG ACT TCA GAA CAT ATG GCG AAG CCA CAC AAT GAA GCA GGG ACT
PK UPPER PRIMER
+3 F I Q T O G L H A M A D V F E L H M
61 TTC ATT CAG ACC CAG CAG CTC CAT GCA GCC ATG GCT GAC ACC TTC CAG GAA CAC ATC
+3 R L D I D S A F I T A R N T G I I C T
121 CSG CTG GAC ATT GAC TCT GGG CCC ATC AGG GCC GGC AAC ACT GGC ATT TGT AGC
+3 G P A S R S V E M L K E M I K S G M N
181 GGG CCT GCT TCC GAA CTG GAG ATT CAG GAG ATG ATT AAC TCT GGA ATG ATG

PK MIDDLE PRIMER

+3 A R L N F S H O T H E Y H A E T I K N
241 GCT CGG CTG ATT TTC TCT CAT GGA ACC CAT GAG TAC CAT GCA GAG ACC ATC AAG AAT

+3 R E A T E S F A S D P I L Y R P V A V
301 COT GAA GCC ACA GAA TTG GCC TCT GAT ACC ATC TAC CTG CTG GAT GGC GTG

+3 L D T K G P E I R T G L I K G S G T A
361 CTG GAT ACA AGG GGA CCT GAG ATG CCG ACT GAA CTC ATG AGC GCC GGC ACC GCT

+3 V E L K K G C A T L F I T L D N A Y M E
421 GTC GAG CTT AAG AAG GGA GCC ACT CTG AAG ATC ACC CTG GAC ACG GAC ACG OCT

+3 C D E N I L R L D Y K N I C K V V E V
481 TGT GAG GAC ACG ATC CTG CGG TGG GAC TAC AAG AAC ATC TGC AAG GTG CTG GAG

+3 S K I Y V D D G L I S L Q V K E K G A
541 ACC AAG ATC TAC TGT GAC GAT GGG CTC ATC TCA CGG CTG AAG TGC AAA

+3 F L V T E V E N G G S L G E K K G Y V N
601 TTC CTG CTG AGG GNG GTC GNG AAT GGT GGC TCC TTC TGG GGC ACG AGC AGG GGC CTG AMC

+3 P G A A V D L P A V S E K D I Q D L K
661 CCC GGC GCT GCT GCT GAT TCT CTC CCC GCT GTC TCC GAA AGG GAC ATC CGG CTG AMG

+3 G V E Q D V D M V F A S F I R K A A D
721 GGC GTG GAG CAG GAT GTC ACG GGT GTG CTG TAC AAC GCC GGC GAC OCT

+3 H E V R K V L G E K K G K N I K I I S K
781 CAT GAA GTC AGG AAG GTC CTG GGA GAG AGG GAG AAC ATC ATG AAC ATG ATC AAG AAA

PK SENSE PRIMER

+3 E N H E G V R R F D E I L E A S D G I
841 GAG AAC HAC GGC UCG TCC AGG AAG TTT GAT GAC ATC TTG GAG GCC AGC GAT GGG ATC

+3 V A R G D L G T E I P A E K V F L Q S
901 GTC GCC ATT GCC GGC ATC GCC AGG ATT CTT GCA GAG ACG TTC TGG TTC TCT GAG

+3 M M I G R C N R A G K P V I C S T Q M
961 ATG ATG ATC GGG CGA TGC AAC GAC GCT GGG AAG GTC ATC TAT TGT CAG ATG CAG AMC

+3 E I M I K K K R P R T R A E G S D V A N
1021 GAG ATC ATG ATC AAG AAG CCA CCG CCC CCC ACC CTT GAA GAT GAT GTC ACG ATG

+3 V L D G A D C I M L S G E T A K G D Y
1081 GTC CTG GAT GGA GCC GGC TCC ATC ATG AGG TCG TCT CTG GAA ACA GCC GCC ACG TAC

+3 A E A A I Y
1141 CTG GAG GCT GCT GCC ATG CAG CAC ATC ATT GCC GCA GGC GAC GCC GCT GCC ATC TAG ATG

B3 SENSE PRIMER

+3 L Q L F E E L R L A P I T S D P T E
1201 TGG CAG CTA TCC GAG GAA CTC GCC GCC GGC GCG GCG GCG ATT ACC AGC GAC CCC ACA GAA

AAG GTC GAT AAG CTC B3 ANTISENSE PRIMER
indicating that it expressed costimulatory molecules (Liu Y and Janeway Jr CA, 1992).
Therefore this cell line was utilized for preparation of Genomic DNA and cDNA. Amplification of the required sequence using the previously described sets of primers was initially attempted. Two approaches were adopted, firstly Genomic DNA was prepared and amplification was done in an effort to get the full-length gene, if any present. Secondly, cDNA was prepared, and a similar amplification was undertaken to obtain the cDNA sequence of B3. For this purpose we chose the PolyATtract system (Promega) to capture the specific mRNA, after lysis with Guanidine thiocyanate.
In order to obtain information regarding the number of B3/PK like sequences present in the cell, the products obtained by PCR were cloned in suitable vectors. Sequencing and restriction analysis was done to characterize these products.
2.2 MATERIALS:

2.2.1 Cell Lines and Media for Cell Culture:
TIB 208/A20
RPMI 1640
FCS (decomplemented)

2.2.2 Bacterial Strains and Media:
JMI09 strain
Luria Bertani Medium: Bactotryptone, Yeast Extract and Agar were obtained from Hi-Media.
M9 Media

2.2.3 Antibiotics:
Cell culture media contained penicillin and streptomycin at a concentration of 60 mg/l and 100 mg/l respectively.
Ampicillin (Sigma) was used as a selection marker in all the plasmids. It was added to liquid Media as well as in plates to a final concentration of 100 μg/ml.

2.2.4 Requirements for mRNA preparation:
DEPC Sigma
Polyattract System 1000 Promega
Random Primers Promega
Rnase free Glycogen Stratagene

2.2.5 Primers synthesized by Gemini Biotech:
PK Upper Primer : 5’ AGG ACT TCA GGA CAT ATG GAG AAG CCA CAC A
PK Lower Primer : 5’ GAG GGG ATC CAG AGG GCC ATC AAG GTA CAG
B3 Sense Primer : 5’ AGG CTG CCA TCT ACC ACT TGC AGC TAT TC
B3 Antisense Primer: 5’ CTC GAA TAG CTG CAA GTG GTA GAT GCC
PK Middle Primer : 5’ GCA GCC GAC GTG CAA GAA GTC AGG

2.2.6 Enzymes:
AMVRT Promega
RNase H Promega
Taq Polymerase Promega
Vent Polymerase New England Biolabs
Restriction enzymes used were from New England Biolabs. SmaI, EcoRI, Hind III, Bam HI, Pst I, Nco I, Nde I, SpeI.

2.2.7 Vectors:

- pGEM TEasy Promega
- pGEM 3zf(+) Promega

2.2.8 Common Chemicals

- Qiagen Gel purification Columns
- Proteinase K Sigma
- RNase A Sigma
- SDS Sigma
- Manganese Chloride Sigma
- HEPES Sigma
- EDTA Sigma
- Tris Base Sigma
- DMSO Sigma

Tissue Culture Grade 50.ml Plastic tubes. All glassware and plasticware used for mRNA preparation were DEPC treated and autoclaved before use.
2.3 METHODS:

2.3.1 Cell Culture:
A20 cells were cultured on a large scale. Cells were cultured in RPMI 1640 containing 10% FCS. Streptomycin and Penicillin were added to a final concentration of 100 mg/l and 60 mg/l respectively. Roller bottles containing 200 ml media were seeded with cells. Cultures were maintained at 37°C on a roller unit until the cell number obtained was $5 \times 10^8$.

2.3.2 Genomic DNA preparation from A20 cells:
Genomic DNA preparation was done essentially by the modified protocol of Blin and Stafford (1979) (Sambrook J et al, 1989).
Each preparation was made from $5 \times 10^7$ cells. Cells were washed twice in 1X PBS and resuspended initially in 1.0 ml of 1 x TE. Cells were resuspended using minimum amount of shearing, and this 1.0 ml was gently mixed into 10.0 ml of Extraction Buffer (10mM Tris pH 8.0, 0.1 M EDTA pH 8.0) containing 0.5% SDS and 20 μg/ml of RNase A. The mixture was maintained at 37 °C for 1.0 hr. This was followed by the addition of Proteinase K to a final concentration of 100 μg/ml. The solution was maintained at 50 °C for a period of three hours and mixed intermittently.
Extraction was carried out with an equal volume of TE equilibrated Phenol, (pH 8.0). The mixture was kept on shaking for 10 min followed by centrifugation at 5,950g for 10 min. Aqueous phase was collected and the extraction was repeated twice more. Care was taken to minimize shearing of the Genomic DNA during all these steps.
Ammonium Acetate was added, 0.2 volumes /ml of 10 M to the aqueous phase alongwith an equal volume of absolute Ethanol. The precipitated DNA was collected, and the pellet was washed with 70 % Ethanol and air-dried. It was resuspended in 500 μl of 1X TE and a 4.0 μl aliquot was analyzed on a 1% Agarose gel containing Ethidium Bromide. 1X TAE was used in the buffer system.

2.3.3 Gene Amplification from Genomic DNA:
To amplify the PK from Genomic DNA, three combinations of primers were used. First, the Pyruvate Kinase upper and Pyruvate Kinase lower primers were used. Second, the Pyruvate Kinase middle and lower primers were used. Thirdly, the B3 sense primer was used in combination with the PK lower primer.
μl of nuclease free water. Precipitation of mRNA was completed using 1/10th volume of sodium acetate and two volumes of RNase free absolute ethanol. The tube was stored at −70 °C for 4 to 5 hrs after addition of 5.0 μg of nuclease free Glycogen. The mRNA was obtained by centrifugation at high speed, 20,800 g for 30 min at 4 °C, the pellet was washed with 70% ethanol and air dried.

2.3.5 First Strand Synthesis:
First strand mix (25 μl) containing 5 mM MgCl₂, 10X RT buffer, 1mM dNTPs and 200ng Random Hexamers was added to the pellet obtained by precipitation. The random primers were annealed to the mRNA by incubation at 70 °C for 10 min. The components were equilibrated for 15 min at 42 °C followed by addition of AMVRT (60 units/3.0μl) and Rnasin (80 u/2.0 μL). The reaction was carried out for 1 hour.

2.3.6 Second Strand Synthesis:
Second Strand components were added to a final volume five times that of the initial volume of first strand synthesis, the reaction was carried out at 16 °C for 4 hrs.
After second strand synthesis the Klenow was inactivated by heating to 65 °C for 10 min and the cDNA was precipitated with 0.1 volume of Sodium acetate and two volumes of ethanol at −70 °C. The DNA was recovered by centrifugation at 20,800 g at 4 °C and the pellet was washed with 70 % Ethanol. The cDNA was reconstituted in a final volume of 10.0 μl in Nuclease Free Water.

2.3.7 Gene Amplification from cDNA:
Using combinations of primers (PK upper/PK lower, B3 Sense/PK lower and PK middle/ PK lower) PCR was carried out, with cDNA as template.
For each reaction cDNA (1.0 μl) was used as template, each cycle consisted of a 95 °C, 3 min denaturation step; 55 °C, 2 min annealing and 72 °C, 3 min extension. The final step was a 10min extension at 72 °C. The remaining PCR conditions were the same as described before.

2.3.8 Cloning of 450 bp product from Genomic DNA and cDNA:
Initially, the 450 bp product obtained by amplification using the B3 sense primer and the PK lower primer, was cloned in the pTEasy vector (Fig 2.2). The TEasy
Fig 2.2: Cloning of 450 bp product from in pGEM 3Zf (+/-)
system utilizes the fact that Taq polymerase leads to the addition of "A" residues at the end of the PCR product. The vector has cohesive ends which are "T" tailed. The cloning of the product from cDNA was done in pGEM 3zf(+) at the Sma I site present in the multiple cloning site. (Fig. 2.3)

A large volume (100 µl) PCR reaction was carried out in order to obtain the product in sufficient amount. The product was analyzed on a 1% agarose gel followed by precipitation with 0.1 volumes of sodium acetate and 2 volumes of Ethanol at −70 °C. After washing the pellet with 70% Ethanol it was reconstituted in 10 µl 1X TE. The entire amount was loaded on a 1% agarose gel. The required bands of DNA were cut and purified using gel purification columns (Qiagen). DNA concentration was determined visually by analysis on a 1% agarose gel.

2.3.9 Ligation Reaction:
The vector to insert ratio used was 1:3. Ligation using the TEasy vector was carried out with 2 to 5 units of Ligase, 1X ligase Buffer containing 1.0 mM ATP at 4°C overnight. For the cDNA fragment, 100 ng of the pGEM 3zf (+) was digested with Sma I as per the manufacturers instructions. The vector and insert were mixed at a 1:3 ratio. Reaction contained 50 units of ligase, 1X ligase buffer containing 1.0 mM ATP. Reaction was completed at room temperature for 6 to 8 hours.

2.3.10 Preparation of Competent Cells and Transformation with Plasmids:
Competent cells were prepared, according to the protocol of Inoue et al (Inoue H et al, 1990). Briefly, JM 109 strain was inoculated in 10.0 ml LB overnight at 37 °C. Approximately, 200 µl was inoculated in 100.0 ml LB at 18 °C until the OD reached 0.4. Cells were pelleted at 2000g for 10 min at 4°C. This was followed by a 10 min incubation with 10.0 ml TSS solution containing 10mM HEPES, 55mM MnCl₂, 250mM KCl, 15mM CaCl₂ pH 6.8 with 7% DMSO. Cells were pelleted and resuspended in 5.0 ml of TSS containing 5% DMSO (v/v). Cell suspension (200 µl) was used for a single transformation. Ligation mix (10 µl) was added and the tube maintained on ice for 30 min, followed by heat shock at 42 °C for 30 seconds. LB media (800 µl) was added and the entire volume incubated at 37 °C with shaking, for 1 hour. A volume of 200 µl was plated on appropriate selection plate and incubated overnight. The remaining 500µl was
Fig 2.3: Cloning of 450 bp PCR product in pGEMTEasy vector
inoculated in 10.0 ml LB containing ampicillin. A plasmid preparation was done using this enmass culture.

**2.3.11 Plasmid Isolation:**
Plasmid isolation was done by the alkali SDS lysis method Sambrook et al. Briefly, a 10.0 ml culture was grown till late log phase and pelleted at 2000g. Cells were lysed by the addition of 100 µl of lysis buffer and the tube maintained on ice for 10 min. This was followed by the addition of 200 µl of the alkali SDS solution and cells maintained on ice for 5 min. Finally, 150 µl of Potassium acetate solution (5 M) was added and cells were maintained on ice for 10 min. The supernatant was collected after high speed centrifugation and equal volume of Isopropanol was added. Precipitation was completed at -20 °C for one hour. The pellet obtained on centrifugation was washed with 70% Ethanol and air dried. After resuspension in 200 µl of TE, DNase free RNase A was added (2.0 µl of 10mg/ml stock), for 1 hour at 37 °C. Extraction was done using Phenol : Chloroform : Isoamyl alcohol (25:24:1), followed by extraction with Chloroform : Isoamylalcohol. DNA was precipitated by the addition of equal volume of Ethanol and 0.1 volume of Sodium acetate, for 2 hours at -70 °C. The pellet was recovered by high speed centrifugation at 4 °C and washed with 70% Ethanol. The pellet was air dried and resuspended in 20 µl of TE and 2.0 µl checked on a 1% agarose gel containing Ethidium Bromide 0.5 µg/ml.

**2.3.12 Screening of recombinants:**
The presence of the required insert in the plasmid was confirmed by restriction digestion analysis with EcoRI, there exist two flanking EcoRI sites in the vector. In the pGEM3zf (+) vector the insert is flanked by the EcoRI and HindIII sites. Digestion was done with restriction enzymes, which were known to have sites with the cloned sequence. These sites were determined based on the known PK M2 sequence.

**2.3.13 Sequencing of positive clones:**
Sequencing was done on the automated sequencer, ABI Prism Model 377. Required clones were sequenced using the T7 and SP6 Primers.
2.4 RESULTS AND DISCUSSION:

2.4.1 PCR of Genomic DNA:
Genomic DNA was amplified with PK upper and PK lower primers and a 1.6 kb PCR product was obtained. Amplification with middle and the lower primer resulted in a fragment of 800 bp. With the third PCR, using B3 sense primer and the PK lower primer a single fragment of 450 bp was visible (Fig. 2.4). The 1.6 kb product was used as template, with the middle and PK lower primer, and the B3 sense and PK lower primer, the two fragments of 800 bp and 400 bp were once again obtained. Previous reports confirmed the presence of a processed pseudogene of Pyruvate Kinase. The fragments that were seen appear to be from this pseudogene. The complete PK gene consists of exons and introns and has a length of 20 kb. It is unlikely that this could be amplified with the methods employed here.

2.4.2 PCR with cDNA:
Amplification using cDNA as template also showed PCR products of similar size, as seen with the Genomic DNA. When the Pyruvate Kinase middle and the C-terminal primers were used a product of 800 bp was seen. Amplification with the B3 sense primer and the PK lower primer of Pyruvate Kinase resulted in a fragment of 450 bp (Fig. 2.7). However, the largest fragment of 1.6 kb could not be amplified, probably because strand synthesis was not extended till the 5' end of the cDNA.

2.4.3 Cloning of the PCR product:
The PCR products were cloned in suitable vectors. Transformation was done using 200 µl of competent cells. Recombinants were identified by blue white selection using 0.5M IPTG and 40 µg/ml of X-Gal. Five white colonies were identified and cultured in a 10.0 ml of LB media. Recombinants were confirmed as positive by insert release using the enzyme Eco RI (Fig. 2.5). In the TEasy system three out of five colonies were found to contain insert. Similarly, recombinants were screened for the insert from cDNA and out of the five colonies screened one was found to be positive. Double digestion using Eco RI and Hind III confirmed the presence of insert (Fig 2.7). It was later confirmed by sequencing that the clone had single base change from C to T at position 1195bp. This resulted in an amino acid change from Leucine to Phenylalanine.
Fig 2.4: PCR amplification from A20 Genomic DNA

Genomic DNA from A20 cells was PCR amplified using a combination of PK prime analyzed on a 1% Agarose Gel. Lanes, 1. PK upper and lower primers, 2. PK midc lower primer, 3. B3 sense and lower primer, 4. Control PCR, 5. lambda-BstEII (size in kb)
Fig 2.5: Insert Release for identification of 450 bp Ins in pTeasy clones.

Plasmid DNA from three individual clones were screened for the presence of ins restriction digestion with EcoRI. Samples were run on a 1% Agarose Gel. Lanes 1. Lambda/BstEII marker (size in kb), 2. Clone 1 / undigested, 3. Clone 1/ EcoRI dig, 4. Clone 2 / undigested, 5. Clone 2/ EcoRI digest, 6. Clone 3 / undigested, 7. Clone 3/ EcoRI digest, 8. 450bp PCR product
Fig 2.6: Restriction Analysis of 450 bp Teasy Clp

Plasmid DNA from Clone 1 was analyzed by restriction digestion to confirm the presence of the desired insert. Restriction enzymes used contained one site in the insert. Samples were run on a 2% agarose gel. Lanes: 1. NcoI digest, 2. SpeI digest, 3. Ncol-Spe I digest, 4. Kpn I digest, 5. Clone 1 undigest, 6. Vector alone, 7. Lambda-BstE II marker (size in kb)
**Fig 2.7 : Insert Release and Restriction Digestion**

450 bp (cDNA) fragment in pGEM 3zf(-).

Plasmid DNA was digested with EcoRI and Hind III to confirm insert release was also digested with Ncol, as the insert contains an Nco I site. Sample analysed on a 1% agarose gel. Lanes, 1. 3zf(-)/Ncol digest, 2. Clone/ NcoI Clone / EcoRI -HindIII digest, 4. 3zf(-)/EcoRI -HindIII digest, 5. 3zf(-)/Eco 6.Clone / EcoRI digest, 7. 450 bp PCR product from cDNA, 8. 800 bp Pci cDNA, 9. Lambda DNA / BstEII markers
The presence of inserts was confirmed by restriction digestion of plasmids. The TEasy vector lacks a Kpn I and Sph I site, digestion resulted in the plasmid being linearized, this indicates that the insert contains a Kpn I / Sph I site.

Digestion was also done using Nco I, the vector backbone has one Nco I site at position 37. The presence of an additional Nco I site (position 1481 bp in PKM2) in the insert yielded a small fragment of 152 bp.

The presence of the Nco I, Kpn I and Sac I sites are comparable of their location in the published Pyruvate Kinase sequence. The vector backbone contains a Spe I site just within the Eco RI at position 64, this enzyme site is not present in the insert. Digestion with Spe I and Nco I resulted in two fragments of approximately 150 bp and 300 bp (Fig 2.6).

2.4.4 Sequencing Results:
Sequencing of the 450 bp fragment obtained by amplification using B3 sense and PK lower primer was done. This product had been amplified from Genomic DNA, and the product cloned in pTEasy vector. This sequence was essentially identical to the sequence 1162 bp to 1596 bp of Pyruvate Kinase M2. There were a few minor differences between the known sequence of PK M2 and the cloned sequence. The differences were as follows: L (CTC) to F (TTC), V (GTG) to L (TTA) and D (GAT) to N (GAT) as indicated (Fig.2.8). An EcoRI site was identified in the Teasy clone at position 1195 bp, due to a single base change from "C" to "T".

An identical sequence was obtained from the cDNA cloned in pGEM3zf (+)
Fig 2.8: PCR amplification using B3 & PK Lower primers.

Sequence data from pTEasy clone.

G G R E F T S D * A A I Y H L Q L F
GCC GCC GCC GAA TTC ACT AGT GAT TAG GCT GCC ATC TAC CAC TTG CAG CTA TT

F R R L A P I T S D P T E A A A V G
TTC GCC GCC CTG GCG CCC ATT ACC AGC GAC CCC ACA GAA GCT GCC GCC GCG TTG G

E A S F K C C S G A I I V L T K S G
GAG GCC TCC TTC AAG TGC TGC AGT GGG GCC ATT ACG ACC CTC ACC AAG TCT G

A H Q V A R Y R F R A P I I A V T R
GCT CAC CAA GTC GCC AGG TAC GCC CCT CGG GCT CCT ATC ATT GCC GTG ACT CG

Q T A R Q A N L Y R G I F P V L C K
CAG ACT GCC CGC AGC GCC CAT CGT TAC CGT GCC ATC TTC CTT CTG TGG TGT AA

L L N A W A R D V D L R V N L A M S
TTA CTG AAT GCC TGG GCT GAG GAT GTC GCC GTT AGA TTC AGG GCC ATG GA

K A R G F F K K G M V V I V L T G W
AAG GCC CGA GCC TTC TTC AGG AGG AGA AAT GTG GTC ATT GTG CTC ACC GGG TG

G S G F T N T M R V V P V P * S N
GCC TCT GGA TCC AAC AGC ATG CTT GTA GTC CCT GCT TTA AGT AAT

Seq. in Pyruvate Kinase

L (CTC)
V (GTC)
D (GAT)

Difference in the cloned product

F (TTC)
L (TTA)
N (GAT)