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**HUMAN INTERFERON REGULATORY FACTOR-2 (IRF-2): CHIMERIC
PROTEIN AND EXPRESSION ANALYSIS**

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ABSTRACT

Interferon Regulatory Factor (IRF-2) is the multifunction mammalian transcription factor involved in antiviral defense. IRF-2 is regarded as a proto-oncogenes found to be mutated or upregulated in certain tumor like pancreatic tumor. This transcription factor binds to element called IRF-E (Interferon Regulatory Element), which is found in the promoter of various genes like IFN and INF inducible genes. The mechanism by which it causes cancer is not fully elucidated, which is attributed to its DNA binding activity. In the present study, we report first time full-length molecular cloning and expression analysis of chimeric human IRF-2 from human blood. We have successfully cloned this gene cDNA in T/A cloning vector by generating cDNA using RT-PCR technique and further sub-cloned in pGEX2TK *E.coli* expression vector. Chimeric IRF-2 protein has been expressed in *E.coli*/BL21 cells as a GST-IRF-2 (~66 KDa) fusion protein. We observed profound full length expression of chimeric IRF-2 protein in *E.coli* adjudged by 12.5 % SDS-PAGE. Furthermore, we observed expression of recombinant IRF-2 after 15 minutes of IPTG induction and expression increased up to 3 hours. We have not observed any low molecular mass bands are getting induced with the full length fusion protein, which indicated that the recombinant protein is stable in nature and not undergoing proteolytic degradation. We also optimized the IPTG concentration 0.5 mM as 0.6 and 0.7 mM concentration of IPTG was toxic to cell and expression level was qualitatively low. This study is first step to analyze this molecule *in vitro*. Further study will pave a way to understand the molecular mechanism of DNA binding

activity of this molecule, which is deregulated in cancerous cells that may help to design drug targeting IRF-2DNA binding domain to cure cancer.

Keywords: Interferons, Recombinant, DNA Binding Domain, cDNA, IRFs, IRF-E, Oncogene, Antioncogene

INTRODUCTION

Interferons (IFNs) are antiviral proteins; activate a group of transcription factors called Interferon Regulatory Factors (IRFs) [1]. IRFs have helix-turn-helix (HTH) motif, tryptophan (w) repeats in their DNA binding Domain (DBD). They regulate transcription of many mammalian genes induced by cytokines and other agents [2]. So far, ten members of the IRF family have been reported, they are IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, ICSBP (IRF-8), IRF-9/p48/ISGF3 γ , IRF-10 and vIRF (coded by the human herpes viruses) [3]. IRFs1/2 recognise certain variants of a consensus hexanucleotide sequence (GAAANN, where N, any nucleotide) present in promoters of mammalian genes induced by virusIFN and IRF-1/2 indicating multiple pathways of virus inducibility of the IFN α/β genes [4]. IRFs actively participate in cellular process like defence against viruses and pathogens, regulation of cell growth and differentiation, antioncogenesis, regulation of immune response and apoptosis [5]. Therefore, IRFs are the important transcription factors for regulation of many genes in mammalian cells and tissues.

IRF-2, which is known as an oncogenic, is a mammalian transcription factor. Recent study has shown that IRF-2 is also act as a tumor suppressor gene in Hepatitis virus B induces hepatocellular carcinoma is belonging to IRF family [6], whose members are performing diverse functions in cell-like apoptosis, specialization of immune cells functions, cell-cycle regulation, virus latency and oncogenesis/antioncogenesis. Among all the members of IRF having conserved DNA binding domain at N-terminal by which these transcription factors bind to IRF-E (IRF-Element), which is present in diverse sets of genes promoters thereby they regulate wide range of gene(s) expression. Major differences observed at C-terminus of these family members by which they show diverse functions. For instance, IRF-1 is an antioncogene whereas IRF-2 is both tumor suppressor and proto-oncogenic.

Recently, umpteen of papers claimed IRF-2 gene is responsible for pancreatic tumor progression, which happens when IRF-2 gets mutated or its level goes up than normal level [7]. From pancreatic tumor having two point mutations reported in the DNA binding domain; 34th no. a.a

phenylalanine to serine and 60th no. a.a histidine by asparagine that leads to made protein functionally inactive [8]. Thus, DNA binding domain is crucial for the function of this molecule. How does a same molecule act as oncogene in pancreatic cell as well as anti-oncogene in liver cell is a mystery?

In this study, we have first time reporting full length human IRF-2 cloning of ORF in T/A vector from human peripheral blood followed by sub cloning in pGEX2TK expression vector for expression of recombinant protein as GST-IRF-2 fusion protein in *E.coli*. Subsequently, we have done chimeric proteins expression analysis in *E.coli*. This study is first step towards analyzing these factors *in vitro*. Functional study is being carried out to explore the functions of these factors *in vitro*. Since these factors are involved in oncogenesis/antioncogenesis so it shall help to decipher the oncogenesis/antioncogenesis mechanism depending upon further analysis.

MATERIALS AND METHODS

Vectors, *Escherichia coli* cells, and reagents: pTZ57R/T- cloning vector (ThermoScientific, USA), pGEX2TK (GE healthcare Life Science, USA) expression plasmid, *E. coli*/ DH5 α and BL21 cells (Zymo Research, Epigenetix, USA) for IRF-2 cloning and expression,

respectively, PCR clean-up kit (Genetix Biotech Asia Pvt. Ltd.), first strand cDNA synthesis kit (Genetix Biotech Asia Pvt. Ltd.) IRF-2 ORF amplification primer pairs: forward primer (FPHUcIRF-2: 5'AAGGATCCATGCCGGTGGAAAGGATGCG 3') and Reverse primer (RPHUcIRF-2P2: 5'TTGGATCCTTAACAGCTCTTGACGGGG 3') of annealing temperature 68°C were designed from human mRNA sequence (Accession No. NM_002199.3) by adding *Bam*HI restriction site in both primers, *Taq*DNA polymerase (G-Biosciences, USA), restriction enzymes (New England Biolabs, UK), T₄ DNA ligase (New England Biolabs, UK) and Calf Intestinal Alkaline Phosphatase (GeNei, UK) and molecular biology grade reagents (Sigma Chemicals Co. USA). The most commonly used molecular biology methods were adopted from the reference [9] and suitably modified.

Total mRNA isolation from human blood: 200 μ l self-donated fresh healthy human (male of age 25 years) peripheral blood collected in a sterile vial, used for total mRNA isolation using RNA isolation kit (Genetix Biotech Asia Pvt. Ltd). After isolation of mRNA, quality of mRNA was checked on 1.2% agarose gel, and quantification was carried out by nanodrop (Thermo Scientific 2000c) instrument.

First strand cDNA synthesis: First strand of cDNA was synthesized from isolated mRNA in following reaction: Twenty microliter reaction was set and following ingredients were taken in PCR eppendorf tube; mRNA 1 µg (10 µl volume), oligodT₁₈ primer 1 µl, 5X reaction buffer 4 µl, RiboLock RNase Inhibitor (20U/ µl) 1 µl, 10 mM dNTP Mix 1 µl and M-MuLV Reverse Transcriptase (20U/ µl) 2 µl followed by incubation for 60 minutes at 37°C. Reaction was terminated by heating at 70°C for 5 minutes.

Human IRF-2 ORF amplification by PCR: IRF-2 ORF specific primers (RPHUcIRF-2 and RPHUcIRF-2 for IRF-2) were used to amplify the desired region. The PCR cycle parameter used for IRF-2 was as follows: 94°C for 5 minutes, 35 cycles for 94°C for 30 seconds, 68°C for 1 minute, 72°C for 1 minute extension, followed by final extension at 72°C for 10 minutes.

T/A-cloning of Human IRF-2 ORF and sequencing: The PCR products were separated on a 1% agarose-ethidium bromide gel, cut out from the gel and purified (Gel Extraction Kit, Qiagen). Two microlitres of purified PCR product (~ 25 ng) was ligated into pTZ57R/T- cloning vector (Fermentas) in the following mix: 2 µl of gel purified IRF-2 ORF, 2 µl pGEX2TK vector (25 ng/ µl), 1 µl T4 DNA

Ligase (3U/ µl), 5 µl Ligation buffer (2X) (30 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 10 mM ATP, 5% polyethylene glycol). Four microliters of ligation mix was added to 200 µl of *E. coli*/DH5α competent cells and plated onto 100 µg/ml Ampicillin Containing LB agar plates. White colonies were picked and checked for the presence of the insert by PCR. PCR-positive colonies were grown overnight in 5 ml of 100 µg/ml Ampicillin containing LB medium. Plasmids were extracted from bacteria, purified (Miniprep purification Kit, Qiagen) and its insert sequenced commercially on an ABI 377 Automated Sequencer (Avantor, Haryana) using M13 reverse and forward primers. Chromatograms were then analyzed with Chromas software.

pGEX2TK cloning of Human IRF-2 ORF: The IRF-2 ORF fragment was sub-cloned into pGEX2TK vector at *Bam*HI site after digesting pTZ57R/T-IRF-2 clone with *Bam*HI restriction endonuclease followed by gel purification. Ligation reaction was set up as follows: 5 µl IRF-2 ORF gel purified insert (~50 ng), 6 µl pGEX2TK vector (150 ng) *Bam*HI digested, dephosphorylated and gel purified, 1 µl of T₄ DNA Ligase (NEB) (400U/µl), 3 µl Ligation buffer (5X) (30 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 200 mM ATP, 5% polyethylene glycol). Four

microlitres of ligation mix was added to 200 µl of *E.coli*/ BL21 competent cells and plated onto 100 µg/ml ampicillin containing LB agar plates. Colonies were picked and checked for the presence of the insert by *Bam*HI Restriction digestion.

Expression of recombinant human GST-IRF-2: Five millilitre LB medium containing ampicillin (100 µg/ml ampicillin) was inoculated with a single colony of pGEX-IRF-2/*E.coli* BL21 cells and grown overnight at 37° C. 100 µl of the overnight grown culture was used to inoculate another 10 ml LB with appropriate antibiotics and grown for 3-4 hrs. at 37° C until O.D₆₀₀ nm reached between 0.6 to 0.8. IPTG (0.5 mM) induction was carried out at 37°C for 3 hours along with the control (*E.coli* BL21 cells harboring empty pGEX2TK vector). Extracts from equal number of cells (~1.0 O.D₆₀₀ nm) was prepared and used to assess GST-IRF-2 expression. Cell pellet from 1.0 O.D. volume of each culture was resuspended in 150 µl of water and 50 µl of 4X loading dye (0.06M Tris-Cl, pH.8.0, 2% SDS, 10% Glycerol, 0.025% Bromophenol blue) and subjected to boiling in a water bath at 95°C for 10 min. The samples were given a spin at 10K rpm, RT for 30 seconds and resolved in 12.5% SDS-PAGE at 100V for 6 hrs. The gel was stained with Commasiae Brilliant Blue R250.

RESULTS

Construction of T/A clones of human IRF-2 ORF: To isolate the Human IRF-2 gene coding region, total RNA was isolated from human peripheral blood (Figure 1-A) and converted into cDNA then gradient PCR was carried out with ORF specific primers to hunt out the IRF-2 coding region. Figure 4 showing total RNA isolated gel, lane 1 showing 28S rRNA and 18S rRNA. Isolated RNA was then subjected to cDNA conversion using manufacturer's protocol. Figure 1-B is showing PCR profile. Lane 2-5 are showing expected band of ~1047 bp amplification at 68°C. We then did gel purification of this band after repeating the PCR at the same temperature in order to get fragment in bulk quantity. After getting purified IRF-2 fragment then it was spliced in T/A vector followed by transformation in *E.coli*/ DH5α cells. Recombinant colony was selected on the basis of colony PCR followed by Restriction digestion (Figure 2-A). Two colonies pT2.1 and pT2.2 were selected for further study. Meanwhile, sequencing was carried out to ensure the cloned fragment is of our interest (data not shown). After getting sequence we analyzed it and found that the insert we have spliced in T/A vector is of our interest without any mutation.

Construction of pGEX2TK expression clones: Clone pT2.1 was subjected to mini

preparation to get recombinant T/A vector in bulk to digest and purify the IRF-2 ORF in order to splice into pGEX2TK vector. Simultaneously, we transform the pGEX2TK vector in *E.coli*/BL21 cells and mini preparation was done followed by *Bam*HI digestion, CIP treatment and gel purification in order to prepare the vector for ligation reaction. Successful ligation reaction was carried out and transformed in *E.coli*/BL21 cells and plated on LB ampicillin plate. Furthermore, colonies were quickly screened by colony PCR. Then probable four colonies (g2.1, g2.3, g2.5, g2.8) were selected and subjected to *Bam*HI digestion to ensure recombinancy of the colonies (Figure 2-B). Three colonies namely were selected (g2.1, g2.2 and g2.3) for expression study. We also checked orientation of recombinant colonies and observed that the all colonies were positively oriented (Data not shown).

Expression of recombinant IRF-2 in E.coli: After verifying orientation of clones we transform the recombinant vector into *E.coli*/BL21 cells followed by 0.5mM IPTG induction for three hours at 37°C at 220 rpm. Figure 3-A is protein profiling of *E.coli* cells harbouring recombinant IRF-2 and control only harbouring only non-recombinant pGEX2TK vector. Lane 1 is 66 kDa BSA marker is used to ascertain the expressed recombinant protein expression

by different clones. Lane 2 is an empty vector without IPTG induction no showing induction of GST (~26 kDa), lane 3 is showing induction of GST moiety after IPTG induction. Similarly, all clones showing expression of ~66 kDa protein in lane 5, 7 and 9 whereas no induction of same band was observed in the IPTG uninduced lanes 4, 6, and 8. We observed profound expression of recombinant IRF-2 protein expression as a GST-IRF-2 fusion protein in *E.coli*.

Expression analysis of recombinant IRF-2: After getting very good expression of all clones in *E.coli* we did IPTG concentration optimization experiment. We observed expression at low concentration as 0.1 mM IPTG but if increased up to 0.7 mM IPTG concentration (Figure 3-B; lanes 5-11). We optimized the IPTG concentration for 0.5 mM because the 0.6 and 0.7 mM IPTG concentration inducing clones with more toxic effect that leads to cells quantitatively low. That's why we optimize 0.5 mM for further study.

Time course study: Next we studied the recombinant IRF-2 protein induction in relation to time. We observed clone g2.1 showing induction of protein after 15 minutes and increased up to 3 hours (Figure 4-B; lane 4-15). We optimize this condition for further study. Since our interest is to purify the recombinant protein

which is close to native protein. We did supernatant and pellet experiment to check whether protein is present in either supernatant or periplasmic space. For this clone g2.1 was induced by 0.5 mM IPTG for 3 hours subjected to sonication, and lysate was cleared by centrifugation. Bradford was done to determine the protein concentration. Five microgram of both supernatant and 5 microgram of pellet was run on 12.5% SDS PAGE for 3 hours and stained by Commassiae brilliant blue followed by destaining. We observed majority of recombinant protein expression in supernatant (Data not shown).

Growth curve analysis: To examine this hypothesis the effect of induced GST-IRF2 protein on the growth curve of different clones. We cultured the clone g2.1, g2.3, g2.5 in 5ml of LB broth containing 10µg/ml ampicillin. All cultures were incubated at 37°C and cell growth was monitored by measuring OD_{600nm} (Table 1) before and after induction of 0.5 mM IPTG concentration at 0 hr and 3hrs of time interval. The experiments were performed in duplicates and data were averaged (Figure 10). We further observed that GST-IRF-2 fusion protein didn't show any toxic or inhibitory effect on the growth of transformed *E.coli*/BL21 cells.

DISCUSSION

IRF-2 is a transcriptional regulator that plays a major role in the regulation of cell growth and immune response. A recent study shows that IRF-2 has dual function tumor inducer as well as the tumor suppressor. The functional consequence of IRF-2 inactivation in HCC cell lines demonstrating that IRF-2 acts as a tumor suppressor, the IRF-2 silencing results increased cell proliferation in contrast its overexpression led to a cell death response by apoptosis.

In this study, we have first time reported that cDNA cloning and expression analysis of chimeric IRF-2 from human blood. IRF-2 gene is induced by virus, IFNs, IRF-1 etc. in order to attenuate the IFN signaling. Malfunctioning serves as continuous and up regulation of type I IFN gene expression leads to psoriasis skin disease [10]. In fact, now days it's various roles have been detected in cell like, apoptosis, cell cycle regulation, immunomodulation, antiviral activity, etc. Information regarding its physiological role is scarce. Moreover, its role in the cancer progression is come in light in early in 2000. Only one report claiming tumor suppressor activity in hepatocellular carcinoma induced by hepatitis B virus (HBV) [6]. This property makes this molecule peculiar and attracts me to do this study. This study is first and small effort that will become the base to do

a further study to explore its unexplored aspect(s).

We have expressed the 349 a.a human IRF-2 fused with GST moiety at N-terminus to form of ~ 66 kDa fusion proteins, showed the IPTG induction in the pGEX2TK expression vector of *E.coli*/BL21 bacterial cells. Figure (3-A) showed the profound expression of recombinant IRF-2 proteins from pGEX-IRF-2 clones namely, g2.1, g2.3 and g2.5. The empty vector pGEX2TK showed expression of GST moiety of ~26 kDa was used as negative control (see lane2 and 3). Furthermore, we did not observe any degradation product(s) of recombinant GST-huIRF-2 fusion proteins in *E.coli*. In contrast to two earlier reports of full length of murine, IRF-2 was expressed in *E.coli* with few degradation products [11]. Similarly, murine IRF-1 was expressed as GST-fusion system in *E.coli* with few degradation products [12]. Interestingly, we observed full-length recombinant protein with same vector and in *E.coli*/BL21 cells. This gives indication that the human IRF-2 protein is more stable than a mouse IRF-1 and IRF-2 proteins.

The expressed fusion protein was further analysed for IPTG concentration optimization, time course induction and effect of fusion protein on bacterial growth. Figure 3-(B) showed the induction of GST-IRF-2 fusion protein from clone g2.1 was

observed at low 0.1 mM IPTG, and the band intensity were increased gradually with increased IPTG concentration viz., 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mM. Although, we observed recombinant protein expression at higher concentrations of IPTG 0.6 and 0.7 mM yet, the numbers of cells were quantitatively low due to toxic nature of IPTG. Thus, we optimized IPTG concentration 0.5 mM IPTG at 37°C for subsequent experiments. This result is same as the paper entitled “chimeric oncogenic interferon regulatory factor-2 (IRF-2): Degradation products are biologically active” [11]. But differ in degradation products means we were fortunate to not observe any degradation products at this IPTG concentration at 37°C. Next we optimized the time for optimal induction of recombinant IRF-2 protein. The figure 4-(A) showed induction within early 15 minutes and increases the induction of fusion GST-IRF-2 protein up to 3 hrs. This result was agreement with the earlier report of [12] in Molecular Biology Reports but we could not observe any degradation products along with full-length recombinant protein.

Further, we have tested whether the recombinant protein is part of soluble fraction or part of insoluble fraction. We observed majority of recombinant protein is part of soluble fraction (data not shown).

This gives advantage to purify the recombinant protein and do further functional analysis. To further elucidate the preliminary activity identification and its biological functions, the IRF-2 protein is successfully expressed and will be purified by one-step glutathione affinity chromatography.

CONCLUSION

In conclusion, this study is the first report to clone the full-length cDNA of human IRF-2 and expression of the complete sequence of IRF-2 ORF from the Human blood. The cloning and characterization of IRF-2 from Human extend our understanding of the evolutionary significance of this molecule from mouse to human that makes this molecule more stable. This report is first report of its own

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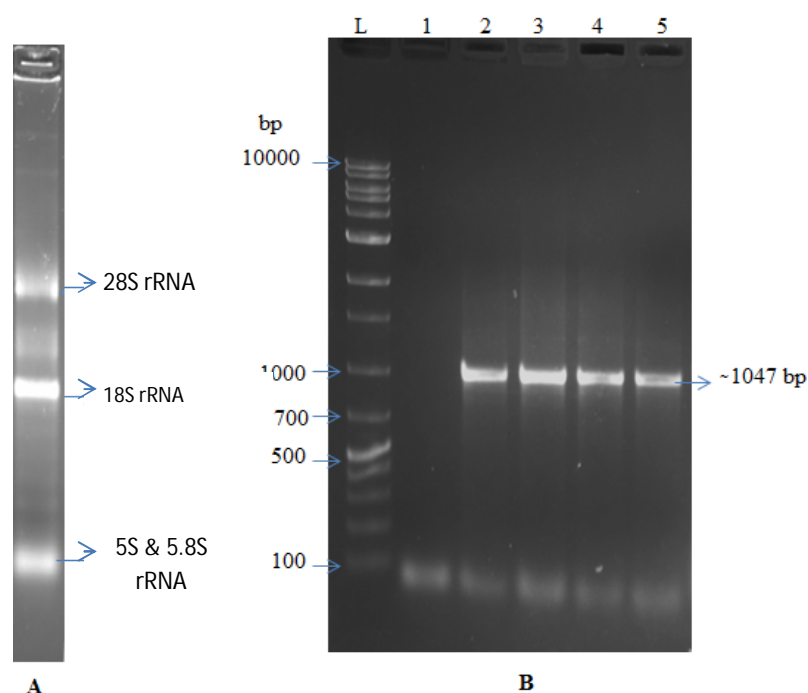


Figure 1: (A) : 1.2% agarose gel showing RNA isolated from human blood (B) 1% agarose gel showing PCR amplification of IRF 2 ORF. L= 1 kb plus DNA ladder, lane 1, (Negative control), lane 1 - 5 showing IRF-2 ORF amplicon of size ~1047 bp.

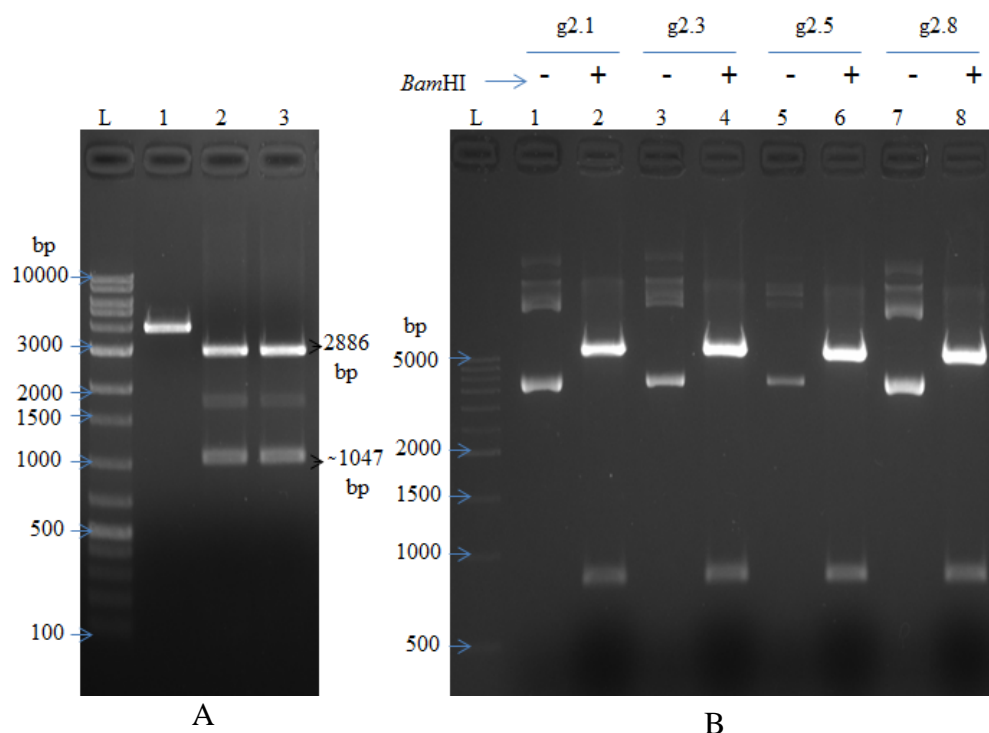


Figure 2: (A) 1% agarose gel profiling of restriction digestion of recombinant plasmid with BamHI restriction enzyme; lane 1, control plasmid DNA digested (pTZ57R/T) + ~1047bp Insert); lane 2, clone pt2.1 digested plasmid; lane 3, clone pt2.2 digested plasmid. (B) 1% agarose gel showing: lane 1, clone g2.1 undigested plasmid; lane2, clone g2.1 digested plasmid ; lane 3, clone g2.3 undigested plasmid; lane 4, clone g2.3 digested plasmid; lane 5, clone g2.5 undigested plasmid; lane 6, clone g2.5 digested plasmid; lane 7, clone g2.8 undigested plasmid; lane 8, clone g2.8 digested plasmid; L, 1 kb plus ladder.

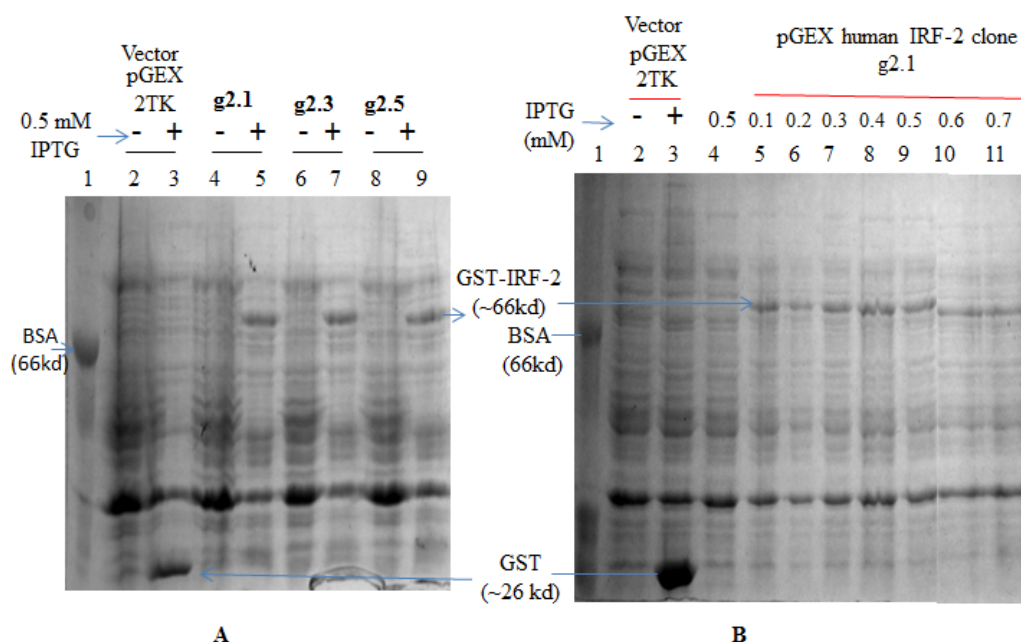


Figure 3: (A) 12.5% SDS PAGE showing expression of recombinant human GST-IRF-2 at (0.5mM) IPTG of different clones; lane 2, uninduced pGEX2TK vector; lane 3, IPTG induced pGEX2TK vector; lane 4, clone g2.1 uninduced; lane 5, clone g2.1 IPTG induced; lane 6, clone g2.3 uninduced; lane 7, clone g2.3 IPTG induced; lane 8, clone g2.5 uninduced; lane 9, clone g2.5 IPTG induced; lane 1, 5 µg BSA(66 kd). (B): 12.5% SDS PAGE showing IPTG concentration optimization: Expression of recombinant GST-IRF-2 after induction of g2.1 clone with increasing concentration of IPTG (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mM) for three hours; lane 1, 5 µg BSA ~ 66 kd; lane 2, uninduced empty vector GST (pGEX2TK); lane 3, 0.5mM IPTG induced vector; lane 4, uninduced clone g2.1; lane 5, 0.1mM IPTG induced clone g2.1; lane 6, 0.2 mM IPTG induced clone; lane 7, 0.3 mM IPTG induced clone; lane 8, 0.4 mM IPTG induced clone; lane 9, 0.5 mM IPTG induced clone; lane 10, 0.6 mM IPTG induced clone; lane 11, 0.7 mM IPTG induced clone

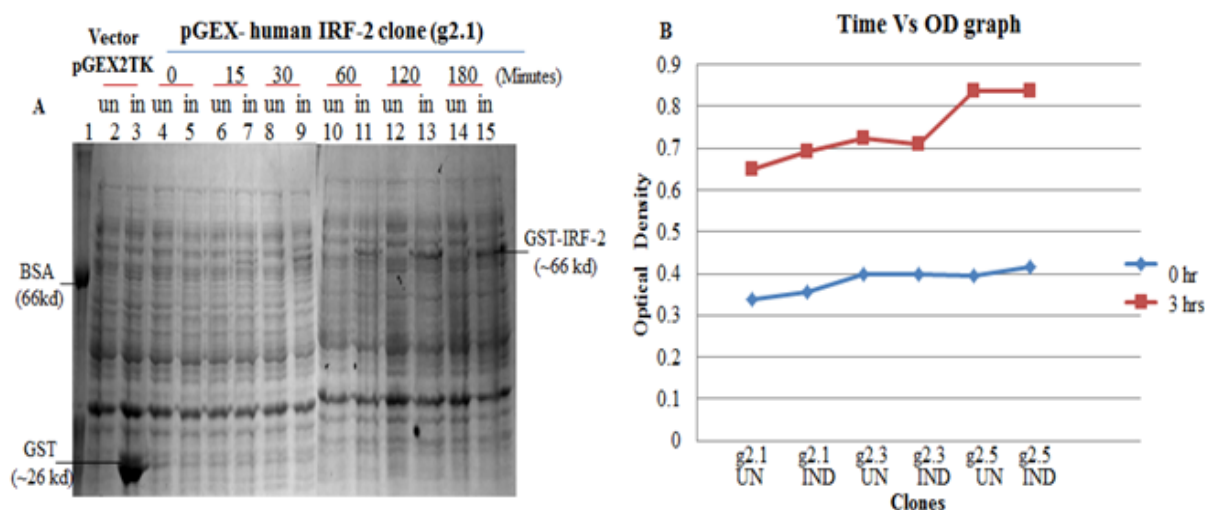


Figure 4: (A) 12.5 % SDS PAGE showing. Expression of GST-IF-2 in relation to time course study of IPTG induction. The GST-IRF-2 was observed in clone g2.1 after induction with 0.5 mM IPTG at different time interval. Induction of GST by vector is shown at 180 min. Lane 1, 5 µg BSA(66 kd); lane 2, , uninduced empty vector GST (pGEX2TK); lane 3, 0.5 mM IPTG induced empty vector; lane 4, uninduced clone at 0 min; lane 5, IPTG induced clone at 0 min; lane 6, IPTG uninduced clone at 15 min; lane 7, IPTG induced clone at 15 min; lane 8, IPTG uninduced clone at 30 min; lane 9, IPTG induced clone at 30 min; lane 10, IPTG uninduced clone at 60 min; lane 11, IPTG induced clone at 60 min; lane 12, IPTG uninduced clone at 120 min; lane 13, IPTG induced clone at 120 min; lane 14, IPTG uninduced clone at 180 min; lane 15, IPTG induced clone at 180 min. (B) Time Vs O.D. of cell growth in pGEX2TK-IRF-2 clones at)hr and 3hrs, after induction with 0.5mM IPTG .

Table 1: Time Vs O.D. Graph

Clones (UN & IND)	O.D. at 0 hr.	O.D. at 3 hrs.
g2.1 UN	0.370	0.650
g2.1 IND	0.355	0.691
g2.3 UN	0.398	0.722
g2.3 IND	0.400	0.711
g2.5 UN	0.394	0.836
g2.5 IND	0.415	0.835

UN: Uninduced, IND: Induced