

*Full Length Research Paper*

# **Nucleofection an efficient non-viral transfection technique for IFN-t-EGFP gene expression study in Sahiwal cattle fibroblast cells**

**Anand Laxmi N.<sup>1</sup>, Gunjan G.<sup>1</sup> and Prateesh M.<sup>2\*</sup>**

<sup>1</sup>DCP, NDRI, Karnal, India.

<sup>2</sup>IVRI, Barrielly, India.

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Viral based techniques were considered to be the most efficient systems to deliver DNA into fibroblast cells as they show high transgene expression in many cellular models. Viral approaches are complicated by immune response, intracellular trafficking potential mutations and genetic alterations due to integration. The nucleofector TM technology is electroporation based gene transfer technique which has been proved to be an efficient tool for transfecting primary cells and hard to transfect cell lines. The present study was designed to examine Sahiwal fibroblast cell to act as competent donor cell in gene expression studies. Using a green fluorescent protein reporter vector, a high transgene expression level was obtained using U-12 and U-23 pulsing programs: 45 and 70% respectively. Cell recoveries and viabilities were 90.5 and 95% respectively for U3-23 program. Overall transfection efficiency was 60% as observed on evaluation by flowcytometry. Further, the cells confirmed to be positive for gene expression when subjected to PCR, RT-PCR and, flow cytometry analysis using pGFP repoter other than supplied by Amaxa system. Hence, the cells were transfected with bIFN-t---GFP reporter gene construct by nucleofection technique and similarly, cells were evaluated for expression of gene by fluorescence microscopy. A sixty percent of cells were positive for fuorescence on enumeration of transfected cells by fluorescence microscopy 72 h post transfection. Further it was confirmed by PCR technique using primers for IFN-t gene. The cell cultures when analysed for IFN-t protein expression, the protein could be detected by silver staining of SDS-PAGE gels. Reports are available where this technology has been successfully applied for the transfection of other cells like monocytic cell lines and neural stem cells. This technique can be further utilised for transfection of genes of economic importance in primary cell lines which be in turn can be utilised for production of transgenic embryos. Through out the study the transfected cell cultures were negative for apoptosis. The Sahiwal fibroblast cells culture system can be utilized for transfection and gene expression studies. Green Fluorescent Protein acts as a useful visual indication system for gene insertion or targeting studies, when conjugated to the gene of interest

**Key words:** Transfection, gene, fibroblast cell.

## **INTRODUCTION**

Gene transfer technology has many potential applications in gene expression studies. Usually most commonly used systems to deliver DNA in to cells and study its expression is based on viral based techniques. This is mainly useful for stable integration in to host cell genome

(Blesch, 2004). The non-viral techniques available are lipofection microinjection, electroporation and gene gun technique which are associated with low gene expression but associated with either mortality or toxicity. The nucleofector technology is a non-viral technology which can be used for transfecting primary cell lines. Transfection of cells is not dependent on cell division, and still yields high transfection efficiency (Naito et al., 2007). This further paves way for studying gene

\*Corresponding author. E-mail: [dna44@yahoo.co.in](mailto:dna44@yahoo.co.in).

expression for longer duration of time. Gene transfection allows, introduction of normal genes in animals with genetic dysfunctions. Growth factors and cytokines also deliver biological agents in considerable amounts. In the present study, we evaluated the efficacy of Nucleofector technology as a gene delivery vehicle in to primary fibroblast cells of Sahiwal cattle and local production or expression of recombinant bIFN-t- EGFP protien. Nucleofection is a transfection method which enables transfer of nucleic acids such as DNA, RNA, Small interfering RNA molecules into cells. Nucleofection, also referred to as Nucleofector Technology, was invented by the biotechnology company *amaxa*. "Nucleofector" and "nucleofection" are trademarks, owned by Lonza Cologne AG. The reported pulsing programs are patented programs set by Amaxa systems. All EGFP positive cell lines were observed by fluorescence microscopy confirmed by PCR, flow cytometry and SDS-PAGE analysis. The objective of this study was to obtain transgenic cell lines available for cloning. Aiming at this p bIFN-t-GFP gene construct was introduced by nucleofection into Sahiwal cattle skin fibroblasts. GFP gene enhanced version EGFP was used in the present study. Studies on expression of GFP in cattle have been carried out by Chan et al. (2002); Rosochacki et al. (2001). Studies have been conducted on expression of IFN-t (Interferon-tau) in bacterial and yeast cell cultures (Fang-Fang et al., 2008).

In the present study, long term culture *in vitro* did not have any effect on the proliferation rate of the cells and also on expression. The bIFN-t-GFP expression could be observed even after six passages post transfection. This is the first study in which expression of recombinant IFN-t protein in fibroblast cells of Sahiwal cattle has been reported for successive passages. The protein fraction extracted from the transfected cells exhibited antiproliferative activity on lymphocyte cell cultures.

## METHODOLOGY

### Harvesting of cell culture

Skin piece was collected from ear pinna of Sahiwal cattle, transported to the lab in PBS supplemented with streptomycin – pencillin antibiotics. Tissue pieces were cultured in petridishes under sterile conditions. When the cells started extruding out of the tissue and became confluent, piece was removed, medium was replaced. The culture was subjected to trypsinization and was transferred to the 75 mm<sup>3</sup> culture flasks.

On attaining 70 to 80% confluency, cultures in the flasks were washed with 2 ml of Ca<sup>++</sup> Mg<sup>++</sup> free D-PBS buffer. One ml. of cold trypsin EDTA solution (trypsin-0.125% and EDTA-0.0125% in D-PBS) was added to each flask. This dose was standardized for Sahiwal cattle. They were incubated at 37°C for eight minutes. On trypsinization, cells appeared spherical in shape which could be observed under inverted microscope. Further 2 ml of complete medium (DMEM+Ham's F12 with 10% FBS) was added to each flask. The suspended cells were immediately transferred to centrifuge tubes and were centrifuged at 1000 rpm for 8 min. The supernatant was discarded and cells were suspended in 1 ml of

medium and cell counts were taken on haemocytometer. After harvesting of primary cell cultures and further on seeding they were designated as passage number one.

### Cell transfection

Nucleofection of fibroblast cells was performed according to optimized protocol provided by the manufacturer (Amata Biosystems, Cologne, Germany, www.amata.com). Briefly cells were suspended in 100 µl of nucleofector solution (Amata Biosystem), mixed with either pEGFP or pIFN-t-EGFP construct [Source Promoter (Toshihiko et al., 1999), and coding regions were generously gifted by Dr. R.M. Roberts Lab, and Dr. Alan D. Ealy Missouri] and were subjected to nucleofection with the U-23 or U-12 program respectively (Figure 1). Immediately after transfection cells were transferred in to prewarmed fresh medium in six well plates. Cells were analysed for viability and GFP expression respectively 36h post nucleofection. The fluorescence of the cells was viewed at different passages post transfection.

### The vector construct

1. The promoter region of IFN-t was PCR amplified using sequence specific primers designed based on cattle genome sequence data. The promoter region was amplified, the amplicon was gel purified and subsequently restriction digested and cloned within a cloning vector (pUC18). A few clones were sequenced to get a clone with no mutations.
2. The coding region of the IFN-t was PCR amplified using sequence specific primers designed based on cattle genome sequence data. The primers had suitable restriction sites for assembling the gene into the final construct. The gene was subcloned into pUC18 for sequence confirmation. A few clones were sequenced to get one with no mutations. The gene was PCR amplified using primers designed in such a way that the gene would be in-frame after it's cloned within the vector backbone (Appendix – Figure 1).

### RNA/DNA / protein extraction

RNA/DNA/protein fractions were extracted from cell culture lysates using Norgen's RNA/DNA/Protein purification kit. Purification is based on spin column chromatography using Norgen's proprietary resin.

The transfected cell culture samples were centrifuged and to the pellet containing 2 x 10<sup>6</sup> no. of cells, 350 µl of lysis solution was added and vortexed for 15 s for complete dissolution. To the lysate 200 µl of 95% ethanol was added and vortexed gently for 10 min.

With the help of DNA, RNA elution solutions and according to the kit's manufacturer's instructions, the RNA was eluted and fraction was stored at -70°C and similarly the extracted DNA fraction was stored at -20°C.

The flow through collected after binding of RNA to the column, the pH of the solution was adjusted and applied on to the column. The flow through obtained was discarded and protein fraction was eluted using protein elution buffer. The fraction was neutralized with the neutralizer.

### PCR Protocol for EGFP gene

Genomic DNA from 2 x 10<sup>6</sup> no. of transfected and non-transfected cells which served as control was extracted with the help of DNA extraction kit. Approximately 1 µg of DNA was used as a template in PCR reactions containing forward and reverse primers.

Template DNA was denatured at 94°C for 1 min. The PCR cycle consisting of denaturation, annealing and extension steps were as follows:

Denaturation at 94°C for 30 s annealing at 56°C for 30 s, extension at 72°C for 1 min. Final extension step was performed at 72°C for 15 min. The PCR amplification was subjected to 30 cycles. The resulting amplified PCR products were analysed on 1.8% agarose gels. A100 bp ladder (Chromous Co., Bangalore) was used as marker and 100 ng of pEGFP served as standard.

ATGGTGAG CAAGGGCGAGGAGCT F- primer:  
: GTACCGTCTGACTGCAGAATTCGAAGCT R- primer

#### PCR protocol for IFN- $\alpha$ gene

Template DNA was denatured at 94°C for 2 min. The PCR cycle consisting of denaturation, annealing and extension steps were as follows:

Denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s Final extension step was performed at 94°C for 2 min. The cycle was repeated 35 times.  
The resulting amplified PCR products were analysed on 1.8% agarose gel with 100 bp ladder as marker. 100 ng of pIFN-t-GFP served as a control.

#### Primers for IFN-t

\*CAGTGACATATGGCCTTCGTGCTCTCTCTACTGATG-F primer  
\*CAGTGACTCGAGAAGTGAGTTCAGATCTCCACCCATCT-R primer.

#### Analysis of EGFP expression

The EGFP fluorescent cells were visualized under fluorescence microscope and analyzed by flowcytometry. For control, cells transfected without vector were considered. Briefly cells were detached from the flasks by incubation with 0.125% Trypsin and 0.012% EDTA for 5-6 minutes, recovered by centrifugation at 800g for 6 minutes. The pellet was suspended in DMEM: Ham's F12 medium. An aliquot of the suspension was visualized under fluorescence microscope. For flow cytometry, the cells were washed with PBS buffer containing 1% BSA. Cells were collected by centrifugation and resuspended in PBS buffer containing BSA and 2% paraformaldehyde before analysis. Both control and pIFN-t-EGFP transfected cells were respectively analyzed on Bector-Dickinson instrument after fixation of cells for analysis by flowcytometry as follows.

Approximately  $1 \times 10^6$  transfected cells were washed with PBS buffer. Samples were centrifuged at 1500 rpm for 5 min. Supernatant was discarded and 500  $\mu$ l of cold PBS was added and vortexed gently. Further, 500  $\mu$ l of cold buffered 2% formaldehyde solution was added. They were incubated for 1h at 2-8°C. The samples were again centrifuged at 1500 rpm for 5 min. and washed once more with cold PBS. To the pellet 1 ml of 40° ethanol chilled at -20°C was added dropwise. The suspension was incubated overnight at 2 to 8°C. The next day the samples were centrifuged and supernatant was discarded. They were incubated with 20  $\mu$ l RNAse at 37°C for 30 min in the dark. The cells were subjected to flowcytometry for measurement of green fluorescence. Fluorescence data was collected using 488 nm excitation and filter combination consisting of filters with 395 and 475 long pass filters. Dot plot scatter for green fluorescence was created using Cell Quest Program.

#### SDS-PAGE

Pellets enriched with cellular proteins were suspended in 500  $\mu$ l of water and 500  $\mu$ l of sample buffer. The preparations were heated for 10 min at 98°C and stored frozen at -20°C. Proteins were visualized by staining with Coosamie brilliant blue R-250 or by silver staining. The apparent molecular weights of the proteins detected were estimated by comparison with a broad-range standard protein marker obtained from Genei Co. (Bangalore).

Separating gel mixture was prepared containing acrylamide monomer, Tris HCl (pH 8.8) and 10% SDS solution. After degassing the gel mixture ammonium per sulphate and TEMED were added to the solution. The resulting acrylamide solution was transferred to the casting chamber with Pasteur pipette. The solution was allowed to run down along the side of the spacers. Stacking gel was also prepared containing acrylamide monomer, Tris buffer (pH 6.8) and 10% SDS. Ammonium per sulfate and TEMED were added just before loading the gel chamber. One ml of freshly prepared stacking gel was poured in to the gel chamber and Teflon comb was placed to form sample wells. The cells and chamber were filled with Tris-Glycine-SDS buffer. Protein standard/markers or extracted protein fractions mixed with sample buffer were placed in to bottom of different wells respectively. The gel was removed from the casting stand and assembled in to appropriate slab for electrophoresis. The proteins were resolved by electrophoresis till the tracking dye reached the bottom of the separating gel. After confirming; the gel was stained with 0.25% Comassie Brilliant Blue R250 in methanol: water: glacial acetic acid (5:5:1).

#### Silver staining technique

To detect the presence of IFN-t-GFP, monoclonal antibodies to EGFP+ were used, and the antigen antibody complex was immunoprecipitated with protein A beads (Chromous Co. Bangalore). The antigen eluted out was separated by SDS-PAGE and further silver stained. Similarly, EGFP was immunoprecipitated from EGFP+ transfected cells and the purified protein was subjected to SDS-PAGE and also subjected to silver staining (Rabilloud, 1992).

#### Reverse transcriptase-PCR

##### Reverse transcriptase – PCR

The gene expression of the recombinant, pIFN-t-GFP construct was monitored by RT-PCR, using BioRT, two step RT-PCR kit (Taurus Scientific, USA). In the first step, cDNA was synthesized from total RNA by reverse transcription using AMV reverse transcriptase and gene specific down stream primers for transfected IFN-t gene, dNTP mixture (10 mM) and Rnase inhibitor. For confirmation the cDNA obtained was subjected to DNase treatment, upon incubation with DNase for 30 min, cDNA band was not observed on 1.8% agarose gel. The program for cDNA synthesis is as follows:

##### RT reaction

60°C - 45 min  
95°C - 5 min  
Ice bath - 5 min

The cDNA obtained in the first step was subjected to amplification by PCR.

#### Apoptosis assay

The genomic DNA was extracted from the fibroblast cell cultures

and was subjected to DNA ladder assay on 0.8% agarose gel. A 1 kbp ladder was used as marker (Genei Co., Bangalore). Briefly the cell cultures were transspined and DNA was extracted from the cells using Norgen's kit (Canada) based on column extraction method. Approx. 500 ng of DNA ladder and 1 µg of DNA extracted from transfected cells with the loading dye were loaded in to separate wells on 0.8% agarose gels. The gels were subjected to electrophoresis for 1h and were further viewed under UV transilluminator (System and Control, US A).

#### Cell culture of lymphocytes and antiproliferative test

The lymphocyte cell suspension was diluted with RPMI 1640 culture media containing 10% FCS, so that final concentration was  $50 \times 10^5$  lymphocytes/ml. The viability of the lymphocytes was evaluated by trypan blue exclusion method. A 200 µl of the medium containing approx.  $10 \times 10^5$  cells were pipetted out into each well in a 96 well plate. For stimulation of mitotic activity phytohemagglutinin (PHA-P) was added at a concentration of 5 µg/ml in a total volume of 200 µl /well. The cell cultures were propagated/ incubated for 24 h in a CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub>. The samples with and without mitogen were run in duplicates. The proliferative response of the lymphocytes was estimated using colorimetric MTT test (Mossman, 1983).

#### MTT assay

MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of pale yellow and form a dark blue formazan crystals. The accumulated formazan in the viable cells is released and solubilized by the addition of DMSO. The lymphocyte cultures were incubated for 48 h with mitogen and immuno-precipitated protein fraction of fibroblast cell culture lysate (1 µg of total protein/well) in DPBS and further with MTT at a conc. of 5 µg/well was also added. The plates were further incubated for 4 h in a CO<sub>2</sub> incubator under controlled conditions. A 100 µl of DMSO was added to each well at the end of 4 h of incubation. The solution in the wells was mixed thoroughly and incubated at room temperature for 15 min. The optical density or absorbance of the solution was recorded at 503 nm with reference to 630 nm.

Lymphocyte stimulation in index = O.D. of the complex formed from stimulated cells/O.D. of the complex formed from unstimulated cells  
The statistical analysis was done by two way ANOVA program.

## RESULTS

#### Fibroblast cell culture

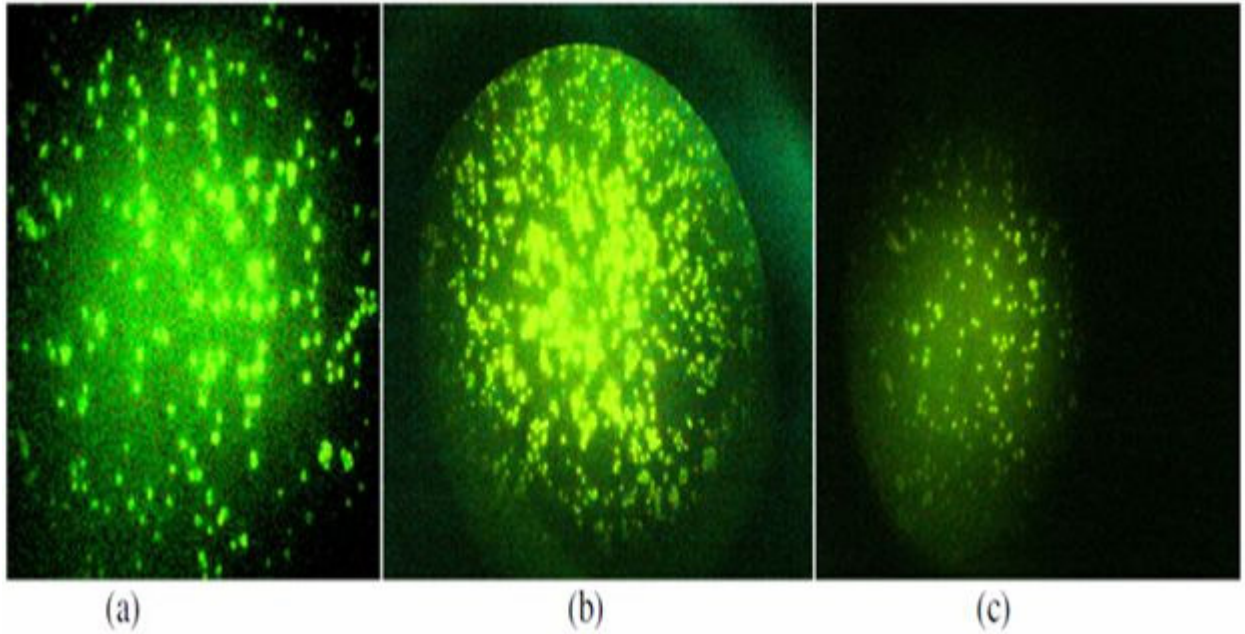
The Sahiwal fibroblast cell cultures were successfully propagated, till sixth passage post transfection. A morphologically homogenous population of confluent fibroblast cell cultures was obtained by 5 to 6 days post harvesting and seeding of cells. At first passage post transfection growing cells were uniformly positive for EGFP fluorescence expression. The Sahiwal fibroblast cells were transfected by the Nucleofector technology following the protocol provided by the manufacturer. The expression of transgene pEGFP and pIFN-t-EGFP

respectively was evaluated by fluorescence microscopy and by fluorescence activated cell sorter (FACS). At 36 h post nucleofection more than 50% were EGFP+ cells which increased to 70% after first passage post-nucleofection (Figure 1a and b). The genomic DNA extracted from cell cultures pre and post transfection respectively when subjected to gel electrophoresis, they were nonapoptotic (Figure 2). Similar were the results obtained with pIFN-t-EGFP gene constructs. But the percentage of bIFNt-EGFP+ cells, at first passage post nucleofection, was 60% (Figure 1c) and thereafter the percentage of EGFP/IFN-t-EGFP expressing nucleofected cells remained constant till sixth passage post nucleofection. Conversely U-12 program, resulted in 40 to 43% EGFP+ cells. U-23 program was found to be superior to U-12 program (Figure 1a and b). The percentage of viable cells estimated by Trypan blue exclusion method was observed to be 90 to 95% at first passage post nucleofection. The results obtained for EGFP+ cells on nucleofection with pIFN-t-EGFP construct was significantly less (60 vs. 70%), ( $P < 0.05$ ) (Figure 2) with U-23 program when compared with pEGFP+ cells. To assess the stability of nucleofection based transfection, the cells expressing EGFP was determined at second, fourth and sixth passage post pIFNt-EGFP nucleofection respectively. It was observed that cells were expressing EGFP fluorescence at all the passages confirming the expression of IFN-t also. On analysis by flowcytometry it was observed that 50% of the cells were expressing fluorescence for the IFN-t transfected cell cultures (Figure 3a and b) when compared with that of the control.

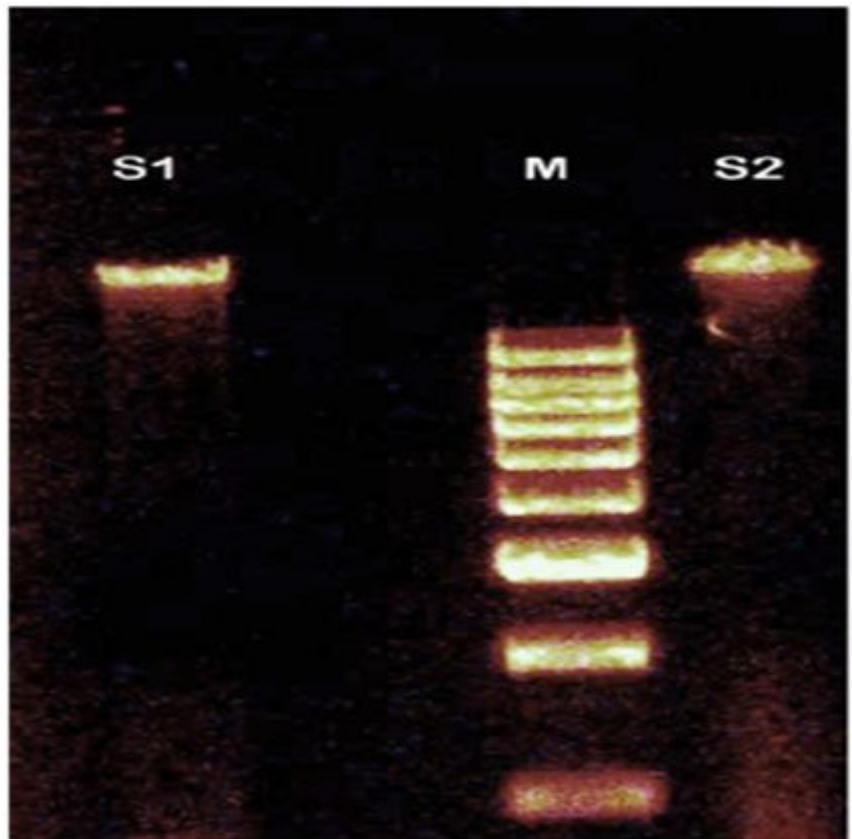
#### PCR and RT PCR studies

The primers specific for EGFP and IFN-t were used for amplification of genomic DNA extracted from EGFP and IFN-t-EGFP gene transfected cell cultures respectively. After amplification of genomic DNA, PCR products were observed at 885 and 585 bp region for the EGFP+ and IFN-t-EGFP respectively confirming presence of the gene [Figure 4a and b]. Though the EGFP fluorescence could be observed post nucleofection, with IFN-t-EGFP gene, the expression of the gene was monitored at transcriptional level using specific primers for IFN-t gene against the exon coding region for the IFN-t transfected cell cultures at second, fourth and sixth passage respectively.

RT-PCR was performed using AMV reverse transcriptase. On amplification of the C-DNA a band was observed on 1.8% agarose gel, at 585 bp region against 100 bp ladder used as marker, which confirmed the expression of gene at transcriptional level and was positive for culture at second, fourth and sixth passage, respectively (Figure 5). Real time PCR studies were conducted using forward and reverse primers for IFN-t



**Figure 1.** 45 and 70% of the cells were observed to be pEGFP<sup>+</sup> with U12; (a) and U23; (b) nucleofector programs respectively; (c) Cells transfected with pIFN-t-EGFP- L-100 bp ladder, 60% fluorescent cells as observed under fluorescence microscope.



**Figure 2.** S1,S2- Genomic DNA of cell cultures pre and post transfection section respectively exhibiting non apoptosis M-Marker in Kbps.

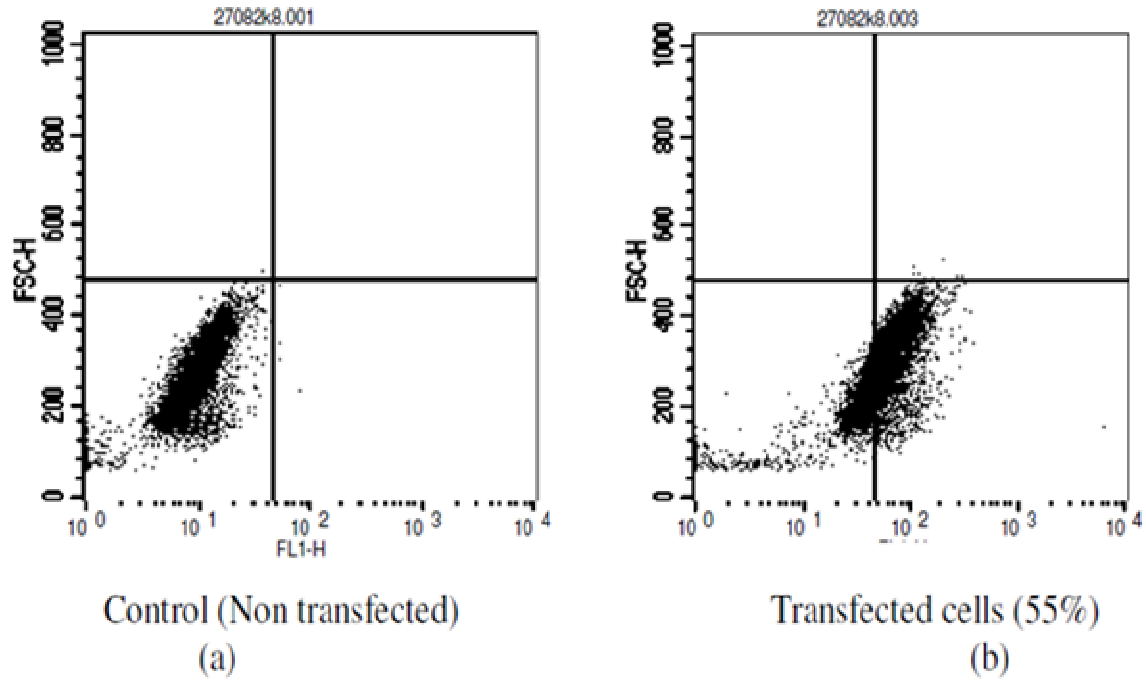


Figure 3. On analysis by flowcytometry.

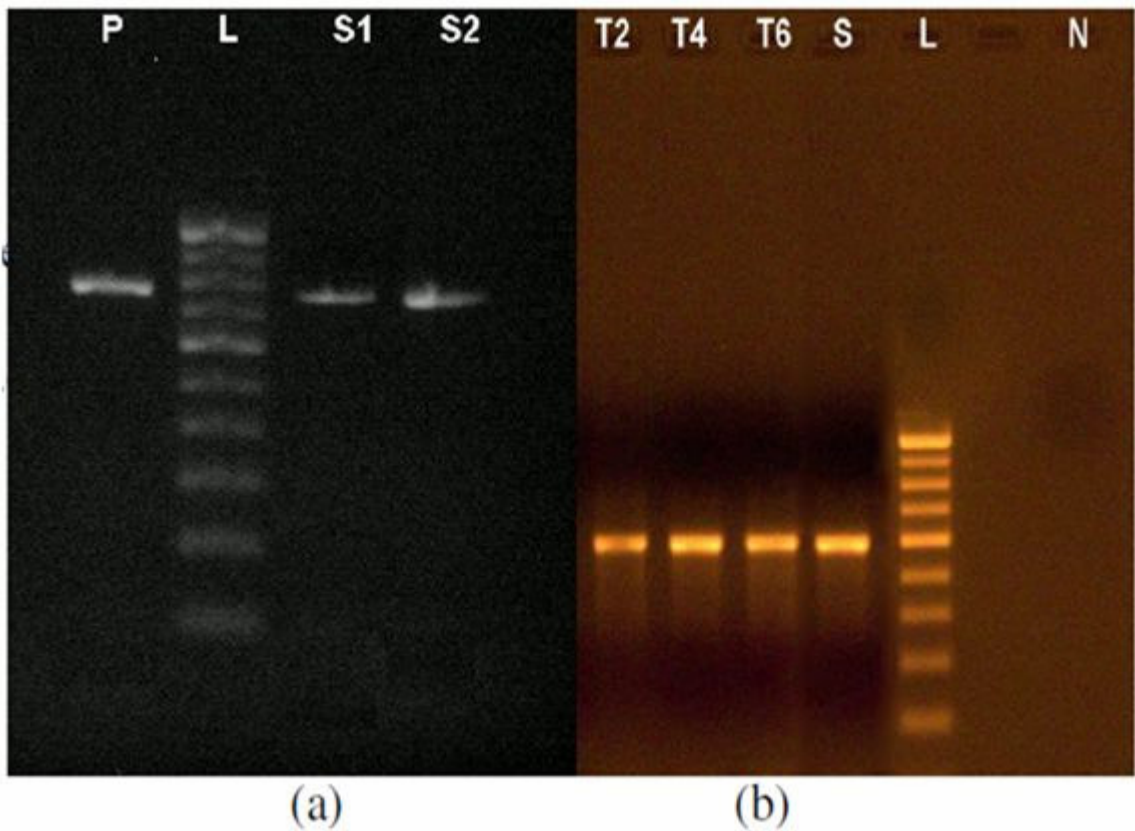
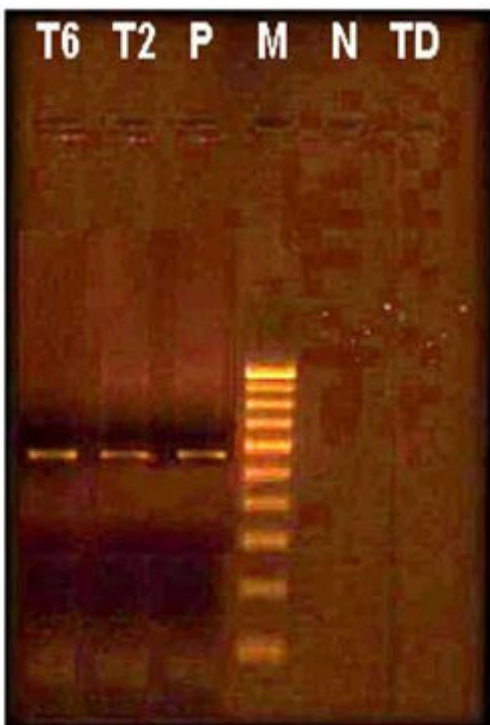


Figure 4. S1,S2 -PCR product of EGFP gene (885 bp) from cells post nucleofection P- Positive marker; L- 100 bp ladder, (b) passages post transfection N- PCR product not observed for DNA from non transfected cells product of IFN-t gene (585 bp) at different.



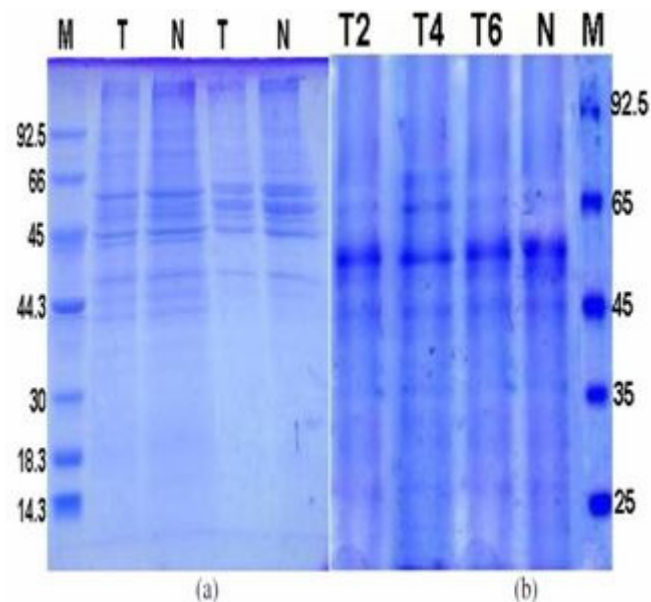


**Figure 5.** RT-PCR of gRNA obtained from transfected cells-cDNA amplified by PCR M – 100 bp marker, T2, T6- Passage no. post transfection P- Positive marker for IFN-t (585 bp), N– Product negative for non transfected cells TD- treatment with DNase.

gene, using SYBR green as fluorochrome using C-DNA obtained from transfected cell cultures, at different passages respectively. No significant amplification for expression of gene from second to sixth passage was observed (Data not given). Hence, transfection of the gene, and expression of the gene at transcriptional and translational level was confirmed.

### SDS PAGE

Protein fraction extracted from the cell cultures containing EGFP/ recombinant fusion protein IFN-t-EGFP and also from non-transfected cell cultures when analysed on SDS-PAGE and further subjected to Coomassie Blue staining, there was no significant difference in the pattern of protein bands, between transfected and non-transfected cell cultures and also between the protein extracts obtained from transfected cell cultures at different passages post transfection [Figure 6a and b]. On silver staining of the SDS gels the protein was detected on SDS-PAGE at 53 and 31 kD region, former for IFN-t-EGFP fusion protein and the latter protein band for EGFP+ respectively which confirms the expression of the transfected gene (lane 1 and 3). In contrast, 31 kD protein

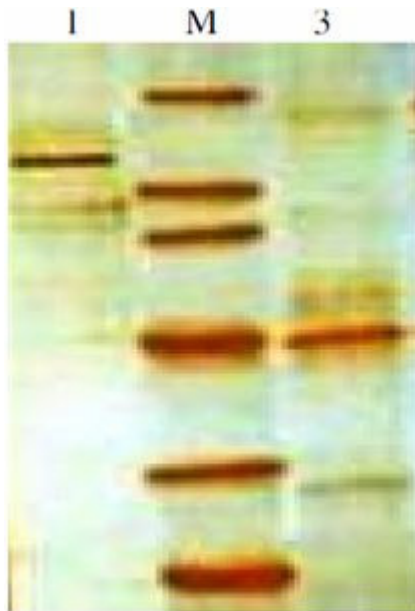


**Figure 6.** Protein fraction obtained from cell lysate subjected to 12% SDS- PAGE and Coomassie blue staining. (a) M – Marker T– transfected protein M- marker (kD) N– Non transfected protein, (b) T2-T6– passage no. for cultures post transfection.

band was not detected from the protein extracted from the cultures transfected with the recombinant IFN-t-EGFP gene. A number of protein bands were observed for the supernatant fraction obtained during the immuno precipitation of proteins. Lane 2 represents the positions of the molecular mass standards (Figure 7).

### Effect on lymphocyte proliferation

The bovine lymphocytes were separated from blood and subjected to mitotic stimulation with phytohaemagglutinin (PHA). Lymphocytes devoid of PHA served as unstimulated cells. The culture medium alone served as blank. These cultures were subjected to MTT test and lymphocyte stimulation index (LSI) was calculated as ratio of the absorbance of the stimulated lymphocytes to the unstimulated lymphocyte cultures. It was observed that LSI of lymphocyte culture treated with immunoprecipitated protein and with elution buffer was 0.745 and 0.945 respectively. The LSI of lymphocyte cultures without addition of any protein was 1.15. The stimulation index with immunoprecipitated protein had significantly decreased ( $P < 0.05$ ) when compared with that of untreated cultures. Similarly LSI of lymphocyte cultures treated with protein fraction of the crude cell lysate obtained from transfected cultures significantly decreased the stimulation index when compared with the protein fraction from the untransfected cells ( $P < 0.05$ ) (Figure 8).

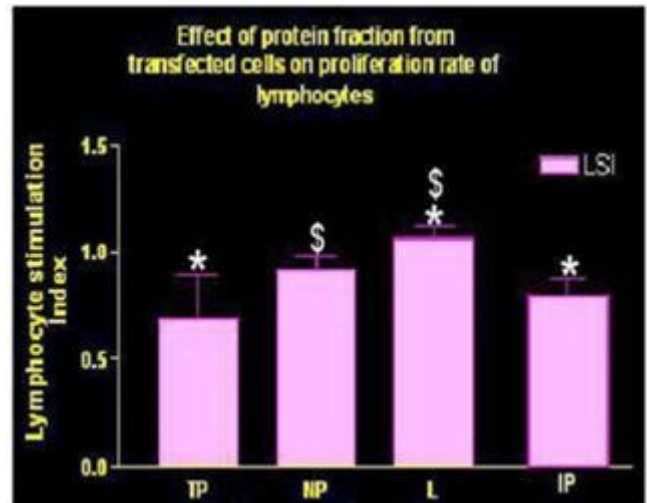


**Figure 7.** Lane-1 Recombinant IFN-tGFP Protein (53 kD) Lane-2 Green fluorescent protein Lane-3 EGFP (31 kD)–Green fluorescent protein Lane-2 M- Standard protein markers from bottom 14, 18, 31, 44.3, 45, 66 (kD). Protein fraction obtained from cell lysate subjected to 12% SDS – PAGE and silver staining.

## DISCUSSION

In this study, we demonstrated the expression of transfected gene IFN-t-GFP in fibroblast cell culture systems. The recombinant IFN-t-EGFP protein could be detected or resolved on SDS-PAGE only after silver staining of the gels.

In this study, we have applied a novel non-viral transfection system, nucleofection to induce high recombinant gene expression in primary fibroblast cell lines. In preliminary experiments, it was tested with different conditions of electrical pulsing to determine the program, inducing the highest transgene expression. By using a IFN-t-GFP and GFP as reporter, plasmid under the transcriptional control of a eukaryotic promoter (Materials and Methods), the highest fraction of GFP+ cattle fibroblast cells was obtained after transfection with U-23 program. Similar were the results on transfection with p EGFP only our results on cell survival and recovery are apparently in confirmation with the results obtained by Aluigi et al. (2006). However, even after six weeks of culture, significant percentage of GFP+ cells could be detected. Nucleofected fibroblast cells could be used in several types of studies where expression of transgene protein is required. There are some reports where low cell recovery was observed (Maasho et al., 2005; Laakshmi et al., 2004).



**Figure 8.** Lymphocyte stimulation index of cell cultures (LSI) When cultures were incubated with protein fraction from Transfected cell culture (TP) ( $P < 0.05$ ), with immune - precipitated protein (IP) ( $P < 0.05$ ), with protein fraction from non transfected cell culture (NP). Control (L) - lymphocyte culture treated with PHA w/o addition of any protein.

Available evidence indicates that IFN-t inhibits transcription of estrogen receptor  $\alpha$  and oxytocin receptor genes to block development of the uterine luteolytic mechanism (Spencer and Bazer, 1996; Spencer et al., 1998). Use of green fluorescent protein (GFP) from the Jellyfish *Acquorea victoria* is a powerful method for non-destructive *in situ* monitoring, since expression of green fluorescence does not require any substrate addition (Anderson et al., 1998). The GFP gene may be transferred to and expressed in a wide range of organisms, e.g., mammals (Ludin et al., 1996).

Because of the central role of IFN-t in gestation, this protein has excited the interest of reproductive physiologists (Demmers et al., 2001). Supplementation of inseminated animals with exogenous IFN-t during the pregnancy recognition period has been considered as a means to compensate for inadequate secretion of IFN-t by conceptuses that are delayed in their development and enhance their chances for survival (Spencer et al., 1999). Hence in the present experiment we attempted to develop culture system for expression of pbIFN-t-EGFP in Sahiwal fibroblast cells. It proves and paves way for studies on transfection of other genes and their functions.

Reports are available, where efficient transfer of DNA expression vectors and SiRNA oligonucleotides into a variety of primary cell types from different species including human B-CLL cells, human CD34+ cells, human lymphocytes, rat cardiomyocytes, human bovine chondrocytes (Greseh et al., 2004). Transfection has been achieved in mural stem cells also (Marchenko and Flanagan, 2007). The versatility and resolving capacity of polyacrylamide gel electrophoresis (PAGE) has resulted



in the most popular method of protein identification and purification post Coomassie blue staining. The sensitive method used by Hochstrasser et al. (1998) and Rabilloud (1992) was used for silver staining procedure. The products of recombinant bIFN-t, expressed in fibroblast cells were analysed by SDS-PAGE both by Coomassie Blue and Silver staining procedure, where we were successful in analyzing protein by silver staining only, this may be due to unstable nature of the protein during and after extraction or low level of expression of protein.

IFN-t can inhibit proliferation of peripheral blood lymphocytes. IFN-t had the ability to inhibit proliferation of PHA stimulated lymphocyte cells (Tekin et al., 2000). In our studies also the extracted protein fraction from the transfected cells could inhibit the proliferation of lymphocytes *in vitro* indicating immunosuppressive activity. This may be due to IFN-t or some upregulated protein which is immunosuppressive in nature.

The current study describes for the first time expression of IFN-t-GFP genes in cattle fibroblast cells at different passages post transfection under the control of eukaryotic promoter specific for IFN-t-expression (Toshihiko et al., 1999), it is necessary for the proliferated transfected cells to show an increase in number of transfected cells. To overcome the above mentioned limitations, the delivery of DNA encoding reporter genes such as EGFP is necessary to promote the expansions capacity and survival potential. As the fluorescence of the protein could be visualized, which was present at the downstream of the recombinant gene construct. It shows that protein IFN-t was expressed in cell cultures.

The detection of protein on silver staining demonstrates the tendency of cell cultures towards less production or less stability of the fusion protein and further recommends improvement in techniques or possibility to enhance the stability of the extracted recombinant protein has to be explored.

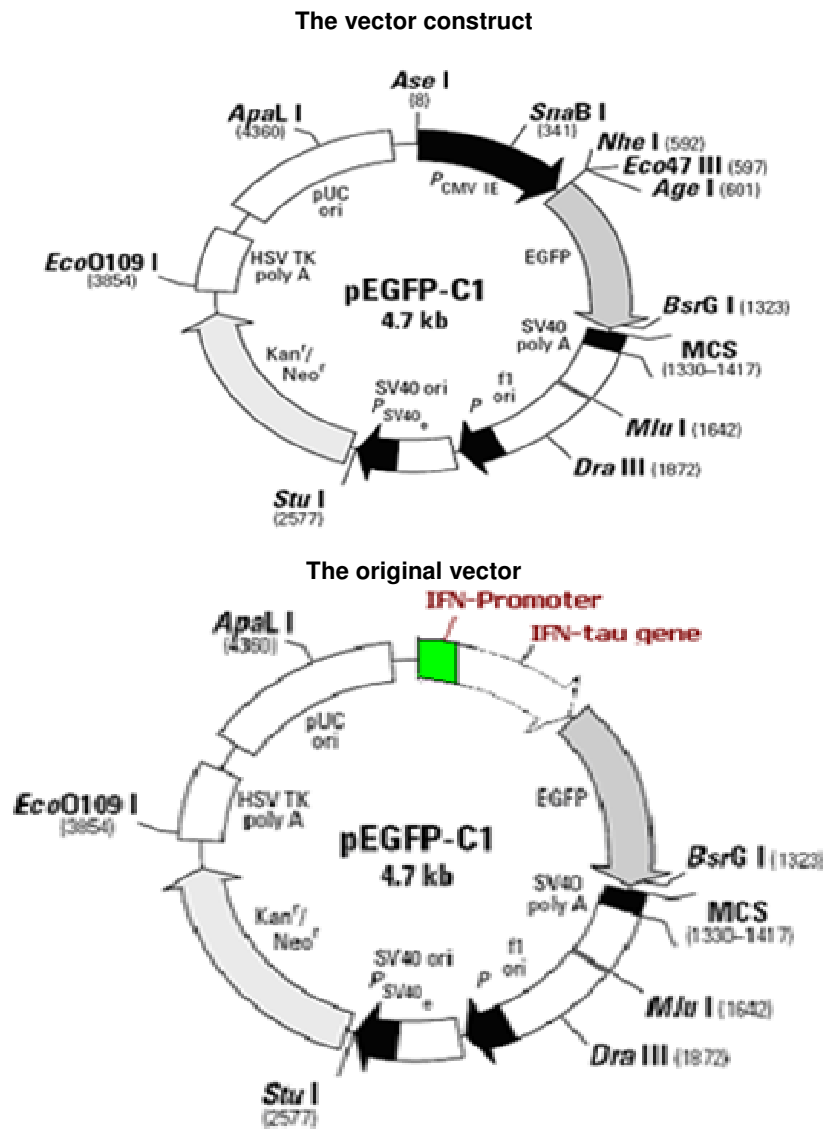
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APPENDIX



**Figure 1.** The cloned construct with IFN-t and EGFP construct at the downstream of IFN-t promoter.