

## The Effect of Linear Versus Circular Vector on Enhanced Green Fluorescent Protein (EGFP) Expression in Transgenic Zebrafish (*Danio rerio*)

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**Abstract:** Green Fluorescent Protein (GFP) has been used as an indicator of transgene expression in living cells and organisms. In this study, a transgene construct containing the Cyto-Megalo-Virus (CMV) promoter sequences, SV40 polyA signal and the Enhanced Green Fluorescent Protein reporter gene (EGFP) was microinjected into the cytoplasm of one-cell zebrafish embryos. About 65 ng  $\mu\text{L}^{-1}$  circular and linearized pEGFP-N1 DNA was used in microinjection. Transgenic founders were detected by Polymerase Chain Reaction (PCR), Slot and Southern blots and Reverse-Transcriptase PCR (RT-PCR). EGFP gene expression was detected by inverted fluorescence microscope in F<sub>0</sub> transgenic zebrafish larvae. About 54 and 25 F<sub>0</sub> transgenic zebrafish were obtained after microinjection of linearized and circular gene constructs, respectively. This is the first study for generation of transgenic zebrafish via cytoplasmic DNA microinjection in Turkey. In conclusion, these results indicate that the gene expression efficiency of circular form was higher than the linearized form in F<sub>0</sub> transgenic zebrafish larvae.

**Key words:** Transgenic fish, transgenic technology, fertilized fish egg, microinjection, *Aequorea victoria*, *Danio rerio*

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### INTRODUCTION

The transgenic technology was first applied to fish in mid 1980's. Several transgenic fish species including rainbow trout, medaka, zebrafish etc. were used in transgenic studies (Chourrout *et al.*, 1986; Brem *et al.*, 1988). Especially, zebrafish has proved to be a useful model for transgenic studies. This is due to the facts that zebrafish embryos are optically transparent, develop rapidly and hatching eggs at 2-3 days postfertilization (Westerfield, 1995).

For about the 20 years, transgenic fish have been actively used in both basic and applied research. Several methods are available to monitor gene activity and protein distribution within the cells. These include the formation of fusion proteins with coding sequences for  $\beta$ -galactosidase and luciferase (Chalfie *et al.*, 1994). Alternatively, fluorescent protein GFP is a simple and sensitive detection method. GFP is a photoprotein of a bioluminescent jellyfish, *Aequorea victoria* (Bagis and Keskinetepe, 2001; Chalfie *et al.*, 1994). GFP used as a

reporter gene in various organisms and did not appear to have a toxic effect on the cells (Bagis and Keskinetepe, 2001; Takeuchi *et al.*, 1999). It does not require an enzyme substrate reaction (Amsterdam *et al.*, 1995; Hamada *et al.*, 1998; Peters *et al.*, 1995; Takeuchi *et al.*, 1999) and fixation procedure, only exposure ultraviolet with suitable excitation wavelength (Chalfie *et al.*, 1994). Thus, it should provide an excellent means for monitoring gene expression and protein localization in living cells (Chalfie *et al.*, 1994). The objective of this study was to investigate comparison of linear versus circular vector on Enhanced Green Fluorescent Protein (EGFP) expression efficiency in transgenic zebrafish (*Danio rerio*).

### MATERIALS AND METHODS

**Zebrafish maintenance and diets:** Fish were spawned and cultured on a light/dark cycle essentially as described by Westerfield (1995). Non-transgenic and transgenic fish were fed on a commercial diet 3 times a day and *Artemia nauplii* once a day.

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**Preparation of vector DNA:** The vector construct, the 4.7 kb pEGFP-N1 plasmid containing the EGFP gene driven by the CMV promoter was purchased from Clontech Laboratories (Clontech Laboratories, Palo Alto, CA). Plasmid DNA was purified by using a commercially midiprep kit (Qiagen), resuspended in water and used in the circular and linearized forms. For linearization, the pEGFP-N1 vector DNA was digested by *VspI* and *AflII*, MBI Fermentas. A linear plasmid fragment containing CMV promoter, the EGFP reporter and the SV40 polyA signal was isolated using the EZ-10 Spin Column DNA Gel Extraction Kit (BioBasic, B S 354). The purified DNAs were quantified and diluted to 65 ng  $\mu\text{L}^{-1}$  in Tris-EDTA buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4).

**Cytoplasmic microinjection and analysis of transgene expression:** Embryos rolled down the agarose ramp and lined up in the groove. Circular or linearized gene constructs were injected into the cytoplasm of fertilized eggs at the one-cell stage. Injections were performed under a stereo microscope (Magnification: X2) (Soif, DA 0671). EGFP expression was detected by observing 3 days old zebrafish larvae with an inverted fluorescence microscope (Carl Zeiss, Axiovert 35M). Photographs were taken by camera system.

**Isolation of genomic DNA and PCR analysis of integrated transgenes:** The DNA was extracted from 3 and 10 days old transgenic and non-transgenic larvae. Isolation of genomic DNA was performed by using a DNA isolation kit (EZ-Spin Column DNA Isolation Kit, Biobasic) according to the manufacturer's instructions. Total genomic DNA samples were analyzed by PCR for the presence of the EGFP transgene. PCR was performed with approximately 100 ng of genomic DNA, 20 pmol of each oligonucleotide (sense and antisense primers), 2X PCR Master Mix (MBI Fermentas, Germany). The PCR cycle parameters were 30 sec at 95°C, 60 sec at 58.7°C and 90 sec at 72°C for 30 cycles. The EGFP specific primers used to amplify a 712 bp fragment of the target sequence were 5'-ATGGTGAGCAAGGGCGAGGAGCTGT-3'(sense) and 5'-TACAGCTCGTCCATGCCGAGAGTGATCC-3'(antisense).

**Slot and southern blot analyses:** Slot and Southern blot analyses were conducted on founder  $F_0$  transgenic and non-transgenic fish. All DNA samples were treated with RNase A (Roche) at 10  $\mu\text{g mL}^{-1}$  for 1 h at 37°C. Slot blot analysis was performed with 10  $\mu\text{g mL}^{-1}$  of genomic DNA from each fish (10 days old). Southern blot analysis was performed 20  $\mu\text{g mL}^{-1}$  of genomic DNA from each fish (10 days old) and genomic DNA was digested *Bgl II* enzymes (MBI Fermentas, Germany), electrophoresed on

a 1% agarose gel and transferred to Hybond-N<sup>+</sup> membrane (Amersham) according to Sambrook *et al.* (1989). Purified circular and linearized DNA were used as a quantification and size control. Linearized EGFP gene construct was labeled with digoxigenin (DIG-DNA Labeling and Detection Kit, Roche, Germany) and used as a probe. The hybridization took place overnight at 48°C in DIG-EASY Hybridization Solution (Roche, Germany).

**Total RNA isolation, first strand cDNA synthesis and RT-PCR:** Total RNA was extracted from the frozen transgenic and non-transgenic larvae by using High Pure RNA Tissue Kit (Roche). First-strand cDNA synthesis was performed using 1  $\mu\text{g}$  of total RNA of larvae according to the RevertAid<sup>TM</sup> First-Strand cDNA Synthesis Kit (MBI, Fermentas). RT-PCR reactions were carried out in 200  $\mu\text{L}$  microfuge tubes containing 10  $\mu\text{L}$  2 $\times$  PCR master mixes and 20 pmol of each EGFP gene specific primers and 1  $\mu\text{L}$  of the synthesized cDNA. RT-PCR reactions were performed at the same PCR reactions conditions given before.

## RESULTS AND DISCUSSION

**Microinjection and production of transgenic zebrafish (*Danio rerio*):** Initially, procedures for obtaining large numbers of zebrafish eggs for microinjection were established and a high frequency of germ-line transmission of injected DNA was obtained. Fluorescent cells were first observed at 3 days post fertilization. Also, fluorescence and non-fluorescence transgenic candidate larvae were investigated for transgene integration and expression using molecular methods. About 54 and 25  $F_0$

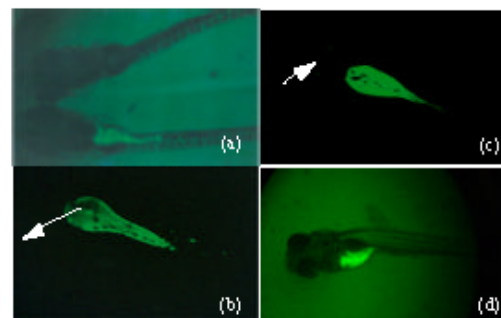


Fig 1: Expression of linearized EGFP was observed 3 days old zebrafish larvae in different tissues. Embryo injected with pEGFP-N1 vector DNA containing EGFP gene under the control of CMV promoter was digested by *VspI* and *AflII* and viewed under a blue excitation light for EGFP observation. The expression of the linearized EGFP gene was observed at the dorsal area (A, B), yolk sack (a, b, c, d) and eyes (C)

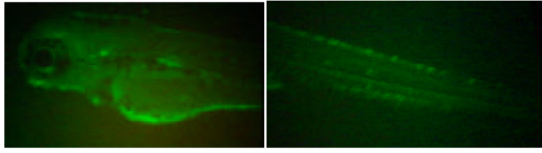


Fig. 2: Expression of circular EGFP was observed 3 days old zebrafish larvae. The expression of the circular EGFP gene construct was observed ubiquitously distributed at different parts of body (yolk sack, head, dorsal muscle of the belly and eyes) in transgenic zebrafish larvae

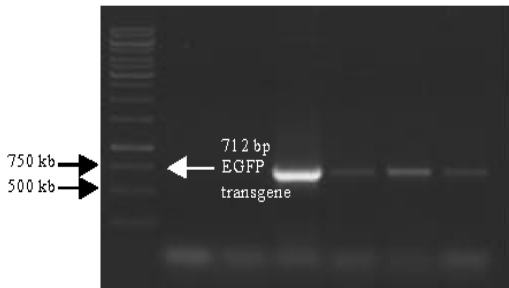


Fig. 3: Results of PCR analyses on a 1% agarose gel. Lane 1, 1 kb DNA ladder; lane 2; not including DNA; lane 3, nontransgenic zebrafish genomic DNA; lane 4, pEGFP-N1 plasmid DNA; lane 5, transgenic zebrafish DNA (line 2), lane 6, transgenic zebrafish DNA (line 3); lane 7 transgenic zebrafish DNA (line 4)

zebrafish were obtained after microinjection of linearized and circular gene, respectively. It was observed that the gene expression efficiency of circular form was higher than the linearized form in  $F_0$  zebrafish larvae. The expression of the linearized gene was observed at the dorsal area, yolk sack and eyes (Fig. 1).

The expression of the circular gene construct was observed ubiquitously distributed at different parts of the body (yolk sack, head, dorsal muscle of the belly and eyes) in transgenic zebrafish larvae (Fig. 2).

After the microinjection of linearized form 6  $F_0$  transgenic fish was obtained. In this study, 520  $F_1$  larvae were obtained. Fluorescent light emission was shown at 102 larvae (20%). Male and female  $F_1$  transgenic fish were obtained by mating of 19 numbered  $F_0$  female with wild-type male fish. Obtained  $F_1$  transgenic fish were mated to each other to produce  $F_2$  transgenic fish in which fluorescent light was observed at 20%. Gene expression of EGFP was confirmed by detection of fluorescent light

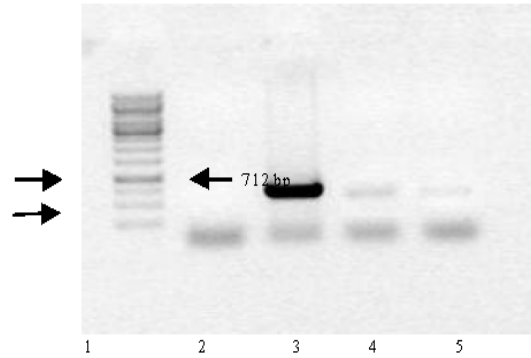


Fig. 4: Results of RT-PCR analyses. Lane 1, 1kb DNA ladder; lane 2, nontransgenic zebrafish as negative control; lane 3, pEGFP-N1 plasmid DNA; lane 4, cDNA of transgenic zebrafish (3 days old) ( $200 \text{ ng } \mu\text{L}^{-1}$ ); lane 5, cDNA of transgenic zebrafish (3 days old) ( $100 \text{ ng } \mu\text{L}^{-1}$ )

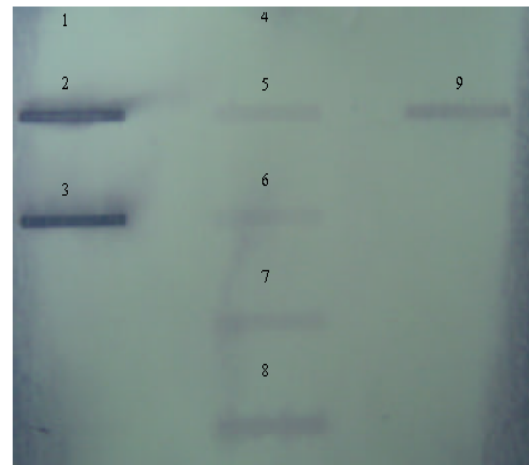


Fig. 5: Results of slot-blot analyses; 1. nontransgenic genomic DNA as negative control ( $10 \mu\text{g}$ ); 2. pEGFP-N1 plasmid DNA; 3. Afl II digested pEGFP-N1 plasmid DNA; 4, 1X copy; 5, 5X copy; 6; 10X copy; 7, 20X copy; 8, 50X copy; 9, transgenic zebrafish DNA ( $10 \mu\text{g}$ ) (line 2)

in zebrafish larvae. The EGFP gene integration to fish genome was detected by PCR analysis (Fig. 3).

Besides, RT-PCR results indicated that EGFP gene was stably transcribed in transgenic zebrafish (Fig. 4). Slot Blot analysis showed that one of the transgenic fish carry about 50 copies of foreign DNA (Fig. 5). Southern Blot analysis was used to test for presence of transgene in the transgenic fish (Fig. 6). Here, we report success in rapidly obtaining large numbers of eggs for injection and also report a high frequency of germ-line transmission of an



Fig. 6: Results of southern-blot analyses. Lanes 1-3 transgenic zebrafish (10 days old) DNA (20 µg); lane 4, *VspI*-*AflIII* fragment of the plasmid (EGFP DNA); lane 5, pEGFP-N1 plasmid DNA. All DNA samples were RNase treated before agarose gel electrophoresis. The filters were hybridized with an EGFP-specific DNA probe. The DNAs were digested with an *BglIII* restriction enzyme

injected linearized and circular EGFP forms. Gene expression efficiency of circular form is higher than the linearized form (Haobin *et al.*, 2000). Moreover, only F<sub>0</sub> transgenic fish using circular gene construct was expressed examinations over the first 48 h following microinjection showed that the linear DNAs and circular forms were expressed at about equivalent rates but thereafter the circular form has given higher rates of expression (Ekici, 2007; Ekici *et al.*, 2007; Haobin *et al.*, 2000). After microinjection of linear form 6 F<sub>0</sub> transgenic fish were obtained which maintained the transgene expression after mating of F<sub>0</sub> transgenic fish with wild-type fish. In concordance with Stuart *et al.* (1988) and Alimuddin (2002) it was found that these F<sub>1</sub> transgenic fish was mating each other to produce F<sub>2</sub> transgenic fish which fluorescent light observed at 20% (Alimuddin, 2002; Culp *et al.*, 1991; Stuart *et al.*, 1988).

Expression of the gene construct containing the coding sequence for the EGFP was determined in whole body of the transgenic zebrafish. Mosaic expression was observed in zebrafish because foreign DNA construct was injected into cytoplasm. Finally, as reported in other studies, we confirm here that EGFP gene expression has no toxic effects in zebrafish embryos.

In this study a transgenic zebrafish, carrying the EGFP gene expression unit was generated. One founder identified by PCR, RT-PCR, Southern and Slot blot analyses were used to establish transgenic zebrafish lineage. Non-radioactive DNA labeling and detection system was applied for the detection of copy number of the transgene sequences. Although, radioactive labelling system is too sensitive for transgene detection it is too harmful for human health. In this study it was shown that non-radioactive DNA labeling and detection system is

sensitive enough for detection of low copy number sequences. Copy number of transgene was detected in 4 and 7 µg of genomic DNA used in Slot Blot experiments.

## CONCLUSION

In this study, transgenic fish production technology was established at the first time in Turkey and EGFP transgenic zebrafish was obtained by using this technology. Procedures for obtaining large numbers of zebrafish eggs for microinjection were established and a high frequency of germ-line transmission of injected DNA was obtained. In the present study, evidence was given for the first time that coding sequence for the GFP gene could be integrated, stably transcribed and expressed in whole body of the transgenic zebra fish. Further breeding programs in the laboratory are being performed to expand EGFP carrying and expressing transgenic lineage to establish a useful model to the scientists for investigating human gene functions. The result of this study will give an opportunity to produce transgenic zebrafish lineages.

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