

Note

Optimisation of transformation in the heterologous fusion gene GAPDH-IFN cloning using DH5α strain of *Escherichia coli*

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Abstract

DNA vaccines are the most essential tool of the disease prevention strategy. In the present study, cloning of a heterologous fusion gene involving glyceraldehyde 3-phosphate dehydrogenase and interferon gamma (GAPDH-IFN) was conceptualised. Cloning was tried using four distinct transformation techniques viz. InstAclone PCR Cloning kit (Fermentas, USA); CaCl_2 transformation protocol; Clontech stellar competent cells protocol and PEG 8000-mediated transformation method, for the heterologous GAPDH-IFN fusion gene using DH5α strain of *Escherichia coli*. The first three methods were found to be unsuitable, and the PEG 8000-mediated transformation method yielded positive clones.

Aquaculture is a rapidly expanding industry with a total production of 122.6 million t, including aquatic animals and algae (FAO, 2022), and bacterial infections are the predominant problem (Austin *et al.*, 2012). In these circumstances, DNA vaccines are a unique and crucial component of the disease prevention approach (Bedekar and Kole, 2022). For one of the fish bacterial pathogens *Edwardsiella tarda*, a viable heterologous fusion-gene DNA vaccine creation, was conceptualised from the prototype bicistronic DNA vaccine pGPD/IFN (Kumari *et al.*, 2018). The heterologous fusion gene inserts GAPDH-IFN (Glyceraldehyde 3-phosphate dehydrogenase of *E. tarda* and Interferon-gamma of *Labeo rohita*) was ligated with the plasmid vector (pGAP/IFN; Valsalam *et al.*, 2023). The vector is injected into competent *Escherichia coli* cells that serve as the source of the clone once a foreign gene has been successfully cloned (Fakruddin *et al.*, 2013). Plasmids are not always stable, especially in cells undergoing several generations of growth. Hence it is critical to address vector stability (Pierce and Gutteridge, 1985). The genotypes of the vector

and host impact plasmid stability; the same plasmid behaves differently in various hosts and *vice versa*. It has been noted that foreign DNA's size and origin impact plasmid stability (Rai and Padh, 2001). GAPDH is the 37 kDa immunogenic outer membrane protein of *E. tarda* (Tu and Kawai, 1999), and IFN- γ is an immune adjuvant gene of *L. rohita* (Bedekar *et al.*, 2018). Together in the DNA vaccine as a chimeric gene combination, GAPDH and IFN- γ provoke a higher immune response than the monocistronic DNA vaccine (Kumari *et al.*, 2018).

In this context, the present study attempted to optimise transformation methods in cloning the heterologous fusion gene plasmid pGAP/IFN, using DH5α strain of *E. coli*. Transformation was carried out using four different methods viz. InstAclone PCR Cloning kit (Fermentas, USA); CaCl_2 transformation protocol; Clontech stellar competent cells protocol and PEG 8000-mediated transformation method, to produce positive clones.

InstAclone PCR Cloning kit (Fermentas, USA) was used for transformation and



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the protocol supplied by the manufacturer was followed wherein the *E. coli* (DH5 α) was inoculated into C-medium (2.0 ml) and incubated overnight (37°C) with shaking. To pre-warmed fresh C-medium (1.5 ml), 150 μ l of the overnight culture was added and incubated (37°C) for 20 min at 200 g. Transformation solution (T-solution) was prepared by mixing equal volumes of T-solution (A) with T-solution (B) and kept on ice. *E. coli* (DH5 α) culture (1.5 ml) was centrifuged (10,000 g) for 1 min, the supernatant discarded, and the cells re-suspended (300 μ l of T-solution) and incubated on ice (5 min). Further centrifugation (2 min at 6000 g), re-suspension (120 μ l T-solution), and incubation on ice (5 min) were done. The re-suspended cells (50 μ l) were added to ice stored 2.5 μ l ligated plasmid and kept for transformation on ice (5 min). Finally, the cells were plated on pre-warmed Luria Bertani (LB) selection plate.

CaCl₂ transformation protocol was adapted from Sambrook and Russell (2006). From the overnight culture of the DH5 α bacteria, 1.0 ml was inoculated in 100 ml of fresh LB broth (pH 7.0) and was grown (37°C) until it had reached 5×10^7 cells ml⁻¹ (time varies depending on inoculum concentration). A 5 ml aliquot of transformation reaction was transferred to centrifuge tubes and chilled on ice for 10 min. The cells were pelleted by spinning (5000 g) for 5 min at 4°C. The supernatant was discarded, and the cells were re-suspended in cold 0.1M CaCl₂ and incubated in ice for 20 min. Centrifugation was repeated, and the cells were re-suspended in 0.2 ml of chilled 0.1M CaCl₂. The suspensions were transferred to tubes to which 100 ng of ligated plasmid was added and incubated on ice for 30 min. The mix was transferred to a 42°C water bath for 2 min and snap chilled on ice. The content of each tube was transferred to 2 ml of LB broth in a tube and incubated (37°C) for 60 min with shaking. Plating was done using 0.1 ml aliquots of the content onto LB selection plates and incubated overnight at 37°C.

In the Clontech stellar competent cells protocol, the *E. coli* HST08, i.e. stellar cells, were used for transformation following the manufacturer's protocol using the readymade stellar competent cells (Takara Bioscience). The competent cells were thawed in an ice bath, and 50 μ l was transferred into a 14 ml round-bottom tube. About 5 ng of DNA for transformation was added to it. The tubes were placed on ice (30 min), and heat shock was given to the cells for 45 s at 42°C. The tubes were again placed on ice for 1-2 min. Pre-warmed SOC medium (provided by the manufacturer) was added to make up the volume to 500 μ l and incubated (37°C) for 1 h at 160 g. Plating was done using 100 μ l of the mixture in the selection media and incubated overnight (37°C).

For PEG 8000-mediated transformation method, DH5 α cells were grown overnight, and the next day the cells were diluted to 1/200 in LB and were grown until the OD₆₀₀ reached 0.2-0.35. The cells were spun at 2500 g for 10 min. The cell pellet was re-suspended in 1/10th volume of PEG 8000 solution (10%). This was incubated on ice for 10 min. The pGAP/IFN ligation DNA (250 ng) was diluted to 200 μ l with

Tris HCl-Mg-Ca solution (pH 7.5) and chilled on ice. This ligation mixture was transferred to the chilled competent cells and incubated on ice for 1 h. Heat shock was given for 2 min at 42°C. This was again incubated on ice for about 5 min, and 600 μ l SOC medium containing bacto-tryptone, bacto-yeast extract, NaCl, KCl, MgCl₂, and glucose was added and incubated at 37°C for 1 h at 150 g. This was poured on an LB selection plate and incubated at 37°C overnight.

The clones were confirmed in each step by colony PCR using IFN- γ primers and the amplification conditions reported by Valsalam *et al.* (2023) with the amplicon size of 574 bp. The colony was picked and used as the template for the PCR reaction.

Following the 'InstAclone PCR Cloning kit (Fermentas, USA) protocol', the transformation was attempted several times, but a successful transformation could not be achieved. So this method was found unsuitable for the transformation of pGAP/IFN. The colonies developed by the CaCl₂ method were found positive for harbouring the insert by colony PCR (Fig 1). Nevertheless, the cells in the subsequent subculture were plasmid negative. It was assumed that colonies excluded the insert from their cells within the next 48 h of transformation. This was repeatedly observed in the subsequent culture plates and in the overnight culture broths, where the procedure was repeated. So the transformation of pGAP/IFN was found unsuccessful by this method.

Following the 'Clontech stellar competent cells protocol', an appropriate amount of culture was plated on the LB selective medium. Some colonies developed were found positive for harbouring the insert by colony PCR (Fig. 2). However, the cells were pGAP/IFN negative in the subsequent subculture. So this method of transformation was also found to be unsuitable.

The PEG 8000-mediated transformation method using DH5 α cells resulted in colony growth in the transformation plate, which was confirmed to be positive for the insert by colony

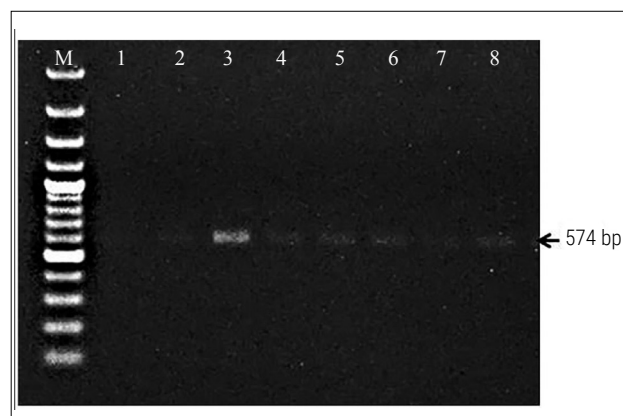


Fig. 1. Agarose gel electrophoresis (1.5%) showing the INF- γ gene amplified by colony PCR from the plate prepared by the CaCl₂ transformation method. Lane M: 100 bp plus molecular weight marker (Thermoscientific Generuler); Lane 1-8: Colony PCR of IFN- γ gene (574 bp)

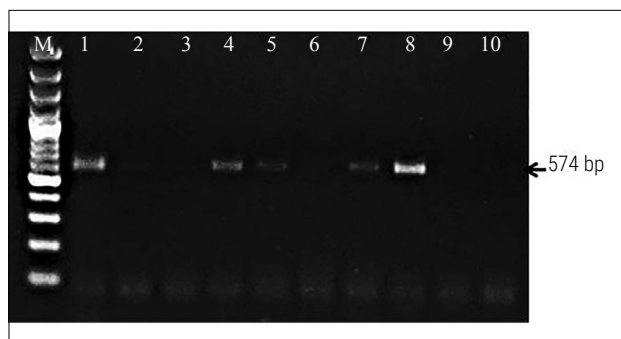


Fig. 2. Agarose gel electrophoresis (1.5%) shows the INF- γ gene amplified by colony PCR from the plate prepared using stellar competent cells transformation protocol. Lane M: 100 bp plus molecular weight marker (Thermoscientific Generuler); Lane 1-10: Colony PCR of IFN- γ gene (574 bp).

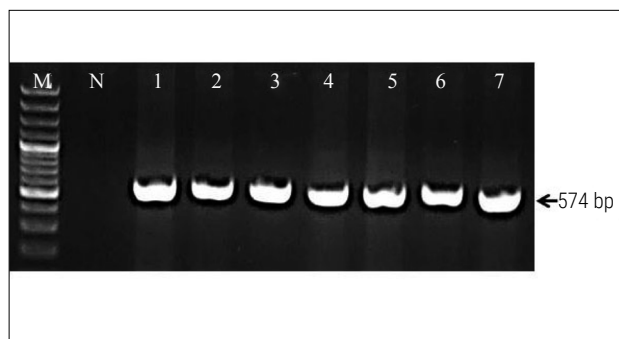


Fig. 3. Agarose gel electrophoresis (1.5%) showing INF- γ gene amplified by colony PCR from the plate prepared using the PEG 8000-mediated transformation method. Lane M: 100 bp plus molecular weight marker (Thermoscientific Generuler); Lane N: Negative control; Lane 1-7: Colony PCR of IFN- γ gene (574 bp)

PCR reaction (Fig 3). The master plate was prepared, and the plasmid extraction was done.

The different types of transformation methods are effective for many of the genes throughout the cloning process; nevertheless, for some types of gene/vector/host, additional aspects affect the efficacy of cloning (Fakruddin *et al.*, 2013). There are selected clones that exert the metabolic burden on the host cells (Aiba *et al.*, 1982), sometimes along with their toxic property (Li *et al.*, 2017) and the formation of inclusion bodies in the host bacteria (Betts and King, 1999). Different methodologies were attempted to get positive clones in the present study on a difficult-to-clone genes. Four chemical transformation methods were compared in a study where DH5 α and stellar cells were used as competent cells. The DH5 α strain was used since it is reported for efficiency to enhance the insert stability and the integrity of plasmid DNA due to its genotype, particularly the *recA1* and *endA1* (EN-English *et al.*, 2023).

The CaCl₂ transformation method was attempted on the DH5 α strain based on the available literature where its suitability for transformation has been reported (Zheng *et al.*, 2006; Smajovic *et al.*, 2021). Some studies contradict these reports, where the CaCl₂ transformation method was best for SCS110, TOP10, and BL21 strains rather than the DH5 α strain (Chan *et al.*, 2013). Similarly, for our study, also CaCl₂ method failed in the transformation.

The vector used in this study was a high copy number plasmid, and it might have caused a severe metabolic burden to the *E. coli* cells, as stated by Jones *et al.* (2000). Only the PEG 8000-mediated transformation method gave the GAPDH-IFN positive clone. Replication and maintenance of plasmid DNA could induce a "metabolic burden" in bacterial cells such as *E. coli* (Silva *et al.*, 2012). The PEG 8000-mediated transformation method was found to contain the nutrients necessary for reducing the metabolic stress in the DH5 α cells, such as bacto-tryptone and bacto-yeast extract along with 2x concentration of LB media (Kram and Finkel, 2015).

The oxidative stress response varies depending on the source of tryptone in the culture media (De Spiegeleer *et al.*, 2004). Also, the use of PEG aided successful cloning and transformation process. It is concluded that out of the four chemical-mediated transformation methods, PEG induced transformation was found to be comparatively efficient and successful.

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