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Expression and characterization of recombinant single chain beta-alpha equine chorionic gonadotropin in prokaryotic host

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ABSTRACT

The aim of present study was to produce recombinant eCG/pituitary glycoprotein in the *Escherichia coli* (*E. coli*) BL21C host cells and to test its diagnostic efficacy. This aim was achieved by optimizing its expression, purification as well as its characterization through the immunoassays and bioassays. A bacterial protein expression vector system based on the phage T_7 promoter and histidine tag was used for the expression and purification. The recombinant single chain beta-alpha equine chorionic gonadotropin (rβαeCG) encoding gene was constructed with beta and alpha sequences according to its biologically active counterpart. It was successfully cloned and when expressed in *E.coli* BL21C host, the purified recombinant protein was found to be active as revealed by enzyme linked immunosorbent assay (ELISA) and Western blotting. However, it was not found to exhibit any significant activity *in vivo* when tested in the mice

Key words: eCG; *Escherichia coli*, Expression, Hormone, Pituitary, Protein, Recombinant.

INTRODUCTION

The equine chorionic gonadotropin (eCG), popularly known as pregnant mare serum gonadotropin (PMSG), belongs to the family of glycoprotein hormones and have important roles in animal reproduction. It is produced by the trophoblast cells of endometrial cups in pregnant mares and is primarily responsible for the maintenance of early gestation (Allen and Moor, 1972). Like the other members of the hormone family including the follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH), eCG is composed of two dissimilar and non-covalently connected α and β subunits. The α-subunit is encoded by a single gene within same species. It is common to all glycoprotein hormones, whereas diverse genes encode β subunits, which confer receptor binding specificity to the glycoprotein hormone heterodimers (Combarnous, 1992).

In horses unlike primates, both the LH and CG β subunits are encoded by the same gene (Sherman *et al*., 1992) and accordingly the recombinant hormone is called eLH/CG. Both the placental eCG and pituitary eLH exhibit dual LH and FSH action in species other than equines with alike FSH/LH activity ratios (Combarnous, 1992). Although eCG and eLH display identical α and β polypeptide chains, their carbohydrate contents are not the same. Certainly, eCG is the most heavily glycosylated of all glycoprotein hormones with 45% carbohydrate by weight versus 30% for eLH (Bousfield and Butnev, 2001). Because of its well reported and experimented biological properties, eCG has been used in the fertilization and fecundity augmentation programs till date. However, the available preparations of partly purified eCG from pregnant mare serum (PMSG) could contain contaminants with impending sanitary risks (Legardinier *et al.*, 2005). Therefore, it is of great interest to produce in large quantities a bioactive substitute for eCG and other gonadotropins to be used as therapeutic agents (Leao and Esteves, 2014). Earlier, several workers reported the production of eCG in varied expression systems, but with limited purification and *in vivo* activity. The tethered eCG/LH (without C-terminal peptide and β26-110 disulphide bridge) was found biologically active *in vitro* (Galet *et al.*, 2000). The expression of a single beta-alpha chain eLH/CG was too reported (Galet *et al.*, 2001). The eLH/CG was also produced in the milk of transgenic rabbits and it was found to be inactive biologically. The biological efficacy of recombinant eLH/CG expressed in Sf9 and Mimic insect cell lines was reported in 2005 (Legardinier *et al.*, 2005). Seo *et al.*, 2015, focused on the biological activities (LH- and FSH-like activities) of amino acids (94, 95, and 96) in the eCG β-subunit. In the present work, we carried out the expression, purification and characterization of single chain beta alpha eCG through bacterial expression system on similar lines of our earlier work on recombinant bovine inhibin alpha (Bhardwaj *et al*., 2012, 2013). Recently, we reported the characterization and molecular docking analysis of eCG alpha molecule (Bhardwaj *et al*., 2017). We further aimed to clone and express pregnant mare serum gonadotropin (PMSG)/eCG/eLH in suitable host system. Further, the recombinant

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protein was also purified for research/commercial application as a diagnostic and/or to study its biological efficacy *in vivo*. Here, it is important to notice that availability of purified hormone is essential to study the structure-activity relationships. It is also important for comprehending the role of equine gonadotropins in the normal equine reproduction as well as in assisted reproductive technologies.

MATERIALS AND METHODS

Ethics statement: All the animal procedures were performed according to the approval by Institute Animal Ethics Committee (IAEC) of ICAR-National Research Centre on Equines.

Bacterial strains and construction of plasmids: For cloning procedures, the cells of *E.coli* strain *DH5alpha* were used and cultivated at 37ºC. The strain *E.coli BL21C* was used for the heterologous expression. Expression study was carried to express the equine chorionic gonadotropin functional gene consisting of two subunits α [(NCBI Gen Bank accession no.: AB000200.1 (Min *et al*., 1994)] and β [(NCBI GenBank accession no.: S41704.1 (Sherman *et al.*, 1992)]. A synthetic DNA (encoding the eCG protein) was designed to optimize the codon usage for protein production in *E. coli* (supplementary Fig. S1). We termed this gene as β*αeCG* and the recombinant protein as rβαeCG. The β subunit signal sequence along with the β subunit mature protein sequence was followed by α subunit protein sequence and α subunit 3' terminal sequence. It was constructed commercially to match its biologically active counterpart as reported (Legardinier *et al*., 2008).The synthetic DNA was cloned into pUC57 vector according to the manufacturer's instructions and propagated in *E. coli DH5alpha cells*. The recombinant plasmids were isolated from the *E. coli DH5alpha* cells (harboring the plasmid pUC57-eCG) by alkaline lysis method and the β*αeCG* gene was amplified by PCR using oligonucleotide primers: (5'-GCGAATTCACCAAGGA TGGAGATGCTCCAGGGACT-3') and (5'-GCTTCGAA CCTTTAAATCTTGTGGTGAT-3').The PCR product of ~700 bp size was purified using Qiagen PCR purification kit and digested with restriction enzymes. The pET-32a (+) vector was used for expression of single chain rβαeCG. The digested products were ligated in the corresponding sites of the pET-32a (+) vector to generate *pET32*-β*αeCG* recombinant plasmid and propagated in *E.coli DH5 alpha* cells. For sub-transformation of recombinant plasmids into the expression host the *E.coli BL21C* cells were made competent by $CaCl_2$ method and the purified plasmids were introduced into competent cells through a general strategy of heat shock at 42°C for 90 sec for transformation reaction (Sambrook *et al*., 2000). The transformed *E.coli BL21C* cells were plated out in Luria Bertani (LB) agar containing 50µg/ml of ampicillin. The transformants were checked for the presence of recombinant expression plasmids by PCR and successfully processed for expression of β*αeCG* towards the recombinant protein production.

Expression and purification of rβαeCG: The *E.coli BL21C* cells harbouring the recombinant plasmid (*pET32*-β*αeCG*) were grown overnight in 100ml LB broth containing ampicillin (50µg/ml) at 37°C with vigorous shaking at 200 rpm in a 1L flask. Approximately 100µl of overnight grown culture was inoculated in a 10ml fresh LB broth containing ampicillin (50µg/ml) at 37°C with 200 rpm shaking until the absorbance (OD_{600nm}) reached ~0.6. At this cell density, 0.5mM Isopropyl-thio-galactoside (IPTG) was added for induction and the cells were harvested at regular intervals (one hourly) to optimize the expression level. The un-induced cells were used as negative control. Five hours after the induction, the cells were harvested by centrifugation (6000xg for 10 min, 4°C) and the pellet was stored at -20°C. The cell pellet was lysed with cell lysis buffer and centrifuged at 4000xg for 5 min and the supernatant was collected and analyzed for protein expression in SDS PAGE (12% polyacrylamide). For obtaining high yield of purified rαβeCG protein, the inclusion bodies were first isolated by sonication and treated with lysozyme (40mg/ml)at room temperature for one hour. Osmotic shock was given by adding 500mM NaCl and 2.5% Triton X-100 at 37°C for one hour. The pellet was obtained by centrifugation at 10,000xg for 10 min at 20°C. The purification of recombinant protein using Ni-NTA affinity chromatography was carried out using Qiagen protein purification kit under denaturing conditions.

Characterization of the rβαeCG by immunoassays: The crude cell lysate expressing the rβαeCG as well as purified protein was analyzed in SDS-PAGE (12% polyacrylamide) along with non-induced and induced host bacterial lysate. The gels were stained with Coomassie brilliant blue and the bands were analyzed for determination of the molecular weight. The protein concentrations were determined through BCA assay and confirmed by spectrophotometry (ND-1000 Spectrophotometer, Nanodrop Technologies Inc. USA). The expressed and purified recombinant protein was characterized by sandwich enzyme linked immunosorbent assay (sELISA) and Western blotting (Towbin *et al.* 1979). For sELISA, the presence of rβαeCG was confirmed based on the color development and intensity. The absorbance was also recorded. The un-induced cell lysate and antigen negative samples were kept as negative control for qualitative characterization of recombinant rβαeCG. For Western blot analysis, the band of purified protein was separated on SDS-PAGE (12% polyacrylamide), electro-blotted to nitrocellulose membrane, probed with specific polyclonal antibodies [the primary antibody (anti-eCG raised in poultry; dilution 1:2000) and secondary antibody HRPO conjugate (anti-goat IgG raised in donkey; dilution 1:1000)]. Diaminobenzidine (DAB) with H_2O_2 was used for detection of rβαeCG band on nitrocellulose membrane corresponding to the band on SDS-PAGE.

***In vivo* bioassay: ovarian hyperaemia reaction:** The standard eCG *in vivo* bioassay was tested for rβαeCG efficacy

in increasing the size and weight of gonads in immature mice through ovarian hyperaemia reaction (OHR) (Cole and Erway, 1941). To test the rβαeCG, 20-24 days old mice (n=6) were inoculated with the purified rβαeCG (~2µg/100µl). The two control mice were administered PBS only. The mice were sacrificed after 72 hours and gonads (uterus, ovary and oviduct) were collected and weighed to differentiate between experimental and control mice responding to rβαeCG.

RESULTS AND DISCUSSION

The primary focus of the present work was to produce the recombinant single chain beta alpha equine chorionic gonadotropin/luteinizing hormone (eCG/LH) with active biological potential as its biological counterpart for further applications in assisted reproductive technologies or diagnostic assays. Therefore, the eCG/LH gene construct was expressed in host and purified for testing its biological efficacy.

Amplification, cloning and expression of rβαeCG in prokaryotic host: The signal sequence of βeCG was reported (Legardinier *et al.,* 2008) to interact with receptors in biological conditions in horses and other farm animals. Therefore the selected region for βαeCG/LH was specifically designed and custom synthesized with codon optimization for expression in prokaryotic host while retaining the original amino acid sequence (supplementary Fig. S1). The synthesized DNA was amplified by PCR with specific primers and the ~700bp product (Fig. 1) was purified and double digested with *EcoRI* and *HindIII* restriction enzyme (to get a ~693bp insert) for ligation with vector (supplementary Fig. S2). This selected region corresponded to 230 amino acid residues of functional β*αeCG* single chain construct. The transformed vectors were propagated in *E.coli DH5alpha* cells for generating multiple copies of cloned plasmids. Recombinant plasmid DNA was isolated and screened. After screening, the clones were sent for custom sequencing for the presence of insert fragment gene in-frame with N-terminal His-tag in pET32a (+) multiple cloning sites. Both the ends of insert were found correct in orientation and sequence. The sequenced clone was preserved in 30% glycerol stock. The purified recombinant pET32a-β*αeCG* vector was transformed into the *E.coli BL21C*expression host strain and the transformed colonies were selected on LB agar with ampicillin (50µg/ml). The *E.coli BL21C* host provides a regulated synthesis of T_7 DNA polymerase required for expression of insert present downstream of T_7 promoter. The recombinant colonies of *E.coli BL21C* host cells containing pET32a-β*αeCG* vector were screened precisely. The small-scale cultures of positive clones (selected based on PCR screening and sequencing) were subjected to IPTG induction for identifying the clones capable of expressing the predicted ~42kDa recombinant protein. On analysis of SDS-PAGE, correct sized recombinant protein was seen (Fig. 2). The higher molecular weight than expected molecular weight of

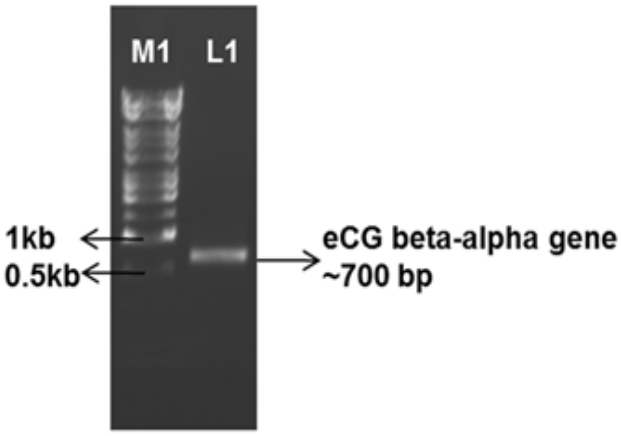

Fig1: PCR Amplification of β*α-eCG*gene: Lane 1: PCR ampli fied β*αeCG* gene construct. M1: DNA molecular weight marker

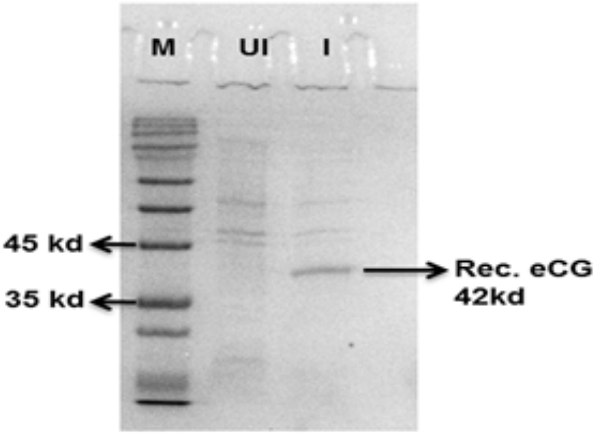

Fig 2: Expression of rβαeCG along with un-induced control culture: SDS-PAGE analysis of rβαeCG: Lane M-molecular weight marker, Lane UI- negative control (un-induced), Lane 1- induced bacterial culture

recombinant protein was due to presence of histidine (*his*) and thioredoxin (*trx*) tags. These were present on the plasmid vector and expressed along with recombinant protein. The results confirmed that the ORF (Open Reading Frame) was selectively expressed in the transformed *E. coli BL21C* cells and apparently constituted a large fraction of the total cellular protein. The over expressed protein was not detected in the controls, (un-induced *E.coli BL21C* cells transformed with the pET32a-β*αeCG* ORF construct). The expression was observed as early as 1 hour post induction and maximum at 5 hours (Fig.3).The property of the expressed rβαeCG protein carrying six histidine residues at the N-terminus was utilized for the purification of expressed protein through Ni-NTA affinity chromatography. The bacterial culture supernatant was also allowed to interact with Ni-NTA affinity column for purification. However, the recombinant protein was recovered from cellular component only, indicating that the expression was confined to cellular compartments and the recombinant

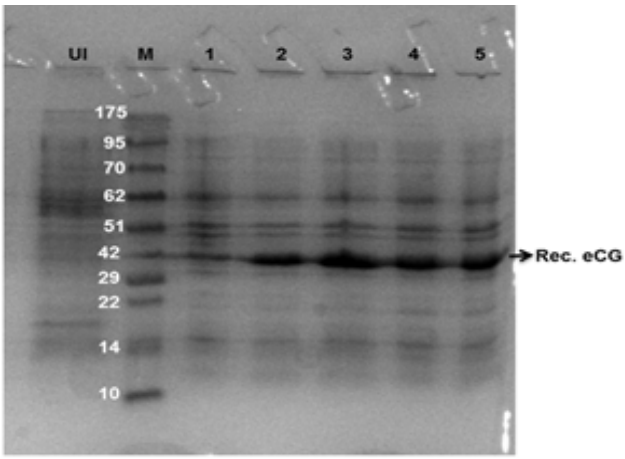

Fig 3: Expression Kinetics of recombinant βαeCG: Kinetics of recombinant βαeCG expression by SDS-PAGE analysis of the bacterial culture after induction with IPTG. UI represents un-induced culture and subsequent lanes correspond to samples taken 1, 2, 3, 4 and 5 hours post induction.

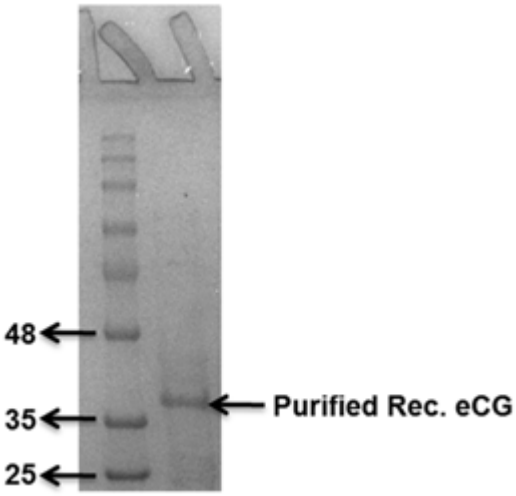

Fig 4: Purified recombinantβαeCG.

protein is non-secretory. The rβαeCG was eluted and analysed in SDS-PAGE (12%) that showed a single band of rβαeCG (~42kDa) (Fig. 4). The expression system employed and the described procedure allowed us to obtain 2-5µg of pure rβαeCG protein from 1L of bacterial culture. This protein preparation was stored at -20°C in 15% glycerol.

Characterization of rβαeCG by immunological assays and *in vivo* bioassay: The immunogenic property of rβαeCG protein was investigated by sELISA and Western blot analysis. The bacterial cell lysates (induced and uninduced) were used as antigens. The uninduced cell lysate was used as negative control. The absorbance obtained was quite comparable to the pregnant mare serum (PMS) which confirmed the presence of rβαeCG protein. In Western blot analysis, the total expressed proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane. The membrane showed the presence of band for rβαeCG at the same position at ~42k Dathus confirming the presence of rβαeCG in the cell lysate (Fig.5).Two minor bands were seen at lower molecular weight positions which may be due to the expression of separate alpha and beta subunits or truncated proteins. No band was revealed in the uninduced culture. These results confirmed the presence of rβαeCG in the induced cell cultures. The ovarian hyperaemia reaction (OHR) was carried out for testing the biological efficacy of rβαeCG for its effectiveness in increasing the size and weight of gonads (uterus, ovary, and oviduct) in immature mice. After 72 hours, the control and experimental mices were observed for increase in the weight of gonads by taking out the gonads and weighing them in comparison to control group. No significant differences were observed in the experimental and control groups of mice.

The last 25 years documented a major advancement in the gonadotropin therapy for ovarian stimulation, pregnancy maintenance and fecundity augmentation including assisted reproductive procedures. The administration of exogenous gonadotropins began as early as 1960s with a substantial breakthrough in the last decade by the use of recombinant products (recombinant FSH, LH, follitropin, hCG). It necessitates the need of more pure forms than urine or serum- derived gonadotropins and offers relative ease of large scale production. Here, we targeted the production of single chain rβαeCG in prokaryotic host along with testing its potential for *in vivo* gonadal effects and probable use towards development of diagnostic assays. The recombinant

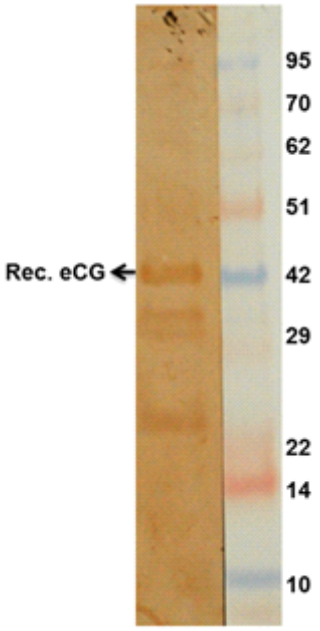

Fig 5:Western blotting analysis of recombinantβαeCG: lane M: SDS-PAGE Molecular weight marker; lane 1: recombinant βαeCG; lane 2: un-induced control showing no band of rβαeCG.

heterodimeric pituitary and gonadal hormones and their subunits are of immense use in the reproductive biotechnology. It may serve as one of the tools to enhance the reproductive potential of farm and dairy animals. However, recent studies exhibited the *in vitro* biological activities with little information concerning *in vivo* potencies (Legardinier *et al.*, 2005, 2008). Earlier, single-chain eLH/CG (Galet *et al*. 2001) and bFSH (Coulibaly *et al.*, 2002) were produced in the milk of transgenic rabbits. *In vivo* biological activity was determined only for eLH/CG, and it was found to be inactive because of its too short half-life (Koles et al. 2004). It has been shown that tethered eCG/LH (without C-terminal peptide and β26-110 disulphide bridge) was biologically active *in vitro* (Galet *et al.*, 2000). Most of the workers reported similar results with very limited *in vivo* potential of recombinant eCG/LH. However, the eCG still finds use in superovulation and gonadotropin based therapy. Recently, a simplified process was reported which required only 1/3 of the solvent and improving the yield from 53 to 65% by use of magnetic micro-adsorbents (Muller *et al.*, 2011).

Our work aimed to produce full length single chain recombinant β*αeCG*. It may provide value to its biological counterpart and eCG based diagnostic tests. Nevertheless, the rβαeCG failed to increase the gonadal weight in immature mice and our result is in accordance with other reported studies for the biological activity assay. The recombinant full length single chain rβαeCG showed good results in ELISA and Western Blot, which emphasized its diagnostic potential and affinity towards antibody binding. Earlier, the lead author had expressed and characterized the bovine inhibin alpha subunit in *E.coli M15* cells (Bhardwaj *et al.*, 2012). There too, difficulties were encountered in purifying the recombinant protein from inclusion bodies. There is need for superior purification protocols with relative ease. Mammalian cells may offer a superior system for production of glycosylated reproductive hormones but till date not much success has been achieved for recombinant eCG for *in vivo* assays (Legardinier *et al.*, 2008).

CONCLUSION

The present work resulted in standardization of protocols for production of recombinant PMSG-eCG and testing through immunoassays with potential diagnostic value. The future perspectives of these recombinant gonadotropins from animals include the extensive study and research to produce large quantities of purified proteins to serve as their native counterparts. In this context, very complex and expensive isolation and purification procedures are required for gonadal hormones from serum or urine. The recombinant DNA technology may provide a grander platform for their production. However, the need of large scale cultures and purification cannot be overlooked, while carrying out these studies.

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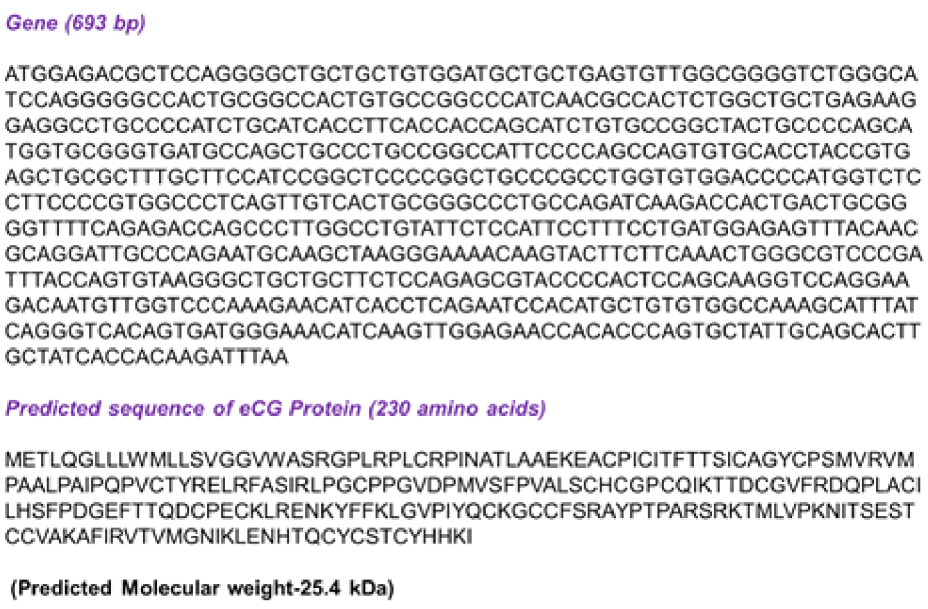
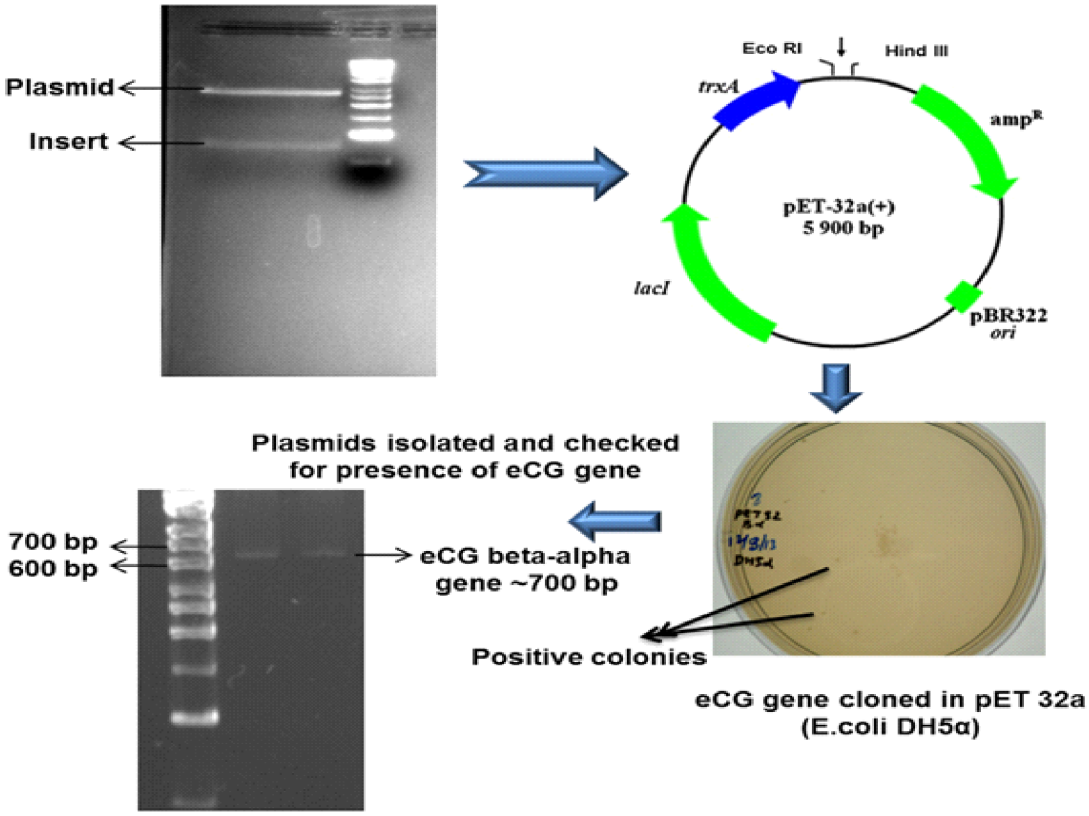
Supporting Information

The β subunit signal sequence along with β subunit mature protein sequence followed by α subunit protein sequence and α subunit 3' terminal sequence was constructed commercially to match its biologically active counterpart.

Supplemental Experimental procedures and results: Supplementary Figures



Supplementary Fig. S1: The synthetic equine chorionic gonadotropin DNA Sequence and the translated protein sequence. The nucleotide sequence consisted of the subunits α (accession no. AB000200.10) and β (accession no. S41704.1).



Supplementary Fig. S2: The flow diagram showing the cloning and screening of clones of βαeCGgene.

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