

# Isolation and cloning of human progesterone receptor isoform-b in CMV-eGFP vector

SAQIBA KHALID & \*NAILA MALKANI

*Department of Zoology, GC University, Lahore, Pakistan*

## ABSTRACT

The progesterone receptor is a type of protein present inside cells, which is activated by the hormone progesterone (type of steroid hormone). In humans, a single PGR gene found on chromosome 11q22 encodes progesterone. There are two main types, A and B the difference lies in their molecular weight. Progesterone receptor isoform B functions tissue-specifically to mediate reproductive functions of progesterone in the present study we did cloning of progesterone receptor isoform B in expression vector CMV-eGFP. Gene cloning provides information about nucleotide sequence. Other motive of this is to manipulate the gene in various ways like genetic transformation. Genomic DNA was isolated from human blood. For cloning of PR-B and CMV-eGFP, restriction and ligation were done using appropriate conditions and concentrations. The resulting DNA was transformed into host organism (*E.coli*, DH5 $\alpha$ ). These were grown on kanamycin resistant agar plated. Colonies containing ligated PR-B with plasmid were then screened, purified and analysed using standard protocol. The desired CMV-eGFPC1-PR-B construct of 7523bp was obtained and stored at -20°C.

**Key Words:** Progesterone receptor II, cloning, CMV-eGFP.

## INTRODUCTION

Progesterone, a natural progestin, is a major hormone released from gonads that is synthesized by the ovary in the female, and in males from testes and adrenal cortex. While progesterone levels are generally higher in female, it is notable that progesterone level during the follicular phase of female menstrual cycle are similar to those in males (Strauss & Barbieri, 2004), so it can be that it is equally important in males. Progesterone affects number of tissues and organs, including the cervix, uterus, breast, ovary, and brain (Graham & Clarke, 1997).

Clinical trials results, and data from animal models, shows that progesterone increase growth and development of breast cancer and uterine fibroids, while progesterone plays a protective role against the development of endometrial cancer (Rossouw *et al.*, 2002). The biological activity of progesterone is mediated via specific receptors. A single hormone can interact with different receptor types. There are 6 different types of receptors belonging two super families, nuclear hormone receptor and membrane-associated receptor super family (Kolmakov *et al.*, 2008). The functions mediated by membrane-associated progesterone receptors are still under

discussion (Krietsch *et al.*, 2006). Primarily Progesterone receptor (PR) is expressed in one cell type e.g mammary gland and uterine cell fibroid muscle, on these tissues it has pro-tumorigenic effect. On the other hand both epithelial and stromal cells in endometrium express PR, its action is mediated by autocrine and paracrine signaling between different cells. This results in preventive effect against carcinogenesis of the epithelium, which is estrogen-dependent (Krietsch, 2006). The objectives of the present research work are to isolate human Progesterone receptor B (hPR-B) from blood and clone hPR-B into an expression vector as fusion protein with eGFP.

## MATERIALS AND METHODS

### Preparation of Chemically Competent Cells

DH5 $\alpha$  Competent cells were prepared according to the protocol described by (Chunge *et al.*1989). Transformation efficiency was determined by the following formula.

$$\text{Transformation Efficiency (cfu/}\mu\text{g)} = \frac{\text{cfu on control plate}}{\text{ng of Competent cells Control DNA plated}} \times \frac{1 \times 10^3 \mu\text{g}}{\mu\text{g}}$$

### Genomic DNA Isolation

Genomic DNA was isolated from human blood following protocol of (Sambrook, 1989). Briefly, sample (human) blood was collected in EDTA coated tube, washed, centrifuged with TE buffer until a white pellet of blood cells was visible. The pellet was dissolved into 375µl of 3M sodium acetate, 25µl of 10% SDS and 5-10µl of proteinase K and incubated at 37°C overnight. Chloroform: isoamyl alcohol (24:1) extraction of DNA was performed and DNA pallet obtained then washed twice with 70% ethanol, airdried, resuspended in 50µl TE buffer. DNA was stored at -20°C after assessing its quality and quantity by nanodrop and 1% agarose gel electrophoresis.

### Primer Designing

The cloning primers were design by using **PRIMER3 PLUS**. The sequence of the resulted primer with restriction sites Xho1 is as follows:

**XhoI**

For= GAAA **CTCGAG** ATGAGCCGT  
 Rev= CTTTT **CTCGAG** CTTTTTATGAA  
**XhoI**

### PCR and Gel Purification

This primer pair was used to extract PGR-B from the genomic DNA by PCR at annealing temperature of 52°C. The PCR product was purified from gel by using Thermo Scientific GeneJET Gel Extraction Kit.

### PGR-B Cloning

The gel purified PCR product and the vector DNA were proceeded for restriction digestion with XhoI to get the compatible ends.

Next the vector DNA and PCR product (insert) were ligated at 1:3 and 1:1 along with a negative control at 16°C overnight. The ligated product then transformed into the DH5α cells on kanamycin resistant plates.

Few colonies were selected from each plate and plasmid DNA was isolated and analysed for the presence of insert. Restriction analysis was carried out with Sal I at 37°C for 2hours. Gel electrophoresis was performed and the colonies having products of appropriate sizes (7523bp) were selected for sequencing. The DNA of the colonies appearing positive in restriction analysis were sequenced. The sequencing positive DNA was named as eGFPC1-PR-B and stored at -20°C.

## RESULTS

### Determination of Efficiency of Competent Cell

After the formation of competent cells, the transformation efficiency was calculated by following equation:

$$\frac{\text{cfu on control plate}}{\text{ng of Competent cells Control DNA plated}} \times \frac{1 \times 10^3 \mu\text{g}}{\mu\text{g}}$$

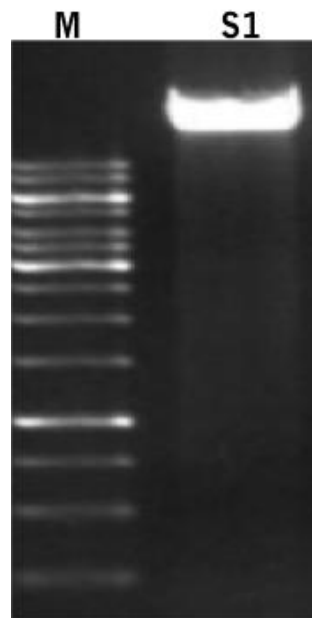
The efficiency of transformed cells was:

$$\begin{aligned} \text{Transformation Efficiency :} &= \frac{120}{0.1} \times \frac{1 \times 10^3 \text{ ng}}{\mu\text{g}} \\ &= 1.2 \times 10^8 \text{ cfu}/\mu\text{g} \end{aligned}$$

Therefore, the transformation with 0.1 ng of DNA gave 120 colonies, hence the transformation efficiency be  $1.2 \times 10^8$  cfu/µg.

### Isolation of Genomic DNA from Blood

The genomic DNA from blood was isolated by the protocol described by. 2ul of isolated gDNA was used for agarose gel electrophoresis. Sharp bands of genomic DNA were visualized through UV transilluminator (Fig., 1).



**Fig., 1:** Gel Electrophoresis of Genomic DNA Isolated From Human Blood Sample; M (Marker 1kb), S1 (Blood sample).

### Designing of Cloning on CLONE MANAGER 7

Before starting the cloning of EGFP1-PR-B in the laboratory, all the steps were designed on

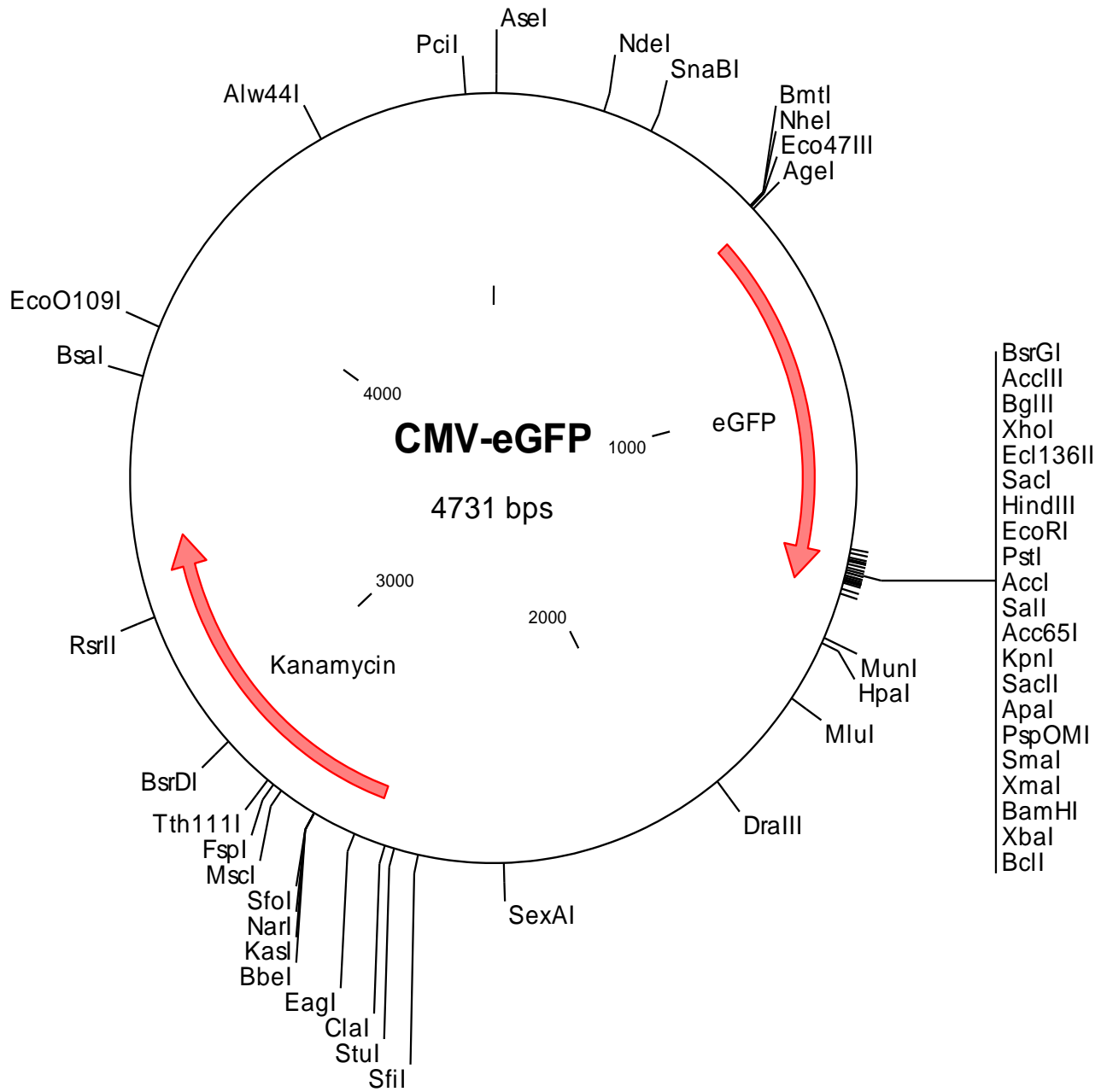
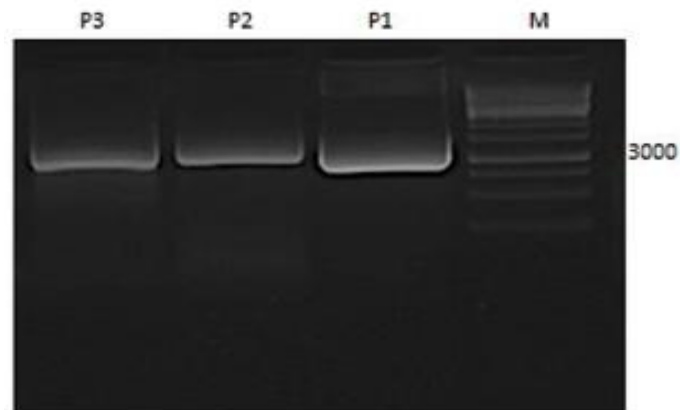
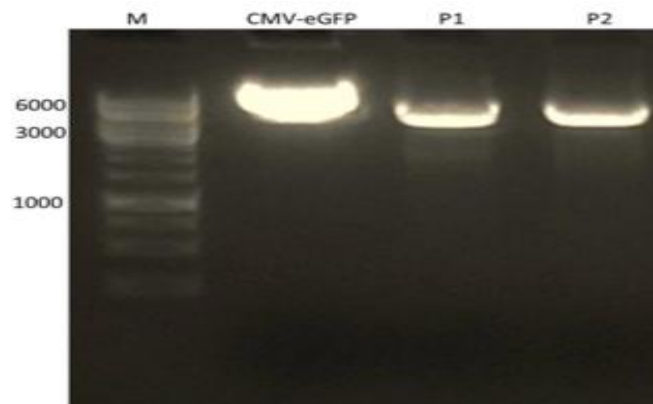


Fig., 2a: CMV-eGFPC1 as designed by Clone Manager Suite 7

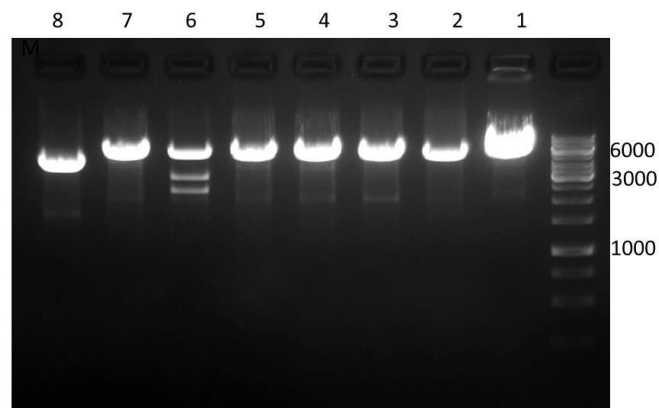




**Fig., 3:** Gel Electrophoresis Of PCR Products Where M= marker (1kb), P1, P2 and P3 are various samples among which P2 was selected PR-B (2820bp).



**Fig., 4:** Restriction Of Vector Cmv-Egfp And Pcr Product Of PR-B With XhoI. where M=marker (1kb), CMV-eGFP(linearizaed vector), P2=PR-B.



**Fig., 5:** Restriction Analysis with SalI of Ligation Products for Detection of Positive Clones Of eGFP1-PR-B. The Positive Clones Shows Bands at 7523bp. Clone No. 1, 2, 3, 4, 5, 7 And 8 Were Positive For Desired Product.



### Screening of Positive Colonies

The resultant gel purified products were than ligated using T4 DNA ligase and transformed. The resulting colonies were screened for the positive clones. For this purpose DNA isolation was done from each colony and then restriction analysis and sequencing was performed.

### Restriction Analysis

The DNA obtained from each colony was subjected to restriction analysis using the enzyme Sal I. The positive clones were supposed to give bands at 7523 bp (Fig., 5). The clones, which were failed to give the required bands were considered negative and were discarded.

### Sequencing

The resulting positive clones from restriction analysis were sent for sequencing. The sequence chromatogram was read using Bioedit software. The nucleotide sequence was aligned to the product designed by Clone Manager7. The clone with no mutation and 100% homology was selected for midiprep and stored at -20 °C (Fig., 6).

The Clone Manager7 software. The vector (CMV-eGFP) and the resulting construct (EGFPC1-PR-B) is shown in figure 2 a,b.

### Cloning Polymerase Chain Reaction (PCR)

Isolated genomic DNA was used as template for the amplification of PR-B gene with the specific primers having restriction sites. PCR product was analysed through 1% agarose gel electrophoresis. Sharp band at the size of 2820bp showed the amplification of PR-B gene (Fig., 3)

### Restriction of PCR Products

The resulting PCR product was gel purified using gel extraction kit (Fermentas) and was subjected to restriction with Xho1 to produce sticky ends. Similarly the vector DNA (CMV-eGFP) was also restricted with the same enzymes to produce sticky ends. After restriction the DNA (both PCR product and vector) was analysed through gel electrophoresis. Required band was cut and purified by gel-purification (Fig., 4).

## DISCUSSION

The progesterone receptor is a type protein found in cells and activated by the hormone progesterone that is steroid in nature (Misrahi, 1987). In humans, A single PGR gene present on chromosome 11q22 encodes human PR (Law, 1987) there are two main types, A and B that differ in molecular weight (Gadkar-Sable, 2005). As hPR-A, hPR-B shares many structural domains, they are

two distinct transcription factors, modulating their own genes and physiological effects (Guiochon-Mantel, 1989). Partial ablation of PR-A in a mouse model, resulted in increase production of PR-B, unexpectedly revealing that PR-B promotes, rather inhibits, proliferation of epithelial cells, in response to estrogen only and progesterone and estrogen both. These results showed that, PR-A isoform in the uterus is required to oppose proliferation by estrogen as well as by PR-B (Guiochon-Mantel, 1989). In recent studies by using PR-B knockout (PRBKO) mice have shown that PR-B ablation do not affect ovarian or uterine, responses to progesterone, rather results in decreased morphogenesis of mammary ducts. So, PR-B is required to induce normal mammary gland proliferation response to progesterone.

In the present study we did cloning of PR-B in expression vector (CMV-eGFP). For cloning of the CMV-eGFP-PR-B genomic DNA first extracted from blood and ligated at appropriate concentrations to covalently link the ends together. The obtained DNA mixture containing randomly joined ends then ready for introduction into the host organism.

The vector used in this work was CMV-EGFP, deposited by Connie Cepko to ADD gene # 11153. In his work, for pCAG-GFP construction cDNAs coded with GFP (EGFP) excised from pEGFP-N1 (Clontech), and cloned into pCAGGS with multiple cloning sites. For pCMV-GFP construction, the promoter site of pCAG-GFP was exchanged by the cytomegalovirus (CMV) promoter extracted from pEGFP-N1. Green fluorescent protein (GFP) is a type of protein consist of 238 amino acid (26.9 kDa) that shows bright green fluorescence when exposed to blue to ultraviolet range (Prendergast, 1978 and Phillips, 2001). The GFP gene is used as an expression reporter. By modifications it used to make biosensors, and many animals have been produced that shows GFP as a proof that a gene can be expressed in an organism (Tsien, 1998). The biggest benefit of GFP is that it can be inherited, while it can be transformed by DNA encoding GFP. In addition, visualizing GFP is noninvasive; so just by throwing light on the protein it can be detected. Further, GFP is a small, inert molecule, that do not interfere with any biological function of interest. (Chalfie, 2009). GFP can express in different structures enables morphological distinction. In these cases, the gene for the GFP production is inserted into the genome of organism in the site of DNA encoding target proteins, controlled by the same sequence of regulation; that is, the regulatory sequence of genes controls the GFP production, in addition to the protein that are tagged (Arun, 2005). In cells GFP

produce at same time where tagged proteins produced, expressing the gene. So, only those cells will show fluorescence where tagged gene express itself. (Chalfie, 2009).

The PR-B gene tagged with eGFP produced green fluorescent, as a complementary DNA for the *Aequorea Victoria*. Green fluorescent protein (GFP) produces fluorescence in prokaryotic (*Escherichia coli*) and eukaryotic (*Caenorhabditis elegans*) cells.

The CMV-eGFP-PR-B is constructed in a way that the gene of interest (PR-B) starts right after GFP and there is no stop codon in between, hence the two genes are under the influence of same promoter and once the replication starts it will go through both the sequences. This technique facilitates equal and simultaneous expression of both the proteins. Therefore, if used in a cell culture or other system where ever the expression of GFP is visualized it means PR-B is there. GFP expression can be used to monitor gene expression and protein localization in living organisms (Chalfie, 1994). EGFP expression served as a marker for pluripotency gene activation and was consistently detected in preimplantation embryos with 9 and more cells (Popken, 2014).

Wang *et al.*, 2015 construct a lentivirus vector, carrying SARI gene tagged with eGFP. Similarly, Silke *et al.*, in 2014 investigated gene delivery to adipose tissue using transcriptionally targeted rAAV8 vectors". pFB-mAP2.2-eGFP was cloned by replacing the CMV promoter in pFB-CMV-eGFP with the minimal adiponectin promoter fragment (mAP2.2) which was generated by gene synthesis. Jiang *et al.*, 2011 did the successful construction of the pKT2/CMV-EGFP-IRES-PURO vector with the insertion of a CMV promoter and EGFP into pKT2/TRE-Tight-BI-GH-rtTA-Advanced in place of the IRES fragment.

Li *et al.*, 2013 published his work entitled "Expression of biologically active human interferon alpha 2b in the milk of transgenic mice" where, the eGFP was used in the construction of expression cassette in the transgene.

Mao *et al.*, 2015 used lentiviral vectors, pTYF-CMV( $\beta$ -globin intron)-eGFP containing CMV promoter and  $\beta$ -globin intron, pTYF-CMV-eGFP containing CMV promoter, and pTYF-EF1 $\alpha$ -eGFP with EF1 $\alpha$  promoter those were packaged, titered, and then transduced into 293T cells (1000 viral genomes per cell). The transduced cells were passaged once every three days at a ratio of 1:10. Expression level and stability of the foreign gene, green fluorescence protein (GFP), was evaluated using fluorescent microscopy and flow cytometry.

**In conclusion**, the cloning of PGR II was done in CMV-eGFP expression vector which, provides an important tool for expression studies and is easy to manipulate further in observing the expression of gene on different cell systems.

## REFERENCES

- Arun, K.H., Kaul, C.L. & Ramarao, P., 2005. Green fluorescent proteins in receptor research: an emerging tool for drug discovery. *J. Pharmacol. Toxicol. Methods.* **51** (1): 1–23.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. & Prasher, D., 1994. Green Fluorescent Protein as a Marker for Gene Expression. *Science*, **263**.
- Chalfie, M., 2009. GFP: Lighting up life. *Proc. Natl. Acad. Sci. USA.*, **106**(25): 10073–10080.
- Gadkar-Sable, S., Shah, C., Rosario, G., Sachdeva, G. & Puri, C., 2005. Progesterone receptors: various forms and functions in reproductive tissues. *Front. Biosci.* **10**:2118–30.
- Graham, J.D. & Clarke, C.L., 1997. Physiological action of progesterone in target tissues. *Endocr Rev.* **18**:502–519.
- Li, H., Liu, Q., Cui, K., Liu, J., Ren, Y. & Shi, D., 2013. Expression of biologically active human interferon alpha 2b in the milk of transgenic mice. *23 Transgenic Res.*, **22**(1):169-78.
- Kolmakov, N.N., Kube, M., Reinhardt, R. & Canario, A.V., 2008. Analysis of the goldfish *Carassius auratus* olfactory epithelium transcriptome reveals the presence of numerous non-olfactory GPCR and putative receptors for progestin pheromones. *BMC Genomics.* **9**: 429.
- Krietsch, T., Fernandes, M.S., Kero, J., Losel, R., Heyens, M., Lam, E.F., Huhtaniemi, I., Brosens, J.J. & Gellersen, B., 2006. Human homologs of the putative G protein-coupled membrane progesterin receptors (mPR $\alpha$ ,  $\beta$ , and  $\gamma$ ) localize to the endoplasmic reticulum and are not activated by progesterone. *Mol. Endocrinol.* **20**: 3146–3164.
- Law, M.L., Kao, F.T., Wei, Q., Hartz, J.A., Greene, G.L., Zarucki-Schulz, T., Conneely, O.M., Jones, C., Puck, T.T. & O'Malley, B.W., 1987. The progesterone receptor gene maps to human chromosome band 11q13, the site of the mammary oncogene int-21. *Proc. Natl. Acad. Sci. U.S.A.* **84** (9): 2877–81.
- Mao, Y., Yan, R., Li, A., Zhang, Y., Li, J., Du, H., Chen, B., Wei, W., Zhang, Y., Sumners, C.,



- Zheng, H. & Li, H., 2015. Lentiviral Vectors Mediate Long-Term and High Efficiency Transgene Expression in HEK 293T cells. *Int J Med Sci.* **12(5)**:407-15.
- Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Meriel, C., Fridlansky, F., Guiochon-Mantel, A., Galibert, F. & Milgrom, E., 1987. Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA., *Biochem. Biophys. Res. Commun.* **143 (2)**: 740–8.
- Phillips, G.J., 2000. Green fluorescent protein--a bright idea for the study of bacterial protein localization. *FEMS Microbiology Letters.* **204 (1)**: 9–18.
- Popken, J., Koehler, D., Brero, A., Wuensch, A., Guengoer, T., Thormeyer, T., Wolf, E., Cremer, T. & Zakhartchenko, V., 2014. Positional changes of a pluripotency marker gene during structural reorganization of fibroblast nuclei in cloned early bovine embryos. *Nucleus.* **5(6)**: 542-54.
- Prendergast, F.G. & Mann, K.G., 1983. Chemical and physical properties of aequorin and the green fluorescent protein isolated from *Aequorea forskålea*. *Biochemistry.* **17**: 3448–53.
- Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M. & Ockene, J., 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA.* **288**:321–333.
- Russell, D.W. & Sambrook, J., 2013. Chapter 1: Plasmids and Their Usefulness in Molecular Cloning. *Molecular cloning: A Laboratory Manual 1 (3rd ed.)*. Cold Spring Harbo. pp: 1–159.
- Schmidt, S.U., Geiger, M., Luippold, G., Birk, G., Mennerich, D., Neubauer, H., Grimm, D., Wolfrum, C. & Kreuz, S., 2014. Gene Delivery to Adipose Tissue Using Transcriptionally Targeted rAAV8 Vectors. *PLoS One.* **9(12)**.
- Strauss, J., Barbieri, R. & Jaff, Y., 2004. *Reproductive Endocrinology*. 5th edition Saunders.
- Tsien, R.Y., 1998. The green fluorescent protein. *Annu. Rev. Biochem.*, **67**: 509–44.
- Yao, M., Gui, Y., Wang, Y., Zhang, B. & Fu, W., 2014. Preliminary study on transgenic cell sheet with cartilage-derived morphogenetic protein. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi.* **28(2)**:142-8.

---

**Received: 13-03-2016**

---

**Revised: 20-04-2016**

---

**Accepted: 25-05-2016**