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*Recebido em abril de 2018.  
Aprovado em agosto de 2018.*

## MOLECULAR CLONING OF A LHB GENE FRAGMENT: PROSPECTS OF GENE THERAPY IN PROSTATIC NEOPLASIA

### RESUMO

Prostate carcinoma is considered, in the United States, the second leading cause of death among men. Numerous therapeutic alternatives are being created in order to maximize the efficiency of treatments commonly used. As an example, we have gene therapy which seeks not only to optimize processes carried out by drugs, but also to explore the influence of certain gene products over the progression of the disease. This work aimed an interspecies test using biotechnology for the molecular cloning of a LHB gene fragment. At the end, it has successfully demonstrated all biotechnological trials needed for the beginning of many interspecies genetic manipulation procedures, basis of gene therapy. Even in the face of growth and improvement of biomolecular techniques, a lot still needs to be done before the solidification of gene therapy as a substitute to the pharmacological interventions.

**Palavras-Chave:** molecular cloning; prostatic neoplasia; lhb.

## CLONAGEM MOLECULAR DE UM FRAGMENTO DO GENE LHB: PERSPECTIVAS DA TERAPIA GÊNICA NA NEOPLASIA PROSTÁTICA

### ABSTRACT

O carcinoma de próstata é considerado, nos Estados Unidos, a segunda maior causa de morte entre homens. Inúmeras alternativas terapêuticas vêm surgindo com o intuito de maximizar a eficiência dos tratamentos comumente empregados. Como um exemplo podemos citar a terapia gênica, que busca não apenas otimizar processos realizados por fármacos, mas também explorar a influência de certos produtos gênicos sobre a progressão das doenças. Este trabalho teve como objetivo a realização de um ensaio interespecies, utilizando técnicas da biotecnologia para a clonagem molecular de um fragmento do gene LHB. Foi demonstrado com sucesso todos os ensaios necessários para o início de muitos procedimentos de manipulação gênica interespecies, base da terapia gênica. Mesmo diante do crescimento e aperfeiçoamento de tais técnicas biomoleculares, muito ainda precisa ser feito antes da solidificação da terapia gênica como uma substituta as intervenções farmacológicas.

**Keywords:** clonagem molecular; neoplasia prostática; lhb.

## INTRODUCTION

Biotechnology is the science which applies biological systems, living organisms or its derivatives, to create or modify products and processes; ranging from simple and traditional techniques such as the fermentation process, until the genetic manipulation techniques, established after the advances of molecular biology (FERRO, 2010; RESENDE; SOCCOL, 2015).

The applications of this science are vast and have attracted the interest not just from scientists, but also from the industry, private investors, public policymakers and, mainly, the health system, being that, the area where biotechnology acts with a greater effectiveness depending on how advances in this field has contributed to the prevention and, above all, to the treatment and study of several diseases (2017), having as one of the main tools of work the recombinant DNA technology, which allows the efficient and safe manipulation of the genome (LINDEN, 2010; RESENDE; SOCCOL, 2015).

Gene therapy modalities in development are normally addressed to somatic cells, not germ cells; this ensures that the manipulation affects only the individual in treatment and not their offspring, which can be summarized in basically 3 principles of applicability: the genetic material that you want to transfer, the transfer method and the cell type that will incorporate this material (LINDEN, 2010; WIRTH; PARKER; YLÄ-HERTTUALA, 2013).

Although initially the focus had been the treatment of monogenic hereditary disorders, later most gene therapy clinical trials focused on the treatment of neoplasms, such as breast, ovary, lung, prostate and leukemia, with the gene silencing by RNA interference (RNAi), for example, being a promising technique (LINDEN, 2010; RESENDE; SOCCOL, 2015; WIRTH; PARKER; YLÄ-HERTTUALA, 2013).

Androgen deprivation as an endocrine treatment in patients with prostatic neoplasia has been described for over 60 years, with luteinizing hormone (LH) suppression being the most prevalent in this type of treatment modality (NISHII et al., 2012).

LH is a heterodimeric protein belonging to a family of glycoprotein hormones. Its structure consists of a common  $\alpha$ -subunit and a specific  $\beta$ -subunit, which confers biological specificity for the hormone receptor. Secreted from pituitary, plays an important role in gonadal function, with the regulation of vertebrate reproduction (DASGUPTA et al., 2012; NYUJI et al., 2015).

This work aimed the interspecies test, using biotechnology and molecular biology techniques for the isolation, amplification and cloning of a LHB gene fragment in prokaryotic systems.

## MATERIAL AND METHODS

### DNA / RNA ISOLATION

It was used 4 whole blood samples (L-a, L-1, L-2 and L-3) from male individuals, obtained as previously approved from CEPESH-HGA (Comitê de Ética em Pesquisas com Seres Humanos do Hospital Guilherme Álvaro - #492/12).

The process of DNA and RNA purification from the whole blood samples was carried out using, respectively, the QIAamp® DNA Mini kit (QIAGEN®) and PureLink™ Total RNA Blood Purification Kit (Invitrogen®) according to the protocol established by the manufacturer.

## PRIMERS DESIGN

The next step of this biomolecular essay was the synthesis of specific primers for the amplification of target gene, located on the long arm of chromosome 19 at band 13 sub-band 32, comprising a sequence of 1111 base pairs - NCBI NG\_011464.

The choice of primers occurred under a literary review (BERGER et al., 1994; HOTAKAINEN et al., 2000; LOFRANO-PORTO et al., 2007; MINEGISHI et al., 1997) and after several attempts, two primers were selected from different papers: forward: 5'-GCACCAAGGATGGAGATGCTCCAG -3' (LOFRANO-PORTO et al., 2007); and reverse: 5'-AGAGCCACAGGGAAGGAGAC -3' (BERGER et al., 1994).

The pair of primers used was responsible for the amplification of a fragment of 914 base pairs. Note that due to the post-transcriptional conservation of primer annealing region, these were also responsible for the amplification of a 326 bp fragment from the LHB cDNA.

## PCR / RT-PCR / SEQUENCING

The following protocol was used in the LHB (luteinizing hormone beta gene) PCR, with final volume of 50  $\mu$ L, consisting of: 5  $\mu$ L of buffer 10 X (Invitrogen®); 2  $\mu$ L of purified DNA; 3  $\mu$ L of forward primer; 3  $\mu$ L of reverse primer; 2  $\mu$ L of dNTPs (10 nM concentration) (Invitrogen®); 1.5  $\mu$ L of MgCl<sub>2</sub> (concentration of 50 nM) (Invitrogen®); 0.5  $\mu$ L of Taq Polymerase (Invitrogen®); 25  $\mu$ L of ultra-pure water (Invitrogen®). Parameters of the thermal cycler (Applied Biosystems®): 94°C for 5m; 94°C for 1m; 63°C for 2m; 72°C for 3m; steps 2 to 4 were repeated 34 times (cycles); 72°C for 15m and 4°C -  $\infty$ .

For reverse transcription, two working solutions were prepared. Solution one, with final volume of 13  $\mu$ L, consisted of: 1  $\mu$ L of Oligo(dT) (Invitrogen®); 5  $\mu$ L of total RNA; 1  $\mu$ L of dNTPs (10 nM concentration) (Invitrogen®) and 6  $\mu$ L of ultra-pure water (Invitrogen®). Solution two, with final volume of 7  $\mu$ L, consisted of: 4  $\mu$ L of buffer 5 X (Invitrogen®); 1  $\mu$ L of DTT (Thermo Scientific®); 1  $\mu$ L of RNaseOUT (Invitrogen®) and 1  $\mu$ L of SuperScript® Reverse Transcriptase (Life Technologies®).

Solution one was heated at 65°C for 5m and, after that, the entire contents of solution two was added. The mix solution was subjected to 1 cycle of 50°C for 60m and 70°C for 15m. In the PCR, 2  $\mu$ L of the cDNA mixture was amplified according to the conditions described above (LHB PCR).

After cDNA amplification, the product was purified with illustra™ ExoStar™ 1-Step (GE Healthcare Life Sciences®) and sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit according to manufacturer's instructions (Applied Biosystems®). All sequences were analyzed in both forward and reverse directions on an ABI Prism 3130XL fluorescent sequencer.

## PRODUCTION OF ELECTROCOMPETENT CELLS

Initially it was performed the pre-inoculation of E. coli cells DH5 $\alpha$  strain (genotype: F' / endA1 hsdR17 (rK-mK+) glnV44 thi-1 recA1 gyrA (NaI<sup>r</sup>) relA1  $\Delta$  (lacZYAargF) U169 deoR ( $\Phi$ 80dlac  $\Delta$ (LacZ)M15) (MESELSON; YUAN, 1968), in 2.5 mL of LB medium (Luria Bertani).

All the pre-inoculum was incubated for 16 hours in shaker with 37°C of temperature and agitation for 180 rpm, and later, inoculated in 250 mL of LB and incubated under the same conditions until it reached an optical density (OD) at 600 nm of approximately 0.5 (2 hours and 30 minutes under agitation at 37°C). The cells were then subjected to a cold shower for 15-30 minutes.

After that, the tube was centrifuged for 15 minutes at 6000 rpm at 4°C. The supernatant was discarded and the pellet re-suspended in 125 mL of Milli-Q cold water and centrifuged again under the same conditions. After discarding the supernatant, the pellet was re-suspended in 25 mL of Milli-Q cold water supplemented with 10% of glycerol.

The last spin cycle was carried out under the same conditions. The supernatant was then discarded and the pellet re-suspended, gently, in 0.5 mL of autoclaved Milli-Q water supplemented with 10% of glycerol, sectioned in 40  $\mu$ L and quickly frozen at -70°C for later use.

## CLONING

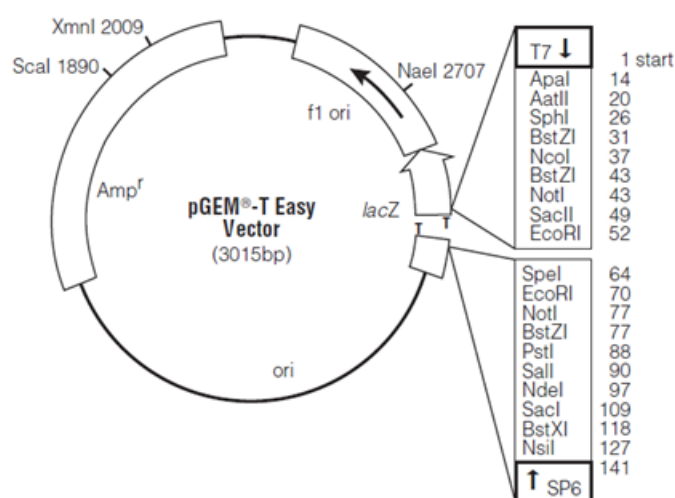
For the elution of the amplified DNA (insert) by PCR targeting a subsequent cloning, the insert related bands were excised from the gel and placed in eppendorf® tubes. The elution protocol was used according to the manufacturer's instructions (QIAquick Gel Extraction - Qiagen®). The eluted DNA was stored at -20°C, following the methodologies of construction of the recombinant DNA molecule according to the manufacturer's instructions (Promega®).

The LB bacterial culture medium was used both for sowing of possible clones as for recovery of *E. coli* cells after the process of electroporation. It was prepared 300 mL of LB growth medium containing the following components: 1% of tryptone; 1% of NaCl; 0.5% of yeast extract; and 2% of agar. After the medium reached approximately 60°C it was added 100  $\mu$ L of the antibiotic Ampicillin in the final concentration of 100  $\mu$ g/mL. Approximately 25 mL of the culture medium, previously prepared, was used for the preparation of the petri dishes.

After complete polymerization of the medium, blue and white selection markers were added (100  $\mu$ L of IPTG in the concentration of 100 nm/mL and 20  $\mu$ L of Xgal at a concentration of 50 mg/mL).

The connection mixture, which is needed for the synthesis of recombinant DNA molecule, (cloning vector + insert) was performed according to the manufacturer's manual (pGEM®-T Easy - Promega®) (figure 1), continuing the practical procedures with the technique of electroporation of competent host cells.

Figure 1 - pGEM®-T Easy Vector System (Promega®).



For bacterial electroporation 40  $\mu$ L of a suspension of competent *E. coli* DH5 $\alpha$  were added to 2  $\mu$ L of the connection mixture previously established. All this content was placed in a refrigerated bucket (Gene Pulser/MicroPulser Cuvettes - BIO

RAD®), very carefully, so that bubbles would not be formed. The bucket was subjected to an electric current, providing a constant 4.4 of transformation efficiency. Immediately after the electric shock, the bucket mixture was re-suspended in 800  $\mu$ L of LB medium and incubated in an oven with an agitation system (shaker) at 37°C for 1 hour, at 200 rpm.

After the incubation was over, 6 petri dishes (previously prepared with LB/Ampicillin/IPTG/X-gal medium) were sown: 3 with 200  $\mu$ L and 3 with 100  $\mu$ L from the cell culture newly transformed, being subsequently incubated in an oven at 37°C for 16 to 24 hours.

After the growth of the cultures, it was initiated the preparation of the plasmidial DNA by the Minipreparation technique.

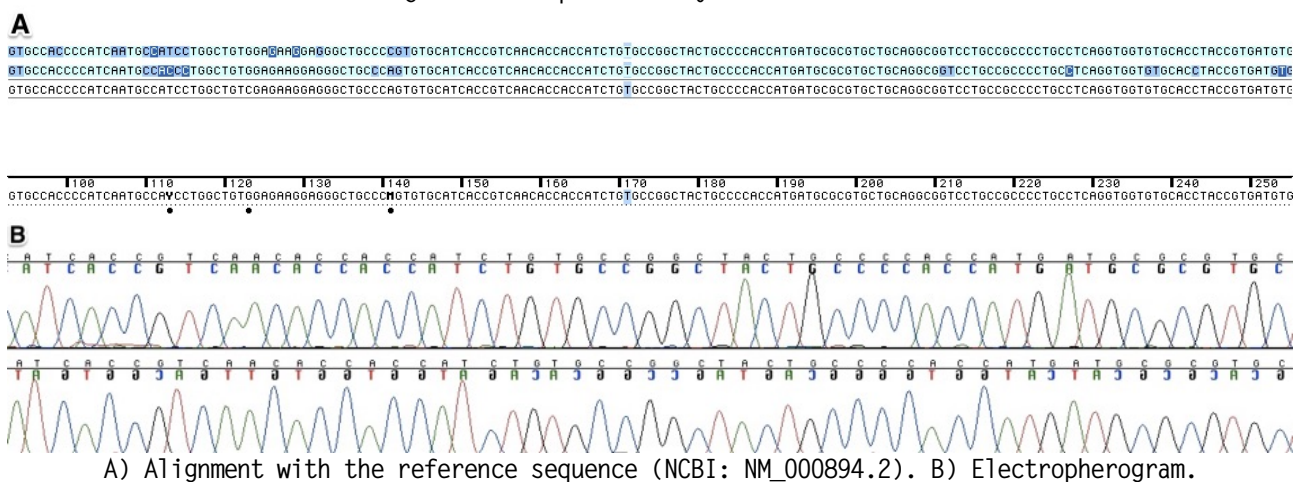
## PLASMIDIAL EXTRACTION AND DIGESTION

The Minipreparation technique, performed according to SAMBROOK; RUSSELL (2001) (RUSSELL; SAMBROOK, 2001), allows the plasmidial DNA isolation in small scale of a bacterial culture through the alkaline lysis procedure. The extracted plasmidial DNA was dissolved in 50  $\mu$ L of buffer (pH 8.0), continuing the process of plasmidial digestion by EcoRI endonuclease according to the manufacturer's instructions (Promega®).

## RESULTS

RNA isolation and cDNA amplification was performed from the 4 selected samples. Sequence analysis confirmed the identity of the isolated LHB mRNA and subsequently amplified LHB cDNA from peripheral blood (figure 2).

Figure 2 - Sequence analysis of LHB cDNA.

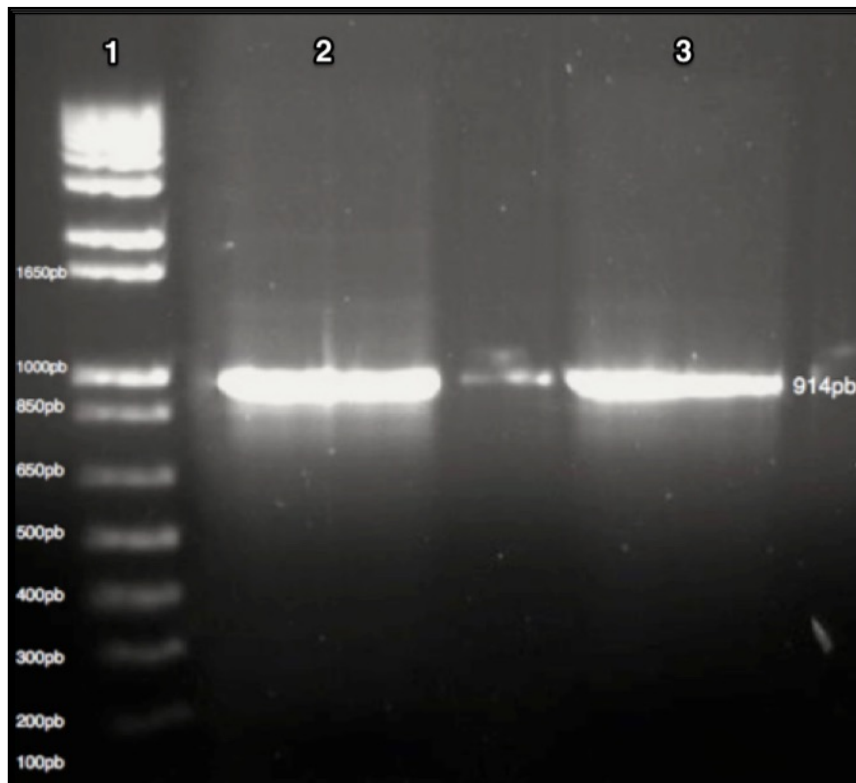


Due to the low concentrations in cDNA amplification, an efficient cloning process was not achieved. We then decided to continue the procedures targeting the genomic DNA. It is important to note that due to post-processing conservation of the sequences used for the annealing of primers, within exons - NCBI NM\_000894, all the gene manipulation performed by us, involving since its amplification until its molecular cloning, could, without any problem, be applied to the corresponding mRNA fragment.

The electrophoretic profile of figure 3 demonstrates the genomic amplicon of LHB gene (PCR product from samples L-a; L-1 and L-2 were obtained, however the photos were not presented in this article). The size of the obtained band corresponds

to the fragment generated during the test of the tool Primer-Blast - NCBI (YE et al., 2012) with the pair of chosen primers.

Figure 3 - PCR product profile from sample L-3 analyzed on a 1% agarose gel, ethidium bromide stained.



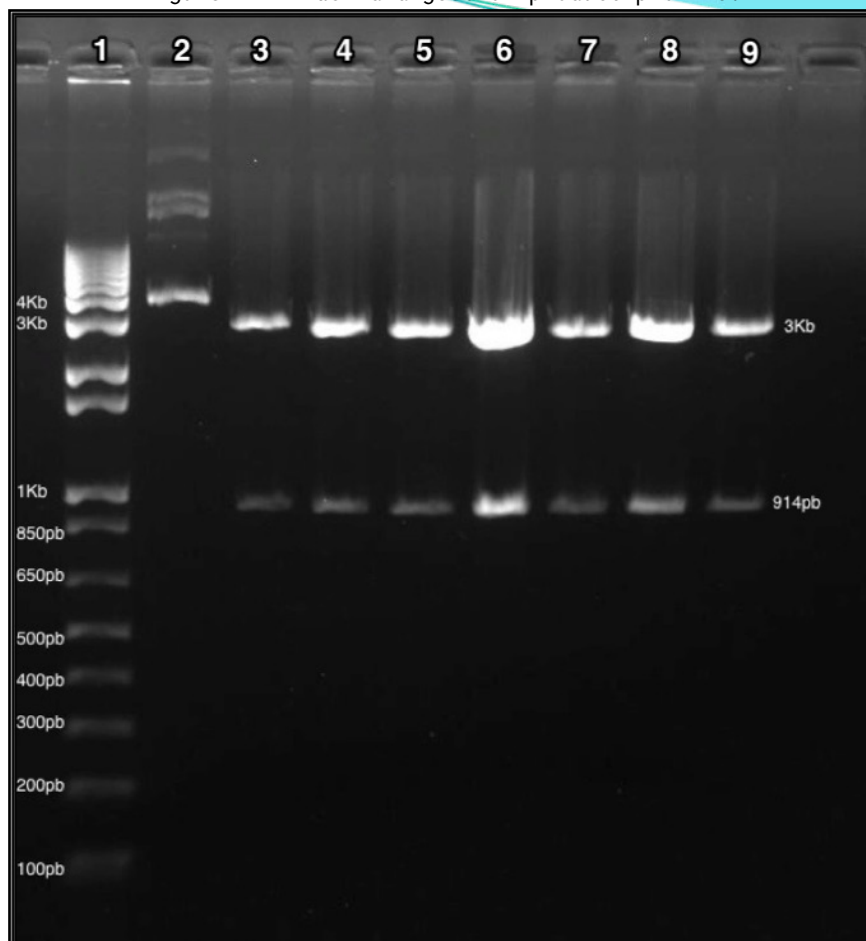
1) 1 Kb Plus DNA Ladder Invitrogen®; 2-3) 914pb fragmente.

While obtaining the gel, proving the amplification of the fragment of interest, the excision and elution of the PCR product were made. All cloning protocol was performed with the sample L-3 because it was the only one that showed a high concentration of the PCR product, ensuring greater efficiency for the synthesis of recombinant DNA molecule.

The bacterial transformation process was 100% achieved. The efficiency calculation of cloning was not performed; however, it was assumed that the process has been extremely effective due to the large number of white colonies in contrast with the blue ones.

After appropriate preparation of the Grid and observation of the white colonies growth, it was performed the plasmidial DNA Minipreparation technique and subsequent digestion, resulting in the electrophoretic profile observed in figure 4.

Figure 4 - Plasmid digestion product profile.



1) 1 Kb Plus DNA Ladder Invitrogen®. 2) Vetor pGEM®-T Easy undigested. 3-9) pGEM®-T Easy digested (3015 pb) and insert corresponding to the excised LHB gene fragment (914pb).

## DISCUSSION

The prostate carcinoma is considered, in the United States, the second leading cause of death among men, most of them caused by bone metastasis. It is the kind of proliferative disorder most commonly diagnosed in male individuals and there are predictions that the number of cases will keep growing year after year.

Androgen deprivation as an endocrine treatment in patients with prostatic neoplasia has been described for over 60 years and the debates about the proper temporal use of hormone therapy are numerous and frequent. In most cases, good initial responses occur to androgen deprivation therapy by depletion of testosterone. On the other hand, some of the current hormonal deprivation methods collide with disadvantages such as: high cost of deployment, in the case of Luteinizing Hormone-Releasing Hormone analogue (LHRHa) and anti-androgen monotherapy; psychological implications, such as those caused by orchiectomy and propensity for developing breast tenderness, gynecomastia and fatal thromboembolism on estrogen therapy (CALABRESE, 2004; NISHII et al., 2012; WILT, 2012).

As the key to generate this disorder includes not only understanding the processes responsible for prostate growth, but also the male hormone homeostasis (NISHII et al., 2012), endocrine therapy has been used in most cases, however the side effects from the treatment still generates great impact on quality of life for these patients (DRUDGE-COATES, 2005; MATTHEWS, 2007; VILAR GONZÁLEZ et al., 2009).

Thus, the current methods of treatment for prostatic neoplasia, lead, in some cases, only to the improvement of the patient survival in addition to cause

several side effects because of the considerable toxicity of medicines. As the surgery, another treatment option, is invasive and cause a series of physical and emotional complications, new therapeutic approaches for the treatment of this disorder are required (DENG et al., 2011; JUNUZOVIC et al., 2011; RAMINDER, 2010).

As conventional non-selective therapies, such as pharmaceuticals, still have importance in everyday practice, most new developed agents aim at specific targets with functional central role in the pathogenesis of the disorder (cytokines, chemokines, transcription factors and molecules expressed on the cell surface) (BARBOSA; LIN, 2004; FRANÇA et al., 2010).

Gene silencing by RNA interference (RNAi), for example, is a promising biotechnological aspect, emerging as an alternative for the treatment of neoplastic disorders, which aims to slow down the tumor development in a less invasive and more efficient way, taking advantage of a pre-existing biological mechanism in the organism (CHOUDHURY et al., 2004; FALTUS et al., 2004; FUJITA; KUWANO; OCHIYA, 2015; MERRITT; BAR-ELI; SOOD, 2010).

As the basis for these and other implementations revolves around classic genome manipulation processes, with the aim of re-establishing a cellular function that was inhibited or defective; to introduce a new function or interfere with an existing one (CAVAGNARI, 2011; LINDEN, 2010; RESENDE; SOCCOL, 2015), this study sought to implement the initial steps of gene manipulation in the main pharmacological target of prostatic neoplasia, LH and its hormonal axis.

With the profile digestion shown in figure 4 we can affirm that the main objective of this work was achieved, that is to say, the fragment initially amplified was cloned successfully, thus demonstrating several biotechnological procedures of gene manipulation.

To verify the testing of similar essays where we could base our thoughts and procedures, it was conducted a review of the scientific literature in databases such as Pubmed, using the following keywords for the search: LHB; cloning; prostate cancer; biotechnology; hormone therapy and LH.

In this research, however, it has not been found any work that performed the entire gene manipulation related to the gene LHB in the way described here. HOTAKAINEN et al., (2000) (HOTAKAINEN et al., 2000) managed to amplify the mRNA sequence of the gene LHB from peripheral blood, proving that there is an ectopic production of LH hormone subunits. The same result was obtained in our laboratories. However, the concentration of the PCR product was very low, demonstrating the need for adjustments in the implementation of RT-PCR technique.

Molecular cloning, such as the one performed here, has great importance because it can be the basis for the development of researches that aim at blocking the expression of LH in gene therapy, using, for example, RNAi molecules.

For the synthesis of these molecules and subsequent realization of more complex tests, it would be required the isolation and amplification of the mRNA molecule from the gene LHB and its gene manipulation, which includes since the amplification technique until the introduction of the insert in an expression vector, such as *Drosophila melanogaster*, widely used due to its advantage to models of expression in mammalian cells (LEMOS et al., 2009).

Once the agonist and antagonist drugs of the hypothalamic-pituitary-gonad axis are widely used, especially in cases of metastatic prostatic neoplasia, symptoms related to the absence of hormones involved in this signaling pathway, such as the LH, are well-known and the advantages of the use of hormonal blockade with gene therapy techniques are justified by the easiness for the production of products to be used (such as RNAi) front of pharmaceuticals engineering, and its high specificity towards the target, something in constant improvement by the pharmacological industry (MENCK; FREDERICO; MENCK, 2007; RESENDE; SOCCOL, 2015).



Despite the accumulated knowledge about the human genetic traits and gene manipulation techniques, the application of this type of therapy as an everyday routine is faced with several problems of a technical nature. It is important, then, that greater efforts are employed to the improvement and development of new procedures (KAUFMANN et al., 2013; LINDEN, 2010; RESENDE; SOCCOL, 2015).

## CONCLUSION

In conclusion, it was successfully sought to demonstrate the biotechnological trials that would be needed for the beginning of many gene handling procedures in order to develop therapeutic alternatives which target the human genome. There is, however, the need for an improvement of the RT-PCR technique, parallel held, in order to transfer the process of cellular cloning to the transcription product (mRNA), because this element would represent the real target of the gene therapy. Even in the face of growth and improvement of current employed biomolecular techniques, much still needs to be done before the solidification of gene therapy as a substitute for pharmacological interventions on prostatic neoplasia or any other neoplastic disorder.

Inherent challenges to the own technique as security of vectors; effectiveness in the expression of exogenous genes or blocking of endogenous genes; and related to the course of prostatic neoplasia, as independent hormone metastasis, are far from being completely solved with the knowledge obtained until today.

However, the gene therapy on prostatic neoplasia, as one of the major fields of biotechnology investment has been the neoproliferative disorders, can act as a tool for research and improvement, working in a complementary way to the common treatment and further contributing to the development of more efficient alternatives; clarification about the pathogenesis of the disorder and improvement of the therapeutic techniques currently employed.

**Disclosure summary:** The authors declare there is no conflict of interest that could be perceived as influencing the impartiality of the research reported.

**Support:** This work was supported by the authors.

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