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Research Article

Optimization of Osteopontin Recombinant Protein as a Candidate Supplementation for Semen Preservation

Tatik Hernawati*, Tita Damayanti Lestari, Suzanita Utama, Rimayanti

ABSTRACT

The recombinant protein of heterologous proteins in *Escherichia coli* strains K12 has various and different systems tested and demands a detailed insight into the multiple factors affecting the encoded protein. One of the crucial factors is the acceptable quality of the DNA copies inserted inside the bacteria. Firstly, the amplification procedure needed to be performed well; thus, designing the primer and selecting the optimum annealing temperature are the focus indicators in this study. This study obtained a reference gene from the NCBI data bank with Reference Sequence: NM_174187.2. Two types of primers (SPP1F-SPP1R and OPN1F - OPN1R) with different targeted bands were designed and selected after being reconstructed using the software. Online software such as addgene.org is also used to identify the right restriction site. The annealing temperature distinguished the PCR system used to amplify each primer. The result of this study revealed the best annealing at 65°C successfully amplified 820 bp of the targeted band. The phenomenon not following the theory of blue-white screening is the empty plasmid control, where not a single colony grows on the media. Competent cells inserted with empty plasmids should still be able to expand on LB-Amp agar media because the presence of these plasmids is capable of providing resistance to antibiotics (in this case, ampicillin). This discrepancy is thought to have been caused by the improper insertion of the empty plasmid so that the plasmid did not enter the competent cell.

Key words: Osteopontin, Primer, DNA clone, PCR amplification.

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INTRODUCTION

Several proteins in seminal plasma modulate essential reproductive functions such as sperm motility and capacitation, cell protection, acrosome reactions, fertilization, and embryonic development. Protein in sperm is also crucial for successful fertilization, egg activation, and embryo development and can be a marker for the status of semen quality in cattle.[1] According to previous research by Samik *et al.* (2014), SPP1 in the epididymis and sperm is essential in regulating sperm calcium levels and the epididymal lumen.[2] SPP1 and its receptors on spermatozoa and seminal fluid indicate that this molecule plays a role in the male reproductive system. Then other studies related to the SPP1 gene polymorphism analysed its impact on milk production and milk composition.[3-5]

Alvarez-Gallardo *et al.* (2013) have reported the effect on fertility of adding recombinant FAA (rFAA) and recombinant TIMP-2 (rTIMP-2) to bovine semen before cryopreservation for use in artificial insemination (AI) program.[6] The result suggested that adding the (rFAA and rTIMP-2 improves the fertility rate, consequently increasing the pregnancy rate in heifers.

Theoretically, the procedure required to produce recombinant protein is relatively straightforward. We take the gene we want, clone it in the expression vector, transform it to the host of choice, and induce it so the protein is ready for purification and characterization. However, in the implementation, many mistakes can occur. Starting from low host growth and inclusion bodies' formation, the protein obtained is inactive and sometimes not getting protein.[7]

Many factors influence the yield of recombinant protein obtained, and the quality of the DNA copies that will be inserted into the bacteria is one of the crucial factors. All procedures in DNA copying, *i.e.*, amplification, must be carried out correctly.[8] Assuming all the reagents have been added in the correct concentration, two essential PCR components will affect gene amplification results, depending on the researcher.[9] The first thing is the nucleic acid template, which must be of adequate quality and not contain Taq DNA polymerase inhibitors, which are enzymes or significant components in the DNA amplification process.[10] The second thing is the selection of oligonucleotide primers. This process is often critical to a PCR trial's overall success because there would be no PCR product without a functional primer set.

Selecting the optimal PCR oligonucleotide primer set can be very tedious, and as such, it often requires assistance with computer analysis. The main factor influencing the function of oligonucleotides - namely, their melting temperature and possible homology among primers - is a well-defined, straightforward task that is efficiently coded in computer software.[11] Once the computer has provided a small number of primary candidates sets, the selection task can (and is still) be done manually. Considering the importance of primer selection, this research focuses on the preliminary design and selection of the optimum annealing temperature to get a functional recombinant protein.

MATERIAL AND METHODS

Sample preparation

The sample was taken from Frisian Holstein cattle and Limousin cattle blood, SPP1 gene primer;

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Forward SPP1: 5' ACC CAG ATG CTG TAG CCA TAT GG 3' and Reverse SPP1: 5' GGC TAT GGA ATT CTT GGC TGA GTT TGG 3', OPN1 gene primer; Forward OPN 1: 5'-ACC CAG ATG CTG TAG CCA TAT GG-3' and Reverse OPN1: 5'-GGC TAT GGA ATT CTT GGC TGA GTT TGG-3', XbaI restriction enzyme, EcoRI restriction enzyme, Quick-DNA™ Miniprep Plus Kit (Zymo Research), agarose, TBE buffer, DNA ladder 1 Kb and 100bp, Nuclease Free Water, Thermo Scientific Green PCR Master Mix**.**

Instruments

Centrifugation tool: Hermle Z216, microwave, incubator, PCR rack, Micro pipette from accumax, Analytical balance, horizontal electrophoresis used from Biorad, thermocycler used C1000 thermal cycler from Biorad, and Gel documentation from Biorad**.**

DNA isolation and PCR program to obtain Osteopontin gene from Cattle blood

DNA isolation was conducted based on the protocol of Quick-DNA™ Miniprep Plus Kit (Zymo Research.[12] Then it was continued with amplifying the DNA target gene with programs of pre-denaturation. at 95°C for 7 min; 35 cycles of denaturation at 95°C for 30s, annealing on 65°C, and elongation at 72°C for 30s. The gene was amplified at 820kb as a targeted band. The gene encoding Osteopontine was then separated by XbaI and EcoRI restriction enzymes, then it was analyzed with agarose gel electrophoresis.

DNA extraction in this study used the "Blood DNA Preparation Kit" by Jena Science. DNA Hydration Solution 50-100µL was added and vortexed at medium speed. Incubated at 65°C for 30 minutes for Accelerate Rehydration. The extraction results were stored at 4°C.

Primer selection and construction of the pET-21a(+) recombinant plasmid

The gene encoding AmpR (Ampicillin Resistance) was amplified with the forward primer AmpR (5'-CTACGGTCTGGCTGCTA-3') and reverse primer AmpR (5'-TGGAGCAAGAGGCGGTA-3') (Olesen *et al.* 2004). PCR conditions included an initial denaturation step at 98°C for 30s, 54°C for 30s, and 72°C for 1 minute, with a final stage of 72°C for 5 minute using Phusion High-Fidelity DNA Polymerase. The PCR product was cloned into the pET-21a(+) vector.

The gene encoding SPP1 (Secreted Phosphoprotein 1) was amplified with forward primer SPP1F (5'-ACCCAGATGCTGTAGCCATATGG-3') and reverse primer SPP1R (5'GGCTATGGAATTCTTGGCTGAGTTTGG-3') with NCBI Reference Sequence (NM_174187.2). The gene coding for OPN (Osteopontin) was amplified with the forward primer OPN (5'-AGCCCACCACCAAATACCTA-3') and reverse primer OPN (5'-TCTGAAGGACTGGCTTAGATTTC-3') at 700bp, as well as the forward OPN primer (5'TCCCTCCCTCTACGTTTTCA-3') and primer reverse (5'-CATCCCAAAAGGGCATAGAA-3') at 850 bp.[13]

The construction and subcloning of the pET-21a (+) recombinant plasmid was carried out in several steps, namely ligase insertion of the AmpR (Ampicillin Resistance) gene with pET-21a(+) expression vector and transformation of the host cell *E. coli* BL21 (DE3) with ligase results. The ligase reaction was carried out according to the pET-21a (+) vector kit procedure. At this ligase stage, the enzyme T4 DNA ligase was used to ligate the DNA fragment inserted into the pET-21a(+) vector. The ligase process can be carried out for 2 hours at room temperature (22°C) or overnight at 4°C. After ligation of the insertion of the AmpR gene with the pET-21a(+) expression vector, the transformation process of *E. coli* BL21 host cells was carried out with the ligase results.

Transformation of plasmids to host cells

The optimized gene was synthesized and inserted into the expression vector plasmid pET-21a(+) using the restriction enzymes Nde1 and BamH1. The restriction fragments were further observed using 1% agarose gel to evaluate the success of the target DNA insertion in the pET-21a(+) plasmid. Competent *E. coli* BL21(DE3) cells with 200L were added 0.26g of pET-21a(+) plasmid and incubated at 4°C (ColdBox) for 30 minute. Followed by incubation at 42°C for 90s, and then 800L of LB medium was added. The cell suspension was incubated at 37°C for 45 minutes, and 50L of transformant cells were spread in LB agar containing ampicillin and incubated at 37°C for 12-16 hours.

A total of 5L of plasmid DNA was mixed into 100L of competent cell suspension (*E. coli* BL21) and then homogenized. The mixture was left on ice for 30 minutes, then incubated (heat-shocked) at 42°C for 90s using a heat block, then placed back on the ice for 30 minutes, then set at 37°C for 1 hour by adding 1mL of LB Broth media (without antibiotics). In this study, the transformation process was carried out three times and only succeeded in showing the success of attaching the OPN gene to the third transformation.

Amplification

The DNA amplification program went through a pre-denaturation stage at 94°C for 5 minutes, followed by 35 cycles consisting of a denaturation step at 94°C for 1-minute, annealing temperature depending on the type of primer (range 58-65 °C) for 30s-1-minute, DNA strand synthesis at 72°C for 1 minute, and DNA strand lengthening at 72°C for 10 minutes, and storage at 4°C.[10,14]

PCR products were separated based on base size using ultrapure™ agarose (Invitrogen) 2% (w/v) gel which had been added with DNA stain - PeqGreen 1 l/30 ml TBE buffer. The DNA in the gel was then run at 100 volts for 35 minutes. The separated DNA results were visualized under UV light using a gel doc (Biorad) and a marker length of base/ladder with ImageLab software.

Characteristics of transformants

The pET-21a(+) plasmid was isolated using a high plasmid mini kit (Geneaid). A total of 1.5mL of *E. coli* suspension containing the plasmid pET-21a(+) was incubated overnight and centrifuged for 1 minute at a speed of 14,000xg at room temperature to form pellet cells. The supernatant was discarded, and the pellet was resuspended using 200µL buffer PD1. The lysis step was carried out by adding 200L of PD2 buffer to the sample, resuspending, mixing by slow inversion ten times, and incubating for 2 minutes at room temperature. The neutralization step was carried out by adding 300L of PD3 buffer and inverting it ten times, followed by centrifugation for 3 minutes at 14,000xg at room temperature. The supernatant was transferred to a PDH column and centrifuged for 1 minute at 14,000xg at room temperature. The supernatant was discarded, and the PDH column was added to 500µL buffer W1 and centrifuged for 1 minute at 14,000xg at room temperature. The supernatant was discarded, and the PDH column was centrifuged for 3 minutes at 14,000xg at room temperature to remove any remaining buffer W1. Then the PDH column was transferred to a new 1.5mL Eppendorf tube and added with 50L of EB buffer, incubated at room temperature for 2 minutes, and centrifuged for 1 minute at 14,000xg at room temperature. The pET-21a(+) plasmid solution was electrophoresed for 30 minutes at 100 V. The purity and concentration of plasmids were measured by Nanodrop 2,000/2,000c Spectrophotometer.

The insertion of the gene encoding the plasmid pET-21a(+) can be detected using the restriction enzymes SacI and HindIII. A total of 0.78g of plasmid pET-21a(+) was added with 2L of Tango buffer, 0.2L of acetylated BSA, 1L of SacI, and 2L of HindIII. The solutions were

mixed and incubated at 37°C for 4 hours. The restriction results were carried out by an electrophoresis process for 30 minutes at 100 V.

Competent *E. coli* cell manufacture

Single colonies of *E. coli* BL21(DE3) from LB agar in Petri dishes that had been incubated for 16-20 hours at 37°C were taken and grown in 30mL LB at 37°C with an agitation speed of 200rpm. After the culture of *E. coli* BL21 (DE3) reached OD600 0.4-0.6 (about 2.5 hours), 15mL was taken and centrifuged at 2,700xg for 10 minutes at 4°C. The supernatant was discarded, and the pellet was suspended in 1mL of cold MgCl2-CaCl2 solution (80mM MgCl2-20 mM CaCl2) and centrifuged at 2,700xg (4,100rpm) for 10 minutes at 4°C. The supernatant was discarded, and the pellet was suspended in 600L of cold 0.1 M CaCl2 solution. Competent *E. coli* BL21(DE3) cells can be used immediately or stored at –80°C.[15]

RESULTS AND DISCUSSION

DNA isolation result

The results of total DNA isolation were also tested for quality using electrophoresis with a 1% agarose concentration. The results of the 1% agarose electrophoresis gel can be seen in Figure 1. The 1% gel electrophoresis results show a band above the 10,000 bp marker, indicating that the isolated total DNA has a size of over 10,000 bp. As a standard of base length, a 1Kb DNA ladder was added to the M code in the first well. The concentration of Agarose was used at 1% because the total DNA size in Bos taurus cattle is known to be relatively large. It requires a low gel density so DNA can move from the negative pole to the positive pole during electrophoresis. According to Zimin *et al.* (2009), the genome of the Bos taurus species reaches 2,857,605,192 base pairs, so the electrophoresis results will show DNA bands, as seen in the electrophoresis results above.[16]

DNA concentration can be calculated accurately through spectrophotometric absorption of ultraviolet light.[17] The method that is often used to determine the purity of DNA is to use the ratio of absorbance at 260 and 280 nm (A260nm/A280nm). The comparison between absorption units of wavelength 260/280 indicates protein or RNA contamination, where 1.8 - 2 is the superior value and below 1.8 indicates protein contamination. In contrast, the above 2 means contamination by RNA. Likewise, the ratio of 260/230 indicates the level of polysaccharide contamination, where more than 2 is the superior value and below 1.8 indicates polysaccharide contamination.[18] From a total of 13 samples that have been extracted, we measured the total DNA average concentration of approximately 30mg/uL.

Amplification

This amplification process is the key to the OPN gene encoding as a candidate gene to be inserted into the plasmid in the test as a recombinant protein. The plasmid selected in this study also plays a role in gene transcription when the gene is inserted and is a transporter into the cell. Gene cloning is also the first step that is always carried out in molecular research that aims to study the characteristics of a gene.[19] Gene cloning is generally done with plasmids. Plasmids have restriction sites that can be used for gene cloning. The compatibility of the plasmid influences the success of gene cloning with the recombinant gene and the compatibility of the host cell with the recombinant plasmid.[20] This study obtained the osteopontin gene amplified at 860-880 kb (target band-annealing temperature at 65°C) using OPN1 primer. Another SPP1 primer, however, failed to produce DNA bands indicating failure of DNA copying during amplification. Based on the results of this study, primers will be used to make rDNA to create recombinant osteopontin through transformation into *E. coli* bacteria.[13]

Two pairs of primers were used to detect the OPN gene in cattle DNA by PCR method, and below is the PCR product from Forward OPN 1: 5'-ACC CAG ATG CTG TAG CCA TAT GG-3' and Reverse OPN1: 5'-GGC TAT GGA ATT CTT GGC TGA GTT TGG-3'. OPN gene characterization by PCR method that has a gene length of 820 bp (Figure 2 and Figure 3).

The SPP1 gene in cows is located on chromosome 6, which consists of the Bovine SPP1 gene composed of 7 exons and stretches of approximately 7 kb of genomic DNA (access number GenBank NW 255516). The sequences database newly assembled the entire cow genome (http://pre.ensembl.org/Bostaurus/index.html based on Btau 3.1). The bovine SPP1 gene (NC 007304.2) consists of 6,961 bp total length: 1,331 bp processed length and 278 protein product length (Figure 2.3 'A').[19]

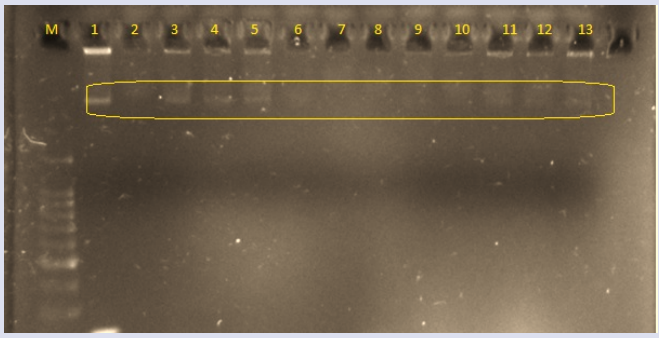

Figure 1: Results of electrophoresis visualization of DNA extract on 1% agarose gel
Description: M = DNA Ladder 1Kb, 1-13 = sample number

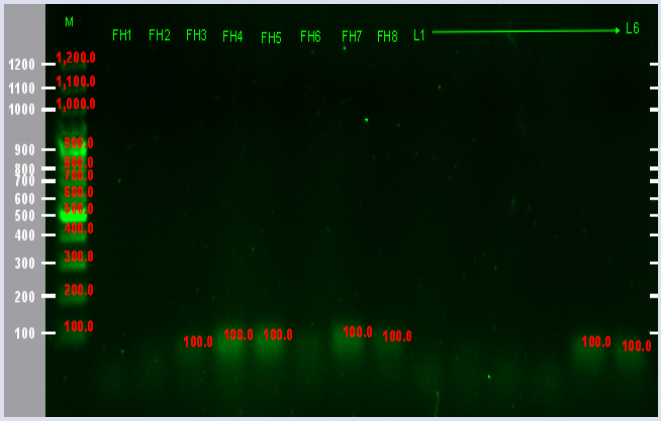

Figure 2: PCR product of Osteopontin gene amplification using SPP1 primer (Agarose 2%)

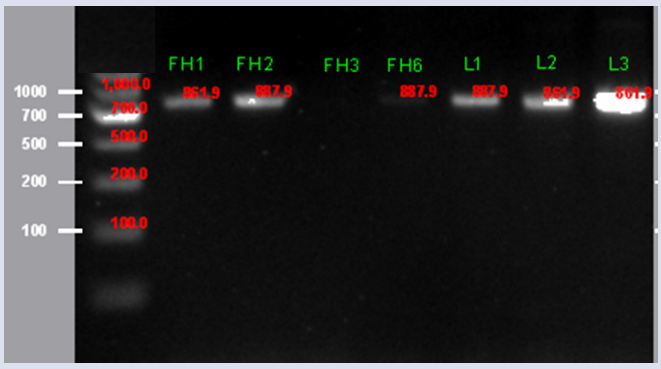

Figure 3: PCR product of Osteopontin gene amplification using OPN primer (Agarose 2%)

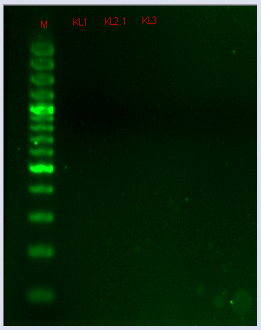

Figure 4: The results of the first transformation: the OPN gene did not successfully enter the *E. coli* BL21. Plasmid

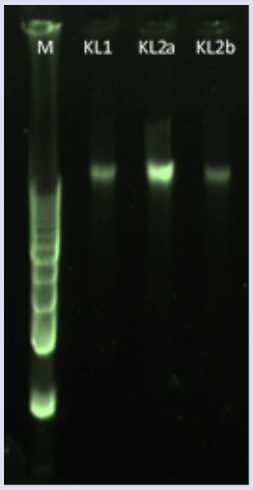

Figure 5: The results of the third transformation: the OPN gene successfully entered the *E. coli* BL21. Plasmid

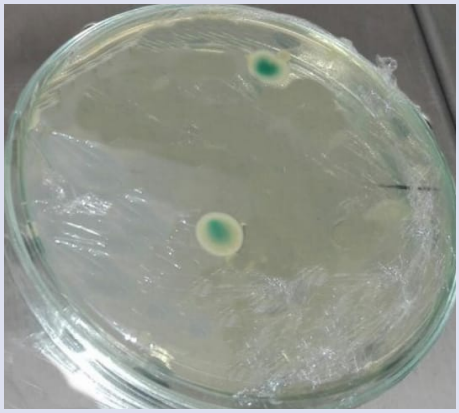

Figure 6: Colonies in blue are bacterial colonies that failed to insert a plasmid so that the lac portion of the bacterial plasmid is damaged and causes a blue colour

Ligase stage

The most critical factor in the ligase process is the enzyme ligase. The ligase enzyme functions to combine DNA fragments that have been cut with restriction enzymes with DNA vector fragments. Ligase enzymes can only join DNA fragments that have a complementary sticky end and a blunt end. Ligases catalyze the formation of covalent bonds between sugars and phosphates from adjacent nucleotides, which requires a nucleotide to have a free 5' phosphate end and an adjacent nucleotide to have a 3' end hydroxyl group. Ligase enzymes do not differentiate DNA from different organisms. Therefore, even two DNA fragments from other organisms can be joined by this enzyme. These two fragments then form a single DNA molecule known as recombinant DNA.[21] This recombinant DNA cannot be seen in the success rate except by multiplying it in the host cell. This process is called transformation or gene cloning.[22]

Plasmid to host cell transformation and transformation efficiency

Transformation of DNA into plasmid

Transformation is one of the abilities of bacteria to take foreign DNA into cells. Researchers use this ability to reproduce a gene. Genes inserted into plasmids are inserted into bacteria by the transformation method.[23] The transformation in the recombinant DNA in this plasmid does not occur naturally but must be induced by certain substances. Bacterial cells that have undergone physical treatment to gain the ability to take up foreign DNA are competent.[23] In addition, these capable cells can also take up circular plasmid DNA. Normal cells can only perform linear DNA transformation.[24]

After 1 hour, 0.3mL of transformed cells were grown on LB Agar medium containing the antibiotic kanamycin (35 g/mL) for selection of positive transformants.[15]

The phenomenon not under the theory of blue-white screening is the empty plasmid control, where not a single colony grows on the media. Competent cells inserted with empty plasmids should still be able to expand on LB-Amp agar media because the presence of these plasmids is capable of providing resistance to antibiotics (in this case, ampicillin). This discrepancy is thought to have been caused by the improper insertion of the empty plasmid so that the plasmid did not enter the competent cell.[25]

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Theoretically, the procedures required to produce recombinant proteins are relatively straightforward. We take the gene we want, clone it in our expression vector, convert it to the host of choice, and coax it, so the protein is ready for purification and characterization. However, in its implementation, many errors can occur. Starting from low host growth formation of inclusion bodies, the protein obtained is inactive, sometimes even getting no protein.[7,27]

Many factors affect the yield of the recombinant protein obtained, and the quality of the DNA copy to be inserted into the bacteria is one of the most decisive factors. All procedures in DNA copying, *i.e.*, amplification, must be carried out correctly.[8] Assuming that all the

reagents have been added in the correct concentration, two essential PCR components will affect the gene amplification results, depending on the researcher. The first is the nucleic acid template, which must be of adequate quality and not contain the DNA polymerase inhibitor Taq, an enzyme or essential component in the DNA amplification process.[14,10] The second thing is the selection of oligonucleotide primers. This process is often critical to the overall success of a PCR trial because there can be no PCR product without a functional primer set. Although selecting a primary site may be trivial, constructing the main stage to apply can be more challenging.[28]

The process of selecting the optimal oligonucleotide PCR primer set can be very laborious and, therefore, often requires the assistance of computer analysis. The main factors influencing the function of oligonucleotides - namely their melting temperature and possible homology between primers - are clear and direct tasks that are easy to code in computer software. Once the computer has provided a small number of primary candidate pools, the selection task can be (and still is) done manually. Given the importance of primer selection, this study focuses on primer design and selecting the optimal annealing temperature to obtain functional recombinant proteins.

CONCLUSION

This research successfully obtained an osteopontin gene amplified at 880 kb (target band- annealing temperature at 65°C) using primer OPN1. However, another primer, SPP1, did not successfully produce any DNA band indicating failure in DNA copying during amplification. Based on the results of this study, the primer will be used to make rDNA to create recombinant Osteopontin in the following process, namely OPN1.

The phenomenon that is not following the theory of blue-white screening is the empty plasmid control, where not a single colony grows on the media. Competent cells inserted with empty plasmids should still be able to expand on LB-Amp agar media because the presence of these plasmids is capable of providing resistance to antibiotics (in this case, ampicillin). This discrepancy is thought to have been caused by the improper insertion of the empty plasmid so that the plasmid did not enter the competent cell.

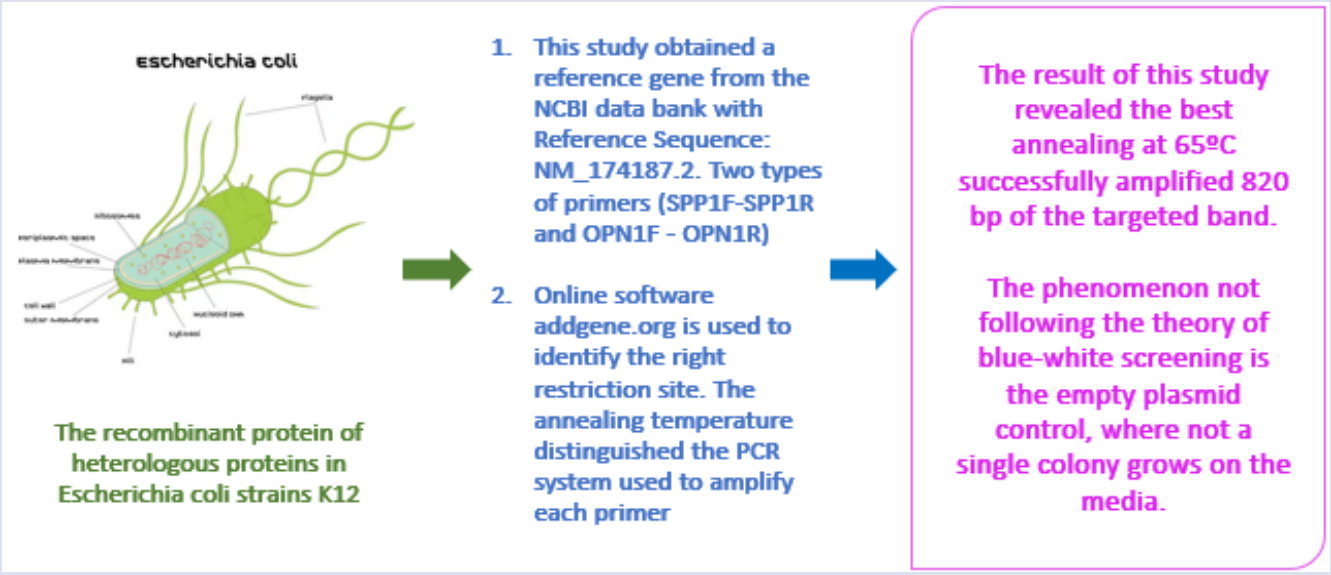
REFERENCES

1. Moura AA, Memili E, Portela AMR, Viana AG, Velho ALC, Bezerra MJB, *et al*. Seminal plasma proteins and metabolites: effects on sperm function and potential as fertility markers. Animal Reprod. 2016;15(1):691-702.
2. Samik A, Oktanella Y, Hernawati T, Ngakan MRW, Intan PD. Addition of Osteopontin in Frozen Semen Diluent of Holstein Friesian Dairy Cattle Increases Expression of B-Cell Cll/Lymphoma-2 Postthawing Spermatozoa. Veterinary J. 2014;15(4):461-6.
3. Boleckova J, Matejickova J, Stipkova M, Kyselova J, Barton L. The association of five polymorphisms with milk production traits in Czech Fleckvieh cattle. Czech J Anim Sci. 2012;57:45-53.
4. Manzoor S, Nadeem A, Babar ME, Shehzad W, Hashmi AS, Imran M, *et al*. Short communication : molecular effects of polymorphism in the 3'UTR of osteopontin gene in Riverine buffalo. Pakistan J Zool. 2017;49:1527-9.
5. Bissonnette N. Short communication: genetic association of variations in the osteopontin gene (SPP1) with lactation persistency in dairy cattle. J Dairy Sci. 2018;101(1):456-61.
6. Alvarez-Gallardo H, Kjelland ME, Moreno JF, Welsh TH, Randel RD, Lammoglia MA, *et al*. Gamete therapeutics: Recombinant protein adsorption by sperm for increasing fertility via artificial insemination. PLoS ONE. 2013;8(6):65083.
7. Rosano GÃL, Ceccarelli EA. Recombinant protein expression in Escherichia coli: Advances and challenges. Front Microbiol. 2014;5:1-17.
8. Dewi DNSS, Soedarsono S, Kurniati A, Mertaniasih NM. The specifc DNA region of esxA gene for the target of PCR to determine Mycobacterium tuberculosis accurately. Bali Med J. 2017;6(1):150-5.
9. Wirawan IGP, Sasadara MMV, Wijaya IN, Krinandika AAK. DNA barcoding in molecular identification and phylogenetic relationship of beneficial wild Balinese red algae, Bulung sangu (Gracilaria sp.). Bali Med J. 2021;10(1):82-8.
10. Lestarini IA, Suryani D, Sabrina Y. An Efficient Polymerase Chain Reaction (PCR) Enhancer for Highly Guanine-Cytosine (GC)-Rich DNA Sequences. Bali Med J. 2019;8(2):415-8.
11. Ariati PEP, Sasadara MMV, Wirawan IGP, Sritamin M, Suada IK, Wijaya IN, *et al*. Application of DNA Barcoding for authentication of Balinese traditional medicinal plant Purnajiwa (Kopsia arborea Blume. and Euchresta horsfieldii) (Lesch.) Benn. Bali Med J. 2022;11(3):1681-5.
12. Effendy E, Loebis B, Amir N, Siregar Y. Snp8nrg433e1006 Neuregulin-1 Genetic Variation In Bataks Ethnic With Schizophrenia Paranoid And Healthy Control. Bali Med J. 2014;3(2).
13. Meher C, Iswara RLK, Roesyanto-Mahadi ID. The correlation TNF-Î± gene promoter region -238G, -308G, -857, and -1031 polymorphism with psoriasis vulgaris. Bali Med J. 2021;10(1):126-31.
14. Rusyanthini E, Wirajana IN, Yowani SC. Optimization of Annealing Temperature for Amplification of 507 bp fragment of rpoB Gene of Clinical Multidrug-Resistant Mycobacterium tuberculosis Isolate 86. Indonesia J Biomed Sci. 2015;9(2).
15. Sambrook J, Russel DW. Molecular Cloning A Laboratory Manual. Cold Spring Harbor Laboratory Press. 2001;1.
16. Zimin AV, Delcher AL, Florea L, Kelley DR, Schatz MC, Puiu D, *et al*. A whole-genome assembly of the domestic cow, Bos taurus. Genome Biol. 2009;10(4):R42.
17. Siswanto JE, Berlian T, Putri CE, Panggalo LV, Yuniani L. DNA Isolation in Peripheral Blood Samples and Buccal Swabs in Infants with ROP: Comparison of Concentration Test Results and Purity Index. Sari Pediatri. 2016;18(4).
18. Kalendar R, Lee D, Schulman AH. Java web tools for PCR in silico PCR, and oligonucleotide assembly and analysis. Genomics. 2014;98(2):137-44.
19. Pareek CS. An Association Between The C>T Single Nucleotide Polymorphism Within Intron Iv of Osteopontin Encoding Gene (Spp1) And Body Weight Of Growing Polish Holstein-Friesian Cattle. An Sci Papers Rep. 2008;26(4):251-7.
20. Souci A, M'Hadheb-Gharbi MB, Gharbi J. Gene Cloning: A Frequently Used Technology in A Molecular Biology Laboratory - Alternative Approaches, Advantages and Limitations. Am J Res Commun. 2013;1(5):18-35.
21. Dwiputri AW, Ratnayani K, Yowani SC. Amplification of 0.7 Kb Fragment katG Gene from Clinical Multi-Drug Resistant Tuberculosis (MDR-TB) Isolate in Bali. Indonesia J Biomed Sci. 2015;9(2):21.
22. Anam, Khairul. Recombinant DNA. Molecular Genetics Practicum Report, Biotechnology Graduate School of IPB. 2009.
23. Brown TA. Gene Cloning and DNA Analysis. Blackwell Publishing. 2010.
24. Sword WE. Chemical Transformation of *E. coli* by: Casali N and Preston A - *E. coli* Plasmid Vectors: Methods and Applications. Humana Press. 2003;235:317-23.
25. Suhito IR, Putra AE. Kloning dan Over-Ekspresi Gen Pengkode Enzim Endo-β-1,3-1,4 Glukanase (BSUW23_10175) dari Bacillus subtilis subsp. Spizizenii W23. Universitas Surabaya. 2015.
26. Hernawati T, Mulyati S, Rimayanti, Suprayogi TW. Identification on Osteopontin Promoter Gene Polymorphism and Post-thawing Quality in Dairy Bull Friesian Holstein breeds. IOP Conf Series Materials Sci Eng. 2019;546(6):062010.

27. Liu X, Liu L, Wang Y, Wang X, Ma Y, Li Y. The Study on the factors affecting transformation efficiency of E. coli competent cells. Pak J Pharm Sci. 2014;27(3):679-84.

28. Dieffenbach CW, Lowe TM, Dveksler GS. General concepts for PCR primer design. PCR Methods Appl. 1993;3(3):S30-7.

GRAPHICAL ABSTRACT



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