2nd Bioinformatics and Biodiversity Conference
Volume 2022
http://dx.doi.org/10.11594/nstp.2022.2101

NST PROCEEDINGS

OPEN ACCESS

Conference Paper

In Silico Analysis of Sox2 Gene for Pluripotency Detection at Mouse Embryonic Fibroblast and induced Pluripotent Stem Cell (iPSC)

Aroem Naroeni[1,2]* Seprianto[1], Kevin Febrianus Moda[1]

[1]Biotechnology Study Program, Faculty of Health Science, Universitas Esa Unggul, Indonesia
[2]Virology and Cancer Pathobiology Research Center, Medical Faculty, Universitas Indonesia

*Corresponding author:
E-mail: aroem@esaunggul.ac.id

ABSTRACT

Stem cells are cells that have not been specialized and have a specific characteristic compared to other cells. There are several transcription factors like Sox2, Oct4, c-Myc, and Nanog to maintain embryonic stem cells. In pluripotent stem cells, sex-determining region Y-box 2 (Sox2) is a critical transcriptional regulator, and for somatic cell reprogramming, which involves returning differentiated cells to a pluripotent embryonic state by reversing their epigenetic arrangement. This study aimed to design primers for detecting the Sox2 gene expressed in Mouse Embryonic Fibroblasts and induced Pluripotency Stem Cell as pluripotency detection in stem cell research. In silico analysis was carried out to detect ofx2gene by using several software such as BLAST to search sequence Sox2 gene from Homo sapiens and *Mus musculus*, Bioedit for sequence alignment, SnapGene for PCR in silico, and PrimerBlast for online primer design. Primer candidates successfully designed were then analyzed for their secondary structure using NetPrimer. The results showed that forward primer (5'- CTACAGCATGTCCTACTCGCA -3') and reverse primer (5'- ACTTGACCACAGAGCCCA -3') were selected primers for *M. musculus*. Also, forward primer (5'-CTACAGCATGTCCTACTCGCA-3') and reverse primer (5'- ACTT-GACCACCGAACCCA-3') for Homo sapiens. Detection by PCR in silico using templates from H. sapiens and *M. musculus* sequences showed that the primers could specifically amplify the Sox2 gene in each species. Nevertheless, laboratory experiments need to be carried out for preliminary validation that has been designed. These primers will be used to measure the gene expression of Sox2 in qRT-PCR to detect the stemness characteristic of stem cells.

Keywords: Bioinformatics, Sox2 gene, primer, in silico analysis, pluripotency detection

Introduction

Stem cells are precursor cells with the ability to self-renewal and differentiate into various types of cells (Chagastelles & Nardi, 2011). Embryonic and adult stem cells are the two types of stem cells. Embryonic stem cells are pluripotent, meaning they can differentiate into any cell type and are derived during early development at the blastocyst stage (Prochazkova et al., 2015). Whereas adults stem cells only can differentiate into limited cell types. MEFs (Mouse Embryonic Fibroblasts) is a type of fibroblast-derived from the embryo of a mouse and commonly utilized as feeder layers to help embryonic stem cells (ESC) proliferate (Amand et al., 2016; Yusuf et al., 2013). Human-induced pluripotent stem cells (hiPSCs) are created from the direct conversion of human somatic cells into a pluripotent state via ectopic expression of particular transcription factors (Fernandez et al., 2013). The reason for using this animal sample is that it will be used as

How to cite:
Naroeni, A., Seprianto, & Moda, K. F. (2022). In Silico Analysis of Sox2 Gene for Pluripotency Detection at Mouse Embryonic Fibroblast and induced Pluripotent Stem Cell (iPSC). 2nd *Bioinformatics and Biodiversity Conference.* NST Proceedings. pages 1-10. doi: 10.11594/ nstp.2022.2101

a model for tissue engineering. *Mus musculus* is easier to manipulate for tissue engineering compared to other animals. Then in the future, it will be applied for a human to overcome the need for organs for transplantation.

Three fundamental transcription factors, Oct4, Nanog, and Sox2, are expressed in cancer stem cell-like cells (CSC-LCs) and embryonic stem cells (ESCs). These transcription factors are essential for CSCLCs and ESCs to maintain their pluripotency and self-renewal abilities (Liu et al., 2013). The Sox2 gene code makes a protein that helps build a variety of tissues and organs during embryonic development (National Library of Medicine, 2021; Karachaliou et al., 2013).

Quantitative reverse transcription PCR (RT-qPCR) is used when the genetic material is RNA. This procedure uses reverse transcriptase to convert total RNA or messenger RNA (mRNA) into complementary DNA (cDNA). After that, the cDNA is employed as a template for the qPCR reaction. Gene expression analysis, pathogen identification, genetic testing, and illness research are among the applications that use RT-qPCR. RT-qPCR is a powerful and sensitive technology for assessing gene expression, and a good RT-qPCR experiment necessitates careful attention to many parameters. First, we need primers that will specifically amplify the mRNA sequence from cDNA because we want to assess its amount (Carson et al., 2012). This is a critical step for gene expression analysis.

The oligonucleotide primer is an essential component of the PCR process. A good primer has unique features that indicate it will amplify a specific region of the genome (Praja & Rosalina, 2021). An in silico study is a study conducted using a computational approach. In general, in silico studies use available databases as research objects (Saraswati et al., 2019). This study aims to design and analyze oligonucleotide primers in silico to amplify the Sox2 gene for pluripotency detection.

Material and Methods

Database search and collection on GenBank

The Sox2 gene sequence was obtained using the "gene" search menu provided by the NCBI GenBank (https://www.ncbi.nlm.nih.gov/). The Sox2 gene sequence used in this study is the sequence with access numbers NM_003106.4 for detection in Homo sapien samples and NM_011443.4 for detection in *Mus musculus*. The obtained Sox2 gene sequences were then stored in FASTA format for further blast analysis.

BLAST Analysis

The gene was subsequently examined using the BLAST (Basic Local Alignment Search Tools) software. The multiple alignment approaches obtain conserved sequences from gene sequences with high identity values.

Multiple Alignments

The Bioedit tool is used to determination of sustainable areas. The ClustalW program is used to align the retrieved base sequence. Then, as a basis for tracking primer attachments, the one with the most similarity in its constituent bases is similar to 90-100 percent (Saraswati et al., 2019).

Designing Primer

The Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to construct the candidate primer pairs amplifying the Sox2gene with specific parameters (Table 1). Prime-BLAST is used to find multiple primer pairs that can amplify the Sox2 gene in each species based on gene sequences in FASTA format containing the Sox2 gene sequence.

Table 1. Parameters used in Primer-BLAST

Parameter	Setting
Product Size	70-150 basepairs
Primer Length	Min: 18, Optimum: 20, Max: 22
Primer TM	Min 58 ^{0}C, Max 60^{0}C
GC Clamp	0
GC Content	47-58 %
Max 3' Stability	9
Max Self Complementary	4.00 (any), 1.00 (3')
Max Pair Complementary	4.00 (any), 1.00 (3')

Secondary structure analysis

Secondary structure analysis on the primary pair was carried out to determine the best primer pair. The NetPrimer application, which can be found at http://www.premierbiosoft.com/NetPrimer/AnalyzePrimerServlet, was used to analyze secondary structures such as a self-dimer, hairpin, repeat, and run.

PCR in Silico

In silico, PCR was performed using the SnapGene software. The template or sequence used is NM_011443.4 *Mus musculus* SRY (sex-determining region Y)-box 2 (Sox2) for mice and NM_003106.4 Homo sapiens SRY-box transcription factor 2 (Sox2) for humans.

Phylogenetic tree construction

The phylogenetic tree was arranged based on the relationship of the Sox2 gene from *Mus musculus* and Homo sapiens species to other genera. This phylogenetic tree is based on sequences from blast nucleotide to see the closest gene relationship using MEGA11 software.

Results and Discussion

SOX2 Gene

This study focused on designing oligonucleotide primers using Primer-BLAST application to amplify the Sox2 gene. The Sox2 gene sequence was obtained through the NCBI Gen-Bank with access numbers NM_003106.4 for Homo sapiens and NM_011443.4 for *Mus musculus.*

BLAST Analysis

The Blast results of the Sox2 gene from *Mus musculus* in GenBank, ten Sox2 gene candidates with 97-100 percent identity were found, each with an accession number NM_011443.4; AK131933.1; X94127.1; U31967.1; BC057574.1; XM_021158544.1; XM_021196361.2; XM_031376603.1; XM_034501276; XM_032898509.1. Meanwnile in the Blast results of the Sox2 gene from Homo sapiens in GenBank, ten Sox2 gene candidates with 97-100 percent iden-tity were found, each with an accession number NM_024865.4; AY230262.1; AK290896.1; JX104845.1; NM_001355281.2; BC098275.1; AY455284.1; JX104837.1; JX104838.1; JX104839.1. Ten candidates for Sox2 gene sequences are aligned with multiple alignments using clustalW in the Bioedit program (v7.0.9 Tom Hall) to obtain conservative sequences as a prereq-uisite for good primer design.

Multiple Alignment

The ten downloaded sequences at NCBI were aligned using BioEdit with ClustalW to obtain consensus sequences. The image below is the result of multiple alignments on the blast analysis of *Mus musculus*, and the exact steps were carried out on Homo sapiens.

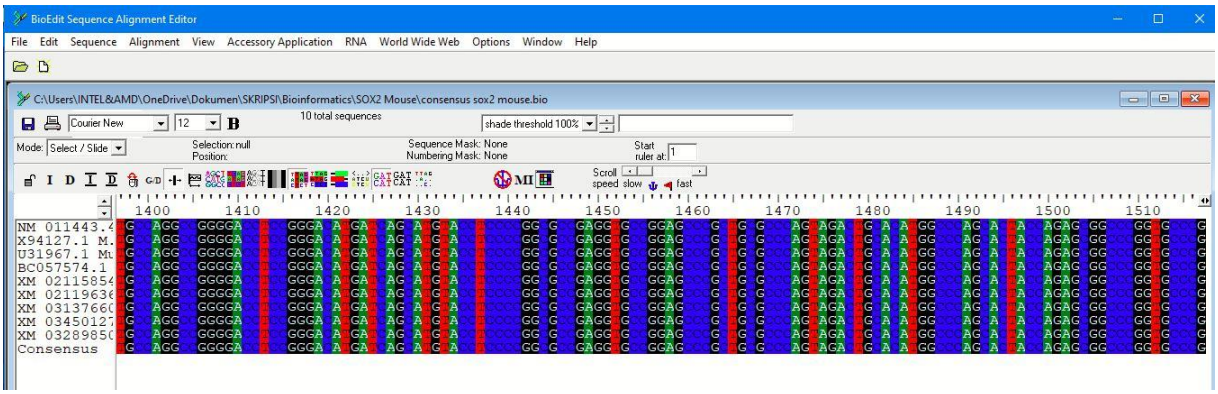

Figure 1. The multiple alignment process of SOX2 gene from *M. musculus* with other species that have 100-97% DNA sequence identity using Bioedit software

Primer

Table 2. Primer Candidates of SOX2 Gene for *Mus musculus*

Sequence (5'->3')	Length Primer	Tm	GC %	Length Product (base-pair)	Self comple-mentarity	Self 3' com-plementarity
Primer 1						
F:CCCACCTACAGCATGTCCTAC	21	59.86	57.14	77	4	1
R : GACTTGACCACAGAGCCCA	19	59.25	57.89		2	1
Primer 2						
F:ATGATGGAGACGGAGCTGAA	20	58.51	50.00	111	4	0
R:CGGGCTGTTCTTCTGGTTG	19	58.75	57.89		2	0
Primer 3						
F:ACCAGCTCGCAGACCTACA	19	60.61	57.89	112	4	0
R:CCTCGGACTTGACCACAGA	19	58.65	57.89		3	0
Primer 4						
F:CTACAGCATGTCCTACTCGCA	21	59.59	52.38	71	4	1
R:ACTTGACCACAGAGCCCA	18	58.01	55.56		2	1

Table 3. Primer Candidates of SOX2 Gene for Homo sapiens

Sequence (5'->3')	Length Pri-mer	Tm	GC %	Length Product (base-pair)	Self com-plementa-rity	Self 3' comple-mentarity
Primer 1						
F: AATGCCTTCATGGTGTGGTC	20	58.45	50.00	70	4	1
R: AGTTGTGCATCTTGGGGTTCT	21	59.89	47.62		4	0
Primer 2						
F:GAGAACCCCAAGATGCACAA	20	58.09	50.00	76	4	0
R:TCTCCGTCTCCGACAAAAGT	20	58.67	50.00		3	1
Primer 3						
F:CTACAGCATGTCCTACTCGCA	21	59.59	52.38	71	4	1
R:ACTTGACCACCGAACCCA	18	58.35	55.56		2	0

The Primer-BLAST software generates several pairs of candidate primers to amplify the Sox2 gene in the consensus sequence region that has been used as a DNA sequence for the gene encoding the Sox2 gene in Homo Sapiens and *Mus musculus*. The results of the alignment of the

Sox2 gene sequences are the conservative region in the CDS (coding DNA sequence) region. The CDS or ORF (open reading frame) sequence is the part of the gene that codes for amino acids in producing proteins (Seprianto et al., 2020). The candidate primers designed by Primer-BLAST showed diversity related to the size of the PCR product, primer length, Tm, GC percentage, Max Self Complementary, and Max Pair Complementary based on the parameters determined in the initial step. In table 2, the shortest product is primer pair number 4, and the longest product is primer pair number 3, while in table 3, the shortest product is primer pair 3, and the longest product is primer pair 2. Furthermore, each primer pair's melting temperature (Tm) has no difference exceeding five 0C with temperatures ranging from 58.00-60.00 C. Values for self-complementary and self 3' complementary also range from 4.00 to 0.00.

The following criteria were considered when designing the primer pairs: (1) Each primer should be 18–24 bp in length. (2) the annealing Tm should be 60 ± 1 °C. (3) The melting temperature difference of all designed primer pairs does not exceed 5 0C (4) The primers should have limited similarity with other sequences in the genome, especially not in the last four bases at the 3′ end of the primer. (5) The amplicon should be between 70 and 150 bp in length. (6) An ideal GC content of 40–60%., and (7) limited self- and heterodimer formation (Lefever et al., 2013; Udvard et al., 2008; Thornton & Basu, 2011; Anika et al., 2019). Therefore, several things must be considered in designing primers, namely primer length, melting temperature, GC percentage, Max Self Complementary, Max Pair Complementary, and other criteria, namely the low number of self-dimer, hairpin, repeat, and run in secondary structure analysis.

In this study, the primer design was designed with 18-22 bp primer length. In theory, primers are suitable in the 18-24 bp range. Longer primers will take longer to hybridize, longer to extend, and longer to remove, thus producing less amplicon. Primer length ranges from 18-30 basepairs, based on consideration of random combinations found in one genome sequence. PCR primers that are too short will tend to experience mispriming (Seprinato & Wahyuni, 2018), and primers that are too long have the potential for hybridization to occur so that it will inhibit the DNA polymerization process (Thronton & Basu, 2011; Anika et al., 2019; Wahyuni et al., 2020). Melting temperature is very important in determining the double-stranded DNA denaturation process. Primers with melting temperatures that are too high (more than 70 0C) tend to mispriming at low temperatures, and primers with low melting temperatures will not be able to work at high temperatures (15). Another criterion for suitable primers is having a low self 3' complementarity so that there is no attachment between primer pairs and forms a structure called a hairpin (Sasmito et al., 2014).

All primers that have been successfully designed in this study have GC percentages ranging from 50.00 to 57.89% (Table 2) in *Mus musculus* primers and between 47.62 and 55.36 (Table 3) in Homo sapiens primers. The results obtained are still good because they are based on a good GC content range of 40-60%. Tm is one of the factors to consider when choosing a primer. The Tm difference between forward and reverse for a pair of appropriate primers is roughly 5°C. The goal is to keep the amplification process from decreasing. The percentage between bases G and C also needs to be considered because the content of the number of bases G and C can affect the Tm of a primer (Dewi et al., 2018). The gene's high GC content causes problems during primer design, such as mismatches and high annealing temperatures, self-dimer formation, and secondary structure. Using standard PCR procedures, amplification of genes is not always possible. The most significant issue is a hairpin loop, which directly interferes with primer annealing on problematic DNA substrates, resulting in no amplification (Kumar & Kaur, 2014).

Furthermore, the percentage of GC is linked to the binding of DNA strands. In comparison to AT (adenine-thymine), it requires more energy and temperature to break the hydrogen bonds of GC. As a result of the high GC content, breaking the double-threaded chain in primers and molds will be challenging to break the bonds. Furthermore, the primers' low GC content causes the primers to be unable to adhere, resulting in decreased PCR efficiency (Sasmito et al., 2014; Maitriani et al., 2015).

NetPrimer

The oligonucleotide primer sequences obtained from Primer-BLAST were further analyzed to identify the most efficient primer. Analysis to identify self dimer, cross dimer, hairpin, repeat, and run was performed using NetPrimer. Other software options besides using NetPrimer, Oligo Calc, Beacon Designer, Oligo Elevator, and others. All primers analyzed showed varying self-dimer, cross dimer, hairpin, repeat, and run results. In addition, NetPrimer also provides an assessment (rating) related to primer quality. The designed primer rating range for Homo sapiens and *Mus musculus* ranges from 85 to 100. Primer pair 4 for the *Mus musculus* and Primer Pair 3 for Homo sapiens are the highest rated primer pair among primers designed using Primer-BLAST.

Table 4. Results of secondary structure analysis of SOX2 primer candidates for *Mus musculus* using NetPrimer

Sequence (5'->3')	Self Dimer	Cross Dimer	Hairpin	Repeat (# of pairs	Run (# of bases)	Rating
	kcal/mol					
Primer 1						
F:CCCACCTACAGCATGTCCTAC	-5.38	-	-	-	3	90.0
R:GACTTGACCACAGAGCCCA	-		-	-	3	100
Primer 2						
F:ATGATGGAGACGGAGCTGAA	-7.34	-4.52	-	-	-	86.0
R:CGGGCTGTTCTTCTGGTTG	-		-	-	3	100
Primer 3						
F:ACCAGCTCGCAGACCTACA	-6.34	-	-	-	-	88.0
R:CCTCGGACTTGACCACAGA	-		-	-	-	100
Primer 4						
F:CTACAGCATGTCCTACTCGCA	-5.38	-	-	-	-	90.0
R:ACTTGACCACAGAGCCCA	-		-	-	3	100

Table 5. Results of secondary structure analysis of SOX2 primer candidates for *Mus musculus* using NetPrimer

Sequence (5'->3')	Self Dimer	Cross Dimer	Hairpin	Repeat (# of pairs	Run (# of bases)	Rating
	kcal/mol					
Primer 1						
F : AATGCCTTCATGGTGTGGTC	-5.38	-6.57	-	-	-	90.0
R : AGTTGTGCATCTTGGGGTTCT	-7.05		-	-	4	87.0
Primer 2						
F : GAGAACCCCAAGATGCACAA	-8.05	-4.75	-	-	4	85.0
R : TCTCCGTCTCCGACAAAAGT	-		-	-	3	100
Primer 3						
F : CTACAGCATGTCCTACTCGCA	-5.38	-6.19	-	-	-	90.0
R : ACTTGACCACCGAACCCA	-		-	-	3	100

The presence of self-dimer will further lead to a decrease in the efficiency of the PCR process. A repeat is a nucleotide sequence (a dinucleotide) that is repeated (e.g., TCTCTCTCTC). These should be avoided because they promote mispriming. If unavoidable, the maximum number

should be four dinucleotides. Runs are repeated nucleotides (e.g.TAAAAAGC has five basepairs run of Adenine). Runs should also be avoided because they are prone to mispriming. The maximum run should be no more than 3–4 bp—GC Clamp refers to the maximum DG of the five bases from the 5'end of the primers. Having 1 to 2 GC clamps is ideal, as it allows the primer to bind strongly to the template strand, making it more specific but avoiding more than 2 GC clamps (Thornton & Basu, 2011).

The presence of run and repeat in the primer sequence can cause mispriming in the PCR process. In general, the maximum tolerated runs and repeats are four. Another factor to consider is the hairpin. The presence of hairpin interactions cannot be tolerated in the primer design process (Sasmito et al., 2014; Sasmitha et al., 2018). Primer pairs for which the homo-dimer and hetero-dimer strength is ΔG≤ −9 kcal/mol (Ruiz-Villalba et al., 2017). If this dimer bond is too strong, it will interfere with the DNA extension process and low DNA concentration.

Based on the theory that has been described, the NetPrimer results in tables 4 and 5 can be analyzed. The higher the run and repeat values on the primer can lower the primer rating. Primer candidate 2 for *Mus musculus* has self-dimer and cross-dimer even though it has run value and repeat below four, making the primer rating value low compared to other candidates. However, there is a tolerance for ΔG in the hairpin, cross dimer, and self-dimer, which is ΔG≤ −9 kcal/mol. Likewise, the candidate primer number 2 for Homo sapiens has a run value and repeat below four but has a ΔG homodimer and a high enough cross dimer value, so it was not chosen as the best primer.

Therefore, primer number 4 was chosen for *Mus musculus* and number 3 for Homo sapiens. Because the repeat and run values are still below four and produce a high primer rating, if the value of self dimers, cross dimers, and hairpins do not appear, the primer rating will be better.

The primer pair number 4 for the selected *Mus musculus* has a very small difference in Tm of 1.58 0C, GC percentages of 52.38 and 55.56%, and does not have cross dimers or hairpins and repeats. This primer was identified as having a self-dimer in the forward primer, but it was still tolerable because it had a ΔG value of -5.38, so it was still at ΔG ≤ -9 kcal/mol. It has no runs on the forward primer but has three runs on the reverse primer. Furthermore, the analysis results using NetPrimer show that this primer pair has the highest value or rating with a value of 90 on the forward primer and 100 on the reverse primer, where this value is the best rating.

The primer pairs number 3 for the selected Homo sapiens have a very small difference in Tm of 1.24 0C, GC percentages of 52.38 and 55.56%, and do not have hairpins and repeats. This primer was identified as having self-dimer in the forward primer but still tolerable because it had a ΔG value of -5.38 and a cross dimer of ΔG -6.19, so it was still at ΔG ≤ -9 kcal/mol. It has no runs on the forward primer but has three runs on the reverse primer. Furthermore, the analysis results using NetPrimer show that this primer pair has the highest value or rating with a value of 90 on the forward primer and 100 on the reverse primer, where this value is the best rating.

PCR in Silico

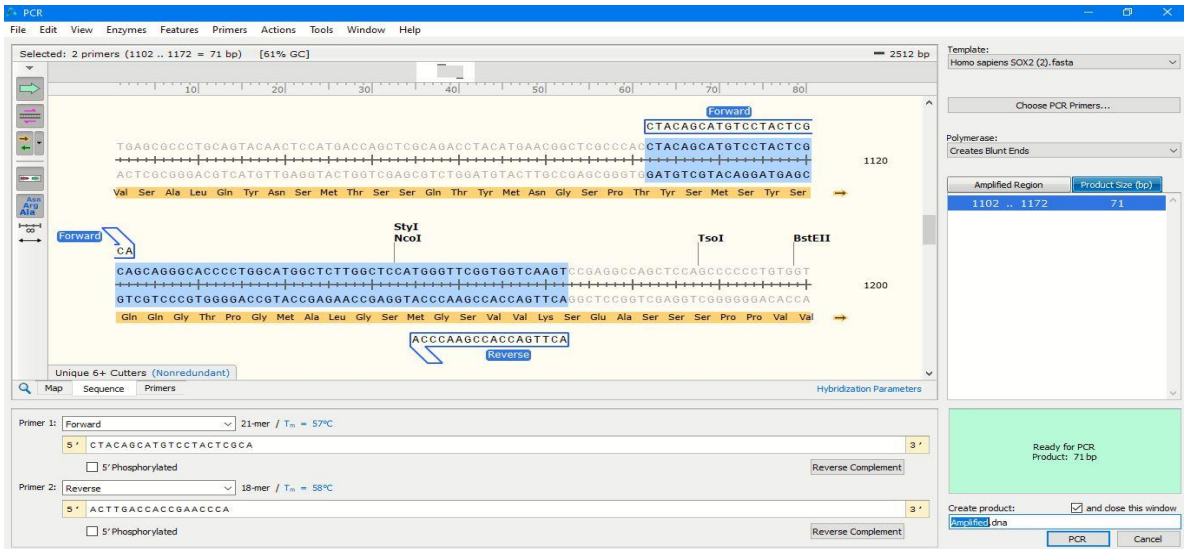

Figure 2. In Silico PCR for *Mus musculus* Primer

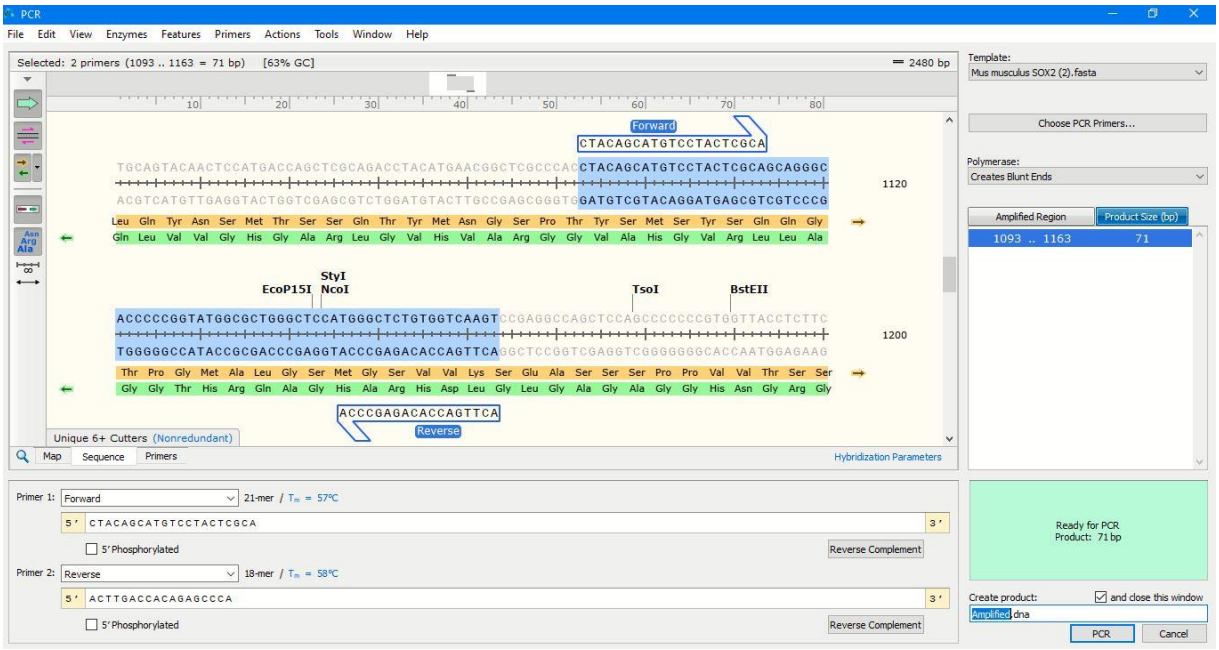

Figure 3. In Silico PCR for *Mus musculus* Primer

The primer pair for Homo sapiens obtained 71 basepair PCR products. This primer amplifies from the 1,093 to 1163 base. Then the primer pair for *Mus musculus* obtained 71 basepair PCR products. This primer amplifies from the 1.102 to the 1.172 bases

Phylogenetic tree

Analyze the phylogenetic tree from several sequences from the previous blast analysis and select sequences that the curator has curated. Based on figure 4. *Mus musculus*'s Sox2 gene is more closely related to Rattus Norvegicus Sox2gene and more distantly related to Ovis Aries Sox2 gene.

Likewise, with Figure 5. Homo sapiens Sox2 gene is more distantly related to *Macaca mulatta* Sox2 gene, and more closely related to Ovis Aries.

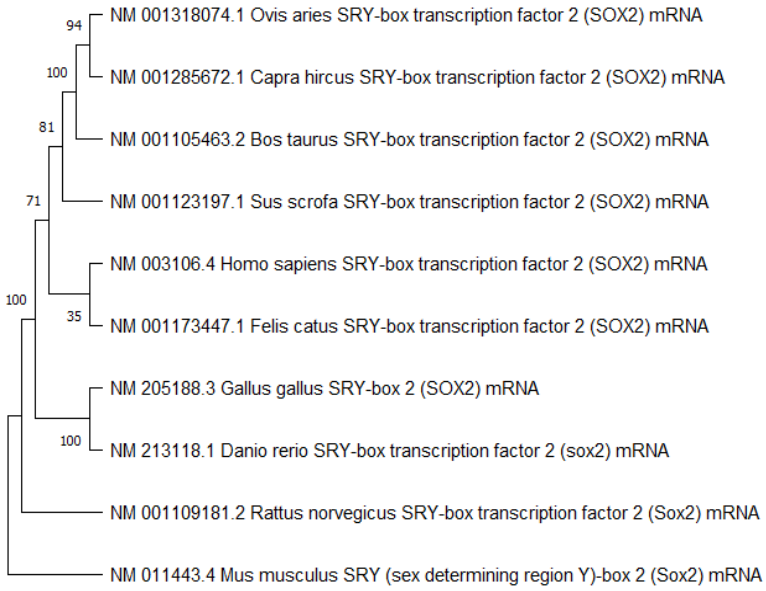

Figure 4. Phylogenetic tree of *Mus musculus* SOX2 gene relationship to other species

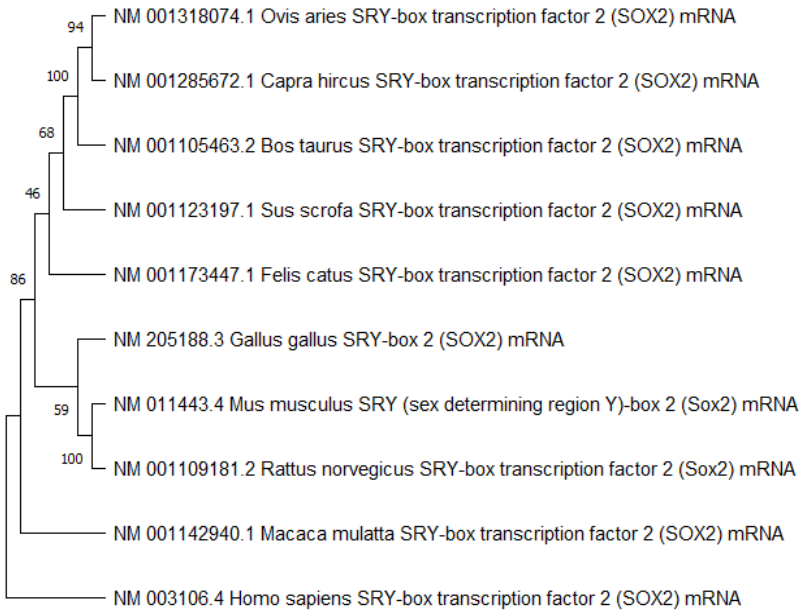

Figure 5. Phylogenetic tree of Homo sapiens SOX2 gene relationship to other species

Conclusion

Specific primer pairs for amplification of the Sox2 gene have been successfully designed. The results showed that forward primer (5'- CTACAGCATGTCCTACTCGCA -3') and reverse primer (5'-

ACTTGACCACAGAGCCCA -3') were selected primers for *M. musculus* with a product size of 71 bp. Also, forward primer (5'-CTACAGCATGTCCTACTCGCA-3') and reverse primer (5'-ACTTGACCACCGAACCCA-3') for H. sapiens with a product size of 71 bp. This primer has succeeded in meeting the main requirements of a good primer in terms of length, melting temperature, and GC percentage to check on NetPrimer. However, a wet laboratory needs to be carried out for primer validation to optimize pluripotency detection.

Acknowledgment

This research is supported by a Grant for Basic Research from the Ministry of the Education Republic of Indonesia.

References

Amand, M. M. S., Hanover, J. A., & Shiloach, J. (2016). A comparison of strategies for immortalizing mouse embryonic fibroblasts. Journal of Biological Methods, 3(2), e41. https://doi.org/10.14440/jbm.2016.110

Anika, M., Putri, D. H., & Wahyuni. (2019). Primer design for identification of beta-carotene encoding genes in cassava. *Bio Sains, 4*(1), 39–47. http://dx.doi.org/10.24036/5320RF00

Carson, S., Miller, H. B., Witherow, D., & Srougi, M. (2012). *Analysis of gst::egfp mRNA Levels by RT-qPCR: Part I*. In: Molecular Biology Techniques.

Chagastelles, P. C., & Nardi, N. B. (2011). Biology of stem cells: an overview. Kidney international supplements, 1(3), 63-67. doi: 10.1038/kisup.2011.15.

Dewi, R. W., Dewi, R. V., Yowanani S. C., Eka N. S., & Yustiantara, Y. S. (2018). Desain primer untuk amplifikasi regio promoter gen inhA Isolat P016 Multidrug Resistance Mycobacterium tuberculosis Dengan Metode Polymerase Chain Reaction. *Jurnal Farmasi Udayana, 7*(1), 34–39. https://doi.org/10.24843/JFU.2018.v07.i01.p01

Fernandez, T. D. S., de Souza Fernandez, C., & Mencalha, A. L. (2013). Human-induced pluripotent stem cells from basic research to potential clinical applications in cancer. *BioMed Research International*, 2013. https://doi.org/10.1155/2013/430290

Karachaliou, N., Rosell, R., & Viteri, S. (2013). The role of SOX2 in small cell lung cancer, lung adenocarcinoma, and squamous cell carcinoma of the lung. *Translational lung cancer research, 2*(3), 172. 10.3978/j.issn.2218-6751.2013.01.01

Kumar, A., & Kaur, J. (2014). Primer-based approach for PCR amplification of high GC content gene: Mycobacterium gene as a model. Molecular biology international, 2014. https://doi.org/10.1155/2014/937308

Lefever, S., Pattyn, F., Hellemans, J., & Vandesompele, J. (2013). Single-nucleotide polymorphisms and other mismatches reduce performance of quantitative PCR assays. *Clinical chemistry, 59*(10), 1470-1480. doi: 10.1373/clinchem.2013.203653.

Liu, A., Yu, X., & Liu, S. (2013). Pluripotency transcription factors and cancer stem cells: small genes make a big difference. *Chinese journal of cancer, 32*(9), 483. doi: 10.5732/cjc.012.10282.

Maitriani, L. K. B., Wirajana, I. N., & Yowani, S. C. (2015). Desain primer untuk amplifikasi fragmen gen inhA isolat 134 multidrug resistance tuberculosis (MDR-TB) dengan metode polymerase chain reaction. *Cakra Kimia (Indonesian E-Journal of Applied Chemistry), 3*(2), 89–96.

National Library of Medicine. (2021). *SOX2 gene*. Retrived from https://medlineplus.gov/genetics/gene/sox2/ on November 21st, 2021

Praja, R. K., & Rosalina, R. (2021). Perancangan primer gen lktB pada Fusobacterium necrophorum untuk analisis PCR. *Jurnal Sains dan Teknologi Peternakan, 2*(2), 47-55.

Prochazkova, M., Chavez, M. G., Prochazka, J., Felfy, H., Mushegyan, V., & Klein, O. D. (2015). *Embryonic versus adult stem cells. In Stem cell biology and tissue engineering in dental sciences,* 249-262. Academic Press.

Ruiz-Villalba, A., van Pelt-Verkuil, E., Gunst, Q. D., Ruijter, J. M., & van den Hoff, M. J. (2017). Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). *Biomolecular detection and quantification, 14*, 7-18. doi: 10.1016/j.bdq.2017.10.001

Saraswati, H., Seprianto, S., & Wahyuni, F. D. (2019). Desain primer secara in silico untuk amplifikasi gen cryiii dari *Bacillus thuringiensis* Isolat Lokal. *Indonesian Journal of Biotechnology and Biodiversity, 3*(1), 33-38.

Sasmitha, L. V., Yustiantara, P. S., Yowani, S. C., MDR-TB, K. S., & Jimbaran, B. (2018). Desain DNA primer secara in silico sebagai pendeteksi mutasi gen gyrA Mycrobacterium tuberculosis untuk metode polymerase chain reaction. *Cakra Kimia (Indonesian E-Journal of Applied Chemistry), 8*(1), 63-69

Sasmito, D. E. K., Kurniawan, R., & Muhimmah, I. (2014). Karakteristik primer pada Polymerase Chain Reaction (PCR) untuk sekuensing DNA: mini review. *In Seminar Nasional Informatika Medis (SNIMed), 5,* 93-102.

Seprianto, F. D. W., Saraswati, H., & Praptiwi, R. A. (2020). In Silico Analysis for Detection of CryII Gene from Local Isolates of *Bacillus thuringiensis*. *In Proceedings of the 1st International Conference on Health (ICOH 2019),* 127-132

Seprianto, S., & Wahyuni, F. D. (2018). Analisis Bioinformatika Gen Potensial Penyandi HalichondrinB Dari Spons Laut Sebagai Kandidat Anti Kanker. *Indonesian Journal of Biotechnology and Biodiversity, 2*(2), 57-66.

Thornton, B., & Basu, C. (2011). Real‐time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education, 39*(2), 145-154. https://doi.org/10.1002/bmb.20461

Udvardi, M. K., Czechowski, T., & Scheible, W. R. (2008). Eleven golden rules of quantitative RT-PCR. *The Plant Cell, 20*(7), 1736-1737.

Wahyuni, F. D., Saraswati, H., & Dewi, K. S. (2020). In-Silico Analysis for cryI Gene Amplification from *Bacillus thuringiensis. BIOEDUKASI: Jurnal Biologi dan Pembelajarannya,* 8-14. https://doi.org/10.1105/tpc.108.061143

Yusuf, B., Gopurappilly, R., Dadheech, N., Gupta, S., Bhonde, R., & Pal, R. (2013). Embryonic fibroblasts represent a connecting link between mesenchymal and embryonic stem cells. *Development, growth & differentiation, 55*(3), 330-340. doi: 10.1111/dgd.12043