Indonesian Journal of Medical Laboratory Science and Technology

Open Access p-ISSN 2684-6748 | e-ISSN 2656-9825 IJMLST

Collagen-VI Specific Primer Design Identification in Rats (*Rattus norvegicus*) Pancreas

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Received: 27 November, 2021
Revised: 15 January, 2022
Accepted: 22 February, 2022
Published: April 28, 2022

DOI: 10.33086/ijmlst.v4i1.2515

Abstract

Type 2 diabetes mellitus, the most common diabetes type characterized by hyperglycemia, is caused by abnormal secretion and activity of pancreatic insulin enzymes. The extracellular matrix (ECM) plays a vital role in keeping β pancreatic cells intact and undissociated. The ECM in the pancreas can play a role in influencing insulin function and production. The most abundant ECM in the pancreas is collagen type VI. Collagen type VI has an essential role in the survival of pancreatic islet cells, including pancreatic β cells. Nowadays, Polymerase Chain Reaction (PCR) technology is widely utilized for molecular biology analysis. One of the most critical factors for successful Polymerase Chain Reaction (PC)R is designing the correct specific PCR primers. The objective of this study was to design a specific primer for collagen VI in the pancreas of *Rattus norvegicus*. The primer was designed and analyzed using MEGA.11, primer three-plus, and primer-BLAST. Five primer pairs were analyzed based on the characteristics of primer length, product amplicon length, Tm value, GC percentage, and secondary structure. Primer pair 3 (F:5'-TGTTTGGCTTTGTCGCGGGC-3' and R:5'-TTGTTGCTGCCGACACTGGC-3'); Col6a2 (F:5'-TGTGGTCAACAGGCTGGGCG-3' and R:5'-TCTGGCGCCGGCTCTCTTTG-3') were considered as the best primer for the Collagen VI expression detection from the pancreas of *Rattus norvegicus*, which produce amplicon about 250pb and 245pb, respectively.

Keywords

Diabetes Mellitus, Primer, Collagen VI, *Rattus norvegicus*.

INTRODUCTION

Type 2 diabetes mellitus (T2DM), a global major health problem, ranks first as a non-communicable disease that causes the highest death in Indonesia (1). DMT2 is closely related to the insulin hormone. This hormone is produced by pancreatic β cells, whose primary function for maintaining glucose homeostasis. Deficiency of insulin secretion by pancreatic β cells is one of the characteristics of the emergence of T2DM, which results in high blood glucose levels (2). The glucose stimulates cellular signaling pathways to synthesize and translocate insulin granules. This series of processes is supported by the pancreatic cell's ability to produce an oscillatory effect that triggers insulin secretion. These oscillatory effects play a role in synchronizing secretory activity between pancreatic cells (3).

The molecular and cellular mechanisms can be studied further by using an *in vitro* model using 3D spheroidal cultures of the iGL cell line. iGL is a cell derived from a subculture of pancreatic β cells of rats capable of expressing insulin-GLase in response to high environmental glucose levels (4). Research by Suzuki *et al*. (4) has proved that iGL cell (insulin-GLase) spheroids exhibit an oscillatory effect of insulin secretion in response to high environmental glucose levels. Furthermore, high glucose stimulation in the 3D culture of iGL cells showed a similar oscillatory effect of insulin secretion as the pancreatic islet of Langerhans *in vivo*. The 3D culture model was considered more representative of natural conditions *in vivo* (5).

The synchronization between cells in the pancreatic β islet will be weak if the cells dissociate. The extracellular matrix (ECM) plays a vital role in keeping pancreatic β cells intact and undissociated. ECM in the pancreas can play a role in protecting cells, influencing insulin function and production, and influencing the susceptibility of pancreatic islets toward cytokines. ECM can be found in large quantities in the pancreas, including laminin, collagen type IV, and collagen type VI (6). The component of type VI collagen was higher than collagen types 1 and 4 in adult human pancreas research (7). Another research stated that the administration of type VI collagen in the pancreatic islets treatment could increase the viability of the pancreatic islet cells when treated *in vitro* (8). It indicates that type VI collagen has an essential role in the survival of pancreatic islet cells, including pancreatic cells. However, how type VI collagen functions on pancreatic islet function and insulin expression is still unknown.

The role of type VI collagen was explored by looking at the expression pattern of type VI collagen at the gene and protein levels. Expression of type VI collagen at the gene level can be detected by the Polymerase Chain Reaction (PCR) method. DNA primer

is DNA sequences complementary to the sequence to be amplified. Therefore, the primer used in the PCR process serves as a barrier to the target DNA fragment to be amplified. A pair of primers consists of a forward primer and a reverse primer (9).

The success of DNA amplification depends on the accuracy of the primer. The primer parameters include melting temperature (Tm), percentage of G and C (% GC), 3′dimer, stability, repeats, and hairpins. The primer designed must meet the criteria used in the PCR process and produce products according to the desired regional range (10). This research was aimed to design the primer with good specificity to detect the expression of the Cx36 gene (type VI collagen gene) in the pancreas of *Rattus norvegicus* by PCR technique.

MATERIALS AND METHODS

This research used bioinformatics software online instruments for designing primer, namely Primer3Plus (website: https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), Integrated DNA Technologies (IDT), BLAST (website: https://blast.ncbi.nlm.nih.gov/Blast.cgi) at the National Center for Biotechnology Information (NCBI), and Molecular Evolutionary Genetic Analysis (MEGA). The material used in this research was DNA sequence data of collagen VI *R. norvegicus* downloaded in FASTA format from NCBI database. The method consists of searching gene sequences on the NCBI site in the gene sub-search, selecting the "RefSeq" menu, and selecting "*Rattus norvegicus*" in the organism option (Figure 1).

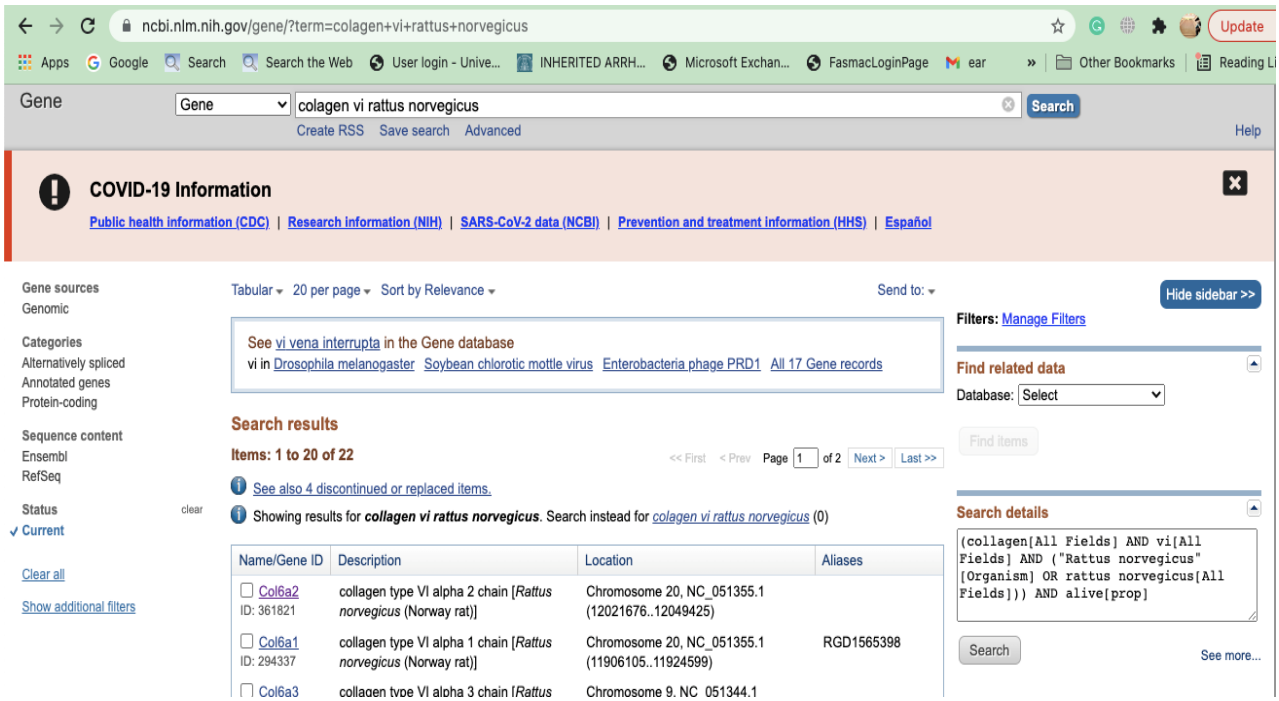

Figure 1. Collagen VI gene search result in NCBI

The gene sequences obtained may have several isoforms. MEGA.11 application was used to carry out alignment to obtain conserved sequences from these isoforms. Then the conserved sequences were used for primer design with Primer3Plus (Figure 2).

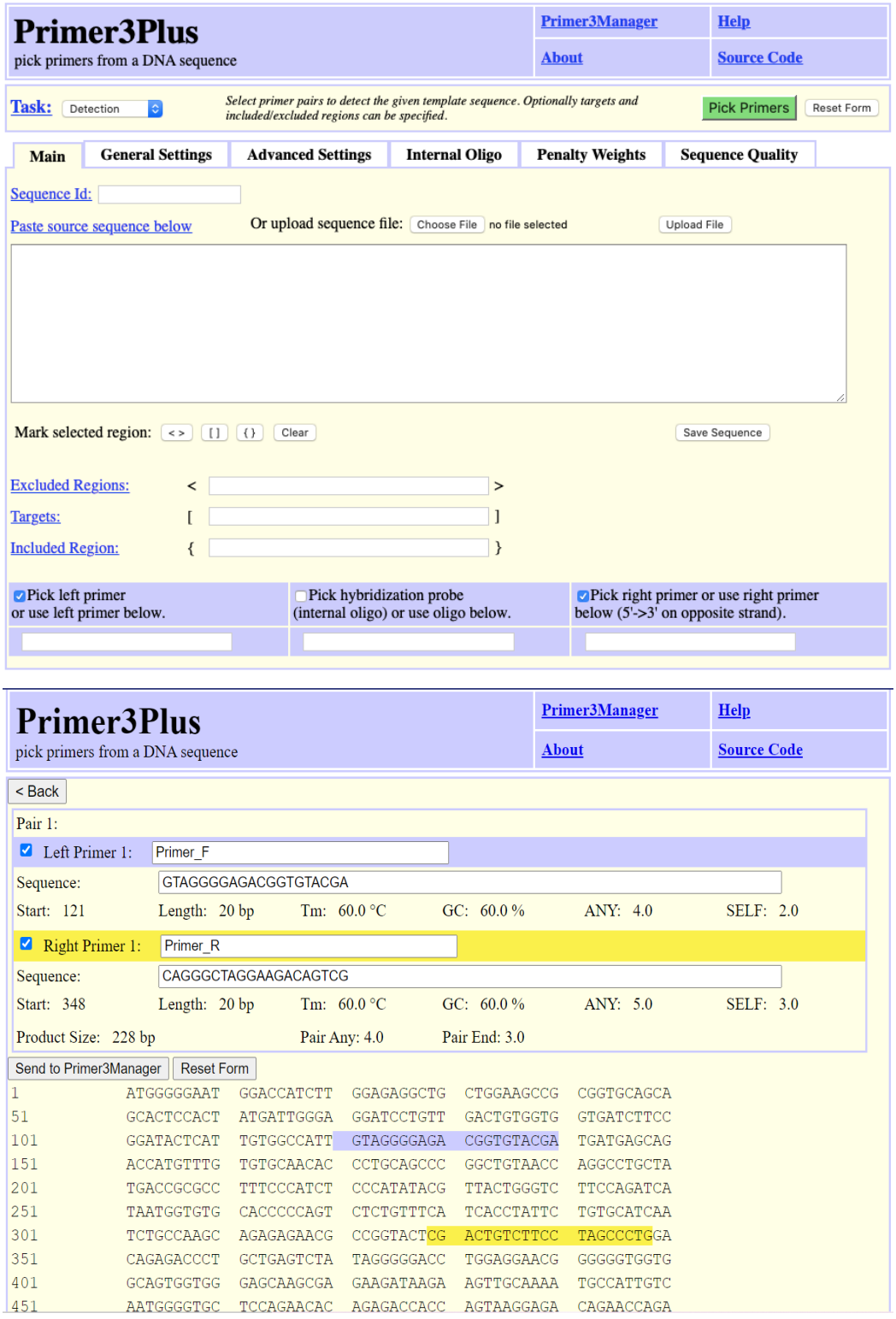

Figure 2. Primer2Plus front page display

Figure 2 shows Primer2Plus setting. Some settings on the "General and Advance Settings" menu for the desired primer criteria. For example, it was related to the primer and amplicon size, Tm, GC composition, etc. Then select "Pick Primers" to get the primer selection. The results displayed were several choices of primer pairs (forward and reverse) accompanied by data on primer length, product or amplicon length, Tm, %GC (11). In order to designing primers for PCR, two types of secondary structures should be analyzed, namely dimers and hairpins. The primers obtained were further analyzed with IDT to determine how big the primers were to form dimer and hairpin primer. Furthermore, the primers obtained were further analyzed with Primer-BLAST to determine the specificity of the primer to the target gene.

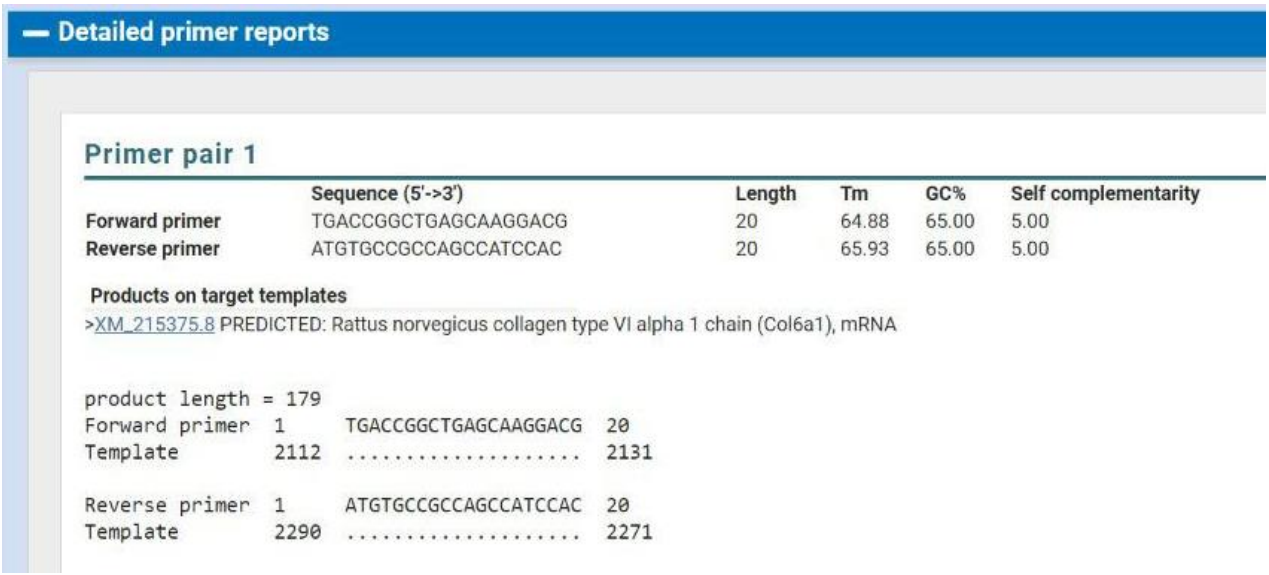

Figure 3. Display of Primer-BLAST. results

RESULTS

The Collagen VI gene (col6a1: ID 294337 and col6a2: ID 361821) in *Rattus norvegicus* is located on chromosome number 20 on NC_051355.1. This gene, which has two isoforms, is aligned using the MEGA application. The primer design result was carried out by using Primer3Plus and further analyzed with IDT and Primer-BLAST, which are summarised in the following Tabel 1.

DISCUSSION

The PCR method has been widely used in the biomolecular and medical world. Good primer design is an important factor for a successful PCR process. Several essential characteristics that need to be considered in designing PCR were Primer length, GC composition, melting temperature (Tm), dimer primer, and hairpin primer (12). The recommended primer length for optimum PCR application is 18-30 base length (bp). If

the primer is too short, it will cause non-specific amplification, while too long primer tends to form secondary structures such as hairpin loops (13). All primers designed for Collagen VI had a length of 20 bp, which was categorized as a good primer.

The GC composition in the primer was also essential to note. The GC proportion was the percentage that described the ratio of G and C nucleotides presented in the primer sequence. The primer GC proportion was 30-80%. However, the recommended optimal GC proportion was at a value of 40-60% (12).

The primer design results obtained had a value of 60-65%. Although this design value was slightly higher, the higher GC composition was considered less adverse on the PCR results (10). The nucleotide composition was also closely related to the melting temperature (Tm) value.

The reaction specificity of the PCR was highly dependent on the primer Tm value. The recommended optimal Tm value was in the range of 50-60°C. The two primers should have the same Tm value or only had a difference of about 2-3°C. Different Tm can cause a decrease in the primer annealing efficiency (13).

All primer pairs obtained from the Collagen VI primer design had a Tm value difference of not more than 3°C between the forward and reversed primer. In addition, almost all primer pairs had Tm values within the recommended range of 58.8-59.6°C. Only col6a1 1 (forward, reverse), 2 (reverse), 3 (reverse), and 4 (forward) primer pairs have a Tm value slightly higher than the recommended value of 60.2°C, and only col6a2 primer pairs 2 (reverse), which had a Tm value slightly higher than the recommended value of 60.0°C.

An important factor to be considered in designing a good primer was the possibility of primer forming a secondary structure. The secondary structure can be formed from one primer sequence itself that forms a hairpin loop structure or between pairs of primer that form dimer primer or heterodimer (12,13). This secondary structure should be avoided because it can reduce the specificity of the primer.

All primers could form self-dimer or heterodimer structures based on the primer design results. The primer pair with the lowest selfdimer, heterodimer, and hairpin numbers was the number four primer pair with selfdimer and hairpin values 1-2 and heterodimer 15.

The five pairs of primer obtained were further analyzed for specificity using Primer-BLAST (11). All tested primer pairs showed specific results with the Col6a1 gene in *Rattus norvegicus* (>XM_215375.8 *Rattus norvegicus* collagen type VI alpha 1 chain (Col6a1), mRNA) with a product amplicon length of 247pb.

Table 1. Collagen VI primer design results

Gene Name	Gene ID	Primer sequences	primary length (bp)	product amplicon length (bp)	Tm (°C)	GC (%)	Self-dimer	Hetero dimer	Hairpin	BLAST result
Col6a1	>lcl|NC_051355.1_cds_XP_215375.5_1	F-TTGGCTTTGTCGCGGGCTCC R-TTGTGGCTGCCGACACTGGC	247	F-20 R-20	F-60,2 R-60,2	F-65 R-65	F-10 R-12	13	F-4 R-3	>XM_215375.8 PREDICTED: *Rattus norvegicus* collagen type VI alpha 1 chain (Col6a1), mRNA. 247bp.
		F-TGACCGGCTGAGCAAGGACG R-ATGTGCCGCCAGCCATCCAC	179	F-20 R-20	F-59,1 R-60,0	F-65 R-65	F-8 R-12	17	F-4 R-1	>XM_215375.8 PREDICTED: *Rattus norvegicus* collagen type VI alpha 1 chain (Col6a1), mRNA. 179bp.
		F- TGTTTGGCTTTGTCGCGGGC R- TTGTGGCTGCCGACACTGGC	250	F-20 R-20	F-59,0 R-60,2	F-65 R-65	F-8 R-9	13	F-5 R-3	>XM_215375.8 PREDICTED: *Rattus norvegicus* collagen type VI alpha 1 chain (Col6a1), mRNA. 250bp.
		F- TGGCTGGCGGCACATTCACC R- ACGTTGAGCTGGTCGGAGCC	223	F-20 R-20	F-60,2 R-59,1	F-60 R-65	F-13 R-10	15	F-1 R-2	>XM_215375.8 PREDICTED: *Rattus norvegicus* collagen type VI alpha 1 chain (Col6a1), mRNA. 223bp.
		F- TGACCGGCTGAGCAAGGACG R- TCCGGTGAATGTGCCGCCAG	187	F-20 R-20	F-59,1 R-59,7	F-65 R-65	F-8 R-10	16	F-4 R-2	>XM_215375.8 PREDICTED: *Rattus norvegicus* collagen type VI alpha 1 chain (Col6a1), mRNA. 187bp.
Col6a2	>lcl|NC_051355.1_cds_NP_001094211.1_1	F-TGAGCTGGCGCTATGGTGGC R-ACGTGCTGCCGGATCTGCTG	172	F-20 R-20	F-59,5 R-59,8	F-65 R-65	F-12 R-10	15	F-2 R-4	>XM_006256300.4 PREDICTED: *Rattus norvegicus* collagen type VI alpha 2 chain (Col6a2), transcript variant X1, mRNA. 172bp.

Gene Name	Gene ID	Primer sequences	primary length (bp)	product amplicon length (bp)	Tm (°C)	GC (%)	Self-dimer	Hetero dimer	Hairpin	BLAST result
	>lcl|NC_051355.1_cds_XP_006256362.1_2	F-TTCCGCAGGGGCACCTTCAC R-ACGGCAAAGAGCCGGATGCC	188	F-20 R-20	F-59,3 R-60,0	F-65 R-65	F-9 R-9	19	F-4 R-1	>XM_006256300.4 PREDICTED: *Rattus norvegicus* collagen type VI alpha 2 chain (Col6a2), transcript variant X1, mRNA. 188bp.
		F- TGTGGTCAACAGGCTGGGCG R- TCTGGCGCCGGCTCTCTTTG	245	F-20 R-20	F-59,6 R-59,4	F-65 R-65	F-11 R-7	13	F-5 R-1	>XM_006256300.4 PREDICTED: *Rattus norvegicus* collagen type VI alpha 2 chain (Col6a2), transcript variant X1, mRNA. 245bp.
		F- TTCCGCAGGGGCACCTTCAC R- TTGGGGGCCACGGCAAAGAG	197	F-20 R-20	F-59,3 R-59,5	F-65 R-65	F-9 R-9	16	F-4 R-1	>XM_006256300.4 PREDICTED: *Rattus norvegicus* collagen type VI alpha 2 chain (Col6a2), transcript variant X1, mRNA. 197bp.
		F- ATGCCCAGCAGCAGGAAGCC R- TAGGCCACCATAGCGCCAGC	248	F-20 R-20	F-59,7 R-58,9	F-65 R-65	F-14 R-15	18	F-5 R-1	>XM_006256300.4 PREDICTED: *Rattus norvegicus* collagen type VI alpha 2 chain (Col6a2), transcript variant X1, mRNA. 248bp.

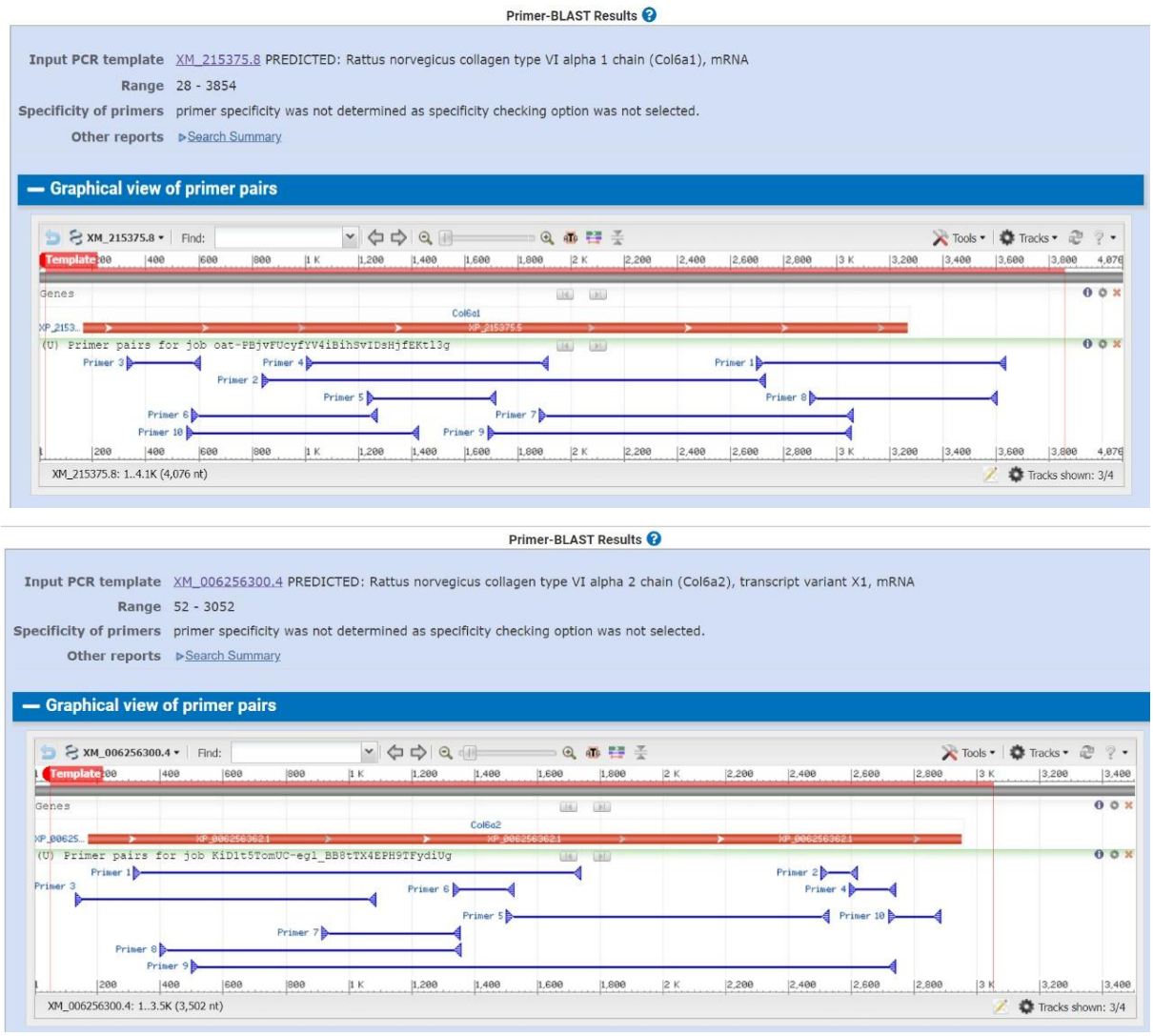

Figure 4. Position of primers in the gene col6a2

Thus, primer pair 3 was considered the closest to a good primer category for detecting Collagen VI expression from the pancreas of *Rattus norvegicus* at the gene level by PCR method with amplicon length. 250pb product. As for the Col6a2 gene in *Rattus norvegicus* (>XM_006256300.4 *Rattus norvegicus* collagen type VI alpha 2 chain (Col6a2), mRNA) with a product amplicon length of 245pb.

Besides all the considerations above, the selection of designed primer outcome must be considered after performing experimental tests in the laboratory.

Researchers can redesign the primer if the PCR results are not good by changing the primary parameter criteria settings. For example, change the GC proportion value, shift the Tm number, increase the resulting amplicon length to 150-259pb [12], or use other primary design applications available online for free.

CONCLUSIONS

The applications MEGA.11, Primer3Plus, and Primer-BLAST can facilitate designing the specific Collagen VI primers. Col6a1 gene primer pairs 3 (F:5'-TGTTTGGCTTTGTCGCGGGC-3' and R:5'-TTGTTGCTGCCGACACTGGC-3'); Col6a2 (F:5'-TGTGGTCAACAGGCTGGGCG-3' and R:5'-TCTGGCGCCGGCTCTCTTTG-3') are the best primer in this study for Collagen VI expression detection in *Rattus norvegicus* pancreas and produce amplicon around 250pb and 245pb, respectively. Future research is required to analyze the specificity of the primer design.

AUTHOR CONTRIBUTIONS

Endin Nokik Stujanna: Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, Drafting the article or revising it critically for important intellectual content. Sri Suciati Ningsih: Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. Rizkyana Avissa: Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data. Novi Putri Ayu: Substantial contributions to acquisition of data. Zahra Nurussofa: Substantial contributions to conception and design
Dewi Jantika Djuarna: Substantial contributions to conception and design
Rini Latifah: Substantial contributions to conception and design. Wawang Setiawan Sukarya: Final approval of the version to be published

ACKNOWLEDGMENTS

The authors would like to thank all supporting staff at the Faculty of Medicine and thank Lemlit UHAMKA.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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