Molecular sexing in *Bos taurus* using quantitative polymerase chain reaction (qPCR) method

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**Abstract.** Cattle are animals that are widely found all over the world which is the domestication of bulls. In Indonesia, domesticated cattle are used as livestock, could be meat cattle or dairy cattle. The sex of the cattle is very influential in terms of selling, especially the type of bull. Calf bull is a product of the process of artificial insemination with spermatozoa resulting in sexing. Mammals have X and Y chromosomes to distinguish their sex. Each chromosome has a special gene such as the SRY gene on the Y chromosome and the PLP gene on the X chromosome. Molecular methods are effective methods of determining the chromosomes carried by spermatozoa. Both special genes are found in the home box (GADPH) of mammals. Primer is made from the mRNA SRY (sex-determining region Y) sequence from *Bos taurus* with a size above 200bp (SRY B) and below 200bp (SRY A) so that optimal results are obtained between the two. The type of sample that has been used in this research was sexing semen from limousin bulls that are treated from 4 pairs of primers each. The results of the qPCR from both primers are viewed from the melt-peak graph and comparison analysis. The differences between SRY A and SRY-B primer indicates that SRY-A produces a more stable melt-peak graph than SRY B. This indicates that the SRY A primer is more stable when used for molecular verification processes using qPCR.

**Keywords:** Bos taurus, Sexing spermatozoa, SRY, qPCR, Primer

1. **Introduction**

Cattle are animals that are widely found all over the world which is the domestication of bulls. In Indonesia, domesticated cattle are used as livestock, could be meat cattle or dairy cattle. The sex of the cattle is very influential in terms of selling, especially the type of bull. Calf bull is a product of the process of artificial insemination with spermatozoa resulting in sexing [1]. Real-time PCR or quantitative PCR (qPCR) is one of the most sensitive methods for detecting and measuring mRNA quantities [2]. The working principle of qPCR is to detect and quantify fluorescent reporters. Fluorescent signal will increase as PCR products increase (amplicon) in reaction. A significant increase in the number of amplicons at an exponential phase is related to
the number of initiations of the target gene. Higher level target gene expression then the detection of fluorescence emissions is faster [3]. The purpose of this study was to see differences in stability and Ct values from primers over 200bp and below 200bp used for molecular rapid detection of sperm sexing results by using qPCR methods.

2. Material and Methods
2.1 Design primers
Bos Taurus sequence sex determining region Y (SRY), mRNA with accession number Nm_01014385.1 from GenBank (http://www.ncbi.nlm.nih.gov/) [4]. Primer is designed using target mRNA and analyzed with primer3plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) [5], where made 2 pairs of primers with sizes above 200 base pairs and 2 primary pairs below 200 base pairs, and checked the primary properties designed including melting temperature (Tm) and GC content using sequence manipulation suite that can be accessed (http://www.bioinformatics.org/sms2/pcr_primer_stats.html) dimer potential and hairpin loop formation is calculated using oligocalc which can be accessed (http://www.basic.northwestern.edu/biotools/oligocalc.html), then simulated by using Primer Blast which can be accessed (http://www blasting.ncbi.nlm.nih.gov/Blast.cgi). Simulations are needed to find out if the designed primer can attach specifically to the target gene, the SRY gene.

2.2 Quantitative polymerase chain reaction (qPCR)
Quantification of gene expression was obtained from Applied Biosystem StepOne Plus™. The PowerUp™ SYBR® GreenMaster Mix (2X) reagent was used according to the manufacturer's recommendations. Each gene target in the quantification reaction was performed individually using its own primer set. For concurrent operations, a constant annealing temperature was used for the amplification reaction and setting the melting curve, although the theoretical melting temperature for each primer set was different. The settings are as follows: 1 step (1 cycle) for 20 s at 95 °C, followed by 3 s at 95 °C; Steps 2, 3, and 4 (40 cycles) end the melting curve at 57 °C for 30 s, then at 65 °C for 5 s and at 95 °C for 5 s.

2.3 Sample preparation
This research was conducted as part of the Biotechnology Program at the Graduate School of Biochemistry, Gazamad University. In the study sample, the sex of the sperm, that is, the sex with the BSA was used. DNA was isolated according to the DNA extraction kit protocol (Geneaid, Taiwan). The result of isolating the DNA used as a DNA template in the amplification process by the qPCR method.

3. Results and Discussion
3.1 Primer design
The resulting list of qPCR primers was created based on the Y Bos taurus mRNA gender region using the Primer3plus program. This gives the results shown in Table 1.

3.2 Threshold cycle (Ct) data
After designing the primers, we established them on PBMCs. Three impartial runs with replica technical replicates, Ct suggest values, delta Ct suggest and delta Ct widespread error (SE) have been calculated as proven in Table 2. The primer units have been evaluated primarily based totally at the back Ct suggest values generated from the Bio-Rad CFX Maestro softwares as proven in Table 2. Primer SRY-A have less Ct mean values compare to SRY-B and SRY control primer.
Table 1. A list of qPCR forward and reverse primers in the form 5’ to 3’ created by the Primer3plus program. Includes corresponding primer length, melting temperature, and GC content.

| Primer name     | Sequence (5’ to 3’)                      | Length (bp) | Tm (°C) | GC content (%) |
|-----------------|------------------------------------------|-------------|----------|----------------|
| SRY 1 A F       | CAGTCCGTGATCATAACCAA                     | 145         | 55.2     | 45.00          |
| R               | CACAAGAAGAGTCCAGGCTCT                    |             | 57.4     | 50.00          |
| SRY 1 B F       | AGCCATACACCGAGACAAT                      | 298         | 56.9     | 45.00          |
| R               | CTCCTTTGAGCAAGTGAA                      |             | 55.6     | 45.00          |
| SRY 2 A F       | GCCAAGACCATACATCTCA                      | 190         | 57.5     | 50.00          |
| R               | CACAAGAAGGTCAGGCTCT                      |             | 57.4     | 50.00          |
| SRY 2 B F       | GAAGAGCAAGTGCTCTCA                      | 235         | 56.9     | 50.00          |
| R               | GAGTATGCTCTTGGCAAGCGA                    |             | 57.5     | 50.00          |
| SRY 3 B F       | AATATCGACCTCGTCGGAGA                     | 216         | 57.7     | 50.00          |
| R               | AGCTGCTCTGATGCTCTTT                     |             | 59.9     | 50.00          |
| SRY control F   | AAGGGGAGAACAGTGGAGAGAG                  | 318         | 60.8     | 52.17          |
| R               | ATCGGGTTGCATAGTATTTAGAG                 |             | 56.3     | 40.91          |

Table 2. CT values for various primer genes with CT mean, delta CT mean, and CT-SE values are taken from independent double runs, and each run contains overlapping technical samples.

| Gene target      | Primer     | Ct mean | ΔCt mean | ΔCt SE |
|------------------|------------|---------|----------|--------|
| Sex determining  | SRY 1 A    | 21.48   | 21.48    | 0.00   |
| region Y         | SRY 1 B    | 21.67   | 21.67    | 0.00   |
|                  | SRY 2 A    | 21.12   | 21.12    | 0.00   |
|                  | SRY 2 B    | 22.11   | 22.11    | 0.00   |
|                  | SRY 3 B    | 26.20   | 26.20    | 0.00   |
|                  | SRY C      | 22.73   | 22.73    | 0.00   |

Real-time PCR technology is considered an effective, accurate and dependable approach for quantifying sperm with the X and Y chromosomes in bovine semen [6]. In present study, validating Bos Taurus semen sexed by BSA was done using SYBR® Green-based Real-Time quantitative PCR (qPCR). The results of the molecular sexing process using qPCR can be seen in Fig.1, Fig.2, and Fig.3 which each show graphs of amplification cycles, melting peaks obtained, and also melting curves. Results in Figures 1 and Figure 2. Showing the graph of the primary SRY 1-A is more stable and has the same peak, while in the primary result SRY 1-B shows a different result both the melting peak of the 2 repeats obtained. It is also found in primary SRY 2-B and SRY 3-B which have a size above 200bp. With a primary size that is above 200bp causes the attachment process to be less specific compared to the primary with a smaller size of 100bp-200bp[7]. According to Nonspecific amplification is proven to depend on primary concentration, template and non-DNA template [8].
Figure 1. SRY 1 A show cycle amplification, melt-peak, and melt curve.

Figure 2. SRY 1 B show cycle amplification, melt-peak, and melt curve.
Figure 3. SRY 2 A show cycle amplification, melt-peak, and melt curve.

Figure 4. SRY 2 B show cycle amplification, melt-peak, and melt curve.
Figure 5. SRY 3 B show cycle amplification, melt-peak, and melt curve.

Figure 6. SRY control show cycle amplification, melt-peak, and melt curve.
4. Conclusion

It conclude that results of the qPCR from both primers are viewed from the melt-peak graph and comparison analysis. The differences between SRY-A and SRY-B primer indicates that SRY-A produces a more stable melt-peak graph than SRY-B. In addition, the Ct value of primer SRY-A is lower than that of SRY-B. This indicates that the SRY A primer is more specific and stable when used for molecular verification processes using qPCR.

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