**AMELX Padlock Probe for the Detection of Amelogenin Gene**

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**Abstract**

Amelogenin is a single-copy gene presented on both X and Y chromosome. This locus is widely used for PCR-based sex determination that amplified a contiguous 106-bp amelogenin gene fragment. Therefore, there will be high chance of allele dropout when analyze highly degraded DNA sample. We have demonstrated the detection of amelogenin gene by using AMELX padlock probe coupled with Rolling Circle Amplification (RCA) technique. The padlock probe was designed that 20 bases of both ends complemented to a string of 40-bases amelogenin X sequence. The RCA technique involved four continuous steps, which are hybridization-ligation, amplification, restriction enzyme digestion, and detection step. Based on perfect hybridization of the AMELX probe on the target sequence, the two complementary-ends of the probe would then be joined together by DNA ligase. Then the circular probe would be amplified by Phi29 DNA polymerase, which has strong stranded displacement activity. Hence, only precisely matched AMELX probe could be ligated and amplified. The reaction yielded a long single stranded DNA product that represents a number of tandem copies of probe complementary sequences. Optimization was carried out using 104-bp AMELX DNA fragment as template for RCA. Results suggested that using AMELX padlock probe coupled with RCA, the target size for amelogenin X gene analysis can be reduced from 106 bp to 40 bp. Therefore, this technique would provide a potential tool for analysis of highly degraded DNA.

***Key word***: Padlock probe, Rolling Circle Amplification (RCA), Amelogenin X gene

**Introduction**

Most of the DNA-based sex determination method is based on the detection of polymorphism of X and Y Amelogenin gene by polymerase chain reaction (PCR), resulting in two DNA fragments of different sizes that can distinguish male from female [1]. In commercial STR typing kits, PCR product sizes of X and Y Amelogenin gene are between 106-bp and 112-bp respectively. However, if highly degraded or ancient DNA is submitted for analysis, the Amelogenin gene product may then be dropped-out.

Padlock probes are highly specific molecular tool that combine advantages of DNA hybridization and PCR amplification. Padlock probe is a linear single-stranded oligonucleotide molecule composed of a nucleotide linker segment flanked by 15-20 bases of DNA sequence at both ends of the probe, which is complementary to the DNA target [2]. Padlock probe has been used as a tool for gene analysis, allele discrimination and SNP detection [3-6] because DNA ligase can join the two ends of the probe together only if there was correct complementary sequence alignment between probe and target during the hybridization stage. This would result in a circular molecule. Consequently, signal of the circular probe would then be amplified by rolling circle amplification (RCA) technique. The amplification generates long ssDNA molecules which each contains thousands of repeats of the complementary probe sequence [7, 8]. However, it would be difficult to amplify padlock probes that remain threads on the target sequence [9]. This problem can be overcome by exonuclease digestion [6, 10-11]. Moreover, exonuclease digestion will also help eliminate excess probe from the reaction. RCA has the advantage over the conventional PCR technique because larger variety of DNA polymerase, such as Φ29 DNA polymerase, Sequenase, Klenow and and the large fragment of *Bst* DNA polymerase can be used to amplify small amount of DNA [8, 12-13]. Moreover, RCA can yield over 10 billion copies of target sequence [14].

In this study, we demonstrated the detection of Amelogenin gene by the utilization of padlock probe coupled with RCA technique. The 90-bases AMELX padlock probe designed carried 40-bases homology of the Amelogenin gene.

**Materials and Methods**

***Primers and Probes***

The 90-bases AMELX padlock probe (Bio basic Inc., USA) and AMELX primers (Bio basic Inc., USA) were designed based on the AMELX gene sequence (accession no.M55418 J04777). AMELX padlock probe was 90 bases long, carrying nucleotide linker segment that included the RCA primer binding site (primer 0) and *Hha*I restriction site (GCG$^{\nabla}$C) flanked by 20-bases homology to the Amelogenin gene at each end of the probe. The predicted PCR product of AMELX forward and reverse primers was 104 bp. Primer 0 was the RCA primer, which *Hha*I

restriction site (GCG$^{\nabla}$C) was incorporated in this primer. Position of AMELX primers and RCA target are shown in figure 1. Probe and primer sequences are shown in table 1.

AMELX padlock probe was phosphorylated prior RCA. The reaction mixture contains 1X T4 Polynucleotide kinase buffer, 1 mM ATP, 5 U kinase enzyme (New England Biolab, USA), and 100 ng of AMELX probe. Phosphorylation was carried out at 37°C for 1 hour.

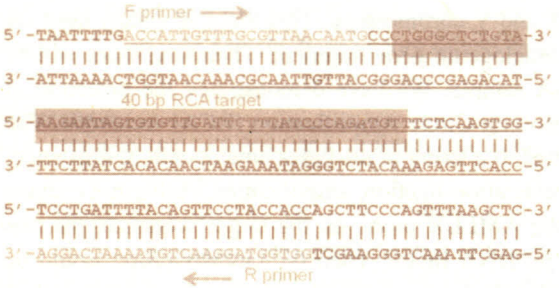



**Figure1.** Positions of primers and target of the RCA probe. Underlined sequence is the 104-bp Amelogenin fragment. Blue are AMELX forward and reverse primers. Red is 40 bases RCA target sequence.

**Table 1 Primers and probe sequences**

| Name | Sequence |
|---|---|
| AMELX forward | 5'-ACCATTGTTTGCGTTAACAATG-3' |
| AMELX reverse | 5'-GTGGTAGGAACTGTAAAATCAG-3' |
| AMELX probe | 5'ACACACTATTCTTTACAGAGTTAGATATGAAGATAGCGCATCG TAGGTACTTTATTTTCGTTAGGTACTTACATCTGGGATAAAATCA3' |
| Primer 0 | 5'- TGCGCTATCTTCA -3' |

Pink highlight are complementary sequence of 40 base RCA target.
Yellow highlight is RCA primer binding site.
Underline is *Hha*I restriction site

***Preparation of DNA template for RCA***

*Genomic DNA*

Genomic DNA was extracted from buccal swab by using QIAamp® DNA Mini Kit (QIAGEN, USA). The extracted DNA was quantified using the NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA).

***104-bp Amelogenin gene fragment***

The 104-bp Amelogenin gene fragment was amplified from human genomic DNA using AMELX primers. PCR was performed in a total volume of 25 µl containing 350 ng genomic DNA template, 20 pmol of each primer, 1.5 mM of MgCl2, 200 µM of dNTPs, 1X PCR buffer, 1 unit of Taq DNA polymerase (Promega, USA), and sterile deionized water. Amplification was carried out using the following condition; initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec, extension at 72°C for 1 min; and final extension at 72°C for 10 min. The amplified product was purified by QAIquick Gel Extraction kit (QIAGEN, USA) following the manufacturer's protocol. The purified fragment was use as RCA template.

***Rolling Circle Amplification (RCA)***

RCA was carried out according to the following steps; hybridization-ligation, amplification, restriction enzyme digestion, and detection. Flowchart in figure 2 demonstrated steps involved in RCA.

***Hybridization-Ligation***

The phosphorylated AMELX probe mixed with denatured 104-bp AMELX DNA fragment and incubated at 37°C for 2 hours to allow annealing of probe and target. After that, ligation was carried out in the reaction containing 26 µM NAD, 100 µg/ml BSA, and 5 U of E.coli DNA ligase (New England Biolab, USA), and incubated at 16°C overnight.

***Amplification***

The amplification reaction comprised 1X Phi29 DNA polymerase buffer, 30 µM of primer 0, 200 µg/ml BSA, 10 mM dNTP, 10U Phi29 DNA polymerase (New England Biolab, USA). The reaction was incubated at 30°C overnight.

***Restriction enzyme digestion***

Prior to detection the RCA product was digested with *Hha*I restriction enzyme (New England Biolab, USA). Digestion reaction carried out by adding the following reagents to the amplification mixture; 1X NE buffer 4, 100 µg/ml BSA, and 5 U *Hha*I. Then, incubated at 37°C for 2.5 hours.

***Detection***

RCA product was separated on 4% (w/v) agarose gel and stained with SYBR Green II dye. A 100-bp DNA ladder (Sibenzyme, USA) was used as DNA marker. DNA fragment was photograph under UV light using GBOX gel documentation system (SynGene, UK)

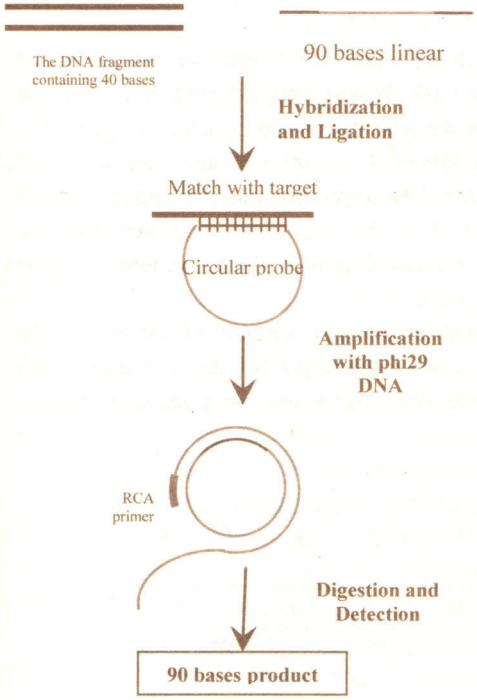



**Figure 2** Flowchart demonstrating steps in Rolling Circle Amplification (RCA).

**Results and Discussion**

In our experiment, the AMELX padlock probe detection of Amelogenin X gene has been first demonstrated by using the 104-bp Amelogenin X fragment as target and RCA product was detected by agarose gel electrophoresis. The photograph of 4%(w/v) agarose gel in figure 3 depicted amplification product from RCA with and without exonuclease digestion step prior to amplification. Exonuclease I and III was added to the ligation mixture in order to remove excess probe and the remaining DNA template. As shown in lane 2 and 4 (figure 3), DNA bands which is the same size of expected product were present in both lanes, however, the band intensities were slightly different. A faint 200-bp DNA band was also present in lane 4. In lane 3, two DNA bands were present when no DNA template was added to the reaction; one is the same size as the probe, the smaller band is likely to be generated from amplification of primer0 using the probe as template. By adding exonucleases, only circular probe would be remained in the reaction and serve as template for RCA. Therefore, intensity of DNA band obtained from RCA without exonuclease digestion would be higher than RCA with exonuclease digestion because of the remaining probes. In addition, incorporation of exonuclease digestion resulted in no DNA bands presented in lane 5 (no DNA template RCA). Hence, it was evident that if not digested with Exonuclease I and III, excess linear probe will be left in the reaction and amplified, and would also compete with circular probe in order to be a template for amplification. These clearly suggested that the product band in lane 4 was genuine RCA product and also ensured that no DNA band would be detected if the probe was not ligated.

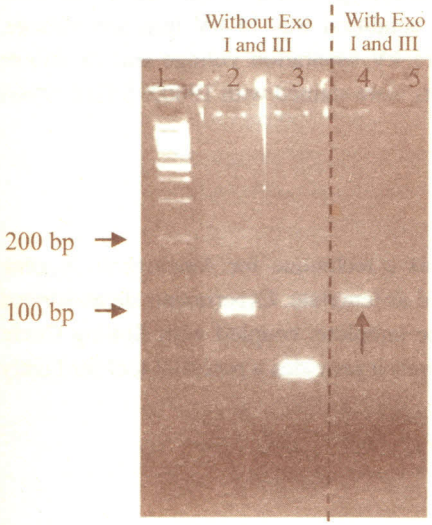



**Figure 3**. A 4% (w/v) agarose gel photograph depicting RCA products generated from 104-bp Amelogenin fragment template, with and without exonuclease I and III digestionprior RCA. Lane 1 represented the 100-bp DNA ladder, Lane 2 and 4 represented RCA with DNA template, and lane 3 and 5 represented RCA without DNA template (negative control). The black arrow represents the real RCA product.

The AMELX padlock probe detection of Amelogenin X gene was then demonstrated by using human genomic DNA as template for RCA. The photograph of 4% (w/v) agarose gel in figure 4 depicted 2 DNA bands, which one is of high molecular weight and the other is approximately 400 bp. The high molecular weight bands on the gel should be the excess genomic DNA which was not completely digested. No 90-bp DNA band was present in the gel photograph. This may be because sensitivity of agarose gel is too low to detect small amount of DNA. This phenomenon can be explained by the limitation

of number copy of target sequence. Because Amelogenin is single copy gene, when used human genomic template there is only one copy of target sequence per genome. Thus, the RCA product can be limited by amount of circularized probe generated from ligation step.

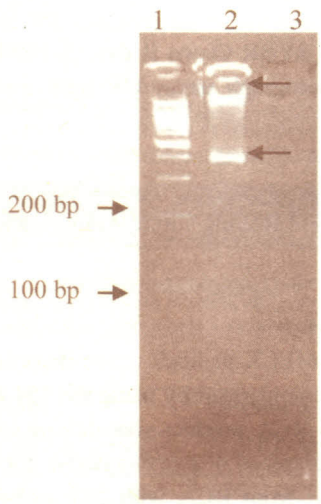
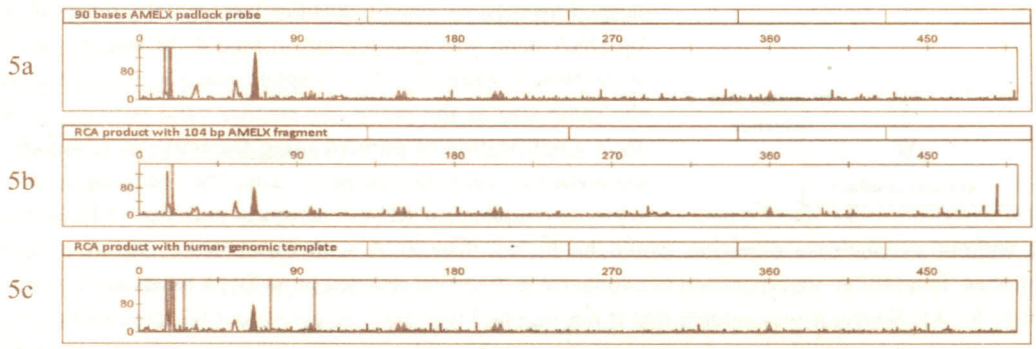



**Figure 4.** A 4% (w/v) agarose gel photograph depicting RCA products generated from human genomic DNA template. Lane 1 represented the 100-bp DNA ladder, lane 2 represented RCA reaction with human genomic DNA template, and lane 3 represented RCA reaction without DNA template (negative control). The black arrows represent the high molecular weight product.

The RCA product was then separated and detected on the automated capillary electrophoresis platform which was coupled with laser induced fluorescent detection (LIF), using the ABI Prism 310 Genetic Analyzer. The product was stained with SYBR Green II dye prior to injection, using the 90-bases AMELX probe as a maker. Electropherograms showed in figure 5 demonstrated peaks (Red arrows) of the RCA product generated from the 104-bp AMELX fragment and human genomic DNA present at same position of the 90-bases AMELX probe.

These may suggested that larger amount of DNA may be added when human genomic DNA was used as template for RCA. However, as for forensic DNA analysis application where the DNA template was limited, multi-cycle ligation with thermostable DNA ligase may help increase ligation efficiency resulting in a larger amount of circular padlock probe molecules.




| Sample | Size | Height | Data point |
|---|---|---|---|
| 90-bases AMELX probe | 66.0 | 135 | 3382 |
| RCA with 104-bp AMELX fragment | 65.55 | 83 | 3393 |
| RCA with human genomic DNA | 65.04 | 79 | 3320 |

**Figure 5.** Electropherograms depicting peaks of the 90 bases-AMELX probe (5a), RCA product generated from 104-bp AMELX fragment (5b) and RCA product generated from human genomic DNA (5c). Electrophoresis condition was 15 kV injection voltage, 30 second injection time, 15 kV separation voltage and 28 minute detection time. The products were stained with 1000X SYBR Green II dye.

## Conclusion

This study demonstrated the use of padlock probe and RCA as a technique for Amelogenin X gene detection. A 104-bp Amelogenin fragment and human genomic DNA were used as template. Experiments demonstrated that AMELX padlock probe, with only 40 bases homologous sequence to the template, coupled with Rolling Circle Amplification (RCA) can be utilized for Amelogenin X gene detection. Moreover, it provides a potential tool for highly degraded DNA analysis.

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