Multiple chemical ligation under thermal cycle

Yuko Kondo¹,², Hiroshi Abe¹, Hiroshi Jinmei¹, Naoko Abe¹, Kyoko Aikawa², Isamu Matsumoto² and Yoshihiro Ito¹

¹Nanomedical Engineering Laboratory, Discovery Research Institute, Riken, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan and ²Department of Chemistry & Biochemistry, Ochanomizu University, 2-1-1 Otsuka Bunkyo-ku, Tokyo 112-0012, Japan

ABSTRACT

Enzymatic ligation methods are useful in diagnostic detection of DNA sequence. Here we describe the investigation of nonenzymatic phosphorothioate-iodoacetyl DNA chemical ligation as a method for detection and identification of RNA and DNA. Specificity of ligation on DNA target is shown to yield discrimination of single point mutation as a drop in two magnitude. Although enzymatic ligation shows very low activity for RNA target, this reaction is found to be very efficient on RNA target. This chemical ligation with RNA target completes 70% within a few seconds, which equal or overcome ligase enzyme-mediated ligation with DNA target. The reaction is also shown to exhibit a significant level of signal amplification under thermal cycle for short time. Further, we found recently that ligation fidelity changed in function of chemical reactivity of probe. This trend was systematically investigated.

INTRODUCTION

There has been much work carried out recently to find molecular strategies for sensing nucleic acid sequence and distinguish single nucleotide difference. Enzymatic methods are one of most common strategies in current use. cDNAs prepared from PCR-based amplification are analyzed in these method. Among enzymes, DNA ligases such as the T4 and T4h are quite sensitive to base mismatches and can amplify DNA signal under thermal cycle condition known as the ligase chain reaction (LCR). Although they are quite useful, enzymatic ligation methods for sequence detection also face some limitations. For example, ligases show low activities with RNAs. In addition, although very short probes exhibit the highest sequence specificity, ligase enzymes are inefficient with shorter oligonucleotides.

As a result, consideration of alternatives to enzymatic methods such as ligases seems justified for some application. DNA template chemical ligation might be one of candidates to general approach to the detection of oligonucleotides. Removing the need for enzyme can lower the cost and RNA sample can be targeted directly without transformation to corresponding cDNA. A number of ligation chemistries have been developed. Some of methods require the addition of ligating reagents to the oligonucleotide probes. Auto ligation chemistries that do not require added reagent are even simpler for the application. Photochemical ligation or 1, 3-dipolor cycloaddition on DNA template has been reported. Nucleophilic displacement of sulfur on bromoacetyl maleimide, tosy, or iodo-nucleoside group. Among these, chemical reaction between phosphorothioate and bromoacetyl group offered fastest ligation within 20min. However, it is still slower reaction compared with ligase enzymatic reaction which completes within 2 min in LCR method. Faster chemical ligation reaction must be developed to achieve thermal cycle amplification.

Figure 1. Signal amplification by multiple chemical ligation. Reaction is carried out in thermal cycler instrument for PCR. DNA probes binds to DNA or RNA target and ligates together. After this step, reaction mixture is heated to promote dissociation of ligated products from target. And then, reaction goes to second round.
Here we described the chemical ligation using pair of phosphorothioate and iodoacetyl group. The reaction almost completed within 60 sec and got turnover under thermal cycle condition (Figure 1). The chemistry was applied to single base discrimination or direct RNA sensing with signal amplification. In addition, we systematically investigated the relationship between ligation fidelity and chemical reactivity.

RESULTS AND DISCUSSION

Design and synthesis of chemical ligation probes. We chose iodoacetyl group and phosphorothioate group for ligation probe, because these functional groups are most reactive and seems to offer best ligation rate (Figure 2). Relatively short probes were designed for high sequence selectivity and for less product inhibition. Iodoacetyl-probe was designed in 8-mer and length of phosphorothioate probe was varied from 5- to 7-mer. Com-DNA and Com-RNA are fully complementary to probes and mutated DNA, mut-1-4 have a mismatch with from one to four base difference to phosphorothioate probe respectively.

![Nucleophilic probe](https://example.com/nucleophilic Probe.png)  
![Electrophilic probe](https://example.com/electrophilic Probe.png)

**Figure 2.** Structures and sequences of DNA probes and target.

Course of ligation on DNA template. Initial slopes of product yields were measured as a function of time, and 6-mer phosphorothioate probe afforded faster ligation rate 24 % yield compared with 7-mer 20 % or 5-mer 2 %. Next, temperature on ligation reaction was varied at from 15°C to 55°C to know optimum temperature. At temperature range from 15°C to 35°C, 6-mer probe showed better yield than 5-mer or 7-mer probe. Ligation of 6-mer probe afforded best yield at 25°C. Thus we convinced that best temperature for ligation reaction was 25°C and used 6-mer probe for further experiments.

Relationship between chemical reactivity and ligation fidelity. We designed and synthesized probe where chemical reactivity gradually increase by functional group. Series of leaving group are primary bromo group (1° Br), primary chloro group (1° Cl), secondary bromo group (2° Br), and secondary chloro group (2° Br). Using these probes, we tested ligation reaction and analyzed the relationship between reactivity and discrimination ability of single base mismatch. Ligations were carried out at 25 °C 1.0 μM halogen probe, 1.0 μM phosphorothioate probe, 1.0 μM com-DNA, in a pH 8.0 Tris-borate buffer containing 10 mM MgCl₂. Phosphorothioate probe were labeled at 5'-terminal end with 32P for the trace. Reactions were followed by polyacrylamide gel electrophoretic analysis (Figure 3).

![Analysis of ligations by polyacrylamide gel electrophoresis](https://example.com/ligations.png)

**Figure 3.** Analysis of ligations by polyacrylamide gel electrophoresis.

CONCLUSION

The present study shows very rapid chemical ligation method which amplifies DNA or RNA signal. The method is applied to the commercially available thermal cycler for PCR for the amplification and use the cycling condition similar as LCR method. A few femto mol of RNA with radio isotope labeling was detected under thermal cycling condition. In addition, ligation fidelity was analyzed in function of chemical reactivity. Consequently, we found that fidelity increase with decrease of chemical reactivity. Ongoing work will be aimed at developing dye-labeled probe for detecting RNA in real time.

REFERENCES


Yuko Kondo. E-mail: yukok@riken.jp