UNIVERSITY OF CALIFORNIA, SAN DIEGO

Use of Padlock Probes and Isothermal Amplification for Genetic Detection

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in

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by

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2008
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LIST OF ABBREVIATIONS

RCA ................................................................. rolling circle amplification
RAM ................................................................. ramification amplification
LAMP ............................................................. loop mediated isothermal amplification
CLL ................................................................. chronic lymphocytic leukemia
IGHV ............................................................... immunoglobulin heavy chain variable region
MRD ................................................................. minimal residual disease
ACKNOWLEDGEMENTS

I would like to acknowledge my committee for their support throughout this process. I specially would like to thank Dr. Esener for giving me the opportunity to join his lab and his guidance throughout this learning experience. Also, without the day to day help of Brad Messmer this work would not be possible. His patience, understanding, and willingness to help me are greatly appreciated. I want to thank god for giving me this opportunity, and my family for their support.
ABSTRACT OF THE THESIS

Use of Padlock Probes and Isothermal Amplification for Genetic Detection

by

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Master of Science in Bioengineering

University of California, San Diego, 2008

Professor Sadik Esener, Chair

Genetic detection, particularly at the single molecule level, has emerged as a leading field in molecular diagnostics since the vast accumulation of genetic information due to DNA sequencing. Genetic detection is particularly important in cancer diagnostics and prognosis, since cancer cells exhibit unique genetic signatures. Padlock probes are particularly suitable for this purpose due to their intrinsic specificity mediated by precise base pairing of 3' and 5' ends for ligation to occur. Moreover, isothermal amplification of ligated padlock probes provides a rapid and sensitive assay for genetic detection. We have investigated two isothermal amplification assays, namely rolling circle amplification (RCA)-loop mediated isothermal amplification (LAMP) and ramification amplification (RAM) in single molecule detection. RCA-LAMP was discovered to successfully detect 10 molecules of ligated padlock probe, and proved a more rapid and sensitive assay than RAM. In addition, a breakpoint in the 20 kb deletion in the 9p21 chromosome of Detroit
562 pharyngeal cancer cells was detected by RCA-LAMP. Herein, we also discuss the limitations and shortcomings of each assay and the application of isothermal amplification of ligated padlock probes in chronic lymphocytic leukemia (CLL).
I. INTRODUCTION

As a result of genome sequencing, the genetic composition of many diseases and conditions have come to light, and the prospects for genetic analyses in clinical and research applications look more promising than ever. Advancement of DNA sequencing technologies and the discovery of genetic polymorphisms in disease phenotypes has necessitated the parallel development of novel molecular genetic diagnostics. Genetic analyses and molecular diagnostics are perhaps most critical in cancer diagnostics and prognosis. Cancer arises when a somatic cell acquires genetic changes that allow it to escape normal regulatory pathways, allowing cells to grow and spread uncontrollably. These genetic changes, in the form of deletions, single nucleotide mutations, translocations, etc, give cancer cells unique genetic signatures that differentiate them from their normal counterparts. This unique signature is a great platform for genetic based detection and monitoring of the cancer following treatment.

In detecting unique genetic sequences, originally short oligonucleotide sequences complementary to the target were used; however, they didn’t offer the sensitivity required for complex genetic analyses. With the advent of the polymerase chain reaction (PCR), the concept of using a nucleic acid based amplification technology for genetic detection proliferated. The emergence of padlock probes -circularizable oligonucleotides compromised of two complementary encoding regions at the 3’ and 5’ ends-signified a novel nucleic acid probing technique. Padlock probes have greater specificity over traditional probes since they require precise base pairing at their 3’ and 5’ ends for ligation, and possess greater hybridization stringency. Despite the greater specificity, padlock probes don’t offer the sensitivity needed for genetic detection. Recently, novel
isothermal nucleic acid amplification methods have added greater sensitivity to padlock probe detection of target nucleic acid sequences.

Here we describe two circularizable probe-based nucleic acid amplification assays. We investigated rolling circle amplification (RCA)-loop mediated isothermal amplification (LAMP) developed in our lab, and ramification amplification (RAM). In both cases, we first investigated the ability of each assay in amplifying and generating signals as we lowered the number of targets. The sensitivity of each assay was assessed by performing each reaction with successively lower number of ligated padlock probes. We also used each assay to detect a breakpoint in the 20 kb deletion on chromosome 9p21 of the Detroit 562 cell line. Here, we also discuss the limitations and problems specific to using these isothermal amplification techniques. Applications to other fields such as chronic lymphocytic leukemia (CLL) are also mentioned.
II. BACKGROUND

*Padlock Probes*

Circularizable oligonucleotide probes (c-probes) or padlock probes are unique oligonucleotide probes that can be circularized upon hybridization to their target. Padlock probes are usually 100 nucleotides long, and are composed of three distinct regions [1], [2]. Target-complementarity regions consisting of 15 nucleotides make up the 5’ and 3’ ends of the probe [1]. Both ends are interspersed by an arbitrary linker region. Upon hybridization to the target, the 5’ and 3’ ends are juxtaposed and in the presence of DNA ligase, the two ends are covalently linked, forming a circular structure [1] [3]. The probe is strictly adhered to the target due to the hybridization and twisting of the complementary regions in a helical manner to the target binding regions [1], [4]. Therefore, part of the probe is wrapped around the target, while the nonbinding region of the probe forms a loop which contains a primer binding moiety for amplification.

*Rolling Circle Amplification*

RCA was developed to offer added sensitivity to target detection by padlock probes [5], [6], [7]. RCA amplifies the closed circular padlock probe, proceeding in a linear fashion [8], [1]. Upon binding of a forward primer to the linker region of the padlock probe, a strand displacing DNA polymerase such as phi29 or Bst polymerase begins extending the primer, using the probe as a template. Therefore, the padlock probe acts as a circular template, allowing the polymerase to go many revolutions around the probe while displacing downstream products. RCA generates multiple copies of the c-
probe in an extended single stranded DNA product, offering several hundred thousand fold amplification [8], [9].

**Padlock probe binding and initiation of RCA**

Figure 1:
A scheme of padlock probe hybridization and RCA initiation.
Ramification amplification

Ramification amplification or hyperbranched rolling circle amplification or cascade rolling circle amplification is an exponential amplification process ensuing the formation of the multimeric RCA product [10], [11], [12], [13]. During this process, the single stranded RCA product serves as a template for multiple reverse primers. These primers are extended by a strand displacing polymerase, and each time an upstream primer encounters a downstream primer, the polymerase displaces the primer and its extended product. These displaced products are single stranded, and act as templates for extension of the initial forward primer. Primer extension, strand displacement, and further primer extension, create a branching or “ramified” complex, resulting in multimeric double stranded DNA of various lengths [10].

Figure 2:
Scheme illustrating padlock probe hybridization, RCA, and generation of double stranded DNA via RAM [10].
**Loop mediated isothermal amplification**

Loop mediated isothermal amplification is a novel nucleic acid amplification mechanism that is highly rapid and sensitive. During this isothermal amplification process, four primers consisting of two outer and two inner primers are used in conjunction with a strand-displacing polymerase [14]. An inner primer containing sense and antisense sequences of the target initiates LAMP; subsequently, this product is displaced by an outer primer [14], [15]. The other inner primer hybridizes to the bumped product, is extended, and displaced by the other outer primer producing a stem-loop DNA structure [14]. The end products of LAMP are concatameric stem-loop and cauliflower like structures [14].

**LAMP**

![Diagram of LAMP](image)

Figure 3: Illustration of LAMP.
RCA-LAMP

A combinatorial exponential amplification scheme was developed in our lab combing RCA with LAMP. Upon padlock probe ligation, the forward primer for RCA, designed like the two inner primers of LAMP, initiates RCA. Another self-complementary loop primer binds the multimeric RCA product, undergoing extension and strand displacement, thereby initiating the starting product for LAMP.

1. Padlock probe encoding LAMP amplicon binds to target DNA

2. Padlock probe is ligated and primed for RCA by reverse loop primer

3. RCA concatamer product is multiply primed by forward loop primers, creating displacement products

4. Displacement products undergo LAMP

Figure 4: Illustration of the RCA-LAMP amplification method.
III. MATERIAL AND METHODS

Genomic DNA isolation from Detroit 562 cells

Genomic DNA from human pharyngeal Detroit 562 cell lines were isolated and purified using two different protocols:

   a. **FUJIFILM Life Sciences QuickGene DNA tissue kit S:**

      Frozen cells were thawed, and counted under a microscope using a hemacytometer. Approximately 1 x 10^6 number of cells were used for the miniprep-80 protocol. The genomic DNA lysate was prepared using the Genomic DNA isolation from Cultures Cell lines protocol provided by FUJIFILM. This lysate was subsequently transferred into the cartridge of the QG-Mini80. Genomic DNA was isolated and eluted in elution buffer (EB) in the final step.

   b. **QIAGEN DNeasy Tissue protocol:**

      Starting from 5 x 10^6 number of cells, genomic DNA was isolated and purified using the DNeasy Tissue Handbook and the kit provided.

Determining DNA concentration

Upon isolation of genomic DNA, the NanoDrop ND-1000 spectrophotometer was used to determine the approximate DNA concentration.
**Padlock probe ligations**

Ligation of padlock probes in titration experiments was carried in 500 µl ligation volumes. A mixture consisting of 100 nM padlock probe, 300 nM DNA template, 50 µl T4 DNA ligase buffer, and 431 µl of water was heated at 95°C and slowly cooled to 37°C. Subsequently, 12.5 µl of T4 DNA ligase was added at 37°C and incubated for 1 hour; thereafter, the mixture was heated to 65°C to inactivate the enzyme. Free nucleotides were removed using the QIAquick Nucleotide Removal Kit after completion of ligation. Unligated padlock probes, template, and other oligonucleotides were removed using the exoIII (ds DNA) and exoT (ssDNA) exonucleases.

**Determining DNA concentration using fluorescence**

In addition to using a spectrophotometer, the Quant-iT OliGreen ssDNA Assay Kit was used to measure the concentration of purified ligated padlock probes.

**Ligation reaction conditions**

Ligation of padlock probes on targets was carried in various volumes, depending on the specific experiment. All reactions contained various concentrations of padlock probe, T4 DNA Ligase, T4 DNA Ligase buffer, water, and DNA template. For synthetic templates, all reagents except ligase were mixed in a tube, heated at 95°C, cooled to 37°C, and incubated at 37°C upon addition of ligase. Finally the mixture was heated to 65°C to heat inactivate the ligase. For genomic templates, the mixture was first heated to 95°C to ensure strand separation. Thereafter, the mixture was slowly cooled to 65°C, incubated for 1 hour, and upon addition of ligase, incubated at 37°C for one hour.
**Phosphorylation of 5’ end of padlock probes**

The ligation process carried by T4 Ligase requires a phosphate group at the 5’ end of the padlock probe. To ensure the presence of a phosphate group, padlock probe, T4 Ligase buffer, and water were added to 1µl of T4 Kinase at 37°C for 30 minutes prior to each ligation reaction.

**Digestion of genomic DNA and amplification products with a restriction enzyme**

Amplification products of Detroit 562 experiments were digested with StuI, a restriction enzyme with the following recognition site:

\[
\text{5'...AGG\textsuperscript{CCT}...3'} \\
\text{3'...TCC\textsuperscript{GGA}...5'}
\]

Restriction was carried with:

2 µl of amplification product

1.5 µl of StuI

1.5 µl of NEBuffer 2

Samples were incubated at 37°C for 1 hour and subsequently analyzed by gel electrophoresis.

**Genomic DNA digested with CviKI-1**

In some ligation experiments using genomic DNA as template, the genome was first cut into many segments using CviKI-1, a restriction endonuclease with the following recognition site:

\[
\text{5'...RG\textsuperscript{C}Y...3'} \\
\text{3'...YGR...5'}
\]
R= A or G, Y= C or T

**RCA-LAMP reaction**

All RCA-LAMP experiments were carried in 25 μl volumes containing 1 μl of 8 U/μl Bst DNA Polymerase, 1.6 μM of each FIP and BIP primer, 2.5 μl of ThermoPol buffer, 1.5 μl of 100 mM MGSO4, 1 μl of 15X SYBR, 0.3 μl reference dye (ROX), 2.7 μl water, 1.4 μM of each dNTP, and 1 μl of ligated padlock probe. In ligations with genomic DNA template, primer and water volumes were adjusted to allow for a higher volume of ligation product to be added to the mixture. Mineral oil (20 μl) was added on top of each reaction and centrifuged to reduce crossover contamination. Reactions were run in the STRATAGENE Mx3005P PCR machine. Amplification took place at 62°C, and enzyme inactivation was carried at 80°C.

**Table 1:**
List of reagents for the RCA-LAMP reaction

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betaine</td>
<td>DNA stabilization</td>
</tr>
<tr>
<td>FIP</td>
<td>Forward primer</td>
</tr>
<tr>
<td>BIP</td>
<td>Backward primer</td>
</tr>
<tr>
<td>ThermoPol Buffer</td>
<td>Polymerase buffer</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Nucleotides for polymerization</td>
</tr>
<tr>
<td>MGSO4</td>
<td>Used for optimum polymerase activity</td>
</tr>
<tr>
<td>SYBR</td>
<td>Double stranded DNA binding dye</td>
</tr>
<tr>
<td>ROX</td>
<td>Reference dye</td>
</tr>
<tr>
<td>Bst Polymerase</td>
<td>Strand displacing DNA polymerase</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Ligated padlock probe added from Ligation reactions</td>
</tr>
</tbody>
</table>
**RAM reactions**

All RAM experiments were carried in 25 µl volumes containing 1 µl of 8 U/µl Bst DNA Polymerase, 1.6 µM of each F and B primer, 2.5 µl of ThermoPol buffer, 1.5 µl of 100 mM MGSO4, 1 µl of SYBR, 0.3 µl reference dye (ROX), 2.7 µl water, 1.4 µM of each dNTP, and 1 µl of ligated padlock probe. In ligations with genomic DNA template, primer and water volumes were adjusted to allow for a higher volume of ligation product to be added to the mixture. Mineral oil (20 µl) was added on top of each reaction and centrifuged, to reduce crossover contamination. Reactions were run in the STRATAGENE Mx3005P PCR machine. Amplification took place at 62°C, and enzyme inactivation was carried at 80°C.

**Table 2:**
List of reagents for the RAM reaction

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betaine</td>
<td>DNA stabilization</td>
</tr>
<tr>
<td>F</td>
<td>Forward primer</td>
</tr>
<tr>
<td>B</td>
<td>Backward primer</td>
</tr>
<tr>
<td>ThermoPol Buffer</td>
<td>Polymerase buffer</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Nucleotides for polymerization</td>
</tr>
<tr>
<td>MGSO4</td>
<td>Used for optimum polymerase activity</td>
</tr>
<tr>
<td>SYBR</td>
<td>Double stranded DNA binding dye</td>
</tr>
<tr>
<td>ROX</td>
<td>Reference dye</td>
</tr>
<tr>
<td>Bst Polymerase</td>
<td>Strand displacing DNA polymerase</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Ligated padlock probe added from Ligation reactions</td>
</tr>
</tbody>
</table>
Table 3:  
Sequence of oligonucleotides used for Detroit 562 cell line experiments (orange represents padlock probe bases complementary to the Detroit template)

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleic acid sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIP primer</td>
<td>gtggcgataatttcaccggttttcattatcgatcagtaccagt</td>
</tr>
<tr>
<td>BIP primer</td>
<td>gtgcgggcaatctgattgacatctgacgtaagagacgagataggacagtacgactttcttactctgta</td>
</tr>
<tr>
<td>F primer</td>
<td>gcattatcgatcagtaccagt</td>
</tr>
<tr>
<td>B primer</td>
<td>acggtgacaatagaagagac</td>
</tr>
<tr>
<td>Detroit 562 padlock probe</td>
<td>tccggtcttgccctcagctcagttcgggcaatctgattgtcgccctatagtgagctgatgaagagacgagagagagtactttcttactctctc</td>
</tr>
<tr>
<td>Detroit 562 template</td>
<td>tccggtcttgccctcagctcagttcgggcaatctgattgtcgccctatagtgagctgatgaagagagagagtactttcttactctctc</td>
</tr>
</tbody>
</table>
IV. RESULTS

RCA-LAMP of ligated padlock probes (circles)

Figure 5:
Blue ~ 1000 ligated padlock probes, Red ~ 100 ligated padlock probes,
Green ~ 50 ligated padlock probes, Gray ~ No ligated padlock probes

*RCA-LAMP with different numbers of ligated padlock probes*

The reaction was done using previously formed ligated padlock probes (circles).

Different starting numbers of circles were added to the reaction to study the sensitivity of
the amplification and its feasibility in single molecule detection. When LAMP begins,
double stranded DNA is generated, which increases exponentially over time. This rise in
amplification product is detected by SYBR green, a double stranded DNA binding dye. The starting time for rise in fluorescence is clearly dependent on the starting number of circles available for amplification. Even though different number of circles saturate at the same level of fluorescence, lower number of circles result in slower rise of signal.
RCA-LAMP of ligated padlock probes (circles)

Figure 6:
Blue ~ 1000 ligated padlock probes, Red ~ 100 ligated padlock probes, Green ~ 50 ligated padlock probes, Gray ~ 10 ligated padlock probes, Gold ~ No ligated padlock probes

*RCA-LAMP with different numbers of ligated padlock probes*

Circles were titrated at the same levels of the previous experiment to investigate the reliability and reproducibility of the results. As seen from figure 6, the amplification plots are more erratic and do not follow the pattern of slower rise in signal with lower
number of circles. The results indicate the reaction is reliable and accurate down to 50 starting number of circles.

**RCA-LAMP of ligated padlock probes (circles)**

![Amplification Plots](image)

**Figure 7:**
Blue ~ 1000 ligated padlock probes, Red ~ 100 ligated padlock probes, Green ~ 50 ligated padlock probes, Gray ~ 10 ligated padlock probes, Gold ~ No ligated padlock probes

**RCA-LAMP with different numbers of ligated padlock probes**

Following the same procedure as before, we observe the erratic nature of the reaction below 50 molecules of padlock probe. The set of samples with 10 starting
number of circles generate signals earlier than higher number of circles. Also, the
negative control with no ligated padlock probe generates a signal, suggesting a
contamination issue.

**Detroit 562 cell line genomic detection via RCA-LAMP**

![Amplification Plots](image)

Figure 8:
Blue ~ Detroit 562 genomic template, Red ~ salmon sperm genomic DNA, Green ~ No
template DNA

A padlock probe was designed to specifically detect a breakpoint in the 20 kb
deletion on chromosome 9p21 of the Detroit 562 cell line. Approximately 150,000
copies of the Detroit 562 template sequence from previously purified genomic DNA were incubated with padlock probe and T4 DNA Ligase. Controls compromising of no template DNA and salmon sperm DNA in place of Detroit template were done. RCA-LAMP was performed with 10 μl of ligated solution. The Detroit and negative samples generate similar signals. A stark contrast exists with the salmon sperm DNA, where the rise in fluorescence signal is much lower.
Restriction enzyme digestion of amplified products

Figure 9:
From Left to Right: 1 kbp ladder, Detroit 562 reaction digested sample 1, Detroit 562 reaction digested sample 2, salmon sperm reaction digested, no template reaction digested sample 1, no template reaction digested sample 2, Detroit 562 reaction undigested sample 1, Detroit 562 reaction undigested sample 2, salmon sperm reaction undigested, no template reaction undigested sample 1, no template reaction undigested sample 2

A restriction site recognized by StuI was inserted in the Detroit 562 padlock probe. Given the identical rise of signal in the negative controls compared to Detroit samples, the amplification products were digested to study their identity. Figure 9 shows digested and undigested samples of each sample. A clear cut point exists in the digested
Detroit samples, where two dark bands are separated. These results suggest that RCA-LAMP detection of the Detroit sequence was successfully performed, and the positive signal in the negatives is an irrelevant amplification product.
Figure 10:
Blue ~ 10,000 template copies, Red ~ 5000 Green ~ 500, Gray ~ 250, Orange ~ 50, Gold ~ 25, Light blue ~ salmon sperm DNA, Purple ~ No template DNA

Restriction enzyme digestion of amplified products
Top lane from left to right:
10,000 copies undigested, 10,000 copies digested, 5000 copies undigested, 5000 copies digested, 2500 copies undigested, 2500 copies digested, 500 copies undigested, 500 copies digested, 250 copies undigested, 250 copies digested, 25 copies undigested, 25 copies digested

Bottom lane from left to right:
Salmon sperm DNA undigested, Salmon sperm DNA digested, no template undigested, no template digested, 50 copies undigested, 50 copies digested
RCA-LAMP with lower number of Detroit 562 target sequences
After successful detection of Detroit genomic template, we attempted to see how low we could go in template copies. Each set of plots in figure 10 represent different number of template copies in the final RCA-LAMP reaction. Ligations were done with titrated amounts of genomic DNA, padlock probe, and T4 DNA Ligase. As we decrease template copy numbers, we do not necessarily observe a corresponding delay in generation of signal. Also, samples for different template copy numbers do not generate a signal in certain cases, i.e. at 2500 copy numbers. Again, the negative control comes up faster than other signals. The former results confirm the unreliability and erratic nature of the reaction as we approach single molecule detection. The latter suggests a contaminant that is causing the reaction to proceed, obviating the need for a template in the process. However, it is important to note the main comparison should be made between the salmon sperm DNA and Detroit DNA samples. Clearly, the salmon sperm DNA is different than our positive targets and gives a much smaller response.

Amplification products were digested with SfuI to confirm the validity of the results. In all cases except at 2500 copies (which didn’t generate a signal), the positive samples display a restriction point when digested with SfuI. The salmon sperm DNA and negative controls don’t exhibit a restriction point, and do not differ with their undigested counterparts. These results confirm we were able to detect and amplify a signal using RCA-LAMP with as low as 25 copies of genomic template.
RAM of ligated padlock probes (circles)

![Amplification Plots](image)

**Figure 11:**
Blue ~ $1 \times 10^7$ ligated padlock probes, Red ~ $1 \times 10^5$ padlock probes, Green ~ 1,000 ligated padlock probes, Gray 100 ligated padlock probes, Gold ~ 50 ligated padlock probes, Light green ~ No ligated padlock probes

Ligated padlock probes were formed using a synthetic Detroit 562 template and its corresponding padlock probe. Various numbers of circles starting from $1 \times 10^7$ and diluting down to 50 circles were used for performing RAM. As seen from figure 11, a clear distinction exists among different circle numbers and the time it takes for the reaction to produce a signal. The reaction appears fairly consistent and reliable down to
100 circles, since at 50 circles, we only have one positive signal which comes up earlier than expected.

**RAM with genomic Detroit 562 template**

![Amplification Plots](image)

**Figure 12:**
Blue ~ 10,000 template copies, Red ~ 5,000 template copies, Green ~ 1,000 template copies, Gray ~ CLL genomic DNA, Gold ~ No template DNA

After demonstrating the assay’s ability to amplify ligated padlock probes at low copy numbers, we decided to investigate its ability in detecting the Detroit 562 template
in genomic DNA. As seen from figure 12, all samples and controls give an identical signal at an early stage. These results indicate template-independent amplification.

**RAM with ligated padlock probes**

![Amplification Plots](image)

**Figure 13:**
Blue ~ $1 \times 10^9$ ligated padlock probes, Red ~ No ligated padlock probes

A control experiment of RAM with pre-formed circles was carried to study for contamination issues. As observed, negative control with no circles don’t produce a signal, disproving the notion of contamination issues with the previous experiment.
RAM with only padlock probe and no template DNA

Figure 14:
Blue ~ 0.1 µM Padlock probe concentration, Red ~ 0.001 µM, Green 0.00001 µM, Gray ~ 0.0000001 µM, Gold ~ Genomic template w/o padlock probe and primers

We proposed that the rise in signal in figure 12 with all samples is due to Bst polymerase activity on a high number of padlock probes. In order to test this hypothesis, ligation of different padlock probe concentration without template DNA were added to the RAM reaction. Figure 14 shows a lower dynamic range and slower rise in signal as we proceed from 0.1 µM to 0.000001 µM concentration of padlock probe. These results indicate that even without template to form ligated padlock probes, Bst polymerase is able to proceed with exponential amplification using only padlock probe and primers.
RAM with only padlock probe and no template DNA

Figure 15:
Blue ~ $1 \times 10^{-5} \mu$M padlock probe concentration, Red ~ $1 \times 10^{-6} \mu$M, Green ~ $1 \times 10^{-7} \mu$M

Dilutions of padlock probe without template DNA, from $1 \times 10^{-5}$ to $1 \times 10^{-7}$ \muM concentrations were added to the RAM reaction. We decided a $1 \times 10^{-6} \mu$M padlock probe concentration, which corresponds to $1 \times 10^7$ molecules was the cut off point to avoid a background signal due to excess padlock probe.
Figure 16:
Blue ~ 1 x 10^10 synthetic Detroit 562 templates, Red ~ No template DNA

Restriction enzyme digestion of amplified products

From Left to Right:
Detroit 562 template digested sample 1
Detroit 562 template digested sample 2
Detroit 562 template digested sample 3
No template digested sample 1
No template digested sample 2
No template digested sample 3
RAM with synthetic Detroit 562 template
Naturally, the next question involved the ability to perform RAM on template sequences using a lower number of padlock probe. First we performed RAM with 1 x 10^10 synthetic Detroit templates and 1 x 10^7 padlock probes. As seen from figure 16, we have a rise in signal after 50 minutes. Even though the negative controls without template give a signal, most of them come up after the positive samples. Second, when digested with Stul, the amplification products of the Detroit template reactions are clearly digested when compared to the negative controls. These results confirm that we were able to detect synthetic template sequences using only 1 x 10^7 padlock probes.
Figure 17:
Blue ~ Detroit 562 genomic template
Red ~ CLL genomic DNA
Green ~ No template DNA
Restriction enzyme digestion of amplified products
From Left to Right:
Lanes 1-3: Digested Detroit 562 samples
Lane 4-6: Digested CLL samples
Lane 7-9: Digested no template samples
RAM with genomic Detroit 562 DNA
Figure 17 shows the amplification plots for RAM carried with 20,000 copies of Detroit genomic template, CLL template, and negative controls without template DNA. The small rise in signal for CLL is simply binding of SYBR to double stranded genomic DNA. However, the Detroit and negative controls produce a significant rise in signal and come up fairly late. The products were digested with Stul, and as seen in figure 17, there is no clear restriction point. This indicates the products were not the result of the correct RAM reaction. The results suggest the lower padlock probe concentration is insufficient for detection in genomic DNA.
V. DISCUSSION

RCA-LAMP

In investigating the use of isothermal amplification of padlock probes for genetic detection, we first studied the single molecule detection characteristics of RCA-LAMP. The RCA LAMP reaction was carried with a titrated number of previously formed ligated padlock probes. The experiments were carried with a high number of circles in one reaction, and successively lower numbers added to other reactions. As seen in figure 5, there is clearly a difference between 1000, 100, and 50 circles for each respective RCA-LAMP reaction. This clearly indicates that with higher number of ligated padlock probes, the RCA-LAMP is able to start amplification more quickly, and thus lead to a faster generation of signal. Also, the experiment indicated detection reliability of the assay to be 50 molecules of ligated padlock probe. To test the sensitivity and reliability of this assay for even lower number of ligated padlock probes, we decreased number of circles down to 10 molecules. As Figure 6 indicates, amplification was successfully carried out with even 10 circles; however, one of the samples with 50 molecules came up later than the 10 molecule samples. The unreliability and erratic nature of RCA-LAMP as we approach single molecule detection became evident when we tried to reproduce these results. As figure 7 shows, not only do the negative control experiments with no ligated padlock probe generate a signal, but there is no clear pattern in the signal time for each set of samples. Lower ligated padlock probe levels do not come up later as expected, and there is clearly an erratic nature to the assay. We believe the positive signal for the negative controls is due to contamination issues involving the LAMP amplicon. Also, as we approach such low number of molecules, naturally, assays
become more unpredictable and can lead to unexpected results. Through these padlock probe titration studies, we can conclude the RCA-LAMP reaction can reliably detect 50 molecules of ligated padlock probe. Also, the detection sensitivity of the assay appears to reach down to 10 molecules of ligated padlock probe.

We were successfully able to detect the deletion breakpoint on the 9p21 chromosome of the Detroit 562 cell line using the RCA-LAMP assay. A padlock probe complementary to 40 bases at this break point was used to carry the ligation process. In figure 8, we used a high copy number of target from purified genomic DNA as a proof of concept experiment. Each reaction in the final set carried approximately 50,000 copies of the target sequence, and the plots come up fairly early after 16 minutes. The same reaction was carried with salmon sperm genomic DNA as a control, and we clearly see a much lower rise in fluorescence, which is mainly due to SYBR binding double stranded genomic DNA. Although the negative control with no target DNA comes up identically to the Detroit sets, we identified the correct product by digesting the amplification products with StuI. As indicated in figure 9, only the Detroit samples show a clear restricted product, thus proving they are the correct product. The negative controls are believed to generate a signal due to contamination issues and are not the correct amplification product. To test the sensitivity of the assay, we carried the same reaction with lower number of target sequence copies. We started with 10,000 copies of the target and diluted down to 25 copies of the target as shown in figure 10. Several notes must be made regarding the results of this experiment. First, although we are able to get a positive signal for as low as 25 copies of the genomic target, we see that there is no clear pattern with different sets of samples according to the number of targets. There is clearly
an erratic nature to this assay, which makes it difficult to reproduce. Also, this assay is extremely sensitive to contamination as seen by the fast rise in signal by the negative controls with no target DNA. However, the samples were analyzed on a gel after digestion by StuI, and only the samples with the Detroit DNA indicate a restriction point, and thus the correct identity of the products were confirmed. Ultimately, the assay can be based on a distinction in the fluorescence signal between the target template and other control templates; thereby, negative signals with no template DNA’s can be ignored as long as the latter holds true. As seen from our results, we had a much higher rise in signal for our positive Detroit 562 target when compared to the salmon sperm target. These results indicate the highly sensitive and powerful nature of the amplification method in RCA-LAMP by detecting only 25 copies of the target genomic DNA sequence. Moreover, the application of RCA-LAMP to detecting a specific genetic signature present in a particular cancer cell, demonstrated the suitability of this assay for genetic detection of cancer cells.

**RAM**

Experiments were carried to study the feasibility of the RAM assay in detecting single molecules. Reactions were carried out with titrated number of ligated padlock probes as in the RCA-LAMP reactions. As seen in figure 11, the assay can clearly detect and distinguish from 1 x 10^7 molecules of target down to 100 molecules. One sample out of a triplicate set for 50 target molecules generates a signal, but it comes up earlier than expected. Therefore, we can infer the RAM assay is reliable and reproducible in detecting down to 100 molecules of target. In comparison, we see a less sensitive assay
than RCA-LAMP, which detected 50 molecules of target reliably. RAM was performed with synthetic Detroit template and the corresponding padlock probe. As seen in figure 12, various numbers of Detroit target, other DNA template (CLL genome) control, and the negative control without DNA template all rise up at the same time and display identical plots. The first suspicion was a contaminant presence; however, this was disproved when the experiment in figure 13 showed that RCA-RAM with pre-ligated padlock probe clearly distinguished between positive and negative samples. We speculated that the high number of padlock probes was creating an artifact product, due to Bst polymerase activity. To test this hypothesis, ligations were carried out with no template DNA, but with different number of padlock probe molecules. As figure 14 and 15 demonstrate, there is a clear cut off point at which the samples do not generate a false signal due to excess padlock probe presence, which is approximately $1 \times 10^7$ padlock probe molecules. This figure is consistent with other published results [12]. We subsequently carried out RAM with $1 \times 10^{10}$ number of synthetic Detroit targets, and $1 \times 10^7$ number of padlock probes. As shown in figure 16, the Detroit samples come up after approximately 50 minutes, with most of the negatives coming up later. To confirm the validity of the product, we ran the StuI digested product on a gel, and as figure 16 shows, only the Detroit samples show a clear restriction point. This was a successful experiment in that with a much lower number of padlock probe molecules compared to template, we were able to successfully detect the target within 1 hour. Although we were successful in detecting the Detroit breakpoint with synthetic DNA template, detection of genomic template was unsuccessful. When using $1 \times 10^7$ number of padlock probe molecules for genomic detection, we could not detect the target as indicated in figures 17.
The presence of double stranded DNA and the shear length of the genomic DNA when compared to using synthetic templates makes it harder for the probe to find the target sequence. Therefore, the necessity to use lower number of padlock probe molecules to avoid false positives proved insufficient for detection of target sequences embedded in genomic DNA. From our results, we conclude the RAM assay can reliably detect 100 molecules of ligated padlock probe. Also, when compared to the RCA-LAMP reaction, RAM was demonstrated to be less sensitive and unable to detect genomic targets.

*Imposing Specificity by Localization*

Many biological processes use localization of substrates and effector molecules to bring about specificity (16). This localization is brought about by the phenomenon of “cooperative binding,” which as the name implies, consists of numerous non-covalent interactions working synergistically to assemble a complex of molecules. By having numerous binding sites to the target and other “locator” molecules, a complex can be localized. Nowhere else, is the notion of localization and cooperative binding more pertinent, than in the mechanisms used for gene regulation and expression (16). We adapted this biological strategy in designing a “bridging primer”. Although experiments with this primer didn’t produce successful results and were not conclusive (results not shown), a brief discussion is mentioned here. This primer which primes the RCA reaction by binding to the padlock probe also has a complementary site to the target. Once the primer is bound to the target, cooperative binding between the target, padlock probe, and primer, can bring about the specificity needed for the padlock probe to more readily find its target. Ideally, by using a bridging primer, one can lower the concentration of padlock
probe needed for both amplification assays. Thus, one would reduce the background noise due to excess padlock probe. Furthermore, this method should allow for lower concentrations of template and primer as well.

Figure 18:
Use of a bridging primer for cooperative binding. Blue and pink represent padlock probe and target complementary regions. Light blue represents bridging primer and target complementary region. Yellow is the RCA forward primer complementary region with the padlock probe.
VI. CONCLUSION AND FUTURE DIRECTIONS

We investigated RCA-LAMP and RAM in their ability to approach single molecule detection. Based on our findings, RCA-LAMP is a far more sensitive and rapid assay than RAM, as we were able to detect down to 10 ligated padlock probes using this assay. At the same time, RCA-LAMP is more erratic as we approach lower numbers of targets and is more prone to contamination due to generation of an intermediate amplicon. In the RCA-LAMP assay, our reliable detection limit was 50 molecules of ligated padlock probe. In using RAM, we could only reliably detect down to 100 molecules of ligated padlock probe. In addition to studying each assay’s single molecule detection characteristics, we applied both amplification schemes for the genetic detection of a breakpoint in the 20 kb deletion of the 9p21 chromosome of the Detroit 562 cell line. We were successfully able to detect down to 25 copies of the genomic target sequence using RCA-LAMP. RAM proved unsuccessful in genomic detection of the Detroit target, although we were able to detect the synthetic template of the breakpoint sequence. Bst polymerase activity in the presence of excess padlock probe generated false signals when doing RAM for genomic detection. By lowering the padlock probe concentration, the chances for detection of genomic DNA were significantly decreased, and thus RAM proved ineffective for this task.

Isothermal amplification of ligated padlock probes offers many advantages over traditional genetic detection methods due to padlock probe specificity, sensitivity of the amplification methods, and isothermal nature of the assays. It must be noted, however, that both RCA-LAMP and RAM are very sensitive to contamination and false positive signal generation. These issues limit the efficacy of each assay in genetic detection, and
necessitate adaptations to accommodate optimum results. The prospect for using isothermal amplification in genetic detection of cancer is an exciting application of this field, particularly in CLL.

**Minimal residual disease (MRD) detection in CLL**

CLL is a type of haematological cancer leading to monoclonal expansion of CD-5 expressing B lymphocytes [17]. CLL is the most prevalent leukemia in the Western hemisphere [17]. Novel therapeutic agents and techniques have prolonged survival in CLL patients by achieving greater remission of the cancer. This progress has been paralleled by development of more sensitive assays to monitor disease state and progression.

MRD can be described as the level of disease diagnosed by the most sensitive methods for detection [18]. Diagnosis of MRD is at the forefront of current research and clinical applications involving CLL. This concept is important, because even though many treatments fall within remission standards set by the National Cancer Institute (NCI), significant populations of CLL monoclonal cells continue to exist within the patient following treatment. To obtain accurate disease state levels, one must detect CLL cells among their normal counterparts well below the level of remission offered by current therapeutic agents. The most sensitive approaches used for MRD detection in CLL include allele-specific oligonucleotide PCR and MRD flow cytometry [18], [19]. Both methods are capable of detecting 1 leukemia cell in 1 x10^4-10^5 leukocytes [18]. The former is based on the amplification of immunoglobulin heavy chain variable (IGHV) region gene segments. It has been established that both assays can reliably
detect 1 CLL cell among 10,000 leukocytes [18]. However, each method has its own limitations. Allele-specific PCR requires sequencing of the heavy chain sequence, and requires unique primers for each patient. Background noise and false positives due to the presence of normal B cells in the sample population limit the efficacy of flow cytometry.

![Changing Definition of Complete Remission](image)

**Hypothetical model comparing National Cancer Institute-defined complete remission and eradication of minimal residual disease.**

**Figure 19:**
Efficacy of modern CLL therapies and detection limits for current methods
Recently, it has come to light that IGHV region sequences among different patients exhibit high similarity, particularly in the CDR3 region [20], [21], [22]:

![Diagram of IGHV region sequences]

Figure 20: Sequences of CLL patients showing high similarity in CDR3 variable region of immunoglobulin heavy chains are demonstrated. Bases with boxes represent identical bases going down the columns within otherwise similar bases in that particular column [20].

We propose designing padlock probes to detect CDR3 regions compromising the V, D, and J coding segments, among subsets of CLL patients. The padlock probes would contain complementary regions to the joining segments between the V, D, J segments. Using this approach, patient specific padlock probes would be obviated for subsets of
patients with similar sequences. Furthermore, we believe our approach will have a sensitivity of detecting 1 CLL cell among $1 \times 10^5$-$10^6$ normal B cells.
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