

# RNA-templated single-base mutation detection based on T4 DNA ligase and reverse molecular beacon

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## Abstract

A novel RNA-templated single-base mutation detection method based on T4 DNA ligase and reverse molecular beacon (rMB) has been developed and successfully applied to identification of single-base mutation in codon 273 of the p53 gene. The discrimination was carried out using allele-specific primers, which flanked the variable position in the target RNA and was ligated using T4 DNA ligase only when the primers perfectly matched the RNA template. The allele-specific primers also carried complementary stem structures with end-labels (fluorophore TAMRA, quencher DABCYL), which formed a molecular beacon after RNase H digestion. One-base mismatch can be discriminated by analyzing the change of fluorescence intensity before and after RNase H digestion. This method has several advantages for practical applications, such as direct discrimination of single-base mismatch of the RNA extracted from cell; no requirement of PCR amplification; performance of homogeneous detection; and easily design of detection probes.

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**Keywords:** Single-base mutation detection; Reverse molecular beacon; T4 DNA ligase; RNA-templated

## 1. Introduction

Many pathogenic and genetic diseases and cancers are associated with the mutation of particular genes [1]. Among these mutations, single nucleotide polymorphisms are the most abundant genetic variation in individuals [2,3]. A majority of these genetic variations can serve as biomarkers for medical diagnosis at early stage of the diseases [4,5]. Therefore, reliable and rapid methods for single-base mutation detection have considerable significance in the biological research, medical science and clinical diagnosis.

In the past decades, a wide variety of techniques and methods have been developed for mutation detection, such as allele-specific oligonucleotides [6], specific primer extension [7], ligation-mediated methods [8], dynamic allele-specific hybridization [9], iFRET [10], and molecular beacon assay [11].

However, most of these methods are incorporated with PCR amplification and gel-based separation.

Molecular beacons are novel hairpin structure nucleotide probes [11–13]. With proper design, it is possible to discriminate between targets that differ by a single nucleotide [14]. However, in practical application, the choices of the probe sequence are limited by target-specific considerations, and a fraction of molecular beacons will not work without optimization, this increases the cost of this method [15,16]. New strategies which combines ligase detection reaction and reverse molecular beacon (rMB) have been established for single-base mutation identification [17,18], but it still need expensive equipments [17] and washing separation steps [18].

Furthermore, compared with cDNA synthesis, transcripts analysis by direct ligation on RNA templates might more accurately report the relative abundance of the RNA [19,20]. In this paper, a novel RNA-templated single-base mutation detection method was established based on T4 DNA ligase and reverse molecular beacon.

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Table 1  
Oligonucleotide sequences

Name	Sequence
F-A	5'-(TAMRA)CGCTGCGGACAGGCACAAACACA-3'
F-T	5'-(TAMRA)CGCTGCGGACAGGCACAAACACT-3'
F-C	5'-(TAMRA)CGCTGCGGACAGGCACAAACACC-3'
F-G	5'-(TAMRA)CGCTGCGGACAGGCACAAACACG-3'
Quenching probe <sup>a</sup>	5'-pCACCTCAAAGCGCAGCG(DABCYL)-3'
Template-A <sup>b</sup>	5'-TCTCCCAGGACAGGCACAAACACTCACCTCAAAGCTGTTCCCTATAGTGAGTCGTATTAGG-3' 5'-CCTAATACGACTCACTATAGGAACAGCTTTGAGGTGAGTGTTTGTGCCTGTCTGGGAGA-3'
Template-C <sup>c</sup>	5'-TCTCCCAGGACAGGCACAAACACGCACCTCAAAGCTGTTCCCTATAGTGAGTCGTATTAGG-3' 5'-CCTAATACGACTCACTATAGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCTGGGAGA-3'
RT-PCR primers	5'-CTGAGGTTGGCTCTGACTG-3' 5'-GTGCTCGCTTAGTGCTCC-3'

<sup>a</sup> 5' end phosphorylation.

<sup>b</sup> DNA templates for *in vitro* transcription template-A.

<sup>c</sup> DNA templates for *in vitro* transcription template-C.

## 2. Experimental

### 2.1. Reagents and instruments

The ligation probes, *in vitro* transcription DNA templates, and RT-PCR primers (shown in Table 1) were synthesized in Takara Biotechnology Co. Ltd. (Dalian, China). The ligation probes were designed according to the sequence around codon 273 on the exon 8 of p53 gene. Quenching probe (Q) was designed to have a six-base arm labeled with DABCYL at 3' terminal and phosphorylated at 5' terminal. Fluorescence probe (F) was designed to have a six-base arm labeled with TAMRA at 5' terminal and a discriminating base at its 3' terminal. The fluorescence probes were named as F-A, F-T, F-C, and F-G, according to their 3' terminal nucleotide which hybridizes to the variable position in the target RNA. Six-base arm at 5' terminal of each fluorescence probe are complementary to the six-base arm at the 3' terminal of the quenching probe but not to the target RNA.

T4 DNA ligase, RNase H, Pyrobest DNA polymerase, DNase I, and dNTPs were purchased from Takara Biotechnology Co. Ltd. Trizol reagent kit for total RNA isolation was purchased from Invitrogen Corporation. RT-PCR reagent kit was purchased from Fermentas Corporation. T7 transcription kit was purchased from Fermentas Corporation. DNA gel extraction kit was purchased from Tiangen Biotech. (Beijing) Co. Ltd.

HeLa (human cervical carcinoma), A549 (human lung carcinoma), and C-33A (human cervical carcinoma) cells were preserved in our lab. RPMI 1640 culture medium and fetal bovine serum were purchased from Gibco. All other chemicals used were of analytical reagent grade without further purification. DEPC-treated purified water was used in experiments.

**Instruments:** All fluorescence measurements were performed on a Hitachi FL-2500 fluorometer (Japan) equipped with a Thermo Neslab water bath circulators (USA). RNA samples' concentration was detected on DU800 Beckman ultraviolet spectrophotometer (USA). PCR amplification was carried out

on PCR system 2700 (Applied Biosystems, USA). Agarose gel electrophoresis was performed using electrophoresis apparatus and electrophoresis tank (Beijing Liuyi Electrophoresis Instrument Factory).

### 2.2. RNA synthesis by *in vitro* transcription reaction

RNA templates were synthesized by *in vitro* transcription. The DNA templates for *in vitro* transcription were designed according to the sequence around codon 273 on the exon 8 of p53 gene, and T7 promoter was added at their 5' terminal (Table 1). *In vitro* transcription reactions were performed using T7 transcription kit according to the manufacturer's protocol. The resultant RNA samples were purified and dissolved in nuclease-free H<sub>2</sub>O. The samples' concentration was detected on Beckman ultraviolet spectrophotometer.

### 2.3. Single-base mismatch detection on RNA templates

Ligation reactions on *in vitro* synthesized RNA templates were performed in a buffer containing 10 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 7.6), 10 μM ATP, 200 nM fluorescence probes, 240 nM quenching probes and 160 nM purified RNA templates. After incubated at 65 °C for 3 min and cooled to room temperature, 7 U T4 DNA ligase was added to the ligation mixture (100 μL) and incubated at 37 °C for 2 h. Then, the reaction was terminated by incubation at 65 °C for 10 min. In order to identify the base type at the mutation site, four separate experiments were performed using different fluorescence probe (F-A, F-T, F-C, and F-G, respectively) for each RNA template. The ligation products were divided into two equal parts. One of them was added 15 μL 5 × RNA degeneration buffer and 1 μL RNase H and the other was added 15 μL 5 × RNA degeneration buffer only. After incubated at 37 °C for 1 h, the reaction mixture was diluted to 400 μL, and the fluorescence was measured on fluorometer with excitation at 521 nm.

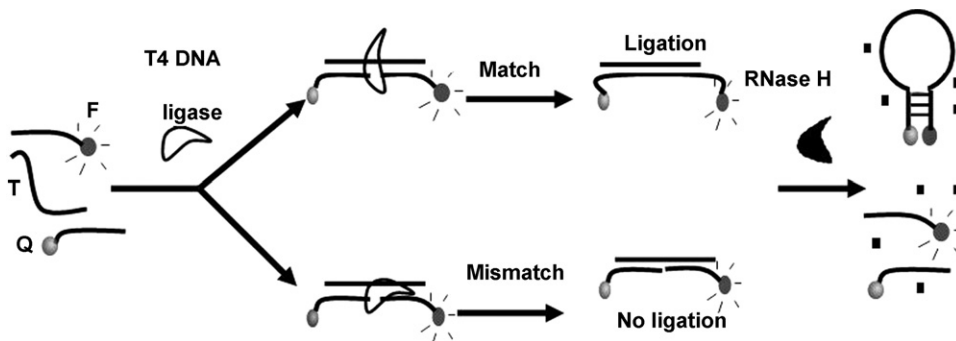


Fig. 1. The scheme of RNA-templated single-base mutation detection. First, the target RNA (T) hybridized with the quenching probe (Q) and fluorescence probe (F). If F and Q perfectly matched to T, the ligation reactions occurred upon adding T4 DNA ligase. After adding RNase H, the target RNA in RNA/DNA duplex was digested and a hairpin structure was formed, causing fluorescence quenching by fluorescence resonance energy transfer. If F and Q not perfectly matched to T, unligated probes could not form a hairpin structure after adding RNase H and could not cause fluorescence quenching. Single-base mutation can be discriminated by analyze the change of fluorescence intensity before and after RNase H digestion.

#### 2.4. Mismatch discrimination based on fluorescence quenching

The change in the fluorescence intensity was measured after 1 h incubation with RNase H. Excitation and emission wavelengths were 521 and 578 nm, respectively. To normalize the difference in different experiments, quenching efficiency ( $Q_e$ ) was defined as follows:  $Q_e = ((F_0 - F_1)/F_0) \times 100\%$ . Where  $F_0$  indicates the fluorescence intensity before RNase H digestion and  $F_1$  indicates the fluorescence intensity after RNase H digestion.

#### 2.5. Single-base mismatch detection on total RNA samples

Cells were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin at 37 °C, and in atmosphere containing 5% CO<sub>2</sub>. Total RNA samples were extracted using Trizol reagent with a standard total RNA isolation protocol from three cell lines, i.e. HeLa, A549, and C-33A. The purified total RNA was dissolved in nuclease-free H<sub>2</sub>O, and the samples' concentration was detected on ultraviolet spectrophotometer. Detections were performed refer to the method previously described in this paper (Section 2.3), and modified as follows. Ligation reactions were performed in a 100  $\mu$ L buffer containing 10 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 7.6), 10  $\mu$ M ATP, 50 nM fluorescence probes, 60 nM quenching probes, and 180  $\mu$ g total RNA sample. After incubated at 65 °C for 3 min and cooled to room temperature, 7 U T4 DNA ligase was added and incubated at 37 °C for 2 h. Then, the reaction was terminated by incubation at 65 °C for 10 min. The ligation products were divided into two equal parts. One of them was added 15  $\mu$ L 5 $\times$  RNA degeneration buffer and 1  $\mu$ L RNase H and the other was added 15  $\mu$ L 5 $\times$  RNA degeneration buffer only. After incubated at 37 °C for 1 h, the reaction mixtures were all diluted to 100  $\mu$ L, and the fluorescence was measured on fluorometer with excitation at 521 nm. In order to identify the base type at the mutation site, four separate experiments were performed using different fluorescence probe (F-A, F-T, F-C, and F-G, respectively) for each total RNA sample.

#### 2.6. RT-PCR and sequencing

Total RNA samples were firstly purified to remove DNA contaminant. Reverse transcription reaction was performed in 20  $\mu$ L volumes with 4  $\mu$ L 5 $\times$  reverse transcriptase buffer, 3  $\mu$ g RNA template, 5 mM dNTPs mixture, 30 U RNase inhibitor, 5 U AMV RTase, and 50 pmole oligo (dT) 18 primer at 42 °C for 1 h. PCR amplification was carried out on PCR system 2700. PCR was performed in 50  $\mu$ L of 1 $\times$  Pyrobest buffer, containing 2.0 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 400 nM PCR primers, 2.5 U Pyrobest DNA polymerase, and 2  $\mu$ L reverse transcription products. The amplification was achieved for 30 cycles at 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

5  $\mu$ L PCR products were analyzed on 2% agarose gels containing ethidium bromide. Agarose gels were examined in UV light vision system after electrophoresis. Target DNA samples were recovered and purified using DNA gel extraction kit. Purified PCR products were sent to Takara Biotechnology Co. Ltd. for sequencing.

### 3. Results and discussion

#### 3.1. Experimental principle

The scheme of this approach is shown in Fig. 1. The detection was carried out by using allele-specific probes, which flanked the point mutation in the target RNA. Quenching probe (Q) was designed to have a six-base arm labeled with DABCYL at 3' terminal and phosphorylated at 5' terminal and fluorescence probe (F) was designed to have a six-base arm labeled with TAMRA at 5' terminal and a discriminating base at its 3' terminal. The arm sequences of the F and Q were complementary to each other but not to the target RNA. After hybridized the target RNA with the F and Q, ligation reaction was carried out in the presence of T4 DNA ligase if the two adjacent probes perfectly matched to the target RNA. Then RNase H was added to cleave the RNA strand in DNA/RNA duplex. The molecular beacon-like hairpin structure (rMB) was formed by the intramolecular hybridization of the complementary arm sequence in the ligation products. This

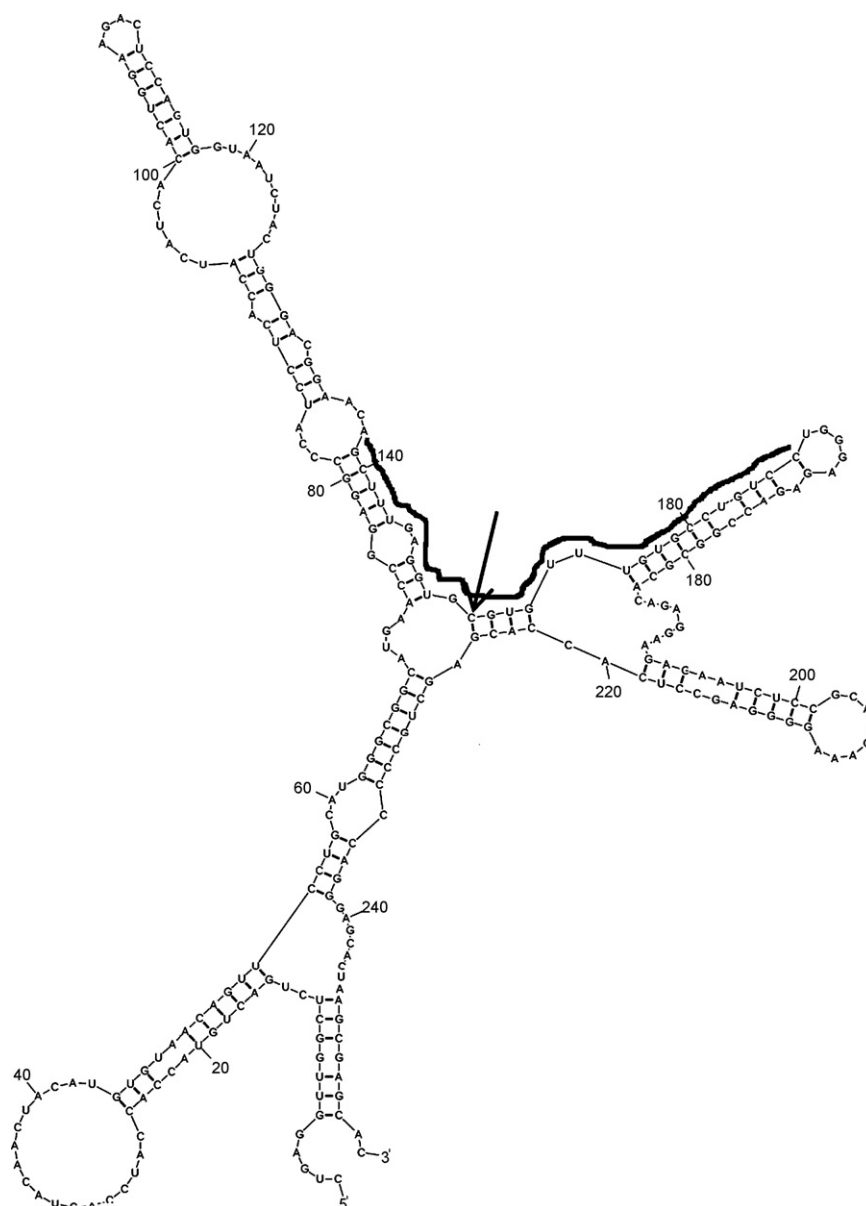


Fig. 2. The most stable secondary structure of partial of p53 mRNA analysis with the Mfold program. The bold line emphasized sequence was chosen for one-base mismatch detection in this paper, and the base variable position was pointed with an arrow.

hairpin structure will keep the fluorophore and quencher in close proximity to each other, causing the fluorescence to be quenched by fluorescence resonance energy transfer. If the two adjacent probes were not perfectly matched to the template, unligated probes could not form a hairpin structure after adding RNase H. One-base mutation can be discriminated by analyzing the change of fluorescence intensity before and after RNase H digestion.

Several computer algorithms such as Mfold [21], primer 5.0, and OligoWalk can be chosen to design the detection primers. The total length comprising the hairpin loop can range between 20 and 35 nucleotides, depending on the target region chosen for probe binding. In order to increase the stability of the DNA/RNA hybridization duplex, the fluorescence probe and quenching probe (not contain the stem sequence) should contain not less than 10 nucleotides. The length of the arm sequences is five or six nucleotides (composed mostly G/C's) to keep the

stable of the intracellular stem loop structure of the molecular beacon-like hairpin structure. Mfold or primer 5.0 can be used to predict the structure of the loop sequence (the sequence in the molecular beacon-like hairpin structure not containing the arm nucleotides) and the molecular beacon; these sequences should not form unexpected secondary structure. The most stable secondary structure of partial of p53 mRNA analysis with the Mfold program is shown in Fig. 2. The bold line emphasized sequence was chosen for one-base mismatch detection in this paper, and the base variable position was pointed with an arrow.

### 3.2. Discrimination of single-base mismatch on *in vitro* synthesized RNA

To validate our postulated mechanism in Fig. 1, two *in vitro* synthesized RNA templates (named template-A and template-C

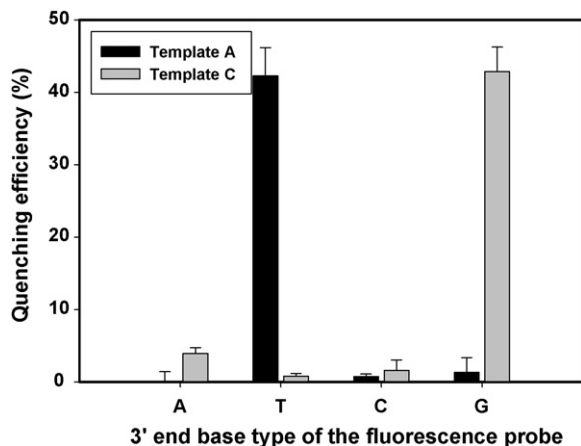


Fig. 3. Single-base mismatch detect of *in vitro* synthesized RNA. The Qe reflected whether the 3' terminal base of fluorescence probe perfectly matched the base at the variable position in the target RNA. Base type at the variable position in the target can be identified by comparing the Qe of relevant fluorescence probes. The concentration of fluorescence probe, quenching probe, and *in vitro* synthesized RNA were 25, 30 and 20 nM, respectively.

according to the base type at the variable position) were used for one-base mismatch detection. The designed probes are shown in Table 1. In order to identify the base type at the mutation site, four separate experiments were performed using different fluorescence probe (F-A, F-T, F-C, and F-G, respectively) for each RNA template.

As shown in Fig. 3, the Qe for template-A were 0.07, 42.24, 0.72, and 1.33% (the corresponding detection probes were F-A, F-T, F-C, and F-G), respectively. The Qe of matched substrate was 31-fold greater than corresponding mismatched substrates. For template-C, the Qe were 3.93, 0.80, 1.60, and 42.8%. The Qe of matched substrate was 10-fold greater than corresponding mismatched substrates. Using this method, we cannot only discriminate one-base mismatch, but also identify the base type at the variable position in the target RNA.

Then, the quantitative relationship between the template RNA concentration and fluorescence quenching efficiency was detected. The perfectly matched fluorescence probe and quench-

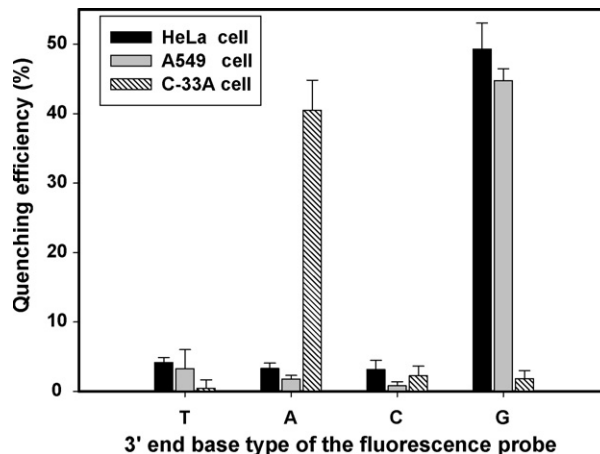


Fig. 4. Single-base mutation detect of extracted total RNA samples. The Qe reflected whether the 3' terminal base of fluorescence probe perfectly matched the base at the variable position in the target RNA. Base type at the variable position in the target can be identified by comparing the Qe of relevant fluorescence probes. The wild-type base at the variable position in the target RNA is C. The concentration of fluorescence probe, quenching probe, and total RNA were 25 nM, 30 nM, and 900 mg L<sup>-1</sup>, respectively.

ing probe were used in all experiments. The Qe (Y) was linearly correlated to the template-A RNA concentrations (X) in the range of 1.25–24 nM. The regression equation is  $Y = 2.241X + 1.068$ ,  $R^2 = 0.9981$ . The limit of detection was estimated to be 0.9 nM, defined by three times of standard deviation ( $\sigma$ ) over the blank measurement.

### 3.3. Discrimination of single-base mismatch on total RNA

To investigate whether this approach has the capability of detecting single-base mutation in RNA samples, total RNA samples extracted using Trizol reagent with a standard total RNA isolation protocol from three cell lines, i.e. HeLa, A549, and C-33A. The results were shown in Fig. 4. These results indicated that the base types at the same position of HeLa, A549 and C-33A RNA were C, C, and U, respectively, by comparing the Qe.

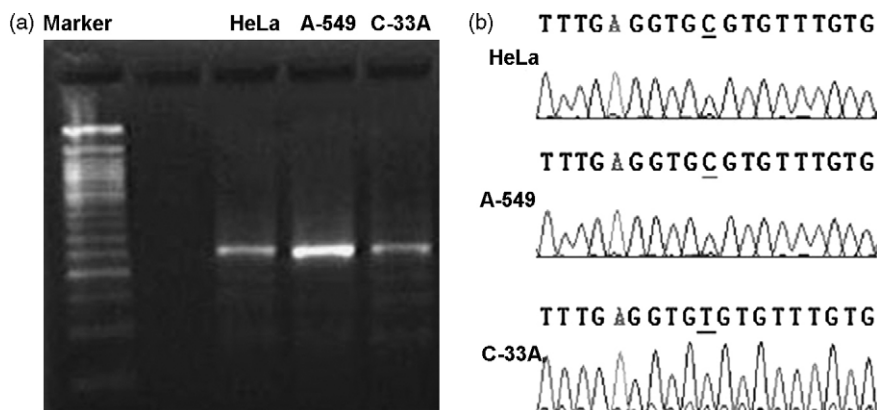


Fig. 5. Sequencing results. (a) The agarose gel electrophoresis image of the RT-PCR productions. (b) The sequencing electropherograms of HeLa, A549, and C-33A cell. The nucleotide at the variable position was indicated with a piece of underline.

### 3.4. Sequence validation

In order to confirm the detection results, a 255 bp DNA fragment containing the target sequence was amplified specifically from three cell lines utilizing the RT-PCR technique. The sequencing results (shown in Fig. 5) were in accordance with the detection results (Fig. 4). By comparing our experimental results with the database in the global bioresource center ATCC (C-33A has the CGT > TGT transition at codon 273, HeLa and A549 have the wild-type codon CGT at codon 273), we thought this method could detect the single-base mutations directly using total RNA samples.

### 4. Conclusions

A novel RNA-template single-base mutation detection method based on T4 DNA ligase and reverse molecular beacon has been developed and successfully applied to identification of single-base mutation in codon 273 of the p53 gene. This method has several advantages for practical applications, including (i) direct discrimination of single-base mismatch of the RNA extracted from cell; (ii) no requirement of PCR amplification, reverse transcription PCR, and gel electrophoresis separation; (iii) performance of homogeneous detection; (iv) easily design of detection probes.

For the limitation of the copy number of the target mRNA in the sample cells, the pitfall of this method is high sample consumption. Further systematic studies should be taken to improve the detection sensitivity and minimize sample consumption, such as combination of the ligase chain technology (LCR). The development of this method will make it suitable for the single-base mismatch detection of the clinical sample containing a small number of disease cells, and provide an attractive alternative to traditional RNA analysis tools.

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