

## Original Article

# Evaluation of efficiency of allele-specific real-time PCR assay for detection of Mycobacterium tuberculosis with isoniazid resistance directly from sputum samples

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**Abstract:** Objective: As drug-resistant tuberculosis is a major threat to the control of tuberculosis, the aim of this study was to evaluate Allele-specific Real-time PCR (AS-RT-PCR) as a effective and accurate assay for the detection of isoniazid (INH) resistance for the treatment and control of multi-drug resistant tuberculosis (MDR-TB). Methods: 230 clinical sputum specimens were detected by AS-RT-PCR and DNA sequencing. To evaluate the sensitivity and specificity of AS-RT-PCR, a series mixtures contained 100%, 10%, 1%, 0.1%, 0.05% and 0.001% MDR mutant plasmids and wildtype genomic DNA were made and tested by designed duplex real-time PCR. Results: The detection result of AS-RT-PCR was over 99% (228/230) consistent with the DNA sequencing. For the sensitivity and specificity, the limit of detection of mutant template was at the concentration of 0.05~0.1% in the mixture and the mutation positive rate was 21.7% according to the AS-RT-PCR assay. Conclusion: Allele-specific real-time PCR not only was a sensitive and specific assay to detect INH drug resistance, but also was a simple, fast method for MDR-TB monitoring and control.

**Keywords:** Allele-specific real-time PCR assay, Mycobacterium tuberculosis, isoniazid resistance

## Introduction

Isoniazid (INH) is a first-line chemotherapeutic agent for anti-tuberculosis with extensive application, which is also most easy to get drug resistance in the treatment of tuberculosis (TB). Especially, the development of INH resistance usually precedes resistance to other anti-tuberculosis drugs [1], so in some way, INH resistance of tuberculosis can be considered as a signal of the emergence of MDR-TB.

In recent years, the global epidemic of drug-resistant tuberculosis is severe. In the 2015 "global TB report", World Health Organization estimated that about 480000 people developed MDR-TB around the world in 2016. Additionally, according to the report, an estimated 3.3% of new cases and 20% of previously treated cases had MDR-TB, globally. Meanwhile, about 9.7% of MDR-TB cases had extensive drug-resistant tuberculosis (XDR-TB).

The increasing popularity and spread of Mycobacterium tuberculosis (MTB) with high resistance rate and drug resistance has become a serious problem for the tuberculosis control [2]. Therefore, detection of INH drug resistance is of great significance for clinical MTB treatment.

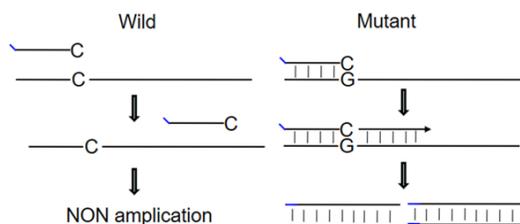
Although the conventional method for drug sensitive detection of Mycobacterium tuberculosis has been developed very well, its detection cycle is too long which lead poor clinical practicality. And the new ways of molecular detections including gene chip are expensive, complex, with large limitations in the application. Thus, it is urgent to find a simple, accurate, fast and sensitive detection method for MDR/XDR-TB detection to meet clinical needs.

Allele-specific real-time PCR (AS-RT-PCR), based on conventional PCR technology, has made a great improvement and developed into a new

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**Table 1.** Primers and probes for detecting Isoniazid Resistance

Name	Sequence (5'-3')
katG-R	ccCACTCGTAGCCGTACAG
katG-P	FAM-CGAGGAAACTGTTGTCCTCCATTTCGTC-BHQ1
katG-AAC-F	ccTAAGGACGCGATCACCAA
InhA-15C-R	ccCACCCCGACAACCTATCG
InhA-15C-P	FAM-TGCTGAGTCACACCGACAAACG-BHQ1
InhA-15C-F	ccGATCCGTCATGGTCAAG
Hsp65-P	HEX-CAAGGATCTGCTGCCGCTGC-BHQ1
Hsp65-F	CCTACATCCTGCTGGTCA
Hsp-65-R	GGATCTTGTGACGACCA
PGEM-katG-F	CGAGACGTTTCGGCGCATG
PGEM-katG-R	CCGTCCTTGGCGGTGTATTG
PGEM-inhA-F	ACATACCTGCTGCGCAATTC
PGEM-inhA-R	CCGATCCCCGGTTTCCTC



**Figure 1.** Show the structure and principle of AS-RT-PCR.

kind of amplification technology. Although there were several reports using AS-RT-PCR to study cancers, such as myeloma [3] and metastatic breast cancer [4], there were rare reports about the AS-RT-PCR application for MTB.

In this experiment, we applied the AS-RT-PCR assay for the detection of INH drug resistance directly from sputum samples to provide a more accurate, convenient and rapid detection method for clinical tuberculosis treatment.

### Materials and methods

#### Ethics statement

The study was approved by ethical committees of Chest hospital of Hebei province, China.

#### Source of samples

A total of 230 clinical sputum specimens which had been requested for AS-RT-PCR analysis were collected from Chest Hospital of Hebei province.

### Experimental methods

#### Sample processing and DNA extraction:

The sputum sample (1 mL) which decontaminated by N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) method [5], were first inactivated by heating at 80°C for 60 min, and then take 200 µL decontaminated sputum sample for DNA extraction. Experiments were performed in accordance with the manufacturer's instruction of Sputum DNAOUT Kit (BTN111201, Beijing Biorab Technology Co., Ltd).

#### Primer and probe design

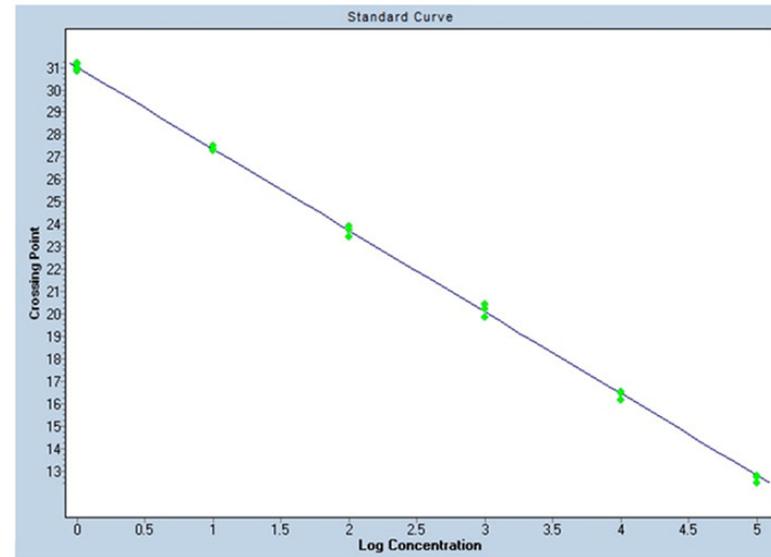
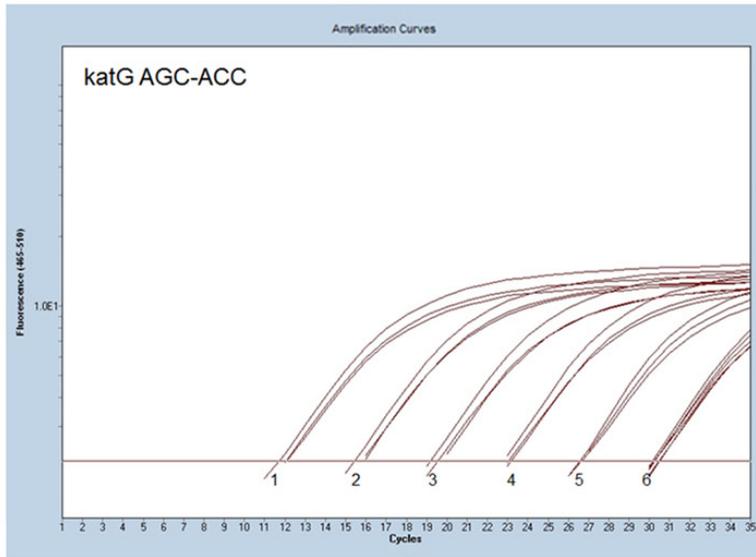
For detection of the INH drug resistance mutant, primers and probes of detecting katG 315 (AGC-ACC, AGC-AAC) and inhA-15 (C-T) were designed (**Table 1**; **Figure 1**). The TaqMan probe was used as a signal system to real-time detect mutations. Allele-specific PCR (AS-PCR) was a dual real-time PCR, and MTB Hsp65 gene designed as internal control (IC) for indicator of MTB DNA contained in test samples (**Table 1**). The function of internal control was to correct error of manual operation. PGEM-katG-F, PGEM-katG-R, PGEM-inhA-F, PGEM-inhA-R were designed as reference primers.

#### Plasmid standards

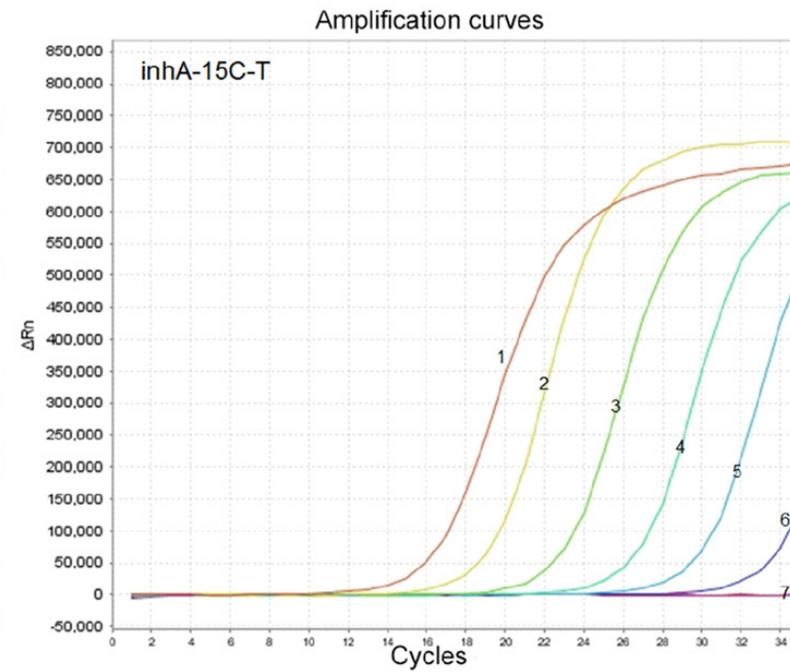
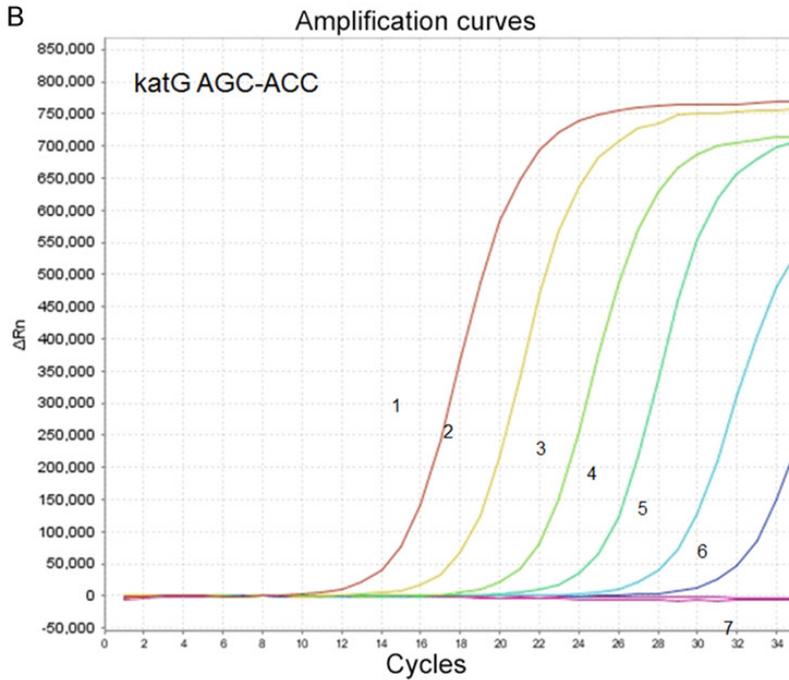
To constructed the standard plasmids of mutation, the mutant (katG 315 (AGC-ACC, AGC-AAC) and inhA-15 (C-T)) strains' DNA was amplified in 50 µl reaction mix which contained: 5 µl MTB complex genomic DNA, 1× Buffer, 500 nM dNTP, 1.5 U Mole Taq DNA polymerase (Shanghai MOLE Diagnostics Co., Ltd.), 100 nM of each primer (**Table 1**); then followed 2 min initial degeneration, 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s, and 5 min final extension. All the PCR products were checked and purified by electrophoresis, and then linked to T-vector (Promega) using a commercial TA Cloning Kit (TaKaRa, Dalian, China). Plasmids were extracted and purified by Qiagen Plasmid Mini Kit (Qiagen, Shanghai, China) and then quantified spectrophotometrically. Use DNA sequencing to confirm the recombination plasmids. The copy number was estimated according to the molecular weight, amount, and length of plasmids. Various concentrations range from  $2 \times 10^7$  to  $2 \times 10^2$  copies/ml were pre-

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A



B



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**Figure 2.** Serial diluting of INH-mutation were detected by AS-RT-PCR. The number “1~6” stands for  $2 \times 10^7 \sim 2 \times 10^2$  copies/ml of the mutation, and “7” for PCR negative control. A. Real-time PCR was performed on Light Cycler 480 II, the Standard curve of katG AGC-ACC mutation, the  $R^2 = 98.87$ . B. Was amplified by ABI 7500 Real-Time PCR System.

pared by diluting the plasmids with TE buffer. Concentrations of each kinds of plasmids were verified by absolute quantitative real-time PCR using standard curves which generated by amplifying the genomic DNA of known concentrations on a system (ABI 7500 Real-Time PCR System).

### Real-time PCR

The AS-RT-PCR assay was performed in a 25  $\mu$ l reaction volume with 1 $\times$  TaqMan Genotyping Master Mix (Applied Biosystems), which contained: 0.15  $\mu$ M of each allele-specific primer, 0.15  $\mu$ M of reverse primer, 0.1  $\mu$ M probe, 0.1  $\mu$ M of Hsp65 primer, 0.08  $\mu$ M of Hsp65 probe (**Table 1**), and 5  $\mu$ l DNA in various concentration. Reference PCR was performed according to the same protocol except the allele specific primers were substituted by the reference primers. The amplification condition was: 95°C 10 minutes, 10 cycles of 95°C for 25 s, 58°C for 50 s, then followed 35 cycles of 95°C for 25 s, 64°C for 50 s, the PCR was carried on Light Cycler 480 II. Record each template's cycle threshold (Ct) values for the AS-PCR assay and the reference assay, and calculate difference in Ct between the AS-RT-PCR assay and the reference assay as  $\Delta$ Ct values in each assay. Especially, accept the result when only internal control reaction failed and discard the both the target gene reaction and the internal control reaction failed result.

### DNA sequencing

DNA sequencing analysis of MDR-TB was performed on the basis of previous report [6], the PCR products were separated by electrophoresis, purified using Qiagen gel extraction kit (Qiagen, Shanghai, China), and sequencing was done with the BigDye terminator cycle-sequencing reaction kit (Thermo Fisher scientific, Beijing, China) and Prism 3730 DNA analyzer (Applied Biosystems).

## Results

### Sensitivity of AS-RT-PCR assay

To test sensitivity of AS-RT-PCR assay, we mix wildtype genomic DNA with a serial dilution of

$2 \times 10^6 \sim 2 \times 10^2$  copies/ml mutant plasmids to get series mixtures contained 100%, 10%, 1%, 0.1%, 0.05% and 0.001% MDR mutant; 100% and 0% presented pure plasmids and wild type genomic DNA, respectively. At least twenty repeats must be performed to test each AS-PCR primer. According to the  $\Delta$ Ct value, the limit of detection of mutant template was at the concentration of 0.05~0.1% in the mixture (**Figure 2**).

### Specificity of AS-RT-PCR assay

To test the specificity of AS-RT-PCR assay, we designed dual real-time PCR: an internal control (IC) real-time PCR, the amplicon of hsp65 gene, and the probe labeled with FAM or BHQ1; Then we confirmed the  $\Delta$ Ct value of AS-RT-PCR and detected INH mutant was as follow: 1) The “S” Curves of FAM and BHQ1 were both observed, and the  $\Delta$ Ct =  $Ct_{IC} - Ct_{Mut} < 15$ , the samples contain mutation; 2) Only the curve of BHQ1 observed, there is no mutation of this sample (**Figure 3**). Among 230 samples, there were 50 cases'  $\Delta$ Ct value over 15 (48 cases had katG-ACC mutants, 2 cases had inhA-15C-T mutants) and 180 cases only had BHQ1 curve indicating the mutation positive rate was 21.7% (**Table 2**).

### Compare AS-RT-PCR and DNA sequencing detection results

Compare AS-RT-PCR and DNA sequencing detection results of a total of 230 clinical samples. 48 (21.7%) samples were detected with isoniazid resistance mutation, including 46 cases with katG S315 mutant (AGC-ACC/AAC), and 2 cases (0.87%) for inhA-15C-T mutation. Only 2 (0.87%) RT-AS-PCR detection results was not in accordance with that of DNA sequencing (**Table 2**).

## Discussion

INH is one of first-line chemotherapeutic drugs used in TB therapy, moreover, INH resistance is the most common forms among Mycobacterium tuberculosis drug resistance. Thereby, performing rapid tests to detect INH resistance would benefit the MTB clinical treatment [7, 8]. It has

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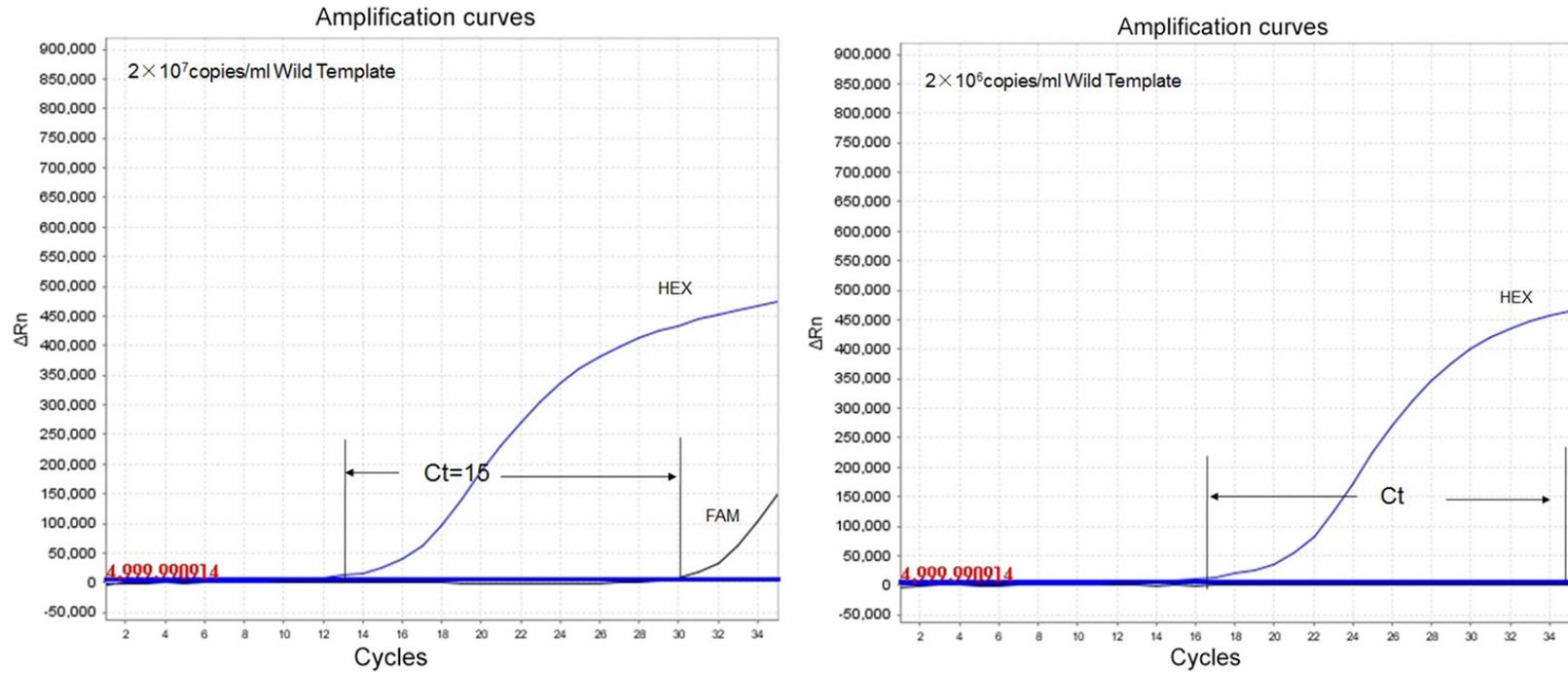


Figure 3. Application of  $2 \times 10^7 \sim 2 \times 10^6$  copies/ml wild template to test AS-RT-PCR.

**Table 2.** Comparison of AS-RT-PCR and DNA sequencing detection of clinical sputum specimens

Detection method	AS-RT-PCR		
	katG-ACC	inhA-15C-T	Wild
DNA Sequencing			
katG-ACC	46		
inhA-15C-T		2	
Wild	2		180

been shown that the mutations in katG S315T (AGC→ACC), S315N (AGC→AAC) and inhA-15C→T were the major reason for drug-resistant of Isoniazid [9-11], which account for 50-95% and 15-34% respectively, and other mutants are rare [1, 6, 12, 13], suggesting that the resistance to INH can be considered as a surrogate marker for MDR-TB.

AS the high resistance rate and drug resistance of MTB has become a serious problem, an effective and accurate assay used to detect drug resistance is crucial for the treatment and control of MDR-TB. Molecular assays, including DNA sequencing [14], AS-PCR [15, 16], MAS-PCR [1, 17-19], ARMS [20], TaqMan-PCR [21-24] and Melting Curve Analysis [25-27], are effective tools for MTB drug resistance monitoring and control. Although, compared with DST, molecular assays does not guarantee monitor all the drug resistance of MTB and the sensitivity and specificity of molecular assays not completely match, by only detecting presence or absence of known MDR mutations, it's a very effective, fast and easy performing method to detect the status of drug-resistance before obtaining the results of DST [1, 10, 11].

AS-RT-PCR, as a novel detection method of mutations of MTB, has high specificity, sensitivity. Compare with traditional polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, AS-RT-PCR is more convenient and simple which only needs the PCR amplification device, and AS-RT-PCR detects gene mutation with less enzyme and shorter time [28]. Meanwhile, AS-RT-PCR which doesn't need the preparation of hybrid membrane and cumbersome steps of reverse line blot (RLB) hybridization assay [29], saves time and efforts, and its result map clear and easy to judge. Therefore, AS-RT-PCR can completely replace the PCR-RFLP and RLB two ways in the clinical diagnosis.

In this study, we developed AS-RT-PCR assay to detect INH drug resistance to monitor MDR-TB. For AS-PCR primer, two bases tail was added to primer 5'-terminal (Table 1) which aim was to enhance Tm value of next amplification and suppress non-specific amplification. Additionally, we designed duplex real-time PCR and involved internal control to investigate the proper concentration range of MTB-DNA for the AS-RT-PCR assay (Figure 2). The result suggested that AS-RT-PCR assay could be used in a very wide range detection of mutants. Most importantly, this assay could be performed in the vast most of clinical labs. Especially, applying this assay to detect INH drug resistance directly from sputum specimens, the result was over 99% (228/230) consistent with those by DNA sequencing (Table 2). So AS-RT-PCR assay was a very effective method to monitor MDR and had high clinical application value, especially in MTB high-burden country.

**Conclusion**

AS-RT-PCR is a sensitive, specific and fast assay to detect INH drug resistance which is also an available and simple method for MDR-TB monitoring, having high potential clinical application value.

**Disclosure of conflict of interest**

None.

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