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Short Communication

Allele specific LAMP- gold nanoparticle for characterization of single nucleotide polymorphisms

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ABSTRACT

Due to their relevance as disease biomarkers and for diagnostics, screening of single nucleotide polymorphism (SNPs) requires simple and straightforward strategies capable to provide results in medium throughput settings. Suitable approaches relying on isothermal amplification techniques have been evolving to substitute the cumbersome and highly specialized PCR amplification detection schemes. Nonetheless, identification of an individual's genotype still requires sophisticated equipment and laborious methods.

Here, we present a low-cost and reliable approach based on the allele specific loop-mediated isothermal amplification (AS-LAMP) coupled to ssDNA functionalized gold nanoparticle (Au-nanoprobe) colorimetric sequence discrimination. The Au-nanoprobe integration allows for the colorimetric detection of AS-LAMP amplification product that can be easily interpreted in less than 15 min. We targeted a clinical relevant SNP responsible for lactose intolerance (-13910C/T dbSNP rs#: 4988235) to demonstrate its proof of concept and full potential of this novel approach.

1. Introduction

Single nucleotide polymorphisms (SNP) are the simplest form of DNA sequence variability, contributing to phenotypic variations between individuals. They are also a relevant class of biomarkers that may be associated to anthropometric traits, risk to a certain disease or response to environmental inputs [1,2]. A good example of a condition associated to a SNP is Lactose intolerance, which has been associated to an SNP −13910C/T (dbSNP rs#: 4988235), located in regulatory element of mini-chromosome maintenance complex component 6 (*MCM6*) gene, playing a key role in the expression of the *LCT* gene responsible for production of the enzyme lactase that degrades lactose. Individuals harboring one or both copies of the T allele (C/T or T/T) at this *locus* have enough enzyme activity in the intestinal cells to digest lactose. Conversely, individuals with no copy of the T allele (C/C) are unable to digest lactose and are classified as lactose non-persistence [3–5].

Standard protocols for screening and tagging SNPs involve conventional PCR for target amplification and SNP identification by Sanger sequencing [6,7]. Recently, the use of isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP) has been shown to be suitable to overcome the turnaround time of target amplification to under 1 h [8]. LAMP presents high specificity, efficiency and is relatively fast under isothermal conditions (60–65 °C). It relies on application of a single polymerase characterized with strand displacement activity (*Bst* DNA polymerase). LAMP uses four specific primers: two outers (F3 and B3 primers) and two inners (FIP and BIP primers) that recognize a total of six distinct sequences on the target DNA. This mechanism creates an initial dumbbell DNA product that undertakes a cyclic exponential amplification cycle producing concatamers with several inverted repeats of the target DNA sequence. An allele-specific LAMP (AS-LAMP) for characterization of a specific SNP (West African-type *kdr* mutation) was specifically designed using BIP primers specific to each allele (wild-type and mutated) [9]. Other examples of LAMP-based SNP detection have been explored in various fields, such as drug resistance screening and CYPs polymorphism analysis [10–12]. In addition to product amplification, an additional step is needed for product identification and sequence characterization.

Gold nanoparticles (AuNPs) show tremendous potential for the detection and characterization of specific DNA sequences with high sensitivity in an affordable and timesaving manner [13,14].

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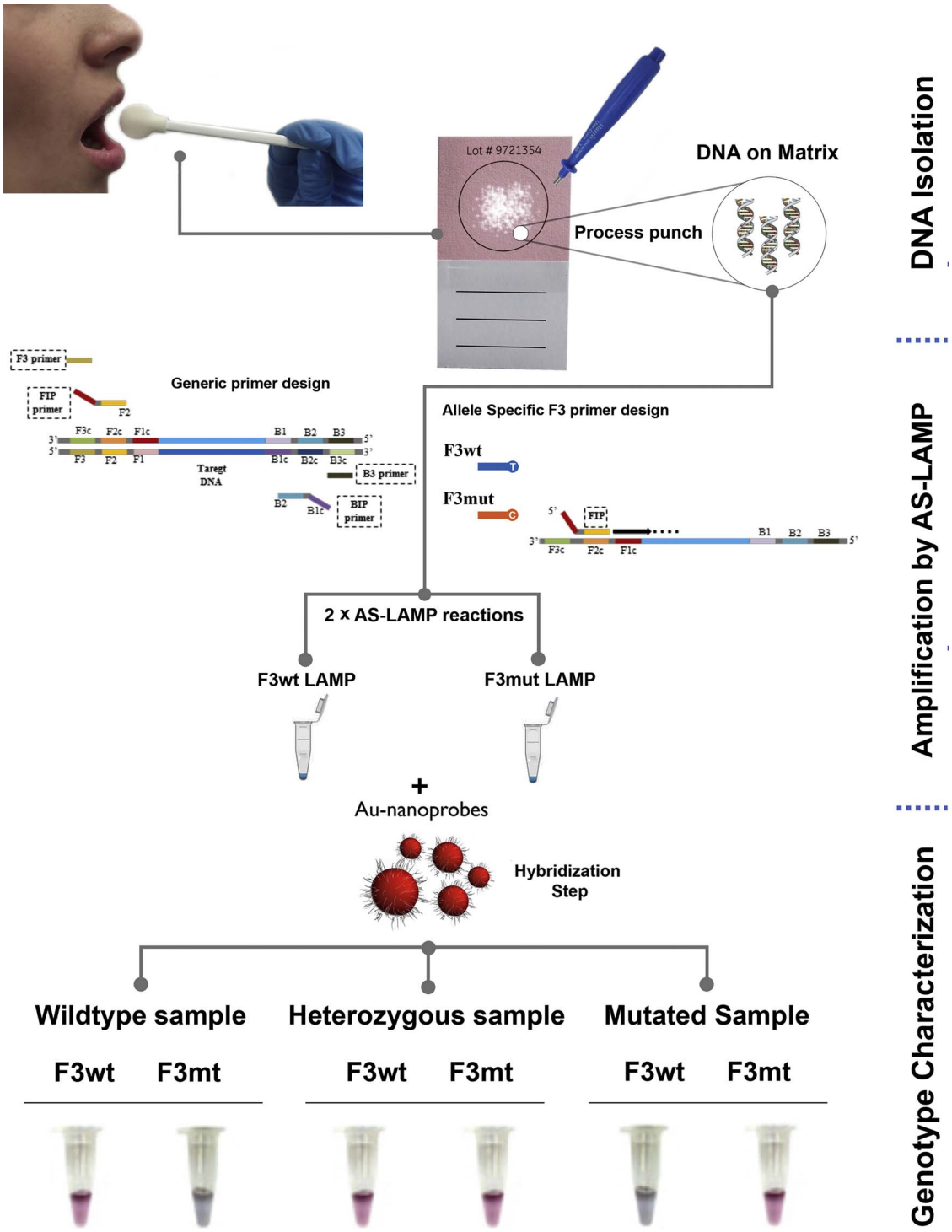

Fig. 1. Allele specific LAMP – Au-nanoprobe for SNP characterization.
Sample collection and storage in FTA® indicating microcard. After DNA isolation two LAMP reactions with allele specific F3 primers are carried out. After isothermal amplification, products are hybridized with a single Au-nanoprobe for colorimetric detection and genotype characterization. Results are attained under 3 h without the need for specialized equipment.

Oligonucleotide functionalized AuNPs – Au-nanoprobes – have been used in a plethora of detection schemes of specific target sequences taking advantage of the optical (colorimetric) properties of colloidal gold solutions [15,16]. The non-cross-linking method relies on the differential aggregation of Au-nanoprobes in presence/absence of a complementary target yielding a color differentiation: a complementary target prevents Au-nanoprobe aggregation (RED color), while non-complementary/mismatched DNA does not prevent Au-nanoprobe aggregation (BLUE color) [17–19].

Here, we combined a novel approach – allele specific LAMP amplification – to Au-nanoprobes for unequivocal screening of one SNP associated to lactose intolerance. Our approach constitutes an affordable new strategy for SNP characterization with faster turnaround time (≈3 h), where LAMP/Au-nanoprobe was validated by screening the target SNP in human samples (Fig. 1).

Table 1
Primers and probes sequences designed for MCM6 SNP −13910C/T (dbSNP rs#: 4988235) characterization.

Name	Sequence (5′-3′)
MCM6Fwd	GAAGATGGGACGCTTGAATG
MCM6Rev	TCAAATGCTTACCAAGCTCT
AS-LAMPMCM6-B3	TAAAACTAGGAAAACGCAGG
AS-LAMPMCM6-F3 wt	GGCAATACAGATAAGATAATGTAGT
AS-LAMPMCM6-F3mut	GGCAATACAGATAAGATAATGTATC
AS-LAMPMCM6-BIP	TCCACGAGGATAGGTCAGTGGAAGATGGGACGCTTGAA
AS-LAMPMCM6-FIP	TGCAGGGCTCAAAGAACAATCTAACTGGCCTCAAAGGAACTC
ASLAMPMCM6-Probe	Thiol-GCAGGGCTCAAAGAACAATC

2. Material and methods

All reagents used were purchased from Sigma Aldrich and were of analytical grade. All oligonucleotide used for target amplification were HPLC purified and purchased from STABVida (Portugal) and used without further purification. Thiolated modified oligonucleotide was used to synthesize the Au-nanoprobe and were also HPLC purified and purchased from STABVida.

2.1. Biological sample collection and characterization

Six samples were purified according to the manufacturer's protocol after collection from buccal swab and preserved in FTA™ Indicated Micro Card (Whatman, UK). All individuals were duly informed about the study and signed an informed consent. For validation, and assess each genotype for SNP −13910C/T (dbSNP rs#: 4988235), samples were amplified via PCR and submitted to Sanger Sequencing. PCR reactions were performed in a final volume of 20 µl containing 1 × of PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 U of Surf HotTaq DNA polymerase (STABVida, Portugal) and 0.4 µM of each specifically designed primer MCM6Fwd and MCM6Rev (see Table 1). PCR amplification involved a first denaturation step at 95 °C for 15 min, followed by 35 cycles, of 94 °C for 1 min, annealing at 60 °C for 30 s. and extension at 70 °C for 30 s., and final extension at 70 °C for 6 min. (See Supplementary information – Fig. S1). Direct sequencing was carried out at STABVIDA with 100 ng/100 bp of the PCR amplicon using the Big Dye V3.1 technology (Applied Biosystems, California, USA) in an Applied Biosystems 3730XL DNA Analyzer.

2.2. AS-LAMP amplification and primer design for the SNP −13910C/T (dbSNP rs#: 4988235)

AS-LAMP amplification and primer design were performed according to Notomi and co-workers [8] with the necessary modification for single nucleotide specificity. Each sample was amplified twice in a final volume of 50 µl: the first amplification was carried out using the F3 wt primer and the second amplification using the F3mut primer. The amplification conditions were: 0.8 µM of each inner primer FIP and BIP, 0.2 µM of each outer primer F3 wt and F3mut and B3, 0.2 mM of dNTP mix, 1 × of the supplied buffer, 1.6 M Betaine (Sigma–Aldrich, MO, USA), 4 mM $MgCl_2$, and 10 ng of DNA. An initial step of 5 min denaturation at 95 °C on a Bio-Rad MyCycler Thermocycler (BioRad, CA, USA) containing AS-LAMP mix and DNA was performed and afterwards, the mix was cooled down to 4 °C for one minute. Afterwards, 1U of Bst DNA polymerase large fragment (New England Biolabs Inc., MA, USA) was added to the reaction mixture and incubated for 75 min at 66 °C. As negative control, ddH2O was used instead of DNA. Following AS-LAMP, all samples were ethanol precipitated and centrifuged for 15 min at 14000 × *g*, the pellet dried under vacuum and suspended in sterile deionized water and submitted to 2% agarose gel electrophoresis.

2.3. Probe design and Au-nanoprobe synthesis

The probe sequence, ASLAMPMCM6-Probe was obtained from the *MCM6* gene sequence (GenBank accession no. NG_008958.1).

AuNPs with an average diameter of 14 nm (See Supplementary information – Fig. S2) were synthesized by the citrate reduction method described by Lee and Meisel [20]. The Au-nanoprobe was prepared as described by Carlos et al. [18]. The Au-nanoprobe solution was centrifuged and resuspended in 10 mM phosphate buffer (pH 8) and 0.1 M NaCl, and stored in the dark at 4 °C.

2.4. Au-nanoprobe colorimetric assay

Each colorimetric assay was performed in a final volume of 30 µl with Au-nanoprobes final concentration of 2.5 nM in phosphate buffer (pH 8.0) as previously described by Carlos et al., 2014. The following test tubes were prepared: a "blank" substituting the DNA with 10 mM phosphate buffer (pH 8) and an "unrelated" containing a non-complementary DNA to the Au-nanoprobe and the AS-LAMP products. Briefly, each colorimetric assay was performed by mixing the generated AS-LAMP product with the Au-nanoprobe solution and incubated 5 min at 95 °C and 20 min at room temperature. After that period, 20 mM $MgCl_2$ was added and the colorimetric change characterized by UV/visible spectroscopy using a microplate reader (Tecan Infinite M200). Each colorimetric assay was performed in a minimum of three independent assays.

UV/visible spectroscopy data allowed to determine the presence or absence of AS-LAMP by the Au-nanoprobe. For that purpose, the ratio between the absorbance of the non-aggregated fraction of Au-nanoprobe (Abs_{525nm}) and the absorbance from the aggregated fraction of Au-nanoprobe (Abs_{585nm}) was calculated: a ratio > 1 indicates a positive result (presence of complementary target to the Au-nanoprobe); ratio < 1 indicates a negative result (absence or unrelated target to the Au-nanoprobe). A one-way analysis of variance (ANOVA) and a *post hoc* Tukey's test was carried and mean differences (P-value < 0.01) between groups determined with a confidence interval (CI) of 99%.

3. Results and discussion

The proposed AS-LAMP required optimization of the primer sequence so as to differentiate between sequences with a single base selectivity. The rationale is that only in presence of the fully complementary target, LAMP will occur; whereas the single base mismatch within the primer will hamper amplification and AS-LAMP will differentiate between genotypes. So, two F3 outer primers (F3 wt and F3mut) were designed to specifically amplify each allele of the SNP −13910C/T (dbSNP rs#: 4988235). Based on the literature [9–12], several configurations were considered, including position of the mismatched nucleobase, primer structure, melting profiles. Single nucleotide discrimination was attained when an additional mismatched nucleotide (thymine) at the neighboring position was used, despite the primer/template mismatch strength could be considered strong (T/C) [21,22].

The temperature at which the reaction occurs is crucial for the

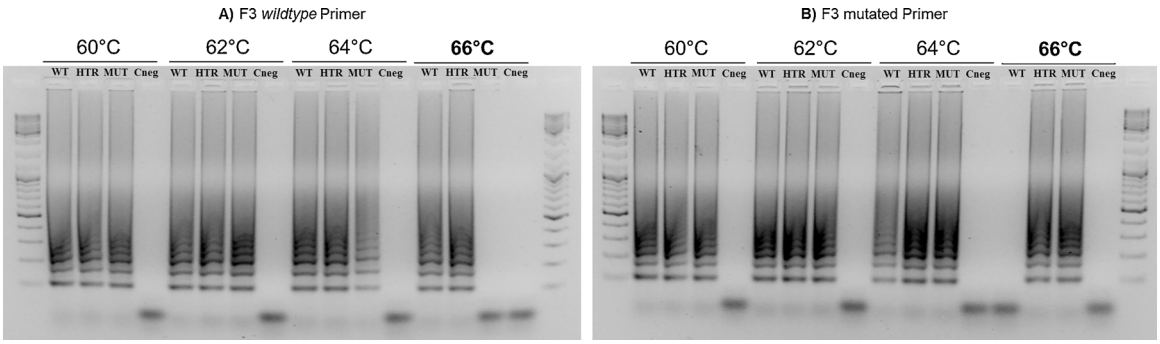

Fig. 2. AS-LAMP temperature gradient analyses for wild type and mutated F3 primers in a 2% agarose gel electrophoresis.
A) AS-LAMP temperature gradient with F3 *wildtype* primer B) AS-LAMP temperature gradient with F3mutated primer. WT – wild type sample, HTR – heterozygous sample, MUT – mutated sample, Cneg – Negative Control.

stringent single base discrimination capability of LAMP. As such, a temperature gradient reaction was performed for the F3 primers – Fig. 2 (see also Supplementary information Fig. S3). Data show that both primers presented optimal specificity at 66 °C. For the F3 *wildtype* primer at 66 °C after 75 min of reaction, only wild type and heterozygous samples yielded an amplification product (Fig. 2A). The opposite was observed for the F3 mutated primer, where amplification only occurs on heterozygous and mutated samples (Fig. 2B).

To validate the concept of AS-LAMP, 6 biological samples of each genotype were used whose sequence had been previously confirmed by direct sequencing (see Supplementary information – Fig. S4). Fig. 3A shows the agarose gel electrophoresis analysis of the panel. Amplification with the F3 *wildtype* primer (Fig. 3A-i), only yielded the characteristic LAMP amplification pattern for wild type (S1 and S3) and heterozygous (S4 and S6) samples, while no amplification was achieved for mutated (S2 and S5) samples. Conversely, AS-LAMP using the F3 mutated primer (Fig. 3A-ii), only amplified the heterozygous (S4 and S6) or mutated (S4 and S6) samples, and no amplification occurred for wild type (S1 and S3) samples.

All amplicons were then screened with the selective Au-nanoprobe via the non-cross-linking method. The purpose of using the Au-nanoprobe is for easy output reading of the presence of the AS-LAMP product (Fig. 3B-i and 3B-ii). Because the allele discrimination for the −13910C/T (dbSNP rs#: 4988235) is obtained by the AS-LAMP specificity, the Au-nanoprobe will only be positive if the correct fragment has been amplified since it will hybridize to the specific LAMP amplicon. The Au-nanoprobe is sequence specific, adding redundancy to the detection approach, increasing the specificity without additional equipment requirements [23]. This way, any unspecific amplification that might occur will not be score positive by the Au-nanoprobe detection. Using this combined approach, we achieve medium-throughput capability with lower cost when compared with the gold-standard

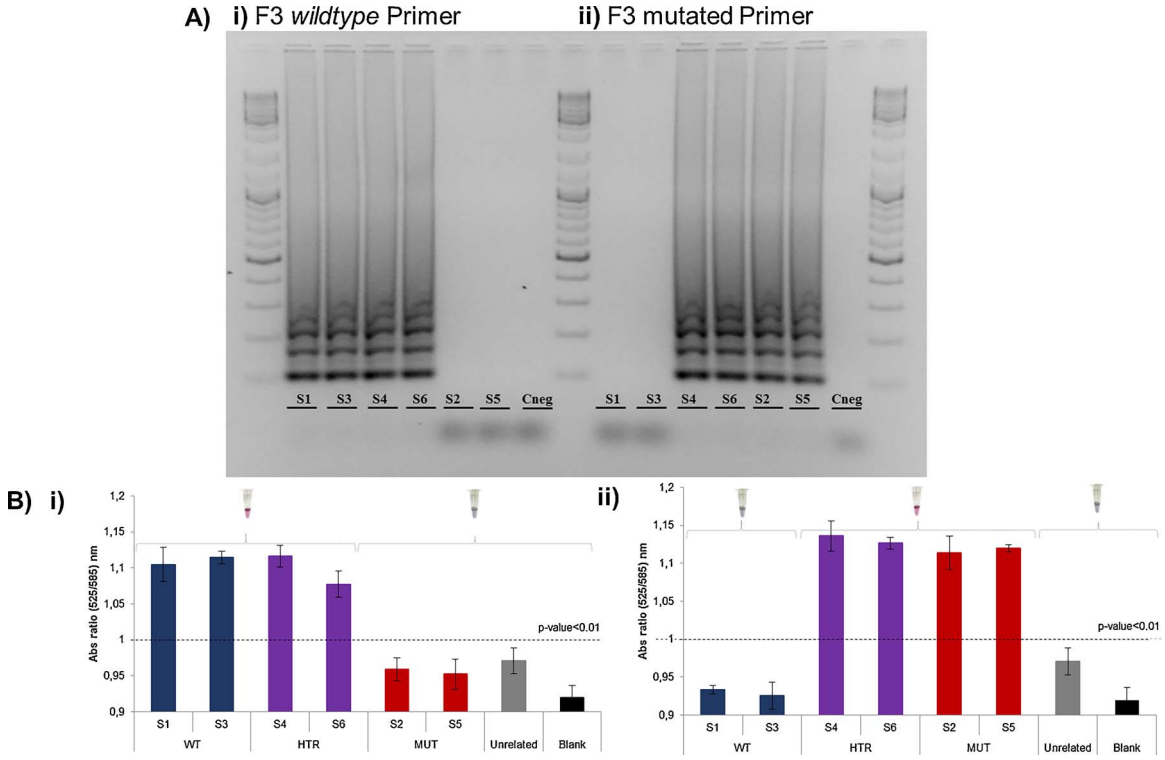

Fig. 3. Characterization of the SNP −13910C/T (dbSNP rs#: 4988235) in biological samples mediated by AS-LAMP and the non-cross-linking approach.
A) i) AS-LAMP biological samples amplification using the F3 *wildtype* primer; ii) AS-LAMP samples amplification using the F3 mutated primer. B) Au-nanoprobe aggregation as measured by ratio of aggregation (ratio of SPR intensity at 525 and 585 nm). Error bars represent the standard deviation of three independent assays. The horizontal line represents the threshold of 1 considered for discrimination between positive (rAbs ≥ 1) and negative (rAbs < 1) result. A representative colorimetric result for the Au-nanoprobe is showed for each result bar – red, positive result; blue/purple, negative result. i) AS-LAMP amplification with F3 *wildtype* detection; ii) AS-LAMP amplification with F3 mutated detection.

technique, Sanger sequencing (average cost of 10–20€ per analysis).

4. Conclusions

We have demonstrated the integration of AS-LAMP and Au-nanoprobes for the detection of SNP in a simple and straightforward approach suitable for medium throughput SNP analysis that requires limited equipment and no specialized personnel. The use of this method to perform a full genotype characterization of a biological sample can be attained under 3 h with an overall cost under €2 per SNP, this value was calculated based on the price of each reagent, how much was used and necessary equipment for the assay. Furthermore, the implementation of isothermal amplification reaction allows the use of this approach at point-of-need, since no thermal cycling apparatus nor dedicated equipment is needed. Integration of AS-LAMP with the Au-nanoprobe detection scheme allowed detection of an SNP associated to lactose intolerance, which may be of use to screen patients with a high degree of sensitivity and specificity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.btre.2017.10.003.

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