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Loop-Mediated Isothermal Amplification and its Application in Rapid Detection of Microorganisms

Review Article

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Abstract:

Loop-Mediated Isothermal Amplification (LAMP) is a new nucleic acid amplification technique developed by Japanese scientist Notomi [1] in 2000, which is concerned by, scholars all over the world and relevant government departments. In just a few years, LAMP has been successfully applied to the detection of diseases caused by various viruses, bacteria and parasites, safety inspection of food and cosmetics and rapid diagnosis of import and export quarantine. The advantages of LAMP method are as follows: high sensitivity (2-5 orders of magnitude higher than traditional PCR method); short reaction time (30~60min can complete the reaction); no special instruments are required for clinical use; easy to operate. Disadvantages of LAMP method: high sensitivity, easy to form aerosol pollution once opened, coupled with the fact that most domestic laboratories cannot be strictly divided, the problem of false positive is more serious, the requirement of primer design is relatively high, and the genes of some diseases may not be suitable for LAMP method. This review mainly describes the principle, types, application fields of loop-mediated isothermal amplification, and the characteristics of Bst DNA polymerase, detection methods of amplification products andother related aspects.

Keywords: Loop-Mediated Isothermal Amplification; Isothermal Amplification Technique; Bst DNA polymerase; Microorganisms; Detection

Introduction

Loop-Mediated Isothermal Amplification (LAMP) is a new nucleic acid amplification technique developed by Japanese scientist Notomi [1] in 2000, which can amplify the target gene quickly and efficiently at a constant temperature (60 ~ 65℃). In 2002, Nagamine [2] proposed to speed up the whole amplification process by adding loop primers to the reaction system, so that the amplification time was reduced by half. After continuous development and improvement, LAMP technology has evolved into a variety of isothermal amplification techniques which could be used as a simple, rapid, accurate and low-cost diagnostic tool for the detection of infectious and non-infectious diseases. It is widely used in foodborne pathogens detection, pathogen detection and other fields. This review mainly describes the principle, types, application fields of loop-mediated isothermal amplification, and the characteristics of Bst DNA polymerase, detection methods of amplification products and other related aspects.

The principle of LAMP technology

The reaction process of LAMP

The Loop-Mediated Isothermal Amplification (LAMP) mainly relies on 4 or 6 specific primers for 6

or 8 independent target gene sequences and Bst DNA polymerase with strand replacement activity to amplify the target gene efficiently and specifically at a constant temperature (60 ~ 65℃). The primers of LAMP are mainly based on F3c, F2c and F1c regions at the 3'end of the target gene, and four specific primers for 6 different loci of B1, B2 and B3 regions at the 5'end, including Forward Inner Primer (FIP), Backward Inner Primer (BIP), Forward Outer Primer (F3), and Backward Outer Primer (B3). FIP is mainly composed of F2 region which is complementary to the F2c region at the 3'end of the target gene and F1C region which is the same as the F1c region at the 5' end of the target gene. BIP is mainly composed of B1C region which is the same as B1c region at 5' end of the target gene and B2 region which is complementary to B2c region at 3' end of target gene. F3 is mainly composed of F3 region which is complementary to F3c region of target gene. B3 is mainly composed of B3 region which is complementary to the B3c region of the target gene.

The LAMP reaction process is mainly divided into two parts: the formation of the initiator of cyclic amplification and the stage of strand replacement reaction. The principle is showed in Figure 1. First of all, the Figure 2 sequence of the forward inner primer (FIP) binds to the F2c region of the template and starts the chain replacement synthesis under the action of Bst DNA polymerase. The forward outer primer (F3) binds and extends the F3c region of the template to replace the complementary strands connected by FIP. The F1C on FIP and F1 on this single strand are complementary structures and form an annular structure with self-base pairing. Using this strand as a template, the backward inner primers (BIP) and backward outer primer (B3) successively initiated a reaction similar to that of FIP and F3 to form a dumbbell-like single strand. Taking the F1 region of 3 'end as the starting point, DNA synthesis was carried out to form a stem-ring structure. Then, using the stem-ring structure as the template, FIP combines with F2c region to start the strand replacement process, and the dissociated single-stranded nucleic acid chain will also form a stem-loop strand structure, and the extension and strand replacement process of DNA synthesis will be carried out with this strand as the template and the B1 region of 3'end as the starting point to form two new DNA single strands with different length of stem-ring structure. BIP hybridize with them to start a new round of amplification. In this way, the target gene can be amplified to 109 copies in a very short period of time.

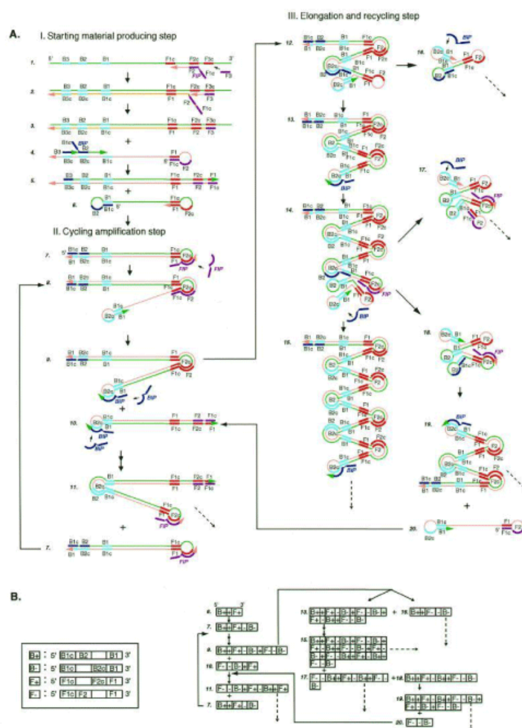

Figure 1: The principle of LAMP.

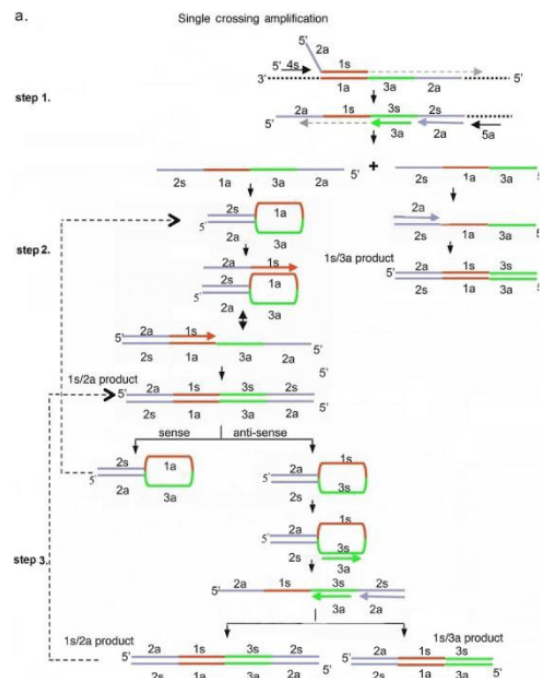

Figure 2: The principle of CPA.

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Bst DNA polymerase

The large fragment of Bst DNA polymerase is part of the DNA polymerase protein of Bacillus stearothermophilus. The large fragment is produced by Escherichia coli, which contains the gene fusion of Bacillus stearothermophilus DNA polymerase gene and Escherichia coli maltose binding protein gene. The region encoded by this gene lacks the 5’→3’ exonuclease structural domain, so the Bst DNA polymerase contains the activity of 5’→3’ polymerase, however, there is a lack of 5’→3’ exonuclease activity. Compared with other DNA polymerase, Bst DNA polymerase has stronger thermal stability, chain replacement activity, high reaction progression, excellent band homogeneity, high accuracy and polymerase activity, so it is more suitable for isothermal amplification [3-6].

Bst DNA polymerase is mainly used in whole genome amplification and isothermal amplification, such as LAMP [1], cross priming amplification (CPA) [7], rolling circle amplification (RCA) [8] and so on. The activity of Bst DNA polymerase varies with temperature. The activity of the polymerase is less than 15%, about 40% and close to100% at the reaction temperature of 37℃ 50℃ and 60 ~ 65℃, respectively. When the reaction temperature is more than 70℃, the polymerase will lose its activity, and the polymerase can be completely inactivated when heated at 80℃ for 10 min.

Types of isothermal amplification techniques

Isothermal Amplification technique based on Bst DNA polymerase: LAMP is an isothermal amplification technique using Bst DNA polymerase with strand replacement function as a key enzyme. Since its advent in 2000, there have been many new techniques based on Bst DNA polymerase, such as loop-mediated isothermal amplification (LAMP) [1], Cross Priming Amplification(CPA) [9], and Rolling Circle Amplification(RCA) [10] and so on. These methods mainly depend on the specially designed primers and the Bst DNA polymerase with strand replacement function for rapid and accurate nucleic acid amplification at a constant temperature. And at present, these techniques have been widely used in pathogen detection, transgenic crop detection, foodborne microbial detection and so on.

The cross priming amplification (CPA) system mainly consists of two cross primers (5’→3’end, 2a1s and 1s2a) which recognize the same sequence, two stripping primers (5’→3’end, 3s and 4a), a probe and a DNA polymerase with the function of strand replacement. The principle of CPA is briefly showed in Figure 2. A brief description is as follows: forward cross primers (2a1s) bind with the master chain and extend. At the same time, the stripping primer (3s) stripped the sub chain from the master chain under the action of strand replacement enzyme to obtain the amplification product with cross primer locus. Using this as a template, forward cross primers (2a1s) and reverse cross primers (1s2a) were hybridized, extended and stripped one after another, which can produce more sub chains containing primer hybridization sites, accelerate the reaction process, and obtain a large number of amplification products in a short time. According to the different number of cross primers in the system, CPA can be divided into single cross priming amplification (Single crossing CAP) and double cross priming amplification (Double crossing CAP). Compared with ordinary PCR, CPA is easy to operate, requires simple instruments and only needs ordinary metal bath or ordinary water bath to complete the reaction. Compared with LAMP, the result of CPA is more objective, direct and observable, which avoids the subjectivity of LAMP in judging the results and avoids the pollution problems in the process of LAMP and ordinary PCR. Gene detection can be extended to grass-roots laboratories or economically underdeveloped areas by using CPA technique. At present, it has been widely used in the detection of agricultural diseases and insect pests, transgenic crops, pathogenic bacteria (such as Staphylococcus [11], Salmonella [12], Vibrio cholera [13] and so on. However, the specificity and sensitivity of CPA are significantly lower than those of LAMP and ordinary PCR, which limits the application of CPA.

Rolling circle amplification (RCA), established in the mid-1990s [10], is an isothermal amplification technique based on the development of rolling loop replication of DNA molecules in circular pathogenic organisms in nature. Using annular DNA as the template, RCA converts dNTPs into single-strand DNA, which contains hundreds of repetitive template complementary fragments, under the catalysis of enzymes with the assistance of a short DNA primer (complementary to part of the cyclic template). According to the number of primers added in the reaction system, RCA can be divided into linear RCA, hyper branched RCA and multi-primer RCA [14]. As one of the isothermal amplification techniques, RCA has been used in circular DNA amplification, single nucleotide polymorphism (SNP), pathogen and miRNA detection [15-17]. RCA can

not only directly amplify DNA and RNA, but also amplify the signal of target genes with a sensitivity of one copy of nucleic acid molecules. Compared with ordinary nucleic acid amplification technology, RCA is carried out at room temperature, which avoids the effect of DNA degradation on the quality of amplification products at high temperature. At the same time, it is widely used because of its high specificity, high sensitivity, rapidity, simplicity and high throughput determination [18]. However, the key of this technology is to construct a single-strand loop, which is complex to construct, and the polymerase consumed in the reaction is expensive. The redundant probes and templates in the reaction system will affect the detection limitation, and the reaction process takes a long time. So it is difficult to meet the needs of rapid detection.

Other isothermal amplification techniques: Enzymatic Unwinding/Primer Annealing Amplification Reactions, such as Recombinase Polymerase Amplification (RPA) [19], Helicase-Dependent Amplification (HDA) [20]. HDA is a method for amplification by simulating DNA replication in vivo, however, HDA is limited by the speed of helicase unwinding and can only be used to amplify short sequences. RPA is not suitable for the detection of short target sequences, and the reaction conditions are harsh. The similarity between HDA and RPA is that the target gene can be amplified by unwinding double-stranded DNA by helicase or by recombination enzyme-primer complex to specifically identify the target sequence on double-strand DNA and promote the combination of primers with template sequence.

Single strand cleavage enzyme-assisted nucleic acid amplification, such as niking endonuclease-mediated amplification (Niking Enzyme Amplification Reaction, NEAR) [21], and Strand Displacement Amplification (SDA) [22]. These methods are mainly assisted by specially designed primer sequences or enzymes with special functions to form new primer binding sites at room temperature, so as to ensure the continuity of the amplification reaction.

Reactions based on RNA transcription, such as Nucleic Acid Sequence-Based Amplification (NASBA) [23], Transcription Mediated Amplification (TMA) [24] and so on. The amplification reaction of NASBA is mediated by two specific primers and catalyzed by three enzymes: AMV reverse transcriptase, phage T7RNA polymerase and ribonuclease H. However, the enzyme involved in this method has poor thermal stability and the effective amplification fragment is short. The technical principle of TMA is basically the same as that of NASBA, but the difference between NASBA and TMA is that TMA uses T7RNA polymerase and MMLV reverse transcriptase but NASBA does not.

Judgement methods and influencing factors of LAMP

There are many methods to judge the results of LAMP reaction, including fluorescence quantitative method, naked eye observation of color change, probe method, agarose gel electrophoresis method, turbidimetric method and so on.

Fluorescence quantitative method: Nucleic acid dye indicators such as SYBR Green I [25], Gene Finder TM and SYTO-9 [26] were added to the reaction system. The reaction system with low concentration of SYBR Green I can be used to judge the reaction results by fluorescence quantitative method (instrument is needed), but SYBR Green I can inhibit the LAMP reaction to a certain extent. Some scholars add SYTO-9 to the reaction system and draw lessons from the real-time fluorescence quantitative PCR to realize the real-time monitoring of LAMP reaction. And the effect of SYTO-9 is similar to that of SYBR Green I.

Naked eye observation of color changes: Metal ion indicators such as Hydroxynaphthol Blue (HNB) [27], Calcein [28] etc., or nucleic acid dyes such as SYBR Green I [25], or PH indicators such as neutral red are added to the reaction system in advance. The color of the reaction system with hydroxynaphthol blue would change from violet to sky blue when the target gene exists. At the same time, hydroxynaphthol blue had no significant effect on the sensitivity of the LAMP. In the LAMP test of the reaction system with Calcein and manganese ions, the negative was brown, the positive was yellowish green, and the end point was clear, which had a great advantage in the naked eye judgment of the detection results. But the addition of manganese ions in the reaction system greatly reduce the sensitivity of the detection. The reaction system with high concentration of SYBR Green I can be judged by the naked eye under ultraviolet lamp or sunlight. If it contains amplification products, the reaction mixture turns green; on the contrary, the orange color of SYBR Green I is kept unchanged. If neutral red was added into the reaction system, the detection results can be visually

judged according to the changes of PH before and after the reaction (the positive result was pink after the reaction, and the negative result was yellow). The idea of this method is novel, and the result judgment is relatively easy, but the operation is more complicated than other methods.

Probe method: Inspired by the TaqMan probe fluorescence quantitative PCR technology, some scholars introduced TaqMan probe into LAMP, thus realizing the direct detection of amplification products and solving the problem of non-specific amplification in LAMP reaction fundamentally. The LAMP amplification system based on probe method (figure 3) reported in the literature adds a probe Fd (usually disconnecting a luminescent group at the 3 'end of F1), while the 5' end of FIP is modified by quenching group, such as IB, BHI and so on [29,30].

Agarose gel electrophoresis: Agarose gel electrophoresis can be used for all the detection results judgement of LAMP reaction. After LAMP amplification, the amplified products were added to agarose gel, and compared with MARKER by gel electrophoresis [31]. The appearance of target bands was observed by gel imaging system, and then the negative or positive results of LAMP reaction were judged.

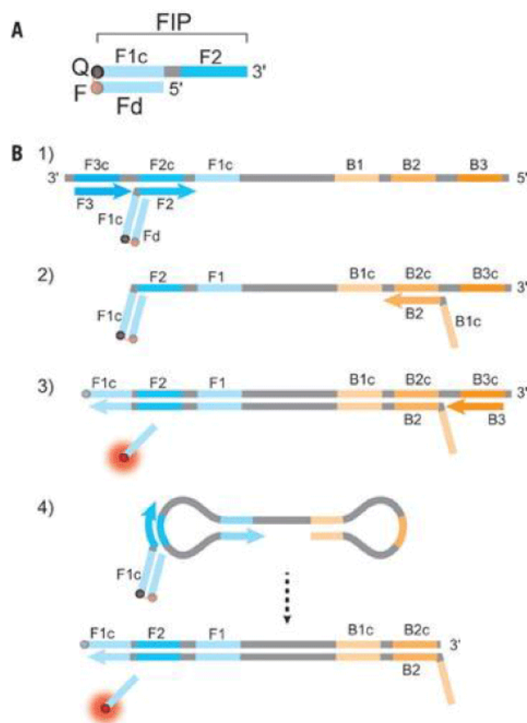

Figure 3: The principle of probe method (29).

Turbidimetric method: Magnesium ion exists in the reaction system, which produces pyrophosphate radical in the process of dNTPs binding. Pyrophosphate radical forms magnesium pyrophosphate precipitation with magnesium ion [32]. After the reaction process is over, magnesium pyrophosphate precipitation can be seen at the bottom of the positive reaction tube by instantaneous centrifugation directly. However, there is a certain subjectivity in naked eye observation. With the development of technology, turbidimeter [33] can be used to judge the results more accurately.

Application and Evaluation of LAMP

Loop-mediated isothermal amplification (LAMP) has been widely used in pathogen detection, foodborne pathogen detection and transgenic crop detection because of its simplicity, rapidity and high specificity.

2C.1 Application in the field of medicine

In the medical field, LAMP is mainly used for the detection of pathogenic microorganisms such as bacteria and viruses. Batule [34] made a ready-to-use virus RNA extraction and detection device based on paper, which can be used to detect mosquito-borne diseases such as Zika, dengue and chikungunya fever from complex clinical samples with limited resources. Yi Zhou [35] et al,. greatly improved the specificity of LAMP by adding a high-fidelity DNA polymerase to the LAMP reaction system, and realized the simultaneous detection of four serotypes of dengue fever. Wang Yichao and others realized the rapid detection of Ureaplasma Urealyticum by designing DNA primers for Ureaplasma Urealyticum, and the sensitivity was 1000 times higher than that of ordinary PCR. Ngan Le Thi [36] et al, developed a real-time reverse transcription loop-mediated isothermal amplification technique using quenching primers, which provides a powerful tool for molecular diagnosis of clinical seasonal influenza virus. Takano Chika [37] established a simple and rapid LAMP method for the specific detection of pneumococcal pcv13 serotype, which is of great help to promote the epidemiological study of pneumococcal serotyping. Fernández-Soto Pedro [38] established a new LAMP method called SmMIT-LAMP for the detection of Staphylococcus mantis in clinical urine samples, which provides a direction for the detection of pathogens in poor and resource-limited areas. Shirzad Fallahi [39] et al, used the LAMP method to detect Candida albicans and compared it with the conventional diagnostic method. Zheng Yingying [40] et al, used LAMP technology

in the detection of Helicobacter pylori vacuolating toxin A (VcaA), which greatly reduce the difficulty of detection and has a good clinical application prospect. Lisa Becherer [41] et al, used TPHD-LAMP technique to detect Treponema pallidum and Plasmodium duodenum at the same time, which provided a new reference direction for molecular diagnosis of yaws. Yuhan Yao [42] et al, established a reverse transcriptional loop-mediated isothermal amplification technique for rapid identification of four tumor stem cell-specific biomarkers to assess the potential risk of metastasis in hepatocellular carcinoma (HCC). Gunjan Goyal [43] and other scholars used LAMP technology to detect the cox1 gene of Taenia saginata, which greatly simplifies the detection method of cerebral cysticercosis and is a promising method for the diagnosis of cerebral cysticercosis by cox1 gene detection. Andrea Vergara [44] and other scholars used LAMP technology to quickly identify the pathogens involved in hospital-acquired pneumonia, which is of great significance for the early diagnosis of Hospital-Aquaired Pheumonia (HAP) patients and the early application of antibiotics. De Paz, H. D [45] and other scholars used LAMP technology to detect invasive Streptococcus pneumonia, its accuracy is similar to traditional PCR, and the detection time can be shortened by five times, so it has a good prospect of clinical application. Cornelia Reuter [46] and other scholars used LAMP technology for the rapid detection of Legionella pneumophila. In addition to the above applications, LAMP can also be used for the detection of other pathogenic microorganisms.

Other applications

By optimizing the reaction system, Deguo Wang [47] et al, used LAMP to detect African classical swine fever virus accurately and specifically. Jeong-Eun Lee [48] et al, established a LAMP method using molecular beacons for the detection of foodborne pathogenic bacteria Vibrio parahaemolyticus. SudaratLedlod [49] et al, established a duplex lateral flow dipstick (DLFD) method based on LAMP technique for the detection of Listeria monocytogenes. This method has high specificity and sensitivity. Simon A [50] et al, used loop-mediated isothermal amplification to detect Bovicola virus in sheep by colorimetry, so that LAMP may become a fixed-point diagnostic tool for monitoring sheep and sheep mycobacteria population. Ji Jun [51] et al, designed four specific primers according to the conserved region of duck hepatitis B virus (DHBV) gene to achieve rapid and low-cost detection of duck hepatitis B virus. Deng Y [52] et al, established a multiloop-mediated isothermal amplification method for rapid detection of Bacillus cereus and Staphylococcus aureus. This is the first time to report the application of multiple loop-mediated isothermal amplification technique to detect Bacillus cereus and Staphylococcus aureus. It can be seen that LAMP technology has been widely used in foodborne pathogens and animal pathogens detection and many other aspects.

Prospect of LAMP

Since the emergence of loop-mediated isothermal technology (LAMP), it has been widely concerned by many scholars. With the deepening of research, other technologies based on LAMP have gradually emerged, such as RT-LAMP, multiple loop-mediated isothermal amplification, multiple internal primer loop-mediated isothermal amplification (MIP-LAMP), real-time fluorescent LAMP, real-time turbidity LAMP, traditional microfluidic LAMP, digital microfluidic LAMP and so on. The methods of judging the amplification results have gradually developed from the initial agarose gel electrophoresis to some more simple methods such as real-time fluorescence method, real-time turbidimetric method, visual colorimetry, probe method and so on. With the development of LAMP technology, it has been widely used in many fields because of its high specificity, high sensitivity and simple operation. However, there are still a series of problems in LAMP. Agarose gel electrophoresis needs to open the tube cover for detection, which is very easy to cause aerosol pollution and false positive results. The primer design of LAMP technology is more complex and time-consuming. Although the operation process of LAMP technology is relatively simple, but the previous nucleic acid extraction process is more complex, which greatly increases the time-consuming of LAMP. Visual colorimetry and visual turbidimetry exists subjectivity, so the judgment of the amplification results is not objective. The target sequence of LAMP amplification is very short, generally within 150-300bp, so long-strand DNA synthesis cannot be carried out. The recovery and sequencing of LAMP products is still very difficult, which cannot be sequenced directly like ordinary PCR products. At the same time, LAMP can only detect the existence of the target gene, but cannot achieve the quantitative detection of the target gene. Moreover LAMP products cannot be used for cloning, because LAMP products are extremely complex and irregular amplification mixture. And the biggest problem of LAMP technology is that it is easy to produce non-specific amplification in the reaction

process, which has a great impact on the results of LAMP reaction. Therefore, the future research direction of LAMP technology can focus on solving the above problems. Although there are a series of problems mentioned above, the ability of clinical promotion of LAMP technology is not optimistic, but LAMP technology still has a broad prospect of clinical application. On the basis of current research, the reaction system can be further optimized. In the future, LAMP technology is expected to become a conventional method of gene amplification, and gradually popularized to clinical practice.

Conclusion

Loop-mediated isothermal amplification (LAMP) is developed as a new type of nucleic acid amplification technology, its outstanding feature is that it can amplify nucleic acid rapidly under constant temperature, omiting the process of denaturation, annealing and extension of ordinary PCR, which not only saves time, but also has relative low requirements for experimental instruments, so it can meet the needs of on-site bedside detection in grass-roots health institutions. With its various advantages gradually discovered by many scholars, it is playing a more and more important role in more and more fields.

Acknowledgments

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