Loop-Mediated Isothermal Amplification Method for Detection of Human Papillomavirus Type 6, 11, 16, and 18

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A new method was developed for detection of human papillomavirus (HPV) by loop-mediated isothermal amplification (LAMP), which was compared with the polymerase chain reaction (PCR), and real-time PCR for specificity and sensitivity. All initial validation studies with the control DNA proved to be type-specific. In order to evaluate the reliability of HPV type-specific LAMP detecting HPV DNA from clinical samples, tissue specimens were obtained from 27 patients with external genital polypoid lesions. The histologic diagnoses included condyloma acuminatum (n = 21), Bowenoid papulosis (n = 2), seborrheic keratosis (n = 2), epidermolytic acanthoma (n = 1), and hairy nymphae (n = 1). HPV-6 DNA and HPV-11 DNA were detected in 18 and 3 of 21 condylomata acuminata, respectively, and there was no simultaneous infection. HPV-16 DNA was detected in one of two Bowenoid papuloses. HPV DNA was not detected in the seborrheic keratoses, epidermolytic acanthoma, and hairy nymphae. These results correlated perfectly with those from real-time PCR analysis. Most positive samples contained high copy numbers of HPV DNA. HPV-11 DNA was detected in one case that could not be detected by PCR. The average reaction time was about 59 min. There was a linear correlation between the genome quantity and reaction time to reach the threshold. The LAMP method has an additional advantage as a quantitative method, and is superior in terms of sensitivity, specificity, rapidity, and simplicity, and can potentially be a valuable tool for the detection of HPV DNA. J. Med. Virol. 79:605–615, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: human papillomavirus (HPV); loop-mediated isothermal amplification (LAMP); polymerase chain reaction (PCR); real-time PCR

INTRODUCTION

Human papillomavirus (HPV) is a small DNA virus which belongs to the family Papovaviridae, and to date, more than 100 types of HPV have been identified [Syrjanen, 2003]. Condyloma acuminatum is a benign tumor caused by low risk/mucous membranes type of HPV such as HPV-6 or 11 infecting the cutaneous regions of the genital and anal areas [Wang, 1993]. A diagnosis is usually made clinically, but there are many tumors that can occur in the skin and mucous membranes. Therefore, clinical diagnosis may not be sufficient, and histopathological diagnosis and virological testing are necessary. Moreover, since high-risk types of HPV associated with cervical cancer and Bowenoid papulosis have been detected, identification of genotypes is preferable [zur Hausen, 2001]. Virological testing includes in situ hybridization (ISH), Southern blot hybridization method, dot blot hybridization, polymerase chain reaction (PCR), and real-time PCR [Lindh et al., 1992; Brown et al., 1993; Oliveira et al., 1994; Qu et al., 1997; Tucker et al., 2001]. However, detectability using ISH is low. In addition, PCR and real-time PCR need specific expensive equipment such as a thermal cycler, and these methods have not yet become common procedures in hospital laboratories. The loop-mediated isothermal amplification method (LAMP) is a cheap, rapid, and simple gene amplification method that was developed originally by Notomi et al. [2000] as an amplification method instead of PCR, attaining amplification efficiency and sensitivity similar to or higher than PCR. The reaction proceed between 63 and 65 °C without thermocycling, and all procedures are completed in one step in about an hour when detecting the
amplification by the appearance of turbidity in the reaction process. Recently published reports have suggested that LAMP could be an effective method for the rapid diagnosis of infectious diseases [Iwamoto et al., 2003; Kuboki et al., 2003; Maruyama et al., 2003; Parida et al., 2004]. In this study, a LAMP-based HPV type-specific DNA amplification method was developed and were compared its specificity and sensitivity with PCR and real-time PCR.

MATERIALS AND METHODS

Patients

Twenty-seven patients (19 males and 8 females; mean age, 39.1 [range, 20–80 years]) with external genital polypoid lesions entered the study at Jikei Aoto Hospital outpatient clinic between 2004 and 2005. The Ethical Review Board of the Jikei University School of Medicine approved the study, and all participants signed a written informed consent. The study was conducted according to the Declaration of the Helsinki Principles.

Tissue Processing

Biopsy specimens measuring 3 mm in diameter were taken from two lesions. One was processed for routine histopathological diagnosis. The other was stored –80°C for DNA extraction.

DNA Extraction

DNA was extracted from the specimen using QIAamp DNA Kit (Qiagen, Chatsworth, CA). After extraction, DNA was eluted in 200 μl distilled water and was stored at –20°C.

A Locations of target sequences in HPV-6, -11, -16, and -18

![Fig. 1. The locations and names of the target sequences used as primers for HPV type-specific LAMP within the E6 region of HPV-6, the E6 region of HPV-11, the E7 region of HPV-16, and the E6 region of HPV-18 (A). Names and sequences of each primer for HPV type-specific LAMP are shown (B). B2c, sequence complementary to B2; F1c, sequence complementary to F1.]

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Preparations of HPV DNA Templates for Control

To determine the specificity of type-specific LAMP method, 13 types of cloned HPV DNAs, HPV-1a, -2, -3, -5, -6, -10a, -11, -16, -18, -31, -33, -35, and -58, were integrated in pBR322 and were cloned by colonic bacillus HB101. The plasmids were refined and extracted using QIAlep Spin Miniplep Kit (Qiagen). After extraction, DNA was eluted in 50 μl distilled water and was stored at -20°C. In addition, to determine the sensitivity of type-specific LAMP method, the DNA concentrations of HPV-6, -11, -16, and -18 were analyzed on a DyNA Quant 200 fluorometer (Amesham Pharmacia Biotech, Piscataway, NJ), and the serial dilutions of each standard (HPV-6, -11, -16, and -18) were prepared to cover the range of 10^7 to 10 copies/tube.

HPV Type-Specific LAMP

Specifically, the LAMP method requires a set of four primers (B3, F3, BIP, and FIP) to recognize a total of six distinct target DNA sequences (B1–B3, F1–F3) within the target DNA. LAMP primers for E6 region of HPV-6, E6 region of HPV-11, E7 region of HPV-16, and E6 region of HPV-18 were designed, using the Primer Explorer V Software (FUJITSU, Tokyo, Japan). The location and sequence of each primer in the target DNA sequences are shown in Figure 1. LAMP reactions were undertaken with a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). After turbidimetry, the LAMP products were subjected to a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). Reaction mixtures (25 μl) contained 1.6 μM each of inner primer (FIP and BIP), 0.2 μM each of outer primer (F3 and B3), 2 × reaction mix (12.5 μl), Bst DNA polymerase (1 μl), and 5 μl of each sample. The mixtures were incubated at 63°C for 120 min. Next, turbidity was measured by TERMAMECS LA200 (Teramecs, Kyoto, Japan). The cutoff value of turbidity used to distinguish negative from positive samples was at 0.1, higher than the mean plus three SD of the turbidity of five negative samples. After turbidimetry, the LAMP products were subjected to a Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). Reaction mixtures (25 μl) contained 1.6 μM each of inner primer (FIP and BIP), 0.2 μM each of outer primer (F3 and B3), 2 × reaction mix (12.5 μl), Bst DNA polymerase (1 μl), and 5 μl of each sample. The mixtures were incubated at 63°C for 120 min. Next, turbidity was measured by TERMAMECS LA200 (Teramecs, Kyoto, Japan). The cutoff value of turbidity used to distinguish negative from positive samples was at 0.1, higher than the mean plus three SD of the turbidity of five negative samples.
to electrophoresis on a 1.5% SeaKem™ ME agarose (Cambrex Bio Science, Rockland, ME) gel stained with ethidium bromide. To avoid contamination between the samples, DNA extraction and LAMP were carried out in different rooms, and pipette tips with filters for aerosol prevention were used.

**PCR**

PCR was used for typing of HPV DNA as described by Yoshikawa et al. [1991]. The consensus primers amplifying at least nine HPV types, HPV-6, -11, -16, -18, -31, -33, -42, -52, and -58 were used for this assay, including L1C1 (5'-CGTAAACGTTTTCCCTATTTTTTTTTT-3'), L1C2 (5'-TACCCTAAAATACTCTGTATTTG-3'), and L1C2M (5'-TACCCTAAATACCCCTATATTG-3'). PCR was performed using 1.25U Taq DNA polymerase (TaKaRaBiomedical, Tokyo, Japan) by TaKaRa thermal cycler (TaKaRaBiomedical) with 40 rounds of thermal cycling conditions; degeneration at 95°C for 1.5 min, annealing at 48°C for 1.5 min, extension at 70°C for 2 min. PCR products were confirmed by electrophoresis through 4% NuSieve™ GTG agarose (Cambrex Bio Science) gel stained with ethidium bromide. Next, they were typed on the basis of restriction fragment length polymorphisms (RFLPs) by Dde I and Rsa I.

**A** HPV-6 type-specific LAMP

![Image of HPV-6 type-specific LAMP](image1)

**B** HPV-11 type-specific LAMP

![Image of HPV-11 type-specific LAMP](image2)

Fig. 2. DNAs extracted from HPV-1a, HPV-2, HPV-3, HPV-5, HPV-6, HPV-10a, HPV-11, HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, and HPV-58 were amplified by using HPV-6 (A), HPV-11 (B), HPV-16 (C), and HPV-18 (D) type-specific LAMP to determine method specificity. The detection of LAMP products was assessed by agarose gel electrophoresis. The numbers indicate HPV types. M, 100-bp DNA ladder marker; N, LAMP reaction without HPV DNA.

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Real-Time PCR for HPV-6, -11, -16, and -18

Type-specific real-time PCR was used to measure the quantity of the DNAs of HPV-6, -11, -16, and -18 in each sample. The sequences of primers and probes for E6/E7 region used have been described by Tucker et al. [2001]. PCR reactions were carried out using the TaqMan PCR Kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer’s directions. Standard curves measuring HPV-6, -11, -16, and -18 DNA concentrations were constructed using CT values obtained from serially diluted plasmids, pBR322, respectively, which contain the target DNA sequences. The CT value from each sample was plotted on a standard curve, allowing automatic calculation of the copy number using Sequence Detector v1.6 software (PE Applied Biosystems). Each sample was tested in duplicate; the copy number of each sample is represented as the mean of the two values.

RESULTS

Specificity

The specificity of HPV-6, -11, -16, and -18 type-specific primers was evaluated. HPV type-specific LAMP was performed by each primer on control DNA of 13 different HPV types. Each tube contained 10⁷ copies of HPV DNA. As LAMP products contained several sizes of inverted-repeat structures, positive samples demonstrate a ladder pattern upon agarose gel electrophoresis. HPV type-specific LAMP primers amplified only the respective type of HPV DNA; no LAMP products were detected in reactions carried out with other type of HPV DNAs (Fig. 2).
Sensitivity

The sensitivity of PCR with consensus primer was reported to detect 0.01 pg in the previous report [Yoshikawa et al., 1991]. The sensitivity of HPV-6, -11, -16, and -18 type-specific LAMP methods were determined. Serial dilutions of the pBR322 plasmid to cover the range of $10^7$ to 10 copies/tube were used to determine the detection limit of HPV type-specific LAMP. The sensitivity of HPV-6, -11, -16, and -18 type-specific LAMP determined by turbidity assay were 1,000 copies/tube (Fig. 3). In contrast, the sensitivity of HPV-6, -11, -16, and -18 type-specific LAMP determined by agarose gel electrophoresis were 100 copies/tube, 100 copies/tube, 1,000 copies/tube, and 100 copies/tube, respectively (data not shown).

Clinical Samples

Twenty-seven biopsy tissue specimens (sample numbers 1–27) collected from patients with genital polypoid lesions were examined (Table I). The histologic diagnoses included condyloma acuminatum (n = 21), bowe-

![A HPV-6 type-specific LAMP](image)

![B HPV-11 type-specific LAMP](image)

Fig. 3. To determine the sensitivities of each assay, serial dilutions of HPV-6, -11, -16, and -18 DNAs were amplified by HPV-6 (A), HPV-11 (B), HPV-16 (C), and HPV-18 (D) type-specific LAMP, respectively. The detection of LAMP products was assessed by turbidity assay using a LA-200. The numbers in the figures are the dilution of $10^n$ copies/tube.
noid papulosis (n = 2), seborrheic keratosis (n = 2), epidermolytic acanthoma (n = 1), and hairy nymphae (n = 1). The results of HPV type-specific LAMP were compared with those of PCR and HPV type-specific real-time PCR. The average reaction time was 59 min 9 sec. Among 21 codylomata acuminata samples, 18 (86%) were positive for HPV-6, and the remaining 3 (14%) were positive for HPV-11 by type-specific LAMP. One bowenoid papulosis specimen was positive for HPV-16 by type-specific LAMP. No HPV-18 DNA was detected in any samples. HPV type-specific LAMP amplified the respective HPV genomes and showed no cross-reactivity. HPV DNAs were detected from 22 of 27 (81%) in total, while they were detected from 21 of 27 (78%) by PCR, consisting of 18 HPV-6s, 2 HPV-11s, and 1 HPV-16 by RFLPs of PCR, identical to the results of LAMP. No HPV DNA was detected in samples that were negative by LAMP. Among the three HPV-11 positive samples by LAMP, only sample No. 21 was negative by PCR. Furthermore, the results of real-time PCR correlated perfectly with those of LAMP. Most of the positive samples by LAMP contained high copy numbers of HPV DNA. The correlation between the time (in sec) to reach the threshold >0.1 of turbidity and copy number was analyzed. The result is shown in Figure 4. There was a linear correlation between the genome quantity and reaction time to reach the threshold: y (copy numbers) = -0.001x (sec) + 9.0025.
DISCUSSION

HPV is detectable in condyloma acuminatum and Bowenoid papulosis, and it often becomes a problem as a sexually transmitted disease [Schwartz and Janniger, 1991; Beutner and Tyring, 1997; Beutner et al., 1998; Leung et al., 2005]. In addition, there is a high-risk type HPV that is associated with cervical cancer. Therefore, it is important to identify the genital HPV type from both an epidemiological and a public health standpoint.

Virologic testings include ISH, Southern blot hybridization, dot blot hybridization, and PCR [Yoshikawa et al., 1991; Lindh et al., 1992; Brown et al., 1993; Oliveira et al., 1994]. Recently, several investigations described real-time PCR, which has the additional advantage of being a quantitative method [Cubie et al., 2001; Seth et al., 2005]. However, these methods are time-consuming, require special expensive equipment such as thermal cyclers, and may not be appropriate for testing in a clinical setting.

LAMP developed by Notomi et al. [2000] has been applied for the detection of various types of infectious agents, mainly varicella-zoster virus [Okamoto et al., 2004], SARS coronavirus [Poon et al., 2004], herpes simplex virus [Enomoto et al., 2005], measles virus [Fujino et al., 2005], mumps virus [Okafuji et al., 2005], and influenza virus [Ito et al., 2006]. LAMP proved to be rapid, highly sensitive, highly specific, and simple, suggesting that it might be used for rapid diagnosis.

LAMP for the detection of HPV was developed and the sensitivity and specificity of LAMP were compared with those of PCR and real-time PCR for the detection of HPV. A pair of primers in the E1 region was designed at first, but since there is 90% homology between the PCR products of HPV-6 and HPV-11, cross-hybridization occurred between HPV-6 and HPV-11. In addition, portions of the E1 gene show greater homology and may be deleted often during HPV integration. Therefore, a pair of primers in the E6/E7 region was designed, since portions of the E6/E7 show smaller homology, and are retained and are expressed usually even when HPV integrates into cellular chromosomes. HPV type-specific LAMP amplified only the respective type of HPV DNA with no cross-reactivity. This specificity was confirmed by two independent detection methods, turbidity assay and agarose gel electrophoresis. The detection limit for HPV type-specific LAMP by the turbidity assay was 1,000 copies/tube. In contrast, the detection limit for HPV-6, -11, -16, and -18 type-specific LAMP by agarose gel electrophoresis was 100 copies/tube, 100 copies/tube, 1,000 copies/tube, and 100 copies/tube, respectively. Although the turbidity assay is less sensitive than agarose gel electrophoresis [Yoshikawa et al., 2004], the ease and rapidity of the turbidity assay make it more appropriate for clinical monitoring. Furthermore, this system also minimizes potential contamination because tubes are closed during amplification and detection of amplified DNA.

The reliability of HPV LAMP was evaluated for the detection of viral DNA from clinical samples. Since the turbidity analysis seemed to be the most appropriate test for clinical laboratory use, this assay was used for clinical sample analysis. HPV-6 DNA and HPV-11 DNA were detected in 18 and 3 of 21 condylomata acuminata, respectively, without concomitant infection. HPV-16 DNA was detected in one of two bowenoid papuloses. HPV DNA was not detected in seborrheic keratosis, epidermolytic acanthoma, and hairy nymphae. These

Fig. 4. LAMP reaction times were monitored to reach the threshold >0.1 in turbidity. The correlation between the logarithmic titers of copy number and reaction time is shown.

\[
y = -0.001x + 9.0025 \\
R^2 = 0.872
\]
| Sample no. | Histopathological diagnosis | Typing by PCR  | Real-time PCR (HPV-6) (copies/tube) | Turbidity (sec) | Electrophoresis | Real-time PCR (HPV-11) (copies/tube) | Turbidity (sec) | Electrophoresis | Real-time PCR (HPV-16) (copies/tube) | Turbidity (sec) | Electrophoresis | Real-time PCR (HPV-18) (copies/tube) | Turbidity (sec) | Electrophoresis |
|-----------|-----------------------------|----------------|-----------------------------------|----------------|----------------|-----------------------------------|----------------|----------------|-----------------------------------|----------------|----------------|-----------------------------------|----------------|----------------|
| 1         | CA HPV-6                    | 6,180,000      | 2,856                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 2         | CA HPV-6                    | 626,000        | 3,204                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 3         | CA HPV-6                    | 835,000        | 3,348                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 4         | CA HPV-6                    | 780,000        | 3,456                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 5         | CA HPV-6                    | 221,000        | 3,516                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 6         | CA HPV-6                    | 641,000        | 3,642                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 7         | CA HPV-6                    | 75,900         | 3,690                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 8         | CA HPV-6                    | 255,000        | 3,720                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 9         | CA HPV-6                    | 451,000        | 3,822                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 10        | CA HPV-6                    | 550,000        | 3,828                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 11        | CA HPV-6                    | 129,000        | 3,924                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 12        | CA HPV-6                    | 541,000        | 3,942                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 13        | CA HPV-6                    | 57,500         | 3,978                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 14        | CA HPV-6                    | 88,700         | 4,242                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 15        | CA HPV-6                    | 37,900         | 4,466                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 16        | CA HPV-6                    | 162,000        | 4,646                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 17        | CA HPV-6                    | 14,100         | 4,770                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 18        | CA HPV-6                    | 22,800         | 4,884                             | +              |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 19        | CA HPV-11                   | 0              | 18,700,000                        | 1,830          | +              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 20        | CA HPV-11                   | 0              | 5,860,000                         | 2,226          | +              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 21        | CA HPV-11                   | 0              | 3,140,000                         | 2,244          | +              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 22        | BP HPV-16                   | 0              | 0                                 |                |                | 0                                 |                |                | 13,300,000           | 2,124          | +              | 0                                 |                |                |
| 23        | BP HPV-11                   | 0              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 24        | SK HPV-11                   | 0              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 25        | SK HPV-11                   | 0              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 26        | EA HPV-11                   | 0              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |
| 27        | HN HPV-11                   | 0              | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                | 0                                 |                |                |

CA, condyloma; BP, Bowenoid papulosis; SK, seborrheic keratosis; EA, epidermolysis acanthoma; HN, hairy nymphae
results correlated perfectly with those from real-time PCR analysis. Most of the positive samples contained high copy numbers of viral DNA. HPV DNA was not detected in samples that were negative by LAMP. Among three HPV-11 positives by LAMP, one was negative by PCR. Therefore, the sensitivity of HPV type-specific LAMP was nearly the same as that of real-time PCR and was greater than that of PCR, for the detection of HPV infection, demonstrating the high sensitivity and specificity of HPV type-specific LAMP in the analysis of clinical samples. As for sample No. 23 of bowenoid papulosis, HPV was not detected by the LAMP method, PCR, and real-time PCR, but HPV-56 was detected by the Hybrid Capture II assay. Recently, the detection of various types of HPV has been reported [zur Hausen and de Villiers, 1994]. This LAMP method may not be useful for detection of broad HPV genotypes. However, there was a linear correlation between the genome quantity and reaction time to reach the threshold by the LAMP method, making quantitation of HPV DNA in clinical samples possible. Several studies showed that an increased HPV-16 viral load correlated with the risk for cervical carcinoma [Peitsaro et al., 2002; Ho et al., 2005]. The presence and quantity of HPV DNA are likely to be a reflection of metastasis and may have a prognostic value. Therefore, this assay may be useful to study the epidemiology, pathogenesis, and monitoring vaccine trials of HPV. The average reaction time of HPV type-specific LAMP was about 59 min, not more rapid than that of other viruses, mainly measles virus [Fujino et al., 2005], influenza virus [Ito et al., 2006], or herpes simplex virus [Sugiyama et al., 2005]. Therefore, modification of primer design or induction of loop primers may be necessary to shorten the reaction time in future analyses, as additional loop primers were necessary for the detection of HPV typing and viral load. Precise determination of viral load is important for the evaluation of vaccine efficacy and predicting disease risk. In summary, by setting a type-specific primer in the E6/E7 region, a new type-specific method for the detection of HPV-6, -11, -16, and -18 was developed. The sensitivity of amplification of LAMP for detection of viral DNA was nearly the same as that of real-time PCR. Cross-reactivity was not observed, and reliability of testing with clinical specimens was demonstrated. The LAMP method is superior in terms of sensitivity, specificity, speed, and simplicity, and can potentially be a valuable tool for the detection of HPV DNA in laboratories.

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