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# Rapid and Simple Method for Detecting the Toxin B Gene of *Clostridium difficile* in Stool Specimens by Loop-Mediated Isothermal Amplification

Haru Kato,[1]* Toshiyuki Yokoyama,[2] Hideaki Kato,[3] and Yoshichika Arakawa[1]

*Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases,*[1] *Kumiai Kosei Hospital, Gifu,*[2] *and Toyokawa City Hospital, Aichi,*[3] *Japan*

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**We applied the loop-mediated isothermal amplification (LAMP) assay to the detection of the toxin B gene (*tcdB*) of *Clostridium difficile* for identification of toxin B (TcdB)-positive *C. difficile* strains and detection of *tcdB* in stool specimens. *tcdB* was detected in all toxin A (TcdA)-positive, TcdB-positive ($A^+B^+$) and TcdA-negative, TcdB-positive ($A^-B^+$) *C. difficile* strains but not from TcdA-negative, TcdB-negative strains. Of the 74 stool specimens examined, $A^+B^+$ or $A^-B^+$ *C. difficile* was recovered from 39 specimens, of which 38 specimens were LAMP positive and one was negative. Amplification was obtained in 10 specimens that were culture negative, indicating that LAMP is highly sensitive. The LAMP assay was applied to detection of *tcdB* in DNA extracted by a simple boiling method from 47 of those 74 specimens, which were cultured overnight in cooked-meat medium (CMM). Twenty-two of 24 culture-positive specimens were positive for LAMP on DNA from the culture in CMM. Four specimens were culture negative but positive by LAMP on DNA from CMM cultures. The LAMP assay is a reliable tool for identification of TcdB-positive *C. difficile* as well as for direct detection of *tcdB* in stool specimens with high sensitivity. Detection of *tcdB* by LAMP from overnight cultures in CMM could be an alternative method of diagnostic testing at clinical laboratories without special apparatus.**

*Clostridium difficile* is well known as a cause of pseudomembranous colitis and a principle causative agent of antibiotic-associated diarrhea. Rapid and sensitive laboratory diagnostic testing is highly desirable for appropriate treatment of *C. difficile*-associated diarrhea (26). Two toxins, toxin A (TcdA) and toxin B (TcdB), are involved in the pathogenicity of this organism. The cell culture assay with the neutralization test is still used as a sensitive and specific method to detect TcdB, although the method is not easy to perform, cost-effective, or highly standardized.

A number of commercial tests are available for rapid and simple immunological detection of TcdA alone or of both TcdA and TcdB. However, these tests were found not to be as sensitive as the cell culture assay (6, 19, 22), and the infection caused by TcdA-negative, TcdB-positive ($A^-B^+$) *C. difficile* could not be diagnosed by using only the TcdA detection kit (1, 3, 16, 17, 18). Culture of *C. difficile* is a sensitive and specific method when cultured isolates are tested for TcdA and TcdB production (7). The PCR assay for detecting the toxin genes has been widely used for identification of types of toxin produced by recovered isolates (3, 12, 14, 27). Detection of *tcdA* and/or *tcdB* in stool specimens by PCR (9, 15), nested PCR (2), and real-time PCR (5) has also been developed and evaluated. Although reported to be rapid and sensitive diagnostic methods, they are not necessarily of practical use in clinical laboratories, where special equipment such as a thermal cycler or detection systems are not available.

Recently, loop-mediated isothermal amplification (LAMP) has been developed as a novel method that amplifies DNA with high specificity and simplicity (20, 21). In this study, we evaluated a LAMP method for identification of TcdB production by recovered isolates as well as for direct detection of *tcdB* in fecal specimens. DNA extraction from stool specimens requires some tedious steps to remove amplification inhibitors, making it difficult to do routinely in clinical laboratories. To further simplify the methods, the LAMP method was applied to detection of *tcdB* in DNA extracted by a simple and quick boiling method from stool specimens which were cultured overnight in cooked meat medium (CMM).

## MATERIALS AND METHODS

**Bacterial strains.** The 40 *Clostridium difficile* strains used in this study were clinically isolated at various hospitals in Japan and previously classified into toxinotypes (Table 1) (24). Strains of *Clostridium* species other than *C. difficile* were obtained from the Japan Collection of Microorganisms except for an enterotoxin-positive *Clostridium perfringens* strain, MRY 05-0166, which was recovered from a case of antibiotic-associated colitis (Table 1).

**Stool specimens.** Stool specimens were obtained with the informed consent of patients admitted to five hospitals in Japan, who were given the diagnosis of antibiotic-associated diarrhea or colitis. The stool specimens were frozen at −80°C until transported and tested at the National Institute of Infectious Diseases. All tests were performed in batches on the same day, after a single thawing of the stored specimen.

**Culture.** *C. difficile* was isolated on cycloserine-cefoxitin-mannitol agar (CCMA) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) from stool specimens, which were treated with alcohol for spore selection. *C. difficile* was identified by colony morphology on CCMA and cell morphology after Gram staining. The latex agglutination test detecting glutamate dehydrogenase (Shionogi Pharmaceutical Co., Ltd., Tokyo, Japan) was used to confirm the identification.

The nonrepeating and repeating sequences of *tcdA* were amplified by PCR with primer sets NK3-NK2 (14) and NK9-NK11-NKV011 (11, 12), respectively. The presence of *tcdB* was examined by PCR with primer set NK104 and NK105 (12).

* Corresponding author. Mailing address: Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, 4-7-1 Musashimurayama,Tokyo 208-0011, Japan. Phone: 81-42-561-0771. Fax: 81-42-561-7173. E-mail: cato@nih.go.jp.

TABLE 1. Strains used in this study and results of detection of *tcdB* by PCR and LAMP

| Species (strain no.) | Tcd type | Toxinotype[a] | Detection[b] of *tcdB* by: | | No. of strains studied |
|---|---|---|---|---|---|
| | | | PCR | LAMP | |
| *Clostridium difficile* | $A^+B^+$ | 0 | + | + | 20 |
| | $A^+B^+$ | I | + | + | 1 |
| | $A^+B^+$ | III | + | + | 1 |
| | $A^+B^+$ | IV | + | + | 1 |
| | $A^+B^+$ | IX | + | + | 1 |
| | $A^+B^+$ | XII | + | + | 1 |
| | $A^+B^+$ | XVIII | + | + | 1 |
| | $A^+B^+$ | XIX | + | + | 1 |
| | $A^+B^+$ | XX | + | + | 1 |
| | $A^-B^+$ | VIII | + | + | 5 |
| | $A^-B^+$ | XVI | + | + | 1 |
| | $A^-B^+$ | XVII | + | + | 1 |
| | $A^-B^-$ | NA | − | − | 5 |
| *Clostridium absonum* (JCM 1381) | − | NA | − | − | 1 |
| *Clostridium bifermentans* (JCM 1386) | − | NA | − | − | 1 |
| *Clostridium beijerinckii* (JCM 1390) | − | NA | − | − | 1 |
| *Clostridium histolyticum* (JCM 1403) | − | NA | − | − | 1 |
| *Clostridium novyi* (JCM 1406) | − | NA | − | − | 1 |
| *Clostridium perfringens* (JCM 1290) | − | NA | − | − | 1 |
| *Clostridium perfringens* (MRY 05-0166) | − | NA | − | − | 1 |
| *Clostridium ramosum* (JCM 1298) | − | NA | − | − | 1 |
| *Clostridium septicum* (JCM 8144) | − | NA | − | − | 1 |
| *Clostridium sordellii* (JCM 3814) | − | NA | − | − | 1 |
| *Clostridium sordellii* (JCM 11011) | − | NA | − | − | 1 |
| *Clostridium sporogenes* (JCM 1416) | − | NA | − | − | 1 |
| *Clostridium tertium* (JCM 6289) | − | NA | − | − | 1 |

[a] NA, not applicable.
[b] +, positive; −, negative.

**Fecal TcdB assay.** Stool specimens were tested for TcdB using a Vero cell cytotoxicity assay with a neutralization test with anti-*C. difficile* TcdB serum (TechLab, Blacksburg, VA). The final dilution of stool specimens in each microtiterplate well was 1:100. The cells were examined after both 24 h and 48 h of incubation.

**Detection of *tcdB* by LAMP.** DNA extraction from cultured isolates for a LAMP assay was performed in the same manner as previously described for PCRs of the toxin genes (12). DNA was directly extracted from stool specimens using the QIAamp DNA stool minikit (QIAGEN, Hiden, Germany) according to the manufacturer's instructions. LAMP was also applied to detection of *tcdB* in DNA which was extracted from overnight cultures of stool specimens with cooked meat medium (Becton Dickinson, Sparks, MD). One swab of stool specimens was inoculated into 5 ml of CMM and incubated at 35°C overnight; 1 ml of inoculated broth was centrifuged at 15,000 × *g* for 2 min, and 500 μl of TES (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 50 mM NaCl) was added to the pellet. The suspension was heated at 95°C for 15 min and centrifuged at 15,000 × *g* for 2 min, and the resultant supernatant was used as the template DNA for the LAMP assay.

The six primers used for the LAMP were derived from *tcdB* (4) (Fig. 1). The outer primers were HK101-F3 (5′-GTATCAACTGCATTAGATGAAAC-3′) and HK101-B3 (5′-CCAAAGATGAAGTAATGATTGC-3′); the inner primers were primer HK101-FIP, consisting of HK101-F1c and HK101-F2 (5′-CTGCACCTA AACTTACACCATCTATCTTCCTACATTATCTGAAGGATT-3′), and primer HK101-BIP, consisting of HK101-B1c and HK101-B2 (5′-GAGCTAAGTGAA ACGAGTGACCCGCTGTTGTTAAATTTACTGCC-3′). The loop primers were primers HK101-FL (5′-AATAGTTGCAATTATAGG-3′) and HK101-BL (5′-AGACAAGAAATAGAAGCTAAGATAGG-3′) (Fig. 1).

The LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. We added 2 μl of DNA template to a total volume of 25 μl buffer consisting of 5 pM of each of the outer primers, 40 pM of each of the inner primers, and 20 pM of each of the loop primers. Amplification was performed at 62°C for 60 min, followed by incubation at 80°C for 2 min to terminate the reaction. The increased turbidity was monitored by a real-time turbidimeter, LA-320C (Eiken Chemical Co., Ltd., Tokyo, Japan). The turbidity was calculated based on the ratio of light intensity (intensity of light received by the photodiode/emitted light intensity). A ratio of 0.1 was defined as positive for the LAMP assay (20).

**Detection of *tcdB* and *tcdA* by nested PCRs in stool specimens.** DNA extracted from stool specimens for the LAMP assay was also used as the template for a nested PCR. The primers used for the nested PCR detecting *tcdB* were NK201 (5′-TTTAGATACTACACACGAAG-3′) and NK202 (5′-GCCATTATACCT ATCTTAGC-3′) for the outer primer set and NK104 and NK105 (12) for the inner primer set (Fig. 1), which were derived from *tcdB* (4). A nested PCR

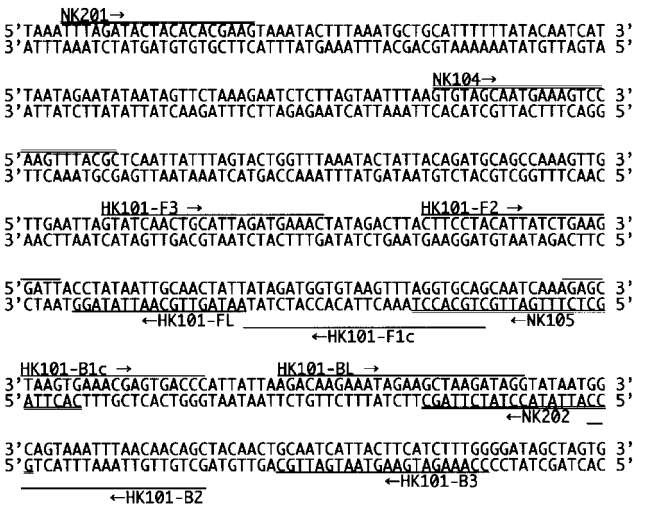


FIG. 1. Oligonucleotide primers used for amplification of *tcdB*. Single-underlined and double-underlined letters indicate the sequences of primers for LAMP and for nested PCR, respectively.

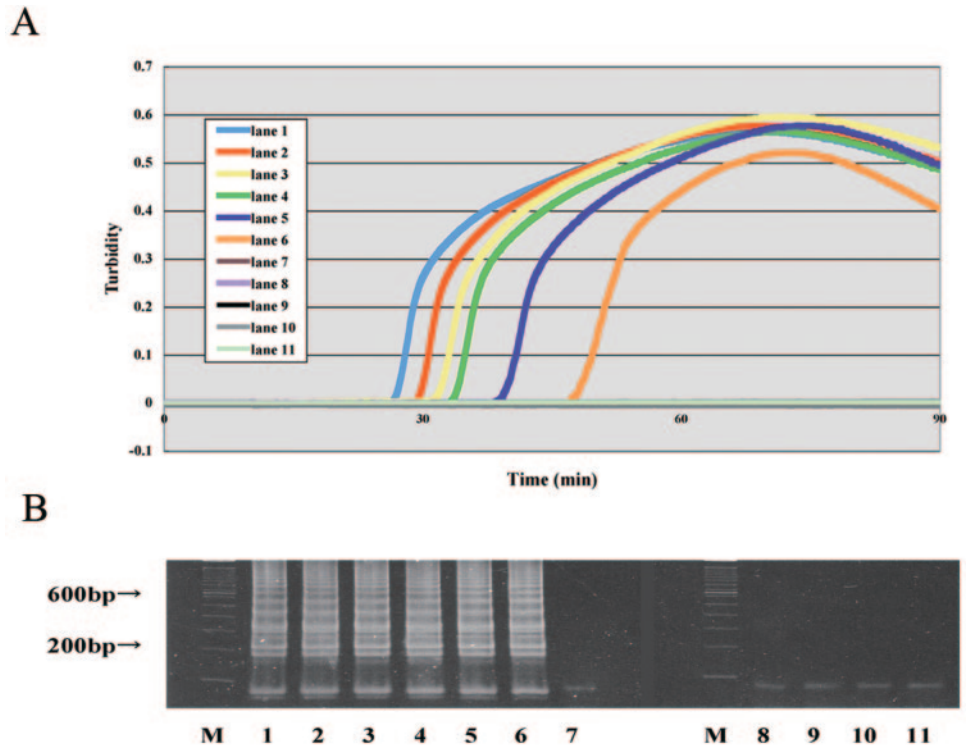


FIG. 2. Real-time detection of turbidity (A) and 5% polyacrylamide gel electrophoresis (B) of amplification products by LAMP. The template extracted from strain VPI 10463 in 10-fold serial dilutions from 50 ng to 50 ag per reaction tube (lanes 1 to 10) was added. Lane 11, negative control; lanes M, 100-bp ladder as a molecular size marker.

detecting the nonrepeating sequences of *tcdA* was performed with primer sets HK5 and HK6 for the outer primer and NK3 and NK2 for the inner primer (13). The nested PCR assay on DNA extracted from stool specimens was performed as described previously (13).

## RESULTS

**Sensitivity and specificity of LAMP.** A total of 40 clinical isolates of *C. difficile* were examined for detection of *tcdB* by LAMP (Table 1). *tcdB* was detected in all 28 $A^+B^+$ and seven $A^-B^+$ isolates representing nine and three different toxinotypes, respectively (23). All five $A^-B^-$ isolates examined were LAMP negative for *tcdB*. The test results by LAMP completely agreed with those by PCR detecting *tcdB* with primer set NK104 and NK105. The LAMP was performed in a 90-min reaction to confirm the specificity in 13 strains of 11 *Clostridium* species other than *C. difficile*, with negative results (Table 1).

DNA was extracted from strain VPI 10463 ($A^+B^+$), and 10-fold serial dilutions from 50 ng to 50 ag of DNA were added to each reaction tube for the LAMP and the nested PCR. Amplification by LAMP was obtained in reaction tubes containing from 50 ng to 0.5 pg of DNA template within 60 min (Fig. 2). Electrophoretic analysis (Fig. 2B) of the final products showed stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops (20, 21). On the basis of the results, the LAMP assay was performed in a 60-min reaction for the following tests. The same serial dilutions of DNA were applied to the nested PCR; the single PCR by primer set NK201 and NK202 was 10-fold less sensitive and the nested PCR was 100-fold more sensitive than the LAMP method (data not shown).

**Direct detection of *tcdB* by LAMP in stool specimens.** The results of detection of *tcdB* by the LAMP assay in DNA extracted directly from stool specimens compared with those of other tests are shown in Table 2. Of 74 stool specimens examined, 68 were available for detection of TcdB by cell culture assay; 32 were positive for the detection of fecal TcdB and 35 were negative, and the test result was nonspecific in the remaining 1. Amplification of *tcdB* by LAMP was obtained in all stool specimens that were positive for fecal TcdB. All 74 stool specimens were cultured for *C. difficile*; 40 were culture positive and 34 were negative. Of 40 isolates recovered from those specimens, 38 were $A^+B^+$, one was $A^-B^+$, and the remaining one was $A^-B^-$. *tcdB* was detected by LAMP on DNA extracted from 38 of 39 stool specimens from which an $A^+B^+$ or $A^-B^+$ *C. difficile* strain was recovered. Direct detection of *tcdB* by LAMP was positive in 10 stool specimens that were negative for *C. difficile* by culture.

The results of direct detection of *tcdB* by LAMP were compared with those by a nested PCR assay. All of the specimens that were positive by LAMP were also positive by nested PCR. The nested PCR detecting *tcdB* generated a PCR product on DNA extracted from 16 stool specimens that were culture negative, of which 10 specimens were positive for LAMP and 6 were negative. A nested PCR detecting *tcdA* was performed on 15 of these 16 specimens, all of which were positive. No specimens were negative for the nested PCR but positive for other tests.

TABLE 2. Results of LAMP assay detecting *tcdB* in DNA extracted from stool specimens and overnight culture in CMM and comparison to other tests

| TcdB detection[a] in stool specimen by cell culture | *C. difficile* culture | | Direct detection of *tcdB* in stool specimen by: | | Detection of *tcdB* from overnight culture in CMM by LAMP | No. of stool specimens |
|---|---|---|---|---|---|---|
| | TcdB detection | Tcd type of isolate | Nested PCR | LAMP | | |
| + | + | $A^+B^+$ | + | + | + | 16 |
| + | + | $A^+B^+$ | + | + | − | 2 |
| + | + | $A^+B^+$ | + | + | ND[c] | 13 |
| Invalid | + | $A^+B^+$ | + | + | + | 1 |
| + | + | $A^-B^+$ | + | + | + | 1 |
| − | + | $A^+B^+$ | + | + | + | 3 |
| − | + | $A^+B^+$ | + | − | + | 1 |
| − | + | $A^-B^-$ | − | − | − | 1 |
| − | − | NA[b] | + | + | + | 2 |
| − | − | NA | + | + | − | 5 |
| − | − | NA | + | + | ND | 1 |
| − | − | NA | + | − | + | 2 |
| − | − | NA | + | − | − | 3 |
| − | − | NA | + | − | ND | 1 |
| − | − | NA | − | − | − | 10 |
| − | − | NA | − | − | ND | 6 |
| ND | + | $A^+B^+$ | + | + | ND | 2 |
| ND | − | NA | + | + | ND | 2 |
| ND | − | NA | − | − | ND | 2 |

[a] +, positive; −, negative. Invalid, test result invalid because of atypical cytotoxic effect.
[b] NA, not applicable.
[c] ND, not done.

**Detection of *tcdB* by LAMP from overnight cultures of stool specimens in CMM.** A total of 47 stool specimens were available for evaluation of the LAMP assay detecting *tcdB* on DNA extracted from overnight cultures of stool specimens in CMM (Table 2). Of the 47 stool specimens examined, 24 were positive for fecal TcdB and/or for culture of *C. difficile* of $A^+B^+$ or $A^-B^+$ on CCMA, of which 22 specimens were positive by LAMP for overnight cultures in CMM and 2 were negative. The two specimens that were LAMP negative in overnight cultures were positive for direct detection of *tcdB* in stool specimens by both LAMP and nested PCR. Amplification by LAMP was obtained on DNA extracted from overnight cultures of four specimens that were negative for both fecal TcdB and *C. difficile* culture on CCMA. The four CMM tubes in which *tcdB* was detected by LAMP were inoculated, and $A^+B^+$ *C. difficile* could be recovered from all four specimens. Of those four specimens, two were positive for direct detection of *tcdB* from stool specimens by both LAMP and nested PCR, and two were positive only by nested PCR.

## DISCUSSION

LAMP is a novel nucleic acid amplification method using DNA polymerase with strand displacement activity and six primers that recognize eight regions on the target nucleic acid, leading to extremely high specificity (20, 21). In the present study, we successfully identified TcdB-positive ($A^+B^+$ and $A^-B^+$) *C. difficile* strains with various toxinotypes by LAMP. Recent reports (1, 11, 16, 17, 18) have demonstrated the clinical significance of $A^-B^+$ strains. Most of the $A^-B^+$ strains are known to belong to toxinotype VIII (23, 24) and produce a variant toxin B ($TcdB_{1470}$) (12, 25). The primers used for the LAMP assay here could detect $tcdB_{1470}$ as well as other variant types of *tcdB* produced by toxinotypes III and IV (24) that could not be detected by real-time PCR (5). No amplification was observed from TcdB-negative ($A^-B^-$) *C. difficile* strains or 13 strains of other *Clostridium* species, including two *Clostridium sordellii* strains, which produce the lethal toxin (TcsL), indicating the specificity of the LAMP. Although the LAMP assay used here cannot distinguish $A^-B^+$ strains from $A^+B^+$ strains, identification of TcdB-positive *C. difficile* should be important for clinical diagnosis.

The LAMP detecting *tcdB* in DNA extracted directly from stool specimens proved to be a reliable assay when the test results were compared with those for detection of fecal TcdB and *C. difficile* culture. Furthermore, amplification was obtained by direct LAMP in 10 specimens that were negative for both fecal TcdB and culture, indicating the LAMP is more sensitive than culture for some specimens. Positive results in nested PCRs for not only *tcdB* but also *tcdA* in LAMP-positive but culture-negative specimens indicate the presence of PaLoc sequences in specimens and the specificity of the LAMP assay. Two of 10 patients from whom the LAMP-positive but culture-negative specimens were obtained were on vancomycin therapy when the specimens were tested, which should be one of the reasons for the lack of *C. difficile* growth. Although the nested PCR proved its high sensitivity, it is not of practical use in clinical laboratories because the procedure is time-consuming and tedious and due concern must be paid to contamination of PCR products.

Although the QIAamp DNA stool minikit is useful for extraction of DNA from stool specimens (2, 9, 13), it is not always practical for clinical laboratories. The LAMP method was applied to the detection of *tcdB* in DNA extracted by a simple

and quick boiling method from stool specimens which were cultured overnight in CMM, and a positive LAMP reaction was successfully obtained for 22 of 24 culture-positive specimens. Two specimens were culture positive but LAMP negative on DNA extracted from CMM culture. This discrepancy might be explained by the existence of amplification inhibitors in samples, because DNA was extracted without any steps for removing inhibitory substances. The heterogeneity of stool specimens also might cause the discrepancy when the specimens contain a low number of *C. difficile* or contain mucus.

Interestingly, four stool specimens that were negative for culture on CCMA were LAMP positive in overnight-cultured CMM. Recovery of *C. difficile* from of these four specimens in CMM indicated that the results of the LAMP with CMM cultures were not false-positives. This simple method using CMM requires neither tedious steps for DNA extraction from stool specimens nor anaerobic incubation equipment, such as an anaerobic chamber, jar, or pouch, making it possible to perform the test at clinical laboratories without special apparatus. In addition, the hands-on time of the procedure is very short, even though the test results are provided on the day after specimen collection.

The LAMP assay is a novel method to amplify DNA under isothermal conditions (20, 21) and has been applied to the identification or detection of some bacteria with high sensitivity and specificity (8, 10). The method is more rapid and easier to perform than a PCR assay and does not require any special equipment, such as a thermal cycler or electrophoresis system. The turbidimeter used in the present study is not needed when the fluorescent detection reagent (Eiken Chemical Co., Ltd., Tokyo, Japan) and UV lamp are available.

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