# Development of an improved rapid BACpro® protocol and a method for direct identification from blood-culture-positive bottles using matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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## ABSTRACT

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been incorporated into pathogenic bacterial identification methods and has improved their rapidity. Various methods have been reported to directly identify bacteria with MALDI-TOF MS by pretreating culture medium in blood culture bottles. Rapid BACpro® (Nittobo Medical Co., Ltd.) is a pretreatment kit for effective collection of bacteria with cationic copolymers. However, the Rapid BACpro® pretreatment kit is adapted only for MALDI Biotyper (Bruker Daltonics K.K.), and there has been a desire to expand its use to VITEK MS (VMS; bioMerieux SA).

We improved the protocol and made it possible to analyze with VMS. The culture medium bacteria collection method was changed to a method with centrifugation after hemolysis using saponin; the cationic copolymer concentration was changed to 30% of the original concentration; the sequence with which reagents were added was changed; and a change was made to an ethanol/formic acid extraction method.

The improved protocol enhanced the identification performance. When VMS was used, the identification rate was 100% with control samples. With clinical samples, the identification agreement rate with the cell smear method was 96.3%.

The improved protocol is effective in blood culture rapid identification, being both simpler and having an improved identification performance compared with the original.

## 1. Introduction

The fatality rate of patients with bloodstream infections is high, so early diagnosis and treatment are needed. The mortality risk rises with a delay in appropriate antimicrobial therapy (Brun-Buisson, 2000; Kumar et al., 2006; Kumar et al., 2009; Martin, 2012). In cases of positive blood culture, rapid estimation or identification of detected bacteria is important, leading to identification of the source of infection and proper antimicrobial therapy. Previously, physicians could only estimate the most likely bacteria from the properties of the positive bottles and from Gram staining of the culture medium. However, in 2011, following approval of mass spectrometers as medical devices, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was incorporated into bacterial identification methods and rapid identification tests became possible. Direct identification methods have also been investigated from blood-culture-positive bottles, and various methods have been reported (Chen et al., 2013; Ferreira et al., 2011; Fothergill et al., 2013; Stevenson et al., 2010). Rapid BACpro® (Nittobo Medical Co., Ltd.) is a pretreatment kit developed for direct identification using positive blood cultures in bottle culture medium. Rapid BACpro® is characterized by effective collection of bacteria in culture medium with the use of cationic copolymers. However, the use of Rapid BACpro® is limited to MALDI Biotyper (MB, Bruker Daltonics K.K.) and improvements are necessary.

We developed a protocol that permits the use of the Rapid BACpro® kit for adaptation to VITEK MS (VMS, bioMerieux SA), and we optimized the protocol with the aim of raising the identification performance and operability.

## 2. Subjects

### 2.1. Blood culture equipment

Blood culture was done using BACTEC FX (Becton, Dickinson and Company) blood culture resin bottles (92F, 93F) (Becton, Dickinson and Company).

### 2.2. Control samples

Control samples were prepared using type strains. The type strains were adjusted to McFarland No. 0.5 with sterile physiological saline, and 100 μL of the suspension was added to blood culture bottles. The blood used was Human Whole Blood-LR "Nisseki" (HLR; Japanese Red Cross Society), of which 10 mL was added to each blood culture bottle. Culture medium from bottles that became positive after culturing were used in the investigation.

### 2.3. Clinical samples

Blood cultures were obtained from the Asahikawa Medical University Hospital between February and December 2016. Of these, 269 samples from blood-culture-positive bottles of patients who consented to secondary use of samples were used in the study. Samples in which two or more types of bacteria were seen with Gram staining or subculturing were excluded. Samples with strains not registered in the database were also excluded.

## 3. Methods

We investigated a change in bacterial collection from the original protocol's use of a serum separator tube to the use of hemolysis of culture medium and centrifugation. In the investigations to develop the improved protocol, spectra and score values were compared using MB. After development of the improved protocol, performance was evaluated using VMS.

### 3.1. Investigation of hemolysis treatment conditions

Saponin (Kanto Chemical Co., Inc.) was used as the lysis buffer surfactant. For the surfactant concentration, a concentration gradient with 11 lines of 0–10% and 1% gradations was prepared. Lysis buffer (500 μL) was added to 1 mL HLR, and after stirring, the red blood cells, white blood cells, and platelets were counted. A XS-500i multi-parameter automated hematology analyzer (Sysmex Corporation) was used to determine blood cell and platelet counts. For saponin concentrations of 2%, 7%, and 10%, the control samples that did not include bacteria were pretreated and measured with MB, and the appearance of blood-derived spectra at the various concentrations were compared. Lysis buffer (500 μL) was added to 1 mL HLR and centrifuged at 2000 ×g for 3 min. Pellets were suspended in sterile 800 μL distilled water, and centrifuged again at 2000 ×g for 1 min. Next, the pellet was resuspended in 800 μL of 70% ethanol, and centrifuged at 2000 ×g for 1 min. After this, 30 μL of 70% formic acid was added and the mixture was stirred. Thirty microliters of acetonitrile was then added, and the mixture was centrifuged at 2000 ×g for 1 min. A supernatant (1 μL) of the resulting supernatant was used in the MS assay.

### 3.2. Investigation of cationic copolymer concentration

The concentration of cationic copolymer used in the original protocol was 100%, and experiments were carried out at concentrations of 100%, 50%, 30% and 10% to determine the most appropriate concentration of cationic copolymer for use in the improved protocol. Changes in the blood-derived spectra of the control samples that did not include bacteria were compared, using the MB assay, for each copolymer concentration. The bacterial collection rate at each concentration was also compared. Lysis buffer (800 μL) was added to 1 mL of each control sample culture medium and centrifuged at 2000 ×g for 3 min.

After the addition of cationic copolymer, samples were centrifuged at 2000 ×g for 1 min, and quantitative culturing was performed using the resulting supernatant. The bacterial collection rate at each concentration was determined. For the control samples, 10 mL HLR was added to a 92F blood culture resin bottle. Colonies obtained by overnight culturing on 5% sheep blood agar were suspended in physiological saline, and 1 mL of the resulting bacterial suspension, adjusted so that the final bacterial load in the blood culture bottle was $1.5 \times 10^8$ CFU/mL, was used in the investigation. These experiments were performed using strains *Escherichia coli* ATCC 25922 and *Staphylococcus epidermidis* NBRC10091.

### 3.3. Comparison of bacterial collection efficiency

For the control samples, 10 mL HLR was added to a 92F blood culture resin bottle, and the bottle was inoculated with 1 mL of a bacterial suspension adjusted to McFarland No. 1; the resulting samples then were used in the investigation. These specific experiments were performed only using strains *E. coli* ATCC 25922 and *S. epidermidis* NBRC10091.

For samples processed according to the original protocol, bacteria were collected using a serum separator tube. Specifically, 3 mL of control sample culture medium was centrifuged in a serum separator tube. The resulting supernatant was transferred to a separate container and the bacteria that accumulated on the separating agent were suspended in 1 mL of trypticase soy medium (Becton, Dickinson and Company). The resulting suspension was subjected to quantitative culturing, and the recovery rate was obtained from the viable cell count. For samples processed using the improved protocol, bacteria were harvested using the lysis buffer. Specifically, 500 μL of lysis buffer was added to 1 mL of culture medium, and the mixture was centrifuged at 2000 ×g for 3 min. The resulting supernatant was decanted; the pellet was resuspended in 1 mL of trypticase soy medium, and the suspension was subjected to quantitative culturing as above.

### 3.4. Control sample identification performance with the improved protocol

The identification performance using control samples was compared between the original protocol and the improved protocol (Fig. 1), and further compared to control samples prepared using the improved protocol without use of cationic copolymers. For these experiments, control samples were prepared using each of 20 strains (Table 1). Obligate anaerobes were grown using 93F blood culture bottles; strains other than obligate anaerobes were grown using 92F blood culture bottles. The control samples were pretreated in accordance with the respective protocol, and identification was performed with VMS using four spots for each strain.

### 3.5. Clinical sample identification performance with the improved protocol

Evaluation of improved protocols using clinical specimens was done using VMS. Results of the cell smear method using colonies obtained by subculture were compared with the results of direct identification by the improved methods.

### 3.6. Handling of measurement results

VMS measurements in the protocol improvement stage were performed using four or more replicate spots. In evaluating the improved protocol, four spots were measured for each sample and results in which the identification results were a single strain at one or more spots and reliability was 99.9% were adopted. In cases when multiple strains