



**HAL**  
open science

## Acceleration of the direct identification of versus coagulase-negative staphylococci from blood culture material: a comparison of six bacterial DNA extraction methods

A. J. M. Loonen, A. R. Jansz, H. Kreeftenberg, C. A. Bruggeman, P. F. G. Wolffs, A. J. C. Brule

### ► To cite this version:

A. J. M. Loonen, A. R. Jansz, H. Kreeftenberg, C. A. Bruggeman, P. F. G. Wolffs, et al.. Acceleration of the direct identification of versus coagulase-negative staphylococci from blood culture material: a comparison of six bacterial DNA extraction methods. *European Journal of Clinical Microbiology and Infectious Diseases*, 2010, 30 (3), pp.337-342. 10.1007/s10096-010-1090-0 . hal-00634809

**HAL Id: hal-00634809**

**<https://hal.science/hal-00634809>**

Submitted on 24 Oct 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Acceleration of direct identification of *Staphylococcus aureus* versus**  
2 **Coagulase Negative Staphylococci from blood culture material: a comparison**  
3 **of six bacterial DNA extraction methods**

4 Anne J.M. Loonen<sup>1,2,3</sup>, Arjan R. Jansz<sup>1</sup>, Herman Kreeftenberg<sup>4</sup>, Cathrien A. Bruggeman<sup>3</sup>,  
5 Petra F.G. Wolfs<sup>3</sup>, and Adriaan J.C. van den Brule\*<sup>1,2,5</sup>

6

7 <sup>1</sup> PAMM Laboratory for Medical Microbiology, Eindhoven/Veldhoven, the Netherlands

8 <sup>2</sup> Fontys University of Applied Science, Centre for Medical Molecular Diagnostics,  
9 Eindhoven, the Netherlands

10 <sup>3</sup> Maastricht University Medical Centre, Department of Medical Microbiology,  
11 Maastricht, the Netherlands

12 <sup>4</sup> Catharina Hospital, Department of Intensive Care, Eindhoven, the Netherlands

13 <sup>5</sup> Catharina Hospital, Department of Molecular Diagnostics, Eindhoven, The  
14 Netherlands

15 **\*Corresponding author:**

16 Dr. Adriaan J.C. van den Brule  
17 Department of Molecular Diagnostics  
18 Catharina Hospital / PAMM Laboratory  
19 Michelangelolaan 2  
20 5623 EJ Eindhoven  
21 The Netherlands  
22 Phone +31-40-2396150  
23 Fax +31-40-2396109  
24 Email a.van.den.brule@pamm.nl

25

26 **Running title:** Accelerated detection of *S.aureus* vs. CNS from blood culture material

27 **Keywords:** Blood culture material, bacterial DNA isolation, real-time PCR, Staphylococci

28 **Abstract**

29 To accelerate differentiation between *Staphylococcus aureus* and Coagulase  
30 Negative Staphylococci (CNS), this study aimed to compare six different DNA  
31 extraction methods from 2 commonly used blood culture materials, i.e. BACTEC and  
32 Bact/ALERT. Furthermore, we analyzed the effect of reduced blood culture  
33 incubation for detection of staphylococci directly from blood culture material.

34 A real-time PCR duplex assay was used to compare 6 different DNA isolation  
35 protocols on two different blood culture systems. Negative blood culture material was  
36 spiked with MRSA. Bacterial DNA was isolated with: automated extractor EasyMAG  
37 (3 protocols), automated extractor MagNA Pure LC (LC Microbiology Kit M<sup>Grade</sup>), a  
38 manual kit MoLYsis Plus, and a combination between MoLYsis Plus and the  
39 EasyMAG. The most optimal isolation method was used to evaluate reduced  
40 bacterial incubation times.

41 Bacterial DNA isolation with the MoLYsis Plus kit in combination with the specific B  
42 protocol on the EasyMAG resulted in the most sensitive detection of *S.aureus*, with a  
43 detection limit of 10 CFU/ml, in Bact/ALERT material, whereas using BACTEC  
44 resulted in a detection limit of 100 CFU/ml.

45 An initial *S.aureus* or CNS load of 1 CFU/ml blood can be detected after 5 hours of  
46 incubation in Bact/ALERT3D by combining the sensitive isolation method and the tuf  
47 LightCycler assay.

## 48 **Introduction**

49 *S.aureus* is a pathogen which can cause both hospital- and community associated  
50 infectious diseases, ranging from minor skin infections to endocarditis, bacteremia,  
51 sepsis and septic shock [1]. Sepsis can result in high morbidity and mortality. In the  
52 United States, blood stream infections develop in approximately 250.000 people  
53 yearly [2]. In the Netherlands, the incidence of patients admitted to the ICU with  
54 severe sepsis ranges around  $8643 \pm 929$  per year [3]. Currently, blood culture is the  
55 gold standard for identification of pathogens from suspected bacterial sepsis patients.  
56 Unfortunately, blood culture is time-consuming taking at least 24-72 hours for final  
57 determination of the bacteria causing the disease. Staphylococci are number one of  
58 Gram positive organisms in blood cultures. Differentiating *S.aureus* from Coagulase  
59 Negative Staphylococci (CNS) is important, because sepsis with *S.aureus* is  
60 common and virulent with mortality rates in the range of 20-30% [4]. CNS are often  
61 considered as being contaminants in blood cultures due to the fact that these species  
62 are members of the normal skin flora and mucous membranes and can contaminate  
63 the sample when it is taken. However, it is known that CNS infections are  
64 increasingly recognized as clinically relevant infections and confirmation on the  
65 presence of these species in blood culture is therefore important (reviewed in [5-8]).  
66 Several molecular methods for rapid accurate detection of bacteria, from positive  
67 blood culture material have been described, including (commercial) real-time PCR  
68 based diagnostic tests [9-11], fluorescence in situ hybridization [12, 13], MALDI-TOF-  
69 MS [14], but also DNA micro arrays [15, 16]. However, all these techniques are used  
70 on positive blood culture material. Reduction in the time to result can be achieved by  
71 applying molecular methods either directly on whole blood or on blood culture  
72 material with reduced incubation times. Ideally usage of whole blood is preferred but  
73 the techniques that are now available are often, clinically, not sensitive enough, as  
74 has been shown by others investigating a commercial real-time PCR test available  
75 [17-19]. Blood culture materials are known to contain inhibiting factors which can  
76 reduce detection in a sensitive real-time PCR [20-23]. It is therefore important to  
77 include a good isolation method, in the molecular diagnostic strategy, which is able to  
78 efficiently remove inhibiting factors and one which still allows sensitive DNA detection  
79 by PCR. In this study we compared 6 different, both manual and automated, bacterial  
80 DNA isolation methods for 2 commonly used blood culture systems, i.e. BACTEC

81 (Becton Dickinson) and Bact/ALERT (bioMérieux), to be able to find the most  
82 sensitive bacterial DNA isolation method. Additionally, we investigated the  
83 occurrence of inhibition in PCR amplification after DNA isolation. A sensitive real-time  
84 PCR assay was designed to be able to detect staphylococci and to differentiate  
85 *S.aureus* from CNS [Loonen A.J.M. *et al.*, manuscript submitted]. Subsequently, this  
86 real-time PCR was used in combination with the optimal DNA isolation method to  
87 investigate the level of time reduction to identify staphylococci from blood culture  
88 material. Results were compared with conventional blood culture techniques used in  
89 diagnostic laboratories.

## 90 **Materials and Methods**

91 **Culture methods:** Negatively cultured blood culture bottles, derived from routine  
92 diagnostics of both BACTEC (Becton Dickinson, the Netherlands) and Bact/ALERT  
93 (bioMérieux, the Netherlands) systems, were used in this study for spiking  
94 experiments (bottles have been cultured for 1 week and remained negative).  
95 Additionally, healthy volunteers donated 10 ml blood as input for the Bact/ALERT  
96 bottles. Bottles were incubated and handled according to manufactures protocol.

97 **DNA isolation methods:** Methicillin-resistant *S.aureus* ATCC 33592 was cultured  
98 overnight on bloodagar. Fresh plates were used to make a 2 McF solution which  
99 was subsequently serially diluted (1:10) in negative blood culture material from both  
100 systems (BACTEC and Bact/ALERT). One hundred µl was plated on bloodagar  
101 plates to check CFU per sample. Three different isolation protocols on the NucliSens  
102 EasyMAG (bioMérieux) were tested (generic, specific A and specific B protocol: main  
103 difference between protocols is the number of washing steps, with least steps in the  
104 generic protocol and most washing steps in the specific B protocol), 1 isolation kit  
105 (LC Microbiology Kit M<sup>Grade</sup>) on the MagNA Pure LC (Roche Diagnostics), the  
106 MolYsis Plus kit (Molzym GmBh, Bremen, Germany) was used according to Molzym  
107 instructions and in combination with the EasyMAG. When MolYsis was used in  
108 combination with the EasyMAG, manufacturer's instructions (Molzym) were followed  
109 until the Buglysis step and subsequently bacterial DNA was extracted according to  
110 the specific B protocol on the EasyMAG. Input volume in each isolation was 200µl,  
111 only for the MagNA Pure LC 100µl was used, and samples were always eluted in  
112 100µl elution buffer provided with each isolation method. Each extraction was  
113 performed at least 2-3 times in independent experiments. Phocine herpes virus type  
114 1 (PhHV-1) was added to each sample eluate as an amplification and inhibition  
115 control. Samples were analyzed with the LightCycler assay described below.

116 **LightCycler assay:** A duplex real-time PCR, detecting the *tuf* gene of both  
117 staphylococci and *S.aureus* specifically, is used as described in detail elsewhere  
118 [Loonen A.J.M. *et al.*, submitted]. In brief, the positions of the primers and probes  
119 were derived from the *S. aureus tuf* gene sequence (GenBank accession no.  
120 AF298796). The sequences of the primers and probes were as follows;  
121 *Staphylococcus* spp. forward primer ccaatgccacaaactcgtga (position 32-51),

122 Staphylococcus spp. reverse primer caccgaccagtgattgagaatacg (position 371-349),  
123 *S.aureus* forward primer tcctgggtcaattacaccacatactg (position 583-608), *S.aureus*  
124 reverse primer ggaaatagaattgtggacgatagttga (position 698-671), Staphylococcus  
125 spp. probe VIC-ccattcatgatgccagttg-BHQ1 (position 326-344) , *S.aureus* probe FAM-  
126 tgataatacgtataacttctgc-BHQ1 (position 637-617). Oligonucleotide primers were  
127 obtained from Eurogentec (Liège, Belgium), Staphylococcus XS probe and the  
128 *S.aureus* XS probe were obtained from Biolegio (Nijmegen, the Netherlands). PhHV-  
129 1 primers and VIC labeled probe [24] were obtained from Applied Biosystems  
130 (Nieuwerkerk aan den IJssel, the Netherlands). PCR mixtures consisted of 5x  
131 LightCycler TaqMan Master mixture (Roche Diagnostics), 0.9µM concentration of  
132 each primer, 0.2µM concentration of each probe, and aquadest in a volume of 15µl to  
133 which 5µl DNA sample was added, leading to a final reaction volume of 20µl in each  
134 capillary. Primers and probe targeting PhHV had a concentration of 0.1µM each and  
135 were used in a separate single real-time PCR. All samples were amplified on the  
136 LightCycler v2.0 (Roche Diagnostics) according to the following cycling parameters:  
137 an initial incubation step for 10 min at 95°C for enzyme activation and DNA  
138 denaturation, subsequently 45 cycli of 15 sec 95 °C followed by 1 min 60°C. Results  
139 were analyzed with LightCycler Software version 4.05 (Roche Diagnostics).

140

## 141 **Results**

### 142 **Evaluation of different bacterial DNA extraction methods**

143 In table 1 the results are presented of the comparison of 6 different bacterial DNA  
144 extraction methods on *S.aureus* spiked blood culture material. The MagNA Pure LC  
145 (Roche Diagnostics) resulted in a detection limit of  $10^4$  CFU/ml for both culture  
146 systems. On the EasyMAG 3 protocols were evaluated to isolate bacterial DNA from  
147 spiked clinical material, generic, specific A and specific B. We tested all 3 protocols  
148 and usage of the specific B protocol resulted in the best detection limit of  $10^2$  CFU/ml  
149 of *S.aureus* in Bact/ALERT material. Although MoYsis Plus did not result in a low  
150 detection limit, we tested this manual kit in combination with the specific B protocol  
151 on the EasyMAG. By combining both methods all human DNA was removed and  
152 bacteria were lysed by the MoYsis Plus kit, and subsequently the EasyMAG was  
153 used for optimal washing and elution of bacterial DNA. It was possible to reduce  
154 hands-on time and avoid possible contamination due to skipping of manual  
155 handlings. This combination of methods resulted in the most sensitive detection limit,  
156 i.e. 10 CFU/ml, in Bact/ALERT material. Time to result for this combined method  
157 followed by real-time PCR is approximately 4-5 hours, but strongly depending on the  
158 number of samples. No inhibition was observed in the real-time PCR, all samples  
159 and controls showed similar Cp values in one run ( $C_p \pm 0.5$ ). All experiments were  
160 performed at least 2-3 times.

### 161 **Reduced blood culture times**

162 To investigate the effect of reduced blood culture incubation for pathogen detection  
163 by PCR a combination between the sensitive *tuf* LightCycler assay and the sensitive  
164 isolation method, the MoYsis Plus Kit in combination with the specific B protocol on  
165 the EasyMAG, was analyzed on *S.aureus* (ATCC 33592) spiked Bact/ALERT blood  
166 culture bottles containing fresh blood of healthy volunteers. First it was investigated  
167 how many CFU *S.aureus* per ml needed to be present to be able to detect a signal  
168 with the LightCycler assay, therefore 1000, 100 and 10 CFU were spiked in different  
169 bottles, both SA (Standard Aerobic) and SN (Standard Anaerobic) Bact/ALERT  
170 bottles. BACTEC bottles were not investigated due to poorer results obtained with all  
171 isolation methods analysed. The results of this experiment are depicted in figure 2  
172 and indicate that 1000 CFU of *S.aureus* (100 CFU/ml blood) could be detected by

173 PCR immediately after spiking indicating that no incubation step was required. The  
174 bottles containing 100 CFU of *S.aureus* (10 CFU/ml blood) resulted in positive real-  
175 time PCR signals after 1 hour incubation in the Bact/ALERT 3D. The 10 CFU bottles  
176 (1 CFU/ml blood) needed a maximum of 5 hours incubation in the Bact/ALERT3D to  
177 be able to detect the *S.aureus* bacteria present using a subsequent PCR. The  
178 experiment with 10 CFU *S.aureus* per bottle was repeated 8 times with both aerobe  
179 and anaerobe bottles and showed reproducible results. Standard deviations (SD) of  
180 the Cp values were calculated for the 10 CFU/bottle experiments (*aureus* probe).  
181 Three hour time point (2/8 positive) showed SD of 3.44, 4hr timepoint (5/8 positive)  
182 indicated SD of 1.09, and the 5 hr time point (8/8 positive) resulted in SD of 1.09.  
183 Clinical strains of *S.aureus* and *S.epidermidis* were also spiked in Bact/ALERT blood  
184 culture bottles (aerobe (SA) and anaerobe (SN)) containing blood of healthy  
185 volunteers. The results of these experiments were similar as the results presented for  
186 the *S.aureus* ATCC 33592 strain. With conventional detection methods, results of the  
187 bottles containing 10 CFU *S.aureus* were available after at least 28 hours.

188

## 189 Discussion

190 In this study, 6 different bacterial DNA extraction protocols were evaluated, by using  
191 a sensitive and specific *tuf* gene based real-time PCR detection assay, which  
192 enabled the differentiation between *S.aureus* and CNS. From this study it can be  
193 concluded that the MoLYsis Plus kit (Molzym) in combination with the specific B  
194 protocol on the EasyMAG (bioMérieux) resulted in the most optimal isolation of  
195 bacterial DNA from Bact/ALERT blood culture material. A detection limit of 10  
196 CFU/ml was obtained in the *tuf* LightCycler assay for Bact/ALERT material. By using  
197 the *tuf* LightCycler assay 1 CFU/ml *S.aureus* or CNS could be detected from  
198 Bact/ALERT blood culture material when an incubation step of a maximum of 5 hours  
199 was applied. By using this reduced incubation time it was possible to identify  
200 *S.aureus* versus CNS within one working day.

201 Six different bacterial DNA extraction methods were compared on two commonly  
202 used blood culture systems (BACTEC and Bact/ALERT). The specific B protocol on  
203 the EasyMAG contains most washing steps compared to the generic and specific A  
204 protocol, possibly explaining the better results obtained with this isolation protocol.  
205 The bacterial DNA isolation performed with the MoLYsis Plus kit resulted in a low  
206 detection limit, for both culture systems. Gebert *et al.*, published a study in 2008 in  
207 which they show that the MoLYsis Plus kit performs well in their hands [25]. They  
208 used 500µl input material, and were able to conclude that PCR analysis in  
209 combination with MoLYsis Plus DNA isolation resulted in accelerated detection of  
210 pathogens in blood culture samples. Inhibiting factors were not found to be present in  
211 the samples in the study of Gebert *et al* [25]. In our study, no PCR inhibition was  
212 observed (all samples and controls showed similar  $C_p \pm 0,5$  values in one run). The  
213 detection limit in the LightCycler assay, after MoLYsis Plus isolation, was low i.e.,  $10^4$   
214 CFU/ml. The MoLYsis Plus kit eliminates human background DNA from the samples  
215 and enrichment of bacterial DNA takes place. Complete isolation with the MoLYsis  
216 Plus kit takes 3 hrs, of which 2 hours hands-on time. By using the EasyMAG specific  
217 B protocol after completion of the MoLYsis Plus Buglysis step, the total isolation time  
218 is not significantly reduced. However, hands-on time and the chance for  
219 contamination are drastically reduced, due to the fact that column handling is  
220 skipped. A comparison between both culture systems resulted in a difference in  
221 bacterial DNA isolation efficiency. Usage of Bact/ALERT blood culture material

222 led in most isolation methods to better results compared to BACTEC material.  
223 We experienced difficulties in the bacterial DNA isolations due to the beads present  
224 in BACTEC bottles, but we can not conclude that the beads led to the weaker  
225 performance of BACTEC material compared to Bact/ALERT material. However, due  
226 to this finding all experiments were continued with Bact/ALERT material.

227 By applying a 5 hour incubation step for blood culture bottles containing  
228 approximately 10 CFU of *S.aureus* or CNS it was possible to detect these bacteria by  
229 the *tuf* LightCycler assay. These results could be produced within 1 working day  
230 when starting early in the morning. Compared to conventional culture techniques for  
231 staphylococci, results were available at least 19hrs faster. Gebert *et al.*[25], also  
232 showed in their paper that by combining a sensitive real-time PCR and a sensitive  
233 isolation method, culture times can be reduced. They showed a 5.0-8.7 hours time  
234 gain in spiking experiments and an average time gain of 10.7 hours was obtained  
235 when the method was applied on blood culture bottles of septic patients. Maximum  
236 time gain can be reached when starting either immediately in the morning or when  
237 the microbiology laboratory has 24 hours service. Laboratories with a 24 hours setup  
238 will always benefit from this method.

239 The experiments described in this paper all focus on accelerated detection of  
240 staphylococci, especially on detection of *S.aureus*. Staphylococci were chosen as a  
241 model system because Gram positive bacteria are frequently detected in blood  
242 culture bottles, making it a relevant group of micro-organisms for such a study [26-  
243 28]. Infections are, however, caused by a variety of pathogens. Staphylococci are  
244 fast growing bacteria and in this study a proof-of-principle is shown. Accelerated  
245 detection, of other pathogens causing blood stream infections (i.e. *E.coli*), directly  
246 from blood culture material in combination with reduced culture time and with this  
247 sensitive isolation method should be investigated in future experiments. It is of  
248 clinical importance in the future to focus especially on fastidious pathogens or fungi,  
249 since those pathogens are difficult to culture and for these pathogens time to result  
250 can than hopefully be drastically reduced. A similar approach as presented in this  
251 study can be applied to investigate reduced incubation times for other pathogens.

252 It is known from literature that staphylococci are the most frequently causative agent  
253 of medical device related and surgical-site infections [7],. Therefore it is interesting to

254 apply the *tuf* LightCycler duplex assay in these patient groups using whole blood  
255 samples. Preliminary results showed that the MoLYsis Complete kit (Molzylm) has  
256 already proven to be successful in our hands (data not shown) as well as in the  
257 hands of others [29].

258 In conclusion, by combining the MoLYsis Plus kit and the specific B protocol on the  
259 EasyMAG 10 CFU/ml could be detected from *S.aureus* spiked Bact/ALERT blood  
260 culture material. By applying this isolation method followed by the sensitive *tuf*  
261 LightCycler duplex assay, using 5 hours incubated spiked blood culture material, 1  
262 CFU *S.aureus* or CNS per 1 ml blood could be detected. Future studies are needed  
263 to address the clinical application of this approach.

264 **Acknowledgements**

265 We thank Wendy Hansen, Jitske Stalpers, Marielle Bierma, Christel van Herk, Paul  
266 van Kaathoven, Gitta Stienen and Jan Michielse for technical support.

267

268 **Conflict of interest**

269 The authors declare that they have no conflict of interest.

270

271 **References**

272  
273 1 Lowy FD. Staphylococcus aureus infections. *N Engl J Med*. 1998; **339**: 520-532.  
274 2 CDC. Report of mortality statistics. *Monthly Vital Statistics Report*. 1992; **40**: 1-23.  
275 3 van Gestel A, Bakker J, Veraart CP, van Hout BA. Prevalence and incidence of severe sepsis in  
276 dutch intensive care units. *Crit Care*. 2004; **8**: R153-162.  
277 4 Saginur R, Suh KN. Staphylococcus aureus bacteraemia of unknown primary source: Where  
278 do we stand? *Int J Antimicrob Agents*. 2008; **32 Suppl 1**: S21-25.  
279 5 von Eiff C, Peters G, Heilmann C. Pathogenesis of infections due to coagulase-negative  
280 staphylococci. *Lancet Infect Dis*. 2002; **2**: 677-685.  
281 6 Piette A, Verschraegen G. Role of coagulase-negative staphylococci in human disease. *Vet*  
282 *Microbiol*. 2009; **134**: 45-54.  
283 7 Huebner J, Goldmann DA. Coagulase-negative staphylococci: Role as pathogens. *Annu Rev*  
284 *Med*. 1999; **50**: 223-236.  
285 8 Kloos WE, Bannerman TL. Update on clinical significance of coagulase-negative staphylococci.  
286 *Clin Microbiol Rev*. 1994; **7**: 117-140.  
287 9 Wellinghausen N, Wirths B, Franz AR, Karolyi L, Marre R, Reischl U. Algorithm for the  
288 identification of bacterial pathogens in positive blood cultures by real-time lightcycler  
289 polymerase chain reaction (pcr) with sequence-specific probes. *Diagn Microbiol Infect Dis*.  
290 2004; **48**: 229-241.  
291 10 Sakai H, Procop GW, Kobayashi N, et al. Simultaneous detection of staphylococcus aureus  
292 and coagulase-negative staphylococci in positive blood cultures by real-time pcr with two  
293 fluorescence resonance energy transfer probe sets. *J Clin Microbiol*. 2004; **42**: 5739-5744.  
294 11 Granger K, Rundell MS, Pingle MR, et al. Multiplex pcr-ligation detection reaction assay for  
295 the simultaneous detection of drug resistance and toxin genes from staphylococcus aureus,  
296 enterococcus faecalis and enterococcus faecium. *J Clin Microbiol*. 2009.  
297 12 Hartmann H, Stender H, Schafer A, Autenrieth IB, Kempf VA. Rapid identification of  
298 staphylococcus aureus in blood cultures by a combination of fluorescence in situ  
299 hybridization using peptide nucleic acid probes and flow cytometry. *J Clin Microbiol*. 2005;  
300 **43**: 4855-4857.  
301 13 Peters RP, van Agtmael MA, Simoons-Smit AM, Danner SA, Vandenbroucke-Grauls CM,  
302 Savelkoul PH. Rapid identification of pathogens in blood cultures with a modified  
303 fluorescence in situ hybridization assay. *J Clin Microbiol*. 2006; **44**: 4186-4188.  
304 14 Stevenson LG, Drake SK, Murray PR. Rapid identification of bacteria in positive blood culture  
305 broths by maldi-tof mass spectrometry. *J Clin Microbiol*. 2009.  
306 15 Yoo SM, Choi JY, Yun JK, et al. DNA microarray-based identification of bacterial and fungal  
307 pathogens in bloodstream infections. *Mol Cell Probes*. 2009.  
308 16 Cleven BE, Palka-Santini M, Gielen J, Meembor S, Kronke M, Krut O. Identification and  
309 characterization of bacterial pathogens causing bloodstream infections by DNA microarray. *J*  
310 *Clin Microbiol*. 2006; **44**: 2389-2397.  
311 17 Avolio M, Diamante P, Zamparo S, et al. Molecular identification of bloodstream pathogens  
312 in patients presenting to the emergency department with suspected sepsis. *Shock*. **34**: 27-30.  
313 18 Dierkes C, Ehrenstein B, Siebig S, Linde HJ, Reischl U, Salzberger B. Clinical impact of a  
314 commercially available multiplex pcr system for rapid detection of pathogens in patients with  
315 presumed sepsis. *BMC Infect Dis*. 2009; **9**: 126.  
316 19 Wallet F, Nseir S, Baumann L, et al. Preliminary clinical study using a multiplex real-time pcr  
317 test for the detection of bacterial and fungal DNA directly in blood. *Clin Microbiol Infect*.  
318 2009.  
319 20 Wilson IG. Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol*.  
320 1997; **63**: 3741-3751.  
321 21 Al-Soud WA, Radstrom P. Purification and characterization of pcr-inhibitory components in  
322 blood cells. *J Clin Microbiol*. 2001; **39**: 485-493.

323 22 Millar BC, Jiru X, Moore JE, Earle JA. A simple and sensitive method to extract bacterial, yeast  
324 and fungal DNA from blood culture material. *J Microbiol Methods*. 2000; **42**: 139-147.  
325 23 Fredricks DN, Relman DA. Improved amplification of microbial DNA from blood cultures by  
326 removal of the pcr inhibitor sodium polyanetholesulfonate. *J Clin Microbiol*. 1998; **36**: 2810-  
327 2816.  
328 24 van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus  
329 infections by real-time amplification and rapid culture. *J Clin Microbiol*. 2003; **41**: 576-580.  
330 25 Gebert S, Siegel D, Wellinghausen N. Rapid detection of pathogens in blood culture bottles  
331 by real-time pcr in conjunction with the pre-analytic tool molysis. *J Infect*. 2008.  
332 26 Alberti C, Brun-Buisson C, Burchardi H, et al. Epidemiology of sepsis and infection in icu  
333 patients from an international multicentre cohort study. *Intensive Care Med*. 2002; **28**: 108-  
334 121.  
335 27 Annane D, Bellissant E, Cavaillon JM. Septic shock. *Lancet*. 2005; **365**: 63-78.  
336 28 Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the united states  
337 from 1979 through 2000. *N Engl J Med*. 2003; **348**: 1546-1554.  
338 29 Hansen WL, Bruggeman CA, Wolffs PF. Evaluation of new preanalysis sample treatment tools  
339 and DNA isolation protocols to improve bacterial pathogen detection in whole blood. *J Clin*  
340 *Microbiol*. 2009; **47**: 2629-2631.  
341  
342  
343

344 **Figures and Tables**

345 **Table 1. Evaluation of 6 different DNA isolation protocols on blood culture**  
 346 **material spiked with 10-fold dilution series of *S.aureus***

347

348 DNA isolation protocol	349 Detection limit in CFU/ml					
	10 <sup>5</sup>	10 <sup>4</sup>	10 <sup>3</sup>	10 <sup>2</sup>	10	1
350 EasyMAG generic	+*	+*	+	-	-	-
351 EasyMAG specA	+*	+*	+*	-	-	-
352 EasyMAG specB	+*	+*	+*	+	-	-
353 MPLC	+*	+*	-	-	-	-
354 MolYsis Plus	+*	+*	-	-	-	-
355 MolYsis Plus + EasyMAG specB	+*	+*	+*	+*	+	-

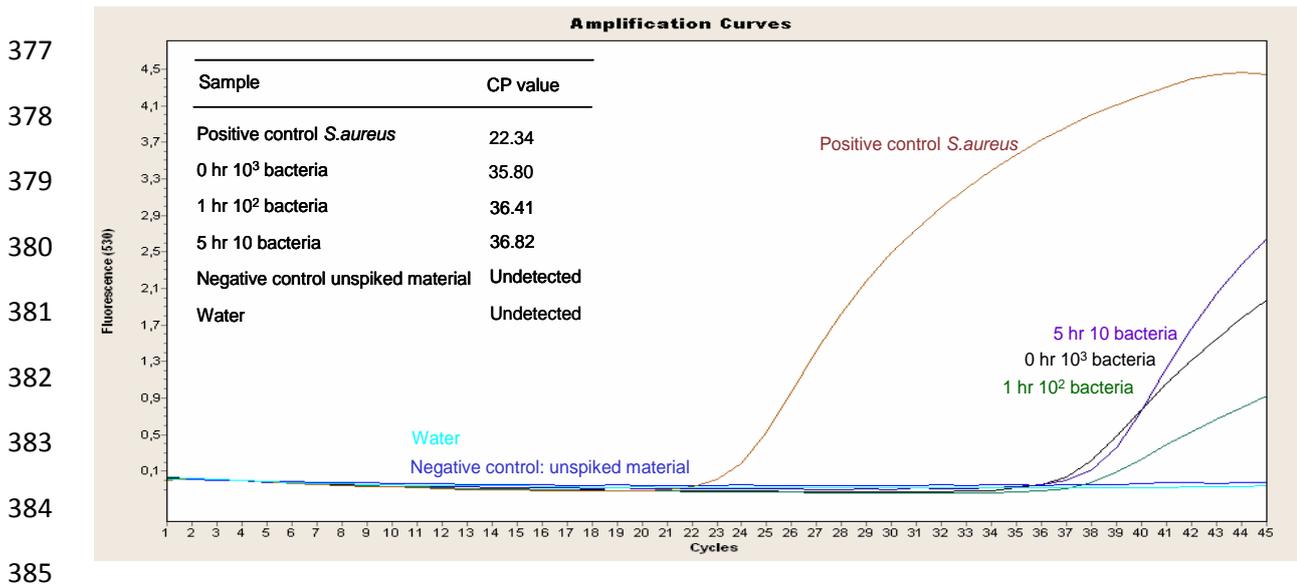
357 \* BACTEC; + Bact/ALERT; - not detected

358  
 359  
 360  
 361  
 362  
 363  
 364  
 365  
 366  
 367  
 368  
 369  
 370

371 A

Bacterial load CFU/bottle	Timepoints (hours)					
	0	1	2	3	4	5
1000	1/1	1/1	1/1	1/1	1/1	1/1
100	0/1	1/1	1/1	1/1	1/1	1/1
10	0/8	0/8	0/8	2/8	5/8	8/8

376 B



386 **Figure 2. *S.aureus* detection in relation to reduced bacterial culture times.**

387 A, Overview of detection of *S.aureus* in relation to reduced culture times.

388 Bact/ALERT blood culture bottles with blood of healthy volunteers were spiked with  
389 *S.aureus* and samples were taken every hour. The experiments with 1000 and 100  
390 bacteria per bottle were performed once. Thousand bacteria/bottle did not need a  
391 pre-culture step, a *tuf* real-time PCR signal could be detected immediately after  
392 spiking (0 hr). Hundred bacteria/bottle needed a pre-culture step of 1 hour in the  
393 Bact/ALERT 3D to obtain a positive signal in the real-time PCR. The experiment with  
394 10 bacteria per bottle (10 ml blood) was repeated 8 times. Real-time PCR signal  
395 could be detected after a pre-culture step of 3-5 hours. All bottles, 8 of 8, were  
396 positive after 5 hours pre-culture. Standard deviations (SD) for 10 CFU/bottle were  
397 calculated; 3 hr SD 3.44, 4 hr SD 1.09, and 5 hr SD 1.09. B, LightCycler *tuf* real-time  
398 PCR result in the 530nm fluorescence channel (*S.aureus* probe).