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1 **Accelerated detection of Gram negative bacteria in blood culture**

2 **by enhanced acoustic flow cytometry (AFC) following peptide**

3 **nucleic acid fluorescence *in situ* hybridization (PNA-FISH)**

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23

24 ## Abstract

25

26   Bacteraemia is a risk factor for subsequent clinical deterioration and death. Current

27   reliance on culture-based methods for detection of bacteraemia delays identification and

28   assessment of this risk until after the optimal period for positively impacting treatment

29   decisions has passed. Therefore, a method for rapid detection and identification of bacterial

30   infection in the peripheral bloodstream in acutely ill patients is crucial for improved patient

31   survival through earlier targeted antibiotic treatment. The turnaround time for current

32   clinical laboratory methods ranges from 12 to 48 hours, emphasizing the need for a faster

33   diagnostic test. Here we describe a novel assay for accelerated detection of bacterial

34   infection in blood culture (BC) using peptide nucleic acid fluorescence *in situ* hybridization

35   enhanced acoustic flow cytometry (PNA-FISH-AFC). For assay development, we used

36   simulated blood cultures (BCs) spiked with one of three bacterial species: *Escherichia coli,*

37   *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* at a low concentration of 10 CFU/mL.

38   Under current clinical settings, it takes a minimum of 12 hours incubation to reach positivity

39   on the BacTEC system, corresponding to a bacterial concentration of 10^7-10^9 CFU/mL

40   optimal for further analyses. In contrast, our PNA-FISH-AFC assay detected $10^3 - 10^4$

41   CFU/mL bacteria in BC following a much shorter incubation of 5 to 10 hours in culture. Using

42   either PCR-based FilmArray® assay or MALDI-TOF for bacterial detection, it took 7-10 and

43   12-24 hours of incubation, respectively, to reach the positive result. These findings indicate

44   a potential time advantage of PNA-FISH-AFC assay over currently used laboratory

45   techniques for rapid bacterial detection in BC with significantly improved turnaround time.

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47

48   **Introduction**

49   Sepsis is one of the leading causes of death from infection, usually accompanied by

50   bacteraemia. In its most fulminant form sepsis has a mortality rate of up to 40-60% [1-3].

51   Sepsis-associated bacteraemia may be mono- or polymicrobial and requires lengthy

52   laboratory processes to identify and characterize the causative agent(s). The time required

53   for initial bacterial detection in the diagnostic laboratory ranges from 12 to 48 hours and

54   depends in part on bacterial growth rate and the number of culture-dependent laboratory

55   procedures required after initial isolation.

56   The standard method for detection of bacteremia boosts bacterial cell numbers by

57   culture of peripheral blood (blood culture, BC) to aid detection and subsequent analysis. To

58   do this, an 8-10mL sample of patient's blood is aseptically inoculated into each of a pair of

59   aerobic and anaerobic BC bottles that contain bacterial growth media, lytic agents to

60   release phagocytosed bacteria from leukocytes and ion exchange resin beads to neutralize

61   the effect of residual antibiotics and improve bacterial recovery [4]. Once inoculated BCs

62   reach the clinical laboratory, they are incubated in a dedicated autoanalyser that

63   continuously monitors changes in pH or carbon dioxide production as indicators of bacterial

64   growth [5]. The autoanalyser signals the BC bottles need further analysis after reaching a

65   pre-set detection threshold. The positive signal threshold is set to correspond to the

66   minimum point at which Gram stain can be used to reliably confirm the presence of bacteria

67   and direct downstream analyses.

68   Growth rates of bacteria vary greatly in BCs. Fast growing bacteria such as

69   *Escherichia coli* and *Klebsiella pneumoniae* may reach the detection threshold in shorter

70   periods of time than slower growers such as *Pseudomonas aeruginosa*, *Staphylococcus*

71   *aureus* and *Streptococcus pyogenes* that may require 19 to 48 hours, or fastidious organisms

72   that can take as long as up to five days. The initial bacterial load in blood plays a critical role

73   in determining time to detection in BC. The bacterial load in the blood of bacteraemic adult

74   patients is typically very low, ranging between 1 to 10 CFU/mL, but in more severe cases

75   may exceed 10^3 CFU/mL. There is a strong correlation between bacterial loads in patients'

76   blood, time to positivity in BC, increased in-hospital stays and mortality in bacteraemic

77   patients [6-9]. This has prompted further development of rapid diagnostic tests to reduce

78   the time taken to detect, identify and perform antimicrobial susceptibility testing in order to

79   optimize antibiotic treatment and improve patients' survival.

80   On detection of a positive BC, aliquots are usually taken for initial presumptive and

81   subsequent definitive identification tests. Three main options for bacterial identification are

82   in current use: (1) testing directly from blood culture bottles; (2) sub-culture to obtain single

83   colony growth; (3) testing after enrichment and subsequent purification (Fig 1). Each of

84   these may use nucleic acid-based identification processes (e.g. probe-based fluorescence

85   microscopy, microarray, PCR, DNA sequencing), protein characterization by matrix-assisted

86   laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, or substrate

87   utilization methods (e.g. VITEK or API), respectively. The final bacterial identification may

88   take an additional 2 to 36 hours for a definitive result, depending on the bacterial species

89   and range of tests performed. Currently, the most rapid turnaround time from BC

90   inoculation to definitive bacterial identification is approximately 16 hours using molecular

91   techniques, while conventional sub-culture based identification may take an additional 48

92   hours or more [10].

93

94   **Fig 1. Timeline of current bacteraemia diagnostics. D**iagram showing major stages in

95   current bacteraemia diagnostics featuring three main test streams with a minimum of 16

96   hours and maximum of 2 or more days for definitive bacterial identification.

97

98   The two rapid, culture-dependent technologies currently used in the laboratory

99   confirmation of bacteraemia are MALDI-TOF mass spectrometry and PCR assays. Although

100   robust enough for use in clinical laboratories, these technologies have shortcomings that

101   may result in failure to detect the target or otherwise misleading data. The minimal

102   bacterial concentration for microbial identification by MALDI-TOF is above 10^7-10^8 CFU/mL

103   for reliable genus or species identifying scores in the range of 1.7-2.0 [11-13]. Bacterial

104   concentrations lower than 10^7 CFU/mL are more likely to generate unreliable identification

105   due to a low signal-to-noise ratio caused by background of media and blood elements.

106   Careful MALDI-TOF result interpretation is required with polymicrobial blood cultures where

107   the error rate can be three times higher [10] and only predominant microorganisms could

108   be identified [12]. Gram negative bacteria are easier to detect in polymicrobial BCs than

109   Gram positive organisms due to their rapid growth rate [14]. MALDI-TOF performs poorly on

110   Gram positive rods and yeasts due to their slow growth, resulting in a low microbial

111   concentration [10]. Alternatively, while molecular detection by PCR can give specific results

112   with a lower concentration of bacteria than MALDI-TOF, the sensitivity of detection is

113   adversely affected by the sample matrix. In complex systems containing blood, urine and

114   sputum, components such as haemoglobin can inhibit the PCR and cause false negative

115   results [15]. In addition, the presence of microbial genetic material as demonstrated by PCR

116   assay does not guarantee the presence of underlying bloodstream infection since it can

117   originate from distant focal points in other tissues. This and carry-over contamination from

118   laboratory equipment or reagents are common causes of false positive results, since a 16S

119   rRNA gene-based detection probe can detect eubacterial DNA found ubiquitously in the

120 environment [16]. Consequently, there is a need to confirm true bacteraemia by specific

121 detection of intact bacterial cells.

122   Peptide nucleic acid (PNA) fluorescence *in situ* hybridization (FISH) has been used

123 extensively in fluorescence microscopy to study pure growth on solid media, identify

124 bacteria and yeast in liquid culture media and in positive blood cultures [17-19]. The

125 repeated units of N-(2-aminoethyl)-glycine give the PNA probe a neutral backbone [20-21]

126 making it more stable and resistant to enzymatic and pH degradation. This, in turn, allows

127 strong binding to DNA and RNA targets under low ionic conditions and easier access to

128 secondary RNA structures, making PNA probes specific, more stable and a better alternative

129 to oligonucleotide probes.

130   Flow cytometry is a technique capable of high throughput analysis of cell

131 suspensions with simultaneous measurement of physical and chemical properties of the

132 cells [22], which has been extensively used for decades in clinical haematology to analyse

133 blood cells. Flow cytometry constitutes three major components: fluidic circuitry for cell

134 alignment, optical signal detection, and electronic conversion of digital signals into

135 interpretable data. The acoustic-enhanced version of the flow cytometer instrument pre-

136 focuses cells into a single stream using ultrasound waves before the interrogation point,

137 resulting in much higher resolution than the hydrodynamic version [24]. Acoustic-enhanced

138 flow cytometers can therefore be used successfully for small particle analysis such as

139 bacteria.

140   We recently reported a flow cytometry assisted susceptibility testing (FAST) assay

141 method to determine a carbapenem minimal inhibitory concentration in pure cultures of

142 carbapenem-resistant *Klebsiella pneumoniae* using the nucleic acid intercalating dye, SYTO®

143 9 [25]. This assay proved applicable to accurate detection of bacteria with the non-specific

144   dye SYTO® 9 in both unexposed and antibiotic-exposed bacteria in pure cultures. However,

145   we believed the same approach could not be reliably used to detect bacteria in BC due to

146   interference from blood-derived elements. Accordingly, we embarked on development of a

147   novel diagnostic approach employing *in situ* hybridization with the bacteria-specific probe

148   prior to acoustic flow cytometry. Here we describe development of the PNA-FISH enhanced

149   acoustic flow cytometry assay that combines the advantages of PNA-FISH probes for fine

150   molecular analysis with high throughput high-resolution cellular analysis of acoustic flow

151   cytometry in order to increase bacterial detection specificity in blood culture and thus

152   reduce the time to bacterial detection.

153

Materials and methods

Bacterial strains and culture media

156   Reference strains of *Klebsiella pneumoniae* (ATCC BAA 1705), *Pseudomonas*

157   *aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were used in the experiments

158   described here. Bacterial stocks were prepared from bacteria by initial culture on blood agar

159   (BA), then inoculation into Brain and Heart Infusion broth (BHIB) containing 10% glycerol

160   and stored in -80°C freezer. These and other culture media including trypticase soy broth

161   (TSB), Hanks Balanced Salt Solution (HBSS) and plate count agars (PCA) were obtained from

162   the PathWest Laboratory Medicine WA (QEII, Nedlands, Western Australia). BacTEC aerobic

163   blood culture bottles were obtained from Becton Dickinson (Franklin Lakes, New Jersey,

164   USA). Fresh, uninfected human blood in EDTA was obtained from healthy adults on the day

165   of the experiment in the specialist sample collection clinic of PathWest Laboratory Medicine

166   WA (QEII, Nedlands, Western Australia) in accordance with institutional standard practice.

167

Fluorescent dye and probe

Direct staining with SYTO® 9

170   One μL of SYTO® 9 dye (ThermoFisher Scientific, Eugene, Oregon, USA) at a final

171   concentration of 5μM was directly added to 1mL samples followed by a 5-minute incubation

172   at room temperature before samples were analysed on the acoustic flow cytometer.

173

PNA-FISH probe design

175   The eubacterial 16S probe (5'-Alexa488-O-TATCTAATCCTGTTT -3') was designed

176   using BioEdit software and NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The

177   peptide nucleic acid probe was conjugated to AlexaFluor 488 (supplied by PNA Bio Company,

178   107 N. Reino Rd., #242 Thousand Oaks, California, USA), and reconstituted prior to use in

179   molecular grade water to 200μM stock concentration.

180

Optimization of hybridization for pure bacterial cultures

182   The hybridization buffer was made in-house and contained 10% (w/v) dextran

183   sulphate, 10mM NaCl, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone,

184   0.2% (w/v) Ficoll, 5mM Na_2EDTA, 0.1% (v/v) Triton X-100, 50mM Tris-HCl (pH 7.5). Wash

185   buffer was also made in-house and contained 25mM Tris-HCl (pH 10.0), 137mM NaCl, and

186   3mM KCl. All reagents were obtained from Sigma Aldrich (St. Louis, Missouri, USA).

187   Three different concentrations of the PNA-FISH probe at 100 nM, 200 nM and 300

188   nM were used during optimization of hybridization. Other reaction parameters that were

189   also optimized included the formamide (Sigma Aldrich, St Louis, Missouri, USA)

190   concentration that varied from 0 - 50% (v/v), hybridization temperature that varied from

191   30°C - 60°C and duration of hybridization, which was either 15 or 30 minutes.

192   Pure bacterial cultures were grown overnight in 10mL TSB at 37°C without shaking

193   and used for hybridization optimization where a 20µL aliquot was added to 480µL of

194   hybridization buffer, followed by vortexing and incubation at varying temperatures and

195   durations as described in figure legends. The sample was centrifuged at 10,000 g for 5

196   minutes and supernatant removed. The pellet was resuspended in a 500 µL wash solution

197   and further incubated for 10 minutes at the same hybridization temperatures. The sample

198   was centrifuged and washed once more in 500 µL wash solution, as described above.

199   Samples were cooled to room temperature before they were analyzed on an acoustic flow

200   cytometer (Attune NxT, ThermoFisher Scientific, Eugene, Oregon, USA). The PNA-FISH probe

201   was added to give a final concentration of 200 nM in hybridization buffer containing

202   formamide 30% (v/v), and was used in all subsequent procedures with simulated BC.

203

204   Preparation of simulated BC

205   Simulated BCs were inoculated with bacteria grown in pure culture as follows: on

206   day 1, stock bacteria were streaked onto the BA plates and incubated overnight at 37°C. On

207   day 2, a single colony was picked, inoculated into pre-warmed TSB and incubated overnight

208   at 37°C for 18 hours. On day 3, 1mL of overnight bacterial culture in TSB was pelleted by

209   centrifugation at 10,000 g for 5 minutes followed by a wash and final resuspension in 1mL

210   HBSS. An aliquot of bacterial suspension in HBSS was taken, diluted 1000-fold in HBSS and

211   directly stained with 1 µL SYTO® 9 for 5 minutes. The aliquot was analyzed on the acoustic

212   flow cytometer (Attune, NxT, Thermo Fisher Scientific) to determine bacterial concentration.

213   Two aerobic BC bottles were prepared in parallel, each with 10 mL of uninfected

214   human EDTA blood obtained from healthy adults. Subsequently, bacteria that were

215   prepared and counted as described above were inoculated into the BC bottles at a final

216   concentration of 10 CFU/mL. Following the BC bottle setup, one aliquot was taken

217   immediately for plate count from one bottle. Both bottles were then incubated in the

218   BacTEC BC autoanalyser. Aliquots were taken at 2-hour intervals for plate counts, PNA-FISH-

219   AFC and other analyses from the same bottle sampled previously while the other bottle

220   stayed untouched and was used for measuring a time to positivity.

221

Hybridization protocol for simulated BC

223   Prior to hybridization, a lysis step was introduced for simulated BC to lyse blood cells

224   and concentrate bacteria. Briefly, 1 mL of inoculated BC was harvested and added to 1 mL

225   10% Triton X-100 for 5 minutes followed by centrifugation at 10,000 g for 5 minutes. The

226   pellet was re-suspended in 1 mL HBSS and centrifuged again followed by re-suspension of

227   the pellet in 20 µL in HBSS. The sample was mixed with 480 µL of hybridization buffer,

228   followed by vortexing and incubation at 40°C for 15 minutes. The sample was centrifuged at

229   10,000 g for 5 minutes and supernatant removed. The pellet was re-suspended in 500 µL

230   wash solution and further incubated for 10 minutes at 40°C. The sample was centrifuged

231   and washed once more in 500µL wash solution as described above. Samples were cooled to

232   room temperature before they were analyzed by AFC.

233   Uninfected BC were lysed as described in the hybridization protocol for simulated BC

234   above and stained with anti-CD 45 PerCP (ThermoFisher Scientific, Eugene, Oregon, USA) to

235   identify blood elements of leukocyte origin by AFC analysis.

236

Instrumentation and software

The acoustic flow cytometer (AFC) was used to analyze the samples. Flow cytometry analysis parameters are shown in Table 1. BacTEC FX blood culture system located in the Department of Microbiology at PathWest Laboratory Medicine WA was used to incubate inoculated blood cultures that rely on CO_2 production as a measurement of bacterial growth. FlowJo software (FLOWJO, LLC, Oregon, USA) was used to analyze the data taken from the AFC. Spectral analysis of bacterial proteins was performed by Bruker MALDI Biotyper (Bruker Daltonics GmbH, Germany). PCR-based detection of bacteria in BC was performed on a FilmArray® instrument (BioFire, version 1, BioMerieux Australia Pty Ltd, Baulkham Hills, NSW).

Table 1. NxT AFC parameters

Bacterial species	PMT voltages	Threshold
K.pneumoniae and *E.coli*	FSC(360), SSC(360), BL1(450), BL(400)	FSC(1.0), SSC(0.1), BL1(1.0)
P.aeruginosa	FSC(500), SSC(360), BL1(450), BL2(400)	FSC(1.2), SSC (0.5), BL1(1.0)

Event gating

The gating method for opto-electronic events produced by fluorescent dyes SYTO® 9, and AlexaFluor 488 incorporated into a bacteria-specific PNA-FISH probe, was similar in pure bacterial cultures. Initially, the population of interest (POI) in the area of events corresponding to the bacterial population was determined by forward and side scatter (FSC-H/SSC-H) to allow detection of clusters of cells according to their size and density (Table 2). The doublet events, if present, were identified and excluded by the forward scatter (FSC-H/FSC-A). Single cells that stained positive with SYTO®9 or AlexaFluor 488 were further identified by their fluorescence intensity on the histogram. This population was then back-

258   gated onto the FSC-H/SSC-H scatter plot to identify intact bacterial cells. This cell population

259   represented pure single bacterial cells that incorporated the label (SYTO® 9 or AlexaFluor

260   488) and was named 'derived population of interest' (dPOI). On all plots, dPOI was used for

261   the final cell count.

262
263   **Table 2. Gating methods flow chart**

Label	Gating directory and names			
	Ungated ->POI	Ungated->POI->single cells	Ungated->POI->single cells->label+	Ungated->POI->single cells->label+->dPOI
SYTO® 9	POI	Single Cells	SYTO9+	dPOI
PNA-FISH	POI	Single Cells	AlexaFluor 488+	dPOI

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265
266

Results

268
Bacterial detection by direct staining on AFC

270   Before developing a FISH-AFC-based assay for detection of bacterial DNA within

271   bacterial cells using a specific probe, we first performed direct staining of pure bacterial

272   cultures. To do this we used a non-specific nucleic acid dye, SYTO® 9, and established

273   instrument parameters as shown in Table 1. The gating method was developed as outlined

274   in Material and Methods for bacterial cells directly stained with SYTO® 9 (Fig 2A). Initially, a

275   combination of forward scatter (FSC) versus side scatter (SSC) analysis was used to identify

276   bacterial cells (so-called, population of interest, POI) based on their physical properties such

277   as size and density (Fig 2A, i). This was followed by application of the FSC-H/FSC-A plot to

278   distinguish intact single cells from doublets (Fig 2A, ii). Eventually, a subset of SYTO® 9

279   positive (SYTO® 9+) single cells was identified in a histogram (Fig 2A, iii) and used to pinpoint

280   intact bacterial cells on the scatter plot by back-gating. This permitted ultimate

281   identification of intact bacterial cells defined by their size and staining properties with

282   SYTO® 9 that were c alled a derived POI (dPOI) (Fig 2A, iv). When using direct staining with

283   fluorescent dye SYTO® 9 the limit of detection was determined as between 10^3-10^4 CFU/mL

284   in pure culture (See Fig S1).

285   The next step was to test whether direct staining with SYTO® 9 could be applied to

286   detect bacteria in simulated blood cultures (BC). The simulated BC was spiked with similar

287   bacterial concentration as in overnight pure culture at the time of detection, although due

288   to its high viscosity and density it had to be either diluted or subjected to a lysis step before

289   analysis on flow cytometer. Use of a dilution step for the BC was abandoned in favour of the

290   lysis method due to reduced loss of bacterial cells and detection sensitivity. However, the

291   lysis method produced an increase in background noise in the sample (Fig 2B). As shown in

292   Fig 2B, the scatter plots of infected (Fig 2B ii) and uninfected (Fig 2B iii) BC following the lysis

293   with 10% Triton X-100 were identical due to a non-specific binding of SYTO® 9 dye to both

294   bacterial cells and to abundant blood cell debris produced by lysis. Hence, detection of

295   bacteria at low concentrations in BC could not be achieved by the direct staining method

296   with SYTO® 9.

297

298   **Fig 2. Bacterial detection by acoustic flow cytometry after direct staining with SYTO® 9 in**

299   **pure bacterial culture and inoculated blood culture. A:** SYTO® 9 gating for bacteria grown

300   in TSB as pure culture: the population of interest (POI) was gated based on the FSC-H/SSC-H

301   scatter plot (i) while single bacterial cell events were identified in the FSC-H/FSC-A plot (ii).

302   Events that were SYTO® 9 positive were gated in the BL1-H channel (iii) and back-gated to

303   FSC-H/SSC-H scatter plot to identify bacterial cells labelled with SYTO® 9, denoted as derived

304   POI (dPOI) (iv) since SYTO® 9 binds to other particles containing nucleic acids. **B:** Bacterial

305   detection in pure culture (i) and blood culture (ii) by SYTO® 9 staining. Uninfected BC was

306   used as a negative control (iii). Both pure culture (i) and blood culture (ii) were inoculated

307   with identical concentrations of bacteria at approximately 8.00 x 10^4 CFU/mL. The pure

308   bacterial culture was stained directly with SYTO® 9, while both spiked and uninfected blood

309   cultures were lysed with 10% Triton X-100 before SYTO® 9 staining. Note the strong

310   background noise produced during the BC lysis in both infected and uninfected BC samples

311   overshadowing dPOI (ii and iii).

312

Development of *in situ* hybridization protocol

314   Due to the inability of SYTO® 9 staining to discriminate bacteria from the background

315   noise in BC samples, a unique bacteria-specific PNA-FISH probe hybridization protocol was

316   developed. For this purpose, a eubacterial 16S PNA probe was designed with BioEdit

317   software and labelled with the fluorophore AlexaFluor 488. Development of the

318   fluorescence *in situ* hybridization (FISH) protocol required optimization of hybridization

319   conditions such as formamide concentration, temperature, probe concentration and

320   hybridization duration in order to obtain good signal-to-noise ratio and preserved cellular

321   integrity. Accordingly, four optimization experiments were performed using pure bacterial

322   cultures of *K.pneumoniae* as a model organism (Fig 3; Fig S1). Optimizations of formamide

323   concentration and hybrization temperature were performed with SYTO® 9 using the gating

324   method that included both single cells and doublets in order to assess a recovery of the

325   overall bacterial population stained with SYTO® 9 (Fig S1). As shown in Fig 3, top two rows,

326   30% formamide in hybridization buffer and 40°C hybridization temperature provided

327   optimal conditions for the best recovery of dPOI (Fig S2) in comparison to lower and higher

328   formamide concentrations and hybridization temperatures, respectively.

329   The hybridization signal was optimised using PNA-FISH probe as described in Fig S1B,

330   altering two assay parameters, PNA-FISH probe concentration from 100nM to 300nM, and

331   hybridization duration, from 15 to 30 minutes (Fig 3). As shown in Fig 3 and Fig S2, optimal

332   hybridization signal intesity was obtained with probe concentration at 200nM following 15

333   minutes of hybridization. In summary, optimal conditions for PNA-FISH hybridization were

334   determined to include 30% formamide (v/v) and 200 nM probe in hybridization buffer, 40°C

335   hybridization temperature and 15 minutes incubation time (Fig 3; flowchart squares with

336   thick frames). These conditions were applied to all subsequent PNA-FISH experiments.

337

338   **Fig 3. PNA-FISH assay optimisation flowchart using pure bacterial culture.** Four

339   optimisation protocols were performed one of each for formamide concentration,

340   hybridization temperature, probe concentration, and hybridization duration. In the first two

341   protocols (top rows), SYTO9 was used to determine changes in the overall bacterial

342   population subjected to variations in formamide concentrations and temperature. Initially,

343   varying concentrations of formamide were added to bacterial suspensions containing equal

344   amounts of bacteria followed by incubation at 40°C for 15 minutes prior to staining with

345   SYTO9. The highest bacterial recovery of 75% was achieved with 30% formamide (v/v, thick

346   frame) compared to other treated and untreated samples. This formamide concentration

347   (30%) was selected for subsequent experiment in which bacterial cells were exposed to

348   varied hybridization temperatures from 30°C to 60°C (second row). The best result with 52%

349   bacterial cell recovery was obtained at 40°C (thick frame). For the last two optimisation

350   steps, PNA-FISH probe was used in hybridization buffer containing 30% formamide at 40°C

351   (third and fourth rows). In the third row, three different probe concentrations from 100 to

352   300 nM were tested by PNA-FISH. The probe concentration of 200 nM turned out to be

353   optimal (thick frame) since no significant increase in hybridisation signal was observed when

354   the probe concentration was elevated to 300 nM. Eventually, hybridization duration of 15

355   minutes was determined as optimal since the majority of the hybridization positive events

356   were detected in the first 15 minutes. The squares with thick frames stacked vertically

357   indicate optimised hybridization conditions applied to all subsequent experiments.

358

359   **PNA-FISH gating method for BC**

360   A gating method for the PNA-FISH assay in BC differed from the gating method

361   applied previously to PNA-FISH in pure bacterial culture (Fig S1B) due to an overlap between

362   blood and probe specific signals in the 530/30nm channel (data not shown). A different

363   approach was developed for the PNA-FISH in BC based on the difference between the

364   specific signal of the AlexaFluor 488-labeled PNA-FISH probe attached to the bacterial DNA

365   (530/30nm) and a non-specific autofluorescence (574/26nm) of the lysed blood. Accordingly,

366   the bacterial population could be found in the BL1 channel (AlexaFluor 488) while the lysed

367   blood elements (autofluorescence) were observed in the BL2 channel (Fig 4, i). The bacterial

368   population was further refined by the FSC-H/FSC-A scatter plot that was used to gate out

369   the doublets (Fig 4, ii). Distribution of signal intensity of the AlexaFluor 488 on the histogram

370   indicated uniform probe hybridization (Fig 4, iii). This was later used as a bacteria-specific

371   signal to gate events representing pure bacterial cell population on the FSC-H/SSC-H scatter

372   plot that was labeled as dPOI (Fig 4, iv).

373   The validity of gating off blood debris using autofluorescence was confirmed in a

374   separate experiment in which the white blood cell-specific antibody anti-CD45 PerCP was

375   used. This antibody bound to the same population observed by autofluorescence but not to

376   pure bacterial population confirming that autofluorescence originated from the lysed blood

377   (data not shown).

378

379   **Fig 4. Gating of PNA-FISH hybridized BC samples.** Bacteria-specific events were separated

380   from the background noise of lysed blood based on a difference between the PNA probe-

381   specific signal and autofluorescence produced by blood (i). This was followed by

382   identification of single cells on the FSC-H/FSC-A scatter plot within the bacterial population

383   (ii). Probe specific signal was gated as AlexaFluor 488 + on the histogram (iii), and all events

384   within this gate were backgated and shown on the FSC-H/SSC-H plot as dPOI (iv).

385

386   **Bacterial detection in BC by PNA-FISH enhanced AFC**

387   A pair of aerobic bottles spiked with approximately 10 CFU/mL of bacteria were set

388   up for three different bacterial species, *E. coli, K. pneumoniae and P. aeruginosa* and

389   incubated in the BacTEC system. Aliquots (1mL) from one bottle of the pair were removed

390   hourly, treated with 10% Triton X-100 and the bacteria concentrated and washed by

391   centrifugation. The samples were then hybridized with the PNA-FISH probe under optimized

392   conditions and analyzed on the NxT AFC. The other BC bottle remained incubating on the

393   BacTEC system until the positivity threshold had been reached.

394   Using *E. coli* as a test species for PNA-FISH flow cytometry, the first positive signal

395   was detected at 5 hours of incubation following hourly sampling and PNA-FISH analysis

396   within the bacteria-specific gate (Fig 5A). The number of events within the bacteria-specific

397   gate increased further after 6 and 7 hours of incubation, indicating actively growing bacteria

398   (Fig 5A). This experiment was repeated three times and time to detection (TTD) with PNA-

399   FISH-AFC for *E. coli* in the spiked BC was determined to be between 5-6 hours.

400   Simultaneously, growth of *E. coli* in spiked BC was monitored by plate count at 2-hour

401   intervals from the same BC bottle (Fig 5B). The time to blood culture-based positivity for *E.*

402   *coli* was determined in the separate, simultaneously incubated BC bottle to be between 13-

403   14 hours with bacterial concentrations reaching values from 4.59 x10^8 to 9.20 x 10^8 CFU/mL

404   at this point (Fig 5B).

405   Similar TTD results were obtained for *K. pneumoniae* using PNA-FISH-AFC analysis

406   (see Fig S3). However, the first positive signal for *P. aeruginosa* by PNA-FISH-AFC was

407   observed after 10 hours of incubation in the BacTEC system (Fig S3). Combined analyses for

408   all three-bacterial species revealed that the first positive signal by PNA-FISH-AFC occurred at

409   a bacterial concentration of 10^3-10^4 CFU/mL reached by *E. coli* and *K. pneumoniae* after 5

410   hours in BC and after 10 hours in BC by *P. aeruginosa*, respectively (Fig 5B). The time to

411   positivity of *E. coli* and *K. pneumoniae* were similar on the BacTEC in the range of 13-14

412   hours of incubation, while the growth of *P. aeruginosa* required 19-21 hours to reach the

413   arbitrary levels due to a slower growth rate (Fig 5B). Since slow growers showed a less steep

414   gradient than fast growers, there was more time gained to detect slow-growing organisms

415   in blood culture when the PNA-FISH assay was applied (Fig 5B).

416

417   **Fig 5. Early bacterial detection in BC with PNA-FISH-AFC. A:** Detection of *E. coli* by PNA-FISH

418   enhanced AFC in spiked BC following incubation on the BacTEC FX system. A 9 time increase

419   in number of events was observed in the bacteria-specific gate from 5 to 7 hours of

420   incubation indicating actively growing bacterial population. **B:** Growth curves of bacteria in

421   spiked BC on the BacTEC system. Two fast growing bacterial species, *E coli* and *K*

422   *pneumoniae*, and one slow grower, *P aeruginosa*, were incubated on the BacTEC FX system.

423   The 2-hourly determined plate counts (*y*-axis, CFU/mL) were used to produce growth curves,

424   while the appearance of bacteria-specific events by PNA-FISH enhanced AFC was monitored

425   hourly for the first 12 hours of incubation. The overall positivity on the BacTEC system was

426   also monitored in parallel. Bacteria-specific signal by PNA-FISH-AFC was first observed at 5

427   and 10 hours of incubation for the fast (*E. coli, K. pneumoniae*) and slower growing bacterial

428   species (*P. aeruginosa*), respectively. This was confirmed by three independent experiments.

429   The positivity on the BacTEC system was reached at approximately 13 hours and 21 hours of

430   incubation for the fast and slow growers, respectively, also confirmed by three independent

431   experiments with slight variations between experiments. The horizontal broken lines

432   indicate the sensitivity of the PNA-FISH-AFC at 10^3-10^4 CFU/mL, and for the BacTEC system

433   at 10^8-10^9 CFU/mL. The time gained by the PNA-FISH assay when compared to BacTEC

434   positivity was 8 hours for fast growers, as indicated by blue arrows, and 10 hours for slow

435   grower, as indicated by red arrows, respectively.

436

437   **Bacterial enumeration - PNA-FISH-AFC *versus* plate count**

438   Each aliquot of spiked BC that was analyzed by PNA-FISH flow cytometry was also

439   enumerated by the plate count method for comparison as indicated in Fig 5B. Biological

440   triplicates were set up for both assays and average bacterial concentrations determined by

441   either PNA-FISH or plate counts are presented in Table 3. As shown on Table 3, the number

442   of events determined by PNA-FISH-AFC was lower than the number of colony forming units

443   (CFU) determined by plate count. Plausible explanations include cell loss during

444   hybridization due to multiple washes and exposure to high temperatures, as well as

445 application of gating methods that removed the bacterial doublets during post analysis.

446 Probe efficiency was very high (97.1%) as expected of peptide nucleic acid probes, hence

447 this did not contribute to the cell loss.

448

449 **Table 3. Bacterial enumeration using PNA-FISH and plate counts at the time of detection**

Bacterial species	BC incubation in the BacTec system (hours)	PNA-FISH-AFC (events/mL±SD)	Plate counts (CFU/mL ± SD)
Escherichia coli	5	$0.17 \times 10^4 \pm 0.11$	$0.21 \times 10^4 \pm 0.27$
	6	$0.40 \times 10^4 \pm 0.26$	$0.57 \times 10^4 \pm 0.72$
	7	$0.28 \times 10^4 \pm 2.72$	$2.94 \times 10^4 \pm 1.05$
Klebsiella pneumoniae	5	$0.25 \times 10^4 \pm 0.40$	$0.44 \times 10^4 \pm 0.62$
	6	$0.64 \times 10^4 \pm 0.79$	$1.41 \times 10^4 \pm 1.44$
	7	$5.29 \times 10^4 \pm 6.24$	$9.24 \times 10^4 \pm 12.2$
Pseudomonas aeruginosa	10	$1.09 \times 10^4 \pm 0.80$	$1.31 \times 10^4 \pm 1.38$
	11	$2.36 \times 10^4 \pm 1.81$	$3.10 \times 10^4 \pm 3.80$
	12	$7.52 \times 10^4 \pm 8.31$	$12.1 \times 10^4 \pm 18.2$

450

451

Performance of PNA-FISH-AFC relative to MALDI-TOF and FilmArray®

453 Two additional culture-independent assays were set up in parallel with PNA-FISH-

454 AFC, MALDI-TOF and FilmArray®, that are routinely used in clinical laboratories. When all

455 three assays were performed in parallel, the TTD using PNA-FISH-AFC was shorter than using

456 MALDI-TOF for all three organisms; FilmArray® and PNA-FISH-AFC were equally sensitive for

457 *K. pneumoniae* and *P. aeruginosa* resulting in similar TTD; while PNA-FISH-AFC was more

458   sensitive than FilmArray**®** for *E. coli* shown by 2-hours shorter TTD (Table 4). This

459   experiment was performed three times, demonstrating consistently shorter TTD by PNA-

460   FISH-AFC than MALDI-TOF for all three bacterial species and a limited advantage of PNA-

461   FISH-AFC over FilmArray**®** assays for *E coli*.

462

463   **Table 4. Earliest time to detection using different diagnostic assays**

Bacterial Species	Time to Detection (hours)		
	PNA-FISH-AFC	**FilmArray®**	**MALDI-TOF**
Escherichia coli	5	7	12
Klebsiella pneumoniae	5	5	12.5
Pseudomonas aeruginosa	10	10	21

464

465   **Discussion**

466

467   Since the early 20th century there has been a search for faster ways to detect

468   bacteria in the bloodstream. While direct detection has thus far been unachievable due to

469   low bacterial starting concentrations of 1-10 CFU/mL of blood, a universal blood culture

470   system that could support growth and increase the concentration of any clinically significant

471   bacterial species has been developed. Unfortunately, nutritional requirements for bacterial

472   species differ, so does the time to achieve a minimum concentration that would give

473   reliable results in subsequent diagnostic tests, ranging from several hours to several days in

474   culture. Bacterial concentrations in BCs at the time of positivity in autoanalyser systems

475   have been established at 10^7-10^9 CFU/mL [13-23]. These figures are in agreement with our

476   data from the BacTec system at the time of positivity, and determined by both flow

477   cytometry and plate counts to be in the range of 4.59-9.20X10^8 CFU/mL for three commonly

478   isolated bacterial species, *E. coli, K. pneumoniae* and *P. aeruginosa*.

479   The introduction of MALDI-TOF analysis has significantly improved the speed of

480   bacterial identification from positive BCs. However, MALDI-TOF relies on a high bacterial

481   concentration and still requires extended incubation time in BCs. It may also require a purity

482   subculture, particularly in polymicrobial and streptococcal bacteraemias where the

483   subculture is mandatory [26, 27]. Recently, a pre-lysis step was introduced prior to bacterial

484   identification by MALDI-TOF in order to avoid the need for subculture [28-31]. This has

485   reduced the turnaround time to bacterial identification to approximately 2 hours after the

486   BC bottle flagged positive although the BC time to positivity had to be extended to allow

487   sufficient bacterial concentration for MALDI-TOF analysis [22].

488   Here we describe a novel approach to bacterial detection with peptide nucleic acid

489   (PNA) fluorescence *in situ* hybridization (FISH) enhanced acoustic flow cytometry (eAFC)

490   that is applied directly to positive BCs at earlier time point without the subculture step. The

491   use of a eubacterial 16S rRNA probe specifically targets bacterial nucleic acids while the flow

492   cytometer determines cellular characteristics of bacteria from their physical and

493   fluorescence properties. In contrast to the PCR-based approaches that do not distinguish

494   between genetic material that is either cell-free or within bacterial cells, the combination of

495   molecular and cellular analyses in PNA-FISH-AFC specifically detects genetic material within

496   intact bacterial cells. Thus, the integrity of bacterial cells becomes another diagnostic

497   feature of this assay that indicates the presence of bacteria in a patient's bloodstream.

498   The fluorophore selected for the FISH assay produces a signal within the green

499   spectrum where much of the autofluorescent background noise produced by leukocytes

500   was observed [32]. Indeed, leukocytic debris was labeled with anti-CD45 antibody

501   conjugated to PerCP in lysed uninfected and spiked BC. In contrast, pure bacterial cultures

502   were CD45 negative (data not shown). The gating method using 530/30nm and 574/26nm

503   scatter plot on the NxT acoustic flow cytometer we developed here proved effective in

504   discriminating lysed blood elements from the bacterial population. This has significantly

505   increased the sensitivity of bacterial detection in BC to the threshold values of $10^3 - 10^4$

506   CFU/mL and decreased time to positivity to only 5 hours for rapidly dividing *K. pneumoniae*

507   and *E.coli*, and 10 hours for slow growers such as *P. aeruginosa*.

508   The newly developed PNA-FISH assay proved more sensitive than MALDI-TOF

509   analysis for detecting three bacterial species in BC, namely *E. coli*, *K. pneumoniae* and *P.*

510   *aeruginosa,* by four to five orders of magnitude. Here, we used a universal eubacterial

511   probe to optimize the assay. In future, we envisage the use of bacterial species-specific

512   probes for the most frequent bacterial species associated with sepsis.

513   The time to detection of FilmArray® was similarly effective as PNA-FISH for early

514   detection of *K. pneumoniae* and *P. aeruginosa,* however, this technology is PCR-based and

515   provides evidence only for the presence of bacterial nucleic acids. In the case of *E. coli*

516   detection, FilmArray® in common with other PCR-based assays, shows decreased sensitivity

517   due to a need to avoid false positives as it is a common contaminant of laboratory reagents.

518   A key advantage of the latter is the detection of bacterial nucleic acids within intact

519   bacterial cells despite a similarity in the time to detection between FilmArray® and PNA-

520   FISH-AFC, and the ability of both methods to provide molecular identification.

521   A further advantage of our assay method is increased specificity and sensitivity of

522   detection of intact bacterial cells in BC that can reduce time to bacterial detection by 6 to 12

523   hours. The optimized protocol allows simultaneous set up of multiple samples with a simple

524   procedure for manual sample acquisition and processing. Overall, it takes 1.5 hours to

525 complete the FISH step with multiple samples, and an additional 3 minutes for AFC reading

526 per sample. In future, we plan to use multiple species-specific probes for early bacterial

527 identification in polymicrobial infections.

528   The novel diagnostic assay, PNA-FISH-AFC, described here has a potential to

529 significantly reduce the turnaround time of bacterial detection and identification in blood

530 culture in large clinical laboratories, and may allow blood culture autoanalyser systems to

531 use lower threshold values for earlier detection of bacteraemia. In septicaemic patients with

532 high bacterial loads in peripheral blood, this novel assay has the potential to identify the

533 causative agent(s) directly from the whole blood. Ultimately, the major potential of PNA-

534 FISH-AFC is in early and enhanced bacterial detection in blood culture that will improve

535 survival and management of bacteraemic patients.

536

537   **Competing interests**

538   XXH receives a PhD scholarship from PathWest Laboratory Medicine WA; TJJI

539 received in-kind support from Thermo Fisher Scientific in the form of loan equipment

540 (acoustic flow cytometer): http://corporate.thermofisher.com/en/home.html

541

542   **Ethics statement**

543 The study was conducted in accordance with the Australian National Health Research Ethics

544 guidelines (NH&MRC), the Declaration of Helsinki and Good Clinical Practice guidelines.

545 Anonymised samples without identifiable clinical data can be used for *in vitro* pathology test

546 development without further Health Research Ethics Committee approval. The work was

547 conducted as a clinical laboratory quality initiative, in which no patient samples were used.

548   The authors did not collect the blood, which was obtained from healthy adult volunteers by

549   the state pathology service's phlebotomists at a designated specimen collection centre.

550   Institutional governance requirements were met by completing a specimen collection

551   request form on each occasion volunteer blood samples were required.

552

553   **Acknowledgement**

557

558   **References**

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673

Supplementary Data

675   **S1. Gating method for PNA-FISH optimization experiments on pure *K.pneumoniae* culture.**

676   **A**: SYTO® 9 was used in the formamide and hybridization temperature optimization

677   experiments to investigate the overall bacterial population that contained both single and

678   multiple events. POI was identified based on FSC-H/SSC-H plots, from which SYTO® 9

679   positive population was identified on the histogram. A 10% contour was applied to the

680   SYTO® 9 positive population and labelled derived POI (dPOI). **B:** PNA-FISH gating on pure

681   bacterial culture where POI was identified based on FSC-H/SSC-H plot followed by FSC-

682   H/FSC-A to exclude doublet events. Probe specific events were identified from the singlet

683   population as AlexaFluor 488+ on the histogram and this population was further gated as

684   dPOI using FSC-H/SSC-H plot.

685

686   **S2. Flow charts of PNA-FISH-AFC optimization.** Four optimization experiments were set up

687   sequentially to identify optimal conditions for bacterial detection by PNA-FISH-AFC as

688   shown in Figure 3. Thick frames indicate the optimal conditions selected for subsequent

689   experiments. Firstly, bacteria in pure culture were exposed to varying concentrations of

690   formamide. The effect of increasing concentrations of formamide from 20 to 40% (v/v) on *K.*

691   *pneumoniae* was monitored by SYTO® 9 staining and presented as dPOI in the FSC-H/SSC-H

692   plots (top row). The best cell preservation with tight bacterial population in the dPOI was

693   observed in 30% formamide when compared to buffers containing 20 and 40% (v/v)

694   formamide. In the second row, bacteria were hybridized in buffer containing 30%

695   formamide (v/v) for 15 minutes at varied hybridization temperatures of 30°C, 40°C and 55°C

696   followed by SYTO® 9 staining. At temperatures of 30°C and 40°C, a better cell preservation

697   was observed with minimal debris to the left of dPOI than at 55°C, where the debris

698   subpopulation to the left of dPOI increased indicating likely degradation of bacterial cells at

699   elevated temperature. In a third row, bacteria were hybridized at 40°C for 15 minutes in

700   hybridization buffer containing 30% formamide (v/v) followed by two washes using wash

701   buffer. Signal intensities of PNA-FISH probe concentrations of 200 nM and 300 nM were

702   compared in a histogram where no significant difference between them was observed.

703   Hence, 200 nM probe concentration was selected for the following experiment to test

704   hybridization duration. In the last experiment (bottom row), two aliquots with the same

705   concentration of bacteria were subjected to PNA-FISH assay for either 15 or 30 minutes

706   under the conditions described above. The signal intensity was 1 log stronger after 15

707   minutes hybridization (red) than after 30 minutes (blue). Hence, the optimal hybridization

708   condition for PNA-FISH-AFC was determined in the presence of 30% formamide (v/v) and

709   200 nM probe at 40°C for 15 minutes.
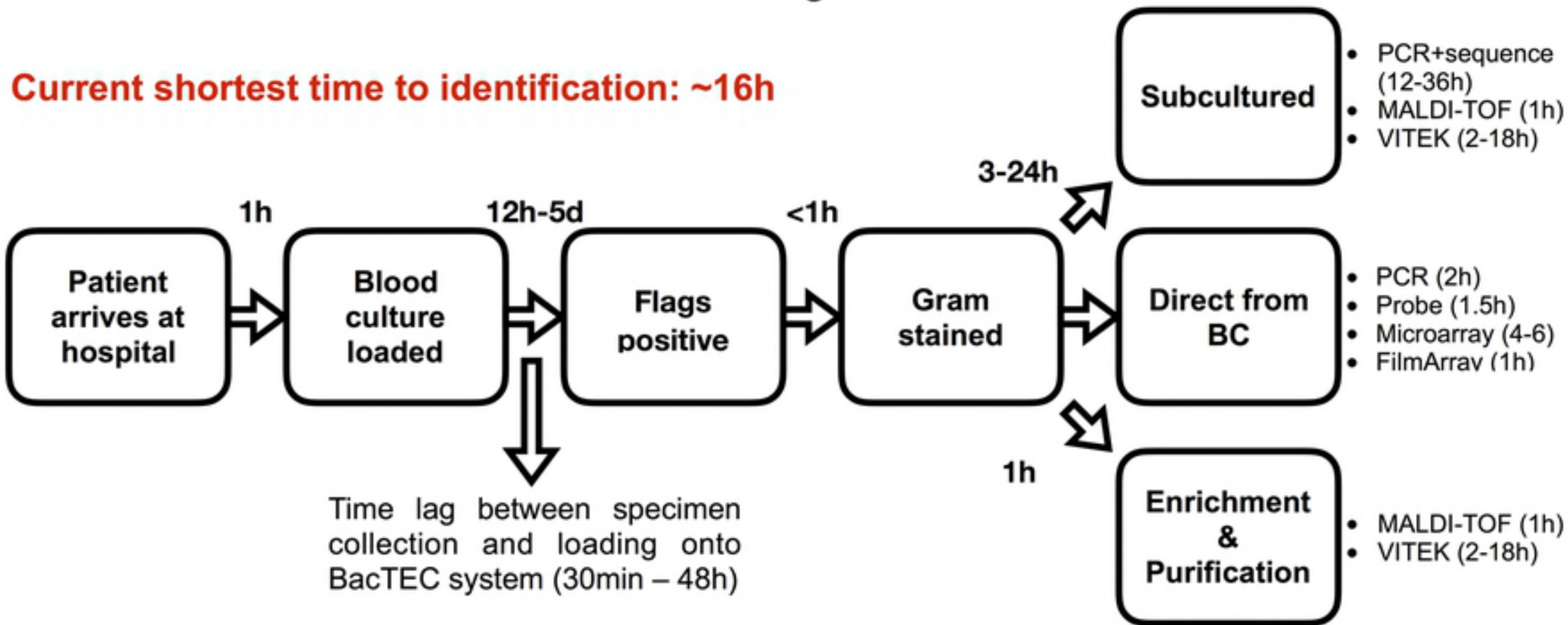
710

711   **S3. Early bacterial detection in spiked BC with *K. pneumoniae* and *P. aeruginosa.*** Similar to

712   experiments with *E. coli* spiked BC (Fig 5), two other bacterial species, *K. pneumoniae* and *P.*

713   *aeruginosa* were subjected to PNA-FISH-AFC following extended incubation in BC. The

714   lowest bacterial concentration detected for both species was between 10^3-10^5 ev/mL as

715   shown below the bacteria-specific population on the plots. While *K. pneumoniae* was first

716   detected by PNA-FISH at 5 hours of incubation on the BacTEC FX system (top row), it took 10

717   hours to detect *P. aerugin*osa (bottom row). Additional 2 hours of incubation resulted in an

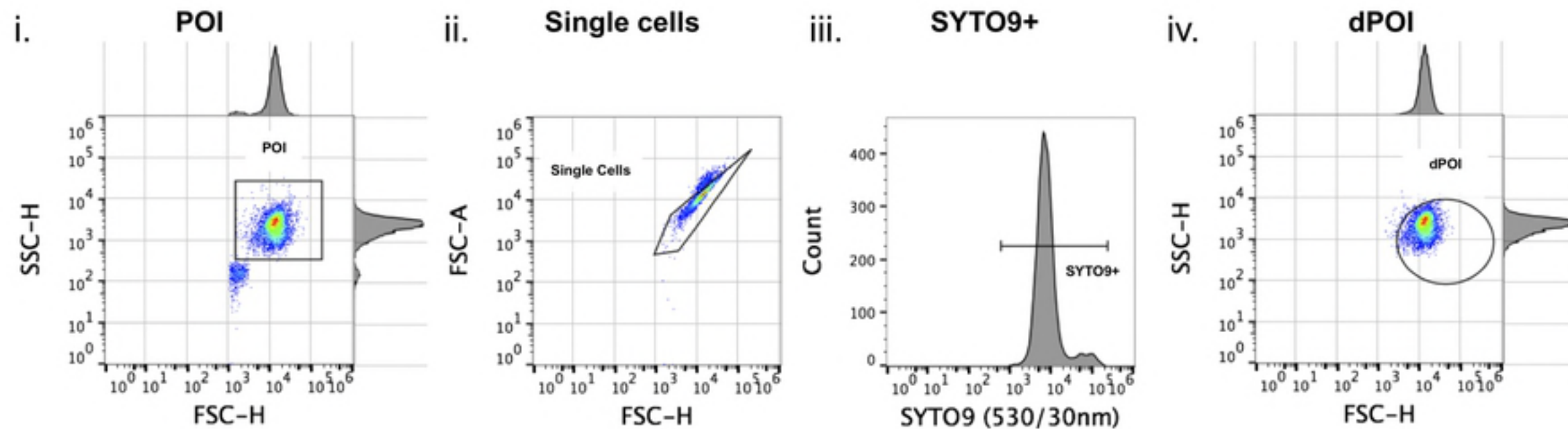718   increase of bacteria specific population for both organisms.
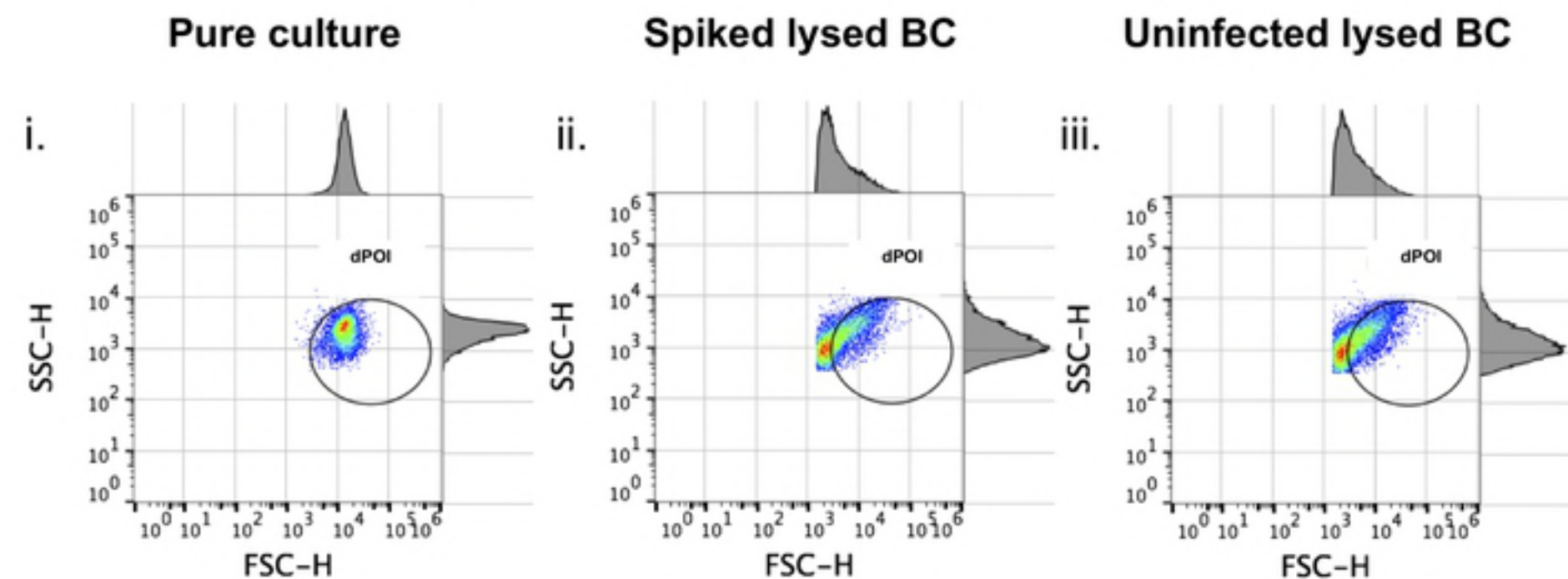
Bacteraemia Diagnostics Timeline

Current shortest time to identification: ~16h

Patient arrives at hospital — 1h → Blood culture loaded — 12h-5d → Flags positive — <1h → Gram stained

Time lag between specimen collection and loading onto BacTEC system (30min – 48h)

Gram stained — 3-24h → **Subcultured**
- PCR+sequence (12-36h)
- MALDI-TOF (1h)
- VITEK (2-18h)

Gram stained → **Direct from BC**
- PCR (2h)
- Probe (1.5h)
- Microarray (4-6)
- FilmArray (1h)

Gram stained — 1h → **Enrichment & Purification**
- MALDI-TOF (1h)
- VITEK (2-18h)

A. SYTO® 9 gating

i. POI

ii. Single cells

iii. SYTO9+

iv. dPOI

B. Bacterial detection with SYTO® 9

Pure culture

Spiked lysed BC

Uninfected lysed BC

i.

ii.

iii.

Cell recovery during PNA-FISH assay optimization
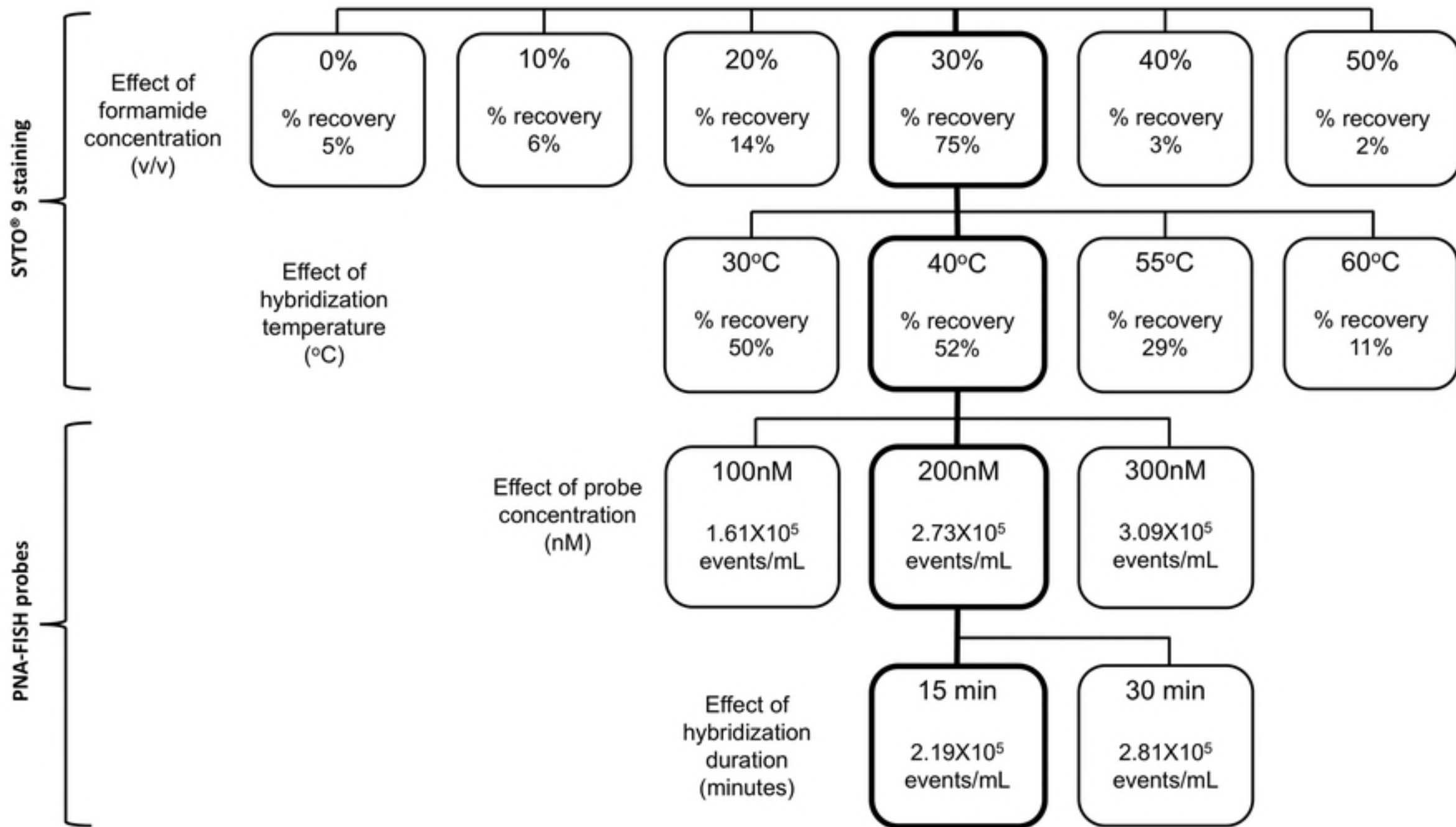
PNA-FISH gating for blood culture

i. Bacterial specific — ii. Single cells — iii. AlexaFluor 488+ — iv. dPOI

A. Early bacterial detection by PNA-FISH AFC

5 hours

Lysed blood 98.4

Bacterial specific 0.63

5.20×10^3 ev/mL

6 hours

Lysed blood 97.7

Bacterial specific 1.34

1.40×10^4 ev/mL

7 hours

Lysed blood 93.0

Bacterial specific 5.24

4.11×10^4 ev/mL

E.coli BC — Autofluorescence (574/26nm)

AlexaFluor 488 (530/30nm)
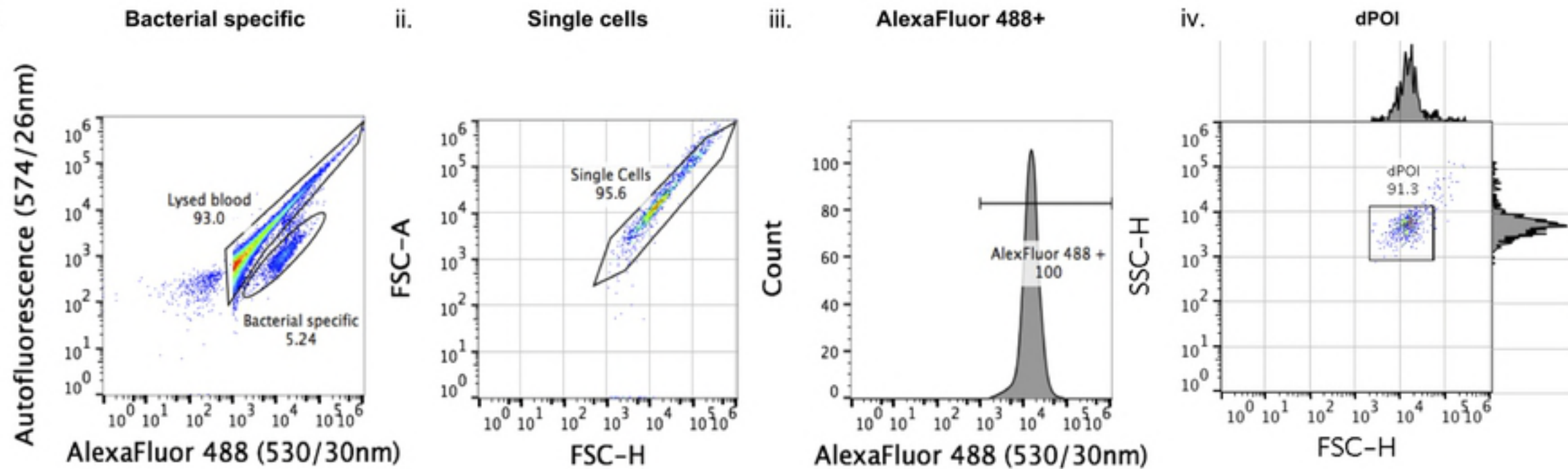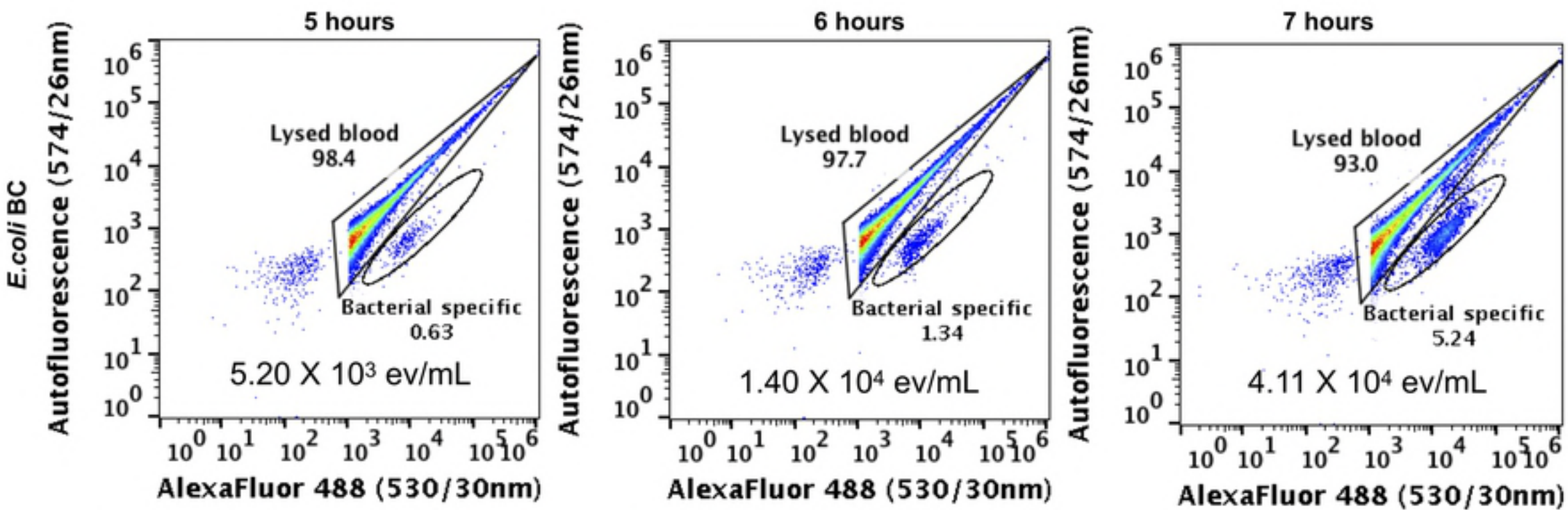
B. Sensitivity and timing of bacterial detection

BacTEC flags positive

PNA-FISH AFC assay

Bacterial Concentration (CFU/mL)

Time (hours)

— P.aeruginosa — E.coli — K.pneumoniae