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1 **Detection of *Candida albicans* DNA from blood samples using a**
2 **novel electrochemical assay.**

3

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5

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8

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12

13

14 Running title: Electrochemical detection of *Candida* DNA in blood.

15

16

17 Subject: Diagnostics, typing and identification.

18

19 **Summary**

20 The genus *Candida* contains a number of yeast species which are
21 opportunistic pathogens and are associated with life-threatening infections in
22 immunocompromised individuals. Provision of appropriate therapy relies on the rapid
23 identification of the infecting species and existing methods of identifying *Candida*
24 species in clinical samples are time and resource intensive and are not always
25 specific enough to differentiate between drug-susceptible and drug-resistant species.
26 We have previously developed a system for the rapid detection of yeast pathogens in
27 clinical samples using PCR followed by hybridisation with a suite of five species-
28 specific, electrochemically labelled DNA probes. The limit of detection of the assay
29 was shown to be 37fg (\approx one genome) per reaction using extracted genomic DNA.
30 We carried out a study to test the limit of detection of one of the probes, CA PR3,
31 using blood samples from a healthy donor that were spiked with genomic DNA or
32 with *C. albicans* cells. Our results demonstrate a limit of detection of 37fg ml⁻¹ (\approx 1
33 genome ml⁻¹) of blood using extracted DNA or 10c.f.u ml⁻¹ of blood using *C. albicans*
34 cells indicating that the assay is capable of detecting *C. albicans* nucleic acid at
35 levels that are encountered in clinical samples.

36

37

38

39 **Introduction**

40 Some species of fungi are capable of causing life-threatening invasive
41 infections most commonly in individuals with a weakened immune system due to
42 underlying disease or immuno-suppressive therapy (Pfaller & Diekema, 2010) and
43 species of the genus *Candida* are by far the most prevalent, being responsible for 70-
44 80% of diagnosed fungal bloodstream infections in the United States (Fridkin and
45 Jarvis, 1996; Trick *et al.*, 2002). *C. albicans* is usually the most commonly isolated
46 species from patients with invasive candidiasis, accounting for approximately 45-65%
47 of cases.

48
49 Diagnosis of invasive candidiasis can be difficult and culture remains the 'gold
50 standard' for definitive diagnosis of invasive fungal infections. However, identification
51 of fungi by culture and subsequent biochemical tests is time consuming (Morris *et al.*,
52 1996) and has been reported to lack sensitivity (Pemán & Zaragoza, 2009). A
53 number of nucleic acid-based assays for detecting fungi have been developed (e.g.
54 Hata *et al.*, 2008; Borman *et al.*, 2008; Lau *et al.*, 2008) which are capable of species-
55 level identification and some have been shown to be capable of direct detection of
56 fungal DNA from blood (e.g. McMullan *et al.*, 2008; Zhao *et al.*, 2009).

57
58 A novel method for detection of nucleic acid was developed by Hillier *et al.* (2004a;
59 2004b) using oligonucleotide probes labelled with the electroactive compound
60 ferrocene and detection was performed using differential pulse voltammetry (DPV) on
61 solid electrodes. We previously extended this work and developed a pan-fungal
62 probe as well as a suite of species-specific probes for the detection of the five most
63 clinically relevant *Candida* species which were capable of detecting DNA equivalent
64 to a single genome from target fungal species in a 50µl reaction (Muir *et al.*, 2009).

65
66 The aim of this work was to develop the assay further in order to optimise conditions
67 to allow detection of clinically relevant amounts of fungal nucleic acid in blood
68 samples. Fungal titres in blood can be extremely low and have been reported to be
69 in the range of 5 – 100 colony forming units (c.f.u) ml⁻¹ of blood (Loeffler *et al.*, 2000).
70 Therefore a study was performed using the *C. albicans* specific probe to optimise
71 reaction conditions for detection of low amounts of *C. albicans* DNA and cells in 1ml
72 spiked blood samples.

73 **Materials and methods.**

74 **Culture acquisition**

75 The type strain of *C. albicans* (CBS 562) species was obtained from the
76 Centraal Bureau voor Schimmelcultures (CBS) and used for all experiments.
77 Cultures were grown on yeast-peptone-dextrose (YPD) agar (1% yeast extract
78 (Oxoid), 2% Mycological peptone (Oxoid), 2% glucose (Sigma), 2% Bacto agar
79 (Difco)) at 28°C for 48 hours. Liquid stocks of *C. albicans* were prepared using the
80 following method: a growing colony was picked from YPD agar using a sterile loop,
81 transferred to 5ml liquid YPD and incubated overnight at 28°C with shaking. 1ml
82 samples of each culture were then aliquoted into 2ml microcentrifuge tubes. Finally,
83 1ml of 20% glycerol added as a cryoprotectant before transfer to -20°C storage.
84

85 **Steps to limit contamination**

86 In order to prevent contamination the setup of PCR reactions was performed
87 in a different room to where the PCR amplification was performed and in a class 2
88 biological safety cabinet which was regularly disinfected with 70% ethanol and
89 sterilized using ultra violet (UV) irradiation. The air flow remained switched off while
90 the mix was prepared to avoid contamination of samples by airborne PCR amplicon.
91 Blood samples were aliquoted at a different site, in a laminar flow hood that was
92 regularly sterilised as above and additionally was regularly swabbed with the DNA
93 degrading reagent DNA-ExitusPlus (Applichem). All stock reagents were aliquoted
94 into working solutions to limit potential contamination and problems associated with
95 freeze-thawing.
96

97 **Whole blood acquisition and treatment**

98 Blood from a healthy donor was collected by a trained individual into 6ml
99 K₂EDTA-coated vacutainers (BD Biosciences) and inverted 8 – 10 times to ensure
100 adequate mixing of the anti-coagulant with blood. 1ml aliquots were then pipetted
101 into 2ml sterile cryovials and the samples stored at -80°C until used.
102

103 **Extraction of DNA from liquid cultures**

104 The QIAGEN DNeasy extraction kit was used for all extractions and the
105 manufacturer's protocol was followed with some modifications that were appropriate
106 for treatment of fungal material. The yeast cell count in a stock cell suspension was

107 estimated by spectrometry according to the method described in Amberg *et al.*
108 (2005) and a solution of approximately 5×10^6 yeast cells was prepared by appropriate
109 dilution of the stock and centrifuged at 10,000 rpm for 10mins to pellet the cells. The
110 supernatant was removed and the cell pellet resuspended in 500ul of a lyticase lysis
111 buffer which comprised 10 U ml^{-1} lyticase from *Arthrobacter luteus* (Sigma), 50mM
112 Tris, pH 7.5, 10mM EDTA and 28mM β -mercaptoethanol; the lyticase digestion was
113 performed at 37°C for 30mins. Following treatment by lyticase the sphaeroplasts
114 were collected by centrifugation and the supernatant was removed. The disruption of
115 the sphaeroplast cell membrane by proteinase and detergent treatments was
116 performed according to manufacturer's instructions and, following the addition of
117 absolute ethanol, the samples were left on ice for 30mins to increase precipitation of
118 DNA and enhance final yield. The samples were then placed in silica-based spin
119 columns and washed according to the manufacturer's instructions. DNA was then
120 eluted twice from each column using 50 μ l of the elution buffer for each elution and a
121 single 1.5ml tube was used to collect both eluates. The concentration of eluted DNA
122 was estimated by spectrometry. All DNA was stored at -20°C.

123

124 **Extraction of DNA from blood samples spiked with *C. albicans* genomic DNA.**

125 The required number of 1ml whole blood samples were removed from storage
126 at -80°C and allowed to thaw. *C. albicans* DNA was removed from -20°C storage
127 and allowed to thaw. A 5ml sterile bijoux tube had the required amount of *C. albicans*
128 genomic DNA added and 1ml of the blood sample was then added to the bijoux. The
129 solution was agitated gently to allow mixing of the DNA in the blood. Extraction of
130 the *C. albicans* DNA from blood was performed using GenElute Bacterial Genomic
131 DNA extraction kit (Sigma) with some modifications which are described below.

132

133 The spiked blood samples had 2ml of a proprietary lysis buffer added and were
134 incubated for 5mins at room temperature with occasional gentle agitation to allow
135 mixing and complete lysis of blood cells. Meanwhile the required number of
136 extraction columns was prepared using Column Preparation Solution as per
137 manufacturer's instructions. After the 5min incubation of blood samples 700 μ l of the
138 lysate was applied to the spin column and centrifuged at 12,000g for 1min to allow
139 DNA to bind to the column. The flow-through was discarded. The above step was
140 repeated until all of the lysed blood sampled had been spun through the column.

141 After the final centrifugation the collection tube was discarded and replaced with a
142 fresh one. Wash steps were then performed as per the manufacturer's instructions.
143 Elution of DNA was initially performed by adding 100µl of elution buffer to the
144 column, incubating at room temperature for 30s and centrifuging at 12,000g for 1min.
145 30µl of this eluate was used as template in PCR reactions. However, to improve the
146 yield when very small amounts of *C. albicans* DNA were spiked into blood the elution
147 buffer was prewarmed to 60°C and 34.25µl was applied to the spin column,
148 incubated for 30s at room temperature and centrifuged at 12,000g for 1min. The
149 eluate was then reapplied to the spin column, incubated and centrifuged for a second
150 time. This entire eluate was then used as template in PCR amplifications.

151

152 **Monitoring for DNA loss and inhibition of PCR**

153 To monitor for potential loss of DNA during the extraction procedure, a
154 comparison was made between assays performed with samples that were extracted
155 from blood spiked with excess *C. albicans* DNA (37pg), and unspiked blood samples
156 which were processed in parallel before having an excess amount (37pg) of *C.*
157 *albicans* DNA added to the eluate. Monitoring for potential inhibition of PCR by
158 excess non-target DNA and/or inhibitory compounds from blood that may have co-
159 eluted with the DNA was performed by comparing the results of assays performed
160 using DNA extracted from spiked blood samples (3.7pg *C. albicans* DNA) and DNA
161 extracted from 1ml samples of sterile H₂O that were spiked with an equal amount of
162 DNA.

163

164 **Extraction of DNA from blood samples spiked with *C. albicans* cells.**

165 To obtain cell suspensions of the appropriate number of cells to spike into
166 blood samples, growing colonies of *C. albicans* were picked with a sterile loop and
167 resuspended in 500µl sterile H₂O. This stock solution was diluted 10-fold and 50-fold
168 and cell-density estimates of the three solutions were made using an improved
169 Neubauer haemocytometer. Estimates of different preparations ranged between
170 $\sim 2 \times 10^7$ cells ml⁻¹ to $\sim 6 \times 10^7$ cells ml⁻¹ and estimates were considered to be in good
171 agreement with one another if the cell density estimates were within 0.5×10^7 cells ml⁻¹
172 ¹ of one another to allow for pipetting inaccuracies. Provided it satisfied this criterion,
173 the 50-fold diluted cell suspension was further diluted to yield solutions of 1 cell µl⁻¹

174 and 0.1 cell μl^{-1} . Viable cell densities were confirmed retrospectively by performing
175 plate counts.

176

177 The extraction procedure was very similar to the method outlined above but differed
178 in the pre-treatment step. After thawing the blood samples, 10 μl of the appropriate
179 *C. albicans* cell suspension was added to a 5ml sterile bijou and then the 1ml blood
180 sample was added and the solution gently mixed. 250 μl of a lysis buffer containing
181 200U ml^{-1} lyticase was added and the reaction mix incubated at room temperature for
182 5min with gentle agitation. After this step the extraction continued with the addition of
183 1ml of the proprietary lysis buffer and was identical to the procedure outlined
184 previously for genomic DNA extraction. Note that 34.25 μl of prewarmed elution
185 buffer was always used for elution of DNA extracted from *C. albicans* cells.

186

187 **Primers and probes**

188 The previously described primers (Muir *et al*, 2009) ITS3.3
189 (TGCCTGTTTGAGCGTCATTTTC) and ITS4.2 (AGTCCTACCTGATTTGAGG) were
190 used for all PCR reactions and the probe CA PR3 (ATCGCTTTGACAATGGCTTA),
191 labelled with a proprietary ferrocene molecule linked to the 5' terminal nucleotide by
192 ATDBio (Southampton) was used to detect amplified target DNA.

193

194 **PCR conditions**

195 PCR amplification conditions used a total volume of 50 μl containing the
196 following reagents: PCR buffer (10mM Tris-HCl (pH 8.3), 50mM KCl), 100nM ITS3.3,
197 500nM ITS4.2, 0.15mM each dNTP, 3mM MgCl_2 and 2.5U Jumpstart *Taq* DNA
198 polymerase (Sigma), extracted nucleic acid (30 μl or 34.25 μl , see above) and
199 molecular biology grade water (Sigma) to the required volume. The cycling
200 conditions used were: 95 $^\circ\text{C}$ for 1min; 94 $^\circ\text{C}$ for 30s plus 58 $^\circ\text{C}$ for 30s plus 72 $^\circ\text{C}$ for
201 1min for 40 cycles; 72 $^\circ\text{C}$ for 3mins. A 10 μl aliquot of the PCR products was added to
202 2 μl of 6X loading dye and visualized on a 1% (w/v) agarose gel made up in Tris-
203 Borate-EDTA (TBE) buffer (0.09M Tris base, 0.09M boric acid, 2mM EDTA pH 8.0)
204 containing 0.5 $\mu\text{g ml}^{-1}$ ethidium bromide to confirm the presence of amplified target
205 DNA. Samples were run at 7V cm^{-1} . 20 μl of the remaining PCR product was used
206 for the electrochemical assay.

207

208 **Electrochemistry**

209 A probe mix was prepared for each sample to be assayed containing the
210 following reagents: 3 μ M of probe CA PR3 and 10U T7 exonuclease (New England
211 Biolabs) made up to 5 μ l with molecular grade water (Sigma). This probe mix was
212 added to 20 μ l of PCR product and incubated for 20mins at 37°C to allow
213 hybridization of the probe to single stranded target DNA and subsequent digestion of
214 the duplex by T7 exonuclease. Once incubation was complete 20 μ l of the probe
215 reaction mix was pipetted onto screen printed carbon electrodes with a silver/silver-
216 chloride reference electrode. The electrodes used for specificity testing were
217 manufactured by Gwent Electronic Materials Ltd. and those used for sensitivity
218 testing were manufactured by G. M. Nameplate Inc. The observed current due to
219 oxidation of the released ferrocene-labeled nucleotide following DPV was measured
220 using a potentiostat (Autolab PGSTAT30, EcoChemie) and dedicated software
221 (GPES version 4.9, EcoChemie). DPV measurements were made using the following
222 settings: modulation time 0.04s; interval time 0.1s; initial procedure -0.1V; end
223 potential 0.5V; step potential 0.003V; modulation amplitude 0.05V.

224

225 **Results**

226 **Detection of excess *C. albicans* DNA and study of inhibitory effects**

227 Assays were carried out using excess *C. albicans* DNA (37pg) spiked into
228 samples in different ways to examine the effects of the extraction procedure on the
229 currents that were generated. Samples that were extracted from blood spiked with
230 excess *C. albicans* DNA produced a mean current of 153.4 \pm 6.6nA (n=3) and this
231 compared with a mean current of 168.9 \pm 17.1nA (n=3) which was produced by
232 samples from unspiked blood which had been processed using the extraction
233 protocol and the eluate spiked with 37pg fungal DNA. The PCR positive control
234 samples - obtained by amplifying the same amount of DNA in a 50 μ l PCR - produced
235 a mean current of 189.9 \pm 20.7nA and an unspiked blood sample gave no current.

236

237 The higher current produced by the positive control was probably as a result of the
238 complete availability of the 37pg template in this reaction, whereas in the other
239 reactions the 37pg template was diluted in 100 μ l of elution buffer. The difference
240 between the results obtained by spiked blood vs. spiked eluate demonstrated the
241 effect of DNA loss due to the extraction procedure on the signals obtained in the

242 assay. The difference in mean currents (15.5nA) was not significant and
243 demonstrated that DNA retention using the procedure was good. The zero current
244 produced by the samples extracted from the unspiked blood confirmed that human
245 DNA from leucocytes did not cross-react with the *C. albicans*-specific probe.

246

247 The assays comparing currents produced by samples extracted from spiked blood or
248 H₂O demonstrated that some inhibition occurred in samples extracted from blood.

249 The mean current produced by samples extracted from 1ml of H₂O spiked with 3.7pg
250 of *C. albicans* DNA was $83.1 \pm 14.9\text{nA}$ (n=3) compared with a mean of $55.5 \pm 11.5\text{nA}$
251 (n=3) produced by assays using samples extracted from 1ml blood spiked with 3.7pg
252 DNA. The difference in current (27.6nA) was statistically significant (P<0.05) and
253 indicated that inhibition, either by sequestration of primers by excess human DNA or
254 due to direct inhibition of *Taq* polymerase, occurred during PCR with samples
255 extracted from whole blood.

256

257 **Detection of clinically relevant levels of fungal DNA from spiked blood**

258 Assays were performed to detect DNA from clinically relevant amounts of *C.*
259 *albicans* spiked into blood samples. The mean currents produced by the samples
260 spiked with 3.7pg and 370fg were $75.6 \pm 8.9\text{nA}$ and $35.6 \pm 6.4\text{nA}$ (≈ 100 and ≈ 10
261 genomes respectively, n=3) and these indicated successful detection but the mean
262 current produced with 37fg DNA was $15.0 \pm 17.2\text{nA}$ (≈ 1 genome, n=3) which
263 demonstrated that detection at this level was unreliable: the mean current was low
264 and the standard deviation high due to one of the samples failing to produce a signal
265 (i.e. 0nA current, fig 1). With a minor adjustment to the elution stage of the extraction
266 procedure (i.e. use of a 34.25 μl eluate as template) more reliable identification of ≈ 1
267 genome of *C. albicans* DNA in a 1ml blood sample was achieved (mean of $26.3 \pm$
268 14.3nA , n=3).

269

270 It was noted that there was a highly significant difference (P<0.001) between the
271 mean current produced by the positive control which contained 3.7pg of DNA in a
272 50 μl PCR reaction compared to the mean current produced by samples which had
273 been extracted from 1ml blood samples spiked with 3.7pg of DNA. It was also
274 observed that the mean current produced by the positive control was approximately

275 twice that of the mean current produced by the same amount of DNA spiked into
276 blood.

277

278 **Detection of *C. albicans* cells from spiked blood samples.**

279 Fungal cells represent the major source of fungal nucleic acid in a clinical
280 sample so it was essential that the assay be capable of their detection. Results of
281 assays attempting to detect 10 c.f.u of *C. albicans* cells present in a 50µl reaction
282 and spiked into a 1ml blood sample showed that detection was successful in both
283 sample types with mean currents of $64.5 \pm 45.7\text{nA}$ (n=3) and $16.3 \pm 12.6\text{nA}$ (n=6)
284 respectively. Attempts were also made to detect suspensions containing a single
285 c.f.u of *C. albicans* added to both PCR samples and 1ml blood samples but these
286 were unsuccessful (data not shown), possibly due to the limitations inherent in the
287 DNA extraction procedure. This may also have accounted for the difference
288 ($P < 0.0001$) between the mean current of $68.5 \pm 3.6\text{nA}$ (n=3) produced by 37fg (≈ 1
289 genome) of *C. albicans* DNA in the PCR positive control and the mean current of
290 $16.3 \pm 12.6\text{nA}$ (n=6) produced by template from the spiked blood sample which
291 contained 10 *C. albicans* c.f.u's.

292

293 **Discussion**

294 The limit of detection of *C. albicans* extracted DNA in blood was shown to be
295 37fg ml⁻¹ (\approx 1 genome ml⁻¹) while the limit of detection of *C. albicans* cells in blood
296 was shown to be 10 c.f.u ml⁻¹. The extraction method was shown to recover a good
297 yield of the spiked *C. albicans* DNA from blood, and minor inhibitory effects due to
298 co-elution of constituents from human blood were demonstrated. There were
299 differences observed in the mean currents obtained from samples containing
300 equivalent amounts of nucleic acid that were prepared in different ways; the currents
301 produced by template extracted from blood spiked with extracted *C. albicans*
302 genomic DNA were higher when compared to the currents produced by assays using
303 equivalent amounts of *C. albicans* c.f.u spiked into blood, while the mean currents
304 produced by genomic DNA and whole cells added directly to PCR were higher than
305 the currents produced by material extracted from spiked blood. Furthermore,
306 detection of a single *C. albicans* c.f.u in blood was not possible using the method.

307

308 These differences could have been due in part to the effects of DNA loss occurring
309 during the extraction procedure which would likely be more critical when lower
310 amounts of DNA were used, or by the potential for PCR primers to be sequestered
311 due to interactions with non-target nucleic acid which would have been provided by
312 the large excess of human genomic DNA and RNA that would have been present in
313 the extracted DNA sample. It was also possible that fungal 5.8S and 26S rRNA
314 molecules that co-eluted with DNA may have inhibited PCR by competing for primers
315 because these molecules possess the ITS3.3/4.2 primer target sites (Li *et al.*, 1990;
316 Metwally *et al.*, 2008).

317

318 The extraction procedure used in this initial study had not been fully optimised for
319 detection of fungal DNA from blood samples. In particular, due to the volume of
320 blood and lysis buffers used, several centrifugation steps were required to process
321 each sample which increased the risk of contamination and loss of nucleic acid and
322 this may have been of particular importance when attempting to detect DNA from a
323 single *C. albicans* cell in a 1ml blood sample. Nonetheless, the fact that detection of
324 fungal cells was possible using the method was a significant result and opportunities
325 exist for optimisation of the DNA extraction procedure that may allow detection of a

326 single cell in blood. Improved methodologies will probably involve immobilisation of
327 the fungal cells on a membrane in order to remove human material and reduce the
328 volume of sample to be processed. Such modifications will be the subject of future
329 research.

330

331 Whole blood was used in the assay both to reduce the turnaround time and because
332 it is a more favourable sample type for the recovery of fungal nucleic acid. Plasma
333 and serum samples, whilst containing fewer inhibitory substances such as human
334 DNA and macromolecules, have the disadvantage of the removal of cell-associated
335 fungal DNA, as well as the extra time required for preparation. Although reports by
336 Bougnoux *et al.* (1999) and Kasai *et al.* (2006) suggested that cell-free fungal DNA
337 can survive in blood for relatively long periods in a rabbit model, and studies have
338 detected cell-free fungal DNA in serum samples (Wahyuningsih *et al.*, 2000;
339 McMullan *et al.*, 2008), other studies using mouse models have shown that naked
340 DNA is rapidly cleared from the blood, mainly due to uptake by the liver but also by
341 the action of serum-associated nucleases (Kawabata *et al.*, 1995; Liu *et al.*, 2007).
342 Therefore cell-free blood fractions may be an unsuitable sample type to use. Despite
343 the difficulties inherent in using whole blood, our results demonstrate that detection of
344 fungal DNA and cells is possible using the assay.

345

346 While spiked blood samples from a healthy patient are useful for preliminary testing
347 of an assay's capability the use of clinical samples would provide much more
348 definitive results. Samples taken from patients with an episode of invasive candidal
349 infection may differ from healthy blood samples, particularly in terms of the leucocyte
350 cell population and can also provide a better measure of the sensitivity of an assay –
351 indicating the likelihood of detection of fungal DNA from a sample taken under clinical
352 conditions. Further testing of the assay using clinical samples would be an essential
353 undertaking for future work to validate this method of detection. Additionally, the
354 remaining probes from fungal detection suite must also be tested to demonstrate a
355 similar range of detection of target DNA in blood samples.

356

357 Nonetheless, the assay was shown to be sensitive enough to detect 37fg (\approx 1
358 genome) of *C. albicans* genomic DNA per millilitre of blood but was less sensitive
359 when intact cells were used, achieving a limit of detection of only 10 c.f.u per millilitre

360 of blood. This detection limit is within the lower clinical range for candidaemia of 5 to
361 100 c.f.u ml⁻¹ (Loeffler *et al.*, 2000) but a lower limit outside of the range would be
362 preferable to avoid the possibility of false negative results and this may be achievable
363 by minor adjustments to the DNA extraction procedure as suggested above.

364

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368

369

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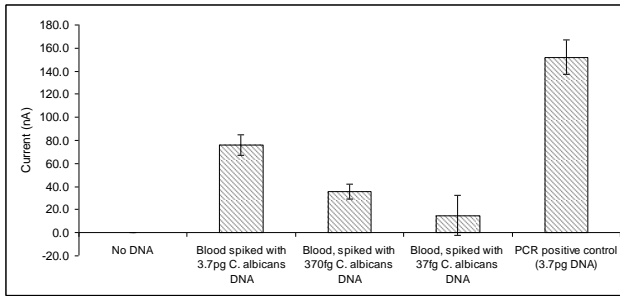
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453 **Figures.**

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456 Figure 1. Mean currents produced in assays with DNA extracted from 1ml blood samples spiked with
457 clinically relevant amounts of *C. albicans* DNA. Error bars represent SD (n=3).