# Development and Optimization of a semi automated rRNA biosensor for the detection of toxic algae

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## ABSTRACT

In order to facilitate the monitoring of toxic algae, a multiprobe chip and a semi-automated rRNA biosensor for the in-situ detection of toxic algae were developed. The biosensor consists of a multiprobe chip with an array of 16 gold electrodes for the detection of up to 14 target species. The multiprobe chip is placed inside an automated hybridization chamber, which in turn is placed inside a portable waterproof case with reservoirs for different solutions. A peristaltic pump transfers the reagents into the flow cell containing the multiprobe chip. For use of the device by laymen, a lysis protocol was successfully developed and manual rRNA isolation is no longer required. Only water sample filtration has to be done manually. The stand-alone system was evaluated using isolated total rRNA from algae cultures and field samples. The device processed automatically the main steps of the analysis and completed the electrochemical detection of toxic algae in less than two hours in comparison to other routine monitoring methods that need at least a day for analysis.

## KEY WORDS

Biosensor, disposable multiprobe chip, monitoring, sandwich hybridization, semi-automated portable device, toxic algae

53 **INTRODUCTION**

54 Coastal areas are an important economic source for fishery, tourism and. Aquaculture. The
55 latter is an increasingly important world-wide industry branch serving as a source of food and
56 employment. Planktonic algae are at the basis of the marine food chain. Thus they are critical
57 food for shellfish and fish. Consequently. in most cases, marine phytoplankton blooming as a
58 natural phenomenon and beneficial for aquaculture and wild fisheries operations. Marine
59 phytoplankton blooming is regarded as a sudden increase in the population and can be
60 activated by suitable growth conditions and cell concentrations can reach up to $10^4$-$10^5$ L-1
61 (Maso and Garces 2006). However, algal blooms can also pose a threat, because about 80
62 algal species have the potential to produce potent toxins that can find their way through the
63 food chain via shellfish (e.g., oysters, mussels) and fish to humans (Hallegraeff 2003). Among
64 the toxic algae, the marine dinoflagellate genus Alexandrium includes a number of species
65 producing saxitoxins, which are potent neurotoxins responsible for paralytic shellfish
66 poisoning (Penna 1999). Also certain Pseudo-nitzschia spp. produce a neurotoxin, which
67 causes amnesic shellfish poisoning (Scholin et al. 1999; Maso and Garces 2006). World-wide
68 monitoring programs have been introduced to observe phytoplankton composition.
69 Monitoring of toxic algae by means of traditional methods, namely light-microscopy, can be
70 time-consuming when many samples have to be routinely analyzed. Reliable species
71 identification requires expensive equipment and trained personnel to carry out the analyses
72 (Tyrrell et al. 2002; Ayers et al. 2005), because unicellular algae are taxonomically
73 challenging and some of them have only a few morphological markers. Various molecular
74 methods are used for the identification of phytoplankton, such as whole cell fluorescent in-
75 situ hybridization (Anderson et al. 2005; Hosoi-Tanabe and Sako 2005; Kim and Sako 2005),
76 PCR-based assays (Penna 1999; Guillou et al. 2002), DNA microarrays (Metfies and Medlin
77 2004; Metfies and Medlin 2005), real time PCR (Galluzzi et al. 2004; Handy et al. 2006) and
78 sandwich hybridization assays (Tyrrell et al. 2002; Ayers et al. 2005). A rapid and potential

79 method for the detection of toxic algae was introduced in the past decade (Metfies et al.
80 (2005) and Scholin) using sandwich hybridization on a biosensor and two oligonucleotide
81 probes that specifically targeted the ribosomal RNA (rRNA) of toxic algae. Usually
82 oligonucleotide DNA probes have a length of 18–25 base pairs and target the complementary
83 sequences of the small and the large subunit ribosomal RNA algal genes. These genes are
84 found in high target numbers in cells and their varying target specificity, which is based on
85 more or less conserved regions, make it possible to design probes on species or clade level
86 (Groben et al. 2004). Electrochemical biosensors combine biochemical recognition with
87 signal transduction for the detection of specific molecules. The detection component (e.g.,
88 probe sequence, antibodies, and enzymes) specifically binds to the target of interest or
89 catalyzes a reaction .. A transducer component transforms this detection event into a
90 measurable signal such as an electrical current (Gau et al. 2005). Single electrode sensors as
91 well as arrays are known from various sectors, such as clinical diagnostic and environmental
92 monitoring. Biosensors have been applied for the detection of biochemical substances as well
93 as of micro-organisms, such as bacteria (Berganza et al. 2006; Lermo et al. 2006; Taylor et al.
94 2006). Phytoplankton communities consist of different species and the temporal and spatial
95 variability in composition in the sea is substantial and therefore a simultaneous detection of
96 multiple species is important. The simultaneous detection of multiple species can be
97 accomplished using arrays of electrodes with different molecular probes. However, molecular
98 techniques for the monitoring of harmful algae usually require transportation of samples to
99 specialized laboratories. The same applies to conventional methods. There are examples for
100 on-site monitoring of toxic algae, such as the environmental sampling processor (Doucette et
101 al. 2006; Silver 2006), but which needs highly trained personnel. The number of samples and
102 the frequency of collection are increasing making an analysis in nearly real time increasingly
103 difficult to manage. As a consequence, results are usually obtained within five working days
104 after receiving the sample and therefore preventative measures are not always possible.

105 Biosensors can be used on-site and therefore circumvent the need to return samples into the
106 laboratory.
107
108 In this regard, a system with two major parts was developed during the EU-project
109 ALGADEC: a multiprobe biosensor with the aim to detect different species of toxic algae
110 simultaneously (Diercks et al. 2008b) in combination with a hand-held device for the in-situ
111 analysis. The proposed use of our device and the method by inexperienced users imply the
112 simplification and automation of the system presented by Metfies et al. (2005). The used
113 sandwich hybridization assay involves a capture probe, immobilized on the working electrode
114 surface of a biosensor that binds to rRNA isolated from the target organism as well as a
115 second digoxigen-labelled probe that also binds to the rRNA but carries the signal moiety. An
116 antibody-enzyme complex directed against digoxigenin is added and incubated. A redox-
117 reaction takes place after substrate addition and the resulting electrical current can be
118 measured with a potentiostat. We present here the second part of the study: the development
119 of a lysis protocol and the adjustment of the semi-automated device for in-situ analysis of
120 toxic algae using a multiprobe biosensor.
121
122 MATERIALS AND METHODS
123 **Probe sets**
124 One set of capture and signal 18S-DNA probes (AMINC: 5'-GAA GTC AGG TTT GGA
125 TGC-3' and AMINC NEXT: 5'-TAA TGA CCA CAA CCC TTC C-3'), specific for the 18S-
126 rRNA of Alexandrium minutum (Diercks et al. 2008a) were applied for the experiments using
127 different lysis buffers and the adaptation of the multiprobe chip to the semi-automated device.
128 The probes and the positive control were synthesized from Thermo Electron Corporation
129 (Ulm, Germany).
130

131 **Culture conditions**

132 The Alexandrium minutum strain AL3T was cultured under sterile conditions in seawater-
133 based media K (Keller et al. 1987) at 15 °C and 120 µEinstein with a light: dark cycle of
134 14:10 hours. Prior to the experiments, the cells were counted using the Multisizer 3 Coulter
135 Counter (Beckman Coulter GmbH Diagnostics, Germany).

136 **Spotting of multiprobe chips**

137 Multiprobe chips (Fig. 1, Gwent Electronic Materials (GEM), UK) were hand-spotted with 10
138 µL of thiolated capture probe (10 µM in 0.5 mol/L phosphate buffer) and incubated for at
139 least 16 hours at room temperature. The sensors were stored in a moisture chamber for all
140 incubation steps to protect the solutions from evaporation. 10 µL of 6-mercapto-1-hexanol
141 (MCH; 1 mmol/L aqueous solution) solution were added and incubated for one hour to
142 minimize the non-specific interaction between the gold surface and the probes. Subsequently,
143 unbound probe and MCH were removed by washing the sensor with 2x saline sodium citrate
144 buffer. The multiprobe chips were blocked with 10 µL of 5 % [w/v] BSA and washed again
145 with 2x saline sodium citrate buffer.

146 **Total rRNA-extraction**

147 The RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used to isolate the total RNA
148 from Alexandrium minutum with modifications of the protocol to enhance the quality and
149 quantity of the RNA by removal of polysaccharides and proteins content. For the achievement
150 of an improved separation of supernatant and cell debris, the centrifugation step of two
151 minutes was extended to 15 minutes. The washing buffer RW1 supplied with RNeasy Plant
152 Mini Kit was applied twice to the RNeasy column, incubated for one minute and centrifuged.
153 The first wash step with buffer RPE supplied with RNeasy Plant Mini Kit was repeated. RNA
154 concentration was measured with a Nanodrop Spectrophotometer (Peqlab, Erlangen,
155 Germany).

156

157 **Fragmentation of total rRNA from Alexandrium minutum**

158 Total rRNA from Alexandrium minutum was fragmented in fragmentation buffer (40 mM
159 Trizma base, pH 8.0/100 mM KOAc/30 mM MgOAc) for 5 minutes at 94 °C prior to
160 hybridization.

161 **Standard hybridization and electrochemical detection**

162 The standard hybridization mixture contained 1x hybridization buffer (75 mM NaCl/20 mM
163 Trizma base, pH 8.0/0.04 % SDS), 0.25 µg/µL herring sperm DNA, 0.1 pmol/µL dig-labeled
164 probe AMIN and target RNA. A positive control contains 0.1 pmol/µL test-DNA (test-DNA
165 is a synthetic oligonucleotide that matches exactly the combined region of the capture and
166 signal DNA probe) instead of target-RNA, whereas the negative control contains no target
167 DNA. The hybridization mixtures were denatured by incubating the hybridization mixtures at
168 94°C for 4 minutes. 10 µL of the solutions were applied to the multiprobe chip to cover the
169 entire electrode array and the multiprobe chip was incubated at 46°C for 30 minutes.
170 Subsequently, the multiprobe chip was washed with POP buffer (50 mM $NaH_2PO4 \times H_2O$,
171 pH 7.6/100 mM NaCl). 10 µL of antibody solution (Anti-DIG-POD, 7.5 U/ml in PBS, pH
172 7.6/0.1 % BSA [w/v]/0.05 % Tween 20 [v/v]) was applied and incubated at room temperature
173 for 30 minutes. Unbound antibody-enzyme complex was removed by washing the multiprobe
174 chip with POP buffer. The multiprobe chip was placed into a substrate reservoir that harbored
175 the substrate solution (4-aminophenylamine hydrochloride (ADPA) [44 µg/ml]/0.44 %
176 ethanol [v/v]/0.048 % $H_2O_2$ [v/v]/50 mM $NaH_2PO_4 \times H_2O$/100 mM NaCl) to carry out the
177 electrochemical detection. Electrochemical signals were measured using a multiplexer, which
178 can measure 8 electrodes simultaneously, and the PalmSens detector (Palm Instruments BV,
179 Houten, Netherlands). Electrochemical detection signals are measured with negative values,
180 but for simplification of analysis, the signals are multiplied by –1 unless otherwise noted.

181

182

183 **Testing of different combinations of lysis buffer and hybridization buffers**

184 Two different lysis buffers and hybridization buffers were tested for the determination of the
185 optimal lysis properties and hybridization signals on the multiprobe chip. Lysis buffer 1 (4 M
186 guanidine-isothiocyanat, 25 mM sodium citrate, 0.5 % sarcosyl [w/v], pH 11) was prepared
187 after Kingston (1998) (Kingston 1998) and the second lysis buffer RLT was taken from the
188 RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). In combination with the two lysis buffers,
189 two different hybridization buffers were tested. The 4x hybridization buffer (0.3 M NaCL, 80
190 mM Trizma base, 0.04 % SDS, pH 8) was described by Metfies et al. (2005) and the second
191 hybridization buffer, named sample buffer (100 mM Trizma base, 17 mM EDTA, 5 M
192 guanidine isothiocyanate, 8.35 % formamide, pH 7.5), was published by Scholin et al. (1999).
193 The experiments were carried out using approximately 400,000 cells of Alexandrium
194 minutum and 450 µL of the lysis buffers. 600 µL of 4x hybridization buffer and sample buffer
195 were added to the different lysis solutions, respectively. Cell debris was removed by filtration
196 through a 0.45 µm filter (Millipore, USA). Detection probe AMINC NEXT and fragmentation
197 buffer were added to the lysis-hybridization solutions, incubated at 94 °C for 5 minutes and
198 applied to the multiprobe chips with the immobilized capture probe AMINC. Negative and
199 positive controls were prepared as described above and total rRNA was isolated from the
200 same cell counts of *A. minutum* and also hybridized for comparison of the signals.

201 **Hybridization and analysis in semi-automated device**

202 The hybridization mixture was prepared as described above, but the amount was amplified.
203 Multiprobe chips consisted of immobilized AMIN probe on all 16 working electrodes. The
204 adjustment of the device was conducted using test-DNA as target of the probes for *A.*
205 *minutum*. Hybridization with different concentrations of target rRNA from *A. minutum*
206 followed instead of the target-DNA. Final adjustments of hybridization mixture and the lysis
207 buffer 1 were carried out using 500,000 cells of A. minutum.

208

209 **RESULTS**

210 **Development of lysis protocol**

211 The identification and quantification of target species with the nucleic acid biosensor is based
212 on the specific detection of ribosomal RNA. The current protocol using a kit for total RNA
213 isolation requires trained and experienced staff to generate a reproducibly high quality RNA,
214 which is prerequisite for a quantification of the target species.. Hence, simplification of the
215 RNA-isolation is crucial for the use of the semi-automated device in the hands of laymen. In
216 this respect, two different lysis buffers were tested for their lysis properties and the signal
217 formation in combination with two different hybridization buffers. For comparison of the
218 signals hybridization with different kinds of target nucleic acids have been carried out. This
219 involved a hybridization with negative and positive controls as well as hybridization with
220 target rRNA (Fig. 2). The signals of all 16 electrodes were averaged for the different
221 experiments and compared. A signal of 238 nA ± 13 was observed for the negative control
222 and 1187 nA ± 41 for the positive control. All experiments with lysis/hybridization buffer
223 combinations and total rRNA showed similar signals. 4x hybridization buffer in combination
224 with lysis buffer 1 achieved the highest mean signal with 554 nA ± 43, whereas in
225 combination with RLT buffer from the Qiagen Kit, the lowest signal of 365 nA ± 48 was
226 detected. Sample buffer in combination with RLT buffer showed a similar signal of 518 nA ±
227 37 to the 4x hybridization buffer/lysis buffer 1 combination. Sample buffer with lysis buffer 1
228 achieved a mean signal of 462 nA ± 39. Hybridization of total rRNA to the multiprobe chip
229 resulted in 365 nA ± 20. Thus, the combination of 4x hybridization buffer with lysis buffer 1
230 was used for further experiments.

231 **Development and adjustment of semi-automated device**

232 A semi-automated portable device, named ALGADEC, was developed by iSiTEC GmbH
233 (Bremerhaven, Germany) and the Alfred Wegener Institute (Bremerhaven, Germany) during
234 the FP7 EU-Project ALGADEC (Fig. 3). The device contains reservoirs for antibody,

235 substrate and washing buffers as well as a flow cell unit for hybridization. A flow cell unit
236 and an additional inlet for applying the samples can be heated and cooled to the required
237 temperatures during the analysis procedure. A peristaltic pump transfers the reagents through
238 the flow cell and finally into the waste reservoir. The main steps of the analysis process, like
239 hybridization, washing and detectionreaction, can be executed automatically in the
240 measurement device. A flow chart was developed for the varying processes (e.g.,
241 hybridization, wash steps, antibody incubation and measurement) and pump times were
242 adapted. Adjustment of the semi-automated device was conducted using multiprobe chips
243 with the probe set for Alexandrium minutum on all 16 electrodes and the respective test-DNA
244 as target for the probes. The disposable multiprobe chip was inserted into the flow cell unit
245 before analysis was started. During measurement of the electrochemical reaction, the signals
246 from the working electrodes with probes were recorded by a microcontroller unit. Process
247 data can be visualized with custom-made software programmed by iSiTEC GmbH if a PC is
248 connected to the system (Fig. 4.). This setup further allows for permanent data storage on the
249 PC storage system for later use. Graphic results and the measured values are stored on the
250 hard disc. The portable ALGADEC device can be operated as a stand-alone system with a
251 build in keypad, display, power supply and memory card. A waterproofed case protects the
252 system and allows its use under adverse conditions.

253 **Hybridization of target RNA and dissolved cells on multiprobe chips**

254 Hybridizations with two different concentrations of target rRNA from A. minutum; a negative
255 and a positive control were carried out in the semi-automated device. For presentation of the
256 results the signals of all 16 electrodes were averaged for the different experiments and
257 compared. A representative pattern of a measurement is presented in figure 5. The
258 measurements were started when washing buffer was still present in the flow cell unit and no
259 signals were observed for washing buffer. After approximately 100 seconds of measurement,
260 substrate buffer arrived in the unit and was pumped continuously through the unit. A redox-

261 reaction took place and in succession the signal of every working electrode decreased. The
262 signals at 500 seconds of measurement were taken for comparison of all experiments, because
263 saturation of the reaction can be observed at this time point. The highest signals were found
264 for the positive control with a mean signal of 265 nA ± 40 for all 16 electrodes after 500
265 seconds of measurement (Fig. 6). At the same measurement point, signals for the negative
266 control, high RNA concentration and low RNA concentration were observed at 104 nA ± 10,
267 201 nA ± 19 and 106 nA ± 10, respectively (Fig. 6). Approximately 500,000 cells from
268 Alexandrium minutum were dissolved in lysis buffer, mixed with hybridization solution and
269 analyzed in duplicate in the device. The mean signal of all 16 electrodes of the analyses at 500
270 seconds was found to be 158 nA ± 22 for the first run and 148 nA ± 27 (Fig. 6) for the
271 replicate.

272 **DISCUSSION**

273 The sandwich hybridization assay described in the first part of our study (Diercks et al.
274 2008b) and by Metfies et al. (2005) involved the isolation of total rRNA from the algal cells.
275 For this method a fume hood, centrifuge and other special laboratory equipment is needed.
276 Prerequisite of a reliable identification and quantification of target species with the
277 ALGADEC –device is reproducibly high quality RNA However, it is possible that separate
278 users isolate different qualities and quantities of rRNA from the same sample with an equal
279 number of algae cells present. The proposed use of our device by inexperienced users meant
280 that we needed to simplify the rRNA extraction method. A lysis protocol independent of
281 expensive laboratory equipment was successfully developed to circumvent manually rRNA
282 isolation with commercial kits. Now, only a filtration of the sample is necessary to collect the
283 algal cells and subsequently lysis buffer can be applied to the cells. Two different lysis and
284 hybridization buffers were tested in combination. Similar signal intensities could be observed
285 for the combination of sample buffer with lysis buffer RLT and the combination of the 4x
286 hybridization buffer with lysis buffer 1. The combination of our 4x hybridization buffer with

287 lysis buffer 1 resulted in slightly higher signals and can be inexpensively produced, whereas
288 the lysis buffer RLT is commercially produced. Consequently the combination of 4x
289 hybridization buffer with lysis buffer 1 was chosen for the other experiments. This simple
290 lysis method can be accomplished by inexperienced users without incorrect handling. Thus,
291 all required steps for the semi-automated detection of toxic algae were achieved.
292
293 A portable device was developed during the EU-project ALGADEC, which can be used as a
294 stand-alone system in the field (e.g., on ships or shores) as well as in the laboratory. The
295 device is easy to handle even for laymen and sample analyses with all required steps can be
296 performed automatically in less than two hours. Only the water sample has to be filtered by
297 hand by the user, incubated with lysis buffer and hybridization buffer and injected into the
298 inlet of the device. The achieved data are stored in the microcontroller unit or, if attached to a
299 PC, can be analyzed directly. In the first part of our study a multiprobe chip with 16 gold
300 electrodes was designed by iSiTEC GmbH and adapted for the use in combination with sand
301 wich hybridization (Diercks et al. 2008b). Multiprobe sensorchips with this design were used
302 for the presented experiments. The design of the multiprobe sensorchip was carried out with
303 respect to an easy handling , thus it was developed with the size of a conventional glass slide
304 and can be stored in standard boxes. Multiprobe chips coated with probes for Alexandrium
305 minutum on all 16 working electrodes and the ALGADEC device were tested using isolated
306 RNA and cells from Alexandrium minutum and the data were compared. The signals for
307 comparison were chosen after 500 seconds of measurement because saturation of the reaction
308 was observed at this point of time. Hybridizations with two different concentrations of target
309 rRNA, high and low, from A. minutum were carried out. Clearly distinguishable signals were
310 determined for low and high concentration of rRNA; a low rRNA concentration resulted in
311 signals in the range of the negative control and was consequently at the detection limit of the
312 probes for A. minutum. A high rRNA concentration gave a mean signal of 201 nA ± 19.

313 When compared to hybridization signals for dissolved cells of A. minutum, decreased signals
314 (mean signal 150 nA ± 25) were observed. The isolated rRNA with a high quality originated
315 from about 260,000 cells, whereas the filtered cell lysate of 500,000 cells also contained
316 proteins and polysaccharides, which can disturb the hybridization. Additionally, a field
317 sample with Pseudo-nitzschia cells from the Orkney Islands, United Kingdom, was tested
318 with a multiprobe chip (data not shown) coated with the genus probe for Pseudo-nitzschia
319 (Diercks et al. 2008c) during a workshop with laymen. The analysis revealed a positive signal
320 for Pseudo-nitzschia. Hence, the semi-automated device in combination with multiprobe chips
321 can also be successful used for the analysis of field samples.
322 A proof of principle is presented here because the sensitivity of the system has to be
323 optimized and the detection limit must be reduced. The detection limit must be far below the
324 fisheries closure number to meet monitoring requirements. To meet these requirements,
325 several adaptations must be made. One possibility for a signal increase is the optimization of
326 flow speeds, incubation times and substrate concentrations (Diercks et al. 2008b).
327 Furthermore, the spotting of the multiprobe chips with probes has to be automated to achieve
328 a regular signal formation. Additionally different probes can be spotted, i.e. species onto the
329 chip, thus chips specific for different geographic areas can be developed. Several specific
330 probe sets for toxic algae have been developed (Diercks et al. 2008c) and need to be adapted
331 to the chips. Subsequently the sensors must be calibrated for each probe set to convert the
332 electronic signal into concentration of toxic cells via the total rRNA concentration per cell
333 with the help of the software. The first results of rRNA isolation experiments were presented
334 by Diercks et al. (2008a). Total rRNA was isolated from three different strains of A. minutum
335 at optimum growth conditions and the mean concentration of RNA per cell of was determined
336 to be 0.028 ng. This is comparable to results presented by Metfies et al. (2005) for
337 Alexandrium ostenfeldii with a mean concentration of RNA per cell of 0.02 ng. Optimum
338 growth conditions are expected to correspond most closely to bloom development in the field

339 (Ayers et al. 2005). Based on the here presented results and results presented elsewhere, the
340 multiprobe chip and the ALGADEC device have the potential to serve as rapid detection
341 system for toxic algae.

342 **CONCLUSION**

343 A portable semi-automated device was developed that automatically processed the main steps
344 of the probe to target hybridization and facilitated the electrochemical detection of toxic algae
345 in less than two hours. The device can be used by laypersons because a manual RNA isolation
346 is no longer required with the development of a lysis protocol. A proof of principle was
347 presented here. The multiprobe chip and the ALGADEC device can be used as a stand-alone
348 system in the field and will contribute to monitoring programs to provide an early warning
349 system for the aquaculture and tourist sectors who are most affected by toxic algal blooms.

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444 **Figure captions:**

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446 **Fig. 1.** Multiprobe chip with 16 gold working electrodes (Diercks et al. 2008b)

447

448 **Fig. 2.** Determination of optimal signal formation using two different lysis and hybridization buffers and probes for Alexandrium minutum

450

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454 **Fig. 4.** Easy to use software from iSiTEC GmbH

455

456 **Fig. 5.** Representative pattern of signals measured with the device

457

458 **Fig. 6.** Hybridization of a positive control (test-DNA), a negative control (without DNA), two
459 different target rRNA concentration and 500,000 lysed cells of Alexandrium minutum in
460 duplicate onto the multiprobe chip in the semi-automated device. All 16 working electrodes
461 are coated with the same capture probe AMINC.
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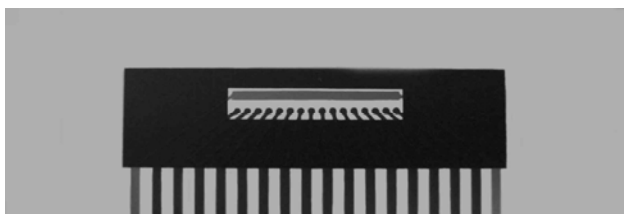

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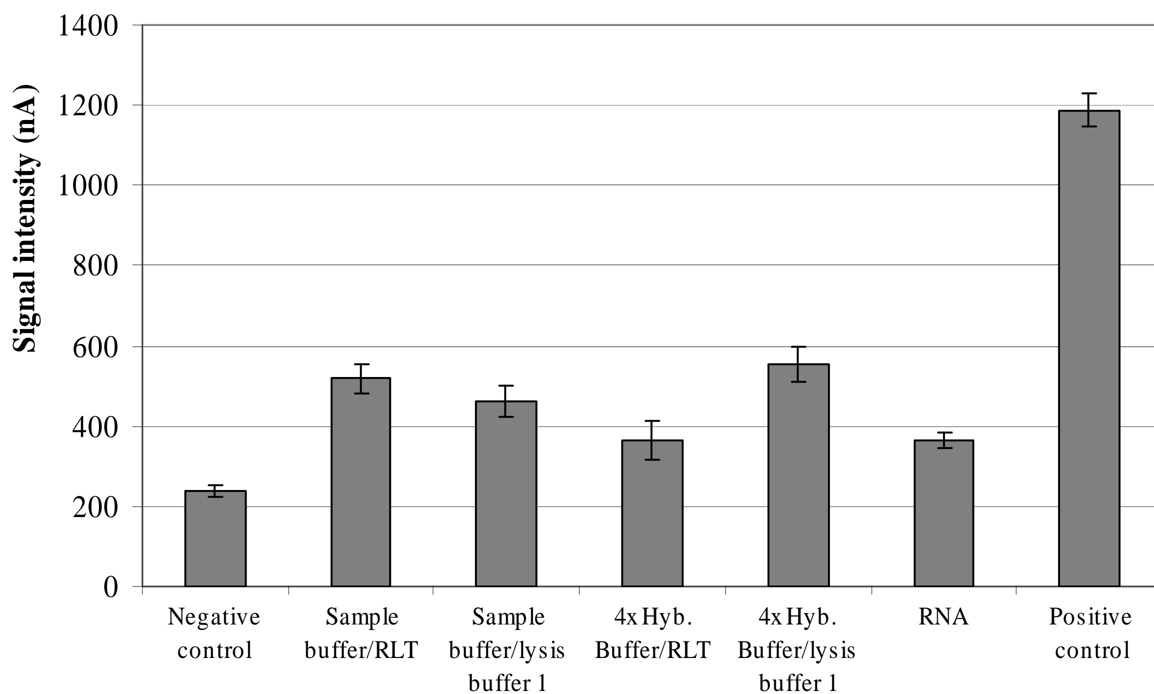


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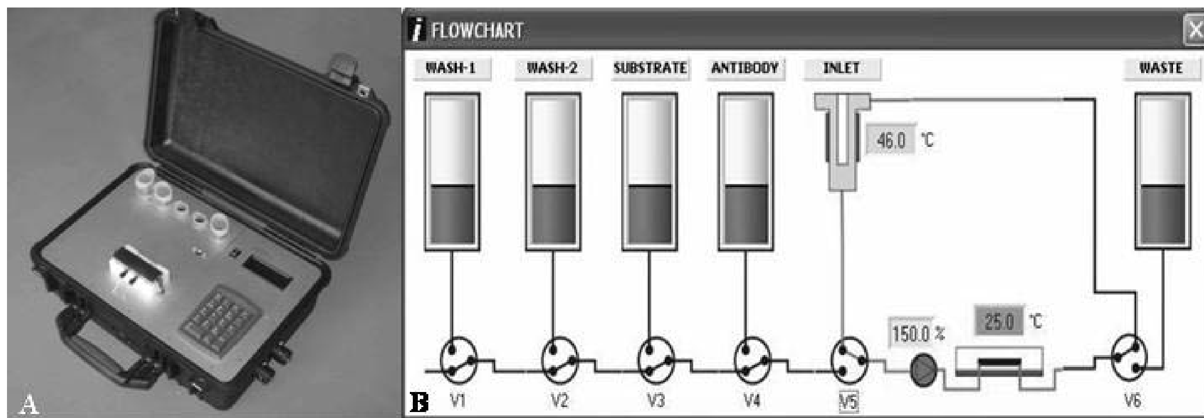


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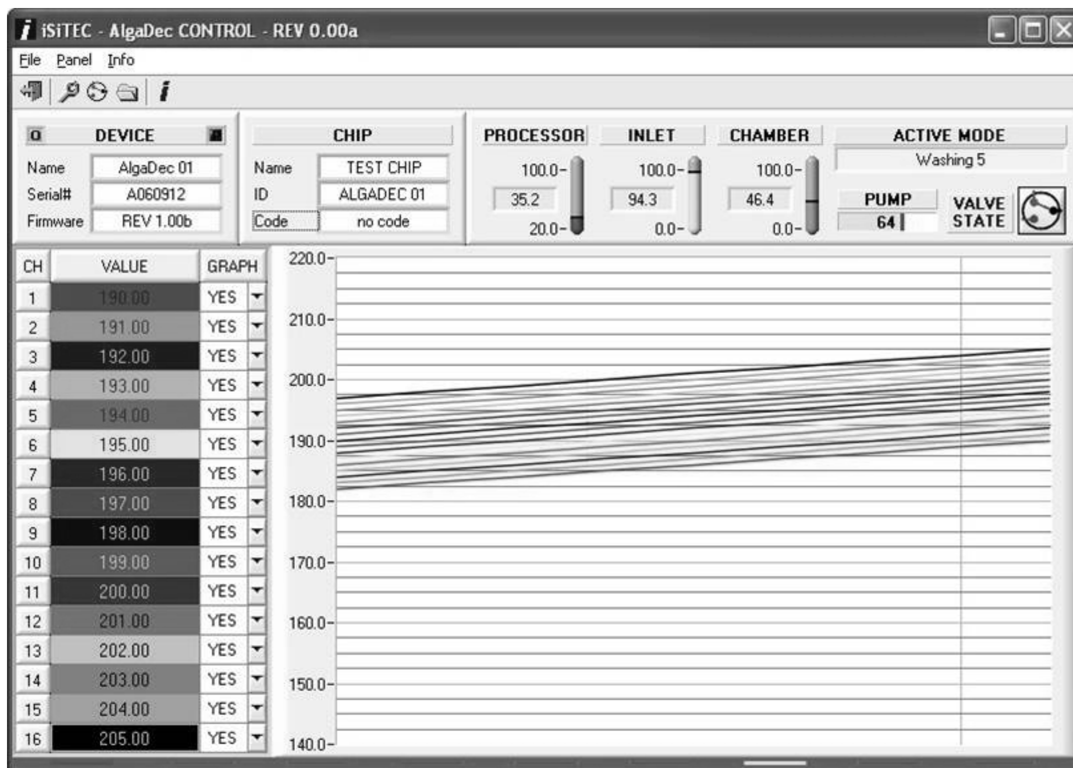


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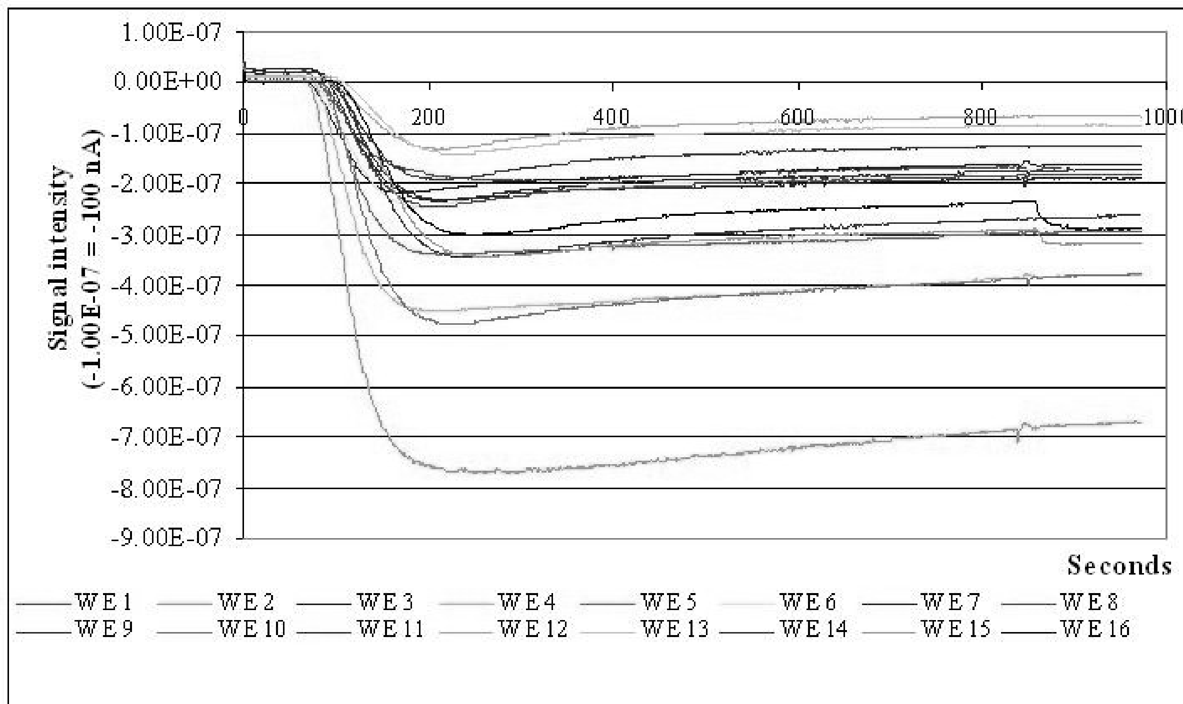


493
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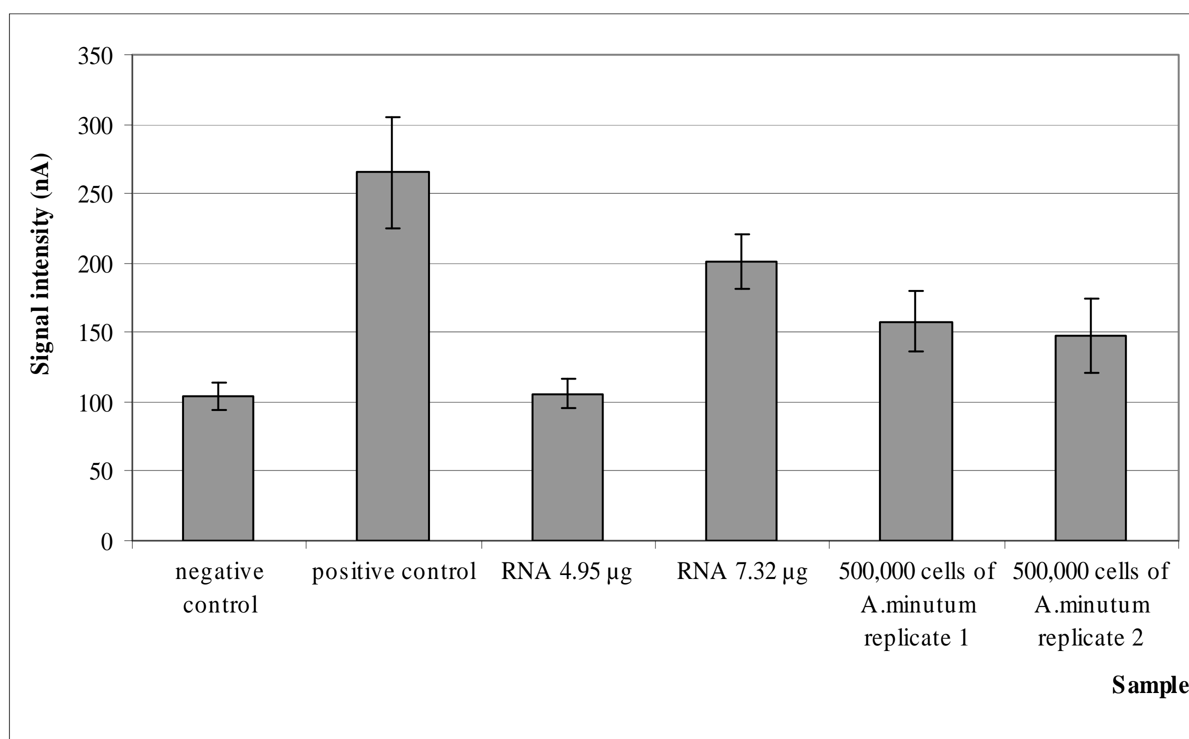


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