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Potentiometric Investigation of the Dynamics of Bacteriophage - Induced Potassium Ion Efflux in *Escherichia coli*

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Abstract

Potassium is a very stable component of biological systems and contributes to maintaining positive turgor pressure of the cells. Although ion-selective electrodes (ISEs) have been used previously for measuring bacteriophage - induced potassium ion efflux in bacteria, the dynamics of this outcome remain unclear. In this study, the effects of incubation time, reaction volume, and phage/bacterial concentration on bacteriophage-induced potassium ion efflux were studied using a commercial ISE. A phage-bacteria reaction volume of 200 µl incubated at 37°C for 30 minutes was found to be optimal for detecting potassium ion efflux using the ISE. Potassium ion efflux was found to be directly proportional to phage multiplicity of infection and ~5 × 10^7 cfu/ml appeared to be the lowest bacterial concentration at which potassium ion efflux could be measured reliably. Furthermore, the differences in potassium ion efflux between control (bacteria + assay buffer) and test (bacteria + phage) samples were statistically significant ($p < 0.05$) and the results were consistent across assays performed on different days. The average of absolute concentrations of effluxed potassium ions was 1.4 mg/L for ~5 × 10^8 cfu/ml of bacteria (MOI (Multiplicity of infection) = 100). The method uses simple bench-top technology, has a rapid turn-around time, is label-free, and has the potential for incorporation in bacterial detection systems.

Keywords: Bacteriophage; *E. coli*; MOI; potassium; ISE; Detection

Introduction

Bacteriophages have several applications in biotechnology, including the typing and epidemiological surveillance of pathogenic bacteria [1]. Reporter bacteriophage technology is advancement over classical phage typing and affords higher sensitivity [2-4]. Further improvements to these assays aimed at making them semi-quantitative require an in-depth understanding of host-bacteriophage interplay. Translocation of the hydrophilic phage DNA across the plasma membrane of the host is a complex process and several models have been proposed to explain this conundrum [5]. One such model is the channel-mediated transport of phage DNA, proposed based on the observation that bacteriophage T1 induces changes in cell membrane permeability and potassium ion efflux [6]. Potassium ion efflux is not specific to lytic bacteriophages and is also shown to occur during temperate bacteriophage infection [7,8].

A potentiometric method that relies on ISEs has been used previously to simultaneously detect the presence of chloride and potassium in beverages [9]. A commercial electrolyte analyzer, which also relies on ISE-based potentiometry and is marketed for clinical laboratory applications, has been used to quantify, trace potassium in water and beverages [10]. Furthermore, bacteriophage-induced potassium ion efflux has been quantified using ISEs [11,12]. An "ion-selective field effect transistor implemented in complementary metal-oxide-semiconductor technology" was used to monitor potassium ion efflux in bacteria [7]. A potassium ion recognizing electrochemical biosensor has also been reported [13]. A micrometer-sized capacitor of titanium electrodes has been used to measure phage-triggered potassium ion cascades in bacterial cells and shown to detect bacteria with a high sensitivity [14].

Although potassium ion efflux in *E. coli* has been measured in relative terms using different methods, no efforts have been made toward absolute quantification of bacteriophage-induced potassium ion efflux. Furthermore, no systematic comparisons of the effects of incubation time, reaction volume, and phage/bacterial concentration on bacteriophage-induced potassium ion efflux have been done. The objective of the present study was to explore the temporal and spatial dynamics of T4 bacteriophage-induced potassium ion efflux in *E. coli* and to validate the sensitivity of a commercially available ISE for potassium ion detection at different concentrations of bacteria and phage.

Methods

Electrode calibration

A commercially available ISE with sensitivity up to 10^{-6} M (Thermo fisher scientific) was used to detect potassium ion efflux. Procedure for calibration of the ISE using potassium standard solutions is given in the supplementary materials.

Bacteria and phage

T4 bacteriophage and its host Escherichia coli strain B were procured from the American Type Culture Collection (ATCC® 11303-B4™ and ATCC® 11303, respectively). *E. coli* strain B was maintained on Luria Bertani (LB) agar plates and a single colony was inoculated into LB broth for culturing at 37°C with 150 rpm shaking. Optical density was measured at 600 nm (OD_{600}) using a spectrophotometer (SpectraMax M2e, Molecular devices). Overnight grown culture was diluted 1:10 and re-incubated at 37°C with 150 rpm shaking. Samples were taken aseptically every 30 minutes for measuring OD_{600} and every hour for spread plating. Plates were incubated overnight at 37°C, colonies were enumerated, and colony forming units (cfu/ml) were calculated. This information was used for estimating cell concentration (at OD_{600} = 1).

Freeze dried bacteriophage T4 was processed as per the procedure provided in the ATCC product sheet. Bacteriophage T4 was initially propagated using the agar overlay method adapted from Adams [15]. After the initial plaque assay on LB agar plates, a single plaque was carefully extricated and mixed with 5 ml fresh culture of *E. coli* strain B (OD_{600} = 2). Bacteriophage T4 was allowed to propagate by incubating this culture for 5 hours at 37°C. The culture was centrifuged and the supernatant was filter sterilized using 0.22 µm syringe filters. This lysate was used to infect a fresh batch of culture and obtain plaques. Final phage lysate was prepared after three serial passages.

For phage stock preparation, E. coli strain B was cultured in LB broth (OD_{600} = 2) and mixed with the phage lysate in a 1:20 ratio (phage:bacteria). This mix was incubated at 37°C for 5-7 hours to facilitate the lytic cycle. Bacterial debris was removed by centrifugation and the supernatant was sterilized using 0.22 µm syringe filters. Filter sterilized phage lysate was precipitated with 10% polyethylene glycol (PEG 8000) at 4°C overnight based on the procedure described for purification of Norovirus [16]. After precipitation, the lysate was centrifuged at 8000 rpm for 15 minutes and the pellets were resuspended in the phage buffer (10 mM Na_3PO_4, 137 mM NaCl, and 0.2% $MgSO_4$; pH 7.2). Phages were concentrated first by 300 kDa and then by 100 kDa Vivaspin ultra filtration units (Sartorius) and equilibration was performed using the phage buffer. Phage concentration was determined by the plaque assay on LB agar plates. Phages were stocked in phage buffer and stored at 4°C for up to six months.

Detection of potassium ion efflux

Since the efflux detection required that the assay be performed in a potassium free system, a buffer containing 10 mM Na_3PO_4 and 137 mM NaCl (pH 7.2) was used. A starter culture (single colony of *E. coli* strain B inoculated into 5 ml LB broth and incubated overnight at 37°C) was used to inoculate 100 ml LB broth and incubated at 37°C for ~2 hours. The cells were harvested by centrifugation and the bacterial pellet was washed three times using ~40 ml of assay buffer (10 mM Na_3PO_4, 137 mM NaCl, and 0.5% glucose; pH 7.2). After the final wash and centrifugation, bacterial pellet was resuspended in the assay buffer such that the OD_{600} = 1 (~5 × 10^8 cfu/ml). The cells were aliquoted (1 ml) into microfuge tubes, centrifuged, and the pellet was placed on ice. Bacterial pellet was resuspended in a suitable volume of assay buffer without glucose, mixed with phage, and incubated at 37°C to facilitate phage-bacteria interaction. After incubation, the phage-bacteria mix was reconstituted to a final volume of 3 ml using the assay buffer without glucose and potassium efflux was monitored using the ISE up to 4 minutes. Assays were done in triplicates for control and test samples.

Statistical analysis

Potassium ion efflux measurement assays for control and test samples were performed on five different days. The results for the control and test samples from the five days were pooled and a paired T-test was used to compare the data. A *p-value* (Two Tail) of *≤ 0.05* was considered statistically significant.

Results and Discussion

Phage lysate preparation

Conventional methods of bacteriophage purification are slower and the recovery rates are variable. Colombet, et al. [17] proposed using PEG (polyethylene glycol) precipitation as an alternative to density gradient ultracentrifugation for purifying viruses. Concentrating bacteriophages by ultrafiltration is a further alternative to ultracentrifugation and has several advantages, including the removal of proteins that could cause bacterial lysis [18,19]. Precipitation using PEG followed by ultrafiltration was therefore the method of choice for preparing the phage stock in this work. Concentration of the bacteriophage stock obtained using this method was ~10^{12} pfu/ml (estimated by the plaque assay). Multiplicity of infection (MOI, "defined as the ratio of phage-to-bacteria") was estimated based on the stock concentration of phage (~10^{12} pfu/ml) and the concentration of bacterial cells in a given experiment.

Effect of incubation time on potassium ion efflux

To optimize the experimental conditions, efflux of potassium ions from *E. coli* strain B upon phage infection needed to be determined across different time points. Pellets of *E. coli* strain B (~5 × 10^8 cfu/ml) were reconstituted using 150 µl assay buffer without glucose and 50 µl phage stock such that the input MOI was ~100. The phage-bacteria mix was incubated at 37°C (for 0, 5, 10, 20, 30, 40, 60, 120, and 240 minutes), the reaction volume was adjusted to 3 ml using assay buffer without glucose, and potassium ion efflux was measured. The differences in potassium ion efflux at 0 and 5 min between control (bacteria + assay buffer) and test (bacteria + phage) samples were very small. However, the differences in potassium ion efflux at 10, 20, 30, 40, and 60 min between control and test samples showed a linear increase (Δ = 0.481, 0.961, 1.144, 1.139, and 1.055, respectively; SD was 0.015, 0.025, 0.051, 0.012, and 0.025, respectively). Furthermore, the differences in potassium ion efflux beyond 30 minutes between control and test samples seemed to plateau and increased efflux could be observed among control samples at 120 and 240 minutes probably due to cell lysis (Figure 1).

Boulanger and Letellier [12], who studied T5 bacteriophage-triggered potassium ion leakage in *E. coli* strain F (~5 × 10^8 cfu/ml, MOI = 3) using an ISE, observed a first efflux (lasting ~2 min) and a second efflux (lasting ~5 min). It is possible that a similar second efflux occurs during T4 bacteriophage infection of *E. coli* strain B and this second efflux is enhanced and prolonged for up to 30 min at higher phage concentrations (e.g., input MOI = ~100), as observed here, and reported elsewhere [7,8]. Moreover, Kasman, et al. [20], quantified the outcome of phage-host interaction after 30 min of incubation at 37°C and opined that the dynamics of these interactions can be more accurately evaluated when the "adsorption period is sufficiently shorter than the doubling time of the cells". Since the doubling time of E. coli strain B in LB broth is ~20 min, and cells are unlikely to replicate in the assay buffer lacking a carbon source, measuring potassium ion concentrations at or after 30 minutes was deemed appropriate. Furthermore, potassium concentrations detected at 30 minutes are most likely due to active efflux and not lysis because T-even bacteriophages lyse cells 25 to 35 minutes after infection [21].

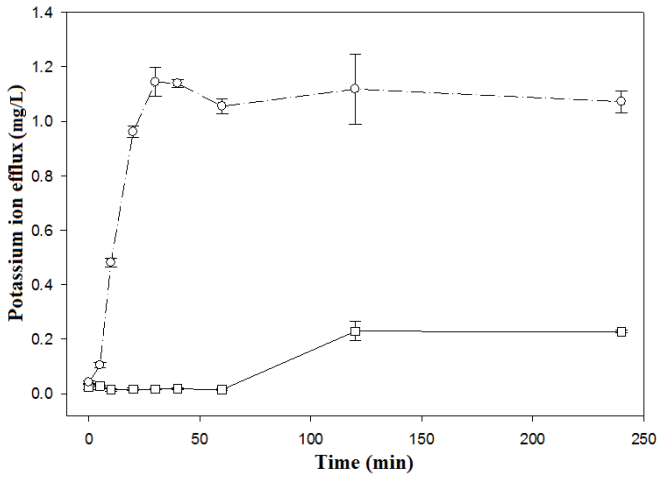

Figure 1: Effect of incubation time on potassium ion efflux. *E. coli* strain B (~5 × 10^8 cfu/ml) and bacteriophage T4 (~10^{12} pfu/ml) were mixed such that the input MOI was ~100 and incubated at 37°C for 0, 5, 10, 20, 30, 40, 60, 120, and 240 min. Potassium ion efflux from control (bacteria + assay buffer, solid line) and test (bacteria + phage, broken line) samples was measured at each time point and plotted. Data represent averages of triplicate measurements.

Effect of reaction volume on potassium ion efflux

Levin and Lenski [22] had opined that host population density determines the form of phage replication. To test the effect of cell density, the efflux of potassium ions from *E. coli* strain B upon phage infection was determined using four different reaction volumes (50, 100, 150, or 200 µl). Pellets of *E. coli* strain B (~5 × 10^8 cfu/ml) were reconstituted using 0, 50, 100, or 150 µl assay buffer without glucose and 50 µl phage stock such that the input MOI was ~100. The phage-bacteria mix was incubated at 37°C for 30 min., the reaction volume was adjusted to 3 ml as before and potassium ion efflux was measured. The differences in potassium ion efflux between control and test samples across the four different reaction volumes showed a linear increase (Δ = 1.044, 1.167, 1.335, and 1.549, respectively; SD was 0.033, 0.048, 0.079, and 0.039, respectively; Figure 2).

When the experiment was repeated using a reduced concentration of *E. coli* strain B (~5 × 10^7 cfu/ml, pellets reconstituted using 0, 50, 100, or 150 µl assay buffer without glucose and 50 µl phage stock such that the input MOI was ~1000), the differences in potassium ion efflux between control and test were 0.048, 0.051, 0.095, and 0.108, respectively; SD was 0.007, 0.010, 0.007, and 0.013 respectively; data not shown). Kasman, et al. [20], had indicated that "concentrating identical mixtures of cells and virus into a smaller volume" improved phage-host interaction due to increased cell density. However, the effect of other factors on this interaction cannot be ruled out and buffer components have been shown to modulate phage-host interaction [23]. Since increased efflux of potassium ions in the current study occurred at higher reaction volumes, measuring potassium ion efflux using a 200 µl reaction volume was deemed appropriate.

Effect of phage and bacterial concentration on potassium ion efflux

The efflux of potassium ions from *E. coli* strain B upon phage infection was determined using four different phage concentrations. Pellets of E. coli strain B (~5 × 10^8 cfu/ml) were reconstituted using 199.5, 195, 175, or 150 µl assay buffer without glucose and 0.5, 5, 25, or 50 µl phage stock such that the input MOI was ~1, 10, 50, or 100, respectively. The phage-bacteria mix was incubated at 37°C for 30 min, the reaction volume was adjusted to 3 ml as before and potassium ion efflux was measured. The differences in potassium ion efflux between control and test samples across four different phage concentrations showed a linear increase (Δ = 0.012, 0.815, 1.132, and 1.549 for input MOI of 1, 10, 50, and 100, respectively; SD was 0.018, 0.248, 0.128, and 0.039, respectively; Figure 3). When the experiment was repeated using a reduced concentration of *E. coli* strain B (~5 × 10^7 cfu/ml, pellets reconstituted using 199.5, 195, 175, or 150 µl assay buffer without glucose and 0.5, 5, 25, or 50 µl phage stock such that the input MOI was ~10, 100, 500, or 1,000, respectively), the differences in potassium ion efflux between control and test were 0.02, 0.087, 0.102, and 0.108, respectively; SD was 0.009, 0.007, 0.013, and 0.013, respectively; data not shown).

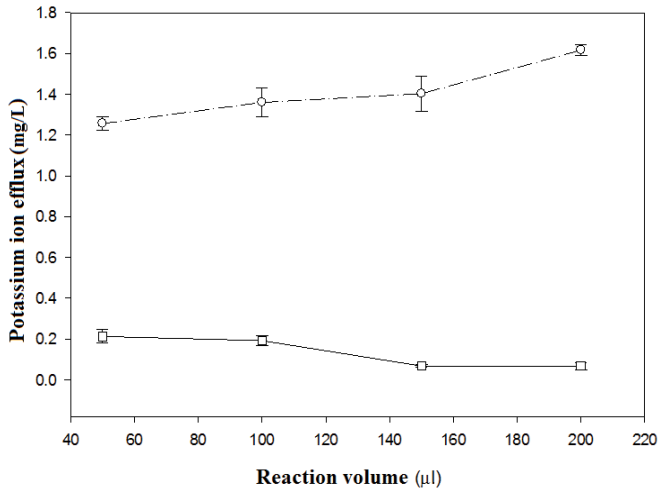

Figure 2: Effect of reaction volume on potassium ion efflux. E. coli strain B (~5 × 10^8 cfu/ml) and bacteriophage T4 (~10^{12} pfu/ml) were mixed such that the input MOI was ~100 and incubated at 37°C for 30 min using four different reaction volumes (50, 100, 150, or 200 µl). Potassium ion efflux from control (bacteria + assay buffer, solid line) and test (bacteria + phage, broken line) samples was measured at each reaction volume and plotted. Data represent averages of triplicate measurements.

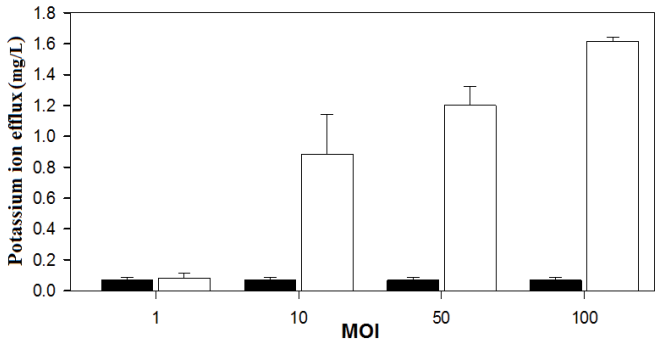

Figure 3: Effect of phage concentration on potassium ion efflux. E. coli strain B (~5 × 10^8 cfu/ml) was mixed with four different volumes (0.5, 5, 25, or 50 µl) of bacteriophage T4 (~10^{12} pfu/ml) such that the input MOI was ~1, 10, 50, or 100, respectively. Potassium ion efflux from control (bacteria + assay buffer, solid bars) and test (bacteria + phage, open bars) samples was measured at each input MOI and plotted. Data represent averages of triplicate measurements.

To further confirm these results, the efflux of potassium ions from *E. coli* strain B upon phage infection was determined using three different bacterial concentrations. Pellets of E. coli strain B (~5 × 10^6 cfu/ml, ~5 × 10^7 cfu/ml, or ~5 × 10^8 cfu/ml) were reconstituted using 150 µl assay buffer without glucose and 50 µl phage stock such that the input MOI was ~10,000, 1000, or 100, respectively. The phage-bacteria mix was incubated at 37°C for 30 min, the reaction volume was adjusted to 3 ml as before and potassium ion efflux was measured. The differences in potassium ion efflux between control and test samples across the three bacterial concentrations (~5 × 10^6 cfu/ml, ~5 × 10^7 cfu/ml, and ~5 × 10^8 cfu/ml) showed a logarithmic increase (Δ = 0.009, 0.102, and 1.469, respectively; SD was 0.006, 0.004, and 0.083, respectively; Figure 4). These results substantiate previous observations that there is a direct correlation between multiplicity of infection and potassium ion efflux [11].

Using mathematical modeling of M13K07 or P1 bacteriophage interaction with E. coli, Kasman, et al. [20] calculated that "an MOI of 10 gives each cell a better than 99.99% chance of being bound and infected by at least one virus particle" during the incubation period. Kasman, et al. [20] also predicted that at higher cell concentrations, the actual MOI experienced by the cells is the same as the input MOI and for a given MOI, more number of transductants would be possible at higher cell concentrations since each phage in the reaction mix will adsorb to a susceptible cell during the incubation period. Therefore, measuring potassium ion efflux using MOI of 100 (for ~5 × 10^8 cfu/ml) or 1,000 (for ~5 × 10^7 cfu/ml) was deemed appropriate. Furthermore, at lower cell concentrations, the actual MOI experienced by the cells was

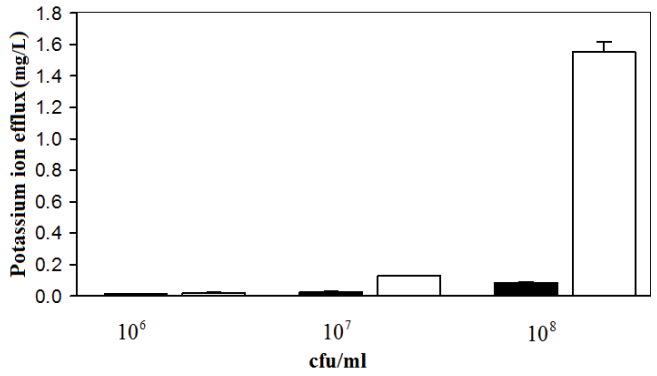

Figure 4: Effect of bacterial concentration on potassium ion efflux. Bacteriophage T4 (~10^{12} pfu/ml) was mixed with three different concentrations of E. coli strain B (~5 × 10^6 cfu/ml, ~5 × 10^7 cfu/ml, or ~5 × 10^8 cfu/ml) such that the input MOI was ~10,000, 1000, or 100, respectively. Potassium ion efflux from control (bacteria + assay buffer, solid bars) and test (bacteria + phage, open bars) samples was measured at each bacterial concentration and plotted. Data represent averages of triplicate measurements.

predicted to be less than the input MOI [20] and in this study ~5 × 10^7 cfu/ml appeared to be the lowest concentration at which potassium ion efflux could be measured reliably. Although in itself MOI cannot be used to describe experimental conditions, it appears to be a decisive parameter in determining experimental outcomes when the bacterial concentrations are low [20].

Experimental repeatability and statistical significance

To assess the repeatability of measurements, the assays were performed on five different days using two cell concentrations. The results among control and test samples were fairly consistent and a ten-fold difference in potassium ion efflux among test samples was apparent between the two cell concentrations (Table 1). The variations in measured efflux among control and test samples at MOI of 100 could be due to fluctuations in bacterial numbers and/or pipetting errors. The average of absolute concentrations of effluxed potassium ions was 0.098 mg/L for ~5 × 10^7 cfu/ml (MOI = 1000) and 1.4 mg/L for ~5 × 10^8 cfu/ml (MOI = 100). The coefficient of variation for the two bacterial concentrations were 7.9% and 12%, respectively, and were well within the specifications (≤ 15%) recommended for bioanalytical assays by the Food and Drug Administration. Comparison of the data using a paired T-test indicated that the difference in potassium ion efflux between control and test samples was statistically significant (two-tailed p-values of 9.65 × 10-15 for ~5 × 10^7 cfu/ml and 2.3 × 10-14 for ~5 × 10^8 cfu/ml).

Conclusion

The assay described here is based on bacteriophage-induced changes in cell membrane permeability and potassium ion efflux, and not on cell lysis. While in principle this label-free assay is similar to that of Boulanger and Letellier [11,12], it emphasizes on absolute quantification (test versus control) of extracellular potassium ions, rather than their intracellular estimation relative to the measured extracellular concentration. Since the quantification relies on measuring an output (potassium ion efflux) that is due to an input (bacteriophage), and has a faster turnaround time (~30 min.), it could be used with appropriate modifications for any pair of interacting phage-bacteria. Hitherto, a comparative temporal and spatial assessment of potassium ion efflux had not been reported in the literature. This study makes an attempt to fill that gap, and is also probably the first to document detection sensitivity in voluminous systems. It presents a detailed protocol for measurement of potassium effluxes, in addition to directly reporting the ion concentrations in ppm without normalization.

Future Prospects

Although other molecular methods (e.g., PCR) are more sensitive than detection of potassium ion effluxes, they also have a selective niche (e.g., in clinical microbiology) and are relatively expensive. The sensitivity of the assay reported here could be improved by immunomagnetic separation of host cells and using novel lytic or lysogenic bacteriophages. Further improvements could include miniaturization of the measurement systems (e.g., micrometer-sized capacitor of titanium electrodes, [7]) and lab on chip techniques (e.g., complementary metal–oxide semiconductor-based potassium selective field effect transistors, [14]) that use lower reaction volumes and produce faster kinetics. The improved assay could be used to detect bacterial contamination of bulk systems such as enzymes, subunit vaccines, and monoclonal antibodies, which are products of the "quality conscious" bioprocess industries.

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Bacterial concentration (MOI)	~5 × 10^7 cfu/ml (1000)					~5 × 10^8 cfu/ml (100)				
Days	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Test 1	0.119	0.139	0.118	0.138	0.121	1.4	1.65	1.640	1.63	1.140
Test 2	0.134	0.121	0.123	0.125	0.133	1.6	1.6	1.620	1.55	1.120
Test 3	0.130	0.119	0.125	0.110	0.128	1.57	1.76	1.590	1.47	1.220
Average^T	0.128	0.126	0.122	0.124	0.127	1.523	1.670	1.617	1.550	1.160
Standard Deviation^T	0.008	0.011	0.004	0.014	0.006	0.108	0.082	0.025	0.080	0.053
Control 1	0.038	0.033	0.035	0.018	0.021	0.218	0.127	0.048	0.086	0.014
Control 2	0.028	0.032	0.027	0.016	0.030	0.195	0.221	0.084	0.075	0.016
Control 3	0.040	0.037	0.030	0.015	0.020	0.232	0.129	0.072	0.091	0.018
Average^C	0.035	0.034	0.031	0.016	0.024	0.215	0.159	0.068	0.084	0.016
Standard Deviation^C	0.006	0.003	0.004	0.002	0.006	0.108	0.082	0.025	0.080	0.053
Average^T - Average^C	0.092	0.092	0.091	0.108	0.104	1.308	1.511	1.549	1.466	1.144

Table 1: Potassium ion efflux detected using the ISE during assays performed on five different days (detection reported as mg/L).

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