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Portable Near-Infrared Fluorometer for a Liposomal Blood Lactate Assay

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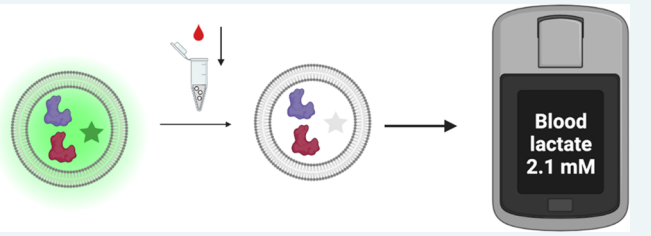
ABSTRACT: In sepsis, plasma lactate is a key biomarker of disease severity, prognosis, and treatment success. However, the median time to result for clinical lactate tests is 3 h. We recently reported a near-infrared fluorescent (NIRF) blood lactate assay that relies on a two-step enzymatic reaction in a liposomal reaction compartment. This assay was optimized in human blood and was capable of quantifying lactate in fresh capillary blood from human volunteers at clinically relevant concentrations in 2 min. However, these studies were performed with a tabletop fluorescence plate reader. For translation to the point of care, the liposomal lactate assay needs to be combined with a small portable NIR fluorometer. Portable NIR fluorometers were successfully used for the analysis of skin and soil samples, but reports for blood metabolite assays are scarce. We aimed at testing the performance of the liposomal lactate assay in combination with a commercial small portable NIR fluorometer. First, we tested the fluorophore of the liposomal lactate assay using the NIR dye sulfo-cyanine 7; we observed strong fluorescence signals and high linearity. Second, we performed the liposomal lactate assay in lactate-spiked human arterial blood using the portable fluorometer as the detector and observed strong and highly linear lactate sensing at clinically relevant lactate concentrations after 2 min. Finally, spiking fresh mouse blood with three clinically relevant lactate concentrations led to a significantly different response to all three concentrations after 5 min. These results highlight the usefulness of the tested portable NIR fluorometer for the liposomal lactate assay and motivate a clinical evaluation of this rapid and easy-to-use lactate assay.



KEYWORDS: *fluorometer, lactate, lactate assay, liposome, point-of-care, diagnostics*

1. INTRODUCTION

In emergency medicine, plasma lactate has gained broad acceptance as a biomarker for hypoxia-associated diseases such as sepsis, traumatic injuries, cardiac arrest, and stroke.[1] In hypoxic cells, there is a metabolic switch from aerobic to lactate-generating anaerobic glycolysis which results in increases in blood lactate concentrations (hyperlactatemia).[2] In patients with sepsis, a generalized immune response leading to vasodilation, hypovolemia, and hypoperfusion leads to hypoxia in tissues and organs.[2] Sepsis has an incidence of over 1.7 million cases per year in the United States and leads to over 270 000 deaths per year.[3] Plasma lactate is a key biomarker in sepsis: current clinical practice guidelines recommend measuring lactate for disease staging and prognosis, and assessing the treatment success of volume replacement therapy.[4] In clinical practice, lactate testing is time-consuming with a median time to result of 3 h, which delays critical clinical decisions.[5] This time loss between the triage and result is related to the need for a peripheral blood draw and analysis in nonportable laboratory analyzers in the hospital laboratory.[5−8] In clinical routine, lactate is measured in plasma using a colorimetric assay.[9,10] The mechanism of action of the clinical gold standard of lactate testing is a two-step enzymatic reaction, in which lactate is oxidized by L-lactate oxidase (LO) and hydrogen peroxide is generated.[10] Hydrogen peroxide is subsequently used by a peroxidase to oxidize a substrate such as 4-aminoantipyrine, leading to a purple product.[10] Other lactate assays such as electrochemical strip-based lactate analyzers have been associated with biased results.[11−14] Moreover, delays between blood collection and lactate testing due to the transport to the hospital laboratory can lead to artifactually elevated lactate concentrations because of erythrocytic glycolysis.[15] To accelerate time to result in patients with sepsis, new portable point-of-care blood lactate assays are required for the inpatient (emergency room and intensive care unit) and outpatient settings (doctor's office, ambulance, home clinic, and pharmacy).

We recently reported a near-infrared fluorescent (NIRF) blood lactate assay that performed well in the fresh capillary blood of human volunteers (Figure 1A).[16,17] This lactate assay relies on a two-step enzymatic reaction in a liposomal reaction

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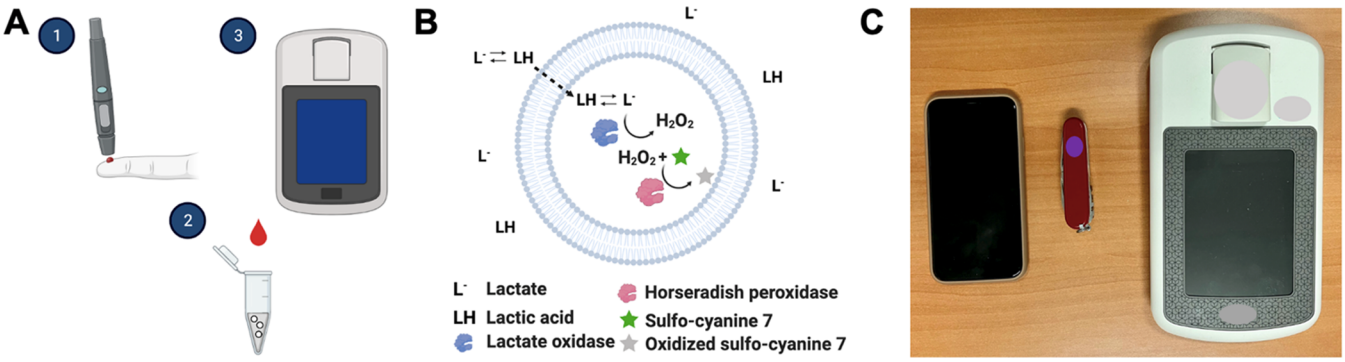

Figure 1. Liposomal lactate assay and portable fluorometer. The proposed lactate measurement includes capillary blood collection by fingerstick, addition of blood to liposomal assay solution, and analysis using a portable point-of-care fluorometer which immediately displays the lactate result (A). Mechanism of action of the liposomal lactate assay: lactate and lactic acid are in equilibrium in solution. After diffusion of lactic acid across the membrane, lactic acid reaches the liposomal lumen where it is in equilibrium with lactate. Lactate is oxidized by lactate oxidase, and hydrogen peroxide is generated, which is subsequently utilized by horseradish peroxidase to oxidize the NIRF dye sulfo-cyanine 7, which leads to a quantifiable fluorescence decrease (B). Portable fluorometer used in this study compared with an iPhone 11 and a Swiss Army Knife as size references (C).

compartment (Figure 1B). Lactate and lactic acid are in equilibrium in solution. Due to its neutral charge, lactic acid readily diffuses across the membrane and enters the liposomal lumen. At physiological pH, lactic acid concentration (pK_a 3.8) is low and the diffusion across the membrane is slow. Decreasing the outer phase pH to 5.4 strongly increased sensor kinetics.[16,17] Therefore, we selected a liposome composition of transmembrane pH-gradient liposomes.[18−21] These tough membranes avoid an acidification of the luminal pH during the sensing process and thus preserve high enzymatic activity.[22−24] Inside the liposome, the exogenous enzyme LO oxidizes lactate to pyruvate and hydrogen peroxide. LO has high substrate specificity to L-lactate and thus confers high analyte selectivity to the assay.[25] Indeed, this enzyme is used in the routine clinical practice plasma lactate assay.[9,10] Hydrogen peroxide is subsequently utilized by horseradish peroxidase (HRP) as an oxidant in the oxidation of the NIRF dye sulfo-cyanine 7 (S7), which leads to a loss of the dye's fluorescence. This enzymatic cascade requires a dedicated reaction compartment in whole blood because erythrocytic catalase and glutathione peroxidase rapidly degrade hydrogen peroxide.[16,26] If the hydrogen peroxide generated by the enzymatic oxidation of lactate is rapidly cleared, it is not available for the enzymatic oxidation of sulfo-cyanine 7 by HRP. Therefore, we developed a liposomal compartment to physically isolate the two-step enzymatic reaction from erythrocytic catalase and glutathione peroxidase. In the past, we optimized this liposomal lactate assay in human arterial blood and validated it in an institutional review board-approved study in fresh capillary blood from human volunteers.[17] However, these studies were performed using a large tabletop fluorescence plate reader. To translate this assay to the point of care, a handheld portable detection system is needed that rapidly quantifies the NIRF intensity of the assay solution and requires low sample volumes.

NIRF assays are a highly promising option for the point of care. As fluorophores and chromophores in blood do not interfere with NIRF, there is no need to separate blood into plasma or serum. Centrifugation is performed for blood separation in clinical laboratories. At the point of care, options for blood separation are limited to filtration. Despite recent progress, filtration methods continue to have important limitations including filter clogging, probe dilution, inadequate characterization of filtered plasma composition, and need for pumps in microfluidics-assisted filtration.[27] Blood separation therefore remains a major bottleneck in the development of point-of-care assays and highlights the potential of NIRF-based assays. The miniaturization of fluorometers poses considerable challenges, however, as acceptable trade-offs between gain, noise, and component count and bulkiness (e.g., photomultipliers) are needed to allow for a sufficient sensitivity with a miniaturized system.[28] While handheld NIR fluorometers have recently been developed and were used to read NIRF probes in the skin[29,30] and in soil samples,[31] we were not able to retrieve reports on blood assays that utilize a commercial small handheld NIRF detector in combination with whole blood NIRF assays. The aim of this study was to combine this liposomal lactate assay with a commercial portable NIRF fluorometer (Figure 1C) and test the assay's performance in whole blood.

2. MATERIALS AND METHODS

2.1. Materials. DPPC, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(methoxy(polyethylene glycol)-2000) (DSPE-PEG(2000)), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Sepharose CL-6B (fractionation range 10−1000 kDa based on dextran), sodium chloride, sodium hydroxide, potassium phosphate monobasic, citric acid, sodium citrate, lactate oxidase (*Aerococcus viridans*, ≥20 U/mg), and HRP (77332, ∼150 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO). S7 was obtained from Lumiprobe (Hannover, Germany). Empty columns (Pierce Centrifuge Columns 89897) were obtained from Thermo Fisher Scientific (Waltham, MA). Arterial whole blood samples from healthy volunteers containing potassium EDTA as an anticoagulant were purchased from Innovative Research (Novi, MI) and stored at 4 °C. Fresh mouse blood was obtained by cardiac puncture from 30-week-old male C57BL/6 mice and stored on ice.

2.2. Methods. *2.2.1. S7 Standard Curve.* S7 was serially diluted in 50 mM isotonic phosphate buffer at pH 7.4. S7 standards (100 μL) were added to 0.5 mL tubes. The fluorescence intensity of the tube was read by a portable fluorometer (Deniro, Detact Diagnostics, Groningen, The Netherlands). The specifications of this fluorometer provided

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by the manufacturer include a processing time of <5 s per sample, an excitation LED source with a maximum at 760 nm, an emission filter at 750−769 nm, and detectors in the range of 800−1250 nm, a warm-up time of <35 s, and a sample holder for 0.5 mL Eppendorf tubes according to the manufacturer. The assay name "CoviTact" was used with a blank of ultrapure water with Excitation "Red" and Emission "Far Red." The "Delta RFU" value was analyzed. The emission and excitation wavelengths, bandwidth, and gain could not be changed.

2.2.2. Liposome Preparation. Liposomes were prepared in accordance with the literature.[16,17] In brief, lipids dissolved in chloroform (15.3 μmol DPPC, 18.4 μmol cholesterol, and 0.3 μmol DSPE-PEO(2000)) were added to a glass vial. The solvent was evaporated using nitrogen flow for at least 2 h and stored under vacuum for at least 2 days. The lipid film was rehydrated using 1 mL of isotonic phosphate buffer 50 mM at pH 7.4 (lipid concentration: 34 mM) containing S7, HRP, and LO and immediately heated to 52 °C for 2 min. Subsequently, two alternating vortexing and heating steps of 1.5 min each were conducted.

2.2.3. Purification Procedure. Liposomes prepared in Section 2.2.2 were purified as reported.[16,17] In brief, empty columns were filled with 6 mL Sepharose CL-6B and subsequently washed with 10 mL of 5 mM isotonic phosphate buffer at pH 7.4. In 0.25 mL steps, 0.5 mL of liposome dispersion and 2.5 mL of buffer were added to the column. Fractions of 0.25 mL were collected. Fractions 7 and 8 were stored at 4 °C and protected from light.

2.2.4. Liposomal Assay in Whole Blood. The liposome assay was conducted as reported.[16,17] In brief, 25 μL of lactate standard consisting of sodium chloride solution 0.9% (w/v) spiked with 1.25−20 mM lactate (final concentration in assay mixture 0.31−5 mM) was added to 25 μL of human or mouse whole blood in a 0.5 mL polypropylene tube. Purified liposome dispersion (24 μL) was added to 66 μL of 98 mM isotonic citrate buffer at pH 5.1 (final citrate concentration in assay mixture 36 mM and pH 5.3), and 50 μL of this mixture was immediately added to the tube containing whole blood and lactate standard. The fluorescence intensity of the tube was read after 30 s, 1, 2, 3, 4, and 5 min by the portable fluorometer described above.

2.3. Statistical Analysis. SigmaPlot (version 13.0) and Microsoft Excel 2016 (linear regression analysis) were used for the statistical analysis. Comparisons of three or more groups were performed by a one-way ANOVA followed by Tukey's post hoc test. To compare the fluorescence response curves, relative fluorescence intensities were compared at the highest lactate concentration. A p-value of <0.05 was deemed statistically significant.

3. RESULTS AND DISCUSSION

3.1. S7 Standard Curve. To determine the linearity of the portable fluorometer, we measured the fluorescence intensity of an S7 dilution series in isotonic phosphate buffer 50 mM at pH 7.4. S7 is a highly hydrophilic dye due to two sulfate groups and exhibits high solubility in water (Figure 2). We observed high linearity (R^2 = 0.998) in the range of 8−1000 nM. The inclusion of the 2000 nM S7 standard resulted in a more sigmoidal curve with decreased linearity (R^2 = 0.992) (Figure S1), potentially due to the self-quenching of the dye.

3.2. Lactate Sensing in Human Blood. In the past, we demonstrated lactate sensing in bovine and human blood using a conventional tabletop fluorescence plate reader.[16,17] To translate this assay to the bedside, we tested its lactate sensing capacity in commercial human blood and measured the NIRF with a portable fluorometer. In a clinically relevant range of 0.3−5 mM, which includes clinically used lactate cutoffs of 2 and 4 mM,[4,32] we observed a significantly decreasing relative fluorescence intensity over 5 min (Figure 3A). The response was strong and highly linear after 2 min with a decrease in fluorescence of about 40% at the highest lactate concentration (Figure 3B). The results were comparable with the performance of the lactate assay using a conventional tabletop fluorometer.[16,17] The blood sample volume of 25 μL is below typical blood sample volumes collected by fingerstick, the most established blood sampling method in clinical routine which yields 40−120 μL of blood depending on the lancet type.[33] These results strongly suggest that the tested portable fluorometer is compatible with our lactate assay in human whole blood.

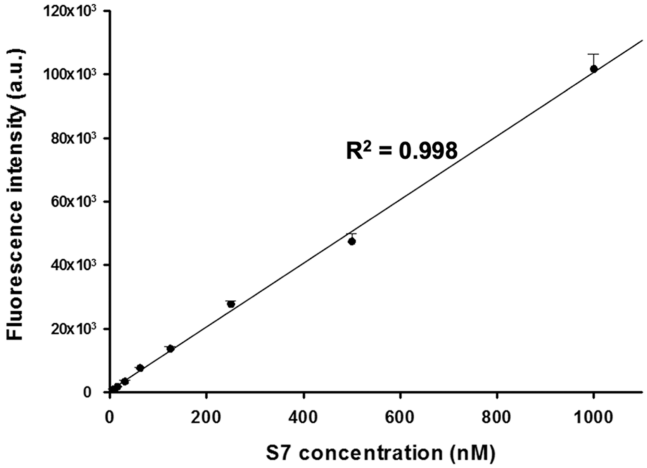

Figure 2. S7 standard curve using a portable fluorometer. Fluorescence intensity of S7 in 50 mM isotonic phosphate buffer at pH 7.4. All results as means ± SDs (n = 3).

3.3. Lactate Sensing in Fresh Mouse Blood. Having previously validated the liposomal lactate assay in fresh blood using a conventional tabletop fluorometer,[17] its performance was tested in fresh blood using the portable fluorometer (Figure 4). Fresh blood is a relevant matrix to investigate due to its lower lactate levels than stored blood, as erythrocytic glycolysis increases lactate levels in the blood during storage.[15] For this experiment, fresh arterial blood from healthy mice was collected and spiked with three clinically relevant lactate concentrations. The liposomal lactate assay was capable of discriminating the 1.25 and 5 mM concentrations after 3 min, and all three concentrations after 5 min. This finding was in agreement with our human capillary blood study, where the samples spiked with 2.5 and 5 mM lactate were significantly different after 2 min.[17] These encouraging results in fresh mouse blood motivate further investigation of this assay in a mouse model of sepsis such as the cecal puncture and ligation model.[34]

Previously, we provided a proof of concept of our investigational lactate assay in an IRB-approved study in fresh blood from human volunteers using a large tabletop fluorometer. In the current study, we provide the next translational milestone: a successful validation of the assay with a portable fluorometer. The motivation behind the use of a portable detection system is that it allows point of care

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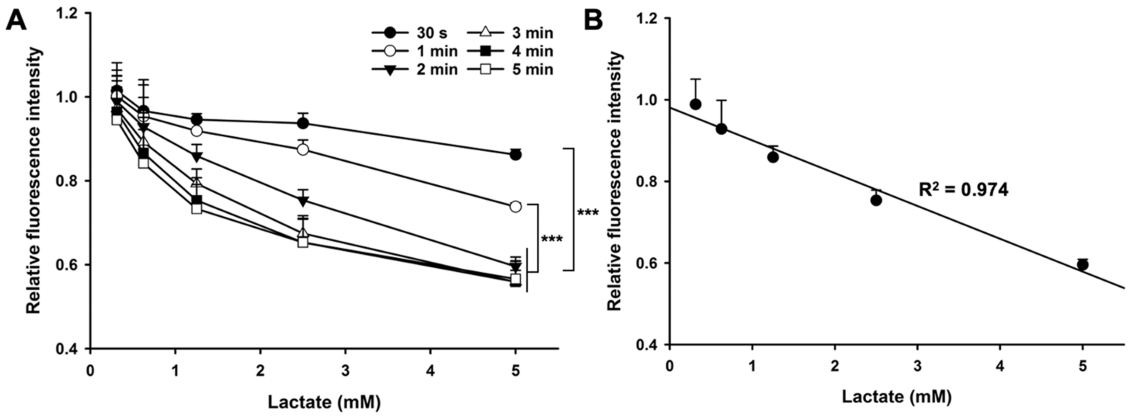

Figure 3. Lactate sensing in spiked human whole blood using a portable fluorometer. Relative fluorescence intensity of LO/HRP/S7-containing liposomes in lactate-spiked human blood at pH 5.4 (A). Relative fluorescence intensity of LO/HRP/S7-containing liposomes in lactate-spiked human blood at pH 5.4 after 2 min with linear regression curve (B). Inner phase composition: S7 concentration: 100 μM; HRP concentration: 2.5 U/mL; LO concentration: 5 U/mL; buffer composition of inner phase: 50 mM isotonic phosphate buffer at pH 7.4; buffer composition of outer phase: 49 mM isotonic citrate buffer at pH 5.4; saline volume fraction 25% (v/v); human whole blood volume fraction: 25% (v/v); incubation at room temperature. All results as means ± SDs (n = 3). ***p < 0.001.

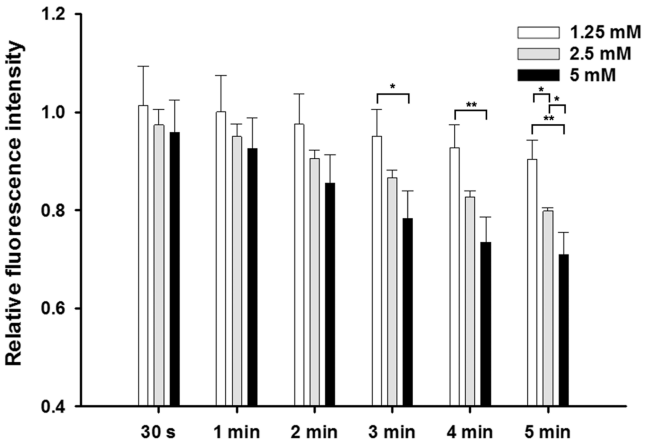

Figure 4. Lactate sensing in spiked fresh arterial blood from healthy mice. Relative fluorescence intensity of LO/HRP/S7-containing liposomes in lactate-spiked fresh arterial blood at pH 5.4. Inner phase composition: S7 concentration: 100 μM; HRP concentration: 2.5 U/mL; LO concentration: 5 U/mL; buffer composition of inner phase: 50 mM isotonic phosphate buffer at pH 7.4; buffer composition of outer phase: 36 mM isotonic citrate buffer at pH 5.4; lactate-spiked saline volume fraction 25% (v/v) at 1.25, 2.5, or 5 mM (final concentration in assay mixture 0.3, 0.6, and 1.3 mM, respectively); mouse arterial whole blood volume fraction: 25% (v/v); incubation at room temperature. Each blood sample was freshly obtained from one mouse and stored on ice until analyzed ∼3 to 4 h after blood sampling. HRP: horseradish peroxidase; LO: lactate oxidase; S7: sulfo-cyanine 7. All results as means ± SDs (n = 3). *p < 0.05; **p < 0.01.

(doctor's office, pharmacy, home clinic, and ambulance truck) and bedside (emergency room and intensive care unit) measurements. When miniaturizing NIRF detectors, the main challenges include finding acceptable trade-offs between gain, noise, component count and dimensions, and current consumption.[28] The NIRF detector investigated here provided fast fluorescence detection in the desired S7 concentration range. In the next steps toward commercialization, we will improve assay stability, validate the assay in sepsis blood, and expand the analyte space. The liposomal formulation is currently stable for at least 5 days at 4 °C.[17] As enzymes degrade in solution over time, a solid formulation would be advantageous to prolong shelf-life. Lyophilization is an established drying procedure for liposomes and proteins.[35,36] The lactate assay in combination with a portable fluorometer will be validated in blood from animal models of sepsis such as the cecum perforation and ligation model and from patients with sepsis. Moreover, digital solutions to facilitate documentation of the results in the patient dossier (e.g., wireless communication with a smartphone app or hospital database) will be developed. Finally, the versatility of the assay will be used to expand the analyte space: by substituting lactate oxidase with another oxidase (e.g., glucose oxidase and alcohol oxidase), other enzymatically oxidizable substrates can be sensed (e.g., glucose and ethanol), as shown previously by us.[16]

4. CONCLUSIONS

Having previously demonstrated rapid and accurate lactate sensing in fresh human blood with tabletop fluorescence plate readers, we aimed at combining our liposomal lactate assay with a small, portable fluorometer that requires very small volumes. The portable fluorometer detected a strong and linear fluorescence increase with increasing S7 concentrations in <5 s per sample. In lactate-spiked human whole blood, we observed a strong, linear, and lactate concentration-dependent fluorescence decrease after 2 min. The liposomal assay in combination with the portable reader also performed well in lactate-spiked fresh mouse blood where three clinically relevant lactate concentrations led to significantly different fluorescence intensities after 5 min. These findings strongly support the usefulness of the portable fluorometer for the liposomal lactate assay and pave the way for the translation of the assay to the point of care.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.3c00055.

Figure S1. S7 standard curve using a portable fluorometer. Figure S2. Lactate sensing in spiked human whole blood using a portable fluorometer. Figure

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S3. Lactate sensing in spiked fresh arterial blood from healthy mice (PDF)

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Notes

The authors declare the following competing financial interest(s): SM is a co-inventor on a patent application related to the lactate sensing technology.

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