Biomolecular Filters for Improved Separation of Output Signals in Enzyme Logic Systems Applied to Biomedical Analysis

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Supporting Information

Summary of experimental results and their statistical treatment for the STI and ABT systems operating in buffer solutions and for the LI system operating in human serum solution

Experimental Section

Chemicals and materials: Alanine transaminase from porcine heart (ALT, E.C. 2.6.1.2), glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (G6PDH, E.C. 1.1.1.49), microperoxidase-11 (MP-11), lactate dehydrogenase from porcine heart (LDH, E.C. 1.1.1.27), pyruvate kinase from rabbit muscle (PK, E.C. 2.7.1.40), creatine kinase from rabbit muscle (CK, E.C. 2.7.3.2), glycyl-glycine (Gly-Gly), *tris*(hydroxymethyl) aminomethane hydrochloride (Tris-buffer), L-alanine (Ala), α-ketoglutaric acid (α-KTG), β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), β-nicotinamide adenine dinucleotide dipotassium salt (NAD^+), L(+)-lactic acid (Lac), D-glucose 6-phosphate sodium salt (Glc6P), creatine anhydrous

(Crt), phospho(enol)pyruvate monopotassium salt (PEP), adenosine 5'-triphosphate disodium salt (ATP, from bacterial source), magnesium acetate tetrahydrate ($MgAc_2$), potassium hydroxide (KOH), hydrochloric acid (HCl) and serum from human male AB plasma were purchased from Sigma-Aldrich and were used as supplied without further purification or pretreatment. Hydrogen peroxide (H_2O_2) (30% w/w) was purchased from Baker. Ultrapure deionized water (18.2 MΩ·cm) from a NANOpure Diamond (Barnstead) source was used in all of the experiments.

Instrumentation and measurements: In order to mimic physiological conditions, optical measurements were done in temperature-controlled 1 mL poly(methyl methacrylate) (PMMA) cuvettes at 37.0 ± 0.2˚C with 1 cm pathway using Shimadzu UV-2450 UV-Vis spectrophotometer (with a TCC-240A temperature-controlled holder). All reagents were incubated at this temperature prior to experimentation. A Mettler Toledo SevenEasy s20 pH-meter was employed for the pH measurements.

Composition and operation of systems for the analysis of injuries

LI system (serum experiment): Human serum was diluted to 50% by the Tris-buffer, pH 7.4. The final concentrations of the logic system "machinery" and components of the filter were Ala (200 mM), α-KTG (10 mM), NADH (150 μM), Glc6P (4 mM) and G6PDH (10 $U \cdot mL^{-1}$). ALT and LDH used as biomarkers of liver injury were dissolved in pure human serum. Because of dilution, logic **0** and **1** levels of ALT (0.01 $U \cdot mL^{-1}$ and 1 $U \cdot mL^{-1}$) and LDH (0.075 and 0.5 $U \cdot mL^{-1}$) were used as half of the physiological and pathophysiological values.[1] Input signals were applied to the logic system in order to realize meaningful circulating levels of these biomarkers and perform the **AND** logic operation with filter. The output signal corresponding to the decreasing concentration of NADH was measured optically at λ = 340 nm.

STI system (buffer experiment): Gly-Gly buffer, 50 mM, with $MgAc_2$ (6.7 mM) was titrated with KOH to the pH value of 7.95 and used as a background solution (note that Mg^{2+} and K^+ cations are essential for activation of CK and PK, respectively). The following components were dissolved in this solution to perform the **AND** logic operation: NADH (0.1 mM), ATP (1 mM),

PEP (1.5 mM), PK (1.6 $U \cdot mL^{-1}$), Crt (7.5 mM). The filtering compounds Glc6P (0.1 mM) and G6PDH (2 $U \cdot mL^{-1}$) were prepared in the same buffer. Logic **0** and **1** levels of CK (0.1 and 0.71 $U \cdot mL^{-1}$) and LDH (0.15 and 1 $U \cdot mL^{-1}$) input signals were applied to the logic system in order to realize meaningful circulating levels of these biomarkers.[2,3] The output signal corresponding to the decreasing concentration of NADH was measured optically at λ= 340 nm.

ABT system (buffer experiment): Gly-Gly buffer, 50 mM, pH 8.5 tuned by KOH, containing $MgAc_2$ (6.7 mM) and NAD^+ (10 mM) was used to perform the **AND** logic operation. MP-11 (50 μM) and H_2O_2 (1.5 mM) were used as filter components. Logic **0** and **1** levels of LDH (0.15 and 1.0 $U \cdot mL^{-1}$) and Lac (1.6 and 6.0 mM) input signals were applied to the logic system in order to realize meaningful circulating levels of these biomarkers.[3,4] The output signal corresponding to the NADH formation was measured optically at λ = 340 nm.

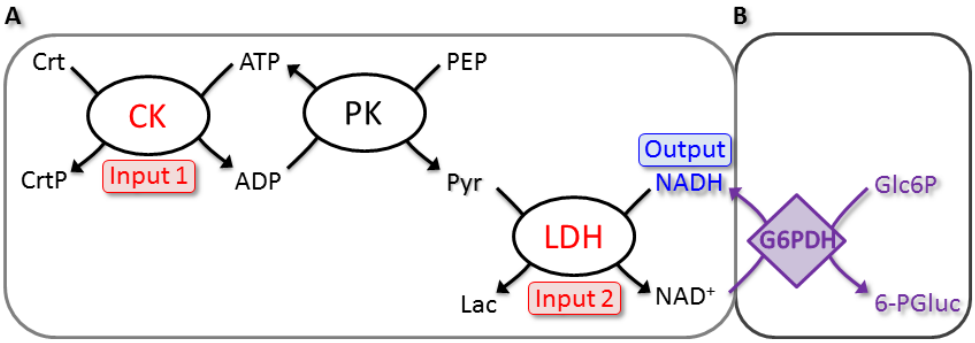

Figure SI 1. The biocatalytic cascade operating as the **AND** logic gate for analysis of the STI without (A) and with presence (B) of the biocatalytic filter. The following abreviations for products and intermediates are used: CrtP = phosphocreatine, ADP = adenosine diphosphate, Pyr = pyruvate, 6-PGluc = 6-phospho-gluconic acid. Other abbreviations are specified in the *Chemicals and materials* section.

Results for the STI and ABT detection systems measured in buffer solutions and for the LI system measured in human serum solutions

Soft Tissue Injury (STI) system operating in buffer: Two enzymes, CK and LDH, were applied as biomarkers characteristic of soft tissue injury.[2,3] Their simultaneous increase from normal to

pathophysiological concentrations provides an evidence of STI conditions. The biochemical cascade catalyzed in the presence of the both enzyme-biomarkers (note the biocatalytic operation of PK being a part of the logic gate "machinery") results in the oxidation of NADH to NAD^+ (Figure SI 1A), thus yielding the corresponding absorbance decrease. The *absolute value of the absorbance change* was used to define the output signal produced by the system. The logic value of the output signal changes from the low **0** value to the high **1** value only upon the concerted work of the both enzyme-inputs (logic inputs combination **1**,**1**), thus mimicking the **AND** logic operation. Since the logic **0** values of the input signals are not physical zero concentrations (they rather correspond to the normal physiological concentrations of the enzymes), the NADH absorbance is also changing upon other combinations of the inputs (**0**,**0**; **0**,**1**; **1**,**0**). Similarly to the LI system described in the main text of the paper, the STI system operation was improved upon addition of the filter system to the analyzing biocatalytic cascade (Figure SI 1). The experimental data obtained in the presence and absence of the filter are summarized in Figure SI 2 and statistically analyzed in Figure SI 3, as further detailed below.

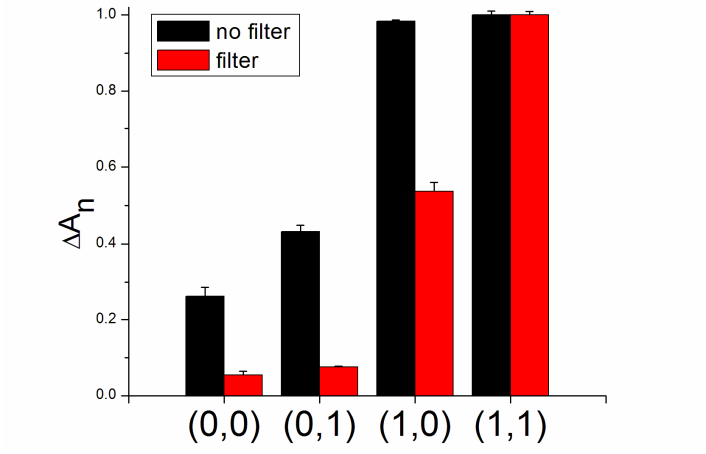

Figure SI 2. Bar chart featuring the **AND** logic operation of the optical system for detection of the STI. The black-colored bars indicate performance of the STI system without filter whereas the red-colored bars are with the applied filter. Optical absorbance measurements were performed at λ = 340 nm at time of 350 sec.

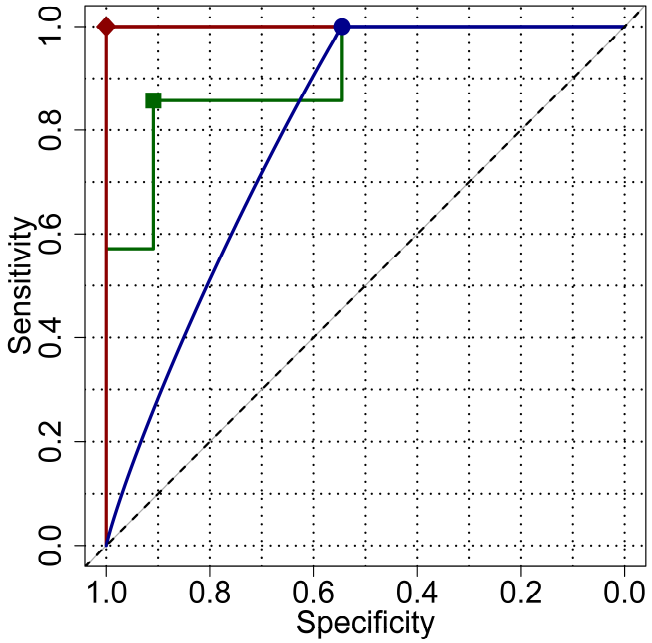

Figure SI 3. Receiver operating characteristic (ROC) empirical (green line) and smoothed (blue line) curve for the non-filtered system in detection of the STI. ROC curves for the filtered system are shown in red color. Note the both empirical and smooth ROC curves for the filtered system correspond to the "perfect performance." Random choice is denoted by the solid diagonal line. The best cutoffs which maximize the accuracy are indicated as solid symbols.

Abdominal Trauma (ABT) system operating in buffer: The enzyme LDH and its substrate Lac appearing together at elevated concentrations can be used as biomarkers of ABT.[3,4] The biocatalytic reaction activated by the enzyme and the corresponding substrate, results in the reduction of NAD^+ cofactor (Figure SI 4), thus leading to increased absorbance at λ = 340 nm corresponding to the formation of NADH. The *absorbance change* was defined as the output signal produced by the system. The logic value of the output signal changes from the low **0** value to the high **1** value only upon the concerted work of both inputs (logic inputs combination **1**,**1**), thus mimicking **AND** logic operation. Since the logic **0** values of the input signals are not physical zero concentrations (they correspond to the normal physiological concentrations of the enzyme and its substrate), the NADH absorbance is also changing upon other combinations of

the inputs (**0**,**0**; **0**,**1**; **1**,**0**). Similarly to the LI system described in the main text of the paper, the ABT system operation was improved upon addition of the filter process to the analyzing biocatalytic cascade (Figure SI 4). The experimental data obtained in the presence and absence of the filter are summarized in Figure SI 5 and statistically analyzed in Figure SI 6.

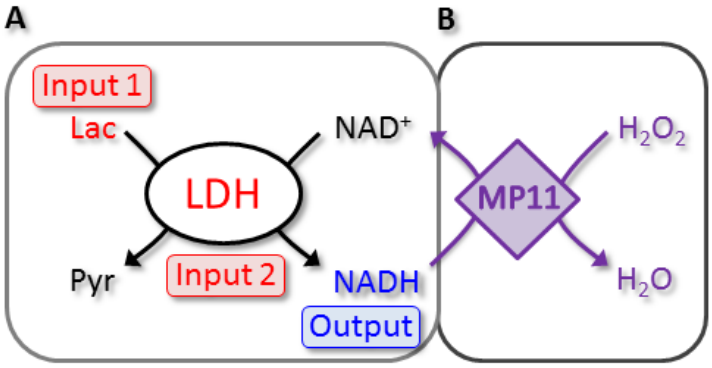

Figure SI 4. The biocatalytic cascade operating as an **AND** logic gate for analysis of the ABT in the absence (A) and in the presence (B) of the biocatalytic filter. The following abreviation for a product is used: Pyr = pyruvate. Other abbreviations are specified in the *Chemicals and materials* section.

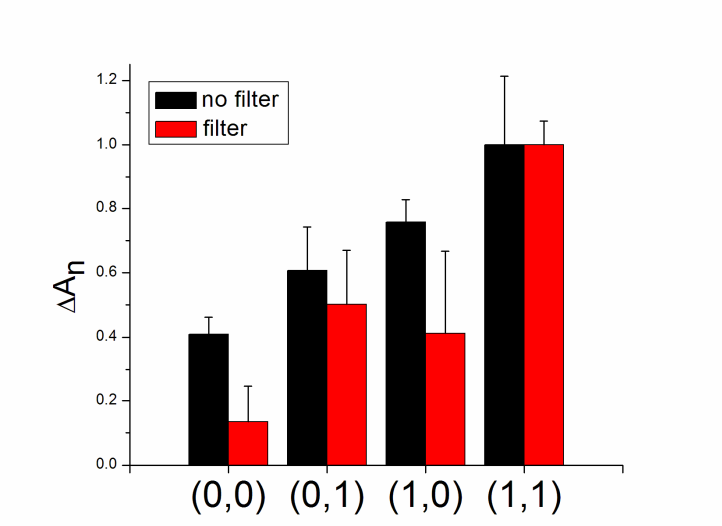

Figure SI 5. Bar chart featuring the **AND** logic operation of the optical system for detection of ABT. The corresponding combinations of input signals without a filter (the black bars) and with a filter (the red bars) are indicated. Optical absorbance measurements were performed at λ = 340 nm at time of 1200 sec.

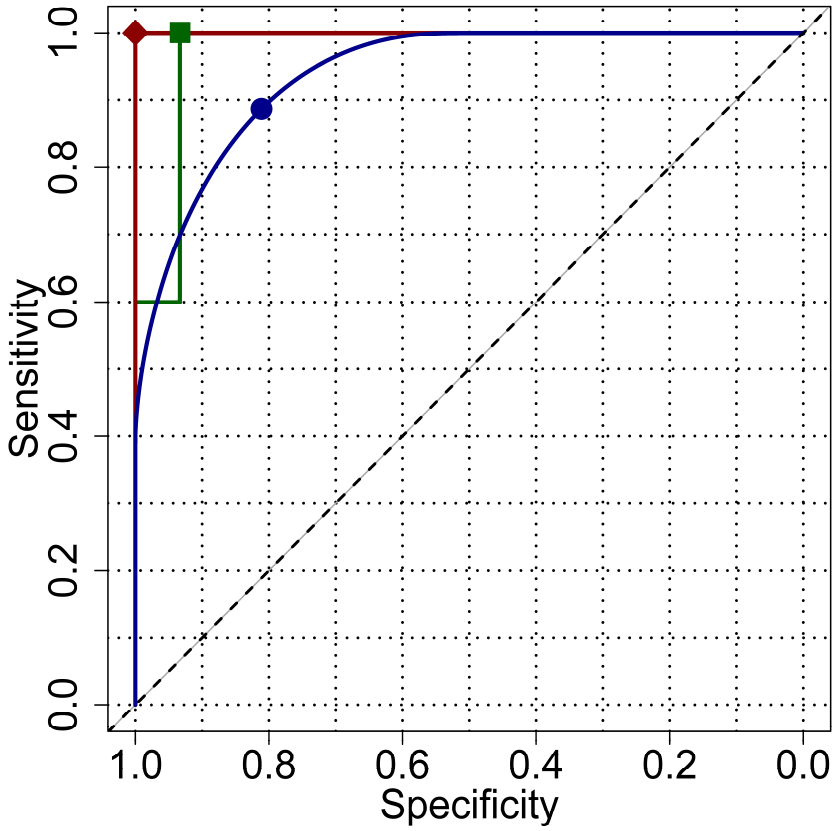

Figure SI 6. Receiver operating characteristic (ROC) empirical (green line) and smoothed (blue line) curve for non-filtered system in detection of the ABT. ROC curves for filtered system are shown in red color. Note the both empirical and smooth ROC curves for filtered system correspond to the "perfect performance." Random choice is denoted by the grey diagonal line. The best cutoffs which maximize the accuracy are indicated as solid symbols.

Liver Injury (LI) system operating in serum: The system is the same as described in the main part of the paper (Scheme 1; Figure SI 7), but its operation was studied in the presence of human serum solution. The experimental data obtained in the presence and absence of the filter are summarized in Figure SI 8 and statistically analyzed in Figure SI 9.

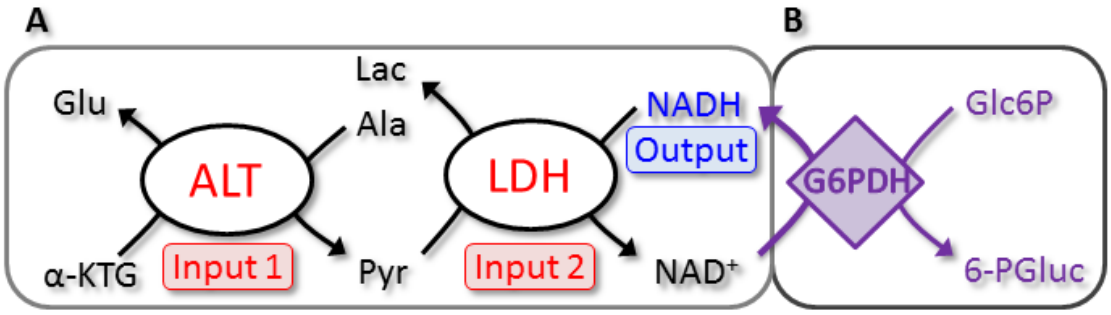

Figure SI 7 (*the same as Scheme 1 in the paper*). The biocatalytic cascade operating as the **AND** logic gate for analysis of LI in the absence (A) and in the presence (B) of the biocatalytic filter. The following abreviation for a product is used: 6-PGluc = 6-phospho-gluconic acid, Pyr = pyruvate, Lac = lactate, Glu = glutamate. Other abbreviations are specified in the *Chemicals and materials* section.

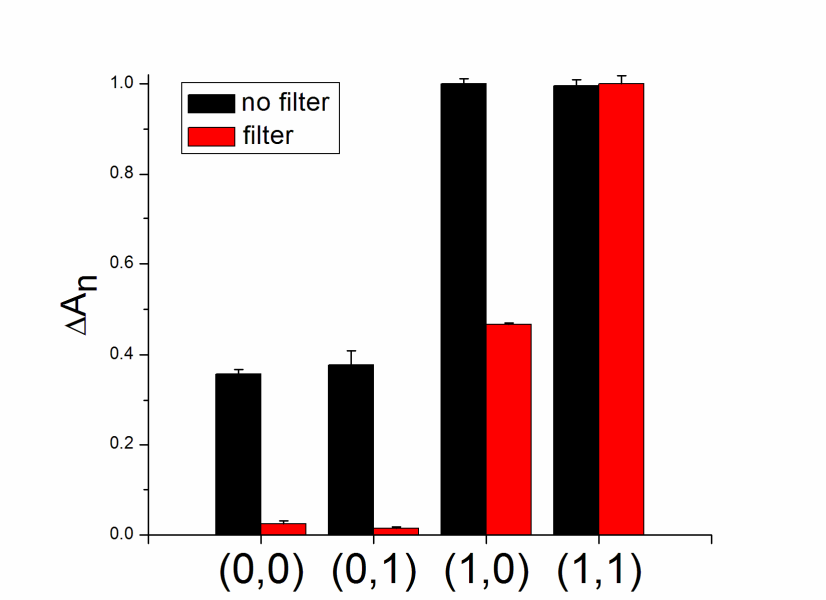

Figure SI 8. Bar charts featuring the **AND** logic operation of the optical system for detection of LI. The corresponding combinations of input signals without a filter (the black bars) and with a filter (the red bars) are indicated.

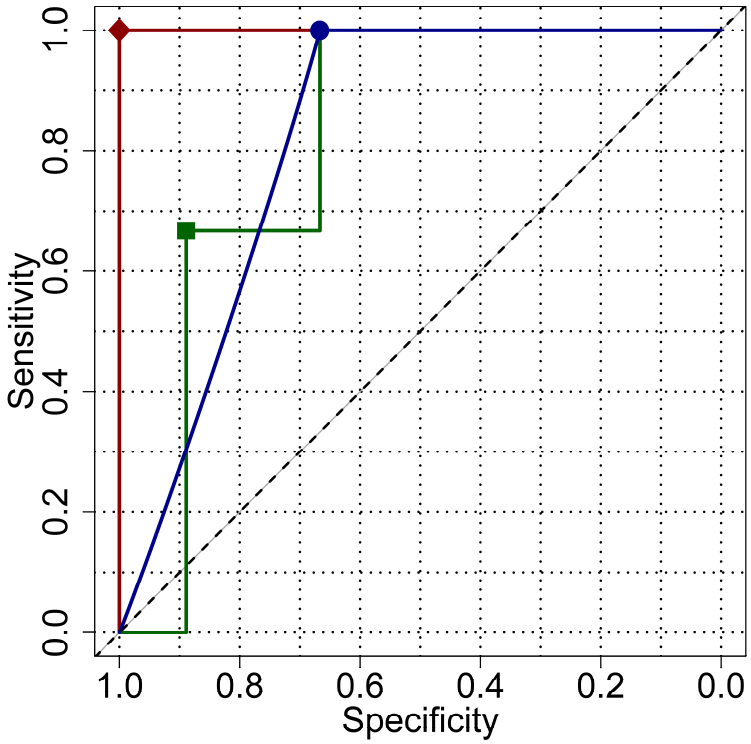

Figure SI 9. Receiver operating characteristic (ROC) empirical (green line) and smoothed (blue line) curve for non-filtered system in detection of LI in serum. ROC curves for filtered system are shown in red color. Note the both empirical and smooth ROC curves for filtered system correspond to the "perfect performance." Random choice is denoted by the grey diagonal line. The best cutoffs which maximize the accuracy are indicated as solid symbols.

Statistical data analysis

The biochemical cascade composed of the specific biocatalytic reactions, results in a distinct change in the output signal (change in absorbance) only in case of the cooperative action of both biomarkers (logic input combination **1**,**1**) and logic value of the output attains **1**. Other combinations of the input signals (**0**,**0**, **0**,**1** and **1**,**0**) represent logic value of the output **0**, but this occurs for non-zero physical values of the inputs, due to their physiological nature. The effect of the enzyme filter on improving the discrimination of logic output **0** and **1** was evaluated by Receiver Operating Characteristic (ROC) curves.

The accuracy of the diagnostic method depends on the ability to distinguish between pathophysiological and physiological samples being tested, which represent the logic output **1** and **0**, respectively. The "area under curve" (AUC) is a summary single measure, defined as an area under an ROC curve, which combines concepts of sensitivity and specificity and is commonly used for quantification of diagnostic test accuracy. The sensitivity—the "true positive rate" (TPR), and specificity—the "true negative rate" (TNR), both depend on the tested thresholds; the TPR and TNR vary as the threshold varies. By considering various possible values of the threshold, an ROC curve can be constructed as a continuous function of sensitivity versus specificity (possibly 1-specificity—the "false positive rate"; FPR).[5] AUC of 1 represents "perfect performance," i.e. 100% TPR, at TNR of 100%; AUC of 0.5 represents a random diagnosis.

The AUCs of empirical ROC curves were estimated by the trapezoidal method of integration, and the corresponding 95% confidence intervals (CI) were estimated with the method described by De Long et al.[6] Using the ROC analysis, the best thresholds (above which the absorbance change is considered as a logic output **1**) that yielded the maximum accuracy were determined and characterized by their specificity and sensitivity with the corresponding 95% CIs. Smoothed ROC curves were additionally estimated by using a non-parametric method. The Kernel density function[5] was used to fit smooth ROC curves to data points because this method is free of parametric assumptions.[7] This smoothed-curve method outperforms the competing methods when pathophysiological and/or control group has a bimodal distribution (see the differences in absorbance changes between logic inputs **0**,**0**, **0**,**1** and logic input **1**,**0**). The bandwidth of the Kernel function is fixed using the robust method developed by Sheather and Jones.[8] The AUCs of smooth ROC curves are indicated with corresponding 95% CIs computed with 2000 stratified bootstrap replicates as described elsewhere.[9] All statistic tests and data plotting were performed using the standard R-project software R 2.1, available online[10].

Three enzymatic systems described above were evaluated by ROC curve analysis before and after the enzymatic filter was included. The cutoff values, sensitivity, specificity, and area under the empirical and smooth ROC curve for all the enzymatic systems without and with filter are presented in Table 1. In non-filtered enzymatic systems, the ability of the systems to distinguish

the logic output **0** from **1** was found as follows: (*i*) liver injury measured in serum (AUC 0.82, 95% CI 0.55-1.00), (*ii*) soft tissue injury measured in buffer (AUC 0.91, 95% CI 0.76-1.00), and (*iii*) abdominal trauma measured in buffer (AUC 0.97, 95% CI 0.91-1.00). The AUCs for smooth ROC curves for non-filtered enzymatic systems were as follows: (*i*) liver injury measured in serum (AUC 0.83, 95% CI 0.65-1.00), (*ii*) soft tissue injury measured in buffer (AUC 0.80, 95% CI 0.73-0.95) and (*iii*) abdominal trauma measured in buffer (AUC 0.94, 95% CI 0.87-1.00). Using the enzymatic filter, we have achieved "perfect performance" in terms of the AUC for both empirical and smooth ROC curve in all diagnostic systems. ROC curves showed a very good discrimination between logical output **0** and **1**, with an AUC of 1.00 (95% CI 1.00-1.00) for all filtered systems; such values correspond with 100% sensitivity and 100% specificity. Notice that in the case of the "perfect performance" the AUCs of empirical and smooth ROCs curve are the same.

Table 1: Receiver Operating Characteristic Curve Analysis of the enzymatic systems for diagnosis of liver injury (performed in buffer and serum), soft tissue injury and abdominal trauma with the enabled/disabled biocatalytic filter.

		AUC 95% CI	**Cutoff**	**Sensitivity 95% CI**	**Specificity 95% CI**	**AUC (smooth ROC curve) 95% CI**
LI (Buffer)	No Filter	0.92 (0.79-1.00)	1.70	0.60 (0.23-0.88)	1.00 (0.80-1.00)	0.90 (0.77-1.00)
	Filter	1.00 (1.00-1.00)	0.84	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
LI (Serum)	No Filter	0.82 (0.55-1.00)	0.77	0.67 (0.21-0.94)	0.89 (0.57-0.98)	0.83 (0.65-1.00)
	Filter	1.00 (1.00-1.00)	0.35	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
STI (Buffer)	No Filter	0.91 (0.76-1.00)	0.58	0.86 (0.49-0.97)	0.91 (0.62-0.98)	0.80 (0.73-0.95)
	Filter	1.00 (1.00-1.00)	0.47	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
ABT (Buffer)	No filter	0.97 (0.91-1.00)	0.76	1.00 (0.57-1.00)	0.93 (0.70-0.99)	0.94 (0.87-1.00)
	Filter	1.00 (1.00-1.00)	0.48	1.00 (1.00-1.00)	1.00 (1.00-1.00)	1.00 (1.00-1.00)

Finally, as stressed in the main text, we emphasize that, our conclusion of "perfect performance" applies only to the analytical variance of the process of the binary **AND** function generation and signal-conversion to digital answers. Indeed, we do not probe the additional noise effects due to actual clinical-testing concentration distributions of the biomarkers involved. The reason has been that the details of the latter distributions[11,12] are simply not well known presently, even though all the considered biomarkers are used in actual biomedical testing, in different assay formats[13,14] than those proposed here. As a precaution, we sat our **0** and **1** "digital"—perhaps more carefully termed "binary"—values safely at the edges of the approximately known[1-5] physiological and pathophysiological ranges: **0** approximately at the highest value of the lower range, **1** approximately at the lowest value of the upper range. In summary, our actual information processing thus involves the binary **AND** function accompanied by analog-to-digital signal conversion for the output, realized and made high-fidelity by adding the "filtering process" to the enzymatic reaction cascade.

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