Electronic Supplementary Material (ESI) for Analytical Methods.


# Nitric oxide quantitative assay by a glyceraldehyde 3-phosphate dehydrogenase/phosphoglycerate kinase/firefly luciferase optimized coupled bioluminescent assay

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# ELECTRONIC SUPPLEMENTARY INFORMATION

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# Experimental

## Preliminary assays

### UV-vis spectrophotometric assays.

**GAPDH and PGK activity assay.** GAPDH and PGK activities were assayed by a spectrophotometric assay based on the 'Enzymatic Assay of 3-Phosphoglyceric Phosphokinase (EC 2.7.2.3) from Baker's Yeast' protocol available from the manufacturer with modifications. Briefly, the test, GAPDH blank and PGK blank reaction mixtures contained 1,725 μL of phosphate buffer 50 mM, pH 6.9 (Pi), 75.0 μL of G3P 0.83 mM, 37.5 μL of $NAD^+$ 0.3 mM, 37.5 μL of ADP 0.2 mM, 188 μL of $MgCl_2$ 4.2 mM, and 750 μL of glycine 133 mM in a 3,500-μL Hellma® QS Suprasil® quartz cuvette (Müllheim, Germany). To the reaction mixtures, 37.5 μL of GAPDH 3.9 μg $mL^{-1}$ (test and PGK blank) or Pi (GAPDH blank) was added. The reagents were mixed by inverting the cuvette and introduced into the spectrophotometer. The absorbance was monitored at $\lambda_{max}$ 340 nm until constant. One hundred and fifty microliters of PGK 0.011 μg $mL^{-1}$ (test and GAPDH blank) or Pi (PGK blank) was added. Absorbance was recorded for 30 minutes (Fig. S1).

**DEA-NONOate releasing assay.** DEA-NONOate releasing was assayed by a spectrophotometric assay. Briefly, 3,325 μL of Pi was pipetted to the cuvette. Then 175 μL of DEA-NONOate in NaOH 10 mM, corresponding to a concentration of 10 μM •NO, was added, the reagents were mixed by inverting the cuvette and introduced into the spectrophotometer. The absorbance was monitored at $\lambda_{max}$ 250 nm until no further changes in the absorbance values were observed (Fig. S2).

### Bioluminescent assays.

**ATP contamination in reagents and enzymes.** ATP contamination in reagents and enzymes was evaluated by luminometry using a homemade luminometer with

a Hamamatsu HCL35 photomultiplier tube (Middlesex, N.J., U.S.A) inside a light-tight dark chamber coupled to a Crison MicroBU 2030 automatic microburette (Barcelona, Spain) equipped with a 2.5-mL Hamilton GASTIGHT® 1002 glass syringe (Bonaduz, Switzerland).

The stock solutions of reagents were diluted in deionized water, DEA-NONOate standard solutions were diluted in NaOH 10 mM, and the enzymes were diluted in HEPES buffer 0.5 M, pH 7.5, and kept on ice until use.

Briefly, 10.00 μL of $MgCl_2$ 4.2 mM was added to 30.0 μL of each of the reagents and enzymes in polypropylene transparent test tubes. Concentrations of the regents and enzymes were as follow: DEA-NONOate 1 mM, Pi 50 mM, G3P 0.83 mM, $NAD^+$ 0.3 mM, ADP 0.2 mM, glycine 133 mM, GAPDH 3.9 μg $mL^{-1}$ and PGK 0.011 μg $mL^{-1}$. Controls were made by assaying ATP 0.2 mM without the reagents or enzymes and adding LUC and D-$LH_2$, by replacing $MgCl_2$, the reagents and the enzymes with deionized water and adding LUC and D-$LH_2$, and by replacing $MgCl_2$, the reagents and the enzymes with deionized water and adding only D-$LH_2$. Ten microliters of either LUC 6 μg $mL^{-1}$ or deionized water (controls) was added to the mixtures, the tubes were introduced into the dark chamber and the baseline register by the equipment was turned on, at an integration interval of 0.1 seconds. After 30 seconds, 50 μL of D-$LH_2$ 8.7 μM was injected from the automatic burette. The light output was recorded for more 30 seconds.

**Reagents and enzymes influence on LUC activity.** The influence of the reagents and enzymes on LUC activity was assayed by luminometry as described in subsection 'ATP contamination in reagents and enzymes' with the following alterations: 10.00 μL of $MgCl_2$ 4.2 mM plus 10.00 μL of ATP 0.2 mM were added to 20.0 μL of each of the reagents and enzymes. NaOH 10 mM was also tested. Controls were made by assaying ATP and $MgCl_2$ without the reagents or enzymes and adding LUC and D-$LH_2$, by replacing

ATP and $MgCl_2$ with deionized water and adding LUC and D-$LH_2$, and by replacing ATP and $MgCl_2$ with deionized water and adding only D-$LH_2$.

**Evaluation of the enzymatic reactions coupling and the effect of •NO on GAPDH. (Fig. 1).** To confirm the reactions coupling and the effect of •NO on GAPDH, a luminometric assay was performed. Briefly, DEA-NONOate solutions with final concentrations corresponding to 1 nM, 1 μM and 1 mM of •NO, considering a ratio of 1.5 mols of •NO *per* parent compound, according to the manufacturer's information, were prepared in NaOH 10 mM in capped polypropylene tubes. The solutions were diluted 1:20 in Pi in capped tubes and incubated at room temperature for 20 minutes before starting the assay to allow the release of •NO. Controls were made by replacing DEA-NONOate with deionized water (positive control) and by replacing GAPDH with deionized water (negative control). Reactions mixtures contained 450 μL of Pi, 25.0 μL of G3P 0.83 mM, 12.5 μL of $NAD^+$ 0.3 mM, 12.5 μL of ADP 0.2 mM, 62.5 μL of $MgCl_2$ 4.2 mM, 250 μL of glycine 133 mM, 12.5 μL of GAPDH 3.9 μg $mL^{-1}$ or deionized water, 50.0 μL of D-$LH_2$ 8.7 μM and 25.0 μL of LUC 6 μg $mL^{-1}$. Fifty microliters of each DEA-NONOate solutions or deionized water was transferred to transparent test tubes, the reaction mixture was added and the tubes were introduced into the dark chamber, one at a time. The baseline register by the equipment was turned on, at an integration interval of 1 second. After 50 seconds, 50 μL of PGK 0.011 μg $mL^{-1}$ was injected from the automatic burette. The light output was recorded for more 10 minutes.

## Experimental designs

**Experimental design execution.** Experimental designs were performed by luminometry. Briefly, a DEA-NONOate solution with concentration corresponding to 5 nM of •NO was prepared in NaOH 10 mM in a capped polypropylene tube. The solution was

diluted 1:20 in Pi in capped tubes and incubated at room temperature for 20 minutes. The reaction mixture contained 25.0 μL of Pi, 2.50 μL of G3P, 1.25 μL of $NAD^+$, 1.25 μL of ADP, 6.25 μL of $MgCl_2$, 20.0 μL of glycine, 5.00 μL of D-$LH_2$ and 2.50 μL of LUC, which were prepared at the concentrations indicated in Table S1. Five microliters of DEA-NONOate solution was transferred to a transparent test tube and preincubated with 1.25 μL of GAPDH at the concentrations and for the times indicated in Table S1. The reaction mixture was added and the tube was introduced into the dark chamber. The baseline register by the equipment was turned on, at an integration interval of 1 second. After 30 seconds, 30 μL of PGK at the concentrations indicated in Table S1 was injected from the automatic burette. The light output was recorded for more 3 minutes. The Box Behnken design was performed using the concentrations in Table S1 for Pi, G3P, ADP, $MgCl_2$ and preincubation time plus the following reagents and enzymes: $NAD^+$ 0.3 mM, glycine 133 mM, GAPDH 3.9 μg $mL^{-1}$, D-$LH_2$ 8.7 μM, LUC 6 μg $mL^{-1}$ and PGK 0.011 μg $mL^{-1}$.

**Coupled bioluminescent assays**

**Samples' assays.**

**ATP quantitation assay.** ATP was quantified in samples by luminometry through a calibration curve of ATP standard solutions. Briefly, ATP standard solutions ranging from 0 to 50 μM were prepared. To 5.00 μL of either samples or ATP standards, 35.0 μL of $MgCl_2$ 4.2 mM was added in polypropylene transparent test tubes. Ten microliters of LUC 6 μg $mL^{-1}$ was added to the mixtures, the tubes were introduced into the dark chamber and the baseline register by the equipment was turned on, at an integration interval of 0.1 seconds. After 30 seconds, 50 μL of D-$LH_2$ 8.7 μM was injected from the automatic burette. The light output was recorded for more 30 seconds.

**Removal of endogenous ATP.** Endogenous ATP in samples was removed by an enzymatic reaction using ATP sulfurylase, PPase and inorganic sulfate.[49] Eight microliters of $MgCl_2$ 3 mM, 4.00 µL of ATP sulfurylase 20 nM, 8.00 µL of PPase 30 nM and 10.00 µL of $Na_2SO_4$ 500 µM were added to 20.0 µL of samples in a capped polypropylene tube for 5 minutes. Reaction was stopped by transferring the tubes to ice. The tubes were boiled in a water bath using an IMLAB ET Basic Yellow Line immersion thermostat (Boutersem, Belgium) for 2 minutes. Afterwards the tubes were allowed to cool, centrifuged (10,000 *x g*, 15 minutes) in an Eppendorf 5415D workbench centrifuge (Hamburg, Germany) and the supernatants were transferred to novel tubes. The remaing ATP was assayed according to the procedure described in subsection 'ATP quantitation assay' with ATP standards ranging from 0 to 50 nM.

**Samples influence on LUC activity.** The influence of the samples on LUC activity was assayed by luminometry as described in subsection 'Influence of the reagents and enzymes on LUC activity' with the following alterations: 17.5 µL of $MgCl_2$ 4.2 mM and 17.5 µL of ATP 0.2 mM was added to 5.00 µL of either samples or deionized water as control.

## Statistical analysis

*Note.* Data treatment and calculations were performed with a Microsoft® Excel® spreadsheet.

**Preliminary assays.**

**ATP contamination in reagents and enzymes.** Evaluation of the amount of ATP in reagents and enzymes was achieved by calculating, for each of them, the corrected peak of bioluminescence, expressed in relative light units (RLU). This value is obtained by selecting the maximum of the luminogram, corresponding to the moment at which D-$LH_2$ is

injected into the mixture, and subtracting the baseline, defined as the average registered bioluminescence in the first 30 seconds of record (Fig. S6 A). Results are presented as mean ± standard deviation (SD) ($n$ = 3), and percent of light emission compared to the control with ATP, of two independent assays (Table S2).

**Reagents and enzymes influence on LUC activity.** Evaluation of the influence of the reagents and enzymes on LUC activity was achieved by calculating, for each of them, the corrected peak of bioluminescence as defined in subsection 'ATP contamination in reagents and enzymes'. Results are presented as mean ± SD ($n$ = 3), and percent of light emission compared to the control with ATP, of two independent assays. Differences between the values were evaluated by one-way analysis of variance (ANOVA) followed by the Dunnett's multiple-comparison test (Fig. S3).

**Samples's assays.**

**ATP quantitation assay.** Calibration curves were set up by the method of least squares by plotting the values of corrected peak of bioluminescence, as described in subsection 'ATP contamination in reagents and enzymes', as a function of the concentration of the ATP standards (Figure S6 B). Results are presented as ATP concentration ± 95% CL of the concentration, of two independent assays.

**Removal of endogenous ATP.** Assessment of the remaining ATP in samples after enzymatic treatment was achieved as described in subsection 'ATP quantitation assay'.

**Samples influence on LUC activity.** Evaluation of the influence of the samples on LUC activity was achieved as described in subsection 'Reagents and enzymes influence on LUC activity'.

**Table S1** Selected factors and the corresponding levels analyzed in the Plackett-Burman screening design and the Box Behnken optimization design

| Factor | Levels | | |
|---|---|---|---|
| | low | central | high |
| **Plackett-Burman Design** | | | |
| Pi concentration / mM | 9 | 50 | 90 |
| G3P concentration / mM | 0.15 | 0.83 | 1.5 |
| $NAD^+$ concentration / mM | 0.05 | 0.3 | 0.50 |
| ADP concentration / mM | 0.036 | 0.20 | 0.36 |
| $MgCl_2$ concentration / mM | 0.76 | 4.2 | 7.6 |
| Glycine concentration / mM | 24 | 133 | 240 |
| GAPDH concentration / μg $mL^{-1}$ | 0.71 | 3.9 | 7.1 |
| D-$LH_2$ concentration / μM | 1.58 | 8.7 | 15.8 |
| LUC concentration / μg $mL^{-1}$ | 1 | 6 | 11 |
| PGK concentration / μg $mL^{-1}$ | 0.0020 | 0.011 | 0.020 |
| Preincubation time / minutes | 0 | 5 | 15 |
| **Box Behnken Design** | | | |
| Pi concentration / mM | 9 | 50 | 90 |
| G3P concentration / mM | 0.15 | 0.83 | 1.5 |
| ADP concentration / mM | 0.036 | 0.20 | 0.36 |
| $MgCl_2$ concentration / mM | 0.76 | 4.2 | 7.6 |
| Preincubation time / minutes | 0 | 5 | 15 |

**Table S2** Evaluation of the ATP contamination in the reagents and enzymes used in the coupled bioluminescent assay

| **Reagent or enzyme** | **Bioluminescence / RLU**[a] | **% of the control (ATP)** |
|---|---|---|
| ATP | 1,062,116 ± 51,808 | 100 |
| D-$LH_2$ without LUC | 12 ± 2 | 0.001 |
| D-$LH_2$ | 13 ± 2 | 0.001 |
| DEA-NONOate | 694 ± 42 | 0.07 |
| Pi | 21 ± 2 | 0.002 |
| G3P | 1,305 ± 34 | 0.12 |
| $NAD^+$ | 81 ± 9 | 0.01 |
| ADP | 7,522 ± 134 | 0.71 |
| $MgCl_2$ | 387 ± 24 | 0.04 |
| Glycine | 98 ± 8 | 0.01 |
| GAPDH | 85 ± 4 | 0.008 |
| PGK | 76 ± 3 | 0.01 |

[a] Values are expressed as mean ± SD ($n$ = 3).

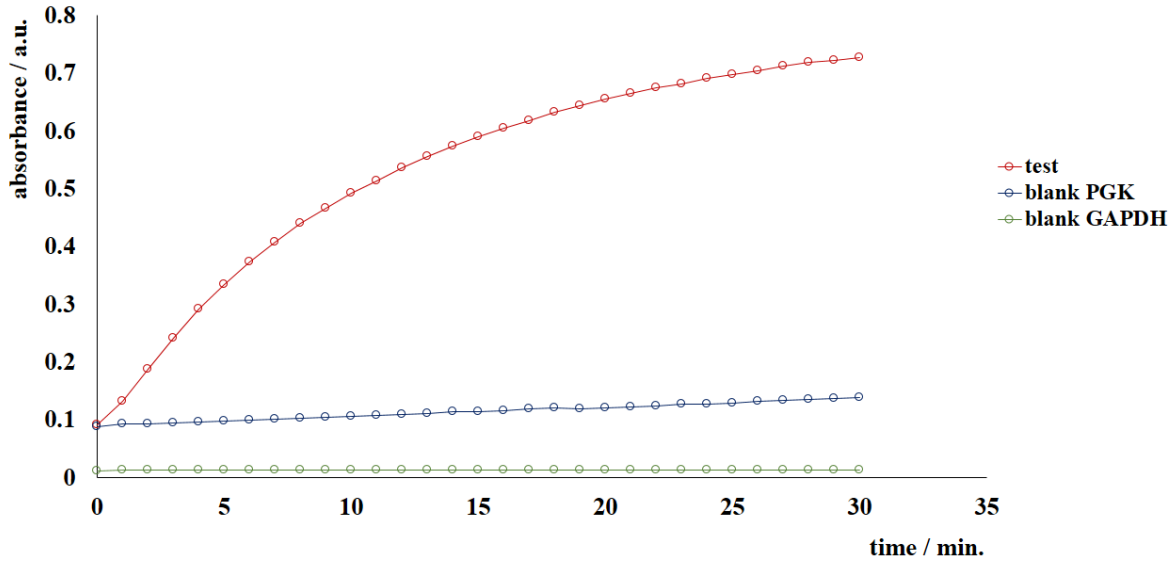


**Fig. S1** UV-vis changes in absorbance along time for GAPDH and PGK coupled reactions generating NADH + $H^+$. a.u., arbitrary units.

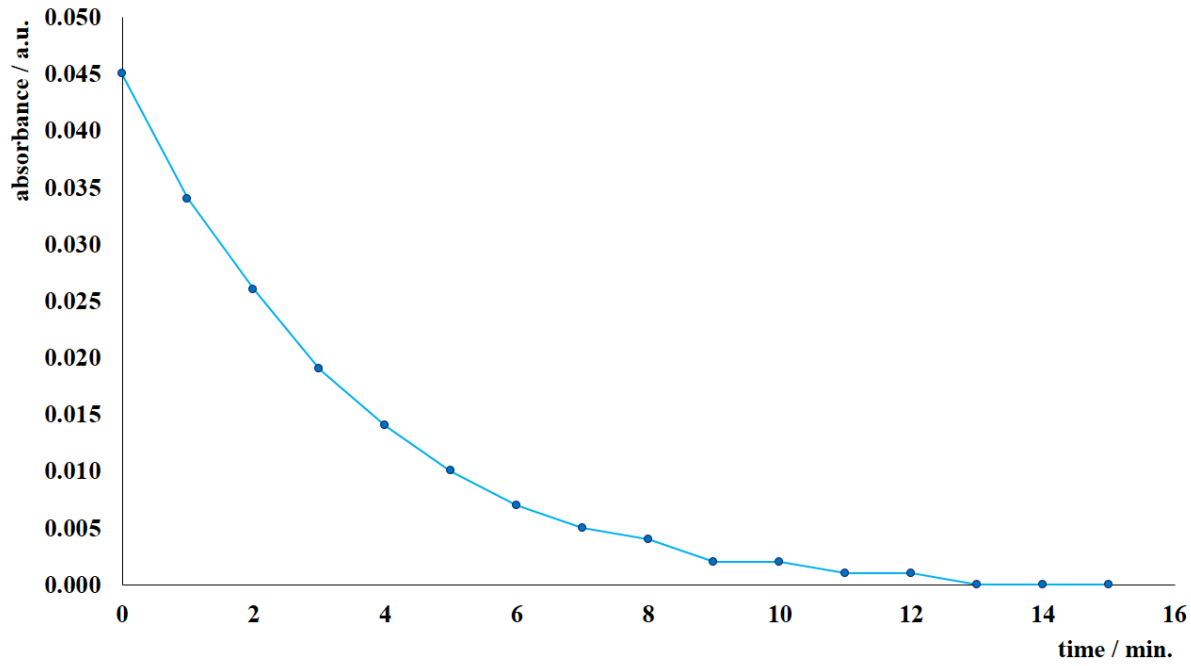


**Fig. S2** UV-vis changes in absorbance along time for the decomposition of DEA-NONOate. a.u., arbitrary units.

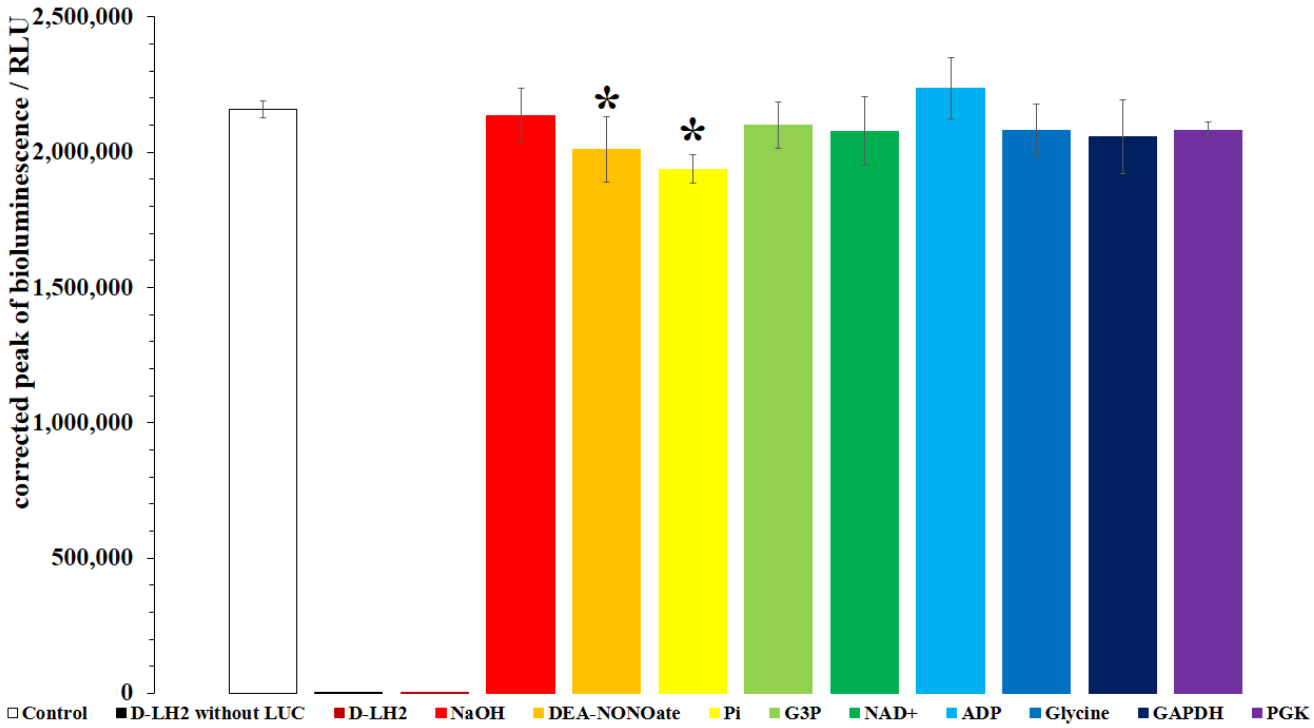


**Fig. S3** Evaluation of reagents and enzymes influence on LUC activity. Control was made in the absence of any reagent or enzyme. Asterisks indicate statistically significant difference as compared with the control ($p < 0.05$). RLU, relative light units.

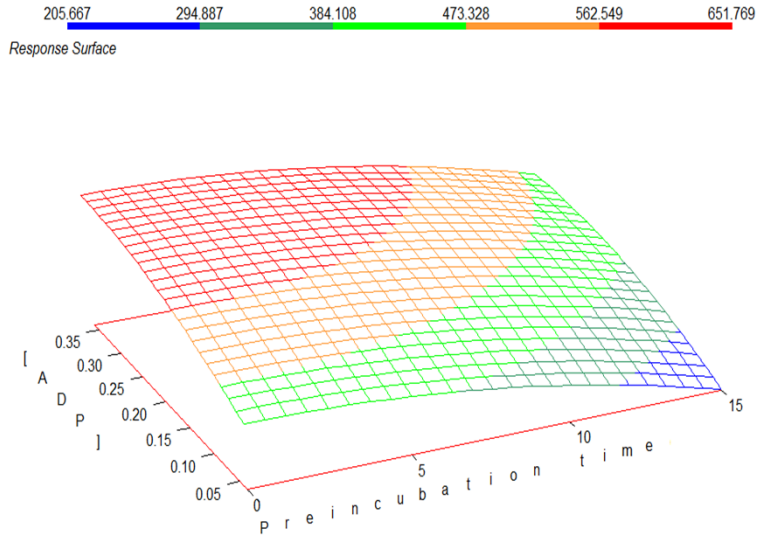


**Fig. S4** Response surface landscape plot for the most important method's factors, the concentration of ADP ([ADP]) and the preincubation time, tested in a Box Behnken design.

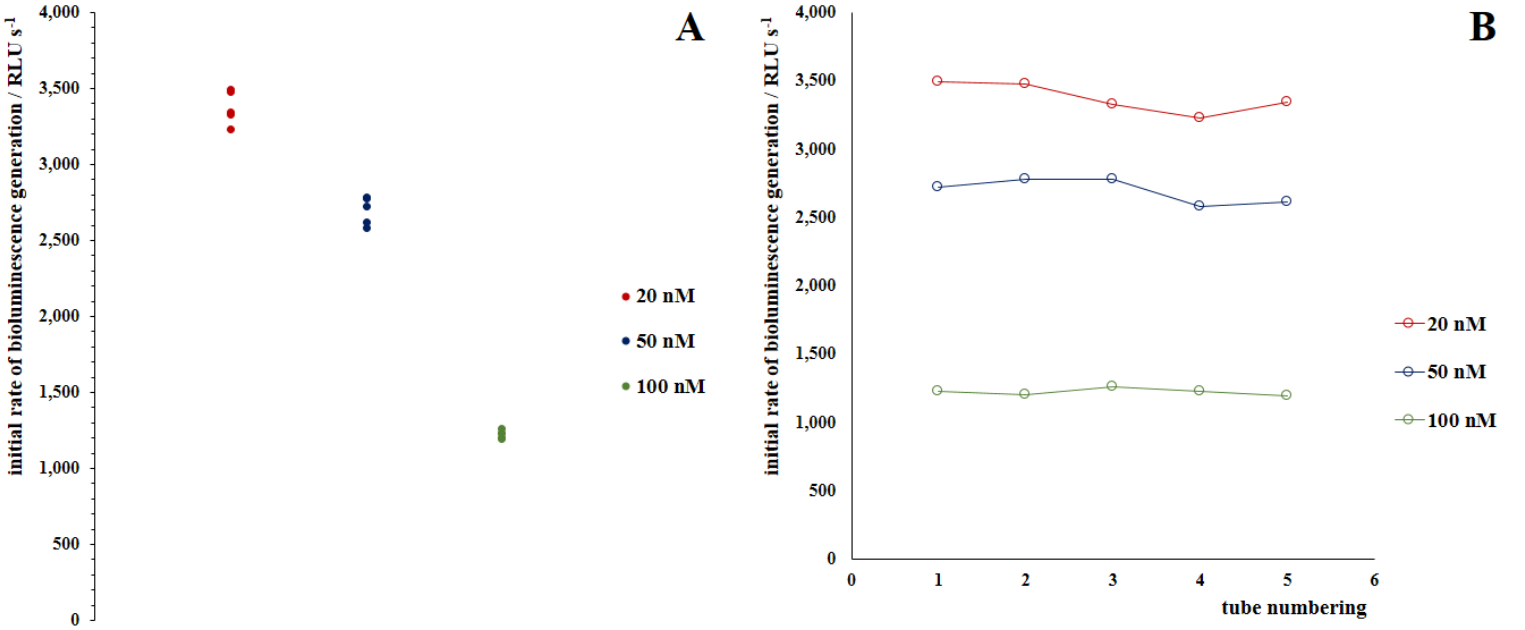


**Fig. S5** Evaluation of the optimized coupled bioluminescent assay repeatability. (A) Presentation of the experimental values by each •NO concentration tested. (B) Presentation of the experimental values by individual tubes within each concentration. RLU, relative light units.

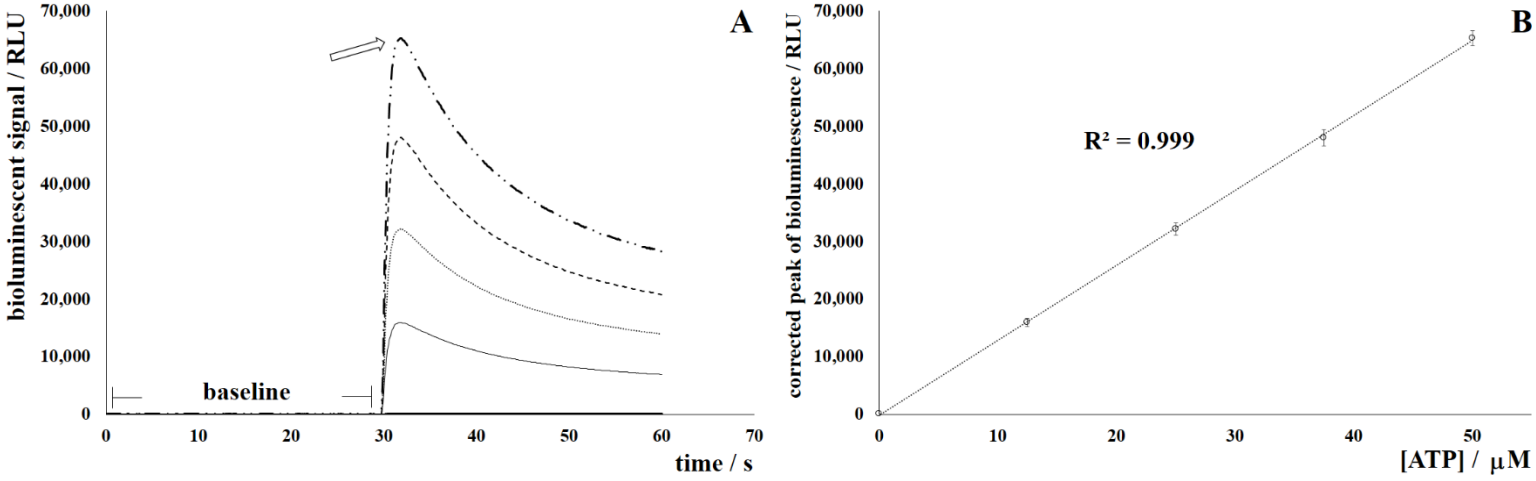


**Fig. S6** Bioluminescent ATP quantitation assay. (A) Typical luminogram and (B) the corresponding ATP calibration curve. The arrow in (A) indicates the peak of bioluminescence due to D-$LH_2$ injection. RLU, relative light units.