Supplementary Information

PeroxyHUB: a modular, cell-free biosensing platform using H_2O_2 as signal integrator.

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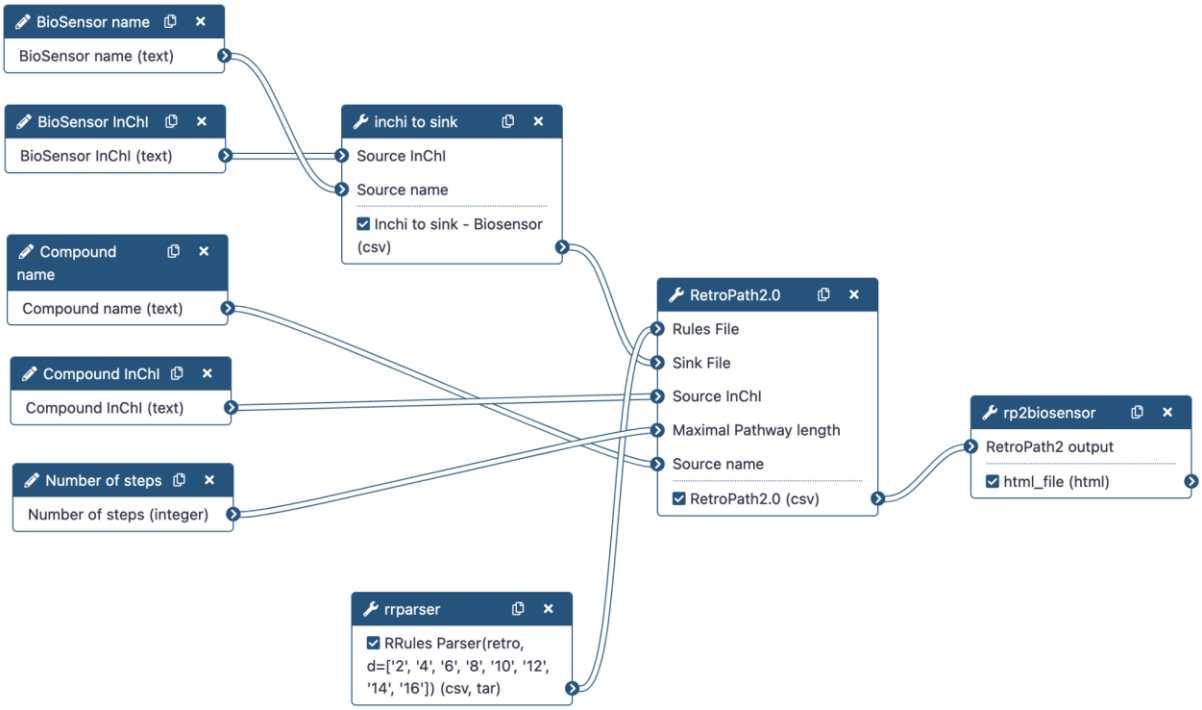
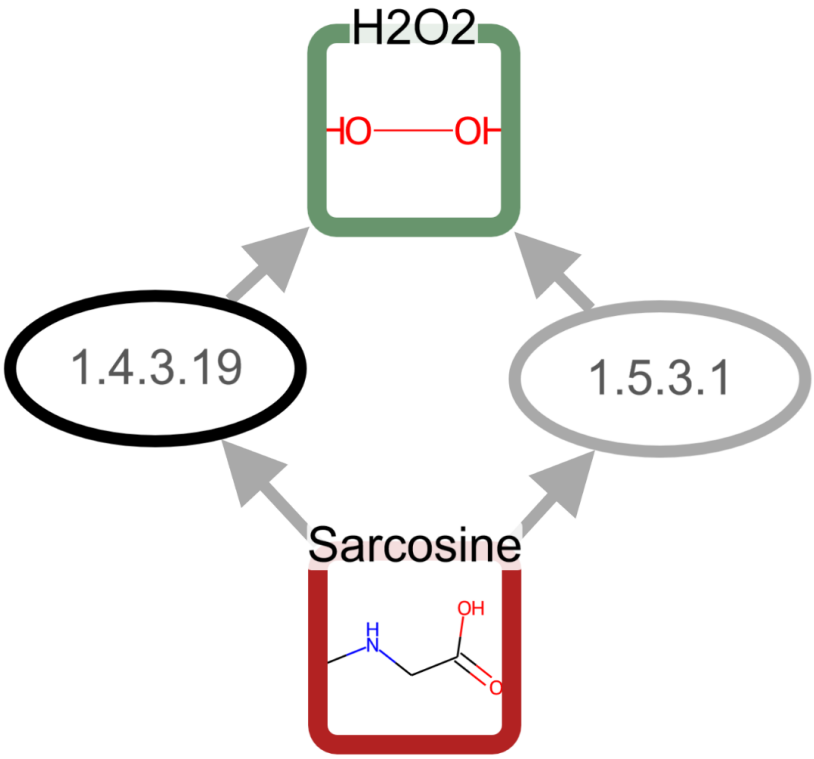
Fig S1:



Supplementary Figure S1: Complete view of the BioSensor Galaxy workflow automatizing biosensor predictions. Characteristics of the main nodes are described in the method section.

Fig S2:



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Reaction template ID(s)
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Enzyme selection using Selenzyme
Crosslink to Selenzyme

Rule score
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Reaction SMILES
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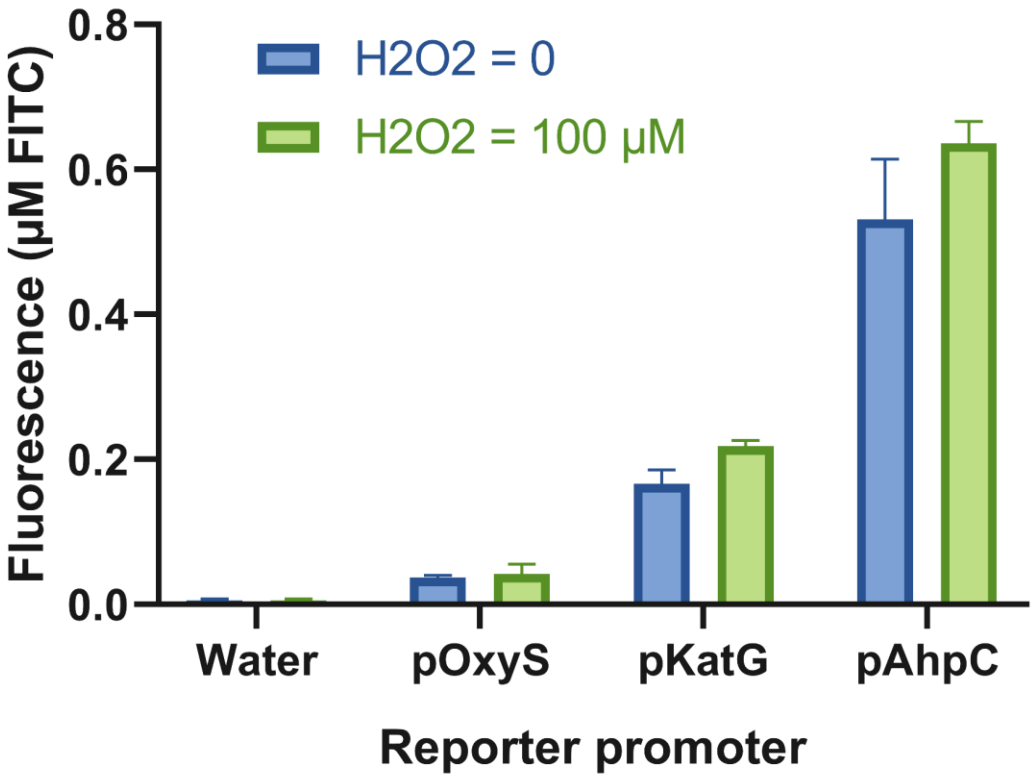
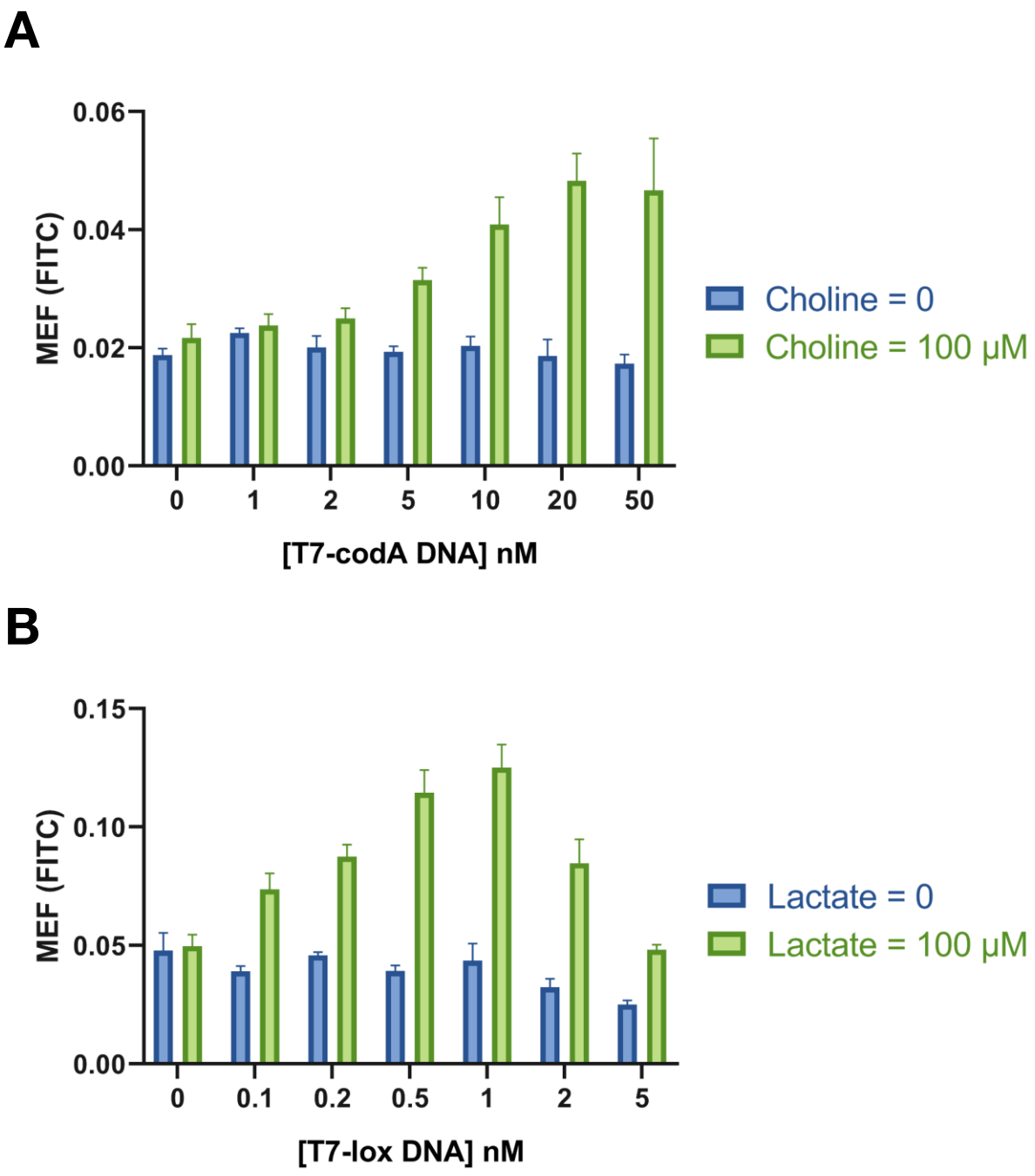
Supplementary Figure S2: Example of result web page output from the Biosensor workflow for sarcosine.

Fig S3:



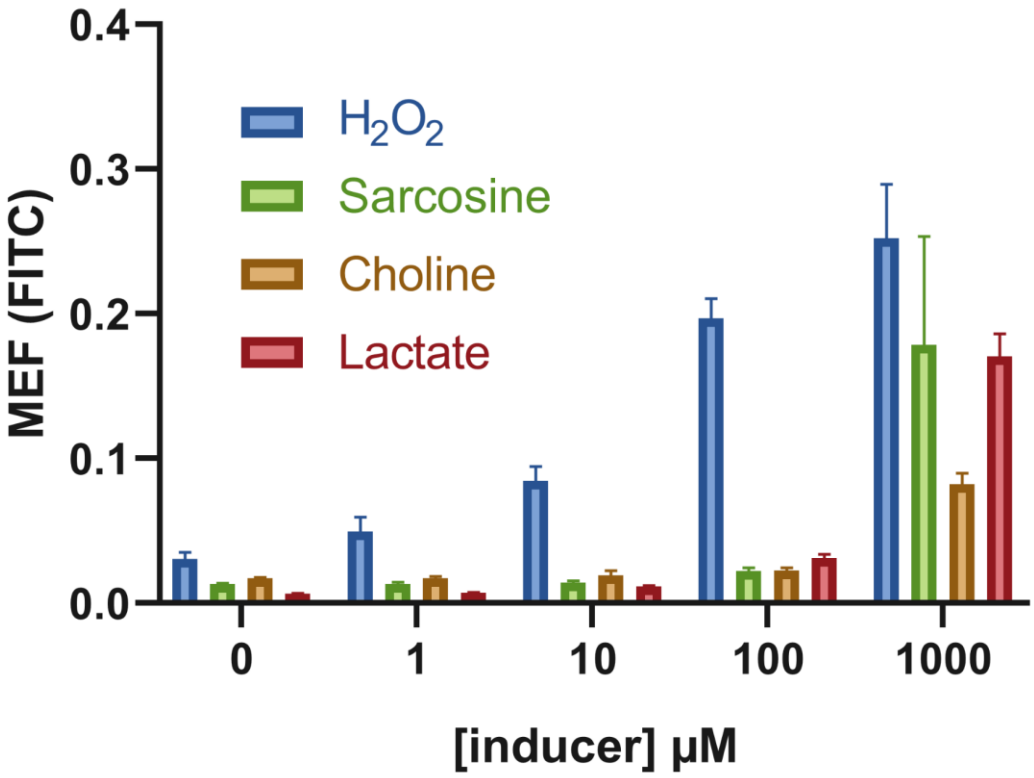
Supplementary Figure S3: Unoptimized H_2O_2 sensor response in cell-free system. Direct cell-free implementation of biosensor candidates derived from *in vivo* design did not show significant response to H_2O_2. 16.5µL of Cell-free mix was supplemented with 1.1µL of TF and Reporter plasmid each, 1.1µL water and 2.2µL of either water or H_2O_2. The final concentrations were of 10 nM for each plasmid added and 100µM for the H_2O_2 inducer. Fluorescence values are MEF measured at an endpoint of 8h

Fig S4:



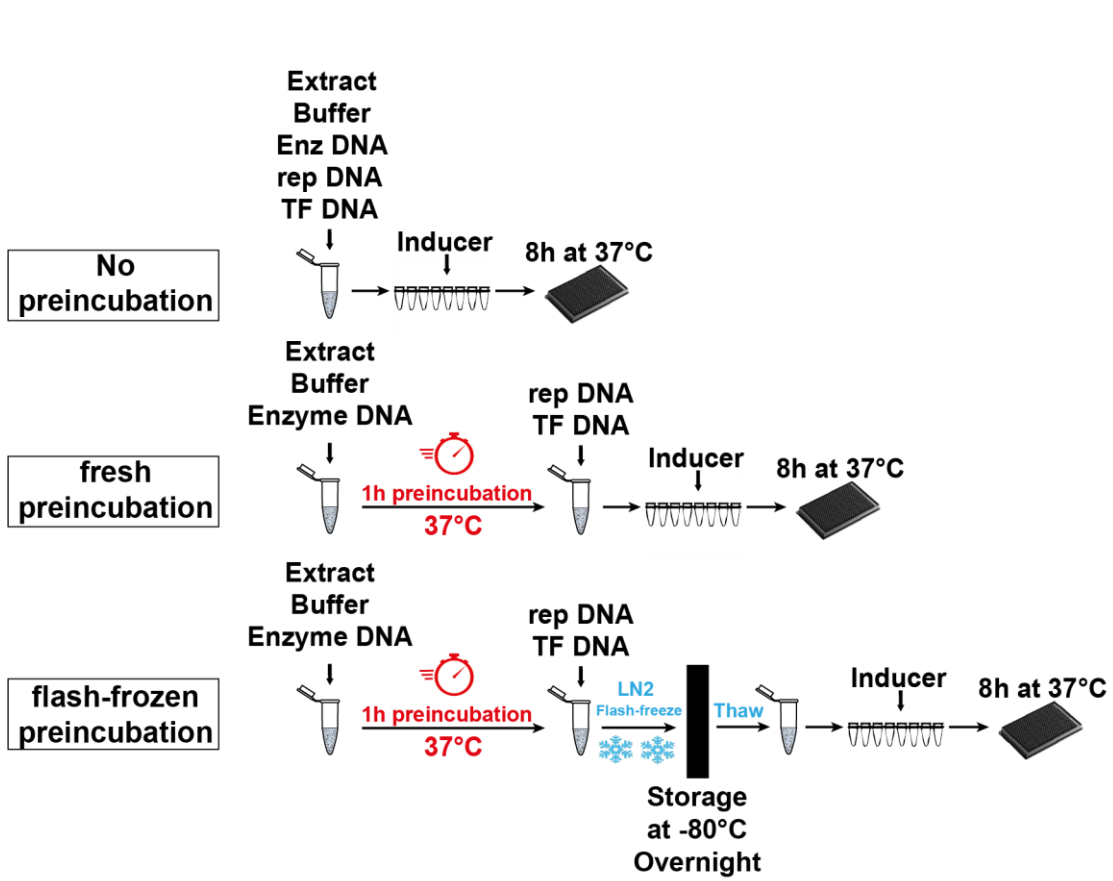
Supplementary Figure S4: Fine tuning of enzyme expression using DNA gradient (A) [T7-codA DNA] optimisation for choline sensing **(B)** [T7-lox DNA] optimisation for lactate sensing

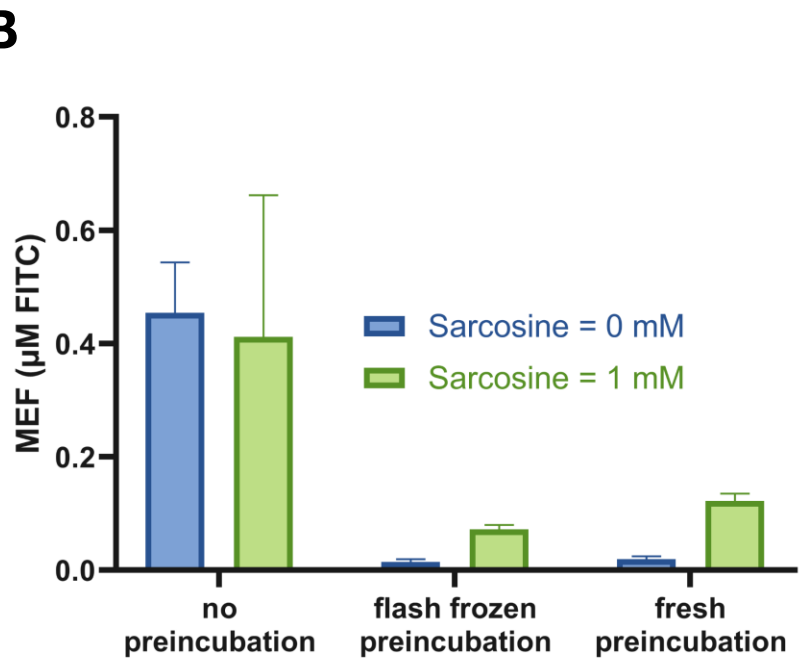
Fig S5:



Supplementary Figure S5: Final sensors fluorescent dose response

Fig S6:

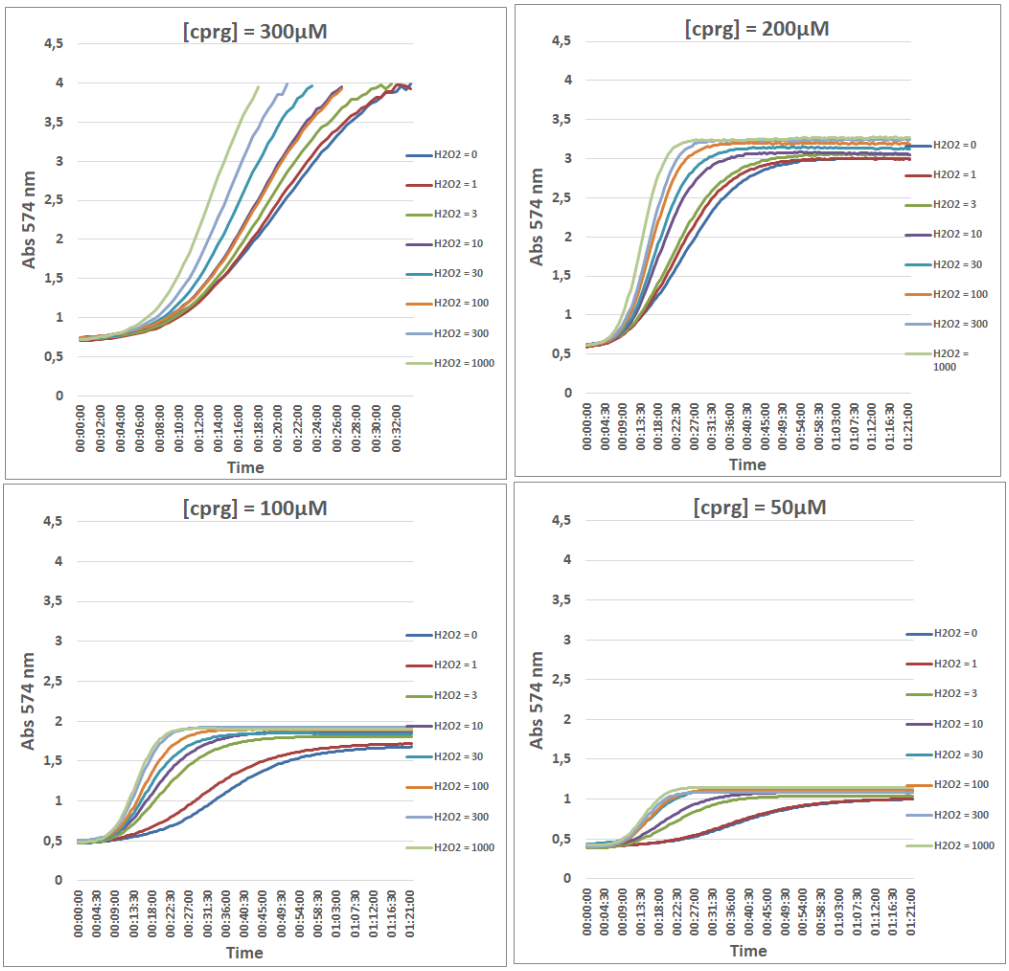




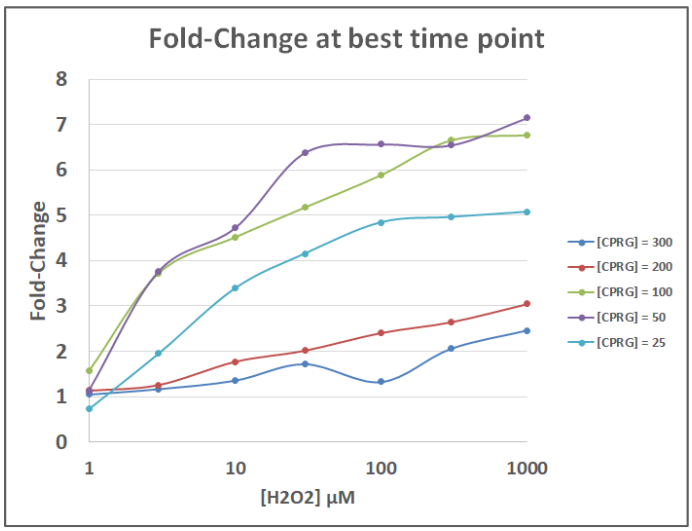
Supplementary Figure S6: Liquid Nitrogen Flash-Freezing of preincubated mix (A) Experimental workflows detailing the no preincubation, the fresh preincubation and the flash frozen preincubation pipelines used for biosensors evaluation **(B)** 2h30 endpoint fluorescence taken in presence and absence of inducer for each pipeline tested reveals few differences of responses between the fresh and the flash frozen preincubation workflows.

Fig S7:

A

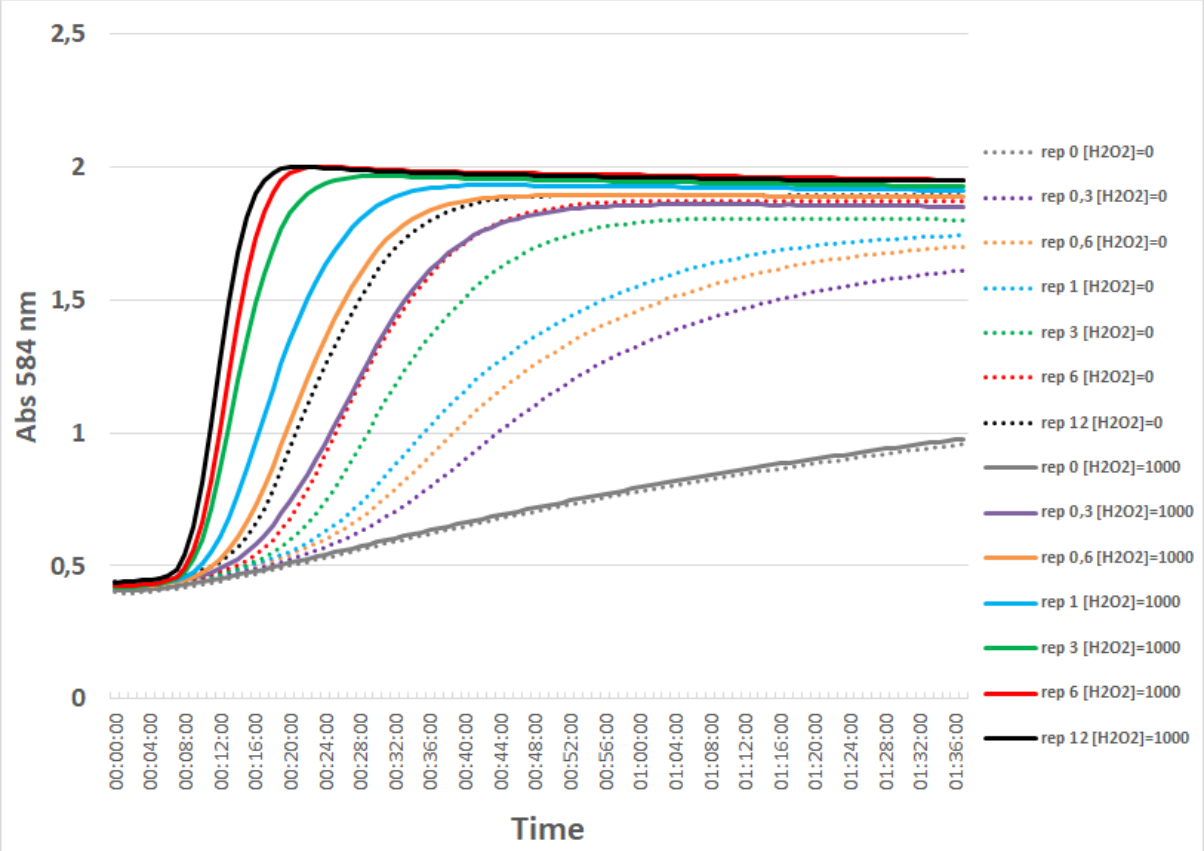


B



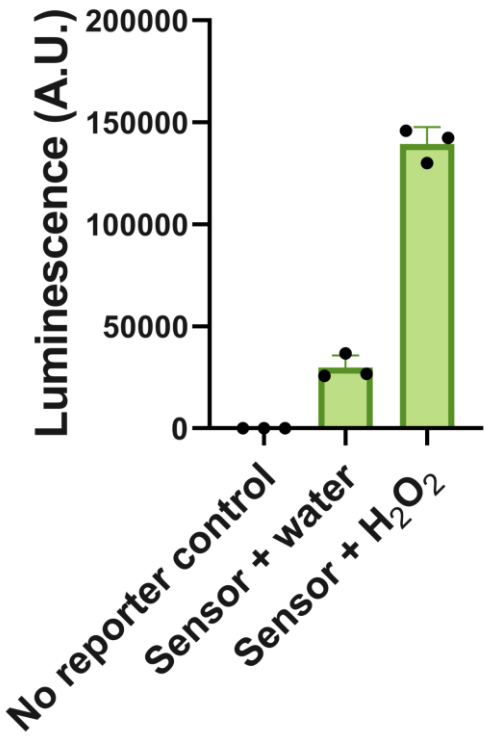
Supplementary Figure S7: CPRG concentration optimization for colorimetric H_2O_2 biosensor (A) 574 nm absorbance time course kinetic of colorimetric H_2O_2 biosensor with various initial concentration of CPRG **(B)** Blank subtracted H_2O_2 dose response curve of colorimetric biosensor with various initial concentration of CPRG

Fig S8:



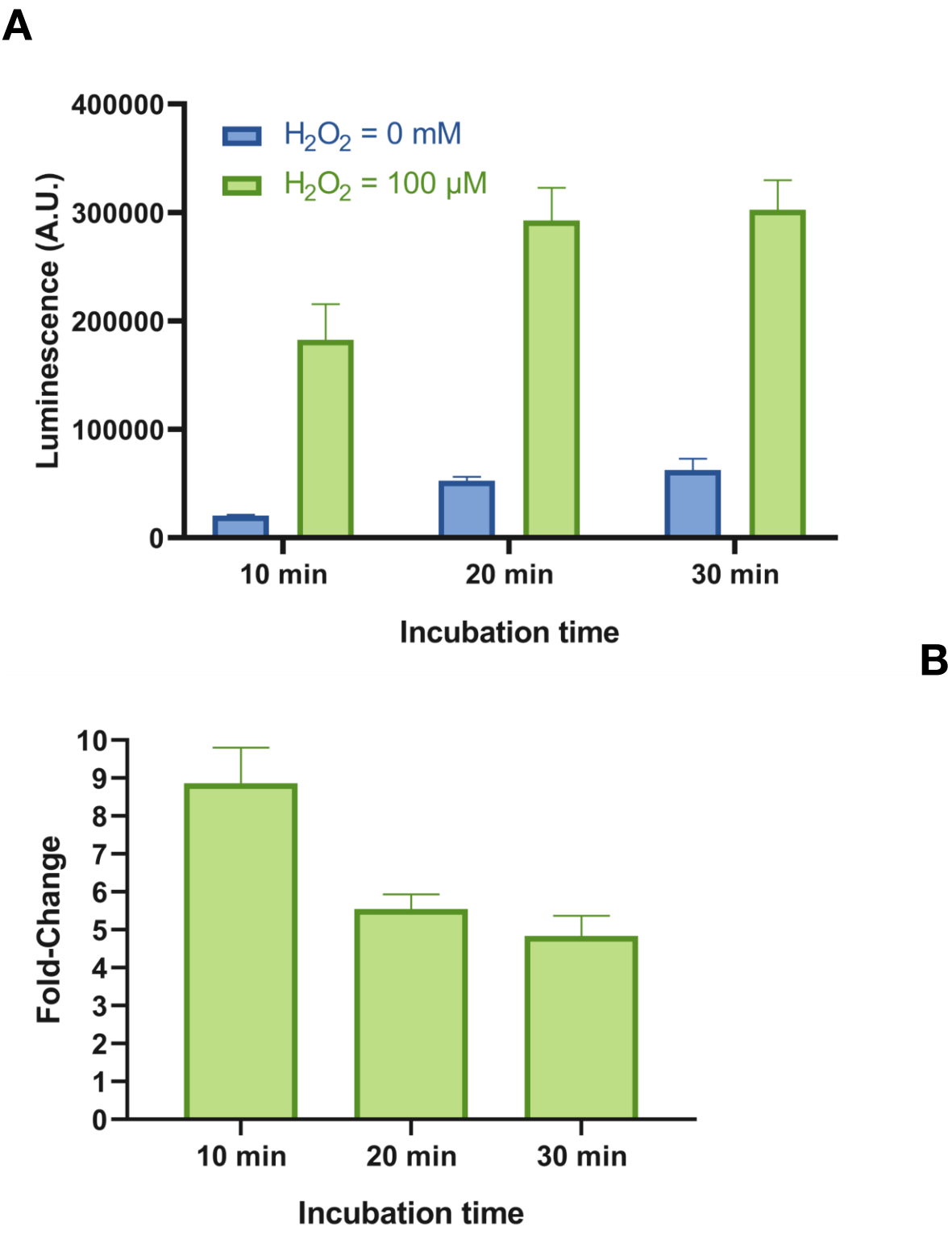
Supplementary Figure S8: [pAhpC-LacZ DNA] concentration optimization for colorimetric H_2O_2 biosensor

Fig S9:



Supplementary Figure S9: Luminescent sensor early evaluation. [pAhpC-Luc DNA] was added at 0 or 6nM and [j23101-OxyR DNA] at 24 nM to 1h preincubated cell-free mix together with either water or 1mM H_2O_2. The reaction was incubated for 30 min at 37°C and 20µL of final reaction was added to 50 µL of Luciferin reagent mix before taking a luminescence point measurement. Dots represent individual values obtained.Error bars represent the standard deviation calculated from the 3 replicates.

Fig S10:



Supplementary Figure S10: Luminescent sensor incubation time optimisation (A) Luminescence absolute values measured in arbitrary units **(B)** Luminescence Fold-Change between induced and non induced condition

Supplementary Table S1

Chemicals identifiers used in the study

Compound	**InChI**
S-lactate	InChI=1S/C3H6O3/c1-2(4)3(5)6/h2,4H,1H3,(H,5,6)/p-1/t2-/m0/s1
choline	InChI=1S/C5H14NO/c1-6(2,3)4-5-7/h7H,4-5H2,1-3H3/q+1
sarcosine	InChI=1S/C3H7NO2/c1-4-2-3(5)6/h4H,2H2,1H3,(H,5,6)

Supplementary Table S2
Characteristics of enzymes used in the study

substrate	**name**	**uniprot ID**	**organism**	**kinetic**		**catalytic efficiency**
				Km	Kcat	Kcat/Km
sarcosine	SoxA	P40859	*Bacillus subtilis*	141,6 mM source	0.204 s-1 source	1.441 s-1.M-1 source
choline	CodA	Q7X2H8	*Arthrobacter globiformis*	0,6 mM source	13.4 s-1 source	22000 s-1.M-1 source
lactate	lox	D4YFM2	*Aerococcus viridans*	0,5 mM source	88 s-1 source	176000 s-1.M-1 source

Supplementary Table S3
Plasmids used in this study:

Plasmid	Antibiotic Selection	Accession/ Catalog reference	Source
pBeast-J23101-RBS-OxyR	Amp^R		This work
pBeast-pAhpC-RBS-sfGFP	Amp^R		This work
pBeast-pOxyS-RBS-sfGFP	Amp^R		This work
pBeast-pKatG-RBS-sfGFP	Amp^R		This work
pBeast-pYjjZ-RBS-sfGFP	Amp^R		This work
pBeast-pZint-sfGFP	Amp^R		This work
pBeast-J23101-RBS-soxA	Amp^R		This work
pBeast-pT7-soxA	Amp^R		This work
pBeast-pT7-codA	Amp^R		This work
pBeast-pT7-lox	Amp^R		This work
pBeast-pAhpC-RBS-LacZ	Amp^R		This work
pBeast-pAhpC-RBS-Luc	Amp^R		This work

Supplementary Table S4
DNA sequences for constructs used in this study:

Plasmid/ DNA construct	Sequence (5'->3')
pBeast-J23101-RBS-OxyR	ATACTAGAGGATGACCCCATCTGTTTACAGCTAGCTCAGTCCTAG GTATTATGCTAGCTAGTAGAGTCACACAGGAAAGTAGTAGATGAAT ATTCGTGATCTTGAGTACCTGGTGGCATTGGCTGAACACCGCCAT TTTCGGCGTGCGGCAGATTCCTGCCACGTTAGCCAGCCGACGCTT AGCGGGCAAATTCGTAAGCTGGAAGATGAGCTGGGCGTGATGTT GCTGGAGCGGACCAGCCGTAAAGTGTTGTTCACCCAGGCGGGAA TGCTGCTGGTGGATCAGGCGCGTACCGTGCTGCGTGAGGTGAAA GTCCTTAAAGAGATGGCAAGCCAGCAGGGCGAAACCATGTCCGG ACCGCTGCACATTGGTTTGATTCCCACAGTTGGACCGTACCTGCT ACCGCATATTATCCCTATGCTGCACCAGACCTTTCCAAAGCTGGAA ATGTATCTGCATGAAGCACAGACCCACCAGTTACTGGCGCAACTG GACAGCGGCAAACTCGATTGCGTGATCCTCGCGCTGGTGAAAGA GAGCGAAGCATTCATTGAAGTGCCGTTGTTTGATGAGCCAATGTT GCTGGCTATCTATGAAGATCACCCGTGGGCGAACCGCGAATGCGT ACCGATGGCCGATCTGGCAGGGGAAAAACTGCTGATGCTGGAAG ATGGTCACTGTTTGCGCGATCAGGCAATGGGTTTCTGTTTTGAAG CCGGGGCGGATGAAGATACACACTTCCGCGCGACCAGCCTGGAA ACTCTTCGCAACATGGTGGCGGCAGGTAGCGGGATCACTTTACTG CCAGCGCTGGCTGTGCCGCCGGAGCGCAAACGCGATGGGGTTGT TTATCTGCCGTGCATTAAGCCGGAACCACGCCGCACTATTGGCCT GGTTTATCGTCCTGGCTCACCGCTGCGCAGCCGCTATGAGCAGCT GGCGGAAGCCATCCGCGCAAGAATGGATGGCCATTTCGATAAAGT TTTAAAACAGGCGGTTTAAactttatctgagaatagtcaatcttcggaaatcccaggtg gcatgctaaaagtctcgtaaagcgttctatcaataacccgttggtgccaggcatcaaataaaacga aaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctctactagagtc acactggctcaccttcgggtgggcctttctgcgtttataccgtctcagaatcggccgtgaacaataaa atagtttcggtattattgaccacttccgagtagaatcgtgcttcagtaagagtcgaccgatgcccttga gagccttcaacccagtcagctccttccggtgggcgcggggcaTGACTATCGTCGCCG CACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACAGGTGCC GGCAGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCG GTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGT AATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACAT GTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATC ACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGA CTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGC TCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTT CTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGG TATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTG CACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACT GGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAG GCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACA CTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTA CCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCA CCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGC

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