Supporting Information

An *E. coli* Cell-free Expression Toolbox: Application to Synthetic Gene Circuits and Artificial Cells

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Content:

Promoter	Description	Source - references
P_{70}	Lambda phage promoter OR_2-OR_1-Pr specific to *E. coli* σ^{70}	GenBank: J02459.1, (*1*)
$P_{70\text{-mut}}$	Same as P_{70} with OR_2 and OR_1 mutated (no repression)	This work
P_{19}	Promoter of the *fec* transport genes (*E. coli*) specific to σ^{19}	GenBank: U00096.2, (*2*)
P_{24}	Promoter of the *htrA* gene (*E. coli*) specific to σ^{24}	GenBank: U00096.2, (*3*)
P_{28}	Promoter of the *tar* gene (*E. coli*) specific to σ^{28}	GenBank: U00096.2, (*4*)
P_{32}	Promoter of the *groE* gene (*E. coli*) specific to σ^{32}	GenBank: U00096.2, (*5*)
P_{38}	Promoter of the *osmY* gene (*E. coli*) specific to σ^{38}	GenBank: U00096.2, (*6*)
P_{54}	Promoter of the *glna* gene (*E. coli*) specific to σ^{54}	GenBank: U00096.2, (*7*)
P_{T3}	Promoter of bacteriophage T3	Genbank: NC_003298
P_{T7}	Promoter of bacteriophage T7	Genbank: NC_001604
araBAD	Promoter specific to *E. coli* σ^{70} and regulated by the *araC* gene product	(*8*)
$P_{L\text{-}lacO1}$	Promoter specific to *E. coli* σ^{70} and regulated by the *lacI* gene product	(*9*)
$P_{L\text{-}tetO1}$	Promoter specific to *E. coli* σ^{70} and regulated by the *tetR* gene product	(*9*)

Untranslated region	Description	Source – references
UTR1	The untranslated region containing the T7 g10 leader sequence for highly efficient translation initiation	GenBank: M35614.1, (*1, 10*)

Transcription terminator	Description	Source – references
T500	Transcription terminator for *E. coli* RNA polymerase	(*11*)

Genes	Description	Source – references
deGFP	The enhanced green fluorescent protein truncated and modified in N- and C- terminal	(*1*)
deCFP	The enhanced cyan fluorescent protein truncated and modified in N- and C- terminal	(*1*)
σ^{19}	*fecI* (*E. coli* σ^{19})	GenBank: U00096.2
σ^{19}-*ssrA*	*fecI* tagged with *ssrA*	This work
σ^{19}-*ybaQ*	*fecI* tagged with *ybaQ*	This work
σ^{24}	*rpoE* (*E. coli* σ^{24})	GenBank: U00096.2

ompA-σ^{24}	*rpoE* tagged with *ompA*	This work
σ^{28}	*rpoF* (*E. coli* σ^{28})	GenBank: U00096.2
σ^{28}-ssrA	*rpoF* tagged with *ssrA*	This work
σ^{28}-ybaQ	*rpoF* tagged with *ybaQ*	This work
σ^{32}	*rpoH* (*E. coli* σ^{32})	GenBank: U00096.2
σ^{32}-ssrA	*rpoH* tagged with *ssrA*	This work
σ^{38}	*rpoS* (*E. coli* σ^{38})	GenBank: U00096.2
ompA-σ^{38}	*rpoS* tagged with *ompA*	This work
σ^{54}	*rpoN* (*E. coli* σ^{54})	GenBank: U00096.2
ntrC	Co-activator of σ^{54} promoters	GenBank: U00096.2
ntrB	Sensory histidine kinase in two-component regulatory system	GenBank: AAC76866.1
T7 RNAP	T7 bacteriophage RNA polymerase	GenBank: FJ881694.1
T3 RNAP	T3 bacteriophage RNA polymerase	GenBank: X02981.1
araC	Arabinose operon regulatory gene	GenBank: AAC73175.1
lacI	Lac operon regulatory gene	GenBank: AAC73448.1
tetR	Tet operon regulatory gene	GenBank: BAG71042.1
αHemolysin-eGFP	Fusion of the pore forming encoding gene *αHemolysin* and *eGFP*	(*12*)
cI	Lambda phage repressor protein CI	GenBank: CAB96428.1
ssrA	SsrA peptide for degradation by AAA+ proteases	(*13*)
ompA	OmpA peptide for degradation by AAA+ proteases	(*13*)
ybaQ	YbaQ peptide for degradation by AAA+ proteases	(*13*)

Table S1. DNA parts used in this work. Except for the pBAD plasmid (araBAD promoter), all the plasmids were constructed from the pBEST-*Luc* plasmid (Promega), with the UTR1 untranslated region.

deGFP [μM]		Transcription factor expressed							
		(σ^{70})*	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P_{70}	1	0.92	0.85	0.62	0.74	0.94	0.95	0.90
	P_{19}	< 0.01	1	0.01	0.02	< 0.01	0.01	< 0.01	< 0.01
	P_{24}	0.01	0.04	1	< 0.01	0.01	0.04	0.01	0.01
	P_{28}	< 0.01	< 0.01	< 0.01	1	< 0.01	< 0.01	< 0.01	< 0.01
	P_{32}	0.04	0.04	0.04	0.02	1	0.04	0.04	0.04
	P_{38}	< 0.01	0.16	0.04	0.01	< 0.01	1	0.01	< 0.01
	P_{T3}	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	1	< 0.01
	P_{T7}	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	1
Rate of deGFP synthesis [nM/min]		Transcription factor expressed							
		(σ^{70})*	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P_{70}	1	0.98	1.07	0.84	0.97	1.13	0.97	0.96
	P_{19}	< 0.01	1	0.01	< 0.01	< 0.01	0.01	< 0.01	< 0.01
	P_{24}	< 0.01	< 0.01	1	< 0.01	< 0.01	0.03	0.01	0.01
	P_{28}	< 0.01	< 0.01	< 0.01	1	< 0.01	< 0.01	< 0.01	< 0.01
	P_{32}	0.02	0.02	0.03	0.02	1	0.3	0.03	0.03
	P_{38}	< 0.01	0.05	0.01	< 0.01	< 0.01	1	< 0.01	< 0.01
	P_{T3}	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	1	< 0.01
	P_{T7}	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	1

*: endogenous sigma factor. All the others are expressed using the endogenous sigma factor 70 (Figure 1a, Supplementary Figure S1).

Table S2. Scaled values of the crosstalk between transcriptional activation units measured in the linear regime of plasmid concentration (0.1 nM of plasmid encoding the sigma factor and 1 nM reporter plasmid, σ^{70} salt conditions, see Table 1). Scaled values of the end-point deGFP productions (upper table) and the maximum deGFP synthesis rates (lower table) for six *E. coli* sigma factors (non-degradable versions), and for the T7 and the T3 RNA polymerases, were measured with respect to each of the specific promoters (shaded in grey) and against each of the other non-specific promoters. This table was obtained from Table 2 by dividing each row by the specific value for each unit. Except for the endogenous σ^{70} present in the reaction, the transcription factors were expressed as shown in the Figure 1a. Expression through the promoter P_{70} is high in all the cases because the endogenous σ^{70} is present in the extract.

deGFP [μM]		Transcription factor expressed							
		(σ^{70})*	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P_{70}	13.75	12.91	11.47	5.68	5.91	12.24	13.74	11.92
	P_{19}	0.26	3.72	0.36	0.26	0.29	0.35	0.28	0.29
	P_{24}	0.26	0.32	5.53	0.26	0.29	0.36	0.29	0.32
	P_{28}	0.24	0.28	0.31	16.73	0.25	0.28	0.24	0.25
	P_{32}	0.75	0.69	0.80	0.45	16.03	0.83	0.90	0.84
	P_{38}	0.27	0.63	0.43	0.26	0.27	4.08	0.27	0.29
	P_{T3}	0.24	0.32	0.50	0.27	0.45	0.46	14.68	0.36
	P_{T7}	0.25	0.31	0.50	0.27	0.34	0.32	0.32	17.04
Rate of deGFP synthesis [nM/min]		Transcription factor expressed							
		(σ^{70})*	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P_{70}	59.61	59.54	56.37	40.12	49.43	57.85	49.73	36.16
	P_{19}	0.23	14.92	0.49	0.15	0.21	0.48	0.26	0.16
	P_{24}	0.26	0.21	16.47	0.09	0.19	0.24	0.32	0.15
	P_{28}	< 0.01	0.08	0.15	71.553	0.07	0.12	0.05	0.03
	P_{32}	2.21	1.64	2.70	1.21	64.74	2.93	2.35	2.40
	P_{38}	0.28	1.50	0.91	0.16	0.25	14.80	0.27	< 0.01
	P_{T3}	0.49	0.50	2.38	0.41	0.24	0.96	71.16	1.20
	P_{T7}	0.31	0.39	1.28	0.13	0.62	0.62	0.83	82.84

*: endogenous sigma factor. All the others are expressed using the endogenous sigma factor 70 (Figure 1a, Supplementary Figure S1).

Table S3. Crosstalk between transcriptional activation units measured in the saturation regime of plasmid concentration (0.5 nM of plasmid encoding the sigma factor and 5 nM of plasmid encoding the reporter protein). The end-point deGFP productions (upper table) and the maximum deGFP synthesis rates (lower table) with the six *E. coli* sigma factors (non-degradable versions), and with the T7 and T3 RNA polymerases, were measured with respect to each of the specific promoters (shaded in grey) and against each of the other non-specific promoters. Except for the endogenous σ^{70} present in the reaction, the transcription factors were expressed as shown in Figure 1A. The magnesium and potassium glutamate concentrations were fixed to 3 mM and 60 mM respectively (optimal conditions for σ^{70}, Table 1). Expression through the promoter P_{70} is high in all the cases because the endogenous σ^{70} is present in the extract.

deGFP [μM]		Transcription factor expressed							
		(σ^{70})*	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P_{70}	1	0.94	0.83	0.41	0.43	0.89	1	0.87
	P_{19}	0.07	1	0.10	0.07	0.08	0.09	0.07	0.08
	P_{24}	0.05	0.06	1	0.05	0.05	0.06	0.05	0.06
	P_{28}	0.01	0.02	0.02	1	0.01	0.02	0.01	0.01
	P_{32}	0.05	0.04	0.05	0.03	1	0.05	0.06	0.05
	P_{38}	0.06	0.15	0.10	0.06	0.07	1	0.07	0.07
	P_{T3}	0.02	0.02	0.03	0.02	0.03	0.03	1	0.02
	P_{T7}	0.01	0.02	0.03	0.02	0.02	0.02	0.02	1
Rate of deGFP synthesis [nM/min]		Transcription factor expressed							
		(σ^{70})*	σ^{19}	σ^{24}	σ^{28}	σ^{32}	σ^{38}	T3RNAP	T7RNAP
Promoters	P_{70}	1	1	0.95	0.67	0.83	0.97	0.83	0.6
	P_{19}	0.02	1	0.03	0.01	0.01	0.03	0.02	0.01
	P_{24}	0.02	0.01	1	< 0.01	0.01	0.01	0.02	< 0.01
	P_{28}	< 0.01	< 0.01	< 0.01	1	< 0.01	< 0.01	< 0.01	< 0.01
	P_{32}	0.03	0.03	0.04	0.02	1	0.05	0.04	0.04
	P_{38}	0.02	0.1	0.06	0.01	0.02	1	0.02	< 0.01
	P_{T3}	< 0.01	< 0.01	0.03	< 0.01	< 0.01	0.01	1	0.02
	P_{T7}	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01	0.01	1

*: endogenous sigma factor. All the others are expressed using the endogenous sigma factor 70 (Figure 1a, Supplementary Figure S1).

Table S4. Scaled values of the crosstalk between transcriptional activation units measured in the saturation regime of plasmid concentration (0.5 nM of plasmid encoding the sigma factor and 5 nM of plasmid encoding the reporter protein). The end-point deGFP productions (upper table) and the maximum deGFP synthesis rates (lower table) with the six *E. coli* sigma factors (non-degradable versions), and with the T7 and T3 RNA polymerases, were measured with respect to each of the specific promoters (shaded in grey) and against each of the other non-specific promoters. This table was obtained from Table S3 by dividing each row by the specific value for each unit. Except for the endogenous σ^{70} present in the reaction, the transcription factors were expressed as shown in Figure 1A. The magnesium and potassium glutamate concentrations were fixed to 3 mM and 60 mM respectively (optimal conditions for σ^{70}, Table 1). Expression through the promoter P_{70} is high in all the cases because the endogenous σ^{70} is present in the extract.

Parameter	*In vivo*	Cell-free reaction	Ratio *in vivo*/cell-free	Footnote
Protein concentration [mg/ml]	200-320	10	20-30	a, b
Transcription				
Core RNA polymerase per cell [nM]	2000	100	20-30	c
Sigma factor 70 [nM]	730	< 35	20-30	d
Sigma factor 19 [nM]	< 1	< 0.05	20-30	d
Sigma factor 24 [nM]	< 10	< 0.5	20-30	d
Sigma factor 28 [nM]	385	< 20	20-30	d
Sigma factor 32 [nM]	< 10	< 0.5	20-30	d
Sigma factor 38 [nM]	< 1	< 0.05	20-30	d
Sigma factor 54 [nM]	115	< 6	20-30	d
NtrC [nM]	73	< 4	20-30	e
Average mRNA elongation rate [nuc/s]	50	-	-	f
Global mRNA mean lifetime [min]	9.8	12-13	1.3	g
Translation				
Ribosome [nM]	46500	< 2300	20-30	h
Peptide chain elongation rate [AA/s]	18	1.5	10-15	i
Doubling time [h]	0.5	50-100	> 100	j

Table S5. Comparison between parameters of *in vivo* and *in vitro* gene expression.

[a] *In vivo* refers to *E. coli* and cell-free reaction to *E. coli* cell-free expression reaction. *E. coli* numbers have been calculated for a division time of 30 minutes at 37°C (*14*). In these conditions, the average cell volume is 1.6 μm^3. The protein concentration in *E. coli* was obtained from (*15*), from which we get the dilution factor 20-30 for the cell-free reaction.

[b] Most of the cell-free reactions are optimum at an extract protein concentration of 10 mg/ml (determined by Bradford assay). Higher extract concentrations have been also used that give similar protein production (*16*).

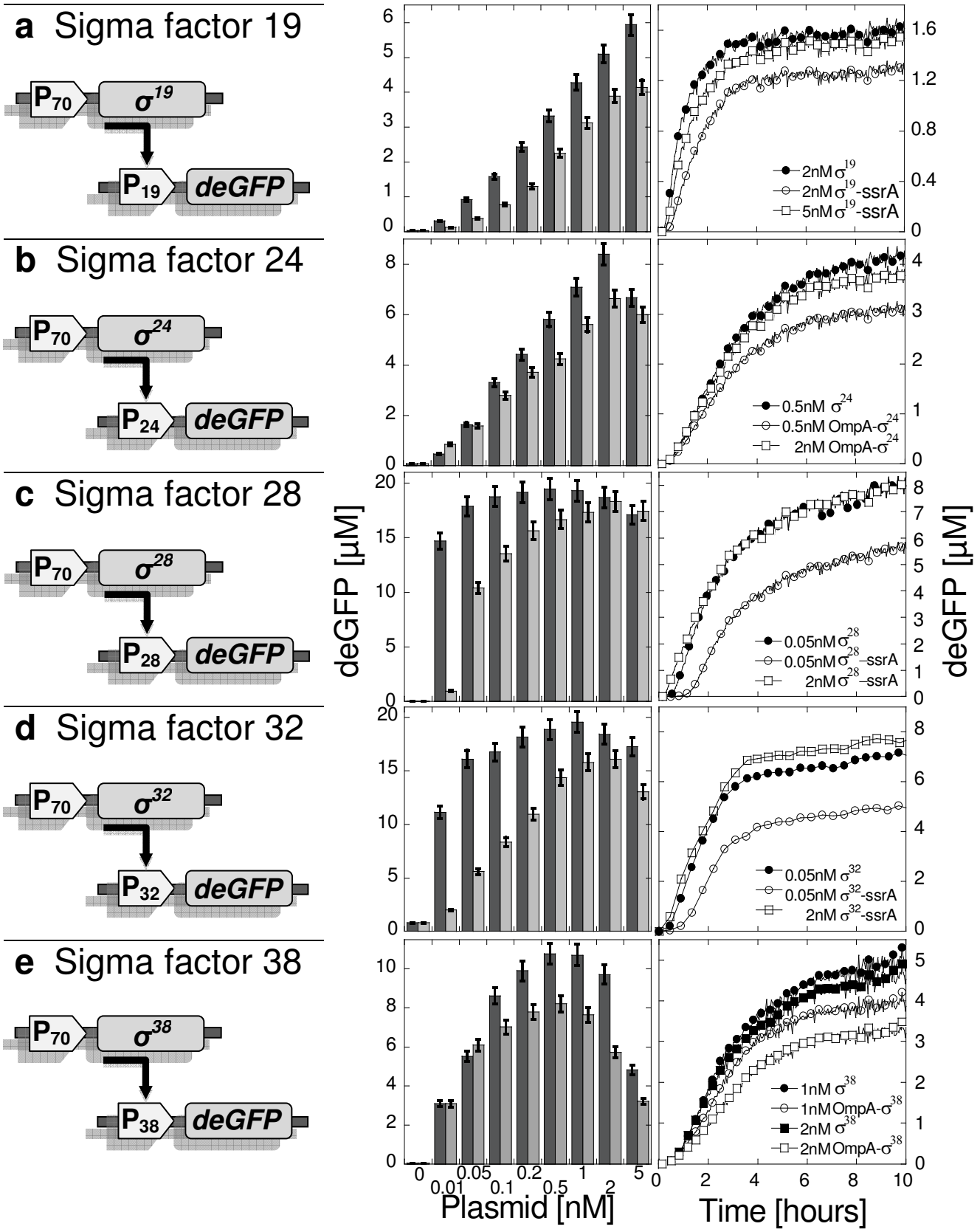
[c] Calculated from (*14*) using Table 3 in part V chapter 96. In a cell-free expression reaction, the concentration of core RNA polymerase was calculated from the protein dilution (20-30) and the amount of active core RNA polymerase present in *E. coli*.

[d] Considering that all sigma factor 70 are free during extract preparation. Sigma factor 70 in a cell-free extract was calculated from the protein dilution (20-30) and the concentration *in vivo* (*17*). The *in vivo* concentration of the other sigma factors were also obtained from (*17*) and diluted by 20 to get the concentrations in cell-free reaction.

[e] The *in vivo* concentration of NtrC was obtained from (*18*).

[f] *In vivo*, the average mRNA elongation rate with the *E. coli* RNA polymerase is 50 nuc/s (*14*). The average mRNA elongation rate with the *E. coli* RNA polymerase has not been estimated in a cell-free system. However, some *in vitro* measurements have been performed, for example in (*19*): 10-30 nuc/s.

[g] *In vivo* from (*20*), *in vitro* from (*21*).
[h] The concentration of ribosomes in a cell-free extract was estimated by Underwood and colleagues (*22*). This concentration can be also obtained from the concentration of ribosomes *in vivo* (*14*) corrected from a dilution of 20-30, considering that the fraction of ribosomes lost during extract preparation is negligible.
[i] *In vivo*, the average peptide elongation rate is 18 aa/s (*14*). *In vitro*, the average peptide elongation rate is 1.5 aa/s (*22*).
[j] The doubling time of a cell-free reaction is referred as to the time required to synthesize a protein amount equal to the amount of protein contained in the extract at the beginning of the reaction (e.g. If the protein concentration of a cell-free reaction is 10 mg/ml at the beginning of cell-free expression, the doubling time is the time required to cell-free synthesized 10 mg/ml of protein). Although no cell-free system has been shown to produce as much protein as in the extract, some estimation about the doubling time can be made from the protein production and the rate of protein production in exchange mode (*16*) and this work.


a Sigma factor 19
P70
σ19
P19
deGFP
b Sigma factor 24
P70
σ24
P24
deGFP
c Sigma factor 28
P70
σ28
P28
deGFP
d Sigma factor 32
P70
σ32
P32
deGFP
e Sigma factor 38
P70
σ38
P38
deGFP
deGFP [μM]
Plasmid [nM]
0
0.01
0.05
0.1
0.2
0.5
1
2
5
2nM σ19
2nM σ19-ssrA
5nM σ19-ssrA
0.5nM σ24
0.5nM OmpA-σ24
2nM OmpA-σ24
0.05nM σ28
0.05nM σ28-ssrA
2nM σ28-ssrA
0.05nM σ32
0.05nM σ32-ssrA
2nM σ32-ssrA
1nM σ38
1nM OmpA-σ38
2nM σ38
2nM OmpA-σ38
Time [hours]
deGFP [μM]

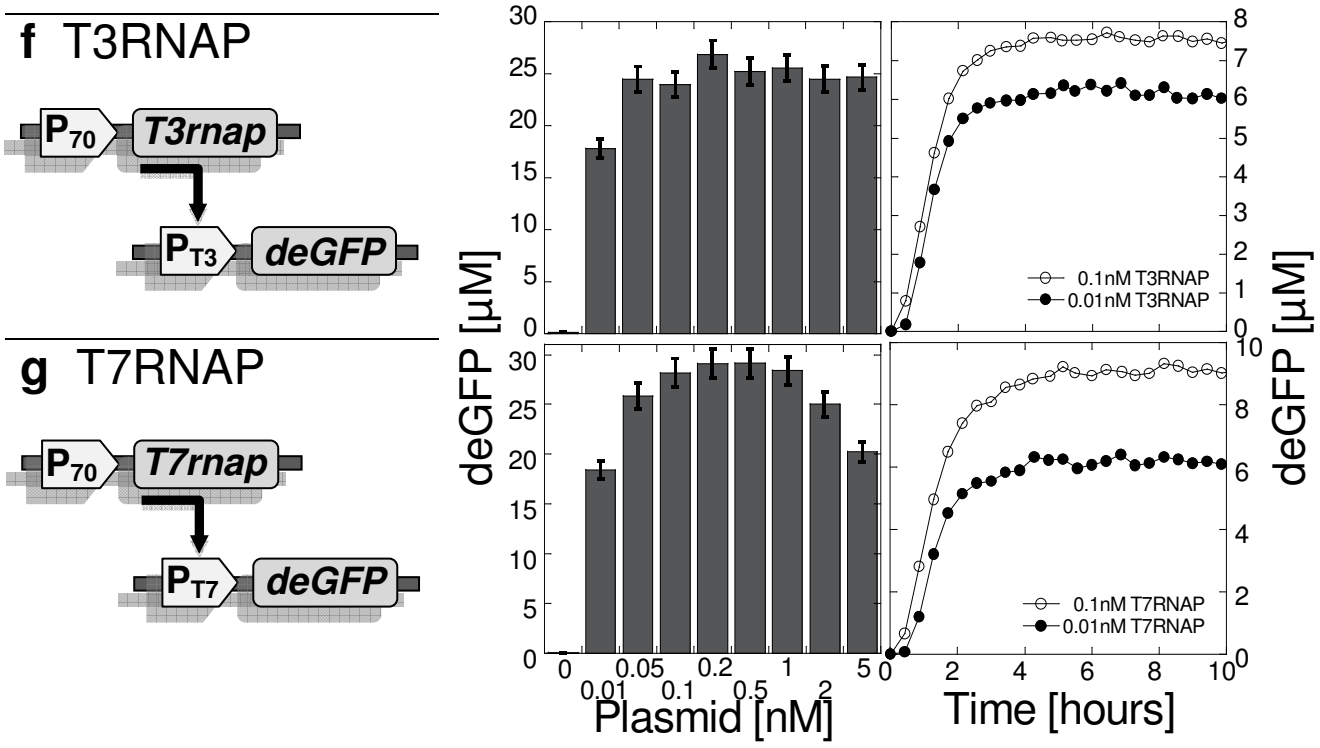


Figure S1. Two-stage transcriptional activation cascades using the *E. coli* σ^{19}, σ^{24}, σ^{28}, σ^{32}, σ^{38} and the bacteriophage RNA polymerases T3 and T7. Left column: schematic of the cascades. Middle column: end-point deGFP production as a function of the concentration of the plasmid carrying the transcription factor gene (symbol: light grey for degradable sigma factors, dark grey for non degradable sigma factors and bacteriophage RNA polymerases). Right column: kinetics of deGFP expression at different plasmid concentrations (the concentration of reporter plasmid was fixed to 5 nM for all of the cascades).

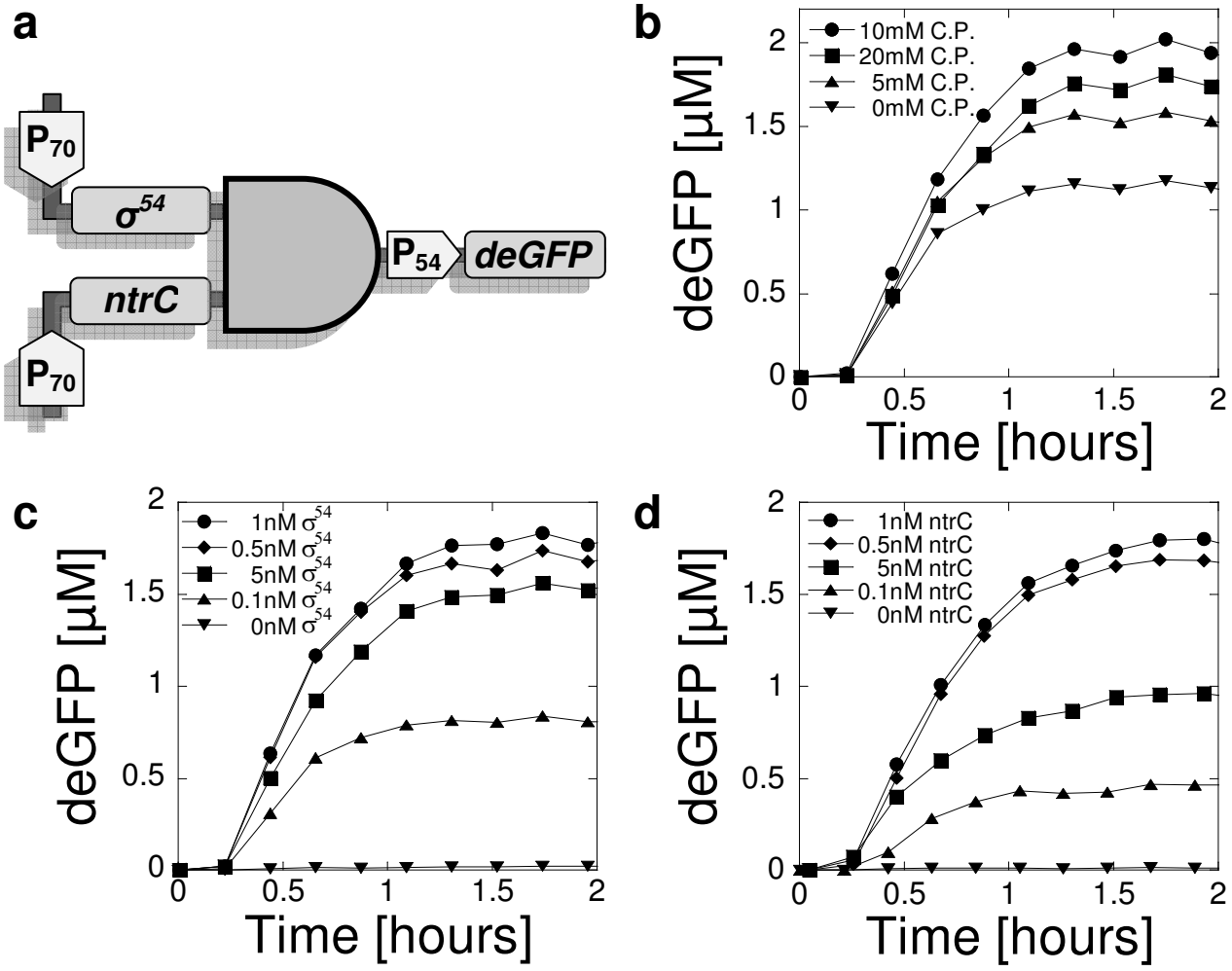


Figure S2. Co-activation of deGFP synthesis by σ^{54} and NtrC. (a) Schematic of the co-activation, shown as an AND gate circuit. *σ^{54}* and *NtrC* were cloned under the same promoter P_{70} in separate plasmids and expressed concurrently to activate the expression of *deGFP* cloned under a promoter P_{54} specific to σ^{54}/NtrC (DNA part list in Supplementary Table S1). (b) Kinetics of deGFP synthesis as a function of the concentration of carbamyl phosphate (C.P.) (1 nM P_{70}-*σ^{54}*, 1 nM P_{70}-*ntrC* and 5 nM P_{54}-*deGFP*). (c) Kinetics of deGFP synthesis as a function of the concentration of plasmid P_{70}-*σ^{54}* (1 nM P_{70}-*ntrC*, 5 nM P_{54}-*deGFP* and 10 mM carbamyl phosphate). (d) Kinetics of deGFP synthesis as a function of the concentration of plasmid P_{70}-*ntrC* (1 nM P_{70}-*σ^{54}*, 5 nM P_{54}-*deGFP* and 10 mM carbamyl phosphate).

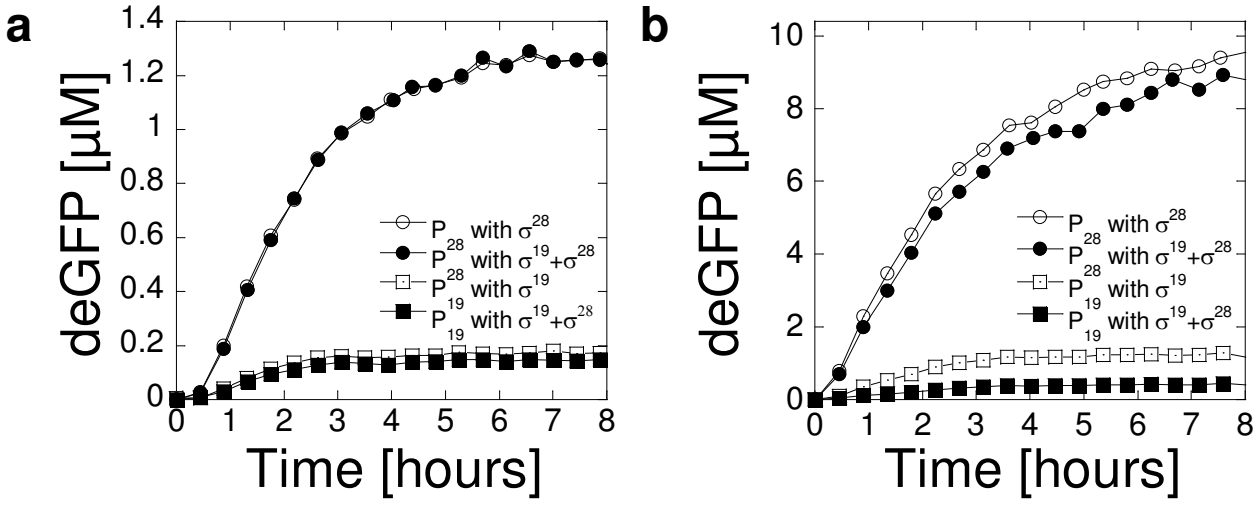


Figure S3. Passive transcription regulation by competition. The magnesium and potassium glutamate concentrations were fixed to 3 mM and 60 mM respectively (optimal conditions for σ^{70}, Table 1). (a) The expression of deGFP through the σ^{28} transcriptional activation unit is not sensitive to the coexpression of σ^{19} (linear regime of plasmid concentration, 0.2 nM of each plasmid). The expression of deGFP through the σ^{19} transcriptional activation cascade is also not sensitive to the coexpression of σ^{28}. (b) The same experiment as in (a) in the saturation regime of plasmid concentration (1 nM sigma factor plasmid and 4 nM reporter plasmid). The expression of deGFP through the σ^{28} transcriptional activation unit is not sensitive to the coexpression of σ^{19}. The expression of deGFP through the σ^{19} transcriptional activation unit decreases by a factor of 4 when σ^{28} is also expressed.

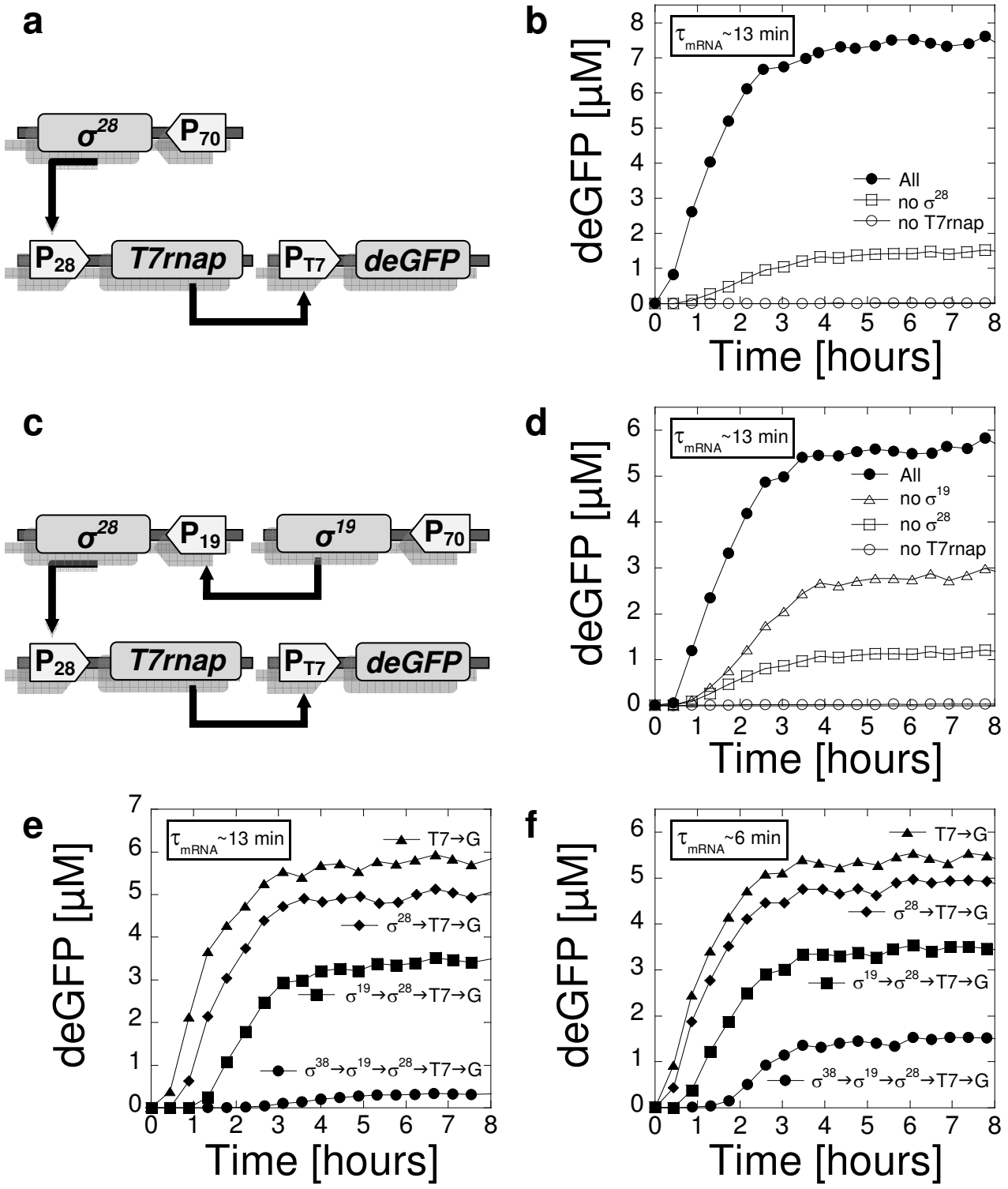
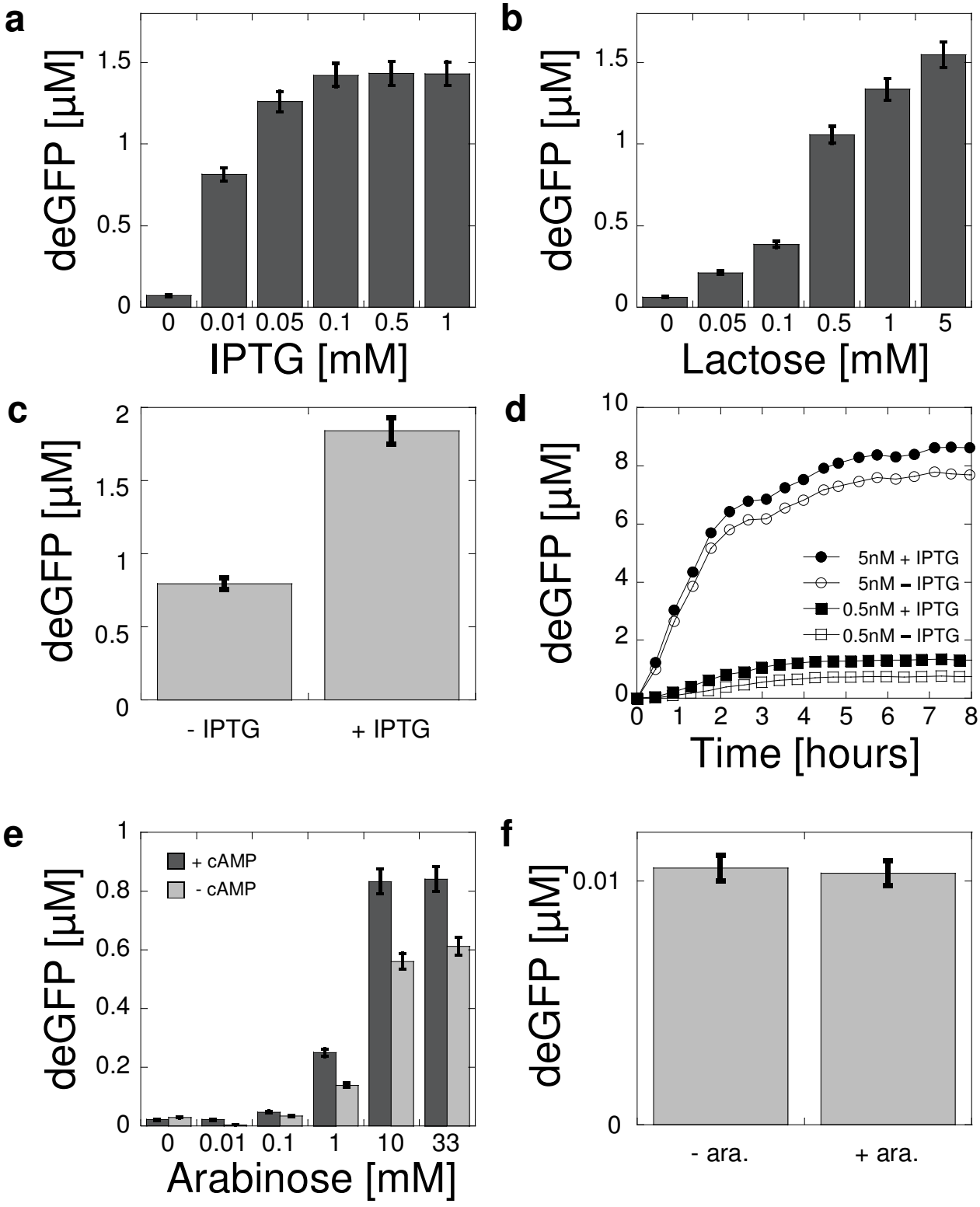


Figure S4. Construction of multiple stages transcriptional activation cascades. The magnesium and potassium glutamate concentrations were fixed to 3 mM and 60 mM respectively (optimal

conditions for σ^{70}, Table 1). (a) Schematic of a three-stage transcriptional activation cascade. (b) Kinetics of deGFP expression through the three-stage transcriptional activation cascade shown in (a) with a global mRNA mean lifetime of 13 min in the saturation regime of plasmid concentration (1 nM of each plasmid). An important leak is already observed for this circuit when the plasmid P_{70}-σ^{28} is removed. (c) Schematic of a four-stage transcriptional activation cascade. (d) Kinetics of deGFP expression through the four-stage transcriptional activation cascade shown in (c) with a global mRNA mean lifetime of 13 min in the saturation regime of plasmid concentration (1 nM of each plasmid). The negative controls show that the output signal of the cascade is not specific. (e) Kinetics of deGFP expression through two- three- four- and five-stage transcriptional activation cascades with a global mRNA mean lifetime of 13 min in the linear regime of plasmid concentration (0.2 nM of each plasmid). In this regime, the output signal is specific for all the cascades (controls below 500 nM, signal to leak ratio > 10). The five-stage cascade, however, barely delivers a relevant output signal (400 nM deGFP produced). (f) Kinetics of deGFP expression through two- three- four- and five-stage transcriptional activation cascades with a global mRNA mean lifetime of 6 min in the saturation regime of plasmid concentration (1 nM of each plasmid). In this regime, the output signal is specific for all the cascades (controls below 500 nM, signal to leak ratio > 10). All the cascades have output signals above 500 nM. Compared to the linear regime of plasmid concentration, the onset of deGFP synthesis appears earlier and the rate of deGFP synthesis is larger by a factor of 10.


a
deGFP [µM]
1.5
1
0.5
0
0
0.01
0.05
0.1
0.5
1
IPTG [mM]
b
deGFP [µM]
1.5
1
0.5
0
0
0.05
0.1
0.5
1
5
Lactose [mM]
c
deGFP [µM]
2
1.5
1
0.5
0
- IPTG
+ IPTG
d
deGFP [µM]
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
Time [hours]
5nM + IPTG
5nM – IPTG
0.5nM + IPTG
0.5nM – IPTG
e
deGFP [µM]
1
0.8
0.6
0.4
0.2
0
0
0.01
0.1
1
10
33
Arabinose [mM]
+ cAMP
- cAMP
f
deGFP [µM]
0.01
0
- ara.
+ ara.

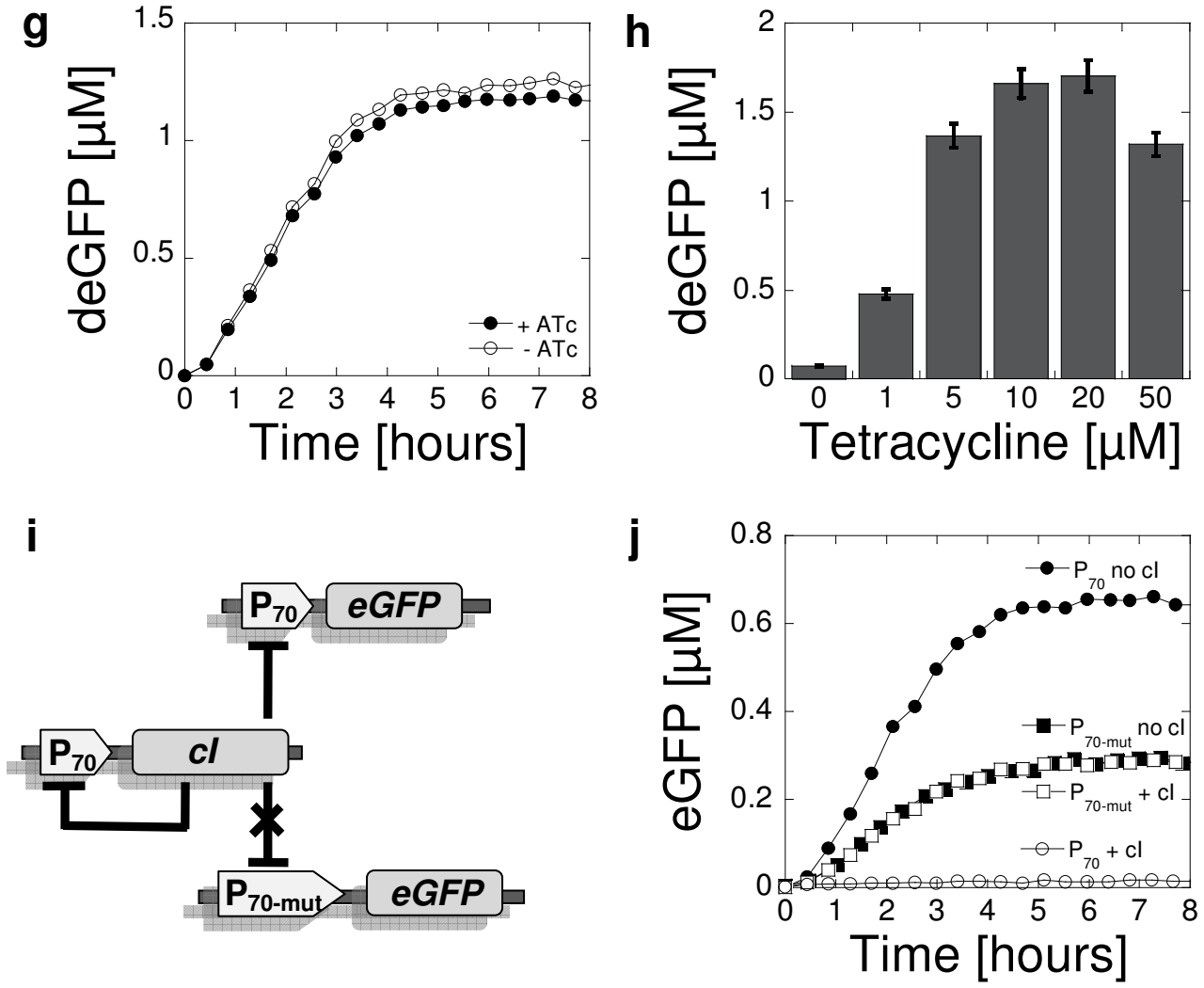


Figure S5. Characterization of the transcriptional repression units. The magnesium and the potassium glutamate concentrations were fixed to 3 mM and 60 mM respectively (optimal conditions for σ^{70}, Table 1). (a) IPTG concentration range for the lac system (1 nM $P_{LlacO-1}$-*lacI* and 0.5 nM $P_{LlacO-1}$-*deGFP*, end-point measurements). (b) Lactose concentration range for the lac system (1 nM $P_{LlacO-1}$-*lacI* and 0.5 nM $P_{LlacO-1}$-*deGFP*, end-point measurements). (c) At low plasmid concentration, a 50% repression is observed when the reporter plasmid only is added to the reaction (0.5 nM $P_{LlacO-1}$-*deGFP*, end-point measurements), which indicates the presence of endogenous LacI repressors in the extract. (d) Repression of the lac system by leftover endogenous LacI repressors in the extract (tested for 0.5 nM and 5 nM $P_{LlacO-1}$-*deGFP*). At high plasmid concentration the repression of the lac system by leftover endogenous LacI repressors is negligible. (e) Arabinose concentration range (5 nM pBAD-*deGFP*, end-point measurements) with and without 0.75 mM cAMP. (f) Expression of deGFP in the absence of the *araC* gene (*araC* was knocked out from the plasmid, end-point measurements). (g) Expression of deGFP with the $P_{LtetO-1}$-*deGFP* plasmid only (1 nM) is the same with and without addition of ATc (10 μM). (h) Anhydrotetracycline concentration range for the tet system (2 nM $P_{LtetO-1}$-*tetR* and 1 nM $P_{LtetO-1}$-*deGFP*, end-point measurements). (i) A non-inducible repression system. deGFP and the lambda repressor Cl were cloned under the P_{70} promoter. The same constructions were made

with a mutated promoter, $P_{70\text{-}mut}$ that cannot be repressed by CI. (j) Kinetic of cell-free expression for the circuit shown in (i) (0.5 nM P_{70}-*eGFP* and 1 nM P_{70}-*cI* or 1 nM $P_{70\text{-}mut}$-*cI*). The strength of the promoter $P_{70\text{-}mut}$ is slightly inferior to the promoter P_{70}.

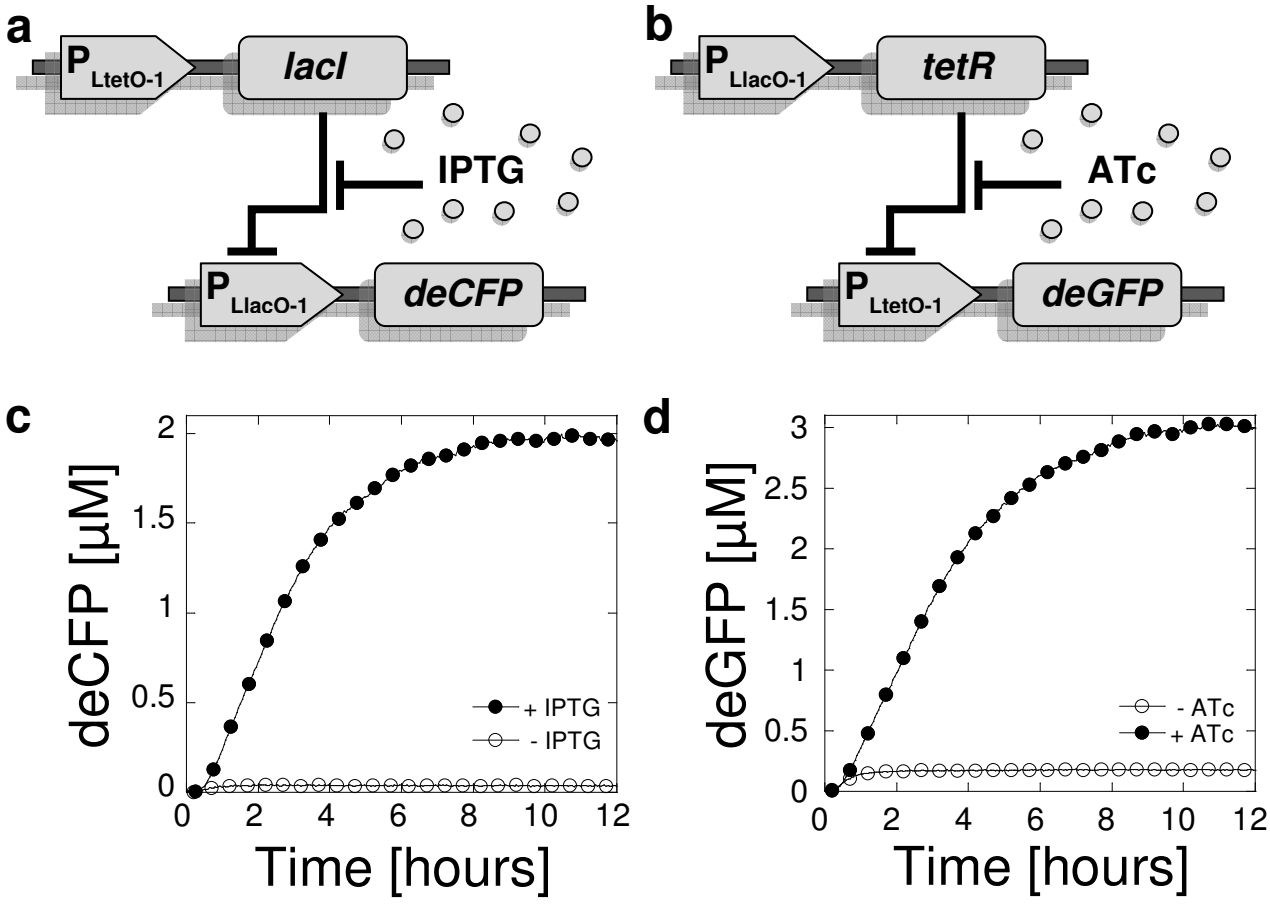


Figure S6. Inducible transcriptional repressions. The magnesium and the potassium glutamate concentrations were fixed to 3 mM and 60 mM respectively (optimal conditions for σ^{70}, Table 1). (a) Schematic of Lac repression. The *lacI* repressor gene is cloned under a promoter $P_{LtetO-1}$. The reporter gene *deCFP* is cloned under a promoter $P_{LlacO-1}$. (b) Schematic of Tet repression. The *tetR* repressor gene is cloned under a promoter $P_{LlacO-1}$. The reporter gene *deGFP* is cloned under a promoter $P_{LtetO-1}$. (c) Kinetics of the Lac repression system (1 nM P_{tetO1}-*lacI*, 4 nM P_{lacO1}-*deCFP*). The open state is obtained by adding 50 µM of IPTG in the reaction. (d) Kinetics of the Tet repression system (2 nM $P_{LlacO-1}$-*tetR*, 2 nM $P_{LtetO-1}$-*deGFP*). The open state is obtained by adding 10 µM of ATc in the reaction.

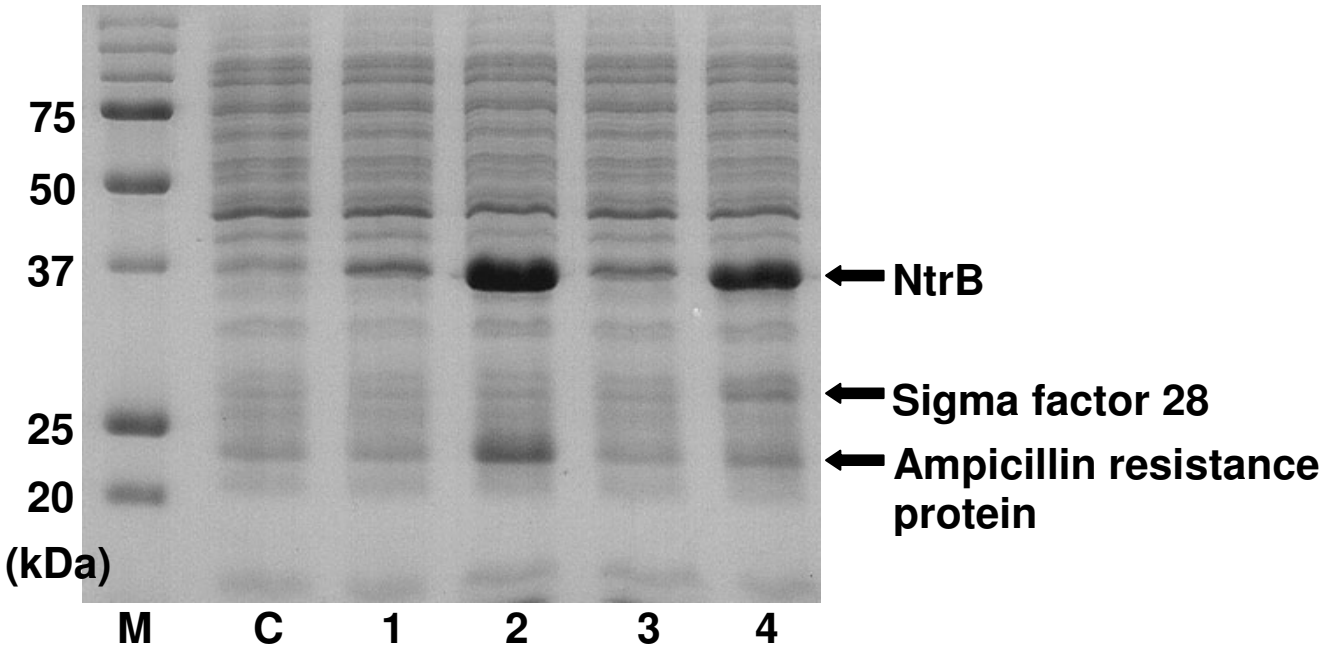


Figure S7. SDS PAGE 13% of cell-free reactions (expression of NtrB protein). M - marker, C - cell-free reaction with no plasmid, 1 – expression of NtrB in batch mode (5 nM P_{70}-*ntrB*), 2 – expression of NtrB in dialysis mode (5 nM P_{70}-*ntrB*), 3 – expression of NtrB through the σ^{28} cascade in batch mode (0.2 nM P_{70}-σ^{28} and 5 nM P_{28}-*ntrB*), 4 – expression of NtrB through the σ^{28} cascade in dialysis mode (0.2 nM P_{70}-σ^{28} and 5 nM P_{28}-*ntrB*). Expression of the ampicillin resistance gene is inhibited by the competition of σ^{28} with the endogenous σ^{70} for the core RNA polymerase (compare sample 2 and 4).

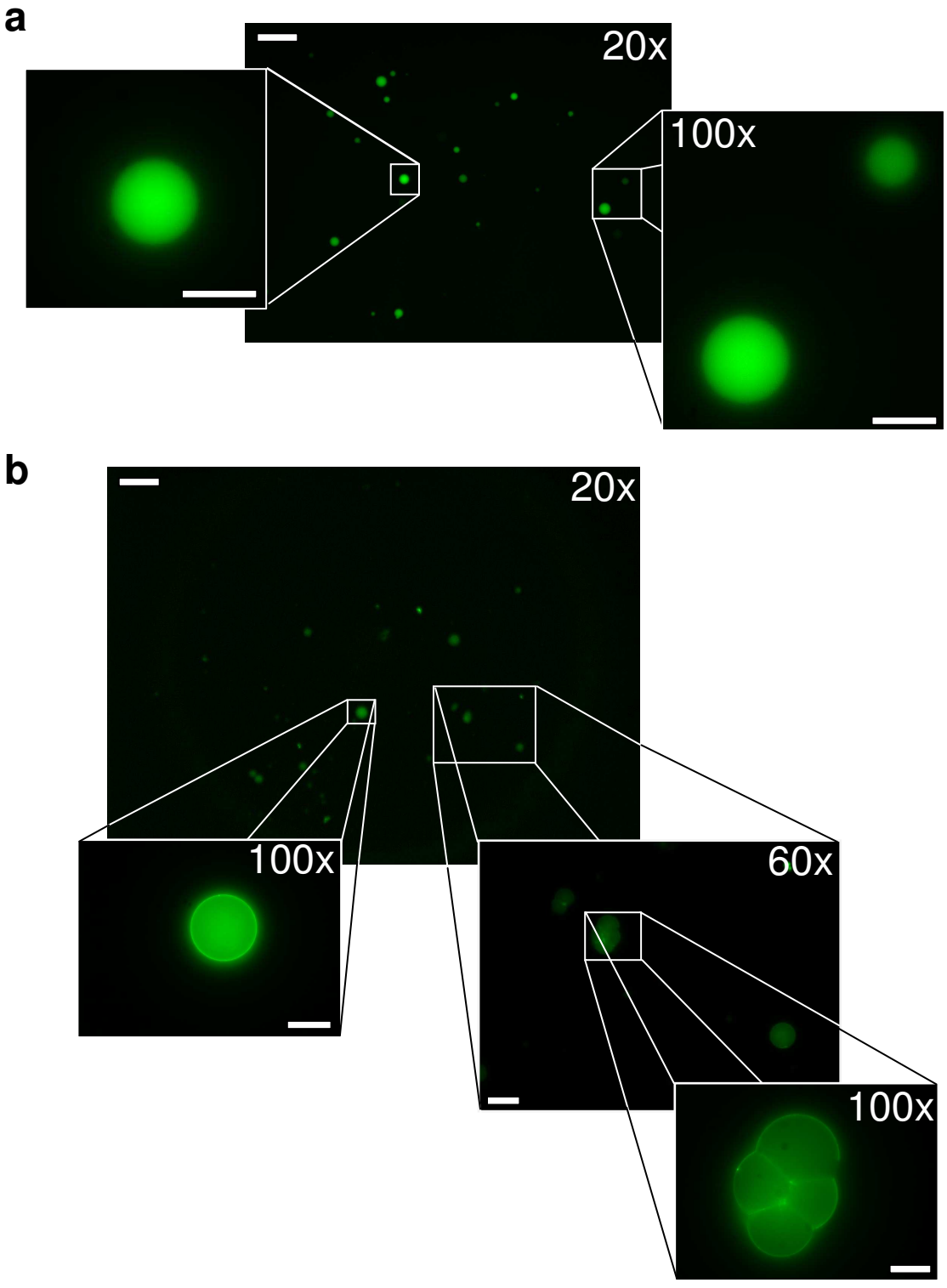

a
20x
100x
b
20x
100x
60x
100x

Figure S8. Fluorescence images of cell-free expression inside phospholipid vesicles after 10 hours of incubation at room temperature. (a) Cell-free expression of deGFP in vesicles (P_{70}-*deGFP*, 20X objective, scale bar: 50 µm, blow-up with 100X objective, scale bar: 10 µm). (b) Cell-free expression of αHemolysin-eGFP in vesicles (0.2 nM P_{70}-σ^{28} and 5 nM P_{28}-*αHemolysin-eGFP*, 20X objective, scale bar: 50 µm, blow-up with 60X objective, scale bar: 25 µm, blow-up with 100X objective, scale bar: 25 µm).

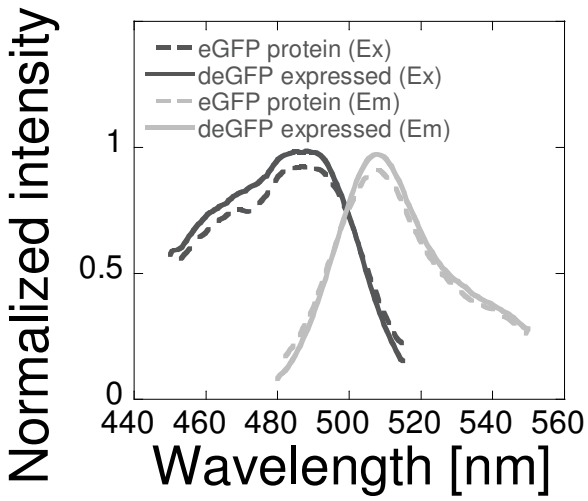


Figure S9. Excitation and emission spectra of deGFP (cell-free synthesized) and pure eGFP (Clontech) scaled from 0 to 1 and from 0 to 0.95 respectively.

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