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TECHNICAL NOTE

A standardized broad host range inverter package for genetic circuitry design in Gram-negative bacteria

by

Huseyin Tas[1], Ángel Goñi-Moreno[2,3] and Víctor de Lorenzo[1]*

Systems Biology Department, Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco, Madrid 28049, Spain, [2]School of Computing, Newcastle University, Newcastle Upon Tyne, NE4 5TG, UK, [3]Centro de Biotecnología y Genómica de Plantas (CBGP, UPM-INIA), Universidad Politénica de Madrid (UPM), Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Campus de Montegancedo-UPM, 28223 Pozuelo de Alarcón, Madrid, Spain

Running Title: Assets for gene circuit design in non-*E. coli* bacteria

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* Correspondence to: Víctor de Lorenzo

Centro Nacional de Biotecnología-CSIC

Campus de Cantoblanco, Madrid 28049, Spain

Tel.: 34- 91 585 45 36; Fax: 34- 91 585 45 06

E-mail: vdlorenzo@cnb.csic.es

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Abbreviations: BHR, broad host range; SD, Shine-Dalgarno sequence; RBS, ribosomal binding sequence; IPTG, isopropyl thiogalactopyranoside; CAD, computer-assisted design; SEVA, Standard European Vector Architecture.

1 **ABSTRACT**

2

3 Genetically encoded logic gates, especially inverters—NOT gates—are the building blocks for designing

4 circuits, engineering biosensors or decision-making devices in synthetic biology. However, the repertoire

5 of inverters readily available for different species is rather limited. In this work, a large whole of NOT

6 gates that was shown to function previously in a specific strain of *Escherichia coli*, was recreated as

7 broad host range (BHR) collection of constructs assembled in low, medium and high copy number

8 plasmid backbones of the SEVA (Standard European Vector Architecture) collection. The input/output

9 function of each of the gates was characterized and parameterized in the environmental bacterium and

10 metabolic engineering chassis *Pseudomonas putida*. Comparisons of the resulting fluorescence

11 cytometry data with those published for the same gates in *Escherichia coli* provided useful hints on the

12 portability of the corresponding gates. The hereby described BHR inverter package (20 different

13 versions of 12 distinct gates) thus becomes a toolbox of choice for designing genetic circuitries in a

14 variety of Gram-negative species other than *E. coli.*

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16 **KEYWORDS:** *Broad host range tools, inverter library, gates, Pseudomonas putida, flow cytometry,*

17 *biosensor, automated circuit design*

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20 Design and implementation of genetic circuits is one of trademarks of contemporary synthetic biology[1].

21 The archetypal approach involves abstracting biological cues (e.g. effectors, metabolites, proteins) and

22 physicochemical signals (e.g. inducers, temperature) as inputs. These are processed through a more or

23 less complex computation layer (most often assembled with regulatory parts: transcriptional factors,

24 riboregulators etc.) which then releases another biological signal as the output. This in turn can be used

25 as the input for another node of the circuit and so on[2, 3]. Further abstraction of biological circuits as

26 wholes of Boolean logic gates enables a superior level of complexity, as shown by a suite of examples

27 involving rewiring of stress responses, detection of environmental contaminants, implementation of

28 cellular calculators and others[4-7]. Yet, as the demand for increasingly complex circuits grows[5, 8, 9], so

29 does the interest in automation of their design and execution. One major landmark in this direction was

30 the publication in 2016 of the CelloCAD platform[5], a complete operative system for virtual assembly and

31 eventual implementation of logic circuits in *E. coli* through combination of series of NOR gates—based

32 themselves on a large collection of well characterized promoter/repressor pairs (i.e. inverters or NOT

1   gates). This tool affords automated design and simulation in seconds of complex genetic networks with
2   successful prediction of > 70% of all states shown by the actual DNA constructs once synthesized and
3   knocked in *E. coli*[5, 7]. Yet, due to the material nature of the building blocks, CAD tools such as Cello are
4   inherently restrictive to single strains or species. While these systems may work well in a given
5   organism, the parameters and general behavior can change very significantly when passed to other
6   hosts. This is a considerable issue when circuits are desired to compute signals under environmental
7   and industrial conditions for which *E. coli* is not an optimal chassis.

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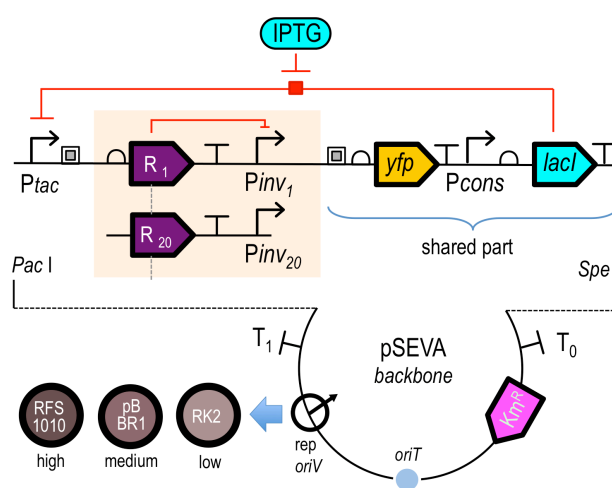
Figure 1. Schematic representation of BHR genetically-encoded inverters. The organization of the functional parts borne by the 20 variants assembled following the SEVA standard is sketched (components not to scale). Similarly to the earlier collection for *E. coli*[5] there are segments shared by all construct i.e. the P*tac*/*lacI*/IPTG-dependent expression system for each of the repressor/SD combinations (R_1 to R_{20}) and the *yfp* gene used for fluorescent readout of inverter performance (the *lacI* gene is expressed through the same constitutive promoter P*cons*). The Km resistance gene and the DNA sequences that punctuate the SEVA backbone (T_1 and T_0 terminators, origin of conjugal transfer *oriT*) are common to all constructs as well. The variable parts include [i] the DNA encoding the SD, the repressor gene (R) and the cognate repressible promoter (P*inv*) upstream of the reporter *yfp* and [ii] the BHR origin of replication: RK2, pBBR1 or RSF1010, each of them supporting different plasmid copy numbers as indicated.

21

22   On this basis we set out to engineer a robust and easy-to-use package of standardized NOT gates that
23   could be used for circuit design in Gram-negative bacteria other than *E. coli* and that—once
24   characterized in the host of interest—could benefit from the CelloCAD software[5] for automatic assembly
25   of the cognate DNA.

26

27   To this end, we recreated the DNA sequence encoding each of the inverters available in the Cello
28   platform and pass them to vectors of the broad host range SEVA (Standard European Vector
29   Architecture) collection with low, medium and high copy numbers. The general organization of the

1 constructs is sketched in Fig. 1. Note that the business part of each plasmid is flanked by upstream and

2 downstream terminators added by the SEVA structure to mitigate potential readthrough from vector

3 promoters. The plasmids backbones were retrieved from the SEVA database and repository[10].

4

5 In practical terms, each of the segments encoding the gates was amplified from the collection of *E. coli*

6 NEB10β strains bearing p15A/Km^R vectors (~15 copies) inserted with the cognate DNA[5]. Primers

7 (Merck Sigma Aldrich, Inc) were designed for adding SEVA-compatible PacI and SpeI restriction sites to

8 the extremes of the amplicons generated with the Q5 High-Fidelity DNA polymerase (New England

9 BioLabs, Inc.). The oligonucleotides used for amplification of the DNA of the NOT gates and verification

10 of the constructs are listed in Supplementary Table S2. Following PCR, the resulting fragments where

11 separately cloned in pSEVA221 (RK2*oriV*, $\leq$ 5 copies/cell), pSEVA231 (BBR1*oriV*, ~ 30 copies) and

12 pSEVA251 (RFS1010*oriV*, $\geq$ 50 copies) and first captured, verified and resequenced in the host strain

13 *P. putida* KT2440. The complete catalogue of constructs is listed in Table 1.

14

15 **Table 1.** Library of available genetically-encoded
16 inverters and auxiliary plasmids in SEVA vectors
17 with 3 different copy numbers (see Supplementary
18 Table S1 for plasmid names and cargoes). Note
19 that 5 inverters caused growth inhibition when
20 cloned in high copy number vectors, plausibly due
21 to toxicity of the repressor protein encoded therein.
22 The repressor borne by each of the gates is
23 described in[5].

| Inverters	Low Copy Number	Medium Copy Number	High Copy Number
AmeR-F1	✓	✓	✓
AmtR-A1	✓	✓	✓
BetI-E1	✓	✓	✓
BM3R1-B1	✓	✓	-
BM3R1-B2	✓	✓	✓
BM3R1-B3	✓	✓	✓
HlyIIR-H1	✓	✓	✓
IcaRA-I1	✓	✓	✓
LitR-L1	✓	✓	✓
LmrA-N1	✓	✓	✓
PhlF-P1	✓	✓	✓
PhlF-P2	✓	✓	✓
PhlF-P3	✓	✓	-
PsrA-R1	✓	✓	✓
QacR-Q1	✓	✓	✓
QacR-Q2	✓	✓	-
SrpR-S1	✓	✓	✓
SrpR-S2	✓	✓	-
SrpR-S3	✓	✓	-
SrpR-S4	✓	✓	✓
Auto-fluorescence	✓	✓	✓
Standardization	✓	✓	✓
Promoter Activity	✓	✓	✓

24

25 Note that some gates (those designated

26 BM3R1-B1, PhlF-P3, QacR-Q2, SrpR-S2

27 and SrpR-S3) could not be cloned in the

28 higher copy number vectors, presumably

29 due to the toxicity effects of the encoded repressor proteins. The collection (Table 1 and Supplementary

30 Table S1) includes a total of 12 inverters, some of them bearing two or more variants of the Shine-

31 Dalgarno sequence of the repressor gene that changes its expression levels. For example, AmeR-F1

32 (AmeR is the gate and F1 is the SD) has only 1 version of the ribosomal binding site of the repressor

33 gene; however, SrpR has 4 versions (SrpR-S1, SrpR-S2, SrpR-S3, SrpR-S4 (SrpR is the gate and S1,

34 S2, S3 and S4 the 4 different SDs)). Note also that the arrangement of functional parts of the gates in

35 the SEVA vectors is identical to the one originally adopted in the p15A/Km^R vectors of the Cello platform

36 (Fig. 1). In addition to the plasmids bearing 20 inverters, we built a set of reference constructs allowing

5

1   relative promoter units (RPU) to be converted and compared between different conditions (see below).

2   These references (Supplementary Fig. S1) include [i] autofluorescence plasmids (recreating the

3   business parts of pAN1201[5] and consisting of each of the backbone plasmids but without any insert e.g.

4   missing the repressor/target promoter segments highlighted in Fig. 1, [ii] RPU standard plasmids

5   derived from pAN1717[5] in which *yfp* is expressed through the constitutive promoter J23101 and which

6   are used in combination with the autofluorescence plasmid for converting YFP readouts of each inverter

7   into RPU (see equation #1 below) and [iii] promoter activity plasmid recreating pAN1818[5], which is used

8   for measuring the promoter activity (pTac-YFP) based on inducer concentrations (Supplementary Fig.

9   S1).

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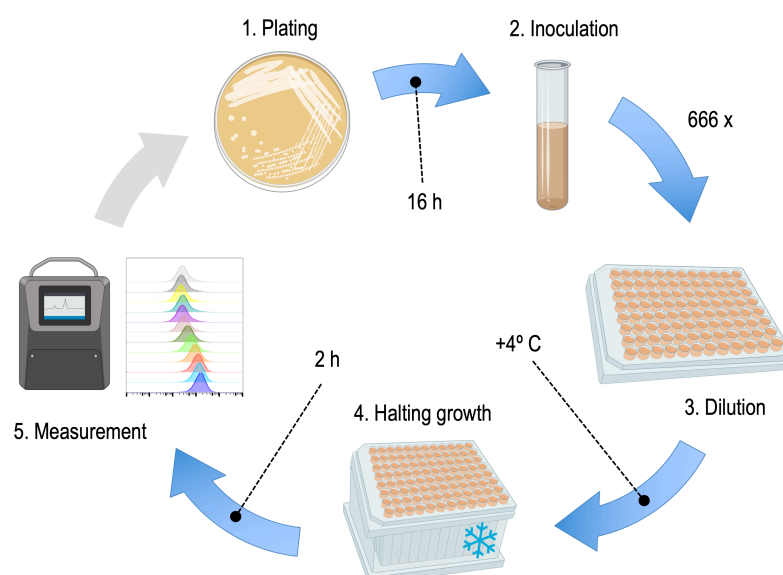
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15   **Figure 2.** Experimental protocol used for measuring performance of the genetic inverters listed in Table 1 in P.
16   putida KT2440. In all experiments bacteria were grown in M9 medium adapted for growth of *P. putida*. 250 ml of
17   liquid culture contained 25 ml x10 M9 salts, 500 µl of 1M $MgSO_4$, 2.2 ml of 20% citrate and milliQ-H_2O to volume.
18   50 µg ml^{-1} kanamycin was also added to secure plasmid retention (all them Km^R). For the experiments, a
19   saturated overnight culture of each of the strains under were diluted over ~ 600-fold in the wells of a microtiter
20   plate with 200 µl per well, plated for 24h, added with IPTG concentrations ranging 0 to 1000 µM and incubated at
21   30 °C with shaking for 24 h. Cultures (typically reaching OD_{600} ~ 0.2-0.3) were then kept in the cold for the rest of
22   the procedure. YFP fluorescence distribution of each sample was measured with a Miltenyi Biotec MACS flow
23   cytometer at channel B1 with an excitation of 488 nm and emission of 525/50 nm. For each sample 30 thousand
24   events were collected with singlet gating. Calibration was done by using MACSQuant Calibration Beads (see text
25   for explanation).

26

27   The library of constructs listed in Table 1 and Supplementary Table S1 is expected to ease utilization of

28   CelloCAD as a genetic programming tool in a suite of Gram-negative bacteria. Note that users have a

1 choice to pick the same gate borne by plasmids with different copy numbers, what may be critical to

2 avoid potential toxicity. Adoption of the standard SEVA format gives two additional advantages. While

3 the construct library of Supplementary Table S1 was built on vectors with a Km resistance gene, the

4 modularity of the SEVA format makes its replacement by an alternative antibiotic marker for selection[10,]

5 [11] easy. Also, the BHR nature of the standardized constructs affords their implantation in diverse Gram-

6 negative hosts, thereby giving a chance to compare their performance in different species and thus

7 learn about interoperability and context dependencies in circuit designs.

8

9 In order to validate these features we tested and parameterized the whole low-copy number versions of

10 the inverter library of Supplementary Table S1 in the environmental bacterium and Synthetic Biology

11 chassis *Pseudomonas putida* KT2440[12, 13]. The experimental workflow to this end (Fig. 2) was designed

12 considering the specific needs of *P. putida* for growth as detailed in the legend of the figure. Once

13 strains bearing each of the constructs were generated, transformants were grown and IPTG

14 concentrations of (5-1000 µM) added to activate each of the devices, for a total 24 h period. YFP

15 fluorescence emission detected with a flow cytometer was recorded after 24 of IPTG addition. Data

16 were then analyzed with FlowJo software (https://www.flowjo.com/). An important detail was that the

17 auto-gating option of the software was set considering at least 50% of the events covered while Forward

18 and Side scatters were plotted. The same gating conditions were applied to all specimens in the same

19 group and repeated for all the samples.

20

21 On the basis of the thereby produced data we quantified the output of the individual devices at each

22 condition as standard RPUs (relative promoter units). This was done by characterizing the fluorescence

23 values emitted by the bacterial population of the cultures exposed to IPTG levels covering an induction

24 ranges from none to saturation (in our case 12 points of growing effector concentrations). On this basis,

25 the RPU value at each point can be calculated with the formula (1):

26

27 $$RPU = \frac{<YFP> - <YFP>_{autofluorescence}}{<YFP>_{standardization} - <YFP>_{autofluorescence}}$$

28

29 Where <YPF> is the median fluorescence value from the gate of interest that is to be converted into

30 RPU (from either the inverter-bearing plasmids or the control promoter activity plasmid),

31 <YPF>autofluorescence is the median fluorescence value of auto-fluorescence plasmid, <YPF>standardization is

32 the median fluorescence value from the standardization plasmid. Next, the output *vs* input values of

7

1   each inverter were represented in a response function plot generated with Hill fits and utilizing the RPU

2   figures as the input to the calculations. Specifically, Hill equation parameters were estimated by plotting

3   each inducer levels with their corresponding standardized median florescence values (Supplementary

4   Data S2) by means of the equation (2):

5

6
$$y = y_{min} + \frac{(y_{max} - y_{min})\, k^n}{k^n + x^n}$$

7

8   where y is the output promoter activity, y_{min} is the minimum observed value for promoter activity, y_{max} is

9   the maximum observed value for promoter activity, k is the input value for which half maximum value for

10   output promoter is reached, n is the Hill coefficient. Experimental response data were thus entered in

11   the above Hill equation. The corresponding Hill parameters are provided in Supplementary Table S3 for

12   both *E. coli* (retrieved from www.cellocad.org) and *P. putida* values (this study). Fitting was performed

13   with MATLAB using the scripts provided in Supplementary Data S1.

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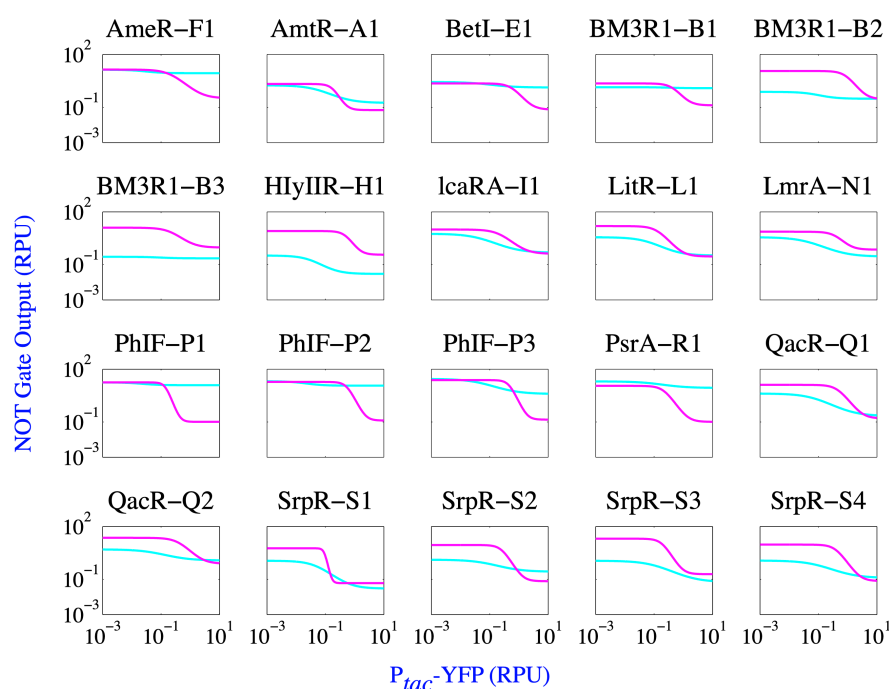
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20   **Figure 3.** Behavior of genetically-encoded inverters in *P. putida* vs. *E. coli.* The panel shows the comparisons of
21   the of the Hill of the same inverters in *E. coli* NEB10β (magenta; data retrieved from[5]) and behavior the same
22   devices in *P. putida* KT2440 (cyan; experimental from this study). X-axes correspond to the activity of the IPTG-
23   inducible pTac promoter and Y-axes indicate the activity of corresponding inverters. Both axes indicate YFP
24   expression in RPUs.
25

8

1   The resulting characterization of the 20 inverters (listed in the Supplementary Table S1) in *P. putida*

2   based on the protocols and calculations above is shown in Fig. 3. The data showed a range of

3   divergences in the behavior of the inverters in either host. In some cases, the patterns were comparable

4   both in terms of the dynamic ranges of the input/output, the contour of the response curves and the

5   specific values of promoter strengths. In other cases, the shape of the curve was kept but the

6   boundaries changed very significantly. And finally, in yet another series of devices there was little if any

7   similarity in their input/output transfer functions between the two types of bacteria While these data

8   expose the limitations of just exporting genetic devices from one species to the other, the large

9   repertoire of gates also enable users to pick the ones whose parameters are compatible with the

10 CelloCAD tool for automation of circuit designs[5]. Furthermore, the dataset associated to Fig. 3 encrypts

11 valuable, quantitative information on the interoperability of parts and circuits between different biological

12 recipients of the same constructs, an issue hardly tackled thus far in the Synthetic Biology literature[14, 15,

13 16]

14

15 In sum, we believe that the hereby described collection of inverters available in a BHR format will help to

16 expand the possibilities of genetic programming towards bacteria other than *E. coli* but still interesting

17 from a SynBio-inspired biotechnological perspective. Furthermore, their testing and parameterization in

18 various hosts may deliver general portability rules that thus far rely on a mere trial-and-error exercise.

19 The whole collection of constructs is available through the SEVA database and vector repository at

20 http://seva.cnb.csic.es .

21

22 Associated content

23

24 **Supporting information**

25 _____

26 Supplementary Figure S1

27 _____

28 Supplementary Table S1: BHR Inverters (XLSX)

29 Supplementary Table S2: Primers (XLSX)

30 Supplementary Table S3: Insulated gate response functions (XLSX)

31 _____

32 Supplementary Data S1: Scripts (MATLAB)

33 Supplementary Data S2: Median Values (XLSX)

34

Notes. The authors declare no competing financial interest

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