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Screening Library of Two-Input, One-Output Genetic Circuits in *E. coli*

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We use this protocol and it's working

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Abstract

Hybrid plate reader/flow cytometry protocol for screening a library of two-input, one-output genetic circuits in *E. coli*. This protocol allows high-throughput screening of 92 circuits per run. The plate reader assay produces 8 hours of bulk growth and fluorescence time series data while the flow cytometry screening, in parallel, enables interrogation of single-cell behaviour.

Guidelines

This is an extensive protocol, spanning across 4 days and with many time-dependent steps, so effective planning beforehand is essential. Booking of equipment well in advance is advised, including plate reader, flow cytometer, laminar flow hood and microplate incubator.

Materials

Reagents:

- Kanamycin (Bio Basic, KB0286)
- L-(+)-Arabinose (Sigma-Aldrich, A3256-25G)
- aTc, anhydrotetracycline hydrochloride (Fisher Scientific Ltd, AC233131000)
- IPTG, isopropyl β -D-1-thiogalactopyranoside (Fisher Scientific Ltd, 10725471)
- 50% Glycerol
- LB broth (Miller)
- LB broth (Miller) with agar
- Stock solutions for making supplemented M9 media (https://benchling.com/biocomputelab/f/lib_Omj7mwAr-media/prt_cIPDFMCb-supplemented-m9-media/edit)
- Glycerol (50%)
- PBS (Fisher Scientific Ltd, 18912014)
- Rainbow Calibration Particles (Spherotech Inc, RCP-30-5A)

Consumables:

- Round bottom 96-well microplates (Fisher Scientific Ltd, AB0796)
- Clear-bottom 384-well microplates (Greiner Bio-one Ltd, 781091)
- Flat-bottom, 384-well microplates (Greiner Bio-one Ltd, 781086)
- Stericup Vacuum Filtration System (Merck, S2GPU02RE)
- Breathe-Easy sealing membranes (Sigma-Aldrich, Z380059-1PAK)
- Aluminium sealing film (Starlab, E2796-0792)
- Reagent reservoirs (Heathrow Scientific, HEAT120642)
- 15 mL & 50 mL Falcon Tubes
- Sterile 12.5 mL, 25 mL & 50 mL Serological Pipettes
- Sterile 1.5 mL Eppendorf microcentrifuge tubes
- Sterile 2 μ L, 200 μ L, and 1000 μ L micropipette tips
- 2.5 μ L, 20 μ L, 200 μ L, and 1000 μ L micropipettes
- 10cm x 10cm Petri dishes

Equipment:

- Microplate incubator
- Laminar flow hood
- Vacuum pump
- -70°C freezer
- Plate reader
- Flow cytometer
- Single & Multichannel pipettes



Before start

This protocol requires a fully assembled, combinatorial library of genetic circuits stored at -20°C *in vitro*.

Firstly, ensure that the plate reader and flow cytometer are booked in advance for days 3 and 4 of the protocol, respectively. It may also be worth booking the laminar flow hood and microplate incubator, particularly on day 3, to avoid any issues.

Before starting, prepare or locate the following stocks:

- DH10 β chemically competent cells (stored at -70°C)
- pRPU DH10 β glycerol stock (relative promoter unit plasmid)
- pL2 DH10 β glycerol stock (destination vector backbone)
- 50 mg/mL kanamycin (1000x stock)
- 500 mM arabinose (100x stock)
- 100 mM isopropyl β -D-1-thiogalactopyranoside (IPTG; 100X stock)
- 100 $\mu\text{g/mL}$ anhydrotetracycline (aTc; 50,000X stock) - diluted 50-fold with nuclease free water to produce 1000X short-term stock on day of use

The following working concentrations of antibiotic and inducers are used throughout this protocol:

- Kanamycin $\rightarrow$ 50 $\mu\text{g/mL}$
- Arabinose $\rightarrow$ 5mM
- IPTG $\rightarrow$ 1mM
- aTc $\rightarrow$ 2ng/ μL



Day 1: Library Transformation

- 1 Following the BCL Benchling heat-shock **E. coli transformation protocol**, transform one aliquot of cells with the final library assembly. Also, streak out glycerol stocks of plasmids pRPU and pL2 as well as DH10B strain without any plasmid.

Note

If performing plate reader run from glycerol stocked 96-well plate, Day 1 steps can be skipped.

- 2 **OPTIONAL:**
It may be worth plating 90% of the transformed cells on one plate and 10% on another to allow a standard plate count to estimate library coverage.
- 3 Leave plates upside down in incubator box at 37°C overnight.

Day 2: 96-Well Overnight Culture

- 4 Check growth of plates and store in fridge at 4°C until required.
- 5 Approximately 30 to 40 minutes before inoculation, prepare a 96-well microplate with 200 µL of LB broth supplemented with arabinose and kanamycin in each well using a multichannel pipette in the laminar flow hood.

Note

If using a 'cell' control with no plasmid, first fill its respective well with 200 µL of LB broth supplemented with only arabinose. Then, add kanamycin to the remaining media and fill all other wells.

Day 2: 96-Well Overnight Culture

- 6 Remove the library plate from the fridge and, using a 2 µL pipette, inoculate wells A1 - H8 each with a **randomly selected, unique colony** from the plate. Discard the pipette tip for a fresh one after each inoculation.

**Note**

If performing a screening run from a glycerol stocked 96-well plate, then remove the plate from -70°C freezer and place it in laminar flow hood. Quickly but carefully remove the aluminium foil cover. Allow cultures to fully defrost and transfer 3ul from each well to its corresponding well in the fresh 96-well plate using a multichannel pipette.

- 7 Inoculate wells H9 - H11 with one colony from each of the following control plates, respectively: pRPU (relative promoter unit plasmid), pL2 (destination vector backbone), and DH10B (cells with no plasmid). Leave well H12 blank.
- 8 Use the following plate layout diagram for reference.

	1	2	3	4	5	6	7	8	9	10	11	12
a	1	2	3	4	5	6	7	8	9	10	11	12
b	13	14	15	16	17	18	19	20	21	22	23	24
c	25	26	27	28	29	30	31	32	33	34	35	36
d	37	38	39	40	41	42	43	44	45	46	47	48
e	49	50	51	52	53	54	55	56	57	58	59	60
f	61	62	63	64	65	66	67	68	69	70	71	72
g	73	74	75	76	77	78	79	80	81	82	83	84
h	85	86	87	88	89	90	91	92 new RPU	pL2	DH10B	Blank	

Overnight culture plate layout. Letters and numbers in red correspond to the well coordinates of the 96-well plate. Numbers and words in black are ID's used to track samples and controls.

- 9 Carefully cover with Breathe-Easy sealing membrane and place in shaking incubator for 16 hours  1000 rpm, 30°C

Day 3: Plate reader and Flow Cytometry Preparation

- 10 Approximately 1 hour before incubation is finished, following the BCL **supplemented M9 media protocol**, prepare 200 mL of M9 minimal media and filter sterilise it using a Stericup and vacuum pump. Store filter sterilised M9 media away from light until needed.
- 11 Approximately 30 minutes before incubation is finished, make a 50 mL aliquot of the M9 media in a falcon tube and place remaining M9 media back out of the light.
- 12 Prepare a 384-well flat bottom microplate with 90 µL of M9 media supplemented with arabinose and kanamycin in each well using a multichannel pipette in the laminar flow hood.

Note

If using a 'cell' control with no plasmid, first fill its respective wells with 90 μ L of M9 minimal media supplemented with only arabinose. Then, add kanamycin to the remaining media and fill all other wells.

- 13 After incubation of the overnight plate is complete, transfer to the laminar flow hood and carefully remove the Breath-Easy sealing membrane, avoiding splashing of cultures between wells.
- 14 Using a multichannel pipette, transfer 10 μ L of culture to each well in the 384-well second seed plate following the layout specified below. Discard the set of pipette tips for a fresh set after each series of inoculations.

Note

Since the spacing between wells is half the distance in a 384-well plate, wells are skipped using a multichannel pipette. Take some time to study the plate layout diagram to understand how to transfer between them. The approach advised involves aligning both plates side by side with the letter coordinates (a-f) of each plate farthest away. Then, from each column (i.e. 1, 2, 3, etc.) of the 96-well plate, transfer 10 μ L to its respective 4 columns of the 384-well plate. For the next column of the 96-well plate, transfer to the same 4 columns of the 384-well plate but in the wells you skipped previously. Repeat this process for all other columns.

- 15 Use the following plate layout diagram for reference.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
a	1	1	1	1	3	3	3	3	5	5	5	5	7	7	7	7	9	9	9	9	11	11	11	11
b	2	2	2	2	4	4	4	4	6	6	6	6	8	8	8	8	10	10	10	10	12	12	12	12
c	13	13	13	13	15	15	15	15	17	17	17	17	19	19	19	19	21	21	21	21	23	23	23	23
d	14	14	14	14	16	16	16	16	18	18	18	18	20	20	20	20	22	22	22	22	24	24	24	24
e	25	25	25	25	27	27	27	27	29	29	29	29	31	31	31	31	33	33	33	33	35	35	35	35
f	26	26	26	26	28	28	28	28	30	30	30	30	32	32	32	32	34	34	34	34	36	36	36	36
g	37	37	37	37	39	39	39	39	41	41	41	41	43	43	43	43	45	45	45	45	47	47	47	47
h	38	38	38	38	40	40	40	40	42	42	42	42	44	44	44	44	46	46	46	46	48	48	48	48
i	49	49	49	49	51	51	51	51	53	53	53	53	55	55	55	55	57	57	57	57	59	59	59	59
j	50	50	50	50	52	52	52	52	54	54	54	54	56	56	56	56	58	58	58	58	60	60	60	60
k	61	61	61	61	63	63	63	63	65	65	65	65	67	67	67	67	69	69	69	69	71	71	71	71
l	62	62	62	62	64	64	64	64	66	66	66	66	68	68	68	68	70	70	70	70	72	72	72	72
m	73	73	73	73	75	75	75	75	77	77	77	77	79	79	79	79	81	81	81	81	83	83	83	83
n	74	74	74	74	76	76	76	76	78	78	78	78	80	80	80	80	82	82	82	82	84	84	84	84
o	85	85	85	85	87	87	87	87	89	89	89	89	91	91	91	91	93 new RPU	93 new RPU	93 new RPU	93 new RPU	95 DH10B	95 DH10B	95 DH10B	95 DH10B
p	86	86	86	86	88	88	88	88	90	90	90	90	92	92	92	92	94 pL2	94 pL2	94 pL2	94 pL2	96 Blank	96 Blank	96 Blank	96 Blank

Transferral of cultures from 96-well to 384-well plate using multichannel pipette. Letters and numbers in red correspond to the well coordinates of the 384-well plate. Numbers and words in black correspond to the cultures from the 96-well plate designated in the figure above.

- 16 Carefully apply a new Breathe-Easy sealing membrane and place plate in shaking incubator for 3 hours  1000 rpm, 37°C .
- 17 Using a multichannel pipette, add 40 μ L of 50% glycerol to each well of the 96-well overnight culture plate and and pipette up and down to mix. Discard the set of pipette

tips for a fresh set each time. Carefully apply an aluminium sealing film, label the film, and place plate in -70°C freezer.

Note

This glycerol stock plate can be used to inoculate a fresh 96-well overnight culture plate for replicate screening runs or for miniprepping and then sequencing of genetic circuits.

- 18 Approximately 1 hour before the incubation of the second seed is finished, make a 100 mL aliquot of the M9 media and supplement with arabinose. Further divide this aliquot into 4X 25 mL aliquots in separate falcon tubes. Label each aliquot 'None', 'aTc', 'IPTG', and 'Both' and supplement with kanamycin and appropriate inducers.
- 19 Prepare 2X 384-well plates (1 flat bottom and 1 clear bottom) with 95 µL of M9 media supplemented with arabinose, kanamycin and the relevant inducers as specified in the plate layout diagram below.

Note

If using a 'cell' control with no plasmid, first fill its respective wells with 95 µL of M9 minimal media supplemented with arabinose and relevant inducers. Then, add kanamycin to the remaining media and fill all other wells.

- 20 Use the following plate layout diagram for reference.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	None	aTc	IPTG	Both	None	aTc	IPTG	Both	None	aTc	IPTG	Both	None	aTc	IPTG	Both	None	aTc	IPTG	Both	None	aTc	IPTG	Both
a	1	1	1	1	3	3	3	3	5	5	5	5	7	7	7	7	9	9	9	9	11	11	11	11
b	2	2	2	2	4	4	4	4	6	6	6	6	8	8	8	8	10	10	10	10	12	12	12	12
c	10	10	10	10	16	16	16	16	18	18	18	18	20	20	20	20	22	22	22	22	24	24	24	24
d	14	14	14	14	16	16	16	16	18	18	18	18	20	20	20	20	22	22	22	22	24	24	24	24
e	25	25	25	25	27	27	27	27	29	29	29	29	31	31	31	31	33	33	33	33	35	35	35	35
f	26	26	26	26	28	28	28	28	30	30	30	30	32	32	32	32	34	34	34	34	36	36	36	36
g	27	27	27	27	29	29	29	29	31	31	31	31	33	33	33	33	35	35	35	35	37	37	37	37
h	38	38	38	38	40	40	40	40	42	42	42	42	44	44	44	44	46	46	46	46	48	48	48	48
i	49	49	49	49	51	51	51	51	53	53	53	53	55	55	55	55	57	57	57	57	59	59	59	59
j	50	50	50	50	52	52	52	52	54	54	54	54	56	56	56	56	58	58	58	58	60	60	60	60
k	61	61	61	61	63	63	63	63	65	65	65	65	67	67	67	67	69	69	69	69	71	71	71	71
l	62	62	62	62	64	64	64	64	66	66	66	66	68	68	68	68	70	70	70	70	72	72	72	72
m	73	73	73	73	75	75	75	75	77	77	77	77	79	79	79	79	81	81	81	81	83	83	83	83
n	74	74	74	74	76	76	76	76	78	78	78	78	80	80	80	80	82	82	82	82	84	84	84	84
o	85	85	85	85	87	87	87	87	89	89	89	89	91	91	91	91	93	93	93	93	95	95	95	95
p	86	86	86	86	88	88	88	88	90	90	90	90	92	92	92	92	94	94	94	94	96	96	96	96

Layout of 384-well induction plates. Letters and numbers in red correspond to the well coordinates of the 384-well plate. Numbers and words in black correspond to the cultures from the 96-well plate designated in the first figure.

- 21 After incubation of the second seed plate is complete, transfer to the laminar flow hood and carefully remove the Breath-Easy sealing membrane, avoiding splashing of cultures between wells.
- 22 Using a multichannel pipette, transfer 5 µL of culture to each well in the first 384-well induction plate (flat bottom) following the layout specified. Discard the set of pipette tips for a fresh set after each series of inoculations.

- 23 After inoculation of first induction plate, carefully apply a new Breathe-Easy sealing membrane and place plate in shaking incubator for 90 minutes  1000 rpm, 37°C .
- 24 Whilst the first induction plate is incubating, as in step 22, transfer 5 µL of culture to each well in the second 384-well induction plate (clear bottom).
- 25 After inoculation of second induction plate, carefully apply a new Breathe-Easy sealing membrane. Carefully transfer plate to plate reader and run the relevant plate reader protocol.

Note

If using SpectraMax iD5 plate reader, open experiment 25_05_05_ML_384_well_experiment as a protocol in SoftMax Pro 7.1 software and simply run the protocol.

- 26 Whilst the first induction plate is still incubating, prepare 4X 96-well round bottom microplates with 95 µL of chilled PBS supplemented with **100X working concentration kanamycin** ('stop solution').
- 27 After incubation of the first induction plate is complete, transfer to the laminar flow hood and carefully remove the Breath-Easy sealing membrane, avoiding splashing of cultures between wells.
- 28 Using a multichannel pipette, transfer 5 µL of culture to each well in the 96-well 'stop solution' plates following the layout specified below. Discard the set of pipette tips for a fresh set after each series of inoculations.

Note

Take some time to study the plate layout diagrams to understand how to transfer between them. The transferal process here is effectively reversing the previous process performed in step 14. This will produce 4 plates (1 for each of the inducer conditions).

- 29 Use the following plate layout diagram for reference.



Plate 1		(None)										
	1	2	3	4	5	6	7	8	9	10	11	12
a	1	2	3	4	5	6	7	8	9	10	11	12
b	13	14	15	16	17	18	19	20	21	22	23	24
c	25	26	27	28	29	30	31	32	33	34	35	36
d	37	38	39	40	41	42	43	44	45	46	47	48
e	49	50	51	52	53	54	55	56	57	58	59	60
f	61	62	63	64	65	66	67	68	69	70	71	72
g	73	74	75	76	77	78	79	80	81	82	83	84
h	85	86	87	88	89	90	91	92 new RPU	pL2	DH10 β	Blank	

Plate 2		(aTc)										
	1	2	3	4	5	6	7	8	9	10	11	12
a	1	2	3	4	5	6	7	8	9	10	11	12
b	13	14	15	16	17	18	19	20	21	22	23	24
c	25	26	27	28	29	30	31	32	33	34	35	36
d	37	38	39	40	41	42	43	44	45	46	47	48
e	49	50	51	52	53	54	55	56	57	58	59	60
f	61	62	63	64	65	66	67	68	69	70	71	72
g	73	74	75	76	77	78	79	80	81	82	83	84
h	85	86	87	88	89	90	91	92 new RPU	pL2	DH10 β	Blank	

Plate 3		(IPTG)										
	1	2	3	4	5	6	7	8	9	10	11	12
a	1	2	3	4	5	6	7	8	9	10	11	12
b	13	14	15	16	17	18	19	20	21	22	23	24
c	25	26	27	28	29	30	31	32	33	34	35	36
d	37	38	39	40	41	42	43	44	45	46	47	48
e	49	50	51	52	53	54	55	56	57	58	59	60
f	61	62	63	64	65	66	67	68	69	70	71	72
g	73	74	75	76	77	78	79	80	81	82	83	84
h	85	86	87	88	89	90	91	92 new RPU	pL2	DH10 β	Blank	

Plate 4		(Both)										
	1	2	3	4	5	6	7	8	9	10	11	12
a	1	2	3	4	5	6	7	8	9	10	11	12
b	13	14	15	16	17	18	19	20	21	22	23	24
c	25	26	27	28	29	30	31	32	33	34	35	36
d	37	38	39	40	41	42	43	44	45	46	47	48
e	49	50	51	52	53	54	55	56	57	58	59	60
f	61	62	63	64	65	66	67	68	69	70	71	72
g	73	74	75	76	77	78	79	80	81	82	83	84
h	85	86	87	88	89	90	91	92 new RPU	pL2	DH10 β	Blank	

- 30 Cover each of the plates with a Breathe-Easy sealing membrane and carefully place in a 4°C fridge overnight to avoid splashing cultures.

Day 4: Flow Cytometry and Data Collection



- 31 When the plate reader run is complete, export the data in a columnar format to a memory stick and then transfer to personal computer.

Note

The Spectramax ID5 plate reader in the Life Sciences Building will sometimes stop recording before the run is complete, seemingly at random. A cold restart before setting up a run can help mitigate this.

- 32 Set up one additional 96-well round bottom microplate with a single well of rainbow calibration beads. In a microcentrifuge tube, add 200 μ L of PBS with 15 μ L of calibration beads and pipette up and down to mix. Transfer to well A1 of the empty 96-well microplate. Cover plate with a Breathe-Easy sealing membrane.
- 33 Carefully transfer flow cytometry plates and calibration plate to flow cytometer. Run calibration well first and then all other plates.

Note

Talk to flow cytometry technicians about copying my existing protocol across. Existing protocol used 150,000 singlet events as a threshold for each well, shaking between measurements. Samples were ran at a medium flow rate of 60 μ L/min. Running all plates took from 10am-6pm (approximately 2 hours per plate).

- 34 Flow cytometry data was exported as FCS files.