Supplementary Information

High-Throughput Optofluidic Screening for Improved Microbial Cell Factories via Real-Time Micron-Scale Productivity Monitoring

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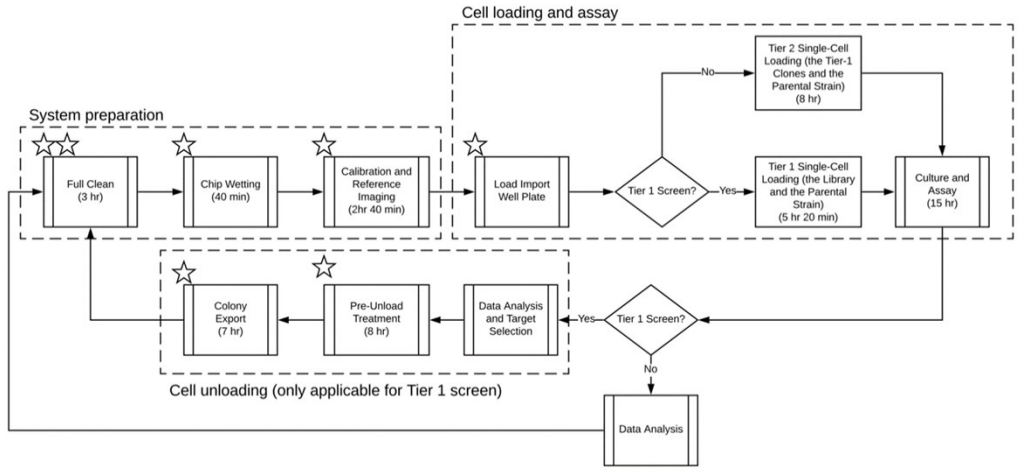
Overview of Beacon screening workflow

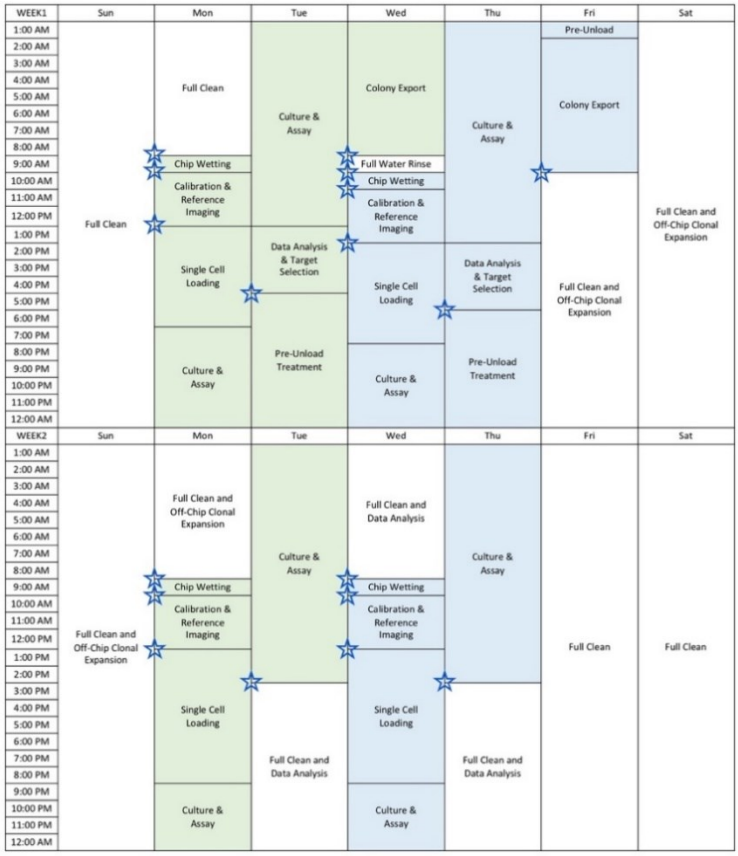
As shown in the flowchart in **Supplementary Figure 1**, the Beacon screening workflow can be divided into 3 major sequences of operations:

- System preparation
  - Instrument cleaning operation: 3 hours of automated cleaning process
  - Chip wetting: 40 minutes
  - Calibration and reference imaging: 2 hours and 40 minutes
- Cell loading and assay
  - Single cell loading: 5 hours and 20 minutes of loading for 4 chips in a Tier 1 screen, and 8 hours of loading for 2 chips in a Tier 2 screen
  - Culture and assay: 15 to 20 hours of on-chip culture and assay
- Cell unloading (only applicable for Tier 1 screen)
  - Data analysis and target selection
  - Pre-unload treatment: 8 hours of overnight pre-unload processes to optimize unload efficiency and reduce clonality risk during unload
  - Unload selected clones: 7 hours for unloading 48 selected clones

The star icons in the flowchart represent human touchpoints such as loading reagents, export plates, etc. Each touchpoint requires less than 10 minutes of involvement. A template of the Beacon screening workflow schedule is demonstrated in **Supplementary Figure 2**. Two independent sets of Tier-1 and Tier-2 screenings can be performed in two weeks with all touchpoints occurring during typical working hours.



Supplementary Figure 1. Beacon screening workflow for the open-pen assay. Stars represent touchpoints.



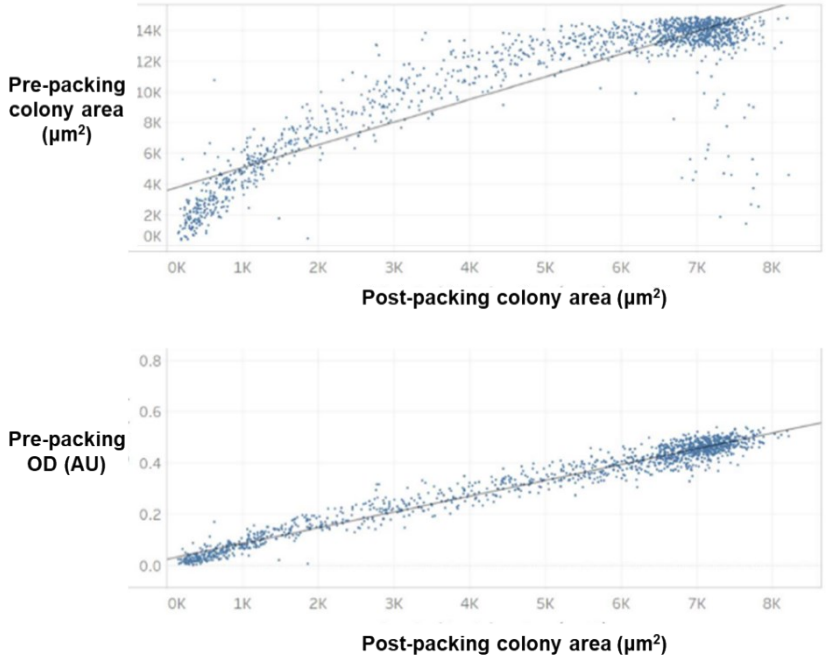
Supplementary Figure 2. Beacon Tier-1 and Tier-2 screening workflow timeline template. In a period of two weeks, two sets of Tier-1 and Tier-2 screenings can be performed. The first and second set of screenings are highlighted in green and blue, respectively. The blue stars represent touchpoints, each with less than 10 min of involvement.

Assessment of measurement error and pen-to-pen phenotypic heterogeneity

Variability in measurements of biomass and productivity among colonies of a single genotype has a number of contributing factors, including:

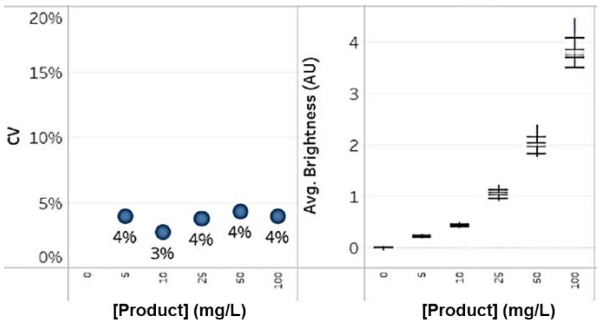
- Biomass measurement error
- Fluorescence measurement error
- Deviation from theoretical steady-state diffusion model (open assay only)
- Intraclonal phenotypic heterogeneity (pen-to-pen, within the same genotype)
    - Process-dominated (e.g., nutrient or temperature gradients)
    - Biological (e.g., epigenetic)
- Intra-colony phenotypic heterogeneity (e.g., spatial condition inhomogeneity)
- Single-cell phenotypic stochasticity (temporal; evident only at very low cell count)

Figure 2 and the corresponding section describe the rough estimation of biomass measurement variability, which amounts to a coefficient of variation (CV) of 10-15% for the range of biomass measured in larger colonies (OD score 0.15-0.30). To maximize assay resolving power, colonies were pre-filtered before analysis, including only those with an OD score above a threshold value of 0.08. Notably, colony area showed poorer linearity with post-packing area, and was not a reliable measurement of biomass (**Supplementary Figure 3**).



Supplementary Figure 3. Comparison of pre-packing colony area and pre-packing OD as measurements of biomass, using post-packing colony area as a reference point for true biomass.

Error in measurement of average fluorescence for a single Nanopen chamber in the sealed-pen assay was estimated by equilibrating an entire OptoSelect chip with the analyte at multiple concentrations and comparing the assay values across the chip. CV values were observed to be < 5% at all relevant concentrations (**Supplementary Figure 4**).



Supplementary Figure 4. Variability measured for fluorescence detection of product using standard solutions perfused through the OptoSelect chip. Every pen in the chip was included in the measurement (n ~ 3500 for each).

Assuming no covariance between fluorescence and OD score, error can be estimated by propagating the errors from the two measurements taken in the assay, which are combined by division in the calculation of normalized score:

$$\sigma_{q_p} \approx \mu_{q_p} \sqrt{(\frac{\sigma_{prod}}{\mu_{prod}})^2 + (\frac{\sigma_{OD}}{\mu_{OD}})^2},$$

where σ and μ respectively represent the standard deviation and mean value of calculated sealed-pen productivity, OD score, or q_p score. This can be equivalently written as:

$$CV_{qP} \approx \sqrt{CV_{prod}{}^2 + CV_{OD}{}^2},$$

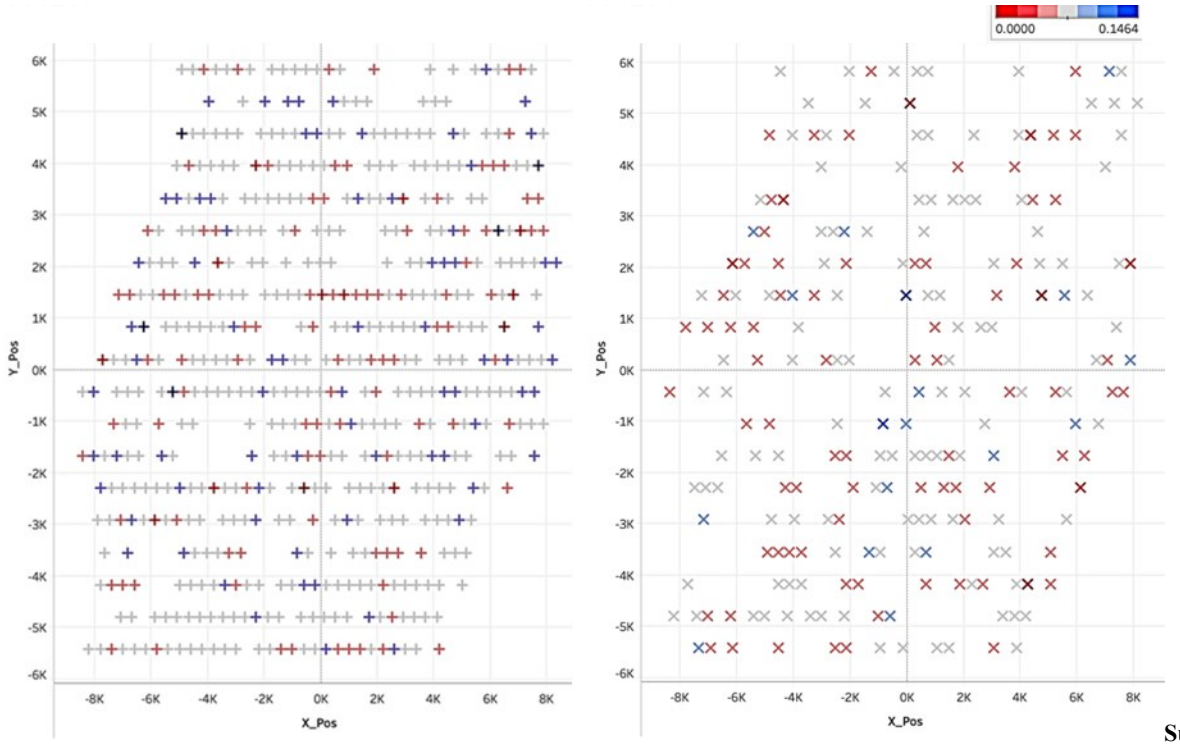
where CV is the coefficient of variation (σ/μ).

We estimated the errors from the biomass and productivity measurements to have a lower bound of 5% and 15%, which gives a rough expected lower bound CV of 16% for the overall assay. This value aligns with the CV values of 15-20% observed for larger colonies of any strain, suggesting that future improvements on the assay noise could probably be achieved by reducing the noise in the biomass measurement.

To estimate residual error due to other sources besides measurement of biomass or fluorescence, the assumption can be made that such errors are additive with the assay variability:

$$\sigma_{obs} \approx \sqrt{\sigma_{assay}{}^2 + \sigma_{residual}{}^2}$$

To assess sources of residual error (σ_{residual} up to ~12% of the mean), tests were performed on several strains for regional bias within an OptoSelect chip. **Supplementary Figure 5** shows representative examples from one experiment. Even under carbon limitation conditions, no obvious spatial bias in the q_p score was observed at any perfusion rate, indicating that media exchange was fast relative to the rate of glucose uptake.



Supplementary Figure 5. Heat maps of relative q_p score under carbon limitation (0.01% glucose), demonstrating no obvious spatial bias in the open-pen assay. The two panels above show special dependence of the performances of two different strains across the same chip.

Only one example of bias was observed during this work, manifested as an edge effect in the sealed-pen assay. This was likely a result of differential access to oxygen, which can easily permeate the walls within the microfluidic chamber, but not the top or bottom electrodes of the chip. It was possible to remove the effect by periodically perfusing air bubbles through the main channel, which ensured an excess of oxygen in the chip.

Long-term sealed-pen yield assay

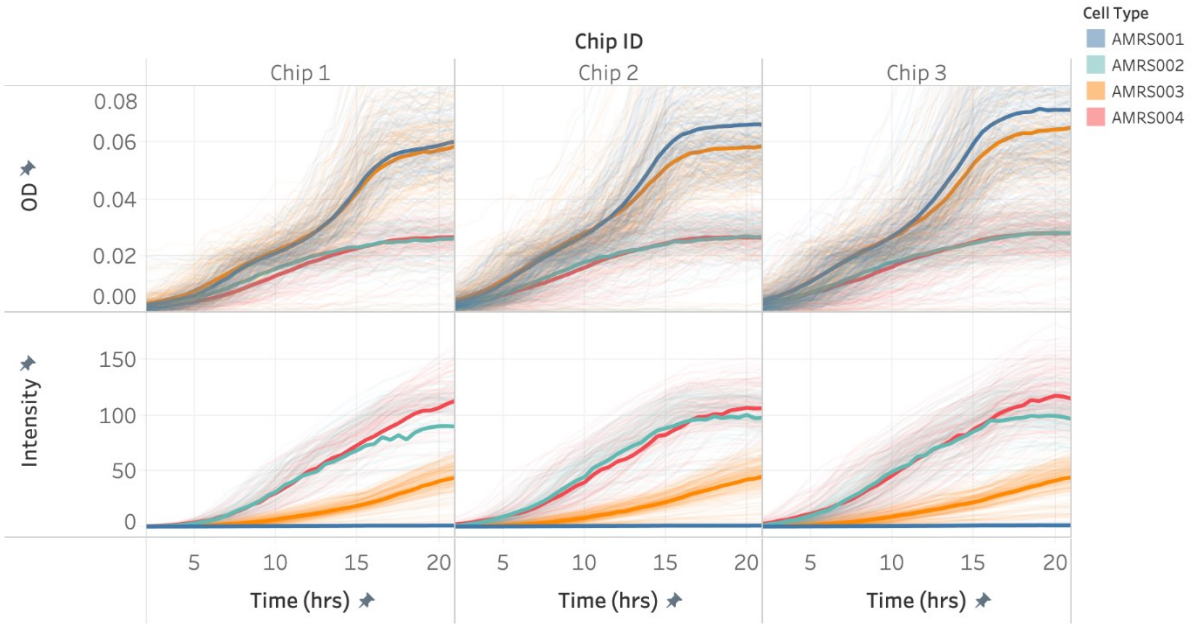
In a proof-of-concept experiment, a long-term sealed-pen assay was introduced to demonstrate the ability to compare average yield of product in an extended batch-like culture model.

After cell loading, the chip was perfused with production media containing 3% sucrose for 60 minutes to supply fresh media to all pens. Fluorinated oil (HFE-7500) was then imported into the main channel to seal each pen for 24 hours. The increases in fluorescence and biomass were monitored over the 24-hour duration by acquiring images every 30 minutes. To supply oxygen to the oil-sealed pens, 5 µL of oil was pushed back and forth through the chip at 0.2 µL/s every 20 minutes. The tubing leading to the chip was highly gas permeable, which allowed reoxygenation of the fluorinated oil. The oxygen solubility of the oil is 100 mL gas/L oil,[1] which is 25-fold higher than that of water (4.8 mL gas/L water).[2] Therefore, the oil perfusion is considerably more effective at oxygenating the pens than media at similar flow conditions.

Supplementary Figure 6 shows the increase of biomass and product concentration in each pen during the 24-hour sealed-pen yield assay. In total, 4 strains were assayed: one wild type (AMRS001), one low producer (AMRS003, ~90 mg/L product titer in microplates), and two

moderate producers (AMRS002 and AMRS004, each with ~450 mg/L titer). Consistent with behavior in plate culture models, the wild type and weak producer accumulated more biomass, while the moderate producers generated a higher fluorescence intensity, indicating that resources were shunted toward product pathway flux.

The long-term sealed-pen assay offers some interesting opportunities that warrant further exploration: (1) tracking growth and productivity under the constraint of limited feedstock provides a direct comparison of resource utilization from strain to strain, a readout complementary to the real-time productivity measurements from the open-pen assay. (2) Because many commercial fermentations are run in fed-batch rather than continuous perfusion conditions, high product and byproduct accumulation can present an obstacle to high performance. Using oil to block the efflux of product may help to apply selective pressure in screening efforts to reduce feedback inhibition and increase product tolerance; likewise, blocking the efflux of toxic byproducts may help screen for strains that produce them at lower concentrations.



Supplementary Figure 6. Sealed-pen yield assay results, showing time-lapse biomass and fluorescence on 3 replicate OptoSelect chips. Each thin line corresponds to a single NanoPen chamber; each thick line shows the average of all NanoPen chambers that contain the same strain within the chip. Biomass is captured by the top row (OD metric), while total accumulated product fluorescence in a NanoPen chamber is shown in the bottom row (Intensity metric).

Loading condition optimization

In the early development phase, we diluted cells for screening in PBS and loaded the sample plate into the well-plate incubator at 4 °C. Cells were then imported onto Chip 1 for the OptoElectroPositioning (OEP) cell loading at 18 °C. The same loading operation was implemented in sequence from Chip 1 to Chip 4. The loaded cells were incubated on chip in PBS at 18 °C until the end of the whole loading process for all 4 chips. However, we consistently observed ascending cell viability across 4 chips in the order of loading, as shown in **Supplementary Figure 7A**. There were 3 major hypotheses:

1. Different temperature cycles between each chip affected cell viability. Cells on Chip 1 were kept in 4 °C PBS for 30 min and 18 °C PBS for the following 6 hours, while cells on nest 4 were in 4 °C PBS for 5 hours and 18 °C PBS for 1.5 hours.
2. Culture conditions immediately following OEP could be important for viability. For instance, post-OEP idle culture using PBS could be detrimental. PBS should be replaced with PR Media quickly after OEP.
3. Increasing PBS exposure prior to OEP improved cell viability. On-chip, cells were only in PBS for 30 min before OEP. It is likely that it takes a few hours for cells to adapt to PBS and experiencing OEP before the adaptation might impact cell viability.

To test the first hypothesis, we changed the loading protocol by importing cells onto all 4 nests prior to penning operation to align temperature conditions across 4 nests. However, the viability gradient was still observed. To test hypotheses 2 and 3, we imported cells that had been incubated in PBS at 4 °C for 4 hours and cells directly transferred from the pre-culture plates in defined yeast growth media. Right after cell loading on each chip, we implemented the post-OEP idle culture at 33.5 °C using defined yeast growth media with 0.1 µL/s perfusion rate, the same as the actual culture and assay condition. The results showed no difference between cells incubated in PBS at 4 °C for 4 hours and cells loaded directly from the preculture plates, which rejected hypothesis 3. Encouragingly, immediate post-OEP culture using PR media at 33.5 °C with 0.1 µL/s perfusion indeed showed promising improvement on overall viability for all chips as well as reduction in chip-to-chip variability in viability, as shown in **Supplementary Figure 7B**.

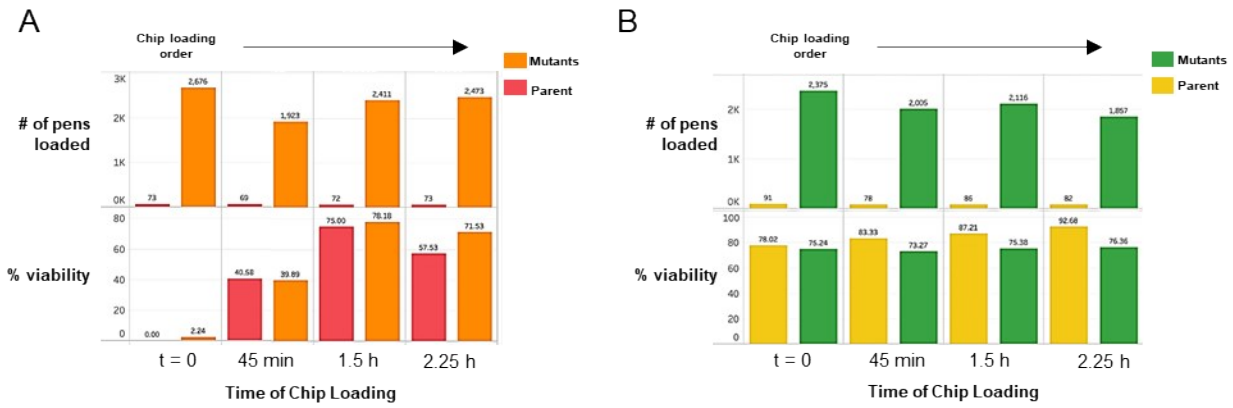
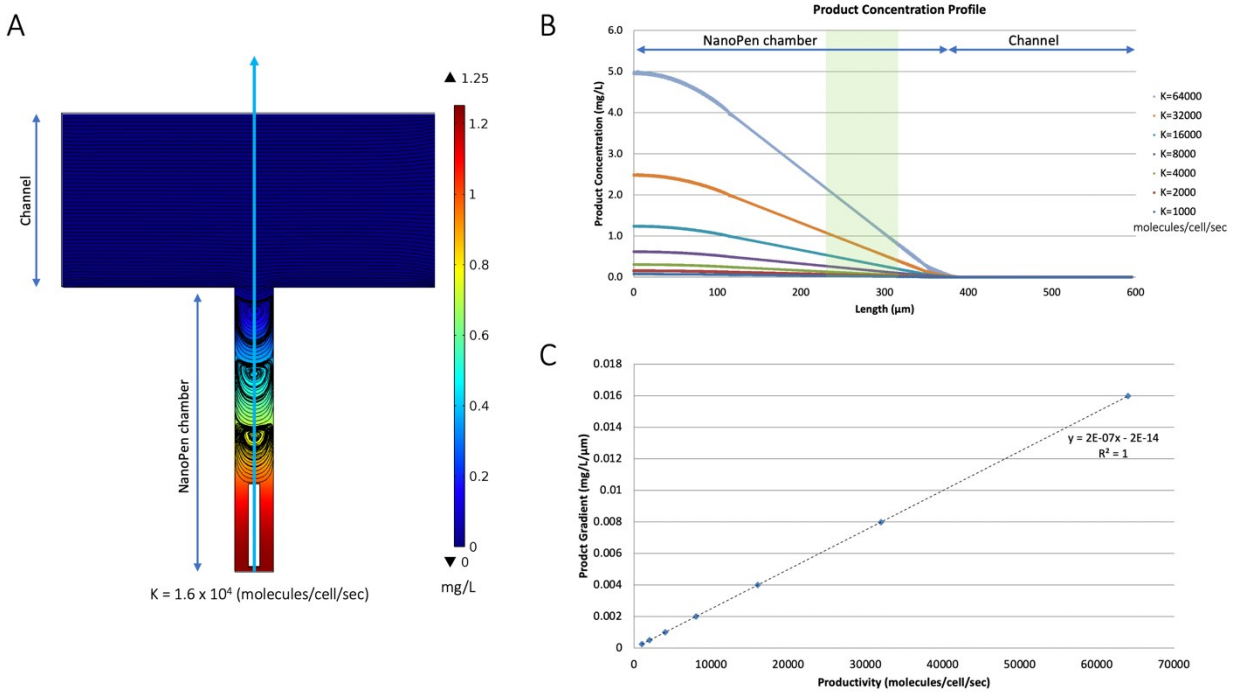


Figure S7. On-chip viability of parent and mutant populations without (**a**) and with (**b**) modification of workflow to initiate prompt perfusion of culture media after OEP in PBS. Note that two different parent strains were used in these experiments. Chips loaded early in panel A were incubated in PBS longer after loading, whereas in panel B there was no relationship between chip loading time and post-loading incubation time in PBS.

Open-pen assay simulation

The goal of the simulation was to verify that product gradient in Nanopen chambers is proportional to the productivity of the cells and therefore can be used to quantify average specific productivity of each clone. A multi-cell model was created using COMSOL Multiphysics software to simulate a colony of cells secreting product of interest at a given rate in a Nanopen chamber during the open-pen assay in which media is flushed through the channels to create an effective sink of product. The colony is approximated by a rectangular source of product, releasing a constant amount of product equivalent to the production by 900 cells with variable productivity *K* (molecules per cell per second).

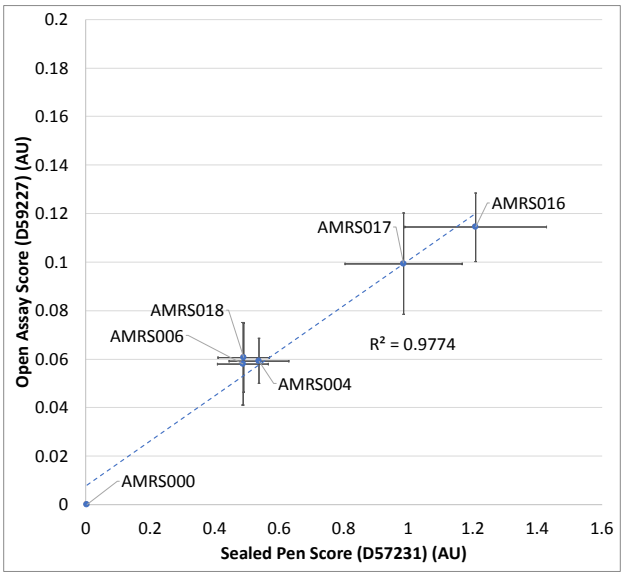
The heat map in **Supplementary Figure 8a** visualizes the distribution of product at steady state in the unit of mg/L during 0.3 µL/s media perfusion, with productivity $K = 1.6 \times 10^4$ molecules/cell/second. **Supplementary Figure 8b** shows the product concentration profiles across the pen, with the x-axis being the distance from the rear wall of the pen (in µm) and the y-axis being product concentration (in mg/L). The concentration increases linearly from the opening of the Nanopen chamber (around 350 µm) to the cell culture region (around 120 µm). The gradients of product in the assay area from 240 µm to 320 µm, marked by the green band in **Supplementary Figure 8b**, were calculated and plotted in **Supplementary Figure 8c** as a function of productivity K from 10^3 to 6.4×10^4 molecules/cell/second. The gradients in the gradient measurement area were indeed linearly correlated with the productivities.



Supplementary Figure 8. Simulation of product distribution during the open-assay using COMSOL Multiphysics. The heat map (**a**) and line profiles (**b**) of product concentration in a single Nanopen chamber. The gradient of concentration in the assay area is proportional to the productivity of cells (**c**).

Correlation between the sealed-pen assay and the open-pen assay

We have quantified the on-chip q_p of a collection of strains using both the open-pen assay and the sealed-pen assay. The average sealed-pen and open-pen assay scores for each strain were well-correlated, with an R-squared of 0.98, as shown in **Supplementary Figure 9**. The open assay was chosen as the primary assay method for correlation analysis and library screening because of its additional advantages, including temporal resolution and minimal interruption during culture.



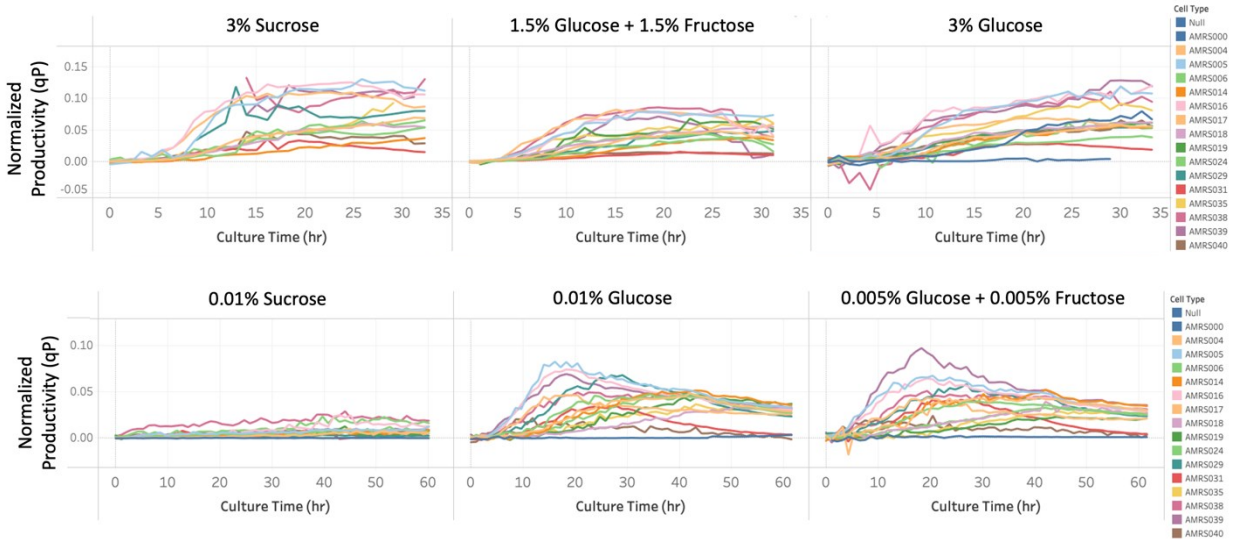
Supplementary Figure 9. The sealed pen assay scores correlate well with the open assay score ($R^2 = 0.98$). The error bars represent the standard deviation of scores of each strain.

Open-pen culture model optimization

Cane syrup largely comprising sucrose is a common feedstock for commercial-scale fermentation, and thus sucrose is frequently used for microtiter plate screens. Glucose, sucrose, and an equimolar mixture of glucose and fructose were tested at 30 g/L total sugar loading in three separate screens (**Supplementary Figure 10**). Similar correlations between Beacon q_p score and bioreactor yield, volumetric productivity, and q_p were observed for all three conditions, with the minimum within-strain variability occurring at assay times between 12 and 20 hours.

In a second set of experiments, the same carbon sources were also tested at 0.1% g/L (**Supplementary Figure 10**). Such conditions are interesting candidates for a culture model because in feedback-regulated and exponentially fed bioreactors, steady-state extracellular sugar concentrations are typically low, a condition that is difficult to sustain in microtiter plate cultures. Growth under carbon limitation required an extended culture period to accumulate sufficient biomass for reliable normalization (> 60 hours), and ultimately yielded poor correlation between Beacon q_p score and bioreactor q_p. Interestingly, analysis of normalized score as a function of colony size indicated a decline in the per-cell productivity in larger colonies under these conditions, which could suggest intra-colony differences in cellular access to sugar.

As summarized in **Supplementary Table 1**, the low sugar culture conditions generally have worse chip-to-bioreactor correlation, lower stability in productivity over time, lower dynamic range, and larger assay CV. The 1.5% glucose + 1.5% fructose was therefore selected to be the carbon source for on-chip production.



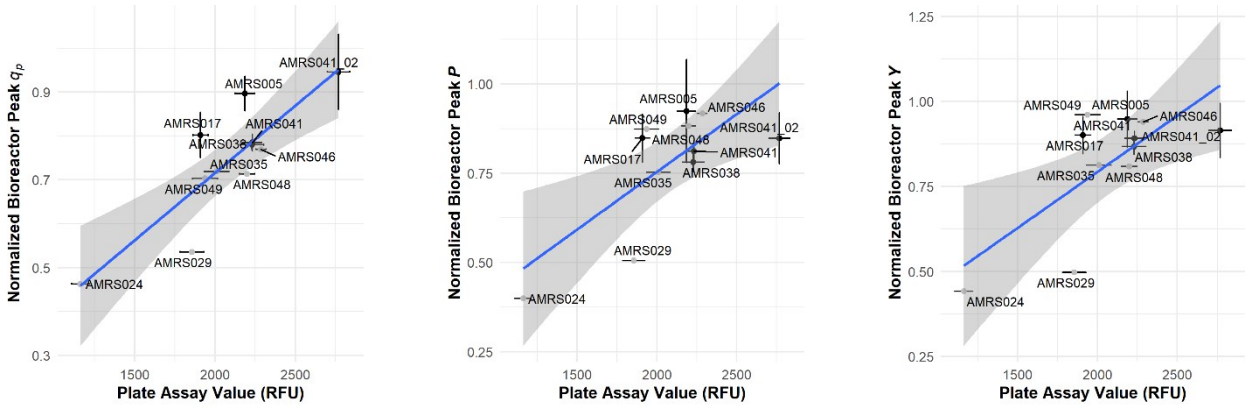
Supplementary Figure 10. The average on-chip q_p score of each strain over time with various carbon sources.

	3% Sucrose	**3% Glucose**	**1.5% Glucose + 1.5% Fructose**	**0.01% Sucrose**	**0.01% Glucose**	**0.005% Glucose + 0.005% Fructose**
Maximum chip-to-bioreactor correlation (observed Pearson's R)	0.80-0.84	0.71-0.77	0.77-0.82	0.23-0.32	0.67-0.70	0.64-0.67
Consistency in chip-to-bioreactor correlation over culture period	High	High	High	Low	Low	Low
Dynamic range of observed q_p scores	High	High	High	Low	Medium	Medium
Typical variability in q_p score for each strain	Low	Low	Low	Medium	Medium	Medium
Assay period	10-24 h	10-24 h	10-24 h	40-50 h	10-20 h	10-20 h

Supplemental Table 1. Head-to-head comparison between various media compositions used in the open-pen assay. 1.5% glucose + 1.5% fructose was selected as the carbon source in the PR media for the final screening conditions shown in the main text of this paper. The maximum chip-to-bioreactor correlation was selected by tracking the Pearson's R observed between Beacon q_p score and bioreactor peak q_p, Y, and P as a function of Beacon assay time.

Plate-tank correlation

As a point of reference for the predictive power of the Beacon assays described, the same set of strains included in the analyses were subjected to evaluation using standard microplate screening workflows at Amyris. Using two different plate models, correlation with any of the three peak bioreactor metrics (q_p, volumetric productivity, or yield) was weaker than the correlation between chip q_p score and bioreactor q_p (**Supplementary Figure 11**).



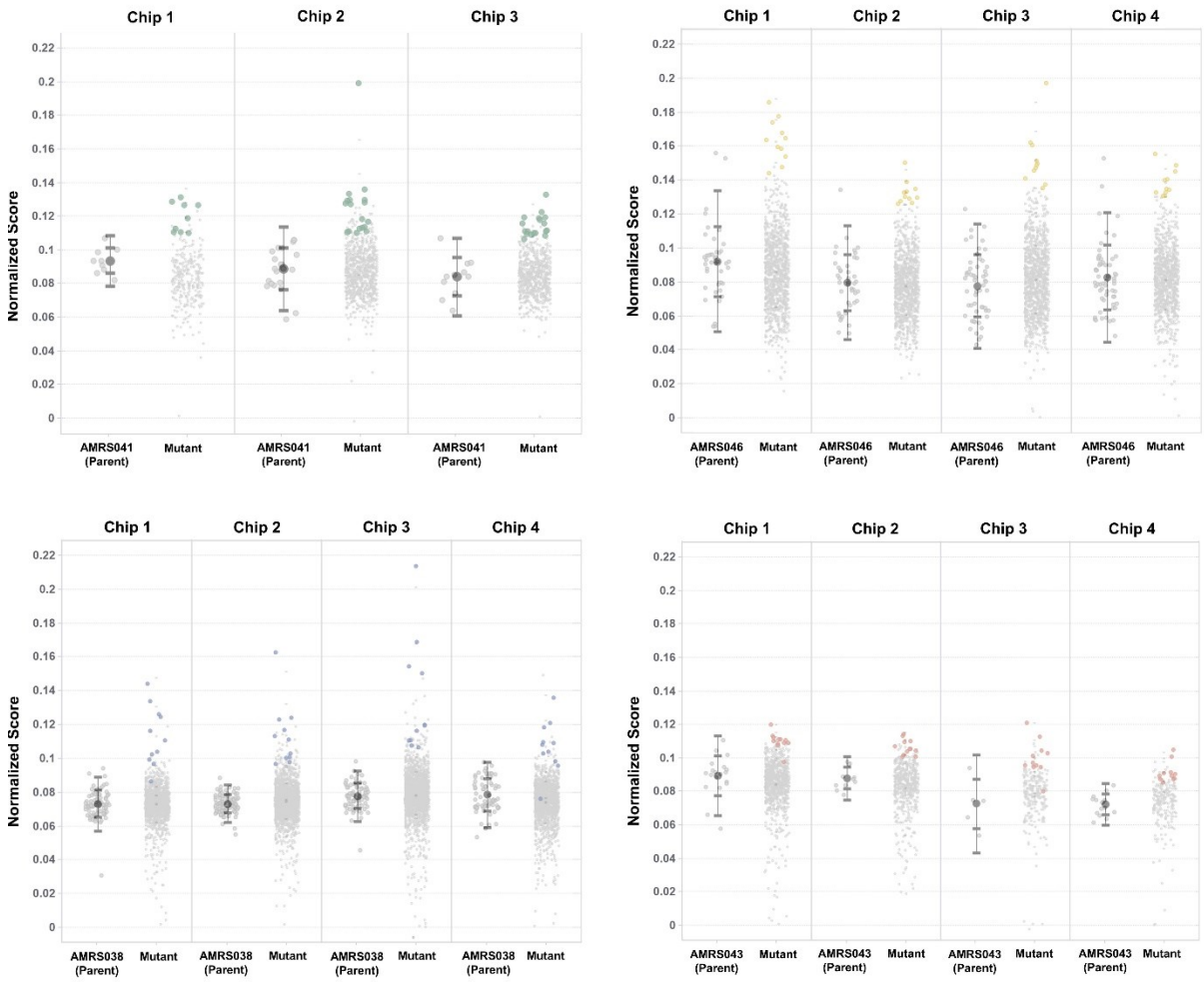
Supplementary Figure 11. Correlation between strain performance in microplate culture models and lab-scale bioreactors. The set of strains and tank data sets are identical to those represented in **Figure 4**. The microplate culture model contained 4% sucrose as the carbon source, and titers were inferred from a fluorescence-based endpoint assay (n = 12 wells per strain, error bars showing standard deviation). Bioreactor data shown are from n = 1-10 independent fermentations, with error bars showing standard deviation. Points in gray indicate that only one bioreactor experiment was run, and error bars are thus omitted. Also shown are least-squares linear regression lines with the 95% confidence intervals shaded. For q_p: $R^2 = 0.73$, $p = 0.00074$; for P: $R^2 = 0.55$, $p = 0.0089$; for Y: $R^2 = 0.47$, $p = 0.012$.

Additional mutagenesis screening results

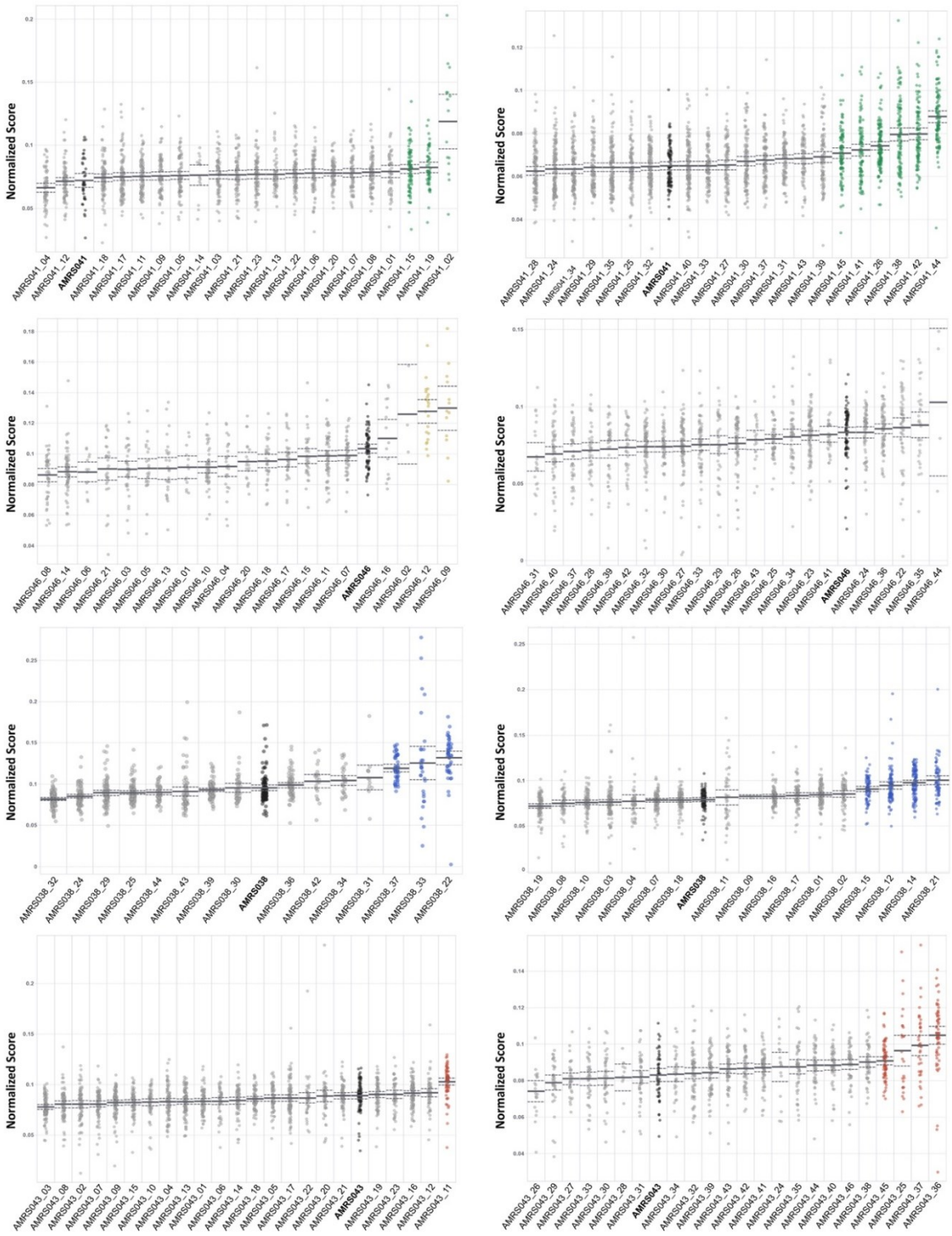
Throughput and data quality measurements from all Tier-1 library screening efforts are summarized in **Supplementary Table 2**. These results indicate that the open-pen assay can deliver throughput and reproducibility comparable to a microplate screen of 2000-6000 mutants, in a variety of background genotypes. The remaining Tier-1 and Tier-2 data not graphically represented in the main text are shown in **Supplementary Figures 12-14**.

Supplementary Table 2. Results of Tier-1 library screening demonstration. Total throughputs exclude colonies from NanoPens in which multiple cells were inadvertently loaded during OEP. On-chip viability indicates the percentage of penned cells that grew into colonies surpassing an OD score threshold of 0.05.

Mutagenesis Parent Strain:	**AMRS038**	**AMRS041**	**AMRS043**	**AMRS046**
Total throughput (monoclonal colonies screened):	**5,861**	**3,306**	**1,969**	**5,144**
Single cells penned:	6,371	6,482	2,661	6,858
On-chip viability (%):	92	51	74	75
Successful exports (%):	**77**	**93**	**100**	**91**
Parent strain CV (%):	**12**	**11**	**10**	**22**

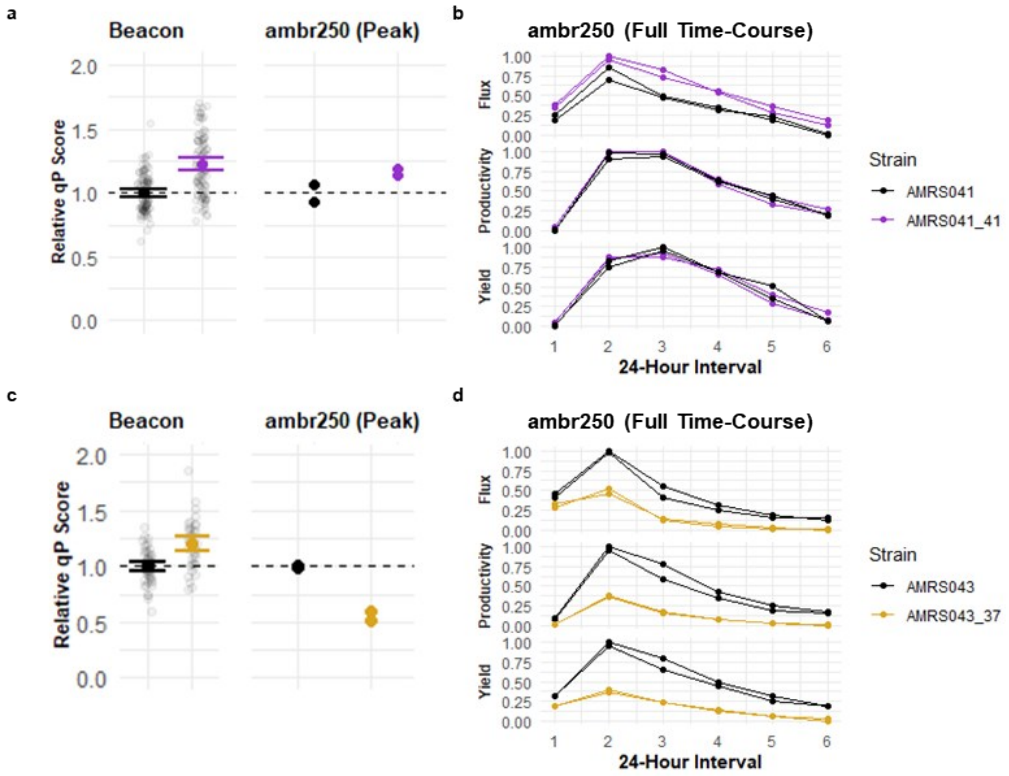


Supplementary Figure 12. Summary of all results from Tier-1 mutagenesis screening data. Isolates chosen for Tier-2 testing are shown in a non-grey color. Error bars show one and two standard deviations above and below the mean for the parent strain on each chip.



Supplementary Figure 13. Summary of all results from high-replication Tier-2 screens from four mutagenesis and screening processes. Each mutagenesis process is split into two screens on separate chips. Mutants shown here were hits from the screens shown in **Supplementary Figure 11**. The parent strain for each screen is shown in black, and all strains statistically improved over the parent (Dunnett's test of multiple comparisons, $p < 0.05$) are shown in

color. Error bars (dashed lines) represent the 95% confidence interval of the population mean. Colors correspond to the Tier-1 screens shown in **Supplementary Figure 11** from which the mutants were chosen.



Supplementary Figure 14. Results from mutagenesis hits tested in ambr250 bioreactors that were not shown in **Figure 6**. (**a** and **c**) Comparison of the q_p scores measured in the Beacon open-pen assay and the peak (Interval 2) q_p measured from each ambr250 bioreactor run. Strains are color-coded, with black representing the parent strain in each screen. Beacon scores for mutants were normalized to the parent strain analyzed on the same chip, shown to their left in black. Bioreactor experiments were performed in duplicate, with each biological replicate shown explicitly and each point normalized to the mean of the peak q_p values measured for the parent strain. (**b** and **d**) Time-course data for ambr250 fermentations run with each parent strain and mutant. Each point indicates the performance metric (specific productivity q_p, volumetric productivity P, or yield with respect to sugar Y) calculated over a 24-hour interval. The bioreactor measurements are normalized to the highest value shown in each graph.

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