Phenotyping an Onyx™ Cell Library A Compendium

The Onyx platform enables the rapid generation of thousands of edited cells, collectively known as an Onyx Cell Library. After an Onyx Cell Library is generated by the Onyx platform, the resulting cell population is ready for phenotypic evaluation. Phenotyping will vary by research program, and the Onyx user should define and design each experiment accordingly. Most broadly, phenotyping of Onyx Cell Libraries will fall into one of two distinct categories:

- Pooled Phenotyping. The goal of a pooled phenotyping experiment is to identify programmed edits that provide a fitness advantage to the strain variants in a population under specific cultivation conditions (e.g. to identify genetic changes that provide resistance to an antibiotic or a growth advantage under certain stress conditions such as extreme temperature, pH or a chemical challenge).

- Isolate Phenotyping. The Onyx Cell library can also be used to phenotype isolates for a variety of end outcomes. One good example is for identifying strain variants producing higher titers of a given analyte or protein (e.g. to identify genetic changes that provide an increased synthesis of a small molecule, peptide or protein, such as lysine, cannabinoid or hemoglobin).

In general, pooled phenotyping workflows are relatively straightforward to perform and require little to no high-throughput equipment. Isolate phenotyping workflows usually require more extensive automation and liquid handling equipment to support a higher throughput.

The goal of this tech note is to provide an overview of pooled and isolate phenotyping workflows. The isolate phenotyping workflow is exemplified for a typical metabolic engineering approach.

How many Onyx Cells are needed for a phenotyping experiment?

The number of cells needed to successfully conduct a phenotyping experiment depends on both phenotyping approach and library composition. Library composition is defined by the diversity of designs in the library and fraction of edited cells. When employing a forward engineering approach for strain optimization, it is not necessary to screen every variant in a population to find everything of value, since the method seeks to improve on an given phenotype through diversity generation and subsequent combinatorial optimization. Importantly, discovery of numerous beneficial hits can be achieved with shallow sampling of a sufficiently large and diverse library when the number of unique variants screened is emphasized rather than allocation of resources to complete coverage of the population. As the single-edit hits obtained from the diversity generation phase are recombined, the number of possible configurations becomes very large very quickly. For example, if a diversity generation library containing 10,000 unique variants returns 100 hits (which is just 1% of the library), the combinatorial complexity of those 100 beneficial variants, would yield 10^{30} possible configurations. However, despite this stupefying large combinatorial space, the opportunity to make large fitness leaps can be achieved rapidly and efficiently by screening a small number of samples (e.g. 1,000 samples) to identify an improved combinatorial variant strain, and then layering in additional combinatorial edits onto this strain in an iterative screening and editing

methodology. In the end, a final winning strain can be generated from having only built and tested a total of a few thousand samples in iterative stages over a period of weeks – saving significant time and money. One approach to combining edits is provided in Figure 1.

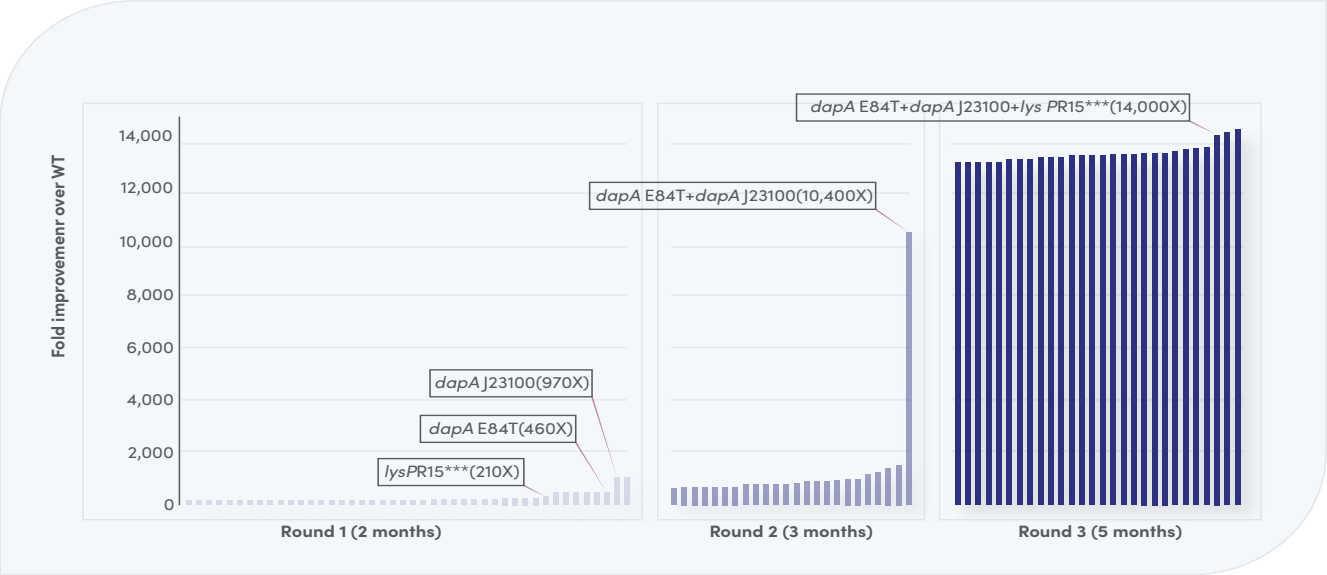

Figure 1. Lysine production: an example of the power of recombination.
This figure shows a massive increase in lysine production through an iterative approach of combining beneficial edits. One variant from Round 1 was selected as the host for building libraries in Round 2 and so on. A 14000X increase of lysine production over WT was achieved under half a year.

To plan the optimal number of cells going into a phenotyping experiment, Inscripta has developed a Selector's Score (for pooled phenotyping) and a Screener's Score (for isolate phenotyping). The scores are generated by InscriptaResolver™ (Resolver) and are based on the data collected using the Onyx genotyping assays. The genotyping assays characterize the library composition. Selector's score estimates the fraction of total Onyx edits likely to be observed in a selection experiment inoculatd wtih 10^6 cells. It is dependent on the distribution of designs in the library and assumes 30% of cells contain an Onyx edit. The Screener's Score estimates the fraction of the total Design Library that is expected to be observed as Onyx Edits in a typical screen (assuming the Onyx Edit Fraction is 30% and the number of cells tested is equal to the number of designs in the Design Library Order).

Resolver also generates the Selector's Curve and the Screener's Curve. These curves are visualizations of the scores based on Onyx Edit Fractions ranging from 5% to 100% plotted as the number of designs by the number of CFUs tested or number of designs by number of isolates, respectively. These curves can be use to estimate the number of cells needed for phenotyping in order to ensure sampling of a certain number of unique edits.

In the example provided in Figure 2, the dark blue represents 100% Onyx Edited Cells, the magenta curve represents 50% Onyx Edited Cells and the light blue curve represents 25% Onyx Edited Cells. The magenta is adjusted using the % of Onyx Edited Cells determined with the edit identification assay. In the example

provided in Figure 2, the dark blue represents 100% Onyx Edited Cells, the magenta curve represents 50% Onyx Edited Cells and the light blue curve represents 25% Onyx Edited Cells. The magenta is adjusted using the % of Onyx Edited Cells determined with the edit identification assay. For a pooled phenotyping experiment (figure 2 left) it is estimated that close to 100% of the edits will be sampled (even with 50% Onyx edit fraction) if 10k CFUs or more are used as a standard inoculation. For an isolated phenotyping screen (figure 2 right), 1,650 colonies are needed to reliably observe approximately 130 unique edits.

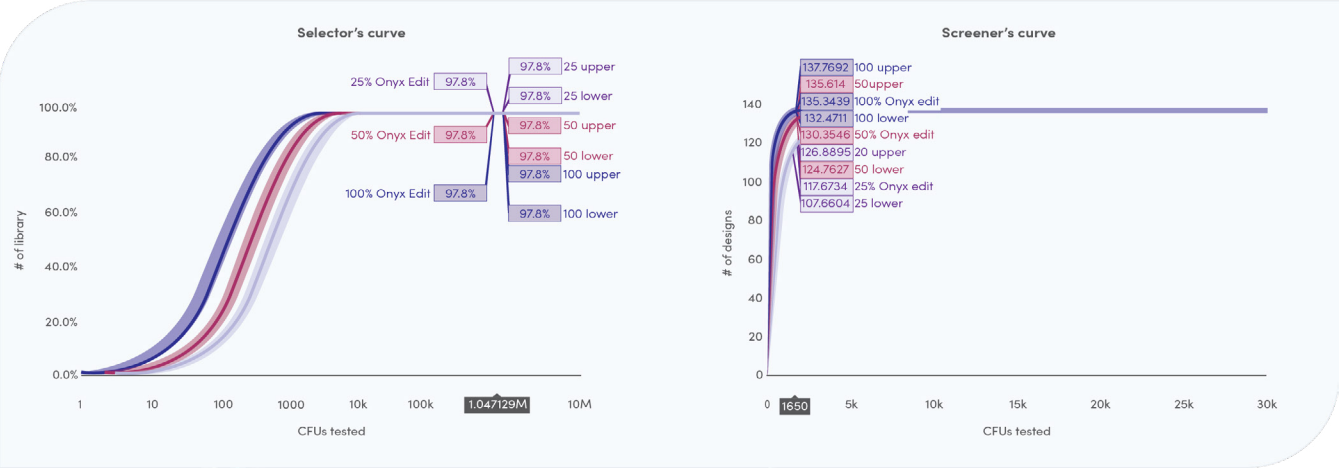

Figure 2. Selector's Curve (left) and Screener's Curve (right).

Pooled Phenotyping Workflow

Pooled phenotyping does not typically require laboratory automation infrastructure. A typical workflow will consist of a relatively simple three- to four-step process, usually involving nothing more than growth media, tubes/flasks and the cell cultivation equipment needed for the specific conditions that will be applied to the samples. An example of pooled phenotyping experiment is described in our application note: Massively Parallel Genome Engineering Followed by Pooled Frowth Selections for Rapid Target Discovery in Microbes (Doc. # 1001836).

Step 1: Prepare Onyx Cell Library aliquots

1. Calculate the number of aliquots of Onyx Cell Library to prepare for the phenotyping experiment based on the number of conditions to be tested and the number of replicates desired. Conditions could be variable temperature, pH or challenge with a particular chemical at a fixed or gradient concentration. Select a range of conditions to test and divide the range into distinct intervals, which can be applied to each of the individual samples separately. The range of conditions should be determined in advance in order to maximize biological signal while avoiding application of too little or too much selective pressure. For example, if resistance to high temperature is the condition being evaluated, but temperatures greater than 50°C are fatal to the strain, a possible temperature testing range and set of intervals might be 30°C, 35°C, 40°C, 45°C and 50°C.

2. Follow the instructions provided in the Onyx Cell Library Handbook (Doc. # 1001942 for *E. coli* and 1001943 for *S. cerevisiae*) for cell library outgrowth and preparation of the appropriate number of aliquots required for your phenotyping experiments.

Step 2: Cell Growth

3. Grow the cells in the specific experimental conditions.

Step 3: Genotype

4. Extract DNA following the instructions provided in the Onyx Cell Library Handbook (see Doc. # 1001942 for *E. coli* and 1001943 for *S. cerevisiae,* for instructions).
5. Genotype the DNA using the Barcode Diversity Assay (see Onyx Genotyping Handbook, Doc. # 1001182, for instructions).
6. Import the NextGen Sequencing Data into Resolver and determine the edits that provide a growth advantage under the specific condition (see Onyx Genotyping Handbook, Doc. # 1001182, for instructions) .

Isolate Phenotyping Workflow

Isolate phenotyping requires more equipment and resources than pooled phenotyping. Here is an outline of the steps in isolate phenotyping, as used in metabolic engineering, and the time and requisite materials for each step (figure 3).

Step 1: Plate cell library for colonies

1. Plate an aliquot of the Onyx Cell Library on a petri dish (low throughput) or QTray (high throughput).
2. Grow cells until colonies are visible.

Step 2: Growth normalization in microtiter plates

3. Create plate and sample ID/barcode tags using LIMS of choice, and apply tags to microtiter normalization plates using an automated workstation.
4. Add growth media to each normalization plate (e.g. 300 uL/well) using the automated workstation.
5. Using a colony picker, pick colonies from the petri dish or QTray and transfer them into the wells of a normalization plate.

Note: Due to the variability of colony picking, some wells may have more initial cells than others. The number of cells is normalized by growing the liquid cultures to stationary phase.

6. Grow the cells in a shaker-incubator (e.g. 250 rpm, 50 mm throw, 85% humidity) until the cultures reach stationary phase.

Step 3: Growth production in microtiter plates

7. Create plate and sample ID/barcode tags using LIMS of choice, and apply tags to microtiter production plates using an automated workstation.
8. Add production media to each production plate (e.g. 285 uL/well) using the automated workstation.
9. Transfer 15-20 uL from the normalization plate to the production plate using a liquid handler (e.g. Tecan or Agilent™ Workstation).
10. Production plates are placed into the shaker-incubator for 1-3 days (e.g. 250 rpm, 50 mm throw, 85% humidity) for both cell growth and production of the analyte of interest.

Step 4: Sample preparation for phenotyping analysis

11. Samples are processed or prepared for a variety of detection techniques (e.g. plate reader, Gas Chromatography (GC), Liquid Chromatography (LC), Mass Spectrometry (MS) etc.). The preparation method depends on the detection technique used.

Step 5: Phenotyping analysis

12. Analyte of interest is detected using the appropriate method (e.g. plate reader, GC, LC, MS etc.). Depending on the instrument and number of samples being analyzed, the detection time can range from a few hours to many days.
13. Library variant strains of interest (typically samples with higher analyte production) are identified as hits.

Step 6: Genotyping

14. DNA is extracted (see Onyx Cell Library Handbook, Doc. # 1001942 for E. coli and 1001943 for S. cerevisiae, for instructions) from each hit strain.
15. The DNA is prepared for sequencing using the Edit Identification Assay (see Onyx Genotyping Handbook, Doc. # 1001182, for instructions).
16. Sequencing data are imported into Resolver to identify the genotype of the hit variant strains (see Onyx Genotyping Handbook, Doc. # 1001182, for instructions).

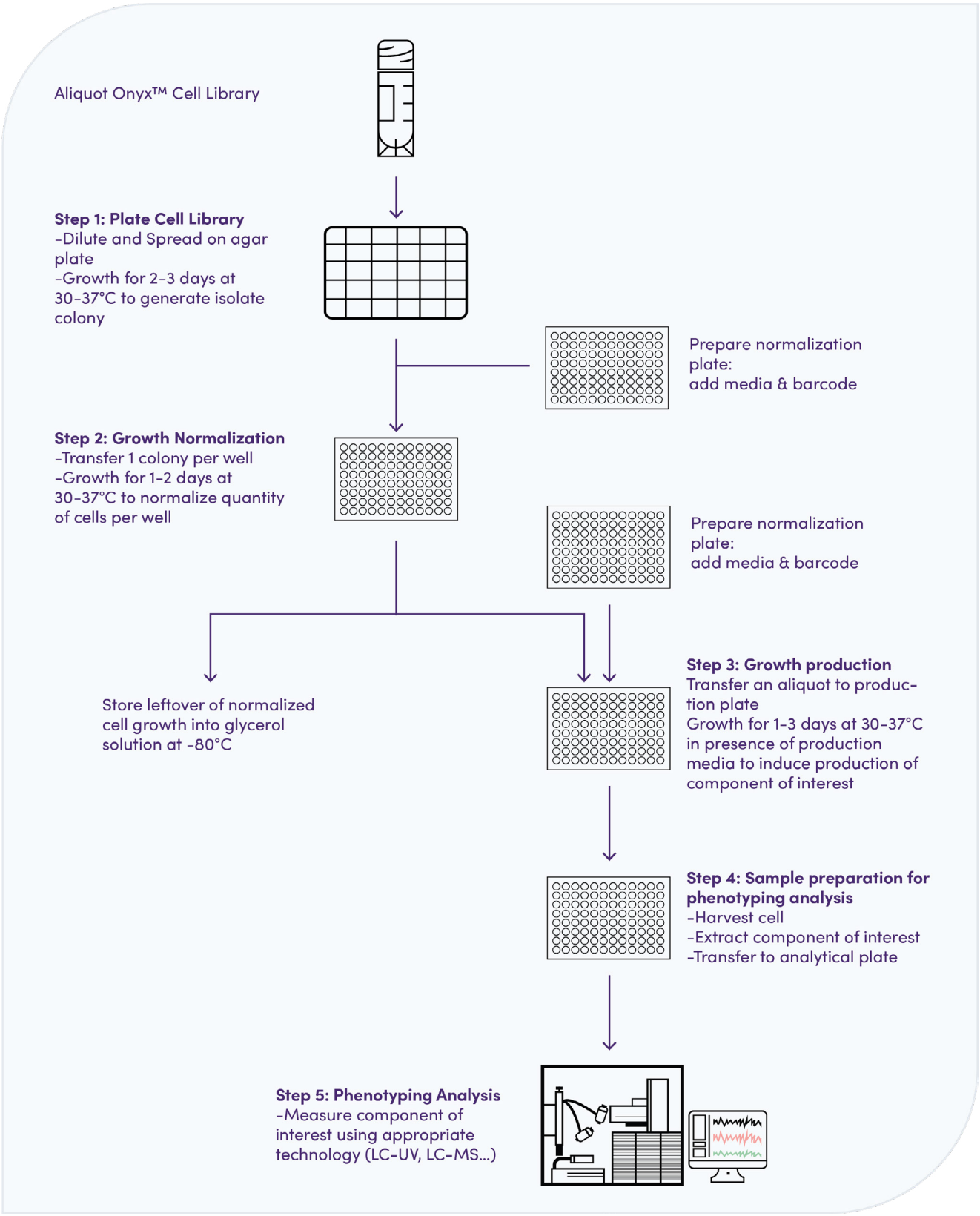

Figure 3. Isolate Phenotyping Workflow.

Step #	Step Name	Sub-step	Duration	Instrument/reagent/consumable for high throughput workflow
1	Colony growth		2-3 days	• QTray enables 1,000-5,000 colonies/agar tray
2	Normalization growth	Prepare plate: add media	1-2 hours	• Square mid-well 96-well plates • Media (e.g. YT, TB or synthetic for E. coli; YPD, YPG or synthetic for S. cerevisiae) • Automated liquid handling (e.g. Agilent Automation Workstation)
		Prepare plate: barcode		• LIMS • Automated liquid handling (e.g. Agilent Automation Workstation)
		Pick colony from Qtray into normalization plates	1-5 hours	• Colony Picker (e.g. Molecular Devices QPix™)
		Growth plate	1-2 days	• Shaker-incubator with 100 plates capacity (e.g. Kuhner Shaker-Incubator)
3	Production growth	Prepare plate: add media	1-2 hour	• Square mid-well 96-well plates • Media : specific to metabolite being produced (e.g. media 1X M9 supplemented with 100uM CaCl2, 10uM FeSO4, 2mM MgSO4, 1% glucose, 100ug/ml carbenicillin, 25ug/ml chloramphenicol, +/- AEC @ 3mM for lysine production – figure 1) • Automated liquid handling (e.g. Agilent Automation Workstation)
		Prepare plate: barcode		• LIMS • Automated liquid handling (e.g. Agilent Automation Workstation)
		Transfer isolates from normalization plates to production plates	1-5 hours	• Automated liquid handling (e.g. Agilent Automation Workstation)
		Growth plate	1-3 days	• Shaker-incubator with 100 plates capacity (e.g. Kuhner Shaker-Incubator)
4	Sample preparation	Extraction - dependent of metabolite of interest	1-5 hours	• Solvents • Plate heat sealer (e.g. Agilent PlateLoc™) • Plate mixer (e.g. Eppendorf MixMate®) • Plate centrifuge (e.g. Eppendorf 5920R) • Automated liquid handling (e.g. Agilent Automation Workstation)

Step #	Step Name	Sub-step	Duration	Instrument/reagent/consumable for high throughput workflow
5	Phenotyping analysis	dependent of metabolite of interest	2-3 hours	Plate reader. Analytes: Small Molecule & Proteins; Sensitivity: ≥ ~1,000 mg/L; Speed: 1-2 min/plate; Measure Type: Direct / Indirect
			1-7 days	LC – UV. Analytes: Small Molecule & Proteins; Sensitivity: ≥ ~1000 mg/L; Speed: 1-4 min/sample; Measure Type: Direct
			7 days or more	LC – MS. Analytes: Small Molecule & Proteins; Sensitivity: ≥ ~0.1 mg/L; Speed: 1-4 min/sample; Measure Type: Direct
			21 days or more	GC – FID/MS. Analytes: Small Molecules; Sensitivity: ≥ ~0.1 mg/L; Speed: 3-5 min/sample; Measure Type: Direct
			1 day (19-27 hours)	RapidFire – MS. Analytes: Small Molecule & Proteins; sensitivity: ≥ ~0.1 mg/L; Speed: 7-10 sec/sample; Measure Type: Direct

Table 1. Instruments, Reagents, Consumables for High-Throughput Isolate Phenotyping.

Phenotyping of genetically diverse libraries is a critical step for biologists, which when executed with appropriate insight and expertise, ensures extraction of maximum value from any library design. Inscripta facilitates the process with the Onyx benchtop digital genome engineering platform and supporting analysis software to enable purposeful test sizes (guided by the Selector's and Screener's Scores and Curves) and efficient resource optimization for goal-driven phenotyping.

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