PHENOS: a high-throughput and flexible tool for microorganism growth phenotyping on solid media

David B. H. Barton[1], Danae Georghiou[1], Neelam Dave[1], Majed Alghamdi[1], Thomas Walsh[1], Edward J. Louis[1±], Steven S. Foster[1*]

[*]Corresponding author: ssf5@le.ac.uk

[±]Co-corresponding author: ejl21@le.ac.uk

[1]Department of Genetics & Genome Biology, University of Leicester, Leicester LE1 7RH, UK.

Other email addresses: D.B.H.B.: dbh8@le.ac.uk; D.G.: dg217@le.ac.uk; N.D.: njd25@le.ac.uk; M.A.: mafa2@le.ac.uk; T.W.: tw164@protonmail.com

Abstract

BACKGROUND Microbial arrays, with a large number of different strains on a single plate printed with robotic precision, underpin an increasing number of genetic and genomic approaches. These include Synthetic Genetic Array analysis, high-throughput Quantitative Trait Loci (QTL) analysis and 2-hybrid techniques. Measuring the growth of individual colonies within these arrays is an essential part of many of these techniques but is useful for any work with arrays. Measurement is typically done using intermittent imagery fed into complex image analysis software, which is not especially accurate and is challenging to use effectively. We have developed a simple and fast alternative technique that uses a pinning robot and a commonplace microplate reader to continuously measure the thickness of colonies growing on solid agar, complemented by a technique for normalizing the amount of cells initially printed to each spot of the array in the first place. We have developed software to automate the process of combining multiple sets of readings, subtracting agar absorbance, and visualizing colony thickness changes in a number of informative ways. **RESULTS** The "PHENOS" pipeline (phenotyping on solid media), optimized for *Saccharomyces* yeasts, produces highly reproducible growth curves and is particularly sensitive to low-level growth. We have empirically determined a formula to estimate colony cell count from an absorbance measurement, and shown this to be comparable with estimates from measurements in liquid. We have also validated the technique by reproducing the results of an earlier QTL study done with conventional liquid phenotyping, and found PHENOS to be considerably more sensitive. **CONCLUSIONS** "PHENOS" is a cost effective and reliable high-throughput technique for quantifying growth of yeast arrays, and is likely to be equally very useful for a range of other types of microbial arrays. A detailed guide to the pipeline and software is provided with the installation files at https://github.com/gact/phenos.

Keywords

Arrays; Yeast; Phenotyping; QTL; SGA; Microbes; Growth

Background

Around the turn of the new millennium, following developments in DNA microarray technology, microbial geneticists began to work with large regularly ordered arrays of colonies of genetically distinct microorganisms. For example, *Saccharomyces cerevisiae* deletion mutant arrays were announced in 1999 [1] and made it possible to rapidly screen the whole yeast genome for synthetic lethal interactions in Synthetic Genetic Array (SGA) analyses [2, 3]. That same technique was soon expanded to other species like the fission yeast *Schizosaccharomyces pombe* [4], and the bacterium *Escherichia coli* [5]. Other labs were also putting gene expression libraries into *E. coli* or in *S. cerevisiae* arrays for high-throughput screening [6, 7].

As these approaches gained ground, robotic colony manipulation systems became commercially viable tools to help create and duplicate these microbial arrays. For example, the VersArray™ Colony Picker and Arrayer Systems from BioRad Laboratories, the QPix 400 Series Microbial Colony Pickers from Molecular Devices, and the easy-to-use ROTOR HDA Colony Manipulation Robot (Singer Instruments in Somerset, UK) which was specifically conceived for SGA analysis.

In just the last few years, the ROTOR HDA system alone has been used for purposes as diverse as screening *S. cerevisiae* for thermotolerance [8], working with *S. pombe* auxotroph deletion libraries [9], phenotyping the wild yeast *Lachancea quebecensis* [10], screening mutagenized strains of the unicellular alga *Chlamydomonas reinhardtii* [11], and generating *E. coli* synthetic genetic arrays [12]. The ROTOR HDA uses disposable plastic 'repads' of pins to print cells from one array to another in a single action. Long-pin repads can be used to transfer material to or from microtitre plates containing liquid cultures and to or from solid agar lawns in rectangular dishes (such as Singer's own proprietory 'PlusPlates™'). 96 spot arrays can easily be combined into 384 arrays, or 384 into 1536, by superimposing each source array with an offset. Larger arrays can easily be broken down into multiple smaller arrays by reversing that process.

64 Microbial growth can be quantified in liquid media in microtitre plates, and analysed using packages
65 such as GrowthRates [13] or GATHODE [14]. It can most accurately be measured by measuring
66 turbidity using specialist machines such the Bioscreen C (Oy Growth Curves Ab Ltd) [15, 16] which
67 holds two 100× 600 µl sample plates with a proprietary honeycomb format meant to create more
68 even heat distribution through the samples. However, it is challenging to simultaneously inoculate
69 these hexagonal-grid wells from the rectangular-grid high-density ROTOR arrays, and to find a level
70 of agitation sufficient to prevent flocculation that does not introduce bubbles or risk cross-
71 contamination between wells. Another solution, the oCelloScope (Philips), uses standard microtitre
72 plates but can only read 96 wells at once. The Omnilog (Biolog) can handle 50x 96-well plates
73 simultaneously, but at a considerable cost and footprint.
74
75 It is becoming more common and is certainly more convenient to measure the growth of whole
76 colonies growing on solid agar lawns in rectangular dishes. This is usually achieved by feeding digital
77 snapshots, taken intermittently, into sophisticated image-analysis software, such as the Screenmill
78 Colony Measurement Engine [17] (as open-source macro for the Java-based ImageJ software),
79 Colonyzer [18] (a command-line app written in Python 2.7), YeastXtract [19] or the Colony-Live
80 imaging system [20]. Specialist instruments have now become commercially available which perform
81 this imaging and analysis in an automated fashion, for example the Phenobooth (Singer Instruments,
82 Somerset, UK). Such an instrument greatly simplifies the process, and incorporating incubation and
83 imaging functions into the same device overcomes limitations in the number of timepoints at which
84 measurements are taken. However, image-based solutions remain vulnerable to confusion by specks
85 and bubbles, particularly when measuring small colonies.
86
87 To overcome problems with these existing approaches and save time and money, we developed an
88 alternative technique which uses a commonplace microplate reader (BMG Labtech's FLUOstar
89 Omega, which can incubate between 25 and 45°C and accommodate 96, 384 and 1536 plates) to

90 measure the thickness of colonies printed with the ROTOR at 20 minute intervals. We have
91 demonstrated that this measure is a reliable proxy for overall colony size. We developed the
92 PHENOS software, with a graphical user interface (GUI), to run on Windows PCs alongside the
93 microplate reader's own software. This software combines multiple output files from the microplate
94 readers and automatically generates a variety of data visualizations, and output files containing
95 various growth curve summary values. The software is written in Python 2.7 and so could be
96 adapted to other operating systems without great difficulty. It was designed to make it relatively
97 easy to add new parser classes to recognise and read new formats of input files that might be
98 generated by different microplate readers. Alternatively, users may prefer to write their own code
99 for analyzing the data, or may find their microplate reader's native software sufficient for their
100 purposes.
101
102 We validated the PHENOS pipeline by comparing its measurements to two additional low-
103 throughput means of measuring colony population: use of a cell counting chamber under a
104 microscope, and absorbance in liquid media using a spectrophotometer. Comparing both
105 absorbance methods to the manual cell count, we found PHENOS to be of comparable accuracy to
106 the spectrophotometer. We also used this data to empirically derive a formula to estimate cell count
107 from PHENOS readings. Because PHENOS measures absorbance through the thickness of a colony (a
108 roughly cylindrical volume) as a proxy for overall colony size (a roughly hemispherical volume), there
109 is not a straightforward linear relationship between the absorbance readings and cell count.
110
111 For additional validation and to demonstrate one of its potential uses, we used PHENOS
112 measurements to reproduce a Quantitative Trait Loci (QTL) analysis previously undertaken using
113 conventional liquid media growth measurements. QTL analysis is a powerful approach to
114 understanding complex genetic architecture by correlating phenotypes with genotypes [21]. It can
115 be substantially automated through the use of large arrays of genetically diverse microorganisms.

116 With sufficient genetic markers determined (e.g. by sequencing) for each strain in the array, growth
117 phenotypes and genotypes can be passed into the statistical analysis package R/qtl [22]. Previously
118 our lab described a set of 576 genetically-diverse haploid F1 *S. cerevisiae* strains derived from
119 crosses between four geographically-diverse parent strains, chosen because they encapsulate much
120 of the genetic diversity discovered in a genomic survey of *S. cerevisiae* [23]. The parents were
121 YPS128 (herein designated 'A' for American), DBVPG6765 ('E' for European), Y12 ('S' for Sake) and
122 DBVPG6044 ('W' for West African), each made genetically tractable with knockouts of the *URA3*
123 uracil-metabolism gene and *HO* gene (thus preventing mating type switching and keeping strains
124 haploid) [24]. From each of the six pairwise crosses between these parental strains, 24 tetrads were
125 dissected, yielding 96 haploid F1s. These progeny are genetically and phenotypically extremely
126 diverse. They were genotyped at over 200 markers using high-resolution melt quantitative PCR [25]
127 to distinguish parental allele types, and then phenotyped for up to 23 different stress conditions in
128 liquid media. 82 significant QTLs were described in total, including 4 distinct regions for resistance to
129 the herbicide paraquat [26]. We phenotyped the same panel on solid YPD media containing 500
130 µg/µl paraquat and used the PHENOS pipeline to automatically generate data for entry into R/qtl
131 [25]. We found the PHENOS method reproduced the QTLs from the earlier study but showed greater
132 sensitivity as 18 additional regions were identified.
133
134 We consider a couple of the distinctive characteristics of PHENOS growth curves: an initial drop in
135 absorbance is commonplace, and as colonies grow and absorbance measurements increase,
136 variance in those measurements also increases. We investigate these properties experimentally and
137 report their probable causes in our discussion and conclusions. Neither affects the utility of PHENOS,
138 which produces growth curves that are both highly reproducible and accurate enough for
139 statistically sensitive analyses such as QTL analysis.
140

141 QTL analysis on genetically diverse populations, by revealing genetic causes of phenotypic variation,
142 is a valuable tool for determining the mode of action of drugs and stresses. But PHENOS will also be
143 useful for measuring population trait variability in general, synthetic genetic interactions, assessing
144 the quality of printed arrays, or for any of the other whole colony array techniques mentioned
145 above.

146 Methods

147 We will first describe, in general terms, the methods used in the PHENOS pipeline itself, including
148 the punch-in technique for normalizing the amount of cells printed to each spot. For much greater
149 detail, users can consult the guide which is available with the source code for the PHENOS software
150 at https://github.com/gact/phenos. As part of this walkthrough, we also show examples of the
151 visualizations that the PHENOS software produces. We then describe two methods used to validate
152 this approach to phenotyping. One of these, using QTL analysis, provides a demonstration of how
153 the PHENOS pipeline can be used in practice. We also describe a simple experiment to investigate
154 properties of the PHENOS growth curves.

155 *Overview*

156 An overview of the experimental side of the PHENOS pipeline is given in Figure 1. The experimental
157 plate is put into the microplate reader before any cells have been printed to it, in order to measure
158 and later subtract out absorbance due to agar. As part of the printing process, starting cell masses
159 can be partially normalized using the 'punch-in' technique described below. The microplate reader is
160 set up to store each set of continuous readings as a different text file, and it is possible to generate
161 growth curves even from intermittent readings. As summarised in Figure 2, the PHENOS software
162 then gathers information from the user about the experiment (for instance the treatment and the
163 name of the array being used, which will be used to look up information about individual strains in
164 the layout, which is stored in a separate file), combines multiple files taken from a single

experimental plate, and provides visualizations and analyses. The PHENOS software is written for Windows (so that it can operate alongside the microplate reader software) in Python 2.7, and has a simple GUI-based interface which can be launched by double-clicking on a desktop icon. It stores readings and other experimental data in HDF5 databases to allow complex queries and comparisons between multiple experiments, for example to measure final growth levels as a treatment/control ratio for use as a phenotype in QTL analysis.

For our experiments, we typically compare normal growth (usually on standard rich YPD media) to growth under a phenotype-discriminating treatment (e.g. the same media containing a genotoxin). Our control experiments are normally arrayed at 384 density, with 2-4 replicates of each strain, on 40ml of YPD agar media: 10g/l Yeast Extract, 20g/l Bacto Peptone, 20g/l D-Glucose, 1% adenine solution (0.5% adenine in 0.05M HCl), adjusted to pH 6.3 using 1M HCl, then autoclaved with 20g/l Bacto agar. Treatment experiments are grown on the same media but with a treatment added (e.g. a toxic compound at a discriminating concentration, or a different incubation temperature). Absorbance readings (optical density, or OD) are taken at 600nm (on a FLUOstar Optima microplate reader, a 595nm filter can be used), every 20 minutes. Control experiments are run for approximately 65 hours (over a weekend) so they can be compared to as many treatment experiments as possible, as some treatment conditions do not produce discriminating growth phenotypes until much later. For many growth phenotypes, however, an overnight program run for ~16 hours is sufficient to discriminate between phenotypes based on final growth levels. Measuring full growth curves gives experimenters the clearest overview from which to judge the best summary values and time window to discriminate between different growth phenotypes. Once this judgement has been made, it is possible to increase productivity by taking only intermittent 'snapshot' measurements of each plate at the appropriate points. Our snapshot protocols take readings of a plate four times in rapid succession (taking under 20 minutes for a 384 array), in order to average

190 over the measurement variance observed at high absorbance values, a phenomenon discussed in
191 more detail below.
192
193 Examples of the visualizations produced by the PHENOS software are given in Figure 3. Additionally,
194 the PHENOS software includes the option to generate time-lapse mp4 movies of colony thickness
195 measurements, which are an effective means for calling attention to any location-related biases in
196 growth. For each experiment (combining any number of sets of measurements of one experimental
197 plate), PHENOS also generates a text file summary of the combined experimental data, a text file
198 containing summary values of the curve for each colony and, if genotype data has been provided, a
199 text file containing both summary values and genotype data suitable for analysis using the R/qtl
200 package.
201
202 The PHENOS software is written with a flexible architecture that allows users to configure which
203 visualizations and summary values are included in the output by editing a text configuration file
204 called 'config.txt'. Each summary value is handled by a specific 'phenotype calculator' subroutine,
205 and new ones can be written into the software with relative ease. These calculator routines are
206 named in 'camel case', following programming convention. The currently available calculators are
207 shown in Figure 4 and listed in 'config.txt'. The calculators used by default can be changed by editing
208 this file, or particular calculators can be specified for particular treatments. The PHENOS software
209 can be used to pair a treatment experiment with a control experiment, creating a 'controlled
210 experiment' database entry that allows for more complex phenotype calculators that compare the
211 two curves (e.g. 'TreatmentRatioCalc', which is the most commonly used for our QTL analysis).

212 ## *Normalization by the punch-in technique*

213 For best results, there should be as little variation as possible in the amount of cells printed to each
214 position in the plate to be phenotyped. We have developed a 'punch-in' technique to normalize
215 these printed masses, which is summarised in Figure 5.

216

217 We create special single-use 'punch-in' plates with double the usual thickness of agar medium (8mm
218 rather than 4mm), and allow the colonies to grow on it overnight. We use long-pin repads and turn
219 the ROTOR pressure and overshoot settings to maximum, in order to drive the pins into the agar and
220 push excess cell mass up the sides of the pin, leaving a more uniform amount on the tip, which is
221 then printed to the target (experimental) plate as normal. When working with 384 arrays and
222 repads, the ROTOR cannot deliver sufficient pressure to punch a 384 repad into normal agar.
223 Therefore, a soft agar punch-in plate, with 25% of the usual agar, must be prepared (and used within
224 24 hours). The yeast array must be printed to this plate, using low pressure and overshoot settings
225 so as to not punch in prematurely. Only 384 repads should be used to print to soft agar plates
226 because 96 repads will punch in to them immediately even at the lowest possible settings, which will
227 prevent proper punch-in normalization when they are later used as a source plate. Printed punch-in
228 plates should be incubated for ~16 hours before use, and then used as a source plate almost
229 immediately. Pinning settings for this source plate must be set to maximum pressure and overshoot,
230 and sufficient repeat pinnings to drive the pins all the way to the bottom of the soft agar precisely
231 once. The detailed PHENOS guide provides extra tips for mastering this technique, and it should be
232 practised before being used for actual experiments. We have observed differences in normalization
233 efficiency are most often due to different makes of agar or inconsistent media pH producing
234 variation in agar hardness, or due to punch-in plates not being thick enough (we recommend 8mm).

235 ## *Cell count validation*

236 We created multiple 96-density arrays on solid media of a single haploid strain with weak
237 flocculation (EW01fc403), and induced a range of colony sizes by incubating the plates from one
238 edge and for different lengths of time. Each plate was measured using PHENOS, with a 'snapshot'
239 program to take four successive readings, and immediately afterwards colonies of different sizes
240 were extracted along with a plug of agar by using a 1 ml pipette tip with 15-20 mm cut off. Colony
241 and agar were deposited into 1 ml deflocculation buffer (50 mM KAc, 5 mM EDTA buffer, pH 4.5),
242 and cells rinsed from the agar by thorough pipetting. The solution was then removed to a cuvette,
243 leaving agar behind, and blanked readings taken in a Helios Gamma spectrophotometer (Thermo
244 Scientific). Thereafter, 5 µl was transferred to a Neubauer improved cell counting chamber for
245 counting at 40x magnification. Number of cells = 10000 × C × (25/S) where C=cells counted,
246 S=subsquares considered. In some cases, freshly printed colonies were extracted into only 100 µl
247 deflocculation buffer for cell counting without also being read in the spectrophotometer. Data was
248 tabulated in Microsoft Excel, and microplate reader measurements were adjusted to remove
249 absorbance due to agar, and to average readings from the snapshot program. For
250 spectrophotometer readings with OD_{600}>1, 1:10 dilutions were used instead and results multiplied
251 by 10 to account for known inaccuracies at high optical densities.

252 ## *QTL validation*

253 The 576 F1 *S. cerevisiae* strains previously described [26], comprising six crosses which we
254 redesignated as 'AE', 'AS', 'AW', 'ES', 'EW' and 'SW', were re-arrayed as three 384-density arrays,
255 each containing two of the crosses, with two biological replicates of each. All were incubated at 30°C
256 for at least 16 hours, both with and without 500 µg/µl paraquat mixed into the YPD agar media
257 (after autoclaving), and measured every 20 minutes. For each replicate, we determined the average
258 OD_{600} (with agar absorbance subtracted) at ~16 hours as a fraction of the control growth (without
259 paraquat) using the TreatmentRatio phenotype calculator in the PHENOS software. Biological

260 replicates were kept separate rather than averaged together in order to improve statistical power.
261 Taking these biological replicates as input, R/qtl was used to calculate genotype probabilities at
262 intervals of 1 centiMorgan, perform single-QTL analysis on the input data, run single-QTL analyses on
263 1000 permuted datasets, and estimate a LOD threshold at 5% significance level based on those
264 permutation analyses. Before being input to R/qtl, each permuted dataset was generated by
265 stratified permutation within tetrads, ensuring that no sample was permuted with its biological
266 replicate. QTL peaks were identified at loci with LOD values exceeding the threshold, and 1.5-LOD
267 support intervals were estimated by extension from the QTL peaks in either direction until the LOD
268 value fell by more than 1.5 LOD units below the peak LOD value.

269 *Analysis of measurement variance*

270 In order to assess the impact of material uniformity on variance in successive measurements, we
271 took multiple successive readings of a range of organic and non-organic substances with different
272 textures and levels of uniformity or granularity: yeast colonies now in stationary phase, artificial
273 spots made from woodglue and yeast extract mixed with biro ink, and layers of printer paper,
274 opaque plastic sheeting (cut from disposable weighing boats), and low-density 2mm foam packing
275 material. The standard deviation of multiple successive measurements was calculated for each
276 material type.

277 Results

278 *Early dips in PHENOS growth curves*

279 A common peculiarity of PHENOS curves is a slight dip that is often observed over the first hour of
280 incubation (clearly visible in Figure 4). We attribute this to microscopic bubbles which we have
281 observed in freshly printed spots (Figure 6), which behave like lenses and diffract the microplate
282 reader's light source until they have evaporated away. While curves can be normalized against each

283 other according to their minimum values, we observe in replicate plots that curves are usually well
284 aligned over most of the time course if the shrinkage is simply ignored.

285 *Cell count validation*

286 A range of differently sized colonies were measured in multiple ways, as described in the
287 corresponding methods section. The measurements and calculations are in Additional File 1, and are
288 displayed in Figure 7. To fit curves to each set of comparisons – spectrophotometer vs cell count,
289 and PHENOS vs $\log_{10}$ (cell count) – Excel's LINEST function was used to determine parameters that
290 minimize the sum of least squares, giving best curve fits relating A (average measurement with agar
291 subtracted) to P (predicted cell count). With parameters rounded, the best fit for PHENOS is
292 $P=77900e^{(1.76A)}$, while for liquid spectrophotometry the relationship is P=8000(725A+7). For average
293 predicted cell counts below 6 million, corresponding to a microplate reader measurement (without
294 agar) of ~2.5, the predicted cell counts for the two methods show linear agreement with R^2=0.77.
295 Following the statistical approach of Bland & Altman for comparing two methods of measurement
296 [27], it can be seen in Figure 8 that at higher absorbance measurements both methods show
297 comparable degrees of inaccuracy, although the PHENOS formula tends to underestimate the size of
298 large colonies.

299 *Variance in measurements at high absorbance values*

300 We observe that higher absorbance readings, above 2.0 (typically reached in 10-20 hours on YPD at
301 30°C) exhibit greater variation between successive measurements, and often in a manner that is
302 synchronised across most colonies on a plate (as can be seen in Figure 3E), but with no discernible
303 periodicity. This variance is equally noticeable with more frequent measurements, with and without
304 incubation, and whether or not the reader is covered up to limit any light leakage or heat flow. We
305 tested the hypothesis that this might be due to microscopic differences in the starting position of
306 each pass of readings, and therefore would be more pronounced for colonies with more elaborate
307 morphologies or, in general, with the granularity of the material being measured. We took multiple

308 successive measurements of a range of organic and non-organic substances with different
309 granularity, but observed no difference between them (Figure 9, Additional File 2).
310
311 These results indicate that reading variation at high absorbance values is not attributable to
312 positioning error and granularity. We conclude that this is simply an unavoidable hardware
313 limitation, though easily overcome by averaging readings within a time window, as in our snapshot
314 protocols.

315 *QTL validation*

316 PHENOS was used to derive treatment ratios for the 576 F1 haploids growing with and without 500
317 µg/µl paraquat. Sample curves are shown in Figure 10.
318
319 QTL analysis with R/qtl yielded 61 QTLs overall, covering 22 distinct regions, including all four found
320 in the previous study [26], with comparable LOD scores, as illustrated in Figure 11. The original study
321 in liquid media, and this reproduction of it, both had low resolution due to large haplotype blocks
322 and low density genetic markers. Much greater resolution, down to individual SNPs, can be achieved
323 by repeating a process of crossing and sporulation to create advanced intercross lines with a high
324 level of genomic admixture, and generating much larger numbers of markers through whole genome
325 sequencing [16].

326 Discussion

327 Despite the reading variation at higher absorbance values described above (and equally problematic
328 in liquid spectrophotometry) PHENOS can provide accurate scores of colony size across a very wide
329 range of sizes, including very small ones that might not be picked up at all by imaging-based
330 approaches. Analyses of inter- and intra-plate variability (Figures 9 and 10 respectively) demonstrate
331 that this is a robust and highly reproducible assay.

332

333 We've already described and investigated a couple of quirks of PHENOS growth curves. These make
334 it difficult to reliably fit the curves to conventional logistic growth curve models [28]. Additionally,
335 we've observed some non-standard yeast growth patterns in response to various genotoxins. Strong
336 doses can produce curves that that show growth initially but then trend downwards as cell death
337 and dispersal thins the colony. Some compounds with lower stability, such as methyl
338 methanesulfonate, can produce curves which plateau and maybe fall before then entering a second
339 phase of growth after the survivors begin to proliferate. These are interesting responses and worthy
340 of study even though they cannot be parameterized as conventional logistic growth models. For the
341 purposes of QTL analysis, the most powerful phenotype measurement is the summary value that
342 shows the greatest variation between sensitive and resistant strains, and is least influenced by
343 experimental factors such as normalization efficiency, or least prone to miscomputation. This may
344 vary on a case by case basis, depending on the growth patterns concerned, so it is best to view the
345 full growth curves before deciding on the best summary values (and time windows) to use.

346

347 As shown in Figure 4, the PHENOS software allows for a range of summary values to be generated
348 from a PHENOS curve, including ones which are analogous to the efficiency (final growth), rate
349 (maximum slope), and lag (growth delay) parameters commonly derived from logistic growth curves
350 in liquid media, [26, 28]. The 'MaxSlopeCalc' value, analogous to growth rate, can be miscomputed
351 when there are multiple growth phases, so replicate plots should be consulted to confirm that
352 inflection points are being correctly identified. The 'LagCalc' value, analogous to growth lag, often
353 correlates with the initial absorbance of cells right after printing (the 'printed mass'). For this reason,
354 by default, PHENOS generates a scatterplot visualization comparing the two values for each curve so
355 that any correlation becomes evident. For the key purpose of discriminating between strains, the
356 growth level in a hand-picked time window ('AverageWithoutAgarCalc'), and its cross-experiment
357 derivative ('TreatmentRatioCalc') are usually the most direct and reliably computed values.

358 So-called 'edge effects' are a common concern of working with microbial arrays. The colonies around
359 the edge of an array might grow differently due to differences in heat-dispersal (in liquids) or
360 nutrient availability (on solid media). In the solid media arrays used for PHENOS, we only observe
361 significant edge effects after about 40 hours in arrays growing on normal YPD media. At this point
362 peripheral colonies continue growing for longer due to greater availability of nutrients at the edge of
363 the array, but we see no evidence of significant differences in earlier growth, or on plates where
364 growth is limited by treatments instead of nutrient availability.
365
366 In some circumstances PHENOS may not be the most suitable strategy for growth phenotyping. If a
367 treatment compound is expensive it will be inefficiently deployed by being dispersed in 40 ml of
368 solid media. Reducing the amount of media to 20ml per plate may mitigate this, but can introduce
369 artefacts due to the agar slightly contracting from the edges of the plate during incubation.
370 Alternatively, it might be possible to dispense hot agar into the wells of a microtitre plate but care
371 would need to be taken to avoid bubbles and unevenness (possibly with the use of additives such as
372 surfactants) that would compromise printing quality, and this would be harder in 384-well plates
373 than in 96.
374
375 Any treatment compound must be soluble of course, and sufficiently stable in solid media, and if it
376 affects the pH too strongly it will also make the agar too soft. If the treatment compound affects the
377 agar colour too much then it may increase the base agar absorbance readings so much that growth
378 curves appear to plateau prematurely, because the maximum possible overall reading, for FLUOstar
379 microplate readers at least, is 3.5. A typical *S. cerevisiae* colony at maximum size in a 384-array
380 growing on normal YPD agar will register an OD_{600} without agar of up to 2.5, so the agar absorbance
381 must be less than 1 when working with this organism. Complex phenotypes such as colony
382 morphology characteristics cannot easily be measured by PHENOS in its current state. Furthermore,
383 we expect that growth on solid media will not always reflect growth under liquid conditions. The

384 latter may be more relevant to applications in brewing and wine-making, although solid media
385 growth is likely to be much more similar to how yeast grow in the wild.
386
387 We have found 384 arrays strike the best balance between scale and reliability but the ROTOR is
388 capable of printing 1536 arrays and, with a minor vendor adjustment, the FLUOstar Omega is
389 capable of reading them. 1536 arrays are challenging to duplicate, especially if they contain diverse
390 growth phenotypes, as slow-growing colonies are easily lost, often due to larger surrounding
391 colonies keeping a repad pin from making full contact. Only short-pin repads are available for 1536
392 arrays, and their pins have a very small cross section which necessitates a much finer alignment
393 between printing and reading coordinates than the machines can usually achieve. This problem may
394 be overcome by using 4 long-pin 384 repads, each normalized from a different punch-in plate, but
395 this reduces the convenience and speed of the method. Furthermore, the greater proximity of
396 colonies in 1536 arrays substantially reduces growth speed and peak colony size, and brings forward
397 the time point at which edge effects become prominent. Also, mating pheromone inhibition or
398 similar effects may complicate growth in mixed arrays.

399 Conclusions

400 In successfully normalized experiments, PHENOS produces proxy growth curves with a high degree
401 of reproducibility between replicates (Figures 3F, 10), and with greater ease and automation than
402 conventional methods. We've reported results that use *S. cerevisiae*, but within our lab the exact
403 same approach has also been used to measure the growth of other yeast strains such as *S.*
404 *eubayanus* and *S. uvarum.* Nor need PHENOS be limited to working with yeast. We have briefly
405 experimented with using PHENOS to measure *E. coli* growth (strain DH5a) on Luria Broth agar, and
406 obtained growth curves with a maximum change in OD_{600} of 0.62 after 16 hours (Additional File 4).
407 While less optically absorbent than yeast, this should still be perfectly sufficient to discriminate

408 growth phenotypes. Provided that an organism can grow on a solid medium in the first place,
409 PHENOS may prove a viable approach to growth phenotyping.
410
411 We have also briefly experimented with measuring whole-colony fluorescence in tandem with
412 optical absorbance and have encouraging preliminary results, although extra software will be
413 needed to handle the data files produced by this procedure.
414
415 For labs in possession of a colony arraying robot, colony thickness as measured by an incubating
416 microplate reader is a very convenient and sensitive alternative to more expensive specialist
417 solutions for quantifying growth. It sidesteps problems with imaging-based solutions such as poor
418 contrast or bubbles or specks which can confuse image analysis, as well as the general difficulty of
419 setting up a high-quality imaging and image analysis pipeline in the first place. It avoids problems
420 with liquid phenotyping such as format conversion, unequal heat distribution, evaporation, and the
421 technical challenge of fine-tuning agitation levels to prevent flocculation without introducing
422 bubbles or cross-well contamination. Furthermore, there are clear safety benefits to having toxic
423 treatment compounds contained within solid agar, and the same will be true if working with more
424 dangerous organisms than yeast

425 List of abbreviations

426 OD: optical density; PHENOS: phenotyping on solid media; QTL: quantitative trait loci; SGA: synthetic
427 genetic array; YPD: yeast extract peptone dextrose growth media.

428 Figure legends

429 **Figure 1 Overview of the PHENOS experimental workflow.** We distinguish between three types of
430 plates: stock plates for array maintenance, punch-in plates for normalization, and experimental
431 plates for measurement.

432

433 **Figure 2 Overview of the PHENOS analysis workflow.** The PHENOS software combines files and
434 visualizes and compares experiments.

435

436 **Figure 3 Examples of visualizations produced by the PHENOS software.** The colour bars and some
437 of the labels have been enlarged for this publication. **A)** Agar thickness of an unprinted plate,
438 showing a low-quality plate with a bulge due to inadequately mixed media. For a good quality plate,
439 all squares would be the same colour (the colour scale shows absorbance at each location in the
440 array—darker colours signifying thicker agar—and is comparable between different visualizations).
441 There should be no pattern to the size of squares either: the largest and smallest squares are the
442 highest and lowest readings on that plate respectively. Squares are also labelled with the array
443 position, although those labels are not legible in these small reproductions. **B)** Printing quality, based
444 on initial readings immediately after printing, with agar subtracted. As with A, the colour scale is
445 absolute and comparable between different experiments, but the spot scaling shows where the
446 largest and smallest printed masses on the plate are located. Each spot is labelled with the strain
447 name, which is legible on full size images. **C)** Final measurements of a plate as measured by the
448 microplate reader (radius being proportional to OD_{600} with agar absorbance subtracted), next to **D)**
449 an actual image of the same plate. In this case, there are four replicates of each strain. **E)** Control
450 growth curves for all 384 colonies on a YPD plate, coloured by the initial printed mass as described
451 for B. In a successful treatment experiment such as is shown in C & D, there would be much greater
452 diversity in these curves. **F)** Replicate plots, showing growth curves of all four replicates of a single
453 strain for which growth has been equally suppressed (compare to E) by hydroxyurea. Dots on these
454 curves indicate where the PHENOS software has calculated the inflection point of each curve to lie,
455 i.e. the point of maximum growth. Curves are also labelled with the array position of the colony.

456

457 **Figure 4 'Phenotype calculators' within the PHENOS software.** Each generates a different summary
458 value. Some, indicated with asterisks, e.g. AverageWithoutAgarCalc, require a particular time
459 window to be specified by the user: for a given curve, all timepoints within that window are then
460 averaged, to account for the variance usually observed at higher absorbance readings. Distinctive
461 properties of PHENOS curves, such as initial reductions in absorbance, are discussed within the text
462 and Figure 6.
463
464 **Figure 5 Punch-in technique for normalizing printed cell masses.**
465
466 **Figure 6 Bubbles in printed cell patches.** These evaporate over the first hour after printing, leading
467 to initial dips in PHENOS growth curves.
468
469 **Figure 7 Accuracy of PHENOS colony measurements. A)** Microplate reader measurements plotted
470 against cell counts. **B)** Liquid spectrophotometry measurements plotted against cell counts; for initial
471 readings >1, 1:10 dilutions were read and their results multiplied by 10 to empirically adjust for non-
472 linearity at high absorbances. Together, these measurements were used to empirically determine
473 formulae for predicting cell counts from measured values.
474
475 **Figure 8 Comparison of predicted cell counts by two methods:** microplate absorbance and
476 conventional liquid spectrophotometry, both at OD_{600}.
477
478 **Figure 9 Variation in multiple successive readings of a variety of materials.** This variation is a
479 function of overall average absorbance, but barely affected by different degrees of granularity.
480
481 **Figure 10 Sample curves from two strains showing extremely different growth responses.** Growth
482 on solid media containing 500 µg/µl paraquat (red curves) compared to normal YPD (blue curves).

483 Curves are labelled with experiment IDs and plate positions, and dots indicate PHENOS-calculated
484 inflection points.
485
486 **Figure 11 QTL validation.** *S. cerevisiae* QTLs for paraquat resistance, determined using PHENOS,
487 compared to QTLs found in the same set of strains grown in liquid media. In both experiments, 576
488 strains were phenotyped, each an F1 progeny from one of six different crosses of four geographically
489 diverse parental strains (A= American, E= European, S= Saki, W=West African). Different crosses
490 produce different QTLs, though one QTL on chromosome XII is common to all the crosses. The
491 results show that PHENOS reproduces the liquid media QTLs and is more sensitive.

492 Additional Files

493 **Additional File 1** Validation by cell counting (xlsx). Raw data and calculations.
494 **Additional File 2** Testing with multiple materials (xlsx). Raw data and calculations.
495 **Additional File 3A** Validation by paraquat QTLs (xlsx). Full list of QTL intervals and their details.
496 **Additional File 3B** Validation by paraquat QTLs measurements (zip). Initial DAT and csv files.
497 **Additional File 4** E coli results (xlsx). Raw data and calculations.

498 Declarations

499 *Ethics approval and consent to participate*

500 Not applicable.

501 *Consent for publication*

502 Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article (and its additional files). The PHENOS software and test files are available from https://github.com/gact/phenos/, under an MIT license. This software is written for Windows, and requires Python 2.7, along with the modules "scipy", "numpy", "tables", "matplotlib", "xlrd", "brewer2mpl" and their dependencies. The yeast strains used in QTL validation are available on request.

Competing interests

The authors declare that they have no competing interests.

Funding

The work was funded by the University of Leicester under a Burgess Centre grant, with additional funding for consumables and genotyping provided by BBSRC grant BB/L022508/1 to EJL. DG was funded by the University of Leicester under a Pritchard PhD studentship award.

Author's contributions

DBHB invented the technique, wrote the PHENOS software and drafted the manuscript. ND tested early versions and provided feedback. MA performed the paraquat phenotyping. TW provided help with R/qtl and Github. DG tested the software in a variety of contexts and drove improvements. EJL and SSF jointly supported the project and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We wish to thank Maria Viskaduraki for statistical advice, and Tuğba Şan and Noor Bafana for testing aspects of the pipeline in its early stages.

525 ## References

526 1. Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, et al. Functional
527 characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science.
528 1999;285:901–6.

529 2. Tong AHY, Evangelista M, Parsons AB, Xu H, Bader GD, Pagé N, et al. Systematic Genetic Analysis
530 with Ordered Arrays of Yeast Deletion Mutants. Science (80-.). 2001;294.

531 3. Scherens B, Goffeau A. The uses of genome-wide yeast mutant collections. Genome Biol. BioMed
532 Central; 2004;5:229.

533 4. Roguev A, Wiren M, Weissman JS, Krogan NJ. High-throughput genetic interaction mapping in the
534 fission yeast Schizosaccharomyces pombe. Nat. Methods. 2007;4:861–6.

535 5. Typas A, Nichols RJ, Siegele DA, Shales M, Collins SR, Lim B, et al. High-throughput, quantitative
536 analyses of genetic interactions in E. coli. Nat. Methods. 2008;5:781–7.

537 6. Holz C, Lueking A, Bovekamp L, Gutjahr C, Bolotina N, Lehrach H, et al. A human cDNA expression
538 library in yeast enriched for open reading frames. Genome Res. Cold Spring Harbor Laboratory Press;
539 2001;11:1730–5.

540 7. Harrington JJ, Sherf B, Rundlett S, Jackson PD, Perry R, Cain S, et al. Creation of genome-wide
541 protein expression libraries using random activation of gene expression. Nat. Biotechnol. Nature
542 Publishing Group; 2001;19:440–5.

543 8. Meersman E, Steensels J, Struyf N, Paulus T, Saels V, Mathawan M, et al. Tuning Chocolate Flavor
544 through Development of Thermotolerant Saccharomyces cerevisiae Starter Cultures with Increased
545 Acetate Ester Production. Appl. Environ. Microbiol. American Society for Microbiology; 2015;82:732–
546 46.

547 9. Malecki M, Bähler J. Identifying genes required for respiratory growth of fission yeast. Wellcome

548 open Res. Europe PMC Funders; 2016;1:12.

549 10. Freel KC, Friedrich A, Sarilar V, Devillers H, Neuvéglise C, Schacherer J. Whole-Genome
550 Sequencing and Intraspecific Analysis of the Yeast Species *Lachancea quebecensis*. Genome Biol.
551 Evol. Oxford University Press; 2016;8:733–41.

552 11. Zhang R, Patena W, Armbruster U, Gang SS, Blum SR, Jonikas MC. High-Throughput Genotyping
553 of Green Algal Mutants Reveals Random Distribution of Mutagenic Insertion Sites and
554 Endonucleolytic Cleavage of Transforming DNA. Plant Cell. 2014;26:1398–409.

555 12. Côté J-P, French S, Gehrke SS, MacNair CR, Mangat CS, Bharat A, et al. The Genome-Wide
556 Interaction Network of Nutrient Stress Genes in Escherichia coli. MBio. American Society for
557 Microbiology; 2016;7:e01714-16.

558 13. Hall BG, Acar H, Nandipati A, Barlow M. Growth Rates Made Easy. Mol. Biol. Evol. 2014;31:232–8.

559 14. Jung PP, Christian N, Kay DP, Skupin A, Linster CL. Protocols and Programs for High-Throughput
560 Growth and Aging Phenotyping in Yeast. Schacherer J, editor. PLoS One. Public Library of Science;
561 2015;10:e0119807.

562 15. Warringer J, Ericson E, Fernandez L, Nerman O, Blomberg A. High-resolution yeast phenomics
563 resolves different physiological features in the saline response. Proc. Natl. Acad. Sci. U. S. A.
564 2003;100:15724–9.

565 16. Cubillos FA, Parts L, Salinas F, Bergström A, Scovacricchi E, Zia A, et al. High Resolution Mapping
566 of Complex Traits with a Four-Parent Advanced Intercross Yeast Population. Genetics. 2013;

567 17. Dittmar JC, Reid RJD, Rothstein R. ScreenMill: A freely available software suite for growth
568 measurement , analysis and visualization of high-. BMC Bioinformatics. 2010;11:353.

569 18. Lawless C, Wilkinson DJ, Young A, Addinall SG, Lydall DA. Colonyzer: automated quantification of
570 micro-organism growth characteristics on solid agar. BMC Bioinformatics. 2010;11:287.

571 19. Shah NA, Laws RJ, Wardman B, Zhao LP, Hartman JL. Accurate, precise modeling of cell
572 proliferation kinetics from time-lapse imaging and automated image analysis of agar yeast culture
573 arrays. BMC Syst. Biol. 2007;1:3.

574 20. Takeuchi R, Tamura T, Nakayashiki T, Tanaka Y, Muto A, Wanner BL, et al. Colony-live —a high-
575 throughput method for measuring microbial colony growth kinetics— reveals diverse growth effects
576 of gene knockouts in Escherichia coli. BMC Microbiol. 2014;14:171.

577 21. Wilkening S, Lin G, Fritsch ES, Tekkedil MM, Anders S, Kuehn R, et al. An Evaluation of High-
578 Throughput Approaches to QTL Mapping in Saccharomyces cerevisiae. Genetics. 2013;

579 22. Broman KW, Sen S. A Guide to QTL Mapping with R/qtl. New York, NY: Springer New York; 2009.

580 23. Liti G, Carter DM, Moses AM, Warringer J, Parts L, James SA, et al. Population genomics of
581 domestic and wild yeasts. Nature. 2009;458:337–41.

582 24. Cubillos FA, Louis EJ, Liti G. Generation of a large set of genetically tractable haploid and diploid
583 Saccharomyces strains. FEMS Yeast Res. 2009;9:1217–25.

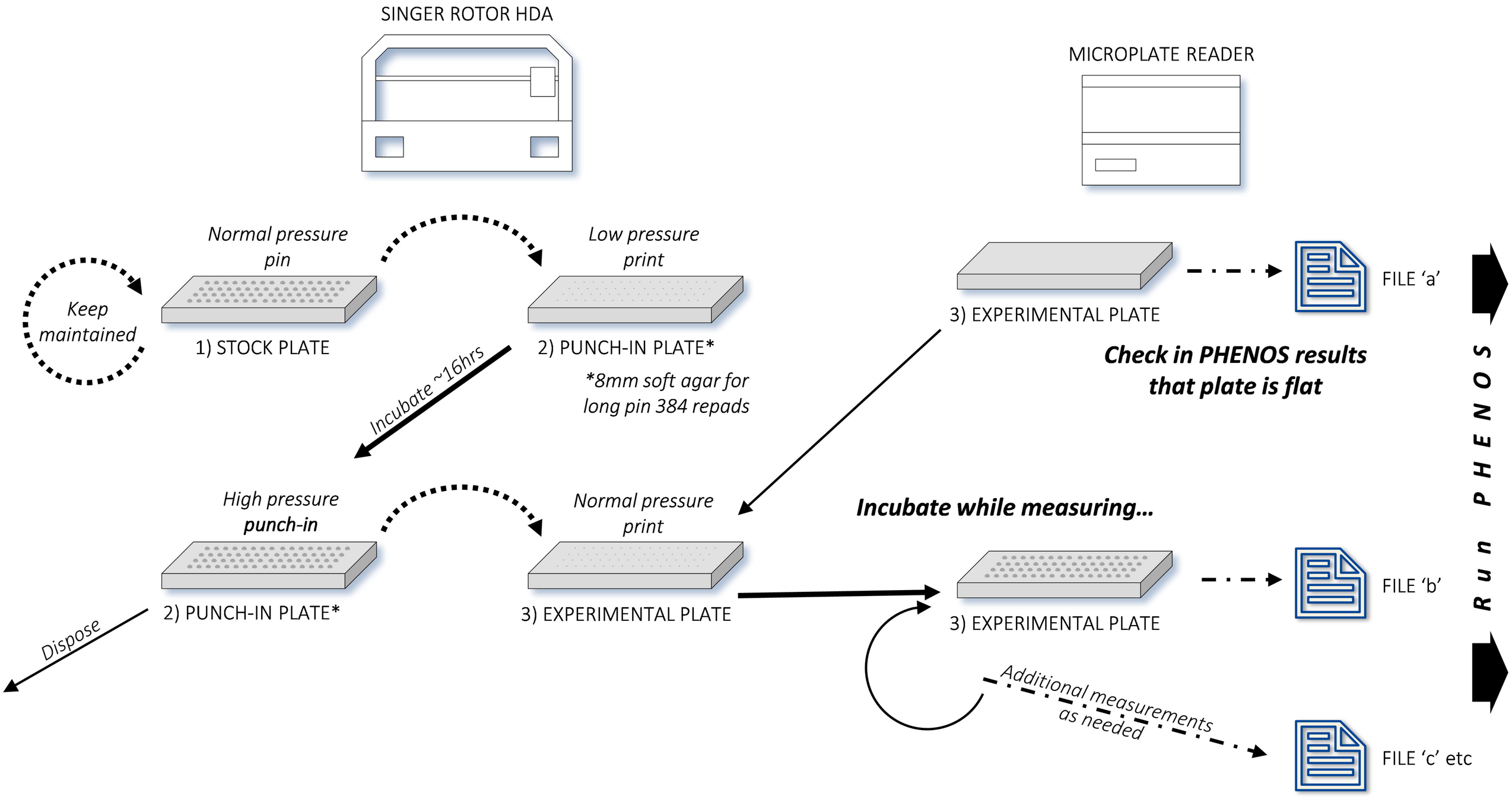
584 25. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-Resolution Genotyping by
585 Amplicon Melting Analysis Using LCGreen. Clin. Chem. 2003;49.

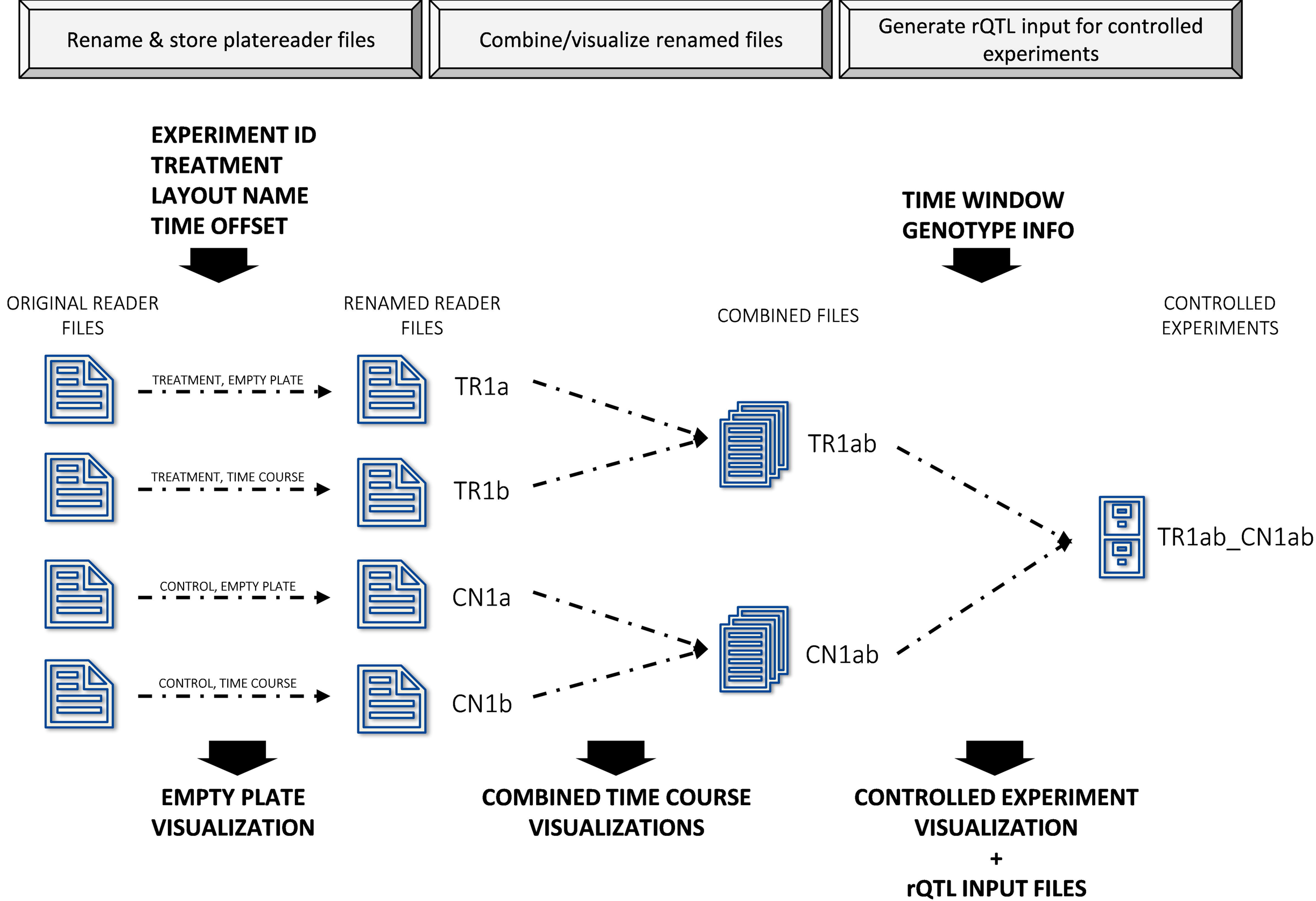
586 26. Cubillos FA, Billi E, Zörgö E, Parts L, Fargier P, Omholt S, et al. Assessing the complex architecture
587 of polygenic traits in diverged yeast populations. Mol. Ecol. 2011;20:1401–13.

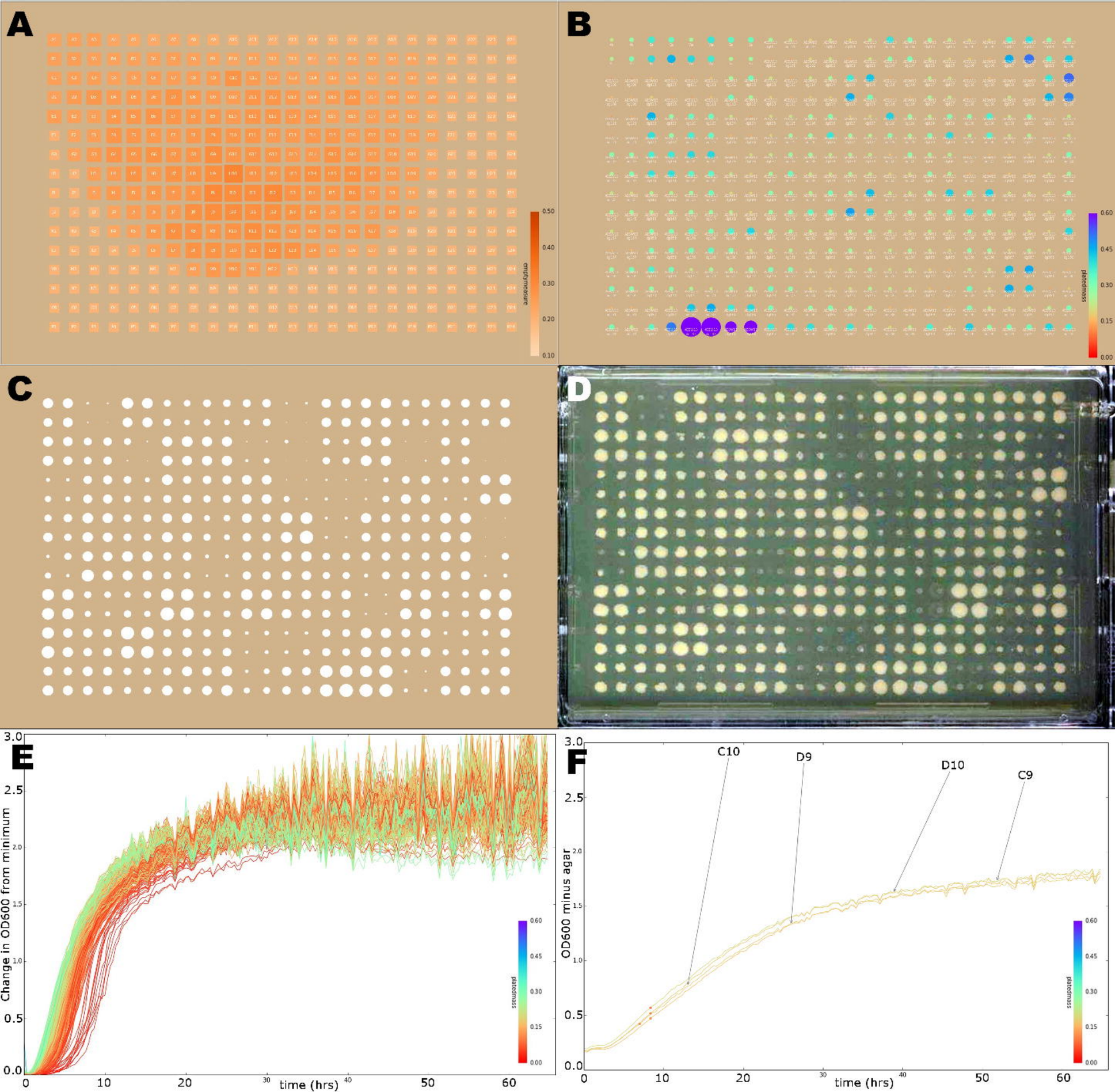
588 27. Martin Bland J, Altman D. Statistical methods for assessing agreement between two methods of
589 clinical measurement. Lancet. 1986;327:307–10.

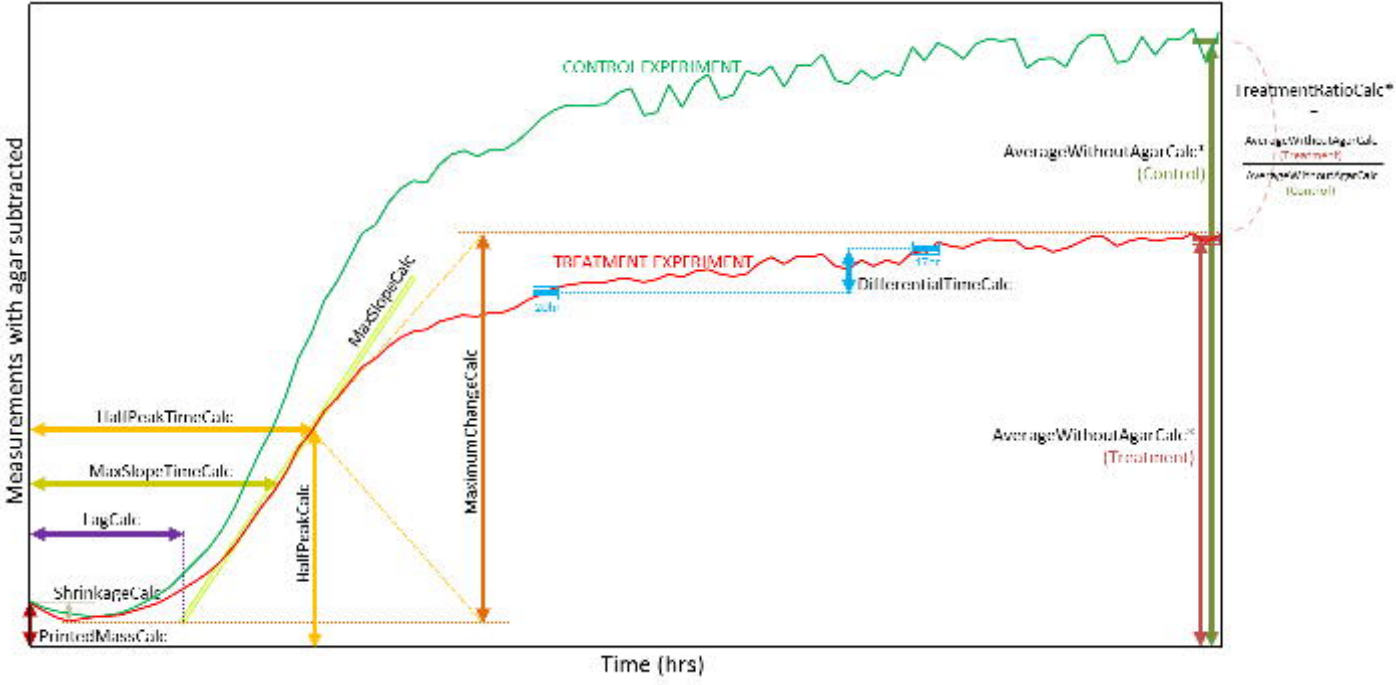
590 28. Richards OW. The Growth of the Yeast Saccharomyces cerevisiae. I. The Growth Curve, its
591 Mathematical Analysis, and the Effect of Temperature on the Yeast Growth. Ann. Bot. Oxford
592 University Press; 42:271–83.

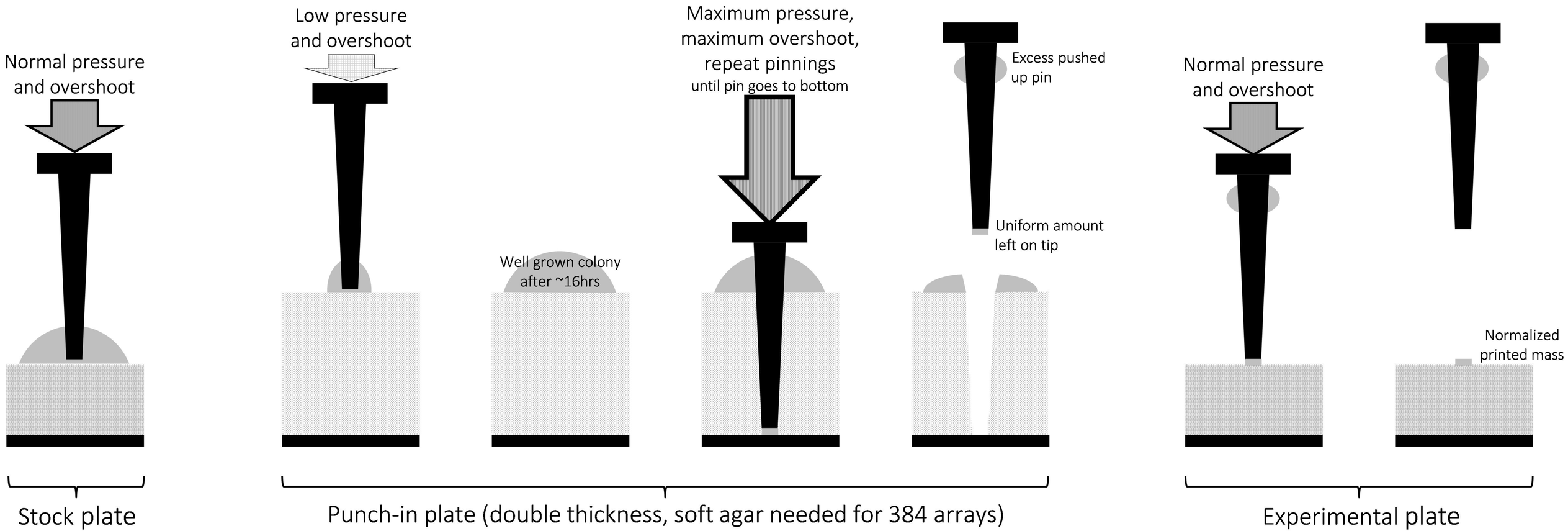
593

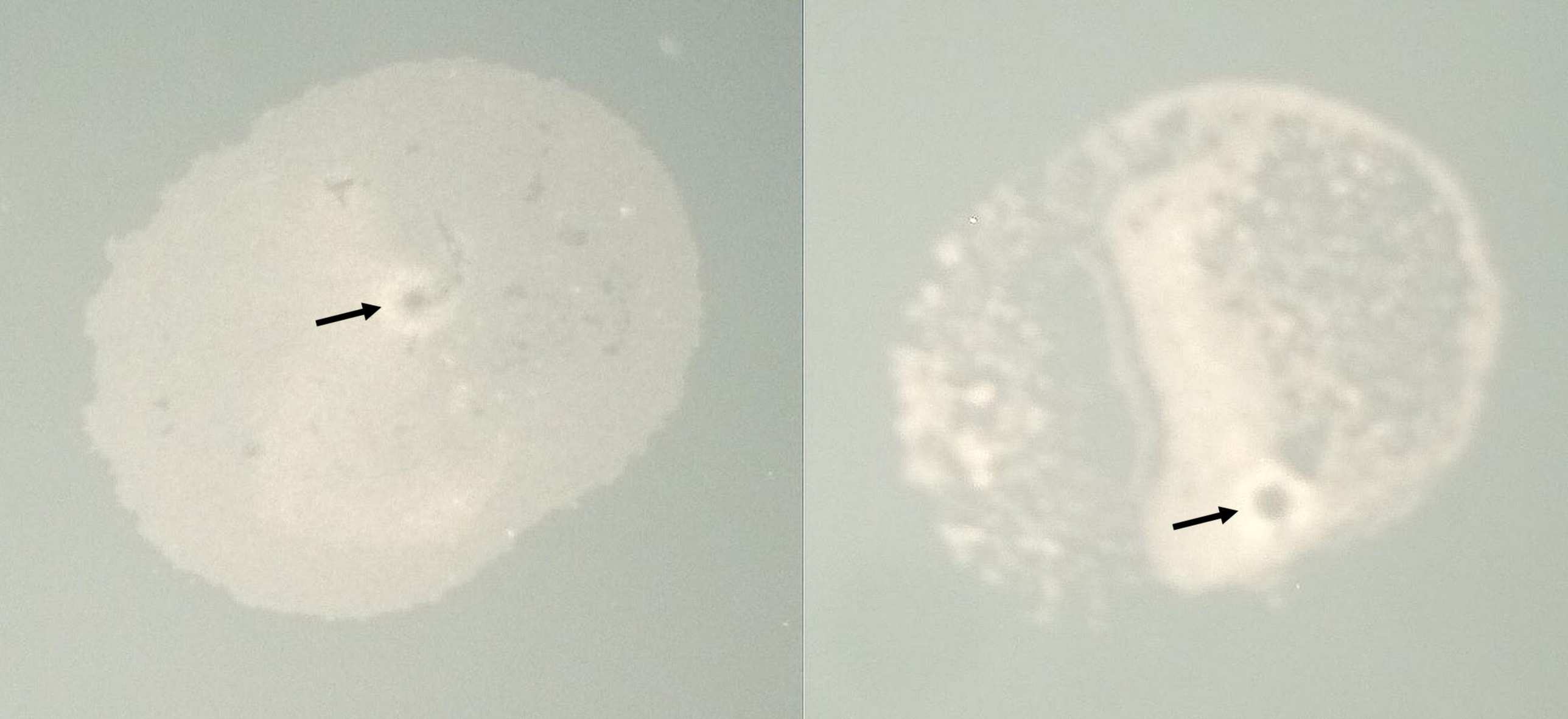

SINGER ROTOR HDA
MICROPLATE READER
Normal pressure pin
Low pressure print
Keep maintained
1) STOCK PLATE
2) PUNCH-IN PLATE*
*8mm soft agar for long pin 384 repads
Incubate ~16hrs
3) EXPERIMENTAL PLATE
FILE 'a'
Check in PHENOS results that plate is flat
High pressure punch-in
Normal pressure print
Incubate while measuring...
2) PUNCH-IN PLATE*
3) EXPERIMENTAL PLATE
3) EXPERIMENTAL PLATE
FILE 'b'
Dispose
Additional measurements as needed
FILE 'c' etc
Run PHENOS

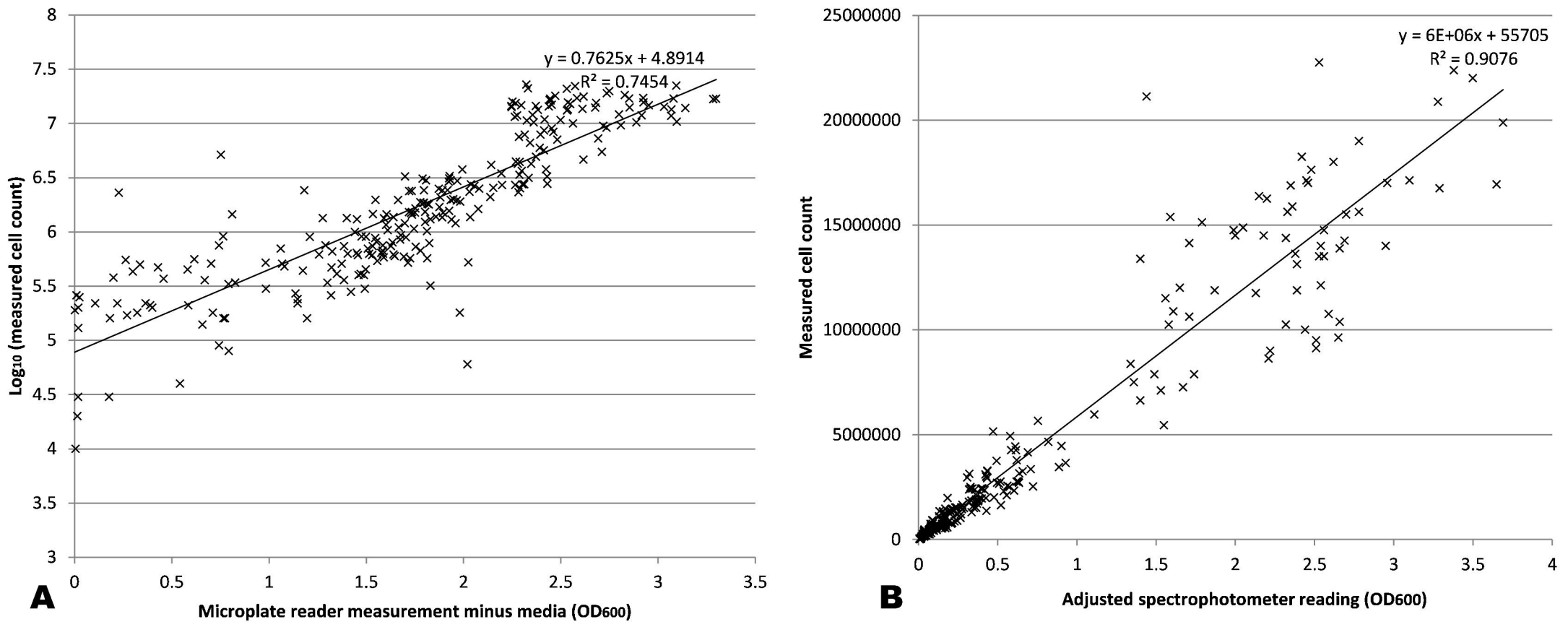

Rename & store platereader files
Combine/visualize renamed files
Generate rQTL input for controlled experiments
EXPERIMENT ID
TREATMENT
LAYOUT NAME
TIME OFFSET
TIME WINDOW
GENOTYPE INFO
ORIGINAL READER FILES
RENAMED READER FILES
COMBINED FILES
CONTROLLED EXPERIMENTS
TREATMENT, EMPTY PLATE
TREATMENT, TIME COURSE
CONTROL, EMPTY PLATE
CONTROL, TIME COURSE
TR1a
TR1b
CN1a
CN1b
TR1ab
CN1ab
TR1ab_CN1ab
EMPTY PLATE VISUALIZATION
COMBINED TIME COURSE VISUALIZATIONS
CONTROLLED EXPERIMENT VISUALIZATION
+
rQTL INPUT FILES

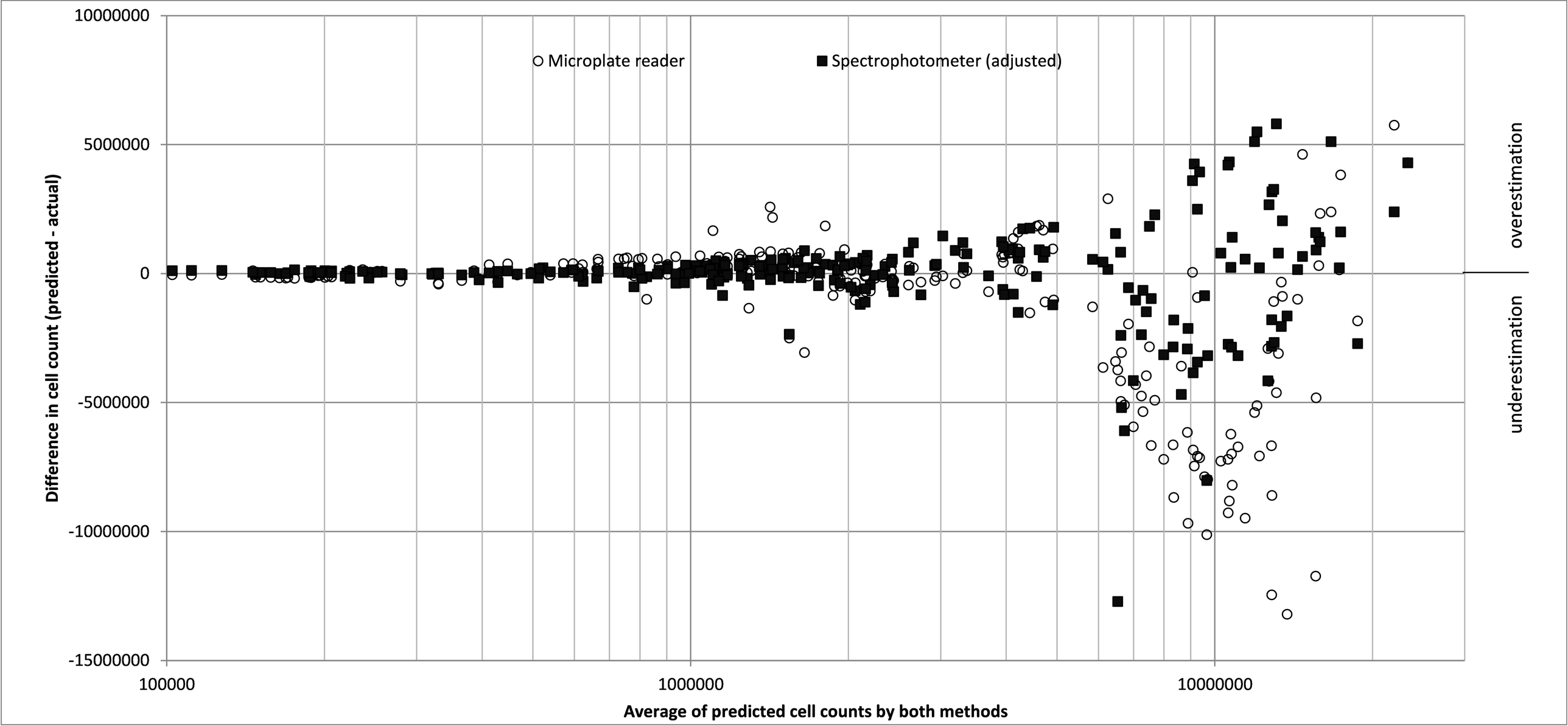

A
B
C
D
E
F
C10
D9
D10
C9
Change in OD600 from minimum
OD600 minus agar
time (hrs)
platedmass

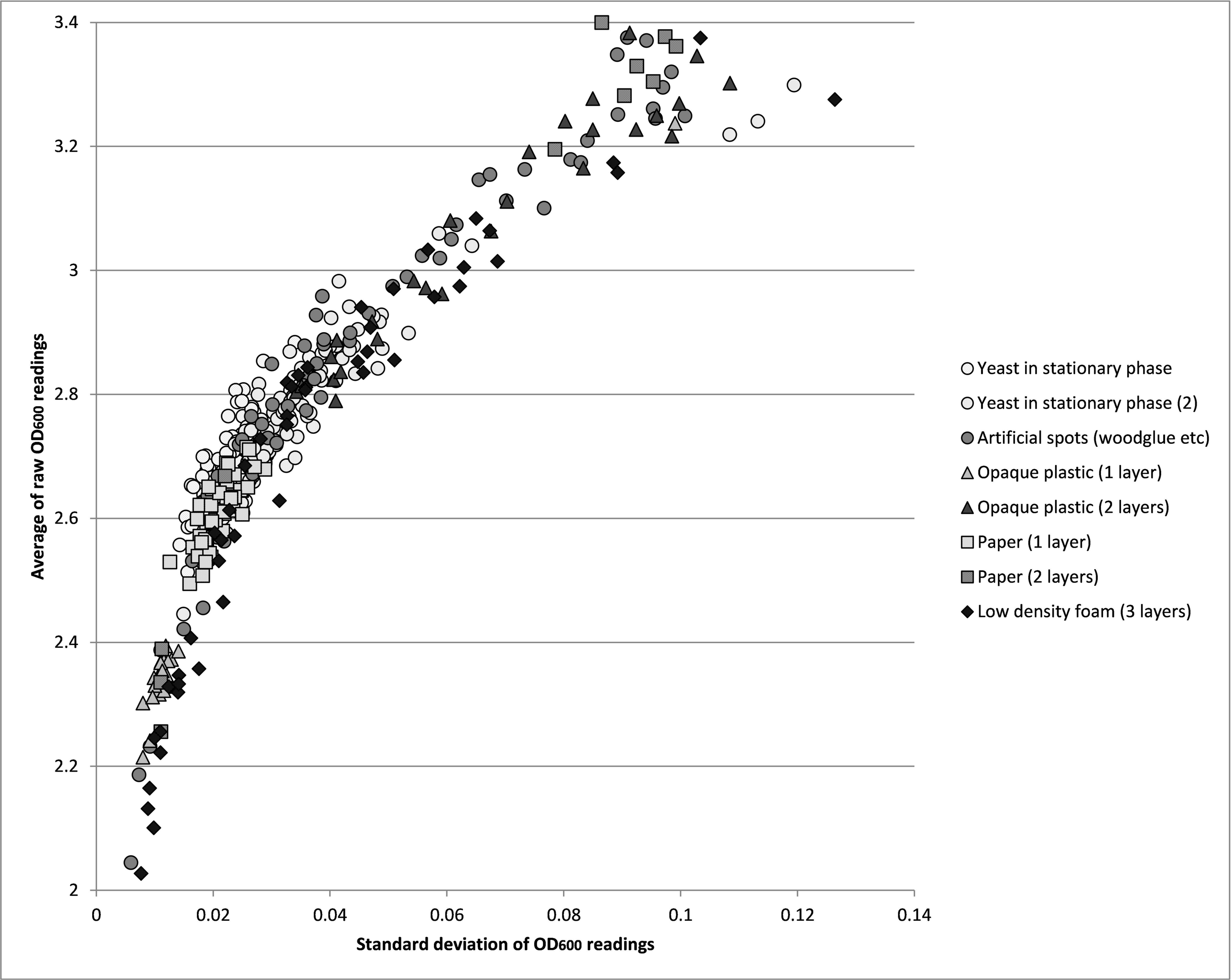

CONTROL EXPERIMENT
TreatmentRatioCalc*
AverageWithoutAgarCalc*
(Control)
TREATMENT EXPERIMENT
DifferentialTimeCalc
MaxSlopeCalc
HalfPeakTimeCalc
MaxSlopeTimeCalc
LagCalc
ShrinkageCalc
PrintedMassCalc
HalfPeakCalc
MaximumChangeCalc
AverageWithoutAgarCalc*
(Treatment)
Measurements with agar subtracted
Time (hrs)

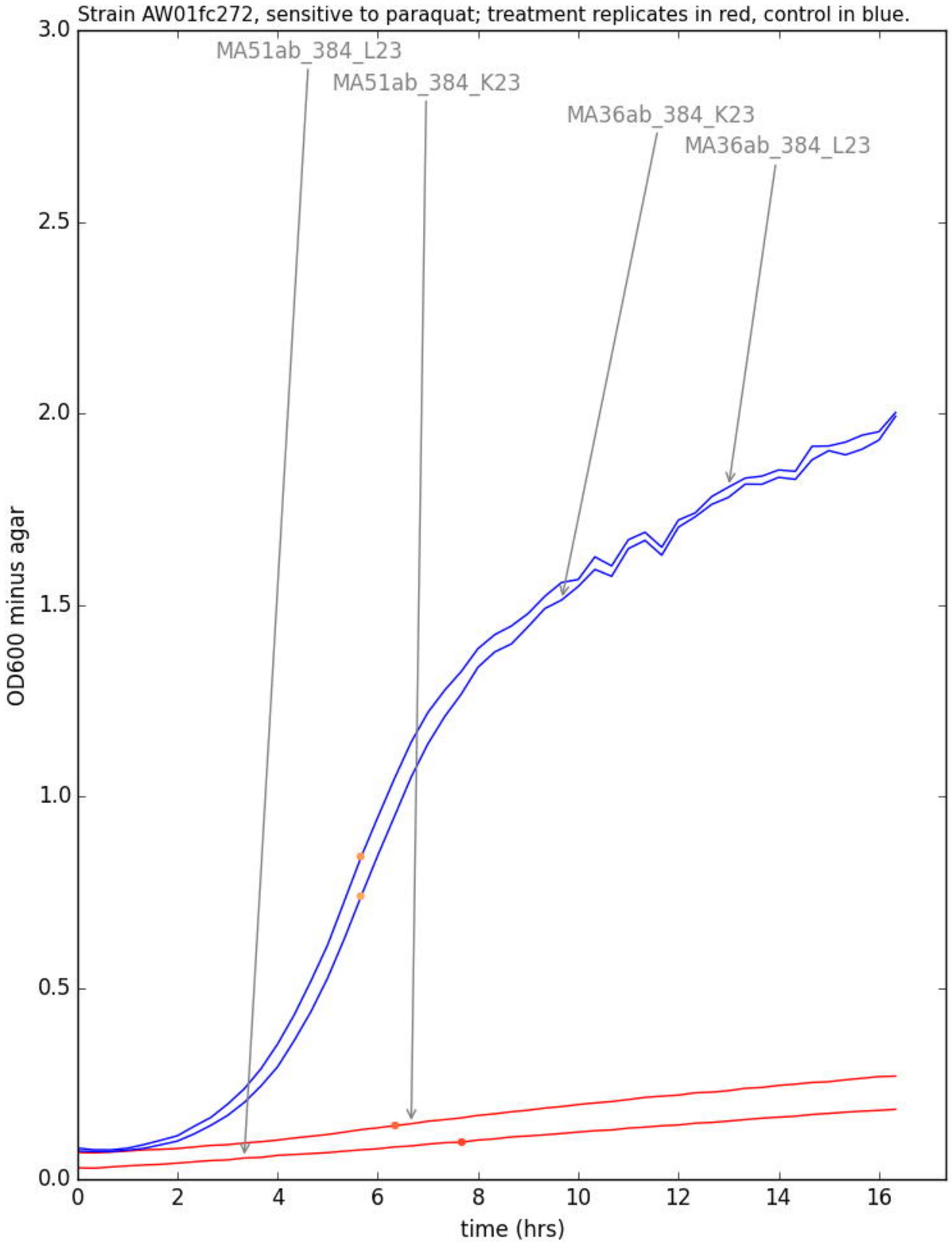

Normal pressure
and overshoot
Low pressure
and overshoot
Well grown colony
after ~16hrs
Maximum pressure,
maximum overshoot,
repeat pinnings
until pin goes to bottom
Excess pushed
up pin
Uniform amount
left on tip
Normal pressure
and overshoot
Normalized
printed mass
Stock plate
Punch-in plate (double thickness, soft agar needed for 384 arrays)
Experimental plate

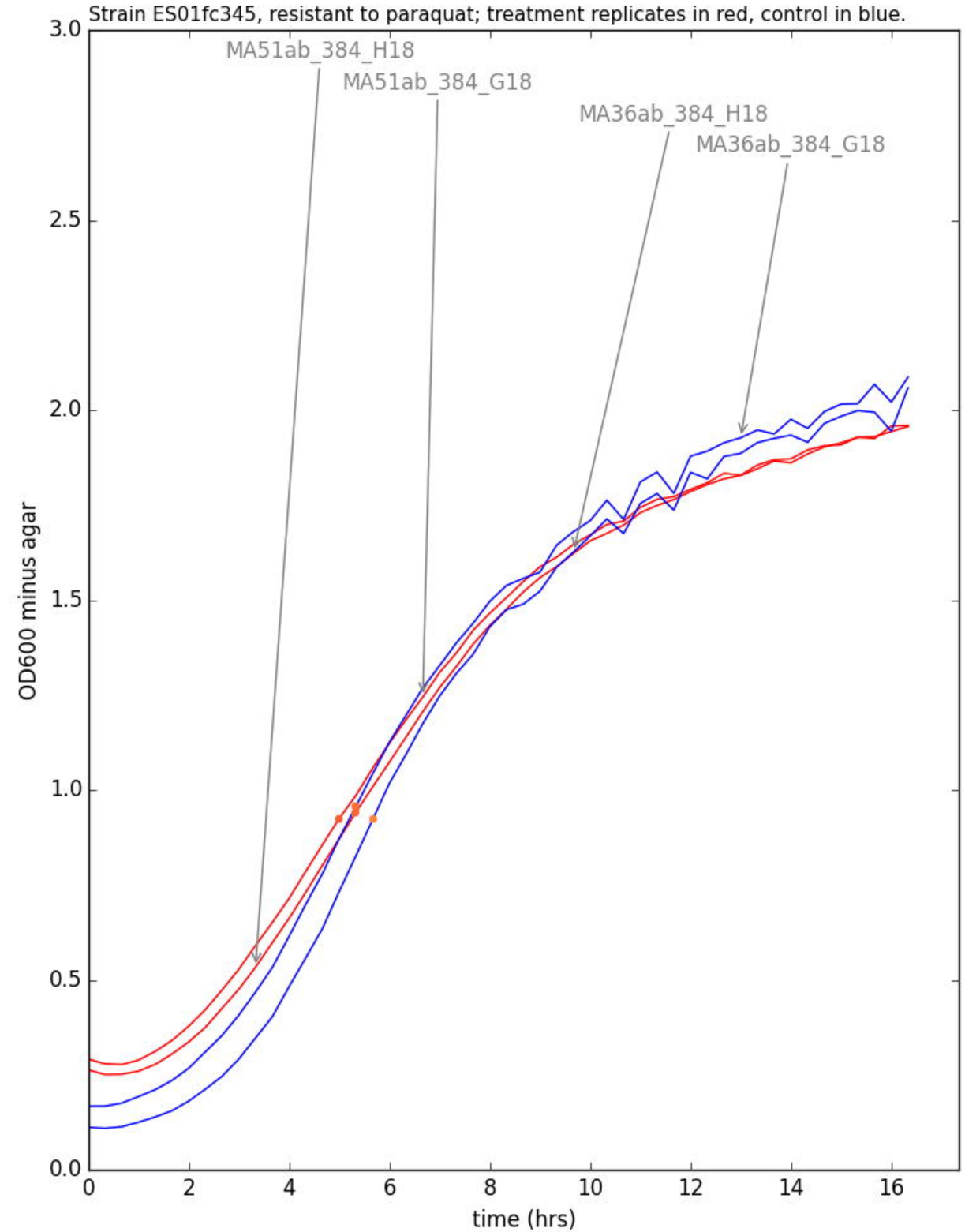


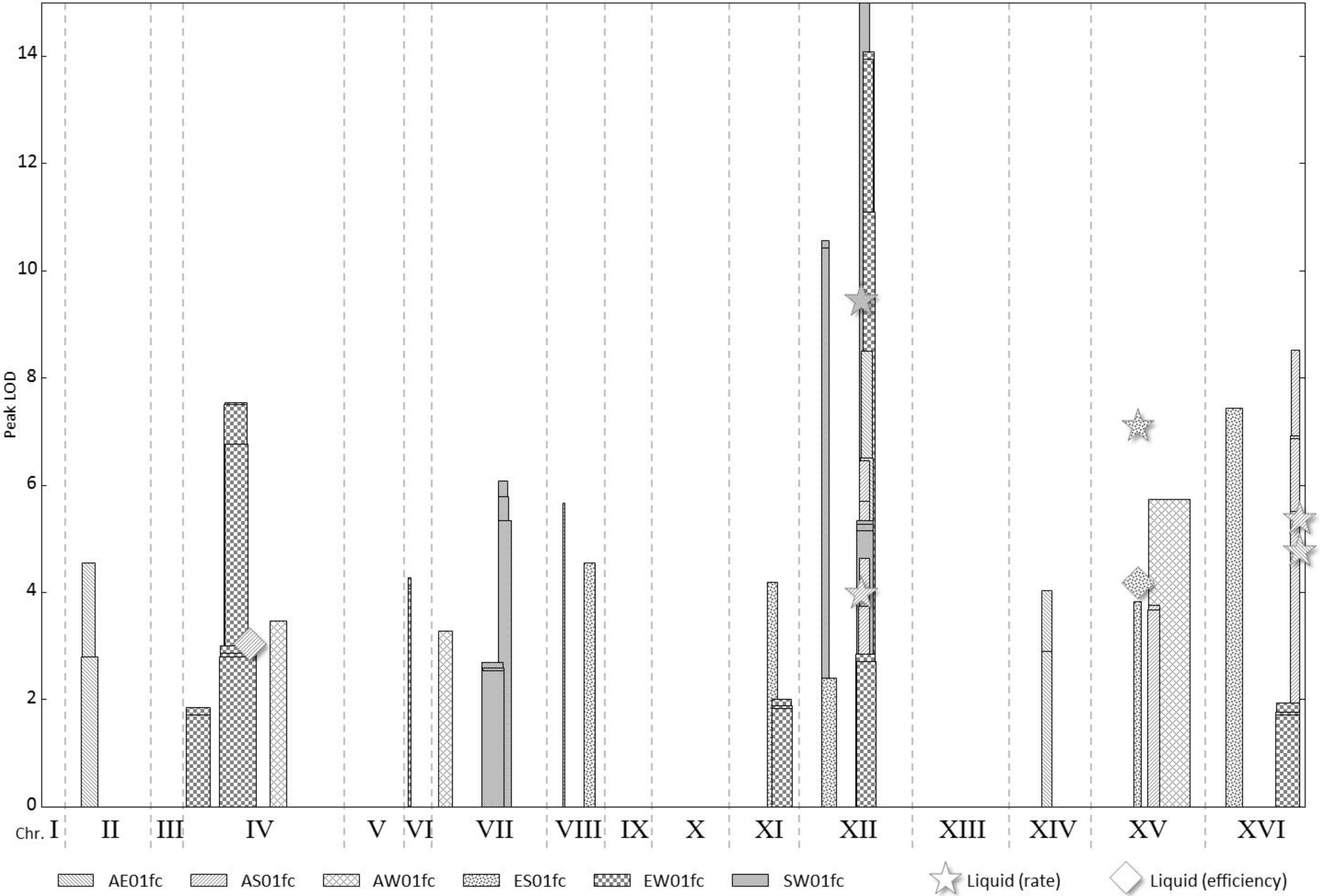

y = 0.7625x + 4.8914
R² = 0.7454
Log10 (measured cell count)
Microplate reader measurement minus media (OD600)
A
y = 6E+06x + 55705
R² = 0.9076
Measured cell count
Adjusted spectrophotometer reading (OD600)
B


Microplate reader
Spectrophotometer (adjusted)
10000000
5000000
0
-5000000
-10000000
-15000000
Difference in cell count (predicted - actual)
100000
1000000
10000000
Average of predicted cell counts by both methods
overestimation
underestimation


3.4
3.2
3
2.8
2.6
2.4
2.2
2
Average of raw OD600 readings
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
Standard deviation of OD600 readings
Yeast in stationary phase
Yeast in stationary phase (2)
Artificial spots (woodglue etc)
Opaque plastic (1 layer)
Opaque plastic (2 layers)
Paper (1 layer)
Paper (2 layers)
Low density foam (3 layers)


Strain AW01fc272, sensitive to paraquat; treatment replicates in red, control in blue.
MA51ab_384_L23
MA51ab_384_K23
MA36ab_384_K23
MA36ab_384_L23
OD600 minus agar
time (hrs)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
2
4
6
8
10
12
14
16


Strain ES01fc345, resistant to paraquat; treatment replicates in red, control in blue.
MA51ab_384_H18
MA51ab_384_G18
MA36ab_384_H18
MA36ab_384_G18
OD600 minus agar
time (hrs)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
2
4
6
8
10
12
14
16


Peak LOD
0
2
4
6
8
10
12
14
Chr.
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
AE01fc
AS01fc
AW01fc
ES01fc
EW01fc
SW01fc
Liquid (rate)
Liquid (efficiency)