Received: 24 September 2019 | Revised: 24 January 2020 | Accepted: 16 March 2020

DOI: 10.1002/bit.27336

COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY AND BIOENGINEERING WILEY

Cell function profiling to assess clone stability

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Funding information
H2020 Innovation In SMEs, Grant/Award Number: 778586

Abstract

In cell line development the identification of stable Chinese hamster ovary cells for production is a critical but onerous task. The stability trial focus upon high-level attributes can mask profound underlying cellular changes, leading to unstable clones mistakenly being chosen for production. The challenge is to assay underlying cell pathways and subsystems without pushing up cell line development costs. ChemStress® cell function profiling is a simple, multiwell plate-based assay that uses a panel of active chemicals to mimic known bioprocess stresses and challenge key pathways. After 3 days of static culture on the plate, functional responses are assayed, for example, titer and growth. Here this approach is used to monitor 131 clones as they change over real stability trials. A novel stability metric is defined over the data to identify stable clones that remain unperturbed across many components of cell function. This allows stability trials to look beneath the titer to identify clones that are internally more stable.

KEYWORDS

bioprocessing, cell function, CHO, clone stability, monitoring, phenotyping, process

1 | INTRODUCTION

Chinese hamster ovary (CHO) cells remain the major mammalian platform for biopharmaceutical production despite a propensity for rapid change (Baik & Lee, 2018; Barnes, Bentley, & Dickson, 2003; Davies et al., 2013; Fernandez-Martell, Johari, & James, 2018; O'Callaghan et al., 2010) that can render a bioprocess uneconomic or generate product deviations incompatible with the clinic. During cell line development (CLD) companies are required to demonstrate production clone stability by monitoring changes to titer, product quality, and other key performance indicators over prolonged sub-culture (ICH Expert Working Group, 1997). These stability trials are immensely time-consuming and resource-intensive.

Aside from certain obviously critical cellular factors, such as integrity of the recombinant gene construct, trials tend to overlook characterization of the underpinning cellular machinery and instead focus upon confirming the stability of high-level process and product quality attributes. To move bioprocessing on from underwriting process performance and product quality by quality control towards a more proactive quality-by-design (QbD) approach, it is critical to identify and understand changes to the cellular machinery driving bioprocess performance (Farrell, McLoughlin, Milne, Marison, & Bones, 2014).

A compelling demonstration of why a deeper understanding of the cell is vital is apparent from the heavy emphasis upon titer in stability trials. In a set of 131 real stability trials of CHO-K1a derived clones within a major biopharmaceutical company, with initial titers in the hundreds to low thousands of mg/L, changes to integral viable cell density (IVCD) and cell-specific protein production (qP) were endemic even amongst supposedly stable clones, where stability was based upon the conventional definition of losing not more than 30% of initial titer. The "stable" clone with the highest rate of qP change exhibited a 63% qP reduction but compensated for this by a 114% increase in IVCD. This clone's radical internal reconfiguration was

not evident from the top-level view afforded by titer, which was reduced by only 21%. Similarly, the clone with the highest rate of IVCD change gained 9% titer while dropping IVCD by 40% and increasing qP by 84%. Again, the titer did not speak to these profound underlying cellular changes. While these are the most extreme responses amongst the conventionally stable clones in the dataset, they cannot be dismissed simply as radical outliers. Across the set, the median qP change is −27%, while the median IVCD change is +18%, yet the median titer change is only −5%. These data indicate that underlying cellular changes are common and can be considerable, but often they are not detected because when qP is lost and IVCD gained, or vice versa, the compensatory changes can lead to apparent stability of titer. While a pragmatic process engineer might accept apparent titer stability regardless of underlying cellular change, this is inadequate for QbD where a deep appreciation of how the production cell works is vital. The challenge is to acquire information about the underpinning cellular machinery driving bioprocess performance, including looking even deeper than IVCD and qP, in a practical and affordable manner.

Omics methods might provide a comprehensive account of molecular abundance for various classes of biomolecules, but even the low cost of modern DNA sequencing is beyond the early stages of CLD where dozens or even hundreds of clones might be assayed multiple times. The ChemStress® cell function profiling array has been developed to directly measure multiple bioprocess-relevant cell functions in one simple and rapid assay. ChemStress® is a 96-well plate containing multiple chemicals selected for their ability to challenge key cellular pathways driving production or to emulate major bioprocess stresses (Figure 1). Static cell culture on ChemStress® plates for 3 days allows time for the chemicals to challenge the cells. Cellular responses to challenges are assayed for viable cell density (VCD) and titer by Valita®TITER (Thompson et al., 2017), or by sampling into other titer assay systems. Taken together, the VCD and titer responses to the different chemicals constitute characteristic fingerprints of deep cell function. Here ChemStress® is deployed to track multiple cell functions as they change throughout stability trials.

ChemStress® assays were conducted at approximately five-generation intervals over the early generations of 131 active stability trials (therefore clones already had good growth and productivity). For each clone, this generated a high-resolution time series of fingerprints. Any pair of fingerprints can be compared by a simple geometric operation (a dot product) that generates an angle describing their overall similarity. Angles of zero correspond to identical fingerprints, small angles to similar fingerprints, and large angles to very different fingerprints. In this study, angles were normalized by the number of generations between fingerprints to quantify the "rate of angle change per generation" as a broad metric of change in cell function over time. For each time series, the normalized angles between subsequent relative growth fingerprints were plotted against their mid-generation (Figure 2). All clones were ranked upon the basis of their maximum rate of angle change across their time series, with the most stable 10% of clones (highlighted in blue) exhibiting consistently low rates of change, implying stability across multiple components of bioprocess-relevant cell function. Figure 3 shows the most (blue) and least (pink) stable clones by the relative growth fingerprint.

Fingerprints can be dissected into individual chemical responses to identify the drivers of cell function change. Linear, low-gradient individual responses over time indicate that a cell does not alter how it responds to a particular chemical. Triaging the most stable clone's individual responses on this basis flags only two chemicals (ammonium chloride and aminotriazole) that change considerably throughout the trial, whereas the least stable clone exhibited changes in ten distinct chemicals. This confirms that the clone ranking heuristic successfully sorts clones by fingerprint stability. Of the ten altered chemical responses in the least stable clone, the ammonium chloride,

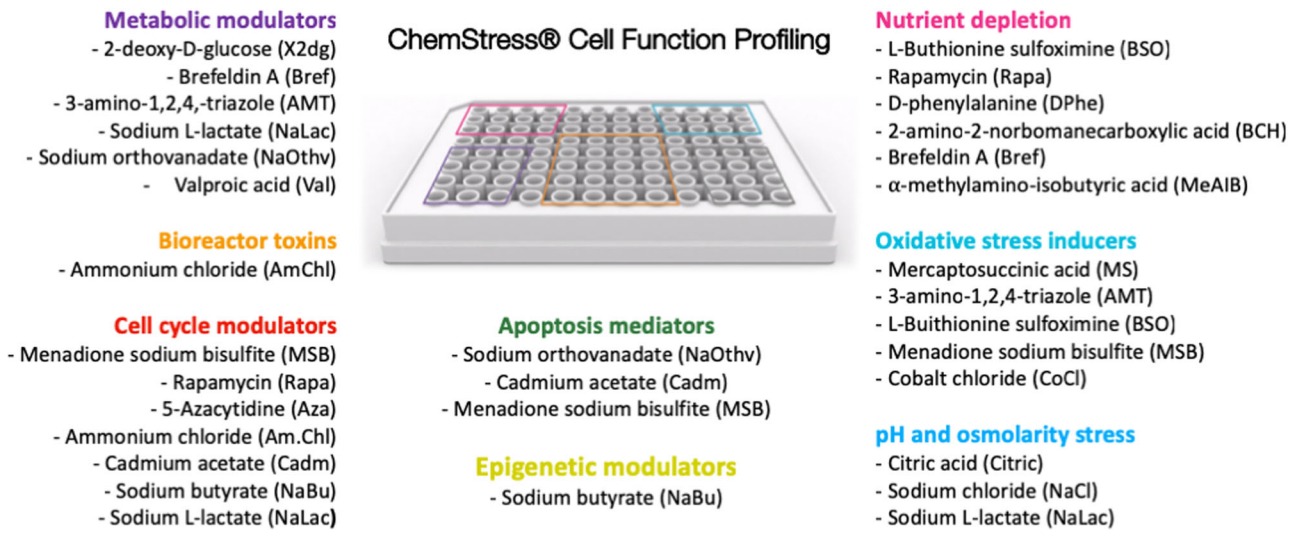

FIGURE 1 ChemStress® plate chemicals and their (nonexclusive) broad mechanistic classes. Cells are incubated on the plate for three days. Growth and titer responses to chemicals are recorded. After combining on-plate replicates, relative growth and titer responses to chemicals are calculated by dividing each by the unstressed value. This gives a rich fingerprint of cell function [Color figure can be viewed at wileyonlinelibrary.com]

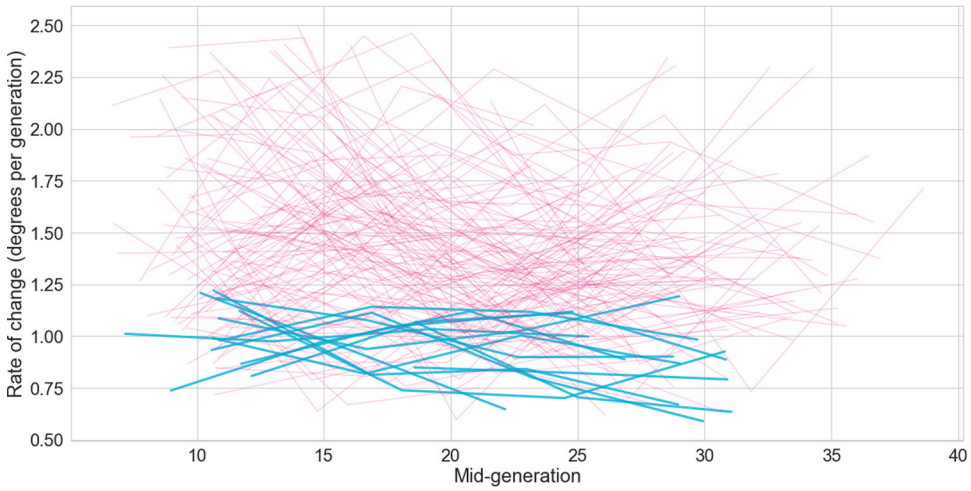

FIGURE 2 Rates of relative growth fingerprint change per generation versus the midpoint of each subsequent fingerprint pair. Start ages vary due to age in the cell bank. The most stable 10% of clones are highlighted in blue [Color figure can be viewed at wileyonlinelibrary.com]

citric acid, and cobalt chloride responses suggest the least stable clone might be particularly sensitive to common bioreactor stresses. Aminotriazole, buthionine sulfoximine, and cadmium acetate dihydrate responses also suggest oxidative stress sensitivity.

The time series plot for relative titer fingerprints (equivalent to Figure 2) is not shown but exhibits the same consistently low rates of change per generation amongst the most stable 10% of clones. The most and least stable clones by relative titer are shown in Figure 4. All chemicals are individually stable for the most stable clone, while the least stable clone exhibits changes across 12 chemicals, also linked to common bioreactor stresses and oxidative stress responses.

In CLD expensive and time-consuming trials are conducted in an effort to identify relatively more stable clones from a background of high CHO instability. The focus upon high-level attributes, such as titer alone does not reveal, and often masks, profound underlying cellular changes. This veneer of clone stability does not bode well for transitioning bioprocessing's guarantor of titer and product quality from quality control to QbD. The challenge is to acquire information about underlying cell functions and pathways without requiring ever more CLD resources. Towards this, ChemStress® profiling was used to track multiple cell functions throughout a stability trial. Angles between fingerprints were used to quantify broad cell function similarity, then time series were ranked by the maximum rate of angle change per generation. This allowed clones to be ranked by cell function stability over the trial, such that top-ranked clones had the most consistent cell function fingerprints. This constitutes a novel, orthogonal stability metric that looks deeper into cell function than titer alone. Whether or not this novel stability metric leads to

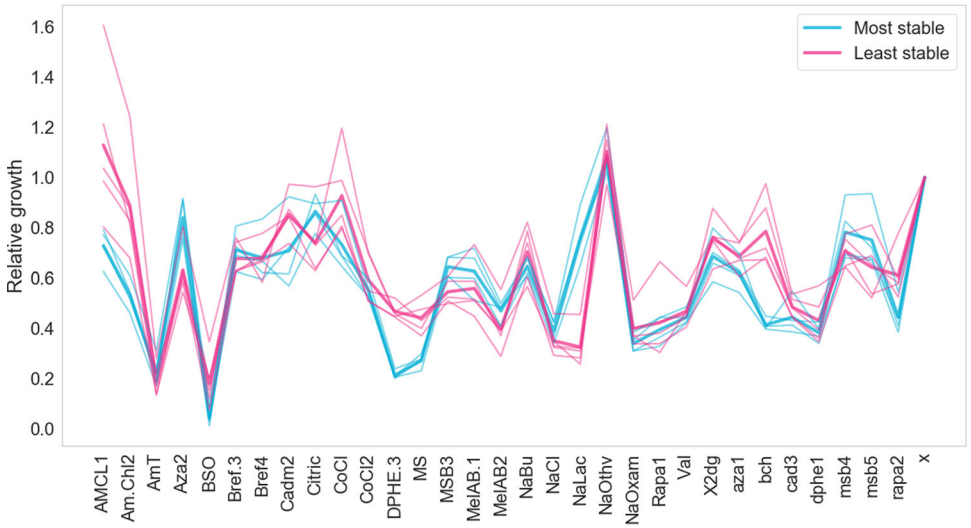

FIGURE 3 The most and least stable clones by relative growth fingerprints. Bold lines indicate the time series mean, with each individual fingerprint indicated in ghosted lines. Mostly tight agreement of the stable (blue) fingerprints contrasts with the more spread unstable (pink) fingerprints [Color figure can be viewed at wileyonlinelibrary.com]

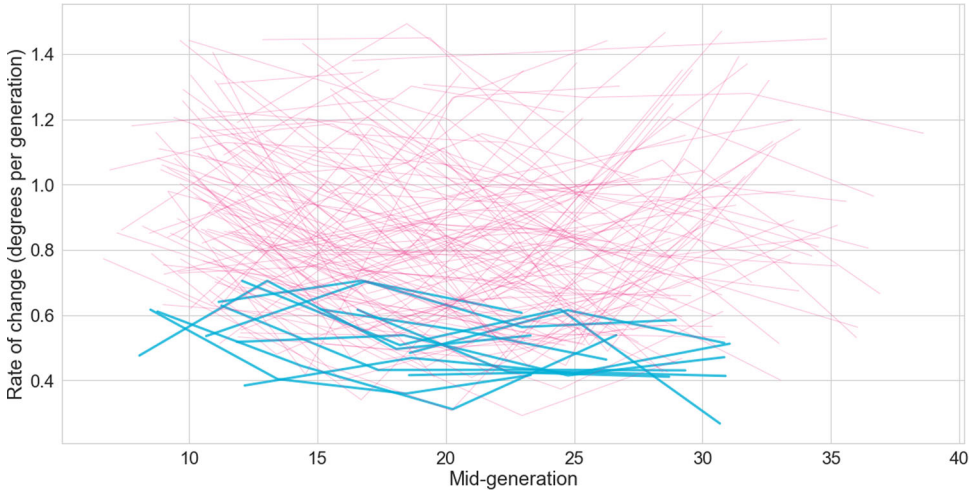

FIGURE 4 Rates of relative titer change per generation versus the midpoint of each subsequent fingerprint pair. Start ages vary due to age in the cell bank. The most stable 10% of clones are highlighted in blue [Color figure can be viewed at wileyonlinelibrary.com]

improved production performance remains to be established as production data is not yet available for the study clones. It can provide confidence that selected clones are functionally (and therefore, by inference, genetically) stable. This provides a rational basis for removing unstable clones early in trials to reduce the cell culture burden.

There is no agreement (all $R^2 < 0.2$) between this novel stability metric and conventional stability trial results (titer was measured out to at least 100 generations), even after recalculation of the conventional but unclear "percentage titer change" trial endpoint to the clearer "rate of titer change per generation" (to adjust for the uneven influence of initial values and account for variable trial lengths). This lack of agreement is unsurprising as apparent titer stability means the conventional trial endpoint is not a good measure of clone stability, while the many changes detected by cell function profiling are difficult to represent within a single summary statistic (the maximum rate of angle change per generation across the time series). This summary statistic is convenient for clone ranking, but only by looking at fingerprints in depth can the many underlying cellular changes be seen.

ChemStress® time series analysis is designed to be a practical tool for ranking clone stability as measured across multiple cell functions. The goal is to drive more informative trials that identify truly stable clones. The metric has some limitations as the biological function is not an objective property (like molecular mass or cell diameter) but a subjective interpretation of role (Shrager, 2003). This subjectivity manifests itself primarily during the calculation of the similarity between fingerprints, where it is embedded in the vector weights. Here a minimal assumption that all components contribute equally (all vector components have equal weight) to overall cell function similarity is implemented, but one could easily argue that the response to osmotic stress inducers should carry more weight than the response to uptake inhibitors, for example. There is no objectively correct answer here, or indeed by any other cell function assay. If certain aspects of function are deemed more interesting than others, this is easily implemented in the angle calculation by assigning higher coefficients to those functions.

Here the ChemStress® time series analysis was interpreted as a novel clone stability metric, but the same approach could be applied whenever cells must be monitored over time, such as continuous bioprocessing, for example. Much has been made of clone stability improvements due to targeted transgene integration, as demonstrated by the transcriptomic studies of Grav et al (Grav et al., 2018), but Chemstress® potentially is an easier way to demonstrate the stability of deep cell function. Broader still, ChemStress® cell function profiling technology could lead to a deeper understanding of how different recombinant products (e.g., easy- vs. difficult-to-express proteins, product classes), expression engineering strategies, and media components impact upon the cell and cell stability.

2 | MATERIALS AND METHODS

Stability trials on 131 clones producing one of four monoclonal antibodies were conducted within a major pharmaceutical company. Clones were maintained on in-house media for at least the duration of an expected production run. Samples were taken early (<15 generations) and late (>80 generations) in subculture were assessed in either shake flasks or Ambr15™ (Sartorius) for titer and IVCD. ChemStress® plates were measured on samples taken from the same stability trials over approximately 30 generations, from 5 to 40 generations, at approximately five-generation intervals. Cells at a density of 200 K per ml were loaded onto ChemStress® plates and statically incubated for 3 days at 37°C (this being the minimum exposure required to generate measurable chemical responses). VCD was assayed by Presto Blue™. Titer was assayed by Valita®TITER (Thompson et al., 2017) against standard curves for each product. Measurements were recorded on a BMG PHERAstar (BMG

LABTECH) plate reader. Negative titer or VCD values were set to zero. On-plate replicates were combined on means, with any exhibiting >15% standard deviation relative to the mean filtered out. Raw fingerprints were transformed into relative fingerprints by dividing each chemical stressor value by the unstressed value. Overall relative fingerprint similarities were quantified by the angle between fingerprints (treated as vectors), calculated via the dot product. Anomalous plates within each time series were detected on the basis of their angle to the mean of the rest of the time series, with plates with angles >15° being discarded from the series (this threshold was determined heuristically and ratified by visual inspection). For each clone, angles were calculated for subsequent fingerprints in the time series. These angles were normalized by the interval in generations between subsequent fingerprints to give the rate of angle change per generation. Clones were ranked relative to each other upon the maximum rate of angle change per generation across their time series.

ACKNOWLEDGMENTS

This work was part-funded through a Horizon 2020 Phase II SME project (778586). All authors were involved in the development of the ChemStress® cell function profiling technology platform. Valitacell® provide ChemStress® on a commercial basis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Dobson PD, Coss KP, Doherty C, Clifford J, Thompson B, James DC. Cell function profiling to assess clone stability. *Biotechnology and Bioengineering*. 2020;1–5. https://doi.org/10.1002/bit.27336