Chinese hamster ovary (CHO) cells, the powerhouse of the biopharmaceutical industry, are inherently variable. This functional heterogeneity is what makes them such a valuable tool for producing biotherapeutics, while also accounting for the considerable cost and time associated with cell line development (CLD). As is the case with other transformed mammalian cell lines maintained in vitro, inherent genomic instability can modulate chromosomal arrangement and complement gene copy number and transcriptional activity. In industry, exploitation of clonal genetic variation is essential to derive cell lines capable of supporting biomanufacturing, as this underpins a variety of prerequisites, most particularly an ability to survive in synthetic environments, achieve high rates of cell proliferation and efficiently manufacture recombinant proteins. However, genetic variation can also give rise to unpredictable behaviour in vitro such as loss of productivity, variations in product modifications, e.g. N-glycosylation, and proliferation rate. Dealing with the consequences of clone-specific genetic and functional variation, and its acquisition during CLD, can be extremely time-consuming and labour-intensive.

CLD requires a significant amount of investment and the instability of cells is the central driver of this lengthy process. It is essential to have the appropriate instrumentation, expertise and time, to assess the performance attributes and stability of hundreds of clonally-derived cell populations in parallel. In addition, it is necessary to culture these cells in an optimum cell environment, to maintain a stable state. Development of cell culture media is therefore another major source of continuing investment. This involves the screening of chemical additives, the functional validation of these enhancer molecules, and the confirmation of the production of a stable, robust, high-quality media product. Moreover, quality control (QC) of the complex medium product itself (post-production, transport and storage) is generally restricted to simple monitoring of cell growth and productivity. There is an urgent requirement for new QC technologies able to measure lot-to-lot variation and functional integrity of this vital part of the bioproduction process.

As the biopharmaceutical market expands to allow for more competitive conditions in the form of biosimilars of costly blockbuster drugs, new technologies which can decrease the time and costs associated with the development of these biotherapeutics will be essential. These technologies must be prepared to understand the molecular mechanisms of the cells at the centre of this industry. To illustrate the financial consequences of defects in any one of the steps in the bioproduction process, contamination alone can result in an estimated $1bn loss in revenue for blockbuster biotherapeutics, along with a projected $1m loss in production costs and up to $20,000 in quality assurance investigations. Companies are therefore ultimately driven towards streamlining CLD and the bioprocessing of biotherapeutics, through the use of informative assays which can generate functional data and determine reliable QC of reagents.

ChemStress®: Generating a Functionally Relevant Biological Signature
ChemStress® is an information-rich, analytical assay supplying data on the functional quality of clones, facilitating the selection of stable producer cells and high-quality media environments through the generation of cellular responses to chemical stressors. The technology draws on the unique biological signatures (e.g. growth and titer for CHO cells producing monoclonal antibodies) a given cell generates when it is grown in the presence of small molecule stressors, which have been selected to simulate toxic environments or stimulate specific stress response pathways. This data gives biological information on how specific clones respond to stressors, and whether these clones have the ability to remain stable and productive in these conditions. These cellular responses are unique identifiers, or ‘fingerprints’. While classic fingerprints are static measurements of identity, these ‘ChemStress® fingerprints’ are markers which, much like cells, can change overtime as a result of e.g. epigenetic silencing events, or loss of recombinant protein expression, giving early indications of loss of specific functionally. The diverse range of responses is a unique way of: identifying cell populations, indicating divergence from its original state and indicating the growth quality of cell culture conditions. ChemStress® employs the use of a novel 96-well microtiter plate, wherein each well is coated with a single specific chemical (Fig. 1), selected based on its published ability to simulate specific conditions, or effect specific cellular pathways. The workflow is illustrated in Fig. 2, where product titer is analysed using our Valita®TITER assay.

![ChemStress® technology](Image)

**Figure 1. ChemStress® technology.** Each well of the 96-well plate is coated with a single specific small molecule stressor, at select concentrations. Each chemical is selected to stimulate a particular cellular pathway, e.g. apoptosis and oxidative stress.
The assay was developed in collaboration with researchers from the University of Sheffield and chemicals were selected based on their published effect on cellular systems (examples included in Fig. 1). Chemicals were tested against a mAb-producing CHO cell line and screened using half maximal inhibitory concentration (IC$_{50}$) assays to determine cell growth inhibition. Several chemicals are present at multiple concentrations to allow for capture of variable responses when exposed to different concentrations of key chemicals.

The wide range of selected chemicals are selected to target a number of pathways stimulated under stressful conditions, including nutrient starvation, cell death and oxidative stress. Rapamycin, which is expressed in mammalian target of rapamycin, is an inhibitor of mammalian target of rapamycin which is suppressed during nutrient starvation\textsuperscript{34}. Rapamycin induces autophagy, a pathway which causes controlled breakdown of cellular compartments, or removal of damaged organelles, generating the components required by the cell for survival during nutrient starvation\textsuperscript{35}. Brefeldin A is often used in flow cytometry to inhibit protein secretion and accumulate cytokines for staining\textsuperscript{14,15}. It is also used to induce endoplasmic reticulum (ER) stress and eventual apoptosis\textsuperscript{16}. In addition to causing disassembly of the Golgi and fusion with the ER\textsuperscript{27}. There are a number of other chemicals on the ChemStress® plate which induce apoptosis, including cadmium acetate and menadione sodium bisulfate, and chemicals which induce oxidative stress, e.g. cobalt chloride and L-1,2,4-triazole.

Applications

ChemStress® targets core issues in biomanufacturing, including:

- Identify parental cell line of clonal isolates (Valita®ID),
- Stability of producer cells (Valita®STABILITY),
- Quality control of cell culture environments (Valita®QC).

Valita®ID

Cross-contamination of cell lines is a major issue, not just for the biopharmaceutical industry, but research in general. HeLa cell contamination of multiple cell lines is one such well-documented case. For the biopharmaceutical industry, regulators insist that the identity of clonal isolates can be traced. There are techniques available to allow companies to meet these requirements, which include karyotyping, short tandem repeat (STR) analysis and DNA sequencing and barcoding. ChemStress® fingerprinting exploits the functional variability of cell populations to generate biologically relevant data on the specific clonal identity of cells, giving users a quick and easy method to keep track of cells (illustrated in Fig. 3).

To test this, ten different mAb-producing CHO cell lines were sourced, derived from a variety of different CHO cell families, including CHO-K1, CHO-S and CHO-DG44. Each cell line was coded anonymously as CHO-A to CHO-J, to prevent any bias. ChemStress® profiling was able to cluster the different groups of coded cell types together as indicated in Fig. 4. The analysis used ‘linear discriminant analysis (LDA)’ and ‘Euclidean distance minimisation’ in addition to ‘Ward’s Hierarchical clustering’ and found that fingerprints were unique and could be used effectively to partition cell types into distinct parental ‘groups’. Also, using LDA, given a query fingerprint, the probability of it being from a specific group can be approximated. This application can be utilised to generate a library of clonal fingerprints to allow for the identification of clones following storage and during CLD.

Given the need to meet the regulatory requirements on clonal identity, it is necessary for the generation of easy to implement, simple, rapid and cost-effective analytical assays which can determine the origin of clones. The ChemStress® platform offers a simple assay which generates biologically relevant data, that predicts the parental identity of clonal isolates.

Valita®QC

There is a substantial lack of informative, cost-effective assays to allow for screening of cell culture medium prior to usage and following long-term on-site storage. Lot-to-lot variations in media have been well-documented, in addition to the consequences of incorrect storage including temperature fluctuations and UV-exposure\textsuperscript{36,37}. Valita®QC can be used to
screen the growth and productivity of a reference cell line when grown in the presence of query lots of media, generating informative data which allows for the acceptance or rejection of media lots based on functionally relevant responses (concept illustrated in Fig. 5).

Figure 5. Valita® QC workflow. A cell line is grown in different lots of a specific media. The resulting fingerprints are analysed to determine acceptable responses (acceptable growth and productivity) to allow for the acceptance of the lots; those with poor fingerprints are rejected.

Figure 6. Growth responses following culturing of cells in different media lots. Three growth response graphs are indicated from three specific chemicals on the ChemStress® plate. The same cells are shown to have unique profiles, despite being cultured in different lots of the same composite media, clearly indicating quality difference between the lots.

The growth and titer responses of cells cultured in different lots results in a distinct fingerprint, as illustrated from select chemicals in Fig. 6. The overall fingerprint generated from the multiple chemicals screened can be used to determine if lots should be rejected based on poor growth and productivity of cells. In addition to determining lot-to-lot variation, ChemStress® aims to detect if any damage has occurred to affect the quality of the media prior to use, e.g. following incorrect storage, including exposure to UV or temperature fluctuations, or during long-term storage (ageing). It is important to have a functionally relevant, reliable assessment of the quality of media before the product is released or used. As a critical component of the end stages of a lengthy process, it is necessary to have stringent QC technology in place which can allow for the accurate assessment of this essential reagent.

Valita® STABILITY
Predictable bioprocesses require stable producer cells that are not prone to sudden changes leading to diminished productivity or quality. To demonstrate stability, clones are sub-cultured and monitored for titer, growth and various CQAs over a period of months. There is a very high workload associated with these trials. To enhance trial efficiency, ChemStress® is being used to identify maximally stable clones pre-trial and to fail unstable clones quickly within a trial.

As a broad measure of stability, companies typically monitor titer over a period roughly equivalent to a production run, with any clone losing more than 30% of its initial titer over this time being considered unstable. Maintaining titer can be a misleading stability metric if a clone’s cell growth rate drops but its protein production rate increases, or vice versa, in such a way that their combined effect on titer change is negligible. This ‘apparent titer stability’, which is masking real underlying biological change, is abundant in our data. Quixotic targets make for challenging prediction problems. An alternative, cleaner formulation of the stability prediction challenge, still aimed at streamlining trials, is to detect clones achieving high titers predominantly through very high growth rates or very high protein production rates as our data indicate such extreme strategies are difficult to maintain over time. By contrast, we observe that high titer clones built from ‘moderate’ growth and protein production rates have a higher chance of stability than these ‘extreme’ clones. Our algorithms leverage ChemStress® to detect extreme clones with a high probability of being unstable.

Figure 7. Valita® STABILITY workflow. Clones are monitored using ChemStress® before or during the early stages of stability trials to assess whether they are extreme and therefore likely to be unstable.

Figure 8. Valita® STABILITY Scoring. Growth and protein production rates for the most extreme clone relative to background distributions for all clones in the same panel, depicted as box-and-whisker plots.
Alongside using ChemStress® to predict industry’s established stability metrics, ChemStress® itself can be used to define a rich, functionally relevant measure of clone stability. A clone can be scored for any stress chemical on the ChemStress® plate by calculating how far removed the clone’s measurement is from the median measurement derived from a background set of clones. A clone that is many deviations removed from the median is, by definition, extreme relative to the background with respect to that particular stress chemical. By summing scores across all stressors, it is possible to rank clones by relative stability.

Predicting stability metrics is challenging as there is considerable variability in how trials are conducted and how stability is defined, with ‘apparent titer stability’ particularly clouding the issue. Deploying ChemStress® to inform ‘extreme’ clone prediction can help streamline trials. Scoring ‘extreme’ clones directly using ChemStress® provides an additional, function-focused stability metric with the option of dissecting the rich fingerprint to scrutinise the underlying biological mechanisms driving instability.

Biomanufacturing Process Control

Exploiting the cellular responses to stressful culture environments is a novel and information-rich approach to addressing the key issues of bioprocessing. The inherent variability of cells is a critical component of the biopharmaceutical industry, which can be exploited to create information-rich data sets. New technologies targeted at decreasing the time and costs associated with the development of biotherapeutics must ultimately be prepared to understand the molecular mechanisms of the cells at the centre of bioprocessing. This is the central approach taken by the ChemStress® platform. The assay generates highly relevant information on how a clone reacts to biological stressors, which can be used to discriminate the variation between clones, the deviation of clones from a stable state and variations in the quality of media environments. This is an assay which offers a rapid and easy way to compile a significant amount of functionally relevant information, which is currently lacking in the biotherapeutic workflow. This kind of strategy is necessary to streamline the CLD process and ultimately decrease the substantial cost and time taken to bring a biotherapeutic to market.

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REFERENCES


