

A publication of
The Williamsburg BioProcessing Foundation

Spring 2007
ISSN 1538-8786

BioProcessing JOURNAL

The Most Trusted Source of BioProcess Technology®

Vol. 6 No. 1

www.bioprocessingjournal.com

Automated Cell Production To Support High-Throughput Screening

By EDWARD MCKILLIP*, NICOLE BRACY-JOHNSON, VERONICA SOLOVEVA, JAMES LAROCQUE, JOHN MORIN and JEFF PASLAY

The search for new and more effective drugs is a complex and challenging endeavor requiring a number of strategies for identifying and processing new drug candidates. A common tool used in this search is high-throughput screening (HTS) of a large library of small molecular weight compounds. In each of our HTS campaigns, more than 500,000 compounds are tested for biological activity against one of our many molecular disease targets in order to identify compounds of potential therapeutic interest.¹

High-throughput screens can be divided into two major types: those using cells, and those not using cells. At least 50% of our screens are cell-based and require a consistent, uniform supply of cells throughout the screening campaign. The provision of the cells is a primary consideration in our cell based-assays and therefore, an automated cell culture laboratory was included in our HTS facility. This laboratory serves two functions: the preparation of cell banks, and the daily provision of microtiter plates containing cells for the HTS.

An average screening campaign requires enough cells to seed 1,500,000 microtiter plate wells. Approximately

3×10^{10} cells are needed per campaign. This is usually accomplished by preparing a single batch of cells grown in a bioreactor system.² The CellCube bioreactor (Corning, Inc., Life Sciences, Lowell, Mass., USA) was introduced in the early 1990s and has been used to produce a variety of cell types.³ The CellCube bioreactor has been used to produce large-scale attachment-dependent cell cultures for a number of studies in gene therapy.⁴⁻⁶

The bioreactor system is capable of producing more than 10^{11} cells per single batch depending on the growth chamber and cell type employed. A single batch of cells is preferable for HTS campaigns, as this will avoid batch-to-batch variation during the screen. The cell batch is aliquoted and placed in cryovials in liquid nitrogen. A sufficient number of these stock vials are thawed

and expanded when necessary for the daily supply of microtiter plates.

Different cell-based assays have different daily requirements with respect to the number of plates that can be processed, cell densities per well, and culture conditions. The SelecT robotic system (The Automation Partnership, Wilmington, Delaware, USA) can be programmed to incubate cells, count cells, harvest cells, determine cell viability, split cell flasks, and inoculate microtiter assay plates.⁷

Once a stock vial of thawed cells, or a flask of cells grown from a stock vial has been introduced into the system, the robot will do all the necessary steps to provide cells and microtiter plates for the screen, unattended, 24/7. We currently have two SelecT systems capable of supporting 25 to 30 cell-based HTS campaigns annually.

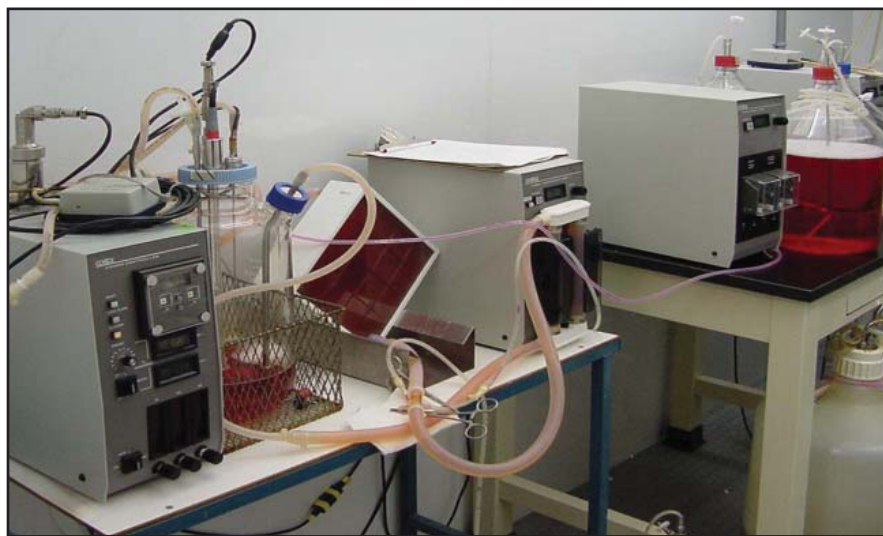


Figure 1. Corning cell cube bioreactor with a 21,250 cm² growth chamber.

Edward McKillip, M.S. (mckille@wyeth.com, phone: 484-865-3883, fax: 484-865-9345), group leader of automated cell culture; **Nicole Bracy-Johnson**, scientist; **Veronica Soloveva, Ph.D.**, group leader of assay development; **James LaRocque**, group leader of high-throughput screening; **John Morin, Ph.D.**, director of high-throughput screening; and **Jeff Paslay, Ph.D.**, vice president of screening sciences; Wyeth Research, Screening Sciences, Collegeville, Pennsylvania, USA. This article is based on a presentation given at The Williamsburg BioProcessing Foundation's conference on Cell Culture Scale-Up, September 18-20, 2006, Thousand Oaks, California, USA. *To whom all correspondence should be addressed.

Preparation of Bulk Cell Cultures

The CellCube bioreactor can provide up to 10^{11} cells per batch. It is a closed system consisting of a circulation loop and a medium exchange loop. Within the circulation loop is a multi-layered chamber for growth of attachment-dependent cells. There are a variety of growth chamber sizes that provide from 10,000 cm² to 85,000 cm² growth area. The 85,000 cm² chamber is ideal for our needs as it produces between 2×10^{10} to 5×10^{10} cells depending on the cell type.

Figure 1 shows the CellCube bioreactor system with growth chamber. Fifty T-175 flasks (total 8,750 cm²) are used to inoculate the 85,000 cm² growth chamber of the bioreactor. The bioreactor is then placed in a 37°C incubator for growth. The pH and oxygen demands are met with the automatic addition of CO₂, O₂ and air. The circulation loop is comprised of a dissolved oxygen probe on either side of the growth chamber.

The difference between the percent oxygen readings of these probes multiplied by the medium circulation rate will provide an oxygen consumption rate (OCR). Table 1 is a record of a typical bioreactor run. The OCR provides an indirect way of measuring cell growth within the growth chamber. When the OCR is plotted versus time, a growth curve can be generated (as seen in Figure 2). When the cells near confluence in the bioreactor they are harvested using 0.025% trypsin/EDTA.

The harvested cells are centrifuged for ten minutes at 250 xg. The supernatant is discarded and the cells are resuspended into freezing medium. The freezing medium consists of the medium the cells were grown in (without selection agents), 10% dimethyl sulfoxide (DMSO) and 20% fetal bovine serum (FBS). The cells are placed in cryovials at 4 ml per vial and a concentration of 1×10^7 to 4×10^7 /ml. The vials are placed in a CBS 2100 rate control freezer (CryoBioSystem, Paris, France) and frozen down to -80°C at a rate of -1°/min. When this process is complete, the vials are placed in a liquid nitrogen freezer until needed.

When an HTS is started, a vial from

Table 1. Oxygen consumption, media circulation rate, and medium exchange during bioreactor run.

Hour	DO #1 %	DO #2 %	Δ DO	Medium Circulation Rate (L/Minute)	Oxygen consumption rate (OCR)	Medium exchange/day (8 liters in circulation)
0	96	94	2	1	2	
24	93	91	2	1	2	
72	96	83	13	1	13	
96	72	54	18	1	18	
120	60	34	26	1.2	31.2	5 liters
144	60	28	32	1.5	48	5 liters
168	63	31	32	2.25	72	

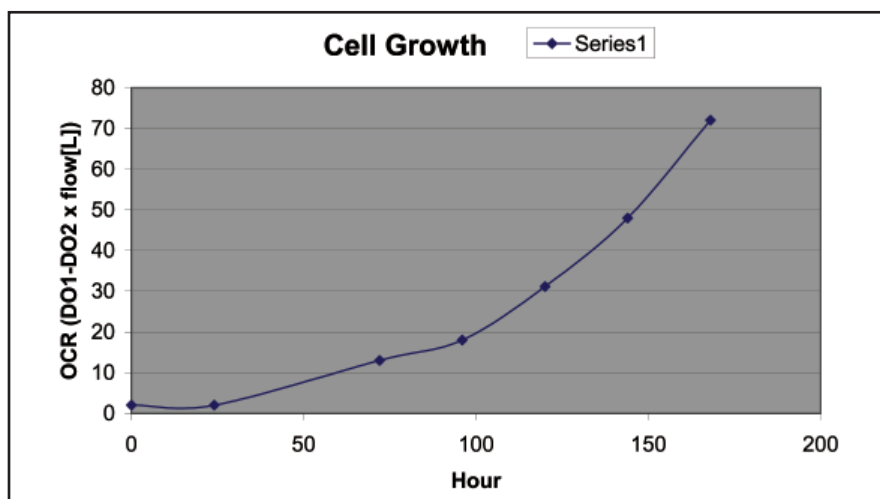


Figure 2. Indirect measurement of cell growth using oxygen consumption versus time.

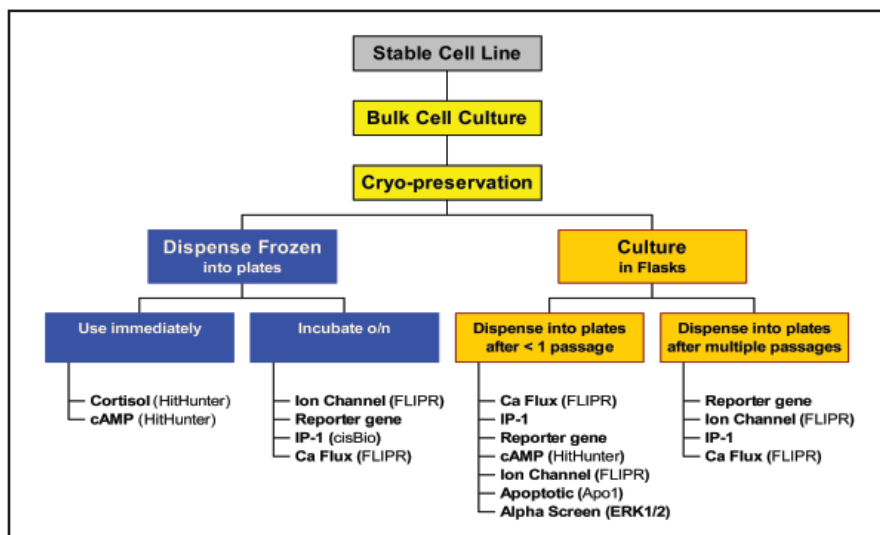


Figure 3. Four procedures used to produce microtiter plates for cell-based assays. Examples of assay types used with each procedure are listed.⁸

the frozen cell bank is rapidly thawed in a 37°C water bath. The cells are suspended in 50 ml of medium and centrifuged at 250 xg for 5 minutes. The cells are then resuspended in the appropriate medium to seed flasks or microtiter plates.

Preparation for Screening Campaign

There are four procedures used to prepare cells in microtiter plates for HTS. A frozen vial of cells can be thawed and used to inoculate microtiter plates. These plates can either be used the same day for the assay or incubated overnight and then assayed. A frozen vial from the cell bank can also be used to inoculate T-flasks. The flasks can be grown and used to seed plates or the flasks can be grown and split to a new passage of flasks that will be used for plating. Figure 3 shows a flow chart of these four

procedures and examples of the HTS assays that they support.

During assay development, a decision is made on which of the four procedures will be employed for a particular campaign. The most direct way, and least expensive, is using freshly thawed cells from the vials to inoculate microtiter plates. However, some cell lines need a recovery period in flasks before they are able to fully express their activity. Several parameters are used in this decision-making process. First and most important are the cellular activity of interest and the consistent reproducibility of that cellular activity in a well-to-well, plate-to-plate, and day-to-day manner. Cells are thawed and used to seed microtiter plates or T-flasks. The T-flasks will be used later to seed microtiter plates. Figure 4 provides three examples of experiments

comparing cellular activity in functional assays of cells prepared according to alternate procedures.

The reproducibility of the functional activity in every well of a microtiter plate is essential for a successful screen. Figure 5 compares activity from microtiter plates made from direct inoculation with freshly thawed cells and microtiter plates prepared from cells that recovered in flasks before plating. The activity from the cells in each well of the plate is tracked independently as a separate trace on the plot. The activity from the microtiter plates inoculated directly from the vials shows widely scattered traces that, in some cases, are very close to the baseline. These plates would not be usable in our assays. The plates inoculated from flasks of recovered cells show a tight, highly reproducible set of traces with a signal-to-background

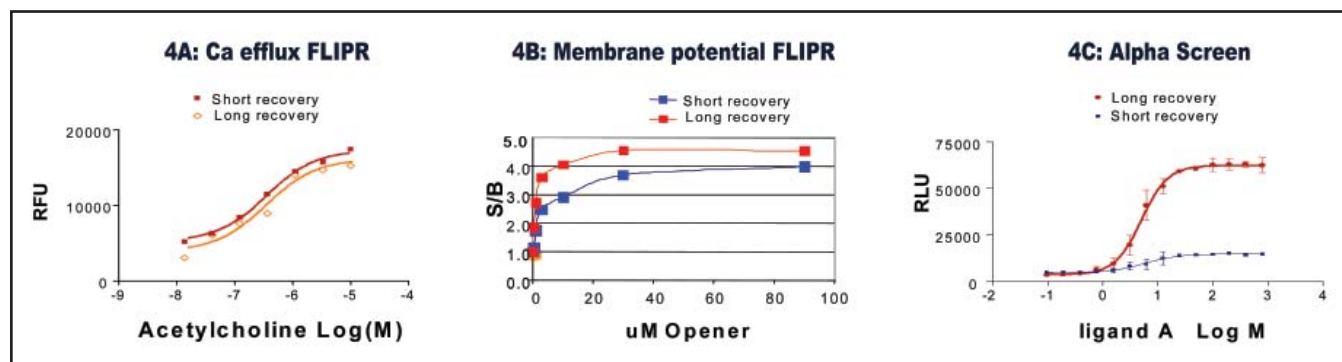


Figure 4. Short recovery uses thawed vials to inoculate microtiter plates. The microtiter plates are then incubated overnight. Long recovery uses thawed vials to inoculate culture flasks to propagate cells being used to inoculate microtiter plates. The microtiter plates are then incubated overnight. A) Illustrates a case where both freshly thawed cells dispensed directly into plates, as well as cells recovered in flasks and then plated, could be used successfully in HTS assay. B) An example of where cells dispensed directly into plates and incubated overnight performed slightly less vigorously than cells that had fully recovered in flasks. In this case, the direct-dispense method was used in order to spare the time, labor and expense of culture in flasks. C) Cell line that must be allowed to recover through at least one passage in flasks before it is used in a functional assay.

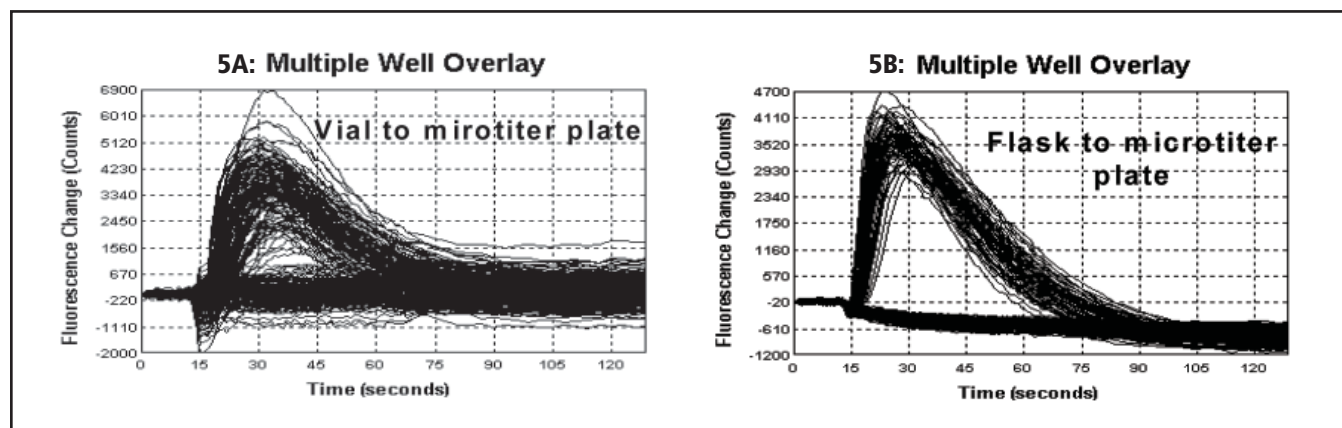


Figure 5. FLIPR assay with: A) freshly thawed cells after overnight incubation in microtiter plate; B) recovered cells passed in flasks prior to plating.

ratio of 14.⁸ These plates would be used in an assay.

Cell Culture in Flasks and Microtiter Plates for HTS Prepared by SelecT System

When it is necessary to grow cells in flasks or to prepare microtiter plates for a screen, the SelecT robotic system is used (Figure 6). The SelecT is capable of growing cells in flasks, trypsinizing flasks to seed new flasks, counting cells and determining viability, adjusting cell concentration, and seeding 96, 384 or 1,536-well microtiter plates. The SelecT has a capacity for 420 microtiter plates and 192 T-flasks. The cell cultures are transported into the SelecT either in a test tube or a T-175 cm² bar-coded flask. The system completes all procedures from this point, including the final plating of the cells for the assay.

If it is determined that cells are to be plated directly from a vial, the vial is thawed in a 37°C water bath, cells are washed with plating medium and then resuspended in plating medium. The cells are transported into the SelecT system for dispensing into plates. The culture volume is recorded in the SelecT program. This system will remove a small amount of the culture to determine cell concentration and cell viability using a Cedex cell counter (innovatis Inc., Malvern, Penn., USA). Once the cell count is completed, the data is recorded, as shown in Figure 7. The culture volume is then adjusted to the required concentration by the SelecT program, and the required number of microtiter plates is produced.

The daily requirement for microtiter plates can be met by inoculating directly from vials (Figure 8). The microtiter plates are either used the same day the vial is thawed or the microtiter plates are incubated overnight. When it is required that the cells recover in flasks, vials from the frozen bank are thawed (as described earlier) and used to inoculate T-175 bar-coded flasks. These flasks are imported into the SelecT for incubation. When the flasks reach the desired confluence, they are used either to seed plates or to seed another batch of flasks.



Figure 6. The automated cell culture laboratory with two SelecT systems.

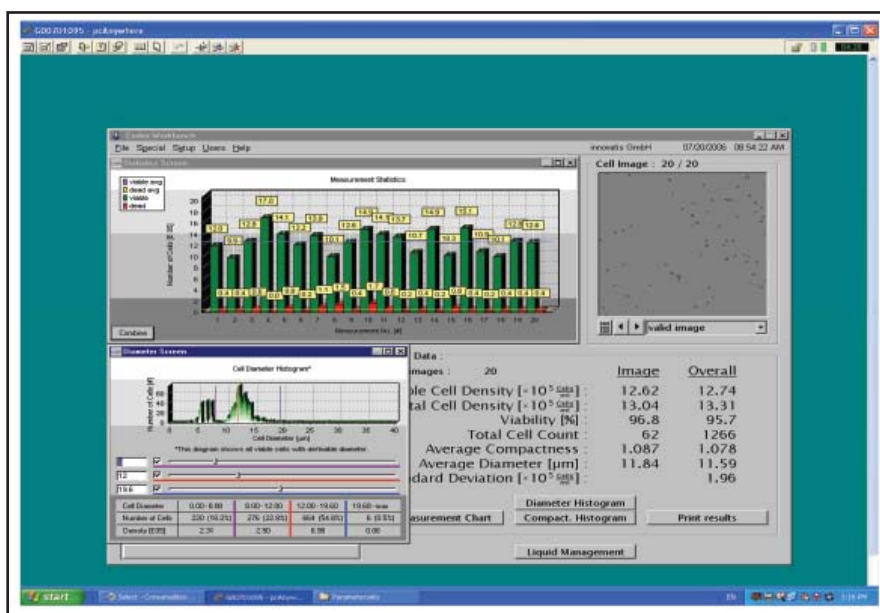


Figure 7. Cedex cell counting program in SelecT system.

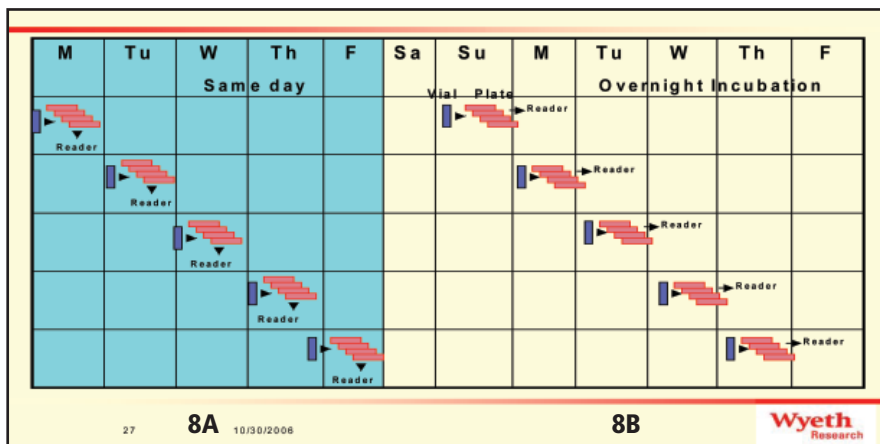


Figure 8. Direct vial-to-plate seeding with: A) same day plate reading; and B) overnight incubation before plate reading.

The growth of the cells in the flasks is carefully monitored offline using the IncuCyte system (Essen Instruments, Ann Arbor, Michigan, USA). The IncuCyte measures cell growth by periodically taking pictures of 32 areas of the growth surface of the T-flask. The areas are evaluated and an average is taken

to provide a percent confluence and a growth curve (Figure 10).

This practice helps to establish appropriate split ratios and time requirements for cell growth and development in the T-175 flasks since it is necessary to maintain certain cell lines at less than 100% confluent.

Daily Microtiter Plate Requirement for HTS Assay

When the microtiter plates have been completed, they are provided to the screening group for assay. There are several steps in the screening process provided. The experimental phase is the first step in the screening campaign. The microtiter plates prepared for this step are used to establish the parameters for the actual screen. A small number of well-established compounds are used to examine the response of the target cell to the compounds and the reproducibility of this response. For most campaigns, the behavior of cells that lack the target gene is also studied to establish the specificity of the assay. It can take several months to complete this phase and is usually done while other assays are performed. When the experimental phase is complete and the automated procedures are validated, the assay enters the primary screen phase.

The primary screen examines all the compounds in the compound library for the cellular activity of interest. The active compounds or 'hits' are then studied in the confirmation phase of the assay. The confirmation phase retests the hits at five concentrations in triplicate. This step confirms the hits and will establish if the activity is dose-dependent. The final phase of the assay is the counter screen which uses an alternative cell line. This step is used to identify non-specific activity. Table 2 shows the approximate number of plates needed for each step of the HTS assay.

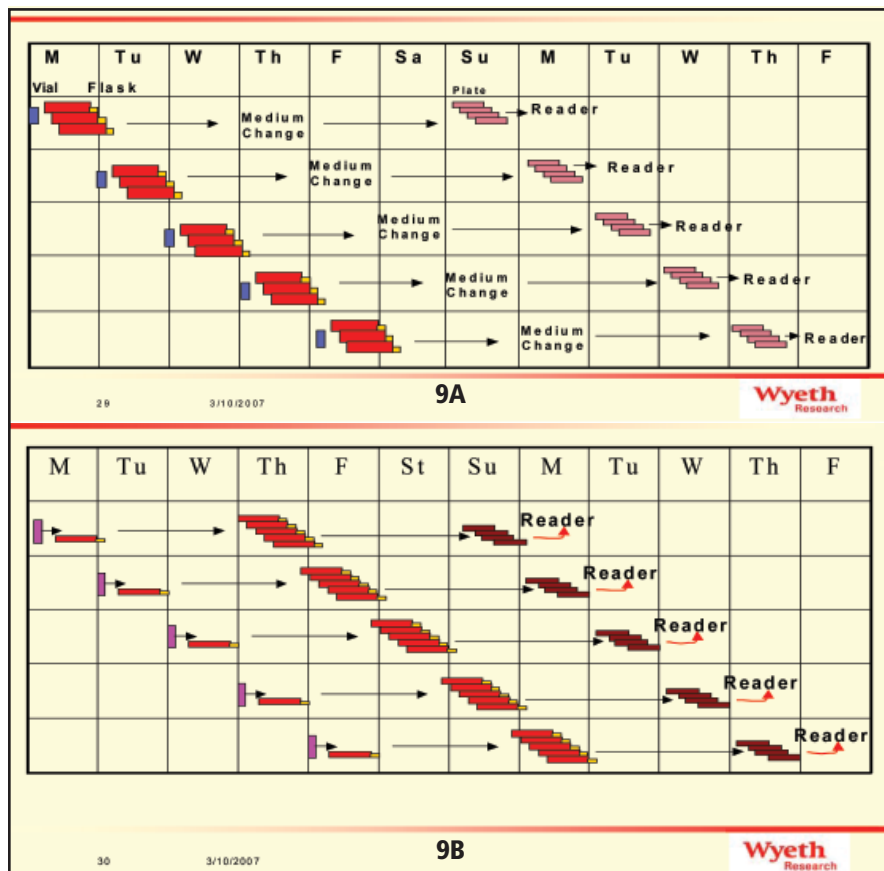


Figure 9. Progression of cells for assay plates using flasks: A) vial to flasks to plates; B) vial to flasks to second passage flasks to plates.

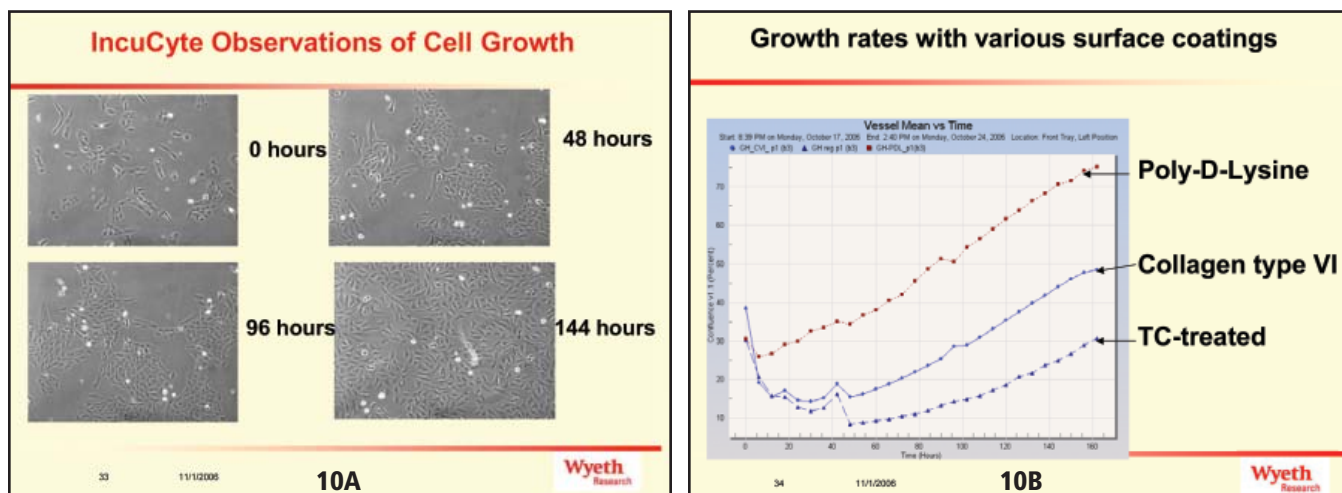


Figure 10. A and B: IncuCyte analysis of T-175 flask.

Conclusion

Two major activities are needed to provide microtiter plates for cell-based high-throughput screens: large-scale cell banks for the provision of a single cell batch for each campaign, and daily production of microtiter plates for that campaign. The cells required for a single campaign can be provided in a single batch with the use of the CellCube bioreactor, eliminating variability between batches of cells. The SelecT system could also be used to produce cells for a cell bank. This system has a capacity of 192 T-175 flasks or 33,600 cm² of growth area.

Three SelecT runs would be needed to provide for a single cell bank, but the use of the SelecT would greatly limit its availability for other needed functions such as daily plating of cells. Therefore, we choose to produce cell banks in the CellCube bioreactor. Our goal is to support 20 to 25 high-throughput screens yearly. The CellCube bioreactor run and turnover time is two weeks, and therefore allows for the production of 26 cell banks annually.

We have established four methods for use of the frozen vials when used for a screen. How the assay is set up, and which method of microplate preparation is used is determined by the desired cellular activity and reproducibility during assay development and the experimental phase of the screen. If the activity is equivalent regardless of the method of cell preparation used, then freshly thawed cells will be used to prepare microtiter plates as this is the least expensive and most efficient option.

The two SelecT systems can be used to provide for microplate production for our screens. Our future aspirations include using 1,536-well plates to reduce the cell and microplate needs; and the addition of selectivity, potency, and specificity assays to our screen.

Table 2. Approximate amount of plates needed to complete an HTS assay.

Campaign Phase	~ Number Of Plates	Assay Days	~ Number Of Wells
Experimental	1000		384,000
Primary	2000	6	768,000
Conformation	750	4	288,000
Counter	250	2	96,000
Total	4000	12	1,536,000

REFERENCES

1. Fox S, Farr-Jones S, Sopchak L, Boggs A, Wang-Nicely H, Khoury R, Biros, M. High-Throughput Screening: Update on Practices and Success. *J Biomol Screen*. 2006. 11(7):864-869.
2. Warnock JN, Al-Rubeai M. Bioreactor systems for the production of biopharmaceuticals from animal cells. *Biotechnology & Applied Biochemistry*. 2006 45(Pt 1):1-12.
3. Blasey H, Isch C, Bernard A, Alain R. CellCube: A New System for Large-Scale Growth of Adherent Cells. *Biotech Tech*. 1995. 9(10):725-728.
4. Wikstrom K, Blomberg P, Islam KB. Clinical Grade Vector Production: Analysis of Yield, Stability, and Storage of GMP-Produced Retroviral Vectors For Gene Therapy. *Biotech Prog*. 2004. 20(4):1198-1203.
5. Newton KH, Zhang S, Chiang YL, Otto E, Weaver L, Blaese RM, Anderson WF, McGarrity GJ. Improved Methods of Retroviral Vector Transduction and Production for Gene Therapy. *Human Gene Therapy* 1994. 5(1):19-28.
6. Ozuer A, Wechuck JB, Goins WF, Wolfe D, Glorioso JC, Ataai MM. Effect of Genetic Background and Culture Conditions on the Production of Herpesvirus-Based Gene Therapy. *Biotech & Bioeng*. 2002. 77(6):685-692.
7. Offin P. Automating Cell Culture for HTS and Lead Optimization. *American Lab* (Shelton). 2002. 34(8):41-43.
8. Soloveva V, LaRocque J, McKillip E. When Robots Are Good: Fully Automated Thermo LAS Robotic Assay System with Dual FLIPR TETRA and TAP SelecT Robotic Cell Culture System. *JALA* 2006; 11:145-156.

Validated Biosystems

www.validated.com

Expert Guidance in downstream processing since 1987

Worldwide consulting services

Hands-on purification process development

Advisory consultation

Practical experience-based courses

Free downstream processing library

Find out more at www.validated.com

Validated Biosystems, 240 Avenida Vista
Montana, Ste 7F, San Clemente, CA 92672
Tel: (949) 276-7477 Fax: (949) 606-1904
E-mail: info@validated.com

