

Development of a Sensitive, High Throughput, Cell Based Assay for Use in Validation of Powdered Cell Culture Medium Blenders

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Assays utilizing biological systems are inherently variable and unpredictable. Conversely, validation protocols require highly precise, repeatable assays. In an ever expanding market it is imperative for a cell culture medium manufacturer to stay abreast of technological advances. It is equally important to achieve and maintain cGMP performance standards. The performance qualification of a pair of new vertical ribbon blending mills, used to manufacture powdered cell culture medium, required that a biological assay be developed to test the final product.

We developed a rapid, high throughput assay utilizing the metabolic dye, Resazurin. Assay optimizing parameters included cell seeding densities, resazurin concentration, incubation time, and medium supplementation. A correlation between increasing metabolism (fluorescence) and increasing viable cell number was established for each of six different cell lines. In both 24-well and 96-well plate formats, the bioassay has acceptable variance and is sensitive enough to detect spiked contaminants. Three different powdered medium blends were assayed using two cell lines per blend, for both of the blenders. Chemical analysis of the same blends showed excellent correlation to the bioassays. Through careful assay development, this rapid cell-based assay was instrumental in the validation of high technology manufacturing equipment. Furthermore, due to its sensitivity, this technology assay has proven useful in the medium development process as a high throughput-screening assay.

Introduction

Traditionally, powdered cell culture medium is manufactured with a ball mill. See figure 1. Ball mills are inherently prone to variability in the homogeneity of the product due to the uncontrollability of the sieve and the manual manipulation required to run the machinery. Additionally, there are concerns of cross contamination of products and concerns regarding chips from the milling stones themselves.



Figure 1: Traditional ball mill requires manual manipulation of product.

We have moved forward with technology that effectively replaces the ball mill in the manufacture of cell culture medium. This new technology incorporates an Air Classifier Mill (ACM) with a vertical ribbon blender to produce superior quality powders, with homogeneity that is unachievable with ball mills (figure 2). Furthermore, cross contamination is much less of a concern and manual interaction with product is nonexistent.

The ACM is based on hammer mill type technology with impactors on a high-speed rotor. An adjustable speed radial separator allows particle size to be tightly controlled. Particles that are too large are directed back into the milling area. The powder then leaves the ACM and goes into one of two interchangeable ribbon blenders. The powder is brought to either the 400-liter blender or the 5000-liter blender via a closed pneumatic system, eliminating the need for manual transfer. Blending is accomplished in a three-dimensional format by a helical rotor of pharmaceutical grade design and contact surfaces. This blending technology offers the flexibility of using 10-100% of fill capacity while allowing 99.99% product discharge without segregation of product.

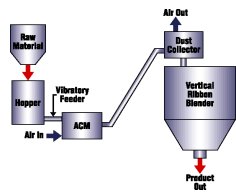


Figure 2: Schematic diagram of the process system for milling and blending.

Cell culture media are classified by the FDA as a class I medical device. The validation of manufacturing processes for this class of product is required by 21 Code of Federal Regulations (CFR) Part 820. To show reproducibility as part of the performance qualification of each new blender, we needed to demonstrate successful functionality of at least three different medium blends. Furthermore, since our customers grow a vast array of different cell lines in them, we decided to grow at least two different cell lines in each of these blends. For this purpose, we chose what we thought were the three most commonly used representative medium types: DME/F12 (Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham), RPMI 1640, and MCDB105. We also chose cell lines based on their availability (ATCC), common industrial usage, and known characterization. For DME/F12 medium we chose CHO-K1 and BHK cells, for RPMI 1640 we chose SP2/0 and HFN7.1 cells, and for the MCDB105 we chose MRC5 and HS68 cells.

Optimizing the growth conditions for the assay balanced two principles. First, we wanted each cell line in each medium to grow successfully with a minimum of supplementation. At the same time, we wanted the conditions to be marginally stressful so that the assay would detect small differences in medium composition. Optimized parameters included cell seeding densities, supplementation (e.g., fetal bovine serum), and the concentration and incubation time of the resazurin solution. All assays were done over a period spanning at least ten days so that the entire growth curve could be observed.

For each cell line, the optimized resazurin assay was verified to be accurate in its correlation of increasing fluorescence with increasing cell numbers. Samples included various time intervals in the blender, as well as from various positions within the blender or within the final powder medium drums. Controls included the traditional blending technology and competitors' corresponding powdered medium.

Materials and Methods

Cells

Stock CHO-K1, BHK, HS68 and MRC5 cells were grown in Basal Medium Eagle (Sigma product number B1522) supplemented with 10% FBS (Sigma product number F 2442). Stock SP2/0 and HFN7.1 were grown in HY (Sigma product number H9014) with 10% FBS. Stock cells were not allowed to proceed over 22 passages from original frozen vials. All stock cultures were grown in 162 cm² flasks with 50 mls medium and were passed twice per week. Attached cell lines were rinsed with Hanks Balanced Saline Solution (HBSS) prior to a 15-minute incubation at 4° C with Trypsin/EDTA (Sigma product number T3924). Trypsin was inactivated by the addition of assay control medium containing 1-2% FBS. After centrifugation the cells were then resuspended in assay control medium.

Cell counts were done on a Particle Data Systems, 280PC. Cell concentrations were adjusted in such a way that a constant volume of 50 ul of cells was required for inoculation of individual wells. Such inoculations were done with an Eppendorf repeating pipettor. Cell inoculum volumes were varied only during studies of seeding densities prior to establishing the final optimized assay. Viability evaluations were done by traditional Trypan Blue (Sigma product number T8154) exclusion methods on a hemacytometer. The minimal cell viability for an assay to proceed was 90%. All cell stocks and assay plates were kept in Thermo Forma model 3110 incubators at 5% CO₂, 95% humidity and 37° C.

Resazurin Dye

Resazurin (Sigma product number R2127) was dissolved in Hanks Buffered Saline Solution (Sigma product number H6648) at 0.1406 grams per liter and sterile filtered. A single preparation of resazurin was used throughout each powder blend assay.

Assay Set Up

All validation assays were done in Corning 24-well plates with 2 mls of medium per well. Each assay plate included blank wells for subtracting background fluorescence values. We used three replicate wells per test sample. A typical assay included 5 competitor media samples, 30 test samples per medium blend, 20 control samples from a ball-mill blend, and various plate controls and blanks. This meant for each assay, we set up ten total plates for each of the ten days of analysis, filling a total of 2400 wells with medium and inoculating 2100 wells with cells.

Much care was taken to minimize any possible bias in assay results that could be linked to uncontrollable parameters. Plates were pre-warmed in the incubator in stacks of ten prior to inoculation. The exact sequence of plate inoculation was systematically randomized to minimize the impact of possible variations within the cell preparation during the time required to inoculate 100 plates. Assigned plate and well locations for all samples were also systematically randomized to minimize possible bias due to inoculation timing and location. After

inoculation, all plates were rearranged within the incubator to match the randomized sequences assigned for each daily reading.

Sample Medium Preparation

Medium samples were dissolved per medium specifications with appropriate sodium bicarbonate supplementation and then sterile filtered. All samples were then supplemented with FBS (and fibroblast growth factor for MCDB 105 medium) as per the assay conditions. Control and competitor medium samples were prepared identically.

Fluorimetric Measurement

All assays were performed on a Packard FluoroCount AF10000 plate reader. The following settings were confirmed: Excitation Filter -560nm, Emission Filter-590nm, PMT-800, Read Length-0.5 seconds and shake time-5 seconds. The addition of resazurin was done with non-sterile technique. However, timing is critical in this step. Resazurin (Sigma Product number TOX-8) was warmed to room temperature prior to using the plate reader. Resazurin additions, were done by removing only one plate from the incubator at a time. 100 ul of resazurin solution was immediately added to the first well (Location A1). Subsequent wells matched the sequence and timing of the fluorometer reading protocol. Upon completion of resazurin additions, a timer was started and the plate was immediately returned to the incubator without its lid. Successive plates were started at fixed intervals (no less than 2 minutes between plates). Each plate was removed from incubator after exactly 30 minutes and read immediately on the plate reader.

Elemental Contaminant Preparation

A 500ng/ml Cupric Chloride (Sigma product number R2127) solution was prepared.

Data Analysis

For each plate, the average blank value was subtracted from the raw data of the other wells to obtain the net Relative Fluorescence Units (Net RFU). For each three well set, the mean, standard deviation and relative percent standard deviation (RSD) was calculated. Whenever an RSD value was greater than 20%, the original data were examined for outliers, defined as individual RFU values greater than 50% different from the daily trending means. Removing an outlier point was very rare (generally less than 0.5% of the samples).

Results and Discussion

Resazurin is a metabolic indicator dye. In its normal oxidized state it shows very little fluorescence. Once the dye penetrates the cell it becomes reduced to the highly fluorescent resorufin by intracellular oxidoreductases and the mitochondrial a-transport chain.

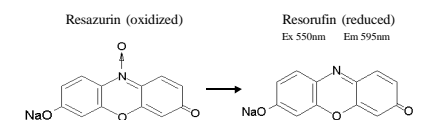


Figure 3: Chemical structures of resazurin and resorufin.

With all other variables held constant, the rate of resazurin reduction should directly correlate to the viable cell density for any given cell line. Thus, it was our goal to find the right set of conditions and constraints by which RFU may substitute as a measure of viable cell number per well. This would then enable us to assay hundreds of wells per day so that we could use solid statistical methods in validating the new blenders.

Figure 4 shows the correlation as established for HFN7.1 cells growing in RPMI 1640, supplemented with 10% FBS. Data was taken from one through 10 days growth, with starting inoculums from same cell stock, at densities ranging from 400 cells per well through 10,000 cells per well. RFU values reflect a 100 ul addition of resazurin with a 30-minute incubation time. Similar correlations were established for all cell lines used. (Data not shown.)

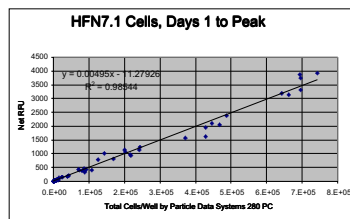


Figure 4: Correlation of HFN 7.1 viable cell density to Net RFU values under final assay conditions. Linear regression equation and correlation coefficient (R²) are indicated.

Having established correlations of cell number and RFUs, the next step was to optimize resazurin concentrations and incubation times. It was obvious from the correlation data that different cell types metabolize resazurin at different rates. Thus it became apparent that we would have to optimize each cell line separately. Finally, we needed to stay below 70,000 total RFUs over the time course of the any hypothetical assay since that is the limit of the fluorimeter.

With all these things in mind, we decided to try 50 ul, 100 ul and 150 ul additions of the resazurin solution and also try incubation times of 20, 30 and 40 minutes. It was decided that longer incubation times would be inconvenient and could likely be shortened by the addition of more resazurin.

Similar studies were done for all cell lines (data not shown) at various cell inoculum densities. The final data suggested that we could allow all cell lines to incubate at 30 minutes with the addition of 100 ul resazurin, thus keeping the measurement protocol for all cell lines the same. Furthermore, these studies suggested some optimal cell seeding densities that could be used to keep the differences between medium types to a minimum.

The third and final step in development of the assay was to evaluate the individual cell lines in positive control medium blends supplemented with various amounts of FBS. This coupled with varying cell inoculum densities would dictate how sensitive the assay would finally be with regard to revealing any differences in the new vs. the old style blenders. The objective was to keep the supplementation as minimal as possible so that the medium performance would not be masked by other factors. Positive control medium was again used, supplemented with 1, 2, and 4% FBS. Cell inocula of 400, 2000, and 10,000 cells per well, were also tested. This was also an ideal time to look at the length of time to achieve highest cell density and overall health of the cells within each assay. This was done to be sure that the assay really was marginally stressful. Therefore, manual cell counts and viabilities were performed again to generate growth curves that could be used in conjunction with the RFU data to evaluate the effectiveness of this assay. This would also give us an indication of how many days we would need to collect data to capture the whole growth curve of each cell line within the assays. Below is an example of the assays we ran for one cell line, BHK.

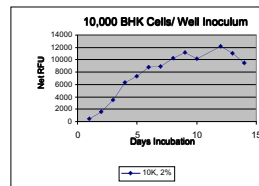
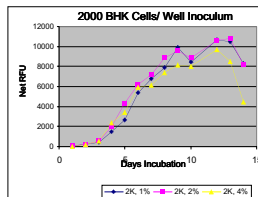
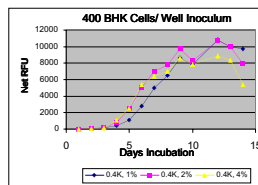


Figure 5: BHK cell growth profile under various seeding cell densities and FBS concentrations.

These experiments revealed that we could use as little as 1% FBS in the DME F12 medium while inoculating 2,000 cells per well for both the CHO and BHK cell lines. Similar data showed that 2% FBS with 10,000 cells/ml seeding density was best for the RPMI 1640 medium with SP2/0 and HFN7.1 cell lines. These numbers were chosen to show both good growth characteristics and to be able to show marginal stress on the cultures in question. They are neither optimal for growth, nor will the cultures die prematurely, given that the base medium is sufficient.

Viability data, (not shown) suggested that all cell lines were beginning to die by day ten. Therefore, the assay would only need to be measured for days 1 through 10. Furthermore, it was becoming obvious that the cell lines grown in the MCDB 105 medium were going to require more supplementation. These cultures were simply not performing to a level that we felt was indicative of a good medium that our positive control medium needed to reflect. MCDB 105 medium is formulated to grow fibroblasts with the addition of Fibroblast Growth Factor. In the stock cells grown in HY medium with 10% FBS present, there was not a need for such factors. However, our assay was sufficiently marginal in FBS supplementation as to warrant the addition of the FGF to get significant, measurable growth at a measurable level. Better growth would allow us to set better criteria for pass/ fail of the final assay.

So we set up another assay with only the HS68 (see figure 6) and MRC5 (data not shown) cell lines in the MCDB 105 positive control medium supplemented with 2% FBS and various amounts of the FGF supplement. The following data shows HS68 cells grown in MCDB105 positive control medium supplemented with 2% FBS and increasing levels of FGF. The cells were planted at 10000 cells per well on day 0.

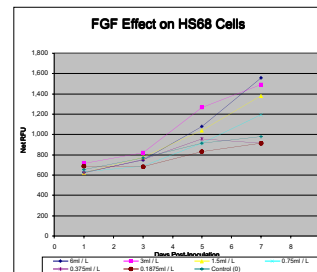


Figure 6: HS68 cell growth profiles (RFU values) at different FGF concentrations.

We decided to use the FGF preparation at 1.5 ml/L, corresponding to approximately 0.2 ng/ml pure FGF. The growth at this level was good enough to allow us to assign solid pass/fail criteria to it, but was not such that we would not be able to see base medium deficiencies due to masking effects by the FGF stimulation. Furthermore, since FGF is simply a stimulant to growth and not a real nutrient, we felt that the addition would serve to further our goal of making the assay stressful upon the cells. Since the cells will be stimulated to grow, any nutrient deficiencies would be magnified by the cultures failure to thrive.

As a final check, copper was added as a contaminant to be sure that small variances such as a trace element could be detected in the base medium. For this purpose an assay was set up with and without the addition of 2 ng/ml cupric chloride. Copper is known to be toxic at higher levels and so was chosen as a good candidate since it should show a slight decrease in RFU to indicate lower cell growth.

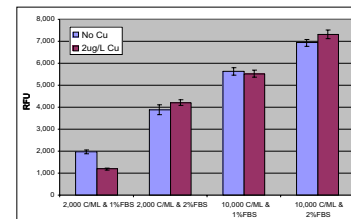


Figure 7: Effect of introducing a low level of toxic trace metal (2 ug/L cupric chloride) on BHK cell growth. Results are at day 7 and show standard error bars (n=10).

As can be seen with higher FBS concentration and/or higher inoculum densities, the toxicity of the low concentration of copper is not evident. However, at the 2,000 cell/ml seeding density and 1% FBS, the stress on the cultures allows detection of toxicity due to the contaminant.

The final assay conditions and passing specifications for the various media and cell lines are shown in table 1. All assays were read for ten days.

Table 1. Final assay conditions for the different media and cell lines.

Medium:	DME/F12	RPMI 1640	MCDB 105
Cell Line:	CHO-K1 BHK	SP2/0 HFN 7.1	HS68 MCR5
% FBS	1%	1% 2%	2% 2%
Seeding density (c/well):	2,000 2,000 10,000	10,000 10,000	10,000 10,000
FGF concentration:	N/A	N/A	2 ng/ml 2 ng/ml
Passing criteria			
Max RFU/day1 RFU:	20 X	20 X	20 X 10 X
or Max RFU/day2 RFU:	10 X	10 X	10 X 5 X

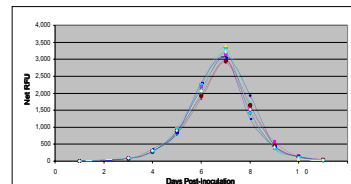


Figure 8: SP2/0 cell growth profiles for 10 individual drum samples of RPMI 1640 during a validation run of one blender. Each point is the average of three wells.

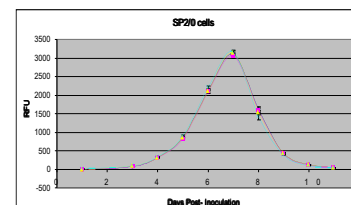


Figure 9: SP2/0 cell growth profiles for four sampling groups of RPMI 1640 during a validation run of one blender. Each point shows the mean value of ten individual samples within the specified group, with standard error bars.

Similar data was generated for all time points with all medium blends and all required cell lines. This data was then tabulated, averaged, and graphed collectively with competitors medium samples.

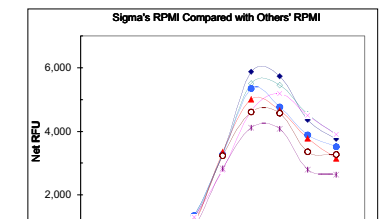


Figure 10: HFN 7.1 cell growth profiles with RPMI 1640 medium, comparing the drum sample groups of the new blender and ball mill blender with five RPMI 1640 samples from non-Sigma sources.

Conclusion

The developed resazurin cell culture assay shows excellent precision, while maintaining sensitivity to slight variations in base medium components. The assay may be used to test a large number of samples under tightly controlled conditions. Therefore, the resazurin assay is a fast and efficient method of screening for toxic or growth-promoting substances. Although this validation work was performed using 24-well plates, we also found it suitable in 96-well formats (Peppers, et al., 2001). Presumably, the assay may also be adaptable to 384-well or smaller formats, making it a valuable high throughput assay in the other fields of proteomics.

The methods for blender validation were carefully developed. After considering all data, including resazurin assay results from 28,800 individual wells and other analytical chemical data (see poster by K. Ray), both 400-liter and 5000-liter blenders were declared suitable for cGMP manufacturing of cell culture medium. Medium from these blenders has been found to perform in the resazurin assay at levels equal to or better than the ball milling blending methods and the tested competitor media.

Acknowledgments

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