

# Homogeneity Analysis and Cell Culture Performance of Powder Cell Culture Media Manufactured with a Novel Continuous Flow Milling Process

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## Abstract

A novel, high-throughput continuous milling process for the manufacture of dry powder cell culture media has been designed, consisting of a pin mill combined with three-dimensional conical mixer technology. The purpose of this study was twofold: first, to demonstrate the homogeneity of key components in powder cell culture media manufactured using the continuous mill; and second, to verify that media manufactured in the continuous mill performs consistently and in a manner equivalent to ball-milled media when used in cell culture. A 2400 kg lot of basal media and an 800 kg lot of serum-free media were formulated, milled, blended and packaged using the continuous milling process. Powder media samples were obtained from various locations and depths within the post-mill blender and final packaging containers and analyzed for amino acids, vitamins and trace metals. The analyses confirm that the milling and blending parameters established for milling process produce homogeneous media formulations. Cell culture studies were performed and indicate that media produced using the continuous milling process performed consistently throughout the batch and in a comparable manner to ball-milled media.

## Introduction

As the demand for powder cell culture media continues to rise, we have designed and developed a new high-throughput milling process to meet the requirements of the pharmaceutical and biotechnology industries. This new system can mill from 800 to 4000 kg of material in a single batch operation and is limited only by the capacity of the blender and a minimum quantity for efficient blending. Milling is a particle size reduction technology that processes materials using machinery which impacts, compresses or shears the particles. Mechanical impact grinding mills such as ball mills or pin mills utilize the impact principle of grinding by either impacting a particle with an outside force or accelerating the particle against a particle. Mechanical-impact mills are ideal for easy-to-grind ("soft") material requiring medium fineness (diameter of the particles < 100 µm).

The pin mill is equipped with a stationary set of pins (stator) and a rotating set of pins. Material enters the mill's grinding chamber and is accelerated by centrifugal force against the rotating pins. The rotational speed and the rate of feed into the mill determine the size of the particles and the amount of heat generated. It is particularly important to control the amount of heat generated due to the number of heat sensitive components in cell culture media. Heat generation is controlled by adjusting the speed of the discs, the rate of feed into the mill and by utilizing chilled nitrogen to feed material into the mill. After milling, the chilled nitrogen carrier gas conveys the material from the pin mill housing to the post-mill blender for final mixing. The amount of time necessary for post-blending has been carefully determined to avoid "deblending" of products.

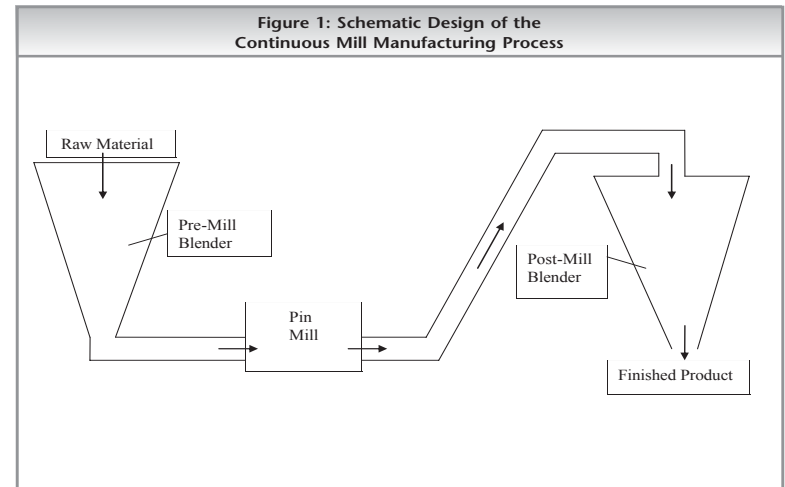
The validation of milling and blending operations are key aspects in the manufacture of consistent powder cell culture media products. Cell culture media are classified by the Food and Drug Administration (FDA) as a Class 1 Medical Device and therefore are governed by 21 Code of Federal Regulations (CFR) part 820. The code states that the process validation program for a powder cell culture medium should be defined and implies that it should include analysis of components that demonstrate the effectiveness of the blending operation.

This study will present the analysis of both basal and serum-free cell culture media to demonstrate that the established processing parameters ensure product homogeneity in both the post-milled bulk material and product in final containers. It will also demonstrate that pin-milled media is comparable to ball-milled media in terms of component concentrations and performance in culture.

## Materials and Methods

### Continuous Mill Process

The process of manufacturing the dry powder media consists of a pre-mill blending step, a milling step and a post-mill blending step, all of which are in a closed, continuous system. A basic diagram of the process is shown in Figure 1.



The individual components were formulated into Flexible Intermediate Bulk Containers (FIBC). The FIBC containing the formulated material was then mechanically lifted up to the top of the pre-mill blender via an FIBC hoist, the material charged into the pre-mill blender and the lid closed. The material was blended for a specified time in the pre-mill blender and conveyed through stainless steel piping to the mill via a volumetric feeder and a nitrogen gas flow. The material enters the center of the pins of the pin mill and passes through concentric circles of pins to be reduced in size and is finally conveyed through stainless steel piping via nitrogen flow to the post-mill blender. Once all the material is milled and conveyed to the post-mill blender, it is blended for a specified amount of time blanketed by nitrogen gas.

### Sampling from the Post-mill Blender

After the specified post-blend time, the nitrogen gas was purged and the top of the blender opened so the material could be sampled. Samples were taken with a sample thief made of two PVC tubes. Samples were taken from five locations at two different depths in the post-mill blender yielding a total of 10 samples.

### Sampling from the Finished Product Containers

After the sampling of the post-mill blender was complete, the entire batch of material was discharged in 11 kg increments into finished product containers using the automated filler. The finished product containers consist of a primary plastic bag, a secondary plastic bag and a tertiary plastic bucket. The batch size determines the number of finished product containers. The number of finished product containers sampled was determined by the following formula:

$$n = \sqrt{N+1}$$

n = the number of product containers sampled  
N = the total number of product containers

Finished product containers from the beginning, middle and end of the fill were sampled. From the first three and last three finished product containers three samples, each at a different height (top, middle and bottom), were taken using a sample thief. From the remaining finished product containers to be sampled, one sample was taken.

### Kinetics Study

Before batches of cell culture media were manufactured and sampled, an initial trial run was conducted to determine an appropriate post-mill blend time and to establish homogeneity kinetics. The trial batch consisted of ~800 kg of sodium chloride (NaCl) spiked with phenol red at 20 parts per million (ppm). The batch was pre-blended for a specified amount of time, milled and then transferred to the post-mill blender. Eight samples were removed from the post-mill blender at 0-, 5-, 10-, 20-, 30-, 45- and 60-minute intervals of blending. Samples were hydrated and analyzed for phenol red using a colorimetric assay.

### Sample Preparation and Analysis

From all of the samples taken from the post-mill blender and finished product containers, a sub-sample of ~1.0 to 2.0 g was weighed, hydrated and sterile filtered. The hydrated samples were then sent to Good Laboratory Practices (GLP) analytical labs where they were quantitatively analyzed for amino acids and water-soluble vitamins by High Performance Liquid Chromatography (HPLC) using a cation exchange column and ultraviolet (UV) or fluorescence detection. Trace metals were analyzed using ion-coupled plasma mass spectrometry (ICP-MS). For comparison, and to serve as controls, samples from basal and serum-free media lots (identical formulations to the pin-milled batches) that had been produced using the current ball-milling process were also hydrated and analyzed in a similar manner.

## Data Analysis and Acceptance Criteria

Dry powder cell culture media is a complex mixture of many components, at varying concentrations. To demonstrate that this manufacturing process will uniformly blend all the components in order to achieve a homogeneous product, percent relative standard deviation (% RSD) was chosen as our metric.

The % RSD is the standard deviation divided by the mean and expressed as a percentage. It is used to indicate the variation between samples in a given population. The smaller the % RSD, the less variation, and in this case the more homogeneous the product. There will be an inherent amount of variability present due to sample preparation and the analytical method itself which will be larger for components which are present at a lower concentration. Based on preliminary work and the concentration of the components being analyzed, a 10% RSD for the amino acids and vitamins, and 15% RSD for the trace metal, Zn were chosen as the acceptance criteria.

## Cell Culture Studies

Dry medium samples from the 800 kg lot of serum-free media were obtained during discharge from the mill, at the beginning, middle and end of the blending period. Liquid media were prepared from a ball-milled control and the pin-milled media samples. Growth and viability were evaluated in each sample using a DXB11-derived cell line in shaker flasks over multiple passages and in a culture longevity study. Aliquots of conditioned medium were obtained daily during the longevity study, and assessed for immunoglobulin (IgG) production using a human IgG ELISA kit (ZeptoMetrix Corporation, Buffalo, New York USA).

## Results and Discussion

### Kinetics Results

Results from the post-blend homogeneity study indicate that the amount of variation in the concentration of phenol red is reduced as the material is blended (see Figure 2). At post-blend times of up to 60 minutes there is no evidence of "deblending" of phenol red.

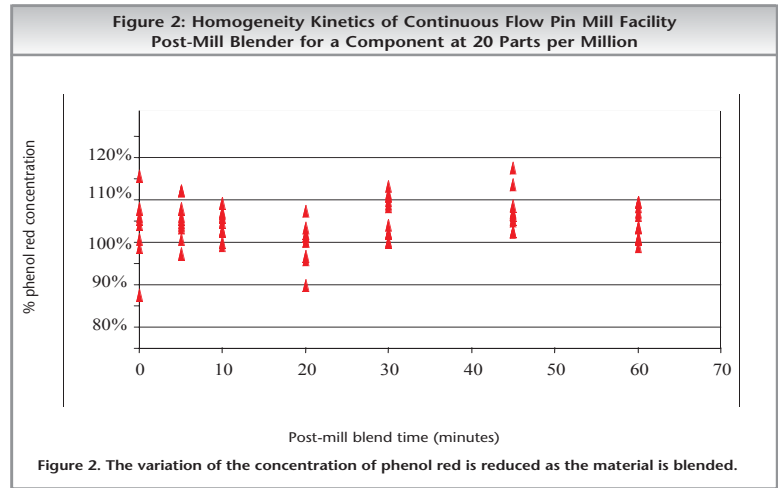


Figure 2. The variation of the concentration of phenol red is reduced as the material is blended.

### Analytical Results

Table 1 reflects the average % RSD of samples analyzed in the 800 kg and 2400 kg media lots. For the 800 kg batch 10 post-mill samples and 22 finished product samples (from 10 finished product containers) were taken, hydrated, analyzed and used to calculate the % RSD values. For the 2400 kg batch 10 post-mill samples and 29 finished product samples (from 17 finished product containers) were taken, hydrated, analyzed and used to calculate the % RSD values. The % RSD values for all of the amino acids and vitamins for both batches are less than 10% and more than half are less than 5%, demonstrating acceptable homogeneity based on the acceptance criteria of < 10% RSD. The % RSD values for Zn are acceptable as well, being less than 15% RSD.

Table 1 reflects the % RSD of components analyzed in the 800 and 2400 kg batch run.

AMINO ACIDS	% RSD for 800 kg batch	% RSD for 2400 kg batch
Aspartic acid	3.27	3.24
Threonine	2.83	2.04
Serine	3.54	2.46
Asparagine	3.20	3.58
Glutamic acid	3.33	3.20
Proline	2.89	4.01
Glycine	2.42	2.48
Alanine	3.70	4.11
Valine	3.03	2.25
Cystine	3.18	5.40
Methionine	5.57	8.90
Isoleucine	6.49	3.18
Leucine	5.43	2.08
Tyrosine	5.01	2.31
Phenylalanine	5.80	2.23
Tryptophan	1.37	2.55
Lysine	5.18	3.10
Histidine	4.31	8.22
Arginine	7.45	3.12
<b>VITAMINS</b>		
Riboflavin	1.27	3.07
Thiamine	1.67	2.41
Nicotinamide	1.66	2.17
Folic acid	0.66	2.08
Cyanocobalamin	3.02	3.66
<b>METALS</b>		
Zinc (~ 20 ppm)	10.64	7.04

A comparison of the amino acid concentrations in ball-milled material compared to material produced in the continuous flow pin mill facility can be found in Table 2. This data is represented as the average percent difference between the ball-milled and pin-milled samples. The difference in concentration of amino acids between ball-milled material and pin-milled material was found to be less than 10% for both batch sizes of pin-milled product.

AMINO ACIDS	% difference for 800 kg batch	% difference for 2400 kg batch
Aspartic acid	4.53	-1.62
Threonine	-0.18	-2.89
Serine	-4.03	-2.44
Asparagine	-3.33	-1.32
Glutamic acid	-7.37	3.04
Proline	0.15	-5.51
Glycine	-3.79	-3.12
Alanine	-3.12	0.73
Valine	0.14	0.63
Cystine	-5.95	-0.31
Methionine	-9.40	-6.73
Isoleucine	-6.82	0.31
Leucine	-7.71	-0.40
Tyrosine	-9.42	-3.52
Phenylalanine	-4.75	-2.25
Tryptophan	-6.10	8.62
Lysine	-5.84	-1.71
Histidine	-4.75	6.67
Arginine	-4.99	-2.86

### Cell Culture Results

The results from the cell culture studies indicate that there were no apparent differences in cell densities, doubling times, culture viabilities, and IgG production in cells grown in the ball-milled control and the pin-mill test media (see Figures 3, 4 and 5). In addition, there is no significant difference in the cell culture performance between the beginning, middle and end samples, further supporting that the media components were homogeneously distributed in the finished product containers during the packaging process. This indicates that media produced in the pin mill performs comparably to ball-milled media.

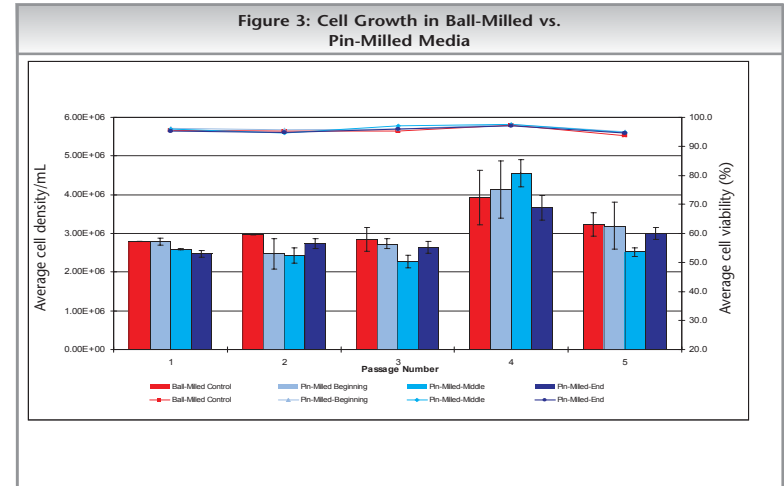


Figure 3: Cell Growth in Ball-Milled vs. Pin-Milled Media

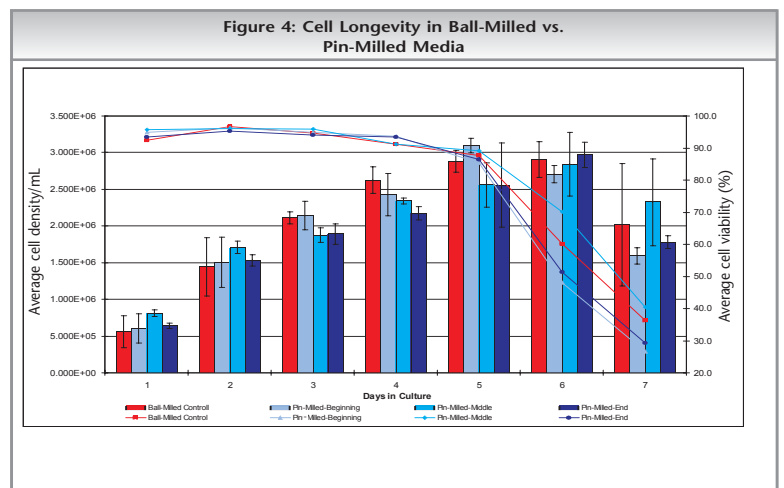


Figure 4: Cell Longevity in Ball-Milled vs. Pin-Milled Media

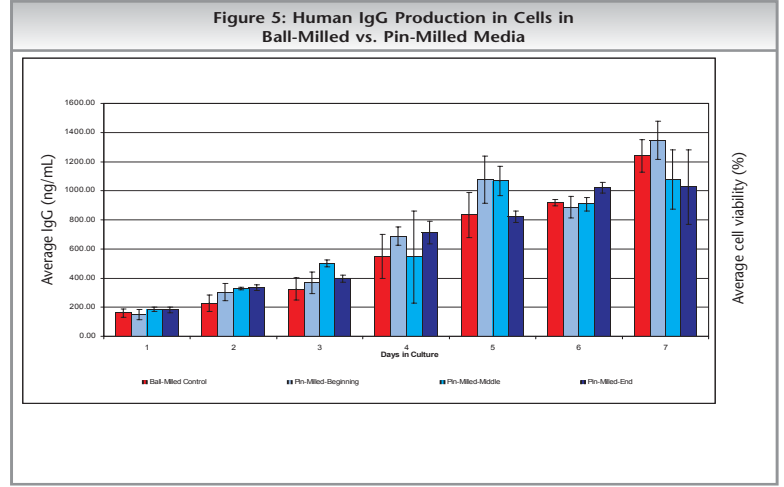


Figure 5: Human IgG Production in Cells in Ball-Milled vs. Pin-Milled Media

## Conclusion

Process parameters, including pre- and post-mill blend times, feeder rates and mill speeds, have been determined for a high-throughput continuous flow process for the manufacture of dry powder cell culture media. The continuous flow process produces a homogeneous distribution of media components in both the post-mill blender and finished product containers. This was verified by analyzing 20 different media components, varying in concentrations, in two different media formulations and batch sizes. Pin-milled powder media was also shown to perform comparably to ball-milled media in cell culture studies. This combined evidence supports the validation of the continuous flow pin mill and blending processes for the production of powder cell culture media.



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