# WHITE PAPER
# TOXICITY TESTING AND THE CFC ASSAY

## Utilizing Colony-Forming Cell Assays In Toxicity Testing

It has been estimated that there are 1 000 000 or more proteins encoded by 20 000 – 25 000 genes in the human genome. Most drugs act by inhibiting a protein in the system and thus the number of potential compounds for therapy is virtually infinite. According to a report by the Tufts Center for the Study of Drug Development, the cost of developing a new drug and bringing it to the market is $802 million over 10 - 15 years. The steps involved in bringing a drug to market include discovery, development, testing and launch. Because it takes more than a decade to bring a drug to the market it is pivotal that the success rate of clinical trials be the priority. Over the past few years there has been an increase in the number of productive discovery programs and better pre-clinical screening methods, which can boost the success rate from 1 in 5 to 1 in 3. The improvement in success rate not only increases the success rates of clinical testing but also significantly reduces total capital cost by $221 – 242 million (~30%) per drug. It is because of these costly factors that the pharmaceutical industry is continually searching for ways to more efficiently eliminate or identify candidate molecules earlier in the development process.

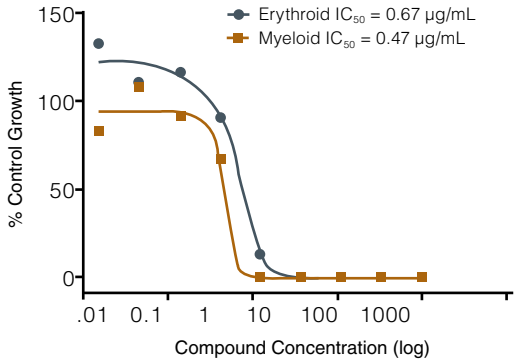
Drug screening typically involves high-throughput assays using myriad distinct cell lines and simple end points such as cell death or proliferation as measured by quantification of chemiluminescence or fluorescence. However, those cell lines usually represent a mature lineage-committed cell type prone to genetic instability that may produce phenotypes not representative of the in vivo situation. One possible way to improve the efficiency of the drug pipeline is to introduce “high-content” assays earlier in the development process as a means of overcoming those limitations. In contrast to cell lines, primary human cells have much more fastidious growth and proliferation requirements, thereby better representing in vivo conditions and increasing the sensitivity of a given assay.

Over the past three decades, a spectrum of standardized in vitro assays has been developed to characterize and measure bone marrow function and assess the multiple cell lineages it contains. In humans, bone marrow is one of the five major organ systems most sensitive to chronic low-dose chemical toxicants, making it an important target to assess in toxicity screening[1].

Colony-forming cell (CFC) assays may be initiated from a number of primary cell sources including mononuclear bone marrow, cord blood as well as from cell populations enriched for $CD34^+$ cells. These assays allow for the detection of an increase or decrease in the frequency of specific hematopoietic progenitor proliferation in response to stimulatory or inhibitory molecules. CFC assays have tremendous flexibility in that they can be used to determine whether a test molecule has sufficient stimulatory activity to be potential therapeutic candidate. By altering cytokine combinations and concentrations, researchers can characterize the effect a test compound has on colony formation, thereby identifying molecules that induce proliferation or alter the lineage commitment of progenitor cells. Such stimulatory molecules could be advantageous in a post-transplantation situation for “kick-starting” cells into division and/or differentiation. That can be significant when cell numbers are critically low and the chance of a life-threatening infection is very high.

Of significant importance, the CFC assay has the ability to predict a direct toxic effect to progenitors since inhibitory concentration ($IC_{50}$) values are calculated on the reduction of colony number whereas $IC_{50}$ value in a proliferation assay is calculated on the reduction of cell number from an expanding cell population.

**FIGURE 1.** Determination of $IC_{50}$ values for 5-Fluorouracil




Dose response curves and $IC_{50}$ values for both human BM derived erythroid and myeloid progenitors incubated with 5-Fluorouracil.

**FIGURE 2.** Colony size changes in the presence of an inhibitory compound

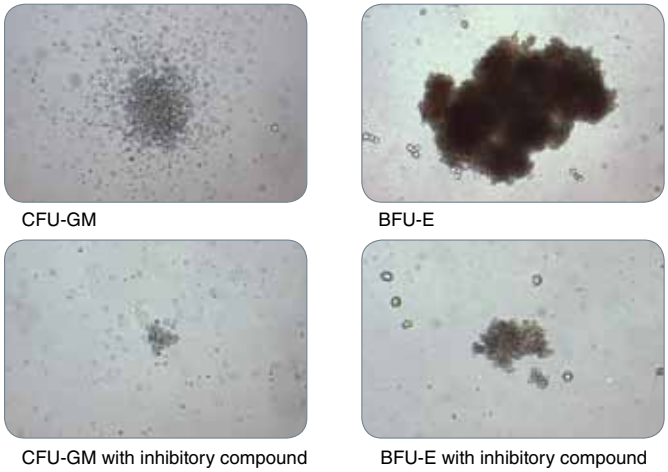






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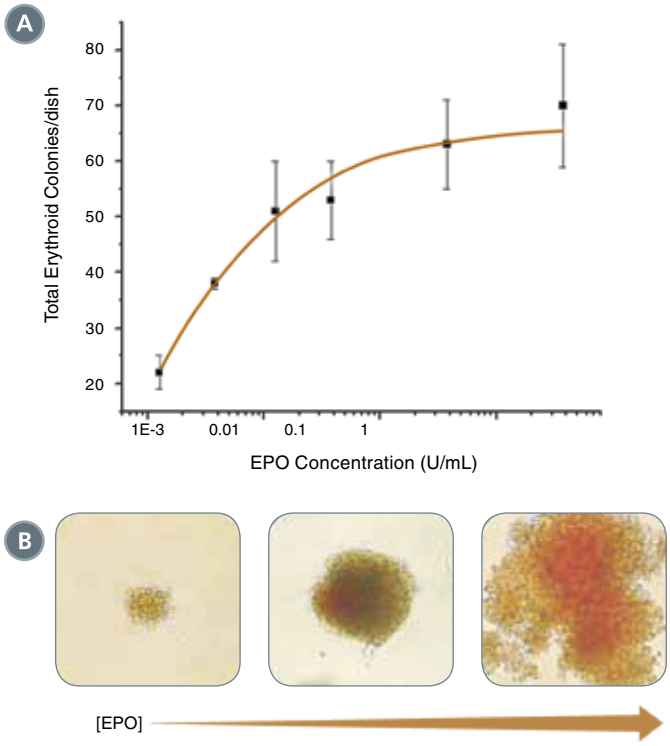
**FIGURE 3.** Stimulation by erythropoietin (EPO)




Stimulation of erythroid progenitor proliferation by erythropoietin (EPO) is both quantifiable (Figure A) and qualitative (Figure B, colony size increases with increasing [EPO]). In vivo models can be used to confirm stimulatory activity of molecules.

## CFC Assay Use In Toxicology

The organs or tissues most susceptible to systemic toxic effects include the blood circulation system, the nervous system, liver, kidneys, lung and skin. Assays using primary bone marrow cells can help evaluate effects to the blood circulation system since it contains the precursors of all cell populations. Although there are many assays that help quantify progenitor, primitive precursor and stem cell populations, a great deal of research has focused on the use of the CFU-GM assay, a measure of the progenitors of the granulocytic/monocytic lineage. The CFU-GM assay has been optimized and validated for use as an in vitro predictor of acute-onset neutropenia by potential hematotoxicants.[2-6] Similar work has been performed to standardize the colony-forming unit–megakaryocyte (CFU-Mk) assay for predicting toxic effects of drugs on progenitors of the megakaryocytic lineage.[13,16] Also of hematologic importance is the erythroid lineage, measured by the burst-forming-unit–erythroid (BFU-E) and colony-forming-unit–erythroid (CFU-E) assays, which detect early and late erythroid progenitor growth respectively. The erythroid lineage has been specifically relevant in studying the effects of environmental toxicants (such as pesticides) and antivirals to the hematopoietic compartment.[1,3,9,10,15]

Use of hematopoietic progenitor cells in CFC assays has been well documented to test the effects of chemotherapeutic agents,[4-8] toxins and environmental compounds,[1,5,9,10] cytokines,[7,11] and other drugs.[5,12-15] Such assays have been used to screen compounds for toxicity before initiating costly clinical trials and also to determine maximum tolerated doses (MTDs). Experiments comparing the drug sensitivity of cord blood– and bone marrow–derived progenitors indicate no significant difference in inhibitory concentration (IC) determination between the two cell sources.[4,6] Researchers may choose their cell source based on availability or experimental goals, cord blood being better suited to growing more immature progenitors and bone marrow for generating more mature ones.

## Differential Effects Of Various Chemotherapeutic Compounds On Human- And Mouse-Derived CFCs

Choice of species is an important consideration when testing a new drug. Mouse CFC assays may be an important tool for determining toxic doses before an in vivo study, but human in vitro assays can also indicate whether a particular animal model is a relevant and accurate predictor of human toxicity. Work by Pessina et al. indicates that for many drugs the human MTDs can be predicted by adjusting mouse-derived MTDs using mouse and human CFC data.[5] However, some studies indicate that significant differences exist between human, rat and mouse hematosensitivity to certain pharmaceuticals and toxins. Human cells are sometimes more sensitive than animal cells to the toxic effects of compounds studied.[1,9,17] Such information could be of great importance before entering clinical trials.

Progenitors in mouse and human bone marrow (in some instances) react very similarly to the toxic effects of drugs. Initial toxicity screening with mouse cells can save time as the development to the different lineages is shorter for mouse cells. The colonies, BFU-E and CFU-GM, can be grown simultaneously in semi-solid media containing stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and erythropoietin (EPO) for human cell assays, or SCF, IL-3, IL-6 and EPO for mouse cell assays. For toxicity testing, cord blood, mobilized peripheral blood or bone marrow cells can be added to methylcellulose-based media along with the compound to be tested. Following culture of the plated cells and incubation at 37°C with 5% $CO_2$ for 14 days (human) or 10 days (mouse), colonies are assessed and enumerated microscopically.

**FIGURE 4.** Effect of compounds on human CFU-GM-derived colonies

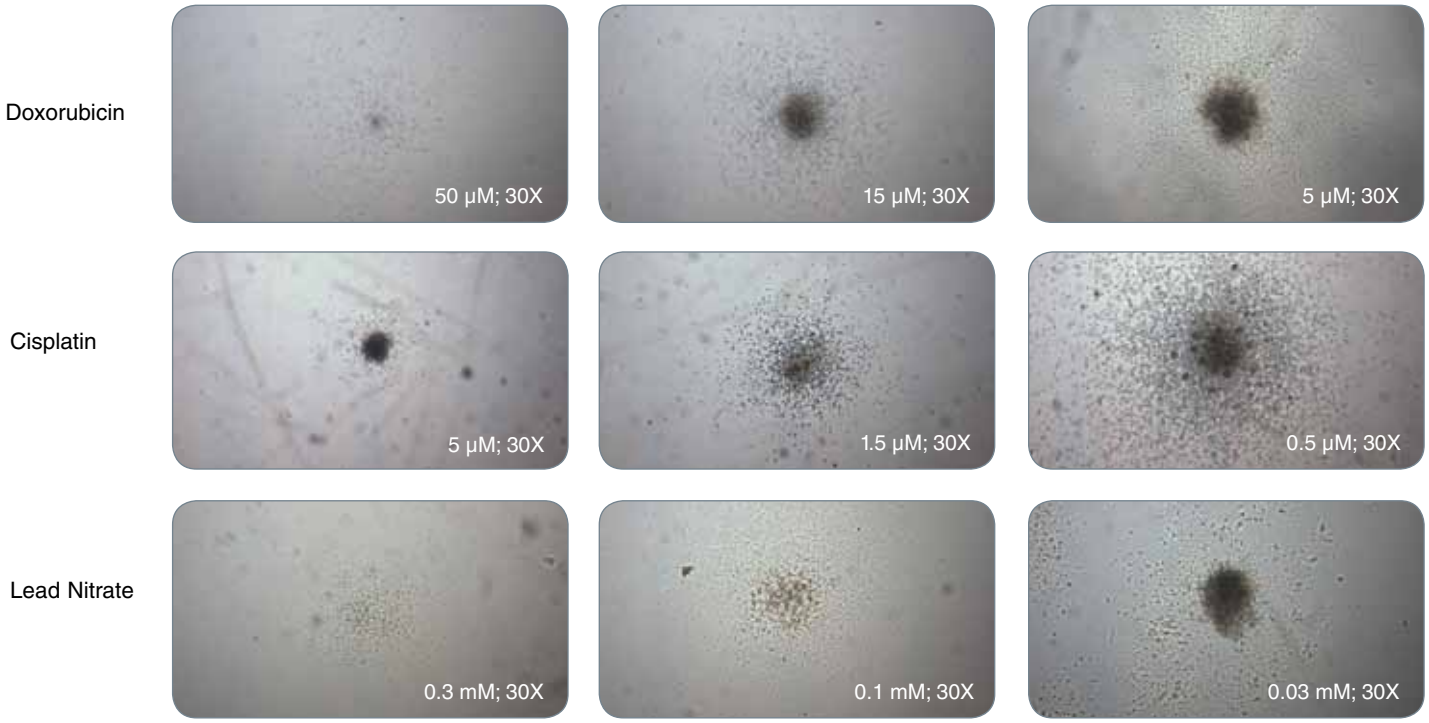
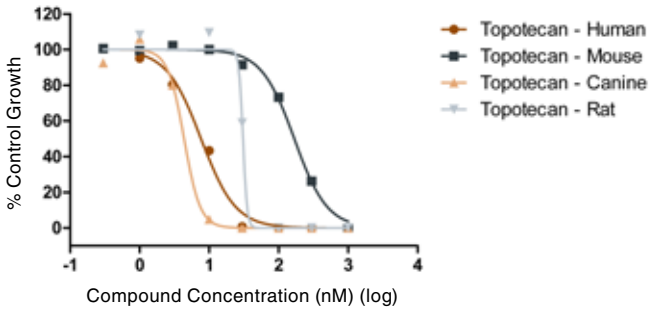



**TABLE 1.** CFU-GM $IC_{50}$ values for compounds in each species

| COMPOUNDS | HUMAN | MOUSE | CANINE | RAT |
|---|---|---|---|---|
| Topoisomerase | | | | |
| Topotecan | **7.69 nM** | 168.80 nM | **4.40 nM** | 30.96 nM |
| Irinotecan | **288.10 nM** | >1000 nM | **358.10 nM** | >1000 nM |
| Camptothecin | **1.03 nM** | 6.16 nM | **0.75 nM** | 9.16 nM |
| Anti-proliferative | | | | |
| Doxorubicin | **0.03 µM** | **0.01 µM** | 0.002 µM | 0.006 µM |
| Cisplatin | **4.21 µM** | **6.79 µM** | 0.97 µM | 2.68 µM |
| 5-Fluorouracil | **3.84 µM** | **3.08 µM** | 0.23 µM | 1.62 µM |
| Tyrosine Kinase Inhibitors | | | | |
| Sunitinib | **0.008 µM** | 1.10 µM | **0.01 µM** | 0.22 µM |
| Imatinib | **2.16 µM** | >30 µM | **1.99 µM** | >30 µM |
| Erlotinib | **15.27 µM** | 19.39 µM | **10.36 µM** | 34.67 µM |
| Environmental Toxin | | | | |
| Lead Nitrate | **0.98 mM** | 2.05 mM | 0.04 mM | **1.20 mM** |

**FIGURE 5.** Comparison of CFU-GM $IC_{50}$ values for Topotecan in each species



### Qualitative And Quantitative

To decrease the cost and time invested in drug development, efforts must be made to improve the chances of a drug successfully completing clinical trials. Although conditions in the human body cannot be completely reproduced in vitro, CFC assays can be used to help bridge the gap between high-throughput screening technologies and in vivo studies. Using primary human or mouse cells, such assays provide a sensitive and reliable method to test for hematotoxic effects. Qualitative and quantitative data can be collected for multiple cell types to confirm high-throughput results and/or predict possible clinical outcomes. Using CFC assays to obtain information on the hematological effects of a new drug is a quick and cost-effective way to make more informed decisions concerning each candidate's progression through the drug pipeline.

## Conclusion

Although bone marrow cell-based assays were initially developed to address questions regarding the function and hierarchy of stem and progenitor cells, these assays have provided us with practical systems to assess toxicity or potential efficacy of candidate molecules. To decrease the cost and time invested in drug development, efforts must be made to improve the chances of a compound successfully completing clinical trials. Validated CFU-GM assays have successfully predicted MTDs in humans and have the bridged the gap between in vitro screening technologies and in vivo studies. Using CFC assays to obtain information on the hematological effects of a new drug is a quick and cost-effective way to make more informed decisions concerning progression through the drug pipeline.

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