WHITE PAPER

Measurement of Hematopoietic Stem Cell Potency Prior to Transplantation

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This White Paper is a forward-looking statement. It represents the present state of the art and future technology in the field of stem cell potency testing. The views expressed in this White Paper are those of HemoGenix®, Inc.

Introduction

The increased number of potential cellular therapies over recent years has necessitated stricter regulations to improve efficacy of the treatment and reduce risk to the patient. One of the regulations that was implemented by the European Medicines Agency (EMEA), Committee for Medicinal Products for Human Use (CHMP) on 15 May 2008 and the publication of a Draft Guidance by the United States Food and Drug Administration (FDA) in October 2008, was the requirement to measure the potency of a cellular product prior to administration to the patient. There have been two primary difficulties with these regulations.

One of the difficulties encountered by the cellular therapy field is the understanding of what potency of a cellular product means. In the United States (U.S.), potency is actually defined in the Code of Federal Regulations (CFR) in Section 21, 600.3 "to mean the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result". In the implemented EMEA "Guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer", potency is simply indicated as a "quantitative measure of biological activity" of the cell based immunotherapy product.

The other difficulty encountered is how potency is measured. Again, in the U.S., a potency test has been defined in CFR Section 21, 610.10 as consisting "of either *in vitro* or *in vivo* tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition" of potency. The EMEA describes measurement of potency as an *in vivo* or *in vitro* test that is "appropriately validated" and "based on a defined biological effect as close as possible to the mechanism(s) of action/clinical response".

Measurement of potency is certainly not a new concept. It has been used for years to determine the activity of drugs, growth factors, vaccines etc. The reason why a measurement of potency is so important is because:

1. It is a measure of consistency during production/manufacture.
2. It shows product stability.
3. It allows product bridging studies to be performed.
4. It predicts product performance and assurance.
5. It allows evaluation and/or correlation with the clinical dose response.
6. It avoids product failure due to toxicity or inadequate potency.

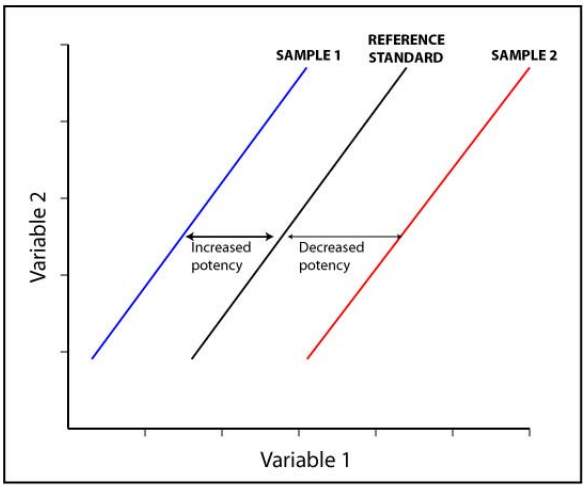
The last reason alone should be sufficient to implement tests of potency for cellular therapy products. However, points 4 and 5 are particularly important because correlation with the clinical response improves quality assurance and outcome prediction and therefore reduces risk to the patient.

How is Potency Measured?

For compounds such as drugs, growth factors or products such as vaccines (not cellular vaccines), potency is measured against a reference standard. Without a reference standard, potency cannot be determined. In the U.S. and many other countries, the U.S. Pharmacopeia provides reference standards for medicines. For many other products such as allergens, antibiotics, cytokines and growth factors, blood products, vaccines and toxins, the World Health Organization (WHO) Expert Committee on Biological Standardization provides reference standards to which the activity of samples can be compared. These "primary reference standards" are then used to establish "secondary, internal reference standards" which are then used for all future standardization of a manufactured product.

To measure potency, a dose response of the sample is compared to that of the reference standard. The potency assay may produce a positive or negative response and in most cases, the response will be a sigmoidal curve. In its simplest form, the linear section of the curve can then used to measure potency. As shown in Fig. 1, the linear section of two theoretical dose response curves is compared to that of the reference standard shown in black. The linear response lines of the samples must be statistically parallel with that of the reference standard. Statistical software has been developed by several companies to test for parallelism and calculate the potency ratio. The potency of the reference standard is set as 1. If the sample is displaced to the left of the reference standard, the potency ratio will be greater than 1 and indicates that the sample has a greater potency than that of the reference standard. It means that less of the sample is required to produce the same response as the reference standard. If, on the other hand, the sample dose response demonstrates a parallel line to the right of the reference standard, the potency of the sample will be less than 1 and more of the sample will be required to achieve the same response as the reference standard.

Figure 1. POTENCY
A Specific Quanitative Measure of Biological Activity



For drugs, growth factors, vaccines etc., parallelism should be obtained for different samples against a reference standard of the same material. The activity of the substance can then be estimated.

Is Cell Potency Different to Estimating the Potency of a Compound?

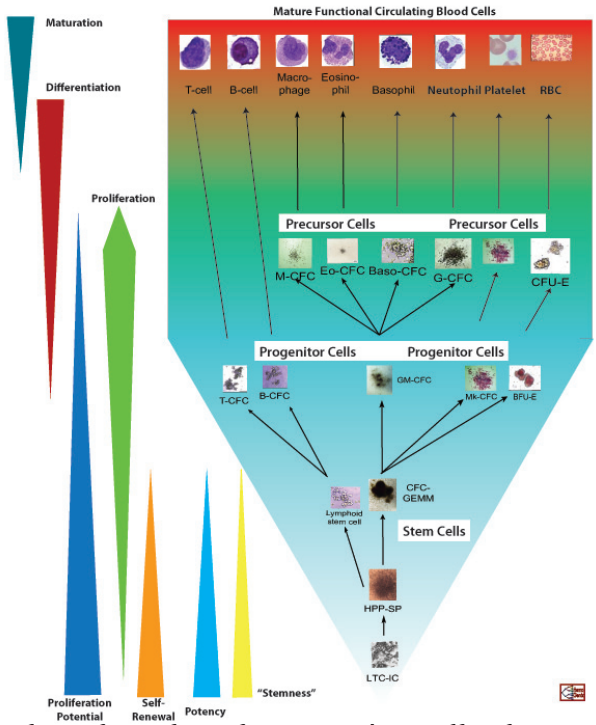
The concept is the same in that the potency of a cell population must be compared to that of a reference standard. However, measurement of cell potency will depend on the specific cellular or molecular function for which the use of the cells was intended. This, in turn, will determine the assay that has to be used to measure potency. An assay may already be available for the function in question or it may require modification. Alternatively, a new assay may need to be developed. In any event, a dose response relationship will have to be measured. If, for a cell population or cellular therapeutic product, the linear section of the dose response curve is parallel to that of the reference standard, it means that the product sample consists of or contains the same material as the reference standard. However, in many instances, demonstrating statistical parallelism between the product and the reference standard will seldom occur. This is because obtaining a population of cells that are exactly the same and respond in a similar manner to that of a reference standard, can only be obtained if the sample and reference standard are essentially identical.

Stem cell products, for example, exhibit different proliferation potentials. As a result, when a stem cell product is compared to that of a stem cell reference standard, the occurrence of a statistically parallel dose response to that of the reference standard means that both cell preparations exhibit the same degree of "stemness" or "primitiveness". Then, the displacement to the left or right indicates that the number of stem cells is either higher or lower than the number of stem cells in the reference standard. To obtain two stem cell populations that are essentially identical in "stemness", and therefore exhibit statistical parallelism with each other, will be the exception rather than the rule.

What is Hematopoietic Stem Cell Potency?

Figure 2 shows a diagrammatic representation of the organizational and hierarchical structure of the hematopoietic system. It can be compared to a tree in that the roots represent the primitive stem cells; the

Figure 2. Stem Cell Potency and its Relationship to "Stemness", Proliferation Potential and the Lympho-Hematopoietic System.



trunk, the mature stem cells; the large branches, the progenitor cells; the smaller branches, the precursor cells and the leaves, the functionally mature blood cells. Just like a tree, the hematopoietic system is a continuum in which one cell stage passes imperceptibly into the next.

By definition, stem cells exhibit self-renewal potential. They are also undifferentiated, exhibit the highest proliferation potential of all other cells and are capable of producing one or more lineage-specific, differentiated cell types. Stem cell biological systems demonstrate a so-called "stem cell hierarchy" in which stem cells can be designated "primitive" or "mature" and everything else in between as determined by a number of characteristics. "Primitive" stem cells are quiescent, while a greater proportion of "mature" stem cells are in cell cycle. "Primitive" stem cells have a greater proliferation potential than "mature" stem cells. Thus, as "primitive" stem cells become "mature" stem cells, their self-renewal capacity, proliferation potential and therefore their "stemness" decreases. This, in turn, means that stem cell potency also decreases. In fact, stem cell potency would actually be zero when the stem cell ceases to be a stem cell and becomes "determined" as a cell that has entered a specific lineage pathway.

WHAT SHOULD BE THE CHARACTERISTICS OF A POTENCY ASSAY?

By definition, a potency assay must demonstrate 3 primary characteristics:

1. It must be specifically designed for the product in question.
2. The assay must be appropriate to demonstrate either directly or in a surrogate form, the biological response that the cellular therapy product should achieve.
3. The assay must be validated.

To demonstrate that the assay is appropriate and validated, the following assay characteristics must be met:

- Accuracy: Closeness of agreement between tests results and the accepted reference value.
- Sensitivity: Responsiveness to a stimulus or proportion of correctly identified samples.
- Specificity: Proportion of negative samples correctly identified. This is a measure of assay

performance.
- Reliability: Objective measure of intra- and inter-laboratory reproducibility. This is part of the validation process.
- Relevance: Ability of the assay to correctly predict or measure the biological effect of interest.
- Robustness: Ability of the assay to withstand changes in the protocol and transferability amongst properly equipped and staffed laboratories.

Can TNC, Viability, CD34 or CFC be used as a Potency Assay?

The answer to this question is simply, no. The goal of a stem cell transplantation procedure, in which the stem cells are derived from bone marrow, mobilized peripheral blood or umbilical cord blood, is to provide engraftment and reconstitution/repopulation of the patient's lympho-hematopoietic system. The cells have to home to the primary hematopoietic site, seed and initiate proliferation (prior to any differentiation taking place) in order to establish a new stem cell compartment. Once the latter has been achieved, stem cells will continue proliferation and some will become "determined" to establish the progenitor amplification compartments. It follows that the most important function the stem cells have to demonstrate is that of proliferation potential. Without proliferation, engraftment and reconstitution will not occur.

Therefore, total nucleated cell (TNC) number, viability, CD34, and even the number of colonies obtained from a colony-forming cell (CFC) assay will not provide the information required that will measure proliferation potential, and thus predict engraftment potential of the stem cell product.

For umbilical cord blood (UCB) in particular, the TNC number has been given special significance, since the greater the TNC/kg transplanted, the greater the chance of engraftment. Taking into account that TNC also includes nucleated cells that cannot and do not proliferate, e.g. granulocytes, using TNC is probably a false parameter. A better parameter would be mononuclear cell number, which contains the majority of cells capable of proliferation, including the stem cells.

Viability and CD34 content are usually measured together by flow cytometry, the former being determined using 7-aminoactinomycin-D (7-AAD). This type of viability is based on a dye exclusion technique, which indicates membrane integrity, but provides no information on cellular or mitochondrial integrity. Therefore, even if a high viability of the cellular product is obtained, this does not mean that the cells will proliferate. Detection of CD34 on the surface of cells has been interpreted to mean the presence of stem cells. Yet, primitive erythropoietic and myelomonocytic progenitor cells also express the CD34 antigen, but do not exhibit self-renewal capability. Furthermore, CD34 is not a proliferation marker. As such, using CD34 as a measure of potency would, by definition, be an invalid potency assay.

Proliferation is the expansion of cells by the continuous division of single cells into two identical daughter cells. Differentiation, on the other hand, is the process whereby an undifferentiated cell acquires the features of a specialized cell. It follows, therefore, that the CFC assay is a differentiation assay and not a proliferation assay. Even though proliferation occurs prior to, and is a requirement for, differentiation the CFC assay measures differentiation potential and therefore cannot be used as a proliferation assay. This, combined with the fact that the CFC assay was designed as a research tool and that it cannot be standardized or validated, also invalidates this assay from being used as a potency assay.

Is there a Compliant Potency Assay Available that does not involve TNC, Viability, CD34 or CFC?

HALO®-96 PQR is one of a family of ATP bioluminescence proliferation assays that was specifically designed

to measure stem cell potency and define acceptance limits for release criteria prior to transplantation. It is also the only assay that incorporates a reference standard, thereby allowing the potency ratio of a sample to be quantified. Furthermore, HALO®-96 PQR complies with all of the aforementioned characteristics of a potency assay and has therefore been validated. As such, HALO®-96 PQR complies not only with the requirements of the Standards Organizations, but more importantly with the guideline requirements of the FDA and EMEA.

HALO®-96 PQR is based on the direct correlation of the intracellular ATP (iATP) concentration with proliferation potential, viability, cell number and cellular and mitochondrial integrity. Thus, a cord blood unit might have a high TNC and a high proportion might be CD34+ and exhibit a high viability by 7-AAD, but if the cellular and/or mitochondrial integrity of the cells has been damaged, the iATP levels will be low or even zero. The cells will not proliferate and therefore not engraft. There is, in fact, a direct correlation between proliferation potential and engraftment.

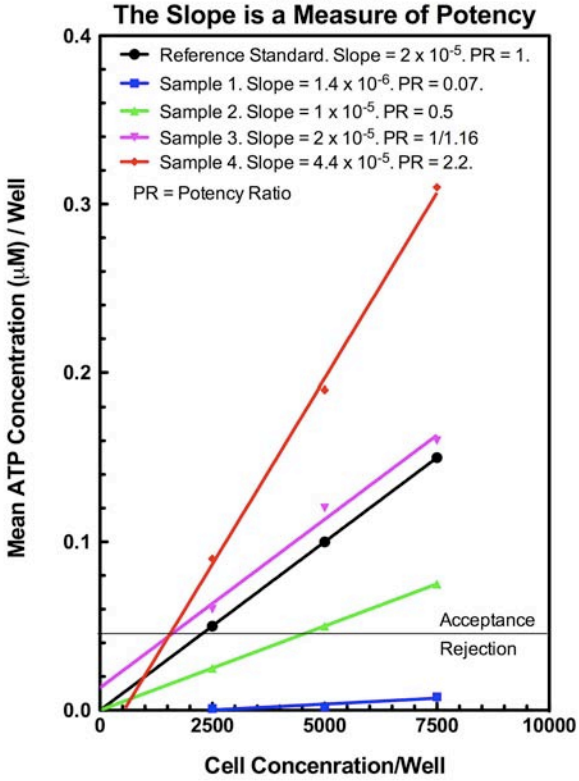
As with any potency assay, a dose response must be performed and HALO®-96 PQR is no exception. Originally designed to determine the potency of cord blood stem cells against a cord blood reference standard, cells from a segment are adjusted to produce a serial cell dose response. The cells from each of 3 doses (minimum requirement) are added to individual tubes containing a HALO® Master Mix with growth factors that will stimulate the in vitro, multipotential stem cells, CFC-GEMM. Inclusion of the more primitive stem cell population, HPP-SP, also adds greater reliability to the potency measurement. Cells from a frozen cord blood reference standard are thawed and a similar cell dose response prepared and added to tubes containing HALO® Master Mix. After mixing the cells with the Master Mix, 6 replicates of the HALO® Culture Master Mix are dispensed into wells of a 96-well plate. The cells are incubated for 5 days. Prior to processing the sample plate, an ATP standard curve is performed. The importance of a standard curve cannot be underestimated. It is probably the single, most important aspect of the assay. Without it, results cannot be compared over time either within or between laboratories. After incubation, the cells are mixed with a single reagent that contains both a lysis reagent and luciferin/luciferase. The released iATP acts as a limiting substrate for the luciferin/luciferase reaction to produce bioluminescence in the form of light. The light is measured in a plate luminometer.

As described above, in some cases, the 3-point cell dose response produced will exhibit a statistically parallel linear regression to the reference standard. This is shown in Fig. 3, where Sample 3 is parallel to the reference standard, so that the displacement to the left provides a potency ratio of 1.16. In the majority of cases, parallelism does not occur. Instead, the samples will exhibit varying linear regression slopes compared to the reference standard. The slope of the cell dose response is the most important parameter of the assay. Not only will it define whether the cell dose response is parallel to that of the reference standard, but also the potency ratio, because the steeper the linear regression slope of the sample:

1. The greater the proliferation potential and “stemness” of the stem cells;
2. The greater the potency of the stem cells and,
3. The greater the probability that stem cells will engraft.

In addition, the concentration of iATP produced will also determine whether the cord blood unit can be accepted or rejected for transplantation purposes. Sample 1 in Fig. 3 is just such a case, in which, not only is the slope near zero, but the iATP concentration is also near zero for all cell concentrations. This unit would be rejected for transplantation. Sample 2 exhibits a cell dose response, but also has a potency lower than the reference standard. Together with the low iATP level, this unit might also be rejected for transplantation purposes. Samples 3 and 4 would be accepted because both demonstrate a greater potency

Figure 3.
The Slope is a Measure of Potency



than the reference standard. However, Sample 5 exhibits a steeper slope than the reference standard and

would therefore be expected to contain stem cells with high proliferation potential and a high expectation of engraftment.

What is the Reference Standard?

The reference standard is the same as the sample. If the potency of a recombinant erythropoietin (EPO) preparation is being measured, then the primary reference standard would be the WHO 2nd recombinant EPO International Standard. Similarly, if the stem cell potency of an umbilical cord blood sample is to be evaluated, then the reference standard also has to be umbilical cord blood. Since only a small number of reference standard preparations can be obtained from a single cord blood unit, it follows that it would be difficult to have a universal umbilical cord blood reference standard. Each cord blood bank or cord blood transplantation unit would have to prepare their own reference standard. However, using an assay that incorporates an external standard, such as ATP used in HALO-96 PQR, allows results from different reference standards and samples to be directly compared with each other at any time. This provides the consistancy, reliability and reproducibility that is required for the assay.

Summary

A potency assay must provide a quantitative measurement of biological activity for the intended purpose. The intended purpose of a hematopoietic stem cell transplantation procedure is to provide the patient with a normally functioning hematopoietic system. To achieve this, the transplanted stem cells must engraft and reconstitute the patient. This can only occur if the stem cells proliferate. Without proliferation of the stem cells, no engraftment and reconstitution will occur. A stem cell product might meet TNC, viability and CD34 criteria, yet low proliferation potential and no possibility of engrafting. Therefore, potency for this specific application must be a measure of stem cell proliferation potential.

HALO®-96 PQR is a simple, 5 day *in vitro* assay that quantitatively measures proliferation potential of mature and primitive hematopoietic stem cells. When compared to the proliferation potential of a reference standard from the same tissue, the potency ratio can be calculated. The potency of a sample is not only directly related to whether a processed product can be used for transplantation purposes, but also provides a tool by which engraftment might be predicted. However, HALO®-96 PQR, like most assays, must be considered part of a collection of available tools which, together, can be used to make a decision that involves as little risk to the patient as possible.

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About HemoGenix®, Inc

HemoGenix® is a privately-held Contract Research Service and Assay Development Laboratory that produces and sells its services and assay kits in the U.S.A. and countries throughout the world. HemoGenix® specializes in developing predictive *in vitro* assay platforms for primary human and animal target cells. The assays have been specifically developed for Contract Research Services and as assay kits for in-house use by our customers. HemoGenix® is responsible for changing the paradigm and bringing stem cell hemotoxicity testing into the 21st century, by developing the HALO® Platforms that allow biotechnology and pharmaceutical companies to detect and predict the effects of large numbers of compounds on up to 14 different cell populations from 5 different species simultaneously. HemoGenix® is also changing the paradigm by providing standardized, instrument-based stem cell quality control and potency assays for transplantation and umbilical cord blood processing laboratories. HemoGenix® prides itself on bringing the best possible *in vitro* assay tools to its clients and customers.