HALO® and the Colony-Forming Cell (CFC) Assay:

Understanding the concepts and relationship between HALO and CFC assays, the information obtained and deciding which assay to use

HALO® was developed from the methylcellulose colony-forming cell (CFC) assay. It was developed for a specific purpose after HemoGenix® was awarded a grant from the National Cancer Institute; a new high throughput screening method was required to determine and predict toxicity to the lympho-hematopoietic system. High throughput screening requires automation. Since the CFC assay could not be automated, a new assay was designed and developed from the ground up. The result was HALO®.

Numerous changes to the traditional or classic CFC assay had to be made in order to produce a patented, *in vitro* assay as advanced as HALO®. Furthermore, validation by small, medium and many of the largest biopharmaceutical companies, such as AMGEN and Hoffmann-La Roche, had to be stringent for them to use the assay.

HALO® is now a 3rd generation assay platform with multiple applications. Indeed, HALO® is the only 384-well plate, cell-based, lympho-hematopoietic, high throughput screening assay available. To achieve this, HALO® had to be converted from a methylcellulose to a methylcellulose-free assay. The HALO® Platform now incorporates Suspension Expansion Culture (SEC) Technology and is completely methylcellulose-free. The original methylcellulose version of the assay is now called CAMEO™-96 and complements the traditional, but miniaturized, classic CFC assay from HemoGenix® called CAMEO™-4.

The HALO® Concept & Principle

The concept underlying HALO® is the measurement of intracellular ATP (iATP). ATP is the cell's energy source. If a cell does not produce ATP it will die. The production of ATP is an indication of cellular and mitochondrial integrity and therefore viability. When cells are stimulated to proliferate, the iATP concentration not only increases proportionately, but several hundred fold. As a result, iATP can be used as a reliable biochemical marker that correlates directly with proliferation. After incubation of lympho-hematopoietic cells to stimulate different cell populations, the iATP is release from the cells and acts as a limiting substrate for a luciferin/luciferase reaction. The result is a bioluminescence output that is measured as light in a plate luminometer. The process by which iATP is measured using bioluminescence is called Bioluminomics™ and has been described in more detail in a separate Information Sheet.

The CFC Concept & Principle

The colony-forming cell assay does not use a biochemical marker as a readout. Instead, the readout is the number of colonies produced by a colony-forming cell that proliferates and differentiates into functionally mature cells that identify the colony's origin. Thus, the CFC assay is a clonal functional assay. Clonality is determined by suspending the cells in a semi-solid viscous medium, usually methylcellulose. In this way, when the cells proliferate they remain stationary and build a colony. These colonies are initiated as small aggregates of proliferating cells, which gradually grow in size. As shown in the diagram on the next page, aggregates start to appear after about 2 days of culture. Providing one aggregate does not "bump" into another, the aggregate will grow into a spherical colonies. Irregular shaped colonies are due to several aggregates growing together. The colony will then have several centers of proliferating cells. These centers are called "Proliferation Units" or PU. If the number of PU are counted on each day, they will parallel the production of ATP as shown in the diagram.

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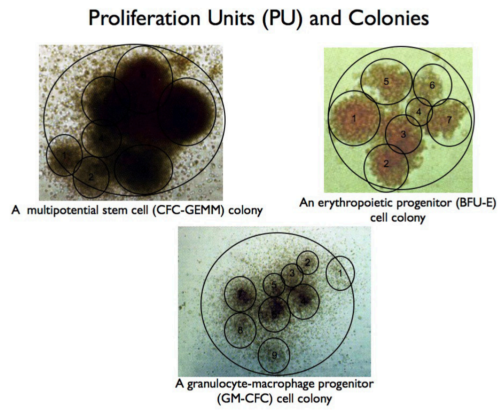
Counting Proliferation Units (PU) and Colonies

The pictures to the right show colonies in methylcellulose derived from multipotential stem cells (CFU-GEMM), erythropoietic progenitor cells (BFU-E) and granulocyte-macrophage progenitor cells (GM-CFC) from human bone marrow. Each one would be counted as a single colony. But each colony contains multiple areas with dark centers. These are individual aggregates of cells that have grown together to build the colony. Each aggregate is a center for proliferation. As a result, each colony can contain multiple proliferation units or PU. As shown in the diagram below, the number of PUs parallels the proliferation.



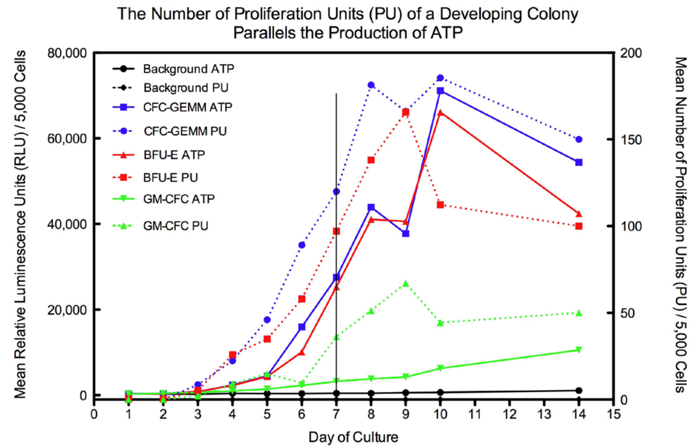
A multipotential stem cell (CFC-GEMM) colony

An erythropoietic progenitor (BFU-E) cell colony

A granulocyte-macrophage progenitor (GM-CFC) cell colony

The Growth of Hematopoietic Colonies

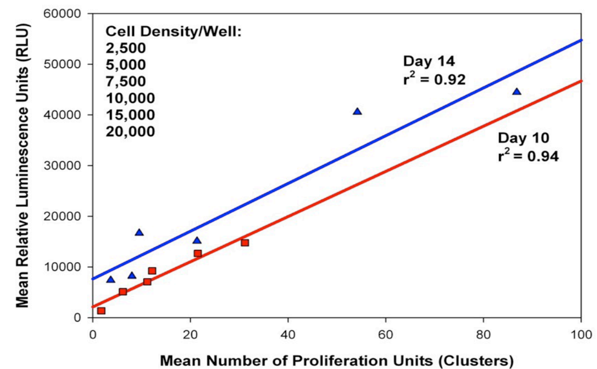
This diagram shows the growth kinetics of human bone marrow aggregates into colonies. The dotted lines show the number of PUs with time. The solid lines show the output from the plate luminometer in relative luminescence units (RLU) as a measure of iATP and therefore proliferation. Notice the difference in RLU between the background and the three hematopoietic cell populations. The increase in PUs and RLUs parallel each other, indicating that it is the iATP in the PUs that is being measured. The vertical line at 7 days represents the time point for measuring iATP in methylcellulose cultures. At this point, proliferation is increasing exponentially, but differentiation is low or non-existent.



Correlation of Proliferation with Proliferation Units

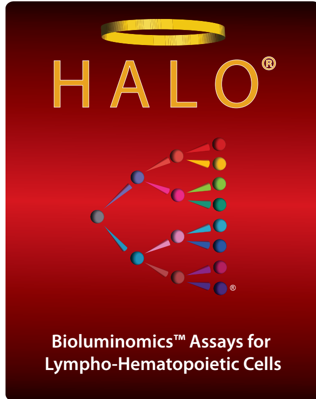
The parallelism between the number of PU and RLU as a measure of iATP and proliferation, indicates that a correlation between the number of PU and cell proliferation should exist. This diagram demonstrates this to be true. When RLU is measured on day 7 of culture and plotted against the number of PUs obtained on days 10 and 14 as a function of cell concentration, a direct correlation occurs. This demonstrates that measuring the proliferation of PUs on day 7 in methylcellulose cultures, will predict the colony growth at day 10 or 14. Although proliferation continues to increase after day 7, differentiation also increases (previous diagram). Since proliferation occurs prior to differentiation, measurement of the former must occur before the latter, but cannot be measured using the same assay.

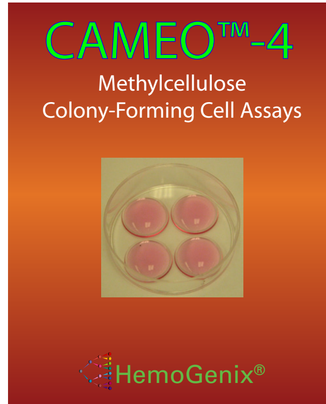
Correlation of Cell Proliferation with Proliferation Units (PU)

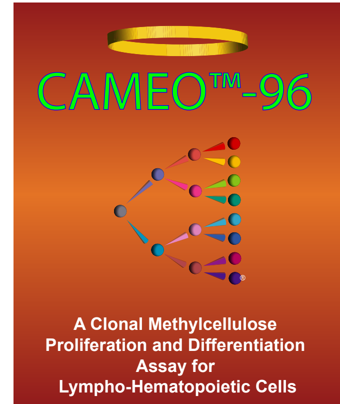


- *Proliferation occurs prior to differentiation.*
- *Without proliferation, differentiation would not occur.*
- *Differentiation is a default program requiring proliferation, but the corollary is not true.*
- *A proliferation assay cannot measure differentiation and a differentiation assay cannot measure proliferation.*

HemoGenix® Proliferation and Differentiation Assays for Lympho-Hematopoietic Cells







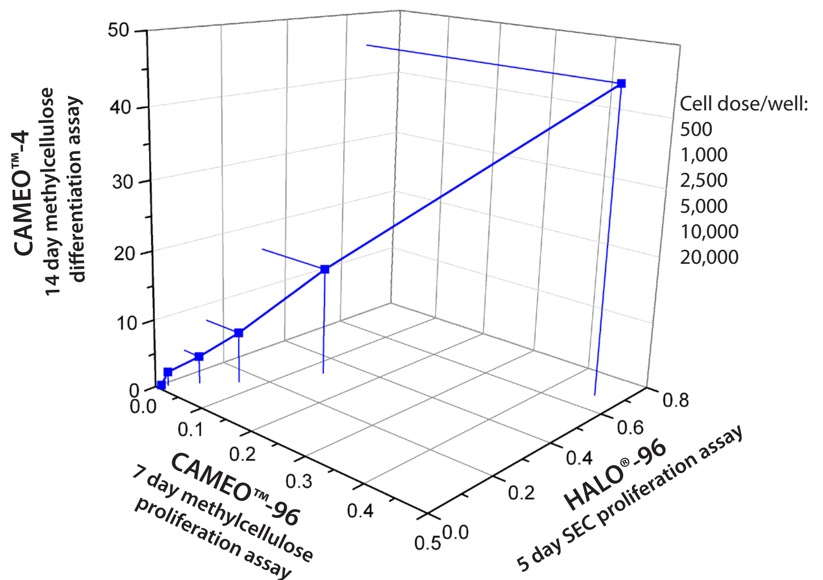
HALO®-96
A methylcellulose-free, proliferation assay for lympho-hematopoietic stem and progenitor cells. HALO®-96 uses Suspension Expansion Culture (SEC) Technology

CAMEO™-4
A classic methylcellulose, CFC differentiation assay for lympho-hematopoietic stem, progenitor and precursor cells in a miniaturized format

CAMEO™-96
A methylcellulose proliferation and differentiation CFC assay for lympho-hematopoietic stem, progenitor and precursor cells in a 96-well plate format

How HALO®, CAMEO™-4 and CAMEO™-96 are Interrelated and how Proliferation and Differentiation are Interrelated.

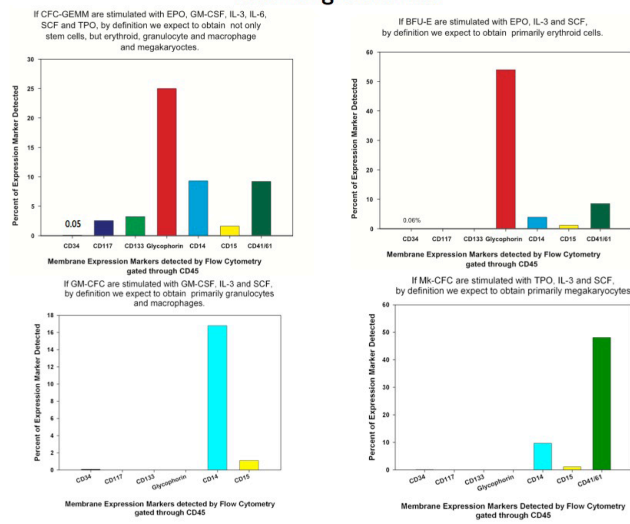
The graph to the right shows the relationship between proliferation and differentiation assays for human bone marrow multipotential stem cells (CFC-GEMM). This same relationship exists for any lympho-hematopoietic cell population from any species provided by HemoGenix® assay kits. The graph demonstrates the direct correlation between the HALO®-96 methylcellulose-free proliferation assay using SEC Technology, CAMEO™-96, a methylcellulose proliferation and differentiation assay and CAMEO™-4, a manual CFC assay as a function of cell dose. Both HALO® and CAMEO™-96 use bioluminomics™ technology to measure proliferation. These results illustrate that when one assay is derived from another, a relationship must exists between the assays. Such is the case with HALO®, CAMEO™-4 and CAMEO™-96. The diagram also demonstrates that the results obtained from the proliferation assay are equivalent to those obtained from the differentiation assay, because these two processes are themselves related when the same cell population is being examined.



If colonies are not formed or are not allowed to differentiate, how is it known that the right cells are produced?

If lympho-hematopoietic stem, progenitor or precursor cells are not stimulated or maintained with growth factors they will enter apoptosis and die. The type of cells produced in a colony using CAMEO™-4, CAMEO™-96 or suspension in a HALO®-SEC assay are dependent upon the growth factor/cytokine cocktail. Therefore, when a multipotential stem cell is stimulated with a cocktail (see below) to produce cells of all three hematopoietic lineages, that is exactly what is produced as shown in the top left graph. The types of cells produced by stimulation with different cocktails was determined by flow cytometry. This demonstrates that the cells expected to be produced are, indeed produced. Therefore it is not necessary to identify the cells in a colony because the growth factor/cytokine cocktail will provide that information.

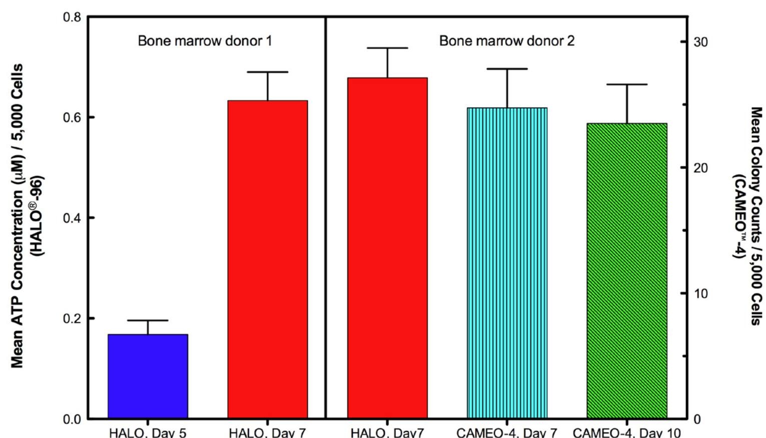
HALO® Cell Culture Produces the Expected Cell Types without Counting Colonies



Difference between HALO® and the CFC Assay

The diagram to the right shows results from HALO® and the CFC assay using CAMEO™-4 for two different human bone marrow donors. The results from donor 1 shows the difference between measuring ATP on day 5 or on day 7. There is >3-fold increase in ATP within 2 days (cf time course on page 2). The results from donor 2 show a similar ATP value on day 7 to that of donor 1. The results also demonstrate a similar response between HALO® and the classic CFC assay. Unlike HALO®, where a significant increase in ATP concentration occurs from day 5 to day 7, there is no such increase in colony counts from day 7 to day 10. This is because once a colony is formed, the only change that occurs is the size of the colony, The size of the colony indicates proliferation and cell number, which cannot be determined using the CFC assay because it is not a proliferation assay. This not only illustrates a major difference between HALO® and the CFC assay, but also demonstrates the importance of using the correct assay to produce the correct interpretation of the results.

Comparison between HALO® performs on Days 5 or 7.
Comparison of HALO® with the CFC Assay (CAMEO™-4).



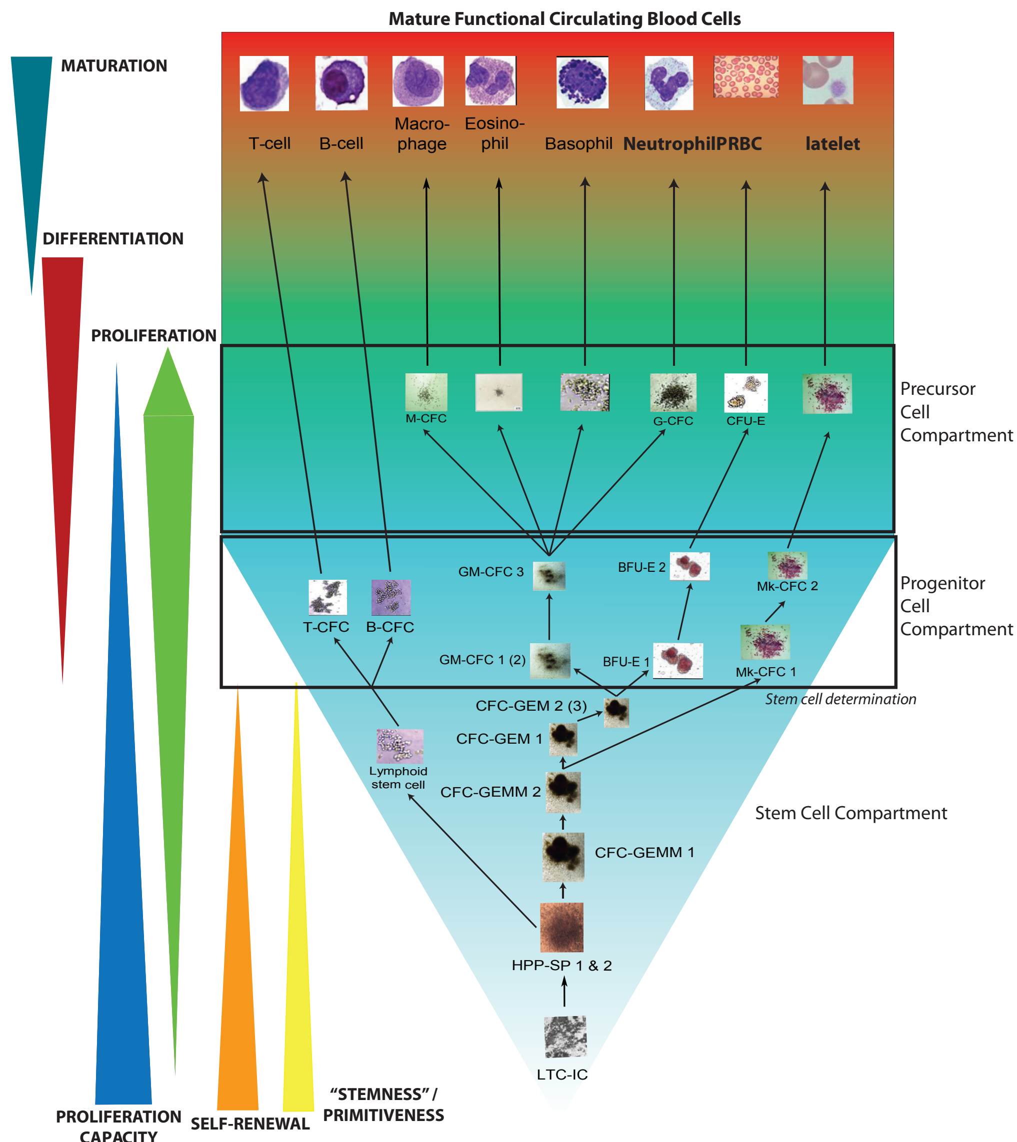
Using the correct type of assay is imperative to obtain results that will lead to the correct interpretation.

All cell-based studies are based on either a proliferation or differentiation process. Although proliferation and differentiation overlap, the two processes cannot be measured using the same assay because they are dependent on different readouts. However, if a readout for each process can be accommodated in the same assay, both proliferation and differentiation can be measured.

LYMPHO-HEMATOPOIETIC CELL POPULATIONS DETECTED with HALO®, CAMEO™-4 and CAMEO™-96

The nomenclature used to identify different lympho-hematopoietic cell populations and to use the correct population to achieve the goal of the experiment or study can sometimes be very confusing. In addition, it might be assumed that with the inclusion of HALO® and CAMEO™-96 to the classic colony-forming cell (CFC) assays (CAMEO™-4), different cell populations might be detected. The diagram below shows the position of the different cell populations within the lympho-hematopoietic organization and the designation of the cell populations detected using HALO®, CAMEO™-4 and CAMEO™-96. It is important to note that the cell populations detected using the CFC assay (CAMEO™-4) are the same as those detected using HALO® and CAMEO™-96, since the same growth factor/cytokine cocktails are used for all three assay systems. ***Please contact HemoGenix by calling (719) 264-6250 or e-mail using info@hemogenix.com to discuss the cell population(s) of interest and the best assay to use to achieve the intended goal.***

Stem, Progenitor and Precursor Cells Detected by HALO®, CAMEO™-4 and CAMEO™-96



	ATP Bioluminescence Proliferation Assay	Classic CFC Differentiation Assay	CFC - ATP Proliferation & Differentiation Assay
Growth Factor / Cytokine Cocktail	**HALO®-96 or -384 SEC**	**CAMEO™-4 MeC colony-forming cell**	**CAMEO™-96 MeC colony-forming cell**
STEM CELL POPULATIONS			
IL-3, IL-6, SCF, TPO, Flt3-L	HPP-SP 1	HPP-SP 1	HPP-SP 1
EPO, GM-CSF, G-CSF*, SCF, TPO, IL-2, IL-3, IL-6, IL-7	HPP-SP 2	HPP-SP 2	HPP-SP 2
EPO, GM-CSF, G-CSF*, SCF, TPO, Flt3-L, IL-3, IL-6	CFC-GEMM 1	CFC-GEMM 1	CFC-GEMM 1
EPO, GM-CSF, G-CSF*, SCF, TPO, IL-3, IL-6	CFC-GEMM 2	CFC-GEMM 2	CFC-GEMM 2
EPO, GM-CSF, G-CSF*, SCF, IL-3, IL-6	CFC-GEM 1	CFC-GEM 1	CFC-GEM 1
EPO, GM-CSF, IL-3, SCF	CFC-GEM 2	CFC-GEM 2	CFC-GEM 2
EPO, GM-CSF, G-CSF*, IL-3, SCF	CFC-GEM 3	CFC-GEM 3	CFC-GEM 3
PROGENITOR CELL POPULATIONS			
EPO, SCF, IL-3	BFU-E 1	BFU-E 1	BFU-E 1
EPO alone (high dose)	BFU-E 2	BFU-E 2	BFU-E 2
GM-CSF, SCF, IL-3	GM-CFC 1	GM-CFC 1	GM-CFC 1
GM-CSF, G-CSF*, SCF, IL-3	GM-CFC 2	GM-CFC 2	GM-CFC 2
GM-CSF alone	GM-CFC 3	GM-CFC 3	GM-CFC 3
TPO, SCF, IL-3	Mk-CFC 1	Mk-CFC 1	Mk-CFC 1
TPO alone	Mk-CFC 2	Mk-CFC 2	Mk-CFC 2
PRECURSOR CELL POPULATIONS			
EPO alone (low dose)	Not available	CFU-E	CFU-E
G-CSF	Not available	G-CFC	G-CFC
M-CSF	Not available	M-CFC	M-CFC
LYMPHOPOIETIC CELL POPULATIONS			
IL-2	T-CFC	T-CFC	T-CFC
IL-7	B-CFC	B-CFC	B-CFC

Which Assay Should I Use For My Research?
HALO®, CAMEO™-4 or CAMEO™-96

To decide which assay will provide the results that will meet the goal of the study, consider the following questions?

1. What is the goal of the study?
2. Is it necessary to measure proliferation, differentiation or both?
3. Is a non-subjective, instrument-based readout necessary or will a subjective, manual readout suffice?
4. Is a standardized assay necessary so that results can be compared directly if the study is to be repeated?

These and other questions will determine which assay should be used. The following table lists the characteristics of each assay that will help make the decision. For help, contact HemoGenix®.

	HALO®-96 SEC	**CAMEO™-4**	**CAMEO™-96**
Type of assay:	Proliferation	Differentiation	Proliferation and differentiation
Type of culture:	Suspension	Methylcellulose	Methylcellulose
Cell growth:	Expansion	Clonal	Clonal
Assay format:	96-well plate	4-well, 35mm Petri dish	96-well plate
Parameters measured:	Intracellular ATP	Proliferation Units (PU) and/or colonies	Intracellular ATP and Proliferation Units and/or colonies
Readout:	Luminometer	Manual	Manual and luminometer
Subjectivity:	Non-subjective	Subjective	Subjective and non-subjective
Incubation time:	4 days animal, 5 days human, variable	2-7 days animal, 7-14 days human	1-7 days animal, 5-10 days human, variable
Calibration & standardization:	Yes. Standards and controls included	No	Yes. Standards and controls included
Conversion of colony number to ATP concentration equivalents:	No	No	Yes
Standardization of CFC/CFU	No	No	Yes
No. of samples:	12-24/plate depending on no. of replicates	1/plate	12-24/plate depending on no. of replicates
Validation:	Yes	No	Yes
Multiplexing capability:	Yes	Difficult	Yes
Kit contents:	HALO®-96 SEC Master Mix Medium. ATP standards. ATP controls. Sterile 96-well plate. Non-sterile 96-well plate. ATP Monitoring Reagent. Instruction Manual.	CAMEO™-4 Methylcellulose Master Mix. Medium. 50, 4-well 35mm Petri dishes. Instruction Manual.	CAMEO™-96 Methylcellulose Master Mix. Medium. ATP standards. ATP controls. Sterile 96-well plate. Non-sterile 96-well plate. ATP Monitoring Reagent. Instruction Manual.

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Innovative Expertise You Can Count On

For more information on HemoGenix® Assays
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