volume of, for example, 1 ml. The test sample is diluted to give an expected concentration of approximately 50 Lf/ml, and, for example, 1 ml aliquots of this dilution are dispensed into each of the tubes containing antitoxin. The tubes are properly mixed by shaking, then placed in a water-bath at a constant temperature between 30 °C and 52 °C, and observed at regular intervals for the first appearance of floccules. This may require the use of a magnifying lens and strong illumination.

The first and the second mixtures to flocculate are recorded as well as the time taken for the first flocculation to appear. 2 tubes may flocculate simultaneously.

The first tube to flocculate is the one that contains the amount of antitoxin closest in equivalence to the amount of antigen in the sample. The antitoxin content of this tube can be used to calculate the Lf value of the sample. If 2 tubes flocculate at the same time, the mean from the tubes are given as the result.

The time taken for the first tube to flocculate (Kf) is a useful indicator of the quality of the antigen. If at a given temperature and concentration of toxoid and antitoxin the Kf value is increased compared with normal, this indicates that the antigen has been damaged. The Kf value may also change with the quality of the antitoxin used.

Example

Tube	A	B	C	D	E	F
Antitoxin added (Lf-eq.)	40	45	50	55	60	65
Antitoxin added (ml)	0.40	0.45	0.50	0.55	0.60	0.65
Saline added (ml)	0.60	0.55	0.50	0.45	0.40	0.35
Diluted sample added	1.0	1.0	1.0	1.0	1.0	1.0

If in this example the first tube to flocculate is tube C then the Lf value of the diluted sample is 50 Lf/ml. However, if the first tube to flocculate is tube A or tube F this does not indicate equivalence at that level. It would be necessary to perform a repeat test using either a different dilution of test sample or selecting a different range of doses of reference antitoxin.

More precision can be obtained by making allowance for the sequence of flocculation after the first tube. Thus, in the example quoted, if the second tube to flocculate had been tube D, the final value for the diluted sample would be 52, whereas if the second tube to flocculate was tube B, the final value would be 48. The test may be performed in duplicate with slightly different dilutions of the test sample.

If there is no indication of the expected Lf value of the sample available, it is advisable to obtain a rough estimate by use of a wider range of antitoxin content in the tubes before proceeding to the final test.

Example

Tube	A	B	C	D	E	F
Antitoxin content (Lf-eq.)	20	30	45	70	100	150

The level of toxin or toxoid and antitoxin concentration in the test may be varied, but this will markedly affect the flocculation time, so that at very low levels the test will take too long, whilst at a high concentration the onset of flocculation may be so rapid as to make it difficult to distinguish the first and second tubes to flocculate.

Assay of low concentrations by blend flocculation

For very low concentrations, it is preferable to measure toxin or toxoid by the method of blend flocculation. This involves comparison of the Lf value of a known toxin or toxoid and that of a mixture of the sample with that toxin or toxoid.

When a toxin or toxoid with a known Lf value and a toxin or toxoid with an unknown Lf value are flocculated together, the mixture will flocculate as the sum of their values if they are homogeneous. If non-homogenous toxins or toxoids are mixed they will produce an aberrant pattern with 2 flocculation maxima.

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2.7.28. COLONY-FORMING CELL ASSAY FOR HUMAN HAEMATOPOIETIC PROGENITOR CELLS

The haematopoietic system represents a continuum of cells whose phenotype and properties change as they progress from stem cells to differentiated cells.

Haematopoietic progenitor cells (HPCs) are capable of forming colonies or ‘cell clusters’ in cultures grown in semi-solid media and are said to be ‘clonogenic’. The determination of the number of colony-forming cells (CFCs) in a cellular product is an indicator of the functional capacity of the progenitor cells and is a predictor of haematopoietic reconstitution. The measured number of CFCs correlates with the minimum number of progenitors present in the sample.

CELL-SURFACE MARKERS

The capacity of colony-forming cells to give rise to haematopoietic colonies *in vitro* and/or to reconstitute the haematopoietic system has been correlated with the expression of specific cell-surface antigens. The expression of the membrane antigen CD34 is an accepted marker for most of the haematopoietic progenitors and stem cells.

COLONY ASSAY SPECIFICITY

Colony-forming cells are identified with a nomenclature based on the lineages of mature cells present in the colony (for example, CFU-Mix, CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E, CFU-E, CFU-Meg) and are a population of progenitors able to give rise to colonies containing one or more lineages of haematopoietic cells. No or low capacity for self-renewal has been ascribed to this population of human HPCs compared with the most immature stem cells.

The amount and type of growth factors supplied during the culture modulate the type and size of colonies that will be formed.

Greater specificity on the general class of HPCs and on their relative proliferative potential is provided by the time required to differentiate *in vitro* into mature cells. The time required by post-natal colony-forming cells to give rise to a colony formed of mature cells *in vitro* is 10-14 days.

QUALITY ASSURANCE FOR A CFC ASSAY

It is paramount for the overall quality of the colony-forming cell assay to apply a strictly standardised approach. It is therefore recommended to carry out intra- and inter-laboratory validations. The source of the materials, including reagents, growth factors and disposables, is identified.

The main factors affecting variability in the CFC assay are the number of cells plated and the identification of colonies. Up to 15 per cent intra-laboratory variability may be observed for the same test. If it is necessary to evaluate the number of colony-forming cells in a purified cell population, it is possible to use a limiting dilution approach where the number of wells positive for cell proliferation is measured with an automated system.

The other main source of variability stems from the use of undefined materials (for example, foetal bovine serum or bovine serum albumin) in the CFC assay. These products derive from pools of source materials and provide a non-specific stimulation of cellular proliferation. However, it is not uncommon to have batches with particular characteristics that selectively stimulate the proliferation of specific haematopoietic lineages.

Finally, a low level of endotoxins (less than 0.01 IU/ml or less than 0.01 IU/mg) in all the materials used for the clonogenic assay is advisable, as higher levels result first in a progressive skewing of the haematopoietic lineages expression in the cultures, and afterwards in a more general inhibition of cell proliferation and clonogenesis.

CFC CLONOGENIC ASSAY

The CFC assay is based on the capacity of progenitor cells to form a colony when plated in a semi-solid medium or in a gel in the presence of specific growth factors. Different types of semi-solid media may be used (for example, methylcellulose, collagen, agar and plasma-clot) depending on the desired readout. Commercially available media usually give more reproducible results.

MATERIALS

A validation is performed at least for the following critical materials.

Growth factors. Both multilineage (such as Kit-ligand or stem cell factor (SCF), interleukin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF)) and lineage-specific (erythropoietin, granulocyte colony-stimulating factor (G-CSF)) growth factors are required to obtain the highest number of colonies from a cell suspension containing a mixed population of HPCs.

Other media components. Media may be supplemented by serum (notably by foetal bovine serum) and/or albumin.

CELL CULTURE

Cells. The sample placed in culture must be representative of the cellular product injected. Cell suspensions are required for this assay. In the case of bone marrow aspirates, such suspensions can be obtained by forcing the bone marrow through a sieve or through progressively smaller calibre needles. Repeated passages through a 21-gauge needle are usually sufficient to disperse cell clusters into a cell suspension.

PLATING AND SCORING

The cells diluted in the culture medium are mixed in the semi-solid medium. It is common to plate 1 ml of the mixture in an untreated sterile Petri dish (Ø 35 mm).

Because of the viscosity of the medium, the solution cannot be plated with air displacement pipettes and the use of syringes equipped with large bore (≤ 18-gauge) needles is required.

The number of cells to be plated depends on the HPC concentration in the sample to be tested. So that no colony is derived from 2 different HPCs, the number of cells plated must allow between 40 and 80 colonies per plate (Ø 35 mm) to be counted. The 'target' number of colonies per plate may be obtained either from the percentage of CD34+ (or concentration of CD34+ cells/ml) determined by flow cytometry (*2.7.24*) or from different dilutions of the cell suspension (usually 2 concentrations are tested).

The plates are incubated in aerobic conditions with a carbon dioxide concentration of 5 per cent, at 37 °C in a humid (saturated) atmosphere for 10-14 days, and the number of colonies is then scored under an inverted microscope. Care must be taken when manipulating the dishes containing the colonies as the methylcellulose-based medium is viscous but not jellified. An inclined plate will result in mixed and 'comet'-shaped colonies making the scoring likely to be incorrect.

IDENTIFICATION OF THE COLONIES

The size and structure of the colonies depend on the type of mature cells that are their constituents. 50 cells per colony is usually considered a minimum. The presence of haemoglobinised cells identifies progenitors of the erythroid lineage. As the amount of mature cells for each lineage largely depends on the growth factors added to the cultures, performing differentiated counts is not recommended unless otherwise prescribed.

EXPRESSION OF THE RESULTS

The results of CFC culture are usually expressed as the arithmetic mean of the number of colonies counted in at least 3 plates in the test. The mean number of colonies is then related to 10^4 or 10^5 viable nucleated cells placed in culture.

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2.7.29. NUCLEATED CELL COUNT AND VIABILITY

The determination of the quality of cell suspensions requires accurate measurements of both cell concentration and percentage of viable cells. These data are essential to the decision-making process for preparing cellular products and for maintaining optimum culture conditions. The cell count may be expressed as the number of cells per volume of cell suspension and the cell viability as the number of viable cells per volume of cell suspension. The cell-count procedure may be performed manually (haemocytometer) or with an automated apparatus (for example, particle counter, flow cytometer). Other methods than that described below may be used.

CELL NUMBER

MANUAL COUNTING

Description of the apparatus and test principle. The following materials are required:

- a haemocytometer: a specialised microscope counting chamber available in different designs. It consists of a thick slide and a coverslip mounted to delimit a chamber with a specific volume for each design. The thick slide of the various haemocytometers consists of counting chambers separated by deep groves to avoid cross-filling. The counting chamber is etched in the glass and contains a grid which is specific for each model;
- a light microscope - low power 10× to 40× magnification;
- pipettes of a suitable volume range.