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ICSH GUIDELINES FOR RETICULOCYTE COUNTING BY MICROSCOPY ON SUPRAVITALLY STAINED PREPARATIONS

Prepared on behalf of the World Health Organization

by

The Expert Panel on Cytometry[1] of the International Council for Standardization in Haematology

Reticulocyte counts can be performed accurately and precisely using flow cytometric devices, but these are not widely available. It is therefore important to improve the reliability and standardization of the visual method using supravital dyes. This document describes a manual method based on the reference method of the International Council for Standardization in Haematology (ICSH). Sources of errors and methods to avoid or minimise them are outlined. This includes the use of the Miller graticule ocular and a table indicating the minimum number of cells to be counted to achieve a coefficient of variation of 10%.

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CONTENTS

1. INTRODUCTION

The reticulocyte count is the simplest practical test available to assess bone marrow erythropoietic activity, and can thus be used to differentiate hyperproliferative from hypoproliferative anaemias. Traditionally this test has been used especially to identify an increase in the count ('reticulocytosis') as occurs following haemorrhage, haemolysis or response to specific haematinic therapy. More recently, however, with the widespread use of cytotoxic chemotherapy, irradiation and bone marrow transplantation, the requirement for precise and accurate reticulocyte counts at low levels has become important. Methods have been developed with flow cytometric devices which result in a procedure which is superior to visual counting methods both in speed and precision. However, this requires special equipment; thus visual methods will continue to be used in many routine laboratories.

Reliability and standardization of the visual method using supra-vital dyes therefore continues to be an important objective (Finch et al, 1985). To achieve this reliability the International Council for Standardization in Haematology (ICSH) is developing a reference method. This document describes a manual method of reticulocyte counting which is based on the ICSH reference method.

It provides a high level of reliability even at low counts, when it may be important to identify a slight reticulocytopenia or to distinguish small differences between sequential counts. It is also the method of choice for reliable counting when the reticulocytes are increased in numbers.

The following aspects are considered:

1. What is a reticulocyte?
2. What is the best routine method for making reticulocyte preparations?
3. What statistical aspects of the counting procedure require consideration?
4. How should the result be expressed?
5. What are the remaining potential sources of error?

2. SOURCES OF ERROR

Many of the sources of error in reticulocyte counting in the routine laboratory relate to differences in definition of the reticulocyte. Other important factors affecting precision and accuracy are the visual acuity and patience of the observer and the quality and resolving power of the microscope. The most accurate counts are performed by a conscientious technologist who has no knowledge of the supposed reticulocyte count, thus eliminating conscious or subconscious bias. Studies by Savage et al (1986) have identified four analytical variables which regularly contribute to technical variation.

(1) The first is variation in the definition of a reticulocyte. Inter-technologist differences in definition of the reticulocyte constitute a major source of imprecision and inaccuracy at all count levels.
(2) The number of cells evaluated during the procedure has an important effect on the precision of the result. Under the assumption that the number of reticulocytes counted follows a binomial distribution, and, since the normal percentage of reticulocytes is low, statistical variation can be great. The limits of the 95% binomial envelope for a 1% reticulocyte count are 0,03 - 5,45% when 100 cells are counted and these limits change to 0,48 - 1,84% when 1000 cells are counted.
(3) The quality of the blood smear in terms of even cell distribution and staining also is important. However, with a properly prepared wedge film a suitable area can usually be found where the cells are evenly distributed.
(4) There is the question whether or not to use a graticule ocular as a counting aid for standardization of area reduction. Brecher and Schneiderman (1950) showed that use of such a device (the Miller graticule ocular) diminished the observed standard error of the reticulocyte count particularly at low levels. Area reducing devices are not widely used.

It has been suggested that reticulocytes mature **in vitro** and that delay in performing the count may introduce a further source of error. A recent study (Lewis et al, 1990) has shown that provided the specimen is kept at 4 °C, the reticulocyte count remains stable for several days. If, however, the specimen is incubated at 37 °C there is evidence of **in vitro** maturation after 24-48 hours.

3. DEFINITON

Reticulocytes are juvenile red cells from which the nucelus has been extruded; they contain remnants of the ribosomes and the ribonucleic acids (RNA) present in more immature cells. Ribosomes have the property of reacting with supravital stains to form a blue precipitate of granules or filaments. While there is no universally agreed definition of a supravitally stained reticulocyte, ICSH accepts the National Committee for Clinical Laboratory Standards (NCCL) definition (1985) as any non-nucleated red cell which contains two or more particles of blue-stained material corresponding to ribosomal RNA.

4. METHOD

4.1 Specimen collection

Specimens of blood should be collected by syringe or evacuated container, taking care to avoid haemolysis, into K_2EDTA (dipotassium ethylene diamine tetra-acetatic acid) anticoagulant in a final concentration of 1,5 ± 0,1 mg/ml. Processing should be undertaken as soon as possible, certainly no later than 4 h following venous sampling if specimens are kept at room temperature (20 °C). Processing may however be delayed for up to 24 h if specimens are stored at 4 °C.

4.2 Apparatus and consumables

The following laboratory equipment is required for performing the test:

- Pasteur pipettes
- 75 x 10 mm glass or plastic tubes
- Glass slides (acid washed)
- Miller graticule ocular (Graticules Ltd., Tonbridge, Kent, England) or equivalent [Figure 1]

Oil immersion microscopy with magnification x 1000 is recommended.

4.3 Stain

While purified dyes such as Azure B have the advantage of not varying from batch to batch, the use of New Methylene Blue for routine purposes is acceptable. To prepare the staining solution 0,1 g of stain (C1 52030) is dissolved in 100 ml of citrate-saline solution (1 volume of 30 g/l sodium citrate to 4 volumes of 9,0 g/l NaCl). The mixture must be well mixed and filtered after the dye has dissolved and then placed in a clean brown bottle. The solution should be filtered immediately before use through Whatman No. 1 filter.

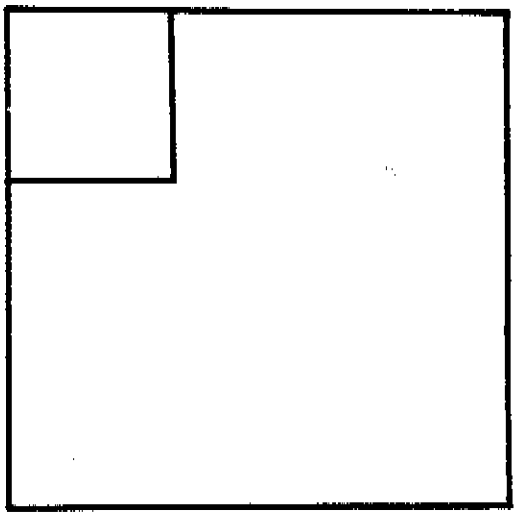

Fig. 1 - Miller graticule ocular

4.4 Procedure

Equal volumes of staining solution and EDTA blood are stoppered and well mixed. The red cells are then gently resuspended and conventional wedge smears made in the normal fashion to produce an evenly spread thin film of stained blood on a 2,5 x 7,5 cm microscope slide. Films should not be fixed or counter-stained since, with the latter, filamentous material is not better defined and precipitated stain may over-lie cells and cause confusion. After the film has dried, areas of the film encompassed by the Miller ocular or graticule where the cells are evenly distributed are selected for counting.

4.5 Cell enumeration

Use of a graticule ocular divides the field for proportional counting. With the Miller graticule ocular [Figure 1] "f = 9"; i.e. "f" is the ratio:

No. of cells on average in large square/No. or cells on average in small square.

It is advisable to check the calibration of the ocular by obtaining the average RBC counts in 5 large squares and in 5 small squares to establish that the proportion is, in fact 9. This assumes that the cells are evenly spread on the blood smear.

Proportional counting is carried out, i.e. the number of reticulocytes in the entire square and the number of red cells in the one-ninth area. Counting is continued in consecutive fields for adequate statistics, from the following formula based on the standard error of a proportion:

Let Se (p) = $[p(1-p)/n]^{1/2}$

where Se is the Standard Error and p the proportion.

Then define the coefficient of variation (CV) as:

$$CV = [Se\ (p)/p]\ 100$$
$$= (100/p)[p(1-p)/n]^{1/2}$$

then: $[CV/100)^2 = (1-p)/np$

and: $n = [100/CV]^2\ (1-p)/p$

where p is the proportion of the reticulocytes, n is the number of red cells in the entire square and n/9 is the number of red cells to be counted in the small square.

Example: Determine the number of red cells to be counted in the small square for a coefficient of variation of 10% and a reticulocyte count of 1%:

$CV = 10$
$p = .01$
$n = [100/10]^2\ (.99)/(.01)$
$= 9{,}900$

Then n/9 = 1,100, the number of red cells to be counted in the small square.

Therefore, to determine the number of red cells to be counted in the small squares using the Miller graticule ocular (f = 9):

Retic count		**No. of cells to be counted in small squares**
%	**P**	**CV = 10%**
1	0,01	1100
2	0,02	550
5	0,05	210
10	0,10	100
25	0,25	34

For practical purposes the total number of red cells to be counted in the small squares in consecutive fields is:

Retic count	Approximate no. of cells to be counted in small squares for CV of 10%	Equivalent to total count of
1 - 2%	1,000	9,000
3 - 5%	500	4,500
6 - 10%	200	1,800
20 - 25%	100	900

The reticulocyte count should always be expressed with the red cell count performed by automated or semi-automated cell counter and it is desirable that it be expressed as an absolute rather than a proportional count, i.e. x 10^9/l. This obviates the need for haematocrit correction.

5. INTERFERENCE

At times it may be difficult to decide what is and what is not a reticulocyte since most mature forms contain only a few dots or threads of reticulo-filamentous material.

In well stained pereparations Pappenheimer bodies usually present as a single small dot, less commonly multiple, and stain a darker shade of blue than the reticulo-filamentous material of the reticulocyte. If there is any doubt, Pappenheimer bodies can be identified by post-staining the preparation for iron by Perls' reaction.

Howell-Jolly bodies are stained in reticulocyte preparations but can usually be correctly identified by their characteristic size and shape. If necessary counter-staining by the Romanowsky method permits identification.

Heinz bodes are also stained by New Methylene Blue but produce a lighter shade of blue than the reticulo-filamentous material of reticulocytes. It may be helpful to use Azure B and examine by phase contrast microscopy if doubt exists; by this procedure the reticular nature of reticulocytes may be better seen.

Hb-H undergoes denaturation in the presence of New Methylene Blue solution, resulting in round inclusion bodies (golf ball) which stain greenish blue. These can be easily distinguished from reticulo-filamentous material.

6. REFERENCES

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