

# ISLH Recommended Reference Procedure for the Enumeration of Particles in Urine

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## 1. FOREWORD

Automated systems for counting urine particles are now being developed and marketed. A reference measurement procedure must be developed to determine the accuracy and validity of these systems and to provide means for instrument calibration.

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A task force of the International Society of Laboratory Hematology (ISLH) has proposed such a procedure for the enumeration of erythrocytes, leukocytes, hyaline casts, and squamous epithelial cells in urine. The proposed standard can be extended to include other urine particles such as the smaller epithelial cells and granular or cellular casts when there is consensus on the morphological description of these entities.

The ISLH Task Force standard is based on ISO/DIS (International Organization for Standardization) draft international standard 78-2, with special consideration given to the requirements for biological materials and for reference measurement procedures, the current model being based on the structures being developed by CEN/TC (European Standardisation Committee, Technical Committee on Health Care Informatics) 140 prEN 12286 and ISO/CD (ISO committee draft) 15193, with reference to ISO/CD 15195. Where relevant the terms and definitions are those provided in the *International Vocabulary of Basic and General Terms in Metrology* (VIM) [1].

The proposed standard draws heavily on previously published guidelines such as the *Finnish Recommendations from the Working Group on Clean Midstream Specimens* [2], the *NCCLS Urinalysis and Collection, Transportation and Preservation of Urine Specimens* (NCCLS Document GP16-A) [3], *JCCLS* (Japanese Committee for Clinical Laboratory Standards Guideline) GP1-P2 [4], and the *ECLM* (European Confederation of Laboratory Medicine) *European Urinalysis Guidelines* [5].

The importance of preanalytical specimen information is usually underestimated, and this information is generally not documented. This information is relevant and must be included in the procedure.

## 2. INTRODUCTION

A reference measurement procedure is required to assess the reliability of laboratory measurement results that are produced in routine service. A reference measurement procedure is defined as a “thoroughly investigated measurement procedure shown to have an uncertainty of measurement commensurate with the intended use, especially in assessing the trueness of other measurement procedures for the same quantity and in characterizing reference materials” (adapted from ISO/CD 15193, prEN 12286). A reference measurement procedure for urine microscopy should provide both correct identification and accurate quantitation of different particles when measuring patient urine specimens. Such a reference procedure does not exist for the enumeration of particles in urine. Standardization of the procedure used is essential to improve accuracy and limits of detection independently of the intended level of final performance [6,7]. Special attention must be paid to elimination of the different sources of error [8-10].

Reference measurement procedures can be used for:

- Assessing performance characteristics of measuring systems (instruments, ancillary equipment, and reagents).
- Demonstrating the interchangeability of different routine measurement procedures for the same analyte.
- Assigning values to reference materials used for the purposes of calibration and control.
- Detecting analytical interferences in patient samples.

A reference measurement procedure is provided in the form of a written standard when it is related to technical requirements:

- In technical specifications or regulations.
- For which values are to be stated by a supplier.
- That have a direct relationship to the performance of a product or process.

Microscopic examination of urine is widely practiced for the identification and enumeration of insoluble urine particles. The purpose of performing urine microscopy is to aid in the diagnosis and monitoring of diseases of the kidney and urinary tract.

In urine sediment preparation the centrifugation step with removal of supernatant is a major tool for concentration of urine specimens, but it is also a major source of error and is thus unsuitable for a reference measurement procedure. Enumeration of particles in the urine is most accurately performed using a chamber-counting method with uncentrifuged native

urine. Quantitative estimations must be based on direct counting and the result expressed in particles per liter.

The errors of the chamber method are related to (1) technique, (2) uneven distribution of the cells in the chamber, and (3) observer recognition errors. When not aggregated, cells in urine conform to an approximate Poisson distribution. However, when present in high numbers, cells adhere to each other, to mucus, and to the borders of the counting chamber, leading into a nonrandom distribution.

### 2.1. Scope

The scope of this standard is definition of the criteria for counting erythrocytes, leukocytes, squamous epithelial cells, and hyaline casts in urine by a procedure consistent with the approved definition of reference measurement. It is intended that an experienced laboratory worker, following this measurement procedure, can be expected to produce analytical results with an uncertainty of measurement not exceeding the stipulated range. Initially this standard will relate to red blood cells (RBCs), leukocytes (WBCs), squamous epithelial cells, and hyaline casts but can be extended to include other particles when their morphological descriptions are unequivocally established, eg, by applying the ECLM-European criteria [5].

It is understood that the reference measurement procedure should be of high metrological order, and the analytical principle of measurement should allow an adequately low uncertainty.

It is the responsibility of the laboratory to comply with the relevant legal health and safety requirements.

### 2.2. Warning and Safety Precautions

All biological specimens are to be treated as infectious and handled according to standard precautions. Standard precautions are new guidelines that synthesize the major features of universal precautions and body substance isolation practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions, which are intended only for prevention of the transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the US Centers for Disease Control and Prevention [11].

### 2.3. Normative References

ISO/DIS 78-2 Chemistry—Layouts for standards—Part 2: Methods of chemical analysis.

CEN/TC 140 prEN 12286 Measurement of quantities in samples of biological origin—Presentation of reference measurement procedures.

ISO/CD 15193 In vitro diagnostic medical devices—Measurement of quantities in samples of biological origin—Presentation of reference measurement procedures.

ISO/CD 15195 Requirements for reference measurement laboratories.

BIPM (International Bureau of Weights and Measures), IEC (International Electrotechnical Commission), IFCC (International Federation of Clinical Chemistry), ISO, IUPAC (International Union of Pure and Applied Chemistry), and OIML (Organisation Internationale de Metrologie Legale): 1993, International vocabulary of basic and general terms in metrology (VIM).

#### 2.4. Principles of Measurement Procedure

**2.4.1. Detection.** A reference method for urine microscopy must provide both correct identification of different particles and their accurate quantitation. Counting of cells in urine is most accurately performed using a hemocytometer. Provided that lysis of particles in hypotonic or alkaline urines can be avoided, the errors in the measurement procedure are related to accuracy of the hemocytometer chamber and errors in detection and identification of observed particles. Phase contrast optics is required for detection and supravital staining for confirmation of identity of some particles [5].

**2.4.2. Count Variance.** In all particle counting procedures there is an inherent error due to the fact that particles are distributed in a random way, following the Poisson distribution, provided that no clumps are present. The following equations apply:

$$\begin{aligned} \text{Standard deviation of the mean count} & s(x) = \sqrt{x} \\ \text{Standard deviation of the total count} & s(n) = \sqrt{n} \\ \text{Coefficient of variation (CV) of the} & \text{CV}(x) = \sqrt{x}/x = 1/\sqrt{x} \\ \text{mean count} & \\ \text{CV of the total count} & \text{CV}(n) = \sqrt{n}/n = \\ & 1/\sqrt{n} = 100\%/\sqrt{n} \end{aligned}$$

If  $p$ -unit volumes, such as squares, are counted,

$$x \text{ (mean count)} = n/p \text{ and } n = px$$

Then,

$$\text{CV}(n) = 1/\sqrt{n} = 1/\sqrt{px} = (1/\sqrt{p}) * (1/\sqrt{x}) = (1/\sqrt{p}) * \text{CV}(x)$$

When small particle concentrations are counted, additional squares improve the estimate of total particle count present in a specimen, because extra squares reduce the CV of  $\text{CV}(n)$  by a factor of  $(1/\sqrt{p})$  compared to a single square, when  $p$  is the number of squares counted.

The Table shows the compilation of 95% confidence intervals of relevant counts to demonstrate the statistical imprecision inherent in particle counting.

### 3. SPECIMEN COLLECTION

The reference measurement procedure is suitable for all common methods of urine collection. The urine specimen should be kept refrigerated at  $+4^\circ\text{C}$  before measurement. The chamber-count procedure must be completed within 2 hours after a documented specimen collection if the origi-

#### Poisson Statistics of Particle Counting

Total Count	Standard Deviation $s(n)$	Coefficient of Variation $\text{CV}(n)$	95% Confidence Interval
50	7	14%	37-66
100	10	10%	81-122
200	14	7%	173-230

nal concentration in the bladder is required. Dilute (relative volumic mass  $< 1.010$ ; osmolality  $< 300 \text{ mOsm/kg}$ ) and alkaline ( $\text{pH} > 7.5$ ) urines should be excluded from comparison studies because of an increased tendency of lysis of erythrocytes and leukocytes.

### 4. STAINING

Staining is not recommended as the first procedure in urine counting, because the colored background may obscure detection of some particles, particularly erythrocytes. On the other hand, staining helps in classification of some nucleated cells and inclusions found in casts. The Sternheimer alcian blue–pyronin B or an equivalent staining method [12] that gives a color contrast with blue and red is recommended. The quality of dyes used should comply with the physicochemical characteristics specified below.

Experimental comment: When counting particles with and without staining, a reduction of 25% to 50% of erythrocyte counts was observed in Bürker chambers after staining, compared to unstained specimens in Fuchs-Rosenthal chambers. Unstained small epithelial cells were confused with WBCs.

#### 4.1. Reagents

The stock solutions are:

- (1) 2% Alcian blue (color index 74240) dissolved in 50 mL laboratory-grade water.
- (2) 1.5% Pyronin B (color index 45010) dissolved in 50 mL water.

Dissolve separately for 2 to 4 hours with magnetic stirring.

Filter overnight at  $20^\circ\text{C}$  (Whatman no. 1 filter paper). Final absorbance may be checked photometrically. Absorption maximum is at 622 nm for alcian blue and 553 nm for pyronin B. Identical classification of particles may be reached with somewhat different absorption characteristics as well. Stock solutions are stable at  $20^\circ\text{C}$  for at least 3 months.

Stock solutions (1) and (2) are mixed together in proportions 1:1 prior to use. Although the mixture is stable for 2 to 4 weeks at  $20^\circ\text{C}$ , bacterial or yeast contamination occurs readily from routine specimens.

#### 4.2. Staining Procedure

Add 1 volume of stain mixture to 9 volumes of well-mixed urine (eg, 500  $\mu\text{L}$  of stain to 4.5 mL urine in a 10-mL tube). Allow the stain mixture to act for 5 minutes before microscopy.

### 5. APPARATUS

#### 5.1. Specimen Containers

Containers for urine specimens should be either plastic or siliconized glass to avoid adherence of particles to the container walls; usual 5- to 12-mL volumes are acceptable.

#### 5.2. Pipettes

Pipettes provide more accurate insertion of specimens into the chamber than capillary tubes. Calibrated pipettes with a volume error less than 5% should be used. Plastic or siliconized glass should be used to prevent particle adherence.

Experimental comment: Repeated filling made it possible to obtain a CV of 9% with a pipette as opposed to a CV of 18% with a traditional capillary tube.

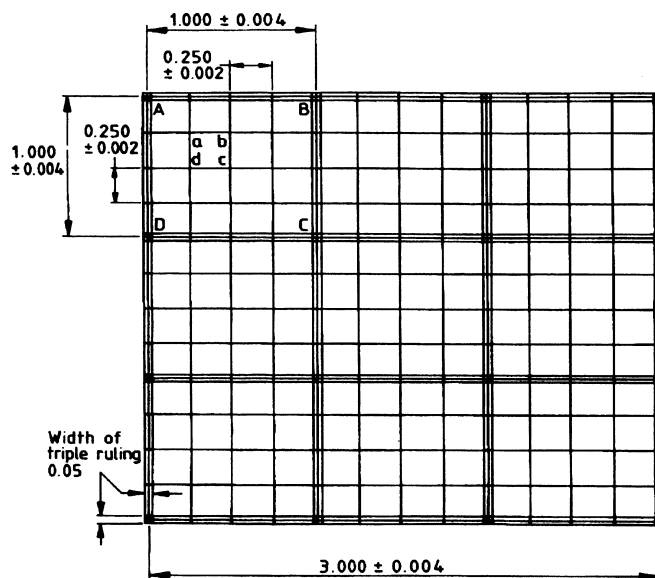
#### 5.3. Counting Chamber

The counting chamber consists of a glass slide of overall dimensions as specified in BS 748 (1982) and DIN12847 and bears a recess that when bridged by a cover glass (*vide infra*) forms a volumetric chamber. The base of the recess carries rulings as illustrated in the Figure.

Because particle concentrations are small, high volumes are needed. That is why a Fuchs-Rosenthal chamber (height, 0.2 mm; total volume,  $2 \times 3.2 \mu\text{L}$  with 16 large squares or  $2 \times 1.8 \mu\text{L}$  with 9 large squares as in Figure) is preferred over smaller chambers, such as a Bürker chamber (height, 0.1 mm; total volume,  $2 \times 0.9 \mu\text{L}$ ). At the 0.2-mm depth, unstained specimens are more easily counted than stained specimens. A nonmirror bottom is recommended because of the phase contrast optics used.

#### 5.4. Cover Glasses

Cover glasses must be optically plane and free from any defect that would interfere with clear vision through the glass when viewed through a microscope. Cover glasses must allow the use of phase contrast optics. The corners should be rounded and the edges smooth ground. Thin cover glasses (<0.4 mm) must be avoided because of the risk of bowing. Suitable cover glass dimensions are 25 mm  $\times$  22 mm with a tolerance of  $\pm 1$  mm. If the cover glass is perfectly flat when placed on the counting chamber, diffraction rings are seen. When the cover glass is placed correctly on the counting chamber, the central ruled area lies in the center of the rectangle to be filled with urine. The counting chamber and the cover glass form the boundaries of the volumetric chamber.



Details of a modified Fuchs-Rosenthal hemocytometer with 9 large squares (dimensions in millimeters). One large square with triple lines has a volume of  $1 \times 1 \times 0.2 \text{ mm} = 0.2 \text{ mm}^3 = 0.2 \mu\text{L}$ . The total volume of the modified chamber illustrated is  $9 \times 0.2 = 1.8 \text{ mm}^3$  (in the classical Fuchs-Rosenthal chamber the total volume is  $16 \times 0.2 = 3.2 \text{ mm}^3$ ).

#### 5.5. Microscope

The microscope should have the following features: phase contrast optics, mechanical stage to allow easy and smooth positioning of the slide, and a set of basic objectives, ie,  $\times 10$ ,  $\times 40$ , and an ocular  $\times 10$ .

### 6. TECHNIQUE OF COUNTING

#### 6.1. Procedure

**6.1.1. Cleansing:** Wash the chamber and the cover glass in running tap water to remove all organic matter. Before use, wash in alcohol and dry.

**6.1.2. Cover Glass:** Place the chamber on a flat surface. After moistening the contact surface, press the cover glass down. Check for the presence of several Newton's rings that will appear on each side if the cover glass is in the correct position. If the rings do not form, reclean the chamber and the cover glass and replace the cover glass. If the rings still do not form, repeat the procedure with another cover glass.

**6.1.3. Mixing:** The native or stained (always uncentrifuged) urine should be well mixed before aspiration into a calibrated pipette. Adequate mixing is achieved by inverting the tube at least 20 to 40 times, preferably with a mixing device (inverting cycles about 20/min). If repeated, 10 to 20 cycles are sufficient.

Experimental note: The efficiency of mixing can be checked by replicate counting. A mixing device may need 40 cycles for a uniform suspension.

**6.1.4. Filling the Chamber:** Both sides of the chamber are filled using a pipette held at an angle of  $45^\circ$  and allowing the urine to flow under the upper edge of the cover glass in a smooth single action. Satisfactory filling volumes are 15 to 16  $\mu\text{L}$  for a Fuchs-Rosenthal chamber and 7 to 8  $\mu\text{L}$  for a Bürker chamber. Filling must not be too rapid. The chamber must be cleaned and the filling process repeated if any of the following occur:

1. Overflow into the moat.
2. Chamber area incompletely filled.
3. Air bubbles anywhere in the chamber area.
4. Debris anywhere in the chamber area.

**6.1.5. Settling:** Leave the filled chamber undisturbed for 5 minutes to allow particles to settle (in isotonic or hypertonic solutions, RBCs might not settle). The bench must be free from vibration and the chamber not exposed to drafts, direct sunlight, or other sources of heat.

**6.1.6. Viewing:** The low-power ( $\times 10$ ) objective is used to scan the chamber to ensure even distribution of particles throughout the ruled grid. The high-power ( $\times 40$ ) objective is used for counting. Large particles (casts and squamous epithelial cells) may, however, be clearly discernible by phase contrast optics even at low-power objective.

**6.1.7. Counting:** An equal amount of squares should be selected for counting from both sides (both grids) of the chamber to balance for different fillings. Edge rules must be strictly applied to ensure that each particle is counted only once: cells touching the left hand and/or upper lines of a square are counted in that square, and those touching the lower and/or right-hand lines are classed as outside the square.

Practical note: For some particles and specimens, a volume of 0.2  $\mu\text{L}$  from both sides may be sufficient to reach a combined total count of 200, whereas for rare particles counting of  $2 \times 3.2 \mu\text{L}$  (full grid area) may be needed for several times, until a total of 50 to 200 particles is reached.

**6.1.8. Time Allowed:** If, during the counting period, the chamber dries out, the preparation must be discarded. Drying at the edges of the preparation initiates currents, which cause movement of the cells after they have settled. A maximum of 60 minutes is allowed for counting of a single specimen if no drying occurs.

Experimental note: No changes in the particle concentrations of most specimens were observed when preserved at  $+4^\circ\text{C}$  for 60 minutes and counted repeatedly every 15 minutes with an automated device. Particles in dilute or alkaline specimens may lyse, however. With a visual chamber, a period of 60 minutes may be

needed to correctly classify and count a sufficient number of particles in a single specimen.

### 6.2. Counting Adequate Total Numbers of Particles

A sufficient volume is required to achieve statistical reliability in counting particles present at low concentrations. A minimum of 50 rare particles, eg, casts and squamous epithelial cells, must be counted to reach a CV of 14%, and 200 particles, eg, WBCs and RBCs, to reach a CV of 7% (Table). Several chamber fillings may be required to obtain these amounts. With a Fuchs-Rosenthal chamber, with each double filling a  $2 \times 3.2 \mu\text{L}$  volume is obtained if the chamber has 16 large squares on both sides or a  $2 \times 1.8 \mu\text{L}$  volume if the chamber has 9 large squares on both sides (Figure). For large particles, such as casts or squamous epithelial cells,  $10 \times 10$  magnification may be sufficient for detection and to allow rapid viewing of large chamber volumes.

### 6.3. Checking the Identity of Counted Particles

To confirm the identity of particles counted from non-stained specimens, a Sternheimer-stained or equivalent preparation should be counted or screened using a Bürker or equivalent chamber with a fluid height of 0.1 mm. This procedure is important, particularly to correctly classify WBCs (granulocytes, lymphocytes, macrophages) and small epithelial cells (transitional epithelial cells, renal tubular epithelial cells).

Staining may also be useful in classification of contents of casts that are not hyaline only.

On the other hand, stained slime in urine may obscure RBCs despite use of phase contrast optics. Because of their large size, some casts may remain outside a chamber with a fluid layer of only 0.1 mm.

## 7. ANALYSIS OF ERRORS

### 7.1. Systematic Errors (Trueness of Measurement)

**7.1.1. Sources of Error.** Chamber volume error should comply with the specified limits. Compliance is achieved by careful placement of the recommended coverslip on top of the counting area.

A representative statistical sample for counting is obtained by carefully mixing the stained urine before counting, by ensuring even distribution of the particles throughout the counting area after filling, and by avoiding extra delays in counting (a maximum of 1 hour/specimen may be accepted). Specimens that are not evenly distributed because of adherence of particles, eg, WBCs to each other or to slime, or are too crowded with particles, bacteria, or precipitate, must be discarded from final comparisons.

Detection of all particles is accomplished by allowing them to settle for 5 minutes in the 0.2-mm-high fluid layer

(Fuchs-Rosenthal chamber) before counting and by focusing at various levels during microscopy.

If the reference counting procedure is used in evaluation of new procedures, the order of counting between the evaluated and the reference procedure must be changed during the evaluation to avoid systematic errors caused by differences between the procedures in delays after specimen collection.

**7.1.2. Between-Observer Variation.** Successful detection and classification must be confirmed by a separate between-observer comparison experiment: At least 3 different observers independently count all particles to be evaluated from the same 5 to 6 specimens, as described above. The observed results usually fall within the 95% confidence intervals for the counts (Table). Any deviations should be discussed and corrected. This consensus process is mandatory to unify classification criteria and detection practice. The results of between-observer comparisons after the training period must be included in the report on performance and expressed as an extra CV to be added into the calculations of total uncertainty if it exceeds that of Poisson distribution. These variations may be related to observers themselves (eg, mixing of the specimens, filling the chamber, detecting and identifying particles) or to other factors as explained above.

### 7.2. Precision (Random Errors)

Repeatability of counting should be addressed by filling a chamber and counting it twice from 20 specimens, both counts being performed within 60 minutes.

Reproducibility from day to day can be evaluated only on preserved specimens, eg, those prepared for external quality assessment. Both the reference measurement and the evaluated procedure should be performed on each day of the reproducibility study. In a multicenter evaluation protocol, preserved specimens can also be used to address reproducibility between laboratories.

### 7.3. Total Uncertainty of Measurements

The current international recommendation [13,14] is to calculate the total uncertainty of measured quantities. This recommendation also applies to particle counting. Experimental SDs should be expanded by estimates of other possible sources of error in the form of assumed or known systematic "uncertainties." It is usually practical to calculate relative uncertainties, ie, CV (%) of combined uncertainty.

The combined uncertainty is defined by the following formula:

$$U(\text{total}) = \sqrt{U_a^2 + U_b^2 + \dots + U_n^2}$$

in which squares (variances) of all different uncertainties from a to n, related to either systematic or random errors, are summarized. The 95% uncertainty interval of results then corresponds to a Gaussian distribution =  $2 \times U(\text{total})$ .

In the proposed reference measurement procedure, at least the following uncertainties should be included in the calculations (see also [10]):

1. Random error (based on Poisson distribution).
2. Observer-related systematic error (based on remaining differences of counts obtained by different observers, exceeding that of Poisson distribution).
3. Error in counting volume (obtained from the manufacturer; may also be measured).
4. Delay within the counting period between the specimens or procedures under comparison (when applicable to estimate the true count in a specimen, because in evaluation protocols this value should be randomized between the procedures).

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