1997 ESACP consensus report on diagnostic DNA image cytometry

Part II: Specific recommendations for quality assurance

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1. Introduction

Quality assurance in medical services has become a more and more important issue. In order to achieve reliable results of DNA image cytometry as described and discussed in detail in the first part of this report [2], the conditions for reproducible preparation, measurement and interpretation have to be worked out, carefully followed and checked at various steps of the process in certain time intervals.

Herein Quality Assurance (QA) is meant as a continuous process in assuring the uniformity and reliability of the diagnostic outcome of the quantitation procedure. It works continuously in a logistic concept with feedback to the sample preparation, the instrumentation settings, the densitometric measurements, and the diagnostic interpretation. In this context Quality Control (QC) is aimed to retrieve the error sources at all stages of the entire methodological and diagnostic process.

From our knowledge [1] we consider the following points to be the most important ones to achieve the desired uniformity and reproducibility of DNA-ICM assessment: specimen preparation, staining, instrumentation, sampling, densitometric measurements, scaling process and measurement interpretation.

Beside sampling procedures all other topics have been conceived in form of quality control tests, presented in detail in the following. Setting "correct" sampling strategy is a prerequisite for reliable interpretation and the importance for this step lies in the fact that the consequences of sampling strategy cannot be corrected at a later stage.
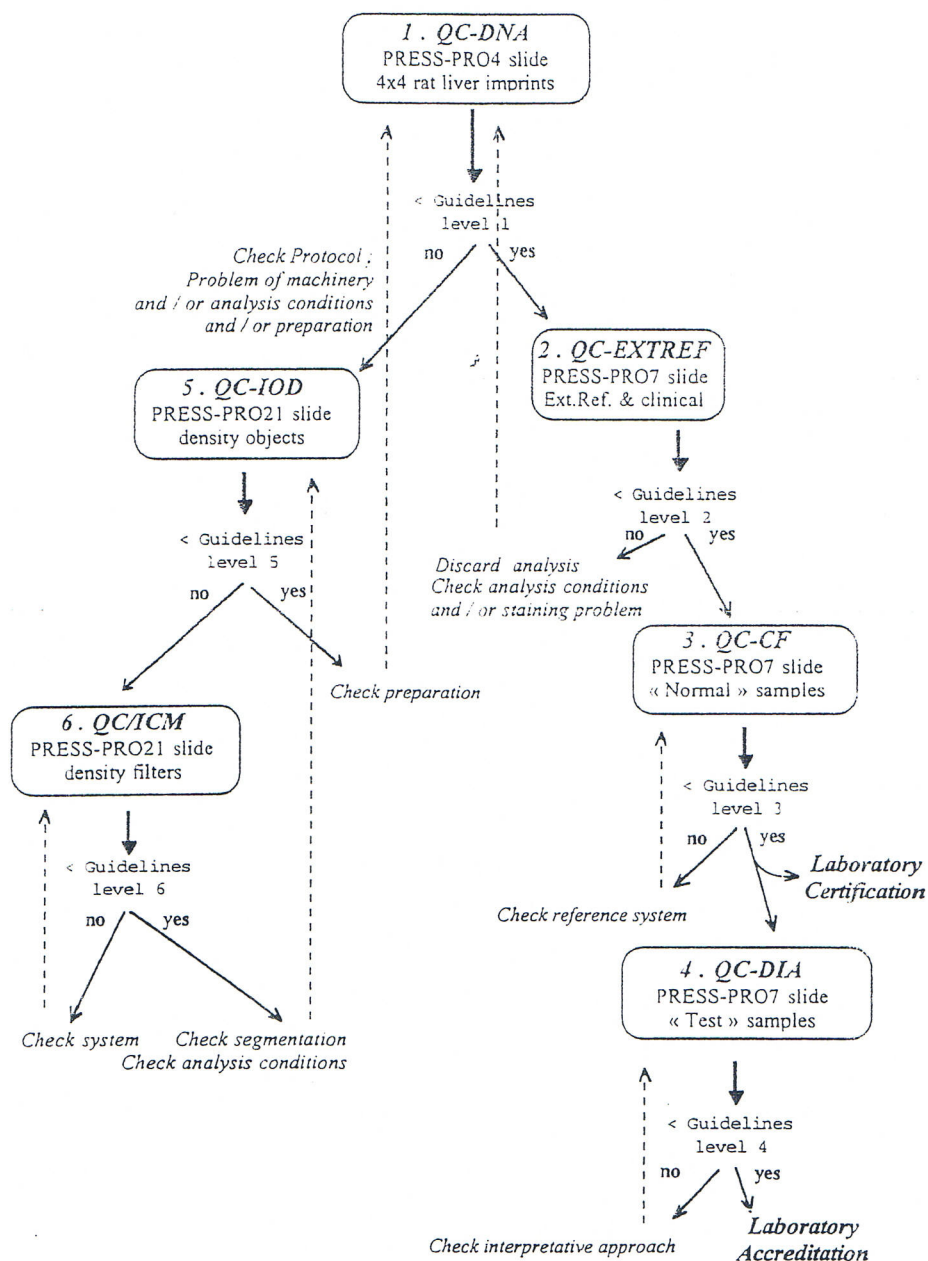
Fig. 1. Flow diagram for use of QC elements.

These quality assurance protocols have been agreed by the task force on DNA image cytometry during the 5th International Congress of the ESACP 1997 in Oslo. The authors are grateful to both users and suppliers of machinery and reagents for hints and advices which can improve the present recommendations.

Quality assurance protocols

Six tests are recommended for quality control by the user: (1) QC for ICM-DNA protocol, (2) QC for preparation stability, (3) QC for corrective factor, (4) QC for diagnostic DNA interpretation, (5) QC for IOD measurements, and (6) QC for ICM instrumentation. In the flow diagram presented in Fig. 1 the feedback mechanisms are demonstrated.

2. QC-DNA: quality control for ICM-DNA protocol

One test has been designed in the frame of the PRESS project [1] to control the quality of the entire methodological process from DNA staining to IOD measurements. It consists of regular measures on biological test slides according to the PRESS-PRO4 slide type (Fig. 2). These slides contain four imprints each from the same rat liver. These test slides should be prepared always with animals of the same species, sex, age, and weight, brought up according to standardised protocols. They may be prepared and stained locally (could be prepared centrally until air drying, but must be post-fixed and stained locally). Four of such test slides should be stained together in the same staining baths, the resulting 16 (4 × 4) imprints should be analysed by the usual DNA cytometry software package. At least 50 cells of each type on each imprint should be analysed.

It is recommended that this test is run at least once a year, and every time a modification in the preparation protocol or in instrumentation has occurred.

The measurements provide information about sample preparation procedure and measurement performance. Resulting information concerns the precision of ANAlysis and REFerence cells peaks, the precision of the ANAlysis/REFerence and ANAlysis-2c/ANAlysis-4c peaks ratio, and the inter-imprint variance within one staining bath.

Guidelines for level 1

- coefficient of variation (CV) of IOD in each peak of any cell type in each of the 4 × 4 imprints <5%;
- coefficient of correlation (r) of nuclear area vs. IOD within any peak $|r| < 0.4$;
- CV of mean IOD values respectively for each cell type (REFerence, ANAlysis-2c and ANAlysis-4c) between the 4 × 4 imprints <3%;
- CV of the ratios of the mean values of the peaks between REFerence and ANAlysis cells of the 4 × 4 imprints <2%;
- CV of the ratios of the mean values of the peaks between tetraploid and diploid analysis cells of the 4 × 4 imprints <1%.

In case of failing the guidelines check preparation (and repeat the QC-DNA protocol) and/or analysis conditions (proceed to QC-IOD protocol) and/or machinery (proceed to QC-ICM protocol).

Impl Imp2 Imp3 Imp4

ANAlysis cells,
e.g. hepatocytes 2c and 4c

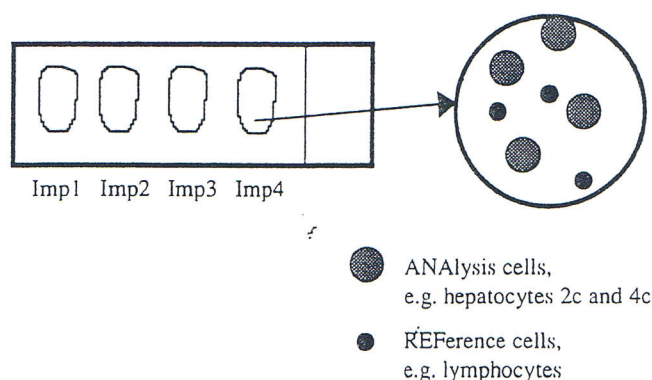REFerence cells,
e.g. lymphocytes

Fig. 2. Schematic representation of a test slide for staining control (PRESS-PRO4 slide type [1]). "EXTernal" reference material, e.g., rat liver, is deposited on four imprints in an order shown above.
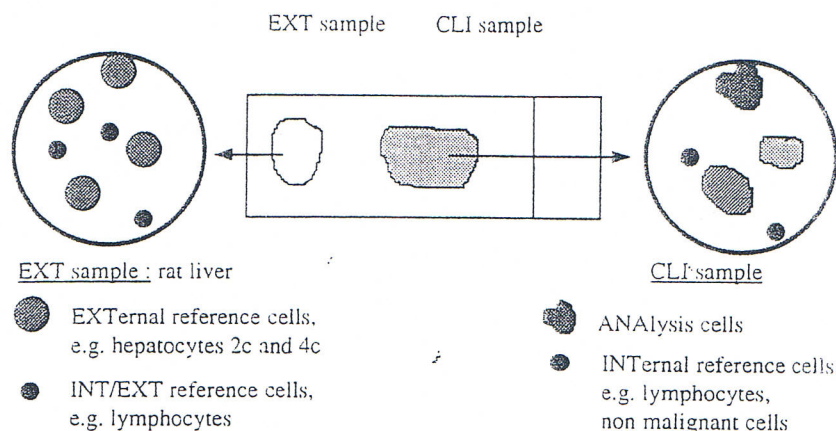
Fig. 3. Schematic representation of a test slide for preparation stability and corrective factors (PRESS-PRO7 slide type [1]). "EXTernal" reference material, e.g., rat liver, is deposited beside the CLInical sample in an order shown above.

3. QC-EXTREF: quality control for preparation stability

Regular day-by-day measures should be performed for keeping the quality of preparation and analysis conditions for each diagnostic sample.

One type of specimen is recommended: "EXTernal" reference material identical to that one used in QC-DNA test, e.g., rat liver. The reference material could be prepared according to the PRESS-PRO4 slide type (one imprint is sufficient) or deposited on the slide together with the CLInical sample according to the PRESS-PRO7 slide type (Fig. 3).

The inclusion and measurement of at least one of these specimens per staining bath is recommended. At least 50 external reference cells should be analysed.

The measurements provide information about day-by-day variations in sample preparation procedure and measurement performance. Results are compared to those one obtained in QC-DNA test.

Guidelines for level 2

- coefficient of variation (CV) of IOD in each peak of any cell type $<5\%$;
- coefficient of correlation (r) of nuclear area vs. IOD within any peak $|r| < 0.4$.

In case of failing the guidelines check the staining conditions and the correct sampling of reference cells. Do not use that specimen or the specimens from the entire staining bath for diagnostic purposes. If the guidelines are failed consecutively check preparation (proceed to QC-DNA protocol) and/or analysis conditions (proceed to QC-IOD protocol) and/or machinery (proceed to QC-ICM protocol).

4. QC-CF: quality control for corrective factor

Using reference cells for rescaling of measurements imply estimation of a corrective factor as already emphasised in Part I as "Basic performance standards". Either EXTernal or INTernal reference cells are used for scaling procedure. In this respect the PRESS-PRO7 slide type is recommended (Fig. 3) including either "EXTernal" reference material or/and "INTernal" reference material depending on the strategy used for calibration. "INTernal" reference material could be routine diagnostic specimens containing DNA-euploid populations and suitable reference cells (e.g., nonmalignant effusions, FNAB from normal

prostate or breast, etc.). The benign character of those specimens must be confirmed by morphological investigations.

The inclusion and measurement of at least one of these specimens per staining bath is recommended. At least 50 reference cells should be analysed.

The measurements provide the essential on-going standard deviation of the so-called corrective factor, necessary for a statistically based diagnostic interpretation.

Guidelines for level 3

Using EXTernal reference sample

- coefficient of variation (CV) of IOD of external reference cells $<5\%$;
- coefficient of correlation (r) of nuclear area vs. IOD of external reference cell peak $|r| < 0.4$;
- deviation of the corrective factor (ratio between the mean IOD values of reference cells and non-malignant diploid cells) from the mean corrective factor for the tissue under investigation obtained under the same laboratory conditions (see guidelines for level 3.3) $<10\%$.

Using INTernal reference sample

- CV of IOD of internal reference cells $<5\%$;
- coefficient of correlation (r) of nuclear area vs. IOD of internal reference cell peak $|r| < 0.40$;
- deviation of the corrective factor (ratio between the mean IOD values of reference cells and non-malignant diploid cells) from the mean corrective factor for the tissue under investigation obtained under the same laboratory conditions (see guidelines for level 3.3) $<10\%$.

For a series of specimens (at least 36) under the same laboratory conditions

- CV of the peak ratios between reference and non-malignant diploid analysis cells of all specimens of the tissue under investigation $<5\%$.

In case of failing the guidelines check the EXTernal or INTernal reference system, especially the correct sampling of internal reference cells, used in rescaling strategy. Do not use that specimen or the specimens from the entire staining bath for diagnostic purposes. If the guidelines are failed consecutively check preparation (proceed to QC-DNA protocol and repeat the QC-CF protocol).

A reference centre has to be established in order to define further the certification procedure. Once a year the test results should be forwarded to this centre for laboratory certification.

5. QC-DIA: quality control of diagnostic DNA interpretation

External, regular measures for testing the diagnostic performance of the laboratory at time intervals dependend on the workload of the laboratory (at least once a year with 10 test slides). Test slides should be provided by the DNA cytometry reference center. They contain biological specimens unknown for the user with usual diagnostic or prognostic problems (e.g., DNA-euploid vs. DNA-aneuploid, degree of DNA-aneuploidy or DNA-ploidy types, etc.).

Guidelines for level 4

- no errors in the discrimination between unequivocal DNA-euploid and DNA-aneuploid cases;
- prognostic DNA-classification according to acknowledged standards.

In case of failing the guidelines check diagnostic approach (proceed to QC-DIA protocol).

The reference centre has to be established in order to define further the accreditation procedure. Once a year the test results should be forwarded to a reference centre for laboratory accreditation.

6. QC-IOD: quality control for IOD measurements

A test has been designed to control the quality of IOD measurements performed on nucleus-like objects, testing object segmentation, densitometric linearity and ratios of IOD measurements [1]. This test consists of rapid and inexpensive measures running the usual DNA cytometry software package with a prototype test slide, the PRESS-PRO21 slide, containing artificial round objects of about 10 μm diameter each having defined optical densities (Fig. 4).

At least 50 objects for each of 8 different densitometric levels between 20 and 80% transmission should be analysed.

This test has to be performed at delivery of the machinery (by the industrial provider) and in yearly intervals by users, unless a problem in measurement precision occurs and each time a new person is running samples on the system.

The variables used for evaluation of these tests are:

- CV of the IOD of objects in each transmission field,
- CV of the area of objects in each transmission field,
- deviation of the measured object area from the real object area (i.e., measured area minus theoretical area) in each transmission field,
- linearity of the mean optical density (MOD = IOD/area) of objects vs. transmission,
- linearity of the measured IOD ratios of objects (with the 50% transmission field as reference field; e.g.: IOD ratio for 80% transmission objects = mean IOD of 80% transmission objects/mean IOD of 50% transmission objects) vs. theoretical IOD ratios.

Guidelines for level 5

- CV of the IOD of objects in each field of different transmission values (from 80 to 20%) <3%;
- CV of the area of objects in each field of different transmission values (from 80 to 20%) <2%;
- deviation of the mean of the measured object area from the real object area in each field of different transmission values (from 80 to 20%) <1%;
- linear regression of the mean of MOD vs. transmission: deviation from slope (=1) <1%;
- linear regression of IOD ratios vs. theoretical ratio: deviation from slope (=1) <1%.

In case of failing the thresholds contact your industrial supplier and/or check segmentation procedure (proceed to QC-IOD protocol) and/or check ICM instrumentation (proceed to QC-ICM protocol).
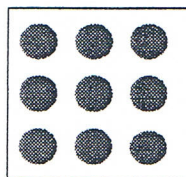

Fig. 4. Schematic view of part of the PRESS-PRO21 slide [1]. Circle pattern – circle: 10 nm diameter; inter-circle: 5 nm. Transmission values in the range: 5%–10%–20%–30%–40%–50%–60%–70%–80%–90%–100%.

7. QC-ICM: quality control of ICM instrumentation

Several tests have been designed in the frame of the PRESS project [1] to control the basic technical characteristics of ICM instrumentation:

- the stability tests for evaluation of random noise and drift during time course,
- the linearity test for evaluation of accuracy of densitometric measurements,
- the shading test for evaluation of non homogeneity of individual fields,
- and glare test for evaluation of light scattering phenomena.

These tests consist of inexpensive measures using defined density filters; the PRESS-PRO21 slide containing density filters is recommended.
The variables to be tested are:

- random noise,
- stability of densitometric measurements during time running,
- linearity of the OD (optical density) measurement,
- homogeneity of the OD measurements within the field of analysis,
- glare.

Reference is given to the final report of the PRESS project [1], where test slides and algorithms for computing such variables, as well as recommendations for users to run the tests have been described. The different tests have to be performed at delivery of the machinery (by the industrial provider) using an appropriate software and in yearly intervals by users, unless an intervening deterioration of the process quality of DNA cytometry deserves an urgent reaction (by the maintenance engineers or by users).

Guidelines for level 6

- stability of grey level values from one pixel during one hour (CV of grey level values <2%);
- stability of grey level values (mean on a whole field) for 8 hours measurement (CV of grey level values <2%);
- linear regression of OD measurements in the whole field of analysis vs. theoretical OD: deviation from slope (=1) <1%, systematic error <5%, standard error at OD 0.3 and OD 0.8 <1.5%, coefficient of regression >0.99;
- linear regression of OD measurements in the 5 windows of the field of analysis vs. theoretical OD: CV of the slopes <2%;
- minimum area accepted for densitometric measurements = 20 μm^2.

In case of failing the guidelines contact your industrial supplier and/or check machinery (proceed to QC-ICM protocol).

8. Conclusions

The above presented recommendations for quality control tests in ICM-DNA are not only thought for users, but are as well important for industrial suppliers of machinery/devices and reagents. Only in a common effort of both users and suppliers will it be possible to achieve high quality ICM-DNA results comparable with respect to precision and accuracy to measurements in clinical chemistry. We

have experienced that the development of QC and QA is a continuous process in which important steps were preceding workshops and projects. Forthcoming ones[1] will have a similar impact.

The principles for quality control recommended above are naturally not restricted to ICM-DNA measurements, but are equally valuable for image cytometry of immunocytochemical reactions, molecular probes, etc. The same is true for assessing changes in nuclear texture as consequences of pathological processes particularly for tumour diagnosis and prognosis. However, for such texture measurements the principles of quality control mentioned above can be only considered as a first step.

References

[1] F. Giroud, 1997 Prototype Reference Standard Slides for quantitative cytometry of nuclear DNA content. Synthesis report. CEE/BCR-DGXII MAT1-CT 930010, September 1996.
[2] G. Haroske, F. Giroud, A. Reith and A. Böcking, 1997 ESACP consensus report on diagnostic DNA image cytometry. Part I: Basic considerations and recommendations for preparation, measurement and interpretation, *Anal. Cell. Path.* **17** (1998), 189–200, this issue.

[1] For example, 6th ESACP congress in Heidelberg, 1999.