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ICSH guidelines for the standardization of bone marrow immunohistochemistry

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SUMMARY

Bone marrow (BM) tissue biopsy evaluation, including trephine biopsy and clot section, is an integral part of BM investigation and is often followed by ancillary studies, in particular immunohistochemistry (IHC). IHC provides *in situ* coupling of morphological assessment and immunophenotype. The number of different IHC tests that can be applied to BM trephine biopsies and the number of indications for IHC testing is increasing concurrently with the development of flow cytometry and molecular diagnostic methods. An international Working Party for the Standardization of Bone Marrow IHC was formed by the International Council for Standardization in Hematology (ICSH) to prepare a set of guidelines for the standardization of BM IHC based on currently available published evidence and modern understanding of quality assurance principles as applied to IHC in general. The guidelines were discussed at the ICSH General Assemblies and reviewed by an international panel of experts to achieve further consensus and represent further development of the previously published ICSH guidelines for the standardization of BM specimens handling and reports.

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1. BACKGROUND INFORMATION, RATIONALE, AND AIMS

Immunohistochemistry (IHC) standards have been evolving during the last few decades as the clinical needs for standardization for IHC laboratory testing have been rising. In particular, IHC testing that provides prognostic and predictive information important for stratification of patients for specific therapies is at the forefront of IHC standardization [1–5]. Although no systematic studies are available to date, it is generally assumed to be correct that false IHC results, related to technical and interpretative pitfalls, may lead to incorrect diagnosis [4, 6]. The risks to patient safety are lower when IHC tests' results are interpreted as a part of the panel and in conjunction with other information (clinical history, morphology, flow cytometry, biochemical testing, etc.) [7]. The need for standardization has been further supported and enhanced by evolving programs for external quality assurance/proficiency testing (EQA/PT) often coupled to requirements for laboratory accreditation. However, bone marrow (BM) IHC, as applied to BM core biopsy specimens and BM aspirate clot sections, is largely unaffected by this trend. In contrast to anatomic pathology, where almost all laboratories fix tissues in formalin (most often in 10% buffered formaldehyde), many laboratories have developed their unique protocols for tissue processing of BM core biopsies as well as aspirate clot preparations [8–12]. Pre-analytical conditions are critical for the IHC outcomes. As long as we cannot standardize pre-analytical conditions, complete standardization of BM IHC will continue to be impossible. The use of different fixatives, unique and variable fixation times, as well as the use of various decalcifying reagents and variation in time for decalcification multiply this problem in BM tissue processing [8]. It is clear from published literature, extensive global experience, and this group's

experimental results (partly included in Appendix) that there is more than one tissue processing protocol (various combinations of various fixatives and decalcifying reagents with significant time range for both fixation and decalcification) that may produce excellent results for IHC testing, as well as good quality of DNA and RNA. However, the large number of protocols prevents standardization, may create difficulties for external consultation/review, and hinders development of EQA [8]. This is similar to anatomic pathology (AP) where fixatives other than formalin are likely to show good or even better performance, but the AP community worldwide selects to use 10% buffered formalin as their primary choice of fixative. Therefore, narrowing choices for fixation and decalcification appears to be one of the most important mandates in the process of developing BM IHC standards.

It is the lack of standardization of the pre-analytical component that prevents building EQA/PT programs for BM IHC because EQA programs cannot provide large number of tissue samples with different pre-analytical conditions that would parallel those methods used in clinical IHC laboratories.

The published literature was the primary basis for the preparation of the recommendations in this study. When such literature was not available, the recommendations were based on the expert consensus. Guidelines summarized in this article do not include proposed IHC panels for workup of various BM diseases/lesions, but rather are focused on technical and other general issues related to BM IHC. Guidelines that include proposed panels for different disorders primarily focus on biology of disease and their diagnostic sensitivity and specificity [9–15]; indirectly, such recommendations also depend on numerous technical parameters that are addressed here.

Although standardization of BM IHC is not fully achievable at this time, it is entirely relevant to define which components can be standardized at present and which components can be harmonized now and possibly standardized in the future.

The aim of these guidelines for BM IHC standardization is to define BM IHC parameters relevant to standardization, set the stage for development of BM IHC EQA/PT, and to develop relevant framework for BM IHC performance characteristics.

2. RECOMMENDATIONS

2.1. IHC test classification

Inspired by The US Food and Drug Administration (FDA) classification of IHC reagents and kits based on the 'risk assessment' and 'level of concern', the Canadian Association of Pathologists (CAP-ACP) developed classification of IHC tests based on the 'risk assessment' and 'level of concern', which was published in 2010 [3]. If the IHC results are to be used by the pathologists, they are classified as Class I (lesser risk to the patient safety) and, if they are to be used by clinicians (higher risk to the patient safety) for stratification for different targeted therapies or any other clinical decision (e.g., the result of IHC testing warrants further genetic testing), they are classified as Class II [3].

The relationship between FDA classification of IHC devices (Class I–III) and CAP-ACP classification of IHC tests (Class I and II) was recently addressed by Torlakovic *et al.* [16]. Although both classifications are based on their intended use and on patient safety/potential to harm principles, the CAP-ACP classification emphasizes that the patient safety/potential to harm are ultimately based on not how the industry markets their products, but on how pathologists and other physicians use the results of IHC testing irrespective of FDA ruling, as follows:

Class I: IHC test results are both interpreted and used by (hemato)pathologists:

- Qualitative IHC tests, which are used for diagnostic purposes; often used for determination of cell lineage.
- Validation design is determined by the medical director of the IHC laboratory.
- Test performance characteristics are descriptive, usually produce categorical data/results and are generally interpreted as 'positive' or 'negative'.

Class II: IHC test results are interpreted by (hemato) pathologists; however, these tests have either prognostic or predictive nature and their results are used by treating physician (oncologists or other nonpathologist physicians, who use this information for stratifying the patients for appropriate therapies or other significant clinical decision-making that may result

in either different treatment approach or different further workup of the patient).

- Qualitative and quantitative IHC used as prognostic and/or predictive markers.
- Validation design based on published guidelines for each marker.
- Test performance characteristics include sensitivity, specificity, reproducibility, and repeatability, and the results may be interpreted as descriptive/qualitative (positive *vs*. negative) or (semi) quantitative (% positivity or H-score or other results of a specific scoring system are reported to treating physicians).
- At this time, only a few Class II IHC markers may occasionally be relevant to BM IHC including breast cancer markers in metastatic breast cancer, ALK-1 in metastatic lung cancer, or NPM-1 when molecular studies may not be available [2, 17, 18]. However, consideration of IHC test class is important for test development by the medical director in the clinical laboratory, test development for clinical trials, as well as researchers using IHC testing of BM samples.

Recommendation 1. CAP-ACP classification of IHC tests into Class I and Class II is recommended to properly design and follow QA requirements relevant to patient safety. Due to higher risk for patient safety, Class II IHC tests need higher level of QA measures, as specifically recommended below.

Recommendation 2. All Class II IHC tests that are used for stratification of patients for definitive targeted therapy need to follow national and/or international guidelines for recommended protocol validation, re-validation, and daily QC, if available.

Recommendation 3. If national and/or international guidelines are not available, it is recommended that the medical director prepare design for validation and re-validation of Class II markers, according to published guidelines for validation [4].

Recommendation 4. If national and/or international guidelines are not available, the Class II IHC tests need to be run with calibrated positive control and reagent negative controls. Calibrated positive controls need to include negative tissues, weakly positive tissues, and strongly positive tissues. For rare disease or marker of low frequency in tissues, it may be difficult or impossible to obtain sufficient tissues for finely calibrated controls; when this is the case, it is a role of medical director to document why calibrated controls are not used. It is recommended that (calibrated) controls are placed on the same slide as the patient's sample (so-called 'on-slide' controls).

Recommendation 5. Reagent negative controls are generally not recommended for Class I IHC tests and are recommended for Class II IHC tests. However, published guidelines for the use of negative controls should be followed whenever possible [16]. Regent negative controls are run on a separate slide without specific primary antibody (Ab) as per reference 16.

2.2. Pre-analytical standards

Pre-analytical standards include standards relevant to the pre-analytical phase. The pre-analytical phase starts at the time of procurement of the BM trephine and aspirate samples and ends by cutting the paraffin-embedded (or plastic-embedded) tissue onto glass slides. This phase includes following components: (i) ischemic time, (ii) type of clot sample preparation, (iii) fixative and fixation time, (iv) type of decalcifying reagent and time of decalcification, (v) embedding media, and (vi) unstained glass slides with tissue. See Section 3 for special considerations relevant to pre-analytical phase.

Recommendations relevant to ischemic time

See Section 3 for special considerations relevant to ischemic time.

Recommendation 1. Containers with fixative need to be included in BM procedure sets so that the sample can be placed in the container with fixative immediately. It is imperative that the ischemic time be predictable and very short, and therefore, the samples need to be placed in fixative at the bedside.

Recommendation 2. It is recommended that BM biopsy imprints (touch preps) be prepared at the bedside by trained professional (a trained professional assisting the procedure or physician who performs the procedure) before the sample is put in the container with the fixative.

Recommendation 3. If transportation of unfixed sample is required, the transportation time of the sample to the laboratory needs to be monitored, and it should be as short as possible.

Recommendation 4. During transportation, dehydration of the BM trephine biopsy needs to be prevented by transporting the sample in a closed container with physiological saline, PBS, or RPMI. Kendall Telfa® (Covidien, MN, USA) or similar nonstick pads are recommended to be placed in the container with the sample. No other transportation medium is recommended.

Recommendation 5. If vented hoods are used for preparation of BM trephine biopsy imprints (of the transported sample), dehydration and loss of the sample in vented hoods due to vacuum forces used for increased air flow need to be prevented.

Recommendations relevant for preparation of clot sample

Recommendation 1. The least possible amount of blood should be included in the clot sample. This allows for more rapid penetration of the fixative and also facilitates cutting of the embedded samples. Although methods of clot section sample preparation may vary in the total amount of clotted blood included in the sample and the clot-/gel-forming medium, only those methods that enable 'clean' collection of BM particles are recommended (e.g., hour-glass dish with manual collection of BM fragments) [19].

Recommendations for fixative and fixation time

See Table 1 for summarized recommendations. It is suggested that for practical reasons, TAT be defined as short, intermediate, and standard. This approach is entirely applicable even for future purposes when new reagents of higher quality may replace currently used reagents. See Section 3 for special considerations relevant to fixation.

Recommendation 1. Heating and stirring both improve fixation and should always be considered.

Recommendation 2. Either 10% buffered formalin (6–72 h of fixation before decalcification) or AZF (Glacial acetic acid, <1%; formaldehyde, 5.6% zinc chloride, 3%) (2–72 h of fixation before decalcification) are recommended.

Recommendation 3. Selection of fixative depends on the desirable turn-around-time (TAT).

Recommendations for decalcification

See Table 1 for summarized recommendations. See Section 3 for special considerations relevant to decalcification.

Recommendation 1. Heating and stirring both improve decalcification and should always be considered.

Recommendation 2. It is recommended that the selection of the type of the decalcifying reagent is based

Table 1. Recommended protocols for bone marrow (BM) fixation and decalcification

Turnaround time (TAT)*	Fixative	Fixation time	Decalcification	Decal time	Comments
Very short TAT	Acetic acid–zinc–formalin (AZF)	2–72 h†	Shandon™ TBD-1™ Decalcifier	30–40 min†	Whenever possible, longer fixation (within the range) is preferred
Intermediate TAT	AZF	Overnight	Gooding and Stewart's decalcification fluid (10% formic acid and 5% formaldehyde)‡	6 h	So-called 'Hammersmith Protocol'
Standard TAT	10% buffered formalin = 3.7% formaldehyde	8–72 h (overnight fixation is preferred)†	14% EDTA	16–24 h†	Preferred protocol for BM biopsy fixation and decalcification

*Consideration of agitation and warming to 37 °C of the decalcifying solutions are recommended for each protocol. Ultrasonic decalcification may also be employed. These methods were shown to significantly shorten TAT.
†The timing may vary based on ancillary use of stirrers, ultrasound energization, microwave or other heating methods, or their combination.
‡Although decalcifying fixative is not recommended to be used alone, decalcifying fixative can produce superior results when used after the BM biopsy was already properly fixed in AZF or formalin.

on allowable TAT as per Table 1. However, shorter TAT methods are not recommended if additional samples for special studies are not available (e.g., separate sample for flow cytometry, molecular studies, and cytogenetic studies) (Figure 1).

Recommendation 3. Decalcification should be followed by careful rinsing of about 10 min to remove decalcification reagent [20, 21].

Recommendation 4. Combined decalcifying fixative solutions are not recommended [21, 22].

Recommendation for embedding

See Section 3 for special considerations relevant to embedding.

Recommendation 1. Embedding in paraffin is recommended.

Recommendations for cutting

Recommendation 1. Whatever embedding and cutting methods are employed, sections must be sufficiently and evenly, thin (generally 2–3 μm) to allow high-quality morphological assessment including cytological evaluation using an oil-immersion lens with × 100 magnification [19, 23, 24].

Recommendation 2. Cutting at a right angle to the long side of the BM cores may decrease some artifacts due to inconsistencies of successive bony trabeculae and marrow spaces compared with cutting parallel to the long side.

Recommendation 3. Unstained slides should be stained as soon as possible or stored at −80 °C individually wrapped in aluminum foil to prevent antigen deterioration at room temperature [25]. For detailed recommendations regarding storage and shipment of unstained slides, see reference 25.

2.3. Analytical standards

The analytical phase pertains to protocols used for IHC staining including antigen retrieval, primary antibody incubation, incubation with detection system, color development for visualization of immunological reaction(s), counterstaining, and it ends with coverslipping. The desired results drive antibody clone selection as well as calibration of the entire protocol by

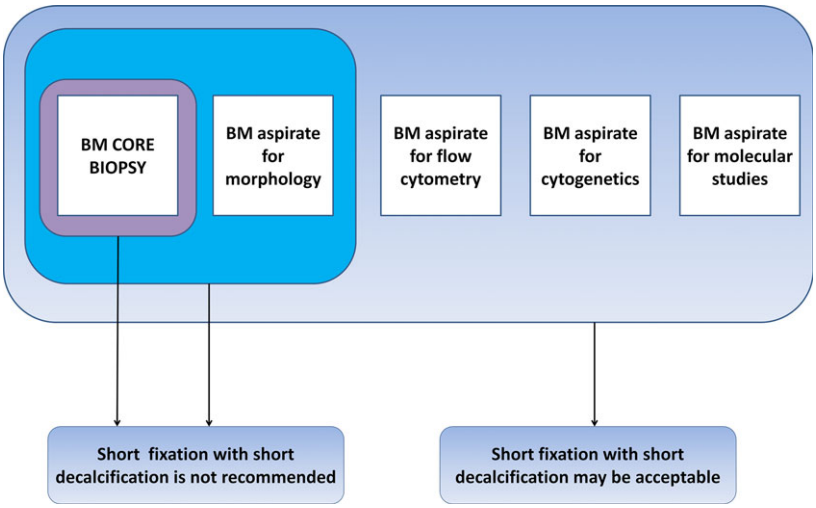

Figure 1. Bone marrow sampling and selection of tissue processing protocol for BM tissue biopsy. Depending on the type of BM samples, short protocol with suboptimal preservation of DNA, RNA, and protein may be acceptable. The selection of the method (short BM TAT *vs*. regular BM TAT) depends on the institutional practice. If BM tissue biopsy +/− smears for morphology are not regularly complemented by additional samples for molecular, cytogenetic, and flow cytometry studies, short BM TAT is not acceptable as diagnostic DNA and RNA testing may not be possible on tissues exposed to harsh acid decalcification. If such additional samples are collected and available to hematopathologists, short TAT protocols may be acceptable.

tweaking various parameters amenable to modification (temperature, time, pH, etc.). As the pre-analytical phase cannot be absolutely controlled and therefore cannot be entirely standardized (acceptable ischemic time is defined as maximum time with a range of T_0–T_{max}, and similarly, there is also an acceptable time range for fixation and decalcification), and this combined with multiple options for selecting other reagents and equipment as well as changes in equipment performance or primary antibody deterioration may lead to changes in IHC protocols to maintain desired sensitivity and specificity of the results. See Section 4 for special considerations relevant to the analytical phase.

Recommendation 1. Immunohistochemistry protocols need to be validated, that is, designed as such to reflect optimal calibration of sensitivity and specificity of the IHC test for particular use depending on the biology of the tested IHC marker and its ultimate use [4]. This assumes that performance characteristics of each IHC test, irrespective of its class, are defined before introduction of the protocol to clinical use.

Recommendation 2. Monitoring of protocol performance characteristics using appropriate calibrated controls is recommended.

Recommendation 3. Separate standard operating procedures (SOPs) for each IHC test that is performed in the laboratory need to be developed, as every IHC test is a different test. All essential components of IHC protocols for each IHC test need to be included in the SOPs (Table 2). This information must be readily available for review for all IHC laboratory staff. The protocols must be regularly updated. Updating of SOPs is required at the time any changes in the protocol are introduced. In addition, periodic review of SOPs is recommended as per laboratory accreditation requirements.

Recommendation 4. To facilitate methodology transfer, when BM IHC results are published (research, case reports, etc.), all information included in SOPs (as shown in Table 2) is recommended to be included in the 'Methods' section.

Table 2. SOPs components required to be specified for each bone marrow immunohistochemistry tests

SOP component	Descriptors
Primary antibody (Ab) type	Monoclonal *vs*. polyclonal, clone or lot name/number, source, concentrated *vs*. prediluted, and dilution (if concentrated Ab), incubation time, temperature
Antigen retrieval method	Type, pH, concentration (for enzyme-based methods), temperature, time, and source
Detection system	Type, name, temperature, time, source
Amplification	Type, temperature, time, source
Chromogen	Type, time
Enhancement	Type, time
Automated stainer platform	Name, source

2.4. Postanalytical standards

Postanalytical standards pertain to the interpretation of the IHC results by the (hemato)pathologist. See Section 5 for special considerations relevant to the postanalytical phase.

Recommendation 1. All BM aspirate results should be available to (hemato)pathologists directly, that is, they need to evaluate them and sign them out, or indirectly, that is, results of cytological evaluation of BM smears, molecular studies, and cytogenetic studies are submitted to (hemato)pathologists who are evaluating BM tissue biopsy optimally before they are made available to other physicians.

Recommendation 2. Interpretation of IHC results should be performed in consideration of the sample adequacy. Proper sampling of the BM cannot be overemphasized. Although IHC is a powerful technique and may help detect pathological cells even when they are present in small numbers and not apparent morphologically, standardization and excellence in BM IHC cannot replace proper BM sampling [26]. If the BM sample does not contain the lesion, no special studies could compensate for the deficiency of proper sampling [27–30]. Therefore, general guidelines for BM biopsy should be followed in the first place [19, 23, 24, 31–33].

Recommendation 3. It is recommended that the interpretation starts with the evaluation of the external control(s), by which it is determined that the proper antibody was applied and the test is properly calibrated.

Recommendation 4. Evaluation of the patient sample starts with detection and evaluation of an internal positive control if such is present. Detection of nonspecific staining should be observed if present.

2.5. Quality assurance standards

Quality assurance (QA) standards pertain to the following: positive and negative controls, participation in PT provided by EQA programs, use of flow cytometry (FCM) to validate IHC, and training and education. See Section 6 for special considerations relevant to QA standards.

Positive and negative controls

Published guidelines relevant to external control selection, design, and use should be followed whenever possible [34].

Recommendation 1. Selection of external control samples needs to reflect desired level of analytical sensitivity and specificity (calibration), which depends on definitions set by the end user (hemato)pathologist and/or medical director.

Recommendation 2. External positive controls need to contain areas with at least two different levels of expression of the target epitope (weak + strong) as well as expected negative areas. Tissue microarray (TMA)-based positive external controls can be used, but are not required for daily QC.

Recommendation 3. Multitissue controls or TMA controls may include a mixture of cores from human tissues, cell-line cell blocks or xenograft tissue. If cell lines are used, positive cell lines could be mixed with negative cell lines to provide expected negative areas.

Recommendation 4. Any exceptions and alterations in the use of positive controls that deviate from general principles of control design and use need to be qualified and approved by the medical director.

Recommendation 5. Tissue processing of control samples needs to be controlled to closely replicate tissue processing of the patients' samples. Importantly, a decalcification step is required for control samples even if they do not contain any bony tissue. Patients' samples have defined range of acceptable fixation and decalcification times. As it is not possible to replicate all potential variations for the acceptable time range in tissue processing, controls could be processed using most frequently used time of fixation and decalcification, but there should be no deviation in the type of fixative and decalcifying reagent.

Recommendation 6. Whenever possible, external controls (multitissue or single tissue) should be mounted on the same slide as patients' samples (so-called 'on-slide controls').

Recommendation 7. BM tissue biopsy often contains elements of normal hematopoiesis and includes many epitopes that are evaluated in the lesional tissue (internal positive control). Internal positive controls should be evaluated before interpretation of IHC test is conducted for the diseased tissue whenever present.

Recommendation 8. When internal controls are present in the BM tissue biopsy, they cannot fully substitute for external controls for some epitopes. When the expression is normally present, the levels of epitope expression in benign BM tissue are usually predictable (useful), but the range is usually limited (not useful).

Recommendation 9. Negative reagent controls are obligatory when biotin-based detection systems are used [16].

Recommendation 10. Negative reagent controls are recommended to be ordered by (hemato)pathologist: (i) when the patient's sample contains internal pigments that may interfere with interpretation of results; (ii) when there is tissue necrosis or dense tissue fibrosis, and (iii) when only one IHC test has been ordered. Standards relevant to negative controls for IHC are detailed elsewhere [16].

Participation in proficiency testing (PT), provided by external quality assurance programs (EQA)

Recommendation 1. It is recommended that laboratories participate in PT schemes for BM IHC only if the EQA program provides samples with identical or nearly identical BM tissue processing. It is not recommended that laboratories participate in PT for BM IHC if the EQA provider does not provide samples with identical or nearly identical BM tissue processing.

Use of flow cytometry to validate IHC

Recommendation 1. If FCM is available and the disease state is such that it enables straight forward interpretation of IHC results (i.e., diffuse involve-

ment of the BM trephine biopsy by lymphoma or leukemia), corresponding IHC testing can be evaluated for QA purposes to compare the number of cells and the levels of expression between the two methods. Therefore, when possible, it is recommended to utilize FCM for QA of BM IHC for commonly used markers.

Education and training in IHC

Recommendation 1. Education for both technologists/laboratory scientists and pathologists alike should include (i) learning the theory of IHC methods and the theory of antigen distribution in normal and diseased tissues, (ii) practical laboratory aspects of assays by manual and automated methods, and (iii) practical aspects of interpretation of IHC results in control tissues and patients' samples. All of the above are required components of education in IHC for both technologists/laboratory scientists and pathologists, but different areas are emphasized by each group.

3. SPECIAL CONSIDERATIONS FOR PRE-ANALYTICAL PHASE

Pre-analytical standards are relevant to both, immunohistochemical methods as well as histochemical methods used for evaluation of BM biopsies. The importance of BM pre-analytical conditions for evaluation of BM core biopsy was highlighted by Buesche *et al.* by showing that pre-analytical conditions of fixation, decalcification, embedding, and marrow tissue shrinkage during biopsy processing, and even the thickness of marrow sections significantly impacted results of diagnosis and quantification of myelofibrosis by Gomori silver impregnation in patients with primary myelofibrosis [35]. These guidelines emphasize the need for radical reduction in BM tissue processing methods because: (i) there is no evidence that many different methods are required for tissue processing of BM samples; (ii) standardized tissue processing of BM tissue could minimize potential technical problems with IHC testing of external BM samples (second opinion, oncology reviews, etc.), and (iii) a limited number of applied methods will enable development of BM IHC EQA/PT. Methodology transfer of IHC testing also depends not only on published analytical parameters, but also on pre-analytical parameters. Many published studies report IHC results on BM tissue biopsy introducing new analytical parameters (e.g., new application of previously widely used antibody, novel antibody (Ab) clones, new antigen retrieval, or new detection systems, etc.) performed on BM tissue biopsy samples that were processed by their unique pre-analytical conditions that cannot directly apply to many diagnostic IHC laboratories [36–38]. Because the pre-analytical conditions are critical for IHC testing, each diagnostic IHC laboratory that would like to develop such new testing often cannot use published methods for methodology transfer, but need to develop protocols independently.

These guidelines make an attempt to reconcile TAT demands and acceptable quality of the BM tissue biopsy samples. Members of the ICSH BM IHC Standardization Working Party agreed that for the great majority of BM core biopsy samples, there is no clinical need for rapid TAT. Further, for most BM core biopsy samples, shorter protocols that are not optimal for DNA or RNA preservation might be acceptable when other types of samples (for FCM, cytogenetics/FISH, and molecular studies) are available (Figure 1). Therefore, recommendations for fixation and decalcification are dependent on acceptable TAT as well as institutional practices regarding collection of samples for molecular, cytogenetic, and FCM studies at the time of BM tissue biopsy. If the most optimal BM work up is desirable, including very short TAT, BM sampling needs to include additional aspirate samples for FCM, molecular, and cytogenetic studies, and these studies need to be under the constituency (control and oversight) of hematopathologists. This may not be achievable even if there is institutional commitment to provide samples of this type because: (i) samples for FCM, molecular, and cytogenetic evaluation may have been submitted to immunologists, molecular scientists, and cytogeneticists, who do not report to hematopathologists, or the results of these studies are instead independently released to or evaluated by hematologists instead of hematopathologists, or (ii) there is BM fibrosis or other pathological conditions that results in so-called 'dry-tap'. When this happens, BM tissue biopsy/core biopsies that have not been fixed in formalin and decalcified in EDTA may not be optimal (see Figure 1).

Ischemic time

The effects of cold ischemic time are important for IHC results even if decalcification is not performed like in clot sections [39–43]. Recommended approach, with partial tissue processing of the BM aspirate and biopsy sample at the bed site, assumes availability of technical help and expertize for preparation of BM biopsy imprints and BM aspirate smears at the bedside. This is the optimal approach and ensures preservation of sample integrity. Due to practice protocols or lack of funds in some institutions, the transport of unfixed samples to the laboratory for further processing, of both trephine biopsy and aspirate samples, is being used as an alternative approach. This is a suboptimal approach that introduces ischemic time. Degradation of protein epitopes relevant to IHC as well as degradation of RNA and DNA integrity is created by delayed fixation as a significant parameter in laboratory testing potentially leading to false-negative results not only by IHC, but also for molecular diagnostics.

Fixation

Insufficient/incomplete/uneven fixation may lead to zoning phenomenon, resulting in areas with absent or suboptimal staining; zoning phenomenon should be considered and recognized at the interpretation stage (postanalytical phase) to prevent misdiagnosis.

Although 10% buffered formalin is widely available and its fixation effects on protein, DNA, and RNA are well understood, AZF was found to provide some greater flexibility for workflow, eliminate labor-intensive steps, save processing time, and improve turnaround time [44–47]. It is essential that fixation time needs to be adjusted to the type of fixative chosen; 10% buffered formalin optimally requires 18 h of fixation, but good results for IHC may also be achieved with shorter fixation even for unstable markers [45, 47, 48]. Fixatives with protein precipitant formulation require shorter time of fixation [46].

There are large numbers of fixatives for BM trephine biopsy and clot preparation that continue to be in use in various institutions [8]. There are also several studies showing that some or many of IHC tests could be performed in BM tissue biopsy fixed in different fixatives. For example, Gala *et al.* [49] showed that there is number of antibodies that can be optimized for BM trephine biopsies that are fixed in Bouin's solution. However, Bouin's solution has very low pH, contains picric acid, which can be explosive, and is sensitive to friction and shock when dry. Further, a recent study showed that fixation in Bouin's solution did not provide IHC results comparable to those obtained with formalin [50]. Fixatives containing mercuric chloride like Zenker's fixative and B5 were also widely shown to be suitable for IHC studies [51–57], but are toxic; their disposal as hazardous waste is costly, and these reagents are not allowed in many countries at this time.

Decalcification

It was already emphasized in 1987 that knowledge of the effects of the various decalcifying agents on the immunoreactivity of cellular antigens is essential for IHC analysis of lesions in calcified tissue [58]. This early study showed that overall EDTA-based decalcification was better for IHC analysis, but also that several differentiation antigens can be detected even in specimens decalcified in strong acid solutions.

Strong mineral acids, such as 10% hydrogen chloride (HCl), or weak organic acids, such as 5–10% formic acid (HCOOH), form soluble calcium salts in an ion exchange that moves calcium in the decalcification solution. Similarly, 10–14% ethylenediaminetetraacetic acid (EDTA) sequesters calcium in aqueous solutions as a chelating factor [20, 21]. Although degradation of proteins that are detected by IHC may have different kinetics than that of DNA, and RNA, current state of practice requires that recommendations for the IHC pre-analytical methods also take into account DNA and RNA integrity. Published evidence suggests that various fixatives and decalcifying agents may be acceptable for preservation of protein antigens, DNA and RNA. However, proper timing of fixation and decalcification is important, and some agents appear to be ultimately superior to others in achieving optimal preservation of the tissue, but the time they require to achieve this may not be compatible with local patient care requirements. Decalcification is best achieved by 14% EDTA that sequesters metallic ions, including calcium, in aqueous solutions as a chelating factor for 16–24 h [38, 59–62]. Although a recent study showed that EDTA and formic acid both perform equally regarding preservation of DNA and RNA,

only effects achieved after 2 h of decalcification with formic acid were studied, which is insufficient for clinical practice [63]. Similarly, another study evaluated effects of SurgiPath Decalcifier II after 24 h, while this particular reagent is more likely to be used for 2–4 h of decalcification in clinical practice as very small samples can be decalcified in <4 h [64].

Therefore, if institutional TAT consideration allows for longer procedures, 14% disodium-EDTA dihydrate is recommended as tissues decalcified in such manner are superior and allow obtaining high-quality protein, DNA, and RNA. The timing may vary based on ancillary use of stirrers, ultrasound energization, microwave or other heating methods, or their combination. In 1958, a systematic study of rate of decalcification using different decalcifying reagents including 5% nitric acid, trichloroacetic acid, and 20% formic acid showed that increasing temperature from room temperature to about 37–40 °C shortens the time about 30–40% and using agitation at RT decalcification is again shortened about 30% [65]. Similarly, newer studies show that excellent results are achieved by shortening EDTA decalcification using a magnetic stirrer in a microwave or hot plate at 37–40 °C [66–69]. Acceleration of the decalcifying process can be achieved by using ultrasound energization at lower temperatures (e.g. 18 °C), which reduces the decalcification time to approximately 6–12 h or, in another study to as short as 2 h [70–72]. Confirmatory studies of the significantly reduced EDTA decalcification time by magnetic stirring in a hot plate or microwave, or ultrasonic energization applied to human bone marrow in diagnostic setting are rare [60]. Currently, if institutional policies ask for 1-day TAT, decalcification of the BM could be carried out after short fixation by AZF using strong mineral acids, such as 10% hydrochloric acid (HCl), or weak organic acids, such as 5–10% formic acid (HCOOH), or similar commercially available solutions; the acids form soluble calcium salts in an ion exchange that moves calcium in the decalcification solution. Formic acid was found suitable for FISH and CGH studies [73]. However, the acid strength and a time of decalcification matters, and 5% formic acid was better than 10% formic acid, and time of <24 h was better than decalcification in excess of 24 h [73]. Also, 10% formic acid was found to preserve protein antigens, DNA, and RNA when used after acetic acid–zinc–formalin fixative as a part of the Hammersmith Protocol [44]. Commercial short decalcifying solutions with decalcification of 1 h or less were found acceptable for frequently used IHC tests (Appendix). In one study, greater success of FISH was achieved when a rapid decalcifier was combined with B5 fixative *vs*. formalin (77% *vs*. 53%) [74]. Although B5 fixative provides good morphology and protein preservation, B5 fixative cannot be not recommended primarily because of environmental concerns, and nucleic acid studies are not recommended on B5-fixed biopsies [75, 76]. Therefore, AZF that allows greater flexibility in fixation times, eliminates labor-intensive steps required for B5 processing is recommended for short TAT protocols instead of either B5 or formalin [46]. AZF fixation was shown to have staining and morphologic detail comparable to B5 and achieved equivalent or superior antigen preservation for IHC studies in the previous studies as well as in the current study (see Appendix). Although 10% buffered formalin is an excellent fixative, it is not recommended for short protocols; optimal fixation will not be achieved with fixation of <8 h, and decalcification of suboptimal fixed BM will further contribute to loss of epitopes (see Appendix).

Shorter protocols for decalcification are more aggressive, which requires that the BM tissues be properly fixed before decalcification and that the time of decalcification is precisely monitored not to exceed the time that is validated for this purpose. Also, in short protocols, it is critical that all reagents are fresh (e.g., commercially available rapid decalcifying reagents will not perform optimally if diluted by large number of samples or if re-used; supplier recommendations regarding recommended volumes and the possibility of re-use should be followed closely). If the core biopsy is not properly decalcified, so-called 'surface decalcification' or 'decalcification on block' is likely to be performed by histotechnologist during cutting of the block, the results of which cannot be fully predicted or controlled, but can be deleterious to some epitopes [20, 21, 77–80]. Decalcification with simultaneous fixation is not recommended. In most of such combinations, acid starts working before the tissue is properly fixed [21, 22]. However, new decalcifying solutions are being developed, and some may be less deleterious to bone marrow tissue even after short fixation [31].

After fixation and decalcification, the tissue needs to be further processed before embedding in paraffin.

The most important problem is inadequate tissue dehydration and clearing prior to paraffin embedding. This can be prevented by preparing all solutions freshly every week, depending on the volume of tissue processed [47].

Embedding

BM trephine biopsy could be embedded in paraffin or in plastic. Both methods have certain advantages and disadvantages (reviewed in references 76 and 77). Under optimal circumstances, morphology is reported as superior in plastic-embedded tissue [81, 82]. However, there is experiential evidence of excellent morphology with appropriate fixation time and thin sectioning (2 μm) of tissue for formalin, B5, and AZF fixatives, and the need for embedding in plastic was challenged in 1987 by Gatter *et al.* [83]. With proper optimization, most special in situ and molecular studies are at least equally amenable to both embedding media as shown in many studies [84–91]. Plastic embedding techniques may obviate the need for the decalcification step and thus eliminate one of the major variables impacting pre-analytical bone marrow IHC quality. However, only a few centers still cultivate expertise required for plastic embedding. Without a resurgence of interest and developmental efforts leading to broader use of plastic embedding, paraffin embedding will likely remain the method of choice in the great majority of laboratories. If plastic embedding could be automated and cost-effective, it could become a preferred choice for embedding of all types of tissue biopsies, not only bone marrow samples.

4. SPECIAL CONSIDERATIONS FOR ANALYTICAL PHASE

The ICSH BM IHC Working Party testing showed that, although many pre-analytical factors show definite effects on preservation of epitopes and success of BM IHC, superior IHC protocols may compensate, at least partly, for unfavorable pre-analytical conditions (see Appendix).

Protocol design

Development of IHC protocols has to be designed not only for bare detection of the epitope of interest, but specifically for how the results of the particular IHC test are used, which drives selection of appropriate clones as well as calibration of the protocols (e.g., ALK-1 IHC in anaplastic large cell lymphoma *vs*. ALK-1 IHC in lung cancer). Therefore, protocols need to be tailored to biologically unique applications. Most IHC tests, including BM IHC, are laboratory-developed tests. As a consequence, each laboratory that is using IHC for clinical purposes needs to define test performance characteristics that are relevant to IHC.

Optimal calibration (analytical sensitivity and specificity) is descriptive for Class I IHC tests. As example, calibration of the CD117 IHC test is descriptive as follows: CD117 needs to be clearly detectable in myeloid and proerythroblasts, and it needs to demonstrate very strong membranous staining in mast cells. For Class II tests, calculation of sensitivity and specificity needs to be performed on the number of samples recommended by national or international guidelines. Therefore, when breast cancer markers are performed in the BM core biopsy, the reported results need to specify if these IHC protocols were fully validated according to international guidelines for this particular use or not. At this time, such guidelines are available for breast cancer markers, but are lacking for other markers that are generally used for BM IHC [2].

Although tweaking of the IHC protocols by changing primary antibody concentration, incubation time, change of detection systems, etc. may improve IHC testing results, no change in antigen retrieval or other protocol components can re-establish epitopes lost to suboptimal pre-analytical conditions related to prolonged ischemic time, drying, or excessive decalcification [41–43, 92–95]. Therefore, analytical parameters are very important and may compensate to some extent for suboptimal pre-analytical parameters, but often even optimal highly sensitive protocols are not able to completely compensate for suboptimal pre-analytical parameters.

Clinical IHC laboratories often need to develop separate protocols for markers that are used for evaluation of tissues with special tissue processing, which prominently includes BM tissue biopsy. Therefore, CD34 test for BM may have different protocol than CD34 test for other tissues that were not decalcified (skin, lymph nodes, other). In addition, due to the broad range of antigen abundance, it can be useful to

use different protocols (usually different antibody concentrations or different antigen retrieval method) even in the same BM Bx depending on how the test is being used (e.g., low-sensitivity and high-sensitivity protocols for Ig light chains depending if surface or cytoplasmic Ig is of interest).

5. SPECIAL CONSIDERATIONS FOR POSTANALYTICAL PHASE

IHC test results can be interpreted only after evaluation of the external and internal controls, which will indicate if the IHC test is properly calibrated and that no internal tissue factors prevent interpretation of specific IHC staining results. The expected results of various IHC tests for BM evaluation are published and updated in peer-reviewed literature as methodology improves as well as our understanding of disease develops [96–107, and http://www.ncbi.nlm.nih.gov]. The overall diagnostic performance of BM samples requires close co-operation between the physician and the laboratory technologist/clinical laboratory scientist, as various different types of samples may be collected, and they each may require different tissue processing, which is often more complex and elaborate than that for usual anatomic pathology samples [37]. Therefore, ideally, interpretation of the BM IHC results should be performed by the (hemato)pathologist familiar with the complexities of the bone marrow tissue processing and their potential effects for the IHC results, who is able to recognize various artifacts that may results in either false-negative or false-positive staining.

6. SPECIAL CONSIDERATIONS FOR QUALITY ASSURANCE

Design of protocols is facilitated and its performance monitored and optimally maintained by the use of suitable positive and negative controls depending on the test application [3, 16].

Positive and negative controls

Recently published guidelines recommend standardization of both, positive and negative controls for IHC [16, 34]. Although general principles apply, controls for specific IHC test for BM IHC are not standardized. Therefore, it is a duty of the medical director to select/design external control samples based on desirable test performance characteristics, which are either described in published literature or, when such published literature is not available, based on the 'fit-for-use' principles.

Internal controls have limitations. For example, myeloperoxidase is very strongly expressed in maturing granulocytes, but even very low levels are also diagnostic of myeloid differentiation. In such circumstances, diseased tissue with low levels of expression is a better choice for test calibration and monitoring. When this is not possible, cell types that show high levels of expression of evaluated marker (e.g., myeloperoxidase) should demonstrate expected high intensity of IHC staining. If only weak reactions are demonstrated, the results are not acceptable. For those markers for which both weak and strong internal control is present, cell types that normally show low levels of expression of certain markers (CD117 in proerythroblasts and myeloblasts) should be demonstrated and the results are not acceptable if only highly expressing cells are detected (mast cells show strong expression of CD117 and are not sufficient evidence of appropriate calibration and sensitivity of the CD117 tests).

External quality assurance and proficiency testing

External quality assurance (EQA) program that would provide PT samples for BM IHC does not exist in the great majority of countries. This is secondary to large number of unique tissue processing protocols that are used for BM biopsy and is linked to large numbers of IHC protocols that are optimized and validated for such tissue biopsy protocols. This document recommends three methods of tissue fixation and decalcification sorted by TAT, which in any combination should achieve comparable results for IHC (Table 1). Once tissue processing is standardized, meaningful BM biopsy PT for IHC and other methods (histochemistry, *in situ* hybridization, or other) will theoretically be possible.

Use of flow cytometry to validate IHC

Theoretically, QA components in BM IHC are largely comparable to anatomic pathology IHC as they are both elements of laboratory medicine quality systems. However, there are some differences that need to be

considered; immunophenotyping using FCM frequently accompanies BM evaluation, which results in: (i) decreased need for IHC and (ii) an additional potentially powerful tool to compare results of IHC to FCM. The latter has been largely underutilized for QA. This is partly due to logistics issues as FCM and biopsy IHC are performed in some countries at different laboratories and interpreted by different specialists. In this document, FCM is also included as a part of BM IHC QA. Thorough understanding of the biology of either benign tissue or neoplastic tissues is required for this exercise as interpretation of the results is dependent on 'expected reactivities'. This is particularly important because these are genuinely different samples, and the number of positive cells and the intensity of staining may not fully correlate in the two samples even if both protocols are working optimally. It has been described that in some conditions, IHC and FCM may not be concordant and on occasion IHC may be more informative than FCM [108–113]. Most common discrepancies are seen when FCM shows weak expression and there is negative staining by IHC. Therefore, the assessment of biologically expected correlation needs to be weighted for parameters related to tissue sampling and primary antibody design. Which samples and which tests are amenable for this type of correlative QA study should be decided by the medical director of IHC in collaboration with the medical director of FCM.

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DISCLAIMER

While the advice and information in these guidelines are believed to be true and accurate at the time of going to press, neither the authors, nor ICSH, nor can publishers accept any legal responsibility for the content of these guidelines.

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APPENDIX

BACKGROUND

ICSH BM IHC Standardization Working Party tested clinically relevant fixation and decalcification conditions for the most frequently used IHC markers. Herein, the results summarize the most important conclusions for CD34 IHC staining.

METHODS

Members of the working party submitted tissues processed according to the BM protocol used in their institution as well as tissues processed specifically to test alternative conditions of fixation and decalcification. Spleen, tonsil, and appendix samples were obtained and TMAs created. Tissues were either prospectively collected or were retrieved from the archives of pathology departments. They included

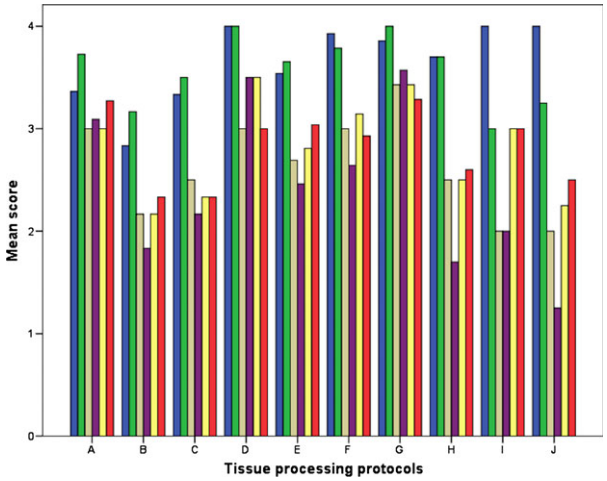

Figure A1. CD34 immunoassay using clone QBEnd/10. Effect of different methods of decalcification and different IHC protocols. Tissue samples were fixed according to individual laboratory methods for clinical samples, except for modification in method B, in which decalcification time was extended for 15 min longer than recommended by the supplier. (A) CD34 staining can be demonstrated to some degree in most tested samples after insufficient fixation (2 h in 10% buffered formalin) and are overall clinically acceptable (score ≥ 3). However, even after 15 min of extended decalcification (B), the results become poor for most samples and most protocols. Optimized protocol with Karnofsky fixative (C) is also overall suboptimal for demonstration of CD34. Formic acid after 2 h of decalcification shows very good to excellent results for most protocols, but samples used in this protocol did not contain bone so this time is not applicable to clinical practice although the results are significantly better ($P = 0.001$ for 2 h *vs.* 6 h or 16 h) (D). Longer decalcification in formic acid between 6 and 16 h (E and F) does not show any differences that appear relevant to clinical practice (the difference between 6 and 16 h are not significant), and most samples will show epitope loss to some degree. Samples that were decalcified in EDTA show excellent epitope demonstration even with suboptimal protocols (G). Another commercial decalcifying agent after formalin fixation (H) as well as one method that employed simultaneous formalin fixation and rapid decalcification (I) showed significant epitope loss in most protocols. The most rapid protocol with 2 h zinc formalin and 30 min TBD1 decalcifying solution showed poor and very poor results with suboptimal protocols, but very good to excellent results with optimal protocols. Note: Different IHC protocols are illustrated by different colors.

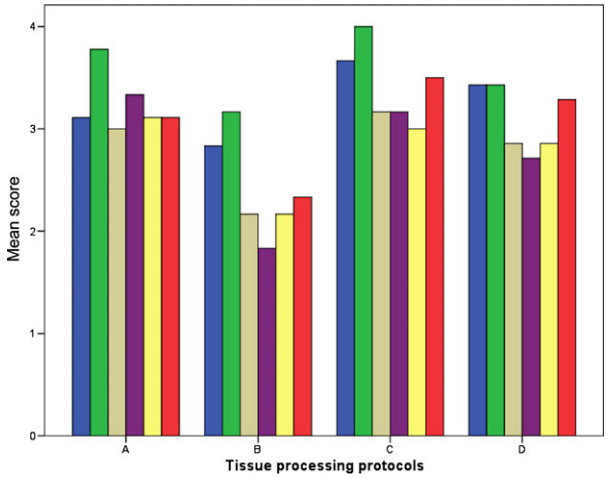

Figure A2. Immunoassay using clone QBEnd/10 (CD34). Effect of different method of decalcification and different IHC protocols. Tissue samples were fixed and decalcified as follows: formalin fixation for 2 and 1 h of rapid decalcification (A), formalin fixation for 2 h and 1′30″ rapid decalcification (B) showing significant difference between recommended and extended decalcification ($P = 0.004$). Formalin fixation for 24 and 1 h of rapid decalcification (C), and formalin fixation for 24 h and 1′30″ rapid decalcification (D); only minimal differences in the results were obtained. The results of this experiment show that proper fixation is essential to stabilize epitopes against potentially harmful decalcification protocols and that optimal protocols may partly compensate for suboptimal tissue processing. It also illustrates that careful timing of decalcification is important if short fixation protocols with formalin are used. Note: Different IHC protocols are illustrated by different colors.

spleen, lymph node, gut, and bone marrow samples. TMAs were constructed and unstained sections sent to five laboratories including laboratories in the USA, Canada, and Europe. Pre-analytical methods were set up as follows: (i) 10% buffered formalin fixation for 2, 4, 18, or 72 h followed by rapid decalcification or 18 h of fixation followed by 36 h EDTA decalcification; (ii) modified Karnofsky fixative 1% glutaraldehyde/4% formaldehyde fixation for 4, 18, or 72 h followed by formic acid decalcification for 6 or 18 h or EDTA for 36 or 72 h; (iii) B5 fixation for 4, 18, or 72 h of fixation followed by 6 or 18 h of formic acid decalcification or rapid decalcification, and (iv) buffered zinc formalin fixation for 4, 18, or 72 h followed by rapid decalcification. Interpretation by an expert panel was done by evaluating the results on the multihead microscope. If any discrepancies in scoring were present, the final score was obtained by majority vote. The semiquantitative scoring was used (0–4+); 4+ was designated for optimal results, 3+ for suboptimal but clinically useful result, while 0–2+ scores were considered unacceptable for clinical practice.

RESULTS

The results of CD34 testing are illustrated in Figures A1 and A2. Proper fixation is important if decalcification is not strictly monitored. Also, some methods were overall more robust than others (proper fixation in formalin followed by standard decalcification in EDTA), which were successful in demonstration of the epitope at clinically significant levels only by highly sensitive protocols.