CYTOPREPARATION FOR THE PATHOLOGIST

"Quality processes for quality outcomes…"

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PREFACE

Successfully detecting abnormal cells is the outcome of a series of interdependent samplings of successively diminishing size. The specimen collection technique samples the biologic process, the cytopreparatory technique samples the specimen, the screening process samples the preparation, and the diagnostic interpretation samples the cellular features. A quality laboratory increases the sensitivity of its cytological method by optimizing and standardizing its materials and methods of specimen collection and preparation.

Cytopreparation is the science of controlling cytomorphology for diagnostic applications. It encompasses all those materials and methods that interact with cells from specimen collection through microscopy, and that substantially influence every cytologic preparation's final number, mix, and distribution of cells, and their final size, shape, thickness, chemical composition, chromatin distribution pattern, color, transparency, and visibility. *Everything presented here is pragmatic, not theoretical, and makes qualitative and quantitative differences that you can see.*

In this era of heightened accountability, it is time that more attention be paid to cytopreparation at all levels — academic, professional, and regulatory — for many cost-effective benefits are realized. Typical benefits can include optimized and standardized preparations, documentation that exceeds regulatory requirements, an institutional memory that helps ensure consistency of outcome as personnel come and go, control of process, cost savings in time and materials, increased screening throughput, tighter diagnostic classifications, enhanced teaching capabilities, more instructive photomicrographs, reduced legal and fiscal exposure, and enhanced professional pride. Obviously, well-prepared specimens constitute one-time investments that pay dividends every time a cytotechnologist or pathologist examines them under the microscope. A technically well-run laboratory is not only good medicine, but also good business.

INTRODUCTION

Emphasizing process outcomes, rather than diagnostic outcomes, makes this contribution somewhat unique, as so very little relevant information has been published previously.[1-8] The wide diversity of body sites and biologic processes exhibited in non-gynecological cytologic specimens requires a simplifying, unifying approach to quality control (QC) and quality assurance (QA). Accordingly, this contribution will:

- define terms,
- compare and contrast gyn and non-gyn cytologic specimens,
- state the goals and objectives of specimen collection and cytopreparation,
- specify the links in the cytopreparatory chain that contribute to cell quality and quantity that are amenable to QC/QA measures,
- provide specific examples of quality control and quality assurance activities in action, and
- summarize the assessment of results with appropriate remedies as needed.

DEFINITION OF TERMS

To promote better understanding of the terms quality control and quality assurance and more effective implementation of such practices, this section:

- defines quality, quality control, quality assurance, and total quality management
- tabulates differential features between quality control and quality assurance, and
- describes two sets of laboratory activities and their QC/QA components.

Quality

"Quality" is derived from the Latin *qualitas,* meaning "of what sort". The set of attributes that allows a product to be used for its intended purpose *defines* its quality. Prerequisite to establishing a quality control and quality assurance program for non-gynecologic cytologic specimens are integrated knowledge of processes and expectation of outcomes.

Quality Control

Quality control activities look *forward*. They define the product's quality, imparting to it the credibility needed for its intended purpose. QC activities are the result of planning and are applied prospectively to everything that contributes to the final product, thereby impacting the outcome. QC activities are deterministic (i.e., lead to expected results when followed).

Quality Assurance

Quality assurance activities look *backward*. They measure the degree to which desired outcomes are successful (i.e., their impact). QA activities, therefore, retrospectively sample outcomes. The findings modify the processes that contribute to the final product (e.g., did the patient have cancer as reported; if not, why?). As a practical matter quality assurance activities

are probabilistic (i.e., have attendant uncertainty relative to reliability), as it not possible to review all product outcomes.

Differential Features of Quality Control and Quality Assurance. To implement an *effective* QC/QA program, laboratory personnel must first understand the *differences* between the two sets of activities. Otherwise, documentation of such activities to meet regulatory requirements becomes primarily an exercise in paperwork compliance, rather than one that makes a real difference in how work is done. Quality control and quality assurance sound so similar that the two terms are often considered to be synonymous. When the terms are used as though interchangeable, the user obviously perceives no difference. When a distinction between the two terms is perceived, it is often applied incorrectly. In either case, the recipient of such information is misinformed. As a result, the planning of QC/QA activities is often confused; the implementation, suboptimal.

Table 1 is intended to help distinguish whether an activity is one of quality control or quality assurance:[9]

Table 1

DIFFERENTIAL FEATURES OF QUALITY CONTROL AND QUALITY ASSURANCE

Differential Feature	**Quality Control**	**Quality Assurance**
Purpose	Defines Quality	Measures Success
Timing	Prospective	Retrospective
Application	All Processes	Sample Outcomes
Impact	Outcomes	Processes
Nature	Deterministic	Probabilistic

Application of Differential Features to 10 Percent Rescreening. The random rescreening of 10 percent of negative gyn cases is often referred to as "QC." While performed prospectively relative to the final reporting, rescreening is performed retrospectively relative to the activity it is intended primarily to measure, that is, the performance of the cytotechnologist. The rescreening samples outcomes; the findings impact the process of screening. The 10 percent of negative gyn cases that are rescreened is a random sample, which means it is probabilistic. Such a set of differential features is characteristic of quality assurance.

On the other hand, routinely rescreening all high risk gyn cases as a matter of laboratory policy is quality control, as it is applied prospectively to all such cases, and determines uniform quality outcomes (i.e., no false negatives).

Total Quality Management

QC activity without associated QA activity is half-action. Documentation *per se* simply constitutes paper compliance of regulations that fails to satisfy the intent. QC and QA activities must be practiced continuously to monitor and maintain the performance of the two sets of contributory processes, recognize problems as they arise, identify corrective actions to be taken, and improve quality. Taken together, these two sets of activities constitute a program of total quality management.

Analyzing Quality Control and Quality Assurance Activities

In the broadest possible sense, QC activities cease and QA activities begin when the laboratory product, the cytological interpretation or consultation, is complete. In other words, everything that precedes sign-out is quality control and everything that follows is quality assurance. Specifically, that point is the moment in time when the cytological interpretation is committed to the laboratory report. That definition is too broad, however, to be instructive at the levels where QC/QA activities are most useful.

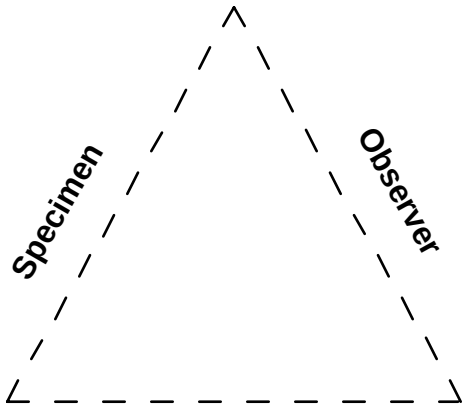
Under the overall umbrella of QC/QA relative to the laboratory's product, there are two sets of activities that have separate and equally valuable quality control and quality assurance components: (1) personnel proficiency and (2) cytopreparation. Each set of activities contributes to the *processes* that determine the outcome. The physical environment in which the work is done (i.e., laboratory space, ventilation, lighting, equipment, preventive maintenance, safety practices, and documentation) have their quality control and assurance aspects as well, though for the most part they do not affect the processes that determine the cytological outcome.

Personnel Proficiency. The first set of activities relates to the training, education, and professional performance of the laboratory personnel. In the case of cytotechnologists, for example, accrediting cytotechnology programs constitutes quality control. The ASCP cytotechnology registry examination is a quality assurance program (i.e., have the new cytotechnologists learned what they are supposed to have learned?). Proficiency testing is a form of outside-imposed quality control; an individual's test score, a measure of quality assurance. This set of activities receives justifiable attention, though its application does not guarantee quality.

Cytopreparation The second set of activities involves cytopreparation in the broadest definition. That is, cytopreparation is the science of collecting, preparing, and analyzing cytologic preparations in ways that optimize and standardize the likelihood of detecting the presence of, and accurately interpreting the cytomorphology of, abnormal cells.

Successfully detecting abnormal cells is the outcome of a series of interdependent samplings of successively diminishing size. The specimen collection technique samples the biologic process, the cytopreparatory technique samples the specimen, the screening process samples the preparation, and the diagnostic interpretation samples the cellular features. A quality laboratory increases the sensitivity of its cytological method by optimizing and standardizing its materials and methods of specimen collection and preparation. The relation of cytopreparation to the whole process of detecting abnormal cells is depicted in Figure 1:

Figure 1
THE CYTOTECT TRIANGLE
— More Probabilistic Than Deterministic —



"Cytotect Triangle" is a portmanteau meaning "cytodetection triangle." Modeled on the deterministic fire triangle, the Cytotect Triangle relates the interdependent roles of the specimen, observer, and microscope usage in the detection of abnormal cells. Relating this model to electronics, the specimen is source of the signal; the observer, the receiver; and the microscope, the transmitter. The many variables that impact the process introduce noise. By optimizing and standardizing the three processes, the signal is strengthened and the noise is reduced. Optimized processes increase the probability of abnormal cell detection (i.e., high sensitivity) and reduce the incidence of missed abnormal cells (i.e., false negatives). In Figure 1, the probabilistic nature of the entire process is represented by dashed lines, rather than solid lines.

ATTRIBUTES OF GYN AND NON-GYN CYTOLOGIC SPECIMENS

From the standpoint of collection and preparation, cytologic specimens are more alike than they are different. All are suspensions of initially viable cells that require similar handling if they are to be useful diagnostically. Differences in cytopreparation arise primarily in the method of effectively transferring the cells from suspension onto a display medium. Nonetheless, it is instructive to compare and contrast various aspects of gyn and non-gyn cytologic specimens (Table 2), as such an analysis provides may account in part for the different levels of attention each receives relative to quality control and quality assurance.

Table 2
GYN VS. NON-GYN CYTOLOGIC SPECIMENS

No.	Attribute	GYN Specimens	Non-GYN Specimens
1	Source	Female genital tract	Both sexes, all body sites
2	Test status	Screening test	Diagnostic work-up component
3	Numbers	60,000,000+/yr	6,000,000/yr (?)
4	Collection method	Spatula and/or brush	Exfoliation, abrasion, aspiration
5	Slides per specimen	Usually 1, often 2, rarely 3	1-10+
6	Preparation	Outside laboratory (usually)	Inside laboratory
7	Slide preparation area	314-mm^2 thru 1,440-mm^2	32-mm^2 thru 1,200-mm^2
8	Slide prep method	Smear or monolayer device	Smear, cytocentrifuge, monolayer device, membrane filtration
9	Fixation status	Spray-fixed usually; sometimes wet-fixed	Fresh, preserved, or spray-fixed
10	CT Responsibility	Signs-out negatives only	Forwards all to pathologist
11	QC/QA	CLIA-mandated 10% negative review	All reviewed by pathologist
12	Problematic	False negatives; 90% all negatives/non-high risk seen only once	False positives; pathologists see everything
13	Reporting system	The Bethesda System	No standardized reporting system
14	Patient management	Result influences	Result may/may not affect
15	Accountability	Unrealistic public expectations	Public relatively unaware
16	Litigation risk	High, 53 lawsuits in 2-1/2 yrs (Drs' Co.)	Low

Consider, for example, that relative to non-gynecologic specimens, CLIA ‘88 requires "(a) The laboratory must assure that —

(2) Effective measures are taken to prevent cross-contamination between gynecologic and nongynecologic specimens during the staining process;

(3) Nongynecologic specimens that have a high potential for cross-contamination are stained separately from other nongynecologic specimens, and the stains are filtered or changed following staining;"

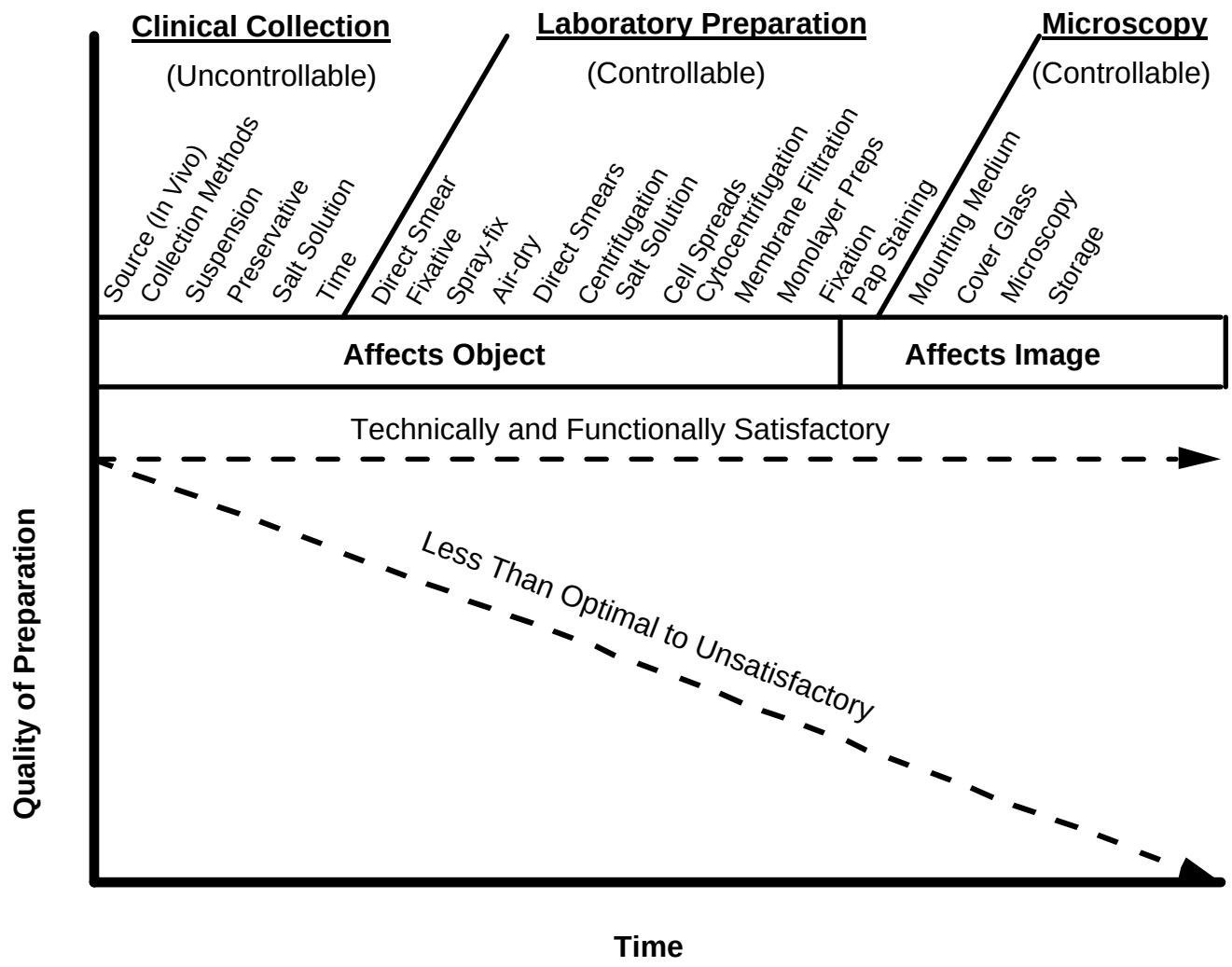
Subparagraph (1) requires that gynecologic smears be stained using a Papanicolaou or modified Papanicolaou staining method, but not that nongynecologic specimens be stained similarly. Note that collection and cytopreparation are not mentioned. Parenthetically, "effective measures…to prevent cross-contamination" are not defined. In fact, the usual measure of filtering stains through coarse laboratory grade filter paper is essentially futile. Such paper consists of cellulosic fibers pressed together, leaving gaping pores through which tissue fragments can pass. An effective cross-contamination control system must incorporate absolute pore size filters.[10]

GOALS AND OBJECTIVES OF COLLECTION AND CYTOPREPARATION

The overarching goal is to make every cell tell, as clearly as possible, its health status. Achieving this goal requires an integrated series of procedures, with interference by possible errors such as air-drying, as shown in Figure 2:

Figure 2

LINKS IN THE CYTOPREPARATORY CHAIN



The collection objectives include using techniques that obtain a representative, well preserved sample of cells from the pathologic biological process. In terms of cytopreparation, the objectives include the specimen's being a representative, well distributed monolayer of well fixed and well stained cells that is properly imaged.

Every cytology laboratory should provide its clients with manuals that describe the principles, and recommended materials and methods, of specimen collection and provide needed telephone numbers, hours of operation, and other laboratory-related information. The recommended materials should be made readily available. Seminars may be presented to groups of clients or new physician residents as appropriate. The laboratory should have an outreach program whereby pathologists and/or cytotechnologists communicate by phone in person with clients as needed.

QUALITY CONTROL AND QUALITY ASSSURANCE IN COLLECTION

Quality control in specimen collection includes specifying the type of salt solution to be used when necessary. Normal saline should not be used; it bursts cells. Balanced electrolyte solutions are satisfactory for use within the laboratory. Balanced salt solutions are indicated for clinical specimen collection (e.g., bronchial lavage specimens, not the lavage itself), though balanced electrolyte solutions will suffice. Cells collected in alcoholic preservative are dead and do not require any salt solution. Table 3 summarizes salient properties of commonly used salt solutions, which properties are reflective of the differences in composition (Table 4). This knowledge exemplifies the level of detail needed to ensure quality cytologic preparations.

Table 3
PROPERTIES OF COMMONLY USED SALT SOLUTIONS

Solution	**Isoosmotic**	**Inorganic Ions**	**pH**	**Buffered?**	**Glucose?**	**Disruptive?**	**Safe Exposure**
Normal saline	Yes	Imbalanced	6.0	No	No	Yes	Seconds
BES*	Yes	Balanced	6.0	No	No	No	Minutes
Hanks' BSS**	Yes	Balanced	7.4	Yes	Yes	No	Hours

* BES = Balanced Electrolyte Solution (*in vivo* use [parenteral injection in humans])
** BSS = Balanced Salt Solution (*in vitro* use [irrigation medium in tissue culture])

Table 4

COMPOSITIONS OF COMMONLY USED SALT SOLUTIONS

No.	Ingredient (gm/L)	Normal Saline	BES	Hanks' BSS
1	Sodium chloride	8.5	4.96	8.0
2	Sodium acetate	—	7.48	NA
3	Potassium chloride	—	0.746	0.4
4	Calcium chloride	—	0.368	0.14
5	Magnesium chloride • $6H_2O$	—	0.305	0.1
6	Magnesium sulfate • $7H_2O$	—	—	0.1
7	Disodium phosphate • $2H_2O$	—	—	0.06
8	Monosodium phosphate	—	—	0.06
9	Sodium bicarbonate	—	—	0.35
10	Dextrose	—	—	1.0

QUALITY CONTROL AND QUALITY ASSURANCE IN CYTOPREPARATION

If a cytology laboratory finds recurring problems in its cytopreparations, it should identify the cause(s) and take corrective action(s). Whether the problem is quantitative or qualitative in nature, the first step is to determine the origin: Is it the specimen or the processing? As a control, examine a drop or two of raw specimen microscopically, taking universal precautions in handling. Place the unstained specimen on a slide and cover it; lower the substage condenser or close the aperture diaphragm to make the cells visible. Assess the specimen's cellularity, composition, and preservation. An absolute deficiency such as total acellularity can not be corrected in cytopreparation, whereas a relative deficiency such as low cell concentrations may be.

Knowing such information in advance shapes expectations and guides actions. Without such information, one can not know whether the cause of an unsatisfactory preparation originated in the specimen, collection, or preparation (Figure 1). Ignoring the opportunity to have such information risks wasting time troubleshooting the wrong problem, needlessly examining unsatisfactory preparations, and incorrectly reporting cases as unsatisfactory or negative. Microscopic examination is not needed with every specimen, as to do so can be unacceptably time-consuming. This procedure, however, is exceedingly instructive, especially if one has never done it before. At the very least, it constitutes quality control at the first step of cytopreparation.

- If the specimen is completely acellular, cytopreparation is pointless.

- If the specimen is hypocellular, centrifuge the specimen to concentrate the cells. Re-examine the button after suspending it in a small volume of salt solution or preservative as appropriate.
- If the specimen is hypercellular, dilute it as needed to avoid overpopulating the preparation.
- If the cells are poorly preserved in a fresh specimen, they may have degenerated because too much time has passed since collection, or the specimen was suspended in normal saline, or storage conditions have been substandard.
- If the cells are poorly preserved in a specimen collected in preservative, the preservative itself may have caused the cells to swell and burst (e.g, certain dilutions of alcohol).
- If particulates are observed, an attempt should be made to redissolve them if possible (e.g., slightly acidifying fresh urine specimens with acetic acid to redissolve phosphate salts) or to separate them by differential sedimentation. Particulates can be problematical for specimens that are processed automatically in a monolayer device. The particulates can clog the filter pores and cause cell collection to stop prematurely.
- If erythrocytes outnumber nucleated cells excessively, they must be removed prior to preparation to prevent their crowding out the nucleated cells and result in a false negative. Bloody fresh specimens may be treated with saponin to hemolyse the red blood cells. Bloody preserved specimens may be suspended in one of at least two proprietary hemolytic preservatives. Hemolysing the RBCs by immersion in a Carnoy-like fixative after the cell spread has been made will not fix the problem.
- If tissue fragments are observed, the preparations should be stained separately to avoid cross-contamination.

Estimating Sample Size. In manual cytopreparation, it is common practice to transfer a portion of cell suspension to the display medium or collection device without first assessing its cellularity and adjusting the volume to assure a satisfactorily cellular preparation. Most of the time, this approach apparently yields satisfactory results as it continues to be used widely. However, problematical specimens point out the shortcomings. Clear urine specimens, for example, appear to be acellular but in fact often contain hundreds if not thousands of cells. Such specimens must first be concentrated by conventional centrifugation, then examined microscopically to determine the appropriate collection method (e.g., membrane filtration, cytocentrifugation, monolayer device) and how much specimen to use. Turbid specimens may appear to be hypercellular to the unaided eye, when in fact the turbidity is due to light-scattering particulates and not cells. Preprocessing to rid the particulates is indicated.

To assess the volume of specimen of varying cell concentrations to add to different cell collection devices, prepare a slide as described: examine a drop or two of raw specimen

microscopically, taking universal precautions in handling. Place the unstained specimen on a slide and cover it; lower the substage condenser or close the aperture diaphragm to make the cells visible. Quickly review the cell distribution using a 4× or 10× objective and select a representative field. Using a 40× objective, compare the observed cellularity with that depicted in the six graphical fields-of-view in Figure 3. Then match the letter of that field-of-view with that in Table 5. The number in the cell below that intersects the row and size of the collection device in use is the recommended volume.

Figure 3
CELLS PER 40X FIELD-OF-VIEW
Refer to Table 5 for Number of Drops Per Display Mode
(Number Within Each FOV = Cell Numbers)

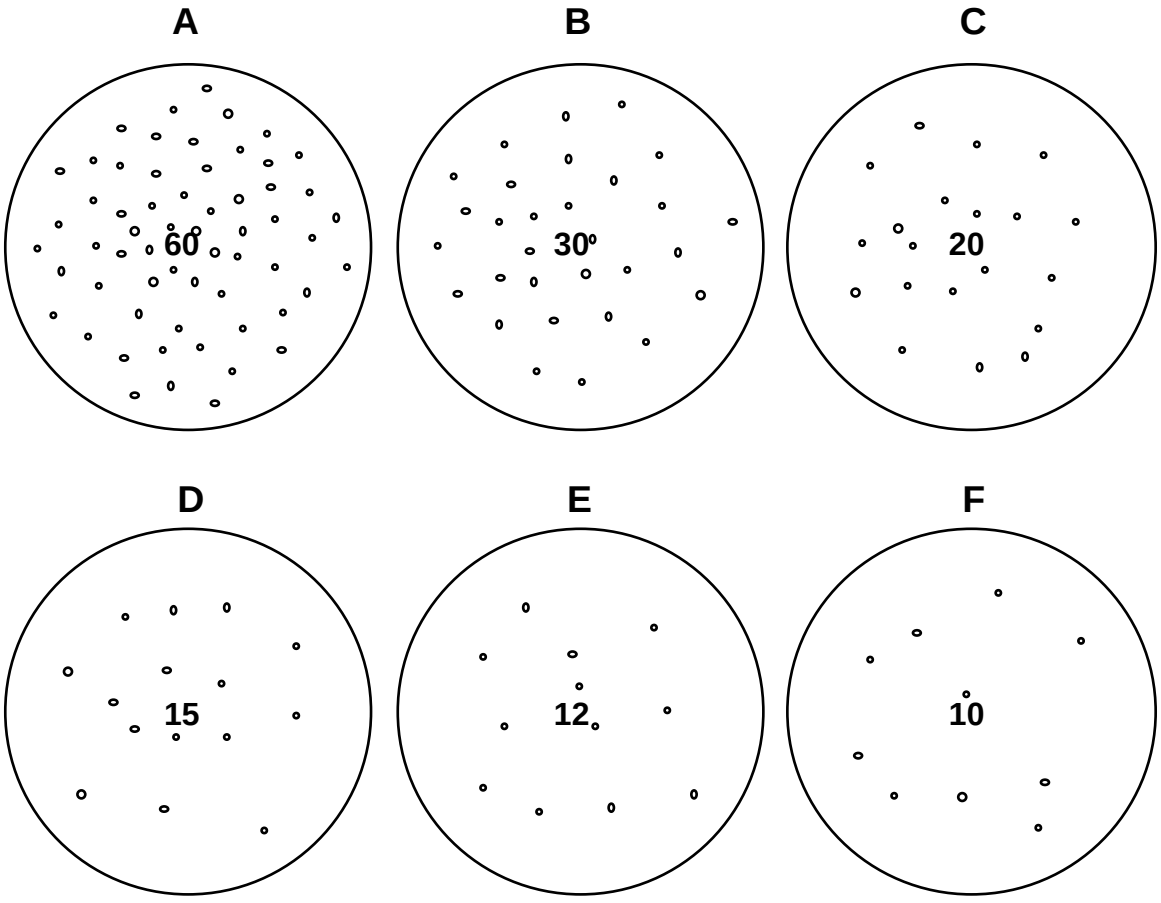


Table 5

SAMPLE SIZE GUIDE

No.	Mode	mm^2	Drops Per Field-of-View Population Density					
			A	**B**	**C**	**D**	**E**	**F**
	Filter							
1	25-mm	250	8	16	24	32	40	48
2	19 × 42-mm	600	18	36	54	72	90	108
3	47-mm	960	30	60	90	120	150	180
	Cytospin							
4	6-mm	32	1	2	3	4	5	6
5	9 × 21-mm	189	6	12	18	24	30	36
	ThinPrep							
6	21-mm	346	10	20	30	40	50	60

Note: There are approximately 16-20 drops per mL, depending on the dropper tip diameter.

Cell Flattening and Retention. So-called watery cytologic specimens such as urine and body cavity fluids are often spread on albuminized slides, frosted slides, or albuminized-frosted slides — all in an effort to keep cells from falling off when immersed in alcohol. While these special surfaces help cells adhere to slides, they prevent cells from flattening individually, like sunny-side-up eggs, and so can not display their chromatin informatively. Instead they remain somewhat rounded-up, like hard-cooked eggs. Such cells display compacted chromatin and therefore are less useful diagnostically than they would if flattened on a plain slide. Qualitative display is compromised for quantitative recovery.

Cells do not flatten and stick to glass simply because the glass is dirty and the cells are prevented from touching the slide by a film of liquid. The following technique promotes cell flattening and retention:

- Immediately before use, immerse each slide in alcohol briefly and wipe it squeaky clean to make it wettable. A rack of slides can be kept in alcohol and the slides withdrawn as needed.
- Whether using raw cell suspension or resuspended cell concentrate, transfer a small amount of cell suspension to the slide. Use less than you might think is needed. Only a single layer (“a light dusting”) of well distributed cells is required, not a heavy layer. The specimen

should not be able to flow to the edges of the slide when a second slide is applied to spread the cells by the 2-slide pull technique. You have succeeded when the slides resist sliding. If the cell suspension is expressed beyond the slide boundaries, too much has been added. The suspension medium will keep the cells from touching the glass, which is essential for flattening and retention.

- Immediately immerse the slide in alcohol.

STAINING

Performance Expectations

Hematoxylin. All chemically competent hematoxylin formulations produce satisfactory results when used properly. Staining times of 1 to 2 minutes are typical for Gill's hematoxylins No. 1 and 2. A blue color is satisfactory; violet, insufficient bluing; gray, borderline; brown, unsatisfactory. The optical density range should be sufficient to make chromatin particles visible in intermediate squamous cell nuclei and to allow resolution of chromatin particles in the lobes of well flattened neutrophils. Slight coloration of the cytoplasm is permissible. Heavy coloration indicates the staining time is too long or the solution is too concentrated. Either decrease the staining time or dilute the solution.

Be certain to blue the stained slides sufficiently. Bluing can occur over a wide range of pHs. Low pHs (e.g., 5-6, distilled water), though acid, are alkaline relative to the isoelectric point of aluminum-hematein, and blue slowly over several minutes. High pHs (e.g., 10-11, 1.5% NH_4OH in 70% alcohol) blue rapidly within seconds and tend to detach cells. Moderate pHs (e.g., 8, Scott's tap water substitute) blue single cells and thick tissue fragments and cellular clumps satisfactorily within 2 minutes.

OG and EA. Orange G should appear yellow to orange; 15 sec to 1 minute is the usual range of staining times. EA is often problematic because of fundamental limitations in its chemical composition. Ideally, one should see clearcut hues of green and red in separate cells. Staining times less than about 3 minutes usually favor the uptake of eosin, with eosin and light green often occupying different areas of the same cells. Most EA formulations perform optimally in the 6-8 minute range. Note particularly that the OG and EA staining times are interdependent: relatively too much time in OG will overload cells with orange G and block the subsequent uptake of eosin.

See References 11-26 for more extensive Pap stain-related information on materials and methods, quality control, quality assurance, and troubleshooting.

Buccal Smears for Quality Assurance and Test Probes

Alcohol wet-fixed buccal smears are invaluable probes to determine the performance of each new lot of stain, to select suitable staining times, to find out how many slides can be stained satisfactorily by a given volume of each stain, to learn when rinses should be changed, and to troubleshoot whether a given stain already in use is the cause of an observed staining problem. Once experience imparts confidence to selected staining times, stain and rinse change schedules, the use of buccal smears is not necessary. However, they should be used when new containers of the same stain with different lot numbers are opened to confirm that the stain does indeed perform as expected. Manufacturers occasionally make bad batches of stain.

The recommendations that follow constitute true quality assurance. Simply looking at one of the first slides stained daily and initialing a stain quality log sheet is valueless if a laboratory has not defined its standards. It is not uncommon to see such sheets dutifully maintained and also to see unsatisfactory staining results.

Quality Assurance. A series of buccal smears should be used to answer three questions for each of the three staining solutions of the Pap stain: (1) what color is produced, (2) what staining time is required to produce the desired optical density, and (3) what is the distribution vis-à-vis the chromatin and cytoplasm. To confirm that each new lot of stain is performing as expected, stain different sets of alcoholic wet-fixed buccal smears in hematoxylin for 30 sec, 1, 2, and 4 min; OG, 15 and 30 sec, 1 min; EA, 1, 2, 4, 6, and 8 min. Rinse the hematoxylin stained slides in two changes of tap water (10 dips each), blue per lab convention, rinse in two changes of tap water (10 dips each), dehydrate, clear, and mount. Rinse the OG and EA stained sets of buccal smears in 95% alcohol, dehydrate in absolute alcohol, clear, and mount. Note that the successive staining times differ from one another by a factor of 2. Seconds-long differences in stain uptake are insufficient to be appreciated visually.

Hematoxylin should be blue, with slight cytoplasmic coloration. The optical density should be light enough to show chromatin detail within the lobes of well flattened PMNs and dark enough to make visible chromatin particles of intermediate squamous cell nuclei. If the stain is too light, stain longer or use a stronger hematoxylin. If the stain is too dark, stain less time or use a weaker hematoxylin. Gray to brown colors indicate overoxidation; don't use the stain.

Orange G looks yellow in thin areas and orange in thicker ones. Light green and eosin are distinctly green and red in properly formulated stains. If the phosphotungstic acid concentration is too low, there may be little or no differential staining—the green and red colors will be muddy and dull.

Preparing buccal smears in this way lets you see the true color of each of the four major dyes without any possible interference by inadvertent misuse of the others. The colors observed

in this reference set should also be seen after Pap smears are stained by a complete Pap stain. If not, then it has problems that should be identified and corrected.

Staining Times. Proceed as above, but stain multiple buccal smears in each stain: hematoxylin for 1, 2, and 4 min; OG for 15 and 30 sec and 1 and 2 min; and EA for 15 and 30 sec, and 1, 2, 4, 6, and 8 min. Choose the staining time that appears best (see the immediately preceding descriptions). Usually the times are close to those recommended. The EA buccal smear series is most instructive, as it shows the shift in stain uptake from the red to green with longer staining times.

Stain Duty Cycles. Duty cycle is the number of slides that can be stained per unit volume of stain before a decline in quality is detectable. Buccal smears can be used to estimate semi-quantitatively how many slides can be stained satisfactorily per given volume of hematoxylin or any other stain for that matter. Since each of the three stains contains different concentrations of dyes, you may find that more slides can be stained in one stain than another, so that all three stains are not changed at the same time. This remains to be determined empirically in your own laboratory.

Simply label a series of alcoholic wet-fixed buccal smears 0, 1, 2, 3, etc. and date them. Put one buccal smear in every fifth rack of slides as a stain is changed. Slide 0 is the first rack, slide 1 is the fifth rack, and so on. Remove the test slides as the rack passes through the counterstains. Examining the entire set of slides will reveal how many slides can be stained satisfactorily. This process need only be done occasionally, as needed, to confirm the continued validity of the practice.

To determine objectively how many slides can be stained satisfactorily per mL, prepare three sets of alcohol wet-fixed buccal smears for every five racks of slides. Identify each slide with the stain name, date and number, beginning with 1 and continuing by fives (i.e., 1, 5, 10, 15…). Put hematoxylin-1 in the first rack of slides to pass through fresh hematoxylin, put OG-1 in fresh OG, and EA-1 in fresh EA. Remove the hematoxylin slide before it enters OG, dehydrate in absolute alcohol, clear, and mount. Rinse the OG slide in the usual three changes of 95 percent alcohol, skip the EA and its rinses, complete dehydration in absolute alcohol, clear, and mount. Rinse the EA slides in its three changes of 95 percent alcohol, dehydrate completely in absolute alcohol, clear, and mount.

You will end up with three sets of buccal smears stained only in hematoxylin, OG, or EA at 5-rack intervals. Compare the first and last slides of each set to see whether there is a visible difference. If there is, find the slide at which you can begin to see an unfavorable difference. That numbered slide represents how many racks you can put through the stain. If you do not see a difference, more slides can be stained before the stain should be changed. Use buccal smears to determine how many more. Usually 1-2 slides can be stained per mL stain, depending on the

concentration of the stain and the cellularity of the preparations. For example, 250 mL of most EAs will stain 250 Pap smears satisfactorily, but not 375. Part of the savings realized by using less alcohol can be applied to the cost of changing the counterstains more frequently.

Rinse Duty Cycles. Pre-hematoxylin water rinses should be changed when cellular debris is visible. First two post-hematoxylin water rinses should be changed after each rack, as the hematoxylin rapidly colors the waters. Next two water rinses can be rotated back two positions and replaced by clean water. The two sets of post-OG/EA alcohols should be changed when the third bath in each set becomes lightly colored: discard the contents of the first dish in each series, move dishes 2 and 3 to positions 1 and 2 in the 3-bath series, and add fresh alcohol to dish 3.

Alcohol rinses work best when clean, deep, and are used for sufficient time to effect removal of excess dye from the Pap smears. Dirty rinses become more like a stain than a rinse and prevent effective rinsing. Buccal smears are also compelling indicators of overused alcohol rinses following OG and EA stains. Simply place a wet-fixed unstained buccal smear in the staining rack as it leaves OG. Remove the buccal smear after it passes through the three alcohol rinses, dehydrate, and clear. Do the same for EA. Dirty rinses are weak stains and can stain the unstained buccal smears dramatically. The cells will look as though they have been immersed in a primary staining solution, rather than in a rinse. They are terrific reality checks.

Troubleshooting. Problems do not occur when quality stains are used for the appropriate staining times, changed at the proper intervals, and used in conjunction with suitable rinses that are also changed at the proper intervals. If a problem does arise, look to the stains for the cause(s). Obscure causes (e.g, the pH of the tap water often being the putative cause) that can not replicate the untoward results experimentally are never the cause in my experience.

Cross-Contamination Control

In regard to the CLIA requirement, *effective measures…to prevent cross-contamination*, it should be noted that the usual measure of filtering stains through coarse laboratory grade filter paper is essentially futile. Such paper consists of cellulosic fibers pressed together, leaving gaping pores through which cells and occasional tissue fragments can pass. This was demonstrated experimentally using an alcoholic suspension of HeLa tissue cultured cancer cells that was passed through conventional laboratory grade filter paper and the filtrate was refiltered through a 5 µm pore size Millipore filter. The tip of the filter paper was cut out, and together with the Millipore filter, stained, mounted, and examined microscopically. The filter paper contained some cells but the Millipore filter contained more.

The cross-contamination control system described below was devised to effectively remove all floaters from suspension.[10] It also allows evaporation-free storage of the filtered stains and subsequent convenient dispensing.

Materials. Figure 4 shows what this system looks like. It consists of three subsystems:

Vacuum Source (not shown)

- In-house line, portable electric pump, or aspirator filter pump
- 1-liter filter flask as trap bottle
- Connecting plastic tubing
- 2 Y-connectors

Stain Storage

- 1-liter separatory flask, globe or pear-shaped, glass or teflon, with teflon plug
- 2-hole stopper
  - — First hole: 3-way stopcock, sidearm connected to trap bottle; free end for vent
  - — Second hole: right angle tubing, connected to Millipore Swinnex 47-mm filter holder
- Support (5x7-inch base with 20-inch rod, ring support, small utility clamp—not shown)

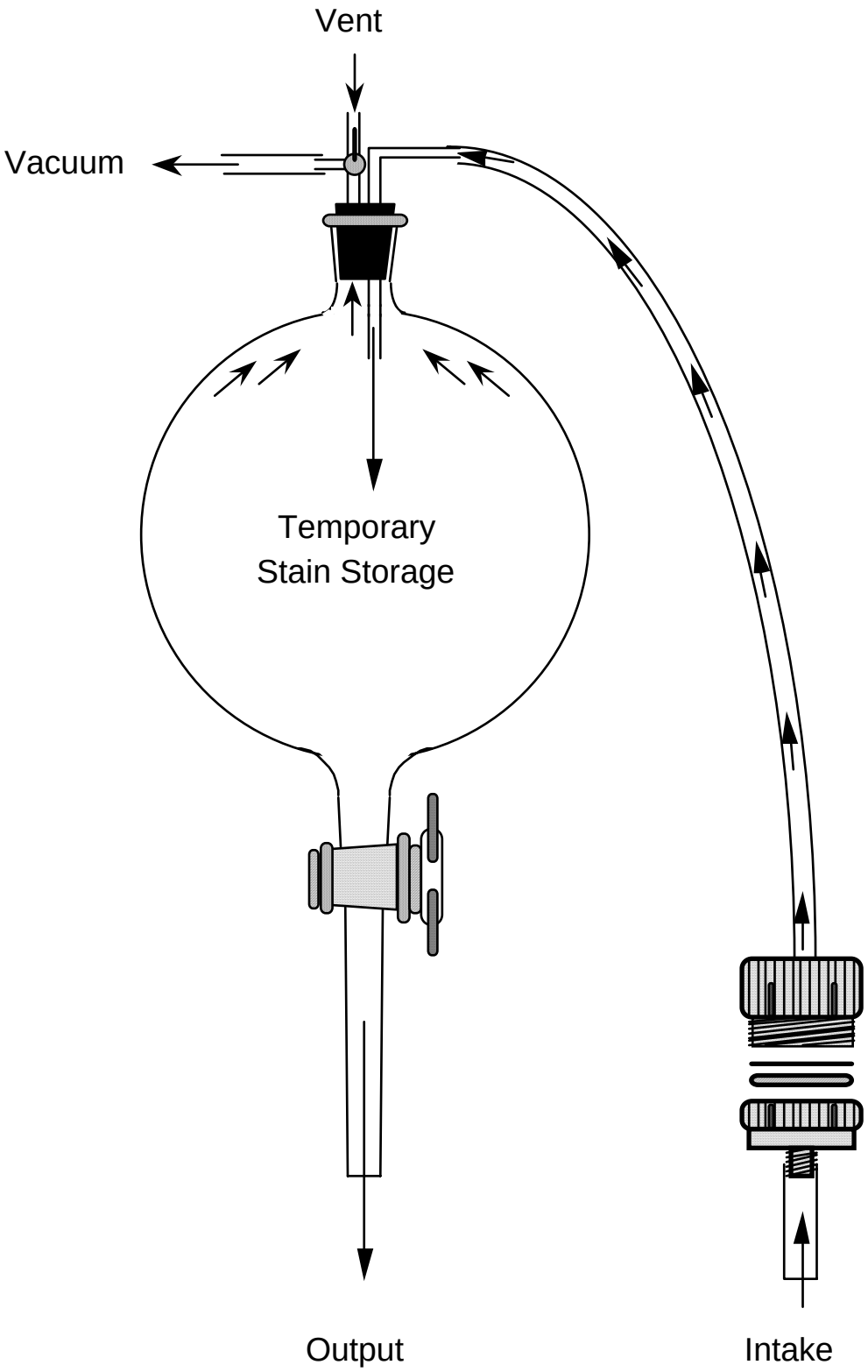
Filtration

- Millipore Swinnex 47-mm filter holder (Cat. No. SX 047 00) with:
  - — Millipore Type AP 47-mm prefilters (Cat. No. AP25 047 00)
  - — Millipore hydrophilic Durapore 47-mm filters, 5 µm pores (Cat. No. SVLP 047 00)
  - — Millipore Parts Kit, Swinnex 47 (Cat. No. SX 00 047 RK)
- 3-inch long plastic tubing to dip into stain for intake into system

These parts must be purchased separately from several vendors. Order enough to assemble three stain storage subsystems, one each for hematoxylin, OG, and EA.

Figure 4

CROSS-CONTAMINATION CONTROL SYSTEM



Methods. The apparatus has three functions: (1) convenient and absolute removal of cells an debris from suspension in any solution, (2) air-tight storage of the filtered solution until it is ready for use, and (3) convenient dispensing of the cell-free and debris-free stain into a clean staining dish.

To filter stains, turn the stopcock of the separatory funnel to its horizontal shut-off position, rotate the lever of the 3-way stopcock to the 3 o'clock position, and turn on the vacuum source. Immerse the intake tubing end in the stain to filter the stain and deposit it in the separatory funnel. Stop filtration by turning the lever of the 3-way stopcock to the 12 o'clock position to vent the system and/or by turning off the vacuum source.

Once filtered into the separatory funnel, a stain may remain there until needed again. Air-tight storage is assured by turning the lever of the 3-way stopcock midway between one of its use positions. If left on overnight, the vacuum will halve the stain volume.

To empty the separatory funnel, turn the lever of the 3-way stopcock to the vent position and open the lower stopcock as shown in Figure 4. The staining dishes are kept under the separatory funnels, not in line with the other staining dishes.

Filtration may be slow initially when using filters still wet from previous use. Wet pores require substantial vacuum to break loose the liquid held in the pores. Once the pores are open again, filtration proceeds smoothly. In other words, slow starting filtration may mimic an overloaded filter that needs replacement. Replace the filters only when filtration becomes labored after several uses. Prefilters extend the useful life of the more expensive Durapore filters.

Discussion. Ancillary cross-contamination prevention measures include: (1) avoiding undue agitation (e.g., running water, rapid dipping), (2) frequent replacement of non-stain solutions, (3) staining GYN specimens before non-GYN preparations, (4) staining high-risk shedder specimens (e.g., body cavity fluids) last, (5) maintaining a separate Pap stain set-up for non-gyn specimens, (6) staining known shedders in separate miniature Pap stain set-ups—using nearly exhausted solutions that can be discarded afterwards where appropriate, and (7) filtering xylene or xylene substitute baths through laboratory grade filter paper to remove particulates.

The price of the component parts has increased substantially since this system was described in 1975. Nonetheless, this cross-contamination control system fulfills the spirit and the intent of the related CLIA regulation.

COVERSLIPPING

Recall that a slide put cover-glass-side-down can still be seen under 10×, and it is not until trying to focus under 40× that one realizes the slide is upside-down. Recall, also, that a freshly mounted preparation looks sharper under 10× than it does under 40× and that older preparations look sharp under all magnifications. These examples demonstrate the fact that

objectives are sensitive to deviations in mounting medium and cover glass thickness as a function of their numerical objectives. Deviations will not be visible under 10× objectives, as their numerical aperture of 0.25 make them insensitive to cover glass thickness. Objectives with numerical apertures equal to or greater than 0.6 (i.e., 40×) require a cover glass thickness of 0.180 mm (i.e., No. 1-1/2 thickness). Freshly mounted preparations rarely have the optimum thickness of mounting medium and cover glass.

The proactive tactic, therefore, is to use No. 1 cover glasses, which are thinner, and as little mounting medium as is consistent with a permanent mount.[27] Some mounting media cause biological dyes to fade even when stored in the dark. Since there is no convenient way of knowing in advance which mounting media will fade dyes, one must simply be aware of this factor as a possibility.

MICROSCOPY

Use clean microscopes illuminated according to the principles of Köhler (Figure 5):[28-30]

1. Focus sharply on the cells of interest throughout the steps that follow.
2. Close the field diaphragm until it is slightly smaller than the field-of-view.
3. Center the field diaphragm within the field-of-view so that the circumference of the illuminated field is equidistant from the outermost circumference of the field-of-view.
4. Close the field diaphragm to its smallest opening. (One could attempt to center it at this step, but centration is difficult to judge.)
5. Close the aperture diaphragm to its smallest opening.
6. Raise or lower the substage condenser until the edges of the field diaphragm are in sharp focus while the cells are also in focus. When in focus, the edges of the field diaphragm will be ringed by a magenta halo and the top lens of the substage condenser will be 1 to 2 mm from the underside of the slide, nearly touching it.

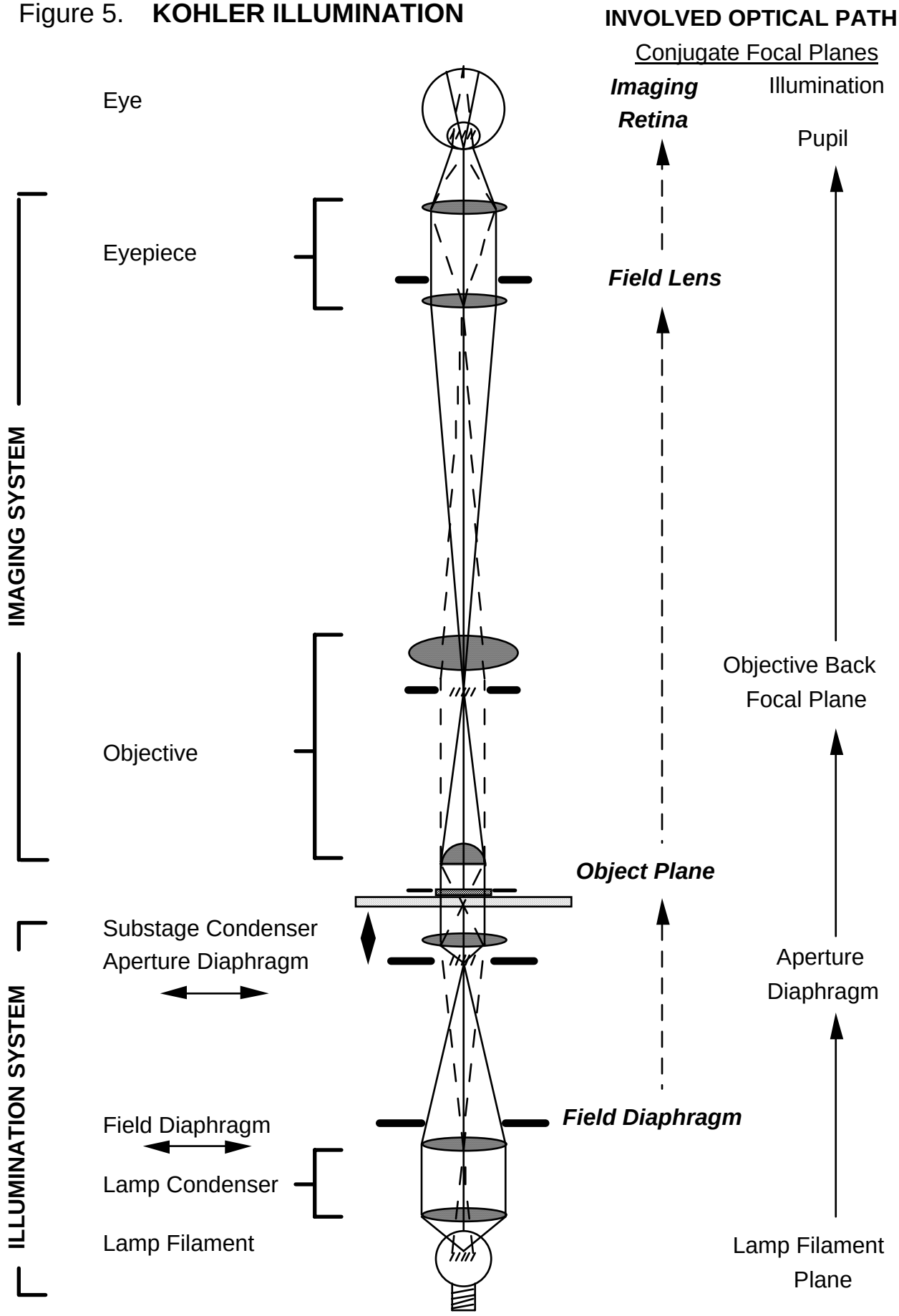
When the substage condenser is raised too much, the halo will be orange; when lowered too far, blue. the intensity of the colors will vary with the opening of the aperture diaphragm. "Raised too much" and "lowered too much" occur within a distance of one to two millimeters of the optimal height.

7. Open the field diaphragm until the edges just disappear from view.
8. Open the aperture diaphragm until best contrast is obtained. This is the last and most important step, as it makes the biggest difference in the quality of the image. It is the only step that requires visual judgment and does not rely on a physical endpoint. The amount of opening will vary with the numerical aperture of the objective, the contrast among the stained objects, and the overall thickness of the mounting medium and cover glass. Opening the aperture diaphragm too little will impart to cells an artifactual glassy look, with so-called optical membranes, as a consequence of diffraction. Opening the aperture diaphragm too far will reduce

contrast as a result of glare and flare, light that does not contribute to image formation. Such images are referred to as washed out, cloudy, hazy, low in contrast, and flat. This is by far the most common mistake. If you look over a microscopist's shoulder and see a bright spot of light at the top lens of the substage condenser, you know he or she is looking at suboptimal images.

Since the combined thickness of mounting medium and cover glass in freshly mounted preparations usually reduces image contrast under 40× objectives, the microscopic technique of last resort is to decrease the opening of the aperture diaphragm to reduce its sensitivity to overly thick mounting medium and cover glass. Doing so changes the working numerical aperture of the objective, as Köhler illumination images the aperture diaphragm at the back focal plane of the objective, thus reducing the objective's sensitivity to mounting medium and cover glass thickness.

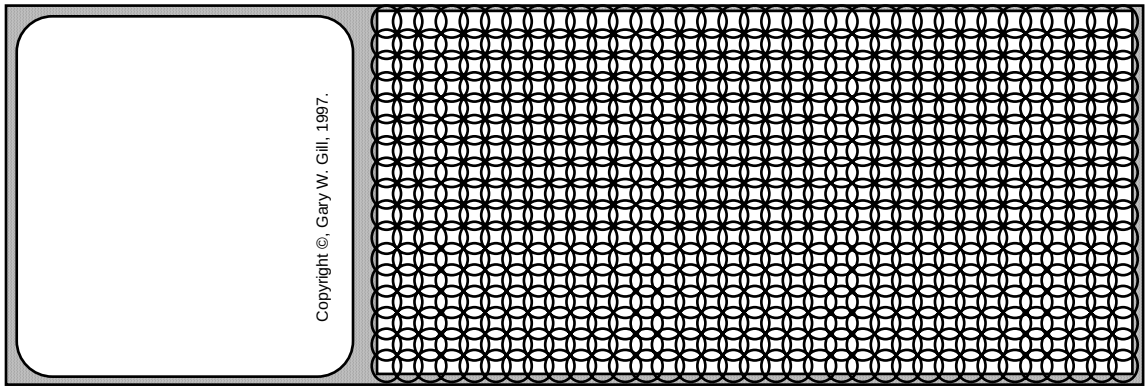
Figure 5. **KOHLER ILLUMINATION**



See References 31-2 for information on cleaning lenses (i.e., quality control) and 33-5, troubleshooting (i.e., quality assurance). To assure complete useful imaging of the entire preparation, the microscope stage must be moved systematically to overlap diameters of successive and adjacent fields-of-view 30 percent at a minimum (Figure 6).[36-43] Without an electronic monitoring device, however, there is no way to assure such overlap.

Figure 6

TOTAL IMAGING SCREENING COVERAGE



Using a 10X objective and 10X eyepieces with a field number of 20, an array of 17x36 2-mm fields-of-view that overlap diameters 30% along the x and y dimensions is required to image an entire conventional preparation. Monolayer preparations occupy 28.8% as much area and require approximately 71% fewer total fields to image the entire preparation.

SUMMARY

Useful QC/QA activities in non-gynecologic cytologic specimen collection and preparation are not practiced widely, though enough information is now available to establish such. Until such practices become required with greater specificity and effectiveness than required currently, quality assurance activities related to outcome will continue to be based on less than optimal processes of specimen preparation. Table 6 gives abbreviated paired examples of QC and QA activities for selected processes of specimen collection and cytopreparation and may serve as a guide to laboratories who want to establish a systematic program.[44]

Table 6

QC/QA IN NON-GYN CYTOLOGIC SPECIMEN COLLECTION AND PREPARATION

Activity	**Quality Control**	**Quality Assurance**
Slide acceptance criteria	Patient-Specimen match	Check at multiple points
Salt solution diluent	Specify balanced salt solution	Assess cellular preservation
Bloody non-gyn specimen	Hemolyse with saponin	Assess degree of hemolysis
Centrifugation speed & time	Calculate necessary RCF	Measure speed
Cytocentrifuge cell recovery	Estimate drops by micro exam	Assess cell recovery post-cent
Cell recovery and flattening	Clean 2-slides, few drops, pull	Evaluate recovery & flattening
Hematoxylin	Specify formulation and time	Buccal smear; blue nucleus
Bluing agent	Specify pH and time	Confirm pH with pH paper
EA counterstain	Specify formulation and time	Buccal smear; red/green cyto
Water-free xylene	Use adsorbent beads & filter	Inspect for water droplets
Cross-contamination control	Specify absolute filtration	Inspect filtrate; floaters?
Cover glass thickness	Specify No. 1 thickness	Measure with micrometer
Mounting medium	Specify minimum thickness	Inspect
Microscopy	Specify Köhler illumination	Test CT knowledge
Screening and detection	Specify 30% 2-way overlap	QA CT performance

CONCLUSION

The impact of differentiating between QC and QA activities is an educated laboratorian who is aware that in the absence of one activity, the other is meaningless, and is able to implement effective QC/QA programs. Without confirmatory QA activities, QC efforts could be ineffective and a waste of laboratory resources. All activities must add value to the laboratory product by building in quality and avoiding waste. For example, stain-related QA activities add value by enhancing the detectability and interpretability of abnormal cells. Examples of waste avoided by QC/QA activities include: (1) time spent examining excessively bloody non-gyn preparations that do not include the abnormal cells present in the raw specimen, (2) reduced

screening productivity caused by low contrast staining, and (3) recovering preparations that contain water droplets.

A program of total quality management must reflect the philosophy and policies of each cytology laboratory. Its implementation at the procedures and practices level is the responsibility of the technical staff. Everyone who examines cytologic specimens is not only an interpreter of cytomorphology but also an inspector of the outcome of the contributory processes. Everyone should be sufficiently skilled to assess the quality of specimen collection and preparation from the microscope. As seen in Table 7, there are four combinations of object and image quality. No.1 is goal. Nos. 2 and 3 are common. No. 4 should be rare in terms of the role played by technical processes.

Table 7
COMBINATIONS OF OBJECT AND IMAGE QUALITY

Object	**Image**	
	Satisfactory	**LTO/Unsatisfactory**
Satisfactory	1	2
LTO/Unsatisfactory	3	4

LTO = Less Than Optimal

No. 1 results when everything goes right and is considered to be technically (i.e., a representative, well distributed monolayer of well fixed and well stained cells that is properly imaged) and functionally satisfactory (i.e., may be technically deficient in some respect, but overall still be suitable for its intended purpose [e.g., no light green]). With one exception, technically satisfactory cytologic preparations are also functionally satisfactory. The one exception is body cavity fluids that are so bloody that the erythrocytes crowd out the nucleated cells, thereby preventing cancer cells that might be present in the raw specimen from being present on the slide.

No. 2 can result when the specimen is satisfactory but the staining is deficient, the mounting medium is too thick, the cover glass is too thick, the microscope is dirty, and the microscopical illumination is not optimal (i.e., not Köhler), or any combination thereof. No. 3 can result when the image is satisfactory but the cells have degenerated due to *in vivo* processes (i.e., biology, time, temperature), *in vitro* processes (e.g., normal saline exposure, air-drying, or unsuitable fixative), or any combination thereof. Nos. 2 and 3 are technically deficient in some observable way but functionally satisfactory overall.

No. 4 is unsatisfactory and simply a combination of extreme degrees of the various factors that contribute to Nos. 2 and 3. Unsatisfactory preparations should be reported as such and not diagnosed. However, quoting: "**CAVEAT**: Any abnormal cell is significant regardless of specimen adequacy. The specimen containing abnormal cells must never be classified as unsatisfactory. CLIA '88 states: THE LABORATORY MUST ENSURE THAT DIAGNOSTIC INTERPRETATIONS ARE NOT REPORTED ON UNSATISFACTORY SMEARS. Similarly the specimen with malignant cells can never be classified as other than satisfactory. Additionally, any clinically suspicious lesion must undergo biopsy irrespective of cytological findings."[6]

And finally, conforming to CLIA '88's QC/QA requirements is sufficient to pass inspection, but it is not enough to ensure effective specimen collection and cytopreparation. CLIA '88 is a regulatory dyslexicon that misspells-out minimal unrelated requirements of marginal effectiveness. Compliance fosters complacency, not competence.[45] Total quality management is everyone's job.

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* Complimentary advertisement-free fax copies of References 17-18 and 20-26 are available only from EM Science, 1-800-557-4367.