Troubleshooting Issues in Cytopreparation Resource Document

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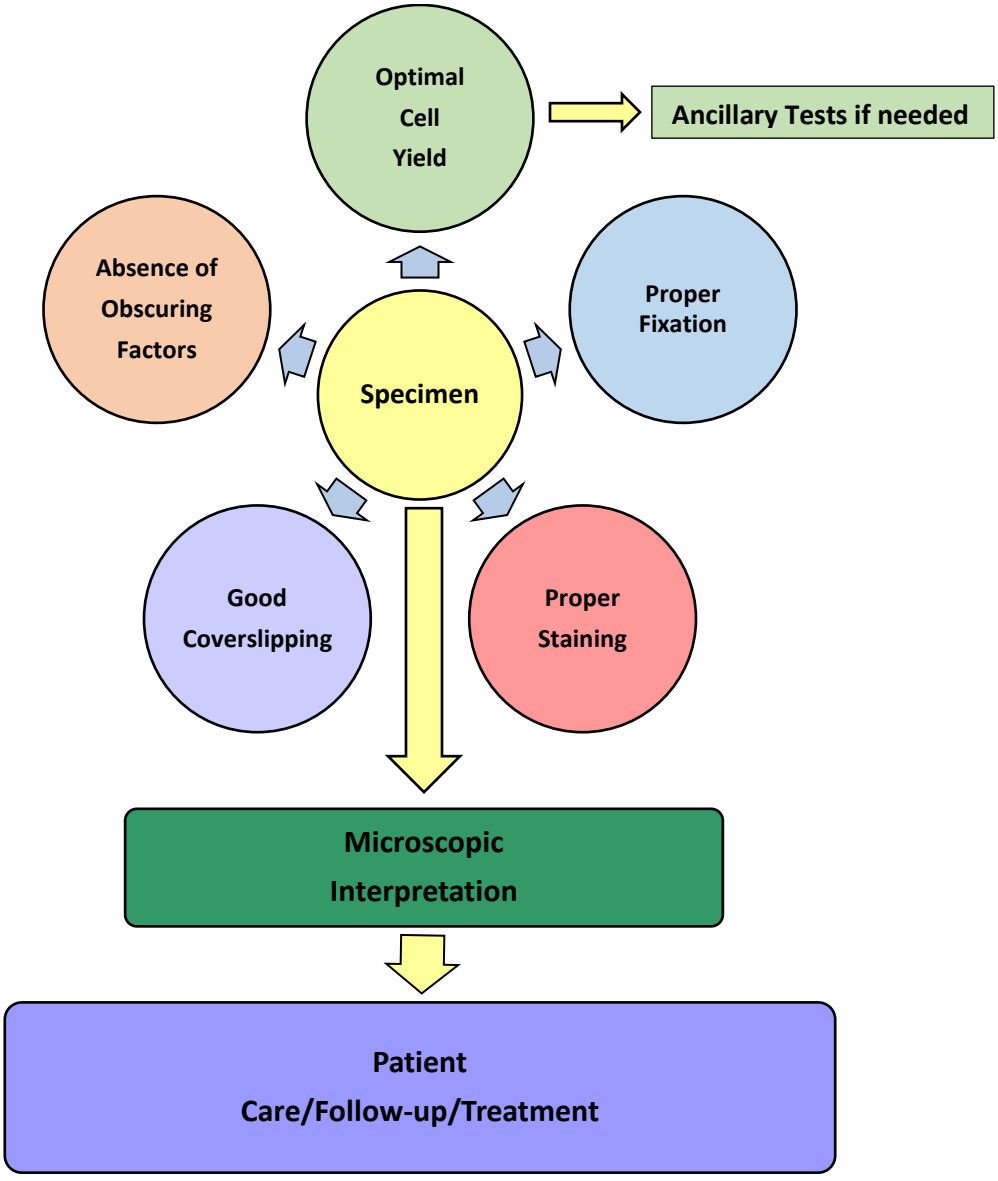
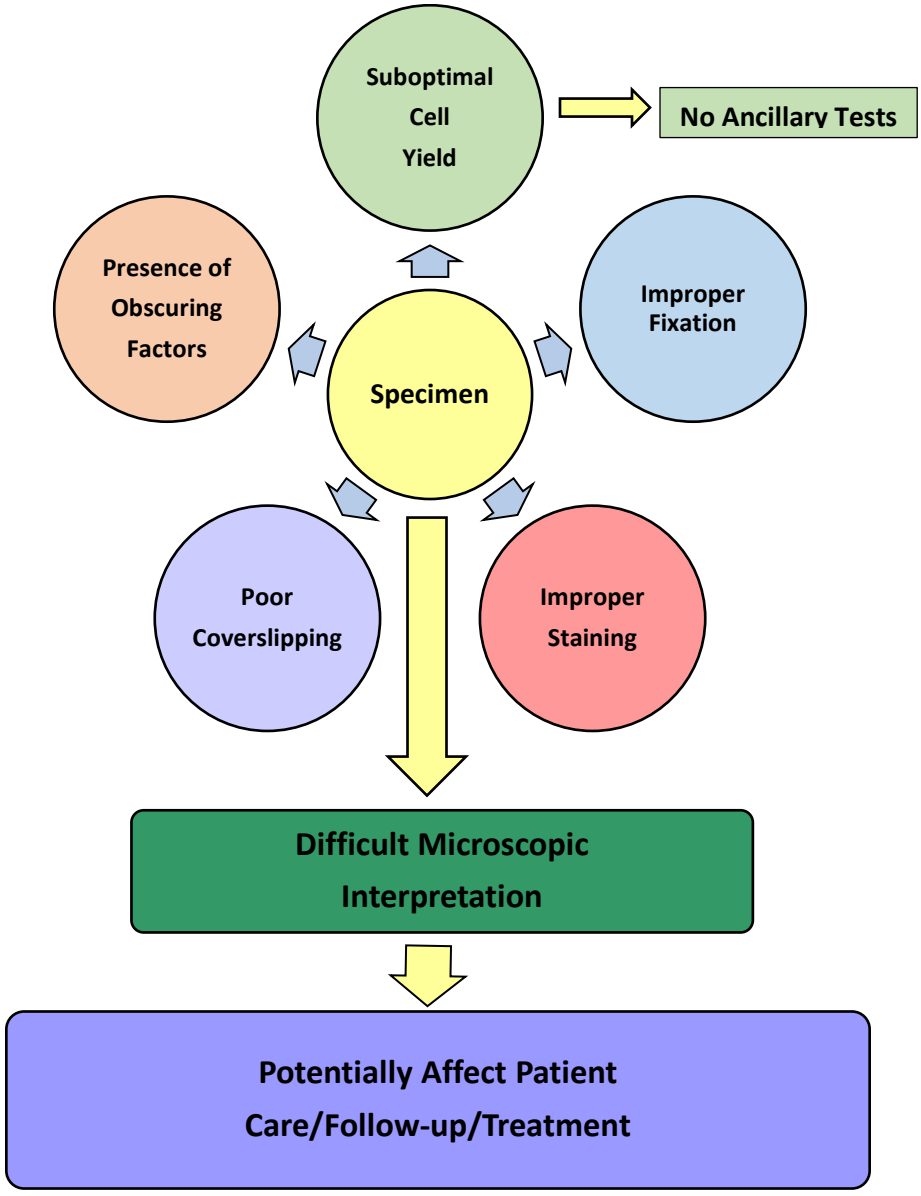
Laboratory procedures and techniques often go along according to protocol with the goal of producing well preserved, well stained gynecologic (Gyn), non-gynecologic (Non-Gyn), and fine needle aspiration (FNA) specimens. The quality of specimen preparation plays an important role in patient care, follow-up and/or treatment depending on the cytologic interpretation. The aim of this document is to serve as a resource tool for troubleshooting various issues in cytopreparation.

Goal of Ideal Specimen Preparation



Specimen processing does not always go as planned. One or more steps may falter and the specimen prepared is not as optimal as it should be.

Potential Specimen Processing Issues



There are potential issues that can affect the specimen prior to submission to the laboratory. Providing in-service education to the clinician/care area staff in regards to any collection, ordering issues, etc. may help to alleviate some of the problems. These issues are as follows:

1. Inadequate specimen volume depending on specimen type, i.e. one cc of body cavity fluid is most likely insufficient for cell block or ancillary tests.
2. Improperly collected specimen, i.e. specimen collected in formalin instead of collecting as fresh specimen.

3. Delay of transporting a fresh specimen or refrigerating a fresh specimen if the specimen cannot be delivered to the laboratory within the laboratory specified time period.
4. Mislabeling of specimens which causes delay in processing.
5. Incorrectly ordered specimens resulting in delay in processing.

Troubleshooting Potential Specimen Processing Issues

Note: This guide applies to Gyn, Non-Gyn, and FNA specimen preparations.

I. Cell Yield

A. Liquid Based Preparations

1. BD SurePath™

Objective: Ensure adequate specimen transfer from specimen vial to centrifuge tube.
Issue: Collection device may impede the syringe from drawing up appropiate specimen aliquot from the specimen vial when utilizing the BD PrepMate™ System.
Remedy: Manually transfer the specified aliquot of specimen from the specimen vial to the centrifuge tube containing 4 ml of the density reagent.

Objective: Ensure adequate number of cells placed on the slide via gravity gradient.
Issue: Insufficient specimen aliquot transferred from centrifuge tube to correlating specimen slide.
Remedy: Check the pressure on the vacuum pump. The vacuum pressure should be between 8-10 Hg. Vacuum pressure that is too low will affect the amount of specimen that will be drawn up in the pipette tip.

Objective: Ensure monolayer of cells on placed on the slide via gravity gradient.
Issue: Large clumps of cells (most often squamous cells in Gyn specimens) with cell overlap.
Remedy: Vortex the cell pellets thoroughly especially the specimens with large thick pellets. Glandular cells will remain intact but the squamous cells will break apart resulting in a monolayer preparation.

Objective: Ensure sufficient number of cells for specimen adequacy and interpretation.
Issue: The processed slide has too few cells and the corresponding cell pellet appears cellular.
Remedy: If the cell pellets are not vortexed prior to placing on the instrument for processing, this may result in an acellular to too few cells processed. The mixing step on the instrument is not strong enough to break the cell pellet apart enough to ensure adequate cell yield.

2. Hologic Thin Prep®

Objective: Ensure sufficient number of cells for microscopic interprepation.
Issue: Non-Gyn specimen slide is sparsely cellular when ample specimen collected and centrifuged cell pellet is visibly adequate.
Remedy: The supernatant may not have been completely poured off resulting in

dilution of the cell pellet. Centrifuge the specimen again and decant the entire supernatant.

Objective: Ensure adequate and even distribution of cells in the cell spot area of the slide.
Issue: Presence of a halo effect on the slide where the cellular material is along the outer edge of the cell spot.
Remedy: This usually results from dense specimens where only the outer edge of the cellular material is transferred from the filter to the slide. Dilute the specimen and reprocess.

B. Cytocentrifugation Preparations (Cytospin)

Objective: Ensure single monolayer deposit of cells via cytocentrifugation.
Issue: Cellular material on this slide is thick with layer(s) of overlapping cells.
Remedy: Check specimen cellularity by placing 1 drop from cell pellet on a slide, mix with 1 drop of methylene blue and coverslip. Examine microscopically. If there are more than 60 cells per high power field, dilute the specimen with a balanced salt solution and add 1 drop of specimen to the cytofunnel. This may vary depending on whether a single or double cytofunnel or a megafunnel is utilized.

Objective: Even dispersion of cells in a monolayer deposition on the slide.
Issue: Circular rim of cells (halo) on the slide with the center of cells absent.
Remedy:
1. Too small volume of specimen added to the cytofunnel so the cells present formed a circular rim. Add more specimen to the cytofunnel and reprocess the cytospin.
2. Cellular material on slide is too thick so center of cytospin slide flaked off upon fixing in 95% alcohol. Dilute the specimen with balanced salt solution and reprocess the cytospin.

C. Direct Smears

Objective: Even dispersion of cellular material on a glass slide with minimal cell overlap.
Issue: Direct smear is thick with multiple overlapping cells.
Remedy:
1. If the drop of specimen appears thick, quickly prepare more than one slide from the original slide which will aid in diluting the specimen. For FNAs, one slide can be air dried for Diff-Quik stain allowing for specimen adequacy and the second slide can be placed in 95% alcohol (or spray fixed) for Papanicolaou (Pap) staining. Prepare additional slides as needed.
2. For Non-Gyn specimens, centrifuge the specimen, pour off the majority of the supernatant leaving some as a diluent or dilute with a balanced salt solution and then prepare direct smear from one drop of specimen.

Objective: Adequate cellular material evenly dispersed on a glass slide.
Issue: Too few cells present for a microscopic interpretation.

Remedy: Note: some Non-Gyn specimens are not suitable for preparing direct smears as the cell yield is typically low. These are primarily cerebrospinal fluids and urines. Select other processing methods better suited for concentrating low yield specimens.

1. For FNA specimens if one drop of specimen placed on slide looks too thin or clear, add one or two more drops on the slide and then prepare the smear.
2. For Non-Gyn specimens, centrifuge the specimen forming a concentrated cell pellet. Pour off the supernatant and prepare the smear directly from the pellet.

II. Cell Fixation

A. Liquid Base Preparations

Note: For Gyn specimens and Non-Gyn specimens collected directly in the appropriate preservative vial for the specific liquid based preparation method, proper fixation is usually not an issue.

1. SurePath™ and Thin Prep®
   Objective: Ensure proper fixation of Non-Gyn specimen upon processing.
   Issue: Liquid based preparation specimen exhibits air drying and cellular degeneration.
   Remedy: a. Specimen should be processed upon receipt in the laboratory following the specific preparation protocol for placing the specimen into the appropriate preservative for the liquid based preparation method. Once the specimen is in the preservative, processing can be done according to laboratory schedule. The sample should be refrigerated until processed.

   b. If the specimen cannot be placed in the specific liquid based preservative fluid upon receipt in laboratory, refrigerate the specimen to maintain specimen integrity.

2. BD SurePath™
   Objective: Ensure prepared slide is well fixed and prepared for automatic staining by PrepStain™ instrument.
   Issue: Processed and stained slide exhibits air drying and cellular degeneration.
   Remedy: a. Check level of Alcohol Blend Rinse. If the alchol level is insufficient, the staining step may not have left the finished slide in alcohol thus potentially allowing air drying and degeneration to occur. Refill the appropriate solution bottle and reprocess the slides.
   b. Ensure the appropriate stain line is in the appropriate stain bottle. If the alcohol line is switched with the Tris buffered DI Water line, air drying, cell degeneration and other inappropriate staining will be observed. Run the "Clean-Up" program which will back flush the solutions into the appropriate stain solution bottles. Do not switch the lines until this step is complete. Run the "Utilities" program to ensure all stain lines and

corresponding syringes are completely clean and free of the incorrect stains. This step may need to be repeated if there is residual stain in the lines or syringe. Now place all the staining lines into the correct stain solution bottles. Reprocess the slides. The instrument will have to be re-primed.

3. Hologic Thin Prep®
   Objective: Ensure slide is well fixed and prepared for staining.
   Issue: Processed and stained slide exhibits air drying and degeneration.
   Remedy: Check 95% alcohol level in the fixative bath on the instrument. Slide can air dry if alcohol level is insufficient to cover the cells. Refill the fixative bath and reprocess the slide.

B. Cytocentrifugation Preparations (Cytospins)

Objective: Ensure well preserved cells prepared for Pap staining.
Issue: The stained cytospin slide exhibits air drying and degeneration.
Remedy:
1. Utilize the appropriate fixative such as Shandon Collection Fluid when placing appropriate volume of sample into cytofunnels. Specimen alone will start the air drying process while undergoing processing in the cytospin.
2. Avoid leaving the cytospin slides in the sealed head for an extended period of time after the instrument stops. The cytofunnels should be capped forming a "wet chamber" but eventually air drying will start.
3. Upon completion of cytocentrifugation, disassemble the cytospin apparatus and immediately place the slides for Pap staining in a container of 95% alcohol or spray fix immediately.

C. Direct Smears

Objective: Ensure well preserved cells on prepared smear ready for Pap staining.
Issue: Cellular material on smear exhibits air drying and degeneration.
Remedy: The smear should be placed immediately in 95% alcohol and remain in this solution for a minimum of 10-15 minutes before staining. The smear can be spray fixed as an alternative to wet fixing in 95% alcohol. The spray fixed slide must be placed in 95% alcohol for a minimum of 15 minutes to remove the spray fixed coating on the cells prior to Pap staining.

III. Cell Staining

A. Liquid Based Preparations – BD SurePath™

Objective: Ensure desired staining hues are achieved during processing on PrepStain™ Instrument.
Issue: Processed slides exhibit incomplete staining, i.e. no nuclear staining but cytoplasmic staining appropriate etc.
Remedy: Check stain solution bottles levels. If stain solution bottle is too low or empty, this will result in inappropriate staining. Replenish the stain solution, re-prime the instrument and reprocess the slides.

Objective: Ensure appropriate staining of all slides processed on the PrepStain™ instrument.

Issue: One of every four slides are not stained appropriately.

Remedy: Some debris may be clogging one of the four staining bundles. Note which slide position the affected slides were in to determine which staining bundle has the clogged line. Place the staining bundle in a container of distilled water. If there is no distilled water uptake in one of the lines then this is where the clogg may be. Take the small guide wire supplied with the instrument and extend up into each small stain line. There are two sizes of guide wires. The wider wire is for the aspirator and the thinner wire is for the stain line. Extend the wire as far up into the line as possible and move up and down several times. Rimming all lines in the staining bundle will ensure all lines are free of clogs. Be careful not to move the staining arm while the instrument is on as this will interfere with the its movement positions while processing. After rimming the stain line with the guide wire, place the staining bundle in distilled water again and ensure that the distilled water is flowing smoothly through the lines. Reprocess the affected specimens.

Objective: Ensure all processed slides exhibit the desired staining intensity.

Issue: Stained slides exhibit inappropriate nuclear and cytoplasmic staining.

Remedy: Check to see that the appropriate stain lines are in the appropriate stain solution bottles and the stain volumes are sufficient. If the stain lines are placed in the wrong staining solution, this will affect the appearance of the slide as will running out of a staining solution during the staining process. Run the "Clean-Up" program which will back flush the solutions into the appropriate stain solution bottles. Do not switch the lines until this step is complete. Run the "Utilities" program to ensure all stain lines and corresponding syringes are completely clean and free of the incorrect stains. This step may need to be repeated if there is residual stain in the lines or syringe. Now place all the staining lines into the correct stain solution bottles. Reprocess the slides. The instrument will have to be re-primed. If a stain solution is low or empty, replace the solution, re-prime the instrument and reprocess the slides.

Objective: Ensure all processed slides exhibit the desired staining intensity.

Issue: The stained slides have a somewhat muddy and overstained appearance.

Remedy: This issue is usually seen quickly if the operator periodically checks the instrument while in operation. The instrument is not suctioning off residual stain from each step resulting in all the solutions backing up in the settling chamber and most often overflowing onto the rest of the slide and slide rack. One of two problems cause this. Either the waste container is full or the waste container lid is not on tight. Empty the waste container into appropriate waste container and ensure the lid is on tight after discarding waste. Reprocess the slides.

Objective: Ensure all processed slides exhibit the desired staining intensity.

Issue: The processed slides exhibit bleaching or fading of the stain around the edges and sometimes through out the slide. At the end of each run of 12 slides, a set volume of Alcohol Blend Rinse is placed in the settling chamber to keep the cells

wet. If this alcohol is not removed in a timely manner, the alcohol will start bleaching the stain. At end of staining run, invert the slide rack discarding the alcohol into the appropriate waste container. Remove the settling chamber and dip each slide 5 times in 100% alcohol to remove excess water and then place in xylene for a minimum of 5 minutes prior to coverslipping to ensure adequate clearing.

B. Automated Staining

Note: There are several automated stainers on the market from various vendors. This guide offers a general issue and remedy. Each model may have its own unique issue(s) that the laboratory should be aware of.

Objective: Ensure consistent well stained cellular material on the prepared slides.
Issue: Suboptimal staining of cellular material.
Remedy:
1. Check solution level in staining dishes. If the solution volume in the staining dish is too low or empty, the resulting stain will be suboptimal or absent. Refill the staining dishes to the appropriate volume. Follow lab protocol for destaining and restaining the slides.
2. Check the staining program to ensure that no staining steps were accidentally deleted. If there are missing steps/staining times, reset the program. Run an empty basket through a staining run to confirm that the program is operating as it should. Follow lab protocol for destaining and restaining affected slides.
3. Check the instrument periodically while it is operating to see that it is running as designed. Occasionally a basket may be dropped or not picked up by the staining arm. Depending on type of operational error and time lapse, the staining process may be continued manually or the slides may need to be destained and restained per lab protocol which is usually the wiser option.

C. Manual Staining

Objective: Ensure consistent, well stained slides.
Issue: Some slides exhibit good staining while others demonstrate inconsistent staining.
Remedy: Follow staining steps and times as outlined in the staining operating procedure. If the slides are left in various solutions for inaccurate times, the staining pattern will be inconsistent. Inconsistent staining patterns may also result when more than one technologist is performing the stain process. Stress the use of a timer even when one step may be 10 seconds.

D. General Pap Staining Guidelines

1. Strictly follow stain/solution change schedule per lab protocol and staining method utilized. Filter all stains (Hematoxylin, Eosin and Orange G) daily, and change and rotate other solutions as required. Determine at what interval to change and replenish all stains as the stain quality will become exhausted and loose staining intensity.
2. If white, cloudy or hazy film appears on coverslipped slides, check the xylene for beads of water. Xylene and water are not miscible. Keep 100% alcohols fresh to avoid water from being carried over into the xylene. Place slide in xylene to remove the coverslip.

Place the slide in 100% alcohol for minimum of 5 minutes to remove the water and then back to xylene for recoverslipping. If the stain is adversely affected, the slide may need to be destained and restained. Keep staining dishes covered they are not in use.

3. For manual staining, avoid carryover of one solution into a different solution by draining excess solution from rack when lifting out of solution dish and then blotting with a towel prior to placing in next solution. This will help keep from diluting the various solutions.

V. Cover-slipping

A. Automated Coverslipping

Objective: Ensure well coverslipped slides with no receding.
Issue: Receding of mounting media on coverslipped slides.
Remedy: 1. This can occurs when there is too much xylene in the mounting media when the slide is coverslipped. The xylene will evaporate when the slide dries leaving air bubbles or receding. If the mounting media dispensing nozzle rests in a container of xylene, ensure it it not submerged in xylene as it will draw the xylene up into the nozzle and then the excess xylene will be dispensed on the slide. As long as it rests above the xylene, the fumes from the xylene will keep the mounting media from building up. The nozzle should be cleaned according to lab protocol or manufacturer's instructions.

2. If the slide is too thick or has thick areas, it is difficult for an automatic coverslipper to adequately coverslip the slide. Most coverslippers work best on a thin, monolayer slides. The volume of mounting media can be increased but this type of slide may need to be manually coverslipped.

Objective: Ensure well coverslipped slides with no excess mounting media.
Issue: Slides are stuck to the slide trays.
Remedy: Check the volume of mounting media dispensed. The volume may need to be decreased. If the volume is too high for the amount of specimen to be covered, the excess will seep out from under the coverslip. Clean the bottom and slides of the slides with xylene. Metal slide trays can also be cleaned.

Objective: Ensure the coverslipper works efficiently without breaking slides or coverslips.
Issue: Slides are getting hung up in the coverslipper and not being coverslipped properly.
Remedy: Mounting media can build up on all surfaces of the coverslipper. Clean all moving surfaces where the slide may sit or touch for coverslipping with xylene. Clean all receiving baskets with xylene. Soaking the baskets overnight in xylene after each day of use assists in keeping the mounting media from building up in the slide wrungs. Adhering to daily cleaning maintenance and all scheduled preventive maintenance will aid in keeping the instrument operating efficiently.

B. Manual Coverslipping

Objective: Ensure well coverslipped slides with no receding.
Issue: Receding of mounting media on coverslipped slides.
Remedy: Any excess xylene on the slide after coverslipping will evaporate when the slide dries leaving air bubbles or receding. Drain the excess xylene off the slide prior to addition of mounting media and coverslip. There is adequate volume of

xylene left on the slide to mix with the mounting media allowing for ease of coverslip placement on the slide.

Objective: Ensure well coverslipped slides with no air bubbles.
Issue: Coverslipped slides often have air bubbles obscuring the cells.
Remedy: Another cause of air bubbles besides the evaporation of excess xylene is the entrapment of air between the coverslip and the slide. Once the mounting media is placed on the slide, the coverslip should be placed on the slide at an angle starting at one edge of the slide. Gently lower the coverslip on the slide following the direction of the angle. Allow the mounting media to start covering the slide by capillary action. When the mounting media has reached the end of the slide, the coverslip should be on top of the slide. If air bubbles are still present, they can be drawn out by gently lifting the coverslip until the air bubble disappears and then slower relower the coverslip.

VI. Obscuring Factors

A. Liquid Based Preparations

1. BD SurePath™

Objective: Ensure well prepared slides with minimal to no obscuring blood.
Issue: Processed SurePath™ Non-Gyn slides have obscuring blood which interferes with the microscopic examination of the specimen.
Remedy: Use of the appropriate CytoRich™ fixative will alleviate this problem. CytoRich™ Red will lyse all of the red blood cells where as CytoRich™ Clear will allow some red blood cells to be visible but not obscure the cells. Use of these fixatives is specimen dependent, i.e. detecting red blood cells can be diagnostic in some specimens. The specimens must be fixed in these solutions for the appropriate amount of time or other issues will result such as air dried blown up appearing cells. Follow all manufacturer recommended processing steps and times for consistent results

Objective: Ensure well prepared slides with minimal to no obscuring mucus.
Issue: SurePath™ slides processed on bronchioloalveolar lavages and bronchial washings often contain obscuring mucus.
Remedy: These specimens often contain abundant mucus. The use of a mucolytic agent is highly recommended prior to placing the specimen in either one of the CytoRich™ fixatives. Double the volume of the mucolytic agent to the specimen volume and vortex for 30 to 45 seconds. Centrifuge the specimen, decant the supernatant and proceed with the fixation steps.

2. Hologic ThinPrep®

Objective: Ensure well prepared Gyn slides with adequate endocervical cell component.
Issue: Processed Gyn slides are yielding a higher specimen inadequate rate due to no endocervical cell component.
Remedy: As the ThinPrep processor utilizes an upside-down filtration method, any endocervical mucus in the sample will be drawn up to the filter followed by the epithelial cells. Endocervical cells may become trapped in the mucus.

Treat the specimen with glacial acetic acid to remove the mucus. Centrifuge the specimen, decant the supernant, vortex and add 30 ml of a 9:1 Cytolyt® to glacial acetic acid solution to the specimen and mix the specimen by inverting the specimen tube 5 times. Centrifuge the specimen, decant the supernatant and vortex to resuspend the cell pellet. Add sufficient quantity of Preservcyt® solution to the specimen tube to bring the total volume up to 20 ml. Mix the sample and process per lab protocol. This same process can be followed for Non-Gyn specimens with excessive mucus.

Objective: Ensure well prepared Non-Gyn slides with minimal to no proteinaceous debris.
Issue: Body cavity fluids processed by ThinPrep® method exhibit excess Proteinaceous debris resulting in lower cell yield.
Remedy: The cell pellet can further be washed in saline following the initial wash in Cytolyt® which will aid in removal of the excess protein.

A. Cytocentrifugation Preparations (Cytospins)

Objective: Ensure well prepared cytospin slides maximizing the cells of interest.
Issue: Prepared cytospin slides exhibit abundant blood with too few cells present.
Remedy: Utilization of a hemolytic agent such as saponin before preparation of the cytospin slides optimizes the cell yield and morphology. Use a 1% solution of saponin as the hemolytic agent followed by a 3% solution of calcium gluconate as an antihemolytic agent. A 2:3 ratio of saponin to calcium gluconate is sufficient lysing the red blood cells but maintaining cellular integrity. Add the saponin to the specimen and vortex. Add the calcium gluconate and vortex. Centrifuge the specimen, decant the supernatant and prepare the cytospins. These solutions must be kept refrigerated or potential fungal growth can form. The expiration date is one week from the date the solution is prepared. Another hemolytic agent is Carnoy's solution. The slide is placed in this solution after the slide is prepared. The downside to using this solution is the potential for cell loss. Cell loss is probably more of an issue with sediment smears than cytospins.

Objective: Ensure well prepared slides with minimal proteinaceous debris.
Issue: Cytospin slides exhibit a rim of cells trapped in proteinaceous debris.
Remedy: Body cavity fluids tend to have a fair amount of protein which can affect the Cytospin preparations. Excess protein can cause the center of the cytospin slide to wash off when it is placed in 95% alcohol for fixation after preparation. Addition of a balanced salt solution to the specimen will help to get rid of the excess protein. Centrifuge the specimen, decant the supernatant and process the cytospins. The balanced salt solution can be added to the cell pellet then centrifuged again.

B. Direct Smears

Objective: Ensure well prepared direct smears with single cell layer and intact cells.
Issue: Cells on the direct smear are broken apart and exhibits both smear and crush artifacts.

Remedy: The proper technique used in preparing the smear will alleviate this artifact. Putting too much pressure on the slides when smearing will result in the crush artifact as well as smear artifact. Place the desired amount of specimen on the slide. Place a second slide on top and allow the specimen to spread by capillary action and then gently pull the slides in opposite direction until the end of the slide is reached.

C. Obscuring Factors Common to All Preparation Methods

Objective: Ensure well prepared and stained slides free of debris.
Issue: Presence of graphite (pencil lead) on processed slides obscures cellular detail.
Remedy: A standard pencil may shed graphite upon staining. A harder lead pencil (#2 pencil) can reduce this shedding but will not necessarily eliminate it. Use a Tissue Tek marking pencil or a xylene resistant pen to write on the frosted end of the glass slide. For using a xylene resistant pen, write on the label of a blank slide and stain it to verify the indelible ink used is solvent resistant.

Objective: Ensure well prepared and stained slides free of other cells.
Issue: A marked number of anucleated squamous cells are seen on the slides such as cerebrospinal fluids, etc.
Remedy: This usually occurs when the preparatory technician is not wearing gloves when handling slides such as writing the patient/specimen identification on the frosted end of the slide before processing. Proper use of gloves will eliminate this artifact. Touching the skin with gloved hands then touching the slides can transfer anucleated squamous cells to the slides. Change gloves often when processing specimens.

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