

Application of Immunohistochemistry to Cytology

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• **Context.**—The uses of monoclonal antibodies via immunohistochemistry have been reported frequently within the literature using various methodologies with applications to cytology specimens. The direct application of immunohistochemistry to cytology may have a variety of pitfalls that the general pathologist familiar with its application to histology may be unaware of when applying it prospectively to patient specimens.

Objective.—To review common pitfalls when applying immunohistochemistry to cytology specimens and to suggest approaches to the more common differential dilemmas that apply to a variety of cytology specimens that could be seen in a general pathology practice.

This article attempts to avoid the repeating of prior organ-specific immunohistochemistry, with the exception of the pancreas, and instead tries to concentrate on our more common cytology sample types in which we are trying to differentiate between metastatic and primary malignancies, such as with effusions and fine-needle aspirations (FNAs) of lymph nodes, lung, and liver. We refer readers to each of the other organ-specific discussions for a more detailed review of expected staining of tumor types that originate from those specific organs. Much of the emphasis in this article is on problems that arise when applying immunohistochemistry within the cytology laboratory and the need for routine quality surveillance. Our approach is to attempt to select minimal panels for a limited sample quantity of the more common diseases that general surgical pathologists are likely to see.

The references given are not meant to be an exhaustive review of the literature but rather information that we have found helpful from abstracts, conferences, and texts, along with the usual peer-reviewed articles in approaching immunohistochemistry's application to cytology specimens. We discuss neither each sensitivity/specificity nor predictive value for every antibody mentioned because those statistics are always in a state of flux dependent on manufacturer, lot number, and methodology and are dependent on the variation and number of specific diagnoses tested and published. Instead we would encourage each reader to routinely use an evidence-based databank of antibodies, as we discuss later. Thus, we hope each reader will find a few practical pieces of information herein to apply to his or her daily practice of cytopathology.

Data Sources.—The authors' own experiences of applying immunohistochemistry to cytopathology specimens within an academic setting along with supportive data from the literature.

Conclusions.—Immunohistochemistry can be used to increase the predictability of a cytology diagnosis if care is taken with the cytology sample preparation methodology and there is judicious use of select monoclonal antibody panels to support a specific cytology diagnosis. Up-to-date evidence-based antibody databases should be used when selecting antibody panels.

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BASICS WHEN APPLYING IMMUNOHISTOCHEMISTRY METHODS IN THE CYTOLOGY LABORATORY

Many cytopathologists routinely do surgical pathology and are quick to adapt ancillary techniques learned in histology to cytology specimens.¹⁻⁴ Unfortunately this jump from a histology section to a cytology specimen for immunohistochemistry is frequently done on a case by case basis without first considering major differences in the specimen preparation that could affect the final interpretation of the immunohistochemistry and ultimately the diagnosis for patient care. Despite published warnings of the dangers of constantly varying methodology on both intralaboratory and postlaboratory testing, we seem to forget the warnings when pressed by the clinician to be as specific as possible with our diagnosis using less and less material.¹⁻⁵ Thus, we would like to begin with common mistakes we and others have found when reviewing publications as well as when consulting on evaluations performed at a variety of outside clinical service laboratories when immunohistochemistry has been applied to cytology specimens. Because we routinely use immunoperoxidase (IP) methodology, we abbreviate generic immunohistochemistry as IP throughout this article unless we refer specifically to the application of monoclonal antibodies to non-formalin-fixed cytology specimens in which case we refer to immunocytochemistry (IC).

The first and probably the most common mistake is improper control specimens and noncustomized antibody concentrations when applying IP to a nonparaffin formalin-fixed specimen (IC). There appears to be a giant leap of faith that it is correct to use an alcohol-fixed, Papanicolaou-stained smear, a destained modified Giemsa air-dried direct smear, a cytospin preparation, or a thin-layered technology slide for immunocytologic testing while using formalin-fixed paraffin sections for the negative and positive controls. Often this is because the pathologist (or referring clinician) has seen a methodology published but does not note that it used a small "n" of samples in its series using the new protocol. Unless the laboratory has a large bank of similarly prepared cytology material for

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positive and negative IC controls (non-formalin-fixed paraffin cytology specimen by the exact same preparation method as the current sample being tested), then criteria for having "proper controls" is not met and any interpretation of IC results should be suspect. It is our observation that this is not mentioned within the materials and methods section of many abstracts or publications in the cytology IC literature (and should be "on the radar" for peer reviewers when other than formalin-fixed cell blocks are used). Controls must be of similarly prepared material for quality assurance. This is usually difficult for the immunohistochemistry laboratory in regard to obtaining sufficient variety and number of controls as well as in meeting storage requirements of cytology specimens.⁶ The antibody concentrations are not always customized for cytology specimens; thus, the antibody concentrations may be much stronger than needed for a true IC specimen (non-formalin-fixed, paraffin-embedded), resulting in a potential for false positives from excess antibody.

Reports suggest some antibodies have been found to become false-negative with alcohol-fixation such as estrogen receptor and S100.⁷ Yet others have reported success with estrogen receptor on alcohol-fixed material and this variation may be based on specific antibody manufacturer and other methods.⁸ Thus, we agree with most immunohistochemists that if one does not have a good cell block then one should not proceed with consideration of IP staining.⁹ Instead, one should make a cytologic diagnosis that indicates that further sampling is indicated so that the best and most reliable IP staining can be performed. We disagree with advice to use "nonadhesive" slides for cytology so that cell transfer technique can be performed for IC staining.¹⁰ Our experience suggests that many cells are lost when using nonadhesive slides dropped into ethanol or other liquid media. Variable cytology preparation methods (air-dried vs fixed, direct vs cytospin, etc) have been reported as better for certain antigens; however, general pathologists should ask themselves if they or their laboratory can keep all of these variables straight and will they have a high enough volume of testing to make results predictable. The answer is "probably not." Table 1 summarizes the pros and cons of various cytology preparation methods as they apply to IP.

The second common mistake is not carefully examining the cytology laboratory's cell block methodology, which could negatively impact immunohistochemistry interpretation. Early on in our laboratory we noted a poor correlation of some nongynecologic formalin-fixed, paraffin-block sections in comparison with later histology tissue samplings of the same tumor. At that time we were using a warm agar methodology for preparing the cytology pellet before placing in formalin. Subsequent to this finding we changed to collection in a nonfixative solution with addition of thrombin and plasma and formation of a cytology pellet prior to formalin fixation and embedding. This resulted in a markedly improved correlation of IP stains between cytology and surgical pathology specimens.^{11,12} Numerous other methodologies have been published, some using commercial agents for producing a cytology pellet. Prior to introducing any new cell block method, we would advise comparison of IP stain results between the cytology IP profile and later or concomitant surgical pathology sample IP profile to be certain that the methodology adopted does not adversely affect IP staining or interpretation.

Table 1. Pros and Cons of Cytology Preparation Methods	
Pros	Cons
Direct Smear	
May do when no extra material No wet material needed Can use what available slides were initially obtained (no expense to extra preparations)	Background artifact severe Panels unlikely Different antibody levels needed Prior staining or ethanol may affect results
Cytospins	
Useful with limited material Panels possible	Background artifact Different antibody levels needed Extra "wet" material needed
Monolayer Preparations	
Possibly decreased background Extra material frequently available and easily stored	Different antibody levels needed Extra "wet" material may still be needed Ethanol in fixative may interfere with some antigen
Cell Block	
Immunohistochemistry laboratory can handle like routine material with proper controls Material easily stored	Limited cellular specimens cannot be used Methodology of cell block preparation must be tested

A third common problem that can occur with surgical pathology, but is more common in cytology because of limited sample size, is the ordering of too few stains (1 isolated stain or an inadequate IP panel). We always attempt to avoid staining with just 1 positive stain to support an uncertain cytologic diagnosis, which would only bias us into favoring our preheld diagnosis. This is further discussed and illustrated later when we have used selective panels for differential diagnoses. It is always helpful to see not only positive but also negative antibody results within a given IP panel. Thus, one should not forget Swanson's editorial "tenets"¹³:

1. Immunohistochemical analyses can only be interpreted in the context of an informed, carefully considered clinical and histologic/cytologic diagnosis.
2. Single immunostains are unlikely to provide a specific diagnosis, even within a limited differential diagnosis.
3. Panels of immunostains are not, in and of themselves, specific for given diagnoses; rather, reproducible immunoprofiles have distinct relative predictive values for different diagnostic alternatives.

Caution must also be exercised, just as with tissue samples, when excessive acute inflammation or when extensive cell necrosis is identified. Publications are mixed in regard to validity of interpretation in these situations.¹⁴ Other than the common pitfalls mentioned previously, other technical factors for the cytology laboratory to consider if they should choose to use direct smears or monolayer technology prepared slides is avoiding protease digestion for non-formalin-fixed material. For routine slides it digests cells off the slides and it is mainly useful for exposing antigens when formalin fixed. The same can be said for avoiding other pretesting methods, such as mi-

Table 2. Antibody Profile for Differentiating Glandular-like Cell Groups in Effusions or Other Metastases*

Likely Origin	Calretinin	CEA	CA 19-9	CA 125	ER/PR	TTF-1	PSA
Mesothelial	+	—	—	?	—	—	—
Lung	—	+	—	—	—	+	—
Breast	—	?	—	—	+/-	—	—
Colon	—	+	—	—	—	—	—
Pancreas	—	+	+	?	—	—	—
Ovary	—	+/-	—	+	+/-	—	—
Prostate	—	—	—	—	—	—	+

* CEA indicates carcinoembryonic antigen; ER/PR, estrogen receptor/progesterone receptor; TTF-1, thyroid transcription factor 1; PSA, prostate-specific antigen; +, suggests greater than 70% are positive; —, suggests greater than 70% stain negative; ?, suggests could easily be positive or negative (35%–65% positive staining); and +/-, dependent on subtype of histology from that organ.

crowaving and so forth, as antigen expression routinely does not need increasing in non-formalin-fixed preparations. Recent reports from a variety of quality assurance programs have revealed considerable variation in quality of staining, as well as in interpretation of the IP stains even with histologic specimens.^{15–18} These variations are often related to variations in methodology, antibody vendor, and lot number with an apparent tendency to overcall weak or nonspecific staining (false positives). It behooves all laboratories performing IP, especially if the result changes patient management, to know from a group of standard samples/controls that recent laboratory changes are not responsible for misinterpretation of IP staining as well as to frequently use interlaboratory programs for quality assurance surveillance of testing validity.

Lastly, it should be remembered that the diagnosis of benign versus malignant must be based on the cytology or the histology. Currently, there remains no one antibody that correctly predicts a cell's malignant potential 100% of the time. On the other hand, the predictive value for malignant potential does improve in a panel combined with newer prognostic markers combined with the clinical features when placed alongside the cytologic/histologic appearance.

Causes of false-positive results include

- Crushed and degenerated cells or marked necrosis
- Acute inflammation in background (neutrophils contain peroxidase)
- Antibody titer too high
- Less specific antibody (vendor or lot variation)

Causes of false-negative results include

- Usually technical problems (ie, wrong antibody, missed procedural step, etc)
- Ethanol fixation for S100 or estrogen/progesterone receptor (possibly others)
- Antibody sensitivity variation (vendor or lot differences)

USEFUL IP PANELS USING MORE COMMON ANTIBODIES

We avoid “predetermined” panels and tend to base the IP panel decision on the cytologic differential diagnosis as combined with the pertinent clinical information plus consideration of the quantity of material available to test. One should consider the following:

- History: patient sex, age, lesion body site, and previous tumor history
 - First: compare with old material if available
- Cytologic characteristics
- Derive a “tight” differential (we often use the DeMay *Gut Course* of cell types¹⁹)

- If the first custom panel does not contribute then:
 - Widen the differential or seek consultation, then select additional IP stains (if material available) and/or obtain more material (advise histologic sampling)
 - Remember time as well as money may be of the essence (do not try to “make a silk purse from a sow’s ear” by repeating stains on a nondiagnostic sample)

COMMON CYTOLOGY CASE EXAMPLES

First, we need a disclaimer that is often missed when applying published IP stain results to specimens prepared in one’s own laboratory. There are many producers of antibodies, variation in antibody lots, and variation in methodologies that can influence the results. The following examples are based mainly on our cytology service’s IP experiences using our IP laboratory’s available antibodies and methodology combined with a variety of published results with specific journal references, with 2 general reference immunohistochemistry texts,^{20,21} or with a Web-based databank that we tend to routinely use. One should remember that the same staining profiles may not have the same predictive value for one’s laboratory results because of differences in methodology or antibody manufacturer.

Pleural Effusion for Exclusion of Malignancy

Probably the most common samples in the cytology laboratory that undergo evaluation by IP are effusions (pleural, pericardial, and ascites). Fortunately, we usually receive a large volume so that cell blocks are routinely available. Epidemiologic information as well as cytologic texts suggest that adenocarcinoma of unknown primary in pleural effusion of adult men is most likely of lung origin. In women, the most likely origin is breast with lung a close second.²² These statistics can probably also be counted on for the differential of adenocarcinoma of unknown primary in pericardial effusions. For ascites with adenocarcinoma of unknown primary in women the source is most likely ovarian, whereas in men it is probably gastrointestinal cancers. However, despite being armed with these “statistical odds,” for any one patient knowing that prognosis and more importantly what treatment regimen is advisable, only IP staining currently offers additional information to the many cytologic look-alikes. This additional IP information, although not necessarily conclusive,²³ can also be helpful to clinicians who are faced with costly²⁴ and time-consuming arrays of possible targets for imaging and invasive procedures and thus can assist in narrowing the search for the primary.

Table 2 contains the common stains that we use for malignant effusions with a “glandular” cytologic appearance that can be used with effusions or suspected metastases elsewhere.

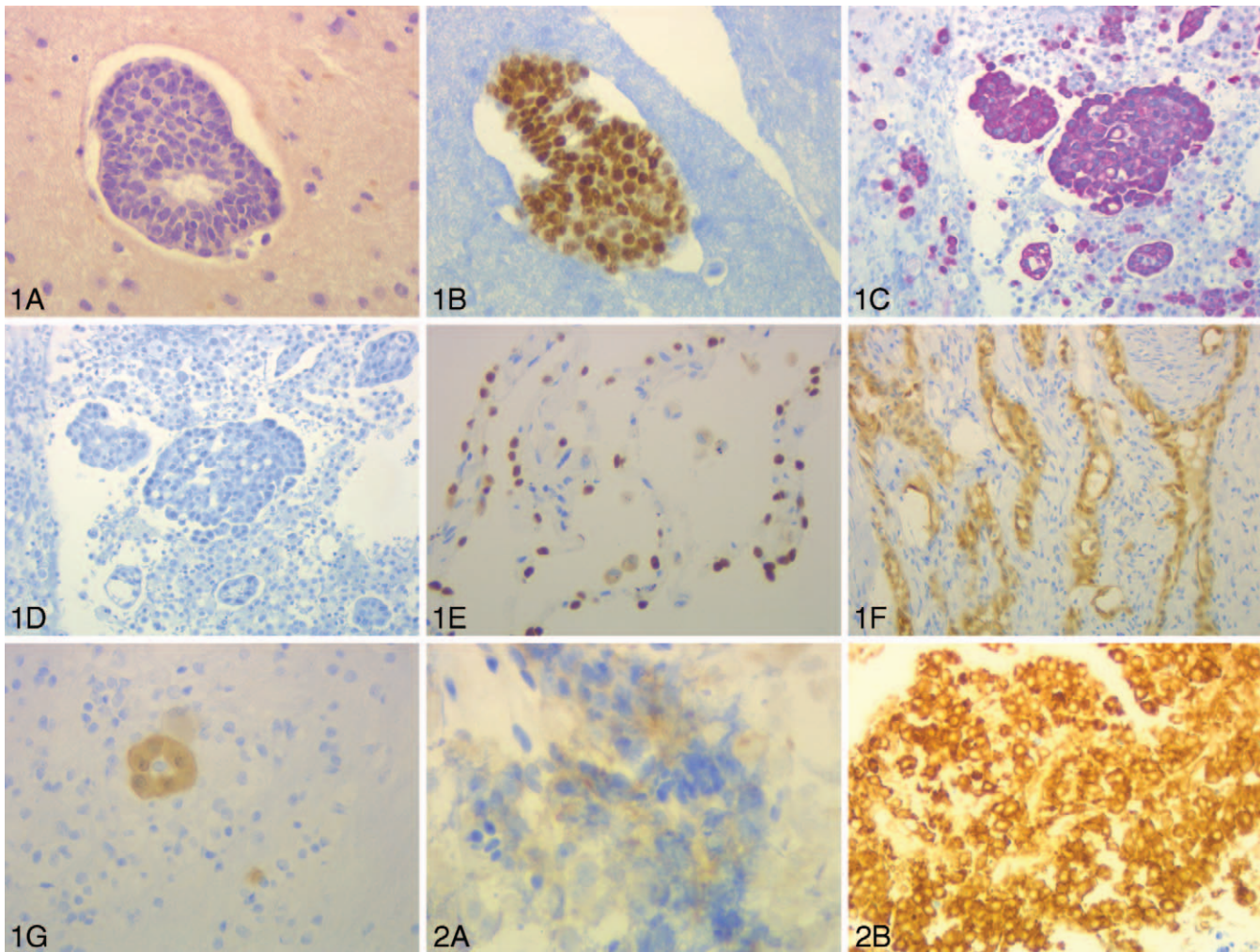


Figure 1. Examples of immunoperoxidase (IP) staining of effusions. A, Adenocarcinoma of breast with negative mucicarmine stain in an ascitic fluid (original magnification $\times 200$). B, The same adenocarcinoma of breast with estrogen receptor antibody positive showing nuclear staining pattern (original magnification $\times 200$). Progesterone receptor should have similar nuclear staining. C, A pleural effusion positive for cytokeratin 7 staining (original magnification $\times 200$). D, The same pleural effusion showing negative cytokeratin 20 (original magnification $\times 200$). The thyroid transcription factor-1 (TTF-1) was also negative. E, If TTF-1 is to be called positive it must have nuclear staining as seen in this control (original magnification $\times 200$). F, Calretinin control reveals both cytoplasmic and somewhat denser nuclear staining (original magnification $\times 200$). G, Calretinin IP in this other effusion has acinar-like cell groups revealing cytoplasmic staining but lacks a definitive denser nuclear stain (original magnification $\times 200$). Thus, the need for an IP panel to assist with equivocal single stains.

Figure 2. Liver fine-needle aspiration immunoperoxidase examples. A, Liver aspirate was weakly positive for epithelial membrane antigen but negative for AE1 and AE3 (original magnification $\times 400$). B, Same liver aspirate with positive CD10, suggesting renal origin (original magnification $\times 200$).

Calretinin remains the single best marker for mesothelial differentiation both in our hands and in the literature.^{20,21} Doglioni et al²⁵ found 100% staining in 44 mesothelial cases but only focal staining of 23% of lung adenocarcinomas. Ordóñez²⁶ later showed the importance of manufacturer when he reported a sensitivity of 74% with Chemicon's calretinin versus a 100% sensitivity using calretinin from Zymed.

Carcinoembryonic antigen (CEA) has long been useful in panels to define adenocarcinoma in a variety of publications and varies in reported sensitivity (90% down to 50%), dependent on the type of adenocarcinomas used in the studies, and specificity in excluding mesothelial origin is usually high (greater than 90%) as long as the better known monoclonal CEA antibodies are used.^{20,21,27,28} When cytology appears glandular but CEA is negative, we frequently use Ber-EP4, which tends to be positive in many nonmu-

cinous adenocarcinomas (serous); however, its routine use for other than lung adenocarcinomas is hit or miss for differentiating from mesothelial origin. Ordóñez²⁹ reviewed the literature in 1998 and found from his laboratory that 18 (26%) of the epithelial mesotheliomas stained with Ber-EP4, 20 (100%) cases of pulmonary adenocarcinomas stained, and 55 (93%) of nonpulmonary adenocarcinomas were reactive. We also use cytokeratin (CK) 7 and CK20 for assisting in determining origins of adenocarcinomas, and references should be reviewed for the "percentages" of confidence as these may assist in this regard.³⁰ Thyroid transcription factor 1 (TTF-1) is reported positive (nuclear staining) in up to 70% to 89% of primary lung adenocarcinomas, and so we often add this to our panel for origin of adenocarcinomas.^{20,31} Figure 1, A through G, reveals the necessary intensity and pattern for a variety of monoclonal antibodies as applied to effusions or ascites.

Likely Origin	Hepar	AFP	CEA	CK7	CK20	AE1	AE3	CA 19-9	ER
Hepatocellular	+	+	–	–	–	+	–	–	–
Colon	–	–	+	–	+	+	+	–	–
Biliary	–	–	+	+	?	+	+	–	–
Pancreas	–	–	+/-	+	?	+	+	+	–
Ovary/endometrial	–	–	+/-	+	–	+	+	–	+
Sarcoma	–	–	–	–	–	–	–	–	–

* AFP indicates α -fetoprotein; CEA, carcinoembryonic antigen; CK, cytokeratin; ER, estrogen receptor; +, suggests greater than 70% are positive; –, suggests greater than 70% stain negative; ?, suggests could easily be positive or negative (35%–65% positive staining); and +/-, dependent on subtype of histology from that organ.

Liver Mass for Evaluation of Malignancy, Primary Versus Metastasis

In recent years we have seen a steady increase of liver FNA related to evaluations for liver transplant as well as for other incidental findings of a liver mass by modern imaging studies. We would refer you to Dr Geller's article in this special issue on liver immunohistochemistry for greater details; however, the following has been our approach in cytology.

Clinical clues that should already favor primary of liver include presence of cirrhosis and solitary versus multiple masses. However, often in advanced disease or in the setting of long-standing cirrhosis, concomitant neoplasms may be noted during workup, which can confuse the differential diagnosis. Although cytologic differences between well-differentiated hepatocellular carcinoma and colon carcinoma are relatively easy, the less differentiated either of the entities becomes, the more one is prone to look to IP staining for assistance. Unfortunately IP staining will not be of much assistance between cholangiocarcinoma and metastatic colon adenocarcinoma although CK7 might assist, as up to 92% of cholangiocarcinomas are positive for CK7, whereas metastatic colon adenocarcinoma is reported as focally positive around only 10% to 17% of the time.^{30,32,33} Thus, review of imaging studies is critical for that differential diagnosis.

Table 3 covers our IP panel for common liver lesions. We tend to use either CK7 and CK20 or AE1 and AE3 but not usually all 4. Prior to CK7 and CK20 being well defined, AE1 was found positive in most cholangiocarcinomas and metastatic colon cancers but was positive in less than 15% of hepatocellular carcinomas.³⁴ If the initial CKs are all negative, we then look for epithelial membrane antigen positivity with a confirmatory CD10-positive staining to identify renal origin (not in Table 3), which can cytologically appear as either epithelioid or sarcomatoid if the classical clear cell characteristics are not present. Epithelial membrane antigen is reported as positive in up to 80% of metastatic renal cell carcinomas, whereas CD10 is reported as positive in 91% of metastatic renal cell carcinomas in a range of studies.^{35,36}

Figure 2, A and B, illustrates epithelial membrane antigen and CD10 IP stains performed on a liver aspirate suspected of being metastatic renal cell carcinoma.

Pancreas Aspirates

The advent of endoscopic ultrasound-guided samplings of the gastrointestinal tract and pancreas has increased the samplings of the pancreas beyond what we previously saw with radiologically guided samplings alone; however, these are frequently scanty samplings. This, in combination with some clinician-written articles on the value of

CA 19-9 levels, CEA levels, and so forth, directly on the fluid obtained, despite the lack of well-established "normal levels" on such samples (as compared with serum samples), we feel has complicated the pathologist's ability to do his or her best at performing an appropriate workup of the pancreas aspirate sample. Frequently, we have found the sample that was being sent to the chemistry laboratory full of cells that made the definitive diagnosis, whereas the sample that was being sent by the clinician to the cytology laboratory lacked any cellularity. The pathologist's communication and education of the clinician obtaining the sample will be the first necessary step if one wishes to use IP staining to assist in more precise diagnoses of pancreatic lesions. The following has been our approach, along with a few pitfalls we have noted, when attempting to placate clinicians' requests.

We feel most diagnoses of solid or partially cystic lesions can be made by the cytology alone with little help from IP staining if the specimen is of sufficient quantity. We have sometimes used IP staining when we wanted reassurance as to the specific pancreatic neoplasm, especially islet cell tumor versus solid pseudopapillary tumor of the pancreas. Less often, IP has been helpful for the neoplasms that would fall into low-grade mucinous neoplasms, acinar cell type, and ductal adenocarcinomas (non-IP mucin stains may be as helpful and cost less). In Table 4, we include CA 19-9 and CEA, which tend to be more helpful in metastatic pancreatic carcinoma situations than in cell differentiation situations from pancreatic aspirates because of the potential for background staining. When obtained from the pancreas, including nonneoplastic cysts, we have found the CA 19-9 to "light up" the entire cell block. In our IP laboratory, CEA appears to have a little less of the background staining with stronger concentration in the cells of interest; however, others have reported a similar problem with CEA.³⁷

Figure 3, A through E, illustrates some pancreatic IP stains useful in the differential diagnosis.

Figure 4, A and B, illustrates why CA 19-9 may be best avoided if the aspirate is directly from the pancreas (because of background artifact) rather than from a metastatic site (where background artifact is avoided).

Additional items to be considered with evaluation of pancreatic tumors include the following:

- The more anaplastic the ductal adenocarcinoma is the less likely IP staining will be helpful. The cytology will be obvious for malignancy, and the imaging studies and clinical information will suggest pancreas primary.
- Acinar tumors may be more strongly positive for trypsin and lipase than for chymotrypsin.³⁸ In addition,

Table 4. Immunoperoxidase Panel for Pancreatic Lesion Differentiation*

Likely Diagnosis	CA 19-9	CEA	Chromogranin	α-Chymotrypsin	Mucicarmine
Ductal adenocarcinoma	+	+	—	—	+
Acinar cell	—	—	?	+	—
Mucinous LMP	+	+	—	—	+
Islet cell type	—	—	+	—	—
Solid pseudopapillary type	—	—	—	+	—
Microcystic adenoma	?	?	—	—	—
Benign pancreatic cysts (if benign cells present)	+	+	—	?	?

* CEA indicates carcinoembryonic antigen; +, suggests greater than 70% are positive; —, suggests greater than 70% stain negative; ?, suggests could easily be positive or negative (35%–65% positive staining); and LMP, low malignant potential.

chromogranin may be positive in up to one third, and some are reported as α-fetoprotein positive.^{39,40}

- All mucinous tumors should be considered of at least low malignant potential when sampled by cytology and deserve resection, although, if well differentiated and mostly unilocular by imaging, a Whipple procedure should only be determined after further histologic sampling.
- Islet cell tumors (or neuroendocrine neoplasms) can be stained for specific hormones, but we tend to mainly stain them for gastrin and somatostatin because these are the ones that may behave more aggressively. Amyloid may be present with the insulin-producing ones (Congo red stain).
- Solid pseudopapillary tumors are often progesterone receptor positive and this may be useful information for therapeutic purposes. The experience of Bardales et al⁴¹ with these tumors mirrors our own experience. CD10 is also consistently expressed.⁴²
- In microcystic adenomas or cystadenocarcinomas, the CEA is usually weaker than in the ductal or mucinous neoplasms and Ber-EP4 is more likely to be positive (serous). Epithelial membrane antigen and lower-molecular-weight CKs are usually positive as well. Amyloid may also be seen accompanying the adenomas (Congo red stain).
- Periodic acid–Schiff without diastase can be very positive and helpful in microcystic adenomas as well as in acinar cell tumors. However, it is advisable to stain both with and without diastase to be certain this is secondary to glycogen in the microcystic adenoma, and the acinar cells should be diastase resistant.
- Pseudocysts return much debris and usually no viable epithelial cells (lack lining). Thus, one should avoid any IP staining of the debris.

Lung Mass for Evaluation of Malignancy, Primary Versus Metastasis

One of our most frequent requests on lung FNA is for the clinician to ask if the squamous carcinoma now present in the lung is related to prior laryngeal or other squamous carcinoma of the head and neck. Although one might attempt a battery of keratin stains comparing the prior with the new, I would find this practice nonconfirmatory because we realize that future metastasis may have different gene expression (a new clonal population of the primary) than the original. However, in regard to adenocarcinoma, we feel evidence shows that if TTF-1 is positive in the tumor cells and that thyroid primary is excluded by history and examination, then this corresponds to a lung primary.³¹ In regard to small cell carcinoma, however, TTF-1 is not helpful because most small cell carcinomas, independent of organ site, are positive.⁴³ Thus, the panel

that one chooses would be similar to that used in regard to adenocarcinomas of effusions. However, for poorly differentiated tumors that one would like to classify further (although management is basically broken down into small cell vs non-small cell tumors currently), the staining properties are as noted in Table 5 as usually noted for lung and pleural primaries. A less common lesion we have received via FNA sampling is solitary fibrous tumor in which CD34 positivity along with the imaging study will be helpful in differentiating from the other lesions in Table 5. A current review of publications on both pulmonary and extrapulmonary solitary fibrous tumors suggests a 95% CD34-positivity rate.³⁵ Flint and Weiss⁴⁴ previously reported a 78.9% rate of positivity for CD34 in pulmonary solitary fibrous tumors that were all negative for CK, and none of their desmoplastic mesotheliomas stained with CD34. We also refer you to the article in this issue by Dr Jagirdar for greater detail in regard to primary pulmonary lesions' immunohistochemistry.

Lymph Node Sampling: Lymphoma Versus Other

Again we would refer you to Dr Kinney's article in this special issue for greatest detail on immunohistochemistry of lymphomas. The following is our approach when limited specimen is available to us in the cytology laboratory. Usually cytologic visual clues such as lymphoglandular bodies, dispersement of cells versus epithelial clumps, and so forth can be counted on to differentiate lymphomas from other malignancies within lymph nodes; however, there are some malignancies, such as melanoma, lobular carcinoma of the breast, small cell carcinoma, or other small round blue cell tumors, that can be confused with lymphomas (both Hodgkin and non-Hodgkin). If during the preliminary/provisional diagnosis of an FNA we are thinking lymphoma, then we immediately consult our hematopathology colleagues for a decision of flow cytometry versus cell block with IP panel. At times there may be sufficient sample to obtain both; however, this is not the routine and one should consider that FNA is meant to be quick and inexpensive. If multiple ancillary tests are used, the cost will become excessive. Because the current standard in the United States for the initial lymphoma diagnosis often includes a whole lymph node excision, if patient condition allows, then the FNA should be used to indicate whether further evaluation is indicated. However, in the clinical situation of probable recurrence, the FNA sample is usually adequate by itself.

Our first 2 IP stains are often a CK and a leukocyte common antigen (CD45RB) although one cannot be faulted if one wishes to add S100 to pick up melanoma on the first battery of stains. When those 3 are negative, then one should begin to look for the rarer entities such as a small round blue

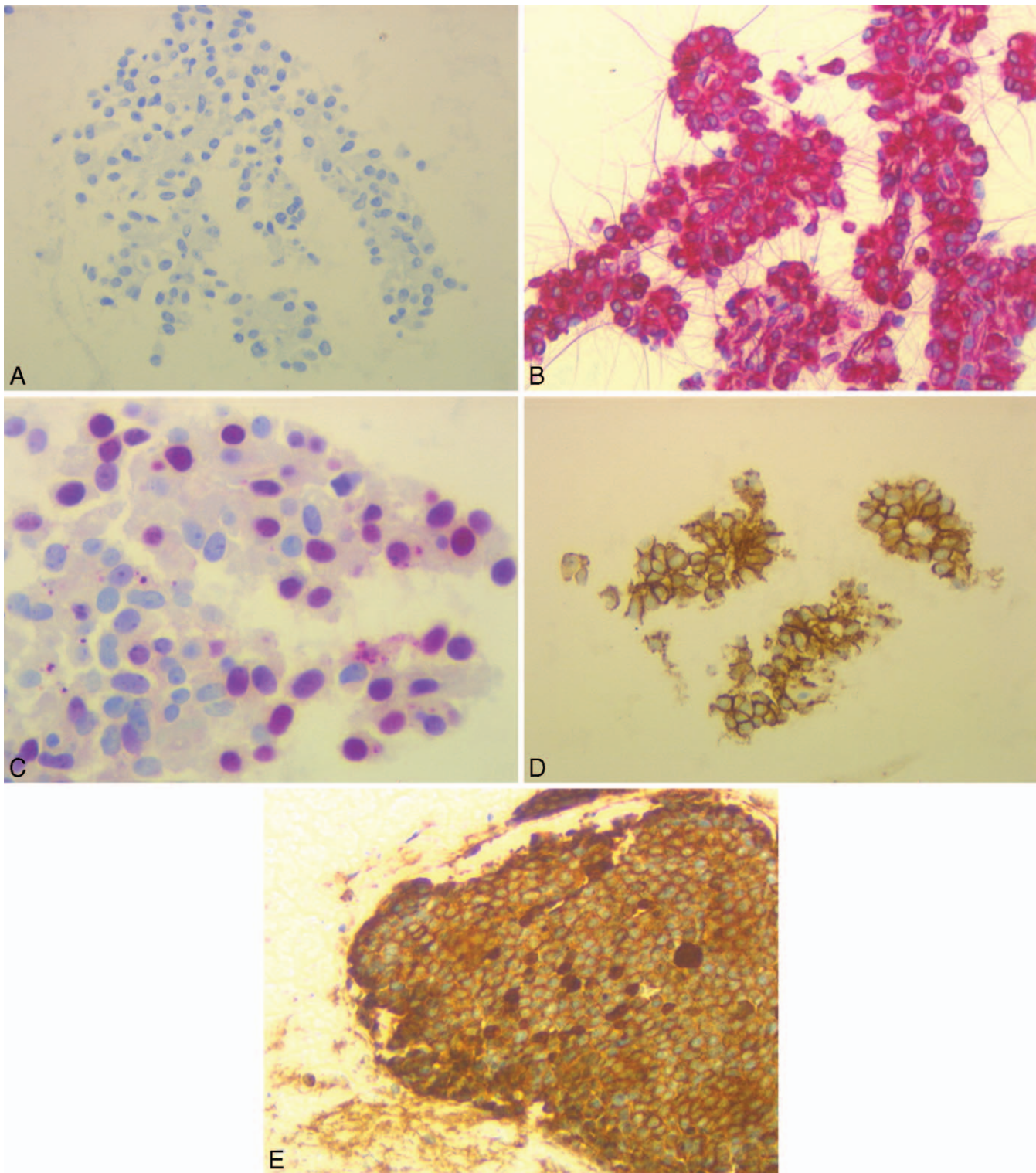


Figure 3. Immunoperoxidase stain examples of pancreas that can be helpful in tumor type differentiation. *A*, Negative AE1/AE3 from a pancreatic mass (original magnification $\times 200$). *B*, Vimentin was positive from the same pancreatic lesion (original magnification $\times 200$). *C*, α -Chymotrypsin was positive from the same pancreatic lesion suggesting solid pseudopapillary pancreatic tumor (original magnification $\times 200$). Progesterone receptor staining also had weak nuclear positivity. CD10 was also strongly positive. *D*, CD56 was positive in the same pancreatic tumor (original magnification $\times 200$). We routinely advise against using CD56 as a substitute for chromogranin or synaptophysin for suspected neuroendocrine tumors because we believe it is too nonspecific. *E*, A different pancreatic lesion appearing more neuroendocrine by cytology reveals strong positivity to chromogranin (original magnification $\times 200$). Keratin (AE1/3) and synaptophysin were also positive. S100 was negative. Further staining with gastrin or somatostatin might be considered for prognostic purposes.

Thyroid FNA for Malignancy

Thyroid aspirates are very common to the cytology laboratory; however, most general pathologists will find it difficult to obtain sufficient cellularity from FNA to make a cell block worthwhile (especially if done by clinicians). We would refer you to the article appearing in this issue on the thyroid by Drs Fischer and Asa for detail on current thyroid monoclonal antibodies. Two IP stains that we have sometimes used on cytopins or monolayer preparations with fairly good success are thyroglobulin and calcitonin, as long as you have 2 extra preparations to stain and can perform both stains concomitantly. Thus, a positive thyroglobulin and a negative calcitonin allow one to confirm a follicular cell differentiation (papillary or follicular types), and a positive calcitonin with negative thyroglobulin allow one to safely confirm a medullary carcinoma, although routinely the regular cytology should be adequate for the differential. A current ImmunoQuery search reveals a cross section of publications report calcitonin staining of 100% of medullary thyroid cancers and of only 2% of papillary or follicular cell carcinomas, and thyroglobulin stained better than 90% of papillary and follicular carcinomas but only 6% of medullary carcinomas of the thyroid.³⁵

Renal Versus Adrenal

There comes a time of uncertainty in some FNA diagnoses as to what organ the image-guided aspirator has procured. Probably one of the more common of these quandaries is adrenal versus renal, especially because adrenal cortex can mimic clear cell carcinoma of the kidney. In this situation, epithelial membrane antigen and CD10 positivity will point toward renal (while negative if adrenal), and if one has inhibin- α (or other sterol antibody), it should be positive if it is adrenal cortical. Of course, chromogranin and synaptophysin would be positive if the source is adrenal medulla. A current ImmunoQuery search reveals a cross section of publications reporting CD10 and epithelial membrane antigen–positive staining in 90% to 80% of renal cell carcinomas versus 0% to 3% in adrenal cortical lesions, respectively, and inhibin was positive in only 8% of renal cell carcinomas but positive in 86% of adrenal cortical lesions.³⁵

Other Newer Applications

Certainly some of the IP stains for infectious agents can be helpful for improving sensitivity and specificity, especially if having to choose Warthin-Starry stain for spirochetes rather than IP. We refer you to the article by Drs Eyzaguirre and Haque on IP uses for infectious organisms for details. However, one should not forget to also send aspirate material for appropriate cultures when infection is suspected.

Although we were able to show fairly good correlation

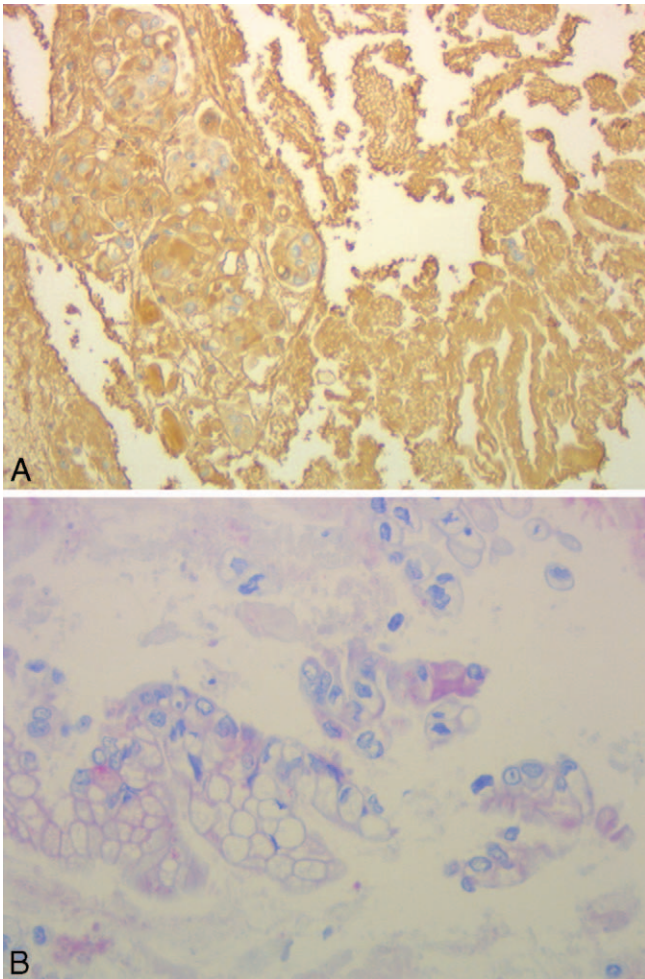


Figure 4. Pitfall of some immunoperoxidase stains with direct pancreatic aspiration sampling. A, Example of why CA 19-9 may not be helpful with aspirates directly from the pancreas (original magnification $\times 100$). B, Focal monoclonal carcinoembryonic antigen staining on same pancreatic case with less background artifact (original magnification $\times 200$).

cell sarcoma with a vimentin or an anaplastic lymphoma with CD30 as seen in Table 6. Salter et al⁴⁵ pointed out a high but variable rate of leukocyte common antigen positivity with plasmacytoid type, and T-cell types have a lower rate of positivity. A current ImmunoQuery search shows leukocyte/lymphoma lesions having a range of 75% or greater positivity for leukocyte common antigen (CD45RB) and almost no positivity for pankeratin in anaplastic and other epithelial-appearing hematologic malignancies.^{35,46}

Table 5. Immunoperoxidase Panel for Common Lung and Pleural Lesions*

Likely Origin	Calretinin	CEA	S100	Synaptophysin	TTF-1	Keratins
Mesothelial	+	–	–	–	–	+
Lung squamous	–	–	–	–	–	+
Lung adenocarcinoma	–	+	–	–	+	+
Lung small cell	–	–	+	+	+	+
Lung large cell neuroendocrine	–	?	+	+	?	+

* CEA indicates carcinoembryonic antigen; TTF-1, thyroid transcription factor 1; +, suggests greater than 70% are positive; –, suggests greater than 70% stain negative; and ?, suggests could easily be positive or negative (35%–65% positive staining).

Table 6. Immunoperoxidase Panel for Lymph Node Aspirates for Lymphoma Versus Other Malignancy*

Likely Origin	Keratins	LCA	S100	HMB-45	Vimentin	TTF-1
Hodgkin lymphoma	—	+	—	—	—	—
Non-Hodgkin lymphoma	—	+	—	—	—	—
Small cell carcinoma	+	—	—	—	?	+
Small round blue cell sarcoma	—	—	-/+	—	+	+
Melanoma	—	—	+	+	+	—

* LCA indicates leukocyte common antigen; TTF-1, thyroid transcription factor 1; +, suggests greater than 70% are positive; —, suggests greater than 70% stain negative; ?, suggests could easily be positive or negative (35%–65% positive staining); and -/+, exception is the neural-derived small round blue cell tumor.

between breast prognostic markers on cell blocks from breast FNAs and later histologic sections of the resected tumor, we would caution that much rests on the patient outcome when selecting therapy choices.⁷ Site selection or sampling variation within large masses, in addition to the other cytology preparation method differences already discussed that may affect IP interpretation, suggests that staining for prognostic marker or other single IP tests that result in therapeutic decision making is probably best avoided. Thus, when testing for estrogen/progesterone receptor (other than for confirming cell differentiation), HER-2/*neu*, and other future antibodies tied to therapeutic choices (exception maybe c-Kit to help make the diagnosis of gastrointestinal stromal tumor), one might better wait for the histology even though we know such staining is possible.⁴⁷ In regard to epidermal growth factor receptor IP testing, it has repeatedly been shown to have extremely poor correlation with current targeted therapy and should probably be avoided, even in tissue samples, and instead one should rely on fluorescent in situ hybridization or other molecular testing methods.⁴⁸

More and more reports are also being noted in the cytology literature that adapt IP panel staining patterns for difficult differentials in histology to cytology preparations. Immunoperoxidase profiles to differentiate dysplasia versus reactive, inflammatory or atrophy conditions of cervix or urothelium, thyroid markers to separate benign follicular thyroid lesions from malignant, and so forth, can be tricky on histology (and for cervix and urothelium may rely on a portion of the epithelial layers staining vs complete layer positivity) and can be even more problematic when used on cytology specimens. Again we would caution against the general pathologist using such IP panels with cytology specimens in these situations to dictate definitive therapy.

Cytopathologists should not give up hope, however, as newer methodology such as proteomic profiling is better used on non-formalin-fixed material and is especially applicable to cytology preparations as long as they are not fixed in ethanol.⁴⁹ Once uniformity of methodology and reproducibility are ensured for these newer ancillary tests, there may come a day when IP staining for selected panels is replaced by the proteomic and genomic profiling methods much like electron microscopy was made to take a “back seat” to IP. For now however, IP staining rules can be a harsh mistress when applied to cytology specimens without due caution.

COMPOSITE IP

Tables 7 and 8 include some of the more common markers that we have used within our cytology laboratory and found to be helpful in characterizing cell differentiation. Table 7 is based on determining a specific cell differenti-

Table 7. Cell Differentiation Types and Their Common Markers*

Cell Type	Monoclonal Antibodies to Consider
Breast	ER, PR, gross cystic fluid protein, CK7, CK20, E-cadherin
Colon and other GI tract	CEA (monoclonal), CK7, CK20
Germ cell	PLAP, α -fetoprotein, β -HCG, AE1/3
Hepatocellular	Hepar, α -fetoprotein, CK7, CK20, AE1 and AE3 (separately)
Lung	TTF-1, CK7, CK20, CEA, Ber-EP4, chromogranin, synaptophysin, S100
Lymphoma	LCA (CD45RB monoclonal), CD3, CD20, CD30, ALK-1, myeloperoxidase, κ and λ light chains, Bcl-2
Mesothelium	Calretinin, AE1, AE3
Melanoma	S100 (when spindled cells), HMB-45 (when epithelioid), MART1
Neuroendocrine	Chromogranin, synaptophysin, NSE, CD56, gastrin, somatostatin
Pancreas	AE1/3, CK7, CK20, CA 19-9, CEA, chromogranin, synaptophysin, α_1 -antichymotrypsin, CD10, PR, Ber-EP4
Prostate	PSA, CK7, CK20
Renal	EMA, CD10, HMB-45, inhibin- α (to exclude adrenal cortical)
Sarcoma	Vimentin, S100, CD117 (c-Kit), CD34, SMA, myogenin, CD31, CD68, desmin, CD1a, CD99
Thyroid	TTF-1, thyroglobulin, calcitonin, CEA
Urothelial	CK7, CK20

* Markers are listed in order of the most frequently used in our cytology laboratory when clinical history and imaging suggests the organ as site of origin. ER indicates estrogen receptor; PR, progesterone receptor; CK, cytokeratin; GI, gastrointestinal; CEA, carcinoembryonic antigen; PLAP, placental-like alkaline phosphatase; HCG, human chorionic gonadotropin; TTF-1, thyroid transcription factor 1; LCA, leukocyte common antigen; ALK-1, anaplastic lymphoma kinase 1; MART1, melanoma-associated antigen recognized by T cells; NSE, neuron-specific enolase; PSA, prostate-specific antigen; EMA, epithelial membrane antigen; and SMA, smooth muscle actin.

ation and the antibodies are listed in frequency of use. It should be remembered that for most anaplastic carcinomas there may be almost no positive marker other than vimentin, which we sometimes use to test if the sample has any antigenicity. Table 8 lists antibodies in alphabetic order that we commonly use in cytology, including whether they are approved for in vitro diagnostic use, as an allied specific reagent, or for research use only; the antibody vendor; our current dilution; and the cytology diagnosis being considered that most commonly prompts the use of the antibody. All are currently run on a Ventana (Tucson, Ariz) XT machine using the manufacturer's recommendations for pretreatment, detection method, and chromogen type.

Table 8. Commonly Used Antibodies At Our Cytology Laboratory*

Antibody	IVD/ASR	Vendor	Dilution	Cytology Diagnosis Being Considered
α ₁ -Antichymotrypsin, polyclonal	IVD	Ventana	PRE	Pancreatic pseudopapillary tumor (SPPT)
Actin muscle, smooth, monoclonal	IVD	Dako	1:200	Skeletal and smooth muscle
α-Fetoprotein (AFP), polyclonal	IVD	Dako	1:4000	Hepatic, yolk sac tumors
ALK-1, monoclonal	IVD	Ventana	PRE	Anaplastic lymphoma
Ber-EP4, monoclonal	IVD	Dako	1:100	Serous tumor
Bcl-2, monoclonal	IVD	Dako	1:50	Lymphoma
CA 19-9, monoclonal	IVD	Ventana	PRE	Pancreas
CA 125, monoclonal	IVD	Ventana	PRE	Ovarian
Calretinin, monoclonal	IVD	Zymed	1:200	Mesothelial
Calcitonin, polyclonal	IVD	Dako	1:200	C cell
c-Kit, CD117, monoclonal	IVD	Dako	1:100	GIST
c-Erb-B2, HER2, monoclonal	IVD	Ventana	PRE	Breast carcinoma
Carcinoembryonic antigen (CEA), monoclonal	IVD	Dako	1:100	Mucinous tumor
CD1a, monoclonal	IVD	Ventana	PRE	Langerhans cell
CD3, monoclonal	RUO	Novo	1:25	T cells
CD5, monoclonal	RUO	Novo	1:50	T cells
CD10, monoclonal	RUO	Novo	1:50	Renal, SPPT
CD15, monoclonal	IVD	Ventana	PRE	Hodgkin
CD20, monoclonal	IVD	Dako	1:2000	B cell
CD30, monoclonal	IVD	Neomark	1:50	Hodgkin and anaplastic
CD31, monoclonal	IVD	Dako	1:20	Vascular
CD34, monoclonal	IVD	Dako	1:40	Solitary tumor
CD45RB, monoclonal	IVD	Dako	1:500	Lymphoid
CD56, monoclonal	IVD	Novo	1:100	Neural
CD68, monoclonal	IVD	Dako	1:2000	Macrophage
CD99, monoclonal	IVD	Neomark	1:50	Ewing tumor
Chromogranin, monoclonal	IVD	Dako	PRE	Neural
Cytomegalovirus (CMV), monoclonal	IVD	Signet	1:10	Viral
Cytokeratin 7, monoclonal	IVD	Dako	1:50	Adenocarcinoma unknown
Cytokeratin 20, monoclonal	IVD	Dako	1:100	Adenocarcinoma unknown
Desmin, monoclonal	IVD	Dako	1:40	Rhabdomyosarcoma
E-cadherin, monoclonal	IVD	Ventana	PRE	Lobular breast carcinoma if negative
Epithelial membrane antigen (EMA), monoclonal	IVD	Dako	1:50	Renal
Estrogen receptor	IVD	Ventana	PRE	Breast and gynecologic
Gastrin, polyclonal	IVD	Ventana	PRE	Neuroendocrine for increased risk
Glucagon, polyclonal	IVD	Dako	PRE	Neuroendocrine
Human chorionic gonadotropin (HCG), polyclonal	IVD	ABCAM	1:50	Choriocarcinoma
Herpes simplex virus type I, polyclonal	IVD	Ventana	PRE	Viral
Herpes simplex virus type II, polyclonal	IVD	Ventana	PRE	Viral
Hepar	IVD	Dako	1:100	Hepatic carcinoma
HMB-45 (melanoma), monoclonal	IVD	Ventana	PRE	Melanoma and angiomyolipoma
Keratin, AE1	IVD	Zymed	1:400	FPS
Keratin, AE3	IVD	Zymed	1:200	FPS
κ Light chains, polyclonal	IVD	Dako	1:20 000	B cell
Ki-67, monoclonal	IVD	Ventana	PRE	Prognosis
λ Light chains, polyclonal	IVD	Dako	1:30 000	B cell
MART1	IVD	Signet	PRE	Melanoma
Myogenin, monoclonal	IVD	Dako	1:500	Rhabdoid
p16	IVD	BCM	1:100	Dysplasia
Pan keratin AE1/AE3	IVD	Ventana	PRE	Epithelial origin
Placental alkaline phosphatase, polyclonal	IVD	Ventana	PRE	Germ cell tumor
Progesterone receptor, monoclonal	IVD	Ventana	PRE	Breast, SPPT
Prostate-specific antigen (PSA), monoclonal	IVD	Dako	1:200	Prostate
S100, monoclonal	IVD	Dako	1:800	Melanoma and neural
Somatostatin, polyclonal	IVD	Ventana	PRE	Neuroendocrine for higher risk
Spirochete	RUO	BCM	1:100	At risk inflammation
Synaptophysin, monoclonal	IVD	Dako	1:10	Neural
Thyroid transcription factor 1 (TTF-1)	IVD	Dako	1:80	Thyroid, lung, and small cell
Thyroglobulin, monoclonal	IVD	Ventana	PRE	Thyroid follicular cell
Vimentin, monoclonal	IVD	Ventana	PRE	Antigenic reactive, stromal

* IVD indicates in vitro diagnostic use; ASR, allied specific reagent; PRE, prediluted; SPPT, solid pseudopapillary pancreatic tumor; GIST, gastrointestinal stromal tumor; RUO, research use only; and FPS, fallopian tube, prostate (skin control). Vendors: Ventana, Tucson, Ariz; Dako, Carpinteria, Calif; Zymed, Carlsbad, Calif; Novo, Novocastro, Burlingame, Calif; Neomark, Fremont, Calif; Signet, Emeryville, Calif; ABCAM, Cambridge, Mass; and BCM, Baylor College of Medicine, Houston, Tex.

We would strongly recommend using a Web-based antibody database that is evidence-based when presented with choosing initial immunostains for a specific differential whether it is histology or cytology. These are usually more frequently updated, collect various abstracts and

publications, and can give overall sensitivity/specificity data for a differential diagnosis, and include easy links to specific publications that also discuss manufacturer and methodology. Thus, general pathologists can review which of the antibodies available to them would best help

in the situation as well as compare the methodology and manufacturer of the antibody that is used in their laboratory. The databases can also reflect recent changes in some of the old tried and true antibodies because of change in lot numbers or differences in manufacturers. Some of these databases are used by larger immunocytochemistry laboratories as a draw, whereas others (some by publishing companies) are sold with a licensing agreement. Both types are valuable in providing evidence-based guidance that can serve one well when sampling is limited, can keep cost to a minimum, and can impact diagnosis quality for the patient's care.

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