Chapter 2
Standardization of Diagnostic Immunohistochemistry

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Abstract Standardization of every step in the pre-analytic, analytic, and post-analytic phases is crucial to achieving reproducible and reliable immunohistochemistry results. This chapter delineates some critical points in the pre-analytic, analytic, and post-analytic phases; reiterates some important questions which may or may not have a consensus at this time; and updates the proposed guidelines on antibody validation from the College of American Pathologists (CAP) Pathology and Laboratory Quality Center. Additionally, the chapter shares Geisinger's experience with (1) testing/optimizing a new antibody and troubleshooting; (2) using cell blocks containing a mixture of cultured cell lines as external positive controls; (3) interpreting and reporting immunohistochemistry assay results; (4) improving and implementing a total immunohistochemistry quality management program; and (5) developing the concept of best practices in immunohistochemistry.

Frequently Asked Questions

1. Summary of common variables impacting immunohistochemistry (IHC) standardization (Table 2.1).
2. What are the most important factors in the pre-analytic phase (Table 2.2)?
3. What are the utilities of multi-tissue tissue microarray (TMA) blocks in a clinical IHC laboratory?
4. Can cultured cell lines be used as IHC-positive controls?
5. What are the advantages of using cultured cell lines instead of multi-tissue TMA blocks as external positive controls?
6. What cell lines are recommended for use as IHC-positive controls (Table 2.3)?
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27. How do you implement best practices in immunohistochemistry (Table 2.12)?

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1. Summary of common variables impacting immunohistochemistry (IHC) standardization.

Numerous variables have been identified in the process of IHC standardization, and some will significantly influence the quality of staining results. These factors may occur in

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Table 2.1 Summary of common variables impacting IHC staining results

Pre-analytic phase
Acquisition (delay in putting samples into fixative)
Fixation type and time
Decalcification type and time
Tissue processing
Slide drying time and temperature
Analytic phase
Antibody selection (different clones, polyclonal)
Antibody optimization (antigen retrieval, antibody dilution, incubation time)
Antibody validation
Instrumentation (different automated platforms, manual stains)
Qualifications of IHC assay personnel
CAP inspection/certification
Post-analytic phase
Positive and negative tissue controls
Interpretation
Result reporting
Pathologist performance
Digital pathology with imaging analysis

pre-analytic, analytic, and post-analytic phases as summarized in Table 2.1 [1–10].

2. What are the most important factors in the pre-analytic phase?

It has been recommended that tissue be fixed in 10% neutral-pH, phosphate-buffered formalin for a minimum of 8 hours [5]. If formalin or a formalin–alcohol mixture is a component solution on the tissue processor instrument, tissue should be fixed in formalin for 6–12 hours before being loaded onto the tissue processor. Non-formalin fixatives and/or alternative fixation methodologies are strongly discouraged [5].

The study by Engel and Moore identified 27 variables that have been examined and reported in published literature [7]. Some of these pre-analytic factors which may or may not impact an IHC assay result are summarized in Table 2.2.

Drying slides can be easily overlooked in an IHC laboratory, and IHC technologists must be educated to pay particular attention to drying time and temperature. It has been recommended that slides be dried at 50–60 °C for a minimum of 1 hour or at room temperature for 24 hours [7].

Decalcification may have a negative impact on an IHC assay for certain antigens [11, 12]. As such, CAP recommends that a disclaimer be included in the surgical pathology or FNA report, which may read as follows: "This IHC assay has not been validated on decalcified tissues. Results should be interpreted with caution given the likelihood of false negativity on decalcified specimens [13]."

Table 2.2 Summary of factors with or without influence on IHC test results

Factors influencing IHC test results
Fixation delay
Fixative type
Fixative concentration
pH and buffer
Time in fixative
Reagents and conditions of dehydration
Clearing reagent and temperature
Paraffin embedding temperature and duration
Condition of slide drying and storage
Factors with no reported effect on IHC test results
Tissue-to-fixative ratio
Type of processor used
Type of paraffin
Post-fixation washing duration
The number and position of specimens during dehydration
The duration of paraffin block storage

3. What are the utilities of multi-tissue tissue microarray (TMA) blocks in a clinical IHC laboratory?

TMA blocks containing various numbers of tumors and/or normal tissues have demonstrated great utilities in clinical IHC laboratories. TMA blocks may be potentially useful for (1) antibody testing and optimization, (2) antibody validation or verification, (3) positive and negative control tissues, (4) quality control, and (5) new biomarker discovery.

Depending upon the need, four different prototypes of TMA blocks can be constructed:

1. A TMA block containing a broad spectrum of tumors and/or normal tissues from various organs, which is useful for screening a new biomarker;
2. A TMA block containing 50–100 tumors with a specific diagnosis such as lung adenocarcinoma, which is useful for antibody validation, revalidation, and research purposes in determining the diagnostic sensitivity and specificity of a newly discovered antibody;
3. A TMA block containing 5–10 cases of a specific type of tumor, which is useful for antibody testing and optimization;
4. A TMA block containing 5–10 cases of selected, mixed tumors and/or normal tissues from various organs, which can be used as external positive and negative control tissues for each antibody.

4. Can cultured cell lines be used as IHC positive controls?

Yes. In fact, cultured cell lines can be a better source for positive control blocks for selected biomarkers. At Geisinger IHC Laboratory, we have used cell blocks containing a mixture of cultured cancer cell lines for a significant number of

antibodies. These cell blocks can be used for (1) external positive and negative control tissues; (2) new antibody testing and optimization; (3) antibody validation; and (4) continuous quality monitoring of commonly used antibodies.

5. What are the advantages of using cultured cell lines instead of multi-tissue TMA blocks as external positive controls?

Depending on the antibodies being ordered, IHC labs in the United States use either tumor tissue blocks or normal tissue blocks as external positive control slides. The positive control blocks can be constructed by each IHC lab or ordered from a commercial company. The cost for each positive control slide varies for a given antibody. Many IHC labs choose to build the majority of their positive control blocks, only purchasing positive control slides for rare antibodies. Multi-tissue TMA blocks are most commonly used as external positive and negative control blocks.

In contrast to TMA blocks, cell blocks containing cultured cancer cell lines provide several advantages. They (1) provide more consistent and reliable quality; (2) save expensive IHC tech time to build TMA blocks; and (3) avoid consuming valuable tumor blocks, which are important for future molecular testing, clinical trials, research, and biospecimen banking, from pathology archives.

6. What cell lines are recommended for use as IHC positive controls?

Geisinger IHC laboratory has tested many cell lines. The ordering information, growth condition, and growth properties of these cultured cell lines are summarized in Table 2.3.

Table 2.3 Ordering information, growth condition, and growth properties of cultured cell lines

ATCC Cat. No.	Designation	Source	Growth condition and growth properties
CCL-253	NCI-H508	Colorectal adenocarcinoma	RPMI160 + 10% FBS
CCL-218	WiDr	Colon adenocarcinoma	EMEM + 10% FBS
CCL-229	LoVo	Colon adenocarcinoma	F12K + 10% FBS
HTB-37	Caco-2	Colon adenocarcinoma	EMEM + 10% FBS
CCL-231	SW48	Colon adenocarcinoma	Leibovitz's L-15 + 10% FBS, CO_2 free
CCL-221	DLD-1	Colon adenocarcinoma	RPMI1640 + 10% FBS
CRL-5822	NCI-N87	Gastric carcinoma	RPMI1640 + 10% FBS
CRL-2547	Panc 10.05	Pancreatic adenocarcinoma	RPMI + 15% FBS + human insulin 10 units/mL
CRL-2549	Panc 03.27	Pancreatic adenocarcinoma	RPMI + 15% FBS + human insulin 10 units/mL
CRL-2551	Panc 08.13	Pancreatic adenocarcinoma	RPMI + 15% FBS + human insulin 10 units/mL
CRL-2279	MS1	Pancreas/islet of Langerhans	DMEM + 10% FBS
CRL-2237	SNU-387	Pleomorphic hepatocellular carcinoma	RPMI1640 + 10% FBS
CRL-2234	SNU-449	Hepatocellular carcinoma (HBV+)	RPMI1640 + 10% FBS
CRL-11233	THLE-3	Liver epithelial SV40 transformed, normal	BEGM + additives[a] + 10% FBS
CRL-2706	THLE-2	Liver epithelial SV40 transformed, normal	BEGM + additives[a] + 10% FBS
CRL-1830	Hepa1-6	Hepatocellular carcinoma (mouse)	DMEM + 10% FBS
HB-8065	HepG2	Hepatocellular carcinoma	EMEM + 10% FBS
CRL-10741	C3A	Hepatocellular carcinoma	EMEM + 10% FBS
CRL-11268	293T/17	Fetus kidney SV-40	DMEM + 10% FBS
CRL-1611	ACHN	Renal cell carcinoma	EMEM + 10% FBS
CRL 1932	786-O	Renal cell carcinoma	RPMI1640 + 10% FBS
HTB-47	Caki-2	Kidney clear cell carcinoma	McCoy's 5a + 10% FBS
CRL-1441	G401	Rhabdoid tumor—Wilms tumor	McCoy's 5a + 10% FBS
CRL-1435	PC-3	Prostate adenocarcinoma	F-12K + 10% FBS
CRL-2505	22Rv1	Prostate adenocarcinoma PSA+	RPMI1640 + 10% FBS
PCS-440-010	N/A	Prostate (normal)	ATCC-PCS-440-030;440-040;30-2200
CCL-185	A549	Lung carcinoma	F-12K + 10% FBS
HTB-184	NCL-H510A	Lung small cell carcinoma	F-12K + 10% FBS, adherent and suspension
CCL-256	NCI-H2126	Lung adenocarcinoma	HITES[b] + 5% FBS
CRL-5826	NCI-H226	Lung squamous cell carcinoma	RPMI1640 + 10% FBS
CRL-5877	NCI-H1573	Lung adenocarcinoma	RPMI1640 + 5% FBS
CRL-5895	NCI-H1792	Lung adenocarcinoma	RPMI1640 + 10% FBS
CRL-5908	NCI-H1975	Lung non-small cell carcinoma	RPMI1640 + 10% FBS
CRL-5869	NCI-H1417	Lung small cell carcinoma	RPMI1640 + 10% FBS, suspension

(continued)

Table 2.3 (continued)

ATCC Cat. No.	Designation	Source	Growth condition and growth properties
CCL-257	NCL-H1688	Lung carcinoma, classic small cell carcinoma	RPMI1640 + 10% FBS
HTB-177	H-460	Carcinoma, large cell lung cancer	RPMI1640 + 0% FBS
CRL-1596	Ramos(RA1)	Burkitt's lymphoma	RPMI1640 + 10% FBS suspension
CRL-2974	MM.1S	B lymphoblast	RPMI1640 + 10% FBS suspension and lightly attached
CCL-159	IM-9	B lymphoblast, Epstein–Barr virus (EBV) transformed	RPMI1640 + 10% FBS suspension
CCL-86	Raji	Burkitt's lymphoma	RPMI1640 + 10% FBS suspension
CRL-1582	MOLT4	Acute lymphoblastic leukemia	RPMI1640 + 10% FBS suspension
CCl-119	CCRF-CEM	Acute lymphoblastic leukemia	RPMI1640 + 10% FBS, suspension
CRL-2264	CEM/C2	Acute lymphoblastic leukemia	RPMI1640 + 10% FBS, suspension
CRL-2294	BCP-1	B lymphoblast lymphoma	RPMI1640 + 20% FBS, suspension
CRL-1585	C32	Melanoma	EMEM + 10% FBS
CRL-1424	G361	Melanoma	McCoy's + 10% FBS
CRL-1872	A375.S2	Melanoma	EMEM + 10% FBS
CRL-2329	HCC1500	Breast ductal carcinoma (ER+/PR+/HER-)	RPMI1640 + 10% FBS
HTB-133	T-47D	Breast ductal carcinoma (ER+/PR+)	RPMI1640 + 10% FBS
CRL-2330	HCC1569	Breast metaplastic carcinoma (HER2+)	RPMI1640 + 10% FBS, suspension and adherent
CRL-2321	HCC1143	Breast carcinoma (ER-/PR-/HER-)	RPMI1640 + 10% FBS
CRL-1902	UACC893	Breast ductal carcinoma	Leibovitz's L-15 + 10% FBS, CO_2 free
HTB-36	JEG-3	Placenta choriocarcinoma	EMEM + 10% FBS
HTB-105	Tera-1	Embryonal carcinoma	McCoy's 5a + 10% FBS
CRL-2073	NCCIT	Pluripotent embryonal carcinoma; Teratocarcinoma	RPMI 1640 + 10% FBS
CRL-2180	L2-RYC	Yolk sac tumor	DMEM + 10% FBS
HTB166	RD-ES	Ewing's sarcoma	RPMI 1640 + 15% FBS
CRL-7556	Hs-822.T	Ewing's sarcoma	DMEM + 10% FBS
CCL-136	RD	Rhabdomyosarcoma	DMEM + 10% FBS
CRL-7822	Hs 5.T	Leiomyosarcoma	RPMI1640 + 10% FBS
HTB-93	SW-982	Synovial sarcoma	Leibovitz's L-15 + 10% FBS, CO_2 free
CRL-2946	UWB1.289 + BRCA1	Ovarian carcinoma	50% RPMI-1640 + 50%MEGM[c] + 3% FBS
CRL-1550	Ca-ski	Cervical carcinoma	RPMI1640 + 10% FBS
HTB35	SiHa	Cervical carcinoma	EMEM + 10% FBS
CRL-5946	NCI-H2452	Mesothelioma	RPMI1640 + 10% FBS
CRL-1803	TT	Thyroid medullary carcinoma	F12 + 20% FBS
CRL-10296	NCL-H295	Adrenocortical carcinoma steroid hormones+	HITES[b] + 2% FBS

Note:

[a]BEGM (Lonza): CC-3170 Kit: BEGM Bullet Kit (CC-3171 & CC-4175); CC-3171: Basal Medium—contains no growth factors, cytokines, or supplements; CC-4175: SingleQuots™ Kit—growth factors, cytokines, and supplements

[b]HITES: HITES medium supplemented with 5% FBS; The base medium DMEM: F12 Medium, ATCC 30-2006; To make the complete growth medium, add the following components to the base medium: (1) 0.005 mg/mL insulin; (2) 0.01 mg/mL transferrin; (3) 30 nM sodium selenite (final conc.); (4) 10 nM hydrocortisone (final conc.); (5) 10 nM beta estradiol (final conc.); (6) Extra 2 mM L-glutamine(for final conc. of 4.5 mM); and (7) 5% fetal bovine serum (final conc.)

[c]MEGM (Lonza): CC-3150: MEGM Bullet Kit (CC-3151 & CC-4136); CC-3151: MEBM Basal Medium 500 mL; CC-4136: MEGM SingleQuots Kit Suppl. & Growth Factors

Other Medium:

RPMI1640: Life Technologies 11875119; DMEM: Life Technologies 11995073; McCoy's 5A: ATCC 30-2007; Leibovitz's L-15: ATCC 30-2008; DMEM-F12 medium: ATCC 30-2006; Eagle's minimum essential medium: ATCC 30-2003

Note: Growth properties: all cell lines were adherent growth unless otherwise mentioned in the table

7. How are cell blocks prepared from cultured cell lines? Cultured cells are harvested, and cell pellets are prepared using standard techniques. The following is a brief example:

When the cell growth is near confluent (adherent growth, about 1×10^7 cells per dish) or near 0.5×10^8 cells per dish (suspended growth), harvest cells by EDTA digestion and centrifuge (adherent growth); or directly move the suspended growth cultures to 50 mL Falcon tubes. Eight large culture dishes (150 × 25 mm each; each dish containing 5–10×10^7 cells) are collected for one cell block preparation.

For the preparation of a cell block with mixed cell lines, we cultured select cell lines simultaneously and mixed these

Table 2.4 Cell lines for construction of a melanoma control block

ATCC Cat. #	Ratio of each cell line	Total cells per block
CRL-1585	About 40%	About 2.50×10^7
CRL-1424	About 40%	About 2.50×10^7
CRL-5895	About 20%	About 1.25×10^7

cells at proper ratios, depending on the purpose of the cell block. Table 2.4 shows an example of a melanoma control block with three different cell lines in the proper ratios and cell counts.

Examples of the steps to prepare a cell pellet are as follows:

(a) Centrifuge the cells to make a cell pellet.
(b) Move the bottom cell pellet to a small glass vial (Cat. #72631-10, Electron Microscopy Sciences, Hatfield, PA), and then place the small glass vial into a 25 mL polyethylene vial (Cat. #72621-62, Electron Microscopy Sciences, Hatfield, PA).
(c) Spin the cells at 1600 rpm for 7 minutes in a Beckman centrifuge with swinging-bucket rotors.
(d) Remove the small glass vial from centrifuge for cell block preparation.
(e) Pour off supernatant completely, and preserve the cell pellet at bottom of the small vial.
(f) Add approximately 5–6 drops of plasma (obtained from the Blood Bank of Geisinger Medical Laboratories) to the cell pellet and re-suspend by gently vortexing; then, add approximately 5–6 drops of bovine thrombin (Cat. #23-306291, Fisher Scientific Pittsburgh, PA) into the cells and mix gently, and then let it stand for 10 minutes.
(g) The cell pellet should become a semi-solid clot at room temperature. Under a fume hood, insert a 23-gauge needle with a syringe containing approximately 2–3 mL of 10% neutral-buffered formalin along the side at the bottom of the vial. While the formalin is slowly pushed through the syringe, the clotted cell pellet is slowly dislodged from the flat-bottom glass vial and floats to the surface.
(h) Place the clotted pellet into a labeled cassette, fix it with 10% neutral-buffered formalin for 6–8 hours (no more than 24 hours), and then send it to the histology lab for tissue processing and paraffin embedding.
(i) The cassette with the clotted cell pellet is processed in the tissue processor using long-run program as routine surgical specimens. After processing, embed the clotted cell pellet in 57–59 °C paraffin on the embedding workstation (Cat. # A81000002, HistoStar™, Thermo Scientific). At this step, the cell block is made and the diameter of the cell block is about 0.5 cm.
(j) Cut the cell block into 4~5μm sections and check the quality of the cell block with hematoxylin and eosin stain (H&E).

8. What specific cell lines are recommended for constructing a set of TMA control blocks for selected biomarkers?

After testing numerous cell lines, those with high-level expression (strongly positive), low-level expression (weakly to moderately positive), and no expression (no staining) for a targeted antigen were selected for six specific cell blocks. These cell blocks include (1) breast cancer; (2) melanoma; (3) lymphoma; (4) germ cell tumor; (5) malignant small round cell tumor; (6) sarcoma; and (7) tumor of unknown primary. These seven cell blocks can potentially cover over 70% of the commonly used diagnostic and predictive markers at Geisinger's IHC laboratory. We have found that identifying a cell line with low-level expression of a targeted antigen can be challenging. Therefore, for some targeted antigens, only cell lines with high-level expression and no expression of these antigens were included.

The specific cell lines, the ratio of each cell line, types of cancer to be covered, and targeted biomarkers with high-level expression are summarized in Table 2.5. Figure 2.1a–h show a mixture of three breast cancer cell lines expressing targeted biomarkers. Figure 2.2a–h show a mixture of three melanoma cell lines expressing targeted biomarkers.

9. What are the recommended immunohistochemistry critical assay performance controls (iCAPCs) for the commonly used IHC markers?

An ideal external positive control for a targeted IHC marker should consist of a tissue/or tissues with high-level expression, low-level expression, or no expression of that antigen. A set of well-characterized primary positive controls (immunohistochemistry critical assay performance controls [iCAPCs]) recommended by the International Ad Hoc Expert Committee to cover 20 commonly used antibodies in clinical IHC labs is summarized in Table 2.6. A tissue with high-level expression of the targeted antigen tends to show a moderate to strong immunohistochemical reaction; in contrast, a tissue with low-level expression of the targeted antigen tends to demonstrate a low to moderate immunohistochemical reaction, or the low limit of detection (LLOD), of this specific antigen. The LLOD in an "optimized" IHC stain is defined by an observed positive reaction (staining) in a tissue/cellular element that is known to express low levels of the evaluated marker [14].

10. How do you select antibodies?

How do you select the "right" antibodies for your IHC lab and your patients? Before you decide to bring a new anti-

Table 2.5 Summary of specific cell lines used to construct a set of TMA control blocks for selected biomarkers

Names of cell lines from ATCC	Ratio of each cell line	Name of control block	Types of cancer to be covered	Targeted biomarkers with high-level expression	Targeted biomarkers with low-level and no expressions
HTB-133 CRL-2330 CRL-2329	HTB-133 -40% CRL-2330 -40% CRL-2329 -20%	Breast cancer	Breast ductal and lobular carcinomas	CK7, ER, PR, HER2, GATA3, GCDFP15, MGB, TFF3, TFF1	ER, PR, HER2, GATA3, GCDFP15, MGB, TFF3, TFF1
CRL-1424(G361) CRL-1585 CRL-5895	G361 -40% CRL-1585 -40% CRL-5895 -20%	Melanoma	Melanocytic tumors	S100, HMB-45, SOX10, MART-1, MiTF, vimentin, SOX2, S100A6, MUM1, AE1/3, CK7	S100, HMB-45, SOX10, MART-1, MiTF, vimentin, SOX2, S100A6, MUM1, AE1/3, CK7
CRL-1582 CCL-86 CRL-5895	CRL-1582 -40% CCL-86 -40% CRL-5895 -20%	Lymphoma	B cell and T cell lymphomas	CD2, CD3, CD5, CD20, CD79a, PAX5, CD10, Tdt, EBV, Bcl-2, Bcl-6, CK, and CK7	CD2, CD3, CD5, CD20, CD79a, PAX5, CD10, Tdt, EBV, Bcl-2, Bcl-6, CK, and CK7
CRL-2073 HTB-36 HB-8065 (HepG2) CRL-1585	CRL-2073 -25% HTB-36 -25% HepG2 -25% CRL-1585 -25%	Germ cell tumor	Seminoma, Embryonal carcinoma, yolk sac tumor, choriocarcinoma, teratoma	SALL4, S100P, PLAP, Beta-HCG, CD10, OCT4, AFP, glypican-3, D2-40, CD30, Nanog, SOX2, cytokeratin	SALL4, S100P, PLAP, Beta-HCG, CD10, OCT4, AFP, glypican-3, D2-40, CD30, Nanog, SOX2, cytokeratin
CCL-136 HTB166 TT (CRL-1803) CRL-1582 CCL-86 CRL-5946	CCL-136 -20% HTB166 -20% TT (CRL-1803) -20% CRL-1582 -20% CCL-86 -20% CRL-5946 -20%	Malignant small round cell tumor	Lymphoma/leukemia, small cell carcinoma/ neuroendocrine carcinoma, Ewing's sarcoma/PNET, rhabdomyosarcoma, neuroblastoma, leiomyosarcoma, desmoplastic small round cell tumor	Desmin, MyoD1, myogenin, smooth muscle actin, CD99, NKX2.2, FLI-1, synaptophysin, chromogranin, CD56, WT1, NSE, vimentin, TTF1, cytokeratin, Tdt, CD3, CD20, EBV	Desmin, MyoD1, myogenin, smooth muscle actin, CD99, NKX2.2, FLI-1, WT1, synaptophysin, chromogranin, CD56, NSE, vimentin, TTF1, cytokeratin, Tdt, CD3, CD20, EBV
HTB166 CCL-136 CRL-2279 CRL-1585 CRL-1550	HTB166 -20% CCL-136 -20% CRL-2279 -20% CRL1585 -20% CRL-1550 -20%	Sarcoma	Leiomyosarcoma, rhabdomyosarcoma, Ewing sarcoma, neurogenic tumors, vascular tumors, sarcomatoid carcinomas	Desmin, myoD1, myogenin, SMA, CD99, NKX2.2, FLI-1, ERG, vimentin, S100, cytokeratin, CK5/6, CK903, p63, p40, p16	Desmin, myoD1, myogenin, SMA, CD99, NKX2.2, FLI-1, ERG, vimentin, S100, cytokeratin, CK5/6, CK903, p63, p16
HTB133 CCL-253 (NCI-H508) TT (CRL-1803) Panc 3.27 (CRL-2549) CRL-1932 CRL-2279 CRL-1550 CRL-5946	HTB133 -10% CCL-253 (NCI-H508) -10% TT (CRL-1803) -10% Panc 3.27 (CRL-2549) -10% CRL-1932 -10% CRL-2279 -10% CRL-1550 -10% CRL-5946 -10%	Tumor of unknown primary	To include a tumor from the lung, breast, pancreas, kidney, thyroid, uterus, uterine cervix, ovary, upper GI tract, colon, bladder, prostate, vascular tumors, and neuroendocrine tumors	ER, PR, GATA3, GCDFP15, CK7, CK20, CK5/6, CK903, p40, p63, CDX2, CEA, MOC31, B72.3, BerEP4, Beta-catenin, SATB2, CDH17, TTF1, calcitonin, chromogranin, synaptophysin, CD56, Maspin, MUC1, MUC2, MUC5AC, IMP3, S100P, PAX8, PAX2, vimentin, P504S, ERG, Fli-1, p16, WT-1, HPV (in situ)	ER, PR, GATA3, GCDFP15, CK7, CK20, CK5/6, CK903, p40, p63, CDX2, CEA, MOC31, B72.3, BerEP4, Beta-catenin, SATB2, CDH17, TTF1, calcitonin, chromogranin, synaptophysin, Maspin, MUC1, MUC2, MUC5AC, IMP3, S100P, PAX8, PAX2, vimentin, P504S, ERG, Fli-1, p16, HPV (in situ)

body to your IHC lab, here is a set of questions that you may want to raise:

1. Why do I need this antibody, and what is its clinical application?
2. What is the diagnostic sensitivity and specificity of this antibody?
3. What is the likely test volume in my IHC lab?
4. Where can I get this antibody?
5. Is more than one antibody (clone) available?
6. Do I have the positive control tissues to test and validate this antibody?
7. How am I going to implement it?

The following combined approach may be helpful in adding a new antibody to your IHC lab:

1. Studying and tracking mature publications in popular peer-reviewed pathology journals, especially those publishing articles on diagnostic surgical pathology and cytopathology;

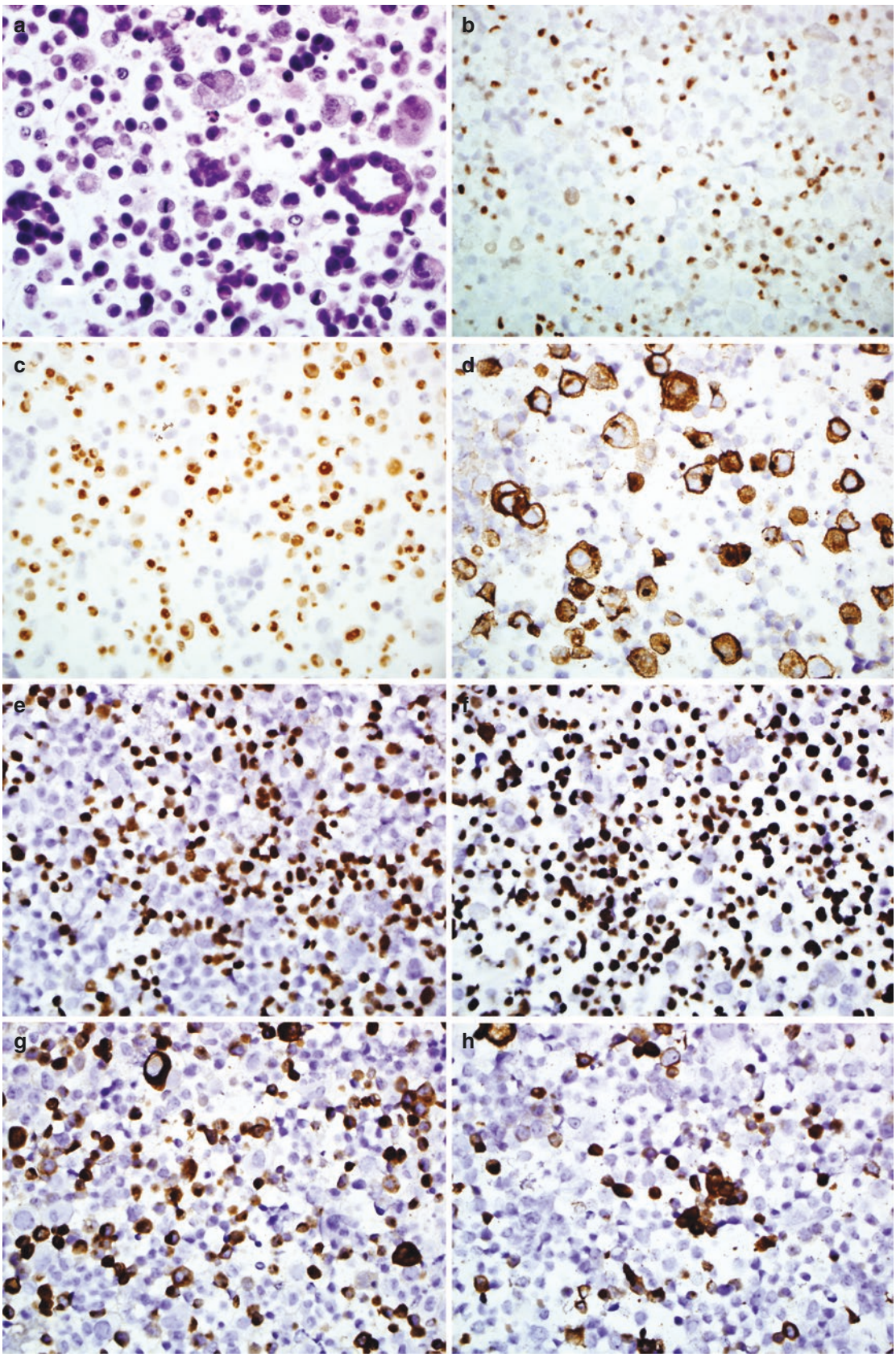

Fig. 2.1 A mixture of cultured breast cancer cell lines on H&E stained section (**a**), and expression of ER (**b**), PR (**c**), HER 2 (**d**), GATA3 (**e**), p53 (**f**), GCDFP15 (**g**), and TFF3 (**h**)

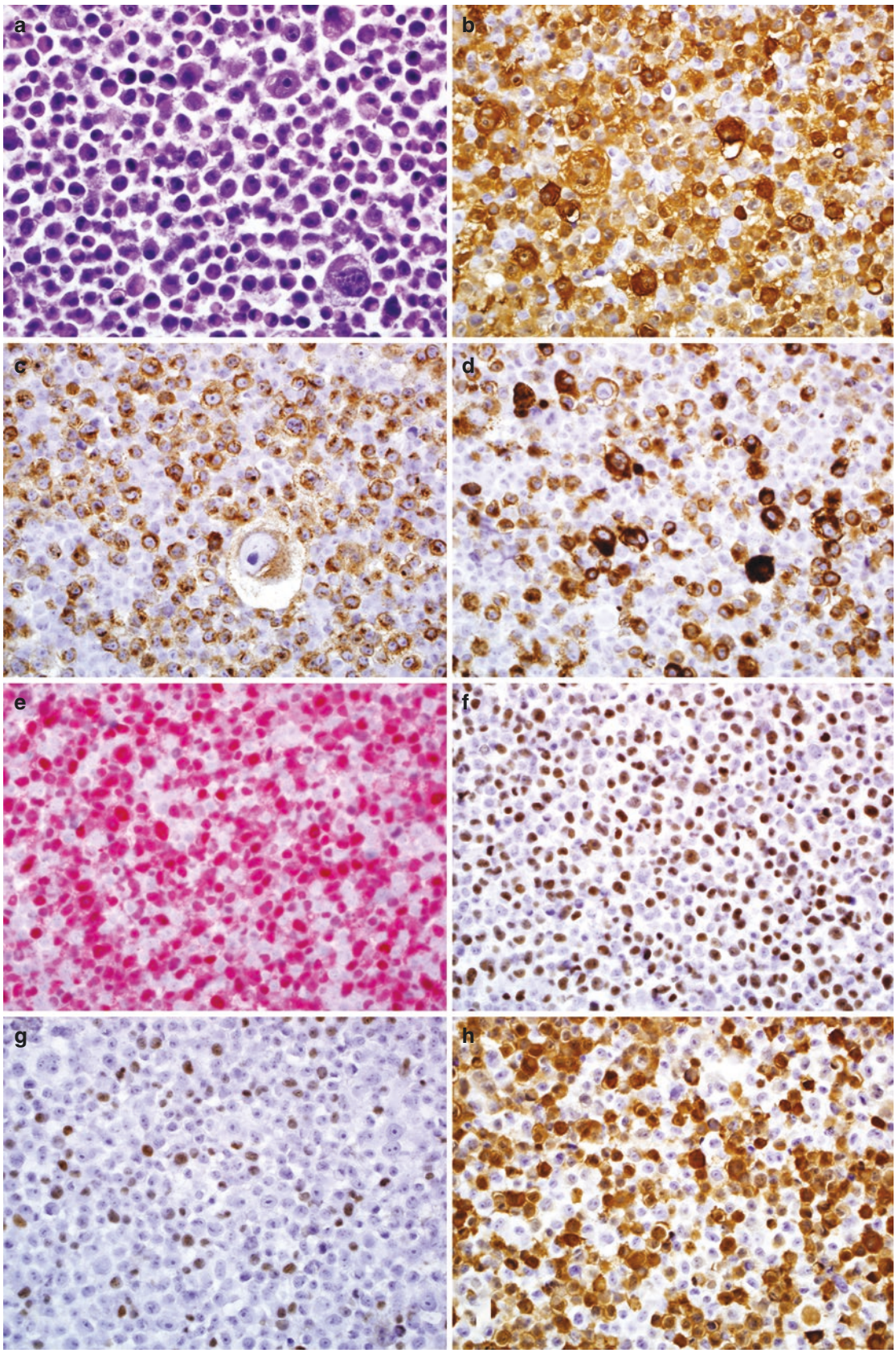

Fig. 2.2 A mixture of melanoma cell lines on H&E stained section (**a**), and expression of S100 (**b**), HMB-45 (**c**), MART-1 (**d**), MiTF (**e**), SOX10 (**f**), SOX2 (**g**), and S100A6 (**h**)

Table 2.6 Summary of iCAPCs for commonly used IHC markers

IHC markers	Tissue with high level of expression	Tissue with low level of expression	Tissue with no expression
Pan-CK	Appendix—Virtually all columnar epithelial cells must show a moderate to strong predominantly cytoplasmic staining reaction	Liver—The vast majority of hepatocytes must show at least weak to moderate cytoplasmic staining reaction with a membranous accentuation	Appendix—Lymphoid tissue
CK8 and/or CK18	Appendix—All columnar epithelial cells must show a moderate to strong predominantly cytoplasmic staining reaction	Liver—The vast majority of hepatocytes must show at least weak to moderate cytoplasmic staining reaction with a membranous accentuation. Tonsil—Scattered squamous epithelial cells show a moderate to strong cytoplasmic staining reaction	Appendix—Lymphoid tissue
CK5 and/or CK14	Tonsil—Virtually all squamous epithelial cells throughout all cell layers must show a moderate to strong cytoplasmic staining reaction	Pancreas—Scattered columnar epithelial cells of intercalated ducts must show a weak to moderate predominantly membranous staining reaction	Liver—No staining
CK20	Appendix—Virtually all surface epithelial cells must show a moderate to strong cytoplasmic staining reaction	Appendix—The majority of basal crypt epithelial cells must show an at least weak cytoplasmic staining reaction	Liver and tonsil—No staining
CK7	Liver—Virtually all epithelial cells of bile ducts must show a moderate to strong cytoplasmic staining reaction	Pancreas—Virtually all epithelial cells of intercalated ducts must show a weak to moderate cytoplasmic staining reaction	Appendix—No staining
Vimentin	Appendix—Endothelial cells of large vessels, stromal cells, macrophages, and lymphocytes in lamina propria must show a moderate to strong cytoplasmic staining reaction	Liver—All endothelial cells and Kupffer cells of the sinusoids must show a weak to moderate cytoplasmic staining reaction	Appendix—No staining on glands
TTF1	Thyroid—Virtually all epithelial cells must show a strong nuclear staining reaction Lung—Virtually all pneumocytes and basal cells of terminal bronchi must show a moderate to strong nuclear staining reaction	Lung—Columnar epithelial cells of terminal bronchi must show an at least weak nuclear staining reaction	Tonsil—No staining
CDX2	Appendix—Virtually all epithelial cells must show a strong nuclear staining reaction	Pancreas—The majority of epithelial cells of intercalated ducts must show a weak to moderate nuclear staining reaction	Tonsil—No staining
mCEA	Colon—A moderate to strong staining reaction must be seen in the brush border of the surface epithelial cells	Colon—Virtually all epithelial cells must show a weak to moderate cytoplasmic staining reaction	Tonsil—No staining in lymphoid tissue
CD31	Pancreas—Virtually all endothelial cells must show a moderate to strong predominantly membranous staining reaction	Liver—Virtually all endothelial cells of the sinusoids must show a weak to moderate predominantly membranous staining reaction	Liver—No staining in hepatocytes
Alpha-SMA	Appendix—All smooth muscle cells in vessels, muscularis mucosae, and muscularis propria of the appendix must show a moderate to strong, distinct cytoplasmic staining reaction	Liver—The majority of the perisinusoidal cells must show an at least weak to moderate, distinct cytoplasmic staining reaction	Tonsil—No staining in lymphoid cells
Desmin	Appendix—All smooth muscle cells in muscularis mucosae and muscularis propria of the appendix must show a moderate to strong, distinct cytoplasmic staining reaction	Appendix—Most smooth muscle cells of vessels must show a weak to moderate cytoplasmic staining reaction	Liver—No staining in hepatocytes
CD3	Tonsil—Virtually all T cells must show a moderate to strong, distinct predominantly membranous staining reaction	Liver—Dispersed T cells must show an at least weak to moderate, distinct predominantly membranous staining reaction	Liver—No staining in hepatocytes
CD20	Tonsil—Virtually all germinal center B cells and mantle zone B cells must show a distinct, moderate to strong membranous staining reaction	Liver—Dispersed B cells must show a distinct, moderate to strong membranous staining reaction	Liver—No staining in hepatocytes
Ki-67	Tonsil—Virtually all germinal center B cells, in both the dark and light zones and parabasal squamous epithelial cells must show a moderate to strong nuclear staining reaction	Not applicable	Liver—No staining reaction must be seen in the vast majority of hepatocytes in normal liver

(continued)

Table 2.6 (continued)

IHC markers	Tissue with high level of expression	Tissue with low level of expression	Tissue with no expression
S100	Tonsil—Dispersed interfollicular dendritic cells must how a moderate to strong nuclear and cytoplasmic staining reaction	Pancreas—The majority of endocrine cells of islets of Langerhans must show a weak to strong nuclear and cytoplasmic staining reaction. All adipocytes should be positive	Liver: No staining reaction in hepatocytes and epithelial cells of bile ducts must be seen
Chromogranin	Pancreas—The majority of the endocrine cells of the islets of Langerhans must show a moderate to strong cytoplasmic staining reaction	Appendix—The majority of ganglion cells and axons of Auerbach's plexus must show a weak to moderate staining reaction	Liver: No staining reaction in hepatocytes and epithelial cells of bile ducts must be seen
Synaptophysin	Appendix—The majority of ganglion cells and axons of Auerbach's plexus must show a moderate to strong staining reaction	Appendix—The majority of goblet cells must show a weak cytoplasmic staining reaction	Liver: No staining reaction in hepatocytes and epithelial cells of bile ducts

Reference: [14]

2. Attending major pathology society conferences, particularly the United States & Canadian Academy of Pathology (USCAP) annual meeting, or reading the abstract book;
3. Using IHC vendor recommendations and online catalogs;
4. Using free websites with a published antibody library such as Geisinger's IHC website (http://www.ihcfaq.com) [15], NordiQC (Nordic Immunohistochemical Quality Control, Aalborg, Denmark, http:/www.nordiqc.org) [16], and IHC menus on the pathology department websites of major medical institutions and hospitals.

11. What are the results and common problems encountered when testing a new antibody?

Troubleshooting problems encountered in an immunohistochemical staining procedure can be straightforward or a very complicated task. Many articles and book chapters have addressed these potential issues in great detail; therefore, this chapter is not intended to be comprehensive or to substitute for published literature. Instead, it attempts to re-emphasize the key points to remember when working on these problems. The nine most likely immunostaining results and potential problems encountered when testing and optimizing a new antibody in a positive control tissue block are summarized in Table 2.7. The possible causes and solutions for each specific problem will be addressed in Question #14.

12. What are the general approaches before getting into a demanding technical issue?

As a general rule, the best approach to avoid the technical issues listed in Table 2.7 is to follow this simple checklist:

1. Follow all steps and instructions in the manufacturer's protocol.
2. Consult the data sheet for general recommendations, such as positive control tissue, antigen retrieval technique, antibody dilution, blocking reagent, etc.

Table 2.7 Summary of possible staining results when testing a new antibody

	Staining signal		
Background	Strong	Weak	None
No background	A	D	G
Weak background	B	E	H
Strong background	C	F	I

3. Confirm the compatibility of a secondary antibody to the species and subclass immunoglobulin of a primary antibody (such as rabbit monoclonal antibody or mouse monoclonal antibody).
4. Be sure to use the "right" tissue or tumor as a positive control; it should contain abundant and well-preserved antigen to be tested.
5. Use a positive control block (such as tissue microarray block) containing multiple tissue sections if it is available.
6. Ensure the oven temperatures do not exceed 60 °C.
7. Perform all relevant blocking steps to eliminate background staining, including endogenous peroxidases and phosphatases.
8. Check all reagents for appropriate preparation, expiration date, and storage condition.
9. Be aware that inadequate fixation (under-fixation), inappropriate fixative (other than 10% neutral-buffered formalin), and high acidity or prolonged decalcification may result in a false-negative result for many antibodies.

13. How do you optimize a new antibody?

The ultimate goal is to achieve a strong staining signal with little or no background staining using the highest primary antibody dilution. A TMA block containing a small number of tumors and/or normal tissues with known positivity and negativity for the target antigen is a good choice to test a new antibody. There are many ways to test a new antibody. Our experience demonstrates that a false-negative result is more

likely due to the wrong antigen retrieval method rather than a suboptimal antibody dilution and/or incubation time. We tend to determine the best antigen retrieval method first and then test the proper primary antibody dilution and incubation time. To achieve this, we start with five different antigen retrieval methods/solutions (heat-induced epitope retrieval with citrate buffer/pH 6.0, with ethylenediaminetetraacetic acid (EDTA)/pH 8, with Target Retrieval Solution (TRS)/pH 6.1, with High pH Target Retrieval Solution (HiTRS)/pH 9, or enzyme digestion such as proteinase K/pH 7.5 solution) and a fixed, high concentration of primary antibody (if a recommended dilution range is 1:100 to 1:500, we will start with 1:100). A range of 1–5 ug/mL of primary antibody concentration is usually recommended for an initial titration [9]. After determining the antigen retrieval method, we will test different antibody dilutions (usually three different dilutions) and adjust the incubation time based on the initial test result. A polyclonal antibody is less specific and may cross-react with other antigens; therefore, using a polyclonal antibody is discouraged unless a monoclonal antibody is not commercially available. The advantage of using a polyclonal antibody, however, is that it can be used at a much higher dilution (>1:1000 for many antibodies), which will reduce the cost.

A standard online tracking table is created for the testing and optimization of each new antibody. The initial testing is labeled as protocol #1. Detailed documentation of changes to the antibody testing parameters, such as retrieval condition, dilution, and incubation time, is kept in an online tracking form and labeled as protocols #2, #3, etc. When the testing condition is optimized, the IHC lab director or the pathologist who oversees the IHC testing process will verify the protocol and proceed to the antibody validation process.

14. What are the possible solutions for each specific technical problem in Table 2.7?

A. *If strong staining signal and no background staining is obtained*
   - The next step is to test the primary antibody in multiple dilutions, obtain the highest dilution with the optimal result, to save the primary antibody and cut down the cost.

B. *If strong staining signal and weak background staining is obtained*
   - Reduce the primary antibody concentration.
   - Shorten the primary antibody incubation time.
   - Shorten the secondary antibody incubation time.
   - Further block the background staining.

C. *If strong staining signal and strong background staining is obtained*
   - Reduce the primary antibody concentration.
   - Shorten the primary antibody incubation time.
   - Shorten the secondary antibody incubation time.
   - Further block the background staining.
   - Try different antigen retrieval methods.

D. *If weak staining signal and no background staining is obtained*
   - Increase the primary antibody concentration.
   - Increase the incubation time for the primary antibody.
   - Increase the incubation time for the secondary antibody.
   - Switch to a more sensitive secondary detecting system.
   - Try different antigen retrieval methods.

E. *If weak staining signal and weak background staining is obtained*
   - Increase the primary antibody concentration and reduce the incubation time.
   - Further block background staining.
   - Increase the incubation time for the secondary antibody.
   - Switch to a more sensitive secondary detecting system.
   - Try different antigen retrieval methods.
   - Use a different primary antibody.

F. *If weak staining signal and strong background staining is obtained*
   - Further block background staining.
   - Switch to a more sensitive secondary detecting system.
   - Try different antigen retrieval methods.
   - Use a different primary antibody.

G. *If no staining signal and no background staining is obtained*
   - Follow the general approaches in Question 12 step by step.
   - Increase the primary antibody concentration and incubation time.
   - Try different antigen retrieval methods.
   - Switch to a more sensitive secondary detecting system.
   - Contact the technical department of the primary antibody supplier for assistance.
   - Use a different primary antibody.

H. *If no staining signal and weak background staining is obtained*
   - Follow the general approaches in Question 12 step by step.
   - Increase the primary antibody concentration and incubation time.
   - Try different antigen retrieval methods.
   - Switch to a more sensitive secondary detecting system.

- Contact the technical department of the primary antibody supplier for assistance.
- Use a different primary antibody.

I. *If no staining signal and strong background staining is obtained*
- Follow the general approaches in Question 12 step by step.
- Try different antigen retrieval methods.
- Switch to a more sensitive secondary detecting system.
- Contact the technical department of the primary antibody supplier for assistance.
- Use a different primary antibody.

15. How do you determine whether or not a primary antibody works?

To get a quick idea of whether a primary antibody works on positive control tissue, refer to Fig. 2.3. If the testing result appears in zone I, II, or III, the primary antibody will work well after fine-tuning. If the testing result falls into zone IV, V, or VI, after additional testing and adjustments of the staining condition, the primary antibody is most likely working. If the testing result ends up in zone VII, VIII, or IX, the primary antibody is unlikely to work; therefore, to save time, a new antibody from a different vendor should be considered.

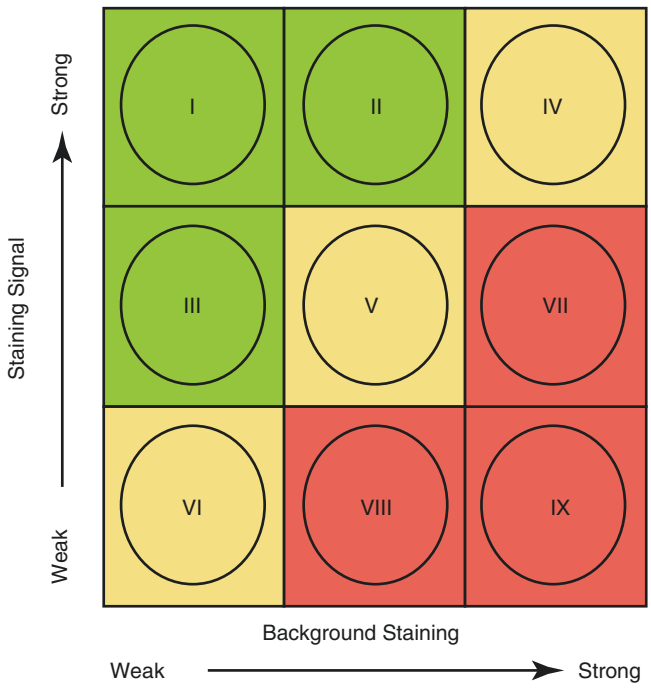

Fig. 2.3 Summary of possible staining results, which gives one a quick idea of whether a primary antibody works on positive control tissue

16. What are the commonly used antigen retrieval methods?

Antigen retrieval technique is a process to unmask an antibody-binding site for a specific antibody on formalin-fixed, paraffin-embedded tissue sections. It can significantly enhance the immunohistochemical staining signal. There are two main antigen retrieval techniques. One is called heat-induced epitope retrieval (HIER). Another method uses enzymatic digestion and is called proteolytic-induced epitope retrieval (PIER) [2, 17, 18].

Many enzymes can be used in PIER, such as trypsin, proteinase K, pepsin A, and pronase. The key factors to obtain an optimal result include enzyme concentration, time of digestion, temperature, and pH. Proteinase K will provide an effective enzymatic digestion for membrane antigens such as pVHL, CD31, and VWF. Over-digestion may result in poor tissue morphology and even a false-positive staining; in contrast, under-digestion may cause a false-negative result.

Microwave oven and water bath are the most commonly used heating devices for HIER in our lab. Other devices may include microwave pressure cooker, vegetable steamer, and decloaker device. A heating and cooling time of 20 minutes each appears to be adequate for many antibodies. EDTA at pH 8.0 is the most frequently used retrieval solution in our practice; citrate buffer at pH 6.0, target retrieval solution at pH 6.1, and target retrieval solution at pH 9.0 (high pH) are also suitable for some antibodies [2, 17, 18].

A combination of HIER and PIER is an alternative approach to unmask difficult antigens when other methods fail. It is especially useful when performing double or triple labeling for two or more antigens simultaneously. However, special attention should be paid because two retrieval methods may cause a false-negative staining result for one of the two antibodies, and sometimes a tissue section may fall off the slide due to the prolonged retrieval time.

17. What are the commonly used antigen retrieval protocols?

- ***Protocol #1: Citrate Buffer Antigen Retrieval Method*** [2, 17, 18]
- **Solutions and Reagents:**

Solution A: Citric acid monohydrate (Fisher Scientific, Catalog# A104-500)

10.505 g, dilute in 500 mL of distilled water, mix to dissolve (0.1 M)

Solution B: Sodium citrate (Fisher Scientific, Catalog # S93364)

14.704 g, dilute in 500 mL of distilled water, mix to dissolve (0.1 M)

Store the solutions at 4 ° C for longer storage. Fresh preparation of citrate buffer before use:

$$\left.\begin{array}{l} 9\,\text{mL of Solution A} \\ 41\,\text{mL of Solution B} \end{array}\right\} \text{Dilute in } 500\,\text{mL of distilled water, mix to dissolve}$$

Adjust pH to 6.0 with 1 N NaOH and 1 N HCl and mix well.

Formalin, or other aldehyde fixation, forms protein cross-linking that masks the antigenic sites in tissue specimens, which in turn gives weak or false-negative staining for immunohistochemical detection of certain proteins. The citrate-based solution is designed to break the protein cross-linking, thereby unmasking the antigens and epitopes in formalin-fixed and paraffin-embedded tissue sections and enhancing staining intensity of antibodies.

- **Procedure:**
  1. Dewax paraffin-embedded tissue sections with two changes of Histoclear® solution (National Diagnostics, Atlanta, GA, product #HS-200) or xylene, 5 minutes each.
  2. Rehydrate the sections in two changes of 100% and 95% ethanol for 30 seconds each and 70% ethanol for 30 seconds; then rinse in distilled water.
  3. Insert the slides in a slide holder and immerse them into a microwave dish containing 500 mL of citrate buffer. Set the lid loosely on top of the microwave dish.
  4. Heat the dish for 5 minutes at high power (level 10) and 10 minutes at medium power (level 5).
  5. Allow the slides to cool for 20 minutes at room temperature.
  6. Rinse sections in cool running tap water; then put the slides into Tris-Buffered Saline with 0.05% Tween (TBST) solution.
  7. Continue with an appropriate antibody staining protocol.
- ***Protocol #2: EDTA Buffer Antigen Retrieval Protocol*** [2, 17, 18]
- **Solutions and Reagents:**

EDTA buffer (1 mM EDTA, pH 8.0)

EDTA (Fisher Scientific, Cat#BP120 – 500)	0.372 g
Distilled water	1000 mL

Mix to dissolve. Adjust pH to 8.0 using 1 N NaOH. Store the solution at room temperature for up to 3 months or at 4 °C for longer storage.

This buffer works well for many antibodies, but sometimes it gives high background staining; therefore, a primary antibody can often be diluted in a lower concentration. It is very useful for low-affinity antibodies or when tissue antigens are not abundant.

The EDTA solution is also designed to break the protein cross-links, thereby unmasking the antigens and epitopes in formalin-fixed and paraffin-embedded tissue sections, thus enhancing the staining intensity of the antibodies.

- **Procedure:**
  1. Dewax the sections in two changes of xylene, 5 minutes each.
  2. Rehydrate the slides in two changes of 100% and 95% ethanol for 30 seconds each and 70% ethanol for 30 seconds; then rinse in distilled water.
  3. Place the slides in a slider holder and immerse them in a microwave dish containing 500 mL of EDTA buffer. Set the lid loosely on top of the microwave dish.
  4. Heat the dish for 5 minutes at high power (level 10) and 10 minutes at medium power (level 5).
  5. Allow the slides to cool for 20 minutes at room temperature.
  6. Rinse sections in cool running tap water; then put the slides into Tris-Buffered Saline with 0.05% Tween (TBST) solution.
  7. Continue with an appropriate antibody staining protocol.
- ***Protocol #3: Target Retrieval Solution Buffer Antigen Retrieval Protocol*** [2, 17, 18]
- **Solutions and Reagents:**

Target retrieval solution, pH 6.1 (Dako, Carpenteria, CA, Catalog# S1700)

Target retrieval solution, pH 9.0 (Dako, Catalog# S2368)

These products are to be used on formalin-fixed paraffin-embedded tissue sections mounted on glass slides for target retrieval prior to IHC procedures.

The retrieval procedure involves incubating the sections in preheated target retrieval solution in a water bath for 20 minutes prior to IHC procedures. This results in an increase in staining intensity for many primary antibodies.

- **Procedure:**
  1. Fill a Coplin staining jar or other suitable containers with a sufficient quantity of target retrieval solution. Place the container in a water bath. Heat the water bath to 95–99 °C (do not boil).
  2. Dewax the sections in two changes of xylene, 5 minutes each.
  3. Rehydrate the slides in two changes of 100% and 95% ethanol for 30 seconds each and 70% ethanol for 30 seconds; then rinse in distilled water.

4. Incubate the sections in preheated target retrieval solution in a water bath for 20 minutes.
5. Remove the entire jar or container with slides from the water bath and allow the slides to cool for 20 minutes at room temperature.
6. Rinse the sections in cool running tap water; then put the slides into Tris-Buffered Saline with 0.05% Tween (TBST) solution.
7. Continue with an appropriate antibody staining protocol.

- ***Protocol #4: Retrieve-All Antigen Unmasking System Retrieval Protocol*** [2, 17, 18]
- **Solutions and Reagents:**

Retrieve-All Antigen Unmasking System 1: Universal, 1X (Covance, Princeton, NJ, Catalog# SIG-31912)

Retrieve-All is an antigen unmasking solution available in the following three pH formulas for use in heat-induced unmasking. Retrieve-All 1 (Universal pH 8) is the most frequently used in our immunohistochemical laboratory. Other Retrieve-All solutions include Retrieve-All 2 (basic pH 10) and Retrieve-All 3 (acidic pH 4.8).

- **Procedure:**
  1. Fill a Coplin staining jar or other suitable containers with a sufficient quantity of Retrieve-All 1 solution. Place the container in a water bath. Heat the water bath to 95–99 °C (do not boil).
  2. Dewax the sections in two changes of xylene solution, 5 minutes each.
  3. Rehydrate the sections in two changes of 100% and 95% ethanol for 30 seconds each and 70% ethanol for 30 seconds; then rinse in distilled water.
  4. Incubate the sections in preheated Retrieve-All 1 solution in a water bath for 10 minutes.
  5. Remove the entire jar or container with slides from the water bath and allow the slides to cool for 10 minutes at room temperature.
  6. Rinse sections in cool running tap water; then put the slides into Tris-Buffered Saline with 0.05% Tween (TBST) solution.
- ***Protocol #5: Proteinase K Antigen Retrieval Protocol*** [2, 17, 18]
- **Solutions and Reagents:**

Proteinase K Solution (Dako, Catalog# S3020)

Proteolytic enzyme solution diluted in 0.05 mol/L Tris-HCl, 0.015 mol/L sodium azide, pH 7.5.

Proteinase K is used for the proteolytic digestion of formalin-fixed, paraffin-embedded tissue sections prior to an immunohistochemical staining protocol.

Enzymatic digestion unmasks certain epitopes/sites which have been masked during the formalin-fixation process; therefore, unmasking the antigens and epitopes in formalin-fixed and paraffin-embedded tissue sections will enhance staining intensity of antibodies. This method may cause tissue damage if the tissue sections are under-fixed. It is crucial to select the appropriate incubation time (5–20 minutes) and temperature (20–60 °C) for a specific application and try to avoid over-digestion of the tissue sections. Proteinase K is a very useful antigen unmasking solution for some cell membrane antigens.

- **Procedure:**
  1. Dewax the sections in two changes of Histoclear® or xylene solution, 5 minutes each.
  2. Rehydrate in two changes of 100% and 95% ethanol for 30 seconds each and 70% ethanol for 30 seconds; then rinse in distilled water.
  3. Transfer the sections into proteinase K working solution and incubate for 5–15 minutes at room temperature in a humidified chamber (optimal incubation time may vary depending on tissue type and degree of fixation).
  4. If you use a Dako Autostainer, after performing peroxidase block (Dako, Catalog# K4007) or avidin/biotin-blocking processing, you can set up a step for proteinase K incubation before primary antibody incubation. Certainly, a polymer detecting system is a more favorable choice.
  5. Continue with an appropriate antibody staining protocol.

18. What are the recommended guidelines for antibody validation?

The CAP Pathology and Laboratory Quality Center gathered a team of pathologists and histotechnologists with expertise in immunohistochemistry to develop guidelines for validation of immunohistochemical assays [19]. Following review of 126 related articles, open comments, panel discussion, and expert opinions, 14 guideline statements, including 4 recommendations and 10 expert consensus opinions, were proposed as listed below [19].

1. Laboratories must validate all IHC tests before placing them into clinical service—Recommendation.
2. For initial validation of every assay used clinically, with the exception of HER2, ER, and PR (for which established validation guidelines already exist), laboratories should achieve at least 90% overall concordance between the new test and the comparator test or expected results. If concordance is less than 90%, laboratories need to investigate the cause of low concordance—Recommendation.
3. For initial analytic validation of non-predictive factor assays, laboratories should test a minimum of 10 positive and 10 negative tissues. When the laboratory medi-

cal director determines that fewer than 20 validation cases are sufficient for a specific marker (e.g., rare antigen), the rationale for that decision needs to be documented—Expert Consensus Opinion.

4. For initial analytic validation of all laboratory-developed predictive marker assays, laboratories should test a minimum of 20 positive and 20 negative tissues. When the laboratory medical director determines that fewer than 40 validation tissues are sufficient for a specific marker, the rationale for that decision needs to be documented—Expert Consensus Opinion.
5. For a marker with both predictive and non-predictive applications, laboratories should validate it as a predictive marker if it is used as such—Recommendation.
6. When possible, laboratories should use validation tissues that have been processed using the same fixative and processing methods as cases that will be tested clinically—Recommendation.
7. If IHC is regularly done on cytologic specimens that are not processed in the same manner as the tissues used for assay validation (e.g., alcohol-fixed cell blocks, air-dried smears, formalin post-fixed specimens), laboratories should test a sufficient number of such cases to ensure that assays consistently achieve expected results. The laboratory medical director is responsible for determining the number of positive and negative cases and the number of predictive and non-predictive markers to test—Expert Consensus Opinion.
8. If IHC is regularly done on decalcified tissues, laboratories should test a sufficient number of such tissues to ensure that assays consistently achieve expected results. The laboratory medical director is responsible for determining the number of positive and negative tissues and the number of predictive and non-predictive markers to test—Expert Consensus Opinion.
9. Laboratories may use whole sections, TMAs, and/or multi-tissue blocks (MTBs) in their validation sets as appropriate. Whole sections should be used if TMAs/ MTBs are not appropriate for the targeted antigen or if the laboratory medical director cannot confirm that the fixation and processing of TMAs/ MTBs are similar to clinical specimens—Recommendation.
10. When a new reagent lot is placed into clinical service for an existing validated assay, laboratories should confirm the assay's performance with at least one known positive case and one known negative case—Expert Consensus Opinion.
11. Laboratories should confirm assay performance with at least two known positive and two known negative cases when an existing validated assay has changed in any one of the following ways: antibody dilution, antibody vendor (same clone), incubation, or retrieval times (same method)—Expert Consensus Opinion.
12. Laboratories should confirm assay performance by testing a sufficient number of cases to ensure that assays consistently achieve expected results when any of the following have changed: Fixative type, antigen retrieval method (e.g., change in pH, different buffers, different heat platforms), antigen detection system, tissue processing or testing equipment, environmental conditions of testing (e.g., laboratory relocation), and laboratory water supply. The laboratory medical director is responsible for determining how many predictive and non-predictive markers and how many positive and negative tissues to test—Expert Consensus Opinion.
13. Laboratories should run a full revalidation (equivalent to initial analytic validation) when the antibody clone is changed for an existing validated assay—Expert Consensus Opinion.
14. The laboratory must document all validations and verifications in compliance with regulatory and accreditation requirements—Expert Consensus Opinion.

At Geisinger, we have established a large TMA bank containing thousands of tumors and normal tissues from various organs. Each TMA block typically contains 50–100 tumors or normal tissues which were fixed and processed under similar or identical conditions as other routine patient samples. Two punched cores of 1.5 mm or 2.0 mm each were usually taken from each case. After antibody testing and optimizing on a small TMA block containing 5–10 cases of tumor/normal tissues, the antibody validation process was followed. For instance, for validation of napsin A monoclonal antibody, three TMA blocks were selected, including lung adenocarcinomas, papillary renal cell carcinomas (RCCs), and lung squamous cell carcinomas. The positive reference range for napsin A was expected to be 75–80% in lung adenocarcinomas, 50–60% in papillary RCCs, and close to zero in lung squamous cell carcinomas. If the validation data were within the reference range, napsin A was included in the antibody library and implemented in our IHC lab. If the validation data showed that the positive percentage was significantly below the reference range (below 70% in this case), we repeated the validation process in 10 cases of lung adenocarcinoma on routine sections to eliminate the possibility of focal staining on TMA sections which consequently resulted in a lower diagnostic sensitivity. If the positive rate (sensitivity) continued to be low, we returned to the antibody testing and optimizing step to increase the positive staining signal and subsequently increase the diagnostic sensitivity to the reference range if possible.

19. How do you select an automated staining platform?

Before you select an automated IHC staining platform, there are several questions you should ask. What are the advantages and disadvantages of automated versus manual IHC? What are the strengths and weaknesses of each automated platform, such as the Ventana Benchmark Ultra (Ventana Medical Systems, Tucson, Arizona), Leica Bond III (Leica Biosystems, Buffalo Grove, Illinois), Dako Omnis (Dako), and Biocare intelliPath FLX (Biocare Medical, Concord Massachusetts)? The parameters that need to be considered are user-friendliness, capacity, turnaround time, amount of reagent/antibody used, waste disposal control, quality of stains, ability to run multiplex and in situ hybridization, and flexibility of integration with other laboratory information systems (LISs). The details are addressed in Chap. 3.

20. How do you interpret IHC assay results?

There is no universal scoring system for an IHC assay result or general agreement on the cutoff point to render a positive or a negative IHC test result. In fact, it is unlikely and impractical to have an absolute cutoff value for all diagnostic immunomarkers. In general, we use 5% as a cutoff point to determine a positive or a negative staining result, especially for cytoplasmic and membranous staining markers. Many factors may influence the interpretation and should be taken into consideration when interpreting an IHC test result: (1) small biopsy and cell block versus large resection specimen; (2) the amount of target antigen in the tested tissue; (3) the specificity of the particular antigen; (4) the sensitivity of a primary antibody; (5) localization of the target antigen, such as nuclear staining versus cytoplasmic staining; (6) the staining intensity of the internal and external positive controls; and (7) how well the tissue has been fixed and processed.

A false-negative result is far more common than a false-positive result. Adequate internal positive staining is the best way to exclude false-negative staining, and a good internal negative staining is the best way to rule out a false-positive staining. In general, nuclear staining is more reliable than cytoplasmic staining. Any nuclear staining, especially in a small tissue biopsy or cell block preparation, should be regarded as a significant finding. For pathogen staining, such as BK virus and cytomegalovirus (CMV), any nuclear staining (even a single nucleus stained) in the right context should be regarded as a positive result. If the known internal and external positive tissues are only weakly positive, then weak staining in the target tissue should be read as positive, or the IHC assay should be repeated. If the target tissue is only weakly positive in the presence of background staining, caution should be taken to render the IHC test result positive. If the known internal and/or external positive controls are negative, the target tissue with no immunoreactivity should be repeated. Some IHC test results, such as integrase interactor 1 (INI1) and mismatch repair (MMR) proteins (MLH1, PMS2, MSH2, and MSH6), are significant when loss of expression occurs. In this instance, the presence of positive internal controls, such as lymphoid cells, endothelial cells, and stromal cells, is imperative before concluding loss of expression.

At Geisinger, we use a scoring system based on the extent and intensity of the stain. The extent of the stain is recorded as 0 (less than 5% of the target cells stained), 1+ (5–25% of the target cells stained), 2+ (26–50% of the target cells stained), 3+ (51–75% of the target cells stained), or 4+ (>75% of the target cells stained). The staining signal is recorded as weak, intermediate, or strong. A strong signal can be easily seen on low magnification; a weak signal is usually observed on high magnification; an intermediate signal border between a strong and a weak staining signal.

21. How do you report IHC assay results?

We report IHC assay results in a tabulated format as in Table 2.8. For example, the following table illustrates a panel of antibodies used to differentiate a metastatic breast carcinoma from a primary lung adenocarcinoma; the IHC assay result below supports the diagnosis of lung adenocarcinoma. The following elements (antibody, result, clone, localization, tissue type, and paraffin block number) are recommended to be included in the pathology report. The positive staining result can range from 1+ to 4+, with weak, intermediate, or strong staining intensity. The detailed staining results (such as 4+, strong) are recorded in our database within the CoPath system and can be potentially used for future research projects and enable one to further understand the clinical significance of the extent and intensity of each IHC stain.

Table 2.8 Summary of IHC assay results on right lung biopsy

Antibody	Target cells	Clone	Localization	Comment	Block
TTF1	Positive	8G7G3/1	N		A1
Napsin A	Positive	EP205	C		A1
GATA3	Negative	L50-823	N		A1
ER	Negative	SP1	N		A1
Ki-67	See comment	MIB-1	N	40% of target cells stained	A1

Note: *C* cytoplasmic staining, *N* nuclear staining

22. How do you improve a total IHC quality management program (quality assurance, quality control, and quality improvement)?

There are many excellent, comprehensive review articles and book chapters discussing the quality assurance, quality control, and quality improvement in the field of immunohistochemistry [2, 4, 5, 9]. The following are some additional steps we have taken in our IHC laboratory to ensure consistent, reproducible, and reliable IHC test results on every antibody, every time.

- ***Quality Control of Each Stain***

To maintain high-quality service, one needs to be proactive rather than reactive to potential quality issues. At Geisinger, every IHC stain (both patient tissues and external positive and negative control tissues) is reviewed for quality control purposes before releasing it to an ordering pathologist. A quality control worksheet containing a set of quality parameters (see Table 2.9) goes with every stain. After the review, the acceptable slides or deficient slides are determined based on these criteria. If a slide is determined to be deficient by an IHC technician, the stain may be repeated to save time. A comment section is left for the ordering pathologist. The QC result and pathologist's feedback for each stain are entered into the IHC database, and the results are collected and reviewed at our weekly laboratory technical specialist meeting. Each issue is analyzed and corrective action is taken. Is the issue caused by the instrument, reagent, staining protocol, personnel, or other factors? More importantly, is this an isolated incident or a trend of poor quality? The IHC technical specialist brings the issues back to IHC technologists to resolve. The Quality Control Worksheet has been well received by both IHC technologists and pathologists. It has proven to be a crucial step in identifying early problems and is an effective way to educate IHC technologists, which in turn will make them more vigilant to poor-quality slides and take corrective action before releasing the slide(s) to an ordering pathologist.

Table 2.9 Quality parameters for the IHC quality control worksheet

Tissue quality	*Staining intensity*
Folds, wrinkles, or chatter	No staining
Part of tissue missing	Weak staining
No tissue present	Overstained
Background staining	*Uniformity of staining*
Everything stained	Gradient staining
Red blood cells stained	Edge artifact
Collagen stained	
Specificity of staining	*Counterstaining*
Incorrect component staining (cytoplasmic, nuclear, membranous)	No counterstain Gradient staining Overstained

- ***Continuous Quality Monitoring***

Each new antibody goes through a vigorous optimizing and validating process before being implemented in our IHC lab. The quality of the IHC test result for a newly introduced antibody is usually excellent at the beginning. However, weeks or months after introduction, the quality of the IHC assay for a particular antibody may be unstable or even deteriorate. The IHC lab begins to receive complaints from ordering pathologists about weak staining, background staining, or incorrect component staining of the antibody. How can we be more proactive than reactive to potential issues? With the growing list of antibodies in our IHC lab, we started a new initiative in monitoring a select number of antibodies on a regular basis using TMA sections. The top 50 antibodies were chosen based on the highest test volumes, the clinical importance of these antibodies, and the target antigen absence from normal tissues, such as TTF1, p40, p63, ER, PR, HER2, GATA3, VHL, arginase-1, glypican-3, PAX8, WT1, CDX2, NKX3.1, NKX2.2, CD31, ERG, CD34, STAT6, desmin, SATB2, S100, HMB-45, MART-1, INSM1, chromogranin, synaptophysin, SALL4, OCT4, calretinin, inhibin-alpha, etc. A special TMA block containing tissues to test all the antibodies mentioned above was constructed. Multiple identical blocks were built. IHC tests were performed on the TMA slides for each antibody, and the slides were scanned and stored online. Monthly, 10–15 selected antibodies are tested on the same TMA block and the results (extent and intensity of the stain) are reviewed, recorded, and compared to the previously scanned images stained with the same antibody. Imaging analysis can be applied to quantitate the staining signal (extent and intensity) if needed. If a suboptimal stain, such as weak staining or background staining, is observed in the newly stained slide, the IHC test will be repeated. If the stain remains suboptimal, the antibody will be withdrawn from the test menu, and a full investigation will be carried out.

23. What is the role of digital pathology in an IHC laboratory?

Digital pathology (whole slide scan), in conjunction with imaging analysis algorithms, is a great innovation in the field of anatomic pathology. This allows pathologists to review the same slides anywhere and anytime with superb and identical quality. Digital pathology has been applied to remote frozen section diagnosis, fine-needle aspiration specimen adequacy assessment, slide consultation, slide archiving, and standardization of educational course material [20–26]. With continued improvement of workflow and integration with the LIS, digital pathology may eventually replace the traditional microscope and revolutionize the field of anatomic pathology. With regard to the field of IHC, digital pathology together with imaging analysis will provide consistent,

reproducible, and quantitative IHC assay results, especially for multiplex staining (such as double staining) and counting nuclear staining signals (such as Ki67). Furthermore, digital pathology will reduce turnaround time in a situation, wherein the IHC slides need to be delivered to another hospital or pathology office. For equivocal HER-2 IHC assay results on gastrointestinal and breast cancer, a fluorescence in situ hybridization (FISH) assay can be ordered before the glass slide is delivered. For a difficult case of tumor of unknown primary, a second panel of antibodies can be ordered before the glass slide is delivered. For quality control purposes, digital pathology enables multiple slides to be viewed simultaneously. A standard TMA slide (for validation, positive control, or continuous quality monitoring purposes) can be aligned side by side with the test slide for easy comparison of the extent and intensity of the stain.

24. What are the available proficiency testing programs?

A CAP-certified IHC lab is required to participate in the CAP External Quality Assessment (EQA) and Proficiency Testing (PT) program. CAP offers a number of PT programs specifically designed for IHC laboratories. The newly developed program, CAP/National Society for Histotechnology (NSH) HistoQIP—IHC (program code HQIHC), is designed to improve the preparation of IHC slides in all laboratories handling GI, skin, and GU biopsies. It requires participants to submit IHC-stained slides for review by a panel of experts. Most other programs are geared to evaluate IHC assay methodology and performance. These programs require participants to perform specific IHC assays (analytes) on centrally prepared slides provided by CAP, interpret the results, and then report to CAP for evaluation. As feedback, participants receive a performance evaluation packet that includes detailed statistical data about the entire survey. The participant should be able to compare its performance with all participating labs. These programs include MK (General Immunohistochemistry), PM2 (ER/PR), PM3 (CD20), PM1 (CD117), mismatch repair proteins (MMR), HER2 (breast), GHER2 (gastric HER2), and PM5 (in which markers vary by year). Among these programs, PM2 and HER2 (breast) are specifically designed to fulfill PT testing requirement of the ASCO/CAP guideline for ER, PR, and HER2 assessment in breast carcinoma. Geisinger also participates in CD30, PD-L1, p16, and BRAF V600E CAP PT testing program. The PM5 program is uniquely designed to evaluate a number of commonly used IHC assays, including chromogranin, cyclin D1, CDX2, CD30, D2-40, CK20, Ki67, PAX2, PAX8, and p63. Nevertheless, for the vast majority of over 200 diagnostic antibodies (class I tests) in a clinical IHC laboratory, there are still no standard CAP proficiency testing programs available.

Other international EQA programs specifically designed for IHC are also available. NordiQC (http:/www.nordiqc.org) [16] and United Kingdom National External Quality Assessment Service (UK NEQAS, Sheffield, England, http://www.ukneqas.org.uk) [27] are two of the most reputable ones.

NordiQC is an independent scientific organization in Denmark that dedicates itself to promoting high-quality IHC by arranging EQA services to IHC laboratories all around the world (mainly European and Asian countries) and providing recommendations for improvement based on testing results. Its website contains useful information about IHC, including free recommended staining protocols for different antibodies on different platforms. Vyberg and Nielsen published the results of a large study of proficiency testing in immunohistochemistry [28]. In their study, more than 30,000 IHC slides evaluated at 700 laboratories from 80 countries during 2003–2015 were collected. Overall, about 20% of the IHC staining results in the breast cancer IHC module and about 30% in the general module have been assessed as insufficient for diagnostic use. About 90% of the insufficient stains were characterized by a too weak or even false-negative staining reaction in one or more cores, while the remaining were insufficient due to poor signal-to-noise ratio, false-positive, or combined false-negative and false-positive results. The most common causes for insufficient IHC staining results are summarized in Table 2.10, which is obtained from this article.

Table 2.10 Major causes of insufficient staining reactions

1. Less successful antibodies (17%)
(a) Poor antibodies[a]
(b) Less robust antibodies[b]
(c) Poorly calibrated RTUs
(d) Stainer platform-dependent antibodies
2. Insufficiently calibrated antibody dilutions (20%)
3. Insufficient or erroneous epitope retrieval (27%)
4. Error-prone or less sensitive visualization systems[c] (19%)
5. Other (17%)
(a) Heat-induced impaired morphology
(b) Proteolysis induced impaired morphology
(c) Drying out phenomena
(d) Stainer platform-dependent protocol issues
(e) Excessive counterstaining impairing interpretation

Note: [a]Consistently gives false-negative or false-positive staining or a poor signal-to-noise ratio in one or more assessment runs; [b]Frequently gives inferior staining results, e.g., due to mouse-anti-Golgi reactions or sensitive to standard operations as blocking of endogenous peroxidase; [c]Biotin-based detection kit for cytoplasmic epitopes, use of detection kits providing a too low sensitivity, or use of detection kits and chromogens giving imprecise localization of the staining signals complicating the interpretation

Reference: [28]

25. What are the required qualifications for IHC personnel?

Minimum standards for the qualifications for immunohistochemistry staff, including laboratory directors, are not yet established by CAP or other regulatory agencies. Certified histotechnologists who receive adequate in-house IHC training are qualified to perform an IHC test. The American Society for Clinical Pathology (ASCP) offers an additional certificate program to histotechnologists who demonstrate advanced knowledge in both theory and practical experience in IHC. Our IHC technologists are encouraged to obtain Qualification in Immunohistochemistry (QIHC) certification from the ASCP. Additionally, competency assessment of each IHC technologist should be performed and documented annually. However, the most critical requirement for the IHC staff is the ability to determine what slides are acceptable and what slides should be rejected before releasing them to the ordering pathologist; this will prevent the release of poor-quality slides and delays in turnaround time. These parameters are listed on the quality control worksheet. Specifically, the staff should be able to recognize appropriate or inappropriate control reactions, tissue quality, and staining artifacts. Equally importantly, the staff should be able to resolve these issues after identifying the problem.

26. What is the CAP checklist for clinical IHC laboratories?

Table 2.11 summarizes the 2019 (09.17.2019) CAP checklist for immunohistochemical laboratories [13]. This checklist is subject to changes in the next updated CAP checklist for anatomic pathology.

Table 2.11 Summary of the revised (9/17/2019) College of American Pathologists (CAP) checklist for immunohistochemistry

Number	Subject header	Declarative statement and comment
ANP.22300	Specimen modification	If the laboratory performs immunohistochemical staining on specimens other than formalin-fixed, paraffin-embedded tissue, the written procedure describes appropriate modifications, if any, for other specimen types *NOTE: Such specimens include frozen sections, air-dried imprints, cytocentrifuge or other liquid-based preparations, decalcified tissue, and tissues fixed in alcohol blends or other fixatives*
ANP.22500	Buffer pH	The pH of the buffers used in immunohistochemistry is routinely monitored *NOTE: pH must be tested when a new batch is prepared or received* Evidence of Compliance: ✓ Written procedure defining pH range for each buffer in use AND ✓ Records of initial and subsequent QC on each buffer
ANP.22550	QC—antibodies	Positive tissue controls are used for each antibody *NOTE: Positive controls assess the performance of the primary antibody. They are performed on sections of tissue known to contain the target antigen, using the same epitope retrieval and immunostaining protocols as the patient tissue. Results of controls must be recorded, either in internal laboratory records or in the patient report. A statement in the report such as "All controls show appropriate reactivity" is sufficient*
ANP.22570	QC—antibodies	Appropriate negative controls are used *NOTE: Negative controls must assess the presence of nonspecific staining in patient tissue as well as the specificity of each antibody with the exception listed below. Results of controls must be recorded, either in internal laboratory records or in the patient report. A statement in the report such as "All controls show appropriate reactivity" is sufficient*
ANP.22615	Endogenous biotin	If the laboratory uses an avidin–biotin complex (ABC) detection system (or a related system such as streptavidin–biotin or neutravidin–biotin), there is a procedure that addresses nonspecific false-positive staining from endogenous biotin *NOTE: Biotin is a coenzyme present in mitochondria, and cells that have abundant mitochondria such as hepatocytes, kidney tubules, and many tumors (particularly carcinomas) are rich in endogenous biotin. Biotin-rich intranuclear inclusions are also seen in gestational endometrium and in some tumors that form morules. If steps are not included in the immunostaining method to block endogenous biotin before applying the ABC detection complex, nonspecific false-positive staining may occur, particularly when using heat-induced epitope retrieval (which markedly increases the detectability of endogenous biotin). This artifact is often localized to tumor cells and may be easily misinterpreted as true immunoreactivity. Blocking endogenous biotin involves incubating the slides with a solution of free avidin (which binds to endogenous biotin), followed by incubation with a biotin solution (which saturates any empty biotin-binding sites remaining on the avidin). Biotin-blocking steps should be performed immediately after epitope retrieval and before incubation with primary antibody*

(continued)

Table 2.11 (continued)

Number	Subject header	Declarative statement and comment
ANP.22660	Control slide review	When batch controls are run, the laboratory director or designee reviews all control slides each day of patient testing *NOTE: Records of this daily review must be retained and should clearly show that positive and negative controls for all antibodies stain appropriately. Batch control records must be retained for 2 years. Immunohistochemical tests using polymer-based detection systems (biotin-free) are sufficiently free of background reactivity to obviate the need for a negative reagent control, and such controls may be omitted at the discretion of the laboratory director following appropriate validation. The batch control slides must be readily available to pathologists who are signing out cases. The location of the slides should be stated in the procedure manual*
ANP.22750	Antibody validation/ verification—non-predictive marker	The laboratory has records of validation/verification of new antibodies, including introduction of a new clone, prior to use for patient diagnosis or treatment *NOTE: The performance characteristics of each assay must be appropriately validated/verified before being placed into clinical use. The initial goal is to establish the optimal antibody titration, detection system, and antigen retrieval protocol. Once optimized, a panel of tissues must be tested to determine the assay's sensitivity and specificity. The scope of the validation/verification is at the discretion of the laboratory director and will vary with the antibody.* *Means of validation/verification may include, but are not limited to, (1) correlating the results using the new antibody with the morphology and expected results; (2) comparing the results using the new antibody with the results of prior testing of the same tissues with a validated/ verified assay in the same laboratory; (3) comparing the results using the new antibody with the results of testing the same tissue in another laboratory with a validated/verified assay; or (4) comparing the results using the new antibody with previously validated/verified non-IHC tests or testing previously graded tissue challenges from a formal proficiency testing program* *For an initial validation/verification, laboratories should achieve at least 90% overall concordance between the new test and the comparator test or expected results* *For validation/verification of a non-predictive assay, the validation/verification should test a minimum of 10 positive and 10 negative tissues. If the laboratory director determines that fewer validation cases are sufficient for a specific marker (*e.g.*, a rare antigen or tissue), the rationale for that decision needs to be recorded. Positive cases in the validation/verification set should span the expected range of clinical results (expression level), especially for those markers that are reported quantitatively* *When possible, laboratories should use tissues that have been processed using the same fixative and processing methods as cases that will be tested clinically. If IHC is regularly done on specimens that are not fixed or processed in the same manner as the tissues used for validation/verification (*e.g.*, alcohol-fixed cell blocks, cytologic smears, formalin post-fixed tissue, or decalcified tissue), the laboratory should test a sufficient number of such tissues to ensure that assays consistently achieve expected results. The laboratory director is responsible for determining the number of positive and negative cases and the number of predictive and non-predictive markers to test* *Refer to the subsection "Predictive Markers" for specific validation/verification requirements for tests that provide independent predictive information (*e.g.*, HER2 and ER/PgR testing in breast carcinoma)*
ANP.22760	New reagent lot confirmation of acceptability	The performance of new lots of antibody and detection system reagents is compared with old lots before or concurrently with being placed into service *NOTE: Parallel staining is required to control for variables such as disparity in the lots of detection reagents or instrument function. New lots of primary antibody and detection system reagents must be compared to the previous lot using at least one known positive control and one known negative control tissue. This comparison should be made on slides cut from the same control block*

Table 2.11 (continued)

Number	Subject header	Declarative statement and comment
ANP.22780	IHC assay performance	Laboratories confirm assay performance when conditions change that may affect performance *NOTE: A change in antibody clone requires full revalidation/verification of the assay (equivalent to initial analytic validation/verification—see ANP.22750)* *Laboratories must confirm assay performance with at least two known positive and two known negative cases when an existing validated/verified assay has changed in any of the following ways: antibody dilution, antibody vendor (same clone), or the incubation or retrieval times (same method)* *A more extensive study to confirm acceptable assay performance in accordance with published guidelines must be performed when any of the following have changed: fixative type, antigen retrieval protocol (*e.g.*, change in pH, different buffers, different heat platforms), antigen detection system, tissue processing or testing equipment, environmental conditions of testing (*e.g.*, laboratory relocation), or laboratory water supply. This study must include a representative sampling of the assays affected by the change and an appropriate number of positive and negative cases per assay, sufficient to confirm acceptable assay performance. The laboratory director is responsible for determining the extent of the study. The rational for the assays selected and number of positive and negative cases checked per assay must be recorded* *For specific validation/verification requirements for tests that provide independent predictive information (*e.g.*, HER and ER/PgR testing in breast carcinoma), refer to the subsection "Predictive Markers"*
ANP.22900	Slide quality	The immunohistochemical stains produced are of acceptable technical quality *NOTE: The inspector must examine examples of the immunohistochemical preparations offered by the laboratory. A reasonable sample might include 10 diagnostic antibody panels*

QC quality control

27. How do you implement best practices in immunohistochemistry?

The majority of large IHC laboratories have an extensive test menu with over 200 antibodies. Diagnostic IHC is an indispensable ancillary technique to anatomic pathology laboratories and has provided ample scientific evidence to objectively support and confirm the histologic impression of some very challenging cases. Due to the large number of antibodies available in a given IHC lab, both underutilization and overutilization of IHC markers have gradually become issues. Here are a few tips which may potentially help an IHC lab implement the concept of best practices in immunohistochemistry. However, most importantly, before ordering any IHC stain, a pathologist should ask what value the stain(s) will add to the final diagnosis and patient care. If the answer is "none at all" or "I don't know," the stain should not be performed. Additionally, if either three stains or five stains will provide the same information, only order three stains. With this in mind, the concept of best practices in immunohistochemistry can be effectively implemented.

- ***Know the Diagnostic Sensitivity and Specificity of Each IHC Marker***

The more you know about the characteristics of each IHC marker, the less likely you will be to overutilize or underutilize it. Importantly, an entirely sensitive and specific IHC marker rarely exists. A small panel of IHC markers rather than a single marker is strongly recommended to avoid a potential diagnostic pitfall. For the step-by-step approach to an undifferentiated neoplasm/tumor of uncertain origin, please refer to Chap. 12.

- ***Begin with a Limited Panel of IHC Markers (First IHC Panel)***

A single IHC marker approach (other than for pathogens such as CMV or BK virus) is strongly discouraged since aberrant expression of a highly specific IHC marker can rarely occur. However, aberrant expression of the entire panel of highly specific IHC markers is statistically nearly impossible. A small IHC panel is recommended. For example, TTF1 and p40 can be applied for distinction of lung adenocarcinomas from squamous cell carcinomas.

Table 2.12 Lung adenocarcinoma versus lung squamous cell carcinoma (first IHC panel)

Marker	Lung adenocarcinoma	Lung squamous cell carcinoma
TTF1	+	–
p40	–	+

Note: "+" – >75% of cases are positive, "–" – less than 5% of cases are positive, *IHC* immunohistochemical, *TTF1* thyroid transcription factor 1

- ***Continue with Second IHC Panels***

If the first IHC panel for the specific differential diagnosis is inconclusive, the follow-up second IHC panel can be considered. For the above example, if both TTF1 and p40 fail to yield the expected result, additional markers such as napsin A, CK7, p63, and CK5/6 can be considered. If the second IHC panel fails to lead to a conclusion, reconsider broadening the differential diagnosis if that clinically makes sense.

- ***Update IHC Panels with New Data***

With additional studies and publications, many IHC markers initially believed to be highly specific gradually lost their specificities. However, additional novel biomarkers are emerging continuously. Both first and second IHC panels for a specific differential diagnosis should be kept updated with more sensitive and specific markers. The less sensitive and specific markers should be deleted from the diagnostic panels. For example, both SALL4 and OCT4 have been proven to be excellent markers to identify germ cell tumor; therefore, the less specific germ cell markers such as PLAP should be removed from the IHC library to reduce an unnecessary cost.

- ***Track and Compare the IHC Utilization Data***

The utilization of immunohistochemical stains should be audited periodically for each subspecialty group and each pathologist. Comparing and contrasting IHC utilization among pathologists within the same subspecialty group should be performed, using the group average or median as the benchmark. For instance, within a group of six GU pathologists, what is the percentage of IHC stains (PIN4) ordered in prostatic core biopsy cases (percentage of cases with IHC stains) for each pathologist? What is the percentage of IHC stains (PIN4) ordered per prostatic tissue core (stain/tissue core) for each pathologist? The pathologists found to excessively use IHC tests should correct the overutilization issue using the group average as a reference. If national benchmarking data are available, they can be used as a reference for the specialty group. The another reason for overutilization is IHC markers being ordered on both FNA and surgical specimens from the same biopsy procedure.

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