EDUCATIONAL

IHC Guidebook | Sixth Edition

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Immunohistochemical Staining Methods

Education Guide | Immunohistochemical Staining Methods
Sixth Edition

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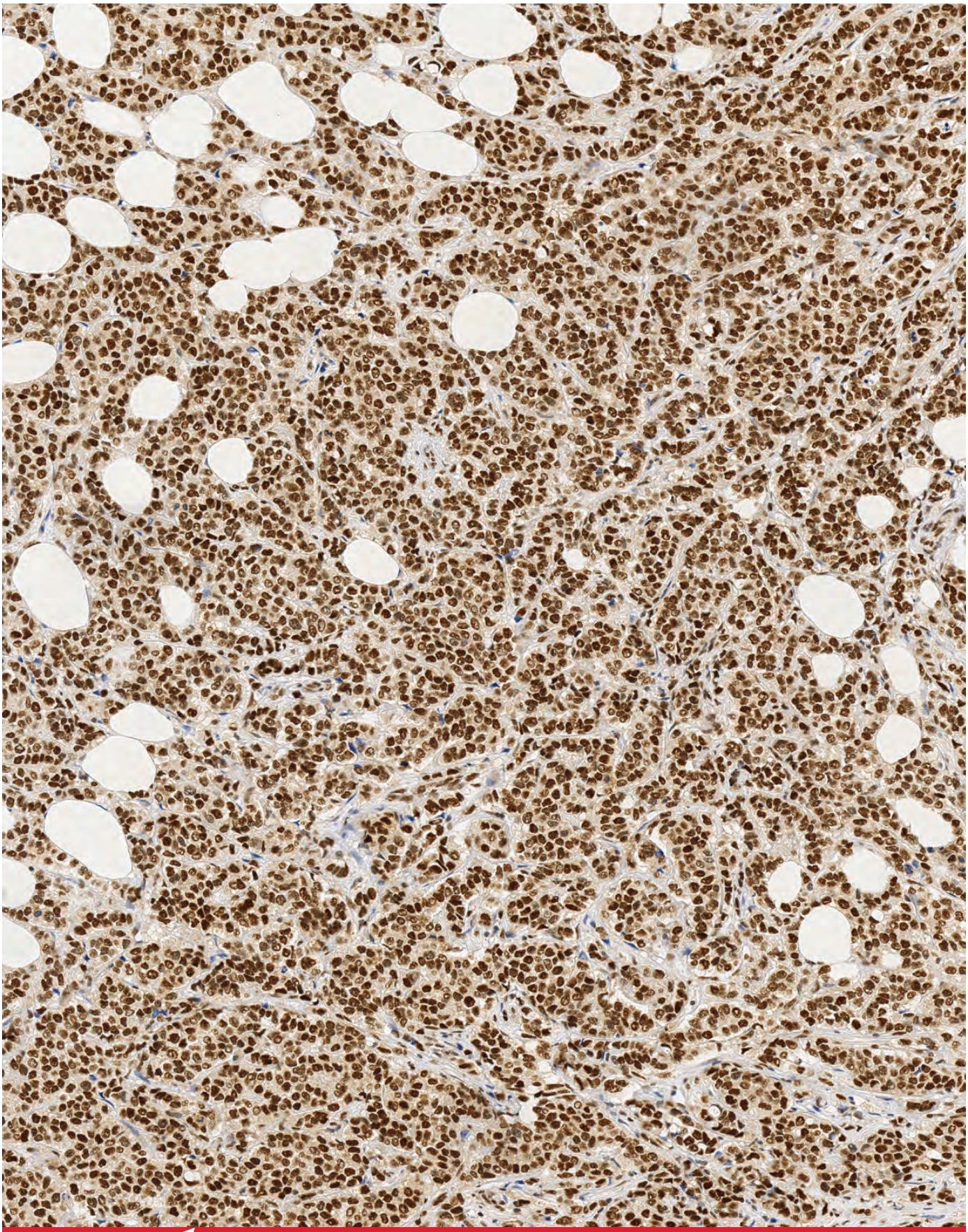

Table of Contents | Immunohistochemical Staining Methods

Part I: The Staining Process

Preface 8

Chapter 1 | Introduction to Immunohistochemistry 10
by C.R. Taylor
1.1 | Introduction 11
1.2 | History of Immunohistochemistry 13
1.3 | Standardization in Clinical Immunochemistry vs. Immunohistochemistry 15
1.4 | Growing Consensus for Standardization 16
1.5 | Standardization Starts in the Surgery Room 16
1.6 | Future Aspects for Standardization of Immunohistochemistry 17

Chapter 2 | Fixation and Other Pre-Analytical Factors 20
by E.C. Colley & R.H. Stead
2.1 | Introduction 21
2.2 | Tissue Handling 21
2.3 | Fixation 23
2.4 | Tissue and Slide Processing 25
2.5 | Special Tissue Preparations 26
2.6 | Control Material 27
2.7 | Validation 28
2.8 | Conclusions 29

Chapter 3 | Antigen Retrieval 30
by S-R Shi & C.R. Taylor
3.1 | Introduction 31
3.2 | Major Factors that Influence the Effect of Antigen Retrieval 32
3.3 | Standardization of AR – The "Test Battery" Approach 34
3.4 | Application of AR Techniques – The Basic Principles 36
3.5 | AR-IHC-based Research and Diagnostics 36
3.6 | Reagents and Protocols 36
3.7 | Water Bath Methods 36
3.8 | Pressure Cooker Heating 38
3.9 | Autoclave Heating 39
3.10 | Microwave Oven Heating 39
3.11 | Proteolytic Pre-treatment 40
3.12 | Combined Proteolytic Pre-treatment and Heat-Induced Antigen Retrieval 40
3.13 | Combined Deparaffinization and Antigen Retrieval 41
3.14 | Conclusions 42

Chapter 4 | Selection of the Primary Antibody 46
by S. Nielsen
4.1 | Introduction 47
4.2 | Seletion of the Proper Antibody 47
4.3 | A New (Replacement) Antibody for an Old Marker 49
4.4 | A New Antibody for New Marker in the Pathology Laboratory 51
4.5 | A New Experiment in the Pathology Laboratory 52
4.6 | Examples of Good and Poor Antibodies 54
4.7 | Current and Future Challenges 57

Chapter 5 | Staining Protocol Optimization 60
by L. Jacobsen, M. Nielsen, S. Månsson & L. Rudbeck
5.1 | Introduction 61
5.2 | The Basis for an Optimal Staining Protocol 62
5.3 | The protocol 64
5.4 | Reproducibility of IHC 70
5.5 | Requirements for Controls 70
5.6 | Verification and Validation of a Protocol 73
5.7 | Guide to the Development of an Optimal Staining Protocol 73
5.8 | Protocol Performance for Abnormal Tissue 73
5.9 | Concluding remarks 76

Chapter 6 | Immunohistochemistry Staining Methods 78
by K. Petersen & H.C. Pedersen
6.1 | Introduction 79
6.2 | Avidin-Biotin Immunohistochemistry 79
6.3 | Polymer-Based Immunohistochemistry 80
6.4 | Catalysed Signal Amplification (CSA) 81
6.5 | Fluorescyl-tyramide Amplification 82
6.6 | Improved Catalysed Signal Amplification (iCSA) 82
6.7 | Multi-Staining Immunohistochemistry 83
6.8 | Selection of Dyes 86
6.9 | Automated Image Acquisition and Analysis in Multiple Staining 87
6.10 | Immunofluorescence 87
6.11 | Future Perspectives 91

Chapter 7 | Digital Pathology 94
by J. Schmidt & C.R. Taylor
7.1 | Microscopy – From Analog to Digital 95
7.2 | Automation of Microscopy 95
7.3 | Slide Scanning 96
7.4 | Digital Slide Review 97
7.5 | Applications in Digital Pathology 97

7.6 | Image Analysis and Quantification 98
7.7 | Analysis of IHC stains – 'Eye vs Algorithm' 99
7.8 | Quality Assurance in IHC 99
7.9 | Future Trends in Digital Pathology 100

Part II: The Potentials and Pitfalls

Chapter 8 | Optimization of Immunohistochemical Reactions 102
by G.E. Pace
8.1 | Introduction 103
8.2 | Tissue Digestion using Antigen or Heat-Induced Epitope Retrieval 103
8.3 | Tissue Digestion using Proteolytic Enzymes 104
8.4 | Endogenous Enzyme Blockers 105
8.5 | Protein Blocking Reagent 106
8.6 | Endogenous Biotin 106
8.7 | Antibody Diluents 106
8.8 | Antibody Concentrations 107
8.9 | Incubation times 107
8.10 | Wash Buffers 108
8.11 | Chromogen Enhancers for DAB 109
8.12 | Type of Glass Slides 109

Chapter 9 | Automation in IHC 110
by O.F. Rasmussen
9.1 | History of IHC Automation 111
9.2 | Key Advantages of IHC Automation 111
9.3 | Staining Technologies 111
9.4 | Requirements for Effective Automated Staining 115
9.5 | Automation vs. Workflow 116
9.6 | Key Features for 'Complete Staining Solutions' 117
9.7 | Next Steps in Automation 119

Chapter 10 | Optimizing Laboratory Workflow 122
by D. MacDonald
10.1 | Introduction 123
10.2 | Specimen Tracking 123
10.3 | Specimen Collection and Identification 124
10.4 | Specimen Accessioning 124
10.5 | Grossing 125
10.6 | Tissue Processing and Microtomy 126
10.7 | Routine Staining and Coverslipping 127
10.8 | Slide/Block Reconciliation 127

10.9 | Laboratory Asset Tracking and Workflow Management 128
10.10 | One Workflow Does Not Fit All 129
10.11 | General Sample Labeling and Tracking 130

Chapter 11 | Companion Diagnostics 132
H.C. Pedersen & J.T. Jørgensen
11.1 | Introduction 133
11.2 | History of Companion Diagnostics 133
11.3 | Companion Diagnostics and 'Personalized Medicine' 134
11.4 | Co-Development of Drug and Companion Diagnostics 136
11.5 | Clinical Validation of the Companion Diagnostics 138
11.6 | Companion Diagnostics and Regulatory Aspects 141
11.7 | Learnings from EGFR 141
11.8 | Conclusion and Future Perspectives 142

Chapter 12 | Tissue Microarray - Construction and QA 144
by R. Saxena & S. Badve
12.1 | Introduction 145
12.2 | Advantages and Disadvantages of TMAs 145
12.3 | Types of TMAs 146
12.4 | Team Required for TMA Construction 147
12.5 | TMA Analysis 150

Chapter 13 | IHC Visualization of Molecular Tests 152
by J. Mollerup & J.T. Jørgensen
13.1 | Introduction 153
13.2 | FISH versus CISH 154
13.3 | Principle of the CISH Procedure 155
13.4 | *HER2* CISH in Clinical Decisions 156

Chapter 14 | Controls 160
by O.F. Rasmussen & R. Jørgensen
14.1 | Introduction 161
14.2 | Purpose of Controls 161
14.3 | Categories of Controls and Control Material 162
14.4 | Use of Controls in Daily Routine Testing 163
14.5 | Tissue Process Control 167
14.6 | Cell Line Controls 167
14.7 | Monitoring the Staining Process 168
14.8 | External Quality Assurance Programs 168
14.9 | Future aspects 169

Chapter 15 | Background 170
by H.G. Wendelboe
15.1 | Introduction 171
15.1 | Detection Methods 171
15.3 | Antigen Retrieval (Heat-Induced Epitope Retrieval) 174
15.4 | General Factors 174
15.5 | Miscellaneous Sources 177
15.6 | General Aspects 177

Chapter 16 | Troubleshooting 180
by H.G. Wendelboe, A. Lykke & G.E. Pace
16.1 | Introduction 181
Section 1 – Common Problems 181
Section 2 – Systematical Approach 187
Section 3 – Troubleshooting Chart 191
Section 4 – Specification Sheets 192
Section 5 – Automated Platform Performance Checks 193

Appendix A | Antibodies 198
Revised by S.S. Jensen
App A.1 | Immunoglobulins 199
App A.2 | Antigens 201
App A.3 | Polyclonal Antibodies 202
App A.4 | Monoclonal Antibodies 202
App A.5 | Polyclonal Antibodies versus Monoclonal Antibodies 203
App A.6 | Antibody Affinity 204
App A.7 | Antibody Cross-Reactivity 204
App A.8 | Antibody Reaction Rates 205
App A.9 | Antibody Stability and Storage 205

Appendix B | Basic Immunochemistry 208
Revised by S.S. Jensen
App B.1 | Antibody Titer 209
App B.2 | Antibody Dilution 209
App B.3 | Incubation Time 210
App B.4 | Incubation Temperature 210
App B.5 | Basic Enzymology in IHC 210

Index 214

Biography | Dako's Guidebook to Immunohistochemical Staining Methods

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MD, D.Phil.

Dr. Clive Taylor started his medical education at the University of Cambridge and completed his doctoral studies in Immunology at University of Oxford. After his education, he accepted a position at University of Southern California where he for 25 years functioned as Chair of the Department of Pathology and Laboratory Medicine, and for 10 years as Dean for Educational Affairs. During his many years of devoted work for improving standardization and quantification of immunohistochemstry for cancer diagnostics, he has published 400 papers and 20 books. Currently he is Editor in Chief of Applied Immunohistochemistry and Molecular Morphology.

"IHC is a precise immunoassay that must be performed only with a high degree of technical rigor and control where ready-to-use reagents, coupled with proven detection systems, fixed and validated protocols, recommended controls and automation, represent a pathway that could, if widely adopted, lead to improved levels of reliability and performance for IHC."

Clive Taylor, MD, D.Phil

Contributors

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Clive Taylor, Elizabeth Colley, Ronald Stead, Shan-Rong Shi, Søren Nielsen, Lars Jacobsen*, Majken Nielsen*, Sofie Månsson*, Kenneth Pedersen*, Hans Christian Pedersen*, Joachim Schmidt*, Gale E. Pace*, Ole F. Rasmussen*, David McDonald*, Jan T. Jørgensen*, Jens Mollerup*, Rashmil Saxena, Sunil Badve, Rikke Jørgensen*, Helle G. Wendelboe*, Anette Lykke*, Susie S Jensen* and Lars Rudbeck*.

Sections, in whole or parts thereof, from the previous editions of this Guidebook are used in the 6th edition. We sincerely thank and acknowledge the contribution of the authors. Special acknowledgements to:
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We truly value everyone's contribution, and appreciate such dedicated participation.

* Current or former Dako employee

PETITIONER EXHIBIT 1012
Page 9 of 218

Preface | Innovation and Quality

Welcome to the Sixth Edition of Dako's Guidebook to Immunohistochemical Staining Methods. Dako earned its reputation for innovation and quality by introducing antibodies with standardized titer almost 50 years ago, and it is still Dako's goal to continue to expand knowledge within the field and continue on our path of scientific advancement. The focus of this book is therefore to provide a comprehensive immunohistochemistry (IHC) resource for lab managers, lab technicians, learning pathologists, and students from around the world.

For readers familiar with the previous editions of this guidebook, it should be noted that the structure of the new edition has changed slightly, so that the first part covers the entire staining process from biopsy to final analysis. The second half is comprised of the many supporting aspects within the field of immunohistochemistry. Since the focus is solely on IHC, the in situ hybridization (ISH) method is not covered in this edition.

Part I covers the immunohistochemical staining process, and includes a general introduction as well as chapters covering pre-analytical factors, antigen retrieval, selection of the primary antibody, staining protocol optimization, IHC staining methods and analysis of IHC stains.

Part II examines the potentials and pitfalls in immunohistochemistry, with chapters on optimization of immunohistochemical reactions, automation in IHC, optimizing the laboratory workflow, companion diagnostics, tissue microarray, IHC visualization of molecular tests, controls, background and troubleshooting.

We sincerely hope that the publication of this book will further enhance the advancement of the field of immunohistochemistry, and will help new and practitioners within the field continue to progress and drive the standardization process within IHC to improve diagnostic certainty. Treatment decisions are heavily influenced by the immunohistochemistry results, thus making IHC important for the ultimate goal of better care of the patient.

Lars Rudbeck
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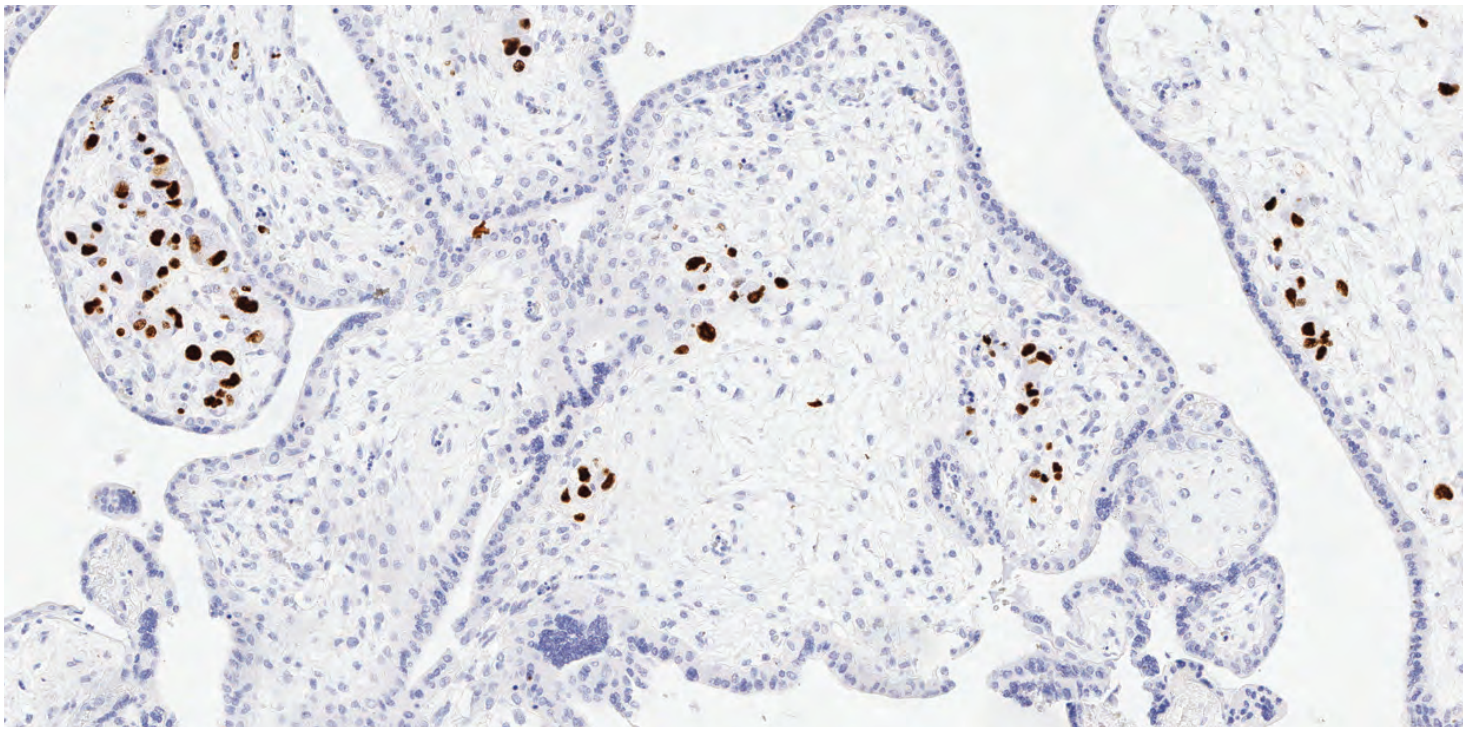
PETITIONER EXHIBIT 1012
Page 10 of 218

Chapter 1

Introduction to Immunohistochemistry

Clive R. Taylor, MD, D.Phil



Im•mu•no•his•to•chem•is•try *(n.)*
Microscopic localization of specific antigens in tissues by staining with antibodies labeled with fluorescent or pigmented material.

The American Heritage® Medical Dictionary

Chapter 1.1 | Introduction

Immunohistochemistry (IHC) is a method used to determine the expression of biomarkers in tissue. This educational guidebook will describe immunohistochemistry as it is used in the pathology laboratory as an aid in the differential diagnosis and classification of cancer, and for certain other diseases, including infections. The factors that influence the immunohistochemical staining result start in the surgery operating room and end at the interpretation of the stain by the pathologist, which ultimately leads to treatment decision by the oncologist.

For those new to the world of immunohistochemistry here is a brief outline of the steps needed to localize antigens in tissues using antibodies for cancer diagnosis:

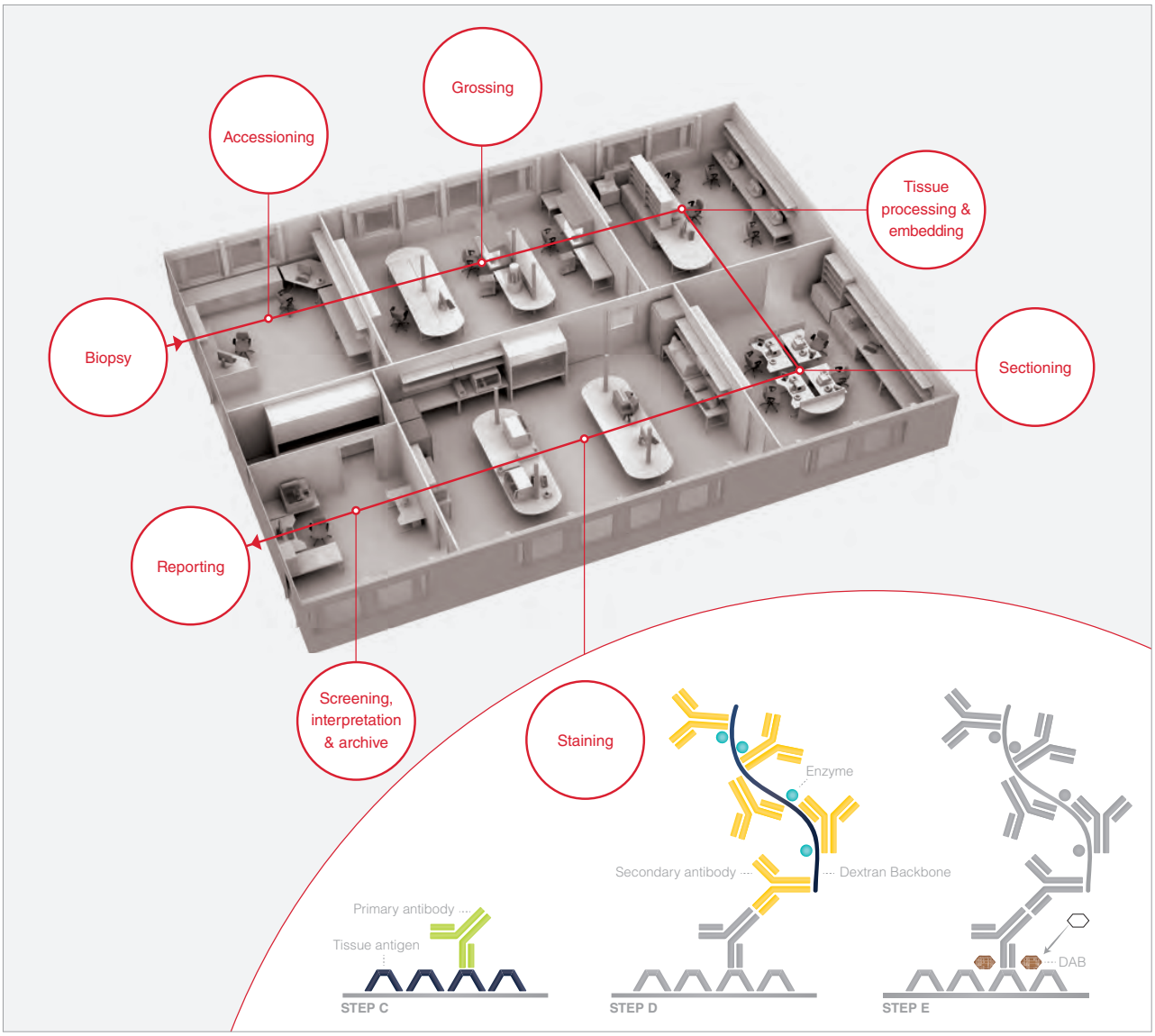


Figure 1.1 From biopsy to reporting.

PETITIONER EXHIBIT 1012
Page 12 of 218

Pre-Analytical Steps

1. A **Biopsy** (surgically removed tissue specimen or needle biopsy) from the surgery room arrives in fixative at the pathology laboratory.
2. In the **Accessioning** room the sample details are entered into the laboratory information system (LIS). A barcoded label can ensure track and trace capabilities.
3. During **Grossing**, the specimen is visually examined for suspicious areas that require further examination. Samples from the specimen that require further microscopic testing are excised as tissue blocks and placed in barcoded cassettes.
4. **Tissue processing and embedding** are the steps where the tissue block is processed into a form and condition suitable for making ultrathin microscopic sections. Typically, the tissue is fixed in formalin then dehydrated before it is embedded in paraffin.
5. **Sectioning** is the fine art of cutting the paraffin-embedded tissue blocks into ultrathin (~4 µm) sections and placing them onto glass slides. A barcode on the slide can ensure traceability and may also contain protocol information for the requested test for that particular section.

Analytical Steps

6. **Staining** is the analytical part of the IHC process. It encompasses antigen retrieval, application of the primary antibody and visualization system, ending with counterstaining:
   a. Antigen retrieval is performed to recover the antigens that may have been altered by fixation;
   b. Endogenous enzymes are blocked (this step can also be performed after primary antibody incubation);
   c. A primary antibody is applied that specifically binds to the antigen of interest;
   d. The secondary antibody carries the label (enzyme); upon application it binds to the primary antibody;
   e. Chromogen is applied to visualize the antibody/antigen complex;
   f. Counterstaining is performed to visualize nuclei and overall tissue architecture;
   g. Sections are dehydrated, mounted and coverslipped.

Post-Analytical Steps

7. In the post-analytical process, the pathologist interprets the stains in context with positive and negative tissue controls, using bright field microscopy.
8. The results are reported to the oncologist for treatment decision.

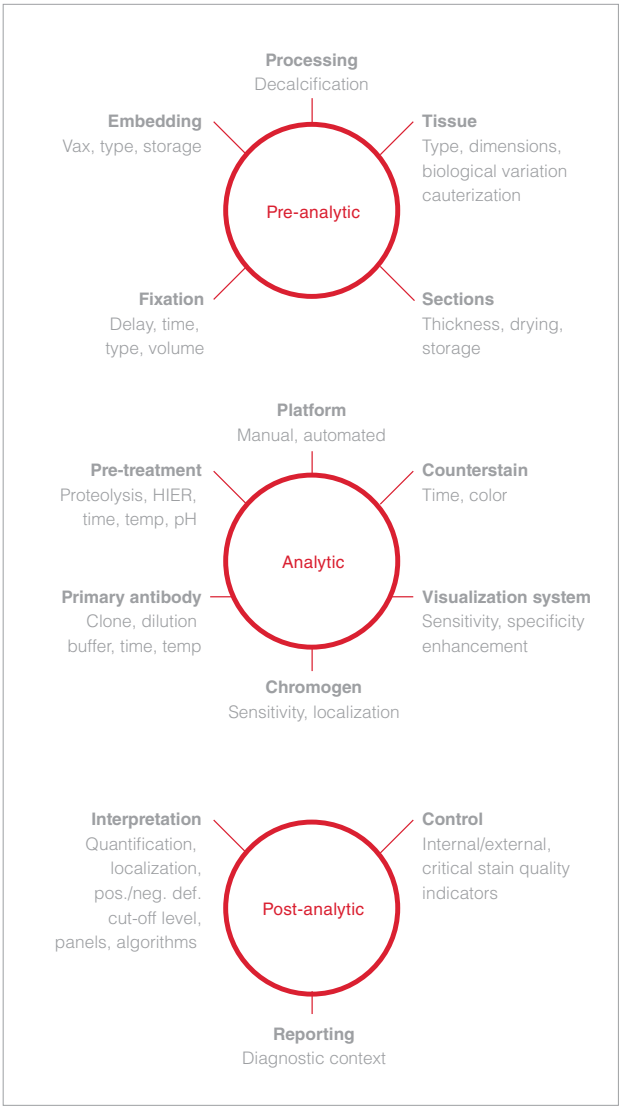


Figure 1.2 Many factors may influence the IHC staining result. With just 3 choices at each of 14 steps there are 4.8 million different procedures!

PETITIONER EXHIBIT 1012
Page 13 of 218

Chapter 1.2 | History of immunohistochemistry

This IHC Educational Guidebook will describe the potentials and pitfalls in the immunohistochemical staining process from biopsy to interpretation, with special attention to the analytical processes and how to improve certainty in the staining result by employing standardization to the processes.

Before immunohistochemistry reached its now widespread use as an important method in routine cancer diagnosis, the technology had a long history of technological developments outlined in the table below.

Table 1.1 The major milestones in the history of immunohistochemistry.

Year	Method	References
1941	Fluorescence-labeled primary antibodies	Coons et al (1)
1967	Enzyme-labeled primary antibodies	Nakane & Pierce (2)
1970	Secondary un-labeled antibodies (PAP)	Sternberger et al (3)
1970	Detection of antigens on ultrathin sections	Kawarai & Nakane (4)
1974	Application to routine formalin paraffin sections	Taylor et al (5-7)
1975	Invention of monoclonal antibodies	Köhler & Milstein (8)
1978	Double staining using un-labeled antibodies (APAAP)	Mason & Sammons (9)
1979	Monoclonal antibodies to human antigens	McMichael et al (10)
1988	Capillary gap semi-automated staining	Brigati et al (11)
1991	Heat-induced antigen retrieval	Shi et al (12)
1993	Standardization efforts as 'Total Tests'	Taylor (13)
1995	Dextran-polymer-based detection system	Dako
1998	Immunohistochemistry as companion diagnostics	Dako (*HER2*)
2007	Recommendations for improved standardization of IHC	Goldstein et al (14) Wolff et al (15)
2008	Molecular HER2 CISH Tests in the IHC lab	Invitrogen

Professor Albert H. Coons and co-workers demonstrated in 1941 that it was possible to localize antigens in tissue slices using antibodies against Streptococcus pneumoniae labeled with fluorescein and visualized by ultraviolet light (fluorescence microscopy) (1). During the next 25 years, the Coons method was used with different modifications, including labeling with heavy metals, but it was not until the introduction of enzyme-labeled antibodies (2) that the method overcame many of the inherent issues with fluorescein and heavy metal labeling of antibodies. In the early 1970s, application of the 'immunoperoxidase' method to formalin paraffin embedded tissues by Taylor, Mason and colleagues in Oxford, was a critical step in extending use of the method into 'routine' diagnosis in anatomic pathology. The direct labeling method had the drawback that each individual primary antibody, or the secondary antibody, had to be labeled with enzyme. That problem was circumvented by the development of an unlabeled antibody enzyme method, the peroxidase anti-peroxidase (PAP) method, which had the further advantage of increased sensitivity, facilitating use in routine tissues. A related parallel development was the introduction of the alkaline phosphatase anti-alkaline phosphatase (APAAP) in 1978 (9). Even with the development of new and improved detection systems for visualization of antigens in tissue, IHC suffered from lack of reproducibility, due in part to poor quality antibody reagents, and in part to the inconsistent and adverse effects of fixation.

Increased demand led to better quality reagents from the commercial sector, with improved quality control of production methods. Polyclonal antibody preparations differ between serum samples in affinity and specificity, as the immune-response changes with time and immunization preparations, and as one animal is replaced by another as the source. Dr. Niels Harboe, founder of Dako, realized in the early 1970s the need for standardized antibody preparations for safe and reproducible diagnoses and began producing purified polyclonal antibodies that had the same strength (as measured by titer) from batch to batch.

Even with the purified and highly specific polyclonal antibodies there was a need for improved specificity of antibodies and a greater variety in terms of target proteins. The invention, in 1975, of hybridomas that could produce monoclonal antibodies (8) resulted in the production of the first monoclonal antibody that was highly specific for human thymocytes using hybridoma technology (10). Monoclonal antibodies paved the way for a rapid growth in the use of IHC in research and diagnosis of cancer.

PETITIONER EXHIBIT 1012
Page 14 of 218

One other consequence of the lack of reproducibility was the development of automated instruments (11). Automation was invented with the fundamental thought that a properly functioning and maintained instrument will consistently perform its pre-programmed instructions in the same way – slide after slide- which is the principal reason why an instrument potentially can give superior reproducibility, compared with manual methods. However, progress was slow until 1991, when Shi et al (12) introduced 'antigen retrieval' (or heat-induced epitope retrieval), thereby facilitating extension of IHC to a much broader range of applications in formalin paraffin sections, but at the cost of adding yet another variable to the process. This important publication on antigen retrieval thus gave new insights and impetus to efforts in standardization of IHC, leading to the introduction of the 'Total Test' concept (13) as a result of a series of meetings sponsored by the Biological Stain Commission and the FDA in the early 1990s.

The standardization efforts, coupled with attempts to use IHC in a semi-quantitative setting raised demands to a new level, exemplified by the introduction, in 1998, of the HercepTest™ (Dako), which was the first cancer companion diagnostic, in this instance designed for selection of breast cancer patients for treatment with the new drug Herceptin® (Genentech/Roche). Clinical trials had shown that patients whose tumors overexpressed HER2 would benefit the most from Herceptin® treatment. The HercepTest™ assay uses IHC on patient samples, in combination with control cell lines having known HER2 expression to determine if a breast cancer overexpresses HER2. Some 15 years later, this assay together with similar HER2 assays from other vendors, still serves as a rare example of a semi-quantitative IHC assay used in routine clinical pathology. The polymer-based visualization system, introduced shortly before HercepTest™, is the most widely used detection method in IHC today, with advantages of stability and high sensitivity.

The technical advances in IHC in the last decade have been incremental, with little impact on the basics of the method. Automation has become more advanced, including laboratory information system integration, with track and trace of samples, while whole slide digital imaging is slowly being integrated into the analysis of stain result. These advances can best be regarded as improvements in standardization; a process that started back

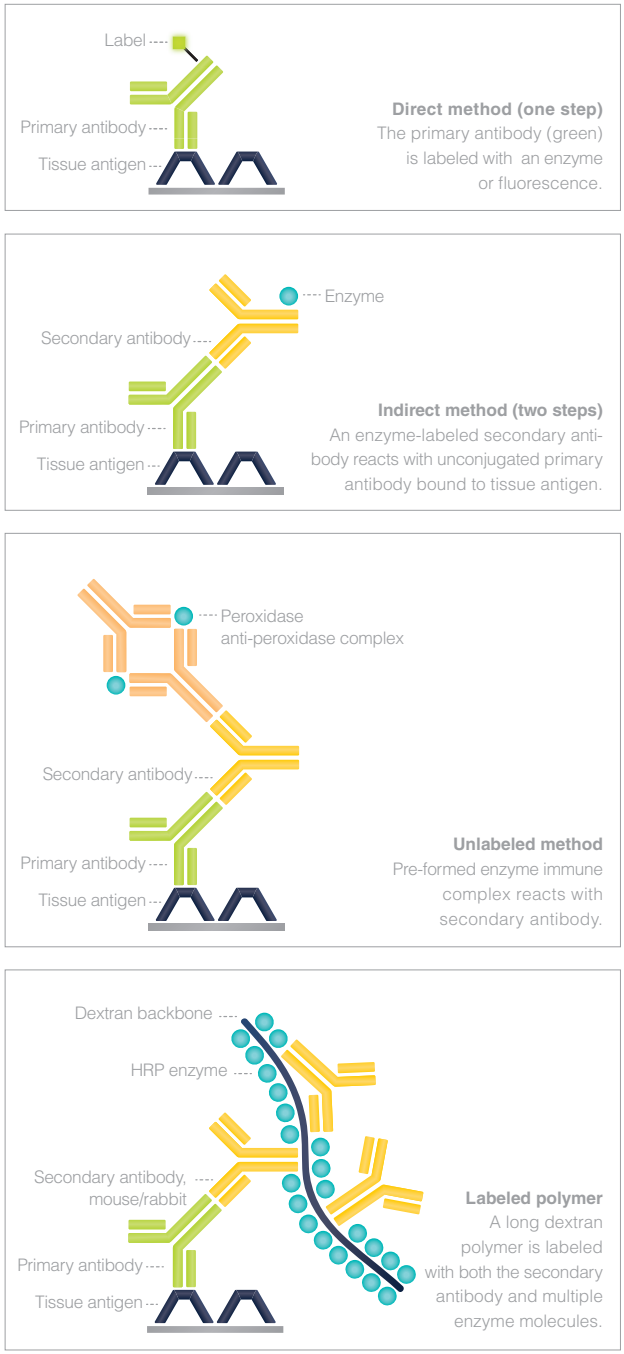


Figure 1.3 The development of detection systems used for IHC. Please see Chapter 6 for a full description of the many different detection methods.

PETITIONER EXHIBIT 1012
Page 15 of 218

in the early 1990s and was re-emphasized in the 2007 publications by Goldstein et al (14) and Wolff et al (15), but also by the work being done e.g. estrogen receptor assessment (16, 17). The critical importance of IHC standardization became evident with the revelation of disturbingly high numbers of false negative or false positive results in IHC determinations of ER (estrogen receptor) and PR (progesterone receptor) expression, and also HER2. In one example, a re-testing in 2007 of 1,023 breast cancer samples from Newfoundland revealed that approximately 1 out of 3 samples was scored falsely ER negative (17). As a consequence of the false negative ER test results, these women were not accorded the potential benefit of anti-hormonal therapy.

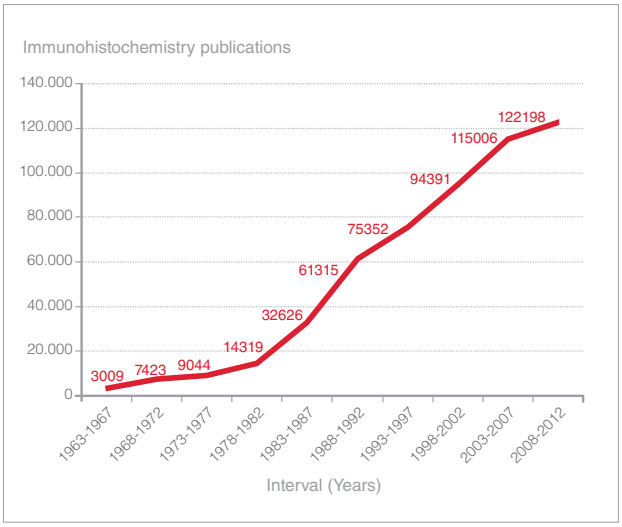


Figure 1.4 The number of IHC publications in the last 50 years. The data are from Pubmed using the search term "immunohistochemistry".

The latest development in cancer diagnosis is the inclusion of molecular tests (FISH/CISH) in anatomic pathology labs, driven by HER2 assessment requirements. Other technologies also are entering into the pathology lab and into routine diagnosis, and technologies such as array comparative genomic hybridization or next generation sequencing will likely be a fundamental part of cancer diagnosis in the future. One ongoing goal is to interface these newer methods of molecular analysis with existing and improved morphologic criteria, a field termed 'Molecular Morphology'.

Chapter 1.3 | Standardization in Clinical Immunochemistry vs. Immunohistochemistry

For more than 30 years, clinical immunochemistry has employed blood or urine samples to determine the concentration of certain biomarkers, e.g. creatinine and cystatin C for evaluation of kidney function, and C-reactive protein as a marker of inflammation. Although clinical immunochemistry covers a multitude of assay types, most of these tests are based on the ELISA (enzyme-linked immunosorbent assay) method, a method that closely parallels IHC in principle. One major difference is that International Reference Materials and Calibrators are used in clinical immunochemistry (ELISA) to achieve quantitative results from these assays.

Immunohistochemistry is based on principles similar to the ELISA method, yet it is at best a semi-quantitative method for determination of the expression of biomarkers in tissue samples. However, IHC should not be regarded as simply another 'special stain', like a PAS stain or a silver stain. IHC is essentially an ELISA method applied to a tissue section. In this respect, when correctly performed, IHC has the potential to perform as a reproducible and quantitative tissue-based ELISA assay; much more than a simple stain. That the IHC method does not perform to this level, reflects deficiencies in the application of the method, specifically inconsistent sample preparation, lack of reference or calibration standards, and inadequate validation of reagents (18, 19). If ELISA can use a standard curve to convert the measured immunoreactivity into a quantitative amount of tested protein, then IHC – in theory – can also convert the IHC intensity observed in FFPE tissue sections into the amount of tested protein by an equivalent standard ruler. Comparative studies of IHC intensity on frozen tissue vs. FFPE tissue have shown identical intensity by using an optimized AR protocol (20, 21), and similar protein quality is evident when examined by mass spectrometry (22), leaving no theoretical reason for lack of true quantitative IHC assays. Nonetheless, today IHC assays are at best no more than semi-quantitative, for reasons that are more of a practical nature.

PETITIONER EXHIBIT 1012
Page 16 of 218

Chapter 1.4 | Growing Consensus for Standardization

From the beginning there has been concern relating to the reproducibility of immunohistochemical methods as applied to formalin-fixed, paraffin-embedded (FFPE) tissue sections. A consequence of not controlling all parameters (in fixation, processing and staining) is poor day to day reproducibility within a single laboratory, and poor reproducibility among different laboratories. In recent years these concerns have increased and lack of standardization, well shown in interlaboratory quality assurance surveys performed by NordiQC and UK NEQAS, is now recognized as a major impediment to basic research, clinical trials, and direct patient care. Over the past three decades a number of conferences have been held to address this topic and to seek constructive resolutions. Among the most productive were a series of meetings sponsored by the Biological Stain Commission and the FDA in the early 1990s, that led to recommendations for manufacturers concerning the precise description and validation of IHC reagents (23), and also highlighted the necessity to pay attention to all aspects of the IHC test procedure. The latter recommendation, borrowed from the much more rigorous protocols applied to immunologic assays in clinical laboratories, became known as the 'Total Test' approach (Table 1) (23, 24). A decade later a meeting of the FDA and NIST (National Institute of Standards and technology) focused upon standardization of HER2 IHC assays, and the need for universal control materials (reference standards) (25).

Chapter 1.5 | Standardization Starts in the Surgery Room

While Table 1.2 only mentions a few of the major steps in a Total Test, the pre-analytical process alone contains at least 62 identifiable steps of which 27 have been examined in published research. Out of these 27 steps, 15 pre-analytical variables are capable of impacting the immunohistochemistry staining result including fixation delay, fixative type, time in fixative, reagents and conditions of dehydration, clearing, paraffin impregnation and conditions of slide drying and storage (26). Pre-analytical variables are described in detail in Chapter 2.

Table 1.2 The Total Test: An IHC stain should be managed in the same rigorous manner as a clinical laboratory analysis. Modified from Taylor (14, 24).

Pre-analytic
Test selection
Specimen type
Acquisition, pre-fixation/transport time
Fixation, type and total time
Processing, temperature
Test selection
Antigen retrieval procedure
Selection of primary antibodies
Protocol; labeling reagents
Reagent validation
Control selection
Technician training/certification
Laboratory certification / QA programs
Post-analytic
Assessment of control performance
Description of results
Interpretation/reporting
Pathologist, experience and CME specific to IHC

In the analytical steps, antigen retrieval is the first challenge. Different antigens require different antigen retrieval for optimal staining results, and the different variations of the AR process add another variable that must be controlled. Antigen retrieval is described in detail in Chapter 3.

Selecting the right antibody for the right marker is one of the key steps in the analytical process. Some monoclonal antibody clones are more specific than others against the same biomarker. In other cases a polyclonal antibody may be the best choice. Selection of the primary antibody is described in detail in Chapter 4.

Using a protocol that is optimized for the detection of the biomarker is vital. The optimal protocol must be able to identify the antigen of interest in cells and structures with both low and

PETITIONER EXHIBIT 1012
Page 17 of 218

high expression. Optimization of the staining protocol is described in detail in Chapter 5.

The final step of the analytical process is the visualization of the antigen/antibody reaction. Here the selection of the detection system must consider the complexity of the visualization and the required amplification needed to visualize the biomarker. The various detection systems are described in detail in Chapter 6.

Table 1.3 Major steps affecting the immunohistochemistry staining result.

Step	Effect on IHC
Biopsy	Depending on the suspected cancer type, tissue samples can be obtained in different ways such as punch/core biopsy, excisional/incisional biopsy, etc. Tissue degradation begins at the time of sample removal.
Fixation	The sample should be fixed as soon as possible after surgery, ideally within less than an hour. The chemical fixation crosslink proteins in the sample thereby stopping the degradation process. Too short or too long fixation can affect the staining result.
Embedding	After fixation, the sample is embedded in paraffin for long-term storage and to enable sectioning for subsequent staining. Once embedded in paraffin, samples can be stored (almost) indefinitely.
Sectioning and Mounting	Formalin-fixed, paraffin-embedded tissues are sectioned into thin slices (4-5 µm) with a microtome. The sections are then mounted onto adhesive-coated glass slides.
Antigen Retrieval	Due to the fixation process, an antigen retrieval treatment is applied to unmask the epitopes, either by heat (heat-induced epitope retrieval; HIER) or enzymatic degradation (proteolytic-induced epitope retrieval; PIER). Incorrect antigen retrieval for the biomarker of interest will adversely affect the staining result.
Primary Antibody	An antibody with specificity for the biomarker of interest is applied. The specificity and sensitivity of the antibody affect the staining result.
Visualization	The antigen/antibody complex signal is amplified and visualized using a detection system. The strength of amplification of the reaction affects the staining result (intensity).
Interpretation	The staining pattern is assessed by a pathologist in context with other biomarkers, controls and other tests (e.g. H&E, special stains. Inter- and intra-observer variability is common, especially for semi-quantitative assays. This variability highlights the importance of training and inter-calibration.

Post-analytical standardization is essential for prognostic or predictive biomarkers, e.g. HER2 and ER/PR, adhering to specified stain interpretation guidelines to give the sample a scaled score (e.g. from 0-3+). However, most biomarkers are used for cell lineage and tissue identification, where expression levels are usually not as critical and interpretation is not linked to a semi-quantitative scoring system, but is reported as a binary 'Yes' or 'No' system (positive or negative) for the tested biomarker. Digital analysis of IHC stains is described in Chapter 7.

Chapter 1.6 | Future Aspects for Standardization of Immunohistochemistry

The consensus arising from the standardization efforts is that the reliability and reproducibility of IHC methods in routine surgical pathology have been greatly hindered by two key factors.

1. While reagents available for IHC have increased in quality, there has been an even greater increase in number of sources and variety of staining methods. This plentitude of reagents contributes to lack of standardization in significant ways, that in theory are manageable by good technique and use of proper controls, but in practice have led to requirements for such high standards of excellence in the technical process, that many laboratories cannot find sufficient, or sufficiently skilled, staff to comply.
2. The usual method of sample preparation for tissue remains as formalin fixation and paraffin embedment (FFPE). This venerable approach may be satisfactory for the preservation of morphologic detail, but does adversely affect the antigenicity of many target molecules in the tissue, to degrees that are unknown. The enormous variation in protocols (including fixation times) employed for FFPE among different laboratories, or within the same laboratory from specimen to specimen, compounds the problem and contributes to the current poor reproducibility.

While several decades have passed, these issues have not been satisfactorily addressed. Legions of investigators, and many manufacturers, have addressed different aspects of the problem, focusing upon better sample preparation (fixation), more effective methods of antigen retrieval, improved

PETITIONER EXHIBIT 1012
Page 18 of 218

reagents, more sophisticated automated platforms, more sensitive detection methods, and the development of reference standards or controls (13, 23-25).

In order to improve the quality and reproducibility from sample to sample, and lab to lab, the accreditation process for many pathology laboratories now includes participation in external quality assurance (EQA) schemes. EQA organizations, like NordiQC, UK NEQAS and CAP, are independent organizations not associated with commercial suppliers. Their role is to promote the quality of immunohistochemistry (and in situ hybridization) by arranging external QA schemes for pathology laboratories. Similar EQA schemes are now available in many countries and regions around the world. The purpose of EQA schemes is to improve the quality of staining results in the participating laboratories; thus it is the individual labs that are being assessed. It is their choice of antibody, visualization system, instrumentation and protocol that is the basis for the EQA organization's evaluation and feedback. A lab volunteers to participate in the assessment runs. Laboratories typically enroll for a year, during which they receive approximately 16 unstained tissue slides (NordiQC), or 7-8 different modules, where each module usually has two tissue slides (UK NEQAS), to stain using their own internal standard protocols for those markers designated by the QA organization. The labs return the stained slides to the QA organization for assessment, which is conducted by experts engaged by the organization. The labs receive either a "Passed" rating or "Not Passed" rating. Both NordiQC and UK NEQAS inform all participants of their individual scores and provide suggestions for protocol optimization when required. Both organizations present the anonymous results on their web sites, with statistics and best method for the particular marker.

CAP (College of American Pathologists) in the US, has a similar QA process, but requires only the return of stain results and interpretation, not the stained slides.

Some broad conclusions are possible:

- Resolution of the problem of pre-analytical sample preparation is not imminent; the practical aspects of developing tissue handling and fixation procedures that fit the daily routine of every hospital are challenging. Importantly the logistical obstacles to implementation of standardized sample preparation procedures worldwide are formidable;
- High-quality reagents are available, with highly sensitive detection methods, but they must be employed properly in controlled fashion, and currently often are not. Participation in EQA schemes can help laboratories improve the reproducibility;
- There is a pressing need for tissue-based IHC controls (or 'reference standards') (19, 25) that can be made available to all laboratories performing IHC assays, somewhat analogous to the international reference standards and calibrators that are available to clinical laboratories performing ELISA testing.

From this brief discussion it follows that to improve standardization to the point that all laboratories would carry out the IHC in identical fashion for every phase of the 'Total Test'; it would require them to use the same fixative and fixation time (adjusted to tissue type), the same antigen retrieval process, the same primary antibodies and detection systems, with the same automated stainer and common controls. Clearly this perfect option will never happen, and we therefore must do what we can to reduce the consequences of the variables in the process.

Ultimately the overriding factor in effecting significant change must be to transform the mindset of pathologists, at least of the next generation, to the view that the end result of an IHC protocol is not just a 'stain', with intensity to be adjusted at the whim of the pathologist. Rather IHC is a precise immunoassay that is strictly quantifiable, and must be performed only with a degree of technical rigor and control that matches any other immunologically-based assay of like principle (namely ELISA). ELISA is a 'gold standard' method for quantitative assays in the clinical laboratory. ELISA reagents are purchased in prepared form, with all of the necessary reagents, defined protocols, and reference or calibration standards, for use with specified instrumentation. Ready-to-use reagents, coupled with proven detection systems, fixed and validated protocols, recommended controls and automation, represent an analogous pathway that could, if widely adopted with appropriate controls, lead to improved levels of reliability and performance for IHC.

PETITIONER EXHIBIT 1012
Page 19 of 218

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PETITIONER EXHIBIT 1012
Page 20 of 218

Chapter 2

Fixation and Other Pre-Analytical Factors

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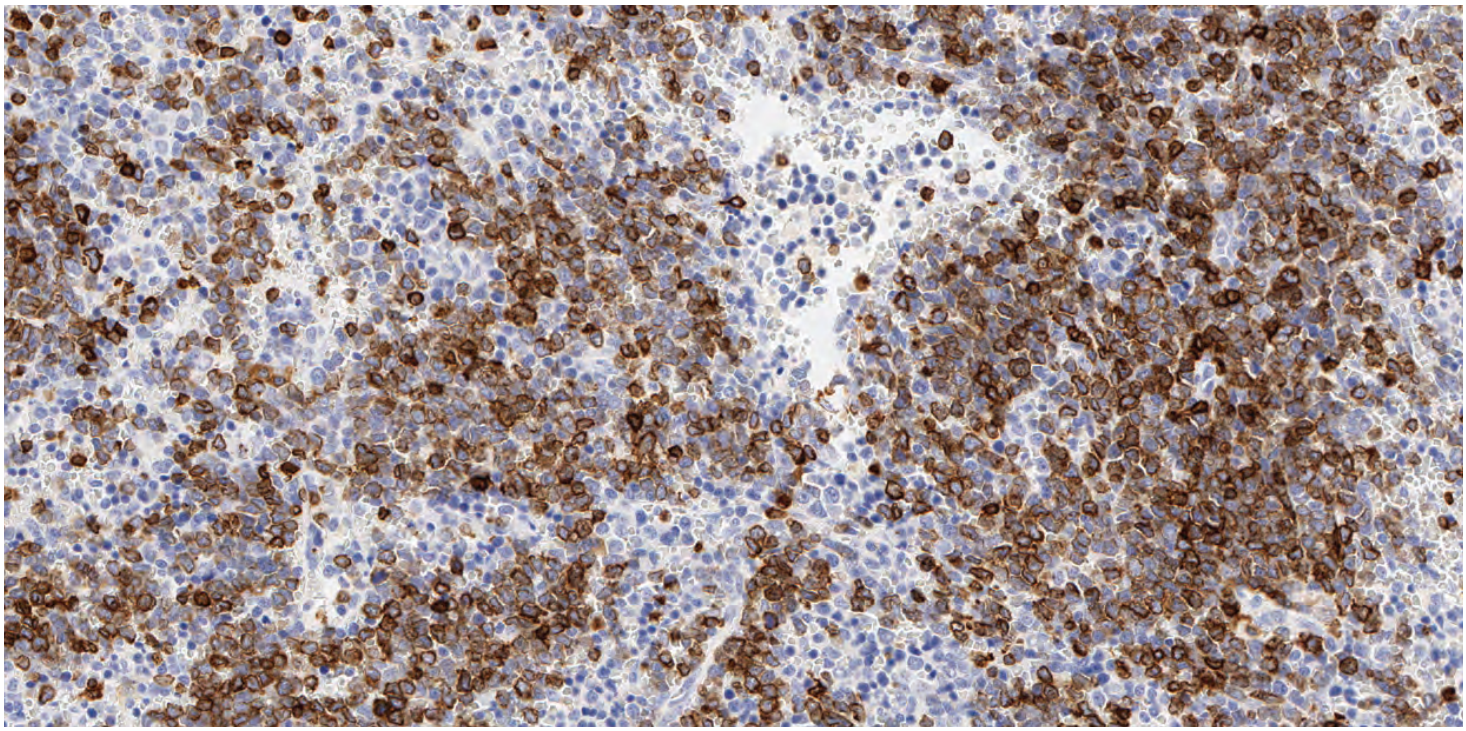

Pre-an•a•lyt•i•cal *(n.)*
Describing any variable whose value can affect the outcome of a subsequent analysis.

The American Heritage® Science Dictionary

Chapter 2.1 | Introduction

Immunohistochemistry (IHC) and in situ hybridization (ISH) techniques have advanced considerably since the first edition of this handbook was published in 1983. The need for standardization was emphasized in that publication and is still very relevant today, to ensure meaningful interpretation and allow quantitative microscopy. The histological process, which begins with the acquisition of tissue samples and continues through to the interpretation of IHC or ISH results, can be broadly broken down into three main stages: pre-analytical, analytical and post-analytical. This chapter will address some of the pre-analytical issues that should be considered when preparing sections for IHC and/or ISH. Pre-analytical variables can significantly and adversely affect the accurate detection of targets. Many of the comments made below are based on the large number of publications about prognostic/predictive (Class II) markers, such as ERBB2 (HER2), however, most of the points are broadly applicable regardless of the target under investigation or the method of choice.

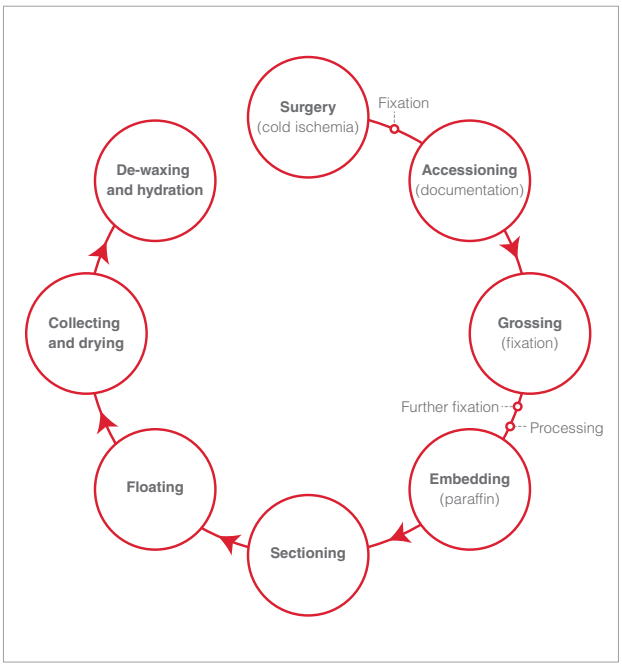

Figure 2.1 An overview of the processing steps included in the pre-analytical phase.

Chapter 2.2 | Tissue Handling

The pre-analytical stage begins as soon as a piece of tissue is removed from its nutritional source (blood supply) and the time to fixation is critical (1). Degeneration is caused primarily by autolysis, which is a process of self-digestion by enzymes contained within cells; and this begins immediately. This process is accelerated by increased temperatures. Fixatives are used to stop degeneration, while preserving the structure and integrity of the tissue elements as much as possible. However, fixation itself introduces artifacts and the ideal fixative would also maintain the structure of all of the epitopes in the tissues. This is not achievable, as the alteration in chemical structure caused by fixation necessarily modifies at least some epitopes. For IHC and ISH procedures it is critical that the tissue does not dry out during any stage of the tissue handling and slide preparation (1). Drying may cause morphological changes, such as poorly defined chromatin; and subsequently alter the structure of the target particularly along the edge of the tissue. This could inhibit ligand binding and is particularly applicable to small specimens such as endoscopic biopsies. Additionally, dry tissue is more adsorbent, which increases the risk of non-specific or unwanted adsorption of reagents during staining procedures, thereby interfering with interpretation of results.

Cold Ischemic Time

Recently, there has been more of a focus on "cold ischemic time" and the impact this may have on IHC and ISH results. The duration of cold ischemia is calculated from when the tissue is removed from the body to when the tissue is placed into fixative. This time should be as short as possible, with published guidelines of one hour or less (2, 3). The deleterious effects of delayed fixation are illustrated in Figures 2.2 and 2.3; and may include increased, decreased or de-localized immunoreactivity. It should be noted that deterioration of an epitope due to ischemia cannot be recovered using antigen retrieval techniques.

Relatively little has been published on the ischemic effects for specific antigens or molecular targets which are Class I. Nonetheless, Figure 2.2 shows that the pattern of staining change with the three Class I targets illustrated. Perhaps, a broader understanding of the interrelationship between ischemic time and different targets will be easier, once the recording of ischemic times becomes a part of required documentation for all specimens.

PETITIONER EXHIBIT 1012
Page 22 of 218

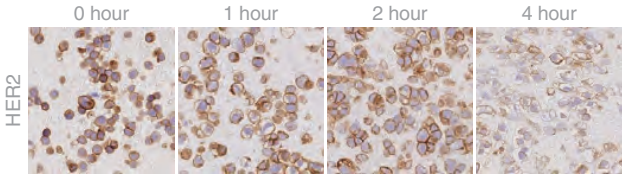

Figure 2.2 Cold ischemia alters the staining intensity of HER2 in MDA-MB-453 cells (2+ cell line). Weak to moderate membrane immunoreactivity on approximately half of the cells is observed in a cell pellet fixed immediately in 10% NBF (0 hour). With as little as one hour cold ischemia (the cell pellet was kept moist under saline-damped gauze), the morphology is already deteriorating and there appears to be increased numbers of cells with membrane staining. After two hours, the staining is even stronger. Following four hours cold ischemia time, much of the membrane staining is lost and the preservation of the cells is poor. This illustrates the need for prompt fixation and that different cold ischemic times can give rise to over staining or under staining of the cell membranes. Cells were stained using IHC and HER2 antibody (Code A0485, Dako) and the Autostainer Link 48, Dako platform.

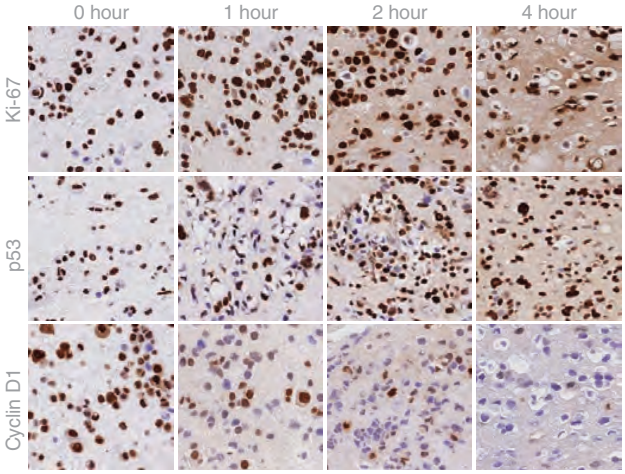

Figure 2.3 MDA-MB-453 (Ki-67 & Cyclin D1) and MDA-MB-231 (p53) cell pellets were fixed immediately in 10% NBF (0 hour) or held for one hour, two hours or four hours before transferring to NBF (the cell pellets were kept moist under saline damped gauze prior to fixation). For Ki-67 and p53 progressively more 'connective tissue' staining is observed with increased cold ischemia time, due to de-localization of the antigens from the nuclei. For Cyclin D1, progressive loss of staining is observed, with some de-localization. Sections were stained using the Autostainer Link 48, Dako platform, with FLEX detection and the following Dako RTU primary antibodies: MIB-1 (Ki-67), DO-7 (p53) and EP12 (Cyclin D1).

Accessioning and Documentation

When the specimen is received in the laboratory it is 'accessioned' and given a unique, traceable number. The documentation (requisition) which accompanies each surgical specimen should include: patient and physician information, date of procurement, clinical information, specimen site and type, collection time, cold ischemic time, type of fixative and duration of fixation (3). If it is necessary to decalcify a specimen, then that information must also be recorded, including: time in fixative before decalcification, the type of decalcification used, the length of decalcification and any post-decalcification treatment (4). Part of the sample verification process during accessioning is to confirm that the information on the requisition matches that on the specimen container. The specimen container should have a minimum of two identifiers such as patient name and date of birth.

It is well published that the length of time in 10% neutral buffered formalin (NBF) has a variable impact on IHC and ISH results. Yet getting the required information for all specimens can be a challenge. For example a survey conducted in 2010, to determine the compliance of 757 laboratories with the ASCO/CAP (American Society of Clinical Oncology / College of American Pathologists) guidelines for HER2 testing, showed approximately 28% of respondents did not include fixation information in case reports (5). Reporting the fixation time is valuable for interpretation and troubleshooting aberrant or unexpected results. It may also influence protocol choice, such as the type or timing of antigen retrieval or enzyme digestion required and the choice of control material (6). It is the responsibility of the collection team (often the clinical or surgical team) to provide the sample information, including the duration of cold ischemia and the time the specimen was placed in fixative. It is the responsibility of the pathology team to set guidelines which clearly outline criteria that determine if a specimen is acceptable for IHC or ISH staining.

Grossing

Once a specimen is deemed acceptable, it is examined macroscopically. This is referred to as grossing and it is a critical pre-analytical step which requires proper training. Larger specimens should be 'bread loafed' (sliced) into approximately 5 mm sections and placed in 10% NBF. Gauze or paper towel may be placed between the slices to facilitate exposure to the fixative. Care must be taken to handle each type of tissue in a standard-

PETITIONER EXHIBIT 1012
Page 23 of 218

ized manner and not to physically damage the tissue. Usually, it is necessary to select areas of interest from a larger specimen. These pieces of tissue, or blocks, should be trimmed such that the size does not exceed 20 mm in length and width, or 4-5 mm in depth. Trimmed tissue is then placed into a processing cassette and submerged immediately into the desired fixative (usually 10% NBF). The volume of fixative should be approximately 10 to 20 times that of the specimen (3, 7). Formalin enters the tissue relatively quickly, but the chemical processes which actually fix the tissue are more time consuming, taking at least 24 hours (8). When calculating total time in fixative, the time the specimen sits in 10% NBF in the grossing area and on the automated tissue processor must be included.

Chapter 2.3 | Fixation

Part of the challenge when choosing a fixative is the fact that the amounts of antigens or molecular targets within tissue specimens are finite. Although fixatives are meant to preserve these elements from elution, migration or degradation by changing the protein structure, they may destroy or mask these targets. There are many fixative recipes, but most of these can be grouped into three main categories: those containing formalin; those containing alcohol; and those containing a combination of both. Regardless of the fixative of choice, the preparation and use of that solution must be consistent. Of the many pre-analytical variables which affect IHC and ISH results, fixation is probably the most significant, impacting many other variables such as antigen retrieval and epitope binding. Unfortunately, to date, no single fixative has proven to be ideal for all targets and detection methods. However, it is generally more deleterious for tissue to be 'underfixed', rather than 'overfixed'.

10% NBF

The most frequently used fixative is 10% NBF with pH 7.0 to 7.4 (9). This fixative is the 'gold standard' and has traditionally been used by pathologists, perhaps because the ingredients are relatively inexpensive and the solution is simple to prepare and stable when stored. Formalin fixes by penetrating the tissue and forming cross linkages between reactive amino groups in proteins. Of course, this is an oversimplification of what is actually happening during fixation. The important point is that the rate at which each of these reactions takes place is different; and the reaction rates are all slower than the penetration rates. It is these differences which have led to confusion about what is an acceptable length of time for 10% NBF properly to fix a variety of specimens. Tissue measuring 4 mm thick should be fixed for at least 24 hours at room temperature (10). The ASCO/CAP recommendation guidelines for fixing tissue state that 10% NBF fixation, prior to IHC or ISH labeling of ER, PgR and HER2 cannot be less than 6 hours and should be no more than 72 hours (2b, 3). The reader should be aware of the recently published update to the 2007 ASCO/CAP HER2 breast cancer guideline (2a) which has changed the fixation recommendations to 6-72 hours for HER2 (2b), aligning this with the recommendations for hormone receptors. The recommendation for HER2 fixation was originally made for breast cancers but has been extended to include gastric cancer. These recommendations represent a compromise for the sake of speed, ignoring data, noted above, that fixation should be for at least 24 hours (also see below).

The ASCO/CAP guidelines also recommended some exclusion criteria for HER2, ER and PR fixation, specific to different types of samples, which are unchanged from 2007 to 2013 (2a, 2b). These exclusion criteria are: "tissues fixed in other than neutral buffered formalin; needle biopsies fixed for less than one hour in neutral buffered formalin; and excisional biopsies fixed in formalin for less than six hours or longer than 48 hours" (2a). It is assumed, however, that the last exclusion criterion should have been changed in the 2013 Guideline Update (2b) to 6-72 hours to be consistent with the new fixation recommendations for HER2. It is further emphasized that fixation outside of the recommended parameters (especially with other fixatives) must be fully validated in the user's laboratory; and that if testing is performed on tissues fixed outside of the recommended ranges, this fact must be included in the report (2a, 2b). In the opinion of the authors, these recommendations and exclusion criteria are inadequate. A minimum of 24 hours fixation should be applied to all samples. It is very important that tissue be "properly" fixed and that sufficient time is given to ensure completion of this process.

Due to its cross-linking characteristic, NBF is an especially good fixative for small molecules such as hormones (1). It is the progressive cross-linking nature of formalin fixation that often leads

PETITIONER EXHIBIT 1012
Page 24 of 218

to the masking of potential IHC or ISH targets, meaning that an end point for fixation is almost as important as a start time. There are many examples of situations that have led to incorrect interpretation of staining patterns caused by the time in fixative. One example is elution of Ki-67 and cyclin D1 protein from the nuclei of 'improperly fixed' samples (Figure 2.4).

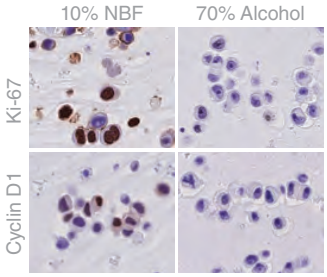

Figure 2.4 SK-BR-3 cell pellets were fixed in 10% NBF overnight before transferring to 70% alcohol (left), as the first step in tissue processing, or directly into 70% alcohol before processing (right). The lack of fixation in the latter resulted in loss of immunoreactivity for Ki-67 and cyclin D1. Sections were stained using the Autostainer Link 48, Dako platform, with Flex detection and the following Dako RTU primary antibodies: MIB-1 (Ki-67) and EP12 (Cyclin D1).

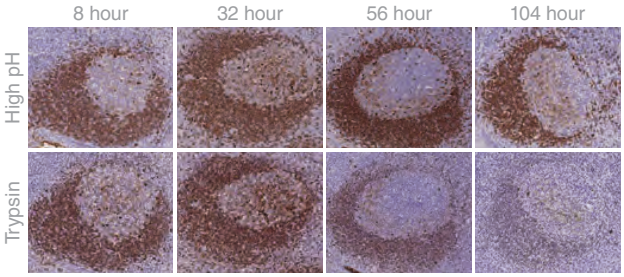

Figure 2.5 Length of fixation affects immunoreactivity for IgM in tonsil mantle zone B cells. This figure illustrates reduced immunoreactivity for IgM in B lymphocytes in the mantle zone of secondary lymphoid follicles in tonsils fixed for several days. It further illustrates the fact that different methods of antigen retrieval produce different results, when attempting to recover the immunoreactivity. In the four images in the top row, the tissues were fixed for 8, 32, 56 and 104 hours and the mantle zone staining is approximately the same in the sections which were treated with Dako target retrieval solution (high pH) for 20 minutes at 97 °C. However, in the bottom row, the target retrieval was performed using trypsin (0.1% for 30 minutes). In this case, the antigen is unmasked when tissue is fixed for 8 and 32 hours but significantly depleted in tissues fixed for 56 and 104 hours. Extending the trypsin digestion time would damage the shorter fixed samples but result in stronger staining for IgM in the tissues fixed for two or four days (not shown). This type of comparison needs to be made for each new antigen being validated in the laboratory.

Antigen retrieval techniques have been developed to help 'de-mask' many targets after NBF fixation; however, without complete information, it is difficult to choose a suitable antigen retrieval protocol that will produce accurate results. Modern heat-retrieval methods seem to work over relatively broad fixation times (Figure 2.5); however, the timing of heat retrieval may need to be adjusted in tissues fixed for short or for excessive times, which is somewhat of a moot point in laboratories using the recommended 24 hour minimum NBF time. With proteolytic retrieval methods, it has been known for years that the necessary duration of trypsinization or pepsinization is directly proportional to the duration of fixation, as illustrated in Figure 2.5. It must be emphasized that the need for retrieval and the optimum protocol may differ from epitope to epitope – not by antigen, so different monoclonal antibodies may require different methods. Since antigen retrieval methods are discussed in Chapter 3, these will not be given additional attention here.

Formalin is considered a Class II carcinogen. Even formalin fumes have the ability to fix. This makes it a prime target for safety concerns. Good laboratory practices should always be employed when handling this product. Many alterative fixatives to 10% NBF have been proposed, and perhaps there will be a trend away from formalin in the future, towards more 'molecular friendly' fixatives. The logistical and practical issues of switching to another fixative are, however, enormous.

Alcohol Fixation

When a tissue is fixed in 10% NBF for less than 6 hours prior to being processed through to paraffin, the tissue is probably fixed in alcohol, or has a variable combination of formalin and alcohol fixation. This non-standardized type of fixation may cause false negative or positive results (see Figure 3). Alcohol fixes by coagulating and precipitating proteins and tends to extract tissue elements such as low molecular weight carbohydrates. It also tends to dehydrate the tissue which causes shrinking and hardening. Alcohol fixation has an advantage over formalin fixation, in that it generally eliminates the need for antigen retrieval. It initially penetrates and fixes tissues more readily than formalin (although penetration slows down subsequently), and is often recommended for nucleic acid work.

PETITIONER EXHIBIT 1012
Page 25 of 218

Enhancing Fixation

There are some fixation methods which incorporate microwave or ultrasound technology. Heat is generated by the excitation of molecules in both methods and accelerates the rate of reactions (1). This effect also speeds up the penetration of the solutions by relaxing the cell structure. Microwave fixation may, however, induce uneven tissue fixation that can vary with the size and composition of the specimens and type of microwave used (10). Directly, microwaving tissue causes protein coagulation and can lead to hard or 'over-cooked' tissue.

Chapter 2.4 | Tissue and Slide Processing

Not all tissue processors are the same and these differences are most apparent when tissues are not fixed adequately. However, most instruments will produce satisfactory results, most of the time. Some of the basic principles of processing and slide preparation are discussed below.

Tissue Processing

During tissue processing, fixation reagents containing water are replaced by wax (polymer, non-polymer and microcrystalline formulas exist) which is done through a series of passages through increasing concentrations of alcohol, up to 100% (absolute) alcohol. This process is followed by clearing the alcohol from the tissue (for example by using xylene) and replacing it with molten wax. Low melting temperature (45 °C) as opposed to higher melting temperature (65 °C) waxes have been reported to produce better staining results for IHC, particularly in T-lymphocyte staining (7). Next, the paraffin infiltrated pieces of tissue are embedded to form blocks, which are easily handled, cut and subsequently stored.

Rapid Tissue Processing

There is increasing pressure to shorten turnaround times (TATs) in tissue pathology, so that patients do not have to wait days to receive their pathological diagnoses. However, the laboratory staff still need to ensure that samples are properly fixed (>24 hours in NBF, even for needle biopsies), to make sure that validated IHC and ISH methods are used. As noted above, tests used after alternative fixation and processing must be fully re-validated. This requirement is particularly applicable to the modern rapid tissue processors which employ alternative fixatives and microwave enhanced processing (as well as small specimen size). This combination allows an H&E diagnosis on paraffin sections the same day. Nonetheless, the morphology will differ from routine FFPE processed samples; and IHC and ISH methods will require complete re-validation, as some of these will not need pre-staining antigen retrieval, whether this is of the heating or proteolytic type (personal observation).

Section Preparation

Generally, unless otherwise specified by a protocol of choice, sections for IHC or ISH are cut at 3 µm, 4 µm or 5 µm. Thicker sections may cause difficulty during staining, and also problems in interpretation due to the multi-layering of cells. After sections are cut they are usually floated on water and picked up onto glass slides that are coated with some adherent material. Sections must lay flat against the glass to prevent lifting during staining or bubble formation, which may trap staining reagents. The more points of adhesion the more likely the tissue will remain fixed to the slide, supporting the need for thinner sections. Some commercially available slides come with a positive charge that attracts the negative charges of tissue proteins. These charged slides are especially effective following formalin fixation of tissues, since formalin blocks amino acids in tissues, rendering the tissue more acidic and therefore more negatively charged. Different manufactures of staining platforms may recommend the use of particular slides to achieve optimal results. As with every other pre-analytical step, cutting and mounting sections onto glass slides, and all steps prior to staining must be standardized. For example, if the slides are to left at room temperature for 15 minutes, in an upright position to allow draining of excess water and then heated in staining rack at 60 °C for 30 minutes prior to staining, this step must be repeated every time sections for IHC or ISH are prepared. Finally, the changes resulting from block and section storage prior to IHC and ISH staining may also affect staining results (11). For example, it is recommended that sections cut for HER2 testing should not be used if they are more than 6 weeks old.

Dewaxing and Hydration

Wax must be removed completely from the tissue sections, so that aqueous antibodies, molecular probes and detection reagents can penetrate and adhere to the tissue. Traditionally,

PETITIONER EXHIBIT 1012
Page 26 of 218

dewaxing was done by immersing the sections into a dewaxing solution (such as xylene), with or without prior brief heating. This step was followed by absolute and graded hydrating solutions (generally alcohols), until the final solution: water. If xylene is used to dewax sections, approximately 50 slides per 50 mL of xylene is the limit, before it is no longer effective and residual wax begins to cause artifacts in the stained tissue.

Today, there are many commercially available staining platforms which include onboard removal of wax and rehydration of the tissue sections. The accumulation of residual wax may be a problem with these instruments, if rinsing is insufficient or if solutions are not replenished regularly.

Chapter 2.5 | Special Tissue Preparations

Biopsy Specimens

There is a trend away from invasive surgical procedures towards less invasive biopsy techniques for pathological diagnosis (12). The cost effectiveness of these procedures and improved imaging capabilities during biopsy procurement, support the notion that these types of specimens will become even more prominent in pathology laboratories. Handling of these biopsy specimens can present challenges for the laboratory. For example, these tiny fragments or cores of tissue require greater dexterity during grossing, embedding, cutting and sometimes staining, making them more time consuming. Also, as noted above, they are particularly susceptible to drying artifacts and often exhibit structural damage from the biopsy procedure per se. They limit the amount of tissue available for microscopic evaluation and limit the tissue elements available for IHC or ISH targeting. These types of specimens are often processed using accessories such as biopsy bags or sponges, in an attempt to reduce the risk of sample loss during processing and to help maintain the architecture of the specimen by mitigating folding or wrinkling, which can interfere with reagent flow, staining and interpretation. If, for example, sponges are used to secure biopsies in tissue cassettes, the sponges themselves will absorb and retain reagents more readily than tissue alone. Hence, excess amounts of absorbed processing fluids may be transferred from one processing container to another, particularly when a large number of sponge-containing specimens are processed simultaneously. Consideration must be given to this contingency when choosing appropriate processing schedules and reagent replenishing practices. To eliminate this variable, the use of nylon biopsy bags, which do not retain significant amounts of reagent, is recommended.

Biopsy specimens can often be very difficult to see, especially after paraffin processing. Adding a small amount of alcoholic eosin to the processing alcohols can make the tissues more visible, by tinting them slightly. This simple procedure does not appear to have any detrimental effect on subsequent IHC or ISH staining, however, the use of eosin or other biopsy coloring fluids should be tested prior to implementation. An example of another stain used for this purpose is Mercurochrome. Notwithstanding the fact that this mercurochrome should not be used for safety reasons, its application diminishes or abolishes the signal in FISH assays and causes excessive background fluorescence (personal observation).

Frozen Sections

If tissue targets cannot be demonstrated following fixation and paraffin processing, then alternative methods such as rapid freezing of the tissues, may be considered, keeping in mind that frozen tissue is not routinely available, and is difficult to obtain for reasons of logistics and expense. As with fixed sections, the protocols for obtaining suitable rapidly frozen tissues sample and preparing frozen sections must be standardized and validated for all antibodies, antigens, or molecular targets; as well as detection methodologies. An example of a situation when a frozen section may be preferred to a formalin fixed specimen is during the investigating of autoimmune or inflammatory diseases or disorders of the skin. Using the Direct Immunofluorescence (DIF) technique and frozen sections, immunoglobulins and complement in skin biopsies can easily be demonstrated. An advantage of frozen sections is that they can be prepared in less time than traditional paraffin processed sections. The most challenging part of preparing these types of sections is freezing the tissue rapidly (quenching or snap-freezing using liquid nitrogen). Freezing, thawing and then re-freezing specimens causes freezing artifacts, which destroy morphology and subsequently may affect the integrity of IHC or ISH. This problem is particularly applicable to control ma-

PETITIONER EXHIBIT 1012
Page 27 of 218

terial, which is often re-cut multiple times. Frozen sections cut for IHC and ISH should be between 4 µm and 6 µm thick, although thicker sections may be required. Fatty specimens, particularly breast tissue, are difficult to cut because fat does not freeze as well as the rest of the tissue (except at temperature low enough to cause shattering of the tissue). Fat continues to be a nemesis of frozen section preparation.

Post-fixation of frozen sections must be standardized for each target under investigation. Many of the colorimetric IHC and ISH protocols used on FFPE sections include the use of an enzyme, such as peroxidase, to produce the final color. Hence, quenching of endogenous enzyme (i.e. peroxidase) activity is often incorporated into IHC and ISH protocols; however, these steps are not usually included when frozen sections are stained. This omission potentially can interfere with interpretation of the results.

Unfixed, frozen tissue may contain viable human pathogens or toxins. Universal precautions must to be employed when handling these types of specimens. Formalin is known to inactivate by far the majority of pathogens that might contaminate human tissue samples (except prions) and archived tissue should be formalin fixed, prior to discard.

Chapter 2.6 | Control Material

Positive Tissue Controls

The interpretation of IHC and ISH results relies on the use of method controls and a general acceptance of what is considered to be appropriate staining. Control material must be fixed and processed in the same manner as the test material, to assure the accuracy of the results. In the authors' laboratory, this requirement includes replicate tissue control blocks fixed in NBF for various times (e.g. 8, 32, 56 and 104 hours; see Figure 2.5); and the control block is selected to match the length of fixation of the test specimen. Traditionally, tissues with known expression of the target are used as controls. For example, breast tumor control tissues with different levels of HER2 expression (as well as normal breast tissue) are employed as controls for HER2 IHC and ISH. A similar approach is taken for all other antigens, and low level expressing normal tissues are particularly useful as method controls. However, it is difficult to maintain a continuous supply of some these types of tissues, especially tumors; and an alternative approach is to use defined cell lines.

Control Cell Lines

The use of cell lines would theoretically, provide a long-term supply of material that contains specified levels of expression for various antigens (11). Using cells as controls is not novel and is incorporated in the HercepTest™ kit (Dako, Denmark) as well as assays from other manufacturers. According to the HercepTest™ kit insert, if the cell controls provided are used in association with the recommended platform and in-house controls, they provide valuable information on assay validity for the semi-quantitative assessment of HER2 overexpression. Cell lines are commercially available and can be cultured, harvested and pelletized, before being fixed and processed in the same manner as test specimens (11, 13; see Figure 2.6). The selection of appropriate cells and the method of processing these for each antibody, molecular marker, detection protocol, and interpretation method must be validated prior to implementation.

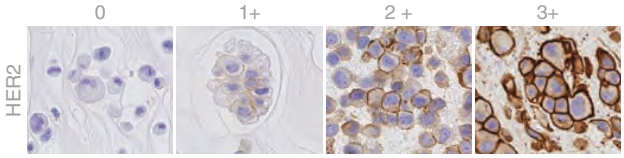

Figure 2.6 Different levels of expression of HER2 on four different breast cancer cell lines. Four cell lines with different levels of HER2 expression, ranging from no expression (0; MDA-MB-231) through 1+ (MDA-MB-175), 2+ (MDA-MB-453) and high expression (3+; SK-BR-3) can be used as controls/standards for HER2 IHC. Other cell lines may be used for different antigens. Cells were stained using IHC and HER2 antibody (Code A0485, Dako) and the Autostainer Link 48, Dako platform.

Unfortunately, while this approach should produce control material that is equivalent to the test samples processed in the same manner, this is not always the case. The authors and Dako Research and Development staff have all observed that control cell lines seem to be inferior to solid tissues as controls for FISH assays (personal communication); and the cell lines may be more easily damaged during staining. This problem serves to emphasize the need for thorough validation of the control materials.

Tissue Microarrays

The use of tissue microarrays (TMAs) has become popular for

PETITIONER EXHIBIT 1012
Page 28 of 218

IHC/ISH method development and research purposes, where multiple different tumors and normal tissues can be combined into a single block, vastly reducing the number of slides needed for staining. TMAs are also used for control material for routine work. Thus small samples of a range of controls (e.g. as noted above for HER2) can be put together into a single small block. Sections from such blocks can be placed alongside every test section, to give the optimum "on-slide" control, assuring that each slide received all reagents during the staining run. The small size of the samples in TMA blocks conserves the control tissue. However, care must be taken to ensure that heterogeneity within tumors does not result in inappropriate controls; and that fixation and processing conditions are matched.

Chapter 2.7 | Validation

Validation is an essential step when establishing protocols and when choosing reagents. Validation ensures that a certain standardized procedure will give consistent and diagnostically useful results on tissues processed in a standardized manner. It also tests the limits of changes in the procedure that will continue to provide optimum results. For example, users can validate the effect of 10% NBF fixation on a particular antigen by preparing multiple, uniform pieces of tissue from the same tissue specimen. These blocks are then fixed in 10% NBF for different lengths of time, spanning multiple days (see Figure 2.5). They are then processed using identical protocols, cut and then stained simultaneously. The results help to establish the range of fixation times that produces acceptable staining for a particular target (epitope), while employing a particular detection method.

The work involved in validation is often difficult, time consuming and expensive. Alternatively, users can choose a system with an existing standardized and validated protocol, the so-called RTU (Ready-to-Use) approach. Commercially available RTU kits, when utilized exactly as described in the kit inserts, are guaranteed (within limits) to provide diagnostically useful results. Examples of such kits are: ER/PR pharmDx™ Kit; and HercepTest™ (c-erb-B2 oncoprotein – HER2 protein) from Dako, Denmark, while similar RTU reagents are widely available from other manufacturers. Another option is to use published information provided by high quality peer-review studies, external organizations such as NordiQC or CAP, and product inserts from manufacturers. This information can narrow the scope of unknown variables, thereby facilitating more efficient, precise testing, and potentially reducing effort and costs. However, such information is not a substitute for in-house validation, which must still occur, albeit with reduced numbers of reference samples. When validating a primary antibody for IHC or an ISH method for Class II targets (prognostic or predictive markers), a minimum of 25 to 100 cases must be tested, according to ASCO/CAP recommendations. Both positive and negative cases are to be included and some of the positive cases should have low expression of the target (4). As part of the ongoing assessment and monitoring for any "drift" in test results, participation in external quality assessment programs is critical and the correlation between the internal and external testing should be very high (90% for positives and 95% for negatives) (4).

Instruments such as tissue processors and automated IHC and ISH staining platforms must also be validated. Methods of validating instruments vary, but the purpose is to prove that the instrument is working as expected, repeatedly and reliably. It is helpful and cost effective when manufacturers of these instruments provide validation documentation to customers upon installation. Customers can then verify that the information provided is reproducible at their own facilities. Different countries and regulatory bodies have different standards regarding validation and these local rules should be consulted.

Examples of when validation would be required include:

- When changes in the fixation or processing protocols are being made
- When an alternative fixative is being introduced
- When a new decalcifying protocol is being introduced
- When a new staining platform is being introduced (automated or manual)
- When a new release of software for a staining platform is being introduced
- When a new IHC or ISH staining detection protocol is being introduced
- When a new antibody or molecular marker is being introduced, including when an alternative clone or nucleic acid probe for an existing test is being introduced
- When new control tissues or cells are being introduced.

PETITIONER EXHIBIT 1012
Page 29 of 218

Once initial validations are complete, it is not necessary to re-validate unless there has been a significant change in the test system, such as a new water supply. However, verification of staining performance is an on-going process. Examples of when verification may be required include:

- When new lot numbers of antibodies or detection reagents are put into use
- When unexpected or aberrant staining results occur; and
- When several different staining platforms are all being used to perform the same IHC or ISH tests (verification should be done at pre-set intervals to determine if each platform is producing comparable staining results, regardless of instrument used)

Chapter 2.8 | Conclusions

Patient safety based on accurate interpretation of results depends heavily on this standardization of all pre-analytical variables. Prognostic tests using IHC and ISH are being developed and they will independently forecast clinical outcomes for patients. HER2, ER and PgR are considered predictive markers that influence the selection of patients who will respond more favorably to therapies, emphasizing further the need for standardization. Even if it is not possible to perfectly optimize every pre-analytical step, it is possible to perform each step in the same manner each time it is done. Rigorous adherence to this approach will yield more meaningful results and will, if necessary, facilitate problem solving.

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PETITIONER EXHIBIT 1012
Page 30 of 218

Chapter 3

Antigen Retrieval

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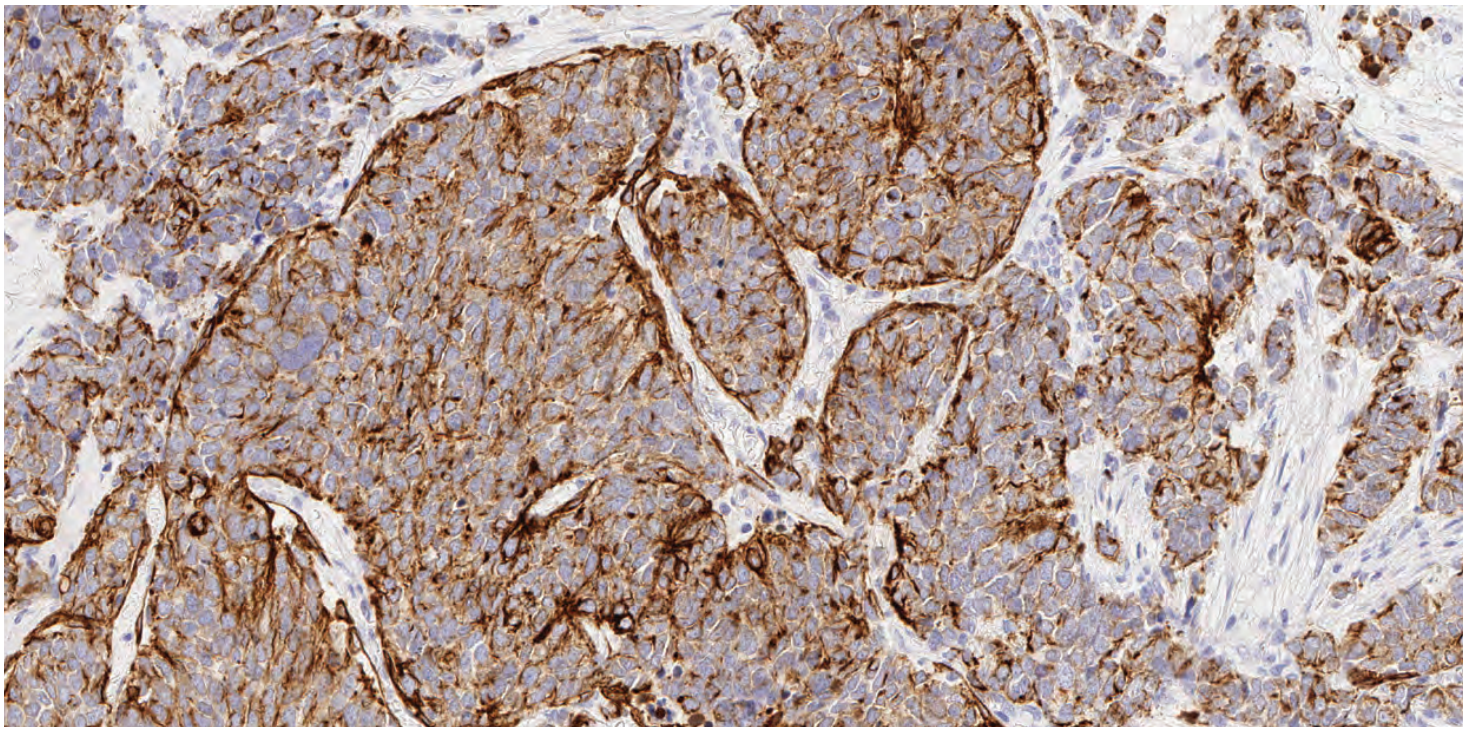

Re•triev•al *(n.)*
The act or process of getting and bringing back something.

Merriam-Webster Online Dictionary

Chapter 3.1 | Introduction

In the majority of cases, tissue specimens for immunohistochemical (IHC) staining are routinely fixed in formalin and subsequently embedded in paraffin. Because of the long history of the use of formalin-fixed, paraffin-embedded (FFPE) tissue sections in histopathology, most of the criteria for pathological diagnosis have been established by the observation of FFPE tissue sections stained by hematoxylin and eosin. Additionally, a great number of FFPE tissue blocks, accompanied by known follow-up data, have been accumulated worldwide, providing an extremely valuable resource for translational clinical research and basic research that cannot easily be reproduced. The major drawback of FFPE tissue is that formalin-induced molecular modification of proteins (antigens) may result in loss of the ability of the antibody to react with the antigen, a loss that can only be corrected by the restoration (retrieval) of the 'formalin-modified' antigen molecular structure. Since the early 1970s, many active pioneers, mostly practicing pathologists who were acutely aware of the need to enhance the capabilities of IHC on FFPE tissue sections while retaining morphologic features, have been searching for an effective retrieval technique (1). Some retrieval methods, such as enzyme digestion, improved IHC staining only for limited antigens. One of the authors (Shi) began a different approach, based upon the practical and theoretical issues to be addressed. A key scientific question was whether fixation in formalin modified the structure of antigens in a reversible or an irreversible manner. To be more specific, was there any prior scientific evidence that the effects of formalin fixation on proteins could be reversed? And if reversed, was the structure of protein restored to a sufficient degree for recovery of antigenicity? With these key questions in mind, Shi spent many days and nights in 1988, prior to online data access, searching the chemical literature the old fashioned way! The answer was finally found in a series of studies of the chemical reactions between protein and formalin, published in the 1940s (2-4). These studies indicated that cross-linkages between formalin and protein could be disrupted by heating above 100 °C, or by strong alkaline treatment. With this knowledge of high temperature heating as a potential retrieval approach, the heat-induced AR technique was developed in 1991 (5).

Subsequently, this AR technique has been applied to in situ hybridization, TUNEL, immunoelectron microscopy, blocking cross-reactions for multiple immunolabeling, aldehyde-fixed frozen tissue sections, mass spectometry on FFPE tissue sections, and the development of a series of novel techniques for successful extraction of nucleic acids and proteins from FFPE tissues (6). Arguably this contribution to protein extraction has proved critical to the development of modern tissue proteomics on FFPE tissues (7, 8).

As a result, FFPE archival tissue collections are now seen as a literal treasure of materials for clinical and translational research, to an extent unimaginable prior to the introduction of heat-induced antigen retrieval two decades ago. The advantages of FFPE tissues in terms of preservation of both morphology and molecules in cell/tissue samples are broadly recognized. For example, there is a growing body of literature demonstrating successful application of FFPE tissue samples for molecular analysis, using AR based methods

Table 3.1 Comparison of frequency concerning application of different terms of heat-induced AR according to OVID Medline data of the 1st week of July & August 2013.

Different terms used	Total articles		Percentage (%)	
	1st week of July	1st week of August	1st week of July	1st week of August
Antigen retrieval	138	140	63.9	63.9
Epitope retrieval	22	22	10.2	10.1
Heat-induced epitope retrieval	15	15	6.9	6.9
Microwave treatment	41	42	19	19.1
Total	216	219	100	100

PETITIONER EXHIBIT 1012
Page 32 of 218

for extraction of DNA/RNA, and proteins from FFPE tissues. Today, twenty years on, the AR technique is widely, almost universally, used in surgical pathology, including veterinary pathology, in all morphology based sciences, and in pharmacology drug related research, with thousands of original articles published worldwide (6). The enormous impact is reflected in the need to divide all publications with respect to IHC on FFPE tissue into two phases: the pre-AR and post-AR eras, with the dividing line in the early 1990s (9). The term "antigen retrieval" (AR) was originally adopted by Shi and colleagues in 1991. Other terms exist, such as heat-induced epitope retrieval (HIER) or antigen unmasking/demasking, but have no particular merit to cause replacement of the original term (10). Table 3.1 is a comparison of frequency with respect to usage of different terms for this technique. Clearly the original term, antigen retrieval, has greatest acceptance and will be employed in this chapter.

The earlier introduction of enzymatic pre-treatment of tissue sections (11) remains in use for certain selected applications, but these methods are much more difficult to control and have been largely replaced by heat-induced AR.

Chapter 3.2 | Major Factors that Influence the Effect of Antigen Retrieval

Following wide application of the heat-induced AR, numerous modifications of the AR technique and various protocols have been documented in literature. As a result, there is a growing need for standardization of the AR technique itself. The critical importance of standardization of AR-IHC has been emphasized by the American Society of Clinical Oncology and the College of American Pathologists in their Guideline Recommendations for HER2 testing in breast cancer, as well as numerous subsequent documents (12a, 12b, 13). In order to understand the key issues with respect to standardization of AR, it is critical first to study the major factors that influence the effectiveness of AR-IHC. The following conclusions are based on our more than twenty year experience of research, and upon literature review.

- Heating is the most important factor: high temperature heating of formaldehyde-fixed proteins in FFPE tissue sections produces hydrolysis that contributes to break down cross-links (14, 15). In the very first article on AR, Shi and colleagues (5) showed a strong keratin-positive staining result simply by boiling sections in distilled water in a microwave oven. While the composition of the AR solution plays a part, it is the presence of heat and water that is critical: immersing FFPE tissue sections in pure 100% glycerine followed by the IHC staining procedure gives a negative result, adding water to the glycerine and boiling again, gives satisfactory IHC staining (16). That high temperature heating is the most important factor for AR technique has been confirmed by numerous subsequent publications (17, 18). There are several critical technical points with respect to the combination of heating temperature and heating time (heating condition = heating temperature x heating time):
- For many antigens, almost any kind of heating treatment, including microwave oven, water bath, pressure cooker, or autoclave may generate similar results, if adjusted appropriately for time
- There is generally an inverse correlation between heating temperature (T) and heating time (t), as expressed by the formula: AR = T x t (19)
- For most antigens, higher temperature heating, such as boiling FFPE tissue sections for 10-20 minutes, may be an optimal heating condition. However, a few antigens require lower temperature heating conditions, over a longer period of time (20).
- It has been recommended that to preserve tissue morphology, a lower temperature (90 °C) with an elongated time may be preferable (21)
- Within the above generalizations, for some antigens the most extreme conditions of temperature and time (e.g. pressure cooker for hours) gives the greatest staining, but at the cost of morphology. Such methods should be considered as a last resort.

pH Value of the AR Solution

The pH value of the AR solution is another factor that significantly influences the result of AR-IHC. In 1995, we (22) tested the hypothesis that pH of the AR solution may influence the quality of immunostaining of a panel of antibodies, by comparing seven different AR buffer solutions at different

PETITIONER EXHIBIT 1012
Page 33 of 218

pH values ranging from 1 to 10. The conclusions of this study are relevant when choosing the optimal AR method for any particular antigen/antibody pairing:

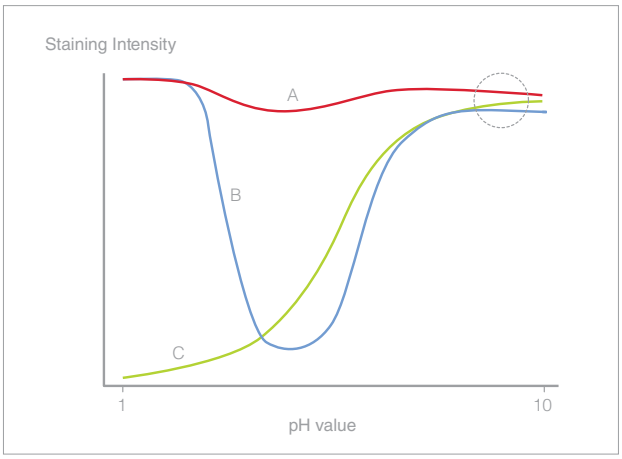

Figure 3.1 Schematic diagram of the three patterns of pH-influenced AR immunostaining. Line A (pattern of Type A) shows a stable pattern of staining with only a slight decrease in staining intensity between pH 3 and pH 6. Line B (pattern of Type B) shows a dramatic decrease in staining intensity between pH 3 and pH 6. Line C (pattern of Type C) exhibits an ascending intensity of AR immunostaining that correlated with increasing pH value of the AR solution. Circle (right) indicates the advantage of using an AR solution of higher pH value. With permission, reproduced from Shi S-R, et al. J Histochem Cytochem 1995;43:193-201.

1. Three types of patterns, reflecting the influence of pH, are indicated in Figure 3.1.
2. **A**, several antigens/clones showed no significant variation utilizing AR solutions with pH values ranging from 1.0 to 10.0 (L26, PCNA, AE1, EMA and NSE); **B**, other antigens/clones (MIB1, ER) showed a dramatic decrease in the intensity of the AR-IHC at middle range pH values (pH 3.0-6.0), but strong AR-IHC results above and below these critical zones; and **C**, still other antigens/clones (MT1, HMB45) showed negative or very weak focally positive immunostaining with a low pH (1.0-2.0), but excellent results in the higher pH range.
3. Among the seven buffer solutions at any given pH value, the intensity of AR-IHC staining was very similar, except that Tris-HCl buffer tended to produce better results at higher pH, compared with other buffers.
4. Optimization of the AR system should include optimization of the pH of the AR solution.
5. A higher pH AR solution, such as Tris-HCl or sodium acetate buffer at pH 8.0-9.0, may be suitable for most antigens (see circle in Figure 3.1).
6. Low pH AR solutions, while useful for nuclear antigens may give a focal weak false positive nuclear staining; the use of negative control slides is important to exclude this possibility.

Numerous investigators have independently reached similar conclusions (23-26).

Chemical Composition of the AR Solution

Other potential factors have been examined for their effect on AR. In considering citrate buffer, it is generally accepted that effectiveness is not dependent so much on the chemical, "citrate", as upon the high temperature heating. Studies have tested various additives to AR solutions, including metal salts, urea and citraconic anhydride; the last of these showed promise in achieving stronger intensity by testing 62 commonly used antibodies, findings confirmed by others (28, 29). In our comparative study between citrate buffer and citraconic anhydride, using 30 antibodies, more than half (53%) showed a stronger intensity of IHC when using citraconic anhydride for AR, whereas 12 antibodies (43%) gave equivalent results; only one antibody gave a stronger intensity using citric buffer alone for AR (28).

Today many commercial retrieval solutions are available, often as part of an RTU approach to an automated platform (see Chapter 5), and some products contain secret ingredients. Under prescribed conditions many of these reagents give good results, but care should be exercised in applying commercial AR solutions, of unknown composition, to targets other than those described by the vendor, or in protocols other than those recommended; both false positive and false negative results can occur.

With the growing use of automated staining platforms, the choice of 'autostainer' to a large degree dictates not only the selection of the primary antibody (see Chapter 4), and its concentration, but also the detection system, and the protocol (see Chapter 5 and Chapter 6), including the method of antigen retrieval. The vendors of automated stainers generally offer recommended AR protocols for (almost) all of the primary antibodies in their portfolio, usually a high pH method (pH 9), a mid/low pH meth-

PETITIONER EXHIBIT 1012
Page 34 of 218

od (pH 6), and an enzyme-based method for a small number of antibodies. The recommendation usually includes the use of proprietary AR solutions, and defined heating conditions, as part of the protocol. As noted above, departure from these recommendations requires a full revalidation process.

For new antibodies (see Chapter 4), and for antibodies produced by other vendors (other than the manufacturer of the particular automated stainer in use) the laboratory must undertake a study to establish the optimal retrieval method. For this purpose it is recommended that the laboratory use some variation of the Test Battery approach introduced by Shi and colleagues.

Chapter 3.3 | Standardization of AR – The "Test Battery" Approach

In 1996, a "test battery" approach was recommended as a method for quick examination of the two major factors that affect the outcome of AR, namely the heating condition and pH value, in order to reach the strongest signal of AR-IHC (maximal level of AR) (30). This test battery serves as a rapid screening approach to optimize the AR protocol and in so doing achieve some degree of standardization (31). In the initial recommendation the test battery included three levels of heating conditions (below-boiling, boiling and above-boiling), and three pH values (low, moderate, and high), such that a total of nine FFPE tissue sections were used (Table 3.2).

Table 3.2. Test battery suggested for screening an optimal antigen retrieval protocol.

Temperature	Tris-HCl buffer		
	pH 1.0-2.0 (Slide #)[a]	pH 7.0-8.0 (Slide #)[a]	pH 10.0-11.0 (Slide #)[a]
Super-high (120 °C)[b]	#1	#4	#7
High (100° C), 10 min	#2	#5	#8
Mid-high (90° C), 10 min[c]	#3	#6	#9

(a) One more slide may be used for control without AR treatment. Citrate buffer of pH 6.0 may be used to replace Tris-HCl buffer, pH 7.0 to 8.0, as the results are similar, and citrate is most widely used.
(b) The temperature of super-high at 120°C may be reached by either auto claving or pressure cooker, or microwave heating at a longer time.
(c) The temperature of mid-high at 90°C may be obtained by either a water bath or a microwave oven, monitored with a thermometer.
Modified from Shi SR, et al. J. Histochem. Cytochem. 45: 327-343. 1997.

In practice, the method may be further simplified in the following ways;

- Test three pH values by using one temperature (boiling), select the best pH value and then test various temperatures; or,
- Test several commonly used AR solutions (or those recommended for the autostainer in use in the laboratory), such as citrate buffer pH 6.0, Tris-HCl + EDTA of pH 9.0

Although this later method is not a complete test, it is more convenient for most laboratories. If satisfactory results are not obtained other variations may be tested, including citraconic anhydride, or enzyme-based digestion methods. Numerous recent articles have emphasized that the application of test battery for establishing an optimal AR protocol is also dependent on the primary antibody and the subsequent detection system. In other words, if an optimal AR protocol is good for antibody clone '1' to protein 'A' employing detection system 'B', it is not necessarily good for antibody clone '2' to protein 'A', using the same or different detection systems; but a different AR protocol might give acceptable results.

Specially prepared tissue microarrays (TMAs), incorporating a range of tissues and tissue cores fixed for differing times, are also of value in helping establish the optimal AR method for a particular antibody, by staining of only a few TMA slides. The advantages are further enhanced by application of recently developed image analysis software (AQUA) that is designed for quantitative IHC in TMA using an automatic scan (32).

PETITIONER EXHIBIT 1012
Page 35 of 218

Table 3.3 Major applications of antigen retrieval technique and principle.

Areas of application of AR	Application of AR technique and/or principle	Reference
Immunoelectron microscopy (IEM)	AR pre-treatment of Epon-embedded ultra-thin sections after etching the grids by solutions[(a)] to achieve satisfactory positive results; or, directly heating the grid and followed by washing procedures including 50 mM NH4Cl and 1% Tween 20	39, 40
In situ hybridization (ISH)	High temperature heating FFPE tissue sections prior to ISH to achieve satisfactory results	41-43
TUNEL	Optimal heating time, as short as 1 min to improve the signal	44, 45
Multiple IHC staining procedures	Adding a microwave heating AR procedure (10 min) between each run of the multiple IHC staining procedure effectively blocks cross-reactions, by denaturing bound antibody molecules from the previous run	33
Human temporal bone collections	Combining sodium hydroxide-methanol and heating AR treatment provides an effective approach for IHC used in celloidin-embedded temporal bone sections. This method is also used for plastic-embedded tissue sections, including IEM	46, 47
Immunofluorescence	To enhance intensity and reduce autofluorescence	48
Cytopathology	AR pre-treatment of archival PAP smear slides promotes satisfactory IHC staining	49
Flow cytometry (FCM)	Enzyme digestion followed by heating AR treatment was adopted to achieve enhancement of FCM of FFPE tissue	50
Floating vibratome section	Microwave boiling of vibratome sections improves IHC staining results; further extended for use with whole mount tissue specimens	51
En Bloc tissue	AR heating of 4% paraformaldehyde-fixed animal brain or testis tissue blocks enhances immunoreactivity for most antibodies tested	52
Frozen tissue section	Aldehyde-fixed frozen tissue section with use of AR treatment gives both excellent morphology and IHC staining	34, 35
DNA extraction from FFPE tissue sections	Boiling AR pre-treatment prior to DNA extraction gives improved results compared to enzyme treatment	53-56
RNA extraction from FFPE tissue sections	Heating AR treatment prior to RNA extraction gives improved results compared to enzyme treatment	57, 58
Protein extraction from FFPE tissue sections	AR pre-treatment with AR solution including 2% SDS and/or other chemicals improves efficiency of protein extraction from FFPE tissue compared to enzyme digestion. Combining with elevated hydrostatic pressure may increase extraction of up to 80-95% of proteins from FFPE tissue sections	59-62
Imaging mass spectrometry (IMS)	AR pre-treatment gives satisfactory results of IMS . Based on comparison among different AR solutions, Gustafsson et al summarized that citrate acid AR method is an important step in being able to fully analyze the proteome for FFPE tissue	36-38

AR = antigen retrieval; FFPE = formalin-fixed paraffin-embedded; IEM = immunoelectron microscopy; ISH = in situ hybridization; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling; FCM = flow cytometry; IMS = imaging mass spectrometry. (a) 10% fresh saturated solution of sodium ethoxide diluted with anhydrous ethanol for 2 min or with a saturated aqueous solution of sodium metaperiodate for 1 hour. Reprinted with permission from Shi SR, et al. J. Histochem. Cytochem. 59:13-32, 2011.

PETITIONER EXHIBIT 1012
Page 36 of 218

Chapter 3.4 | Application of AR Techniques – The Basic Principles

In addition to its use in IHC, AR has increasingly been adopted in the following related applications:

- In situ hybridization (ISH) and in situ end-labeling (TUNEL) of apoptotic cells in FFPE tissue sections; as well as in flow cytometry to achieve stronger positive signals while reducing non-specific background noise
- In IHC multi-stains, AR has been used to block the cross-reaction from the previous run (33)
- In addition to FFPE tissue sections, AR has been adopted for aldehyde-fixed fresh tissue sections, plastic-embedded tissue sections, cell smear samples for cytopathology, and floating vibratome sections (33)
- Modified AR methods have been used successfully for extraction of DNA and RNA from FFPE tissue sections for PCR-based methods and sequencing
- Imaging mass spectrometry (IMS) has been applied to proteins extracted from FFPE tissue sections by AR approaches, providing an avenue to fully analyze the proteome of archival FFPE tissue (36-38)

Chapter 3.5 | AR-IHC-based Research and Diagnostics

Over the past two decades AR has found extensive application, not only for IHC, but also for molecular methods applied to FFPE tissues, so called tissue proteomics, as well as standardization and quantification of IHC. For further details the reader is referred to the multi-author text edited by Shi and colleagues (6), which includes discussion of a proposal for quantitative IHC, based upon the use of AR. This hypothesis proposes to minimize the variation in IHC that is observed in clinical FFPE tissue sections, by using optimal antigen retrieval (AR) in a test battery approach. The intent is to use AR to reduce the loss of antigenicity observed for many proteins, following variable fixation, to a level comparable to frozen tissue sections, at which point a standard calibration curve could be developed using internal proteins. This approach is similar to that of enzyme-linked immunosorbent assays (ELISA) where a standard curve is used to convert the immunoreaction signal into a quantitative amount of protein (63).

Chapter 3.6 | Reagents and Protocols

Sections 3.6-3.12 will describe the following retrieval protocols:

- Water Bath Methods
  - Dako PT Link
  - Water Bath (conventional) Heating
- Pressure Cooker Heating
- Autoclave Heating
- Microwave Oven Heating
- Proteolytic Pre-treatment
- Combined Proteolytic Pre-treatment and Antigen Retrieval
- Combined Deparaffinization and Antigen Retrieval

The composition and the pH of retrieval buffers are crucial for optimal retrieval. Although citrate buffers of pH 6 are widely used retrieval solutions, high pH buffers have been shown to be widely applicable for many antibodies, as discussed previously (22, 64). It is the responsibility of the individual laboratory to determine which of the available buffers perform optimally for each antigen/antibody and then to use them consistently. Although 0.01 M citrate buffers of pH 6 have historically been the most widely used retrieval solutions, high pH buffers have started being implemented when showing improved end results for some antigens. The following protocol descriptions should serve as guidelines only. It is the responsibility of the individual laboratory to compare methods and select the optimal protocol for consistent use. It is recommended for the AR methods to control temperature settings and to measure the actual temperature at regular intervals. The following protocols focus mostly on Dako reagents and systems, with detailed input from Dako; other manufacturers supply similar reagents and protocols, which should be followed scrupulously.

Chapter 3.7 | Water Bath Methods

A. Dako PT Link

Dako PT Link instrument simplifies the water bath antigen retrieval process by performing automated retrieval using specified protocols, which incorporate preheat temperature, antigen retrieval temperature, and time as well as cool down settings. Typically, antigen retrieval is performed for 20 minutes at 97 °C.

PETITIONER EXHIBIT 1012
Page 37 of 218

Figure 3.2 Dako PT Link is a water bath method for antigen retrieval

Materials Required

- Dako PT Link*
- Dako Autostainer Slide Rack
- Retrieval solution
- FLEX IHC Microscope Slides or slides coated with other suitable adhesives
- Personal protective equipment

*Dako Omnis has onboard pre-treatment module. See User Manual for protocol.

Protocol

Wear chemical-protective gloves when handling parts immersed in any reagent used in PT Link.

1. Deparaffinize and rehydrate tissue sections.
2. Prepare a working solution of the selected target retrieval solution according to specifications.
3. Fill tanks with 1.5 L of desired target retrieval solution.
4. Place tank lids on tanks. Close and lock main lid with external latch.
5. See Operator's Manual for instrument set-up details:
   a. Recommended time is 20-40 minutes.
   b. Set antigen retrieval temperature to 97 °C.
   c. Set preheat temperature to 65 °C (allows up to 85 °C).
6. Press [RUN] button for each tank and the CYCLE will show PREHEAT. Allow solution to reach the selected preheat temperature.
7. Open the PT Link and immerse the Autostainer Slide Rack with deparaffinized tissue sections into the preheated target retrieval solution.*
8. Place tank lids on tanks. Close and lock main lid with external latch.
9. Press [RUN] button for each tank to start run. CYCLE will show WARM-UP and the lid lock will engage.
10. PT Link will warm up to preset temperature and then start the countdown clock for target retrieval cycle.
11. When target retrieval cycle is finished, CYCLE will show COOL. The COOL cycle is finished when temperature reaches Preheat SET temperature, even if Preheat is disabled.
12. When COOL cycle is finished, CYCLE will show DONE and lid will unlock automatically.
13. Open the PT Link and remove each slide rack with the slides from the PT Link Tank and immediately immerse slides into the PT Link Rinse Station containing diluted, room temperature Dako Wash Buffer (10x).
14. Leave slides in the diluted, room temperature Dako Wash Buffer for 1-5 minutes.
15. Proceed with IHC staining.

*As an alternative, a 3-in-1 solution can be used for both deparaffinization and target retrieval. See Section 3.13 I Combined Deparaffinization and Antigen Retrieval.

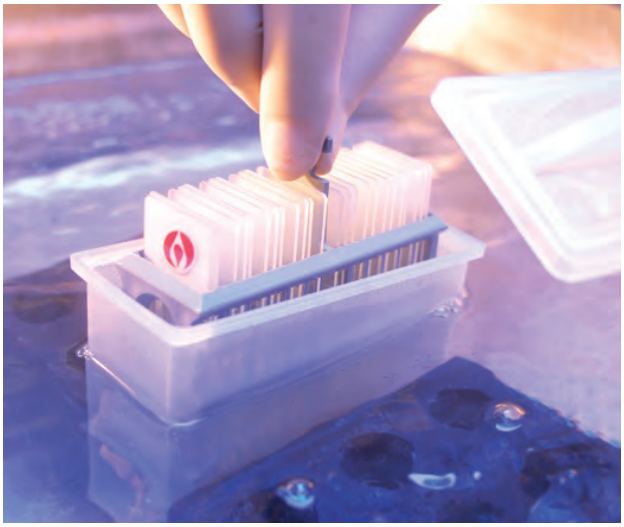

Figure 3.3 Antigen retrieval in conventional water bath.

PETITIONER EXHIBIT 1012
Page 38 of 218

B. Water Bath (conventional) Heating

One of several advantages of the water bath heating method is the ready availability of water baths in most clinical laboratories. Temperature settings just below the boiling point of water (95-99 °C) are most commonly used.

Materials Required

- Temperature-controlled water bath
- Slide rack
- Incubation container and cover
- Retrieval solution
- Tris-Buffered Saline
- Silanized Slides or slides coated with other suitable adhesives
- Thermometer
- Personal protective equipment

Protocol

It is recommended to wear insulated gloves when handling parts immersed in any reagent used in a water bath.

1. Deparaffinize and rehydrate tissue sections.
2. Fill container with enough retrieval solution to cover slides and equilibrate to 95-99 °C in water bath.
3. Immerse racked slides in preheated retrieval solution, cover container with lid, and incubate for specified time within the 20-40 minutes range after the set temperature has been reached.
4. Remove the container from the water bath and cool the contents with the lid in place for 20 minutes at room temperature.
5. Rinse with Tris-Buffered Saline (TBS) or distilled water at room temperature.
6. When removing the slides from the container it is very important that the slides do not dry out.
7. Transfer slides to TBS.
8. Proceed with IHC staining.

Chapter 3.8 | Pressure Cooker Heating

Pressure cookers set to approximately 103 kPa/15 psi will achieve a temperature of approximately 120 °C at full pressure. Alternatively, setting at 125 °C can be used for antigen retrieval. Stainless steel pressure cookers are recommended as the aluminum models are susceptible to corrosion by some retrieval solutions. As an alternative, individual plastic container(s) can be filled with retrieval solution and placed in reagent grade water in the pressure cooker pan.

Figure 3.4 Pressure cooker for antigen retrieval.

Materials Required

- Stainless steel pressure cooker, preferably electrically programmable
- Metal or plastic slide racks
- Retrieval solution
- Silanized Slides or slides coated with other suitable adhesives
- Tris-Buffered Saline
- Incubation container (optional)
- Personal protective equipment

Protocol

It is recommended to wear a safety face shield and insulated gloves.

1. Deparaffinize and rehydrate tissue sections.
2. Fill the pressure cooker with enough retrieval solution to cover slides. Alternatively, fill individual plastic container(s) with retrieval solution and add at least 500 mL of reagent grade water to pressure cooker chamber.

PETITIONER EXHIBIT 1012
Page 39 of 218

3. Bring contents to near boiling point, place racked slides into retrieval solution, seal the pressure cooker, and again bring the solution to a boil. For programmable pressure cookers, set target temperature and heating time, place racked slides in retrieval solution, seal the pressure cooker, and begin antigen retrieval procedure from room temperature.
4. Boil for 30 seconds to 5 minutes and allow the pressure cooker to cool for 20 minutes prior to opening. (Note: Vent any residual pressure before opening). Open programmable pressure cooker when antigen retrieval procedure is completed.
5. Transfer slides to room temperature Tris-Buffered Saline. When removing the slides from the container it is very important that the slides do not dry out.
6. Proceed with IHC staining procedure

Chapter 3.9 | Autoclave Heating

When set to 15 psi, an autoclave, like a pressure cooker, will achieve a temperature of about 120 °C at full pressure (65, 66).

Materials Required

- Bench top autoclave
- Plastic or metal slide rack
- Incubation container
- Retrieval solution
- Silanized Slides or slides coated with other suitable adhesives
- Tris-Buffered Saline
- Personal protective equipment

Protocol

It is recommended to wear safety face shield and insulated gloves.

1. Deparaffinize and rehydrate tissue sections.
2. Place slides in plastic or metal slide rack.
3. Fill the incubation container with enough retrieval buffer (typically 250 mL) to cover slides. Insert the slide rack and cover.
4. Place the container in the autoclave and follow Autoclave Manufacturer's Operating Instructions.
5. Set the temperature to 120 °C/15 psi and the time to 10-20 minutes. Start operation.
6. After venting pressure, open the lid and remove the slide container from the autoclave.
7. Rinse slides in Tris-Buffered Saline (TBS) or reagent grade water. When removing the slides from the container it is very important that the slides do not dry out.
8. Transfer slides to TBS.
9. Proceed with IHC staining procedure.

Chapter 3.10 | Microwave Oven Heating

Microwave ovens are very efficient for the heating of aqueous solutions, however, the standardization of procedures is important when used for antigen retrieval (and for the retrieval of DNA for in situ hybridization, i.e. target retrieval). In an effort to maintain consistency of AR protocols and to ensure reproducibility of staining results, the following elements should be standardized:

- Wattage of the microwave oven
- Presence of a turntable
- Volume of retrieval buffers per container
- Number of slides per container
- Number of containers

Materials Required

750-800 W microwave oven with turntable (please note that the effective power may decrease over time)

- Incubation container for microwave oven
- Plastic slide holder for microwave oven
- Retrieval solution
- Silanized Slides or slides coated with other suitable
- adhesives
- Tris-Buffered Saline
- Personal protective equipment

Protocol

Never use the microwave oven with metallic material present. It is recommended to wear insulated gloves when handling parts immersed in any reagent.

1. Deparaffinize and rehydrate sections.
2. Place slides in slide holder. Fill empty positions with blank slides.
3. Fill incubation container with enough retrieval solution to cover slides and insert slide holder.
4. Cover the container to minimize evaporation. Use a lid with minimal opening to avoid build-up of pressure and reduce evaporation.

PETITIONER EXHIBIT 1012
Page 40 of 218

5. Place container in the middle of the turntable and heat to near boiling point.
6. Incubate for fixed amount of time, typically 10 minutes.
7. Remove the container from the microwave oven, remove the lid, and allow to cool at room temperature for 15-20 minutes.
8. Rinse with distilled water.
9. Place in Tris-Buffered Saline.
10. Proceed with staining protocol.

Chapter 3.11 | Proteolytic Pre-treatment

As with other pre-treatment methods, procedures for proteolytic pre-treatment vary due to laboratory-specific differences in formalin fixation. Proteolytic pre-treatment must be optimized (dilution and time – specific elevated temperature may also be selected) according to the particular fixation process used in each laboratory. Examples of antigens most often treated with proteolytic enzymes include cytokeratins and immunoglobulins.

Materials Required

- Humidity chamber
- Silanized Slides or slides coated with other suitable adhesives
- Proteolytic Enzyme, Ready-to-Use
- Tris-Buffered Saline

Protocol

1. Deparaffinize and rehydrate tissue sections.
2. Place slides horizontally and apply enough enzyme working solution to cover tissue section(s), typically 200-300 µL.
3. Incubate for defined time, typically 5-15 minutes.
4. Stop enzymatic reaction by rinsing with distilled water or Tris-Buffered Saline.
5. It is recommended that enzyme digestion is included in the relevant Autostainer protocols. For the RTU series antibodies, enzyme digestion is included.

For Dako Proteolytic Enzymes, the following guidelines apply:

Proteinase K, Concentrated and Ready-to-Use:

Digestion for 6 minutes at room temperature is generally sufficient, but may be prolonged to 15 minutes.

Pepsin:

Digestion for 10 minutes at room temperature is generally sufficient, but may be prolonged to 15 minutes.

Proteolytic Enzyme, Ready-to-Use:

Digestion for 5-10 minutes at room temperature is sufficient. For details, please refer to the product specification sheets.

Chapter 3.12 | Combined Proteolytic Pre-treatment and Antigen Retrieval

Some antigens are more efficiently retrieved by a combination of heating and enzyme digestion, e.g. some cytokeratins and immunoglobulin light chains. The protocol below describes a method of first treating with Proteinase K and then AR by either water bath or microwave method.

Materials Required

- Silanized Slides or slides coated with other suitable adhesives
- Target Retrieval Solution, pH 6, Dako*
- Tris-Buffered Saline
- Tris-buffered NaCl Solution with Tween 20 (TBST), pH 7.6

*Other target retrieval solutions will work with a similar protocol optimized according to individual laboratory requirements.

Protocol

It is recommended to wear insulated gloves when handling parts immersed in any reagent.

1. Deparaffinize and rehydrate tissue sections.
2. Cover tissue sections with Proteinase K and incubate for 5-10 minutes.
3. Rinse with distilled water and place in Tris-Buffered Saline.
4. Proceed to antigen retrieval using either PT Link, another water bath or microwave method below.

AR – Water Bath

5. Fill container with enough retrieval solution (200 mL) to cover slides and equilibrate to 95-99 °C in water bath. Place the incubation container into the water bath and incubate for 20-40 minutes.
6. Remove the container from the water bath and cool the contents with the lid removed for 20 minutes at room temperature.

PETITIONER EXHIBIT 1012
Page 41 of 218

7. Rinse with Tris-Buffered Saline (TBS) or distilled water at room temperature.
8. Transfer slides to Tris-Buffered NaCl Solution with Tween 20 (TBST), pH 7.6 Wash Buffer.
9. Proceed with IHC staining.

AR – Microwave

5. Fill incubation container with enough retrieval solution (200 mL) to cover slides and insert slide holder. Insert slides in holder and cover.
6. Place the incubation container into microwave oven and incubate for 2 x 5 minutes.
7. In between steps 4 and 5, fill up the container with enough distilled water (50 mL) to cover slides.
8. After the second treatment, leave the sections in the retrieval solution at room temperature to cool for 15-20 minutes.
9. Rinse with distilled water.
10. Proceed with IHC staining.

Chapter 3.13 | Combined Deparaffinization and Antigen Retrieval

Combining deparaffinization and AR reduces slide handling time significantly and provides added convenience without sacrificing staining quality. Using Dako PT Link instrument simplifies the combined deparaffinization and target retrieval process by performing automated deparaffinization and retrieval in a single step.

Materials Required

- PT Link
- PT Link Rinse Station
- Silanized Slides or slides coated with other suitable adhesives
- Target Retrieval Solution, pH 9, 10x Concentrated, (3-in-1)*
- Dako Wash Buffer (10x)

*When used in PT Link for 3-in-1 specimen preparation procedure, the diluted deparaffinization / target retrieval solution can be used three times within a five day period, if stored at room temperature.

Table 3.4 Dako Products for Antigen Retrieval**

Product Name	Dako Code
Target Retrieval Solutions	
FLEX Target Retrieval Solution, High pH	K8004
FLEX Target Retrieval Solution, Low pH	K8005
Target Retrieval Solution, pH 6.1, 10x Concentrated	S1699
Target Retrieval Solution, pH 6.1, Ready-to-Use	S1700
Target Retrieval Solution, pH 9, 10x Concentrated	S2367
Target Retrieval Solution, pH 9, Ready-to-Use	S2368
Target Retrieval Solution, pH 9, 10x Concentrated, (3-in-1)	S2375
Proteolytic Enzymes	
Proteinase K, Concentrated	S3004
Proteinase K, Ready-to-Use	S3020
Pepsin	S3002
Proteolytic Enzyme, Ready-to-Use	S3007
Buffers	
Dako Wash Buffer (10x)	S3006
Tris-Buffered Saline	S3001
Tris-buffered NaCl Solution with Tween 20 (TBST), pH 7.6, 10x Concentrated	S3306
Instruments and Other Products	
Dako Omnis	GI100
Dako PT Link	PT100/PT101
PT Link Rinse Station	PT109
PT Link Tank	PT102
Dako Autostainer Slide Rack	S3704
FLEX IHC Microscope Slides	K8020
Silanized Slides	S3003

**Note that other manufacturers provide similar products; the user should bear in mind that commercial products generally are designed and tested to be used in the specified format, within a defined protocol, and specified instrumentation. Products are not freely interchangeable across detections systems, and any change from the recommended protocol requires complete revalidation.

PETITIONER EXHIBIT 1012
Page 42 of 218

Protocol

Wear chemical-protective gloves when handling parts immersed in any reagent used in PT Link. Recommended 3-in-1 specimen preparation procedure using PT Link and above target retrieval solution:

1. Prepare a working solution of the selected target retrieval solution according to the specifications.
2. Fill PT Link Tanks with sufficient quantity (1.5 L) of working solution to cover the tissue sections.
3. Set PT Link to preheat the solution to 65 °C.
4. Immerse the mounted, formalin-fixed, paraffin-embedded tissue sections into the preheated target retrieval solution (working solution) in PT Link Tanks and incubate for 20-40 minutes at 97 °C. The optimal incubation time should be de termined by the user.
5. Leave the sections to cool in PT Link to 65 °C.
6. Remove each Autostainer Slide Rack with the slides from the PT Link Tank and immediately dip slides into a jar/tank (PT Link Rinse Station) containing diluted, room temperature Dako Wash Buffer (10x).
7. Leave slides in the diluted, room temperature Wash Buffer for 1-5 minutes.
8. Place slides on an automated instrument and proceed with staining. The sections should not dry out during the treatment or during the immunohistochemical staining procedure.
9. After staining, it is recommended to perform dehydration, clearing and permanent mounting.

Chapter 3.14 | Conclusion

As discussed above, an effective AR protocol is based on the major factors that influence the effect of AR-IHC. Thus, for new antibodies, a test battery approach is recommended for establishing the optimal AR protocol for each antigen/antibody pair in FFPE tissue sections. Although citrate buffer of pH 6 is a widely used retrieval solution, high pH buffers have been shown to be widely applicable for many antibodies. It is the responsibility of the individual laboratory to determine which of the listed AR solutions perform optimally for each antigen/antibody pair. In an automated system a new antibody can be 'plugged' into an existing automated protocol, and run with whatever two or three choices of antigen retrieval are programmed for the instrument, with the appropriate AR recommended reagents. If satisfactory results are not obtained, it is advised then to revert to a test battery approach.

PETITIONER EXHIBIT 1012
Page 43 of 218

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PETITIONER EXHIBIT 1012
Page 44 of 218

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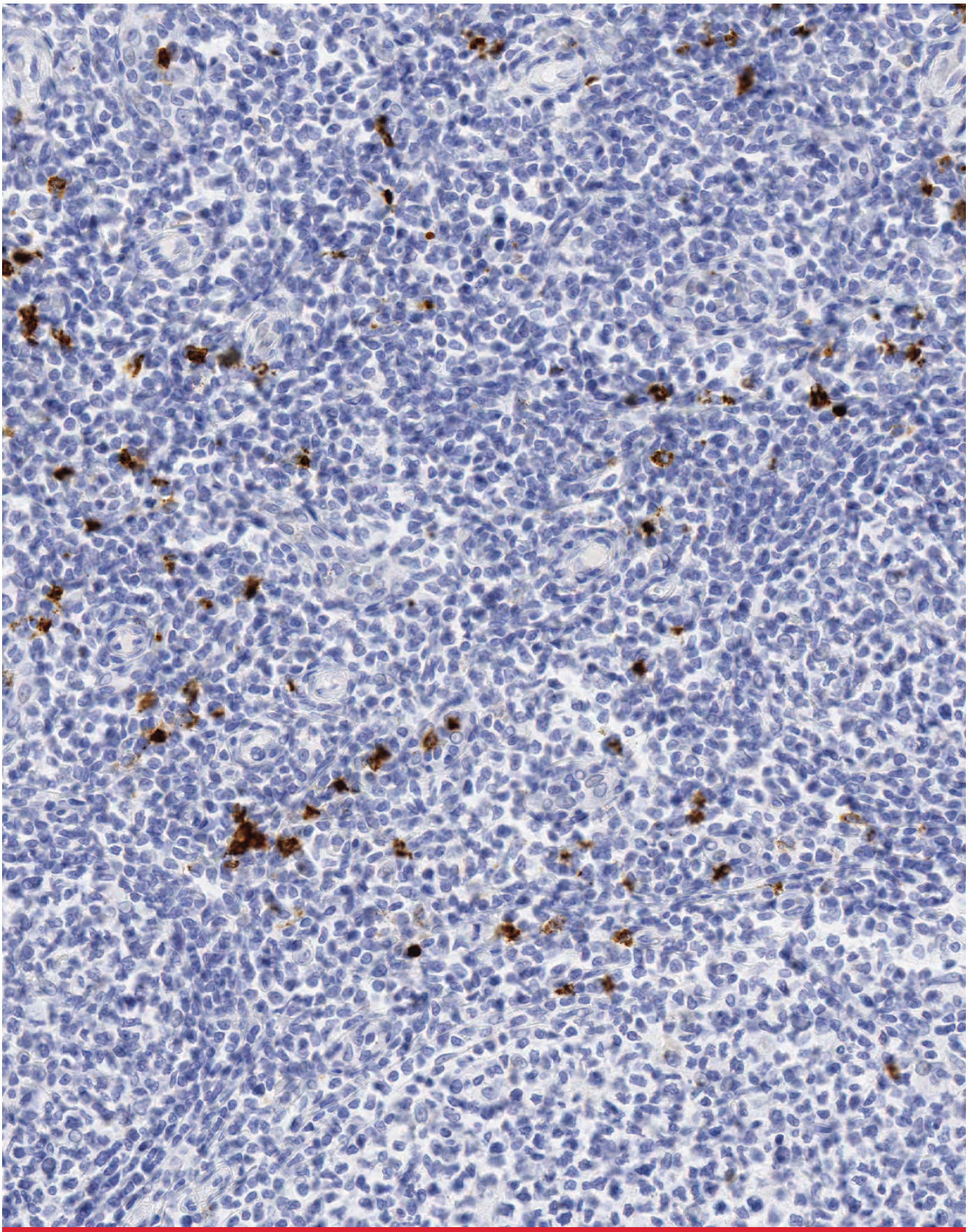
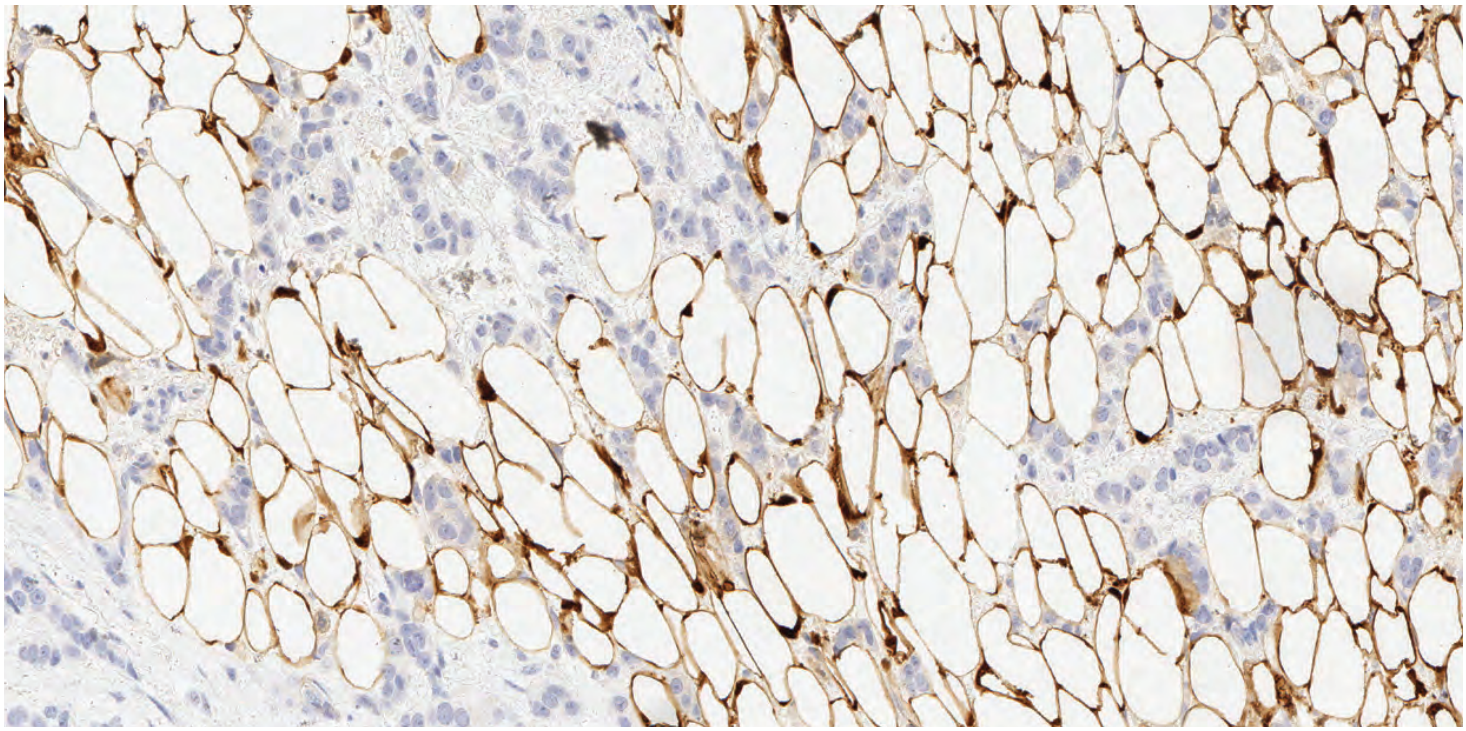
PETITIONER EXHIBIT 1012
Page 45 of 218



Chapter 4

Selection of the Primary Antibody

Søren Nielsen, Scheme Manager, NordiQC



An•ti•bod•y *(n.)*
Any of numerous proteins produced by B lymphocytes in response to the presence of specific foreign antigens, including microorganisms and toxins.

The American Heritage® Science Dictionary

Chapter 4.1 | Introduction

Immunohistochemistry (IHC) has now become an indispensable assay and is consistently performed in anatomic pathology in order to give a specific diagnosis and subclassification of neoplasms. IHC serves at present as a diagnostic, prognostic and predictive assay and the results contribute to the choice of treatment of patients in a clinical setting.

IHC is a complex assay, where the end result is influenced by multiple parameters in the pre-analytic phase, the analytic phase and the post-analytic phase (1). At least 4 million different protocols can be generated for one IHC analysis of one specific antigen (Figure 4.1, see also Chapter 1).

Depending on the selection and performance of these parameters, the final IHC result using the same primary antibody can show a range from negative to positive for the target antigen. In order to provide a robust and diagnostically useful IHC assay, it is important that the assay is based on a solid foundation, anchored by the most important factors influencing the assay. Special attention should be given to the following five parameters:

- The tissue fixation process
- The antigen retrieval method
- The primary antibody selection
- The detection system applied
- The choice of the tissue control material

This chapter will focus on the considerations to which users must pay special attention when selecting the right antibody for a given test.

Chapter 4.2 | Selection of the Proper Antibody

The selection of the primary antibody and the analytical conditions applied for the analyte have a significant impact on the IHC result, regarding the diagnostic sensitivity and specificity of the test.

Laboratories are consistently introduced to new antibodies from scientific publications and manufacturers, and in each situation the benefits of the new antibody must be carefully considered before implementation in a diagnostic setting.

First of all the diagnostic potential and application areas must be evaluated. For example, is the antibody labeling a new target, a supplementary antibody for a well-known target, a replacement for an existing antibody within a certain area, or a new antibody clone replacing an old clone, etc.? From a questionnaire performed by the College of American Pathologists and submitted to American laboratories, it was seen that typically 4-12 new markers were implemented annually in the 727 laboratories responding to the survey (2).

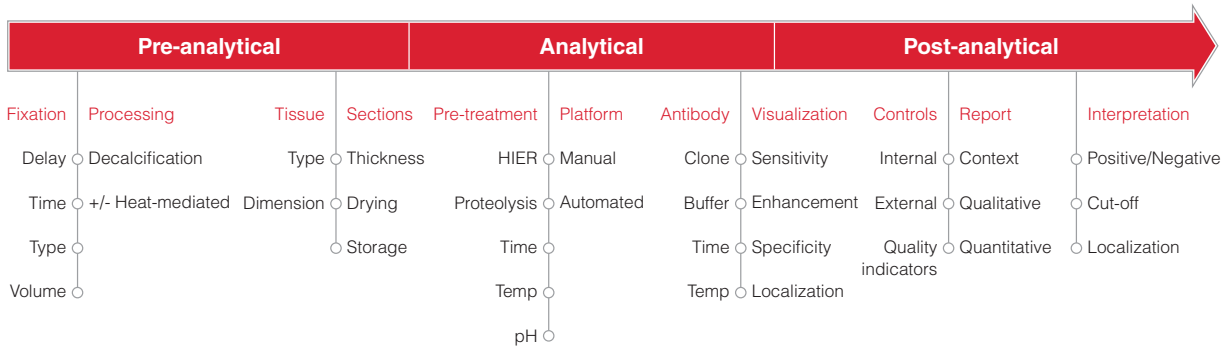


Figure 4.1 The multiple parameters influencing the IHC result.

PETITIONER EXHIBIT 1012
Page 48 of 218

Each and every new antibody introduced and implemented for diagnostic use in a laboratory must be technically optimized to identify the 'best practice' protocol that gives the highest signal-to-noise ratio. The antibody must subsequently be validated, providing data of the diagnostic sensitivity and specificity, using the protocol identified and selected (3). For new antibodies replacing old clones or existing markers, the laboratories may exploit data from established protocols and publications, facilitating the optimization and validation process, and supporting the final evaluation. For antibodies against new targets, both the technical optimization and the validation process can be much more challenging, especially if no benchmark data are available, and there is only limited information about positive and negative controls.

The ongoing development of new and improved antibodies, introduced to the laboratories by the manufacturers, is very valuable and contributes to the continuous enhancement and expansion of IHC. At the same time, the many choices for the selection of the primary antibody also contribute to a lack of standardization of IHC and require increased resources from laboratories to comply with the antibody optimization and validation process. The test process gives rise to an increased workload in the laboratory, and also requires a high level of technical expertise to interpret the tests performed.

At present more than 180 companies offer in excess of 350,000 primary antibodies for clinical and research use (4), a huge number which underlines the complex issue. For one target antigen more than 100 primary antibodies may be candidates for implementation.

For primary antibodies the sensitivity and specificity are the core elements. Ideally, the primary antibody must provide both a high sensitivity and a high specificity to produce an accurate and robust IHC assay.

Monoclonal antibodies have become widely used because of their high specificity, consistency, purity and commercial availability. Monoclonal antibodies, produced in mice according to the in vitro hybridoma method developed by Köhler (5), or by molecular engineering, contain a single 'species' of antibody molecule, where every antibody molecule is identical by idiotype, with a single specificity and single affinity. High specificity may occasionally be accompanied by a low affinity, consequently reducing the sensitivity of the antibody. Polyclonal antibodies, typically produced in rabbits by traditional immunization techniques, with booster immunizations to maximize the reactivity against the target antigen, frequently give a higher sensitivity (avidity) compared to monoclonal antibodies, as the many antibody 'species' present react with more antigen sites. Polyclonal antibodies may thus minimize the deleterious impact of tissue fixation and processing, thus providing a more robust assay. However, because more antigen sites are recognized by polyclonal antibodies, the risk of cross-reaction to other proteins is increased.

Recently, rabbit monoclonal antibodies have been introduced to IHC (6). Some scientists hold that these antibodies combine the high specificity of monoclonal antibodies, being generated by the hybridoma technique, with greater sensitivity that results from improved recognition of human antigens by the immune systems of rabbits. Certainly, some rabbit monoclonal antibodies have significantly improved the quality for the immunohistochemical demonstration of challenging antigens, such as cyclin D1 (7), estrogen receptor (8) and CDX2 (9), providing increased sensitivity and robustness, but also an analogous specificity comparable to the corresponding mouse monoclonal antibodies. The final choice whether to use a mouse monoclonal antibody, a rabbit monoclonal antibody, or a polyclonal antibody must be determined by the individual laboratory, as the final performance is highly dependent on many pre-analytical and analytical parameters in the total test.

In the IHC external quality program, NordiQC (www.nordiqc.org), it has been shown that for certain epitopes, many different antibodies, including both mouse monoclonals and rabbit polyclonals, may be used to obtain similar good staining results. For other epitopes the choice of the antibody is much more critical.

For prostate specific antigen (NordiQC, run 27, 2009), the selected IHC protocol could be based on many monoclonal and polyclonal antibodies to provide an optimal staining result, whereas for cyclin D1 (run 33, 2011) only the rabbit monoclonal antibodies, clones SP4 and EP12, could be used to generate an optimal result.

PETITIONER EXHIBIT 1012
Page 49 of 218

The choice of the primary antibody is highly dependent on the various steps of the general analytical protocol and the IHC stainer platform used by the laboratory. The chemical composition of the reagents, sequence of reagent application, and mechanical handling of the sections by the stainer platform, can have a deleterious effect on some antibodies, in particular for some mouse monoclonal antibodies. For example, the mouse monoclonal antibody clone 1F6 for CD4 may be adversely affected by the blocking step for endogenous peroxidase. If this step is performed by using a standard 3% hydrogen peroxidase solution after heat-induced epitope retrieval (HIER) and immediately before incubation with the primary antibody, the detection of antigen is significantly impaired. This protocol setting has typically been the backbone for many fully automated IHC stainer platforms and consequently this clone will not provide the desired staining result under these circumstances.

Due to the complexity of identifying the most appropriate primary antibody and the high demands of optimizing and validating the IHC protocols, many laboratories choose to apply ready-to-use (RTU) antibodies and RTU IHC systems as an alternative. It is also possible to purchase a total IHC system where the primary RTU antibody is calibrated, along with the detection system and a protocol which is optimized and validated by the manufacturer. The laboratories must still verify and validate the end results on their own tissue samples, but the optimization process to identify the best epitope retrieval method, antibody titre, choice of detection system etc., has been carried out by the manufacturer, an approach which can lead to a better standardization of IHC.

Chapter 4.3 | A New (Replacement) Antibody for an Old Marker

The continuous focus to develop and to optimize IHC frequently encourages laboratories to test and evaluate new antibodies, as replacements for existing and already implemented antibodies in their daily diagnostic practice. The incitements to test and compare data concerning a new antibody can be manifold and relate to technical, diagnostic or economic issues.

Examples of technical issues and problems of an existing antibody and reasons to replace this:

- An antibody from a mouse ascites harvest may give a positive staining reaction in human tissue with blood group A (10, 11) complicating the interpretation
- An antibody against a nuclear antigen may give an aberrant staining reaction in the membranous or cytoplasmic compartment, a reaction not anticipated from available data
- An antibody may be unstable in the diluted format, showing a significant loss of affinity after storage for a few days
- An antibody may require enzymatic proteolysis as the method of epitope retrieval, compromising the robustness of the protocol, because performance often is greatly influenced by fixation time in formalin (in contrast to protocols based on HIER, which is less influenced by the formalin fixation time) (12)

With the use of proper external and internal tissue controls the above mentioned issues and similar technical issues should not have a diagnostic impact. However, they do present a daily challenge and may warrant replacement with another antibody having superior performance characteristics.

Concerning practical utility in diagnosis, a new antibody may provide an improved sensitivity and/or specificity compared with the old antibody. Many antibodies have been commercially available for years or decades and still provide excellent staining results, whereas other 'troublesome' antibodies should be replaced when a new and improved antibody becomes available. The mouse monoclonal antibody cocktail AE1/AE3 against 'Pan-cytokeratin' and a rabbit polyclonal antibody against S100 are both examples of excellent antibodies, which have been commercially available for more than 20 years and yet still provide optimal IHC results (www.nordiqc.org). They have thus maintained their utility through the general transition and development of the IHC reagents and methods, including introduction of antigen retrieval/HIER and IHC automation.

Other antibodies with low specificity and/or sensitivity have remained for diagnostic use for a long time because no better alternatives were available. With new immunogens and improved antibody production methods many improved antibodies have become available, a fact that may be overlooked by labora-

PETITIONER EXHIBIT 1012
Page 50 of 218

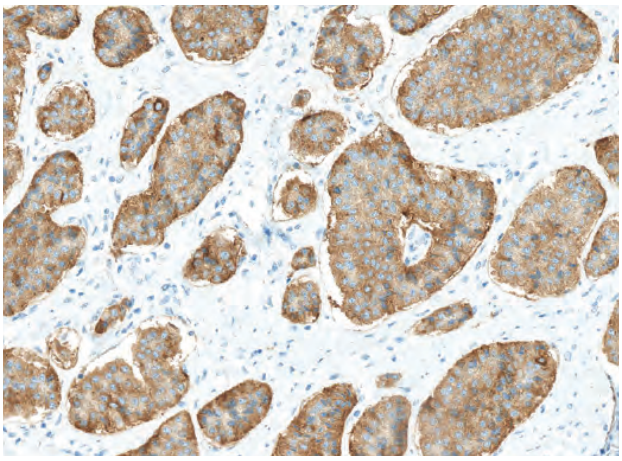


Figure 4.2 A) Intestinal neuroendocrine tumor. Staining for synaptophysin using the mouse monoclonal antibody DAK-SYNAP. Virtually all the neoplastic cells are distinctively demonstrated. The staining reactivity is as expected and confirms the neuroendocrine differentiation of the neoplasm.

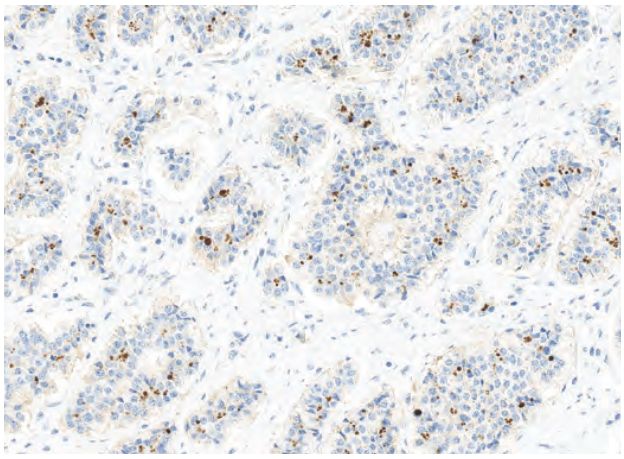


Figure 4.2 B) Intestinal neuroendocrine tumor. Staining for synaptophysin using the mouse monoclonal antibody clone SY38. An inferior staining pattern is seen compared to the result expected and obtained by new antibodies against Synaptophysin (Fig 4.2A). From a technical aspect, the antibody was optimized to give the best possible result, but still is inferior, underlining the low affinity of the clone SY38.

tories. The mAb clone SY38 against synaptophysin and the mAb clone 35βH11 against cytokeratin 8 were also introduced to the laboratories more than 20 years ago. However, when comparing the performance of these two antibodies with new antibodies, the data (www.nordiqc.org) clearly indicate that the new antibodies for these two target antigens provide superior staining performance and should replace the old ones (Figure 4.2 and Figure 4.4).

Due to the comprehensive workload and demands for technical expertise associated with antibody selection and protocol optimization, some laboratories hesitate to perform evaluations and comparisons of new antibodies (typically new clones) with existing antibodies, while other laboratories perform antibody evaluations regularly.

When a new antibody is tested and compared to an existing reagent, the laboratory must carefully design the frames and conditions of the test. The technical optimization process must be performed on material/tissue processed in the same way as the diagnostic material. Processing factors to include are fixative(s) used, time range in fixation, decalcification methods, if relevant, and dehydration conditions used in the laboratory. This means that if the laboratory uses fixatives other than formalin (e.g. B5) then tissues fixed with both reagents must be included to monitor the performance of both. It is essential that tissues fixed for different times are included in the technical optimization process, in order to evaluate the impact of the formalin fixation time. In a routine situation, the material sent to pathology departments may undergo an enormous range of fixation times, as much as 2-168 hours, with clear effects on the performance of some antibodies (see Chapter 2).

For the technical optimization process it is very valuable to perform the tests on tissue microarrays (TMA) composed of cores of different normal tissues. These should be processed and fixed for different times according to the routine and standard conditions used by the laboratory.

Using serial sections of a TMA as shown in Figure 4.3, identification of the protocol giving the best technical result is facilitated. The antibody can be applied in different concentrations e.g. 1/50, 1/200 and 1/800 as a starting point, and for all the titers selected the different epitope retrieval methods used by the laboratory can be tested (this method corresponds to the 'test battery' approach advocated by Shi and colleagues to deter-

PETITIONER EXHIBIT 1012
Page 51 of 218

mine optimal retrieval conditions – see Chapter 3). In this testing phase, the laboratory should also include the protocol settings recommended by the manufacturer of the primary antibody.

Figure 4.3 TMA for the technical optimization process of primary antibodies. All specimens are fixed in 10% neutral buffered formalin and processed accordingly to the SOP in the laboratory.

When the laboratory has identified the protocol giving the best technical result for the new antibody, this protocol and the currently used antibody should be tested simultaneously and validated on the same material. In this context, it is of high importance that different tissues/diseases/neoplasms with a wide range of expression levels of the target antigen are tested. Tissues with high level, low level, and no expression must be represented in order to evaluate both sensitivity and specificity. A key question is; how many samples are needed to secure a solid validation? No specific number can be pre-determined, as this will depend on the marker in question and its usage. If the marker is only used within a restricted diagnostic area, or the target antigen is rarely encountered, then the number might be less compared to a marker used extensively in a number of different areas. A minimum of 25 different samples are recommended for inclusion in the validation set (3), reflecting the range from no expression to high expression of the antigen of interest. In both the optimization and validation process it is highly recommended that normal tissues are included as these tissues in general will express a more consistent level of antigen when compared with neoplasms.

During this process, it is critical that the staining patterns, the distribution and sub-cellular localization of staining, the number of cells demonstrated and the staining intensity are evaluated for the antibodies being compared. In this evaluation phase, focus should be on the reasons for initiating the test; whether related to technical issues or diagnostic issues. It is also important that other observations are registered and taken into consideration. If the new antibody meets predetermined expectations, and functions well within the basic laboratory protocols, the antibody can be implemented. However, the replacement of an existing antibody that has a well-described and long history of performance will at least for a period compromise intra-laboratory standardization until more experience is gained with the new reagent.

When a new antibody has been implemented it is recommended to monitor the staining results and register any aberrant results on a regular basis. Communication with vendors and with other laboratories, in addition to studies of the literature describing the antibody in question, is crucial to gain reliable information of performance and interpretation of a new primary antibody.

Chapter 4.4 | A New Antibody for a New Marker in the Pathology Laboratory

IHC is an indispensable assay in the armamentarium of diagnostic tools for the pathologist, primarily to immunophenotype and classify the neoplasms concerning lineage or origin (such as carcinoma, melanoma, lymphoma etc.). Secondary IHC is used as a predictive and prognostic asset (e.g., in breast carcinoma to demonstrate and quantify hormone receptors). IHC is also rapidly finding application in theranostics/companion diagnostics where the therapy selected for the individual patient in part is based on the outcome of the IHC tests. A related use is for 'genomic IHC', where primary antibodies are used to identify specific proteins that are indicative for a genomic

PETITIONER EXHIBIT 1012
Page 52 of 218

abnormality or mutation. As a consequence of these novel demands and possibilities, many new markers are being introduced to IHC. Laboratories must be prepared to establish and validate these central and vital assays.

When setting up and evaluating an IHC marker as replacement for an existing marker, the laboratory has a set of benchmark data that facilitates interpretation of the comparative study (Section 4.2). For a brand new marker with no history and no benchmark data in the laboratory, the optimization and validation process is much more challenging and a number of additional considerations have to be taken into account. The clinical and diagnostic application of the new marker must be defined and relevant literature and publications must be carefully reviewed concerning both diagnostic potential and the IHC methodological parameters applied in the published studies.

If available, detailed data must be collected concerning the IHC staining results reported in the literature, with focus on the diagnostic sensitivity and specificity. The number, selection, and diversity of samples included in the reported studies all have an impact on the results obtained. For a laboratory considering implementation of an antibody to a new marker, which is described of value in a specific differential diagnosis, it is crucial to have information about the reactivity of the antibody in a broad range of clinically relevant samples. Descriptions of staining patterns, both for the sub-cellular (nuclear, cytoplasmic, membranous) and tissue distribution are essential. These data are used for the internal optimization and validation process, including the identification of positive and negative tissue controls. It is also central to search for guidelines or discussions concerning the 'cut-off' value or staining threshold, for interpretation and how to integrate and report the result in the final description to the clinicians. Additionally, focus must be centered on details of the IHC methods given in reported studies. First of all, the studies must be performed on samples processed identically, or in a manner similar to the conditions used by the laboratory. The IHC analytical parameters will have a significant impact on the staining results. For this reason, focus should be on three central issues:

- Choice of antigen retrieval method
- The selection of primary antibody
- IHC stainer platform adopted (or manual protocol if that is the choice)

These central topics must be thoroughly studied before the marker is validated against control tissues and finally implemented for clinical use.

When new diagnostic markers are introduced, the focus areas and utility of these markers are frequently directed towards very specific fields, and they highlight the diagnostic benefits and challenges within these fields. Additional studies must be performed to elucidate the utility of the marker both within in the initial areas described and in a broader perspective. It has often been shown that new emerging markers initially have been reported to be diagnostically very valuable, providing high diagnostic sensitivity and/or specificity for a certain target. However, subsequent testing by other groups may generate radically different results due to IHC methodological issues, including the choice of a different primary antibody or clone. In the review paper by Ordonez (13), it is shown that the first IHC studies published for PAX8 (paired box protein 8) reported B-cell lymphomas and neuroendocrine carcinomas as being positive, while later studies reported these neoplasms to be negative. The different conclusions and staining patterns were mainly related to the reactivity of the antibodies used in the studies. The reason appears to be that antibodies reacting with the N-terminal of the PAX8 antigen also show a cross reaction with PAX5 expressed by normal and neoplastic B cells, as the antigen site on the N-terminal is shared by all PAX family members. If antibodies directed towards the C-terminal of the PAX8 antigen are utilized, B cells and neuroendocrine carcinomas test negative.

Chapter 4.5 | A New Experiment in the Pathology Laboratory

Having focused on the issues and considerations related to the requirements for the test set-up of an antibody as replacement of an existing antibody, and how to implement a new marker, this section will describe the considerations of how to start the process of implementing a marker, for which there is only limited or no data in the literature. The focus will be on the pitfalls associated with this process.

As mentioned previously, benchmark data and antibody performance history are of high value when laboratories evalu-

PETITIONER EXHIBIT 1012
Page 53 of 218

ate new clones and markers to serve as replacements, or new tests in a well-described area. When a new undescribed marker is evaluated for possible incorporation as a laboratory test many other obstacles and methodological issues arise. Some of the challenges and questions are as follows:

- Which samples (tissues/cells) can be used as negative and positive controls?
- Does the antibody work on formalin-fixed, paraffin-embedded (FFPE) material?
- What is the expected sensitivity and specificity of the antibody?
- How should the staining results be interpreted?
- What is the reproducibility of the antibody and the test protocol?

In addition to the challenges listed, it is difficult to determine in which order to approach these questions. It is not possible to evaluate the overall performance of the marker on FFPE material or the impact of the pre-analytical conditions, if the proper negative and positive controls are not identified. In practice this usually means that the laboratory, or the researcher, will start the evaluation process on one set of materials and during the optimization and validation process they may have to go back and repeat some studies on other materials, as more knowledge about these selections is generated.

The identification of appropriate tissue samples suited to be included in the test set-up may be difficult for antibodies with no solid reference data concerning tissue expression. As an alternative, cell lines with known expression levels of the antigen can be the best, or only, type of material that is useful in the initial phase of the process. In order to evaluate how pre-analytical conditions will affect the affinity of the primary antibody, the cell lines can be processed by different methods e.g. adjusting the type and length of fixation etc., and FFPE cell line pellets with the characteristic of tissue processing standards can be generated.

It may be beneficial to test, side by side, more than one antibody/clone for the same epitope. For example, two or three antibodies from different vendors may be selected as candidates for optimization and validation tests. Vendors typically give information about the specificity of their antibodies by showing western blot (WB) panels and information about the functionality in different applications such as IHC, WB, immunoprecipitation (IP) and enzyme-linked immunosorbent assays (ELISA). Occasionally, they may also provide information on documented cross-reactivity in different species. If no information is listed about the functionality of the antibody for IHC on FFPE material, this does not mean that the antibody will not work on this type of material, but the laboratory must perform studies to confirm (or disprove) effectiveness. For these purposes antibodies showing narrow bands for the molecular weight of the expected target antigen by WB should be preferred; in most instances such antibodies will provide a higher signal-to-noise ratio in the IHC assay. In this context, it has been shown that only about 50% of antibodies that are effective in other assays, will function in IHC (4).

Cell lines can also be used as control material in the initial test phase for antibodies that purport to demonstrate proteins generated by gene alterations, such as translocation proteins (anaplastic lymphoma kinase (ALK) in lung carcinoma and anaplastic large cell lymphoma), or fusion proteins (ASPL-TFE3 in renal papillary carcinoma), or other proteins not expressed in normal adult tissues, such as fetal transcription factors (myogenin). The cell lines can be used as checkpoints to evaluate the affinity and functionality of the antibody for the target antigen, as expressed in the cell lines processed under selected conditions. Subsequently, the antibody must be evaluated on TMAs with a wide range of normal and neoplastic samples, potentially showing the antigen at different expression levels ranging from negative to high. The complexity of the implementation and optimization process of an antibody against a protein not expressed by normal cells is reflected in the set-up for the ALK translocation protein. No normal cells express this protein and in the neoplasms a high-level of expression is typically seen in anaplastic large T-cell lymphomas, whereas lung adenocarcinomas only express low-levels of ALK protein (14). To ensure the development of a protocol demonstrating ALK in a wide range of tissues and different tumor types, the laboratory must have access to samples with this range of antigen expression to perform a test of the final protocol.

When testing a new antibody with no or only limited history, it is also important to establish data for the reproducibility of the antibody test. For the individual laboratory this includes inter-run reproducibility, using same reagents, protocol, tissue, and inter-lot

PETITIONER EXHIBIT 1012
Page 54 of 218

reproducibility to see if the antibody provides identical results when different lots and aliquots of the antibody are used.

It is also necessary to evaluate the specificity of a new antibody. This aspect is described in depth in section 4.2, and only supplemental considerations are listed here. A standard method is to employ absorption by blocking peptides. Identical peptide sequences, to those used to generate the antibody, are incubated with the antibody in great excess. The antibody with and without the blocking peptide is then applied to two serial sections of tissue samples expressing the target of interest. If the antibody is specific, the addition of the blocking peptide will result in loss or at least a major reduction of staining on the tissue sample, compared to the positive result obtained by the antibody without blocking reagent. This test documents the specificity of the antibody to the immunogen, but it does not test for any staining in 'off-target' (cross reactive) sites, and aberrant (unexpected) staining may still occur. Negative cell lines and negative tissue controls are essential and must be included in the evaluation of antibody specificity.

New initiatives from research groups have generated publicly available tools that facilitate the validation of new markers. Antibodypedia (www.antibodypedia.com) is a searchable database of antibodies against human proteins. It aims to provide the research community and scientists with information on the effectiveness of specific antibodies in specific applications, including IHC. The database is generated by academic groups submitting their validation data, and by information from the commercial providers of the antibodies.

Another resource is the Human Protein Atlas project (www.proteinatlas.org) where information on IHC-based protein expression profiles are available for a large number of normal human tissues, cancers and cell lines. For the majority of proteins the sub-cellular distribution and transcription expression levels in cell lines are also available.

Chapter 4.6 | Examples of Good and Poor Antibodies

The selection and choice of the primary antibody will have a significant impact on the IHC result. Consequently, this choice can influence the final diagnosis and management of patients in a clinical setting, which underlines the importance of using high quality antibodies in clinical laboratories. As described, the overall goal is to use antibodies that are specific, sensitive, robust (not influenced by pre-analytical parameters) and reproducible (inter- and intra-laboratory), as these prerequisites are the foundation for standardization in the field of IHC. The standardization of IHC is, in fact, compromised by the large number of reagents, including primary antibodies, that are available from the many manufacturers. It can be difficult, or virtually impossible, for the individual laboratory to consistently select and use the optimal antibodies in their total IHC armamentarium. As mentioned in the previous sections, new antibodies are continuously being introduced to the market and a balance must be found for replacement of existing antibodies in current use. From a practical point or view, the skills and expertise necessary to conduct optimization and validation studies are considerable. In consequence, laboratories might refrain from performing complete validation due to lack of resources, therefore they continue to use already implemented antibodies, even though they may produce inadequate staining results.

When some laboratories rapidly integrate new antibodies, and especially new clones for the same target antigen, while other laboratories continue to use the old well-established antibodies, huge differences in the IHC results can be obtained by the two scenarios. The differences can be related to all the core issues of specificity, sensitivity, robustness and reproducibility. Some antibodies were initially designed and brought to the market for one target area but later studies may have revealed other possible areas of use. This aspect, in combination with the continuous optimization of IHC technical methods, with more efficient antigen retrieval methods, and detection systems having improved sensitivity, continues to expose previously undescribed reaction patterns for many antibodies.

One of the first antibodies against cytokeratin, high molecular weight (CK-HMW), was the mouse monoclonal antibody clone 34βE12. It was introduced for IHC in FFPE material in 1982 (15) and primarily used to demonstrate the CK-HMW subtypes 1, 5, 10 & 14. After 30 years, this antibody is still the most widely used marker for CK-HMW, and diagnostic utility has been documented in a remarkably high number of

PETITIONER EXHIBIT 1012
Page 55 of 218

publications. In particular, it has been shown to be valuable for the demonstration of CK-HMW in basal cells of prostate glands for the differential diagnosis of non-invasiveness and invasiveness. The demonstration of CK-HMW is now also performed in breast pathology to identify the basal cell like subtype of breast carcinoma and to differentiate this entity from other subtypes. The specificity of the primary antibody for this different use is critical. It has been shown that the mAb clone 34βE12 is less specific for this diagnostic context, compared with newly introduced antibodies to CK-HMW (run 16 and 38, www.nordiqc.org); a cross reaction with a cytokeratin low molecular weight is seen. Consequently, the mAb clone 34βE12 cannot be recommended to be used as a general antibody for CK-HMW in breast (see Figure 4.4A and 4.4B).

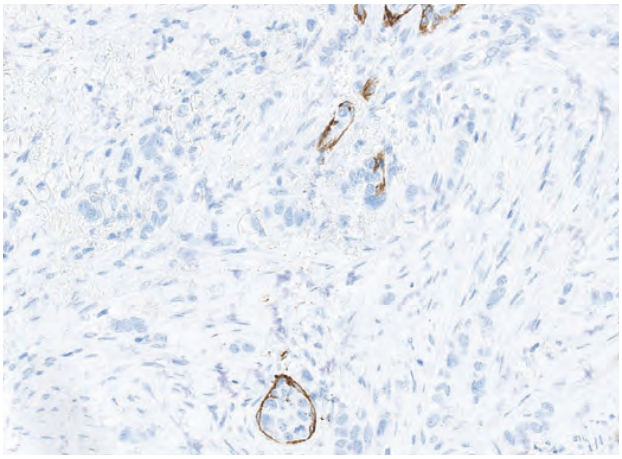


Figure 4.4 A) Breast ductal carcinoma. Staining for CK-HMW using the mouse monoclonal antibody clone D5/16B. The myoepithelial cells decorating the carcinoma in situ component show a cytoplasmic staining reaction, while the infiltrating tumor cells are negative.

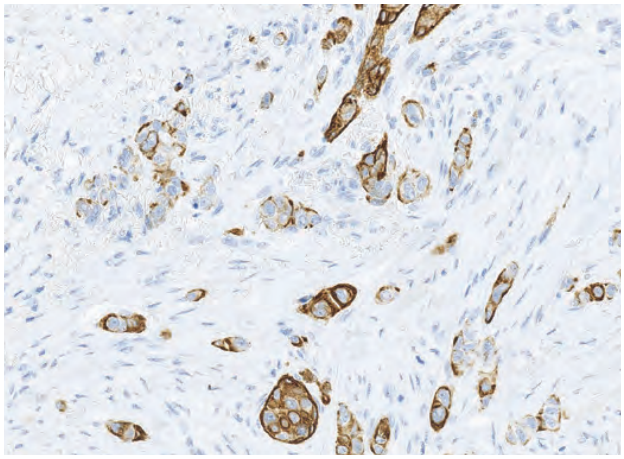


Figure 4.4 B) Breast ductal carcinoma. Staining for CK-HMW using the mouse monoclonal antibody clone 34βE12. The infiltrating tumor cells show a positive staining reaction due to a cross reaction of the primary antibody to an unidentified target antigen.

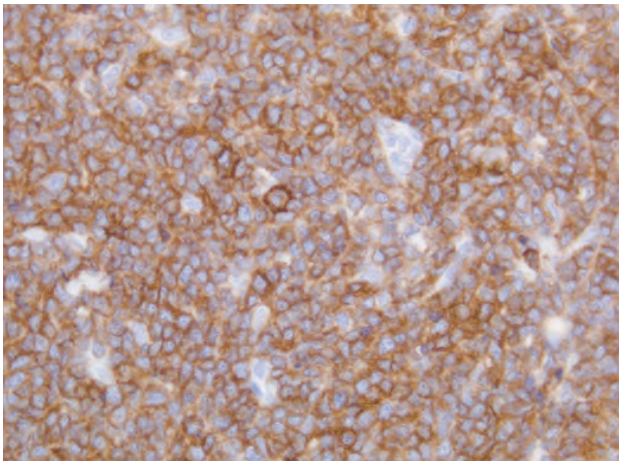


Figure 4.5 A) Mantle cell lymphoma. Staining for CD5 using the rabbit monoclonal antibody clone SP19. Virtually all the neoplastic B cells show a moderate staining reaction. In the centre a normal T cells show a strong staining reaction.

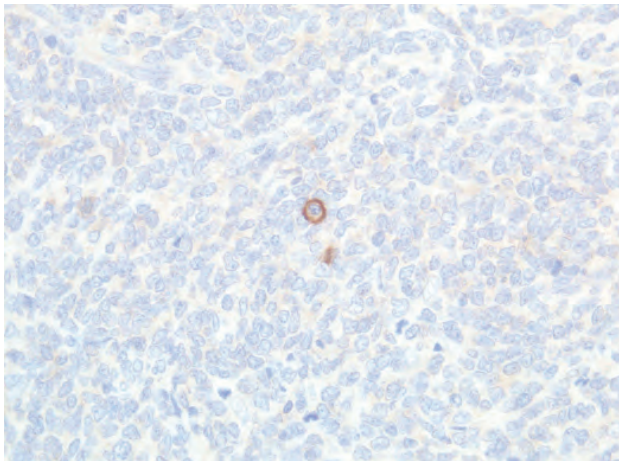


Figure 4.5 B) Mantle cell lymphoma. Staining for CD5 using the mouse monoclonal antibody clone CD5/54/F6. Only the normal T cells are demonstrated, while all the neoplastic B cells are false negative.

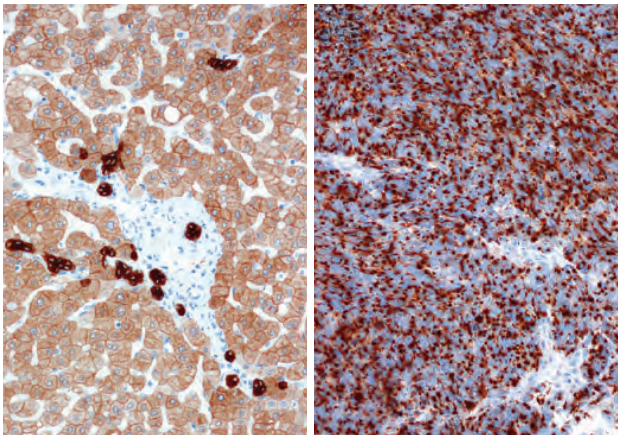
PETITIONER EXHIBIT 1012
Page 56 of 218

For cells with low expression of a target antigen, the central issue in a diagnostic setting is to provide high sensitivity. In the immunophenotyping of small B-cell lymphomas the demonstration of CD5 is important. The neoplastic B cells will typically only express limited amounts of CD5, compared with the high-level CD5 expression in any admixed normal Tcells. It is therefore mandatory to visualize the entire range of expression of the target antigen in the tissue sample tested. Low affinity antibodies may show an acceptable IHC staining result in cells with high levels of the target antigen (T cells), but inferior performance in cells with low levels (neoplastic B cells). This observation further emphasizes the need to optimize and validate the total IHC protocol on tissue with both high- and low-level antigen expressions.

Many CD5 antibodies are commercially available and a huge difference regarding the effective sensitivity is seen for the most commonly used antibodies. Using optimized protocols, the mouse monoclonal antibody clone CD5/54/F6 detects CD5 in high-level expression sites but not in low-level expression (see Figure 4.5A and 4.5B). The mouse and rabbit monoclonal antibodies clone 4C7 and SP19, respectively, detect CD5 in both low- and high-level expression sites.

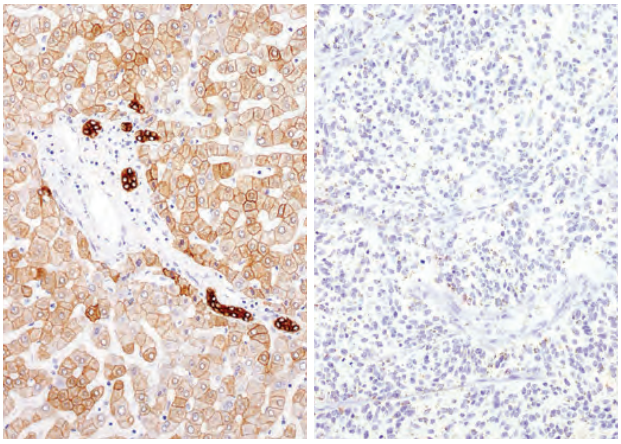
The selected antibody must also provide high robustness and give a consistent and reliable result only minimally influenced by the variations in the tissue handling process, and other fluctuations inevitably seen in an IHC analysis. Antibodies that require heat-induced antigen retrieval (AR or HIER – Chapter 3) should be preferred to antibodies that require enzymatic pre-treatment, as HIER reduces the impact of variations in formalin fixation time compared with enzymatic pre-treatment. Enzymatic pre-treatment must be adjusted to the duration of formalin fixation to provide a consistent IHC result, which can be difficult to accomplish in a routine setting, where the tissue samples inevitably show a wide variation in the fixation time. When comparing the data sheets of the two mouse monoclonal antibodies against broad spectrum cytokeratin, clones AE1/AE3 and MNF116, both react with the most relevant subtypes of cytokeratin and can be used on FFPE material. However, the IHC protocol for the clone MNF116 must be based on enzymatic pre-treatment, reducing the robustness of the IHC assay compared with the IHC assay using clone AE1/AE3 (see Figure 4.6A and 4.6B).

A



Liver. Small cell lung carcinoma.

B



Liver. Small cell lung carcinoma.

Figure 4.6 A) Staining for broad spectrum cytokeratin using the mouse monoclonal antibody clones AE1/AE3. The IHC protocol is based on HIER. In the liver sample hepatocytes and the bile epithelial cells are demonstrated and in the small cell lung carcinoma, all the neoplastic cells are distinctively demonstrated. A consistent and optimal staining reaction is seen in both samples tested using the same IHC protocol. **B)** Staining for broad spectrum cytokeratin using the mouse monoclonal antibody clone MNF116. The IHC protocol is based on enzymatic pre-treatment. The staining reaction in the liver sample is as expected and comparable to the staining obtained by the clone AE1/AE3. As the hepatocytes are distinctively demonstrated, the staining result is optimal according to e.g. NordiQC criteria. The staining of the small cell lung carcinoma is inadequate, as only few cells are demonstrated. The IHC protocol based on enzymatic pre-treatment is in this scenario less robust as the sensitivity is significantly reduced in the lung sample and the protocol must be adjusted for the individual samples tested to provide an optimal result.

PETITIONER EXHIBIT 1012
Page 57 of 218

Chapter 4.7 | Current and Future Challenges in Pathology Laboratories

It has been recognized in several publications that standardization of IHC is fundamental for reproducible and reliable results. In order to fully exploit IHC in a diagnostic setting, consistency must be achieved both in the individual laboratory and in between different laboratories. Because "IHC is technically complex, and no aspect of this complexity can be ignored, from the moment of collecting the specimen to issuance of the final report" (1), attention must be given to all variables influencing the result. This chapter has mainly focused on the selection of the primary antibody and the challenges associated. Due to the many pitfalls and extensive requirements for laboratories to accomplish the optimization and validation process, implementation of RTU antibodies has expanded in the field of diagnostic IHC. RTU antibodies are typically accompanied by specific protocols and guidelines concerning the choice of reagents and the tissue controls to be used in conjunction with the RTU product, thereby facilitating implementation in the laboratory for clinical use. For optimal performance, a RTU antibody must be used within a total IHC system, where the product is used strictly according to the guidelines given by the vendor. It is important to stress that if a RTU antibody is used with a modified protocol, then a full optimization process must be performed by the laboratory.

The class II and III IVD tests, such as HercepTest™ and PATHWAY® for HER2 demonstration in breast cancer, have for years been used by the laboratories as RTU systems, and have proven to be superior to laboratory developed tests (LDT). A general transition to convert class I antibodies from LTD to RTU is now an ongoing process. Related to the standardization of diagnostic IHC, RTU systems from the vendors should be aligned to give comparable results for a specific target antigen, irrespective of different reagents, methods and IHC automated platforms being used by the individual vendors of the RTU systems. In principle, the exact methodology applied to generate the final IHC result is of minor importance, providing that the result is concordant to the expected, regarding the overall sensitivity and specificity of the test. The combination of reagents and procedural parameters that generates the best IHC result is often unique to each individual IHC automated system. Typically, the different IHC systems provide different sensitivity levels depending on, e.g. the detection systems and HIER settings that are used. For the RTU antibodies in general, the vendors can adjust the concentration and/or incubation time of the primary antibody to regulate the results of IHC tests, such that the end results on their different stainer systems will be closely comparable.

Transition from manual IHC and LDT to fully automated IHC, using RTU antibodies in closed IHC stainers (see chapter 9), with vendor-determined preset analytical parameters, is essential to standardize the field of IHC. In general, overall IHC performance will be improved as a result of higher reproducibility, and the use of high-quality and state-of-the art reagents being offered by the vendors. However, it is recognized that for some antibodies performance might be impaired as preset analytical conditions and reagents selected for the IHC stainer system may fail to optimize performance of certain antibodies under certain conditions of processing and fixation. The mAb clones 1F6 for CD4 and PG-B6 for BCL-6, are examples of antibodies that are adversely impacted by some aspects of reagent composition and by the protocol used to quench endogenous peroxidase. If the quenching step is performed after HIER and before the incubation with the primary antibody, and by the use of 3% hydrogen peroxide (a standard setting in many fully automated IHC systems) then the affinity of these two antibodies is significantly compromised and the IHC result is unreliable. For other markers, different staining patterns can be seen when applied on different IHC stainer platforms, even though comparable protocols regarding sensitivity levels, antigen retrieval conditions and detection reagents are being used (see Figure 4.7A and 4.7B). Such differences may be the result of several small differences, not having a significant impact in isolation, but in combination antibody binding to target antigen is impaired.

In these circumstances, alternative antibodies to the same target might resolve the issue. Laboratories changing from manual to automated IHC, or making a change from one IHC stainer platform to another must take such parameters into consideration and should monitor and manage any differences observed.

PETITIONER EXHIBIT 1012
Page 58 of 218

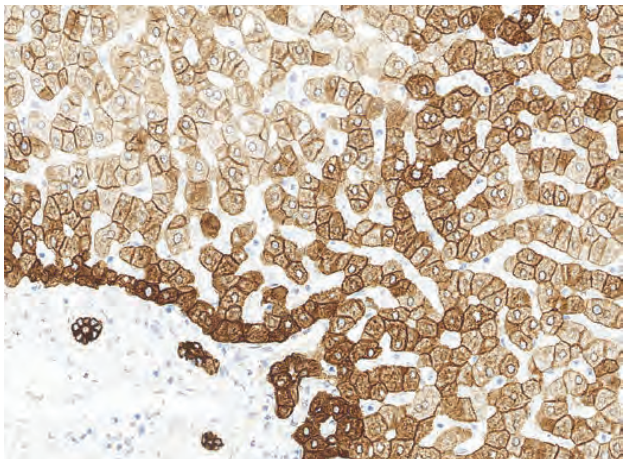


Figure 4.7 A) Liver. Staining for low molecular weight cytokeratin using the mouse monoclonal antibody clone 5D3. The IHC protocol is performed on a semi-automated IHC stainer from vendor A using the reagents and protocol settings linked to the system. All hepatocytes and bile duct epithelial cells are demonstrated as expected.

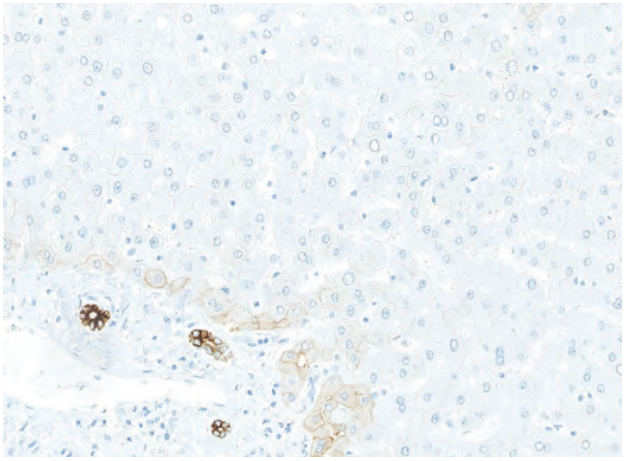


Figure 4.7 B) Liver. Staining for low molecular weight cytokeratin using the same mouse monoclonal antibody clone 5D3. The IHC protocol is performed on a fully-automated IHC stainer from vendor B using the reagents and protocol settings linked to the system. The bile duct epithelial cells are demonstrated, whereas the hepatocytes are negative.

From a practical point of view, and in relation to issues as seen for the endogenous peroxidase blocking step, it is important that the 'backbone' of the closed RTU stainer system still has some flexibility and is modular. Adjusting the protocol should be the rare exception for a 'difficult' antibody. Other antibodies and markers should first be explored. If no other marker is available, the laboratory must then be able to modify the manufacturer's preset protocol settings to establish a modified antibody-directed protocol to give the desired staining result. If this approach fails, the particular test may be performed manually or by another stainer system.

Typically, RTU systems will support standardization of IHC, but occasionally it can be impossible to develop a single test protocol that is applicable to all tissue samples presented to the laboratory, for all diagnostic purposes. Some antigens show such a wide range of expression levels that one protocol will not fit all diagnostic applications. As an example, the demonstration of immunoglobulin kappa (IgK) light chains can be based on use of the same primary antibody, for quite different purposes, e.g. visualization of membranous IgK located on lymphocytes, intra-cytoplasmic IgK in plasma cells, and extracellular IgK deposits in autoimmune diseases. However, for these different purposes the primary antibody concentration and protocol must be optimized separately for each of the three areas. Using a protocol optimized for surface IgK on lymphocytes produces a much too intense and un-interpretable result for demonstration of plasma cells in bone marrow samples.

No vendor can offer all primary antibodies as RTU, and RTU systems from different vendors frequently give different IHC staining results. This underlines the need for some degree of flexibility and modularity retained in the IHC stainer systems to be used judiciously by the laboratories. At present, there are no uniform guidelines concerning the staining requirements and no descriptions for the expected levels and staining patterns of the vast majority of immunoreactions. Dako Atlas of Stains gives examples, but the lack of a complete library with recommended tissue controls complicates the development of properly calibrated RTU systems, as well as the final validation and implementation of the markers cross all laboratories.

PETITIONER EXHIBIT 1012
Page 59 of 218

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PETITIONER EXHIBIT 1012
Page 60 of 218

Chapter 5

Staining Protocol Optimization

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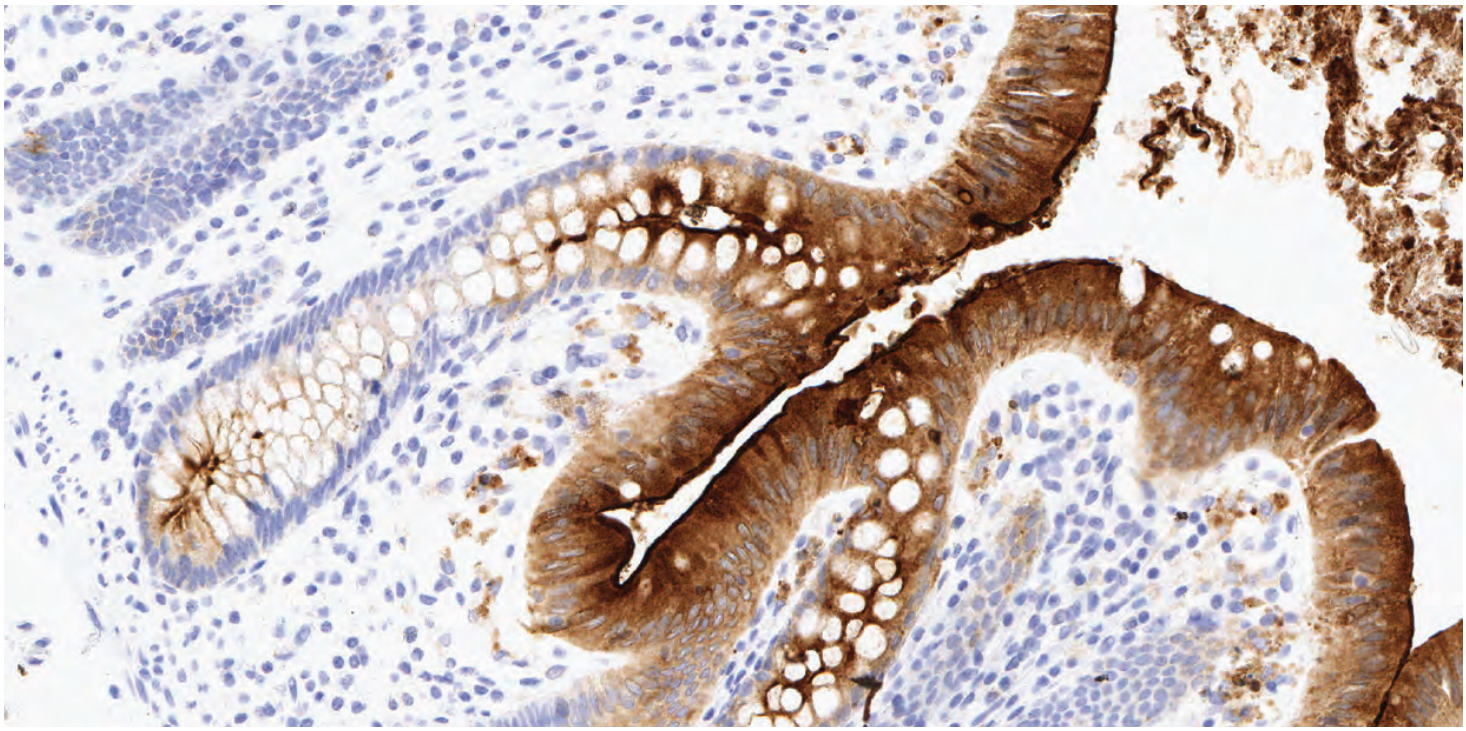

Pro•to•col *(n.)*
The plan for a course of medical treatment or for a scientific experiment.
The American Heritage® Medical Dictionary

Chapter 5.1 | Introduction

Ever since the first use of immunohistochemistry (IHC) for the diagnosis of cancer, reproducibility has been a key concern. Lack of reproducibility, both inter- and intra-laboratory, shows the need for improved standardization, yet many attempts to improve the quality of IHC staining worldwide are limited by two main factors; pre-analytical and analytical factors in the form of the quality of IHC reagents and the staining protocols used.

Diagnostic IHC tests can be divided in two main types; those that are qualitative and those that are semi-quantitative. The majority of IHC tests are qualitative, where the resulting stain is interpreted only as positive or negative. These types of stains can to some extent involve quantitation in the form of a cut-off point or threshold for positivity (e.g. >10% stained cells is indicative of a positive result). Interpretation of the qualitative IHC tests is focused on the correct cellular localization of the staining reaction and staining of the correct tissue structures. Appropriate positive and negative controls are pivotal for optimization of these tests.

Semi-quantitative IHC tests are interpreted according to an arbitrary scoring range (e.g. from 0 to 3+) that reflects antigen expression by means of the staining intensity and distribution, as well as the percentage of positive cells. Ideally, the semi-quantitative tests are optimized and calibrated against reference control materials, having known levels of expression of the target antigen. This way the staining result of a patient sample can be fitted into the scoring range.

This chapter focuses on the optimization of qualitative IHC tests, where the purpose of the IHC protocol is to ensure that the IHC staining unambiguously determines if the antigen is, or is not, expressed in the tissue. Optimal staining protocols are fundamental to reduce the risk of false negative results, while at the same time not introducing a false positive result. An optimized protocol for a qualitative IHC test should thus not aim to accurately reflect the expression level (quantitative) of the antigen in the tissue, but should ensure that the staining accurately reflects whether or not the antigen is expressed at all. In a recent survey of 727 American pathology laboratories, it was reported that one out of three laboratories does not have written validation procedures for introducing new, non-predictive antibodies. The survey also showed that 14% of the laboratories did not validate the performance of the most recently introduced IHC test. Of those laboratories that did validate, 75% used fewer than 21 cases, and 42% did not include weak or focally positive cases (1).

The survey points to the unclear definitions of the terms validation and verification as one reason for the relatively high percentage of labs that do not validate new IHC assays. The FDA definition of validation is “confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use can be consistently fulfilled” whereas verification is “a study used to determine whether a test system meets specifications”. CAP guidelines (2) recommend the use of 10 positive and 10 negative neoplasm samples for previously well-described antibodies, but do not specify the need to include weak or focally positive samples. Either way, there seems to be a lack of testing (verification and/or validation) of new assays to ensure optimal staining; not only when introducing new antibodies, but also when changing fixative, antigen retrieval, detection system, and even instrumentation.

Data from NordiQC EQA schemes also suggest that the concept of optimal staining protocols is not implemented throughout pathology laboratories. Accumulated assessment run data show that ~30% of all stains assessed by NordiQC are scored as insufficient, with some of the reasons being new antibodies, new techniques and new platforms. A weak or false negative stain accounts for 85-90% of the insufficient stains (3).

This chapter will describe the requirements for an optimal staining protocol with focus on signal transfer and signal generation in each step from antigen retrieval to visual chromogenic deposition. The chapter is based on the research done for Dako’s FLEX Ready-to-Use solution and automation, on both Dako Omnis and Autostainer Link instruments. Other vendors may have different approaches to protocol optimization.

PETITIONER EXHIBIT 1012
Page 62 of 218

Chapter 5.2 | The Basis for an Optimal Staining Protocol

An optimal staining protocol is characterized by two main properties:

- Robustness
- Correctness

Robustness

Robustness is the protocol's ability to minimize variation in the staining result by 'absorbing' variations related to the pre-analytical factors, including the degradation and recovery of the antigen (Chapter 2 and Chapter 3), as well as the analytical factors, by ensuring optimal signal transfer in the staining reactions. As illustrated in Figure 5.1, the pre-analytical variations in tissue handling (red-colored bar) are carried through the analytical steps (grey-colored bars) adding to the total variation. When non-optimized analytical protocols are used, the total variation becomes even greater. Optimal signal transfer reactions in the analytical steps ensure that all available targets in the tissue are detected and thereby all contribute to the visual signal. This optimized staining reduces the contribution from analytical variation, thus increasing the robustness of the IHC test.

Correctness

Correctness relates to the protocol's ability to correctly visualize the antigen in the tissue, at both low and high expression levels. A correct representation of antigen expression in a qualitative IHC test is not a linear representation of staining intensity as a function of antigen expression levels. Correctness in a qualitative IHC test depends on whether the antigen is truly expressed or not, thus balancing on the fine line of specific signal versus noise.

Optimal Signal Transfer

It is essential that the visualization of the antigen is performed with little or no signal loss, meaning that all antigen/antibody complexes are converted into a signal. A signal becomes visible when enough antigen/antibody complexes are converted into a chromogenic deposit at a single site in the tissue. By selecting incubations that result in visually saturated signals, the varying factors such as time and efficiency are not transferred into the visual signal.

Each step in an IHC protocol is necessary for the conversion of signal from the antibody/antigen/enzyme complex into a chromogenic deposit that the human eye can see. Each active step (not including wash/blocking steps) in this conversion to a

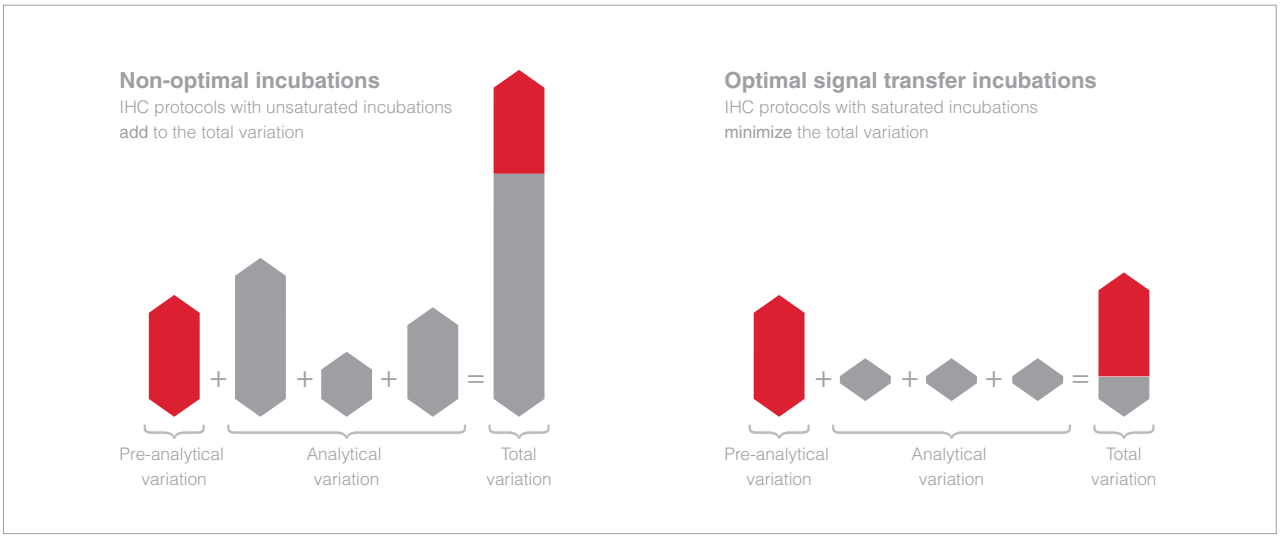

Figure 5.1 Optimal signal transfer protocols ensure that analytical variation has as little as possible influence on the total variation. "Unsaturated/saturated" refers to the protocols' ability to convert available targets into signal.

PETITIONER EXHIBIT 1012
Page 63 of 218

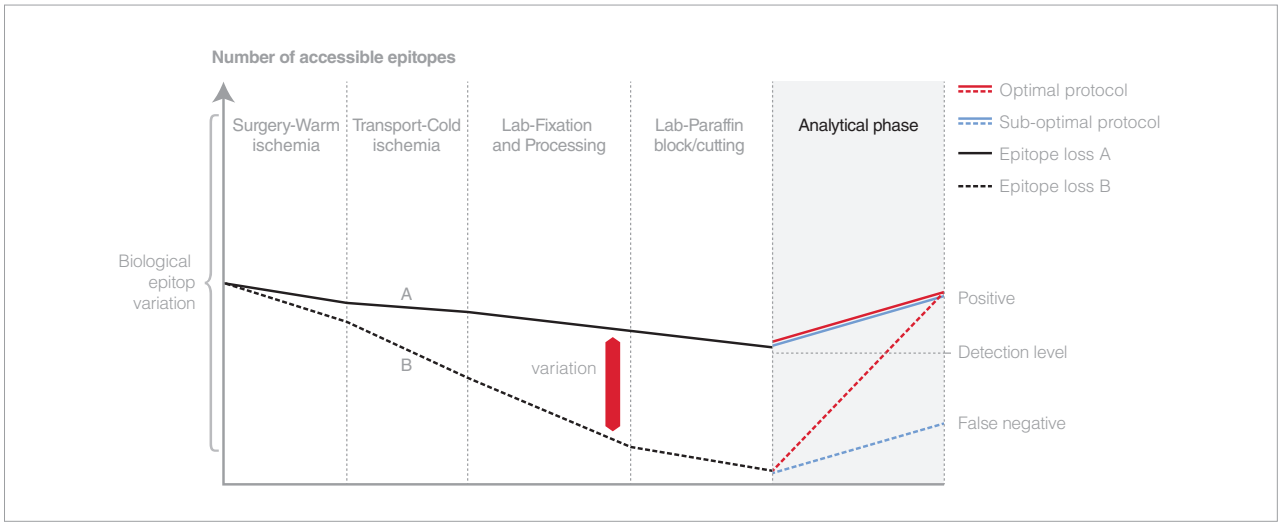

Figure 5.2 An optimal staining protocol must ensure that as many available epitopes as possible are converted into visible signals to avoid false negative staining results while not introducing false positive results. In scenario A, the minor loss of epitopes in the pre-analytical phase does not affect the result which is above the detection level for both the optimal (red line) and sub-optimal protocol (blue line). However, in scenario B the loss of epitopes is not recovered by the sub-optimal protocol, which could result in a false negative result. The same could occur when having a sample with low expression due to biological epitope variation. The figure illustrates the challenges when creating an optimal staining protocol that is affected by pre-analytical factors, which may differ from laboratory to laboratory, together with the biological nature of the protein being examined.

visible signal consists of incubations which basically follow the saturation curve shown in Figure 5.3.

Visual 'Saturation'

Obtaining intense staining without background is a delicate balance between chemical saturation parameters in the antigen/antibody reaction, plus the subsequent visualization reactions, versus the visual saturation of the brown end-product for the DAB reaction. As shown in Figure 5.3, the FLEX RTU protocol has been developed with the goal of always reaching the best signal to noise ratio possible. Some pathologists may consider the resulting stain to be "overstained", but the proper philosophy is to stain as intensely as possible without introducing background staining. The intense staining is a result of optimal signal transfer, where all available targets contribute to the signal. This is the aim of the protocol in a qualitative IHC test. Correctness in a qualitative IHC test relates to whether the antigen is truly expressed or not. In this context, correctness does not imply any direct proportional measurement of antigen expression.

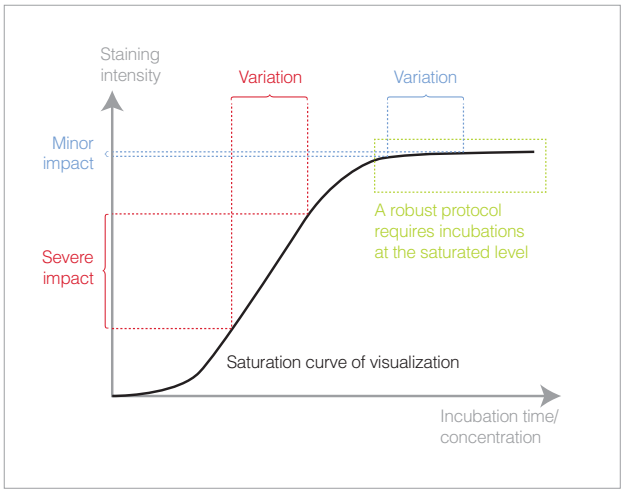

Figure 5.3 Each step in the analytical phase basically follows an incubation/concentration curve. A robust protocol ensures optimal signal transfer in all steps of the analytical phase. The green box represents the visual saturation area of a FLEX RTU protocol which is the goal in order to minimize the impact of variation.

PETITIONER EXHIBIT 1012
Page 64 of 218

With the saturation curve in mind, it seems obvious that the visualization (detection system) part of the staining protocol should not be used to compensate for sub-optimal antigen retrieval, or insufficient antibody concentration and incubation time, i.e. sub-optimal chemical signal transfer. Thus, the addition of extra amplification steps should only be used if the previous steps in the protocol have been optimally conducted and the specific protocol has not reached the optimal signal to noise ratio.

Chemical 'Saturation'

An optimal protocol should ensure that all available targets in the tissue contribute to the signal. Chemical 'saturation' in this context relates to ensuring that all available targets in the tissue have been found and are subsequently converted equally into a signal. Visual 'saturation' is the conversion of these chemical targets into chromogenic deposits.

Chapter 5.3 | The protocol

In order for a stain to be optimal a number of requirements must be fulfilled. The variation in the staining intensity is a sum of the total variation of all possible influencing factors.

- Biological variances. The protocol must be able to identify the antigen in normal tissue, with both high and low expression, and more importantly unknown levels of expression in abnormal tissue. Tumors are known to exhibit very heterogeneous antigen expression.
- Ischemic time. The protocol should ideally 'iron out' different degrees of antigen degradation and retrieval (to a certain point) (Chapter 3).
- Time of fixation. The protocol must be able to identify the same level of antigen expression, independent of the time of fixation when in the overall validated timeframe.
- Fixative. The protocol must be usable with commonly used fixatives. The widespread use of formalin fixation has narrowed this requirement.
- Tissue thickness. The protocol should encompass the effects of differences in section thickness on staining intensity.

The aim when developing optimal IHC protocols is to achieve a robust and correct visualization of the target antigen in clinical samples with unknown levels expression, thereby contributing to a valid diagnosis. The analytical part of the IHC process is controllable from a protocol point of view. It consists of antigen retrieval (Chapter 3), antibody selection and incubation (Chapter 4), and the visualization (Chapter 6).

In-House Protocols vs. RTUs From Vendors

Creating an optimal protocol is – in theory – quite simple: Optimize the protocol parameters so that the antigen of interest is stained in normal tissue elements with high expression of the antigen and at the same time – using the same protocol settings – will stain the antigen in normal tissue elements with low expression of the antigen. Abnormal tissues of interest should preferably express the antigen within the upper and lower limits of the control tissue elements. Background should not cause risk of misinterpretation of any positive signal (false positive). As noted in Chapters 4 and 6, this result may be achieved either by use of RTU reagents, or by in-house optimization of antibody concentrates and detection systems, using either automated platforms or manual methods.

FLEX RTU Core Protocol

As mentioned in the Introduction, this chapter is based on the research and development of the FLEX RTU series of antibodies. In this development, a Dako core protocol is the basis for the protocol adjustments for each of the individual antibodies in the series. The core protocol has been developed by testing a panel of antibodies and thereby finding the common denominator that ensures labeling of all available targets in the tissue. From here, each ready-to-use antibody is tested for low or high pH target retrieval requirement and finally the incubation time of the primary antibody. If needed for visual saturation, an amplification step (Linker) may be added. Concentrated antibodies have one other parameter to consider and to adjust, namely the concentration (see also Chapter 4).

The aligned core protocol will ensure an efficient workflow with minimal risk of false positive or negative results.

PETITIONER EXHIBIT 1012
Page 65 of 218

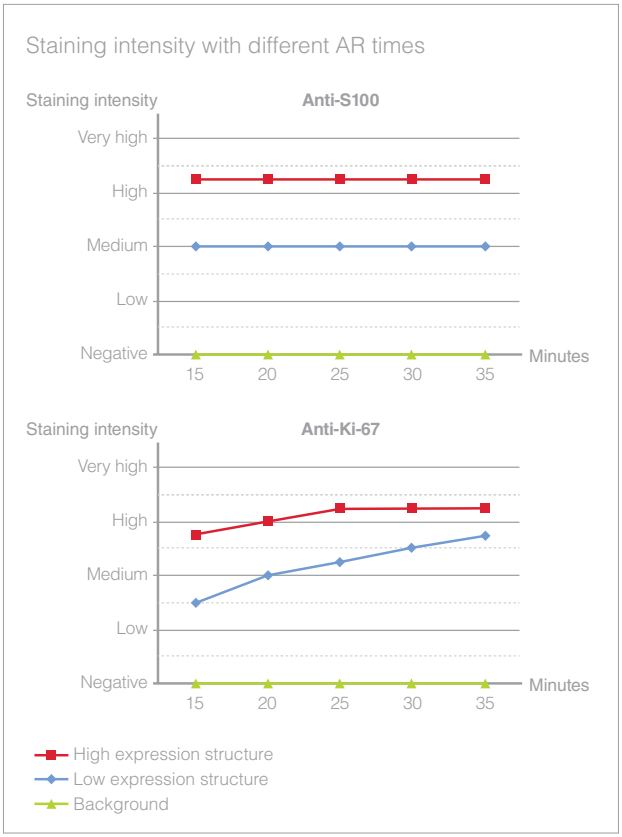

Figure 5.5 No variation in target retrieval efficiency from 15 min to 35 min for Anti-S100 (top). However, due to the low expression (LE) structures labeled by Anti-Ki-67 (bottom) and other antibodies (not shown) an incubation of 35 min at 97 °C is necessary for effective target retrieval of a broad range of antibodies.

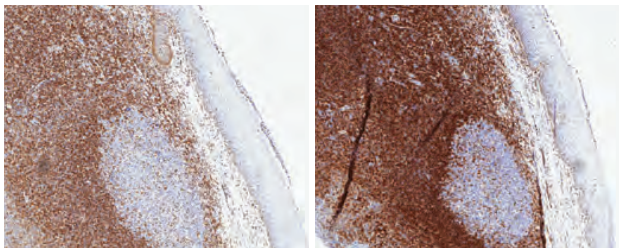

Figure 5.6 Anti-BCL2 staining of tonsil (FFPE). Target retrieval performed for 15 min (left) and 30 min (right).

Antigen Retrieval

As discussed in Chapter 3, antigen retrieval is a process that is influenced by many factors; most importantly heat and time. However, factors such as pH, molarity, content and concentration of the retrieval buffer, may all have major impact on the efficiency of the antigen retrieval process, which further affects the choice of clone and dilution of the primary antibody (4).

Time and Temperature

The selected antigen retrieval heating time (and temperature) secures optimal target retrieval for groups of antibodies that respond better to either low or high pH target retrieval solution (Chapter 3). The antibodies are grouped and tested over varying incubation times. In the FLEX RTU method, one incubation time is finally selected for a group of antibodies, in order to maximize throughput in the laboratory, while maintaining optimal or near optimal retrieval for each antibody.

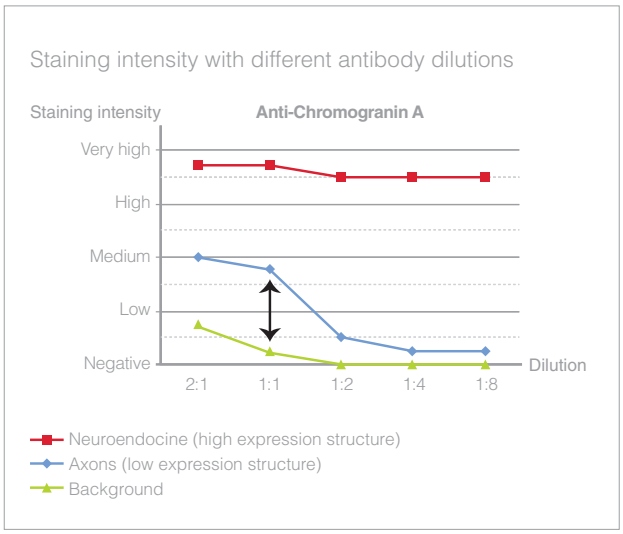

Figure 5.7 Control tissue, normal colon stained with Anti-Chromogranin A. Staining intensity of the high expression (HE) structures is strong and stable across the dilutions. The low expression (LE) structures are moderate in staining intensity at the 2:1 and 1:1 dilution with a steep fall off at higher dilutions. The optimal antibody dilution (1:1) is selected as the dilution that delivers crisp staining with the strongest possible intensity, while maintaining acceptable background. The 2:1 dilution provides a more intense staining but background staining is unacceptably high. The 1:2 dilution is not chosen as the LE structure is too weakly stained. See also Figure 5.8.

PETITIONER EXHIBIT 1012
Page 66 of 218

Figure 5.5 exemplifies some of the issues associated with obtaining the optimal target retrieval setting and applying it to the daily workflow in a busy pathology laboratory. While the target retrieval time for Anti-S100 is optimal at just 15 minutes, the optimal time for Anti-Ki-67 is 35 minutes. When many different antibodies are used in the lab, an alignment of incubation time is needed in order to keep the most efficient workflow in the daily routine. Antibodies should be tested with a wide range of incubation times so that optimal workflow is obtained, without unduly sacrificing the optimal staining result. This can be a cumbersome task for a laboratory when introducing new concentrated antibodies. An RTU version of the antibody will have been tested for these factors before release, and the vendor should have aligned the incubation times, while ensuring optimal staining quality.

Antibody Dilution

Definition of the optimal antibody dilution and protocol must take both specific staining intensity as well as background staining into consideration (see also Chapter 4). The overall principle is to define an optimal antibody dilution and protocol, resulting in a crisp staining with the strongest possible intensity, but without generating background staining. During development each FLEX RTU antibody is tested in different dilutions along with its recommended protocol, and then tested for sufficient and robust staining performance on a broad range of clinical samples with variable antigen expression.

Antibody Incubation Times

– 'Adjust the antibody incubation time, not the core protocol'

The rationale behind optimal signal transfer reactions in the analytical step of an IHC stain is to minimize the impact of sample variation (Figure 5.1). The visualization (detection) part of

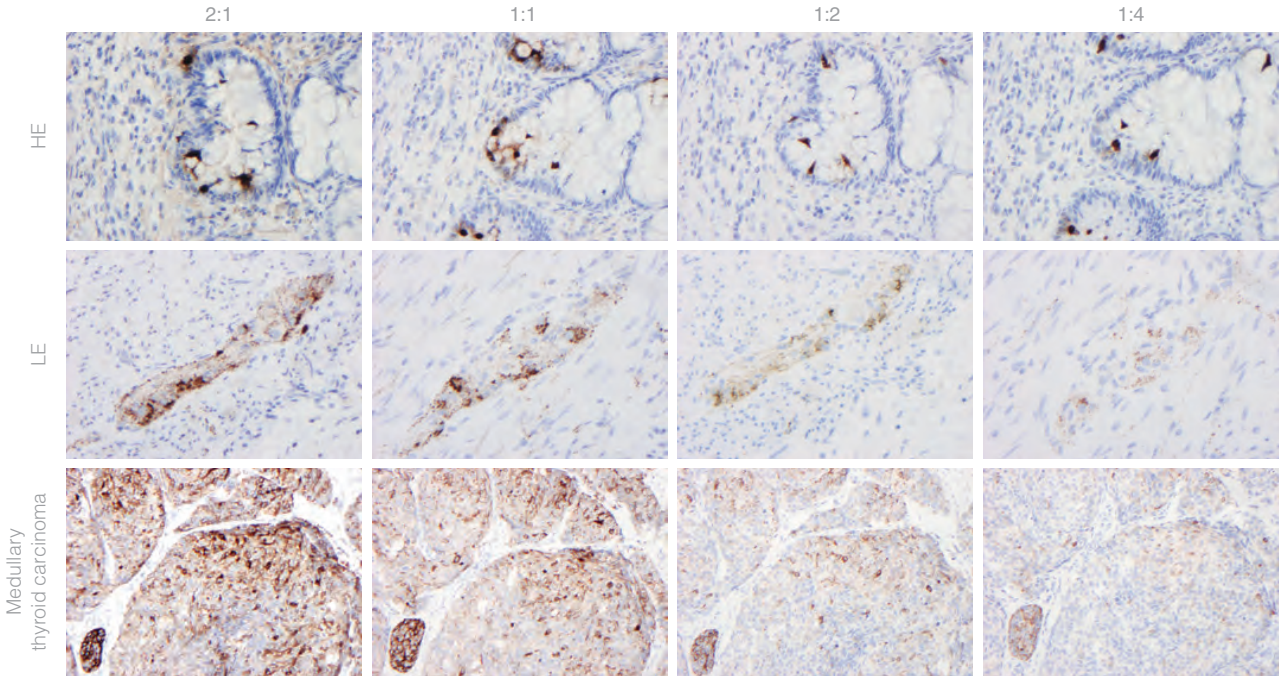

Figure 5.8 The impact on staining intensity when altering the concentration of the primary Chromogranin A antibody. The optimal dilution was selected as 1:1. HE: The neuroendocrine cells in colon show a strong cytoplasmic staining reaction independent of antibody dilution. The epithelial cells are negative. Note that there is some background around the crypts when staining with 2:1 concentration. LE: The axons and ganglion cells in colon show an increased staining intensity when using 2:1 concentration and a profound decrease in staining when using a 1:2 or 1:4 dilution. Medullary thyroid carcinoma: The majority of the neoplastic cells show an increased staining intensity when staining with 2:1 concentration and a profound decrease in staining intensity when using a 1:2 or 1:4 dilution.

PETITIONER EXHIBIT 1012
Page 67 of 218

the protocol should not be used to compensate for suboptimal antigen retrieval, insufficient antibody concentration, or insufficient incubation time. Once the optimal conditions for antigen retrieval, visualization and counterstaining (the core protocol) have been determined, only the incubation time of the RTU primary antibody is adjusted to ensure proper staining of both low and high antigen-expressing cells and structures in the tissue (Figure 5.9).

Visualization System Incubation Times

The overall aim for selecting the right incubation time is to ensure that all primary antibodies associated with the specific target are transferred into signal as effectively and with as little variation as possible. As shown in Figure 5.10, some LE structures are visually saturated after just 7.5 minutes horseradish peroxidase (HRP) polymer incubation (CD20, ST-M3-02), while the LE structures for S100 staining requires 17.5 minutes before visual saturation is obtained. When testing a wide panel of sensitive antibodies even the most challenging structure obtains a staining intensity plateau at 20 minutes of HRP incubation.

When testing the impact on intensity of IHC staining of chromogranin A using an alternative protocol, the most profound reduction in staining intensity was seen in the LE structures, as well as in the clinical tissue.

Using the FLEX RTU protocol, the high expression structures may seem "over-stained" (Figure 5.11, top left), but this visual saturation is needed to minimize the risk of false negative results (Figure 5.11, bottom right). Again, these results highlight the importance of evaluating not only the HE structures, but also the LE structure since they are generally more sensitive to changes in the protocol than the HE structure.

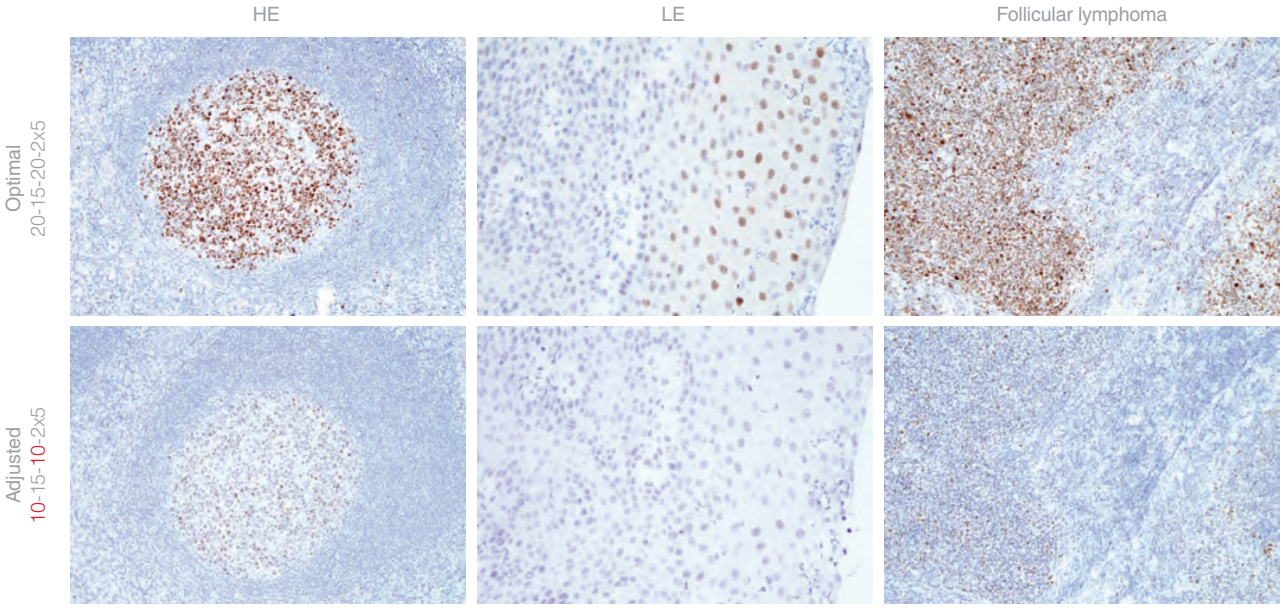

Figure 5.9 The impact of reducing the antibody incubation time (from 20 min to 10 min (red text)) together with a shorter amplification step (from 20 min to 10 min text) for EnVision™ FLEX+ with Linker amplification. Note that changing two steps at the same time, in isolation, is usually not a good idea, because any observed effect may be due to either one, or both, of the changes. In some instances where the primary antibody does not saturate, then the detection system may also be adjusted as part of a larger experiment, often termed a chess board or chequer board titration, to achieve mutual optimization and saturation (Chapters 4 and 6).

PETITIONER EXHIBIT 1012
Page 68 of 218

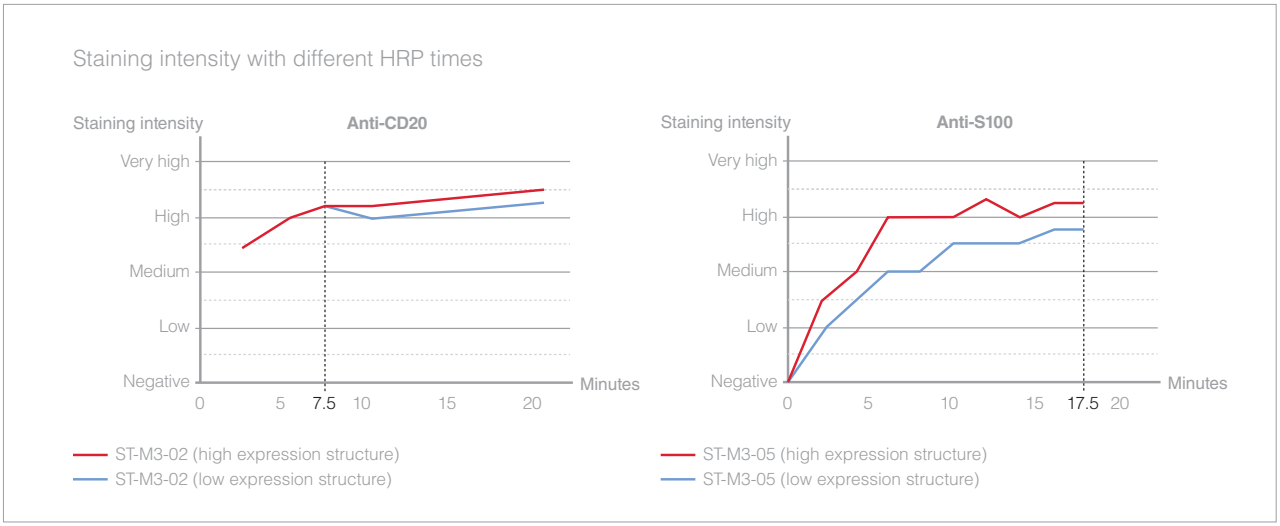

Figure 5.10 Some specific structures are visually saturated faster than others (7.5 min HRP incubation for CD20 versus 17.5 min for S100. However to obtain optimal workflow the incubation times for FLEX RTU protocols are aligned while still securing optimal signal transfer and thereby robust protocols.

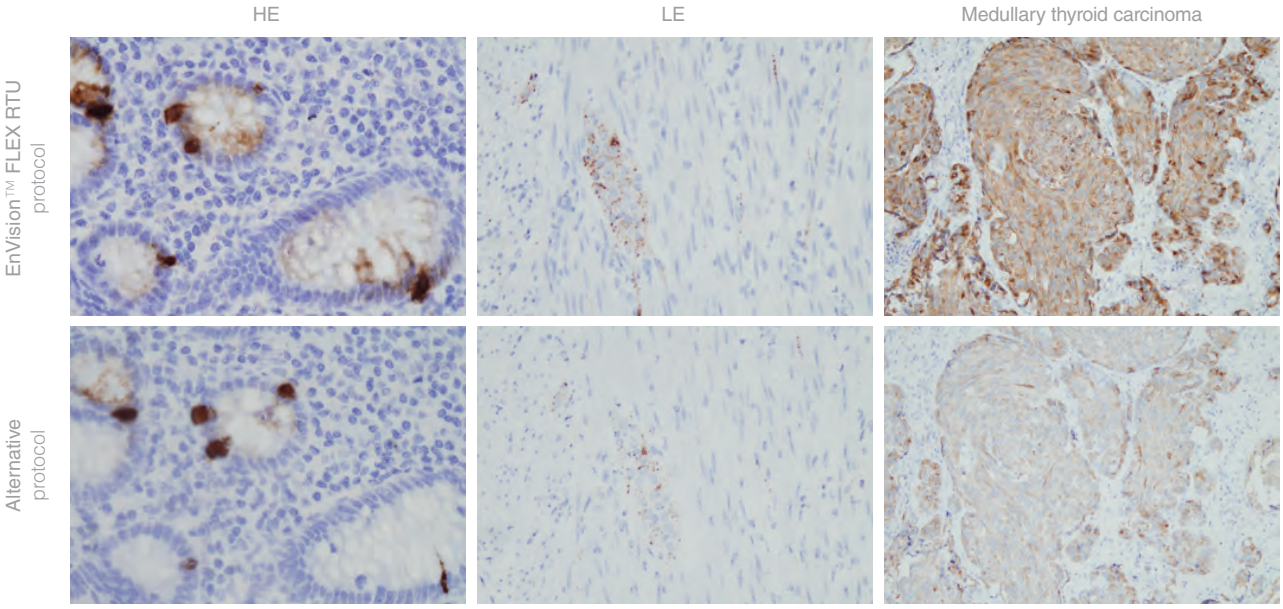

Figure 5.11 The impact on the staining intensity of the quality indicators and on clinical tissue when altering the protocol of the FLEX RTU chromogranin A antibody. HE: The neuroendocrine cells in colon show a negligible reduction in staining intensity when using the alternative protocol. The alternative protocol is optimized using the HE structures, whereas the FLEX RTU protocol is optimized using the LE structures. LE: The ganglion cells and axons in colon, as well as the medullary thyroid carcinoma show a profound reduction in staining intensity when using the alternative protocol compared to using the FLEX RTU protocol.

PETITIONER EXHIBIT 1012
Page 69 of 218

Section Thickness

The fine art of tissue sectioning requires skilled technicians, and consistent tissue thickness may sometimes be an overlooked factor in the IHC standardization efforts. The standard section thickness of 4-5 µm used in IHC may only partially include the nucleus, which is ~6 µm on average. This variation in depth of included nucleus can potentially lead to reduced staining intensity of a nuclear IHC stain, but is rarely important in the diagnostic result since the typical scoring criterion is qualitative (yes/no). However, it may be important in semi-quantitative scoring, where a tangential cut of the nucleus may not register as positive, potentially leading to a lower score. The tissue section thickness is important when the protocol settings are being optimized. A thicker section will tend to give stronger staining intensity than a thin section, simply because there is more tissue, more antigen, and more 'space' into which the DAB end-product may precipitate. Also, uneven thickness over the section may be encountered, but is usually only detectable with quantitative digital imaging (5). As illustrated in Figure 5.12, the same protocol can give remarkably different staining intensity depending on the thickness of the tissue. Frequent microtome calibration and skilled technicians are necessary for obtaining consistent tissue section thickness.

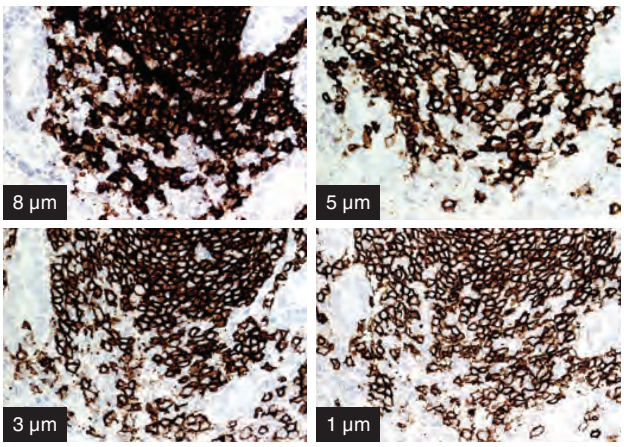

Figure 5.12 The impact of section thickness. A thicker tissue section can give stronger staining. All stains are done with the same protocol on serial sections. Courtesy of Søren Nielsen, NordiQC.

Counterstaining Incubation Times

Hematoxylin is commonly used as a counterstain for IHC due to a number of advantages it offers in the interpretation phase of the IHC stain. The blue nuclear coloring by hematoxylin produces a high level of contrast for the human eye, between the brown DAB chromogen, or red AEC chromogen. This facilitates improved visualization of tissue on the slide, assessment of tissue morphology, and determination of stained structures and cells. The counterstaining step of the protocol must also be optimized to reduce variability in intensity, which may otherwise lead to difficulty in recognizing nuclear localization of the chromogen.

Too intense counterstaining can lead to an optical distortion, especially for nuclear antigens, and too weak counterstaining may impair the tissue morphology assessment. For some assays the counterstaining can directly affect the diagnostic outcome, such as Ki-67 assessment, where the number of negative nuclei (denominator) directly affects calculation of the percentage of Ki-67-positive cells, i.e. a weak counterstain can lead to overestimation of the Ki-67 index (6).

Workflow Alignment

Laboratories have to meet the increasing demand for more tests and faster results, with fewer resources. The use of pre-defined protocols for FLEX RTUs is a balance between workflow and turnaround time without compromising optimal staining result. As shown in Figure 5.5, it is possible to use an antigen retrieval time of just 15 minutes for Anti-S100, while the optimal time for Anti-Ki-67 is 35 minutes. For the alignment of workflow, a 35 minute incubation is used without negatively impacting the staining of S100.

Depending on the instrumentation in the laboratory, the use of different target retrieval solutions for antibodies that are used often, or used in the same panel, can be a challenge for the optimal workflow. For example, a frequently used antibody may give optimal staining using a low pH target retrieval buffer, but acceptable staining can also be achieved using a high pH target retrieval buffer. Many labs are tempted to align the target retrieval protocol to the high pH protocol, used for 85-90% of all antibodies. The result is a better aligned workflow in the laboratory, at the expense of a less optimal, but still ac-

PETITIONER EXHIBIT 1012
Page 70 of 218

ceptable staining result for the frequently used antibody. Alternatively, laboratories would have many different protocols each suitable for only a few antibodies, which would thereby negatively affect the workflow.

Chapter 5.4 | Reproducibility of IHC

The issues of reproducibility and standardization of IHC have been addressed for years by researchers, pathologists and manufacturers using different approaches to solve one or more of the factors affecting the staining result (Chapter 1). Similar pre-analytical preparation methods, uniform antigen retrieval methods, high quality reagents, sensitive visualization systems and use of positive and negative tissue controls are central elements for improving staining quality. Despite these efforts, high level of reproducibility and reliability has not yet been achieved, as evidenced by data from EQA schemes, e.g. UK NEQAS and NordiQC).

Finding the cause of inconsistent IHC results can be addressed in different ways. Is variation caused by biological, technical, or observational differences? Very few studies have been designed to find the actual root cause of inconsistency in IHC staining results. One study comparing five antibodies at two institutions - each with two observers - found that the largest source of variation comes from differential expression of the molecule being tested (biological), rather than technical or observational differences (7). This conclusion differs from other studies, e.g. the ER reproducibility issues where both technical and observational issues contributed to unreliable results (8).

These two papers illustrate some of the challenges emerging from both the pre-analytical and post-analytical phases, together with the biological nature of the protein under examination. When a pathologist evaluates a stained tissue sample, the staining protocol must be sufficiently robust to minimize variations in the pre-analytical phase and give a visual representation of the expression of the antigen in the tissue that the human eye will be able to interpret comparably across various patient samples. To date, standardization approaches are still needed for an overall IHC system that assures uniform, high-quality staining, with a level of reproducibility and reliability that is sufficiently robust to allow comparison of IHC results, both intra- and inter-laboratory.

As discussed in Chapter 2, the influence of pre-analytical factors now has a higher attention in the standardization efforts than previously, e.g. the issues with fixation are being addressed with practical workflow efforts in most laboratories, to ensure immediate and proper fixation time of tissue specimens. This step is important for correct diagnosis because epitopes where the primary amino acid structure is degraded cannot be restored. Not even an optimal protocol with the most specific and sensitive reagents can reflect the in vivo antigen expression when epitopes are lost prior to fixation. In other words, garbage in = garbage out.

Chapter 5.5 | Requirements for Controls

For many antibodies, a variety of tissue structures are positive with high and low expression of the antigen in the normal control tissues. It is therefore important to choose structures that are both stable and reflect changes in protocol performance. A proper control tissue should preferably fulfill the following parameters:

- Show stable antigen expression between samples of the same tissue type
- Reflect changes in protocol performance by changes in staining intensity

In normal tissue, antigen expression is generally relatively stable between different samples of the selected control tissues, whereas clinically relevant abnormal tissues often show a large variation in antigen expression, between samples as well as within the same tissue. As a consequence of these requirements, abnormal tissue samples are usually not suitable for protocol optimization studies, as they by nature have unknown and unstable antigen expression. Instead optimization must be conducted on normal samples that 'resemble' the diagnostic material as closely as possible, including weak or focally positive cases. Interestingly, only 40% of laboratories include such weak/focally positive cases in validation procedures (1).

For the technical optimization process, it is very valuable to perform the tests on TMAs (see Chapter 12) with different nor-

PETITIONER EXHIBIT 1012
Page 71 of 218

mal tissues processed according to the routine and standard conditions used by the laboratory. The use of RTU systems where both reagents and matching protocols are validated by the vendor, can make the introduction of new assays much simpler. The laboratory can simply select positive control tissue with pre-analytical factors matching the anticipated diagnostic samples, and stain these strictly according to the manufacturer's validated protocol. The protocol has already been tested with different pre-analytical and analytical factors on an automated staining system, but variations in sample preparation in individual laboratories may influence the staining result compared with the vendors' tested settings. When an optimal staining result has been successfully achieved, then the robustness and correctness of the assay can be further validated on additional cases in the user laboratory. If the vendor's validated protocol does not give the preferred staining result, the first option should be to identify pre-analytical factors in the laboratory that could potentially have a negative effect on the staining result. Only when the laboratory cannot identify the cause (other than personal preference of staining intensity) should protocol modifications be attempted. If the laboratory dilutes the RTU, changes reagents or adjusts the protocol outside the manufacturer's guidelines, the laboratory must do a full revalidation of the assay.

Using too few or inappropriate controls, which do not reflect variations in the pre-analytical and biological factors, jeopardizes the diagnostic staining result. As illustrated in figure 5.13, an optimized protocol should have a large 'window', where samples with variable expression levels and variable pre-analytic factors will be positively stained. If an altered protocol is tested with only a few controls or with cases that only represent few variables, the risk of having samples that fall outside the acceptable window increases.

In order to serve as a reliable control tissue, the antigen expression must be predictable and stable between independent samples. Normal tissue that fulfills these criteria

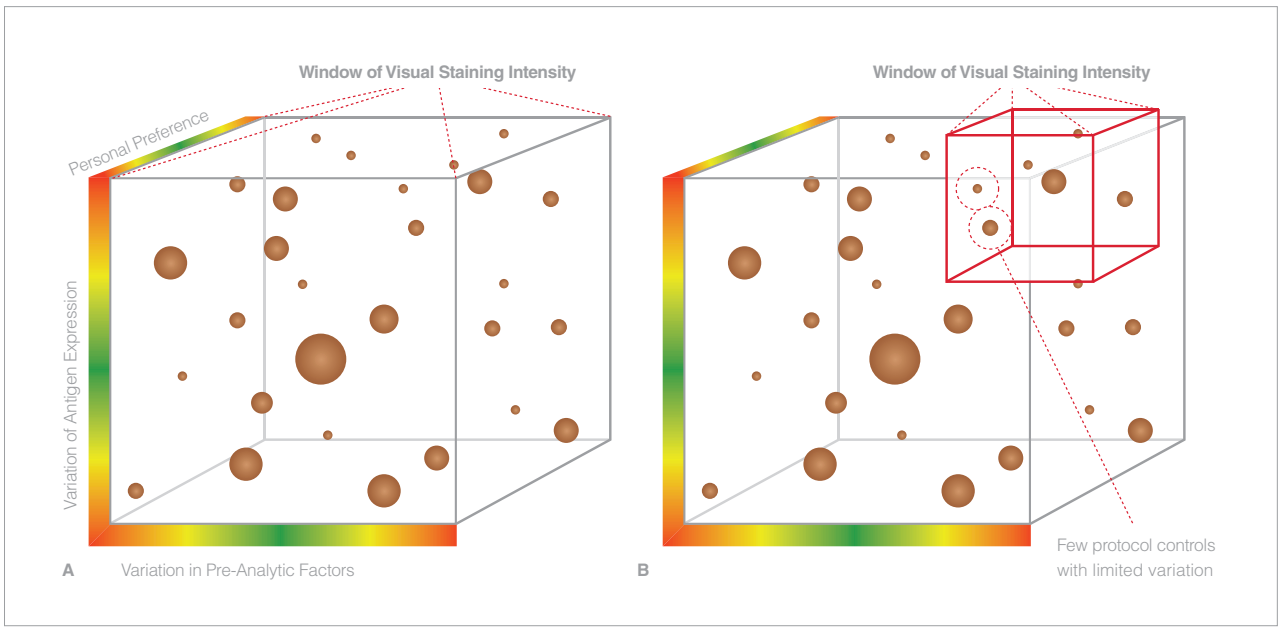

Figure 5.13 A) An optimized protocol ensures that samples with variation in antigen expression and pre-analytical factors produce a visible signal, due to a rigorous validation procedure. Many samples with differing expression levels from different sources in optimal signal transfer incubation reactions are tested. **B)** When only a few samples with little variation and poor signal transfer reactions are used to optimize a staining protocol, the window in which the protocol generates visual signals is reduced and some samples may fall outside the window, increasing the risk of false negatives and false positives results.

PETITIONER EXHIBIT 1012
Page 72 of 218

should be chosen as control tissue. When optimizing the protocol it is important to identify normal tissues that express the antigen in high expression (HE) and low expression (LE) structures. Below is an example of Anti-Actin, clone HHF35, where the defined HE and LE structures are present in different tissue types. In colon, all smooth muscle cells in vessel wall, muscle layers and lamina muscularis mucosa were defined as HE (Figure 5.14A), whereas LE was observed in the myoepithelial cells of the mucous/salivary glands in tongue (Figure 5.14B).

However, evaluation of various normal tissues may identify cases where antigen expression varies too much to be defined as stable. Using the same Anti-Actin antibody as an example, normal liver tissue shows staining of perisinusoidal smooth muscle cells – but only in some samples (Figure 5.14C). This finding serves to underline that all normal tissues may not, by default, be suitable as control tissue. Detailed analysis should be carried out when selecting a normal tissue that is optimal as control tissue, by using a validated protocol that is able to identify variations in antigen expression.

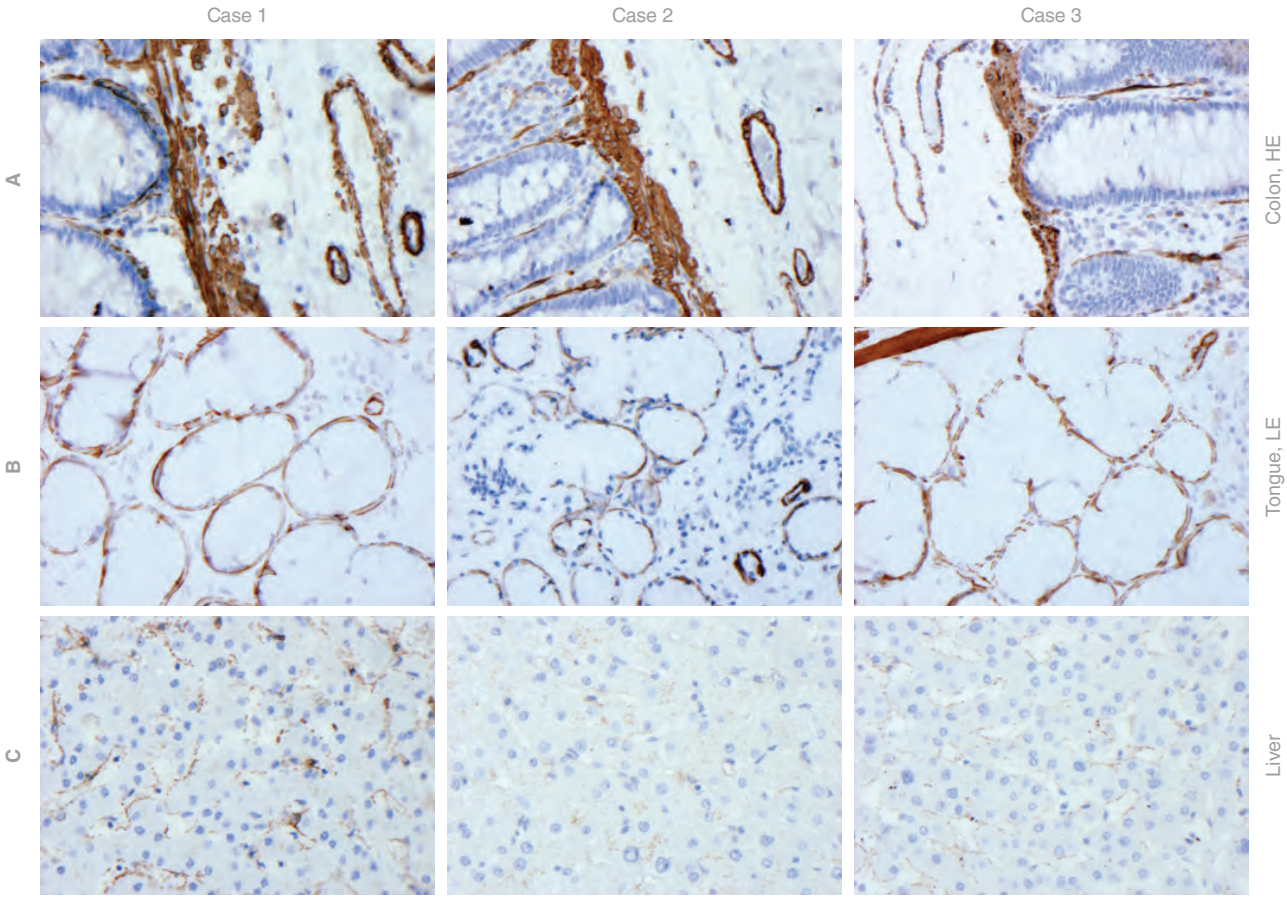

Figure 5.14 A) Normal colon from three different patients stained with Anti-Actin, clone HHF35. Smooth muscle cells in vessel wall, muscle layers and lamina muscularis mucosa, defined as high expression (HE) structure show comparable staining intensity in all three samples. **B)** Normal tongue from three different samples Anti-Actin, clone HHF35. The myoepithelial cells of the mucous/salivary glands, defined as low expression (LE) structure, show comparable staining intensity in all three samples. **C)** Normal liver from three different patients stained with Anti-Actin, clone HHF35. The staining intensity of perisinusoidal smooth muscle cells varies from weak to negative, and is consequently a poor control tissue due to the variable antigen expression between tissue samples.

PETITIONER EXHIBIT 1012
Page 73 of 218

Having a 'catalog' with normal external control tissues with high and low expression of the antigen is useful for a number of critical requirements for the optimal staining:

- High expressers are required to evaluate the process (right antibody)
- Low expressers are required to evaluate sensitivity (right protocol)
- Non expressers are required to evaluate specificity (right signal to noise)
- Variation is required to evaluate robustness (right staining, repeatedly)

Insufficient staining results in NordiQC assessment runs are often related to the use of inappropriate positive tissue controls (3). Increased focus on external tissue controls is needed to standardize and optimize IHC. See Chapter 4 and Chapter 14 and for more information on the use of controls in IHC.

Chapter 5.6 | Verification and Validation of a Protocol

Verification

Once the protocol has been optimized and 'locked' using the optimal signal transfer incubation principles explained above, the antibody enters the verification phase where both the antibody and the protocol are tested to ensure that they meet specifications related to analytical specificity and sensitivity.

The FLEX RTU antibody is tested on a minimum of 30 different normal tissues from three independent sources, according to requirements from the FDA (9). This test is evaluated by an external, qualified pathologist and the results are included in the instructions for use for the antibody (package insert). Relevant stains are cataloged in the Dako Atlas of Stains book.

The antibody undergoes extensive precision testing to ensure reproducibility and repeatability of antibody performance. The test is performed as a Gauge R&R study and includes intra-run reproducibility, inter-run reproducibility and inter-instrument reproducibility, to confirm that the antibody performance is the same on the same day, between days, and when used on different (Dako) platforms. Normal control tissue with well-defined cellular structures/elements, as well as clinically relevant (abnormal) control tissue are included in the tests.

Validation

Validation is performed on production lots and validates the intended use of the product. The validation testing is performed according to the instructions for use of the antibody on an extensive set of positive and negative clinical specimens related to the intended use of the product. The test is performed as a method comparison study, and the positive and negative agreement is compared to a second antibody of similar specificity and documented performance, if obtainable.

Chapter 5.7 | Guide to the Development of an Optimal Staining Protocol

This chapter has focused on minimizing the risk of false negative and false positive results by ensuring that the protocol transfers all available targets into a visual signal without introducing background staining. The cornerstone of FLEX RTUs is the core protocol. To reach the core protocol the antibody has been tested using a protocol decision tree. If the staining is not optimal the protocol is adjusted in the next step and so forth (Figure 5.15).

Chapter 5.8 | Protocol Performance for Abnormal Tissue

When evaluating the defined HE/LE structures in variable protocol settings, variation in protocol performance should be reflected by changes in staining intensity. Typically, staining intensity of the HE structure is fairly stable, whereas the LE structure varies in intensity. When fine-tuning a protocol, intensity changes in HE structure are usually minor, whereas the intensity changes in the LE structure are generally larger and easier to detect.

The changes in IHC performance monitored by the HE/LE structures are also reflected in the staining intensity in the clinical tissue. In Figure 5.16, two different T-cell lymphoma cases show variation between samples of clinical tissues with respect to antigen expression. Compared with the optimal pro-

PETITIONER EXHIBIT 1012
Page 74 of 218

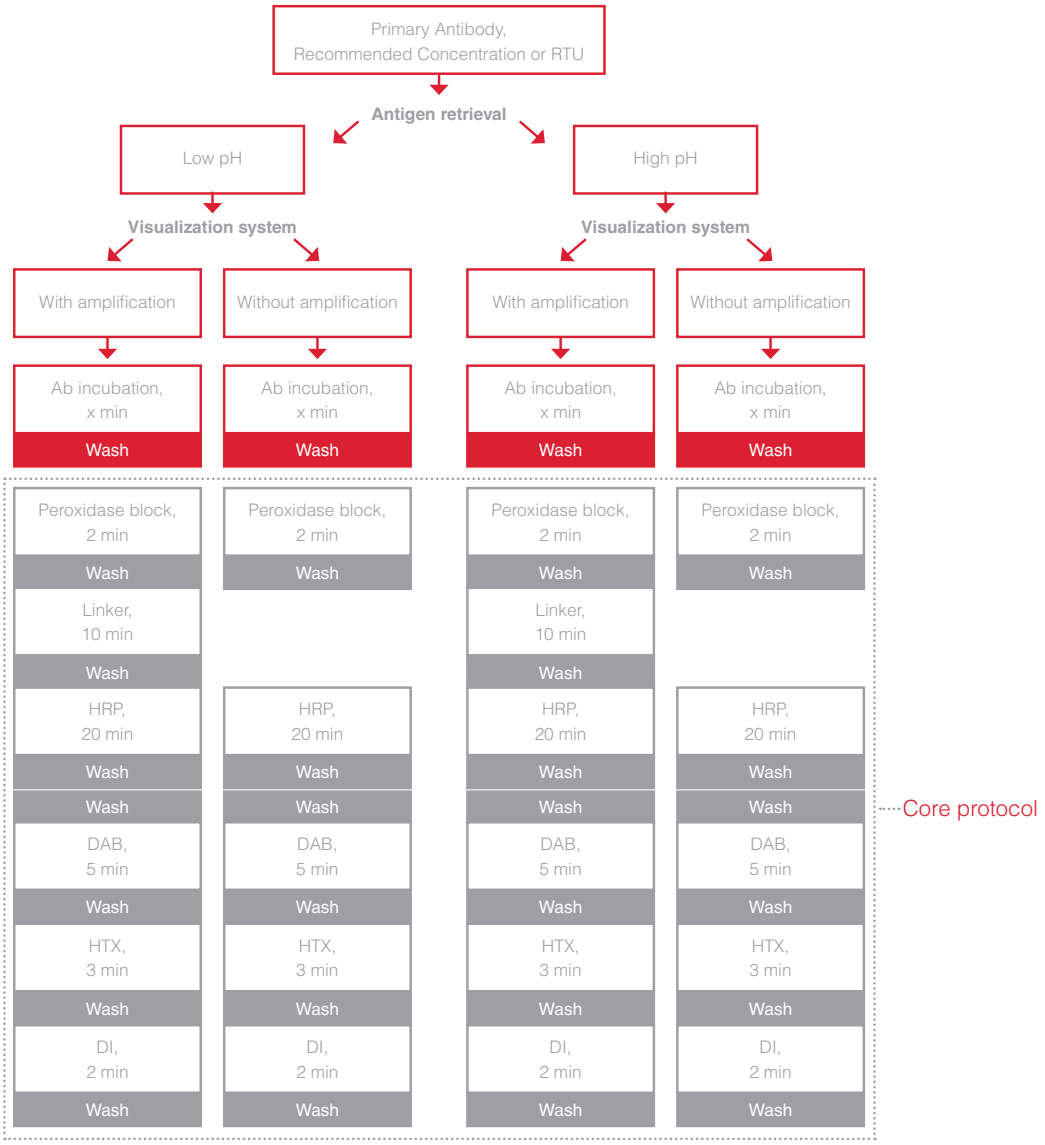

Figure 5.15 The core protocol is reached by testing the parameters using a protocol decision tree. **Step 1:** Assess the optimal AR. Test both high pH and low pH AR using the vendor's recommended primary antibody concentration (incubate for 20 min) and a Linker visualization system. Select the antigen retrieval solution which retrieves most targets. **Step 2:** Assess staining protocol. Titrate primary antibody concentration (never for RTUs) or adjust incubation time. Test with and without amplification in the visualization protocol. Select the protocol that stains both HE and LE expression structures with optimal signal to noise ratio.

PETITIONER EXHIBIT 1012
Page 75 of 218

tocol, the staining intensity of the HE structure is only slightly weaker in the sub-optimal protocol, while staining intensity of the LE structure is dramatically decreased in the sub-optimal protocol. Thus, in the sub-optimal protocol, Case 2 might be interpreted negative – a false negative result that would only be recognized by evaluating the HE and LE structures in the normal control tissue.

Without including the normal control tissue with associated HE/LE structures, it would be impossible to identify whether the staining quality of the clinical tissue was due to the biology of the cancer or influenced by the quality of the protocol. These results emphasize the importance of monitoring IHC protocol performance by carefully evaluating the defined HE and LE structures within the defined normal control tissue in order to ascertain optimal staining in clinical tissues. This process in turn helps to ensure the correct diagnosis. Some laboratories also monitor the monthly distribution of HER2 score percentages. In the case that the percentages differ too much from the average, both the assay and the protocol are scrutinized to identify possible issues with the test.

Using an amplification step (Figure 5.17) is sometimes necessary to increase the sensitivity of the protocol to minimize the risk of false negative results. Amplification steps should only be considered when adjustment of parameters outside the core protocol (Figure 5.15) does not result in optimal signal to noise reaction. Therefore, amplification should not to be used to create a more intense staining when the problem of weak signal may be due to a non-optimal protocol.

Amplification may produce a very intense staining of structures in the control tissues, which some pathologists find undesirable ("over-stained"). However, as exemplified in figure 5.17, a protocol that produces intense staining of control tissue structures is sometimes needed to minimize the risk of false negative results.

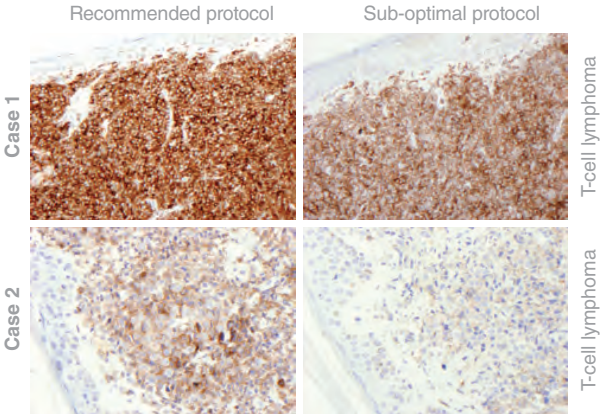

Figure 5.16 Staining intensity of the LE structure is dramatically decreased in the sub-optimal protocol. Thus, in the sub-optimal protocol, Case 2 is false negative. Stained with Anti-CD4, Clone 4B12 using the recommended Linker amplification (EnVision™ FLEX+) versus a protocol that is sub-optimal for this antibody (EnVision™ FLEX).

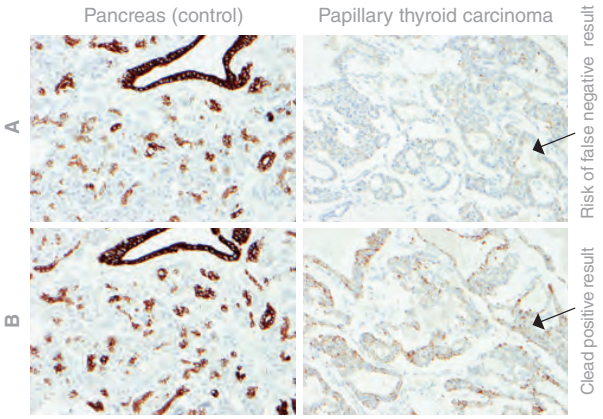

Figure 5.17 Using an amplification (Linker) step for the protocol will produce strong, intense staining of the control tissue (bottom, left), and can reduce the risk of false negative results (bottom, right). **Protocol A:** Primary Ab: 20 min, HRP: 20 min, DAB: 2x5 min. **Protocol B:** Primary Ab: 20 min, **Linker: 10 min**, HRP: 20 min, DAB: 2x5 min.

PETITIONER EXHIBIT 1012
Page 76 of 218

Chapter 5.9 | Concluding Remarks

This chapter has described the requirements for creating an optimal protocol with focus on robustness and correctness, using proper tissue controls with high and low expression of the antigen. The FLEX RTU solution, with a defined visualization system and validated protocol has been used to exemplify the many contributing factors to which laboratories must pay attention, when trying to optimize protocols for concentrated antibodies. The use of an RTU solution reduces the resources necessary to verify performance when introducing new antibodies in the laboratory. It also forces the standardization of reagents, dilutions, detection systems and staining protocols among those different laboratories using the same system. Optimal protocols minimize the impact of pre-analytical factors, due to optimal signal transfer incubations, and thus increase the diagnostic confidence, by detection of cells and structures with both low and high expression of the antigen.

While an optimal protocol, with optimal signal transfer incubations, can to some extent 'absorb' or 'iron out' pre-analytical vari-ations, these variations are still one of the most important hurdles to overcome for achieving standardization among pathology laboratories (10, 11). Neutral buffered formalin has become the standard choice of fixative in most laboratories worldwide but many laboratories still do not have standard procedures for the preparation of formalin, the source (vendor/home-made), or even the fixation time. Practical daily procedures in the hospital can cause fluctuations in fixation time, which can influence efficacy of antigen retrieval, and ultimately the staining result. Standardization starts in the operating room, where prolonged duration of pre-fixation time (ischemic time) may permit degradation of tissue resulting in loss of epitopes. Not even the best protocol can restore the primary sequences of an epitope lost in this manner. Similarly, the effect of other steps in the pre-analytical phase, including processing, dehydration, paraffin embedding, storage before staining, de-paraffinization and rehydration are not fully understood (12). Something as simple as the water, or type of paraffin, used in the laboratory may have an impact on the parameters in the optimal staining protocol.

A more widespread use of RTUs can contribute to some improvement in standardization, but cannot solve the pre-analytical standardization issues. RTU reagents are extensively tested internally by the manufacturers, and vendors should provide protocols that establish optimal performance of the system, including guidance to correct control tissue. In-house testing, within the user laboratory, must include a wide range of FFPE tissues that have been subject to different pre-analytical factors, including time and conditions of fixation. A correct and robust RTU protocol, from antigen retrieval to counterstaining, can and should minimize the effects of lack of standardization by 'absorbing' the variations. To ensure visual saturation of both the LE and HE structures in different tissue types, the IHC stain may sometimes appear "over-stained" for HE structures. This high intensity is an intended 'drawback' (the 'price to pay') when improving diagnostic certainty in tumor samples with heterogeneous antigen expression.

Skilled and experienced staff in many laboratories are no doubt capable of matching a concentrated antibody to a detection system. They can perform the necessary titrations, and test incubation times to establish the optimal protocol for the FFPE tissues available to each antibody. However, the resources and experience necessary to conduct such optimization studies are substantial, and may not be available in smaller laboratories. Larger institutions, on the other hand, may not easily find the required time to conduct such extensive optimization, validation and verification tests. RTUs are an option representing a path to improved diagnostic certainty for both experienced, high volume laboratories as well as for smaller laboratories without skilled and experienced staff.

When up to 90% of insufficient staining results are due to weak staining intensity (3), it seems obvious that a greater focus on appropriate tissue controls and optimal staining protocols is a step in the right direction for standardization of IHC.

PETITIONER EXHIBIT 1012
Page 77 of 218

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PETITIONER EXHIBIT 1012
Page 78 of 218

Chapter 6

Detection Methods

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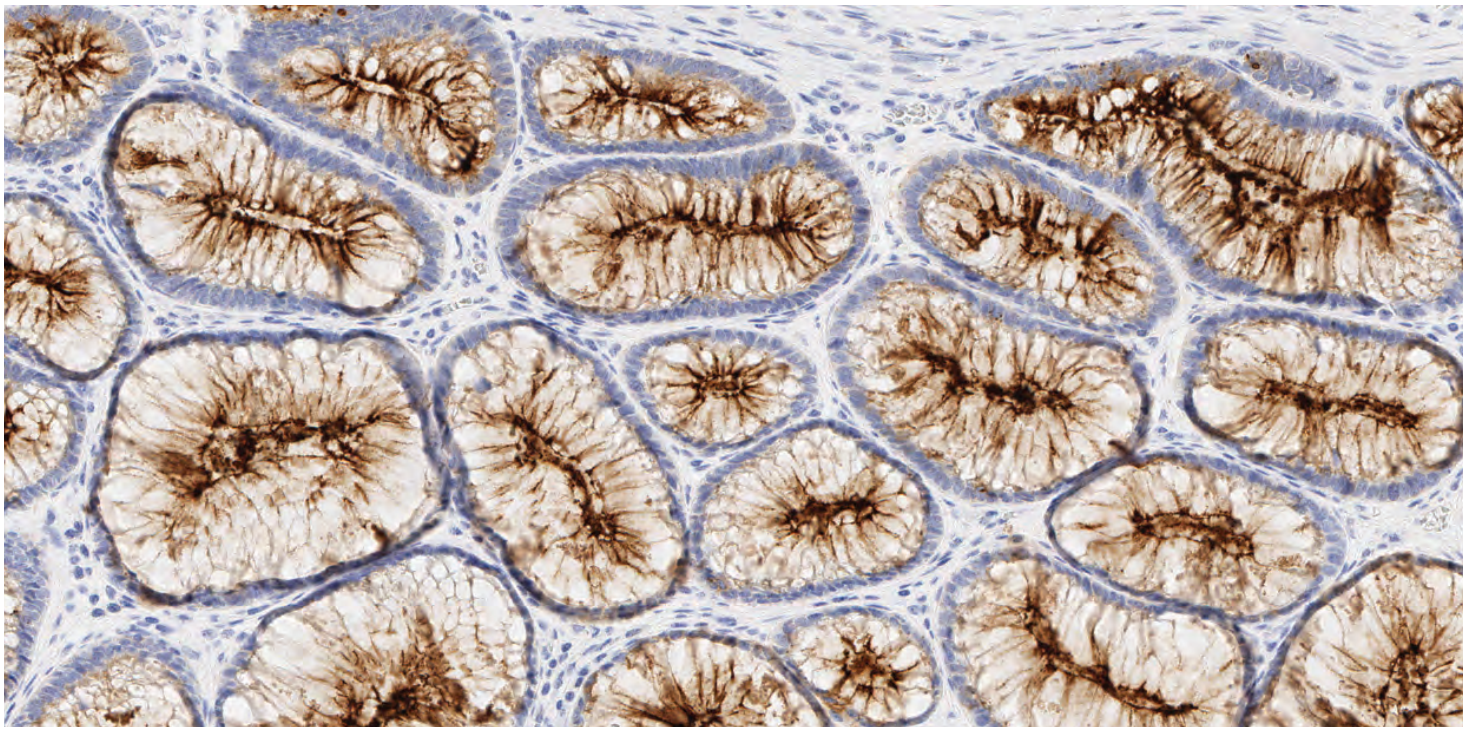
Hans Christian Pedersen, MSc



De•tect *(n.)*
To discover or determine the existence or presence of <something>.

Merriam-Webster Online Dictionary

Chapter 6.1 | Introduction

Immunohistochemistry (IHC) has emerged as a powerful investigative tool that can provide supplemental information to the routine morphological assessment of tissues. The use of IHC to study cellular markers that define specific phenotypes has provided important diagnostic, prognostic, and predictive information relative to disease status and biology. The application of antibodies to the molecular study of tissue pathology has required adaptation and refinement of IHC techniques, particularly for use in fixed tissues. In contrast to solution-based immunoassays that detect relatively abundant native proteins, in fixed tissues the preservation of antigen is variable and unpredictable. Thus, the history of IHC has evolved so that we today are able to detect proteins in tissue with great sensitivity, and also provide a semi-quantitative assessment, with the ultimate goal of integrating tissue-based analysis with proteomic information.

Immunohistochemistry: In the Beginning

The first staining with an antibody to find an antigen in tissue was reported in 1941, using a fluorescence-labeled antibody (1). Twentyfive years later, the enzyme horseradish peroxidase (HRP) together with 3,3'-diaminobenzidine (DAB) was used to study mouse kidneys (2). The following year, an antibody linked to HRP was used to visualize antigens in tissue using the indirect method, where a second antibody is used to recognize the first or primary antibody which is attached to the antigen (Figure 6.1). The secondary antibody recognize the constant part (Fc) of the primary antibody, which makes it possible to recognize all primary antibodies as long as they are from the same species.
These pioneering studies using enzyme labels instead of fluorescent dyes and the application to formalin-fixed, paraffin-embedded tissue (FFPE) (3) opened the door to the use of immunoperoxidase methods for routine diagnosis in anatomic pathology (4, 5), and led to the development of modern methods of IHC (see Chapter 1).

The good preservation of features and improved morphology of FFPE of tissues, makes this method the preferred choice in almost every clinical pathology laboratory. The indirect staining methods are likewise the preferred staining methods because labeling of the primary antibody is avoided, and they give a more intense staining. The secondary antibodies used in the indirect methods are typically raised in goat against either mouse (GaM) or rabbit (GaR) antibodies. With the successful application of IHC methods to formalin-fixed specimens, new staining methods were rapidly developed including the immunoperoxidase bridge method (6) and the peroxidase anti-peroxidase (PAP) complex method (7).

Table 6.1 Complexity vs. Sensitivity of Detection Systems.

Number of Steps						
5						iCSA
4			Polymer (Doublestain)		CSA II	
3		LSAB/ABC		Polymer + Linker		
2		Indirect	Polymer			
1	Direct					
	1	2	3	4	5	6
Sensitivity						

LSAB=Labeled streptavidin-biotin; ABC=Avidin-biotin complex; CSA II=Catalyzed signal amplification II, iCSA = Improved catalyzed signal amplification.

Chapter 6.2 | Avidin-Biotin Immunohistochemistry

The next generation of IHC methods emerged in 1981 with the avidin-biotin-based methods (Figure 6.2) (8). These methods are still used to a limited degree in some pathology laboratories and rely on the strong affinity of avidin or streptavidin for the vitamin biotin.

Streptavidin (from the bacteria *Streptomyces avidinii*) and avidin (from chicken egg) both have four binding sites for biotin. The biotin molecule is easily conjugated to antibodies and enzymes. In the avidin-biotin complex (ABC) method secondary antibodies are conjugated to biotin and function as links between tissue-bound primary antibodies and an avidin-biotin-peroxidase complex. The four binding sites for biotin make 'lattice' complexes possible, where the avidins are linked together via the enzyme (8). The only requirement is that the enzyme has at least two biotin molecules attached so that it can function as a link between the avidins. A colorless substrate,

PETITIONER EXHIBIT 1012
Page 80 of 218

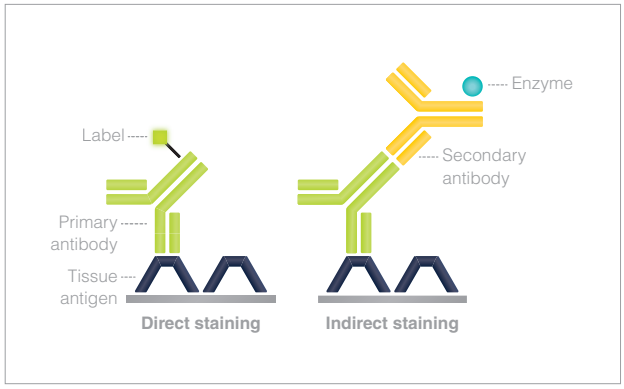


Figure 6.1 Direct vs. indirect staining.

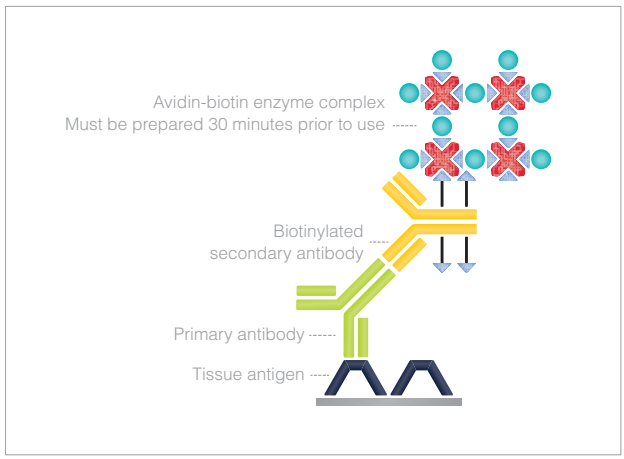


Figure 6.2 Avidin-Biotin Complex (ABC) method.

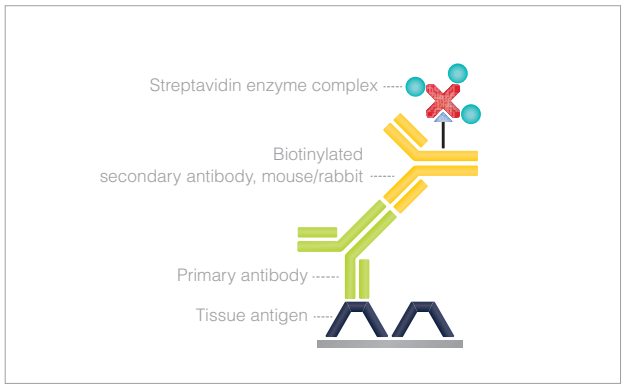


Figure 6.3 Labeled Streptavidin-Biotin (LSAB) Method

e.g. DAB, is subsequently added, and is converted to a brown end-product by the multiple peroxidase enzyme molecules now attached at the site of the target antigen.

In a similar method the labeled streptavidin-biotin (LSAB) method also utilizes a biotinylated secondary antibody that links primary antibodies to a streptavidin-peroxidase conjugate (Figure 6.3). This approach has the advantage that preassembly of the ABC complex is not needed. In both methods a single primary antibody is subsequently associated with multiple peroxidase molecules, and because of the large enzyme-to-antibody ratio, a considerable increase in sensitivity is achieved compared to direct peroxidase-conjugate methods.

When using these methods it is important to be aware of their limitations. Avidin has a tendency to bind non-specifically to lectin-like and negatively charged tissue components at physiological pH. For streptavidin less non-specific tissue binding is observed. Another challenge is the presence of endogenous biotin in tissues. Formalin fixation and paraffin embedding has been shown to significantly reduce the level of endogenous biotin, but residual activity can still be observed in tissues such as liver and kidney. Methods to block endogenous biotin are partially effective, but add another layer of complexity to an already complex procedure. In frozen tissue sections, the level of endogenous biotin is usually even higher than that encountered in FFPE specimens, giving troublesome non-specific binding of the avidins.

Chapter 6.3 | Polymer-Based Immunohistochemistry

The limitations associated with the avidin-biotin system, led to the development of detection systems with higher sensitivity and specificity, employing polymer-based IHC techniques (9). These methods utilize a polymer backbone to which multiple antibodies and enzyme molecules are conjugated. As many as 70 enzyme molecules and about 10 primary antibodies can be conjugated to a single dextran backbone. This construct allowed the entire IHC staining procedure, from primary antibody to enzyme, to be accomplished in a single step (10). On the other hand, one limitation of this method was its restriction to a select group of primary antibodies provided

PETITIONER EXHIBIT 1012
Page 81 of 218

by the manufacturer, and lack of utility for many user-supplied primary antibodies.

To overcome this limitation a new type of visualisation system, EnVision™, was introduced (Figure 6.4). This indirect visualization system also contains a dextran backbone to which multiple enzyme molecules are attached. However, the EnVision™ system contains secondary antibodies with anti-mouse Ig and anti-rabbit Ig specificity. This 'universal' reagent could be used to detect any tissue-bound primary antibody of mouse or rabbit origin. The broad applicability of this method opened the door to a new family of polymer-based IHC methods. The sensitivity of these methods when compared to LSAB and ABC methods is comparable or even slightly greater in most cases (11). By adding an additional linker step, the sensitivity can be improved further. However, because of the large molecular size of the polymer conjugates, accessibility to certain epitopes can be a challenge, presumably due to steric hindrance.

Chapter 6.4 | Catalysed Signal Amplification (CSA)

This amplification technique is based on the ability of peroxidase enzyme to oxidize phenolic compounds to highly reactive and unstable intermediates called radicals (12). The commonly used substrate in this technique is tyramine. It has a phenol in one end, used by peroxidases, and an amine in the other end of the molecule. The amine can be used to add biotin, or other molecules of interest, to tyramine through an amide bond, hence the tyramide amplification name also used for this method. When tyramide is oxidized, it will react rapidly with electron-rich aromatic compounds, such as the amino acid tyrosine found in protein molecules (13). This reaction can be used in IHC to bind biotinyl-tyramide to protein molecules in the immediate vicinity of peroxidase enzymes. This reaction results in the deposition of numerous biotin signals around the primary antibody.

In a typical CSA-based IHC procedure, peroxidase enzymes are first associated with primary antibodies by any of the standard IHC methods (Figure 6.5). Biotinyl tyramide and hydrogen peroxide are applied as a substrate to generate numerous biotin signals. These biotin molecules can then be used to capture subsequent streptavidin-peroxidase enzymes to produce the desired staining by addition of the appropriate substrate (14). Another possibility is repetition of the biotinyl-tyramide reaction, which will increase the numerous biotin signals even further. This cycling of the reaction is practically limited to two or three cycles before background staining becomes too high. CSA is a highly sensitive amplification technique, but has several disadvantages that prevent its general use. The method is time consuming, results can be hard to reproduce, and as in previous biotin-based methods endogenous biotin can give a high background staining.

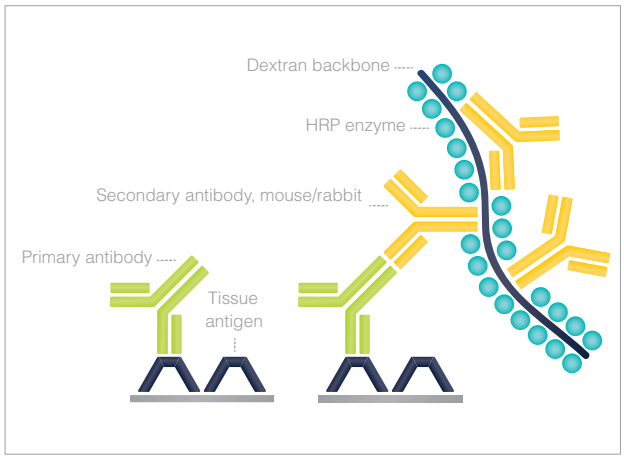


Figure 6.4 Two-step polymer method (EnVision™).

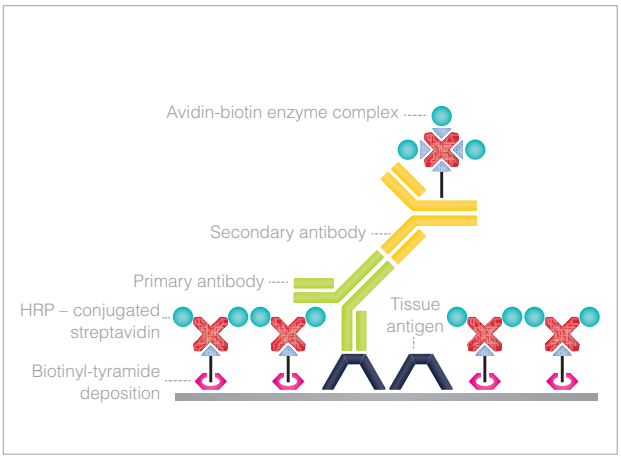


Figure 6.5 The CSA system.

PETITIONER EXHIBIT 1012
Page 82 of 218

Chapter 6.5 | Fluorescyl-tyramide Amplification

Fluorescyl-tyramide can replace biotinyl-tyramide to avoid endogenous biotin background. In this procedure peroxidase is associated with a tissue-bound primary antibody by application of a secondary anti-mouse antibody to which peroxidase has been conjugated. The peroxidase catalyzes the conversion and deposition of fluorescyl-tyramide onto the tissue section. At this point the reaction can be terminated and viewed by fluorescence microscopy, or the signal can be converted to a colorimetric reaction by the sequential application of an anti-fluorsecein antibody conjugated to peroxidase followed by a diaminobenzidine-hydrogen peroxide substrate.

In comparison to standard IHC methods, tyramide amplification methods have typically increased sensitivity by at least 50-fold or greater (15). As with any amplification method, background tends to increase along with signal.

Chapter 6.6 | Improved Catalysed Signal Amplification (iCSA)

The latest improvement of the CSA method to increase sensitivity and improve signal to noise ratio introduces a new more soluble substrate. It entails a background-reducing effect, combined with a crosslinker that enhances the precipitation of the substrate in step 3 (Figure 6.6) The fluorescein is conserved in the substrate while the tyramine is substituted with ferulic acid, which is a much better peroxidase substrate. Together these changes improve CSA method by maintaining high sensitivity and reducing background, giving high signal-to-noise ratio. Furthermore, the incubation time in each step can be reduced significantly making it possible to stain a tissue in less than one hour.

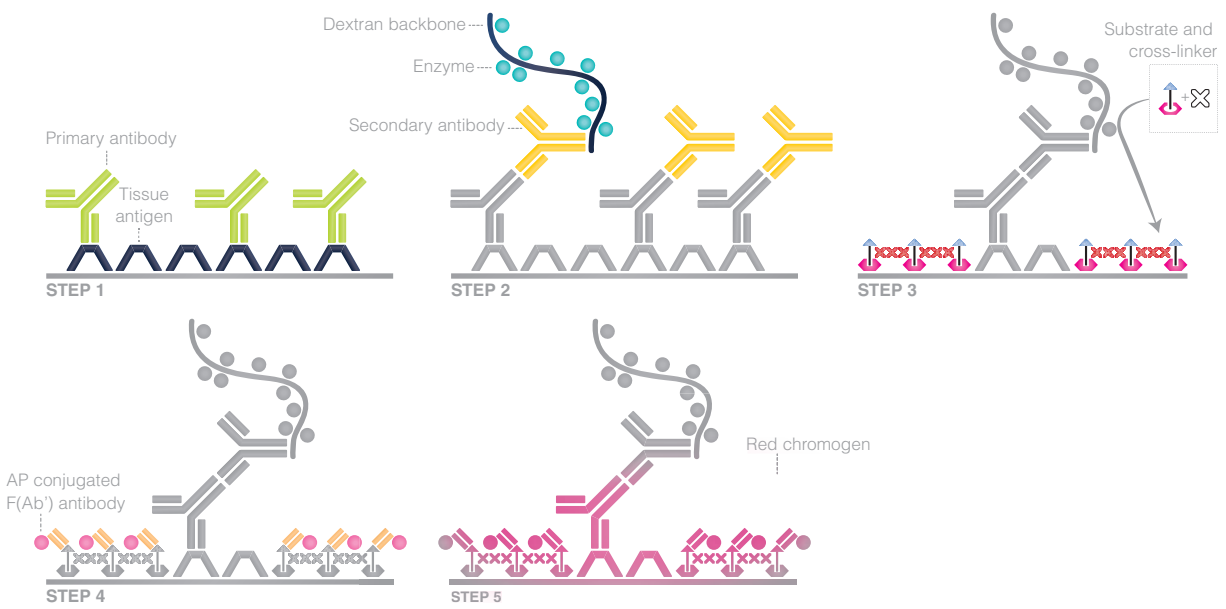


Figure 6.6 Improved CSA system (iCSA). A proprietary methodology developed by Dako.

PETITIONER EXHIBIT 1012
Page 83 of 218

Chapter 6.7 | Multi-Staining Immunohistochemistry

In some cases there is a need for knowledge about the relative localization of targets, which context can only be obtained by visualizing multiple targets in one slide. In other cases, the material available for staining is scarce and there is a need for multiplexing to retrieve all possible information out of material available.

Definition of Multi-Staining IHC

Multiple staining can be defined as the detection of two or more targets on one slide, thus increasing the information obtained from each slide and reducing turnaround time, compared to single staining or sequential staining (see definition below). This technique also makes it possible to assess the topographic relationship of two or more targets, for example, to determine whether targets are present in different cell populations, in different cells, in the same cell, or even in the same cellular compartment. In addition, multiple staining allows the combination of in situ hybridization (ISH) and IHC, giving information about a particular target both at protein level and DNA/mRNA level. Information can also be obtained on possible cell-to-cell spatial contacts of different cell types. Furthermore, with an increasing demand for less invasive sampling techniques and smaller and fewer specimens available, multiple staining has the advantage not only of conserving tissue, but also saving time and reagents.

Examples of Multiple Staining

The diagnosis of prostatic intra-epithelial neoplasia (PIN) is just one example of the clinical importance of multiple staining. Prostate needle biopsy is the preferred method for diagnosing early prostate cancer, but in some cases the diagnosis is uncertain because the biopsy includes only a few malignant glands, or a few hyperplastic or dysplastic glands that are difficult to distinguish from cancer (16, 17). Since basal cells typically are present in hyperplastic, and dysplastic glands, as well as around 'in situ' (PIN) lesions, but absent in malignant invasive glands, the demonstration of basal cells can be used to assist recognition, or exclusion, of invasive cancer. Basal cells are labeled using high molecular weight cytokeratin, cytokeratin (e.g. CK5/6 - cytoplasmic) or p63 (nuclear) immunostaining, or both. In addition, AMACR/P504S, is expressed in a high percentage of prostate carcinomas, but is negative or only weakly expressed in benign prostate tissue. Thus it is used as a positive cancer marker, often in a multiplex stain with keratin and p63 (see example in Figure 6.8). If single stains are done on serial sections, interpretation is much more difficult and ambiguous lesions may be absent in adjacent cuts, especially when dealing with small foci, with the result that some malignancies may remain undiagnosed. In this context, multiple staining protocols significantly improve the ability to distinguish between benign and malignant lesions. This approach, which reduces the percentage of ambiguous lesions and the need for additional biopsies, is being extended to facilitate recognition of other invasive cancers, as in breast.

Technical Challenges

Before embarking on a multi-staining project, some important issues should be considered:

- Most primary antibodies used today originate from either mouse or rabbit and are visualized using systems based on anti-mouse and anti-rabbit secondary antibodies. The challenge of distinguishing between two primary antibodies of the same species (mouse-mouse, or rabbit-rabbit) must be addressed, because separate mouse and rabbit primary antibodies to the chosen targets often are not available. Utilizing two primary antibodies of the same species can require quite elaborate protocols.
- Spectral differentiation of stain colors may be difficult, especially if the targets are co-localized leading to a mixture of colors (18). The 'mixed' color should contrast well with the two basic colors. In the case where a rare target is co-localized, the color reaction of the more abundant target will tend to dominate the other.
- Even if targets are not co-localized it is difficult to balance signals so as to enable visualization of a rare target in the same slide as highly expressed targets. An adjustment in concentration of the primary antibodies may solve this problem.
- If different targets are viewed under different magnifications, it may be difficult to obtain the desired topographic information.
- Image analysis approaches, such as 'spectral separation', are generally superior to the human eye in segregating the different color reactions in a multiplex stained slide.

PETITIONER EXHIBIT 1012
Page 84 of 218

Pre-treatment

Multiple staining, like single staining, can be performed on any of FFPE tissue sections, frozen sections, cell smears and cytospin preparations. Multiple staining may be constrained by the fact that it may not be possible to find a single tissue pre-treatment (retrieval) protocol that is optimal for all targets. In this case, it may be necessary to determine a method that allows all targets to be stained, although the method may be sub-optimal for some targets.

Multi-Staining Method Selection

To ensure success, IHC staining using multiple antibodies must be carefully planned. If primary antibodies of the desired specificity for the two (or more) targets are commercially available, and made in different species, then there are several different staining methods that one can choose. However, very often the choice may be limited by the reagents available (19). Care must be taken to avoid cross-reactivity between reagents; in the event that avoidance is not possible, then measures must be taken to minimize the risk, including additional controls to detect significant cross reactivity if present.

In general, staining methods can be divided into the following classes:

Sequential staining

By this method, one staining procedure succeeds another. For example, the first antibody is applied to the tissue section followed by a labeled detection system such as streptavidin-biotin horseradish peroxidase (HRP), with a chromogen such as DAB. The second primary antibody is applied only after the excess DAB is rinsed off, followed by labeling with a streptavidin-biotin alkaline phosphatase (AP) detection system and a colored chromogen. The biggest advantage of sequential staining is that by this procedure problems related to cross-reactivity are minimized, possibly due to steric interference.

A sequential staining is shown in Figure 6.7. Here, the primary and secondary antibodies from the first staining were eluted before the staining of the next target was performed. The disadvantages of sequential staining are: the method cannot be used for co-localized targets, the technique often leads to a long staining protocol and carries an inherent risk of incorrect double staining due to incomplete elution of unreacted reagents from the first staining sequence, before application of the next reagents.

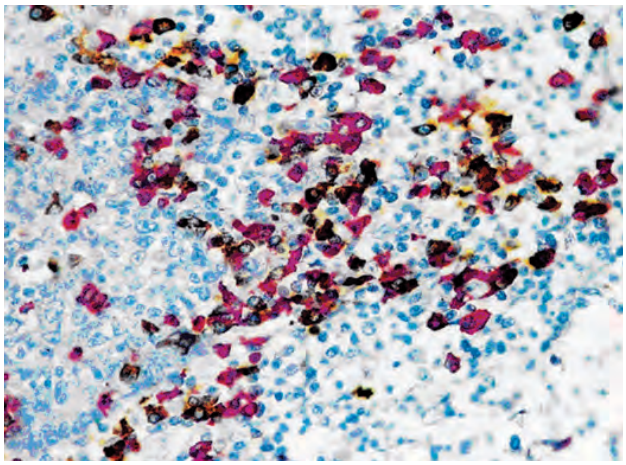


Figure 6.7 Sequential double staining method performed with the EnVision™ GI2 Doublestain Kit using polyclonal anti-kappa light chains (red) and polyclonal anti-lambda light chains (brown) as primary antibodies. FFPE tissue sections from tonsils.

Elution may become an issue with some high-affinity primary antibodies, as these may remain at their binding-site, leading to spurious double stained structures. Elution also risks denaturing epitopes of antigens to be visualized subsequently. Furthermore, for some chromogens there is a risk that the first chromogen (DAB in particular) may shield other targets. This technique is, therefore, not recommended for evaluation of mixed colors at sites of co-localization, because not all reaction products are capable of surviving the rigorous washing required to remove the antibodies. To avoid such problems and blurry staining results, it is recommended to use the most 'robust' dyes such as DAB, Fast Red, AEC and Blue chromogen first, followed by other less 'robust' dyes.

Simultaneous staining

In a simultaneous double stain, the primary antibodies can be applied simultaneously. The advantage of this method is that it is less time-consuming because the reagents can be mixed together. However, the technique can only be used if the primary antibodies are from different species, or are directly labeled with different enzymes (20).

PETITIONER EXHIBIT 1012
Page 85 of 218

A simple example of the direct method is when the primary antibodies are fluorescence-labeled with fluorochromes emitting different colors to allow direct visualization of two or more targets. This avoids cross-reactivity, but is rarely practical since some form of amplification is necessary to get sufficient fluorescent signal. Alternatively, the primary antibodies may be conjugated directly with enzymes, biotin or haptens, subsequently employing the corresponding secondary antibody or streptavidin reagent. This approach is less time-consuming than the sequential method, because primary and secondary antibodies can be mixed together in two incubation steps. However, it requires avoiding all cross-reactivity.

With the indirect method it is also possible to apply time-saving antibody 'cocktails' because the primary antibodies are recognized by different secondary antibodies. Generally, it is advantageous to use secondary antibodies raised in the same host in order to prevent any unexpected interspecies cross-reactivity at the level of the secondary antibody. One example of such a system is the EnVision™ DuoFLEX from Dako. This system applies a mixture of primary antibodies of mouse and rabbit origin, followed by a mixture of the secondary goat-anti-mouse and goat-anti-rabbit antibodies labeled with HRP and AP, respectively. Finally, the chromogens are applied sequentially. The result is a double stain where the primary mouse antibodies are stained brown with DAB and the primary rabbit antibodies are stained red with Permanent Red (for an example, see Figure 6.8). The system has been developed for Dako's line of RTU cocktails of primary antibodies, but may also be used with other antibody cocktails or individual antibodies that are sequentially incubated on a single slide.

Multi-step technique

This is an indirect/direct method combining unlabeled primary antibodies with directly-conjugated antibodies (3). The method starts with staining of the unlabeled antibody/antibodies with the appropriate detection system, but without performing the final enzymatic staining reaction. The tissue is blocked with normal serum from the host of the first primary antibody before the second, directly-labeled primary antibody is added. The staining ends with the two enzymatic reactions being performed sequentially.

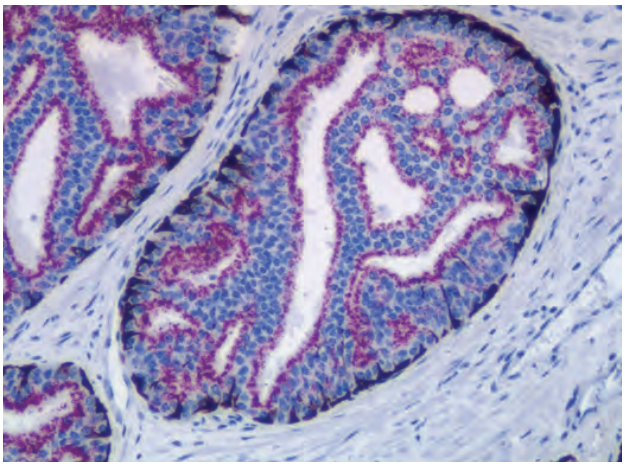


Figure 6.8 Simultaneous double staining performed with EnVision™ DuoFLEX using an antibody cocktail containing monoclonal rabbit anti-AMACR (red), monoclonal mouse anti-HMWCK and monoclonal mouse anti-CK 5/6 (brown/black). FFPE tissue sections from prostate.

Multi-step staining can be used when the selection of primary antibodies is limited. However, when using this method it is not possible to mix reagents. Users will often find that the choice of staining method is limited by the availability of the primary antibodies with respect to species origin or label.

Difficulties arise when targets are known or suspected to be co-localized, and the only available primary antibodies are unlabeled monoclonal mouse antibodies of the same IgG subclass. In that case, none of the techniques described above are applicable.

One solution for such circumstance is the Dako Animal Research Kit (ARK™), which contains reagents for labeling mouse primary antibodies with a biotinylated anti-mouse Fab fragment, followed by blocking of the remaining reagent with normal mouse serum. This approach can be applied to the tissue as part of the multi-step technique (21). The kit uses a non-covalently labeled antibody, thus avoiding the risk of reducing affinity. In addition, only small amounts of primary antibody are needed and the kit does not require time-consuming purification steps.

Another solution is Zenon Technology (Invitrogen) developed for flow cytometry. It essentially uses the same technique and offers labeling kits for mouse primary antibodies, available as enzyme conjugates or conjugated to one of a wide variety of fluorescent dyes.

PETITIONER EXHIBIT 1012
Page 86 of 218

Finally, it is important to be aware of the fact that visualization systems with dual recognition such as the EnVision™+ Dual Link system do not discriminate between species, and thus are only suitable for multiple staining when using the sequential method. Visualization kits with amplification layers that are not clearly specified should be avoided, since possible cross-reactivity cannot be predicted.

Chapter 6.8 | Selection of Dyes

The primary choice to make when deciding how to make the targets visible is whether to use immunoenzyme staining or fluorescence. Both have advantages and disadvantages and in the end, decisions should be made based on conditions of the individual experiment.

Chromogenic Dyes

Table 6.2 Examples of enzyme/chromogen pairs suitable for triple staining.

Enzyme	Chromogen	Color
Gal	X-Gal	Turquoise
AP	Fast Blue BB	Blue
HRP	AEC	Red
HRP	DAB	Brown
Gal	X-Gal	Turquoise
AP	Liquid Fast Red	Red
HRP	DAB	Brown
AP	New Fucsin	Red
HRP	TMB	Green

Gal (beta-galactosidase); X-Gal (5-bromo-4-chloro-3-indolyl β-galactoside); AP (alkaline phosphatase); HRP (horseradish peroxidase); AEC (3-amino-9-ethylcarbazole); DAB (3,3'-diaminobenzidine); TMB (3,3',5,5'-tetramethylbenzidine)

When selecting color combinations for multiple staining with chromogenic dyes, it is advisable to choose opposing colors in the color spectrum such as red and green to facilitate spectral differentiation. If using a counterstain, this color must also be included in the considerations. When working with co-localized targets, dyes must be chosen so that it is possible to distinguish the mixed color, from each individual color. Double staining using chromogenic dyes is well-established, but if the targets are co-localized then a percentage of the single colors cannot be easily identified. For triple staining, it is naturally more difficult to choose colors that can be unambiguously differentiated, and even more so if targets are co-localized. In such cases, a technique known as spectral imaging may be applied (18,19). Spectral imaging allows images of the single stains to be scanned and by using specialized software algorithms the colors are unmixed, thereby displaying the distribution and abundance of the individual chromogens.

Visualizing Low Expressed Targets

A narrow dynamic range is a disadvantage for immunoenzymatic staining. The precipitation process, which is crucial for this method, is only triggered at a certain threshold concentration of substrate and product. On the other hand, at high concentrations the precipitated product may inhibit further reaction. Therefore, it is difficult to visualize rare targets and highly abundant targets in the same slide. To ease this problem, catalyzed signal amplification - an extremely sensitive IHC staining procedure can be used (Figures 6.5 and 6.6). The method can bring low expressed targets within the same dynamic range as high expressed targets.

Fluorescent Dyes

Double immunofluorescence labeling is quite well established (22). Some of the same considerations as for chromogenic dyes apply when working with immunofluorescence. It is equally necessary to select dyes with distinguishable spectral properties. However, there are more colors available and the emissions spectra of the fluorescent molecules are narrower than the spectra of the chromogenic dyes. It is possible to have more stains on one slide with fluorescent dyes than it is with chromogenic dyes, which is one of the main advantages of fluorescent dyes in multistaining. The use of multiple fluorescent colors is also well established in FISH and flow cytometry. When using fluorescence dichroic excitation/emission, filters are employed to separate the different fluorescent signals. The spectral separation can be aided by digital compensation for overlapping emission spectra. In addition, new fluorescence microscope systems can separate the spectral signatures of up to eight fluorochromes without any problems, using multi-spectral imaging techniques such as emission fingerprinting (23).

PETITIONER EXHIBIT 1012
Page 87 of 218

When staining co-localized targets, fluorescent dyes may allow separate identification of targets. This makes it possible to discern targets even in very different concentrations, whereas subtly mixed colors from chromogenic dyes may easily pass unnoticed with immunoenzyme staining.

Thus immunofluorescence has some advantages, but there are also inherent problems; mainly loss of morphologic detail, which may determine the choice technique for a multi-staining application.

Alternative dyes

Alternatives to the conventional chromogenic dyes are colloidal gold-labeled antibodies that can be used with bright field microscopy, with silver enhancement, Green Fluorescent Proteins (GFP and their variants), and Quantum dots. The latter, especially, has been found to be superior to traditional organic dyes on several counts, such as brightness (owing to the high-quantum yield), as well as their higher stability (owing to less photodestruction). They can be linked to antibodies or streptavidin as an alternative to fluorochromes (24). However, the size of these conjugates poses problems of steric interference and diffusion, in terms of getting these inorganic particles into cells or organelles.

Chapter 6.9 | Automated Image Acquisition and Analysis in Multiple Staining

Digital image analysis will increase the number of usable dyes because it does not rely on the human eye for detection and differentiation. A digital image is acquired at excitation wavelengths relevant for the dyes applied, and separate detectors record individual colors. Thus, digital image analysis will allow the combination of both fluorescent and immunoenzyme dyes (25). Detectors, however, have biased color vision. They amplify colors differently than does the human eye. Therefore, dyes used in image analysis should be optimized for the best fit possible with the detector's filter properties.

Image analysis systems incorporate algorithms that allow compensation for overlapping emission spectra, comparable to flow cytometry. They also allow signal gating within a range of wavelengths of interest, enabling users to see only signals within the desired range. Visualizing a combination of several gates, with the color selected independently of the dyes used for staining, may clarify pictures and facilitate interpretation. This capability also makes it possible to set a threshold on signal intensity, to exclude non-specific staining or background staining from final images. A more thorough discussion of image acquisition and analysis can be found in Chapter 7.

Chapter 6.10 | Immunofluorescence

Immunofluorescence (IF) is a common laboratory technique used in almost all aspects of biology. This technique, based on pioneering work by Coons and Kaplan (26, 27), and later by Osborne (28), has been widely both in research and clinical diagnostics. Applications include the evaluation of cells in suspension, cultured cells, frozen tissue, FFPE tissue, beads, and microarrays for the detection of specific proteins. In IF techniques, antibodies are chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). As in the enzymatic methods these labeled antibodies can be use directly or indirectly to bind to the antigen of interest, which allows for antigen detection through fluorescence techniques. The degree of fluorescence can then be quantified using a flow cytometer, array scanner, or automated imaging instrument, or visualized using fluorescence or confocal microscopy. IF techniques can be used on both fresh and fixed tissue samples, though the latter present problems of autofluorescence.

Table 6.3 Advantages and disadvantages of direct and indirect immunofluorescence.

	Direct Immunofluorescence	Indirect Immunofluorescence
Pros	Simpler	Higher signal (amplified)
	Antibodies from the same species	Flexibility (array of fluorescent colored secondary antibodies)
		Low costs
Cons	Lower signal	More steps
	Higher costs	Antibodies from the same species cannot be used together
	Less flexibility	Background may be amplified

PETITIONER EXHIBIT 1012
Page 88 of 218

Principle of Fluorescence

Fluorescence and phosphorescence are both types of luminescence. When molecules with luminescent properties absorb light, they emit light of a different wavelength. With fluorescence the emission of light occurs extremely rapidly after the absorption of excitation light, whereas with phosphorescence emission continues for milliseconds to minutes after the energy source has been removed. Fluorescent materials give off light because of their atomic structure. Electrons are arranged in discrete energy levels surrounding the atom's nucleus, with each level having a predetermined amount of energy. When an electron absorbs the energy from a photon of light (Figure 6.10) it becomes 'excited' and jumps to a higher, less stable, energy level. The excited state does not last long. The half-life of the excited state is generally less than 10 seconds. The electron loses a small amount of energy as heat, and the remainder of the extra energy is given off in the form of a photon. The emitted fluorescence has a lower energy than the absorbed light, so the wavelength of the emitted light is longer than that of the excitation light.

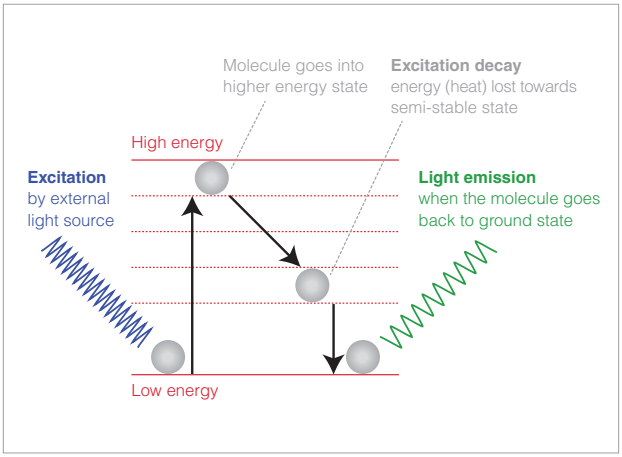


Figure 6.10 Principle of fluorescence.

A range of wavelengths of light can excite the electrons of a fluorochrome. For example, fluorescein will fluoresce when hit by light with any wavelength between 450 nm and 520 nm. However, the closer the excitation wavelength is to 495 nm, the

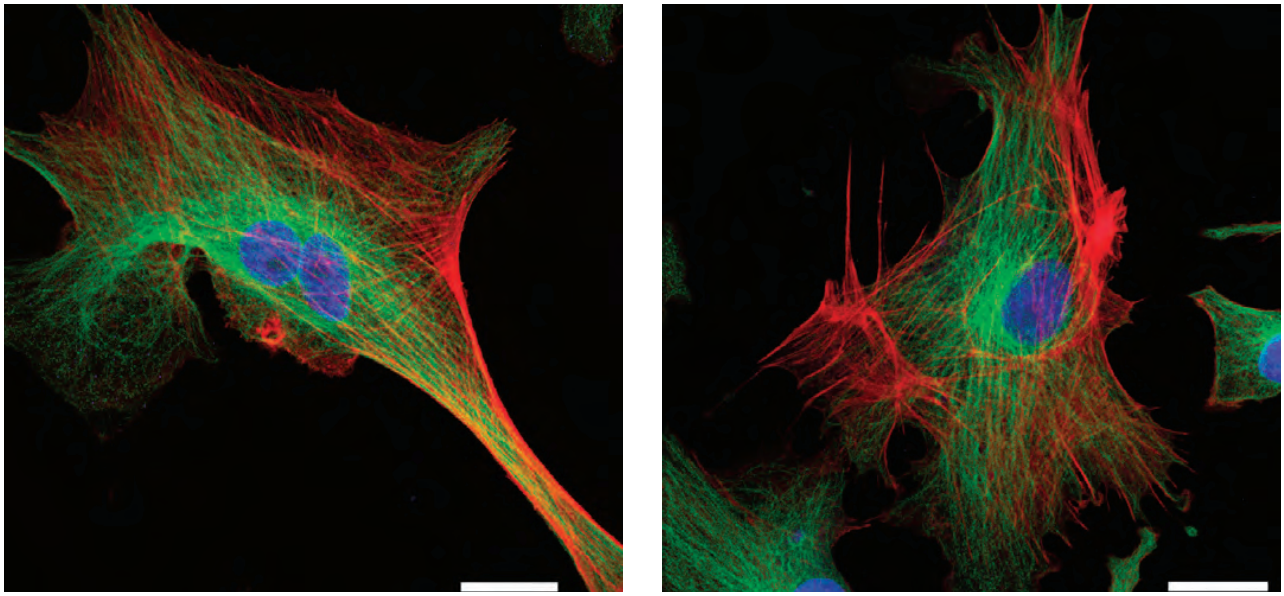


Figure 6.9 Cultured pulmonary artery endothelial cells stained for tubulin (red), actin (green) and DNA (blue). The dual immunofluorescence procedure used rabbit-anti-actin and mouse-anti-alpha tubulin as primary antibodies. The secondary antibodies used were Texas Red-conjugated goat, anti-rabbit IgG and FITC-conjugated goat, anti-mouse IgG. The sample was also stained with the DNA-specific dye Hoechst 33342. Scale bar is equal to 20 microns.

PETITIONER EXHIBIT 1012
Page 89 of 218

more fluorescence will be produced. This optimal wavelength is called the excitation peak. Similarly, the light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein ranges from 490 nm to 630 nm, and the emission peak is approximately 515 nm. Since the phenomenon of fluorescence was first explained in 1852 by a British scientist, Sir George Stokes, the shift in wavelength from short to long during fluorescence is called "Stokes shift" (Figure 6.11).

Some fluorochromes have a small Stokes shift, while other fluorescent compounds have large Stokes shifts. For example, the fluorochrome fluorescein can be excited by blue-green light, and its Stokes shift is only about 20 nm, which means that the light emitted is green. This contrasts with another fluorochrome, phycoerythrin, which also can be excited by blue-green light, but has a large Stokes shift and thus the light will be emitted in a different color (yellow).

Photobleaching

As with most fluorescence-based techniques, a significant problem with immunofluorescence is photobleaching. Photobleaching is when the fluorophore looses its ability to fluoresce. This photochemical destruction is due to the generation of reactive oxygen species in the specimen as a byproduct of fluorescence excitation (Figure 6.12). Photobleaching can be minimized by: (a) decreasing the excitation light in both intensity and duration, (b) reducing the availability of singlet oxygen ($^1O_2$) by the addition of singlet oxygen scavengers (= antifade reagents), and (c) using a low concentration of a fluorochrome with high-quantum efficiency.

Autofluorescence

Biological autofluorescence in mammalian cells due to flavin coenzymes (FAD and FMN: absorption, 450 nm; emission, 515 nm) and reduced pyridine nucleotides (NADH: absorption, 340 nm; emission, 460 nm) can be problematic in the detection of fluorescence probes in tissues and cells. Fixation with aldehydes, particularly glutaraldehyde, can result in high levels of autofluorescence. This can be minimized in fixed cells by washing with 0.1% sodium borohydride in phosphate-buffered saline (29) prior to antibody incubation. Problems due to

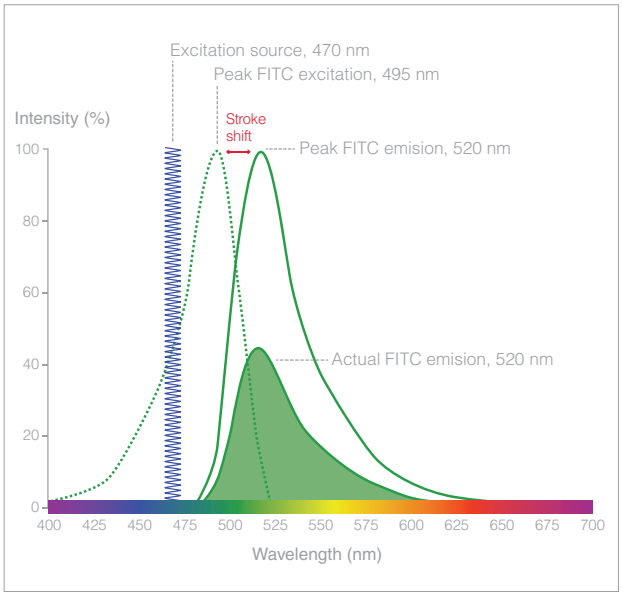


Figure 6.11 Excitation and emission spectrum of fluorescein. When fluorescein is excited at a wavelength other than its peak excitation (470 nm in this example), the shape of the emission curve (darker green) remains the same, but the relative intensity is reduced. The efficiency of the excitation at 470 nm is 45% of peak excitation.

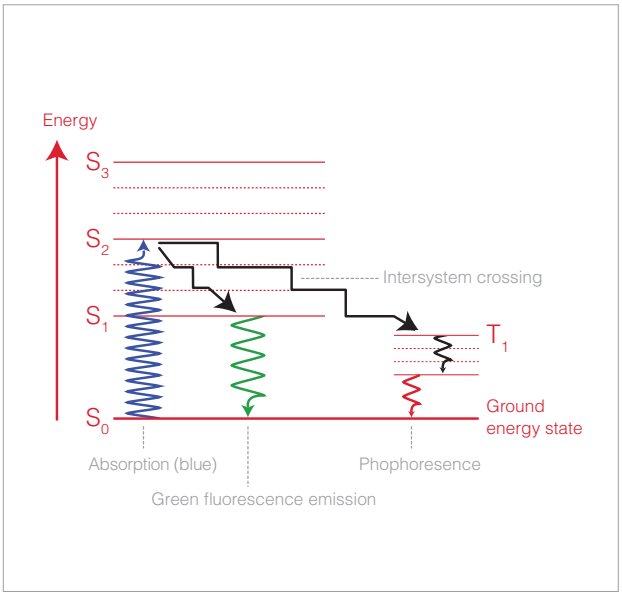


Figure 6.12 Illustration of how a singlet-excited state can convert to a triplet-excited state. Photobleaching is the irreversible decomposition of the fluorescent molecules in the exited state because of their interaction with molecular oxygen prior to emission.

PETITIONER EXHIBIT 1012
Page 90 of 218

autofluorescence can be minimized by selecting probes and optical filters that maximize the fluorescence signal relative to the autofluorescence. Other factors that limit IF include the performance of the detection instrument (i.e. how well the microscope has been calibrated and set), the specificity of the antibodies, and the specimen preparation.

Fluorescence Overlap

One of the problems that must be dealt with when measuring fluorescence of more than one color is the possibility that the emission signals overlap. It is necessary to remove the overlapping signal or it will give a false level for one or more colors. For example, as shown in figure 6.14, there is significant overlap when using FITC and PE. A range of wavelengths will be collected for each detection channel. In the figure, these are identified as the FITC detector bandwidth and the PE detector bandwidth. These band-pass optical filters will allow photons within this wavelength range to reach the detector. However, as can be seen in Figure 6.14, there is a very small amount of PE fluorescence, which is within the FITC band, and similarly a small amount of FITC fluorescence within the PE band. These unwanted signals must be electronically removed or the measurement for each detector will overestimate the actual signal. This process is called fluorescence compensation and can be automatically calculated in many detection systems using single positive controls.

Applications of IF in Pathology

Some practical applications of IF in diagnostic pathology are:

- Analysis of protein antigens in fresh, frozen or, less often, fixed tissues; sub-cellular localization of protein antigens in tissue culture monolayers; and observation of bacterial or parasitic organisms. Immunofluorescence is primarily used in the research setting, or in clinical research setting, on frozen tissue. In particular where antibodies compatible with formalin fixation and paraffin embedding have not been developed.
- A major practical use is for fluorescence in situ hybridization (FISH), fluorescent labeled DNA is used to detect gene aberrations in cells.

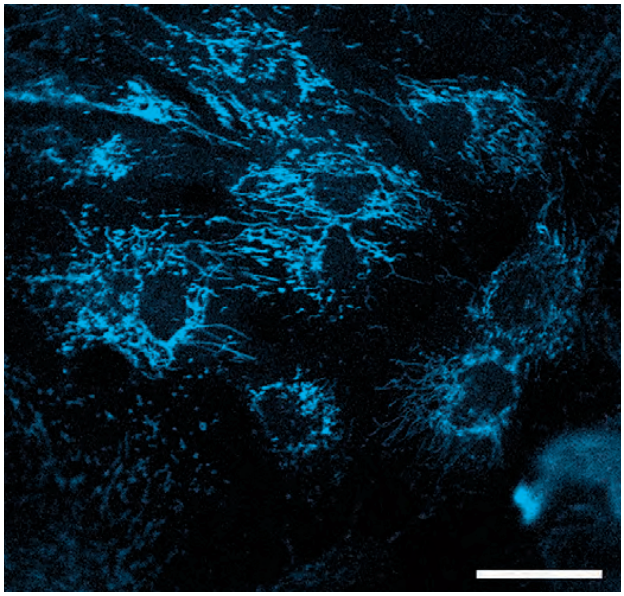


Figure 6.13 NADH autofluorescence in a human colon carcinoma cell line (HCT116). Ultra-violet excitation at 363 nm was used and the emitted fluorescence greater than 440 nm was collected. Scale bar is 10 microns. Courtesy of Giselle M. Knudsen, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA.

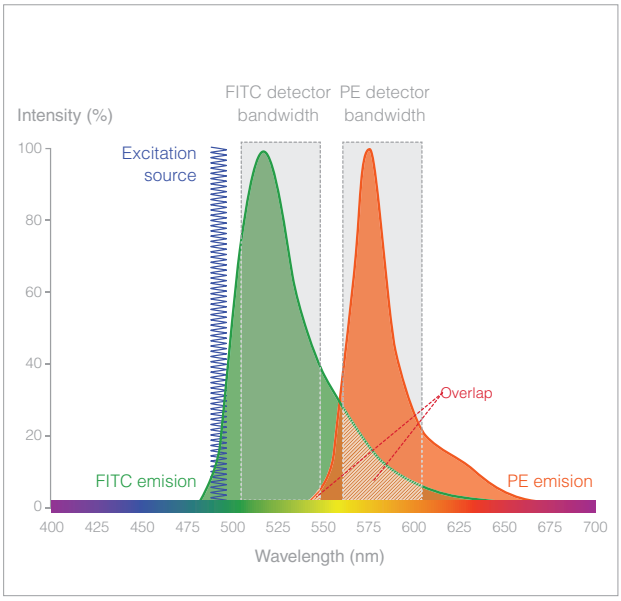


Figure 6.14 Fluorescence overlap of FITC and PE.

PETITIONER EXHIBIT 1012
Page 91 of 218

- Immunofluorescence potentially has a wider dynamic range than immunoenzyme staining, as there is no enzymatic amplification involved and thus the dynamic range is determined solely by the sensitivity of the detectors (25). Quantitative immunofluorescence staining coupled with digital scanning of slides and image analysis algorithms have been utilized to create an automated quantitative immunofluorescence technique which has been applied in various studies (30).
- Multi-staining (see multi-staining section)
- Visualization of cell structures by super resolution microscopy

Chapter 6.11 | Future Perspectives

The IHC technique continues to undergo evolution and improvement, driven by ongoing demands of reproducibility, sensitivity and quantification. Today, automated systems enable standardized visualization of targets in tissue with increased sensitivity and improved signal to background ratio. Chromogen, fluorescence and multistain technologies are being employed. Increasingly, stained slides are submitted for digital scanning and signals quantified using image analysis algorithms. The demand for more information from each slide, to conserve available tissue, will inevitably lead to increasing use of multistaining technologies in the pathology laboratories.

In addition, targeted therapies have created a need for more quantitative biomarker information, launching a rapidly growing range of new types of IHC tests, variously termed 'prognostic markers', 'predictive markers', 'companion diagnostics' or 'advanced personalized diagnostics' (Chapter 11). Thus, future IHC-based tests will increasingly rely upon standardized, approved kits and reagents, in combination with an automated image analysis system for the evolution into quantitative pathology.

Acknowledgements

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PETITIONER EXHIBIT 1012
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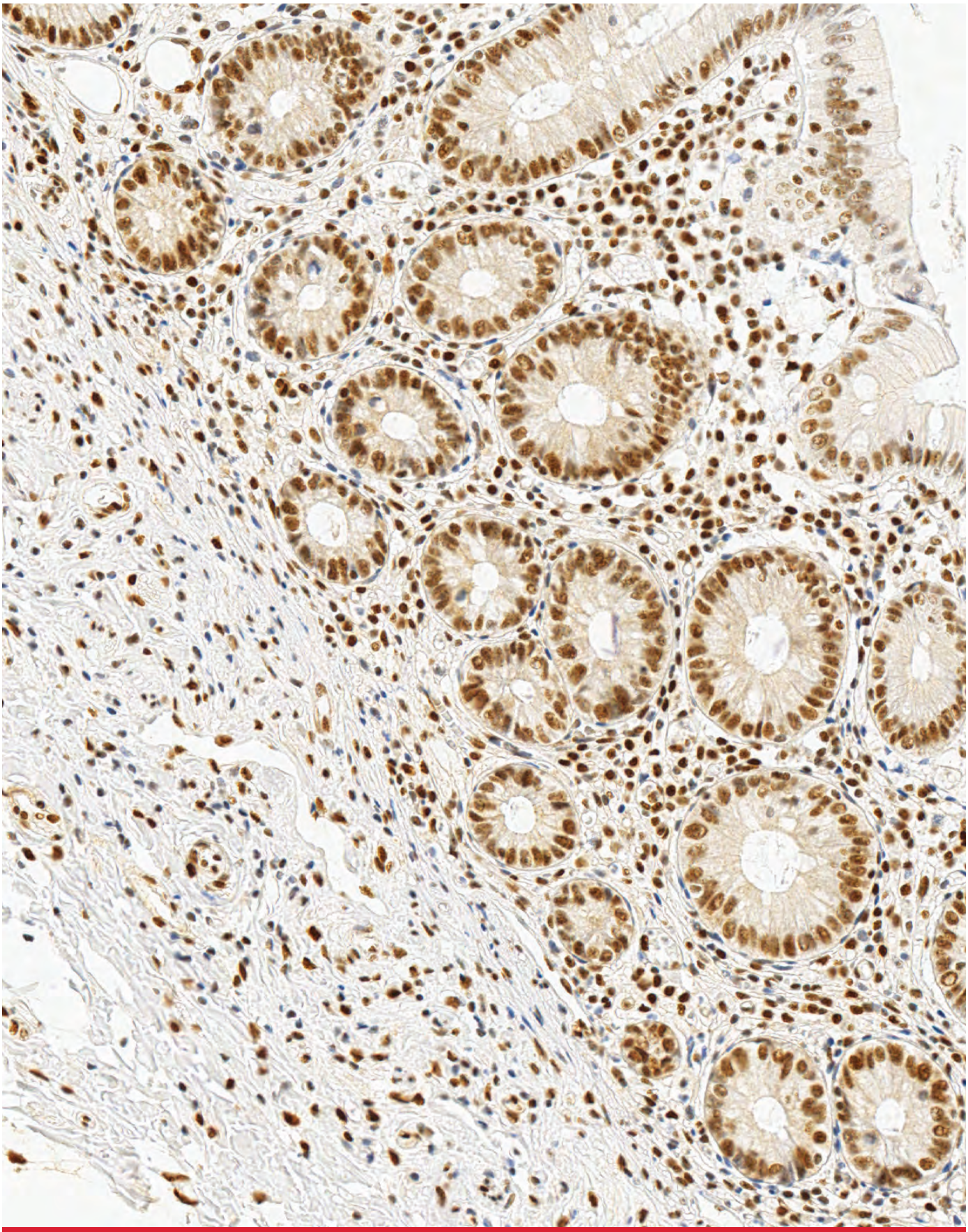
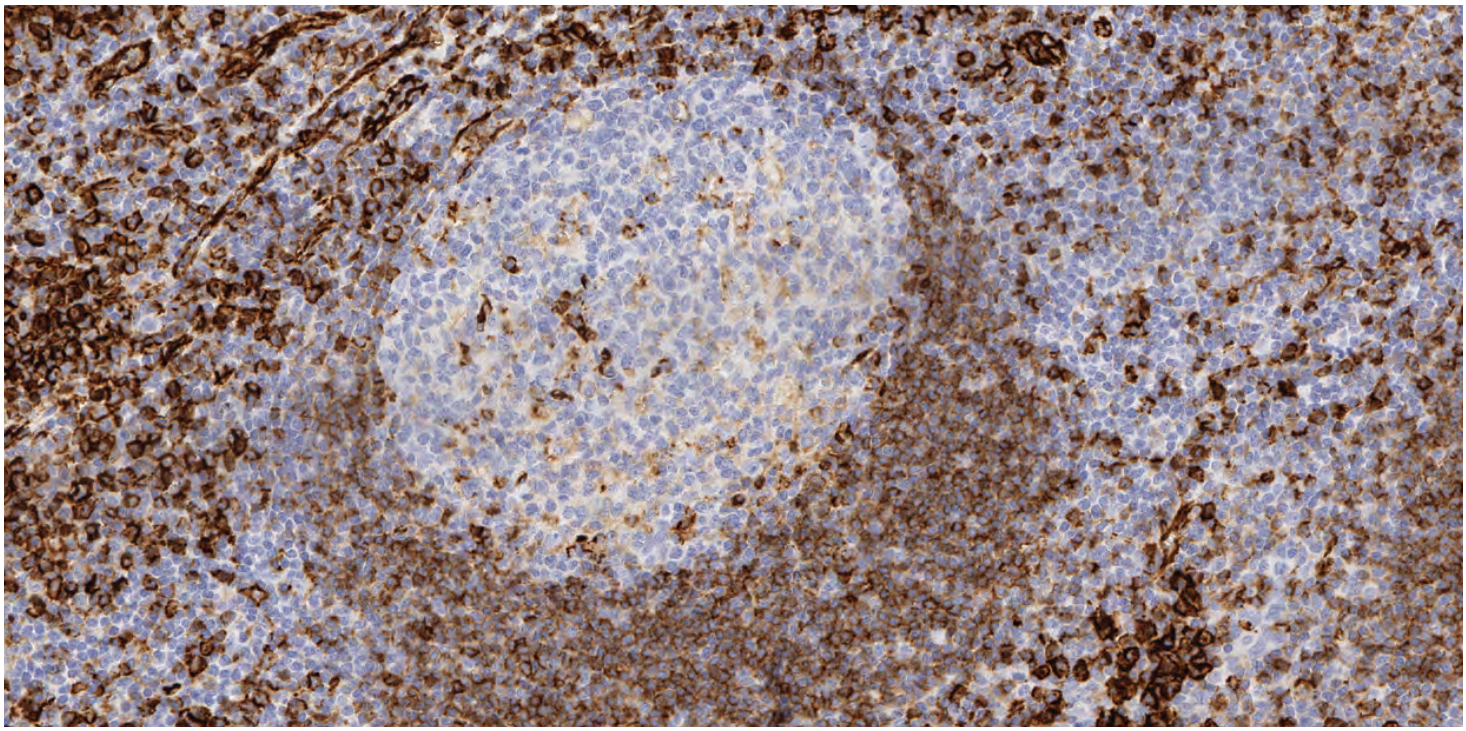
PETITIONER EXHIBIT 1012
Page 93 of 218



Chapter 7

Digital Pathology

Joachim Schmid, PhD
Clive R. Taylor, MD, DPhil



Dig•i•tal Pa•thol•o•gy *(n.)*
A dynamic, image-based environment that enables the acquisition, management and interpretation of pathology information generated from a digitized glass slide.

The Digital Pathology Association web site

Chapter 7.1 | Microscopy – From Analog to Digital

Early in the 19th century, improvements in the manufacture of lenses allowed production of affordable, high quality, compound microscopes (Figure 7.1). The impact on the biological sciences and on medicine was enormous. Pathology, a new branch of medical sciences was born, with surgical pathologists effectively practicing 'image analysis' on a personal, subjective basis.

Figure 7.1 Early image analysis device.

One hundred and fifty years later, the accumulated literature and experience of surgical pathology has grown to such an extent that it is the 'gold standard' for diagnosis of many diseases. Even in this molecular age, cancer treatment is rarely initiated without a tissue diagnosis. However, the methods of microscopic examination have changed relatively little in more than a century (1). Hematoxylin and eosin, and other stains in common use today, were first introduced in the 1850s. Immunohistochemistry was utilized in routinely processed FFPE tissues in 1974, and entered general use in the next two decades. In 2013, pathologists are still performing cancer diagnosis by examining glass slides at a microscope. However, new computer-based technologies offer the real prospect of radical change.

Radiology and pathology share a foundation of interpreting images for diagnostic purposes. A little over a decade ago, radiology converted to a digital format over a remarkably short period of time. The change was technology driven. It radically transformed the way in which radiology is practiced today, and as such provides a glimpse into the future of digital slides in pathology. To date, digital imaging has found only limited application in pathology, primarily for education and research, with diagnostic use limited to isolated telepathology applications, and a few immunohistochemical 'quantitative analyses'. A crucial difference from radiology is that pathologists begin, not with a digital file, but with a piece of tissue, that must be fixed, embedded, cut and stained, prior to obtaining a digital image. Current pathology practice is that glass slides are distributed to the pathologist, and diagnosed by the usual light microscopic approach, as they have been for a hundred and more years. Selected slides may then be digitally scanned on an elective basis, for research or educational purpose, or for performance of quantitative algorithms. Until recently, the scanning time has been prohibitively slow using digital imaging methods, adding greatly to the time required for a pathologist to complete a case. But all this is changing in the face of rapid improvements in scanning technology, both hardware and software.

Chapter 7.2 | Automation of Microscopy

Automated manual microscopy has been evolving since the first demonstration of telepathology in 1968. The concept has evolved along two distinct pathways, one driven by technology, the other by need. The technology field focused on adapting new knowledge developed for other disciplines in an effort to prove that automated microscopy was possible. Improvements in digital camera resolution, speed and fidelity, the invention of the robotic microscope and stage, the development of the Internet and the exponential advancements in computer technology, including processor speed, memory and storage, all have contributed to eliminating the hurdles that delayed development of a viable automated microscopy system. The second group focused on need, with the goal of responding to demands in anatomic pathology, such as rapid second opinion, pathology staffing of remote frozen sections, cost reduction for reference laboratories, medical student and resident teaching, continuing medical education, and improved storage and retrieval of slides (2-8).

PETITIONER EXHIBIT 1012
Page 96 of 218

The concept of developing a functional robotic telepathology network, with the aim of providing real-time expert opinions for frozen sections and difficult cases was put forth by Ronald Weinstein in the mid 1980s (9, 10). Performance studies supported the feasibility of telepathology, and a demonstration project for the U.S. Department of Defense showed how slides prepared in El Paso, Texas could be read remotely via satellite, at the Armed Forces Institute of Pathology in Washington, D.C. Although the prototype was successful, there were many limitations to commercialization, most involving a lack of telecommunication infrastructure and standards. Since it was not cost effective to purchase a satellite, cheaper alternatives were sought, and the concept of "static telepathology" was introduced as an alternative. In static telepathology, a pathologist captures and saves a digital image (photomicrograph), using a camera mounted on a microscope, and then forwards one or more images to a remote computer, where they may be reviewed by a second pathologist. E-mail and servers were available to facilitate the transfer of images, and standards in image formats evolved rapidly. However, the static nature of the images and the inability of the viewing pathologist to examine other fields and to change magnification, severely limited clinical use. In practice, thousands of static images or photomicrographs are required to fully represent a standard pathology slide, and a method was required to organize and display them, so as to represent a facsimile of the original slide.

As a result, only few pathologists used these systems diagnostically, although many used digital photomicrographs for tumor boards, teaching and other educational purposes.

The next major technical step was the creation of 'stitching' software, which allowed the digital representation of an entire microscopic slide by digitizing individual microscopic fields and then stitching them together to create a virtual slide. This process was laborious and time consuming and the computer processing and storage requirements pushed up against the limits of then available technology. As a result of these limitations, the next systems created were hybrids, containing elements of both static and dynamic systems. These systems digitized an entire slide at low power magnification, creating a tissue map for the pathologist to select areas of interest, which would then be re-digitized at higher magnification and forwarded as a series of static images.

Chapter 7.3 | Slide Scanning

The first step in digital pathology is to obtain a digital representation of a pathology slide. The scanned image or images are saved as two-dimensional digital files. However, pathology slides and the tissue on pathology slides are three-dimensional structures, albeit that the third dimension is only a few microns. With a low power magnification lens (such as a 5x lens), scanning a single focal plane is often sufficient for capturing this three-dimensional tissue with acceptable focus. With higher magnification (such as a 40x) lenses, the depth of focus is shallow, necessitating that the scanning system has the capability to automatically focus on, select and capture a single focal plane. In addition, more sophisticated systems in fact capture several single focal planes, a process known as Z-stacking, that allows the viewing pathologist a good replicate of focusing up and down through the tissue section.

All modern systems have the ability to autofocus; however, they all do it in a slightly different manner. A second camera can be used to monitor and adjust the focus continuously as the slide is scanned. Or a map of the tissue can be made available on the slide to be scanned, and 'points of focus' can be created. Then, as the slide is scanned, the system performs an autofocus at each calculated focus point. In this way, systems may overcome problems such as variations in thickness of the tissue or tissue folds.

The process of scanning a slide also differs between various systems. All processes involve acquiring multiple images in some fashion and then stitching them together to create a representation of the slide. Some systems acquire images as tiles, while other systems use methods such as line scanning.

Scanning systems generally are judged by two criteria: speed and resolution. The total speed of acquisition involves not only acquiring the image (scan time), but also entails stitching the images together, image compression and storing the resulting image on a computer for viewing. Since the purpose of acquiring a virtual slide is to view or analyze it in the future, some additional processing is often implemented to facilitate this viewing process. For example, to implement the full functionality of a standard light microscope, the scanned image must be viewable at similar mag-

PETITIONER EXHIBIT 1012
Page 97 of 218

nifications found on a standard microscope, namely, 1x, 2.5x, 5x, 10x, 20x and/or 40x. Assuming the slide is scanned, stitched and stored at the equivalent of a 40x objective, viewing the 40x image does not require additional processing. However, to view the image at the equivalent of a 5x objective, the 5x image must be derived from the 40x image before it can be displayed. This process results in a significant delay between the time an area is selected and the time it is displayed on a monitor. To reduce this delay, the acquired image is processed to a format where the intermediate magnification levels have already been calculated and stored. While this format, known as a pyramidal format, decreases the time to load intermediate magnification views, it increases the image processing (time) necessary following image acquisition and produces a larger file to be stored (11). An image format for digital pathology has been developed as a DICOM format (Digital Imaging and Communications in Medicine), a standard for distributing and viewing medical images.

The ability of the image acquisition system to resolve features present in the microscopic slide is known as the absolute or "point-to-point resolution" and is dependent on the microscope objectives, the camera lens and the analog to digital conversion process. What is more important is the actual resolution, as appreciated by the observer, which is also dependent on hardware and software compression techniques and on the characteristics of the monitor used to display the image. In radiology these monitors are subject to standards, not yet established and accepted for pathology.

Chapter 7.4 | Digital Slide Review

A digital slide is a digital representation of a glass slide that can be viewed on a screen at any location. Different systems have different approaches to display information. Not only is the digital slide information displayed, but patient and case information can be displayed, as well as multiple images and/or thumbnails of multiple slides. These interfaces have been termed 'pathology cockpits' as they allow the pathologist to work in one location on all aspects of a case. The user interface allows to perform similar tasks such as changing the magnification, moving the slide in any direction, saving regions as image files, annotating specific areas of the slide, launching image analysis applications and generating reports.

Chapter 7.5 | Applications in Digital Pathology

Like applications (apps) for smartphones, new software for image analysis will be utilized. Routine use of these apps (mostly algorithms) will extend the capabilities of pathologists beyond subjective morphologic criteria, and beyond the present compass of the human eye (1).

Applications for digital pathology today include, but are not limited to:

- Quality assurance of routine and IHC slides, using objective measurements of color and intensity

Figure 7.2 Philips Ultra Fast Scanner, an example of a digital pathology scanner with a time to display of less than one minute at 40x magnification.

Figure 7.3 Pathology cockpit by Omnyx, LLC, A GE Healthcare and UPMC Joint Venture. An example of a digital pathology interface.

PETITIONER EXHIBIT 1012
Page 98 of 218

- Image analysis and quantification
- Telepathology; remote viewing and sharing of digital slides
- Obtaining a second opinion in difficult cases (consults)
- Obtaining a rapid second opinion (as for frozen sections)
- Reports including images for the diagnostic report (archiving and recovery)
- Tumor boards
- Internal and external quality assurance at the interpretative level (diagnostic review)
- Research; drug development, clinical trials, 3D pathology
- Education; medical students, residents and CME

This growing range of applications will be considered here primarily with reference to immunohistochemistry.

Chapter 7.6 | Image Analysis and Quantification

Collecting an image for subsequent analysis has more rigorous requirements than the capturing of images for viewing only. Since the image will be 'analyzed', procedures must be established to ensure that the image is captured reproducibly, and that the system is operating in the detectable range for the image that is analyzed. For example, if an image is acquired for quantifying the amount of HER2/neu protein, then the exact same image must be produced whether the slide is scanned today, tomorrow or six months from now. The reproducibility of such an image depends on a number of factors such as stage and camera drift over time, fluctuation/variation in the light source, and variation in the analog-to-digital conversion process. In addition, further complexity may be introduced by the use of different objective magnifications. To avoid these complications, calibration must be performed on a regular basis (most systems today perform system calibration automatically).

Image analysis represents a significant step in standardizing the interpretation of slides in pathology (12). The creation of an image analysis application is not an easy task. Just as it takes considerable time and effort to create and validate a pharmacodiagnostic assay, it takes significant effort to create and validate a clinically useful image analysis algorithm. These algorithms are meant to complement pathologists, not replace them. Accordingly, algorithms should not attempt to emulate those skills and capabilities in which pathologists excel, but rather should aid pathologists in tasks where the human eye does not excel. The pathologist performs well at selecting relevant areas on the slide using morphology; an algorithm performs well at quantifying intensities and counts.

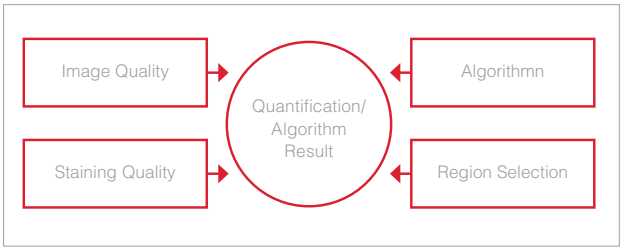


Figure 7.4 Quantification/Algorithm results are dependent on staining quality, image quality, the algorithm, and region selection.

Additionally, the principle of garbage-in, garbage-out cannot be overstated. There is no point in attempting to use image analysis to quantify IHC slides unless one is sure that the immunohistochemical procedure has been performed in a reproducible fashion. Automation (Chapter 9), standardization and the proper use of controls (Chapters 4, 5 and 14) in the process of preparing the IHC slides are essential prerequisites for successful image analysis.

As shown in Figure 7.4, staining quality, image quality, algorithm and region selection are important components to achieve good quantification/algorithm results. Staining quality can be optimized by using ready-to-use reagents and automated staining equipment with defined staining protocols. Additionally, linking specific pre-treatment, antibodies, and staining protocols to an algorithm is key for a consistent result. Having consistent, standardized staining is necessary for successful image analysis because differences in staining lead to differences in acquired digital images, thereby affecting image analysis. The image quality of a digital slide must meet rigorous standards in order to apply image analysis methods for the purpose of quantification, or to differentiate between components of the morphology of the tissue. Clearly the algorithm must also be developed to produce results that are relevant for the clinician. Areas to be evaluated (such as the tumor regions) must be selected for the algorithm to be included in the analysis. Selection of the region(s) for analysis should be made based upon predetermined guidelines, relying on the pathologist's expertise. Thus, image analysis is an aid to the pathologist.

PETITIONER EXHIBIT 1012
Page 99 of 218

Algorithms for the following areas are in use today:

- HER2 quantification
- ER/PR quantification
- DNA ploidy quantification
- Ki-67 quantification
- p53 quantification

In these quantitative algorithms the image analysis process is intended to mimic the pathologist, producing semi-quantitative results on an arbitrary scoring system, of 0 to 3+ , or similar. A number of the software programs designed for this purpose have been cleared as algorithms by the FDA, as Class II devices. ASCO/CAP guidelines for HER2 and ER/PR recommend the use of image analysis for the evaluation of these stains (13, 14). The use of digital pathology for reading of H&E slides is currently under discussion and will probably be a Class III device.

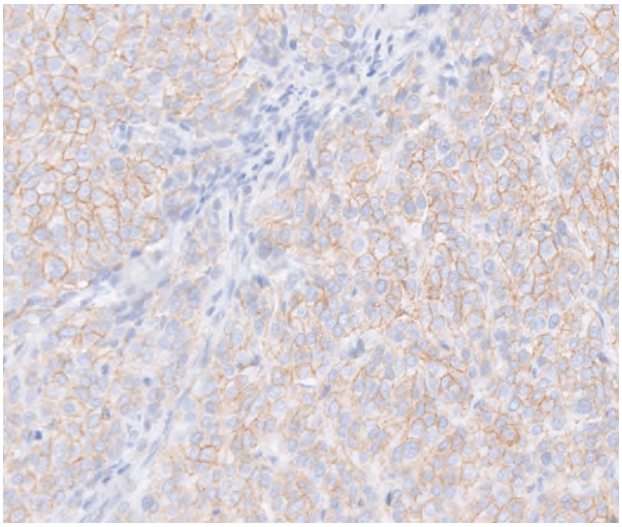


Figure 7.5 Pathologists can now use a digital system from Philips to help them score Her2-neu stained slides to assess treatment options for breast cancer patients.

Dako HercepTest™	Region Score	Slide Score
Staining intensity	3	3
HercepTest™ Score	3+	3+
Results		Strongly Positive

Figure 7.6 Dako HercepTest™ algorithm result displayed on the Omnyx system.

Chapter 7.7 | Analysis of IHC stains – 'Eye vs Algorithm'

With the exception of these few semi-quantitative IHC tests and algorithms, the great majority of IHC stains performed in the surgical pathology laboratory are performed for the purposes of assisting in the identification of cell types, and ultimately tumor diagnosis and classification. In this context, the question 'asked' of the IHC stain is whether or not there is a significant positive reaction (in relation to the controls), and whether it is positive or negative in the context of cell identification, not 'how much positivity' is present? As noted above, the experienced trained human eye is relatively good at distinguishing patterns as 'normal' or not, and grouping the 'abnormals' into known disease categories. The human eye is much less reliable at counting by number and calculating percentage values, and is quite poor at judging variations in intensity, unless major. In many respects the computer, given the appropriate digital slides, is almost the converse, good at assessing intensity, reproducibly, good at counting, good at event detection, but less good at categorizing complex patterns. Given the high-quality, high-resolution images, and standards against which the computer (software) can be calibrated, including 'training' in pattern recognition, a great number of these shortfalls in digital image analysis are diminishing, and diminishing rapidly. So much so that the use of 'intelligent' trained software to assist the pathologist in interpretation of IHC images (as well as perhaps H&E stains) is now a reasonable expectation in the very near future. For accurate quantification, the use of computer driven algorithms will be indispensible, but at this point in time still awaits the general availability of IHC staining methods that deliver a known and defined amount of signal (stain) to target (antigen), as well as the development of quantifiable internal reference or calibration controls (Chapter 1).

Chapter 7.8 | Quality Assurance in IHC

Presently, the results of IHC staining, including control slides, are assessed by technologists and/or pathologists, on an individual subjective basis, without direct comparison to prior control slides from earlier runs or slides from validation studies. Digital images of control slides allow for direct and accurate comparison of positive (and negative) control slides for spectral pattern and in-

PETITIONER EXHIBIT 1012
Page 100 of 218

tensity, providing a more precise means of quality assurance of automated staining platforms (or manual methods). It is likely that these types of programs will become an intrinsic component of the monitoring process in future generations of IHC autostainers.

While reproducibility of stain color and intensity is important in IHC in general, it is absolutely critical that precise measurement of performance occurs if IHC is to be used for true quantification, extending beyond current semi-quantitative visual or automated scoring approaches (0, 1+, 2+, 3+, etc.). Under these circumstances, the human eye might not appreciate subtle changes in intensity that easily may affect whether a slide is scored as 1+ or 2+ (and hence negative or positive), whereas a digital slide can be evaluated for intensity by appropriate algorithms with great accuracy.

Chapter 7.9 | Future Trends in Digital Pathology

As already noted the experience of 'digitized radiology' provides insight into future developments in pathology. Most of the current applications described above will continue and will expand and new, yet unimagined applications will emerge.

As has occurred in radiology, digital pathology will be used to review complete cases including H&E, special stains and IHC and ISH slides. In scenarios where the complete workflow in the pathology laboratory is digitized the information will all be collected in the pathologist cockpit and used to generate comprehensive patient reports. The impact upon workflow (Chapter 10) in pathology laboratories will be profound.

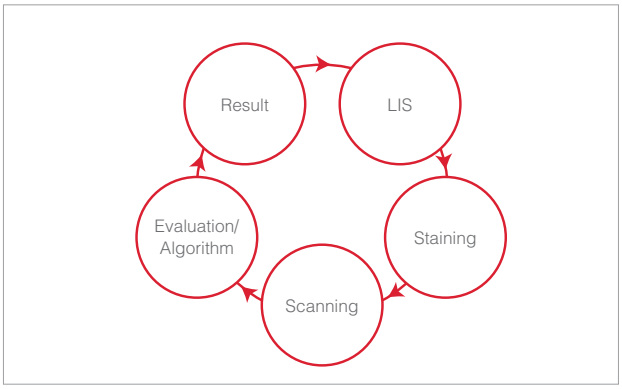


Figure 7.7 Complete digital workflow.

Additionally, the acquisition and assembly of multiple registered parallel slides from single tissue blocks may provide a 'virtual 3D tissue block', that will provide for levels of morphologic examination and manipulation far beyond current capabilities, all of course contingent upon ability to manage huge amounts of data and control costs. With the recent speed of progress in these areas almost anything seems possible!

New methods to combine morphologic analysis and staining expression evaluation will also support the integrated analysis of different markers. Especially for cases where 'multiplex staining', combining IHC, ISH and fluorescence methods will be used to review and analyze slides that have been stained with multiple markers, where the evaluation of patterns of staining of single cells and colocalization information will become critical.

In summary, digital pathology is an important new technology that already has penetrated pathology education significantly. With recent improvements in the technology, other uses are sure to follow quickly.

Acknowledgements

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PETITIONER EXHIBIT 1012
Page 101 of 218

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PETITIONER EXHIBIT 1012
Page 102 of 218

Chapter 8

Optimization of Immunohistochemical Reactions

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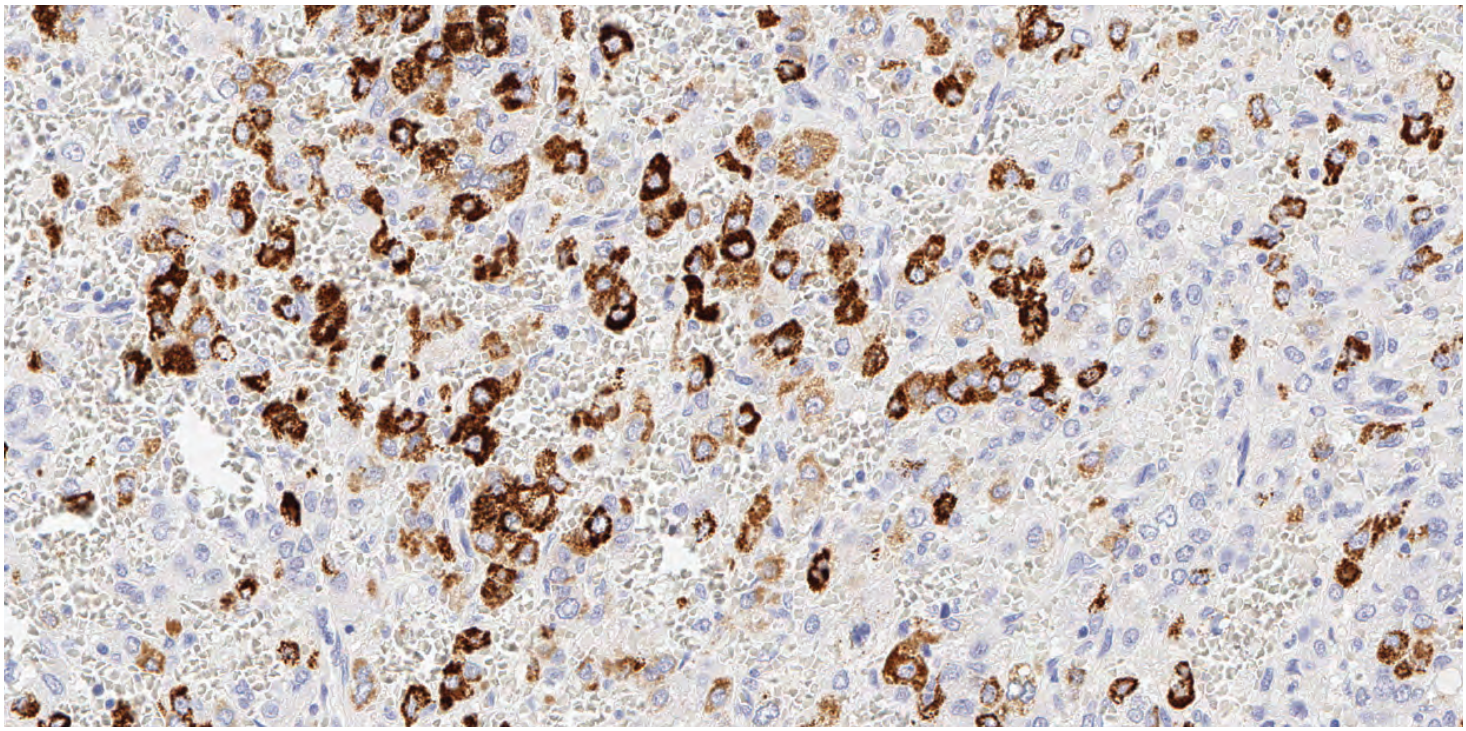


Op•ti•mi•za•tion *(n.)*
An act, process, or methodology of making something (as a design, system, or decision) as fully perfect, functional, or effective as possible.

Merriam-Webster Online Dictionary

Chapter 8.1 | Introduction

The process of implementing immunohistochemical tests in the diagnostic laboratory has been greatly simplified by the availability of standardized reagents, instruments, and assay protocols from commercial manufacturers. However, researchers and diagnosticians who wish to develop new immunohistochemical assays or to explore new applications for existing tests must carefully consider all of the different steps in the process, including the methods of tissue preparation and the reaction conditions for each assay step in order to obtain clear, specific antigen signals and to minimize non-specific (background) reactions, as well as interpretation and reporting (see Chapter 1).

The complex nature of tissue specimens presents both an aid and a challenge to the development of any new immunohistochemical test. Tissue morphology can be tremendously helpful for interpreting the specificity of staining signals in a new immunohistochemical reaction; on the other hand, tissues are notoriously prone to non-specific binding artifacts, and are also sources of enzymatic and other biochemical activities that can generate false signals from assay reagents and confound even the most expert interpretation. The goal of assay optimization is to enhance the strength and specificity of the signal generated by the immunological and enzymatic staining reactions while suppressing noise and artifacts.

This chapter will discuss the formulation and use of several basic immunohistochemical reagents, including proteolytic enzymes for tissue pre-treatment, antibody diluents, blocking and enhancing solutions, and wash solutions as they relate to assay optimization. Complementary information on the topics of tissue preparation, antigen retrieval, background suppression, and other aspects of assay optimization may also be found in the related chapters in this Guidebook.

Chapter 8.2 | Tissue Digestion using Heat-induced Epitope Retrieval

Antigen retrieval, often referred to as the heat-induced epitope retrieval technique (HIER) is predominantly based on heating slide-mounted specimens in a buffer solution, followed by a cooling-off period.

High-temperature heating of tissues is used as a non-enzymatic pretreatment for immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections. HIER is commonly used to overcome the effects of covalent cross-links that are formed in tissues during formalin fixation (see Chapter 3). While controlling the temperature and incubation time in retrieval solution is vital in this process, pH is also very important in maintaining optimal morphology and consistent immunoreactivity. Common pH's in clinical IHC applications range between

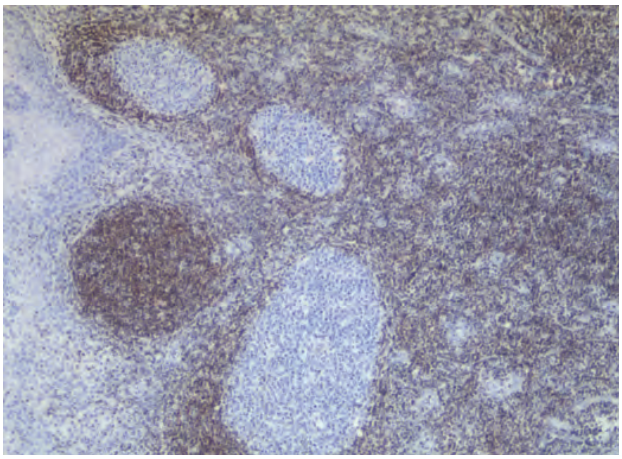


Figure 8.1 A) Anti-BCL2 1:200 with pH 6.1 retrieval.

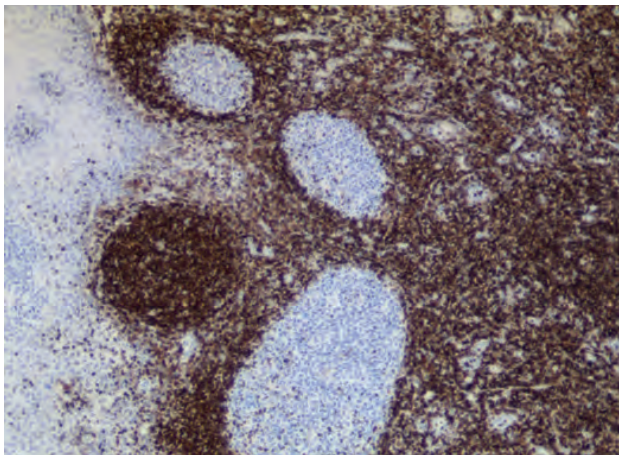


Figure 8.1 B) Anti-BCL2 1:200 with pH 9.0 retrieval.

PETITIONER EXHIBIT 1012
Page 104 of 218

6.0-9.0. Low pH solutions have been observed to preserve tissue morphology while demonstrating a more distinct nuclear pattern staining intensity. High pH retrieval solutions depending on the chemical composition tend to increase staining intensity of most antibodies, with target epitope in membrane and cytoplasm structures. For IVD-labeled antibodies the specification sheet from the manufacturer provides the appropriate HIER protocol which demonstrates the most consistent result. For example BCL2 demonstrates a weak staining in lymphoid tissues when pH 6.1 citrate retrieval solution is used, but very strong and specific appropriate immunoreactivity results when using pH 9.0 EDTA retrieval solution (Figure 8.1).

For more information on antigen retrieval or HIER, please refer to Chapter 3.

Chapter 8.3 | Tissue Digestion using Proteolytic Enzymes

Proteolytic digestion is commonly used to overcome the effects of covalent cross-links that are formed in tissues during formalin fixation. Controlled proteolysis can improve the penetration of reagents into the tissue structures and restore the immunodominant conformation of epitopes of interest, thus allowing the primary antibody to reach and bind its intended target. If the enzymatic cleavage points for the protease are in proximity to a formalin cross-link, digestion may induce a relaxation of the rigid protein structure and facilitate contact between the primary antibody and the corresponding antigenic determinant.

Proteolytic enzymes cleave specific amino acid sequences within the peptide chain of the digested protein; since proteases differ in their cleavage specificities, they can have markedly different effects upon tissues depending upon the type of fixative used for processing the tissue, the antigenic target, and the epitope recognized by the antibody.

Because the effectiveness of proteolytic digestion is dependent upon multiple factors, optimal digestion conditions must be determined empirically for each tissue and antigen/antibody combination. Carbohydrate epitopes, for instance, being non-proteinaceous, may be expected to be unaffected by proteolytic digestion; however, glycoproteins that contain carbohydrate epitopes may benefit from proteolysis if the epitope is 'unmasked' by cleaving and de-constraining the peptide backbone, allowing the antibody access to the epitope for binding. Non-protein antigens may also benefit from the improved reagent penetration resulting from tissue digestion.

Preliminary experiments should be conducted to determine the appropriate choice of enzyme, incubation time, temperature, and concentration for optimal digestion. Most of the proteolytic enzymes commonly used for IHC display their highest activities near 37 °C, and many protocols use this reaction temperature in order to achieve the digestive effect in the shortest possible time. In some cases, however, the use of a lower temperature may be preferable. By reducing the rate of the digestion reaction, one may extend the reaction time and thus exert more control over the digestion process – a method that is especially useful for situations requiring very mild digestion. Table 8.1 lists several commonly used enzymatic reagents and the typical incubation conditions used in IHC.

In general comparison studies by Quality Assurance Organizations such as UK NEQAS and NordiQC have revealed that enzymatic methods perform less well than antigen retrieval or HIER and are much less reproducible. So much so that their use is recommended only in those rare circumstances where HIER does not give satisfactory results.

Table 8.1 Proteolytic enzymes and typical incubation conditions.

Enzyme	Approximate activation temperature	Incubation time
Proteinase K	25-37 °C	5 min
Trypsin	37 °C	10 min
Pepsin	37 °C	5-20 min
Protease XXIV	37 °C	5-20 min
Pronase	25-37 °C	30 min

NOTE: Formalin does not preserve tissue proteins by coagulation but it is thought to form cross-links with basic amino acids. Ethanol and mercuric chloride-based fixatives are based on coagulation. With few exceptions retrieval should not be performed on ethanol-fixed tissues. It should only be conducted with limited controlled protocols in mercuric–chloride-based fixatives.

PETITIONER EXHIBIT 1012
Page 105 of 218

Chapter 8.4 | Endogenous Enzyme Blockers

The two most common enzyme activities that are used to generate chromogenic signals in immunohistochemistry, horseradish peroxidase and alkaline phosphatase, are also both encountered as endogenous activities in a variety of cellular and tissue specimens. If the endogenous enzymes are similar in specificity to the enzymes used in the immunohistochemical detection system, the endogenous activity can produce false-positive signals that interfere with, and even overwhelm, the signals from the immunohistochemical reactions. Enzyme blockers are used to inhibit the activity of such endogenous enzymes within cells and tissue specimens.

Table 8.2 lists some of the common sources of these endogenous activities. Simple reagents may be used to completely block these endogenous enzymes by either competitive inhibition or acid inactivation (see Chapter 15, for further information on enzyme inhibitors)

Table 8.2 Endogenous enzymes found in a variety of cells and tissue types.

Enzyme: Peroxidase	Enzyme: Alkaline Phosphatase*
Red blood cells	Placenta Intestine - situated between cellular components of mucosa
Granulocytes	Proximal tubules of kidney
Eosinophils	Osteoblast in bone
Hepatocytes	Arterial & capillary endothelial cell surfaces
Muscle	Stromal reticulum cells
Kidney	Neutrophils
Monocytes	Follicle and mantle zones in most lymphoid tissue

*Alkaline phosphatase is destroyed by routine fixation and paraffin-embedding procedures.

Generally speaking, enzyme blockers are applied prior to the addition of antibody reagents in the staining protocol. However, the enzyme blocking reagent may interfere with the immunohistochemical reaction, which may alter sensitive epitopes to the extent that they are no longer recognized by their cognate antibody. In such cases the blocking reaction should be attempted after the primary antibody has been applied, but before the application of the enzyme-labeled immunohistochemical detection reagent. This principle is used onboard the Dako Omnis instrument.

Table 8.3 Common endogenous enzyme blocking reagents for horseradish peroxidase and alkaline phosphatase systems.

Blocker	Enzyme
Dual endogenous enzyme block	Horseradish peroxidase and alkaline phosphatase labels
Hydrogen peroxide	Horseradish peroxidase label
Levamisole + chromogen except intestinal alkaline phosphatase	Alkaline phosphatase label
Weak acid (0.3 N HCl), including intestinal alkaline phosphatase	Alkaline phosphatase label

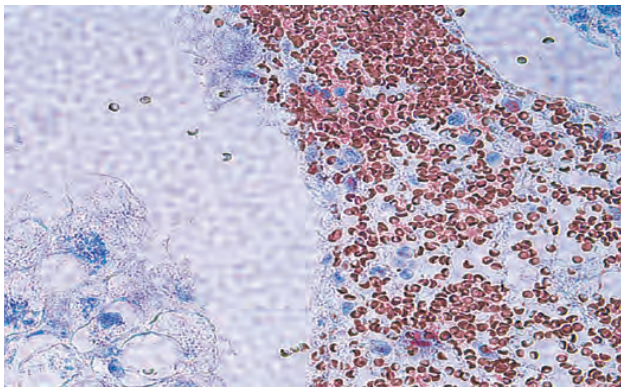


Figure 8.2 Example of endogenous peroxidase in red blood cells of kidney stained with DAB.

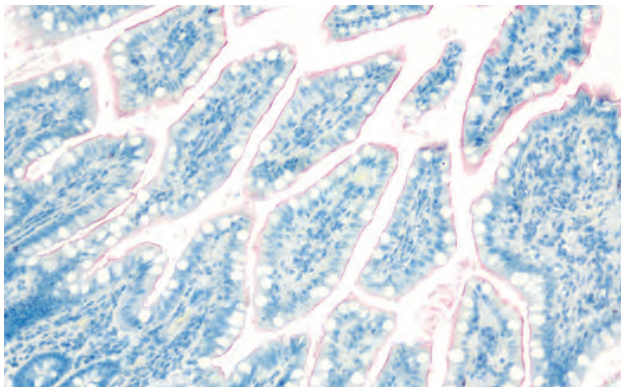


Figure 8.3 Example of endogenous alkaline phosphatase in ileum stained with Permanent Red.

PETITIONER EXHIBIT 1012
Page 106 of 218

Chapter 8.5 | Protein Blocking Reagent

These protein-containing reagents are used to reduce non-specific reactions that may result from the binding of antibodies and other assay reagents to various tissue components. Antibodies and detection systems used in immunohistochemistry are primarily protein-based reagents, and they are susceptible to non-specific binding. Protein blockers act to minimize non-specific protein adsorbance by competing for the non-specific protein binding sites on the specimen. By occupying the tissue binding sites with blocking protein prior to (or during) the incubations with the other immunohistochemical reagents, the non-specific signals that would otherwise develop can be greatly reduced. A more thorough discussion of the various non-specific protein-protein interactions that occur between immunohistochemical reagents and tissues, and the protein blockers that may be used to prevent such interactions, is given in Chapter 15.

Chapter 8.6 | Endogenous Biotin

Biotin, may be protein-bound to tissue and can interfere with proper interpretation of staining patterns, when using a labeled (strept) avidin-biotin (LSAB) or ABC complex-based visualization systems. Kidney and liver contain high amounts of endogenous biotin, while lesser amounts may be found in the GI tract, lung, spleen, and cells grown in culture media containing biotin (RPMI). Biotin blocking is based on the extreme affinity of (strept)avidin for biotin. Once the avidin stage is saturated, free biotin is added the system is essentially closed to further interaction with subsequent free or conjugated (strept)avidin or biotin stages. Excess avidin is applied to the tissue. Each biotin molecule is capable of binding to only one avidin molecule. Once the avidin stage is saturated, free biotin is added. The system is essentially closed to further interaction with subsequent free or conjugated (strept)avidin or biotin stages. The need for biotin blocking solutions has decreased due to the development of labeled polymer visualizations. Polymer visualizations provide the technician with fewer protocol steps due to the conjugation of the enzyme and secondary antibody on a dextran backbone. Polymer systems have the potential for greater numbers of horseradish peroxidase enzymes to produce increased staining intensity. For these reasons the use of traditional ABC systems today is limited to specific research and clinical needs.

Chapter 8.7 | Antibody Diluents

Antibody diluents are buffered solutions that are used to formulate working solutions of antibodies for use in IHC assays. Because antibody conformations are highly dependent upon the aqueous environment, diluent formulations can significantly alter the stability and binding properties of antibodies, affecting both epitope specificity and “non-specific” interactions with the Fc region of the antibody molecule. Variations in charge, hydrophobicity, glycosylation, and other physico-chemical properties between antibodies can make it difficult to accurately predict the behavior of antibody-diluent combinations, but the availability of several effective commercial diluents has simplified the empirical process of diluent selection.

Ionic interactions are one of the primary forces controlling the immunochemical binding of antigens with antibodies, and these interactions are pH-dependent. Generally speaking, buffers that approximate pH (7.3-7.4) are often suitable for dilution of primary antibodies, and are a good starting point for optimization. The isoelectric point, or pI (the pH at which the net electric charge of a molecule is zero), for immunoglobulins can range from 5.8 to 8.5 for a given antibody. If the pH of the diluent is too close to the pI of the antibody, solubility can be diminished, with negative effects upon both signal and background. If low signal and high background are observed at the intial pH tested, try raising or lowering the pH of the diluents buffer by 0.5 pH units. Antibody interactions are also affected by ionic strength; most diluents contain millimolar quantities of sodium chloride or other salts which serve to reduce non-specific interactions among charged molecules. Excessive ionic strength should be avoided, since it may overcome the specific charge interactions required for high avidity antibody-epitope binding.

Several pre-formulated antibody diluents are now available from commercial manufacturers; these diluents can simplify the optimization process when selecting diluents for a new antibody or assay, and may also confer increased stability to the working primary antibody solution. Many of the commercially available antibody diluents are based upon Tris-HCl buffers containing a detergent and proprietary stabilizers. Some diluents also contain protein-based background-reducing components such as bovine serum albumin or serum proteins. Se-

PETITIONER EXHIBIT 1012
Page 107 of 218

rum-containing diluents should be used with caution; serum binding to the primary antibody can cause a reduction in sensitivity, while binding to the secondary antibodies used in IHC detection systems can result in false positive results.

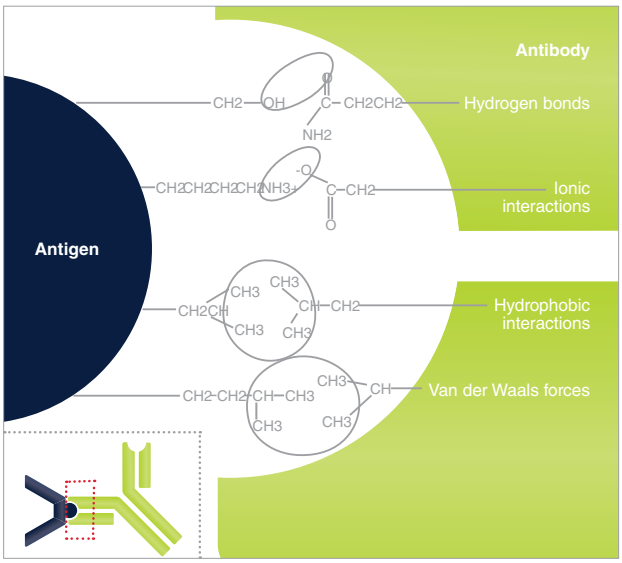


Figure 8.5 Antibodies are attracted to antigens initially through electrostatic interactions, and subsequently through Van der Waals and hydrophobic interactions.

Antibody stability in solution cannot be predicted without thorough stability studies; technicians are advised to follow proper quality control procedures for stability validation if primary antibodies are to be diluted in the laboratory and utilized for extended periods of time. An advantage to using commercially diluted primary antibodies is the built-in customer protection provided by the regulatory mandates that govern reagent manufacturers. Manufacturers must demonstrate the stability of commercially produced reagents for defined periods to establish a predictable shelf life for their antibody products.

Important Points to Consider Regarding Antibody Diluents:

- Diluent pH can be an important determinant of effectiveness
- High concentrations of sodium chloride and azides are used frequently as preservatives in commercial preparations, but these components can reduce antibody reactivity
- Excessive ionic strength can decrease specific staining by interfering with antibody-antigen binding
- Generally speaking, the more dilute the antibody, the less stable the working solution
- Highly diluted antibody solutions should not be used for more than a few days unless validated stability data is available
- The pH's of Tris-based buffers are sensitive to temperature changes. Always prepare buffers at the same temperature in which they will be used, and be aware that refrigeration and heating can cause changes in the pH of temperature-sensitive buffers. Always allow reagents to equilibrate to room temperature before use.
- Phosphate buffered saline (PBS) should generally not be used as a diluent unless specifically recommended by the manufacturer for a particular antibody

Chapter 8.8 | Antibody Concentrations

Antibodies belong to a group of proteins called immunoglobulins (Ig) which are found in the plasma or serum. Five major classes of immunoglobulins exist – IgM, IgG, IgA, IgD and IgE. Most antibodies in IHC are from the IgG class. Optimal reactivity can be achieved when using the appropriate diluents. The optimization process includes diluting the antibody at the recommended dilution and one serial dilution above the recommended dilution and one below to ensure the appropriate staining reaction. More on this topic can be found in Chapter 4 and Chapter 5.

Chapter 8.9 | Incubation times

All other factors held constant, incubation times affect every step in immunohistochemistry protocol. Insufficient primary antibody or visualization incubation times have a direct effect on the colorimetric expression. Shorter incubation times results in partial staining or weak stain intensity, while longer incubation times can increase staining intensity. Optimum is usually not achieved during incubation times less than 20 minutes. Typically, manufactures' specifications provide recommended incubation ranges for tissue demonstrating a high level of targeted epitope with high affinity antibodies. However, optimal incubation time, i.e. saturated incubation reactions should give sufficient staining intensity to identify high expression and, especially, low expression of immunoreactivity. The importance of

PETITIONER EXHIBIT 1012
Page 108 of 218

identification of low expression cells and structures is covered in more detail in Chapter 4 and Chapter 5.

Chapter 8.10 | Wash Buffers

Wash buffers are used to remove excess reagents from the specimen after each incubation step in the assay protocol. Commonly used wash buffers, including those that are commercially available, include Tris-buffered saline (TBS) and phosphate-buffered saline (PBS).

Tris-Buffered Saline

Tris(hydroxymethyl)aminomethane-based wash buffers are often utilized in combination with the non-ionic determent Tween 20. Commercial wash buffer preparations may also include a preservative, such as 0.01% sodium azide, to prevent the growth of microorganisms and extend the shelf life of the reagent.

Tris-buffered solutions are pH-sensitive; as the solution decreases in temperature, pH increases at a rate of approximately 0.03 units per degree centigrade, so care should be taken to equilibrate buffer solutions to the proper working temperature in order to maintain consistent pH. This effect is illustrated in Table 8.4.

Table 8.4 Effects of temperature on pH of Tris-buffered saline.

Temperature	pH
5 °C	8.18
25 °C	7.6
37 °C	7.30

Wash buffers can be employed to counteract the effects of non-specific reagent binding to tissue specimens. When conditions require very high specificity reactions, such as when using highly sensitive detection methods, an increase in the saline and detergent content of the wash buffer can be used to minimize non-specific binding. The addition of high concentrations of salts (e.g. sodium chloride) and detergents (e.g. Tween 20) to wash buffers will significantly reduce the non-specific binding of many immunohistochemical reagents, including antibodies and detection reagents such as labeled polymers.

Phosphate-Buffered Saline (PBS)

PBS is also commonly used as a wash buffer for IHC. PBS's advantages are reduced auto fluorescence in immunofluorescent assays, and it is relatively inexpensive compared to Tris-based buffers. However, in some cases PBS can cause higher levels

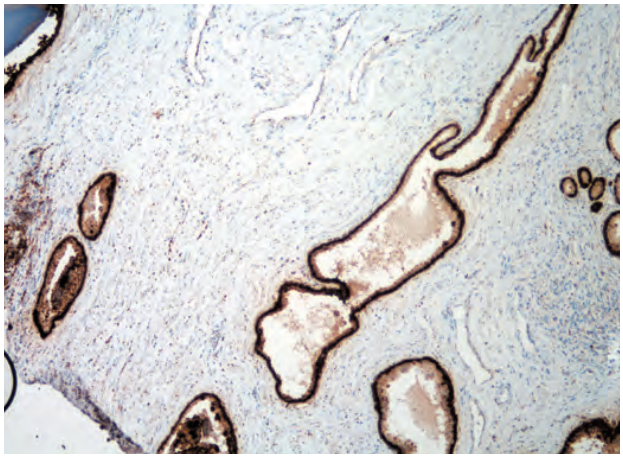


Figure 8.6 A) Anti-Cytokeratin, Clone 34βE12, Dako Code M0630, on human prostate tissue, rinsed with 150 mM NaCl, 0.05% Tween 20, Tris-Buffered Saline, Dako Code S3006, pH 7.6 at 25 °C. Non-specific staining is evident in the lumen and the connective tissue.

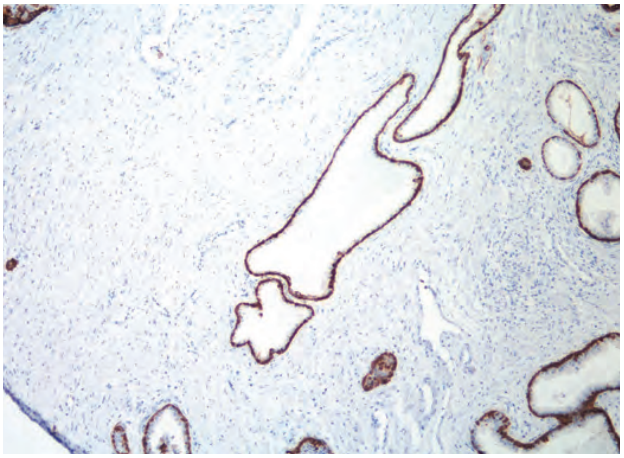


Figure 8.6 B) Anti-Cytokeratin, Clone 34βE12, Dako Code M0630, on human prostate tissue, rinsed with 300 mM NaCl, 0.1% Tween 20 Tris-Buffered Saline, Dako Code S3306, pH 7.6 at 25 °C. Staining is confined to specific signals in the cells of the prostatic epithelium.

PETITIONER EXHIBIT 1012
Page 109 of 218

of nonspecific staining, and it has been observed to reduce the specific binding abilities of certain monoclonal antibodies (Anti-CD30, for example).

Suggestions for Making Wash Buffers

- Always replace buffers after expiry, and do not combine old buffers with new
- Do not dilute reagents beyond the manufacturer's recommendations; buffering ability may be compromised, and ionic strength will no longer be optimal if the recommended dilution is exceeded
- Clearly label all new solutions with the date when they are opened and made
- Do not mix different types of buffers with one another
- Use distilled, organically filtered deionized, or high quality reagent grade water only to make and dilute buffers. Do not use tap water, as the presence of trace quantities of metals, inorganic ions, and other contaminants in tap water may interfere with immunohistochemical reactions

Chapter 8.11 | Chromogen Enhancers for DAB

The DAB chromogen is a popular choice for signal generation with peroxidase-based immunohistochemical detection systems. The DAB reaction product, which normally appears as a light brown precipitate, may be enhanced by reaction with any one or a combination of several types of metals. The metals enhance the reduction of the DAB reaction product and darken its appearance. Copper, silver, nickel, gold and cobalt have all been used as enhancers.

Metal enhancers may be added directly to the chromogenic DAB reaction, or they may be applied in a separate incubation step following the development of the DAB reaction product and a rinse in high-purity water. The latter approach is recommended when it is desirable to fine-tune the degree of enhancement; the optimal incubation time for the enhancement reaction may be determined empirically by monitoring the development of the enhanced chromogen under the microscope. The hue and intensity of the final DAB reaction product will vary depending upon the type and concentration of the metal(s) used in the enhancing reaction, but in general the enhanced product will appear darker and more intense than the non-enhanced DAB product. Since the enhancer metals work only upon the reduced chromogen that has been deposited by the peroxidase reaction, the enhancer reaction generally does not contribute significantly to background. Incubation times for enhancement should be determined by the individual laboratory, based on the desired hue of the chromogen.

The use of enhancers adds yet another uncontrolled variable to the entire staining process, and their use should be reserved for those cases where a satisfactory intensity cannot be achieved by adjusting antibody or label concentrations or incubation times.

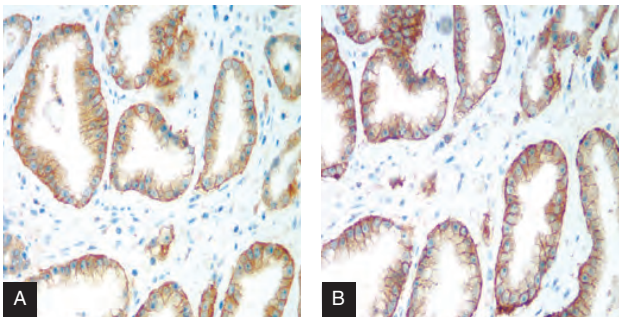


Figure 8.7 **A)** Standard DAB chromogen without enhancement. **B)** DAB enhanced with Dako DAB Enhancer.

Chapter 8.12 | Type of Glass Slides

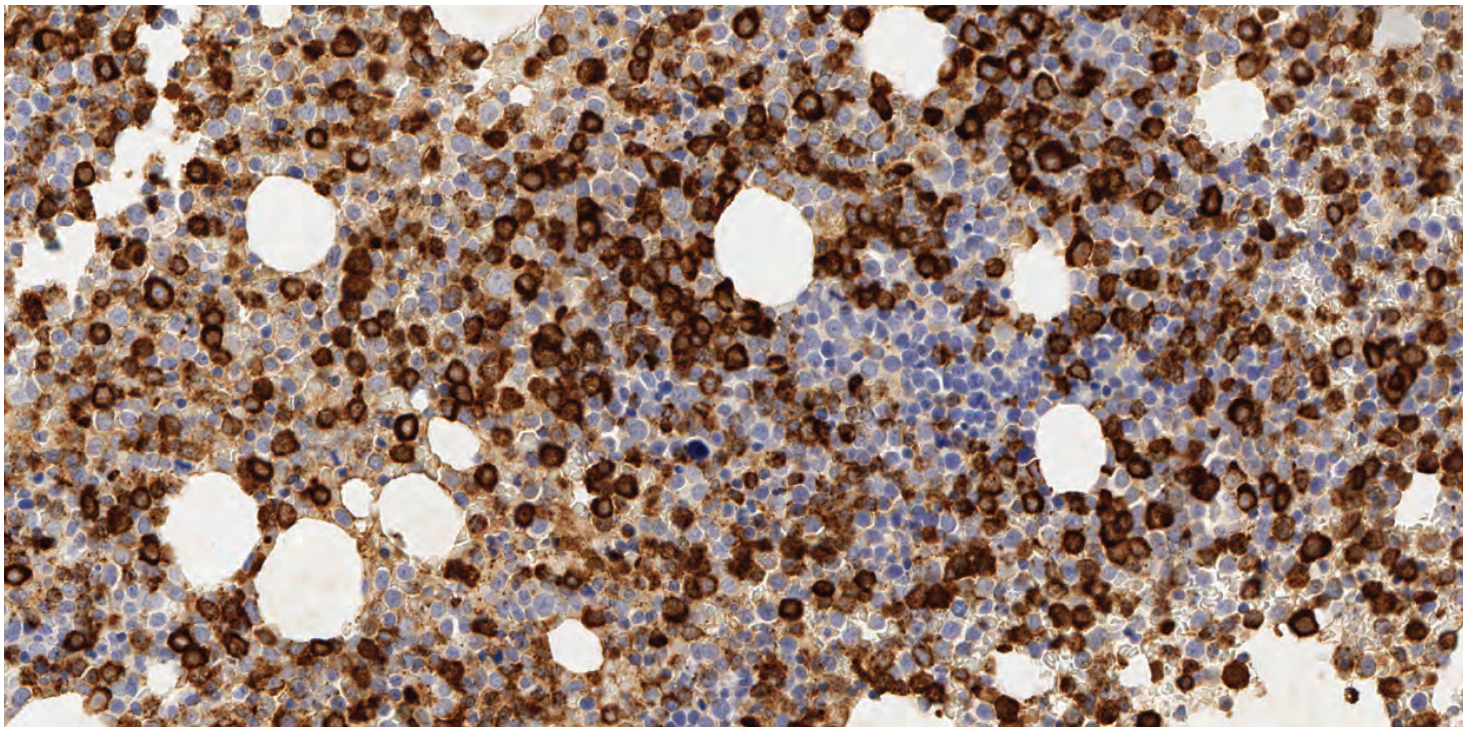
FFPE tissue sections bind to positive charged glass slides by the negatively charged carboxyl end of cellular proteins. There are a number of glass slides on the market that address tissue adherence for immunohistochemistry and insitu-hybridization. Poly-L-Lysine, Silanized slides, and plus slides have the common characteristic of binding tissue sections. There are also a number of additives which can be used in the water bath during sectioning that cause tissue to adhere to plain or non-charged slides. It should be mentioned that using the combination of tissue adhesives and charged slides results in tissue falling off the slide. Other causes of tissue loss include hand lotion contaminating the waterbath, insufficient section drying temperature or time where water is trapped between the glass and the tissue, extended heat induced retrieval time, and tissue with high adipose content where there is low cellular protein content.

PETITIONER EXHIBIT 1012
Page 110 of 218

Chapter 9

Automation in IHC

Ole Feldballe Rasmussen, PhD, MSc



Au•to•ma•tion *(n.)*
Automatically controlled operation of an apparatus, process, or system by mechanical or electronic devices that take the place of human labor.

Merriam-Webster Online Dictionary

Chapter 9.1 | History of IHC Automation

The first automated devices for immunohistochemistry (IHC) appeared in mid-late 1980s and were based on differing technologies (1-3). One device used totally enclosed slide chambers with a computer-controlled microfluid transport system and reagents placed on a carousel (3), and another was based on capillary action between two slides with tissues facing each other (1). A key driver for implementation of automation was to avoid the labor-intensive and thus expensive manual staining. It has been estimated that during the manual staining procedure each slide must be manipulated over 100 times, and, depending on the skill set, one technician can manually handle up to 50 slides per day (4). The first automated device capable of both IHC and in situ hybridization (ISH) was described in 1990 (5). The staining was performed in a capillary gap-based reaction chamber with the slides placed specimen-side down.

Automation of IHC quickly caught on – one might compare that development with what we currently see with the entrance of Next Generation Sequencing into cancer diagnostics – and already in 1995, several IHC instruments were commercially available. A total of five systems were compared by Le Neel et al. (6) who concluded that automation is possible in routine laboratory setting, and that the different systems served different laboratory needs. These stainers were all semi-automated, with deparaffinization and antigen retrieval performed off instruments, and all had limited user interface.

Some instrument generations later, today's most advanced IHC staining instruments are now fully automated, handling all steps from baking to counterstaining. Some instruments can perform ISH, the graphical user interface is designed for user-friendliness, and they can be fully integrated with the hospitals' laboratory information systems.

Chapter 9.2 | Key Advantages of IHC Automation

Today, automated IHC staining is commonplace. However in the 1990s it was a major step forward with manual IHC being highly complex as described by Moreau in 1998 (7): "outline for manual staining that before beginning the staining process the specimens should be circled with a delimiting pen that serves as a guide when wiping away excess liquid to prevent the specimen from being accidentally wiped off; that to avoid evaporation of solution, the slides should be laid flat with the specimen facing upward in a humidity chamber; that during buffer wash excess liquid should be wiped off using an absorbent tissue and consequently that when processing a large number of specimens, only 3 to 5 slides should be wiped at one time before applying the appropriate solution."

The quote also serves as an illustrative background to highlight some of the key advantages of IHC automation:

- Reduced hands-on time that may compensate for increased staining volume, frees up skilled resources to perform other essential tasks such as cutting and embedding or slide review, and may compensate for increased difficulties in recruiting skilled staff as seen in many regions of the world.
- Standardization, which ensures that staining is repeatedly performed in the same way and does not depend on differences in personal skills. Furthermore, standardization combined with ease-of-use facilitates use of multiple technicians at multiple shifts, thereby increasing laboratory flexibility.
- High reproducibility by ensuring that any given step of the staining procedure is continuously performed as specified by any given protocol
- Optimized use of reagents
- Improved error control via process control (e.g. control of slides to be stained and correct selection of reagents via barcode tracking) and process monitoring (e.g. reporting of correct liquid level or process temperature), plus alarm notification if action is needed.

Chapter 9.3 | Staining Technologies

There are currently at least four different IHC staining principles being used on commercially available instrument systems.

Open Individual Slide System

The slides are arranged horizontally with reagents being applied using a dispenser that typically directs reagents to one or more zones on the slides depending on the location and size

PETITIONER EXHIBIT 1012
Page 112 of 218

of the tissue. These types of systems are the closest to mimic manual staining.

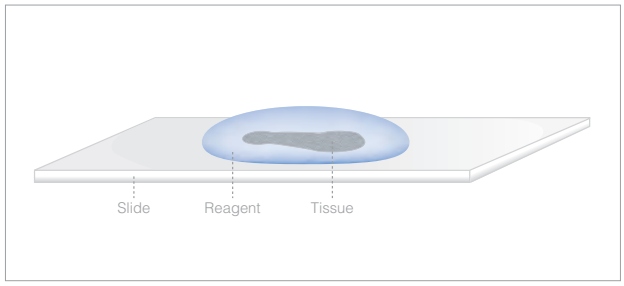


Figure 9.1 Open individual slide system. Reagents are applied on top of the tissue by an automated dispenser system. The principle has been and is used on a range of slide stainers, including Dako Autostainer Link 48 and intelliPATH from Biocare Medical.

Liquid Overlay Technology

With this technology, an inert fluid is deposited over the entire slide. Reagents are either overlaid or deposited into the overlay fluid. This technology allows reagent mixing on the slide as well as reactions at elevated temperature as the overlay fluid restricts evaporation. Completed slides must be cleaned off the overlay fluid before coverslipping.

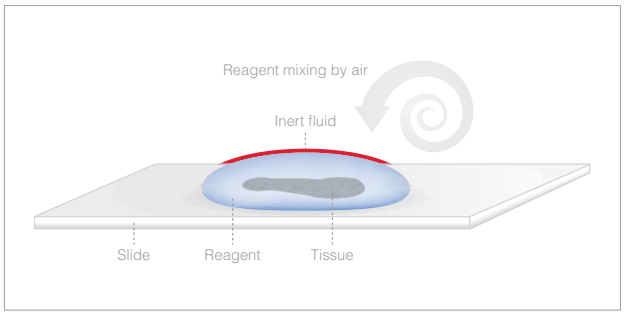


Figure 9.2 Liquid overlay technology. Reagents are either overlaid with an inert overlay fluid or dispensed into/under the overlay fluid. This principle is currently being used by Ventana Medical BenchMark systems.

Capillary Gap Staining

The capillary gap technology utilizes capillary forces to draw and/or keep liquid between two planar units that may be either two microscope slides with tissue facing each other, or a slide and a cover plate. This requires a definite spacing between the two units to ensure equal and correct capillary forces across the entire slide. The capillary gap system exists in a vertical version where reagents are applied by placing the slides in a reagent jar and reagents are drawn out by blotting, with washing being performed by repeated reagent draw up and out. Capillary gap staining is also used in a horizontal version using a replaceable covertile that can be moved back and forth by the instrument and in this way create a pseudo-capillary gap.

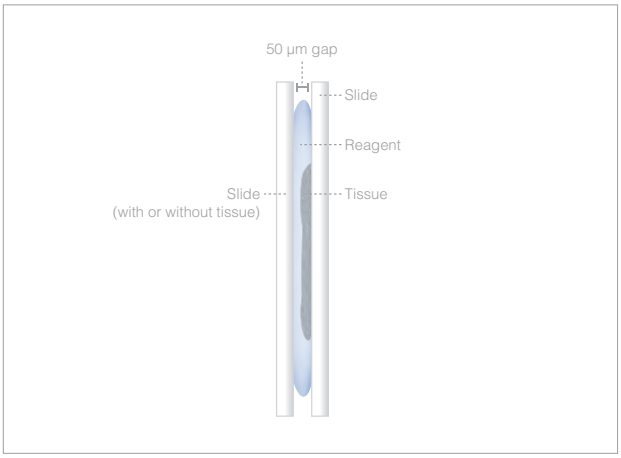


Figure 9.3 Vertical capillary gap staining. A definite and very small spacing between the two surfaces creates capillary forces to draw and/or keep liquid between the two planar units. This principle has been and is being used on a range of stainers including the Dako TechMate instruments (vertical orientation of slides) and Bond from Leica Biosystems (horizontal orientation of slide using an overlay covertile).

Dynamic Gap Staining

The dynamic gap staining technology uses capillary forces to secure homogeneous spreading of reagent throughout the staining area during reagent application. During reagent incubation, dynamic movement of the coverlid ensures continuous movement of reagents across the staining area. In this way the dynamic gap staining technology provides homogeneous reaction conditions across the entire staining area throughout the full incubation time. In addition, the technology decreases incubation time and a tight humidity control of the system facilitates use of elevated temperature to further decrease incubation time. Moreover, no slide cleaning is required post staining.

An intermediate technology between capillary and dynamic gap staining is the wave-based system from Celerus where

PETITIONER EXHIBIT 1012
Page 113 of 218

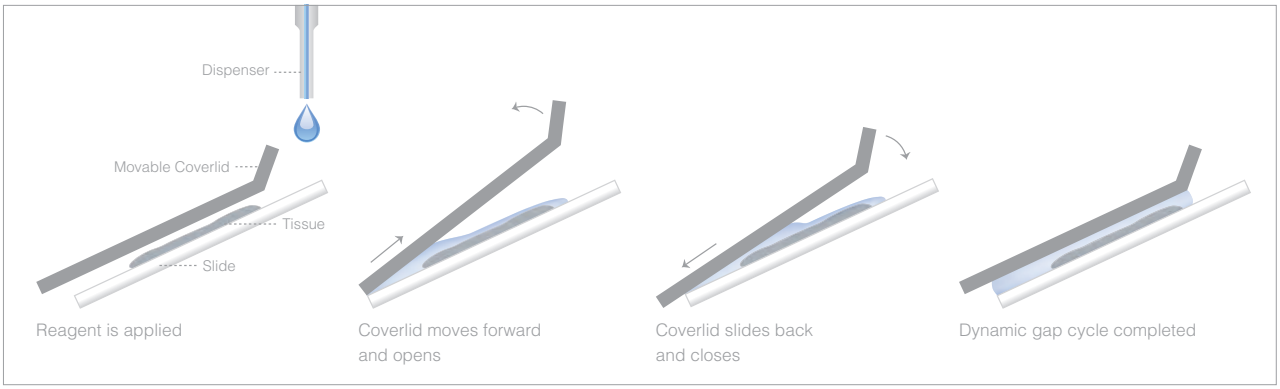


Figure 9.4 Dynamic gap staining. The dynamic gap uses two motions. The lid is moved back and forth along the glass slide, and the upper end of the glass lid is moved up and down. When the glass lid and glass slide ends are aligned, the dynamic gap is created. Each cycle takes 16 seconds.The dynamic gap principle is implemented in the IHC staining module of the Dako Omnis staining instrument.

paired slides are repeatedly being opened and closed during reagent incubation.

Open vs. Closed Systems

Automated IHC staining systems are commonly referred to as being 'open' or 'closed'. In open IHC staining systems, the users may select any reagent (incl. target retrieval buffer, antibody, and visualization system) and staining protocol (incl. temperature, incubation time, wash time) that they prefer and thus fully design the staining according to specific needs. A primary advantage of open systems is that they offer a very high degree of flexibility. Any lab, pathologist or scientist may design an assay with any antibody, visualization system or protocol to fulfill their specific needs. However, it is critical to note that in order to ensure consistent and high staining quality, such systems require highly skilled technicians.

In closed systems, the visualization reagents and protocols are typically locked (often termed Ready-To-Use (RTU)). In addition, other protocol steps, typically antigen retrieval, may also be locked or restricted. The primary antibody and antibody incubation time may also be available in RTU format, but also flexibility and use of other antibodies is allowed, as it is not realistic that one vendor can supply all antibodies required in any given clinical laboratory. Closed systems offer a much higher degree of standardization and flexibility in use of staff with varying skill sets.

There is obviously no strict line between open and closed systems. Fully open systems are particularly relevant in research settings to investigate the expression of the growing number of new biomarkers for biomedical research. In contrast, there is a strong tendency to move towards more closed systems in clinical settings where standardized and high-quality performance for a defined number of antibodies is required.

Table 9.1 The advantages and disadvantages of open and closed systems.

	Open systems	Closed systems
Pros	Free choice of visualization system	High level of standardization through use of RTU reagents
	High antibody flexibility	High level of staining consistency; many available RTU antibodies
	Research application friendly	Reduced hands-on time
Cons	Increased need for internal protocol optimization	Reduced staining protocol options
	Require highly skilled technicians or increased risk of manual errors	Antibody restrictions; some may not work optimally
	Reduced staining consistency	

PETITIONER EXHIBIT 1012
Page 114 of 218

Sequential vs. Parallel Staining Processes

For most automated systems, the staining is fully sequential in the sense that any given slide stays at the same slide position in the instrument and all staining processes, in principle from baking to coverslipping, are carried out at that position. Such a system puts huge demands on the capability of each staining unit, as it has to perform very different tasks. The advantage is that once the slide is placed in the instrument, all subsequent staining actions are “hands off”.

An alternative is found in 'parallel processing' where one or more of the staining protocol steps are carried out on separate instruments or instrument modules. The very basic example is baking done in a separate oven or in a seperate module onboard the instrument. Another example is a separate unit for deparaffinization, antigen retrieval and rehydration. A newly introduced staining system benefits from having a number of modules, each performing one or more of the process steps. Overall, the benefits of parallel processing are two-fold: 1) Each module can be optimized to perform the specific process step(s) and 2) It allows a higher degree of flexibility for initiating staining of a new set of slides. Furthermore, by having specialized units, new ways of handling process steps are facilitated. One such example is the pre-treatment unit that combines antigen retrieval and rehydration into one single step. The primary drawback of parallel processing – when separated on different instruments – is the addition of an extra manual step compared to full automation.

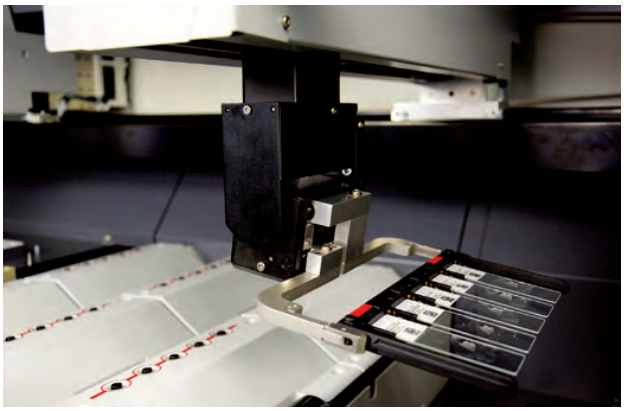


Figure 9.5 Inside Dako Omnis, a robotic arm moves the slide rack from one module to the next allowing for staining protocol steps to be carried out in separate instrument modules.

Batch vs. Continuous Loading

Previously, all automated IHC stainers were 'batch stainers'. Here, a large number of slides are loaded and off-loaded at the same time with no manual intervention, and typically with a pre-set completion time defined by the instrument according to the number of slides and complexity of the selected staining protocols. The primary advantage is that a large number of slides can be completed in one run within a defined time; thus enabling staff to perform other laboratory tasks during the fixed run-time. Another advantage is that these instruments typically offer significant capacity for overnight runs to ‘catch up’ on days with high slide volume.

In recent years, automated stainers with options for continuous loading and unloading have appeared. In this context the term 'continuous' covers a spectrum of options for independently loading smaller batches; typically 5-10 slides that will be completed at different times. The completion time will depend on loading time, protocol complexity and potentially also priority. In the extreme case, single slides are loaded and unloaded independently. The primary advantages of continuous loading include reduced time to first result, the possibilities to continuously prioritize patient cases, and increased flexibility in the lab workflow. Potential drawbacks include increased hands-on time per slide or patient case, and less overall efficient use of the instrument.

Some continuous IHC stainers depend on “instrument free time”, so introduction of new slides – and possible new reagents required for new protocols – does not interfere with planned protocol steps for slides already in process on the instrument.

One option to circumvent this limitation is to include designated slide loading and unloading stations, which creates full flexibility for slide loading – as long as there are open positions in the loading station. It is still possible to apply priority to specific patient cases and the instrument will continuously identify the best solution for effective staining according to the priorities selected. Likewise, there is increased flexibility in unloading, either removal as soon as a small batch has been completed to facilitate rapid assessment, or removal in larger batches to reduce overall hands-on time. A system with loading and unloading stations requires some level of parallel processing capability of the instrument.

PETITIONER EXHIBIT 1012
Page 115 of 218

Figure 9.6 Separate loading and unloading stations enable true continuous loading of slides.

Chapter 9.4 | Requirements for Effective Automated Staining

Today's IHC staining instruments are generally of high standard and capable of delivering high-quality staining results. Nonetheless, there are a number of requirements that must be fulfilled to realize the full potential of automated IHC staining.

High Quality Tissue

An absolute requirement for good staining quality is that the pre-analytical process has been correctly performed, including short ischemic time, controlled transport to the pathology lab and, not least, that the tissue has been fixed correctly. The importance of fixation on the staining quality has been documented in several reports (8, 9, and references therein). Furthermore, correct baking is also important. Williams et al. (10) reported that baking for 30 min on a 70 °C hot plate had a negative impact on the staining quality for 4 out of 12 antibodies compared to standard baking for 1 hour at 60 °C.

Reagents and Staining Protocols

First of all, the reagents must be of high quality with high specificity and sufficient sensitivity to give correct staining. Also, the staining protocols must be optimized to ensure that high-quality staining results are obtained consistently and reproducibly, see details in Chapter 5. In this context, it is important to note that staining protocols must be optimized for a specific set of reagents together with a specific instrument type. The same protocol might not be optimal for two different instrument types, even though they may be using the same staining principle. Moreover, we have seen examples where specific antibodies perform very well on one instrument system, but less optimally to very poorly on another when different staining principles apply. One factor may be staining temperature, but other factors may be of importance as well.

The Instrument

From the above it is obvious that automation by itself does not guarantee high staining quality, many other factors must be in place as well. However, it is critical that the instrument is able to perform each protocol step in a precise and reproducible manner. Important elements include:

- Correct reagent application: there must be sufficient reagent to cover the tissue, both to ensure that the whole tissue is indeed stained and to avoid drying out which will give detrimental artifacts. Application of too much reagent will negatively impact the number of tests per reagent vial.
- Correct incubation times: the instrument must be able to control the incubation time precisely; too short a time may give insufficient staining, while too long a time may give staining that is too intense, impacting readability and increasing the risk of background staining.
- Temperature control: this is a key parameter, particularly for antigen retrieval, and to some degree also for reagent incubation. The extent of temperature control on an instrument has direct impact on staining protocol optimization.

An important instrument feature with major influence on instrument efficiency is its 'scheduler'. A scheduler is the software that in detail organizes the sequence of each individual step

PETITIONER EXHIBIT 1012
Page 116 of 218

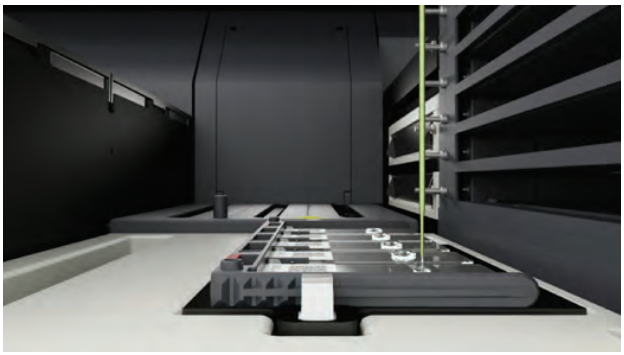


Figure 9.7 Correct reagent application: there must be sufficient material to cover the tissue to ensure that the whole tissue is indeed stained.

Figure 9.8 The scheduler software organizes the sequence of all individual steps an instrument must perform from start to end of the staining process for the given load of slides. For some instruments, the scheduler also calculates when slides will be completed.

that an instrument must perform for each slide from start to end of the staining process. The more complex the staining protocol is, the greater the diversity of protocols in the same run, and the more slides to be stained simultaneously, the greater are the demands placed on the scheduler. The efficiency and dynamics of a scheduler have a direct impact on the total staining time; both for batch staining and when slides are loaded continuously. In the latter case, there are two options: either the staining sequence for the new slides is added to the queue, or the scheduler creates a new plan if some of the new slides have high priority, which often prolongs the completion time of current slides. In order to give the scheduler as good a basis for an as effective action as possible, it is important that the incubation and wash times allow the maximum flexibility that is consistent with good staining quality.

Some of today's staining instruments are offered as part of a 'staining solution' that consists of an instrument, the software, and a large portfolio of ready-to-use (RTU) reagents (antigen retrieval buffer, antibodies, visualization reagents) that comes with optimized staining protocols. This not only gives high staining quality, but also provides short overall staining time (turnaround time). Many laboratories make modifications to staining protocols to obtain a staining pattern according to in-house material (tissue and reagents), internal needs or personal preferences. However, when doing internal modifications it is important to consider potential impact on the overall staining time – and most importantly the risk of introducing false negative or false positive results. As noted elsewhere (Chapters 1, 4, 5 and 14), it is vital that revalidation is performed when any changes are made to reagents or protocols.

Last, but not least, the instrument must be maintained according to the manufacturer's instructions, both with respect to daily/weekly/monthly internal maintenance tasks and the regular service intervals. In this regard, it is important always to be aware of potential irregularities, e.g. extensive or reduced buffer use, deviation in quality of control slides and change in instrument noise. Following major repair, or if instruments are moved, it is necessary to again establish with controls that the instrument is working properly. For example, it is not uncommon to identify incorrect instrument leveling as the root cause for incorrect staining results. Some vendors offer service contracts to ensure timely response and minimal downtime with fixed costs.

Chapter 9.5 | Automation vs. Workflow

Workflow describes the method and sequence in which an activity is performed. Today, there is a lot of focus on establishing efficient workflow in the pathology laboratory from sample receipt, through processing and analysis, to final reporting and storage of slides and data. Creating an efficient workflow is highly dependent on the situation in each labora-

PETITIONER EXHIBIT 1012
Page 117 of 218

tory. It will differ among laboratories depending on a number of factors including type of samples received, sample load, number of staff and staff competence, and not least variation in working hours. A critical element in an efficient workflow is selection of the type and level of automation that best supports the workflow. Choice of instrumentation is thus one of several important factors for an efficient laboratory.

There are many examples of how instrumentation and/or changed use of instrumentation have improved the workflow in pathology laboratories. Examples include the use of rapid processing instruments that have the potential to significantly reduce time to result, and the use of IHC staining instruments in a continuous loading mode rather than a batch mode, facilitating both shorter time to result and a balanced workload. Also, automation of special stains can free up laboratory resources significantly. In short, automation has great potential to improve the laboratory workflow provided it increases efficiency in key parameters including:

- reduction of time to analysis
- reduction of hands-on time from data entry to slide storage
- reduction of human errors
- increase of information from a specimen – the actual parameters being highly dependent on the focus of and requirements to the laboratory

Figure 9.9 An overview of all slides being processed on all instruments in the laboratory can improve the workflow by increased information, control and overview of the processes.

Chapter 9.6 | Key Features for Complete Staining Solutions

When looking for a new IHC staining instrument, there is a large range of features to consider; some of which have been mentioned above, e.g. the ability to perform batch and/or continuous staining. In this context, it should be noted that it is important to compare instruments in the context of the laboratory in which they will be used. Also, it is useful to consider the 'complete staining solution' provided by the vendor, including reagents, protocols, software, service. Each of the separate elements – not least how well they work together – impacts the overall performance in terms of quality, efficiency and cost. Below, a range of important solution features are briefly discussed. The level of importance of each feature may differ among laboratories; what is really important is that the combination of features has the best fit to the needs of the individual laboratory.

High Quality Staining

The most important feature for an IHC staining solution is that it consistently can deliver high-quality staining, with high sensitivity and specificity for all relevant combinations of staining protocols and tissues. This outcome can only be achieved via effective interaction between high-quality reagents and staining protocols that work seamlessly with the instrument.

Turnaround Time

The turnaround time (TAT), the time from loading of slides to completion of staining, has great impact on the laboratory workflow, including when the pathologist receives the stained slides for evaluation. Consequently, a short TAT facilitates a rapid answer to the oncologist, and by that may have direct impact on when patient treatment can be initiated. The TAT will also have a significant impact on the total slide throughput. A small, but significant, detail is the scheduler's ability to accurately predict when a slide or slide run is completed, to facilitate rapid post-processing of the slide(s) and timely preparation and loading of the next slide(s).

The TAT of a specific staining solution is influenced by multiple parameters, including how many slides are loaded onto a given instrument. Depending on the instrument design and

PETITIONER EXHIBIT 1012
Page 118 of 218

scheduler (see section 9.4), even a small change in the number of slides per batch may significantly alter how efficiently the instrument performs. Likewise, changes in incubation times are likely to impact the overall staining time, directly in case of prolonged incubation, or indirectly by affecting the scheduler. Manufacturers of complete staining solutions will typically aim to optimize their instrument, software and reagents to give a low TAT; thus any in-house laboratory modification of protocols may increase the TAT.

Capacity

With the constantly increasing number of slides in the routine diagnostic laboratory, without concomitant increase in staff, the capacity of the staining solution is a major parameter. The capacity can be measured in several ways. It is important that capacity is assessed according to laboratory needs such as the length of the workday, whether the solution is used throughout the 24 hours of the day, average daily workload, and the slide number peaks and distribution. Special consideration should be made on need for overnight capacity, as that may be a very important option for management of increasing slide volume and heavily fluctuating slide volumes between days.

Hands-On Time

With increasing workload, laboratories are under pressure to reduce the hands-on time in order to process the daily volume of slides. Factors such as duration of daily start-up procedures, time spent on slide loading and un-loading, as well as reagent, bulk fluid and waste handling times are important. Each of these factors may include several important components, according to the specific needs of the laboratory; one example being the reagent capacity of the instrument. The lower the reagent capacity, the greater the need to change reagents between runs, whereas a high reagent capacity may require little or no reagent handling during the day. An additional important instrument feature is related to maintenance, including daily, weekly and long term maintenance.

Regarding hands-on time, it is truly a question of assessing not only the instrument itself but the complete staining solution, including how instrument, software and reagents interact. Important parameters include a wide variety of factors that are not isolated to the instrument alone, e.g. remote access to instrument status, report generation, and need for cleaning of stained slides or instrument accessories. Another important parameter to assess is the total reagent package, including handling of reagent vials and bulk fluid bottles, as well as the ease of use of the software (user-friendliness), e.g. when setting up new staining protocols or requesting new slides for staining.

Figure 9.10 More onboard capacity for reagents and bulk fluid bottles can reduce hands-on time due to little or no reagent handling during the day.

Functionality

Today, all IHC staining instruments can perform the complete staining process from peroxidase blocking to counterstaining. Many instruments include deparaffinization and antigen retrieval whereas other approaches have separated the two processes. Onboard deparaffinization and antigen retrieval reduce the number of times that slides need to be handled and the risk of manual errors. A split of these functionalities allows for parallel processing so that deparaffinization/antigen retrieval can be performed in parallel on a dedicated instrument and thus be ready for immediate staining when the IHC staining instrument has completed its run. Efficient use of this approach has a positive impact on total efficiency and throughput. Furthermore, some instruments have the capability to bake slides, which reduces the number of slide handling steps. This advantage must be measured against the quality of baking, the efficiency/throughput of the instrument, and not least which method has the best fit in overall laboratory workflow.

PETITIONER EXHIBIT 1012
Page 119 of 218

The final functionality to be mentioned is the possible application of the instrument to fluorescence in situ hybridization (FISH), or a bright field microscope version, such as chromogenic in situ hybridization (CISH). As hybridization assays are significantly different from IHC assays, dual capability puts increased demands on instruments and integrated staining solutions. It is therefore important to carefully evaluate the quality of both IHC and ISH stainings and whether the instrument can effectively run both types of assays in parallel without significantly impacting TAT and capacity. Further, the overall fit of dual IHC and ISH stainers into the laboratory workflow must also be evaluated. Important elements in this context are the distribution and volume of IHC and ISH slides and the need for specific report times (e.g. can either assay types be run overnight, or can ISH slides be batched and run on set days only?).

Laboratory Accreditation Support

More and more clinical laboratories are subject to accreditation requirements, and in some countries laboratories must be accredited to perform clinical testing. In the USA, accreditation is also a requirement for reimbursement through the Medicare system. Accreditation is a mechanism of value to verify that laboratories have an appropriate quality management system, can properly perform specific assays, and are able to properly document test results. In this context automated IHC stainers, that incorporate complete staining solutions, represent an approach to monitoring important assay parameters, which at the same time can include a reporting format that suits the laboratory needs according to its quality management system. Required accreditation data vary according to the country in which the laboratory is situated, as well as the quality management system of the individual laboratory. Requirements may be quite detailed, down to the level of specific information of the IHC staining on a per slide basis, or the specification of which lot of wash buffer was used. Likewise, instrument maintenance and service must typically be carefully logged, often with an 'on instrument' record.

Chapter 9.7 | Next Steps in Automation

Looking 5-10 years ahead, new technologies and new types of biomarkers will be commonplace in routine pathology laboratories. Examples of new types of biomarkers include mRNA, miRNA and other non-coding RNAs, DNA methylation variants, and post-translationally modified proteins. With regard to new technologies, digital imaging is certain to occupy a central position, fully integrated with the IHC staining solutions and other slide-based staining solutions (see Chapter 7). New, non-slide-based tests, e.g. next generation sequencing, are in the process of entering routine diagnostic application providing new important information for cancer diagnostics, but at the cost of morphological information. Thus, IHC and other slide-based staining techniques will in 10 years time continue to play an important role in cancer diagnostics, however, in an environment that is quite different from today's pathology laboratory.

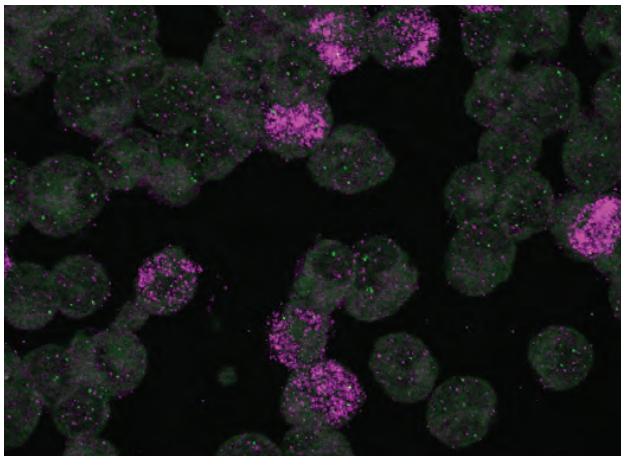


Figure 9.11 New technologies and new types of biomarkers will be commonplace in routine pathology laboratories in the next 5-10 years. This will put new demands on future instrumentation. The image shows RNA FISH demonstrating differential RNA expression within a cell population. Courtesy of Robert A. Arch, Agilent Laboratories.

PETITIONER EXHIBIT 1012
Page 120 of 218

The future staining solutions will need to be able to generate much more information from less sample material, in a more effective and faster manner without compromising the requirement for quality; that surely will increase over the next 10 years.

- More information will be derived from new biomarkers and biomarker types, with growing use of dual- and multiplex assays with several antibodies being applied to the same slide as well as antibody and F/CISH combinations.
- There will be demand for accurate quantification, with ongoing, perhaps growing, demand for quantification at an individual cell level within a heterogeneous tumor cell population.
- A drive towards less invasive sampling methods will reduce the amount of sample material, at least for some cancer types, and the solutions must provide more information from less sample material.
- Complete staining solutions will support new multiplex and quantitative assays and become significantly more effective with a reduction in hands-on time per slide. Efficiency may not be measured on a per slide basis but rather on a per patient case basis. Increased efficiency will be implemented via an increase in the functionality of complete staining solutions as well as of the whole laboratory We will probably see improved alignment between tissue cutting and slide loading, as well as integrated slide scanning and distribution of Whole Slide Images (Chapter 7).
- There will be a continued drive towards reduced time to diagnosis that will be translated to the staining part of the workflow, as reduced time to result or TAT. This goal may be affected by adoption of rapid staining protocols, employing new visualization chemistry that can also handle several biomarkers simultaneously.

Again, it is important to stress the concept of a complete staining solution, incorporating instrumentation, reagents and protocols in an optimized system. In order to be able to live up to the new customer needs, it will be imperative that instruments, reagents and software are developed to work optimally together. For example the introduction of more rapid, more sensitive staining methods will not only require new visualization technology, but also instruments and software schedulers that support short incubation times for efficient use of the instrument modules or units. Finally, it will be very important that the new staining solutions will be able to work seamlessly with whole slide digital scanners and laboratory/hospital information systems.

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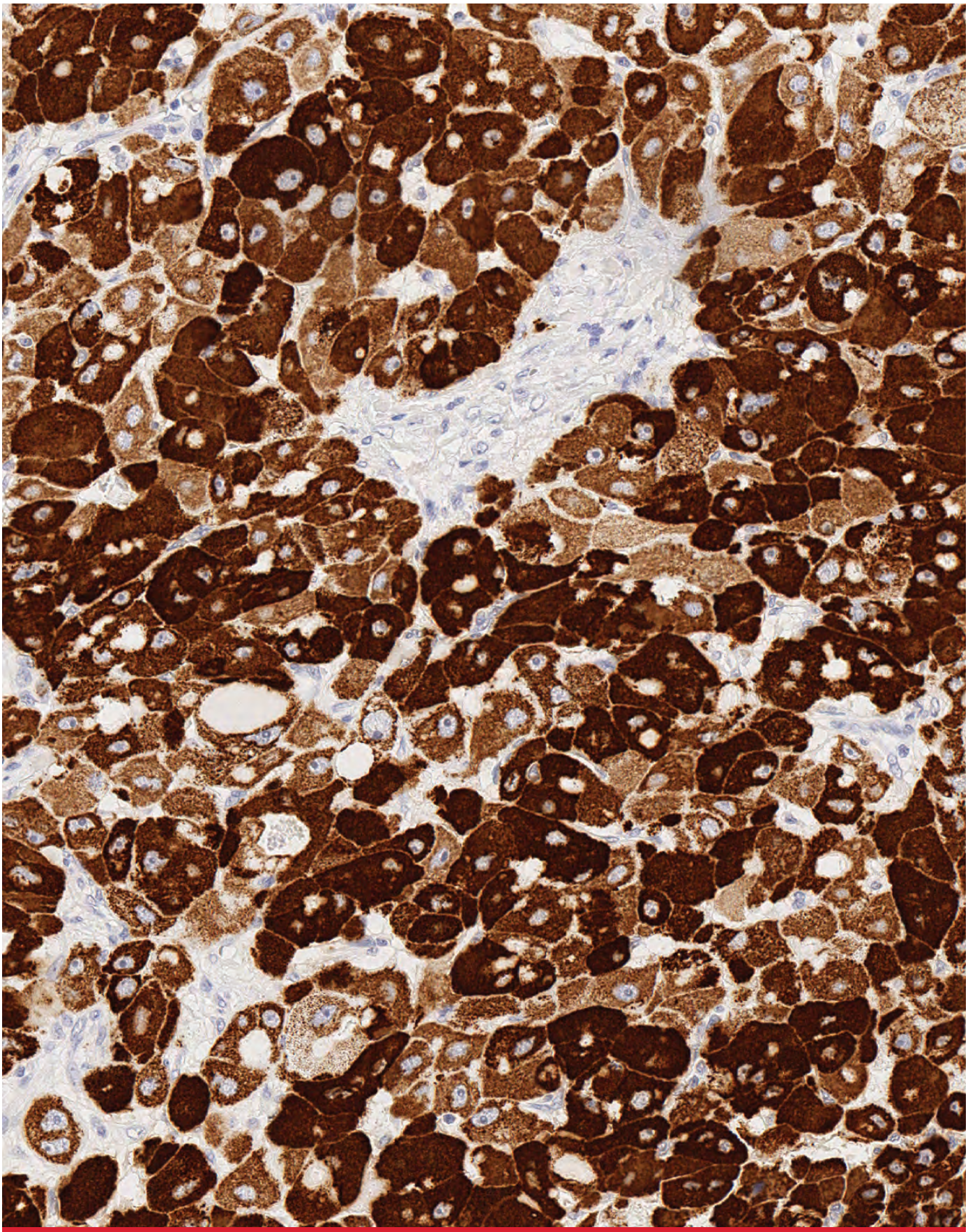
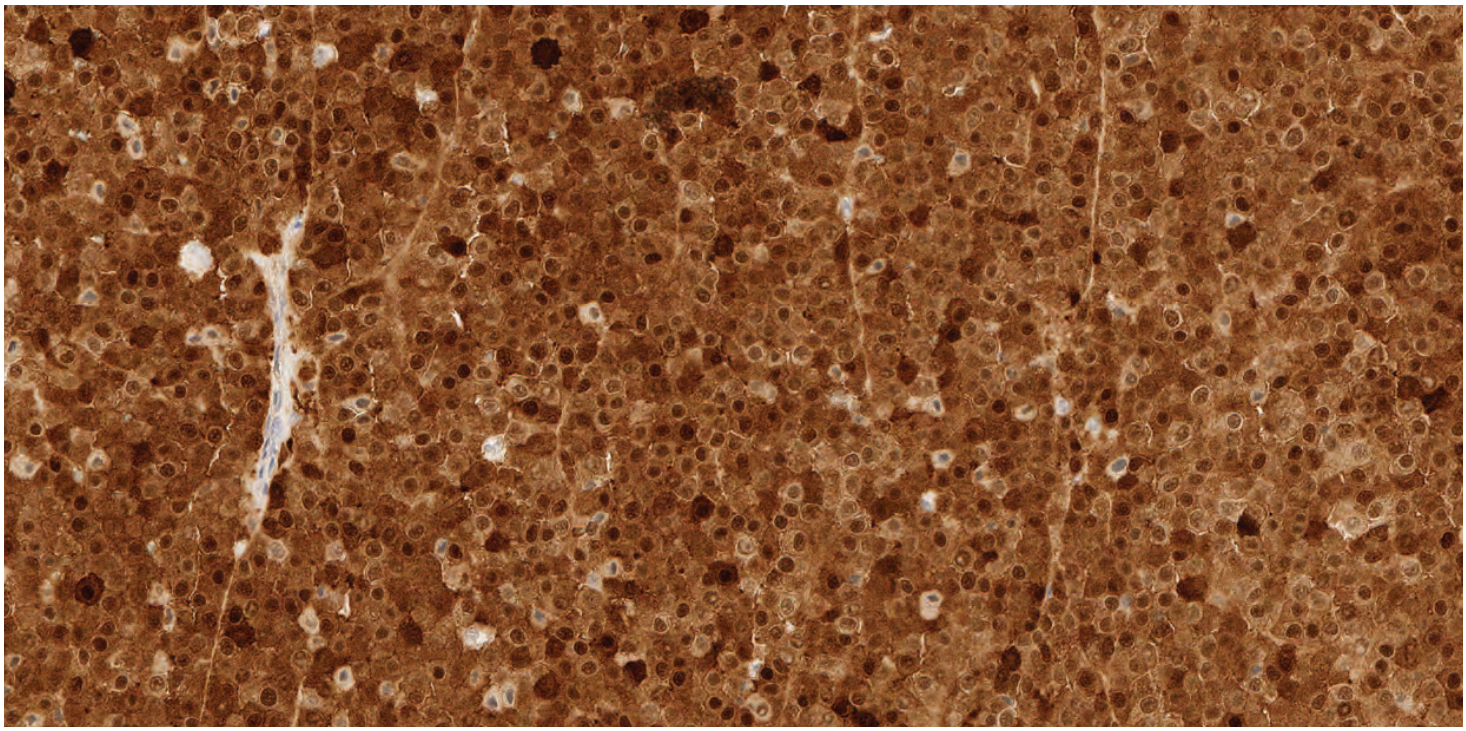
PETITIONER EXHIBIT 1012
Page 121 of 218



Chapter 10

Optimizing Laboratory Workflow

David McDonald, BSc, MLT, LSSBB



Work•flow *(n.)*
1. The flow or progress of work done by a company, industry, department, or person.
2. The rate at which such flow or progress takes place.

The American Heritage® Science Dictionary

Chapter 10.1 | Introduction to Workflow

The term "Workflow" has been defined in numerous ways and scope of activity over time and across the evolution of sophisticated observation and statistical analysis techniques. Ultimately any definition will have merit, based upon the ability to demonstrate the information required to make an educated and fact-based decision as to future direction. That direction must be aligned with the defined criteria.

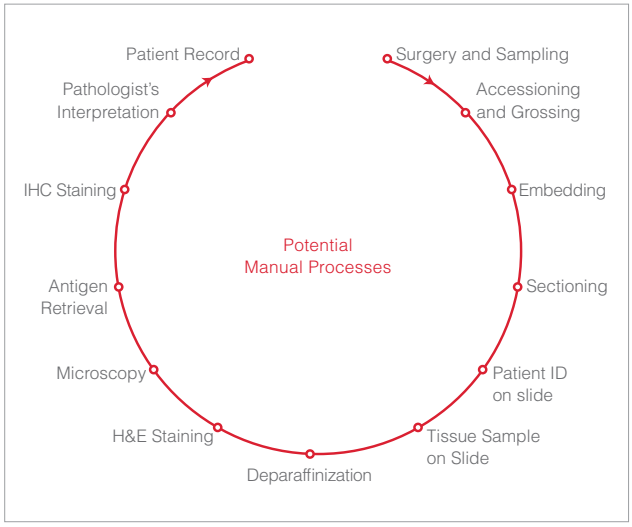


Figure 10.1 No two labs are the same. Manual handing in one or more of the illustrated steps is frequent in most labs.

Workflow is based upon the simple principle of deriving a complete and thorough understanding of the entire current process, the multiplicity of steps inherent within that process and all of the factors which impact the current achievable outcome. An important rule of workflow resides in the belief that only by having a complete understanding of the current processes can an alternative, more effective process be implemented. The understanding of an IHC pathology 'facility specific' workflow resides in the assessment of the following parameters:

- Slide availability – how do current processes in the laboratory affect at what point in the shift slides become accessible for staining; and subsequently how do they impact at what time slides become available for delivery to the pathologist for review?
- Stainer load capacity – how many slides can be loaded without compromising stainer throughput capacity and consistency?
- Slide load interval – how frequently can slides be loaded onto a stainer without compromising throughput consistency?
- Batch size – does the current process enable the maximum number of slides to be loaded with each load event?
- Process complexity – to what extent do manual processes such as documentation, written tracking of slides and stains, post stain labeling of slides, case assignment rules and details of protocol impede and delay the overall process?

Workflow must be designed around a series of individual solutions that respect and exploit the unique characteristics and needs of each operation. After all, no two labs are the same.

Chapter 10.2 | Specimen Tracking

Within the scope of daily workload processing, one of the single most pervasive forms of delay incorporated into an overall process is the need to identify and track individual specimens/blocks/slides as they advance through each subsequent process step, i.e. initial accessioning, embedding, microtomy, labeling of slides, drying, staining, cover slipping and delivery to the pathologists. Many of these steps are still manually performed in most laboratories today, greatly increasing the risk for mislabeling errors at one or more of these checkpoints, thereby leading to potential misidentification of patient samples.

In the US, both the College of American Pathologists (CAP) (1) and The Joint Commission (JCAHO) (2) have issued directives that address the importance of a specimen identification system throughout the clinical laboratory. Across the world, hospital labs have implemented various Quality and Safety protocols as part of their accreditation systems. However, the direct connection between patient identification in the hospitals' laboratory information system and the IHC staining process steps are not yet fully implemented in all labs.

Historically, sample tracking is accomplished through the use of manual documentation logs. In recent years, vendors have developed specific software and hardware solutions to address and attempt to eliminate the requirement of manual documentation logs within the scope of Anatomic Pathology. When investigating such a solution for a specific laboratory, it

PETITIONER EXHIBIT 1012
Page 124 of 218

is important to consider the following criteria:

- Overall system flexibility – ease of connectivity
- Individual process step points of contact – where in the process is it important and/or desired to capture specimen/block/slide handling and transfer advance
- Scope of system implementation – single site versus multiple site

The tracking solution usually consists of software that links the facility's LIS to a server-software that collects and feeds label information to slide label printers and assay information to instruments. This connection enables tracking of multiple interaction points within the process, such as accessioning, grossing, tissue processing, embedding, microtomy, staining, slide distribution and archiving; thus capturing the entire scope of daily operations. The tracking capability is enabled through a specific barcode attributed to specimens, blocks and slides. It is a huge benefit for an optimized tracking system to be deployed, such that the total number of access points into the system is virtually unlimited. Only in this manner can clerical staff, technical staff and pathologists have the ability to perform search functions on specific specimens, blocks and slides. Tracking systems can ultimately eliminate the need for manual documentation, streamline the process flow and provide a means of error-proofing the entire system.

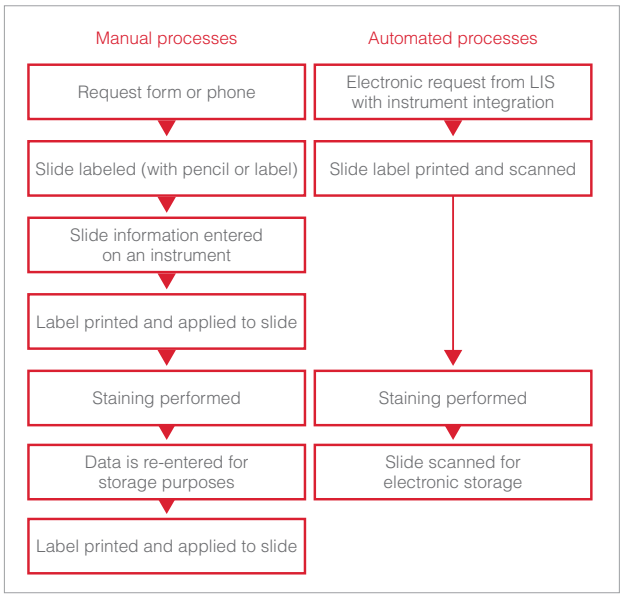


Figure 10.2 Example of workflow challenges involving slide labeling and data entry. LIS integration with IHC instrumentation can potentially save many manual labeling steps.

Chapter 10.3 | Specimen Collection and Identification

Specimens are collected and delivered to the anatomic pathology lab through a variety of mechanisms and can take the form of wet specimens, blocks and slides. These mechanisms will vary in complexity based upon the catchment area of the specific testing facility and may include in-house, multi-site affiliated and non-affiliated referral specimens. Requisitions may be of either a standardized format, or a combination of multiple form and requisition types, adding a further layer of complexity.

LIS connectivity among sites offers a distinct advantage in the process of specimen collection and identification through site-specific prefixes in the specimen identification number. The lack of integrated LIS connectivity among testing referral sites, however, need not be a barrier to connectivity for staining purposes. Through wide area networks (WAN), some systems are able to connect separate multiple sites to enable remote ordering, such that incoming slide orders are able to populate instrument management software.

Obstacles/Challenges

There is a perceived limitation that only through LIS interfaces can multiple sites achieve the level of connectivity required to transmit orders to an individual testing site facility. There may also be the misconception that all sites are required to use the same LIS and version software.

Improvements/Solutions

In the absence of LIS interface connectivity, WAN solution software can enable multiple sites to achieve the level of communication required in order to permit direct transfer of slide order requests into a single test site instrument software.

Chapter 10.4 | Specimen Accessioning

Reducing the complexity of specimen accessioning resides in the ability to translate a physical specimen order request into a visible slide protocol that is recognized by the staining instrumentation in use. By utilizing a suitable interface connectivity between the

PETITIONER EXHIBIT 1012
Page 125 of 218

Figure 10.3 A busy lab can benefit from systems that are able to connect separate multiple sites to enable remote ordering.

facility LIS and the instrument server software, users can place orders into the LIS that can then be assigned to individual instrument staining runs directly from workstations in the laboratory.

Orders are typically placed within the LIS directly by the pathologist, or additionally can be submitted via separate requisitions, sometimes hand written in a separate order log book. One specific advantage of interface connectivity between the facility LIS and the instrument server software is elimination of the delays associated with multiple layers of specimen accessioning, at the same time reducing opportunities for error.

Obstacles/Challenges

Translation of order requests into the instrument stain protocol can also be a source for delay in the daily initiation of staining runs. Historically, Histology departments have developed time specific cut-offs, after which staining runs are initiated with the intent of capturing all of the pending orders up to that point in the day. Outstanding slides are confirmed by pulling pending logs and/or checking separate request logs, and cutting/retrieving the associated slides, all of which may require up front manual activity.

Improvements/Solutions

Through the advent of direct download capabilities into the instrument software, with accompanying order request information and notations, pending slide runs can be planned and executed much sooner in the daily routine. The result is that slides become available for review sooner into the shift, and overall turnaround time is reduced.

Chapter 10.5 | Grossing

At the point of grossing, as in the many other aspects of specimen processing, delays and errors may occur as a result of inherent complexity of multiple sequential steps within that particular process. Ideally, the generation of specimen cassettes is optimized when each cassette is printed/etched automatically as a direct result of data entry at the requisitioning step.

Most tracking software enables the generation of cassettes with case and specimen specific identification within a unique barcode assignment, as a parallel activity directly associated with requisition and ordering. This capability eliminates the need to switch from specimen requisitioning to either a separate soft-

PETITIONER EXHIBIT 1012
Page 126 of 218

ware application to generate specific cassettes, or a manual keying in of each individual cassette at the cassette printer. The optimization of specimen entry, transfer to grossing, and then on to histology, augments and maximizes the ability for effective use of load capacity and short tissue processing cycles.

Obstacles/Challenges

Typically, specimen requisitioning involves the batch registration of multiple specimens, either delivered to the laboratory, or collected during a sweep of associated specimen generating locations within the facility (operating rooms, day surgery sites, specialty clinics). Once these specimens have been registered, the resulting batches transition from the gross lab into properly labeled cassettes, matched with the specimen. This crucial step requires planning and dedicated lead time in advance of grossing. Additionally, specimen registration is not always localized adjacent to the grossing stations, a situation that can impede the progress of specimens to the stage of cutting, as well as limit accessibility for the request and generation of additional cassettes as required.

Improvements/Solutions

The design of a process whereby cassettes are printed in association with, and as a direct result of, specimen registration expedites the progression of specimens through grossing as single integrated flow, such that individual specimens can be accessed in real time, rather than as part of a batch transfer event between sequential steps in the process.

Additional direct benefit results from the placement, wherever possible, of specimen registration directly adjacent to grossing, and the provision of at least one supplemental cassette printer within the immediate grossing area. This arrangement allows for additional cassette requests without a separate request to the registration clerk, or interruption of the registration and grossing processes.

Chapter 10.6 | Tissue Processing and Microtomy

The optimization of workflow within tissue processing and microtomy is a factor of batch size and sequential versus parallel activity. Batch event or “all-or-nothing” processing is a non-optimal activity that often becomes the rate limiting step of all daily activity. One manner by which optimization of workflow may be addressed is covered by the workflow term “trigger point transfer” – namely the rapid shift from one activity to the next based upon accumulation of inventory (work), such as embedded blocks or cut slides. In contrast, standard batch event processing is a function of arbitrary individual shift start/stop times, established hours of operation and current process capability limitations. Common scenarios can be characterized by the following practices:

Tissue Processing

- Specimens are grossed as they come in throughout the day and tissue processors are all loaded at the same time for an overnight cycle, up to a specified ‘cut off’ time

Batch Event Obstacles

- Delays in loading processor baskets into appropriate short cycle processors for certain tissues (rapid biopsies)
- All cassettes become available at the same time the following day, the large batch of cassettes for sorting, embedding and microtomy creating a bottleneck effect
- Loading subsets of appropriate tissue cassettes into short cycle processors, as they become available, removes a portion of the workload from the remaining process batch and improves throughput of the overall process

Embedding/Microtomy

- Typically multiple staff members embed all cassettes from the overnight processing cycles and once all embedding is completed, then microtomy begins
- Slides are hand-written or etched at the ‘front end’ of microtomy, in advance of cutting, and re-labeled at the back end, post routine or special staining

Batch Event Obstacles

- By waiting until embedding is complete, microtomy, which is an inherently long and labor intensive process, becomes the next rate limiting step, impacting the time available to load and stain slides. By identifying and setting an available block count, which triggers some staff members to begin moving from embedding to microtomy, the overall process is shortened and stain throughput, case assembly and distribution are optimized.

PETITIONER EXHIBIT 1012
Page 127 of 218

- 'Hand writing' slides at the front end and labeling at the back end means that slides are handled and identified twice. With tracking software capabilities, labeled slides can be generated in real time prior to microtomy, thereby eliminating one of these slide labeling interactions, reducing time, while simultaneously removing an opportunity for error.

Figure 10.4 Exchanging hand-written slides with barcoded labels and software with tracking capabilities reduce time and error opportunity.

Chapter 10.7 | Routine Staining and Coverslipping

The staining of routine slides generally is accomplished either by a manual transfer staining process, or by use of an integrated staining platform. With integrated staining methods, the processes of slide baking, staining and coverslipping are performed on a 'single load' instrument platform.

Manual Transfer Processes

Manual transfer processes rely on the transfer of slide racks across each of the individual and separate steps of baking in a stand-alone oven, deparaffinization, staining and dehydration (often on an automated stainer), followed the manual load and unload activity associated with a stand-alone coverslipping device. In each of these scenarios, workflow optimization becomes a function of reducing the number of steps, load intervals and batch size.

Manual transfer processes are subject to the rate-limiting factors of:

- The number of staff manning the workstation
- Other duties which intermittently take staff away from the workstation
- Slide load intervals, which can be inconsistent and discontinuous
- Time required for 'off-line baking' and transfer to the staining step
- Slide rack capacity and the inherent time required to cut sufficient slides to fill a rack
- Sequence timing limitations in the X-Y-Z motion of the robotic rack transfer arm, affecting overall throughput time

Integrated Staining Platforms

Integrated staining platforms remove the requirement of having trained laboratory staff immediately available at the appropriate times for transfer between subsequent steps. Efficiency remains dependent upon;

- The ability to take advantage of all online features, while ensuring that they perform as intended (for example, does the oven actually perform an adequate baking process)
- Making optimal use of inherent process-balanced design features, such that bottlenecks are avoided on the unit in any of the process steps (does baking or staining take a disproportionate time relative to how slide racks can move through the remaining processes and dip tanks?)
- Manual oversight and intervention may still be required (how many coverslips can be loaded at once to alleviate the constant need for reloading?)
- Performing the required frequencies of onboard reagent filtering, swap-out exchange and/or position transfer (replacing the first xylene or alcohol dip tank and moving the others in sequence to the next position as required).

Chapter 10.8 | Slide/Block Reconciliation

Case assembly for verification and distribution is another time consuming process and a source of imparted delay into overall turnaround time. It is therefore important to design a functional system that facilitates case assembly with a minimum of manual activity and time commitment.

PETITIONER EXHIBIT 1012
Page 128 of 218

Obstacles/Challenges

The organization and structure of the slide sorting and case assembly process presents a significant obstacle to workflow. Typically sorting, assembly and distribution is an end of shift batch event, and not a continuous process. Also the number of people brought to the task requires a large laboratory footprint to accommodate multiple racks of 'routine' H&E slides for sorting to multiple case folders, accompanied by an additional degree of disassembly or reorganization of cut slides in order to carry out further staining processes (such as IHC and special stains, grouped by antibody, pre-treatment protocol or stain group).

Improvements/Solutions

Introducing more frequent (ideally continuous) checking of blocks and sorting of routine slides minimizes occurrence of "all-or-nothing" batch events during the shift, or at the end of the shift, and expedites the flow of completed slides to the pathologist for review. In addition, reducing disassembly of sets of cut slides for IHC and Special Stains, by advance planning and organization not only facilitates staining by case, but also minimizes hands-on manipulation on the part of the technologist. This process can be accomplished by choosing staining equipment with high slide and onboard reagent capacity, plus the ability to combine slides with differing protocol requirements side-by-side within the same run.

Chapter 10.9 | Laboratory Asset Tracking and Workflow Management

Within any anatomical pathology environment, the ultimate goal should be to achieve maximum access to all the relevant information in the course of managing daily workflow.

The ultimate management of workflow is a function of three specific factors;

- Tracking capabilities – the ability to trace specimens, blocks and slides throughout the entire process, from requisitioning to archival. Tracking should ideally also provide for attribution of process-specific activities to individual staff within the scope of the entire process.
- Connectivity – the ability to place orders locally as well as remotely, and to generate a barcode that identifies and marries specimen, cassette and slide. A slide barcode should be specific down to the individual stain and protocol level. There should be an unlimited number of access entry points to enable the monitoring of workload status. Connectivity should also remain flexible in order to accommodate third party ancillary devices as required, (barcode label printers, for example).
- Reporting – Reports should be easily configurable and custom tailored to the needs of the facility for capture and organization of information as required

Figure 10.5 Integration from a Laboratory Information System (LIS) to the stainer instruments can be accomplished through a single point of connection using an integration module, e.g. the Universal LIS Agent (ULISA). The ULISA manages communication with a LIS server and provides the various instruments with the data received.

PETITIONER EXHIBIT 1012
Page 129 of 218

Obstacles/Challenges

Historical challenges include the ability to interface fully with the facility LIS provider, proprietary limitations with respect to all associated ancillary devices and standard versus configurable reporting structures, which may or may not meet the needs of the facility.

Improvements/Solutions

Ideally, the most flexible solution will enable a facility to achieve all of the following;

- Provide an independent test order module for pathologists
- Enable the generation of barcode labels at the microtome station, to cut and label slides in real time and as single piece flow
- Enable deployment at the reagent fridge for log in and management of reagent inventory
- Facility for full installation on any user PC, wherever it makes sense to track workload freeing technologists from instrument specific workstations
- Provide for workload monitoring at multiple sites in an integrated delivery network
- Facilitate the ability to generate orders remotely and stain locally
- Facilitate a single barcode label throughout the entire process
- Enable the configuration of facility specific report format

Chapter 10.10 | One Workflow Does Not Fit All

When it comes to addressing workflow, it is important to acknowledge that each pathology laboratory, while performing

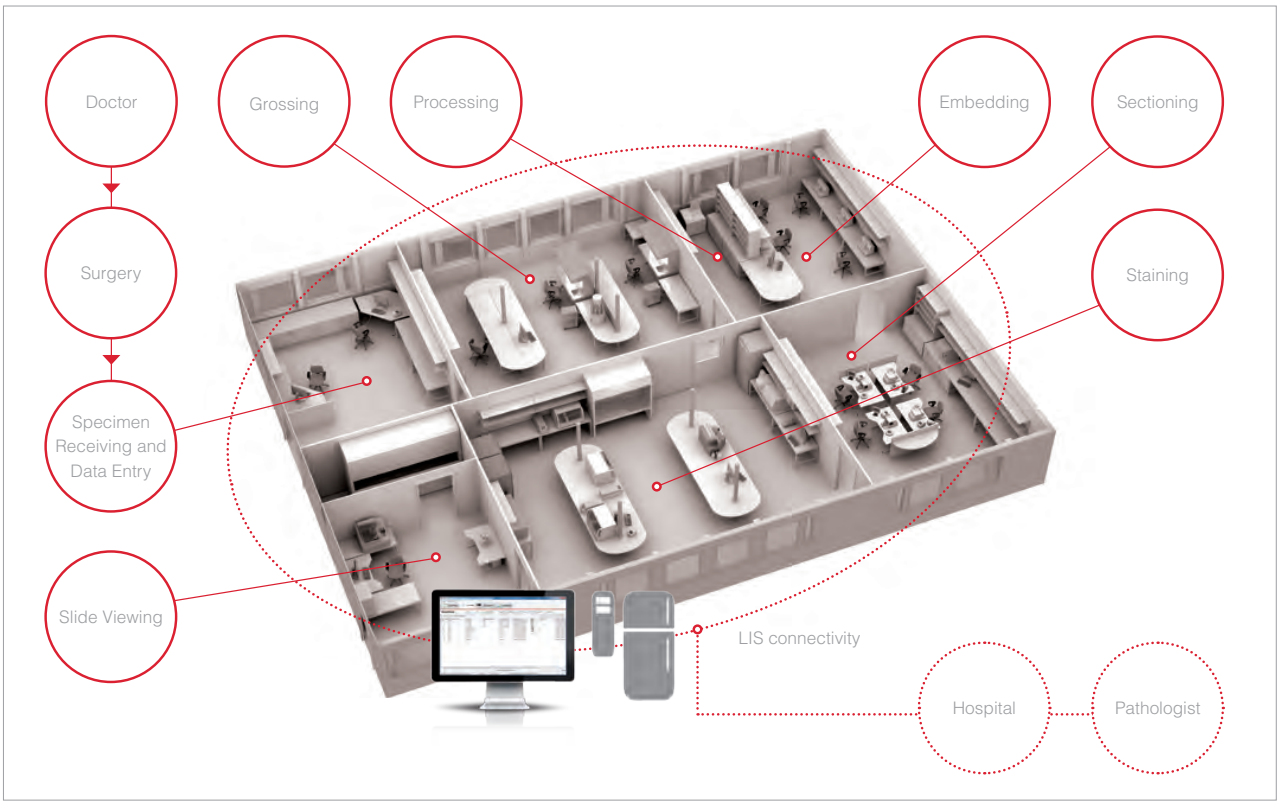


Figure 10.6 Some laboratories are disconnected; the process steps are all critical to the final result, yet they are not integrated with one another.

PETITIONER EXHIBIT 1012
Page 130 of 218

similar activities, differs in specific workload processes, with respect to absolute slide volumes, stain menu diversity, hours of operation, staffing levels, catchment area, and interface with one or more institutions for which service is provided. The most effective strategy to approach workflow evaluation will incorporate the following capabilities:

- Assessing and cataloguing those functions that perform well versus those that need improvement
- Fully understanding the current workflow, identifying specific obstacles to success and deriving criteria by which alternative process capabilities may be assessed
- Deriving the proper key performance indicators to measure process activity and capability
- Facilitating statistical analysis to identify historical growth rate, current achievable throughput against demand, vendor-related capabilities with respect to instrument productivity, slide capacity, waste generation, and the ability to accommodate incremental slide volume over time
- Methods for aligning input from all key stakeholders, from bench Technologist, department Managers, Pathology Director, and Pathologists, and together with hospital administrators and clinical users as necessary

Chapter 10.11 | General Sample Labeling and Tracking

(by James Happel, DLM (ASCP) HTL, from IHC Staining Methods, 5th Edition, published by Dako.)

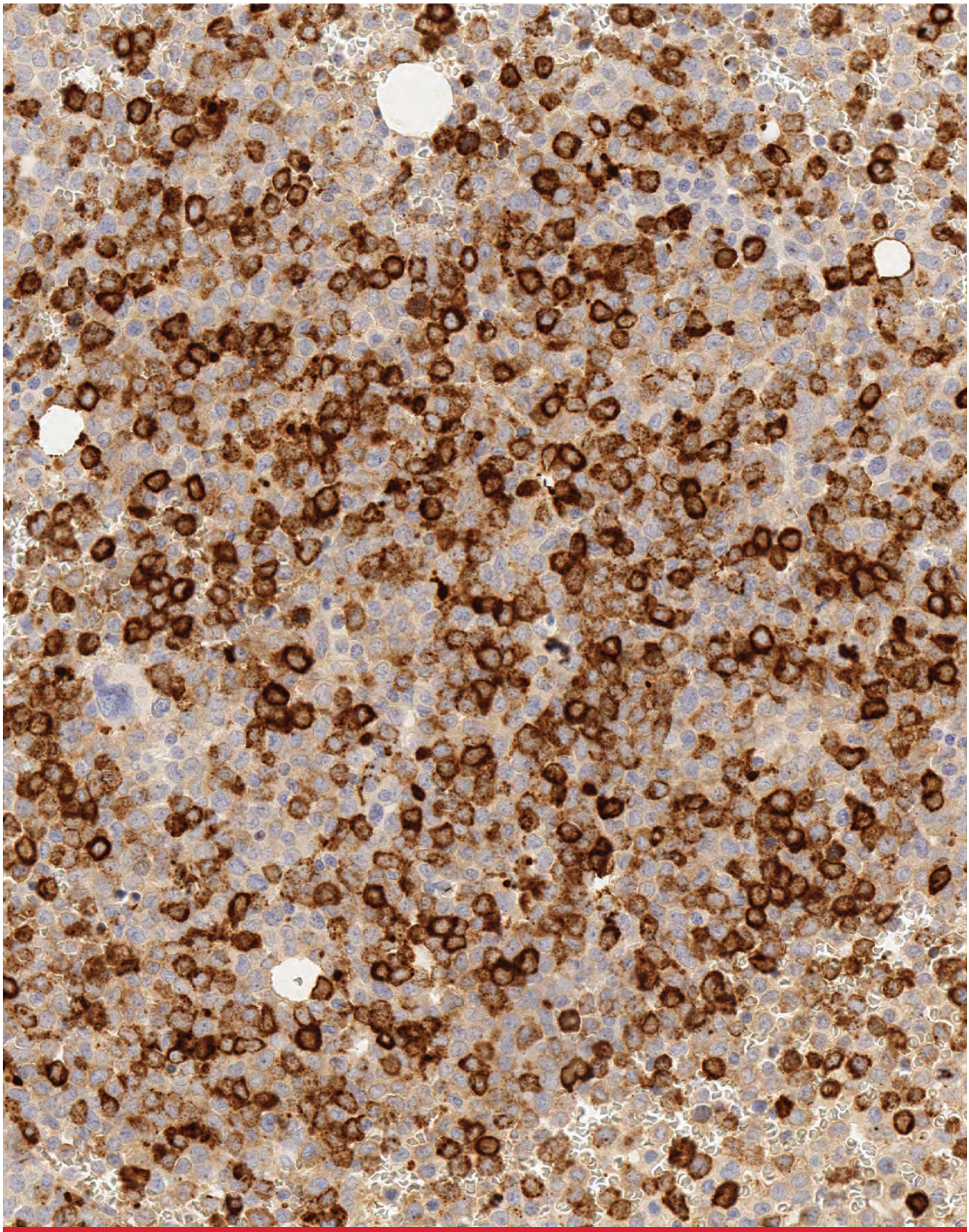
Do

- Have clear and concise procedures based on CAP and JCAHO specimen identification recommendations
- Remove waste baskets from the specimen collection areas
- Retain all trash for a minimum of three working days
- Label only one case at a time
- Keep all materials from a single case together (requisition, specimen containers, cassettes, etc.)
- Have the staff responsible for placing the specimen in the transport vessel (i.e. specimens collected during a surgical procedure, cultures to be sent for microbiological analysis, tubes of blood, etc.) be the same individual who labels all materials generated for that case
- Have a second staff member review and verify what was sent to the laboratory
- Have only the tissue processing cassettes for the case being grossed at the prosection station during that cases dissection. Immediately remove from the work bench and discard all cassettes that are not utilized for the processing of the case.
- When grossing, only have the tissue cassettes for the current case on the cutting board at the time of dissection. All other tissue cassettes should be with their appropriate specimen and not on or near the prosection station.
- When embedding, be certain to wipe off forceps after every use to insure that any residual tissue is removed thereby preventing carryover from one cassette to another
- Work in tandem with all those who come in contact with the specimen including the Operating Room Team, Transporters, Specimen Accessioning staff, Pathologists' Assistants, Residents, Pathologists, Histologists, Secretaries, Transcriptionists, slide and block filing room staff, etc.

Do not

- Do not pre-label requisitions, specimen containers or tissue cassettes
- Do not accession similar case types (tissue) in sequence
- When embedding, do not open more than one cassette at one time
- When cutting on the microtome, never cut a second paraffin block until the first block has been cut to completion
- Do not leave ribbons on the water bath after picking up sections on slides
- Do not leave printed labels in the work area. Discard all unused labels as soon as it is identified that they are not to be used.

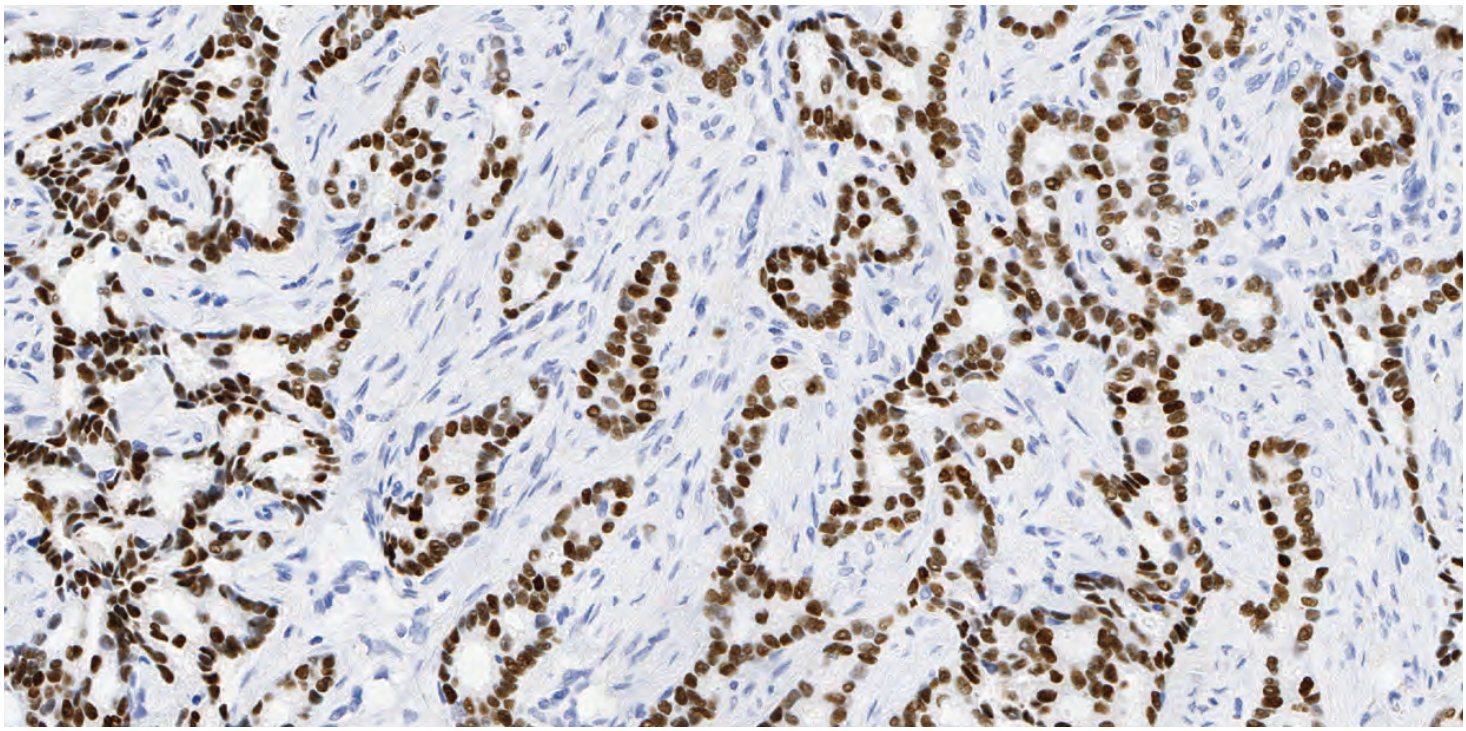
PETITIONER EXHIBIT 1012
Page 131 of 218



Chapter 11

Companion Diagnostics

Hans Christian Pedersen, MSc
Jan Trøst Jørgensen, PhD, MSc



Com•pan•ion di•ag•nos•tics *(n.)*
A companion diagnostic device is an in vitro diagnostic (IVD) device that provides information that is essential for the safe and effective use of a corresponding therapeutic product. also known as Predictive Markers, Theranostics, or Advanced Personalized Diagnostics.

www.fda.gov

Chapter 11.1 | Introduction

Predicting the response to a pharmacological intervention is an optimal goal for any healthcare professional working with pharmacotherapy, especially when it comes to serious and life-threatening diseases such as cancer. Early correct diagnosis and effective intervention are two elements of key importance in the treatment of cancer. In the event of a wrong treatment decision the disease may become disseminated, with little or no chance of cure. Companion diagnostics (CDx) hold the promise of improving predictability of outcome of a specified pharmacological intervention, thereby constituting an important tool for the oncologist in relation to choice of treatment. In a related sphere there is an urgent need to improve the development pathway of new drugs, with better predictability of the outcomes of R&D in terms of approved and useful product. CDxs have already been shown to be useful tools in this respect, especially when it comes to clinical development (1). The way that many drugs are being developed today is far from optimal and, in general, the productivity of the R&D departments in the pharmaceutical industry and biotech companies seems to be decreasing. Recently, it has been estimated that around USD 60 billions, of the USD 85 billions spent on R&D globally every year, are wasted due to failures during the drug development process (2).

Chapter 11.2 | History of Companion Diagnostics

The idea of combining drugs and diagnostics is not new. When the selective estrogen receptor modulator tamoxifen (Nolvadex®, AstraZeneca) was developed in the 1970s for the treatment of advanced breast cancer, data on estrogen receptor status was correlated with the treatment outcome. Based on a phase II study performed in patients with metastatic breast cancer, published in 1976, the investigators concluded: “A high degree of correlation between response and positive estrogen-receptor assay suggests the value of the diagnostic test as a means to select patients for tamoxifen treatment” (3). Despite the fact that this conclusion was reached as early as the mid seventies, more than 20 years would pass before the next predictive or selective diagnostic assay saw the light of day, namely the HercepTest™ (Dako), an immunohistochemical (IHC) assay for detection of overexpression of the human epidermal growth factor receptor 2 (HER2) protein.

In the 1990s, the US-based biotechnology company, Genentech, ‘humanized’ the monoclonal antibody trastuzumab (Herceptin®, Roche/Genentech) and placed it under development for treatment of women with metastatic breast cancer. Trastuzumab is a drug that targets the external domain of the HER2 receptor. Genentech very early on realized that only patients with cancer cells that had a high expression of HER2 receptor protein responded to treatment with trastuzumab, pointing to the need for a companion diagnostic selecting such patients for therapy. Data from the subsequent phase III study with trastuzumab in HER2-positive patients with breast cancer showed that this was a wise decision (5). Furthermore, this decision later led directly to the development of the CDx HercepTest™, designed to detect HER2 in a semi-quantitative manner (4).

A few years after the finalization of the phase III trial, a retrospective statistical analysis of the data showed some rather remarkable results with respect to the design of this study. Based on this analysis, it was concluded that the use of the HER2 IHC assay as a pre-selection tool had played a key role. If this assay had not been available it would have required enrolment of several thousand patients in order to reach a conclusive result, instead of the 469 patients that made up the total phase III study population (5, 6). It has also been said that if the HER2 IHC assay had not existed, we would likely not have access to trastuzumab today, due to the fact that it would have been discarded during clinical development because of lack of efficacy in an unselected patient population (7). The development of trastuzumab showed for the first time how crucial a CDx assay can be in relation to the development of a targeted anti-cancer drug. The HercepTest™ assay was approved for routine clinical use in September 1998 simultaneously with trastuzumab in a novel coordinated process between two divisions of the Food and Drug Administration (FDA) (4). The parallel development of Herceptin® and HercepTest™ is outlined in Figure 11.1.

Within the last decade, HER2 has proven not only to be an important target in the treatment of breast cancer, but more recently also gastric cancer (8, 9). In 2010 the FDA approved use of HercepTest™ and the *HER2* FISH pharmDx™ Kit (Dako) for the assessment of gastric cancer patients for whom trastuzumab treatment is being considered. Further, within the last couple of years the HER dimerization inhibitor pertuzumab

PETITIONER EXHIBIT 1012
Page 134 of 218

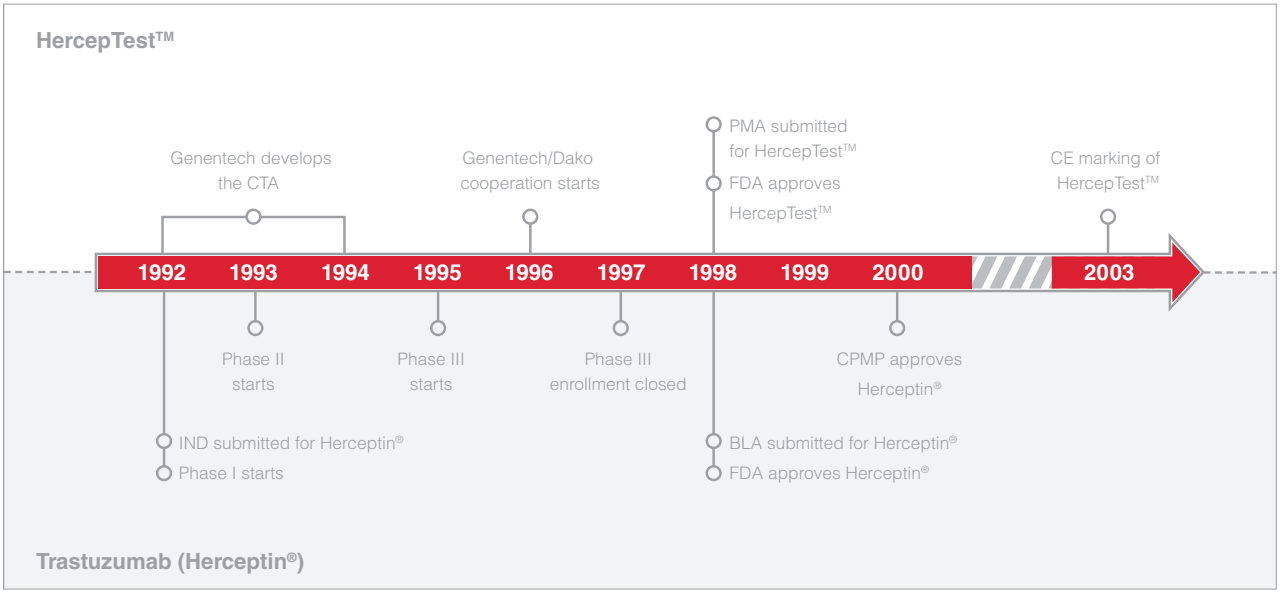


Figure 11.1 Co-development of the drug Herceptin® and the companion diagnostic, HercepTest™. CTA: Clinical Trial Assay; PMA: Premarket Approval; CE: Conformité Européene; IND: Investigational New Drug; BLA: Biologic License Application; CPMP: Committee for Proprietary Medicinal Products.

(Perjeta™, Roche/Genentech), and the antibody-drug conjugate ado-trastuzumab emtansine (Kadcyla™, Roche/Genentech) have both been approved for treatment of breast cancer. During the clinical development of these two compounds both HercepTest™ and the *HER2* FISH pharmDx™ Kit were used for patient selection, resulting in the FDA approval of updates to the intended use of these assays, so as also to include selection of patients under consideration for treatment with pertuzumab or ado-trastuzumab emtansine.

As shown in Table 11.1 only two assays have obtained FDA approval for use in relation to gastric cancer and the two new HER2 targeting compounds; pertuzumab and ado-trastuzumab emtansine.

Chapter 11.3 | Companion Diagnostics and 'Personalized Medicine'

There is lack of consensus regarding the terminology of a diagnostics assay that is developed in parallel to a targeted drug and used to guide the treatment decision. A number of different names are used in the literature and by the regulatory authorities, such as pharmacodiagnostics, theranostics, pharmacogenomic biomarkers, advanced personalized diagnostics, and companion diagnostics. Within the last few years 'companion diagnostics' has been used more and more frequently and this name has also been adapted by the FDA; however, theranostics is still used quite frequently especially in the academic medical literature (1).

For this book chapter, we will adhere to the definition of CDx that the FDA recently published in a draft guideline (12). According to this definition a CDx assay is an in vitro diagnostics device that provides information that is essential for the safe and effective use of a corresponding therapeutic product. The FDA further specified three areas where a CDx assay is essential:

- To identify patients who are most likely to benefit from a particular therapeutic product
- To identify patients likely to be at increased risk of serious adverse reactions as a result of treatment with a particular therapeutic product
- To monitor response to treatment for the purpose of adjusting treatment (e.g., schedule, dose, discontinuation) to achieve improved safety or effectiveness.

PETITIONER EXHIBIT 1012
Page 135 of 218

According to the FDA, a CDx can be used both to predict outcome (efficacy and safety) and to monitor the response.

The predictive or selective characteristics of CDxs have attracted the most attention so far. Use of a CDx assay facilitates the design of clinical trials with a smaller number of subjects, which in turn has a clear positive effect on the resources spent on clinical development (1). A definition that focuses on the predictive or selective characteristics of the CDx assay and makes a link to personalized medicine is: "A pre-treatment test performed in order to determine whether or not a patient is likely to respond to a given therapy. This type of test is classified as a predictive or selective test and is a prerequisite for implementation of personalized and stratified medicine" (13).

Table 11.1 Companion diagnostics assays approved through the premarket approval (PMA) process by the FDA. For further information see (10).

Companion Diagnostic	Type of Assay	Indication(s) mentioned in the Indications for Use	Drug(s) mentioned in the Indications for Use
PathVysion HER-2 DNA Probe Kit (Abbott Molecular)	FISH	Adjuvant treatment of breast cancer	Trastuzumab (Herceptin)
Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular)	FISH	Non-small cell lung cancer (NSCLC)	Crizotinib (Xalkori)
InSite HER-2/neu Kit (Biogenex Laboratories)	IHC	Breast cancer	Trastuzumab (Herceptin)
HercepTest™ (Dako)	IHC	Breast cancer Metastatic gastric or gastroesophageal junction adenocarcinoma	Trastuzumab (Herceptin) Pertuzumab (Perjeta)[1] Ado-trastuzumab emtansine (Kadcyla)[1, 2]
HER2 IQFISH pharmDx™ (Dako)	FISH	Breast cancer Metastatic gastric or gastroesophageal junction adenocarcinoma	Trastuzumab (Herceptin) Pertuzumab (Perjeta)[1] Ado-trastuzumab emtansine (Kadcyla)[1, 2]
HER2 CISH pharmDx™ Kit (Dako)	CISH	Breast cancer	Trastuzumab (Herceptin)
EGFR pharmDx™ Kit (Dako)	IHC	Colorectal cancer	Cetuximab (Erbitux) Panitumumab (Vectibix)
c-Kit pharmDx™ Kit (Dako)	IHC	Gastrointestinal stromal tumors (GIST)	Imagines (Gleevec/Glivec)
Bond Oracle Her2 IHC System (Leica Biosystems)	IHC	Breast cancer	Trastuzumab (Herceptin)
SPOT-Light HER2 CISH Kit (Life Technologies)	CISH	Breast cancer	Trastuzumab (Herceptin)
Therascreen KRAS RGQ PCR Kit (Qiagen)	RT-PCR	Colorectal cancer	Cetuximab (Erbitux)
COBAS 4800 BRAF V600 Mutation Test (Roche Molecular Systems)	RT-PCR	Melanoma	Vemurafenib (Zelboraf)
Inform Her-2/Neu (Ventana Medical Systems)	FISH	Breast cancer	None (Prognostic)
INFORM HER2 Dual ISH DNA Probe Cocktail (Ventana Medical Systems)	CISH	Breast cancer	Trastuzumab (Herceptin)
PATHWAY Her2 (Ventana Medical Systems)	IHC	Breast cancer	Trastuzumab (Herceptin)

[1]Breast cancer only, [2]Approved March 2013, in US only

PETITIONER EXHIBIT 1012
Page 136 of 218

Chapter 11.4 | Co-Development of Drug and Companion Diagnostics

Companion diagnostic development requires long term commitment from the parties involved. In an ideal scenario a diagnostics manufacturer and a drug developer engage in a long term partnership that if successful leads to the launch of the drug and the companion diagnostic assay. The diagnostics partner brings the assay forward in parallel to the drug development stages, ensuring that the assay has the appropriate level of evidence at each stage, in order to support required testing during each phase of the clinical trial. The combination of a relevant biomarker and the clinical outcome data into a predictive or selective assay demands close collaboration between the diagnostic industry and the pharmaceutical industry during the development of companion diagnostics (Figure 11.2). This process is also supported by the FDA's Critical Path Initiative, launched in March 2004 with the white paper Innovation-stagnation: Challenge and Opportunity on the Critical Path to New Medical Products, plus the FDA's release in April 2005 of its Drug-Diagnostic Co-Development Concept Paper and Draft Guidance paper on companion diagnostics (12, 18). The main activities involved in each stage are reviewed in the following pages. The order and timing for each of these stages depends on the biomarker, the clinical trial design and the extent to which the diagnostic/pharmaceutical companies are willing to invest, prior to having proof of concept of their predictive biomarker hypothesis in patients. In accordance with the theme of this book we will assume that the selected biomarker for companion diagnostic development is an immunohistochemistry-based assay.

Feasibility Stage

Feasibility has two parts, identifying a relevant biomarker for predicting efficacy of the drug in patients and establishing an assay for the relevant predictive biomarker. The first part involves many years of work understanding the biology of the disease and how the drug target interacts with the cancer cell,

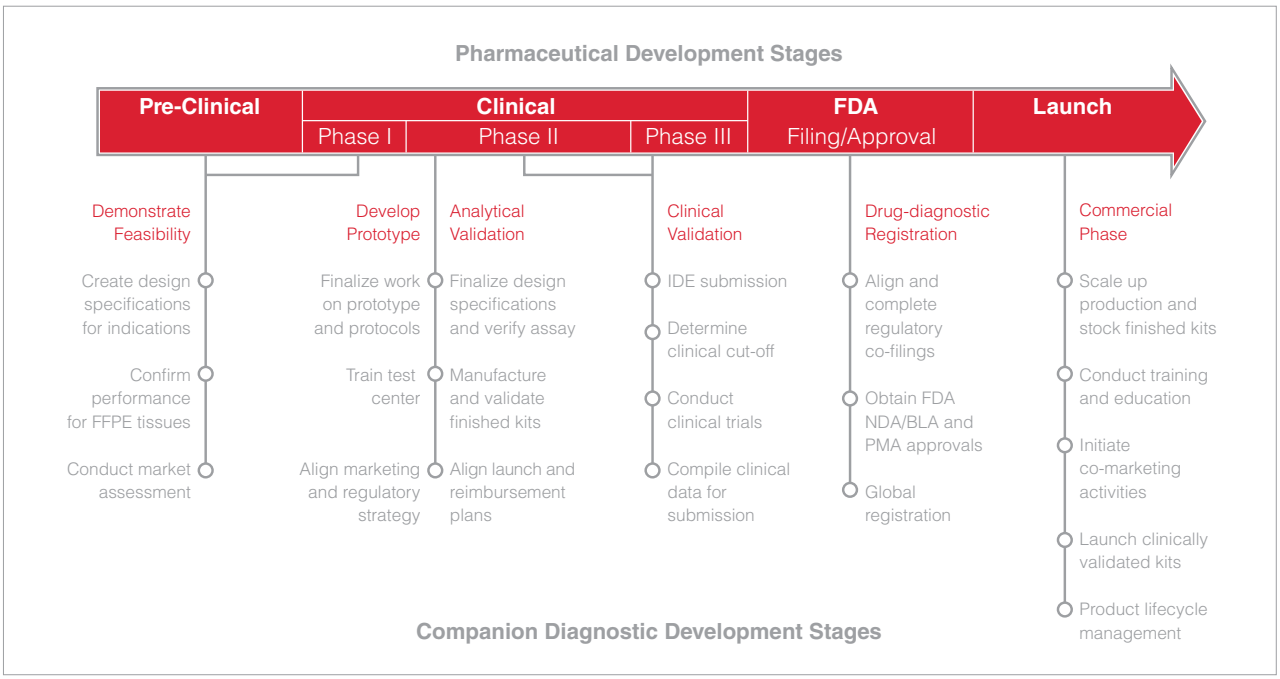


Figure 11.2 Collaboration between the diagnostic industry and the pharmaceutical industry during the development of companion diagnostics. FFPE: Formalin-Fixed, Paraffin-Embedded; FDA: Food and Drug Administration; NDA: New Drug Application; BLA: Biologic License Application; PMA: Pre Market Approval.

PETITIONER EXHIBIT 1012
Page 137 of 218

identifying the critical determinants that dictate if a cell is likely to respond to the drug. The second part aims to establish whether it will be possible to develop a companion diagnostic assay as desired. Feasibility studies cover development or identification of suitable antibodies for the target protein that work on clinically relevant tissues, e.g. if the drug targets a protein deregulated in non-small cell lung cancer (NSCLC) the antibody must be able to detect the protein of interest in clinical NSCLC samples. Important points to consider are that the assay should be functional within the relevant clinical entities, so that if the assay is used for prospective or retrospective testing in early stage clinical trials, it will work effectively in all disease entities being tested (lung, melanoma, kidney, etc), and will be compatible with the sample types and sample processing methods present in the patient population, i.e. formalin-fixed, paraffin-embedded surgical resection specimen or biopsies from the tumors. Another important aspect at this stage is to establish the specificity of the assay and sensitivity for detection of the relevant protein in the tissue. It should be noted that the terms 'specificity' and 'sensitivity' in the above context refer to technical aspects of the test and are not the same as when used to describe the ability of the validated assay to detect 'non-responders' and 'responders' in the patient population (clinical specificity and sensitivity – see below).

Prototype Stage

Once the points above have been covered in the prototype assay stage, a feasibility assay/kit can be developed. The assay description (protocol) should encompass how the entire immunohistochemical staining is performed from obtaining and preparing the patient sample, through staining, interpretation and reporting of assay results. Under design control, the assay design specifications should include definitions sensitivity and specificity, interpretation rules and criteria, and clinical indications. These specifications are typically setup and tested during this stage. In respect to the interpretation system, a data report form is established to make sure all relevant interpretation data are collected when the prototype assay is later applied to clinical samples. A limited number of relevant assay robustness studies may also be performed during this stage; however, the majority of studies establishing assay robustness and reproducibility are performed following development of the analytically validated assay.

In addition, in the prototype assay stage relevant positive and negative control material should be established, controls which can be used by laboratories running the test in order to accept or reject an assay run. This control material can be either normal histologic structures in the tissue being stained, that are positive/negative for the protein (internal control), or screened normal/cancer tissue with known protein status (external control). The development and inclusion of positive and negative control material by the laboratory is a requirement for testing in order to assure the validity of the testing procedure (36). Thus, laboratories that will deploy testing using the prototype assay have to be appropriately trained in performing the assay procedure, establishing controls and reporting results.

During the early clinical trials the prototype assay can be employed retrospectively or prospectively depending on the level of evidence for association between biomarker and response to therapy. A 'go/no go' decision is made based on the study of both the drug and the biomarker in question. Prototype assays are typically labeled RUO as the prototype assay application in early stage drug development is only used as a research tool for exploratory purposes in terms of investigating the relationship between biomarker status and response to therapy (14, 15, 18).

Analytical Validation Stage

In order to deploy an assay for prospective selection of patients in a phase II/III it is recommended that the assay must previously have been validated (18). Based on earlier stage clinical trials, a cut-off or threshold for positivity for the target protein is established and if possible linked to the efficacy of the drug. The cut-off value needs to be established before the finale clinical validation of CDx assay in phase III. This cut-off is built into final configuration of the device, and validation studies are performed using kits that have been manufactured in accordance with final production procedures in a GLP controlled setting. In addition, if control materials, such as paraffin embedded cell lines that express known levels of target protein, are included as part of the analytically validated assay, then these have to be developed at this stage. Final device design and configuration are first verified, including testing of accuracy, technical sensitivity, and specificity, robustness (tolerance), and precision (intra-assay run, inter-assay run, inter-lot variability, inter-reader variability, inter-instrumentation variability). Finally, external analytical validation studies are performed to doc-

PETITIONER EXHIBIT 1012
Page 138 of 218

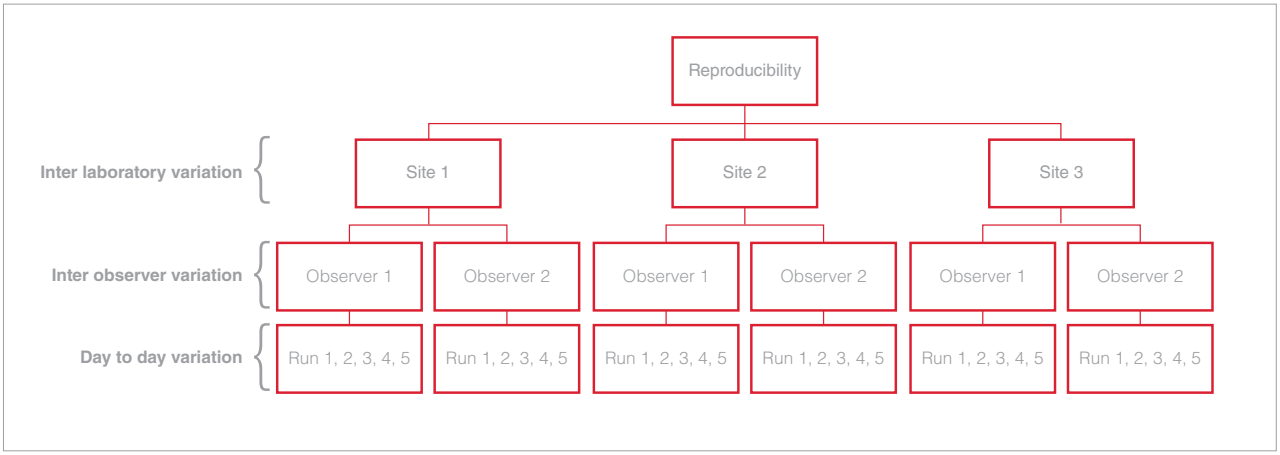


Figure 11.3 Example of reproducibility study.

ument reproducibility (day to day, inter-observer, inter-laboratory). See Figure 11.3 for example of reproducibility study.

The sensitivity of the assay to detect the target protein is established (limit of detection), together with pattern of reactivity in patient material; non-pathological as well as pathological. The specificity of the assay is demonstrated, along with showing the absence of cross-reactivity to closely related targets. Testing will define the stability of the actual reagents comprising a kit, but will also demonstrate working stability of the assay as well as onboard stability of the kit. Today, the FDA only recognizes real time stability data when approving companion diagnostic assays to be released on the IVD market (18).

The purpose of the activities performed during the analytically validated assay stage is to ensure that a robust assay with stringent requirements can be deployed in a safe and effective manner in clinical trial testing. The analytically validated assay is manufactured in compliance with the applicable FDA Quality System Regulations (QSR) and labeled IUO, supplemented by a text that clearly defines that "The performance characteristics of this product have not been established." Laboratories that will perform testing during clinical trial must be trained in deploying the assay according to the instructions for use, and pathologist(s) must be trained in performing interpretation of staining pattern and reporting of results. Training includes criteria for reporting if a patient is positive or negative for the target protein as defined by the cut-off. If testing of a 'significant risk device' is to be deployed on US patients, an Investigational Device Exemption (IDE) is required in order to ensure that the device is safe and effective for patient selection in the clinical trial setting and does not put patients participating in the clinical trial at risk. The FDA will inspect the sponsor to conduct the trial under full IDE regulations. An IDE application is submitted by the sponsor responsible for deployment of the companion diagnostic during the clinical trial and typically contains: investigational plan, report of prior investigations, device description, device performance, investigators' information, monitoring, Institutional Review Board information, and informed consent documents. An IDE may be required during the prototype phase if prospective selection of patients is based on the test result.

Upon IDE approval, the analytically validated assay will be used in conjunction with phase III/pivotal clinical trials for the medical drug, to establish clinical validation of the device, and to establish the assay as an IVD companion diagnostics.

Chapter 11.5 | Clinical Validation of the Companion Diagnostics

The traditional way to evaluate safety and efficacy of a new drug is to perform a randomized clinical trial, in which the performance of the new drug is compared to standard treatment.

PETITIONER EXHIBIT 1012
Page 139 of 218

If the result of such a comparative trial shows superiority of the new drug over standard treatment, the usual interpretation would be that the new drug is the preferred choice for patients with the disease. However, a new drug with 10-15% superiority over the current standard treatment may still be the wrong choice for many patients. Diseases are heterogeneous and the traditional randomized clinical trial only takes this variation into account to a limited extent. This type of study does not answer the question about the efficacy of the new drug in the individual patient, and extrapolation of the average study result to all patients may often be a wrong decision (16, 17). The drug-diagnostic co-development model is a way to obtain insight into disease heterogeneity, thereby making any extrapolation from clinical research to clinical practice much more accurate.

In the drug-diagnostic co-development model the clinical trials should be designed in such a way that both safety and efficacy of the drug, and the performance of the CDx assay are assessed at the same time. As described in Figure 11.2 it is desirable that the CDx development starts early during preclinical development of the drug, so an analytical validated assay can be ready before start of phase III. Further, the clinical cut-off value for the assay needs also to be established, which requires detailed access to both the diagnostic test results as well as clinical outcome data from the patients treated with the drug in question. The clinical outcome data that are collected from the phase II studies, which, for oncology studies, typically will be objective response rate, and sometimes time to progression and progression-free survival. If a relationship can be established between the assay result and the clinical outcome data, the next step will be to select the cut-off value for the assay that defines a result as test positive (CDx+) or test negative (CDx–). The ability of an assay to discriminate between CDx+ and CDx– results at a given cut-off value will depend both on the analytical performance of the assay and the strength of the clinical response to treatment with the drug under development. The clinical cut-off value for the assay should be selected prior to performing the pivotal phase III trials, which will provide the final evidence of safety and efficacy for the new drug as well as the clinical validation of the CDx assay (17).

A CDx assay will only be useful if it provides information that can discriminate between patients who are likely responders versus non-responders, and in this respect the clinical diagnostic accuracy of the assay is important. Data on clinical sensitivity, clinical specificity, positive predictive value (PPV) and negative predictive value (NPV) for the assay are important diagnostic metrics to consider in this regard. Several designs for clinical drug-diagnostic co-development have been proposed and they can be broadly categorized into three groups (17-19):

- Untargeted/all-comers
- Targeted/enrichment
- Stratification/marker-by-treatment-interaction

In the untargeted or all-comers design, all patients meeting the eligibility criteria of the study protocol will be entered into the trial. Despite the patients being tested at study entry, the treatment assignment is independent of the test result from the CDx assay and the patients are either randomized to the new treatment or to the standard treatment (Figure 11.4).

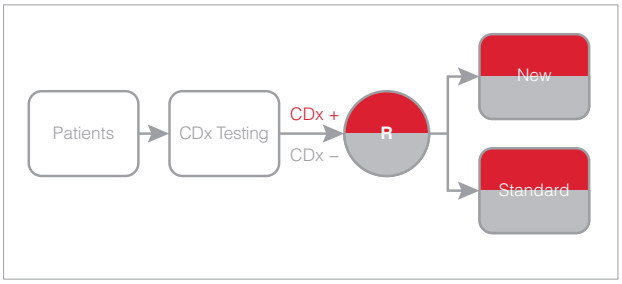


Figure 11.4 The untargeted/all-comers design. After inclusion in the study all the patients are randomized (R) to either the new treatment (New) or the current standard treatment (Standard) independent of the CDx assay result. CDx+ indicates test positive patients; CDx– indicates test negative patients. Despite the patients being tested at study entry, the treatment assignment is independent of the test result from the CDx assay.

If the relationship between known pathophysiology and the mechanism of the drug is unclear, this type of design addresses the question of whether the new treatment is beneficial for all patients, or only a subset of patients, especially the CDx+ group (17, 19, 20). However, an untargeted design will not be very useful if the prevalence of the CDx+ patients is low, because the treatment effect in the overall population will be diluted (21). If the prevalence of the CDx+ patients is sufficiently high the untargeted design has the advantage that it allows for a preliminary calculation of the clinical diagnostic sensitivity,

PETITIONER EXHIBIT 1012
Page 140 of 218

specificity, PPV and NPV for the assay. In general the untargeted design must be regarded as explorative, and is often used during early clinical development in phase I/II.

If there is clear evidence of a strong relationship between a CDx+ status and treatment-outcome with the drug (e.g. from previous phase I and/or II studies), a targeted or enrichment design can be used (12, 17). With this design, all the patients are tested by means of a CDx assay, but only the CDx+ patients are enrolled in the study and subsequently randomized to either the new treatment or to the standard treatment groups (Figure 11.5).

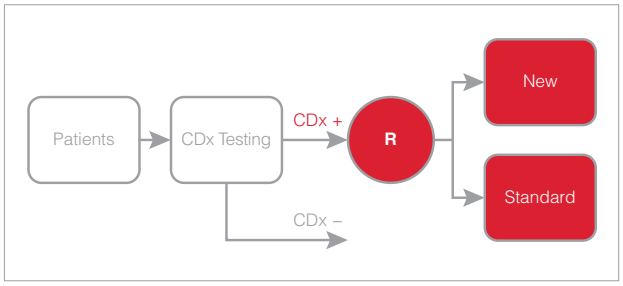


Figure 11.5 The targeted/enrichment design. With this design only patients that have a positive CDx+ result are randomized (R) to either the new treatment (New) or standard treatment (Standard). CDx+ indicates test positive patients; CDx– indicates test negative patients.

This design have been used more often recently, for example to demonstrate safety and efficacy of anti-cancer drugs, such as vemurafenib (Zelboraf™, Roche/Genentech), crizotinib (Xalkori®, Pfizer), pertuzumab and ado-trastuzumab emtansine, and to validate clinically the corresponding CDx assay (22-25). A targeted design study was also used when trastuzumab went through final phase III testing in women with advanced breast cancer in the 1990's (5). This design generally requires randomization of a smaller number of patients as compared with the untargeted designs, due to the fact that only CDx+ patients are enrolled in the trial, which makes the study population more homogeneous. However, this design allows only PPV to be calculated and not sensitivity, specificity and NPV.

The targeted design approach has one drawback as it does not provide insight into the efficacy of the new treatment in CDx– patients. However, this aspect is included in the stratification design, also called the marker-by-treatment-interaction design (12, 17, 19). With this design the patients are stratified based on the result of the CDx assay and subsequently ran-domized to either the new treatment or the standard treatment (Figure 11.6).

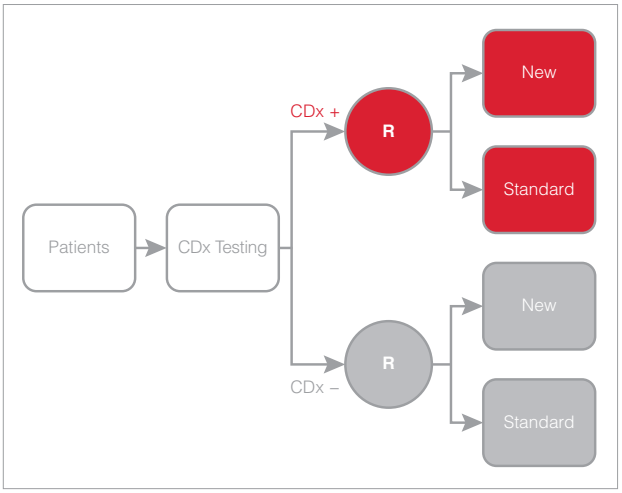


Figure 11.6 The stratification/marker-by-treatment-interaction design. With this the patients are stratified based on the result of the CDx testing and subsequently randomized (R) to either the new treatment (New) or standard treatment (Standard). CDx+ indicates test positive patients; CDx– indicates test negative patients.

In fact, this design is to some extent similar to conducting two independent randomized trials, one in CDx+ patients and one in CDx– patients. However, with the stratified design these trials are conducted under one umbrella, and differ from one large single randomized study in a couple of ways; in that both sample size calculation, and randomization are stratified based on results from the same CDx assay in the stratified design approach. The advantage of this design is that it allows the calculation of the clinical diagnostic sensitivity, specificity, PPV and NPV (17). So far very few trials have been conducted using this design, but one of these is the Marker Validation for Erlotinib in Lung Cancer (MARVEL) study. This trial was designed to evaluate whether epidermal growth factor receptor (EGFR) gene status, measured by fluorescence in situ hybridization (FISH), could be used to predict the response to treatment with erlotinib (Tarceva®, Roche/Genentech). Based on the FISH testing result patients with advanced non-small cell lung cancer (NSCLC) were divided into two groups, EGFR+ and EGFR-, and subsequently randomized to either erlotinib or to pemetrexed (Alimta®, Lilly) (26).

PETITIONER EXHIBIT 1012
Page 141 of 218

Chapter 11.6 | Companion Diagnostics and Regulatory Aspects

If a diagnostic CDx assay is developed in conjunction with a targeted cancer drug, the CDx assay may later end up determining the conditions for use of the drug after approval. The CDx assay then becomes a kind of gatekeeper in relation to which patients to treat with the drug in question. This gatekeeper role requires that the CDx assay must live up to certain standards during development, both in relation to analytical and clinical performance. This aspect has long been recognized by the FDA in the US, where most CDx assays must go through the PMA process. In the European Union (EU) these types of assays are not subject to any premarket approval process by the regulatory authorities, but only a conformity assessment and CE marking, which normally is performed by the manufacturers themselves. However, the growing number of CDx assays under development, and their increasing importance in relation to the clinical use of targeted anti-cancer drugs, has raised questions as to whether the regulatory framework for these types of assays ought to be changed in the EU (1). In fact, very recently the European Parliament has suggested changes in the regulation for medical devices. With this newly proposed regulation, which is expected gradually to come into force from 2015 and onwards, it is anticipated that a type of premarket approval process will be introduced, where an independent notified body will be involved in conformity assessment relating to in vitro diagnostic medical devices, which of course will include CDx assays (27).

From a medical and regulatory point of view CDx assays are considered high risk medical devices, because clinical decisions and actions are taken based directly on the test results. For example, an incorrect diagnostic result from a CDx assay could lead to a wrong treatment decision, which for a cancer patient could have serious consequences. In general, in US, the classification of a diagnostic device depends on the intended use of the assay and its associated risk to the patient. An intended use with a higher risk will direct the classification of the device towards a high risk class III device. Intended uses judged to carry moderate risk allow the lower risk classification, of class II, while devices of low risk are classified as class I devices (28). In the US, the FDA normally classifies CDx assays as high risk class III diagnostic devices. A similar risk-based classification system will also be part of the new in vitro diagnostic regulation to be introduced in the EU, and CDx assays will likely be classified as high risk products (27).

When it comes to the regulations for CDx assays, the FDA has been at the forefront compared to other national health agencies. Already in 2005 the FDA issued a draft concept paper on drug-diagnostic co-development, where both the analytical and the clinical requirements for in vitro diagnostics assays developed in conjunction with a specific drug were discussed (18). In this draft concept paper a model for the parallel development of a drug and a diagnostic assay was suggested. This model was very much influenced by the process through which the HER2 IHC assay and trastuzumab were developed by Genentech in the 1990's. On several occasions the FDA has indicated that a draft guideline would be issued, based on this drug-diagnostic co-development concept paper from 2005, and input from the different stakeholders, but at the time of the present writing such guidance has not yet been released.

Overall, within the last few years the regulatory framework for development of CDx assays has gradually been changed to reflect the important role that they play in relation to both drug development and care of the individual patient. For a number of targeted anti-cancer drugs the CDx assay result has now become the decisive factor in relation to the treatment decision in the clinic. This key role of the CDx assays has not only been recognized by the FDA, but also now by other regulatory health agencies worldwide.

Chapter 11.7 | Learnings from EGFR

Upon the success of trastuzumab in clinical trials it was anticipated that other targeted agents against growth factor receptors would follow and that similar correlations of overexpression of a growth factor and benefit of treatment would be found. Epidermal growth factor receptor (EGFR) was shown to be overexpressed, acting as an oncogene in several cancers, rendering it an attractive target for therapeutic intervention (32). Several clinical trials were initiated with EGFR inhibitors such as cetuximab (Erbitux®, Bristol-Myers Squibb / Eli Lilly) and panitumumab (Vectibix®, Amgen). Patients were tested with IHC-based assays for detection of EGFR expression by tumor, however, a clear cor-

PETITIONER EXHIBIT 1012
Page 142 of 218

relation between overexpression of protein and the patient's response to treatment was not found (30-33). Instead, exploratory testing for mutations in patients from the clinical trials revealed a linkage between K-RAS mutation and resistance to EGFR inhibitors, suggesting that inhibition of EGFR expression was bypassed by downstream signaling (34). Subsequently, specific mutations in the EGFR receptor have been linked to response to therapy with erlotinib and been used in clinical trials as predictive biomarkers. Consequently, the COBAS EGFR Mutation Test has recently received FDA approval as a companion diagnostic for erlotinib (35). This example illustrates the need for thorough understanding of the biology of the disease and the pathway(s) being targeted in order to devise the best strategy for a companion diagnostic, prior to engaging in clinical trials.

Chapter 11.8 | Conclusion and Future Perspectives

IHC or FISH-based assays are commonly used as companion diagnostic tests in routine pathology laboratories, making them the current methods of choice for an increasing number of oncological drug development programs. The use of a CDx assay to select patients for treatment during clinical development is clearly a way to fulfill the promise of personalized medicine, from both a patient and regulatory perspective. In this chapter we have tried to summarize the efforts necessary for development and regulatory approval of an IHC-based companion diagnostic. In order to successfully employ a companion diagnostics strategy, a concerted effort from both the pharmaceutical company and the diagnostic manufacturer is needed, which clearly requires major commitments of time and resources by both parties.

The development of trastuzumab is often held up as an example of successful development of a targeted anti-cancer drug and has served as the main inspiration for the current drug-diagnostic co-development model. It is now 15 years ago since the approval of HercepTest™. Looking at the list of CDx assays that have gone through the premarket approval process at the FDA, reveals a relatively short list, with modest diversity. Ten of the 15 assays on the list are measuring HER2, either as protein overexpression or as gene amplification (10) (see Table 11.1). However, this situation is very likely to change dramatically in the years to come, as it has been estimated that as many as 80 targeted cancer drugs could be introduced for clinical use before 2018, and that most of these will likely have a CDx linked to their use (11).

Immunohistochemistry-based companion diagnostics may be limited in their precision to measure the target protein, for reasons that include non-standard sample preparation, assay variation, or poor observer-to-observer reproducibility, all of which point towards a need to move into more standardized controlled quantitative assay methods, that also incorporate digital image analysis to measure accurately the amount of target protein in the tissue.

Technological advances in sequencing methods have also made significant progress in the past decade and comprehensive sequencing efforts using next generation sequencing methods have revealed the genomic landscapes of the a number of common human cancers (37). These findings already have had profound effects on our understanding on the genetic background of different cancers types and will inevitably lead to new therapeutic development, and thereby companion diagnostic development. It is likely that sequencing of critical genes performed in combination with measurement of the functional output of the deregulated pathways by immunohistochemistry and other protein based technologies will lead to increasingly complex companion diagnostic testing schemes that in turn will result in new and innovative trial designs and approval procedures.

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PETITIONER EXHIBIT 1012
Page 143 of 218

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PETITIONER EXHIBIT 1012
Page 144 of 218

Chapter 12

Tissue Microarray – Construction and Quality Assurance

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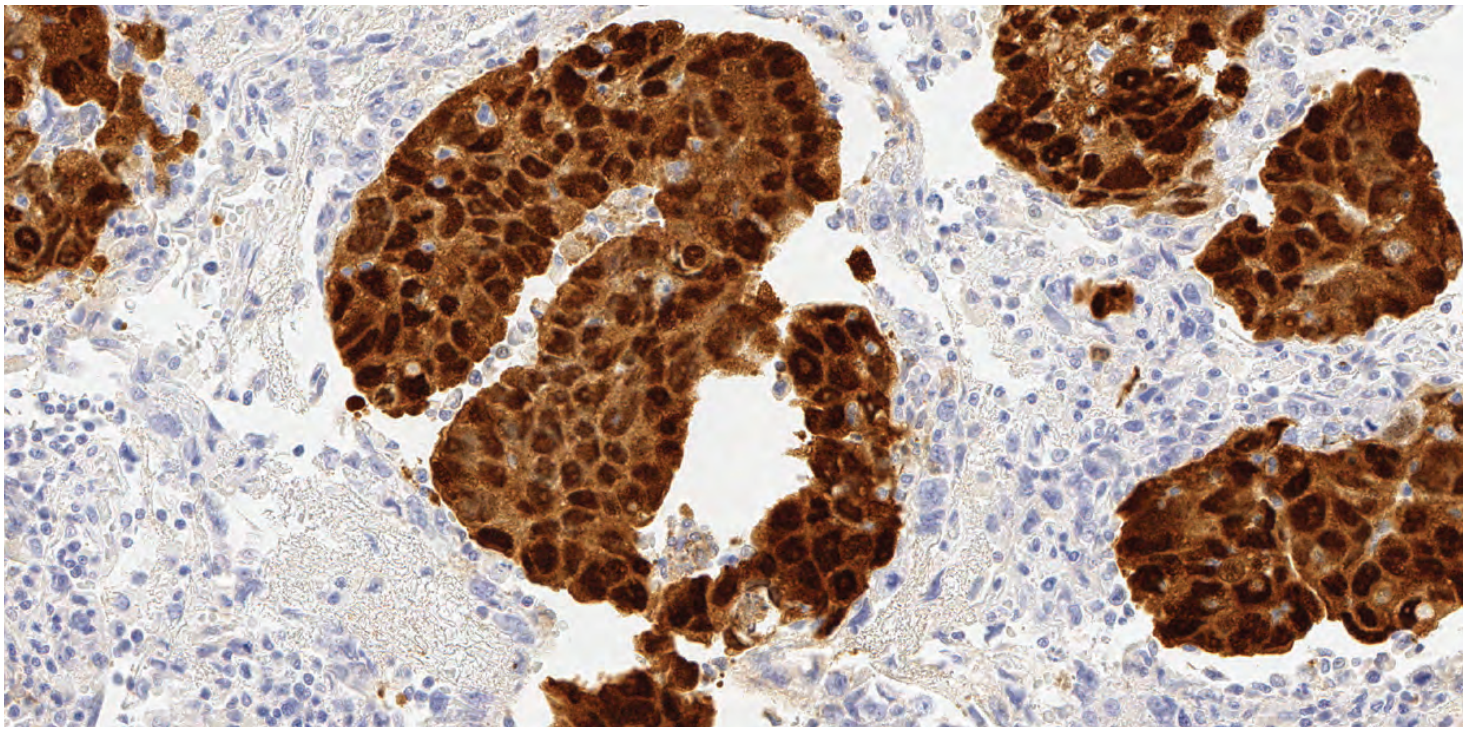


Mi•cro•ar•ray *(n.)*
A supporting material (as a glass or plastic slide) onto which numerous molecules or fragments usually of DNA or protein are attached in a regular pattern for use in biochemical or genetic analysis.

Merriam-Webster Dictionary of Medical Terms

Chapter 12.1 | Introduction

Recent times have seen the advent of high throughput assays such as array comparative genomic hybridization, cDNA microarray and Next Generation Sequencing techniques, which have led to the rapid discovery of thousands of potential biomarkers. However, these need to be validated in tissue-based studies in large datasets to prove their potential utility. As these datasets are typically present in the form of formalin-fixed, paraffin-processed tissue blocks, immunohistochemical (IHC) methods are ideal for validation. However, performing whole-section IHC on hundreds to thousands of blocks requires a lot of resources in terms of reagents and time. Analysis needs to be batched; batch to batch variability could interfere with the analyses. In addition, an average block will yield less than 300 slides of 5 µm each. The tissue microarray (TMA) technique circumvents some of these problems.

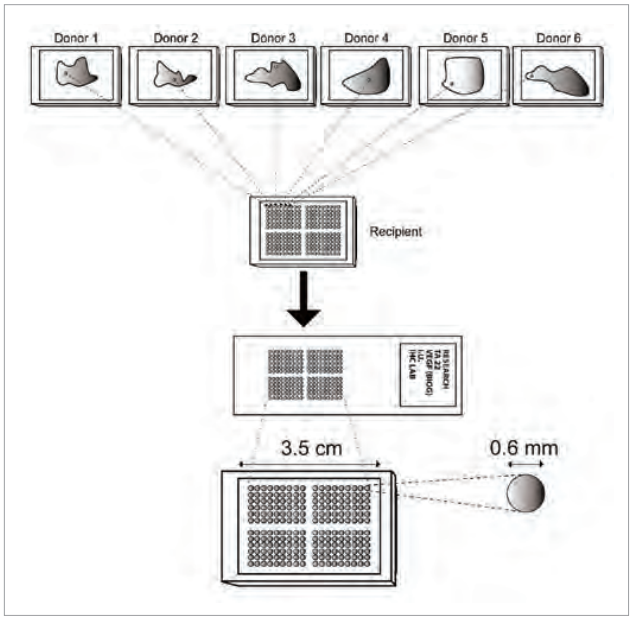


Figure 12.1 A) Principle of tissue microarray (TMA) analysis. Cylindrical cores are obtained from a number (up to 1,000) of individual formalin-fixed, paraffin-embedded tissue blocks. These are transferred to a recipient TMA block. Each TMA block can be sectioned up to 300 times. All resulting TMA slides have the same tissues in the same coordinate positions. The individual slides can be used for a variety of analyses saving labor and reagent costs while maintaining uniformity of assay. Typically a minimum of three cores for each case are used for 0.6 mm cores.

The origin of TMAs can be attributed to Dr Hector Battifora's humble 'sausage' blocks (1), in which a number of tissues, typically from different organs, were thrown together in the same block and the tissue distribution of a particular antigen/protein was assessed. A significant disadvantage of this technique was that when tumors or tissues from the same site were put together it was difficult, if not impossible, to trace them back to the patient. This prevented meaningful analysis of prognostic markers. However, many laboratories, small and large (including our own), adopted this technology to generate multi-tissue or multi-organ tissue blocks. The next step in the development of TMA was described by Wan et al. (2) who used a 16-gauge needle to manually bore cores from tissue blocks and array them in a multi-tissue straw in a recognizable pattern. This method was further modified by Kononen et al. (3) using a 4 mm skin biopsy punch. They used a cast of a small amount of melted paraffin to record the position of each punch specimen. This landmark study lead to the development of a TMA precision microarray instrument with an x-y guide by Beecher Instruments (Sun Prairie, WI). This device enabled real high-throughput analysis, with arraying of up to 1,000 cores in the same block.

Chapter 12.2 | Advantages and Disadvantages of TMAs

Advantages

The major advantage of TMAs is that they allow the performance of tissue-based assays (immunohistochemistry, histochemistry, in situ hybridization, etc.) on a large number of patient samples in an efficient and cost-effective manner. With TMA technology, several hundred representative cores from several hundred patients may be included on a single glass slide for assay. Thus, significantly more tissue can be conserved than if the blocks were to be sectioned serially. TMAs have been generated from all tissue types, including decalcified bone and core biopsies. The latter are usually rotated 90 degrees and embedded vertically to ensure presence of tissue of interest in multiple cuts. In addition, methods for generating TMAs from fresh frozen tissue using blocks made from either optimal cutting temperature compound, or from a mix of gelatin-sucrose, have been described (4). More recently, a technique for developing 'patch' TMAs from unstained slides has been described (5).

PETITIONER EXHIBIT 1012
Page 146 of 218

Disadvantages

The major disadvantage of TMAs is that each core (or set of cores) represents a fraction of the lesion. This was considered a major weakness, particularly in the early days of the TMA. However, multiple studies in different organ systems have now demonstrated that consistent and comparable results can be obtained using TMA cores as with whole sections. In order to obtain comparable results two main strategies have been used. The first is increasing the number of cores from each case. It is typical in breast cancer to use at least two cores from each case when using a 1 mm core; a minimum of three cores from each case for 0.6 mm cores. The number of cores may vary according to the disease site; for example, it is typical to use five cores in colon cancer. Although a 2 mm core might be theoretically considered better than multiple smaller cores, in practice this is usually not true. Smaller cores permit sampling of different tumor areas and are, therefore, more likely to be representative of the entire tumor. In addition, smaller cores tend to inflict a lesser degree of damage on the original tissue blocks. The second strategy consists of increasing the number of tumors included in the study. This method averages out the errors that might result from tumor heterogeneity associated with the use of tissue cores.

Figure 12.2 Photograph of low density TMA block.

When Not to Use TMAs

TMAs are not recommended for certain types of studies. In certain tumors such as glioblastoma, there is such marked heterogeneity within tumors that this feature may not be adequately captured in TMA studies. In addition, TMAs are also not very useful to study rare or focal events, such as number of immune cells in tumors. It is also difficult to study certain facets of tumor biology, such as interactions between the tumor and it's stroma, as these stromal components may not be adequately represented in the cores. The use of large cores (2 mm) has been advocated for these types of studies.

Chapter 12.3 | Types of TMAs

The type of TMA to be generated depends on the question being asked within the study. The following are the commonly used types:

Cell Line Arrays

These arrays consist of normal or cancer cell lines that are grown in culture. The major function of these arrays is to survey the presence of proteins that are known to be present in one or more of the cell lines. In addition, cell line arrays can be used to analyze the utility (plus sensitivity and specificity) of an antibody in detecting proteins. The most common example of this type of array is the 3-cell line control that is used with HER2 testing in breast cancer.

Random Tissue/Tumor Arrays

These arrays contain tissues from multiple sites and contain tumor and/or non-tumor tissues. Small arrays of this kind can be used for quality control measures, such as monitoring of existing reagents/antibodies, as well as work-up of novel re-agents. In addition, they can also be used as discovery tools. For example a survey of CD10 in tumors arising in multiple tissue sites lead to the discovery of its utility in diagnosing uterine stromal tumors (6).

Consecutive Case Array

This type of array is constructed using consecutive cases belonging to a single tissue site. These types of arrays are extremely useful for quality control purposes, including identifying shifts and drifts in reagent quality. They are also useful in studying the prevalence of a protein/antigen in a given tumor type, and analyzing the relationships between different biomarkers.

Tumor Characteristic-based Array

This is a special type constructed solely on the basis of a given characteristic, such as patient age or tumor grade. The latter is useful for evaluating the frequency of a marker throughout the spectrum of tumor differentiation. Similarly TMAs can be gen-

PETITIONER EXHIBIT 1012
Page 147 of 218

erated based on the expression of a biomarker, such as estrogen receptor or HER2/neu positive or triple negative breast cancers. These types of biomarkers are useful in analyzing interrelationships between different cellular pathways.

Progression Arrays

These types of arrays are used to analyze the role of protein(s) in cancer progression and consist of normal tissues from patients without cancer, normal tissue from patients with cancer, pre-invasive lesions and tumor (from local and metastatic sites). The addition of normal tissue from close to the tumor and those much further away from the tumor site might enable study of 'field effect'.

Outcome-based Arrays

These special arrays are the most valuable and most difficult to generate, as they involve collation of tissues from patients that have the same disease and have been more or less similarly treated and followed up for a significant period of time. The period of follow-up depends on the type of disease or tumor being studied. These types of arrays are mostly used to evaluate prognostic or predictive biomarkers. The presence of biomarkers in tumor subtypes might then be used to design novel therapeutic strategies.

Other Special Types

TMAs can be generated based on specific question being asked, whether it be race (Caucasians versus African Americans), sex (male versus female) or more tissue-oriented questions such as center of the tumor versus invasive edge of the tumor.

Chapter 12.4 | Team Required for TMA Construction

Construction of TMA is a team effort (7). The first and foremost question that needs to be answered is: why is the TMA being constructed? This will decide the composition of the team. For the generation of the simplest of TMA, a technologist might be all that is required. However, most TMA synthesis will require close collaboration with the pathologist, who will identify the areas of interest and mark them for the technologists to obtain cores. It is a good idea to involve biostatisticians from the onset rather than just asking them to do the data analysis. They can help with deciding the number of cores from each case, total number of cases needed, how they should be distributed across the array to avoid bias. In general the number of cases required in a TMA is dependent on the size of the difference expected in the outcome and the degree of variance in the samples; this sample size calculation is best done with the assistance of a statistician. Outcome-based TMAs may need input from, or participation of, treating physicians/oncologists. As an example, it took two years for the design and construction of TMAs in a study that involved 200 cases at six sites in the United States and Canada. The study was conducted to validate definitively tissue biomarkers of prostate cancer recurrence after radical prostatectomy. The scientific team involved pathologists, clinicians, statisticians and cancer researchers (7).

The following steps are recommended for breast TMA construction:

Step 1: Define the question

As described above TMAs are created to answer specific questions. It is important to define clearly this question at the outset. The question will help define the number of cases and cores that need to be used in the generation of the TMA. For example, a TMA containing 20 cases might be sufficient for routine quality control/ assessment, but is not enough for biomarker assessment.

Step 2: Review the cases to be included in the TMA

Pull all the cases to be included in the TMA together. If the blocks have been previously cut into for other clinical or research purposes, it is prudent to review a fresh H&E slide to ensure that the slide is representative of the block. Review all the slides and mark areas of interest. It is useful to mark multiple areas from more than one block, as blocks may be depleted or misplaced. Areas to be sampled (tumor, normal, and pre-malignant tissues) should be identified.

Step 3: TMA core size and number of cores

Size of the cores: The typical core sizes used for TMA constructs are 0.6 mm, 1.0 mm, 1.5 mm and 2.0 mm. Many workers consider the small 0.6 mm cores as the standard of practice. Use of smaller core diameters, however, allows for a greater number of cores to be extracted from the lesion and a greater number of cores that can fit into the TMA block. In addition, they tend to inflict little damage on the donor and recipient blocks and the cores are easier to remove and replace from these blocks. The larger core sizes have the advantages of being more robust and the cores are more resistant to damage during handling. However, these larger sizes can lead to increased likelihood of difficulty in extracting the

PETITIONER EXHIBIT 1012
Page 148 of 218

cores from the blocks, as well as greater chance of the blocks being broken or cracked during the TMA generation process.

Number of Cores

The optimal number of cores, to be included in the TMA, is marker dependent and can vary depending on the degree of tumor size and heterogeneity. In general, the greater the degree of intratumoral heterogeneity for any given marker, the greater is the number of cores that will be required. When using 0.6 mm sized cores, it is typical to use a minimum of three cores per case. Three 0.6 mm cores are still better than one 1.0 mm core, even though the tissue surface area is essentially identical. For small tumors, three 1.0 mm cores could result in destruction of the donor block; so tumor size would also drive number/size of cores to be taken. Studies that have used 1 mm core punches have tended to use two cores (8).

Density

The maximum number of cores that should be placed on a single block will vary depending on core size, block size, and IHC methodology, among other factors. It is best to avoid placing so many cores on a TMA that the surface section of cores becomes larger than the antibody coverage area on the slide programmed by the autostainer (e.g. Dako Autostainer). Similarly, too many cores diminish the amount of paraffin at the edge of the block creating difficulties in sectioning. Cores should start at least 3 mm away from the block edges, to prevent the paraffin from cracking. Maximum number of cores per block should therefore depend on the comfort level of the technician, as well as the pathologist, who is ultimately going to read the slides. For these reason, it is typical for most workers to put somewhere between 100 and 300 of 0.6 mm cores in a TMA block.

Distance

The distance between cores should NOT exceed the core diameter. It is easier for the microscopist to follow the rows and columns if he/she can "lead" from one core to another. If the distance between cores is large, it difficult to follow the chain of cores and may result in skipping of lanes and false recording of data when performing manual interpretation.

Step 4: Identify control tissues to be included in the block

Controls should be placed on each TMA block, for quality control and to address tumor heterogeneity. Three types of control tissues may be used:

- Tissue-specific controls: Normal tissues and cell lines from the organ site can help in comparative analysis of the marker expression status, in addition to helping ensure standardization.
- Biology-associated controls: It is useful to insert 'pathway associated' controls to ensure that the reagents are working well; thus functioning as good internal controls within the TMA block. Common examples include endometrium for hormone receptor, testes or lymph node or tonsils, for proliferation.
- Organ system controls: examples include adrenal gland, brain, breast, colon, kidney, liver, lung, pancreas, placenta, prostate, testes, salivary gland, uterine myometrium (smooth muscle). These controls are particularly useful when the TMA is being used to analyze novel markers, as one or more of these tissues can serve as internal controls. Normal tissue TMAs, at a minimum, should contain: liver, kidney, endometrium, lymph node, colon, and testis.

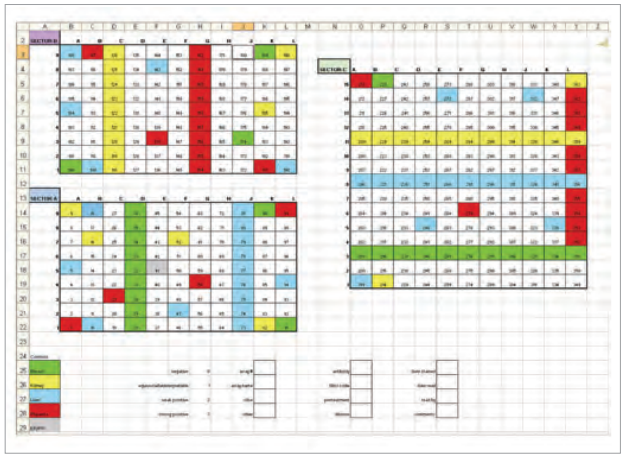


Figure 12.3 TMA map and block design: TMA layout should be asymmetric and irregular so that it is relatively easy to orient the TMA block. This irregularity should be obvious to the histotechnician who is cutting the block, so that all the cuts from the block are taken on the slides in an identical manner. In addition, locating the controls in an asymmetric manner is also helpful when reading the slides. For example, the following features may be included: 1) Blank rows and columns that do not run down the center lines of the TMA blocks, but to one side, so that the block is cut into two-third and one-third grids. 2) A blank corner for orientation or a tail coming out from close to one of the corners. 3) Asymmetric distribution of control cell lines and tissue controls. Placing stained cores of control tissues at the edge of the grid can be useful to mark orientation.

PETITIONER EXHIBIT 1012
Page 149 of 218

Step 5: Make a TMA map depicting the layout

The TMA map may consist of a simple Excel sheet, or may be a more sophisticated datasheet made using one of the TMA generation programs. This map also serves as a guide in order to arrange blocks in the sequence in which they need to be arrayed. Thus, the TMA map will contain the exact location of each case, including the duplicate samples, and where controls are located. Mini-arrays ("City Blocks") of the cores (3x5, 4x5, 5x5, 6x5) can be spaced for easy orientation, with control tissue in the rows between the mini-arrays.

Issues Related to Layout

- TMA layout should be asymmetric and irregular to assist orientation (see Figure 12.3)
- If multiple TMA blocks are being made for the same project, one consideration is to carry a small proportion of cases onto other blocks (e.g., 10%)
- Cores from the same case: Ideally, if same-patient cores are to be placed on the same block, they should be dispersed on the block. This will decrease the risk of interpretation bias. However, some researchers prefer this arrangement since it permits immediate 'normalization' or 'confirmation' of the results of the different cores from the same patient. If same patient cores are to be dispersed across multiple blocks, it is better to place them in different regions of the array (outer and inner); (outer in one block and inner in the other), with random placement, rather than placing them in the same location in each block. This is done to prevent similar artifacts affecting all cores.

Step 6: Creating the TMA itself

Instrumentation

The need for specialized instrumentation for creating TMAs is entirely based on the number of cores and value of the tissue being inserted in these TMAs. For TMAs being constructed for quality control/quality assessment or work-up of new reagents, the number of cores inserted is relatively low. This enables use of larger cores and diminishes the need for specialized instrumentation. However, for TMAs to be made from valuable cases with scant materials, it is necessary to use these instruments. The simplest of these devices consists of hand-held punches and is generally not very useful for a serious TMA project, where it is necessary to use at least an intermediate grade device. These intermediate grade devices consist of a stand, in addition to a positioning apparatus, and ensure vertical punching of the blocks and proper placement within the grid. Fully automated devices additionally have integrated computers that can be programmed to select the donor sites from different blocks and transfer them in the recipient block.

Donor Block

The block from which a core will be taken is referred to as the donor block. The area of the donor block to be cored for TMA should be selected by a pathologist. Although it is intuitive, it must be stated that the donor blocks should be optimally processed and should not contain any poorly processed areas. Similarly, cores should be obtained from the block before the block gets depleted. The thicker the donor blocks the greater the number of useful sections that can obtained from the TMA. Core punches should be pushed gently into the TMA block, and not too deeply as this can damage the needle as well as the block. When using semi-automated devices it is easier to mark the depth of the punch to the level of the plastic of the cassette. It has been suggested that heating the tissue core for 10 minutes, before inserting it into the recipient block, allows better fusion of the paraffin within the core and that surrounding the core. Fusion avoids loss and folding of tissue cores during sectioning of the TMA (9).

Recipient Block

The block into which the cores are placed is referred to as the recipient block. It is best to place the cores towards the center of this block in order to prevent cracking of the block. After the cores are inserted, place the TMA at 37 °C degree overnight, and then on the cold plate of the tissue embedding station, with subsequent two to three 1-hour cycles of hot/cold to temper the array. Multiple sections from the block should be cut at the same time to prevent wastage of tissue. Incomplete sections should not be discarded; these can be used for standardization of staining technique (see below).

Staining TMAs

When performing staining of the TMA, the step is to ensure that the staining procedure actually works in the laboratory and the procedure has been standardized. If the TMA has been obtained from an outside institution, it is important, if possible, to get other tissue processed in that laboratory, or alternatively

PETITIONER EXHIBIT 1012
Page 150 of 218

poor-quality sections (incomplete or discards) from the TMA, for practice and standardization; following standardization good quality TMA sections should be used for analysis. As TMA sections are usually larger, they require special care to ensure that the entire section (the whole array) is covered with reagents, otherwise uneven staining will be observed.

One of the limitations of the TMA is that the tissues in the tumor cores have been processed at different times and often with different protocols. This will lead to optimal staining of some tumors, but also sub-optimal staining (over or under-staining) of quite a few tumor cores. However, the large number of cases included in the TMA can to some extent compensate for this limitation.

Step 7: Validation and quality assurance

Measures for the TMA should include the following:

Validation

The use of TMAs enables analysis of large datasets, however this ability does not by any means suggest that the dataset is not skewed (10). This skewing may be the result of the institution's location (population distributions with regards to race, ethnicity, and access to health care), or type of practice (community hospital versus referral center). These biases collectively might influence the tumor size, grade and subtype composition of the cases in the dataset. Such abnormalities of the dataset need to be recognized and allowed for in interpretation of findings; the involvement of a biostatistician from the start (i.e. at case selection) helps to prevent the creation of biased TMAs. It is useful to perform common biomarker analysis on sections from the created TMA to confirm the "normal" distribution of known parameters. Comparison of this data with prior clinical data (e.g. ER analysis) obtained from whole section analysis is particularly useful to validate utility of the TMA. Alternatively the incidence of expression of a number of biomarkers in the TMA should be compared to that in published literature (using whole sections).

Quality Assurance Measures

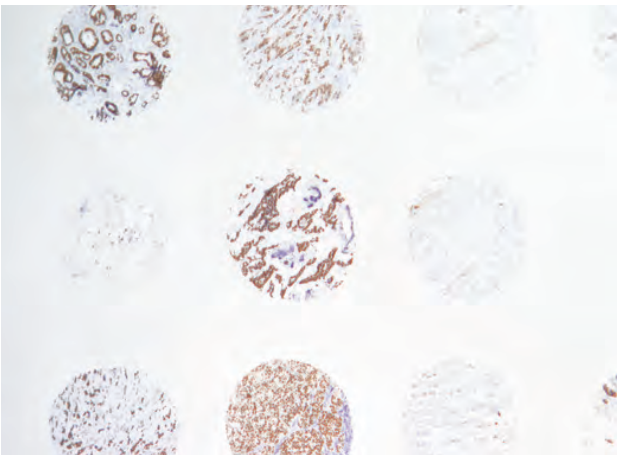
It is critical to perform and analyze H&E sections from the TMA to confirm the presence of tissue of interest (usually tumor) in the TMA sections. In addition, H&E should be performed at regular interval (e.g. on every 25th slide) from the TMA blocks. The above tests should be reviewed by a pathologist familiar with the study.

Chapter 12.5 | TMA Analysis

The analysis of the TMA has two components. The first involves analysis of the slides and recording of the data. The second involves data analysis.

Slide Analysis

The TMA slides can be analyzed manually; alternatively automated image analysis programs that can assist with the analysis are also available. The need for these programs is based



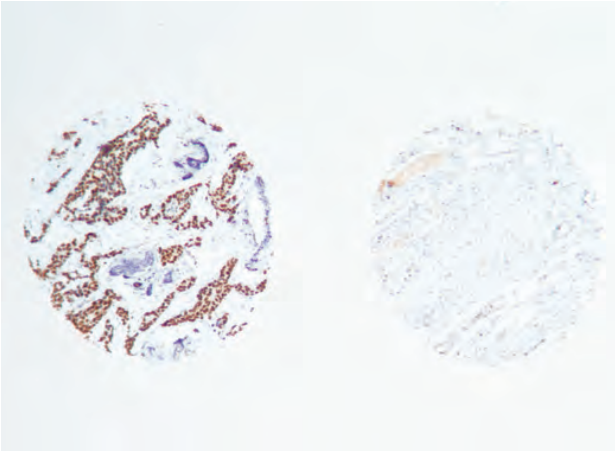


Figure 12.4 A) Low power photograph of TMA stain. **B)** High power photograph of TMA stain.

PETITIONER EXHIBIT 1012
Page 151 of 218

on the work volume as well as density of the TMAs. Some programs generate a virtual slide (Whole Slide Image – WS) of the TMA and further analysis can be done using a computer screen. Use of a WSI has the advantage of avoiding burn to the slides and all the cores can be analyzed at the same "optical and illumination" conditions. It additionally permits electronic storage of the fresh images and later re-analysis if required. This capability is particularly beneficial for FISH sections, which fade with viewing and time.

Data Analysis

Step 1: Data cleaning

Given the large number of samples in a typical TMA study, analysis of the data can become quite a challenge. One needs to exclude the cases that are not informative; it is not unusual to lose up to 10% of cases due to insufficient representation of tissue of interest. On the informative cases, strategies for conversion of multiple values (one per core) for each case into a single data point have to be devised. The commonly used strategies include using the highest value or a numerical mean of the values obtained (for review see (11). Each method used for normalization has its own advantages, as well as limitations.

Step 2: Statistical analysis

The tests used to determine the p value will be dependent on the type of data (i.e. nominal or categorical), as well as the degree of variance within the data. For simple analyses of relationships, contingency tables, and chi-squared tests are used. For demonstration of survival distributions, most researchers use the Kaplan-Meir plot and then apply Log-rank analysis to test survival differences between groups. The most frequently used analytical strategy is to subdivide patient material into high- and low-risk groups, based on the expression of novel biomarkers. Some commercially available computer programs, such as X-tile program (11), may assist the selection of the best cut-off point. This cut-off point needs to be confirmed in a separate series of cases to validate its utility. The NCI – EORTC group has developed the REMARK (Reporting recommendations for tumor marker prognostic studies) (12) guidelines which should be followed whenever possible.

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PETITIONER EXHIBIT 1012
Page 152 of 218

Chapter 13

Immunohistochemical Visualization of Molecular Tests

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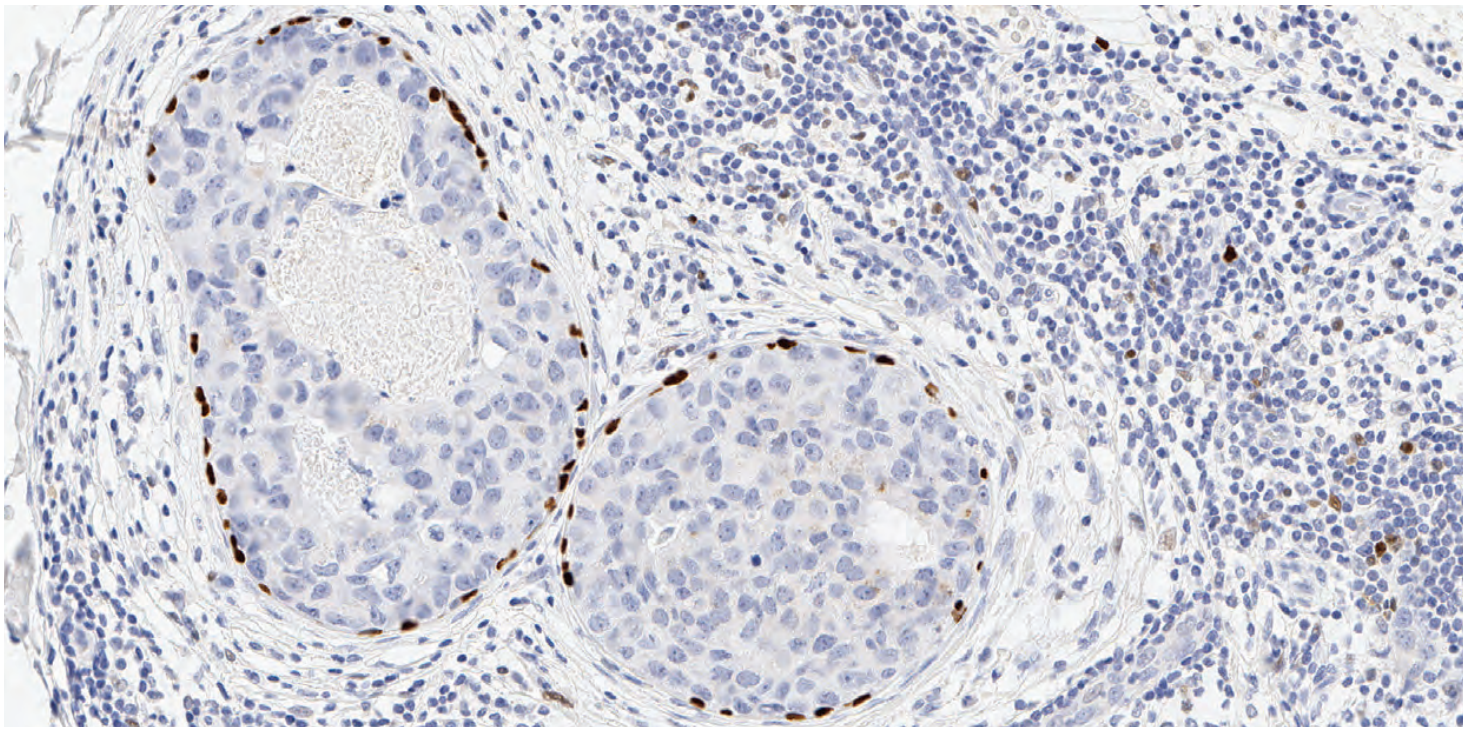
Jan Trøst Jørgensen, PhD, MSc



Mo•le•cu•lar test *(n.)*
The analysis of human DNA, RNA, and chromosomes to detect heritable or acquired disease-related genotypes, mutations, phenotypes, or karyotypes for clinical purposes, which include predicting risk of disease, identifying carriers, and establishing prenatal or clinical diagnoses or prognoses.

McGraw-Hill Concise Dictionary of Modern Medicine

Chapter 13.1 | Introduction

Molecular tests are increasingly used in the diagnosis of cancers. The information gained from looking at the genetic characteristics of the cancer can be of importance for the treatment decision. Many of the molecular tests, involving PCR (polymerase chain reaction) or sequencing techniques, are performed by specialized genetic laboratories or cytology laboratories. However, with the introduction of the chromogenic technique that transforms the fluorescent signals to signals that are visible in a standard bright field microscope, some of these tests have moved back to the pathology laboratory. This chapter will focus on the immunohistochemical (IHC) aspects of molecular testing for genetic aberrations in cancer.

ISH (in situ hybridization) is the common name for a sensitive and robust technique that is mostly used to evaluate gene amplifications, deletions, and translocations, as well as chromosomal copy number changes, in tissue or cell material. The ability to visualize genetic rearrangements and quantify copy numbers of specific genes in the morphological context of the tissue has widespread importance. ISH has been used as a research tool for more than 40 years (see 1) and the method has also gained popularity as a diagnostic tool in clinical oncology, exemplified by determination of epidermal growth factor receptor 2 (ERBB2 /HER2) status in breast and gastric cancer (2, 3). ISH methods allow the specific recognition of DNA target sequences in the nuclei of target cells using fluorescence- or hapten-labeled sequence pairing probes. The techniques are referred to as FISH, CISH or SISH depending on whether fluorochromes, chromogens or silver is used to visualize the probe.

In FISH, fluorescent probes are directly visible using fluorescence microscopy. In CISH, fluorochromes or other labels attached to the probes are used as haptens for antibody-directed enzymatic deposition of chromogen that can be viewed using bright field microscopy. The principle is illustrated in Figure 13.1. In practice, the FISH procedure should be followed until just prior to the final dehydration step. Instead of proceeding with dehydration, the slides are immersed in CISH wash buffer and are ready for CISH staining. CISH staining is a standard IHC staining with initial blocking of endogenous peroxidase activity, addition of a primary antibody mix containing antibodies for the fluorescent labels and visualization of the signals by deposition of chromogen in the tissue. Finally, counterstaining with hematoxylin is performed to enhance and visualize the nuclear borders.

Haptens can be fluorochromes such as FITC, Texas Red or Cy-3 or they can be non-fluorescent such as dinitrophenyl, biotin or digoxygenin. The enzymes most often used to generate the deposition of chromogens are horseradish peroxidase (HRP) or alkaline phosphatase (AP). In silver-enhanced in situ hybridization (SISH) the probe location is visualized as a black coloration due to silver precipitation (4).

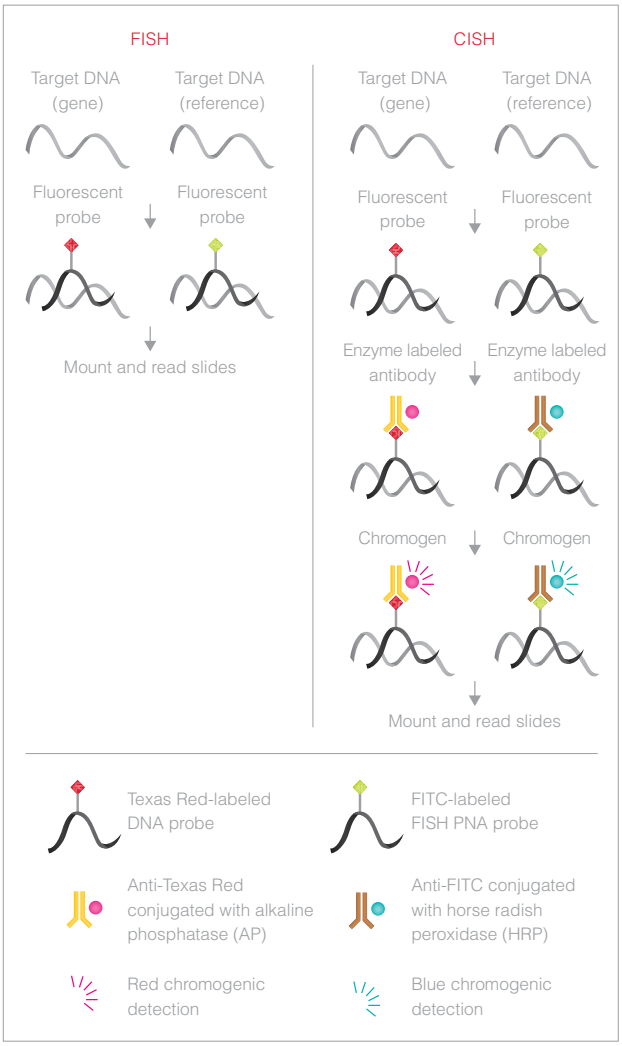


Figure 13.1 A schematic illustration of the procedures for FISH and CISH.

PETITIONER EXHIBIT 1012
Page 154 of 218

Chapter 13.2 | FISH versus CISH

FISH has been used as a sensitive and reliable technique for evaluating gene status since 1986 (5). The publication of a chromogenic *HER2* ISH procedure in 2000 was the first to show that quantification of HER2 copy numbers was possible in formalin-fixed, paraffin-embedded breast cancer tissue using CISH (6). To obtain information regarding tumor aneuploidy at that time, a reference marker was visualized on a serial slide from the same specimen. Today, it is possible to get information regarding both HER2 and a reference marker (which typically is the centromere of chromosome 17) on the same slide using dual color CISH. This simplifies interpretation of cases with borderline gene copy number by offering simultaneous visualization of the two probes, e.g. the ability to distinguish between true gene amplification/deletion and chromosomal aneuploidy on the same slide. The combination of a dual color procedure and bright field evaluation is attractive, because it allows for easy interpretation of staining without the use of fluorescence microscopes. Studies have shown that inter-laboratory and observer-to-observer variability is reduced for interpretation of HER2 status using CISH, when compared to FISH; this advantage also applies when it comes to inexperienced observers (7-9). Compared to FISH, CISH has several other advantages:

- Bright field microscopes used for CISH are generally available in diagnostic laboratories, whereas fluorescence microscopes are more complex, more expensive and require the use of dark rooms/light-restricted rooms
- Bright field CISH allows for good visualization of tissue structures with good ability to distinguish appropriate tumor areas
- CISH signals do not fade over time and stained slides may be archived and re-evaluated, for retro-spective studies or educational purposes at a later point in time
- Documentation by image acquisition of bright field images is simpler compared to fluorescence images

Furthermore, the evaluation time for CISH specimens have been shown to be considerably shorter than for identical specimens evaluated by FISH (10, 11). This observation is likely to be due to the good visualization of tissue morphology in the bright field microscope. Normal cells within the tissue may serve as an internal control for success of the staining procedure, as properly processed nuclei should expose the red to blue signal ratio expected of normal diploid cells. The results of a CISH test is an almost complete 1:1 conversion ratio of FISH signals to CISH signals (Figure 13.2), resulting in a high level of concordance between the two analyses.

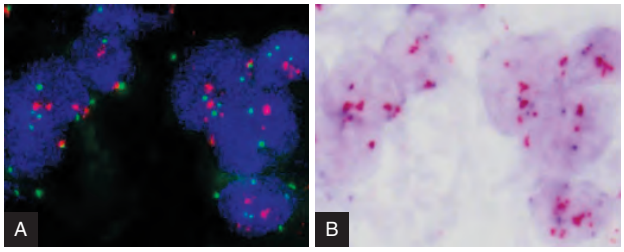


Figure 13.2 The images illustrate a "dot-to-dot" conversion of FISH signals **(A)** to CISH signals **(B)**. The red florescent signals are converted to red chromogenic signals and the green fluorescent signals to blue chromogenic signals (Dako DuoCISH™). In practice the sample was stained in the FISH procedure, evaluated by fluorescence microscopy and subsequently converted to CISH. Due to the nature of the image capture, i.e. by using an already mounted FISH slide to create the CISH stain, the CISH staining quality is compromised (12).

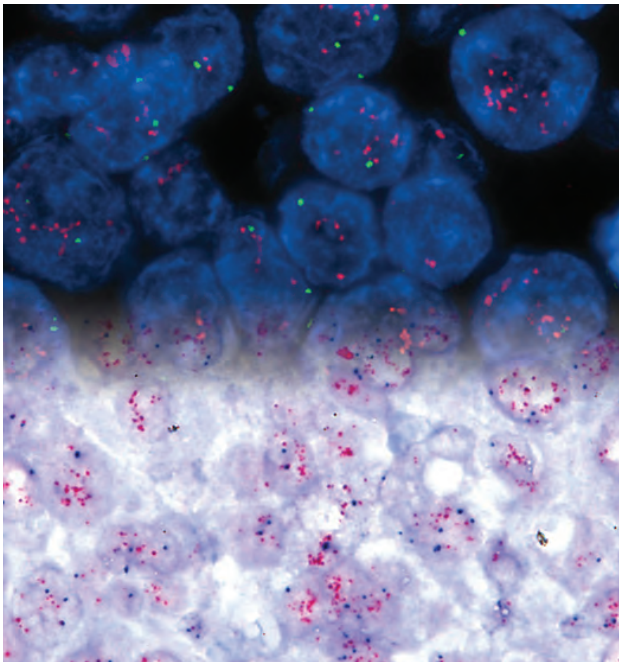


Figure 13.3 A graphical morphing of two sections stained with *HER2* FISH pharmDx™ and *HER2* CISH pharmDx™ kits, respectively.

PETITIONER EXHIBIT 1012
Page 155 of 218

Chapter 13.3 | Principle of the CISH Procedure

CISH is considered an easy way to do molecular testing, but it should be kept in mind that the staining procedure has several reagent and protocol steps that are critically important for robust and correct staining results. The procedure will vary depending on the vendor and the specific marker kit. The main steps in the procedure for Dako's *HER2* CISH pharmDx™ Kit are briefly explained below, serving as an example of the general principles of converting FISH signals into CISH signals.

After deparaffinization and rehydration, the sections are heated in pre-treatment solution. The next step involves proteolytic digestion using pepsin either at room temperature or at 37 °C. The optimal pepsin incubation time depends on the fixation history of the tissue and should be determined by the user. Following the proteolytic step, sections are dehydrated and the probes applied. Following co-denaturation of section and probes, the hybridization is carried out. The specific hybridization to the two target regions result in formation of a distinct fluorescent signals at each *HER2* gene locus and distinct fluorescent signals at each chromosome 17 centromere, for example red for *HER2* when using Texas Red fluorochrome and green for CEN-17 when using FITC fluorochrome. After a stringency wash and rinses, the fluorescent probe signals are converted to chromogenic signals by an immunohistochemical staining procedure. First, endogenous peroxidase is inactivated and then slides are incubated with an antibody mix, comprising HRP-labeled anti-FITC and AP-labeled anti-Texas Red. Following antibody incubation, red and blue signals are generated by incubation with the red and blue chromogen solutions. The enzymatic activity and the presence of the reactive chromogens result in formation of visible red and blue end-products at the target sites of the Texas Red- and FITC-labeled probes. Thus, red fluorescent signals are converted to red chromogenic signals and green fluorescent signals are converted to blue chromogenic signals (13). The chromogenic signals are clear and distinct and are supported by a hematoxylin counterstain to enhance morphological features. The red and the blue signals are insoluble in water and are mounted in a water-based permanent mounting media. In Figure 13.4, representative images of two breast cancer specimens are shown. The signals emanating from a CISH staining are readily apparent using low power objectives (10x and 20x) or can be clearly visualized and counted using high power objectives (40x, 60x or 100x).

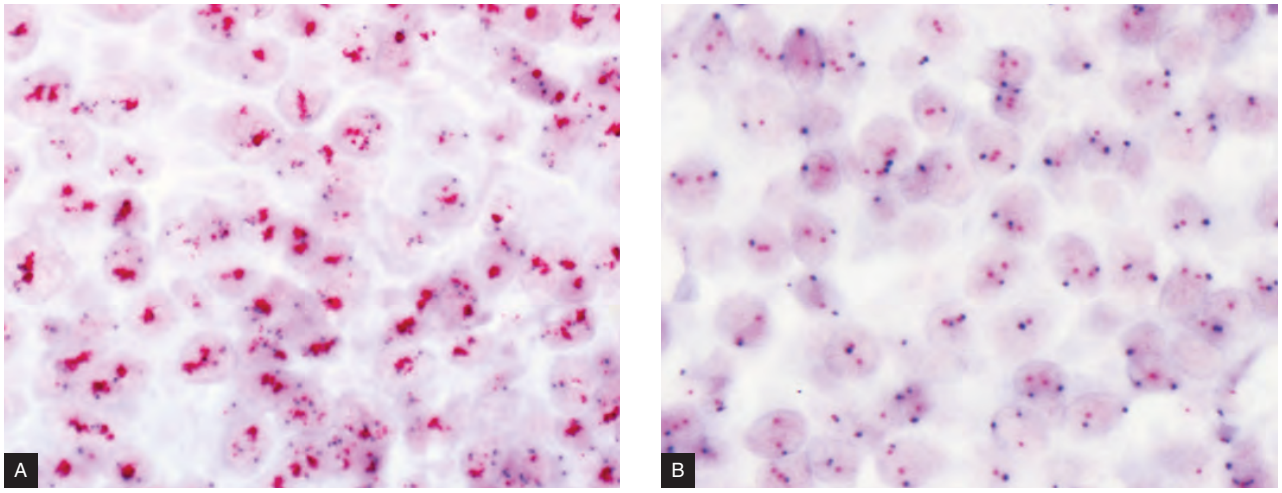


Figure 13.4 Examples of HER2 amplified **(A)** and non-amplified **(B)** FFPE breast cancer sections visualized with a 60x objective. Stained with Dako *HER2* CISH pharmDx™ Kit.

PETITIONER EXHIBIT 1012
Page 156 of 218

To determine the *HER2* gene status, the invasive component of the tumor cells is located in the bright field microscope, and enumeration of the red (*HER2*) and blue (CEN-17) signals is conducted. The *HER2*/CEN-17 ratio is calculated based on the counting of at least 20 nuclei. Normal cells within the analyzed tissue section serve as an internal positive control of the staining process and the pre-analytical treatment of the specimen. When performing the staining reaction described above, a complete one to one conversion of FISH signals to CISH signals takes place as illustrated in Figure 13.2. In this particular staining, a FISH staining was first completed, fluorescence pictures taken, coverslip removed and the section was washed prior to performing the CISH immunohistochemical staining reaction. Then, new bright field pictures were taken to allow comparison of identical tissue areas as illustrated. Many publications have shown a very high level of agreement between the ratios for *HER2*/CEN-17 by FISH and CISH (see Table 13.1).

Table 13.1 Concordance between FISH and CISH/SISH. All the studies listed in the table have evaluated for overall agreement with respect to *HER2* gene status between the two ISH methods. CISH: Chromogenic in situ hybridization, SISH: Silver-enhanced in situ hybridization.

Study	Sample size (n)	Tissue	Assay	Concordance (% Overall Agreement)
Park YS, et al (14)	588	Gastric	SISH	98.3
Lee Y, et al (15)	543	Breast	SISH	96.7
Riethdorf S, et al (16)	399	Breast	CISH	94.9
Mollerup J, et al (10)	348	Breast	CISH	98.3
Hyun CL, et al (17)	309	Breast	CISH	96.8
Hanna WM, et al (18)	254	Breast	CISH	95.3
Hwang CC, et al (19)	227	Breast	CISH	100.0
Gong Y, et al (20)	226	Breast	CISH	98.5
Brügmann A, et al (21)	201	Breast	CISH	93.0
Sàez A, et al (22)	200	Breast	CISH	94.8

Chapter 13.4 | *HER2* CISH in Clinical Decisions

The use of validated products and procedures are important to provide the best possible assay result and subsequent treatment guidance in clinical decisions. Following the introduction of molecular testing using CISH, a large number of studies have reported on the agreement between FISH and CISH/SISH with respect to *HER2* gene status in breast and gastric cancer tissue. However, many of these studies are small and include a relatively low number of tissue samples. In order to identify the larger published concordance studies between FISH and CISH/SISH a PubMed search was undertaken. Studies that contained 200 or more breast or gastric cancer tissue samples were selected if the publication was in English. Since the main purpose of the study was a direct comparison between FISH and CISH/SISH, the percent overall agreement should have been calculated. In Table 13.1 the result of the literature search is presented. Ten concordance studies were indentified that fulfilled the search criteria and the number of tissue samples in these studies ranged from 200 to 588 with the overall agreement percentage ranging from 93.0% to 100.0%. The literature survey showed that the overall agreement between the FISH and CISH/SISH assays is generally very high, with 7 out of 10 studies having an agreement percentage of 95% or higher.

For many years, HER2 testing in the clinic has been done using IHC with reflex testing using FISH, or with FISH as the initial test and most of the important clinical studies done with HER2-targeted compounds in HER2-positive cancer patients have used the traditional IHC and/or FISH assays for patient selection (23-28).

However, correlation between HER2 status measured by CISH and response to trastuzumab (Herceptin®, Roche/Genentech) has now been demonstrated in clinical studies (29, 30). One of these studies was the FinHer Study in primary breast cancer, which showed an improved three-year, recurrence-free survival for the HER2-positive patients treated with trastuzumab. Currently, four different ISH products are approved by the FDA as in vitro companion diagnostic devices that can be used to provide predictive information prior to the use of a particular therapeutic product (see Table 13.2).

PETITIONER EXHIBIT 1012
Page 157 of 218

Table 13.2 Overview of dual color in situ hybridization products currently assigned as companion diagnostic devices by the FDA. Accessed May 31, 2013. (www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm)

Trade Name	Manufacturer	Assay type	Tissue	Drug tradename
HER2 CISH pharmDx™ Kit	Dako, Agilent Technologies	CISH Dual color	Breast	Herceptin® (trastuzumab)
INFORM HER2 DUAL ISH DNA Probe Cocktail	Ventana Medical Systems, Inc.	SISH/CISH Dual color	Breast	Herceptin® (trastuzumab)
HER2 IQFISH pharmDx™	Dako, Agilent Technologies	FISH Dual color	Breast, Gastric	Herceptin® (trastuzumab); Perjeta™ (pertuzumab)[1]; Kadcyla™ (ado-trastuzumab emtansine)[1,2]
PathVysion HER-2 DNA Probe Kit	Abbott Molecular Inc.	FISH Dual color	Breast	Herceptin® (trastuzumab)

[1]Breast cancer only, [2]Approved March 2013, in US only

Finally, from an analytical and a clinical viewpoint, *HER2* CISH assays have shown to provide results similar or identical to FISH assays and it must be concluded that CISH is a reliable alternative to FISH for HER2 testing.

Acknowledgements

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PETITIONER EXHIBIT 1012
Page 158 of 218

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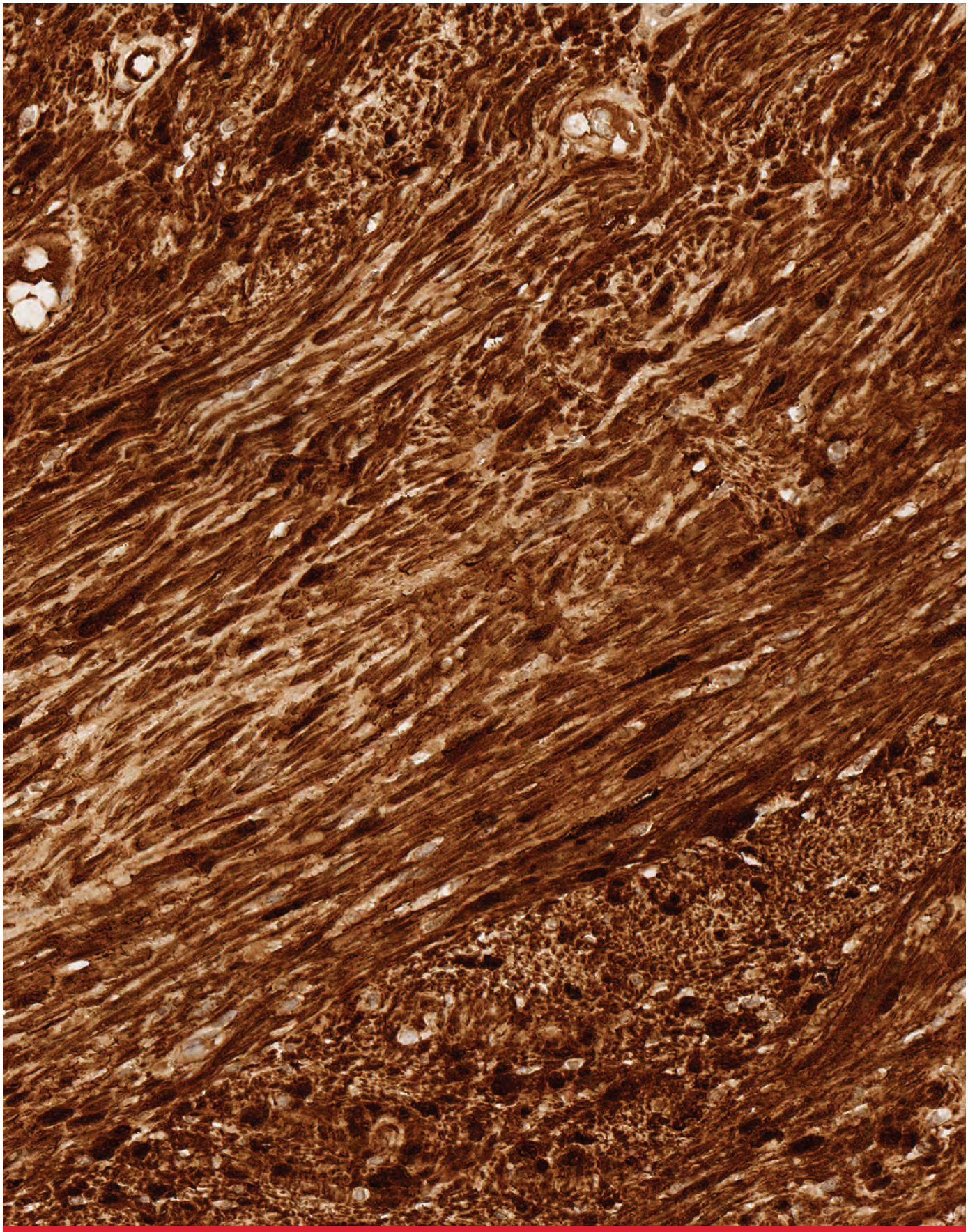
PETITIONER EXHIBIT 1012
Page 159 of 218



PETITIONER EXHIBIT 1012
Page 160 of 218

Chapter 14

Controls

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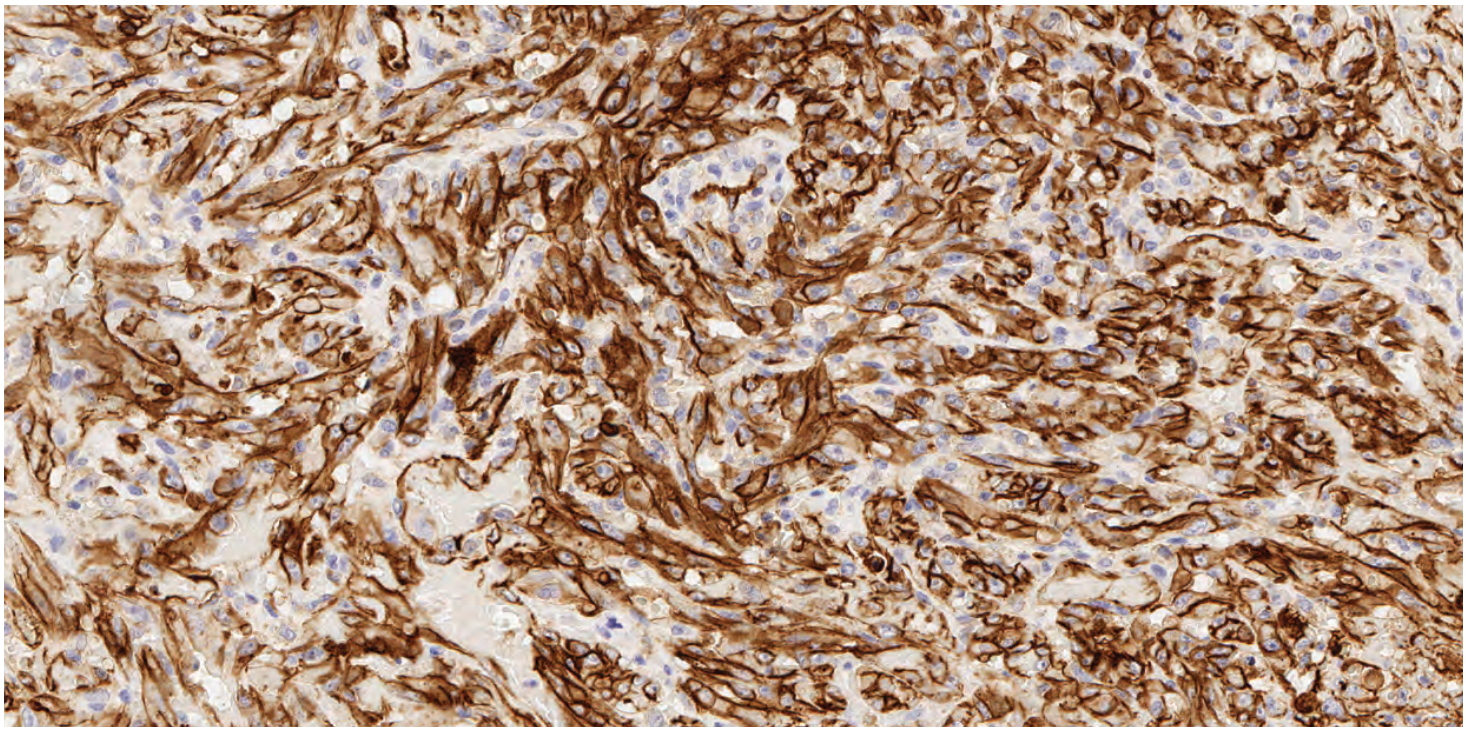

Con•trol *(n.)*
A standard of comparison for checking or verifying the results of a scientific experiment.

The American Heritage® Dictionary of the English Language

Chapter 14.1 | Introduction

Many factors may introduce variations in immunohistochemistry including ischemic time, tissue fixative and fixation time, tissue processing, efficiency of epitope retrieval, selection of antibody/antibody clone, detection system, instrumentation – and not least interaction among the many different sources of potential variation.

All major suppliers of diagnostic systems to the pathology laboratories have implemented measures to safeguard the quality of their systems. These measures are implemented in development and validation as well as during manufacturing and supplier quality control. However, with the many potential factors that may influence an immunohistochemical staining, it cannot be assumed that any given IHC staining is correct. Consequently, it is important to include controls for verification of results for in vitro diagnostic use. It is also important to understand what information a given control can provide or not provide.

Figure 14.1 This chapter will describe the range of controls that should be used in a diagnostic laboratory.

Chapter 14.2 | Purpose of Controls

In short, IHC staining controls are required for validation of staining results to document and/or ensure that each and every IHC test is performed correctly and can be used to support the diagnosis. The controls will show whether all reagents are in good order, and very importantly, they will also show if the staining system is working correctly and thus demonstrate potential day-to-day, operator-to-operator and/or instrument-to-instrument variations.

For many of the potential variations there are other measures in place to safeguard; unfortunately, it is not always possible to account for extreme situations or equipment defects. Reagent expiry is one example. Reagent expiry dates are determined experimentally and extensively documented during development and verification; testing includes extensive transportation scenarios, with changes in temperature and prolonged storage at increased temperature. However, such investigation will never be able to encompass all situations, e.g. that a package is lost for weeks during shipment. Likewise, a defective refrigerator can impact reagent quality. Sub-optimal reagent quality should be detected by appropriate controls as part of the staining process.

Many countries have regulatory requirements for diagnostic methods, reagents, instruments and software, as well as vendor and laboratory responsibilities. For each laboratory it is important to understand and implement these requirements accordingly. For reference, a thorough description on Quality Management and Regulation in the US is given in (1).

Vendor responsibilities

In addition to country-specific requirements, the site of manufacture may also impact vendor responsibilities. In the EU, vendor responsibilities are determined by a specific IVD directive (98/79/EC). Basically, there are requirements for development and validation, as well as for manufacturing and quality control of IVD products. Key parameters that must be documented are included in the data/specification sheet or corresponding product specific documentation. For example, antibody vendors must ensure appropriate specificity using a range of immunochemical techniques, including immunoelectrophoresis, Western blot, double diffusion immunoassays and ELISA. Testing on transgenic cells expressing the specific, as well as closely related, antigens may also be performed. It is, however, imperative also to include testing on tissue; both cancer tissue and normal tissue, for high and low expression structures (see also Chapter 5). For primary antibodies, manufacturers typically first test antibodies on a range of positive tissues to identify the op-

PETITIONER EXHIBIT 1012
Page 162 of 218

timal antibody dilution in combination with the chosen staining protocol. Next, immunohistochemistry testing is extended to an expanded panel of additional tissues known to either contain, or not contain, targeted antigens. For new antibody lots, manufacturers typically perform quality control to verify the specificity and sensitivity that were documented during development.

Laboratory responsibilities

Laboratories performing IHC assays hold key responsibilities relating to controls:

- Laboratory validation of new antibodies, protocols and systems
- New reagent lot verification
- Performance control for each run

The exact responsibilities vary depending on the country and accreditation program. If the laboratory is enrolled in the US CAP's (College of American Pathologists) laboratory accreditation program, the laboratory must comply with a specific checklist (2). Here it is specified that "The performance characteristics of each assay in the immunohistochemistry laboratory must be appropriately validated before being placed into clinical use". The goal is to verify vendor specifications and the optimal staining protocol, as part of validating overall clinical performance. It is important to test a panel of positive and negative tissues to determine assay sensitivity and specificity. With respect to the number of tissues to be included for validation of an antibody, 10 positive and 10 negative tissues are suggested for well-characterized antibodies with a limited number of targets/applications. A recent study investigated the use of immunohistochemistry validation procedures and practices for non-predictive assays (3). The study was based on 727 responders to a questionnaire composed of 32 items. Key results were that 68% of the laboratories had written validation procedures, and 86 % had validated the most recently introduced antibody. With respect to the number of different tissues included in the validation, 75% used 21 or fewer cases for the validation.

New reagent lot verification is also addressed in the CAP Anatomic Pathology Checklist, where it is stated that "the performance of new lots of antibody and detection system reagents is compared with old lots before or concurrently with being placed into service".

For every immunohistochemistry staining run, at least one control should be included. This is straightforward when staining is carried out in batch mode (see Chapter 9), however with continuous slide loading, "a staining run" must be defined in a different way, e.g. once per day, or once per x number of slides. The staining run control may be expanded to at least one per antibody, and over the past years there has been an increasing use of control material on every slide (see below for further discussion).

The saying, "if it isn't documented, it didn't happen", directly applies to the staining control mentioned. It is important that all procedures and results – as well as procedural changes, are documented and proper record-keeping procedures are applied. In addition, it is advisable to implement a trend-tracking procedure to capture small changes over time, e.g. running a standard at regular intervals. Many labs track the relative percentages of HER2 scores (0, 1+, 2+, 3+) to use as a indirect measure of assay performance over time. Changes in the relative percentage of HER2 scores may warrant further investigation of the cause. If results may be influenced by changing environmental conditions, it is also advisable to keep track of the relative humidity and temperature.

Chapter 14.3 | Categories of Controls and Control Material

The terminology for the various control types are in some parts of the literature not clearly defined. This chapter uses the terminology emphasized by Torlakovic (4).

Control material can be categorized in different ways:

- Reagent controls
  - Positive reagent control is the actual reagent; e.g. the primary antibody when tested on control material. Testing is done during development and product validation to ensure reagent specificity and sensitivity.
  - Negative reagent control is a reagent substitute for the primary antibody used to assess potential specificity issues/false positive staining reaction
- Tissue controls
  - Positive tissue control is tissue with the specific antigen at known, relevant and stable level. Purpose is to document correct staining.

PETITIONER EXHIBIT 1012
Page 163 of 218

- Negative tissue control is tissue without the specific antigen present – or not present in specific regions. Purpose is to document specificity of the staining.
- Tissue blocks/tissue microarrays, consisting of a few to several different tissues that may serve as control material for a range of antibodies (see Chapter 12)

- Internal tissue controls
  - The presence of the target antigen (protein) within normal elements of the tissue under investigation is an internal positive control
- Tissue process control
  - Constitutively expressed proteins generally expressed in all tissues at the same level. Such a protein may serve as control for not only the staining process but also for the pre-analytical steps.
- Cell line controls
  - Control material based on cultured cells with specific antigenic characteristics. This type of control typically is made for very specific purposes, in particular predictive assays.

Chapter 14.4 | Use of Controls in Daily Routine Testing

The many sources of variations, as well as the potentially critical consequences thereof, call for standardization, assay validation and use of controls. Although awareness of the complexity of immunohistochemical assays and the inevitable variation is increasing, a meta-analysis of 100 peer-reviewed papers on immunohistochemistry revealed that only 13% of the articles described positive and negative controls run on identically prepared samples. More than half of the articles either did not mention controls, or did not run controls as separate specimens (5). The papers included in the meta-analysis have both diagnostic and research focus, underlining the importance of paying proper attention to inclusion of controls in both types of IHC applications.

This section will elaborate on the different types of controls used in IHC and their applications in the diagnostic laboratory. Controls are a necessity for proper interpretation of staining, and omitting them will entail a risk. Although it is impractical to devise and run controls for every combination of pre-analytical and analytical factors, data on controls in diagnostic immunohistochemistry, as well as in research, must be included for valid and reproducible test results. Correctly used controls can reveal if the staining protocols were run correctly, and help determine day-to-day and operator-to-operator variations.

Figure 14.2 On-slide positive tissue controls, where specific controls are placed on the same slide as the test specimen are strongly preferred. **A)** Illustration of the principle of multi-core blocks. Tissue controls are the cores in red, yellow, gray and green. Patient sample is placed below **B)** Example of on-slide tissue controls. **C)** Example of cell line controls.

Reagent controls

Negative reagent control for polymer-based detection systems

Since the start of IHC, it has been good practice to include a negative reagent control to check for specificity of the reagents. In recent years, there has been a significant improvement in both sensitivity and specificity of detection systems. A key element has been development of polymer-based, biotin-free visualization systems, which has reduced the need for negative reagent controls for such detection systems. In fact, recently, the US CAP organization changed its recommendation for US laboratories, now stating that for polymer-based (biotin-free) systems, negative reagent controls may be omitted at the discretion of the laboratory director (2).

PETITIONER EXHIBIT 1012
Page 164 of 218

Currently, most antibody specification sheets from major vendors include suggestions for appropriate negative reagent controls, with no reference to choice of detection system; and as stated in the US CAP recommendation, the negative reagent control should be omitted only when given appropriate consideration.

Thus, laboratories must still pay attention to potential artifacts or special cases, for example occurrence of non-specific DAB precipitates when performing staining of small bacteria or viruses. DAB precipitates may resemble small stained elements, which could complicate interpretation of the staining result. A negative control would help determine if the staining seen in the patient sample is a false positive result.

Negative reagent control for biotin-based detection systems

For tests using biotin-based detedtion systems, a negative reagent control test should still be included in all runs, due to the inherent possibility of endogenous reactivity. This test has to be done on a separate section of patient tissue, using the same staining protocol with same target retrieval and detection system, except that the primary antibody is replaced by the negative reagent control.

For monoclonal mouse primary antibodies, the negative reagent controls may be developed by different methods. The optimal method is an antibody of the same isotype, present in the same immunoglobulin concentration, using the same diluent and exhibiting no specific reactivity with the given human tissues tested. A less optimal alternative is to use mixtures of antibodies representing all or most relevant IgG subtypes. Finally, the diluent itself may also be used as an alternative which, however, is neither efficient nor desirable.

For polyclonal antibodies, negative reagent controls should be a dilution of immunoglobulin fractions, or whole normal (non-immune) serum of the same animal source. Again, the negative reagent control should be applied in the same concentration as the test antibody, and the same diluent should be used.

Using the same protocol as the primary antibody, the negative reagent control should be applied to a sequential section of each patient specimen, to evaluate nonspecific staining in that particular tissue.

NOTE: A special situation worthy of note is when a panel of two or more antibodies (of the same species) is applied to serial sections, using the same detection system. In this instance, negatively stained areas of one slide may serve as the negative/non-specific binding background control for other antibodies in the panel.

In cases where it is necessary to evaluate non-specific binding, potentially caused by sources other than the primary antibody, additional patient tissue sections may be stained with selected reagents. For example, tissues may be stained with just the secondary antibody and/or the enzyme, followed by application of the substrate/chromogen. In cases where the suspected non-specific staining may be the result of endogenous enzyme present within the tissue, this possibility can be confirmed by application of the substrate/enzyme only.

Tissue Controls

As noted in above, both positive and negative tissue controls provide important information and must be included in daily routine regardless of type of detection system used. Typically, the tissues being used for controls are carefully selected normal or cancer tissues previously analyzed in the laboratory. However, in some cases the actual patient tissue being tested can be used as tissue control. For all tissue controls, if the staining does not perform as expected, results from the respective test specimen should be considered invalid.

Positive tissue control

The positive tissue control must contain the target antigen at relevant, known and stable expression level. It serves to document that proper staining has been performed and confirms that the target retrieval procedure has been carried out correctly. Thus, a positive tissue control assesses correct staining protocol performance (temperature, time and correct application of reagents).

As positive tissue controls are indicative of properly prepared tissue, they should be as accurate as possible in the same manner as the patient samples. Optimally, autopsy/biopsy/surgical specimens should be fixed, processed and embedded as soon as possible for best preservation of antigens. Generally, autopsy tissue is least preferred because of inevitable delays before fixation (cold ischemia, with degradation of some antigens, see also Chapter 2).

PETITIONER EXHIBIT 1012
Page 165 of 218

Furthermore, the positive controls should be properly documented, including sectioning date and storage conditions for the control material. Special attention should be given to the stability of the specific antigen(s) to be controlled, as antigen degradation occurs in cut, unstained slides, at different rates dependent on the antigen (6). A different challenge may be access to optimal tissue control material; in particular in cases where normal tissue may not be used, e.g. for certain viral infections. Finally, the use of patient material for control tissue must comply with local or regional regulations.

Ideally these controls should contain a spectrum of weak to strongly positive staining reaction. If such tissue is not available, at least a weakly positive tissue should be used, as this provides the best basis to evaluate whether a particular staining reaction is optimal (see also Chapter 4 and Chapter 5).

On-slide positive tissue controls

In the daily routine, positive tissue controls may be run on a separate slide - as a batch control or daily control, or they may be included on the same slides as the test specimens. Using batch run controls, with the control section on a separate slide, the control slide will be indicative that the staining process has run correctly. However, batch controls will not be able to identify a missing reagent (false negative), wrong antibody (false positive) or inappropriate protocol (false negative or false positive) on any other slides run on the instrument in that batch other than on the control slide itself. Consequently, on-slide positive controls, where specific controls are placed on the same slide as the test specimen are strongly preferred (Figure 14.2).

More and more laboratories are using TMA cores as on-slide controls. It is possible to build a few basic multi-tissue control blocks each containing a small number of control tissues to cover most of the markers used in clinical diagnostics. NordiQC, an independent scientific organization arranging quality schemes for pathology laboratories, has made a suggestion for set of four multi-tissue control blocks each containing four

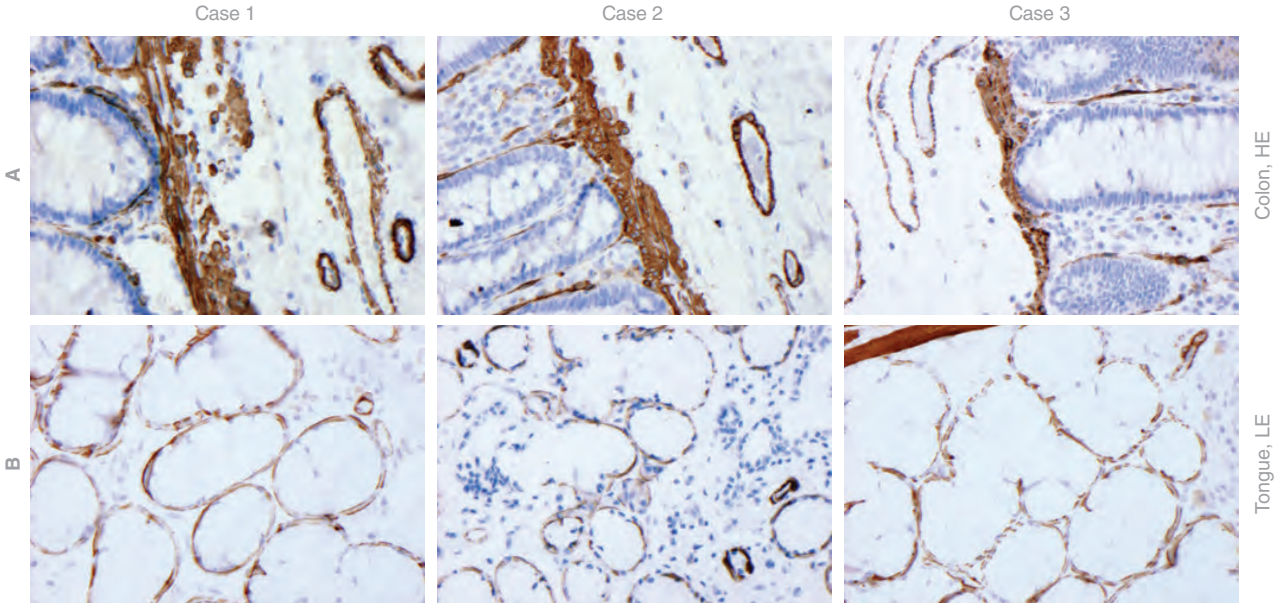

Figure 14.3 Normal colon from three different cases stained with Anti-Actin, clone HHF35. Smooth muscle cells in vessel wall, muscle layers and lamina muscularis mucosa, defined as high expression (HE) structure, show comparable staining intensity in all three samples. **B)** Normal tongue from three different cases stained with Anti-Actin, clone HHF35. The myoepithelial cells of the mucous/salivary glands, defined as low expression (LE) structure, show comparable staining intensity in all three samples.

PETITIONER EXHIBIT 1012
Page 166 of 218

different – and preferably normal – tissues that can be used for most of the markers used in clinical diagnostics. For each block, the specific antibodies covered are specified, together with the reaction pattern on each control tissue, as well as quality indicators for diagnostic use if available (7). As an example, one of the multi-tissue control blocks contains appendix, tonsil, pancreas and liver, and the reaction patterns are described for approximately 100 different antibodies.

Dako has created an Atlas of Stains book that for each antibody in the FLEX RTU (Ready-To-Use) series specifies recommended control tissues with low expression (LE) and high expression (HE) quality indicator structures to use. In addition,matching descriptions and images of the expected staining patterns are included.

Example of positive tissue controls

The shown example is for Anti-Actin, clone HHF35, where the defined HE and LE structures are present in different tissue types; in colon all smooth muscle cells in vessel wall, muscle layers and lamina muscularis mucosa are defined as HE (Figure 14.3A), whereas LE is the myoepithelial cells of the mucous/salivary glands in tongue (Figure 14.3B).

However, evaluation of various normal tissues can show cases where antigen expression varies too much to be defined as stable. Using the same Anti-Actin as example, normal liver tissue shows staining of perisinusoidal smooth muscle cells – but only in some samples (Figure 14.4). This variability emphasizes that all normal tissues may not, by default, be suitable as control tissue. Detailed analysis should be carried out when selecting the normal tissue that is optimal as control tissue, by using a validated protocol that is able to identify variations in antigen expression.

Negative Tissue Control

A negative tissue control is tissue that lacks the specific antigen – or where the antigen is not present in specific regions. It serves to ascertain specificity of the staining. If using the 'on-slide TMA method', one of the cores should be negative, or contain structures/cells that are expected to be negative for the antibody that is being used. The negative tissue control must be included to identify the correct specificity of the antibody; showing no staining of structures or cells that are known to lack the antigen. The negative tissue control also serves for identification of sub-optimal protocol (high background). Just as for positive controls, tissue used for negative controls should be prepared in the same manner as the patient sample.

Internal Tissue Controls

Positive internal controls, also known as 'built-in' or intrinsic controls, contain the target antigen within normal tissue elements, in addition to the tissue elements to be evaluated. This circumstance appears ideal, as the tissue elements to be evaluated have been treated exactly as the positive control. However, the level of target in the internal positive control is not predetermined, and may or may not be as stable as the external tissue control. Thus, when analyzing the slide, careful assessment of the internal positive control is important. Obviously, if the test slide only contains tumor tissue, an internal control is not an option. One example of a positive internal tissue control is weak expression of the estrogen receptor in normal breast

Liver

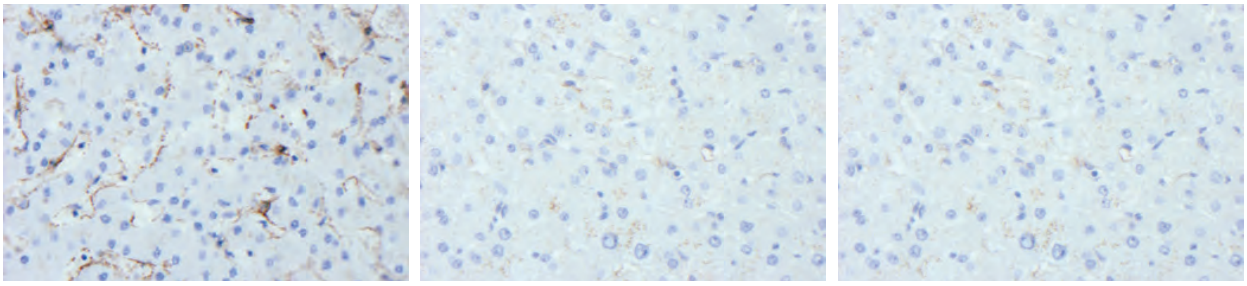

Figure 14.4 Normal liver from three different cases. The staining intensity of perisinusoidal smooth muscle cells varies from weak to negative, and is consequently a poor control tissue due to the variable antigen expression between tissue samples.

PETITIONER EXHIBIT 1012
Page 167 of 218

ducts. Thus, if the section to be tested is selected to include normal benign duct structures, these cells will be an excellent positive control. Another example is presence of S-100 protein in both melanoma and in normal tissue, such as peripheral nerves and dendritic reticulum cells (Figure 14.5). Internal controls can of course also function as negative controls when cells known to lack the antigen in question are used.

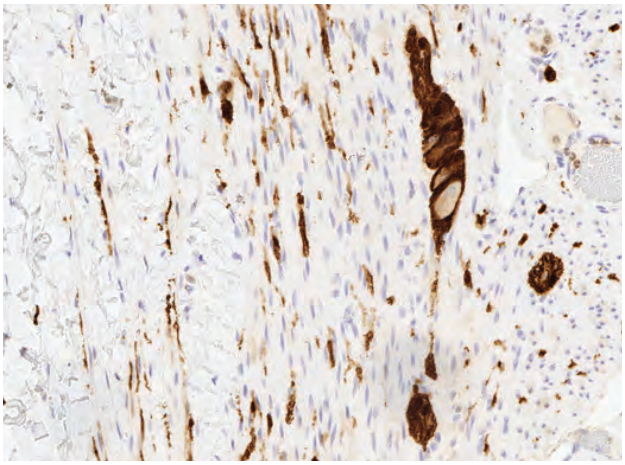

Figure 14.5 Normal appendix stained with Anti-S-100. The satellite cells and the Schwann cells of the peripheral nerves show a moderate to strong staining reaction.

Quantified, 'built in', or internal, proteins may potentially serve as calibrators for quantification in IHC (Quantifiable Internal Reference Standard – see Chapter 1, and (9), in much the same way as housekeeping genes, such as actin, serve as internal reference controls for expression studies (8, 9).

Chapter 14.5 | Tissue Process Control

The controls described in section 14.4 all serve to document the staining process. However, the pre-analytic steps, in particularly the ischemic time and fixation, can have significant impact on the capability of the tissue to provide good staining. Unfortunately, it has not yet been possible to develop efficient controls that are optimal for the pre-analytic process. It has been speculated to use constitutively expressed proteins present in the test sample (8, 9). Battifora (8) suggested the use of vimentin for this purpose. However, no broad system of controls that makes tissue process control effective in daily routine exists.

Chapter 14.6 | Cell Line Controls

Several FDA-approved companion diagnostics contain cell line controls as part of the kit, or sold separately. These cell lines are specifically developed to monitor staining of the antigen of interest and should be included in all staining runs as an additional protocol control.

Just as with tissue controls, cell line controls may be positive or negative. Positive cell line controls monitor staining performance by assessing target retrieval, blocking, antibody incubation and visualization. Negative cell line controls assess specificity and, depending on the characteristics of the chosen cell line, may also provide information on performance. It must be emphasized that because processing differs for these cell lines, they do not serve as control for pre-analytic variables; only internal controls do that.

An ideal negative cell line control will contain an amount of target antigen that is sufficiently low to produce no staining if the procedure has been performed correctly. At the same time, the amount may be sufficiently high to produce a weakly positive stain if the run has been performed under conditions that produce an excessively strong staining.

An ideal positive cell line control would contain a number of target antigens producing staining of medium intensity. This would allow the control to assess both stains that are too weak and stains that are too strong.

An example of the way in which cell line controls can be used is illustrated by Dako's HercepTest™ kit, which contains three cell line controls: 0 (negative), a 1+ (weak staining) and a 3+ (strong staining). All are placed on the same microscope slide. The scale used for HER2 scoring (0, 1+, 2+, 3+) is arbitrary, and does not represent the proportional difference in target molecules per cell. The control cell line with 3+ score has an estimated number of 1,400,000 to 2,390,000 receptors per cell, up to a 30-fold increase over the number of receptors expressed by the 1+ cell

PETITIONER EXHIBIT 1012
Page 168 of 218

line (12, 13) (Table 14.2). It is important to note that the staining intensity of the control slides should not be used to interpret the staining intensity in patient samples by comparison. The cell lines are used only to indicate the validity of the staining run.

Table 14.2 The correlation between the number of HER2 receptors per cell and the resulting IHC score (9, 10).

Cell line	HER2 receptors	IHC Score	Staining Pattern
MDA-231	21,600	0	No discernible membrane staining
MDA-175	92,400	1+	Faint, discontinuous membrane staining
SK-BR-3	1,400,000-2,390,000	3+	Moderate/strong, complete membrane staining

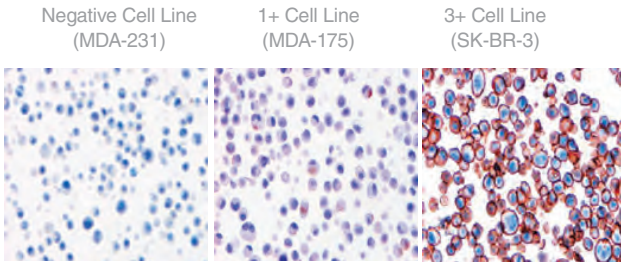

Figure 14.6 Cell line controls for Dako HercepTest™.

Chapter 14.7 | Monitoring the Staining Process

New staining instruments from several manufacturers, exemplified by the Dako Omnis and Autostainer Link 48, collect information concerning a range of the key staining parameters; either on a batch, slide rack, or single slide basis. The type of information collected may include onboard reagent temperature and time, to monitor reagent stability, target retrieval time and temperature. The data include temperature ramp-up and ramp-down time, staining incubation time, and temperature at each individual step, as well as reagent use.

Today, monitoring of the staining process cannot replace the controls discussed above; however, it can provide information prior to inspection of control slides that might indicate invalidity of the staining run, or indicate that special attention must be paid to specific slides. In addition, should the reading of control or test slides give any reason for concern, the staining process data are likely to indicate which step may have introduced an error. Furthermore, the staining process data may be used to assess the stability of the instrument and indicate if one parameter may be drifting, so that service can be applied if necessary (see also Chapter 16).

Chapter 14.8 | External Quality Assurance Programs

Most routine IHC staining results have no common quantitative measures. Instead, diagnostic IHC results are typically qualitative (yes/no answer) and are based on subjective interpretation by pathologists of varying experience (11). Quality control and assurance therefore remain crucial, and warrant high attention by both manufacturers and the pathology laboratory.

Internal quality control procedures in the individual laboratory are important for the reproducibility of the IHC performance, i.e. they will determine if the protocols, reagents and interpretations are working the same way, from day to day, and from individual to individual. However, internal quality control procedures will not necessarily identify if the staining is sub-optimal and if the IHC test is sub-optimally calibrated an IHC result may be obtained, it just may not be correct (12). Consequently, it is important for laboratories to participate in proficiency testing through an external quality assessment (EQA) scheme. The EQA organization independently assess staining results from a range of laboratories, and identify good staining quality as well as insufficient or poor staining, plus inappropriate protocols and possible interpretation problems. EQA schemes assess staining results by sending out tissue samples to be stained using the laboratory's routine IHC staining procedures. Laboratories then return their results, and in some cases examples of the stained slides, which are compared with all other participating laboratories and summarized in a final report.

Selected EQA schemes

- Canadian Immunohistochemistry Quality Control (Canada) – www.ciqc.ca
- College of American Pathologists (CAP) (United States) – www.cap.org
- NordiQC (Based in Denmark) – www.nordiqc.org

PETITIONER EXHIBIT 1012
Page 169 of 218

- UK NEQAS (United Kingdom) – www.ukneqas.org.uk

Similar programs are introduced in Australia, Austria, Germany, and other countries or regions around the world.

Chapter 14.9 | Future Aspects

To ensure diagnostic certainty, IHC quality control will remain a very important and integrated part of the daily routine. Across the world, there is an increasing requirement for laboratories to be accredited, and often accreditation includes a need for participation in proficiency testing. Thus the EQA schemes will see increased participation – and importance – becoming more widespread across the world. We also see that on-slide controls will be commonplace to provide a slide specific control, the challenge here being the workload and associated cost in making and placing the on-slide controls.

As discussed in Chapter 7, there is an increasing use of digital whole slide imaging, and it is expected that this will become a widespread tool in the routine laboratory. In the context of this chapter, it is important to point out that both manufacturers and laboratories must comply with the requirements described in section 14.2, and ensure proper validation, both from the vendor side and at the laboratory, of the intended use of the system. Regulations for digital pathology are more extensively discussed in Chapter 7 (13). Moving further along, it is interesting to speculate to which extent digital imaging will be used in the process of automating the reading of control material, namely the tissue controls, cell block controls or protein spots.

New technologies on the horizon will likely facilitate more efficient – and standardized – means of generating tissue control substitutes, one example being peptide IHC controls, where formalin-fixed peptide epitopes resembling the target protein are covalently attached to the glass microscope slides (14). Another possibility that may approach a more complete control of the IHC 'total test' (Chapter 1) is the 'faux tissue' or 'Histoid', which if fully developed has the potential also to control fixation variables, if not cold ischemia time (18, 19). When the performance of such systems has been documented to be comparable to tissue controls, they could be an effective tool relieving the laboratories for the burden of both identifying proper tissue and internal manufacture of the control slides. However, such systems serve to validate only the analytic phase of IHC, and do not serve to control for pre-analytic error such as internal reference controls – if developed to routine use may do. A final note is that availability of such systems is likely to speed up the adoption of on-slide controls – one critical factor, of course, being the price.

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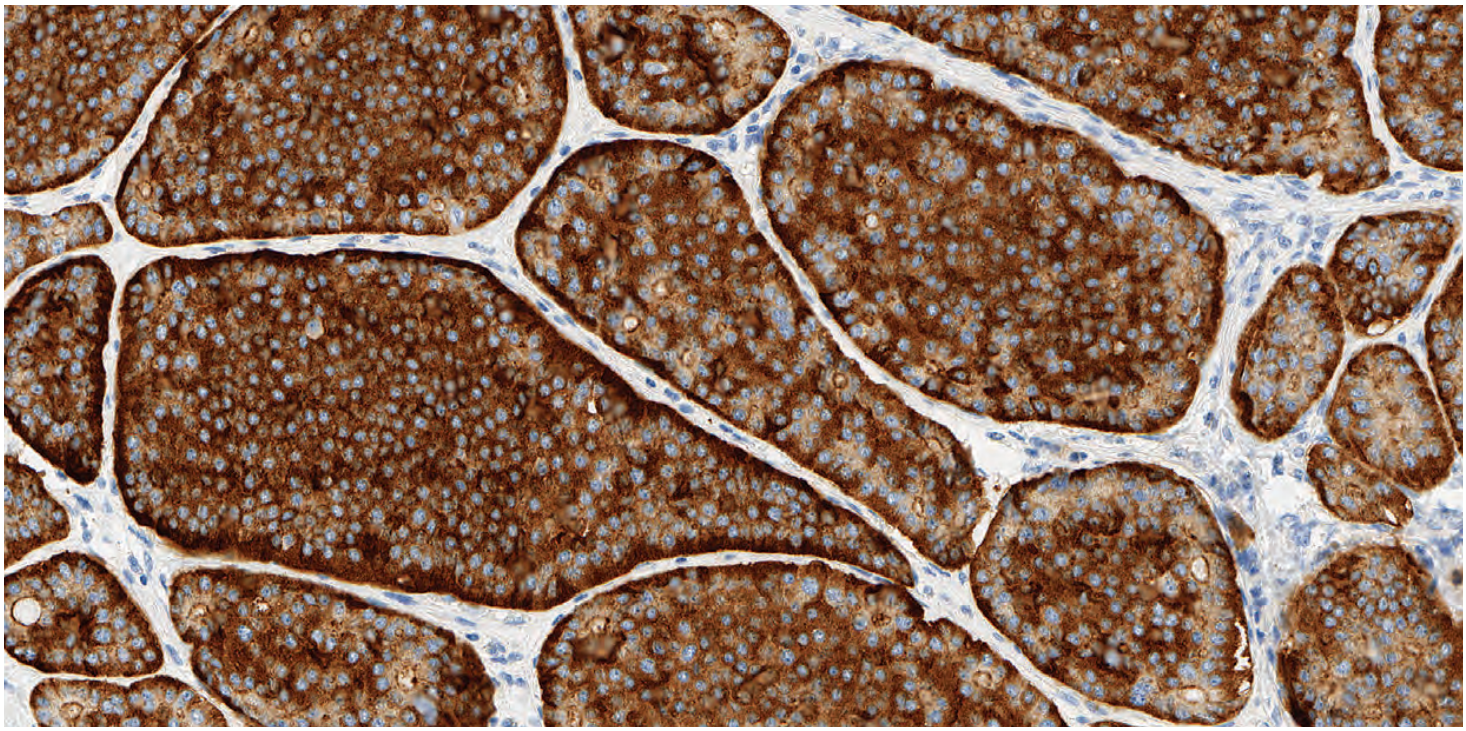
PETITIONER EXHIBIT 1012
Page 170 of 218

Chapter 15

Background

Helle Grann Wendelboe, MSc



Ba•ck•gro•und *(n.)*
Extraneous signals that can be confused with the phenomenon to be observed or measured.

The Free Online Dictionary

Chapter 15.1 | Introduction

As immunostaining of histological tissue specimens becomes more diversified in methodology and more sensitive in detection, background staining has develop into one of the most common problems in immunohistochemistry. Background staining in tissue sections may be due to several factors, some of which are specific to the antigen and antibody reaction or detection method, and others, which are of a more general character. The terminology used in this chapter uses the term "unwanted specific staining", if the staining is mediated by interactions between any antibodies and their respective epitopes, and "non-specific staining" for all other interactions. The following description will cover the major causes of background staining related to antibodies, detection methods, and other general factors and will offer possible solutions to these problems.

Chapter 15.2 | Detection Methods

Polymer-Based Detection Methods

Non-specific staining caused by endogenous avidin/biotin can be completely avoided by use of polymer detection systems. However, polymer-based detection systems may induce general non-specific staining if insufficient washing is performed after polymer application. Due to the large size of some polymer conjugates, the diffusion rate of these molecules is lower than for low-molecular weight conjugates. In addition, polymer conjugates based on a hydrophobic backbone have a tendency to be "sticky". This can be resolved by applying multiple wash steps, adding detergent to the wash buffer, and by prolonging washing time.

The latest guidelines from CAP (1) leave it up to the individual laboratory to evaluate whether the selected detection method poses a negligible risk for non-specific staining, such that the negative reagent control for the polymer-based detection methods can be eliminated (Chapter 14). However, some staining situations still justify the use of negative reagent control for polymer-based detection methods, e.g. if instrumentation is used where a reaction between the polymer-based detection system and the substrate-chromogen can occur in an error situation, or where the substrate-chromogen by itself can be captured by foreign items like graphite or dust. Capturing of chromogens such as DAB pose a challenge when the target structure is small, for example *helicobactor pylori*. For small structures it is therefore necessary always to include a negative reagent control.

Vendors of IVD reagents for IHC will continue to recommend the use of negative reagent control for polymer-based detection methods to comply with FDA regulations.

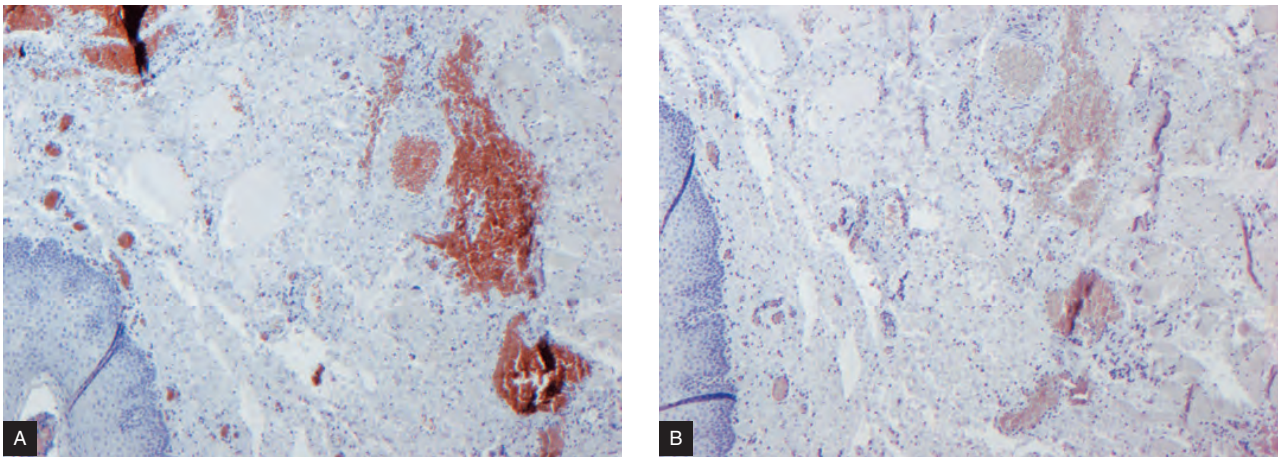


Figure 15.1 Red blood cells showing endogenous peroxidase activity. **A)** before, and **B)** after blocking with 3% hydrogen peroxide.

PETITIONER EXHIBIT 1012
Page 172 of 218

Horseradish Peroxidase-Based Detection Methods

For practical purposes in immunohistochemistry, endogenous peroxidase activity can be defined as any activity that results in the decomposition of hydrogen peroxide (H_20_2). Such activity is a common property of all hemoproteins, such as hemoglobin (red cells), myoglobin (muscle cells), cytochrome (granulocytes, monocytes), and catalases (liver and kidney). Peroxidase activity may also be encountered in tissue adjacent to vascularized areas, due to the diffusion of blood cells prior to fixation.

The most commonly used procedure for suppressing endogenous peroxidase activity in formalin-fixed tissue is the incubation of sections in 3% H_2O_2 for 5-10 minutes (Figure 15.1).

Methanolic H_2O_2 treatment (11 parts 3% H_2O_2 plus 4 parts absolute methanol) for 20 minutes is also used, but is not recommended for specimens where cell surface markers are to be stained. Methanolic treatment may also detach frozen sections from their carrier glass. Endogenous peroxidase activity can also be suppressed by a mixture of sodium azide and H_2O_2 (1). However, in most work with formalin-fixed tissue sections, the interpretation of specific staining is not impaired by endogenous peroxidase activity. If the formalin-fixed tissue is rich in blood-containing elements then it is a good idea to quench endogenous peroxidase activity. In cell preparations and frozen sections, routine quenching of endogenous peroxidase is also advisable.

Specimens rich in endogenous peroxidase activity may be processed using an alkaline phosphatase detection method instead of a peroxidase method, eliminating this type of background.

Alkaline Phosphatase-Based Detection Methods

Endogenous alkaline phosphatase activity is frequently encountered in intestine, kidney, osteoblasts, endothelial cell surfaces, neutrophis, stromal reticulum cells, lymphoid tissues, and placenta. In frozen tissue, where endogenous alkaline phosphatase activity is most pronounced, routine quenching of endogenous alkaline phosphatase is recommended. In most formalin-fixed tissue sections, interpretation is not usually impaired by endogenous alkaline phosphatase, which makes quenching an optional choice. Most forms of endogenous alkaline phosphatase can be quenched by including 5 mM levamisole in the chromogen substrate solution (Figure 15.2). The intestinal form of alkaline phosphatase is the exception and resists this treatment, but it can be quenched by treating the tissue sections with a weak acid wash prior to the application of the primary antibody.

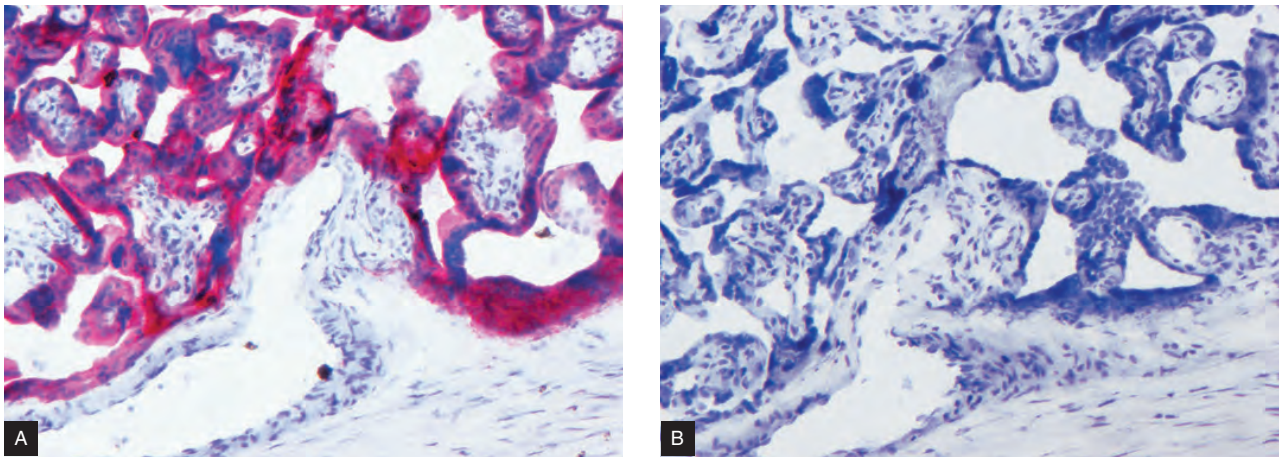


Figure 15.2 Placenta showing endogenous alkaline phosphatase activity. **A)** before, and **B)** after blocking with levamisole.

PETITIONER EXHIBIT 1012
Page 173 of 218

Combined Peroxidase and Alkaline Phosphatase (Double Staining)

Double staining using these enzymes requires quenching of both endogenous activities. To achieve this, use the H_2O_2 method for endogenous peroxidase and the weak acid method for endogenous alkaline phosphatase. The sequence of blocking endogenous activities is optional: the procedure will work effectively conducting either step first. Also, reagents that block both endogenous peroxidase and alkaline phosphatase in one step are available.

Biotin/Streptavidin-Based Detection Methods

Endogenous avidin-binding activity (EABA) has been observed with all biotin-based techniques, due to the presence of biotin in a wide variety of tissues. Biotin is bound to enzymes and other protein especially in the liver (hepatic nodules), kidney (tubular epithelia) and lymphoid tissue (paracortical histiocytes) (Figure 15.3).

EABA is usually observed within cytoplasm and is most pronounced when using frozen tissue sections. Paraffin-embedded tissues also retain substantial endogenous biotin. Other examples of EABA include the nonimmunochemical staining of myelin (3) and mast cells (Figure 15.4), in both frozen and paraffin-embedded tissue (4). Guesdon et al (5) found EABA in granulocytes from mouse spleen.

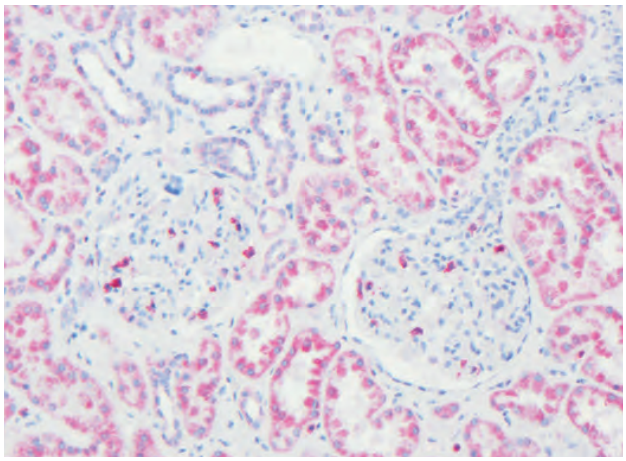


Figure 15.3 Endogenous biotin expression in the kidney.

EABA is best suppressed by sequential incubations of 10 to 20 minutes of tissue sections, first with 0.01% to 0.1% avidin, followed by 0.001% to 0.01% biotin prior to the staining protocol (6). Avidin has four binding sites for biotin while each biotin molecule can bind to only one avidin molecule. The first incubation with avidin effectively blocks endogenous biotin but simultaneously adds three more potential biotin-binding sites to the specimen. This means there are extra biotin binding sites open to link antibodies or detection systems that can give background staining. Therefore it is important to block these extra biotin binding sites with

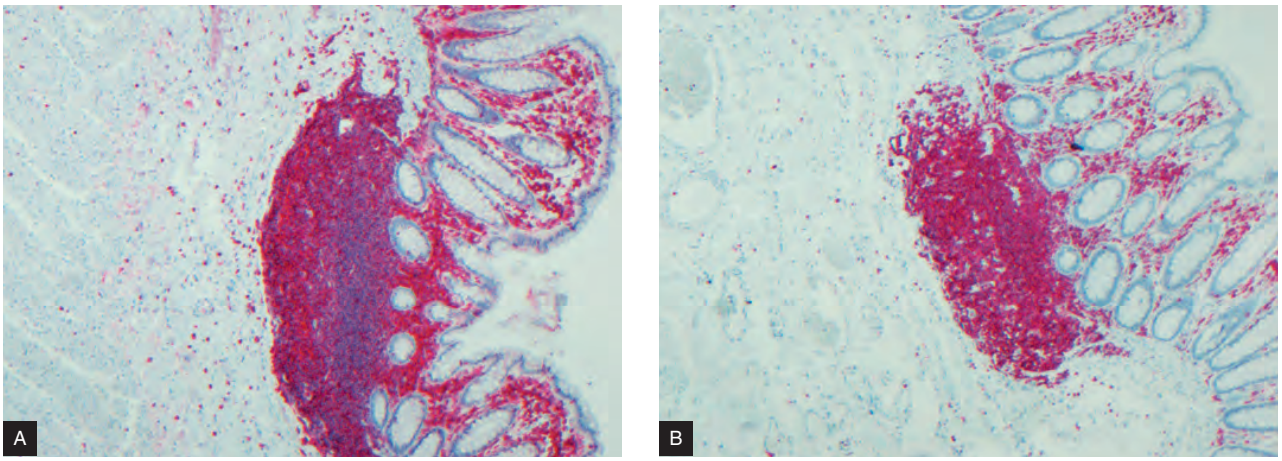


Figure 15.4 Avidin-biotin-complex (ABC) binding to mast cells in submucosa. **A)** before, and **B)** after blocking for endogenous avidin binding activity (EABA).

PETITIONER EXHIBIT 1012
Page 174 of 218

subsequent biotin incubation. Before blocking products became commercially available, egg white (avidin) and skimmed milk (biotin) could be used, at the risk of inconsistent performance.

Because avidin is a glycoprotein containing 10% carbohydrates and has a pI of 10, it tends to bind nonspecifically to lectin-like and negatively charged tissue components at physiological pH. Streptavidin contains no carbohydrates and has a pI of five. Its introduction to IHC has largely eliminated these problems. A sugar solution can block the lectin-like elements. Many commercially available avidin detection systems contain modified avidin to minimize non-specific avidin binding. Polymer-based detection systems, as noted, circumvent problems of background caused by avidin-biotin binding.

Chapter 15.3 | Antigen Retrieval (Heat-Induced Epitope Retrieval)

Antigen retrieval has been reported both to eliminate and introduce cytoplasmic and nuclear background in immunohistochemical procedures (7) (see also Chapter 3). A possible explanation is that antigen retrieval influences antigen-antibody binding activity, and thereby affects binding of the antibody to tissue proteins. Different types of antigen retrieval solutions, with different buffer compositions, pH and chelating abilities exist. Citrate pH 6.0, Tris/EDTA pH 9.0, and TRS pH 6.0 retrieval solutions vary in the way they influence antigen-antibody binding. Retrieval time and heating temperature can also influence antigen-antibody binding, so for new antibodies it is advisable to investigate which antigen retrieval solution and time are best to optimize signal and minimize background (Chapter 3).

Chapter 15.4 | General Factors

Antigen Diffusion

Unwanted specific background staining may occur when the tissue marker to be stained has diffused from its sites of synthesis or storage into the surrounding tissue. Because many fixatives penetrate tissues slowly, it is important to keep tissue specimens as small as possible and to fix immediately (Chapter 2). Otherwise the antigens may not be adequately fixed and may be extracted or displaced by the subsequent tissue processing steps. Extracellular antigens, or those of low molecular weight, are more likely to diffuse than high molecular weight antigens.

A typical example is the diffusion of thyroglobulin from thyroid follicular epithelium and colloid into surrounding stromal tissue. Similarly, specific background may result when the tissue marker is also present in high concentrations in blood plasma, and has diffused in the tissue prior to fixation. This effect can be seen when tonsil tissue is stained for immunoglobulin heavy and light chains (Figure 15.5), particularly when fixation was not performed promptly, and when antisera were not diluted sufficiently. Ingestion of target antigens by phagocytes may also produce specific background staining, resulting in staining patterns not normally seen in such cells.

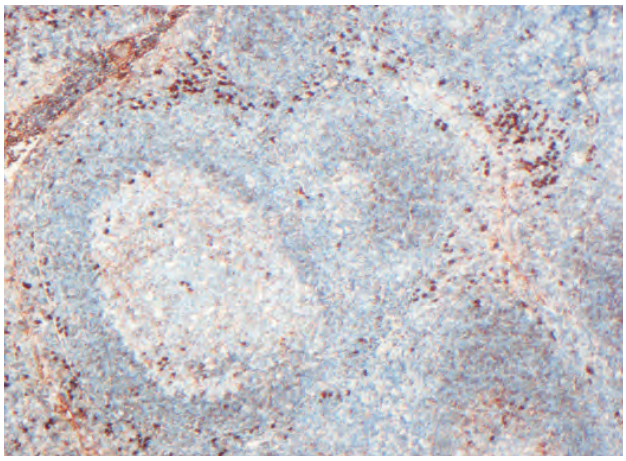


Figure 15.5 Undesirable staining of plasma proteins with anti-kappa light chains. Plasma cells stain specifically.

Natural and Contaminating Antibodies in Polyclonal Antibodies (Antisera)

Natural Antibodies

Low-level natural antibodies present in a polyclonal antibody or antiserum, as a result of prior environmental antigenic stimulation, may increase in titer during immunization with use of adjuvants. As a consequence, they can give rise to non-specific staining. In 1979, Osborn et al (8) reported that sera from non-immunized rabbits and goats, but not from guinea pigs, contained environmental antibodies to keratins. This finding

PETITIONER EXHIBIT 1012
Page 175 of 218

may be an example of 'specific epithelial background' staining caused by natural antibodies. Although also observed by others, attempts to isolate or remove these antibodies from the antiserum were not successful (9).

Most natural antibodies are of the non-precipitating type and occur only in relatively low concentrations. Binding of these antibodies usually is not detected on tissue if the antiserum is used at a sufficiently high dilution, or with the incubation periods.

Contaminating Antibodies

Isolated antigens used for immunization are rarely pure. If a host's immune system reacts to the impurities, antibody reagents containing immunoglobulins of unwanted specificity (contaminating antibodies) will result. Usually these contaminating antibodies are present in low concentration and will not detract from the functional immunohistochemical specificity of high-titer antisera, provided they are diluted sufficiently.

Contaminating antibodies may be related to infectious agents; other animal species kept in the same facilities, or carrier proteins used for immunization. These antibodies may be of special concern when dealing with antisera against synthetic peptide. Small peptides are not antigenic, and must therefore be coupled to carrier proteins prior immunization. The antisera produced will therefore contain antibodies against the carrier protein and the peptide.

However, if contaminating antibodies potentially may interfere with specificity, affinity absorption of the antiserum is usually performed. 'Batch-absorbed' antisera almost always contain residual levels of contaminating antibodies (mostly of the non-precipitating type), and will cause nonspecific staining of tissue if used at excessively high concentration (9).

Monitoring and evaluating the results of absorption by use of such techniques as immunodiffusion, immunoelectrophoresis and rocket immunoelectrophoresis can only be used to determine non-specificity. This monitoring cannot establish the specificity of an antiserum in a tissue section environment, where a multiplicity of antigens are present. Ultimate mono-specificity must be demonstrated by use of the designated technique, and by extensive use of tissues.

Problems stemming from natural and contaminating antibodies, of course, do not occur with monoclonal antibodies produced in tissue culture, but may be present in monoclonal antibodies prepared from ascites fluid.

Cross-Reactivity

Background staining due to antibody cross-reactivity may result when target tissue antigen epitopes are shared with other proteins. A typical example is the use of unabsorbed antiserum to carcinoembryonic antigen (CEA). Because CEA shares epitopes with some normal tissue proteins and blood group antigens, non-specific staining may result. Careful absorption of such antisera, or in the case of monoclonal antibodies careful screening of clones, will eliminate this type of background staining.

Non-specific antibody cross-reactivity with similar or dissimilar epitopes on different antigens may also be the cause of confusing background staining. This effect is rare however, and can be avoided by using antibodies from hyper-immunized animals, or carefully selected clones.

Cross-reactivity of antigens from related species is a common problem in multi-staining methods. This difficulty can often be avoided by using affinity-purified antibodies, subtype specific antibodies or site/region specific antibodies (see also Appendix A).

Fc Receptors

Fc receptors (FcR) are a family of detergent-soluble membrane glycoproteins with approximate molecular weights of 50-70 kDa. They comprise less than one percent of the total membrane proteins and are most frequently present on macrophages and granulocytes. They have also been reported on B cells and some T cells. The intrinsic affinity of the FcR for monomeric IgG is approximately $1x10^6$ to $1x10^8$ M^{-1}, but is higher for polymers and immune complexes of IgG. There is considerable class/subclass and species specificity among different FcR's. For example, the FcR on some human cells was found to bind mouse monoclonal IgG_{2a} and IgG_3, but not other IgG subclasses (10). Goat sera do not react with FcR's of human leucocytes (11).

Background staining due to FcR is more common in frozen sections, smears, and in lightly fixed tissues, than in tissues fixed by harsher procedures. It can be avoided by use of

PETITIONER EXHIBIT 1012
Page 176 of 218

F(ab')2 fragments instead of whole IgG molecules, and by careful screening of monoclonal antibodies.

Hydrophobic Interaction

In aqueous media, hydrophobic interactions between macromolecules occur when surface tensions are lower than that of water (called van der Waals forces). These interactions can be interatomic as well as intermolecular, and originate through the fluctuating dipolar structure within these macromolecules.

Hydrophobicity is a property shared to varying degrees by most proteins and is imparted primarily through the side chains of neutral aromatic amino acids phenylalanine, tyrosine and tryptophan. By their lower attraction for water molecules, these amino acids tend to link to one another, thus expelling water from the molecule. While hydrophobicity is one of the natural forces that confer stability on the tertiary structure of peptides, it also imparts stability to formed immune complexes, and depending on environmental factors, can exist also between different protein molecules.

Tissue Proteins

In tissue, proteins are rendered more hydrophobic by fixation with aldehyde-containing reagents, such as formalin and glutaraldehyde. Increased hydrophobicity often results from cross-linking reactive epsilon- and alpha-amino acids within and between adjacent tissue proteins. The extent of this hydrophobic cross-linking during fixation is primarily a function of time, temperature and pH. Changes in these factors will likely result in variable hydrophobicity due to variable cross-linking of tissue proteins. Therefore, once optimized, fixation procedures must be maintained and controlled. Tissues that commonly have the most background staining as a result of hydrophobic, as well as ionic, interactions are connective tissue: collagen laminin, elastin, proteoglycans and others, squamous epithelium (keratins), and adipocytes (due to lipids, if incompletely removed during processing with xylene). Excessive background staining due to overfixation with formalin may sometimes be remedied by postfixation with Bouin's, Zenker's or B5 fixative (12), but at a potential cost of decreased antigenicity, and poor performance of antigen retrieval methods.

Antibodies

Of the major serum proteins, immunoglobulins unfortunately are particularly hydrophobic. In general, mouse antibodies of subclass IgG_1 and IgG_3 are more hydrophobic than those belonging to subclasses IgG_2 and IgG_4. Furthermore, some isolation procedures for IgG class antibodies promote the formation of aggregates, thereby further increasing their hydrophobicity. Storage of immunoglobulins (antibody reagents) may also increase their hydrophobicity and lead to aggregation and polymerization. This effect frequently leads to a diminution in, or loss of, immune reactivity. Attendant increase in non-specific background staining by use of a polyclonal IgG fraction, when compared to that obtained by use of the original whole antiserum, has been observed (13).

The diluent buffer's formulation can also influence hydrophobic binding between monoclonal IgG and tissue proteins: the greater the proximity of diluent pH and the isoelectric point (pI) of antibodies, the stronger hydrophobic interaction will be. The lower the ionic strength of the diluent, the weaker will be the strength of hydrophobic attraction. The following anions and cations are arranged in order of their diminishing effect on hydrophobicity:
Anions: PO_4^{3-}, SO_4^{2-}, Cl^-, NO^{3-}, SCN^-
Cations: NH_4^+, K^+, Na^+, Ca^{2+}

Other possible methods to reduce hydrophobic interactions between tissue and reagent proteins include adding detergent, for example Tween 20, or ethylene glycol to the diluent, or raising the pH of the diluent used for polyclonal antibodies only.

The most widely practiced measure to reduce background due to hydrophobic interaction is to use a protein blocking solution, either in a separate step, or by adding it to the antibody diluent. However this strategy will only be successful if the blocking protein is a type that can compete effectively with IgG or its aggregates or conjugates, for hydrophobic binding sites. Separate incubation with a solution containing blocking protein is best carried out immediately prior to application of the primary antibody. The solution should contain proteins identical to those present in the secondary link or labeled antibody, but not to those in the primary antibody, in order to prevent non-specific binding of the secondary antibody.

The addition to the primary antibody diluent of one percent bovine serum albumin (BSA) is probably the most widely practiced step for reducing non-specific binding due to hydrophobic inter-

PETITIONER EXHIBIT 1012
Page 177 of 218

action. Use of non-fat dry milk (14) or of casein (15) for reducing background staining is also recommended. Casein, when used as a blocking agent, an antibody diluent and in the wash buffer, was found to result in significantly less background staining compared with normal swine and sheep sera (15).

Because of the different uses of biotinylated antibodies today, it should be of interest to note that biotinylation can change the pI of the antibody in excess of three units, for example from a pI of 8 for the antibody to less than pI 5 for the conjugate (16). This change may have a marked effect on the solubility of these conjugates, possibly due to increased hydrophobicity.

Ionic and Electrostatic Interactions

Ionic interactions are among the prime forces that control immunochemical interaction between antigens and their corresponding antibodies. They may, however, also contribute to non-specific background.

The pI of the majority of polyclonal IgG ranges from approximately 5.8 to 8.5. At physiological pH and at the pH commonly used for diluents, antibodies can have either net negative or positive surface charges. Ionic interaction of some antibodies with tissue proteins can be expected if the latter possess opposite net surface charges. Negatively charged sites on endothelia and collagen fibers have been reported to interact with cationic conjugates composed of rabbit Fab fragments and horseradish peroxidase type VI (pI 10.0) (17). In general, interactions of the ionic type can be reduced by use of diluent buffers with higher ionic strength. Addition of NaCl to the diluent buffer can reduce background staining stemming from ionic interactions, but its routine use in diluents for monoclonal antibodies is not recommended (18).

Unfortunately most diffuse background staining results from a combination of ionic and hydrophobic interactions. Remedies for one type of interaction may aggravate the other.

Complement-Mediated Binding

Complement-mediated binding may occasionally be a cause of background in frozen tissue when whole antisera are used. However by the time large pools of antisera have been prepared for use, several of the complement factors are usually inactivated.

Chapter 15.5 | Miscellaneous Sources

Physical injury to tissue, drying out prior to fixation, or incomplete penetration of fixative, may cause diffuse staining of all or most tissue elements within an affected area. Similar diffuse background staining of both the section and the glass slide, usually limited to the area of antibody incubation, has been observed and may be due to residual embedding medium. Sections mounted routinely in water baths containing protein additives, such as Knox gelatin or Elmer's glue, may also show this type of diffuse background, especially in procedures of high staining sensitivity. Water baths should be free of bacterial or yeast contamination.

Non-specific staining due to undissolved chromogen granules may on occasion also be encountered.

Non-immunologic binding of horseradish peroxidase (either in free form or as a conjugate) to HbsAg in hepatocytes was reported by Omata et al (19). The precise nature of this binding is not known.

Necrotic areas of tissue may stain with all reagents. Nadji and Morales (20) provide an excellent collection of color plates illustrating background staining, with accompanying explanations.

Excessive counterstaining may compromise visualization of the specific staining signal.

Sub-optimal operation and maintenance of automated platforms can compromise the effectiveness of the wash between reagent incubations, or of the reagent-handling components, e.g. the dispensing probe. It can potentially lead to unwanted non-specific staining reaction, if two reagents react at the wrong step in the IHC staining reaction due to inefficient washing of the slide, or the reagent-handling probe. It is, therefore, important to adhere to the recommended service schedule and maintenance checks for any automated platform performing IHC staining.

Chapter 15.6 | General Aspects

While it is clear that background staining can be caused by the factors outlined above, it is also important to work with

PETITIONER EXHIBIT 1012
Page 178 of 218

well-characterized reagents and established protocols, in order to avoid background, or the necessity to troubleshoot background staining. Many vendors of IHC reagent offer "system solutions," which are IHC product lines with carefully optimized buffers, target retrieval reagents, primary antibodies, detection reagents and substrates to be run on an automated platform (the RTU approach – Chapters 4 and 5). These products are designed to provide users with consistent, optimal staining. Several countries have established national quality programs, such as United Kingdom National External Quality

Assessment Service (UK NEQAS) (21), Nordic Immunohistochemical Quality Control (NordiQC) (22), the Canadian Immunohistochemistry Quality Control (CIQC) (23) and the College of American Pathologists (CAP) (24). These organizations, formed in response to the need for raised awareness of quality control and best practices in IHC laboratories, have fostered higher performance in many laboratories, including reduction of background staining. See Chapter 14 for further discussion. Information on individual national programs can be found on each program's Web site.

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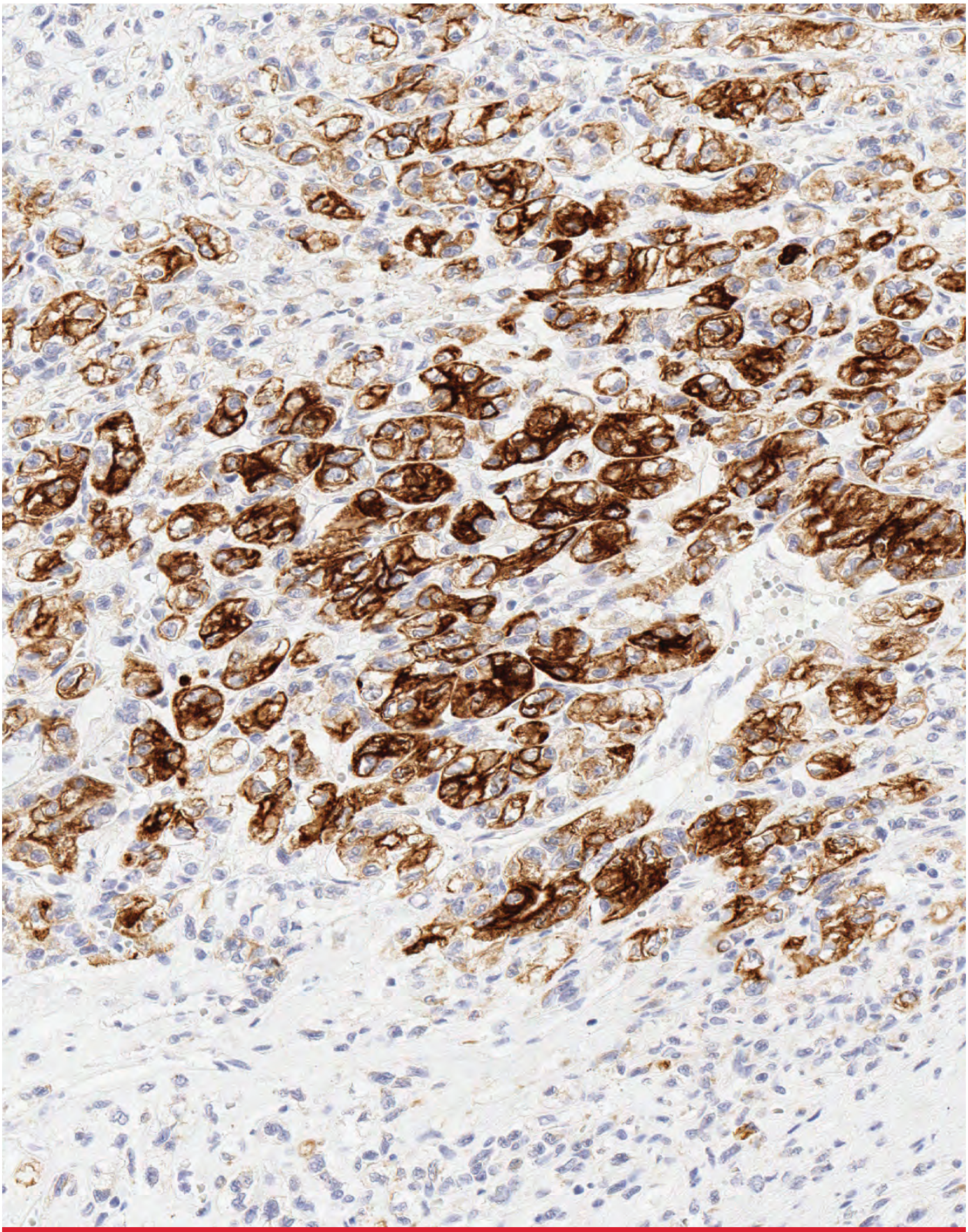
PETITIONER EXHIBIT 1012
Page 179 of 218



Chapter 16

Troubleshooting

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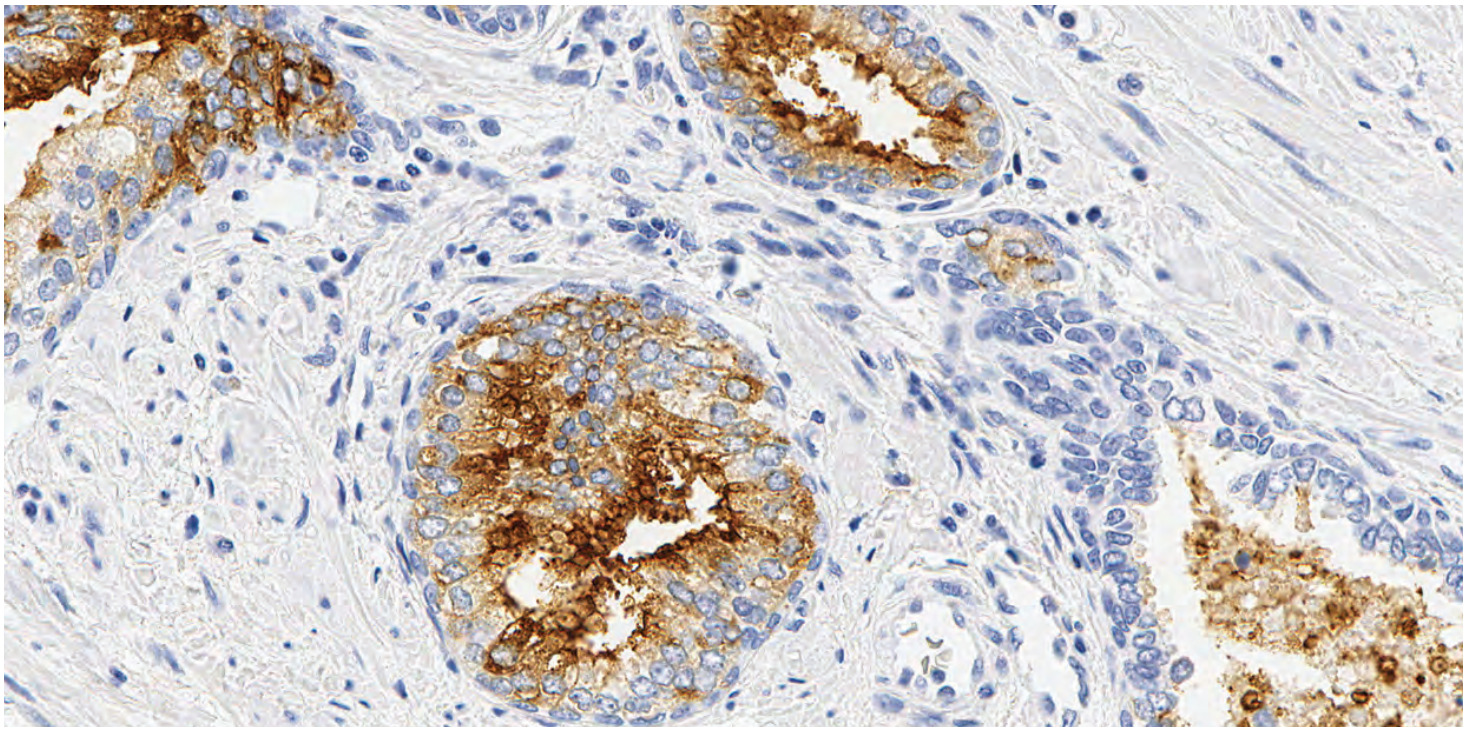

Trou•ble•shoot•ing *(n.)*
Discovering why something does not work effectively and making suggestions about how to improve it.

Cambridge Advanced Learner's Dictionary

Chapter 16.1 | Introduction

Immunohistochemistry (IHC) is a multi-step process that requires specialized training in the processing of tissue, the selection of appropriate reagents and interpretation of the stained tissue sections. In general, IHC staining techniques allow for the visualization of antigens by sequential application of a specific antibody to the antigen, a secondary antibody to the primary antibody, an enzyme complex and a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. Because of its highly complex nature, the causes of unexpected negative reactions, undesired specific staining, or undesired background may be difficult to isolate. The information contained in this chapter should enable the user rapidly to pinpoint and resolve problems encountered during the staining procedure.

Section 1 is a compilation of common problems encountered when using IHC staining reagents, including the underlying causes of staining failure and the recommended corrective actions. The chart is divided into sections describing inadequate staining, general background staining, and limited background staining.

Section 2 presents a method of systematically adding one IHC reagent at a time to determine at which stage in a staining protocol non-specific or undesired staining may be occurring.

Section 3 is a simple chart used to define the type of tissue specimen, the IHC reagents, and the staining protocol already in use by the laboratory personnel. The user is encouraged to copy this chart and use it to help troubleshoot any problems that may be encountered in the IHC staining process.

Section 4 is a guide to reading a manufacturers' specification sheet for IVD (in vitro diagnostic) antibodies. The guide includes general information for use in immunohistochemistry, including fixation, recommended visualization systems, recommended titer and diluent, pre-treatment methods, and selection of required controls.

Section 5 is a guide to check that the automated platform used to perform the staining has operated correctly during the staining process.

Section 1 | Common Problems

Inadequate Staining (little or no specific staining)

Possible cause of poor staining	Solution
A) Both the positive controls and the specimen tissue show little or no specific staining, except for counterstain. The tissue section may show little or no background staining.	
Primary antibody or labeled reagent omitted. Reagents used in wrong order.	Repeat the procedure using the manufacturer's staining system specification sheet, or the standard operating procedure reagent checklist as established by the individual laboratory.
Excessively diluted or excessively concentrated reagents; inappropriate incubation time and or temperature.	Determine correct concentration for each reagent (see Chapter 4 and Chapter 5). Depending on the degree of staining obtained, if any, a 2- to 5- fold increase in concentration may be needed. Incubation temperature and incubation time are inversely proportional and will affect results. To determine optimal incubation protocol, vary either the time or temperature for each reagent in the IHC staining system. Generally, incubation times can be extended if little or no background is detected. Overnight incubation at higher dilution may also be effective.
Primary antibody diluted with inappropriate buffer. Use of PBS or TBS as an antibody diluent. Lack of stabilizing or carrier protein. Detergent in diluent.	Check formula and compatibility of antibody diluent. A change of ion content and/or pH of the antibody diluent can cause a diminution in the sensitivity of the antibody. Addition of NaN_3 should be avoided. This problem is primarily seen with monoclonal antibodies.

PETITIONER EXHIBIT 1012
Page 182 of 218

Possible cause of poor staining	Solution
Primary antibody defective; one or several secondary or ancillary reagents defective. Do NOT use product after expiration date stamped on vial.	Replace defective or expired antibody; repeat staining protocol, replacing one reagent at a time with fresh, in-date reagents. ■ Store products according to each product specification sheet or package insert ■ If using a neat or concentrated antibody, and directed by the manufacturer to store frozen, the reagent may be aliquoted to avoid repeated freezing and thawing ■ Do not freeze ready-to-use (RTU) or customer diluted products ■ Follow manufacturer recommendations on product specification sheets, package inserts, and reagent labels
Dissociation of primary antibody during washing or incubation with link antibodies.	Particularly a feature of low affinity antibodies: ■ Polyclonal primary antiserum: Attempt staining at lower dilutions (higher concentrations) ■ Monoclonal primary antibody: Replace with higher affinity antibody of identical specificity ■ Re-optimize incubation times for washing buffer and link antibody
Use of alcohol-based counterstain and/or alcohol-based mounting media will remove aqueous–based chromogens.	■ Repeat staining, using water-based counterstain and mounting media ■ Use a permanent chromogen, such as DAB/DAB+, that is not affected by organic solvents
Excessive counterstaining may compromise proper interpretation of results.	Use a counterstain that: ■ Will not excessively stain tissue sections ■ Can be diluted so as not to obliterate the specific signal ■ Reduce incubation time of the counterstain
Incorrect preparation of substrate-chromogen mixture.	■ Repeat substrate-chromogen treatment with correctly prepared reagent ■ Staining intensity may be decreased when excess DAB/DAB+ is present in the working reagent
Incompatible buffer used for preparation of enzyme and substrate-chromogen reagents: Use of PBS wash buffer with an alkaline phosphatase staining system. Sodium azide in reagent diluent or buffer baths for immunoperoxidase methodologies.	Check compatibility of buffer ingredients with enzyme and substrate-chromogen reagents. Repeat staining: ■ Commercial phosphate buffers may contain additives that will inhibit alkaline phosphates activity ■ Avoid sodium azide in diluents and buffers. A concentration of 15 mM/L sodium azide, which is routinely added to IHC reagents to inhibit bacterial growth, will not impair HRP conjugated labels
Antigen levels are too low for detection by the employed visualization system. May be due to loss of antigenic differentiation in some tumors or loss of antigenicity due to sub-optimal tissue fixation.	■ Utilize a higher sensitivity staining system ■ Prolong incubation time of primary antibody ■ Re-optimize incubation times and concentrations of ancillary reagents ■ Perform antigen retrieval, if applicable, using a range of pH buffers (see Chapter 3)
Steric hindrance due to high antigen level and possible prozone effect.	Re-optimize concentration of the primary antibody and ancillary reagents. Antibody concentration of the primary antibody may be too high.
Use of inappropriate fixative. Use of certain fixatives may damage or destroy antigens or epitopes in the tissue specimen. Use of non-cross linking fixatives may allow the elution of antigens soluble in IHC reagents. Different fixatives may affect standardization of cells.	Check manufacturer's specifications regarding recommended fixatives known to be effective with antibody and protocol in use.
Immunoreactivity diminished or destroyed during embedding process.	Use a paraffin wax with a melting temperature ~55-58 °C. Wax used for embedding should not exceed 60 °C.
Immunoreactivity diminished or destroyed during dewaxing at high oven temperature.	Oven temperature not to exceed 60 °C. NOTE: The intensity of immunostaining may be diminished when tissue is exposed to prolonged heat at this stage in the protocol. Paradoxically heating of the section in aqueous solution is used to recover antigenicity in the AR process (See Chapter 3). Refer to the primary antibody specification sheet for additional information.
Immunoreactivity diminished or destroyed on pre-cut tissue sections.	The intensity of immunostaining may be diminished when pre-cut tissue sections are exposed to air. Use freshly cut sections and re-seal paraffin-embedded blocks.
Immunoreactivity diminished or destroyed by the enzyme blocking reagent altering a specific epitope.	More common on frozen sections: apply the primary antibody prior to the enzymatic block to ensure its reaction. In such cases the blocking reagent can be applied at any point after the primary and before the enzyme-labeled components.

PETITIONER EXHIBIT 1012
Page 183 of 218

Possible cause of poor staining	Solution
Excessive wash buffer or blocking serum remaining on tissue section prior to application of IHC reagents.	Excess residual reagent will dilute the next consecutive reagent. Repeat staining, making sure to wipe away excess washing buffer and blocking serum.
Antigen retrieval protocol is inappropriate or has been omitted.	Many tissue antigens require proteolytic enzyme digestion or heat-induced antigen retrieval performed prior to staining (Chapter 3). The need for pre-treatment depends on the type and extent of fixation, specific characteristics of the antigen and the type of antibody used. Use the pretreatment method recommended by the manufacturer. No single pre-treatment is suitable for all applications.
Repeated reuse of antigen retrieval buffer.	Do not reuse buffer.
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol, using fresh xylene or xylene substitute.
Failure to achieve the optimal temperature required for heat induced antigen retrieval.	■ When using a waterbath or steamer, allow sufficient time for the retrieval buffer to equilibrate to a temperature range of 95-99 °C ■ At high altitude (greater than ~4,500 feet), the buffer will boil at less than 95 °C ■ Utilize a closed heating system such as a pressure cooker, autoclave or Pascal, or utilize a low temperature protocol if standardization of the validated procedure is not affected
Excessive or incomplete counterstaining.	Re-optimize concentration of counterstain and incubation time.
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specifications.

B) Positive control tissue shows adequate specific staining with little or no background staining. Specimen tissue shows little or no specific staining with variable background staining.

Specimen held for too long in a cross-linking fixative, usually in formalin, causing "masking" of antigenic determinants. Control appropriately fixed.	Standardize routine fixation, matching test specimens to control tissues. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive (Chapter 3). Refer to the primary antibody specification sheet for additional information.
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor.	Serum proteins diffuse through tissue and are fixed in place. Cut new tissue block if available, using sharp blade.
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.
Uneven fixation of section; portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration. Unfixed tissue tends to bind all reagents non-specifically.

General Background

Background is seen in both control tissue and specimen tissue. Background staining may affect several tissue elements, such as connective tissue, adipose tissue and epithelium.

Excessive incubation with substrate-chromogen reagent.	Reduce incubation time.
Substrate-chromogen reagent prepared incorrectly.	Repeat incubation with correctly prepared chromogen reagent.
Secondary or link antibody cross-reacts with antigens from tissue specimen.	Absorb link antibody with tissue protein extract or species-specific normal serum from tissue donor.
Secondary or link antibody and/or tertiary reagents too concentrated.	Repeat staining. Determine correct concentration for each reagent. Incubation temperature and incubation time will affect results. To determine optimal incubation protocol, vary both the time and temperature for each reagent in the IHC staining protocol.
Substrate-chromogen reagent prepared incorrectly.	Repeat incubation with correctly prepared chromogen reagent.

PETITIONER EXHIBIT 1012
Page 184 of 218

Possible cause of poor staining	Solution
Slides inadequately rinsed.	Gently rinse slide with wash buffer bottle and place in wash bath for 5 minutes. Gentle agitation of the wash bath may increase effectiveness when used with cytoplasmic or nuclear staining protocols.
Insufficient saline or detergent in wash buffer.	High-sensitivity staining systems may require higher concentrations of saline or detergent in the wash buffer. Refer to the staining system specification sheet for optimal formulation.
Blocking serum or wrong blocking serum used.	Block with serum from the host of the secondary or link antibody. Avoid serum that contains auto-immune immunoglobulins. Alternatively, a serum-free protein block, lacking immunoglobulins, may be substituted for the serum block.
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol using fresh xylene or xylene substitute.
Non-specific binding of the secondary antibody with an animal tissue specimen.	Use a secondary antibody that has been absorbed against a species specimen, or use a secondary antibody produced in a host that exhibits little or no cross-reactivity with the tissue source.
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.

Specimen tissue and negative reagent control slides show background staining. Positive and negative control tissue show appropriate specific staining. May involve several tissue elements such as connective tissue, adipose tissue and epithelium.

Specimen held for too long in a cross-linking fixative, usually in formalin, causing 'masking' of antigenic determinants due to aldehydes cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.
Sectioned portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue. Unfixed tissue tends to bind all reagents nonspecifically.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration.
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Serum proteins diffuse through tissue and are fixed in place. Re-cut tissue using sharp blade.
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.
Excessive or unevenly applied subbing agent on poly-L-lysine, charged, or silanized slides.	Some IHC reagents may bind to these products, resulting in a light stain over the entire slide surface. Some slides may be unevenly coated, and will exhibit the above problems on only a portion of the tissue or glass.
Antigen diffusion prior to fixation causing specific background outside the expected antigen site.	Avoid delays in fixation of the tissue.
Tissue sections too thick.	Cut tissue sections thinner. Formalin-fixed paraffin-embedded tissue sections should be approximately 4-6 µm; cryostat section 4-6 µm or less.
Incomplete permeabilization of tissue sections.	Seen in frozen sections, cell smears and non-paraffin embedded tissue: incomplete permeabilization of cells allows unattached reagents to become trapped within the cells and resistant to removal by wash buffer.

PETITIONER EXHIBIT 1012
Page 185 of 218

Possible cause of poor staining	Solution
Negative reagent control slide shows background. Positive control tissue, negative control tissue and specimen tissue show expected specific staining.	
Negative control serum insufficiently diluted.	Use properly diluted negative reagent control serum: ■ For polyclonal antibodies, dilute the negative reagent control serum until the protein concentration is equal to that of the primary antibody ■ For monoclonal antibodies, dilute the negative reagent control serum until the Ig concentration is equal to that of the primary antibody
Contaminating antibodies in the negative control serum are cross-reacting with proteins from the specimen tissue.	Replace the negative reagent control serum; repeat staining protocol.
Negative reagent control serum contaminated with bacterial or fungal growth.	Replace product with non-contaminated serum.

Limited Background

Areas of inconsistent staining on controls, specimens and glass slides.	
Protein trapped beneath the tissue during the mounting process will allow partial lifting of the section. Pooling of IHC reagents beneath the section, or partial detachment of the tissue from the slide may occur.	Avoid the use of commercial adhesives, glue starch or gelatin in water baths when mounting tissue sections. Avoid allowing water from an initial section mounting to flow over an area where additional sections will be mounted. This is particularly important when using charged or silanized slides.
Undissolved granules of chromogen.	Ensure that chromogen in tablet or powder form is completely dissolved, or switch to a liquid chromogen.
Incomplete dezenkerization of tissue fixed with B5 or mercury containing reagents.	Remove embedding medium thoroughly, using fresh reagents.
Incomplete dezenkerization of tissue fixed with B5 or mercury containing reagents.	Perform dezenkerization with fresh reagents.
Bacterial or yeast contamination from mounting waterbath.	Clean and refill waterbath.
Partial drying of tissue prior to fixation. Unaffected areas show normal staining.	■ Immerse tissue promptly in fixative or holding reagent ■ Keep moist during the entire staining process ■ Use a humidity or moist chamber during incubation steps ■ When using an automated staining instrument, addition of wet towels to the sink may prevent drying of slides
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.
Adipose or connective tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Background in connective and epithelial tissue.	
Hydrophobic and ionic interactions between immunoglobulins and lipoid substances in fatty tissue.	Non-specific staining of fatty tissue rarely interferes with interpretation of specific staining and can usually be disregarded.
Primary antibody and negative reagent control serum are insufficiently diluted.	Reoptimize the dilution of the primary antibody and negative control serum.
Epithelial tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Staining is moderate to marked, especially in epidermal epithelium. Background in epithelia accompanies background in connective tissue.	
Both the primary antibody and negative control serum contain contaminating antibodies to epithelial elements, possibly cytokeratins.	■ Use a higher dilution of the primary antibody and negative control serum ■ Increase the incubation time ■ Replace the antibody
Excessive formalin fixation of tissues may increase protein cross-linking, resulting in tissue hydrophobicity.	Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody and/or the negative reagent control specification sheet for appropriate pre-treatment.

PETITIONER EXHIBIT 1012
Page 186 of 218

Possible cause of poor staining	Solution
Focal cytoplasmic staining observed in epithelium in the specimen tissue.	
Focal cytoplasmic staining is seen, particularly in intermediate and superficial layers of the epidermis. May be caused by passive absorption of plasma proteins into degenerating epidermal cells.	This observation is rare and should not interfere with interpretation of specific staining.
Background seen in all control and specimen tissue when using an immunoperoxidase staining system.	
Unquenched endogenous peroxidase activity may be seen in all hemoprotein-containing specimens, including hemoglobin in erythrocytes, myoglobin in muscle cells, cytochrome in granulocytes and monocytes and catalases in liver and kidney.	■ Use alternate or prolonged peroxidase blocks or use another enzyme label such as alkaline phosphatase ■ Eosinophils and mast cells are particularly resistant to peroxidase quenching. Use a peroxidase blocker ■ Use special stains: eosin will stain eosinophils a bright red-orange
Background seen in all control and specimen tissue when using an alkaline phosphatase staining system.	
Unquenched endogenous alkaline phosphatase activity may be seen in leucocytes, kidney, liver, bone, ovary bladder, salivary glands, placenta and gastro-intestinal tissue.	Add levamisole to the alkaline phosphatase chromogen reagent or use another enzyme label such as horseradish peroxidase. Intestinal alkaline phosphatase is not quenched by the addition of levamisole. Pretreat the tissue with 0.03 N HCl.
Background seen in all control and specimen tissue when using a biotin-streptavidin staining system.	
Endogenous protein-bound biotin (water-soluble B vitamin). High amounts of biotin are found in adrenal, liver, and kidney. Lesser amounts are found in the GI tract, lung, spleen, pancreas, brain, mammary gland, adipose tissue, lymphoid tissue, and cells grown in culture media containing biotin as a nutrient.	Use a biotin block or chose another non-biotin based staining system.
Background of skeletal or smooth muscle tissue in positive control tissue, negative control tissue, specimen tissue and negative reagent control.	
Cause is not understood. It is possibly due to antibodies to muscle antigens in primary and negative reagent control serum.	Should not interfere with interpretation of specific staining.
Undesired 'Specific' Staining. Positive staining of leucocyte membranes in specimen tissue, positive control, negative tissue control and negative reagent control.	
Binding of the Fc portion of Ig by Fc receptors on the cell membrane of macrophages, monocytes, granulocytes and some lymphocytes.	■ Use $F(ab')_2$ or F(ab) fragments for the primary and secondary antibodies rather than intact antibodies ■ Add detergent to the wash buffer
Positive staining of histiocytes and granulocytes in the specimen tissue only, with a marker not normally reactive with these cells.	
Phagocytosis of antigens may render phagocytes positive for the same.	Rare. Should not interfere with interpretation of specific staining.
Positive membrane staining of specimen tissue and negative reagent control tissue when using a horseradish peroxidase staining system.	
Tissue from persons infected with Hepatitis B virus and expressing Hepatitis B surface antigen may exhibit undesired staining.	Utilize a non-peroxidase staining system.

Miscellaneous

Loss of viability of cell cultures.	
Some manufacturers produce antibodies and reagents for in vitro use only. These products may contain preservatives, usually sodium azide, which is a known poison.	Utilize an in vivo product for application on viable cells. For use on cell cultures only: sodium azide may be dialyzed out of some reagents. Contact Dako Technical Support for additional information.

PETITIONER EXHIBIT 1012
Page 187 of 218

Section 2 | Systematical Approach Using a Troubleshooting Flow Chart

This flow chart can be used to determine source(s) of non-specific staining that has been encountered when using an IHC staining protocol. Each step (Slide box to the left) is a suggestion for reagents to be tested on the indicated tissue type with known positive or negative expression pattern. In the first step (Slide #1) the tissue is only counterstained with hematoxylin. If the described result (to the right) does not match the observed staining pattern when using the suggested setup, proceed to the next step in the flow chart. In the next step (Slide #2), a chromogen is added to the staining protocol, before counterstaining and so forth.

Background Staining Encountered with Peroxidase Reagents

Reagents	Result/Action
Slide 1 **Positive Control Tissue:** Counterstain with hematoxylin If Result/Action does not match the observed staining: Go to next step	Brown endogenous pigment (such as melanin) observed: ■ To distinguish melanin pigment from DAB chromogen, Azure B can be used as a counterstain. The melanin stains blue-green, while the DAB remains brown. ■ An alternate method is to use AEC as the chromogen. However, if high levels of pigment exist in the tissue, the red chromogen may be partially obscured. Since bleaching protocols to remove melanin may compromise tissue antigenicity, it should be avoided if at all possible.
Slide 2 **Positive Control Tissue:** DAB/AEC chromogen and counterstain If Result/Action does not match the observed staining: Go to next step	Brown/red color observed: ■ Indicates endogenous peroxidase activity in the tissue sections. It is present in all hemoprotein containing tissue including erythrocytes, muscle, liver, kidney, granulocytes and monocytes. ■ Block with 3% hydrogen peroxide or other peroxidase blocking reagent. Using a new bottle of hydrogen peroxide, perform a 3% H_20_2 peroxidase block, followed by DAB and an appropriate counterstain.
Slide 3 **Positive Control Tissue:** Peroxidase Block + Secondary Antibody + Streptavidin-HRP DAB/AEC Counterstain If Result/Action does not match the observed staining: Go to next step	Brown/red color observed: ■ Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cell grown in culture media containing biotin (RPMI, NCTC, MEME). ■ Block with a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction

PETITIONER EXHIBIT 1012
Page 188 of 218

Reagents	Result/Action
Slide 4 **Positive Control Tissue:** Peroxidase Block + Biotin Block (if required) + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/red color observed: ■ Indicates non-specific or undesired binding of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. ■ To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10 µL of normal serum (from the species of tissue to be stained) per 100 µL of the secondary antibody
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 5 **Positive Control Tissue:** Peroxidase Block + Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-HRP + DAB/AEC	Brown/red color observed: ■ May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody. Add 0.05-0.1% Tween 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 6 **Negative Control Tissue:** Perform complete staining protocol	Brown/red color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections

Background Staining Encountered with Alkaline Phosphatase

Slide 1 **Positive Control Tissue:** Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/blue color observed: ■ Indicates endogenous alkaline phosphatase activity in the tissue sections. It is present in liver, kidney, GI tract, bone, bladder, ovary, salivary gland, placenta, leukemic, necrotic or degenerated cells. ■ Block with levamisole (Intestinal alkaline phosphatase may be quenched by the addition of 0.03 N HCl prior to the addition of the alkaline phosphatase)
If Result/Action does not match the observed staining: Go to next step ▼	

PETITIONER EXHIBIT 1012
Page 189 of 218

Reagents	Result/Action
Slide2 **Positive Control Tissue:** Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: ■ Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cells grown in culture media containing biotin (RPMI, NCTC, MEME). ■ Block with a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 3 **Positive Control Tissue:** Biotin Block (if required) + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/blue color observed: ■ Indicates non-specific or undesired binding of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. ■ To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10 µL of normal serum (from the species of tissue to be stained) per 100 µL of the secondary antibody
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 4 **Positive Control Tissue:** Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain.	Red/blue color observed: ■ May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or a protein block; add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue or as specific staining in pancreatic sections
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 5 **Negative Control Tissue:** Perform complete staining protocol	Red/blue color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections

PETITIONER EXHIBIT 1012
Page 190 of 218

Negative Reagent Control

Reagents

Negative Control Reagent:
Perform complete staining protocol.

Result/Action

- (Human tissue) Perform the peroxidase blocking protocol from Slide #2 under "Background Staining Encountered with Peroxidase Reagents"
- Perform a biotin block if required, protein block if required, apply the appropriate negative reagent control (see below), apply biotinylated secondary antibody, apply streptavidin/HRP reagent and DAB
- Prepare a negative reagent control
  - Polyclonal: non-immunized sera from the same species, diluted to the same protein concentration as the primary antibody
  - Monoclonal: negative reagent control that matches the isotype as the primary antibody. Additionally, the diluent used to manufacture a monoclonal primary antibody and isotypic negative control should contain the same ions. Diluents containing sodium or phosphate ions may change the sensitivity of some monoclonal antibodies.
  - Calculation:
    - Ig or total protein concentration of primary antibody divided by dilution factor of primary antibody = x
    - Ig or total protein concentration of negative reagent control divided by x = dilution factor of negative reagent control

PETITIONER EXHIBIT 1012
Page 191 of 218

Section 3 | Troubleshooting Chart

Tissue Specimen: Successful staining of tissue with an IHC marker is dependent on the type and preparation of the specimen. The chart below provides a convenient check list, as to [procedure at each step in the 'Total Test'.

Species: _____
(important to note in research studies).

Organ/tissue source: _____

Collection:

- ☐ Surgical specimen/biopsy
- ☐ Post-mortem specimen
- ☐ Fine needle aspirate
- ☐ Peripheral blood (include anti-coagulant)
- ☐ Brushing
- ☐ Biologic fluid
- ☐ Cell culture
- ☐ Other

Tissue preparation:

- ☐ Paraffin embedded
- ☐ Plastic embedded
- ☐ Cryostat section
- ☐ Cytospin
- ☐ Cell smear
- ☐ Mono-layer cultured cells
- ☐ Other

Tissue fixation:

- ☐ Type of fixative
- ☐ Total length of time in fixative, including during transport, grossing and on the tissue processor
- ☐ Size of specimen ; size of block; wheterh additional blocks are available if needed

Tissue mounting:

- ☐ Slide mount
- ☐ Tissue thickness
- ☐ Gelatin, glue commercial adhesive or starch in the water bath
- ☐ Other

Blocking of endogenous components that may produce spurious staining.

Background staining is defined as unexpected or undesirable staining seen on the test or control tissue, which does not represent the target antigen. Frequent causes of background staining are endogenous enzyme activity and endogenous biotin.

Peroxidase is an enzyme of the oxido-reductase class that reacts with a substrate containing hydrogen peroxide as the electron acceptor. To block this activity, a variety of hydrogen peroxide reagents can be applied to cells producing this enzyme.

Alkaline phosphatase is an enzyme having various isoforms, which are produced in the leukocytes, liver, bone, intestine, placenta and Regan (carcinoma). Addition of levamisole to the chromogen/substrate will inhibit endogenous alkaline phosphatase activity, with the exception of the intestinal isoform. If necessary, this can be blocked with a weak acid wash, such as 0.03-0.5 N HCl.

Biotin, a B vitamin, may be protein-bound to tissue and can interfere with proper interpretation of staining patterns when using a streptavidin or avidin reagent. To block this binding, a biotin/avidin block.

Peroxidase block:

- ☐ 3% H_2O_2
- ☐ Methanol/H_2O_2
- ☐ Sodium azide
- ☐ Peroxidase Block (e.g. Dako Code S2003)
- ☐ Other

Alkaline phosphatase block:

- ☐ Levamisole
- ☐ 0.03 N HCl (not for use on cryostat tissue)
- ☐ Other

Biotin block:

- ☐ Biotin Block (e.g. Dako Code X0590)
- ☐ Other (e.g. skimmed milk)

Protein block:

- ☐ Protein Block (e.g. Dako Code X0909)
- ☐ Normal sera or Ig from host species of the secondary antibody
- ☐ Other

PETITIONER EXHIBIT 1012
Page 192 of 218

Section 4 | Specification Sheets

Below is an example of the information supplied in a typical Dako package insert for an IVD (in vitro diagnostic) concentrated antibody. The information and placement in the package insert will vary.

Information You Need to Know	Information Located on the Specification Sheet/Package Insert*	Comments
Regulatory Status of the Primary Antibody	Intended use ■ For in vitro diagnostic use	Indicates that a product meets the FDA requirements as a clinical diagnostic product. Likewise, a CE icon indicates the reagent meets European Union requirements.
Tissue Preparation	**Specimen preparation** Paraffin sections: The antibody can be used for labeling formalin-fixed, paraffin-embedded tissue sections. Tissue specimens should be cut into sections of approximately 4 µm. Pre-treatment: Pre-treatment of formalin-fixed, paraffin-embedded tissue sections with heat-induced epitope retrieval (HIER) is required. Optimal results are obtained by pretreating tissues with HIER using diluted EnVision™ FLEX Target Retrieval Solution, High pH (50x) (Codes K8000/K8004). Deparaffinization, rehydration and epitope retrieval can be performed in Dako PT Link (Code PT100/PT101). For details, please refer to PT Link User Guide. The tissue sections should not dry out during the treatment or during the following immunohistochemical staining procedure. For greater adherence of tissue sections to glass slides, the use of FLEX IHC Microscope Slides (Code K8020) is recommended. After staining the sections must be dehydrated, cleared and mounted using permanent mounting medium.	Indicates the type of specimen that was used during validation studies. In many cases this would include formalin-fixed tissue and frozen sections. Use of other fixatives requires validation by each individual laboratory. This section also indicates the optimal epitope retrieval procedure and warns against procedures that may destroy the epitope. Specimen preparation and staining procedure sections can and will change periodically, to reflect changes in technology. So remember to retain copies of each version of the reagent specification sheet. Version numbers are usually found on each page.
Choosing the Visualization System	**Staining procedure** Visualization: The recommended visualization system is EnVision™ FLEX, High pH (Code K8000/K8010) using a 20 minute incubation at room temperature. Follow the procedure enclosed with the selected visualization system(s). Automation: The antibody is well-suited for immunohistochemical staining using automated platforms, such as Dako Autostainer, Autostainer Plus and Autostainer Link as well as PT Link for pre-treatment.	Indicates the recommended visualization system to be used with the antibody. Conditions will differ if other detection systems are used. It also indicates that the antibody can be used for automated staining. NOTE: If your state regulatory agency requires written documentation that a reagent can be used for automated staining and this indication is not listed on the specification sheet, you may wish to contact the manufacturer's technical support group for further information.
Diluting the Primary Antibody	**Staining procedure** Dilution: The recommended dilution of Monoclonal Mouse Anti-Human PSMA, Clone 3E6, Code M3620, is 1:50. Dilute the antibody in Dako Antibody Diluent (Code S0809). Incubate pretreated tissue sections for 20 minutes at room temperature. These are guidelines only. Optimal conditions may vary depending on specimen and preparation method, and should be validated individually by each laboratory.	Includes a suggested dilution for the antibody and the recommended diluent. The dilution is a suggested starting point, but may require further optimization depending on specimen, preparation method, temperature of the laboratory or automated instrumentation.

* Examples included. The information given will vary depending on the individual antibodies. Always refer to the actual package insert for specific information

Information You Need to Know	Information Located on the Specification Sheet/Package Insert*	Comments
Controls	**Staining procedure** Controls: Positive and negative control tissues should be run simultaneously using the same protocol as the patient specimens. The positive control tissue should include prostate and the cells/structures should display reaction patterns as described for this tissue in the "Performance characteristics" section. Negative control: The recommended negative control reagent is Dako Negative Control, Mouse IgG1 (Code X0931), diluted to the same Ig concentration as the primary antibody. Unless the stability of the diluted antibody and negative control has been established in the actual staining procedure, dilute these reagents immediately prior to use. Positive and negative controls should be run simultaneously with patient specimens.	Use of a negative reagent control (ANP.22570) is no longer required by the College of American Pathologists (CAP), based on Clinical Laboratory Improvement Amendments (revised July 31 CLIA 2012), for each patient or patient block in a staining run when using polymer detection systems. The latest guidelines from CAP (5) leave it up to the individual laboratory to evaluate whether the selected detection method poses a negligible risk for non-specific staining that the negative reagent control for the polymer-based detection methods can be eliminated.
Positive Control Tissue	**Performance characteristics** Normal tissues: In prostate, glandular epithelial cells show a moderate to strong cytoplasmic and/or membranous staining reaction. Abnormal tissues: In 92/102 prostate adenocarcinoma, glandular epithelial cells showed a moderate to strong cytoplasmic and/or membranous staining reaction.	**CLIA 2003 Sec. 493.1273 (3)** Mandates that fluorescent and immunohistochemical stains must be checked for appropriate positive and negative reactivity each time they are used. Most IVD antibody specification sheet will list tissue that will exhibit positive and negative staining patterns in the Performance Characteristics section. NOTE: abnormal tissue will not necessarily be labeled. Both negative and positive tissue controls should be processed using the same fixation, embedding, mounting, drying, epitope retrieval and immunostaining protocols as the patient tissue.

Section 5 | Automated Platform Performance Checks

Information You Need to Know	Information Located at the Instrument	Comments
The right protocol has been applied to the slide	Right protocol ■ Find the location for the completed slides in the automation software and look up the slide ID of the slide in question and check that the applied protocol is the correct one	The information can be located in different places based on your automated solution. If the location is not known then contact your automated platform supplier

PETITIONER EXHIBIT 1012
Page 194 of 218

Information You Need to Know	Information Located at the Instrument	Comments
Target retrieval has been performed under the right conditions	**Target retrieval** Target retrieval solution: ■ Find the location of the specific data for the target retrieval procedure – this information can be a part of the slides' log file under completed slides ■ Check that the right target retrieval solution has been used. Sub-optimal results can be seen if a high pH target retrieval solution is used for an antibody that according to the specification sheet requires a low pH target retrieval solution. ■ Check that the target retrieval solution has been within the expiration limits when used for the slide Target retrieval temperature: ■ Check that the temperature has been held within the limits of the target retrieval equipment throughout the course of the target retrieval process. A too high temperature can lead to impaired morphology and "over retrieval" of the antigen epitopes. Low temperature can lead to inadequate retrieval of the epitopes and thereby reduced staining intensity or lack of stained epitopes. The temperature data for the slide can be located at different places in the instrument software based on which automated solution is used. It is recommended to consult the user guides for the automated platform or to contact the supplier. ■ High altitude installations need to provide information in the datalog that appropriate temperature was achieved Target retrieval time: ■ Find the location of the specific data for the target retrieval time. The target retrieval time for the slide can be located at different places in the automated platforms software based on which automated solution is used. It is recommended to consult the user guides for the automated platform to find the location or to contact the supplier. ■ Check that the time the slide actually received target retrieval is within the allowed limit for the assay performed ■ If positive control has been run for the assay on the slide in question an evaluation of the effect of any deviations can be made based on the positive control. Based on this evaluation it can be determined if the slide can be used even though not processed inside the allowed limits. Special care shall be taken if the time has been reduced because the level of positivity in the samples is not known and can differ from the positive control and thereby potentially result in a false negative result.	If any of the checks performed for the target retrieval step show irregularities it is recommended to re-run the sample and/or to get the automated equipment serviced by the manufacturer. If a failure of either use of the target retrieval reagents or the target retrieval platform has been identified, remember to search for other slides which potentially have been submitted to the same failure and perform a quality check of these related slides. Check of the instrument overall performance is not based on a single slide. However, if a given automated platform is the main denominator between failing slides then it should be considered to make a check of the automated platform and potentially get it serviced by the supplier.
LIS Protocols align with workstation test	Protocols from the server ■ Verify that correct protocols are received from the server ■ Find the appropriate test name for the protocol. Verify that this name is mapped in the list of IHC or ISH test protocols. Contact the vendor technical support or bioinformatics department for assistance.	Important to check this whenever new protocols or tests have been added to the server of the system. LIS may not automatically update and map new test protocols from the server.
Staining process has been performed under the right conditions	**Staining process** Reagents: ■ Find the location of the specific data for the executed protocol – this can be separate or a part of the slides' log file under completed slides. The location of the information can be looked up in the user guides for the automated platform or provided by the supplier. ■ Check that the right protocol including the right reagents have been used to stain the slide in question. Sub-optimal results may be seen if another reagent than the validated for the assay either by the laboratory itself or the supplier and the laboratory in combination is used. ■ Check that the right reagent volume has been applied according to the protocol ■ Check that all the reagents used have been within the expiration limits when used for the slide ■ Check that the label of the reagents used is actually in agreement with liquid in the bottles used on the automated platform. This can be done by looking at what the bottle previously has been used for in the bottle history (can be located different places dependent of automated platform used). If the bottle history shows successful use of the reagent prior to this then the right reagent is in the bottle.	If you use reagents that you dilute from concentrate then it is important to check that the dilution has been done correctly. This can be done by validating the new dilution against a previous dilution still within the expiry limits. It is recommended that these checks are done to eliminate other variables like different automated platform or other reagents in the process. It is recommended that controls are applied on every slide to ensure that it has received the exact same treatment as the sample being evaluated which supports the trouble shooting for the samples to a higher degree than a separate run or daily control will do.

PETITIONER EXHIBIT 1012
Page 195 of 218

Information You Need to Know	Information Located at the Instrument	Comments
	■ Check whether the reagent has been stored according to recommendation from the supplier ■ Check that the reagent volume usages have been adequate compared to the calculated use. This can be done by checking the bottle history together with the weight of an empty container and compare this to the container used making it possible to estimate the consumption. Staining temperature: ■ If temperature control is used during the staining process then check that the temperature has been held within the limits of the staining process. The location of the information can be found in the user guides for the automated platform or provided by the supplier. ■ Check that the operating conditions for the automated platform are within specifications. The specified operating conditions for automated platforms are listed in the user guides. Incubation time: ■ Find the location of the executed incubation times for the slide in question. This location can vary based on which automated platform is used. The location of this information is accessible either from the user guides or can be provided by the supplier. ■ Check that the incubation times the slide actually received are within the allowed limits for the assay performed. Variation in incubation time can result in variation in staining results. Too long incubation time can potentially lead to increased level of background staining which can influence the interpretation of the slide. Too short incubation times can lead to false negative results. Wash: ■ Find the location of the executed wash incubation times for the slide in question. This location can vary based on which automated platform has been used. The location of this information is accessible either from the user guides or can be provided by the supplier. ■ Check that the incubation time the slide actually received in the wash steps are within the allowed limits for the assay performed. Wide variation in wash times can be allowed for most assays. However, too short wash time potentially can result in unwanted specific as well as non-specific background due to inadequate wash. Extensive wash times can for special assays lead to reduced intensity. ■ Check that the wash buffer volume has been within the acceptable limits. How this is done can be different based on the automated platform used. Most platforms have estimated buffer consumption per run which can be checked against the actual usages. Furthermore, description of how to check the wash volume per slide may be provided in the user guides or can be requested from the supplier. ■ Check that the wash buffer used is the buffer specified for the assay or has been validated for the assay by the laboratory ■ Check that the wash buffer used for the slide has been within the expiry limits when used for the slide in question General: ■ If a positive control has been run for the assay on the slide in question an evaluation of the effect of any deviations can be made based on the positive control. Based on this evaluation it can be determined if the slide can be used even though not processed inside the allowed limits. Special care shall be taken if the time has been reduced because the level of positivity in the samples is not known and can differ from the positive control and thereby potentially result in a false negative result. ■ Check of leveling of the slide during the staining process. How this is done will vary based on the automated platform used. The information can be obtained either from the user guides for the automated platform or requested from the the supplier. It is important to check the level of the slide as a slide which is not in level during staining can lead to the area of interest not receiving the necessary reagents. This will result in inconsistent staining.	If problems with the automated platform is indentified it is recommended to check other samples processed in the same period on the same platform or platforms in order to identify potential relations. To confirm or discharge the automated platform as the reason to problems with the staining quality it is a good idea to search for related samples e.g. other samples treated on the same automated platform run or in the same slide position, this can help lead in the right direction when doing the troubleshooting. If problems with the automated platform is identified, a service of the instrument is recommended

PETITIONER EXHIBIT 1012
Page 196 of 218

Information You Need to Know	Information Located at the Instrument
Automated IHC staining platform has been performing as expected	**Automated IHC staining platform** Reagent volume: ■ Make sure that the volume your automated platform is set to use is adequate to cover the entire area of your sample over the total duration of the incubation time. It is important to take into account the potential evaporation of the reagent over time. Drying out of the tissue during the staining process can result in staining effects including no staining, inconsistent staining, extensive background and other staining artifacts Contamination: ■ Contamination of the probe on the automated platform can lead to mixing of reagents in the probe and/or on the slide. This can lead to false positive staining or background on the slide. The inclusion of negative tissue controls can help identify if a contamination has taken place. A contamination of the visualization system in the substrate-chromogen will not be identified by the negative control as it will be present both on the positive control and the negative control. In order to identify this contamination it is necessary to make an investigational test where the first dispense of substrate chromogen can be removed and examined by mass spectrometry or ELISA for small quantities of visualization component. ■ Contamination with bacterial and/or fungal growth can be seen in automated IHC platforms when the recommended maintenance schedule is not followed and/or some parts has been defective which increase the risk of bacterial and/or fungal growth. Inspection of the visual parts of the instrument as well as keeping the maintenance schedule can prevent the contamination from happening. If growth is expected normal microbiological methods can be used to determine the presence of both bacteria and fungus. After identification the automated platform has to be cleaned according to specifications listed in the user guides or the supplier can be contacted for advice on how to clean. ■ Contamination caused by inadequate wash of the automated platforms probe. After the probe aspirates reagent it has to be washed before transferred to another reagent bottle. If this wash is not adequate then the second bottle can be contaminated with reagent from the previous bottle. This will be identified as an unspecific reaction for a given marker e.g. CD20 staining in the nuclei if the first aspiration wash from Ki67. This contamination can be confirmed by having the probe go into a bottle of wash buffer or other neutral fluids after the first aspiration and then measure the content of the previous reagent by either mass spectrometry or ELISA. ■ Contamination of the tissue by the probe dropping a drop of unrelated reagent onto the slide. This will be recognized as a false positive reaction either being in the wrong structure or wrong location. Use of positive tissue controls including multiple organ types on each slide will make it possible to identify contamination of this origin. Waste separation: ■ Automated platform separates hazardous from non-hazardous waste. Failure of this separation does not impact the staining process and thereby should not influence the staining result. Bulk fluid supply: ■ The automated platforms normally have a function which makes it possible to check whether the supply of bulk fluids is working adequately. This check can be a prime of the bulk fluid trough the system securing that there are no leakages or clots preventing the fluid from flowing. Check of the bulk fluid supply is described in the user guides or can be requested from the supplier. General: ■ If in doubt about the performance of the instrument, thorough observation of the automated platform during the staining process can give an indication of whether the individual steps is being executed as expected. ■ Check that the instrument is closed correctly as the performance depends on the surrounding conditions for optimal staining. Improper closing can result in inability to start or create staining conditions which are sub-optimal do to the air getting into the instrument through the open cabinet. ■ Instrumentation should be installed away from direct sunlight. Sunlight can affect the staining conditions and viability of reagents.

Acknowledgements

Sections, in whole or parts thereof, from the previous editions of this Guidebook are used in the 6th edition. We sincerely thank and acknowledge the contribution of the authors. Special acknowledgements to: Karen N. Atwood and Dako Technical Support Group

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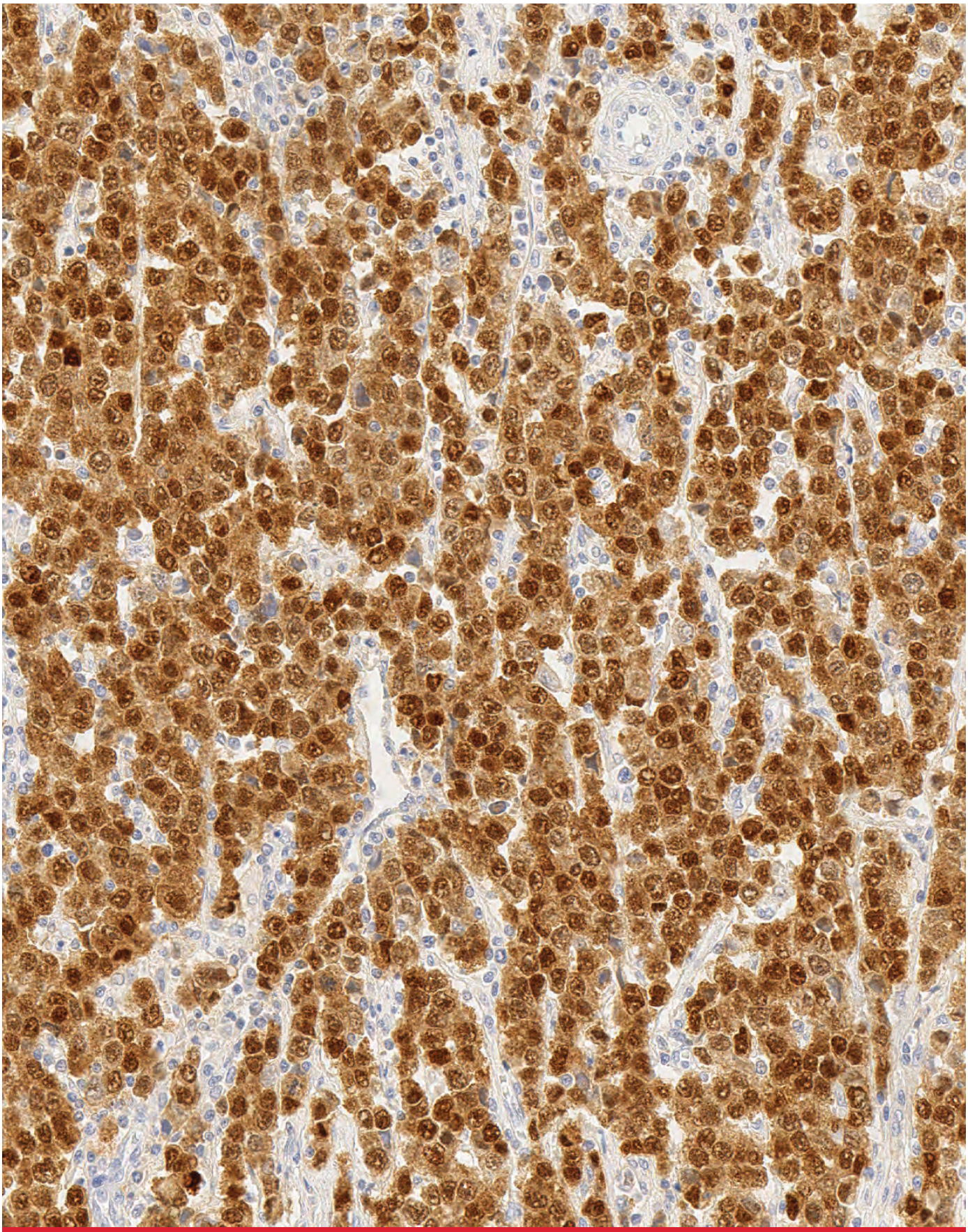
PETITIONER EXHIBIT 1012
Page 197 of 218



Appendix A

Antibodies

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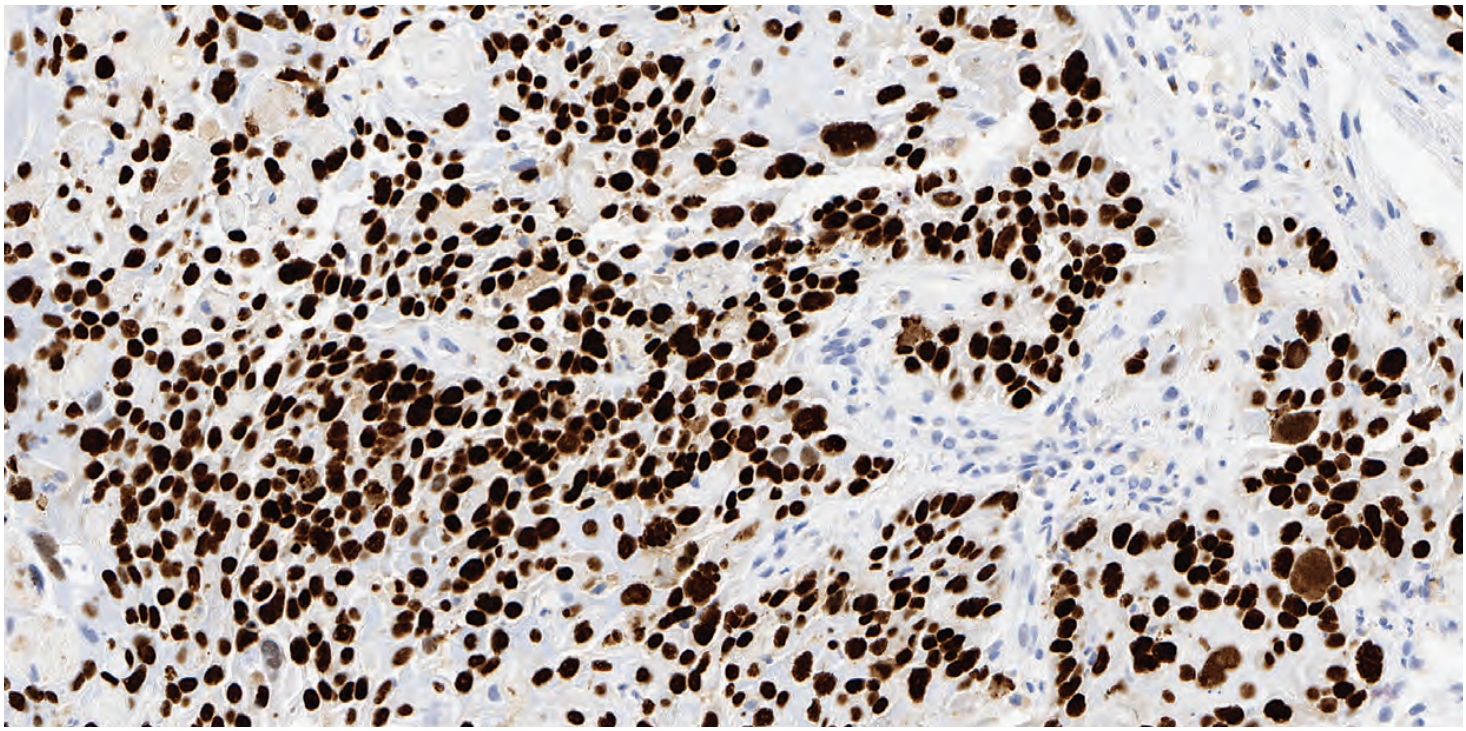
Original version by: **Thomas Boenisch**



An•ti•bod•y *(n.)*
A Y-shaped protein on the surface of B cells that is secreted into the blood or lymph in response to an antigenic stimulus, such as a bacterium, virus, parasite, or transplanted organ, and that neutralizes the antigen by binding specifically to it; an immunoglobulin.

The American Heritage® Dictionary of the English Language

The central reagent common to all immunohistochemical techniques is the antibody. The availability of new monoclonal antibodies, antisera and their immunoglobulin fractions to an ever-increasing number of clinically useful tissue biomarkers (antigens) has enormously expanded the quantity and quality of the immunohistologic repertoire. This Appendix A briefly covers the structural and biochemical features of antibodies and antigens and is as such not a necessity in order to understand the immunohistochemical staining procedure. However, to better comprehend the potential of immunohistochemical as used in clinical research and clinical pathology as well as associated problems, it is helpful to have a basic knowledge of antibodies, their potentials and their limitations.

Appendix A.1 | Immunoglobulins

Antibodies belong to a group of proteins called immunoglobulins (Ig) that are present in the blood of immunized individuals. The removal of cells and fibrin from blood is used to collect the serum fraction frequently referred to as antiserum. Listed in order of decreasing quantity found in plasma or serum, immunoglobulins comprise five major classes: immunoglobulin G (IgG), IgA, IgM, IgD and IgE. Each is composed of two identical heavy chains (H) and two identical light chains (L). The H chains differ in antigenic and structural properties, and determine the class and subclass of the molecule. The two L chains are either of type kappa (κ) or lambda (λ). Distribution of κ and λ chains differs in all Ig classes and subclasses, as well as between different species. Covalent bounds join L to H and H to H chains. By participating in the tertiary structure, they confer greater stability to the immunoglobulin molecule.

Of the five classes of immunoglobulins, IgG and IgM will be considered in more detail here, as these are by far the most frequently utilized antibodies in immunohistochemistry. Unless otherwise noted, most of what is described of the IgG structure in this text was learned from studies with human IgG of subclass IgG1.

IgG

IgG has the general formula of one molecule of IgG is composed of two γ heavy chains, and two light chains of either type κ or type λ ($\gamma_2\ \kappa_2$ or $\gamma_2\ \lambda_2$) (Figure A.1).

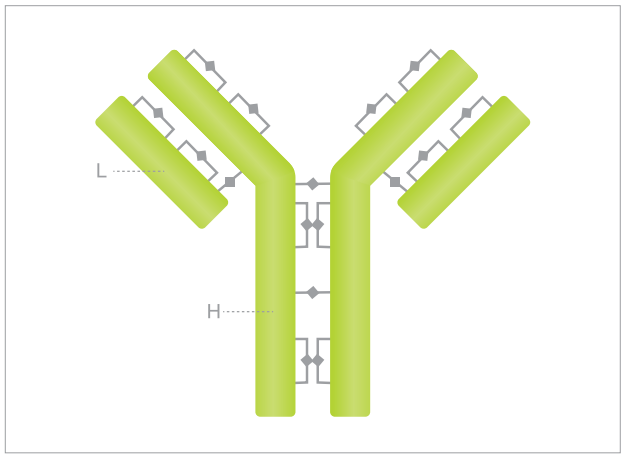


Figure A.1 Diagram showing the structure of an immunoglobulin molecule. It comprises two identical heavy (H) chains and two identical light (L) chains. The heavy chains of IgG are denoted as gamma (γ) chains. The two L chains are either of type kappa (κ) or lambda (λ). Inter- and intrachain disulphide bonds (—♦—) contribute to the structure and stability of the molecule. One molecule of IgG has a molecular weight of ~150 kDa.

Much of the structure of the IgG molecule has been determined in part by proteolytic digestions and chemical dissociation of the molecule (Figure A.2). When the IgG molecule is digested by the enzyme papain it results in the cleavage of a covalent bond on the N-terminal side of the inter-heavy chain disulfide bridges. This yields two antigen-binding fragments (Fab, monovalent) and one crystalline fragment (Fc). The digestive enzyme pepsin cleaves the γ chains on the C-terminal side of the inter-heavy chain disulfide bridges, resulting in one antigen-binding fragment ($F(ab')_2$, bivalent). In this case, the Fc fragments are destroyed. Using chemical reductive dissociation of an IgG molecule will split interchain disulfide bridges, and if the free sulfhydryl groups are blocked, it results in the formation of two H chains (molecular weight of 50 kDa each) and two L chains (25 kDa each).

The IgG molecule can be further divided into so-called domains, namely the variable domains (V) and the constant domains (C). Each domain contains 110 to 120 amino acids and one intrachain disulfide bond. On the variable domain of the light chain (V_L), and on the variable domain of the heavy chain (V_H), the amino terminals of the immunoglobulin molecule are located. Together, V_L and V_H form the antigen-combining site.

PETITIONER EXHIBIT 1012
Page 200 of 218

Several hypervariable (H_V) regions are located within the V_L and V_H domains of the antibody.

It is these hypervariable regions that recognize the antigen. During the formation of the antibody/antigen complex, the HV regions are brought into close proximity to the antigenic determinant (epitope). The distance between the antigen and HV regions of the antibody is approximately 0.2 to 0.3 nm.

In this region, unique structural specificities called idiotypic determinants are located. Each antibody clone expresses its own idiotype. Each L chain also has one constant domain (C_L) in addition to the V_L domain. The H chain has three constant domains (C_H1, C_H2 and C_H3). The hinge regions are located between the C_H1 and C_H2 domains of the H chains. Minor differences within these hinge regions contribute to the subclass specificity of immunoglobulin G. The same are designated by subscripts as in IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2C} and IgG_3 for mice and IgG_1, IgG_2, IgG_3 and IgG_4 for humans. Whereas in human IgG the overall ratio of κ to λ is 2:1, mice have approximately 95% κ chains, and therefore most monoclonal IgG antibodies from this species have κ chains. The number of disulfide bridges linking the heavy chains also varies among the IgG subclasses. IgG_1 and IgG_4 each have two, while IgG_2 and IgG_3 have four and five, respectively. Because of the flexibility of the hinge region, the angle that both Fab fragments form can vary to accommodate varying distances between epitopes.

Rabbits have only one IgG subclass which, like the human and mouse IgG, also has a molecular weight of ~150 kDa, with two heavy chains (~50 kDa each) and two light chains (~25 kDa each) under non-reducing conditions.

IgM

IgM is a pentamer (MW ~900 kDa) consisting of five subunits of ~180 kDa each (Figure A.3). The general formula can be expressed as (μ_2 κ_2) or (μ_2 λ_2)[5]. Each subunit is linked by a sulfhydryl-rich peptide, the J chain (15 kDa), and consists of two heavy chains μ and two light chains of type κ or λ. The J-chains contribute to the integrity and stability of the pentamer. As with IgG, IgM subunits can be fragmented by enzymatic and reductive cleavage into $F(ab')_2$, Fab and Fc portions, as well as heavy and light chains, respectively.

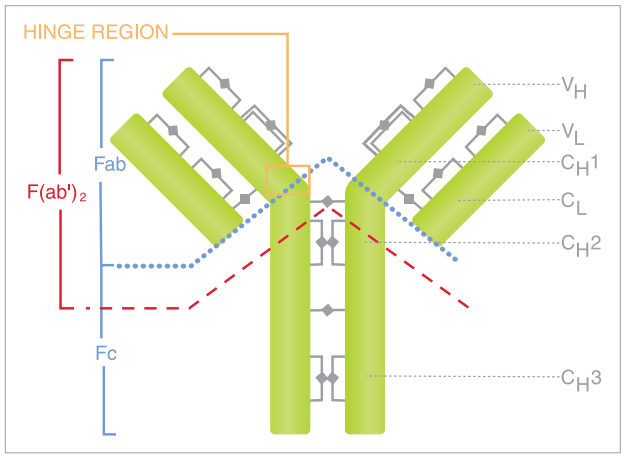


Figure A.2 Diagram showing the structure of rabbit IgG (which exists as a single major subclass). The heavy (H) and light (L) chains are composed of variable (V) and constant (C) domains and are linked by inter- and intrachain disulfide bonds (—♦—). Proteolytic digestion with papain (• • • • •) yields two antigen-binding fragments (Fab) and one crystalline fragment (Fc), whereas digestion with pepsin (– – – –) yields one F(ab')2 fragment.

Whereas IgG is the most abundant antibody in the hyperimmunized host, in the newly immunized animal, IgM is the first humoral antibody detectable. The primary antibody formation proceeds in several major stages. The antigen first reaches equilibrium between extra- and intravascular spaces, then undergoes catabolism resulting in smaller fragments, and finally is eliminated from the intravascular spaces by the newly formed antibodies. The period from the introduction of an antigen until the first appearance of humoral IgM antibodies is called the latent period and may last approximately one week. Within two weeks, or in response to a second antigen challenge, IgG class antibodies usually predominate. Like all proteins, antibodies are subject to catabolism. Whereas antibodies of class IgM have a relatively short half-life of only four to six days, IgG antibodies have a mean survival of approximately three weeks. The serum antibody level will decrease after this period.

Antibody formation on the molecular level is a complex process, and a detailed account of it is beyond the scope of this guidebook.

Immunoglobulins in different species

Human, mouse and rabbit have different sets of immuloglobulins

PETITIONER EXHIBIT 1012
Page 201 of 218

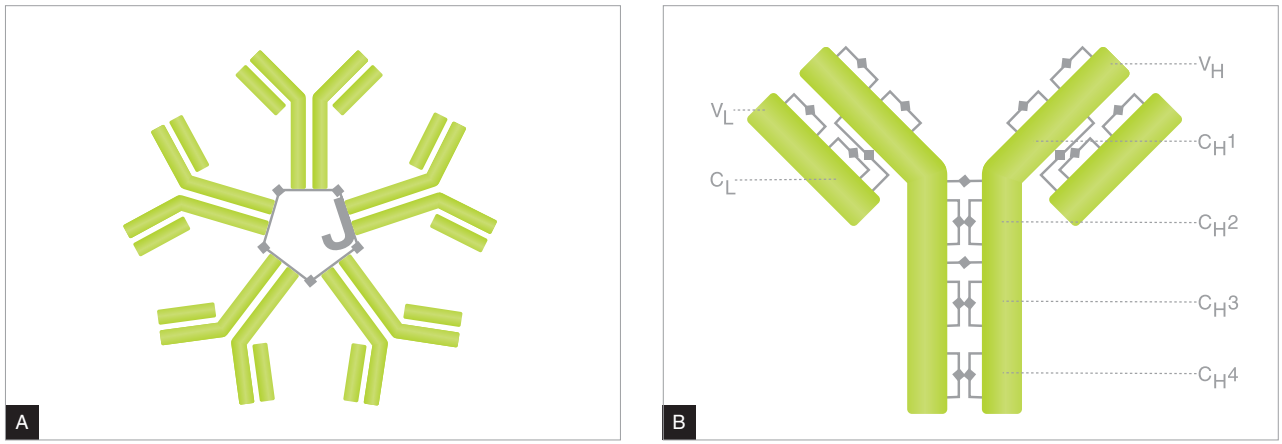


Figure A.3 Diagram showing **A)** the five subunits of mouse IgM linked by disulfide bridges (—♦—) and the J chain to form a pentameric ring structure. **B)** Each subunit comprises two μ heavy (H) chains and two light (L) chains each composed of constant (C) and variable (V) domains.

and subtypes within each immunoglobulin class. Humans and mice have five antibody classes (IgA, IgD, IgE, IgG, and IgM), but in rabbits only four classes have been identified (IgA, IgE, IgG, and IgM). The most commonly used immunoglobulin for immunohistochemistry, IgG, exists in five subclasses in mouse but only one in rabbit. Humans have two IgA subclasses, mice have only one IgA subclass, but 14 IgA subclasses have been found in rabbits.

Table A.1 List of immunoglobulin subclasses in human, mouse and rabbit.

Ig Class	Human	Mouse	Rabbit
IgA	IgA1, IgA2	IgA	IgA1, IgA2, IgA3, IgA4, IgA5, IgA6, IgA7, IgA8, IgA9, IgA10, IgA11, IgA12, IgA13, IgA14
IgD	IgD	IgD	
IgE	IgE	IgE	IgE
IgG	IgG1, IgG2, IgG3, IgG4	IgG1, IgG2a, IgG2b, IgG2c*, IgG3	IgG
IgM	IgM	IgM	IgM

*IgG2c is found in some inbred mice strains

Appendix A.2 | Antigens

When a biomarker of interest is used for immunizing an animal, the biomarker is referred to as an antigen, a name derived from its ability activate an antibody generation response. In immunohistochemistry, the biomarkers of interest are proteins, and full length or fragments of proteins are often used as these are the most potent antigens. Shorter peptide sequences (10-20 amino acids) can be used with advantage in terms of higher specificity and reduced likelihood of cross-reactivity, but requires that the peptide is coupled to a carrier protein (e.g. keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or ovalbumin (OVA)). The requirement of a carrier protein also applies to carbohydrates, DNA and smaller molecules like dinitrophenol (DNP) and biotin. These smaller substances – often referred to as haptens - can also elicit an immune response, but are less potent antigens compared with protein and their derivatives.

The part of the antigen that is specifically recognized by the immune system is called an epitope; this is the antigenic determinant that is bound by the antibody. One protein can have multiple epitopes, but a monoclonal antibody only recognize one of these epitopes in the protein. The epitopes of proteins are divided into two categories, conformational epitopes and linear epitopes, based on their structure and interaction with the antibody (1). Linear epitopes are defined by the primary amino acid sequence of a particular region of a protein and vary typically from 5 to 20 amino acids in length. Conformational epitopes are defined by the spatial structure of the native protein and are situated on disparate parts of in the protein

PETITIONER EXHIBIT 1012
Page 202 of 218

sequence, but are brought close to each other in the folded native structure. In IHC, the conformation of the proteins in a tissue sample will be affected by the formalin fixation and the antigen retrieval process, which may be the reason that some antibodies directed against linear epitopes perform better (2).

To determine which part of the biomarker is most suited for generating a specific antibody, the biomarker should be thoroughly investigated. Features like the amino acid sequence, structure and spatial position of the epitope in the protein can be examined by integrated computational and microarray-based analysis to find the most immunogenic epitopes and to prevent cross-reactivity (3).

Appendix A.3 | Polyclonal Antibodies

Polyclonal antibodies are a heterogeneous mixture of antibodies directed against various epitopes of the same antigen (Figure A.4). The antibodies are generated by different B-cell clones of the animal and as a consequence are immunochemically dissimilar. The antibodies in a polyclonal mixture can have slightly different specificities and affinities (see Antibody Affinity). Polyclonal antibodies are most frequently produced in rabbits but also made in other mammals including goat, swine, guinea pig and cow. Rabbits are frequently the species of choice in polyclonal antibody production due to the appreciable amount of anti-serum and relative rarity of human antibodies to rabbit proteins when compared to other species such as goats. Additionally, rabbit antibodies precipitate human proteins over a wider range of either antigen or antibody excess.

Polyclonal antibodies are produced by immunizing with antigen (also known as immunogen) using doses ranging from 10 ug to 200 ug. Antigen can be prepared with or without an adjuvant such as Freund's Complete or Incomplete Adjuvant which can boost the immune response. For smaller or less immunogenic proteins or peptides, the immunogen should be coupled to carrier proteins. The immunization period lasts from 3 to 8 months with biweekly immunogenic boots. The polyclonal antibody preparation can be used in the form of stabilized antisera or further purified. Purification of the immunoglobulin; to eliminate other serum proteins can be performed through ammonium sulfate precipitation and ion exchange chromatography, Protein A or G purification or affinity isolation.

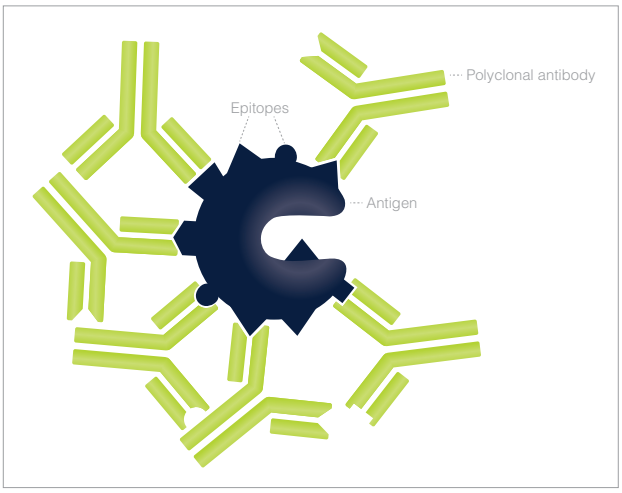


Figure A.4 Schematic diagram of polyclonal antibodies binding to various epitopes on an antigen.

Appendix A.4 | Monoclonal Antibodies

Monoclonal antibodies are a homogeneous population of immunoglobulin directed against a single epitope. The antibodies are generated by a single B-cell clone from one animal and are therefore immunochemically similar. Monoclonal antibodies are most commonly produced in mice and rabbits, but may also be produced in other species such as rat and camel.

Mouse Monoclonal Antibodies

The mice are immunized and boosted every two weeks over a period of two months. The animal's immune response is monitored through periodic testing of the serum. Upon achieving an acceptable immune response, the B lymphocytes are isolated from the spleen and fused with an immortal cell line (myeloma cell line/fusion partner). The B lymphocytes confer the capacity to produce specific immunoglobulin while the fusion partner cell line enables immortality and indefinite growth in culture. The fused and immortalized cell line is called a hybridoma. The hybridoma cell line is cultured, selected and sub-cultured by limiting dilution to isolate a stable clone with a high antibody production capacity. For production of tissue culture supernatant, the

PETITIONER EXHIBIT 1012
Page 203 of 218

hybridoma cell line is cultured in multiple tissue culture flasks. Bioreactors can be used for large scale hybridoma growth and antibody generation. A bioreactor is a system that continually replenishes cells with fresh media and promotes growth for cultivation of concentrated amounts of antibody. In mice, ascites fluid has also been used for production due to a very high concentration of antibody compared with tissue culture supernatant; however contaminating antibodies will be present in ascites preparations. Most monoclonal antibody preparations are from stabilized tissue culture supernatant. Further purification of immunoglobulin can be performed through the use of protein A or G chromatography or affinity purification.

Rabbit Monoclonal Antibodies

While the vast majority of monoclonal antibodies are produced in mice, a growing number of rabbit monoclonal antibodies are being manufactured. The process for generating a rabbit hybridoma is comparable to the mouse hybridoma generation described in Figure A.6. The use of rabbits for monoclonal antibody production confers some advantages over mouse monoclonals. These advantages are also shared by rabbit polyclonals. Rabbits are reported to have more diverse epitope recognition than mice (less immunodominance) and an improved immune response to small-sized epitopes. There is also a tendency with rabbits to generate antibodies with higher affinity and overall avidity. The resulting rabbit antibodies also have enhanced binding properties due to heavy glycosylation. Mouse hybridomas however tend to generate a higher yield of immunoglobulin than rabbit hybridomas and the mouse hybridoma cell lines are typically more stable in culture.

Appendix A.5 | Polyclonal Antibodies versus Monoclonal Antibodies

When comparing the advantages and disadvantages of polyclonal and monoclonal antibody preparations, there are benefits to both. Due to their multiclonality, polyclonal antibodies are typically more robust reagents when used on routinely-processed tissue specimens. The ability of a polyclonal reagent to recognize multiple epitopes on a single molecule means that the reagent is not as subject to the deleterious effects on epitopes of pre-analytic processing of specimens, as are monoclonal antibodies which are directed against a single epitope. The presence of antibodies to multiple epitopes however can increase the chance for cross-reactivity (see Antibody Cross-Reactivity) with other proteins.

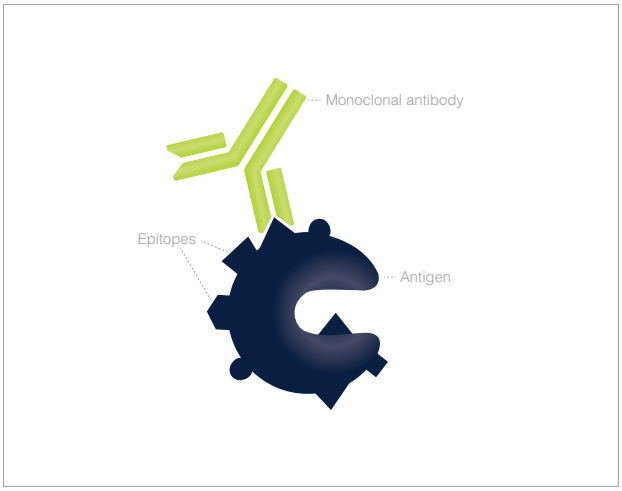


Figure A.5 A given monoclonal antibody clone reacts with only one specific epitope on an antigen.

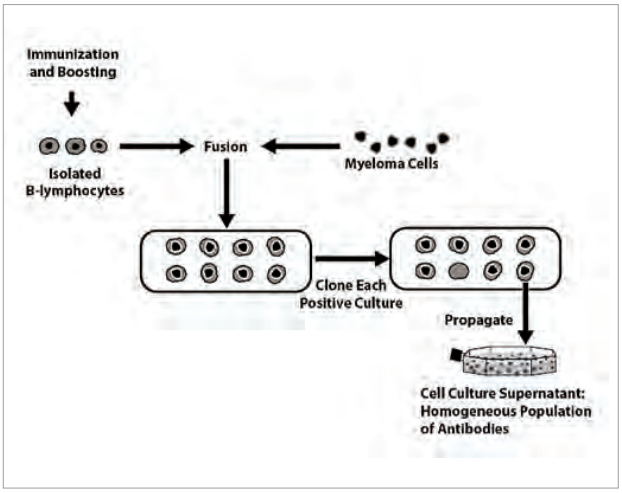


Figure A.6 The process of monoclonal antibody production.

PETITIONER EXHIBIT 1012
Page 204 of 218

Monoclonal antibodies have the advantage of lot-to-lot consistency and lack the inherent variability of polyclonal antibodies due to the immunological state of the animal. The use of a hybridoma in monoclonal antibody production enables a sustained production of antibody.

Appendix A.6 | Antibody Affinity

Antibodies from hyperimmunized animals not only differ with regard to the determinants they recognize on multivalent antigens, but also differ in their affinities for the same. The term "affinity" has been used to describe both intrinsic and functional affinities (4).

The intrinsic affinity of an antibody resides in the H_V region and is determined by the same sequence of amino acids that determines specificity. In immunohistochemistry, the functional affinity of an antibody or an antiserum can be very loosely defined by the time required to reach equilibrium with the tissue antigen. If equal aliquots of two antibodies or antisera of identical titer are incubated for increasing periods of time with the antigen on the tissue, the antibody that reaches a plateau of maximum staining intensity first is of a higher functional affinity. The term "avidity" has been used synonymously to describe functional affinity (5), but has also been used to denote the strength of the binding reached between antibody and its antigen (6). The term avidity has also been used to describe the sum total of all intrinsic affinities found in a polyclonal antibody population.

Because antigen-antibody reactions are reversible, the simple immune complexes formed on tissue may occasionally dissociate during the washing cycles used in immunohistochemistry. The ease and extent of this dissociation vary from antibody to antibody, and low salt concentrations as well as low temperatures will reduce the likelihood of weak staining due to dissociation of an already formed immune complex. Thus, high affinity antibodies are desirable and have the advantage that during washing, dissociation is less likely to occur than with low-affinity antibodies. As mentioned before, a polyclonal population of antibodies contains a more or less continuous spectrum of low to high affinities against several epitopes on a given antigen. Therefore after incubation with a primary antibody of this type, excessive washing is unlikely to result in any appreciable loss of staining.

On the other hand, monoclonal antibodies are of uniform affinity and loss of staining may be due to the dissociation of the antibody from its epitope if the affinity is low. Therefore monoclonal antibodies of high affinity should be selected, if possible. As indicated above, factors that weaken the antigen-antibody bond such as high salt concentrations, high temperature and very low pH during the washing of the specimens should be avoided. Experience in the handling of antibodies in immunohistochemistry has shown that the washing and incubation in buffer baths can be safely reduced and that gentle agitation helps to reduce background staining (7).

The affinity of antibodies is also related to their capacity to form insoluble immune complexes. Generally, the higher the affinity of an antibody, the greater its tendency to form a precipitate. Precipitation proceeds through a rapid stage in which soluble antigen-antibody complexes form, followed by slower aggregation and, eventually, precipitation. Non-precipitating antibodies are mostly of lower affinity and are incapable of forming the lattice required for precipitation to occur.

Monoclonal antibodies, regardless of whether they are of high or low affinity, do not form a lattice with antigen, and, hence only rarely form insoluble precipitates. However, in immunohistochemistry, the capability of a primary antibody to form precipitating immune complexes is of little importance because reaction with immobilized tissue antigen entails antibody capture onto tissue rather than precipitation.

Appendix A.7 | Antibody Cross-Reactivity

The term "cross-reactivity" denotes an immunochemical activity that can occur either between an antibody and two or more antigens or vice versa, when an antigen reacts with several different antibodies. Typical examples are when anti-λ (or -κ) chain antibodies interact with all five Ig classes or when carcinoembryonic antigen (CEA) reacts with antibodies against CEA, blood group antigens and normal tissue proteins, respectively. The common denominator in each case is the sharing of at least one common epitope between several antigens.

Another valid use of the term cross-reactivity denotes the experimentally or accidentally induced changes within one or

PETITIONER EXHIBIT 1012
Page 205 of 218

several epitopes, through antigen retrieval (8), leading to a possible loss of specificity by a given monoclonal antibody for this antigen. The term cross-reactivity also describes the interaction of an antibody with similar or dissimilar epitopes on unrelated antigens. This latter phenomenon however is frequently a property of low affinity antibodies, and is usually subject to change because of affinity maturation during immunization.

Cross-reactivity of antibodies to human antigens with identical or similar antigens of other species, or "cross-species cross-reactivity," can be of interest to the researcher and veterinarian because of the scarcity of animal-specific antibodies. To overcome this, two publications reported the results of cross-species reactivity studies using commercially available antihuman polyclonal and monoclonal antibodies (9, 10). It was demonstrated that the majority of animal antigens selected showed strong reactivity with antihuman antibodies. However, for more technical detail on the use of a given mouse primary antibody on animal tissues, the reader is referred to animal research kit products.

The terminology of cross-reactivity however is misplaced when describing any observed staining by the same antibody of different cells or tissue components, regardless whether they contain common antigens, as this would distort the strict immunochemical definition of the term.

Appendix A.8 | Antibody Reaction Rates

Although under ideal conditions antibodies react with their antigens very rapidly, in immunohistochemistry the conditions are rarely ideal. However, very short incubation periods are made feasible by the relatively rapid reaction rates that occur when higher concentrations of high-affinity primary and link antibodies are used. Generally, the size and shape of the antibody molecule and its conjugates or complexes appear to be of little consequence in immunohistochemistry. Insufficient tissue penetration, even when staining nuclear or cytoplasmic antigens, has never been observed, regardless of whether primary antibodies of class IgM (900 kDa), large complexes like PAP (400-430 kDa) or APAAP (~560 kDa) or dextran-polymer-linked reagents were used. However, it is reasonable to assume that gross overfixation of tissue may make penetration more difficult for antibodies and their complexes.

Appendix A.9 | Antibody Stability and Storage

Polyclonal antibodies, when stored unfrozen and used subsequently in immunohistochemistry, are somewhat less stable as immunoglobulin fraction compared to whole antiserum (7). However, this reduced stability was found to depend largely on the method of purification and storage as well as on the method of application. Exposure of antibodies to extreme pH, as well as high or very low concentrations of salts during purification tends to decrease their stability more than does exposure to mild conditions such as ion exchange chromatography.

Monoclonal antibodies also have been shown to be influenced in their performance by methods of purification and storage; 42% of monoclonal antibodies investigated by Underwood and Bean showed changes in specificity, affinity and cross-reactivity (11). Antibodies of class IgM and subclass IgG_{2b} were especially sensitive.

Antibody stability in commercially produced reagents is determined best by real-time and real-temperature testing by each manufacturer. Most manufacturers demonstrate stability by testing during a pre-determined period of time, i.e. the "shelf life". While many antibodies may retain activity longer, the only regulatory requirement for the manufacturer is to certify the period of time that the antibody has been tested. There is no requirement to continue testing until the antibody loses activity. Sometimes, the conditions for the storage of reagents in the user's laboratory are not identical to those that prevailed during the manufacturer's shelf life studies. Because of the possibility of adverse storage conditions after the purchase of the product, the manufacturer can only offer a limited liability instead of predicting the actual demise of a reagent.

The only possible corollary to these requirements is to allow laboratories to document the activity of the product until the loss of the same. Alternatively, laboratories may aliquot and freeze undiluted antibody at -20 °C for later use. At this time, laboratories must confirm activity prior to the use of the antibody in any test.

Finally, expiration dating as practiced today also serves the purpose of conforming to regulatory requirements. Regulatory guidelines in place in the US for clinical laboratories have

PETITIONER EXHIBIT 1012
Page 206 of 218

been mandated by the Clinical Laboratory Improvement Act of 1988 and by the College of American Pathologists. These regulations mandate that expired reagents cannot be used in the clinical diagnostic laboratory on human tissue.

Handling of Antibodies

In order to achieve optimal performance from reagents used in immunohistochemistry, it is imperative to observe basic rules for their handling and storage. If properly maintained, most reagents will remain stable for months or even years. Recommendations given by the manufacturer on specification sheets and on vial labels should always be followed.

Receiving

Although many commercially produced immunochemicals are guaranteed to be stable for up to several years, ready-to-use (RTU) antibodies have a shorter shelf life. Upon receipt, immunochemicals should be promptly stored according to the manufacturer's recommendations. The users should log reagents by entering the manufacturer's lot numbers, expiration date, date of receipt and invoice number. These entries provide valuable information for the user, especially if later reclamations should become necessary.

Storage Containers and Temperature

Perhaps the two most important considerations when storing antibodies are the storage container and the temperature. Ideally, preferred materials for storage containers of protein solutions should have negligible protein adsorptivity. Polypropylene, polycarbonate or borosilicate glass are recommended and are used widely. Solutions containing very low concentrations of protein (i.e. less than 10-100 µg/mL), should receive an addition of immunochemically inert protein. Generally, 0.1% to 1.0% bovine albumin is used to reduce loss through polymerization and adsorption onto the container. Containers made of clear and colorless materials are preferred, as these will allow ready inspection of contents. Container labels also should allow access for inspection.

Probably more than any other factor, observe proper storage temperature as recommended by the manufacturer. Monitor refrigerators and freezers used for storage of immunochemicals for accurate and consistent temperatures. Store valuable or large quantities of immunochemical reagents in equipment with temperature alarm and emergency back-up power systems.

Store most RTU antibodies and their conjugates solutions at 2-8 °C, because freezing and thawing is known to have a deleterious effect on their performance. This also applies to entire kits that contain ready-to-use reagents, including monoclonal antibodies. Store concentrated protein solutions such as antisera and immunoglobulin fractions in aliquots and frozen at -20 °C or below, in order to prevent cycles of repeated freezing and thawing. Bring frozen protein solutions to room temperature slowly, and avoid temperatures above 25 °C.

Use and Care

Proper reagent care can reduce problems stemming from contamination, heat or excessive light exposure. Reagent contamination can be avoided by the use of clean pipette tips. Prompt return of reagents to proper storage conditions will prolong their shelf life. Familiarity with the nature of antibodies, their capabilities and limitations, will allow the user to better utilize these reagents and to more efficiently solve problems, if they occur.

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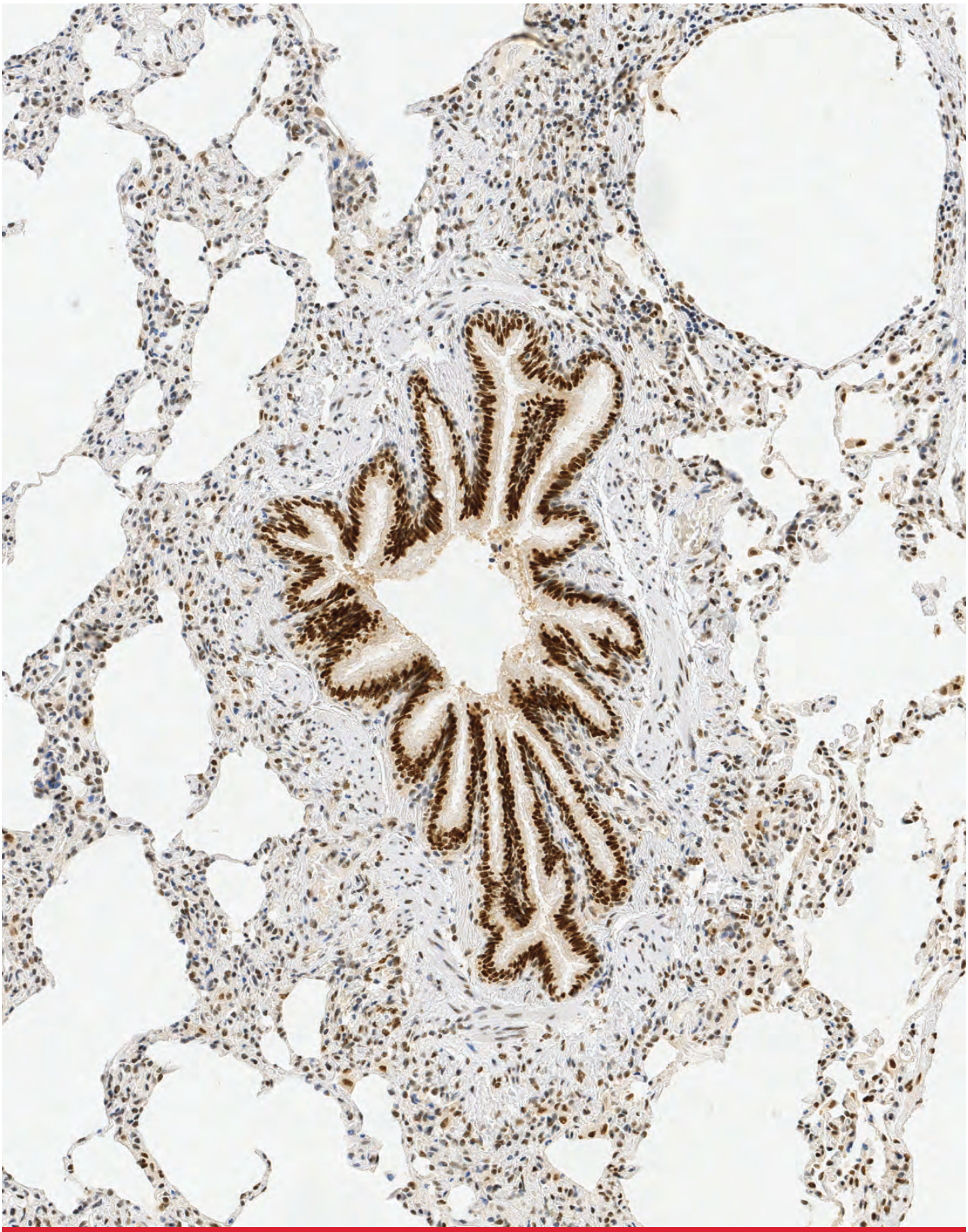
PETITIONER EXHIBIT 1012
Page 207 of 218



Appendix B

Basic Immunochemistry

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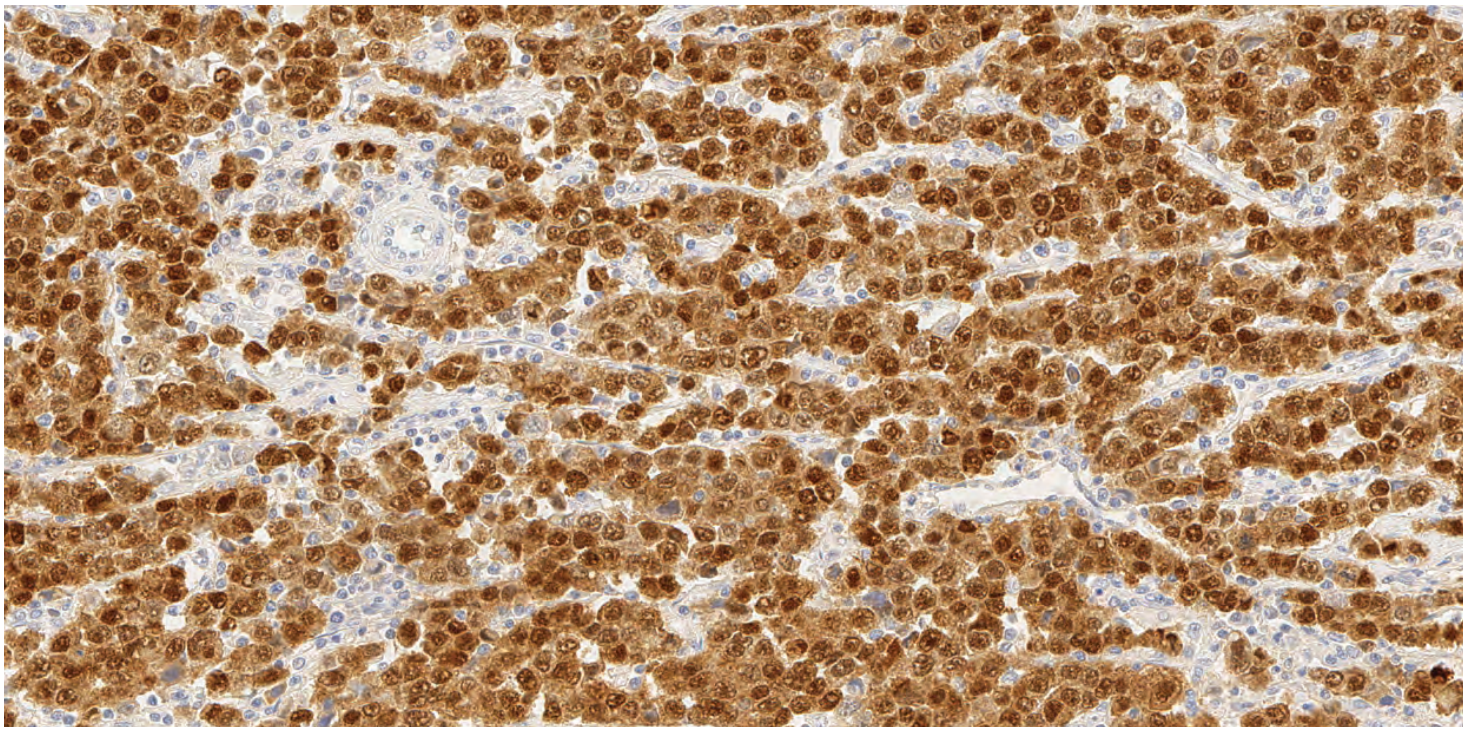
Original version by: **Thomas Boenisch**



Im•mu•no•chem•is•try *(n.)*
A method for the detection and localization of proteins and other cellular components using antibodies that specifically label the materials.

Collins English Dictionary – Complete and Unabridged

In immunohistochemistry (IHC), antibody titer and dilutions as well as incubation time, temperature and pre-treatment of tissue samples are tightly interwoven in their effect on staining quality. These factors can be changed independently, or as is more often the case, in complementary fashion to bring about positive differences. The predominant goal of an IHC staining is to achieve optimal specific staining accompanied by minimal interference from background staining. This Appendix B will briefly describe how changes to titer, dilution, incubation time and temperature may influence the staining reaction. See Chapters 4 and 5 for detailed discussion of the implications when changing these parameters in an IHC staining protocol.

Appendix B.1 | Antibody Titer

Optimum antibody titer may be defined as the highest dilution of an antibody (monoclonal or polyclonal) that results in maximum specific staining with the least amount of background under specific test conditions. This highest dilution is determined primarily by the absolute amount of specific antibodies present.

The amount of antibody required for optimal staining in any given test has to be determined by different antibody dilutions. For polyclonal antisera the amount of specific antibodies is often not measurable, so the optimal staining titer is determined by a series of antiserum dilutions. Affinity purification of polyclonal antisera produces little benefit for immunohistochemical applications, because non-specific antibodies and soluble aggregates – frequent sources of non-specific background become enriched also. For monoclonal antibody preparations, the absolute concentration of specific antibodies can be readily determined, and frequently forms the basis for making required dilutions.

An optimal antibody dilution is also governed by the intrinsic affinity of an antibody. If the titer is held constant, a high-affinity antibody is likely to react faster with the tissue antigen and give more intense staining within the same incubation period than an antibody of low affinity.

In more practical terms, titers may vary from 1:100 to 1:2000 dilution of polyclonal antisera and from 1:10 to 1:1000 dilution of monoclonal antibodies in cell culture supernatants. These dilutions may likely be exceeded in the future due to ever-increasing sensitivities of newer detection methods, including the use of more effective antigen retrieval procedures.

Appendix B.2 | Antibody Dilution

Correct dilutions will contribute to the quality of staining if they are prepared accurately and consistently. Often a manufacturer offers ready-to-use (RTU) reagents ready for use, or recommends dilution ranges compatible with other variables such as method, incubation time and temperature. If this information is not provided, optimal working dilutions of immunochemical reagents must be determined by titration. Correct dilutions are best determined by first selecting a fixed incubation time and then by making small volumes of a series of experimental dilutions. Depending on specimen size, applications of 0.1 0.4 mL of solution per section is generally adequate. It should be noted that at least on paraffin sections optimal dilutions of primary antibodies are not only signaled by a peak in staining intensity, but also by the presence of minimal background (maximal signal to noise ratios). Once the optimal working dilution has been found, larger volumes can be prepared according to need and stability.

The extent to which monoclonal antibodies can be diluted is subject to additional criteria. Because of their restricted molecular conformation and well defined pI, monoclonal antibodies are more sensitive to the pH and ions of the diluent buffer (1). Indeed, it has been demonstrated that almost all monoclonal antibodies could be diluted higher and stained more intensely at pH 6.0, especially after the use of heat-induced antigen retrieval and almost all monoclonal antibodies stained more intensely in the absence of NaCl (1). Of several diluents used in this investigation, phosphate buffered saline (PBS), although still widely used as a diluent for primary antibodies, was found to suppress the reactivity of all monoclonal antibodies tested (1). Differences in the net negative electrostatic charges of the target antigen are likely the explanation for these pH- and ion-related observations (1, 2).

Dilutions are usually expressed as the ratio of the more concentrated stock solution to the total volume of the desired

PETITIONER EXHIBIT 1012
Page 210 of 218

dilution. For example, a 1:10 dilution is made by mixing one part of stock solution with nine parts diluent. Two fold serial dilutions are made by successive 1:2 dilutions of the previous dilution. In order to make a very small volume of a highly diluted solution, it may be necessary to make it in two steps. For example, to prepare 1.0 mL of a 1:1000 dilution, first make 100 µL of a 1:10 dilution (10 µL + 90 µL), and then 1000 µL of a 1:100 dilution using 10 µL of the intermediate dilution (10 µL + 990 µL).

Antibody Incubation

As mentioned above, incubation time, temperature and antibody titers are interdependent. A change in one factor may affect the others.

Appendix B.3 | Antibody Dilution

There is an inverse relationship between incubation time and antibody titer: the higher the antibody titer, the shorter the incubation time required. In practice however, it is important to consider the alignment of protocol incubation times for the antibodies used the laboratory in order to achieve optimal workflow (see Chapter 5).

Incubation times for the primary antibody may vary within up to 24 hours, with 10-30 minutes probably being the most widely used incubation time. For an antibody to react sufficiently strongly with the tissue antigen in a short period of time, it must be of high affinity and concentration, as well as have the optimal reaction milieu (pH and diluent ions). Variables believed to contribute to increased non-specific background staining should be kept to a minimum (see Chapter 15). Low affinity and/or low titer antibodies must be incubated for longer periods than antibodies of high affinity and concentration. But nothing can be gained by prolonging primary antibody incubation beyond the time at which the tissue antigen is saturated with antibody. Consistent timing and temperature (see below) of the antibody incubation step is important to reduce variations in overall staining quality and intensity, which otherwise may lead to incorrect interpretation of results. These criteria are particularly essential in efforts that attempt to assess the degree of tumor differentiation.

Appendix B.4 | Incubation Temperature

Because antigen antibody reactions happen more quickly at 37 °C compared with room temperature, some workers prefer to incubate at the higher temperature. However, while increases in incubation temperature allow for greater dilution of the antibody and/or a shortened incubation time, consistency in incubation time becomes even more critical. It is not known whether an increased temperature promotes the antigen antibody reaction selectively, rather than the various reactions that give rise to background.

In some cases, e.g. a research/experimental setting, a temperature of 4 °C is may be used in combination with overnight or longer incubations. Slides incubated for extended periods, or at elevated temperature, should be placed in a humidity-controlled environment to prevent evaporation and drying of tissue sections. Similarly, tissue incubated at room temperature in a very dry or drafty environment will be at risk of drying out. Most automated staining instruments used in the pathology laboratories today are designed to take into account the potential issues of drying of tissue sections.

Appendix B.5 | Basic Enzymology in IHC

Immunoenzymatic staining methods utilize enzyme substrate reactions to convert colorless chromogens into colored end products. Of the enzymes used in these applications, only horseradish peroxidase and calf intestine alkaline phosphatase will be described briefly. Detailed descriptions and information for the preparation of the most commonly used substrate-chromogen mixtures and their appropriate use and advantages or disadvantages are available in references 4-7.

Horseradish Peroxidase (HRP)

The enzyme horseradish peroxidase (HRP) has a molecular weight 40 kDa and is isolated from the root of the horseradish plant (Cochlearia armoracia). HRP has an iron-containing heme group (hematin) as its active site, and in solution is colored brown. The hematin of HRP first forms a complex with hydrogen peroxide (H202), and then causes it to decompose, resulting in water and atomic oxygen. HRP oxidizes sever-

PETITIONER EXHIBIT 1012
Page 211 of 218

al substances, two of which are polyphenols and nitrates. It should be noted that similar to many other enzymes, HRP and some HRP like activities can be inhibited by excess substrate. The complex formed between HRP and excess hydrogen peroxide is catalytically inactive, and in the absence of an electron donor (eg, chromogenic substance), is reversibly inhibited. It is the excess hydrogen peroxide and the absence of an electron donor that brings about quenching of endogenous peroxidase activities. Cyanide and azide are two other strong (reversible) inhibitors of peroxidase.

HRP can be attached to other proteins either covalently or non-covalently. Covalent binding of HRP is used in the dextran-polymer visualization systems, such as EnVision™ FLEX.

HRP Substrate – DAB

3,3' diaminobenzidine tetrahydrochloride (DAB) is a HRP substrate that produces a brown end product which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerization, resulting in the ability to react with osmium tetroxide, and thus increasing its staining intensity and electron density. Of the several metals and methods used to intensify the optical density of polymerized DAB, gold chloride in combination with silver sulfide appears to be the most successful (4). DAB has been classified as a potential carcinogen and therefore should be handled and disposed of with appropriate care.

HRP Substrate – AEC

3 amino-9-ethylcarbazole (AEC) is a HRP substrate that forms a rose red end product which is alcohol soluble. Therefore, specimens processed with AEC must not be immersed in alcohol or alcoholic solutions (for example Harris' hematoxylin). Instead, an aqueous counterstain and mounting medium should be used. AEC is unfortunately susceptible to further oxidation and when exposed to excessive light will fade in intensity. Storage in the dark is therefore recommended.

Alkaline Phosphatase (AP)

The enzyme calf intestine alkaline phosphatase (AP) has a molecular weight of 100 kDa and removes (by hydrolysis) and transfers phosphate groups from organic esters by breaking the P 0 bond; an intermediate enzyme-substrate bond is briefly formed. The chief metal activators for AP are Mg^{++}, Mn^{++} and Ca^{++}.

AP had not been used extensively in IHC until publication of the unlabeled alkaline phosphatase-antialkaline phosphatase (APAAP) procedure (8, 9). The soluble immune complexes utilized in this procedure have molecular weights of approximately 560 kDa. The major advantage of the APAAP procedure compared to the earlier peroxidase techniques was the lack of interference posed by endogenous peroxidase activity. Because of the potential distraction of endogenous peroxidase activity, the alkaline phosphatase techniques were particularly recommended for use on blood and bone marrow smears. Endogenous alkaline phosphatase activity from bone, kidney, liver and some white cells can be inhibited by the addition of 1 mM levamisole to the substrate solution (10), although 5 mM has been found to be more effective (11). Intestinal alkaline phosphatases are not adequately inhibited by levamisole.

In the immunoalkaline phosphatase staining method, the enzyme hydrolyzes naphthol phosphate esters (substrate) to phenolic compounds and phosphates. The phenols couple to colorless diazonium salts (chromogen) to produce insoluble, colored azo dyes. Several different combinations of substrates and chromogens have been used successfully.

AP Substrate – Naphthol AS-MX Phosphate

This can be used in its acid form or as the sodium salt. The chromogens Fast Red TR and Fast Blue BB produce a bright red or blue end product, respectively. Both are soluble in alcoholic and other organic solvents, so aqueous mounting media must be used. Fast Red TR is preferred when staining cell smears.

AP Substrate – New Fuchsin

This substrate also gives a red end product. Unlike Fast Red TR and Fast Blue BB, the color produced by New Fuchsin is insoluble in alcohol and other organic solvents, allowing for the specimens to be dehydrated before coverslipping. The staining intensity obtained by use of New Fuchsin is greater than that obtained with Fast Red TR or Fast Blue BB. Additional substrates include naphthol AS-BI phosphate, naphthol AS-TR phosphate and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP). Other possible chromogens include Fast Red LB, Fast Garnet GBC, Nitro Blue Tetrazolium (NBT) and iodonitrotertrazolium Violet (INT).

PETITIONER EXHIBIT 1012
Page 212 of 218

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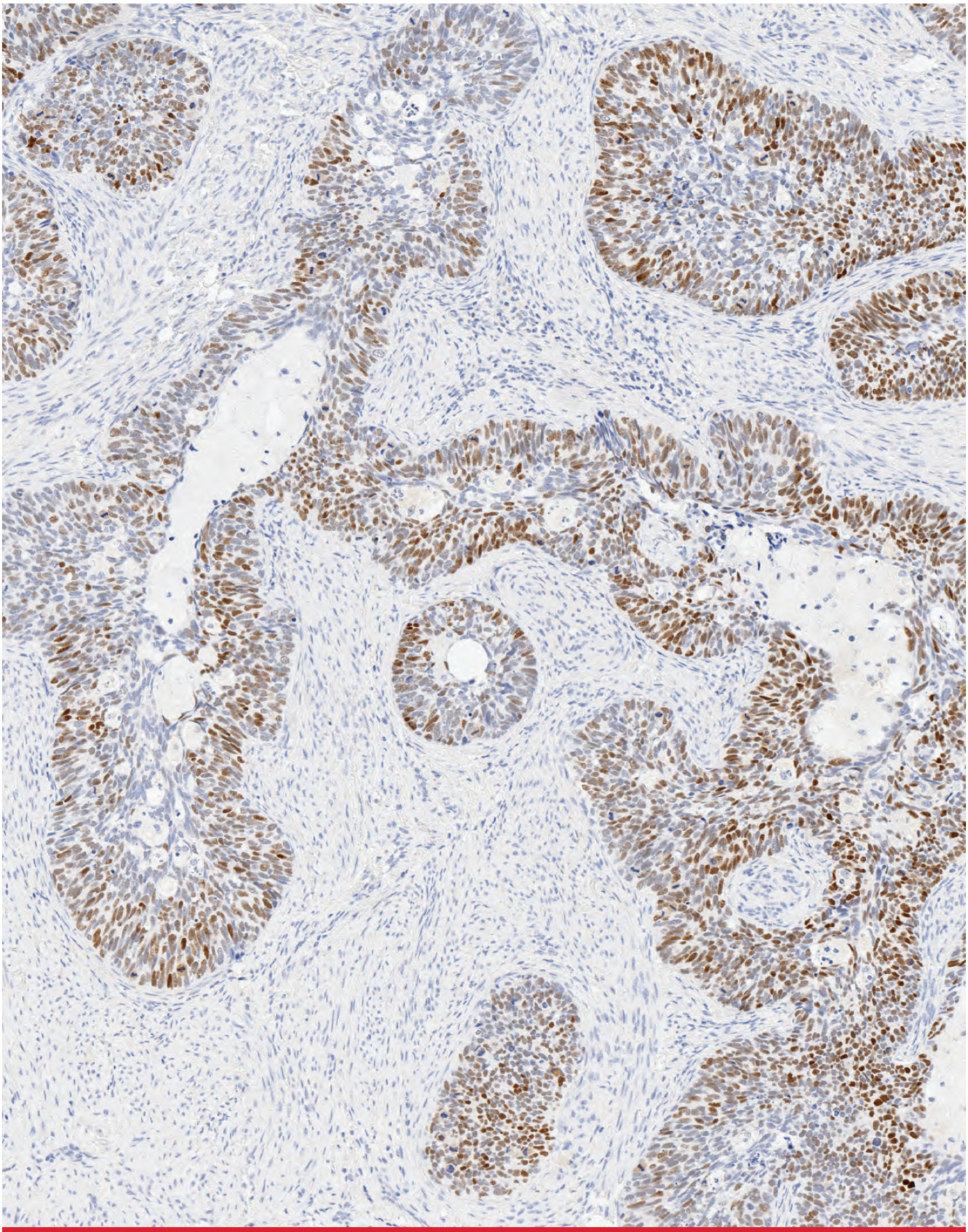
PETITIONER EXHIBIT 1012
Page 213 of 218



Index

ABC 79, 80, 81, 106, 173
Accessioning 11, 12, 21, 22, 123-125, 130
Adjuvant 135, 174, 202
AEC 69, 84, 86, 187, 188, 211
Affinity 13, 48-50, 53, 56, 57, 79, 84, 85, 106, 107, 175, 182, 202-205
Algorithm Chap 7 and 12, 86, 87, 91
Alkaline phosphatase 13, 84, 86, 105, 153, 172, 173, 182, 186, 188, 189, 191, 210, 211
Animal Research Kit 85, 205
Antibodies
adsorption 206
aggregates 176, 209
cocktail 49, 85
dilution 12, 65, 66, 106-109, 162, 175, 181
incubation temp. 104, 181, 183, 209, 210
incubation time Chap 5
polymerization 176, 206, 211
secondary 11-14, 79-87, 106, 107, 164, 176, 181-191
Antigen(s) (see also Epitope(s)) Chap 2, Chap 3, Chap 4, Chap 5, App A and 80-84, 87, 90, 99, 103, 104, 106, 107, 111, 113, 114, 115, 116, 118, 123, 145, 146, 160-167, 171, 174-177, 181-189, 191, 194, 209, 210
Antigen retrieval Chap 1, Chap 2, Chap 3 and 47, 49, 52, 54, 56, 57, 61, 64, 65, 67, 69, 70, 74, 103, 104, 111, 113-116, 118, 123, 174,176, 182, 183, 185, 188, 189, 202, 205, 209
APAAP 13, 205, 211
Artifact(s) 21, 26, 103, 115, 149, 164, 196
ASCO or CAP guidelines 21-23, 32, 61, 99, 171, 193
Avidin-Biotin 79, 80, 81, 84, 106, 173, 174
Avidity 48, 106, 203, 204
Autofluorescence 35, 87, 89, 90
Automation, IHC Chap 9 and 8, 9, 12-14, 18, 28, 33, 34, 36, 41, 42, 47, 49, 57, 58, 61, 64, 71, 91, 95, 98, 100, 127, 177, 178, 181, 183-185, 192-196
B5 fixative 176
Background Chap 15 and 9, 26, 36, 63-66, 73, 81, 82, 87, 91, 103, 106, 109, 111, 115, 142, 164, 166
181, 183-91, 195, 196, 209, 210
Bacterial 90, 177, 182, 185, 196
B cell(s) 24, 52, 55, 175, 198
Basal cell(s) 55, 83
Batch staining 114, 116, 117, 123
Biopsy 9, 11-13, 17, 26, 83, 145, 164, 183, 184, 191
Biotin (See also avidin-biotin) 79, 80-82, 84, 85, 106, 153, 163, 164
Biotinylated 80, 85, 177, 190
Breast cancer 14, 15, 23, 27, 32, 51, 55, 57, 83, 99, 133-135, 140, 146-148, 153-157
BSA 106, 176, 201, 206
Buffer(s) Chap 3 and 12, 47, 65, 69, 103, 106-109, 111, 113, 116, 119, 153, 171, 174, 176, 177, 181-184, 186-189, 195, 196, 204, 209
CAP 18, 22, 23, 28, 61, 99, 123, 130, 162-164, 168, 171, 178, 193
Capillary Gap 13, 111, 112
Cell line(s) 14, 17, 22, 27, 29, 53, 54, 90, 137, 146, 148, 163, 167, 168, 202, 203
Cell smear(s) 35, 84, 184, 211
Chromogen(s) 12, 61, 62, 64, 69, 82, 84-87, 91, 105, 109, 153, 155, 164, 171, 172, 181-183, 185-187, 191, 196, 210, 211
CISH Chap 13 and 15, 119, 120, 135, 153
Class II/Class III 21, 24, 28, 57, 99, 141
Clone(s) Chap 4 and 12, 16, 28, 33, 34, 65, 72, 75, 108, 161, 165, 166, 175, 192, 200, 202, 203
Companion diagnostics Chap 11 and 9, 13, 14, 51, 91, 157, 167
Conjugation 79-82, 85, 87, 88, 106, 134, 153, 171, 176, 177, 182, 205, 206
Connective tissue 22, 108, 176, 183-185
Connectivity Chap 10
Controls, Reagent Chap 14 and 16, 171, 184-190, 193
Controls, On-slide 28, 163, 165, 166, 169
Controls, Tissue Chap 14, Chap 16 and 12, 27, 28, 47, 49, 52, 54, 57, 58, 64, 65, 70-76, 148, 149
Cores Chap 12 and 26, 34, 50, 163, 165, 166,
Counterstain 12, 67, 69, 76, 86, 111, 118, 153, 155, 177, 181-183, 187-189, 211
Cross-links 23, 31, 32, 103, 104, 176, 183-185
Cross-reaction 31, 35, 48, 52-55, 84-86, 138, 175, 183-185, 201-205
Cryostat 184, 191
CSA 79, 81, 82
Cut-off 12, 47, 52, 125, 126, 136-139, 151
Cytology 153
Cytoplasmic 49, 52, 55, 58, 66, 83, 104, 173, 174, 184, 186, 193, 205
Degradation 17, 23, 62, 64, 76, 164, 165
Dextran 11, 13, 14, 80-82, 106, 205, 211
DAB 11, 63, 69, 74, 75, 79, 80, 84-86, 105, 109, 164, 171, 182, 187, 188, 190, 211
Dako Omnis 37, 41, 61, 105, 113, 114, 168
Digital imaging Chap 7 and 14, 69, 119, 169
Diluent 103, 106, 107, 164, 176, 177, 181, 182, 190, 192, 209, 210
Disulfide bridges 199, 200
DNA 32, 35, 36 , 39, 83, 88, 90, 99, 119, 135, 145, 152, 157, 201
Double staining 13, 79, 84-86, 173
Drying 12, 16, 21, 26, 47, 109, 115, 123, 177, 185, 193, 196, 210
Dynamic gap 112, 113
EGFR 135, 140-142
ELISA 15, 18, 36, 53, 161, 196
Embedding 11, 12, 17, 21, 26, 76, 80, 90, 105, 111, 123, 124, 126, 129, 130, 149, 177, 182, 185, 193
EnVision™ 67, 68, 75, 81, 85, 86, 192, 211
Enzyme(s) 12-14, 21, 22. 27, 34, 35, 39, 62, 69, 80-82. 84-87, 89, 91, 103-106, 120, 153, 164, 173, 181-183, 191, 210, 211
EQA 18, 61, 70, 104, 168, 169
Epitope(s) 21, 23, 24, 28, 48, 49, 53, 63, 70, 76, 81, 84, 104-107, 169, 171, 175, 182, 194, 200-205
Epitope retrieval (see Antigen retrieval)
ER/PR 15, 17, 28, 48, 99, 133, 147, 166
Ethanol 35, 104
Expression Chap 5 and 17, 27, 28, 51, 53, 54, 56, 58, 107, 108, 133, 161, 165, 166
Expiry 109, 161, 182, 194, 195, 205, 206
Fab/F(ab')2 fragment 82, 85, 176, 177, 186, 199, 200,
Fade 151, 154, 211
False, negative or positive 15, 24, 33, 55, 61, 63, 64, 67, 71, 73, 75, 105, 107, 116, 162, 164, 165, 194-196
Fast Red/Blue 84, 86, 188, 189, 211
Fc fragment/receptor 79, 106, 175, 186, 199, 200
FDA 14, 16, 61, 73, 99, 133-136, 138, 141, 142, 156, 157, 167, 171, 192
Fixatives Chap 2 and 12, 16, 18, 50, 61, 64, 76, 104, 161, 174, 176, 177, 182-185, 191, 192
FITC 13, 82, 87-90, 153, 155
FISH Chap 13 and 15, 26, 27, 86, 90, 119, 133-135, 140, 142
FLEX, RTU Chap 5 and 22, 24, 166
Flow cytometry 35, 36, 85-87
Fluorescent dye 79, 85-89
Fluorescent probe Chap 13 and 90
Fluorescyl-tyramide amplification 82
Formalin fixation 12-14, 16, 17, 22-27, 31, 35, 40, 42, 49-51, 53, 56, 64, 76, 79, 90, 103, 104, 169, 176, 183-185, 202
Frozen sections 15, 26, 27, 31, 35, 36, 80, 84, 87, 90, 95, 96, 98, 145, 172, 173, 175, 177, 182, 184, 192
FFPE tissue 13- 17, 31-36, 42, 53-56, 65, 76, 79, 80, 84, 85, 87, 95, 103, 109, 136, 137, 154, 155, 172,
Grossing 11, 12, 21-23, 26, 123-126, 129, 130, 183, 184, 191

PETITIONER EXHIBIT 1012
Page 215 of 218

HER2 Chap 11, Chap 13 and 13-17, 21-23, 25, 27-29, 32, 57, 75, 98, 99, 146, 147, 162, 168
HercepTest™ 14, 27, 28, 57, 99, 133-135, 142, 167, 168
HIER Chap 3 and 12, 13, 17, 47, 49, 56, 57, 103, 104, 174, 183 192, 209
HRP 14, 67, 68, 74, 75, 79, 81, 84-86, 105, 106, 153, 155, 172, 177, 182, 186-188, 190, 210, 211
Hybridoma 13, 48, 202-204
Hydrophobicity 106, 107, 171, 176, 177, 184, 185
iCSA 79, 82
Image analysis Chap 7 and 83, 87, 91, 142, 150
Immune complex 12, 14, 62, 79-81, 106, 173, 175, 176, 200, 204, 205, 211
Immunization 13, 48, 174, 175, 202, 205
Immunofluorescence 26, 35, 86-91, 108
Immunogen (see also antigen) 49, 54, 202
Ischemic time 21, 22, 63, 64, 76, 115, 164, 167, 169
LDT 57
Liquid Overlay 112
LIS 12, 14, 111, 120, 123, 128, 129, 194
LSAB 79-81, 106
Lymphocyte(s) 24, 25, 46, 58, 186, 202
Lymphoid tissue 24, 104, 105, 172, 173, 186, 187, 189
Macrophages 175, 186
Membrane/membranous 22, 49, 52, 58, 104, 168, 175, 178, 186, 193
Microscope/microscopic Chap 7 and 10, 12, 13, 21, 26, 31, 35, 82, 85-87, 90, 91, 109, 112, 119, 123, 153, 154, 156, 160, 167, 169
Monoclonal antibodies App A and 13, 16, 24, 48-50, 54-56, 58, 85, 109, 133, 164, 175-177, 181, 182, 185, 188-190, 209
Morphology 15, 17, 21, 22, 25, 26, 31, 32, 35, 69, 79, 87, 97, 98, 100, 103, 104, 119, 153-155, 194
Mounting 17, 25, 42, 185, 191, 193
Mounting medium 155, 182, 192, 211
Multi-Staining 34, 83-85, 87, 91, 175
Naphthol 211
NBF 22-25, 27, 28
Next generation sequencing 15, 111, 119, 142, 145, 153
New Fuchsin 188, 211
NordiQC 16, 18, 28, 48-50, 55, 56, 61, 69, 70, 73, 104, 165, 168, 178
Nuclear 33, 49, 52, 69, 83, 104, 153, 174, 184, 205
Overexpression 14, 27, 133, 141, 142
PAP 13, 14, 79, 205
Parallel processing 114, 118
PCR 36, 135, 153
Peroxidase, endogenous 49, 57, 58, 105, 153, 155, 171-173, 186, 187, 211
Pepsin 40, 41, 104, 155, 199, 200
Photobleaching 89
pH value 12, 23, 24, 32-34, 36, 40, 41, 47, 64, 65, 69, 74, 80, 103, 104, 106-108, 174, 176, 177, 181, 192, 194, 204, 205, 209
Polyclonal antibodies App B and 13, 16, 48, 49, 84, 164, 174, 176, 177, 182, 185, 188-190, 209
Polymer-based detection systems 13, 14, 25, 67, 79, 80, 81, 106, 108, 153, 163, 171, 174-176, 193, 205, 206, 211
Post-analytic Chap 7 and 12, 16, 17, 21, 47, 70
Pre-analytic Chap 2 and 9, 12, 16, 18, 47, 48, 53, 54, 61-63, 70, 71, 76, 115, 156, 163, 167, 169, 203
Precipitation 53, 82, 86, 153, 202, 204
Predictive 17, 21, 28, 29, 47, 51, 79, 91, 132, 133, 135, 136, 139, 142, 147, 156, 163
Pressure Cooker/Autoclave Chap 3 and 183
Pre-treatment Chap 3 and 47, 56, 84, 98, 103, 114, 128, 135, 155, 181, 183, 185, 192, 209
Primary antibody Chap 1, Chap 4, Chap 5, Chap 6, App A and 22, 24, 28, 33, 34, 104-107, 153, 161, 162, 164, 172, 176, 178, 181-190, 209, 210
Prognostic 17, 21, 28, 29, 47, 51, 79, 91, 135, 145, 147, 151
Probes 25, 89, 90, 153-155
Proteolytic (enzymatic) digestion Chap 3 and 17, 24, 25, 49, 56, 103, 104, 155, 183-185, 199, 200
Protocol Chap 4, Chap 5, Chap 8 and 8, 9, 12, 15-18, 22, 25-28, 32-34, 36-42, 83, 84, 98, 111, 113-118, 120, 123-125, 128, 136, 137, 139, 150, 155, 162, 164-168, 173, 178, 181-185, 187, 189, 190, 193, 194, 209, 210
Quantitative/quantification 8, 12, 15, 18, 21, 34-36, 47, 91, 98-100, 120, 142, 154, 167, 168
Quality control 13, 107, 146-149, 161, 162, 168, 169, 178
Red 84-86, 105, 188, 189, 211
Retrieval solution Chap 3 and 24, 74, 103, 104, 174, 192, 194
Robustness 47- 49, 54, 56, 62, 71, 73, 76, 137, 138, 153, 155
RNA/mRNA/miRNA 32, 35, 36, 83, 119, 153
RTU Chap 5 and 22, 24, 28, 33, 39, 49, 85, 96, 113, 116, 166, 178, 182, 206, 209
Saturation 63, 64, 67, 76
Semi-quantitative 14, 15, 17, 27, 61, 69, 79, 99, 100
Sensitivity 13, 14, 17, 47-49, 51-54, 56, 57, 73, 75, 79-82, 91, 107, 115, 117, 137-140, 146, 162, 163, 177, 181, 182, 184, 190
Serum/Serum proteins 13, 85, 106, 107, 164, 176, 183-186, 188, 189, 199-202, 204
Signal-to-noise 48, 53, 62-64, 73-75, 82, 209
Specificity 12, 13, 17, 47-49, 51-55, 57, 73, 80, 81, 84, 90, 103, 105, 106, 108, 115, 117, 135, 138-140, 146, 161-163, 166, 167, 175, 182, 200, 201, 204, 205
Stability 14, 87, 106, 107, 138, 165, 168, 176, 193, 199, 200, 205, 209
Standardization Chap 1 and 8, 9, 21, 32, 34, 36, 39, 48, 49, 51, 54, 57, 58, 61, 69, 70, 76, 98, 111, 113, 148-150, 182, 183
Staining intensity Chap 4, Chap 5 and 15-18, 22, 33, 35, 87, 89, 90, 97, 99, 100, 104, 106, 108, 165-168, 182, 194, 195, 204, 209-211
TAT Chap 10 and 25, 69, 83, 116-119, 125, 127
T cells 53, 55, 56, 73, 75, 175
Telepathology 95, 96, 98
Test battery 34, 36, 42, 50
Theranostics (see companion diagnostics)
Thickness, section 12, 47, 64, 69, 96, 191
Tissue blocks 12, 31, 35, 100, 145, 146, 163, 183
TMA Chap 12 and 27, 28, 34, 50, 51, 53, 70, 165, 166
Tissue processing 11, 12, 24, 25, 53, 124, 126, 130, 161, 174
Total test 13, 14, 16, 18, 48, 169, 191
Trastuzumab 14, 133-135, 140-142, 156, 157
Troubleshooting Chap 16 and 22, 178
UK NEQAS 16, 18, 70, 104, 169, 178
Virtual microscopy (see digital imaging) Chap 7
Validation 15, 16, 25, 27, 28, 29, 34, 41, 48, 51-54, 57, 61, 70, 71, 73, 76, 99, 107, 116, 136-140, 145, 150, 161-163, 169, 192
Water bath Chap 3 and 109, 130, 177, 185, 191
Verification 22, 29, 49, 61, 73, 76, 127, 161, 162
Wash buffers 37, 41, 42, 108, 109, 119, 153, 171, 177, 182, 184, 186, 188, 189, 195, 196
Wavelength 87-90
Wax/Dewax 21, 25, 26, 182-184
Western blotting 53, 161
Workflow Chap 10 and 64, 66, 68-70, 100, 114, 116-120, 210

PETITIONER EXHIBIT 1012
Page 216 of 218

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