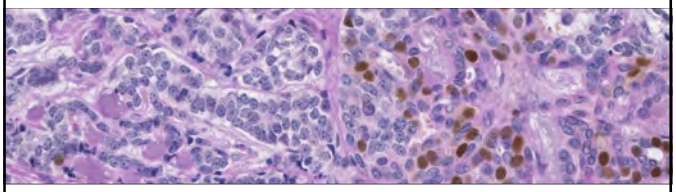
## Counterstains and IHC Interpretation



Dominique Trudel MD PhD FRCPC

September 20th, 2018

## Conflicts of interest

- Janssen-Ortho, Scientific advisor, unrelated work
- ODS Medical, Investigator initiated trial, unrelated work

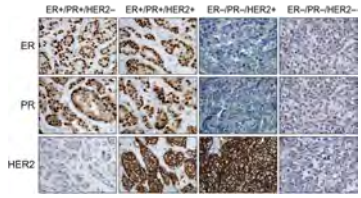
## Objectives

**At the end of this session the pathologist should be able to:**

- describe the validation process for a new stain
- identify areas in which the immuno-HE could be used
- to compare the pros and cons of this technique compared to standard immunohistochemistry

## Standard immunohistochemistry (IHC)

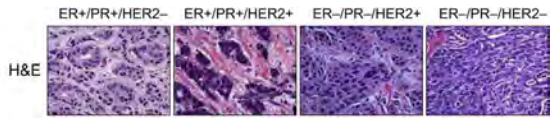
**A clear-cut contrast between signal and background:**
Breast cancer classification




Modified from Rivenbark *et al.*, Am J Pathol 2013

## Hematoxylin and Eosin (H&E) stain

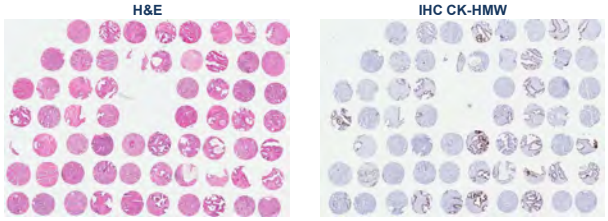
**Routinely used to provide accurate morphological diagnosis:**
Breast cancer grading




Modified from Rivenbark *et al.*, Am J Pathol 2013

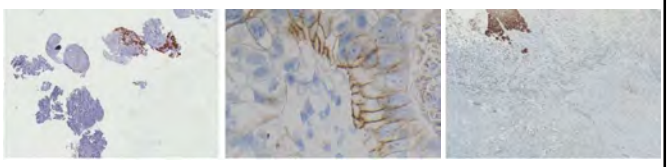
## The need of IHC and H&E in serial sections

**Tissue microarrays: Research and quality controls**



## The need of IHC and H&E in serial sections

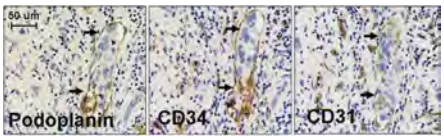
**HER2 in malignant glands of gastric adenocarcinoma**



Ruschoff *et al.*, Mod Pathol 2012

## The need of IHC and H&E in serial sections

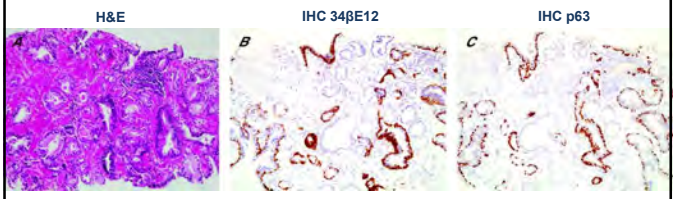
**The malignant phenotype of the cells floating in a vessel**




Mohammed *et al.*, Mod Pathol 2011

## The need of IHC and H&E in serial sections

**Difficult prostate biopsies**




Humphrey *et al.*, J Clin Pathol 2007

## IHC and H&E on the same slide?

TECHNICAL ARTICLE

Hematoxylin and Eosin Counterstaining Protocol for Immunohistochemistry Interpretation and Diagnosis

*Andrée-Anne Grosset, PhD,*†‡ Kevin Loayza-Vega, BSc,*† Éloïse Adam-Granger, BSc,*† Mirela Birlea, BSc,*† Blake Gilks, MD,§ Bich Nguyen, MD,‡‖ Geneviève Soucy, MD,‡‖ Danh Tran-Thanh, MD,‡‖ Roula Albadine, MD,‡‖ and Dominique Trudel, MD, PhD*†‡‖*

Grosset *et al.*, Appl Immunohistochem Mol Morphol 2017

## Tested parameters

- Hematoxylin: Harris, Gill I or Mayer
- Bluing reagent: $Li_2CO_3$, $NH_4OH$ or without
- Alcoholic eosin Y alone or with phloxine B
- Ethanol dilution: 100%, 95%/100% or 70%/95%/100%
- Time for each reagent and water

## Tested counterstaining protocols on tonsil

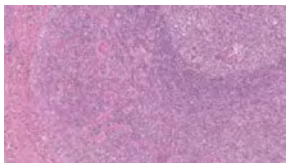
**Mayer Hematoxylin + $Li_2CO_3$**

| $Li_2CO_3$ | 30 sec |
|---|---|
| Hematoxylin | 30 sec |
| Eosin Y | 30 sec |
| Ethanol 70%/95%/100% | 180 sec |

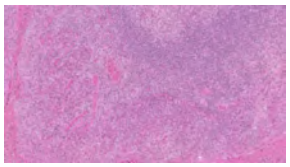
| $Li_2CO_3$ | 5 sec |
|---|---|
| Hematoxylin | 45 sec |
| Eosin Y | 45 sec |
| Ethanol 95%/100% | 90 sec |

## Tested counterstaining protocols on tonsil

**Mayer Hematoxylin without bluing reagent**



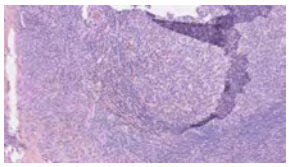
| Hematoxylin | 120 sec |
|---|---|
| **Eosin Y** | **6 sec** |
| Ethanol 70%/95%/100% | 90 sec |



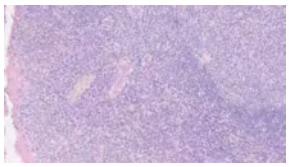
| Hematoxylin | 120 sec |
|---|---|
| **Eosin Y** | **12 sec** |
| Ethanol 70%/95%/100% | 90 sec |

## Tested counterstaining protocols on tonsil

**Gill I Hematoxylin without bluing reagent**



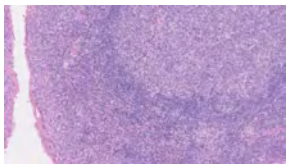
| Hematoxylin | 60 sec |
|---|---|
| **Eosin Y + Phloxine B** | **1 sec** |
| Ethanol 70%/95%/100% | 90 sec |



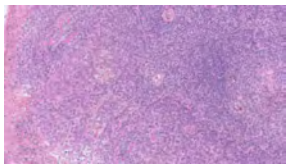
| Hematoxylin | 60 sec |
|---|---|
| **Eosin Y** | **1 sec** |
| Ethanol 70%/95%/100% | 90 sec |

## Tested counterstaining protocols on tonsil

**Gill I Hematoxylin without bluing reagent**



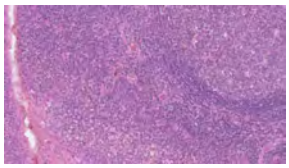
| Hematoxylin | 60 sec |
|---|---|
| **Eosin Y + Phloxine B** | **6 sec** |
| Ethanol 70%/95%/100% | 90 sec |



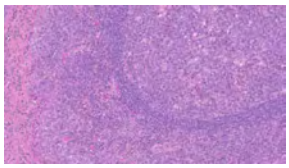
| Hematoxylin | 60 sec |
|---|---|
| **Eosin Y** | **6 sec** |
| Ethanol 70%/95%/100% | 90 sec |

## Tested counterstaining protocols on tonsil

**Gill I Hematoxylin without bluing reagent**



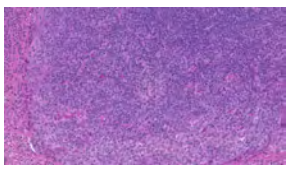
| **Hematoxylin** | **60 sec** |
|---|---|
| **Eosin Y + Phloxine B** | **12 sec** |
| Ethanol 70%/95%/100% | 90 sec |



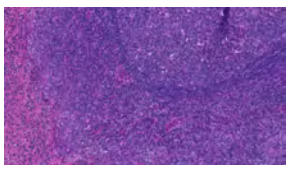
| **Hematoxylin** | **30 sec** |
|---|---|
| **Eosin Y** | **12 sec** |
| Ethanol 70%/95%/100% | 90 sec |

## Tested counterstaining protocols on tonsil

**Harris Hematoxylin + $Li_2CO_3$**



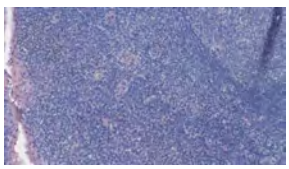
| Hematoxylin | 25 sec |
|---|---|
| Eosin Y + Phloxine B | 10 sec |
| Ethanol 100% | 45 sec |



| Hematoxylin | 120 sec |
|---|---|
| Eosin Y + Phloxine B | 120 sec |
| Ethanol 95%/100% | 60 sec |

## Tested counterstaining protocols on tonsil

**Harris Hematoxylin + $NH_4OH$**



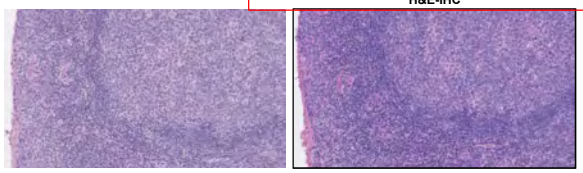
| Hematoxylin | 60 sec |
|---|---|
| **Eosin Y + Phloxine B** | **1 sec** |
| Ethanol 95%/100% | 60 sec |

| Hematoxylin | 60 sec |
|---|---|
| **Eosin Y** | **1 sec** |
| Ethanol 95%/100% | 60 sec |

## Tested counterstaining protocols on tonsil

**Harris Hematoxylin without bluing reagent**

**Selected protocol with 50% (vol/vol) solution of eosin: H&E-IHC**



| | |
|---|---|
| Hematoxylin | 60 sec + 60 sec water |
| Eosin Y | 1 sec |
| Ethanol 70%/95%/100% | 90 sec |

| | |
|---|---|
| Hematoxylin | 60 sec + 300 sec water |
| Eosin Y | 1 sec |
| Ethanol 95%/100% | 60 sec |

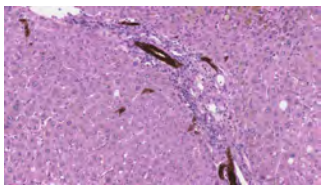
## Recommendations for validation of a new AB

- As many as 50 to 100 samples for a class II testing
- Assay accuracy of a 95% concordance rate is recommended
- Preferably by using 50% cases that are unequivocally positive and 50% cases that are the mixture of weakly positive and unequivocally negative.

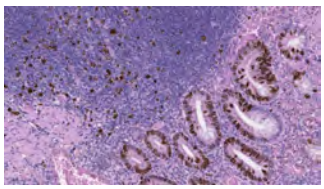
(**Canadian Association of Pathologists–Association canadienne des pathologistes National Standards Committee/Immunohistochemistry: Best Practice Recommendations for Standardization of Immunohistochemistry Tests, Torlakovic et al 2010**)

## H&E-IHC: Class I IHC tests

**Cytokeratin 7 in liver**

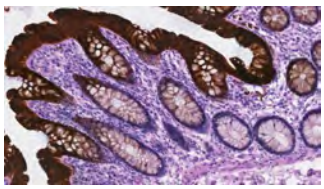


**Ki-67 in appendix**

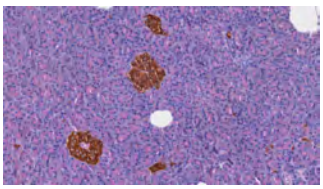


## H&E-IHC: Class I IHC tests

**Cytokeratin 20 in rectum**

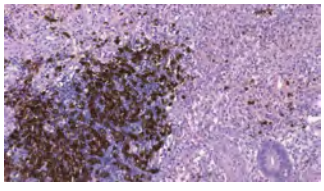


**Synaptophysin in pancreas**

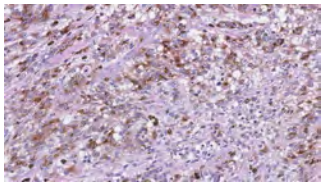


## H&E-IHC: Class I IHC tests

**CD20 in appendix**

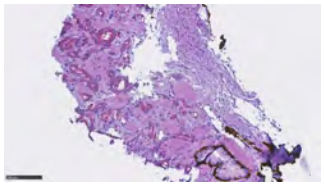


**HMB45 in melanoma**

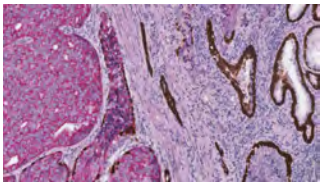


## H&E-IHC: double IHC staining

**Prostate biopsy**



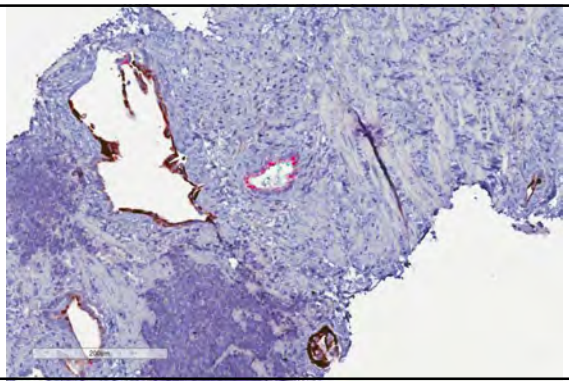
**Radical prostatectomy**

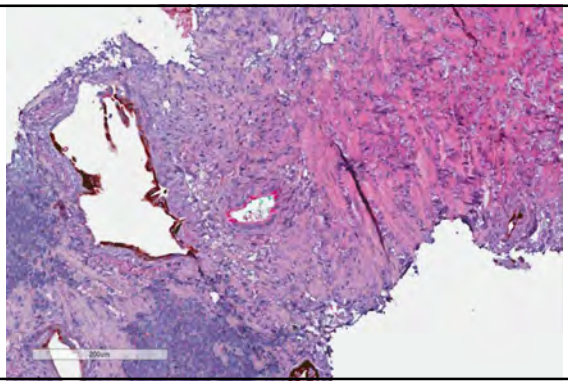


- high molecular weight cytokeratins and p63 (basal cell markers) with 3,3'-diaminobenzidine(DAB)
- α-methylacyl-CoA racemase (cancer cell marker) with alkaline phosphatase

## Validation on incident cases

- Standard IHC
- Slide scanning
- Coverslip removal
- Eosin staining

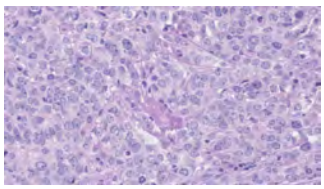




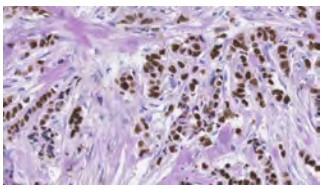
## H&E-IHC: Class II IHC tests

**Breast cancer clQc TMA: 40 cores (run 59)**

**Triple negative**



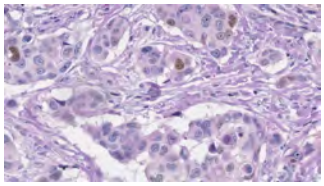
**Estrogen receptor positive**



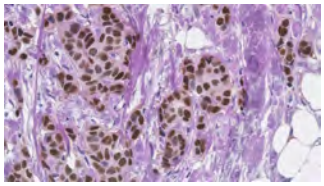
## H&E-IHC: Class II IHC tests

**Breast cancer clQc TMA: 40 cores (run 59)**

**Weak progesterone receptor positive**



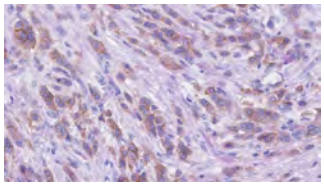
**Strong progesterone receptor positive**



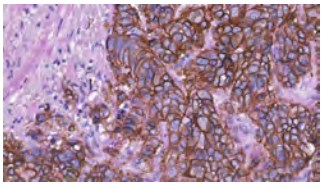
## H&E-IHC: Class II IHC tests

**Breast cancer clQc TMA: 40 cores (run 59)**

**HER2 2+**



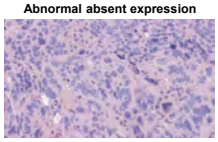
**HER2 3+**



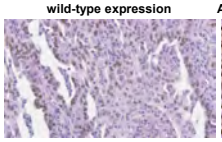
## H&E-IHC: Class II IHC tests

**Ovarian cancer cIQc TMA: 42 cores (run 54)**
**p53**

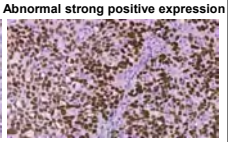
**Abnormal absent expression**



**Normal weak/moderate wild-type expression**



**Abnormal strong positive expression**



## Agreement between H&E-IHC, IHC, cIQc values

**Breast and ovarian cancer cIQc TMA**

**TABLE 2.** κ Values for Agreement Between H&E-IHC, Standard IHC, and cIQc Reference Values

| | Standard IHC and H&E-IHC | H&E-IHC and cIQc Reference Values | Standard IHC and cIQc Reference Values | Interval for cIQc Participants Mean (min-max) |
|---|---|---|---|---|
| ER | 1.00 | 0.93 | 1.00 | 0.96 (0.53-1.00) |
| PR | 0.87 | 0.83 | 0.77 | 0.88 (0.29-1.00) |
| HER2 | 0.93 | 1.00 | 1.00 | 0.98 (0.85-1.00) |
| p53 | 1.00 | 0.95 | 0.88 | 0.90 (0.76-1.00) |

cIQc indicates Canadian Immunohistochemistry Quality Control; ER, estrogen receptor; H&E-IHC, hematoxylin and eosin counterstaining of immunohistochemistry; PR, progesterone receptor.

## Inter/Intraobserver agreement

**Breast and ovarian cancer cIQc TMA**

**TABLE 3.** Interobserver Agreement Between Pathologists for H&E-IHC Interpretation

| | n | κ | P |
|---|---|---|---|
| ER | 32 | 1.00 | <0.001 |
| PR | 33 | 0.94 | <0.001 |
| HER2 | 32 | 0.94 | <0.001 |
| p53 | 34 | 0.89 | <0.001 |

ER indicates estrogen receptor; H&E-IHC, hematoxylin and eosin counterstaining of immunohistochemistry; n, the number of cores that were interpretable for all pathologists; PR, progesterone receptor.

**TABLE 4.** Intraobserver Agreement for H&E-IHC Interpretation

| | n | κ | P |
|---|---|---|---|
| ER | 32 | 1.00 | <0.001 |
| PR | 32 | 0.87 | <0.001 |
| HER2 | 33 | 1.00 | <0.001 |
| p53 | 37 | 0.95 | <0.001 |

ER indicates estrogen receptor; H&E-IHC, hematoxylin and eosin counterstaining of immunohistochemistry; n, the number of cores that were interpretable for all pathologists; PR, progesterone receptor.

## Cons

- Morphology is not exactly as crisp as for a standard H&E
  - Antigen retrieval
- Dual stain is not necessarily efficient in every organ
  - Racemase (alk-phos reveal) in kidney tumours

## Pros

- Every slide for which you would overlap the H&E to the IHC
  - Research TMAs
  - Prostate biopsies
  - Gastric HER2
  - Microinvasive breast carcinoma
  - Etc
- Two step validation process enhances security
- Seems to work with any hematoxylin/eosin combination
  - Needs validation

## Conclusions

**H&E-IHC in the clinical workflow…**

**For technicians**
- Addition of only ~10 seconds to the actual protocol
- Reagents available in laboratories
- Standardized clinical protocol

**For pathologists**
- Diagnosis and IHC interpretation on the same slide
- Faster and more accurate than serial sections

## Acknowledgments


**CRCHUM**

**Andrée-Anne Grosset**
**Kevin Loayza-Vega**
**Éloïse Adam-Granger**
**Mirela Birlea**
Mame Kany Diop
Noémi Roy
Vincent Quoc-Huy Trinh

**CHUM**

Roula Albadine
Danh Tran-Thanh
Geneviève Soucy
Bich Nguyen
Mélanie Girouard

**Vancouver General Hospital**

Blake Gilks