The Royal Children's Hospital Melbourne

Validation of ready to use antibodies

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Ready to use antibodies

- RTU
- Standardised by the manufacturer
- Come with a data sheet
  - Tested at low and high pH
  - Optimised concentration
  - Rigorous validation procedure
- Recommended methods and protocol
- Plug and play (in theory)

Table 1. Recommended Staining Protocol for anti-BRAF V600E (VE1) antibody with OptiView DAB IHC Detection Kit on BenchMark XT, BenchMark ULTRA and BenchMark GX instruments.

Procedure Type	Method
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, 64 minutes
Enzyme (protease)	Not required
Pre-primary antibody peroxidase inhibition	Selected
Antibody (Primary)	BenchMark XT instrument 16 minutes, 37°C BenchMark ULTRA instrument 16 minutes, 36°C BenchMark GX instrument 28 minutes, 37°C
Counterstain	Hematoxylin II, 4 minutes
Post Counterstain	Bluing, 4 minutes

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- Often vendors test the antibody on a wide range of tissue types
  - So we don’t have to!
  - Complex in house validations are not necessary
  - Following protocol exactly means they are usually classed as a commercial IVD
  - Verification with in house controls with documentation necessary

BRAF V600E (Roche)

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Specificity

Table 2. Specificity of anti-BRAF V600E (VE1) antibody was determined by testing formalin-fixed, paraffin-embedded normal tissues.

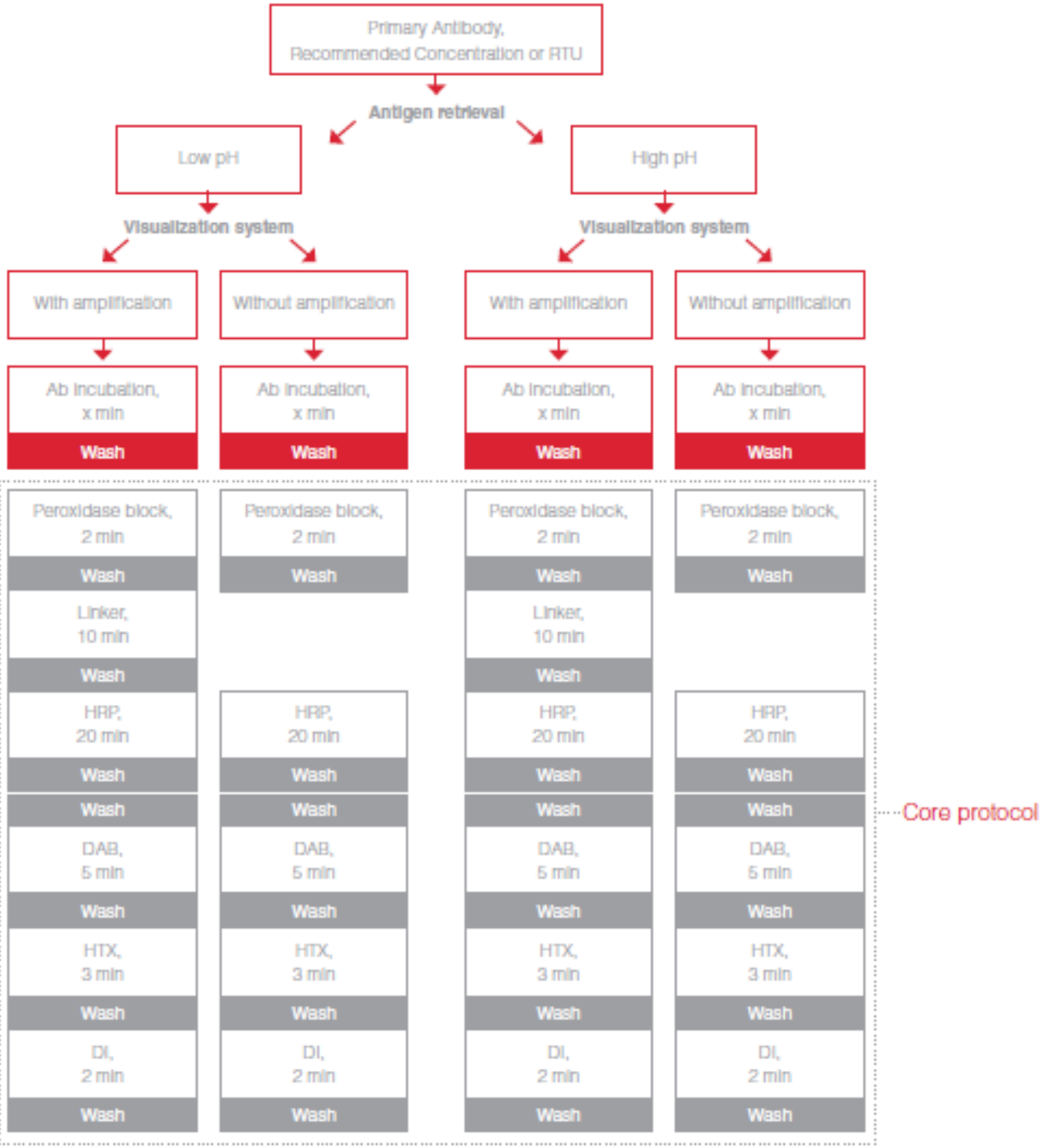
Tissue	#positive/ total cases	Tissue	#positive/ total cases
Cerebrum	0/3	Thymus	0/3
Cerebellum	0/3	Myeloid (bone marrow)	0/3
Adrenal gland	0/3	Lung	0/13
Ovary	0/3	Heart	0/3
Pancreas	0/3	Esophagus	0/3
Thyroid	0/4	Stomach	0/3
Parathyroid gland	0/3	Small intestine	0/3
Hypophysis	3/3*	Colon	0/3
Testis	2/3*	Liver	0/3
Breast	0/16	Salivary gland	0/3
Spleen	0/3	Kidney	0/3
Tonsil	0/3	Prostate	0/3
Endometrium	0/3	Cervix	0/3
Skeletal Muscle	0/2	Skin	0/3
Nerve (sparse)	0/3	Mesothelium	0/2
Bladder	0/3	Pleura and lung	0/1

* nuclear staining

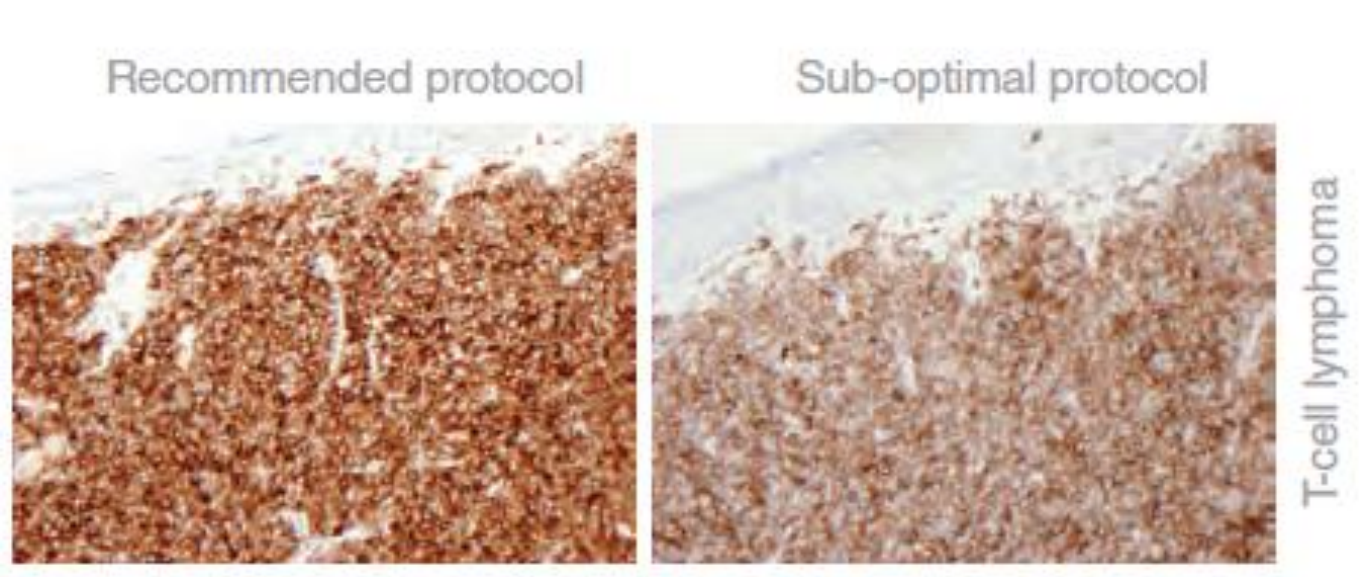
Sensitivity

Table 3. Sensitivity of anti-BRAF V600E (VE1) antibody was determined by testing a variety of formalin-fixed, paraffin-embedded neoplastic tissues.

Pathology	# positive / total cases
Glioblastoma	0/1
Atypical meningioma	0/1
Malignant ependymoma	0/1
Malignant oligodendroglioma	0/1
Serous papillary adenocarcinoma (ovary)	0/1
Mucinous papillary adenocarcinoma (ovary)	0/1
Islet cell carcinoma	0/1
Pancreatic adenocarcinoma	0/1
Seminoma	0/1
Embryonal carcinoma (testis)	0/1
Thyroid medullary carcinoma	0/1
Thyroid papillary carcinoma	21/28
Breast intraductal carcinoma	0/1
Breast invasive ductal carcinoma	2/132
Breast medullary carcinoma	1/9
Diffuse B-cell lymphoma	0/1
Lung small cell undifferentiated carcinoma	0/7


Primary Antibody,
Recommended Concentration or RTU
Antigen retrieval
Low pH
High pH
Visualization system
Visualization system
With amplification
Without amplification
With amplification
Without amplification
Ab incubation, x min
Wash
Peroxidase block, 2 min
Linker, 10 min
HRP, 20 min
DAB, 5 min
HTX, 3 min
DI, 2 min
Core protocol

- Stain recommended positive and negative tissues as indicated by the manufacturer
- Assess staining



Do your homework first

- RTU's aren't always the answer
- Check performance on Nordiqc if available
- Demonstrate sensitivity on low expressing tissue eg Fallopian tube in serous ca
- Don't calibrate with a strong Serous ca
- We failed QA with an RTU PAX8 (false negative)

What was done?

- We reviewed our protocol against the data sheet
- We also reviewed NordicQC
- Re-ran QA slides with no improvement
- Trialled the concentrate version
- Calibrated easily and demonstrated weak staining in the QA slide
- Switched from RTU to concentrate

- The use of an RTU solution
  - Reduces the resources necessary to verify performance when introducing new antibodies in the laboratory.
  - 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.

- Even with the use of a standardised method, there are variables which can affect the staining:
  - Fixation; time, type
  - Wax;
  - Slides; type, adhesion type
  - Adhesive in waterbath
  - Water quality in buffers, waterbaths etc
  - Bacterial growth
  - Expired reagents
  - Improper transport conditions
  - Improper storage (due to blackouts, equipment failure etc)

Not all RTU are robust

- Some antibodies can start to “go off” before their expiry date
- We find that some antibodies are sensitive to temperature
- PMS2, Myogenin and WT-1 are our worst offenders
- Start to notice aberrant background staining with poor nuclear signal

Changing the RTU protocol

- This occurs if the published method isn't sufficiently sensitive enough for what you are testing for
- Or if the signal is too strong with heavy background
- Changing the recommended protocol means it is now a laboratory developed test
- A more rigorous validation is recommended

Example: BRAF on brain tumours

- The recommended protocol was not sufficiently sensitive enough to detect BRAF V600E mutant protein at RCH
- Our cases were confirmed by PCR
- Increasing the retrieval and adding a short amplification step calibrated the staining
- We now only send equivocal or negative BRAF IHC cases for PCR

BRAF on brain tumours

Validation / Verification Report

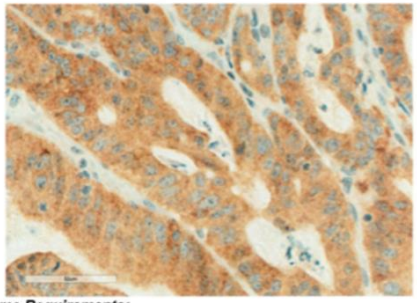
***SPECIMEN REQUIREMENTS:**

Proposed Sample Type:

- Formalin fixed, paraffin embedded tissue, 3um thick

Specimen Acceptance/Rejection Criteria

- Strong cytoplasmic staining in positive cases.



Sample Volume Requirements:

- 3um paraffin section on adhesive slide.

Sample Storage Requirements:

- Room temp for paraffin sections and blocks

DATA EVALUATION

CORRELATION DATA:

Case #	IHC result	BRAF V600E mutation analysis
18952932	Positive	Detected
15950952	Positive	Detected
15952086	Positive	Detected
17954377	Positive	Detected
16953017	Positive	Detected
18950135	Equivocal	Not Detected
18950340	Negative	Not Detected
18950552	Negative	Not Detected
18951202	Equivocal	Not Detected

INTRA RUN REPLICATE TESTING:

N/A

INTER RUN REPLICATE TESTING:

N/A

Sensitivity

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BRAF at RCH

- So BRAF in our lab is an (LDT)
- Our validation set used 9 cases (mixture of positive and negative)
- We knew of no lab to correlate our cases with, so correlation was confirmed with PCR
- For other antibodies e.g OLIG2, we sent a duplicate set to another local lab

Controls

- BRAF is commonly used for colorectal ca along with the MSI panel
- It is also used for Thyroid papillary carcinoma
- The controls you select should reflect the entity you are demonstrating if possible eg HER2 breast should have a mix of breast control

Controls

- In our lab we use BRAF for brain tumours
- We also use it to confirm Langerhans cell histiocytosis on skin biopsies
- It is hard to source controls as our paediatric brain biopsies are small and tissue is needed for diagnosis and clinical trial work
- So we compromise and use colon ca

BRAF on brain biopsies

- The plan is to source brain tumour (pos)
- Take a small punch of tissue
- Embed it on end to preserve tissue
- We have done this for LIN28

IHC Critical Assay Performance Controls (iCAPCs)

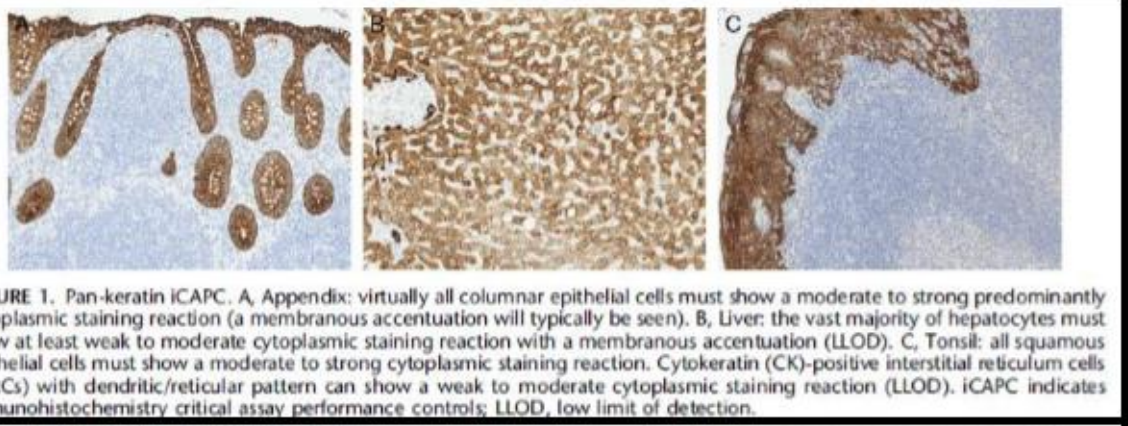

FIGURE 1. Pan-keratin iCAPC. A, Appendix: virtually all columnar epithelial cells must show a moderate to strong predominantly cytoplasmic staining reaction (a membranous accentuation will typically be seen). B, Liver: the vast majority of hepatocytes must show at least weak to moderate cytoplasmic staining reaction with a membranous accentuation (LLOD). C, Tonsil: all squamous epithelial cells must show a moderate to strong cytoplasmic staining reaction. Cytokeratin (CK)-positive interstitial reticulum cells (CIRCs) with dendritic/reticular pattern can show a weak to moderate cytoplasmic staining reaction (LLOD). iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

FIGURE 7. TTF-1 iCAPC. A, Thyroid: virtually all epithelial cells must show a strong nuclear staining reaction. B, Lung: virtually all pneumocytes and basal cells of terminal bronchi must show a moderate to strong nuclear staining reaction. Columnar epithelial cells of terminal bronchi must show an at least weak nuclear staining reaction (LLOD). C, Tonsil: no staining reaction must be seen. iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

FIGURE 8. CDX-2 iCAPC. A, Appendix: virtually all epithelial cells must show a strong nuclear staining reaction. A weak cytoplasmic staining reaction in addition to strong nuclear staining is often present. B, Pancreas: the majority of epithelial cells of intercalated ducts must show a weak to moderate nuclear staining reaction (LLOD). C, Tonsil: no staining reaction must be seen. iCAPC indicates immunohistochemistry critical assay performance controls; LLOD, low limit of detection.

Examples for 17 markers

Generel expected patterns

High expression
(Right antibody)

Low expression
(Appropriate sensitivity)

No expression
(Appropriate specificity)

Which tissue
Which cells
Which extension
Which intensity

- Analytical validation
  - Laboratory developed tests (concentrates and RTU formats being applied modified to official protocol)
  - Non-predictive markers (- ER, PR, HER-2..)
    - CLSI: 20 cases per entity relevant (pos, neg)
    - CAP: 10 positive, 10 negative

      The validation set should include high and low expressors for positive cases when appropriate and should span the expected range of clinical results (expression levels) for markers that are reported quantitatively.
    - Ad-Hoc: 10 strongly pos, 10 interm. to low, 5 neg.

Number less important compared to use of tissue with full range of expression patterns reflecting the diagnostic use

In summary

- Ready to antibodies are usually ronust and simple to integrate into your lab
- Their application could dictate that you change the parameters making it a LDT
- Control selection is as important as the antibody itself

Any questions?

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