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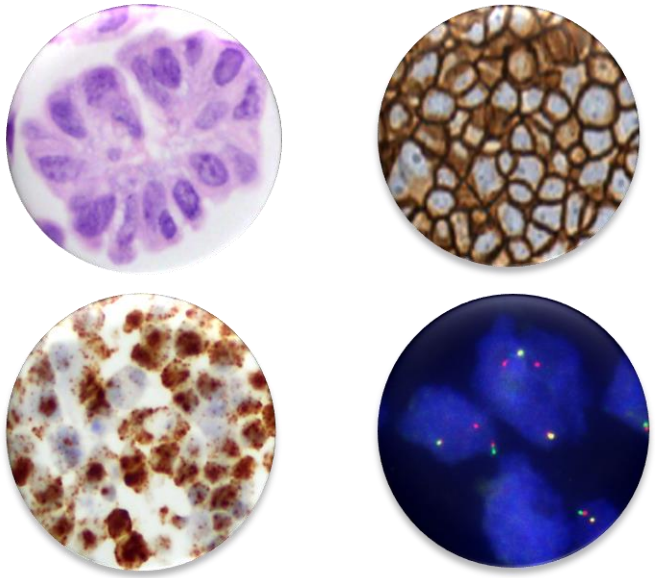

HPV/p16 Analyte Control

Utility review and ring study results

Colin Tristram, Director

bsi. ISO 13485 Medical Devices Quality Management

MD 659011

2017

HPV/p16 Analyte Control

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Collaboration:

- Dr Max Robinson at Newcastle University a leading pathologist in head and neck cancer.
- Keith Miller, United Kingdom National External Quality Assurance Scheme for ICC and ISH (UKNEQAS).

Drivers:

- Clinical trials – large volume assessments. No consistency of control material across all cases when using tissue as same slide controls.
- EQA – varying quality of assays in HPV ISH and p16 despite "standardisation".
- Unmet needs assessment:

  Testing varies by country. HPV ISH vs p16. Assessment of cervical carcinoma often different to oral and oropharyngeal squamous cell carcinoma.

  Significant demand in USA.

HPV and Cancer

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- While vaccines are reducing rates of HPV infection and associated cancers this is primarily in females.
- Oral cancer is still the 6th leading cancer by incidence globally (WHO).
- Virtually all cases of cervical cancer are caused by HPV infection, with HPV 16 & 18 detected in 70%.[1,2]
- HPV 16 is responsible for around 85% of anal cancers and HPV 16 & 18 account for approximately 50% of vaginal, vulval and penile cancers.[3]
- Within the last 20 years, the incidence of HPV-related oropharyngeal squamous cell carcinoma (OPSCC) has increased, particularly among men.
- HPV 16 has been identified in around 50% of OPSCC in the US.[4]
- It has been estimated that, by 2020, HPV will cause more OPSCC than cervical cancers in the US.[5]
- p16 positivity is a useful surrogate marker of oncogenic HPV infection. p16 negative oral cancer are typically caused by tobacco.
- HPV-related OPSCC tend to have a better prognosis.
- There is emerging evidence to suggest that HPV positive patients may benefit from de-escalated treatments.

1. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. *Lancet* 2007; 370(9590):890–907.
2. Muñoz N, Bosch FX, Castellsagué X, et al. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *International Journal of Cancer* 2004; 111(2):278–285.
3. Watson M, Saraiya M, Ahmed F, et al. Using population-based cancer registry data to assess the burden of human papillomavirus-associated cancers in the United States: overview of methods. *Cancer* 2008; 113(10 Suppl):2841–2854.
4. Jayaprakash V, Reid M, Hatton E, et al. Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis, 1985–2010. *Oral Oncology* 2011; 47(11):1048–1054.
5. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of Clinical Oncology* 2011; 29(32):4294–4301.
6. Bar-Ad V, Wag ZX, Leiby B, Tuluc M. Combination of p16 levels and pre-radiotherapy factors predicts outcome in patients treated for oropharyngeal carcinoma. *J BUON*. 2013 Oct-Dec;18(4):982-8.

HPV/p16 Analyte Control for use in:

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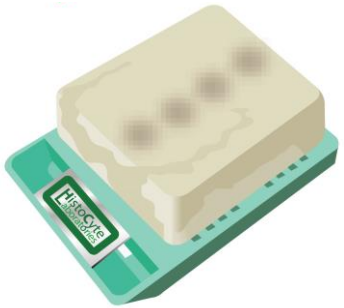
- **HistoCyte Laboratories Ltd developed: High-Risk Human Papilloma Virus (16, 18) Control Slides for same slide use in:**
  - HPV DNA in situ hybridization
  - E6/E7 mRNA in situ hybridisation
  - p16 immunohistochemistry

The following slides show typical staining achieved with the HPV/p16 Analyte Control^{DR} using:

- Ventana/Roche
  - CINtec® p16 Histology assay. Ready to use (RTU) antibody.
  - INFORM III HPV ISH assay
- ACDBio RNAScope E6/E7 mRNA assay



p16 immunohistochemistry

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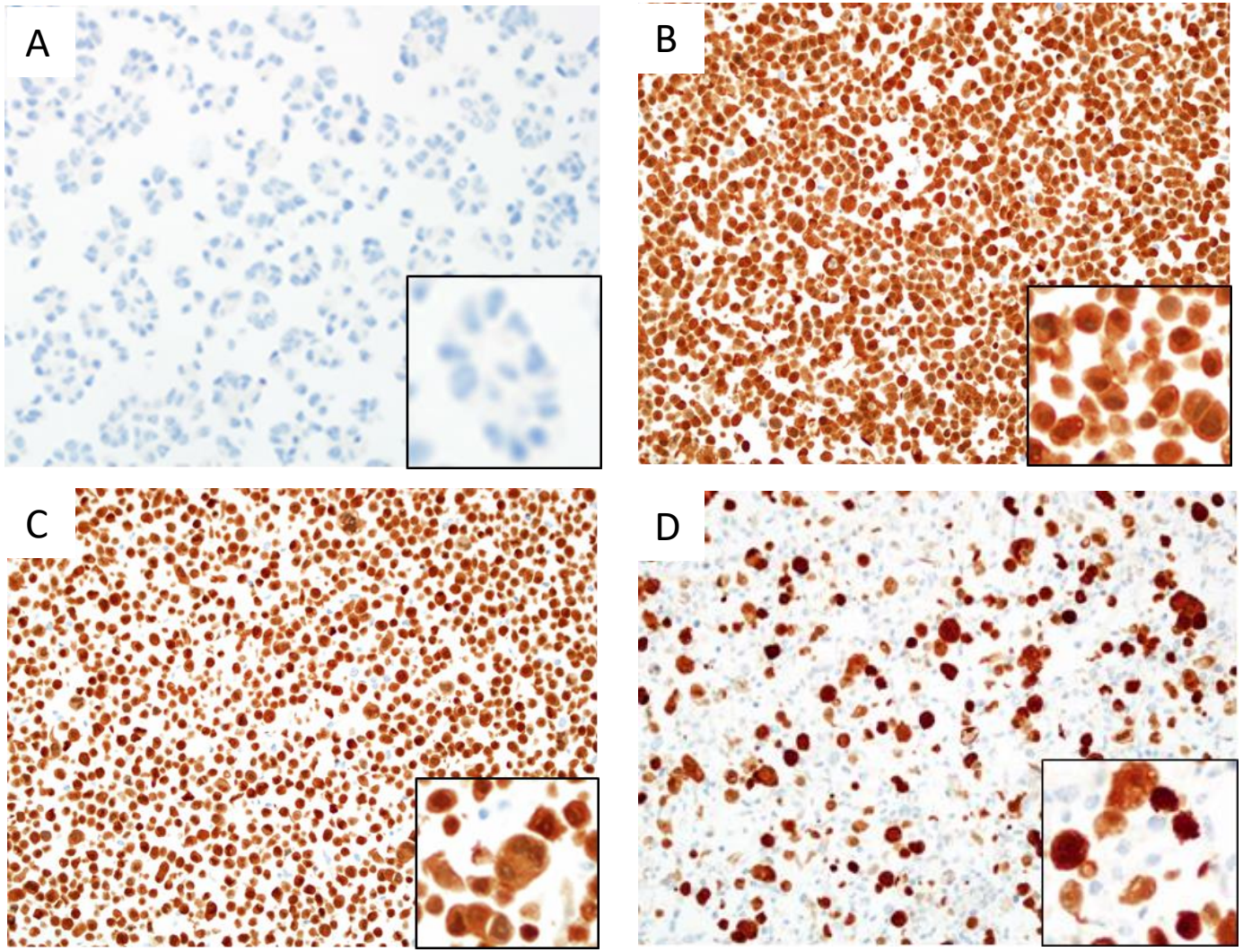

- p16 protein expression strongly associated with HPV infections.
- Cell (A) negative (no HPV)
- Cells (B) and (C) have high homogeneous expression throughout cell population.
- Cell (D) has a high heterogeneous expression (Cell D).

Ventana/Riche CINtec® p16 Histology assay

HPV DNA in situ Hybridization

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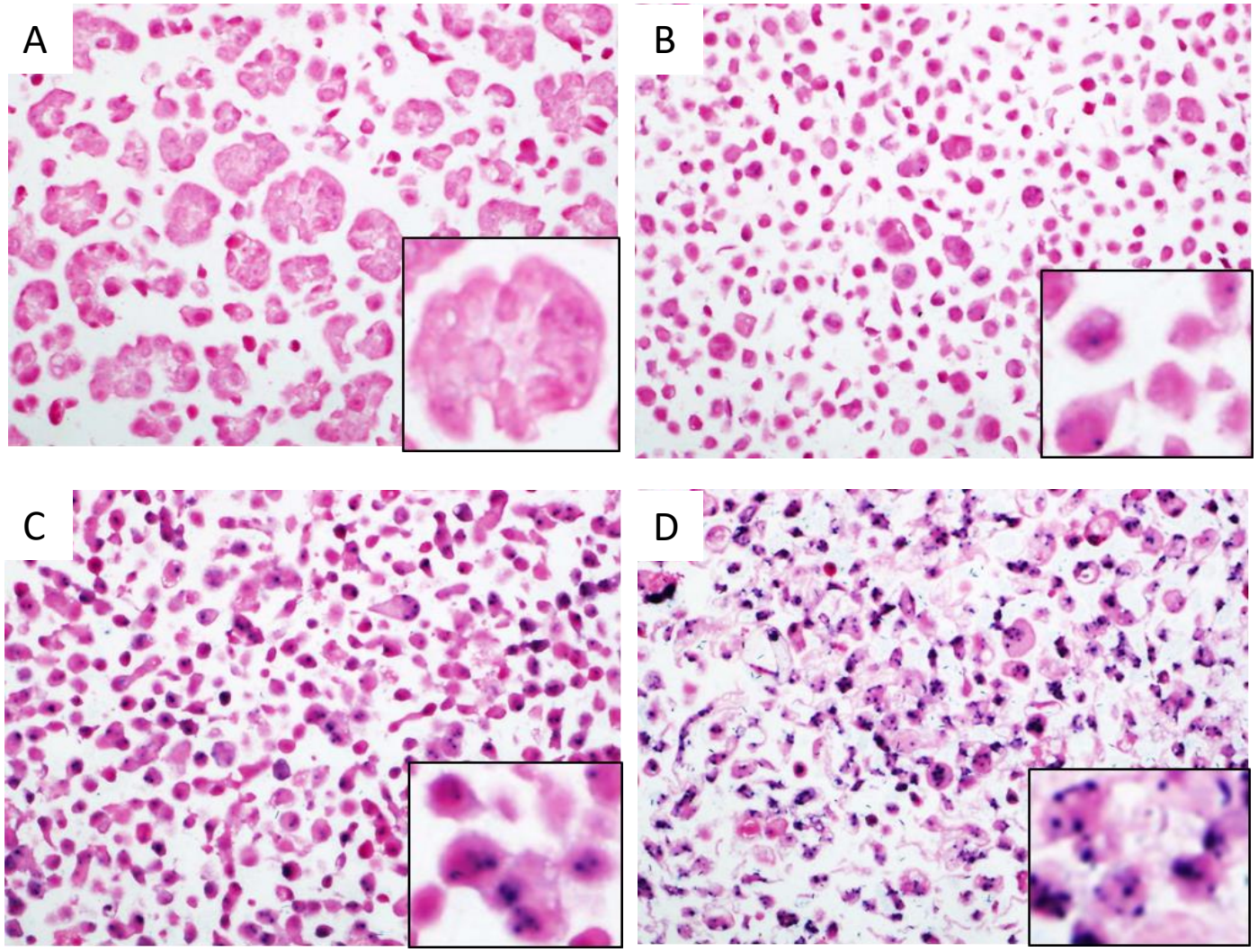

- HPV DNA in-situ hybridisation.
- Cell (A) is HPV negative.
- Cell (B) has very low HPV16 gene copies.
- Cell (C) has medium HPV18 gene copies.
- Cell (D) has high HPV16 gene copies.

Ventana/Roche INFORM III HPV ISH assay

E6/E7 mRNA in situ Hybridization

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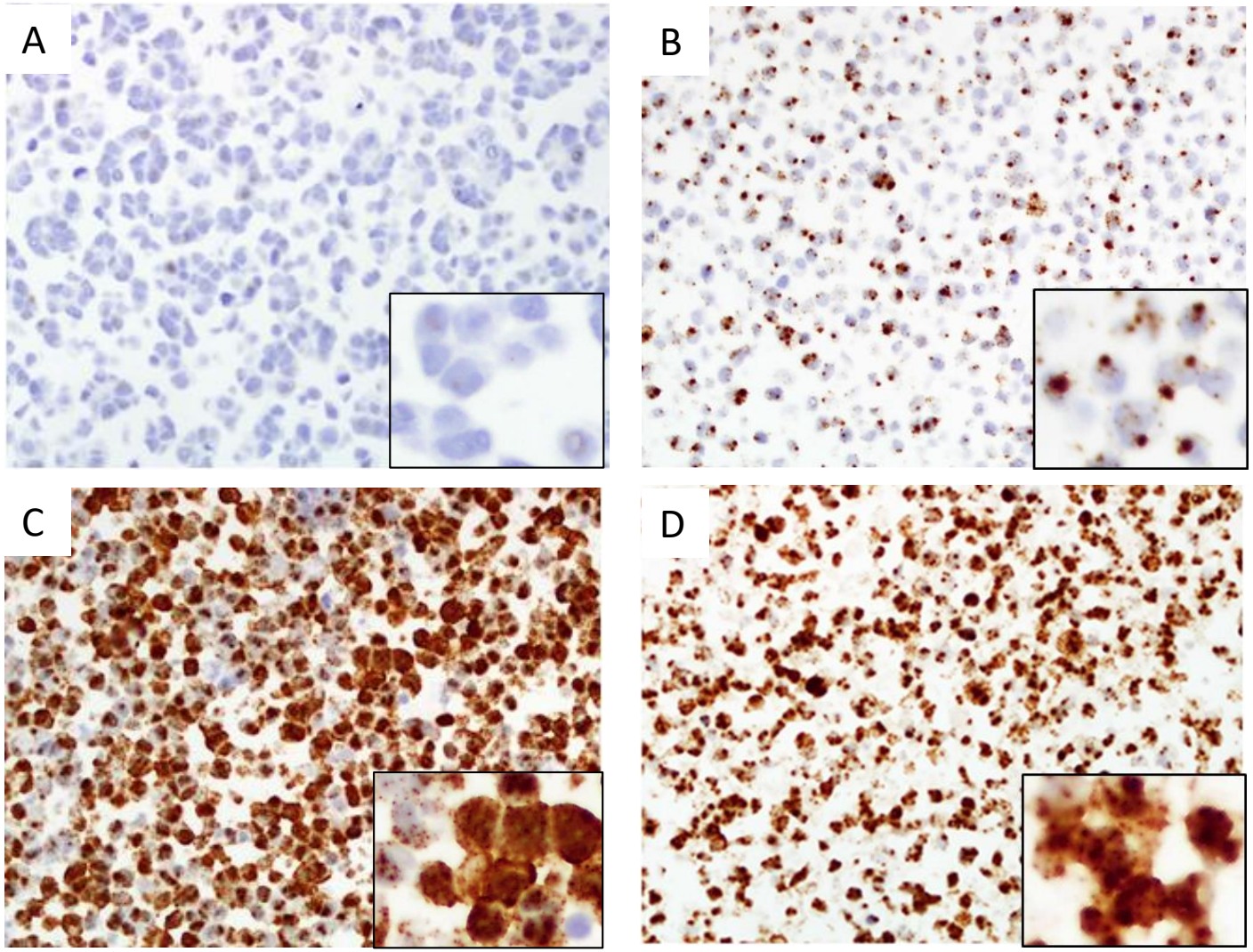

- mRNA in-situ hybridisation for E6/E7. ACD RNAScope
- Cell (A) is negative, though there is occasional back ground staining, it is not a genuine signal.
- Cell (B) is a low positive cell line, which reflects the low HPV gene copies.
- Cells (C) and (D) have high levels of HPV mRNA.

ACDBio RNAScope E6/E7 mRNA assay

Verification and validation

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- As part of verification and validation HistoCyte Laboratories Ltd assessed multiple batches over an extended period to determine stability and reproducibility. All testing was done with Roche/Ventana assays.
- To determine their utility in the market HistoCyte Laboratories Ltd conducted a “Ring Study” across 8 clinical sites.
  - 5 tested both HPV and p16
  - 8 tested for p16
- All HPV ISH assays were with Ventana/Roche
- X7 sites used p16 from Ventana/Roche:
  - 4/7 on Ventana Benchmark
  - 2/7 on Leica Bond
  - 1/7 on Dako Autostainer.
- X1 site used Santa Cruz Ab on Ventana Benchmark.

The results are summarised in the following slide

Ring study results: p16

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Summary of results from ring study: p16

Site No.	1	2	3	4	5	6	7	8
Platform	Ventana/Roche	Dako/Agilent	Ventana/Roche	Ventana/Roche	Ventana/Roche	Ventana/Roche	Ventana/Roche	Ventana/Roche
Assay	p16 IHC	p16 IHC	p16 IHC	p16 IHC	p16 IHC	p16 IHC	p16 IHC**	p16 IHC
Cell A	0	0	0 (blush)	0	0	0 (blush)	0	0
Cell B	5+ N/C >99%	4+ N/C >99%	4-5+ N/C >99%	3-5+ N/C >95%	3-4+ N/C >99%	5+ N/C >99%	5+ N/C >99%	3-5+ N/C >95%
Cell C	5+ N/C >99%	4-5+ N/C >99%	5+ N/C >99%	5+ N/C >99%	4-5+ N/C >99%	5+ N/C >99%	5+ N/C >99%	3-5+ N/C >99%
Cell D	3-5+ N/C 30-40%	3-5+ N/C 30-40%	3-5+ N/C 30-40%	3-5+ N/C 30-40%	2-4+ N/C 40-50%	4-5+ N/C 50-60%	4-5+ N/C 30%	3-5+ N/C 30-40%
	C Blush 2+	No Blush	C Blush 2+	No Blush	No Blush	C Blush 2+	C Blush	No Blush

* Cells appear over digested, morphology disrupted to some degree. Still interpretable. ** p16 from Santa Cruz Biotechnology used on the Roche platform. Clts: clusters. C: cytoplasm. Intensity scored on a scale of 0: negative to 5+: very strong.

Excessive cytoplasmic staining in Cell D

Clean cytoplasm in Cell D

All slides were anonymised and scored independently by two assessors.

Ring study results: HPV

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Summary of results from ring study: ISH					
Site No.	1	5	6	7	8
Platform	Ventana/Roche	Ventana/Roche	Ventana/Roche	Ventana/Roche	Ventana/Roche
Assay	HPV ISH	HPV ISH	HPV ISH	HPV ISH	HPV ISH
Cell A	0	0	0	0	0
Cell B	<5% (1-2 sigs)	<5% (1-2 sigs)	<5% (1-2 sigs)	<5% (1-2 sigs)	<5% (1-2 sigs)
Cell C	>50% (>2 sigs)	>50% (>2 sigs)	>60% (>2 sigs)	>80% (>2 sigs)	>60% (>2 sigs)
Cell D	>80% (sigs in clts)	>90% (sigs in clts)	>99% (sigs in clts)*	>99% (sigs in clts)*	>80% (sigs in clts)

* Cells appear over digested, morphology disrupted to some degree. Still interpretable. Clts: clusters.

All slides were anonymised and scored independently by two assessors.

Ring study review

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- The most striking result was the difference in sites that had both used the same assay and yet cell line D was generally providing two different results.
    1. Strong nuclear staining in 30-60% of cells with cytoplasmic staining.
    2. Strong nuclear staining 30-50% of cells with no cytoplasmic staining.
- The ISH results were consistent, cell C had signals in 50-80% of cells. The higher percentage correlated with excessive digestion demonstrated by damaged cell architecture (site 7).
- To determine the consistency of the p16 scoring done manually, the slides were scanned and assessed using Visiopharm image analysis. Performed by Visiopharm we were able to demonstrate consistency in the scoring (see next two slides) regardless of the excessive cytoplasm

Image Analysis Assessment Site #5

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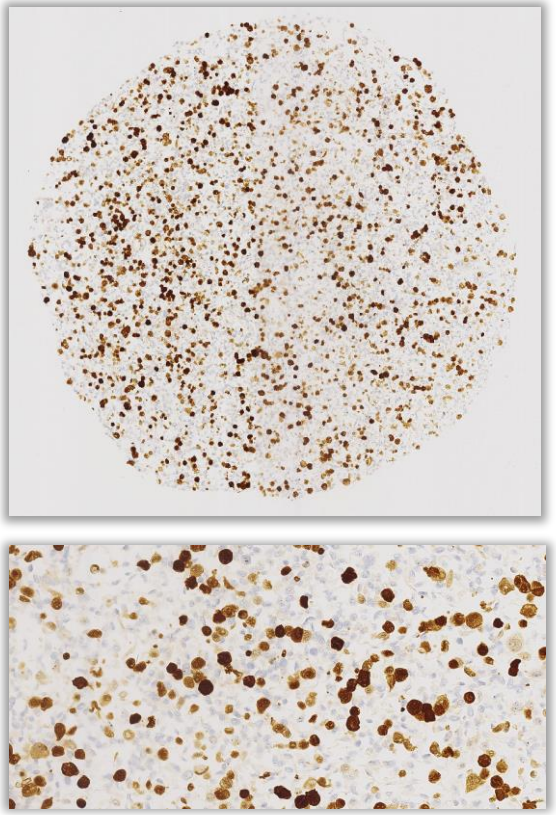
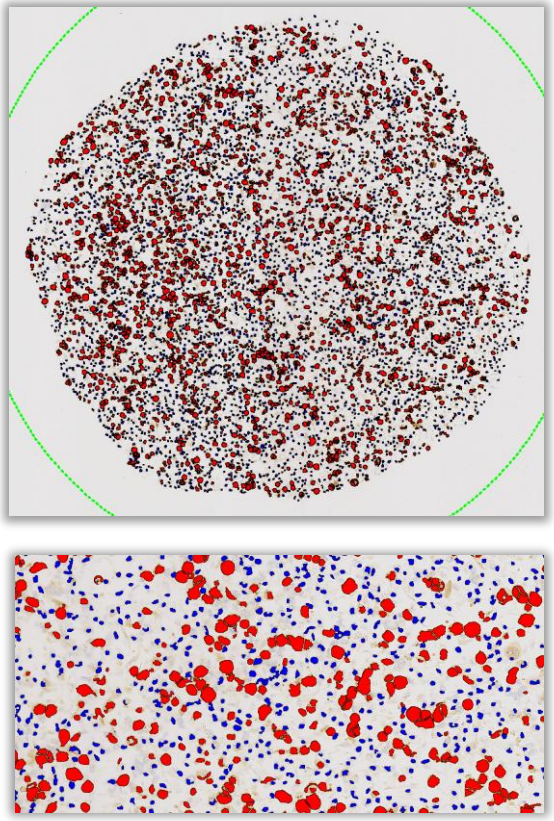
- Case 5 – “HCL standard” No staining in the cytoplasm of cell D. As seen in our development.





HCL Assessment	Ventana/Roche
Assay	p16 IHC
Cell A	0
Cell B	3-4+ N/C >99%
Cell C	4-5+ N/C >99%
Cell D	2-4+ N/C 40-50% No Blush

VisioPharm Analysis
Cells Counted
6,326
Positive Percentage
45%

VISIOPHARM®

Image Analysis Assessment Site #6

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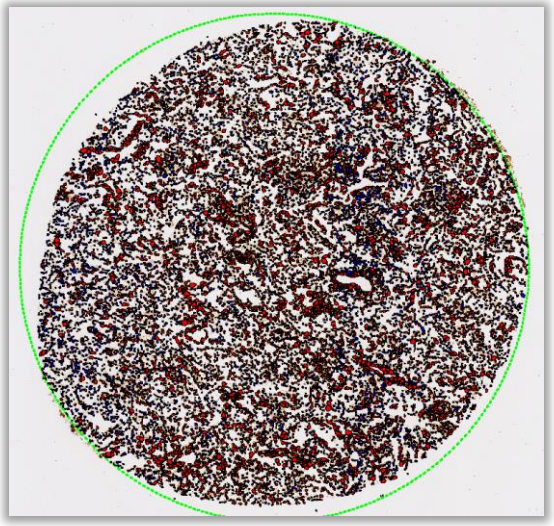
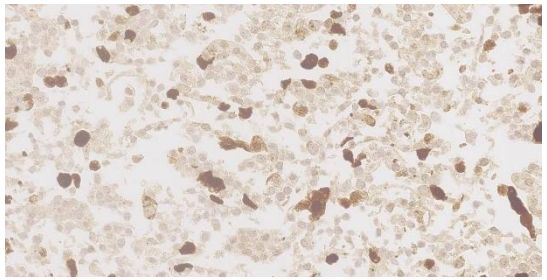
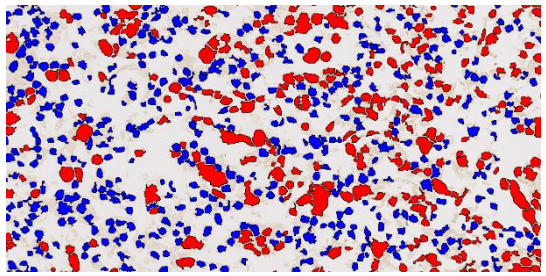
- Site 6 – Typical of strong staining and excessive staining in the cytoplasm of cell D.







HCL Assessment	
	Ventana/Roche
Assay	p16 IHC
Cell A	0 (blush)
Cell B	5+ N/C >99%
Cell C	5+ N/C >99%
Cell D	4-5+ N/C 50-60% C Blush 2+

VisioPharm Analysis
Cells Counted
14,737
Positive Percentage
62%

VISIOPHARM®

Observations from HPV in ISH

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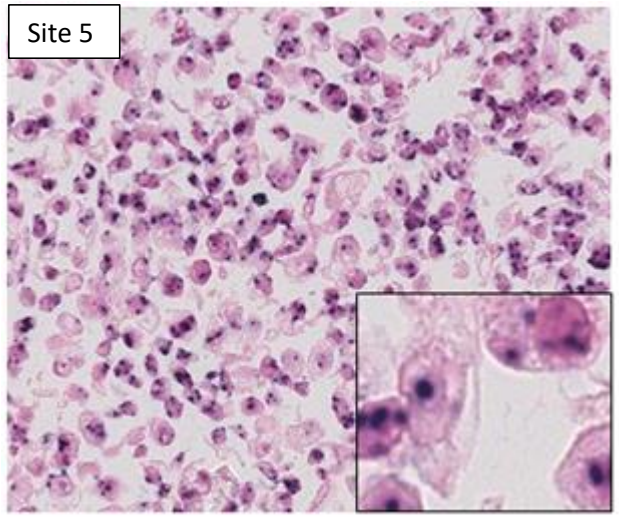
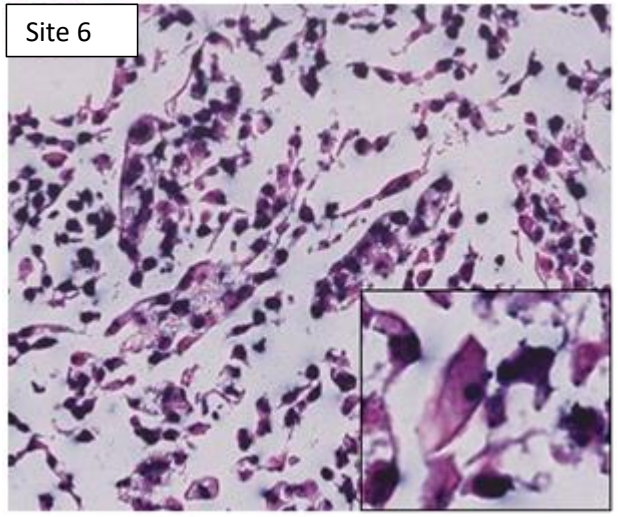
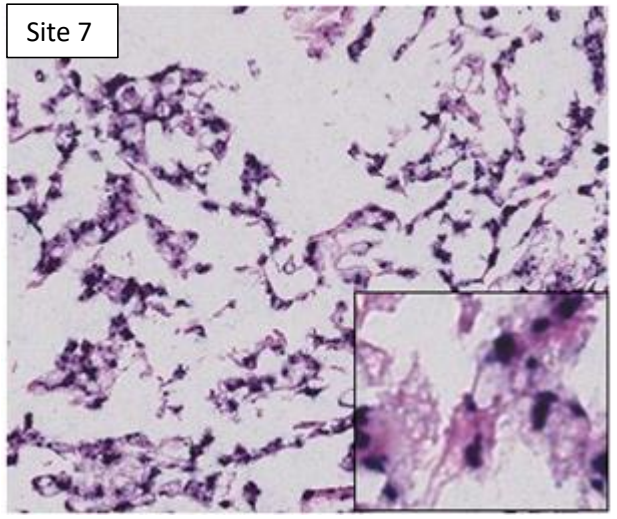
- Only 5 of the sites performed HPV ISH – All Roche Inform.
- All state they're using the "standard" protocol from Roche.







- Over digested Cell line D at Sites 6 and 7. Site 5 is a typical result.
- Site 6 protease step is 24 minutes compared to 8 minutes at Site 7 and 5.
- Denaturation is "standard" 2 hours with CC1.
- Sometimes the reasons for the differences are less obvious. In each of these cases the assay has worked as signal is very clearly seen, however, the cell integrity was clearly compromised.

Why differences in staining

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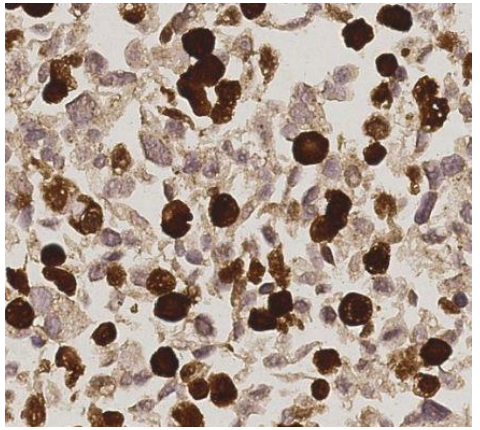
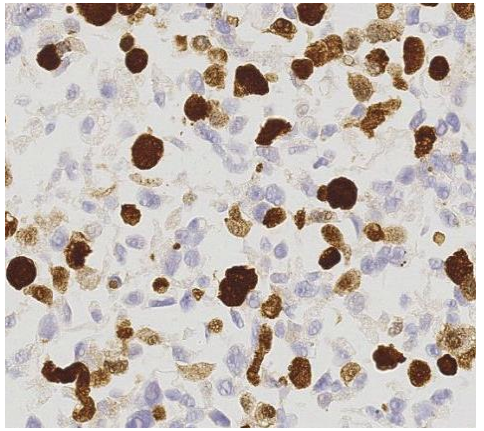
While each site had assays that provided appropriate results in terms of determining p16 expression and gene copy numbers, the quality clearly varied.

Variations in protocols

- UltraView versus OptiView
- Amplification versus no-amplification
- Antibody incubation times
- RTU dilution
- Automation

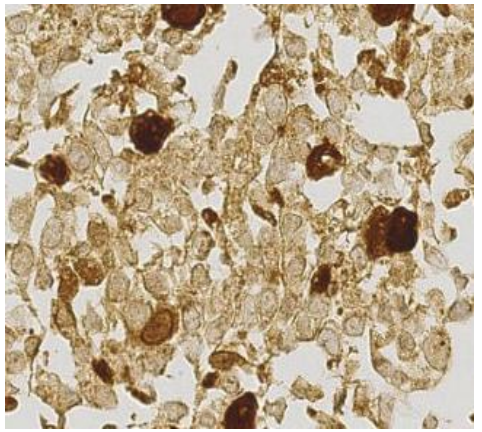


Site 1: Roche p16 on Leica BondIII



Site 2: Roche p16 on Dako Autostainer

Some variation is due to platform and/or associated chemistries or antibody



Site 7: Santa Cruz p16 on Benchmark

Why differences in p16 staining

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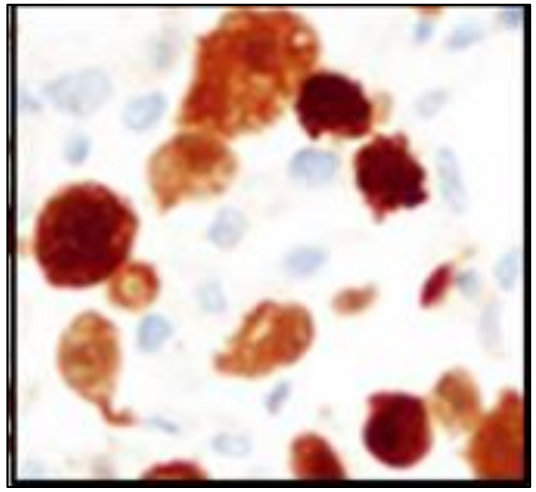
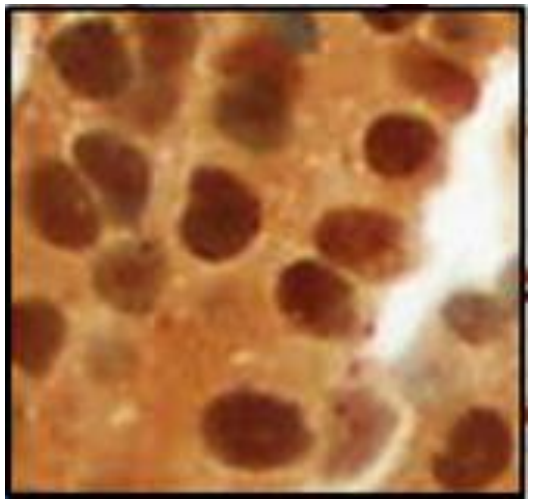
RTU diluted 1:2



RTU Neat



- The difference was solved on further enquiry with the sites. It appears that some laboratories dilute the Roche RTU clone.
- Those sites that do dilute loose the cytoplasmic staining.
- It was done because the RTU staining was excessive and they could get satisfactory staining by diluting the RTU.
- The risk is that the product is used outside of the manufacturers recommendations.
- It appears from this small study that cell line D can determine how this antibody is being used. Importantly both results are correct in as much as the cell line is p16 positive. The ultimate use of the antibody is defined by the laboratory and not by these controls.

HPV ISH sensitivity

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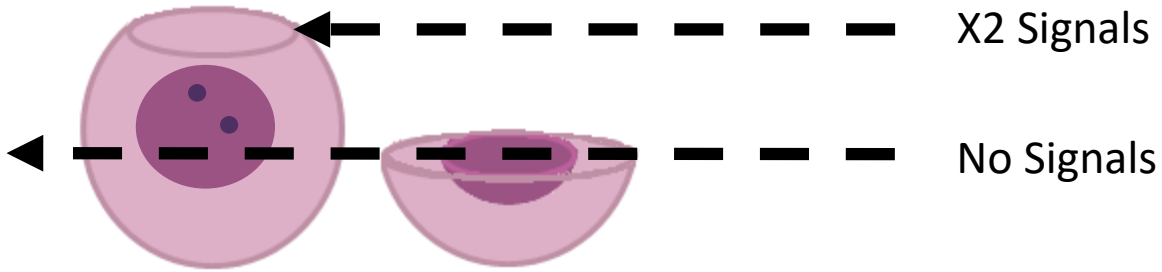
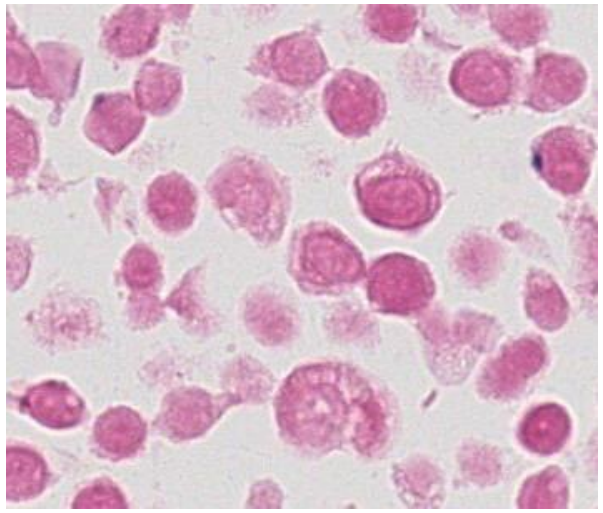
- Cell line B is a well characterised cell line with 1-2 signals per cell.
- Affected by plane in which the cell is cut and sensitivity of the assay:





- Can be easily overlooked!
- In US field trials they were called negative. Slide review showed they were positive.
- Created three core version of the product which was considered easier to use in the USA.

Summary of HPV/p16

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- Created a very good QA/QC tool. Through phenotype and genotype the cells are able to demonstrate a number of things:
  - IHC: Antibody usage and suitable protocol
  - ISH: Slide treatment/digestion and efficacy of the probes
- Allows some degree of trouble shooting. In future we hope to determine specific issues based on cell performance.
- Standardised appears anything but!
- p16 may become more important as a Companion Diagnotic as a surrogate marker for targeting of CDK4 in a variety of cancers[1-3].
- For more information: info@histocyte.com

Product Name	Format	Code
HPV/p16 Analyte Control[DR] (Four cores with dynamic range of HPV gene copies and p16 expression)	Slide(2)	HCL001
	Slide(5)	HCL002
	Block	HCL003
HPV/p16 Analyte Control (Three cores with dynamic range of HPV gene copies and p16 expression)	Slide(2)	HCL004
	Slide(5)	HCL005
	Block	HCL006

1. Eilers G, Czaplinski J, Mayeda M, et al. Mol Cancer Ther. 2015 Jun;14(6):1346-53
2. Sherr CJ, Beach D, Shapiro G. Cancer Discov. 2016 Apr;6(4):353-67.
3. Sheppard KE, McArthur GA Clin Cancer Res. 2013 Oct 1;19(19):5320-8

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