Introduction to automated platforms for IHC. Manual staining Vs Automated staining

Hazel Chambers-Smith

Sri Lanka Workshop on Basics of Immunohistochemistry, June 2019

Manual Staining

- Can be fully manual
  - Dewaxing
  - Blocking
  - Antigen retrieval
  - Antibody application
  - Secondary antibody
  - Tertiary antibody/conjugated chromogen
  - Buffer washes
  - Counterstaining

Manual IHC is very labour intensive

From deparaffination to counterstaining the IHC procedure at minimum requires 60-100 manual interactions and handling procedure on each slide to be stained. Capacity ?? (50-100 slides pr tech.*)


CELL MARQUE
CD10 (56C6)

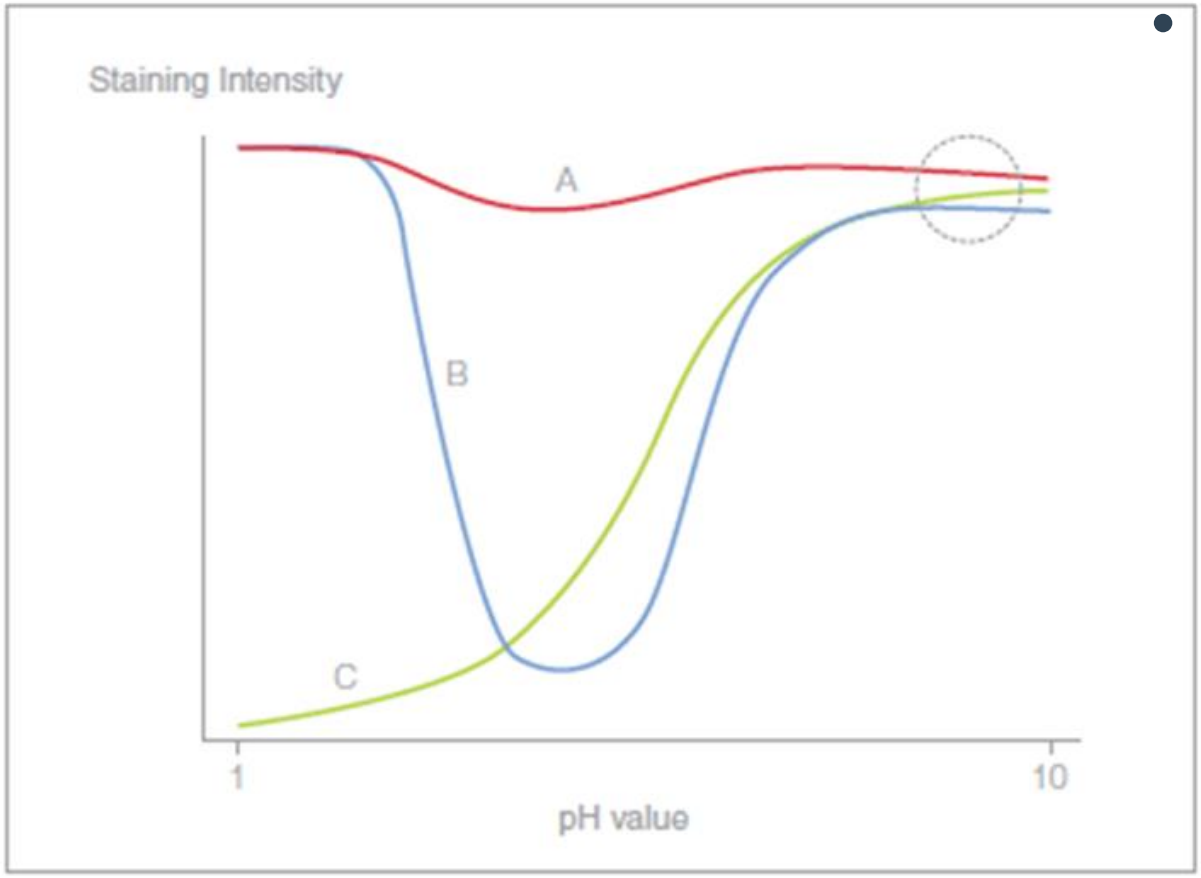
Dewaxing and Epitope Retrieval

- Ensure slides are cut on adhesive slides
- Ensure water is removed from sections by drying at room temp
  - **Slides can be heated to 65 °C for 10-15 mins (but be cautious of heating for an extended time – particularly for hormone receptors).**
- Dewax as per convention

Unmasking epitope sites

- Protelolytic enzyme digestion (usually performed at 37°C)
- Heat induced epitope retrieval (HIER) using antigen retrieval buffers at varying pH
  - EDTA
  - Citrate
  - Tris/EDTA

A universal retrieval buffer?



- Optimal staining of the vast majority of antibodies is with an alkaline pH such as EDTA pH9 (Ventana CC1 is based on this)

Manual HIER

- Manual HIER unmasking can be performed using:
  - Microwave oven irradiation
  - Pressure cooker heating
  - Waterbath
  - Autoclave
  - Steamer
- Some antigens are not obscured by fixation and do not need unmasking.

Blocking

- Endogenous peroxidase is present in bone marrow and lymphoid tissue and needs to be inactivated before staining with peroxidase-based detection systems
  - Hydrogen Peroxide/methanol solution is commonly used
  - It can be done before or after antigen retrieval
  - Common and convenient to perform after dewaxing

- Endogenous biotin is present in liver, kidney and spleen
  - Needs to be inhibited
  - Can use skim milk for 10 mins
  - Specific avidin-biotin blocking reagent

Antibody application

- Apply antibody at optimised dilution
  - Usually 30mins is standard
- Use a covered incubation tray for best results
- Antibody can be incubated overnight at 4°C
  - Dilute antibody a further 1/10 for O/N as a rule of thumb

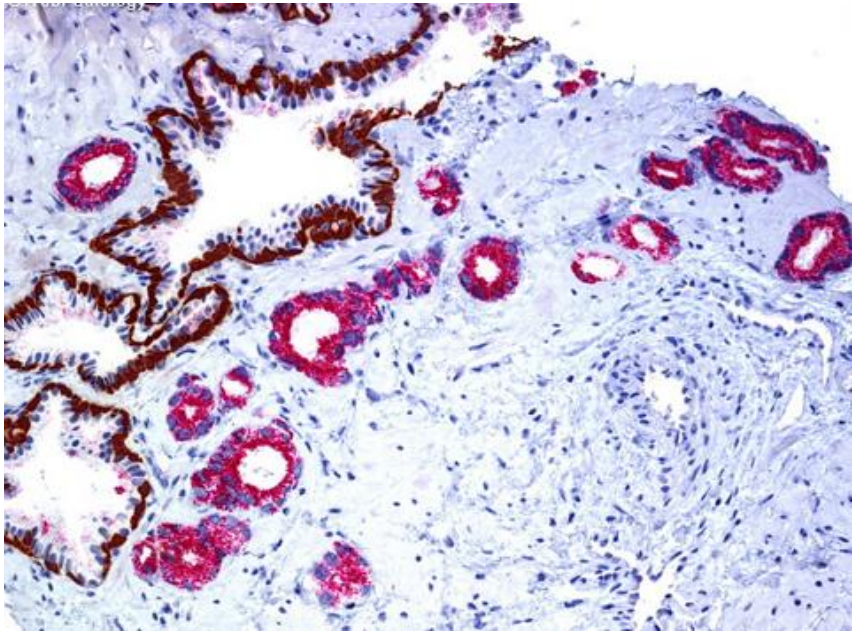
Detection reagents

- Recommended dropper style
- Ensure link antibodies are appropriate for primary antibody
- 30mins at room temperature is usually recommended

Chromogen

- A range of chromogens are available
- Conjugated for a specific detection method
  - **Alkaline phosphatase**
  - **Horseradish peroxidase**
- Some chromogens are soluble in alcohol/solvents and require mounting in an aqueous media

Red Chromogens



- Example PIN4
- The brown DAB is insoluble in alc and xylene but the red will wash away
- Potential for false negatives

Enzyme	Chromogen	Color	Mounting media	Advantages (+) and disadvantages (-)
Horseradish peroxidase (HRP)	AEC	Red	Aqueous	+ Intense color, contrasts well with blue in double staining.
Horseradish peroxidase (HRP)	DAB	Brown	Organic	+ Intense color; permanent.
Alkaline phosphatase (AP)	BCIP/NBT	Blue	Organic	+ Intense color.
Alkaline phosphatase (AP)	Naphthol AS-MX phosphate + Fast Red TR	Red	Aqueous	+ Less intense, good for double staining. - Fast Red TR prone to fading.

Buffer washes

- Ensure buffers are made up with quality water, are pH tested
- Must contain a detergent eg Tween
- This helps reagents spread over the entire slide, not stick in one area
- Thorough rinsing in buffer is important!
- Do not spray buffers directly onto sections

Timers

- Timing of steps can be critical
- Use timers to minimise over incubation

Manual staining hints

- Use a moistened covered chamber for incubation steps
- Leave gaps between slides to prevent cross contamination
- Circle sections with a wax pen
- 2-3 drops of reagent per slides is usually sufficient

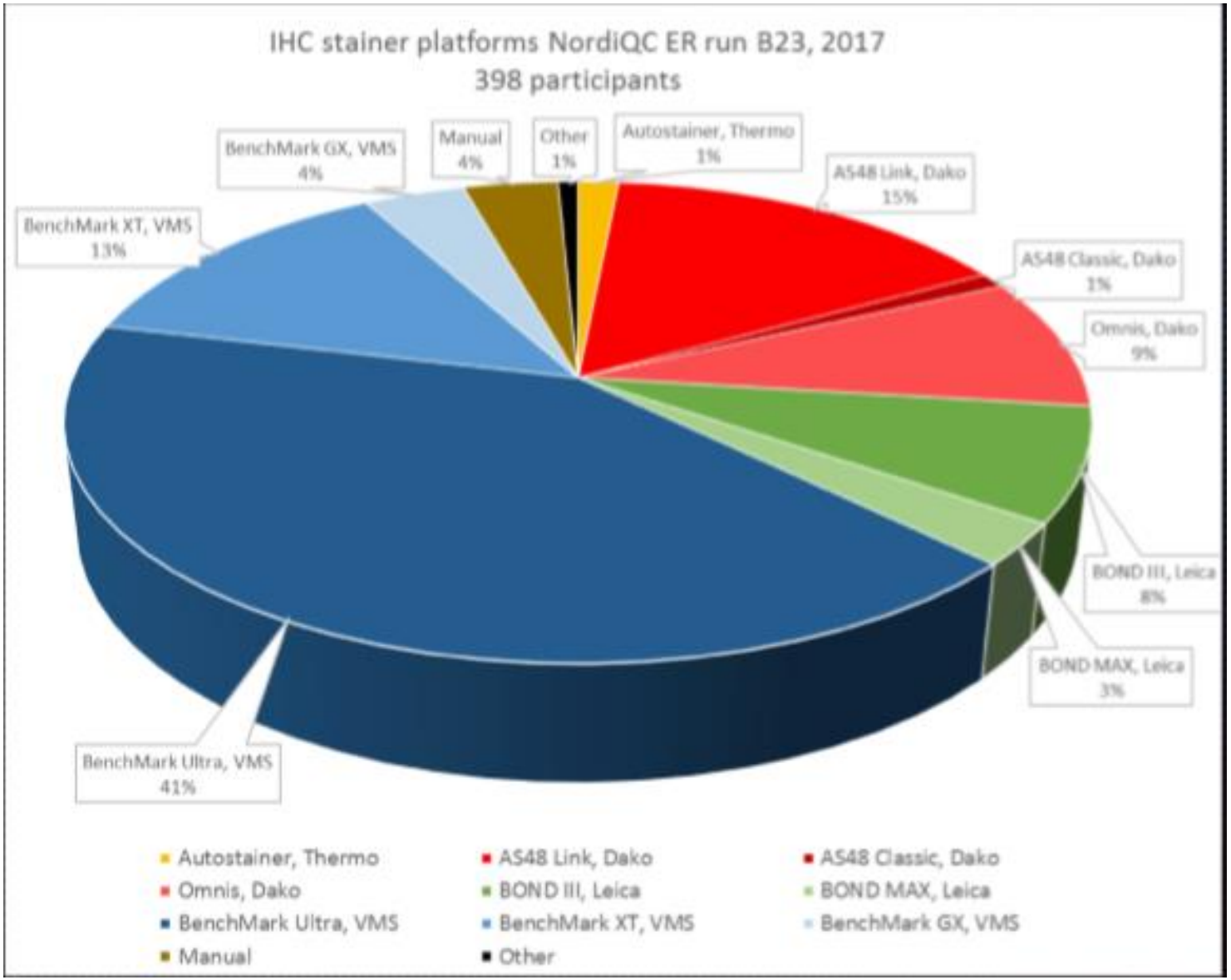
Time management

- Creating a system to make the manual process more efficient is worthwhile
- The fewer the steps you can create, the less room there is for error
- Having similar incubation times makes life easier (this can be factored in to calibration)
- Have detection step commence at the same time.
- Must run in batches (too hard to run continuous)

Automated methods

Meet Edward and Jacob at RCH Melbourne ☺

Benchmark XT and GX


IHC stainer platforms NordiQC ER run B23, 2017
398 participants
Autostainer, Thermo 1%
A548 Link, Dako 15%
A548 Classic, Dako 1%
Omnis, Dako 9%
BOND III, Leica 8%
BOND MAX, Leica 3%
BenchMark Ultra, VMS 41%
BenchMark XT, VMS 13%
BenchMark GX, VMS 4%
Manual 4%
Other 1%
Autostainer, Thermo
AS48 Link, Dako
AS48 Classic, Dako
Omnis, Dako
BOND III, Leica
BOND MAX, Leica
BenchMark Ultra, VMS
BenchMark XT, VMS
BenchMark GX, VMS
Manual
Other

- Can range from semi-automated (still requiring dewaxing and antigen retrieval) to fully automated (whole IHC process including baking and dewaxing through to counterstaining).

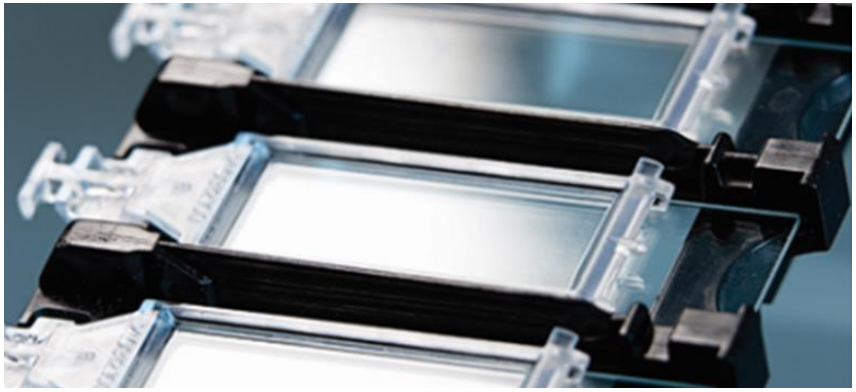
- Automated IHC strainers started to appear in the early 1980’s
- Software design, hardware improvements and laboratory demands saw automated instruments appearing in diagnostic labs
- Manual IHC in diagnostic labs is almost unheard of now in the UK and Australia

- Repetitive, large number of operator dependant steps may lead to parts of the method being omitted or performed in the incorrect sequence
- Slides can dry out, incubation times too short/long
- Lead to variation in staining

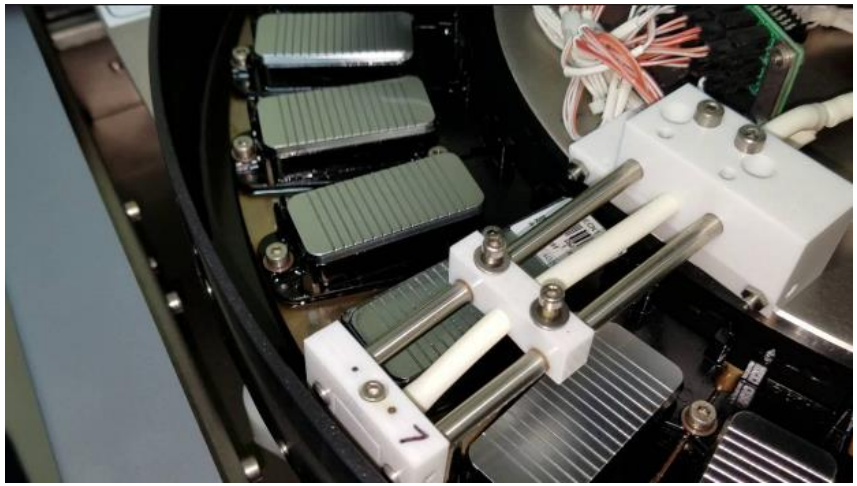
Styles of automation

- Vertical placement of slides with reagents fed by gravity from the top of the slide
- Uses capillary action to displace the liquid
- Can also have the reagents climb up from the bottom-to-top with the liquids blotted from the bottom of the slide

- With the capillary action, the efficiency is related to the capillary gap to fill or empty.
- May require a specific slide so that the exact spacing of the gap between the slide and covertile is correct



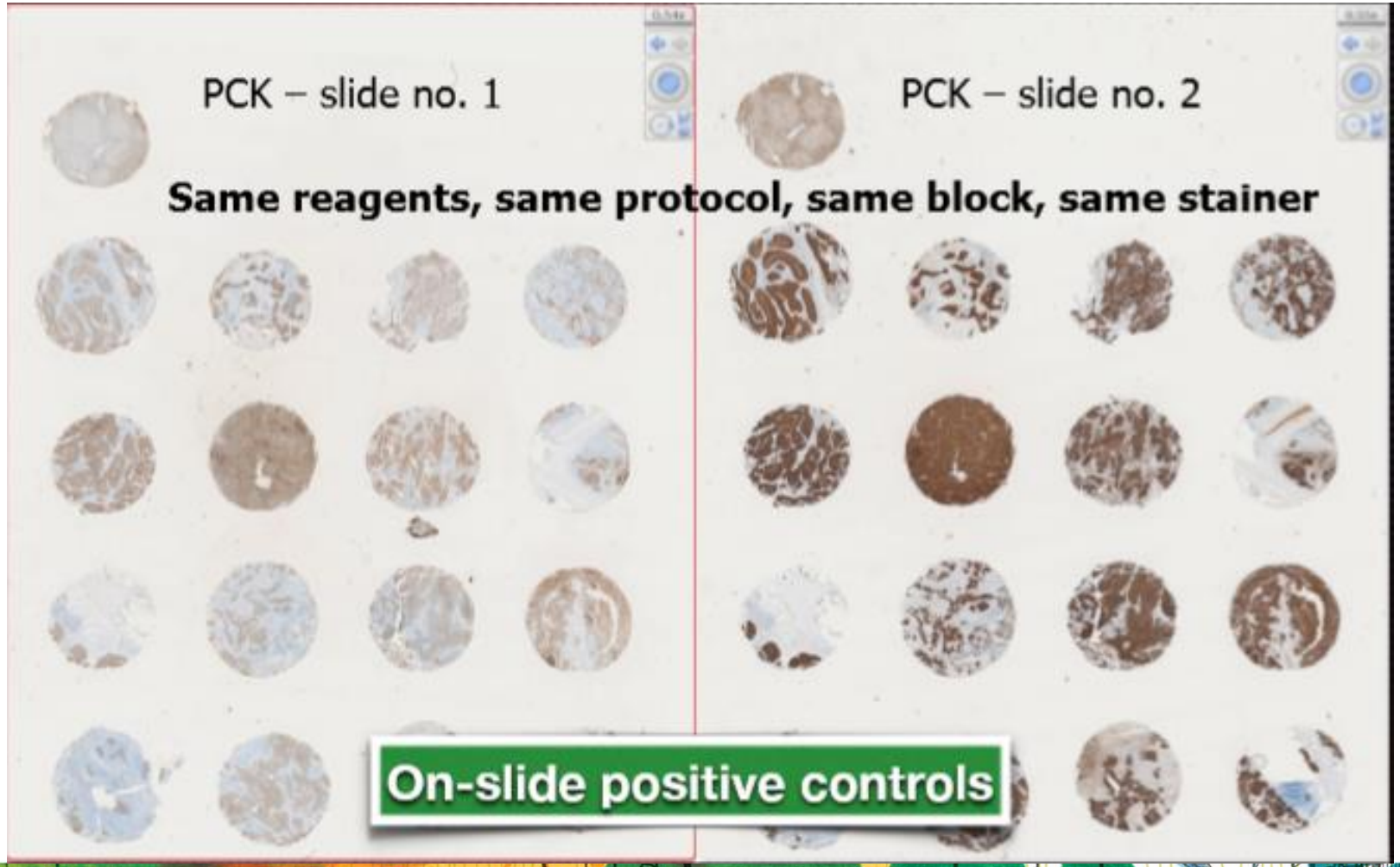
- Horizontal method whereby reagents and buffers are administered from above by a probe/disposable tip
- Air jets mix and eliminates reagents from slides



Fully automated stainers

Stainer	Company	Principle	Capacity
BenchMark Ultra	Ventana/Roche	Flat labelling	30 slides
Bond III/Max	Leica	Capillary	30 slides
OMNIS	Dako/Agilent	Dynamic capillary	60 slides
Oncore	Biocare	Kinetic chamber	36 slides
Tissue-Tek Genie	Sakura	Capillary	30 slides
Xmatrx ELITE	BioGenex	Flat labelling / Micro-chamber	40 slides

The importance of same slide controls



Wait! Before jumping to automation…

- Run parallel comparisons – confirm staining results are comparable or better than the manual method.
- Demonstration of run-to-run reproducibility
- Able to hold multiple antibodies and detection systems
- Random access is an advantage

- Software should be easy to use
- Error tracking present and be able to report the problem
- Precise, reproducible quantities dispensed
- Evaporation loss and carryover should be minimal

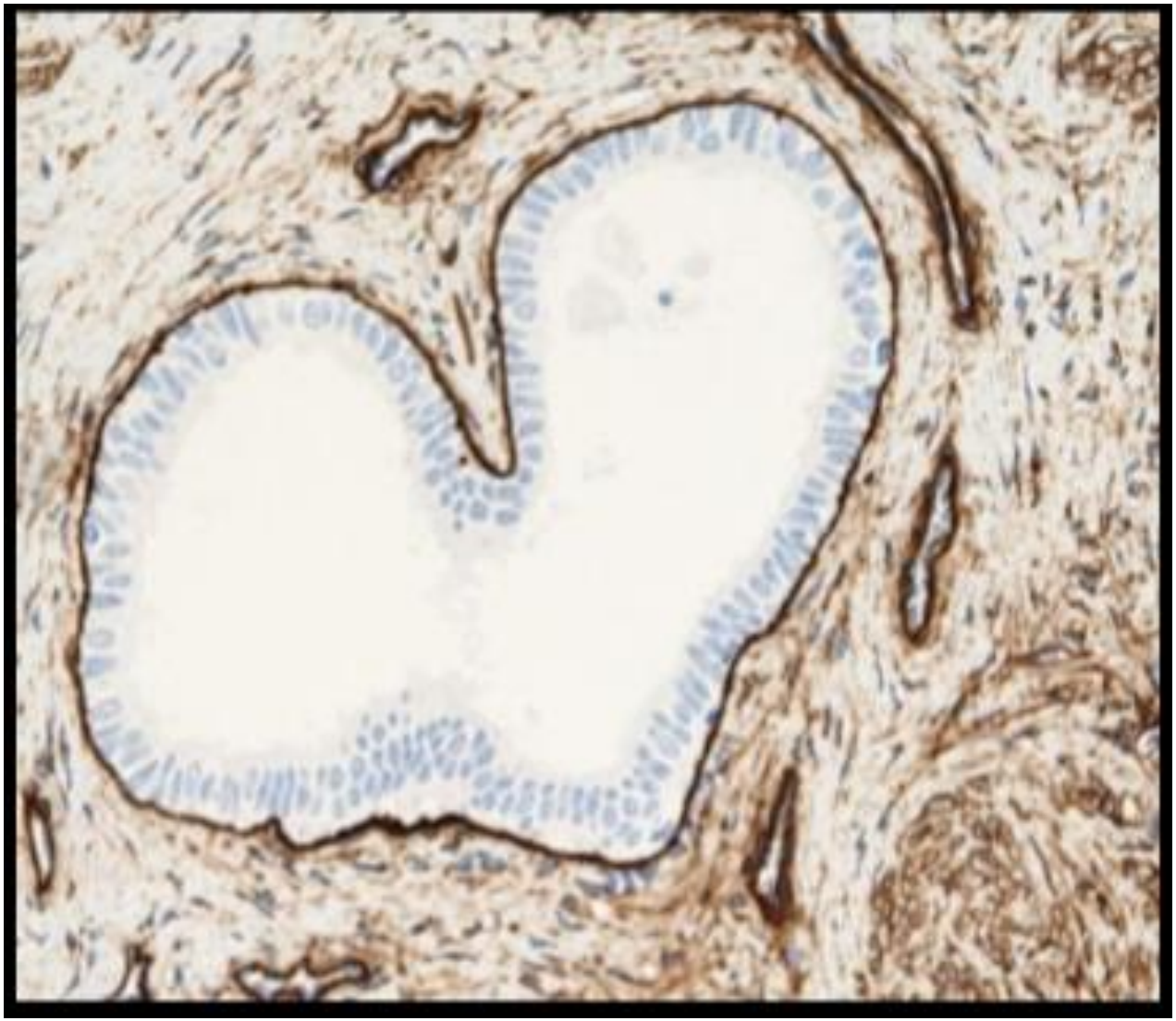
Advantages of automation over manual methods

- Proprietary products ensure quality of result
- Uniform, standardise method each time
- Enclosed instruments ensure operator safety
- Accountability and reportability of each step of the staining method
- Xylene-free processing
- Technical support from vendor

Automation will not overcome:

- Poor fixation and processing
- Pathologist/scientific experience in troubleshooting and interpretation
- User error: wrong antibody made up, incorrect protocol chosen, wrong control section placed on slide.

Thanks for your attention ☺




The Royal
Children's
Hospital
Melbourne