

Immunohistochemistry and in situ hybridization: practical aspects

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Overzicht

- Pre-analytische fase
- Immuunhistochemie
- In-situ hybridisatie

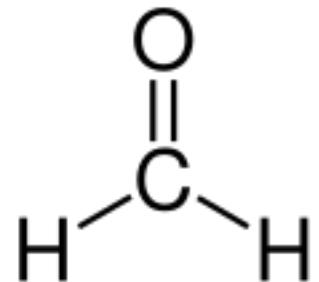
Pre analytische fase

- Staatname
- Fixatie
- Doorwerken
- Paraffine inbedding
- Coupes maken

- Analytische fase

Fixatie

- Gebufferde formaline 10%
 - (4% formaldehyde)
 - pH 6,8-7,2
 - Carcinogeen
-
- Niet gebufferde formol geeft geleidelijke drop in pH met mogelijke precipitatie als gevolg



Fixatie

- Water oplosbaar fixatief
- Crosslinks tussen eiwitten en tussen eiwitten en nucleïnezuren
- Behoud van de secundaire eiwitstructuur
- Kan een antigen-antilichaam interactie hinderen
- Deels reversibel (antigen retrieval)

Fixatie

- Fixatie wordt o.a. beïnvloed door
 - Het type weefsel, penetrantie snelheid
 - B.v. vet versus spier
 - Grote van het weefsel
 - Temperatuur
 - Volume

Fixatie

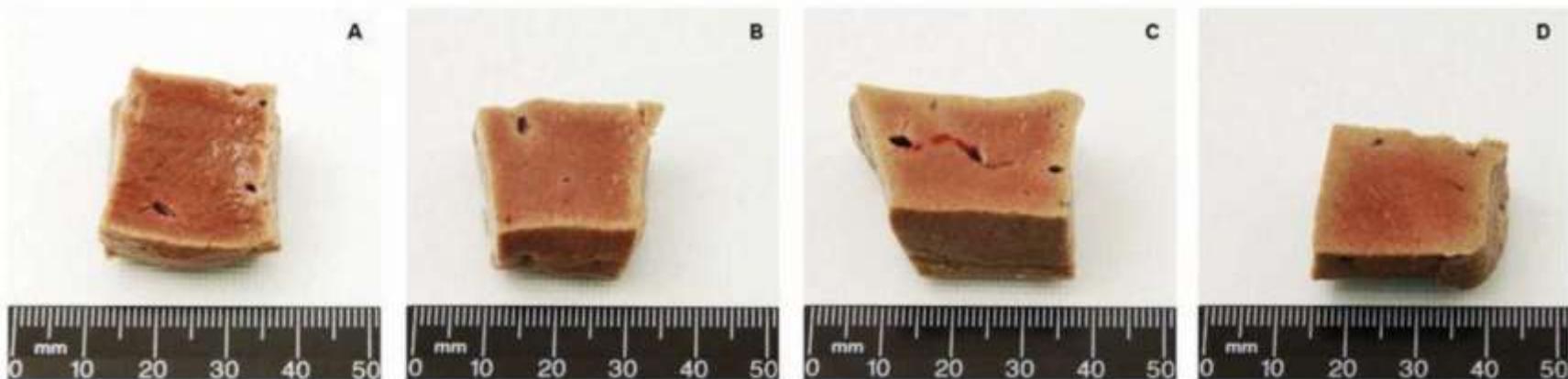


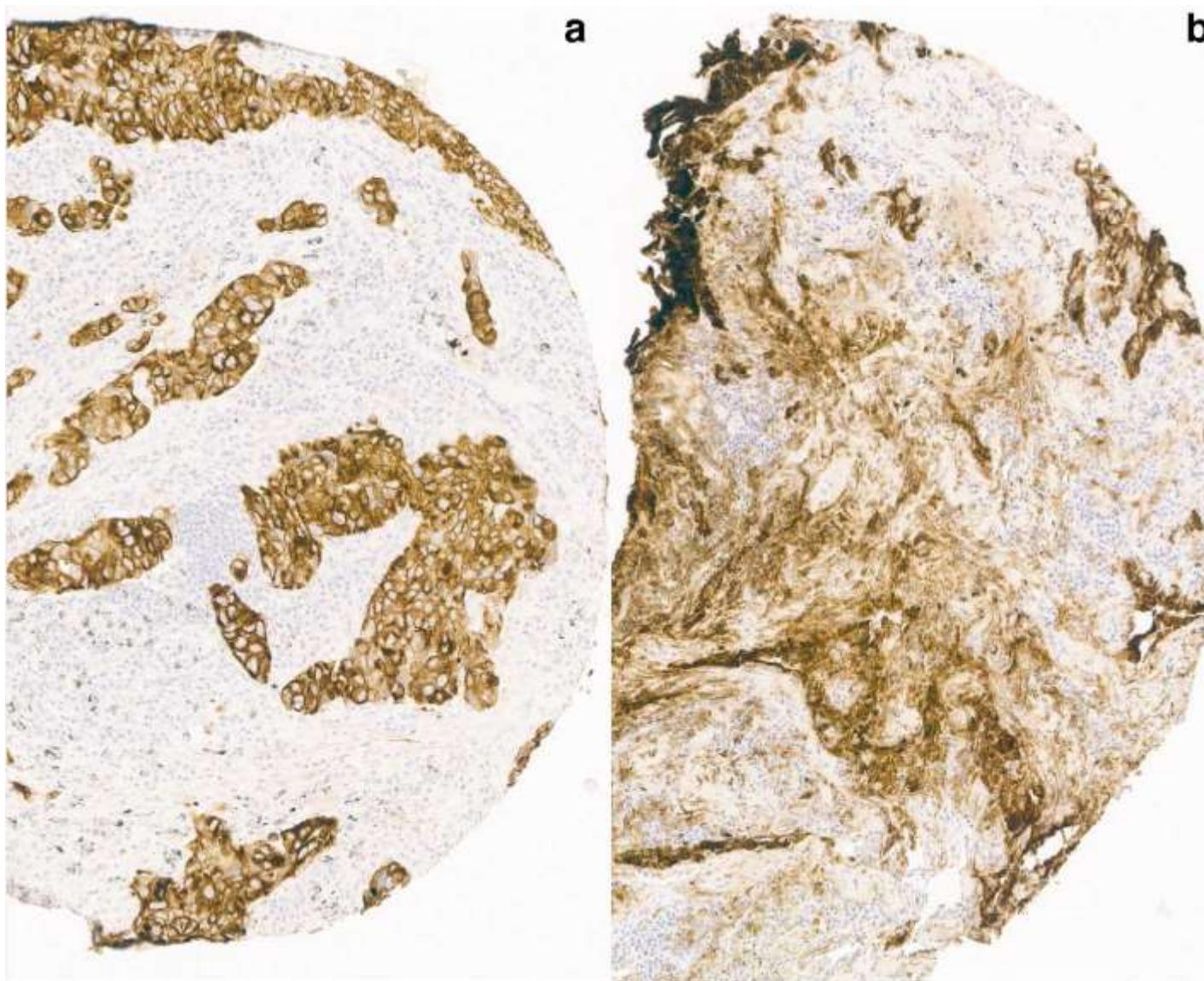
Figure 1: A composite photograph showing the rate at which 10% neutral buffered formalin penetrates into 25 mm cubes of liver. At the end of each time period a cube has been sliced to reveal the advancing fixation front. A: one hour (approximately 0.8 mm penetration), B: two hours (approximately 1.2 mm penetration), C: four hours (approximately 1.6 mm penetration) and D: eight hours (approximately 2.2 mm penetration). Note that after eight hours the centre of the specimen remains unfixed.

<https://www.leicabiosystems.com/en-be/knowledge-pathway/fixation-and-fixatives-2-factors-influencing-chemical-fixation-formaldehyde-and-glutaraldehyde/>

Fixatie

- Vertraagde start van fixatie en te korte fixatie
 - Autolyse
 - Onvoldoende cross links
 - Alcoholische (coagulatieve) fixatie bij de dehydratatie
- Te lange fixatie
 - Overmaat aan crosslinks

Example of a CK 7 (Monosan) staining after normal fixation (**a**) and after 96 h delay in fixation (**b**). A is scored as sufficient and B as insufficient quality, due to prominent non-specific (background) staining (for both objective $\times 15$)



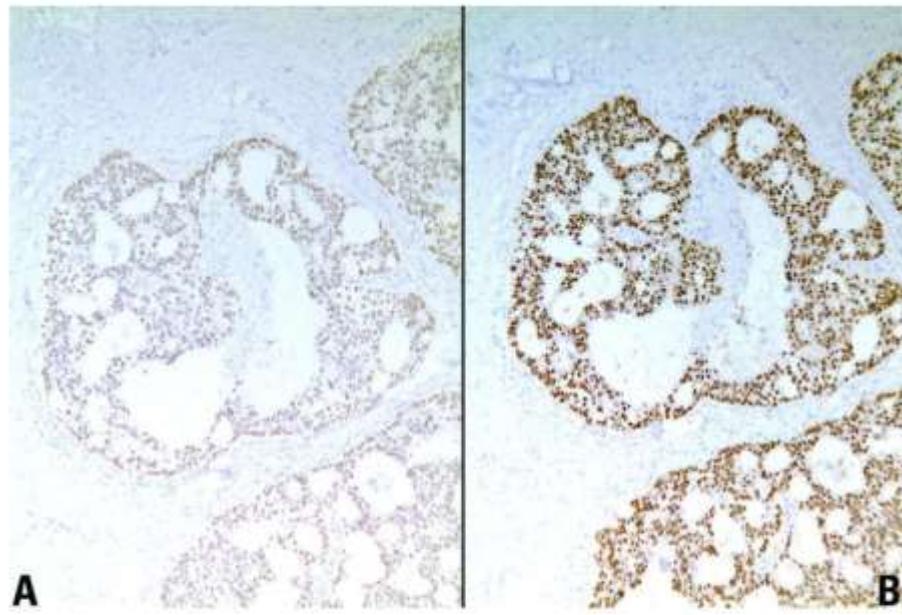


Figure 1. Estrogen receptor (ER) staining of breast carcinoma. A. Tissue block fixed for 3 hours in 10% formal saline showing weak demonstration of ER positive cells. B. Tissue block fixed 8 hours in 10% formal saline showing strong demonstration of ER positive cells.

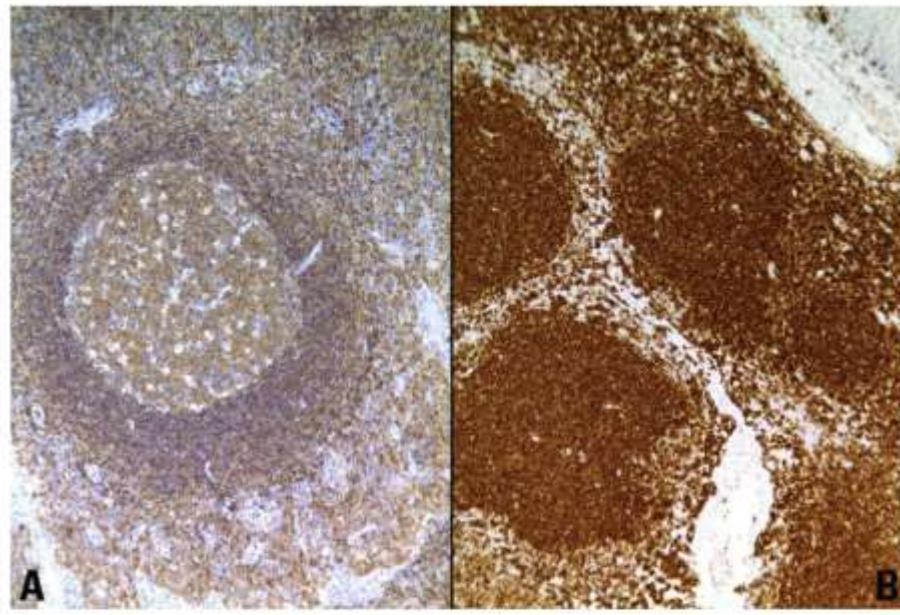
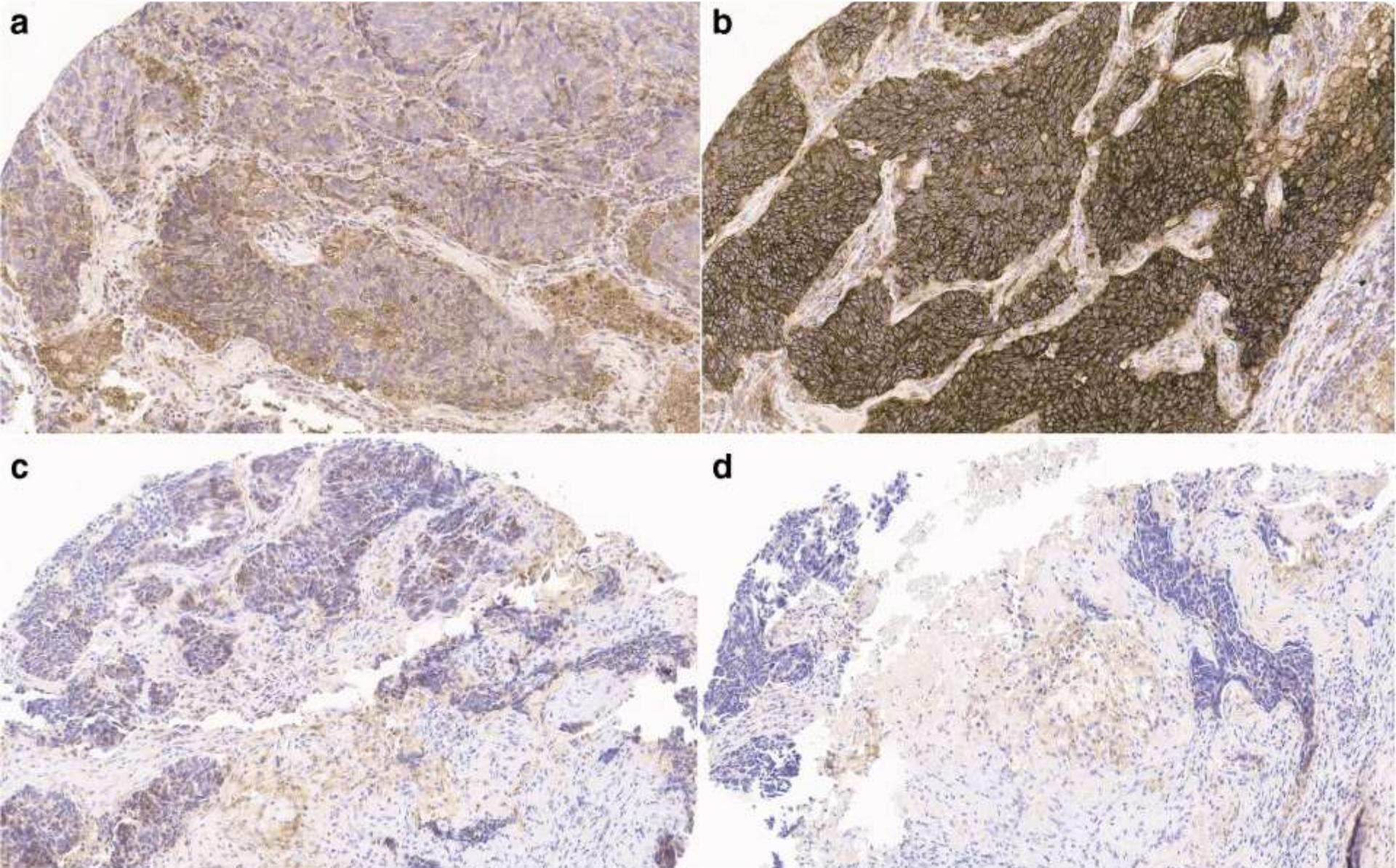


Figure 2. LCA (CD45) staining on reactive tonsil. A. Block fixed for 3 hours in 10% formal saline showing weak demonstration of lymphocytes. B. Block fixed for 8 hours in 10% formal saline showing strong staining of lymphocytes.

Presentation of PD-L1 staining in a tumor sample after normal fixation (**a**), after 6 h (**b**), 48 h (**c**), and 96 h (**d**) of delay in fixation (for all objective $\times 20$).

Note: deterioration of membrane staining in 48+ h delayed fixation and increase of non-specific staining



Fixatie

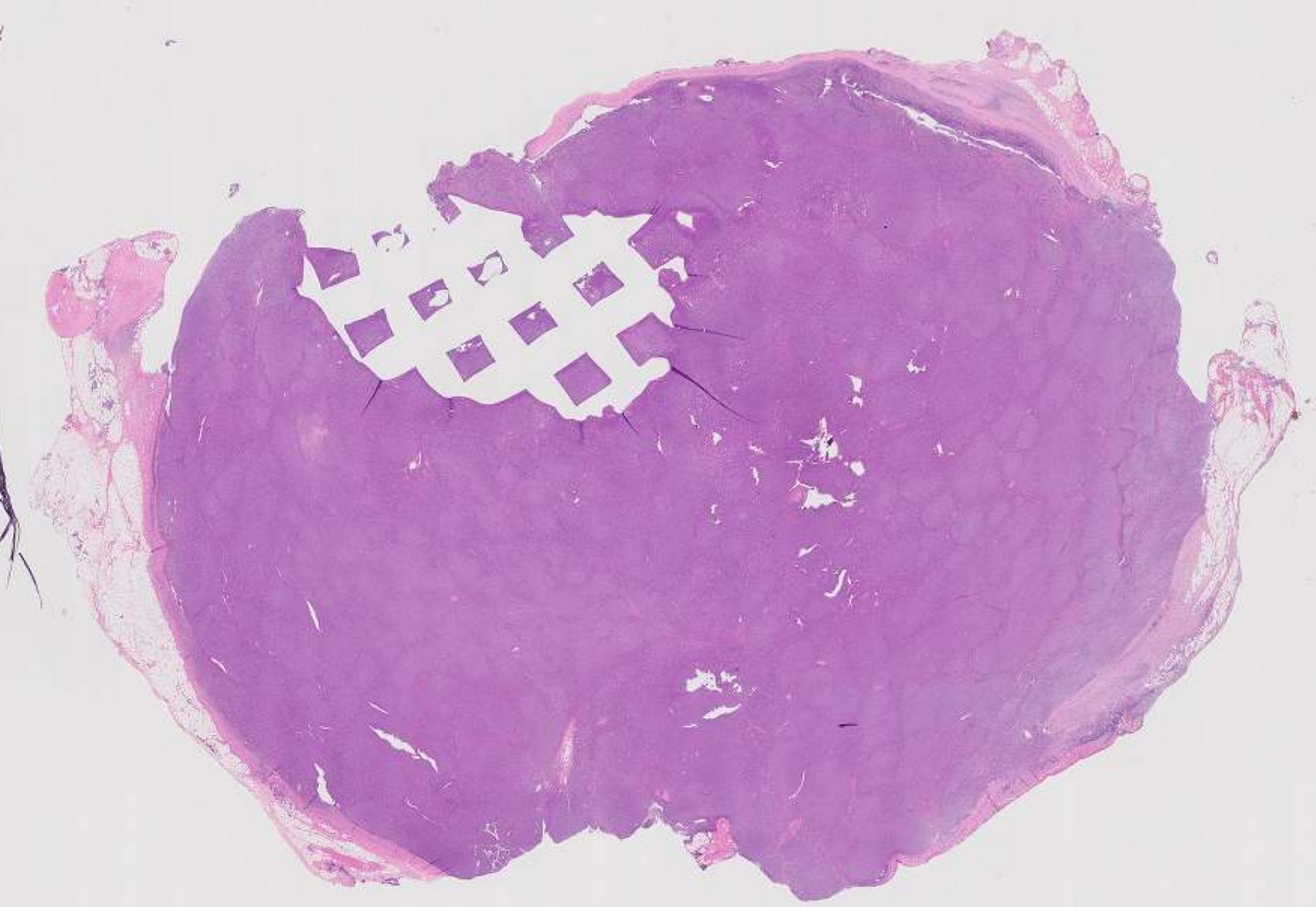
- Advies
 - Omkapselde of vetrijke organeninsnijden bij ontvangst en dan laten fixeren
 - Oplamelleren borst, lever, milt, testis, ...
 - Opspuiten van longresecties
 - Openknippen van darmen
 - Overmaat aan gebufferde formol
 - 6-48 (72) u fixatie
 - Weefsel prelevenementen 2-3 mm dik
 - Zorg dat de cassettes niet 'uitpuilen'
- Te volle cassettes geven slechte HE-coupes en slechte IHC

Doorwerken van het weefsel

- Doel: dehydratatie en impregnering met paraffine om het snijden van coupes mogelijk te maken
- Dehydratatie met alcohol
 - Oplopende concentraties
- Clearing
 - Xyleen
- Paraffine impregnering op temperatuur (smeltpunt 57°C)

Snijden van de coupes

- Dikte, +- 4 micron
- Wijze van drogen
 - Oven
 - Warme plaat
 - Duur (blanco's ?)
- Paraffinecoupe
 - Deparaffineren en rehydrateren
 - Xyleen en alcohol (in dalende concentratie)



Ki67

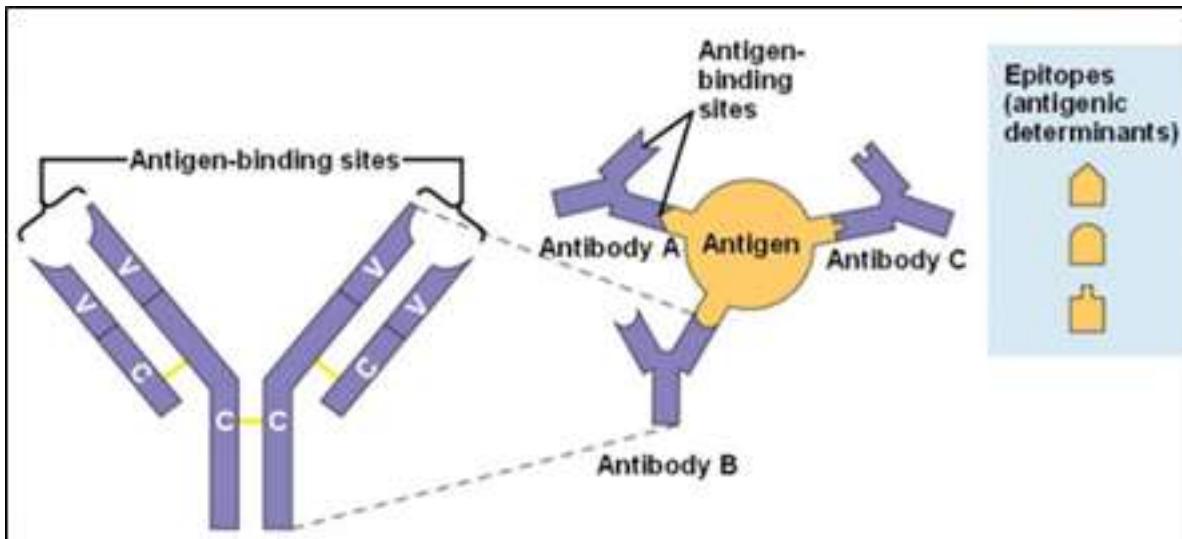


CD10



Immunohistochemie

- Basis:
 - Gebaseerd op de antigen– antilichaam interactie



P, Sharda & R, Shruthy & Priya, N & Hostur, Sreelatha & Jali, Pramod. (2021). Basic principles of immunohistochemistry and epithelial immunohistochemical markers. IP Archives of Cytology and Histopathology Research. 6. 1-6. 10.18231/j.achr.2021.001.

Immunohistochemie

- Epitope (antigen) – paratope (antilichaam)
 - Epitope: klassiek een 5-8 aminozuren of 1-6 monosacchariden
 - Niet-covalente, reversibele binding (ion-binding, H-bruggen, hydrofobe interactie, Van der Waals krachten)
 - Afhankelijk van o.a.
 - pH
 - Concentraties
 - Tijdsduur
 - Temperatuur
 - Ruimtelijke toegankelijkheid van het antilichaam

Immunohistochemie

- Polyclonaal
 - Verschillende antilichamen gericht tegen verschillende epitopen op eenzelfde antigen
- Monoclonaal
 - 1 type antilichaam gericht tegen 1 enkel epitoot

All differences at a glance

	Polyclonal antibodies	Monoclonal antibodies	Recombinant antibodies
Reproducibility	low	Limited – dependent on cell line stability	Very high
Timeline	months	0.5 – 1 year	At evitria: 5 weeks
Cost	low	Medium-high	Medium in development, low in production
Host animals	Mostly rabbit, goat, sheep	Mouse, rat, hamster	<i>In vitro</i> process
applications	Diagnostic applications (e.g. Western Blots, FACS, immunoassays, etc.)	Therapeutic antibodies, diagnostic applications	Therapeutic antibodies, diagnostic applications

Immunohistochemie

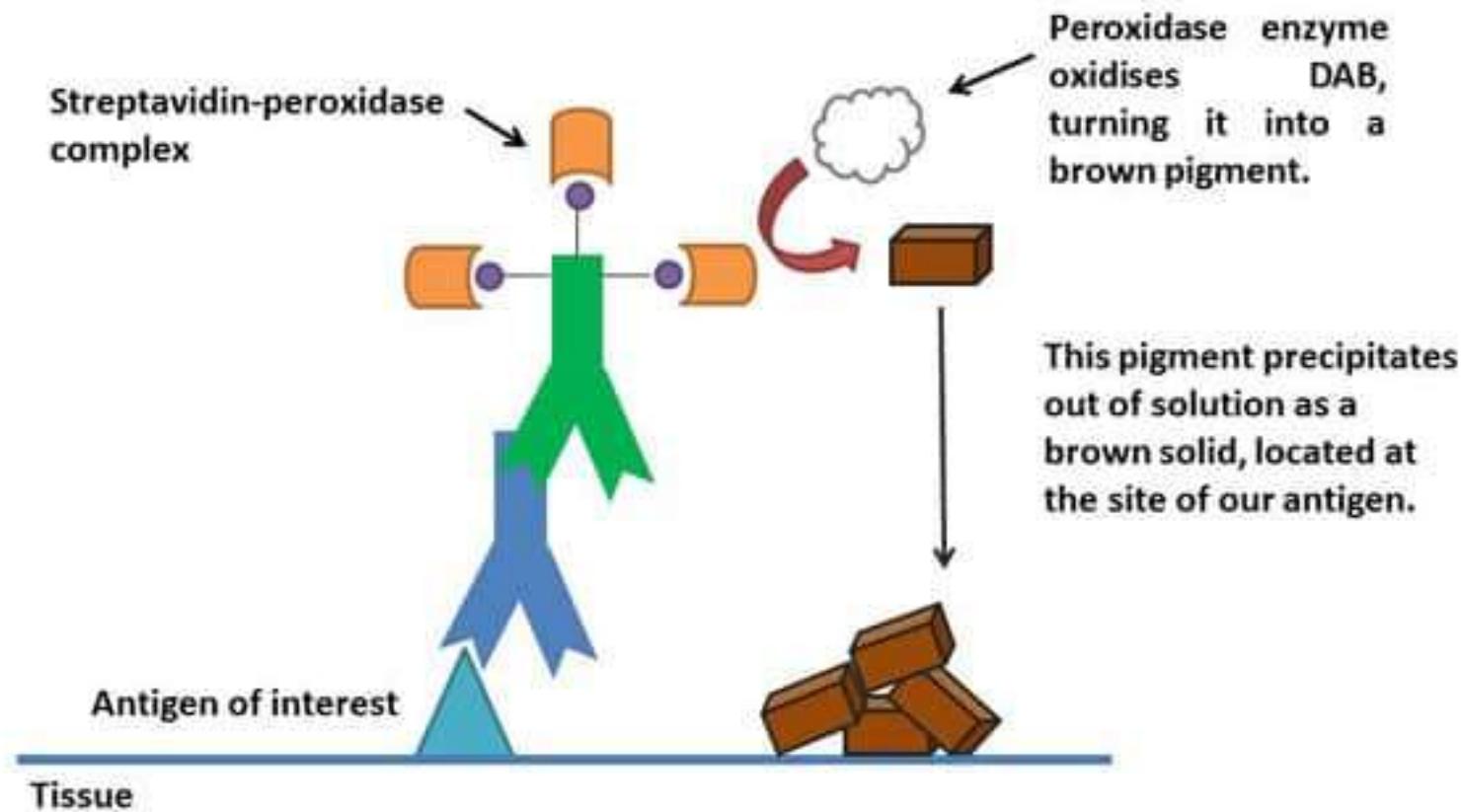
- Het epitoot van het antigen moet ruimtelijk toegankelijk zijn voor de interactie met het paratoot van het antilichaam
- Antigenretrieval
 - Doel: de cross-linken, t.g.v. de formolfixatie, deels afbreken
 - Thermisch
 - Enzymatisch

Immunohistochemie

- Detectiemethoden
 - Fluorescent
 - Functionele nier- en huid pathologie
 - Op vriescoupes
 - Nadeel:
 - Nood aan fluorescentie microscoop
 - Tijdelijk signaal
 - Chromogeen (meest toegepast)
 - DAB (bruin)
 - Oxidatie via HRP > bruin kleur
 - Niet oplosbaar in water of ander veel gebruikte solventen
 - AEC (rood)
 - Oxidatie via HRP > rode kleur
 - Niet oplosbaar in water, wel in alcohol
 - Enzym
 - HRP
 - AP
- Direct
 - Rechtstreeks gelabelde antilichamen
- Indirect
 - signaalversterking

Immunohistochemie

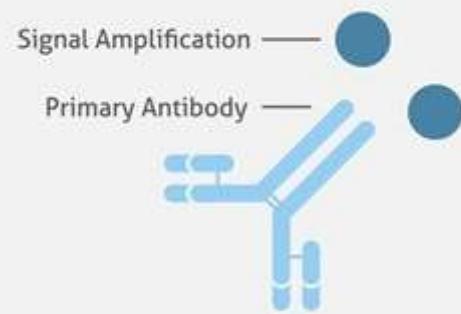
- block
 - Endogene biotin
 - Endogene peroxidase/alkalische fosfatase
 - Niet specifieke bindingsplaatsen



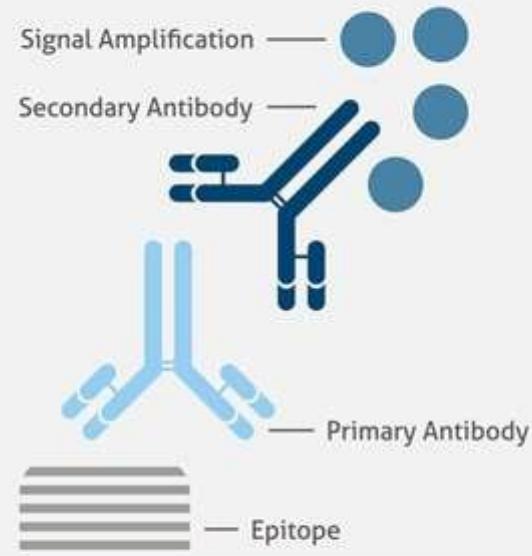
Immunoistochemie

Expression level of protein of interest

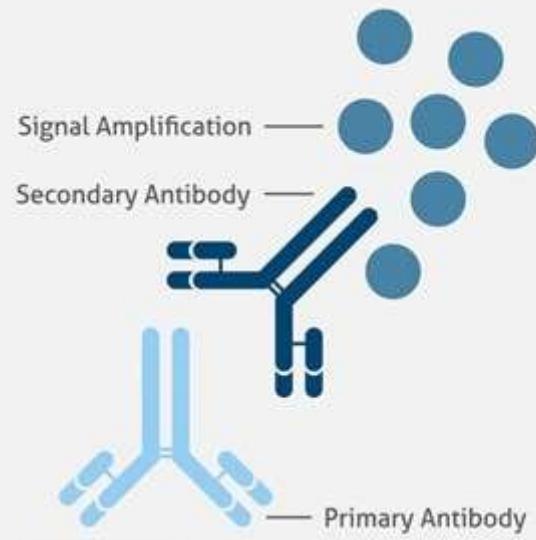
High



Medium



Low



Direct Labeled First Antibody

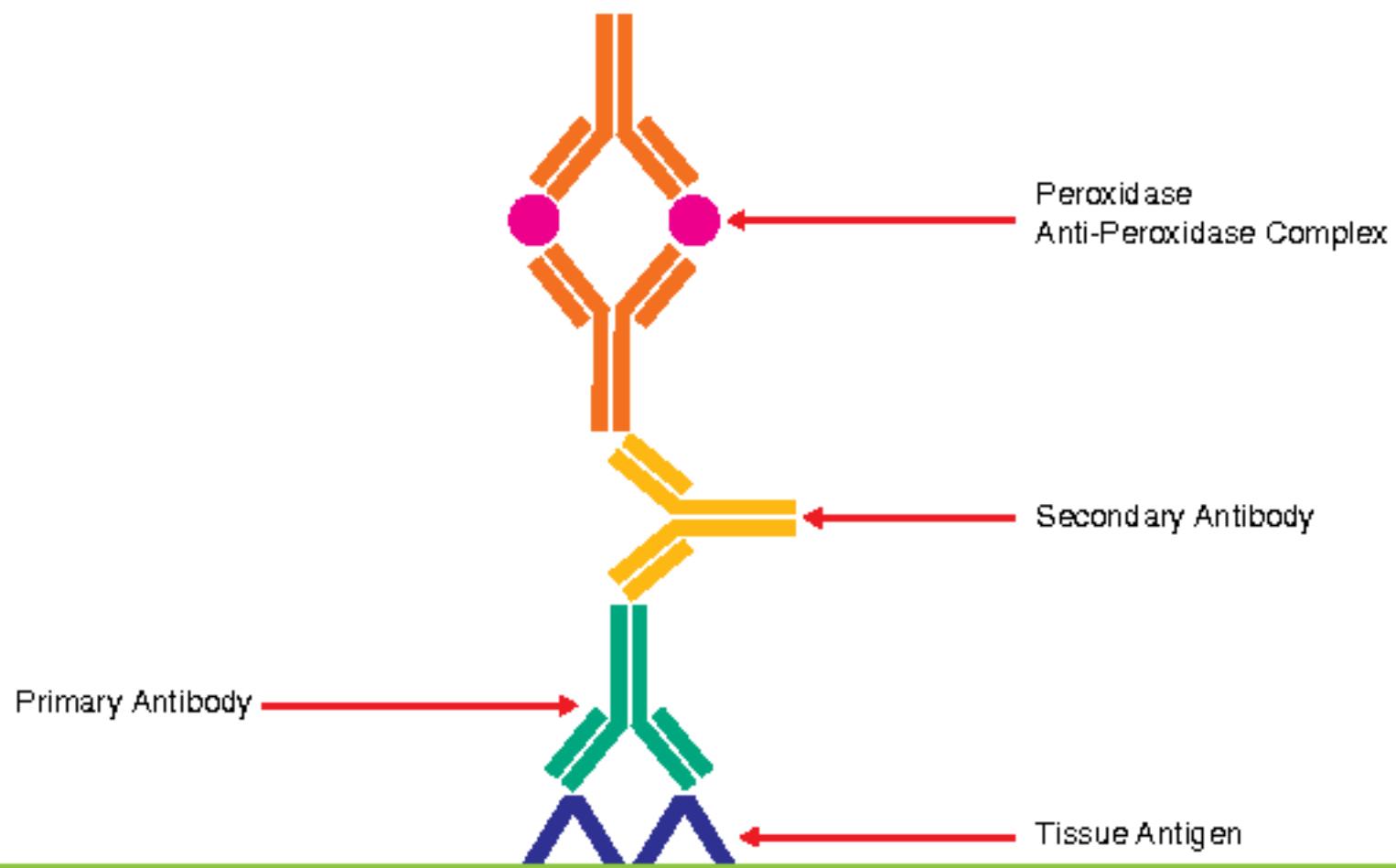
Secondary Antibody Labeled

Secondary Antibody Labeled
Plus Enhancer

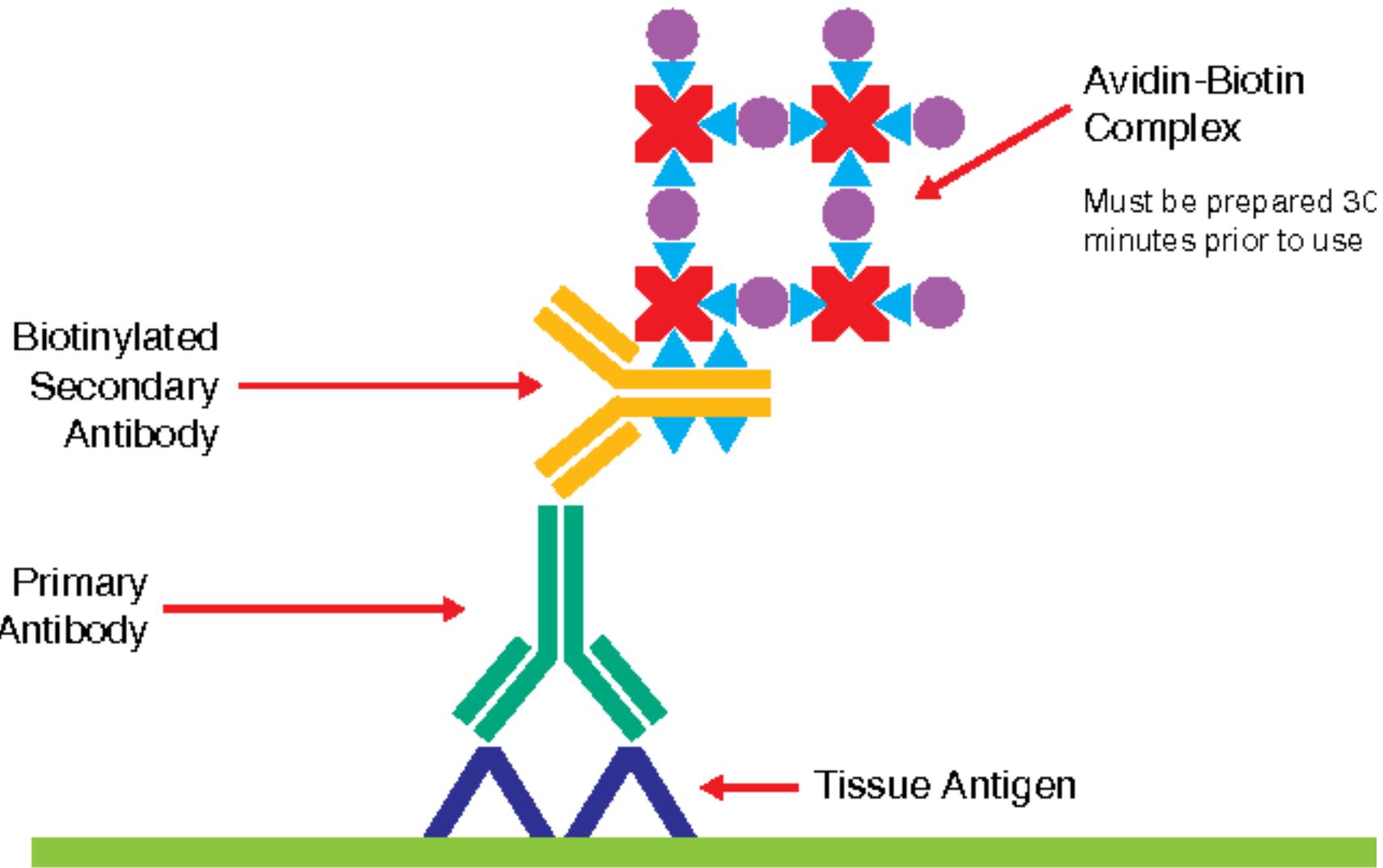
Immuunhistochemie

- Directe immuunhistochemie:
 - Nier en huid pathologie, fluorescente detectie
- Indirecte immuunhistochemie:
 - Meest frequent
 - Chromogene detectie methode
 - Hoe groter de ‘kerstboom’ hoe sterker het signaal
 - Balans tussen signaal amplificatie en achtergrond
- PAP (Peroxidase anti-peroxidase)
- ABC (avidin/biotin complex)
- LSAB (Labeled Streptavidin Biotin)
- Polymeer/multimeer methode

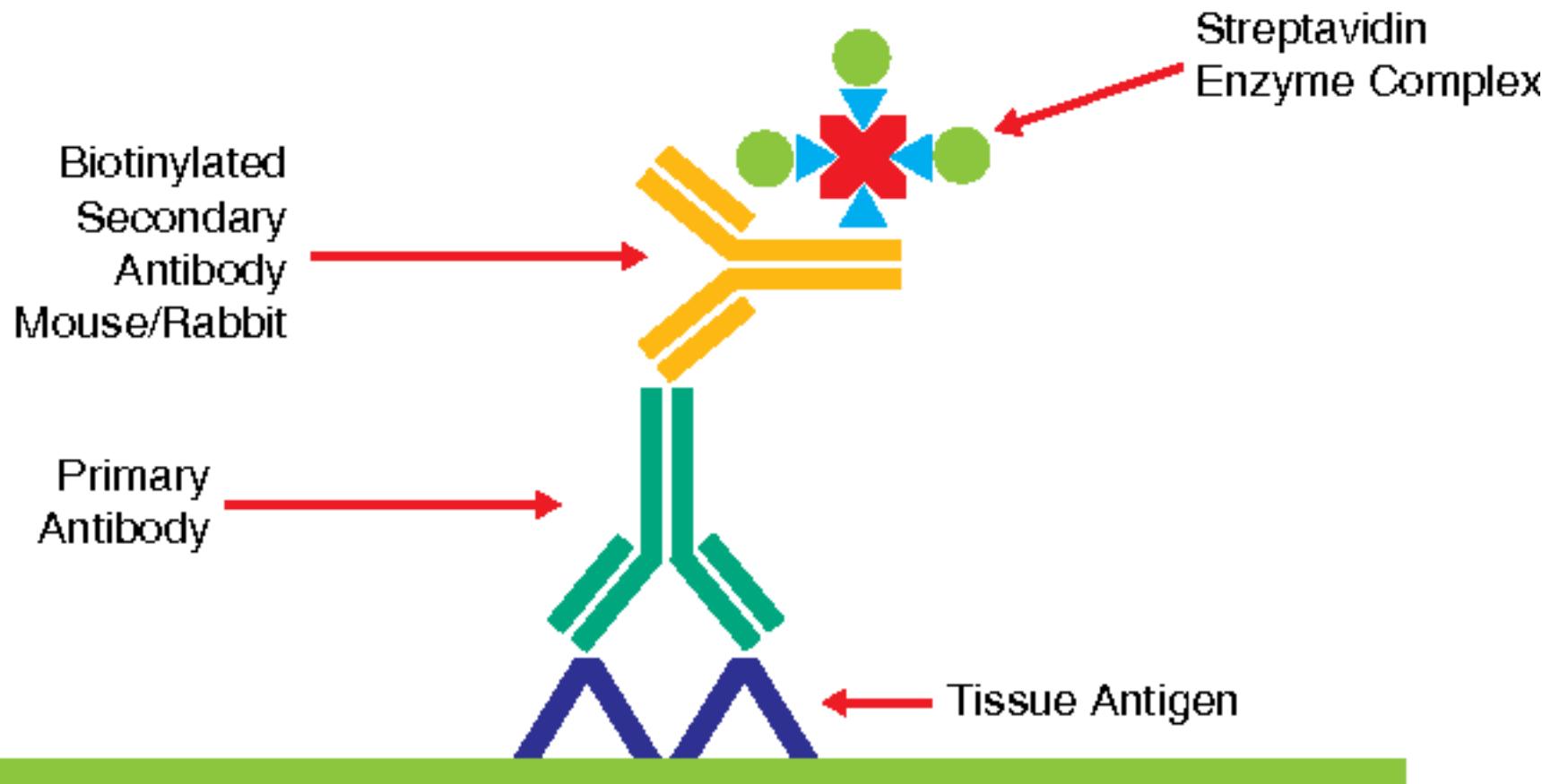
Indirect Method — PAP



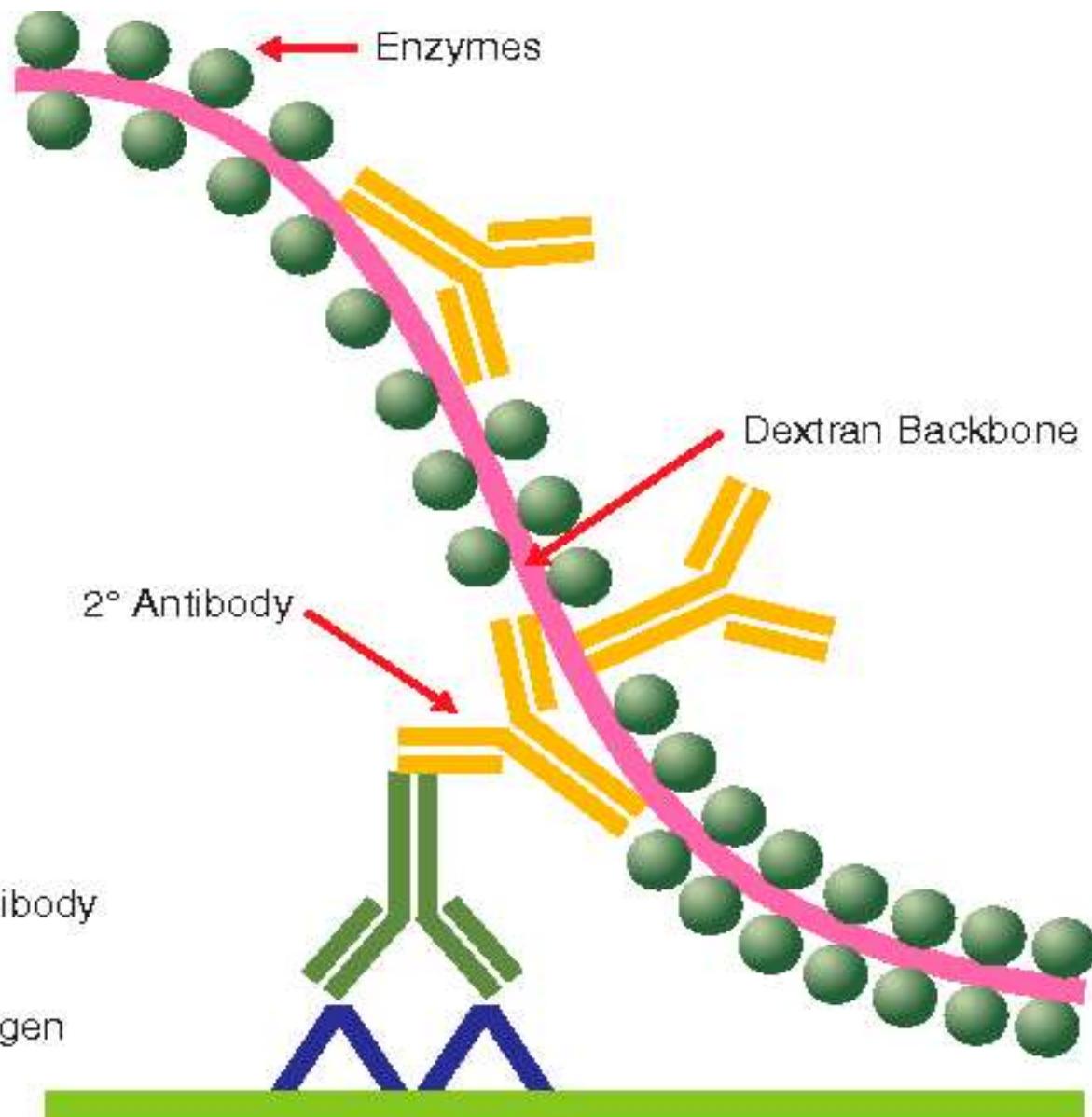
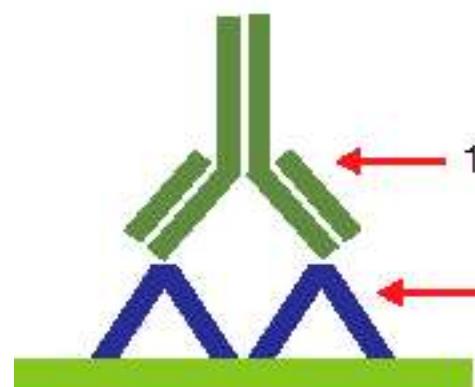
Indirect Method — ABC



LSAB Methodology



-  Antigen
-  1° Antibody
-  2° Antibody
-  Enzyme (HRP or AP)
-  Dextran Backbone



Step Two

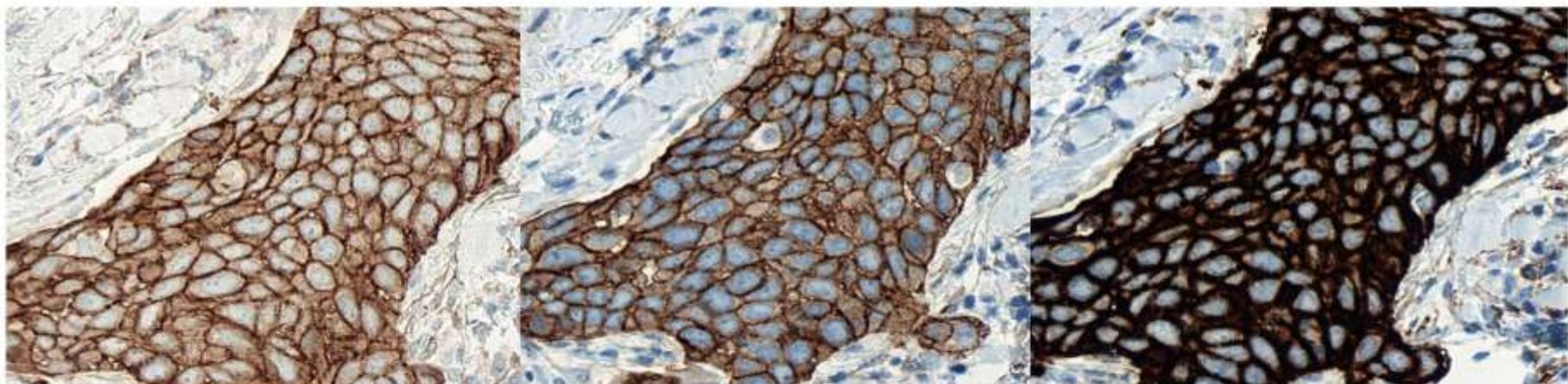
Immunohistochemie

- Tegenkleuring
 - Hematoxyline
- Monteren en afdekken

***ultraView DAB* vs OptiView DAB**



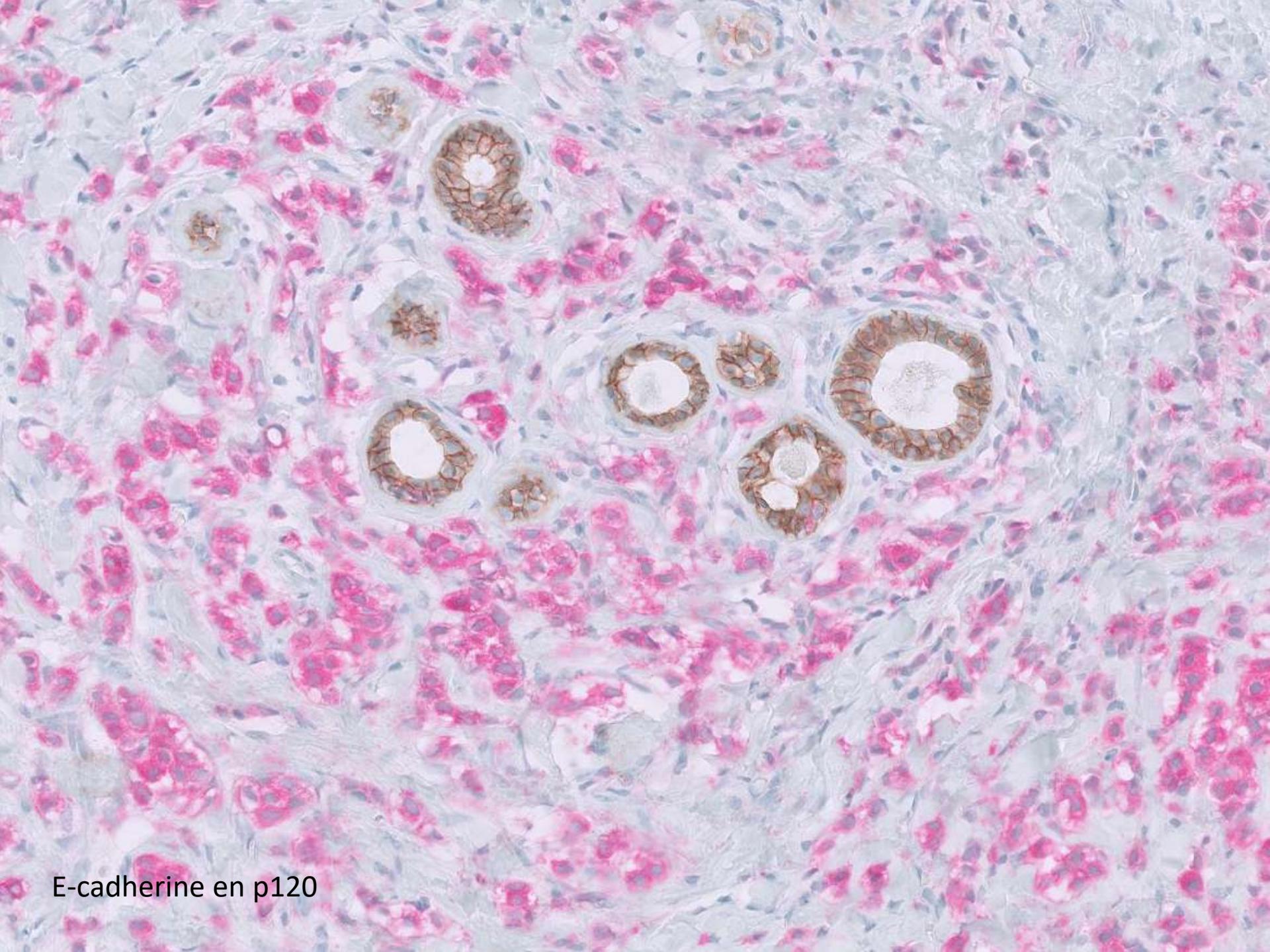
Rabbit α EGFR Primary Antibody



ultraView

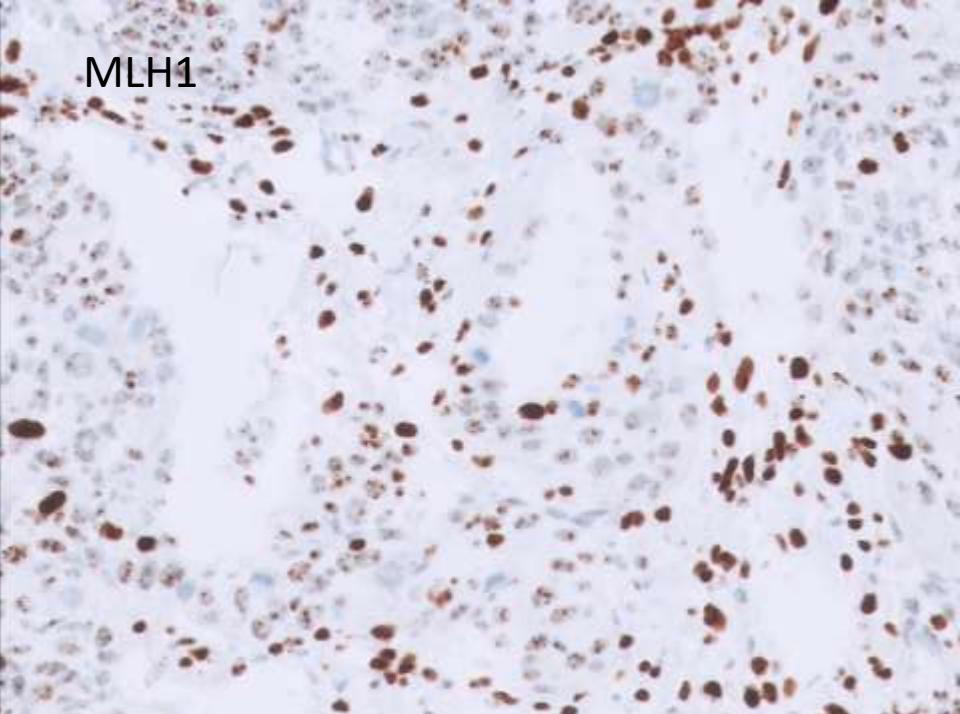
OptiView

**OptiView +
Amplification**

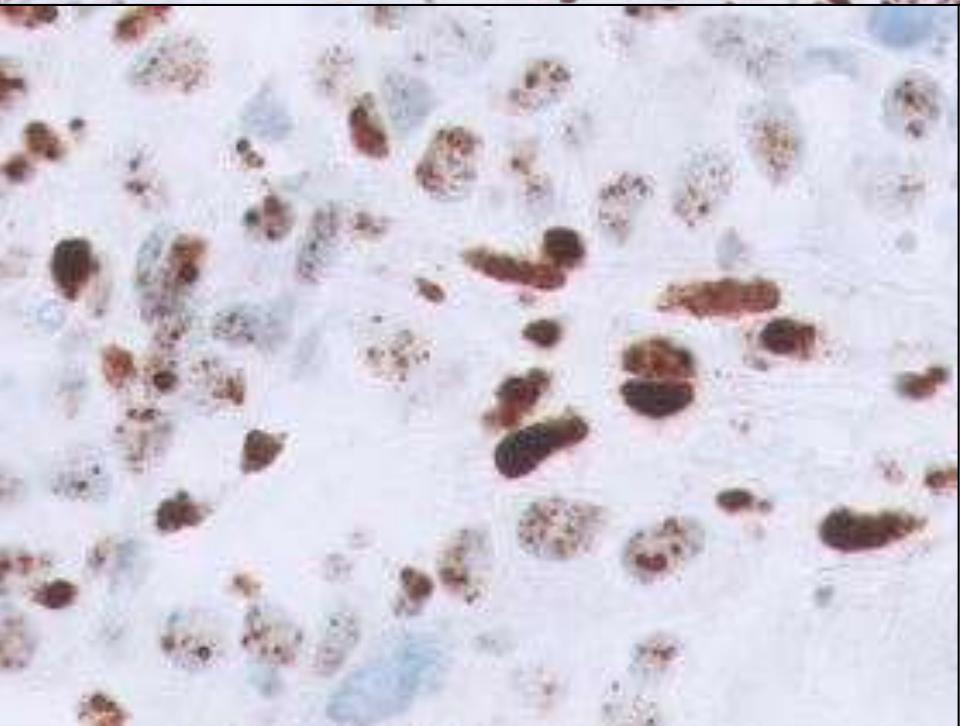
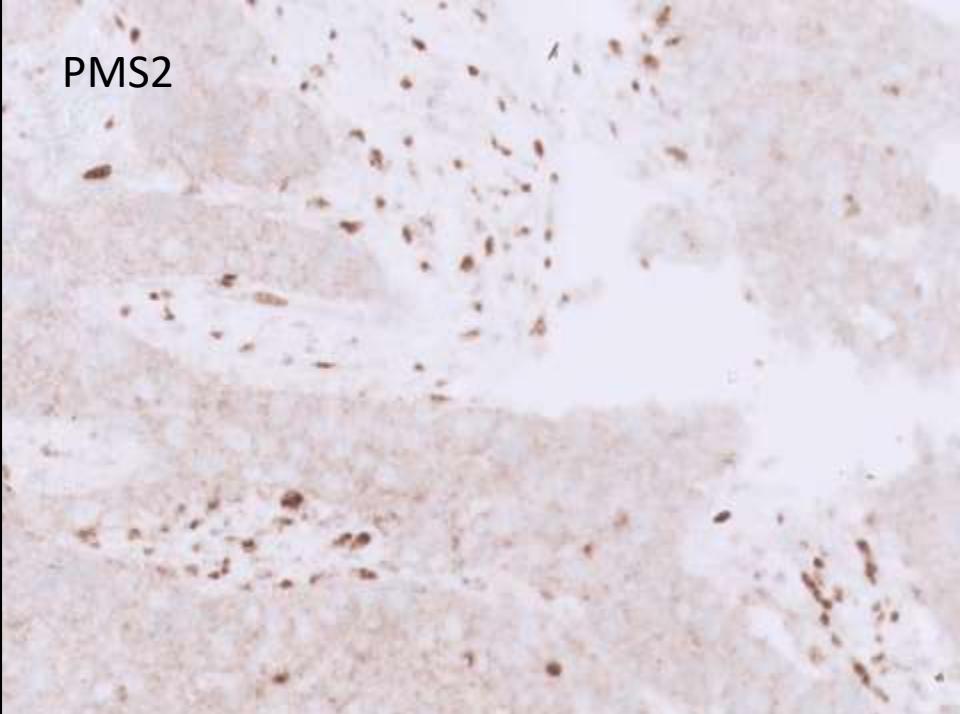


E-cadherine en p120

MLH1



PMS2



Immunohistochemie

- Optimalisatie
 - Concentraties
 - Incubatietijden
 - Incubatietemperatuur
 - Block
 - Antigenretrieval
- CE/IVD, RTU, RUO
- IVDR en de nieuwe praktijkrichtlijn

Immunohistochemie

- Diagnostisch
 - Cytokeratines, TTF1, CDX2, ...
- Prognostisch
 - Ki67, ...
- Therapeutisch
 - ER, PR, ...
- ‘Moleculair’
 - IDH1, BRAF V600E, ALK, ROS, pan-TRK
 - EPM2AIP voor MLH1 promotor methylatie
 - RAS Q61R
- Relatief snel
- Relatief goedkoop
- **Correlatie met de histologie**

In situ hybridisatie

- Detectie van nucleïnezuur sequenties door middel van een probe
 - DNA of RNA
- Directe of indirecte labelling
- Target sequentie moet toegankelijk zijn
 - Enkelstrengig
- FISH (fluorescente)
- CISH (chromogene)
- SISH (silver stain)

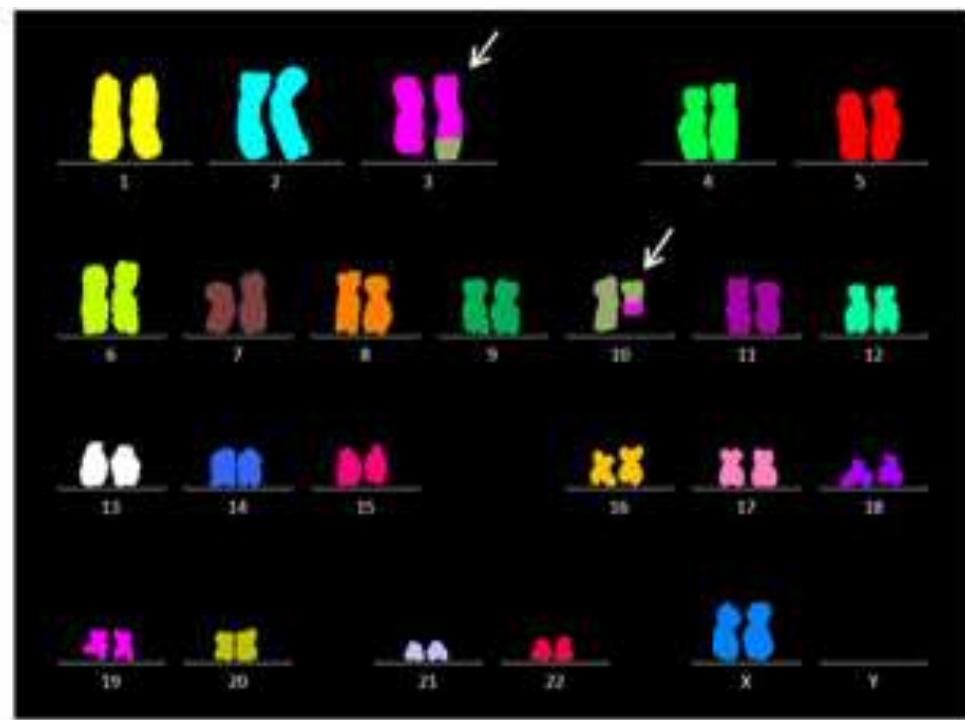
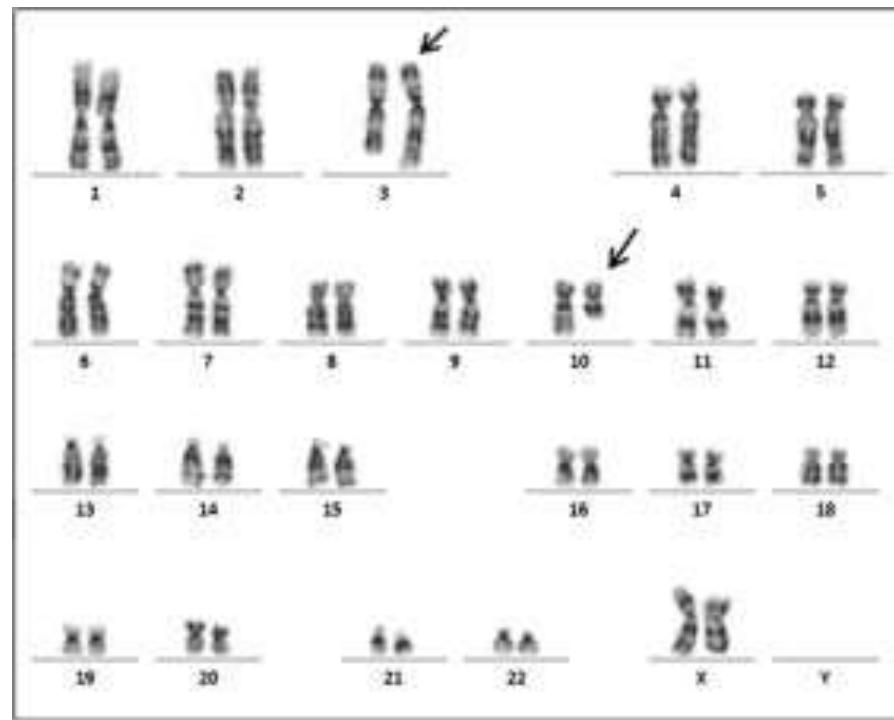
Basis principe ISH

- Coupe
- Deparaffinisatie bij FFPE
- Proteolyse (enzymatisch, b.v. pepsine of proteïnase K) en denaturatie (thermisch)
- Probe hybridisatie met target sequentie, DNA of RNA
- Signaal detectie d.m.v fluorescentie in FISH of chromogenen in CISH en SISH
- Tegenkleuring, DAPI bij FISH (enkel kern), haematoxyline bij CISH/SISH

Basis principe ISH

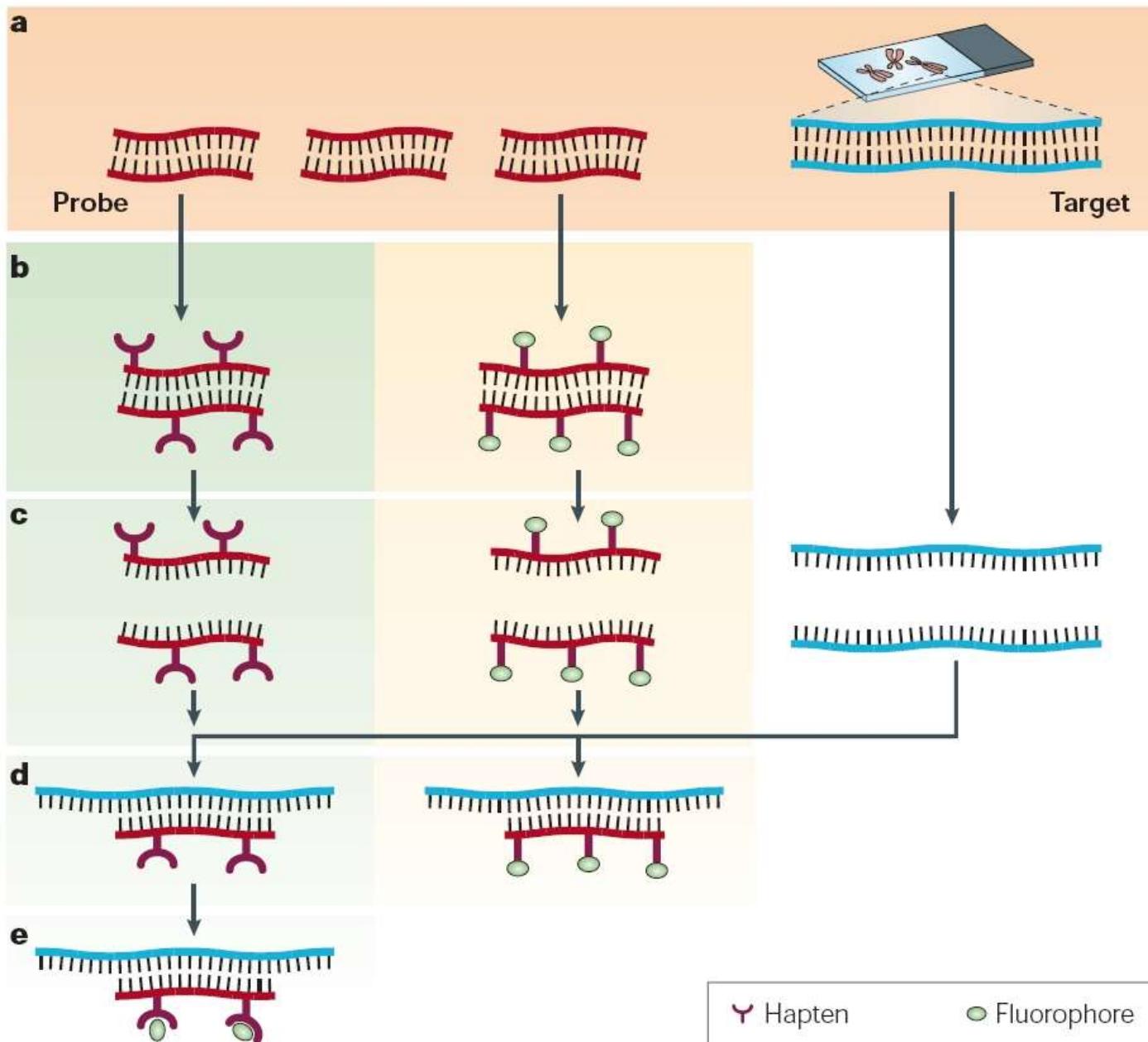
- Probes zijn meestal 200-300 nucleotiden lang maar kunnen variëren tussen 20-30 baseparen en 1Mb
- Hoe langer, hoe specifieker
- Voordelen t.o.v. IHC:
 - Specificiteit
 - Minder invloed van de formolfixatie
 - De hybridisatie tussen probe en target is sterker dan de binding tussen AG-AL

Verschillende fluorochromen zijn mogelijk



aberrant karyotype 46,XX,t(3;10)(q26;q21) in AML

Jancuskova, Tereza & Plachy, Radek & Zemankova, Lucie & Hardekopf, David & Stika, Jiri & Zejskova, Lenka & Praulich, Inka & Kreuzer, Karl-Anton & Rothe, Achim & Othman, Moneeb & Kosyakova, Nadezda & Pekova, Sona. (2014). Molecular characterization of the rare translocation t(3;10)(q26;q21) in an acute myeloid leukemia patient. *Molecular cytogenetics*. 7. 47. 10.1186/1755-8166-7-47.

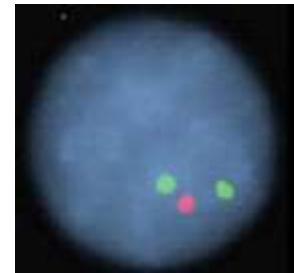
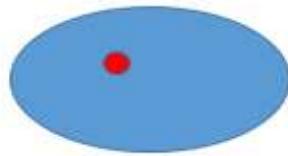
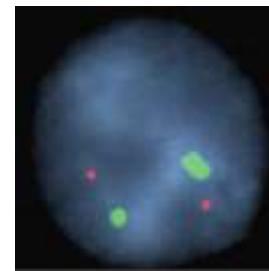
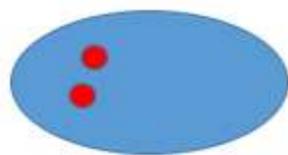


Toepassingen van ISH

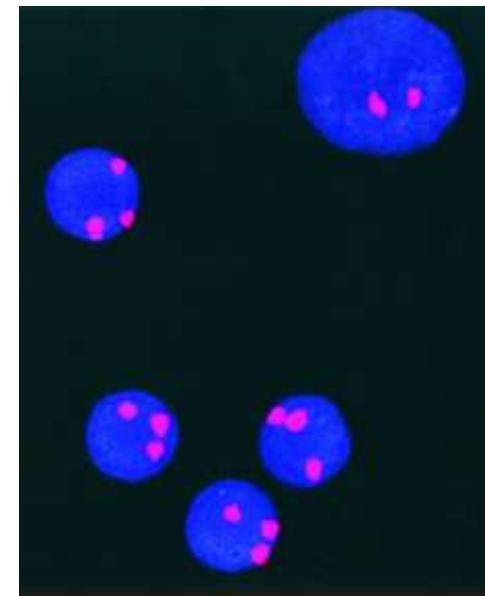
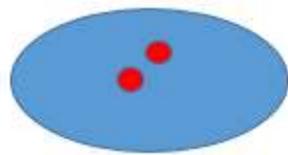
- **Detectie van viraal RNA or DNA**, b.v. EBV in hematopathologie of HPV in head & neck en cervixpathologie
- **Numerical changes:**
 - Gen amplificatie, b.v. MDM2 in liposarcoma, HER2 in borstcarcinooma
 - Polysomie, b.v. trisomie 12 in CLL
 - Gen verlies/deletie, b.v. Rb verlies in spindle cell lipoma
- **Gen translocatie** (break-apart FISH), b.v. translocation van EWSR1 in Ewing sarcoma.
- **Gen fusie**, e.g. EWSR1-FLI1 fusie in Ewing sarcoma

Numerical changes: polysomy/deletion

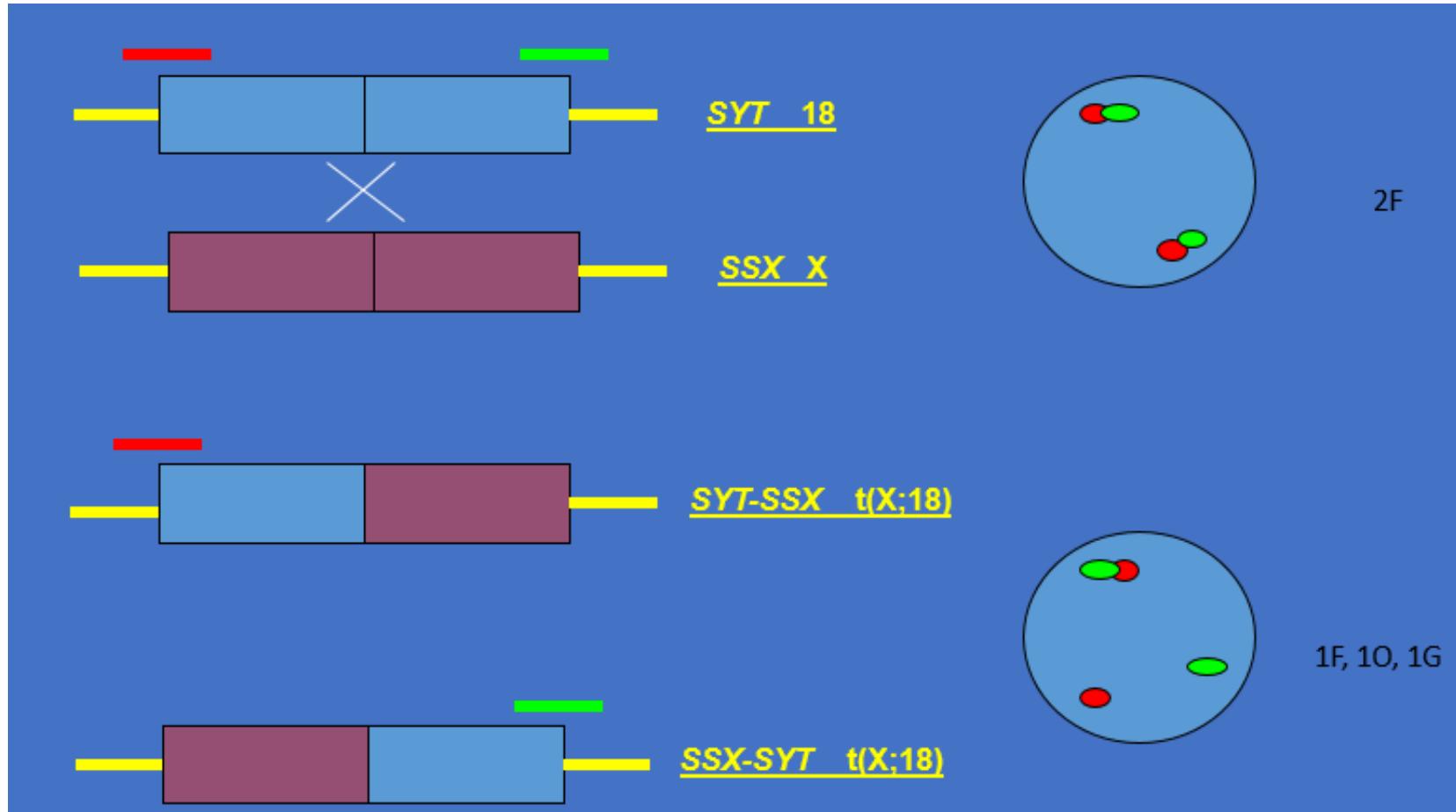
Deletie TP53 in CLL



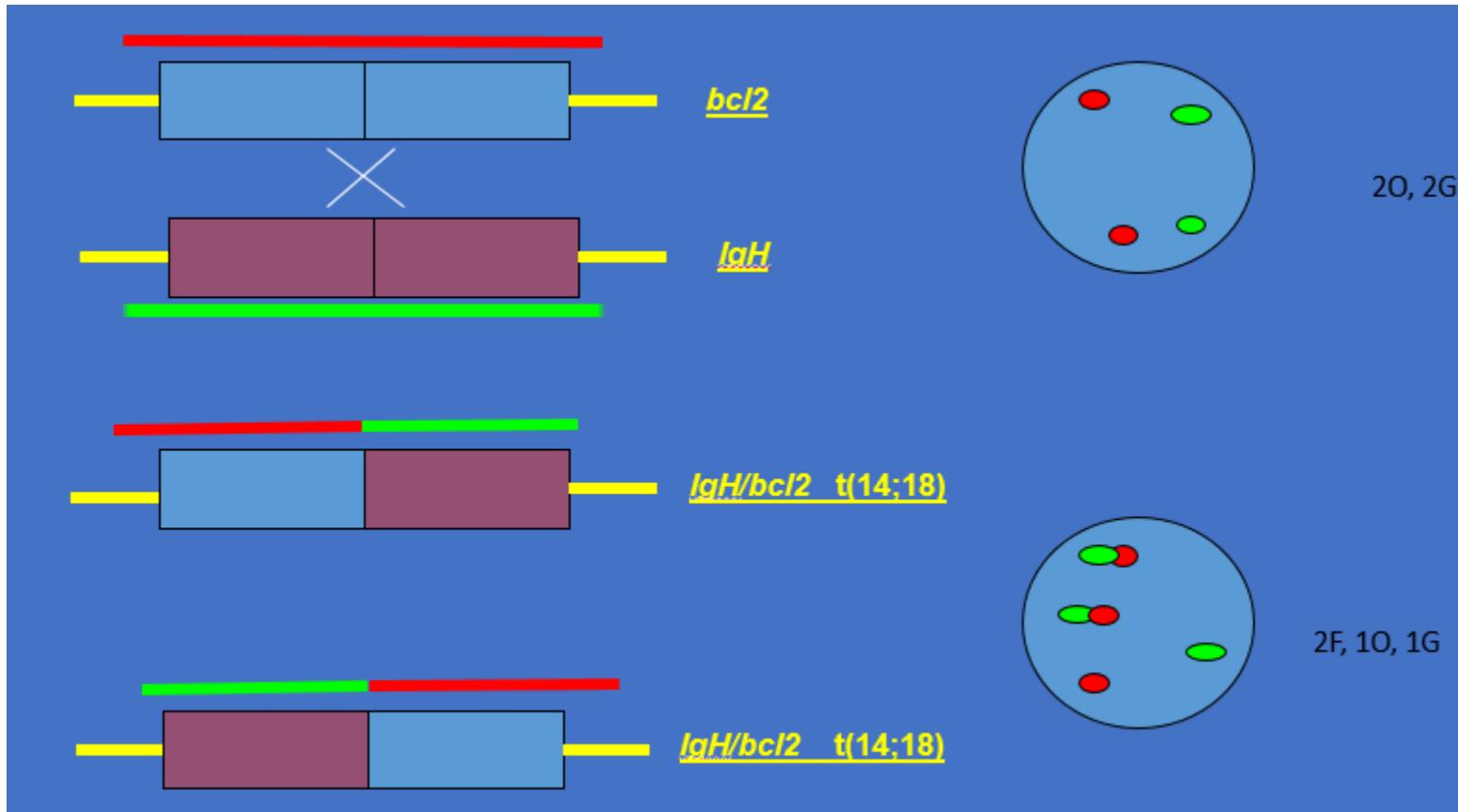
Trisomie 12 in CLL



Detectie van gen translocaties met break-apart probes

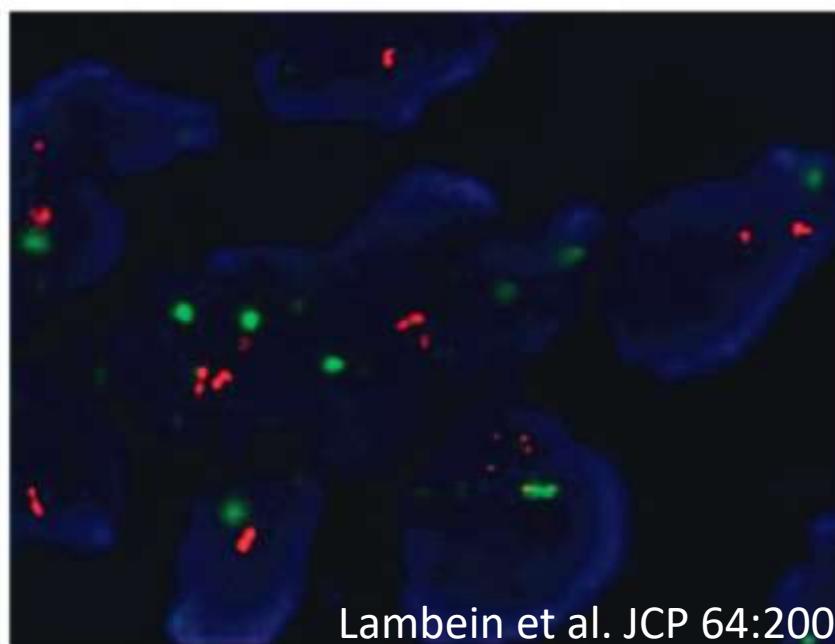
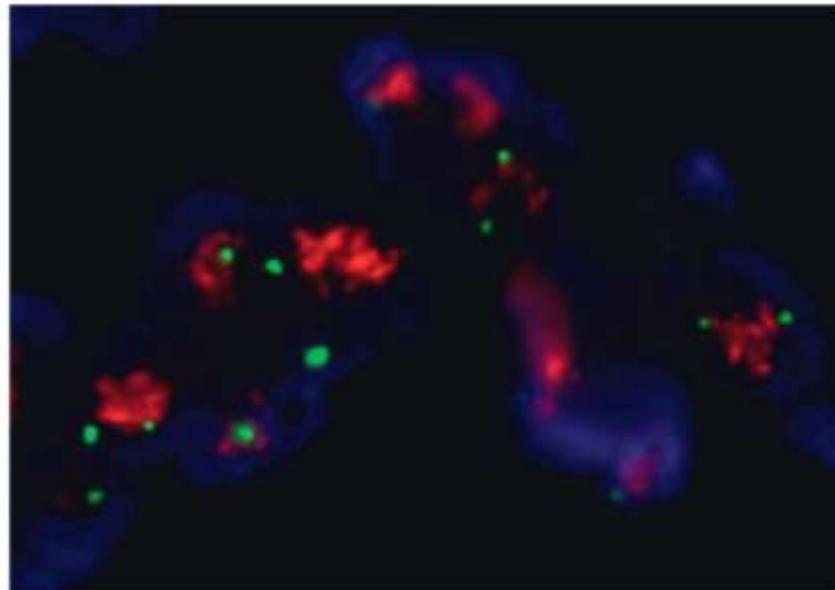
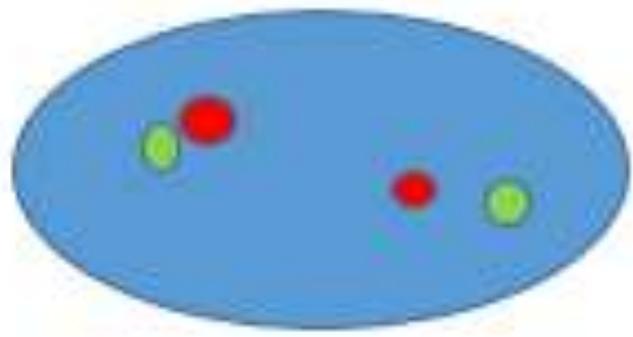
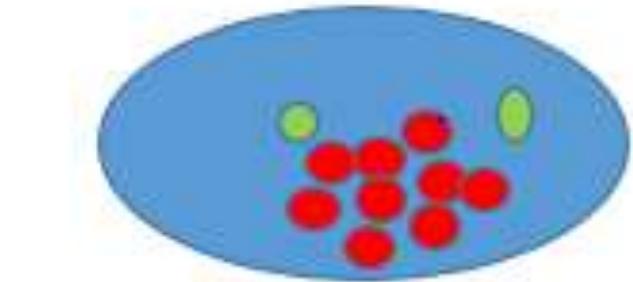


Detectie van gen fusies

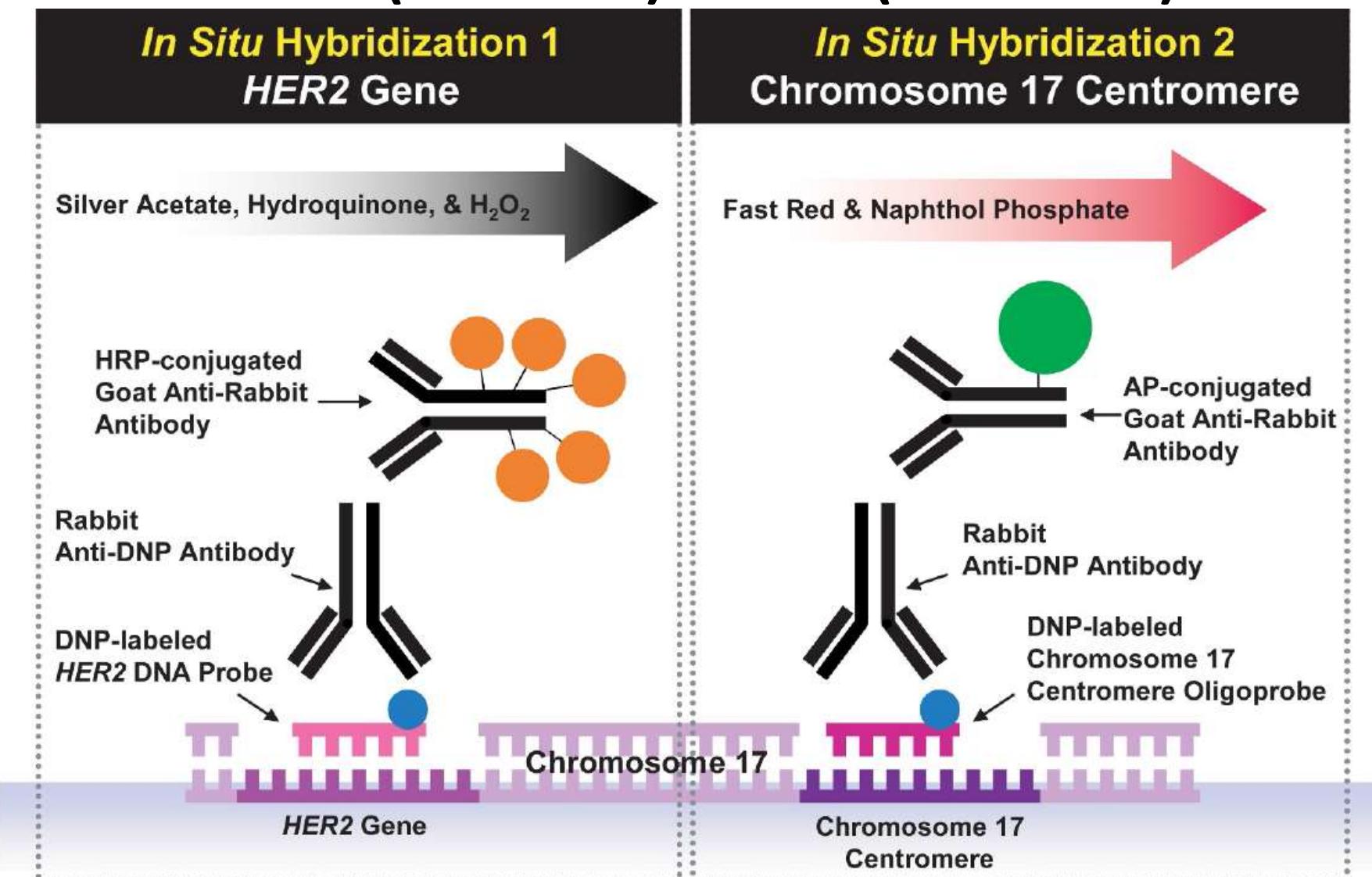


HER 2 Dual Color FISH bij borstcarcinoom

17

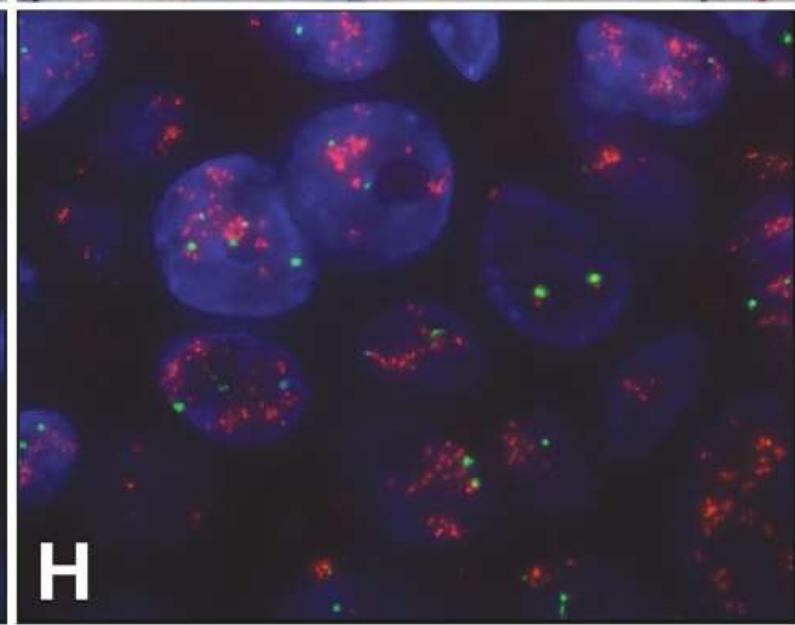
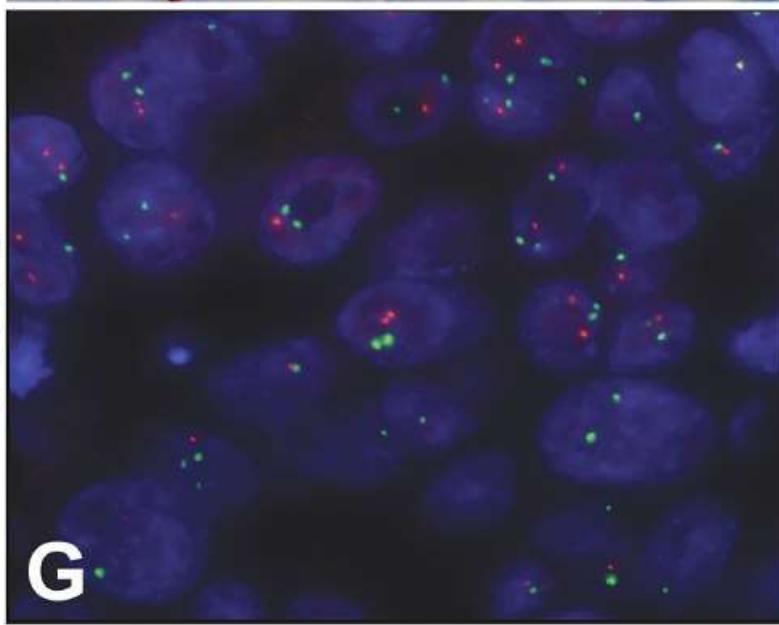
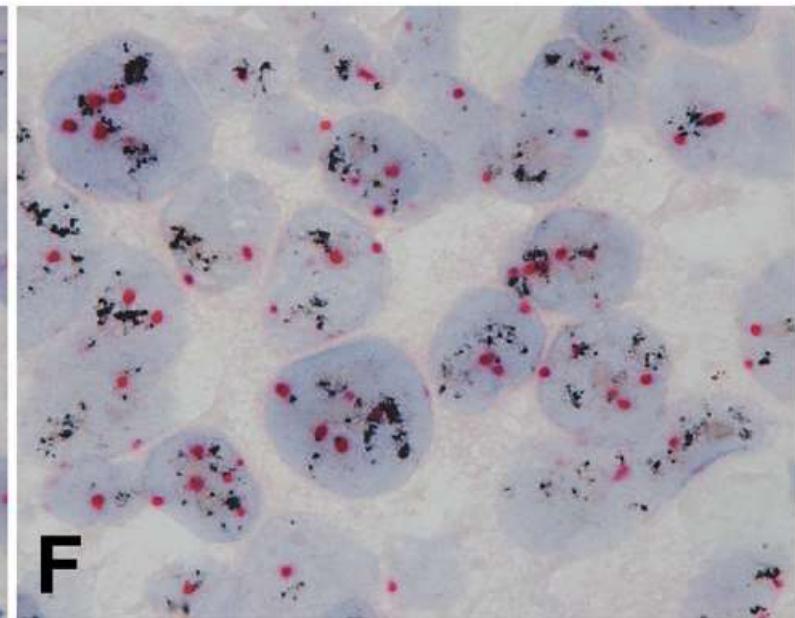
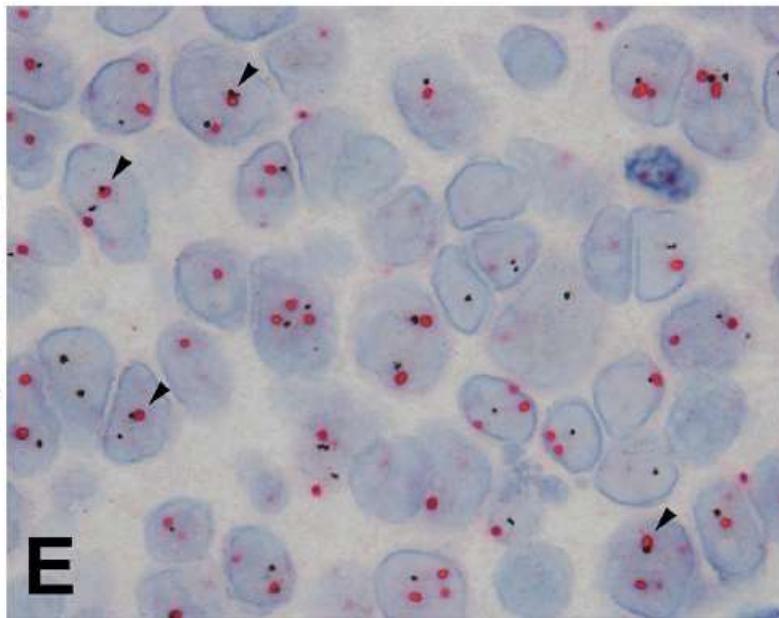


Bright-field double *in situ* hybridization (BDISH): SISH (for HER2) + CISH (for CEP17)



HER2 & CEN 17

FISH BDIISH



Voordelen van FISH

- Hogere resolutie
- Vandaar het gebruik voor de evaluatie van fusies en translocaties

Voordelen van SISH/CISH

- Geen signaal fading
- Geen nood aan een fluorescentiemicroscoop
- Betere weefsel correlatie

Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer

American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update

Antonio C. Wolff, M. Elizabeth Hale Hammond, Kimberly H. Allison, Brittany E. Harvey, Pamela B. Mangu, John M.S. Bartlett, Michael Bilous, Ian O. Ellis, Patrick Fitzgibbons, Wedad Hanna, Robert B. Jenkins, Michael F. Press, Patricia A. Spears, Gail H. Vance, Giuseppe Viale, Lisa M. McShane, Mitchell Dowsett

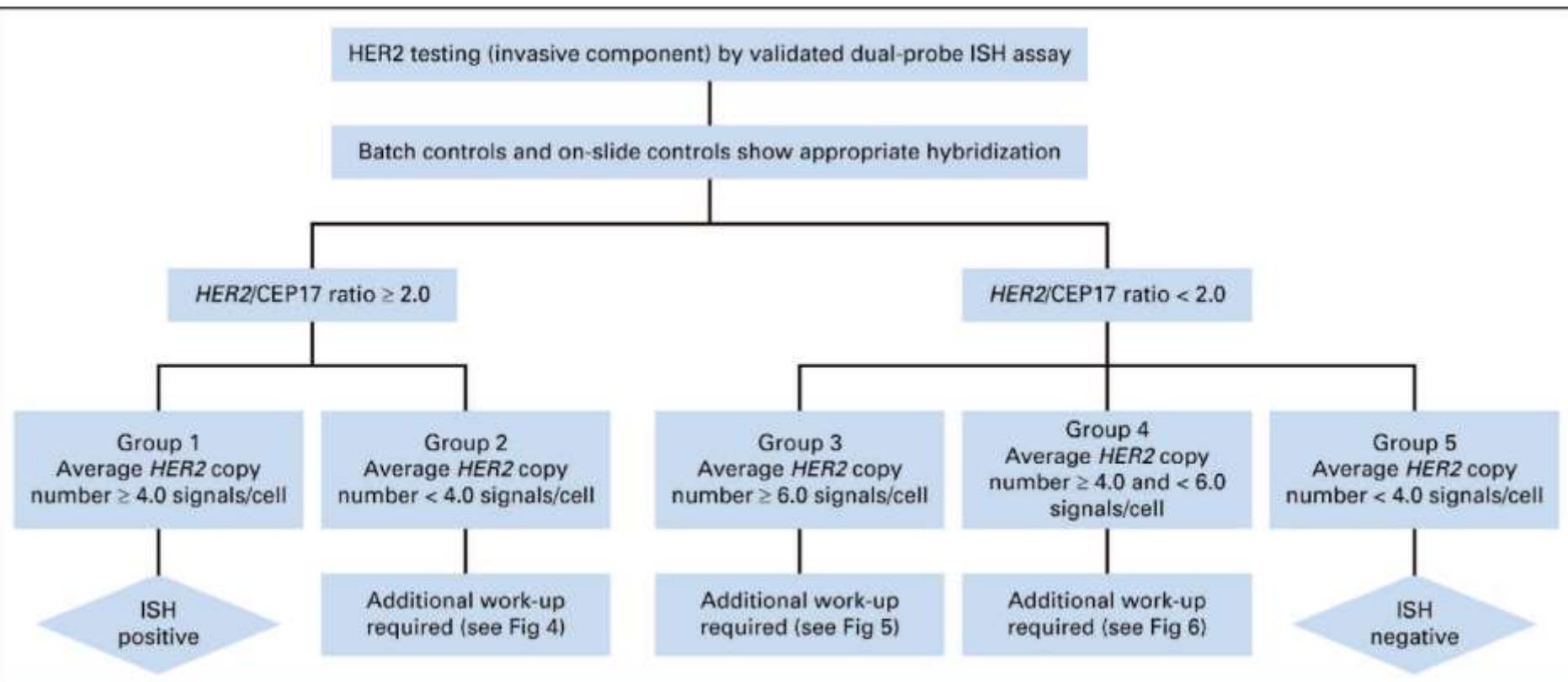


Figure 4

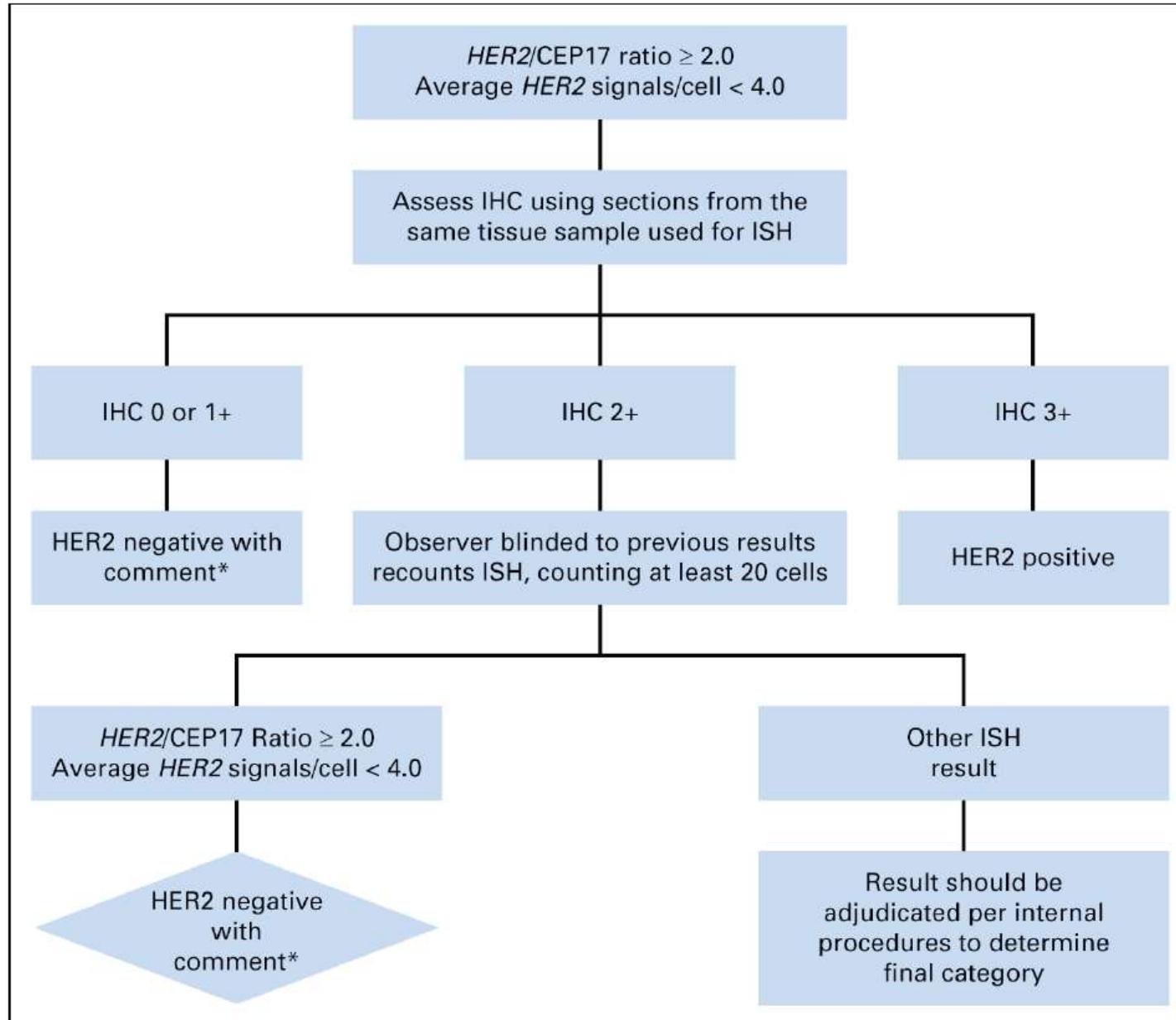


Figure 5

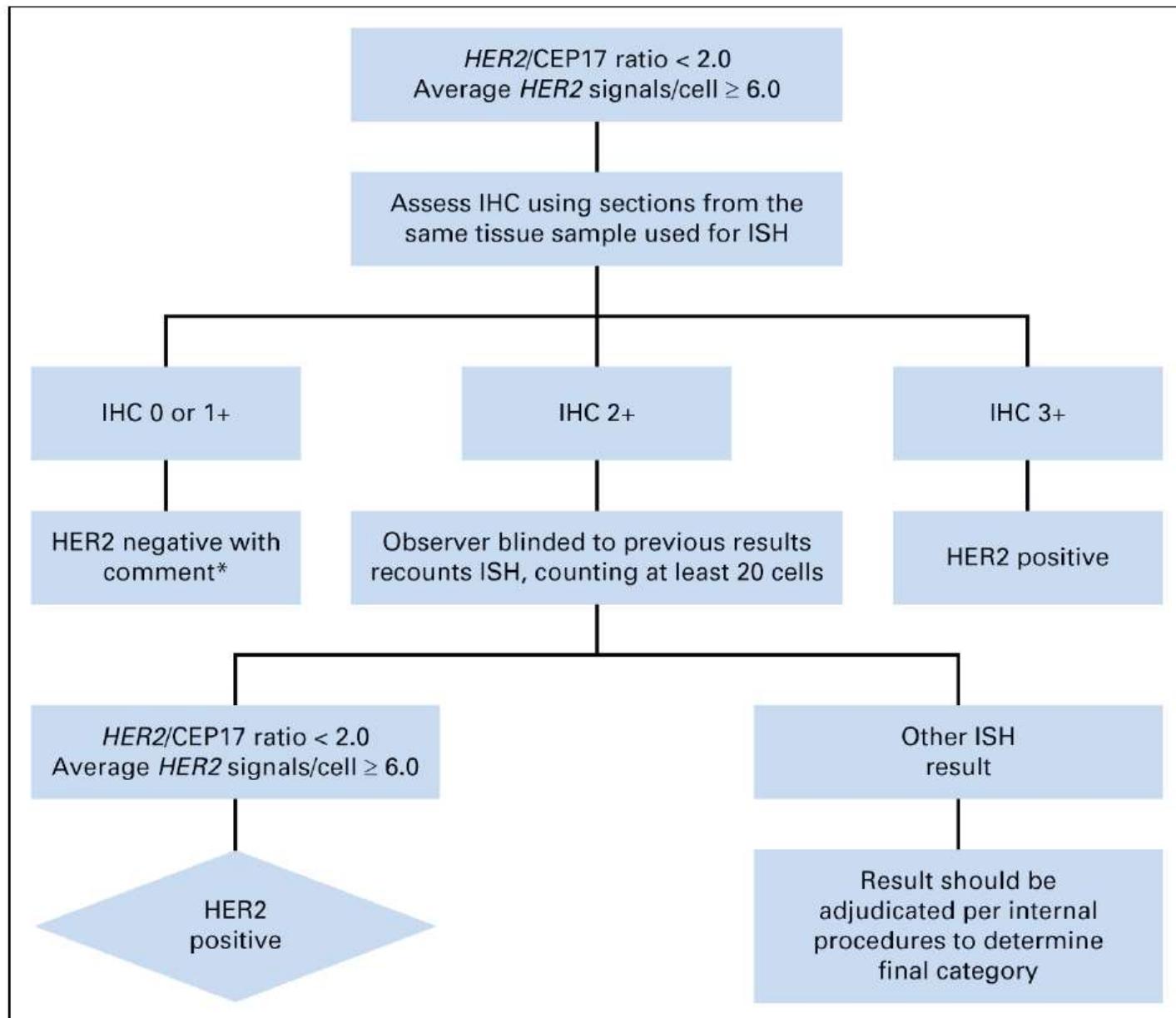
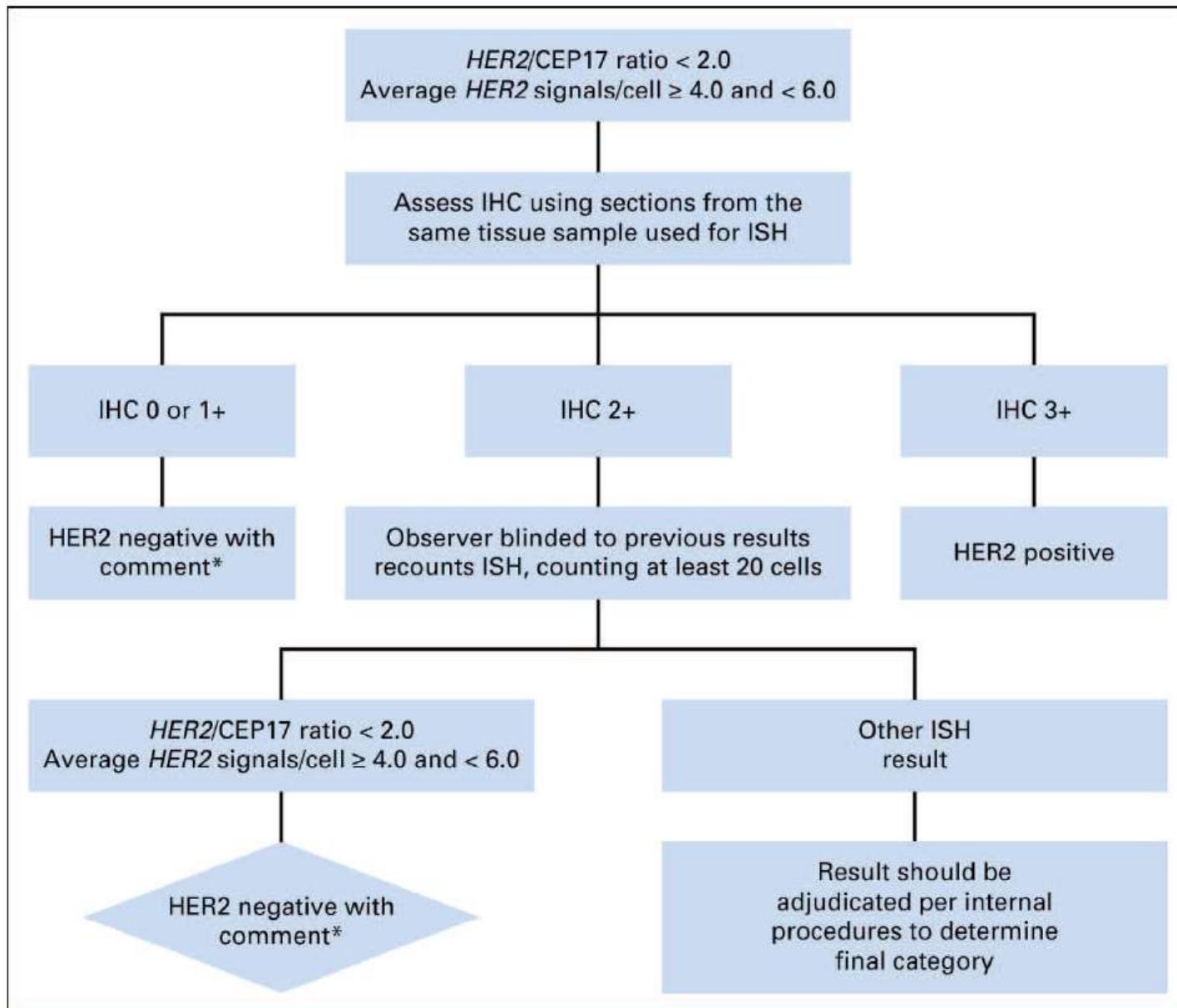
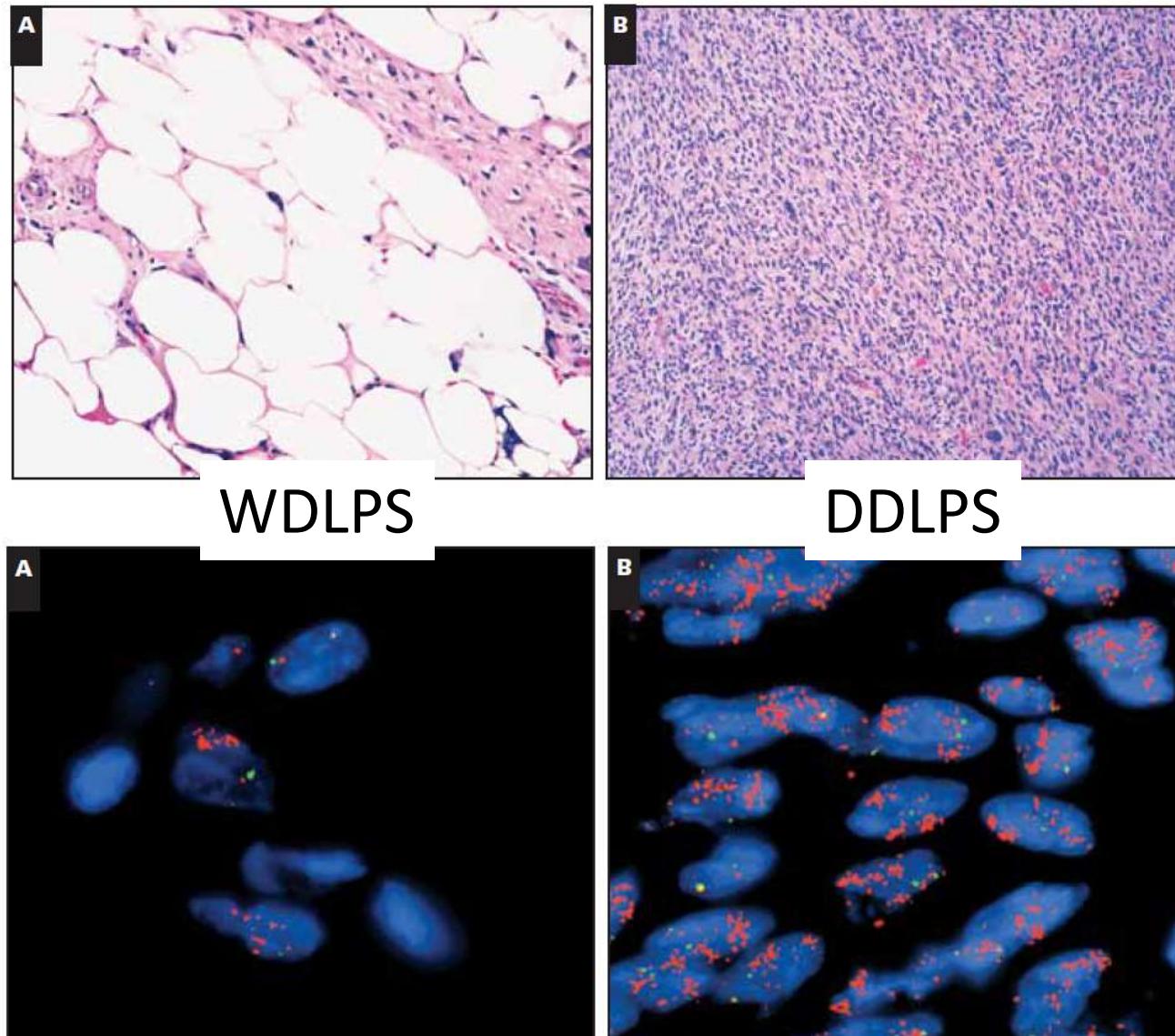


Figure 6

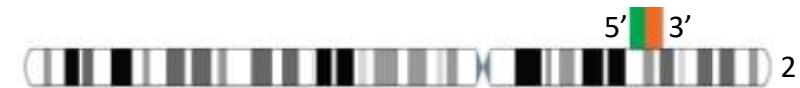
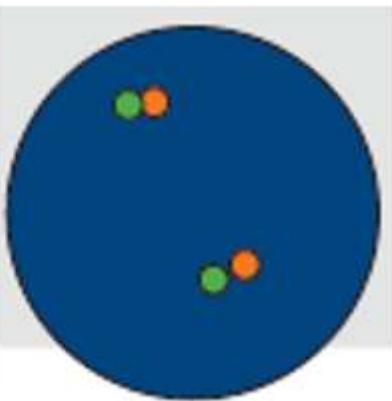
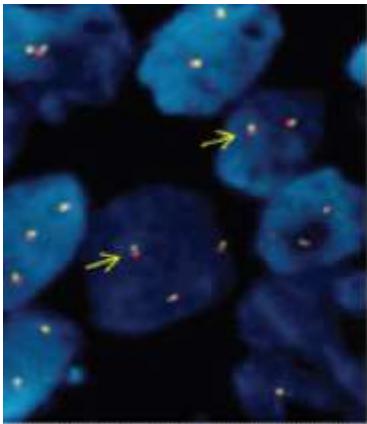


Toename van het MDM2 copynumber bij de evolutie van WDLS naar DDLPS

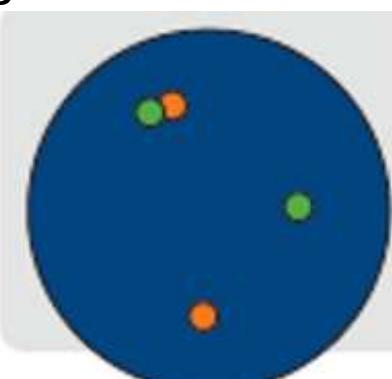
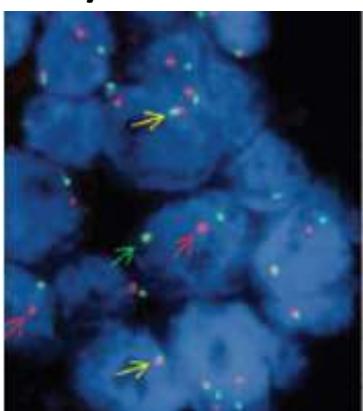


Long adenocarcinoma: ALK en ROS1

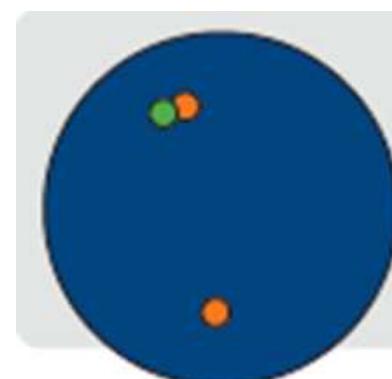
Normal pattern



ALK/ROS1 rearrangements



1 fused signal and 1
split signal



Isolated 3' signal pattern
and 1 fused signal



Lung adenocarcinoma: ALK and ROS1

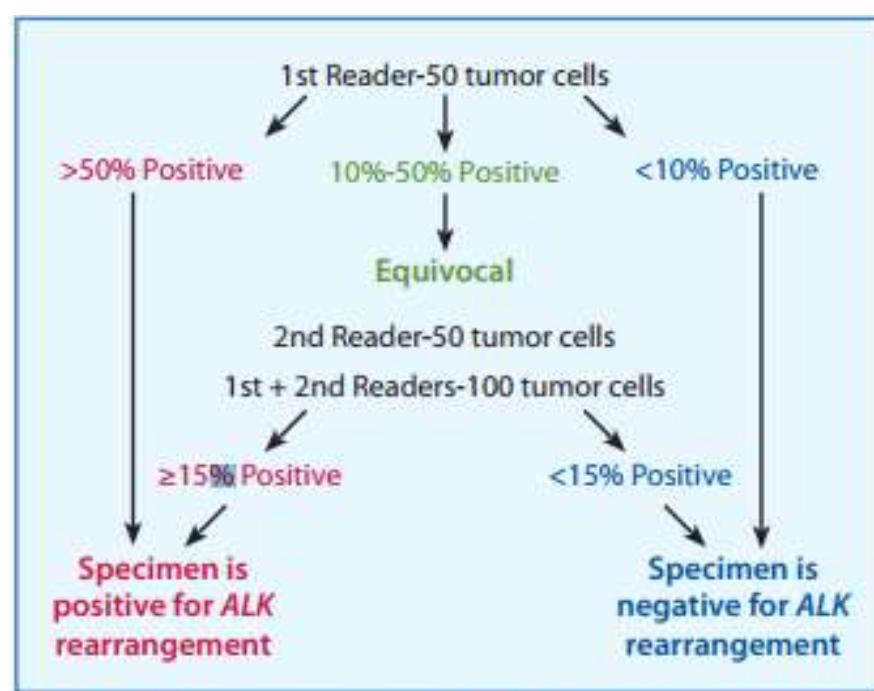


Figure 11. Recommended scoring algorithm for ALK FISH.

SECOND EDITION

IASLC ATLAS OF ALK AND ROS1 TESTING IN LUNG CANCER



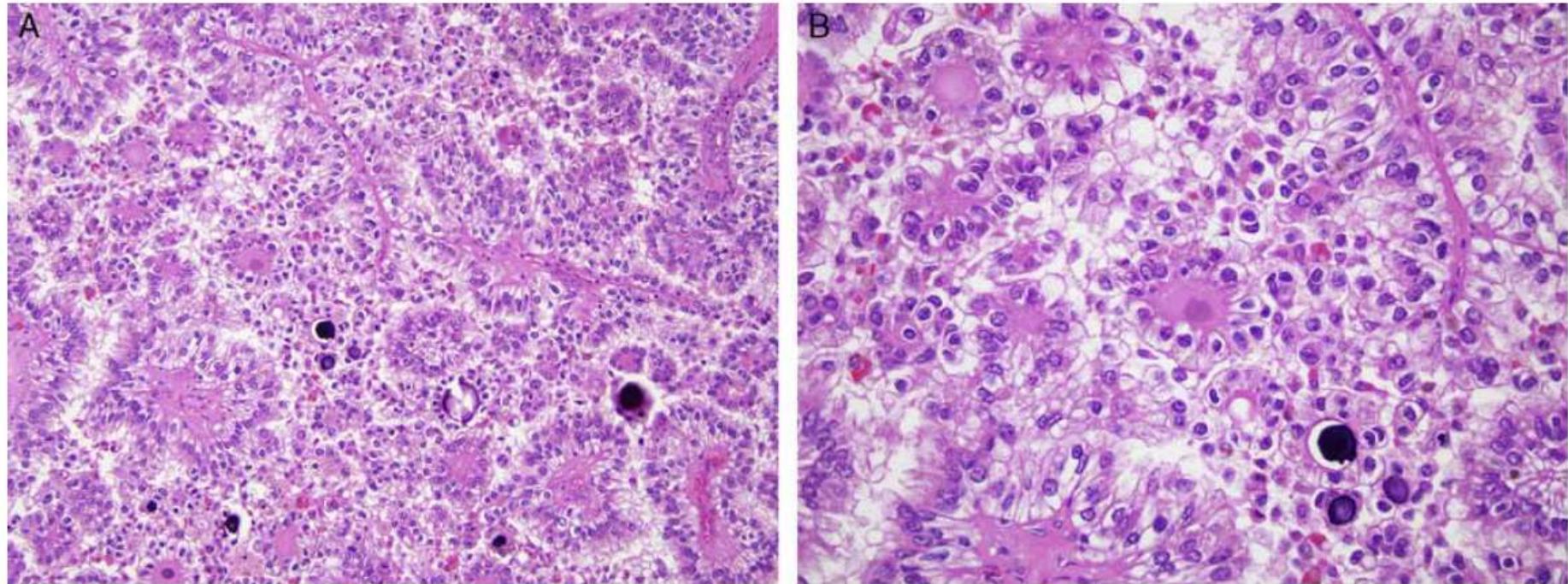
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Pathological diagnosis and
molecular testing in non-small cell
lung cancer: Belgian guidelines

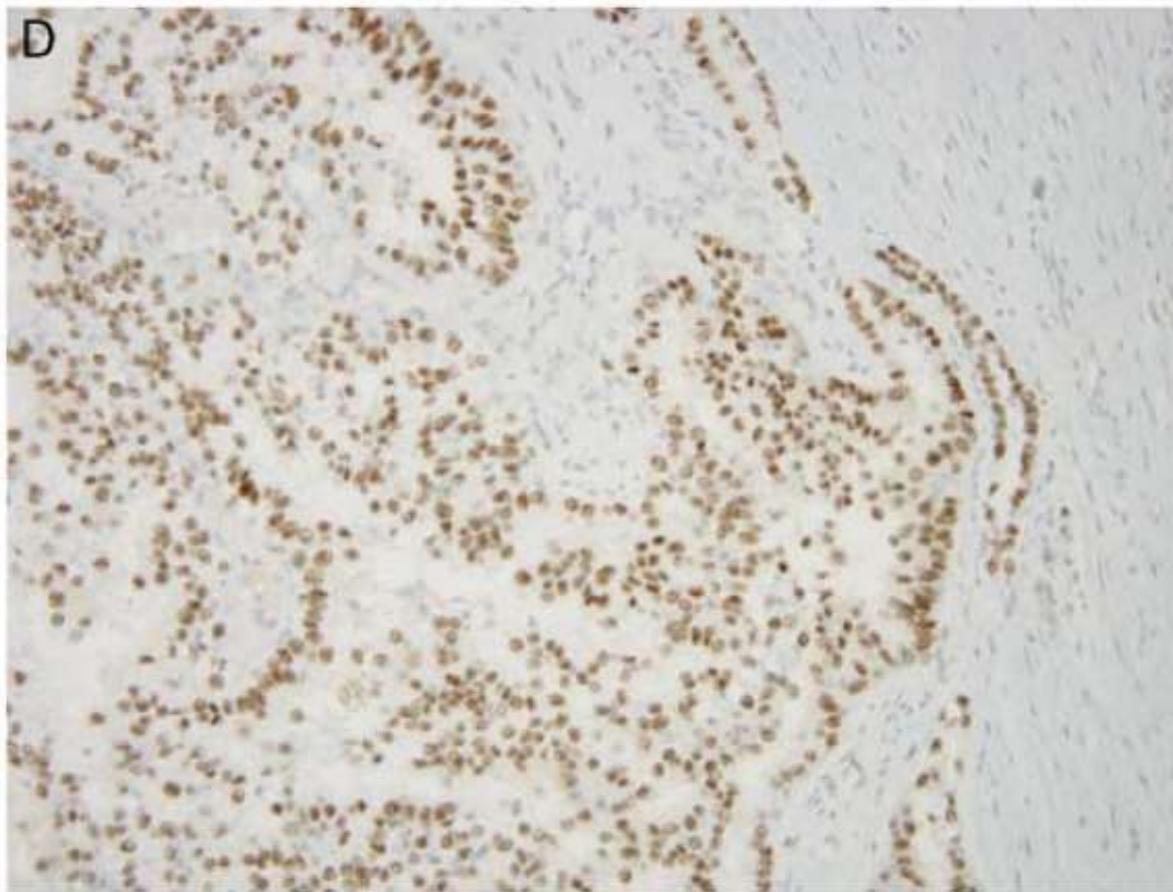
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On behalf of the Working Group of Molecular Pathology and the Belgian Society of Pathology

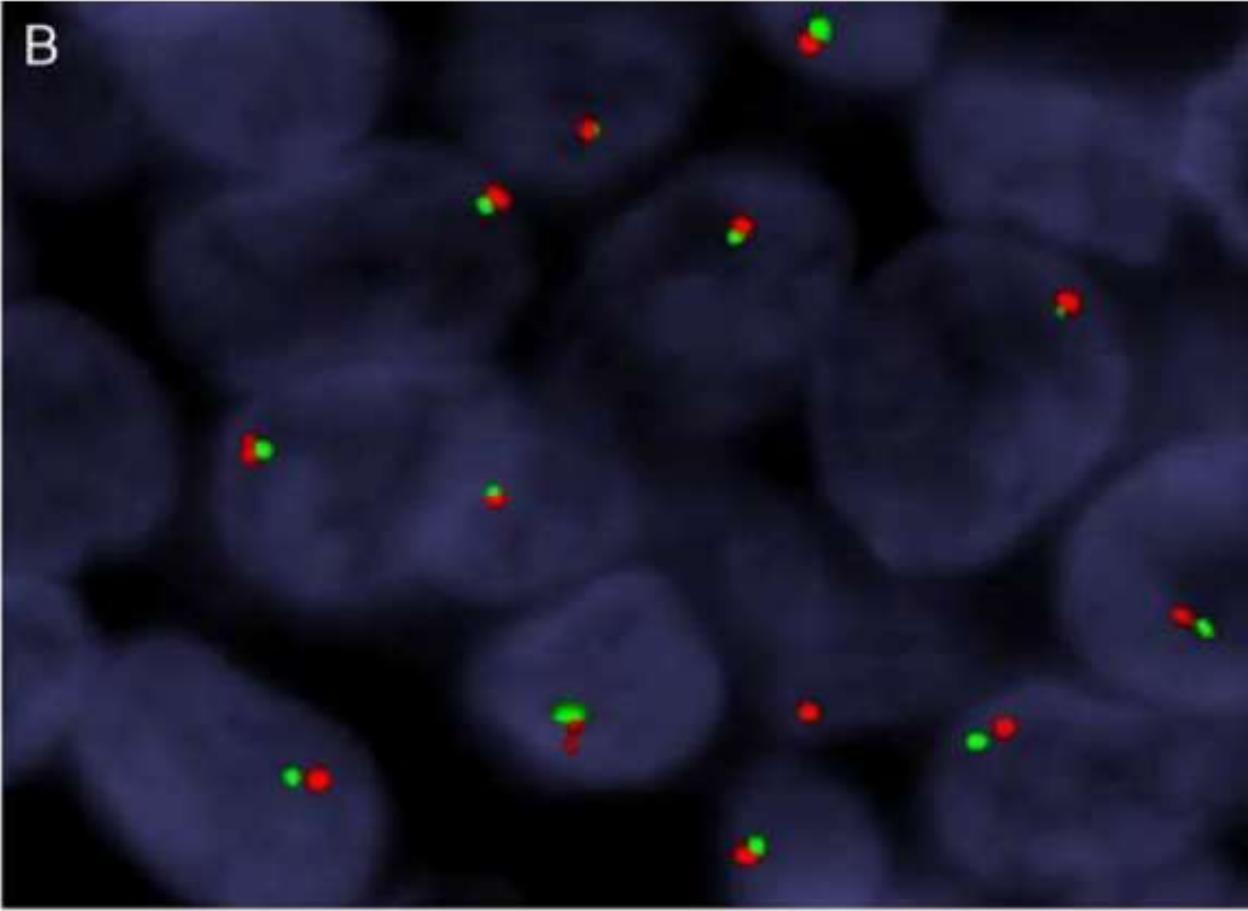
Voorbeeld



A renal cell carcinoma with papillary structures,
clear cells and psammoma bodies:
HE strongly suggestive for
TFE3 translocation renal cell carcinoma



TFE3 IHC diffusely positive



TFE3 FISH:

red and green signal together, so TFE3 is not displaced

RBM10-TFE3 Renal Cell Carcinoma

A Potential Diagnostic Pitfall Due to Cryptic Intrachromosomal Xp11.2 Inversion Resulting in False-negative TFE3 FISH

Pedram Argani, MD,*† Lei Zhang, MD,‡ Victor E. Reuter, MD,‡ Satish K. Tickoo, MD,‡ and Cristina R. Antonescu, MD‡

- In this tumor, the partner of TFE3 is RBM10.
- RBM10 is normally only 1.8 Mb away from TFE3.
- So, TFE3 “moves” only a very little bit, via inversion.
- On a normal break-apart FISH, this subtle move can not be seen; a specific RBM10-TFE3 FISH was positive.

Take home message

- Weet waar je naar kijkt en wat je kan verwachten
- Weet wat je moet zien
- Weet wat je niet mag zien
- Ken de beperkingen van de gebruikte methode
- Wees altijd kritisch bij het beoordelen van kleuringen, IHC of ISH, zelfs HC

