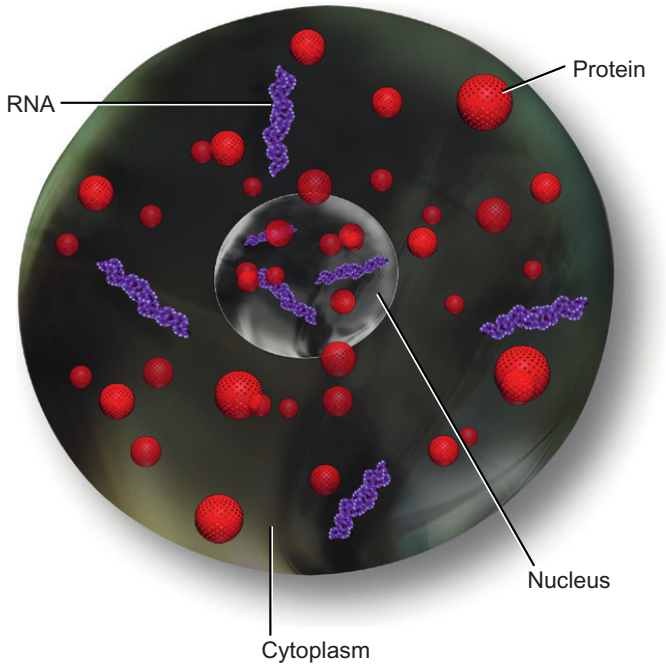
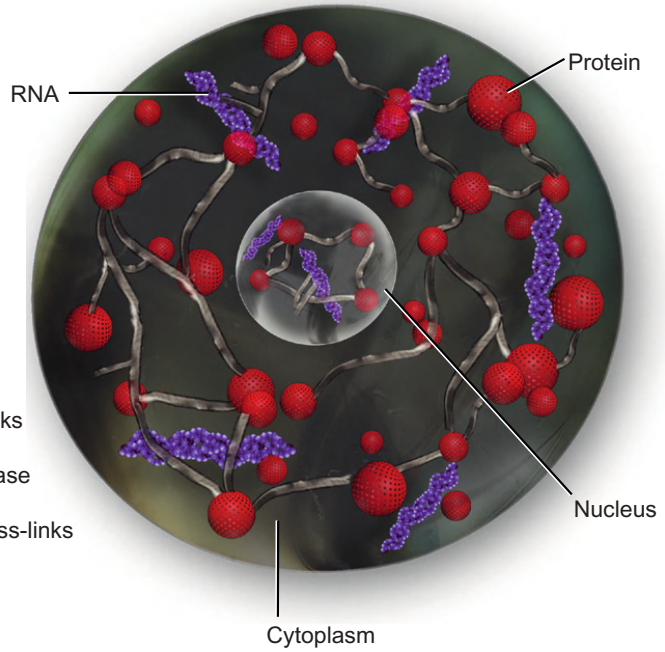
A



UNFIXED CELL

FORMALIN ADDED

B



FIXED CELL

Formalin fixation gives more resistance to cells, but nucleic acids become fragile

Consequences:

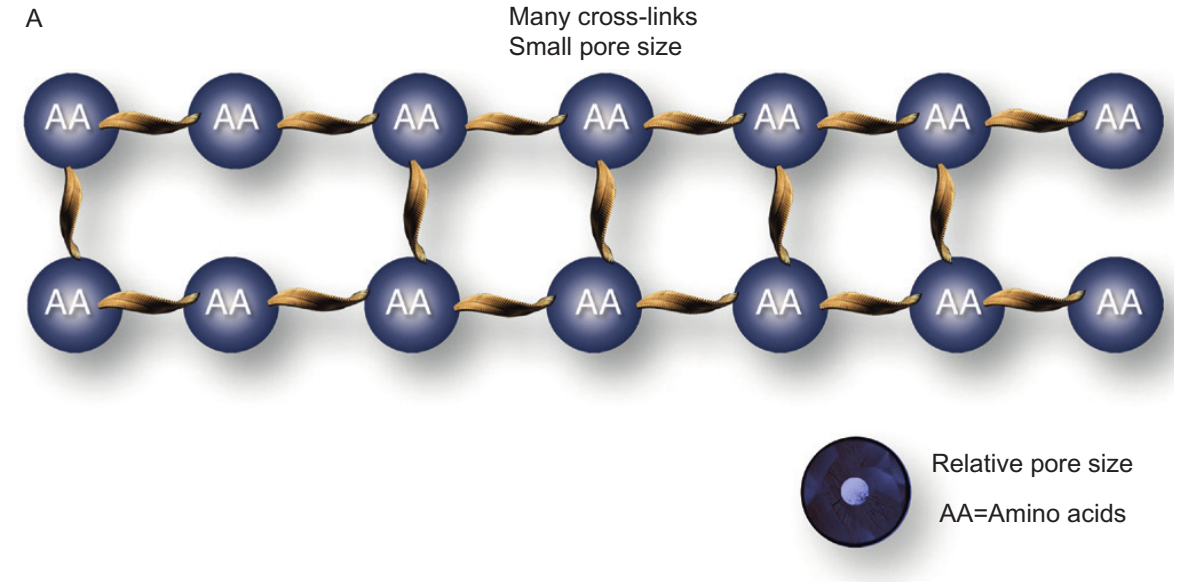
1) Extensive Protein to Protein cross-links

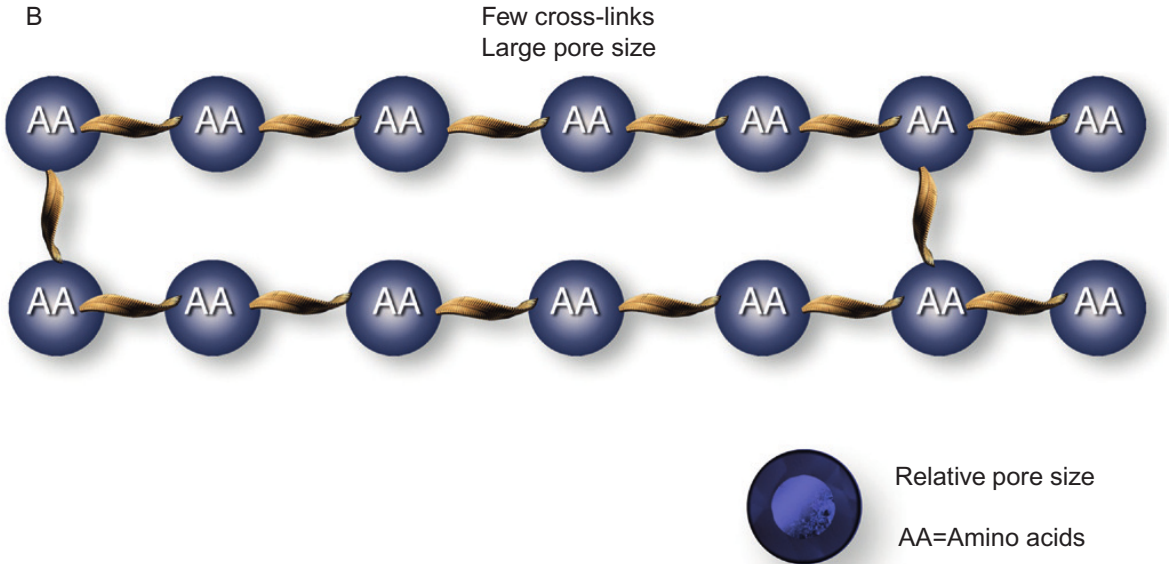
2) Inactivation of RNase, DNase, Protease

3) Extensive Protein to DNA + RNA cross-links

In Situ Molecular Pathology and Co-Expression Analyses

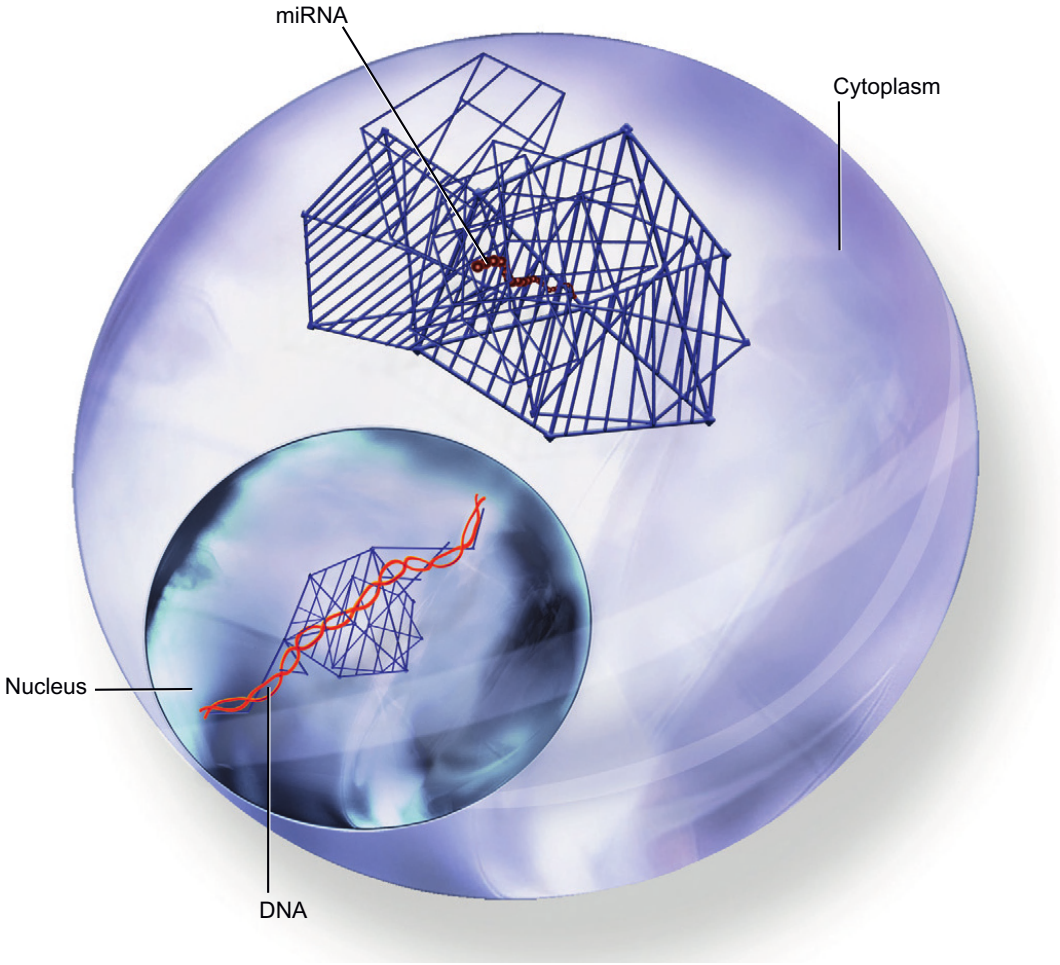
Gerard J. Nuovo

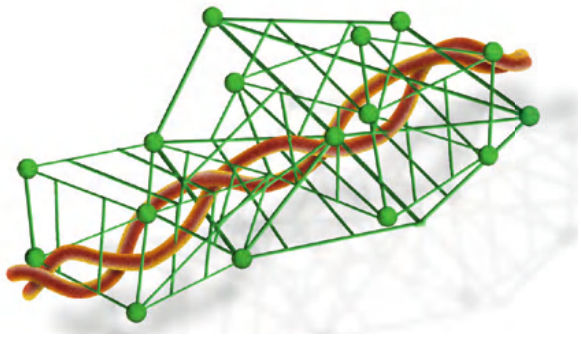




The longer is fixation the higher is number of crosslinks

VARIABLE SIZED CAGES FOR DIFFERENT TARGETS IN THE SAME CELL





- The "cages" would represent the different protein density that surrounds any DNA, RNA, or protein epitope in the living state after variable cross-linking with formalin.
- This explain the marked differences in optimal pretreatment conditions for different targets in the same cell. It is to hypothesize that they are surrounded by variably sized "cages."

Ibridazione in situ-ISH

Sfrutta la reazione fra 2 sequenze di acidi nucleici a singolo filamento complemetari fra loro ⇒ interazione fra le 2: legami H

Rilevazione di ibridi in cellule o sezioni tissutali ⇒ morfologia

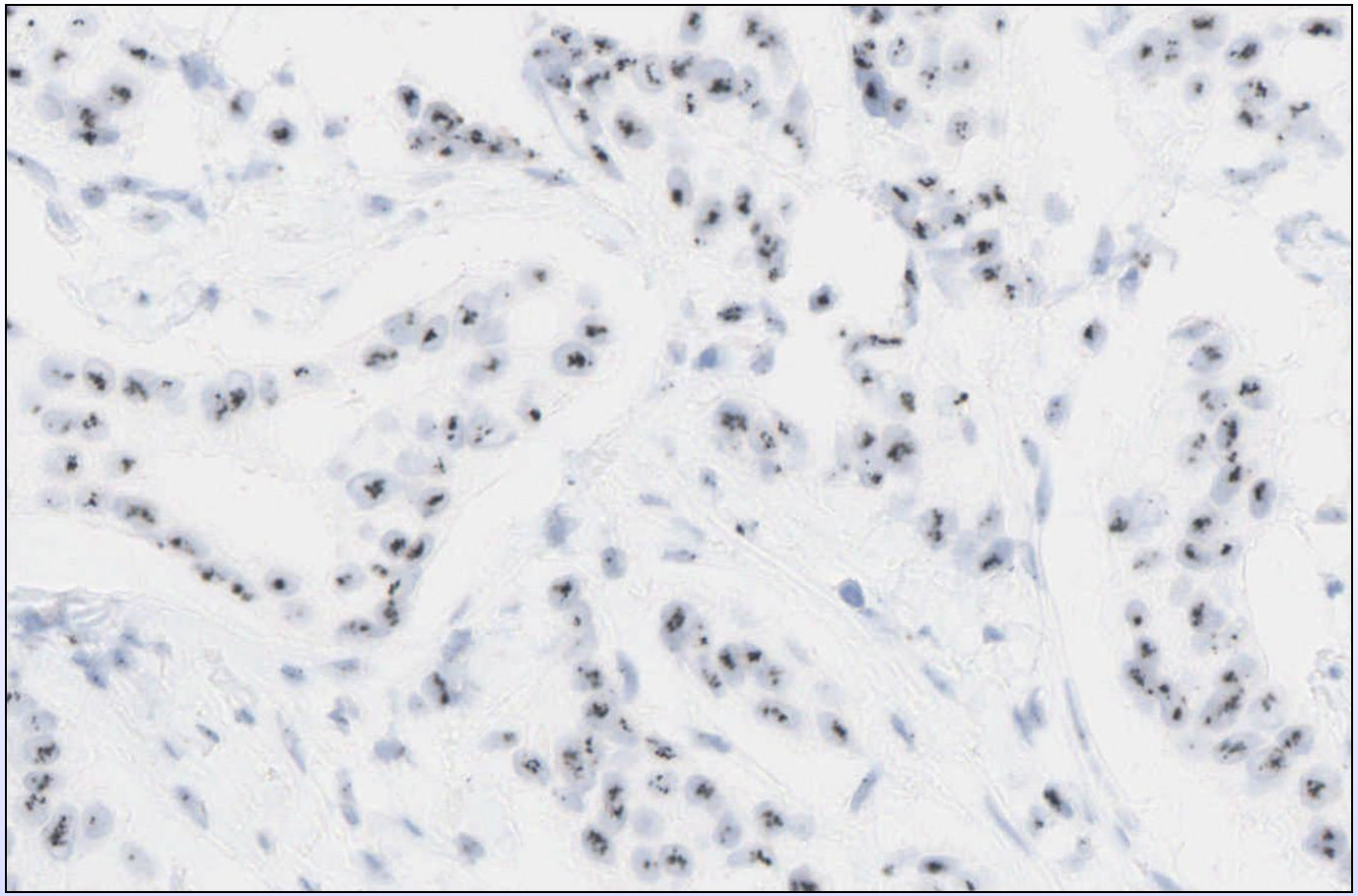
Utilizzata la prima volta per la rilevazione di sequenze di DNA o geni amplificati (RNA) in nuclei cellulari.

FISH: evidenzia siti specifici su nuclei o cromosomi mediante l'impiego di sonde che emettono in fluorescenza.
ISH: per RNA citoplasmatico usata la prima volta per rilevare sequenze virali in tessuti infetti.

FISH (Ibridazione In Situ Fluorescente)

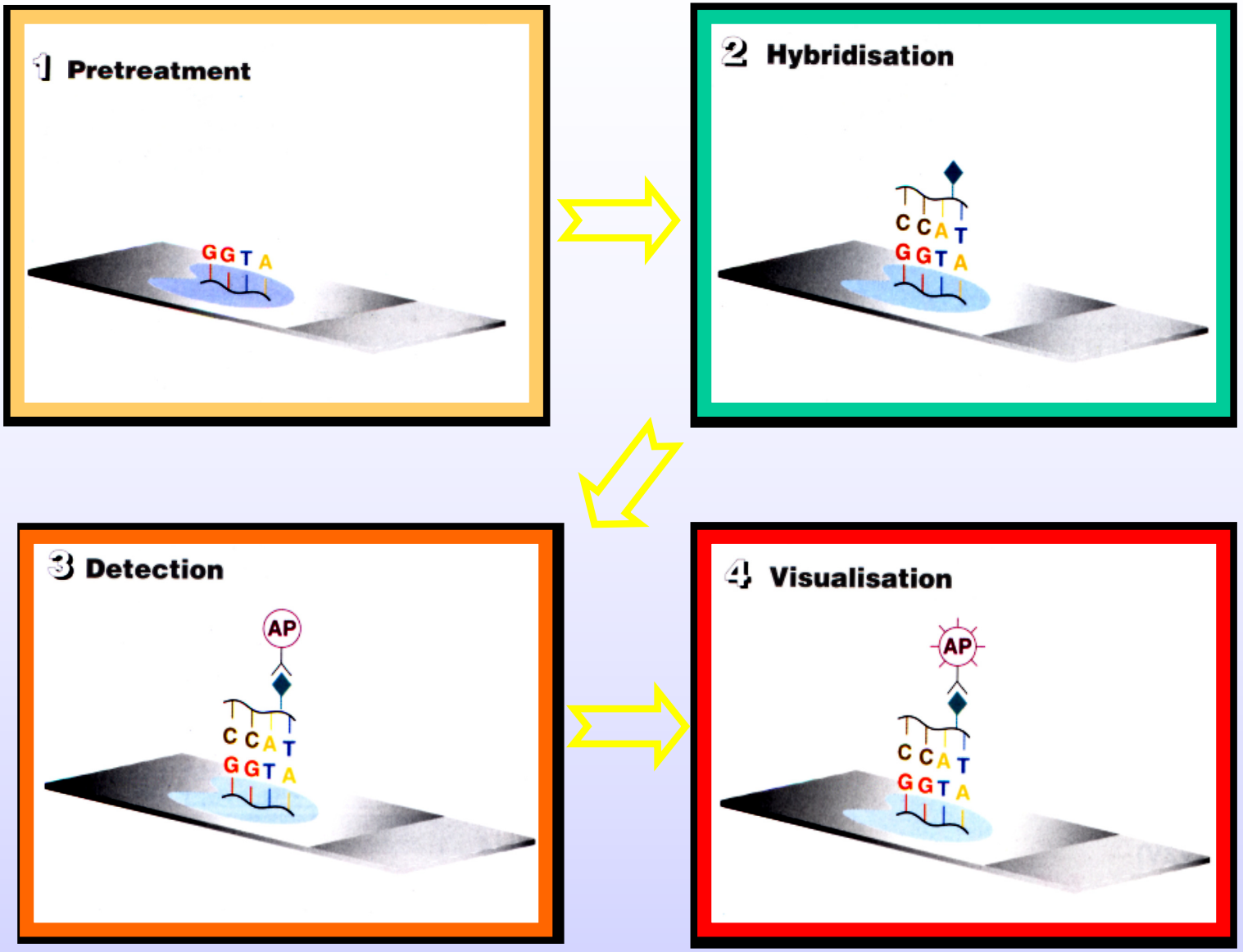
CISH (Ibridazione In Situ Cromogenica)

SISH (Ibridazione In Situ Argentica)



SISH per gene HER2 positiva con *clusters* di segnali nei nuclei delle cellule neoplastiche

IN SITU HYBRIDIZATION



From Novocastra laboratories Ltd product catalogue 1997

ISH

Una volta impiegava sonde radiomarcate ⇒ *laborioso.*

Ora si usano sistemi alternativi ad elevata efficienza. Coinvolgono l'impiego di ***sonde cDNA o cRNA marcate con biotina o digossigenina****.*

Visualizzazione: sandwich con reagenti o sistemi enzimatici.

- *Nel sistema non radiomarcato l'oligo complementare biotinilato o la sonda cRNA ibridizza con il mRNA e la rilevazione viene evidenziata da sandwich perossidasi-streptavidina coniugata.*
- *Nel sistema con digossigenina la rilevazione viene fatta con metodi immunoistochimici, usando Ab anti-dig, coniugati a molecole reporter fluorescenti o enzimatiche.*

La varietà di metodidi rilevazione consente di fare doppie analisi: ≠ rilevazioni immunoistochimiche o combinazione di sonde marcate dig o biotina nella medesima reazione.

Tutti gli steps devono essere condotti in condizioni Rnase free

(marcatura dei vetrini a matita!!)

PROTOCOLLO TIPO

- ✓ Su sezioni di paraffina, deparaffinizzare e trattare con EtOH
- ✓ Reidratare le sezioni in PBST (0.2%) per 15', lavare 3x con PBS.
- ✓ **Permeabilizzazione**: 37° C, 15-20' con [proteinasi K] 1 µg/ml in Tris 10mM pH 8, EDTA 5 mM. Bloccare la proteolisi per immersione in Glicina 0.1 M in PBS.
- ✓ **Postfissazione degli ac nucleici**: para-formaldeide 4% 3'. Risciacquare in PBS 2x per eliminare la PFA (fissativo crosslinkante).
- ✓ **Acetilazione**: trietanol amina 0.1M anidride acetica 0.25%, 10' risciacquare con ddH_2O e asciugare a 37° C. L'acetilazione serve a ridurre il background (blocca OH).
- ✓ **Diluizione della sonda**: diluire la sonda 2.5 ng/µl nel tampone preriscaldato a 50° C.

↓

Formamide deionizzata 50%, 5x SSC, 10% dextran sulfate , 5x Denhart's sol, salmon sperm DNA denaturato 100 µg/ml di soluz, SDS 2%.

IBRIDAZIONE in situ

✓ **Ibridazione**: applicare 10 µl di sonda diluita su ogni sezione, coprire con coprioggetto evitando bolle d'aria. V> per sezioni >. Incubare in camera umida 16-20 h alla T opportuna, con tamponi d'ibridazione al 50% in formamide il range di T è ~ 42-48° C per il cDNA, 42-55° C per il cRNA (sonde oligonucleotidiche).

✓ **Lavaggi:** dopo rimozione del coprioggetto si immergono i vetrini in SSC 2x, SDS 0.1% (4x 5'). Lavare alla T_{ibr} per 2x per 10' con 0.1X SSC e 0.1% SDS. Per sonde RNA si risciacqua con SSC 2x per 2x per eliminare l'SDS che blocca le Rnasi, si incuba poi con Rnasi A in SSC2x a 37° C per 15'. Risciacquo con SSC 2x per 2x e con PBS, procedere con IHC.

IBRIDAZIONE in situ-IHC

✓ Incubazione con l'Ab: blocco dei siti aspecifici con BSA 3% T= 37° C, t= 10' in 0.1 M Tris pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$. Asciugare il vetrino mantenendo umido il tessuto. Sul tessuto mettere l'Ab antidig-coniugato ad AP, diluito nel medesimo tampone di bloccaggio (~1:500) per 2h a RT

✓ Lavaggi: nello stesso tampone d'incubazione 3x 3'

✓ Equilibrare le sezioni: 10' in 0.1 M Tris pH 9.5, 0.1 M NaCl, 2 mM $MgCl_2$ Immergere le sezioni nel tampone per il substrato (100 ml buffer + levamisole + NBT/BCIP) per 10' al buio. Controllare al microscopio lo sviluppo del colore e bloccare la reaz immergendo per 5' in Tris 20 mM pH 7.5, EDTA 5mM.Lo sviluppo del colore varia da tessuto a tessuto e dipende dalla presenza dell'ac. Nucleico. In alcuni casi ci vogliono ore.

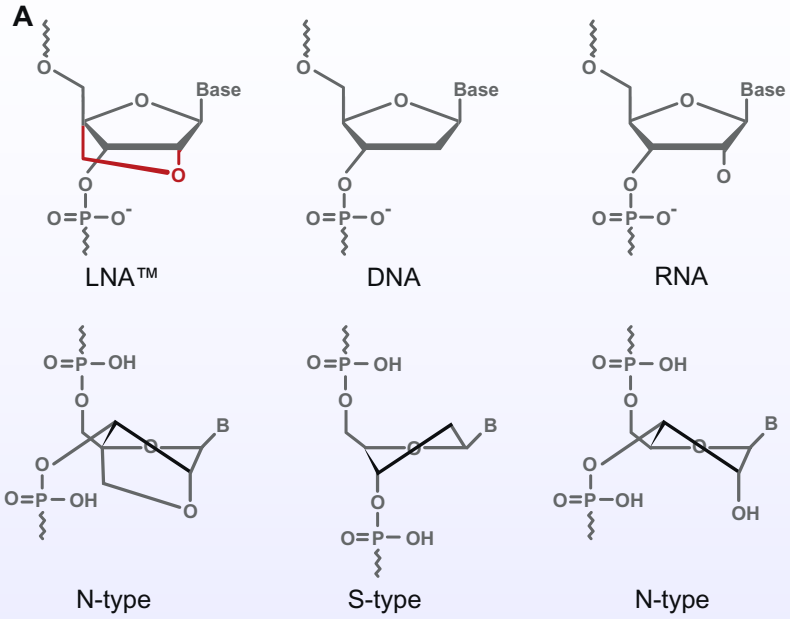
Ibridazione in situ-ISH

Vantaggi:
- *Studio di espressione in sistemi cellualari eterogenei*
- *Morfologia*

Svantaggi:
- *Laborioso*
- *Poco sensibile per geni poco espressi*
- *Analisi quantitativa complessa con uso di sonde radiomarcate*

Why LNA?

1. High affinity RNA
2. Ribose ring is “locked” in the ideal conformation for Watson–Crick binding.
3. As a result, LNA™ oligonucleotides exhibit thermal stability when hybridized to a complementary DNA or RNA strand.
4. For each incorporated LNA monomer, the melting temperature (Tm) of the duplex increases by 2° C–8° C (Figure A-2).
5. LNA oligonucleotides can be made shorter



B

Probe \ Target	Perfect match 3′-acgaccac-5′	Single mismatch 3′-acggccac-5′	Δ T_m
DNA 8-mer 5′-tgctggtg-3′	T_m = 35° C	T_m = 25° C	10° C
LNA™ 8-mer 5′-TGCTGGTG-3′	T_m = 71° C	T_m = 45° C	26° C

Figure A-1 Structure and conformations of nucleotides (A) and the effect of LNA™ on melting temperature of duplexes (B).

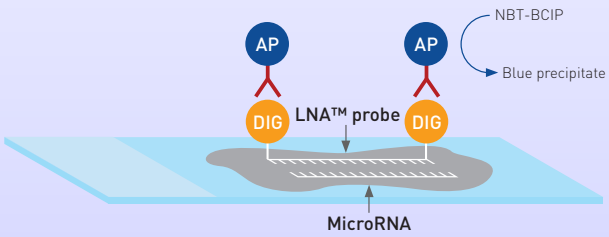
From: G.J. Nuovo (Eds) In Situ Molecular Pathology and Co-expression analyses (2013)

Graz, 1st April 2014

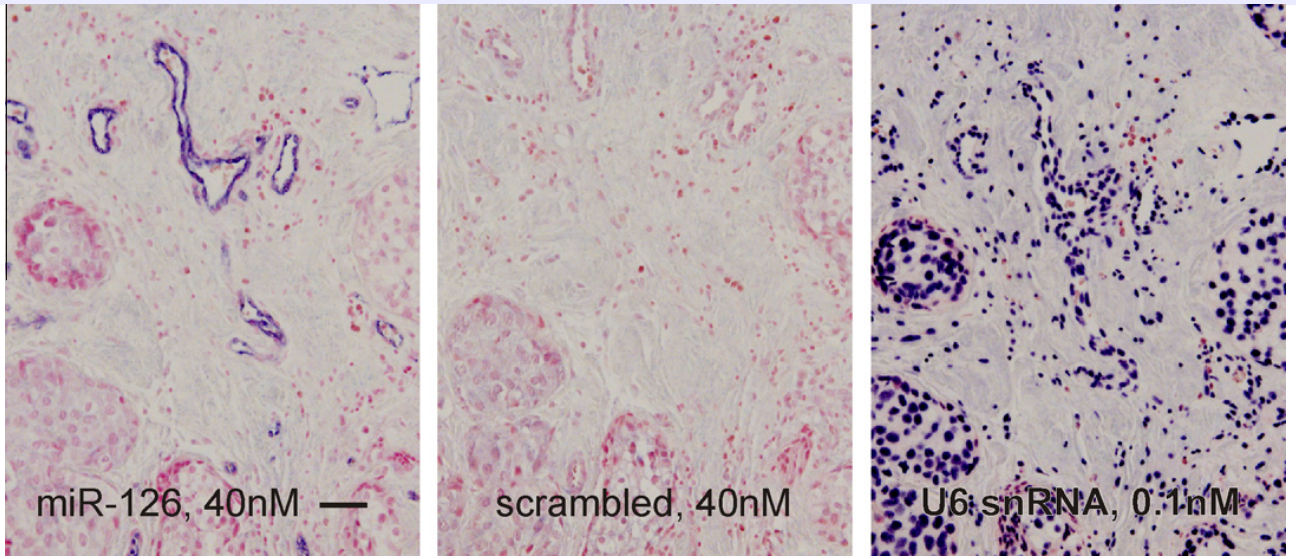
ISH for mRNA and miR detection

- ✓ Morphology
- ✓ Sensitivity
- ✓ miRs have high degree of similarity between the sequences. Some micro- RNA family members vary by a single nucleotide.
- ✓ Use LNA-enhanced oligonucleotides.
- ✓ The use of LNA™ in probes enables highly sensitive detection and analysis of the short miRNA sequences, but also mRNA.
- ✓ The use of LNA probe allows a better control of Tm even in AT rich sequences and for short stretches (miR)

Methods 52 (2010) 375–381



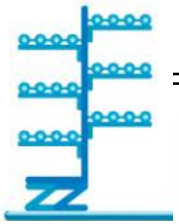
miRCURY LNA™ microRNA ISH
Exiqon



RNAscope

Fay Wang, et al *The Journal of Molecular Diagnostics* Volume 14, Issue 1, Pages 22-29 (January 2012) DOI: 10.1016/j.jmoldx.2011.08.002

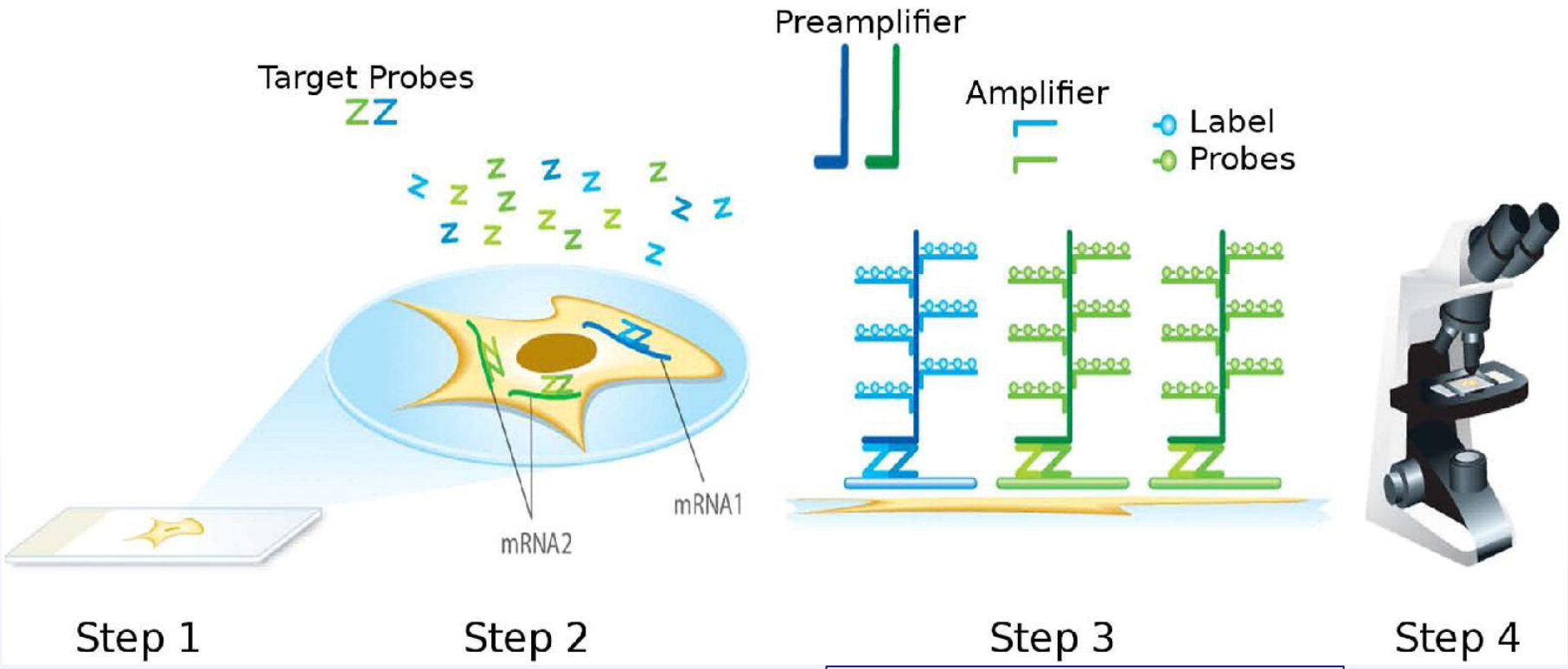
- ✓ Single-molecule visualization in individual cells
- ✓ Novel probe design strategy and a hybridization-based signal amplification system to simultaneously amplify signals and suppress back- ground.
- ✓ multiplex detection for up to 4 target genes (number of spectrally discernible fluorescent dyes)
- ✓ A series of target probes are designed to hybridize to the target RNA molecule.
- ✓ Each target probe contains an 18- to 25-base region complementary to the target RNA, a spacer sequence, and a 14-base tail sequence (conceptualized as Z). A pair of target probes (double Z), each possessing a different type of tail sequence, hybridize contiguously to a target region (☐ 50 bases).



RNAscope

Wang, et al *The Journal of Molecular Diagnostics* Volume 14, Issue 1, Pages 22-29 (January 2012) DOI: 10.1016/j.jmoldx.2011.08.002

- ✓ The two tail sequences together form a 28-base hybridization site for the preamplifier, which contains 20 binding sites for the amplifier, which, in turn, contains 20 binding sites for the label probe.
- ✓ Typically, 1-kb region on the RNA molecule is targeted by 20 probe pairs; thus, sequential hybridizations with the preamplifier, amplifier, and label probe can theoretically yield up to 8000 labels for each target RNA molecule.
- ✓ The probe can be either fluorescently labelled for direct visualization or conjugated to an alkaline phosphatase or horseradish peroxidase (HRP) molecule for chromogenic reactions
- ✓ Multiple RNA species can be measured simultaneously in two ways: the target probes for different genes can have the same tail sequence recognized by the same signal amplification system, generating a pooled signal; alternatively, multiple signal amplification systems with different label probes can be used to detect each RNA species, allowing for multiplex detection of multiple target RNAs.



Step 1: fixed cells or tissues are permeabilised to allow for target probe access.

Step 2: target RNA-specific oligonucleotide probes (Z) are hybridized in pairs (ZZ) to multiple RNA targets

Step 3: multiple signal amplification molecules are hybridized, each recognizing a specific target probe, and each unique label probe is conjugated to a different fluorophore or enzyme

Step 4: signals are detection with a epifluorescent microscope or standard bright-field microscope.

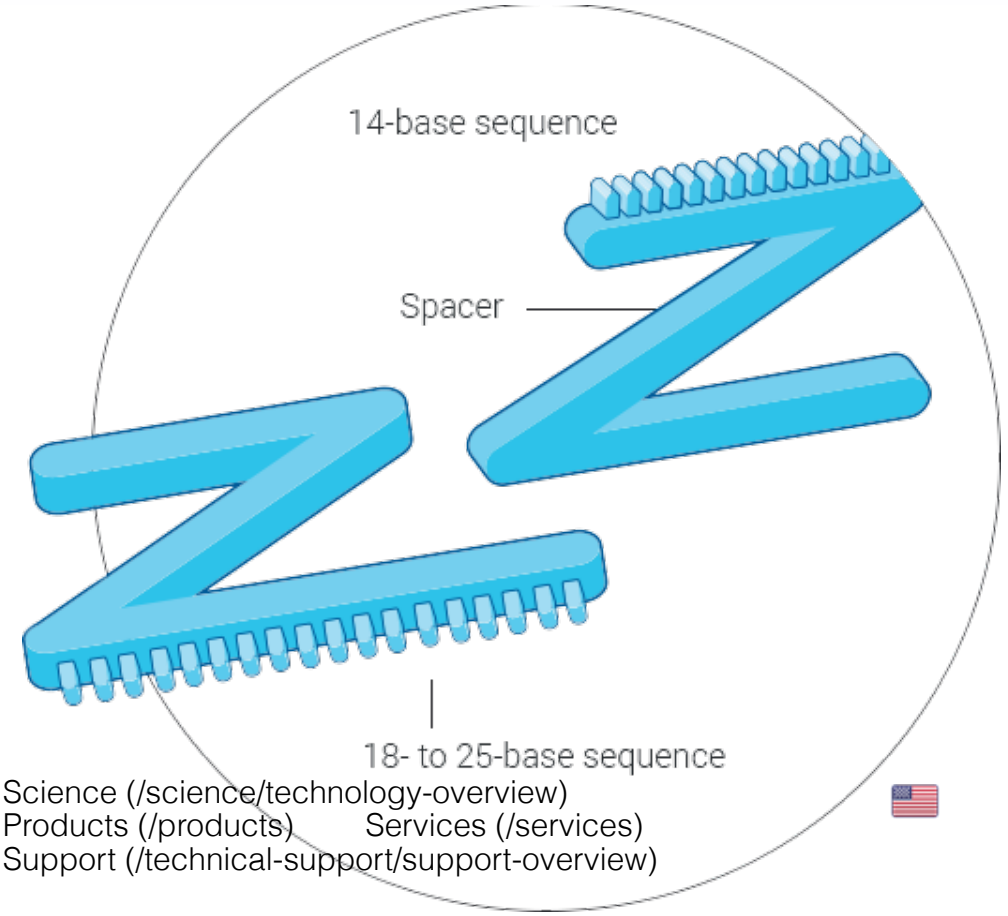
The Journal of Molecular Diagnostics 2012 14, 22-29DOI: (10.1016/j.jmoldx.2011.08.002)

Contains Three Elements

The lower region of the Z is an 18-to 25-base region that is complementary to the target RNA. This sequence is selected for target specific hybridization and uniform hybridization properties.

A spacer sequence that links the two components of the probe. The upper region of the Z is a 14-base tail sequence.

The two tails from a double Z probe pair forms a 28 base binding site for the pre-amplifier.



Signal Amplification is Achieved by a Cascade of Hybridization Events

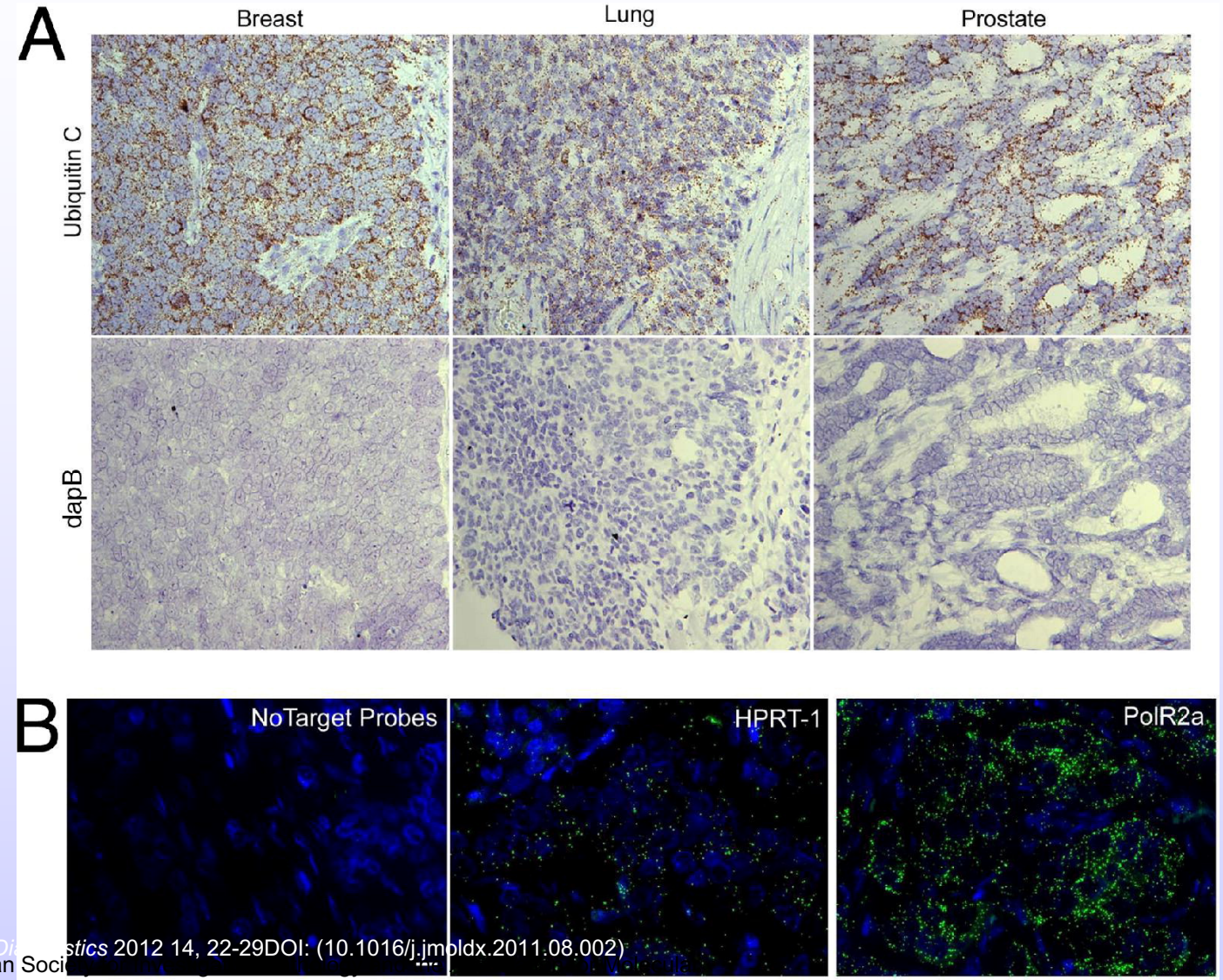
Step 1. Double Z target probes hybridize to the RNA target (~1kb)

Step 2. Pre-amplifiers hybridize to the 28-base binding site formed by each double Z probe

Step 3: Amplifiers are then binding to the multiple binding sites on each preamplifier.

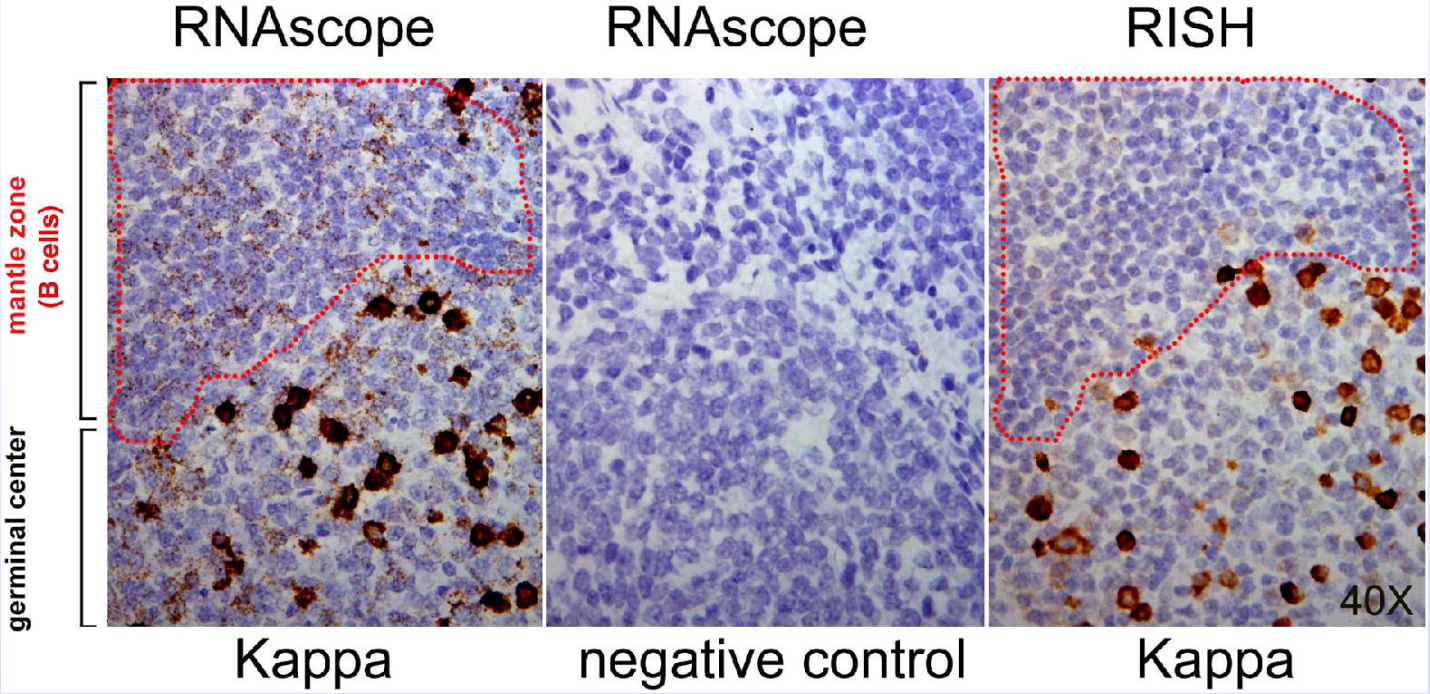
Step 4: Labeled probes, containing a fluorescent molecule or chromogenic enzyme, bind to the numerous binding sites on each amplifier.

Detection of RNA in FFPE tumor tissues. **A:** Chromogenic staining (DAB) hybridized with either probes to ubiquitin C (*UBC*) or probes against the bacterial gene *dapB* as negative control. Nuclei were counterstained with hematoxylin. **B:** Fluorescent detection of low-copy transcripts in FFPE samples. Breast tumor tissue section was hybridized with either no probes or with Alexa Fluor 488-labeled probe sets (green) to HPRT1 or POLR2A. Nuclei were counterstained with DAPI (blue). Scale bar = 10 μm.



The Journal of Molecular Diagnostics 2012 14, 22-29DOI: (10.1016/j.jmoldx.2011.08.002)

Detection of Ig κ chain expression in B lymphocytes in FFPE human tonsil tissue. κ light chain mRNA transcripts were stained using RNAscope or a commercial non-radioisotopic RNA ISH kit. For RNAscope, a negative control (bacterial gene *dapB*) was also included. The **dotted line** outlines the mantle zone. Original magnification, ×40.



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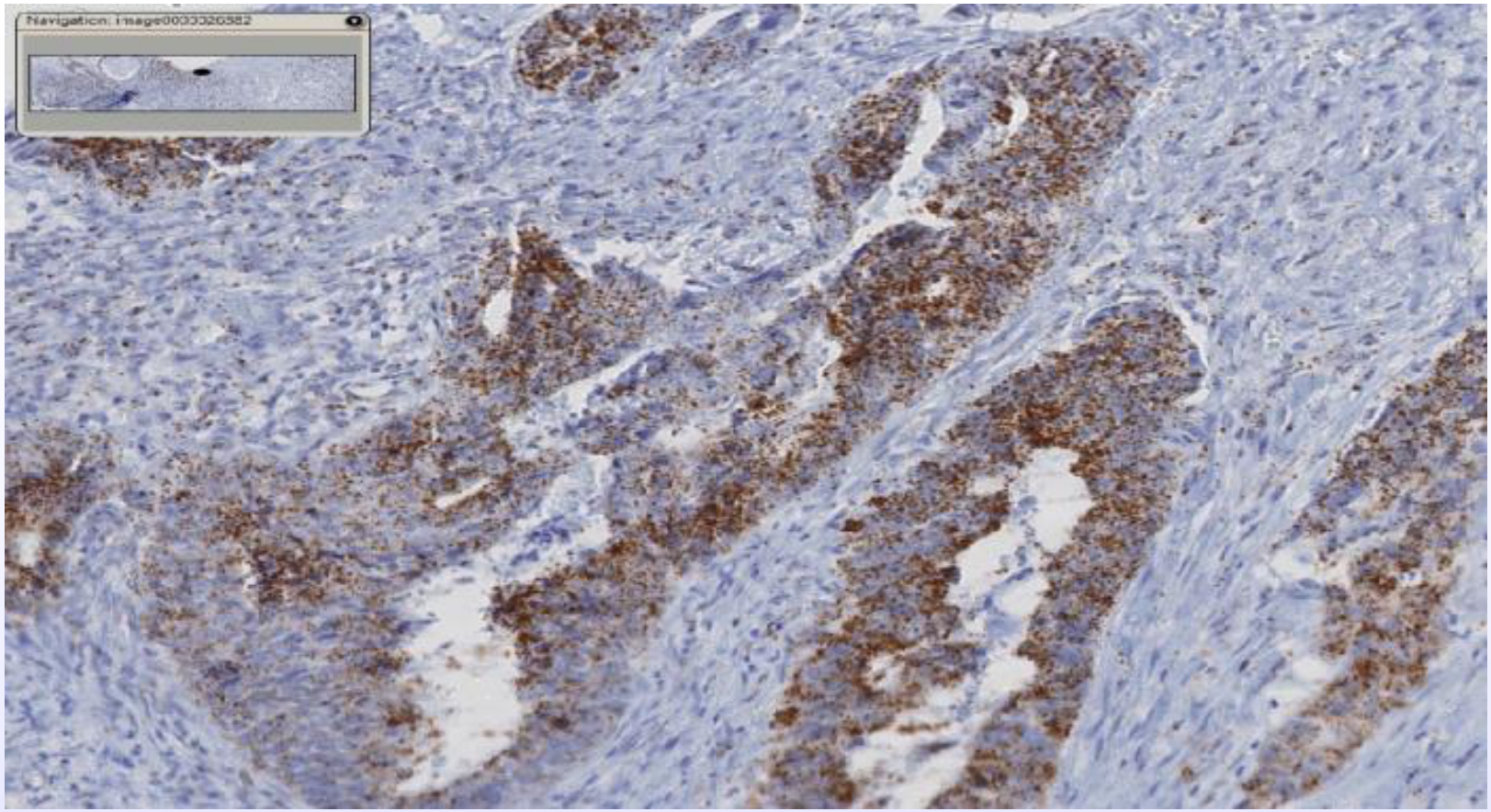
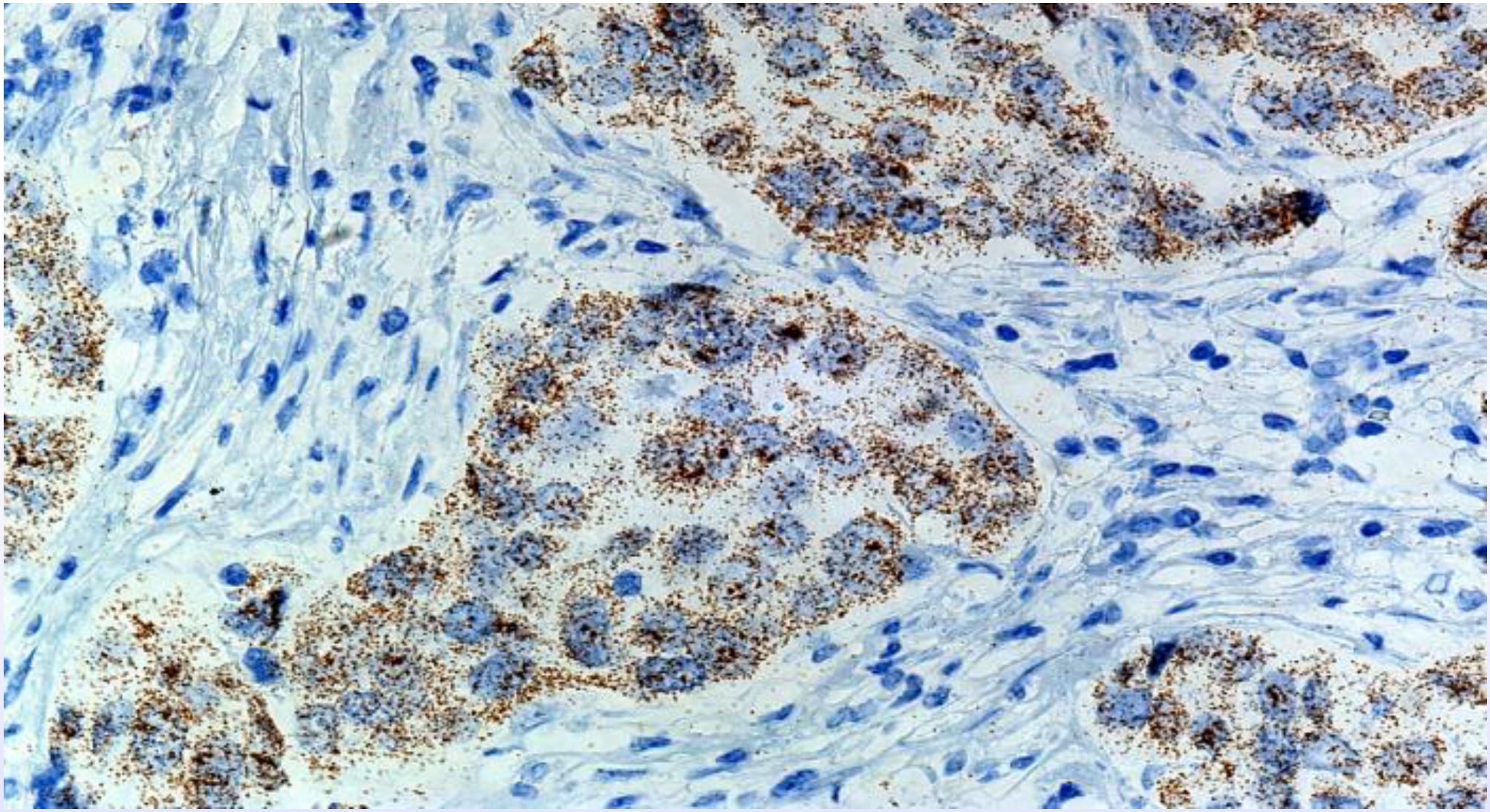
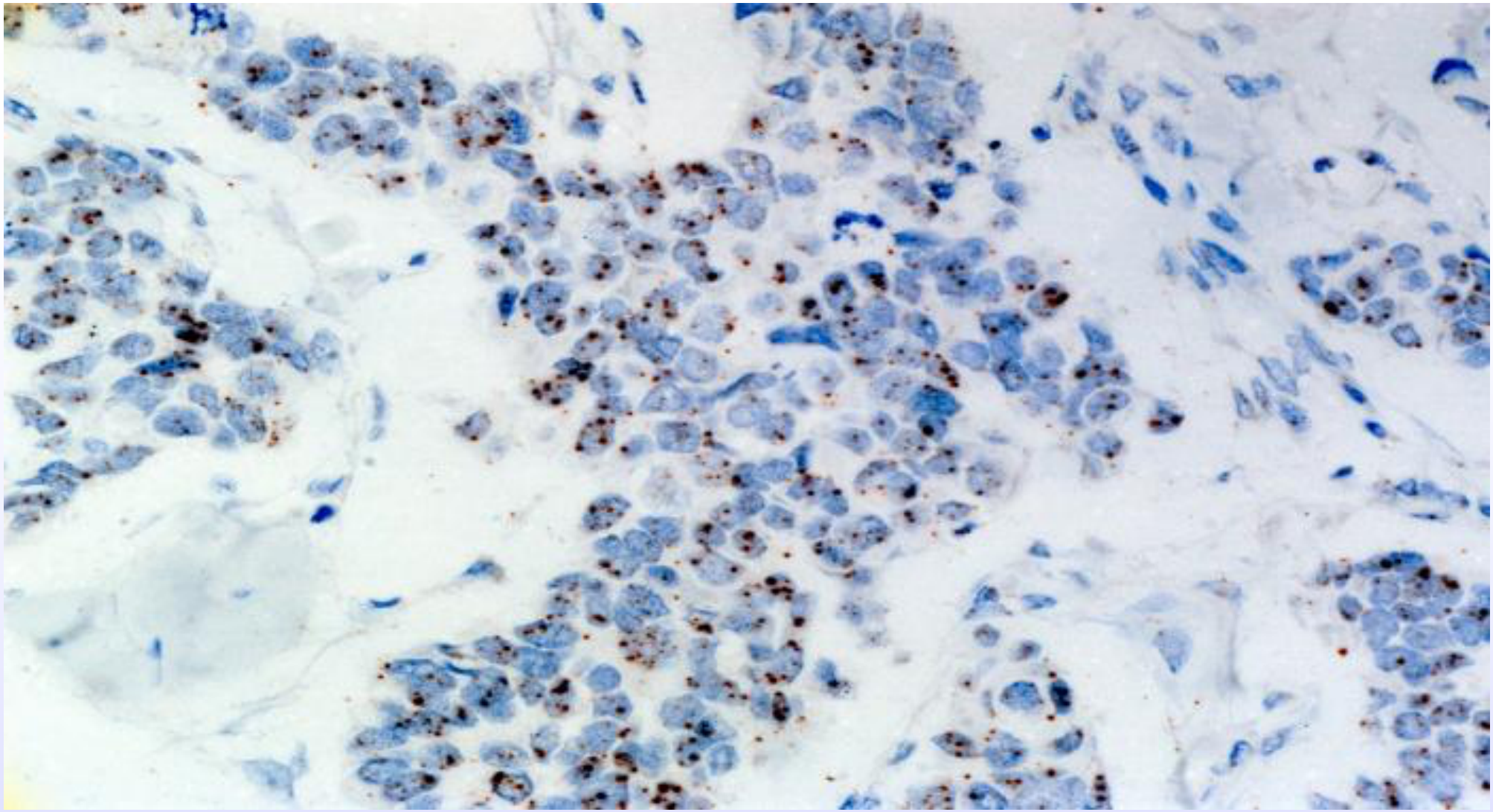


TP53 mRNA expression in human colon FFPE tissue with Automated RNAscope® VS Brown

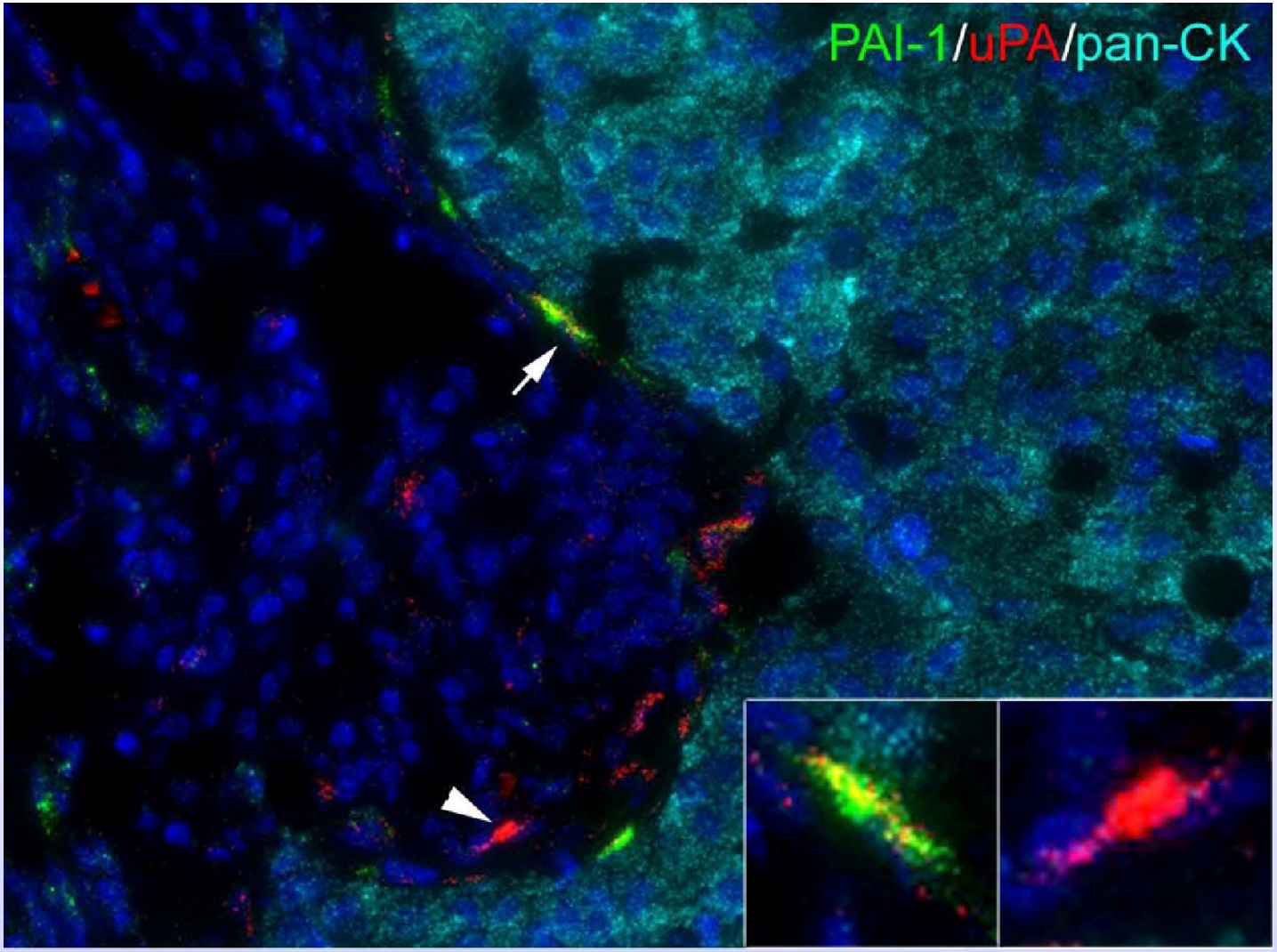


Human breast cancer FFPE tissue: HER2 expression using RNAscope® 2.0 HD Reagent Kit-BROWN



Expression of HOTAIR RNA (brown dots) in human breast cancer FFPE tissue, RNA in situ hybridization (ISH) using RNAscope® 2.0 HD Reagent Kit-BROWN

Multiplex fluorescence detection of uPA and PAI mRNAs in breast cancer. Merged pseudo-colored image of a metastatic breast cancer tissue section stained with probes specific to cytokeratins [PanCK (CK-8, CK-18, and CK-19), labeled with Alexa Fluor 647], uPA (labeled with Alexa Fluor 546), and PAI-1 (labeled with Alexa Fluor 488).



Both uPA expression (**arrowhead** and **right inset**) and coexpression with PAI-1 (**arrow** and **left inset**) were detected. Nuclei were counterstained with DAPI (blue). Original magnification, ×40.

The Journal of Molecular Diagnostics 2012 14, 22-29DOI: (10.1016/j.jmoldx.2011.08.002)