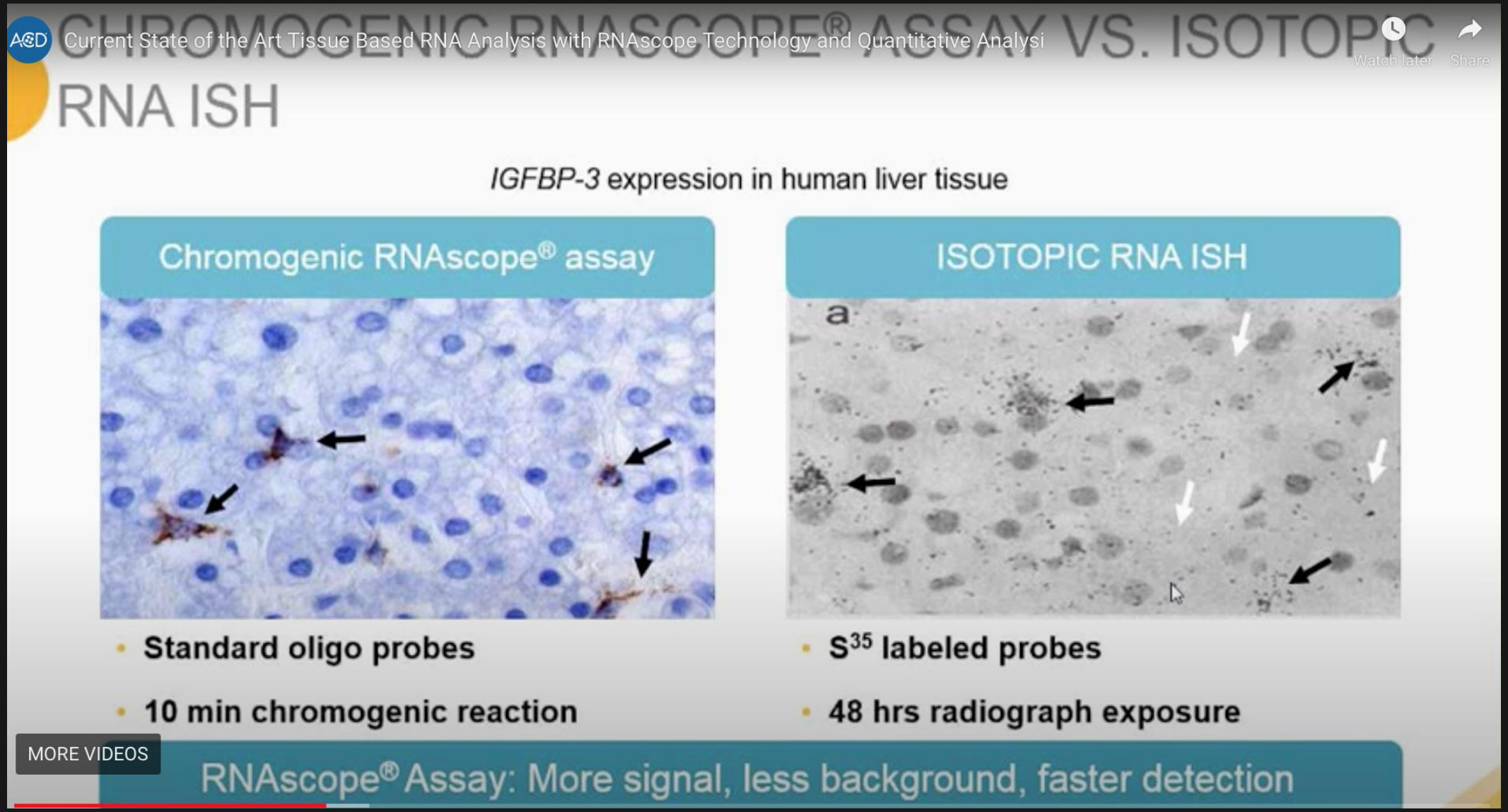
TOPICS

- **RNAscope Introduction and Overview:** Embryonic and postnatal skeletal development
  - RNAscope technology: A novel RNA ISH technology, *Wang et al. J. Mol.Diagn. 2011.*
  - Chromogenic Red and the Multiplex Fluorescent Assays (Advanced Cell Diagnostics: ACD)
  - RNAscope assay workflow

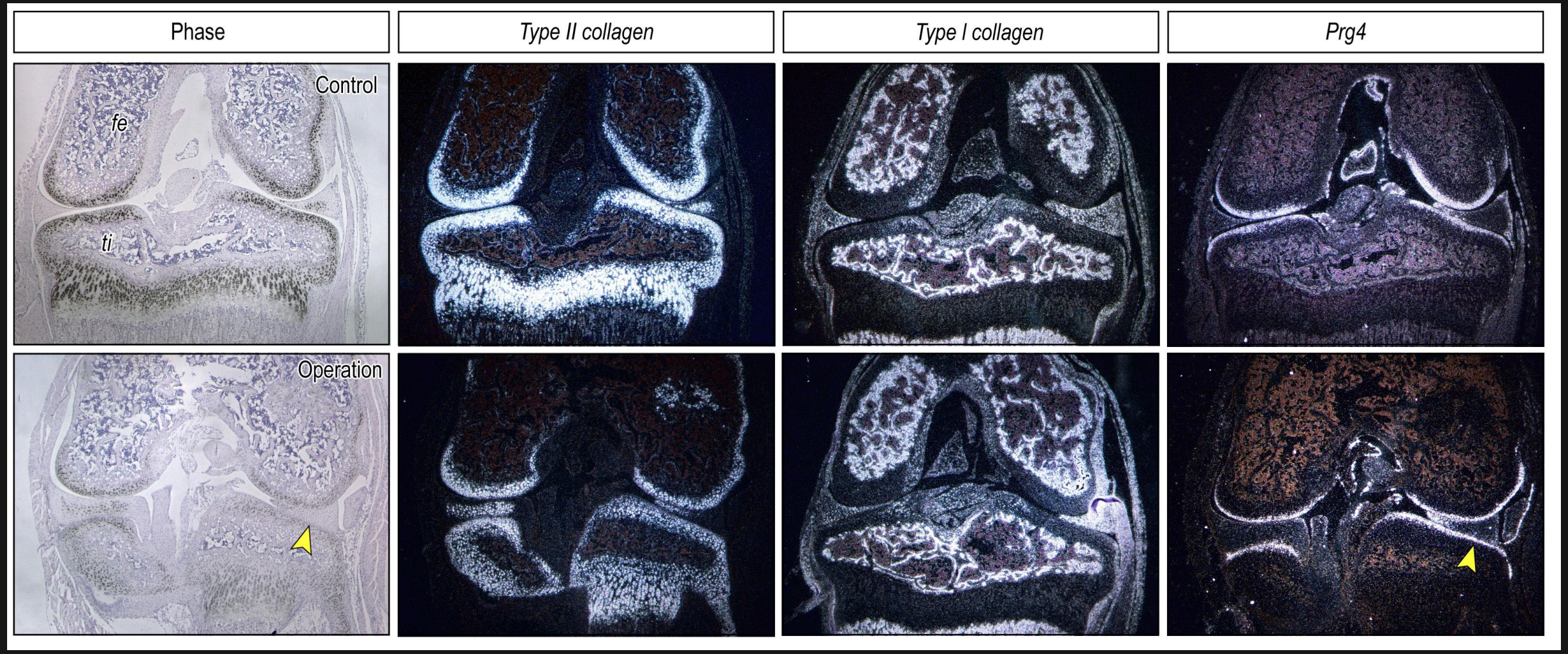
- **Tips for an RNAscope Assay**
  - Sample fixation
  - Pretreatment



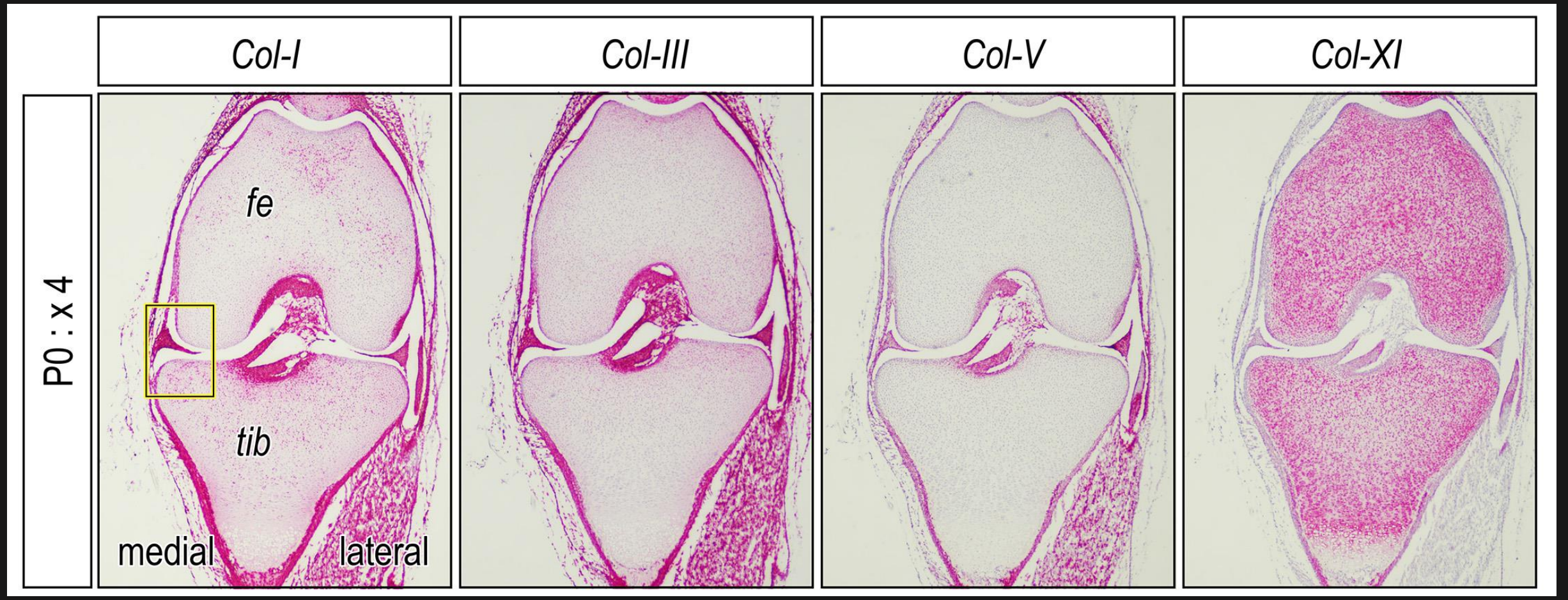
Obtained from ACD

Monitoring, Inspection, Isotope, Dry & Liquid Waste, Emulsion, Dark room……

ISH with 35S-labeled RNA probes



RNAscope 2.5 HD Assay: RED

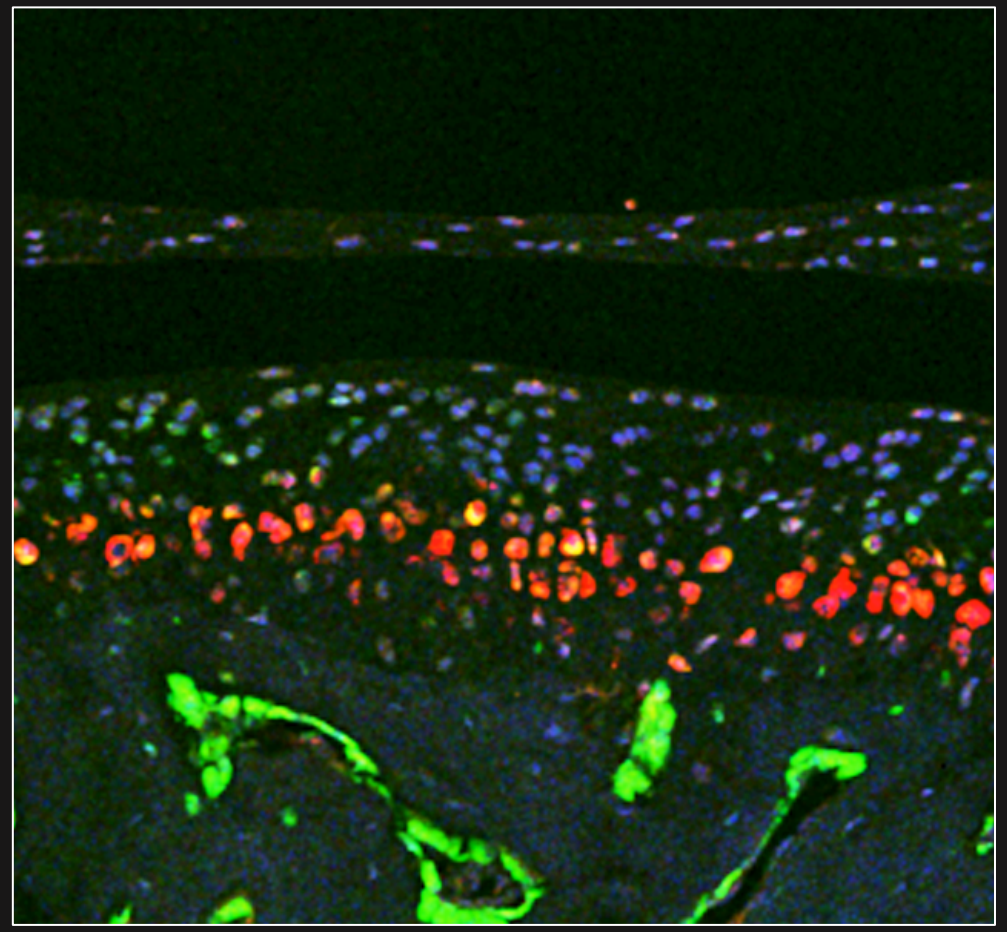


RNAscope Multiplex Fluorescent Assay

Articular Disc

Condylar (Fibro)Cartilage

Subchondral bone



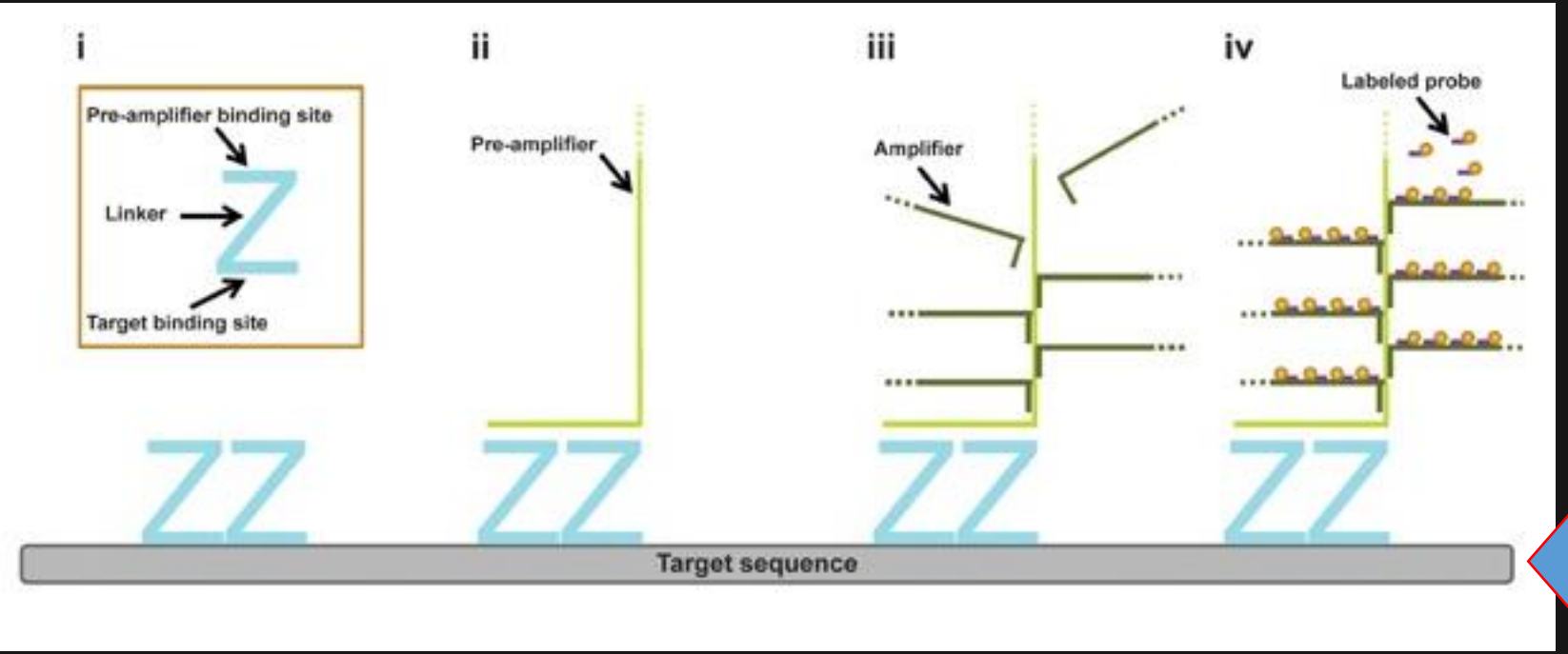
Fibrochondrocytes

Immature chondrocytes

Osteoblasts and osteocytes

Type I collagen-Green, Type II collagen-Red, Dapi-Blue

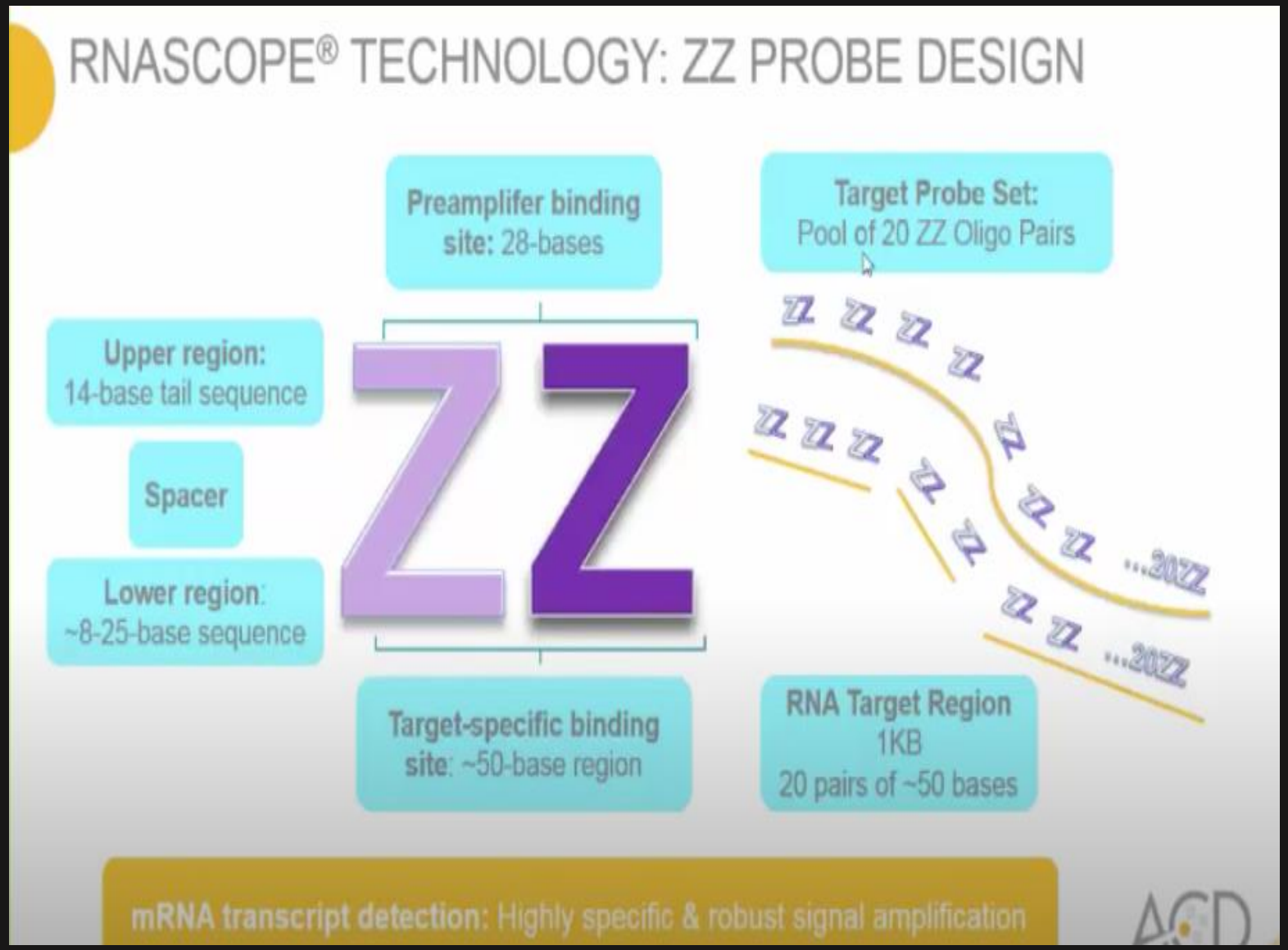
RNAscope Technology Overview



Obtained from ACD

- **Step1**: Double Z target probes hybridize to the RNA target.
- **Step2**: Pre-amplifiers hybridize to the 28-base binding site formed by each double Z probe.
- **Step3**: Amplifiers are then bound to the multiple biding sites on each preamplifier.
- **Step4**: Labeled probes, containing a fluorescent molecule or chromogenic enzyme, bind to the binding sites on each amplifier.

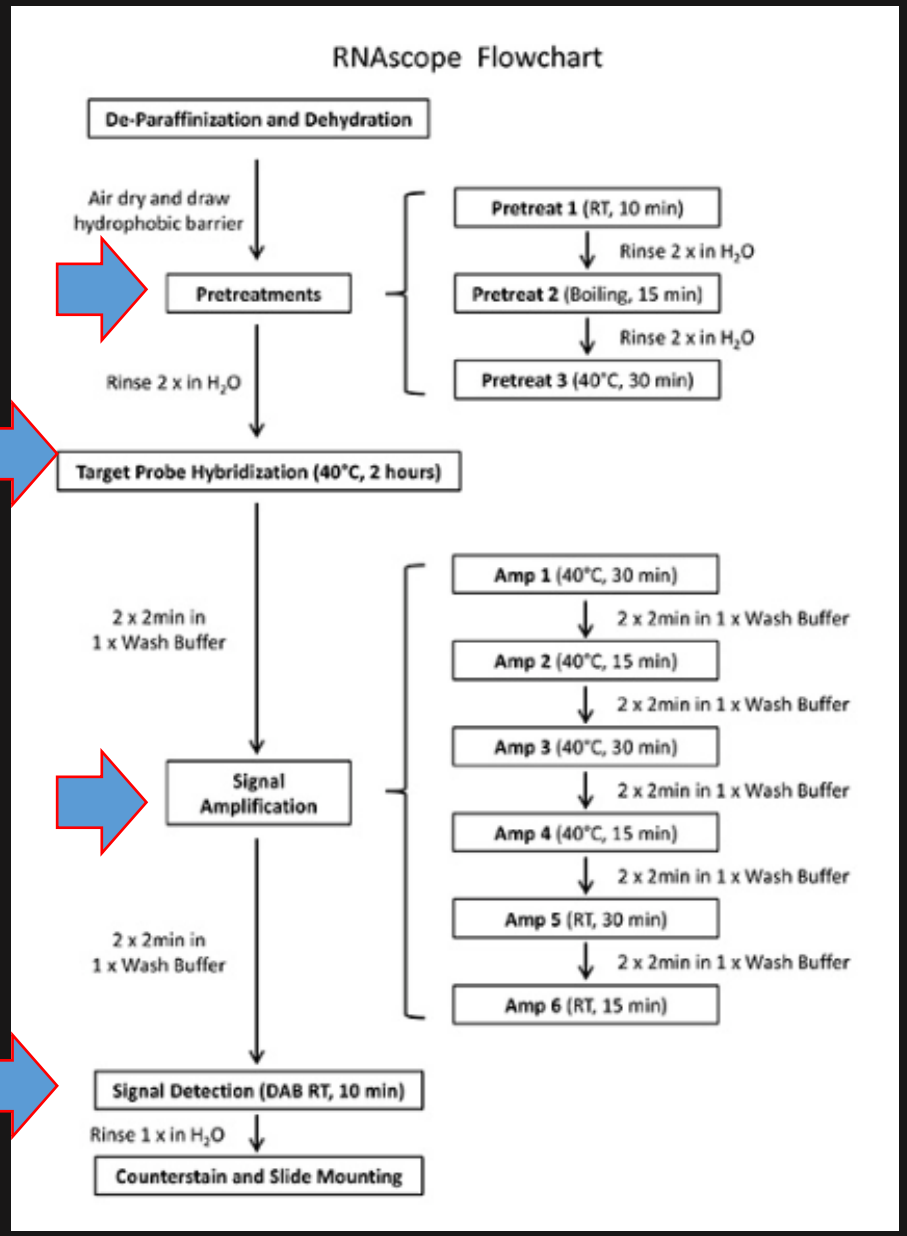
Each Target Z Probe Contains Three Elements



- The lower region of a double Z probe pair is about 50 bases long and is complementary to the target RNA.
- A spacer sequence links the upper and lower regions.
- The upper region of a double Z probe pair forms a 28 base biding site for the preamplifier.
- The Z probe pair needs to hybridize to the target RNA in tandem.

Obtained from ACD

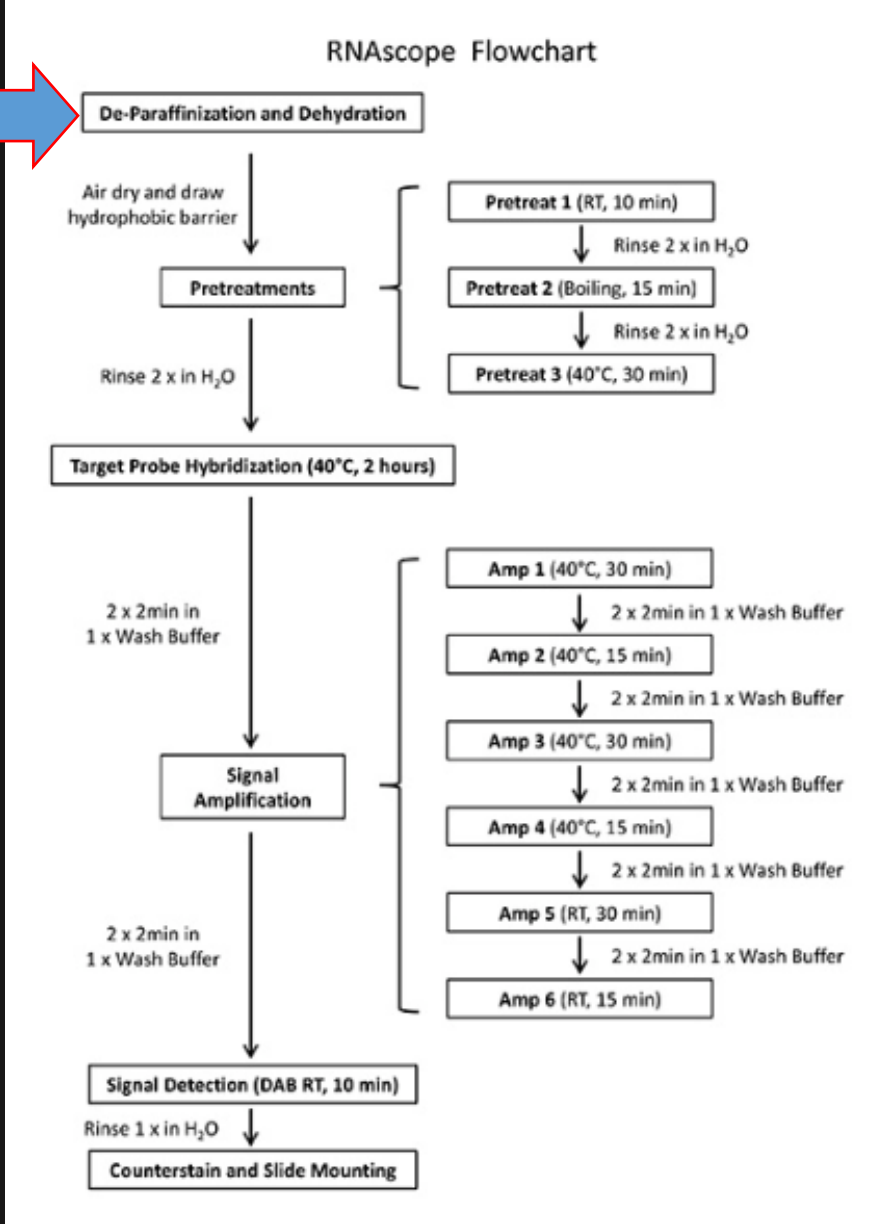
Flowchart of RNAscope assay with FFPE sections, Chromogenic Red or Brown



- RNA scope assay has a highly streamlined workflow that is similar to IHC. It consists of four major steps:
  - Pretreatments
  - Hybridization
  - Signal amplifications
  - Detection

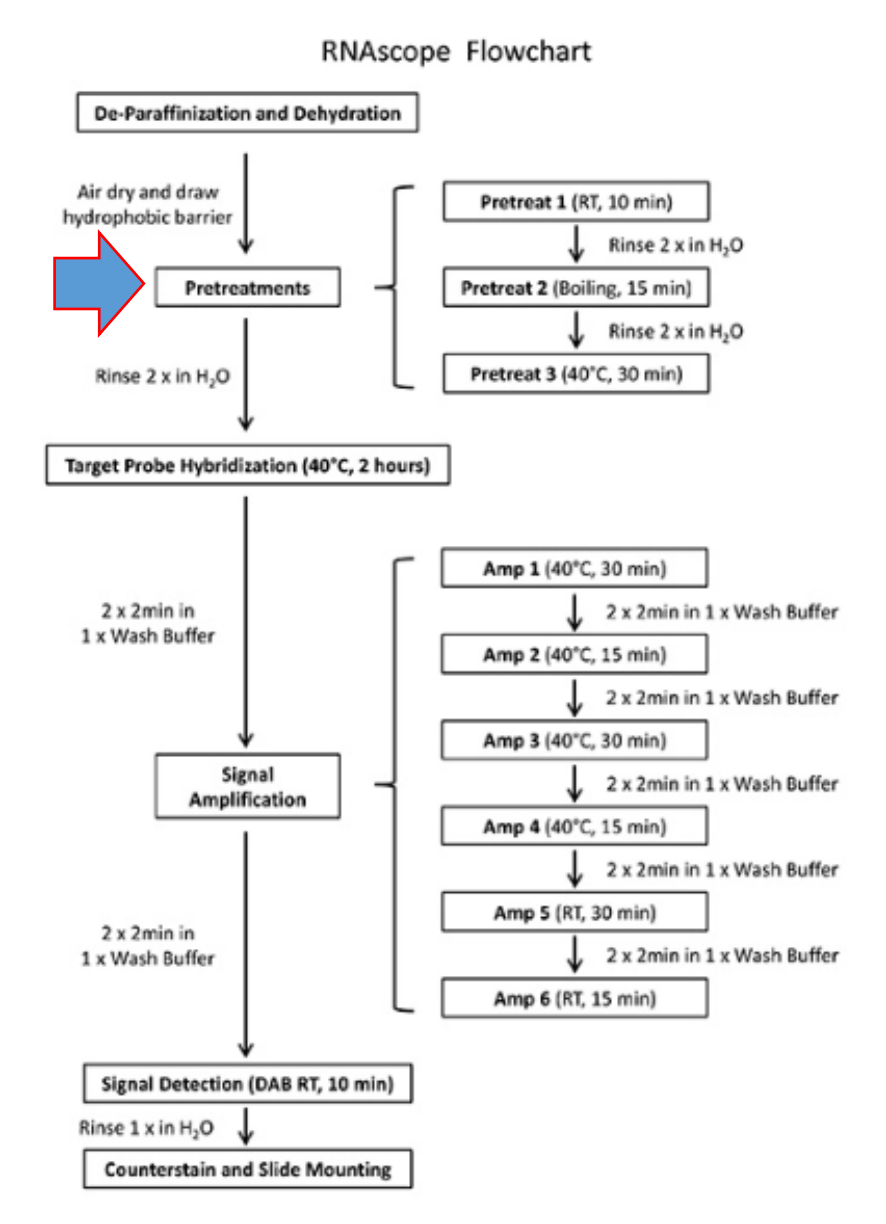
Wang et al. JOVE. 2014

Flowchart of RNAscope assay with FFPE sections



- **Deparaffinization and Dehydration**
- After **baking** (for 10 min at 60◦C), deparaffinize tissue sections in Xylene for 2x5 min with agitation, and dehydrate in 100% Et-OH for 2x5 min with agitation.
- Air dry for 10 min at 40◦C and draw a hydrophobic barrier around the tissue section with a Hydrophobic Barrier Pen. **Dry the hydrophobic barrier completely at 40◦C for 30 min.**

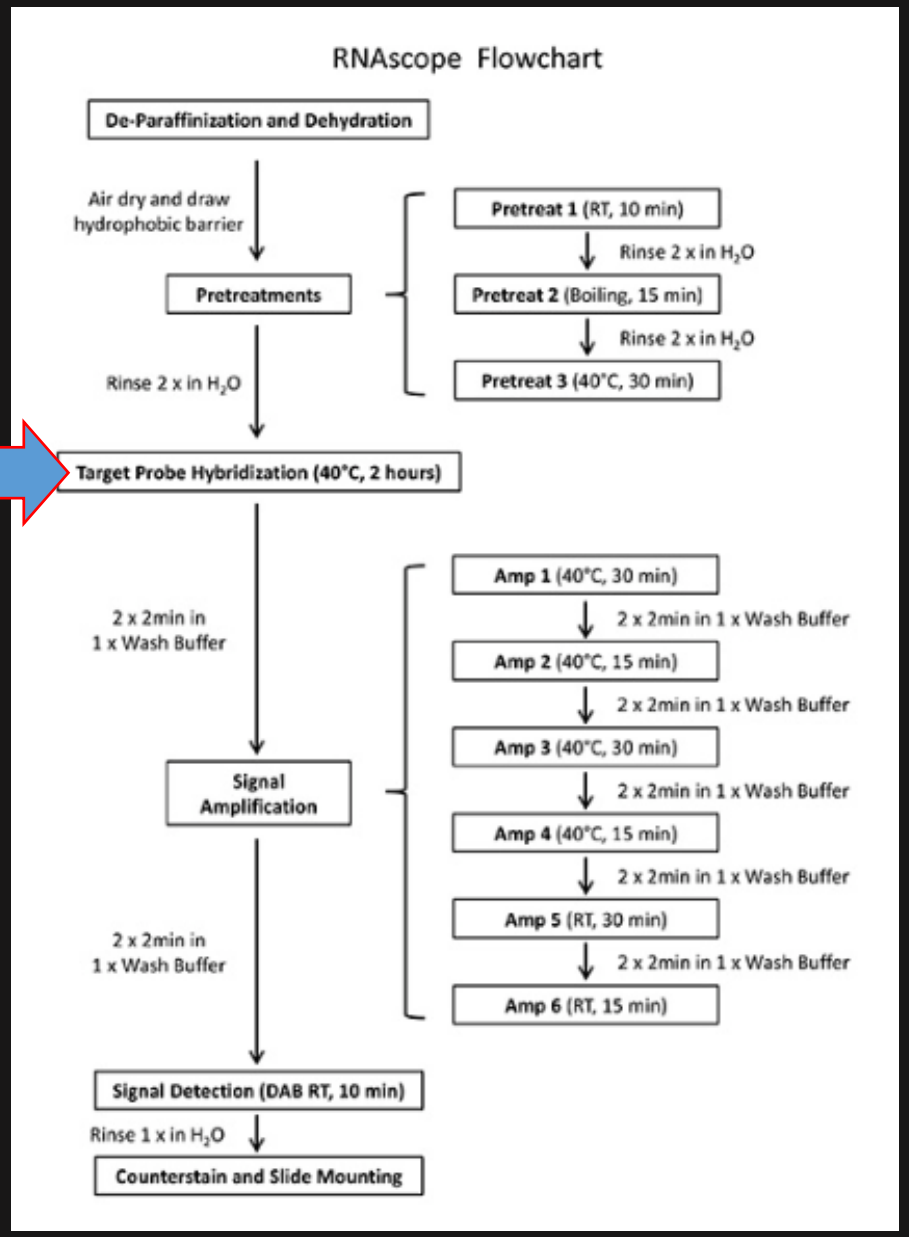
Flowchart of RNAscope assay with FFPE sections



- **Pretreatments: ACD**
  - H2O2 block: RT, 10 min
  - Epitope retrieval (pH6, citrate buffer): boiling, 10min
  - Protease digestion: 40◦C, 30min

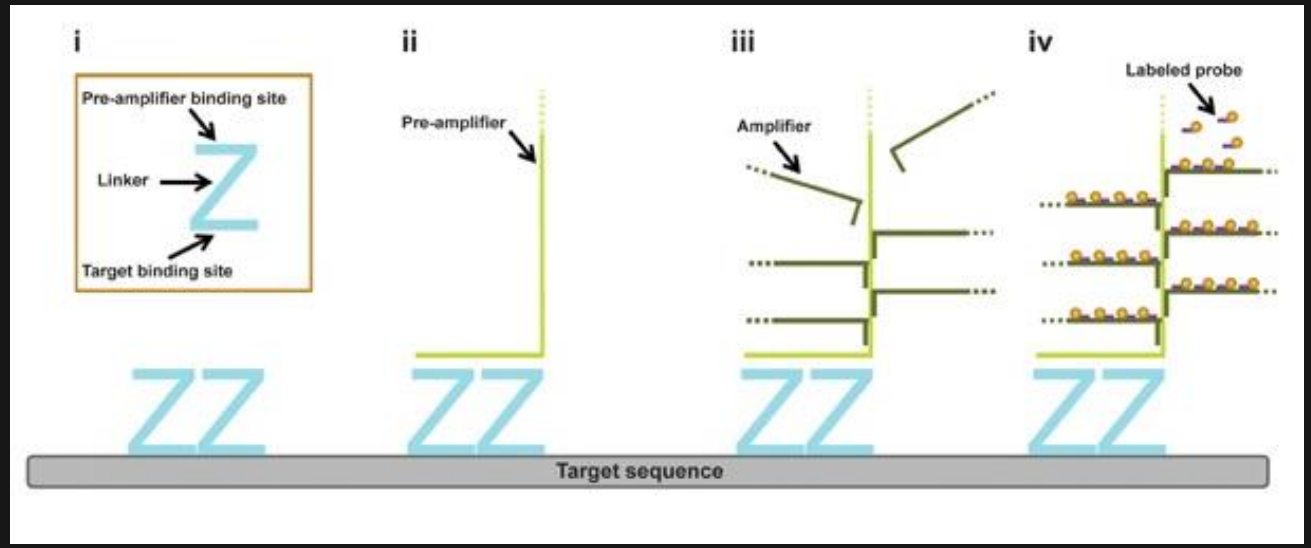
- **Embryonic/Postnatal Bone/(fibro)cartilage**
  - H2O2 block: RT, 10 min
  - ~~Epitope retrieval~~
  - ~~Protease digestion~~
  - **Custom pretreatment reagent (Cat. 300040, $90) @ 40◦C for 30 min.**

Flowchart of RNAscope assay with FFPE sections

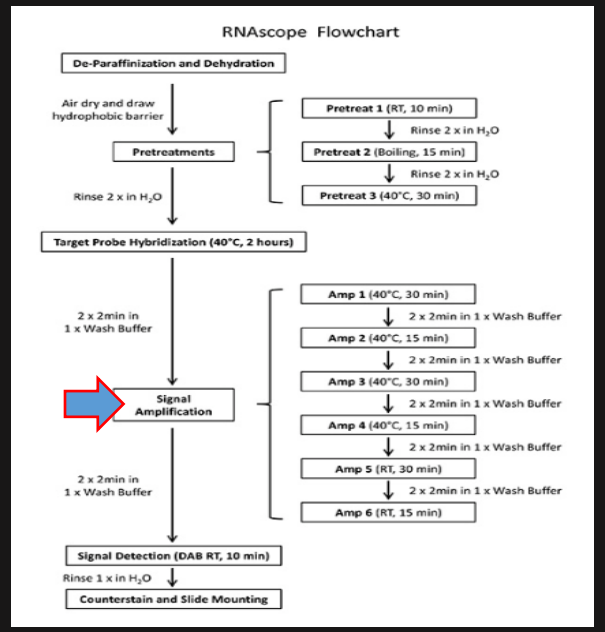


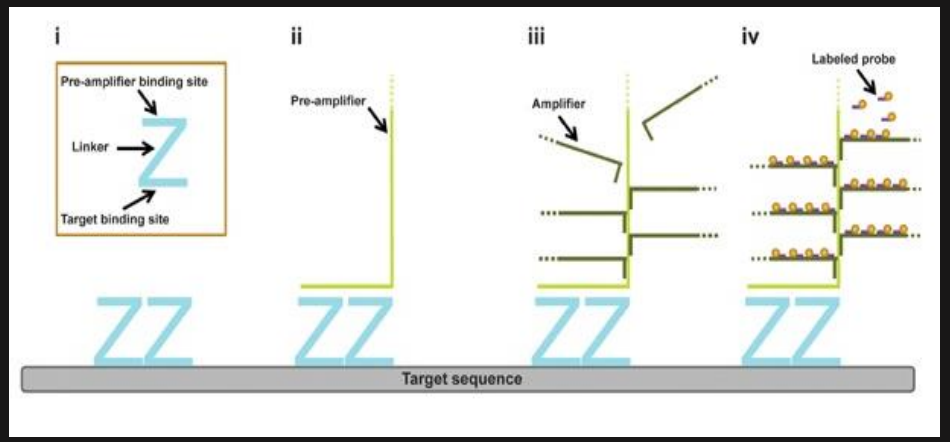
• Target Probe Hybridization:

- Pre-warm probes @ 40◦C, and then 40◦C, 2hr
- 3-4 drops of probe solution: ~~the solution still contains large amounts of un-hybridized Z probes.~~
- Do not use PARAFILM to cover the sections



Flowchart of RNAscope assay with FFPE sections

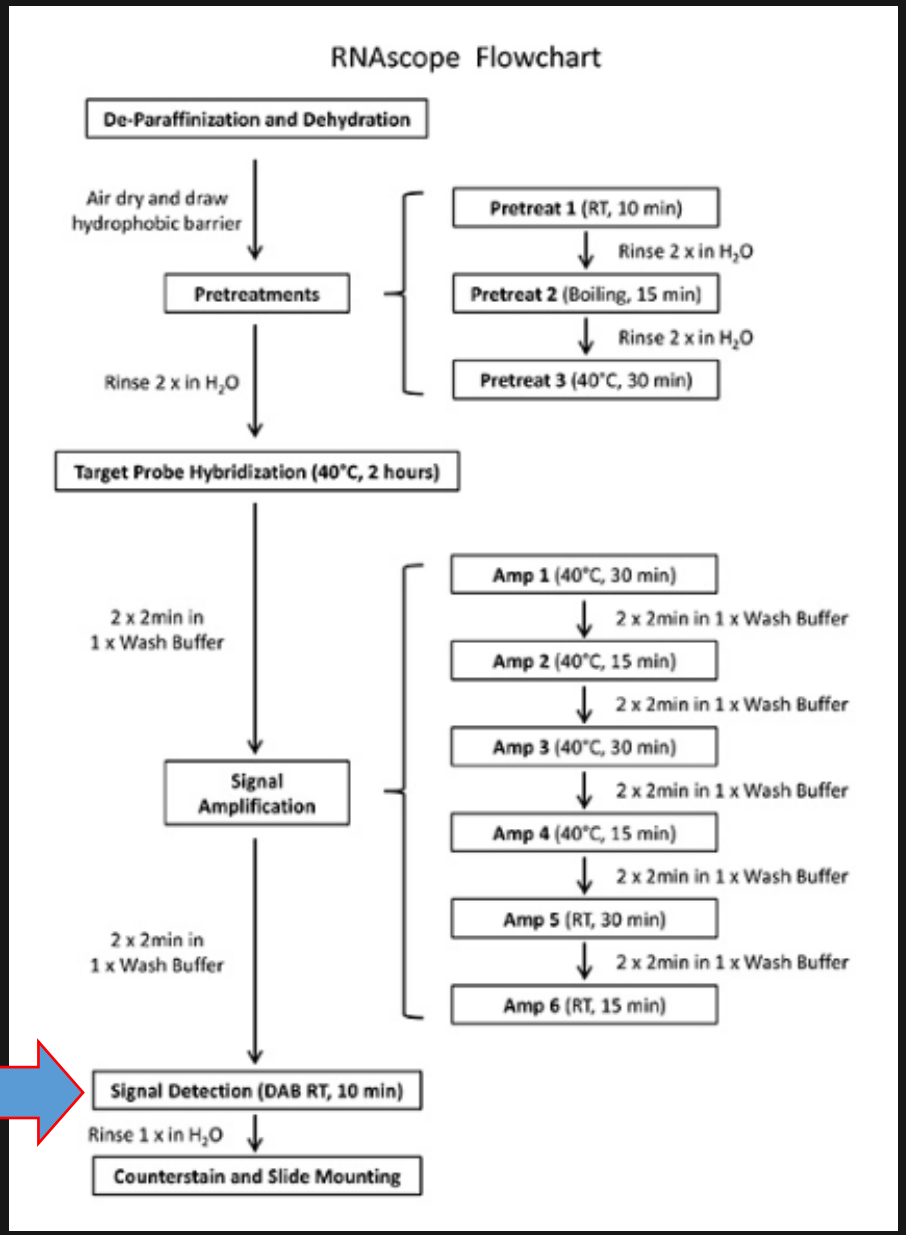




- **Signal Amplification is achieved through Amp1-6.**
  - **Bring Amp1-6 to RT**
  - **Step 1**: **Pre-amplifier** binds to the upper region of each double Z probe pair, 40◦C, 30min
  - **Step 2**: Background reducer, 40◦C, 15min
  - **Step 3**: **Amplifiers** bind to the binding site on each Pre-amplifier, 40◦C, 30min
  - **Step 4**: Labeled probes, containing chromogenic enzymes or fluorescent molecules, adhere to the binding site on the amplifiers, 40◦C, 15min

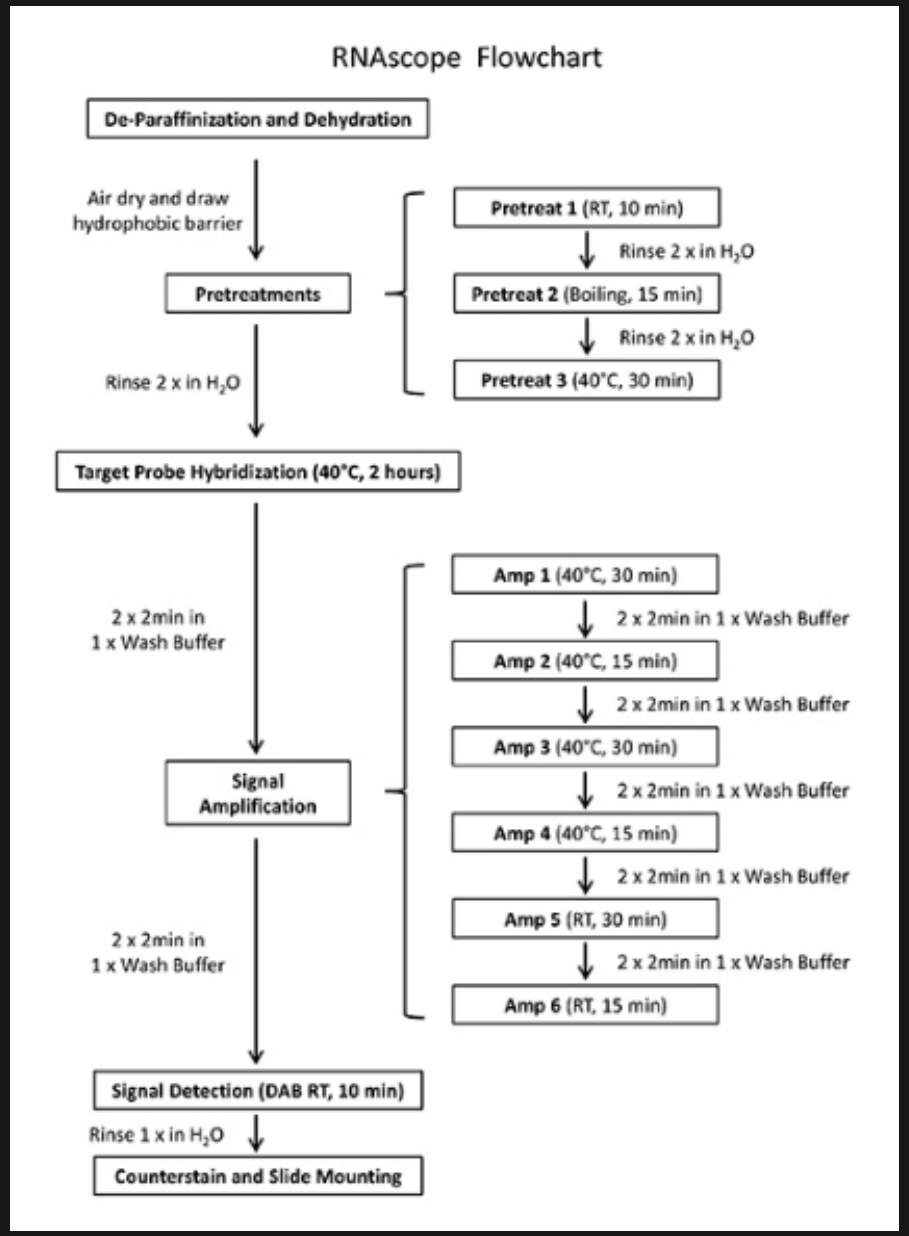
  - Amp5: **RT**, 30min or **2hr**
  - Amp6: **RT**, 15min

Flowchart of RNAscope assay with FFPE sections



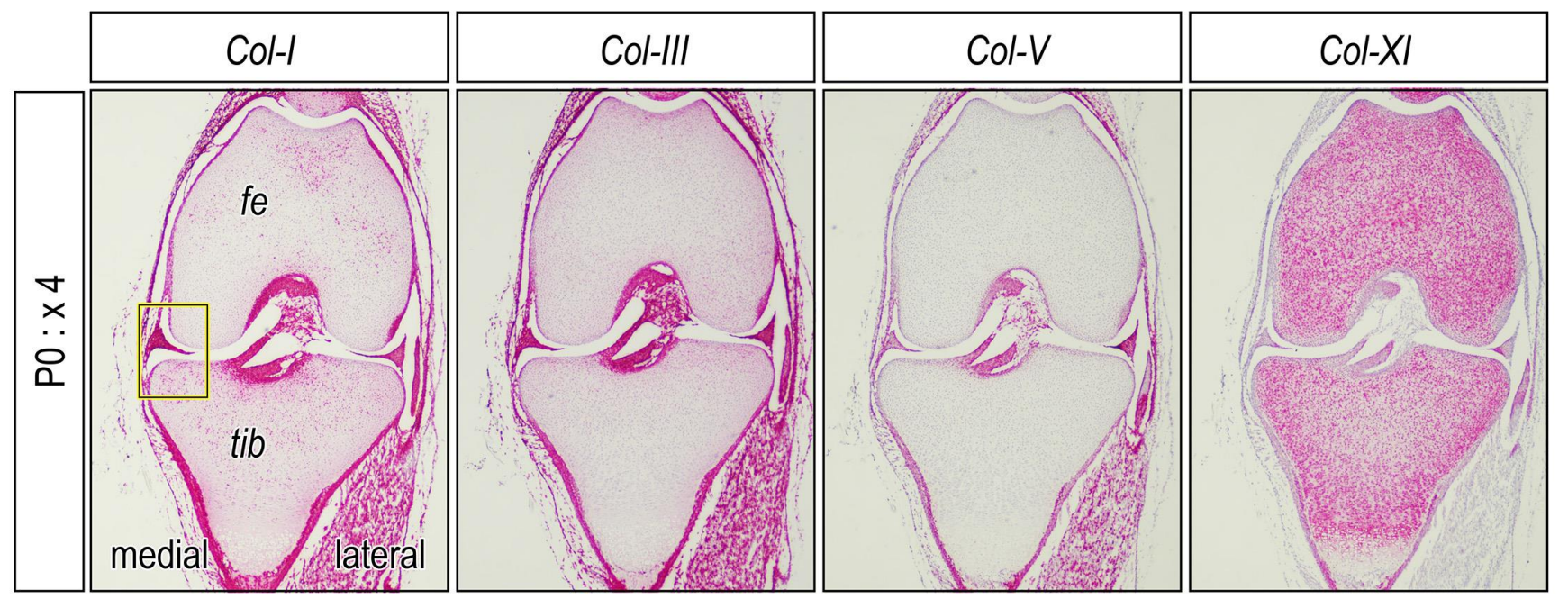
- **Signal Detection**
  - Enzyme reaction is very quick in some cases (Prg4 2-3 min, Collagens 2-4 min), you need to observe the slide under the microscope.
  - Dip the slide in H2O, postfix with 1-2%PFA for 10 min, and then counterstain with hematoxylin.

Flowchart of RNAscope assay with FFPE sections



- **Counterstaining**
  - Stain tissue section with hematoxylin solution (Hematoxylin QS, Vector: 1:125 with H2O) for 1 min at RT, rinse with H2O until slides are clear. Dry slide O/N, dip slides in xylene and mount with xylene-based mounting media.

RNAscope 2.5 HD Assay: RED



- ACD developed universal negative control probes targeting the DapB gene from the Bacillus. You can also order the sense probe with the sense direction.
- Cyclophilin B (PPIB) is used as a positive control probe. It is expressed at a low level , and so provides a rigorous control for sample quality. If PPIB is positive, then any target probe will detect your target RNAs.

ONE DAY / TWO DAY ASSAY

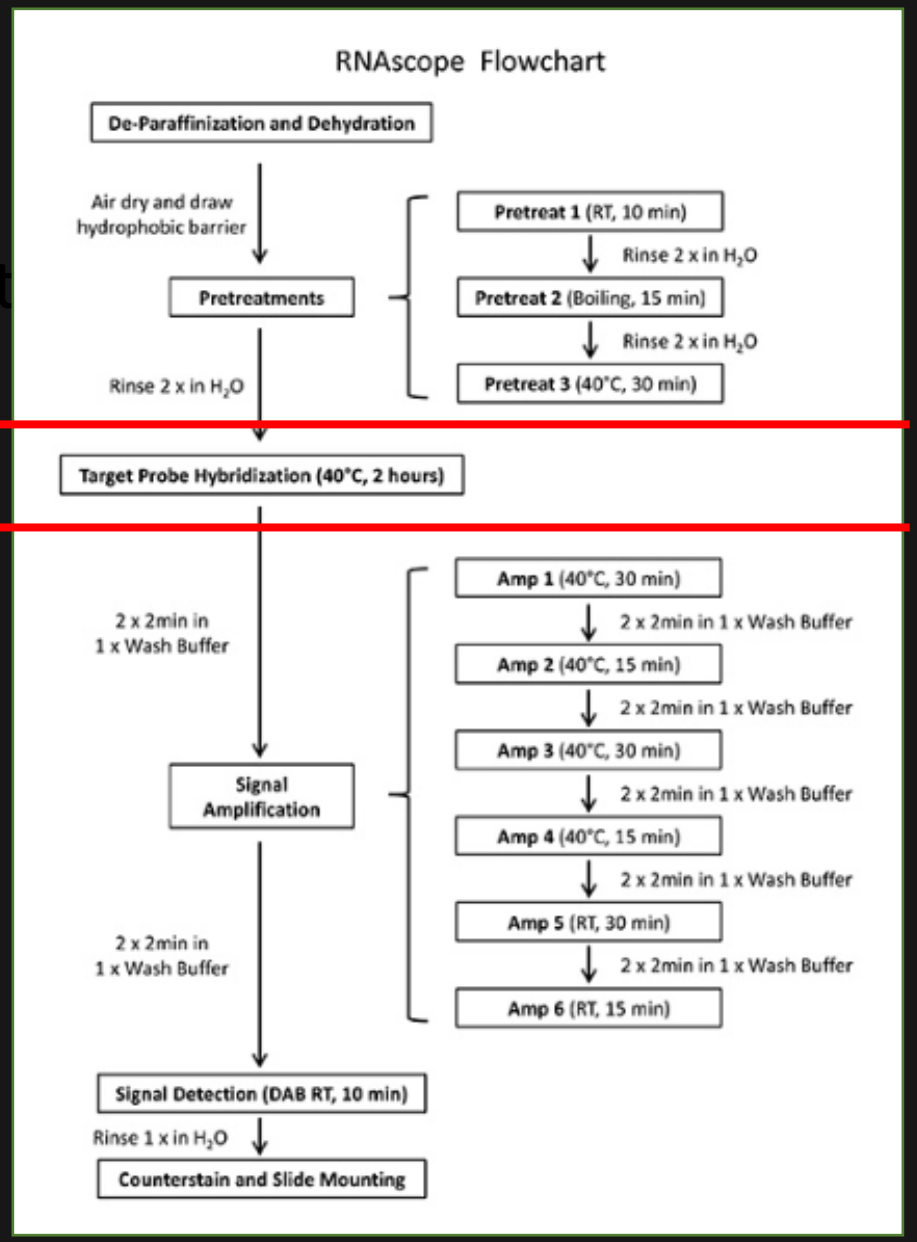
~1.5hr

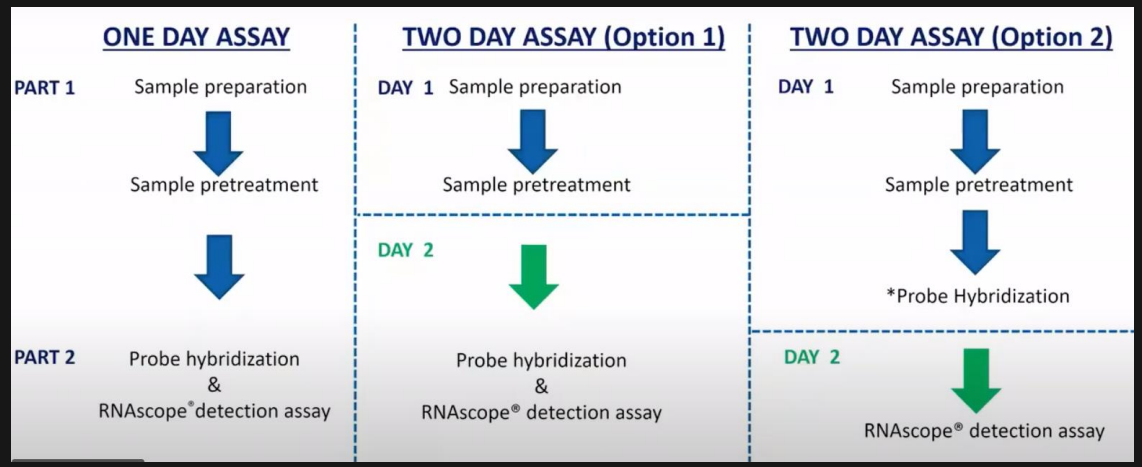
~1hr

~2hr

~3-5hr

~0.5hr





Obtained from ACD

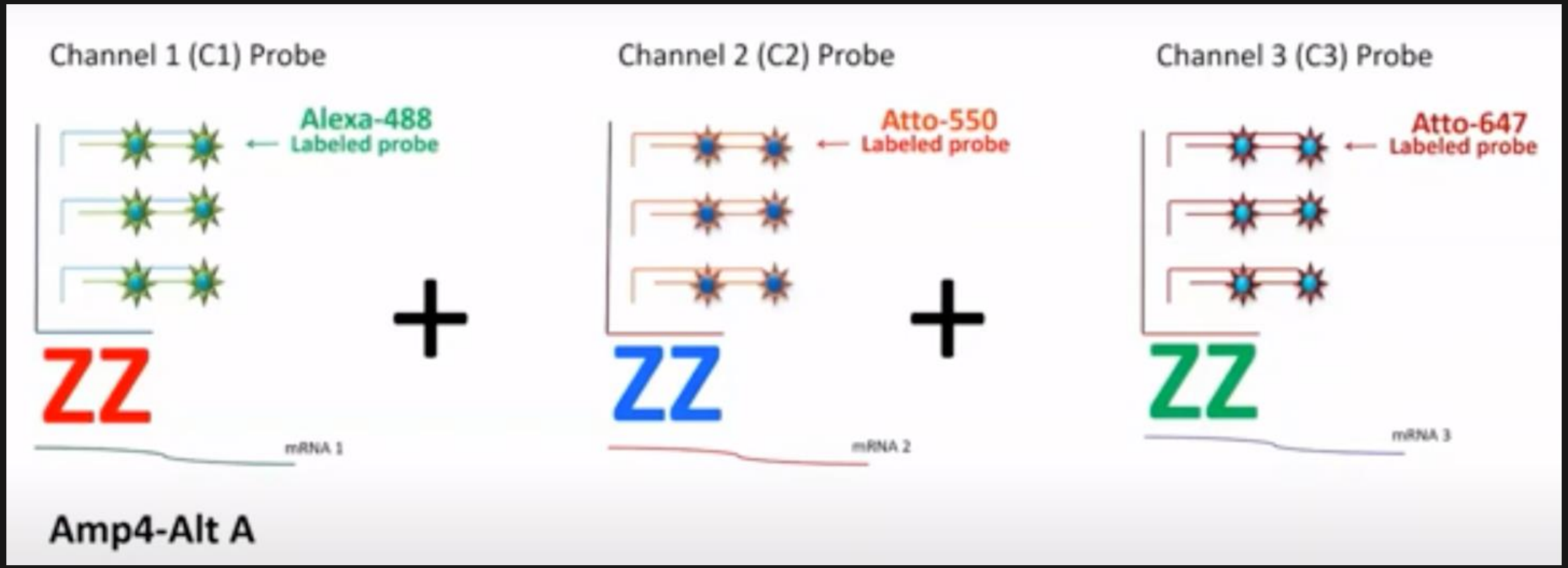
After hybridization
RT, 2 min in 1xWash Buffer (0.1xSSC)
Store @RT, O/N in 5xSSC (low stringency, keep slide from drying)

Next day, wash with 1xWash Buffer, then start amplification.

Critical Factors for Success of the RNAscope assay

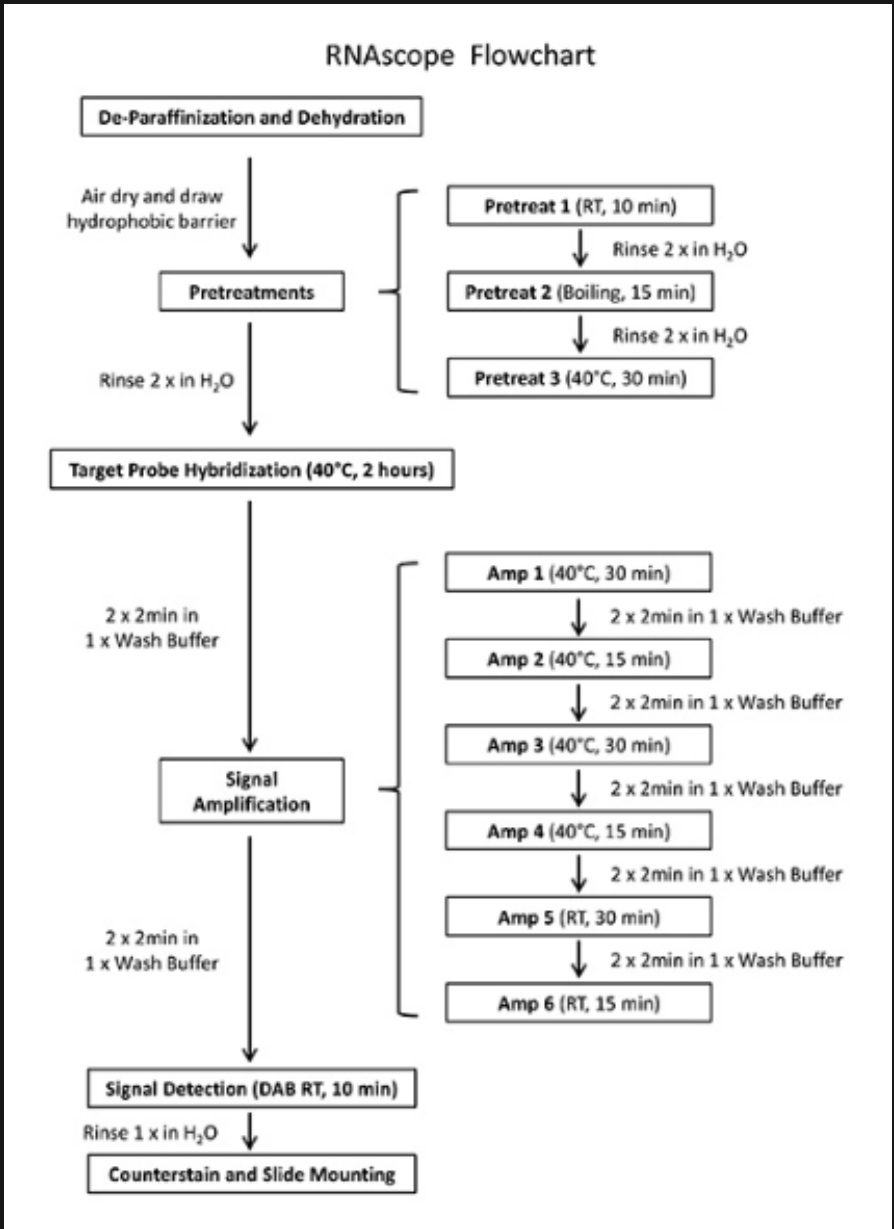
- Tissue should be fixed with fresh 4%PFA by injection. ACD recommends fresh 10% neutral buffered formalin at RT for 16-32 hr.
- Tissue decalcification: 10%EDTA with 2%PFA for 2-14 days, Formic acid (96%, Spectrum, F1089) @RT, for 2 days. But, reporter activity, IHC…….
- HybEZ oven is highly recommended since it enables optimal control of temperature and humidity for probe hybridization and signal amplification.
- Keep the tissue section from drying after hybridization.

Multiplex Fluorescent Assays

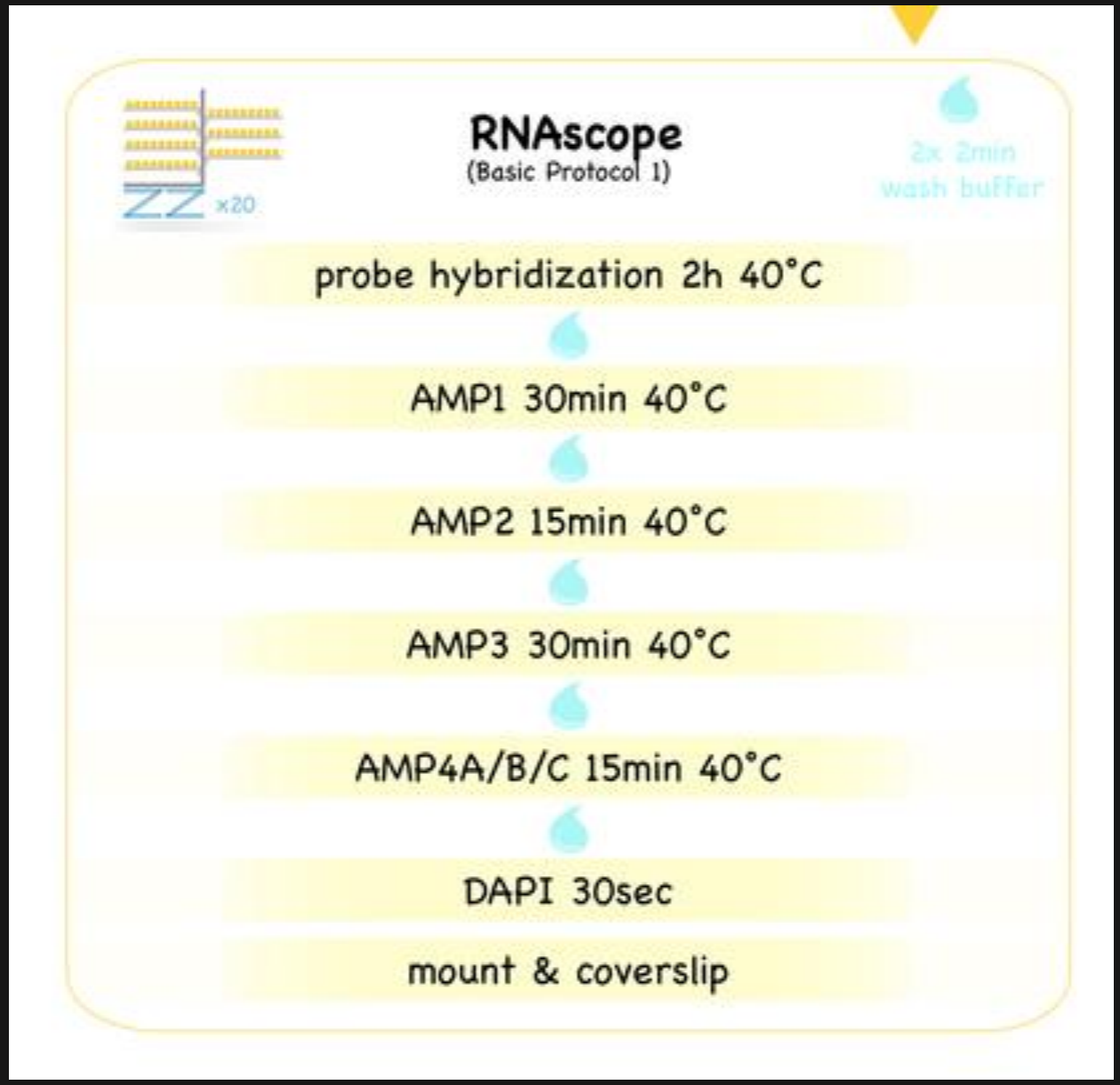


Obtained from ACD

Chromogenic Red/Brown



Multiplex Fluorescent



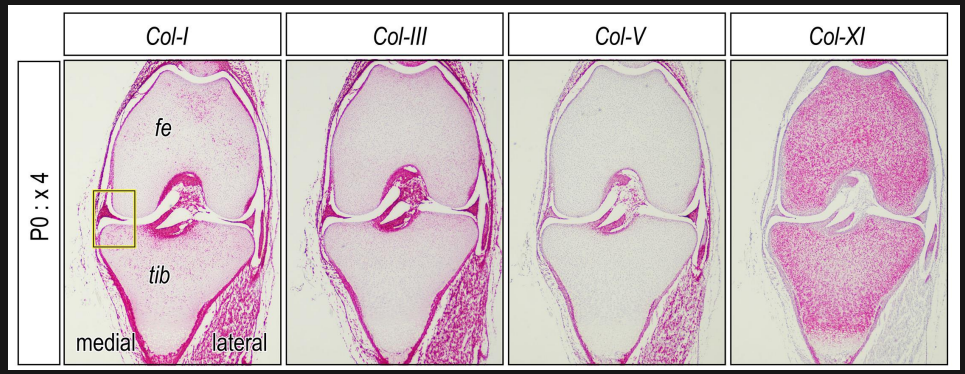
probe channel	Channel 1 (C1)	Channel 2 (C2)	Channel 3 (C3)
channel sensitivity	highest	weakest	high
cell type analysis of target gene expression	gene of interest	cell type marker 1 (e.g. vGLUT1/2)	cell type marker 2 (e.g. GAD1/2)
AMP4A	Alexa488	Atto550	Atto647
AMP4B (recommended)	Atto550	Alexa488	Atto647
AMP4C	Atto550	Atto647	Alexa488

Figure 2.

Multiplexing of the three channels in RNAscope. Detected fluorophores of the three channels (C1, C2, C3) can be adjusted by distinct amplification solution 4 (AMP4A, AMP4B, AMP4C). The sensitivity of the three channels is C1 > C2 >> C3. Therefore, we recommend examining the expression of a target gene (lowest expected expression) in different cell types using a channel 1 (C1) probe against this gene of interest and cell type-specific marker genes in Channel 2 & 3.

Obtained from ACD

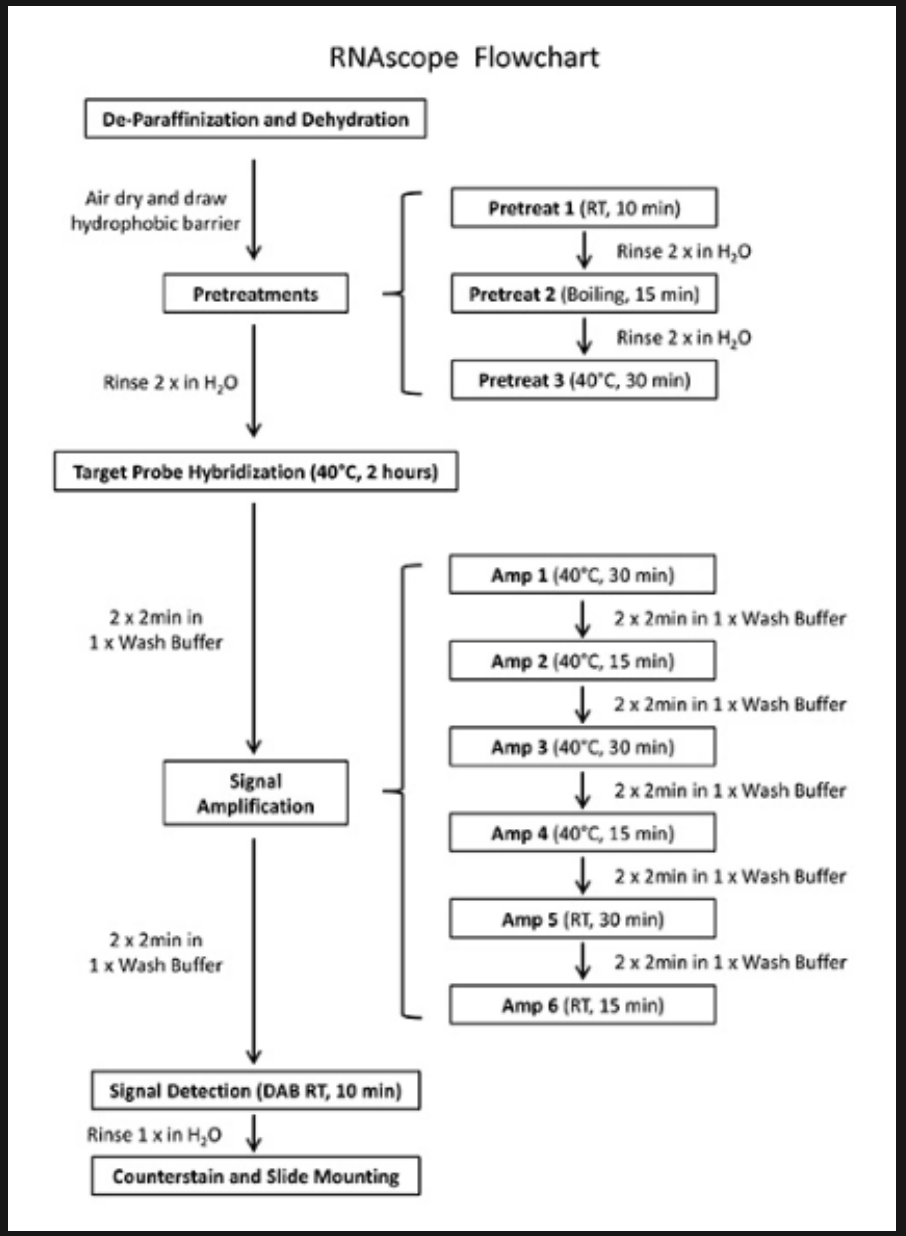
RNAscope 2.5 HD Assay: RED



- The manual RNAscope procedure discussed here has been fully automated. This should greatly facilitate standardization of assay conditions and save precious manual labor.

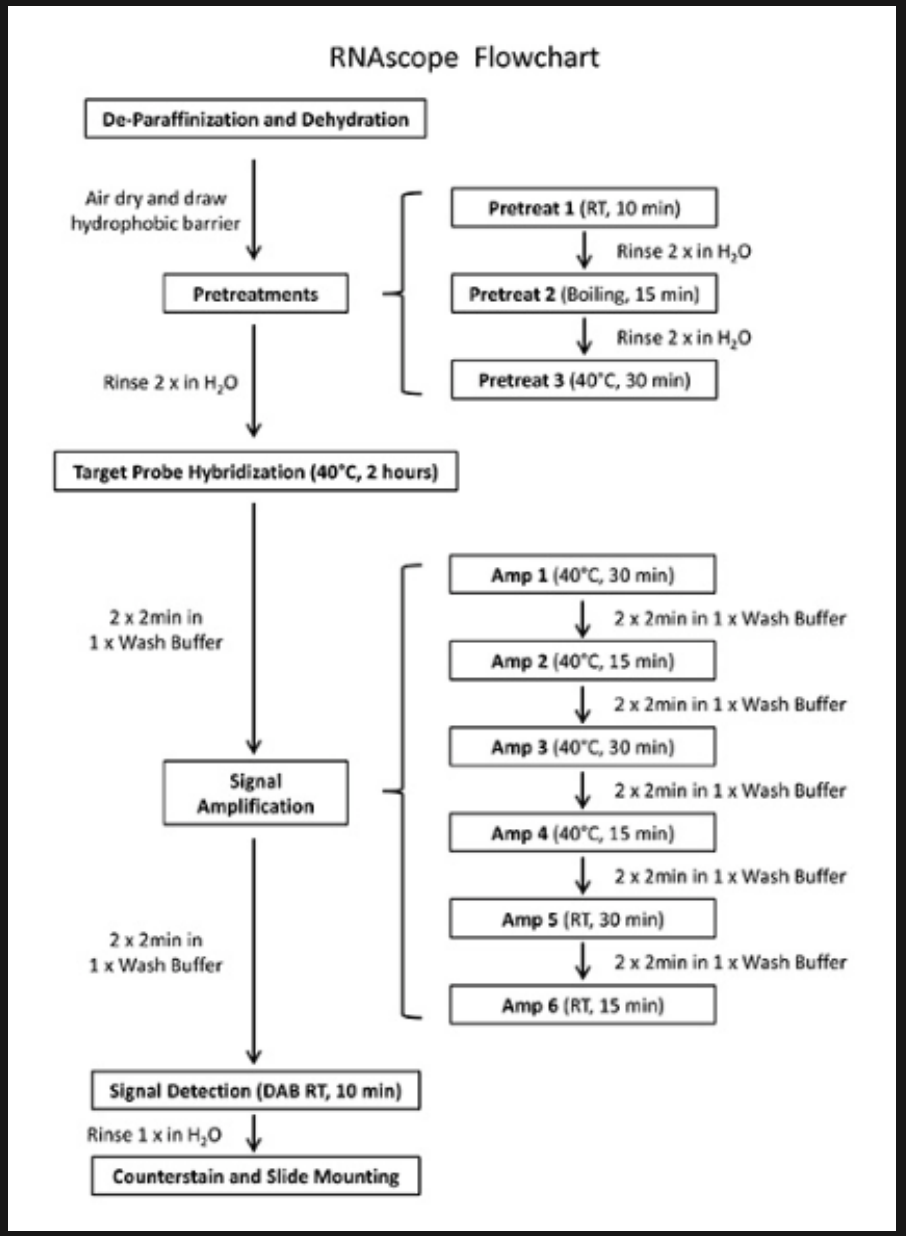
ffffgggggg

Flowchart of RNAscope assay with FFPE sections



- **Signal Detection**
  - Amp1: preamplifier
  - Amp2 background reducer
  - Amp3 amplifier
  - Pam4 labeled probe
  - Amp5
  - Amp6

Flowchart of RNAscope assay with FFPE sections



- **Signal Detection**
  - Amp1: preamplifier
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  - Amp5
  - Amp6

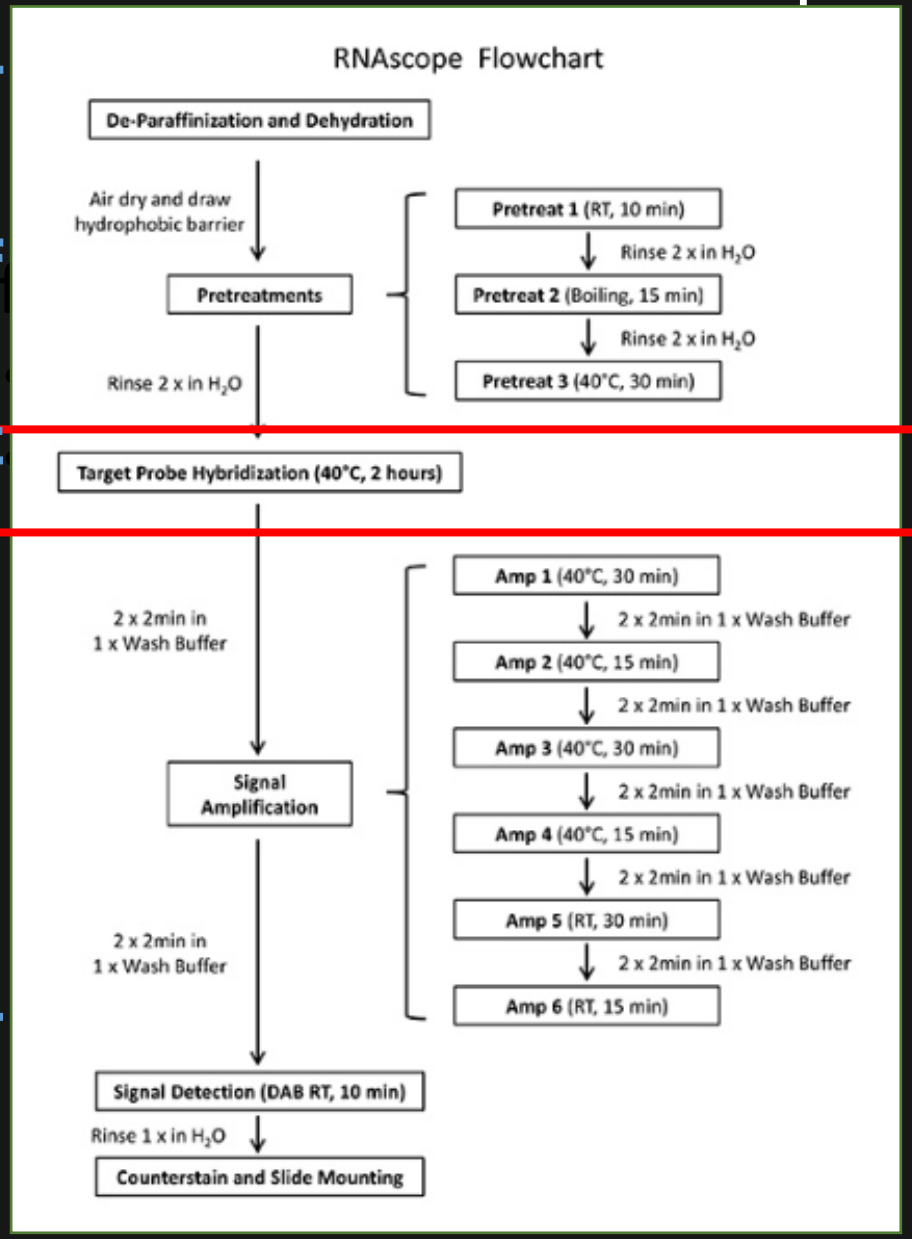
Flowchart of RNAscope assay

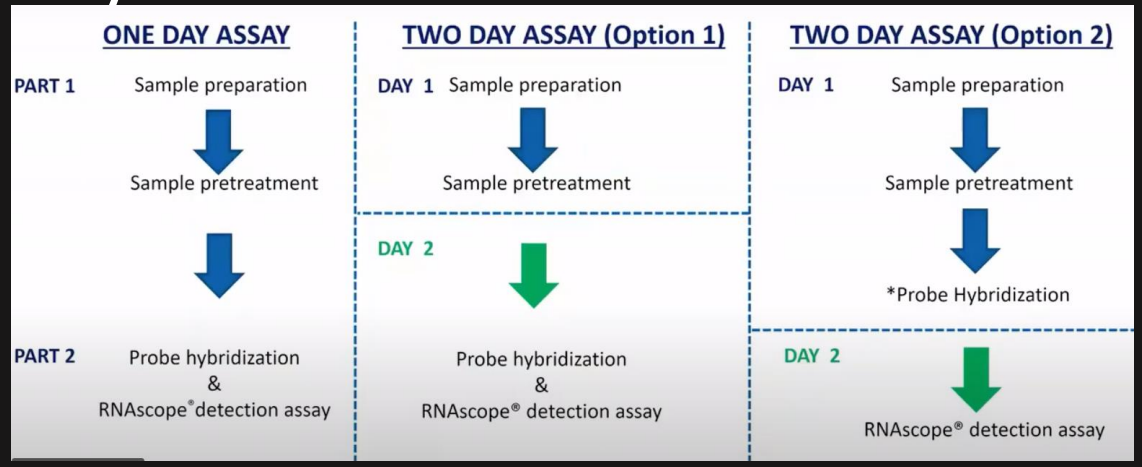
~1.5hr

~2hr

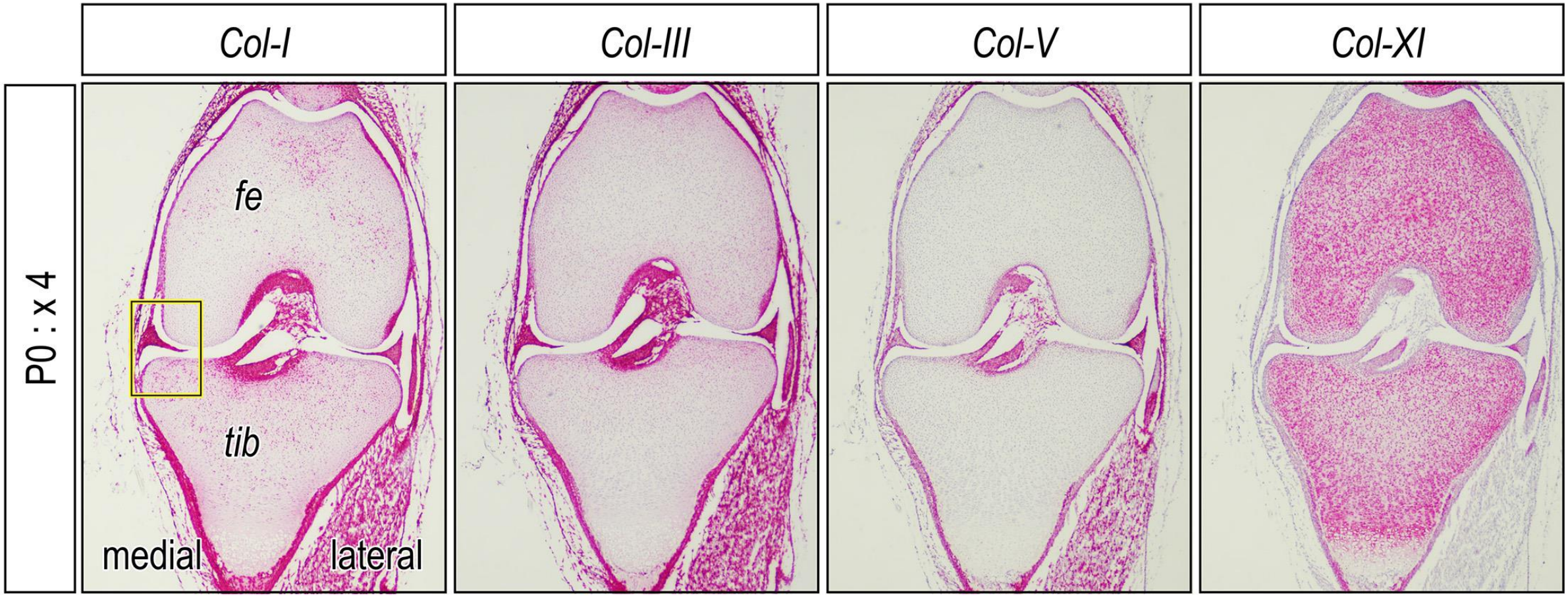
~3-5hr

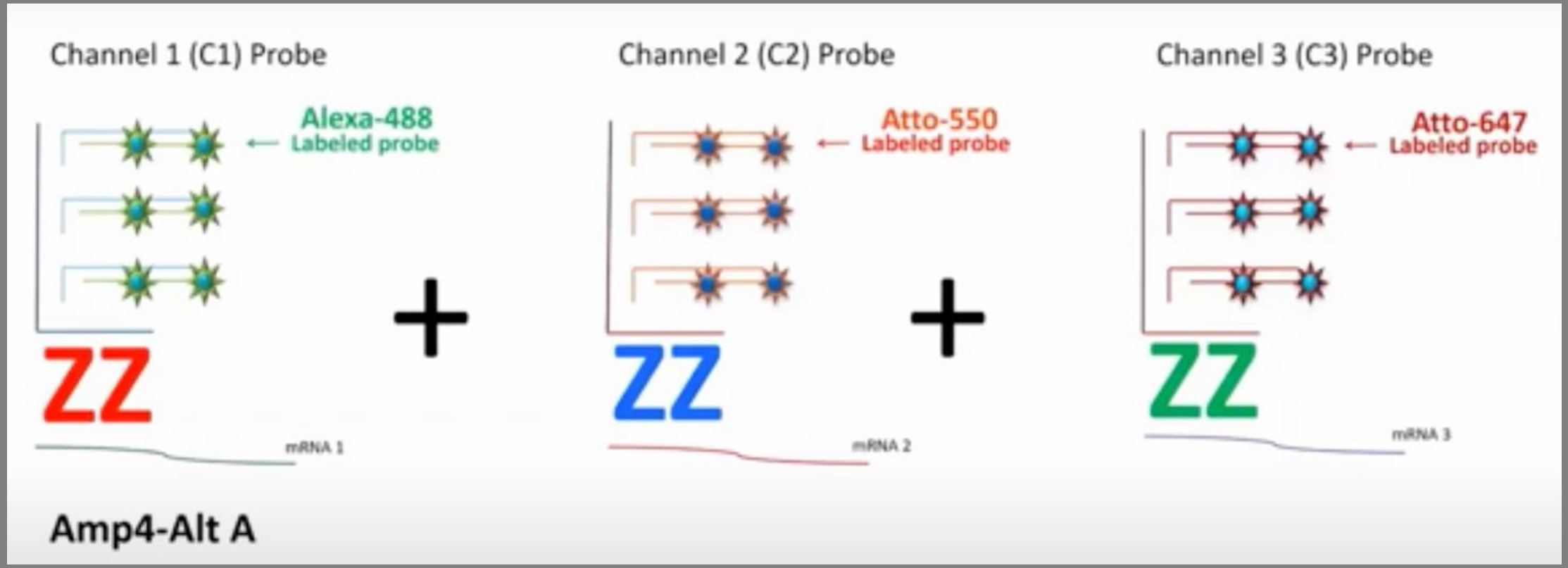
~0.5hr



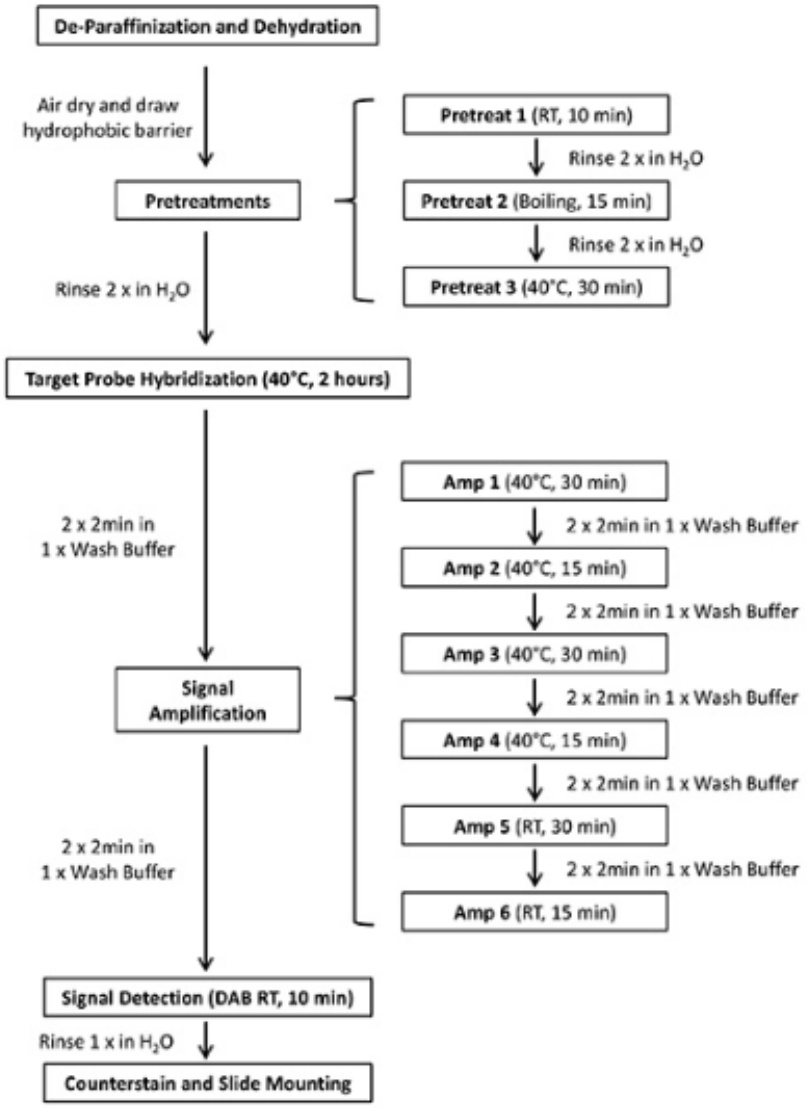


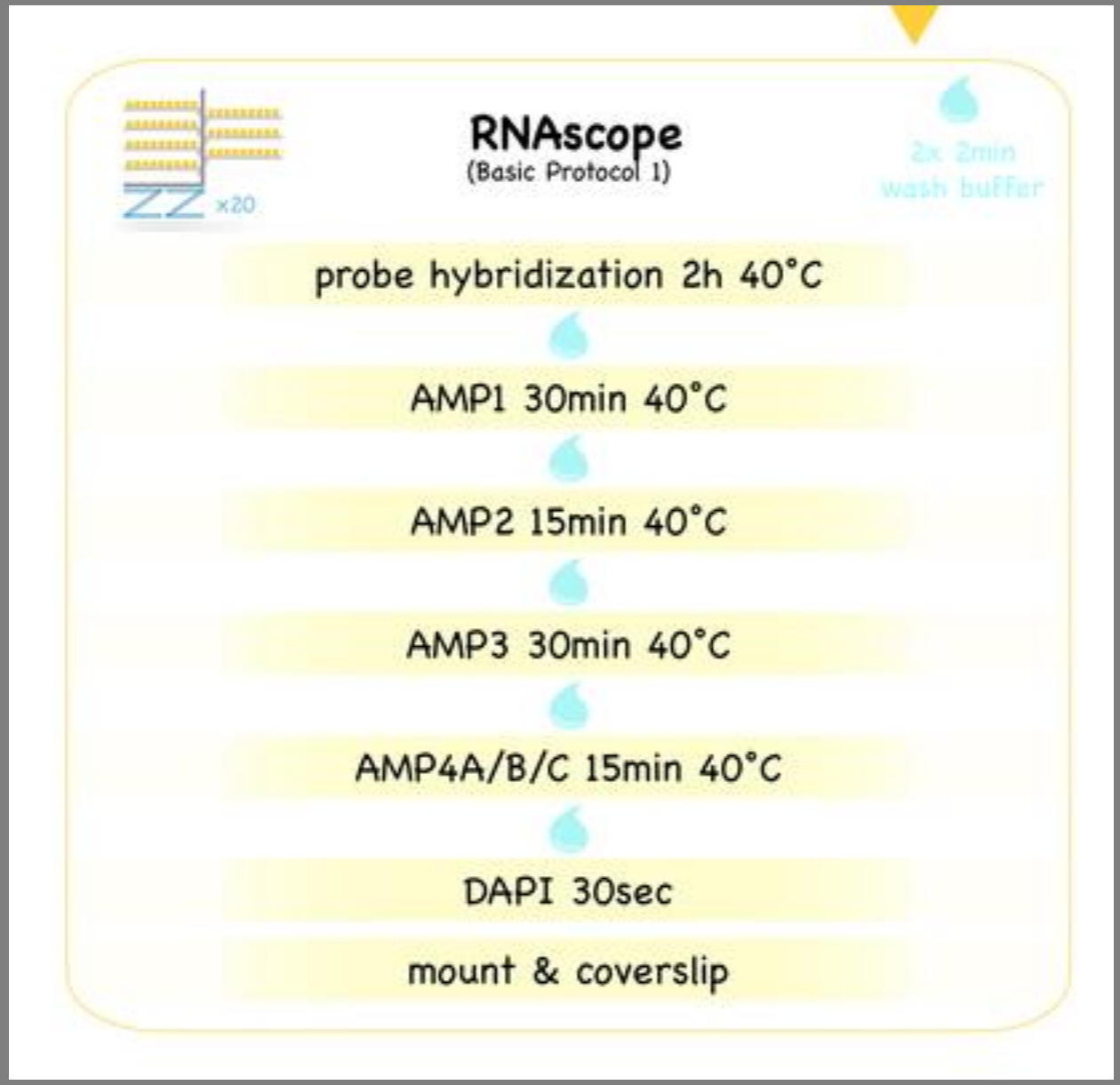
After hybridization
RT, 2 min in 1xWash Buffer
Store @RT, O/N in 5xSSC


Col-I
Col-III
Col-V
Col-XI
P0 : x 4
fe
tib
medial
lateral


Channel 1 (C1) Probe
Alexa-488
Labeled probe
ZZ
mRNA 1
+
Channel 2 (C2) Probe
Atto-550
Labeled probe
ZZ
mRNA 2
+
Channel 3 (C3) Probe
Atto-647
Labeled probe
ZZ
mRNA 3
Amp4-Alt A

RNAscope Flowchart





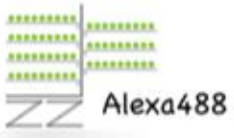
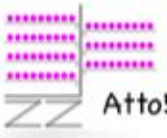
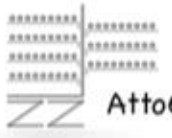
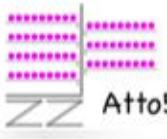
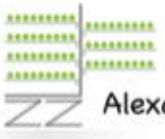
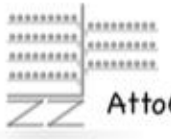
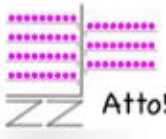
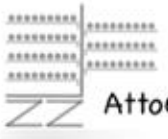
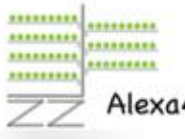
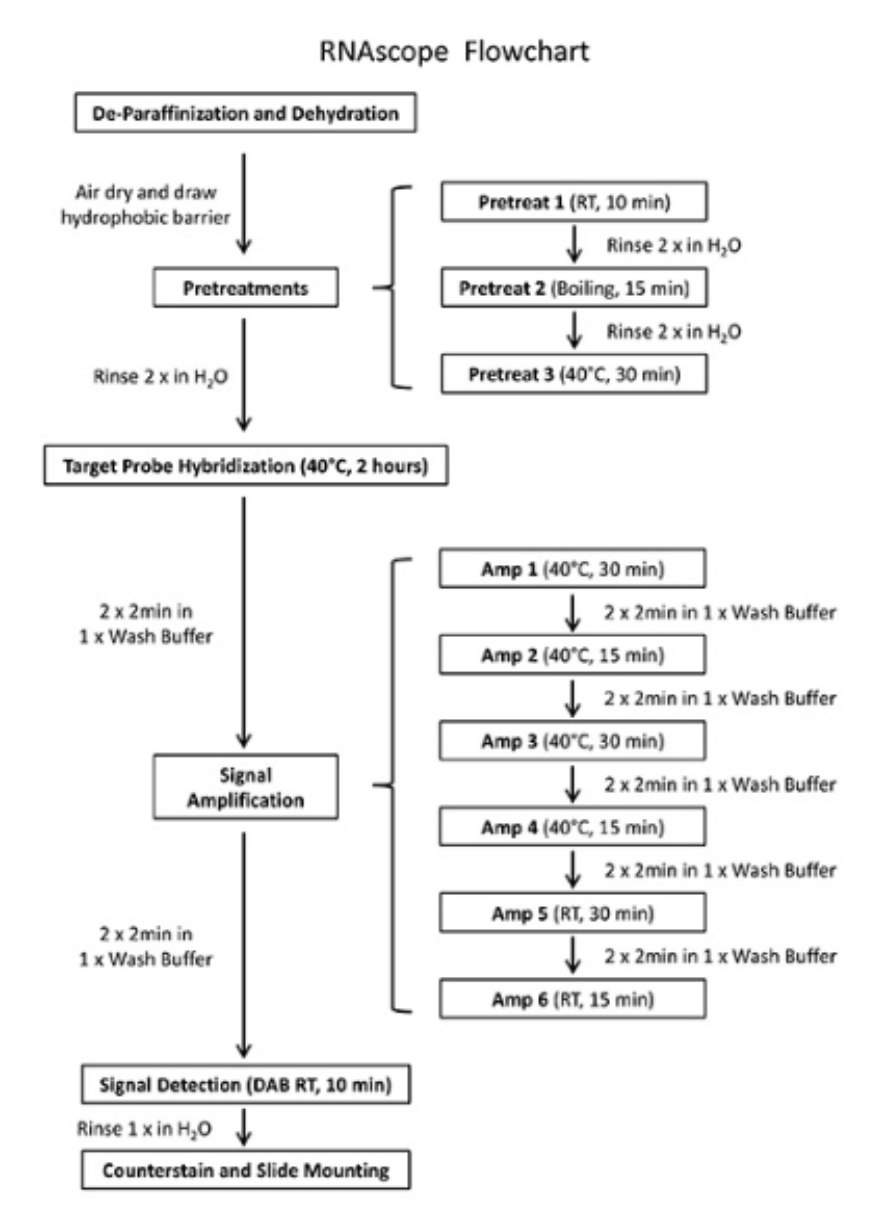
probe channel	Channel 1 (C1)	Channel 2 (C2)	Channel 3 (C3)
channel sensitivity	highest	weakest	high
cell type analysis of target gene expression	gene of interest	cell type marker 1 (e.g. vGLUT1/2)	cell type marker 2 (e.g. GAD1/2)
AMP4A	 Alexa488	 Atto550	 Atto647
AMP4B (recommended)	 Atto550	 Alexa488	 Atto647
AMP4C	 Atto550	 Atto647	 Alexa488

Figure 2.

Multiplexing of the three channels in RNAscope. Detected fluorophores of the three channels (C1, C2, C3) can be adjusted by distinct amplification solution 4 (AMP4A, AMP4B, AMP4C). The sensitivity of the three channels is C1 > C2 >> C3. Therefore, we recommend examining the expression of a target gene (lowest expected expression) in different cell types using a channel 1 (C1) probe against this gene of interest and cell type-specific marker genes in Channel 2 & 3.

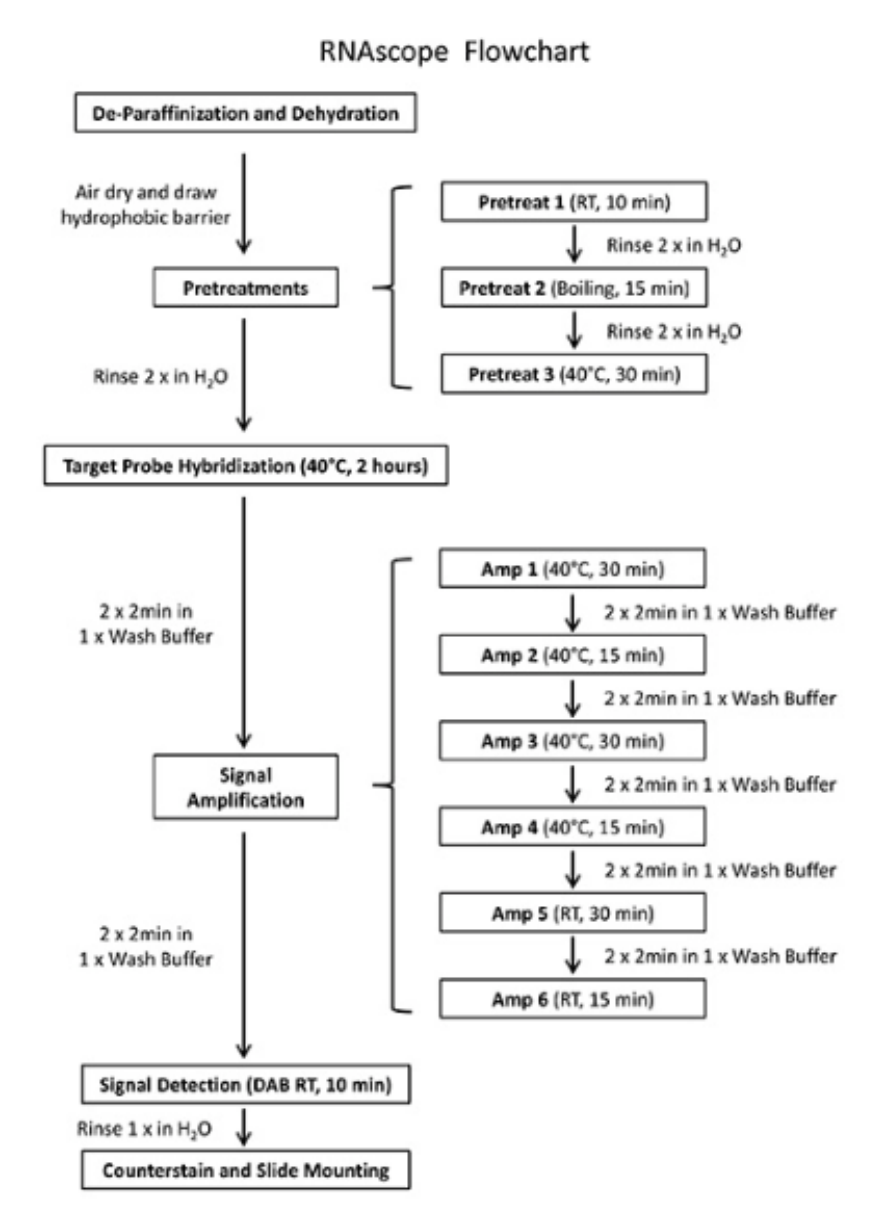
Flowchart of RNAscope assay



- **Signal Detection:**
  - Incubate tissue sections with **1:1 DAB** Mixture by mixing equal volume of **Brown-A** and **Brown-B** for 10 min at RT, rinse twice in dH_2O.

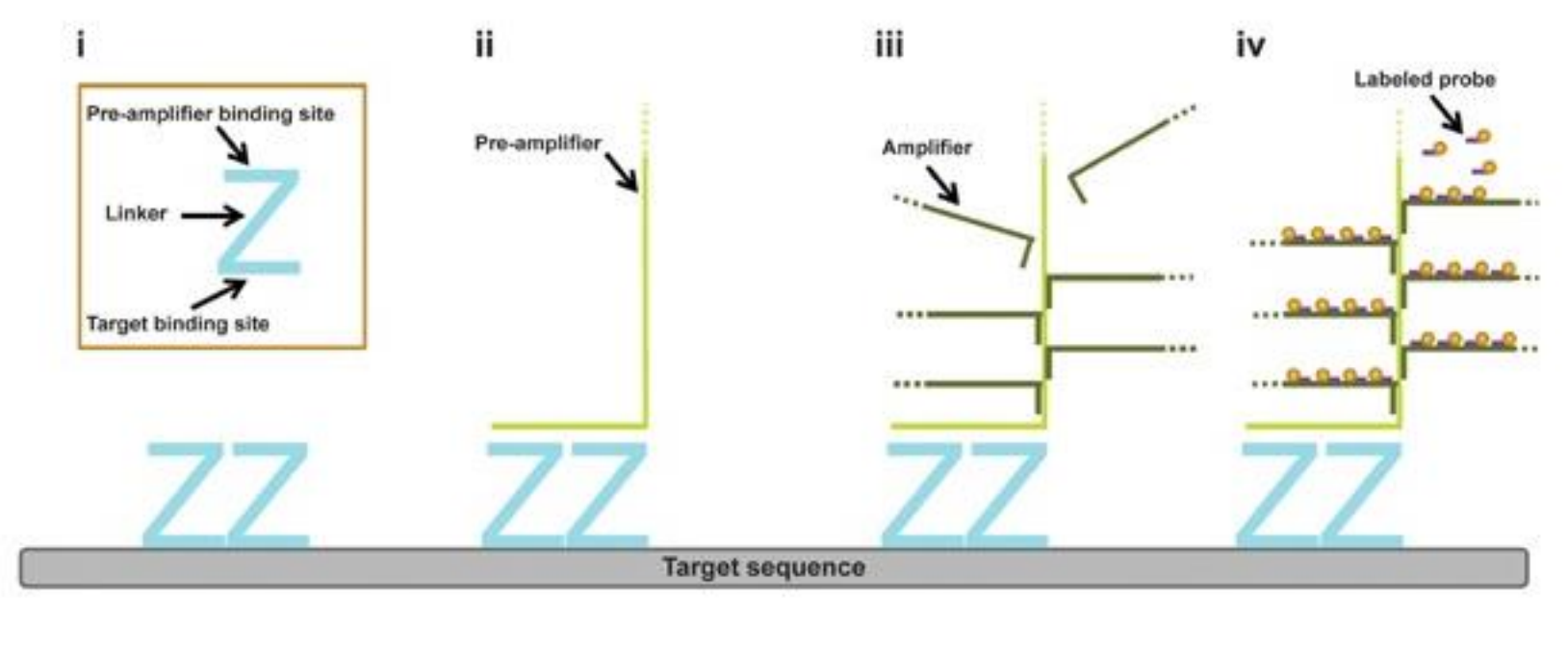
  - Stop the substrate reaction to prevent overproduction of a colored precipitate. When the enzyme substrate is added, you need to observe the reaction very often.

Flowchart of RNAscope assay



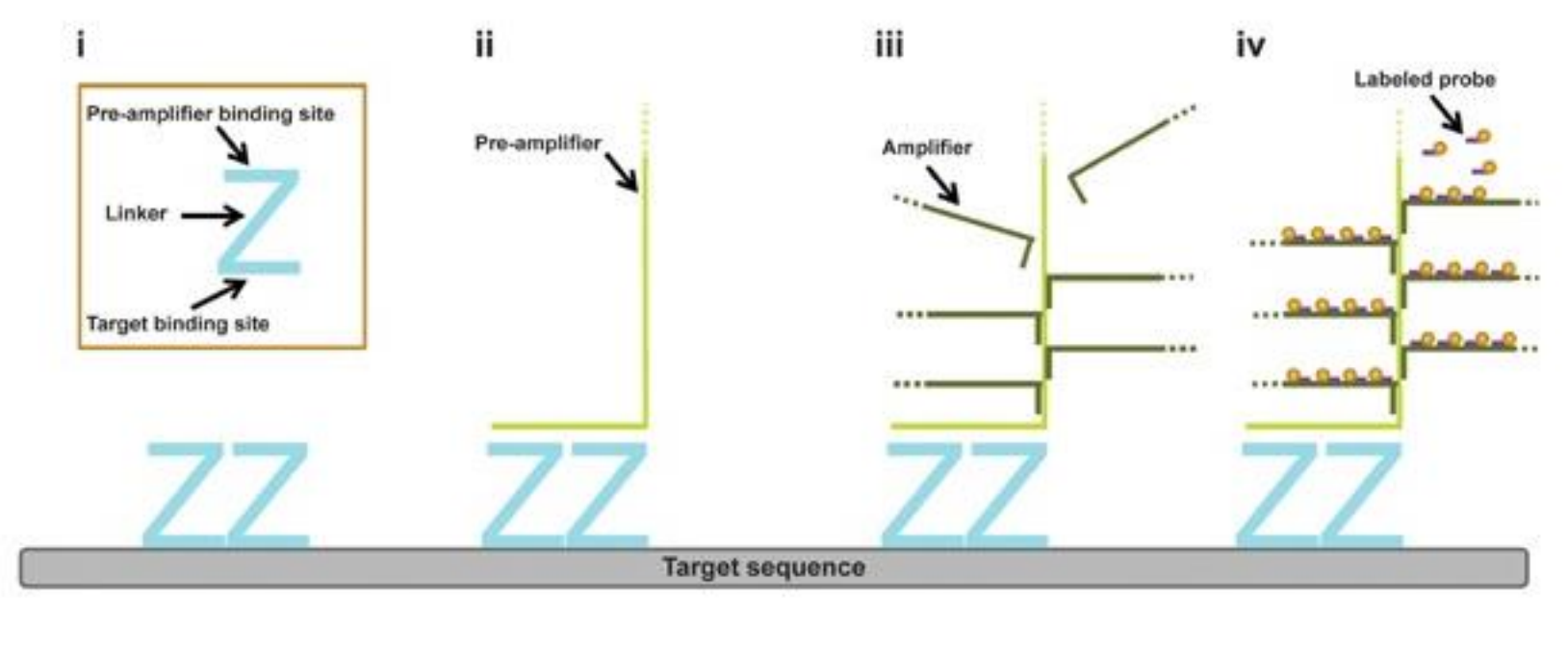
- Fix tissue specimen with 4% PFA for 24 hr.
- 5 um thickness
- Slides are stored at 4 C
- The mounted tissue slide should be baked ~~in a~~ at 60 C prior to the assay.
- Pretreatment: for bone and cartilage: Hybridization of multiple amplifiers to preamplifier.
- Pre-warm target probes at 40 C for 10 min.

RNAscope technology overview



- (i) Hybridization of each Z-shaped target probe hybridizes to the RNA target.
- (ii) Hybridization of the pre-amplifier to the upper portion of the Z-probe pairs.
- (iii) Hybridization of multiple amplifiers to the preamplifier.
- (iv) Hybridization of multiple labeled probes to amplifier.

RNAscope technology overview



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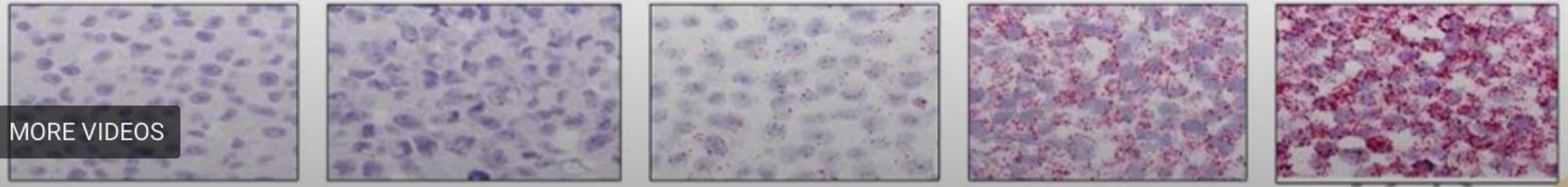
DATA ANALYSIS: SEMI-QUANTITATIVE METHOD

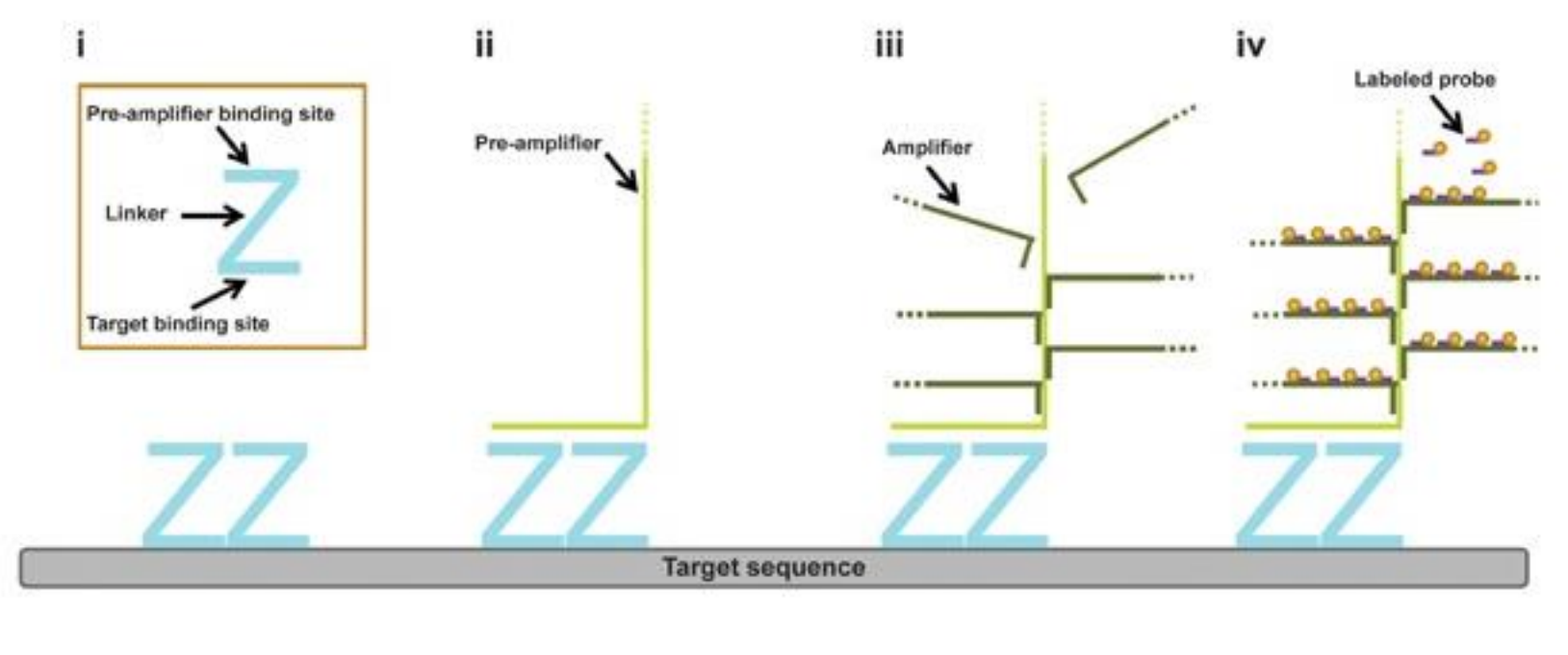
Semi-quantitative image analysis

Score assigned to a sample based on average number of target dots/cell performed manually

Score	Criteria
0	No staining or <1 dot/ 10 cells
1	1-3 dots/cell
2	4-9 dots/cell. None or very few dot clusters
3	10-15 dots/cell and <10% dots are in clusters
4	>15 dots/cell and >10% dots are in clusters



RNAscope technology overview



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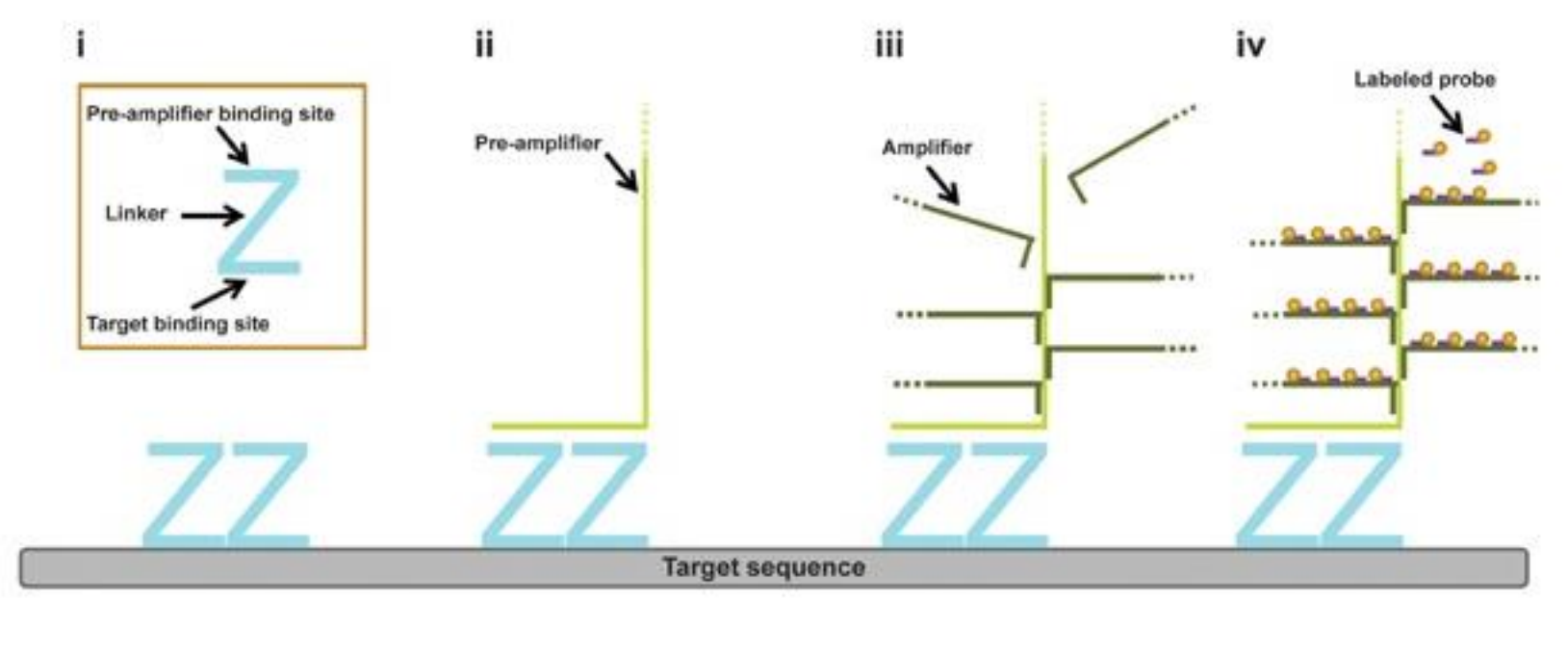
RNAscope technology overview



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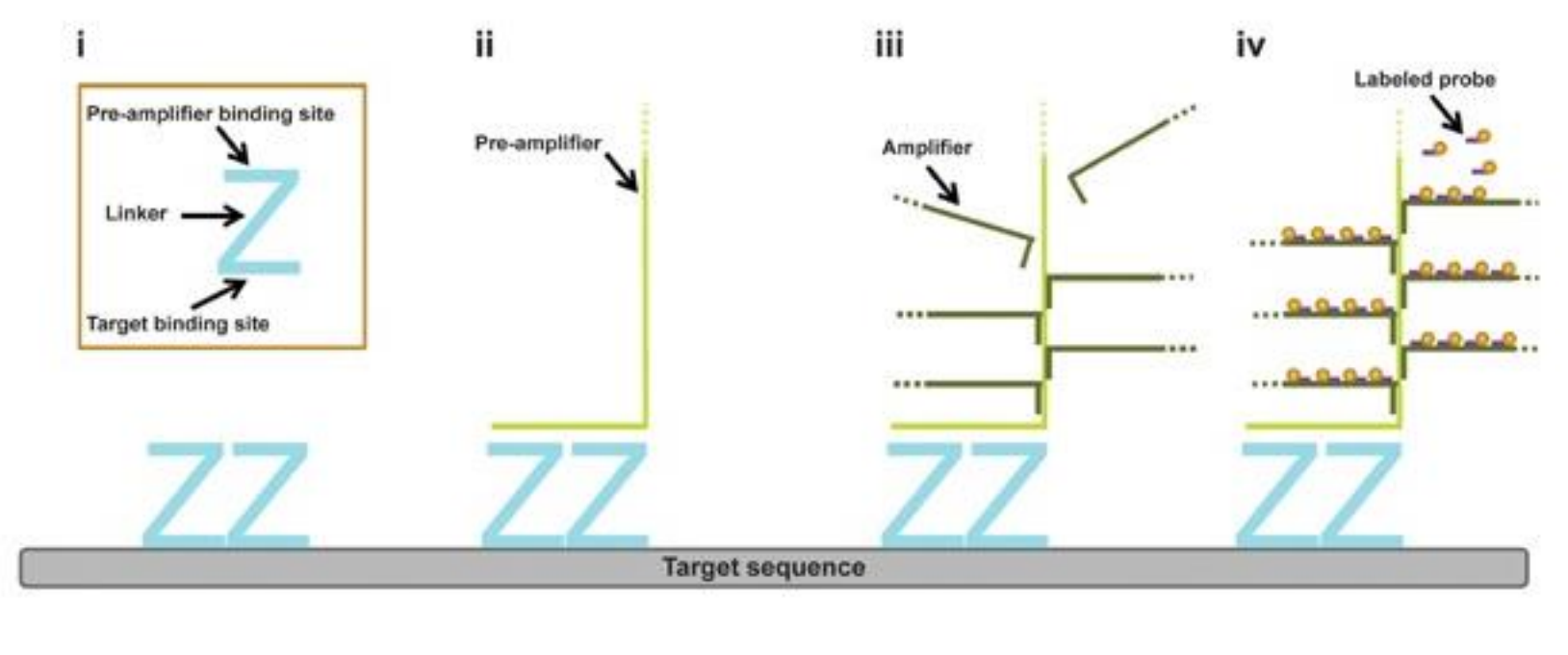
RNAscope technology overview



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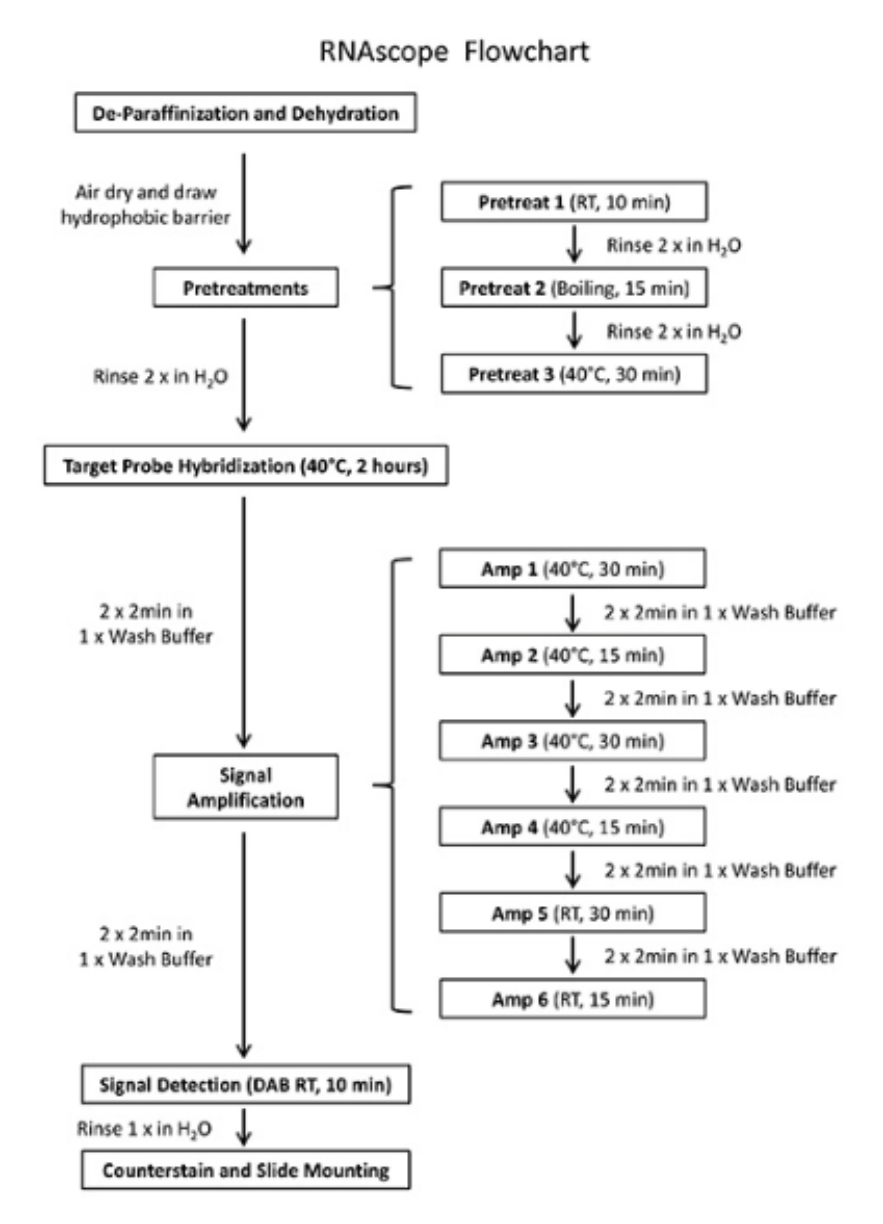
Flowchart of RNAscope assay



- Fix tissue specimen with 4% PFA for 24 hr.
- 5 um thickness
- Slides are stored at 4 C
- The mounted tissue slide should be baked ~~ina~~ at 60 C prior to the assay.
- Pretreatment: for bone and cartilage: Hybridization of multiple amplifiers to preamplifier.
- Pre-warm target probes at 40 C for 10 min.

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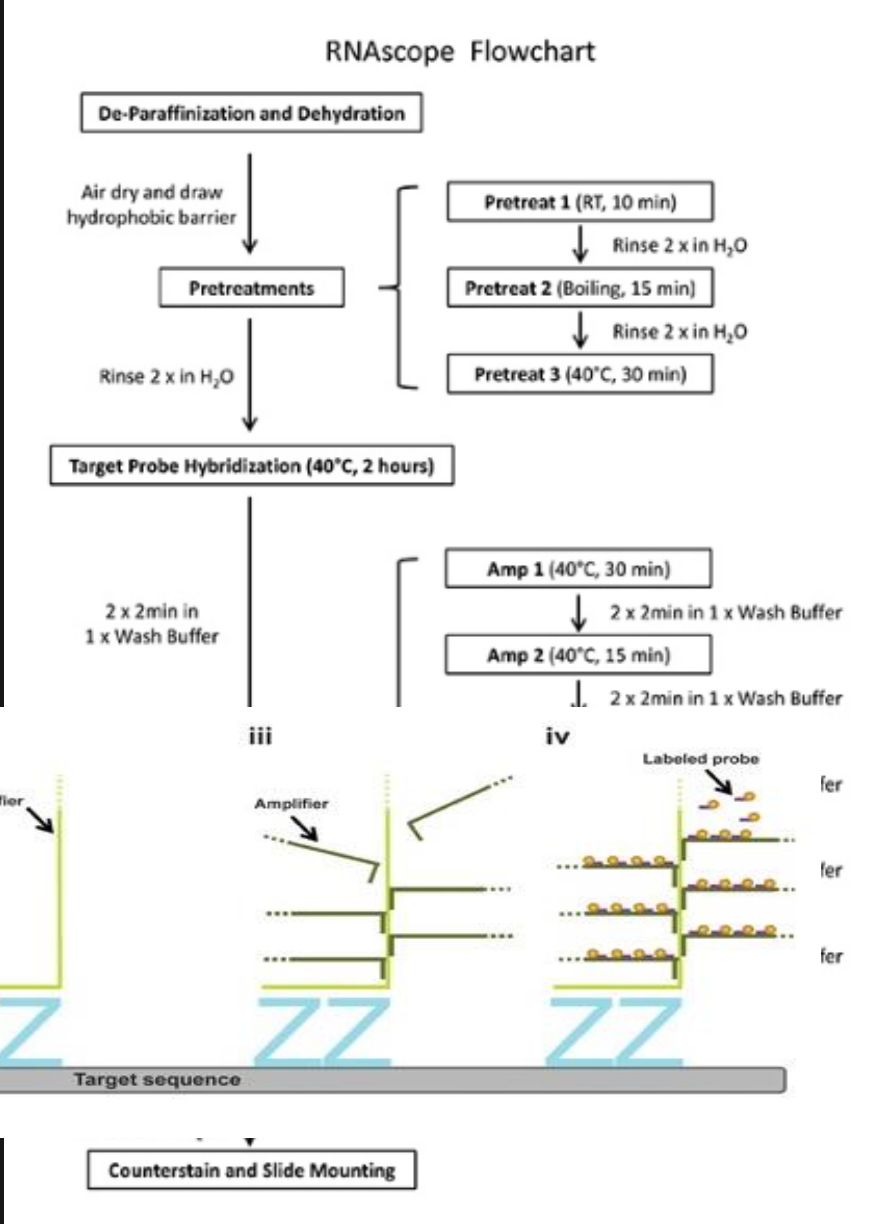
Flowchart of RNAscope assay with FFPE sections



- **Signal Amplification is achieved through Amp1-6.**
  - Amp1: **Pre-amplifier** binds to the upper region of each double Z probe pair, 40◦C, 30min
  - Amp2: Background reducer, 40◦C, 15min
  - Amp3: **Amplifiers** bind to the binding site of Preamplifier, 40◦C, 30min
  - Amp4: Background reducer, 40◦C, 15min
  - Amp5: **Amplifiers** bind to the binding site of Preamplifier, RT, 30min or 2hr

  - Amp5: **RT**, 30min or **2hr**
  - Amp6: **RT**, 15min