miRNAscope™ RED Assay combined with Immunohistochemistry - Integrated Co-Detection Workflow (ICW)

Introduction

This Technical Note provides guidelines for performing *in situ* hybridization (ISH) using miRNAscope HD Reagent Kit RED (Cat. No. 324500) with immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) tissue sections. Protocols for other sample types are available in Appendix B and Appendix C. This technical note is for advanced users who are familiar with the procedures in the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM), available at www.acdbio.com/technical-support/user-manuals. For every chemical, read the Safety Data Sheet (SDS) and follow handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For the latest services and support information, go to www.acdbio.com/support.

Required Reagents

The following reagents are required specifically for miRNA-protein co-detection. For a complete list of materials and equipment, please refer to the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM).

ACD Reagents:

RNA-Protein Co-Detection Ancillary kit (Cat. No. 323180)		
Reagent	**Quantity**	**Storage**
Co-Detection Target Retrieval (10X)	70 mL x 4 bottles	Room temp (15–30°C)
Co-Detection Antibody Diluent	120 mL x 1 bottle	2–8°C
Co-Detection Blocker	4.5 mL x 1 bottle	2–8°C

Additional ACD Reagents	Ordering Info
RNAscope™ Hydrogen Peroxide and Protease Reagents	Cat No. 322381
miRNAscope HD Detection Reagents-RED	Cat. No. 324500
miRNAscope Target and Control Probes	Various

Additional ACD Reagents	Ordering Info
2.5 LS Green Accessory Pack (optional for green IHC)	Cat. No. 322550

Additional Reagents:

Reagents	Ordering Info
Phosphate Buffered Saline w/0.1% Tween-20 (PBS-T) (1X)	User provided
Primary Antibody	User provided
10% Neutral Buffered Formalin	User provided
Secondary Antibody	User provided

Workflow

Prepare Tissues

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Post-fix samples ~2 HRS (12% formaldehyde) or OVERNIGHT (10% NBF)

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H_2O_2 Block (10 MIN) and Target Retrieval ~15 MIN

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Primary Antibody Incubation ~OVERNIGHT

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Post-Primary Fixation ~30 MIN

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Protease Treatment ~30 MIN

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miRNAscope ISH

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Secondary Antibody Staining ~30 MIN

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Counterstain and mount the slides ~10 MIN

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Evaluate the samples

IMPORTANT! Standard antibody diluents negatively impact miRNA signal and are not recommended for use with this workflow. Co-Detection Antibody Diluent is formulated for maximum compatibility with the miRNAscope assays and will best preserve miRNA signal.

Note: For optimal protein detection, you may want to use a higher primary antibody concentration than you would normally use for immunohistochemistry alone.

Part 1: Prepare and Pretreat Tissues

For sample preparation details, please refer to the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM).

Prepare Slides

1. Bake slides in a dry air oven for **60 MIN** at **60°C**.
2. In a fume hood, fill 2 Tissue-Tek® clearing agent dishes with ~200 ml fresh xylene and fill 2 Tissue-Tek staining dishes with ~200 ml fresh 100% ethanol.
3. Place slides in a Tissue-Tek slide rack in xylene for **5 MIN** with slight agitation. Repeat in second xylene dish for an additional **5 MIN**.
4. Incubate slides in 100% ethanol for **2 MIN** with slight agitation. Repeat in second 100% ethanol dish for **2 MIN**.
5. Remove slides from rack and let air dry for **5 MIN** to **OVERNIGHT** at **RT**.

Post-fix Samples

For optimal performance of the miRNAscope Assay, post-fix with either 10% NBF or 12% formaldehyde by choosing one of the following procedures:

Post-fixation with NBF

1. In a fume hood, fill a Tissue-Tek Clearing Agent dish with ~200 mL fresh 10% NBF.
2. Place slides in a Tissue-Tek Slide Rack and submerge them in the 10% NBF.
3. Incubate slides **OVERNIGHT** (16-18 HRS) at **RT**.
4. Remove slides from the 10% NBF and wash them for **2 MIN** in distilled water.
5. Dry slides for **5 MIN** at **60°C** or until completely dry.

Post-fixation with formaldehyde

1. In a fume hood, prepare 12% formaldehyde in a Tissue-Tek Clearing Agent dish by combining 65 ml fresh 37% formaldehyde with 135 mL of 1X PBS.
2. Place slides in a Tissue-Tek Slide Rack and submerge them in the 12% formaldehyde.
3. Incubate slides for **2 HRS** at **RT**.
4. Remove slides from the 12% formaldehyde and wash them for **2 MIN** in distilled water.
5. Dry slides for **5 MIN** at **60°C** or until completely dry.

Apply Hydrogen Peroxide

1. Add 2–4 drops of RNAscope Hydrogen Peroxide to each section for **10 MIN** at **RT**. Use enough solution to completely cover the sections.
2. Place slides in a Tissue-Tek Slide Rack and wash twice with distilled water.

Apply Target Retrieval

1. Cover a beaker containing 700 mL fresh 1X Co-Detection Target Retrieval and a beaker containing 700 mL of distilled H_2O with foil and bring temperature to **98–102°C** using a hotplate. Maintain this temperature.
2. Add the slides to the container containing distilled H_2O for **10 SEC** to acclimate slides.
3. Remove the slides with a pair of forceps *very slowly* and submerge the slide rack into the hot 1X Co-Detection Target Retrieval solution for **15 MIN**.

Note: Depending on tissue type, you may need to adjust the boiling time.

Note: Maintain temperature at **98–102°C** for the duration of target retrieval.

Note: You can also use the steamer method for target retrieval. For details on target retrieval methods, please refer to Chapter 3 of the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM). Make sure that you use 1X Co-Detection Target Retrieval.

3. *Immediately* transfer the hot slide rack to a staining dish containing distilled water.
4. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water.
5. Wash slides in 1X PBS-T by moving the rack up and down 3–5 times.

Create Barrier

1. Working quickly to prevent the sample from drying out, use a tissue to carefully dry the glass surrounding your sample. Draw 2–4 times around the section using the ImmEdge™ hydrophobic barrier pen. Let the barrier dry **~30 SEC**.

IMPORTANT! Do not allow the sample to dry out completely as this may negatively impact IHC signal.

Part 2: Perform Immunohistochemistry Part A

Apply Primary Antibody

1. Place slides in the HybEZ™ Slide Rack and add 150–200 µl of primary antibody diluted in Co-Detection Antibody Diluent to each section. Use enough solution to cover each tissue section completely.
2. Place the HybEZ Slide Rack in the Humidity Control Tray lined with damp humidifying paper and incubate **OVERNIGHT** at **4°C**.

Prepare Materials

1. Bring HybEZ Oven to 40°C.
2. Place a wet humidifying paper in the Humidity Control Tray, leaving the HybEZ Slide Rack on the bench. Insert the covered tray into the oven and close the oven door. The tray should be pre-warmed for at least **30 MIN** before use. Keep tray warm during the assay.

Post-primary Fixation

1. Following primary antibody incubation, wash slides in PBS-T for **2 MIN**. Repeat twice with fresh PBS-T each time.
2. In a fume hood, place slides in a Tissue-Tek Slide Rack and submerge in 10% Neutral Buffered Formalin (NBF) for **30 MIN** at **RT**.
3. In a fume hood, wash slides in PBS-T for **2 MIN**. Repeat wash with fresh PBS-T buffer.
4. Wash slides in PBST for an additional **2 MIN**. Repeat wash with fresh PBS-T buffer.

IMPORTANT! Formalin that has been stored for more than six months or opened for more than one month can result in suboptimal fixation.

Apply Protease

1. Place slides in the HybEZ Slide Rack and add 2–4 drops of RNAscope Protease III to each section. Use enough solution to cover each tissue section completely.

IMPORTANT! Be sure to use the correct protease for each assay. Using the incorrect protease can result in suboptimal miRNA or protein detection.

2. Place the HybEZ Slide Rack in the pre-warmed HybEZ Humidity Control Tray. Seal tray and insert back into the HybEZ Oven.
3. Incubate at 40°C for the amount of time specified by the table in Appendix A. Tissue Pretreatment Recommendation on page **Error! Bookmark not defined.** of the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM).

Note: Prepare miRNAscope HD Reagent Red assay materials during this incubation.

4. Place slides in a Tissue-Tek Slide Rack submerged in distilled water.
5. Wash the slides by moving the rack up and down 3–5 times. Repeat with fresh distilled water.

Part 3: In Situ Hybridization

To run the miRNAscope assay, follow the instructions in Chapter 4 of the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM) (Cat. No. 324510-USM).

IMPORTANT! After developing the RED solution in the miRNAscope assay, proceed directly to Part 4: Immunohistochemistry Part B. Do not counterstain your sample until after completing the IHC procedure.

Part 4: Perform Immunohistochemistry Part B

Tissue Blocking

1. Wash slides in 1X Wash Buffer for **2 MIN**. Repeat with fresh Wash Buffer.
2. Apply Co-Detection Blocker to the sections and incubate for **15 MIN** at **40°C**.
3. Wash slides in 1X Wash Buffer for **2 MIN**. Repeat with fresh Wash Buffer.
4. Wash slides with PBS-T for **2 MIN**.

Secondary Antibody Staining

1. Add secondary antibody diluted in Co-Detection Antibody Diluent to the sections and incubate for **30–60 MIN** at **RT**. Use enough solution to completely cover the sections.
2. Wash slides with PBS-T for **2 MIN**. Repeat with fresh PBS-T.

Detect the IHC Signal and Counterstain Using Green Chromogen

1. Wash slides in 1X Wash Buffer for **2 MIN**.
2. Prepare 200 µl of working Green solution per slide using a 1:50 ratio of Green-B to Green-A. Mix well. Combine reagents immediately before use.

IMPORTANT! Use the Green working solution within **5 MIN** of mixing. Do not expose to direct sunlight or UV light.

3. Remove excess liquid from slides and pipette ~200 µl Green solution onto each tissue section, covering each section entirely.
4. Incubate in sealed tray containing HybEZ slide rack for **15–30 MIN** at **RT**.
5. Remove solution and insert slides into a Tissue-Tek Slide Rack. Submerge slides in a Tissue-Tek Staining Dish filled with distilled water.
6. Quickly wash slides with fresh distilled water for no longer than **30 SEC**.

IMPORTANT! Work quickly as Green signal may fade when left in water or hematoxylin for longer than 30 seconds.

7. Place slides in 50% Gill's Hematoxylin I for **30 SEC** at **RT**.
8. *Immediately* transfer the slide rack into a staining dish filled with tap water. Do not let the slides remain in the water for more than 30 seconds. Briefly repeat tap water rinse once or twice.
9. Wash slides **10 SEC** in 0.02% Ammonia water.
10. Transfer the slide rack into a staining dish filled with tap water. Do not let the slides remain in the water for more than 30 seconds. Briefly repeat tap water rinse once or twice.

IMPORTANT! Use only 0.02% Ammonia water for the bluing step. Commercial bluing solutions may degrade the green signal.

Dry and Mount the Slides

1. Dry slides in a **60°C** oven for **30 MIN.**
2. Cool the slides for **5 MIN** at **RT**.
3. Dip the slides into fresh pure xylene and immediately place 1-2 drops of EcoMount on the slide before the xylene dries. Place a coverslip over the section.
4. Air dry for **5 MIN**.

Evaluate the Results

Examine tissues under a standard bright field microscope. The miRNAscope assay should produce clear and intense, red, punctate dots. Puncta can fill a large portion of the cytoplasm when a robust signal is detected.

Obtaining Support

For the latest services and support information, go to: https://acdbio.com/technical-support/support-overview.

At the website, you can:

- Access telephone and fax numbers to contact Technical Support and Sales.
- Search through FAQs.
- Submit a question directly to Technical Support.

Appendix A. Integrated Co-Detection Troubleshooting Guide

To optimize protein detection, we recommend titrating antibody concentration within the co-detection workflow. You may want to use a higher primary antibody concentration than you would normally use for IHC alone.

If non-specific IHC staining is observed, consider adding a pre-secondary blocking step. For pre-secondary blocking, cover tissue sections with co-detection antibody diluent for **1 HR** at **RT**. After blocking, remove excess liquid but do not rinse tissues. Proceed with secondary antibody staining.

The post-primary fixation and pretreatment conditions in this Tech Note provide optimal miRNA and protein detection across most tissue samples. If you need to optimize the protocol for a specific sample or target of interest, you can adjust the following parameters:

Reagent	Incubation Temp.	Recommended Incubation Time	Optimization Range
Target Retrieval	40°C	15 MIN	15-30 MIN
Primary Antibody	4°C	OVERNIGHT	60–120 MIN at RT or OVERNIGHT at 4°C
10% NBF	Ambient	30 MIN	15–60 MIN
Protease III	40°C	30 MIN	15–30 MIN
Protease IV	40°C	30 MIN	15–30 MIN*
Secondary Antibody	Ambient	30 MIN	15–60 MIN

* If stronger protease treatment is required, two 30 minute incubations may be performed. Fresh reagent must be applied prior to the second incubation.

Appendix B. Integrated Co-Detection for Fresh Frozen Tissue

Part 1: Prepare Tissue

To prepare fresh frozen tissue sections, follow the instructions in Chapter 3 of the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM). After preparing tissue sections, fix your samples using the following procedure.

Sample Fixation

1. In a fume hood, pre-chill 200 mL of 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) in 1X PBS to **4°C**.
2. Remove fresh frozen tissue slides from **–80°C**. In a fume hood, immediately immerse the slides in the pre-chilled 10% NBF or 4% PFA.
3. Incubate the slides for at least **30–60 MIN** at **4°C**.

Dehydrate the Tissue

1. Prepare 200 mL 50% EtOH, 200 mL 70% EtOH, and 400 mL 100% EtOH.

2. Remove the slides from NBF or PFA. Immerse in 50% EtOH. Incubate for **5 MIN** at **ROOM TEMPERATURE (RT)**.
3. Remove the slides from 50% EtOH. Immerse in 70% EtOH. Incubate for **5 MIN** at **RT**.
4. Remove the slides from 70% EtOH. Immerse in 100% EtOH. Incubate for **5 MIN** at **RT**.
5. Remove the slides from 100% EtOH. Immerse in fresh 100% EtOH. Incubate for **5 MIN** at **RT**.

Create Barrier

1. Remove slides from 100% EtOH. Leave slides for **5 MIN** at **RT**.
2. Draw 2–4 times around tissue using the ImmEdge hydrophobic barrier pen. Let the barrier dry **~30 SEC**.

Part 2: ICW Pretreatment and Immunohistochemistry Part A

Apply Hydrogen Peroxide

1. Add 2–4 drops of RNAscope Hydrogen Peroxide to each section for **10 MIN** at **RT**. Use enough solution to completely cover the sections.
2. Place slides in a Tissue-Tek Slide Rack and wash twice with distilled water.

Prepare Materials

1. Prepare working dilution of primary antibody using Co-Detection Antibody Diluent.

IMPORTANT! You must use antibodies diluted in Co-Detection Antibody Diluent (Cat. No. 323160). Standard antibody diluents negatively impact miRNA signal and are not recommended for use with this workflow. Co-Detection Antibody Diluent is formulated for maximum compatibility with the miRNAscope assays.

Note: For optimal protein detection, you may want to use a higher primary antibody concentration than you would normally use for IHC alone.

Apply Primary Antibody

1. Place slides in the HybEZ Slide Rack and add 150–200 µl of primary antibody diluted in Co-detection Antibody Diluent to each section. Use enough solution to cover each tissue section completely.
2. Place the HybEZ Slide Rack in the Humidity Control Tray lined with damp humidifying paper and incubate at **4°C OVERNIGHT**.

Post-primary Fixation

1. Following primary antibody incubation, wash slides in PBS-T for **2 MIN**. Repeat twice with fresh PBS-T each time.
2. In a fume hood, place slides in a Tissue-Tek Slide Rack and submerge in 10% Neutral Buffered Formalin (NBF) for **30 MIN** at **RT**.
3. In a fume hood, wash slides in PBS-T for **2 MIN**. Repeat with fresh PBS-T.
4. Wash slides in PBST for an additional **2 MIN**. Repeat with fresh PBS-T.

IMPORTANT! Formalin that has been stored for more than six months or opened for more than one month can result in suboptimal fixation.

Apply Protease

1. Place slides in the HybEZ Slide Rack. Add 2–4 drops of RNAscope Protease IV to each section. Use enough solution to cover each tissue section completely. Incubate for **30 MIN** at **RT**.

IMPORTANT! If over-digestion is observed, first reduce the protease digestion time. Otherwise, use RNAscope Protease III instead of RNAscope Protease IV.

Note: Prepare miRNAscope Reagent RED assay materials during this incubation.

2. Place slides in a Tissue-Tek Slide Rack submerged in distilled water
3. Wash the slides by moving the rack up and down 3–5 times. Repeat with fresh 1XPBS.

Immediately proceed to Part 3: In Situ Hybridization on page 4 and complete the remaining steps.

Appendix C. Integrated Co-Detection for Fixed Frozen Tissue

Part 1: Prepare Tissue

To prepare fixed frozen tissue sections, follow the instructions for *"Fixed frozen tissue sample preparation"* in Chapter 3 of the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM). After preparing tissue sections, fix your samples using the following procedure.

Sample Preparation

1. In a fume hood, pre-chill 200 mL of 10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA) in 1X PBS to **4°C**.
2. Remove fixed frozen tissue slides from **–80°C**. Wash the slides with 200 mL 1X PBS in a Tissue Tek slide rack for **5 MIN** while moving the rack up and down to remove Optimal Cutting Temperature (OCT) embedding media.
3. Bake the slides for **1 HR** at **60°C**.
4. In a fume hood, post-fix the slides by immersing them in the pre-chilled 10% NBF or 4% PFA in 1X PBS for **15 MIN** at **4°C**.

Dehydrate the Tissue

1. Prepare 200 mL 50% EtOH, 200 mL 70% EtOH, and 400 mL 100% EtOH.
2. Remove the slides from NBF or PFA. Immerse in 50% EtOH. Incubate for **5 MIN** at **ROOM TEMPERATURE (RT)**.
3. Remove the slides from 50% EtOH. Immerse in 70% EtOH. Incubate for **5 MIN** at **RT**.
4. Remove the slides from 70% EtOH. Immerse in 100% EtOH. Incubate for **5 MIN** at **RT**.
5. Remove the slides from 100% EtOH. Immerse in fresh 100% EtOH. Incubate for **5 MIN** at **RT**.
6. Remove slides from EtOH and let air dry for **5 MIN** to **OVERNIGHT** at **RT**.

Post-fix Samples

For optimal performance of the miRNAscope Assay, post-fix with either 10% NBF or 12% formaldehyde by choosing one of the following procedures:

Post-fixation with NBF

1. In a fume hood, fill a Tissue-Tek Clearing Agent dish with ~200 mL fresh 10% NBF.
2. Place slides in a Tissue-Tek Slide Rack and submerge them in the 10% NBF.
3. Incubate slides **OVERNIGHT** (16-18 HRS) at **RT**.
4. Remove slides from the 10% NBF and wash them for **2 MIN** in distilled water.
5. Dry slides for **5 MIN** at **60°C** or until completely dry.

Post-fixation with Formaldehyde

1. In a fume hood, prepare 12% formaldehyde in a Tissue-Tek Clearing Agent dish by combining 65 mL fresh 37% formaldehyde with 135 mL of 1X PBS.
2. Place slides in a Tissue-Tek Slide Rack and submerge them in the 12% formaldehyde.
3. Incubate slides for **2 HRS** at **RT**.
4. Remove slides from the 12% formaldehyde and wash them for **2 MIN** in distilled water.
5. Dry slides for **5 MIN** at **60°C** or until completely dry.

Part 2: ICW Pretreatment and Immunohistochemistry Part A

Prepare Materials – Day 1

1. Prepare 700 mL fresh 1X Co-Detection Target Retrieval in a beaker. Cover with foil and bring temperature to **98–102°C** using a hotplate. Maintain this temperature.
2. Place a wet humidifying paper in the Humidity Control Tray, leaving the HybEZ Slide Rack on bench.
3. Prepare working dilution of primary antibody using Co-Detection Antibody Diluent.

IMPORTANT! It is important to use antibodies diluted in Co-Detection Antibody Diluent (Cat. No. 323160). Standard antibody diluents negatively impact miRNA signal and are not recommended for use with this workflow. Co-Detection Antibody Diluent is formulated for maximum compatibility with the miRNAscope assay.

Note: For optimal protein detection, you may want to use a higher primary antibody concentration than you would normally use for IHC alone.

Note: You can also use the steamer method for target retrieval. For details on target retrieval methods, please refer to Chapter 3 of the *miRNAscope HD (RED) Assay with Sample Preparation and Pretreatment User Manual* (Cat. No. 324510-USM). **Make sure to use the1X Co-Detection Target Retrieval.**

Apply Hydrogen Peroxide

1. Add 2–4 drops of RNAscope Hydrogen Peroxide to each section for **10 MIN** at **RT**. Use enough solution to completely cover the sections.
2. Place slides in a Tissue-Tek Slide Rack and wash twice with distilled water.

Apply Target Retrieval

1. With a pair of forceps *very slowly* submerge the slide rack into the hot 1X Co-Detection Target Retrieval solution for **5 MIN**.

Note: Depending on tissue type, boiling time may need to be adjusted.

Note: Maintain temperature at **98–102°C** for the duration of target retrieval.

2. *Immediately* transfer the hot slide rack to a staining dish containing distilled water.
3. Wash slides in the distilled water by moving the rack up and down 3–5 times and repeat with fresh distilled water.
4. Wash slides in 1X PBS-T by moving the rack up and down 3–5 times.

Create Barrier

1. Working quickly to prevent the sample from drying out, use a tissue to carefully dry the glass surrounding your sample. Draw 2–4 times around the section using the ImmEdge hydrophobic barrier pen. Let the barrier dry **~30 SEC**.

IMPORTANT! Do not allow the sample to dry out completely as this may negatively impact IHC signal.

Apply Primary Antibody

1. Place slides in the HybEZ Slide Rack and add 150-200 µl of primary antibody diluted in Co-Detection Antibody Diluent to each section. Use enough solution to cover each tissue section completely.
2. Place the HybEZ Slide Rack in the Humidity Control Tray and incubate at **4°C OVERNIGHT**.

Prepare Materials

1. Bring HybEZ Oven to **40°C**.
2. Place a wet humidifying paper in the Humidity Control Tray, leaving the HybEZ Slide Rack on bench. Insert the covered tray into the oven and close the oven door. The tray should be pre-warmed for at least **30 MIN** before use. Keep tray warm during the assay.

Post-primary fixation

1. Following primary antibody incubation, wash slides in PBS-T for **2 MIN**. Repeat twice with fresh PBS-T each time.
2. In a fume hood, place slides in a Tissue-Tek Slide Rack and submerge in 10% Neutral Buffered Formalin (NBF) for **30 MIN** at **RT**.
3. In a fume hood, wash slides in PBS-T for **2 MIN**. Repeat with fresh PBS-T.
4. Wash slides in PBS-T for an additional **2 MIN**. Repeat with fresh PBS-T.

IMPORTANT! Formalin that has been stored for more than six months or opened for more than one month can result in suboptimal fixation.

Apply Protease

1. Place slides in the HybEZ Slide Rack. For miRNAscope, add 2–4 drops of RNAscope Protease III to each section. Use enough solution to completely cover each tissue section.
2. Place the HybEZSlide Rack in the pre-warmed HybEZ Humidity Control Tray. Seal tray and insert back into the HybEZ Oven.

NOTE: Depending on tissue type and IHC target, you may need to adjust protease treatment strength and/or time.

NOTE: Prepare miRNAscope HD Reagent RED assay materials during this incubation.

3. Place slides in a Tissue-Tek Slide Rack submerged in distilled water
4. Wash the slides by moving the rack up and down 3–5 times. Repeat with fresh distilled water.

Immediately proceed to Part 3: In Situ Hybridization on page 4 and complete the remaining steps.

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