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BaseScope™ Detection Reagent Kit v2 – RED User Manual

With FFPE Sample Preparation and Pretreatment

Document Number 323900-USM

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Contents

1 Chapter 1. Product Information

Before using this product, read and understand the safety information in **Appendix D. Safety** on page 29.

IMPORTANT! We recommend reading the entire user manual before beginning any protocols.

About this guide

This user manual provides guidelines and protocols to use the BaseScope™ Detection Reagent Kit v2 – RED (Cat. No. 323900) on properly prepared formalin-fixed, paraffin-embedded (FFPE) tissues mounted on slides. BaseScope™ v2 Assays are also compatible with fresh frozen and fixed frozen tissues.

Product description

Background

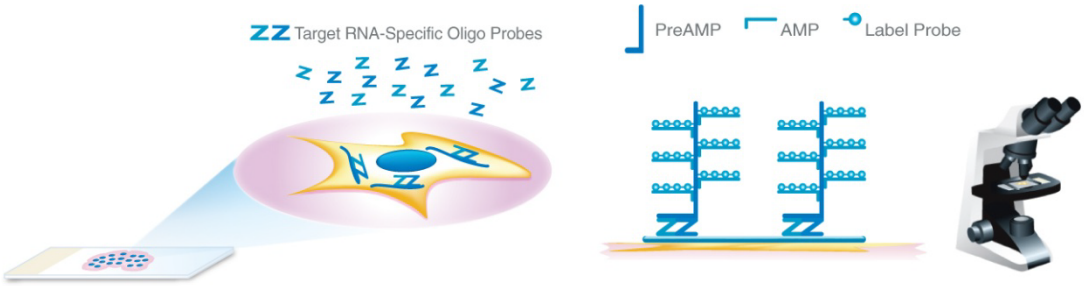
The BaseScope™ v2 Assays use a novel and proprietary method of *in situ* hybridization (ISH) to visualize single RNA molecules for splice variants and short targets in slide-mounted samples. BaseScope™ v2 Assays do not require the RNA-free environment used for traditional ISH. The assays are based on ACD's patented signal amplification and background suppression technology. Compared with the RNAscope® 2.5 Assay, the BaseScope™ v2 Assay incorporates an additional signal amplification step, which makes it possible to detect RNA splicing variants, point mutations, small insertions or deletions, and short RNA targets (50–300 nucleotides).

Overview

Figure 1 on page 6 illustrates the BaseScope™ v2 Assay procedure. The procedure can be completed in eight to nine hours or conveniently divided over two days. Most of the BaseScope™ v2 Assay reagents are available in convenient Ready-To-Use (RTU) dropper bottles and provide a simple, nearly pipette-free workflow.

Starting with properly prepared tissue samples, sections mounted on glass slides are first pretreated, and then RNA-specific probes are hybridized to target RNA. The signal is amplified using a multi-step process, and detected using a red chromogenic substrate. Each single RNA transcript appears as a distinct dot of chromogen precipitate visible using a common bright field microscope at 40–100X magnification.

Figure 1. Procedure overview



1: Tissue section	2: Hybridize to target RNA	3: Amplify signal	4: Image
Start with properly prepared sections and pretreat to allow access to target RNA.	Hybridize gene-specific probe pairs to the target mRNA.	Probes are hybridized to a cascade of signal amplification molecules. The BaseScope™ Assay enhances signal further with additional amplification steps. Add Fast Red substrate to detect target RNA.	Visualize target RNA using a standard bright field microscope.

Kit contents and storage

The BaseScope™ v2 Assay requires the BaseScope™ Probes and the BaseScope™ Detection Reagent Kit v2. Probes and Reagent Kits are available separately.

IMPORTANT! BaseScope™ Probes must be used with the BaseScope™ v2 Detection Reagent Kit. RNAscope® probes are incompatible with the BaseScope™ Detection Reagent Kit v2.

BaseScope™ Probes

The BaseScope™ Probes consist of the user-specified Target Probe and the Positive and Negative Control Probes.

Each bottle contains enough probe to stain ~20 sections with areas of approximately 20 mm x 20 mm (0.75" x 0.75") each. Larger tissue sections will result in fewer tests. The probes have a shelf life of two years from the date of manufacturing when stored as indicated in the following table:

Target Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	BaseScope™ Target Probe – *[species]*–*[gene]*	Various	Probe targeting specific RNA	3 mL x 1 bottle	2–8°C
Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	BaseScope™ Positive Control Probe-Human (Hs)-PPIB-3ZZ	701031	Probe targeting common housekeeping gene	3 mL x 1 bottle	2–8°C
	BaseScope™ Positive Control Probe-Mouse (Mm)-PPIB-3ZZ	701071	Probe targeting common housekeeping gene	3 mL x 1 bottle	2–8°C
	BaseScope™ Positive Control Probe-Human (Hs)-PPIB-1ZZ	701041	Probe targeting common housekeeping gene	3 mL x 1 bottle	2–8°C
	BaseScope™ Positive Control Probe-Mouse (Mm)-PPIB-1ZZ	701081	Probe targeting common housekeeping gene	3 mL x 1 bottle	2–8°C

Control Probes					
☑	Reagent	Cat. No.	Content	Quantity	Storage
	BaseScope™ Negative Control Probe-DapB-3ZZ	701011	Probe targeting bacterial gene dapB	3 mL x 1 bottle	2–8°C
	BaseScope™ Negative Control Probe-DapB-1ZZ	701021	Probe targeting bacterial gene dapB	3 mL x 1 bottle	2–8°C

IMPORTANT! When running the BaseScope™ v2 Assay, ensure that your control probes contain the same number of ZZ pairs as your target probe. If using more than 1 ZZ target probe, use the 3 ZZ control probes. Consult support at **support@acdbio.com** for additional information.

BaseScope™ Detection Reagent Kit v2– RED

Each BaseScope™ Detection Reagent Kit v2 – RED (Cat. No. 323900) provides enough reagents to stain ~20 tissue sections with areas of approximately 20 mm x 20 mm (0.75" x 0.75") each. Larger tissue sections will result in fewer tests. Each kit contains the following components: Pretreatment Reagents, Target Retrieval Reagents, Wash Buffer Reagents, and Detection Reagents.

The reagents have a shelf life of nine months from the date of manufacturing when stored as indicated in the following table:

Pretreatment Reagents (Cat. No. 322381 and 322000)			
☑	Reagent	Quantity	Storage
	RNAscope® Hydrogen Peroxide	4 mL x 2 bottles	2–8°C
	RNAscope® Protease Plus	4.5 mL x 2 bottles	2–8°C
	RNAscope® Protease III*	4.5 mL x 2 bottles	2–8°C
	RNAscope® Protease IV*	4.5 mL x 2 bottles	2–8°C
	RNAscope® 10X Target Retrieval	70 mL x 4 bottles	Room temp (15–30°C)
BaseScope™ Detection Reagents v2– RED (Cat. No. 323910)			
☑	Reagent	Quantity	Storage
	BaseScope™ v2 AMP 1	3 mL x 1 bottle	2–8°C
	BaseScope™ v2 AMP 2	3 mL x 1 bottle	2–8°C
	BaseScope™ v2 AMP 3	4.5 mL x 1 bottle	2–8°C
	BaseScope™ v2 AMP 4	3 mL x 1 bottle	2–8°C
	BaseScope™ v2 AMP 5	3 mL x 1 bottle	2–8°C
	BaseScope™ v2 AMP 6	4.5 mL x 1 bottle	2–8°C
	BaseScope™ v2 AMP 7	4.5 mL x 1 bottle	2–8°C
	BaseScope™ v2 AMP 8	3 mL x 1 bottle	2–8°C
	BaseScope™ Fast RED-A	3 mL x 1 bottle	2–8°C
	BaseScope™ Fast RED-B	50 µL x 1 vial	2–8°C
RNAscope® Wash Buffer Reagents (Cat. No. 310091)			
☑	Reagent	Quantity	Storage
	RNAscope® 50X Wash Buffer	60 mL x 4 bottles	Room temp (15–30°C)

* You can use RNAscope® Protease IV for all tissue types. Use RNAscope® Protease III with FFPE cell pellet samples.

IMPORTANT! ACD kits share some of the same reagents including Hydrogen Peroxide, Target Retrieval, Protease, and Wash Buffer. Only these reagents may be interchanged among kits. Do not interchange other reagents, even if they have the same name.

Required materials and equipment

The following materials and equipment are needed to perform the BaseScope™ v2 Assay.

HybEZ™ Hybridization System

IMPORTANT! The BaseScope™ v2 Assay has been verified using this system only.

Use the HybEZ™ Hybridization System to perform BaseScope™ v2 Assay hybridization and incubation steps. These steps require humid conditions to prevent sections from drying out.

For instructions on how to use the HybEZ™ Hybridization System, refer to the *HybEZ™ Hybridization System User Manual* available at **https://acdbio.com/documents/support-documents** and view the training video at **https://acdbio.com/technical-support/learn-more**. The system contains the following components:

☑	Component	Quantity	Cat. No.
	HybEZ™ Oven (110 or 220 VAC) or HybEZ™ II Oven (110 or 220V)	1 oven	310010 or 310013 (HybEZ™) 321710 or 321720 (HybEZ™ II)
	HybEZ™ Humidity Control Tray (with lid)	1 tray	310012
	ACD EZ-Batch™ Slide Rack (20 slide capacity)	1 rack	310017
	HybEZ™ Humidifying Paper	2 sheets	—

Note: To order HybEZ™ Humidifying Paper Pack, 15 sheets, use Cat. No. 310015.

User-supplied materials

IMPORTANT! Do not substitute other materials for the VectaMount or EcoMount listed in the following table.

☑	Description	Supplier	Cat. No.
	ImmEdge™ Hydrophobic Barrier Pen (required)	Vector Laboratory	H-4000
	SuperFrost® Plus Slides (required)	Fisher Scientific	12-550-15
	10% neutral-buffered formalin (NBF)	MLS*	—
	Paraffin wax	MLS	—
	Microtome	MLS	—
	Gill's Hematoxylin I	American Master Tech Scientific/MLS*	HXGHE1LT
	Xylene	Fisher Scientific/MLS	X3P-1GAL
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek® Staining Dishes	American Master Tech Scientific/MLS	LWT4457EA
	Tissue-Tek® Clearing Agent Dishes, xylene resistant	American Master Tech Scientific/MLS	LWT4456EA
	100% alcohol (EtOH)	American Master Tech Scientific/MLS	ALREACS
	VectaMount or EcoMount	Vector Labs Biocare Medical	H-5000 EM897

☑	Description	Supplier	Cat. No.
	Cover Glass, 24 x 50 mm	Fisher Scientific/MLS	12--545-F
	Ammonium hydroxide, 28–30% (1N)	Sigma-Aldrich/MLS	320145-500mL
	Carboy (>3L)	MLS	—
	Oster® Steamer Model 5712, Black and Decker Steamer HS3000, or the Braun Multiquick FS 20 Steamer	—	—
	Aluminum foil (Optional)†	MLS	—
	Forceps, large (Optional)†	MLS	—
	Hot plate (Optional)†	MLS	—
	Glass beaker 1 or 2 L (Optional)†	MLS	—
	Digital thermometer	MLS	—
	Water bath or incubator, capable of holding temperature at 40 +/– 1°C	MLS	—
	Pipettors and tips, 1–1000 µL	MLS	—
	Distilled water	MLS	—
	Tubes (various sizes)	MLS	—
	Fume hood	MLS	—
	Graduated cylinder	MLS	—
	Parafilm	MLS	—
	Paper towel or absorbent paper	MLS	—
	Microcentrifuge	MLS	—
	Microscope and accessories	MLS	—
	Drying oven, capable of holding temperature at 60 +/– 1°C	MLS	—

* Major Laboratory Supplier in North America. For other regions, please check Catalog Numbers with your local lab supplier.
† Required for the alternate target retrieval method in **Appendix B** on page 27.

2 Chapter 2. Before You Begin

Prior to running the BaseScope™ v2 Assay on your samples for the first time, we recommend that you:

- View the video demonstrations available at **https://acdbio.com/technical-support/learn-more**.
- Run the assay on FFPE BaseScope™ Control Slides (Cat. No. 310023) for Human control slide, HeLa; Catalog No. 310045 for Mouse control slide, 3T3) using positive and negative control probes (see the following information).
- Use the BaseScope™ Control Probes Pack (Human Cat. No. 322975 or Mouse Cat. No. 322976) to perform the assay on control slides.

IMPORTANT! When running the BaseScope™ v2 Assay, ensure that your control probes contain the same number of ZZ pairs as your target probe. If using more than 1 ZZ target probe, use the 3 ZZ control probes. Consult support at **support@acdbio.com** for additional information.

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Appendix A. Tissue Pretreatment Recommendation for FFPE Samples** on page 25 and to our sample preparation and pretreatment user guides available at **https:// acdbio.com/technical-support/user-manuals**.
- Use only samples mounted on SuperFrost Plus® Slides (Fisher Scientific, Cat. No. 12-550-15).
- Follow the recommended pretreatment guidelines for your sample. Refer to our sample preparation and pretreatment user guides available at **https:// acdbio.com/technical-support/user-manuals**.
- Always run positive and negative control probes on your sample to assess sample RNA quality and optimal permeabilization.
- Do *not* substitute required materials. Assay has been validated with these materials only.
- Follow the protocol exactly for best results.
- Do not let your sections dry out during the procedure.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix D. Safety** on page 29 for more information.

3 Chapter 3. Prepare and Pretreat Samples

The following protocols describe formalin-fixed, paraffin-embedded (FFPE) sample preparation and pretreatment. For other sample types and preparation methods, contact **support@acdbio.com** for the latest protocols and guidelines.

IMPORTANT! We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

For suboptimally treated samples, you may need to optimize pretreatment conditions. Refer to **Appendix A. Tissue Pretreatment Recommendation for FFPE Samples** on page 25 and to **https://acdbio.com/technical-support/solutions**.

Prepare FFPE sections

Materials required

- 10% neutral buffered formalin (NBF)
- 1X PBS
- Paraffin wax
- Tissue-Tek® Clearing Agent Dishes
- Tissue-Tek® Staining Dishes
- 100% alcohol (EtOH)
- Xylene
- Microtome
- Water bath
- SuperFrost® Plus slides

Fix the sample

1. Immediately following dissection, fix tissue in 10% NBF for **16–32 HRS** at **ROOM TEMPERATURE (RT)**. Fixation time will vary depending on tissue type and size.

⚠ CAUTION! Handle biological specimens appropriately.

IMPORTANT! Fixation for **<16 HRS** or **>32 HRS** will impair the performance of the BaseScope™ v2 Assay.

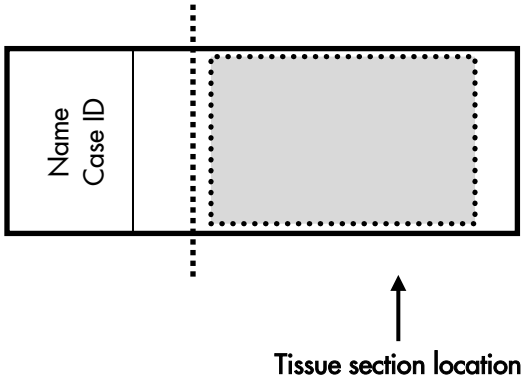
Dehydrate, embed, and cut the sample

IMPORTANT! Use fresh reagents.

1. Wash sample with 1X PBS.
2. Dehydrate sample using a standard ethanol series, followed by xylene.
3. Embed sample in paraffin using standard procedures.

Note: Embedded samples may be stored at room temperature with desiccants. To better preserve RNA quality over a long period (>1 yr), storing at 2–8°C with desiccants is recommended.

4. Trim paraffin blocks as needed, and cut embedded tissue into 5 +/– 1 µm sections using a microtome.
5. Place paraffin ribbon in a **40–45°C** water bath, and mount sections on **SUPERFROST® PLUS SLIDES**. For optimal staining, place tissue in the location indicated in the following figure:



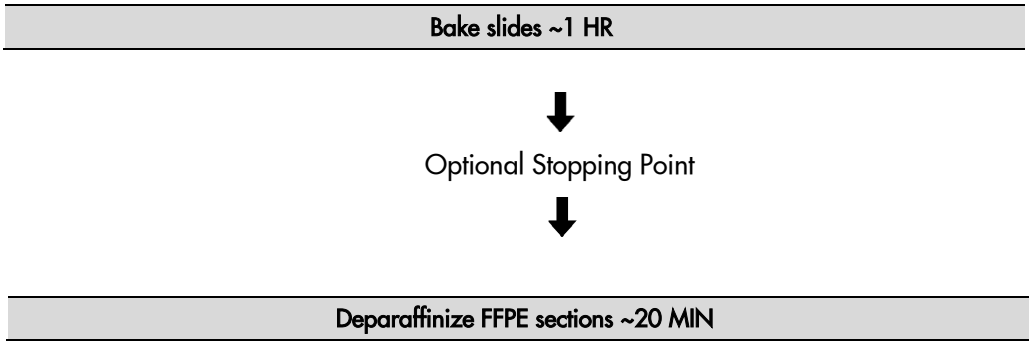
IMPORTANT! Do not mount more than one section per slide. Place sections in the center of the slide.

6. Air dry slides **OVERNIGHT** at **RT**.

OPTIONAL STOPPING POINT 1. Use sectioned tissue within three months. Store sections with desiccants at room temperature.

Prepare FFPE slides for the BaseScope™ v2 Assay

Workflow



Materials required

- Drying oven
- Prepared FFPE slides
- Tissue-Tek® Vertical 24 Slide Rack
- Distilled water
- Fume hood
- Xylene
- 100% alcohol (EtOH)
- Tissue-Tek® Clearing Agent Dishes
- Tissue-Tek® Staining Dishes

Bake slides

1. Bake slides in a dry oven for **1 HR** at **60°C**.

OPTIONAL STOPPING POINT 2. Use immediately, or store at **RT** with desiccants for ≤1 week. Prolonged storage may degrade sample RNA.

Deparaffinize FFPE sections

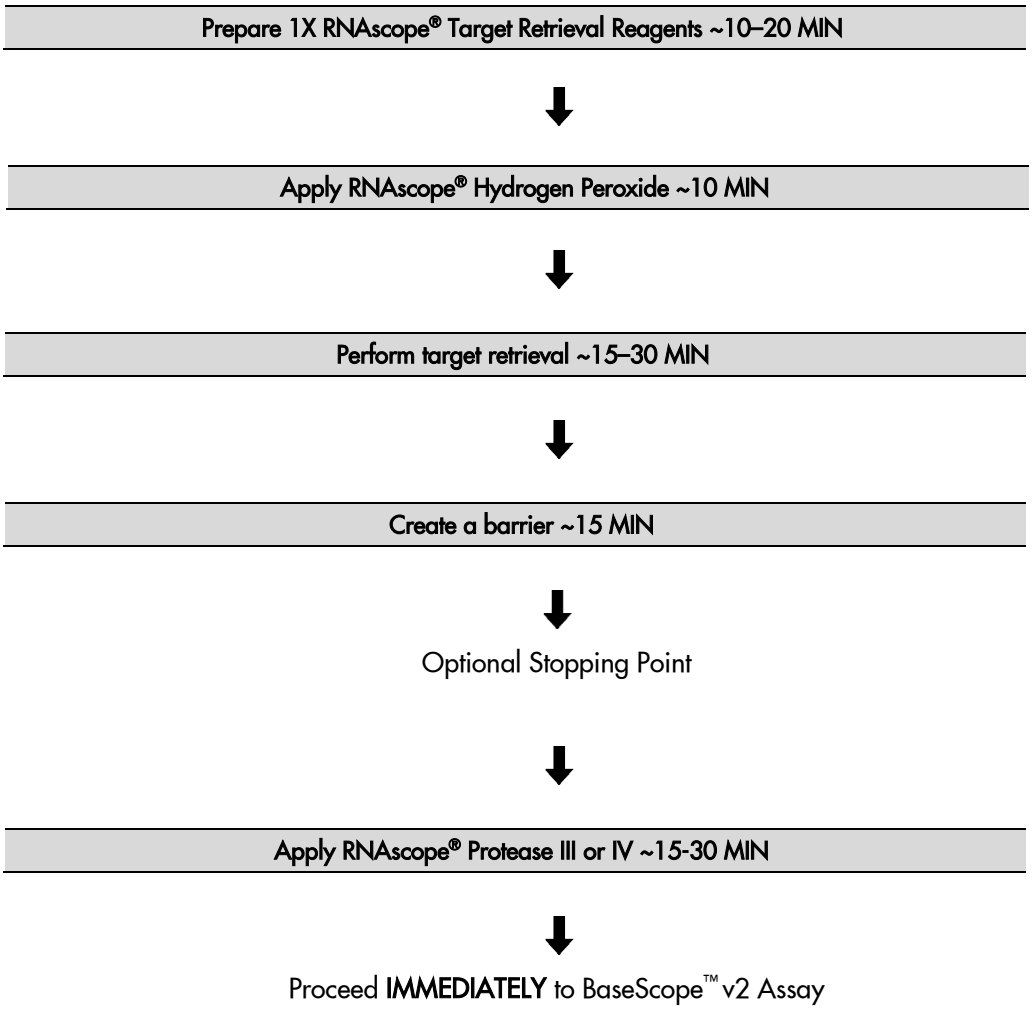
Reagents may be prepared ahead of time. Ensure all containers remain covered.

1. In a fume hood:
   - Fill two Tissue-Tek® Clearing Agent dishes with ~200 mL fresh xylene.
   - Fill two Tissue-Tek® Staining dishes with ~200 mL fresh 100% alcohol.
2. Place slides in a Tissue-Tek® Slide Rack and submerge in the first xylene-containing dish in the fume hood.
3. Incubate the slides in xylene for **5 MIN** at **RT**. Agitate the slides by occasionally lifting the slide rack up and down in the dish.
4. Remove the slide rack from the first xylene-containing dish, and *immediately* place in the second xylene-containing dish in the fume hood.
5. Incubate the slides in xylene for **5 MIN** at **RT** with agitation.
6. Remove the slide rack from the second xylene-containing dish, and *immediately* place in a dish containing 100% alcohol.
7. Incubate the slides in 100% alcohol for **2 MIN** at **RT** with agitation.
8. Remove the slide rack from the first alcohol-containing dish, and *immediately* place in the second alcohol-containing dish.
9. Incubate the slides in 100% alcohol for **2 MIN** at **RT** with agitation.
10. Remove the slides from the rack, and place on absorbent paper with the section face-up. Air dry slides for **5 MIN** at **60°C**, or until completely dry.

Pretreat samples

IMPORTANT! Before you begin, make sure you know the pretreatment conditions specific to your sample type from **Appendix A. Tissue Pretreatment Recommendation for FFPE Samples** on page 25.

Workflow



Materials required

Materials provided by the Universal Pretreatment Kit	Other Materials and Equipment
• RNAscope® Hydrogen Peroxide • RNAscope® Protease III or IV • RNAscope® 10X Target Retrieval Reagents	• Prepared slides • Distilled water • HybEZ™ Humidifying System/ACD EZ-Batch™ Slide Rack • Paper towel or absorbent paper • Steamer • Digital thermometer • Tissue Tek® Slide Rack • Tissue Tek® Staining Dishes • ImmEdge™ Hydrophobic Barrier Pen

Equilibrate equipment

1. Turn on HybEZ™ Oven and set temperature to **40°C**.
2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.
3. Insert covered tray into oven and close the oven door. Warm the tray for **30 MIN** at **40°C** before use. Keep the tray in the oven when not in use.

Prepare 1X RNAscope® Target Retrieval Reagents

- Prepare 250 mL of fresh RNAscope® 1X Target Retrieval Reagents by adding 225 mL distilled water to 25 mL 10X Target Retrieval Reagents. Mix well.

Apply RNAscope® Hydrogen Peroxide

1. Lay deparaffinized slides on the bench and add ~5–8 drops of RNAscope® Hydrogen Peroxide to cover the entire section.
2. Incubate slides for **10 MIN** at **RT**.
3. Remove RNAscope® Hydrogen Peroxide solution from one slide at a time by tapping and/or flicking the slide on absorbent paper. Immediately insert the slide into a Tissue-Tek® Slide Rack submerged in a Tissue-Tek® Staining Dish filled with distilled water.
4. Wash slides 3–5 times by moving the Tissue-Tek® Slide Rack up and down in the distilled water.
5. Repeat the wash step with fresh distilled water.

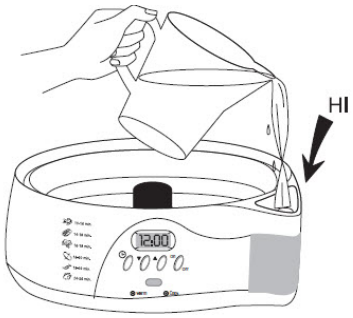
Perform target retrieval

We highly recommend using an Oster® Steamer for target retrieval. For an alternate method, see **Appendix B. Manual Target Retrieval** on page 27.

Note: You may also steam with the Braun Multiquick FS 20 Steamer or the Black and Decker Steamer HS3000. To use the Braun Multiquick FS 20 Steamer, fill the water to the maximum level before starting and do not refill water during the steaming process.

1. Fill the water reservoir with cold tap water to the "HI" marking line.

IMPORTANT! Do not overfill.

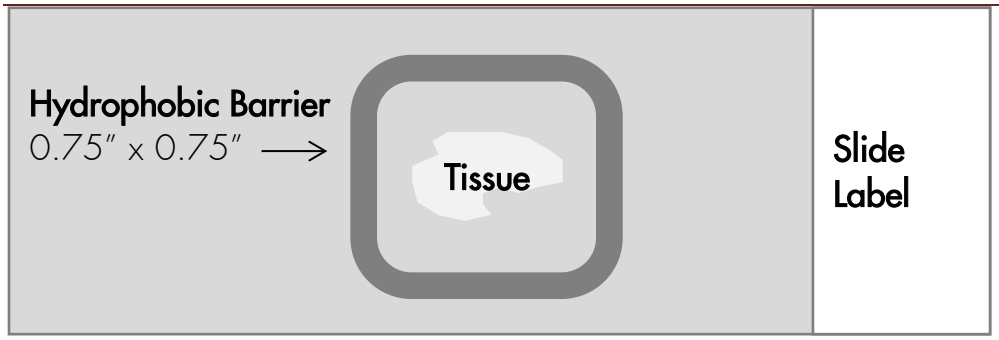


2. Place a clear Steaming Bowl onto the base.
3. Place two slide holders in the steam bowl. Fill one slide holder with 200 mL of RNAscope® 1X Target Retrieval Reagent. Fill the other slide holder with 200 mL of distilled H_20.
4. Turn on the Steamer. Set the steamer timer by turning the black knob clockwise. Set heating time to **95 MIN**.
5. Insert a digital thermometer through the holes of the lid and into the container containing RNAscope® 1X Target Retrieval Reagent. Allow temperature to rise to at least **99°C**.
6. Add slides to the container containing distilled H_20 for 10 seconds to acclimate slides.
7. Remove slides and move them to the container containing RNAscope® 1X Target Retrieval Reagent. Cover the Steamer with lid.
8. Start timer for **15 MIN** for mild and standard conditions, and **30 MIN** for extended pretreatment. For pretreatment times, consult **Appendix A. Tissue Pretreatment Recommendation for FFPE Samples** on page 25.
9. Remove slides from steamer and transfer to a separate rinse container with 200 mL of distilled water. Allow slides to rinse for **15 SEC**.
10. Transfer slides to 100% alcohol for **3 MIN**.
11. Dry slides in **60°C** incubator for **5 MIN**, or at room temperature.

Create a barrier

1. Use the following template to draw a barrier 2–4 times around each section with the Immedge™ hydrophobic barrier pen.

IMPORTANT! Do not let the barrier touch the tissue section. An Immedge™ hydrophobic barrier pen is highly recommended. An alternative type of pen may result in suboptimal results.



Note: We do not recommend drawing a smaller barrier and using less than the recommended volume amounts, even for smaller sections. Larger barriers will result in fewer tests per kit.

2. Let the barrier dry completely **~1 MIN** or **OVERNIGHT** at **RT**.

Note: If you need to reapply the hydrophobic barrier during the following procedures, dry the appropriate area of the slide with a kimwipe. Do not touch the tissue section.

OPTIONAL STOPPING POINT 3. Dry slides overnight for use the following day, or proceed directly to the next section.

Apply RNAscope® Protease IV (tissue) or Protease III (cell pellets or under-fixed tissue)

1. Place dried slides on the HybEZ™ or ACD EZ-Batch™ Slide Rack, and add ~5 drops of RNAscope® Protease IV or III to entirely cover each section.
2. Remove the HybEZ™ Humidity Control Tray from the HybEZ™ Oven and place the slide rack in the tray. Close the lid, seal, and insert tray back into the oven.
3. Incubate at **40°C** for the amount of time specified by the table in **Appendix A. Tissue Pretreatment Recommendation for FFPE Samples** on page 25.

Note: You may preparethe BaseScope™ v2 Assay materials during this step. See **Prepare the materials** on page 19.

4. Remove the HybEZ™ Humidity Control Tray from the oven. Remove the slide rack from the tray. Place tray back into the oven.
5. Take each slide one at a time from the slide rack and tap/and or flick to remove the excess liquid. Immediately place each slide in a Tissue-Tek® Slide Rack submerged in a Tissue-Tek® Staining Dish filled with distilled water.
6. Wash slides 3–5 times by moving the Tissue-Tek® Slide Rack up and down in the distilled water.
7. Proceed *immediately* to the next chapter.

4 Chapter 4. BaseScope™ v2 Assay

This procedure flows directly from sample preparation and pretreatment. Refer to **Chapter 3. Prepare and Pretreat Samples** on page 11, or the appropriate sample preparation and pretreatment user manual for your specific sample type.

Workflow

Prepare the materials ~10–30 MIN

↓

Run the assay ~6HRS

Hybridize probe ~2 HRS

↓

Hybridize BaseScope™ v2 Amp 1 ~30 MIN

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Hybridize BaseScope™ v2 Amp 2 ~30 MIN

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Hybridize BaseScope™ v2 Amp 3 ~15 MIN

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Hybridize BaseScope™ v2 Amp 4 ~30 MIN

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Hybridize BaseScope™ v2 Amp 5 ~30 MIN

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Hybridize BaseScope™ v2 Amp 6 ~15 MIN

↓

Hybridize BaseScope™ v2 Amp 7 ~30 MIN

↓

Hybridize BaseScope™ v2 Amp 8 ~15 MIN

↓

Detect the signal ~10 MIN

↓

Counterstain the slides ~2 MIN

↓

Mount samples ~5 MIN

↓

Evaluate the samples

Materials required for the assay

Materials provided by BaseScope™ Detection Reagent Kit v2 – RED	Materials provided by BaseScope™ Probes	Other Materials and Equipment
• RNAscope® 50X Wash Buffer • BaseScope™ v2 AMP 1 • BaseScope™ v2 AMP 2 • BaseScope™ v2 AMP 3 • BaseScope™ v2 AMP 4 • BaseScope™ v2 AMP 5 • BaseScope™ v2 AMP 6 • BaseScope™ v2 AMP 7 • BaseScope™ v2 AMP 8 • BaseScope™ Fast RED-A • BaseScope™ Fast RED-B	• Target Probe • Positive Control Probe • Negative Control Probe	• Prepared sections • Distilled water • Carboy (>3L) • Fume hood • Xylene • Tissue-Tek® Staining Dishes • Tissue-Tek® Clearing Agent Dish, xylene-resistant • Gill's Hematoxylin • Ammonium hydroxide, 28–30% • Graduated cylinder • Parafilm • HybEZ™ Humidifying System/ACD EZ-Batch™ Slide Rack • Water bath or incubator • Tissue-Tek® Vertical 24 Slide Rack • Tubes (various sizes) • Paper towel or absorbent paper • Pipettors and tips, 1–1000 µL • Dry oven • VectaMount • Cover Glass, 24 mm x 50 mm

Prepare the materials

You may prepare the reagents at the same time you prepare pretreatment reagents. See **Pretreat samples** on page 14.

Some of the materials may be prepared in advance and stored at room temperature.

Prepare 1X Wash Buffer

Note: Warm RNAscope™ 50X Wash Buffer up to **40°C** for **10–20 MIN** before preparation. 1X Wash Buffer may be prepared ahead of time and stored at room temperature for up to one month.

- Prepare 3 L of 1X Wash Buffer by adding 2.94 L distilled water and 1 bottle (60 mL) of RNAscope™ Wash Buffer (50X) to a large carboy. Mix well.

Prepare counterstaining reagents

1. In the fume hood, prepare 50% Hematoxylin staining solution by adding 100 mL Gill's Hematoxylin I to 100 mL distilled water in a staining dish.

Note: 50% Hematoxylin staining solution can be reused for up to 1 week.

2. In the fume hood, prepare 0.02% (w/v) Ammonia water (bluing reagent) by adding 1.43 mL of 1N Ammonium Hydroxide to 250 mL distilled water in a graduated cylinder or other container.
3. Seal the cylinder with parafilm. Mix well 3–5 times.

Note: For assay quantitation, it is critical to use Ammonium Hydroxide.

Equilibrate reagents

1. Remove AMP 1–8 reagents from refrigerator and place at **RT**.
2. Ensure HybEZ™ Oven and prepared Humidity Control Tray are at **40°C**.
3. Before each use, equilibrate the probes for **30 MIN** at **RT**.

Run the assay

IMPORTANT! Do **NOT** let sections dry out between incubation steps. Work *quickly* and fill barrier with solutions.

IMPORTANT! View the wash step video at **http://www.acdbio.com/technical-support/learn-more** before proceeding.

Hybridize probe

1. Tap and/or flick to remove excess liquid from slides, and place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack located in the HybEZ™ Humidity Control Tray.
2. Add ~4 drops of the appropriate probe to entirely cover each section.

Note: Refer to **Appendix C. Reagent Volume Guidelines** on page 28 to determine the recommended number of drops needed per slide. For example, for a 0.75" x 0.75" barrier add 4 drops of the appropriate probe.

3. Cover the HybEZ™ Humidity Control Tray with lid and insert into the oven for **2 HRS** at **40°C**.

IMPORTANT! To prevent evaporation, make sure the turn nob is completely turned to lock position.

4. Remove the tray from the oven and remove the slide rack.
5. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT**. Agitate slides by moving the slide rack up and down in the dish.
7. Repeat Step 6 with fresh 1X Wash Buffer.

Hybridize BaseScope™ v2 AMP 1

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid from slides.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 1 to entirely cover each section.
3. Close tray and insert into the oven for **30 MIN** at **40°C**.
4. Remove the tray from the oven and remove the slide rack.
5. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
7. Repeat Step 6 with fresh 1X Wash Buffer.

Hybridize BaseScope™ v2 AMP 2

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid from slides.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 2 to entirely cover each section.
3. Close tray and insert into the oven for **30 MIN** at **40°C**.
4. Remove the tray from the oven and remove the slide rack.
5. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
7. Repeat Step 6 with fresh 1X Wash Buffer.

Hybridize BaseScope™ v2 AMP 3

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid from slides.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 3 to entirely cover each section.
3. Close tray and insert into the oven for **15 MIN** at **40°C**.
4. Remove the tray from the oven and remove the slide rack.
5. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
7. Repeat Step 6 with fresh 1X Wash Buffer.

Hybridize BaseScope™ v2 AMP 4

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid from slides.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 4 to entirely cover each section.
3. Close tray and insert into the oven for **30 MIN** at **40°C**.
4. Remove the tray from the oven and remove the slide rack.
5. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
7. Repeat Step 6 with fresh 1X Wash Buffer.

Hybridize BaseScope™ v2 AMP 5

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid from slides.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 5 to entirely cover each section.
3. Close tray and insert into the oven for **30 MIN** at **40°C**.
4. Remove the tray from the oven and remove the slide rack.
5. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
7. Repeat Step 6 with fresh 1X Wash Buffer.

Hybridize BaseScope™ v2 AMP 6

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid from slides.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 6 to entirely cover each section.
3. Close tray and insert into the oven for **15 MIN** at **40°C**.
4. Remove the tray from the oven and remove the slide rack.

IMPORTANT! Do not insert tray into the HybEZ™ Oven for the rest of the procedure.

5. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
6. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
7. Repeat Step 6 with fresh 1X Wash Buffer.

Hybridize BaseScope™ v2 AMP 7

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 7 to entirely cover each section.
3. Seal tray and incubate for **30 MIN** at **RT**.
4. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
6. Repeat Step 6 with fresh 1X Wash Buffer.

IMPORTANT! Staining intensity can be modified by adjusting the AMP 7 incubation time.

Hybridize BaseScope™ v2 AMP 8

1. Take each slide one at a time from the Tissue-Tek® Slide Rack, and tap and/or flick to remove the excess liquid.
2. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray, and add ~4 drops of AMP 8 to entirely cover each section.
3. Close tray and incubate for **15 MIN** at **RT**.
4. One slide at a time, *quickly* remove excess liquid and place slide in a Tissue-Tek® Slide Rack submerged in the Tissue-Tek® Staining Dish filled with 1X Wash Buffer.
5. Wash slides in 1X Wash Buffer for **2 MIN** at **RT** with occasional agitation.
6. Repeat Step 6 with fresh 1X Wash Buffer.

Detect the signal

1. Briefly spin down the contents of the BaseScope™ Fast RED-B tube to be sure content is at the bottom of the tube before opening the cap.
2. Depending on the size of your hydrophobic barrier, prepare sufficient RED working solution per section by using a 1:60 ratio of BaseScope™ Fast RED-B to BaseScope™ Fast RED-A. For example, for a 0.75" x 0.75" barrier, add 2 µL of Red B to 120 µL of Red A into a tube. Mix well.

IMPORTANT! Use the Fast RED-B solution within **5 MIN**. Do not expose to direct sunlight or UV light.

3. Take each slide one at a time from the Tissue-Tek® Slide Rack and tap and/or flick to remove the excess liquid.
4. Place slides in the HybEZ™ or ACD EZ-Batch™ Slide Rack in the HybEZ™ Humidity Control Tray.
5. Pipette ~120 µL RED solution onto each tissue section. Ensure sections are covered.
6. Seal tray and incubate for **10 MIN** at **RT**.
7. Remove the slide rack from the tray.
8. To remove the RED working solution from the slides, tilt each slide one at a time over a waste container and tap the corner on the edge of the container. *Immediately* insert the slide into a Tissue-Tek® Slide Rack submerged in a Tissue-Tek® Staining Dish filled with tap water. Rinse again with fresh tap water.

Counterstain the slides

1. Move the Tissue-Tek® Slide Rack into the staining dish containing 50% Hematoxylin staining solution for **2 MIN** at **RT**. Slides will be purple.
2. *Immediately* transfer the slide rack back into the staining dish containing tap water, and wash slides 3–5 times by moving the rack up and down. Keep repeating with fresh tap water until the slides are clear, while sections remain purple.
3. Replace tap water in the staining dish with 0.02% Ammonia water. Move rack up and down 2–3 times. Section should turn blue.
4. Replace Ammonia water with tap water. Wash slides 3–5 times.

Mount the samples

1. Remove the slide rack from the staining dish and dry slides in a **60°C** dry oven for at least **15 MIN**, or until the slides are completely dry.

IMPORTANT! The RED substrate is alcohol sensitive. Do not dehydrate the slides in alcohol, and make sure your reagents are not contaminated with alchohol.

2. Place 1–2 drops of VectaMount or Ecomount on the slide.
3. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
4. Repeat steps 2 and 3 for each slide.
5. Air dry slides for **≥5 MIN**.

Evaluate the samples

Examine tissue sections under a standard bright field microscope at 20–40X magnification:

- Assess tissue and cell morphology.
- Assess positive control signal strength. Positive control signal should be visible as punctate dots within cells at 20–40X magnification.
- Assess negative control background. One dot to every 20 cells displaying background staining per 20X microscope field is acceptable.
- Evaluate target probe signal using the scoring guidelines in the next section.

Scoring guidelines

The BaseScope™ v2 Assay enables a semi-quantitative scoring guideline utilizing the estimated number of punctate dots present within each cell boundary.

An example of how to develop such a guideline for semi-quantitative assessment of BaseScope™ staining intensity is presented below for a gene with expression level varying between 1 to > 10 copies per cell.

Note: If your gene expression level is higher or lower than this range, you may need to scale the criteria accordingly.

Categorize staining into five grades: **0**, **1+**, **2+**, **3+**, and **4+** according to the following table:

Staining Score	Microscope Objective Scoring*
0	No staining or less than 1 dot to every 20 cells (40X magnification)
1	1 dot/cell (visible at 20–40X magnification)
2	2–3 dots/cell. No or very few dot clusters (visible at 20–40X magnification)
3	4–10 dots/cell. Less than 10% positive cells have dot clusters (visible at 20X magnification)
4	> 10 dots/cell. More than 10% positive cells have dot clusters (visible at 20X magnification)

* Discount cells with artificially high nuclear background staining.

Troubleshooting

For troubleshooting information, please contact technical support at **support@acdbio.com**.

A Appendix A. Tissue Pretreatment Recommendation for FFPE Samples

Follow the recommended pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissue types
- Samples prepared differently than the sample preparation protocol found in Chapter 3.

Tissue pretreatment recommendation

1. Stain representative samples using the positive and negative control probes.
2. Fix sample in fresh 10% NBF for **16–32 HRS** at **RT**.

Note: Perform tissue fixation step using the recommended amount of time. Over or under-fixation will result in significant signal loss when performing the BaseScope™ v2 Assay.

3. Depending on your tissue type, vary the amount of time for the RNAscope® Target Retrieval Reagents and/or RNAscope® Protease IV (see the following section).

Reagent	Mild	Standard	Extended
RNAscope® Target Retrieval Reagents	15 MIN	15 MIN	15–30 MIN
RNAscope® Protease IV	15 MIN	30 MIN	30 MIN

Note: Sample types, such as certain Xenografts and Cell Pellets, require less time. For these tissue types, vary the RNAscope® Target Retrieval Reagents time to **8 MIN** and RNAscope® Protease III time to **15 MIN**. For the ACD Cell Pellet sample, we recommend a **15 MIN** treatment with RNAscope® Target Retrieval Reagents, and a **15 MIN** treatment with RNAscope® Protease III. If you have a tissue type not listed, contact support at **support@acdbio.com**.

Note: For under-fixed tissues, we recommend using Protease III.

Tissue-specific pretreatment conditions

If your sample fixation is successful in fresh 10% NBF (Step 2 above), then refer to the following table for tissue-specific pretreatment conditions. For information about species or tissue type not listed here, contact support at **support@acdbio.com**.

Species	Tissue Type	Pathology	Pretreatment Condition
Mouse/Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Eye/Retina	Normal	Standard/Mild
	Liver	Normal	Extended
	Kidney	Normal	Standard

Species	Tissue Type	Pathology	Pretreatment Condition
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	Colon	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Mild
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard
	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard
	Brain	Normal	Standard
	Head	Cancer	Standard
	Neck	Cancer	Standard
	Liver	Cancer	Standard
	Kidney	Normal	Standard
	Skin	Normal	Standard
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Skin (TMA*)	Normal	Standard
	Breast (TMA)	Normal	Standard
	Melanoma (TMA)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Normal	Standard
	Stomach (TMA)	Tumor	Standard
	HeLa (ACD controls) or Cell pellets, fixed with 10% NBF or 10% Formaldehyde	—	Mild/Protease III

B Appendix B. Manual Target Retrieval

Materials required

Materials provided by the Universal Pretreatment Kit	Other Materials and Equipment
• RNAscope® 10X Target Retrieval Reagents	• Prepared slides • Distilled water • Glass beaker (1 or 2 L) • Paper towel or absorbent paper • Hot plate, isotemp brand • Aluminum foil • Thermometer • Forceps, large • Tissue Tek® Slide Rack • Tissue Tek® Staining Dish • ImmEdge™ Hydrophobic Barrier Pen

Prepare 1X RNAscope® Target Retrieval Reagents

IMPORTANT! Do not boil the 1X RNAscope® Target Retrieval Reagents more than **15 MIN** before use.

1. Prepare 700 mL of fresh RNAscope® 1X Target Retrieval Reagents by adding 630 mL distilled water to 1 bottle (70 mL) 10X Target Retrieval Reagents in the beaker. Mix well.
2. Place the beaker containing RNAscope® 1X Target Retrieval Reagents on the hot plate. Cover the beaker with foil and turn the hot plate on high for **10–15 MIN**.
3. Once 1X RNAscope® Target Retrieval Reagents reaches a mild boil (**98–102°C**), turn the hot plate to a lower setting to maintain the correct temperature. Check the temperature with a thermometer.

Apply RNAscope® Target Retrieval Reagents

1. With a pair of forceps *very slowly* submerge the slide rack containing the slides into the mildly boiling RNAscope® 1X Target Retrieval Reagents solution. Cover the beaker with foil and boil the slides for the amount of time specified by the table in **Appendix A. Tissue Pretreatment Recommendation for FFPE Samples** on page 25.
2. Use the forceps to *immediately* transfer the hot slide rack from the RNAscope® 1X Target Retrieval Reagents to the staining dish containing distilled water. Do not let the slides cool in the Target Retrieval Reagents solution.
3. Wash slides 3–5 times by moving the Tissue-Tek® Slide Rack up and down in the distilled water.
4. Wash slides in fresh 100% alcohol and allow the slides to dry completely at **RT**.
5. Draw hydrophobic barrier and continue with BaseScope™ v2 Assay.

C Appendix C. Reagent Volume Guidelines

Determine reagent volume

Before starting your experiment, measure the inner edge of the hydrophobic barrier to determine the recommended number of drops needed per slide (see table below).

Size of Hydrophobic Barrier* (in)	Recommended Number of Drops per Slide	Recommended Volume per Slide (µL)	Relative Template Size
0.75″ x 0.75″ †	4	120	
0.75″ x 1.0″	5	150	
0.75″ x 1.25″	6	180	

* Hydrophobic barrier measured at inner edge. References in this user manual are for the 0.75″ x 0.75″ hydrophobic barrier size.

† Recommended hydrophobic barrier size is 0.75″ x 0.75″. With this barrier size, each probe is sufficient for staining ~20 sections. Larger tissue sections will result in fewer tests.

D Appendix D. Safety

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see **https://acdbio.com/technical-support/user-manuals**.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at: **https://www.cdc.gov/biosafety/**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: **https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_id=10051&p_table=STANDARDS**

- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: **https://www.cdc.gov/biosafety/**

In the EU:

- Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at:
  http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
- Information about the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) can be found at: **https://echa.europa.eu/regulations/reach**

Documentation and Support

Obtaining SDSs

Safety Data Sheets (SDSs) are available at: **https:// acdbio.com/technical-support/user-manuals**. For the SDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

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 facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Search for user documents, SDSs, application notes, citations, training videos, and other product support documents.
- Find out information about customer training events.

Contact information

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Information: **info@acdbio.com**
Orders: **orders@acdbio.com**
Support Email: **support@acdbio.com**

Limited product warranty

Advanced Cell Diagnostics, Inc. and/or its affiliate(s) warrant their products as set forth in the ACD General Terms and Conditions of Sale found on the ACD website. If you have any questions, please contact Advanced Cell Diagnostics at **https://acdbio.com/about/contact**.

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