



DNAscope™ HD Duplex Detection Kit
(Chromogenic)
With Sample Preparation and Pretreatment

Document Number UM 324700

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Citing RNAscope in Publications

When describing a procedure for publication using this product, please refer to it as the RNAscope Assay and cite: Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J and Luo Y. RNAscope: A Novel *In Situ* RNA Analysis Platform for Formalin-Fixed Paraffin-Embedded Tissues. *J. Mol. Diagnostics*, 2012, 14:22–29.

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Chapter 1. Product Information



Before using this product, read and understand the safety information in **Appendix E. Safety.**

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 DNAscope HD Duplex Reagent Kit (Cat. No. 324700) on formalin-fixed, paraffin-embedded (FFPE) samples. DNAscope Assays are compatible with a variety of sample types including tissue microarray (TMA) and cell samples.

Visit www.acdbio.com/technical-support/user-manuals to download a technical note for other sample types or contact support.acd@bio-techne.com for more information.

Product description

Background

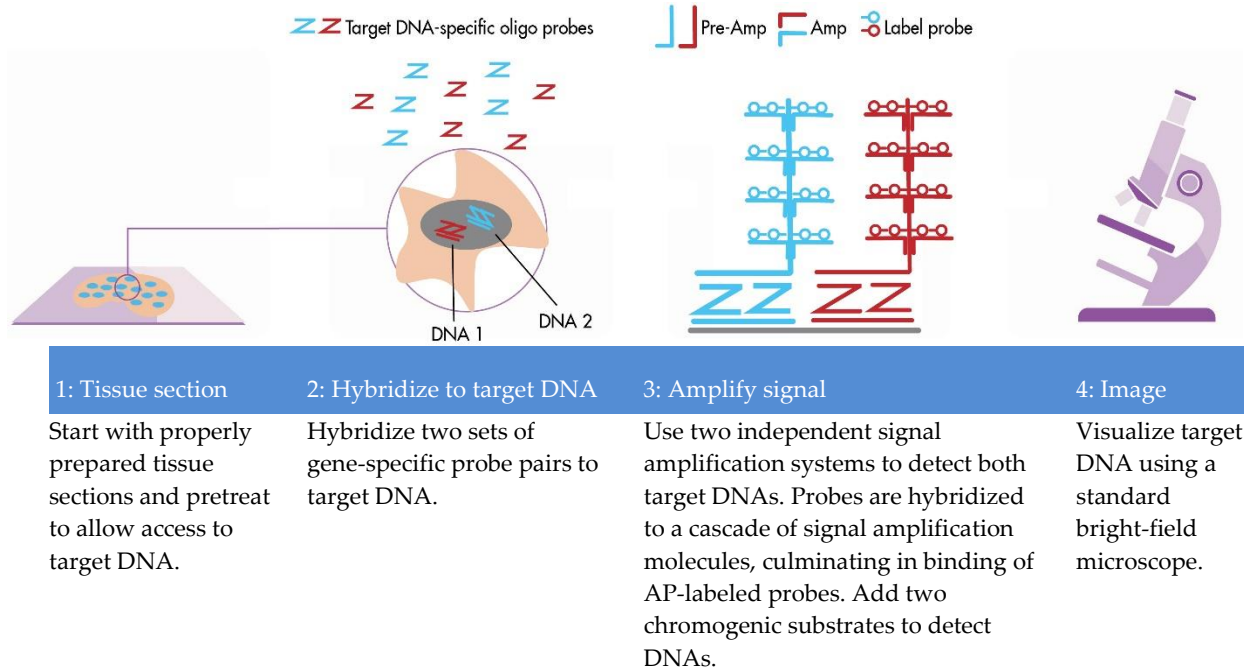
The DNAscope HD Duplex Chromogenic Assay uses a novel and proprietary method of *in situ* hybridization (ISH) to simultaneously visualize two DNA targets in samples mounted on slides. The assay is based on ACD's patented signal amplification and background suppression technology and incorporates multiplexed signal amplification systems, which enable users to investigate expression as well as positional relationship between two different genes within a cellular context.

Overview

The DNAscope HD Duplex Chromogenic Assay procedure can be completed in 24 hours, conveniently divided over two days (see Figure 1). Most of the DNAscope Assay reagents are available in convenient Ready-To-Use (RTU) bottles.

Starting with properly prepared tissue samples, sections are first pretreated, and then DNAscope-specific probes are hybridized to target DNA. The DNAscope HD Duplex Chromogenic Assay employs two independent signal amplification systems, each using the same chromogenic enzyme. Two target genes appear as punctate dots of two distinctly colored chromogen precipitates, visible using a common bright-field microscope at 40–60X magnification.

Figure 1. Procedure overview



Kit contents and storage

The DNAscope HD Duplex Chromogenic Assay requires the DNAscope Probes and the DNAscope Detection Kit. Probes and Detection Kits are available separately.

DNAscope Probes

Visit <https://acdbio.com/products> to find a gene-specific target probe. Each target probe contains a mixture of short oligonucleotides designed to bind to a specific target DNA and is detectable in one of two color channels, C1 or C2.

| Probe Channel ID | Chromogenic Labels | |
|------------------|--------------------|-------|
| | Enzyme | Color |
| C1* | AP | RED |
| C2 | AP | BLUE |

* Default channel

Channel C1 target probe is available as a Ready-To-Use solution, while the Channel C2 is available as a 50X stock. To independently detect two target DNAs in a duplex assay, each target probe must be in a different color channel and there must be a C1 probe in the mixture.

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



| Target Probes | | | | | |
|-------------------------------------|---|----------|---|-----------------|---------|
| <input checked="" type="checkbox"/> | Reagent | Cat. No. | Content | Quantity | Storage |
| | DNAscope Target Probe – [species] – [gene]-C1 | Various | Ready-To-Use (RTU) probe for color channel C1 | 3 mL x 1 bottle | 2–8 °C |
| | DNAscope Target Probe – [species] – [gene]-C2 | Various | 50X probe for color channel C2 | 60 µL x 1 tube | 2–8 °C |

DNAscope HD Duplex Reagent Kit

Each DNAscope HD Duplex Reagent Kit (Cat. No. 324700) provides enough reagents to stain ~20 tissue sections, each with an area of approximately 20 mm x 20 mm (0.75" x 0.75"). Larger tissue sections will result in fewer tests.

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

| Pretreatment Reagents (Cat. No. 323750) | | | | |
|---|---------------------------------|----------|-------------------|---------------------|
| <input checked="" type="checkbox"/> | Reagent | Cat. No. | Quantity | Storage |
| | RNA Removal Solution | 323748 | 4.5 ml x 1 bottle | 2–8°C |
| | Hydrogen Peroxide | 322335 | 3 mL x 2 bottles | 2–8°C |
| | Protease Plus | 322331 | 4.5 mL x 1 bottle | 2–8°C |
| | DNAscope Target Retrieval (10X) | 323745 | 70 mL x 4 bottles | Room temp (15–30°C) |

| DNAscope HD Duplex Detection Kit (Cat. No. 324710) | | | |
|--|---------------------------------|-------------------|---------|
| <input checked="" type="checkbox"/> | Reagent | Quantity | Storage |
| | DNAscope HD Duplex AMP 1 | 3 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 2 | 3 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 3 | 4.5 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 4 | 4.5 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 5 | 4.5 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 6 | 3 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 7 | 4.5 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 8 | 4.5 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 9 | 4.5 mL x 1 bottle | 2–8°C |
| | DNAscope HD Duplex AMP 10 | 3 mL x 1 bottle | 2–8°C |
| | DNAscope Fast Red-A – diluent | 3 mL x 1 bottle | 2–8°C |
| | DNAscope Fast Red-B – substrate | 60 µL x 1 tube | 2–8°C |
| | DNAscope Blue-A – diluent | 3 mL x 1 bottle | 2–8°C |
| | DNAscope Blue-B – substrate | 60 µL x 1 vial | 2–8°C |



| Wash Buffer Kit (Cat. No. 310091) | | | |
|-------------------------------------|-------------------|-------------------|---------------------|
| <input checked="" type="checkbox"/> | Reagent | Quantity | Storage |
| | Wash Buffer (50X) | 60 mL x 4 bottles | Room temp (15–30°C) |

IMPORTANT! DNAscope HD Reagent Kits share the same Wash Buffer, Hydrogen Peroxide, and Protease Plus as other ACD Reagent Kits, but the rest of the components are unique. Do not interchange these reagents between different types of ACD Kits.

Required materials and equipment

The following materials and equipment are needed to perform the DNAscope Assay.

HybEZ Hybridization System

IMPORTANT! The DNAscope Assay has been developed using this system only.

Use the HybEZ™ Hybridization System to perform DNAscope 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 II Hybridization System User Manual* available at <https://acdbio.com/documents/product-documents> and view the training video at <https://acdbio.com/technical-support/learn-more>. The system contains the following components:

| <input checked="" type="checkbox"/> | Component | Quantity | Cat. No. |
|-------------------------------------|--|-----------|-----------------------------|
| | HybEZ II Oven (110 or 220V) | 1 oven | 321710 or 321720 (HybEZ II) |
| | HybEZ Humidity Control Tray (with lid) | 1 tray | 310012 |
| | ACD EZ-Batch™ Slide Holder (20 slide capacity) | 1 rack | 310017 |
| | HybEZ Humidifying Paper Pack | 15 sheets | 310015 |

User-supplied materials

IMPORTANT! Do not substitute other materials for the ImmEdge™ Hydrophobic Barrier Pen or the VectaMount listed in the following table.

| <input checked="" type="checkbox"/> | Description | Supplier | Cat. No. |
|-------------------------------------|---|--------------------------------------|-------------------------|
| | ImmEdge Hydrophobic Barrier Pen (required) | Vector Laboratory | H-4000 |
| | SuperFrost® Plus Slides or SuperFrost Excell Slides | Fisher Scientific | 12-550-15 22-034-985 |
| | Gill's Hematoxylin I | American Master Tech Scientific/MLS* | HXGHE1LT |
| | Xylene | Fisher Scientific/MLS | X3P-1GAL |
| | 100% alcohol (EtOH) | American Master Tech Scientific/MLS* | ALREAGAL |



| <input checked="" type="checkbox"/> | Description | Supplier | Cat. No. |
|-------------------------------------|---|--|----------------|
| | 10% Neutral Buffered Formalin (NBF)/ 4% Paraformaldehyde (PFA) / 37% Formaldehyde | MLS Sigma | — 252549-1L |
| | 1X Phosphate Buffered Saline (PBS) | MLS | — |
| | 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 Dish, xylene resistant | American Master Tech Scientific/MLS | LWT4456EA |
| | VectaMount Permanent Mounting Medium (requir | Vector Labs | H-5000 |
| | Cover Glass, 24 x 50 mm | Fisher Scientific/MLS | 12--545-F |
| | Ammonium hydroxide, 28–30% | Sigma-Aldrich/MLS | 320145-500mL |
| | Carboy (>3L) | MLS | — |
| | Paraffin wax | 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 | — |
| | Microtome | MLS | — |
| | Water bath or incubator, capable of holding temperature at 40 +/- 1°C | MLS | — |
| | Oster® Steamer Model 5712, Black and Decker Steamer HS3000, the Braun Multiquick FS 20 Steamer, or the Hamilton Beach Steamer | — | — |
| | Microscope and accessories | MLS | — |
| | Drying oven, capable of holding temperature at 60 +/- 1°C | MLS | — |
| | Aluminum foil (Optional)† | MLS | — |
| | Forceps, large (Optional)† | MLS | — |
| | Hot plate (Optional)† | MLS | — |

* Major Laboratory Supplier or preferred vendor in North America. For other regions, please check Catalog Numbers with your local lab supplier.

† Required for the alternate target retrieval method in Appendix C on page 29.

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Chapter 2. Before You Begin

IMPORTANT! The DNAscope assay has an overnight probe hybridization step. Total assay run time including overnight probe hybridization is ~24 hours.

Prior to running the DNAscope 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>.

Important procedural guidelines

- Start with properly fixed and prepared sections. Refer to **Appendix A. Tissue Pretreatment Recommendation, Chapter 3. Prepare and Pretreat Samples**, and pretreatment user guides available at www.acdbio.com/technical-support/user-manuals.
- Use only samples mounted on SuperFrost Plus Slides or SuperFrost Excell Slides (Fisher Scientific; Cat. Nos. 12-550-15 and 22-034-985).
- Follow the recommended pretreatment guidelines for your sample. Refer to **Chapter 3. Prepare and Pretreat Samples** and pretreatment user guides available at www.acdbio.com/technical-support/user-manuals.
- Do *not* substitute required materials. Assay has been developed with these materials only.
- Follow the protocol exactly for best results.
- Do not let your sections dry out unless it is part of the procedure.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix E. Safety** for more information.

Chapter 3. Prepare and Pretreat Samples

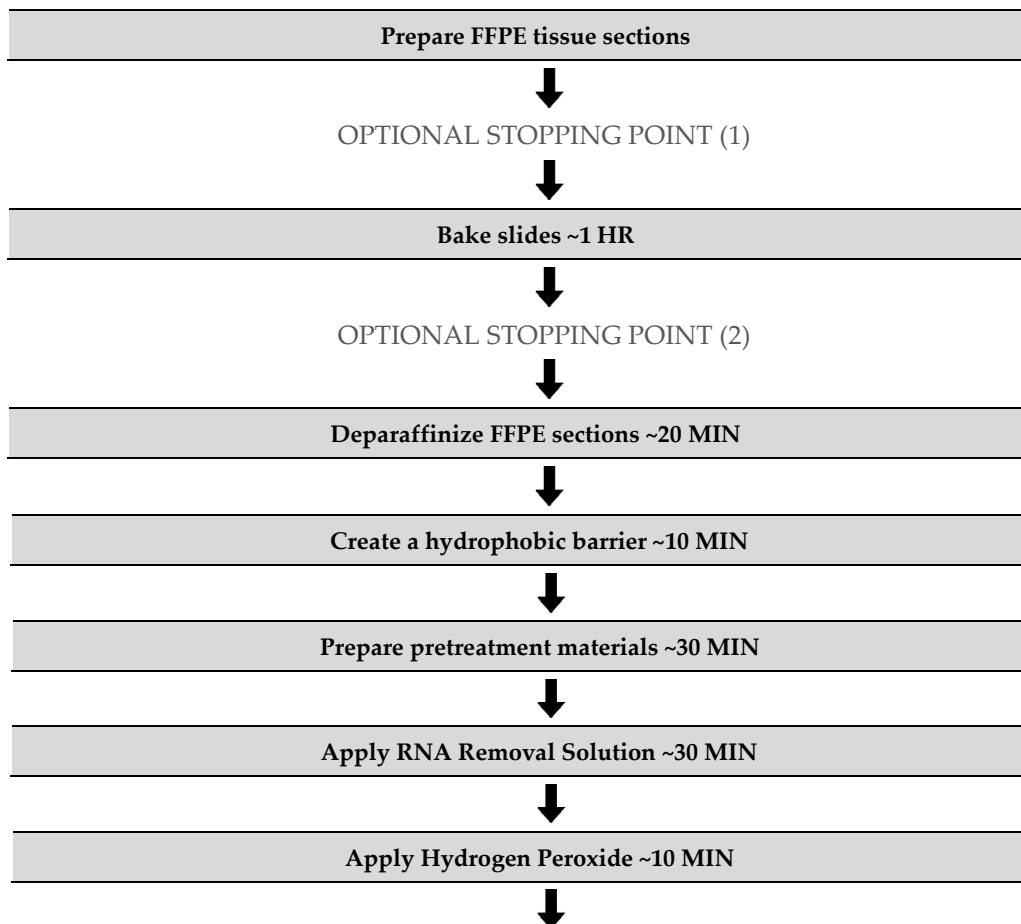
This chapter describes preparation and pretreatment of formalin-fixed, paraffin-embedded (FFPE) samples.

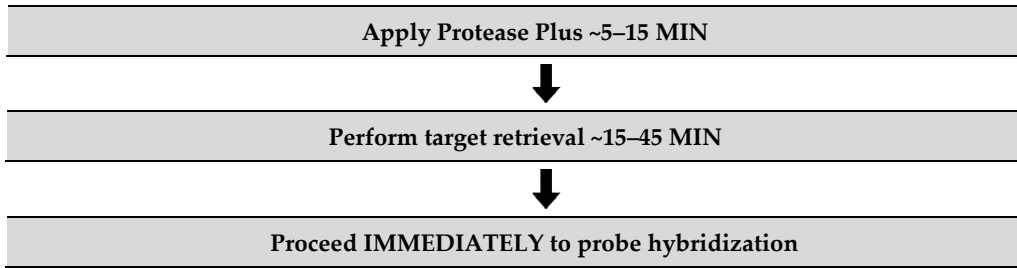
IMPORTANT! We highly recommend following these guidelines. We cannot guarantee assay results with other preparation methods.

FFPE sample preparation and pretreatment

For suboptimally prepared samples, you may need to optimize pretreatment conditions. Refer to **Appendix A. Tissue Pretreatment Recommendation** and to <https://acdbio.com/technical-support/solutions>.

Workflow





Materials required

| Materials provided by Pretreatment Reagents (Cat. No. 323750) | Other Materials and Equipment |
|--|--|
| <ul style="list-style-type: none"> • RNA Removal Solution • Hydrogen Peroxide • Protease Plus • DNAscope10X Target Retrieval Reagent | <ul style="list-style-type: none"> • 10% Neutral Buffered Formalin (NBF) or fresh 4% PFA • 1X PBS • Paraffin wax • Tissue-Tek Clearing Agent Dishes • Tissue-Tek Staining Dishes • Tissue-Tek Vertical 24 Slide Rack • 100% alcohol (EtOH) • Xylene • Microtome • Water bath • SuperFrost Plus slides • ImmEdge Hydrophobic Pen • Drying oven • Distilled water • Fume hood • HybEZ Humidifying System/ACD EZ-Batch Slide Holder and Wash Tray • Paper towel or absorbent paper • Steamer • Digital thermometer |

Prepare FFPE tissue sections

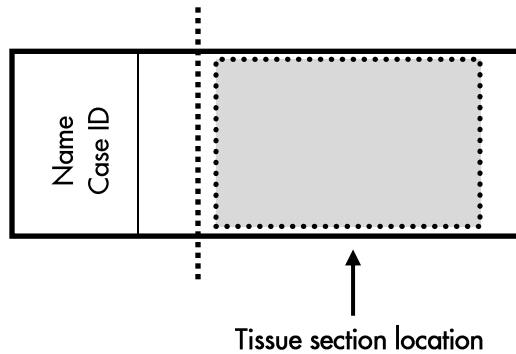
1. Immediately following dissection, fix tissue in 10% NBF for **16–32 HRS** at **ROOM TEMPERATURE (RT)**. Fixation time will vary depending on tissue type and size.



CAUTION! Handle biological specimens appropriately.

IMPORTANT! Fixation for <16 HRS or >32 HRS will impair the performance of the assay.

2. Wash sample with 1X PBS.
3. Dehydrate sample using a standard ethanol series, followed by xylene.
4. Embed sample in paraffin using standard procedures.
5. Trim paraffin blocks as needed, and cut embedded tissue into 5 +/- 1 μm sections using a microtome.
6. Place paraffin ribbon in a 40–45°C water bath, and mount sections on **SUPERFROST PLUS SLIDES**. Place tissue as shown below for optimal staining:



IMPORTANT! Some tissue specimens are more prone to detachment than others. Please use Superfrost Excell slides for mounting samples if increased tissue detachment is observed.

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

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

OPTIONAL STOPPING POINT (1). You can store sections with desiccants at room temperature.

Bake slides

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

OPTIONAL STOPPING POINT (2). Use immediately, or store with desiccants at room temperature.

Note: If you continue with the procedure, you can prepare materials for the next steps while the slides are baking.

Deparaffinize FFPE sections

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

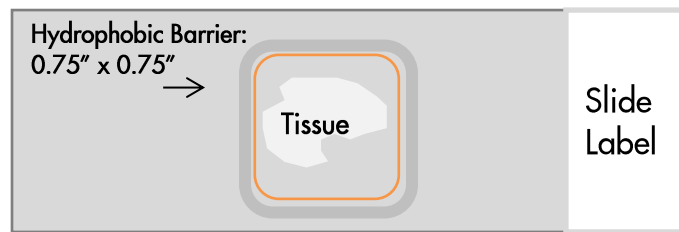
1. In a fume hood:
 - Fill two Tissue-Tek Clearing Agent dishes with ~200 mL fresh xylene.
 - Fill two Tissue-Tek Staining dishes with ~200 mL fresh 100% ethanol.
1. Place slides in a Tissue-Tek Slide Rack and submerge in the first xylene-containing dish in the fume hood.
2. Incubate the slides in xylene for **5 MIN** at **RT**. Agitate the slides by occasionally lifting the slide rack up and down in the dish.
3. Remove the slide rack from the first xylene-containing dish, and *immediately* place in the second xylene-containing dish in the fume hood.
4. Incubate the slides in xylene for **5 MIN** at **RT** with agitation.

5. Remove the slide rack from the second xylene-containing dish, and *immediately* place in a dish containing 100% ethanol.
6. Incubate the slides in 100% ethanol for **2 MIN** at **RT** with agitation.
7. Remove the slide rack from the first ethanol -containing dish, and *immediately* place in the second ethanol -containing dish.
8. Incubate the slides in 100% ethanol for **2 MIN** at **RT** with agitation.
9. Remove the slides from the rack, and place on absorbent paper with the section face-up. Dry slides in a drying oven for **5 MIN** at **60°C** (or until completely dry).

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. Other pens may cause 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 **~10 MIN**.

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.

Prepare the materials for day one

Prepare 1X Wash Buffer

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

Note: Warm 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 probes

1. Warm probes for at least **10 MIN** at **40°C** in a water bath or incubator.
2. Briefly spin the C2 probe to collect the liquid at the bottom of the tubes.
3. Mix 1:50 ratio of C2 probe to C1 probe by pipetting 1 volume of C2 probe to 50 volumes of C1 probe into a tube. Invert the tube several times. Warm probes for at least **10 MIN** at **40°C** in a water bath or incubator.



Note: Do not mix probes of the same color channel. The mixed target probes can be stored at 4°C for up to six months.

Prepare pretreatment materials

1. Turn on the HybEZ Oven, and set the temperature to 40°C.
2. Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.
3. Insert the covered tray into the 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.
4. Prepare 1X DNAscope Target Retrieval Reagents (for example, add 180 mL distilled water to 20 mL 10X DNAscope Target Retrieval Reagents). Mix well.

Apply DNAscope RNA Removal Solution

1. Load the dry slides into the ACDEZ-Batch Slide Holder by opening the swing clamp (see page 30 for detailed instructions).
2. Add ~5–8 drops of DNAscope RNA removal solution to each slide. Make sure each section is completely covered.
3. Place the ACDEZ-Batch Slide Holder in the pre-warmed HybEZ Humidity Control Tray. Close the lid, seal, and insert the tray back into the oven.
4. Incubate for **30 MIN** at 40°C.
5. Pour at least 200 mL distilled water into the transparent ACDEZ-Batch Wash Tray.
6. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
7. Place the ACDEZ-Batch Slide Holder into the wash tray containing water. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation (see page 31 for details).
8. Repeat the wash step with fresh distilled water.

Apply Hydrogen Peroxide

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-Batch Slide Holder, and add ~5–8 drops of Hydrogen Peroxide to cover each section.
2. Incubate slides for **10 MIN** at **RT**.
3. Pour at least 200 mL distilled water into the transparent ACDEZ-Batch Wash Tray.
4. Place the ACDEZ-Batch Slide Holder into the wash tray containing water. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation.
5. Repeat the wash step with fresh distilled water.

Apply Protease Plus

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-Batch Slide Holder, and add ~5 drops of Protease Plus to entirely cover each section.
2. Place the ACDEZ-Batch Slide Holder in the pre-warmed HybEZ Humidity Control Tray for incubation at 40°C or on the bench for incubation at **RT** depending on your sample (see **Appendix A. Tissue Pretreatment Recommendation** on page 26).

Note: If needed, prepare DNAscope Assay materials during this step.

3. Pour at least 200 mL distilled water into the transparent ACDEZ-Batch Wash Tray.
4. Remove the HybEZ Humidity Control Tray from the oven if you were incubating the slides at 40°C. Remove the slide holder from the tray. Place the tray back into the oven.
5. Place the ACDEZ-Batch Slide Holder into the clear plastic wash tray containing water. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation.
6. Repeat the wash step with fresh distilled water.

Perform target retrieval using a steamer

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

We highly recommend using a steamer for target retrieval. For target retrieval using a hot plate, see **Appendix C. Manual Target Retrieval** on page 29.

Note: For each steamer, fill the water to the maximum level before starting and do not refill water during the steaming process. Refilling water during steaming process will drop the temperature and interfere with Target Retrieval.

1. Fill the water reservoir with cold tap water to the MAX fill marking line.

IMPORTANT! Do not overfill.



2. Place two slide holders in the steam bowl. Fill one slide holder with 200 mL of 1X DNAscope Target Retrieval Reagent. Fill the other slide holder with 200 mL of distilled H₂O.
3. Turn on the steamer. Set the heating time to the maximum so steamer does not shut off during Target Retrieval.
4. Insert a digital thermometer through the holes of the lid and into the container containing 1X DNAscope Target Retrieval Reagent. Allow the temperature to rise to at least 99°C.
5. Add the slides to the container containing distilled H₂O for **10 SEC** to acclimate the slides.
6. Remove the slides and move them to the container containing 1X DNAscope Target Retrieval Reagent. Cover the steamer with the lid.
7. Start the timer for **10–15 MIN** for mild conditions and **30–45 MIN** for standard conditions. For pretreatment times, consult **Appendix A. Tissue Pretreatment Recommendation**.
8. Remove the slides from the target retrieval reagent and transfer to the container with hot distilled water (100°C). Allow slides to rinse for **5–10 SEC**.



9. Transfer the slides to a separate container filled with 1X Wash buffer and keep the slides immersed in wash buffer at **RT** until the DNAscope probes are added. Add the probes as quickly as possible.

Proceed to the DNAscope Assay

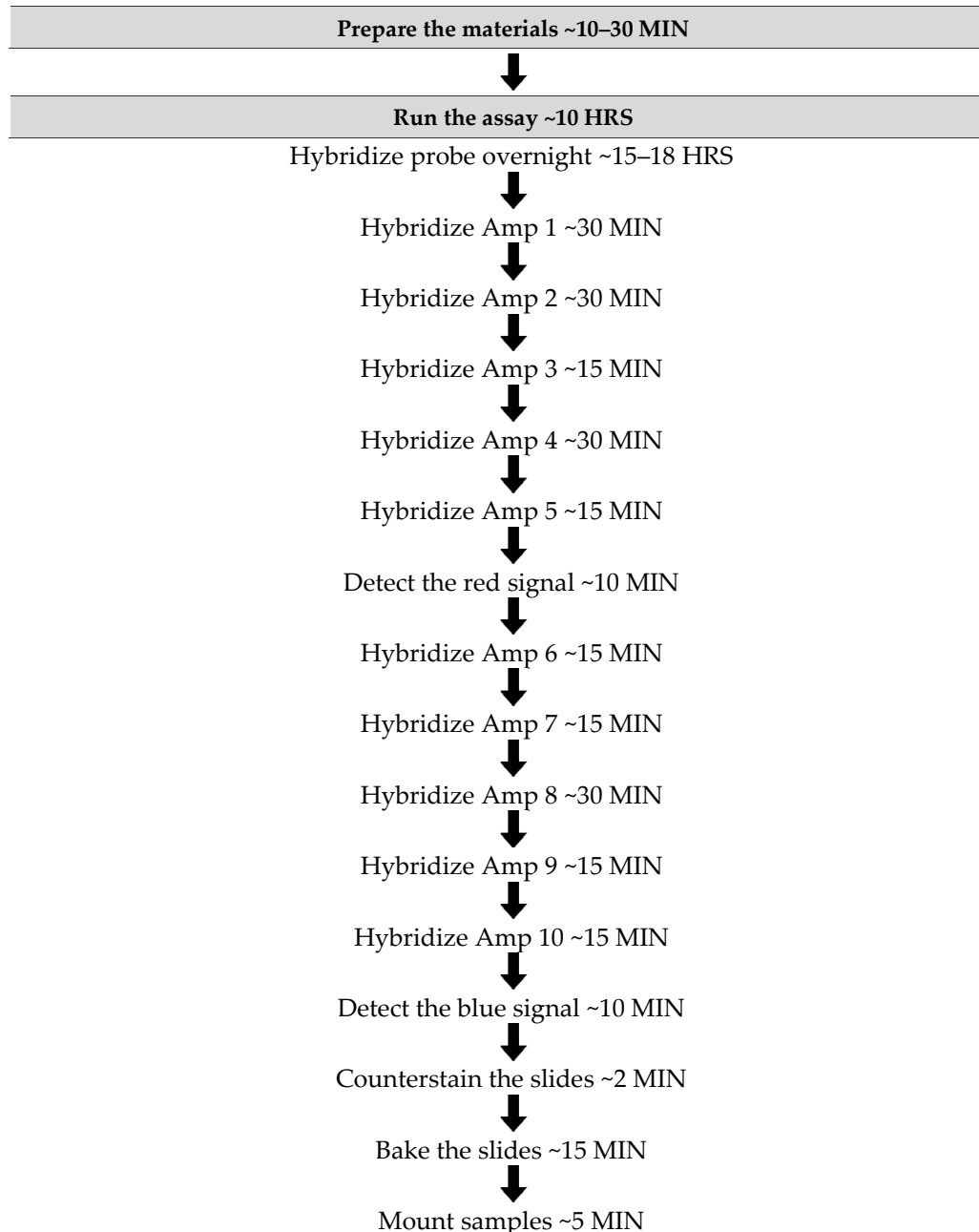
Proceed *immediately* to **Chapter 4. DNAscope HD Duplex Assay**.

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Chapter 4. DNAscope HD Duplex Assay

This procedure flows directly from sample preparation and pretreatment. Refer to **Chapter 3** and pretreatment user guides available at www.acdbio.com/technical-support/user-manuals.

Workflow





Evaluate the samples

Materials required for the assay

| Materials provided by DNAscope HD Duplex Detection Kit | Materials provided by DNAscope Probes | Other Materials and Equipment |
|--|--|---|
| <ul style="list-style-type: none"> • DNAscope Wash Buffer (50X) • DNAscope HD Duplex Amp 1 • DNAscope HD Duplex Amp 2 • DNAscope HD Duplex Amp 3 • DNAscope HD Duplex Amp 4 • DNAscope HD Duplex Amp 5 • DNAscope HD Duplex Amp 6 • DNAscope HD Duplex Amp 7 • DNAscope HD Duplex Amp 8 • DNAscope HD Duplex Amp 9 • DNAscope HD Duplex Amp 10 • DNAscope Fast Red-A • DNAscope Fast Red -B • DNAscope Blue-A • DNAscope Blue-B | <ul style="list-style-type: none"> • C1 Target Probe • 50X C2 Target Probe | <ul style="list-style-type: none"> • Prepared sections • Distilled water • Carboy (>3L) • Fume hood • Xylene • 100% alcohol (EtOH) • Tissue-Tek Staining Dishes • Tissue-Tek Clearing Agent Dishes, xylene-resistant • Gill's Hematoxylin I • Ammonium hydroxide, 28–30% • Graduated cylinder • Parafilm • HybEZ Humidifying System/ ACD EZ-Batch Slide Holder and Wash Tray • Water bath or incubator • Tissue-Tek Vertical 24 Slide Rack • Tubes (various sizes) • Paper towel or absorbent paper • Pipettors and tips, 1–1000 μL • VectaMount • Cover Glass, 24 mm x 50 mm • Dry oven |

Prepare the materials

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

Prepare the materials for day two

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, you must use Ammonium Hydroxide.

Prepare mounting reagents

IMPORTANT! Do not reuse deparaffinization reagents to dehydrate the slides after the assay.

- In the fume hood, add ~200 mL xylene to a clearing agent dish.

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

Equilibrate reagents

- Remove Amp 1–10 reagents from refrigerator and place at **RT**.
- Ensure HybEZ Oven and prepared Humidity Control Tray are at **40°C**.

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 <https://acdbio.com/technical-support/learn-more> before proceeding.

Hybridize probes overnight

IMPORTANT! Ensure probes are prewarmed to dissolve any precipitation prior to use.

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-Batch Slide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add ~4 drops of the probe mixture to entirely cover each slide.

Note: Refer to **Appendix C. Reagent Volume Guidelines** on page 29 to determine the recommended number of drops needed per slide. For example, add 4 drops of the appropriate probe to a 0.75" x 0.75" barrier.

3. Close the tray and insert into the HybEZ Oven for **15–18 HRS** at **40°C**.
4. Next day pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
6. Place the ACDEZ-Batch Slide Holder into the wash tray containing 1X Wash Buffer. Make sure all the slides are submerged. If needed, carefully add more buffer. Wash the slides with slight agitation for **2 MIN** at **RT** (see page 30 for details). Repeat the wash step with fresh 1X Wash Buffer.



Hybridize Amp 1

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 1 to cover each section.
3. Close the tray and insert into the HybEZ Oven for **30 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
6. Place the ACD EZ-BatchSlide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Hybridize Amp 2

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 2 to cover each section.
3. Close the tray and insert into the HybEZ Oven for **30 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
4. Place the ACD EZ-BatchSlide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Hybridize Amp 3

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 3 to cover each section.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Keep the tray at **RT**.
6. Place the ACD EZ-BatchSlide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Hybridize Amp 4

1. Remove excess liquid from the slides while keeping the slides locked in the ACD EZ-Batch Slide holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 4 to cover each section.
3. Close the tray and incubate for **30 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
5. Remove the ACDEZ-BatchSlide Holder from the HybEZ Humidity Control Tray.
6. Place the ACD EZ-BatchSlide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.



Hybridize Amp 5

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 5 to cover each section.
3. Close the tray and incubate for **15 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
5. Remove the ACDEZ-BatchSlide Holder from the HybEZ Humidity Control Tray.
6. Place the ACD EZ-BatchSlide Holder into the wash tray and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Detect the red signal

1. Briefly spin down the contents of the Red-B tube to be sure that the contents are at the bottom of the tube before opening the cap.
2. Depending on the size of your hydrophobic barrier, make RED working solution per section by using a 1:50 ratio of Red-B to Red -A. For example, for a 0.75" x 0.75" barrier, add 3 µL of Red-B to 150 µL of Red-A into a tube. Mix well.

IMPORTANT! Use the RED solution immediately. Do not expose to direct sunlight or UV light.

3. Remove excess liquid from the slides while keeping them locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
4. Pipette ~150 µL RED solution onto each tissue section. Ensure that the sections are covered.
5. Close the tray and incubate for **10 MIN** at **RT**.
6. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
7. Remove the ACDEZ-BatchSlide Holder from the HybEZ Humidity Control Tray, and place the tray back into the oven.
8. Place the ACDEZ-BatchSlide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Hybridize Amp 6

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder.
2. Remove the HybEZ Humidity Control Tray from the oven, and insert the slide holder into the tray.
3. Add enough AMP 6 to cover each section.
4. Close the tray and incubate for **15 MIN** at **40°C**.
5. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
6. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray. Place the tray back into the oven.
7. Place the ACD EZ-BatchSlide Holder into the wash tray and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.



Hybridize Amp 7

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 7 to cover each section.
3. Close the tray and insert into the HybEZ Oven for **15 MIN** at **40°C**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray
5. Remove the HybEZ Humidity Control Tray from the oven. Remove the slide holder from the tray.

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

6. Place the ACDEZ-BatchSlide Holder into the wash tray, and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Hybridize Amp 8

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 8 to cover each section.
3. Close the tray and incubate for **30 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACDEZ-Batch Wash Tray.
5. Remove the ACDEZ-BatchSlide Holder from the HybEZ Humidity Control Tray.
6. Place the ACD EZ-BatchSlide Holder into the wash tray and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Hybridize Amp 9

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 9 to cover each section.
3. Close the tray and incubate for **15 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
5. Remove the ACDEZ-BatchSlide Holder from the HybEZ Humidity Control Tray.
6. Place the ACD EZ-BatchSlide Holder into the wash tray and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.

Hybridize Amp 10

1. Remove excess liquid from the slides while keeping the slides locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
2. Add enough AMP 10 to cover each section.
3. Close the tray and incubate for **15 MIN** at **RT**.
4. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray
5. Remove the ACDEZ-BatchSlide Holder from the HybEZ Humidity Control Tray.
6. Place the ACD EZ-BatchSlide Holder into the wash tray and wash the slides for **2 MIN** at **RT** with slight agitation. Repeat the wash step with fresh buffer.



Detect the blue signal

1. Briefly spin down the contents of the Blue-B tube to be sure that the contents are at the bottom of the tube before opening the cap.
2. Depending on the size of your hydrophobic barrier, make BLUE working solution per section by using a 1:50 ratio of Blue-B to Blue-A. For example, for a 0.75" x 0.75" barrier, add 3 μ L of Blue-B to 150 μ L of Blue-A into a tube. Mix well.

IMPORTANT! Use the BLUE solution immediately.

3. Remove excess liquid from the slides while keeping them locked in the ACDEZ-BatchSlide Holder. Insert the slide holder into the HybEZ Humidity Control Tray.
4. Pipette ~150 μ L BLUE solution onto each tissue section. Ensure that the sections are covered.
5. Close the tray and incubate for **10 MIN** at **RT**.
6. Pour at least 200 mL 1X Wash Buffer into the transparent ACD EZ-Batch Wash Tray.
7. Remove the ACDEZ-BatchSlide Holder from the HybEZ™ Humidity Control Tray.
8. Place the ACDEZ-BatchSlide Holder into the wash tray and wash the slides for **5 MIN** at **RT** with slight agitation.
9. Rinse the slides quickly in water to remove excess wash buffer.

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 turn purple.
2. Transfer the slide rack into a staining dish filled with tap water. Repeat wash step once or twice until there is no remaining hematoxylin left.
3. Replace tap water in the staining dish with 0.02% ammonia water. Move the rack up and down five times. Sections should turn blue.
4. Replace ammonia water with tap water and wash the 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 **10–15 MIN**. Avoid prolonged dehydration.
2. Cool the slides for **5 MIN** at **RT**.

IMPORTANT! We do not recommend dehydrating the slides in alcohol.

IMPORTANT! The BLUE substrate is partially soluble in xylene. If using xylene-based automated coverslipping, minimize xylene exposure by using one rack at a time.

IMPORTANT! Use the VectaMount mounting medium for manual coverslipping.

3. Briefly dip one slide into fresh pure xylene and immediately place 1–2 drops of VectaMount on the slide before the xylene dries.
4. Carefully place a 24 mm x 50 mm coverslip over the tissue section. Avoid trapping air bubbles.
5. Repeat steps 3 and 4 for each slide.
6. 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 signal strength. Signal should be visible as punctuate dots within the cell nucleus at 40–60X magnification.

Troubleshooting

For troubleshooting information, please contact technical support at support.acd@bio-techne.com.



Appendix A. Tissue Pretreatment Recommendation

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. 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 DNAscope Assay.

2. Depending on your tissue type vary the amount of time for the Target Retrieval Reagents and/or Protease Plus.

| Reagent | Mild | Standard |
|------------------|---------------|----------------|
| Target Retrieval | 10–15 MIN | 30–45 MIN |
| Protease Plus | 0–5 MIN at RT | 15 MIN at 40°C |

Note: Sample types, such as certain xenografts and cell pellets, require less time. For these tissue types, vary the Protease Plus time to **0-5 MIN** depending on the fixation condition, and the Target Retrieval time to **15 MIN**. If you have a tissue type not listed, contact support at support.acd@bio-techne.com.

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.acd@bio-techne.com.

| 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 |



| Species | Tissue Type | Pathology | Pretreatment Condition |
|---------|---|-----------|------------------------|
| | Prostate | Normal | Standard |
| | Pancreas | Normal | Standard |
| | Cervical | Cancer | Standard |
| | Cervical | Normal | Standard |
| | Brain | Tumor | Standard |
| | Brain | Normal | Standard |
| | Head | Cancer | Standard |
| | Neck | Cancer | Standard |
| | Liver | Cancer | Standard |
| | Kidney | Normal | Standard |
| | Skin | Normal | Standard |
| | Skin (TMA*) | Normal | Standard |
| | Breast (TMA) | Normal | Standard |
| | Stomach (TMA) | Normal | Standard |
| | Stomach (TMA) | Tumor | Standard |
| | HeLa (ACD controls) or Cell pellets, fixed with 10% NBF or 10% Formaldehyde | — | Mild |




* Tissue Microarray

B

Appendix B. 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.

| 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.



Appendix C. Manual Target Retrieval

Materials required

| Materials provided by the Pretreatment Reagents Kit | Other Materials and Equipment |
|--|---|
| <ul style="list-style-type: none"> 10X DNAscope Target Retrieval Reagents | <ul style="list-style-type: none"> 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 Target Retrieval Reagents

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

1. Prepare 700 mL of fresh 1X DNAscope Target Retrieval Reagents by adding 630 mL distilled water to 1 bottle (70 mL) 10X DNAscope Target Retrieval Reagents in the beaker. Mix well.
2. Place the beaker containing 1X DNAscope 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 the 1X DNAscope Target Retrieval Reagents reach 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 Target Retrieval Reagents

1. With a pair of forceps *very slowly* submerge the slide rack containing the slides into the mildly boiling 1X DNAscope 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** on page 26.
2. Use the forceps to *immediately* transfer the hot slide rack from the 1X DNAscope Target Retrieval Reagents to the staining dish containing hot distilled water at **100°C**. 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 hot distilled water.
4. Store slides in 1X Wash Buffer and immediately add probes.

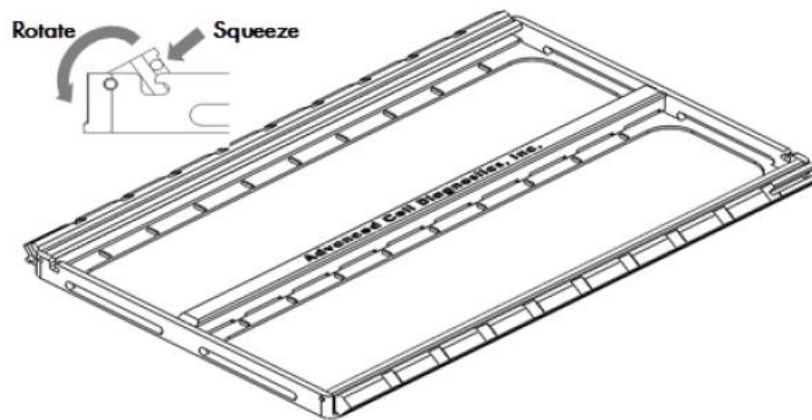
D

Appendix D. Using the EZ-Batch Slide Holder and Wash Tray

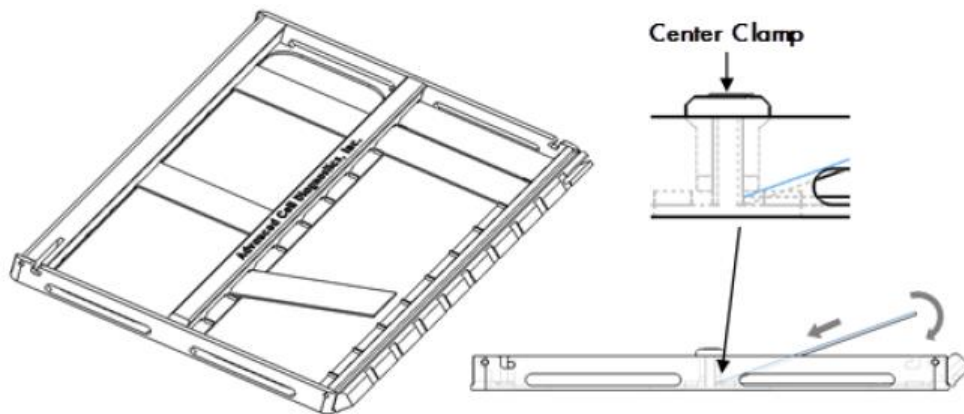
Load the slides in the ACD EZ-Batch Slide Holder

The ACD EZ-Batch Slide Holder can hold up to 20 standard glass slides in secure, lock-down positions arranged in two parallel columns. Lock-down is achieved by two lockable swing clamps, one per column, along both sides of the slide holder. Clamp locking mechanisms are located at the slots found at one end of each clamp.

1. Open the swing clamps one at a time by simultaneously squeezing (pressing and holding) the slotted portion of each clamp and rotating it up then outwards, as shown.

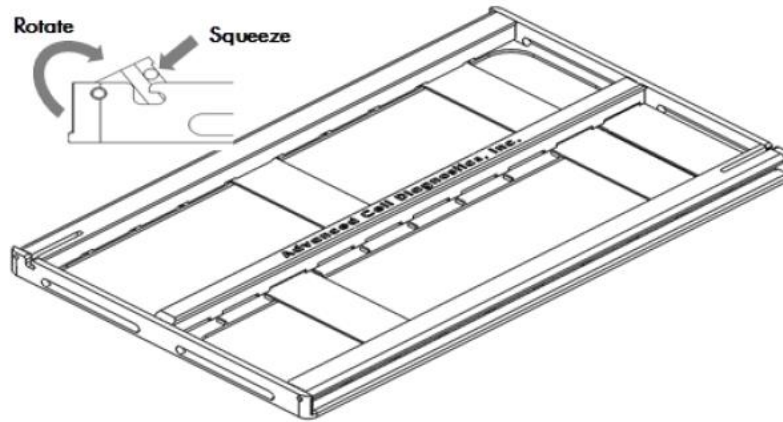


2. Insert the slides one at a time into the holder (up to 10 slides per column). The non-label end of each slide should be aligned toward the center of the holder and inserted under the fixed clamp, as shown. Place the rest of the slide down into the holder.



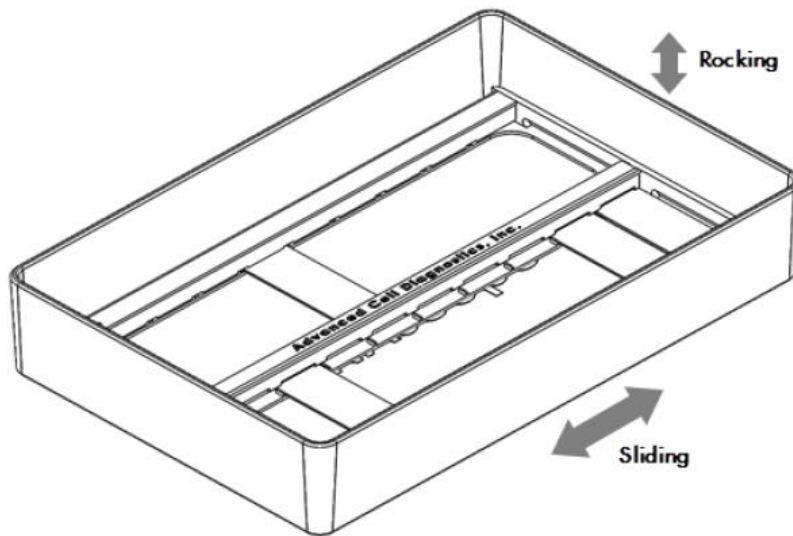


3. Close and lock the swing clamp of the column by simultaneously squeezing the slotted portion of each clamp and rotating it in then downwards in the direction opposite to the direction used to open the clamp, as shown.



Wash slides in the Wash Tray

1. Place the ACD EZ-Batch Slide Holder into the clear plastic wash tray containing water. Make sure all the slides are submerged. If needed, carefully add more water. Wash the slides with slight agitation.



E

Appendix E. 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, Authorisation 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

Advanced Cell Diagnostics, Inc.

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Newark, CA 94545

Toll Free: 1-877-576-3636

Direct: 1-510-576-8800

Fax: 1-510-576-8801

Information: info.acd@bio-techne.com

Orders: orders.acd@bio-techne.com

Support Email: support.acd@bio-techne.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>.

Headquarters

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