



# RNAscope<sup>®</sup> Sample Preparation and Pretreatment Guide for Fresh Frozen Tissue

(For Manual RNAscope<sup>®</sup> Assay)

## PART 1

Catalog Number 320513

*Compatible with the RNAscope<sup>®</sup> Fluorescent Multiplex Detection Kit Part 2 (Catalog No. 320293).*

## **For Molecular Biology Applications.**

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### **Citing RNAscope<sup>®</sup> 2.0 in Publications**

When describing a procedure for publication using this product, please refer to it as the RNAscope<sup>®</sup> 2.0 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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# 1

## Chapter 1. Product Information



Before using this product, read and understand the information in **Appendix B. Safety** on page 19 in this document.

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

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### About this guide

This user guide provides guidelines and protocols for the proper preparation and pretreatment of fresh, frozen tissues mounted on slides. The slides can then be assayed using an RNAscope® Reagent Kit.

RNAscope® Reagent Kits come with a separate RNAscope® Assay User Manual. Do not perform an RNAscope® Assay without the correct user manual.

### Product description

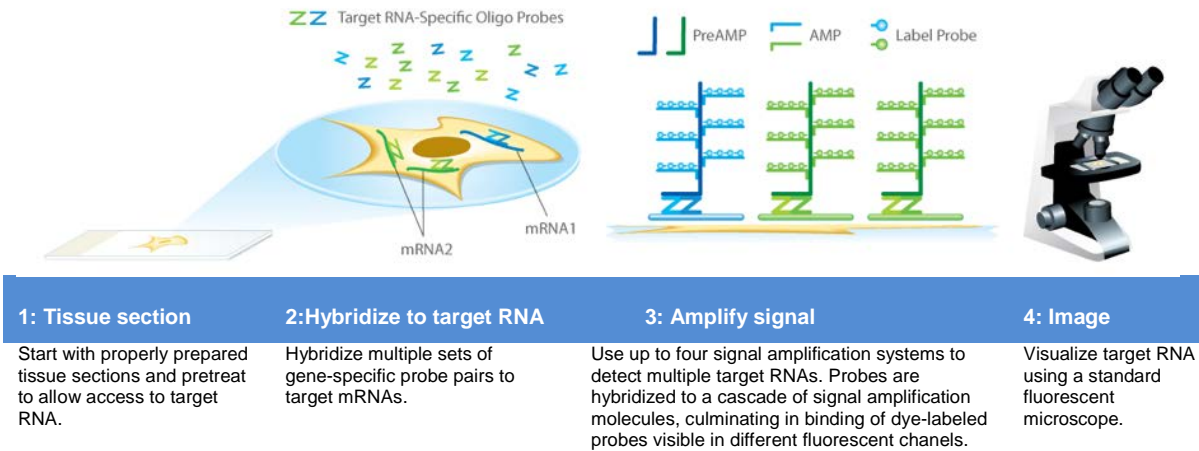
#### Background

The RNAscope® Assays use a novel and proprietary method of *in situ* hybridization (ISH) to visualize single RNA molecules per cell in samples mounted on slides. The assays are based on ACD's patented signal amplification and background suppression technology. Proprietary RNA-specific probes are hybridized to target RNA, and are then bound to a cascade of signal amplification molecules culminating in signal detection (Figure 1). Single-plex, 2-plex, multiplex, and automated assays are all available. The RNAscope® Assay procedure is illustrated in Figure 1 on page 6 and can be completed in 6–10 hours depending on the assay, or conveniently divided over two days. Most RNAscope® Assay reagents are available in convenient Ready-To-Use (RTU) dropper bottles and provide a simple, nearly pipette-free workflow. Results are observable using standard bright field or fluorescent microscopy.

#### Sample types

In order to perform the RNAscope® Assays, you must start with properly prepared and pretreated samples. Multiple sample types are now compatible with RNAscope® Assays and include: formalin-fixed, paraffin-embedded (FFPE) tissue, fresh frozen (FF) tissue, fixed frozen tissue, tissue microarray (TMA), and cell samples. Visit [www.acdbio.com](http://www.acdbio.com) and contact [support@acdbio.com](mailto:support@acdbio.com) for more information.

**Figure 1 Procedure overview**



## RNAscope® Assay reagents

RNAscope® Assays require the RNAscope® Probes and an RNAscope® Reagent Kit. If you are performing an automated assay using the Ventana® DISCOVERY XT or ULTRA Systems, you must use RNAscope® VS Probes and the RNAscope® VS Reagent Kit. Probes and Reagent Kits are available separately.

Visit [www.acdbio.com/products/target-probes/search-product](http://www.acdbio.com/products/target-probes/search-product) to find a gene-specific probe from a searchable catalog of >27,000 predesigned Target Probes, or order a custom probe. Visit [www.acdbio.com/products/target-probes/controls-housekeeping](http://www.acdbio.com/products/target-probes/controls-housekeeping) to find appropriate Control Probes.

## RNAscope® Reagent Kit

Each RNAscope® Reagent Kit provides enough reagents 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.

Each kit contains three sub-kits including a Pretreatment Kit, which come with up to three reagents. Fresh frozen tissue (Cat. No. 320842) requires only Pretreat 4. Pretreatment Kit instructions are provided in this guide. For information on the other sub-kits and directions for use, refer to an RNAscope® Assay User Manual. The reagents have a shelf life of six months from the shipment date when stored as indicated in the following table:

Pretreatment Kit (Cat. No. 320842)			
<input checked="" type="checkbox"/>	Reagent	Quantity	Storage
	Pretreat 3—1X protease	4.5 mL x 1 bottle	4°C
	Pretreat 4—2X protease	4.5 mL x 2 bottle	

**Note:** Use Pretreat 3 with Cultured Adherent Cells and Peripheral Blood Mononuclear Cells (PBMC). Use Pretreat 4 for Fresh Frozen Tissues.

**IMPORTANT!** RNAscope® Reagent Kits share the same Pretreatment Kit and Wash Buffer, but have unique Detection Kits. Do not interchange the reagent components of the Detection Kits, even those having the same name.

## Required materials and equipment

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

### HybEZ™ Hybridization System

**IMPORTANT!** The RNAscope® Assay has been validated using this system only.

The HybEZ™ Hybridization System (110 VAC, Cat. No. 310010; 220 VAC, Cat. No. 310013) is designed for the hybridization and incubation steps in the RNAscope® Assays. Incubation steps in the RNAscope® Assay 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 [www.acdbio.com/product\\_literature.html](http://www.acdbio.com/product_literature.html) and view the training video at [www.acdbio.com/demo\\_video.html](http://www.acdbio.com/demo_video.html). The system contains the following components:

<input checked="" type="checkbox"/>	Component	Quantity	Cat. No.
	HybEZ™ Oven (110 or 220 VAC)	1 oven	310010 or 310013
	HybEZ™ Humidity Control Tray (with lid)	1 tray	310012
	HybEZ™ Slide Rack (20 slide capacity)	1 rack	310014
	HybEZ™ Humidifying Paper	2 sheets	—
	HybEZ™ Humidifying Paper Pack	15 sheets	310015

### User-supplied materials

**IMPORTANT!** Do not substitute other materials for the ImmEdge™ Hydrophobic Barrier Pen and the SuperFrost® Plus Slides 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 (required)	Fisher Scientific	12-550-15
	100% ethanol (EtOH)	American Master Tech Scientific/MLS	ALREAGAL
	1X PBS	MLS	—
	Scalpel	MLS	—
	Forceps	MLS	—
	Dry ice, liquid nitrogen, or isopentane	MLS	—
	Cryo-embedding medium (e.g. OCT)	Fisher Scientific/MLS	NC9806257

☑	Description	Supplier	Cat. No.
	Cryomold	Fisher Scientific/MLS	NC9511236
	10% neutral buffered formalin (NBF) or 4% paraformaldehyde (PFA)	Fisher Scientific/MLS	23-245-684
	Cryostat, capable of cooling down to at least -20°C.	MLS	—
	Slide boxes	MLS	—
	Aluminum foil or zip-lock bags	MLS	—
	Tissue-Tek® Vertical 24 Slide Rack	American Master Tech Scientific/MLS	LWSRA24
	Tissue-Tek® Staining Dish (6 required)	American Master Tech Scientific/MLS	LWT4457EA
	Water bath or incubator, capable of holding temperature at 40 +/- 1°C	MLS	—
	Distilled water	MLS	—
	Paper towel or absorbent paper	MLS	—

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



# 2

## Chapter 2. Before You Begin

Prior to running the RNAscope® Assay on your samples for the first time, we recommend that you:

- View the video demonstrations available at [www.acdbio.com/support/online-training-videos](http://www.acdbio.com/support/online-training-videos).

### Important procedural guidelines

- Start with properly fixed and prepared sections.
- 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.
- Use good laboratory practices and follow all necessary safety procedures. Refer to **Appendix B. Safety** on page 19 for more information.



# 3

## Chapter 3. Prepare and Pretreat Samples

Fresh frozen sample preparation and pretreatment are described in the following protocols. For other sample types and preparation methods, contact [support@acdbio.com](mailto:support@acdbio.com) for the latest protocols and guidelines.

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

---

### Prepare fresh frozen sections

#### Materials required

- 
- Scalpel
  - Forceps
  - Cryo-embedding medium (OCT)
  - Dry ice, liquid nitrogen, or isopentane
  - Cryostat
  - Slide boxes
  - SuperFrost® Plus slides
  - Aluminum foil or zip-lock bags
- 

#### Prepare the block

1. Remove tissue and cut to fit into cryomolds.



**CAUTION!** Handle biological specimens appropriately.

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2. Freeze the specimen on dry ice or in liquid nitrogen within **5 MIN** of tissue harvest.
3. Embed frozen tissue in cryo-embedding medium (OCT):
  - a. Add two drops of OCT into a cryomold.
  - b. Place the frozen tissue on the OCT in the correct orientation for cutting.
  - c. Add more OCT to fill the cryomold. Do not allow any air bubbles to form.
  - d. Hold the block with forceps on the surface of liquid nitrogen or isopentane cooled by dry ice or liquid nitrogen, or place the cryomold on dry ice.
4. Store the frozen block in an air-tight container at **-80°C** prior to sectioning.

**Note:** Embedded tissue may be stored for at least **3 MONTHS**.

#### Section the block

1. Equilibrate block to **-20°C** in a cryostat **~1 HR**.

2. Cut 10–20  $\mu\text{m}$  sections and mount onto SuperFrost® Plus slides.
3. Keep the sections at  $-20^{\circ}\text{C}$  to dry.
4. Store the sections in slide boxes wrapped air-tight with aluminum foil or zip-lock bags at  $-80^{\circ}\text{C}$  until use.

**Note:** Sections may be stored for at least **3 MONTHS**.

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**IMPORTANT!** Do not fix the slides prior to this step.

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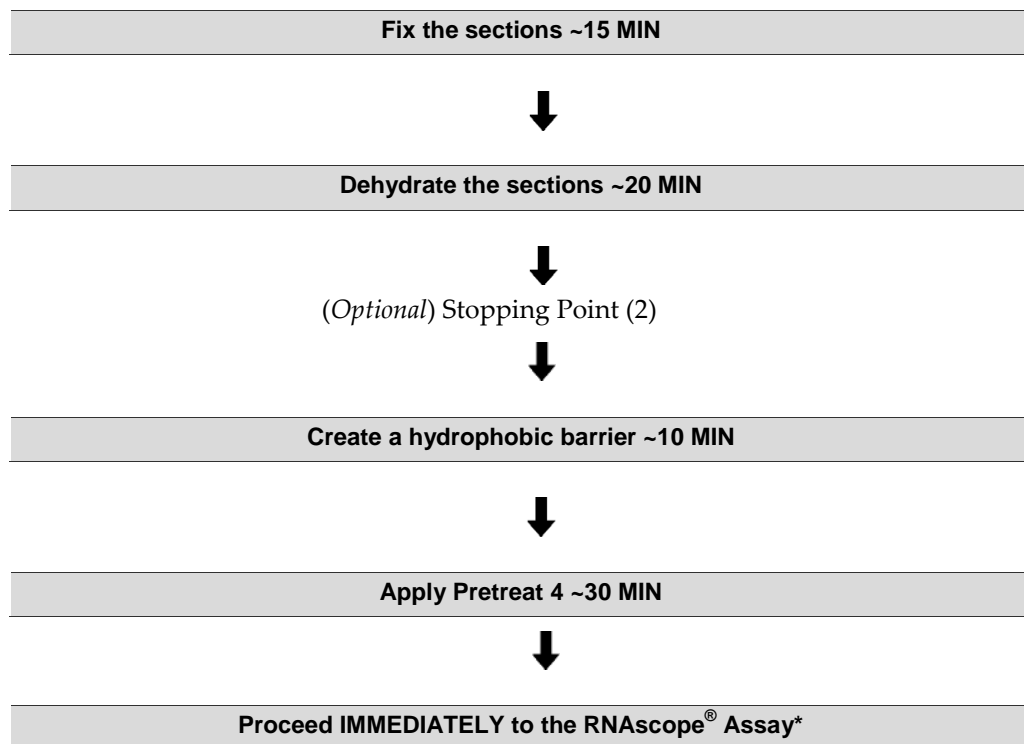
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OPTIONAL STOPPING POINT (1). Use sectioned tissue within 3 months.

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## Prepare the slides for the RNAscope® Assay

### Workflow



\* *Immediately* proceed to the RNAscope® Fluorescent Multiplex Detection Kit Part 2 User Manual (Catalog No. 320293).

## Materials required

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- 1X PBS
  - 10% NBF or 4% PFA
  - 100% ethanol (EtOH)
  - Tissue-Tek® Vertical 24 Slide Rack
  - Tissue-Tek® Staining Dishes (5 required)
  - ImmEdge™ Hydrophobic Barrier Pen
- 

## Fix the sections

1. Chill **200 mL** 10% NBF (fresh 10% NBF or 4% PFA in 1X PBS) to **4°C** in a Tissue Tek® Staining Dish.
2. Remove slides from **-80°C**, and place in a Tissue Tek® Slide Rack.
3. *Immediately* immerse slides in the pre-chilled fixative. Fix for **15 MIN** at **4°C**.

**Note:** Formalin that has been stored for more than 6 months, exposed to air for more than a week, or used repeatedly may result in suboptimal tissue fixation.

## Dehydrate the sections

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

1. Prepare **200 mL** 50% EtOH, **200 mL** 70% EtOH, and **600 mL** 100% EtOH in Tissue Tek® Staining Dishes.
2. Place the slides in 50% EtOH for **5 MIN** at **ROOM TEMPERATURE (RT)**.
3. Place the slides in 70% EtOH for **5 MIN** at **RT**.
4. Place the slides in 100% EtOH for **5 MIN** at **RT**.
5. Repeat step 6 with fresh 100% EtOH.

---

OPTIONAL STOPPING POINT (2). Slides may be stored in 100% EtOH at **-20°C** for up to **1 WEEK**. Prolonged storage may degrade sample RNA.

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## Create a hydrophobic barrier

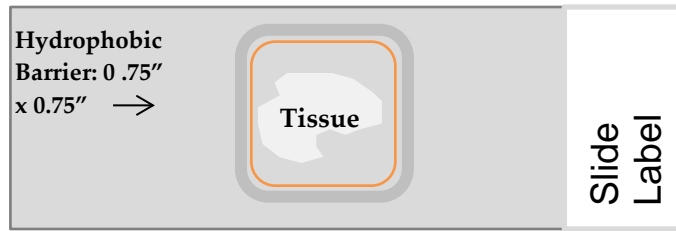
1. Take slides out of 100% EtOH and place on absorbent paper with the section face-up. Air dry for **5 MIN** at **RT**.
2. Use the following template to draw a barrier 2–4 times around each section with the Immedge™ hydrophobic barrier pen. See example below (size of this hydrophobic barrier is 0.75" x 0.75").

**Note:** Refer to **Appendix A. Reagent Volume Guidelines** on page 17 to determine the recommended number of drops needed per slide.

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

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**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.

3. Let the barrier dry completely ~ 1 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.

## Pretreat samples

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**IMPORTANT!** Do not apply Pretreat 1 or Pretreat 2 to fresh, frozen sections when using the RNAscope® Fluorescent Multiplex Kit. Other sample types may need these treatments.

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### Materials required

Materials provided by the RNAscope® Reagent Kit	Other materials and equipment
<ul style="list-style-type: none"> <li>• Pretreat 3 &amp; 4</li> </ul>	<ul style="list-style-type: none"> <li>• Prepared slides</li> <li>• Distilled water</li> <li>• Paper towel or absorbent paper</li> <li>• HybEZ™ Humidifying System</li> <li>• Tissue Tek® Slide Rack</li> <li>• Tissue Tek® Staining Dish</li> </ul>

### Prepare the equipment

- Turn on HybEZ™ Oven and set temperature to 40°C.
- Place a Humidifying Paper in the Humidity Control Tray and wet completely with distilled water.

### Apply Pretreat 4

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**IMPORTANT!** View the wash step video at [www.acdbio.com/support/online-training-videos/wash-slides](http://www.acdbio.com/support/online-training-videos/wash-slides) before proceeding.

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1. Place the dried slides on the HybEZ™ Slide Rack, and add ~5 drops of Pretreat 4 to entirely cover each section.
2. Incubate for 30 MIN at RT.

3. Take each slide one at a time from the HybEZ™ 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 1X PBS.
4. Wash slides with 1X PBS by moving the rack up and down 3–5 times and repeat with 1X PBS.

## Proceed to the RNAscope® Assay

While slides are incubating, proceed *immediately* to the appropriate RNAscope® Assay protocol. Ensure you have the appropriate user manual. User manuals are available at [www.acdbio.com/support/technical-doc](http://www.acdbio.com/support/technical-doc). For any questions, please contact [support@acdbio.com](mailto:support@acdbio.com).

**Note:** Slides can stay in 1X PBS for up to **15 MIN**.

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**IMPORTANT!** Slides should not stay in 1X PBS for longer than **15 MIN**. Proceed to the RNAscope® Assay using the appropriate user manual.

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## Troubleshooting

For troubleshooting information, please contact technical support at [support@acdbio.com](mailto:support@acdbio.com).








# A

## Appendix A. 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.



# B

## Appendix B. Safety

### Chemical safety




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**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 Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain MSDSs, see **Documentation and Support** in this document.
- 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




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**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: [www.cdc.gov/biosafety](http://www.cdc.gov/biosafety)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030), found at: [www.access.gpo.gov/nara/cfr/waisidx\\_01/%2029cfr1910a\\_01.html](http://www.access.gpo.gov/nara/cfr/waisidx_01/%2029cfr1910a_01.html)
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at: [www.cdc.gov](http://www.cdc.gov)

**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: [www.who.int/csr/resources/publications/biosafety/who\\_cds\\_csr\\_lyo\\_2004\\_11/en/](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: [eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:133:0001:0043:EN:PDF](http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:133:0001:0043:EN:PDF)

# Documentation and support

## Obtaining MSDSs

Material Safety Data Sheets (MSDSs) are available at: [www.acdbio.com/support/technical-doc/category/msds](http://www.acdbio.com/support/technical-doc/category/msds). For the MSDSs of chemicals not distributed by Advanced Cell Diagnostics, contact the chemical manufacturer.

## Obtaining support

For the latest services and support information, go to: [www.acdbio.com/support](http://www.acdbio.com/support)

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, MSDSs, application notes, citations, training videos, and other product support documents.
- Find out information about customer training events.

## Contact information

Advanced Cell Diagnostics, Inc.

3960 Point Eden Way  
Hayward, CA 94545

Toll Free: 1-877-576-3636

Direct: 1-510-576-8800

Fax: 1-510-576-8801

Information: [info@acdbio.com](mailto:info@acdbio.com)

Orders: [orders@acdbio.com](mailto:orders@acdbio.com)

Support Email: [support@acdbio.com](mailto: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 at [www.acdbio.com/tos/terms-and-conditions-of-sale](http://www.acdbio.com/tos/terms-and-conditions-of-sale). If you have any questions, please contact Advanced Cell Diagnostics at [www.acdbio.com/support](http://www.acdbio.com/support).

**Headquarters**

3960 Point Eden Way | Hayward, CA 94545 | Phone 1-510-576-8800 | Toll Free 1-877-576-3636

**For support, email [support@acdbio.com](mailto:support@acdbio.com).**

**[www.acdbio.com](http://www.acdbio.com)**

