BD Pharmingen™

# Anti-Ig HRP Detection Kits

## Instruction Manual

| Detection Kit | Cat. No. |
| --- | --- |
| Anti-Mouse Ig HRP | 551011 |
| Anti-Rat Ig HRP | 551013 |
| Anti-Hamster Ig HRP | 551012 |

BD

BD Biosciences

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# Kit Contents

| Kit | Size | Cat. No. |
|---|---|---|
| **Anti-mouse Ig HRP Detection Kit** | **200 slides** | **551011** |
| Goat anti-mouse Igs, biotinylated | 0.5 ml | 51-7446KC |
| Antibody Diluent Buffer | 50 ml | 51-7099KC |
| Streptavidin-HRP | 20 ml | 51-7547KC |
| DAB Buffer | 20 ml | 51-7548KE |
| DAB Chromogen | 1.0 ml | 51-7549KE |
| **Anti-rat Ig HRP Detection Kit** | **200 slides** | **551013** |
| Goat anti-rat Ig, biotinylated | 0.5 ml | 51-7605KC |
| Antibody Diluent Buffer | 50 ml | 51-7099KC |
| Streptavidin-HRP | 20 ml | 51-7547KC |
| DAB Buffer | 20 ml | 51-7548KE |
| DAB Chromogen | 1.0 ml | 51-7549KE |
| **Anti-hamster Ig HRP Detection Kit** | **200 slides** | **551012** |
| Anti-hamster IgG Cocktail, biotinylated | 0.5 ml | 51-7444KC |
| Antibody Diluent Buffer | 50 ml | 51-7099KC |
| Streptavidin-HRP | 20 ml | 51-7547KC |
| DAB Buffer | 20 ml | 51-7548KE |
| DAB Chromogen | 1.0 ml | 51-7549KE |

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# Introduction

Immunohistochemistry is a powerful technique that allows a researcher to detect molecules within a tissue microenvironment. Protein expression can be studied in relation to tissue or cell compartments, and changes due to either normal or pathologic stimuli can be evaluated. Detection of an antibody bound to antigen can be accomplished either by direct or indirect methods. The direct method uses a primary antibody labeled with a fluorescent tag or linked with an enzyme. The indirect method uses an enzyme-linked secondary antibody that binds to the primary antibody. For even greater sensitivity, a biotinylated secondary antibody is used with a tertiary step such as an enzyme-linked avidin or streptavidin. While the indirect methods involve more assay steps, the amplification process at each step leads to increased sensitivity.

Although very simple in principle, immunohistochemistry involves many steps such as proper fixation of tissue, tissue processing, and sectioning. Antigen retrieval is often required for optimal antigen detection. Each of these steps are crucial for optimal immunostaining and for maintaining good tissue morphology, both of which are important to understand the biological process *in situ*. The direct method uses a primary antibody labeled with a fluorescent tag or an enzyme.

# Overview

## Anti-Ig HRP Detection Kits

The BD Pharmingen™ Anti-Ig HRP Detection Kits are designed for immunohistochemical staining of acetone-fixed frozen sections, formalin- or zinc-fixed paraffin sections, and cytospins/smears. The detection kit can be used with any purified primary antibody. Each kit includes a biotinylated anti-Ig antibody that is species-specific (anti-mouse, anti-rat or anti-hamster), antibody diluent, pre-diluted Streptavidin-horseradish peroxidase (SAv-HRP), and DAB substrate.

The affinity-purified, polyclonal, biotinylated secondary antibody is adsorbed against multiple sera of related species, reducing non-specific staining, while providing maximum reactivity. Antibody diluent is a buffered solution containing protein stabilizers to enhance antibody reactivity, detergent for permeabilization, and blocking agents to reduce non-specific staining. The ultra-sensitive SAv-HRP is pre-diluted for convenience. DAB substrate is provided for signal development. Consistent results are assured with all these matched reagents. A comprehensive series of protocols, for immunostaining in different types of tissues, is provided with the kit.

## Storage and Handling

All the components of the Anti-Ig HRP Detection Kit must be stored at 4°C. Some solutions require diluting or mixing prior to use. Instructions for handling, preparation, and storage of kit components follow.

### Anti-Ig Antibody (500 µl)

One vial of stock biotinylated anti-Ig (anti-mouse, anti-rat or anti-hamster) antibody sufficient for staining 200 slides is provided. Prior to use, dilute the stock antibody solution 1:50 with the antibody diluent. Store at 4°C. Do not freeze.

### Antibody Diluent (50 ml)

The antibody diluent is provided to dilute the primary and secondary antibodies to a working concentration. This solution contains both blocking reagents to reduce background and detergents to permeabilize the cells. Component contains BSA. Store at 4°C.

### Streptavidin-HRP (20 ml)

SAv-HRP, a pre-diluted solution, is provided for the detection system. The Streptavidin moiety binds to the biotinylated anti-Ig antibody and the HRP enzyme is used with the DAB substrate system, for colorimetric visualization of the results. Store at 4°C.

*Note:* Do not use sodium azide as a reagent preservative. Sodium azide inactivates the horseradish peroxidase enzyme over time.

### DAB Buffer and Chromogen

DAB (Diaminobenzidine) substrate system is provided to visualize the immunostaining pattern via the HRP enzyme. DAB buffer (20 ml) and chromogen (1 ml) are provided separately to prevent complex formation. To prepare a working solution, add one drop of the DAB chromogen (50 µl) for every 1 ml of the DAB buffer. DAB buffer and chromogen must be stored separately at 4°C.

*Note*: Working DAB substrate solution must be mixed just prior to use, and must be discarded after use.

# Additional Reagents Required

### Primary Antibody

The primary antibody is not provided with the kit. Any antibody appropriate for the antigen to be detected may be used. BD Biosciences – Pharmingen has a portfolio of over 250 antibodies against the human, mouse, and rat species for IHC. Please refer to our website, **www.bdbiosciences.com** for a complete listing.

### $H_2O_2$ Solution

A stock solution of $H_2O_2$ (30%) is required for endogenous peroxidase blocking.

### Phosphate Buffered Saline (PBS)

**Recipe for 1× PBS (1 liter)**

| | |
|---|---|
| KCl | 0.2 g |
| $KH_2PO_4$ | 0.2 g |
| NaCl | 8.0 g |
| $Na_2HPO_4$ $7H_2O$ pH 7.2-7.4 | 2.16 g |


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# Warnings and Precautions

1. Each of the biotinylated anti-Ig antibodies (51-7446KC, 51-7605KC, 51-7444KC, and 51-7447KC) contains 0.09% Sodium Azide. Sodium Azide yields a highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
2. DAB Buffer contains 5% hydrogen peroxide and is harmful.
   R 36/38 Irritating to eyes and skin.
   S3 Keep in cool place.
   S26 In case of contact with eyes, rinse immediately with water and seek medical advice.
   S28 After contact with skin, wash immediately with plenty of water.
3. DAB Chromagen contains 1.74% diaminobenzidine and is harmful.
   R40 Limited evidence of a carcinogenic effect.
   S36/37 Wear suitable protective clothing and gloves.
4. Source of all serum proteins is from USDA inspected abattoirs located in the United States.

# Background and Protocols

## Tissue Processing

The goal of tissue processing is to get the tissues into a solid medium so they can be sectioned. The most common embedding media, including paraffin wax and plastic resins are hydrophobic substances that will not permeate wet tissue. Tissue processing removes water from tissues and replaces it by organic solvents. The basic process involves soaking the tissues in graded alcohols usually starting around 70% and eventually reaching 100%. The alcohol is then removed with a clearing agent, usually xylene, and the tissue is then transferred to molten paraffin.

## Fixation

The goal of fixation is to preserve the *in vivo* structure of cells and tissues. Paraformaldehyde, formalin, zinc, and acetone are each various fixatives that can be used for different applications. Paraformaldehyde, a polymer of formaldehyde, is usually used as a 4% solution. It is a gentle fixative, however it penetrates cells quickly. 10% neutral buffered formalin is the most commonly used fixative for paraffin-embedded tissue. Formalin preserves tissue morphology, destroys most infectious agents in tissues, and preserves stable antigens for prolonged periods of time. However, formalin, through its crosslinking chemistry, tends to mask or destroy many epitopes especially on the cell surface. Very few monoclonal antibodies representing the common CD markers can be used in formalin-fixed paraffin sections. Heavy metals such as mercury and zinc can be used to fix tissue before paraffin embedding. BD Biosciences has developed a zinc fixative for IHC (Cat. No. 550523) that helps alleviate some of the problems encountered during formalin fixation. The zinc fixative is a milder fixative than formalin and preserves approximately 80% of human CD markers and 75% of mouse CD markers tested in lymphoid tissue while preserving good tissue morphology (please refer to our website, **www.bdbiosciences.com** for a listing of antibodies reactive with zinc-fixed tissue). Snap freezing is the most common method for preparing tissues for immunohistochemistry procedures. Quickly freezing tissues preserves the basic morphology (cellular detail and nuclear detail are lost), preserves most antigens, and produces a hard block of material that can be cut into sections for immunostaining.

## The Cryostat and Microtome

Tissues are cut using equipment with extremely sharp blades. For frozen sections, the cryostat is employed, while for paraffin sections, a microtome is used. For routine histologic work and immunostaining, sections are usually 4 – 5 µm thick.

# Frozen Sections

## Preparation of Frozen Tissues for Sectioning

### Materials Needed:

- 2-methylbutane (isopentane)
- Liquid nitrogen
- Dry ice
- Peel-Away® base molds
- Frozen tissue matrix (OCT® or Cryomatrix®)
- Long forceps
- Necropsy tools
- Superfrost Plus® slides

1. Label base mold and partially fill the mold with frozen tissue matrix.
2. Sacrifice animal by prescribed and approved euthanasia techniques.
3. Remove desired tissues, trim and cut tissue to no more than 5 mm in thickness. Place in pre-labeled base molds filled with frozen tissue matrix. Arrange tissue in the matrix near the bottom so tissue is easily exposed when sections are cut.
4. Place a stainless steel beaker of 2-methylbutane in liquid nitrogen and allow to cool adequately. Place base mold with tissue into the beaker of cold 2-methylbutane and quickly immerse the block. Allow the tissue matrix to solidify completely and remove block from 2-methylbutane and place on dry ice or in the –20°C cryostat.

   *Note*: If block is left in 2-methylbutane too long, the block may crack.
5. Store blocks in the –80°C freezer until ready for sectioning.

## Standard Cryostat Protocol

The following is a general protocol:

1. Before cutting sections, allow the temperature of the block to equilibrate to the temperature of the cryostat (–20°C).
2. Place the tissue block on the cryostat specimen disk. Adjust the positioning of the block to align the block with the knife blade. Face tissue block until the desired tissue is exposed.
3. Cut sections of the desired thickness (usually 5 µm) and place the sections on a Fisher Superfrost slide.


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## Fixation

1. Fix slides by immersion in cold acetone or other suitable fixative (eg, alcohol, formal alcohol, formalin, etc.) for 2 minutes (–20°C). Let air dry, then proceed to staining.
2. Alternatively, the dry, frozen-sectioned slides can be stored for a short period of time at –70°C in a sealed slide box. When ready to stain, remove slides from freezer and warm to –20°C in the cryostat or –20°C freezer, fix for 2 minutes in cold fixative (acetone or other suitable fixative) and allow to come to room temperature to continue with the staining.

## Standard Immunohistochemical Staining Procedure for Frozen Sections

1. Label slides with a solvent resistant pen and demarcate the tissue if required.
2. Rinse slides 3× in PBS, to remove the tissue-freezing matrix.
3. Block endogenous peroxidase activity by incubating the slides in **0.3%** $H_2O_2$ solution in PBS for 10 minutes.
4. Rinse slides 3× in PBS, 2 minutes each time.
5. Dilute the primary antibody to the desired concentration in the Antibody Diluent. Apply the diluted antibody to the tissue sections on the slide. Incubate for 1 hour at room temperature in a humidified chamber. Longer incubation with primary antibody may be required.

   *Note*: It is important to titrate the primary antibody before deciding on the correct dilution for each experiment.
6. Rinse slides 3× in PBS, 2 minutes each time.
7. Dilute the provided biotinylated anti-Ig secondary antibody 1:50 in the Antibody Diluent. Apply to the tissue sections on the slide and incubate for 30 minutes at room temperature.
8. Rinse slides 3× in PBS, 2 minutes each time.
9. Apply the Streptravidin-HRP to the tissue sections on the slide and incubate for 30 minutes at room temperature.
10. Rinse slides 3× in PBS, 2 minutes each time.
11. Prepare DAB substrate solution by adding 1 drop of DAB chromogen to every 1 ml of DAB buffer.

    *Note*: DAB is a suspect carcinogen. Handle with care. Wear gloves, lab coat, and eye protection.
12. Drain PBS from slides and apply the DAB substrate solution. Allow slides to incubate for 5 minutes or until the desired color intensity is reached.
13. Rinse slides 3× in water, 2 minutes each time.

14. Counterstain slides:
    a. Stain in hematoxylin solution for 30 – 60 seconds.
    b. Rinse slides thoroughly in water.
    c. Dip slides twice in Bluing Reagent or dilute ammonia water.
    d. Rinse slides thoroughly in water.
15. Dehydrate through 4 changes of alcohol (95%, 95%, 100% and 100%), for 5 min. each. Clear in 3 changes of xylene (or xylene substitute) and coverslip.

# Paraffin-embedded Sections

## Preparation of Tissues for Paraffin-embedded Sections

### Fixation of Tissues in 10% Neutral Buffered Formalin

Sacrifice animal by prescribed and approved euthanasia techniques. Tissues to be fixed and processed should be cut to a size no larger than 3 mm thick. Fix tissues in 10% formalin at room temperature for 8 hours but not to exceed 24 hours. For small rodent tissue, it is recommended to fix tissues for 4 – 8 hours prior to processing the tissue.

### Fixation of Tissues in Zinc Fixative

Many antigenic epitopes are masked or even destroyed by 10% formalin fixation. In some cases, fixation in a milder fixative such as Zinc fixative for IHC (Cat. No. 550523) is helpful to preserve the antigenic epitopes. Place freshly dissected tissues trimmed 3 mm thick into Zinc Fixative and allow tissues to fix for 24 – 48 hours at room temperature prior to processing.

## Paraffin-embedding Technique

### Standard Microtome Protocol

1. Paraffin sections are usually cut by trained histotechnologists in a histology or pathology laboratory.
2. Section paraffin blocks at the desired thickness (usually 4 – 5 µm) on a microtome and float on a water bath containing deionized or distilled water.
3. Transfer the sections onto a Superfrost Plus slide. Allow the slides to dry overnight and store slides at room temperature until ready for use.


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### Deparaffinization and Re-hydration of Tissue Slide

1. Place the slides in a 55°C oven for ten minutes to melt the paraffin. Deparaffinize slides in 2 changes of xylene or xylene substitute for 5 minutes each.
2. Transfer slides to 100% alcohol, 2 changes for 3 minutes each and transfer once through 95% alcohol for 3 minutes.
3. Block endogenous peroxidase activity by incubating sections in **3%** $H_2O_2$ solution in methanol for 10 minutes.
4. Rinse in PBS 2× for 5 minutes each time.
5. Continue with the standard immunohistochemical protocol for frozen sections listed on page 9 starting at step 5, unless antigen retrieval is necessary (see ***Protocol for Antigen Retrieval using BD Retrievagen A,*** page 11).

## Antigen Retrieval

Formalin fixation is used in routine tissue processing because it provides good morphology and architectural detail. 10% neutral buffered formalin fixes the tissue by cross-linking proteins that may cause a conformational change or “masking” of the antigenic epitope leading to reduced or absent antibody reactivity. Antigen retrieval with citrate, EDTA, urea, and Tris are commonly used to overcome epitope masking seen with formalin-fixed, paraffin-embedded sections. BD™ Retrievagen A (pH 6.0) (Cat. No. 550524) or BD™ Retrievagen B (pH 9.5) (Cat. No. 550527) can be effectively used to unmask many antigens in formalin-fixed paraffin sections.

## Protocol for Antigen Retrieval using BD™ Retrievagen A (pH 6.0) or BD™ Retrievagen B (pH 9.5)

1. Deparaffinize slides.
2. Place slides in a plastic coplin jar or staining dish with the BD Retrievagen A working solution and heat to 89°C (193°F) in a microwave.

   *Note*: Alternatively, pre-treat slides with Retrievagen A or B in a pressure cooker or autoclave at 120° – 125°C and 17 – 25 PSI for 5 minutes. When completed, open pressure cooker or autoclave. Allow slides to cool down for 20 – 30 minutes before removing them from the coplin jar. Wash slides in PBS for 5 minutes. Proceed with ***Standard Immunohistochemical Staining Procedure for Frozen Sections*** page 9 starting at step 5.
3. Mix the solution and incubate the slides at the desired temperature of 89°C for 10 minutes.
4. Remove the staining dish or coplin jar with slides, cover tightly, and allow the solution to slowly cool to room temperature for 20 min.

   *Note*: It is important to allow the temperature to slowly ramp down.
5. Wash slides with 2 – 3 changes of water and continue with application of the primary antibody, step 5 of the ***Standard Immunohistochemical Staining Procedure for Frozen Sections***, on page 9.

# Examples of Immunohistochemical Staining

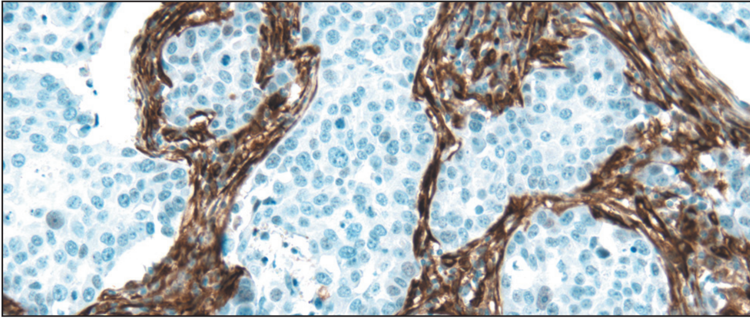

***Figure 1. Immunohistochemical staining for MMP-2.*** Paraffin sections of human breast cancer tissue were stained with the A-Gel VC2 antibody specific for human MMP-2. The BD Pharmingen™ Anti-Mouse Ig HRP Detection Kit (Cat. No. 551011) was used to develop the signal. Cells positive for MMP-2 can be identified by the brown staining. Magnification 20×.

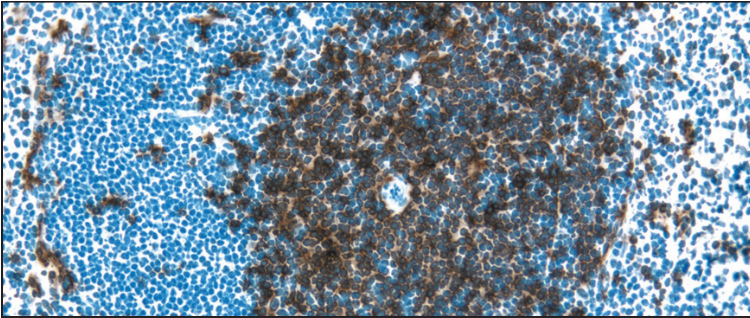

***Figure 2. Immunohistochemical staining of $CD8^+$ T lymphocytes.*** Frozen sections of normal mouse spleen were stained with 53-6.7 antibody specific for mouse CD8a. The BD Pharmingen™ Anti-Rat Ig HRP Detection Kit (Cat.No. 551013) was used to develop the signal. $CD8^+$ T lymphocytes can be identified by the intense brown labeling of their cell-surface membranes. Magnification 20×.

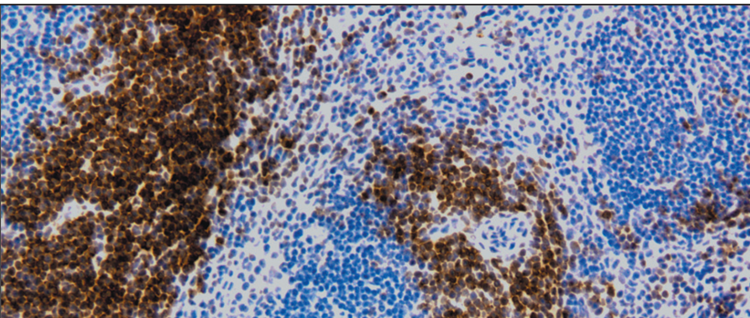

***Figure 3. Immunohistochemical staining of $CD3^+$ T lymphocytes.*** The frozen section of normal mouse spleen was stained with 145-2C11 mAb specific for mouse CD3e. The BD Pharmingen™ Anti–Hamster Ig HRP Detection Kit (Cat. No. 551012) was used to develop the signal. Note the brown labeling of cell-surface membranes of lymphocytes in the periarteriolar sheath. Magnification 20×.



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# Artifacts and Troubleshooting

Artifacts can be grouped into fixation, processing, or staining artifacts. The most common fixation artifacts are inadequate fixation that results in poor tissue morphology, over fixation that causes loss of antigenicity, and the use of non-buffered formalin that causes a brown formalin precipitate to be formed. Processing artifacts result from the tissue being too thick or the processing time being too short. Failure to remove all the water from the tissue results in areas of the tissues that are incompletely permeated by paraffin and these regions tear and fall from the slide leaving holes in the section. Some of the staining problems are listed below.

## Overstaining or Weak Staining

Overstaining or weak staining are the most common staining difficulties encountered in immunohistochemistry. It is crucial to titrate the antibody in the recommended range before performing the experiment. The incubation time and the temperature also affect staining intensity. Routine incubations with primary and secondary antibodies are performed at room temperature for 1 hour.

Weak staining may be due to a variety of other problems such as:

a. The primary antibody does not recognize the antigen after the fixation.

b. Incomplete embedding or antigen retrieval procedures.

c. Incompatibility of counterstain and mounting media (chromogen reaction product is dissolved).

d. Reagents are past their expiration date.

## Non-specific Background Staining

Excessive staining in structures of the tissue that should be negative is termed non-specific background staining and may be due to any of the following reasons:

a. Rinsing between steps was inadequate.

b. Tissue was allowed to dry with reagents on.

c. Tissue contains endogenous biotin or endogenous enzyme.

To avoid non-specific background staining, ensure adequate slide washing between steps. If tissue has endogenous biotin, it may be blocked by an avidin-biotin block or a suitable protein block may be used.

# Appendix

## Reagents for Immunohistochemical Staining*

### Kits

| Specificity | Application | Cat. No. |
|---|---|---|
| Anti-hamster Ig HRP Detection Kit | Fr, F, Zn | 551012 |
| Anti-mouse Ig HRP Detection Kit | Fr, F, Zn | 551011 |
| Anti-rat Ig HRP Detection Kit | Fr, F, Zn | 551013 |
| BrdU *In-Situ* Detection Kit I (50 slides) | Fr, F, Zn | 550803 |
| BrdU *In-Situ* Detection Kit II (200 slides) | Fr, F, Zn | 551321 |

### Detection Systems and Substrates

| Specificity | Application | Cat. No. |
|---|---|---|
| AEC Substrate Kit | Fr, F, Zn | 551015 |
| BCIP/INT Substrate Kit | Fr, F, Zn | 551229 |
| BCIP/NBT Substrate Kit | Fr, F, Zn | 551009 |
| DAB Substrate Kit | Fr, F, Zn | 550880 |
| Streptavidin-AKP Pre-diluted | Fr, F, Zn | 551008 |
| Streptavidin-HRP Pre-diluted | Fr, F, Zn | 550946 |

### Buffers and Fixatives

| Specificity | Application | Cat. No. |
|---|---|---|
| Antibody Diluent for IHC | Fr, F, Zn | 559148 |
| BD Retrievagen A (pH 6.0) | F, Zn | 550524 |
| BD Retrievagen B (pH 9.5) | F, Zn | 550527 |
| BrdU | | 550891 |
| Immunocytochemistry Diluent Buffer | ICC | 550009 |
| Immunocytochemistry Fixation Buffer | ICC | 550010 |
| IHC Zinc Fixative | Zn | 550523 |

**Fr**: Frozen
**F**: Formalin–fixed paraffin
**ICC**: Immunocytochemistry
**Zn**: Zinc–fixed paraffin

***:** For current listing please visit us at **www.bdbiosciences.com**


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