invitrogen™

BioModule™ Immunohistochemical (IHC) Staining for Tissues

For highly sensitive and specific immunohistochemical staining of tissues

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User Manual

Table of Contents

Experienced Users Procedure

Introduction

A brief experienced user's procedure for immunohistochemical staining of tissues is described below. Refer to this manual for a detailed protocol. Perform all steps at room temperature unless another temperature is specified.

Step	Procedure	Incubation Time
Sample Preparation		
Prepare Slides	Pre-coat slides with HistoGrip™ (page 25), 0.1% poly-L-lysine, or purchase commercially available pre-coated glass slides.	
Preparing Frozen Tissue Samples	A sample protocol for preparing frozen tissue samples is described below. If you have optimized protocols in the laboratory for your sample type, use the optimized protocol. 1. Snap freeze fresh tissues in cryomolds containing OCT® (Optimal Cutting Temperature) compound. 2. Cut 4-6 µm cryostat sections and mount on coated glass slides. 3. Dry tissue sections at room temperature. 4. Place the slides in 100% acetone at 4ºC to fix the sections. 5. Air dry slides. 6. Circle each tissue section using the Mini PAP Pen. 7. Rinse slides in 1X PBS. 8. Proceed immediately to **Peroxidase Quenching**, next page.	30 min. 10 min. 10-30 min. 10 min.
Deparaffinization and Rehydration	A sample protocol for deparaffinization and rehydration is described below. If you have optimized protocols in the laboratory for your sample type, use the optimized protocol. Perform this step for formalin-fixed paraffin embedded tissues only. 1. Place slides in xylene. 2. Place slides in 100% ethanol. 3. Place slides in 95% ethanol. 4. Place slides in 80% ethanol. 5. Remove slides and place in 1X PBS. 6. Circle each tissue section using the Mini PAP Pen after wiping the area near the tissue sections with a laboratory wipe. 7. Proceed immediately to **Peroxidase Quenching**, next page. If you need to perform Epitope Retrieval Protocol, see page 16.	2 x 5 min. 2 x 5 min. 5 min. 5 min. 10 min.

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Experienced Users Procedure, Continued

Step	Procedure	Incubation Time
Immunohistochemical Staining Procedure		
Peroxidase Quenching (optional)	1. Treat **frozen tissue** slides with 100-200 µl Peroxo-Block™. Treat **formalin-fixed paraffin embedded tissue** slides with 100-200 µl Peroxo-Block™. 2. Wash slides with 1X PBS.	45 seconds 1-2 min. 3 x 2 min.
Incubate with Primary Antibody	3. Apply 100 µl of the appropriately diluted primary antibody to slides. 4. Incubate the slides in a humidified chamber. 5. Wash slides with 1X PBS containing 0.05% Tween-20.	30 min. 3 x 2 min.
Incubate with HRP Polymer Conjugate	6. Apply 2 drops (100 µl) of the ready-to-use HRP (horse radish peroxidase) Polymer Conjugate (Reagent A) to slides. 7. Incubate the slides in a humidified chamber. 8. Wash slides with 1X PBS containing 0.05% Tween-20.	10 min. 3 x 2 min.
Add DAB Chromogen	9. In a sterile microcentrifuge tube, add 1 drop Reagent B1, 1 drop Reagent B2, and 1 drop Reagent B3 to 1 ml distilled water to prepare diluted DAB (3, 3′ diaminobenzidine) Chromogen just prior to use. Mix well. Protect from light and use within 1 hour. 10. Apply 2 drops (100 µl) diluted DAB chromogen to slides. **Caution:** DAB is a known carcinogen; handle with care. 11. Incubate the slides in a humidified chamber. 12. Rinse thoroughly with distilled water.	5 min.
Counterstaining	13. Apply 2 drops (100 µl) Hematoxylin Counterstain Reagent to slides. 14. Incubate the slides in a humidified chamber. 15. Rinse thoroughly with distilled water. 16. Dip the slide in ammonia water (0.25% NH_3) or 1X PBS to blue the nuclei. 17. Rinse thoroughly with distilled water. 18. Dehydrate through increasing concentrations of ethanol (95%, 100%) for 10-20 dips each. Clear in 2 changes of xylene or xylene substitutes for 10-20 dips each.	1-3 min.
Mounting	19. Apply 2-4 drops (100-200 µl) of Histomount™ mounting medium to slides. 20. Apply a cover slip on the slide. Allow the medium to dry at room temperature overnight.	
Microscopy	Evaluate the results by examining the slides using a light microscope at 20x magnification. Interpret the results using various controls included in the experiment.	

Kit Contents and Storage

Shipping and Storage

The shipping condition for each component is listed in the table below. Upon receipt, store the components as described below.

Box	Components	Shipping	Storage
1	Peroxo-Block™	Blue ice	2ºC to 8ºC
	SuperPicTure™ Polymer Detection Kit	Blue ice	2ºC to 8ºC
	Antibody Diluent Solution	Blue ice	2ºC to 8ºC
	Phosphate Buffered Saline (PBS), Powder	Blue ice	2ºC to 8ºC or room temperature
	50% Tween-20	Blue ice	Room temperature
	Histomount™	Blue ice	2ºC to 8ºC or room temperature (protect from light and flame)
	Hematoxylin Counterstain Reagent	Blue ice	2ºC to 8ºC or room temperature (protect from light)
2	Mini PAP Pen	Room temperature	Room temperature

Kit Contents

The components included with the BioModule™ Immunohistochemical (IHC) Staining for Tissue are described below. Sufficient reagents are provided to perform staining for 150 slides.

Item	Composition	Amount
Peroxo-Block™	Ready-to-use solution of an oxidizing agent	2 x 18 ml
SuperPicTure™ Polymer Detection Kit	Kit contains: Reagent A: Ready to use HRP (horse radish peroxidase) Polymer Conjugate Reagent B1: 20X Buffer/Substrate Reagent B2: 20X DAB (3, 3' diaminobenzidine) chromogen in methanol Reagent B3: 20X 0.6% H_2O_2	 15 ml 3 ml 3 ml 3 ml
Phosphate Buffered Saline (PBS), Powder	10 mM phosphate buffer, pH 7.2-7.3, 150 mM NaCl	12 packages
Antibody Diluent Solution	1X PBS, pH 7.4, BSA, and 0.05% sodium azide	2 x 50 ml
50% Tween-20	50% Tween-20 solution	20 ml
Histomount™	Organic mounting medium	2 x 15 ml
Hematoxylin Counterstain Reagent	Ready-to-use solution	2 x 18 ml
Mini PAP Pen	--	1

Note

Some reagents in the unit may be provided in excess of the amount needed.

Introduction

Overview

Introduction

The BioModule™ Immunohistochemical (IHC) Staining for Tissue provides qualified reagents and validated protocols to perform highly sensitive and specific immunohistochemical staining of tissues. For details on IHC, see next page.

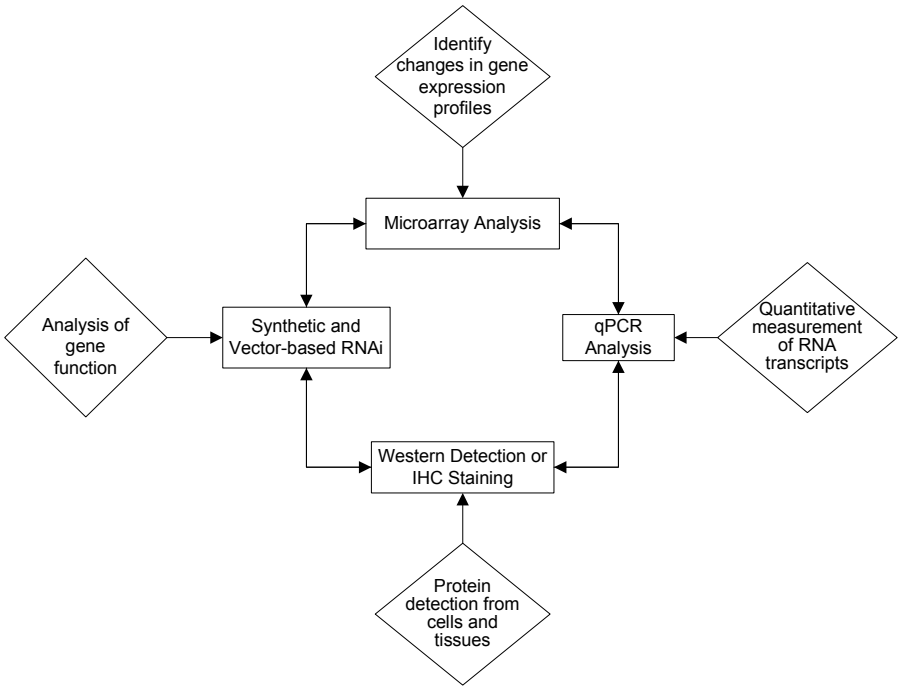
The key component of Bio-Module™ IHC Staining Unit for Tissues is the *SuperPicTure*™ Polymer Detection Kit employing HRP detection system to detect mouse, rabbit, rat, or guinea pig primary antibodies bound to tissue antigens (see page 4 for details). The BioModule™ IHC Staining for Tissues is designed for use with frozen and formalin-fixed paraffin embedded tissues.

In addition to the immunohistochemical staining reagents, the BioModule™ IHC Staining Unit for Tissues also includes several key reagents for peroxidase quenching, counterstaining, and mounting.

BioModule™ Units for Gene Expression Profiling

The Bio-Module™ IHC Staining Unit for Tissues is one of the several BioModule™ Units available from Invitrogen (page 25) for gene expression profiling. Each of the BioModule™ Units for gene expression profiling includes high-quality reagents and validated protocols with relevant controls for each step of the workflow (see below). Each unit is designed to provide an integrated workflow that allows you to perform various steps seamlessly during expression analysis.

Gene expression profiling comprises multiple steps employing various technologies such as microarray analysis or quantitative PCR (qPCR) for analysis at the nucleic acid level; western immunodetection and immunohistochemistry for analysis at the protein level; and RNAi for functional analysis.



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Overview, Continued

IHC

Immunohistochemical (IHC) Staining allows you to detect antigens in a tissue fixed on a glass slide. The BioModule™ IHC Staining Unit utilizes indirect staining method to detect antigens and includes the following steps:

1. Specific antigen on the tissue binds to unlabeled primary antibody.
2. Labeled secondary antibody conjugated to an enzyme (HRP) binds to the antigen-antibody complex, thus amplifying the signal greater than direct staining methods.
3. Enzyme (HRP) forms a colored (brown), insoluble precipitate at the antigenic sites in the presence of a substrate (H_2O_2) using a chromogen, DAB (3, 3'-diaminobenzidine) that is easily visualized with light microscopy.

IHC staining is simple and easy to perform at the benchtop without the need for any specific instrumentation and allows you to detect antigens in context of tissue morphology. The colored precipitate from DAB is stable for several years providing a permanent record.

System Components

The BioModule™ IHC Staining Unit includes:

- *SuperPicTure*™ Polymer Detection Kit for highly sensitive and specific immunohistochemical staining of tissue samples using HRP.
- Peroxo-Block™, a specific inhibitor of endogenous peroxidase activity, to effectively eliminate endogenous peroxidase activity without interfering with specific immunostaining.
- Antibody Diluent for diluting primary antibodies.
- PBS Powder and 50% Tween-20 to prepare washing buffer.
- Mini PAP pen draws a water repellent circle around slide mounted tissue, preventing any wasting of valuable reagents by keeping the liquid pooled in a single droplet.
- Hematoxylin Counterstain Reagent for brilliant staining of nuclei without interfering with the chromogen signal.
- Histomount™, an organic based mounting medium, for immunohistochemical procedures.

For more information about each component, see page 4.

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Overview, Continued

SuperPicTure™ Polymer Detection Kit

The *SuperPicTure*™ Polymer Detection Kit features Zymed's proprietary and enhanced HRP polymer technology to provide excellent sensitivity, high specificity, and a faster protocol than conventional immunohistochemical staining methods. The enhanced HRP polymer technology provides intense nuclear, cytoplasmic, and membrane antigen staining compared to other polymer systems which produce weak nuclear staining due to compromised cell penetration. The *SuperPicTure*™ Polymer Detection Kit includes all reagents for immunohistochemical staining including buffer, HRP Polymer Conjugate, DAB chromogen, and substrate. See next page for details.

System Overview

After formalin-fixed, paraffin-embedded tissue sections are deparaffinized in xylene and dehydrated in a graded series of ethanol, an endogenous peroxidase quenching step is performed using Peroxo-Block™. No blocking step is required. After incubating the primary antibody on the tissue, the HRP polymer conjugate is added, and excess reagents are removed with a wash step. The DAB chromogen is added, and the peroxidase catalyzes the substrate (hydrogen peroxide) and converts the chromogen to a brown deposit, allowing you to visualize the location of the antigen.

Advantages

Using the BioModule™ IHC Staining Unit for Tissues offers the following advantages:

- Includes qualified reagents and validated protocols to provide consistent results
- Use of the *SuperPicTure*™ Polymer Detection Kit allows fast and easy IHC staining protocol than conventional LAB-SA protocol
- Provides highly sensitive and specific immunohistochemical staining using HRP detection system
- Use of HRP Polymer conjugated to the second antibody eliminates one incubation step, resulting in a shorter, faster protocol
- Absence of biotin or streptavidin avoids potential background problems associated with endogenous biotin activity

Purpose of this Manual

This manual provides the following information:

- An overview of immunohistochemical staining
- General guidelines for sample preparation
- Immunohistochemical staining protocol
- Example of expected results
- Troubleshooting

Description of Components

Introduction

Brief description of the components included with the BioModule™ IHC Staining Unit for Tissues is described in this section.

SuperPicTure™ Polymer Detection Kit

SuperPicTure™ Kit is one-step polymer detection kit using HRP and DAB for increased sensitivity. The kit employs a stable amino acid polymer conjugate containing multiple HRP's that react with mouse, rabbit, guinea pig, and rat primary antibodies. Since the *SuperPicTure*™ Kit does not contain biotin or streptavidin, there is no background due to endogenous biotin activity. The *SuperPicTure*™ Kit is designed for use with frozen or formalin-fixed paraffin embedded tissues and user supplied primary antibodies.

The *SuperPicTure*™ Kit contains the HRP Polymer Conjugate, 20X DAB substrate solution, 20X buffer for diluting the substrate, and 20X 0.6% H_2O_2.

Peroxo-Block™

Peroxo-Block™ is a ready-to-use solution containing specific inhibitor of endogenous peroxidase activity. The Peroxo-Block™ is specially formulated for frozen or formalin-fixed paraffin embedded tissues and effectively eliminates endogenous peroxidase activity in less than a minute without interfering with specific immunostaining.

Hematoxylin Counterstain Reagent

Hematoxylin Counterstain Reagent is a ready-to-use, bluish/purple counterstain for routine nuclear staining and can be used with many chromogens including DAB. Hematoxylin Counterstain Reagent stains nuclei brilliantly without interfering with the chromogen signal. The reagent produces a brilliant blue nuclear counterstain for immunohistochemistry.

Histomount™

Histomount™ is a ready-to-use organic based mounting medium for immunohistochemical procedures and ideal for preserving organic insoluble chromogens such as DAB. Samples preserved in Histomount™ are stable for several years.

Mini PAP Pen

The Mini PAP Pen allows you to draw a water repellent circle around the tissue mounted on a slide, keeping the liquid pooled in a single droplet. This prevents any wastage of valuable reagents and ensures even staining of the tissue. The Mini PAP Pen is especially useful when working with multiple sections on a single slide. Each pen can be used to draw about 400 circles.

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PBS and Antibody Diluent

PBS

Use the PBS powder included with the kit to prepare 1X PBS (10 mM phosphate buffer, pH 7.2-7.3 with 150 mM NaCl). The PBS with 0.05% Tween-20 is used for some washing steps during the staining protocol.

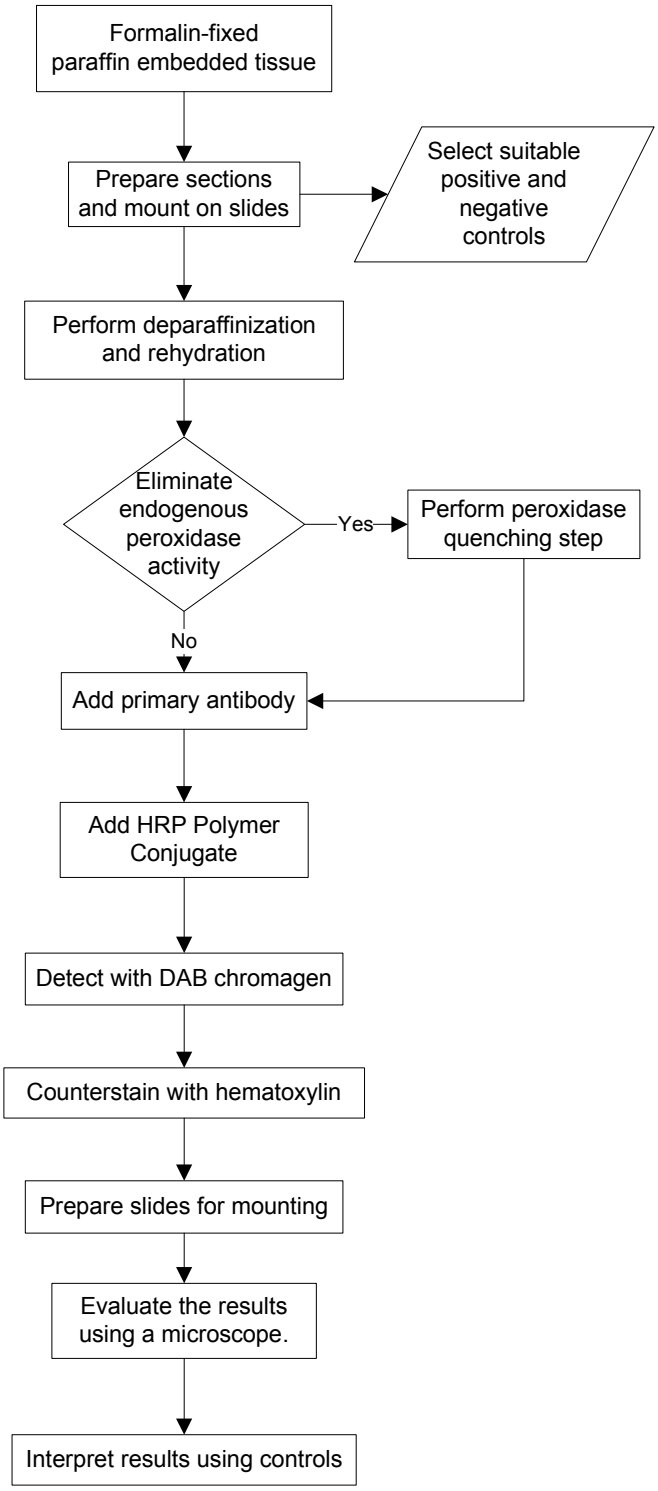
Antibody Diluent

The Antibody Diluent is a ready-to-use solution of 1X PBS with BSA (for stabilizing the antibody) and preservative. Dilute your primary antibody using the Antibody Diluent, if you are not using a pre-diluted antibody such as the Zymed® *2nd Gen* antibodies (page 25).

Experimental Overview

Workflow

An experimental workflow for using the BioModule™ IHC Staining Unit with formalin-fixed paraffin embedded tissues is shown below.



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Experimental Overview, Continued

Materials Needed

Materials supplied with the BioModule™ IHC Staining Unit for Tissues and User Supplied materials are listed below. Ordering information is on page 25.

Step	Supplied in the kit	User Supplied
Sample Preparation	PBS	• Tissue Sample (fresh, frozen, or formalin-fixed paraffin embedded tissue) • Coplin jars or staining containers **For Frozen sections** • OCT® (Optimal Cutting Temperature) Compound • Chilled 100% acetone for fixing • Pre-coated glass slides **For formalin-fixed paraffin embedded sections** • Xylene • Graded series of ethanol (80%, 95% and 100% ethanol) • 55ºC oven
Peroxidase Quenching	• Peroxo-Block™ • PBS	• *Optional:* 3% hydrogen peroxide in methanol
Immunohistochemical Staining	• *SuperPicTure*™ Polymer Detection Kit • PBS and 50% Tween-20 • Antibody Diluent • Mini PAP Pen	• Primary antibody • Distilled water • Coplin jars or staining containers • Humidified chamber
Counterstaining	• Hematoxylin Counterstain Reagent	• Xylene or xylene substitutes • Ethanol (95% and 100%)
Mounting	Histomount™	Coverslips
Microscopy		Light Microscope

Methods

General Guidelines

Introduction

General guidelines for using the BioModule™ IHC Staining Unit for Tissues are described in this section. Review the information in this section prior to performing immunohistochemical staining to obtain the best results.

Note

- Most of the reagents included with the BioModule™ IHC Staining Unit for Tissues are supplied as ready-to-use solutions without the need for any preparation or dilution and are supplied in a dropper bottle that allows easy dispensing of reagents. We do not recommend diluting the reagents beyond the concentration supplied or removing the reagents from dropper bottles. Do not pipette reagents directly from the bottle.
- If you are performing immunohistochemical staining using mouse or rat primary antibodies on mouse or rat tissues, respectively, we recommend that you use HistoMouse™-Max Kit (page 25) to prevent background problems and obtain best results.

Handle human origin products according to biosafety practices as outlined for your institution.

Starting Material

The BioModule™ IHC Staining Unit for Tissues is designed for use with frozen or formalin-fixed paraffin embedded tissue sections mounted on slides using primary antibodies.

Based on your starting sample material, you may need to perform some sample preparation steps described on page 11, prior to staining the tissue sections.

Fixatives

Appropriate tissue and antigen fixation is required to obtain reproducible performance and reliable interpretations.

Suitable fixatives for most antigens of clinical significance include 10% neutral buffered formalin, B5, Bouin's, Zinc formalin or alcohol-base fixatives. Formalin-fixed tissues post-fixed in B5 before paraffin embedding may show improved stain.

Cell smears prepared from body fluids should be made to assure a monolayer of cells. Multilayers of cells can trap staining reagents and interfere with the interpretation of results. Fix smears immediately after preparation. Depending on the properties of the antigen, cell smears are usually stable for 1-2 weeks when stored at 4°C.

Continued on next page

General Guidelines, Continued

Primary Antibody

The BioModule™ IHC Staining Unit for Tissues is compatible for use with any mouse, rabbit, guinea pig, and rat primary antibodies. A large variety of high-quality antibodies including the Zymed® Antibodies is available from Invitrogen for use in immunohistochemistry. Prediluted *2nd Gen* primary antibodies that are ready-to-use and titered for use with the *SuperPicTure*™ Polymer Detection Kit are also available. *2nd Gen* primary antibodies contain a general protein blocker eliminating the need for a separate blocking step. For details, visit www.invitrogen.com or contact Technical Service (page 24).

Polyclonal or monoclonal antibodies can be used with the kit. For higher specificity, we recommend using monoclonal antibodies. Select antibodies that can detect low antigen levels and can recognize epitopes from formalin-fixed paraffin embedded tissues.

The optimal antibody concentration for use with immunohistochemistry is usually recommended by the antibody manufacturer or you may determine the optimal concentration using a checkerboard titration experiment.

Peroxidase Quenching

Since the BioModule™ IHC Staining Unit for Tissues utilizes HRP detection system, tissues exhibiting high endogenous peroxidase activity will cause high background. Usually the endogenous peroxidase activity is inhibited (quenched) using H_2O_2 pre-treatment. The BioModule™ IHC Staining Unit for Tissues includes Peroxo-Block™, a ready-to-use efficient peroxidase inhibitor.

Because different tissues have different levels of endogenous peroxidase activity, you may need to perform the peroxidase quenching step, only if your tissue exhibits high endogenous peroxidase activity.

To assess the endogenous peroxidase activity in your tissue, hydrate the slide containing tissue sections in PBS. Apply the DAB substrate, incubate for 10 minutes, and wash with distilled water. Examine the slide under the microscope to see any staining. If there is staining, you need to perform the peroxidase quenching step as described on page 15 using Peroxo-Block™.

Epitope Retrieval

Antigens that are masked by formalin fixation and embedding procedures can be retrieved using standard proteolytic or heat treatment procedures prior to performing the immunohistochemical staining. Some antibodies require epitope retrieval as recommended by the antibody manufacturer while staining with some antibodies is enhanced by epitope retrieval. See page 16 for sample epitope retrieval protocols.

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Appropriate Controls

When performing immunohistochemical staining, it is important to include proper positive and negative controls to help evaluate your results.

We recommend including the following three control slides that are necessary for interpreting results. Additional controls can be added based on the experimental design.

- **Positive Tissue Control**

  A specimen processed in the same way as the unknown and contains the antigen to be stained.

- **Reagent Control**

  An additional slide that is treated with a non-immune serum or isotype control antibody that matches the isotype of the experimental antibody instead of the same concentration of primary antibody. Any staining observed on the specimen is probably due to non-specific protein binding or non-specific binding of other reagents.

  Rabbit and mouse primary antibody isotype controls are available from Invitrogen (page 25).

- **Negative Control**

  A specimen processed in the same way as the unknown but does not contain the antigen to be stained (optional).

Sample Preparation

Introduction

This section provides general guidelines for sample preparation to perform immunohistochemical staining. Proper sample preparation is key to the success of an immunohistochemical staining experiment. Based on your starting sample material, you may need to perform some sample preparation steps described below, prior to staining the sample.

Detailed protocols for preparing frozen or formalin-fixed paraffin embedded tissues or tissue sectioning are not included in this manual.

Materials Needed

You will need the following materials:

- Fresh, frozen tissue, or paraffin embedded tissue of choice
- Microtome and cryostat for tissue sectioning
- Pre-coated slides (see below)
- Coplin jars or equivalent
- PBS (supplied with the kit)

For Frozen sections

- OCT® Compound
- Chilled 100% acetone for fixing

For formalin-fixed paraffin embedded sections

- Xylene
- Graded series of ethanol (80%, 95% and 100% ethanol)
- 55ºC oven

Slide Preparation

If you are preparing your own slides, pre-coat slides with HistoGrip™ (page 25) or 0.1% poly-L-lysine in water, then air dry. Commercially available pre-coated glass slides are available and can be used to mount frozen or formalin-fixed paraffin embedded tissue sections.

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Sample Preparation, Continued

Frozen Tissue

A sample protocol for preparing frozen tissue samples is described below. If you have optimized protocols in the laboratory for your sample type, use the optimized protocol.

1. Chill 100% acetone at 4ºC (required for Step 7, below).
2. Obtain fresh tissue.
3. Snap freeze fresh tissues in cryomolds containing OCT® (Optimal Cutting Temperature) compound (a solution of glycols and resins which provides an inert matrix for sectioning). Store frozen tissue blocks at -70ºC until you are ready for tissue sectioning.
4. For sectioning, allow the frozen tissue block to equilibrate to the cryostat temperature.
5. Cut 4-6 µm cryostat sections and mount on coated glass slides.
6. Dry tissue sections at room temperature for 30 minutes. If desired, store slides at -70ºC before fixing. If slides are stored at -70ºC, warm the slides to room temperature before the fixing step.
7. Place the slides in 100% acetone at 4ºC for 10 minutes to fix the sections.
8. Remove slides from acetone and air dry for 10-30 minutes.
9. Circle each tissue section using the Mini PAP Pen.
10. Store at -70ºC until use or wash the slide in PBS for 10 minutes and proceed immediately to **Peroxidase Quenching** (page 15).

Paraffin Embedded Sections-- Deparaffinization and Rehydration

To use the formalin-fixed paraffin embedded sections for immunohistochemical staining, you need to perform the deparaffinization with xylene and rehydration in a graded series of alcohol as described in the sample protocol below.

1. Obtain or prepare the formalin-fixed paraffin embedded sections of choice.
2. Dry slides containing 4 µm formalin-fixed paraffin embedded sections in a 55ºC oven for 2 hours or overnight (do not allow the temperature to exceed 60ºC). Store the slides containing the formalin-fixed paraffin embedded tissue sections at room temperature until needed.
3. Place slides in xylene for 5 minutes at room temperature.
4. Remove slides and place in xylene a second time for an additional 5 minutes.
5. Remove slides and place in 100% ethanol 2 times for 5 minutes each time.
6. Remove slides and place in 95% ethanol for 5 minutes.
7. Remove slides and place in 80% ethanol for 5 minutes.
8. Remove slides and place in PBS for 10 minutes.
9. Drain any excess reagent by tapping the edge of the slide on paper towels and wipe the area near the tissue sections with a laboratory wipe.
10. Circle each tissue section using the Mini PAP Pen.
11. Proceed immediately to **Peroxidase Quenching** (page 15).

Immunohistochemical Staining

Introduction

Immunohistochemical staining procedure using the *SuperPicture*™ Polymer Detection Kit is described below.

The total staining time including counterstaining is ~80 minutes.

Experimental Outline

For immunohistochemical staining of your samples, you will:

1. Perform peroxidase quenching step using the Peroxo-Block™, if your sample exhibits high endogenous peroxidase activity.
2. Incubate the samples with an appropriate dilution of the primary antibody.
3. Wash any unbound antibody.
4. Incubate the samples with HRP Polymer Conjugate.
5. Wash any unbound polymer conjugate.
6. Add diluted DAB chromogen to develop the signal.
7. Perform counterstaining with hematoxylin.
8. Mount the slides using Histomount™.
9. Visualize the staining using a light microscope.

To obtain the best results, follow these recommendations:

- Perform all steps at room temperature, unless a different temperature is specified.
- Always wear protective clothing, safety glasses, and gloves while handling the slides and reagents.
- **Do not** allow the slides to dry once the staining procedure has started.
- To avoid dislodging the tissue cores, handle the slides gently, especially during the washing steps.
- Perform the staining procedure using Coplin jars, staining dishes or automated instruments.
- Perform all incubation steps with various solutions in a humidified chamber.
- Be sure to run appropriate controls (see page 10 for details on controls).
- Determine the optimal titer of the antibody through serial dilutions (page 9).
- Apply enough reagents to cover the tissue during reagent incubation steps; usually ~100-200 µl.
- Drain any excess reagents after washing by tapping the edge of the slide gently on the jar or on paper towels for a few seconds.

Continued on next page

Immunohistochemical Staining, Continued

Materials Needed

You will need the following materials:

- Tissue sample (see page 11 for sample preparation)
- Primary antibody
- Distilled water
- Xylene or xylene substitutes
- Ethanol (95% and 100%)
- Coplin jars or equivalent
- Humidified chamber
- Coverslips
- *Optional:* 3% hydrogen peroxide in methanol

For Epitope Retrieval

- Hot plate
- 20X Citrate buffer, pH 6.0 (page 25)
- 20 X EDTA Solution, pH 8.0 (page 25, for antibodies that require EDTA instead of citrate buffer)
- *Optional:* Digest-All™ Kit (page 25) for enzymatic digestion

Components Supplied with the Kit

The following components are supplied with the kit:

- Peroxo-Block™
- *SuperPicTure*™ Polymer Detection Kit
- PBS and 50% Tween-20
- Antibody Diluent
- Hematoxylin Counterstain Reagent
- Histomount™

Mini PAP Pen

Preparing PBS

PBS

PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.2-7.3) is supplied as a dry powder in the kit. To prepare 1X PBS, dissolve one envelope (package) in 1000 ml distilled water and mix well. Store at room temperature until use.

PBS with 0.05% Tween-20

To prepare 1 L PBS with 0.05% Tween-20, add 1 ml 50% Tween-20 supplied with the kit to 1000 ml 1X PBS. Mix well and store at room temperature until use.

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Peroxidase Quenching

Peroxidase quenching is optional. Perform this step if elimination of endogenous peroxidase activity is necessary (see page 9 for details).

Frozen Tissues

1. Treat frozen tissue slides (Step 9, page 12) with 100-200 µl Peroxo-Block™ or enough to cover the tissue section for 45 seconds. **Do not incubate for more than 45 seconds.**
2. Wash slides 3 times with 1X PBS for 2 minutes, each time.
3. Proceed immediately to **Incubating with Primary Antibody**, page 17.

Formalin-fixed paraffin embedded Tissues

1. Treat formalin-fixed paraffin embedded tissue slides (Step 10, page 12) with100-200 µl Peroxo-Block™ or enough to cover the tissue section for 1-2 minutes.
2. Wash slides 3 times with 1X PBS for 2 minutes, each time.
3. Proceed immediately to **Incubating with Primary Antibody**, page 17 or proceed to **Epitope Retrieval Protocol**, next page.

Note

Peroxo-Block™ works efficiently on many tissues, especially with formalin-fixed paraffin embedded tissues, but with some frozen tissues, you may need to use 3% H_2O_2 to obtain optimal results (better morphology or lower background). To use 3% H_2O_2 for blocking endogenous peroxidase activity, incubate the slides in 3% H_2O_2 in methanol for 10 minutes. Wash slides 3 times with 1X PBS for 2 minutes, each time and then proceed to primary antibody incubation.

Continued on next page

Epitope Retrieval Protocol

A sample epitope retrieval protocol developed for some Zymed® Antibodies is described below. The epitope retrieval protocol is used to reverse the loss of antigenicity that occurs with some epitopes in formalin-fixed paraffin embedded tissues. Some Zymed® antibodies may require epitope retrieval protocol (*e.g.*, estrogen receptor, and Ki-67 antibodies), while the staining of many other antibodies may be enhanced by epitope retrieval protocol (*e.g.*, S-100, cytokeratin, and synaptophysin antibodies).

You may use any other epitope retrieval protocol recommended by the supplier of your primary antibody.

Heat Induced Epitope Retrieval Protocol

1. Perform this epitope retrieval protocol after **Peroxidase Quenching** (page 15).

   Note: You may use citrate buffer (page 25) or EDTA solution (page 25) depending on the antibody manufacturer's recommendation.

2. Based on the reagent that you are using, dilute the reagent to 1X as follows:

   To 25 ml 20X Citrate Buffer, pH 6.0 solution, add 475 ml distilled water to obtain a 1X Citrate Buffer, pH 6.0 solution.

   To 25 ml 20X EDTA solution, pH 8.0, add 475 ml distilled water to obtain a 1X EDTA solution, pH 8.0.

3. Place the slides in a slide rack and place the rack in a 1 L glass beaker containing 500 ml diluted citrate buffer (1X), pH 6.0 or diluted EDTA (1X) solution, pH 8.0.
4. Place the beaker with slides on a hot plate. Heat the solution until it boils and continue boiling the solution for 15 minutes.
5. Remove beaker from the hot plate and allow the contents to cool for 25 minutes at room temperature.
6. Rinse slides with 1X PBS and proceed immediately to **Incubation with Primary Antibody** (next page).

Epitope Retrieval Protocol Using Enzymatic Digestion

Brief protocol using Digest-All™ Kit (page 25) is described below. The Digest-All™ is a flexible and standardized system for tissue digestion and contains trypsin, ficin, and pepsin proteolytic enzymes. The degree of digestion is based on a standardized 10 minute incubation at 37°C. The trypsin solution is supplied with diluent so that different trypsin concentrations can be made to generate a range of digestion activity between that of Ficin and Pepsin.

1. Perform this epitope retrieval protocol after **Peroxidase Quenching** (page 15).
2. Add the digestion enzyme of choice to your tissue sections

   Ficin and pepsin may be applied directly from the bottle. Trypsin requires dilution: 1 drop Trypsin concentrate to 3 drops Trypsin Diluent. Mix well and apply to tissue sections.

3. Incubate for 10 minutes at 37°C.
4. Wash slides in several changes of 1X PBS and proceed immediately to **Incubation with Primary Antibody** (next page).

Continued on next page

Immunohistochemical Staining, Continued

Incubation with Primary Antibody

For best results, primary antibody dilution and incubation times need to be determined empirically and are dependent on sample preparation, antibody affinity, amount of antigen present, and antigen accessibility. Refer to the **Primary Antibody** section on page 9 for more details.

The HRP Polymer Conjugate will react with mouse, rabbit, guinea pig, and rat primary antibodies.

1. Dilute primary antibody in Antibody Diluent as recommended by the antibody manufacturer or as determined by a titration experiment. Alternatively, you may use prediluted *2nd Gen* primary antibodies (page 25).
2. Apply 100 µl or enough of the diluted primary antibody to completely cover the tissue on each section.
3. Incubate the slides in a humidified chamber for 30 minutes.
4. Wash slides 3 times with 1X PBS containing 0.05% Tween-20 for 2 minutes, each time.
5. Proceed immediately to **Incubation with HRP Polymer Conjugate**, below.

Incubation with HRP Polymer Conjugate

1. Apply 2 drops (100 µl) or enough of the ready-to-use HRP Polymer Conjugate (Reagent A) to completely cover the tissue on each section.
2. Incubate the slides in a humidified chamber for 10 minutes.
3. Wash slides 3 times with 1X PBS containing 0.05% Tween-20 for 2 minutes, each time.
4. Proceed immediately to **Signal Development**, below.

Signal Development

1. Dilute the 20X DAB Chromogen supplied with the kit in a sterile microcentrifuge tube just prior to use as follows:

Distilled water	1 ml
Reagent B1	1 drop
Reagent B2	1 drop
Reagent B3	1 drop

   Mix well. Protect from light and use within 1 hour.
2. Apply 2 drops (100 µl) or enough of the diluted DAB chromogen (prepared in Step 1) to completely cover the tissue on each section.

   Caution: DAB is a known carcinogen; handle with care.
3. Incubate the slides in a humidified chamber for 5 minutes or until the desired signal intensity is reached.

   Incubation time can vary depending on the antibody.
4. Rinse thoroughly with distilled water.
5. Proceed immediately to **Counterstaining**, next page.

Continued on next page

Immunohistochemical Staining, Continued

Counterstaining

1. Apply 2 drops (100 µl) or enough of the Hematoxylin Counterstain Reagent to completely cover the tissue on each section.
2. Incubate the slides in a humidified chamber for 1-3 minutes.
3. Rinse thoroughly with distilled water.
4. Dip the slide in ammonia water 1X PBS or (0.25% NH_3) to blue the nuclei.
5. Rinse thoroughly with distilled water.
6. Dehydrate through increasing concentrations of ethanol (95%,100%) for 10-20 dips each. Clear in 2 changes of xylene or xylene substitutes for 10-20 dips each.
7. Proceed immediately to **Mounting**, below.

Mounting

1. Apply 2-4 drops (100-200 µl) of Histomount™ mounting medium to the slide.
2. Apply a cover slip on the slide.
3. Allow the medium to dry at room temperature overnight.

Microscopy

Evaluate the results by examining the slides using a light microscope at 20x magnification. Interpret the results as described below.

Interpreting the Results

Interpret the results using various controls as described in the table below. If immunohistochemical staining is performed on patient samples, always interpret the results within the context of the patient's clinical history and other diagnostic tests.

Positive Tissue Control is a specimen processed in the same way as the unknown and contains the antigen to be stained.

Reagent Control is a slide that is treated with a non-immune serum or isotype control antibody that matches the isotype of the experimental antibody instead of the same concentration of primary antibody.

Negative Control is a specimen processed in the same way as the unknown but does not contain the antigen to be stained [optional].

See page 20 for an example of expected results. For troubleshooting, see page 21.

Case No.	Positive Control	Reagent Control	Negative Control	Sample	Analysis
1	-	-	-	-	Procedure incorrect.
2	+	+	+	+	Non-specific staining due to protein binding or endogenous peroxidase activity.
3	+	-	+	+/-	Negative control contains the antigen.
4	-	-	-	+	Positive control does not contain the antigen.
5	+	-	-	-	Sample does not contain the antigen.
6	+	-	-	+	Sample contains the antigen.

Continued on next page

The Next Step

After detecting the antigen in the tissue of interest, you may wish to perform functional analysis using RNA interference (RNAi) studies, Western analysis, or another functional assay.

A variety of BioModule™ Units including units for Western and RNAi analysis are available from Invitrogen (page 25).

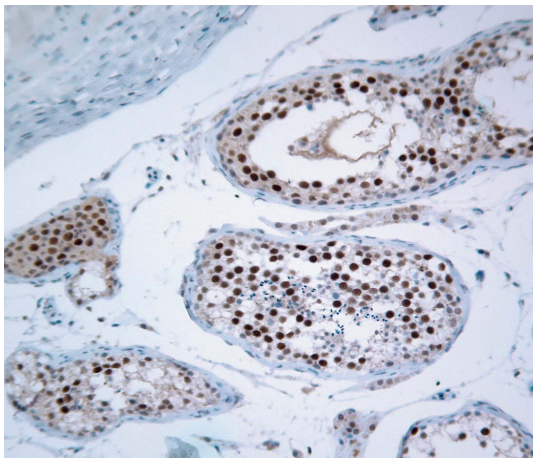
Expected Results

Example of Results

An example of results obtained after performing an immunohistochemical staining experiment with the BioModule™ Immunohistochemistry (IHC) Staining for Tissue is shown below.

In this experiment, formalin-fixed paraffin-embedded human testis tissue was subjected to the immunohistochemical staining protocol using *2nd Gen* predilute (ready-to-use) rabbit anti-phospho-MAP kinase ERK1/2 antibody from Invitrogen (cat. no. 08-1389) as described in this manual.

The image shows phospho-MAP kinase-positive cells that are stained brown from the DAB chromogen, displaying the expected nuclear staining pattern.



Troubleshooting

Introduction

Review the information in this section to troubleshoot your immunohistochemical staining experiments.

Problem	Reason	Solution
Weak or no staining	Primary antibody was inactive or too dilute	Perform staining using a new batch of antibodies. Be sure to store the antibody at the recommended temperature. Aliquot the antibody into smaller aliquots and store as recommended avoiding repeated freezing and thawing that may result in loss of activity. Check the activity of the antibody using ELISA or Western blotting. Be sure to perform a titration experiment to determine the optimal antibody concentration.
	Insufficient incubation times	Be sure to perform all incubation steps as indicated in the protocol. You may increase the antibody incubation time to improve staining or increase the substrate incubation time.
	Missed steps or steps not performed in the correct order	Make sure the staining protocol was followed as described on page 13.
	Incomplete deparaffinization	Perform the deparaffinization step for a longer time. Use fresh xylene.
	Epitope not accessible	For formalin-fixed paraffin embedded tissues, perform an appropriate epitope retrieval protocol (page 16). For frozen tissues, reduce the fixation time.
	Substrate prepared incorrectly	**Do not** use the 20X DAB chromogen directly from the bottle. Dilute the 20X DAB chromogen as described on page 17.
	Tissue sections may have dislodged	Always use pre-coated slides to mount tissue sections. We recommend treating the slides with HistoGrip™ or 0.1% poly-L-lysine, or you may use commercially prepared pre-coated slides. Handle the slides gently especially during the washing steps.
	Samples retain excess liquid after rinsing steps	Be sure to remove any excess liquid using paper towels without allowing the tissue sections to dry.
	Using sections thicker than normal	Thicker sections require longer incubation times for optimal staining.

Continued on next page

Troubleshooting, Continued

Problem	Reason	Solution
High background	Endogenous peroxidase activity not blocked or blocking is incomplete	Perform peroxidase quenching step (page 15) using Peroxo-Block™. For some frozen tissues, you may need to use 3% H_2O_2 to obtain optimal results (page 15).
	Incomplete deparaffinization	Perform the deparaffinization step for a longer time. Use fresh xylene.
	Inadequate washing of slides	Perform the recommended washing steps for the appropriate time. Do not skip any washing step.
	Longer incubation times used	Reduce the incubation time for antibody or substrate.
	Over-development of substrate	Reduce the incubation time with the DAB chromogen.
	High antibody concentration	Use diluted primary antibody. Perform a titration experiment to determine optimal primary antibody concentration.
	Sections on the slide have dried	Do not allow the sections to dry out during the staining protocol. Be sure to add enough reagents to completely cover the sections on the slide (usually 100-200 µl). Perform all incubations in a covered humidified chamber to prevent drying.
	Using mouse/rat antibodies on mouse/rat tissues	Use HistoMouse™-Max Kit (page 25) for best results.
	Faint background all over the slide	Perform an additional blocking step using 10% normal goat serum blocking solution (page 25) in PBS for 10 minutes prior to the primary antibody incubation step.
Edge of tissue staining intensely	Reagents have accumulated under the tissue	Increase the washing time after antibody incubations.
Bubbles on the slide	Cover slip not placed properly	Avoid trapping any bubbles when placing the cover slip on the slide. If bubbles are already trapped, remove the cover slip by incubating the slide in a 37ºC water bath until the cover slip can be removed easily. Place a clean cover slip on the slide without trapping any bubbles.

Continued on next page

Troubleshooting, Continued

Problem	Reason	Solution
Negative staining on positive slides	Specimen was improperly fixed or processed	Refer to page 11 for the recommended sample preparation protocols.
	Missed primary antibody/HRP Polymer Conjugate incubation steps or steps not performed in the correct order	Make sure the staining protocol was followed as described on page 13.
	Sections on the slide have dried	Do not allow the sections to dry out during the staining protocol. Be sure to add enough reagents to completely cover the sections on the slide (usually 100-200 µl). Perform all incubations in a covered humidified chamber to prevent drying.
Weak or no staining for the antigen in question but there is a precipitate on the slide	Primary antibody contaminated	Use fresh batch of primary antibody. Use sterile pipette tips while handling reagents to prevent contaminating the antibody solution.

Appendix

Technical Service

Web Resources

Visit the Invitrogen Web site at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our Web page (www.invitrogen.com).

Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
tech_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail:
jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail:
eurotech@invitrogen.com

Material Data Safety Sheets (MSDSs)

MSDSs are available on our Web site at www.invitrogen.com. On the home page, click on Technical Resources and follow instructions on the page to download the MSDS for your product.

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, contact our Technical Service Representatives. Invitrogen warrants that all of its products will perform according to specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order. Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives. Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Accessory Products

BioModule™ Units

Additional BioModule™ Units that can be used for further experiments are available separately from Invitrogen. Ordering information is provided below. For more information, visit www.invitrogen.com or call Technical Service (page 24).

Product	Amount	Catalog no.
BioModule™ qRT-PCR Unit	100 reactions	WFGE01
	1000 reactions	WFGE02
BioModule™ Microarray Analysis		
with indirect labeling	15 arrays	WFGE03
with direct labeling	15 arrays	WFGE04
BioModule™ Transfection and Control Unit with BLOCK-iT™ Technology	1 unit	WFGE06
BioModule™ BLOCK-iT™ Unit with Pol II miR RNAi Expression Vector	20 reactions	WFGE07
BioModule™ BLOCK-iT™ Unit with Lentiviral Pol II miR RNAi Expression System	20 reactions	WFGE08
BioModule™ Western Analysis Unit		
for chromogenic detection	20 transfers	WFGE09
for chemiluminescent detection	20 transfers	WFGE10

Additional Products

Additional reagents for use with the BioModule™ IHC Staining Unit are available separately from Invitrogen. Ordering information is provided below. For more information, visit www.invitrogen.com or call Technical Service (page 24).

Product	Amount	Catalog no.
HistoGrip™	10 ml	00-8050
Citrate buffer, pH 6.0 (20X)	100 ml	00-5000
EDTA Solution, pH 8.0 (20X)	100 ml	00-5500
Digest-All™ Kit	1 kit	00-3006
HistoMouse™-Max Kit (DAB, Broad Spectrum)	1 kit	87-9551
10% Normal Goat Serum Blocking Solution	100 ml	50-062Z
Isotype Control for Rabbit Primary Antibody	18 ml	08-6199
Isotype Control for Mouse Primary Antibody	18 ml	08-6599
MaxArray™ Human Normal Tissue Microarray Slides	5 unstained slides	75-4013
MaxArray™ Human Carcinoma Tissue Microarray Slides	5 unstained slides	For details, visit www.invitrogen.com

Antibodies

A large variety of high-quality antibodies including the Zymed® Antibodies is available from Invitrogen for use in immunohistochemistry. Prediluted *2nd Gen* primary antibodies that are ready-to-use with the *SuperPicTure*™ Polymer Detection Kit are also available. For details, visit www.invitrogen.com or contact Technical Service (page 24).