# Naveni pY PD1, HRP

KIT INSTRUCTIONS

## GENERAL GUIDELINES:

- Do not mix NaveniBright reagents with other Naveni™ product lines.
- Reaction volume depends on the sample size.
- Use best practices when pipetting to minimize reagent consumption.
- Centrifuge vials before pipetting.
- Thoroughly defrost all buffer mixtures at room temperature, vortex well and spin down before use.
- Vortex and spin down all enzymes (1 and 2) before use.
- Keep enzymes on ice or a frozen cold block.
- Wait to add enzymes until immediately before adding to the sample.
- Remove excess washing buffer from samples before adding reagent.
- Do not allow slides/samples to dry.
- Preheat the humidity chamber before each step.
- Incubation times or temperatures other than those specified may give erroneous results.
- As with any product derived from biological sources, proper handling procedures should be used.
- Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
- Chromogens may be carcinogenic and should be handled with care.
- Unused solutions should be disposed of according to local regulations.

## REQUIRED BUT NOT SUPPLIED

- VectaMount® Express Mounting Medium (H-5700-60) from Vector Laboratories.
- Isopropanol 99,5 %.
- Endogenous horseradish peroxidase quenching solution.
- TBS and TBS-T – Tris-buffered saline and Tris-buffered saline supplemented with 0,05% Tween, respectively.

## IMPORTANT:

Appropriate precautions should be taken to avoid antibody cross-contamination. Cross-contamination is the primary source of unspecific background due to the high sensitivity of the assay.

**Avoid bulk washing methods when multiple antibodies are used.**

**Wash any technical controls separately.**

KIT COMPONENTS:

Box 1.1:

| Material | Article Number | Amount | Storage* |
|---|---|---|---|
| **NaveniBright Blocking Buffer (1x)** | NB.1.100.01 | 4000 µl | +4 to +8°C<br>**DO NOT FREEZE** |
| **NaveniBright Supplement 1** | NB.1.100.03 | 500 µl | |
| **NaveniBright Antibody Diluent (1x)** | NB.1.100.02 | 8000 µl | |
| **NaveniBright Supplement 2** | NB.1.100.04 | 1000 µl | |
| **Probe Diluent (1x)** | NF.1.100.03 | 4000 µl | |
| **NaveniBright Probe Anti-M (40x)** | NB.1.100.06 | 100 µl | |
| **NaveniBright Probe Anti-R (40x)** | NB.1.100.07 | 100 µl | |

Box 1.2:

| Material | Article Number | Amount | Storage* |
|---|---|---|---|
| **NaveniBright HRP Reagent (800x)** | NB.1.100.05 | 100 µl | +4 to +8°C<br>**DO NOT FREEZE** |
| **NaveniBright HRP Substrate 1** | NB.1.100.13 | 170 µl | |
| **NaveniBright HRP Substrate 2** | NB.1.100.14 | 100 µl | |
| **NaveniBright HRP Substrate 3** | NB.1.100.15 | 100 µl | |
| **NaveniBright HRP Substrate 4** | NB.1.100.17 | 176 µl | |

Bag 1.3:

| Material | Article Number | Amount | Storage* |
|---|---|---|---|
| **NaveniBright AP/HRP diluent** | NB.1.100.08 | 8000 µl | +4 to +8°C<br>**DO NOT FREEZE** |
| **Nuclear Stain** | NB.1.100.16 | 6000 µl | |

Bag 2:

| Material | Article Number | Amount | Storage* |
|---|---|---|---|
| **Navenibody PD1 (200x)** | PD1.2.01 | 20 µl | at<br>-25 to -15°C |
| **Navenibody pTyr M (200x)** | PD1.2.02 | 20 µl | |
| **NaveniBright Buffer 1 (5x)** | NB.2.100.17 | 800 µl | |
| **NaveniBright Enzyme 1 (40x)** | NF.2.100.11 | 100 µl | |
| **NaveniBright Buffer 2 (5x)** | NB.2.100.18 | 800 µl | |
| **NaveniBright Enzyme 2 (40x)** | NF.2.100.15 | 100 µl | |

* When stored as directed, the product is stable at least for 3 months after receipt

**1. Sample preparation**

1.1 After antigen retrieval, add enough horseradish peroxidase blocking solution (for quenching) (not provided) to cover each sample. Incubate for 5 min at room temperature, or according to manufacturer's user guide.
1.2 Wash slides for 2x5 min in 1x TBS-T**.

**2. Blocking**

2.1 Prepare **blocking solution** by adding 5 µl of **Supplement 1** to every 40 µl of **Blocking Buffer** (1x).
2.2 Add the prepared blocking solution to the entire sample area (approximately 40 µl for each 1 $cm^2$ area).
2.3 Incubate for 60 min at +37 °C in a preheated humidity chamber.

**3. Navenibody incubation**

3.1 Prepare **Navenibody solution** by adding 5 µl of **Supplement 2** to every 40 µl of **Antibody Diluent** (1x).
3.2 Use the prepared **Navenibody solution** to dilute **Navenibody PD1** and **Navenibody pTyr M** to 1x (dilute 1:200 each).
3.3 Decant the blocking solution from slides and wash for 2x3 min in 1x TBS-T.
3.4 Add enough of Navenibodies to cover the sample area.
3.5 Incubate overnight at +4 °C in a humidity chamber.
3.6 Decant the antibody solution and wash slides for 3x5 min in 1x TBS-T** in a staining jar under gentle agitation.

| Kit component | Blocking Solution | Navenibody solution |
|---|---|---|
| **Blocking Buffer** | 40 µl | - |
| **Supplement 1** | 5 µl | - |
| **Primary antibody diluent** | - | 40 µl |
| **Supplement 2** | - | 5 µl |
| **Total**: | 45 µl | 45 µl |

**4. Probe incubation**

4.1 Prepare the probes by diluting **Probe anti-M** and **Probe anti-R** (dilute 1:40 each) in **Probe Diluent** (1x).
4.2 Add enough of the probes to cover the sample area.
4.3 Incubate for 60 min at +37 °C in a preheated humidity chamber.
4.4 Decant the solution and wash slides for 3x5 min in 1x TBS-T in a staining jar under gentle agitation.

**5. Reaction 1**

5.1 Dilute **Buffer 1** 1:5 in distilled water. Vortex and spin down.
5.2 Prepare **Reaction 1** by adding **Enzyme 1** (dilute 1:40) to the diluted buffer. Mix gently by pipetting and spin down.
5.3 Add enough Reaction 1 to cover the sample area.
5.4 Incubate for 30 min at 37 °C in a preheated humidity chamber.
5.5 Wash slides for 2x3 min in 1x TBS-T in a staining jar under gentle agitation.

**6. Reaction 2**

6.1 Dilute **Buffer 2** 1:5 in distilled water. Vortex and spin down.
6.2 Prepare **Reaction 2** by adding **Enzyme 2** (dilute 1:40) to the diluted buffer. Mix gently by pipetting and spin down.
6.3 Add enough Reaction 2 to cover the sample area.
6.4 Incubate for 90 min at +37 °C in a preheated humidity chamber.

**7. HRP Incubation**

7.1 Decant the solution and wash slides for 2x5 min in 1x TBS, followed by 1x10 min in 0,1x TBS in a staining jar under gentle agitation.
7.2 Dilute the **NaveniBright HRP reagent** 1:800 in **AP/ HRP diluent.**
7.3 Decant wash buffer from the slides.
7.4 Add enough HRP solution to cover the sample area.
7.5 Incubate for 30 min at room temperature with slow agitation.

**8. Substrate development**

8.1 Decant the solution and wash slides for 2x2 min in 1x TBS in a staining jar under gentle agitation.

8.2 Prepare the substrate solution by mixing **HRP Substrate 1** (dilute 62x), **HRP Substrate 2** (dilute 100x), **HRP Substrate 3** (dilute 100x) and **HRP Substrate 4** (dilute 62,5x) in distilled water. [+]
See calculation example for minimal volume:

| Kit Component | Substrate Solution |
|---|---|
| **Distilled water** | 100 µl |
| **HRP Substrate 1** | 1,6 µl |
| **HRP Substrate 2** | 1,0 µl |
| **HRP Substrate 3** | 1,0 µl |
| **HRP Substrate 4** | 1,6 µl |
| Total: | 105,2 µl |

8.3 Decant wash buffer from the slides.

8.4 Add enough substrate solution to cover the sample area.

8.5 Incubate the slides at room temperature for 5 to 10 min. [++]

8.6 Decant of the substrate solution from the slides and wash slides for 2x2 min in deionized water under gentle agitation.

**9. Nuclei staining**

9.1 Decant wash buffer from the slides.

9.2 Add enough **Nuclear stain** to cover the sample area.

9.3 Incubate for 2 to 10 seconds at room temperature. [+++]

9.4 Rinse the slides under running tap water (not deionized water).

**10. Dehydration and mounting ¤**

10.1 Wash slides in water for 5 min with gentle agitation.

10.2 Rapid dehydrate slides with 2x1 min wash in isopropanol.

10.3 Blot excess isopropanol from slides and apply VectaMount® Express Mounting Medium (H-5700-60).

10.4 Apply coverslip and allow slides to dry flat at room temperature for 10 to 20 min.

10.5 Analyze using a brightfield microscope, using at least a 20x objective.

10.6 After imaging, store the slides at room temperature. Signal is stable for years.

[+] For alternative substrates, prepare according to manufacturer's user guide.

[++] Substrate incubation time should be optimized for each assay.

[+++] Excessive nuclear staining may obscure developed signals.

¤ Slides must be mounted with **VectaMount® Express Mounting Medium (H-5700-60)** from Vector Laboratories. Usage of other dehydration methods or mounting medium may lead to reduced signal intensity.