NPT.PDL1.AP.100

Naveni® PD1/PD-L1 AP

General guidelines

- Do not mix Naveni® PD1/PD-L1 reagents with other Naveni® product lines.
- A volume of approximately 40 µL/cm^2 is recommended. For example, for an FFPE tissue section that covers an area of 5 cm^2 on a slide, we recommend that you use a reaction volume of 200 µL/slide.
- Use best practices when pipetting to minimize reagent consumption.
- Thoroughly defrost all buffers at room temperature. Vortex well before use.
- Centrifuge vials before pipetting.
- Gently mix and spin down Enzymes 1 and 2 before use.
- Keep enzymes on ice or on a frozen cooling block.
- Add enzymes right before applying reaction mix to sample.
- Preheat humidity chamber before each step.
- Incubation times or assay temperatures other than those specified may negatively impact results.
- All washing steps are performed on a platform shaker with gentle agitation.
- Warm washing buffer is only required for step 4.5, additional warm washing steps may lead to signal deterioration.
- Remove excess washing buffer from samples before adding the reagent mix.
- Do not allow slides/samples to dry.
- As with any product derived from biological sources, proper handling procedures should be used. Wear appropriate personal protective equipment to avoid contact of reagents with eyes and skin.
- Unused solutions should be disposed of according to local regulations.
- Chromogens may be carcinogenic and should be handled with care.

Additional reagents, not provided

- Antigen retrieval buffer.
- Endogenous alkaline phosphatase/ horseradish peroxidase blocking solution.
- Tris-buffered saline (TBS) and Tris-buffered saline supplemented with 0,05% Tween.
- VectaMount® Express Mounting Medium (H-5700-60) from Vector Laboratories.

Application

Naveni®PD1/PD-L1 is an *in situ* proximity ligation assay for the study of PD1 and PD-L1 interaction in formalin-fixed paraffin-embedded human tissues and cells samples. For research use only. Not for use in diagnostic procedures.

Detection enzyme and substrate

- Horseradish peroxidase.
- Red precipitating reaction product.

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.

Kit components

Box 1.1:

Storage: 4 to 8°C

DO NOT FREEZE

Material	Art.no	Amount
Blocking Buffer (1x)	NB.1.100.01	4000 µL
Supplement 1	NB.1.100.03	500 µL
Antibody Diluent (1x)	NB.1.100.02	8000 µL
Supplement 2	NB.1.100.04	1000 µL
Navenibody Diluent (1x)	NB.1.100.02	4000 µL
Navenibody M1 (40x)	NB.1.100.06	100 µL
Navenibody R2 (40x)	NB.1.100.07	100 µL

Box 1.2:

Storage: 4 to 8°C

DO NOT FREEZE

Material	Art.no	Amount
AP Diluent	NB.1.100.08	8000 µL
AP Reagent	NB.1.100.10	100 µL
AP Substrate Diluent	NB.1.100.09	8000 µL
AP Substrate 1	NB.1.100.11	140 µL
AP Substrate 2	NB.1.100.12	100 µL

Box 1.3:

Storage: 4 to 8°C

DO NOT FREEZE

Material	Art.no	Amount
Nuclear Stain	NB.1.100.16	6000 µL

Box 2:

Storage: -25 to -15°C.

FREEZE

Material	Art.no	Amount
PD1 antibody (40x) based on clone EH33 CST	PPI.2.01	100 µL
PD-L1 antibody (40x) based on clone SP142 Abcam RabMAb®	PPI.2.02	100 µL
Buffer 1 (5x)	NB.2.100.17	800 µL
Enzyme 1 (40x)	NF.2.100.11	100 µL
Buffer 2 (5x)	NB.2.100.18	800 µL
Enzyme 2 (40x)	NF.2.100.15	100 µL

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

For more information, or to place an order, visit
www.navinci.se/products
Email: contact@navinci.se

Instructions for use

1. Sample preparation

1.1. After antigen retrieval, add enough alkaline phosphatase/ horseradish peroxidase blocking solution (for quenching; not provided) to cover each sample. Incubate according to manufacturers´ user guide.
1.2. Wash slides for 2x5 min in 1x TBS-T.
1.3. Prepare two bottles of 1X TBS-T* and pre-warm one bottle to 37°C together with the required number of staining jars (warm wash is required after Navenibody incubation at Step 4.5).

2. Blocking

2.1. Prepare blocking solution by adding 5 µL of Supplement 1 to every 40 µL of Blocking Buffer (1x) (table 1).
2.2. Add the prepared blocking solution from step 2.1 to the entire sample area (approximately 40 µL for each 1 cm² area).
2.3. Incubate for 60 min at 37 °C in a preheated humidity chamber.

3. Primary antibody incubation

3.1. Prepare primary antibody solution by adding 5 µL of Supplement 2 to every 40 µL of Antibody Diluent (1x) (table 1).
3.2. Use the prepared primary antibody solution from step 3.1 to dilute PD1 and PD-L1 antibody to 1x (dilute 1:40 each).
3.3. Decant the blocking solution from slides and wash for 2x 3 min in 1x TBS-T.
3.4. Add enough of the diluted antibodies to cover the sample area.
3.5. Incubate for 60 min at 37 °C or overnight at +4 °C in a humidity chamber.
3.6. Decant the antibody solution and wash slides for 3x 5 min in 1x TBS-T in a staining jar.

Table 1: Blocking solution and primary antibody solution.

Kit component	Blocking solution	Primary antibody solution
Blocking Buffer	40 µL	-
Supplement 1	5 µL	-
Antibody diluent	-	40 µL
Supplement 2	-	5 µL
Total volume:	45 µL	45 µL

4. Navenibody incubation

4.1. Dilute Navenibody M1 and Navenibody R2 1:40 in Navenibody Diluent (1x).
4.2. Add sufficient volume of the Navenibody working solution from step 4.1 to cover the sample area.
4.3. Incubate for 60 min at 37 °C in a preheated humidity chamber.
4.4. Decant the Navenibody solution.
4.5. Wash slides for 2x 10 sec and 1x 15 min with the pre-warmed 1x TBS-T in the staining jar from step 1.3. Wash the controls separately.

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 1:40 to the diluted buffer from step 5.1. Mix gently by pipetting and spin down.
5.3. Add sufficient volume of 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 2x 3 min in 1x TBS-T in a staining jar.

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 1:40 to the diluted buffer from step 6.1. Mix gently by pipetting and spin down.
6.3. Add sufficient volume of Reaction 2 to cover the sample area.
6.4. Incubate for 90 min at 37 °C in a preheated humidity chamber.

7. AP incubation

7.1. Decant the solution and wash slides for 2x 5 min in 1x TBS, followed by 1x 10 min in 0,1x TBS in a staining jar.
7.2. Dilute the AP reagent 1:300 in AP diluent.
7.3. Decant wash buffer from the slides.
7.4. Add sufficient volume of AP solution to cover the sample area.
7.5. Incubate for 30 min at room temperature with slow agitation on a platform shaker.

8. Substrate development

8.1. Decant the solution and wash slides for 2x 2 min in 1x TBS in a staining jar.
8.2. Prepare the substrate solution by mixing AP Substrate 1 (dilute 62,5x) and AP Substrate 2 (dilute 80x) in AP Substrate Diluent +.

For an example calculation for a minimal reaction volume see table 2.

Table 2: Example calculation.

Kit Component	Substrate solution (µL)
AP Substrate Diluent	80 µL
AP Substrate 1	1,3 µL
AP Substrate 2	1,0 µL
Total:	82,3 µL

8.3. Decant wash buffer from the slides.
8.4. Add first substrate solution to cover the sample area.
8.5. Incubate the slides with substrate at room temperature for 25 min. ++
8.6. Decant of the substrate solution from the slides and wash slides for 2x 2 min in deionized water.

9. Nuclear 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.
10.2. Rapid dehydrate slides with 2x 1 min wash in isopropanol.
10.3. Blot excess isopropanol from slides and apply VectaMount® Express Mounting Medium (H-5700-60).
10.4. Apply the coverslips 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. The signal is stable for years.

* TBS-T (Tris-buffered saline supplemented with 0,05% Tween 20).
+ For alternative substrates, prepare according to manufacturers' 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.

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