Art.nr 60000

Naveni® Plex

Instructions For Use

For research use only

Manufactured by:

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Intended use

Naveni® Plex enables the simultaneous detection of 3 protein-protein interactions and/or single protein expression with high specificity and sensitivity in FFPE human tissue.

Principles of the procedure

Naveni® Plex is an *in situ* proximity ligation multiplex assay with a fluorescent readout. With the assay up to 3 separate targets, protein-protein interactions and/or single protein expression, can be imaged simultaneously. Each target has a unique pair of Navenibodies, either directed against two separate proteins, for detecting interactions, or two separate epitopes on the same protein, for specific single protein detection. Each target uses separate fluorophores to make simultaneous detection possible.

Warnings and precautions

- For research use only. Not for use in diagnostic procedures.
- Not for internal or external use in humans or animals.
- As with any product derived from biological sources, proper handling procedures should be used.
- Wear appropriate personal protective equipment to avoid contact with reagents with eyes and skin.
- Always work in a fume hood when handling Xylene and hydrophobic pens.

General guidelines

- Reaction volume depends on sample size and should be sufficient to cover the entire sample area. 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 tissue section, we recommend you use a reaction volume of 200 µL/tissue section.
- 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 vortex and spin down Enzymes 1 and 2 before use.
- Keep enzymes on ice or on a frozen cooling block.
- Dilute enzymes and mix well right before adding reaction mix to sample.
- Incubation times or assay temperatures other than those specified may negatively impact results.
- Warm washing buffer is only required for step 4.4, additional warm washing steps may lead to signal deterioration.
- Detection Mix is light sensitive. Always keep it protected from light.
- Remove excess washing buffer from samples before adding the reagent mix.
- Do not allow slides/samples to dry.
- Preheat the humidity chamber before each step.
- Unused solutions should be disposed of according to local regulations.

Kit components

Naveni®, Box 1

Art.nr 60001
Storage: +2 to +8°C

DO NOT FREEZE

Name	Art.no	Amount
Naveni Block	50014	6 mL
Naveni Diluent	50001	8 mL

Naveni® Plex, Box 2

Art.nr 60002
Storage: -25 to -15°C

FREEZE

Name	Art.no	Amount
Naveni Enzyme 1 (40x)	50002	100 µL
Naveni Enzyme 2 (40x)	50003	100 µL
Naveni Plex Buffer 1 / ABC (5x)	50004	800 µL
Naveni Buffer 2 (5x)	50005	800 µL
Naveni Post Block (40x)	50006	100 µL
Naveni Plex Detection Mix / ABC (5x)	50007	800 µL

Navenibodies

The Navenibodies used in the Naveni® Plex assay are packed separately to make it possible for the user to customize the assay. By choosing different combinations of Navenibodies different panels can be created.

The different antibodies are conjugated to different sets of oligonucleotides named A, B, and C. It is important to note that each oligonucleotide set (A, B, and C) should only correspond to one target. Choosing Navenibodies for two different targets conjugated to the same set will result in indistinguishable signals from the different targets.

Available Navenibodies can be found in the table below.

Storage: +2 to +8°C

DO NOT FREEZE

Name	Art.no	Amount
Naveni® Plex Navenibodies A PD1/ PD-L1	60003	40 µL x2
Naveni® Plex Navenibodies B CD3	60004	40 µL x2
Naveni® Plex Navenibodies C CD8/ MHC-I	60005	40 µL x2

Required but not provided

Materials

- Pipettes and tips with appropriate volumes
- Tubes, beakers and cylinders for reagent preparation
- Incubators 37°C and 60°C
- Pressure cooker
- Hydrophobic pen
- Vortex mixer
- Microcentrifuge
- Shaker
- Humidity chamber
- Staining jars
- Freeze block for enzymes
- Coverslips
- Fluorescence microscope

Reagents

- Xylene
- Ethanol, 70, 95 and 99.5%
- EDTA antigen retrieval buffer *Sigma-Aldrich, E1161-1000ML*
- Tris Buffered Saline with 0.05% Tween 20 (TBS-T)
- Tris Buffered Saline (TBS)
- Phosphate Buffered Saline (PBS)
- Deionized water
- Fluoroshield mounting medium *Thermo-Fisher Scientific, F6182*
- DAPI (Diluted in PBS to 1 µg/mL) *Thermo-Fisher Scientific, 62248*

Instructions for use

1. Pre-treatment

1.1 Bake the slides in a pre-heated incubator at 60°C for 45 minutes.
1.2 Deparaffinize the slides via a Xylene-Ethanol series. Move the slides between different staining jars containing the chemical and for the time stated in the list below. When lowering the slide holder into the staining jar move it up and down three times to ensure proper coverage of the sample. After each incubation lift out the slide holder and let it drop off before moving it to the next jar.

Xylene-Ethanol series:

Xylene	5 minutes
Xylene	5 minutes
Xylene	1 minute
Ethanol 99.5%	3 minutes
Ethanol 99.5%	3 minutes
Ethanol 95%	5 minutes
Ethanol 95%	3 minutes
Ethanol 70%	3 minutes

1.3 Move the slides to a staining jar with DI water. Exchange the water twice for a total of 3 washing steps.
1.4 Perform antigen retrieval (HIER, Heat-Induced Epitope Retrieval) in EDTA Antigen retrieval buffer (Sigma-Aldrich cat.no. E1161-1000ML) with a pressure cooker at 110°C for 10 minutes.
1.5 Let the slides stand in the antigen retrieval buffer at room temperature for 30 minutes for a slow decrease in temperature.
1.6 Gradually exchange the warm antigen retrieval buffer with DI water by pouring out half of the antigen retrieval buffer from the staining jar and adding DI water. Repeat this step twice for a total of 3 2x dilutions with water.
1.7 Move the slides to a new staining jar with DI water. Exchange the water 4 times for a total of 5 washing steps.
1.8 Move the slides to a new staining jar with 1x PBS. After this the slides can be kept in 1x PBS in a refrigerator at +4-8°C overnight if needed.
1.9 Use a hydrophobic pen to draw a border around each tissue sample.

2. Preparations

2.1 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 4.4).

3. Blocking

3.1 Add Block to the entire sample area.
3.2 Incubate for 60 min at 37 °C in a preheated humidity chamber.

4. Navenibody incubation

4.1 Dilute each of the chosen Navenibodies 1:100 into one tube with Diluent. An example calculation for a total volume of 200 µL, sufficient for a sample area of ~5 cm^2 can be seen in table 1 below.

Table 1. Example calculation for a 200 µL Navenibody working solution

Reagent for 5 cm^2 sample area	Volume
Diluent	188 µL
N1 A PD1/PD-L1 (100x)	2 µL
N2 A PD1/PD-L1 (100x)	2 µL
N1 B CD3 (100x)	2 µL
N2 B CD3 (100x)	2 µL
N1 C CD8/MHC-I (100x)	2 µL
N2 C CD8/MHC-I (100x)	2 µL
Total volume	200 µL

4.2 Decant the Block and add a 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 solution and wash the slides with the pre-warmed 1x TBS-T from Step 2.1. Wash for 2x 10 sec and 1x 15 min in the pre-warmed staining jar under gentle agitation. Wash the controls separately.

5. Reaction 1

An example calculation for a total reaction volume of 200 µL, sufficient for a sample area of ~5 cm^2 can be seen in table 2.

5.1 Start preparing Reaction 1 by diluting **Buffer 1** 1:5 in water. Vortex and spin down.
5.2 Add **Enzyme 1** so that it is diluted 1:40. Mix gently by pipetting and spin down.

Table 2. Example calculation for a 200 µL reaction volume.

Reagent for 5 cm^2 sample area	Volume
H_2O	155 µL
Buffer 1 (5x)	40 µL
Enzyme 1 (40x)	5 µL
Total volume:	200 µL

5.3 Add a 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 Decant the solution, wash slides 1x 10 sec and 1x 5 min with 1x TBS-T in a staining jar under gentle agitation.

6. Reaction 2 (protect from light!)

6.1 Start preparing Reaction 2 by diluting **Buffer 2** 1:5 in water. Vortex and spin down.
6.2 Add **Enzyme 2** so that it is diluted 1:40. Mix gently by pipetting and spin down.

6.3 Add a sufficient volume of Reaction 2 to cover the sample area.
6.4 Incubate for 90 min at 37 °C in a preheated humidity chamber. Proceed to step 7.1 before the incubation is over.

7. Post-block

7.1 While Reaction 2 is incubating, thaw and dilute **Post-block** 1:40 in **Diluent**. Vortex and spin down.
7.2 After the incubation step is complete, decant Reaction 2 and add a sufficient volume of the **Post-block** working solution from step 7.1 to cover the sample area.
7.3 Incubate for 30 min at 37 °C in a preheated humidity chamber.

8. Detection (protect from light!)

8.1 Prepare the Detection working solution by diluting **Detection reagent** 1:5 in water. Vortex and spin down.
8.2 Decant the **Post-block** solution. Do not wash but make sure the **Post-block** solution has been removed. Add a sufficient volume of Detection working solution from step 8.1 to cover the sample area.
8.3 Incubate for 30 min at 37 °C in a preheated humidity chamber.
8.4 Decant the solution and wash slides for 2 min with 1x TBS in a staining jar under gentle agitation.

9. Nuclear stain and mounting (protect from light!)

9.1 Decant excess washing buffer from the slides.
9.2 Add DAPI or a nuclear stain of your choice with a similar emission spectrum diluted in 1x PBS to a concentration recommended by the vendor. Incubate for 5 min at room temperature in a humidity chamber.
9.3 Decant the solution, wash slides 2x 5 min in 1x TBS under gentle agitation.
9.4 Perform a final 5 min wash in 0.1x TBS under gentle agitation. Dry slides in a slide centrifuge or air-dry them, and mount them with a coverslip using an antifade mounting medium.

10. Imaging

10.1 Image your slides on a fluorescence or confocal microscope using a 20x objective or higher.
10.2 For imaging, a filter set corresponding to your chosen nuclear stain and the detection fluorophore's excitation/emission spectra is required, see table 3.

Table 3. Filter sets for imaging.

Filter set	Detecting	λ Excitation	λ Emission
DAPI	Nuclei		
FITC	Proximity signal detected with set B	480-490 nm	525-535 nm
Cy3	Proximity signal detected with set A	545-555 nm	575-585 nm
Cy5	Proximity signal detected with set C	635-645 nm	665-675 nm

Example of results

Figure 1. Human FFPE tonsil tissue at 4x magnification. PD1/PD-L1 is shown in magenta, CD3 in cyan, CD8/MHC-I in yellow, and DAPI in gray.

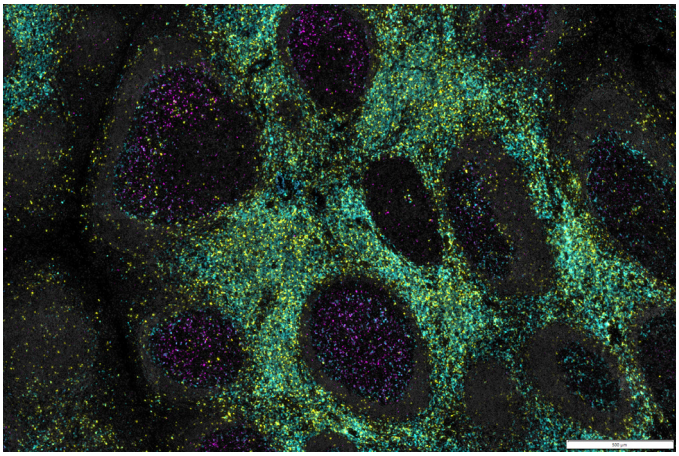
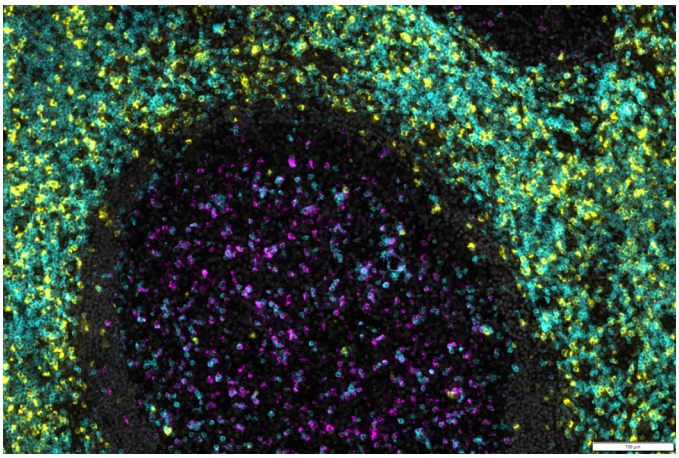


Figure 2. Human FFPE tonsil tissue at 15x magnification. PD1/PD-L1 is shown in magenta, CD3 in cyan, CD8/MHC-I in yellow, and DAPI in gray.



Warranty

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