Item Numbers 39505 & 39504 (Red/ Atto647N)

NaveniFlex Cell MR

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.
- Use best practices when pipetting to minimize reagent consumption.
- Thoroughly defrost all buffers at room temperature. Vortex well before use.
- Centrifuge vials before pipetting.
- Keep enzymes on ice or on a frozen cooling block.
- Vortex and spin down Enzymes 1 and 2 before use.
- Add enzymes right before adding the reaction mix to the sample.
- The recommended Navenibody working dilution is 1:40. Depending on target abundance, Navenibodies can be titrated down to 1:80.
- The NaveniFlex Cell MR kit can be combined with traditional immunofluorescence (IF) provided that all primary antibodies are either raised in different species or labelled with fluorophores different from the ones used in the kit.
- Buffer 2 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.
- Incubation times or assay temperatures other than those specified in the instructions for use may negatively impact results. Warm washing buffer is only required for step 4.4. Additional warm washing steps may lead to signal deterioration.
- 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.
- The NaveniFlex Cell MR assay is also applicable for 96-well plates. For more information and protocol, please visit www.navinci.se/products.

Kit components

Box 1:

Storage: 4 to 8°C

DO NOT FREEZE

Material	Art.no	Amount
Block (1X)	NT.1.100.01	4 ml
Diluent 1 (1X)	NB.1.100.02	8 ml
Diluent 2 (1X)	NB.1.100.02	4 ml
Navenibody M1 (40X)	NB.1.100.06	100 µl
Navenibody R2 (40X)	NB.1.100.07	100 µl

Box 2:

Storage: -25 to -15°C. Protect from light!

FREEZE

Material	Art.no	Amount
Buffer 1 (5X)	NB.2.100.17	800 µl
Enzyme 1 (40X)	NF.2.100.11	100 µl
Buffer 2 (5X) Red or Atto647	NF.2.100.12/.14	800 µl
Enzyme 2 (40X)	NF.2.100.15	100 µl

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 different primary antibody pairs are used, or when washing different technical controls.

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Instructions for use

1. Pre-treatment (reagents not provided)

1.1. Fix and permeabilize cells according to the immunofluorescence protocol optimized for the primary antibodies utilized in this assay.
1.2. Where applicable, use a hydrophobic barrier pen around the sample to prevent spillage and sample drying.
1.3. Prepare two bottles of 1X TBS-T*; pre-warm one bottle to 37°C together with the required number of staining jars (a warm wash is required after Navenibody incubation at Step 4.4)

*TBS-T (Tris-buffered saline supplemented with 0.05% Tween 20).

2. Blocking

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

3. Primary antibody incubation

3.1. Use the provided Diluent 1 to dilute your primary antibodies to working concentration. Start with a concentration optimized for/recommended by the antibody vendor for ICC.
3.2. Decant Block and add a sufficient volume of the antibody working solution from step 3.1 to cover the sample area.
3.3. Incubate in a humidity chamber for 60 min at 37 °C, or at 4 °C overnight.
3.4. Decant the antibody solution, wash slides 2x 10 sec and 1x 15 min with 1X TBS-T in a staining jar under gentle agitation. Wash controls separately.

4. Navenibody incubation

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

5. Reaction 1

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, see example calculation in Table 1 below.

Table 1. Example Calculation for a 40 µl reaction volume, sufficient for a 1 cm^2 area.

Reagent for 1 cm^2 sample area	Volume
H_2O	31 µl
Buffer 1 (5x)	8 µl
Enzyme 1 (40x)	1 µl
Total volume:	40 µl

5.3. Add a sufficient volume of Reaction 1 to cover the sample area.
5.4. Incubate in a preheated humidity chamber for 30 min at 37 °C.
5.5. Decant the solution, rinse once with 1 X TBS-T, then wash 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 in a preheated humidity chamber for 90 min at 37 °C.
6.5[1]. Decant the solution and wash slides for 2 min with 1X TBS in a staining jar under gentle agitation.

7. Nuclear stain and mounting (reagents not provided; protect from light!)

7.1. Remove excess washing buffer from the slides.
7.2. Add DAPI or a nuclear stain of your choice with a similar emission spectrum; dilute and incubate as recommended by the vendor.
7.3. Decant the solution, wash slides 2x 10 min in 1X TBS in a staining jar under gentle agitation.
7.4. Perform a final 15 min wash in 0.1X TBS in a staining jar under gentle agitation. Dry slides in a slide centrifuge or air-dry them, and mount slides with a coverslip using an antifade mounting medium.

8. Imaging

8.1. Image your slides with a fluorescence or confocal microscope using a 20x objective or higher.
8.2. For imaging, a filter set corresponding to DAPI, Cy3.5 and Cy5 is required. See table 2 below.

Table 2. Filter sets for imaging.

Filter set	Detecting	$\lambda_{Excitation}$	$\lambda_{Emission}$
DAPI	Nuclei		
Cy3.5/ TexasRed	Proximity signal detected by Buffer 2 Red	585-595 nm	615-625 nm
Cy5	Proximity signal detected by Buffer 2 Atto647	635-645 nm	665-675 nm

[1] (Optional) Fluorescently labelled primary antibodies may be diluted in diluent 1 and added after this step if co-staining is desired. Dilute and incubate as recommended by the manufacturer.

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