Navinci Diagnostics™
Navinci Universal - 100 & Navinci Universal - 25
Protocol Guide

Table of Contents

Table of Contents 1

Introduction 2

1. Contents and storage 2
2. Required materials and equipment not provided 3
3. General guidelines 3
4. Workflow 4
5. Procedure 5
   - Samples 5
   - Blocking 5
   - Primary Antibody Incubation 5
   - Probe Incubation 5
   - Reaction A 5
   - Reaction B 6
   - Reaction C 6
   - Mounting 6
6. Troubleshooting 7
7. References 11
8. Limited product warranty 11

Introduction

Navinci Diagnostics™ Unfolding Proximity Probes Technology was developed to increase the sensitivity and efficiency of *in situ* detection and quantification of any protein, protein interactions and protein modifications in tissue and cells. The core features of our technology are dual epitope tagging, probe activation, and DNA-based signal amplification.

The benefits of Unfolding Proximity Probes technology are clear target detection, consistency in staining, reproducibility, possibility to detect low abundant proteins, detection of protein-protein interactions, and specific detection of post-translational modification.

The technology is tailored for academic and industrial researchers working in fields of immune-profiling, signaling profiling, drug testing, treatment validation, biomarker discovery, and diagnostic.

1. Contents and storage

Navinci Universal kits enable you to detect proteins, protein interactions or protein modifications in cultured cells as well as fresh-frozen and FFPE samples.

Navinci Universal kits are designed for dual epitope recognition by two primary antibodies, one of rabbit and one of mouse origin, using immunofluorescence detection with a filter for emission wavelength 615 nm.

There are two sizes of **Navinci Universal** kits. The standard kit **Navinci Universal 100** is for 100 tests á 40 µL and the smaller **Navinci Universal 25** allows for 25 tests á 40 µL. The reaction volume of 40 µL should be adequate to cover 1 cm^2 sample on a slide. The volume might be adjusted according to the reaction area and the number of samples.

Product details	**Navinci Universal 100**	**Universal 25**
Catalog No	**ND0100**	**ND0200**
Product size	100 tests	25 tests
Final working volume	4 ml	1 ml
Species	α-Mouse, α-Rabbit	α-Mouse, α-Rabbit
Detection method	Fluorescent	Fluorescent
Emission wavelength	615 nm	615 nm
	For use with filters as e.g. Texas Red™	

Navinci Universal kits consist of two boxes that are shipped separately: at 4-8 °C (Box 1) and frozen on dry ice (Box 2). Upon receipt, store the Box 1 at 4-8 °C and Box 2 at -15— -25 °C.

2. Required materials and equipment not provided

Materials and/or equipment	Specification
Primary antibodies of choice reactive to the protein(s) of interest	Mouse and rabbit origin to be used in association with **Navinci Probes**
Reagents required for antigen retrieval of the sample	According to your antibody specification
High purity water	Sterile filtered, Milli-Q or similar
Washing buffers	1x TBS (Tris-buffered saline) 1x TBS supplemented with 0.05 % Tween 20
Calibrated pipettes	From 0.5 to 1000 µl
Low retention filter tips	From 0.5 to 1000 µl
Incubator	37 °C
Vortex Microcentrifuge Shaker Humidity chamber Freeze block for enzymes Hydrophobic pen Staining jars	Major laboratory supplier
Fluorescence microscope	Equipped with appropriate filters, camera, and software for image acquisition
Coverslips	Compatible with fluorescence microscopy
Mounting media	Water-based anti-fade mounting medium (with/without DAPI)

3. General guidelines

- Reaction volume depends on the sample size.
- Use best practices when pipetting to minimize reagents consumption.
- Completely defrost all buffer mixtures (A, B, C) at room temperature and vortex well before use.
- Vortex and spin-down all enzymes (A, B and C) before use.
- Keep enzymes on ice or on a frozen cold block.
- Wait to add enzymes until immediately prior to adding to the reaction solution.
- All incubations should be performed in a humidity chamber.
- Preheat the humidity chamber before each incubation step.
- All wash steps should be performed at room temperature in a staining jar with the specimens fully covered by the washing buffer and with gentle agitation.
- Remove excess washing buffer from samples before adding reagents.
- Do not allow slides/samples to dry.
- Buffer C is a light-sensitive reagent. Always keep it protected from light.
- 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.
- Take an appropriate precaution when handling reagents. Use disposable gloves when handling suspected carcinogens or toxic materials. Avoid contact with skin and eyes.
- Unused solution should be disposed according to local regulation.

4. Workflow

Sample	Step
	**Primary Antibody incubation** Two primary antibodies bind to their target epitopes, located on a single protein or on two separate proteins.
	**Probe incubation** To complete the dual binding, **Navinci Universal Probes** recognize their respective primary antibodies.
	**Reaction A** The locked probes are activated by an enzymatic step that increases the efficacy of signal generation, thus improving assay sensitivity.
	**Reaction B** Only the probes that are within close proximity connect, guaranteeing high specificity by reducing nonspecific background staining
	**Reaction C** Fluorescent signals are amplified. The high signal strength enables the detection of separate proximity events, allowing for a resolution down to single protein or protein interaction.
	**Visualization and data processing** The results can be visualized with a fluorescence microscope, and the subsequent quantification can be done with various image software.

5. Procedure

Samples

- Tissue: embedded or frozen.
- Cells: adhered cell cultures or cell suspensions.
- The samples should be deposited on glass slides and pre-treated with respect to fixation, antigen retrieval and/or permeabilization.

Blocking

- Vortex the **Navinci Diagnostics** **Blocking Buffer/Primary Antibody Diluent (1x)**
- Add **Blocking Buffer** to the entire sample area.
- Incubate for 60 min at 37 °C in a pre-heated humidity chamber.

Primary Antibody Incubation

Please note that conditions and dilutions needed for primary antibodies are variable from system to system. Please refer to appropriate literature or to your primary antibody provider for more specific details.

IMPORTANT: Appropriate precautions should be taken to avoid antibody cross-contamination. Cross-contamination is the main source of unspecific background due to the high sensitivity of the assay.
Avoid bulk washing methods when multiple antibodies are used.

- Use the provided **Blocking Buffer/Primary Antibody Diluent (1x)** to dilute your primary antibodies.
- Decant the Blocking Buffer and add enough of your antibodies to cover the sample area.
- Incubate in a pre-heated humidity chamber.
- The time and temperature depend on the primary antibody used. This information can be found in the database and should be verified experimentally by the user.
- Decant the antibody solution and wash slides for 3x5 min with 1x TBS-T in a staining jar under gentle agitation.

Probe Incubation

- Prepare the probes by diluting **Probe M1** and **Probe R2** in **Probe Diluent (1x)**, dilute 1:40 each.
- Add enough of the probes to cover the sample area.
- Incubate for 60 min at 37 °C in a pre-heated humidity chamber.
- Decant the solution and wash slides for 3x 5 min with 1x TBS-T in a staining jar under gentle agitation.

Reaction A

- Start preparing Reaction A by diluting **Buffer A (5x)** 1:5 in water. Vortex and spin down.
- Add **Enzyme A** (dilute 1:40). Mix gently by pipetting and spin down.
- Add enough Reaction A to cover the sample area.
- Incubate for 60 min at 37 °C in a pre-heated humidity chamber.
- Decant the solution and wash slides for 2x 3 min with 1x TBS-T in a staining jar under gentle agitation.

Reaction B

- Start preparing Reaction B by diluting **Buffer B (5x)** 1:5 in water. Vortex and spin down.
- Add **Enzyme B** (dilute 1:40). Mix gently by pipetting and spin down.
- Add enough Reaction B to cover the sample area.
- Incubate for 30 min at 37 °C in a pre-heated humidity chamber.
- Decant the solution and wash slides for 2x3 min with 1x TBS-T in a staining jar under gentle agitation.

Reaction C

IMPORTANT: Light sensitive reagents – keep protected from light.

- Start preparing Reaction C by diluting **Buffer C (5x)** 1:5 in water. Vortex and spin down.
- Add **Enzyme C** (dilute 1:40). Mix gently by pipetting and spin down.
- Add enough Reaction C to cover the sample area.
- Incubate for 90 min at 37 °C in a pre-heated humidity chamber.
- Decant the solution and wash slides for 2x 10 min with 1x TBS in a staining jar under gentle agitation and protected from light.
- Decant the solution and wash slides for 15 min with 0.1x TBS in a staining jar under gentle agitation and protected from light.

Mounting

IMPORTANT: Light sensitive reagents – keep protected from light.

- Tap off excess wash buffer from the slides.
- Mount the slides with a coverslip using a water-based anti-fade mounting medium with DAPI.
- Wait for 15 minutes.
- Image your slides in a fluorescence or confocal microscope, using 20x objective or higher and filter set for DAPI and Texas red.

6. Troubleshooting

Hight amount of background signal (in the technical control)	
Insufficiently washed samples and/or antibody cross-contamination	• Due to the high efficiency of detection, it is important to wash different antibody conditions separately to reduce cross-contamination between samples. • Increase the number of washes, the washing time, and/or the wash volume. • Use wash buffers indicated in the protocol. • Use a fresh wash solution. If it has become cloudy or there are any salt precipitates, prepare a new washing buffer.
A too high concentration of the primary antibody	• Titrate both antibodies individually while keeping the other constant and select the concentration for each of the antibodies that resulted in the highest signal-to-noise ratio.
Wrong fixation method	• Use fresh fixation reagents. • Use a fixation method recommended/optimized for the chosen antibodies. Recommendations for antibodies can be found in the datasheets.
Inadequate blocking	• Use the Navinci blocking buffer/AB diluent for blocking and for diluting the primary antibody. • Cover the entire sample in the blocking solution. • Dilute the probes in the provided Navinci probe diluent. • Increase blocking incubation time. • In certain cases, an alternative blocking protocol might be needed for a specific set of antibodies.
Bad/dysfunctional probes	• Do not let the probes freeze. • Do not leave the probes in room temperature for extended periods. • Expired probes might not perform as expected.
Drying of sample	• Keep samples in a pre-heated humidity chamber during incubations. • Do not let samples dry out in between steps. To reduce the risk of drying, prepare the reaction mixtures before taking samples out of washing buffer and only remove a subset of your samples at a time.
Precipitate in buffer B	• Make sure that the buffer B is completely thawed. • The buffer can be left for 30 min at room temperature. If the precipitate has not been dissolved after vortexing, it can be heated in the palm of your hand until completely dissolved. • Always vortex reagents before use.

Non-RCA based fluorescent particles	• If growing cells: Wash off any culture medium before fixation. • Prepare fresh fixation reagents. • Use a fresh wash solution; if it has become cloudy or there are any salt precipitates, prepare a new washing buffer. • Ensure to wash the sample in 0.1x TBS before mounting. If the problem persists, dip the sample in pure water to remove any excess salt.
Unspecific primary antibodies	• If background persists despite the critical determination of conditions (fixation, permeabilization, antibody titter, etc.), try an alternative primary antibody against the target(s).
Diffuse staining and/or autofluorecence	• Cell and tissue autoflurecence is often caused by the fixation procedure and can be reduced by investigating alternative fixation methods or by incubating with autofluorecence blocking reagents e.g. glycin. • Autoflurencent-like staining can be caused by unspecific binding of detection reagents in Reaction C. It can be removed by increasing the washing time after Reaction C and, in certain cases, by leaving the sample in 0.1x TBS overnight at 4°C. • Optimize the sample mounting as certain mounting media can cause haziness of the staining. Choosing a mounting medium without nuclei stain e.g. DAPI have showen to give a clearer nuclei stain.

No or low amount of signal	
Primary antibody from incorrect species	• Use primary antibodies suitable for the two probes. The two probes will bind an antibody from mouse and rabbit origin, respectively.
No or insufficient binding of primary antibodies	• Optimize sample preparation such as fixation, permeabilization, and antigen retrieval to ensure binding of both primary antibodies. • Titrate both antibodies individually while keeping the other constant and select the concentration for each of the antibodies that results in the highest signal. • For single protein detection, make sure that the antibody pair selected do not interfere with each other's epitope binding.
Incubation of reaction at the incorrect temperature	• Perform all incubations at the indicated temperatures, especially during the enzymatic steps (Reaction A, B, and C).
Excess wash buffer left on the sample	• Dilution of antibodies and/or enzymes by residual wash buffer will increase variability between samples/experiments and reduce the efficiency. • Remove any excess wash buffer by aspiration or taping it off, before adding the reagent mixtures
Incorrect washing buffers	• Use wash buffers according to the protocol specification. Exchanging type of buffer can reduce efficiency. • Ensure the wash buffers are at room temperature.

Inefficient Reaction A, B or C	• Keep to the incubation times and temperature (37 °C) as stated in the protocol. • Remove any excess of wash buffer from the sample before adding the reagents. • Defrost the buffer at room temperature and vortex before use. • Always keep the enzyme at -20 °C for storage and on ice or on a frozen cold block when pipetting to ensure enzyme activity. • Prepare Reaction A, B and C fresh before use and add enzyme immediately prior to adding to the sample. • Incubate in a pre-heated humidity chamber.
Incompatible filter set during acquisition	• Use the appropriate filter set during image acquisition. • Navinci Universal have a fluorophore with an excitation wavelength of 596 nm and an emission wavelength of 613 nm and would be compatible with a filter set suitable for e.g. TexasRed fluorophores.

Poor imaging and analysis	
Coalescing Signal	• Set exposure time to avoid over-exposure of any image. Never use auto-expose when acquiring images of the signal channel and use the same exposure time for all samples within an experiment. • One or both primary antibody concentrations could be too high. Consider titrating them. • Keep the incubation time (90 min) and temperature (37 °C) according to the protocol for reaction C, but for certain assays reducing the incubation time could reduce signals coalescing.
Autofluorescence	• Set exposure time to avoid over-expose of any image. Never use auto-expose when acquiring images of the signal channel. • Cell and tissue autoflurecence is often caused by the fixation procedure and can be reduced by investigating alternative fixation methods or by incubating with autofluorecence blocking reagents e.g. glycin. • For certain assays, increasing the incubation time for Reaction C can increase signal strength over autofluorescence. • Increase the number of washes after Reaction C. • For certain assays, leaving the sample in 0.1x TBS overnight at 4°C will reduce autofluorescence-like background from fluorophores present in Buffer C.

The variation between replications/experiments	
Experimental set-up	• Different experimental set-ups e.g. a change in cell line/tissue, antibody or cell treatment can introduce variation. To control for assay variability, always keep one of your conditions identical across all experiments.
Sample preparation	• Cell fixation, permeabilization, and cell density all affect the signal and should be constant between experiments. • Use the same tissue and tissue antigen retrieval protocol when assessing variation.
Deviation from the protocol	• Follow the protocol incubation times and temperatures. • If a deviation from the protocol is required, make the change consistently across all the experiments and change one experimental contidion at a time.
Buffer mixing and pipetting errors	• Defrost buffers completely and allow to come to room temperature. Vortex reagents to ensure a homogeneous solution. • Use same reagents across experiments in case of batch alteration due to suboptimal storage or contamination of specific tubes. • Use best practices when pipetting to minimize variation between experiment. • Immerse the pipette tip to the appropriate depth. Too much immersion causes enzymes to stick to the outside of the tip and increase the enzyme volume in the reaction.
Sample drying and insufficient removal of wash buffer	• Do not let the sample dry out in between steps. • Remove any wash buffer left before adding reaction buffer to avoid dilution. • Work with fewer samples at a time if the two suggestions above are difficult to perform successfully.

7. References

Klaesson A, Grannas K, Ebai T, Heldin J, Koos B, Leino M, Raykova D, Oelrich J, Arngården L, Söderberg O, Landegren U. Improved efficiency of in situ protein analysis by proximity ligation using UnFold probes. Sci Rep. 2018 Mar 29;8(1):5400. PMID: 29599435

8. Limited product warranty

Navinci Universal kits is not liable for property damage, personal injury, or economic loss caused by this product.
This product includes a license for non-commercial use of the **Navinci Diagnostics™** Product. Commercial users will require additional licenses and/or permissions.
Please contact **Navinci Diagnostics™ AB** for details.

To learn more about and search for specific **Navinci kits**, please visit **www.navinci.se** or contact as at **contact@navinci.se**