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SARS-CoV-2 Membrane Protein Proximity Ligation Assay (PLA) Kit (Cy3)

(For Research Use Only. Not for Use in Diagnostic or Therapeutic Applications)

GTX537371-23

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Introduction

Proximity ligation assay (PLA) is an antibody-based method that allows localized detection of proteins or protein complexes with superior specificity and sensitivity (1). In short, when a pair of oligonucleotide (oligo)-labeled antibodies (PLA probes) bind to their target epitopes in close proximity (<40 nm), the oligonucleotides will direct the formation of a circular DNA molecule (2). This circular DNA molecule undergoes rolling circle amplification to generate a localized, long concatemeric DNA product, which is then detected using fluorescent dye- or HRP-conjugated detection probes. Because of the requirement for dual recognition of the target by both antibodies and the subsequent localized DNA amplification, PLA offers researchers a highly specific and sensitive diagnostic tool to detect individual proteins or interacting protein pairs *in situ*.

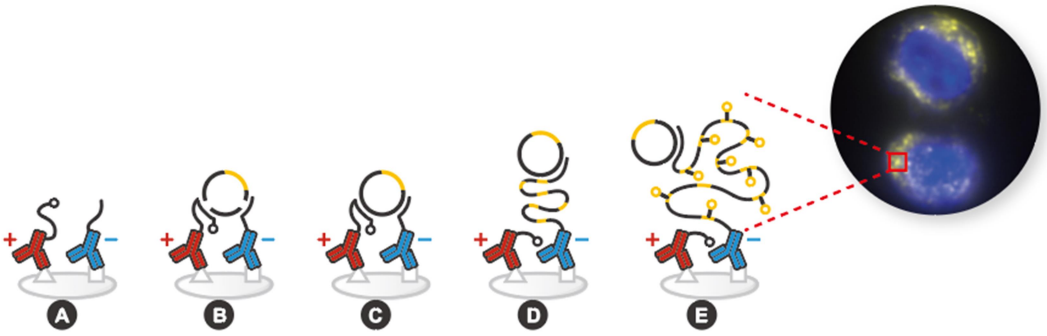
Advantages of Proximity Ligation Assay

- Detects endogenous protein
- Visualizes protein-protein interactions
- Visualizes protein modifications (mutations, phosphorylation, etc.)
- High specificity
- Single-molecule sensitivity

GeneTex's SARS-CoV-2 Membrane Protein Proximity Ligation Assay (PLA) Kit (Cy3) enables the detection of SARS-CoV-2 membrane protein in virus-infected tissue or infected/transfected cell block samples. Membrane protein is bound by two oligo-conjugated primary antibodies and visualized through fluorescence detection.

Principle of PLA

A. The samples are incubated with the two PLA probes that bind to their target epitopes.
B. The ligation solution, consisting of long and short connector oligos, is added to the sample. With the two PLA probes bound in close proximity, the linked oligos hybridize with the connector oligos to form a single-stranded DNA circle.
C. The gaps between the two connector oligos on the DNA circle are then sealed by DNA ligase.
D. The amplification solution and DNA polymerase are added to the sample. One of the PLA oligos serves as a primer while the closed DNA circle acts as the template for the rolling circle amplification (RCA) reaction.
E. A Cy3 fluorescent dye-conjugated oligonucleotide probe is then added to hybridize to the RCA product. The resulting signal is detected as a bright fluorescent spot under the fluorescence microscope.



Positive PLA Result Example

A successful PLA reaction will generate discrete fluorescent signals seen through a fluorescence microscope in different locations in cells or tissue sections. Each fluorescent spot represents an individual PLA pair recognizing a single protein molecule.

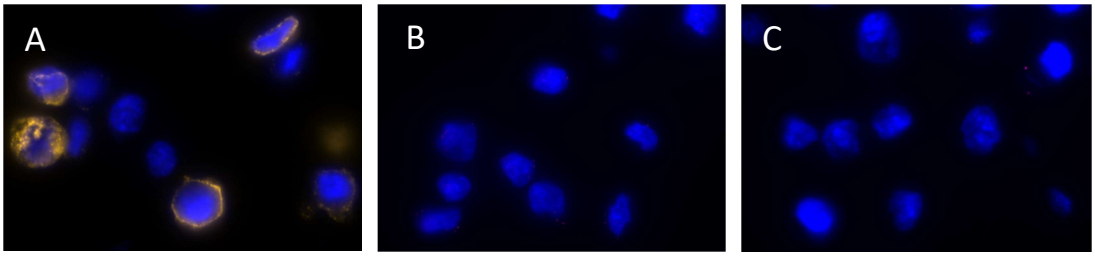


Figure. Detection of SARS-CoV-2 (COVID-19) membrane protein in a transfected HEK293T cell FFPE Cell Pellet Block (GTX435640) section using the SARS-CoV-2 Membrane Protein Proximity Ligation Assay (PLA) Kit (Cy3). PLA signals appear yellow and nuclei are blue. A) Positive PLA reaction in transfected HEK293T cells. B) Negative control with mock-transfected HEK293T cells. C) Negative control using one non-specific PLA antibody probe in transfected HEK293T cells.

Kit Contents (Five (5) reactions)

Item	Name	Quantity	Storage
A1	Blocking Solution	10 mL, 2 bottles	At -20°C
A2	Antibody (Ab)-Oligo A	40 µL, 1 tube	At 4°C for 1-2 weeks or at -20°C for long-term storage
A3	Antibody (Ab)-Oligo B	40 µL, 1 tube	At 4°C for 1-2 weeks or at -20°C for long-term storage
A4	Ligase (400 U/µL)	10 µL, 1 tube	At -20°C
A5	5x Ligation Solution	500 µL, 1 tube	At -20°C
A6	Polymerase (10 U/µL)	10 µL, 1 tube	At -20°C
A7	5x Amplification Solution	500 µL, 1 tube	At -20°C
A8	Detection Probe (Cy3)	50 µL, 1 tube	At -20°C
B1	10x Probe Solution	200 µL, 1 tube	At 4°C
B2	20x Wash Buffer	50 mL, 2 bottles	At 4°C

Reagent Description

1. **Blocking Solution (A1) – store at -20°C**

- For blocking and dilution of PLA antibodies A2 and A3.
- Ready to use.

2. **Oligo-conjugated antibodies A and B (A2, A3) – store at -20°C for long-term storage or at 4°C for 1-2 weeks**

- Avoid multiple freeze-thaw cycles.
- Spin down and gently pipette before use.
- Dilute required amount 1:100 in Blocking Solution and mix well by gentle pipetting before adding to the sample.

3. **20x Wash Buffer (B2) – store at 4°C**

- Dilute required amount of 20x stock in ddH_2O to make 1x wash buffer.

4. **5x Ligation Solution (A5) – store at -20°C**

- Thaw at room temperature and vortex before use.
- Dilute required amount 1:5 in ddH_2O immediately before use to make 1x ligation solution.

5. **Ligase (400 U/µl) (A4) – store at -20°C**

- Ligase should be kept at -20°C at all times. Use a freezing block on ice when removing the enzyme from the freezer.
- Add required amount of Ligase to the 1x ligation solution at a 1:400 dilution immediately before adding to the sample.
- Ensure that the ligation-ligase reaction mix is thoroughly but gently pipetted before adding to the sample.

6. **5x Amplification Solution (A7) – store at -20 °C**

- Thaw at room temperature and vortex before use.
- Dilute required amount 1:5 in ddH_2O immediately before use to make 1x amplification solution.

7. **Polymerase (10 U/µl) (A6) – store at -20 °C**

- Polymerase should be kept at -20°C at all times. Use a freezing block on ice when removing the enzyme from the freezer.
- Add required amount of Polymerase to the 1x amplification solution at a 1:200 dilution immediately before adding to the sample.
- Ensure that the amplification-polymerase reaction mix is thoroughly but gently pipetted before adding to the sample.

8. **Detection Probe (Cy3) (A8) – store at -20 °C**

- Always store in dark.

9. **10x Probe Solution (B1) – store at 4°C**

- Dilute required amount 1:10 in ddH_2O to make 1x probe solution.

Materials required but not provided

- Ultrapure or sterile-filtered ddH_2O
- Formalin-fixed paraffin-embedded (FFPE) samples (cells or tissues) on a slide
- Mounting medium

Equipment required

- Fluorescence microscope with appropriate filters, camera, and software for image acquisition
- 30°C incubator
- Humidity chamber
- Hydrophobic barrier pen for delimitation of reaction area on slide
- Freezer block (for enzymes)
- Staining jars
- Forceps
- Pipettes and tips (from 0.2 µL to 1000 µL)
- Coverslips compatible with fluorescence microscopy

PLA Immunofluorescence Protocol

The following protocol is for paraffin-embedded tissue sections or cell blocks mounted on a glass slide. The samples need to be deparaffinized, rehydrated, and have undergone an antigen-retrieval process before starting the protocol.

A. Delimitation of reaction area

1. Use a hydrophobic barrier pen or silicone wax to encircle a reaction area encompassing the sample(s) on the slide.
2. Use open droplet reactions without a coverslip.
3. Use volumes appropriate for the designated reaction area.

Reaction Volume Guide

Area	Reaction Volume
1 cm^2	40 µl
2 cm^2	80 µl
3 cm^2	120 µl
4 cm^2	160 µl
5 cm^2	200 µl
6 cm^2	240 µl
8 cm^2	320 µl
10 cm^2	400 µl

For this kit, one (1) reaction assumes a 400 µl reaction volume.

B. Blocking

1. Add sufficient Blocking Solution (A1) to cover the entire reaction area.
2. Incubate slide in a humidity chamber for 30 min at room temperature.

C. PLA probe incubation

1. Dilute both (A2, A3) PLA antibody probes 1:100 in Blocking Solution (A1) (i.e., for each 100 µl of a reaction mix, add 1 µl of each antibody to 98 µl of Blocking Solution).
2. Tap off the Blocking Solution from the slides.
3. Add the diluted PLA antibody solution to each slide.
   Note: Do not allow the samples to dry out as this may result in significant background.
4. Incubate in a humidity chamber overnight at 4°C.

D. Ligation

1. Tap off the PLA antibody solution from the slides.
2. Wash the slides in 1x wash buffer 3 times for 5 min each. Tap off the wash buffer from the slides.
3. During the wash, prepare 1x ligation solution by diluting the 5x Ligation Solution (A5) 1:5 in ddH_2O and mix well.
   Note: The Ligase (A4) enzyme should be added to the final mix at a 1:400 dilution immediately before dispensing onto the samples. Please take the volume of Ligase into account when calculating the amount of ddH_2O needed to dilute the ligation mix (e.g., for a 200 µl reaction, use 40 µl of the 5x Ligation Solution (A5) and 159.5 µl ddH_2O).
4. Add Ligase (A4) to the 1x ligation solution at a 1:400 dilution (i.e., 0.5 µl for the 200 µl reaction mix example in step 3 above) and mix well by gentle pipetting.
5. Tap off the wash buffer from the slides and add the ligation reaction mix from step 4 to each sample.
6. Incubate in a humidity chamber for 45 min at 30°C.

E. Amplification

1. Tap off the ligation reaction mix from the slides.
2. Wash the slides in 1x wash buffer 3 times for 5 min each. Tap off the wash buffer from the slides.
3. During the wash, prepare the 1x amplification solution by diluting the 5x Amplification Solution (A7) 1:5 in ddH_2O and mix well.

Note: The Polymerase (A6) enzyme should be added to the final mix at a 1:200 dilution immediately before dispensing onto the samples. Please take the volume of Polymerase into account when calculating the amount of ddH_2O needed to dilute the amplification mix (e.g., for a 200 µl reaction, use 40 µl of the 5x Amplification Solution (A7) and 159 µl ddH_2O).

4. Add Polymerase (A6) to the 1x amplification solution at a 1:200 dilution (i.e., 1.0 µl for the 200 µl reaction mix example in step 3 above) and mix well by gentle pipetting.
5. Tap off the wash buffer from the slides and add the amplification reaction mix from step 4 to each sample.
6. Incubate in a humidity chamber for 100 min at 30°C.

F. Probe hybridization

1. Tap off the amplification reaction mix from the slides.
2. Wash the slides in 1x wash buffer 3 times for 5 min each. Tap off the wash buffer from the slides.
3. During the wash, prepare the 1x probe solution by diluting the 10x Probe Solution (B1) 1:10 in ddH_2O and mix well.
4. Add the Detection Probe (Cy3) (A8) to the 1x probe solution at a 1:40 dilution (e.g., add 5 µl to 195 µl of 1x probe solution for a 200 µl reaction mix) and mix well by gentle pipetting. Wrap tube in foil to protect from light.
5. Tap off the wash buffer from the slides and add the detection probe reaction mix from step 4 to each sample.
6. Incubate in a humidity chamber for 30 min at room temperature in the dark.

G. Mounting and imaging

1. Tap off the detection probe reaction mix from the slides.
2. Wash the slides in 1x wash buffer 3 times for 5 min each. Tap off the wash buffer from the slides.
3. Wick the residual wash buffer from the sample using a Kimwipe.
4. Mount the slide with a coverslip using a minimal amount of aqueous mounting medium. Ensure there are no air bubbles under the cover slip. Seal the coverslip edge with nail polish and allow it to dry before imaging.
5. To obtain enough spatial resolution, we recommend using a 40x or higher resolution objective.
6. The sealed slide can be stored in the dark at 4°C for up to 5 days or at -20°C for up to 6 months.

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Poor signal in expected positive samples	Insufficient binding of PLA probes	Reduce the dilution factor of the two PLA probes to 1:80 or 1:50.
	Insufficient reaction volume	Ensure that the droplet covers the reaction area during the incubations. Place the slide in a humidity chamber to prevent evaporation.
	Inefficient ligation	Ensure no wash buffer remains on the slide. Prepare fresh ligation mix just prior to use.
	Inefficient amplification	Ensure no wash buffer remains on the slide. Prepare fresh amplification mix just prior to use.
High background	Incomplete deparaffinization	Incomplete removal of paraffin may cause background.
	Insufficient washing	Ensure sufficient washing during the washing steps.
	Drying of sample	Ensure constant humidity during all incubation steps and never let slides dry out.
High non-amplification-related background	Tissue autofluorescence	Use autofluorescence quencher to quench tissue autofluorescence.
	Dust, salt or fixation precipitates causing fluorescent particles	Use freshly prepared fixatives and wash cultured cells at least twice to ensure the complete removal of culture medium before adding fixatives. Sterile filter the 1x wash buffer.

References

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2. Bagchi S, Fredriksson R, Wallén-Mackenzie Å. In Situ Proximity Ligation Assay (PLA). *Methods Mol Biol.* 2015;1318:149-59. doi: 10.1007/978-1-4939-2742-5_15. PMID: 26160573.