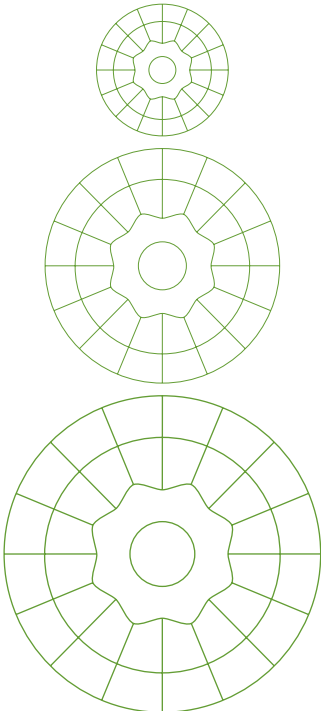


# Duolink<sup>®</sup> In Situ

INSTRUCTION – PROBE MAKER





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# 1. Introduction

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Duolink® In Situ Probemaker enables quick and convenient conjugation of the PLA® oligonucleotide arms directly to primary antibodies or secondary antibodies raised against haptens or species other than mouse, rabbit or goat.

The Duolink In Situ reagents are based on *in situ* PLA, which is a proximity ligation assay technology. A pair of oligonucleotide labeled antibodies (PLA probes) generates a signal only when the two PLA probes, one PLUS and one MINUS, have bound in close proximity. The signal from each detected pair of PLA probes is visualized as an individual spot. These PLA signals can be quantified (counted) and assigned to a specific subcellular location based on microscopy images.

With the Probemaker reagents you can create your own PLA probes by conjugating PLA oligonucleotides to antibodies. To perform a complete Duolink In Situ assay you need one PLA probe PLUS and one PLA probe MINUS and in addition your choice of Detection reagents, Wash Buffers and Mounting Medium. For a complete list of products, please visit [www.olin.com](http://www.olin.com) or contact your local distributor.

## 2. Applications

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### 2.1 USE ANTIBODIES FROM THE SAME SPECIES

Study protein-protein interactions, protein modifications, or single proteins, using two primary antibodies derived from the same species. Label one of the antibodies with the PLUS oligo and the other with the MINUS oligo (Fig. 1).

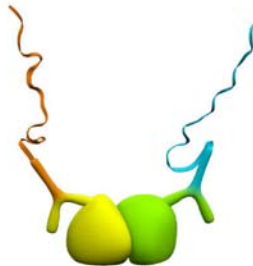


Fig 1. Detection of protein interactions using primary antibodies from the same species.

### 2.2 USE ANTIBODIES FROM ANY SPECIES

Study protein-protein interactions, protein modifications, or single proteins, using one or both primary antibodies derived from species other than mouse, rabbit or goat. Label one of your secondary antibodies with the PLUS oligo and the other with the MINUS oligo. Or, combine one labeled secondary antibody with a standard secondary Duolink In Situ PLA probe (Fig. 2).



Fig 2. Detection of protein interactions using one or both primary antibodies derived from species other than mouse, rabbit or goat.

# 3. Antibody requirements

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## 3.1 CONCENTRATION AND VOLUME

The antibody to be conjugated must have a concentration of 1 mg/ml. 20 µg (=20 µl) of antibody is needed per conjugation.

*Note: If a monoclonal antibody is used, it should be Protein A or Protein G affinity purified.*

## 3.2 BUFFER

The antibody must be in an amine free buffer, ideally PBS. The buffer should be carrier and preservative free but may contain up to 0.1% BSA, 5% trehalose and 0.02% sodium azide.

If you are unsure about the composition of the buffer your antibodies are stored in, Olink strongly recommends dialysis or buffer exchange prior to conjugation. Antibodies from various sources and suppliers vary in residual primary amine content often in the form of remaining glycine from the acidic elution of the antibody during the antigen-affinity purification step. Even if the supplier states PBS content, it is likely that there are residual amounts of primary amines.

Olink recommends the following standard procedure for buffer exchange:

*Note: Not for reducing high concentrations of BSA or other macromolecules.*

- ▶ Pre-equilibrate an illustra MicroSpin G-50 Columns spin column (GE Healthcare, Art. no. 27-5330-01) with 1xPBS by first spinning the column at 3000 rpm for 1 min, then add 400 µl of 1xPBS and spin again for 1 min and repeat 4 times. Place the column in a new microfuge tube.
- ▶ Add your antibody (12–50 µl) to the column and spin again for 2 min at 3000 rpm. The concentration of the collected antibody should be verified by OD. 1 mg/ml should have an OD 280 nm of 1.4.

Concentrating very diluted antibodies prior to Duolink In Situ Probemaker conjugation is not recommended unless you have large amounts since losses are very high with filter type concentrators.

## 4. Reagent preparation and storage

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Store the unused Probemaker kit at -20°C. Expiration date is marked on each individual lot. Once you have conjugated your antibody we recommend storage at +4°C. The Storage Solution contains buffer and reagents for stabilizing and preserving the conjugated antibody. Please note that you might need to add other components to preserve your specific antibody.

Each kit contains reagents to conjugate 20 µg antibody at a concentration of 1 mg/ml.

### Duolink In Situ oligonucleotide PLUS or MINUS – store at -20°C

- ▶ One vial with lyophilized activated oligonucleotide for one conjugation of 20 µg antibody at a concentration of 1 mg/ml.

### Conjugation Buffer – store at -20°C

- ▶ Reagents for buffering the conjugation reaction
- ▶ Vortex before use
- ▶ Ready-to-use solution

### Stop Reagent – store at -20°C

- ▶ Reagents for stopping the conjugation reaction
- ▶ Vortex before use
- ▶ Ready-to-use solution

### Storage Solution – store at -20°C

- ▶ Buffer for preserving the conjugated antibody (PLA probe)
- ▶ Vortex before use
- ▶ Ready-to-use solution

### Assay Reagent (20x) – store at -20°C

- ▶ Specific reagent to be added to your own optimized antibody diluent
- ▶ Vortex before use
- ▶ Dilute 1:20 in your optimized antibody diluent prior to diluting the conjugated antibodies (PLA probes).

*Note: If you do not have a previously optimized blocking solution or antibody diluent for your antibody, please use the following Blocking Solution and PLA probe Diluent included in the Probemaker kit.*

### Blocking Solution – store at -20°C to +4°C

- ▶ For blocking of your sample prior to staining with Duolink In Situ
- ▶ Vortex before use
- ▶ Ready-to-use blocking solution, one drop equals approximately 40 µl

### PLA probe Diluent – store at -20°C

- ▶ Buffer for diluting the conjugated antibody (PLA probe) to the final assay concentration
- ▶ Vortex before use
- ▶ Ready-to-use solution

## 5. Protocols

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This folder consists of two sections; 5.1 Conjugation protocol and 5.2 PLA probe protocols. The first, Conjugation protocol in section 5.1, describes how to conjugate your antibody with either the PLUS or MINUS oligonucleotide, to create a PLA probe. Subsequent to the conjugation procedure, proceed to any of the four PLA probe protocols in section 5.2: Custom solutions and conjugated primary antibodies (5.2.1), Custom solutions and conjugated secondary antibodies (5.2.2), Duolink In Situ solutions and conjugated primary antibodies (5.2.3) or Duolink In Situ solutions and conjugated secondary antibodies (5.2.4). This depends on if you have your own custom blocking solution and antibody diluent or if you use the supplied Duolink In Situ solutions and if you have used Probemaker to conjugate your primary or secondary antibodies. To perform a complete Duolink In Situ assay and generate PLA signals you also need your choice of Detection Reagents, Wash Buffers and Mounting Medium. The Detection protocol is described in the Duolink In Situ User Manual (Doc no. 0650 for Fluorescence and Doc no. 0656 for Brightfield). Please visit [www.olk.com](http://www.olk.com) for more information.

### 5.1 CONJUGATION PROTOCOL

Ensure that your antibody to be conjugated has a concentration of 1mg/ml. 20 µg (=20 µl) of antibody is needed per conjugation. The antibody buffer should be carrier and preservative free but may contain up to 0.1% BSA, 5% trehalose and 0.02% sodium azide.

#### CONJUGATION OF PLA OLIGONUCLEOTIDE TO ANTIBODY

- 1) Add 2 µl of Conjugation Buffer to 20 µl of the antibody to be conjugated. The antibody concentration should be 1 mg/ml.
- 2) Mix gently with a pipette.
- 3) Transfer the antibody solution to one vial of lyophilized oligonucleotide (PLUS or MINUS).  
*Note: After opening the vial, add the antibody solution immediately.*
- 4) Mix gently with a pipette.
- 5) Incubate at room temperature over night.
- 6) Add 2 µl of Stop Reagent to the reaction.
- 7) Incubate at room temperature for 30 minutes.
- 8) Add 24 µl of Storage Solution and possibly other reagents for stabilizing your specific antibody.
- 9) The conjugation is now completed and your PLA probe is stable in the Storage Solution at +4°C.

» Proceed to any of the four PLA probe protocols described in section 5.2 depending on your blocking solution and antibody diluent and if you have conjugated your primary or secondary antibodies.



## 5.2 PLA PROBE PROTOCOLS

It is recommended that you use your previously optimized blocking solution and antibody diluent, or follow the recommendations from the antibody vendor, when blocking and diluting your conjugated antibodies (PLA probes). Note that you have to add the Assay Reagent (20x) to your custom antibody diluent before diluting your conjugated antibodies. When using your own custom blocking solution and antibody diluent, please follow the PLA probe protocol given in section 5.2.1 if you have conjugated your primary antibodies and 5.2.2 if you have conjugated your secondary antibodies.

If you do not have a previously optimized blocking solution or antibody diluent for your conjugated antibody you should use the Blocking Solution and the PLA probe Diluent included in the Probemaker kit. These reagents use BSA as blocking agent. When using these reagents, please follow the PLA protocol given in section 5.2.3 if you have conjugated your primary antibodies and 5.2.4 if you have conjugated your secondary antibodies.

Before you start, your samples should be deposited on glass slides and pre-treated with respect to fixation, retrieval and/or permeabilization.

Your reaction area must be delimited e.g. with a hydrophobic barrier pen. Use reaction volumes corresponding to your delimited reaction area, see the Reaction Volume Guide (Art. no. 80520). Use open droplet reactions *without a cover slip* and perform all incubations in a humidity chamber.

## 5.2.1 PLA PROBE PROTOCOL – CUSTOM SOLUTIONS AND CONJUGATED PRIMARY ANTIBODIES

Follow this protocol if you use your own custom blocking solution and antibody diluent and if you have used Probemaker to conjugate primary antibodies.

### 1. BLOCKING

*Use your previously optimized blocking solution. If you are using Duolink In Situ Detection Reagents Brightfield we recommend a peroxidase quenching step before blocking.*

- a) Add your custom blocking solution to each sample.
- b) Incubate the slides.

### 2. PLA PROBES

*Use your previously optimized antibody diluent to dilute your conjugated antibodies (PLA probes).*

- a) Dilute the Assay Reagent (20x) 1:20 in your custom antibody diluent.
- b) Dilute your conjugated antibodies (PLA probes), one PLUS and one MINUS, to a suitable concentration in your antibody diluent containing 1x Assay Reagent. Allow the mixture to sit for 20 min at room temperature.  
*Note: You may need to titrate your PLA probes to a suitable concentration; we recommend you to start with a higher concentration than what you usually use for your primary antibodies in IF or IHC.*
- c) Tap off the blocking solution from the slides. Try to obtain an equal residual volume on each slide as this will affect reproducibility.  
*Do not allow the samples to dry before adding the PLA probes as this will cause background.*
- d) Add the diluted PLA probes to each sample.
- e) Incubate in a humidity chamber.  
*Use temperature and time optimal for your antibodies.*

### 3. DETECTION PROTOCOL

Proceed to section 7.3 in the Duolink In Situ User Manual (Doc no. 0650 for Fluorescence and Doc no. 0656 for Brightfield).

## 5.2.2 PLA PROBE PROTOCOL – CUSTOM SOLUTIONS AND CONJUGATED SECONDARY ANTIBODIES

Follow this protocol if you use your own custom blocking solution and antibody diluent and if you have used Probemaker to conjugate secondary antibodies.

### 1. BLOCKING

*Use your previously optimized blocking solution. If you are using Duolink In Situ Detection Reagents Brightfield we recommend a peroxidase quenching step before blocking.*

- a) Add your custom blocking solution to each sample.
- b) Incubate the slides.

### 2. PRIMARY ANTIBODIES

*Use your previously optimized antibody diluent to dilute your primary antibodies.*

- a) Dilute your primary antibodies to a suitable concentration in your custom antibody diluent.
- b) Tap off the blocking solution from the slides. Try to obtain an equal residual volume on each slide as this will affect reproducibility.  
*Do not allow the samples to dry before adding the primary antibodies as this will cause background.*
- c) Add the primary antibody solution to each sample.
- d) Incubate in a humidity chamber.  
*Use temperature and time optimal for your primary antibodies.*

### 3. PLA PROBES

*Use your previously optimized antibody diluent to dilute your conjugated antibodies (PLA probes).*

- a) Dilute the Assay Reagent (20x) 1:20 in your custom antibody diluent.
- b) Dilute your conjugated antibodies (PLA probes), one PLUS and one MINUS, to a suitable concentration in your antibody diluent containing 1x Assay Reagent. Allow the mixture to sit for 20 min at room temperature.  
*Note: You may need to titrate your PLA probes to a suitable concentration; we recommend you to start with the same concentration as you usually use for your secondary antibodies in IF or IHC.*
- c) Tap off the primary antibody solution from the slides.
- d) Wash the slides in a wash buffer suitable for your primary antibodies.  
*Washing should be performed in a staining jar, with a minimum volume of 70 ml on a shaker (cradle) with gentle orbital shaking. Bring the wash buffers to room temperature before use.*
- e) Add the diluted PLA probes to each sample.
- f) Incubate the slides in a pre-heated humidity chamber for 1 h at +37°C.

### 4. DETECTION PROTOCOL

Proceed to section 7.3 in the Duolink In Situ User Manual (Doc no. 0650 for Fluorescence and Doc no. 0656 for Brightfield).

### 5.2.3 PLA PROBE PROTOCOL – DUOLINK IN SITU SOLUTIONS AND CONJUGATED PRIMARY ANTIBODIES

Follow this protocol if you use the Blocking Solution and PLA probe Diluent included in the Probemaker kit and if you have used Probemaker to conjugate primary antibodies.

#### 1. BLOCKING

*If you are using Duolink In Situ Detection Reagents Brightfield we recommend a peroxidase quenching step before blocking.*

- a) Add one drop of Blocking Solution per 1 cm<sup>2</sup>. One drop equals approximately 40  $\mu$ l.
- b) Incubate the slides in a pre-heated humidity chamber for 30 min at +37°C.

#### 2. PLA PROBES

- a) Dilute your conjugated antibodies (PLA probes), one PLUS and one MINUS, to a suitable concentration in the PLA probe Diluent.

*Note: You may need to titrate your PLA probes to a suitable concentration; we recommend you to start with a higher concentration than what you usually use for your primary antibodies in IF or IHC.*

- b) Tap off the Blocking Solution from the slides. Try to obtain an equal residual volume on each slide as this will affect reproducibility.  
*Do not allow the samples to dry before adding the PLA probes as this will cause background.*
- c) Add the diluted PLA probes to each sample.
- d) Incubate in a humidity chamber.  
*Use temperature and time optimal for your primary antibodies.*

#### 3. DETECTION PROTOCOL

Proceed to section 7.3 in the Duolink In Situ User Manual (Doc no. 0650 for Fluorescence and Doc no. 0656 for Brightfield).

## 5.2.4 PLA PROBE PROTOCOL – DUOLINK IN SITU SOLUTIONS AND CONJUGATED SECONDARY ANTIBODIES

Follow this protocol if you use the Blocking Solution and PLA probe Diluent included in the Probemaker kit and if you have used Probemaker to conjugate secondary antibodies.

### 1. BLOCKING

*If you are using Duolink In Situ Detection Reagents Brightfield we recommend a peroxidase quenching step before blocking.*

- a) Add one drop of Blocking Solution per 1 cm<sup>2</sup>. One drop equals approximately 40 ul.
- b) Incubate the slides in a pre-heated humidity chamber for 30 min at +37°C.

### 2. PRIMARY ANTIBODIES

- a) Dilute your primary antibodies to a suitable concentration in the Blocking Solution.
- b) Tap off the Blocking Solution from the slides. Try to obtain an equal residual volume on each slide as this will affect reproducibility.  
*Do not allow the samples to dry before adding the primary antibodies as this will cause background.*
- c) Add the primary antibody solution to each sample.
- d) Incubate in a humidity chamber.  
*Use temperature and time optimal for your primary antibodies.*

### 3. PLA PROBES

- a) Dilute your conjugated antibodies (PLA probes), one PLUS and one MINUS, to a suitable concentration in the PLA probe Diluent.  
**Note:** *You may need to titrate your PLA probes to a suitable concentration; we recommend you to start with the same concentration as you usually use for your secondary antibodies in IF or IHC.*
- b) Tap off the primary antibody solution from the slides.
- c) Wash the slides in 1x Wash Buffer A or in a wash buffer suitable for your primary antibodies, preferably 2 x 5 min.  
*Washing should be performed in a staining jar, with a minimum volume of 70 ml on a shaker (cradle) with gentle orbital shaking. Bring the wash buffers to room temperature before use.*
- d) Add the diluted PLA probes to each sample.
- e) Incubate in a pre-heated humidity chamber for 1 h at +37°C.

### 4. DETECTION PROTOCOL

Proceed to section 7.3 in the Duolink In Situ User Manual (Doc no. 0650 for Fluorescence and Doc no. 0656 for Brightfield).





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