Return to Web Version SIGMA-ALDRICH®

Duolink® PLA Troubleshooting Guide

Tips & Tricks
- Upfront Considerations
- Common Experimental Parameters
- Sample Storage and Analysis

Troubleshooting
- Too High of a Background Signal
- Low or Lack of Signal
- Poor Imaging & Analysis

Frequently Asked Questions
- General Questions
- Protocol and Analysis
- Specific Requirements
- Duolink® Probemaker

Additional Support
Materials

Tips & Tricks

The Duolink® PLA technology for protein detection offers a simple and straightforward protocol that has a set of defined steps. This section will delineate important reminders that will ensure your Duolink® PLA experiment proceeds smoothly.

Upfront Considerations

- Use conditions for optimal primary antibody performance within the sample to be tested. These include sample processing parameters (fixation, permeabilization, antigen retrieval, etc), primary antibody titer, and incubation time.
- Always include both technical and, when possible, biological controls in your experiment to properly evaluate the results.
- The **Duolink® PLA Control Kit** is a convenient tool to test the technology and obtain successful results. It includes microscope slides with pre-plated cells and a pair of antibodies to look at protein-protein interaction using the **Duolink® PLA reagents**. The PLA signals are guaranteed. Use this Control Kit to gain confidence with the **Duolink® PLA technology** or to add additional controls to your experiment.

Common Experimental Parameters

- Never let your sample dry out. Use a humidity chamber during incubation steps.
- Make sure to remove excess wash solutions from samples. Residual wash buffer can cause further dilution of antibodies and/or decrease ligation or amplification efficiency.
- Use a hydrophobic pen to surround the sample. When using chambered slides, this will help prevent cross-contamination of solutions between wells. When using tissue sections, this will minimize required reagent volume.
- Perform all steps at the appropriate temperatures and incubation times for best results, in particular the enzymatic steps (ligation and amplification).
- For detection of low-abundance proteins, extended amplification times may be required. If background increases under these conditions, perform the amplification and detection steps separately by using the Duolink® Brightfield Amplification Buffer (does not contain detection oligos), followed by a 30-minute incubation with the Duolink® Fluorescent Amplification Buffer (contains detection oligos).
- Perform washes in ample wash buffer and ensure the samples are fully covered. Use Wash Buffers A and B where specified. Perform washes at room temperature.
- Keep enzymes in a freezer block while in use. Make sure other reagents are completely thawed (e.g., ligation buffer and amplification buffer) and vortexed prior to usage.

Sample Storage and Analysis

- All Duolink® PLA Probes must be stored at 4°C; this includes those generated using Duolink® Probemaker. **Do not freeze** or results will significantly decrease.
- For fluorescent applications, store slides in the dark at 4°C after mounting with Duolink® Mounting Media with DAPI for up to 4 days. Seal and store at -20°C for up to 6 months.
- Do not overexpose. Fluorescent signals can coalesce making it difficult to obtain accurate quantitative analysis. Of note, coalescence will not affect flow cytometry analysis.
- Images should be taken with the same acquisition parameters between experimental and control samples.

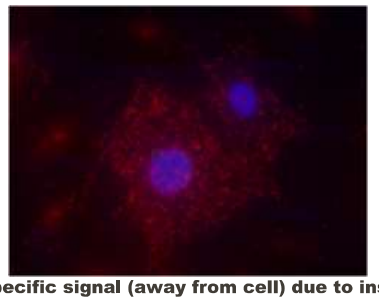
back to top

Troubleshooting

Despite all precautions, and the use of some excellent tips and trick, sometimes the results are not as expected. Lack of signal in a positive control or high background are two of the most common problems that are often seen when performing immunodetection experiments, and may also occur with the Duolink® PLA technology. The tables below list the probable causes and suggested solutions to get around these common problems. If problems remain, contact us at **sigma-aldrich.com/techservice** or (800) 325-5832.

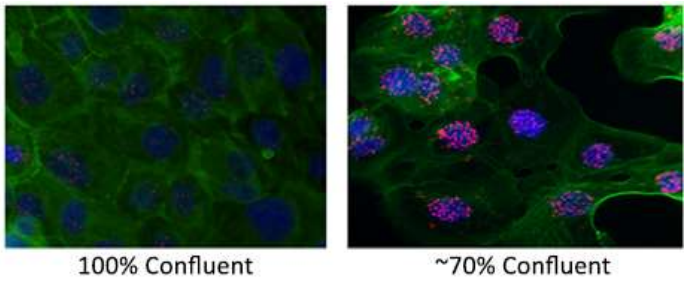
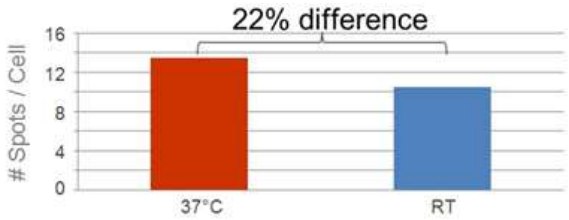
A. Too High of Background Signal

Concentration of primary antibody is too high	• Titrate each primary antibody separately using the single recognition PLA method as each may contribute differently to the background signal.
Unspecific binding of primary antibodies	• If background persists despite critical determination of conditions (fixation, permeabilization, antibody titer, etc.), try an alternative primary antibody to the same target.
Insufficient blocking	• Ensure entire sample is covered in blocking reagent. • Increase blocking incubation time.

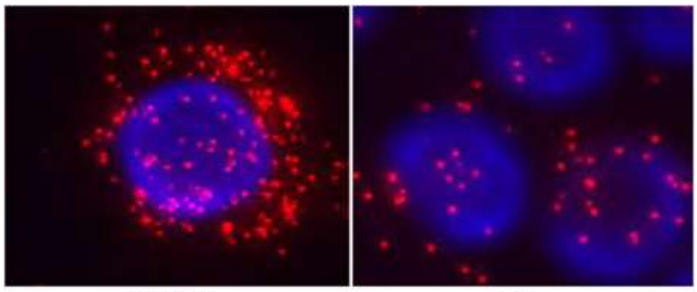
	• Dilute the primary and PLA probes in the provided antibody diluent which contains blocking agents optimized for Duolink® PLA. • If using your own blocking solution or antibody diluent, consider adding 20x Assay reagent (available in Duolink®Probemaker kits).
Insufficient washing of reaction	• Increase number of washes, wash times, and/or wash volumes • Use appropriate Wash Buffers A and B when specified. • Use freshly made wash solutions  **High level of nonspecific signal (away from cell) due to insufficient washing.**
Drying of sample	• Ensure good humidity during all incubation steps and never let filters dry out after washes and before addition of reagents.
Precipitate in ligation buffer	• Make sure ligase buffer is completely thawed and vortexed prior to using.
Dust, salt, or fixation precipitates cause highly fluorescent particles	• Wash your cells at least twice to ensure that the culture medium is removed before adding the fixative. • Use freshly made wash solutions. If the problem remains, sterile filter all washing solutions. • Make sure to use a 0.01x diluted Wash Buffer B prior to mounting..

back to top

B. Low or Lack of Background Signal

Cells at suboptimal density	• The optimal cellular confluence for Duolink®PLA experiments is 50-70% confluence. Less reagent penetration when overly confluent, resulting in lower signal.  100% Confluent ~70% Confluent
No or insufficient binding of primary antibodies	• Ensure parameters, such as fixation, epitope retrieval, and permeabilization conditions, are optimal for primary antibody performance. When using two primary antibodies, sample processing needs to be compatible for both primary antibodies. • Titrate each primary antibody separately using the single recognition PLA method.
Inappropriate incubation temperatures	• Perform all steps at the appropriate temperatures, in particular the enzymatic steps (ligation and amplification). 
Insufficient removal of excess wash buffers	• Residual wash buffer can cause further dilution of antibodies and/or decrease ligase and polymerase function. Aspirate or gently tap off excess solution prior to addition of new reagent.
Wash buffers used incorrectly	• Bring wash buffers to room temperature. • Use wash buffers A and B when specified.

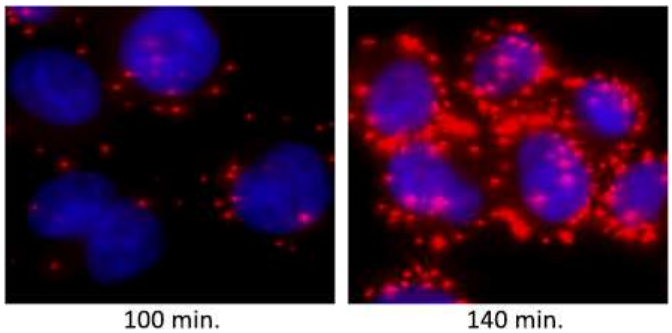
Effects of Wash Buffer Temperature



Wrong filter used for acquisition	• The fluorophore in the Amplification Green has an excitation wavelength of 495 nm and an emission wavelength of 527 nm and can be detected using the same filters as for e.g. Cy2 or FITC. • The fluorophore in the Amplification Orange has an excitation wavelength of 554 nm and an emission wavelength of 579 nm and can be detected using the same filters as for e.g. Cy3. • The fluorophore in the Amplification Red has an excitation wavelength of 594nm and an emission wavelength of 624 nm and can be detected using the same filters as for e.g. Texas Red. • The fluorophore in the Amplification Far Red has an excitation wavelength of 644 nm and an emission wavelength of 669 nm and can be detected using the same filters as for e.g. Cy5. • Make sure that you use appropriate filters.
Inefficient ligation	• Keep ligation incubation time and temperature. • Ensure that no excessive amount of wash solution remains on the filter before addition of ligation reagents. • Ensure that the Ligase is active (i.e. has been kept at -20 °C) and that correct dilution of the reagents have been used. • Prepare fresh dilutions just before use; do not allow mix, with enzyme, to stand for more than five minutes before use.
Inefficient amplification	• If the interactions are very low abundance, the signal can be very weak, as only a fraction above the negative controls. • Ensure that no excessive amount of wash solution remains on the filter before addition of amplification reagents. • Adjust amplification time for low-abundance interactions (can go as long as overnight at 37°C). • Ensure Polymerase is active (i.e., has been kept at -20 °C) and that correct dilution of the reagents have been used. • Prepare fresh dilutions just before use; do not allow mix, with enzyme, to stand for more than five minutes before use.

back to top

C. Poor Imaging and Analysis

Coalescence of Signal	• Extended amplification times can cause coalescence of signal. Follow recommended times for ligation and amplification steps.  • Too high of primary antibody titer can also cause coalescence of signal. Titrate each primary antibody using the single recognition PLA method. • Over-exposure during imaging can also result in coalescent signals. Do not use auto-exposure setting during image capture.
Autofluorescence	• Over-exposure during imaging can result in autofluorescence. Do not use auto-exposure setting during image capture. • Sometimes autofluorescence is inherent to the cells or tissue sample. Try changing the detection color, e.g., from green to far red, if possible. • Increase wash times or volumes with Wash Buffer B.

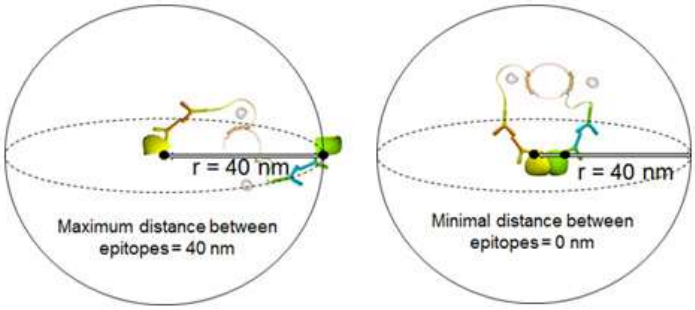
back to top

Frequently Asked Questions

This section provides answers to some frequently asked questions (FAQs). However, some information is proprietary and cannot be disclosed. This includes questions pertaining to specific composition of reagents and buffers, oligonucleotide length and sequences, or the conjugation chemistries of the Duolink® PLA Probes and Duolink® Probemaker kits.

GENERAL QUESTIONS

1. **What are the minimum and maximum distances for proximity ligation to work?**
   All Duolink® PLA products are designed to detect proximity. When using the secondary antibody approach, the theoretical maximum distance between your two target proteins (epitopes) is 40 nm to be able to create a signal. These numbers are based on the average diameter of an antibody being around 10 nm. Duolink® PLA can in theory detect epitopes that are within zero nm of each other, as long as the two primary antibodies can bind and there is no steric hindrance.



2. **How long do the Duolink® PLA products/reagents last?**
   - All the reagents in the detection kits must be kept at -20°C at all times. This is especially critical for the Ligase and the Polymerase enzymes, which need to be kept in a pre-chilled ice-block if taken out from the freezer (an ice-block keeps the enzymes at -20°C; you cannot achieve that by just having the enzymes on an ice bath). Duolink® PLA probes, antibody diluents, blocking solutions and mounting medium must be kept at +4°C. **Do not freeze the PLA probes or those generated by Probemaker.**
   - We have a 6-month guarantee upon arrival to your laboratory for the Duolink® PLA Probemaker, as long as it is kept at -20°C. Once the conjugation has taken place, we have noticed that for most conjugated antibodies the shelf life at +4°C is three to six months. In this case it will be very much dependent on if the antibody requires special preservatives or not.
3. **Can I apply Duolink® PLA to live cells or non-fixed tissue samples?**
   - Duolink® PLA has been optimized for fixed cells and tissues.
   - Permeabilization is necessary for the antibodies and probes to find their targets.
   - Furthermore, some of the Duolink® PLA reagents (enzymes, DNA oligos) will probably be destroyed by active mechanisms in living cells.
4. **Can I combine a PLA probe and traditional immunofluorescence?**
   Yes. It is possible to combine Duolink® PLA and traditional immunofluorescence techniques, as long as all primary antibodies are from different species and the fluorescence detection systems use different colored fluors.

back to top

DUOLINK PLA PROTOCOL OR ANALYSIS

1. **What are the Duolink® PLA Probes?**
   - Duolink® PLA Probes are secondary antibodies conjugated with either the PLUS or the MINUS oligonucleotides.
   - The secondary antibodies are derived from farmed donkey (*Equus asinus domesticus*).
   - Made using anti-mouse, anti-rabbit, and anti-goat polyclonal antibodies.
   - Minimal cross-reactivity with other species.
2. **What is the minimum and maximum number of cells / tissue needed?**
   - In principle, the minimum number of cells required for a Duolink® PLA experiment is one cell. The actual minimum number of cells used will be dependent on your particular application.
   - The maximum is difficult to establish. Antibodies and reagents may have difficulty to reach cells in the center of the sample if the cell density is too high. **50-70% confluent is optimal.**
   - When it comes to tissue samples, there is no minimum number. As for the maximum, up to 30 µm thick tissue slices have been used with successful results. **5-10 µm thick is optimal.**
   - The success of the experiment will depend both on the tissue sections and its pretreatment (e.g. fixation, permeabilization, epitope retrieval).
3. **When can I stop the procedure? OR What is a good stopping point in the protocol?**
   - There are several stopping points in the Duolink® PLA protocol. These include:
     - After fixation, samples can be left in 1x PBS at 4°C for several days
     - Blocking and primary antibody incubations can be run overnight. Depending on the primary antibody, incubation times can vary from half an hour to overnight. Observe that even if the recommended incubation time is shorter for a particular primary antibody, it is possible to prolong the incubation time to overnight at 4°C.
   - After the procedure is finished.
     - For the fluorescence detection: the drying of the slides at room temperature after the final wash step and before mounting the slides can be done overnight. Mounted slides can be stored at +4°C up to four days or sealed and stored at -20 °C for months before analysis. Please note that slides should be stored in the dark!
     - For the HRP detection (Brightfield): after the last dehydration step, slides can be left in fresh xylene or one can proceed with the mounting of the slides. Once slides are mounted they need to get dry, usually overnight.
   - Note: The ligation and amplification conditions have been optimized and should not be varied. Detection of a very low abundance protein or flow cytometry application may be an exception but may result in increased background if the amplification and detection steps are not separated.

4. **What is the effect of changing incubation times and temperatures?**
   The Duolink® PLA protocol has been optimized to obtain results in the shortest time possible without compromising the quality of results. Optimization of incubation times and temperatures has been an important factor in that respect, and therefore, best results are acquired when the protocol is followed with the recommendations given therein.

5. **How do I perform a single recognition experiment?**
   Performing a single recognition experiment, you will need one primary antibody against your particular target protein and two PLA probes, both directed towards the species of your primary antibody (e.g. if your primary antibody was raised in rabbit, then you will need the PLA probes anti-Rabbit PLUS and anti-Rabbit MINUS). The rest of the Duolink® PLA experiment is performed as usual.

6. **How long does the PLA signal last? OR Is the PLA signal stable? OR How should I save my glass slide in order to keep the PLA signals?**

- We highly recommend using **Duolink® PLA Mounting Media with DAPI** for fluorescence applications, because they help to preserve PLA signals. PLA signals can fade when other mounting media is applied, sometimes even immediately after their application on the sample.
- Slides mounted with **Duolink® PLA Mounting Medium with DAPI** can be stored at 4 °C in darkness up to 4 days before they are analyzed with the microscope.
- Unmounted slides can also be saved at -20 °C over the weekend.
- For long term storage, we recommend applying transparent nail polish around the borders of the cover slip. In this way, the slide can be stored at -20 °C for up to half a year, although normally the signal will fade a little bit with time. If the slide has already been analyzed with the microscope, additional fading may be expected.
- When slides are mounted with an non-aqueous (xylene based), permanent (hard-set) mounting media for the brightfield application, samples can be stored at room temperature and PLA signals will last for years.

back to top

SPECIFIC REQUIREMENTS

1. **What are the Primary Antibody requirements when using Duolink PLA Probes? OR Which antibodies are recommended?**
   - The primary antibodies should be:
     - IgG-class
     - Specific for the target to be detected, preferably affinity purified
     - Host must be mouse, rabbit or goat
     - Monoclonal or polyclonal
     - Validated by IF and/or IHC; PLA validated antibodies are now available.
   - MilliporeSigma offers over **70,000 primary antibodies for you to consider**.
   - MilliporeSigma has partnered with Bethyl Laboratories to provide **validated primary antibody pairs for PLA.**
2. **What concentration of my primary antibody should I use?**
   If you already have a working assay for IHC or IF, use the same primary antibody concentration to start with. Sometimes it may be necessary to perform a titration of your primary antibody.

back to top

DUOLINK® PROBEMAKER

1. **My antibodies are from a species other than mouse, rabbit and goat. Can I still apply Duolink® PLA?**
   Yes. We offer the Duolink® PLA Probemaker kit that enables conjugation of the PLA oligonucleotide arms PLUS or MINUS to your antibodies of interest. In that way, you build your own PLA probes that can later be applied to detect your proteins of interest in combination with Duolink® PLA Reagents.
2. **Both my primary antibodies are from the same species. Can I still apply Duolink® PLA?**
   Yes. We offer the Duolink® PLA Probemaker kit that enables conjugation of the PLA oligonucleotide arms PLUS or MINUS to your primary antibodies of interest. In that way, you build your own PLA probes that can later be applied to detect your proteins of interest in combination with Duolink® PLA Reagents. This allows for the detection of protein homodimers.
3. **When should the Probemaker Kit be used?**
   The Probemaker kit enables conjugation of a PLA oligo (PLUS or MINUS) to any antibody. Thus, PLA probes can be generated using Probemaker from primary antibodies for direct detection or from secondary antibodies for indirect detection.
   - When the host of a primary antibody is different than mouse, rabbit or goat
   - When using two primary antibodies from the same species
   - When using a primary antibody from the same host species as the sample (e.g., “mouse on mouse”)
4. **Are there antibody recommendations for the Probemaker Kit?**
   - Antibody concentration of 1 mg/ml
   - Amount of antibody is 20 mg per conjugation
   - Antibody needs to be free of additives
5. **When using Duolink® PLA Probemaker, can I use a lower concentration of the primary antibody than 1 mg/ml?**
   - Each kit of Duolink® PLA Probemaker contains reagents to conjugate 20 ug of antibody at a concentration of 1 mg/ml. The use of antibodies at this concentration is strongly recommended.
   - Good results cannot be guaranteed with a lower or higher concentration.
   - If the antibody concentration is too low, concentrate it by ultra-filtration in a centrifuge device. Note that in this process buffer changes can also be performed by repeated additions of the desired buffer with subsequent centrifugations.
   - If the antibody concentration is too high, dilute in an amine-free buffer (e.g., PBS)
6. **My primary antibodies contain many additives. What should I do?**
   - For pre-treatment of the antibody before using Duolink® PLA Probemaker, we refer to standard procedures.
   - To change buffer and/or to remove low molecular weight additives like azide or Trehalose, you can either dialyze against PBS or perform gel filtration on a spin column (Sephadex G25) equilibrated with PBS.
   - To remove high molecular weight additives such as BSA or gelatin, we recommend purification with Protein A or Protein G. The antibody is eluted with low pH which might affect antibody activity. We recommend to immediately add a strong buffer of neutral pH. There is always a loss of antibody with this purification method.
7. **What dilution of the conjugated antibody should I use?**
   The working concentration of the antibody will be dependent mainly on the sensitivity of the antibody, the sample type and the sample pretreatment. Our general recommendation is to use a starting concentration that gives good results with IF or IHC. Sometimes, titration may be necessary.
8. **Can I combine a directly conjugated primary antibody and a PLA probe?**
   Yes. It is possible to combine a directly conjugated antibody and a Duolink® PLA probe as long as one of them is PLUS and the other is MINUS and there is no species cross-reactivity (that is, the PLA probe should be directed against a species different from your directly conjugated primary antibody).

Additional Support

Contact us for further support: **techserv@sial.com**

When contacting us, please, provide the following information:

- Description of your assay
- Images of your results
- Controls (positive/negative, biological/technical) that have been performed (if any)
- Previous IF/IHC results (if any)

Materials

Product No.	Description	Add to Cart

Duolink® PLA Starter Kits	
DUO92101	Duolink® In Situ Red Starter Kit Mouse/Rabbit
DUO92102	Duolink® In Situ Orange Starter Kit Mouse/Rabbit
DUO92103	Duolink® In Situ Red Starter Kit Mouse/Goat
DUO92104	Duolink® In Situ Orange Starter Kit Mouse/Goat
DUO92105	Duolink® In Situ Red Starter Kit Goat/Rabbit
DUO92106	Duolink® In Situ Orange Starter Kit Goat/Rabbit
DUO92202	Duolink® PLA Control Kit
Duolink® PLA Accessories	
DUO80102	Duolink® In Situ Brightfield Mounting Medium
DUO82040	Duolink® In Situ Mounting Medium with DAPI
DUO82047	Duolink® In Situ Wash Buffer, Brightfield
DUO82049	Duolink® In Situ Wash Buffers, Fluorescence
DUO82064	Duolink® In Situ Microplate Nuclear Stain, Anti-Fade
DUO82065	Duolink® In Situ Microplate Heat Transfer Block
Duolink® PLA Detection Reagents	
DUO92007	Duolink® In Situ Detection Reagents Orange
DUO92008	Duolink® In Situ Detection Reagents Red
DUO92012	Duolink® In Situ Detection Reagents Brightfield
DUO92013	Duolink® In Situ Detection Reagents FarRed
DUO92014	Duolink® In Situ Detection Reagents Green
Duolink® PLA Probes	
DUO92001	Duolink® In Situ PLA® Probe Anti-Mouse PLUS Affinity purified Donkey anti-Mouse IgG (H+L)
DUO92002	Duolink® In Situ PLA® Probe Anti-Rabbit PLUS Affinity purified Donkey anti-Rabbit IgG (H+L)
DUO92003	Duolink® In Situ PLA® Probe Anti-Goat PLUS Affinity purified Donkey anti-Goat IgG (H+L)
DUO92004	Duolink® In Situ PLA® Probe Anti-Mouse MINUS Affinity purified Donkey anti-Mouse IgG (H+L)
DUO92005	Duolink® In Situ PLA® Probe Anti-Rabbit MINUS Affinity purified Donkey anti-Rabbit IgG (H+L)
DUO92006	Duolink® In Situ PLA® Probe Anti-Goat MINUS Affinity purified Donkey anti-Goat IgG (H+L)
DUO92009	Duolink® In Situ Probemaker PLUS
DUO92010	Duolink® In Situ Probemaker MINUS

back to top