Duolink®
Tips and Tricks
Duolink® Tips and Tricks

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1. Experiment

Sample and antibodies
Sample fixed on glass slides

Optimized:
- Pretreatment
- Blocking
- Primary antibodies, diluent, titer

Protocol: Your own or take a working one and optimize
E.g. DAKO Education Guide, IHC world, Nordiq, Abcam etc.

Primary Antibodies:
- Affinity purified polyclonal
- Monoclonal (avoid ascites)

Duolink In Situ assay: equipment needed

- Grease pen
- Humidity chamber
- Freeze block for enzymes
- Incubator 37 °C
- Staining jar and shaker
Biological controls
Best and recommended controls include:

Positive control:
- Known protein interaction or modification
- Overexpression, stimulation

Negative controls:
- Irrelevant protein (e.g. non-interacting, present in different compartment)
- Sample not expressing target protein or down-regulated/repressed, silencing, mutation etc.

Lower HER2 in MDA cells
Duolink In Situ assay: technical controls

Technical negative controls:

- Leave one primary antibody out
  - Information about unspecific binding of primary antibodies
  - Helpful to find an optimal titer
- Leave both primary antibodies out
  - Information about non specificity of PLA® probes
- Do not use unspecific sera!

Duolink In Situ Control Kit:

- Useful to check assay conditions in your lab
- Positive control of Detection Reagent

Common parameters to consider

Check antibody titer to obtain specific and distinct staining
Common parameters to consider

- Use appropriate blocking agent. Dilute PLA probes in a buffer containing the blocking agent
- Never let your sample dry out during the Duolink In Situ assay
- Gently tap-off excess solutions
- Wash in at least 70 mL of wash buffers
- Use Wash Buffers A and B where specified
Common parameters to consider

Incubation temperature:

Keep humidity chamber preheated inside incubator

Incubator always at 37 °C
Common parameters to consider

Wash temperature:
- Bring wash buffers to room temperature
- Perform wash at room temperature

![Graph showing PLA signals per cell intensity at different temperatures](image)

**RT**

**+4 °C**

**+30 °C**

Common parameters to consider

Amplify for 100 min to avoid coalescent signals

![Images showing amplified samples at different times](image)

**100 min**

**140 min**

Thaw reagents but use freeze block for Ligase and Polymerase

![Images of reagent thawing and freezing](image)
Image interpretation: common autofluorescence interference

- Use Wash B and Duolink In Situ Mounting Medium with DAPI
- Do not overexpose

Red nuclei artifact

No artifact

Image interpretation: common autofluorescence interference

Autofluorescence

- Inherent to sample
- Can be observed in green channel
- Alternative: Use either FarRed or Brightfield Detection Reagents

Red channel: both PLA signals and autofluorescence.

Superimposed image with three color channels: PLA signals can be distinguished from autofluorescence background.

Green channel: autofluorescence from blood.
2. Sample analysis and storage

Mounting and storage

- Duolink In Situ Brightfield Mounting Medium: non-aqueous, xylene based
- Duolink In Situ Mounting Medium with DAPI: aqueous, contains anti-fade and DAPI for Fluorescence application

Storage: O/N in dark at RT before mounting

< 4 days in dark at 4 °C after mounting

Seal and freeze at -20°C, for months after mounting

Notes:
We have tested many other media and PLA signals fade away with many of them. Add a few drops and press out any excess media.

Image acquisition: filters

FarRed:
- Focus on cell nuclei plane
- Obtain z-stacks if necessary
**Image acquisition: parameters**

**Brightfield application**

- Good contrast
- White background/unstained tissue

**Fluorescence application**

- 20x or 40x objective
- Do not overexpose
  - Signals can coalesce
  - Can give rise to autofluorescence
- Z-stacks if possible

| t
  | t
  | mph/Objective | 20x | 40x |
|---|---|---|---|
| 60 min | | | |
| 100 min | | | |
| 140 min | | | |

Tip: Look with 40x if difficulties finding signals with 20x
3. Image analysis and presenting data

Quantification
Compare only samples that have been run in parallel
• Images taken with same acquisition parameters under same session
Use 20x or 40x
• 63x or 100x can give nice images for publication but not worth quantifying
Quantification is relative, e.g.
• Positive vs. negative controls
• Signals in nuclei vs. signals in cytoplasm

Counterstaining
Add counterstaining after Wash buffer B step in the protocol, wash and mount
Presenting data

- Zoom in and scale up
- Increase brightness and contrast for presentations and publications

Example on how increasing brightness/contrast (numerical values above) can be useful when presenting data.

Zoom in on a region of interest (above) and scale up (left).
4. Probemaker

Probemaker: antibody requirements

Good quality antibodies

- Affinity purified polyclonals
- Non-ascites monoclonals
- Non-modified (e.g. no biotinylation)
- Stock 1 mg/mL
- Stock buffer additive free, ideally PBS. Pretreatment

<table>
<thead>
<tr>
<th>Buffer exchange</th>
<th>Affinity purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>G25, G50</td>
<td>Protein A, G</td>
</tr>
<tr>
<td>Small molecules: e.g. azide, Tris</td>
<td>Macromolecules: e.g. gelatin, BSA</td>
</tr>
</tbody>
</table>

Probemaker: assay

Concentration of conjugated antibody

Primary: higher concentration than for IHC/IF.

Secondary: start with concentration as for IHC/IF.

Dilution of conjugated antibody

- Custom solutions: blocking agents + Assay Reagent
- Duolink In Situ solutions: PLA probe Diluent
Contact for support
You are very welcome to contact us for further support requests
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)