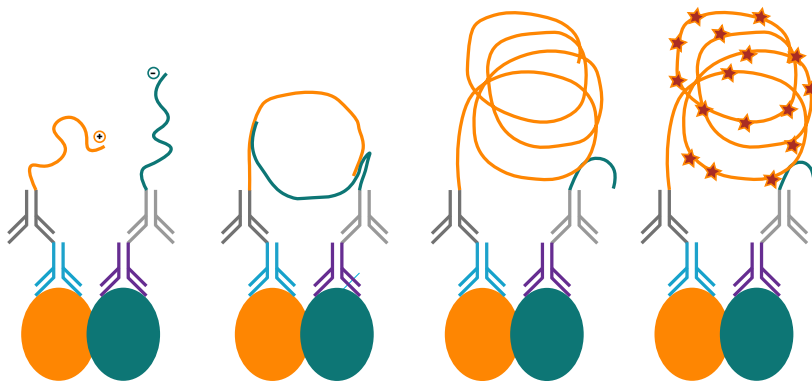
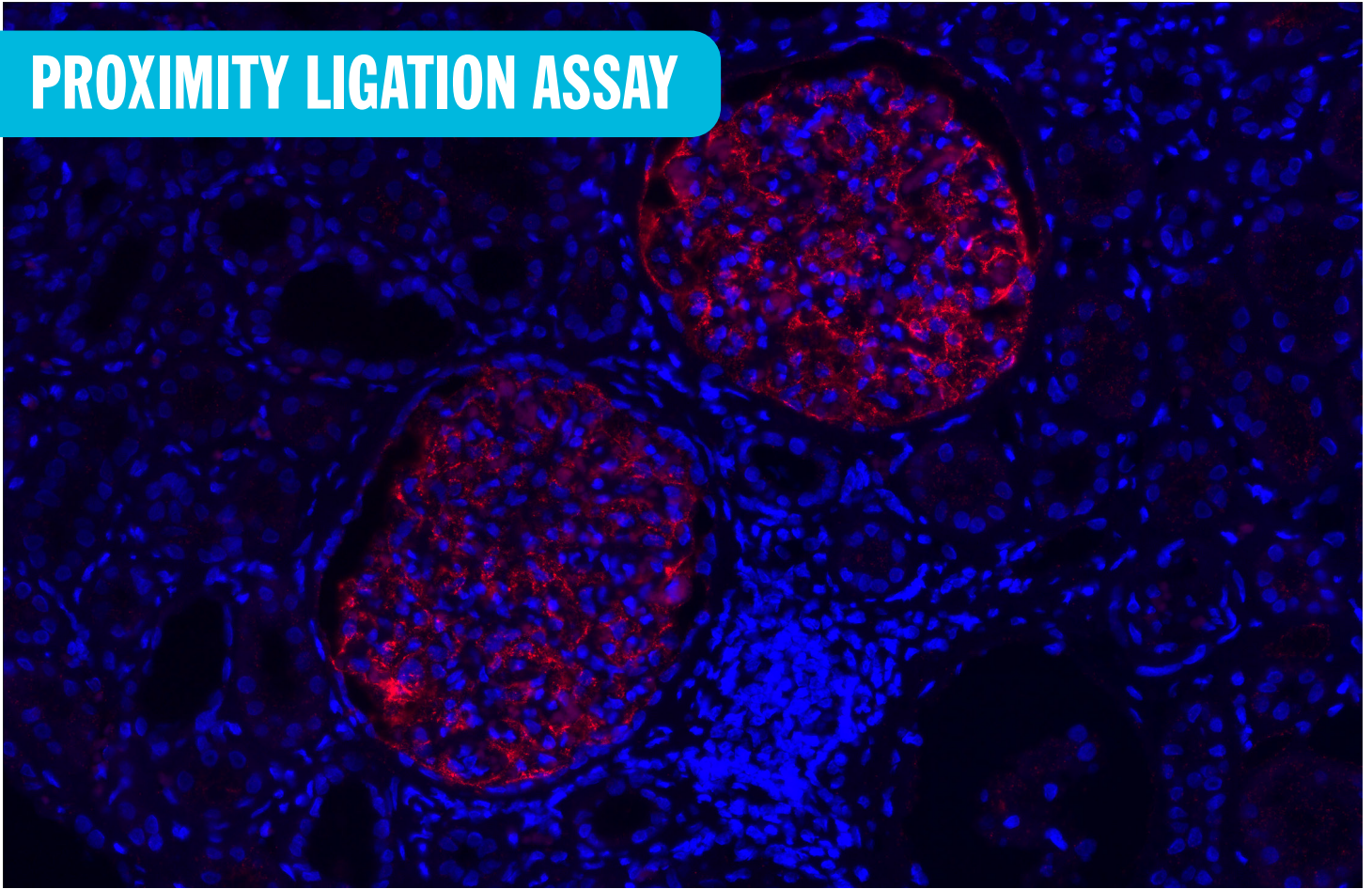


PROXIMITY LIGATION ASSAY



IN SITU PLA - APPLICATION EXAMPLE

Detection of protein-protein interactions by *in-situ* PLA (*is-PLA*) using PreciSA Monoclonals™ and Triple A Polyclonals™

Proteins are complex biological molecules essential for cellular structure and functions. The majority of proteins commonly interact with various molecules, including other proteins, in order to exert their functions. These protein-protein interactions are involved in a wide range of cellular processes, including e.g. protein modifications, transport, signaling and cell cycling.

Studies of protein interactions are critical for the characterization of protein functions in the context of cell biology in both normal state and disease.

A number of techniques for assessing the protein interactions are available, such as co-immunoprecipitation, pull-down assay, crosslinking and far-Western blot analysis.

Although these techniques provide powerful tools for studying interactions of the proteins, they lack the subcellular resolution possible with microscopy-based analysis of native tissues or cells.

A proximity ligation assay (PLA) (Duolink®, Sigma-Aldrich) is a technique which allows for studying protein-protein interactions in tissues or cells *in situ* (*is-PLA*)¹.

This method can be used for detection, visualization and quantification of protein-protein interactions, as well as for detection of individual proteins and protein modifications in cells or tissue sections.

The data presented in this white paper demonstrates how the Triple A Polyclonals and PreciSA Monoclonals can be used for detection of protein-protein interactions in human tissues *in situ* using a powerful PLA technique. Studies based on such data can further contribute to our understanding of protein functions in normal and pathological states.

In situ PLA principle

*is-PLA*³ is based on the use of oligonucleotide-labelled antibodies (PLA probes), which generate a signal only when the two probes are in a very close proximity (about 30 nm). One of the DNA probes in the created DNA circle serves then as a primer for the rolling circle amplification (RCA) (Fig.1A,B). By adding a DNA polymerase, a long DNA product is formed and remains attached to the PLA probe (Fig.1C).

After finalizing the RCA, the concatemeric repetitions of the same sequence enable hybridization of multiple detection oligonucleotides (Fig.1D). The signal from each detected pair of PLA probes is an individual fluorescent spot which can be visualized under a microscope and quantified. The PLA signals (blobs, spots) can be assigned to a specific subcellular location and quantified using image analysis software, e.g. BlobFinder⁴.

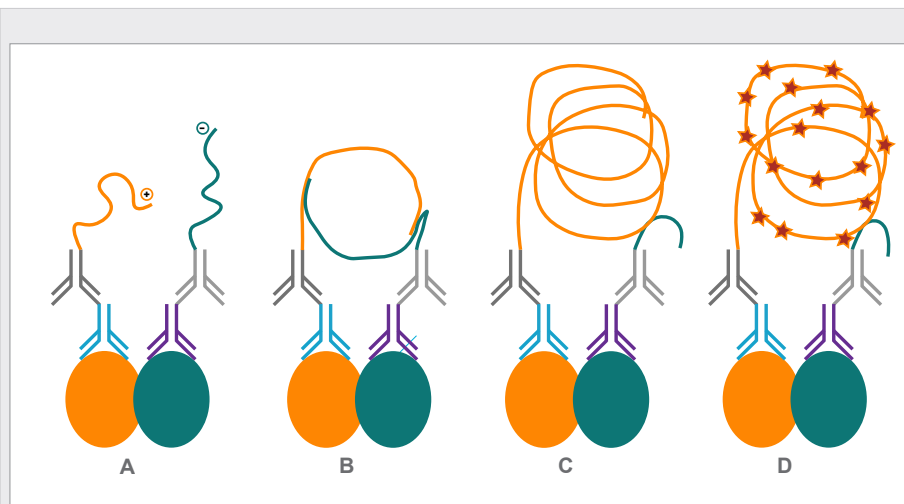


Figure 1. Schematic drawing of PLA reaction. Following incubation with primary antibodies, incubate with PLA probes PLUS and MINUS (A). Hybridize connector oligos and use ligase to complete the DNA circle (B). Rolling circle amplification (C). Hybridize fluorescence-labeled oligonucleotides for signal detection (D).

isPLA – a Short Protocol

1. Tissue pre-treatment for optimal antibody performance (e.g. HIER, pH=6)
2. Blocking
3. Primary antibodies incubation
4. Wash
5. PLA® probes incubation
Incubate for 60 min at 37°C.
6. Wash
7. Ligation
Incubate for 30 min at 37°C.
8. Amplification
Incubate for 100 min at 37°C.
9. Wash
10. Imaging
Mount the samples with Duolink In Situ Mounting Medium with DAPI.

For achieving reliable results with the PLA assay, the choice of primary antibodies is crucial.

Only specific and selective antibodies will enable a successful PLA reaction. General requirements for the primary antibodies are that they should be of IgG class, affinity-purified and not least, target-specific. In addition, for the detection of protein-protein interaction, the two primary antibodies must be raised in two different species, i.e. rabbit and mouse.

Atlas Antibodies provides a great resource of primary antibodies which can be used for PLA assay. Over 21,000 polyclonal (TripleA Polyclonals™) and 600 monoclonal (PrecisA Monoclonals™) antibodies are available in the catalog, together targeting more than 75% of the human protein coding genes.

Both the Triple A Polyclonals and PrecisA Monoclonals are developed using carefully designed antigens.

The highest levels of specificity, reproducibility and versatility are achieved by a unique antibody purification process using the recombinant antigen as affinity ligand for the Triple A Polyclonals. PrecisA Monoclonals are purified on protein A columns. Importantly, the antibody specificity is validated by an extensive IHC analysis in 44 normal and 20 cancerous human tissues.

The main advantages of *is*PLA in comparison to other techniques is the possibility to detect protein-protein interactions with subcellular resolution in native tissue sections or cells using microscopy.

This can be particularly important in e.g. cancer research, including disease diagnostics, patient stratification and biomarker studies².

Careful consideration of the fixation protocol is necessary to ensure the optimal preservation of morphology of the specimen and the target antigen. Formalin-fixed paraffin embedded tissue (FFPE) sections usually provide an optimal preservation of tissue morphology.

All antibodies in the Atlas Antibodies catalog are validated and approved for use in human (and in some cases rodent) FFPE samples following heat-induced epitope retrieval.

Examples of protein-protein interactions detection in FFPE human tissue sections by *is* PLA using PrecisA Monoclonals and Triple A Polyclonals from Atlas Antibodies.

Emerin-lamins interactions in the nuclear membrane

The nuclear envelope delineates nucleus from cytoplasm, preserves the structural integrity of the nucleus and controls molecular passage between nucleus and cytoplasm⁵. Emerin (EMD) is an inner nuclear membrane protein, involved in both mechanical integrity of the nucleus and tissue-specific gene regulation⁶.

The nuclear lamins are major components of the nuclear lamina underlying the inner nuclear membrane. They act as a bridge between the inner nuclear membrane and chromatin⁷ providing mechanical support to the nuclear envelope, maintaining proper chromatin organization and transcriptional regulation⁸. Interactions between emerin and lamins are important for maintenance of nuclear integrity⁹. Mutations in emerin and lamin genes produce pathologies known as 'nuclear envelopathies'¹⁰.

Here, we have used the Anti-EMD mouse monoclonal antibody AMAb90560 (1:5000, **2A**) and Anti-LMNA rabbit polyclonal antibody (HPA006660, 1:30, not shown) or LMNB1 rabbit polyclonal antibody (HPA050524, 1:150, **2B**) respectively to visualize EMD-LMNA (**2C**) and EMD-LMNB1 (**2D**) interactions in the nuclear membrane of stratified squamous epithelium cells.

Note the red immunofluorescence PLA signal located in the nuclear membrane area (**2C,D**) corresponding to the IHC staining (**2A,B**). Nuclei are counterstained with DAPI (**2C,D**).

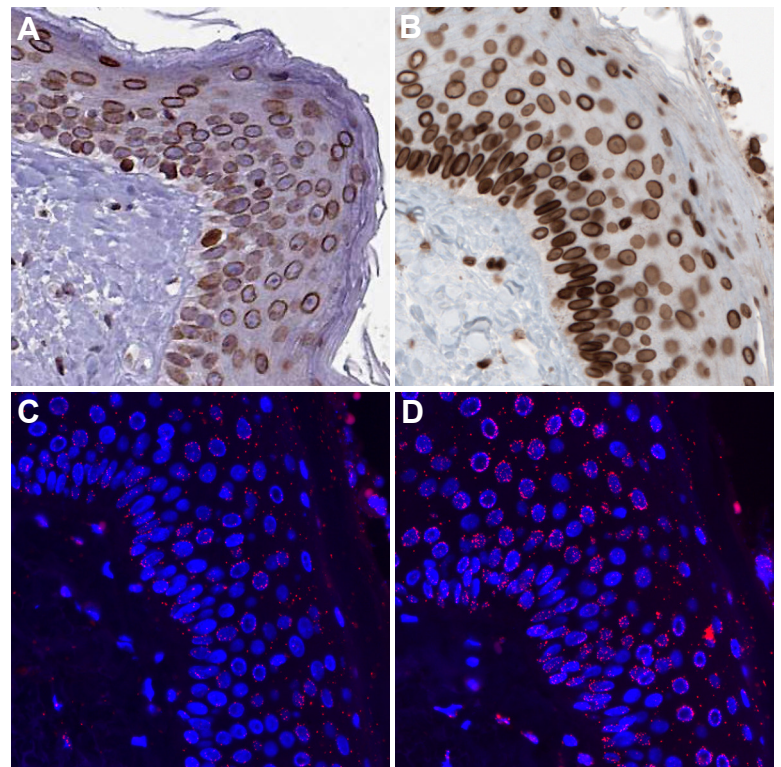


Figure 2
Emerin-lamins interactions in the nuclear membrane. The IHC staining using the Anti-EMD antibody AMAb90560 (**A**) and the Anti-LMNB1 antibody HPA050524 (**B**) shows distinct nuclear membrane immunoreactivity in the skin epithelial cells. Immunofluorescence *is*-PLA signal shows protein-protein interactions between EMD and LMNA (**C**) and EMD and LMNB1 (**D**) in the nuclear membrane of skin epithelial cells, respectively.

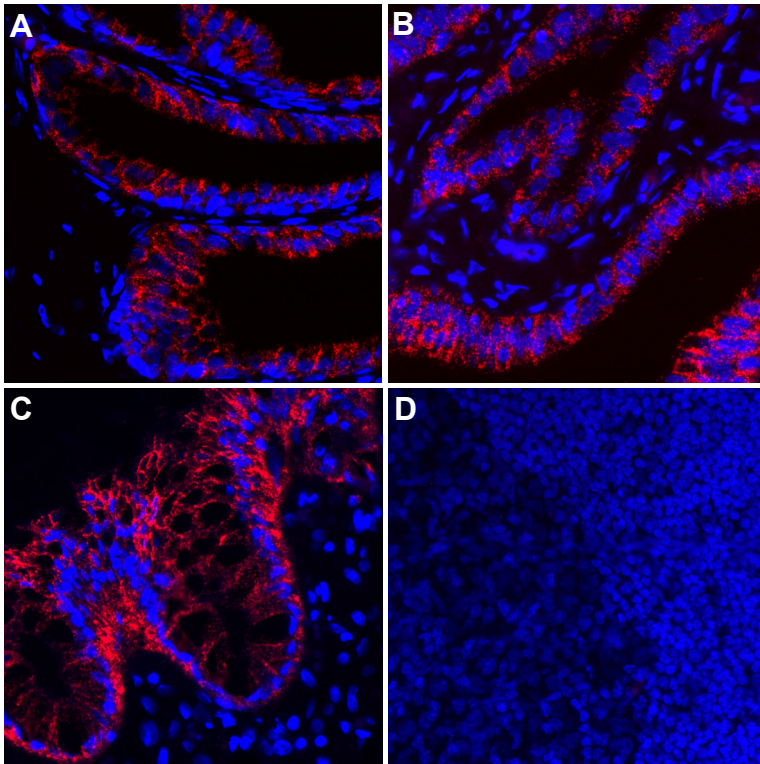


Figure 3
E-cadherin – beta-catenin interactions in the epithelial cells membrane.
 Immunofluorescence *is*-PLA signal showing CDH1 and CTNNB1 protein-protein interactions in the membranes of the epithelial cells in prostate (**A**), fallopian tube (**B**) and rectum (**C**). Tonsil is used as negative control (**D**).

E-cadherin – beta-catenin interactions in the epithelial cells membrane.

Epithelial cells serve as permeability barriers, separating underlying cells from the environment. This function requires that the cells are tightly connected to each other. E-cadherin (CDH1), a transmembrane protein forming complex with beta-catenin (CTNNB1), plays a key role in cellular adhesion in the epithelial cells.

The loss of catenin from the cadherin-mediated cell-cell contacts is an important factor in the epithelial-mesenchymal transition (EMT) process^{11,12}. The EMT process has been proposed as an important event in the metastatic spread of tumor cells, where epithelial tumour cells acquire a more motile and invasive phenotype and disseminate from the primary tumor¹³.

Here, the Anti-CDH1 monoclonal antibody AMAb90865 (1:1000) and the Anti-CTNNB1 polyclonal antibody HPA029159 (1:600) were used to visualize the CDH1-CTNNB1 interaction in the membrane of normal epithelial tissues, such as prostate (**3A**) fallopian tube (**3B**) and colon (**3C**).

Note the strong membranous positivity in the epithelial cells (**3A-C**) and the absence of PLA signal in the lymphoid tissue of tonsil (negative control, **3D**). Nuclei are counterstained with DAPI.

Podocalyxin-like – ezrin interaction in the membranes of renal glomeruli

Cellular plasma membrane contains numerous types of proteins, involved e.g. in maintenance of the cell structure, transmembrane transport and signal transduction. Podocalyxin-like protein (PODXL) is a transmembrane sialomucin protein with anti-adhesive properties.

In normal kidney, PODXL function is to maintain an open filtration pathway between neighbouring foot processes in the podocyte. PODXL was also shown to play a role in cancer development and aggressiveness¹⁴⁻¹⁷, at least partly through its interaction with the actin-binding protein ezrin (EZR)^{18,19}.

Both PODXL and EZR are expressed in the normal renal glomeruli membranes, as well as in the ciliated epithelium of fallopian tube. Here, we show the interaction of these proteins by *is*-PLA, using the Anti-PODXL monoclonal antibody AMAb90644 (1:5000) and the Anti-EZR polyclonal antibody HPA021616 (1:2500).

Note the strong PLA signal in renal glomerular membrane (**4A,B**), as well as in the apical membranes of the epithelial cells in fallopian tube (**4C**). Liver is used as a negative control (**4D**). Nuclei are counterstained with DAPI.

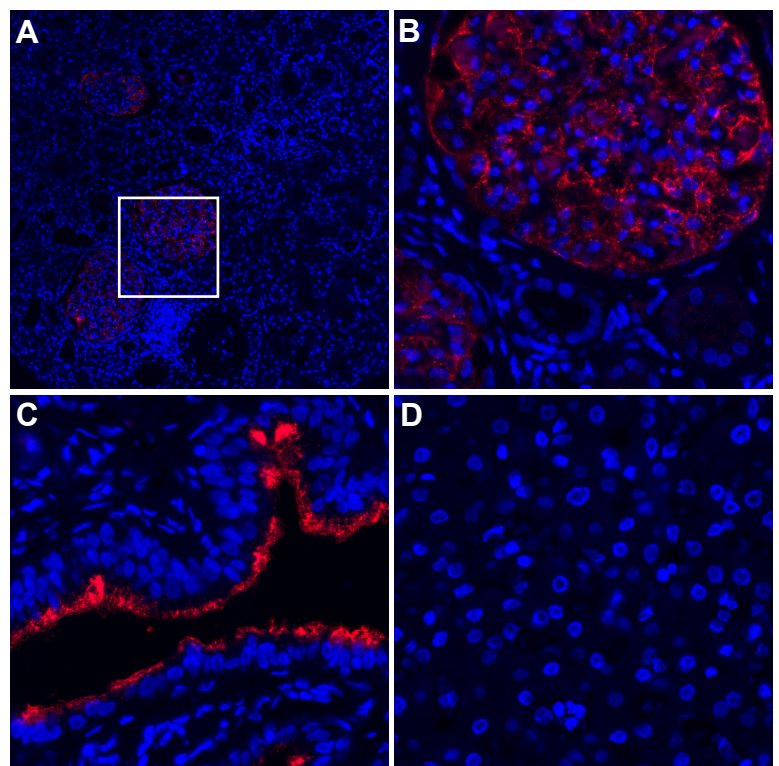


Figure 4
Podocalyxin-like – ezrin interaction in the membranes of renal glomeruli
 Immunofluorescence *is*-PLA signal showing PODXL and EZR protein-protein interactions in the membranes of renal glomeruli (**A,B**), as well as in the ciliated epithelium of the fallopian tube (**C**). Note the absence of PLA signal in renal tubules (**A,B**), as well as in the underlying connective tissue in the fallopian tube (**C**). Liver is used as negative control (**D**).

References:

1. Fredriksson S *et al.* Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol.* 2002 20(5):473-477.
2. Blokzijl A *et al.* Profiling protein expression and interactions: proximity ligation as a tool for personalized medicine. *J Intern Med.* 2010 268(3):232-245.
3. Söderberg O *et al.* Direct observation of individual endogenous protein complexes *in situ* by proximity ligation. *Nat. Methods.* 2006 3(12):995-1000.
4. Allalou A, Wählby C. BlobFinder, a tool for fluorescence microscopy image cytometry. *Comput Methods Programs Biomed.* 2009 94(1):58-65.
5. Gerace L, Burke B. Functional organization of the nuclear envelope. *Annu Rev Cell Biol* 1988 4:335-374.
6. Yorifuji H *et al.* Emerin, deficiency of which causes Emery-Dreifuss muscular dystrophy, is localized at the inner nuclear membrane. *Neurogenetics* 1997 1(2):135-140.
7. Ciska M, Díaz de la Espina M. The intriguing plant nuclear lamina. *Front Plant Sci.* 2014 5:166.
8. Luperchio TR *et al.* Genome regulation at the peripheral zone: lamina associated domains in development and disease. *Curr Opin Genet Dev.* 2014 25:50-61.
9. Sakaki M *et al.* Interaction between emerin and nuclear lamins. *J Biochem.* 2001 129(2):321-327.
10. Muchir A, Worman HJ. Emery-Dreifuss muscular dystrophy. *Curr Neurol Neurosci Rep.* 2007 7(1): 78-83.
11. De Wever O *et al.* Molecular and pathological signatures of epithelial-mesenchymal transitions at the cancer invasion front. *Histochemistry and cell biology.* 2008 130(3):481-494.
12. Stemmer V *et al.* Snail promotes Wnt target gene expression and interacts with beta-catenin. *Oncogene.* 2008 27(37):5075-5080.
13. Christiansen JJ, Rajasekaran AK. Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer research.* 2006 66(17):8319-8326.
14. Casey G *et al.* Podocalyxin variants and risk of prostate cancer and tumor aggressiveness. *Hum Mol Genet.* 2006 15(5):735-741.
15. Larsson A *et al.* Overexpression of podocalyxin-like protein is an independent factor of poor prognosis in colorectal cancer. *Br J Cancer.* 2011 105(5):666-672.
16. Binder ZA *et al.* Podocalyxin-like protein is expressed in glioblastoma multiforme stem-like cells and is associated with poor outcome. *PLoS One.* 2013 8(10).
17. Boman K *et al.* Membranous expression of podocalyxin-like protein is an independent factor of poor prognosis in urothelial bladder cancer. *Br J Cancer.* 2013 108(11):2321-2328.
18. Orlando RA *et al.* The glomerular epithelial cell anti-adhesin podocalyxin associates with the actin cytoskeleton through interactions with ezrin. *J Am Soc Nephrol.* 2001 12(8):1589-1598.
19. Sizemore S *et al.* Podocalyxin increases the aggressive phenotype of breast and prostate cancer cells *in vitro* through its interaction with ezrin. *Cancer Res.* 2007 67(13):6183-91.

PLA Reagents Used

Duolink In Situ PLA Probe Anti-Rabbit PLUS,
Sigma-Aldrich # DUO92002

Duolink In Situ PLA Probe Anti-Mouse MINUS,
Sigma-Aldrich # DUO92004

Duolink In Situ Detection Reagents Red,
Sigma-Aldrich # DUO92008

Duolink In Situ Mounting Medium with DAPI,
Sigma-Aldrich # DUO82040



Contact us: contact@atlasantibodies.com

Visit us: atlasantibodies.com

Follow us: [@atlasantibodies](https://www.instagram.com/atlasantibodies)

We are continuously updating our catalogs.
Please refer to the online version for the latest updates of this document.

Atlas Antibodies logo, Triple A Polyclonals™, PreciSA Monoclonals™, and PrEST Antigens™ are trademarks or registered trademarks of Atlas Antibodies AB. All other trademarks are the property of their respective owners. Products are for research use only. Not for use in diagnostic procedures. © Atlas Antibodies AB 2023.

016-Jan 2023