Proximity Ligation Assay

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ELISA Guidelines Related to Performing PLA in Cells

The proximity ligation assay is a robust assay, and most users will have no difficulty obtaining appropriate results in various cell lines. Once you have defined antibodies that will function in the assay, then you may deviate from the basic protocol in order to optimize it for your specific cell line and/or experimental design. Bethyl recommends taking into consideration the following issues when performing the assay with cells.

1. Choice of cell line is important to obtain good results. Certain proteins may not be expressed in particular cells, and certain protein-protein interactions may be non-existent or at such a low level as to be indistinguishable from the background fluorescent signal produced by a particular antibody pair. The assay is highly sensitive and, if a good signal is obtained on a western blot, then the PLA should function appropriately (although steric hindrance & epitope presentation are possible problems).

2. Chambered microscope slides can greatly ease the processing of your samples. We recommend tissue-culture treated slides with eight (8) chambers/wells, which allow cultures of 0.2-0.4 mL of media and have a surface area of ~0.8cm2. Typically, this type of slide uses a silicone mounting base to hold the chamber divider in place and prevent leakage between wells. This silicone base may be left in place to allow for different treatments/processing for the different wells, and then removed immediately prior to application of the mounting medium and coverslip.

3. If you have access to an inverted microscope system, then the use of 96-well or 384-well microtiter plates are also very good for increasing throughput of the assay. Please refer to the Protocols section of our website and the document entitled "Guidelines related to PLA for HTS and Microtiter Plates" for additional information.

4. If at all possible, we recommend choosing a cell line that grows adherently. There are various methods that allow the use of suspended cell cultures on slides (e.g., a centrifugal cytospin protocol), but these are much less amenable to high throughput processing of the assay. However, if there are no other options, then this process can be used for performing PLA, although the number of samples able to be processed will be reduced.

5. Cells should be cultured so as to avoid becoming too confluent. Overgrown cells are much more difficult to image properly, and typically show very different expression patterns. Bethyl recommend harvesting cells for processing at ~50-70% confluency, which will vary somewhat depending on the particular cell line.

6. After you have completed your experimental protocol, wash your cells 3X in PBS. This should always be followed by fixation steps before beginning the PLA assay. Fixation is very important for two reasons, first to help keep the cells on your slides through the many washing steps, and second to retain the current state of protein folding/interaction(s). However, you should avoid fixing for too long; 30 minutes at room temperature in 10% formalin (in PBS, pH7.0) works very well for most cell lines. The fixation step should be followed by washing 3X in PBS.

7. For most cell lines, a short permeabilization step greatly improves assay results. After the post-fixation PBS washes, permeabilize with 0.25% Triton-X100 for 10 minutes at room temperature. Afterwards, wash again 3X in PBS prior to application of Olink Blocking Solution, and proceeding with the remainder of the assay.

General Guidelines for Improving PLA Results

The proximity ligation assay is robust, and most users will have no difficulty obtaining appropriate results, especially if you begin by following the basic protocol outlined in the documents supplied with the various reagents. Once you have defined antibodies that will function in the assay, then you may deviate from the basic protocol in order to optimize it for your specific cell line/tissue type and/or experimental design. Bethyl recommends taking into consideration the following issues as part of your optimization process

1. Antibody choice is critical to the success of the assay. Antibodies that function well against epitopes in a western blot may not function in PLA. Generally, if an antibody has already been qualified for immunohistochemistry, then it should also perform well in PLA. If the assay is used to recognize a single protein target, then the epitopes recognized by the antibodies should not be so close together that steric hindrance becomes a problem. In addition, the epitope(s) must be presented on the surface of the folded protein and consideration should be given to whether they are known to be involved in binding or interacting with other molecules, i.e., the biochemistry of a protein will define in which cases a particular epitope is an acceptable target for PLA.

2. PLA relies on oligonucleotides and the products of rolling circle amplification. Therefore, care should be taken to eliminate the introduction of DNases during the various steps of the protocol. Always use clean glassware and quality reagents for making buffers and/or performing the wash steps.

3. Care should be taken to ensure that cells/tissue samples are appropriately plated or mounted. For slides/tissue culture plates of cells, we have found the best results are obtained by processing them at ~50-70% confluency. For tissue sections, you should make an effort to have them done as thin as reasonably possible – obviously, this will vary depending on the tissue type. Thinner sections affect both the quality of imaging obtained, as well as the ability of the antibodies to permeate the tissue. Note that the assay results are significantly improved by performing an overnight incubation of the primary antibody when using tissue sections.

4. The blocking and/or the primary antibody incubation steps can be done overnight, in order to accommodate scheduling issues associated with performing the assay. When performing these steps overnight, they can be done at room temperature (rather than at 37 degrees C).

5. The blocking solution and antibody diluent supplied with the reagent sets are critical to optimizing the performance of the assay. Bethyl strongly recommends that users restrict the assay development process by only using these reagents. Once the assay development process is completed, then you may decide to further optimize the assay by replacing these reagents with standard materials you produce, rather than relying on commerical materials.

6. If you are performing the assay on slides, it is recommended to use a large vessel (containing ~250mL solution) to hold the slides during washes, rather than a small coplin jar. The vessel can be easily placed on a platform shaker, and the larger volume of wash buffer produces superior imaging results due to lower background fluorescence and less non-specific binding of the antibodies.

7. Once you begin processing your slides/plates, it is very important to avoid drying them in between steps as this greatly increases background/non-specific fluorescence. However, you also need to avoid carry-over of wash buffer(s), especially into the ligation and amplification steps of the assay as the dilution effect can greatly reduce the efficiency of these reactions. Bethyl recommends careful aspiration of residual liquids from the slides/wells of the plate, followed by immediate application of the ligation or amplification solution. If you process one slide (or one row of a plate) at a time, then you can avoid over-drying the material as well as avoid diluting these important enzyme-containing solutions.

Guidelines related to Proximity Ligation Assay for HTS & Microtiter Plate

The proximity ligation assay (PLA) is very robust, and can be developed for high-throughput screens (HTS). However, it is recommended that you first optimize the assay in chambered slides, especially focusing on defining the appropriate antibody concentrations. Once you have established the experimental parameters for your antibodies, then you can scale-up to microtiter plates in a stepwise manner; first, to a single plate and then on to planning for the logistics associated with larger numbers. For general considerations regarding the use of cells in performing proximity ligation assay, which are equally applicable here, please refer to the Protocols section of the Bethyl website and the document entitled "Guidelines Related to Performing PLA in Cells". Below is a set of guidelines specific to HTS and microtiter plates:

1. Choice of cell line is especially important when developing PLA for HTS. For the microtiter plate format, Bethyl recommends cell lines that grow adherently. Cells grown in suspension are simply not amenable to this format due to the many wash steps associated with the assay.

2. The use of ELISA-style plate washing systems is not recommended for high-throughput PLA. These systems do not have adequate control over the liquid dispensing pressures, such that they will typically remove most of the cells from your plates. If more advanced robotic liquid handling systems are unavailable, then we recommend doing liquid transfers with a multichannel micropipette. Although this will restrict the number of plates that can be processed in each experiment, you will obtain far superior results and have fewer repeat experiments to perform.

3. When performing the assay on slides, it is important not to allow your samples to dry in between the different steps, as this can greatly increase non-specific fluorescence. Generally, when robotic systems are being employed for liquid handling, this is a less important consideration. However, if liquid transfers are done using a multichannel micropipette, this becomes much more important and should be addressed as part of assay development.

4. Choice of 96-well or 384-well format will depend on the availability and capability of image-capture and robotic/automated liquid handling systems. Bethyl recommends restricting the assay format to 96-well for several reasons, mostly related to problems with liquid handling. Researchers often experience significant problems with bubble-formation when using the 384-well format due to the repeated steps involving wash solutions. However, if good robotic platforms are available for plate processing, and the imaging system can accommodate them, then the 384-well format is possible.

5. Choice of microtiter plates is critical for both good imaging and avoiding problems with automated liquid handling systems. Please refer to the guidelines published by the manufacturer of your imaging system as well as any robotic system to ensure conformity with both plate dimensions and optics. Bethyl recommends avoiding the use of lift-arms for moving plates. Moving them by hand both on and off the stage/platform of a robotic system will help reduce imaging problems due to scratched plates.

6. Reaction volumes for the different incubation steps can be optimized based on your experimental set-up and plate choice, but care needs to be taken to avoid drastic volume changes that may negatively affect the enzyme-containing assay steps. Potential steps for volume optimization includes primary antibody, secondary antibody (for secondary-conjugate form of the assay), ligation, and amplification. As a starting point, we recommend using 40 uL reaction volumes for 96-well plates, and 20 uL reaction volumes for 384-well plates. Washing steps should be done using 200 uL volumes for 96-well plates, and 75 uL volumes for 384-well plates.

7. Bethyl recommends performing the primary antibody incubation over-night for all HTS formats, regardless of whether the assay being performed is a primary- or secondary-conjugate form. For most antibody pairs, this extended primary incubation can be done at room temperature, and the over-night scheduling will usually provide a better work flow for performing the assay.

8. The reactions involving the ligation & amplification steps are especially critical to obtaining good results. Prior to applying these solutions it is important to try to remove as much residual liquid as possible from the wells of your plates, but to do so without allowing them to fully dry. Residual liquids will affect the buffering conditions of these enzyme-containing solutions, reducing their effectiveness. A good method of addressing these conflicting needs is to invert the plates after the last wash step, and tap them gently on a paper towel before adding the reaction

solution.

9. For several steps of the assay, Bethyl recommends that plate processing be done in stages to avoid large differences in timing between the first and last plates of the experiment. This is especially critical for the following steps: length of time for secondary antibody incubation (for secondary-conjugate form of the assay), length of time for the ligation incubation, length of time for the amplification incubation.

10. Attention should be paid to avoid variations in temperature associated with how the microtiter plates are situated during the 37 degree C incubations for the ligation and amplification steps of the assay. Variations in temperature can greatly affect the quality of your results, especially if there is well-to-well variability that could affect your final data analysis. Plates should be either placed individually, or stacked in pairs, directly on the shelves within the 37 degree C incubator. Various commercial heat-transfer blocks are available for reducing temperature variability, but these are not practical when assays involve more than a few plates.

Proximity Ligation Assay (PLA) FAQs - General Questions

What is the distance for the proximity ligation to function?
All the reagent sets for PLA detection are designed to detect proximity of the antibodies in the assay. When using the secondary-conjugate approach the theoretical maximum distance between your two target epitopes is 30-40 nm to create a signal. The assay can theoretically detect epitopes that are very close to each other, as long as the two primary antibodies are not sterically hindered from simultaneously binding their respective recognition sites.

What is the effect of changing the basic protocol incubation times and temperatures?
The proximity ligation assay 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 initial results are acquired when the protocol is followed with the recommendations provided. However, results will vary depending on your own experimental system and choice of cell/tissue type. Once you have obtained baseline results, you may choose to spend time optimizing the results you achieved using the standardized protocol.

From which source animals can antibodies be derived to perform the PLA?
If your two primary antibodies are derived in two different animals, either rabbit, mouse, or goat, then you can use commercially available pre-conjugated secondary antibodies to perform the secondary-conjugate form of the assay.

Both my primary antibodies are from the same species; can I still perform PLA?
Yes. There are commerically available conjugation kits that enable conjugation of the PLA oligonucleotide arms PLUS or MINUS directly to your primary antibodies.

When performing the secondary-conjugate form of the assay, what concentration of the primary antibody should I use?
The working concentration of the primary antibodies will be dependent mainly on the sensitivity of the antibody, the sample type (cells or tissues), and the sample pretreatment. Our recommendation for primary antibodies is to use a starting concentration that gives good results with IHC, although a titration series may be necessary as part of optimizing your results. For Bethyl primary antibodies, we recommend using a 1:1000 dilution of the original antibody concentration (i.e., 1 µg/mL for most antibodies) as a starting point, and then optimize based on results obtained for each particular pair of antibodies.

Which fluorescent colors are available for PLA detection, and what are their absorption & emission spectra?
The fluorophores in the commercially available detection reagents have the following colors & spectra:

orange:	λexc. 554nm, λem. 579nm
red:	λexc. 594nm, λem. 624nm
green:	λexc. 495nm, λem. 527nm
far red:	λexc. 644nm, λem. 669nm

FAQs - Related to Conjugation

What is the basis of the chemistry associated with the conjugation reaction?
The conjugation chemistry of the standard commercially available kits is proprietary information, and is not disclosed by the manufacturer. The antibody used in the reaction should be concentrated to 1 mg/ml and it should be in a neutral pH, amine-free buffer, preferably PBS. In addition, the antibody buffer should NOT contain any glycerol, BSA, trehalose, or preservatives of any kind.

Can I use a lower concentration of the antibody than 1 mg/ml in the conjugation reaction?
Each kit of standard commercially available kits contains reagents to conjugate 20 µg of antibody at a concentration of 1 mg/ml. Bethyl strongly recommends using antibodies at this concentration, because the manufacturer does not guarantee good results with a lower concentration. If your antibody is at a lower concentrate it by ultra-filtration in a centrifuge device. Note that, in this process, buffer exchange can usually be performed by repeated additions of the desired buffer with subsequent centrifugations. Suitable filters are available from several manufacturers.

My primary antibody contains many additives: what should I do?
For pre-treatment of the antibody before using one of the commercially available conjugation kits, Bethyl refers users to standard purification procedures. To change buffer and/or remove low molecular additives like azide or trehalose you can either dialyze against PBS or perform gel filtration on a spin column equilibrated with PBS. To remove high molecular additives like BSA or gelatin Bethyl recommends purification by protein G or A columns. The antibody is eluted with low pH, which might affect antibody activity, and therefore it is recommended to compensate with the immediate addition of strong buffer of neutral pH. There is always a loss of antibody amount in this procedure— which could be quite substantial.

Can I obtain pre-conjugated primary antibodies from Bethyl?
Yes. As a custom service, Bethyl will provide pre-conjugated primary antibodies, thus saving the extra steps associated with user-generation of conjugates. Conjugation is available for any of our high quality primary antibodies (i.e., custom conjugations are not available for non-Bethyl antibodies). The resulting Bethyl conjugate is typically sufficient to perform at least 200 PLA assays (depending on the concentration and reaction size necessary for the slide/plate format chosen), and is stable for at least 3 months when stored at +4 degrees C.

What dilution of primary-conjugate antibody should I use for this form of the assay?
The working concentration of the conjugated primary antibodies will be dependent mainly on the sensitivity of the antibody, the sample type (cells or tissues), and the sample pretreatment. A general recommendation for primary antibody conjugates you generate yourself is to use a starting concentration that gives good results with IHC, although a titration series may be necessary as part of optimization. For pre-conjugated or user conjugated Bethyl antibodies, use a 1:1000 dilution of the original antibody concentration (i.e., 1 µg/mL) as a starting point, and then optimize based on results obtained for each particular pair of antibodies.