TROUBLESHOOTING GUIDE

Glysite™ Explorer *in situ* PLA Glycan Detection Kit

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This guide will take you through potential issues you may encounter while using the Glysite™ Explorer *in situ* PLA Glycan Detection Kit (GEK-1000) and necessary steps to troubleshoot these problems.

Controls:

Users will gain the most benefit from this troubleshooting guide if they include control samples in their assay. There are two main controls that are recommended for every user to run alongside their positive samples. These controls will help identify non-specific binding.

1 Control 1:

Omit the primary antibody (single deletion). Run a slide with all components except for the primary antibody. This will show the non-specific binding of the probes.

2 Control 2:

Omit both the primary antibody and the lectin (double deletion). Run a slide with all components except for the primary antibody and the Explorer lectin. This will show the non-specific binding of the system.

→ Start primary antibody optimization at concentrations that work well in traditional IHC.

ISSUE Background – Puncta are present in either the single deletion or double deletion control

Cause	Recommendation
Samples are not washed sufficiently	▶ Use wash buffers indicated in the protocol. ▶ Increase the number of washes, the washing time, and/or the wash volume. Ensure wash buffers are used at temperatures specified in the protocol. ▶ Use a fresh wash solution. If it has become cloudy or there are any salt precipitates, prepare a new washing buffer.
Antibody/probe cross-contamination	▶ Due to the high efficiency of detection, it is important to wash different probe conditions separately to reduce cross-contamination between samples in step 18. ▶ Try to wash samples where the primary antibody or lectin is omitted in separate washing jars.
Inadequate blocking	▶ Make sure and use the proper blocking solutions for the specified amount of time. ▶ Cover the entire sample in the blocking solution. ▶ Dilute the primary antibody, the lectin and the probes in the provided Protein Diluent.
Compromised probes	▶ Do not let the probes freeze. ▶ Do not leave the probes at room temperature for extended periods of time. ▶ Expired probes may show reduced performance.

Cause	Recommendation
Drying of sample	▶ Keep samples in a pre-heated humidity chamber during incubations. ▶ Do not let samples dry out in between steps. To reduce the risk of drying, prepare the reaction mixtures before taking samples out of the washing buffer, and only remove a subset of your samples at a time.
Precipitate in Buffer 1 and Buffer 2	▶ Make sure that the Buffer 1 & 2 are completely thawed. ▶ The buffer can be left for 30 min at room temperature ahead of the reaction. If the precipitate has not been dissolved after vortexing, it can be heated in the palm of your hand until completely dissolved. ▶ Always vortex reagents before use.
Nonspecific binding of primary antibodies	▶ Titrating your primary antibody down may also help if the primary has some off-target binding. ▶ If the background persists despite the optimization of conditions (fixation, antigen retrieval, antibody titer, etc.), try an alternative primary antibody against the target(s).

ISSUE Diffuse background staining or toning

Cause	Recommendation
Inadequate washing	▶ Diffuse staining or toning will appear smooth and not as distinct puncta like true signals. In these instances, additional washing can help reduce the background.
Poor fixation of sample	▶ Optimize sample fixation procedure.
Drying of sample	▶ Keep samples in a humidity chamber during incubations. ▶ Do not let samples dry out in between steps.

ISSUE Reduced or absent signal in positive samples

Cause	Recommendation
Ensure your target protein is glycosylated	▶ While overlapping IHC staining of a lectin and a primary antibody is a good clue that the protein may be glycosylated, it does not mean the glycan is necessarily proximal to the target protein. ▶ Use UniProt or other theoretical analysis to ensure the target protein is glycosylated if other data is not available.
Primary antibody from incorrect species	▶ Make sure the correct probe is used with your primary antibody so that the species match. The Mouse Probe (50×) should only be used with mouse primary antibodies. The Rabbit Probe (50×) should only be used with rabbit primary antibodies.
No or insufficient binding of primary antibodies	▶ Optimize sample preparation such as fixation, permeabilization, and antigen retrieval to ensure the binding of the primary antibody. ▶ Titrate the antibody and select the concentration that results in the best signal as determined by IHC.

Cause	Recommendation
Incubation of reaction at the incorrect temperature	▶ Perform all incubations at the indicated temperatures.
Excess wash buffer left on the sample	▶ Dilution of antibodies/lectins and/or enzymes by residual wash buffer can lead to reduced sensitivity and increased variability between samples/experiments. ▶ Remove any excess wash buffer by aspiration or taping it off, before adding the reagent mixtures.
Incorrect wash buffers	▶ Use wash buffers indicated in the protocol. Using alternative wash buffers can impact performance. ▶ Ensure the wash buffers are at indicated temperatures.
Positive signals (puncta) are small	▶ Puncta can be quite small so if your epitopes are not abundant, isPLA signal can be difficult to see. Always zoom in to 20x or 40x to look for puncta if it cannot be seen with a lower magnification.
Counterstain obscures positive signals	▶ Ensure counterstain is not too dark or prominent. Hematoxylin can easily occlude isPLA signals and needs to be optimized before use. Having one section stained with Hematoxylin and one section without Hematoxylin works well to ensure signals are not being occluded by counterstain.

ISSUE Excessive variation between experiments

Cause	Recommendation
Experimental set-up	▶ Changes to experimental set up (such as incubation methods) may lead to variations.
Changes to specimens & reagents	▶ Different sample types or primary antibodies can introduce variation. ▶ Specimen fixation and antigen retrieval methods can affect the signal and should be constant between experiments.
Mixing and pipetting errors	▶ Use best practices when pipetting to minimize variation between experiments. ▶ Defrost buffers completely and allow them to come to room temperature. Vortex reagents to ensure a homogeneous solution. ▶ Immerse the pipette tip to the appropriate depth. Too much immersion causes enzymes to stick to the outside of the tip and increase the enzyme volume in the reaction.
Sample drying and insufficient removal of wash buffer	▶ Do not let the sample dry out between steps. ▶ Remove excess wash buffer from the specimen before adding reagents to avoid dilution.

VUS-LBL-03444

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