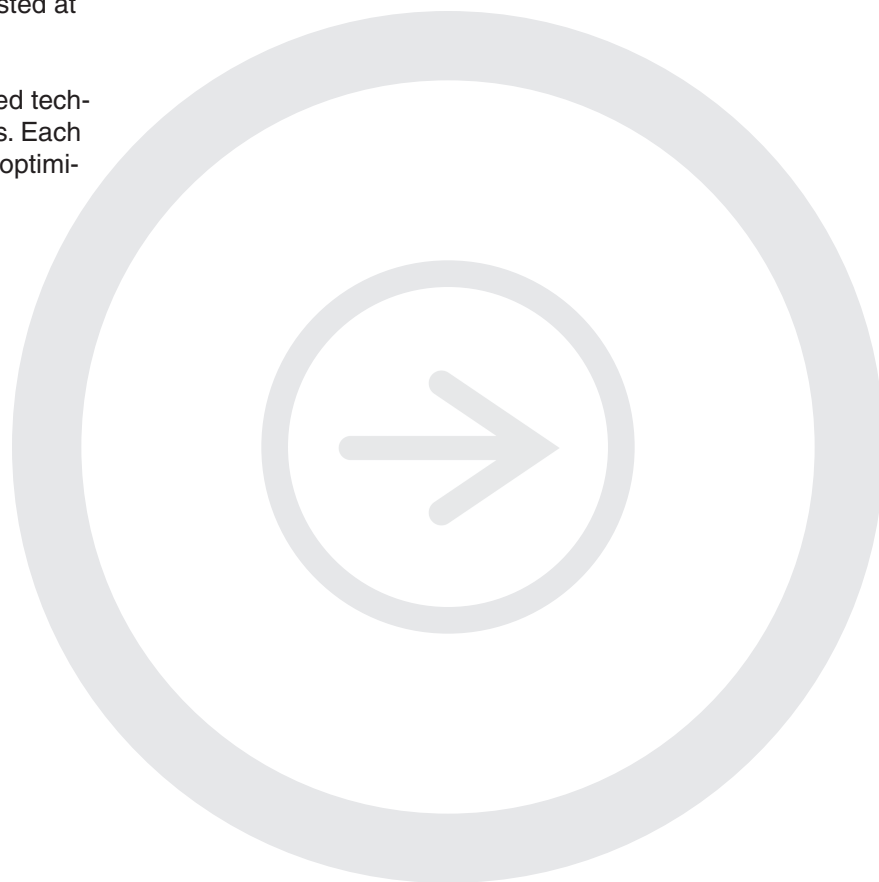


Technical Note

Fluorophore-Linked Immunosorbent Assay (FLISA) Recommendations

Published January, 2006. The most recent version of this Technical Note is posted at <http://biosupport.licor.com/support>.

Notice: LI-COR provides only limited technical support for FLISA applications. Each FLISA assay will require individual optimization and performance may vary.



The following should be used only as a guideline for adapting a Fluorophore-Linked Immunosorbent Assay (FLISA) to the Odyssey® Infrared Imaging System. LI-COR provides only limited technical support for FLISA (ELISA) application.

I. Assay Components

Microplates

Many commercially available microplates and strip wells designated for ELISA or EIA/RIA use are compatible with Odyssey. A few recommended examples are given below. Consider the following characteristics when deciding to use a particular microplate: whether to use clear or opaque microplates, the diameter at the base of each well, and the distance from the bottom of the wells to the bottom of the supporting edge of the plate.

Clear vs. Opaque: LI-COR recommends black microplates with optically clear bottoms to facilitate quantification. Clear microplates perform well, but produce significant laser light scatter around the well edges; extra care must be taken to exclude these edges during quantification.

Well Diameter: It is necessary to know the well diameter for a particular microplate or 8-well strip; this diameter is used by the Odyssey application software for grid placement and determining well area for quantification. Well diameter can be determined by physically measuring the inside diameter of a well, searching the microplate manufacturer's specifications, or using the selection drawing tool within the application software (dimensions of a selected area are listed at the bottom of the window).

Focus Offset: For microplates, the focus offset required in Odyssey application software is the distance from the inner surface at the base of the wells to the plate base that contacts the Odyssey scanning surface. Focus offset can be determined either physically, by measuring the distance (in millimeters); or empirically, by incrementing the focus offset over multiple scans of the same plate containing experimental or control samples. Quantify the same well, or wells, on each scan and determining the focus offset with the highest mean integrated intensity.

Microplate Examples:

- Greiner Bio-One 96-well, polystyrene microplate; high binding capacity
Black with flat, optically clear well bottoms
Manufacturer's part number: 65509
- Greiner Bio-One 384-well, polystyrene microplate; high binding capacity
Black with flat, optically clear well bottoms
Manufacturer's part number: 781097
- Greiner Bio-One 96-well, polystyrene strip plate; 12 x 8-well removable strips; high binding capacity
Flat, clear strips/wells in a microplate frame
Manufacturer's part number: 762071
- Corning Costar 96-well, polystyrene microplate; high binding capacity
Black with flat, optically clear well bottoms
Manufacturer's part number: 3601
- Corning Costar 96-well, polystyrene StripWell™ strip plate; 12 x 8-well removable strips
Flat, clear strips/wells in a microplate frame; high binding capacity
Manufacturer's part number: 2592
- Corning Costar 96-well, clear polystyrene microplate; high binding capacity
Manufacturer's part number: 9018

Suggested Reagents

Wash Buffers

- 1X PBS: This is commonly made from a 10X solution (LI-COR Biosciences Cat# 928-40018 or 928-40020) containing 1 M sodium phosphate and 1.5 M sodium chloride, but can also include potassium phosphate and/or potassium chloride depending on your preference. Check pH prior to use and adjust to 7.2 - 7.4 as necessary.
- Alternate: 1x PBS + 0.05% Tween[®]-20 may be used when greater stringency is required for washing. A final rinse with 1X PBS helps alleviate some of the frothing effect of the Tween-20. Take care when adding detergents to the wash buffer (e.g. SDS) as they can adversely affect other reagent components in the assay.

Blocking Buffer

- 1% BSA
- 5% Sucrose
- 0.05% Sodium Azide
- Dilute in 1x PBS
- Store at 4 °C

Alternate #1

0.1% Casein
0.1% Tween-20 (optional)
Dilute in 1X PBS
Store at 4 °C

Alternate #2

Odyssey Blocking Buffer (LI-COR Biosciences Cat# 927-40000)
0.1% Tween-20 (optional)

Note: There are many other commonly used blocking reagents used for ELISA, each with its own advantages and disadvantages. If the aforementioned blocking buffers do not perform well in your system (i.e. the assay produces an exorbitant amount of nonspecific background signal), then you may want to experiment with other blocking agents and detergents.

Reagent Diluent

- 0.1% BSA
- 0.05% Tween-20
- Dilute in 1X TBS (0.02M Tris base, 0.15M Sodium Chloride)
- Adjust to pH 7.2 - 7.4 with HCl
- 0.2 µm filtered
- Store at 4 °C

Alternatively, dilute with an appropriate blocking buffer or simply 1X PBS. Reagent diluent should only be used to dilute protein sample and detection antibody; dilute dye-labeled streptavidin and dye-labeled secondary antibody with 1X PBS.

Capture Buffer

- 15 mM sodium carbonate
- 35 mM sodium bicarbonate
- 0.02% sodium azide
- Dilute in water
- Store at room temperature

Detection Reagents

Streptavidin Conjugates

- IRDye® 800CW labeled Streptavidin (LI-COR Biosciences Cat# 926-32230)

Secondary Antibody Conjugates

- IRDye 680 and 800CW labeled secondary antibodies are available from LI-COR Biosciences.

Protein Labeling Kits

- IRDye 800CW Protein Labeling Kit (LI-COR Biosciences Cat# 928-38040, 928-38042, or 928-38044)
- EZ-Link® Sulfo-NHS-LC Biotinylation Kit (Pierce Biotechnology Cat# 21430)

II. Optimization Considerations

Reagent Titration

Ideally, each reagent involved in the assay should be titrated in pairs; start by titrating the capture antibody and antigen, followed by titrating the detection antibody and secondary antibody or streptavidin conjugate. Realistically, however, the amount of antibody and/or antigen available may be a limiting factor in such an extensive titration. An example of a more efficient, though less thorough, titration of capture antibody, antigen, and detection antibody, is outlined in Figure 1. In addition, incubation temperatures and times can have significant effects on assay performance, and should be considered as part of the experimental design. In general, the temperature of incubation will determine the length of incubation time for each step in the assay. For example, standard/sample incubation performed at 4 °C will usually require an overnight incubation time, while incubation at 37°C will require a much shorter incubation time to obtain an equivalent fluorescent signal.

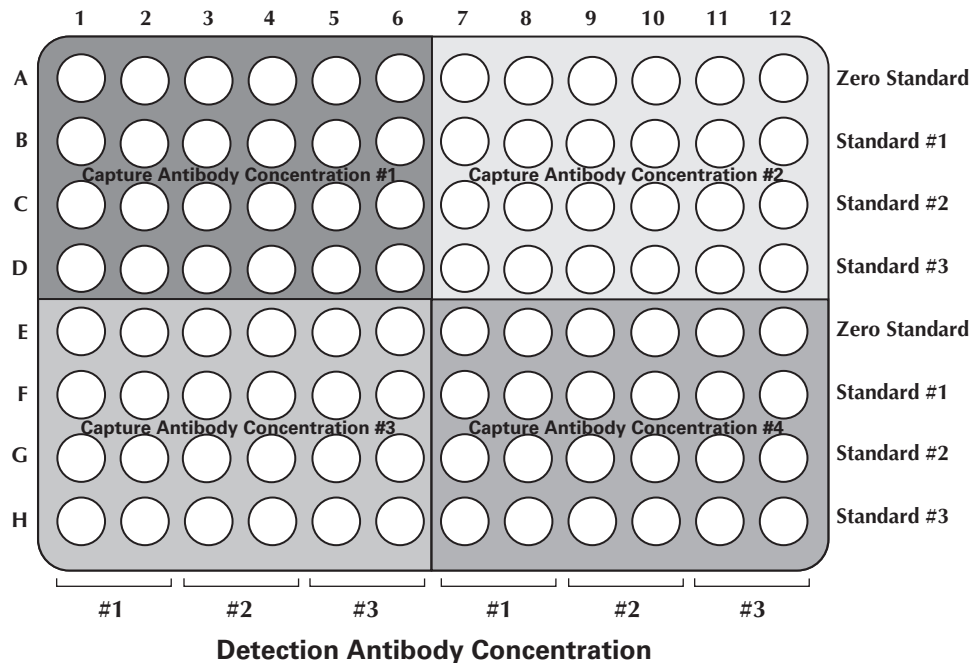


Figure 1. Example of a plate outline for reagent titration. Each quadrant of this particular plate tests three different detection antibody concentrations, each with four standard concentrations in duplicate (including a zero standard). These titrations are repeated in each quadrant for four distinct capture antibody concentrations. Antibody/streptavidin conjugate is maintained at a fixed concentration.

Analyte

Standards: A calibration curve, run in duplicate, should be generated for each experiment. Serial dilution range can be determined after an initial standard titration (see above) and should include a zero standard.

Samples: If possible, samples should be filtered, column purified, dialyzed, or otherwise purified for best results. Dilute samples in Reagent Diluent (recommended), blocking buffer, or 1X PBS.

Fluorescent Detection

The recommended method of detection for FLISA assays is biotin/streptavidin because many commercially available ELISA kits use biotinylated detection antibodies along with streptavidin-conjugated horseradish peroxidase or alkaline phosphatase. This method allows you to purchase an ELISA kit and substitute the HRP/AP-streptavidin with a fluorescent IRDye-labeled streptavidin.

For cases in which a biotin conjugated detection antibody is either unavailable or undesirable, an alternative detection option would be to utilize a dye-labeled secondary antibody against an unlabeled detection antibody; however, this format generally does not provide the level of sensitivity of biotin/streptavidin. Another detection alternative is to employ a dye-labeled antibody conjugate directly; this option has the advantage of requiring one less reagent, but may introduce additional problems with non-specific fluorescent signal.

Wash Procedure

The wash steps in the FLISA assay are critical to obtaining good results. Use the following guidelines for best results:

- Standard laboratory wash bottles work well for washing. Automatic plate washers may be used when higher throughput is necessary, as long as cross-contamination between wells can be prevented and the guidelines below can be adhered to. Wells can be overfilled and sprayed vigorously, but not harshly. Make sure each well is filled completely with buffer for each wash (exception: for the last set of washes, use a pipet to dispense and aspirate wash buffers. This will prevent extraneous signal caused by dye conjugate spilling over between wells.).
- For more stringent washing, first wash 2-3 times with 1X PBS-T, then rinse with 1X PBS. After the final wash, briefly centrifuge the plate upside-down on a clean paper towel at low speed (~600 x g); this will ensure all liquid is removed and allow for better reagent contact in the microplate wells for the successive steps. If a centrifuge is not available, rigorously (but not harshly) blot the plate on paper towels until all liquid is removed from the wells.
- Optionally for each wash, allow the wash buffer to sit in the wells for 1-2 minutes with gentle rocking/shaking.

III. Odyssey Settings

Scan

Start by defining a Scan Preset to use with the FLISA assay. Choose **Settings > Scan Presets** in Odyssey application software and edit the default "MicroPlate2" scan preset. Change the focus offset to match the distance measured from the well bottom to the base of the plate. Click **Save As** and save the Scan Preset using a new name. Consult the Odyssey User Guide for complete information on using the Scan Preset settings.

Sample Quantification

Quantification in Odyssey application software is performed by applying a grid to the microplate image (choose **Analyze > Add Grid**). If one of the default Grid Templates does not fit your microplate, use the Grid Template settings (choose **Settings > Grid Templates**) adjust the well diameter and other parameters to correspond with the microplate you are using. Quantification data can be viewed in the grid sheet (choose **Analyze > Grid Sheet**). Consult the Odyssey User Guide for more detailed microplate quantification procedures.

IV. General FLISA Protocol

The following is an example protocol for performing a 96-well microplate sandwich FLISA with the Odyssey Infrared Imaging System. Reagent concentrations, incubation times and temperatures, and method of fluorescent detection are given only as a typical FLISA example; these characteristics may vary considerably with the samples and antibodies in your assay system.

1.	Bring all reagents to room temperature before use.
2.	Prepare an appropriate amount of 2 µg/ml capture antibody. Pipet 100 µl into each desired well. Incubate microplate at 37 °C for 30 minutes, room temperature for 2 hours, or 4 °C overnight (16-18 hours). Gently agitate plate on a shaker or rocker during incubation.
3.	Remove capture antibody solution from wells. Wash 3 times with 1X PBS-T and once with 1X PBS; use enough wash buffer to fill each well. Gently agitate wash buffer for 1-2 minutes before removing. Ensure wash buffer is removed completely before proceeding to the next step.
4.	Pipet 300 µl of blocking solution into each well. Incubate at room temperature with gentle agitation for at least one hour. Prepare samples and standards while waiting for blocking to proceed.
5.	Remove blocking buffer completely.
6.	Add 100 µl of the appropriate samples/standards dilutions to each well. Incubate at 37 °C for 30 minutes, room temperature for two hours, or 4 °C for 4-6 hours. Gently agitate plate during incubation.
7.	Remove samples/standards from wells. Wash 3 times with 1X PBS-T and once with 1X PBS as in step (3).
8.	Prepare an appropriate amount of 200 ng/ml biotinylated detection antibody. Pipet 100 µl into each well. Incubate at 37 °C for 30 minutes, room temperature for 1 hour, or 4 °C for 2-3 hours.
9.	Remove detection antibody solution from wells. Wash 3 times with 1X PBS-T and once with 1X PBS as in step (3).
10.	Prepare an appropriate amount of 1 µg/ml streptavidin conjugate. Add 100 µl to each well. Protect the plate from light and incubate at room temperature with gentle agitation for 30 minutes.
11.	Carefully decant the streptavidin solution with a pipet. Wash 4 times with 1X PBS-T and once with 1X PBS; take extra care during this set of wash steps so as not to allow wash buffer to spill between wells (and thereby introduce a potential source for extraneous fluorescent signal).
12.	Place the microplate on the Odyssey scanning surface with well A1 in upper left orientation. Use the microplate scanning guide as described in the Odyssey Operator's Manual. Scan the microplate following the aforementioned guidelines and instructions in the Odyssey User Guide.

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