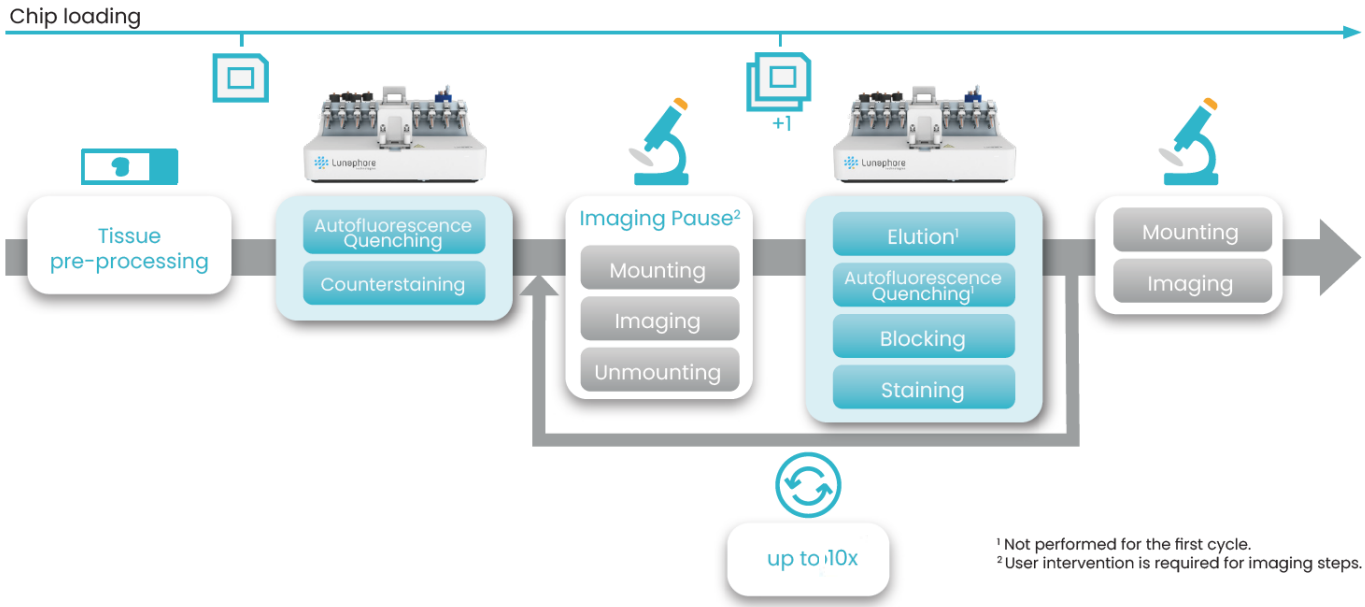
Short Guide: FS Sequential IF staining on LabSat®

This short guide will help you get started with protocols for FS Sequential immunofluorescence stainings on LabSat®. LabSat® handles the staining steps from autofluorescence quenching to counterstaining without any user intervention.
LabSat® is CE/UKCA/UL marked and is For Research Use Only. Not for use in diagnostic procedures.



Material needed

	Description	Brand	Catalog number
Equipment	LabSat®	Lunaphore	LS01
	Microfluidic Chips (Microfluidic Kit, LabSat® Distribution Chips)	Lunaphore	MK01, MK02
	Scanner/microscope	N/A	N/A
	Image analysis software	N/A	N/A
	Orbital shaker capable of speed of 30 rpm	N/A	N/A
Staining reagents[1]	Primary antibodies	N/A	N/A
	Alexa Fluor™ Plus 555 Goat anti-Mouse IgG[5]	Lunaphore	DR555MS
	Alexa Fluor™ Plus 647 Goat anti-Rabbit IgG	Lunaphore	DR647RB
	Counterstaining DAPI	Thermo Fisher Scientific	62248
	Ethanol 70% (diluted in DIW, for automated in-protocol washes)	Fischer Chemicals	E/0650DF/15
	Multistaining Buffer[2]	Lunaphore	BU06
	Elution Buffer Kit, small[2]	Lunaphore	BU07
	Quenching Buffer Kit, small[2]	Lunaphore	BU08
	Blocking Buffer Kit[2]	Lunaphore	BU10
Pre- & post-processing reagents[3]	NBF™	Thermo Scientific	5701
	Formaldehyde	N/A	N/A
	Triton	N/A	N/A
	Staining Buffer[2]	Lunaphore	BU01
	IF mounting solution SlowFade™ Gold antifade reagent[4]	Thermo Fisher Scientific	S36936
	Deionized or demineralized water (DIW)	N/A	N/A

[1] The system is open and can be used with various detection systems.
[2] Use of Lunaphore buffers (BU01, BU06, BU07, BU08, and BU10) is recommended, but other buffer solutions can be used.
[3] The reagents listed are required to perform the pre-processing procedure for FS slides recommended by Lunaphore. Other reagents and methods can be used as long as the slide is fixed before being processed by LabSat®.
[4] Warning: not to be used with directly labeled primary antibodies. In that case, use ProLong™ Diamond mounting solution instead.
[5] Not compatible with mouse tissues.

Lunaphore standard pre-processing IF procedure for FS slides

For optimal results with FS samples, Lunaphore recommends the following pre-processing steps for Sequential IF protocols.

1. Thaw the samples for 20 minutes at room temperature (RT).
2. If using fresh FS samples: incubate the samples for 40 minutes at RT in NBF 10% for human samples, or formaldehyde 4% for mouse samples, on an orbital shaker under the hood.
3. Wash the samples for 5 minutes at RT in Staining Buffer on an orbital shaker under the hood.
4. Incubate the samples for 20 minutes at RT in Staining Buffer with 0.2% Triton on an orbital shaker under the hood.
5. Store the samples in Staining Buffer until they are loaded on LabSat®.

Protocol creation and loading

1. Check that all the necessary reagents are present in the "Reagents database". If necessary, create new reagents from the Reagents tab.
2. Create a new protocol by clicking "Add new" in the Protocols tab, then follow the steps below. If a protocol has already been created, go to step 5.
3. "Create a new protocol" window:
    a. Select FS > Sequential IF.
    b. Select the number of staining cycles and whether to image the tissue's autofluorescence.
    c. Click "OK" to create the template.
4. "New protocol" window:
    a. Edit name of the protocol if needed (it is "FS Sequential IF" by default).
    b. Complete the "Reagent kit" and "Description" fields that are below the estimated "Total time" (optional).
    c. Select a "Washing buffer" (Multistaining Buffer by default) from the drop-down list.
    d. Input the "Protocol base temperature" (37 °C by default). This will be the execution temperature of the protocol for all steps except Elution.
    e. Configure the protocol steps by selecting reagents, incubation times, and temperatures by expanding each step with the button. You can also add or remove steps using the buttons or change their order via drag and drop.
    f. Status dots are displayed for each step and for the whole protocol. If all of them are green, no problem has been detected. Hovering the mouse over an orange dot (warning) or red dot (error) reveals the error message.
    g. Click "Save" to save your protocol. Protocols with errors can be saved but cannot be executed.
5. Click "Add to queue" on the row of the desired protocol in the Protocols tab. This will add the protocol to the bottom of the "Queue" on the Home tab from where it can be loaded to the protocol area by double-clicking on its name.
6. The "Required actions" on the right side of the Home tab will guide you through the allocation of reagents and buffers to reservoirs, will indicate the volume to add to each reservoir, and will indicate when the waste needs to be emptied. For more details on the volume of reagents and buffers to prepare and on which reservoirs to allocate reagents and buffers, see the next section of this short guide.
7. Once all the "Required actions" have been performed, insert the chip and slide and then, click "Start".

Reagent preparation

1. The full list of reagents and buffers that are required for the protocol to execute can be displayed by clicking the button on the right of the protocol's row in the Protocols tab. The list of required reagents and their required volumes are displayed. Priming volumes are not included in this list, they are 120 µL for small reservoirs and 500 µL for large reservoirs.
2. Determine the reagent and buffer allocation to the reservoirs. Keep in mind that:
    a. DIW (reservoir A), washing buffer, and alcohol (reservoir D) are loaded in large reservoirs. Lunaphore recommends loading elution buffer in a large reservoir to prevent the formation of bubbles that may occur when pipetting elution buffer into a small reservoir.
    b. All other reagents are loaded in small reservoirs. Reagents for which more than 2 mL is necessary for the whole protocol can nonetheless be loaded in a small reservoir and refilled during the pause step.
    c. Reagents that have Dynamic incubation turned on must be placed in small reservoirs.
    d. All reservoirs except A (DIW) and D (Ethanol 70%) can be washed during the dedicated pause, to replace the loaded reagent.
3. Once the allocation of reagents and buffers has been determined, add the corresponding priming volume to the "Required volume" seen in step 1 and prepare the total volume of reagent or buffer (total volume of X = required volume of X + priming volume of reservoir to which X has been allocated).

Tip: If non-specific binding of the primary and/or secondary antibodies is observed when using protein block in the Blocking step, try to dilute the primary and secondary antibodies in protein block.

Large reservoirs should always be loaded with at least 10 mL. Even if less is required, prepare more of a given reagent/buffer and reuse it for your next protocols.

Reservoir allocation and the necessary reagent and buffer volumes will vary depending on the protocol. For a multiplex protocol with n cycles, each detecting two markers, using the default parameters for all steps, two secondary antibodies and the Lunaphore buffers listed in page 1, Lunaphore recommends the following configuration:

		Description	Dilution and diluent	Volume to prepare and load
Large reservoirs	A	DIW	N/A	50 mL
	B	Multistaining Buffer	1:20 in DIW	50 mL
	C	Elution Buffer	Dilute Sol. 2 (20X) in Sol. 1 (1:20)	At least 10 mL
	D	Ethanol	70% in DIW	50 mL

		Description	Dilution and diluent	Volume to prepare and load (priming volume + dispense volume)
Small reservoirs	1	Quenching Buffer	1. Dilute Sol. 1 (10X) in DIW (1:10) 2. Dilute Sol. 2 (10X) in Sol. 1 (1X) (1:10)	120 µL + n * 350 µL
	2	DAPI	Dilute 1:1000 in Multistaining Buffer[1]	120 µL + n * 280 µL
	3,4	Secondary antibodies [3]	Dilute to 10 µg/mL in Multistaining Buffer[1]	120 µL + n * 180 µL or 120 µL + n * 280 µL [2]
	5,6	Primary antibodies [3]	Dilute in Multistaining Buffer or antibody diluent to the appropriate concentration.	120 µL + 180 µL or 120 µL + 280 µL [2]
	7	Blocking Buffer	1. Dilute 1 volume of Sol. 1 (10X) in 8 volumes of DIW. 2. Add 1 volume of Sol. 2 (10X).[1]	120 µL + n * 230 µL

[1] If using Lunaphore products described in page 1.
[2] If Dynamic incubation is on.
[3] Lunaphore recommends loading cocktails of the primary antibodies used in the same cycle and of the secondary antibodies used in the same cycle. This shortens the protocol time and does not change the reagent consumption.

Protocol Optimization

The staining parameters can be optimized, and the epitope stability and elution efficiency can be determined for FS Sequential IF protocols on LabSat®. The steps below will guide you through the first two. For more details, and instructions on how to obtain the elution efficiency, see the FS Sequential IF annex of the user manual (Protocol Optimization section).

1. First screening
    a) Create a new protocol using protocol "A" below as a template. Use antibodies according to the manufacturer's recommendations.
    b) Run the protocol created in step 1a) on a slide pre-processed according to the guidelines above.
    c) Image the slide and evaluate the staining quality.
    d) Keep the stained slide to continue the optimization process.
2. Staining optimization
    a) Based on the staining performed in step 1, identify which parameters need to be adapted and define the conditions to test.
        i. To increase or decrease the signal, adjust antibody dilution and incubation time, and enable/disable Dynamic incubation.
        ii. To reduce non-specific binding, add a Blocking step before the Staining step or dilute the antibodies in blocking solution and/or use blocking solution as antibody diluent.
        iii. To improve elution efficiency, increase the number of dispenses and/or the incubation time of the Elution step.
    b) Create a new protocol using protocol "B" below as a template. Adjust the steps according to the conditions defined in step 2a). Each protocol can test up to 5 conditions.
    c) Run the protocol on the slide kept in step 1d).
    d) Qualitatively assess the staining results to determine which condition gave the best staining in terms of signal intensity, signal-to-background ratio and elution efficiency. If the results are unclear by eye, conduct a quantitative analysis.
3. Epitope stability and elution efficiency characterization
    a) Create a new protocol using protocol "C" below as a template. If you plan to use your optimized marker in a multiplex protocol with less than ten cycles adjust the number of Elution steps between stainings to mimic a protocol with fewer cycles.
    b) Run the protocol on a new slide.
    c) Quantitatively assess the elution efficiency of the 1st cycle.
    d) Qualitatively assess the epitope stability between the 1st, 5th and 10th cycle. If the results are unclear by eye, conduct a quantitative analysis and compute the decrease in signal over cycles.

Protocol A	Protocol B	Protocol C
Autofluorescence imaging	Staining with 1st condition	Autofluorescence imaging
1. Initialization 2. Autofluorescence quenching 3. Counterstaining 4. Imaging pause	1. Initialization 2. Staining [Condition 1] 3. Imaging pause	1. Initialization 2. Autofluorescence quenching 3. Counterstaining 4. Imaging pause
Negative control (optional)	Elution of first condition	Epitope stability at cycle 1
5. Initialization 6. Secondary antibody 7. Counterstaining (if necessary) 8. Imaging pause	4. Initialization 5. Elution 6. Autofluorescence quenching 7. Counterstaining 8. Imaging pause	5. Initialization 6. Staining [Optimized before] 7. Imaging pause
Staining	Staining with 2nd condition	Elution of optimized staining
9. Initialization 10. Primary antibody 11. Secondary antibody 12. Counterstaining (if necessary) 13. Imaging pause	9. Initialization 10. Staining [Condition 2] 11. Imaging pause	8. Initialization 9. Elution 10. Autofluorescence quenching 11. Counterstaining 12. Imaging pause
Elution		Epitope stability at cycle 5
14. Initialization 15. Elution 16. Autofluorescence quenching 17. Counterstaining 18. Imaging pause	Repeat cycles 2 and 3 for up to 5 conditions.	13. Initialization 14. Elution 15. Elution 16. Elution 17. Autofluorescence quenching 18. Staining [Optimized before] 19. Imaging pause
Negative control (optional)	Elution of last condition	Epitope stability at cycle 10
19. Initialization 20. Secondary antibody 21. Counterstaining (if necessary) 22. Final wash	12. Initialization 13. Elution 14. Autofluorescence quenching 15. Counterstaining 16. Final wash	20. Initialization 21. Elution 22. Elution 23. Elution 24. Elution 25. Elution 26. Autofluorescence quenching 27. Staining [Optimized before] 28. Final wash