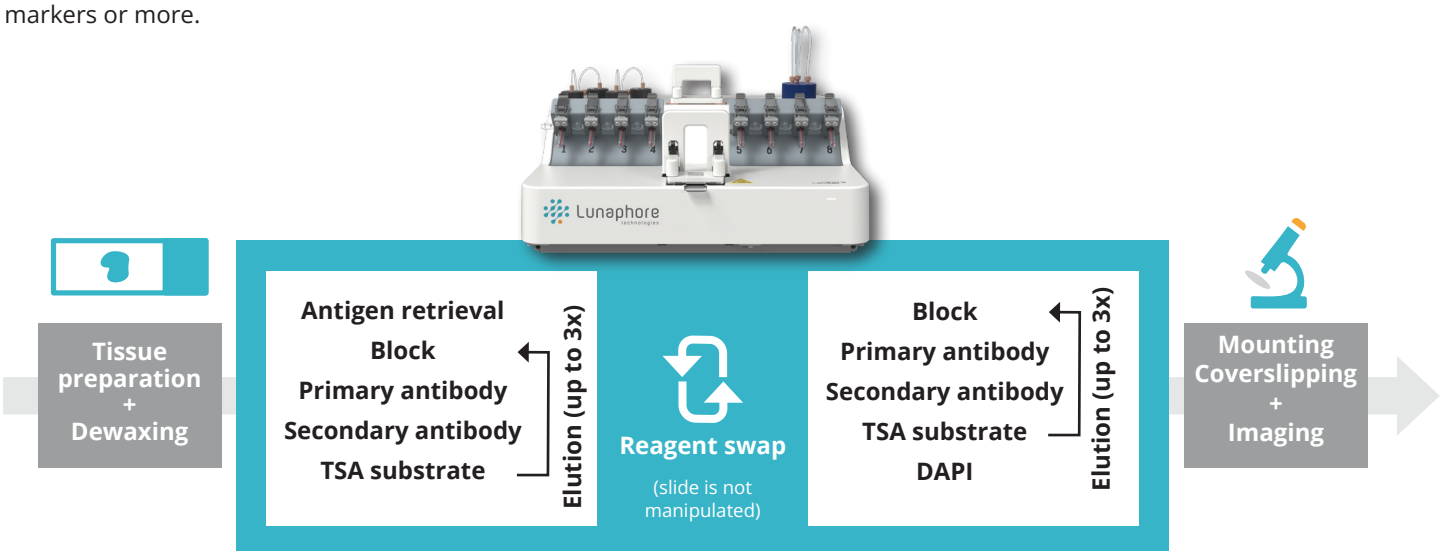
Short Guide TSA-based Multiplex IHC on LabSat®

This short guide will help you get started with TSA-based multiplex protocols on LabSat®. LabSat® handles the staining from antigen retrieval to counterstaining and only requires a 2-minute user intervention to load new reagents when staining four markers or more.



Material Needed

	Description	Brand	Catalogue number
Equipment	LabSat®	Lunaphore	LS01
	Microfluidic Chips (Microfluidic Kit, LabSat Distribution Chips)	Lunaphore	MK01, MK02
	Fluorescent scanner/microscope	N/A	N/A
	Image analysis software (if spectral unmixing is needed)	N/A	N/A
	Oven capable of 65°C incubation	N/A	N/A
Staining reagents	Primary antibodies	N/A	N/A
	TSA Reagent Kit (Secondary antibodies and Substrate)	N/A	N/A
	DAPI	N/A	N/A
	Antigen Retrieval Solution (pH6 or pH9)	Lunaphore	BU04 or BU05
	Multistaining Buffer	Lunaphore	BU06
	Cleaning Ethanol, 70%	Lunaphore	BU02
Pre-/Post-processing reagents[1]	Histoclear™ (Xylene substitute)	National Diagnostics	HS-200
	Ethanol 100% (will be used at several concentrations)	Fischer Chemicals	E/0650DF/15
	NBF™ 10%	Thermo Scientific	5701
	Tap water	N/A	N/A
	ProLong™ Diamond Antifade Mountant	ThermoFisher	P36970
	Deionized or demineralized water (DIW)	N/A	N/A

[1] The reagents listed are required to perform the pre-processing procedure for FFPE slides recommended by Lunaphore. Other reagents and methods can be used to deparaffinize and re-hydrate the slide before processing on LabSat®.

Lunaphore standard pre-processing procedure for FFPE slides

1. Bake samples for 60 minutes at 65°C in an incubation oven.
2. Dewax samples for 10 minutes in Histoclear™ solution on a shaker.
3. Perform the following sequential washing steps in separate containers:
    a. Histoclear™, 30 seconds
    b. Ethanol 100%, 30 seconds
    c. Ethanol 100%, 10 seconds
    d. Ethanol 95%, 10 seconds
    e. Ethanol 70%, 10 seconds
    f. Ethanol 40%, 10 seconds
    g. Tap water, 30 seconds
    h. NBF 10%, 20 minutes on shaker
    i. DIW, 5 minutes on shaker
    j. Multistaining Buffer 0.05%, leave until staining

Other reagents and methods can be used to deparaffinize and re-hydrate the slide before processing 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 FFPE > TSA-based multiplex IHC.
    b. Select the number of markers.
    c. Click “OK” to create the template.
4. “New protocol” window:
    a. Edit name of the protocol if needed (“TSA-based multiplex IHC” 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. 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.
        i. By default, the reservoirs of the primary antibody and substrate are not washed during the following “Elution step”. Activate the “Wash previous Abl and Substrate reservoirs” option to automatically wash these reservoirs if you do not want to recover the leftover reagents.
        ii. The “Reservoir exchange step” is added automatically when staining more than 3 markers. This step pauses the protocol to let the user change the reagents and refill buffer reservoirs. It also performs the remaining washes that may be needed. By default, the option to “Retrieve leftover reagents” will be “On” if the primary antibody and substrate reservoirs were not washed during the “Elution step”. This option will allow you to access the reservoirs before they are washed to retrieve any reagents that you wish to keep.
    e. 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 a respective message.
    f. 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. It 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 slide, place the chip, position the slide to select the staining area, close the stainer lid and then, click “Start”.
8. If the protocol includes Reservoir exchange steps, return to LabSat at the indicated time to retrieve the leftover reagents (if selected in the protocol) and load the new reagents.
    a. Leftover reagent retrieval: Remove the reagents that you want to save and load empty reservoirs instead. Click “Resume” to start the washes.
    b. Load the reservoirs needed for the second part of the protocol onto the instrument. Click “Resume” to continue.

Reagent preparation

1. The full list of reagents and buffers that are required to execute the protocol 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 respective volumes are displayed. Priming volumes are not included, 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. Washing buffer and alcohol (reservoir D) are loaded in large reservoirs.
   b. Primary antibodies and TSA substrates must be loaded in small reservoirs.
   c. Reagents that have "Dynamic incubation" turned on must be placed in small reservoirs.
   d. Only small reservoirs can be washed during the dedicated pause and during the "Elution" with reservoir wash steps to replace the loaded reagent.
   e. Reagents for which more than 2 mL are necessary for the protocol can nonetheless be loaded in a small reservoir and refilled during the pause step.
3. Once the allocation of reagents and buffers has been determined, add the corresponding priming volume to the "Required volume" described 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 6 cycles (using the default parameters for all steps, one secondary antibody, and the Lunaphore buffers listed in page 1) Lunaphore recommends the following configuration.

At the start of the protocol:

Large reservoir A	**Large reservoir B**	**Large reservoir C**	**Large reservoir D**
50 mL Washing buffer	10 mL Antigen Retrieval Solution	10 mL Blocking Buffer	50 mL 70% Ethanol
Small reservoir 1	**Small reservoir 2**	**Small reservoir 3**	**Small reservoir 4**
1800 µL Secondary antibody	450 µL AbI - 1	450 µL TSA substrate - 1	450 µL AbI - 2
Small reservoir 5	**Small reservoir 6**	**Small reservoir 7**	**Small reservoir 8**
450 µL TSA substrate - 2	450 µL AbI - 3	450 µL TSA substrate - 3	-

During the reservoir exchange pause:

Large reservoir A	**Large reservoir B**	**Large reservoir C**	**Large reservoir D**
30 mL Washing buffer	No change	No change	No change
Small reservoir 1	**Small reservoir 2**	**Small reservoir 3**	**Small reservoir 4**
No change	450 µL AbI - 4	450 µL TSA substrate - 4	450 µL AbI - 5
Small reservoir 5	**Small reservoir 6**	**Small reservoir 7**	**Small reservoir 8**
450 µL TSA substrate - 5	450 µL AbI - 6	450 µL TSA substrate - 6	450 µL DAPI