USER MANUAL

LabSat® Research

Autostainer

Document

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Identification of instrument

Instrument Name: LabSat® Research

Version: LS4.0

Software identification: LabSat Research

Software version: 2.4.0

Manufacturer

The manufacturer is **Lunaphore Technologies SA**, with its registered office at **Route de Lully 5C, CH-1131 Tolochenaz, Switzerland.**

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Contents

1. Introduction

The LabSat® instrument, along with the consumable microfluidic chip is an automated stainer that performs immunohistochemistry (IHC) protocols on tissue samples (both chromogenic and fluorescent), meant for Research Use Only applications. It is compatible with IHC reagents, for staining on fixed frozen sections (FS), and deparaffinized formalin fixed paraffin-embedded sections (FFPE).

It has been designed to automate and perform rapidly IHC protocols of FFPE and FS tissues, allowing transfer of manual procedures to the stainer, and perform automated multiplex protocols on FFPE and FS samples.

This document is the user manual of the Lunaphore automated stainer LabSat®.

Intended use

The LabSat® instrument is designed to perform automated staining of tissue section samples in histological applications.

The system is to be used with the consumable chips from Lunaphore. In addition, a list of recommended and validated products to be used with LabSat® is available in the user manual. The instrument is intended for use by professional users, such as researchers, technicians and physicians trained in staining techniques and the operation of the LabSat®.

For Research Use Only. Not for use in diagnostic procedures.

2. Important Information

This user manual is an important part of the product and instructs on the safe operation and maintenance of the instrument. These instructions must be read before use and must be kept close to the instrument. Make sure you read the following sections carefully: Warnings (Section 2.1), Symbols and indicators (Section 2.2), Limitations of use (Section 2.3) and Important statements (Section 2.4).

2.1. Warnings

2.1.1. Use

1. Do not use the instrument in any way other than the intended use. Any use that deviates from the intended use is considered a misuse, which may lead to injury or damage to the instrument. Any use which deviates from these instructions is also considered a misuse. In case of misuse, the manufacturer is not liable for any injury, damage, or staining results.
2. Comply to the washing and maintenance instructions and follow the Troubleshooting guide (Section 9) in case of issues. When instructed, report issues by contacting Customer Support.
3. Only operate LabSat® if you are trained personnel. All users must be familiar with the instrument features presented in this manual.
4. Lunaphore has tested and approved some commercially available detection kits to be used in combination with LabSat®. Please contact Customer Support for more information. Only use the recommended cleaning solutions (see recommended solutions in Section 12.2). Do not use dewaxing reagents for dewaxing steps and solvent-based solutions with LabSat® or its consumables. Contact Customer Support before using solutions other than those recommended. Also note that Quenching buffer (BU08) and Fluidics Cleaning Kit (BU03) show limited compatibility with some parts of LabSat®. Therefore, it is particularly important to use these buffers only as instructed in this User Manual.
5. Wash all reservoirs according to washing protocols (see Section 8.14). Do not install third party software products or screen savers without previously consulting Customer Support. Keep Windows up to date but do not install updates packages while LabSat Research software is running.
6. Avoid generating bubbles inside the reservoirs during preparation and loading and favour non-surfactant solutions.
7. Do not use reagents and buffers which have expired.
8. Take notice of the heat induced steps in the protocol, they may lead to the unwanted degradation of heat sensitive reagents.

2.1.2. Safety

1. Use disposable protective gloves and protective glasses when handling LabSat®, the consumable chips and reagents.
2. Normal operation of LabSat® may involve the use of solutions that are pathogenic, toxic, flammable, or irritant. Handle the solutions according to good laboratory procedures and methods to prevent exposure, and always refer to the product labels for safety information. Because spills may generate aerosols, observe proper safety precautions for aerosol containment. Always follow your local safety regulations.
3. Do not lean on the autostainer or place items on it.
4. Never open stainer, Distribution System, reservoirs, or waste bottle during ongoing protocols, to prevent risk of contact with reagents inside LabSat® while the system is pressurized (the locked reservoirs symbol (see Section 11) is present during this time). If liquids do come into contact with eyes or mucous membranes, wash with copious amounts of water, and consult a medical professional.
5. Do not attempt to touch the stainer until the "Hot stainer" icon is no longer displayed in the UI (see Section 11).
6. Do not pour liquids on the instrument as it is not designed to drain large volumes. If alcohol or hazardous material has been spilled into the device, immediately turn it off using the switch button at the back of the machine. If Quenching Buffer has been spilled into the device, immediately clean it with a paper towel and with DIW to prevent rust from forming. The user is responsible for the appropriate decontamination of the instrument and for the use of a cleaning agent that does not cause a device hazard. Contact Customer Support in case of doubt about the compatibility of a cleaning agent with the instrument.
7. Do not make any hardware and software changes before consulting a Customer Support. If for a specific reason, a part of the instrument's cover needs to be removed, always switch off the instrument and unplug it from the power source first.
8. Do not close the software or unplug any of the machine's cables and tubes during an ongoing process.
9. Do not exchange the waste bottle with a regular laboratory bottle. The supplied bottle is compatible with LabSat®'s operating pressure. Contact Customer Support if you need to change it.
10. Do not expose the power supply to liquids. In case of an emergency, detach the main power supply to disconnect the device.
11. Do not lift the instrument by the stainer or Distribution system handles.
12. Do not use flammable solutions as washing buffer solutions.

2.2. Symbols and indicators

Symbol	Meaning
	Catalogue Number
	Caution
	CE Marking European Conformity
	UL marking North America Conformity
	UKCA Marking Great Britain Conformity
	Consult Instructions for Use

	Correct Disposal of WEEE (Waste Electrical and Electronic Equipment) Marking of electrical and electronic equipment in accordance with article Directive 2012/19/EU.
	Direct Current
	Keep dry
	Manufacturer
	On (Power)
	Off (Power)
	Serial Number
	Fragile, handle with care The instrument may break if not handled with care.
	Hot Surface Hazard The symbol is located next to the stainer, to indicate that there is a risk of burn because of hot surfaces. Do not touch the stainer during ongoing processes.
Indicators	Meaning
	SAP (Systems, Applications and Products) Code for SAP Enterprise Resource Planning software.
	Shock indicator Indicator detects if the shipment has been transported and stored safely. If a severe shock was endured, it can be detected and proven.
	This way up Must be transported in the indicated direction.

Table 1) Symbol and indicators, and their signification

2.3. Limitations of use

The instrument users performing IHC/IF stainings must be trained personnel, capable of selecting appropriate reagents, preparing tissue samples, and reading IHC/IF results. Staining results greatly depend on tissue handling and treatment before staining processes. Improper handling, cutting, freezing, and thawing, deparaffinization, fixation of the tissue, reagent storage and preparation, and/or contamination, may produce false positive or negative results and artifacts which may lead to staining failure.

It is paramount that the operating personnel is trained for proper use of LabSat® to obtain accurate and consistent results.

It is the user's responsibility to evaluate the quality and result of the staining, as well as to maintain the instrument clean and functional as instructed in this user manual and during the user training.

It is possible that new reagents show unexpected activity in some tissues, hence the need for positive and negative controls (see Section 8.6). Variations within tissues of the same origin may be due to antigen expression variations.

Limitations of LabSat®

1. Used reagents and buffers shall not exceed a viscosity of 2.5 cP.
2. Tissue/cell samples shall always be fixed prior to being used with LabSat®.
3. For the reservoirs, it is recommended to use the 2 mL microtubes listed in section 12.2.
4. If performing protocols with "Antigen retrieval" steps, it is best not to stain more than 10 samples in a day to avoid overheating of the device and distortion of further results.
5. Only use reagents and buffers which have not expired.
6. It is recommended to use tissue sections of thickness: 3-10 µm.
7. It is recommended to use positively charged slides with LabSat®, for a better tissue adherence to the glass slide.

2.4. Important statements

Federal Communications Commission (FCC) Statement

This device complies with part 15 of the FCC Rules. Operation is subject to the following two conditions:

(1) This device may not cause harmful interference, and

(2) This device must accept any interference received, including interference that may cause undesired operation

Caution: Changes or modifications not expressly approved by Lunaphore could void the user's authority to operate the equipment.

Note: The operator shall be required to stop operating the device upon a finding by the Commission or its representative that the device is causing harmful interference. Operation shall not resume until the condition causing the harmful interference has been corrected.

This equipment has been tested and found to comply with the limits for a Class B digital device, pursuant to part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference in a residential installation. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instructions, may cause harmful interference to radio communications. However, there is no guarantee that interference will not occur in a particular installation. If this equipment does cause harmful interference to radio or television reception, which can be determined by turning the equipment off and on, the user is encouraged to try to correct the interference by one or more of the following measures:

- Reorient or relocate the receiving antenna.
- Increase the separation between the equipment and receiver.
- Connect the equipment into an outlet on a circuit different from that to which the receiver is connected.
- Consult the dealer or an experienced radio/TV technician for help.

3. Product Specifications

3.1. LabSat®

The LabSat® instrument is a benchtop stainer and performs rapid immunohistochemistry tissue staining protocols. It is composed of both the autostainer instrument and the software, run on an adjacent computer. It offers a user-made reagent database, staining protocols and reports, as well as operations for the system's upkeep.

Unmatched Speed

The working principle of LabSat® is based on a microfluidic technology, where reagents are delivered to the sample in a closed chamber. Molecule deposition on the sample depends on convection rather than solely on diffusion, which allows rapid delivery and short incubation times.

It is a single-slide stainer, which can perform automated and rapid IHC (chromogenic and fluorescent) protocols of fixed frozen sections and deparaffinized paraffin-embedded sections.

The Staining chip allows unprecedented staining protocol times.

User Interface

The system includes protocol templates for different applications, that can be edited by the user.

The system provides a comprehensive program for instrument preparation and upkeep, protocol execution and report generation, with little handling time.

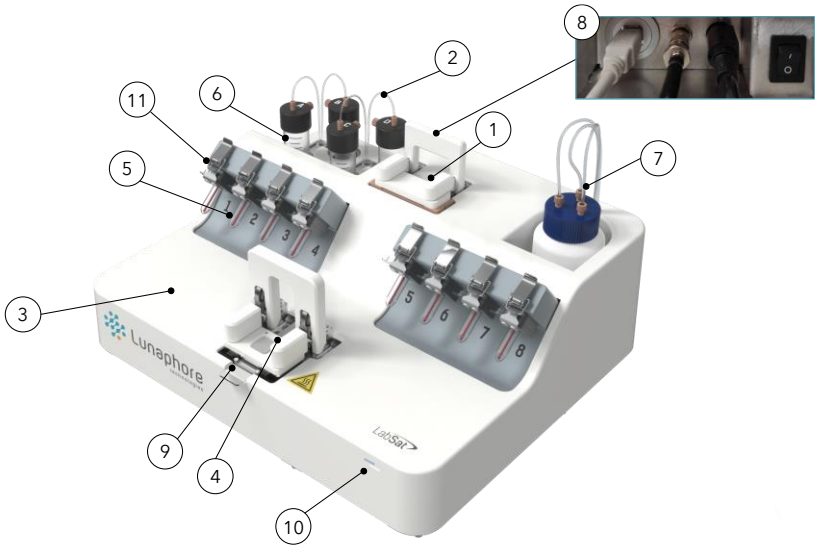


Figure 1) LabSat® components

1	Distribution system	Loading area for the consumable Distribution chip. The handles allow the user to open and close the system.
2	Fluidic tubes	Tubes in which reagents and buffers flow through the instrument and onto the sample.
3	Housing	Protective cover of the instrument.
4	Stainer	System to load the single-use consumable Staining chip and tissue sample. The handles allow user opening and closing of the stainer and loading of the consumable Staining chip.
5	Small reservoirs	Loading docks for eight consumable microtubes with reagents. Each dock is numbered to designate reagent position.
6	Large reservoirs	Loading caps for four consumable conical tubes with washing buffers and cleaning solutions. Each cap is labelled to designate buffer position.
7	Waste bottle	Glass bottle to collect waste from the executed protocols.
8	Connectors and power switch	Located on the back of the instrument: USB, pressure, and power connections, switch to turn the instrument on and off.
9	Glass slide	Standard histological slide with fixed sample is loaded in the stainer.
10	Status LED	LED displaying the status of LabSat®: in red when it is in idle state, and white when connected to the software.
11	Small reservoir latch	Metal latch used to lock the small reservoirs tightly into place.

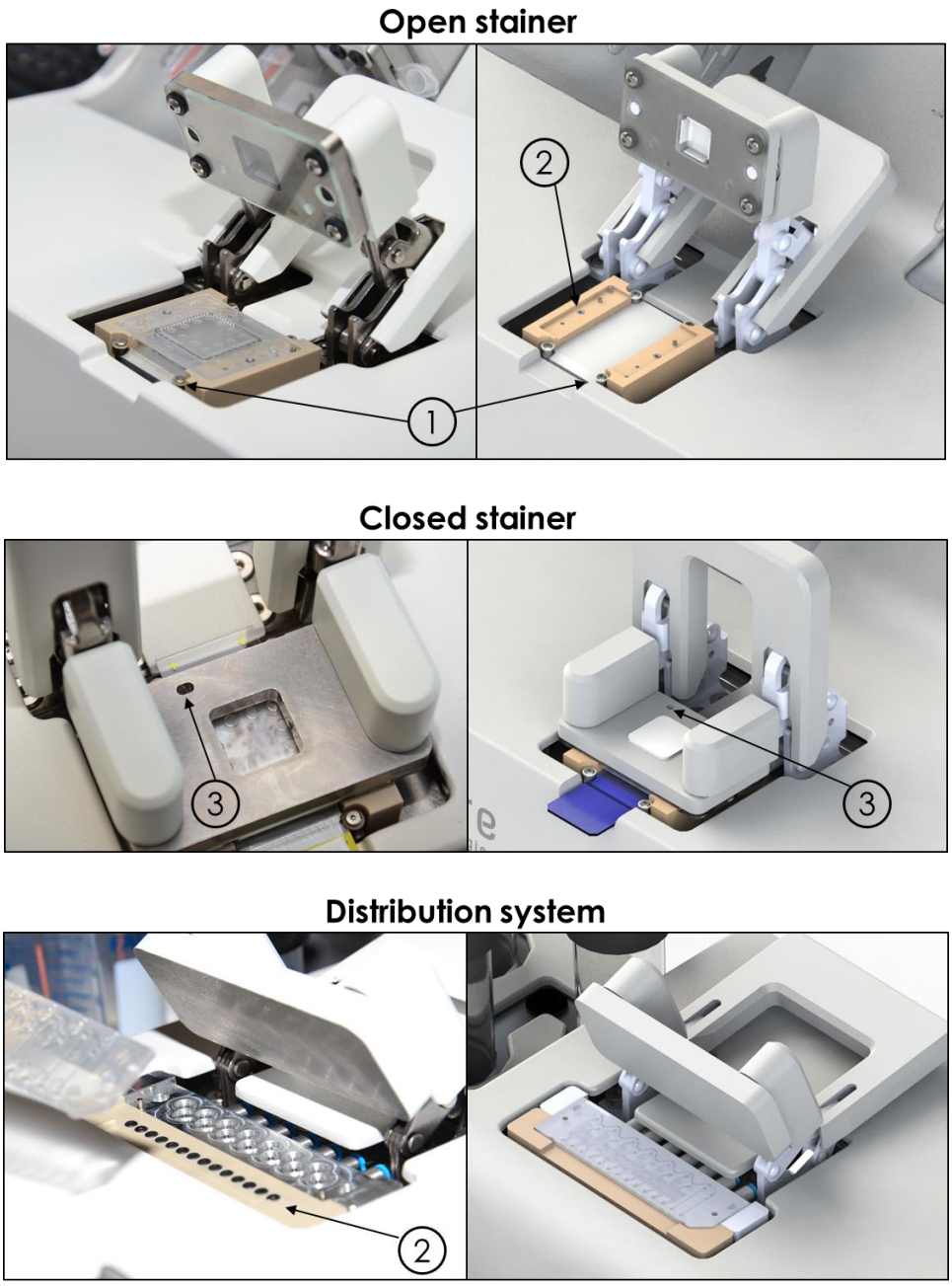


Figure 2) Stainer and distribution system components

1	**Slide centring system:** rotating pins that help the slide glide into the chamber and ensure that the slide is aligned with the centre of the stainer.
2	**Stainer and distribution system rings:** inlets and outlets of the loaded microfluidic chips. The rubber rings ensure a tight seal during operation.
3	**Stainer's visibility hole:** see-through portion of the stainer's cover that allows the user to check that the glass slide has been inserted far enough into the stainer.

Specifications

LabSat® dimensions	45 cm W x 37 cm D x 22 cm H (tube radius not included)
LabSat® weight	12.5 kg
Power supply information	24 VDC (160 W), Switching Mode Power Supply for 100-240VAC / 50/60Hz, 120 cm. MeanWell GST160A24-R7B
Power cord	Power cord (IEC320-C13), 180 cm Only use a Listed power supply cord set for US and Canada type SJT or similar type, 3x18 AWG: one end terminated in parallel blade (NEMA 5-15P) grounding-type attachment plug rated 10 A, 125 VAC, opposite end terminated in IEC320 style connector rated min. 10 A, 125 VAC.
USB	USB 2.0 (type B, male), 150 cm
Pneumatic plug	Quick Disconnect, Valved, 1/8" Tube ID, Acetal
Operating temperature	18 °C – 27 °C (64 °F – 82 °F)
Operating pressure input	5-8 bars (0.5-0.8 MPa) or 20 L/min
Slide capacity	1
Reagent capacity	8 (2 mL each)
Buffer capacity	4 (50 mL each)
Waste reservoir capacity	250 mL
Dispense volume reagents	Standard dispense: 180 µL Can be higher for specific reagents or for the Boost and Dynamic Incubation options.
Dispense volume buffers	Standard dispense: 500 µL Can be higher for specific protocol steps.

Table 2) Hardware specifications

Reagent database	Generation of a database of reagents and buffer solutions for continued use in protocols.
Reservoir allocation	Allocation status of reagents and buffers in reservoirs located on the instrument.
Reservoir volume calculation	Volumes in all reservoirs are constantly updated. The software informs the user when to empty the waste or refill reservoirs.
Protocol creation	Selection of staining parameters during the editing of protocol templates.

Calibration	Upon changing the Distribution chip, automatic calibration of the system runs. User can also choose to run a machine calibration only.
Daily Wash	Walk-away automated daily washing with alcohol and deionized water allows upkeep of the system's cleanliness for the next day's stainings and the exchange of the reservoirs' contents.
Full Wash	Walk-away automated Full Wash protocol keeps the system's fluidics fully functional over time.
Priming	Reservoirs loaded with reagents and buffers that have not been used in a protocol yet are automatically primed during the initialization step at the start of the protocol execution. Reservoirs can also be selected manually for priming.
Wash and Distribution chip change countdowns	The countdowns indicate when to change the Distribution chip or perform wash protocols. The countdowns decrement at a different rate depending on the application (see the application annexes for details).

Table 3) Software specifications

Staining chip **(25 units in MK01)**	Single-use microfluidic chip. Loaded in the stainer by the user before a protocol. Staining area: 23 x 23 mm
Distribution chip **(5 units in MK01 and 20 units in MK02)**	Daily use microfluidic chip. Loaded in the Distribution System at the beginning of each day after software start up, or when Distribution chip change countdown reaches zero.
Waste bottle	250 mL glass bottle that is rated for up to 1.5 bars. To be emptied when the software warns the user.
Instructions for use	User Manual with operating procedures.
Stainer cover	Cover to hide the chamber window (protection from light exposure)
Black rings for stainer and Distribution system	Bag of spare black rings to replace in case of need.
Air compressor **(CP01)**	Pressure: 5 – 8 bars Minimal flowrate: 20 L/min Air quality (ISO 8573): 1.4.4 Noise level: ~60 dB
Computer, keyboard, and mouse. **(AC01)**	Intel i5 3.5GHz 8 GB RAM 256 GB SSD 21.5-inch screen Windows 10 (with .Net Framework 4.5.2)

Table 4) Device accessories and spare parts (provided by Lunaphore)

Small reservoirs*	2 mL microtubes for reagent loading in the instrument.

Large reservoirs*	50 mL conical tubes, for washing buffer and cleaning solutions loading in the instrument.
Glass slide	Positively charged slide with dimensions: - Thickness: 0.9 to 1.1 mm - Width: 24.5 to 26.5 mm - Length: 74.5 to 76.5 mm

Table 5) Device consumables (not provided by Lunaphore), *Compatible brands can be found in the Annex 1.

3.2. Microfluidic chips description

Two consumable chips are used in LabSat®.

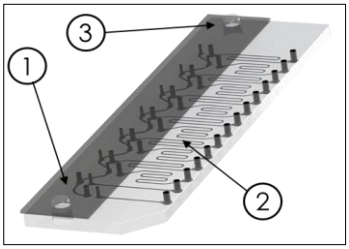


Figure 3) Distribution chip. 1. Membrane. 2. Microfluidic channels. 3. Pins.

The Distribution chip is a consumable to replace daily, and which delivers the reagents in the desired order. The top part contains a black membrane attached to the chip with pins, and the bottom surface shows the microfluidic channels where the reagents are delivered.

The Distribution chip is inserted in the Distribution System, with the membrane and pins facing down into the Distribution System (Figure 2).

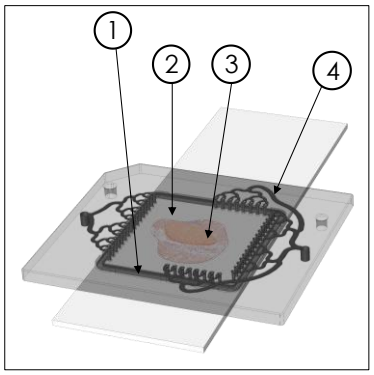


Figure 4) Staining chip. 1. Seal. 2. Chamber area. 3. Tissue sample. 4. Microfluidic channels

The Staining chip is a single-use consumable which is inserted in the stainer of LabSat®. It is composed of the chamber area where the staining process occurs with the inserted sample, and the microfluidic channels that lead reagents in and out of the chamber and a sealing membrane. The seal is the border between chamber and exterior.

Both chips are made of plastic material and taped with a sticky tape.

4. Installation

4.1. LabSat®

To ensure proper function, the transportation, unpacking, installation and assembly of LabSat® system shall be done only by a Lunaphore representative or distributor, and the installation requirements must be met (Section 0).

It must be installed on a laboratory wet bench, next to the computer supporting the Lunaphore software and user interface. It must also be connected to a compressed air line. An air compressor may be installed under the wet bench.

Once LabSat® is correctly installed, all users must receive proper training by a Lunaphore representative. In no case shall the device be used without prior training.

4.2. Microfluidic chips

Microfluidic chips are packaged in kits. Upon reception of the kits (product code: MK01 and MK02), the user must perform an incoming check to verify the conformity of the accessories (Figure 5). There are 25 Stainings chips and 5 Distribution chips in the MK01 and 20 Distribution chips in MK02.

For MK01, remove the chips from the primary cardboard packaging and check the secondary plastic packaging (blister) for any damage. Open the blister and verify the number of chips indicated on the label are inside the package.

For MK02, verify that the bag is not damaged and that the number of chips indicated on the label are inside the package.

Figure 5) MK01 kit with packaging

Before using the microfluidic chips, verify their conformity:

- No cracks
- No detached tape
- No tape edge too close to the fluidic channels
- Membrane attached to the Distribution chip
- Seal attached to the Staining chip.

Do not use a chip which does not conform to the above specifications. If a chip kit is not compliant to the order, contact Customer Support.

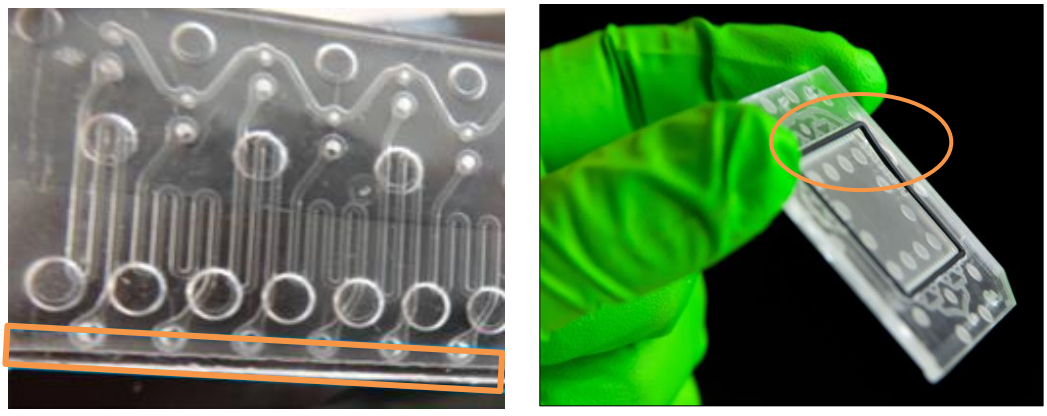


Figure 6) Tape too close to the fluidic channels (left), Seal removed in one corner (right)

4.3. Installation Requirements

Space	The surface must be solid and level, must resist to vibrations, and support the instrument's weight. Space size: at least 50 cm large, 40 cm deep (with an additional 5 cm free space available at the front of the instrument). Allow at least 5 cm on each side of the instrument to ensure proper circulation of air around it, and to allow access to power switch and cables in the back. Plan for enough space for a computer or laptop adjacent to the instrument. The space shall not be next to heat-producing laboratory equipment, nor in an area with relative humidity exceeding 85% (non-condensing).
Computer	The instrument must be placed next to a computer for USB connection.
Power source	The instrument must be placed close to a power outlet, and not used with an extension cord. The Manufacturer recommends using a dedicated power source to prevent interferences from other equipment. The instrument shall be placed in such a way that the power cord can be easily disconnected. The required voltage range is: 100 to 240 VAC 50 to 60 Hz The Supply voltage shall comply with the following conditions: - 10% fluctuations of the nominal voltage - Transient overvoltage up to levels of category II - Pollution degree 2. The instrument shall only be used with the power supply that is provided with it (MeanWell GST160A24-R7B). The power supply shall be protected from any exposure to liquids. For all the system equipment, there shall be 3 available power outlets. External computing devices connected to the USB port must be limited power source and SELV circuit according to the standards ANSI/UL 60950-1 and CAN/CSA-C22.2 No. 60950-1 or similar standards.
Environment	The instrument shall only be operating indoors and in a well-ventilated laboratory space, with ambient temperature between 18 °C to 27 °C, and not face direct sunlight. It is functional up to 1500 m above sea level.
Pressure	The instrument must be in a laboratory with access to compressed air. The pressurized air input for LabSat® is: 5-8 bars (0.5-0.8 MPa) If necessary, an air compressor can be purchased to fulfil this requirement.
Air supply	The air supply shall be of minimum 20 L/min.
Compressed air quality	The pressurized air shall comply with the norm ISO 8573-1 purity classes: - Solid particles: 1 - Water: 4 - Oil: 4

Table 6) LabSat® instrument's installation requirements

5. Storage and handling

5.1. LabSat®

Use and store LabSat® and its accessories at room temperature and in a non-humid environment. When stored, protect LabSat® from dust (using a plastic bag for example), water damage (do not store directly on the floor) and keep it safe from fire.

Install LabSat® on a laboratory bench top and handle it carefully. Unplug it from the power source and pressure when moving it. We recommend that you only move LabSat® after a wash has been performed.

The start-up procedure is always as follows:

- Make sure all three connections are plugged in (electric cable, USB, and pneumatic tube).
- Turn the pressure on and wait until the pressure reaches at least five bars.
- Switch the instrument ON (switch button in the back).
- Turn on computer and software.

To clean the instrument, follow instructions in Section 8.14. Clean the housing of the device once a week to remove stains and dust. If there is liquid on the instrument, immediately remove it with a paper towel and 70% ethanol (or 70% isopropanol) and then with a paper towel and water.

5.2. Microfluidic chips

Store the consumable microfluidic chips in the provided packaging until they are used, to protect them from damage caused by humidity, dust, and exposure to light.

6. Warranty and Service

Lunaphore guarantees that the product delivered has been subject to its internal quality control procedures, and that it is compliant with the technical specifications.

The scope of the warranty is dependent on the contents of the contract made. The terms in the contract with your Lunaphore sales representative, or official dealer, shall apply exclusively.

For any servicing and maintenance requests please contact your Lunaphore representative or dealer's customer support and prepare the following information:

- Instrument model, and serial number
- Software model and version
- Reason for service request
- Customer name
- Installation date

Do not attempt to service or replace parts without support.

7. Disposal information

All instruments, parts, and reagents are subject to local and regional regulations, as well as applicable laboratory specifications.

7.1. Disposal of consumables

- Dispose of the consumables, such as **chips** and used **reservoirs** in the hazardous waste.
- Dispose of **glass slides** in contact with biological samples in the appropriate sharp waste.
- We recommended to check with local authorities regarding the proper waste disposal of **IHC/IF consumables**.
- Dispose of the **waste and unused reagents** in the biohazard liquid waste.

7.2. Waste Electrical and Electronic Equipment disposal

As indicated by the crossed-out wheeled bin symbol (also shown on the right) present on LabSat®'s identification plate, LabSat® must not be disposed of with other waste. It must be taken to an approved treatment facility or to a designated collection point for recycling, according to local laws and regulations.

In the European Union, the European Directive 2012/19/EC on Waste Electrical and Electronic Equipment (WEEE) requires the proper disposal of electrical and electronic equipment when it reaches its end of life.

The separate collection and recycling of waste electronic equipment at the time of disposal helps to conserve natural resources and ensures that the product is recycled in a manner that protects human health and the environment.

Contact Customer Support for removal, repair, or disposal of LabSat® at the end of its working life. This must be handled by Lunaphore representatives or distributors and must not be mixed with other commercial waste. The instrument must be cleaned before removal and disposal.

8. Instrument Operation

8.1. Performance of the instrument

The instrument can perform a single plex staining or a cycle of a multiplex staining usually between 15 and 30 minutes, depending on the protocol and samples used.

LabSat® is compatible with FS and FFPE samples of any human tissue. It is also possible to use tissue samples of animal origin. The performance of all the applications is guaranteed on human tissues, except FS sequential IF for which the performance is guaranteed on mouse FS tissues.

8.2. Preparation prior to operation

Before operating the LabSat® instrument, the user must be trained by Lunaphore or a representative. The training includes handling LabSat®, preparing and loading reagents and buffers, inserting/changing the consumable chips, using all the functions of the software and troubleshooting.

LabSat® must be used in combination with the Microfluidic Kit consumable chips and the cleaning of the instrument is performed with Fluidics Cleaning Kit (BU03). Make sure to have these products prior to operation.

For optimal staining results, Lunaphore also provides washing buffers (BU01 - Staining Buffer and BU06 - Multistaining Buffer), an autofluorescence quenching buffer (BU08 – Quenching Buffer), an elution buffer (BU07 – Elution Buffer) and an alcohol-based cleaning solution (BU02 - Alcohol).

Material to prepare before starting a staining protocol:

- Up to date LabSat Research software
- List of protocols to run
- Numbered and annotated tissue samples on glass slides prepared according to the steps described below depending on the sample type (see Section 8.2.1. Pre-processing steps for FFPE samples or 8.2.2. Pre-processing steps for FS)
- Reagents and buffers that will be used during the protocol. The user can refer to the list of solutions and volumes needed from the Protocols tab (see Section 8.4.6).
- DIW in all reservoirs on LabSat® (except EtOH 70% in reservoir D). Washing buffer may be left in reservoir B.

8.2.1. Pre-processing steps for FFPE samples

For optimal results with FFPE samples, Lunaphore recommends the following dewaxing protocols depending on the application. These pre-processing steps are to be performed before moving onto LabSat®.

IHC, Single IF and Sequential IF protocols

1. Bake samples for 10 minutes at 65 °C.

2. Dewax samples for 10 minutes in Histoclear™ solution (see Section 12.2) on a shaker under the hood.
3. Perform the following sequential washing steps in separate containers under the hood:
    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
4. Store samples in washing buffer (such as Staining Buffer or Tris-Buffered Saline (TBS)) until loaded into LabSat®.

Multiplexing TSA (Tyramide Signal Amplification) protocols

1. Bake samples for 1 hour at 65 °C.
2. Perform the following sequential washing steps in separate containers under the hood:
    a. Histoclear™, 10 minutes
    b. Histoclear™, 30 seconds
    c. Ethanol 100%, 30 seconds
    d. Ethanol 100%, 10 seconds
    e. Ethanol 95%, 10 seconds
    f. Ethanol 70%, 10 seconds
    g. Ethanol 40%, 10 seconds
    h. Tap water, 30 seconds
    i. Neutral buffered formalin (NBF) 10%, 20 minutes
    j. DIW, 5 minutes
3. Store samples in washing buffer (such as Staining Buffer or Tris-Buffered Saline (TBS)) until loaded into LabSat®.

Note: It is recommended to avoid using Multistaining Buffer for this step.

8.2.1. 8.2.2. Pre-processing steps for FS samples

For optimal results with FS samples, Lunaphore recommends the following pre-processing steps depending on the application. These pre-processing steps are to be performed off LabSat®.

IHC stainings

1. Place the samples on a hot plate at 45 °C for 1 minute under the hood or let them thaw for 15 minutes at room temperature (RT).
2. Incubate for 3 minutes at RT in NBF 10% on an orbital shaker under the hood.
3. Wash in DIW for 1 minute at RT.
4. Store samples in washing buffer (such as Staining Buffer or Tris-Buffered Saline (TBS)) until loaded into LabSat®.

If NBF fixation does not give good results, acetone fixation might work better with some markers (CD45 LCA marker especially). The recommended pre-processing steps for acetone fixation are the following:

1. Place the samples on a hot plate at 45 °C for 1 minute under the hood or let them thaw for 15 minutes at room temperature.
2. Incubate for 3 minutes at RT in acetone on an orbital shaker under the hood.
3. Dry samples for 1 min at 45 °C under the hood, or 15 minutes at room temperature.
4. Store samples in washing buffer (such as Staining Buffer or Tris-Buffered Saline (TBS)) until loaded into LabSat®.

Sequential IF stainings

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 washing 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 washing buffer until they are loaded on LabSat®.

8.3. Standard Operating Overview

STANDARD OPERATING OVERVIEW

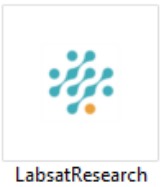
The standard workflow to perform a staining using LabSat® consists of the following steps:

1. Open LabSat Research (see Section 8.4) and follow the instructions on the pop-ups.
2. Change the Distribution chip (see Section 8.9). After the exchange procedure, the system will initialize (calibrate). Once finished, LabSat® is ready to be used.
3. Create new reagents if needed (see Section 8.4.7).
4. Create or edit a protocol using the reagents and buffers created in the previous step (see Section 8.4.6).
5. Add the protocol to be run to the Queue (see Section 8.4.6).
6. Load the protocol you wish to execute to the Protocol Area (see Section 8.4.4).
7. Allocate the reagents and buffers to reservoirs by clicking “Add” in the Required Actions.
8. Prepare the reagents and buffers (see Section 8.8), load them into the reservoirs and click “Fill” or “Empty” in the Required Actions.
9. Insert a Staining chip in the stainer and close the handles (see Section 8.10).
10. Load a sample (see Section 8.11).
11. Start the protocol (see Section 8.4.4). During protocol execution, an intervention may be necessary to wash and/or refill reservoirs or image the slide. In this case, the time until the next intervention is indicated in the software. During the intervention follow required actions and resume the protocol.
12. At the end of execution: Open the stainer and remove the slide (see 8.11).
13. Mount the slide and visualize it under a microscope to assess the staining result.
14. Verify that the protocol was executed properly by checking the Report.

8.4. Software Operation

The LabSat® instrument is controlled by LabSat Research, a software installed on the computer connected by USB.

To start the software, double-click on the Lunaphore Technologies icon on your desktop:



When the software opens, a pop-up may appear that recommend performing certain actions before using the device (Figure 7). Depending on previous use of LabSat®, some of the following actions may be recommend:

- Changing the Distribution chip
- Running a Full Wash
- Running a Daily Wash
- Changing buffers

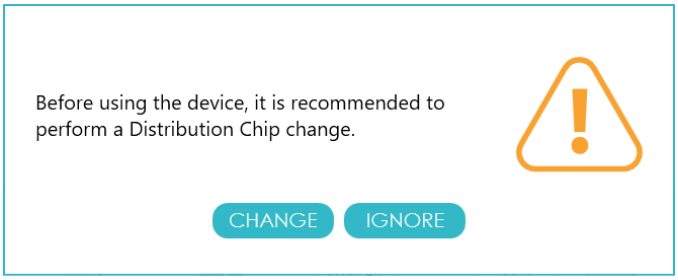


Figure 7) Example of a pop-up that could appear when opening LabSat Research. In this example, a Distribution chip replacement is being recommended.

8.4.1. Home Tab Features

After closing the pop-up, the software opens onto the Home tab (Figure 8). This tab is used to check the instrument status, execute protocols and launch washes and primings.

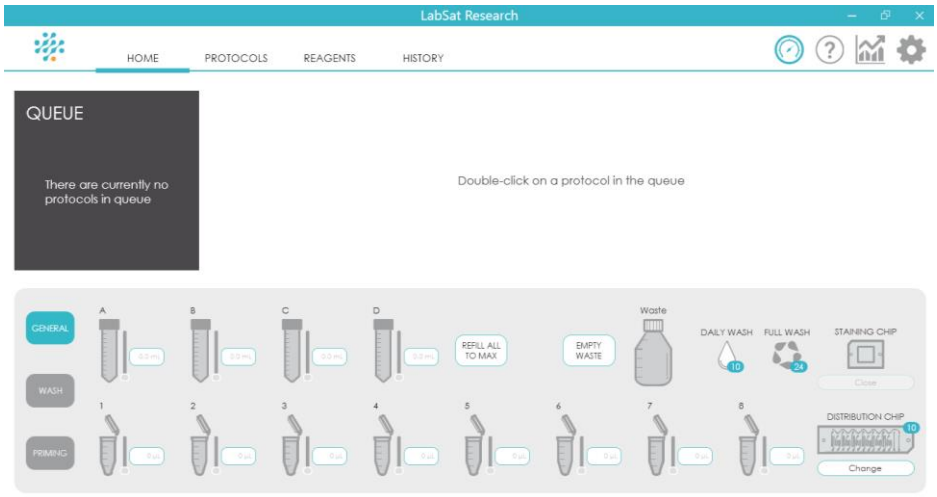


Figure 8) Home tab

From the Home tab the user has access to:

- **Queue and Protocol areas** (top half of the Home tab): the queue displays a list of selected protocols that can be loaded to the protocol area (on the right) for execution by double clicking on their name in the Queue.
- **Instrument Overview area** (bottom half of the Home tab). The user can toggle between the three following boxes:
    - "General" box: to view and edit information on instrument status
        - Volume of reagents and buffers in reservoirs
        - Volume in waste reservoir
        - Number of stainings before Daily Wash, Full Wash or Distribution chip change
    - "Wash" box: to load wash protocols
    - "Priming" box: to load priming protocol
- Protocols, Reagents and History tabs can be accessed from the menu bar.
- Instrument **Settings, Statistics and About** windows can be accessed on the right side of the menu bar.

The following sections go into detail for these areas, tabs, and windows.

8.4.2. Settings Window

Settings are accessed by clicking the gear icon in the menu bar.

The following settings can be changed from the window that opens (Figure 9):

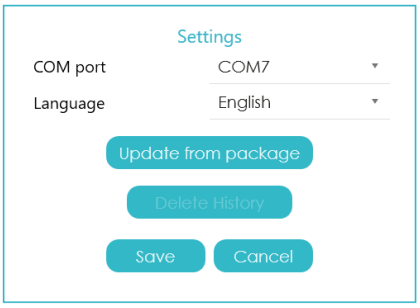


Figure 9) Settings Window

- COM port

Select which USB COM port on the computer is connected to LabSat® from the drop-down list. Once the correct COM port is selected, the LED on the device will turn from red to white.

- Language

Select the software language from the drop-down list. Currently only English is available.

- Update from package

If a new package for a protocol template is available, click "Update from package" and select the corresponding XML file from the browser.

- Delete History

Click "Delete History" to delete all the history files of the protocols that have been run.

- Click "Save" to keep any changes made, otherwise click "Cancel".

8.4.3. Instrument Overview

Instrument overview is available at the bottom part of the Home tab. There are three boxes that can be viewed:

General box

The General box displays the volume in all of the reservoirs, their allocated reagents and buffers, the volume in the waste bottle, and the countdowns for the Distribution chip change and the washes protocols.

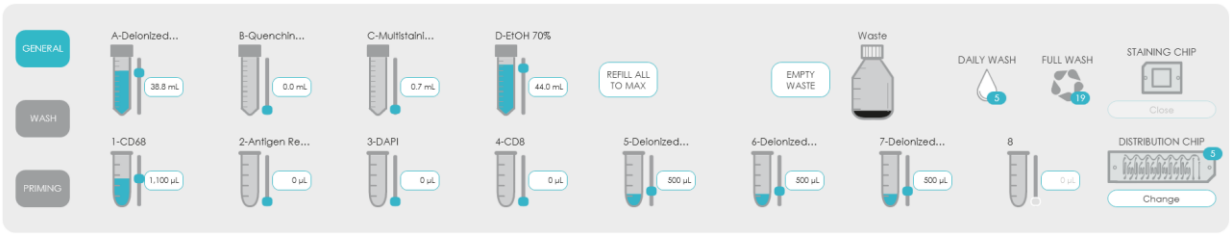


Figure 10) General box of the Instrument Overview area: Reservoirs statuses on the left, Distribution chip change and wash countdowns and stainer status button on the right.

- Setting reservoir volumes
  - Reservoir volumes can be adapted using the slider next to each reservoir or inserted manually in the text box.
  - The volume for large reservoirs is in millilitres, the volume for small reservoirs is in microliters.
  - All reservoirs can be automatically filled to the maximum volume, by clicking "Refill all to max".
  - Click "Empty waste" to reset the waste volume to 0 mL.
  - The refilling and emptying of reservoirs can also be done directly from the Required Actions or the Reagents tab.
- Wash countdowns

- Daily Wash and Full Wash countdowns inform the user when the respective washes must be performed (Figure 11).
- The countdowns start at 10 for the Daily Wash, and 24 for the Full Wash.
- Wash protocols must be performed when the software recommends it. Protocol execution is disabled until the required wash is performed. A wash protocol may need to be performed before the counter reaches zero to execute multiplex protocols, the software will indicate when a wash is required.

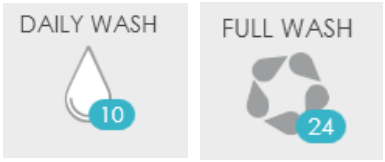


Figure 11) Daily Wash and Full Wash countdowns indicating when the washes must be performed

- Staining chip Opening
  - Clicking "Open" opens the stainer to access the loaded slide (Figure 12).
  - "Open" is deactivated once a protocol is launched to prevent unwanted chamber opening.
  - For applications that require user intervention (e.g. reservoir refilling, imaging), "Pause" steps (see section 8.4.5) allow the user to safely retrieve the slide from the machine during a protocol execution.
  - It is not possible to open the stainer if its temperature is above the safety limit. The user must wait for the system to cool down before being able to open the stainer. The temperature warning icon will disappear when stainer has cooled down.
  - The stainer closes automatically at the start of a protocol and when resuming a protocol after a pause.

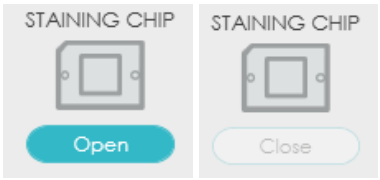


Figure 12) Staining chip button indicating when the stainer can be opened (left) and when it is open (right)

- Distribution chip change (see Section 8.9)
  - The Distribution chip can be used until the countdown reaches 0. Protocol execution is disabled until the required Distribution chip change is performed. A Distribution chip change may need to be performed before the counter reaches zero to execute multiplex protocols, the software will indicate when this is required.
  - The chip icon has a countdown badge as shown in Figure 13. The badge is displayed with different colours.
    - Blue: many counts left
    - Orange: 2 counts left
    - Red: chip needs to be replaced
  - Protocol execution is disabled until the exchange is done.

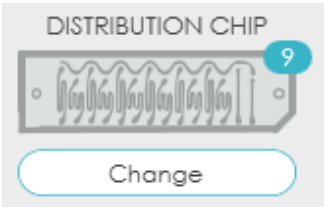


Figure 13) Distribution chip display on Instrument General Screen

  - The Distribution chip "Change" button has 2 options:

        - "Start": Carry out a full Distribution chip change (replacement + calibration)
        - "Calibration only": Carry out the instrument calibration only
        - Click "Cancel" to return to the Home tab
    - The system calibrates after each chip change, or if "Calibration only" has been selected. Verify that DIW is loaded in all reservoirs before starting the calibration procedure to avoid wasting reagents. Solutions in reservoirs B and D do not need to be replaced with DIW for this procedure.

Wash box

In the Wash box, there are 3 options to choose from:

- **Daily Wash** generates an automated wash protocol using 70% Ethanol (or BU02 – Alcohol) and DIW to wash all the reservoirs used during the day. It must be performed when the Daily Wash countdown reaches zero and before closing the software. The presence of a slide or a Staining chip in the stainer is not required for a Daily Wash.
- **Full Wash** must be performed when the Full Wash countdown reaches zero. Launching a Full Wash loads an automated wash protocol for all reservoirs and dispense and outlet tubing, using Lunaphore's Fluidics Cleaning Kit (see 8.14.3). The presence of a slide and a Staining chip in the stainer is mandatory for a Full Wash.
- **Select & Wash** generates a wash protocol for all the selected reservoirs using 70% Ethanol (or BU02 – Alcohol) and DIW. It can be performed on any reservoir to exchange its content or when requested by an alert in the software. Click to select the reservoirs to wash. The presence of a slide or Staining chip in the stainer is not required for Select & Wash.

When one of the wash protocols is selected, click "Load wash protocol" to load it in the Protocol area for execution. The system will inform you to load washing solutions if necessary. If a slide and a Staining chip must be loaded for the wash, a reminder will pop-up before the start of the protocol.

Priming box

From the Priming box, a priming protocol can be set-up and launched:

1. Click the reservoirs you wish to prime to select them.
2. Click "Load protocol" to load the priming protocol to the Protocol area for execution.
3. Complete the Required Actions and click "Start".

Primed reservoirs have a blue border on their icon (Figure 15). The volume consumed for priming is 120 µL for small reservoirs and 500 µL for large reservoirs. The presence of a slide or a Staining chip in the stainer is not required for priming. A primed reservoir can be primed again if needed.

Figure 14) Wash box, Daily Wash automatic selection

Figure 15) Priming box of the Instrument Overview area. Top: Reservoirs selected for priming are in blue. Bottom: Reservoirs that have been primed have a blue border, reservoirs with black borders are not primed.

8.4.4. Queue and Protocol area

The Queue and Protocol area (including Required Actions area) are in the top half of the Home tab.

Queue

This is a list of pre-selected protocols to be run. Double-click on a protocol to load it to the Protocol area. Protocols in the queue can be deleted by clicking on the bin icon.

Protocol area

When a protocol is selected from the Queue it is loaded into the Protocol area. Below the protocol's name there are the protocol's steps, on the left, and the Required Actions, on the right. When the staining starts, the protocol execution progress bar will appear above the Protocol area and the remaining time, and the total

Figure 16) Queue, Protocol, and Required Actions areas.

protocol time will be displayed.

- The protocol's steps are presented in a table with the following details for each step: Step number, Step name, Reagent, Incubation time and Parameters.
- The Required Actions need to be completed before the user can click "Start". The Required Actions that are displayed include the reagents and volumes required until the next user intervention. The user can Add, Fill and Create directly from the Required Actions panel without going to the Reagents tab. Required Actions are described in more detail in Section 10.3.

Once the user has completed all the Required Actions, the "Start" button will become available and will replace the Required Actions area (Figure 17). The protocol can be started after inserting the Staining chip and loading the slide.

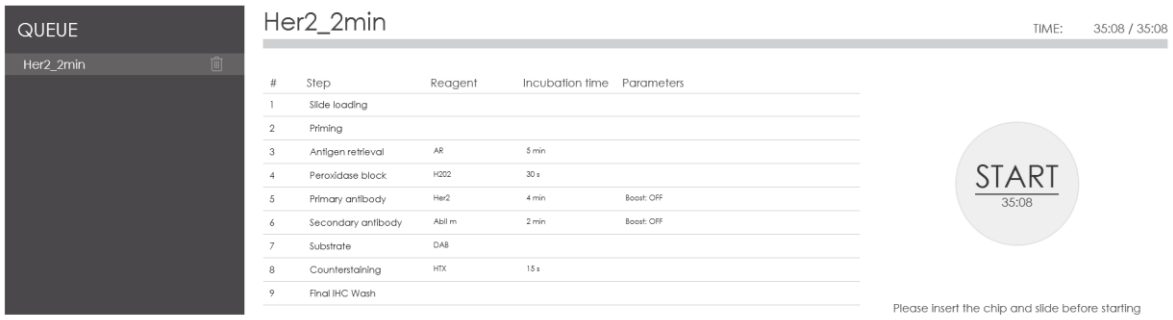


Figure 17) Queue and Protocol area. START button and duration are displayed once the user has completed all the required actions.

- Click “Start” to execute the protocol (Figure 17).
- Click the Abort button (square icon) to stop the protocol (Figure 18).
- Click the Reload button (circular arrow) to reload the protocol for another execution of the same protocol (Figure 18).

Figure 18) Abort and Reload buttons

8.4.5. Pause windows and pop-ups

Some applications require user intervention steps, called “Pause” steps. When a protocol reaches a “Pause” step, the user will need to follow the instructions on the screen and complete the Required Actions to resume the protocol. The instructions and Required Actions depend on the application and will include one or more actions (Figure 19):

- The “Open stainer” pop-up allows to open the stainer to retrieve the slide from LabSat® for imaging (Figure 19, A). The Staining chip used for the previous part of the protocol must be discarded after opening the stainer.
- The “LabSat status” window allows to retrieve tubes containing precious reagent from the machine and replace them with empty ones before washing of the reservoirs. This windows also displays the current reagent configuration of LabSat® (Figure 19, B).
- The “Wash” window allows to wash reservoirs containing reagents that are not needed anymore in the protocol to allocate new reagents required to continue with the next cycle of the protocol (Figure 19, C). Two counters on the right side of the window guide the user to choose the appropriate reservoirs to wash. The washing procedure can only start if enough reservoirs are selected to accommodate new reagents needed for the next cycle (the two counters should read 0).
- The “Reagent management” window allows to replace clean reservoirs with new reagents or to top-up the volume of reagents already loaded on the machine (Figure 19, D). The volumes of the reagents are editable in this window to allow the user to adapt them to match the real reagent configuration on LabSat®. Before clicking “Continue”, make sure that the reagent configuration on the software is matching what is physically loaded on the device.
- The “Resume protocol” pop-up reminds the user that the protocol will resume and that the slide should be inserted back on the stainer after imaging and unmounting, along with a new Staining chip, before closing the handles of the device and clicking “Continue” (Figure 19, E).

Warning: To prevent contact with hazardous reagents, do not open the stainer during “Pause” steps unless specifically asked to do so by the instructions on the screen (Figure 19, A).

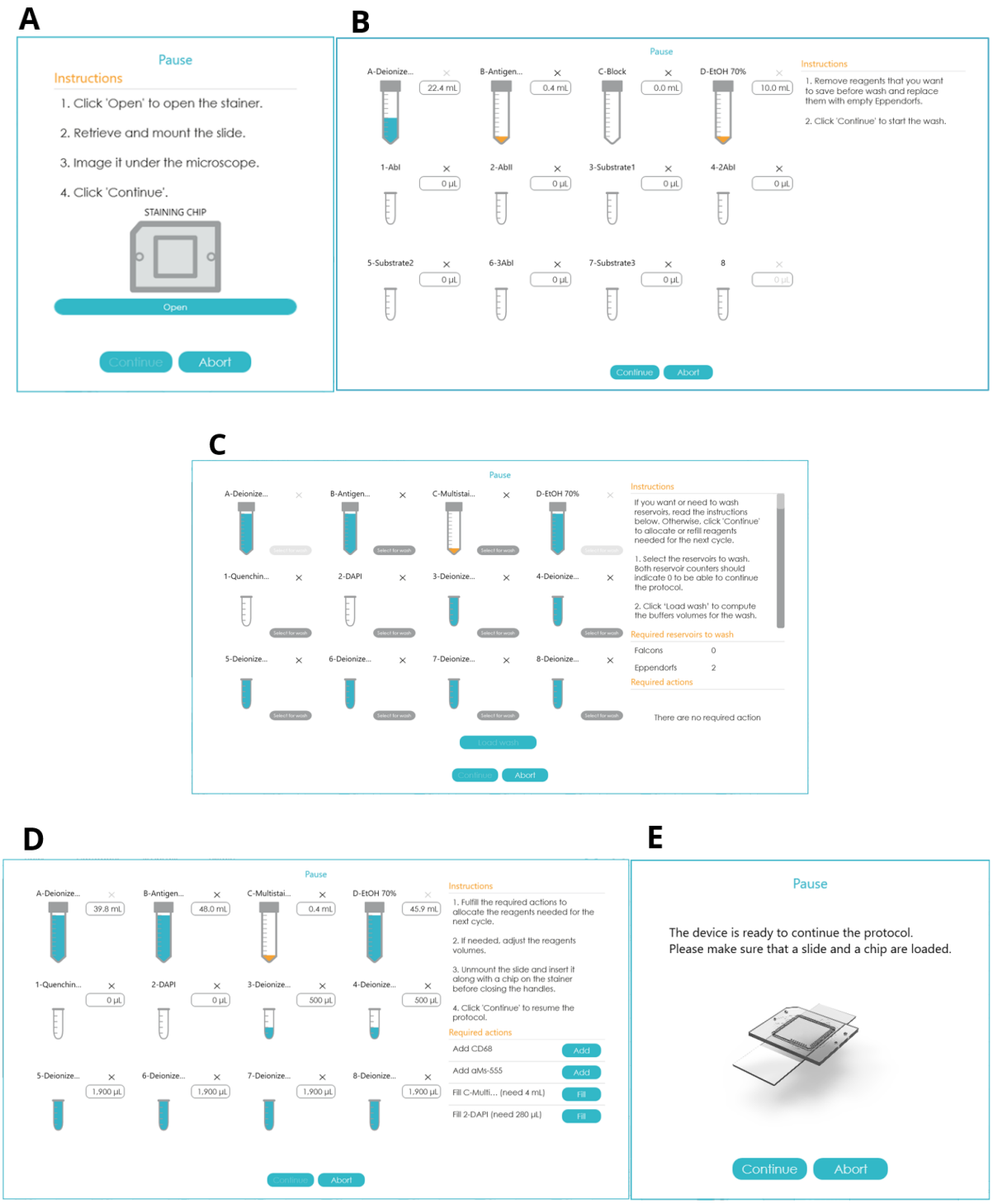


Figure 19) Pause windows and pop-ups. A) "Open stainer" pop-up. B) "LabSat status" window. C) "Wash" window. D) "Reagent management" window. E) "Resume protocol" pop-up.

8.4.6. Protocols tab

From the Protocols Tab the user has access to all the functions related to protocols (create, edit, add to queue, etc.) and can see the list of saved protocols (Figure 20).

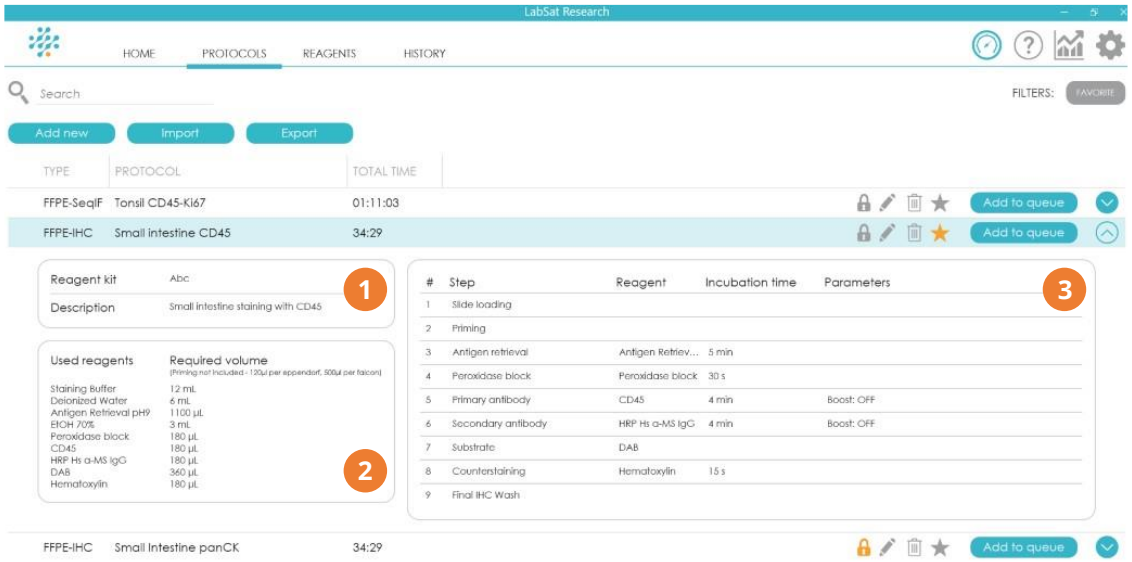


Figure 20) Protocols tab with full protocol details expanded: 1. Reagent kit used and protocol description. 2. List of required reagents and corresponding required volumes (excluding priming volume). Priming volumes are indicated above the list. 3. Protocol steps details: step number, step name, reagent used, incubation time, and parameters.

Protocol list

All protocols that have been saved or imported can be found in the protocol list. For each protocol, the following information is displayed (Figure 20):

- Protocol template type ("Type")
- Protocol name ("Protocol")
- Total execution time ("Total time")

Protocols can be protected, edited, deleted, and added to favourites by clicking on the corresponding buttons (Figure 21).

Note: "Protect protocol" and "Add to queue" buttons are only available for valid protocols (protocols that do not contain errors).

Figure 21) Protect protocol, Edit, Delete, Add to favourite buttons

Display protocol information

Click the arrow on the right of the protocol line (see Section 11 and Figure 20) to expand the line and display the full details of the protocol.

Create a new protocol

To create a new protocol, follow these steps:

1. Click "Add new" above the protocol list. The "Create a new protocol" window (Figure 22) will open.
2. Select the desired protocol template from the menu on the left.
3. Fill in the parameters that appear on the right to shape the default template. The parameters that will appear are application-dependent (there may also not be any parameters to fill in). See the corresponding application Annex for more information on the parameters.
4. Click "OK". The "New protocol" window will open (Figure 23).
5. Fill in the general parameters and information:
6. Protocol name (click on the default name and type)
7. Reagent kit (indicate the detection kit used, if applicable)
8. Description

9. Washing buffer (the selected buffer will be used for tissue washes and intra-protocol reservoir washes).
10. Fill in the details for the protocol steps. Depending on the step you may need to select certain reagents and/or definition certain parameters (incubation time, temperature, toggle options on/off, etc.).
11. Click “Save”. If there are red error dots a pop-up will inform you that the protocol will be saved as a draft. See below how to handle red, orange, and green dots.

Errors and warnings

Red, orange, and green dots indicate the status of the overall protocol or of individual protocol steps. They correspond to “error”, “warning”, and “ok” respectively. Protocol dots appear next to the protocol name and protocol step dots appear next to the step name. The cause of an error or a warning can be displayed by hovering the cursor over the coloured dot. Keep the following rules in mind, when editing a protocol:

- Protocol error: the protocol can be saved as a draft but cannot be loaded to the queue
- Protocol warning: the protocol can be saved and loaded to the queue
- Step error: leads to a protocol error
- Step warning: leads to a protocol warning

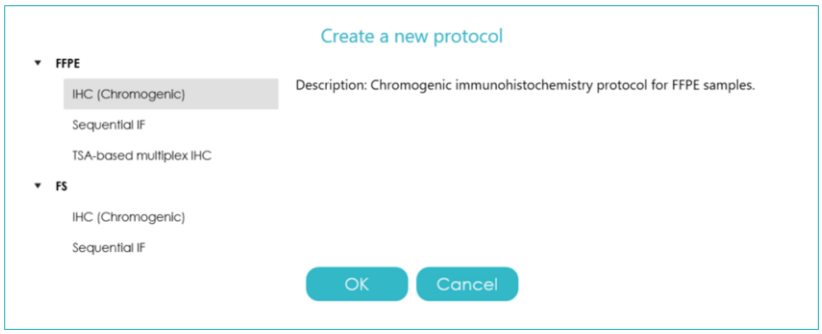


Figure 22) “Create a new protocol” window

Note: Protocols may generate errors when they are loaded to the queue if there is no reservoir configuration that can accommodate the protocol’s needs or if a reagent used in the protocol does not exist in the reagent database. In the case of the first error, adapt your protocol by cocktailing reagents (e.g., secondary antibodies and counterstain), if possible. In the case of the second error, the reagent can either be created in the Reagents tab or directly in the Required Actions by clicking “Create”.

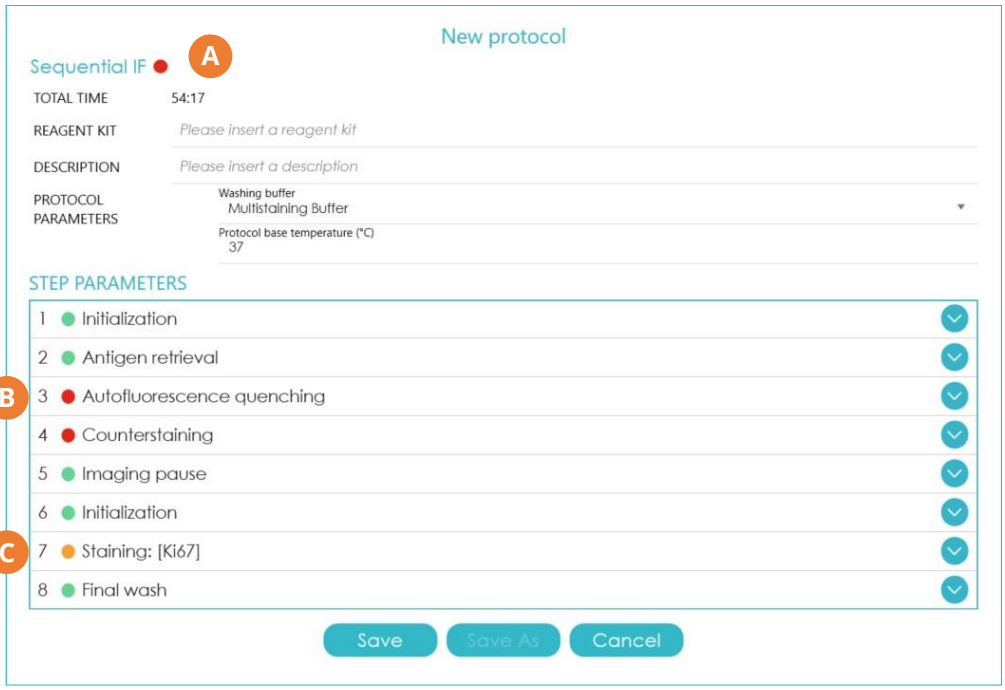


Figure 23) "New protocol" window showing a protocol error (A). Step error (B) and step warning (C).

Export

Click "Export" (Figure 20) to export all the protocol files locally into a folder on the computer. Use the checkboxes to select the protocols to export (multiple protocols can be exported at once), select the export directory, then, click "Export". A draft protocol can be exported and imported back as a draft. The properties of the reagents, buffers and cocktails used in the exported protocol will be embedded in the protocol file.

Import

Click "Import" (Figure 20) to import protocols from a folder.

If an imported protocol contains reagents, buffers, or cocktails that are not present in the database, those reagents will be imported. The same rules as those described in Section 8.4.7 apply to reagents imported through the protocol import function.

Reagents, buffers, and cocktails that are not imported during protocol import can either be created from the Reagents tab and then selected in the protocol by editing the protocol from the Protocols tab or created with the required actions (see Section 10.3). They will be indicated during protocol editing with a small attention symbol (Figure 24).

Figure 24) Imported protocol containing a reagent that was not imported (Ki67 on the figure), indicated with an orange warning symbol.

Imported protocols are never protected, even if the initial exported protocol was protected. The protocol can be protected again after import.

Edit

Click on the pencil icon (see Section 11 and Figure 21) to edit an existing protocol. Click "Save as" to create a new protocol out of the selected protocol with a new name, or "Save" to edit the selected protocol.

Delete

Click the bin icon (see Section 11) and confirm the protocol deletion by clicking "Yes" in the pop-up. If the protocol is protected the user will need to type "DELETE" (case-sensitive) in the pop-up and then click "OK".

Deleting a protocol that is loaded in the Queue will remove it from the Queue. Deleting a protocol that is being executed is not possible.

Favourite

Click on the star icon (see Section 11 and Figure 21) to add a protocol to the favourites.

Add to queue

Click “Add to queue” (Figure 20) to add the protocol to be run to the Queue of the Home tab.

Search

Insert a word or number in the Search tool (see Section 11 and Figure 20) to filter the protocols based on the reagents that are used in the protocol, the protocol name, the protocol estimated time, the protocol type, and reagent kit field content.

Filters

Click “Favourite” in the top right corner to only display the protocols that have been added to favourites.

8.4.7. Reagents tab

From the Protocols Tab the user has access to all the functions related to the reservoir configuration and the reagent and buffer database saved in the software (Figure 25).

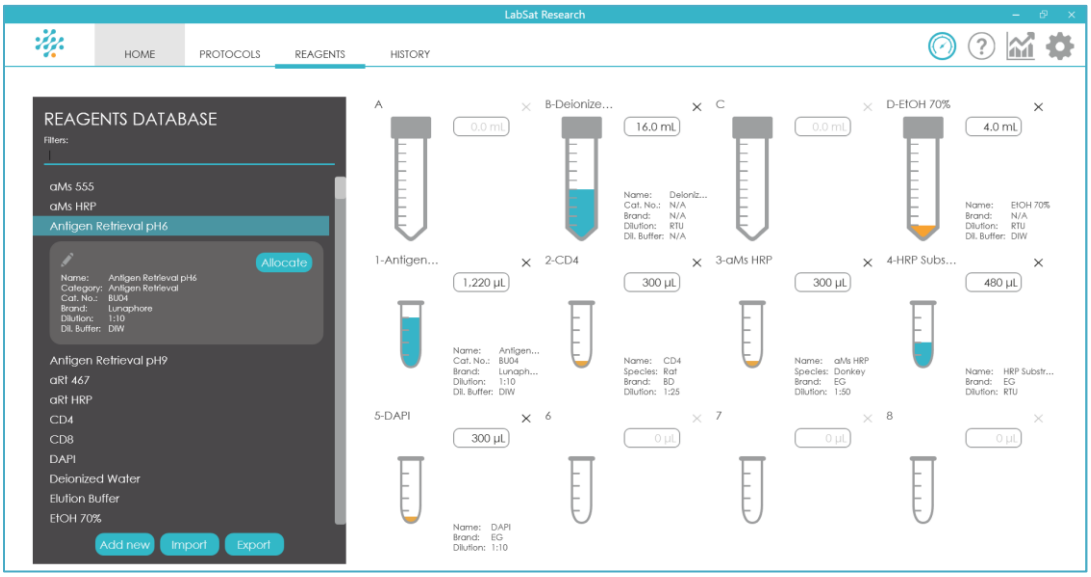


Figure 25) Reagents tab

Reagent and Buffer Database

All the reagents and buffers in the database are displayed in the black box on the left side of the Reagents tab. Scroll with the bar on the right to access all the entries. The Lunaphore buffers are in the database by default and cannot be deleted but can be edited to change the lot number and expiration date.

Add a new reagent, buffer, or mix

1. Click “Add new”
2. Fill in the mandatory fields (“Name” and “Category”). The available categories are Antigen Retrieval, Autofluorescence Quencher, Cleaning Buffer, Counterstaining, Custom, Custom Buffer, Elution Buffer, Peroxidase Block, Primary Antibody, Primary Antibody Mix, Protein Block, Secondary Antibody, Secondary Antibody and Counterstaining mix, Secondary Antibody Fluo-Labeled, Secondary Antibody Mix, Secondary Antibody, Substrate, and Washing Buffer.
3. Optional: fill in the optional fields (Catalogue number, Brand, Lot, Dilution, Dilution buffer, Species (for antibodies), Clone (for primary antibodies) and Expiration date).
4. For mixes: select the individual reagents from the database to combine using the drop-down menus.

Note: If the expiration date is input for a reagent / buffer then the software will display a warning when the reagent / buffer is used past expiration.

Expanded details view

Click on a reagent or buffer in the list to expand it and visualize its details (Figure 26).

Edit

Click on the pencil icon (see Section 11) to edit a reagent or buffer. The default buffers and reagents are Antigen Retrieval pH 6, Antigen Retrieval pH 9, Deionized Water, Elution Buffer, EtOH 70%, Multistaining Buffer, Quenching Buffer and Staining Buffer. They cannot be deleted and only the lot number and expiration date can be edited.

For all other reagents, once a reagent is created, it is not possible to edit its Name, Category, Brand and Dilution.

When a mix is being edited, if it contained a reagent that has been deleted, the software will display a warning symbol next to the deleted reagent.

Delete

Click on the waste bin icon (see Section 11) to delete the reagent or buffer from the database. Default buffers and reagents cannot be deleted.

Import

Click “Import” to import reagents saved locally (multiple reagents can be imported at once).

Reagents being imported that have the same name as reagents in the database will trigger warnings and actions according to the situations below:

- If there is a reagent/buffer in the database that has the same name, brand, dilution, and category as a reagent/buffer being imported then the user will be informed and asked to confirm whether to replace the reagent/buffer with the one being imported.
- If there is a reagent/buffer in the database that has the same name as a reagent/buffer being imported, then the reagent will not be imported, and a warning will be displayed.
- If there is a cocktail in the database that has the same name, category, and components (name, brand, dilution, and category) as a cocktail being imported, then the user will be informed and asked to confirm whether to replace the cocktail with the one being imported.
- All components of cocktails are imported according to the same rules as for other reagents / buffers.

Export

Click “Export” and use the checkboxes to select the reagents you wish to export (multiple reagents can be exported at once), select the export directory, then click “Export”.

Filter

Search the database for a reagent or buffers name or filter by category by typing the name or category in the “Filters” tool. To remove a filter, click on the **x** next to it.

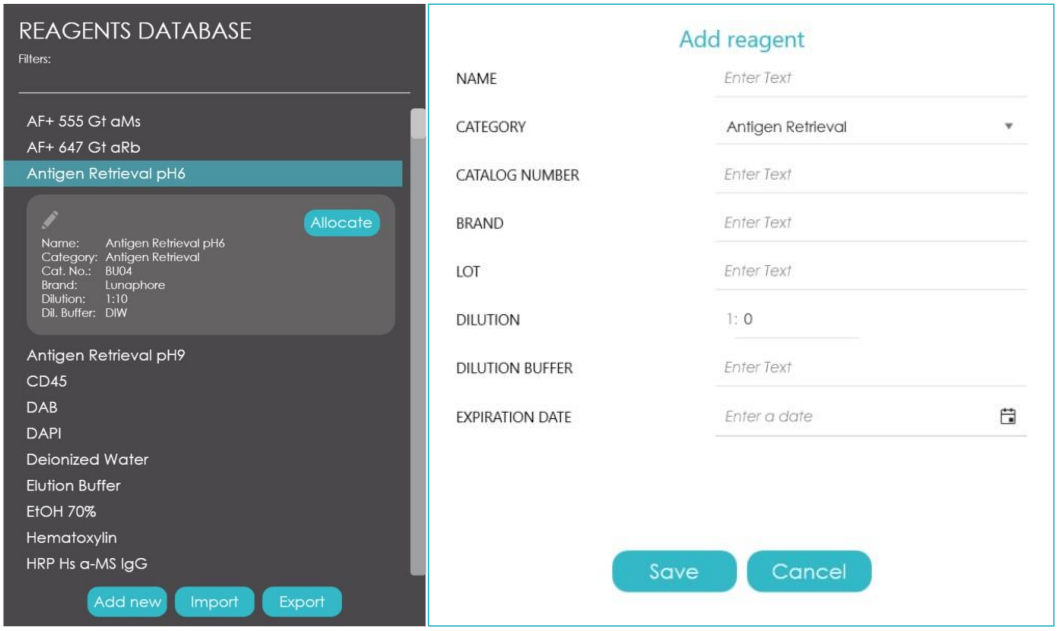


Figure 26) Expand reagent in the database to see details as well as "Add new", "Import", "Export" buttons (left), Add reagent (right)

Reservoir Management

There are four large reservoirs (50 mL conical tubes) labelled A, B, C, D, and eight small reservoirs (2 mL microtubes) numbered 1 to 8 (Figure 27).

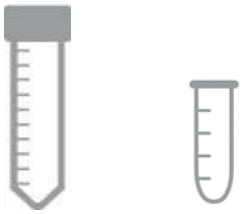


Figure 27) 50 mL conical tube (left), and 2 mL microtube (right)

The name of the reagent or buffer in each reservoir is indicated next to the label of the reservoir. Other details on the reservoir's content is written to the right of each reservoir (Figure 28).

Click in the grey box next to the reservoir to manually enter the volume of reagent or buffer present in the reservoir. Volumes are in millilitres (mL) for large reservoirs and in microliters (µL) for small reservoirs.

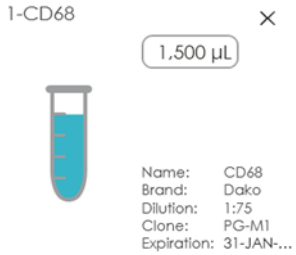


Figure 28) Allocated reservoir with details

Allocate a reagent / buffer to a reservoir

From the black box on the left, double-click on the reagent / buffer you wish to allocate or click on "Allocate" from the expanded details view and select the reservoir from the pop-up.

Remove a reagent / buffer from a reservoir

Click on the **X** to the right of the reservoir to remove its allocated reagent / buffer. The allocated reagent / buffer cannot be directly removed from a reservoir if:

- The allocated reagent / buffer is not a washing buffer
- The reservoir is primed

If the reservoir has been primed, the reservoir will need to be washed (either with Select & Wash or with a Daily Wash) before another reagent can be allocated to it.

Note:

- **Only washing buffers can be allocated to more than one reservoir.**
- **All used reservoirs must be washed at the end of the day, even if they contain washing buffer. Only previously washed reservoirs filled with DIW do not have to be washed.**

8.4.8. History tab

From the History Tab the user has access to the list of all executed protocols (Figure 29).

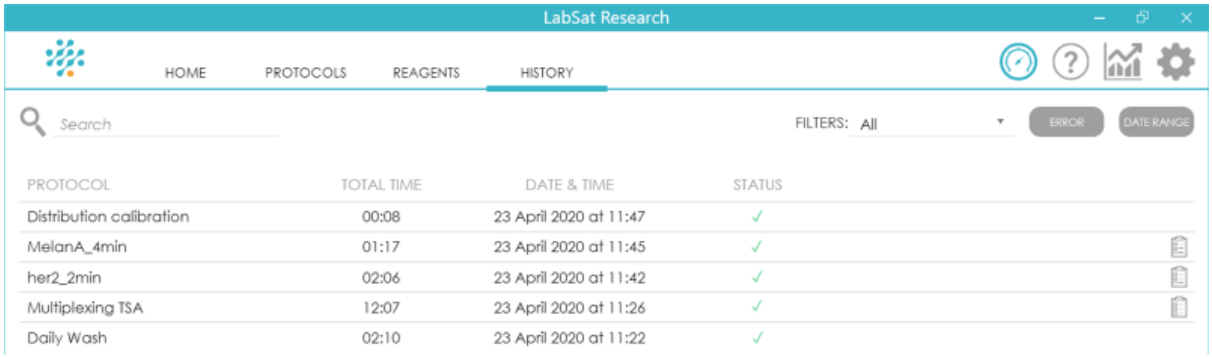


Figure 29) History tab

History list

Performed stainings, primings, calibrations and washes are listed in the history. Protocols are listed in inverse chronological order (most recent at the top). For each protocol the name, length, date and time of protocol execution and status are displayed. The history can be deleted from the Settings window (see Section 8.4.2).

Report

Reports are available for staining protocols. Click on the report icon (see Section 11) on the right of the protocol row to open the report in a PDF reader. The report can be saved to the computer.

Search

Search the history by typing the protocol name in the Search bar (see Section 11).

Filters

Protocols can be filtered by:

- Type (All, Staining, Wash, Calibration or Priming) – select from the drop-down list
- Error (protocols that had an error / warning during execution) – click “Error”
- Execution date range – click “Date range”, enter the desired date range and click “Save” (Figure 30)

To remove the error and date range filters, click the corresponding filter button again.

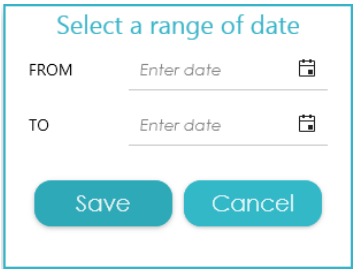


Figure 30) Filter reports with a date range

8.4.9. Statistics window

Click on the statistics icon  to open the "Statistics" window (Figure 31). All completed protocols are counted in the statistics (protocols that are aborted before the first primary antibody step are not included).

Available Charts

Different types of charts can be generated:

- Data: the total number of markers stained, in a specific time period (either week or month).
- Count vs Time: the number of markers stained over time is shown in a plot.
- Count per marker: a histogram shows the number of stainings performed for each marker.
- Count per marker vs Time: the number of stainings performed over time for each marker is shown in a plot.
- Count per Plex: a histogram shows the number of stainings performed for each plex (on the x axis, 1 refers to single plex, 2 to 2-plex …).

Plot settings

Plot parameters that can be adjusted and filtered:

- Period length (week or month) – select from the drop-down list
- Date range – click "Date range", enter the desired date range and click "Save" (Figure 30)
- Marker – type the name of the marker of interest in the "Enter Text" field
- Template – type the name of the template of interest in the "Enter Text" field

To remove the marker and template filters, click on the **x**. To remove the date range filter, click "Date range" again.

Export

To export the statistics in excel format click "Export".

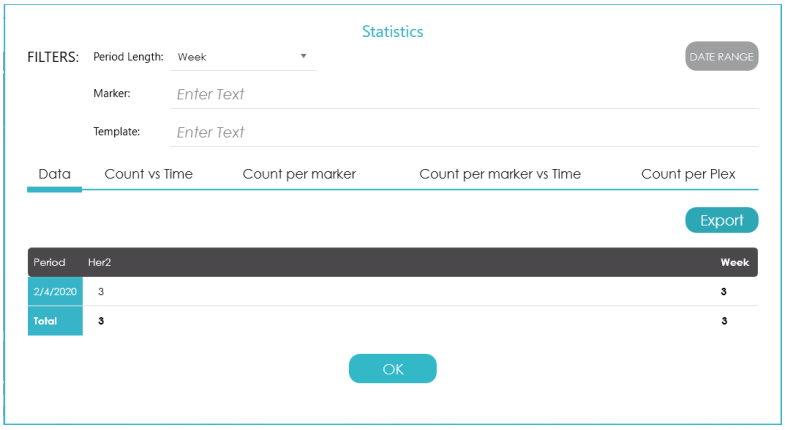


Figure 31) Statistics window

8.4.10. About window

Click on the About icon to open the "About" window (Figure 32). This window shows the software version used and the Lunaphore contact information. Click "Show details" to display the Product version, firmware version, serial number, database version and size, installation date and last service.

During remote support, this information may be asked for.

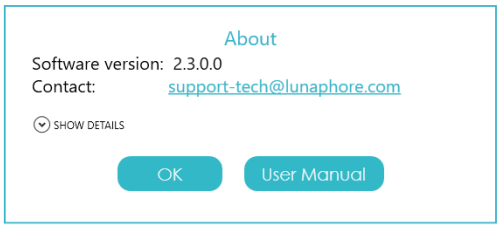


Figure 32) About window

8.5. Protocol creation and editing

Template protocols for various applications have been carefully designed step-by-step for optimal results using LabSat®. Below are instructions on how to create and edit protocols in general. However, the initial options, general parameters, and protocol steps available are application dependent. Refer to the corresponding application annex for the full details. Protocols can be edited and saved once they have been edited according to the user's needs.

To open a blank protocol template for editing, go to the Protocols tab and follow the steps below:

1. Click "Add new" above the protocol list. The "Create a new protocol" window (Figure 22) will open.
2. Select an application from the list on the left of the window. The following applications are available:
    - FFPE Chromogenic (Single-cycle Immunohistochemistry for FFPE tissues)
    - FS Chromogenic (Single-cycle Immunohistochemistry for FS tissues)
    - FFPE Sequential IF (Sequential-cycles Immunofluorescence for FFPE tissues)
    - FS Sequential IF (Sequential-cycles Immunofluorescence for FS tissues)
    - Multiplexing TSA (Tyramide for Signal Amplification-based Multiplex Immunofluorescence)
3. If present, select your preferences for the options on the right side of the window.
4. Click "OK". The "New protocol" window will open (Figure 23).
5. Fill out the general parameters at the top of the protocol template.
    a. [Optional] Protocol name: click on the default name and type the new name
    b. [Optional] Reagent kit: indicate the detection kit used
    c. [Optional] Description: insert a short description of the protocol being created
    d. [Required] Washing buffer: the selected buffer will be used for tissue washes and for intra-protocol reservoir washes in the Multiplexing TSA application.
    e. Additional parameters may be available depending on the application selected. See corresponding application annex for details.
6. Expand each step with the down-arrow button and fill in the parameters and options available. The details on the parameters that can be selected, the rules that must be followed and the volumes of reagents that are dispensed for each application are explained in the corresponding annex. Warnings and errors will appear to guide the user.
7. Add above/below and delete steps using the corresponding buttons on the right of each step.
8. Steps can be moved around by drag-and-drop. However, if the order of the steps goes against the rules of the protocol template an error or warning will be displayed (see corresponding annex for details).
9. Click "Save" to the save the protocol. A new protocol can be created by changing the protocol name and clicking "Save as". When clicking "Save as" the original protocol will not be modified. Click "Cancel" to cancel the changes made and exit the protocol editing window.

The total time of the protocol is updated as the protocol template is edited, it is displayed below the protocol name.

Note:

- **Two protocols cannot have the same name.**
- **A protocol loaded in the queue or execution area of the Home tab can only be saved under a different name once edited (by clicking "Save as").**

8.6. Quality controls

At the end of each protocol execution LabSat Research software will generate a report. This report will indicate whether the protocol was executed as expected or whether certain steps failed to execute correctly (see Section 8.13).

The report generated and results obtained have no diagnostic value. Results must be interpreted and evaluated by scientific personnel with experience in the immunohistochemical procedures along with the necessary controls.

The correct execution of the protocol on LabSat® does not guarantee interpretable results. Users are responsible for setting up the appropriate quality control measures that apply with laboratory regulations. Lunaphore recommends regularly performing positive and negative controls on control tissue sections using the same reagents as used for the stainings.

Before using new reagents (primary antibody, detection kits, etc.), perform both biological and technical positive and negative tissue controls to ensure they are functional. Using control tissues with known responses to IHC and IF staining is essential to determine the new reagent's performance.

8.6.1. Positive Tissue controls

Aim

Positive tissue controls are important to assess the quality of new reagents or protocols used in combination with LabSat® and accessories, as well as for the titration of primary antibody dilution.

Tissue selection

Select a tissue with known positivity as the positive control tissue. Good positive control tissues are tissues with a known weak positive signal, as fresh as possible and with a preparation as similar to the preparation of the final sample to be tested as possible.

Experiment

Stain the positive control tissue with the primary antibody of interest using the same protocol that is used for experimental stainings.

Result interpretation

If the positive control fails (i.e., no specific signal is present on the tissue), all results obtained with the same reagents and tissue are considered invalid.

8.6.2. Negative Tissue Controls

Aim

Negative tissue controls are important to assess the specificity of the primary antibodies used to the target antigen.

Tissue selection

Select a known negative tissue (tissue that does not express the antigen recognized by the primary antibody of interest) as the negative control tissue. The same tissue can be used as the positive and the negative control tissue if the sample contains an internal negative control (e.g., the sample contains cell types know to not react with the antibody being tested).

Experiment

Perform the negative control after the positive control. Stain the negative control tissue using the same primary antibody and protocol as the positive tissue control.

Result interpretation

Evaluate the background signal level and the presence/ absence of specific staining in the negative tissue controls. If there is no staining in the negative control, it shows that there is no antibody reactivity to other (undesired) cellular components.

Nonspecific staining may be present in the negative tissue control. Non-specific stains can be identified by trained users because they present a diffuse appearance (as opposed to the specific stain signal), and might be present in, for example, necrotic or degenerated cells, fatty tissue or connective tissue.

Negative Reagent Control

The specificity of secondary antibodies can be tested by substituting the primary antibody with a negative reagent (e.g., primary antibody diluent only or a primary antibody that does not target any antigen expressed by the tissue) to evaluate non-specific staining.

To run a negative control reagent using LabSat®, you must:

- Add the negative control reagent to the reagent database in the "Primary antibody" category (See section 8.4.7). In this way, you can use the same protocol parameters (incubation time) as for the positive control protocol. A possible negative control reagent could be primary antibody diluent with immunoglobulin G (IgG).
- Create a negative control protocol from the Protocols tab. Choose the same parameters as the positive control protocol but select the negative control reagent instead of the primary antibody. The negative control protocol can also be created by opening the positive control protocol for editing and replacing the primary antibody with negative control reagent. Change the protocol name and click "Save as".

Note: Other than using a negative control reagent instead of the primary antibody of interest, use the same protocol for the negative control as for the positive control

8.7. Reservoir Management

Prepare the reagents and buffer solutions in the order displayed by default in the Protocol Area. This will help to avoid mistakes.

The software will calculate how much volume is required for each solution once the protocol is loaded from the Queue. Make sure you check the required actions on the right-hand side of the Home tab and check if any reservoir is in orange (reaching critical low limit) (see Section 8.4.3).

DIW and Alcohol (Ethanol 70%) reservoirs can be left for up to one week loaded on LabSat® without needing to be changed or washed.

Never open the system when the locked reservoir icon is displayed (see Section 11).

8.7.1. Reagent / buffer preparation

Prepare the reagents/buffers to load on LabSat® in 2 mL microtubes for reagents/buffers that will be loaded in small reservoirs or in 50 mL conical tubes for reagents/buffers that will be loaded in small reservoirs. Always use new tubes for reagent/buffer preparation and wear protective gloves when handling reagents.

The volume of reagent/buffer to prepare is:

$$V_{priming} + (V_{dispensed} \times n)$$

Where:

- $V_{priming}$ is the priming volume: 120 µL for small reservoirs and 500 µL for large reservoirs
- $V_{dispensed}$ is dispense volume (see application annexes for dispense volumes)
- n is the number of protocols that will be executed or cycles (in the case of multiplex protocols) using this specific reagent.

The volume of reagent used in a protocol excluding the priming volume can also be found in the details that appear in the Protocols tab when expanding the protocol line with the down-arrow.

After loading the reservoir, immediately adapt the volume in the software. Go to the General box in the Home tab, or the Reagents tab, and indicate the volume that was entered in the reservoirs (see sections 8.4.3 or 8.4.7). The volume update in the software must be as precise as possible so that the software can always estimate the remaining volume and alert you in case a reagent is running low or missing.

8.7.2. Small reservoirs

Reagent/buffer Loading

1. Open the reservoir latches on LabSat® and tilt the bottom piece to remove it (Figure 33).
2. Place the microtube through the hole in the bottom piece with the cap open and position the cap in the notch.
3. Clip the reservoir in by closing the latch. Do not tilt the reservoir too much to prevent damage to the fluidic tube, to prevent spilling the reservoir's contents and to avoid exposure to the reagents.
4. Make sure the microtube is well inserted on the machine. If not, there will be an air leakage during protocol execution that will impair LabSat®'s performance (see Section 9.3).
   To prevent contamination of the tube, do not touch the tube with any other solutions or materials during the exchange procedure. To clean the tube in case of accidental contact, see Troubleshooting Section 9.8.
5. Keep the reagent loading time below five minutes to prevent the tube's contents from drying.

Note: To prevent contamination of the instrument, do not overfill the reservoirs. Respect the maximum limit of 1.9 mL for 2 mL microtubes.

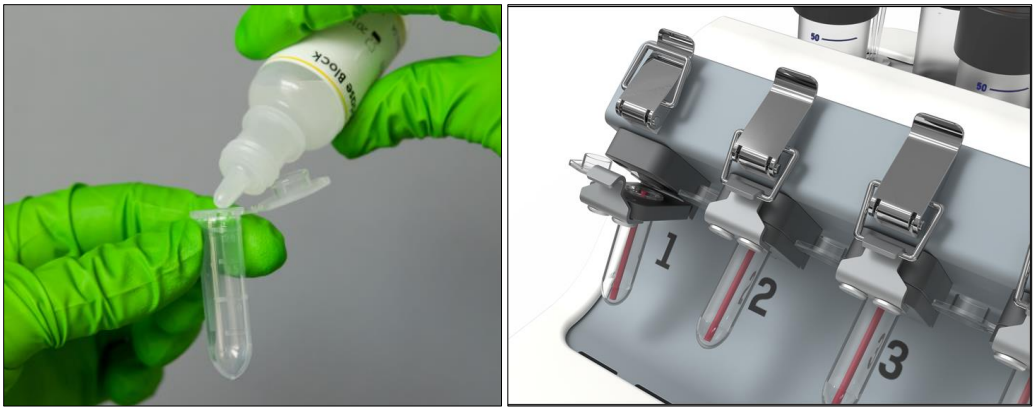


Figure 33) Loading reagent into microtube (left) and reservoir loading on LabSat® (right)

Refilling

Open the reservoir's latch and tilt the reservoir downwards (Figure 34) then use a dropper bottle or a pipette to directly refill the reservoir, without fully removing it.

Figure 34) Refilling reservoir loaded on LabSat®

Light-sensitive reagents

When using light-sensitive reagents, cover the tubes with foil. This will protect the reagents from light and preserve their fluorescent properties. Alternatively, use opaque microtubes to protect the reagents from light (see Annex 1).

8.7.3. Large reservoirs

Reagent/buffer loading and refilling

1. Keep the newly prepared conical tube close by and remove its lid.
2. Lift the conical tube currently loaded on LabSat® upwards and unscrew the tube from the cap. Twist the conical tube, do not twist the reservoir lid with the tubings.
3. Remove the lid, being careful not to touch the fluidic tube with any other parts.
4. Replace the old conical tube with the new one.
5. Insert the new conical tube in the slot, place the fluidic tube inside and screw the lid on tightly, without tilting the tube to prevent the contents from spilling and liquids from entering the pneumatic tube (Figure 35). If the lid is not screwed on properly, there will be an air leakage during protocol execution that will impair LabSat®'s performance (see Section 9.3).
6. Update the software according to the volume loaded.

To prevent contamination of the tube, do not touch the tube with any other solutions or materials during the exchange procedure. To clean the tube in case of accidental contact, see Troubleshooting Section 9.8.

Keep the reagent loading time below five minutes to prevent the tube's contents from drying.

Warning: Do not pinch, twist, or pull strongly on the fluidic tubes; these actions could result in damage to the fluidic tubes.

Note: To prevent contamination of the instrument, do not overfill the reservoirs. Respect the maximum limit of 50 mL for conical tubes.

Change the buffer solutions in the instrument if bubbles form in the reservoirs.

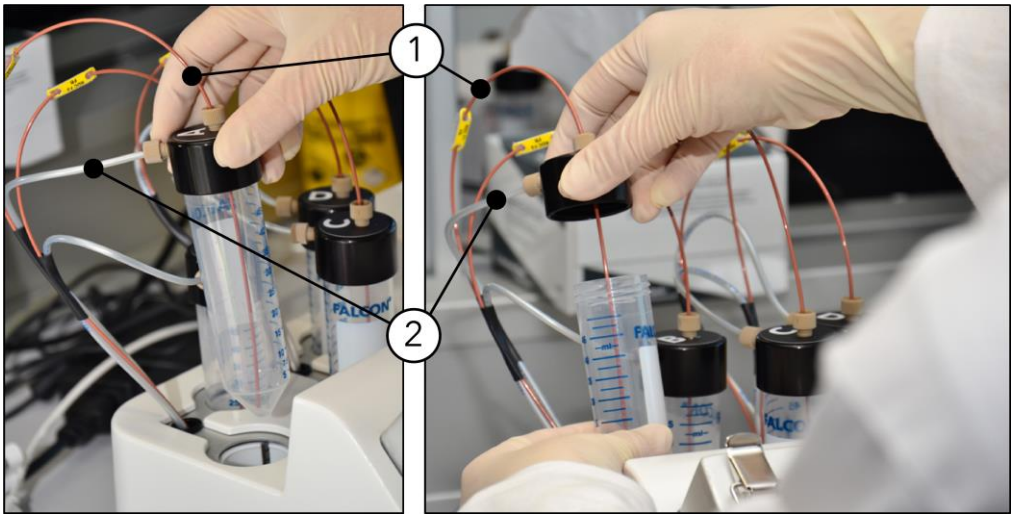


Figure 35) Conical tube loading for large reservoirs. 1. Fluidic tube. 2. Pneumatic tube.

8.7.4. Waste reservoir

The maximal volume the waste bottle can contain is 250 mL. To empty the waste of LabSat®, follow these steps:

1. Remove the cap of the waste bottle (Figure 36).
2. Take the bottle out of its slot, without tilting it to avoid spilling the contents and to prevent liquid from entering the pneumatic tube
3. Empty the bottle in a chemical liquid waste according to your local regulations. **The waste contains products of IHC/IF protocols and cleaning solutions; it shall be dealt with appropriately**.
4. Put the empty bottle back in place and screw the cap on tightly again.
5. Click “Empty waste” in the General box of the Home tab to update the waste bottle’s volume in the software (see Section 8.4.3).

⚠ Always wear protective gloves when handling the waste.

Figure 36) Waste bottle with cap (in blue)

Before executing a protocol, the software assesses the total waste volume generated by this protocol, and it checks that the volume in the waste bottle will not exceed 250 mL during protocol execution. If the software detects that the waste will fill to more than 250 mL the user will be blocked from executing the protocol until the waste bottle is emptied. Always visually double-check the level of the waste before executing a protocol.

Never try to open the waste bottle during any ongoing protocol, and when the locked reservoir icon is displayed (see section 11).

Never exchange the waste bottle with a regular laboratory bottle,

The bottle provided is a special bottle that is compatible with the LabSat operating pressure. If your waste bottle is damaged, immediately contact Customer Support for replacement and do not use LabSat® until the bottle is replaced.

8.8. Queue: Protocol selection

Up to 30 protocols can be loaded in the Queue for fast access from the Home tab (see section 8.4.4).

- Load a protocol to the Protocol Area: double-click on it in the Queue
- Change the protocol loaded in the Protocol Area: select another protocol from the Queue. It will replace the loaded protocol in the Protocol Area but the changed protocol will stay in the Queue.
- The order in which the protocols are added to the Queue does not define the execution order.
- Once a protocol has been executed, even partially, it is removed from the Queue list.
- One protocol can be added multiple times to the Queue.
- Protocols can be removed from the Queue at any time by clicking the bin icon.

8.9. Distribution chip exchange

The Distribution chip must be changed when the countdown reaches zero and at least once a day. It is inserted in the Distribution System. To change the chip, make sure DIW is loaded in all the reservoirs (washing buffer and EtOH 70% can remain in reservoirs B and D, respectively, for this procedure), and then:

1. From the General box in the Home tab, click “Change” under the Distribution chip icon and then click “Start”.
2. Follow the instructions on the screen to turn the pressure off.
3. Manually open the Distribution System with the handles (Figure 37).
4. Remove the chip and wipe any remaining liquid away from the Distribution System with a clean paper towel.

Never pour liquid directly onto the Distribution System.

5. Take a new Distribution chip and place it in the system with the membrane of the chip facing down, and the cut corner of the chip placed in the bottom right corner of the chip holder.
6. Manually close the system with the handles and follow the instructions on the screen to turn the pressure back on.
7. The device will automatically start to calibrate the tubes with DIW.
8. Once the calibration is finished and successful, the device is ready to be used.

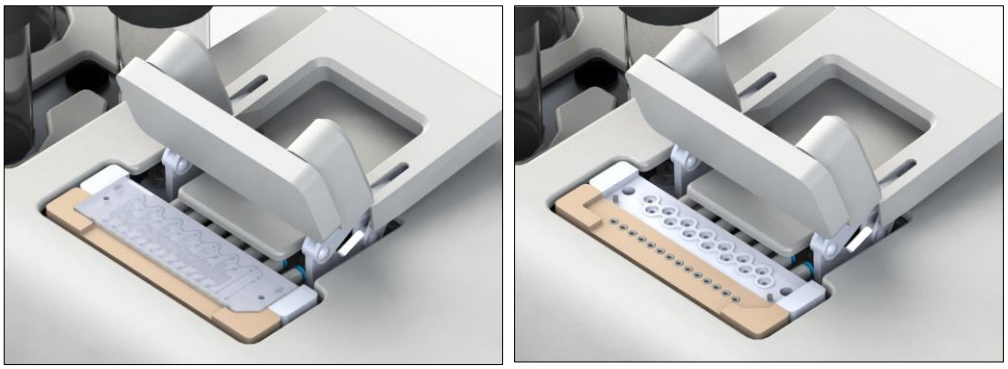


Figure 37) Distribution System opening with chip inside (left), open without chip (right)

If the black rings of the Distribution System (Figure 2) come off the device during the Distribution chip exchange procedure, refer to the Troubleshooting section (see section 9.8 step 02).

The Distribution chip should always be changed when starting the software, either after switching the instrument “On” or in the case of a power cut to the instrument or computer.

Always wear protective gloves when handling the Distribution chip, and never open the system without performing the Distribution chip exchange procedure. Do not force the opening and closing of the Distribution System handles, if they do not work properly, contact Customer Support.

8.10. Staining chip loading

When the protocol is ready to be executed and the start button is available in the Home tab, you can load a Staining chip (Figure 38). To do so, follow these steps:

1. Take a new Staining chip from the package.
2. If not already done, open the stainer from the software by clicking “Open” below the Staining chip icon.
3. Manually open the stainer using the handles with both hands.
4. Remove the previous Staining chip if one is present.
5. Insert the new Staining chip. The cut corner of the chip must be placed in the top left corner of the Stainer, with the seal of the chip facing down (Figure 38, right).
6. Manually close the stainer using the handles with both hands, until its clips shuts.

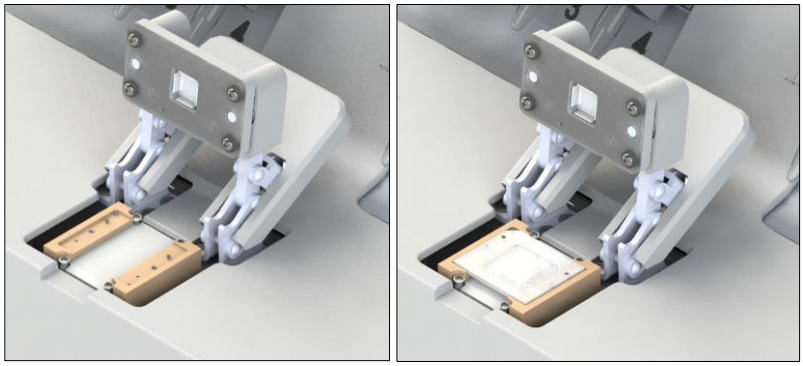


Figure 38) Stainer open without chip (left). Staining chip insertion in stainer (right).

Important notes:

- **Every time the stainer is opened, the chip must be changed.**
- **If the black rings of the stainer (Figure 2) are lost during Staining chip exchange procedure, refer to the Troubleshooting Section (see Section 9.8 step 01).**
- **Always use protective gloves when handling the Staining chip.**
- **Do not open the stainer until the end of the wash or protocol execution and always open the stainer on the software (by clicking “Open” under the stainer icon) before manually opening it.**
- **Do not force the opening and closing of the stainer, if it does not work properly, contact Customer Support.**

8.11. Slide loading and removal

Once a protocol has been loaded, all the corresponding Required actions have been performed and a new Staining chip loaded on the stainer, the slide can be inserted in the stainer (Figure 39):

1. Insert the sample slide from the front of the stainer between the two pins at the entrance, with the tissue facing upwards.
2. Carefully push the slide horizontally into the chamber and align the tissue with the reaction chamber by checking through the visibility hole.

3. Place the slide so that the top edge reaches at least the small visibility hole (Figure 2). If the slide's edge does not reach the visibility hole, then the seal between the slide and chip will not form and there may be leaking, and the slide may break. If the slide does not reach the two pins at the back of the stainer, visually check that the slide is centred in the stainer. A slide positioned diagonally in the stainer will lead to a leak.
4. If performing a protocol using fluorescent reagents, place the stainer cover on the stainer's see-through window (Figure 39).

Once the slide is inserted in the stainer, do not wait more than 10 seconds to start the protocol. This will prevent the tissue from drying out before the protocol starts.

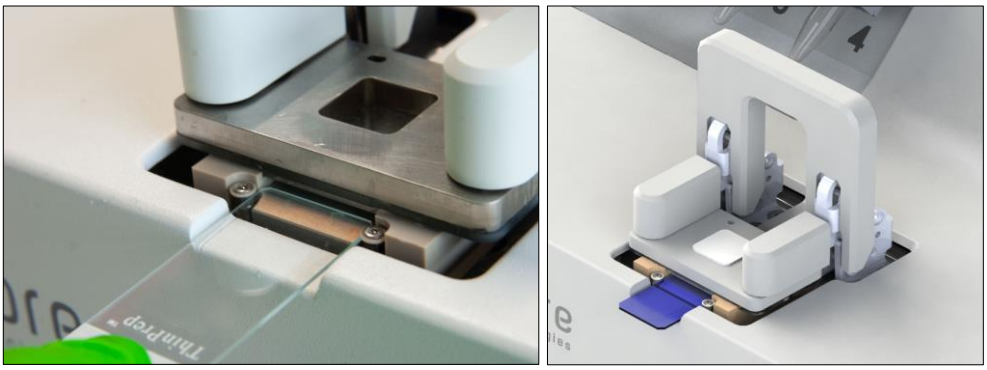


Figure 39) Slide insertion into the stainer (left). Stainer cover on stainer's see-through window (right).

To remove the slide from the stainer at the end of a protocol or during a "Pause" step, follow these steps:

1. Open the stainer from the software, either from the pop-up at the end of a protocol or at the beginning of a "Pause" step, or from the stainer button of the General box in the Home tab (see section 8.4.3).
2. Wait for the release of the pressure on the stainer and open the stainer's handles.
3. Remove and discard the Staining chip. If the seal is stuck on the slide, remove it carefully with tweezers.
4. Gently pull horizontally on the slide to take it out of the stainer.
5. Immediately coverslip the slide or store it in a washing buffer solution or DIW. Do not expose the tissue to air for more than 10 seconds to prevent the tissue from drying. Make sure to select the appropriate mounting medium type for the application. Some mounting media might impair the staining results (see section 12.2, table 23).

8.12. Protocol Execution and Abortion

Protocols are launched from the Protocol area on the Home tab.

1. Load the desired protocol into the Protocol Area (see section 8.8 for details).
2. The right side of the Protocols Area display the Required Actions that need to be performed before the protocol can be launched. The following actions may have to be performed
    a. "Add": allows the user to allocate the reagent/buffer to a reservoir
    b. "Fill": updates the volume in the reservoir, remember to load the displayed volume of reagent/buffer into the corresponding reservoir
    c. "Empty waste": sets the waste volume to zero, remember to dispose of the liquid in the waste bottle
    d. "Create": allows the user to add the missing reagent to the Reagent database
3. A sentence describing an error in the reagent allocation might also appear in the Required actions (Figure 40). Go to the Reagent tab to remove the reagent wrongly allocated. Go back to the Home tab and re-allocate it to the correct reservoir through the Required actions to be able to run the loaded protocol.

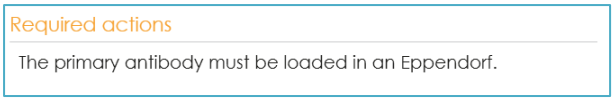


Figure 40) Example of a reagent allocation error in the Required actions.

4. Before clicking "Start":
    a. Visually confirm that reagent/buffer volumes are correctly loaded.
    b. The protocol summary table on the left side of the Protocol Area is correct.
    c. Load a Staining chip and the slide into the stainer.
5. Click "Start" to initiate the protocol.
6. The following things will happen after the user clicks start:
    a. The stainer will close and lock the sample inside, until the next "Pause" step or until the end of the protocol.
    b. All the reservoirs which have not already been primed will be primed.
    c. The steps of the protocol will be executed in order.
7. If "Pause" steps are scheduled, the protocol will pause and will not resume until the user intervention has taken place. Follow the instructions on the screen to resume the protocol.
8. At the end of the protocol execution, release the slide by clicking "Yes" on the pop-up (Figure 41), or by clicking "No" and then clicking "Open" below the Staining chip icon in the General box on the Home tab (see Section 8.4.3). Do not manually open the stainer or remove the slide until this step has been performed.
9. Follow the steps from section 8.11 to physically remove the slide from the stainer.

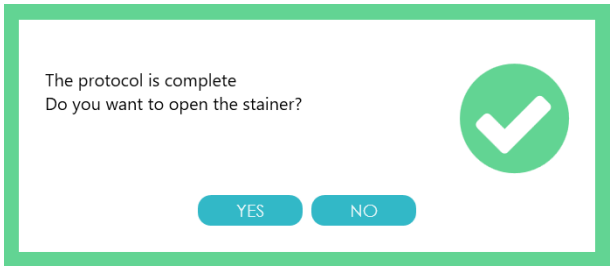


Figure 41) Stainer opening pop-up at the end of protocol execution.

Note: During execution the remaining time is displayed above the protocol area, as well as the total time of the protocol, and a progress bar with the percentage of completion of the full protocol. If a "Pause" step is upcoming then the time until the next user intervention will also be displayed.

Protocol abort

If the protocol needs to be interrupted before its scheduled end, follow these steps:

1. Click the Stop button to abort the protocol execution and confirm this action in the pop-up (see section 8.4.4 and Figure 42).

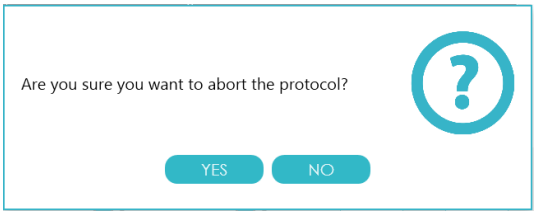


Figure 42) Abort confirmation pop-up

2. The following things will happen after the user clicks stop:
    a. The sample is automatically washed with washing buffer. This will prevent the user from coming into contact with harmful reagents when the stainer opens.

b. If the protocol was aborted during the “Antigen retrieval” step, the system will wait for the sample to have cooled down before the stainer can be opened and the slide can be accessed. During this time, the Reagent and Protocol tabs will remain available.

3. Retrieve the slide (see section 8.11) and make sure the reservoir volumes on the machine match the reagent configuration on the software after the abort.
4. The aborted protocol can be reloaded to be executed again (see Section 8.4.4). If re-using the sample, make sure to follow these steps:
   a. Remove the chip and slide.
   b. Dry the excess liquid.
   c. Load a new chip and the same slide.
   d. Restart the protocol.

Note: If a wash protocol is aborted, it must be executed again.

8.13. Report Generation

At the end of a protocol’s execution, a report is automatically generated. Go to the History tab and click on the report icon to open it in a PDF reader. The report can then be saved to your computer.

The report contains all the executed protocol steps, with their status (passed, error generated, warning generated or aborted). For failed steps the report will either display “Warning – failed” or “Error – failed” depending on the failure. The report will give you directions in case of warning and errors, for further troubleshooting steps.

It contains the protocol name, date, and time of execution and all the reagents that were loaded on the instrument and used.

Before evaluating the outcome of a protocol on LabSat®, always check the status of the protocol, and of each of its steps.

8.14. Washing and Cleaning Protocols

Three different automated wash protocols are available to wash reservoirs and tubing.

Go to the Wash box in the Home tab to customize and launch the wash protocols (see section 8.4.3).

LabSat® offers a walk-away solution for the end of the day (Figure 43). Before starting a wash protocol, you can activate the “Shut down system after wash” option. At the end of the wash, the software and computer will automatically turn off. If you are using an air compressor, it will need to be manually switched off at the end of the wash protocol.

At the end of the wash, a pop-up will indicate its status (successful execution or failed execution). This window appears either at the end of the wash protocol or at the next start-up of the system if the option “Shut down system after wash” was selected.

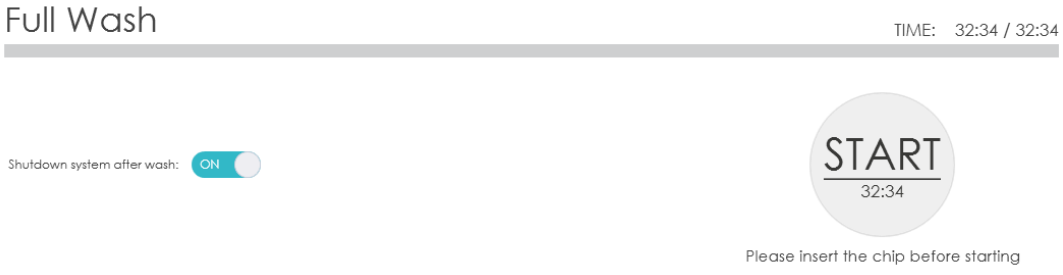


Figure 43) Walk-away solution, with shutdown after wash activated

8.14.1. Select and Wash

The Select and Wash protocol is used when:

- The reservoir content must be changed.
- There is a need to wash a specific reservoir based on the Troubleshooting section.

This washing procedure uses DIW and EtOH 70% (or BU02 – Alcohol), to wash away the remaining reagents or buffers in the selected reservoirs.

Follow these steps when performing the Select & Wash procedure:

1. If you want to keep the remaining reagent or buffer for future use, first remove the reservoir and replace it with an empty one.
2. Make sure there is DIW in reservoir A, and Ethanol 70% (or BU02 – Alcohol) in reservoir D.
3. Click “Select & Wash” in the Wash box (see Section 8.4.3).
4. Select the reservoirs to be washed.
5. Click “Load wash protocol” to load the wash protocol to the Protocol area.
6. Optional: toggle the “Shutdown system after wash” switch to ”On”.
7. Clamp the lid of the stainer shut and make sure that no chip and no slide are inserted (Figure 44).
8. Click “Start” to launch the wash.

At the end of the protocol, the washed reservoirs contain DIW and can be replaced with reservoirs loaded with new reagents/buffers.

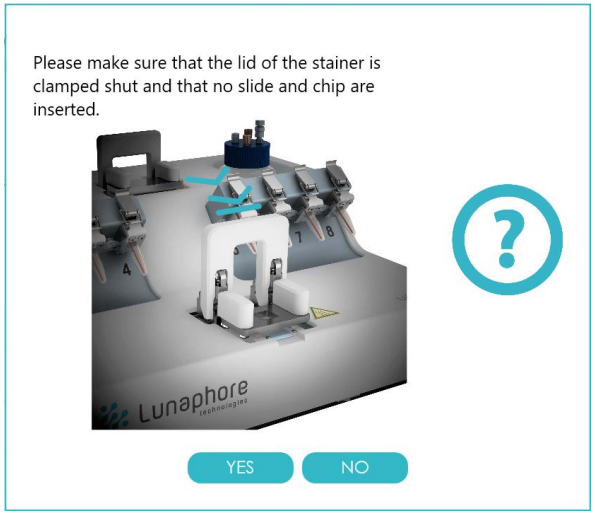


Figure 44) Pop-up reminding the user to clamp the lid of the stainer shut before Select and Wash protocols and Daily Wash protocols.

8.14.2. Daily Wash

The Daily Wash protocol is used:

- At the end of the day and when the Daily Wash countdown reaches 0.
- If the software indicates that a Daily Wash is required.

All the small reservoirs that have been used during the workday will be washed by the Daily Wash. If a Daily Wash is launched when no reservoir has been used the system will perform a calibration.

During the Daily Wash protocol, fluidic tubes of reservoirs B and C are always washed.

Follow these steps when performing the Daily Wash procedure:

1. If you want to keep the remaining solution for future use, remove the reservoir and replace it with a new one.
2. Make sure that there is DIW in reservoirs A B, and C, and Ethanol 70% (or BU02 – Alcohol) in reservoir D to run the protocol.
3. Click “Daily Wash” in the Wash box (see Section 8.4.3).
4. Click “Load wash protocol” to load the wash protocol to the Protocol area.
5. Optional: toggle the “Shutdown system after wash” switch to ”On” to shut down the computer once the Daily Wash is finished.
6. Clamp the lid of the stainer shut and make sure that no chip and no slide are inserted (Figure 44).
7. Click “Start” to launch the wash.

At the end of the protocol, the washed reservoirs contain DIW and can be replaced with reservoirs loaded with new reagents or buffers.

8.14.3. Full Wash

The Full Wash protocol is used:

- When the Full Wash countdown reaches 0.
- If the software indicates that a Full Wash is required.

The Full Wash uses Lunaphore's Fluidics Cleaning Kit (see Section 12.2) and DIW to wash all the reservoir tubes and tubing in the system. Reservoirs A and D must contain DIW, reservoir B must contain Fluidics Cleaning Kit solution 1 & 2 and reservoir C must contain Fluidics Cleaning Kit solution 3.

Follow these steps when performing the Full Wash procedure:

1. If you want to keep the remaining solution for future use, first remove the reservoir and replace it with an empty one.
2. Click "Full Wash" in the Wash box (8.4.3).
3. Click "Load wash protocol" to load the wash protocol to the Protocol Area.
4. Follow the instructions in the software and the product's datasheet for solution preparation. Load the following solutions into the reservoirs:
    a) Reservoir B: 1.8 mL Fluidics Cleaning Kit Solution 1 + 1.8 mL Fluidics Cleaning Kit Solution 2 + 14.4 mL DIW.
    b) Reservoir C: 18 mL Fluidics Cleaning Kit Solution 3.
    c) Reservoirs A and D: DIW
5. Empty the waste.
6. Load a dummy slide and chip before starting the protocol (Figure 45). This will allow the system to also wash the stainer and connected tubing.
7. Click "Start" to launch the wash.

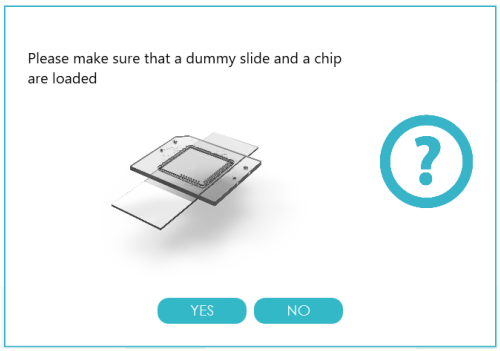


Figure 45) Pop-up reminding to load a dummy slide and chip

At the end of the wash protocol, the staining chamber will open automatically. The washed reservoirs contain DIW and can be replaced with reservoirs loaded with new reagents/buffers. Before loading new reagents/buffers, wipe the fluidic tube inside the large reservoirs with a paper towel with Ethanol 70% (or BU02 – Alcohol), and then with DIW (Figure 46).

Figure 46) Cleaning fluidic tube to change a buffer

8.15. Maintenance

The user is responsible for performing the wash protocols according to the instructions in section 8.14. In addition to the washing and cleaning protocols the other maintenance that must be performed is detailed in the following sections.

8.15.1. Daily maintenance

If liquid is spilled on the instrument's parts, wipe them, with a paper towel with EtOH 70% (or BU02 – Alcohol) or isopropanol 70%, and then with a paper towel dampened with DIW. Contact a Lunaphore representative or distributor when in doubt about the compatibility of cleaning agents with specific instrument parts.

Remove dirt and dust which may have accumulated on the instrument daily. Make sure the working area around the instrument is clean.

8.15.2. Weekly maintenance

At least once per week, clean the inside of the waste bottle cap, the large reservoir caps, and small reservoir holders with a paper towel with Ethanol 70% (or BU02 – Alcohol) followed by a paper towel with DIW (Figure 47).

8.15.3. Annual maintenance

An annual maintenance is required for LabSat® to continue operating reliably. The reliable operation of LabSat® may be compromised if this maintenance is not performed. The maintenance must be performed by a trained technician. It is the user's responsibility to contact Lunaphore or an official representative to organize the annual preventive maintenance.

There are no internal parts of the instrument which may be serviced by users. Only the spare rings provided with the instrument may be changed.

Figure 47) Cleaning inside the large reservoir caps (left), and microtube holders (right)

8.16. Shut-down procedure

Only shut-down LabSat® if there is no ongoing process, and only once a Daily Wash has been performed (see Section 8.14).

To shut down LabSat® follow the procedure below:

1. Shut-down the software on the computer. When the software has shut down, the LED on the instrument turns red indicating that LabSat® is in idle mode.
2. If the stainer is closed, it will open automatically once the pressure is released.
3. Remove the sample from the stainer if one is present.
4. Place a clean paper towel in the stainer to prevent fluid leaking into the stainer from the tubes.
5. Close the lid without clamping it (Figure 48).

Figure 48) End of day procedure, place paper towel in the stainer

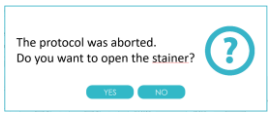
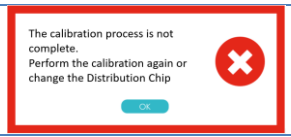
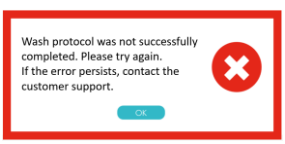
To disconnect LabSat® from any electrical inputs, switch the button at the back of the device to off (zero) (Figure 1).

When moving LabSat® or when LabSat® will not be used for a prolonged period of time, switch off the instrument (flip the switch on the back to zero) and unplug the electric cable as well as the pressure and USB cables.

9. Troubleshooting

For any issue encountered while operating LabSat®, check the troubleshooting guidelines below. Follow the solutions proposed below in the given order until the issue is resolved. If none of the solutions resolve the issue, contact Lunaphore's Customer Service (8.4.10).

9.1. Flow

#	What	Detail	Solutions
01	There is an error related to the flowrate	The flowrate for one or more of the steps is out of range (Error 202, or 302).	1. If not aborted automatically, abort the protocol, retrieve the slide, and store it in buffer if you wish to conserve it. Before executing the staining again, check that: • Staining chip and slide are not damaged. • All reservoirs are properly sealed (no air or liquid leak, see Troubleshooting Sections 2 and 3). • Pneumatic tubes of the large reservoirs are not pinched. • The tubes being used are of are on the list of consumables recommended by Lunaphore (see Annex 1: Reservoir compatibility). • The black rings of the stainer are inserted properly in their slots. 2. Wash all reservoirs with a Daily Wash protocol and change the Distribution chip. Check that the black rings of the Distribution system are properly inserted in their slots. Then execute the staining again. Check that the slide and the chip are correctly inserted in the Stainer. Then execute the staining again. 3. Execute a Full Wash protocol. Then execute the staining again. 4. Change the black rings of the stainer (inlet and outlet) by pushing them firmly into the hole. Then execute the staining again. Try several different rings if necessary. 5. Change the 13th black ring (second last from the right) of the Distribution system. Then execute the staining again.
02	The protocol automatically aborted	The protocol did not completed and there was no user intervention that interrupted the protocol (Error 500). 	
03	Calibration is not completed		1. Check that: • The device is connected to the software (LED is white) • The software's instructions were followed correctly before starting the calibration. • All reservoirs are well sealed. • The pneumatic tubes of the large reservoirs are not pinched. • The low-pressure icon is not displayed. 2. Change the Distribution chip. During this procedure, check that all the Distribution
04	The Daily Wash or Full Wash is not completed		

			System rings are in place. Restart the calibration once or twice if necessary. 3. Restart the LabSat® instrument and software. Restart the calibration. 4. If the system allows it, execute a Full Wash protocol.
05	There is a warning or error related to the dynamic incubation	Error 701 or 702.	1. Abort the protocol, retrieve the slide, and store it in buffer if you wish to conserve it. Then check that: • The reservoirs were filled with the correct required volumes. • No reservoir was open during the protocol. • The microtubes being used are compatible with LabSat®. • No reservoirs became empty during the protocol. • The reagents being used are in the viscosity range defined in Section 2.3.
06	Protocol aborts during dynamic incubation	The protocol aborts during a Dynamic incubation. Error 500.	

Table 7) Troubleshooting guidelines for flow-related issues

9.2. Liquid leak

#	What	Detail	Solutions
01	Visible liquid leak around the stainer during protocol	Fluid is leaking out of the stainer during the execution of a protocol (Note: visible liquid leak around the Stainer during chamber closing is normal).	1. Abort the protocol, retrieve the slide, and store it in buffer if you wish to conserve it. Then check that: 2. The slide was inserted far enough (at least until the visibility hole). 3. The chip and slide are not damaged and were correctly inserted in the stainer. 4. The stainer was properly closed. 5. The slide was properly sealed in the stainer (if sealed then the slide cannot be moved back and forth). 6. The low-pressure icon is not displayed. 7. Change Staining chip. During this procedure, make sure that the two rings of the stainer are present. Place the Staining chip correctly, with the seal facing down. Then execute the staining again. 8. If the Quenching Buffer has leaked, clean immediately with a wet paper towel to prevent rust from forming.
02	Liquid leak from reservoirs	There is liquid leaking out of the reservoirs during a protocol.	1. For large reservoirs, tighten the seal. Before carrying out the following solutions, abort the protocol, retrieve the slide, and store it in buffer if you wish to conserve it. Once the pressure icon has turned off you may proceed. 1. Do not fill more than the recommended maximum volumes. 2. Use the reservoir brands recommended by Lunaphore.

03	Leak from the stainer when not in use	When there is no Staining chip and slide loaded, there may be liquid leaking out of the stainer inlet.	Always insert a paper towel in the stainer when it is not in use, to prevent liquid from leaking (Figure 48).
04	Liquid leak from the Distribution System	During use, there is fluid leaking out of the Distribution System.	Abort the protocol, retrieve the slide and store it in buffer if you wish to conserve it. Proceed with the following solutions. 1. Perform a Daily Wash and then change the Distribution chip. Make sure all the black rings are present in the system, that the Distribution chip is correctly inserted, and that the Distribution System is properly closed. 2. If the low-pressure icon was present during the issue and is not disappearing after the Distribution chip change, contact the Customer Support. 3. If the Quenching Buffer has leaked, clean immediately with a wet paper towel to prevent rust from forming.
05	The reservoirs overflow	During the automated wash protocols, the reservoirs overflow (are filled too much).	1. Abort the protocol and immediately and contact Customer Support. 2. In the future, make sure that the correct microtubes were used and solutions being used are the correct ones and that they are correctly diluted (EtOH 70%, and Fluidics Cleaning Kit solutions 1 & 2).

Table 8) Troubleshooting guidelines for liquid leak-related issues

9.3. Air leak

#	What	Detail	Solutions
01	Air leak out of the Distribution System	There is an air leak noise coming out of the Distribution System after changing the chip.	Abort the protocol, retrieve the slide, and store it in buffer if you wish to conserve it. Proceed with the following solutions. 1. Perform a Daily Wash if necessary and then change the Distribution chip. Make sure all the black rings are present in the system, that the Distribution chip is correctly inserted, and that the Distribution System is properly closed. 2. If the low-pressure icon was present during the issue and is not disappearing after the Distribution chip change, contact the Customer Support.
02	Air leak from the small reservoirs	There is an air leak noise coming out of a small reservoir.	Abort the protocol, retrieve the slide, and store it in buffer if you wish to conserve it. Proceed with the following checks. 1. The small reservoir holder is not sealed properly: check that the microtube is inserted correctly and the clip is closed. If the issue persists, replace the microtube. 2. If all the small reservoirs are leaking, change the microtube brand to a brand recommended by Lunaphore.

03	Air leak from a large reservoir	There is an air leak noise coming out of a large reservoir.	1. Screw large reservoir cap on tightly. 2. Clean the reservoir cap with water and change conical tube. 3. Change conical tube brand to a brand recommended by Lunaphore.
04	Air leak from waste bottle	There is an air leak noise coming from the waste bottle cap or pneumatic tubes connected to it.	1. Screw the cap tightly onto the bottle. 2. Screw the tubes tightly into the cap.

Table 9) Troubleshooting guidelines for air leak-related issues

9.4. Bubbles

#	What	Detail	Solutions
01	Bubbles in the chamber from the start	There is a bubble trapped at the start of protocol in the chamber.	1. Abort the protocol, retrieve the slide, and store it in buffer if you wish to conserve it. • Make sure there is enough reagents and buffers to run the protocol and that there are no empty reservoirs. If needed, refill the reservoirs, and update the volumes in the software accordingly. 2. Reprime all the lines by using the priming menu. 3. Change the Staining chip and start the protocol over again. 4. If the issue is not solved, change the inlet black ring of the stainer by pushing it firmly into the hole. Try several different rings if necessary. 5. If the issue is not solved, perform a daily wash, and change the Distribution chip.
02	Bubbles forming during protocol	New bubbles appear in the reaction chamber during the protocol.	1. Check that: • No reservoir is empty. • There is more than 10 mL in all buffer reservoirs. • If the two previous checks are positive, abort the protocol and change the buffers with fresh solutions. 2. Perform the priming and staining again, using a new Staining chip.

Table 10) Troubleshooting guidelines for bubbles-related issues

9.5. Temperature

#	What	Detail	Solutions
01	There is a warning related to temperature	The report indicates the temperature is out of range for a step (Error 101).	1. Wait for the software to inform you that the device has cooled down and is ready to be used again before repeating the staining. Lunaphore recommends performing at most 10 stainings a day with heating steps (antigen retrieval, elution). 2. Check that the room temperature is within the range of operation (Section 0).
02	There is a temperature error	The report indicates the step temperature has failed (Error 102).	If needed, wait for the software to inform you that the device has cooled down and is ready to be used again and then follow these steps: 1. Restart the system (LabSat®, software and computer). 2. Repeat the staining.

Table 11) Troubleshooting guidelines for temperature-related issues

9.6. Pressure

#	What	Detail	Solutions
01	There is a low-pressure error	The software indicates that the pressure in the system is low (Error 401 on the report). 	1. Check that: • All the reservoirs are properly sealed and connected. • The device is connected to the software (LED is white). • The device is connected to the pressure line and that the pressure entering the device is between 5-8 bars. 2. Restart the system (LabSat®, software and computer). When turning on LabSat®, if the red light continues to blink, contact Customer Support.

Table 12) Troubleshooting guidelines for pressure-related issues

9.7. Protocol optimization

Troubleshooting guidelines for protocol optimization-related issues are presented in Annexes 2.

9.8. Part replacement and cleaning

#	What	Detail	Solutions
01	Distribution system black ring lost	During Distribution chip exchange, a black ring is lost.	Follow these steps: 1. Take a new black ring from the stock provided by Lunaphore. 2. Using tweezers, place a new ring inside the empty slot. 3. Make sure the ring is fully inserted into its slot by pushing it down.
02	Stainer ring lost	During Staining chip exchange, a ring is lost.	Contact Customer Support.
03	Reservoir tube is contaminated by mishandling	During the exchange procedure for a small or large reservoir, the fluidic tube got contaminated by another solution or material.	Follow these steps: 1. Remove the microtube or conical tube, and do not use the remaining solution inside. 2. Wipe the tube as explained in section 8.15.2. 3. Perform a Select & Wash protocol of the corresponding reservoir. 4. Prepare a new solution and load it in a new reservoir.

04	Stainer or Distribution System contaminated	After: - Opening the stainer or Distribution System clamps while LabSat® was under pressure - A power cut - Switching off the software The system is no longer sealed and gets dirty.	Follow these steps: 1. If the automatic abort was not triggered, abort the protocol. 2. Remove the Staining chip and tissue sample if necessary. 3. Run a Daily Wash. 4. Wipe away the remaining liquid in and around the stainer/Distribution System and lid with a paper towel. 5. Take a clean paper towel and wet it with either Isopropanol or EtOH 70%. 6. Wipe carefully all the stainer and lid parts that were contaminated, then repeat with a paper towel with DIW. 7. Wait for the parts to dry before reusing. 8. Change the Distribution chip. 9. Restart the staining by using a new Staining chip.
05	Broken chip (Distribution chip or Staining chip)	Chip breaks during insertion and/or clamping.	Follow these steps: 1. Remove the broken chip. 2. Check if any debris is left and remove it. 3. Take a new chip and insert it. 4. Make sure to close the clamps properly.
06	Stainer contaminated by sample	Sample was placed upside down and contaminated the stainer.	Follow these steps: 1. If necessary, abort the protocol. 2. Remove the Staining chip and sample. 3. Take a clean paper towel and wet it with either Isopropanol or EtOH 70%. 4. Wipe carefully all the stainer and lid parts that were contaminated, then repeat with a paper towel with DIW. 5. Wait for the parts to dry before reusing. 6. Use a new Staining chip to repeat the staining.
07	Contamination due to reservoir spillage	Spilling reagent or buffer or waste creates contamination of the device parts	Follow these steps: 1. Take a clean paper towel and wet it with either Isopropanol or EtOH 70%. 2. Wipe carefully the contaminated parts, then repeat with a paper towel with DIW. 3. Wait for the system to dry before reusing.

08	Broken part	Pneumatic or fluidic tubes, reservoir latches, handles, screws, waste bottle, reservoir caps, stainer or distribution system lid are broken	Immediately contact Customer Support. Do not attempt to replace the parts.
09	Tissue is scratched	After removal of the sample, the tissue is scratched	Follow these steps: 1. Ensure that the steps of slide removal were executed in the proper order. 2. Repeat the staining with a new Staining chip. Ensure that the Staining chip is intact and that the seal is well-placed.
10	Stainer or Distribution System cannot be closed	The handles do not clamp properly	Change the chip or place it correctly. Contact Customer support if the problem persists.
11	Slide breaks	The slide breaks during or at the end of the protocol	Follow these steps: 1. If a protocol is running, abort it. 2. Remove the sample when the software allows it. 3. Open the stainer, remove the chip and remove all the glass residues with a paper towel. Be careful not to drop any glass residues inside the holes of the stainer. 4. Flush some DIW on the stainer and dry it with a paper towel. 5. Repeat the staining with another Staining chip. 6. Contact Customer support if another slide is broken.
12	Stainer does not clamp the slide	The slide is not clamped at the beginning of the protocol.	Follow these steps: 1. Check that the low-pressure icon is not displayed. 2. Make sure the software is connected to LabSat®, if not, change the COM port. 3. Restart the computer, then start the software.
13	Stainer does not open	The stainer does not go down completely when opened from the software.	Follow these steps: 1. Retrieve the sample from the stainer. 2. Push the piston down gently to be able to reinsert another slide. 3. If not possible to push it by hand, turn the software and LabSat® off and on again.

Table 13) Troubleshooting guidelines for part replacement or cleaning-related issues

9.9. Software

#	What	Detail	Solutions

01	Software does not start	The user clicks on the software icon, but it does not start.	1. Delete the Database folder (you can keep a copy in case you need to recover data later) and restart the software. 2. Restart the computer, then start the software.
02	Error message	An instance of this application is already running.	Restart the computer, then start software.
03	Error message during protocol loading	The software cannot load a protocol for execution.	Restart the software.
04	Error message during protocol export	The software cannot export the protocols.	Check the disk space and permissions on target location.
05	There is a device error	The device error message appeared.	Restart the device.
06	Error message during priming protocol loading	The software cannot load a priming protocol for execution.	Restart the software.
07	An error occurred while saving the reagent/protocol	Something went wrong while saving the reagent/protocol.	1. Check there is enough space on the disk. 2. Restart the software, computer and device.
08	An error occurred during reagent/protocol update.	Something went wrong while updating the reagent/protocol.	
09	The protocol could not be imported.	Protocol cannot be imported.	
10	The report could not be saved	Something went wrong while saving the report.	
11	Error while updating the software with an update package	There is an issue with updating the software.	
12	The reagent could not be imported	Something went wrong while trying to import a reagent.	
13	The reagent could not be opened	Something went wrong while trying to edit a reagent.	
14	The update package could not be opened	Something went wrong while trying to open an update package.	
15	Error message during reagent export	The software cannot export the reagent.	1. Check that there is enough space on the disk. 2. Retry to export the reagent. 3. Restart the computer and the software.
16	The protocol could not be opened	Protocol cannot be opened.	Make sure the selected protocol is not corrupted (see entry 9.9.18).
17	Templates incompatible with software version	Issue with trying to import an update package that is	Make sure that you are using the correct software version and that you

		incompatible with the software version.	are trying to update with the appropriate update package.
18	Protocol cannot be edited or protected	Protocol is corrupted.	Restart the computer. If it does not help, delete the corrupted protocol, and create a new one.
19	Software does not respond	The software crashes or is frozen.	Restart the software, computer, and device.
20	No connection with LabSat®	There is a loss of connection between LabSat® and the software .	1. Verify power, power button and LED of the device. 2. Verify USB connection or try another USB port. If LabSat® was working before and there is a connection loss afterwards, follow these steps: 1. Go to the Settings in the software. 2. If a COM port is already selected, click "Save". 3. If the device is still not connected, change the COM port, and save.
21	Error codes in the report	101, "Temperature out of range"	See entry 9.5.01.
		102, "Temperature error"	See entry 9.5.02.
		202, "Flowrate error"	See entry 9.1.01.
		302, "Incubation flow error"	See entry 9.1.01.
		401, "Low pressure error"	See entry 9.6.
		500, "Aborted"	The step was aborted. Discard the staining. See entry 9.1.02.
		601, "Duration out of range"	Contact Customer support.
		602, "Duration error"	
		701, "Dynamic incubation warning"	See entry 9.1.05.
		702, "Dynamic incubation error"	

Table 14) Troubleshooting guidelines for software-related issues

If the troubleshooting steps and protocol optimization do not resolve the issues, contact Customer Support.

10. Notifications, Warnings, and required actions

The important safety messages that appear in the LabSat Research software are available in multiple languages and are provided along with this user manual. For an additional copy of the translated safety messages, contact Customer Support.

10.1. Notifications

Notification	Description
A maintenance is required before starting any protocol.	Contact Customer support to perform the maintenance.
A protocol cannot be loaded while LabSat® is busy.	It is not possible to load protocols in the Protocol Area during an ongoing process. Wait until the end of the execution to load a new protocol.

An error occurred during protocol editing/reagent update.	An issue occurred while saving in the database. Check Troubleshooting Section 9.9.08.
An error occurred while (re)loading the (washing) protocol.	Something went wrong while loading a protocol. Check Troubleshooting Section 9.9.03.
An error occurred while adding the update to the database	Something went wrong while updating the software with an update package. Check Troubleshooting Section 9.9.11.
An error occurred while exporting.	The software is not able to export the protocols. Check Troubleshooting Section 9.9.04.
Data initialization failed. **Error code: E-00005**	Backup the LabsatSys.bin file (can be found in the software folder) and remove it from the folder. Start the software again.
Impossible to edit the protocol	The protocol cannot be edited. To save the changes, modify the protocol name and click "Save As".
It is impossible to unprotect a protocol. If the current protocol is outdated, please create a new protocol, and delete the current one.	This notification appears when clicking on the protect icon of a protected protocol.
LabSat® requires a Daily Wash/Full Wash before starting another protocol.	When trying to start a protocol when the Daily Wash countdown has gone to 0 and 2 additional protocols have already been run / When trying to start a protocol when the Full Wash countdown has gone to 0 and 2 additional protocols have already been run.
Priming protocol failed to load	Check Troubleshooting section 9.9.06.
Protocol export failed.	The protocol cannot be exported. Check Troubleshooting section 9.9.04.
Reagent export failed	The protocol cannot be exported. Check Troubleshooting section 9.9.15.
Reagents could not be imported	Check Troubleshooting section 9.9.12.
Report(s) could not be saved	There was an issue while saving the report. Check Troubleshooting Section 9.9.10.
Reservoirs initialization error. **Error code: E-00006**	Backup the Data directory (can be found in the software folder), delete its content, and start the software again.
Some templates are incompatible with this software version	Check Troubleshooting section 9.9.17.
The configuration file could not be loaded, please contact support to fix the issue. **Error code: E-00001**	In the software folder, replace the LabSatResearch.exe.Config file with a backup one.

The Distribution chip has to be changed before starting another protocol.	See section 8.4.3.
The protocol could not be imported.	Check Troubleshooting section 9.9.09.
The protocol could not be opened.	Check Troubleshooting section 9.9.16.
The protocol has errors. You can save it as a Draft, but it has to be finished before being executable.	The protocol contains errors. See Section 8.4.6.
The protocol is corrupted. Please create a new one.	Check Troubleshooting section 9.9.18.
The protocol template selected cannot be loaded. Please make sure having updated all the packages. If the error persists, contact the customer support.	See Section 8.4.2.
The update package could not be opened	An error occurred while trying to update the software with an update package. Check Troubleshooting section 9.9.14.
The wash calibration has failed. If this error persists, please refer to the troubleshooting section in the User Manual.	Check Troubleshooting section 9.1.05.
There is an issue with the flowrate. Please refer to the troubleshooting section in the User Manual.	The system detected an issue with the flowrate. Check Troubleshooting Section 9.1.
This change will take effect the next time you launch the software.	Restart the software to implement the change.
This LabSat® device is not compatible with this software version. **Error code: E-00008.**	Contact customer support to get another software version.
This LabSat® version cannot open this category of protocol	A software update is required to open the protocol category. Contact Customer support.

This reagent must be present if you want to execute a protocol that uses it directly or through a cocktail. **Cocktail reagents using this reagent:** **[list of cocktail names]** **Protocols using this reagent:** **[list of protocol names]** **Are you sure you want to delete [reagent name]?**	If you delete this reagent, you will have to create it again before being able to execute any protocol that is using it or that is using a mix containing this reagent.
This reagent must be removed from the reservoir before being deleted	Once a reagent is loaded in a reservoir, it cannot be deleted from the reagent database in the Reagents tab. To delete it, first remove the reagent from the reservoir where it is loaded.
Unable to connect to database. **Error code: E-00004**	1. Make sure that the Data directory exists in the software folder and that you can write in it. 2. Backup the Data directory, delete its content and start the software again.
Unable to create the database. **Error code: E-00003**	Make sure that the Data directory exists in the software folder and that you can write in it.
Unable to start the device. **Error codeE-00007**	1. Restart the device and retry. 2. Check if the COM port is correct in the LabSatResearch.exe.Config file.
Unable to start the recorder. **Error code: E-00002**	Check that the logs directory exists in the software folder and that you can write in it.
You cannot delete a reagent that is used in a loaded protocol	Once a reagent is used in a protocol that is loaded in the execution area in the Home tab, it cannot be deleted from the reagent database in the Reagents tab. To delete the reagent, first remove the protocol from the execution area.

Table 15) Software notifications and their description

10.2. Warnings

Warning	Description
A Daily Wash is necessary. **Do you still want to start the protocol without performing a Daily Wash?**	See Section 8.14.2.
A Daily Wash shall be done before changing the Distribution chip. **Click "OK" to continue or "Cancel" to stop the process.**	See Section 8.14.2.
A Daily Wash shall be done before closing the application. **Click "OK" to close the application or "Cancel" to keep it open.**	Always run a Daily Wash after using LabSat®. See Section 8.14.2.
A Full Wash is necessary. **Do you still want to start the protocol without performing a Full Wash?**	See Section 8.14.3.
A new name needs to be set.	The protocol name already exists. Change the name.

A protocol is running, are you sure you want to abort the protocol and close the application?	If you click "Close", the protocol will abort. It is recommended to first finish the protocol, and execute a Daily Wash, before closing the software.
A reagent with the same name already exists	Two reagents cannot have the same name. Choose a different name for the new reagent or edit the previous one.
A reagent with the same name already exists "[reagent name]". Please delete it before continuing.	It is not possible to create a reagent that has the same name as an existing one. To create the new reagent, you have to delete the existing reagent first.
At least one reagent might be expired, do you want to continue?	According to the reagent database, the reagent loaded in the reservoir is expired. It is recommended to not use expired reagents. Update the reagent's expiration date in the database when a new reagent is being used.
Enter a name	A name must be indicated when saving a reagent.
If you continue, all the reagents in the selected Eppendorf® tubes will be lost.	When executing wash protocols, reagents are flushed to the waste. If you want to store them for later use, remove the corresponding reservoirs and replace them with empty ones.
Invalid range	The selected range is not valid, for example the end date may be before the start date.
Please select at least one reservoir	To execute the Select&Wash protocol, at least one reservoir must be selected. Click on the reservoirs to select them.
Reagents with the same name already exist. To be able to import all the reagents in the file, please delete the following reagents: [list of reagent names]	Not all the reagents have been imported. To import the remaining reagents, delete the reagents listed in the message and re-import the file.
The Distribution chip shall be changed. Do you want to start the protocol without changing the Distribution chip?	Change the Distribution chip when the countdown reaches zero, and at least once a day. See Section 8.4.3.
The name/brand/dilution cannot contain a "§"	It is not possible to save a reagent if a "§" sign is used in the brand/dilution field. Do not use a "§" sign for brand/dilution.
The pressure will be turned back ON.	This warning appears during Distribution chip change procedure. After clicking "OK", the system's pressure is turned on. The Distribution System is now under pressure and should not be opened.
The pressure will be turned OFF.	This warning appears during Distribution chip change procedure. After clicking "OK", the pressure on the system is turned off. The reservoir and the distribution chip are not under pressure anymore. The Distribution System can be safely opened to change the Distribution chip.
The waste is full. Please empty the waste before changing the Distribution chip/the calibration	See Section 8.7.4.
This LabSat® has not been used for a while, please change the buffers	The buffers have been left on the machine for at least one week. Change them with a new solution to avoid any risk of contamination.
This reagent is already loaded in a reservoir	One reagent cannot be loaded in two different reservoirs. Choose another reagent to load or remove the reagent from the other reservoir.

You cannot load a reagent in this reservoir. In order to load the reagent, please carry out a wash protocol on the reservoir first.	The selected reservoir cannot be used to load another reagent. You must first wash the reservoir with a Select & Wash protocol before loading another reagent. Follow instructions in Section 8.14.1.
Warnings in the report	**Description**
Protocol aborted because the device was not ready.	See 9.9.05.
Protocol aborted due to a device error.	See 9.9.05.
Protocol aborted due to flowrate problem.	See 9.1.01.
Protocol aborted due to incubation flowrate problem.	See 9.1.01.
Protocol aborted due to lack of pressure.	If the symbol  is displayed, check that the pressure input is on. If there is no symbol displayed, contact Customer Support.
Protocol completed with failed step(s).	Check the report and identify the errors/warnings. Refer to the troubleshooting guide for the error/warning types.
Protocol completed with warning(s).	Check the report and identify the errors/warnings. Refer to the troubleshooting guide for the error/warning types.
Protocol incomplete.	See 9.9.05.

Table 16) Software and report warnings, and their description

10.3. Required actions

Required actions	Description
Create [Reagent/Buffer]	The reagent/buffer is missing from the database and needs to be created before the protocol can be started. The reagent/buffer can either be created by clicking “Create” or it can be created from the Reagents tab. The reagent/buffer must be similar to the original one, which means that the Name, Category, Brand and Dilution have to be identical to the reference reagent/buffer. These fields are filled automatically when using the “Create” button in the Required Actions list.
Add [Reagent/Buffer]	The reagent/buffer must be allocated to a reservoir before the protocol can be started. Click “Add” in the Required Actions area from Home tab. Then select a reservoir for reagent allocation. Reagent/buffer allocation can also be done through the Reagents tab (see Section 8.4.7).
Fill [Reagent/Buffer]	The reagent/buffer’s reservoir does not contain enough volume for the loaded protocol. Refill the reservoir and click fill. The volume will be automatically updated to the volume stated next to the “Fill” button. Alternatively, refill the reservoir and manually enter how much volume is in the reservoir after refill from the General box in the Home tab (see Sections 8.4.3 and 8.4.7).
Empty Waste	The waste reservoir is too full for the loaded protocol to start. Empty the waste bottle and put the reservoir back in place, click “Empty waste” to confirm that the waste was manually emptied. The volume will be reset to 0 mL.
Change Distribution chip	The Distribution chip countdown has reached 0 or the Distribution chip is not functioning properly. Click “Change” under the Distribution chip icon in the Home tab (General box) and follow the instructions to change the chip (see Section 8.4.3).

Table 17) Software Requires actions and their description

11. Icons

Icon	Name / Description
	About
	Daily Wash countdown
	Delete
	Protect protocol
	Edit protocol / reagent
	Delete protocol / reagent
	Add to favourites, unselected (grey), selected (orange)
	Distribution chip countdown
	Small reservoir
	Warning
	Expand protocol
	Add a step above / below
	Valid step / The protocol is valid
	Step / Protocol editing error
	Step / Protocol editing warning
	Large reservoir
	Full Wash countdown
	Hot stainer: Do not touch the stainer until this icon is displayed
	Open the report (pdf)
	Protocol completed successfully
	Protocol failed
	Pressurized reservoirs: Do not open reservoirs, nor open the stainer, Distribution System or waste bottle
	No connection to LabSat® detected
	No / low pressure detected
	Reload protocol
	Search

	Settings
	Staining chip
	Statistics
	Abort protocol
	Waste
	Maintenance mode

Table 18) Icons and their meaning

12. Additional information

12.1. Acronyms

Ab	Antibody
AR	Antigen retrieval
CK	Cytokeratin
D	Depth
DAB	Diaminobenzidine
DI	Dynamic incubation
DIW	Deionized water
DPX	Dibutyl phthalate Polystyrene Xylene
EtOH	Ethanol
FCC	Federal Communications Commission
FFPE	Formalin Fixed Paraffin Embedded
FS	Fixed Frozen section
H	Height
HRP	Horse-Radish Peroxidase
IF	Immunofluorescence
IHC	Immunohistochemistry
LED	Light-Emitting Diode
MUX	Multiplexing
NBF	Neutral Buffered Formalin
RT	Room temperature
RTU	Ready-to-Use
SAP	Systems, Applications and Products
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween
TCEP	Tris (2-carboxyethyl) phosphine
TSA	Tyramide for Signal Amplification
W	Width
WEEE	Waste Electrical and Electronic Equipment

Table 19) Acronyms and their signification

12.2. Required and recommended material to use with LabSat®

The tables below list the material that is must be used with LabSat® and the material that Lunaphore recommends using with LabSat®. Refer to the material's safety datasheets to get all the information on the material's hazard indications, hazardous components, and safety measures to adopt when using the material.

Solution Name	**Company**	**Product Code**	**Dilution**

Fluidics Cleaning Kit	Lunaphore	BU03	Preparation: Reservoir B: -10% Fluidics Cleaning Kit Sol. 1 + -10% Fluidics Cleaning Kit Sol. 2 + -80% DIW Reservoir C: Fluidics Cleaning Kit Sol. 3 (RTU)

Table 20) Buffers required for use with LabSat®

Solution Name	**Company**	**Product Code**	**Dilution**
Staining Buffer	Lunaphore	BU01	1:10 in DIW
Alcohol	Lunaphore	BU02	RTU
Antigen Retrieval Solution pH6[1]	Lunaphore	BU04	1:10 in DIW
Antigen Retrieval Solution pH9[1]	Lunaphore	BU05	1:10 in DIW
Multistaining Buffer	Lunaphore	BU06	1:20 in DIW
Elution Buffer Kit, small[2]	Lunaphore	BU07	Dilute 1 volume of Solution 2 (20X) in 19 volumes of Solution 1.
Quenching Buffer Kit, small[3]	Lunaphore	BU08	Dilute 1 volume of Solution 1 (10X) in 8 volumes of deionized or double distilled water. Mix well. Then, add 1 volume of Solution 2 (10X). Mix well.
Blocking Buffer Kit[4]	Lunaphore	BU10	Dilute 1 volume of Solution 1 (10X) in 8 volumes of deionized or double distilled water. Mix well. Then, add 1 volume of Solution 2 (10X). Mix well.

[1] Recommended for FFPE IHC application.
[2] Recommended for Sequential IF protocols, to be used only for the dedicated “Elution” step.
[3] Recommended for Sequential IF protocols, to be used only for the dedicated “Autofluorescence quenching” step.
[4] Recommended for Sequential IF protocols, to be used only for the dedicated “Blocking” step as the blocking solution.

Table 21) Solutions recommended to use in LabSat® protocols

Material	**Use**	**Specifications**
Orbital shaker	Washing samples in container with solution	Speed: 30-65 rpm depending on the tissue type

Hot plate	Thaw frozen sections	Temperature: 45 °C
Oven	Baking FFPE samples during pre-processing steps	Capable of 65 °C incubation temperature

Table 22) Material recommended for use in combination with LabSat®

Recommended solutions to be used for pre/post-processing or cleaning steps (to be used outside of LabSat®)

Solution Name	**Company**	**Product Code**	**Dilution**	**Comment**
DPX™	Sigma-Aldrich	101729518	RTU	Mount slide with DPX™ under the hood. Only for IHC stainings. Do not use for IF stainings.
ProLong® Diamond Antifade Mountant	Thermo Fisher Scientific	P36965	RTU	Use for permanent mounting of IF stainings.
SlowFade® Gold antifade	Thermo Fisher Scientific	S36936	RTU	Use for non-permanent mounting of IF stainings. Do not use with directly conjugated primary antibodies.
Histoclear™	National Diagnostics	HS-200	RTU	
Isopropanol	Acros Organic	389710025	70% (diluted in DIW)	
NBF™	Thermo Scientific	5701	RTU	Preparation under the hood
Ethanol	Fisher Chemical	E/0650DF/15	100%, 95%, 70%, 40% (dilutions in DIW) for tissue rehydration 100% and 95% for slide mounting	Preparation under the hood

Table 23) Recommended solutions not to be used on the LabSat®, but for steps outside of the instrument (pre/post-processing or cleaning steps)

Annex 1: Reservoir compatibility

List of brands of 2 mL microtubes and conical tubes that are compatible with LabSat®.

Only use tubes by the following brands with the following references. Contact Lunaphore Customer Support (support-tech@lunaphore.com) if you do not have access to these brands.

Type	Size	Brand	Ref/Model	Comment
Small reservoir	2 mL	Clearline®	Microtube with cap 2 ml, in PP [CL0269]	N/A
Small reservoir	2 mL	Sarstedt	SafeSeal reaction tube, 2 ml, PP, Biosphere® plus [72.695.200]	N/A
Small reservoir	2 mL	Eppendorf®	Eppendorf Safe-Lock Tubes, 2,0 mL, PCR clean, ambra [30120248]	Opaque tube
Small reservoir	2 mL	Eppendorf®	Protein LoBind® Tubes, 2.0 mL, PCR clean, colorless [30108132]	N/A
Large reservoir	50 mL	Corning®	Falcon® 50 mL High Clarity PP Centrifuge Tube, Conical Bottom [352070]	N/A
Large reservoir	50 mL	Fisherbrand™	Polypropylene Centrifuge Tubes [11829650]	[1]
Large reservoir	50 mL	Gosselin™	Conical Base Polypropylene Test Tubes [TC50-04]	[1]
Large reservoir	50 mL	Greiner Bio-One™	Tube, 50 ml, PP, Screw Cap [227261]	[1]
Large reservoir	50 mL	TPP®	Conical centrifuge tube with rim [91051]	[1]

[1] This reagent/buffer reservoir may not be compatible with LabSat® TSA-based multiplex IHC protocols.

Annex 2.1: FFPE IHC (Chromogenic) protocol template

This annex presents the information regarding the FFPE IHC (Chromogenic) protocol template of LabSat®.

Protocol creation and editing

To open a blank protocol template for editing, go to the Protocols tab and follow the steps below:

1. Click “Add new” above the protocol list. The “Create a new protocol” window will open.
2. Select “IHC (Chromogenic)” in the FFPE branch. The protocol description will be displayed on the right side of the window.
3. Click “OK”. The “New protocol” window will open (Figure 49).

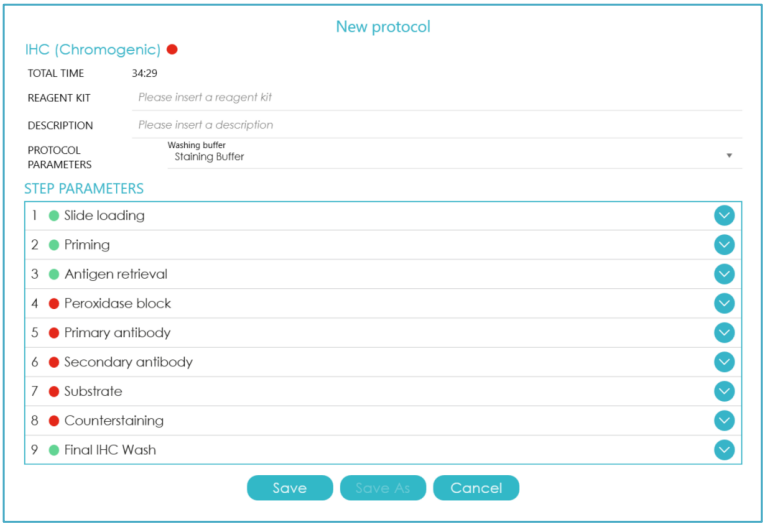


Figure 49) "New protocol" window for FFPE IHC (Chromogenic) protocols

The “New protocol” window is divided in two sections, the general protocol parameters section (under the protocol name in blue) and the protocol steps section (under “Step Parameters” title in blue).

Editing the general protocol parameters

Fill in the general protocol parameters as follows:

1. [Optional] Protocol name: click on the default name (“IHC (Chromogenic)”) and type the new name
2. [Optional] Reagent kit: indicate the detection kit used, if applicable
3. [Optional] Description: insert a short description of the protocol being created
4. [Required] Washing buffer: the selected buffer will be used for slide loading and tissue washes. The default reagent is “Staining Buffer”.

Editing the protocol steps

The steps displayed by default are “Slide loading”, “Priming”, “Antigen retrieval”, “Peroxidase block”, “Primary antibody”, “Secondary antibody”, “Substrate”, “Counterstaining” and “Final IHC Wash”. “Custom reagent”, “Protein block” and “Wash” steps can be added to the template by clicking . The steps present by default can also be added with these buttons. The details and parameters to fill and for each type of step are presented below.

Slide loading

The slide loading is performed when the protocol starts. The Staining chip and the slide loaded in the stainer are pushed together creating a closed chamber.

Rules:

- The “Slide loading” step is mandatory and must be the first step of a protocol.
- The protocol cannot contain more than one “Slide loading” step.

Priming

During this second step all the reservoirs with reagents that have not yet been used are primed.

Rule:

- The “Priming” step is mandatory and must be placed after the “Slide loading” step.

Antigen retrieval

Parameters:

1. Select an antigen retrieval reagent from the drop-down list.
2. Select the incubation time (5 min or 10 min).

For best results, Lunaphore recommends the use of the following solutions:

- Antigen Retrieval solution pH 6 (10X), Lunaphore, ref: BU04
- Antigen Retrieval solution pH 9 (10X), Lunaphore, ref: BU05

Do not touch the Stainer or slide during the “Antigen retrieval” steps. The system is heating to very high temperature and can be painful upon touch.

The hot surface symbol (to the left) is placed next to the Stainer to identify the area surrounding the heat source.

Rules:

- The “Antigen retrieval” step is not mandatory, but it is recommended for FFPE tissue.
- The “Antigen retrieval” step cannot be placed after the primary antibody.
- The protocol can only contain one “Antigen retrieval” step.
- An antigen retrieval reagent must be selected.

Dispense volume:

The dispense volume of antigen retrieval reagent is 1100 µL.

Peroxidase block

Parameters:

1. Select a peroxidase block solution from the drop-down list.
2. Select the incubation time (30 s or 2 min).

Rules:

- The “Peroxidase block” step is not mandatory, but it is recommended for FFPE tissue and IHC protocols.
- A peroxidase block reagent must be selected.

Dispense volume:

The dispense volume of peroxidase block is 180 µL.

Primary antibody

Parameters:

1. Select a primary antibody from the drop-down list.
2. Select the incubation time (1 min, 2 min, 4 min, or 8 min).
3. Toggle the Boost option “On” or “Off” (“Off” by default).

Rules:

- The “Primary antibody” step is not mandatory, but it is recommended for IHC protocols.
- A primary antibody reagent must be selected.
- The “Primary antibody” step should be placed before the “Secondary antibody” step.

Dispense volume:

The dispense volume of primary antibody is 180 µL (standard dispense), or 280 µL (Boost).

Secondary antibody

Parameters:

1. Select a secondary antibody from the drop-down list.
2. Select the incubation time (1 min, 2 min, 4 min, or 8 min).
3. Toggle the Boost option “On” or “Off” (“Off” by default).

Rules:

- The “Secondary antibody” step is not mandatory, but it is recommended for IHC protocols.
- A secondary antibody reagent must be selected.

Dispense volume:

The dispense volume of secondary antibody is 180 µL (standard dispense), or 280 µL (Boost).

Substrate

Parameters:

1. Select the substrate from the drop-down list.

Rules:

- The “Substrate” step is not mandatory, but it is recommended for IHC protocols.
- A substrate reagent must be selected.
- The “Substrate” step should be after the “Primary antibody” and “Secondary antibody” steps.

Dispense volume:

The dispense volume of substrate is 360 µL.

Counterstaining

Parameters:

1. Select the counterstain from the drop-down list.
2. Select the incubation time (15 s, 30 s, 1 min, or 2 min)

Rules:

- The “Counterstaining” step is not mandatory, but it is recommended for IHC protocols and imaging.
- A counterstaining reagent must be selected.

Dispense volume:

The dispense volume of counterstain is 180 µL.

Final IHC Wash

The final wash of the tissue uses the Washing Buffer and Cleaning Buffer (EtOH 70%).

Rules:

- The “Final IHC Wash” step is mandatory and should be placed at the end of the protocol.
- The protocol can contain only one “Final IHC Wash” step.

Custom reagent (not present by default)

Parameters:

1. Select a reagent from the drop-down list.
2. Select the incubation time: (15 s, 30 s, 1 min, 2 min, 4 min, or 8 min)

Rules:

- The “Custom” step can be placed anywhere in the protocol between the “Priming” step and the “Final IHC Wash” step.

Dispense volume:

The dispense volume of the reagent selected in the “Custom” step is 180 µL.

Protein block (not present by default)

Parameters:

1. Select a protein block solution from the drop-down list.
2. Select the incubation time (30 s or 2 min).

Rules:

- The “Protein block” step can be placed anywhere in the protocol between the “Priming” step and the “Final IHC Wash” step.

Dispense volume:

The dispense volume of Protein block is 180 µL.

Wash (not present by default)

The same washing buffer is used as for the rest of the protocol.

Rules:

- The “Wash” step can be placed anywhere in the protocol between the “Priming” step and the “Final IHC Wash” step.

Distribution chip change and wash countdowns

For FFPE IHC (Chromogenic) protocols, the countdowns decrement by one unit after the primary antibody dispense.

Troubleshooting: Protocol optimization

#	What	Detail	Solutions
01	No specific signal	There is no specific signal in the sample.	1. Check all the reagents were dispensed correctly by verifying status of each step in the report. 2. Check that the species reactivity of primary and secondary antibodies matches. 3. Check no reagent is expired. 4. Check the correct reagents were used and loaded in LabSat®. 5. Repeat staining with fresh reagents. 6. Test another antigen retrieval solution (change the pH). 7. Test a longer incubation time of the “Antigen retrieval” step. 8. Make sure the antigen retrieval process was successful (no bubbles in the chamber). 9. Increase concentration of primary and/or secondary antibodies. 10. Increase incubation time of primary and/or secondary antibodies. 11. Perform manual staining with the reagents to check their validity. 12. Perform the protocol on a known positive control tissue. 13. Change the reagents use a new batch or try different brands, species, etc).

02	Weak signal	The signal is present but weak	1. Check the staining steps were performed correctly by verifying status in the report. 2. Increase the incubation time of the primary antibody. 3. Increase the incubation time of the secondary antibody. 4. Turn ON the boost option one by one in the protocol. 5. Increase the concentration of primary and/or secondary antibodies. 6. Change the primary antibody clone. 7. Repeat staining with fresh reagents. 8. Perform the protocol on a known positive control to check the validity of the reagents. 9. Test another antigen retrieval solution (change the pH). 10. Increase incubation time of the “Antigen retrieval” step. 11. Add post-staining signal enhancers (example: copper sulphate). 12. Change antibody diluent with appropriate buffer. 13. Reduce incubation time of counterstain. 14. Check that the species reactivity of primary and secondary antibodies matches.
03	Non-uniform staining	The staining is not uniform within the staining area	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check all the reagents were dispensed correctly by verifying status of each step in the report. 3. Check that the used reagents are in the viscosity range defined by the user manual. 4. Repeat the staining with a new Staining chip and make sure: a. the staining process was successful (no bubbles in the chamber) b. there is enough volume of the reagents used c. there is DIW or Washing Buffer in all the unused reservoirs.

04	High general background	The signal to noise ratio is too low	1. Make sure the tissue does not dry during handling (before loading and after retrieving the slide from LabSat®). 2. Activate the blocking steps. 3. Repeat staining with fresh reagents. 4. Reduce the incubation time of the antibody steps. 5. Dilute the antibody solutions more. 6. Change the primary antibody clone. 7. Perform a Full Wash. 8. Add additional washing steps in between the protocol steps sequentially. 9. Change the reagents (use a new batch or try different brands, species, etc). 10. Change the antigen retrieval incubation time. 11. Change the antigen retrieval solution. 12. Use a solvent-based permanent mounting solution (example: DPX™).
05	One area is not stained	One area in the Stainer is never stained properly	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check the staining steps were performed correctly by verifying status in the report. 3. Repeat the staining with a new Staining chip and make sure: a. there is enough volume of the reagents used b. there is DIW or washing buffer in all the unused reservoirs 4. Do not use highly concentrated detergent solutions. 5. Check that the used reagents are in the viscosity range defined by the user manual. 6. Perform a Daily or Full wash and change the Distribution chip.
06	Signal is too strong	The specific signal is too strong	1. Dilute the primary and/or secondary antibodies more. 2. Reduce the incubation time of the primary and/or secondary antibody. 3. Repeat the staining using fresh reagents. 4. Change the reagents (use a new batch or try different brands, species, etc).
08	Weak counterstain	The counterstain is too weak	1. Increase the incubation time of the “Counterstaining” step. 2. Check that the “Counterstaining” step was performed correctly by verifying status in the report. 3. Change the counterstaining reagent (use a new batch or try a different brand).

09	Counterstain is too strong	The counterstain is too strong	1. Decrease the incubation time of the counterstain. 2. Dilute the counterstain solution. 3. Add an additional wash step after the "Counterstaining" step. 4. Change the counterstain (use a new batch or try a different brand). 5. Repeat the staining using fresh reagents.
10	Tissue morphology	The tissue morphology is altered	1. Decrease the incubation time of the "Antigen retrieval" step. 2. Test another antigen retrieval solution (change the pH). 3. Improve pre-processing steps of tissue fixation.
11	Tissue detaching	The tissue detaches after the process	1. Use positively charged slides for tissue fixation. 2. Use coated histological slides for tissue fixation. 3. Improve pre-processing steps of tissue fixation. 4. Decrease the incubation time of the "Antigen retrieval" step.
12	Overstaining	The signal is spreading out (the signal is not sharp) The signal is leaking out the structures (the signal is not sharp)	1. Reduce the incubation times of the antibody steps. 2. Dilute the primary antibody solution more. 3. Change the clone of the primary antibody. 4. Change the secondary antibody. 5. Change the substrate. 6. Use a solvent-based permanent mounting solution (example: DPX™).
13	Non-specific staining	There is non-specific staining in the sample	1. Add or increase the "Peroxidase block" step. 2. Change the clone of the primary antibody. 3. Dilute the primary antibody solution more. 4. Decrease incubation time of the secondary antibody. 5. Change the secondary antibody solution. 6. Change the substrate. 7. Add additional washing step after the staining steps in the protocol. 8. Perform a negative control of the same tissue to understand from which antibody the non-specific staining may come from. 9. Change the antigen retrieval incubation time. 10. Test another antigen retrieval solution (change the pH). 11. Use fresh reagents.

Table 24) Troubleshooting guidelines for FFPE IHC (Chromogenic) protocol-related issues

Annex 2.2: FS IHC (Chromogenic) protocol template

This annex presents the information regarding the FS IHC (Chromogenic) protocol template of LabSat®.

Protocol creation and editing

To open a blank protocol template for editing, go to the Protocols tab and follow the steps below:

1. Click “Add new” above the protocol list. The “Create a new protocol” window will open.
2. Select “IHC (Chromogenic)” in the FS branch. The protocol description will be displayed on the right side of the window.
3. Click “OK”. The “New protocol” window will open (Figure 50).

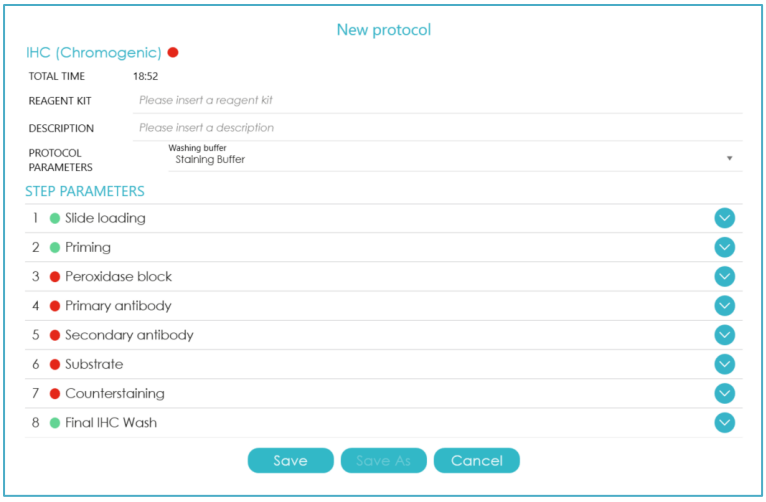


Figure 50) "New protocol" window for FS IHC (Chromogenic) protocols

The “New protocol” window is divided in two sections, the general protocol parameters section (under the protocol name in blue) and the protocol steps section (under “Step Parameters” title in blue).

Editing the general protocol parameters

Fill in the general protocol parameters as follows:

- [Optional] Protocol name: click on the default name (“IHC (Chromogenic)”) and type the new name
- [Optional] Reagent kit: indicate the detection kit used, if applicable
- [Optional] Description: insert a short description of the protocol being created
- [Required] Washing buffer: the selected buffer will be used for slide loading and tissue washes. The default reagent is “Staining Buffer”.

Editing the protocol steps

The steps displayed by default are “Slide loading”, “Priming”, “Peroxidase block”, “Primary antibody”, “Secondary antibody”, “Substrate”, “Counterstaining” and “Final IHC Wash”. “Custom reagent”, “Protein block” and “Wash steps” can be added to the template by clicking . The steps present by default can also be added with these buttons.

The details and parameters to fill and for each type of step are presented below.

Slide loading

The slide loading is performed when the protocol starts. The Staining chip and the slide loaded in the stainer are pushed together creating a closed chamber.

Rules:

- The “Slide loading” step is mandatory and must be the first step of a protocol.
- The protocol cannot contain more than one “Slide loading” step.

Priming

During this second step all the reservoirs with reagents that have not yet been used are primed.

Rule:

- The “Priming” step is mandatory and must be placed after the “Slide loading” step.

Peroxidase block

Parameters:

1. Select a peroxidase block solution from the drop-down list.
2. Select the incubation time (30 s or 2 min).

Rules:

- The “Peroxidase block” step is not mandatory, but it is recommended for FS tissue and IHC protocols.
- If used, a peroxidase block reagent must be selected.

Dispense volume:

The dispense volume of peroxidase block is 180 µL.

Primary antibody

Parameters:

1. Select a primary antibody from the drop-down list.
2. Select the incubation time (1 min, 2 min, 4 min, or 8 min).
3. Toggle the Boost option “On” or “Off” (“Off” by default)

Rules:

- The “Primary antibody” step is not mandatory, but it is recommended for IHC protocols.
- A primary antibody reagent must be selected.
- If used, the “Primary antibody” step should be placed before the “Secondary antibody” step.

Dispense volume:

The dispense volume of primary antibody is 180 µL (standard dispense), or 280 µL (Boost).

Secondary antibody

Parameters:

1. Select a secondary antibody from the drop-down list.
2. Select the incubation time (1 min, 2 min, 4 min, or 8 min)
1. Toggle the Boost option “On” or “Off” (“Off” by default)

Rules:

- The “Secondary antibody” step is not mandatory, but it is recommended for IHC protocols.
- A secondary antibody reagent must be selected.

Dispense volume:

The dispense volume of secondary antibody is 180 µL (standard dispense), or 280 µL (Boost).

Substrate

Parameters:

1. Select the substrate from the drop-down list.

Rules:

- The “Substrate” step is not mandatory, but it is recommended for IHC protocols.
- A substrate reagent must be selected.
- If used, the “Substrate” step should be after the “Primary antibody” and “Secondary antibody” steps.

Dispense volume:

The dispense volume of substrate is 360 µL.

Counterstaining

Parameters:

1. Select the counterstain from the drop-down list.
2. Select the incubation time (15 s, 30 s, 1 min, or 2 min)

Rules:

- The “Counterstaining” step is not mandatory, but it is recommended for IHC protocols and imaging.
- A counterstaining reagent must be selected.

Dispense volume:

The dispense volume of counterstain is 180 µL.

Final IHC Wash

The final wash of the tissue uses the Washing Buffer and Cleaning Buffer (EtOH 70%).

Rules:

- The “Final IHC Wash” step is mandatory and should be placed at the end of the protocol.
- The protocol can contain only one “Final IHC Wash” step.

Custom reagent (not present by default)

Parameters:

1. Select a reagent from the drop-down list.
2. Select the incubation time: (15 s, 30 s, 1 min, 2 min, 4 min, or 8 min).

Rules:

- The “Custom” step can be placed anywhere in the protocol between the “Priming” step and the “Final IHC Wash” step.

Dispense volume:

The dispense volume of the reagent selected in the “Custom” step is 180 µL.

Protein block (not present by default)

Parameters:

1. Select a protein block solution from the drop-down list.
2. Select the incubation time (30 s or 2 min).

Rules:

- The “Protein block” step can be placed anywhere in the protocol between the “Priming” step and the “Final IHC Wash” step.

Dispense volume:

The dispense volume of Protein block is 180 µL.

Wash (not present by default)

The same washing buffer is used as for the rest of the protocol.

Rules:

- The “Wash” step can be placed anywhere in the protocol between the “Priming” step and the “Final IHC Wash” step.

Distribution chip change and wash countdowns

For FS IHC (Chromogenic) protocols, the wash countdowns decrement by one unit after the primary antibody dispense.

Troubleshooting: Protocol optimization

#	What	Detail	Solutions
01	No specific signal	There is no specific signal in the sample.	1. Check all the reagents were dispensed correctly by verifying status of each step in the report. 2. Check that the species reactivity of primary and secondary antibodies matches. 3. Check no reagent is expired. 4. Check the correct reagents were used and loaded in LabSat®. 5. Repeat staining with fresh reagents. 6. Increase concentration of primary and/or secondary antibodies. 7. Increase incubation time of primary and/or secondary antibodies. 8. Perform manual staining with the reagents to check their validity. 9. Perform the protocol on a known positive control tissue. 10. Perform pre-processing steps shortly before loading the slide in LabSat®. 11. Decrease time of fixation. 12. Try alternative fixation method. 13. Change the reagents use a new batch or try different brands, species, etc).
02	Weak signal	The signal is present but weak	1. Check the staining steps were performed correctly by verifying status in the report. 2. Increase the incubation time of the primary antibody. 3. Increase the incubation time of the secondary antibody. 4. Turn ON the boost option one by one in the protocol. 5. Increase the concentration of primary and/or secondary antibodies. 6. Change the primary antibody clone. 7. Repeat staining with fresh reagents. 8. Perform the protocol on a known positive control to check the validity of the reagents. 9. Add post-staining signal enhancers (example: copper sulphate). 10. Change fixation method (try lower incubation in NBF 10%). 11. Decrease time of fixation. 12. Check that the species reactivity of primary and secondary antibodies matches.

03	Non-uniform staining	The staining is not uniform within the staining area	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check all the reagents were dispensed correctly by verifying status of each step in the report. 3. Check that the used reagents are in the viscosity range defined by the user manual. 4. Repeat the staining with a new Staining chip and make sure: a. the staining process was successful (no bubbles in the chamber) b. there is enough volume of the reagents used c. there is DIW or Washing Buffer in all the unused reservoirs.
04	High general background	The signal to noise ratio is too low	1. Make sure the tissue does not dry during handling (before loading and after retrieving the slide from LabSat®). 2. Activate the blocking steps. 3. Repeat staining with fresh reagents. 4. Reduce the incubation time of the antibody steps. 5. Dilute the antibody solutions more. 6. Change the primary antibody clone. 7. Perform a Full Wash. 8. Add additional washing steps in between the protocol steps sequentially. 9. Change the reagents (use a new batch or try different brands, species, etc). 10. Use a solvent-based permanent mounting solution (example: DPX™). 11. Make sure tissues are well dried (hot plate or air dry) before fixation. 12. Make sure slides do not come from a very old block.
05	One area is not stained	One area in the Stainer is never stained properly	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check the staining steps were performed correctly by verifying status in the report. 3. Repeat the staining with a new Staining chip and make sure: a. there is enough volume of the reagents used b. there is DIW or washing buffer in all the unused reservoirs 4. Do not use highly concentrated detergent solutions. 5. Check that the used reagents are in the viscosity range defined by the USER MANUAL. 6. Perform a Daily or Full wash and change the Distribution chip.

06	Signal is too strong	The specific signal is too strong	1. Dilute the primary and/or secondary antibodies more. 2. Reduce the incubation time of the primary and/or secondary antibody. 3. Repeat the staining using fresh reagents. 4. Change the reagents (use a new batch or try different brands, species, etc).
08	Weak counterstain	The counterstain is too weak	1. Increase the incubation time of the "Counterstaining" step. 2. Check the that the "Counterstaining" step was performed correctly by verifying status in the report. 3. Change the counterstaining reagent (use a new batch or try a different brand).
09	Counterstain is too strong	The counterstain is too strong	1. Decrease the incubation time of the counterstain. 2. Dilute the counterstain solution. 3. Add an additional wash step after the counterstaining step. 4. Change the counterstain (use a new batch or try a different brand). 5. Repeat the staining using fresh reagents.
10	Tissue morphology	The tissue morphology is altered	1. Improve pre-processing steps of tissue fixation.
11	Tissue detaching	The tissue detaches after the process	1. Use positively charged slides for tissue fixation. 2. Use coated histological slides for tissue fixation. 3. Improve pre-processing steps of tissue fixation.
12	Overstaining	The signal is spreading out (the signal is not sharp) The signal is leaking out the structures (the signal is not sharp)	1. Reduce the incubation times of the antibody steps. 2. Dilute the primary antibody solution more. 3. Change the clone of the primary antibody. 4. Change the secondary antibody. 5. Change the substrate. 6. Use a solvent-based permanent mounting solution (example: DPX™).
13	Non-specific staining	There is non-specific staining in the sample	1. Add or increase the "Peroxidase block" step. 2. Change the clone of the primary antibody. 3. Dilute the primary antibody solution more. 4. Decrease incubation time of the secondary antibody. 5. Change the secondary antibody solution. 6. Change the substrate. 7. Add additional washing step after the staining steps in the protocol steps. 8. Perform a negative control of the same tissue to understand from which antibody the non-specific staining may come from. 9. Use fresh reagents.

Table 25) Troubleshooting guidelines for FS IHC (Chromogenic) protocol-related issues

Annex 2.3: TSA-based multiplex IHC protocol template

This annex presents the information regarding the TSA-based multiplex IHC protocol template of LabSat®. Up to six markers plus counterstaining (seven colours) can be used within a single protocol to stain FFPE tissue samples. The template has been optimized using the Manual Opal™ 7-Color IHC Kit from Akoya Biosciences; however, this template provides the flexibility to be used with TSA reagent kits from other suppliers.

Protocol creation and editing

To open a blank protocol based on the template, go to the Protocols tab, and follow the steps below:

1. Click “Add new” above the protocol list. The “Create a new protocol” window will open.
2. Select “TSA-based multiplex IHC” in the FFPE branch. The protocol description will be displayed on the right side of the window.
3. Select the number of markers to be detected on the slide (from one to six, three by default).
4. Toggle the “Advanced template” option “On” (“Off” by default) to access a more flexible protocol template. The advanced template splits the “Marker detection” step into 4 individual steps: “Blocking”, “Primary antibody”, “Secondary antibody” and “Substrate” steps.
5. Click “OK”. The “New protocol” window will open (Figure 51) with the selected number of marker detection steps.

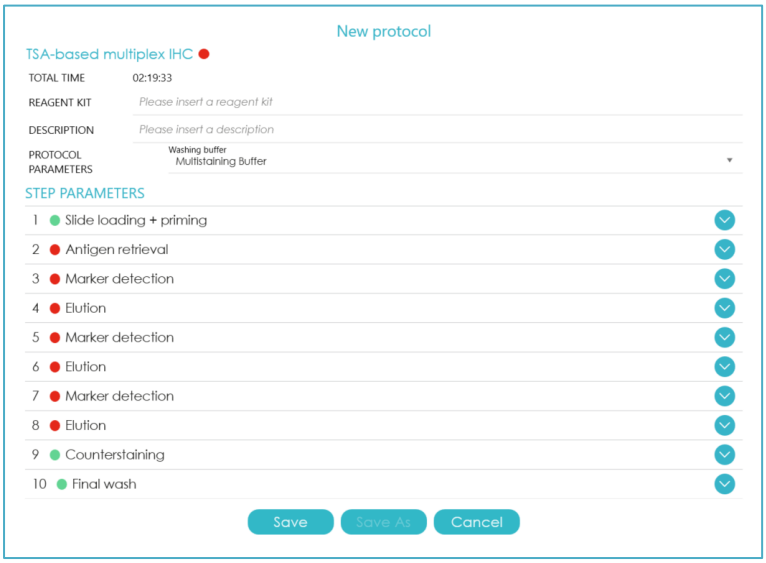


Figure 51) "New protocol" window for TSA-based multiplex IHC protocols (one marker, “Advanced template” option “Off”)

The “New protocol” window is divided in two sections, the general protocol parameters section (under the protocol name in blue) and the protocol steps section (under “Step Parameters” title in blue).

Editing the general protocol parameters

Fill in the general protocol parameters as follows:

- [Optional] Protocol name: click on the default name (“TSA-based multiplex IHC”) and type the new name
- [Optional] Reagent kit: indicate the detection kit used, if applicable
- [Optional] Description: insert a short description of the protocol being created
- [Required] Washing buffer: the selected buffer will be used for tissue washes and intra-protocol reservoir washes. The default buffer is “Multistaining Buffer”.

Editing the protocol steps

The steps displayed by default are “Slide loading + priming”, “Antigen retrieval”, “Marker detection”, “Elution”, “Counterstaining” and “Final Wash”. “Blocking”, “Custom”, “Primary antibody”, “Priming”, “Reservoir exchange”,

"Reservoir wash", "Secondary antibody" and "Substrate" steps can be added to the template by clicking  . The steps present by default can also be added with these buttons. The details and parameters to fill in for each type of step are presented below.

Slide loading + priming

This step performs the slide loading and priming for the loaded sample. During this step the Staining chip and the slide loaded in the stainer are pushed together creating a closed chamber and all the reservoirs with reagents that have not yet been used are primed.

Rules:

- The "Slide loading + Priming" step is mandatory and must be the first step of a protocol.
- The protocol cannot contain more than one "Slide loading + Priming" step.

Dispense volume:

The priming volume is 120 µL for reagents loaded in small reservoirs and 500 µL for reagents loaded in large reservoirs.

Antigen retrieval

Parameters:

1. Select an antigen retrieval reagent from the drop-down list.
2. Select the temperature using the slider (from 1 to 5, 3 by default).
3. Select the incubation time (2 min, 5 min, or 10 min, 10 min by default).

Do not touch the Stainer or slide during the "Antigen retrieval" step. The system is heating to very high temperatures and can cause pain and burns.

The hot surface symbol (depicted to the left) is placed next to the Stainer to identify the area surrounding the heat source.

Rules:

- An antigen retrieval reagent must be selected.
- The antigen retrieval reagent selected in the first "Antigen retrieval" step of the protocol will be automatically assigned to all the following "Antigen retrieval" and "Elution" steps of the protocol. The reagent for these can still be changed by simply selecting a different reagent from the drop-down menu.
- The "Antigen retrieval" step is not mandatory, but it is recommended for FFPE samples.
- There can be at most one "Antigen retrieval" step per protocol.
- d

Disclaimer: The Staining chip has been fully validated for Antigen retrieval at temperature 3 for 10 minutes, followed by 6 "Elution" steps at temperature 2 for 5 minutes. When using higher temperature settings, minor deformations of the chip's plastic may occur leading to reagent leaking during protocol execution.

Dispense volume:

The dispense volume of antigen retrieval reagent is 1160 µL.

Marker detection

During the "Marker detection" step protein block, primary antibody, secondary antibody, and substrate are dispensed sequentially. This step is named "Marker detection" by default and it is automatically renamed "[Primary antibody name] – [Substrate name]" once both the primary antibody reagent and the substrate are selected. "Marker detection" steps should be followed by an "Elution" step.

Parameters:

1. Toggle the "Protein blocking" sub-step "On" or "Off" ("On" by default).
2. If "On", Select a blocking reagent from the drop-down list and select the incubation time (30 s, 2 min, or 4 min; 2 min by default).

3. Select a primary antibody from the drop-down list (only primary antibodies that have not been selected in the previous steps of the protocol will appear) and select the incubation time (2 min, 4 min, 8 min, or 10 min; 8 min by default).
4. Select a secondary antibody from the drop-down list and select the incubation time (2 min, 4 min, 8 min, or 10 min; 4 min by default).
5. Select a substrate from the drop-down list (only substrates that have not been selected in the previous steps of the protocol will appear) and select the incubation time (2 min, 4 min, 8 min, or 10 min; 4 min by default).

Rules:

- The blocking reagent selected in the first "Marker detection" step of the protocol will be automatically assigned to all the following "Marker detection" and "Blocking" steps of the protocol. The reagent for these steps can still be changed by simply selecting a different reagent from the drop-down list.
- Make sure to have enough reservoirs before using more than one protein blocking solution.
- Primary antibodies must be loaded in small reservoirs.
- The secondary antibody solution selected in the first "Marker detection" step of the protocol will be automatically assigned to all the following "Marker detection" steps of the protocol. The reagent for these steps can still be changed by simply selecting a different reagent from the drop-down list.
- Make sure to have enough free reservoirs before using more than one secondary antibody solution.
- The substrate must be loaded in a small reservoir.

Dispense volume:

	Dispense volume
Protein block	180 µL
Primary antibody	330 µL
Secondary antibody	280 µL
Substrate	330 µL

Elution

The "Elution" step removes the primary-secondary antibody complex from the previous marker to be able to perform a new marker detection on the same sample.

This step includes an optional automatic reservoir washing sub-step ("Wash previous AbI and Substrate reservoirs" toggle) to clean the reservoirs containing primary antibody and substrate used in the previous "Marker detection" step. Toggle this option "On" to save time when performing a protocol that requires one or more "Reservoir exchange" steps because the user intervention will only require the loading of reagents (no further wash will be needed).

Note: When the "Wash previous AbI and Substrate reservoirs" toggle is "On", all the leftover reagents will be lost after the "Elution" step. If you expect leftover reagents and wish to retrieve them, switch the toggle "Off". To avoid leftovers, load the exact reagent volume indicated in the Required action into the reservoir.

Parameters:

1. Select a reagent from the drop-down list (reagents categorized as "Antigen retrieval" or "Elution buffer" appear in the list).
2. Select the temperature using the slider (from 1 to 5; 2 by default).
3. Select the incubation time (2 min or 5 min; 5 by default).
4. Toggle the "Wash previous AbI and Substrate reservoirs" option "On" or "Off" ("Off" by default). This toggle only appears once a primary antibody or substrate has been selected in the previous protocol steps.

Disclaimer: The Staining chip has been fully validated for Antigen retrieval at temperature 3 for 10 minutes, followed by 6 "Elution" steps at temperature 2 for 5 minutes. When using higher temperature

settings, minor deformations of the chip's plastic may occur leading to reagent leaking during protocol execution.

Rules:

- It is recommended to add an "Elution" step after each "Marker detection" step.
- Make sure to have enough reservoirs if using more than one antigen retrieval solution across the same protocol (i.e., different solutions for the "Antigen retrieval" step and "Elution" steps).

Dispense volume:

The dispense volume for the "Elution" step reagent is 1160 µL. If the "Wash previous AbI and Substrate reservoirs" toggle if "On", an additional 10 mL of protocol washing buffer and 4 mL of 70% ethanol will be dispensed per washed reservoir.

Counterstaining

Parameters:

1. Select the counterstain from the drop-down list (reagents categorized as "Counterstaining" or "Secondary Antibody and Counterstaining Mix" appear in the list, if a reagent with the name "DAPI" is present in the database it will be selected by default).
2. Select the incubation time (2 min, 4 min, or 6 min for Counterstaining reagents and 2 min, 4 min, 8 min or 10 min for Secondary Antibody and Counterstaining Mix reagents).

Note: Digoxigenin (DIG)-labeled substrates, along with corresponding anti-DIG fluorescent conjugates (e.g., Opal 780 when using the Opal™ kit), can be used with this application. To do so, create a substrate reagent for the DIG-labeled substrate and a secondary antibody and counterstaining mix reagent comprising the anti-DIG fluorescent conjugate and the counterstain. In the protocol, choose the DIG-labeled substrate in the "Marker detection" step and the secondary antibody and counterstaining mix reagent in the "Counterstaining" step. Make sure there are no heating steps after the "Counterstaining" step to avoid the elution of the anti-DIG fluorescent conjugate.

Rules:

- The "Counterstaining" step is not mandatory, but it is recommended for imaging.
- It is not recommended to place a "Counterstaining" step before a heating step ("Antigen retrieval" or "Elution" steps).
- The counterstain must be placed in a small reservoir.

Dispense volume:

The dispense volume of the counterstain is 330 µL.

Final wash

The final wash of the tissue uses the washing buffer that is selected in the general parameters of the protocol.

Rules:

- The "Final wash" step is mandatory and must be placed at the end of the protocol.
- The protocol can contain only one "Final wash" step.
- If there are multiple Secondary antibodies, Protein blocks or Custom reagents before this step, make sure that there will be enough free reservoirs to resume the protocol. If not, add a "Reservoir exchange" step to the protocol.

Dispense volume:

The dispense volume of the protocol washing buffer is 1160 µL.

Reservoir exchange (present by default when creating a protocol for 4 markers)

During this step the reservoirs containing the primary antibodies and substrates used in the first part of the protocol will be washed and the reagents necessary for the next part of the protocol are loaded onto the instrument. This step also provides the opportunity to retrieve reagent leftovers from the instrument via the "Retrieve reagents leftover" toggle. If the toggle is "On", the user will be able to remove the reservoirs containing reagent leftovers and load empty microtubes in their place before the reservoirs are washed. A second intervention will be needed to load the new reagents for the second part of the protocol. If the "Retrieve reagents leftover" switch is toggled "Off", the reservoirs will be washed before the protocol pauses and therefore there will only be one user intervention to load the new reagents onto the instrument.

When it is time for the user intervention, a “Pause” window will open to guide the user (Figure 19).

Steps to follow:

1. First user intervention (only if the “Retrieve reagents leftover” switch is “On”)
    - Follow the instructions provided in the “Pause” window.
    - Fulfil all the Required actions displayed.
    - Click “Continue”.
2. Second user intervention
    - Complete the Required actions.
    - Click “Continue” to resume the protocol.

Note: To prevent contact with hazardous reagents, do not open the stainer during “Pause” steps if not indicated by the software.

Parameters:

1. Toggle the “Retrieve reagents leftover” switch “On” or “Off”. This toggle only appears once a primary antibody or substrate has been selected in the previous protocol steps and if there are “Elution” steps with the “Wash previous AbI and TSA” toggle switched “Off”.

Rules:

- If there are multiple Secondary antibodies, Protein blocks or Custom reagents before this step, make sure there will be enough free reservoirs to resume the protocol.

Dispense volume:

The dispense volume of the protocol washing buffer is 500 µL plus an additional 3.6 mL per reservoir washed.

Additional available protocol steps

Blocking

The “Blocking” step dispenses and incubates protein blocks. When used in combination with the “Primary antibody”, “Secondary antibody” and “Substrate” steps, marker detection is more flexible than when using the combined “Marker detection” step. A “Wash after blocking step” toggle is available to allow the user to wash the protein block out of the reaction chamber before moving on to another step. It is recommended to toggle it “Off” when dispensing primary and secondary antibodies directly after the “Blocking” step.

Parameters:

1. Select a protein block from the drop-down list. Reagents in the “Protein block” category are displayed.
2. Select the incubation time (30 s, 2 min or 4 min; 2 min by default).
3. Toggle the “Wash after blocking step” option “On” or “Off” (“Off” by default).

Rules:

- The “Reagent” field must be filled.

Dispense volume:

The dispense volume of protein block is 180 µL.

Primary antibody

The “Primary antibody” step dispenses and incubates primary antibodies. When used in combination with the “Blocking”, “Secondary antibody” and “Substrate” steps, marker detection is more flexible than when using the combined “Marker detection” step.

Note: the “Primary antibody” step is considered an advanced step, therefore little guidance is provided during protocol creation. See the Advanced template section for more information.

Parameters:

1. Select a primary antibody reagent from the drop-down list. Reagents in the “Primary antibody” category are displayed.
2. Select the incubation time (2 min, 4 min, 8 min or 10 min; 8 min by default).

Rules:

- There cannot be more than one “Primary antibody” step in the same marker detection cycle.

- The “Primary antibody” step cannot coexist with the “Marker detection” step in the same marker detection cycle.
- The “Reagent” field must be filled.
- The primary antibody reagent must be loaded in a small reservoir.

Dispense volume:

The dispense volume of primary antibody reagent is 330 µL.

Secondary antibody

The “Secondary antibody” step dispenses and incubates secondary antibodies. When used in combination with the “Blocking”, “Primary antibody” and “Substrate” steps, marker detection is more flexible than when using the combined “Marker detection” step.

Note: the “Secondary antibody” step is considered an advanced step, therefore little guidance is provided during protocol creation. See the Advanced template section for more information.

Parameters:

1. Select a secondary antibody reagent from the drop-down list. Reagents in the “Secondary antibody” category are displayed.
2. Select the incubation time (2 min, 4 min, 8 min or 10 min; 4 min by default).

Rules:

- There cannot be more than one “Secondary antibody” step in the same marker detection cycle.
- The “Secondary antibody” step cannot coexist with the “Marker detection” step in the same marker detection cycle.
- The “Reagent” field must be filled.

Dispense volume:

The dispense volume of secondary antibody reagent is 280 µL.

Substrate

The “Substrate” step dispenses and incubates substrates. When used in combination with the “Blocking”, “Primary antibody” and “Secondary antibody” steps, marker detection is more flexible than when using the combined “Marker detection” step.

Note: the “Substrate” step is considered an advanced step, therefore little guidance is provided during protocol creation. See the Advanced template section for more information.

Parameters:

1. Select a substrate from the drop-down list. Reagents in the “Substrate” category are displayed.
2. Select the incubation time (2 min, 4 min, 8 min or 10 min; 4 min by default).

Rules:

- There cannot be more than one “Substrate” step in the same marker detection cycle.
- The “Substrate” step cannot coexist with the “Marker detection” step in the same marker detection cycle.
- The “Reagent” field must be filled.
- The substrate must be loaded in a small reservoir.

Dispense volume:

The dispense volume of substrate is 280 µL.

Custom step

During this step, the selected reagent will be dispensed into the staining chamber.

Parameters:

1. Toggle the Dynamic Incubation (DI) option “On” or “Off” (“On” by default).
2. Select a reagent from the drop-down. Reagents of all categories except Primary antibody, Substrate, Cleaning buffer, Protein block and Counterstaining will be available to select from the drop-down list. Additionally, if “Dynamic incubation” is “On”, the washing buffer selected in the general settings of the protocol will not be available as a reagent for this step.
3. Select the incubation time:

- If DI is “On”: any whole number between 1 and 10 min
- If DI is “Off”: any whole number between 1 and 60 min

Rules:

- If “Dynamic incubation” is “On” then the selected reagent must be loaded in a small reservoir.

Dispense volume:

The dispense volume of the reagent selected in the “Custom” step is 330 µL (DI “On”) or 180 µL (DI “Off”).

Reservoir wash

During this step any reservoir can be washed. The reservoirs of the reagents selected in this step will be washed with the washing buffer selected in the general parameters of the protocol. It is not possible to retrieve reagent leftovers before this step; therefore, make sure you are loading the exact amount of reagent indicated in the Required actions.

Parameters:

1. Select the reagents that you wish to remove from the instrument by clicking on “Please select reagents” and typing their name. Reagent names will appear as a drop-down list and by clicking the reagent name they are added to the wash list. To remove a reagent from the list, click the “**x**” to the right of the reagent’s name. Only reagents that are used in the previous protocol steps and which have not been previously selected in a “Reservoir wash” step can be added to the wash list.

Rules:

- The “Reservoir wash” step can only be used to wash small reservoirs. Plan the reagent allocation accordingly.
- Reagents which have been selected in a “Reservoir wash” step cannot be selected as the reagent in any of the following protocol steps until after the next “Reservoir exchange” step.

Dispense volume:

The dispense volumes of protocol washing buffer and 70% ethanol are 4 mL and 1.2 mL respectively per reservoir washed.

Proposed reservoir configuration

The reagent allocation rules for TSA-based multiplex IHC protocols are the following. All the rules will be enforced by the software with warnings and errors that will prevent the user from starting the protocol until all the rules are respected.

- Reagents for which the Dynamic incubation option is on must be loaded in small reservoirs.
- Only small reservoirs can be washed during the protocol. Load reagents that you wish to exchange during the protocol in small reservoirs.
- The reservoirs containing washing buffer must be filled up to the maximum volume (50 mL) at the beginning of the protocol and during Reservoir exchange steps.

Lunaphore recommends the following reservoir configuration for a standard 6-plex:

	Large reservoirs				Small reservoirs							
Part 1	A	B	C	D	1	2	3	4	5	6	7	8
	Washing buffer	Protein block	Antigen retrieval	Ethanol 70%	AbII	AbI 1	AbI 2	AbI 3	Sub 1	Sub 2	Sub 3	-
RESERVOIR EXCHANGE												
Part 2	A	B	C	D	1	2	3	4	5	6	7	8
	Washing buffer	Protein block	Antigen retrieval	Ethanol 70%	AbII	AbI 4	AbI 5	AbI 6	Sub 4	Sub 5	Sub 6	DAPI

Table 26) Recommended reservoir configuration for a standard 6-plex protocol. AbI: Primary antibody. AbII: Secondary antibody. Sub: Substrate (Tyramide for Signal Amplification reagent).

Advanced template

The advanced template offers more flexibility during protocol design by splitting the combined “Marker detection” step into four individual steps: “Blocking”, “Primary antibody”, “Secondary antibody” and “Substrate”. Additional steps can be added in between them to customize the protocol.

Note: A protocol created using the standard template (advanced template option "Off") can always be partially or completely turned into an advanced protocol by replacing the "Marker detection" step in one or more cycles with advanced steps.

To offer more freedom to the user, the step order is not subject to strict control. Therefore, pay close attention when building your protocol and double-check that it matches your experimental plan.

Distribution chip change and wash countdowns

The countdowns decrement by n+1 units after the full completion of a n-plex.

Extra "Antigen retrieval" or "Elution" steps that are added to the default protocol template will decrement the countdowns by one additional unit per step added.

Delayed slide retrieval

A slide can be left clamped overnight after the completion of a TSA-based multiplex IHC staining. However, slides should not be left overnight during a "Reservoir exchange" step.

The user can walk away after launching a protocol that does not contain "Reservoir exchange" steps or after all the "Reservoir exchange" steps have been completed.

Lunaphore does not guarantee the same results for slides that are left on LabSat® overnight and slides that were removed from the instrument and imaged directly at the end of the protocol. It is the user's responsibility to verify that the overnight incubation of their tissue in washing buffer does not impair morphology, signal, background and sharpness of the staining, and the integrity of the glass slide.

TSA-based IHC for higher than 6-plex

During creation of a TSA-based multiplex IHC protocol, the maximal number of markers that can be detected on a slide is six. It is not possible to create a protocol for more than a 6-plex. If the user wants to detect more than six markers on the same slide, they can run two consecutive TSA-based multiplex IHC protocols.

For example, to run an 8-plex follow the steps below:

1. Run a 6-plex without counterstaining.
2. Retrieve the slide and store it in in buffer.
3. Run a Select & Wash protocol, if necessary, to free enough reservoirs to run the second protocol.
4. Change the Staining chip.
5. Run a 2-plex protocol on the same slide without "Antigen retrieval" step before the first "Marker detection" step (i.e., delete the "Antigen retrieval" step from the protocol).

Important notes:

- **At the end of the first 6-plex protocol, there is a mandatory "Final Wash" step. Keep in mind that this step might have an impact on the staining of the 7th marker.**
- **Lunaphore does not guarantee the performance of stainings obtained with this method. Only assays up to 6-plex have been validated by Lunaphore.**

Troubleshooting: Protocol optimization

#	What	Detail	Solutions
01	No specific signal	There is no specific signal in the sample.	1. Check all the reagents were dispensed correctly by verifying status of each step in the report. 2. Make sure that selected fluorophores are compatible with excitation/emission set up of image acquisition system. 3. Check that the species reactivity of primary and secondary antibodies matches. 4. Check no reagent is expired. 5. Check the correct reagents were used and loaded in LabSat®. 6. Repeat staining with fresh reagents. 7. Test another antigen retrieval solution (change the pH). 8. Test a longer incubation time of the “Antigen retrieval” step. 9. Make sure the antigen retrieval process was successful (no bubbles in the chamber). 10. Increase concentration of primary and/or secondary antibodies. 11. Increase incubation time of primary and/or secondary antibodies. 12. Perform manual staining with the reagents to check their validity. 13. Perform the protocol on a known positive control tissue. 14. Reduce substrate concentration in previous steps. 15. Change the reagents use a new batch or try different brands, species, etc).

02	Weak signal	The signal is present but weak	1. Check the staining steps were performed correctly by verifying status in the report. 2. Increase exposure time of the acquisition. 3. Make sure to perform image acquisition shortly after staining. 4. Make sure that the staining was not bleached before imaging: ensure that the light sensitive reagents / stainer were protected from light during staining and protect the slide from direct light once mounted. 5. Make sure to match the fluorophore with the optimum excitation/emission set up for image acquisition. 6. Increase the incubation time of the primary antibody. 7. Increase the incubation time of the secondary antibody. 8. Increase the concentration of primary and/or secondary antibodies. 9. Change the primary antibody clone. 10. Repeat staining with fresh reagents. 11. Perform the protocol on a known positive control to check the validity of the reagents. 12. Test another elution solution. 13. Test another antigen retrieval solution (change the pH). 14. Increase the temperature of the “Antigen retrieval” step. 15. Increase substrate concentration. 16. Increase substrate incubation time. 17. Decrease the incubation time of the blocking reagent or remove the blocking step. 18. Check that the species reactivity of primary and secondary antibodies matches. 19. Remove the NBF fixation in slide pre-processing. 20. Reduce substrate concentration in previous steps. 21. Move the marker position further in the panel sequence. 22. Mount the samples with an anti-fade solution. 23. Change reagents (use a new batch or try different brands, species, etc).

03	Non-uniform staining	The staining is not uniform within the staining area	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check all the reagents were dispensed correctly by verifying status of each step in the report. 3. Check that the used reagents are in the viscosity range defined by the user manual. 4. Repeat the staining with a new Staining chip and make sure: a. the staining process was successful (no bubbles in the chamber) b. there is enough volume of the reagents used c. there is DIW or Washing Buffer in all the unused reservoirs.
04	High general background	The signal to noise ratio is too low	1. Make sure the tissue does not dry during handling (before loading and after retrieving the slide from LabSat®). 2. Activate the blocking steps. 3. Reduce the incubation time of the antibody steps. 4. Dilute the antibody solutions more. 5. Change the primary antibody clone. 6. Perform a Full Wash. 7. Change the antigen retrieval incubation time and temperature. 8. Change the antigen retrieval solution. 9. Lower substrate incubation time. 10. Lower substrate concentration.
05	One area is not stained	One area in the Stainer is never stained properly	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check the staining steps were performed correctly by verifying status in the report. 3. Repeat the staining with a new Staining chip and make sure: a. there is enough volume of the reagents used b. there is DIW or washing buffer in all the unused reservoirs 4. Do not use highly concentrated detergent solutions. 5. Check that the used reagents are in the viscosity range defined by the user manual. 6. Perform a Daily or Full wash and change the Distribution chip.

06	Signal is too strong	The specific signal is too strong	1. Reduce exposure time of acquisition. 2. Dilute the primary and/or secondary antibodies more. 3. Reduce the incubation time of the primary and/or secondary antibody. 4. Repeat the staining using fresh reagents. 5. Reduce substrate concentration. 6. Reduce substrate incubation time. 7. Test a shorter incubation time of the “Antigen retrieval” and/or “Elution” step. 8. Test a lower temperature of the “Antigen retrieval” and/or “Elution” step. 9. Change the reagents (use a new batch or try different brands, species, etc).
07	Signal crosstalk	Strong signal is detected in other channels	1. Put the strong marker at the beginning of the Multiplex protocol. 2. Put an “Elution” step before the detection of the strong marker instead of an “Antigen retrieval” step. Use the “Antigen retrieval” step before the weaker markers.
08	Weak counterstain	The counterstain is too weak	1. Increase the exposure time. 2. Increase the incubation time of the counterstain step. 3. Dilute the counterstain less. 4. Check the that “Counterstaining” step was performed correctly by verifying status in the report. 5. Repeat the staining using fresh reagents. 6. Change the counterstaining reagent (use a new batch or try a different brand).
09	Counterstain is too strong	The counterstain is too strong	1. Decrease the incubation time of the counterstain. 2. Dilute the counterstain solution. 3. Add an additional wash step after the counterstaining step. 4. Change the counterstain (use a new batch or try a different brand). 5. Repeat the staining using fresh reagents. 6.
10	Tissue morphology	The tissue morphology is altered	1. Decrease the incubation time of the “Antigen retrieval” step. 2. Test a lower temperature of the “Antigen retrieval” step and the “Elution” step. 3. Test another antigen retrieval solution (change the pH). 4. Improve pre-processing steps of tissue fixation.
11	Tissue detaching	The tissue detaches after the process	1. Use positively charged slides for tissue fixation. 2. Use coated histological slides for tissue fixation. 3. Improve pre-processing steps of tissue fixation. 4. Decrease the incubation time of the “Antigen retrieval” step. 5. Test a lower temperature of the “Antigen retrieval” step and the “Elution” step.

12	Overstaining	The signal is spreading out (the signal is not sharp) The signal is leaking out the structures (the signal is not sharp)	1. Reduce exposure time. 2. Reduce the incubation times of the antibody steps. 3. Dilute the primary antibody solution more. 4. Change the clone of the primary antibody. 5. Change the secondary antibody. 6. Reduce substrate concentration. 7. Reduce substrate concentration time. 8. Test a shorter incubation time of the "Antigen retrieval" step and the "Elution" step. 9. Test a lower temperature of the "Antigen retrieval" step and the "Elution" step.
13	Non-specific staining	There is non-specific staining in the sample	1. Add or increase the "Peroxidase block" step. 2. Change the clone of the primary antibody. 3. Dilute the primary antibody solution more. 4. Decrease incubation time of the secondary antibody. 5. Change the secondary antibody solution. 6. Add additional washing step after the staining steps in the protocol. 7. Perform a negative control of the same tissue to understand from which antibody the non-specific staining may come from. 8. Change the antigen retrieval incubation time. 9. Test another antigen retrieval solution (change the pH). 10. Use fresh reagents. 11. Re-balance the assay (by adjusting "Primary antibody" or "Substrate" steps) to have the intensities of the different markers in the same range.
14	Autofluorescence	The tissue shows autofluorescence	1. Lower the antigen retrieval pH solution. 2. Use unstained tissue as negative controls. 3. Use fresh fixative solution or change it. 4. Add a "Custom" step with an autofluorescence quenching buffer as the reagent before the staining step.

Table 27) Troubleshooting guidelines for FFPE TSA-based multiplex IHC protocol-related issues

Annex 2.4: FFPE Sequential IF protocol template

This annex presents the information regarding the FFPE Sequential IF protocol template of LabSat®.

Note: Only Sequential IF assays up to 10-plex on the same slide have been validated by Lunaphore. Additionally, slide integrity has been validated for up to 10 cycles.

Protocol creation and editing

To open a blank protocol template for editing, go to the Protocols tab and follow the steps below:

1. Click “Add new” above the protocol list. The “Create a new protocol” window will open.
2. Select “Sequential IF” in the FFPE branch. The protocol description will be displayed on the right side of the window.
3. Toggle the “Autofluorescence imaging” switch “On” or “Off” (“On” by default). When turned “On”, there will be a pause for the user to image the autofluorescence of the sample.
4. Select the desired number of cycles, between 1 and 10. A cycle is defined as a set of steps leading to a staining of up to 4 markers simultaneously followed by a pause in the protocol for slide imaging.
5. Toggle the “Advanced template” option “On” (“Off” by default) to access a more flexible and less guided protocol template. The advanced template splits the “Staining” step into 3 individual steps: “Primary antibody”, “Secondary antibody” and “Counterstaining” steps.
6. Click “OK”. The “New protocol” window will open (Figure 52) with the selected number of cycles.

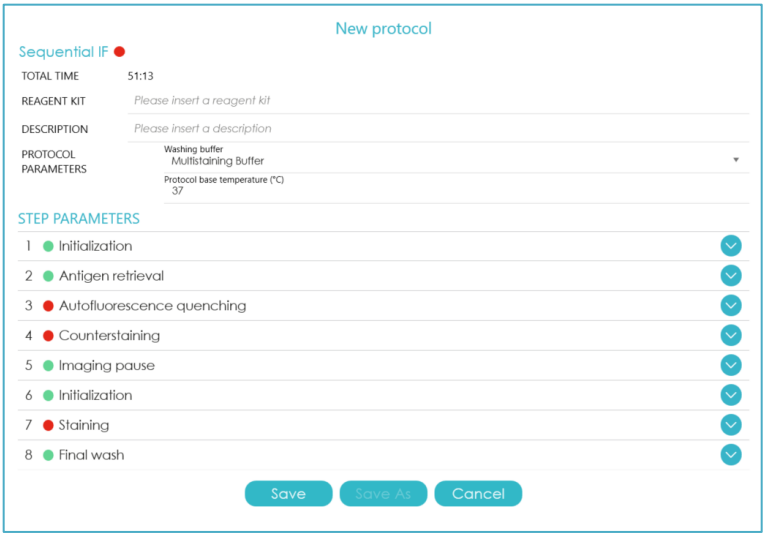


Figure 52) "New protocol" window for FFPE Sequential IF protocols

The “New protocol” window is divided in two sections, the general protocol parameters section (under the protocol name in blue) and the protocol steps section (under “Step Parameters” title in blue).

Editing the general protocol parameters

Fill in the general protocol parameters as follows:

1. [Optional] Protocol name: click on the default name (“Sequential IF”) and type the new name.
2. [Optional] Reagent kit: indicate the detection kit used.
3. [Optional] Description: insert a short description of the protocol being created.
4. [Required] Washing buffer (buffer used for tissue washes and intra-protocol washes): select the washing buffer from the drop-down list (Multistaining Buffer by default).
5. [Required] Protocol base temperature (temperature at which the protocol will be executed): input a temperature in the text field (between 27 °C and 42 °C, 37 °C by default). The “Antigen retrieval” and “Elution” steps will be performed at a temperature chosen separately.

Editing the protocol steps

The steps displayed by default are “Initialization”, “Antigen retrieval”, “Autofluorescence quenching”, “Counterstaining”, “Imaging pause”, “Staining”, “Elution” and “Final Wash”. “Blocking”, “Custom”, “Primary antibody”, “Secondary antibody”, and “Wash” steps can be added to the template by clicking  . The steps present by default can also be added with these buttons.

If the advanced template is selected in the “New protocol” window, the “Primary antibody”, “Secondary antibody”, and “Counterstaining” steps will be displayed by default instead of the “Staining” step for each cycle. The Advanced template section below goes into the specific details of this option.

The details and parameters to fill for each step are presented below.

Default steps

Initialization

During this step slide loading is performed (the piston closes and clamps the chip and slide together creating a closed chamber, then the chamber is filled) and all the reservoirs containing reagents that have not been used are primed.

Parameters:

1. Select a buffer from the drop-down list. This buffer will be used as slide loading buffer. The available reagents are the protocol washing buffer and deionized water (DIW).

Note: To ensure the optimal upkeep of LabSat®, Lunaphore recommends the use of DIW as slide loading buffer when possible.

Rules:

- A washing buffer must be selected.
- The “Initialization” step is mandatory and must be the first step of the protocol and the first step after each “Imaging pause” step.
- The protocol cannot contain more than one “Initialization” step per cycle.

Antigen retrieval

The “Antigen retrieval” step is a heat-induced epitope un-masking procedure for FFPE samples.

Parameters:

1. Select an antigen retrieval buffer from the drop-down list. Reagents in the Antigen retrieval category are displayed. The default antigen retrieval buffer is Lunaphore’s Antigen Retrieval solution pH 9 (BU04).
2. Select the antigen retrieval temperature level: from 1 (lowest) to 6 (highest). The default temperature level is 5.
3. Enter an antigen retrieval incubation time from within the following range: 02:00 – 10:00 (mm:ss). The default incubation time is 10:00.

Do not touch the Stainer or slide during the antigen retrieval steps. The system is heating to very high temperature and can be painful upon touch.

The hot surface symbol (to the left) is placed next to the Stainer to identify the area surrounding the heat source.

Lunaphore recommends the use of the following antigen retrieval solutions with LabSat® to obtain the best results.

- Antigen Retrieval solution pH 6 (10X), Lunaphore, ref: BU04
- Antigen Retrieval solution pH 9 (10X), Lunaphore, ref: BU05

Rules:

- A cycle cannot have more than one "Antigen retrieval" step.
- An antigen retrieval buffer must be selected.

Dispense volume:

The dispense volume of antigen retrieval buffer is 1160 µL.

Autofluorescence quenching

The "Autofluorescence quenching" step is intended to reduce the intrinsic tissue autofluorescence level.

Parameters:

1. Select a quenching buffer from the drop-down list. Reagents in the Autofluorescence quencher category are displayed.

Note: Once you have selected an Autofluorescence quencher in the first "Autofluorescence quenching" step of the protocol, the same reagent will automatically be selected for all the subsequent "Autofluorescence quenching" steps. To use a different quencher in subsequent steps, simply change it to the desired one from the drop-down list.

2. Enter an autofluorescence quencher incubation time from within the following range: 01:00 – 59:59 (mm:ss). The default incubation time is 02:00.

Lunaphore recommends the use of the following autofluorescence quenchers with LabSat® to obtain the best results.

- Quenching Buffer, Lunaphore, ref: BU08

Rules:

- The "Reagent" field must be filled.

Dispense volume:

The dispense volume of autofluorescence quencher is 350 µL.

Counterstaining

The "Counterstaining" step performs the counterstaining of the sample.

Parameters:

1. Select a counterstaining reagent from the drop-down list. Reagents in the "Counterstaining" category are displayed.

Note: Once you have selected a Counterstaining reagent in the first "Counterstaining" step of the protocol, the same reagent will automatically be selected for all subsequent "Counterstaining" steps and Counterstaining options of "Staining" steps. To use a different counterstain in subsequent steps, simply chang e it to the desired one from the drop-down menu.

2. Enter a counterstaining reagent incubation time from within the following range: 01:00 – 10:00 (mm:ss). The default incubation time is 02:00.

Rules:

- The "Reagent" field must be filled.
- The counterstaining reagent must be loaded in a small reservoir.

Dispense volume:

The dispense volume of counterstaining reagent is 280 µL.

Staining

The "Staining" step includes the dispense and incubation of primary antibody, secondary antibody, and counterstain. The counterstain dispense can be turned off; this is recommended when the secondary antibody reagent contains a counterstain. The dispense of primary and secondary antibody can be complemented with "Dynamic incubation", a feature designed to enhance the staining quality. Up to 4 consecutive dispenses of primary and secondary antibodies are possible during a "Staining" step.

Notes:

- **To use directly labelled primary antibodies, refer to the Advanced template section below.**
- **Antibodies and counterstain are heat-sensitive reagents. Make sure that the "Staining" step is not followed by a heating step (i.e., "Antigen retrieval" step).**

Parameters:

1. Primary antibody section

- Toggle the Dynamic incubation option “On” or “Off” (by default “On”). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).
- Select a primary antibody solution from the drop-down list. Reagents in the “Primary antibody” and “Primary antibody mix” categories are displayed.
- Enter a primary antibody incubation time from within the following range:
- 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is “On”.
- 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is “Off”.
- The default incubation time is 04:00 in both cases.

2. Secondary antibody section
    - Toggle the Dynamic incubation option “On” or “Off” (by default “On”). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).
    - Select a secondary antibody reagent from the drop-down list. Reagents in the “Secondary antibody” and “Secondary antibody mix” categories are displayed if Dynamic incubation is “Off”. If the Dynamic incubation option is “On”, the reagents in the “Secondary antibodies and counterstaining mix” category are displayed as well.
    - Enter a secondary antibody incubation time from within the following range:
    - 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is “On”.
    - 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is “Off”.
    - The default incubation time is 02:00 in both cases.
3. Counterstaining section
    - Toggle the Counterstaining option “On” or “Off” (by default “On”). By deactivating this option, the “Staining” step will skip the counterstaining dispense and incubation.
    - Select a Counterstaining reagent from the drop-down list. Reagents in the “Counterstaining” category are displayed.
    - Enter a counterstaining incubation time from within the following range: 01:00 – 10:00 (mm:ss). The default incubation time is 02:00.

Rules:

- A protocol cannot have more than one "Staining" step per cycle.
- The “Reagent” field of the primary antibody section must be filled.
- The “Reagent” field of the secondary antibody section must be filled.
- If the Counterstaining option is “On”, the “Reagent” field of the counterstaining must be filled.
- At most four primary antibodies can be used during a single “Staining” step (antibodies in mixes are counted individually).
- At most four secondary antibodies can be used during a single “Staining” step (antibodies in mixes are counted individually).
- Counterstaining is not mandatory but recommended for imaging. A warning is shown if the Counterstaining option is disabled, and if the reagent selected as secondary antibody does not belong to the category Secondary antibody and counterstaining mix.
- If using antibody mixes, it is recommended to have a matching total number of antibodies between primary and secondary antibody sections (antibodies in mixes are counted individually).
- The “Staining” step cannot coexist with a “Primary antibody”, “Secondary antibody” or “Counterstaining” step in the same cycle.
- Reagents with the “Dynamic incubation” option turned on and the counterstaining reagent must be loaded in small reservoirs.

Dispense volume:

	Primary antibody reagent	Secondary antibody reagent	Counterstaining
If Dynamic incubation option is on	280 µL	280 µL	280 µL
If Dynamic incubation option is off	180 µL	180 µL	N/A

Imaging pause

The "Imaging pause" is an interactive step primarily intended to retrieve the slide from the stainer for imaging between marker detection cycles (Figure 19). The Staining chip used during the previous cycle must be discarded.

The "Imaging pause" step also provides the opportunity to wash reservoirs and allocate new reagents necessary for the following cycles. During this step, reservoirs can also be refilled if they contain reagents required in multiple cycles with a total protocol volume exceeding the reservoir's capacity.

Once the reagents for the next cycle are loaded on LabSat®, the imaging procedure is done and the slide is unmounted, insert the slide in the stainer along with a new Staining chip before closing the handles and resuming the protocol.

Parameters:

There are no parameters to set in the "Imaging pause" step.

Rules:

- The "Imaging pause" step must always be followed by an "Initialization" step.

Elution

The "Elution" step is intended to remove the signal from a previous marker detection to be able to perform a new one on the same sample.

Note: In a standard protocol, an "Elution" step should be present at the beginning of each cycle, starting from the second cycle, to remove the signal from the previous cycle.

Parameters:

1. Select an elution buffer from the drop-down list. Reagents in the "Elution Buffer" category will be displayed.

Note: Once you have selected an elution buffer in the first "Elution" step of the protocol, the same reagent will automatically be selected for all subsequent "Elution" steps. To use a different elution buffer in subsequent steps, simply change it to the desired one from the drop-down menu.

2. Enter an Elution temperature from within the following range: 27°C to 50°C. The default temperature is 37°C.
3. Toggle the High flow option "On" or "Off". This feature can enhance the elution efficiency but might not be suited for fragile samples.
4. Choose the number of Elution buffer dispenses you want to perform from the drop-down list.
5. Enter elution buffer incubation times after each dispense from within the following range: 00:30 – 10:00 (mm:ss). The default incubation time is 00:30.

Rules:

- There cannot be more than 5 "Elution" steps in the same cycle.
- The "Elution buffer" field must be filled.
- The total incubation time over all the elution buffer dispenses of the same "Elution" step cannot exceed 10 minutes.

Dispense volume:

The dispense volume of elution buffer is 230 µL per dispense.

Final wash

The “Final wash” step washes the tissue and rinses the dispense line at the end of the protocol.

Parameters:

1. Select a buffer from the drop-down list. The available reagents are the protocol washing buffer and DIW. The default buffer is DIW.

Note: To ensure optimal upkeep of LabSat®, Lunaphore recommends the use of DIW as Final wash buffer when possible.

Rules:

- The “Final wash” step is mandatory and must be placed at the end of the protocol.
- The protocol can only contain one “Final wash” step.
- The “Buffer” field must be filled.

Additional available protocol steps

Primary antibody

The “Primary antibody” step dispenses and incubates primary antibodies and primary antibody mixes. When used in combination with the “Secondary antibody” and “Counterstaining” steps, marker detection is more flexible than when using the combined “Staining” step. This step can also be used with the “Counterstaining” step only when using directly labelled primary antibodies.

Note: the “Primary antibody” step is considered an advanced step, therefore little guidance is provided during protocol creation. See the Advanced template section for more information.

Parameters:

3. Toggle the Dynamic incubation option “On” or “Off” (by default “On”). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).
4. Select a primary antibody reagent from the drop-down list. Reagents in the “Primary antibody” and “Primary antibody mix” categories are displayed.
5. Enter a primary antibody incubation time from within the following range:
    a. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is “On”.
    b. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is “Off”.
    c. The default incubation time is 04:00 in both cases.

Rules:

- There cannot be more than 4 “Primary antibody” steps in the same cycle.
- The “Primary antibody” step cannot coexist with the “Staining” step in the same cycle.
- The “Reagent” field must be filled.
- Reagents with the “Dynamic incubation” option turned on must be loaded in small reservoirs.

Dispense volume:

The dispense volume of primary antibody reagent is 280 µL if “Dynamic incubation” is on and 180 µL if “Dynamic incubation” is off.

Secondary antibody

The “Secondary antibody” step dispenses and incubates secondary antibodies and secondary antibody mixes. When used in combination with the “Secondary antibody” and “Counterstaining” steps, marker detection is more flexible than when using the combined “Staining” step.

Note: the “Secondary antibody” step is considered an advanced step, therefore little guidance is provided during protocol creation. Go to the Advanced template section for more information.

Parameters:

1. Toggle the Dynamic incubation option “On” or “Off” (by default “On”). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).

2. Select a secondary antibody reagent from the drop-down list. Reagents in the “Secondary antibody” and “Secondary antibody mix” categories are displayed if Dynamic incubation is “Off”. If the Dynamic incubation option is “On”, the reagents in the “Secondary antibodies and counterstaining mix” category are displayed as well.
3. Enter a secondary antibody incubation time from within the following range:
    a. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is “On”.
    b. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is “Off”.
    c. The default incubation time is 02:00 in both cases.

Rules:

- There cannot be more than 4 “Secondary antibody” steps in the same cycle.
- The “Secondary antibody” step cannot coexist with the “Staining” step in the same cycle.
- The “Reagent” field must be filled.
- Reagents with the “Dynamic incubation” option turned on must be loaded in small reservoirs.

Dispense volume:

The dispense volume of secondary antibody reagent is 280 µL if “Dynamic incubation” is “On” and 180 µL if “Dynamic incubation” is “Off”.

Wash

The “Wash” step is an extra wash of the tissue that can be placed anywhere in the protocol, except between the “Imaging pause” and the “Initialization” steps.

Parameters:

1. Select a buffer from the drop-down list. Reagents in the “Washing buffer” category are displayed. The default buffer is the protocol’s washing buffer.

Rules:

- The “Buffer” field must be filled.

Blocking step

The “Blocking” step dispenses and incubates blocking reagents.

Parameters:

1. Select a blocking reagent from the drop-down list. Reagents in the “Protein block” category are displayed.

Note: Once you have selected a blocking reagent in the first “Blocking” step of the protocol, the same reagent will automatically be selected for all subsequent “Blocking” steps. To use a different blocking reagent in subsequent steps, simply change it to the desired one from the drop-down menu.

2. Enter an incubation time from within the following range: 01:00 – 59:59 (mm:ss). The default incubation time is 02:00.

Rules:

- The “Reagent” field must be filled.

Dispense volume:

The dispense volume of blocking reagent is 230 µL.

Custom step

The “Custom” step dispenses and incubates any reagent needed for the assay that does not have a dedicated step in the FFPE Sequential IF template; for example, permeabilization reagent, custom buffer, etc.

Parameters:

1. Toggle the Dynamic incubation option “On” or “Off” (by default “On”). This feature can improve the homogeneity of the reagent dispense in the chamber but will increase the reagent volume consumption (see volume consumption table below).
2. Select a reagent from the drop-down list. Reagents in the “Custom” or “Custom buffer” categories are displayed if Dynamic incubation is “Off”. If the Dynamic incubation option is “On”, only the reagents in the “Custom” category are displayed.
3. Enter an incubation time from within the following range:

a. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is “On”.
b. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is “Off”.
c. The default incubation time is 02:00 in both cases.

Rules:

- If the “Dynamic incubation” option is turned on, the custom reagent must be loaded in a small reservoir.

Dispense volume:

The dispense volume of custom reagent is 280 µL if “Dynamic incubation” is “On” and 180 µL if “Dynamic incubation” is “Off”.

Advanced template

The advanced template offers more flexibility during protocol design by splitting the combined “Staining” step into three individual steps: “Primary antibody”, “Secondary antibody” and “Counterstaining”. This feature allows the user to repeat up to 4 times in the same cycle both the “Primary antibody” and the “Secondary antibody” steps. The “Primary antibody”, “Secondary antibody” and “Counterstaining” steps can also be individually added in a cycle, allowing for negative controls or the use of directly labelled primary antibodies.

Note: a protocol created using the standard template (advanced template option “Off”) can always be partially or completely turned into an advanced protocol by replacing the “Staining” step in one or more cycles with advanced steps. The advanced steps rules then apply to this cycle.

To offer more freedom to the user, the number of warnings that are displayed is reduced in the advanced template compared to the standard one. Keep in mind the following points when building your desired protocol:

- If you plan to image a slide at the end of a cycle, make sure a counterstaining is performed at some point in the cycle, after “Elution” and “Antigen retrieval” steps.
- When using advanced steps in a cycle, the total number of primary and secondary antibodies used in this cycle is not checked by the software.

Note: Lunaphore has tested and approved the use of up to 4 primary and secondary antibodies within the same cycle. If more antibodies are used within the same cycle, Lunaphore does not ensure satisfactory staining quality.

- Pay close attention to the order of the steps in each cycle and double-check that the final protocol matches your experimental plan.
- Verify that there are no heating steps (i.e., “Elution” or “Antigen Retrieval”) after steps dispensing heat-sensitive reagents (ex. “Counterstaining” or “Staining”), unless this is an intentional choice.

Negative control

Negative controls are important to ensure the validity of the results obtained during an experiment. They allow the user to assess if the sample used is particularly subject to non-specific binding of the secondary antibody, or to which extent the elution of the primary antibody was successful.

To build a negative control cycle within your protocol, follow these steps:

1. Create a cycle with the following steps: “Initialization”, “Elution” (optional), “Secondary antibody”, “Counterstaining”, and “Imaging pause” (Figure 53). This can be done by deleting the “Primary antibody” step from a cycle in an advanced template. If the secondary antibody reagent you plan to use for the negative control contains a counterstain, delete the “Counterstaining” step as well.
2. Make sure that the “Elution” step, if required, is placed before the “Secondary antibody” and “Counterstaining” steps.

3.

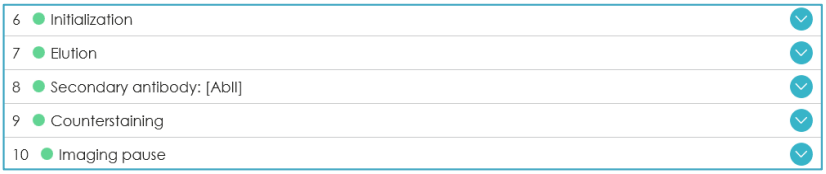


Figure 53) Negative control cycle. The "Elution" step is optional and should be added only if signal from a previous cycle needs to be removed before performing the negative control.

Directly labelled primary antibodies

Directly labelled primary antibodies are a useful tool in situations when the usual primary antibody-secondary antibody technique cannot be used; for example, when primary antibodies raised in the same species need to be used on a sample simultaneously.

To build a cycle compatible with directly labelled primary antibodies, follow these steps:

1. Create a cycle with the following steps: “Initialization”, “Elution” (optional), “Primary antibody”, “Counterstaining”, and “Imaging pause” (Figure 54). This can be done by deleting the “Secondary antibody” step from a cycle in an advanced template. Make sure to keep the “Counterstaining” step.
2. Make sure the “Elution” step, if required, is placed before the “Primary antibody” and “Counterstaining” steps.

Figure 54) Cycle using a directly labelled primary antibody. The "Elution" step is optional and should be added only if signal from a previous cycle needs to be removed.

Distribution chip change and wash countdowns

The Wash and Distribution chip countdown decrements by one unit after each “Initialization” step.

Protocol optimization and characterization

On LabSat® you can optimize the staining of a marker or cocktail of up to four markers from the pre-processing conditions for optimal epitope retrieval to the elution parameters for efficient signal removal. Additionally, the elution efficiency and epitope stability can be characterized. Below, is the three-part workflow to follow for protocol optimization and characterization.

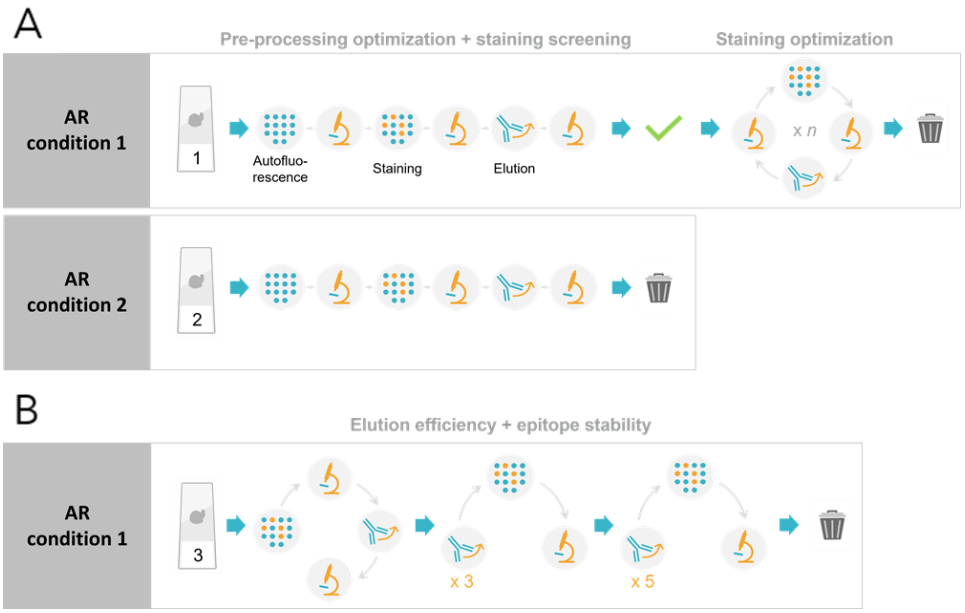


Figure 55) Protocol optimization workflow for a single marker that will be used in a 10-plex. A) Two first parts of protocol optimization: pre-processing (2 different conditions pictured in this example) and staining optimization (n being the number of relevant staining conditions to test). After the pre-processing optimization, the slide with optimal pre-processing is kept for the staining optimization part and the other slide(s) are discarded. B) Third part of protocol optimization: determining the elution efficiency and epitope stability over 10 cycles.

Part 1: Antigen retrieval and first screening

The starting point of the optimization process is the optimization of the sample pre-processing. Pre-processing determines how accessible the epitope of the marker of interest will be to the primary antibody.

Additionally, you will also run a first staining screening using the antibody dilutions recommended by the antibody provider to get preliminary results on the staining performance before starting the staining optimization process.

The following steps will guide you through the creation and execution of the protocols for antigen retrieval optimization and staining screening:

a) Determine the relevant antigen retrieval conditions to test. Conditions that can be compared are:
   - Different antigen retrieval solutions: pH 6 and pH 9
   - Different antigen retrieval temperatures
   - Different incubation durations
   - We recommend starting with the default antigen retrieval temperature and incubation parameters: temperature level 5 and 10:00 incubation.

b) Find the recommended primary and secondary antibody dilution provided by the antibody supplier for immunofluorescence applications and create the corresponding primary and secondary antibody reagents in the Reagents tab.

c) Create a new FFPE Sequential IF protocol for each antigen retrieval condition selected in part a), with the following protocol parameters:
   - Autofluorescence imaging: “On”
   - Number of cycles: 4
   - Advanced template: “On”
   - Select the washing buffer and protocol parameters of your choice.

d) The first cycle of the protocol created in c) is dedicated to antigen retrieval and autofluorescence imaging to **assess background fluorescence**.
   - Select the parameters of the “Antigen retrieval” step according to the conditions determined in part a).
   - Fill the reagent fields of the “Autofluorescence quenching” and the “Counterstaining” steps with the reagents that are routinely used for experiments.

e) The second cycle is a **negative control for specificity**. Only the secondary antibody is dispensed to check if the secondary antibody interacts in a non-specific way with the tissue.
   - Delete the “Primary antibody” step.
   - If the secondary antibody reagent you plan to use contains a counterstain, delete the “Counterstaining” step as well.
   - Select your secondary antibody reagent and keep the default incubation time.
   - This cycle is optional and can be skipped.

f) The third cycle is the **staining** itself.
   - Select the primary and secondary antibody reagents and the counterstaining reagent (if needed) created in b).
   - Leave Dynamic incubation on and the default incubation times (04:00 for the primary antibody and 02:00 for the secondary antibody) for the first screening.
   - Delete the “Elution” and “Autofluorescence quenching” steps.
   - To be able to compare the different pre-processing conditions, keep the staining conditions the same for all the pre-processing optimization protocols.

g) The fourth cycle is the **elution cycle**, to assess signal removal.
   - Select an elution buffer of your choice for the “Elution” step.
   - Leave the default number of dispenses and incubation time after the dispense (1 dispense followed by 00:30 incubation) for the first screening.
   - Delete the “Primary antibody” and “Secondary antibody” steps but keep the “Counterstaining” step to be able to image the slide after this cycle.

h) The last cycle is a **second negative control**, to make sure the elution of the primary antibody was successful.
   - As with the second cycle, use the “Secondary antibody” step to dispense only the secondary antibody reagent.
   - Delete the “Elution”, “Autofluorescence quenching” and “Primary antibody” steps.
   - This cycle is also optional and can be skipped.

i) Deparaffinize and rehydrate the number of slides corresponding to the number of pre-processing conditions to test.

j) Load the protocol corresponding to the first condition to test and run it on the corresponding slide.

k) Mount and image the slide at each “Imaging pause” step.

l) Once the imaging is done, unmount the slide and reload it on the machine along with a new Staining chip.

m) Repeat steps j) to l) for all the conditions that you wish to test.

n) Do not discard the slides until the screening is finished. Once the optimal pre-processing condition is found, keep the corresponding slide for part 2.

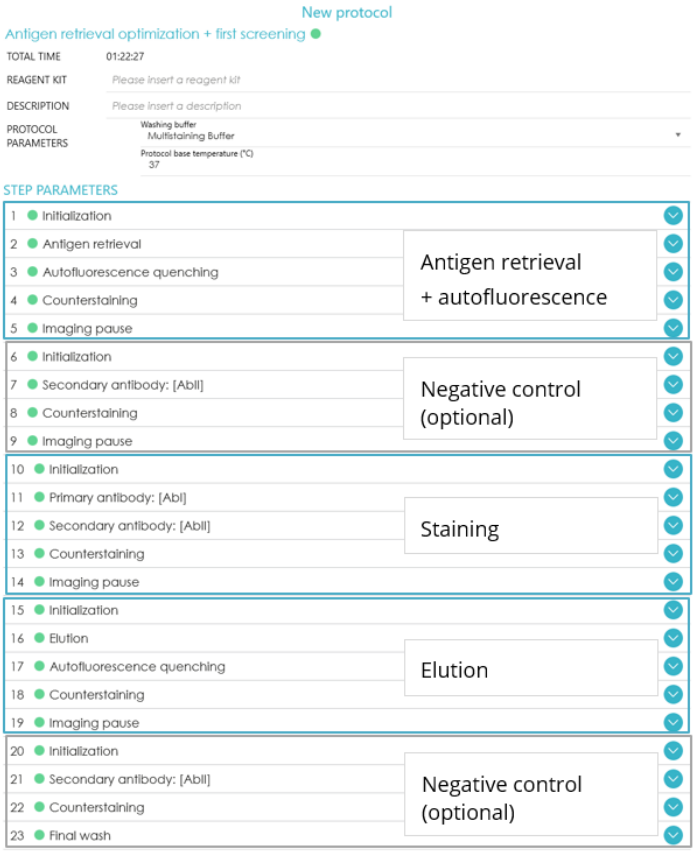


Figure 56) Protocol for pre-processing optimization and first staining screening. Cycles 2 and 5 are optional. Create as many versions of this protocol as the number of pre-processing conditions you wish to test. Only the antigen retrieval parameters should change between the different versions of the protocol.

Part 2: Staining optimization

Determining the optimal dilution and incubation time of primary and secondary antibodies for each marker is crucial to obtain a good staining signal with a satisfactory signal-to-background ratio and limited antibody non-specific binding.

The following steps will guide you through the creation and execution of the protocols for staining optimization:

a) Based on the result of the first staining screening performed in part 1, determine which parameters need to be adapted.

b) If the signal was too weak:
- Decrease the primary antibody dilution and/or increase its incubation time.
- Decrease the secondary antibody dilution and/or increase its incubation time (note: non-specific signal might increase when making these changes).
- Turn “On” the Dynamic incubation option for primary and secondary antibody incubations if it was “Off”.

c) If the signal was too strong:
- Increase the primary antibody dilution and/or decrease its incubation time.
- Increase the secondary antibody dilution and/or decrease its incubation time.
- If non-specific binding was observed, add a “Blocking” step before the “Staining” step or dilute the primary and/or secondary antibody in blocking buffer.

d) If the elution was suboptimal:
- Increase the number of dispenses of elution buffer.
- Increase the incubation time after each dispense of elution buffer.
- Increase the temperature of the “Elution” step.
- Refer to the Troubleshooting section if none of the suggestions above give satisfactory results.

e) Create the reagents corresponding to the relevant conditions to test, determined in step a) in the Reagents tab.
f) Create a new FFPE Sequential IF protocol, with the following protocol parameters:
   - Autofluorescence imaging: "Off"
   - Number of cycles: 2x the number of conditions to test (the staining and the elution are imaged for each condition).
   - Advanced template: "Off"
   - Select the washing buffer and protocol parameters of your choice.
g) The sample with the optimal pre-processing from part 1 should be re-used for the staining optimization. No antigen retrieval is therefore required and the "Antigen retrieval" step, along with the "Autofluorescence quenching" step, should be deleted from the first cycle.
h) The first cycle of the protocol is therefore a "Staining" step with the staining parameters selected in step a) that are expected to produce the lowest signal (i.e., the highest dilution the shortest incubation time of the primary/secondary antibody).
i) The second cycle will perform the elution of the first staining.
j) Delete the "Staining" step.
k) Fill the reagent field of the "Elution" step with your desired elution buffer.
l) The elution parameters (number of dispenses and incubation duration after dispensing) should be adapted if the elution was not satisfactory during part 1, as discussed in a).
m) Repeat steps e) and f) with the other conditions selected in a), in the order of increased expected signal. A maximum of 5 conditions can be tested because the maximum number of cycles in a protocol is 10.
n) Once the protocol is created, load it to the Protocol Area and perform the required actions.
o) Unmount the selected slide from part 1, load it on the device along with a Staining chip and close the handles.
p) Mount and image the slide at each "Imaging pause" step. Once the imaging is done, unmount the slide and reload it on the machine along with a new Staining chip. Repeat until the protocol is finished.
q) Qualitatively assess the staining results to determine which condition gave the best staining in terms of signal intensity and signal-to-background ratio. If the results are unclear by eye, conduct a quantitative analysis.

Note: At this stage of optimization, the effect of elution on the epitope stability remains uncharacterized. Test conditions in increasing order of expected signal, so that if a condition displays a weaker staining signal than the previous one, discard the current slide and continue the optimization on a new sample starting from the condition that gave a weaker staining signal than expected.

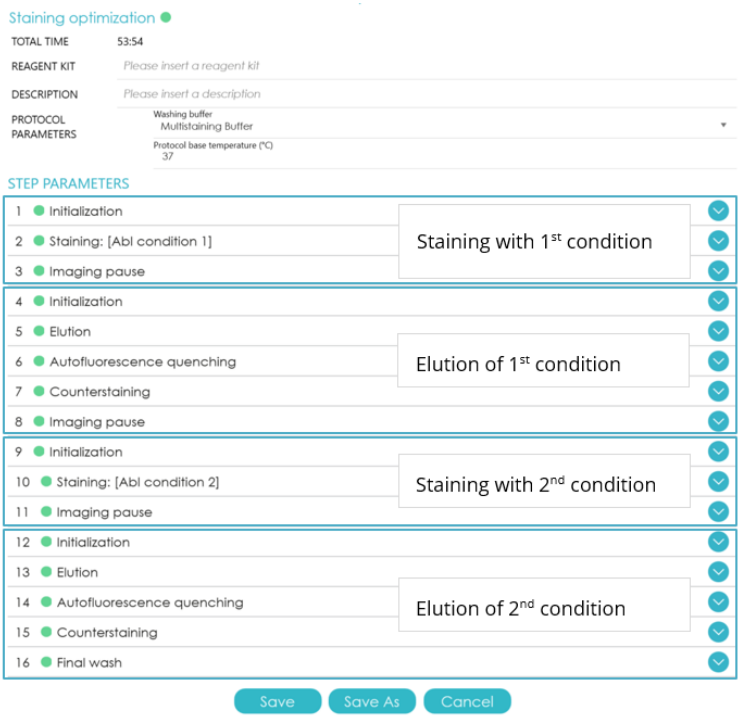


Figure 57) Example of protocol for staining optimization, with two different primary antibody conditions tested.

Part 3: Elution efficiency & epitope stability characterization

At this point, the optimal staining conditions have been established and only the elution efficiency and the epitope stability remain to be determined to define the position of the marker of interest in a multiplex protocol. This workflow will describe how to obtain the elution efficiency of your marker as well as the epitope stability at cycles 1, 5 and 10.

The elution efficiency can be computed by two different methods:

A) **Autofluorescence and elution method**: the autofluorescence image and the image after elution are taken into consideration to determine the elution efficiency. This technique does not assess the elution efficiency of the primary antibody. The results obtained using this technique should therefore be treated with caution.

B) **Negative controls method:** This technique takes into account the elution of both the primary and secondary antibodies but has a higher reagent consumption. With this technique, the negative control images before and after the staining cycle are used to determine the elution efficiency. These images are taken after dispensing secondary antibodies without having dispensed primary antibodies before. Since secondary antibodies do not have any primary antibodies to bind specifically to, they are more likely to bind non-specific targets and increase the background signal. Therefore, the elution efficiency determined using this technique may be lower than the actual one.

The protocols created to assess the epitope stability and elution efficiency differ if method A) or B) is chosen.

Protocol for method A)

1) Create a new FFPE Sequential IF protocol, with the following protocol parameters:
   a) Autofluorescence imaging: "On"
   b) Number of cycles: 4
   c) Advanced template: "Off"
   d) Select the washing buffer and protocol parameters of your choice.
2) The first cycle performs antigen retrieval and autofluorescence imaging to assess **background fluorescence**.
   a) Fill the reagents fields of the "Antigen retrieval", "Autofluorescence quenching" and the "Counterstaining" steps.

3) The second cycle is the first staining and will allow you to compute the **epitope stability at cycle 1** of your marker or cocktail of markers of interest
   a) Fill in the parameters of the "Staining" step with the optimal staining conditions identified in part 2. These conditions will be used for all the "Staining" steps of this protocol.
4) The third cycle is the first elution cycle. This cycle, along with the two previous ones, will allow you to **characterize the elution efficiency**.
   a) Fill the parameters of the "Elution" step with the optimal elution condition determined in part 2. These conditions with be used for all the "Elution" steps of this protocol.
   b) Delete the "Staining" step.
5) The fourth cycle will allow you to compute the **epitope stability at cycle 5**.
   a) Add 2 "Elution" steps after the default "Elution" step already present in the default template. This will mimic the effect of 3 cycles on the tissue (since the elution is the harshest step on the sample) while reducing the protocol duration.
   b) Keep the "Staining" step after the four "Elution" steps.
6) The fifth cycle will allow to compute the **epitope stability at cycle 10**.
   a) Add 4 "Elution" steps after the default "Elution" step already present in the default template. This will mimic the effect of 5 cycles on the tissue.
   b) Keep the "Staining" step after the four "Elution" steps.

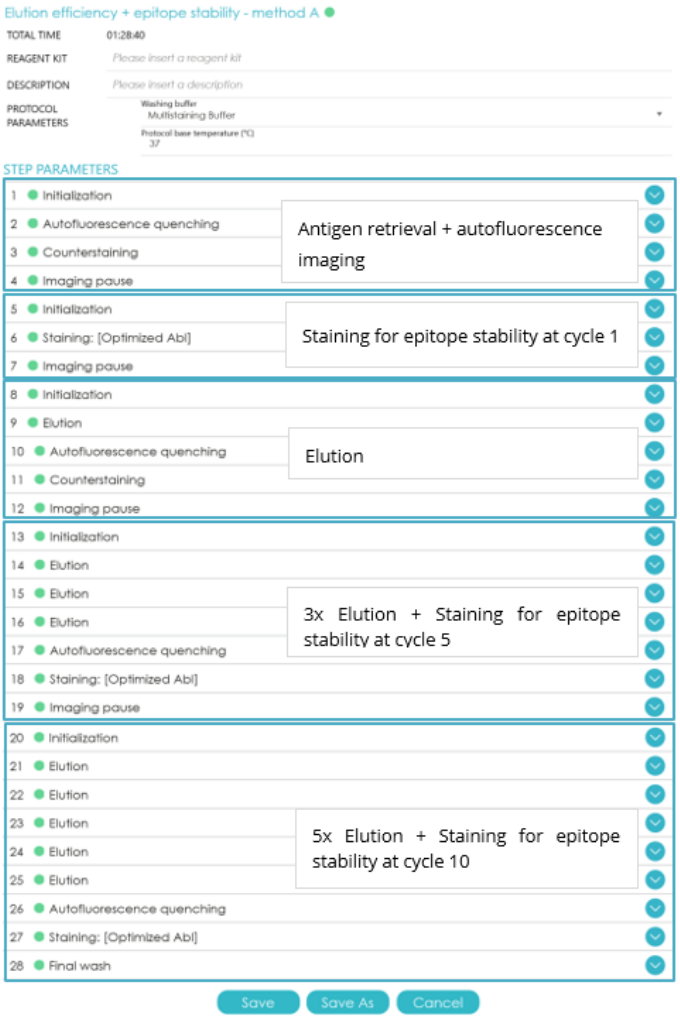


Figure 58) Protocol for elution efficiency and epitope stability determination with method A).

Protocol for method B)

Create the same protocol as for method A), except for the following parameters:

- When creating the protocol, turn the advanced template on (all the “Staining” steps will be split into “Primary antibody”, “Secondary antibody” and “Counterstaining” steps).
- In the third cycle, create a negative control cycle by deleting the “Primary antibody” step and filling the “Secondary antibody” step (and “Counterstaining” step if the counterstain is not cocktailed with the secondary antibody) with the optimal staining parameters.

Figure 59) Protocol for elution efficiency and epitope stability determination with method B).

Protocol execution for both methods

1. Deparaffinize and rehydrate one slide.
2. Load the new protocol to the Protocol area and perform the required actions.
3. Load the slide on the device along with a Staining chip and close the handles before starting the loaded protocol.
4. Mount and image the slide at each “Imaging pause” step. Once the imaging is done, unmount the slide and reload it on the machine along with a new Staining chip. Repeat until the protocol is finished.
5. Compute the elution efficiency using the first 3 cycles.
6. 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 the cycles.

Note: if you plan to use your optimized marker in a multiplex protocol with less than 10 cycles, it might not be useful to compute its epitope stability until cycle 10. Adapt this protocol to your needs by reducing the number of elutions in between stainings to mimic protocols with less cycles. For example, if your multiplex protocol has 8 cycles, remove one "Elution" step from cycle 4 and another one from cycle 5 to obtain data for epitope stability at cycles 4 and 8.

Troubleshooting for FFPE Sequential IF protocols

#	What	Detail	Solutions
01	No specific signal	There is no specific signal in the sample.	1. Check all the reagents were dispensed correctly by verifying status of each step in the report. 2. Make sure that selected fluorophores are compatible with excitation/emission set up of image acquisition system. 3. Check that the species reactivity of primary and secondary antibodies matches. 4. Check no reagent is expired. 5. Check the correct reagents were used and loaded in LabSat®. 6. Repeat staining with fresh reagents. 7. Test another antigen retrieval solution (change the pH). 8. Test a longer incubation time of the "Antigen retrieval" step. 9. Make sure the antigen retrieval process was successful (no bubbles in the chamber). 10. Increase concentration of primary and/or secondary antibodies. 11. Increase incubation time of primary and/or secondary antibodies. 12. Perform manual staining with the reagents to check their validity. 13. Perform the protocol on a known positive control tissue. 14. Change the reagents (use a new batch or try different brands, species, etc.)

02	Weak signal	The signal is present but weak	1. Check the staining steps were performed correctly by verifying status in the report. 2. Increase exposure time of the acquisition. 3. Make sure to perform image acquisition shortly after staining. 4. Make sure that the staining was not bleached before imaging: ensure that the light sensitive reagents / stainer were protected from light during staining and protect the slide from direct light once mounted. 5. Make sure to match the fluorophore with the optimum excitation/emission set up for image acquisition. 6. Increase the incubation time of the primary antibody. 7. Increase the incubation time of the secondary antibody. 8. Check that dynamic incubation option is toggled ON when it is recommended. 9. Increase the concentration of primary and/or secondary antibodies. 10. Change the primary antibody clone. 11. Repeat staining with fresh reagents. 12. Perform the protocol on a known positive control to check the validity of the reagents. 13. Test another antigen retrieval solution (change the pH). 14. Increase the temperature of the “Antigen retrieval” step. 15. Check that the species reactivity of primary and secondary antibodies matches. 16. Mount the samples with an anti-fade solution. 17. Change reagents (use a new batch or try different brands, species, etc).

03	Non-uniform staining	The staining is not uniform within the staining area	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Make sure the tissue did not partially dry during protocol execution (only the tissue fully in the chamber during all staining cycles should be considered). 3. If the signal in the non-uniform area is non-specific and at the edge of the tissue, it might be remaining signal from the previous staining cycle which was not eluted since this part of the tissue was outside the reaction chamber. Do not take this part of the tissue into consideration. 4. Check that dynamic incubation option is toggled ON when it is recommended. 5. Check all the reagents were dispensed correctly by verifying status of each step in the report. 6. Check that the used reagents are in the viscosity range defined by the user manual. 7. Repeat the staining with a new Staining chip and make sure: a. the staining process was successful (no bubbles in the chamber) b. there is enough volume of the reagents used c. there is DIW or Washing Buffer in all the unused reservoirs.
04	High general background	The signal to noise ratio is too low	1. Make sure the tissue does not dry during handling (before loading and after retrieving the slide from LabSat®). 2. Use a protein blocking solution (see “Blocking” section). 3. Reduce the incubation time of the antibody steps. 4. Dilute the antibody solutions more. 5. Change the primary antibody clone. 6. Perform a Full Wash. 7. Change the antigen retrieval incubation time and temperature. 8. Change the antigen retrieval solution.
05	One area is not stained	One area in the stainer is never stained properly	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check the staining steps were performed correctly by verifying status in the report. 3. Do not use highly concentrated detergent solutions. 4. Check that the used reagents are in the viscosity range defined by the user manual. 5. Perform a Daily or Full wash and change the Distribution chip.

06	Signal is too strong	The specific signal is too strong	1. Reduce exposure time of acquisition. 2. Dilute the primary and/or secondary antibodies more. 3. Reduce the incubation time of the primary and/or secondary antibody. 4. Repeat the staining using fresh reagents. 5. Test a shorter incubation time of the “Antigen retrieval” and/or “Elution” step. 6. Test a lower temperature of the “Antigen retrieval” and/or “Elution” step. 7. Change the reagents (use a new batch or try different brands, species, etc).
07	Signal crosstalk	Strong signal is detected in other channels	1. Use different species of primary antibodies when cocktailed together. 2. Use secondary antibodies with adequate reactivity with respect to the used primary antibodies. 3. Ensure that secondary antibody species are different than primary antibody species, to avoid secondary antibody recognizing the other secondary antibodies of the mix cocktail. 4. Ensure that each secondary antibody recognizes only one primary antibody in the mix.
08	Weak counterstain	The counterstain is too weak	1. Increase the exposure time. 2. Increase the incubation time of the counterstain step. 3. Dilute the counterstain less. 4. Check the counterstain step was performed correctly by verifying status in the report. 5. Repeat the staining using fresh reagents. 6. Change the counterstaining reagent (use a new batch or try a different brand).
09	Counterstain is too strong	The counterstain is too strong	1. Decrease exposure time. 2. Decrease the incubation time of the counterstain. 3. Dilute the counterstain solution. 4. Add an additional wash step after the staining step (if it includes counterstaining) or after the counterstaining step. 5. Change the counterstain (use a new batch or try a different brand). 6. Repeat the staining using fresh reagents.
10	Tissue morphology	The tissue morphology is altered	1. Decrease the incubation time of the “Antigen retrieval” step. 2. Test a lower temperature of the “Antigen retrieval” step and the “Elution” step. 3. Test another antigen retrieval solution (change the pH). 4. Improve pre-processing steps of tissue fixation.

11	Tissue detaching	The tissue detaches after the process	1. Use positively charged slides for tissue fixation. 2. Use coated histological slides for tissue fixation. 3. Improve pre-processing steps of tissue fixation. 4. Decrease the incubation time of the “Antigen retrieval” step. 5. Test a lower temperature of the “Antigen retrieval” step and the “Elution” step.
12	Overstaining	The signal is spreading out (the signal is not sharp) The signal is leaking out the structures (the signal is not sharp)	1. Reduce exposure time. 2. Reduce the incubation times of the antibody steps. 3. Dilute the primary antibody solution more. 4. Change the clone of the primary antibody. 5. Change the secondary antibody. 6. Test a shorter incubation time of the “Antigen retrieval” step and the “Elution” step. 7. Test a lower temperature of the “Antigen retrieval” step and the “Elution” step.
13	Non-specific staining	There is non-specific staining in the sample	1. Add a “Blocking” step in the protocol. 2. Change the clone of the primary antibody. 3. Dilute the primary antibody solution more. 4. Decrease incubation time of the secondary antibody. 5. Change the secondary antibody solution. 6. Add additional washing step after the staining steps in the protocol. 7. Perform a negative control of the same tissue to understand from which antibody the non-specific staining may come from. 8. Change the antigen retrieval incubation time. 9. Test another antigen retrieval solution (change the pH). 10. Use fresh reagents.
14	Autofluorescence	The tissue shows autofluorescence	1. Lower the antigen retrieval pH solution. 2. Use unstained tissue as negative controls. 3. Use the autofluorescence quenching buffer (BU08) in the dedicated steps.
15	Strong fluorescent dots (non-specific signal)	Secondary antibodies aggregates are visible on the tissue and on the glass surface	1. Repeat the staining using fresh secondary antibody solution. 2. Perform a Full Wash of the machine and change the Distribution chip. 3. Change the secondary antibody (use a new batch or try different brands, species, etc...)
16	Signal bleeding	The staining shows extremely deteriorated sharpness, with a halo-like distribution of the signal around targeted structures.	1. Change mounting medium (see section 12.2, table 23). 2. Change the secondary antibody solution.

17	Inefficient elution	The staining signal from a previous cycle is still appearing in subsequent ones after elution.	1. Increase the number of dispenses of Elution buffer. 2. Add TCEP to your Elution buffer.
18	Bubbles during elution	Bubbles appear in the chamber during the “Elution” step.	1. Decrease the temperature at which the elution is performed. 2. Decrease the incubation time of the Elution buffer.

Table 28) Troubleshooting guidelines for FFPE Sequential IF protocol optimization-related issue

Annex 2.5: FS Sequential IF protocol template

This annex presents the information regarding the FS Sequential IF protocol template of LabSat®.

Note: Only Sequential IF assays up to 10-plex on the same slide have been validated by Lunaphore. Additionally, slide integrity has been validated for up to 10 cycles.

Protocol creation and editing

To open a blank protocol template for editing, go to the Protocols tab and follow the steps below:

1. Click “Add new” above the protocol list. The “Create a new protocol” window will open.
2. Select “FS Sequential IF” in the FS branch. The protocol description will be displayed on the right side of the window.
3. Toggle the “Autofluorescence imaging” switch “On” or “Off” (“On” by default). When turned “On”, there will be a pause for the user to image the autofluorescence of the sample.
4. Select the desired number of cycles, between 1 and 10. A cycle is defined as a set of steps leading to a staining of up to 4 markers simultaneously followed by a pause in the protocol for slide imaging.
5. Toggle the “Advanced template” option “On” (“Off” by default) to access a more flexible and less guided protocol template. The advanced template splits the “Staining” step into 3 different steps: “Primary antibody”, “Secondary antibody” and “Counterstaining” step.
6. Click “OK”. The “New protocol” window will open (Figure 60) with the selected number of cycles.

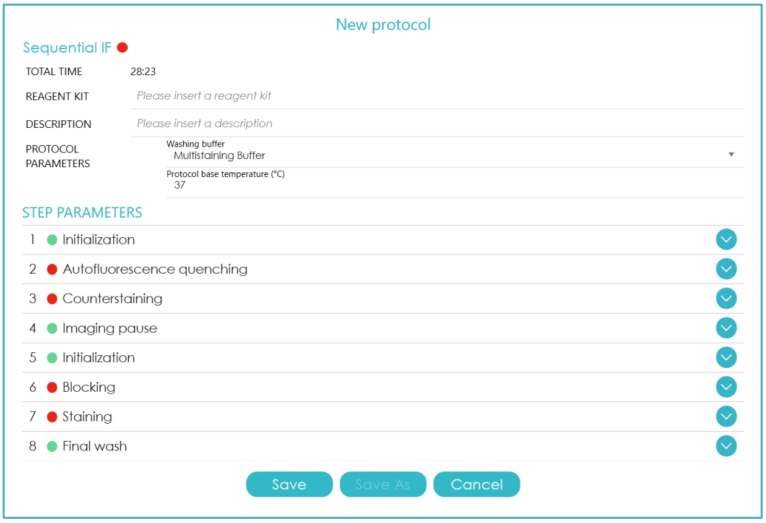


Figure 60) “New protocol” window for FS Sequential IF protocols

The “New protocol” window is divided in two sections, the general protocol parameters section (under the protocol name in blue) and the protocol steps section (under “Step Parameters” title in blue).

Editing the general protocol parameters

Fill in the general protocol parameters as follows:

- [Optional] Protocol name: click on the default name (“Sequential IF”) and type the new name.
- [Optional] Reagent kit: indicate the detection kit used.
- [Optional] Description: insert a short description of the protocol being created.
- [Required] Washing buffer (buffer used for tissue washes and intra-protocol washes): select the washing buffer from the drop-down list (Multistaining Buffer by default).
- [Required] Protocol base temperature (temperature at which the protocol will be executed): input a temperature in the text field (between 27 °C and 42 °C, 37 °C by default). The “Elution” step will be performed at a temperature chosen separately.

Editing the protocol steps

The steps displayed by default are “Initialization”, “Autofluorescence quenching”, “Counterstaining”, “Imaging pause”, “Blocking”, “Staining”, “Elution” and “Final Wash”. “Custom” step, “Primary antibody”, “Secondary antibody”, and “Wash” steps can be added to the template by clicking  . The steps present by default can also be added with these buttons.

If the advanced template is selected in the “New protocol” window, the “Primary antibody”, “Secondary antibody”, and “Counterstaining” steps will be displayed by default instead of the “Staining” step for each cycle. The Advanced template section below goes into the specific details of this option.

The details and parameters to fill for each step are presented below.

Default steps

Initialization

During this step slide loading is performed (the piston closes and clamps the chip and slide together creating a closed chamber, then the chamber is filled) and all the reservoirs containing reagents that have not been used are primed.

Parameters:

1. Select a buffer from the drop-down list. This buffer will be used as slide loading buffer. The available reagents are the protocol washing buffer and Deionized water (DIW).

Note: To ensure the optimal upkeep of LabSat®, Lunaphore recommends the use of DIW as slide loading buffer when possible.

Rules:

- A washing buffer must be selected.
- The “Initialization” step is mandatory and must be the first step of the protocol and the first step after each “Imaging pause” step.
- The protocol cannot contain more than one “Initialization” step per cycle.

Autofluorescence quenching

The “Autofluorescence quenching” step is intended to reduce the intrinsic tissue autofluorescence level.

Parameters:

1. Select a quenching buffer from the drop-down list. Reagents in the Autofluorescence quencher category are displayed.

Note: Once you have selected an Autofluorescence quencher in the first “Autofluorescence quenching” step of the protocol, the same reagent will automatically be selected for all the subsequent “Autofluorescence quenching” steps. To use a different quencher in subsequent steps, simply change it to the desired one from the drop-down list.

2. Enter an autofluorescence quencher incubation time from within the following range: 01:00 – 59:59 (mm:ss). The default incubation time is 02:00.

Lunaphore recommends the use of the following autofluorescence quenchers with LabSat® to obtain the best results.

- Quenching Buffer, Lunaphore, ref: BU08

Rules:

- The “Reagent” field must be filled.

Dispense volume:

The dispense volume of autofluorescence quencher is 350 µL.

Counterstaining

The “Counterstaining” step performs the counterstaining of the sample.

Parameters:

1. Select a counterstaining reagent from the drop-down list. Reagents in the "Counterstaining" category are displayed.

Note: Once you have selected a Counterstaining reagent in the first "Counterstaining" step of the protocol, the same reagent will automatically be selected for all subsequent "Counterstaining" steps and Counterstaining options of "Staining" steps. To use a different counterstain in subsequent steps, simply change it to the desired one from the drop-down menu.

2. Enter a counterstaining reagent incubation time from within the following range: 01:00 – 10:00 (mm:ss). The default incubation time is 02:00.

Rules:

- The "Reagent" field must be filled.
- The counterstaining reagent must be loaded in a small reservoir.

Dispense volume:

The dispense volume of counterstaining reagent is 280 µL.

Blocking step

The "Blocking" step dispenses and incubates blocking reagents.

Parameters:

1. Select a blocking reagent from the drop-down list. Reagents in the "Protein block" category are displayed.

Note: Once you have selected a blocking reagent in the first "Blocking" step of the protocol, the same reagent will automatically be selected for all subsequent "Blocking" steps. To use a different blocking reagent in subsequent steps, simply change it to the desired one from the drop-down menu.

2. Enter an incubation time from within the following range: 01:00 – 59:59 (mm:ss). The default incubation time is 02:00.

Note: To prevent non-specific binding of secondary antibodies on the tissue, we recommend using Lunaphore Blocking Buffer Kit (see Table 21). It efficiently blocks nonspecific electrostatic interactions related to anionic fluorescent dyes such as Alexa Fluor® dyes.

Rules:

- The "Reagent" field must be filled.

Dispense volume:

The dispense volume of blocking reagent is 230 µL.

Staining

The "Staining" step includes the dispense and incubation of primary antibody, secondary antibody, and counterstain. The counterstain dispense can be turned off; this is recommended when the secondary antibody reagent contains a counterstain. Primary antibody and secondary antibody dispenses can be complemented with "Dynamic incubation", a feature designed to enhance the staining quality. Up to 4 consecutive dispenses of primary and secondary antibodies are possible during a "Staining" step.

Note: To use directly labelled primary antibodies, refer to the Advanced template section below.

Parameters:

1. Primary antibody section
    a. Toggle the Dynamic incubation option "On" or "Off" (by default "On"). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).
    b. Select a primary antibody solution from the drop-down list. Reagents in the "Primary antibody" and "Primary antibody mix" categories are displayed.
    c. Enter a primary antibody incubation time from within the following range:
        i. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is "On".
        ii. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is "Off".
        iii. The default incubation time is 04:00 in both cases.
2. Secondary antibody section

a. Toggle the Dynamic incubation option “On” or “Off” (by default “On”). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).
b. Select a secondary antibody reagent from the drop-down list. Reagents in the “Secondary antibody” and “Secondary antibody mix” categories are displayed if Dynamic incubation is “Off”. If the Dynamic incubation option is “On”, the reagents in the “Secondary antibodies and counterstaining mix” category are displayed as well.
c. Enter a secondary antibody incubation time from within the following range:
    i. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is “On”.
    ii. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is “Off”.
    iii. The default incubation time is 02:00 in both cases.

3. Counterstaining section
    a. Toggle the Counterstaining option “On” or “Off” (by default “On”). By deactivating this option, the “Staining” step will skip the counterstaining dispense and incubation.
    b. Select a Counterstaining reagent from the drop-down list. Reagents in the “Counterstaining” category are displayed.
    c. Enter a counterstaining incubation time from within the following range: 01:00 – 10:00 (mm:ss). The default incubation time is 02:00.

Rules:

- A protocol cannot have more than one "Staining" step per cycle.
- The “Reagent” field of the primary antibody section must be filled.
- The “Reagent” field of the secondary antibody section must be filled.
- If the Counterstaining option is “On”, the “Reagent” field of the counterstaining must be filled.
- At most four primary antibodies can be used during a single “Staining” step (antibodies in mixes are counted individually).
- At most four secondary antibodies can be used during a single “Staining” step (antibodies in mixes are counted individually).
- Counterstaining is not mandatory but recommended for imaging. A warning is shown if the Counterstaining option is disabled, and the reagent selected as secondary antibody does not belong to the category Secondary antibody and counterstaining mix.
- If using antibody mixes, it is recommended to have a matching total number of antibodies between primary and secondary antibody sections (antibodies in mixes are counted individually).
- The “Staining” step cannot coexist with a “Primary antibody”, “Secondary antibody” or “Counterstaining” step in the same cycle.
- Reagents with the “Dynamic incubation” option turned on and the counterstaining reagent must be loaded in small reservoirs.

Dispense volume:

	Primary antibody reagent	Secondary antibody reagent	Counterstaining
If Dynamic incubation option is on	280 µL	280 µL	280 µL
If Dynamic incubation option is off	180 µL	180 µL	N/A

Imaging pause

The “Imaging pause” is an interactive step primarily intended to retrieve the slide from the stainer for imaging between marker detection cycles (Figure 19). The Staining chip used during the previous cycle must be discarded.

The “Imaging pause” step also provides the opportunity to wash reservoirs and allocate new reagents necessary for the following cycles. During this step, reservoirs can also be refilled if they contain reagents required in multiple cycles with a total protocol volume exceeding the reservoir’s capacity.

Once the reagents for the next cycle are loaded on LabSat®, the imaging procedure is done and the slide is unmounted, insert the slide in the stainer along with a new Staining chip before closing the handles and resuming the protocol.

Parameters:

There are no parameters to set in the “Imaging pause” step.

Rules:

- The “Imaging pause” step must always be followed by an “Initialization” step.

Elution

The “Elution” step is intended to remove the signal from a previous marker detection to be able to perform a new one on the same sample.

Parameters:

1. Select an elution buffer from the drop-down list. Reagents in the “Elution Buffer” category will be displayed.

Note: Once you have selected an elution buffer in the first “Elution” step of the protocol, the same reagent will automatically be selected for all subsequent “Elution” steps. To use a different elution buffer in subsequent steps, simply change it to the desired one from the drop-down menu.

2. Enter an Elution temperature from within the following range: 27°C to 50°C. The default temperature is 37°C.
3. Toggle the High flow option “On” or “Off”. This feature can enhance the elution efficiency but might not be suited for fragile samples.
4. Choose the number of Elution buffer dispenses you want to perform from the drop-down list.
5. Enter elution buffer incubation times after each dispense from within the following range: 00:30 – 10:00 (mm:ss). The default incubation time is 00:30.

Rules:

- There cannot be more than 5 “Elution” steps in the same cycle.
- The “Elution buffer” field must be filled.
- The total incubation time over all the elution buffer dispenses of the same “Elution” step cannot exceed 10 minutes.

Dispense volume:

The dispense volume of elution buffer is 230 µL per dispense.

Final wash

The “Final wash” step washes the tissue and rinses the dispense line at the end of the protocol.

Parameters:

1. Select a buffer from the drop-down list. The available reagents are the protocol washing buffer and DIW. The default buffer is DIW.

Note: To ensure optimal upkeep of LabSat®, Lunaphore recommends the use of DIW as Final wash buffer when possible.

Rules:

- The “Final wash” step is mandatory and must be placed at the end of the protocol.
- The protocol can only contain one “Final wash” step.
- The “Buffer” field must be filled.

Additional available protocol steps

Primary antibody

The “Primary antibody” step dispenses and incubates primary antibodies and primary antibody mixes. When used in combination with the “Secondary antibody” and “Counterstaining” steps, marker detection is more flexible than when using the combined “Staining” step. This step can also be used with the “Counterstaining” step only when using directly labelled primary antibodies.

Note: the "Primary antibody" step is considered an advanced step, therefore little guidance is provided during protocol creation. See the Advanced template section for more information.

Parameters:

1. Toggle the Dynamic incubation option "On" or "Off" (by default "On"). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).
2. Select a primary antibody reagent from the drop-down list. Reagents in the "Primary antibody" and "Primary antibody mix" categories are displayed.
3. Enter a primary antibody incubation time from within the following range:
    a. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is "On".
    b. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is "Off".
    c. The default incubation time is 04:00 in both cases.

Rules:

- There cannot be more than 4 "Primary antibody" steps in the same cycle.
- The "Primary antibody" step cannot coexist with the "Staining" step in the same cycle.
- The "Reagent" field must be filled.
- Reagents with the "Dynamic incubation" option turned on must be loaded in small reservoirs.

Dispense volume:

The dispense volume of primary antibody reagent is 280 µL if "Dynamic incubation" is "On" and 180 µL if "Dynamic incubation" is "Off".

Secondary antibody

The "Secondary antibody" step dispenses and incubates secondary antibodies and secondary antibody mixes. When used in combination with the "Secondary antibody" and "Counterstaining" steps, marker detection is more flexible than when using the combined "Staining" step.

Note: the "Secondary antibody" step is considered an advanced step, therefore little guidance is provided during protocol creation. Go to the Advanced template section for more information.

Parameters:

1. Toggle the Dynamic incubation option "On" or "Off" (by default "On"). This feature can enhance the quality of the marker detection but will increase the reagent volume consumption (see volume consumption table below).
2. Select a secondary antibody reagent from the drop-down list. Reagents in the "Secondary antibody" and "Secondary antibody mix" categories are displayed if Dynamic incubation is "Off". If the Dynamic incubation option is "On", the reagents in the "Secondary antibodies and counterstaining mix" category are displayed as well.
3. Enter a secondary antibody incubation time from within the following range:
    a. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is "On".
    b. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is "Off".
    c. The default incubation time is 02:00 in both cases.

Rules:

- There cannot be more than 4 "Secondary antibody" steps in the same cycle.
- The "Secondary antibody" step cannot coexist with the "Staining" step in the same cycle.
- The "Reagent" field must be filled.
- Reagents with the "Dynamic incubation" option turned on must be loaded in small reservoirs.

Dispense volume:

The dispense volume of secondary antibody reagent is 280 µL if "Dynamic incubation" is "On" and 180 µL if "Dynamic incubation" is "Off".

Wash

The "Wash" step is an extra wash of the tissue that can be placed anywhere in the protocol, except between the "Imaging pause" and the "Initialization" steps.

Parameters:

1. Select a buffer from the drop-down list. Reagents in the "Washing buffer" category are displayed. The default buffer is the protocol's washing buffer.

Rules:

- The "Buffer" field must be filled.

Custom step

The "Custom" step dispenses and incubates any reagent needed for the assay that does not have a dedicated step in the FS Sequential IF template; for example, permeabilization reagent, custom buffer, etc.

Parameters:

1. Toggle the Dynamic incubation option "On" or "Off" (by default "On"). This feature can improve the homogeneity of the reagent dispense in the chamber but will increase the reagent volume consumption (see volume consumption table below).
2. Select a reagent from the drop-down list. Reagents in the "Custom" or "Custom buffer" categories are displayed if Dynamic incubation is "Off". If the Dynamic incubation option is "On", only the reagents in the "Custom" category are displayed.
3. Enter an incubation time from within the following range:
    a. 01:00 – 10:00 (mm:ss) if the Dynamic incubation option is "On".
    b. 01:00 – 59:59 (mm:ss) if the Dynamic incubation option is "Off".
    c. The default incubation time is 02:00 in both cases.

Rules:

- If the "Dynamic incubation" option is turned on, the custom reagent must be loaded in a small reservoir.

Dispense volume:

The dispense volume of custom reagent is 280 µL if "Dynamic incubation" is "On" and 180 µL if "Dynamic incubation" is "Off".

Advanced template

The advanced template offers more flexibility during protocol design by splitting the combined "Staining" step into three individual steps: "Primary antibody", "Secondary antibody" and "Counterstaining". This feature allows the user to repeat up to 4 times in the same cycle both the "Primary antibody" and the "Secondary antibody" steps. The "Primary antibody", "Secondary antibody" and "Counterstaining" steps can also be individually added in a cycle, allowing for negative controls or the use of directly labelled primary antibodies.

Note: a protocol created using the standard template (advanced template option "Off") can always be partially or completely turned into an advanced protocol by replacing the "Staining" step in one or more cycles with advanced steps. The advanced steps rules then apply to this cycle.

To offer more freedom to the user, the number of warnings that are displayed is reduced in the advanced template compared to the standard one. Keep in mind the following points when building your desired protocol:

- If you plan to image a slide at the end of a cycle, make sure a counterstaining is performed at some point in the cycle, after "Elution" steps.
- When using advanced steps in a cycle, the total number of primary and secondary antibodies used in this cycle is not checked by the software.

Note: Lunaphore has tested and approved the use of up to 4 primary and secondary antibodies within the same cycle. If more antibodies are used within the same cycle, Lunaphore does not ensure satisfactory staining quality.

- Pay close attention to the order of the steps in each cycle and double-check that the final protocol matches your experimental plan.
- Verify that there are no heating steps (i.e., "Elution" steps) after steps dispensing heat-sensitive reagents (ex. "Counterstaining" or "Staining"), unless this is an intentional choice.

Negative control

Negative controls are important to ensure the validity of the results obtained during an experiment. They allow the user to assess if the sample used is particularly subject to non-specific binding of the secondary antibody, or to which extent the elution of the primary antibody was successful.

To build a negative control cycle within your protocol, follow these steps:

1. Create a cycle with the following steps: “Initialization”, “Elution” (optional), “Secondary antibody”, “Counterstaining”, and “Imaging pause” (Figure 61). This can be done by deleting the “Primary antibody” step from a cycle in an advanced template. If the secondary antibody reagent you plan to use for the negative control contains a counterstain, delete the “Counterstaining” step as well.
2. Make sure that the “Elution” step, if required, is placed before the “Secondary antibody” and “Counterstaining” steps.

Figure 61) Negative control cycle. The "Elution" step is optional and should be added only if signal from a previous cycle needs to be removed before performing the negative control.

Directly labelled primary antibodies

Directly labelled primary antibodies are a useful tool in situations when the usual primary antibody-secondary antibody technique cannot be used; for example, when primary antibodies raised in the same species need to be used on a sample simultaneously.

To build a cycle compatible with directly labelled primary antibodies, follow these steps:

1. Create a cycle with the following steps: “Initialization”, “Elution” (optional), “Primary antibody”, “Counterstaining”, and “Imaging pause” (Figure 62Figure 54). This can be done by deleting the “Secondary antibody” step from a cycle in an advanced template. Make sure to keep the “Counterstaining” step.
2. Make sure the “Elution” step, if required, is placed before the “Primary antibody” and “Counterstaining” steps.

Figure 62) Cycle using a directly labelled primary antibody. The "Elution" step is optional and should be added only if signal from a previous cycle needs to be removed.

Distribution chip change and wash countdowns

The Wash and Distribution chip countdown decrements by one unit after each “Initialization” step.

Protocol optimization and characterization

On LabSat® you can optimize the staining of a marker or cocktail of up to four markers from the antibody incubation parameters to the elution parameters for efficient signal removal. Additionally, the elution efficiency and epitope stability can be characterized. Below, is the three-part workflow to follow for protocol optimization and characterization.

Figure 63) Protocol optimization workflow for a single marker that will be used in a 10-plex. A) Two first parts of protocol optimization: staining screening and optimization (n being the number of relevant staining conditions to test). The slide used for staining screening can be re-used for the staining optimization. B) Third part of protocol optimization: determining the elution efficiency and epitope stability over 10 cycles.

Part 1: First screening

The starting point of the optimization process is a first screening using the antibody dilutions recommended by the antibody providers to get preliminary results on the staining performance before starting the staining optimization process.

The following steps will guide you through the creation and execution of the protocols for the first staining screening:

a) Find the recommended primary and secondary antibody dilution provided by the antibody supplier for immunofluorescence applications and create the corresponding primary and secondary antibody reagents in the Reagents tab.

b) Create a new FS Sequential IF protocol with the following protocol parameters:
- Autofluorescence imaging: "On"
- Number of cycles: 4
- Advanced template: "On"
- Select the washing buffer and protocol parameters of your choice

c) The first cycle of the protocol created in c) is to **assess background fluorescence**.
- Fill the reagent fields of the "Autofluorescence quenching", "Blocking", and the "Counterstaining" steps with the reagents that are routinely used for experiments.

d) The second cycle is a **negative control for specificity**. Only the secondary antibody is dispensed to check if the secondary antibody interacts in a non-specific way with the tissue.
- Delete the "Primary antibody" step but keep the "Blocking" step.
- If the secondary antibody reagent you plan to use contains a counterstain, delete the "Counterstaining" step as well.
- Select your secondary antibody reagent and keep the default incubation time. Select the blocking reagent of your choice.
- This cycle is optional and can be skipped.

e) The third cycle is the **staining** itself.
- Select the primary and secondary antibody reagents created in a) and the blocking and counterstaining (if needed) reagents.
- Leave Dynamic incubation "On" and the default incubation times (04:00 for the primary antibody and 02:00 for the secondary antibody) for the first screening.
- Delete the "Elution" and "Autofluorescence quenching" steps.
- To be able to compare the different pre-processing conditions, keep the staining conditions the same for all the pre-processing optimization protocols.

f) The fourth cycle is the **elution cycle**, to assess signal removal.
- Select an elution buffer of your choice for the "Elution" step.

- Leave the default number of dispenses and incubation duration after dispensing (1 dispense followed by 00:30 incubation) for the first screening.
- Delete the "Blocking", "Primary antibody" and "Secondary antibody" steps but keep the "Counterstaining" step to be able to image the slide after this cycle.

g) The last cycle is a **second negative control**, to make sure the elution of the primary antibody was successful.
- As with the second cycle, use the "Secondary antibody" step to dispense only the secondary antibody reagent and keep the "Blocking" step.
- Delete the "Elution", "Autofluorescence quenching" and "Primary antibody" steps.
- This cycle is also optional and can be skipped.

h) Perform the fixation on one FS slide.

i) Load and run the protocol.

j) Mount and image the slide at each "Imaging pause" step.

k) Once the imaging is done, unmount the slide and reload it on the machine along with a new Staining chip. Perform steps j) and k) until the end of the protocol.

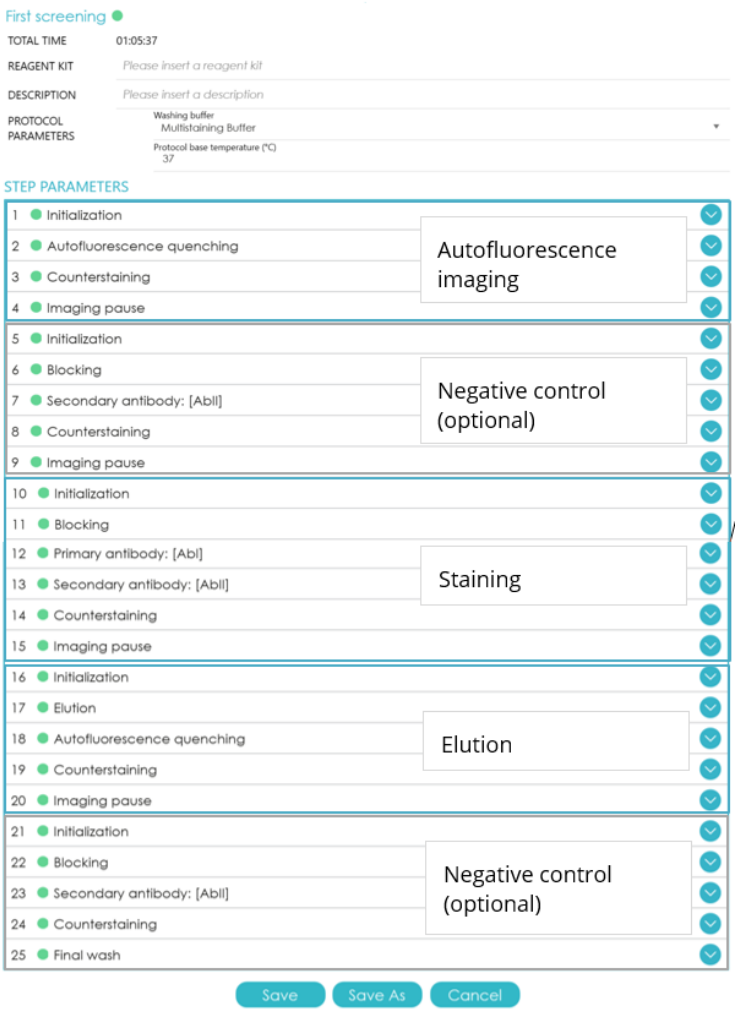


Figure 64) Protocol for the first staining screening. Cycles 2 and 5 are optional.

Part 2: Staining optimization

Determining the optimal dilution and incubation time of primary and secondary antibodies for each marker is crucial to obtain a good staining signal with a satisfactory signal-to-background ratio and limited antibody non-specific binding.

The following steps will guide you through the creation and execution of the protocols for staining optimization:

a) Based on the result of the first staining screening performed in part 1, determine which parameters need to be adapted.
b) If the signal was too weak:
   - Decrease the primary antibody dilution and/or increase its incubation time.
   - Decrease the secondary antibody dilution and/or increase its incubation time (note: non-specific signal might increase when making these changes).
   - Turn "On" the Dynamic incubation option for primary and secondary antibody incubations if it was "Off".
c) If the signal was too strong:
   - Increase the primary antibody dilution and/or decrease its incubation time.
   - Increase the secondary antibody dilution and/or decrease its incubation time.
   - If non-specific binding was observed, change the blocking reagent, increase the blocking reagent incubation, or dilute the primary and/or secondary antibody in blocking reagent.
d) If the elution was suboptimal:
   - Increase the number of dispenses of elution buffer.
   - Increase the incubation time after each dispense of elution buffer.
   - Increase the temperature of the "Elution" step.
   - Refer to the Troubleshooting section if none of the suggestions above give satisfactory results.
e) Create the reagents corresponding to the relevant conditions to test, determined in step a) in the Reagents tab.
f) Create a new FS Sequential IF protocol, with the following protocol parameters:
   - Autofluorescence imaging: "Off"
   - Number of cycles: 2x the number of conditions to test (the staining and the elution are imaged for each condition).
   - Advanced template: "Off"
   - Select the washing buffer and protocol parameters of your choice.
   - Delete the "Autofluorescence quenching" step from the first cycle.
g) The **first cycle** of the protocol is therefore a "Staining" step with the staining parameters selected in step a) that are expected to produce the lowest signal (i.e., the highest dilution the shortest incubation time of the primary/secondary antibody).
h) The **second cycle** will perform the elution of the first staining.
   - Delete the "Blocking" and "Staining" step.
   - Fill the reagent field of the "Elution" step with your desired elution buffer.
   - The elution parameters (number of dispenses and the duration of the incubation) should be adapted if the elution was not satisfactory during part 1, as discussed in a).
i) Repeat steps e) and f) with the other conditions selected in a), in the order of increased expected signal. A maximum of 5 conditions can be tested because the maximum number of cycles in a protocol is 10.
j) Once the protocol is created, load it to the Protocol Area and perform the required actions.
k) Unmount the selected slide from part 1, load it on the device along with a Staining chip and close the handles.
l) Mount and image the slide at each "Imaging pause" step. Once the imaging is done, unmount the slide and reload it on the machine along with a new Staining chip. Repeat until the protocol is finished.
m) Qualitatively assess the staining results to determine which condition gave the best staining in terms of signal intensity and signal-to-background ratio. If the results are unclear by eye, conduct a quantitative analysis.

Note: At this stage of optimization, the effect of elution on the epitope stability remains uncharacterized. Test conditions in increasing order of expected signal, so that if a condition displays a weaker staining signal than the previous one, discard the current slide and continue the optimization on a new sample starting from the condition that gave a weaker staining signal than expected.

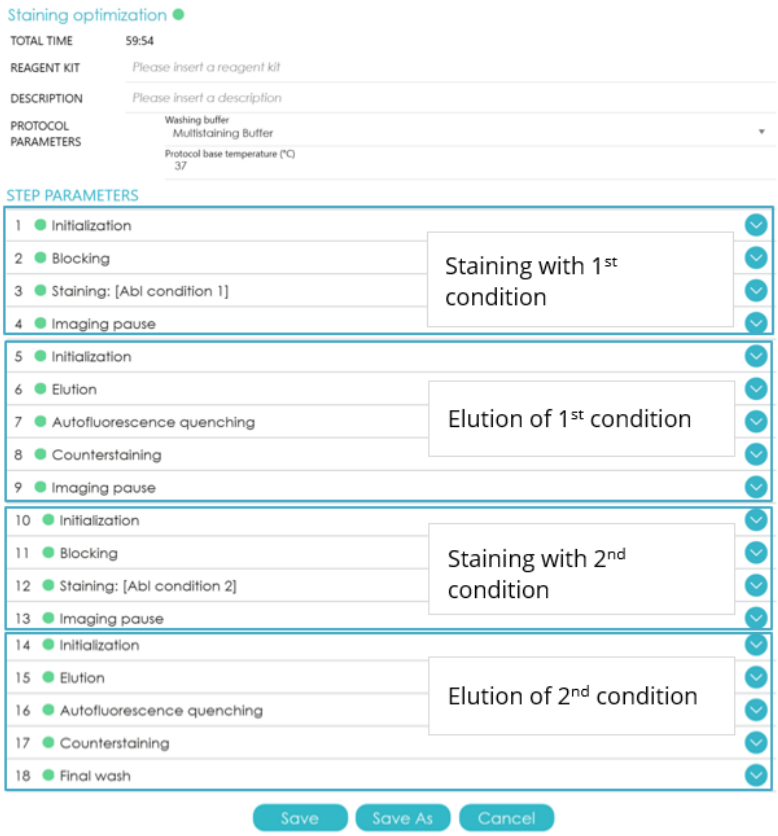


Figure 65) Example of protocol for staining optimization, with two different primary antibody conditions tested.

Part 3: Elution efficiency & epitope stability characterization

At this point, the optimal staining conditions have been established and only the elution efficiency and the epitope stability remain to be determined to define the position of the marker of interest in a multiplex protocol. This workflow will describe how to obtain the elution efficiency of your marker as well as the epitope stability at cycles 1, 5 and 10.

The elution efficiency can be computed by two different methods:

A) **Autofluorescence and elution method**: the autofluorescence image and the image after elution are taken into consideration to determine the elution efficiency. This technique does not assess the elution efficiency of the primary antibody. The results obtained using this technique should therefore be treated with caution.

B) **Negative controls method:** This technique takes into account the elution of both the primary and secondary antibodies but has a higher reagent consumption. With this technique, the negative control images before and after the staining cycle are used to determine the elution efficiency. These images are taken after dispensing secondary antibodies without having dispensed primary antibodies before. Since secondary antibodies do not have any primary antibodies to bind specifically to, they are more likely to bind non-specific targets and increase the background signal. Therefore, the elution efficiency determined using this technique may be lower than the actual one.

The protocols created to assess the epitope stability and elution efficiency differ if method A) or B) is chosen.

Protocol for method A)

a) Create a new FS Sequential IF protocol, with the following protocol parameters:
- Autofluorescence imaging: “On”
- Number of cycles: 4
- Advanced template: “Off”
- Select the washing buffer and protocol parameters of your choice.

b) The first cycle performs autofluorescence imaging to assess **background fluorescence**.

- Fill the reagents fields of the “Autofluorescence quenching” and the “Counterstaining” steps.

c) The second cycle is the first staining and will allow you to compute the **epitope stability at cycle 1** of your marker or cocktail of markers of interest
   - Fill in the parameters of the “Blocking” and “Staining” steps with the optimal staining conditions identified in part 2. These conditions will be used for all the “Blocking” and “Staining” steps of this protocol.

d) The third cycle is the first elution cycle. This cycle, along with the two previous ones, will allow you to characterize **the elution efficiency**.
   - Fill the parameters of the “Elution” step with the optimal elution condition determined in part 2. These conditions with be used for all the “Elution” steps of this protocol.
   - Delete the “Blocking” and “Staining” steps.

e) The fourth cycle will allow you to compute the **epitope stability at cycle 5**.
   - Add 2 “Elution” steps after the default “Elution” step already present in the default template. This will mimic the effect of 3 cycles on the tissue (since the elution is the harshest step on the sample) while reducing the protocol duration.
   - Keep the “Blocking” and “Staining” steps after the four “Elution” steps.

f) The fifth cycle will allow to compute the **epitope stability at cycle 10**.
   - Add 4 “Elution” steps after the default “Elution” step already present in the default template. This will mimic the effect of 5 cycles on the tissue.
   - Keep the “Blocking” and “Staining” steps after the four “Elution” steps.

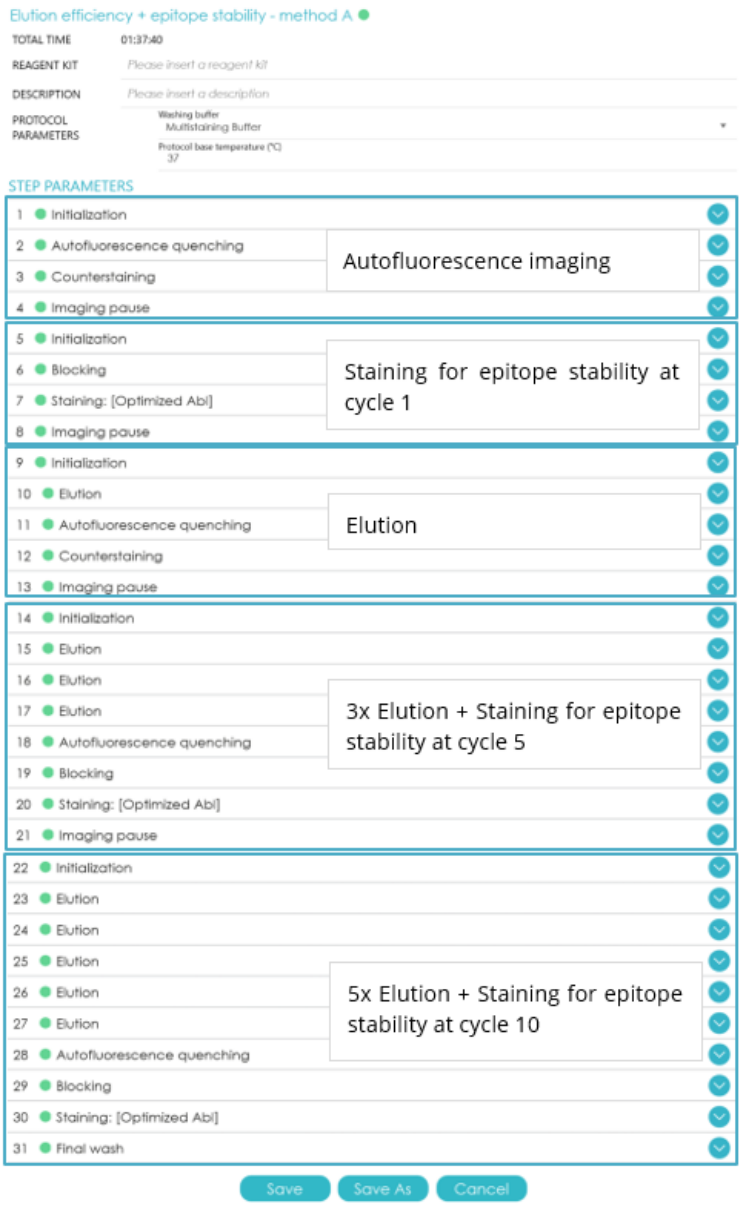


Figure 66) Protocol for elution efficiency and epitope stability determination with method A).

Protocol for method B)

Create the same protocol as for method A), except for the following parameters:

- When creating the protocol, turn the advanced template “On” (all the “Staining” steps will be split into “Primary antibody”, “Secondary antibody” and “Counterstaining” steps).
- In the third cycle, create a negative control cycle by deleting the “Primary antibody” step and filling the “Secondary antibody” step (and “Counterstaining” step if the counterstain is not cocktailed with the secondary antibody) with the optimal staining parameters.

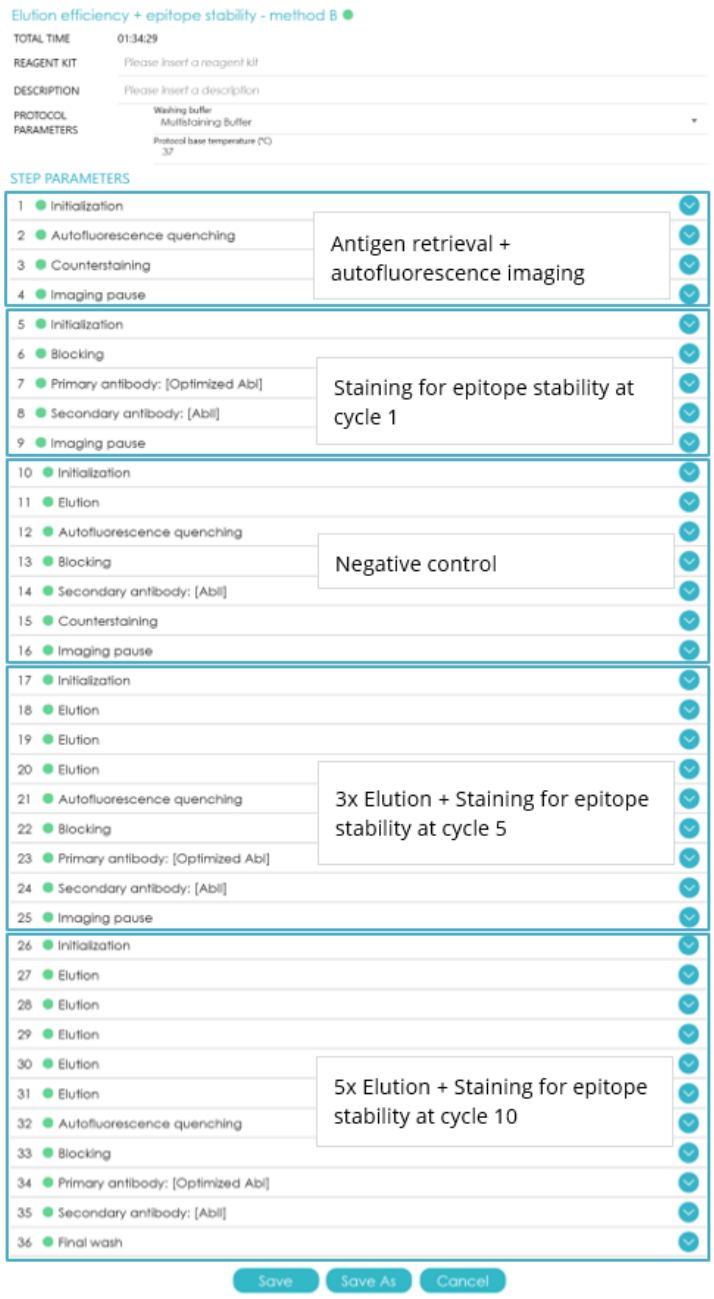


Figure 67) Protocol for elution efficiency and epitope stability determination with method B).

Protocol execution for both methods

1. Deparaffinize and rehydrate one slide.
2. Load the new protocol to the Protocol area and perform the required actions.
3. Load the slide on the device along with a Staining chip and close the handles before starting the loaded protocol.
4. Mount and image the slide at each “Imaging pause” step. Once the imaging is done, unmount the slide and reload it on the machine along with a new Staining chip. Repeat until the protocol is finished.
5. Compute the elution efficiency using the first 3 cycles.
6. 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 the cycles.

Note: if you plan to use your optimized marker in a multiplex protocol with less than 10 cycles, it might not be useful to compute its epitope stability until cycle 10. Adapt this protocol to your needs by

reducing the number of elutions in between stainings to mimic protocols with less cycles. For example, if your multiplex protocol has 8 cycles, remove one "Elution" step from cycle 4 and another one from cycle 5 to obtain data for epitope stability at cycles 4 and 8.

Troubleshooting for FS Sequential IF protocols

#	What	Detail	Solutions
01	No specific signal	There is no specific signal in the sample.	1. Check all the reagents were dispensed correctly by verifying status of each step in the report. 2. Make sure that selected fluorophores are compatible with excitation/emission set up of image acquisition system. 3. Check that the species reactivity of primary and secondary antibodies matches. 4. Check no reagent is expired. 5. Check the correct reagents were used and loaded in LabSat®. 6. Repeat staining with fresh reagents. 7. Increase concentration of primary and/or secondary antibodies. 8. Increase incubation time of primary and/or secondary antibodies. 9. Perform manual staining with the reagents to check their validity. 10. Perform the protocol on a known positive control tissue. 11. Perform pre-processing steps shortly before loading the slide in LabSat®. 12. Decrease time of fixation. 13. Try alternative fixation method. 14. Change the reagents use a new batch or try different brands, species, etc.).

02	Weak signal	The signal is present but weak	1. Check the staining steps were performed correctly by verifying status in the report. 2. Increase exposure time of the acquisition. 3. Make sure to perform image acquisition shortly after staining. 4. Make sure that the staining was not bleached before imaging: ensure that the light sensitive reagents / stainer were protected from light during staining and protect the slide from direct light once mounted. 5. Make sure to match the fluorophore with the optimum excitation/emission set up for image acquisition. 6. Increase the incubation time of the primary antibody. 7. Increase the incubation time of the secondary antibody. 8. Check that dynamic incubation option is toggled ON when it is recommended. 9. Increase the concentration of primary and/or secondary antibodies. 10. Change the primary antibody clone. 11. Repeat staining with fresh reagents. 12. Perform the protocol on a known positive control to check the validity of the reagents. 13. Change fixation method (try lower incubation in NBF 10%). 14. Decrease time of fixation. 15. Check that the species reactivity of primary and secondary antibodies matches. 16. Mount the samples with an anti-fade solution. 17. Change reagents (use a new batch or try different brands, species, etc.).

03	Non-uniform staining	The staining is not uniform within the staining area	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Make sure the tissue did not partially dry during protocol execution (only the tissue fully in the chamber during all staining cycles should be considered). 3. If the signal in the non-uniform area is non-specific and at the edge of the tissue, it might be remaining signal from the previous staining cycle which was not eluted since this part of the tissue was outside the reaction chamber. Do not take this part of the tissue into consideration. 4. Check that dynamic incubation option is toggled ON when it is recommended. 5. Check all the reagents were dispensed correctly by verifying status of each step in the report. 6. Check that the used reagents are in the viscosity range defined by the user manual. 7. Repeat the staining with a new Staining chip and make sure: a. the staining process was successful (no bubbles in the chamber) b. there is enough volume of the reagents used c. there is DIW or Washing Buffer in all the unused reservoirs.
04	High general background	The signal to noise ratio is too low	1. Make sure the tissue does not dry during handling (before loading and after retrieving the slide from LabSat®). 2. Use a protein blocking solution (see “Blocking” section). 3. Reduce the incubation time of the antibody steps. 4. Dilute the antibody solutions more. 5. Change the primary antibody clone. 6. Perform a Full Wash. 7. Make sure tissues are well dried (hot plate or air dry) before fixation. 8. Make sure slides do not come from a very old block.
05	One area is not stained	One area in the stainer is never stained properly	1. Make sure the tissue did not partially dry during handling (before loading and after retrieving the slide from LabSat®). 2. Check the staining steps were performed correctly by verifying status in the report. 3. Do not use highly concentrated detergent solutions. 4. Check that the used reagents are in the viscosity range defined by the user manual. 5. Perform a Daily or Full Wash and change the Distribution chip.

06	Signal is too strong	The specific signal is too strong	1. Reduce exposure time of acquisition. 2. Dilute the primary and/or secondary antibodies more. 3. Reduce the incubation time of the primary and/or secondary antibody. 4. Repeat the staining using fresh reagents. 5. Change the reagents (use a new batch or try different brands, species, etc.).
07	Signal crosstalk	Strong signal is detected in other channels	1. Use different species of primary antibodies when cocktailed together. 2. Use secondary antibodies with adequate reactivity with respect to the used primary antibodies. 3. Ensure that secondary antibody species are different than primary antibody species, to avoid secondary antibody recognizing the other secondary antibodies of the mix cocktail. 4. Ensure that each secondary antibody recognizes only one primary antibody in the mix.
08	Weak counterstain	The counterstain is too weak	1. Increase the exposure time. 2. Increase the incubation time of the counterstain step. 3. Dilute the counterstain less. 4. Check that the counterstaining step was performed correctly by verifying status in the report. 5. Repeat the staining using fresh reagents. 6. Change the counterstaining reagent (use a new batch or try a different brand).
09	Counterstain is too strong	The counterstain is too strong	1. Decrease exposure time. 2. Decrease the incubation time of the counterstain. 3. Dilute the counterstain solution. 4. Add an additional wash step after the “Staining” step (if it includes counterstaining) or after the “Counterstaining” step. 5. Change the counterstain (use a new batch or try a different brand). 6. Repeat the staining using fresh reagents.
10	Tissue morphology	The tissue morphology is altered	1. Improve pre-processing steps of tissue fixation.
11	Tissue detaching	The tissue detaches after the process	1. Use positively charged slides for tissue fixation. 2. Use coated histological slides for tissue fixation. 3. Improve pre-processing steps of tissue fixation.
12	Overstaining	The signal is spreading out (the signal is not sharp) The signal is leaking out the structures (the signal is not sharp)	1. Reduce exposure time. 2. Reduce the incubation times of the antibody steps. 3. Dilute the primary antibody solution more. 4. Change the clone of the primary antibody. 5. Change the secondary antibody.

13	Non-specific staining	There is non-specific staining in the sample	1. Add a “Blocking” step in the protocol. Use the recommended blocking solution: Lunaphore Blocking Buffer (see “Blocking” section). Change the clone of the primary antibody. 2. Dilute the primary antibody solution more. 3. Decrease incubation time of the secondary antibody. 4. Change the secondary antibody solution. 5. Add additional washing step after the staining steps in the protocol steps. 6. Perform a negative control of the same tissue to understand from which antibody the non-specific staining may come from. 7. Use fresh reagents.
14	Autofluorescence	The tissue shows autofluorescence	1. Use unstained tissue as negative controls. 2. Use fresh fixative solution or change it. 3. Use the autofluorescence quenching buffer (BU08) in the dedicated steps.
15	Strong fluorescent dots (non-specific signal)	Secondary antibodies aggregates are visible on the tissue and on the glass surface	1. Repeat the staining using fresh secondary antibody solution. 2. Perform a Full Wash of the machine and change the Distribution chip. 3. Change the secondary antibody (use a new batch or try different brands, species, etc...)
16	Signal bleeding	The staining shows extremely deteriorated sharpness, with a halo-like distribution of the signal around targeted structures.	1. Change mounting medium (see section 12.2, table 23). 2. Change the secondary antibody solution. 3. Use fresh fixative solution or change it.
17	Inefficient elution	The staining signal from a previous cycle is still appearing in subsequent ones after elution.	1. Increase the number of dispenses of Elution buffer. 2. Add TCEP to your Elution buffer.
18	Bubbles during elution	Bubbles appear in the chamber during the “Elution” step.	1. Decrease the temperature at which the elution is performed. 2. Decrease the incubation time of the Elution buffer.

Table 29) Troubleshooting guidelines for FS Sequential IF protocol optimization-related issue

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