Aluora™ Spatial Amplification Kits

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

Product description

Aluora™ Spatial Amplification is a highly sensitive method used in multiplex immunohistochemical (mIHC) experiments. It allows for the simultaneous detection of up to 8 fluorescent labels in a single experiment, with each label specifically optimized for spatial proteomics tissue imaging. This technology combines the optimized brightness of Aluora™ dyes with polyHRP-mediated tyramide-like signal amplification, which results in an excellent signal to background ratio, providing a high level of precision, sensitivity, and requiring less primary antibody.

The sensitivity achieved with Aluora™ Spatial Amplification Kits is 10–200 times greater than standard IHC techniques and 2–10 times greater than other tyramide amplification and catalyzed reporter deposition (CARD) techniques. The Aluora™ Spatial Amplification reagents are optimized for use with Invitrogen Prolong Glass Antifade Mountants and 1mg/mL Thermo Scientific DAPI Nucleic Acid Stains for multiplex analysis.

Key features of Aluora™ Spatial Amplification Kits include the following:

- **Signal amplification using Aluora™ kits:** These kits employ Aluora™ dyes, which have the ability to react with HRP (horseradish peroxidase) to deposit bright and photostable Aluora™ dyes onto proteins and similar molecules in the immediate area. The Aluora™ Spatial dyes work together to enhance signal amplification, specifically optimized for spatial proteomics tissue imaging and color unmixing. This technology ensures a significant improvement in signal to background ratio, and reduces the amount of primary antibody required.
- **Comprehensive spatial analysis and imaging in research:** The Aluora™ Spatial dyes, provides the possibility to achieve up to 8-plex multiplexing for spatial imaging on spectral imaging systems. This means that up to 8 different fluorophores, each with similar brightness, can be utilized simultaneously to enable the detection of multiple markers in a single sample.
- **Automation compatible fluorescent system:** These kits are specifically designed to be compatible with automated slide staining instruments without requiring any additional modifications. This allows for seamless integration and automation of the staining process.
- **Workflow flexibility:** The Aluora™ reagents offer the advantage of being covalent labels. This means that they can be used in combination with a primary conjugated antibody labeling step or a primary/secondary labeling step. This flexibility allows researchers to choose the most suitable modality to visualize each protein of interest in the tissue, based on their specific experimental needs and preferences. The Aluora™ system can be used with all types of FFPE tissues that undergo labeling with standard IHC techniques.
- **PolyHRP antibody enhancement:** The anti-IgG Aluora™ Spatial Amplification Kits contain upgraded, highly cross-absorbed polyHRP-conjugated antibodies specific to the primary antibody being used for antigen labeling. In the Aluora polyHRP kits, several HRP enzymes are conjugated to each antibody, enhancing the signal several fold over regular HRP systems, and reducing the quantity of primary antibody needed.
- **Highly cross-adsorbed specific secondary antibodies:** These kits employ highly-cross-adsorbed secondary antibodies, which are then conjugated to form the polyHRP. When performing multiple antibody labeling, this high cross-adsorption helps ensure specificity with minimal cross-labeling.
- **Reduction of background:** These kits include blockers for the elimination or reduction of endogenous peroxidase and fluorescent background signals. These blockers help ensure that only specific targets result in fluorescent signals, while keeping non-specific and background signals in check.
- **Primary antibody flexibility:** The Aluora™ kits come with secondary detection reagents for use with Mouse and Rabbit primary antibodies, as well as for use with Biotin labeled antibodies from any species with the Aluora™ Streptavidin-HRP kits. These kits enable use of biotin conjugated primaries, and can be used in conjuction with Invitrogen™ antibody labeling kits (Cat. No. R10711) to add biotin as needed to primary antibodies of interest.

Contents and storage

Table 1 Aluora™ Spatial Amplification Kits

Material	Amount	Concentration	Storage
Dimethylsulfoxide (DMSO) (Component A) [2, 4]	200 µL	N/A	2–8°C [2]
Blocking buffer (10% Goat Serum) (Component B)[4]	22.5 mL	1X	2–8°C
PolyHRP-conjugated secondary antibody or HRP-conjugated streptavidin	22.5 mL	1X	
Hydrogen peroxide (Component C) [4]	28.5 mL	Stabilized 3% solution	
Reaction buffer (Component D) [1, 4]	6 mL	20X	
Reaction stop reagent (Component E) [4]	2×8 mg	N/A	2–8°C [2]
Aluora™ Spatial dye	1 vial [3]	N/A	–25°C to –5°C [2,4]

[1] Reaction Buffer can be replaced with Tris Buffer, pH 7.4 for similar performance.
[2] Desiccate
[3] Sufficient material is provided for up to 100 slides based on the protocol.
[4] Protect from light

Table 2 Aluora™ Spatial dyes provided in Aluora™ Spatial Amplification Kits

Labeled Dye	Excitation (Ex)	Emission maxima (Em)	PolyHRP-Goat Anti-Mouse IgG	PolyHRP-Goat Anti-Mouse Rabbit IgG	HRP-Streptavidin	Kit size
Aluora™ 430	427 nm	499 nm	A40001329	A40001337	A40001345	100 slides
Aluora™ 488	493 nm	518 nm	A40001330	A40001338	A40001346	
Aluora™ 514	512 nm	529 nm	A40001331	A40001339	A40001347	
Aluora™ 555	553 nm	567 nm	A40001332	A40001340	A40001348	
Aluora™ 594	589 nm	615 nm	A40001333	A40001341	A40001349	
Aluora™ 647	652 nm	670 nm	A40001334	A40001342	A40001350	
Aluora™ 700	687 nm	706 nm	A40001335	A40001343	A40001351	
Aluora™ 750	757 nm	783 nm	A40001336	A40001344	A40001352	

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier. Catalog numbers that appear as links open the web pages for those products.

Item	Source
Tissue	MLS; use positive and negative controls as needed
Slides, coverslips, containers	MLS
Primary antibodies	MLS
PBS (phosphate buffered saline), pH 7.4 (without calcium, magnesium, or phenol red)	10010031
PBS tablets	003002
95% ethanol	MLS
Distilled water, highly purified	15230-147
Hydrophobic Barrier Pap Pens	R3777
Image-iT™ Fixation/Permeabilization Kit	A5818101
1 mg/mL Thermo Scientific™ DAPI Nucleic Acid Stain	62248
ReadyProbes™ Streptavidin/Biotin Blocking Solution (1X)	R37628
IHC Antigen Retrieval Solution – Low pH (10X)	00-4955-58
IHC Antigen Retrieval Solution – High pH (10X)	00-4956-58
BlockAid™ Blocking Solution	B10710
ProLong™ Glass Antifade Mountant	P36982
SlowFade™ Glass Soft-set Antifade Mountant	S36917
eBioscience™ StainTray	44-0404-10

Procedural guidelines

- When using Aluora™ Spatial Amplification Kits for the first time, optimize the protocols following the guidelines in **signal optimization**.
- A hydrophobic barrier PAP pen (wax pen) can be used to hold liquid reagents on the sample slide or coverslip.
- For longer incubations, a humidified chamber (for example, the eBioscience StainTray) can be used.
- To ensure the optimal pairing of Aluora™ Spatial dyes refer to **factors for optimal pairing** .
- For use with an automated slide stainer both 100 µL and 150 µL of reagent and dye per slide can be used.
- When using biotinylated ligands or primary antibodies on tissues, ensure to block the endogenous biotin, biotin receptors, and streptavidin binding sites.

Before first use

- The Reaction Stop Reagent stock solution should only be prepared if needed, based on sample preparation type and quantity.
- Before treating the tissue for endogenous peroxidase activity in **step 1**, it is necessary to deparaffinize and dehydrate the tissue according to standard IHC protocols.
- Optimize sample preparation and input by running a dilution series to ensure all targets are in the assay's dynamic range.

Workflow

- The Aluora™ Spatial Amplification Kits featuring Aluora™ dyes, consist of all the essential components required for the labeling and detection of tissue samples using standard IHC techniques.
- Each kit provides ample Aluora™ reagents, allowing for the labeling of 100 slides with a recommended reaction volume of 100 µL per slide during critical incubation steps.
- The specific volumes provided may vary depending on the kit size, as outlined in **table 2** of the kit documentation.
- It is important to note that the reaction volume can be adjusted as needed to accommodate samples of different sizes.
- The Aluora™ core reagent kits can include additional volumes to support the use of automated slide staining instruments, further enhancing the convenience and versatility of the Aluora™ Spatial Amplification Kits.

Typical labeling and detection workflow using Aluora™ Spatial Amplification Kits

Prepare reagents

- Prepare 100X Aluora™ Spatial dye stock solution
- Prepare 100X Hydrogen Peroxide (H_2O_2) solution
- Prepare 1X Reaction Buffer
- Prepare Reaction Stop Reagent stock solution as needed

Pretreat the tissues using Hydrogen Peroxide Solution

Label the tissues using Aluora™ Spatial dyes

- Prepare an Aluora™ Spatial dye working solution
- Using Aluora™ Spatial dye working solution and incubation
- Apply Reaction Stop Reagent if required
- Rinse with PBS and multiplex
- Multiplex tissue samples

Counterstain nuclei in the labeled tissue

- Counterstain the tissue with DAPI or other nuclear dye
- For optimal results mount the coverslips using a ProLong™ or SlowFade™ glass mountant
- Analyze the tissue

Guidelines to multiplex with primary antibodies from the same species

- To prevent cross-reactivity, it is necessary to strip the antibodies from the tissue in between staining cycles.
- There are several methods available for stripping/antigen retrieval in immunohistochemistry (IHC), depending on the equipment and resources at hand.
- Heat the slides to 90–98°C (without boiling) for 10–20 minutes in 1x IHC Antigen Retrieval Low pH Solution (10 mM Sodium Citrate, pH 6.0).
- We highly recommend optimizing the experimental conditions to acquire the most specific signal and minimal background.
- Once the antibodies have been stripped, if desired, repeat **step 1** from (Peroxidase labeling) to **step 4** from (Aluora™ dye labeling),with a primary antibody of the same species.

  Note: It is important to use a different Aluora™ dye that is spectrally compatible with the Aluora™ Spatial dyes used in the previous rounds.

Guidelines to multiplex with primary antibodies from different species

To multiplex, use a primary antibody from a host different from the one used in **step 5**, and a fluorescent label that is spectrally compatible with the first fluorescent label. Repeat the protocol from **step 1**.

Experimental protocol

- Do not let the tissue samples dry out throughout the IHC standard procedure.

1 Prepare reagents

1. **Prepare 100X Aluora™ dye stock solution**
   a. Dissolve the Aluora™ dye reagent in 100 µL of DMSO (Component A) (for 100 slides).
   b. Invert the vial several times to dissolve any tyramide that might coat the sides of the vial.
   c. Prepare single use aliquots.
   d. Store the 100X dye stock solution at –25°C to –5°C for up to 6 months in sealed tubes, away from moisture.

   Note: Allow vials to reach room temperature before opening and adding DMSO, which is critical to getting a homogenous solution.

2. **Prepare 100X Hydrogen Peroxide (H_2O_2) solution —** Add 1 drop (approximately 50 µL) of 3% Hydrogen Peroxide solution (Component C) to 1 mL of distilled water.

   Note: Prepare the 100X H_2O_2 solution fresh on the day of use.

3. **Prepare 1X Reaction Buffer —** Add 1 drop (approximately 50 µL) of 20X Reaction buffer (Component D) to 1 mL of distilled water.

   Note: Prepare the 1X Reaction buffer fresh on the day of use. Tris buffer at pH 7.4 can be substituted for Reaction buffer for similar performance.

4. **Prepare Reaction Stop Reagent stock solution (as needed)**
   a. Add 1.45 mL of 95% ethanol to one vial of Reaction Stop Reagent (Component E).
      The Reaction Stop Reagent stock solution is diluted 1:11 in PBS before use, to prepare Reaction Stop Reagent working solution.
   b. Vortex the vial to dissolve any stop reagent coating the sides of the bottle.

   Note:
   - All these solutions and buffers need to be prepared fresh before use.
   - The unused portion of the stock solution can be stored at –20°C for 6 months.

2 Label the tissues with primary antibodies and secondary HRP reagents

1. Quench the endogenous peroxidase activity of the sample by adding enough drops of 3% Hydrogen Peroxide Solution (Component C) to cover the sample. Incubate the sample in a humidified staining tray, at room temperature for 15–60 minutes , depending on the tissue type. Do not let the tissue samples dry out.

2. Rinse the tissue three times with 1X PBS at room temperature.

3. **If using HRP-conjugated streptavidin**, block the endogenous biotin in the sample using ReadyProbes™ Streptavidin/Biotin Blocking Solution (1X) (Cat. No. R37628). Rinse the tissue three times with 1X PBS at room temperature before proceeding to the next step.

4. Add 2–3 drops (approximately 100–150 µL) of Blocking Buffer (Component B) to the sample and incubate for 30–60 minutes at room temperature.

5. To label the tissue with primary antibody with mouse or rabbit as the host, dilute the antibody or biotin-conjugated ligand in Blocking Buffer (Component B) or another compatible blocking solution such as 2% BSA or BlockAid™ Blocking Solution (Cat.No. B10710). Incubate with the tissue in a humidified staining tray for 60 minutes at room temperature or overnight at 2–8°C.

   Note: The concentration of the primary antibody in **step 5** plays a crucial role in obtaining high-resolution images with specific signal. We highly recommend optimizing the antibody concentration by using positive and negative control slides at various concentrations when conducting this experiment for the first time.

6. Rinse the tissue for 5–10 minutes with PBS at room temperature. Repeat this step three times.

2 *(continued)*

7. Add 2–3 drops (approximately 100–150 µL) of poly HRP-conjugated secondary antibody or HRP-conjugated streptavidin to the tissue then incubate for 30–60 minutes in a humidified staining tray, at room temperature or overnight at 2–8°C.

   Note: If you observe non-specific signal, you can shorten this incubation period.

8. Rinse the tissue for 5–10 minutes with PBS at room temperature. Repeat this step three times.

3 Label the tissues using Aluora™ dye solution

1. **Prepare an Aluora™ dye working solution according to the below table:**

Number of coverslips (18 mm × 18 mm)	100X dye stock solution	100X H_2O_2 solution	1X Reaction buffer
5	5 µL	5 µL	500 µL
10	10 µL	10 µL	1 mL
20	20 µL	20 µL	2 mL
50	50 µL	50 µL	5 mL
100	100 µL	100 µL	10 mL

   Note: The volumes in this table are calculated based on the requirement of 100 µL of Aluora™ dye working solution per 18 mm × 18 mm coverslip. You can adjust this volume according to the size of the coverslip or the volume needed for automated slide staining.

2. Apply 100 µL of the Aluora™ dye working solution to the tissue then incubate in a humidified staining tray, for 10 minutes at room temperature.

3. If required, apply 100 µL of Reaction Stop Reagent prepared in **step 4**.

   Note: The Stop Reagent inhibits the HRP labeling reaction, and its use can help ensure similar signal intensities when preparing multiple samples simultaneously. However, if samples are moved directly into a PBS wash immediately after staining, this step is not required.

4. Rinse the tissue three times with PBS.

5. Following **step 4**, it is possible to multiplex tissue samples with another Aluora™ dye or by employing standard IHC protocols.

   Note: The Aluora™ Spatial Amplification Kits are designed to be compatible with automated slide stainers. Some systems allow a 100 µL dispensing per slide, however, it can be necessary to adjust the reagent volumes based on the specific system being used.

4 Counterstain nuclei in the labeled tissue

1. Counterstain the tissue as needed using standard protocols. For optimal results, we recommend 1mg/mL DAPI (Cat. No. 62248). Rinse the tissue three times with 1X PBS at room temperature.

2. For optimal results mount the coverslips using a mountant with superb antifade properties and a refractive index (RI) above 1.51 after curing such as ProLong™ Glass Antifade Mountant (Cat. No. P36980) or SlowFade™ Glass Antifade Mountant (Cat. No. S36917- 5 x 2 mL).

3. Analyze the tissue using EVOS S1000 or a compatible imaging instrument. The Aluora™ Spatial Amplification system is compatible with all types of fluorescent microscopes equipped with compatible fluorescent filters - see Table 3 for reference.

Factors to ensure optimal pairing of Aluora™ Spatial dyes

To ensure the optimal pairing of Aluora™ dyes with markers, we recommend considering the following factors during the selection process:

- **Co-expression:** If the research involves studying the co-expression of markers on a specific cell type, choose fluorophores that are not spectrally adjacent to each other. This facilitates, effective distinction and visualization of different markers.
- **Rare vs. Abundant markers:** For markers that are expressed at lower levels, it is beneficial to assign them to brighter fluorophores. This enhances their detection and visibility, hence the more abundant markers can be assigned to dimmer fluorophores as their higher expression levels compensates for the lower fluorescence intensity.

Table 3 Pairing factors for Aluora™ Spatial dyes

Channel	Aluora™	Multiplex order	Brightness ranking	Primary antibody dilution concentration	Ex/Em	EVOS cube
430	Aluora™ 430		Lowest	Most	427/499	CFP
488	Aluora™ 488	Stain earlier	High	Least	493/518	GFP
514	Aluora™ 514		Medium	Least	512/529	YFP
555	Aluora™ 555	Stain earlier	Medium	Low	553/567	RFP
594	Aluora™ 594	Stain earlier	High	Least	589/615	TexasRed
647	Aluora™ 647		High	Low	652/670	Cy5
700	Aluora™ 700	Stain later/last	Low	Medium	687/706	Cy5.5
750	Aluora™ 750		Low	Medium	757/783	Cy7

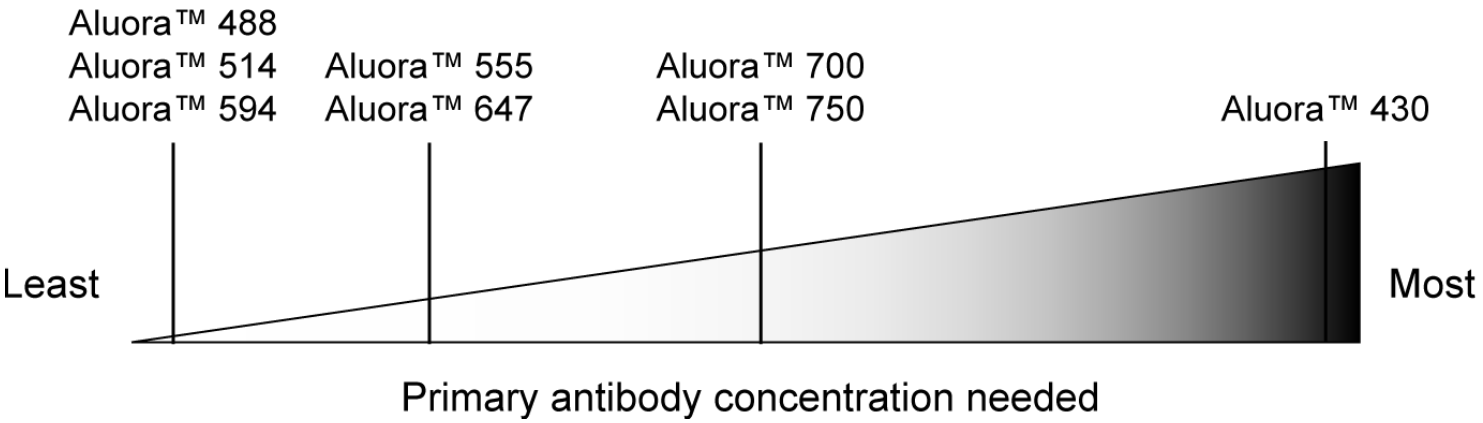

Figure 1 Aluora™ Spatial dyes versus antibody concentration

Aluora™ Spatial Amplification Kit applications

The applications of Aluora™ Spatial Amplification Kits include:

- Signal amplification by using the Aluora™ Spatial kits can lead to cost savings and increased experimental efficiency, since it allows the user to use significantly lower amounts of primary antibody compared to standard IHC experiments although still achieving the same level of signal intensity.
- Valuable in situations where high endogenous autofluorescence is observed or when the detection of low-abundance targets is critical. This is because the kits greatly enhance the specific signal intensity over background, enabling clear and reliable detection even in challenging experimental conditions.
- Multiplexed images can be achieved with up to 8 Aluora™ dye labels and a DAPI counterstain, resulting in an 8+1 plex.
- Provide a user-friendly solution that can seamlessly expand standard 3–4 color IHC experimental protocols to 9 plex multiplex immunohistochemical (mIHC) spatial tissue imaging protocols, applicable to various tissue types. The method shares similarities with other enzymatic IHC detection procedures like diaminobenzidine (DAB), making it easy for current IHC users to adapt and integrate into their workflows.
- Compatible with multiple primary antibodies from the same host species. This facilitates easier multiplexing, which is often challenging to achieve with standard IHC labeling techniques.
- Provide a straightforward and adaptable solution for expanding the capabilities of IHC experiments, enabling the generation of comprehensive multiplex spatial images with enhanced data resolution.
- Expands spatial labeling options with the Streptavidin kits being able to amplify any biotin labeled antibody or ligand.

Signal optimization—experimental conditions for specific signal and minimal background

To optimize the amount of primary antibody or ligand used in **step 5**, we recommend testing the following conditions:

- **Slide 1:** 10-fold dilution of the amount used for the primary antibody dilution as the standard fluorescent IHC method or as recommended by the manufacturer (for example, a suggested dilution of 1:100 becomes 1:1000)
- **Slide 2:** 10-fold dilution of the amount used for Slide 2 (further dilution may be necessary especially for sensitive antibodies and/or abundant markers)
- **Slide 3:** Negative control (antibody or ligand omitted)

You can dilute the antibody or ligand in Component B (10% goat serum) or another compatible blocking solution such as 2% BSA or BlockAid™ Blocking Solution (Cat. No. B10710).

Incubation time for Aluora™ dye labeling:

- We do not recommend adjusting the Aluora™ dye incubation time as a first step to address signal issues.
- Optimizing the primary antibody concentration can address many issues. However, if further optimizing is needed, perform 0, 2.5, 5, 7.5 and 10 minute incubations using positive and negative control slides.
- In case of non-specific signal found in negative controls or if the signal is blurry in positive controls, decrease the incubation time.
- In case dim or no signal is found in positive controls, increase the incubation time.

Troubleshooting

Observation	Recommended action
Excess signal	• Optimize the primary antibody dilution • Shorten the incubation time with the dye • Decrease the dye reagent concentration
Low signal	• Optimize the primary antibody dilution and incubation time • Lengthen the incubation time with the tyramide reagent working solution • Use antigen retrieval techniques to unmask the signal
Low resolution or blurry signal	• Optimize the primary antibody dilution and incubation time • Check the dilution of the Stop reagent
High background	• Lengthen the incubation time with the H_2O_2 solution (step 1) to decrease endogenous peroxidase activity. • Decrease the primary antibody concentration • Lengthen the incubation time for the blocking step (step 4). • Increase the number and/or the length of the wash steps • Shorten the incubation time with the tyramide reagent working solution • Use a lower concentration of secondary antibody than recommended • Check for endogenous biotin (if using streptavidin conjugates) and use ReadyProbes™ Streptavidin/Biotin Blocking Solution (1X) (Cat. No. R37628) to minimize interference from endogenous biotin.

Documentation and support

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN1000360 A

Revision	Date	Description
B	12 February 2025	Storage temperature is changed from 2-8°C to –25°C to –5°C in reagent prepration.
A	19 September 2024	New document created for Aluora™ Spatial Amplification Kits in CCMS. Converted the legacy document, to the current document template, with associated updates to the publication number, limited license information, warranty, trademarks, and logos.