KIT COMPONENTS

PRODUCT CODE	DESCRIPTION
XTM4001KT	PhenoFlamma™ 6-Plex mIHC Kit

Components

XTR1031	10X TR1 Buffer
XTR1032	10X TR2 Buffer
XTR1041	Blocking Buffer
XTR2001	Astra Diluent
RSA4011	Mouse/Rabbit HRP 2nd Antibody
XTD1001	Astra DAPI
XTR1021	DMSO
XTR1022	H_2O_2
XTD1011	ASTRA - 480
XTD1031	ASTRA - 520
XTD3011	ASTRA - 570
XTD2051	ASTRA - 620
XTD3041	ASTRA - 690
XTD3051	ASTRA - DIG
XTA3051	ASTRA - 780 (Anti-DIG)

Box 1	
10X TR1 Buffer	1 × 100 mL
10X TR2 Buffer	2 × 250 mL
Blocking Buffer	1 × 100 mL
Astra Diluent	1 × 60 mL
Mouse/Rabbit HRP 2nd Antibody	1 × 7 mL
Astra DAPI	1 × 10 mL
DMSO	1 × 0.5 mL
H_2O_2	1 × 0.5 mL

Box 2	
10X TR2 Buffer	2 × 250 mL
Blocking Buffer	1 × 100 mL
Astra Diluent	1 × 60 mL
Mouse/Rabbit HRP 2nd Antibody	1 × 7 mL
- MiniBox	
ASTRA - 480	1 vial
ASTRA - 520	1 vial
ASTRA - 570	1 vial
ASTRA - 620	1 vial
ASTRA - 690	1 vial
ASTRA - DIG	1 vial
ASTRA - 780 (Anti-DIG)	1 vial

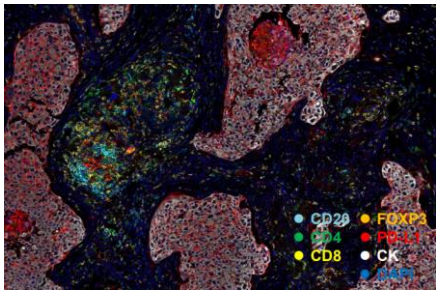
PhenoFlamma™ 6-Plex mIHC Kit

Catalog number : XTM4001KT

PRODUCT CODE	DESCRIPTION
XTM4001KT	PhenoFlamma™ 6-Plex mIHC Kit

Cat. No.	Component	Amount	Storage
XTR1031	10X TR1 Buffer	1 × 100 mL	• 2–8°C • Protect from light • Avoid repeated freezing and thawing cycles
XTR1032	10X TR2 Buffer	4 × 250 mL	
XTR1041	Blocking Buffer	2 × 100 mL	
XTR2001	Astra Diluent	2 × 60 mL	
RSA4011	Mouse/Rabbit HRP 2nd Antibody	2 × 7 mL	
XTD1001	Astra DAPI	1 × 10 mL	
XTR1021	DMSO	1 × 0.5 mL	
XTR1022	H_2O_2	1 × 0.5 mL	
XTD1011	ASTRA - 480	1 vial	
XTD1031	ASTRA - 520	1 vial	
XTD3011	ASTRA - 570	1 vial	
XTD2051	ASTRA - 620	1 vial	
XTD3041	ASTRA - 690	1 vial	
XTD3051	ASTRA - DIG	1 vial	
XTA3051	ASTRA - 780 (Anti-DIG)	1 vial	

OVERVIEW



PhenoFlamma™ mIHC is an immunostaining kit designed for multi-fluorescent tissue staining in FFPE tissue (Formalin-fixed, paraffin-embedded tissue). PhenoFlamma™ is able to perform multiple immunostaining by staining the target biomarker via primary-secondary antibody fluorescence labeling, and then removing all antibodies except fluorescence-labeled biomarker through microwave treatment (MWT). The characteristics of stained biomarker is not significantly affected under MWT, and after MWT, primary antibody of the same species (Host) can be reused for the next target detection without the contamination by cross-reactions.

PhenoFlamma™ mIHC allows simultaneous staining of up to 6 biomarkers that allowing correlation analysis between target molecules that are difficult to analyze with conventional methods. In addition, construction of an efficient antibody panel is useful for immune profiling analysis such as cancer tissue microenvironment and detection of expression of specific markers.

PRODUCT INFORMATION

Stability : See kit label on outside of box for expiration date.

Application : PhenoFlamma™ mIHC Kits are dedicated to multiplex IHC staining.

Safety Note : DMSO is classified as hazardous and combustible. DAPI is considered corrosive to the skin and an irritant to the e ye. All other reagents are classified as non-hazardous. It is strongly recommended that you wear disposable glove s and eye protection while working with the components of this kit.
Thorough washing of hands after handling is recommended.

Quality Control : We certify that QC results of these reagents meet our quality release criteria.

Slider Number : When used under the recommended conditions of this kit (Astra-dye, 1:150 dilution), 4 or 7 colors for 50 slides(3 or 6-plex + DAPI) staining is possible.

PhenoFlamma™ 6-Plex mIHC Kit

Catalog number : XTM4001KT

MATERIALS REQUIRED BUT NOT PROVIDED

- Xylene : Deparaffinization of FFPE tissue
- Ethanol (Histological grade) : Rehydration of FFPE tissue
- Microwave : Microwave treatment

Note : Rating of 1,000W or more, 10 or more power settings are required.

- Standard staining dishes
- Slide incubation/humidity tray
- Hydrophobic barrier pen
- Glass coverslips
- Control tissues
- Charged slides
- TBST wash buffer (0.05% Tween20)
- Neutral buffered formalin (NBF)
- Peroxidase-free water

Note : This specification may be met by commercial "cell culture grade" water or ultra-pure (eg. Milli-QTM) water.

- Primary antibodies
- Mounting medium (for fluorescent)

PREPARATION OF SOLUTION

- TBST Wash Buffer
- : After diluting 10X Tris-HCl (250mM, pH7.5) in peroxidase-free water at a ratio of 1:10, add 0.05% Tween20.
- TR1 or TR2 Buffer Working Solution
  : Prepared by diluting 10X TR1 or TR2 Buffer in peroxidase-free water at a ratio of 1:10.
- Primary Antibody Working Solution
  : Prepared by diluting the primary antibody in blocking buffer to the optimal concentration for PhenoFlamma™ mIHC.
- Secondary Antibody Working Solution
  : Prepared by diluting Ms/Rb HRP 2nd antibody in blocking buffer at a ratio of 1:8.
- Astra-dye
  : Prepared by dispensing 75 µL of DMSO into Astra-dye.

Note : Note : Astra-dye dissolved in DMSO is stored at 2-8℃.

- Astra Diluent Solution (0.0015%) H_2O_2
  : By diluting H_2O_2 (hydrogen peroxide) as follows, prepare Astra Diluent solution with final concentration 0.0015% H_2O_2.

1) Prepare 0.15% H_2O_2 solution (A) by adding 1 µL of 3% H_2O_2 to 19 µL of Astra Diluent.
2) Add 10 µL of 0.15% H_2O_2 solution (A) to 990 µL of Astra Diluent to make a final concentration of 0.0015% H_2O_2.

- Astra-dye Working Solution
  : Prepare Astra-dye Working Solution by diluting Astra-dye in Astra Diluent Solution (0.0015% H_2O_2) at a ratio of 1:150. 100~300 µL of Astra-dye Working Solution is required per slide, and the remaining Astra-dye Working Solution is discarded.

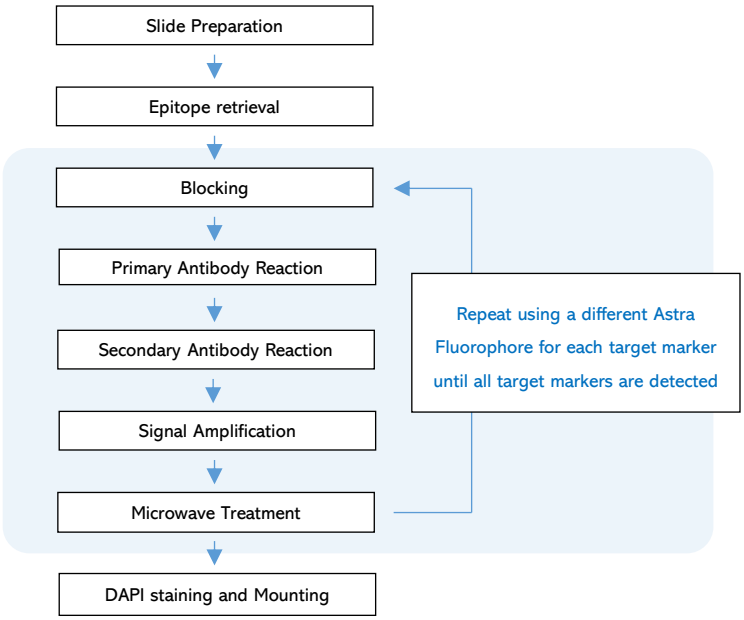
RECOMMENDATIONS

- Xylene is used to remove paraffin from FFPE tissue sections, and slides between steps must not dry out.
- Humidified chambers are recommended for all reaction steps.
- To avoid reagent dilution and uneven staining, before adding the next solution, drain as much of the reaction solution as possible and use absorbent paper to remove any remaining solution around the tissue section (not over the tissue).
- Use enough of each reagent to completely cover tissues or cells.
- Before multi-plex staining, the assay conditions (dilution factor) for each primary antibody are optimized through single-plex staining.
- In order to remove endogenous peroxidase, pre-treatment with 3% H_2O_2 Blocking buffer, followed by blocking step.
- When using Astra-dye (in DMSO), ensure that all solution is at the bottom of the tube by spinning the tube in a standard microcentrifuge.
- Microwave treatment (MWT) performs antigen retrieval, removes antibodies from previous steps, and quenches endogenous peroxidases. The time for each step of the protocol should be used after being optimized for the performance of the microwave. The microwave chamber must be kept clean and free of debris.

1) Place slides vertically into Slide Processing Jar, then fill to top level (~140 mL) with 1X TR1 or 1X TR2 Buffer and loosely cover. Place one Jar at a time near the edge of the turntable in the microwave so that the energy is evenly distributed.
2) Process about 45~90 seconds using 100% power to reach boiling point. The time for this step may need to be increased or decreased depending on the performance of the microwave.
3) Process for 15 minutes at 20% power.

- As this protocol was developed with the specified reagents, other options require independent validation.

EXPERIMENTAL PROCEDURE



PhenoFlamma™ mIHC PROTOCOL

Before proceeding with multiplex IHC, the IHC conditions for each single antibody should be optimized. The concentration for each primary antibody is optimized with Astra-dye yielding within an exposure time of 50~250 ms. This protocol details a method for single antibody IHC, which can be used for multiplex IHC staining. For multiplex IHC, antibodies and Astra-dye detection sequences should be independently validated.

- Slide Preparation

1. Prepare tissues or cells using standard fixation and embedding techniques.
   ※ For each experiment, it is recommended to simultaneously use an isotype control sample corresponding to the primary antibody.
2. After incubating each slide in a dry oven at 60℃ for 1 hour, dewax (3 x 10 minutes) with xylene.
3. Proceed with rehydration (100%, 10 minutes; 95%, 10 minutes; 70%, washing) using Ethanol.
4. After rehydration, the slides are briefly washed in distilled water and then fixed in 10% neutral buffered formalin (NBF) for 20 minutes.
   For certain tissues, such as skin, longer fixation times in NBF may be required.

5. Wash slides with distilled water (Briefly).
6. Wash slides with TR1 (Briefly).

• Epitope retrieval

1. Place the slides in the Slide Processing Jar and completely fill the TR1 buffer.
2. Cover the container loosely with a lid and microwave for 50 seconds at 100% power.
※ Optimization required.
3. Microwave for 15 minutes at 20% power.
4. Cool the slides at room temperature for 15~30 minutes.
※ Be careful not to dry out the tissues on slides.
5. Wash slides with TBST. (Briefly)
6. Wash slides with distilled water. (Briefly)

• Blocking

1. Using a hydrophobic barrier pen, mark around the tissue section to ensure complete tissue containment.
2. Place the slides in a humidified chamber, dispense 100~300 µL Blocking buffer on each tissue, and incubate at room temperature for 10 minutes.

• Primary Antibody Reaction

1. Drain the blocking buffer and dispense the Primary Antibody Working Solution on each tissue. ※ Using a humidified chamber.
2. Incubate according to antibody manufacturer's guidelines for incubation time and temperature requirements, or under optimized conditions in the laboratory. (Example: 1 hour at room temperature)
3. Use an appropriate amount of reagent to cover the tissue. (100~300 µL /slide)
4. Wash slides with TBST. (3 x 2 minutes)

• Secondary Antibody Reaction

1. Dispense Second Antibody Working Solution and incubate the slide at room temperature for 10 minutes.
※ Using a humidified chamber.
2. Use an appropriate amount of reagent to cover the tissue. (100~300 µL /slide)
※ Ms/Rb HRP 2nd antibody is recommended for human tissue experiments using primary antibodies of mouse or rabbit origin. Other options must be independently verified.
3. Wash slides with TBST. (3 x 2 minutes)

• Signal Amplification (Astra-dye Reaction)

1. Dispense 100~300 µL of Astra-dye Working Solution to each tissue.
2. Incubate for 20 min at room temperature.
※ Using a humidified chamber.
3. Wash slides with TBST. (3 x 2 minutes)
4. Wash slides with TR2 buffer. (Briefly)

• Microwave treatment

1. Place the slides in the Slide Processing Jar and completely fill the TR2 buffer.
2. Cover the container loosely with a lid and microwave for 50 seconds at 100% power.
※ Optimization required.
3. Microwave for 15 minutes at 20% power.
4. Cool the slides at room temperature for 15~30 minutes.
※ Be careful not to dry out the tissues on slides.
5. Wash slides with TBST. (Briefly)
6. Wash slides with distilled water. (Briefly)
7. After removing the primary-secondary-HRP complex through MWT, the introduction of the next primary antibody is allowed. Repeat steps 3~7 for the next antibody staining, performing step 8 after all staining is complete. (Except Astra-780)

• DAPI staining & Mounting

1. After dropping DAPI 3~5 times on the tissue, incubate for 5 minutes at room temperature.
※ Using a humidified chamber.
2. Wash slides with TBST. (2 minutes)
3. Wash slides with distilled water. (2 minutes)
4. Dispense mounting medium (1~2 drops) on the tissue and cover the tissue with a coverslip

ASTRA-DYE EXCITATION AND EMISSION

Fluorophore	Excitation	Emission
DAPI	360 nm	460 nm
Astra-480	450 nm	500 nm
Astra-520	494 nm	525 nm
Astra-540	523 nm	536 nm
Astra-570	550 nm	570 nm
Astra-620	588 nm	616 nm
Astra-650	627 nm	650 nm
Astra-690	676 nm	694 nm
Astra-780	750 nm	770 nm

OPTIMIZING SINGLE-PLEX IHC

The first step in designing a multi-plex IHC panel is to properly set the combination for each antibody and Astra-Dye with single-plex staining of the tissue. Once a user successfully completes panel development, other panels can be more easily optimized.

1. Optimization of primary antibody composition
1) As with standard IHC, the antigen retrieval method, primary antibody dilution rate, and incubation time can be adjusted.
2) Adjust the brightness of fluorescence and signal intensity by titrating the concentration of Astra-Dye.
3) Serial sections of tissue are used to establish the antigen retrieval condition with the highest staining intensity.

2. Primary antibody dilution and antigen retrieval condition
1) Antigen retrieval with TR1 or TR2 buffer.
2) Test using the optimal concentration of the primary antibody verified in standard IHC and additional dilution concentrations of 1:2 and 1:4.
3)

Antigen retrival BF	Standard IHC	1:2 Dilution	1:4 Dilution
TR1	Slide 1	Slide 2	Slide 3
TR2	Slide 4	Slide 5	Slide 6

4) Use TR2 and 1:2 additional dilution if there is no significant difference in staining results under all conditions.

3. Positive control tissue staining
1) Positive control tissue for each antibody is optimized and stained, as standard IHC development typically validates appropriate staining pattern.
2) Comparison with standard IHC staining (Specific, Background, Staining pattern).

PhenoFlamma™ 6-Plex mIHC Kit

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OPTIMIZING SINGLE-PLEX IHC

Astra-dye must be paired with each antibody. Consideration of the following points may be helpful in selecting pairs.

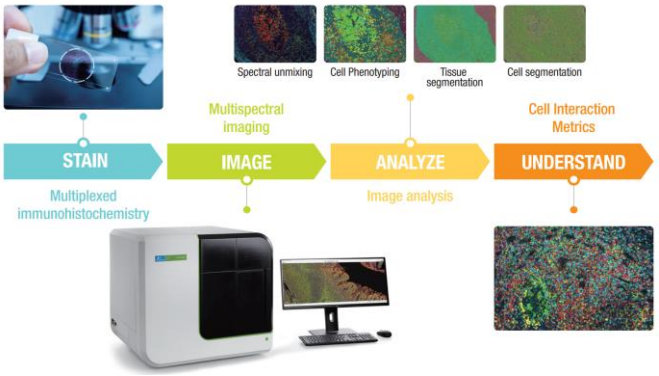
1. In the case of co-expression of markers in a specific cell type, select Astra-dye that is not spectrally adjacent.
2. Markers with low expression paired with brighter Astra-dye, whereas markers with high expression matched relatively weaker Astra-dye.

Astra-dye	Brightness
Astra-480	Medium
Astra-520	High
Astra-540	Medium
Astra-570	High
Astra-620	Low
Astra-650	High
Astra-690	Medium
Astra-780	Low

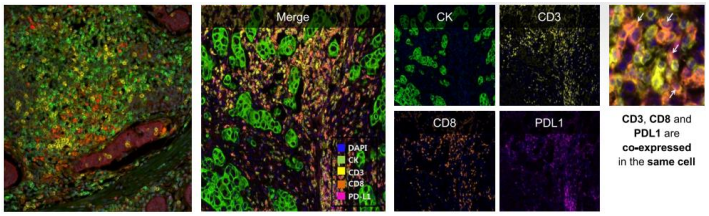
TROUBLESHOOTING

Problem	Suggestion
Low Signal	· Readjust the concentrations of the Primary Antibody and Astra-dye to determine the optimal concentration for fluorescence detection. - Use by increasing the concentration of Primary Antibody. - Use by increasing the concentration of Astra-dye. · Increase the incubation time of the Primary Antibody and Astra-dye Working Solution. (Recommended range is 10~30 minutes).
High Signal	· Readjust the concentrations of the Primary Antibody and Astra-dye to determine the optimal concentration for fluorescence detection. - Use by reducing the concentration of Primary Antibody. - Use by reducing the concentration of Astra-dye. · Reduce the incubation time of the Primary Antibody and Astra-dye Working Solution.
High Background	· Check whether endogenous peroxidase is completely quenched by microwave treatment. · In order to completely quench the endogenous peroxidase, pre-treatment with 3% H_2O_2 · Blocking buffer (TBS dilution) and then the blocking step is performed. Readjust the concentrations of the Primary Antibody and Astra-dye to determine the optimal concentration for fluorescence detection. - Use by reducing the concentration of Primary Antibody. - Use by reducing the concentration of Astra-dye. · All buffers and solutions are freshly prepared and used. · Check laboratory water sources for HRP contamination. · Check if the background is caused by endogenous biotin (If using streptavidin conjugate). · Readjust the wash frequency or time.

MULTIPLE IHC WORKFLOW



- ❖ Simultaneous visualization and quantification of cell relationships and distribution within the tumor microenvironment on the same slide
- ❖ Simultaneous visualization and quantification of multiple biomarkers in one cell on the same slide



RELATED PRODUCTS

Cat. No.	Product name
XTM4001KT	PhenoFlamma™ 6-Plex mIHC Kit
XTM4002KT	PhenoFlamma™ 3-Plex mIHC Kit

TECHNICAL SUPPORT

ADDRESS

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+1-510-561-9916

FAX

+82-32-88-8206

ASTRA-780 STAINING PROTOCOL

※ Astra-780 always uses last after other Astra-dye dyes are finished.

※ Do not perform MWT after Astra-780 antibody reaction.

(Astra-780) 1. Blocking

1) Using a hydrophobic barrier pen, mark around the tissue section to ensure complete tissue containment.

2) Place the slides in a humidified chamber, dispense 100~300 µL Blocking buffer on each tissue, and incubate at room temperature for 10 minutes.

(Astra-780) 2. Primary Antibody Reaction

1) Drain the blocking buffer and dispense the Primary Antibody Working Solution on each tissue.

※ Using a humidified chamber.

2) Incubate according to antibody manufacturer's guidelines for incubation time and temperature requirements, or under optimized conditions in the laboratory. (Example: 1 hour at room temperature)

3) Use an appropriate amount of reagent to cover the tissue. (100~300 µL per slide)

4) Wash slides with TBST. (3 x 2 minutes)

(Astra-780) 3. Secondary Antibody Reaction

1) Dispense Second Antibody Working Solution and incubate the slide at room temperature for 10 minutes.

※ Using a humidified chamber.

2) Use an appropriate amount of reagent to cover the tissue. (100~300 µL per slide)

※ Ms/Rb HRP 2nd antibody is recommended for human tissue experiments using primary antibodies of mouse or rabbit origin. Other options must be independently verified.

3) Wash slides with TBST. (3 x 2 minutes)

(Astra-780) 4. Signal Amplification (Astra-DIG Reaction)

1) Dispense 100~300 µL of Astra-DIG Working Solution to each tissue.

2) Incubate for 20 min at room temperature.

※ Using a humidified chamber.

3) Wash slides with TBST. (3 x 2 minutes)

4) Wash slides with TR2 buffer. (Briefly)

(Astra-780) 5. Microwave treatment

※ Optimization required.

3) Microwave for 15 minutes at 20% power.

1) Place the slides in the Slide Processing Jar and completely fill the TR2 buffer.

2) Cover the container loosely with a lid and microwave for 50 seconds at 100% power.

4) Cool the slides at room temperature for 15~30 minutes.

※ Be careful not to dry out the tissues on slides.

5) Wash slides with TBST. (Briefly)

6) Wash slides with distilled water. (Briefly)

(Astra-780) 6. Astra-780 Antibody Reaction

1) It is prepared by diluting Astra-780 antibody 1:75 in Blocking buffer.

2) Dispense Astra-780 Antibody and incubate the slide at room temperature for 1 hour.

※ Using a humidified chamber.

3) Use an appropriate amount of reagent to cover the tissue. (100~300 µL per slide)

4) Wash slides with TBST. (3 x 2 minutes)

(Astra-780) 7. DAPI staining & Mounting

1) After dropping DAPI 3~5 times on the tissue, incubate for 5 minutes at room temperature.

※ Using a humidified chamber.

2) Wash slides with TBST. (2 minutes)

3) Wash slides with distilled water. (2 minutes)

4) Dispense mounting medium (1~2 drops) on the tissue and cover the tissue with a coverslip

Solution	Time	Temperature
Antibody diluent/block	5m	
Primary antibody	30m (adjustable)	
Ms/Rb HRP secondary antibody	10m	
Astra-DIG	10m	
TR2 (pH6.0)	20m	95℃
Astra-780	60m	
DAPI	5m	