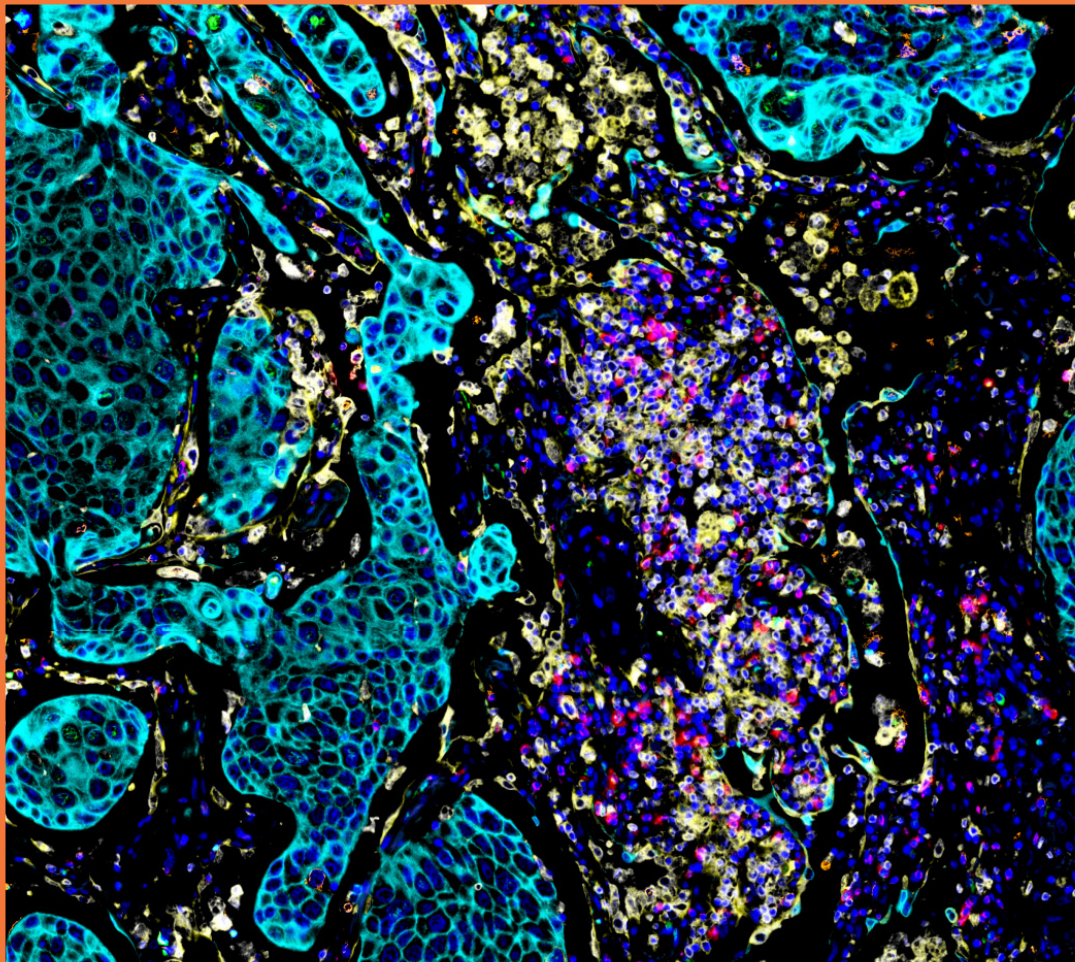


Multiplex IHC



Solutions for Multiplex IHC (mIHC)

Research on tissue microenvironments, such as the tumor immune microenvironment, can help accelerate the discovery of novel therapeutic targets and prognostic/predictive biomarkers. This area of research is of growing importance in basic/translational medicine research, clinical research, and diagnostic fields. Studies on the nature of the tissue microenvironment require the synergistic development of technologies in genomics, transcriptomics, proteomics, and spatial genomics. However, more modern histological analyses are required to achieve this, such as analysis of the co-localization of different biomarkers, spatial distribution and interaction of different cell clusters, and analysis of the heterogeneity of the tissue microenvironment, etc. These require the simultaneous detection of multiple targets on one tissue section to mine deeper biological information of the tumor microenvironment (TME), which assists in discovering predictive and prognostic biomarkers for clinical treatments.

Traditional tissue diagnosis relies on single-marker analysis through hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). This approach, while valuable, has limitations:

- Tissue samples can only be used once for H&E and singleplex IHC, reducing the amount of information that can be obtained from them
- Evaluating multiple markers simultaneously is difficult
- Quantitative information and spatial analysis of target proteins can be hard to obtain

In the case of a cancer, tumors reside within a complex ecosystem called the TME. This TME is comprised of a range of cell types, such as immune cells, stromal cells, and vasculature. Understanding the abundance of each cell type and their spatial distribution is crucial for obtaining insights into tumor progression, immune response, and therapy.

Multiplexing, the simultaneous detection of multiple proteins in a single assay, addresses these limitations. mIHC has several benefits:

- **Tissue conservation:** Gain **multiple insights** from a **single sample**; particularly valuable for rare diseases
- **Comprehensive analysis:** Ideal for **complex tumors** requiring multiple IHC stains for diagnosis with limited **tissue samples** available
- **Quantitative power:** Enables quantification of protein levels within the TME
- **Proximity analysis:** Reveals **spatial relationships** between different cell populations
- **Phenotypic profiling:** Provides a **comprehensive view** of protein expression and cell states
- **Combined analysis:** Enables **visualization of protein and RNA** within a single cell
- **Improved diagnostics:** Identifies activation states of various cell types, leading to a more **comprehensive understanding** of the TME and **disease heterogeneity**

mIHC technology can simultaneously detect multiple targets on one section *in situ*. It can be used to evaluate cell (cluster)-cell (cluster) interactions, analyze sample heterogeneity, and screen biomarker combinations in complex samples. Currently, low-plex mIHC (4-11 plex) staining can be achieved by the following methods:

- Tyramide Signal Amplification™ (TSA)
- Cyclic/non-cyclic immunofluorescence staining with conjugated antibodies

Recent advancements in high-plex (>40-plex) imaging technologies enable the comprehensive characterization of the cellular phenotype, composition, function, and spatial organization (cell-cell/cell-TME interactions) within tissues. These novel imaging techniques are based on the following, and the more common approaches are discussed below:

- Cyclic immunofluorescence
- Tyramide-based mIHC/IF using the Vectra platform (Akoya Biosciences)
- Epitope-targeted mass spectrometry
- RNA detection
- Metal-based mIHC/IF
- DNA barcoding-based mIHC/IF using the CODEX platform (Akoya Biosciences)

TSA method and recommended antibody panels

TSA is an enzyme-mediated detection method that can simultaneously detect multiple targets in one section *in situ* in a stepwise manner. It involves the sequential application of a primary antibody specific to the target of interest and an HRP-conjugated secondary antibody, followed by the application of fluorophore-conjugated tyramide. The HRP-conjugated secondary antibody catalyzes tyramide into an active form, leading to the covalent binding of tyramide radicals with the tyrosine moieties close to the epitope of the target. Next, the bound antibodies are removed by microwave heating. This staining cycle is repeated, and multiple primary/secondary antibody complexes are sequentially applied and removed until all the targets of interest are stained (Figure 1).

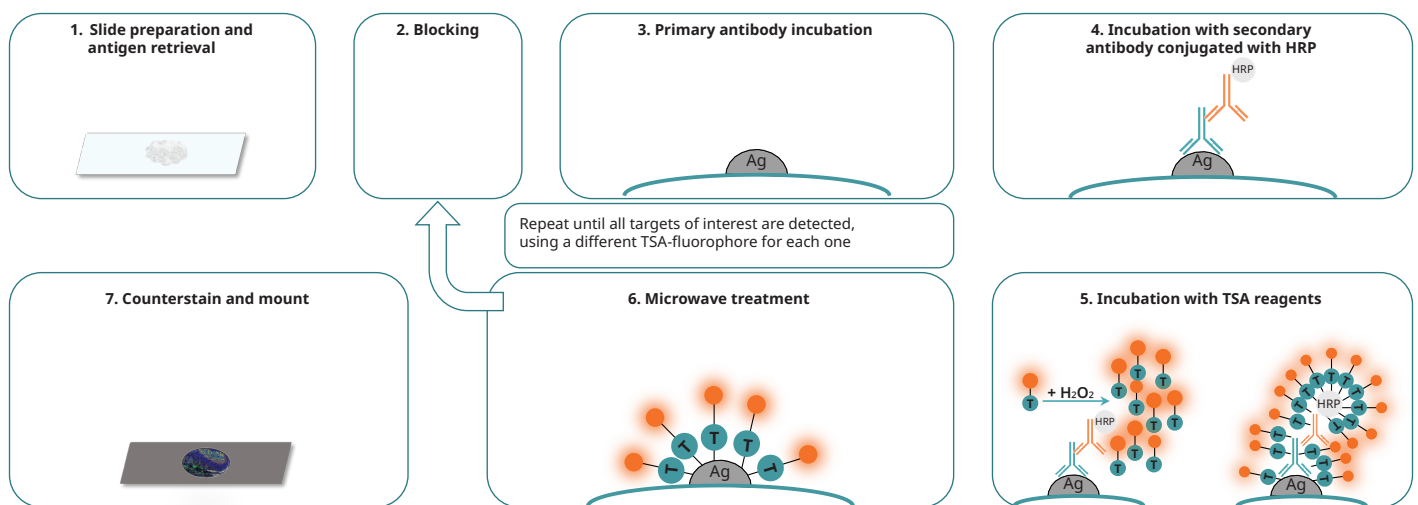


Figure 1. Principles of the TSA method. Ag: antigen, HRP: horse-radish peroxidase, TSA: Tyramide Signal Amplification™.

Advantages of the TSA method:

- Enables 7-10 plex staining (6-9 plex for targets of interest, plus staining of the nucleus)
- Multiple primary antibodies from the same host species can be used
- Higher sensitivity than direct fluorescence, conventional IHC, and indirect IF methods

Ensuring the antibody against each target to be detected is specific, sufficiently sensitive to provide an accurate signal within the dynamic range of the assay, and provides reproducible results with consistent robust performance is essential to the successful development of a TSA-based assay.

To better meet your needs for accurate, in-depth analysis of the tissue microenvironment and long-term reproducible results, [we have a catalog of over 33,000+ recombinant monoclonal antibodies, including 13,000+ conjugation ready carrier free antibodies](#), and a wide range of conjugation kits, including our innovative Lightning-Link® kits to get you there faster with the following benefits:

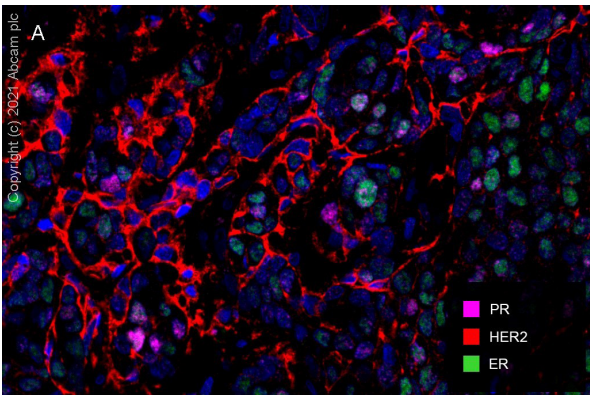
- Unrivaled specificity, with over 4,600+ knock-out validated antibodies
- Improved sensitivity
- Consistent results from batch to batch
- Reliable and scalable long-term supply
- Choice and flexibility of assay design

In addition, we have developed **47+** Opal™ TSA-based mIHC panels with **300+** recombinant antibodies validated in mIHC.

Below are some examples of low-plex mIHC of cancer and neuronal tissue.

Human triple-positive breast carcinoma

4 colors 3 targets

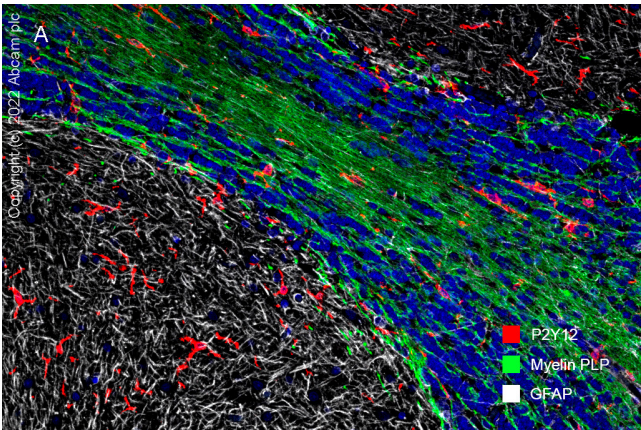


Round of staining	Target	Target description	Clone	Dye	Catalog number	Color
1	PR	Steroid hormone receptor. Lack of ER and PR expression in breast cancer cells predicts earlier recurrence and poorer survival in breast cancer patients	SP2	Opal™690	ab16661	Magenta
2	ER		SP1	Opal™520	ab16660	Green
3	HER2	An RTK and a member of the EGFR family. It is amplified and overexpressed in ~20%–25% of breast cancer cases	SP101	Opal™570	ab231438	Red

Immunostaining was performed on a Leica Biosystems BOND® RX instrument with an Opal™ 4-color kit. Image acquisition was performed using a Leica SP8 confocal microscope. DAPI (dark blue) was used as a nuclear counter stain. PR: progesterone receptor, ER: estrogen receptor, HER2: receptor tyrosine-protein kinase erbB-2, RTK: receptor tyrosine-protein kinase, EGFR: epidermal growth factor receptor.

Human cerebellum

4 colors 3 targets



Round of staining	Target	Target description	Clone	Dye	Catalog	Color
1	GFAP	Astrocyte marker	EPR1034Y	Opal™690	ab68428	Grey
2	Myelin PLP	Oligodendrocyte myelin sheath marker	EPR23504-106	Opal™520	ab254363	Green
3	P2Y12	Microglial cell marker	EPR23511-72	Opal™570	ab254347	Red

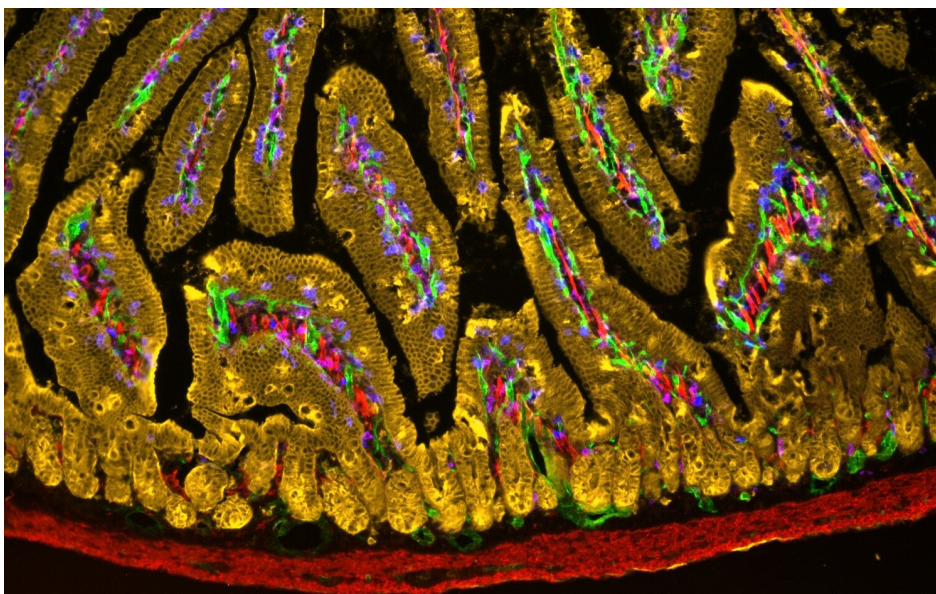
The immunostaining was performed on a Leica Biosystems BOND® RX instrument with an Opal™ 4-color kit. Image acquisition was performed using a Leica SP8 confocal microscope. DAPI (dark blue) was used as a nuclear counter stain. GFAP: glial fibrillary acidic protein; myelin PLP: myelin proteolipid protein.

TSA-based mIHC kit – recommended for frozen sections

We offer a TSA-based mIHC detection kit (ab312827) specific for frozen sections. The features and benefits of this kit include:

- Optimised for TSA-based mIHC on **frozen tissue sections**
- **No heat treatment** between staining cycles, which allows multiplex TSA to be performed on frozen sections to **preserve tissue morphology**
- The **fast-staining** protocol allows 7-color mIHC (DAPI + 6 biotinylated antibodies) using TSA to be **completed in one day**

The figure below provides an example of staining with the TSA-based mIHC detection kit using a frozen section.



mIHC analysis of mouse small intestine tissue mounted in Optimal Cutting Temperature compound using the mIHC Detection kit (TSA amplification) (ab312827) with the following antibodies: biotin rat-anti CD31 (clone MEC13.3), biotin mouse-anti human alpha-actin (clone 1A4), biotin rat-anti CD326 (clone G8.8), biotin rat-anti mouse CD8a (clone 53-6.7). TSA colors were developed with Alexa Fluor 488 tyramide, Cy3 tyramide, Alexa Fluor 594 tyramide, and Cy5 tyramide. Images were taken with a 10x objective on a standard microscope equipped with an appropriate set of filters (SpectraSplit7) for optimal fluorochrome separation. TSA: Tyramide Signal Amplification™.

Case study: Nine-target, 10-color TSA-based mIHC staining of a lung cancer tissue

Working with TissueGnostics, we aimed to determine the expression levels and location of nine different immune checkpoints and immune cell markers in a human lung cancer tissue. We also assessed the multidimensional biological information contained in the sample, including tissue structure, spatial distribution of cells, and co-expression of signaling pathway-related or cellular functional targets. To achieve this, nine Abcam antibodies were selected, a 10-color multiplex IHC reagent kit from TissueGnostics, and StrataQuest software for precise *in-situ* quantitative analysis of the tissue at the single-cell level were used.

In total, nine rounds of TSA mIHC staining were performed on the formalin-fixed paraffin-embedded (FFPE) lung cancer tissue section. Before each round of signal amplification, antigen retrieval was performed to remove antibodies from the previous round of staining to avoid cross reactivity. TG470SN channel (dark blue) was used for nuclear counterstaining.

After the staining was complete, the tissue section was scanned using TissueFAXS Spectra. Depending on the excitation and emission peaks of the dyes, scanning parameters and channels were set to continuous full-spectrum high-resolution imaging to construct a whole-slide multispectral digital slide. The clear multicolor images and individual signals from each channel allow for the deciphering of the complex TME.

Meet the collaborator

Tissue Gnostics

TissueGnostics Asia Pacific Limited



TissueGnostics Asia Pacific Limited is a subsidiary of TissueGnostics (TG) in Austria.

Committed to developing a total solution for quantitative analysis of *in situ* single-cell imaging on tissues and developing tools that can serve researchers and assist clinical diagnosis of difficult diseases and *in situ* screening of drug targets on tissues.

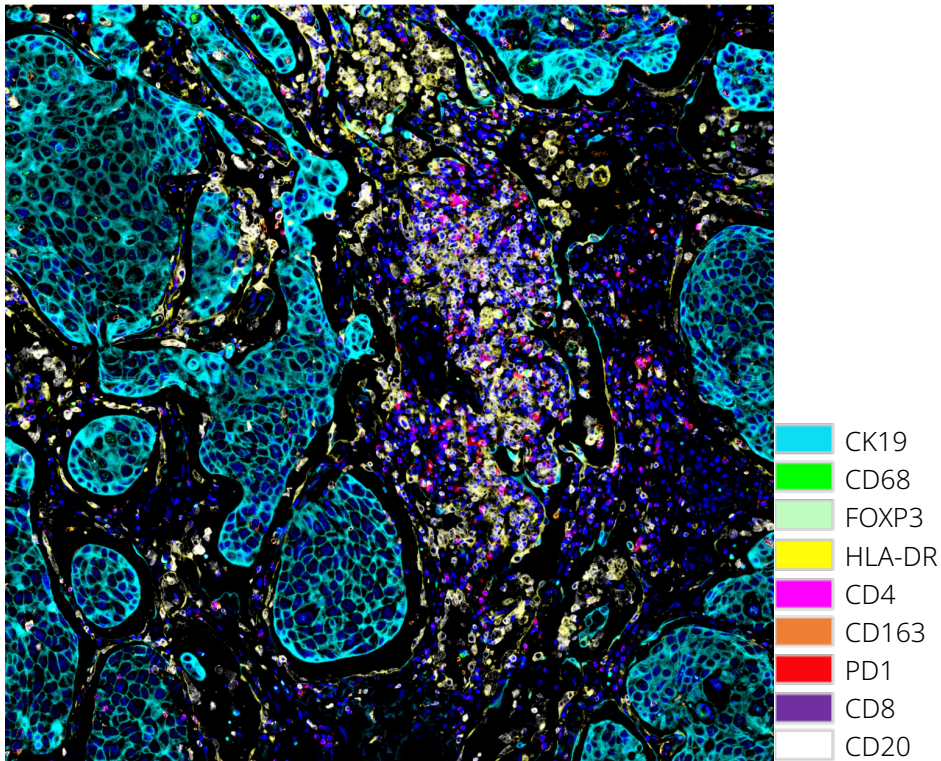
TissueGnostics Asia Pacific Limited (TG-AP)

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Tel: +86/400/898 1980

Website: www.tissuegnostics.com/cn

10-color mIHC analysis of human lung cancer tissue (FFPE section)



Merged staining of anti-FOXP3 (ab215206; TG540N), anti-PD1 (ab52587; TG700N), anti-CD163 (ab182422; TG650N), anti-HLA-DR (ab92511; TG570N), anti-CD4 (ab133616; TG620N), anti-CD8 alpha (ab101500; TG540S), anti-CD20 (ab9475; TG660S), anti-CD68 (ab192487; TG520N), anti-Cytokeratin 19 (ab52625; TG440N). TG470SN (dark blue) was used as a nuclear counter stain. Image acquisition was performed using TissueFAXS Spectra (TissueGnostics).

Cyclic multiplex fluorescent IHC with conjugated primaries and validated antibodies

Multiplex fluorescent IHC can be accomplished by cyclic staining with conjugated primaries. This approach does not require the incubation of secondary antibodies or an enzymatic reaction for signal amplification. After each cycle of cyclic multiplex fluorescent IHC staining and imaging, the fluorescence is quenched, followed by a new round of staining with a conjugated primary antibody. Multiple conjugated primaries can be used for each staining cycle. The staining cycles are repeated until all targets are detected. Our growing portfolio of **7,000+** fluorescent conjugated recombinant antibodies features **790+** conjugates directly validated in fluorescent IHC, making developing your cyclic multiplex panels easy. The benefits of using our fluorescent conjugated recombinant monoclonal primary antibodies for the development of mIHC panels include:

- **Choice and flexibility:** Abcam offers the most comprehensive range of conjugated recombinant antibodies
- By **eliminating the need for a secondary antibody**, our antibodies help **reduce** the chances of false positive results, providing reliable outcomes
- We have an **extensive selection of fluorophores** to help you stay at the forefront of conjugation innovation
- **Specificity:** Our conjugated recombinant primary antibodies bind directly to the target, providing **specific and accurate results**
- **Batch-to-batch consistency:** Our recombinant manufacturing process and stringent quality checks ensure **reproducible results** with reduced variability
- **High sensitivity:** our quality assurance process ensures a **high signal-to-noise ratio** during your experiments involving the removal of free dye

High-plex mIHC and validated antibodies

High-plex mIHC can provide considerable information on a wide range of interactions between cells (clusters)-cells (clusters) and the spatial distribution of different cell populations. Currently, common high-plex mIHC methods are based on oligonucleotide or metal-conjugated antibodies, which detect >40 targets simultaneously. The key to these approaches is the conjugation of carrier-free antibodies with specific labels.

We offer **5,400+** carrier-free, IHC-validated recombinant antibodies suitable for ultra-high-plex mIHC technologies based on oligonucleotide-conjugated antibodies (InSituPlex®, CODEX®, GeoMx®DSP, etc.) and technologies based on metal-conjugated antibodies (IMC™).

Features and advantages of carrier-free recombinant antibodies

Our range of recombinant antibodies offers high batch-to-batch consistency, meaning you can achieve highly reproducible results and move your project forward faster. They also offer:

- **BSA, sodium azide, and glycerol-free format** that supports consistent and successful conjugation
- **1 mg/mL** aliquots to eliminate the need to concentrate before conjugation
- **Purified IgG** to increase conjugation yield
- **Improved sensitivity** and **confirmed specificity**
- **High consistency**, with **high affinity** across batches
- **Reliable long-term supply** for easy scalability

View our range of IHC-validated antibodies and multiplex validated antibody panels

View our full list of antibodies that have been validated for use in IHC by clicking [here](#). To view our range of antibody panels for specific use cases, click [here](#).

You can refer to our product datasheets to view the key experimental parameters and the IHC images of our automated-IHC-validated antibodies. Alternatively, you can contact our Scientific Support if you have questions about the automated-IHC validated antibodies. For all technical inquiries please go to:

www.abcam.com/contactus

Note: InSituPlex® is a registered trademark of Ultivue, Inc.; Opal™ is a registered trademark of Akoya Biosciences, Inc.; CODEX® is a registered trademark of Akoya Biosciences, Inc. (CODEX re-branded to 'PhenoCycler'; GeoMx® is a registered trademark of NanoString Technologies, Inc.; IMC™ is a registered trademark of Standard BioTools Inc.; Bond® is a registered trademark of Leica Biosystems Melbourne Pty Ltd.; Alexa Fluor® is a registered trademark of Life Technologies. Alexa Fluor® dye conjugates contain(s) technology licensed to Abcam by Life Technologies; Lightning-Link® is a registered trademark of Abcam.

Let's work together:
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