ab134000 – Human Angiogenesis Antibody Array - Membrane

Instructions for Use

For the simultaneous detection of 20 Human Angiogenic Factor concentrations in all sample types

This product is for research use only and is not intended for diagnostic use.

Version 6 Last Updated 28 December 2018

Table of Contents

1. Introduction

New techniques such as cDNA microarrays have enabled us to analyze global gene expression. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows a disparity between the relative expression levels of mRNA and their corresponding proteins. Therefore, analysis of the protein profile is critical.

The conventional approach to analyzing multiple protein expression levels has been to use 2-D SDS-PAGE coupled with mass spectrometry. However, these methods are slow, expensive, labor-intensive and require specialized equipment. Thus, effective study of multiple protein expression levels can be complicated, costly and time-consuming. Moreover, these traditional methods of proteomics are not sensitive enough to detect most cytokines (typically at pg/ml concentrations).

Cytokines, broadly defined as secreted cell–cell signaling proteins and peptides distinct from classic hormones or neurotransmitters, play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation. They are involved in most disease processes, including cancer, obesity and inflammatory and cardiac diseases.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cytokines. Regulation of cellular processes by

cytokines is a complex, dynamic process, often involving multiple proteins. Positive and negative feedback loops, pleiotrophic effects and redundant functions, spatial and temporal expression of or synergistic interactions between multiple cytokines, even regulation via release of soluble forms of membrane-bound receptors, all are common mechanisms modulating the effects of cytokine signaling. As such, unraveling the role of individual cytokines in physiologic or pathologic processes generally requires consideration and detection of multiple cytokines rather than of a single cytokine.

Abcam Cytokine Antibody Arrays have several advantages over detection of cytokines using single-target ELISA kits:

1. More Data, Less Sample: Antibody arrays provide high-content screening using the same sample volume as for ELISA.

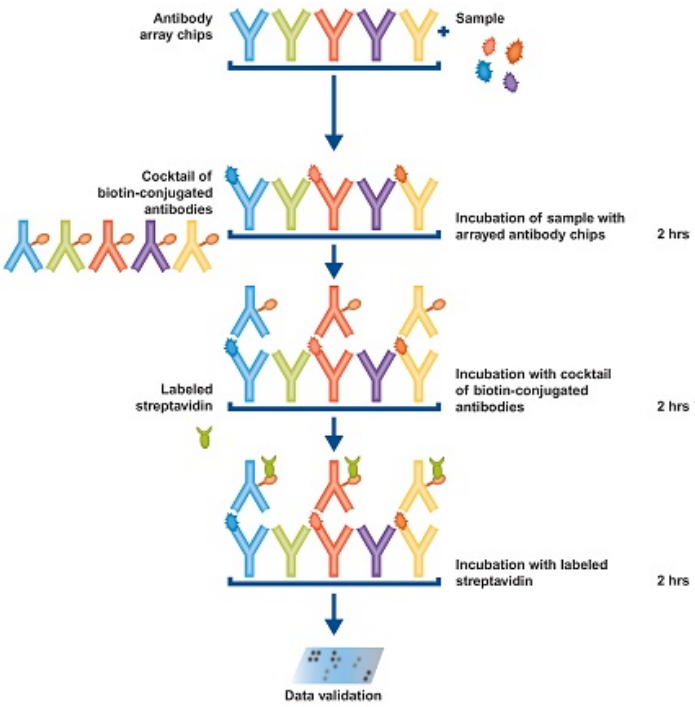
2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms or biomarkers related to cytokine signaling.

3. Greater Sensitivity: As little as 4 pg/ml of MCP-1 can be detected using the Cytokine array format. In contrast, our similar MCP-1 ELISA assay has a sensitivity of 40 pg/ml of MCP-1.

4. Increased Range of Detection: ELISA assays typically detect a concentration range of 100- to 1000-fold, however, Abcam

arrays can detect IL-2 at concentrations of 25 to 250,000 pg/ml, a range of 10,000-fold.

5. Better Precision: As determined by densitometry, the inter-array spot-to-spot Coefficient of Variation (CV) ranges from 5 to 10%, which compare favorably with the typical CV in ELISA testing of 10-15%.



2. Storage and Components

A. Storage

For best results, store the entire kit at ≤ –20°C upon arrival.

Once thawed, store array membranes and 1X Blocking Buffer at ≤ –20°C, and all other component at 4°C. Array kits are robust and will retain full activity even if stored for up to 24 hours at room temperature.

B. Components

Item	Quantity	
Angiogenesis Antibody Array Membranes (C1)	4 membranes	8 membranes
Biotinylated Antibody Cocktail (C8)	2 vials	4 vials
1000X HRP-Conjugated Streptavidin	50 µl	50 µl
1X Blocking Buffer	25 ml	2 x 25 ml
20X Wash Buffer I	10 ml	20 ml
20X Wash Buffer II	10 ml	20 ml
2X Cell Lysis Buffer	10 ml	16 ml
Detection Buffer C	1.5 ml	2.5 ml
Detection Buffer D	1.5 ml	2.5 ml
8-Well Plastic Tray	1 unit	1 unit

*Plastic sheets also included.

C. Additional Materials Required

- Small plastic boxes or containers
- Pipettors, pipet tips and other common lab consumables
- Distilled or De-ionized Water
- Tissue Paper, Blotting Paper or Chromatography Paper
- Orbital shaker or oscillating rocker
- Saran Wrap or similar plastic film
- A chemiluminescent blot documentation system
- X-ray Film and a suitable film processor, or other chemiluminescent detection system.

3. Preparation and Storage of Samples

A. General Considerations

- Freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot your samples prior to initial storage.
- Spin samples hard (5-10 minutes at 10K to 15K RPM) immediately prior to incubation of samples with array.

- Optimal sample concentrations may need to be determined empirically based on the signal intensities of spots and background signals obtained with each sample.

- If spot intensities are weak, increase sample concentration in subsequent experiments.

- If background or spot intensities are too strong, decrease sample concentration in subsequent experiments.

- Most samples will not need to be concentrated. If concentration is required, we recommend using a spin-column concentrator with a chilled centrifuge.

- Unless otherwise noted, dilute all samples using the same dilution factor in 1X Blocking Buffer.

B. Recommended Sample Volumes and Dilution Factors

NOTE: All sample dilutions should be made using the 1X Blocking Buffer provided in this kit. For all sample types, final sample volume = 1.0 ml per membrane

- Cell Cultured Media: Neat (no dilution needed)

- Serum & Plasma: 2-fold to 10-fold dilution

- Most other Body Fluids: Neat or 2-fold to 5-fold dilution

- Cell and Tissue Lysates: Minimum 5-fold to 10 fold to equal concentrations of total protein in lysate. Optimal sample concentration of cell and tissue lysates should be determined empirically. For more details, please see Section E (Cell and Tissue Lysates/Homogenates) below.

- Other Liquid Sample Types: Most often Neat or 2-fold to 5-fold. However, optimal dilutions should be determined empirically. For more details, please see Other Liquid Samples section (F), below.

C. Preparing Serum/Plasma

- Prepare samples according to established protocols or collection tube manufacturer's instructions. Sub-aliquot into plastic tubes. Store at –20°C or –80°C.

- We do not recommend comparing results between serum and plasma samples or between plasma prepared using different anticoagulants.

- For most applications, you may test plasma samples prepared using any anticoagulant (ie, Heparin, EDTA or Citrate). However, EDTA-prepared plasma may interfere with optimal detection of MMPs and other metal-binding proteins.

- If possible, avoid testing hemolyzed Serum or Plasma samples, as these samples may generate anomalous

cytokine expression patterns and/or high background signals.

D. Preparing Cell-Cultured Media

- Expression of proteins in cell culture may depend on many variables, including cell type, starting cell number, media composition and growth conditions.

- To start, we recommend seeding ≥1 x 10^6 cells in a 100 mm culture dish. However, you should consult the scientific literature for tips on how to cultivate your particular cell type.

- Cell type and experimental protocols can profoundly influence cytokine expression. Please consult the scientific literature for details on the effectiveness of various treatments to elicit a desired response, optimal timeframes for growing cells prior to treatment, optimal concentrations and exposure times for treatments and the timing of sample collection

- If possible, use media that is free of recombinant or purified growth factors. If you must add them, we strongly recommend testing an uncultured media aliquot as a sample "blank" to assess baseline signal response for comparison with cultured media samples.

- Serum-containing media rarely produce a baseline signal response with this product. Nevertheless, an ideal

experimental design would be to test uncultured media as a sample “blank” to assess baseline signal responses.

E. Preparing Cell and Tissue Lysates/Homogenates

- IMPORTANT: Lysate sample volumes required must be determined empirically and will depend upon the total protein concentration of each lysate and the intensity of background signals for each sample.

- You must determine the total protein concentration of each lysate/homogenate. We recommend using the BCA method, as it is insensitive to detergents commonly found in lysis buffers.

- Minimum Recommended Total Lysate Protein Concentration (prior to sample dilution) = 1.0 µg/µl

- Minimum Recommended Dilution of Lysates (prior to sample incubation): 5-fold to 10 fold with 1X Blocking Buffer. Dilute all lysate samples to the same final concentration of total lysate protein in 1X Blocking Buffer to 1 ml final volume.

- For your first experiment, we recommend using 200-250 µg of total protein in 1 ml of 1X Blocking Buffer (final volume) for each array membrane.

- Optimal amounts of total lysate protein may range from 50-1000 µg per array membrane. Based upon the signal

intensities of background and spots obtained with each sample, you may need to increase or decrease the volume of lysate used in subsequent experiments.

- We recommend preparing cell or tissue lysates using 2X Cell Lysis Buffer which is provided. Be sure to properly dilute Cell Lysis Buffer (1:1 with deionized H_2O) before use.

- Other lysis buffers can be used if they are non-denaturing, non-reducing, total salt concentration ≤700 mM), contain ≤2% total detergent (v/v) and are free of sodium azide (NaN_3).

- *We strongly recommend adding protease inhibitors to your cell lysis buffer.* Any broad-spectrum protease inhibitor cocktail intended for preparation of mammalian cell/tissue lysates should be sufficient, but please consult the scientific literature before deciding upon the exact composition of cocktail to use.

- Optimal protocols for mechanical disruption vary for different cell and tissue types. Please consult the scientific literature for examples of successful detection of proteins using ELISA or multiplex ELISA techniques in lysates made from cell or tissue samples similar to yours.

- BEWARE! Sonication can quickly heat volumes of 1 ml or less and denature proteins in your samples!

- After extraction, clarify your lysates by centrifugation and save the supernatant for your experiment. Preparing sub-aliquots is strongly recommended.

F. Other Liquid Samples

- Abcam Cytokine Antibody Arrays are compatible with most liquid samples, including extracts, perfusates and lavages, as well as body fluids, such as CSF, Sputum, Saliva, Tears, and Urine.

- Be sure to measure the total protein concentrations of these samples prior to sample incubation.

- For samples that have fairly consistent concentrations of total protein between samples (CV ≤20%), dilute samples using equal volumes for each sample (ie, use the same dilution factor, v/v).

- For samples exhibiting wider ranges of total protein content, dilute to equal concentrations of total protein (eg, 200 µg of total protein) in 1X Blocking Buffer to a final volume of 1 ml.

4. Handling Array Membranes

- Array membranes are fragile when dry. Handle with care.
- Wet or dry, grasp membranes by the edges using forceps.
- Do not allow membranes to dry out during experiments.
- The printed side of each membrane is denoted by a dash mark (-) or array number in the upper left corner.
- Unused membranes should be stored at -20°C or -80°C.
- Completed membranes may be stored up to 5 days in Wash Buffer II or Blocking Buffer at 4°C. Be sure to cover container to prevent evaporation during storage.
- For longer term storage of completed membranes, place wetted membranes between 2 plastic sheets (provided in kit), wrap in Saran Wrap and store at -20°C or -80°C

5. Incubations and Washes

- Perform all incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/sec) to ensure complete and even solution coverage as well as to avoid foaming or bubbles from appearing on the membrane surface.

- All washes and incubations (except for detection buffers incubation step) should be performed using the 8-Well Incubation Tray provided in the kit.

- Cover the 8-Well Incubation Tray with the lid for all incubation steps to avoid evaporation.

- Completely cover the membranes with sample or reagent during each incubation. Avoid forceful pipetting directly onto the membrane, instead gently pipette in a corner of each well.

- The sample, biotin-conjugate antibody, and HRP-Streptavidin incubation steps may be performed overnight at 4°C. Overnight incubations are the most effective method of increasing spot intensities but may also increase background noise.

- If you perform overnight sample incubations, we recommend adding the optional “Large Volume Wash” described in Step 7.B.4 to minimize background signals.

6. Chemiluminescence Detection

- Trying multiple exposure times is recommended to obtain optimum results. Anywhere from a few seconds to 10 minutes is common with 30 seconds to 1 minute being suitable for most samples.

- If the signals are too weak, increase exposure time (e.g. 2-10 mins). If the signals are too strong, reduce exposure time (e.g. 3-30 seconds).

- Blot documentation systems that use CCD cameras to detect chemiluminescence are ideal for imaging Abcam array membranes. They can easily be programmed to take multiple exposures, and the dynamic range of these detectors tends to be 2-3 orders of magnitude greater than that of X-ray film or and much more sensitive to chemiluminescence than phosphorimaging systems.

7. Protocol

A. Preparation and Storage of Reagents

NOTE: Keep all reagents on ice during preparation. Reagents should only be used in their 1X working concentration.

1. Blocking Buffer is supplied as 1X working concentration.

2. Wash Buffers I and II are supplied as 20X concentration.

   a) Dilute each Wash Buffer 20-fold with distilled or deionized water to prepare the 1X working concentration.

   b) 1X Wash Buffers can be stored at 4°C for up to 1 month.

3. Biotinylated Antibody Cocktail (C1) are supplied at 2000X concentration as a small liquid bead. 1 vial is enough to test 2 membranes.

   a) Briefly centrifuge each vial prior to reconstitution as the concentrated liquid bead can adhere to the inside walls and cap during transit.

   b) Reconstitute by pipetting 2 ml of 1X Blocking Buffer into the Biotinylated Antibody Cocktail (C1) vial to prepare the 1X working concentration.

4. HRP-Conjugated Streptavidin is supplied at a 1000X stock concentration.

    a) Mix the 1000X HRP-Conjugated Streptavidin vial well before use as precipitants may form during storage.

    b) Dilute 1000X HRP-Conjugated Streptavidin 1000-fold with 1X Blocking Buffer to prepare the 1X working concentration.

5. Detection Buffers C & D are supplied as 1X working concentration.

B. Blocking and Incubations

NOTE: Please prepare all reagents immediately prior to use. All incubations and washes must be performed under gentle rotation/rocking.

1. Place each membrane printed side up (see Section 4) into the 8-well tray provided in the kit.

2. Block membranes by incubating with 2 mL 1X Blocking Buffer at room temperature (RT) for 30 min.

3. Aspirate 1X Blocking Buffer from each well.

4. Pipette 1 ml of diluted or undiluted sample into each well and incubate for 1.5 - 2 hours at RT.

NOTES: When trying the protocol for the first time or when the signal is low, we strongly recommend incubating samples **overnight at 4°C**.

5. Aspirate samples from each well.

   NOTES: **After the overnight incubation**, we strongly recommend an additional step with Large Volume Wash: After Step 4 and before Step 5, place membranes into clean container(s). Add 20-30 ml of Wash Buffer I per membrane, and wash at RT with gentle shaking or rocking for 30-45 min. Return membranes to the 8-well tray.

6. Wash Buffer I Wash: Pipette 2 ml of 1X Wash Buffer I into each well and incubate for 5 minutes at RT. Repeat this 2 more times for a total of 3 washes using fresh buffer and aspirating each time.

7. Wash Buffer II Wash: Pipette 2 ml of 1X Wash Buffer II into each well and incubate for 5 minutes at RT. Repeat this 1 more time for a total of 2 washes using fresh buffer and aspirating each time.

8. Pipette 1 ml of 1X Biotinylated Antibody Cocktail (C1) into each well and incubate for 1.5 - 2 hours at RT or overnight at 4°C.

9. Aspirate 1X Biotinylated Antibody Cocktail (C1) from each well.

10. Wash membranes as directed in Steps 6 and 7.

11. Pipette 2 ml of 1X HRP-Conjugated Streptavidin into each well and incubate for 2 hours at RT or overnight at 4°C.

12. Aspirate 1X HRP-Conjugated Streptavidin from each well.

13. Wash membranes as directed in Steps 6 and 7.

C. Chemiluminescence Detection

NOTE: Do not allow membranes to dry out during detection.

1. Transfer and place each membrane printed side up onto a sheet of chromatography paper, tissue paper, or blotting paper lying on a flat surface, such as a benchtop.

2. Remove any excess wash buffer by blotting the membrane edges with another piece of chromatography, blotting, or tissue paper.

3. Transfer and place each membrane printed side up onto a plastic sheet (provided) lying on a flat surface.

4. Into a single, clean tube, pipette equal volumes (1:1) of Detection Buffer C and Detection Buffer D. Mix well.

5. Gently pipette 500 µl of the Detection Buffers mixture onto each membrane (e.g., 500 µl = 250 µl of Detection Buffer C and 250 µl of Detection Buffer D) and incubate for 2 minutes

at RT (do not rock or shake). Immediately afterwards, proceed to Step 6.

NOTE: Exposure should ideally start within 5 minutes after finishing Step 5 and completed within 20 minutes as chemiluminescence signals will fade over time.

6. Place another plastic sheet on top by starting at one end and gently "rolling" the flexible plastic across the surface to the opposite end to smooth out any air bubbles. The membranes should now be "sandwiched" between two plastic sheets.

   NOTE: Avoid sliding the plastic sheet along the membranes' printed surface.

7. If using a CCD camera (recommended), transfer the sandwiched membranes to the imaging system and expose. (See tips for obtaining array images in Section C).

   NOTE: If using X-ray film remove the top plastic sheet covering the printed side so that the membranes can be directly exposed to the film.

8. Try multiple exposures to obtain an image with low background and strong positive control signal spots that do not bleed into one another. Typical exposure times are between few seconds to 2 minutes.

9. To store, without direct pressure, gently sandwich the membranes between 2 plastic sheets (if not already), tape the sheets together or wrap in plastic wrap to secure them, and store at -≤20°C

8. Interpretation of Results

Typical results obtained with Abcam Antibody Arrays:

The preceding figure presents typical images obtained with Abcam Human Cytokine Antibody Array. These membranes were probed with conditioned media from two different cell lines. Membranes were exposed to film at RT for 1 min.

Note the strong signals of the Positive Control spots, provided by biotin-conjugated IgG printed directly onto the array membrane in the upper-left and lower-right corners. These Positive Control spots are useful for proper orientation of the array image.

The signal intensity for each antigen-specific antibody spot is proportional to the relative concentration of the antigen in that

sample. Comparison of signal intensities for individual antigen-specific antibody spots between and among array images can be used to determine relative differences in expression levels of each analyte sample-to-sample or group-to-group.

Obtaining Densitometry Data:

Visual comparison of array images may be sufficient to see differences in relative protein expression. However, most researchers will want to perform numerical comparisons of the signal intensities (or more precisely, signal *densities*), using 2-D densitometry. Gel/Blot documentation systems and other chemiluminescent or phosphorescent detection systems are usually sold as a package with compatible densitometry software.

To obtain densitometry data from an X-ray film, one must first scan the film to obtain a digitized image using an ordinary office scanner with resolution of 300 dpi or greater. Any densitometry software should be sufficient to obtain spot signal densities from your scanned images. One such software program, ImageJ, is available for free from the NIH (for more info, visit http://rsbweb.nih.gov/ij/).

We suggest using the following guidelines when extracting densitometry data from our array images:

- For each array membrane, identify a single exposure that the exhibits low background signal intensity and strong Positive Control signals that do not "bleed" into one another. Exposure times do not need to be identical for each array,

but Positive Control signals on each image should have similar intensities.

- Measure the density of each spot using a circle that is roughly the size of one of the largest spots. Be sure to use the same circle (area and shape) for measuring the signal densities on every array for which you wish to compare the results.

- For each spot, use the summed signal density across the entire circle (i.e., total signal density per unit area)

Once you have obtained the raw densitometry data, you should subtract the background and normalize to the Positive Control signals before proceeding to analysis.

Background Subtraction:

On each array, several “Negative Control” and/or “Blank” spots will be included. Blank spots are literally blank; nothing has been printed there. Negative Control spots are printed with the same buffer used to dilute antibodies printed on the array. Thus, the signal intensities of the Negative Controls represent the background plus non-specific binding to the printed spots. We recommend subtracting the mean of 4 or more Negative Control spots for background correction.

Normalization of Array Data:

The amount of biotin-conjugated IgG protein printed for each Positive Control spot is consistent from array to array. As such the intensity of these Positive Control signals can be used to normalize signal responses for comparison of results across multiple arrays, much like housekeeping genes and proteins are used to normalize results of PCR gels and Western Blots, respectively.

To normalize array data, one array is defined as "reference" to which the other arrays are normalized. This choice can be arbitrary. You can calculate the normalized values as follows:

$X(Ny) = X(y) * P1/P(y)$

Where:

P1 = mean signal density of Positive Control spots on reference array

P(y) = mean signal density of Positive Control spots on Array "y"

X(y) = mean signal density for spot "X" on Array for sample "y"

X(Ny) = normalized signal intensity for spot "X" on Array "y"

After normalization to Positive Control signal intensities, you can compare the relative expression levels, analyte-by-analyte, among or between your samples or groups. By comparing these signal intensities, one can determine relative differences in cytokine expression in each sample.

9. Array Maps

Detects 20 angiogenic factors in one experiment

A	B	C	D	E	F	G	H
POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

10. Troubleshooting Guide

Problem	Cause	Recommendation
No signal for any spots, including Positive Controls	Global detection failure	Repeat incubation with HRP-Streptavidin and Detection Buffers.
Weak or no signals antigen-specific spots	Sample is too dilute	Repeat experiment using higher sample concentration.
	Improper dilution of HRP-Streptavidin	Tube may contain precipitants. Repeat detection, mix 1000X HRP-Streptavidin well before diluting reagent.
	Waiting too long to detect chemiluminescent signals	Repeat detection, making sure to complete this process within 20 min.

Weak or no signals antigen-specific spots	Other Tips	Incubate with sample O/N at 4°C.
		Increase concentration of HRP-Streptavidin.
		Increase concentration of Biotinylated Antibody Cocktail (C1).
		Extend exposure time (may go overnight).
Uneven signal or background	Bubbles present on membrane during incubations	Be sure to completely remove all bubbles from membrane surface.
	Membranes were not evenly covered during washes/incubations or allowed to dry out	Completely cover membranes with solution, use a rocker or shaker during washes and incubations.
High background signals	Overexposure	Decrease exposure time.
	Sample is too concentrated	Repeat experiment using more dilute sample.
	NOTE: To reduce background on completed membrane, wash O/N @ 4°C in Wash Buffer II, then re-incubate with HRP-Streptavidin and repeat detection.	

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp