CTA Protein Array

Instruction Manual

This product is intended for

Research Use Only

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1. Product Description

The CTA Protein Array is a slide based high-density protein microarray based on Sengenics patented KREX™ protein folding technology [1]. The product enables highly multiplexed detection and relative quantification of autoantibodies circulating in human blood and is intended primarily for disease biomarker discovery. The array content comprises 200+ immobilized, full-length, correctly folded human proteins. The proteins are immobilized on a proprietary, planar hydrogel surface supported by a glass slide. KREX™ technology (1) ensures that only correctly folded proteins are immobilized onto the surface and the aqueous environment of the hydrogel helps the proteins to maintain their native conformation. The arrayed proteins represent major protein classes such as protein kinases and transcription factors, signalling molecules as well as proteins acting at the extracellular environment, such as cytokines.

The immobilized native proteins serve as surrogate autoantigens which capture any autoantibodies present in the sample. The non-specifically bound material is removed by washing and the captured autoantibodies are detected using anti-human IgG coupled to Cy-3 fluorophore. Native protein conformation and correctly folded epitopes lead to a highly specific signal and low assay background. The fluorescent readout ensures wide dynamic range of >3 logs, and low pg/ml sensitivity. Image acquisition is achieved using Agilent Microarray scanner and data analysis is performed using Genepix Pro7 analysis software.

2. Background

Recombinant proteins are mainstay not only in basic biomedical research but are also widely used as tools in the field of proteomics and in drug-discovery. The three-dimensional structure of proteins is critical to their biochemical function. Correct folding of recombinant proteins, however, is difficult to ensure and conducting experiments with misfolded proteins may lead to misleading results thus compromising research or negatively impacting discovery projects. The fundamental principle behind Sengenics' patented KREX technology is that when the protein of interest is correctly folded, it co-translationally drives the correct folding of a genetically fused protein, called biotin carboxyl carrier protein (BCCP). The biotin ligation site within BCCP becomes exposed and available for biotinylation, only when properly folded [1]. Therefore, only correctly folded recombinant fusion proteins will be covalently biotinylated. This biotinylation is not chemical but occurs post-translationally *in vivo*, in cell culture. The solid support of the protein array contains Streptavidin and only biotinylated proteins bind to the surface with an extremely high affinity. All other proteins, including misfolded recombinant proteins are washed away. Moreover, Sengenics' proprietary streptavidin-coated hydrogel surface chemistry provides an aqueous environment, preserving the native structure and function of the protein. KREX technology also ensures that the proteins are immobilized on the array surface in oriented fashion at a single attachment point. With the BCCP protein also serving as a linker, the recombinant proteins are tethered to the surface at a distance which allows them to interact with other large proteins, such as antibodies, without steric hindrance [1].

References

1. Beeton-Kempen, N., Duarte, J., Shoko, A., Serufuri, J.-M., John, T., Cebon, J., & Blackburn, J. (2014). Development of a novel, quantitative protein microarray platform for the multiplexed serological analysis of autoantibodies to cancer-testis antigens. International Journal of Cancer, 135, 1842–1851

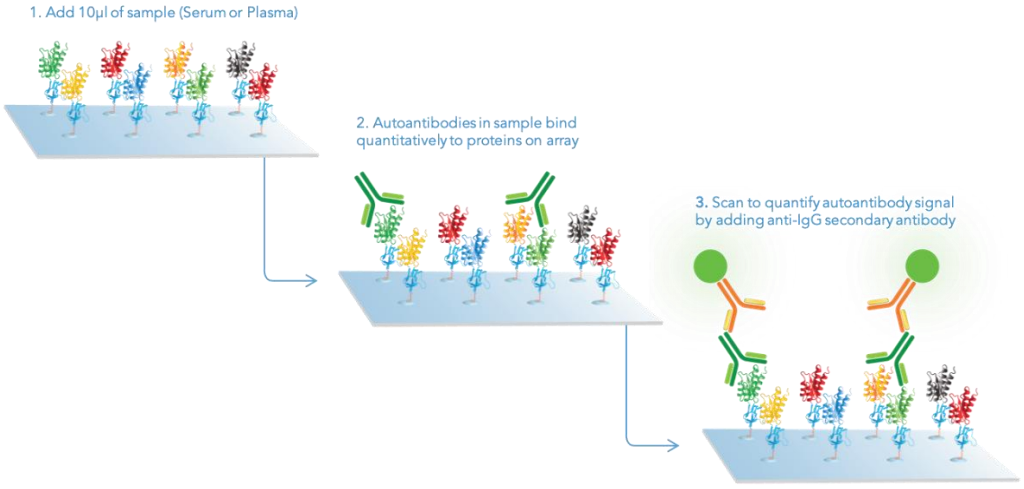


Figure 1. Graphic overview of the CTA Protein Array assay. Autoantibodies in the sample are captured by the immobilized, native recombinant human proteins. The unbound material is removed by washing and the captured autoantibodies are detected by anti-human IgG coupled to Cy-3 fluorescent dye.

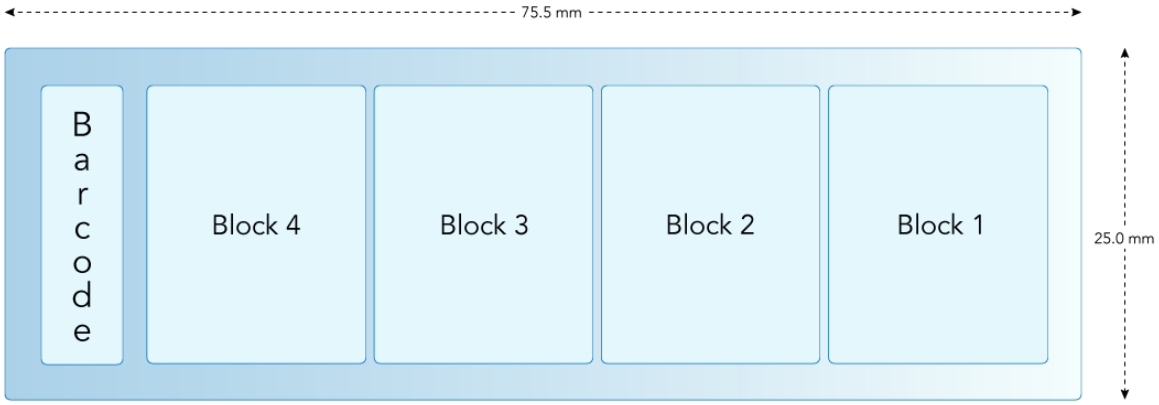


Figure 2. Slide Layout. The CTA Protein Array slide has 4 blocks (see scheme above), each having a content of >200 recombinant proteins. In addition, each block consists of two identical sub-arrays comprising the full protein array content, creating a duplicate spot per sub-arrays. One sample is applied per block, creating a quadruplicate measurement for each target analyte. The quadruplicate measurement makes the data considerably more robust and reliable. The slides are provided in Pap jars and are submerged in a storage solution.

3. Product Specifications

Table 1. Specifications.

Category	Specifications
Product Name	CTA Protein Array
Cat #	CTA-BUN-004
Product Type	Slide-based high density protein microarray
Content	200+ immobilized full-length recombinant human proteins
Sample type	The assay was optimized for serum and plasma samples. Other sample types may require further optimization.
Sample volume and dilution	30 µl per assay. Recommended dilution 1:400
Performance characteristics	Sensitivity (limit of detection): low pg/ml Dynamic range: >3 logs. Semi-quantitative assay
Readout	Fluorescence (Green channel, e.g. Cy3). Relative Fluorescence Intensity (RFU)
Equipment needed for data capture and analysis	**Agilent SureScan Microarray** - equipped with a green channel and a minimum resolution (pixel size) of 10 µm. Data analysis is performed with a **Genepix Pro7** analysis software.
Storage and stability	CTA Protein Arrays can be stored for up to 12 months at -20°C. ***Note: Do not reuse arrays – single use only. Opening a jar and removing only one slide will not affect the use by date or the shelf life of the remaining slide.***

4. List of Required Equipment, Reagents and Disposables

Table 2. Reagents needed to make the assay buffer.

Materials	Suggested Manufacturer	Catalogue Number	Storage
10X Phosphate Buffer Saline, pH 7.4	General	N/A	RT
Skim milk powder	Sigma Aldrich	70166-500G	RT
Triton X-100	Sigma Aldrich	T9284-100ML	RT

Table 3. Major equipment needed to perform the assay.

Materials	Suggested Manufacturer	Catalogue Number	Storage
Refrigerated incubator shaker	JeioTech/Medline	IST-4075R	RT
Shaker	JeioTech/Medline	OS-3000	RT
Biological Safety Cabinet	General	NA	RT
30 mL laboratory dispenser	General	NA	RT
Microarray scanner	Agilent Technologies	G4900DA/ G2505 C	RT
10/200/1000 µL Pipette	General	NA	RT
4 multi-channel pipette, 1000 µL	General	NA	RT
Volumetric flask glass 200 mL	General	NA	RT
Slide staining dish and rack for 25 slides (staining trough, complete with tray, black)	BRAND™	BR471800-5EA	RT
ProPlate® 4 Well Multi-Array Chambers, 10 pcs/pack	Grace Bio-Labs	246854	RT
ProPlate® Stainless Steel Clip, Numbered, 2 pcs/pack	Grace Bio-Labs	204837	RT

Table 4. List of other required reagents

Materials	Suggested Manufacturer	Catalogue Number
Cy3-anti-human IgG (h-IgG) (Stock Concentration: 1 mg/mL)	Sengenics	OTH-CYG-220

Table 5. List of required consumables.

Materials	Suggested Manufacturer	Catalogue Number
96 deep well plates (1 mL)	General	NA
1.5 mL microcentrifuge	General	NA
10/200/1000 µL tip	General	NA
30 mL Pap jars	Evergreen Scientific	FIS#05-557-2
Pap jar racks (24 places)	General	NA
Solution basins/reservoir	General	NA

5. Handling and Disposal

Handling

Follow good laboratory practice guidelines when handling slides and samples. Glass slides should be handled with extra care. Remove each slide from the storage container by holding the slide at the barcode labelled end. The proteins are printed on the same side of the slide as the barcode. Do not touch the array surface area on the glass slide. The barcode must be oriented at the bottom of the slide with the array facing upward in every step.

Disposal

Follow local environmental regulatory requirements for disposal of the sample and reagents used in running the slides.

6. Assay Procedure

6.1 Preparation of Serum Assay Buffer (SAB)

Serum Assay Buffer (SAB)		
Reagent	**% (v/v; w/v)**	**Volume; Weight for 3L**
Triton X-100	0.1 %	3 mL
Skim milk powder	0.1 %	3 g
10X Phosphate Saline (PBS)	10 %	300 mL
High Purity Water (18.2 MΩ-cm)	Make up to a final volume of 3 L	

Pour approximately 200 mL of SAB into a slide staining dish and rack and put it aside at 4°C to be used for the first slide washing step. Equilibrate the rest of SAB at room temperature (20-22°C).

Note: 3 L of buffer is sufficient to run an assay of 24 slides of CTA Protein Array

6.2 Sample Dilution

1. Dispense 600 µL of Serum Assay Buffer (SAB) into labelled 1.5 mL micro centrifuge tubes or 96 deep well plate. If using 1.5 mL tube, label each tube referring to the number of slides running on the day following block number. For example, 1_1 & 1_2 referring to slide number 1, block 1 and slide number 1, block 2, respectively.
2. Equilibrate at room temperature (20-22°C) for at least 30 minutes.
3. Thaw samples and mix by brief vortexing. Inspect each sample visually to ensure sufficient volume and homogeneity. Minimum sample volume required per assay is 30 µL.
4. Centrifuge the samples for 3 minutes at 13,000 x g to pellet any particles or cell debris.
5. Dilute the samples by adding 1.5 µL of sample into a tube containing 600 µL SAB and briefly vortex. The 400-fold dilution is an optimal dilution for plasma/serum.

   Note: Handling of undiluted human samples should be carried out in a Class II Biological Safety Cabinet using locally mandated PPE requirements.

6.3 Preparation of the Slides and Sample Application

1. Take out the slide staining dish and rack pre-filled with 200 mL of cold (4°C) SAB.
2. Remove the CTA Protein Array slides from the storage and randomly pick Pap jars containing CTA slides according to the total slide number to be utilized. (Each Pap jar contains two slides).
3. Remove the required number of slides from the Pap jar(s) by holding the slide at the labelled end of the slide.

   Note: The proteins are printed on the barcode labelled side.

4. Scan the slide barcodes pasted on Pap jar to log into Sample Annotation.
5. Drain excess liquid from the slide by touching the edge of the slide on the rim of the Pap jar. Record or scan the barcode number of each slide (Figure 3).
6. Lift the rack from the slide dish and place the first slide in slot 2 from the left with the barcoded side facing towards slot 1. Then place the rack back in the slide dish to prevent the slide from drying out.
7. Add each slide to the rack in turn from left to right, making sure the slides are all in the same orientation.
8. When all the slides have been added, gently move the rack up and down five times in the buffer.
9. Put the lid on the slide dish and wash on an orbital shaker at 50 rpm, for 5 minutes at room temperature (20-22°C).
10. Place several layers of white laboratory tissue onto the bench surface and cover this with three layers of lint free tissues.

    Note: Always place the rack back in the buffer in between removing slide.
11. Assemble the ProPlate® 4 Well Gasket (Figure 4) by following the illustrated instructions in Appendix 1.
12. Grip the slide between index finger and thumb and wipe back of the slide once with laboratory tissue. Then blot the long edge of the array three times on the wad of lint free tissue paper.
13. Place the CTA Protein Array slide (barcoded-side facing down) over the ProPlate® 4 Well's silicone (Figure 4) and press gently to ensure slide assembly is properly aligned. Insert the ProPlate® Stainless Steel Spring Clips (Figure 5) onto slide assembly by snapping onto long edge of module (refer Appendix 1). Prior to this step, check the slides number in sample annotation to ensure that the slides are sequentially assembled.
14. Place the assembled slide into any container with a flat surface. Before adding the samples, please ensure the slide barcode is at the bottom.
15. For 96 well plate, use 4 multi-channel 1000 µL pipette to aspirate and dispense 500 µL of diluted samples into assembled slide. Dispense the diluted samples by columns. Ensure the position of multichannel pipette reflect the position of diluted samples in 96-well plate. For single pipette 1000 µL, dispense the diluted samples sequentially.

    Important Note: Ensure that the barcode is at the bottom of slide before start dispensing diluted samples.
16. Repeat step 11 to 15 for the remaining of slides.

    Note: It is advisable to assemble and process ONE slide at a time. Please ensure that sample plate and slide is in a correct position.
17. Place the cover and incubate in the shaking incubator at 50 rpm, 20 °C for 1 hour.

Note: Ensure that the arrays are always kept horizontal to prevent slopping of solutions between wells. Handle the arrays very gently to prevent slopping or splashing of contents between wells/chambers.

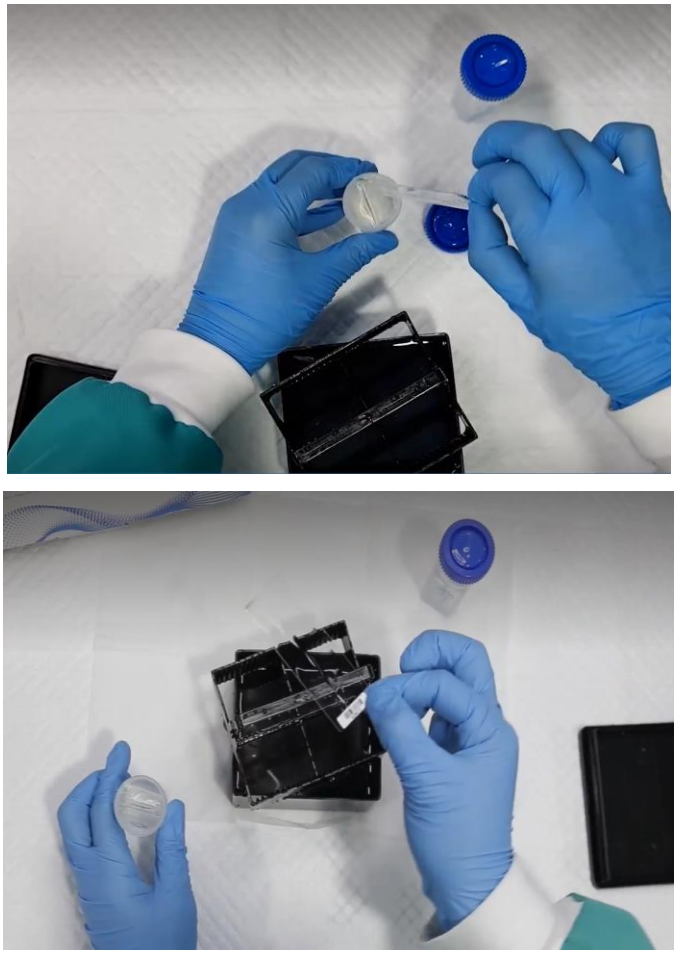


Figure 3. Removal of slides from the Pap jars. The slide staining dish and rack is used in several wash and incubation steps throughout the assay procedure. The rack can hold up to 25 slides and has a lid (BRAND™; Cat# BR471800-5EA).

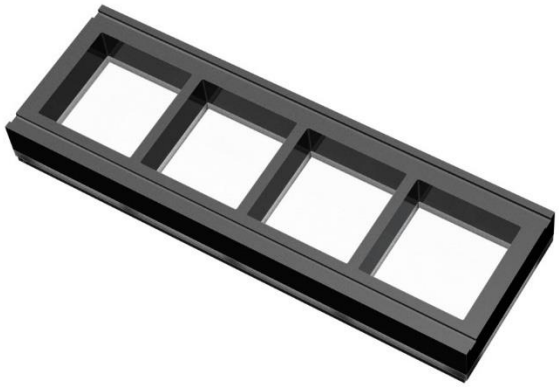


Figure 4. ProPlate® 4 Well Multi-Array Chamber. Grace Bio-Labs (Cat# 246854).

Figure 5. ProPlate® Stainless Steel Spring Clips. Grace Bio-Labs (Cat# 204837).

6.4 Washing after Sample Incubation

1. Towards the end of the incubation period, pre-fill Pap jars with 30 mL of SAB.

2. **Wash 1:** When the incubation is complete, gently discard the diluted samples. Subsequently, wash the individual wells with 500 µL of SAB buffer, three times.
3. Remove each clip from the gasket and detach the CTA slides from gasket. Wash each array individually in a Pap jar containing 30 mL of SAB. Invert a few times before placing it in the Pap jar rack on the shaker and shake at 50 rpm (Figure 6).
4. Process the remaining slides in order and place each in the Pap jar rack on the shaker whilst shaking at 50 rpm as they are prepared.
5. Start a timer to countdown 5 minutes after processing the last slide.

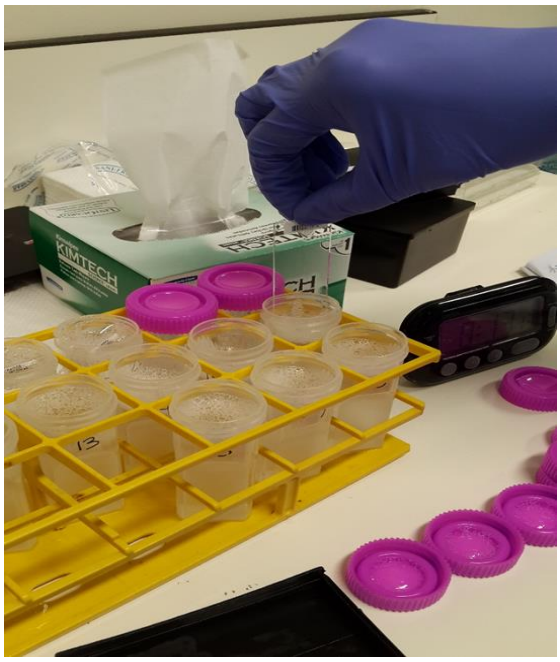


Figure 6: Wash 1: Wash the slide in a pre-filled Pap jar of 30 mL SAB (one slide per Pap jar).

NB: *After Wash 1, take out Cy3-anti human IgG tube from -20°C and place it in 4°C fridge to thaw.*

6. **Wash 2**: After the 1st wash is completed, take the first Pap jar and pour out the wash solution into an empty beaker. Dispense 30 mL of fresh SAB at the back of the slide. Cap the Pap jar, invert it four times and place it in the Pap jar rack on the 50 rpm shaker. Process the remaining slides in order and start the timer to count down 5 minutes.
7. **Wash 3**: When the 2nd wash step is nearly finished, prepare a slide staining dish with a rack and add 200 mL of SAB. When the second wash has finished, take the first Pap jar and pour out the buffer.

Grip the slide between the index finger and thumb and place in slot 2 of the slide rack with the barcoded side facing towards slot 1. Place the rack back in the SAB.

8. Add the remaining slides sequentially until all slides have been transferred. Ensure the slides are all in the same orientation and order. Replace the slide rack in buffer between the additions of each slide (Figure 7).
9. When all the slides have been added, gently shake the slide rack up and down five times to aid mixing. Place the slide staining dish on a shaker for 5 minutes at 50 rpm. It is recommended to cover the slide staining dish with the lid.

Figure 7: Wash 3: Transfer all slides into slide staining dish with a rack containing 200 mL of SAB.

6.5 Incubation with Cy3-Anti Human IgG

1. When the 3rd washing step is nearly complete, add 200 µL of Cy3-Anti-Human IgG into 200 mL of SAB (Cy3-Anti-Human IgG final concentration: 1 µg/mL / 1:1000 dilution) and mix well. Pour the solution into a clean slide staining dish (without the rack) and cover until required (Figure 8).
   NB: Minimize exposure of Cy3-Anti-Human IgG to light.
2. Place several layers of paper towel on the bench surface and cover this with layers of laboratory tissue. After the 3rd wash is completed, lift the rack of slides from the wash solution and place them on the laboratory tissue to dry.
3. Tap the slide rack gently on the tissue five times to remove excess SAB. Immediately place the slide rack in the slide staining dish containing the mixture of Cy3-Anti-Human IgG solution.

4. Move the rack up and down five times to aid mixing.
5. Place the lid on the slide staining dish and incubate the slides in 20°C incubated shaker at 50 rpm for 1 hour.

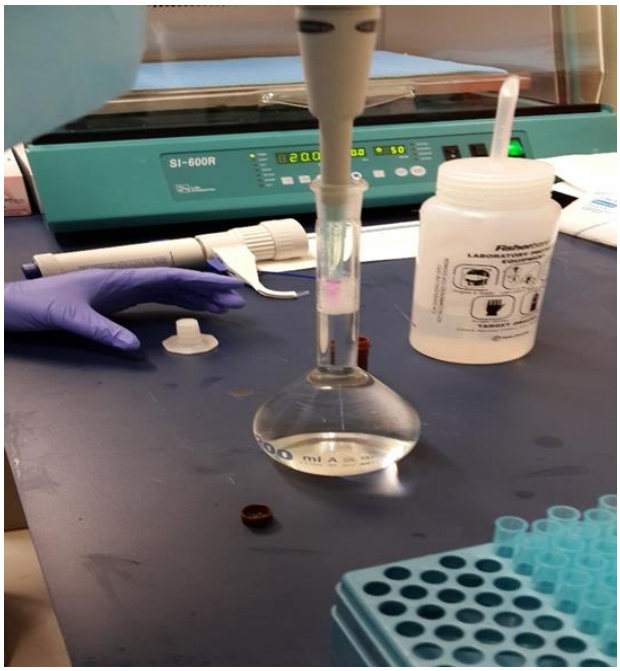


Figure 8: Add 200 µL of Cy3 – anti human IgG into 200 mL of SAB (1:1000 dilution) in volumetric flask. Invert a few times to mix.

6.6 Washing after Cy3-Anti Human IgG Incubation

1. After the secondary antibody incubation period, wash the slides three times with SAB for 5 minutes. Perform each wash in a clean slide staining dish pre-filled with 200 mL of SAB. The detailed steps of the washing step are described below:

1st wash:

- Lift the slide rack from its incubation solution and place it into 200 mL of fresh SAB wash solution.
- Move the rack gently up and down five times. Replace the lid and shake for 5 minutes at 50 rpm at room temperature.

2nd wash:

- Prepare 200 mL of SAB for the 2nd wash in a clean slide staining dish. After the 1st wash is completed, lift the slide rack out and place it into 200 mL of SAB wash solution. Discard the old wash buffer.
- Move the rack gently up and down five times. Replace the lid and shake for 5 minutes at 50 rpm at room temperature. Discard the old wash buffer.

3rd wash:

- Prepare 200 mL of SAB for the 3rd wash in a clean slide staining dish. After the 2nd wash is completed, lift the slide rack out and place it into 200 mL of SAB wash solution. Discard the old wash buffer.
- Move the rack gently up and down five times then replace the lid and shake for 5 minutes at 50 rpm at room temperature.

2. Prepare a new slide staining dish with distilled and filtered water. When the 3rd wash is complete, lift the slide rack out of the dish and place the slide rack in the water. Shake gently up and down five times.
3. Repeat Step 2 twice (3 total washes) to ensure the buffer components are completely washed away from the slide rack and arrays.
4. Place 2 layers of laboratory tissues inside a clean, dry slide staining dish. Additionally, place several laboratory tissues on a clean bench for the drying step.
5. Remove the slide rack from the dish and tap gently five times on the laboratory tissues to remove excess water.
6. Place the slide rack back in the dry slide staining dish and cover with the lid.

6.7 Drying the Slides

Prior to scanning, the slides need to be dried. The slides can either be air dried overnight, protected from light or by gentle centrifugation for 4 minutes at 400 x g using a centrifuge microplate adaptor.

NB: If drying slides by centrifugation, make sure to balance the centrifuge with a slide staining dish filled with blank glass slides.

6.8 Scanning the Slides

1. Insert the dry slides into the fluorescence microarray scanner. Refer to the scanner manufacturer's instruction manual and safety information on the correct use of the scanner.
2. General guidelines for scanner settings are as follows:

Wavelength	532 nm
Channel	Green (G)
Resolution	10 µm
TIFF	16-bit
G/R PMT (%)	40 – 80 %

3. PMT percentage/Laser Power and Scan Region are scanner dependent. It is recommended to perform scanning optimization. Use the lower PMT settings for the initial scan. Preview the microarray. Adjust PMT (%), if needed. The scan region determines the area of the slide that is scanned. The scan region should cover the protein printed area and exclude the barcode or other non-transparent areas of the slide.
4. Select an output path for storing TIFF images to a dedicated local drive before proceed with scanning.
5. Rotate the images to a vertical position and save. See Figure 9 below showing vertically oriented scanned slide image. The orientation markers will appear at the top of the array.

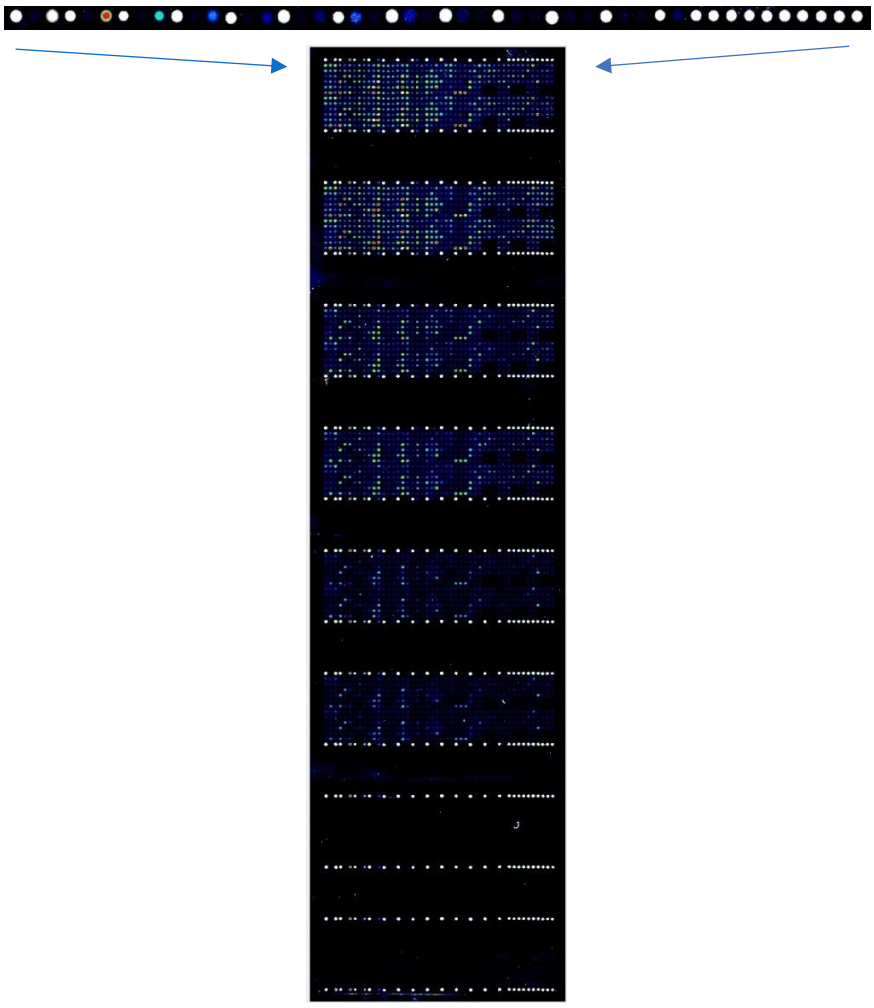


Figure 9. Image of the CTA Protein Array scanned slide. Arrows indicate the positions of the slide orientation control markers.

6. Save the images of the scanned slides as an electronic file in 16-bit TIFF format. At minimum, please ensure the TIFF images are saved in the following format: "***SlideID.tiff***". The slide IDs can be obtained

by scanning the barcodes on the protein array slides. You may refer to the scanner manufacturer's instruction manual to set this automatically.

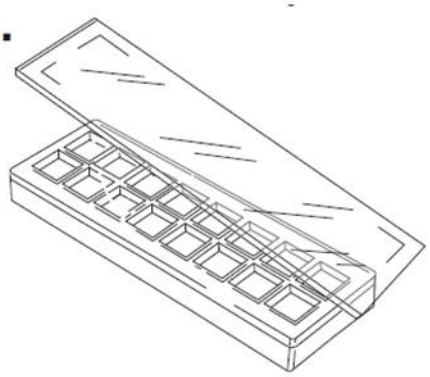
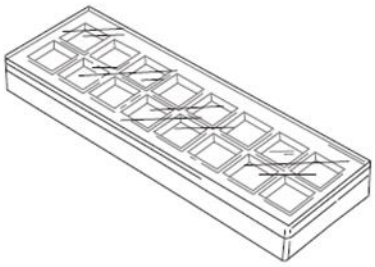
To obtain the Relative Fluorescence Intensity (RFU) for each spot on the array, you will need to analyse each TIFF image using a compatible microarray image analysis software*. A GenePix Array List (GAL) file will be required to perform the image analysis. The GAL file contains the names and positions of all the proteins and control probes on each array. The GAL file for the CTA Protein Array can be downloaded from the product page on the Sengenics website. Please refer to CTA Protein Array (Image Analysis) Instruction Manual for a step-by-step guide on how to perform the image analysis*.

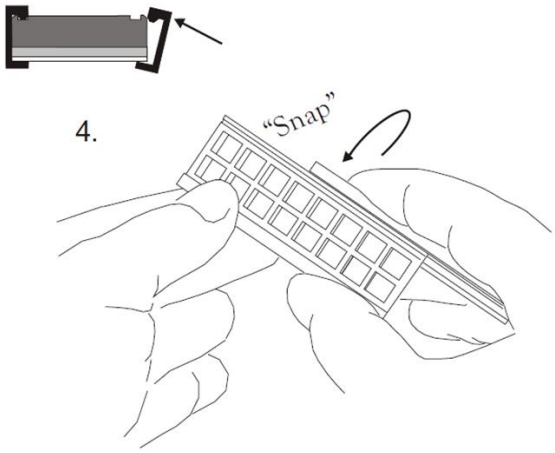
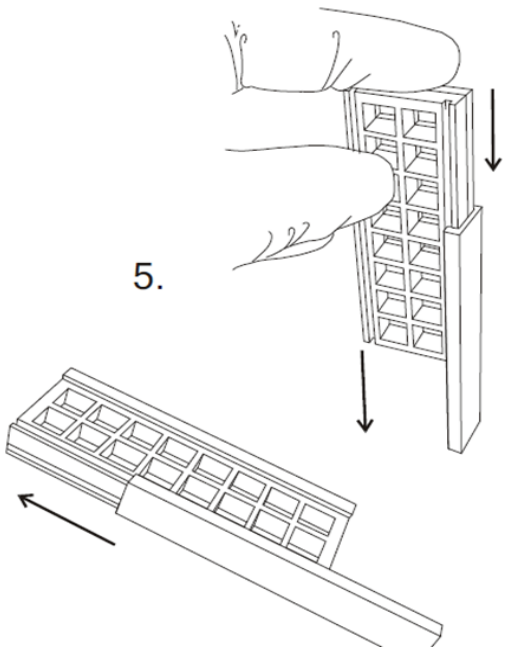
If you need assistance with data analysis, contact us at support@sengenics.com and we will provide a secure link for you to upload the TIFF files with the images of the scanned slides.

** Image analysis software is not part of the product. We recommend using one of the following software packages to perform image analysis: GenePix® Pro7.*

Appendix 1.

Illustrated instructions to assemble ProPlate Slide Module

1. 	Remove release liner to expose silicone gasket.
2. 	Place CTA Protein Array slide (barcoded-side facing down) over silicone gasket, aligning edges of slide with the edges of the upper structure.
3. 	Press gently on back side of the slide to adhere slide to gasket.

	Place stainless steel clip onto slide assembly by snapping onto long edge of module. Grasping assembly, place the flat inner edge of the clip over the glass slide and press the clip into the groove in the upper structure surface.
	Slide clip with the assembly until end. Alternatively, clips may be pressed against the bench top to facilitate application. Repeat assembly step for each gasket.

7. Troubleshooting

High background on protein printed area	Slides were not properly washed. Increase the wash time. Any wash containers used should be cleaned with copious amounts of deionized, distilled water or high purity water.
No signal on positive control spots	Ensure the scanner settings are correct as instructed.
Barcode sticker on slide slips off during washing	There is a gray dot at the bottom-right of each slide. The gray dot is printed on the same side as the array. This dot can be used as an orientation indicator if the barcode comes off.

Contact Information

Sengenics Corporation

Technical support email: support@sengenics.com
www.sengenics.com