Affinity-Based Immobilization Tools For Functional Genomics

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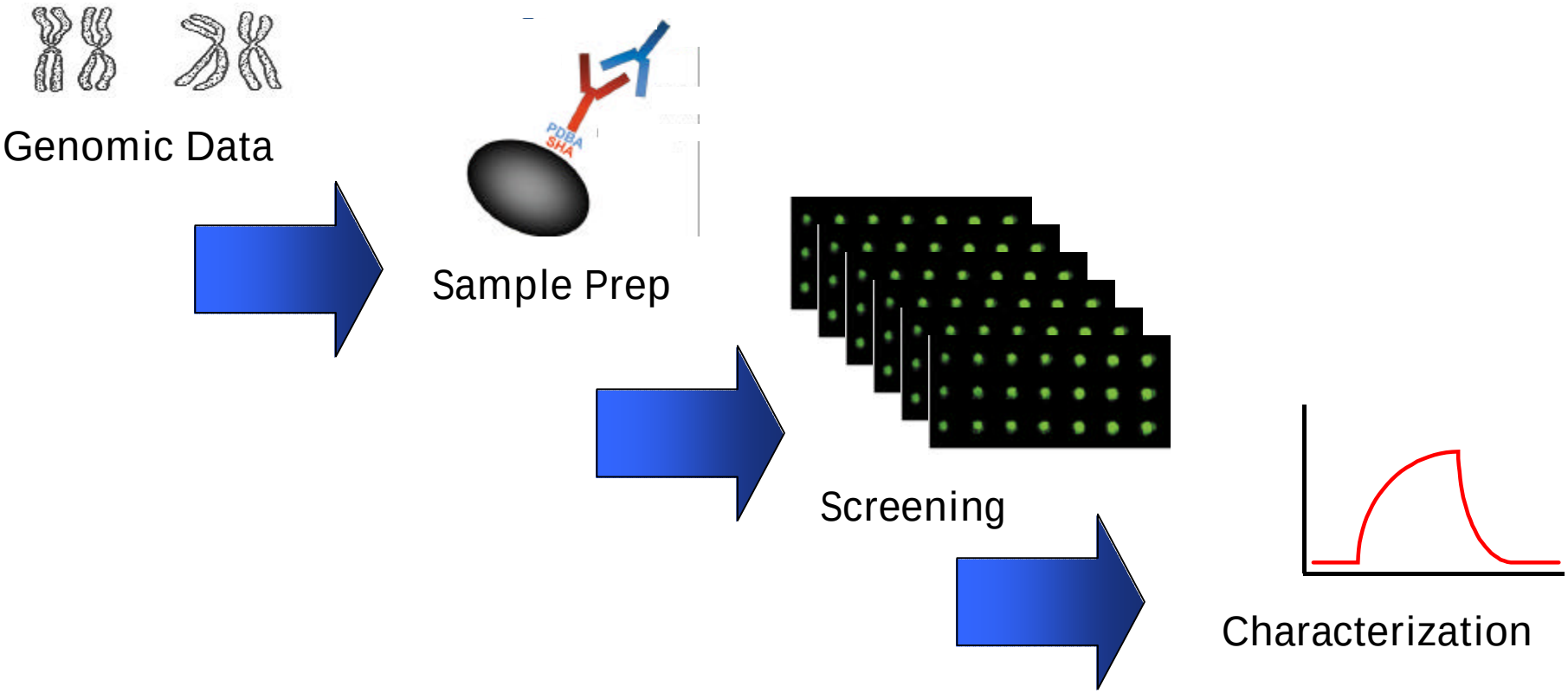
ABSTRACT

Studies of gene expression and subsequent interactions of gene products on a systems scale require methods that are robust, simple to implement and yield data that can be analyzed efficiently. Such methods often require immobilization of biomolecules for capture and detection of analytes, impurities, secondary products or metabolites. Immobilization methods such as direct conjugation to surfaces (e.g., glutaraldehyde coupling) or biologically-based affinity systems (e.g., (strept)avidin/biotin), can be limited by poor reproducibility, low surface capacities, essential purification steps and significant non-specific binding. Prolinx®, Inc. has developed a small molecule affinity system suitable for immobilization of nucleic acids and proteins on a variety of surfaces. This technology is based on the reversible complexation of phenyl(di)boronic acid (P(D)BA) with salicylhydroxamic acid (SHA). Surfaces can be reproducibly modified with SHA resulting in high capacities for P(D)BA-modified biomolecules and excellent assay sensitivities. P(D)BA-modification is performed in solution, independent of immobilization, and PDBA-conjugates can be directly immobilized on an SHA-modified solid support without purification; any excess reagent is removed by washing. As a result, multiple conjugations may be performed in an automated format suitable for high-throughput applications such as protein microarrays. The advantages demonstrated using this system make P(D)BA-SHA technology a convenient platform for systems-scale research.

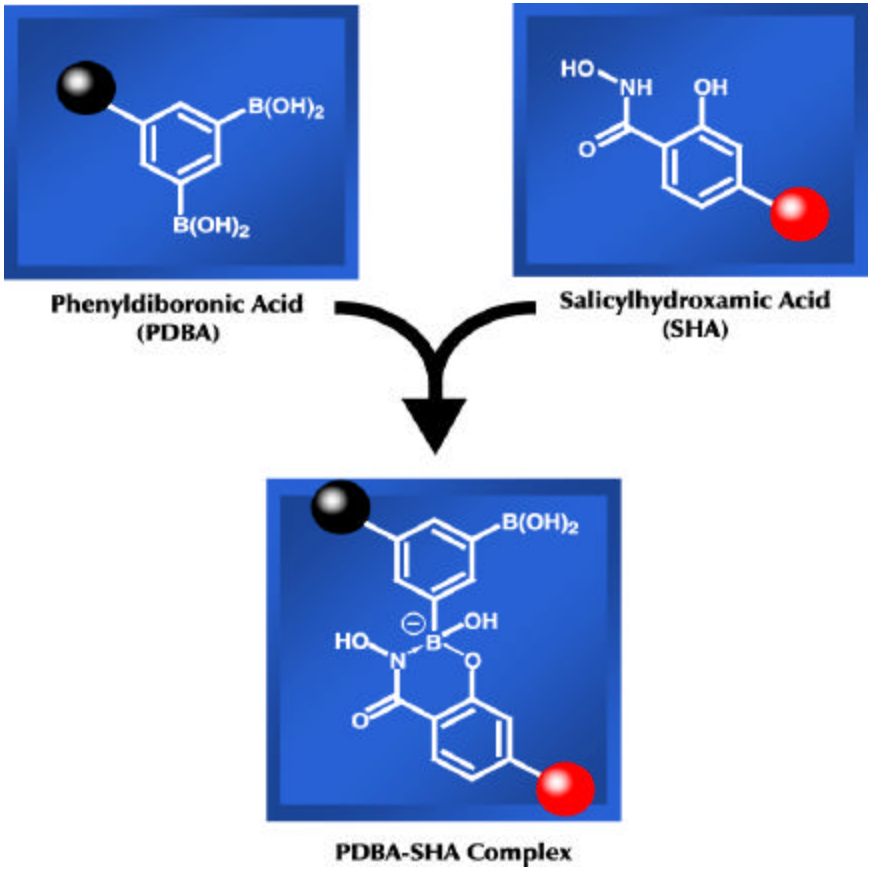
Challenges in Post-Genomics Research: Proteomics

Reality	Desired Tools Characteristics
Large number of samples	Compatible with: ♦ Automation ♦ Miniaturization ♦ High throughput analysis ♦ Bioinformatics
Varied nature of proteins	Universal platforms
Fragile nature of proteins	Utilize mild reaction and sample prep conditions
Maintenance of biological activity critical	Little perturbation of structure

Progression From Genomics to Characterization of Function



Versalinx Chemistry - Platform Technology for Functional Genomic Studies



- Synthetic, small molecule, low molecular weight affinity pair
- Complexation used for the facile immobilization and detection of nucleic acids, proteins and other macromolecules
- Interaction is reversible under controlled and mild conditions

Attributes of Versalinx Tools

- Easy to use and compatible with common assay conditions
- Exhibit fast complex formation with high retention of ligand activity
- Afford specific complex formation
  - Low non specific binding
  - High signal:noise
- Provide “universal” surfaces for immobilization of ligands
  - Preparative, analytical
  - e.g., beads, plates, slides, biochips, arrays, separation media,
  - microfluidic devices
- Offer extraordinary flexibility due to independent reagent modification and immobilization
  - Solution phase modification affords accessibility to all conjugation sites
  - Better distribution of orientation of immobilized ligand
- Post-modification purification is not required
  - Eliminates steps, increasing efficiency
  - Facilitates automated and HTP applications

Versalinx Tools for Protein Modification: Mix and Match

Protein Modification Reagents

Amine Modifying Reagent (NHS ester)
Carbohydrate Modifying Reagent (hydrazide)
Sulfhydryl Alkylating Reagent (maleimide)
Disulfide Forming Reagent (dithiopyridyl)

Assay Surfaces

High Capacity Superparamagnetic Beads
Microtiter Plates
Microscope Slides

Separation Media

High Capacity Superparamagnetic Beads
Chromatography Media
- Crosslinked Agarose, 4%
- Cellulose

Detection Reagents

Rapid Protein-Enzyme Conjugation Kit, HRP
Rapid Protein-Enzyme Conjugation Kit, AP

Other

Protein Releasing Reagent

Nucleic Acid Modification Reagents also available
Custom services available upon request.

Attributes of Versalinx Complex Formation

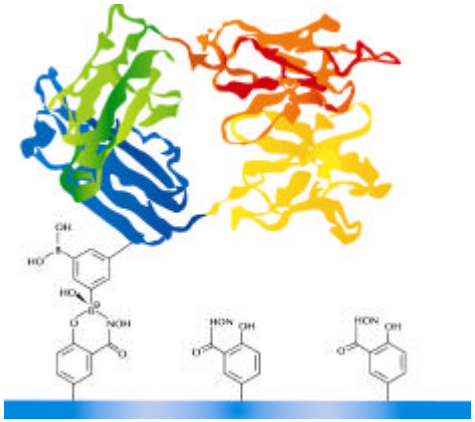
Complex formation is compatible with:

- Wide range of buffers (e.g., acetate, phosphate, Tris, carbonate)
  - pH 5 to 9
- Ionic strength to 1.5 M (monovalent and divalent cations)
- Organic co-solvents (formamide, dimethyl sulfoxide, alcohols)
- Detergents to 1% (SDS, Triton X100, Tween 20)
- Chaotropes (urea to 8 M, guanidine hydrochloride to 4 M)

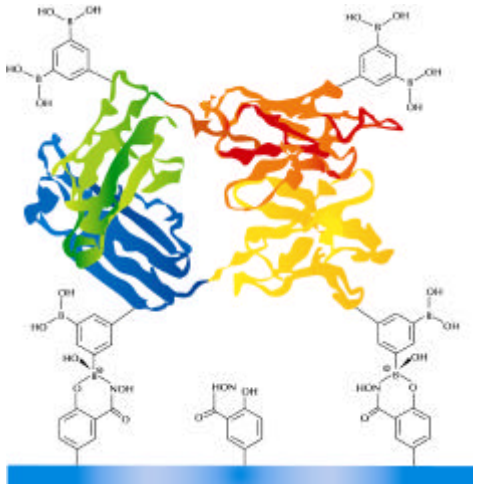
Once formed, the PBA:SHA complex is stable to an even greater variety of conditions.

Attributes of Versalinx Tools

Optimized Avidity



$K_{affinity} \cong 10^6\ M^{-1}$



$K_{observed} \geq 10^{10}\ M^{-1}$

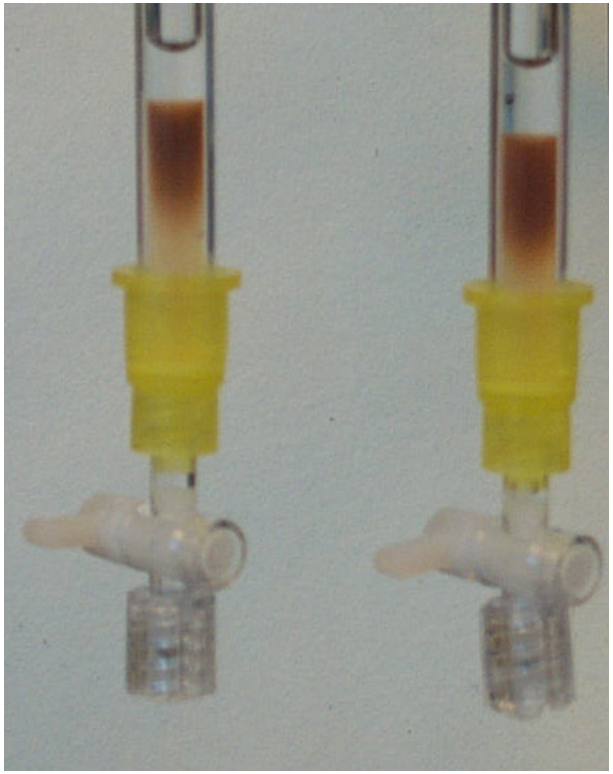
- Modest 1:1 affinity but high avidity through multiple labels means proteins will bind strongly and excess conjugating reagent will bind weakly
- Crude reaction mixtures can be used directly without purification

Sample Preparation Using Versalinx Tools

Fewer Steps Than Other Methods

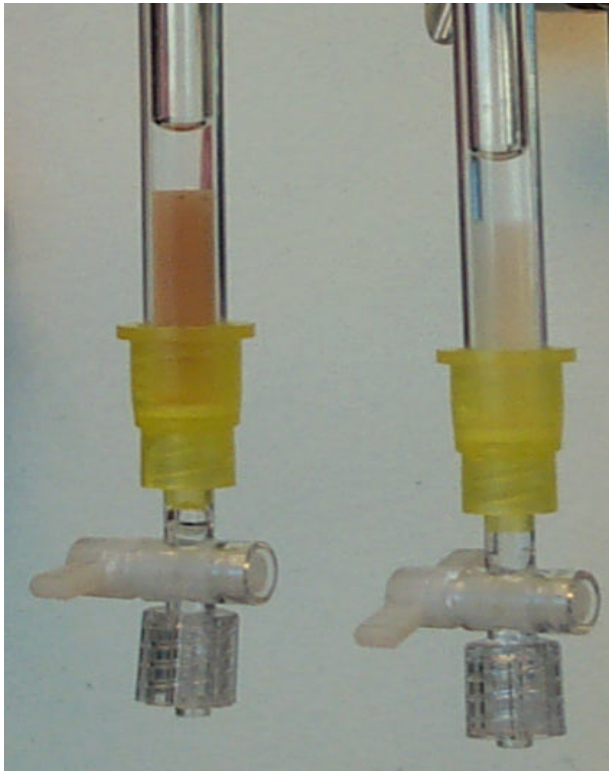
A comparison of **PDBA:SHA** and **biotin:streptavidin** affinity chromatography systems demonstrating that Versalinx Tools require fewer steps because purification of PDBA-conjugates is not necessary.

SHA agarose, 4%



Left: PDBA-HRP, purified (96% retained)
Right: PDBA-HRP, unpurified (95% retained)

streptavidin agarose, 4%

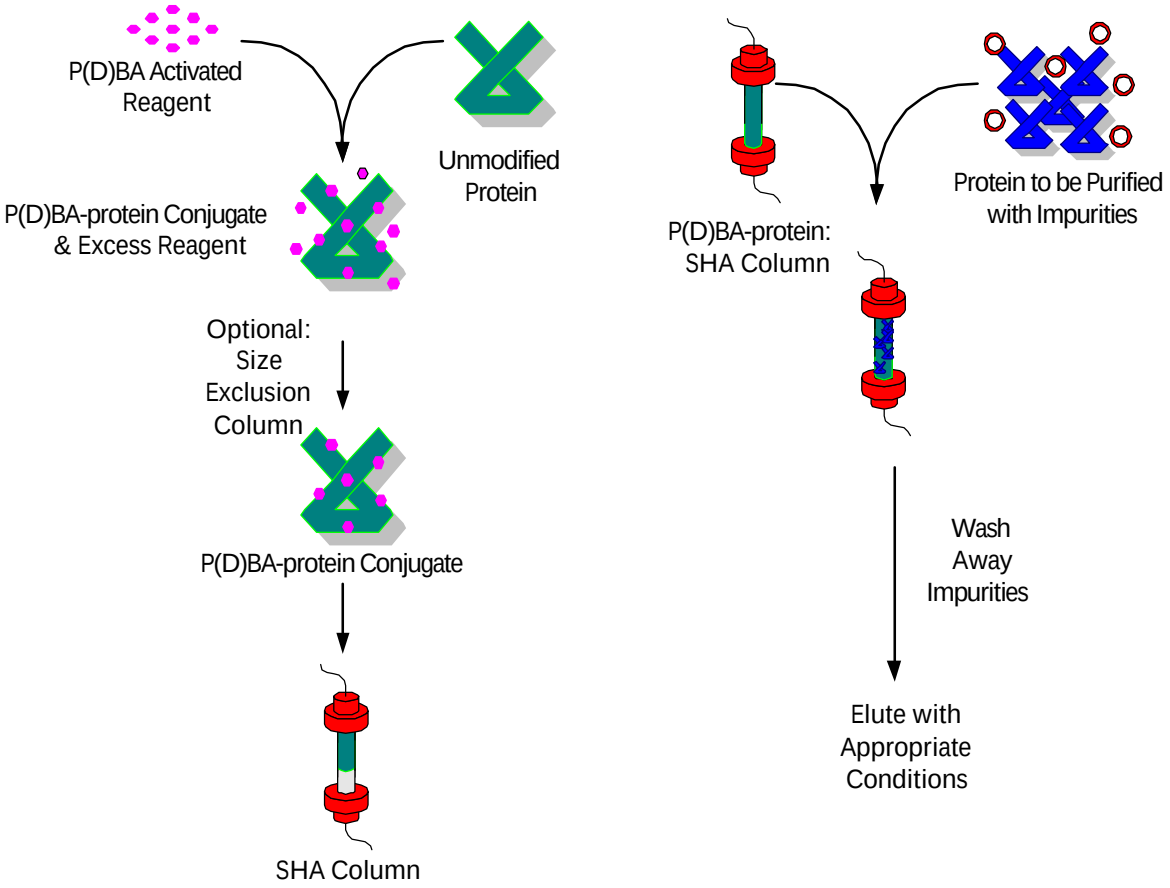


Left: biotin-HRP, purified (68% retained)
Right: biotin-HRP, unpurified (25% retained)

PDBA-horseradish peroxidase (HRP) or biotin-HRP (4 mg each) were applied to affinity chromatography columns containing 0.5 mL SHA-agarose or streptavidin-agarose, respectively. Retention was calculated by measuring protein in flow through and comparing this value to protein input. HRP contains heme (λ max = 405nm), so the differences in the extent of immobilization of the HRP conjugates can be observed visually.

Sample Preparation Using Versalinx Tools

A Facile Affinity Chromatography System

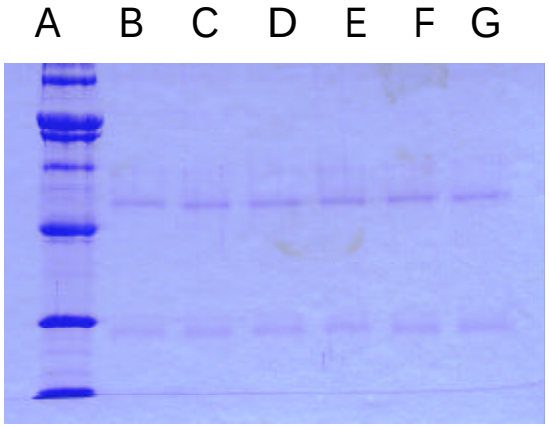


Sample Preparation Using Versalinx Tools: High Throughput Applications

Superparamagnetic Particles

Method

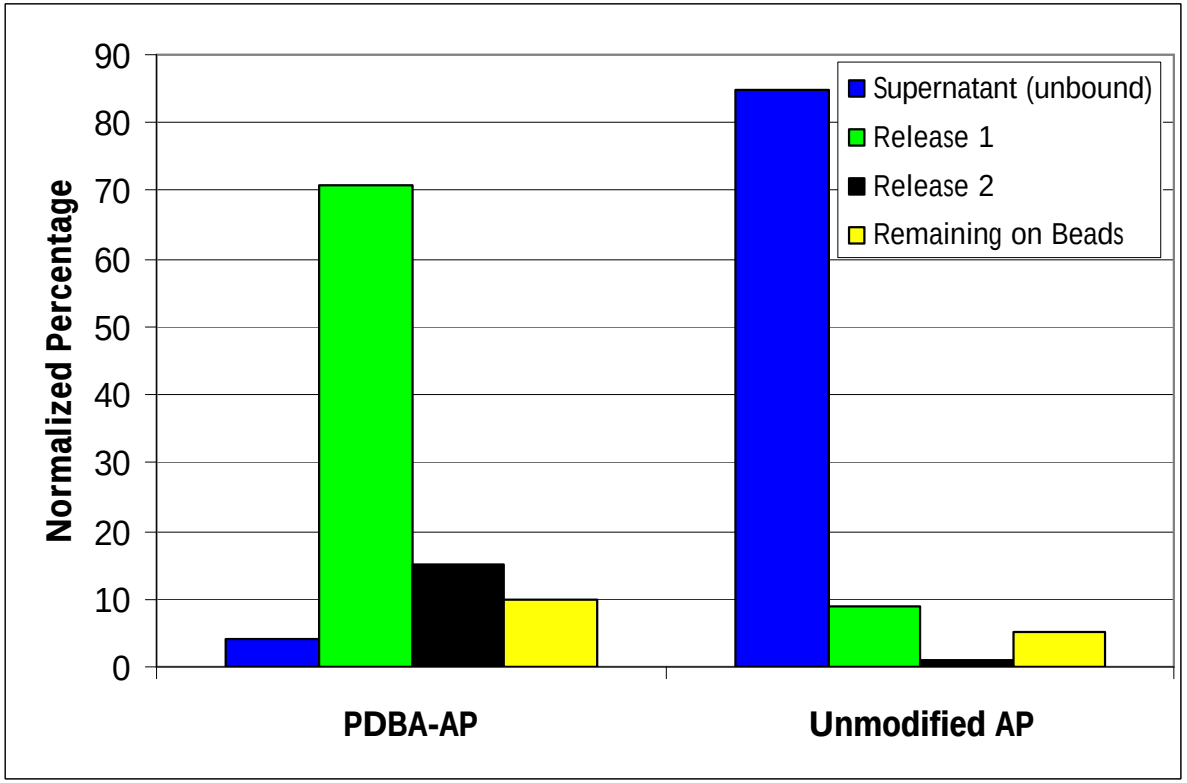
- Add 0.3 mg high capacity superparamagnetic beads to each well of a 96-well plate, wash twice in phosphate buffered saline, pH 7(PBS)
- Add P(D)BA-conjugated protein A - titration range 25-400pmoles, 1 hr room temp, wash as above
- Add 100 pmole human IgG (100 µL), incubate 1 hr, wash as above
- Release human IgG in 0.1 N NaOH, 15 min, pellet beads on a magnetic separator, collect supernatant
- Neutralize with HCl to pH 7
- Analyze released human IgG in PAGE gel and Coomassie blue stain



A = protein standard
IgG Fraction Captured and Released from:
B = 400 pmoles PDBA-protein A
C = 300 pmoles PDBA-protein A
D = 200 pmoles PDBA-protein A
E = 100 pmoles PDBA-protein A
F = 50 pmoles PDBA-protein A
G = 25 pmoles PDBA-protein A

Sample Preparation Using Versalinx Tools

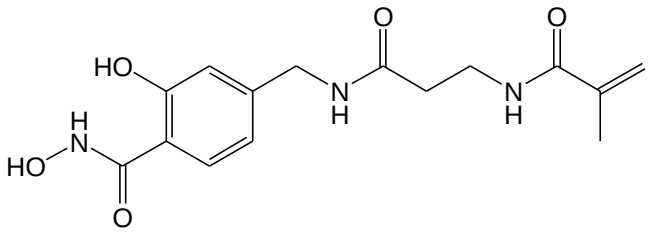
Capture and Release of Protein Complexes is Efficient



PDBA alkaline phosphatase (AP) or unmodified AP (1 mg) were added to 0.3 mg of Versalinx high capacity superparamagnetic beads in PBS. Reactions were incubated 1 hour at room temperature, then beads were washed twice in PBS. To recover PDBA-conjugated protein, Versalinx Protein Releasing Reagent was added and reactions were incubated 1 hour at 37°C. Beads were pelleted on a magnetic separator, and supernatant was removed ("release 1") and this release step was repeated ("release 2") with little effect on the total recovery. After both release steps, the beads were washed 2X and resuspended in PBS, pH 7.2. Protein was quantified by incubating with p-nitrophenylphosphate (pNPP) for 45 minutes at 37°C, then measuring absorbance at 405 nm.

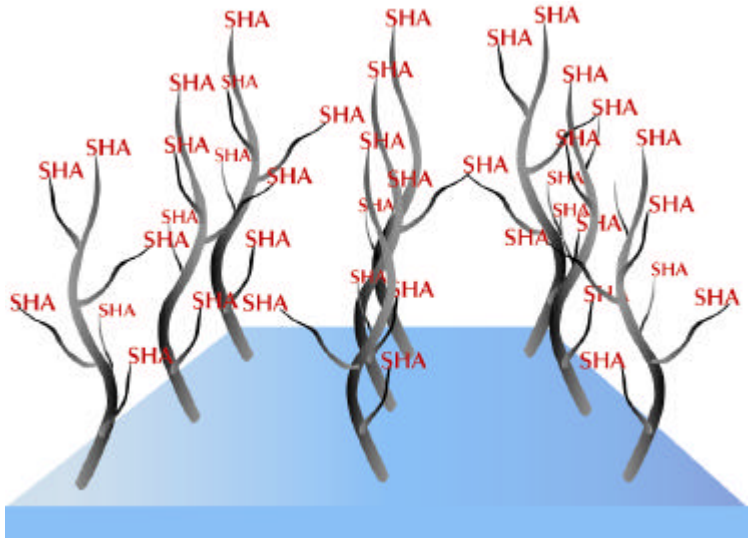
Versalinx Tools for Screening: Protein Microarrays

3D Presentation of SHA, Polymer Brushes



+

co-monomer

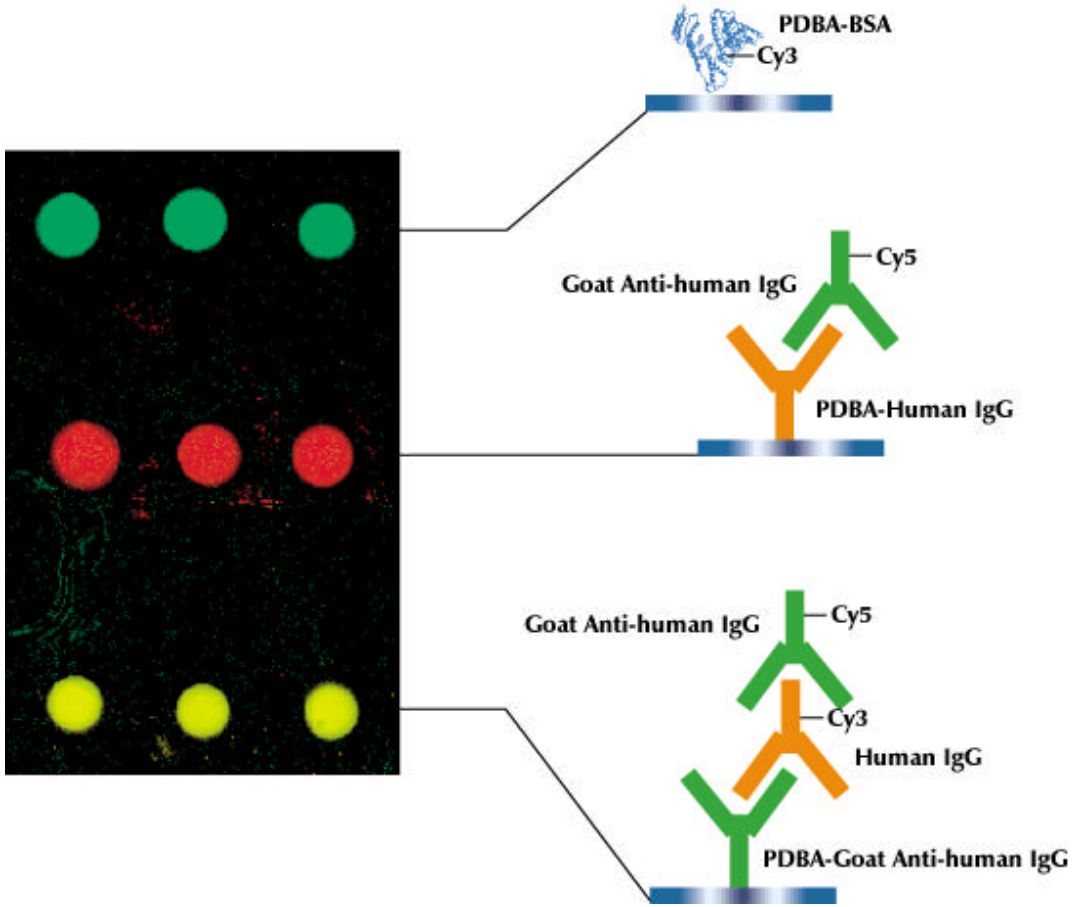


Direct polymerization on slide surface

SHA is incorporated into a 3-D matrix bound to a glass microscope slide. P(D)BA is covalently bound to proteins using known chemistries. Proteins are immobilized on the surface by the specific complex formation of P(D)BA with SHA

Versalinx Tools for Screening

Performance of SHA-Modified Polymer Brush Glass Slides for Protein Microarrays



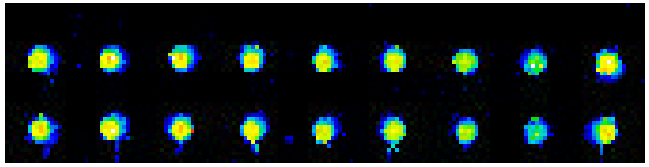
Method

- Spotted proteins manually
- Incubated 60 minutes
- Washed
- Developed with Cy3-human IgG
- Washed
- Developed with Cy5-Goat anti-human IgG
- Washed
- Detected at:
  - *Cy3, 90% laser, 75% PMT gain, 10 mm resolution*
  - *Cy5, 90% laser, 50% PMT gain, 10 mm resolution*

Green = Cy3 signal; Red = Cy5 signal; Yellow = Cy3 + Cy5 signal

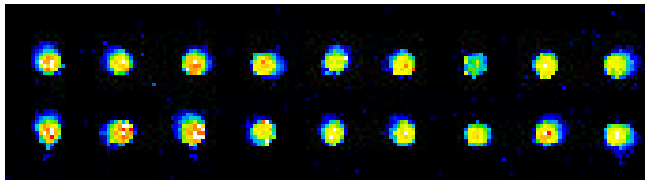
Versalinx Protein Microrray Tools Enable High Throughput Protein Labeling

Purification of Versalinx-Modified Proteins is NOT Required



Dialyzed PDBA-Human IgG

- Modified at 15:1 input ratio (PDBA-X-NHS), 1 hours, 4 °C then dialyzed overnight
- Spotted at 150 µg/mL, 1 hour



Crude PDBA-Human IgG

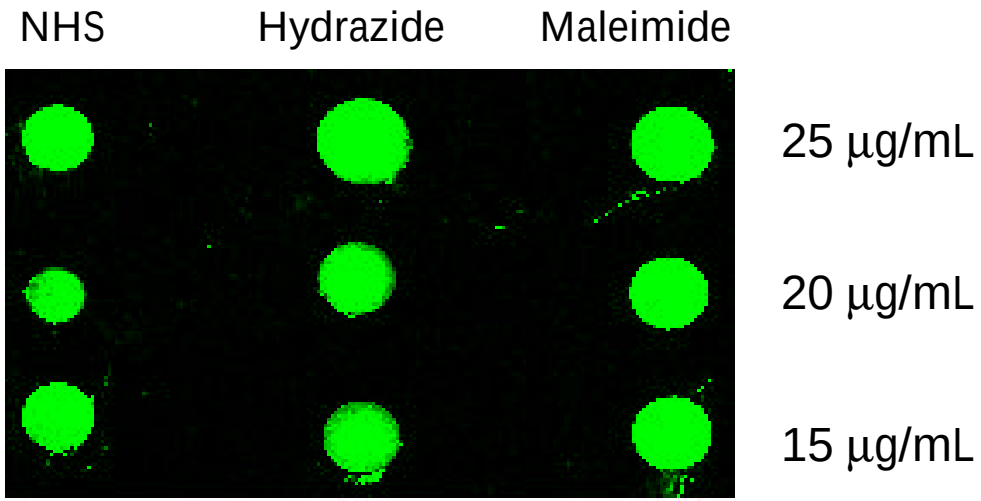
- Modified at 15:1 input ratio (PDBA-X-NHS), 1 hours, 4 °C
- Spotted at 150 µg/mL, 1 hour

- *Developed with Cy3-Goat anti-Human IgG, 4 mg/mL, 30 min*
- *Cy3 channel, 80% laser, 75% PMT gain, 5mm resolution*

Performance of Versalinx Protein Arrays

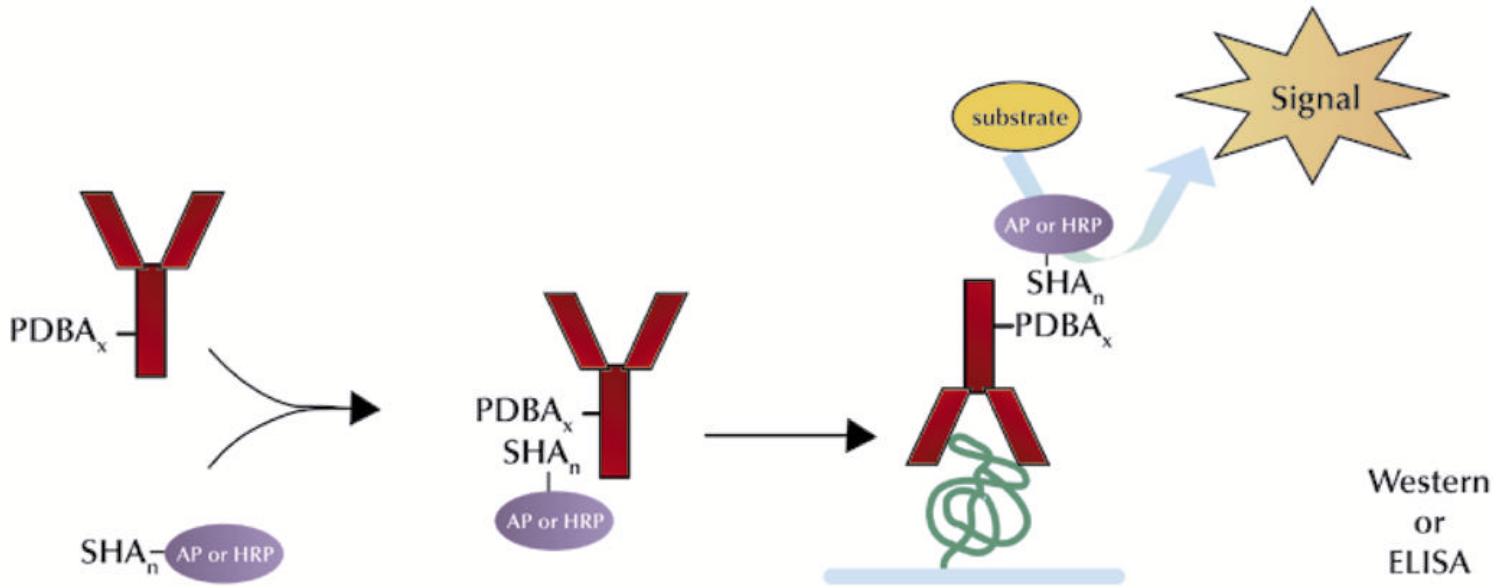
3D Surface Exhibits Strong Signal/Low Noise Regardless of Conjugation Reagent Used

Human IgG Samples Modified Using Different Versalinx Protein Modification Reagents



- *Human IgG modified at 10:1 input ratio PDBA-X-NHS, PDBA-X-hydrazide or PDBA-X-maleimide*
- *Incubated 1 hour, 4 °C*
- *Unpurified protein conjugation reactions were spotted manually, 1 hour incubation*
- *Developed with Cy3-Goat anti-Human IgG, 0.1 mg/mL, 30 min*
- *Cy3 channel, 100% laser, 90% PMT gain, 50 mm resolution*

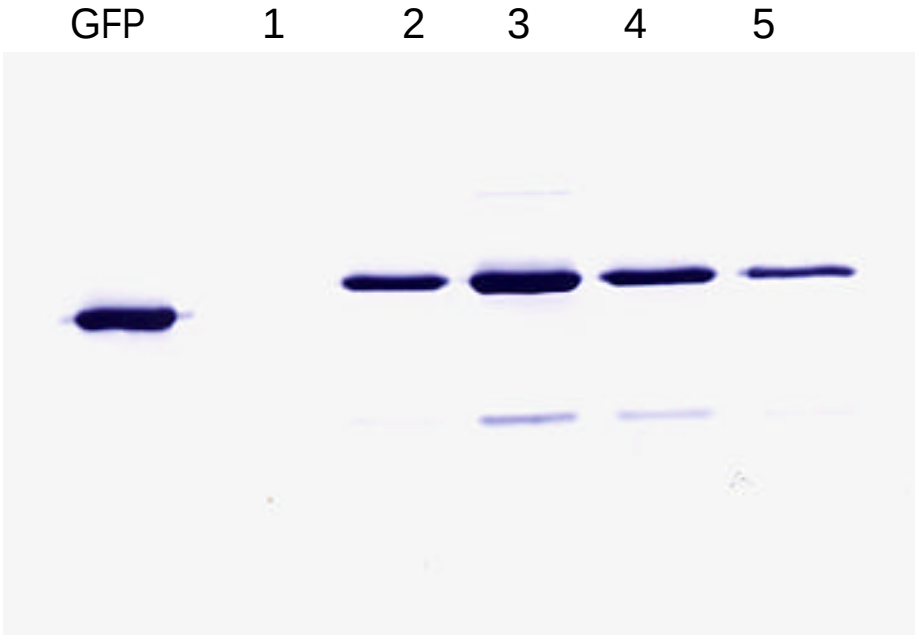
Versalinx Tools May Be Used for Protein Detection



Versalinx Tools May Be Used for Protein Detection

Western Blot

Detection Using PDBA-modified Antibody and SHA-modified Alkaline Phosphatase



GFP: Unmodified (control
1. 6xHis GFP Fraction 1, 3 pmole
2. 6xHis GFP Fraction 2, 17 pmole
3. 6xHis GFP Fraction 3, 33 pmole
4. 6xHis GFP Fraction 4, 23 pmole
5. 6xHis GFP Fraction 5, 8.5 pmole

His-tagged green fluorescent protein (6xHis GFP) was purified from crude cell lysate using Ni-NTA column (Pierce). Fractions from purification were run on an acrylamide gel and blotted to a nylon membrane. The membrane was hybridized with pre-conjugated PDBA-anti GFP:SHA-AP and hybridization was visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT)

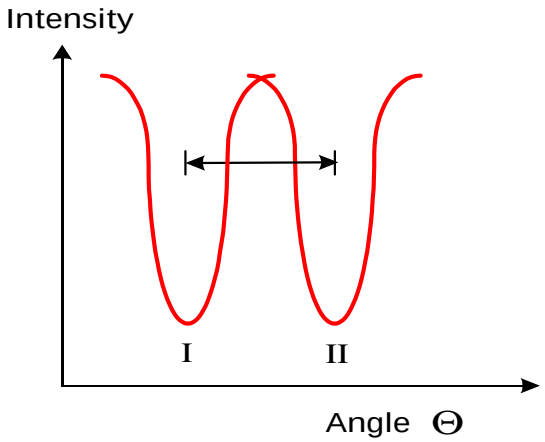
Versalinx Tools for Protein Characterization

Octave™ Molecular Interaction Analysis System

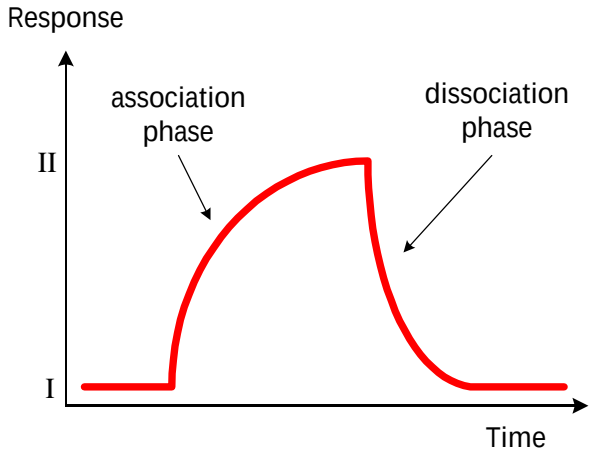
Based on Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) Enables Real-time, Label-free Detection of Molecular Interactions

- Result of the interaction of energy from light photons interacting with a thin metal (gold) surface
- An evanescent wave propagates through the gold, exciting the plasmons on the surface
- Changes in the mass at the surface (state "I" to state "II") result in changes in the angle of reflection of minimum intensity (Θ)
- These changes can be monitored to produce sensorgrams, plots of angle vs. time, yielding information about kinetics of the transition from I to II
- This enables real-time, label-free detection of molecular interactions



Raw Data



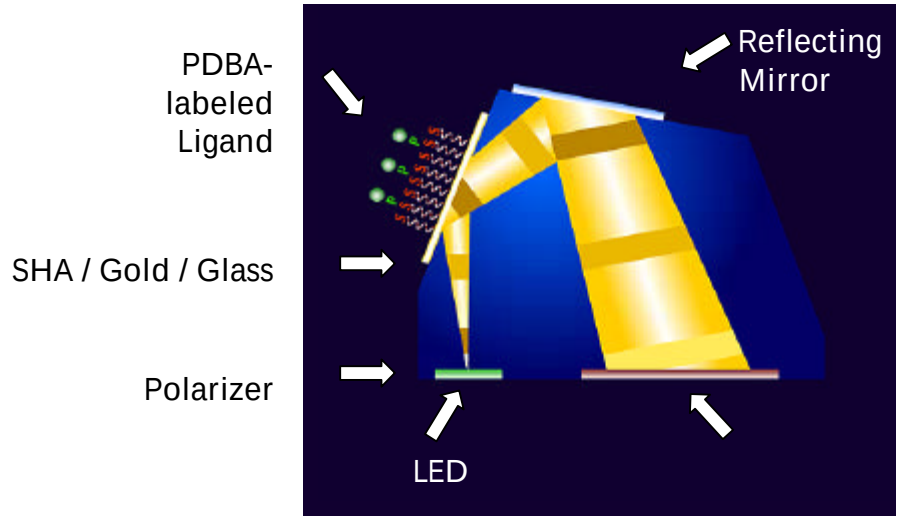
Sensorgram

Versalinx Tools for Protein Characterization

Octave Molecular Interaction Analysis System

Cross-Section of the Spreeta 2000 Sensor Chip

- Complete SPR sensor in a unit about the size of a dime
- Miniature device that can be arrayed along 9 mm centers (standard 96-well plate)



Versalinx Chemical Affinity Tools

- Eliminate the need for several different surface chemistries for ligand immobilization on sensor surface
- Provide sensor surfaces that exhibit very low non-specific binding
- Provide sensor surfaces that can be regenerated by displacing bound analyte and, in some cases, allow intact complexes to be recovered for further analysis

Prolinx Octave System
Biosensor Biomolecular Interaction Analyses

- Protein-protein
- Peptide-protein
- DNA-protein
- Nucleic acid hybridization
- Biomolecule-cell receptor
- Receptor-ligand
- Small molecule-target molecule
- Hormone-receptor
- Protein-carbohydrate
- Peptide-carbohydrate
- Lipids-vaccine
- Bacteria-cell surface receptor
- Cells-drug candidate
- Viruses/virus-like particles

Versalinx Tools for Functional Genomics

Conclusions

- Easy to Use
  - stable, robust surface chemistry
  - universal substrates
  - Simple to implement
  - solution phase modification affords accessibility to all conjugation sites, better distribution of correctly oriented immobilized ligands
- Post-modification purification is not required
  - eliminates steps, increasing efficiency
  - facilitates efficient analyses
    - automated and HTP applications
- Ideal for functional genomics and proteomics
  - sample prep, screening and characterization
  - higher-throughput and automated analyses
    - protein microarrays
    - protein detection
    - molecular interaction analysis