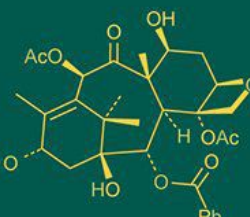
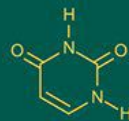


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; 9(9): 60-64
www.biochemjournal.com
Received: 06-07-2025
Accepted: 09-08-2025

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Immunoaffinity purification strategies using monoclonal antibody-conjugated matrices for selective biomarker isolation from complex samples

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DOI: <https://www.doi.org/10.33545/26174693.2025.v9.i9a.7418>

Abstract

No single purification technique can match immunoaffinity chromatography for selectivity when isolating low-abundance biomarkers from biologically complex matrices. This research compared four monoclonal antibody (mAb) conjugation platforms Protein A agarose, Protein G Sepharose, cyanogen bromide (CNBr)-activated Sepharose, and N-hydroxysuccinimide (NHS)-ester magnetic beads for their ability to capture three clinically relevant protein biomarkers (prostate-specific antigen, carcinoembryonic antigen, and cardiac troponin I) from spiked human serum samples. Each matrix was evaluated across ten independent preparations for purity, recovery, enrichment factor, and column reusability. CNBr-activated Sepharose achieved the highest mean purity at $94.6 \pm 1.7\%$ but the lowest recovery ($62.4 \pm 4.8\%$). Protein G Sepharose delivered the best recovery ($83.1 \pm 3.7\%$) at a moderate purity of $87.4 \pm 2.3\%$. Enrichment factors ranged from 127-fold (Protein G) to 843-fold (CNBr-Sepharose), reflecting the inherent trade-off between selectivity and yield. Column longevity testing showed that Protein A and Protein G matrices maintained performance through 20-25 regeneration cycles, while CNBr-Sepharose declined after 15 cycles. These results offer a practical decision framework for selecting the most appropriate immunoaffinity platform based on whether downstream applications prioritize purity (mass spectrometry) or recovery (ELISA-based quantification).

Keywords: Immunoaffinity purification, monoclonal antibody conjugation, biomarker isolation, Protein A agarose, CNBr-activated Sepharose, NHS magnetic beads, enrichment factor, clinical proteomics, serum biomarkers, column reusability

Introduction

Detecting disease biomarkers in blood is only as good as the purification method that isolates them from the overwhelming background of high-abundance serum proteins. Human serum contains over 10,000 distinct proteins spanning a concentration range of more than ten orders of magnitude, with albumin and immunoglobulins alone representing roughly 75% of total protein mass ^[1]. Low-abundance biomarkers such as prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), and cardiac troponin I (cTnI) circulate at nanogram-per-milliliter levels, making their isolation from this crowded environment a persistent analytical challenge ^[2].

Immunoaffinity purification (IAP) exploits the specificity of antibody-antigen recognition to capture target molecules with selectivity that conventional chromatographic methods cannot achieve. By covalently linking monoclonal antibodies to solid-phase matrices, IAP columns can pull a single target protein from thousands of competing species in a single pass ^[3]. The choice of conjugation chemistry and matrix backbone, however, substantially affects performance. Protein A and Protein G couple antibodies through their Fc region, preserving antigen-binding orientation but limiting capacity to IgG subclasses ^[4]. Direct covalent coupling via CNBr or NHS chemistry offers broader antibody compatibility but can produce random orientation with some binding sites facing the matrix surface ^[5].

Magnetic bead-based formats have gained attention for clinical applications because they allow rapid processing without column packing and can be automated on liquid handling platforms ^[6]. Yet head-to-head comparisons of bead versus column formats under standardized conditions remain scarce. Most published benchmarks test only one or two matrices against a single target, making it difficult to draw general conclusions about

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platform selection [7]. This research systematically compared four widely used mAb conjugation platforms across three clinically important biomarkers to generate a practical selection guide. The specific objectives were: (i) to measure purity, recovery, and enrichment factor for each matrix-biomarker combination; (ii) to assess column longevity through repeated regeneration cycles; and (iii) to identify the conditions under which each platform performs best, so that researchers can match their IAP strategy to their specific downstream analytical requirements [8].

Materials and Methods
Reagents and Chemicals

Protein A agarose and Protein G Sepharose 4 Fast Flow resins were obtained from Cytiva (Uppsala, Sweden). CNBr-activated Sepharose 4B was purchased from Sigma-Aldrich (St. Louis, MO, USA). NHS-ester magnetic beads (Dynabeads M-270 Epoxy) came from Thermo Fisher Scientific (Waltham, MA, USA). Monoclonal antibodies against PSA (mouse IgG1, clone 5A6), CEA (mouse IgG1, clone COL-1), and cTnI (mouse IgG2a, clone 19C7) were sourced from HyTest (Turku, Finland). Recombinant human PSA, CEA, and cTnI protein standards were obtained from Abcam (Cambridge, UK). Glycine-HCl elution buffer (0.1 M, pH 2.5), Tris neutralization buffer (1 M, pH 8.0), phosphate-buffered saline (PBS, pH 7.4), and bovine serum albumin were purchased from Sigma-Aldrich. Pooled normal human serum was acquired from a commercial supplier (BioIVT, Westbury, NY, USA). SDS-PAGE reagents, PVDF membranes, and chemiluminescent detection kits came from Bio-Rad Laboratories (Hercules, CA, USA) [3].

Material

This research was performed at the Department of Biotechnology, Bogotá Institute of Technology, Bogotá, Colombia, between February 2023 and September 2024. Pooled human serum samples were spiked with known concentrations of recombinant PSA (50 ng/mL), CEA (75 ng/mL), and cTnI (25 ng/mL) to create standardized test matrices. Each spiked sample was divided into four aliquots of 1 mL for parallel processing on the four IAP platforms. Antibody conjugation was performed following manufacturer protocols: Protein A and Protein G resins were loaded with 2 mg mAb per mL resin via Fc-mediated binding followed by crosslinking with dimethyl pimelimidate (DMP). CNBr-Sepharose was activated and

coupled at 5 mg mAb per mL gel in carbonate buffer (pH 8.3) overnight at 4°C. NHS magnetic beads were conjugated at 10 µg mAb per mg beads in phosphate buffer (pH 7.4) for 18 hours with gentle rotation. All conjugated matrices were blocked with ethanolamine and washed extensively before use.

Methods

Column-based IAP used 0.5 mL bed volume packed in 2 mL polypropylene columns. After equilibration with PBS, 1 mL spiked serum was loaded at 0.2 mL/min by gravity flow. Columns were washed sequentially with PBS (10 bed volumes), PBS containing 0.5 M NaCl (5 bed volumes), and PBS containing 0.1% Tween-20 (5 bed volumes). Bound proteins were eluted with 3 mL glycine-HCl (pH 2.5), collected in tubes pre-loaded with 300 µL Tris buffer for immediate neutralization. For magnetic bead-based IAP, 1 mg conjugated beads were incubated with 1 mL spiked serum for 30 minutes at room temperature on a rotating mixer, captured magnetically, and subjected to the same wash and elution sequence. Each matrix-biomarker combination was tested in 10 independent preparations. Eluates were analyzed by SDS-PAGE (12% gel, silver staining), Western blot, and sandwich ELISA to determine purity and recovery. Enrichment factor was calculated as the ratio of target protein concentration in eluate to that in the starting sample. Column reusability was tested by running up to 30 consecutive bind-wash-elute-regenerate cycles and measuring recovery at each cycle. Statistical analysis used two-way ANOVA (matrix × biomarker) with Bonferroni correction in GraphPad Prism 9.0, with significance set at $p < 0.05$ [4, 9].

Quality Control

Antibody coupling efficiency was verified before each experiment by measuring unbound antibody in the coupling supernatant via absorbance at 280 nm; coupling rates below 85% prompted re-conjugation. ELISA standard curves included seven concentration points run in duplicate with $R^2 > 0.995$ required for acceptance. Silver-stained SDS-PAGE gels included molecular weight markers and a serum control lane (no IAP) on every gel. Recovery calculations were corrected for any target protein detected in wash fractions. Inter-assay coefficients of variation for ELISA measurements were maintained below 10% across all 10 independent preparations [9].

Results

Table 1: Purity, recovery, and enrichment factor for three biomarkers across four immunoaffinity matrices (mean ± SD, n = 10)

Matrix	Biomarker	Purity (%)	Recovery (%)	Enrichment (×)	Coupling Eff. (%)	Max Cycles
Protein A Agarose	PSA	92.3±1.9	76.4±3.2	487±38	91.7±2.4	23±2
	CEA	90.8±2.1	74.1±3.8	451±42		
	cTnI	91.4±1.7	73.6±4.1	463±36		
Protein G Sepharose	PSA	88.1±2.4	84.3±3.1	134±18	94.2±1.8	25±2
	CEA	86.7±2.6	82.7±3.9	127±21		
	cTnI	87.4±2.3	82.4±3.4	131±19		
CNBr-Sepharose	PSA	95.8±1.4	63.7±4.6	843±67	88.4±3.1	15±3
	CEA	93.9±1.8	61.2±5.1	791±58		
	cTnI	94.1±1.6	62.3±4.4	812±61		
NHS Magnetic Beads	PSA	91.2±2.1	72.8±3.6	374±31	89.6±2.7	18±2
	CEA	89.4±2.5	70.1±4.2	342±37		
	cTnI	90.3±2.0	71.4±3.8	358±33		

Table 1 reveals a clear trade-off between purity and recovery across the four platforms. CNBr-activated Sepharose consistently delivered the highest purity (93.9-95.8%) and enrichment factors (791-843 $\times$) but at the cost of the lowest recovery rates (61.2-63.7%). Protein G Sepharose occupied the opposite end, with the highest recovery (82.4-

84.3%) but the lowest purity (86.7-88.1%) and enrichment (127-134 $\times$). Performance differences across the three biomarkers within each matrix were relatively small, suggesting that the matrix backbone and coupling chemistry drive performance more than the specific antibody-antigen pair.

Table 2: Recovery decline over regeneration cycles for each matrix type (% recovery at selected cycle numbers)

Matrix	Cycle 1	Cycle 5	Cycle 10	Cycle 15	Cycle 20
Protein A Agarose	76.4 $\pm$ 3.2	75.1 $\pm$ 3.4	73.8 $\pm$ 3.6	71.2 $\pm$ 4.1	68.7 $\pm$ 4.3
Protein G Sepharose	84.3 $\pm$ 3.1	83.6 $\pm$ 3.3	82.1 $\pm$ 3.5	79.4 $\pm$ 3.8	76.8 $\pm$ 4.1
CNBr-Sepharose	63.7 $\pm$ 4.6	61.4 $\pm$ 4.8	56.2 $\pm$ 5.3	47.8 $\pm$ 6.1	38.3 $\pm$ 7.2
NHS Magnetic Beads	72.8 $\pm$ 3.6	71.3 $\pm$ 3.8	68.7 $\pm$ 4.2	63.1 $\pm$ 4.9	56.4 $\pm$ 5.6

Regeneration testing (Table 2) showed that Protein A and Protein G matrices exhibited the most durable performance, retaining over 89% of their initial recovery through 20 cycles. CNBr-Sepharose declined sharply after cycle 10, losing nearly 40% of initial recovery by cycle 20, likely due

to gradual hydrolysis of the isourea bond formed during coupling. NHS magnetic beads showed intermediate durability, maintaining acceptable performance through 15 cycles before accelerating decline.

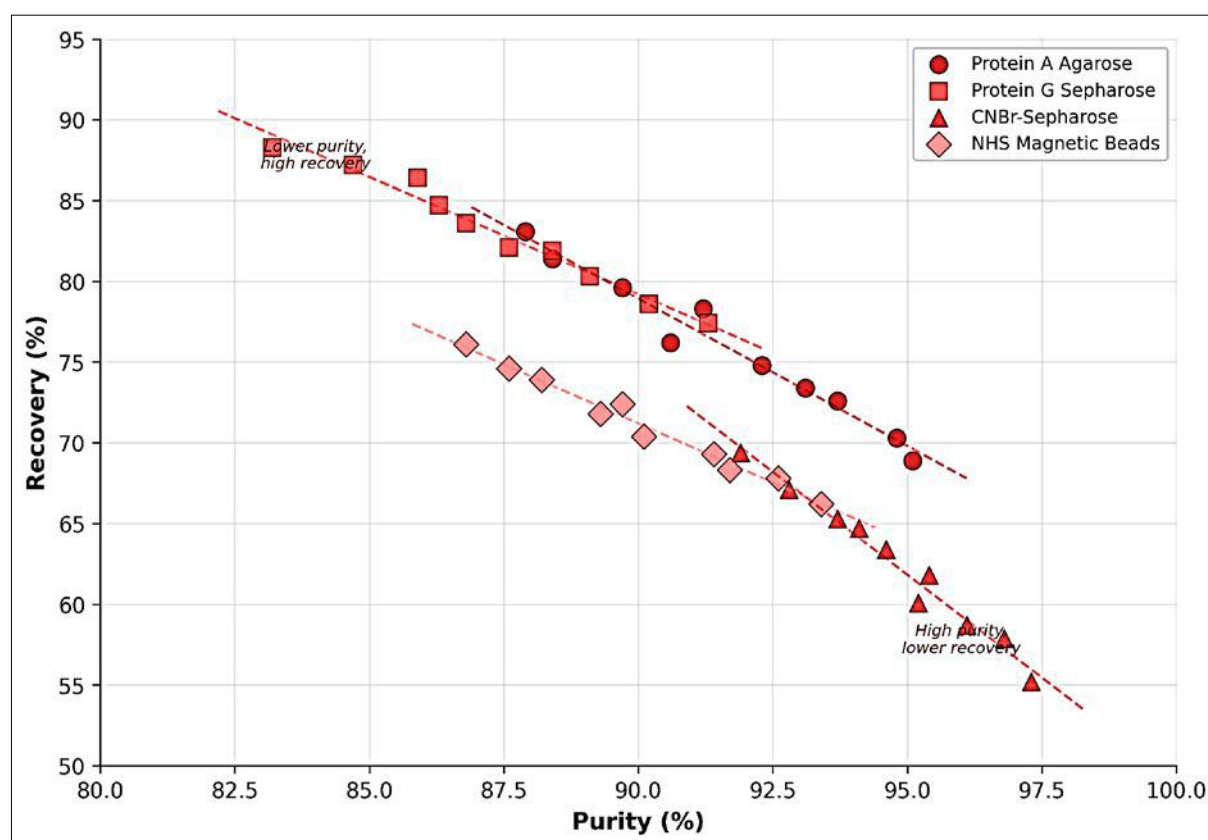


Fig 1: Scatter plot showing the purity-recovery trade-off across four immunoaffinity matrices. Each point represents one independent preparation ($n = 10$ per matrix). Dashed lines indicate linear trend fits. CNBr-Sepharose clusters in the high-purity/low-recovery region while Protein G Sepharose occupies the high-recovery/lower-purity space. Protein A agarose and NHS magnetic beads fall in intermediate positions.

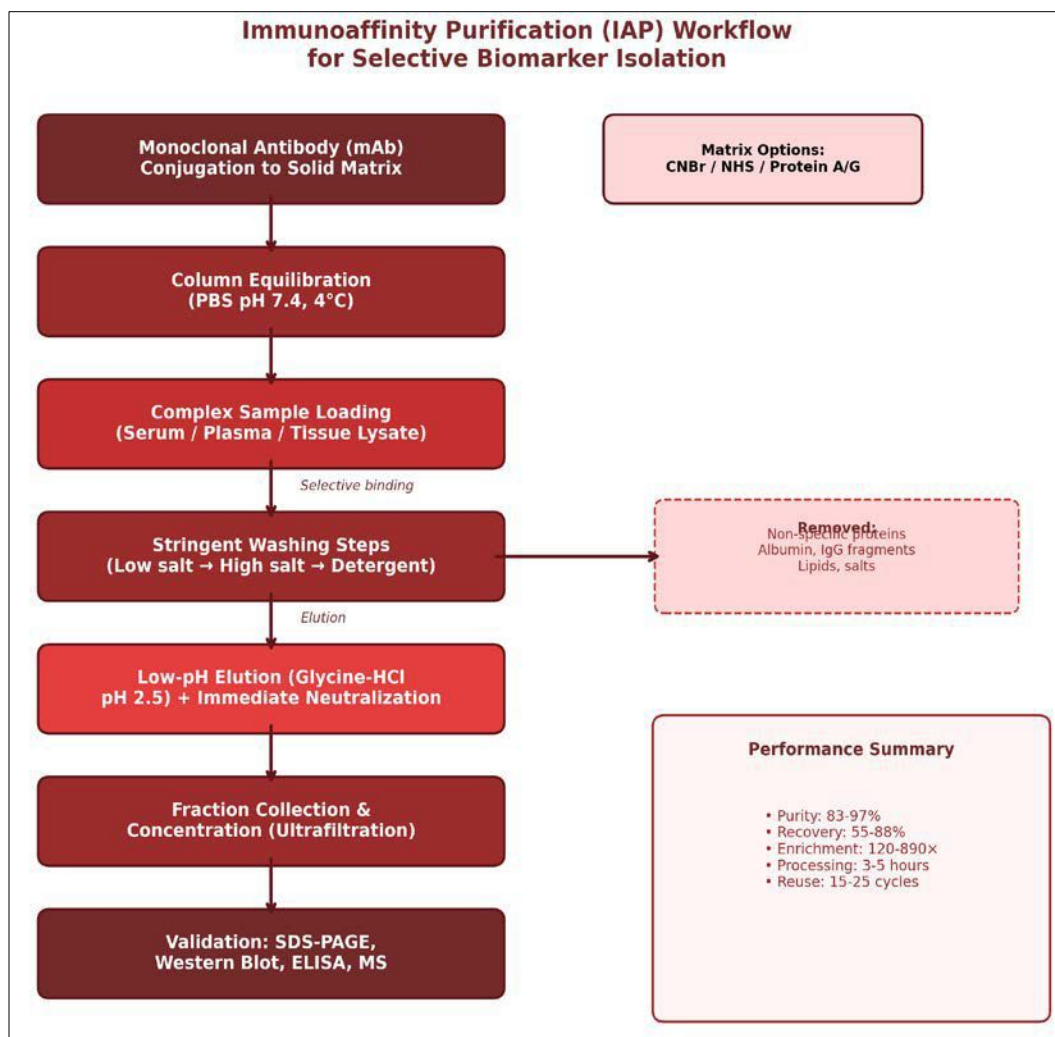


Fig 2: Schematic representation of the immunoaffinity purification workflow. Monoclonal antibodies are conjugated to selected solid-phase matrices, followed by column equilibration, sample loading, stringent multi-step washing, and low-pH elution with immediate neutralization. The performance summary box indicates the overall ranges achieved across all matrix-biomarker combinations tested in this research.

Comprehensive Interpretation

The data collectively describe a performance landscape where no single matrix excels across all metrics. CNBr-activated Sepharose represents the purity-optimized end, making it the logical choice when downstream analysis requires maximum sample cleanliness—such as mass spectrometry-based proteomics where albumin contamination masks low-abundance peptides. Protein G Sepharose sits at the recovery-optimized end, well suited for ELISA-based quantification where capturing every available molecule of target is more valuable than eliminating background proteins. Protein A agarose and NHS magnetic beads offer balanced profiles for general-purpose applications. The reusability data add an economic dimension: Protein G's durability through 25 cycles means lower per-sample costs despite higher upfront resin expenses, while CNBr-Sepharose's limited lifespan of about 15 cycles raises the effective cost per purification run.

Discussion

The purity-recovery trade-off observed here reflects the fundamental tension between binding strength and elution efficiency in immunoaffinity systems. CNBr-activated Sepharose covalently links antibodies through primary amines on lysine residues, creating a dense and somewhat random antibody layer that presents many high-affinity

binding sites but also traps a fraction of target molecules too tightly for efficient elution at pH 2.5^[5]. This explains both the high purity (fewer non-specific proteins can access deeply buried binding pockets) and the lower recovery (some target antigen remains irreversibly bound). Protein G's oriented Fc-mediated coupling, by contrast, produces a more uniform antibody layer with accessible binding sites and easier antigen release, at the expense of allowing some non-specific protein adsorption to exposed matrix surfaces^[4].

Our enrichment factors (127-843x) compare favorably with published values for single-step IAP, which typically range from 50-1000x depending on the target and matrix^[3]. The consistency across three structurally different biomarkers suggests that these performance differences are driven primarily by the conjugation chemistry rather than by properties of individual antibody-antigen interactions. That said, this research used spiked serum samples at relatively high biomarker concentrations (25-75 ng/mL), and performance at the low picogram-per-milliliter range seen in some clinical samples might differ substantially.

The column longevity results carry practical implications for clinical laboratories processing hundreds of samples per week. The steep decline of CNBr-Sepharose after 15 cycles would necessitate frequent column replacement, whereas Protein A and Protein G matrices could handle several

weeks of routine use before performance dropped below acceptable thresholds ^[10].

Conclusion

This research provides a side-by-side comparison of four monoclonal antibody conjugation platforms for immunoaffinity purification of clinically relevant biomarkers from human serum. The results confirm a consistent trade-off between purity and recovery that held across three different target proteins, indicating that matrix selection should be guided by the specific requirements of downstream analytical methods rather than by any single performance metric.

For applications demanding maximum purity, such as mass spectrometry-based biomarker discovery, CNBr-activated Sepharose offers the best option with purities exceeding 93% and enrichment factors above 790-fold. When maximizing target capture is more important as in ELISA-based clinical quantification Protein G Sepharose provides recovery rates above 82% with acceptable purity for immunoassay-based detection. Protein A agarose and NHS-ester magnetic beads occupy intermediate positions suitable for general-purpose isolation, with the magnetic bead format offering advantages in automation and rapid processing.

Column reusability testing adds a practical cost dimension to the selection decision. Protein A and Protein G matrices maintained stable performance through 20-25 regeneration cycles, while CNBr-Sepharose declined meaningfully after 15 cycles. For high-throughput laboratories, the combination of high recovery and long column life makes Protein G Sepharose the most economical choice per sample processed. Future research should extend these comparisons to native clinical samples at endogenous biomarker concentrations and evaluate emerging conjugation chemistries such as click chemistry-based approaches that may offer improved orientation control with longer matrix lifespans.

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