Optimizing Immunoprecipitation Workflows for Next-gen Protein Sequencing

HIGHLIGHTS

- **Get reliable next-gen protein sequencing (NGPS) results with IP-compatible buffers:** Common elution buffers directly impact NGPS performance
- **Confidently identify proteins across diverse IP conditions:** NGPS enables accurate protein identification from low-abundance targets to highly enriched samples
- **Gain sequence- and isoform-resolved insight from IP samples:** With compatible workflows, NGPS extends IP analysis beyond detection to achieve sequence-resolved characterization of biologically relevant protein forms
- **Enable high-confidence sequencing through IP workflow optimization:** Careful control of buffer composition, sample complexity, and enrichment specificity supports robust NGPS performance

ABSTRACT

Next-gen protein sequencing (NGPS) on the Platinum® Pro platform enables comprehensive characterization of proteins enriched by immunoprecipitation (IP), including isoforms, sequence variants, and post-translational modifications. NGPS performance depends on optimization of upstream IP workflows and sample preparation conditions. In this study, we evaluated key variables influencing NGPS performance, including elution buffer composition and target protein abundance. Using recombinant human serum albumin (HSA) as a model, we found that commonly used low-pH tris/glycine and citrate/phosphate buffer systems were incompatible with NGPS, resulting in reduced alignments and peptide identification.

NGPS was then applied to proteins immunoprecipitated from mammalian cell lysates using on-bead digestion workflows. Immunoprecipitation of heat shock protein 90 (HSP90) yielded robust sequencing of both isoforms, with high alignments, peptides, and inference likelihood, while immunoprecipitation of integrin alpha-1 (ITGA1) demonstrated that NGPS can correctly infer low-abundance mature protein forms.

Together, these results show that NGPS can successfully sequence immunoprecipitated proteins across a range of abundances when buffer compatibility, sample composition, and enrichment specificity are carefully controlled.

INTRODUCTION

Immunoprecipitation is widely used to enrich proteins of interest from complex biological samples, enabling focused analysis of low-abundance targets and associated protein complexes. Following IP, target recovery and specificity are commonly assessed by Western blotting or mass spectrometry (MS). Although these readouts are effective for many applications, IP workflows are typically optimized for binding and recovery rather than for downstream sequencing-oriented library preparation. As a result, sample preparation choices made during IP can strongly influence the quality and interpretability of subsequent sequence-level analyses.

NGPS on the Platinum Pro platform provides targeted sequencing with single-amino acid resolution at the peptide level,[1] potentially enabling detection of sequence variants, mutations, and post-translational modifications. Beyond confirming protein presence, NGPS supports detailed characterization of molecular heterogeneity, proteoform diversity, and modification states that may not be readily accessible with traditional protein analysis methods.

NGPS relies on bottom-up library preparation, including proteolytic digestion, and is therefore sensitive to upstream handling and sample composition. Samples derived from IP workflows may contain components introduced during enrichment and processing, including residual elution buffers, bead-associated reagents, and co-enriched species (e.g., antibody, nonspecific binders, or formulation additives). These components can influence digestion efficiency, library preparation chemistry, and downstream protein inference, potentially lowering the sequence coverage of the intended target. Consequently, IP conditions that perform well for Western blotting or MS may require additional optimization to ensure compatibility with NGPS.

In this application note, we demonstrate the use of NGPS on the Platinum Pro platform to sequence proteins enriched by IP from biological samples. We examine factors that influence sequencing performance, such as the compatibility of common IP elution buffers. Additionally, we present examples of successful NGPS of immunoprecipitated proteins from mammalian cell lysates, illustrating how the relative abundance of the target protein influences both sequence coverage and inference confidence. Together, these results highlight key considerations for optimizing IP workflows to enable robust, sequence-level characterization by NGPS.

MATERIALS AND METHODS

MODELING IP ELUTION BUFFER CONDITIONS

Recombinant human serum albumin (HSA) was used as a model protein to simulate post-IP sample conditions for low-pH tris/glycine and citrate/phosphate buffer systems. HSA was prepared at a final input of 200 ng per sample (2 µL of a 100 ng/µL stock) and combined with NGPS library preparation reagents, including acetonitrile and surfactant from Quantum-Si's Library Preparation Kit V3 (catalog no. 910-00012-03). Buffer components and concentrations were selected to approximate conditions used during IP elution and neutralization.

For tris/glycine modeling, four HSA samples were prepared. Two control samples (Ctrl 1 and Ctrl 2) were prepared in sample buffer from Library Preparation Kit V3 containing surfactant to control for variability in handling and buffer volume. Two experimental samples contained tris and glycine with surfactant and acetonitrile. For control samples, HSA was combined with Library Preparation Kit V3 sample buffer and surfactant, with acetonitrile adjusted to match experimental conditions. Experimental samples contained either 110 mM glycine with 55 mM tris or 130 mM glycine with 65 mM tris, achieved by addition of 0.2 M glycine and 1 M tris stock solutions. Final sample compositions were normalized to comparable

acetonitrile and surfactant concentrations across all conditions.

For citrate/phosphate modeling, two HSA samples were prepared: a control sample in the sample buffer from Library Preparation Kit V3 with surfactant and an experimental sample containing citrate and phosphate. The experimental sample included 32.5 mM citrate and 65 mM phosphate, achieved by combining citrate buffer (0.1 M, pH 3.0) and phosphate buffer (0.2 M, pH 8.0), reflecting concentrations commonly used during IP elution and neutralization. Both samples contained identical amounts of HSA, surfactant, and acetonitrile to isolate the effect of buffer composition.

GENERATION OF BIOLOGICAL MATERIALS

For ITGA1 (integrin alpha-1), HEK293 cells were expanded in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin to generate ~20 million cells. Cells were seeded in 10 cm dishes and transfected with an ITGA1-FLAG plasmid (5 µg per dish) using PolyJet transfection reagent (SignaGen Laboratories catalog no. SL100688) according to the manufacturer's instructions. Cells were incubated for 48 hours post-transfection, harvested by detachment with PBS containing EDTA, pelleted by centrifugation, and stored at -80°C until processing.

For HSP90 (heat shock protein 90), MCF7 cells were expanded in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin to generate ~20 million cells for protein extraction. Cells were seeded in 15 cm dishes to >90% confluency, harvested with a cell lifter, pelleted by centrifugation, and stored at -80°C until processing.

CELL LYSIS AND PROTEIN IMMUNOPRECIPITATION

For ITGA1, cell pellets were lysed in lysis buffer (50 mM tris-HCl, pH 7.5; 300 mM NaCl; 0.5% NP-40; 1× Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific catalog no. 78429) on a rotating mixer at 4°C for 30 minutes. Lysates were clarified by centrifugation at 20,000 × g for 20 minutes at 4°C. Anti-FLAG M2 magnetic beads (Sigma-Aldrich, M8823) were prepared as a 50% slurry in lysis buffer and washed three times before use. Clarified lysates were incubated with 25 µL of bead slurry per sample for 2 hours at 4°C with rotation. Beads were washed four times with lysis buffer followed by four washes with PBS. After the final wash, 20 µL of Library Preparation Kit V3 sample buffer was added to the beads.

For HSP90, cell pellets from six 15 cm plates were resuspended in 2 mL lysis buffer (50 mM HEPES, pH 8.0; 150 mM NaCl; 5 mM EDTA; 5% glycerol; 0.1% NP-40; 1× Halt protease inhibitors) and rotated at 4°C for 30 minutes. Lysates were briefly sonicated on ice and clarified by centrifugation at 15,000 × g for 15 minutes at 4°C. Protein concentration was adjusted to ~5 mg/mL, and 1 mL of lysate was incubated with HSP90 alpha/beta antibody (F-8) agarose conjugate (Santa Cruz Biotechnology, sc-13119 AC) overnight at 4°C with end-over-end mixing. Beads were washed five times with lysis buffer followed by two washes with final wash buffer (50 mM HEPES, pH 8.0; 150 mM NaCl) and stored at -20°C until downstream processing.

LIBRARY PREPARATION

For bead-bound samples, beads were briefly centrifuged and the supernatant was removed. On-bead digestion and library preparation were performed using Library Preparation Kit V3 with minor modifications. Briefly, 18 µL of sample buffer and 2 µL of surfactant were added to the beads. Samples were reduced with 2 µL of TCEP at 37°C for 30 minutes with shaking at 1,100 rpm and then alkylated with 2 µL of CAA at room temperature for 30 minutes with shaking at 1,100 rpm. Digestion was initiated by adding 2 µL of Lys-C solution, and samples were incubated overnight (16–18 hours) at 37°C with shaking at 1,100 rpm. Following digestion, the supernatant was transferred to a new tube.

Samples then underwent diazo-transfer by incubation with 2 µL of K_2CO_3, 2.6 µL of $CuSO_4$, and 2 µL of ISA for 90 minutes at room temperature. The entire volume of each sample was transferred to a tube containing beads for quenching at room temperature for 30 minutes with end-over-end mixing. After quenching, samples were filtered using the provided spin columns and acidified with 3 µL of acetic acid to adjust pH. Linker conjugation was performed by adding 2 µL each of EDTA, CTAB, and K-Linker to 47 µL of each sample, followed by incubation at 37°C overnight (16–18 hours).

For recombinant protein samples, library preparation was performed using Library Preparation Kit V3 and workflow with minor modifications. Starting from an 18 µL input containing 200 ng of HSA, 2 µL of surfactant was added, followed by reduction (2 µL TCEP; 37°C; 30 minutes) and alkylation (2 µL CAA; room temperature; 30 minutes). Digestion was initiated by adding 2 µL of Lys-C solution and incubating overnight (16–18 hours) at 37°C. Diazo-transfer, quenching, filtration, acidification, and linker conjugation were performed as described above.

NGPS ON PLATINUM PRO AND DATA ANALYSIS

After conjugation, samples were removed from 37°C and kept on ice until sequencing on the Platinum Pro instrument using the Quantum-Si Sequencing Kit V4 (catalog no. 910-00038-04), following its protocol. All samples were loaded at 0.133 nM using a split-chip configuration (one side of the chip).

Sequencing data were analyzed using *Primary Analysis* v2.16.0, *Peptide Alignment* v2.18.2, and *Protein Inference* v2.17.0, with an inference panel containing human proteome proteins ranging from 10–85 kDa. For the HSP90 experiment, the inference panel was curated to remove putative HS90B forms (H90B2 and H90B3), whose high sequence similarity to HSP90β (HS90B) can reduce inference likelihood despite correct peptide-level evidence. This curation was performed to evaluate protein inference under conditions that more closely reflect biologically validated isoforms.

For *Primary Analysis*, high-quality reads were defined as sequencing reads supported by at least three unique recognizers and four or more recognition segments (RS, defined as filtered regions of interest per read). For *Protein Inference*, the inference score was calculated by aggregating the false discovery rate (FDR) of all *in silico*–digested peptides generated from a protein sequence, and the likelihood inferred reflects confidence that a given protein is present in the sample.

SDS-PAGE AND WESTERN BLOT ANALYSES

Samples were prepared in Novex™ tris-Glycine SDS Sample Buffer (2×; Thermo Fisher Scientific, LC2676), heated at 95°C for 5 minutes, and loaded onto a 4–20% Novex tris-Glycine Mini Protein Gel (Thermo Fisher Scientific, XP04200BOX). Gels were run in Novex tris-Glycine SDS Running Buffer (Thermo Fisher Scientific, LC2675) at 200 V for 55 minutes.

For SDS-PAGE visualization, gels were stained and destained using SimplyBlue™ SafeStain (Thermo Fisher Scientific, LC6060) following the manufacturer's microwave protocol.

For HSP90 Western blotting, proteins were transferred to PVDF membranes (Thermo Fisher Scientific, PB5210) using a Power Blotter Station (Invitrogen, PB0010) with the mixed-MW protocol. Membranes were blocked in SuperBlock™ Blocking Buffer in TBS (Thermo Fisher Scientific, 37535) and probed with HSP90 alpha/beta antibody (F-8) HRP (Santa Cruz Biotechnology, sc-13119 HRP) at 1:200 dilution in blocking buffer. Detection was performed using 1-Step™ Ultra TMB-Blotting Solution (Thermo Fisher Scientific, 37574). Stained gels and blotted membranes were imaged using an Azure 300 Imaging System (Azure Biosystems, AZI300-01).

RESULTS AND DISCUSSION

ELUTION BUFFER COMPONENTS USED IN IP WORKFLOWS COMPROMISE NGPS PERFORMANCE

To evaluate how common IP elution buffer components affect NGPS performance, we performed a series of experiments using recombinant HSA. We tested buffers containing tris and glycine or citrate and phosphate at concentrations representative of typical IP elution conditions (see *Materials and Methods*). All experiments used the standard Library Preparation Kit V3 protocol and were sequenced on the Platinum Pro instrument with a half chip and Sequencing Kit V4 at 0.067 nM loading concentration.

We first examined the impact of tris and glycine. Four HSA samples were prepared: two controls in the Library Preparation Kit V3 sample buffer with surfactant and two experimental samples containing 55–65 mM tris and 110–130 mM glycine. As shown in Table 1, control samples produced robust sequencing metrics, including 38,141–59,241 high-quality reads, 4,124–5,669 alignments, and 13–15 peptides identified, with correct protein inference (rank 1; 99.99% likelihood). In contrast, tris/glycine-containing samples showed a near-complete loss of signal (227–332 high-quality reads), with zero alignments and no peptides identified, precluding protein inference. This effect was observed at both tested concentrations, indicating that even moderate levels of tris and glycine are incompatible with NGPS.

This outcome aligns with the presence of primary amines in tris and glycine, which are likely to disrupt the ISA derivatization chemistry used in NGPS library preparation. Therefore, when IP workflows involve buffers containing tris or glycine, it is advisable to perform buffer exchange or desalting into an NGPS-compatible buffer that is free of primary amines before preparing the library.

Sample	High-quality reads	Alignments	Peptides identified (FDR 10% or lower)	Inference rank	Inference score	Inference likelihood
Ctrl 1	38,141	4,124	13	1	28.63	99.99%
Ctrl 2	59,241	5,669	15	1	26.16	99.99%
55 mM tris, 110 mM glycine	227	0	0	N/A	0	0
65 mM tris, 130 mM glycine	332	0	0	N/A	0	0

Table 1. Effect of tris and glycine on NGPS performance. HSA samples were prepared in the Library Preparation Kit V3 sample buffer (Ctrl 1 and Ctrl 2) or with tris and glycine added at two concentrations. Tris/glycine-containing samples showed near-complete loss of reads, alignments, and peptides, preventing protein inference, while controls yielded robust sequencing and correct protein identification.

We next evaluated citrate and phosphate. A control sample in the sample buffer from Library Preparation Kit V3 with surfactant was compared with an experimental sample containing 32.5 mM citrate and 65 mM phosphate (with identical surfactant and acetonitrile concentrations). Although HSA was still detected and correctly inferred at 99.99% likelihood in the citrate/phosphate sample, all sequencing metrics were substantially reduced relative to the control (Table 2). The control sample yielded 93,171 high-quality reads, 3,963 alignments, and 15 peptides identified, whereas the citrate/phosphate sample produced 4,606 high-quality reads, 300 alignments, and 7 peptides identified.

Collectively, these results demonstrate that buffer components commonly used in IP workflows (tris, glycine, citrate, and phosphate) can substantially compromise NGPS library preparation, reducing reads, alignments, and peptide coverage. To preserve sequencing quality and inference performance, proteins should be eluted into or exchanged into NGPS-compatible buffers prior to library preparation.

Sample	High-quality reads	Alignments	Peptides identified (FDR 10% or lower)	Inference rank	Inference score	Inference likelihood
Ctrl 1	93,171	3,963	15	1	24.39	99.99%
32.5 mM citrate, 65 mM phosphate	4,606	300	7	1	2.85	99.99%

Table 2. Effect of citrate and phosphate on NGPS performance. HSA samples were prepared in the sample buffer from Library Preparation Kit V3 or with citrate and phosphate. The citrate/phosphate sample showed reduced reads, alignments, and peptide identifications relative to the control.

NGPS SEQUENCING OF IMMUNOPRECIPITATED ITGA1 DEMONSTRATES SENSITIVITY UNDER LIMITED MATURE PROTEIN ABUNDANCE

To evaluate NGPS performance when the biologically relevant form of a protein is present at low abundance, ITGA1 was immunoprecipitated from HEK293 cells transiently expressing an ITGA1-FLAG construct. Immunoprecipitation was performed using an anti-FLAG antibody, and enriched material was analyzed by SDS-PAGE (Figure 1A).

NGPS identified 193 peptide alignments corresponding to three unique ITGA1 peptides. Protein inference correctly ranked ITGA1 as the top protein with a likelihood of 77.26% (Figure 1B and C). These results demonstrate that NGPS can correctly identify proteins even when the target species is present in low abundance, although inference likelihood may be reduced under low target-to-background conditions.

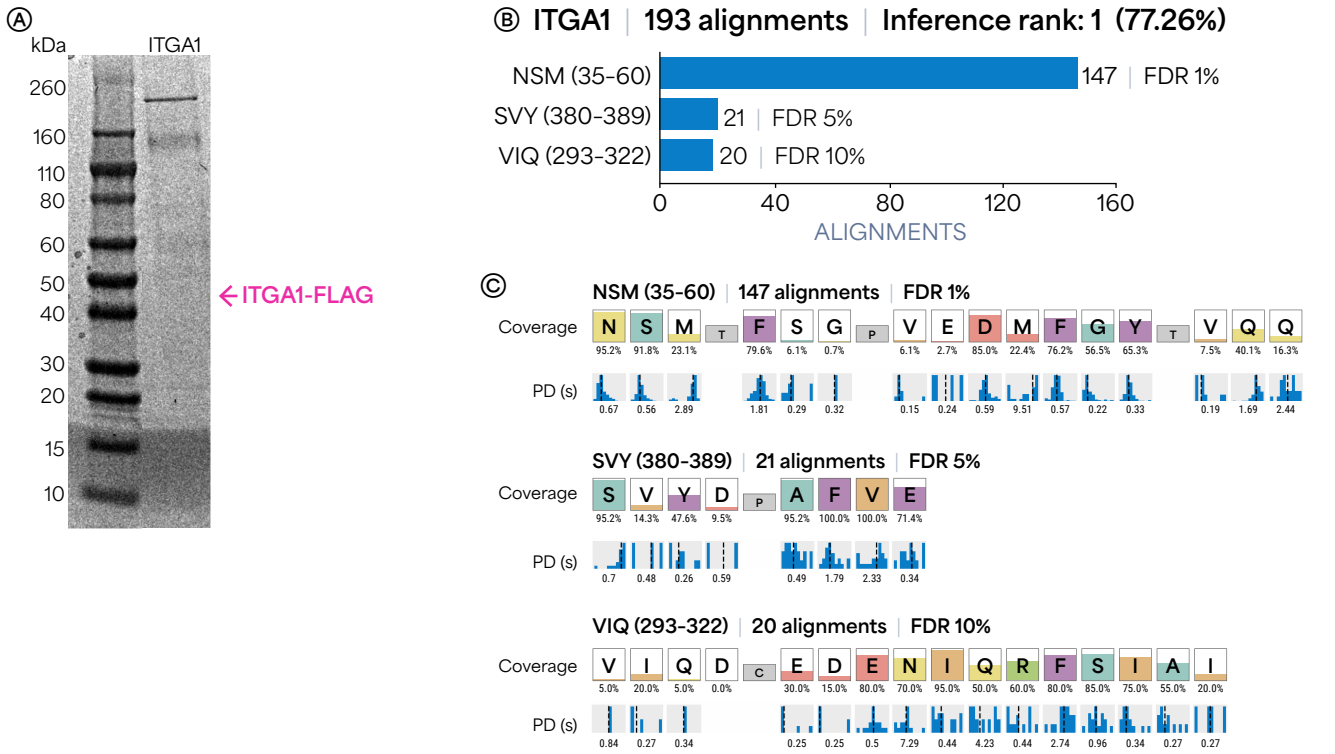

Figure 1. NGPS analysis of immunoprecipitated ITGA1 from HEK293 cells. Ⓐ SDS-PAGE analysis of ITGA1-FLAG IP with Simple Blue staining showing that the ITGA1 protein was present at a very low level. Ⓑ Sequencing results from the NGPS *Peptide Alignment* and *Protein Inference* workflows identified a total of 193 peptide alignments corresponding to 3 unique peptides. The mature ITGA1 form is inferred as the top-ranked protein (rank 1) with an inference likelihood of 77.26%. Peptide-level alignment information confirmed correct mapping to ITGA1, despite the low relative abundance of the mature form. Ⓒ Kinetic signatures for each ITGA1 peptide, showing sequence coverage and pulse duration information supporting confident identification.

NGPS SEQUENCING OF HSP90 HIGHLIGHTS ISOFORM-RESOLVED ENRICHMENT AND HIGH-CONFIDENCE INFERENCE

We next evaluated NGPS performance on a target enriched with higher abundance and specificity. HSP90 was immunoprecipitated from MCF7 cell lysates using beads pre-conjugated with an anti-HSP90 antibody designed to enrich both HSP90α (HS90A) and HSP90β (HS90B). An isotype IgG control was processed in parallel to assess nonspecific binding. SDS-PAGE and Western blotting confirmed efficient capture of HSP90α/β in the antibody-conjugated sample, while no HSP90α/β signal was detected in the IgG control (Figure 2).

NGPS analysis of the HSP90 IP sample revealed robust sequencing of both isoforms. HSP90β was supported by 1,406 peptide alignments and six unique peptides, while HSP90α was supported by 438 alignments and six unique peptides (Figure 3 and Table 3). Protein inference ranked HS90B first (99.99% likelihood) and HS90A second (99.67% likelihood). In contrast, the IgG control yielded minimal alignments, no peptides identified at FDR 10% or lower, and neither HSP90 isoform was inferred (Table 3), confirming enrichment specificity.

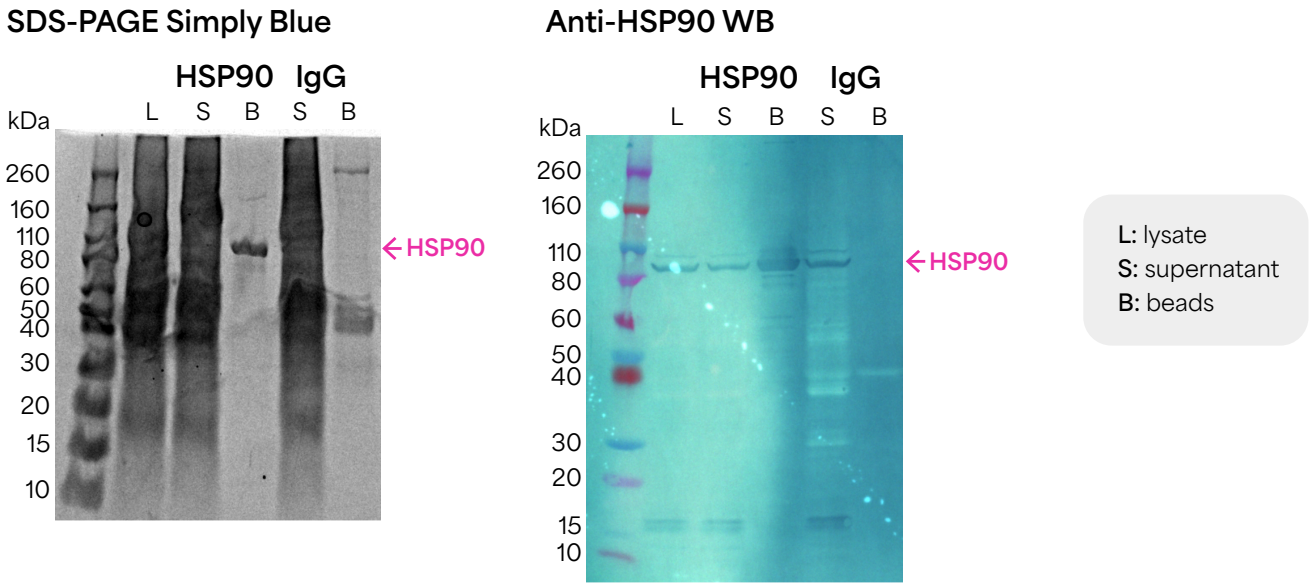

Figure 2. SDS-PAGE and Western blot analysis of HSP90 immunoprecipitation from MCF7 lysates. The beads fraction from the anti-HSP90 IP shows a single prominent band at ~90 kDa, corresponding to HSP90α/β, indicating successful enrichment. In contrast, no HSP90α/β band was observed in the beads fraction of the isotype IgG control, demonstrating the high enrichment specificity.

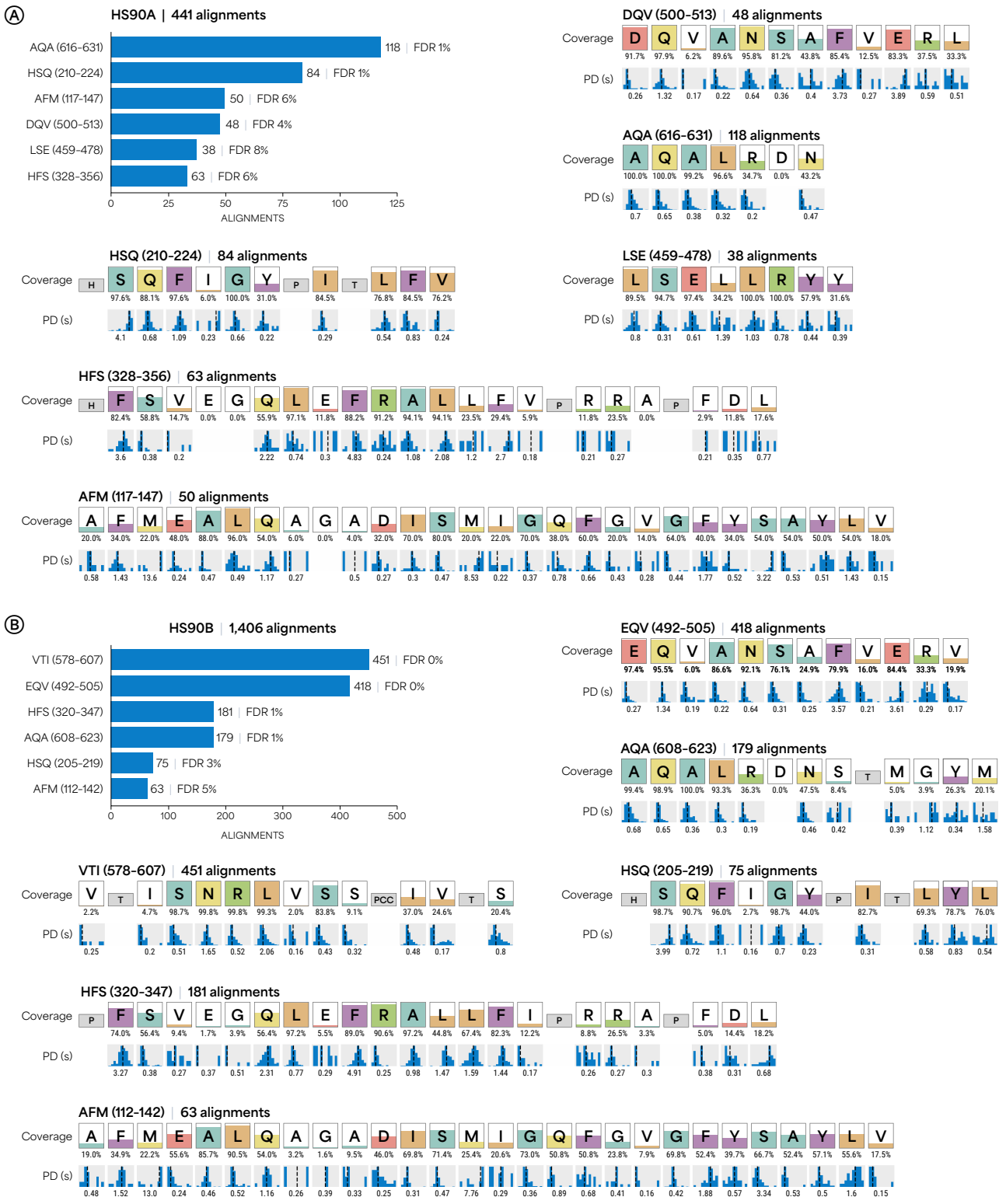

Figure 3. Peptide-level NGPS analysis of HSP90 isoforms following immunoprecipitation. Peptide alignment results for Ⓐ HSP90α (HS90A) and Ⓑ HSP90β (HS90B) showing peptide-level alignment counts and corresponding kinetic signatures, including sequence coverage and pulse duration information. These data provide strong evidence for the confident identification of both HSP90 isoforms from the immunoprecipitated sample.

	HS90A				HS90B			
Sample	Alignments	Peptides identified FDR ≤10	Inference rank	Likelihood inferred	Alignments	Peptides identified FDR ≤10	Inference rank	Likelihood inferred
HSP90	441	6	2	99.67%	1,406	6	1	99.99%
Isotype IgG Ctrl	60	0	N/A	N/A	54	0	N/A	N/A

Table 3. NGPS sequencing metrics for HSP90 isoforms in immunoprecipitated and control samples. Both HSP90α (HS90A) and HSP90β (HS90B) were robustly detected in the anti-HSP90 IP sample, with 441 and 1,406 peptide alignments respectively, each having 6 unique peptides. Protein inference ranked HS90B first (99.99% likelihood) and HS90A second (99.67% likelihood). In contrast, the isotype IgG control resulted in minimal alignments and no peptides identified at FDR of 10% or lower, confirming the specificity of the enrichment.

Together, the ITGA1 and HSP90 case studies illustrate how NGPS performance scales with enrichment efficiency and target-to-background ratio. ITGA1 demonstrates that low-abundance targets can be correctly inferred, albeit with reduced likelihood, while HSP90 highlights high-confidence, isoform-resolved sequencing when the protein is efficiently enriched.

CONCLUSION

This application note demonstrates that NGPS on the Platinum® Pro platform can generate detailed, sequence-level information from immunoprecipitated proteins in complex biological samples. Elution buffer composition emerged as a critical determinant of performance: moderate concentrations of tris, glycine, or citrate/phosphate – commonly encountered in IP workflows – substantially reduced peptide recovery, alignments, and protein inference. Therefore, careful selection and optimization of IP elution conditions are crucial for compatibility with downstream NGPS.

In addition, although on-bead digestion is convenient and compatible with NGPS, antibody formulation components, and nonspecifically bound proteins can contribute disproportionately to the peptide pool, reducing the relative abundance of the target and complicating protein inference (data not shown).

Across multiple case studies, NGPS reliably identified immunoprecipitated targets spanning a range of enrichment levels. The ITGA1 example shows that low-abundance mature protein forms can be correctly inferred, albeit with reduced confidence, while the HSP90 example highlights high-confidence, isoform-resolved sequencing when enrichment specificity and target abundance are sufficient. Together, these findings emphasize the importance of minimizing incompatible buffer components, controlling contaminants, and validating enrichment specificity with orthogonal quality control for successful NGPS.

By enabling sequence-resolved analysis of immunoprecipitated proteins, NGPS provides a powerful complement to traditional IP readouts and is well-suited for studies of proteoform diversity and post-translational modifications. With appropriate sample preparation and workflow optimization, NGPS extends the analytical value of IP experiments beyond protein detection toward comprehensive, sequence-level characterization.

REFERENCES

1. Reed BD, Meyer MJ, Abramzon V, et al. Real-time dynamic single-molecule protein sequencing on an integrated semiconductor device. *Science.* 2022;378(6616):186-192. doi:10.1126/science.abo7651

Ordering information

Product	Catalog number
Platinum® Pro instrument	910-10904-00
Sequencing Kit V4 (4 chips)	910-00038-04
Library Preparation Kit V3 (4 libraries)	910-00012-03