

## ORIGINAL ARTICLE

# Preparation and Application of an Antibody Specifically Targeting Tyrosine-Phosphorylated PI3K p85 at Position 452

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### ABSTRACT

**Background:** The current invention pertains to the development and utilization of an antibody that specifically recognizes tyrosine-phosphorylated PI3K p85 at position 452. The process encompasses antigen preparation, immunization, and the construction of affinity chromatography columns.

**Methods:** To facilitate efficient peptide conjugation to a carrier protein and subsequent peptide-based affinity purification, a cysteine residue (C) was incorporated at the C-terminus of the peptide as a linker. The final peptide sequence was identified as KLHEY(p)NTQFQE. The antibody was purified through a two-step affinity purification protocol: initially, the antiserum was passed through a phosphorylated peptide column to enrich phospho-specific antibodies, followed by passage through a non-phosphorylated peptide column to eliminate non-specific binders.

**Results:** This methodology enables the scalable production of the anti-phospho-PI3K p85 (Tyr452) antibody, which demonstrates high specificity for phosphorylation and strong affinity.

**Conclusions:** In comparison to traditional protein A purification, this approach is markedly more efficient. (Clin. Lab. 2026;72:1-3. DOI: 10.7754/Clin.Lab.2025.250804)

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## Supplementary Data

### MATERIALS AND METHODS

#### Antigen design

The amino-acid sequence of the PI3K protein was scanned for epitopes using Abdesigner software, with emphasis on hydrophilicity, antigenicity, surface accessibility, and conformational flexibility [1]. Guided by prior antibody-generation experience, we further evaluated structural complexity, oxidative susceptibility, synthetic feasibility, and the type and distribution of each residue. These analyses converged on the phosphotyrosine-containing PI3K epitope KLHEY(p)NTQFQE. To enable efficient conjugation to carrier proteins and subsequent peptide-based affinity purification, a cysteine residue was appended to the C-terminus, yielding the final synthetic peptide sequence KLHEY(p)NTQFQE-C.

#### Peptide conjugation

To prepare the peptide-KLH conjugate, first dissolve 100 mg KLH in 12.5 mL degassed ultrapure water, then add 25 mg sulfo-SMCC per 100 mg KLH and stir gently at room temperature for 60 minutes to activate the carrier. After activation, remove free sulfo-SMCC by desalting on a column pre-equilibrated with 3 column volumes of conjugation buffer, loading and eluting the KLH-SMCC solution while monitoring  $A_{280}$  to collect and pool the protein peak. Determine the KLH-SMCC concentration with the Lowry assay, aliquot at 3 mg per tube, and prepare an equimolar amount of peptide (peptide: KLH mass ratio = 1:1). Dissolve the peptide in sterile, degassed water to  $20 \text{ mg mL}^{-1}$  (brief sonication or warming may be used if needed), combine with the KLH-SMCC solution, and incubate the mixture overnight at  $4^\circ\text{C}$  on a gentle rotator. The next day, cap remaining maleimide groups by adding cysteine at twice the KLH mass (dissolved in water) and incubate at  $4^\circ\text{C}$  with gentle shaking for 2 hours. Transfer the capped conjugate into a pre-treated dialysis bag and dialyze against 0.01 M PBS, exchanging the buffer at least twice with  $\geq 4$  hour-intervals. Collect all contents, including any precipitate, rinse the bag with 0.01 M PBS, and pool the fractions. Based on an empirical coupling yield of  $\sim 30\%$  (e.g., 10 mg KLH + 10 mg peptide  $\approx$  13 mg conjugate), dilute the peptide-KLH conjugate to  $1 \text{ mg mL}^{-1}$  in 0.01 M PBS, dispense into clearly labeled 300  $\mu\text{g}$  and 200  $\mu\text{g}$  aliquots, and store in designated sample boxes. This procedure has been improved upon the previous [2].

#### Rabbit immunization

After antigen preparation, label and set up syringes according to the immunization schedule. Thaw the peptide-KLH antigen from  $-70^\circ\text{C}$ , dilute it to 1 mL per rabbit with 0.01 M PBS, and emulsify with an equal volume of Freund's adjuvant (complete for priming, incomplete for boosts) by repeated back-and-forth pump-

ing through a double-ended connector until a drop remains intact on cold water. Transfer the emulsion to a glass syringe, recheck labels, then restrain each rabbit by ear tag, clip and disinfect fur on the left dorsal flank, and administer the primary immunization subcutaneously at 8 - 10 sites (0.3 - 0.5 mL each). For boosts, repeat on the right flank 4 - 6 cm away from any ulcer or granuloma, using five subcutaneous sites (0.1 - 0.3 mL each) plus two intramuscular injections into the medial thigh muscles (0.1 - 0.3 mL each), avoiding any scarred areas throughout. This procedure has been improved upon the previous [3].

#### Serum collection

Following the immunization schedule, blood is collected from each rabbit via the marginal ear vein on days 7 after the first and second booster immunizations (samples A and B) and on day 7 after the third booster (positive serum). Tubes are pre-labeled and cross-checked against ear numbers. After gently restraining the rabbit, fur on the ear margin is clipped, the surface is wiped with an alcohol swab, and the vein is lightly tapped to engorge it. A 75% ethanol-sterilized needle is used to puncture the vein, and 0.3 - 0.4 mL of whole blood is collected into a 0.5 mL tube. The wound is disinfected again, and the rabbit is returned to its cage. Whole blood is allowed to clot at room temperature for 1 hour, then centrifuged in a palm-size centrifuge after balancing the tubes. Serum is aspirated with a pipette and transferred to sterile 0.5 mL tubes for storage. This procedure has been improved upon the previous [4].

#### Preparation of the affinity chromatography column

To prepare the affinity column, allow sulfolink gel to reach room temperature, mount the column vertically, rinse it with one bed volume of coupling buffer, and insert the bottom frit. Transfer 2 mL of well-mixed sulfolink gel slurry into the column, equilibrate with  $\geq 20$  mL coupling buffer, cap the bottom, dissolve 2 mg peptide in 2 mL coupling buffer, load this solution onto the gel, cap the top, label the column, and rotate overnight at  $4^\circ\text{C}$ . The next day, let the column stand at room temperature for 30 minutes, remove both end caps in top-to-bottom order, collect the effluent, wash the gel with  $\geq 5$  bed volumes (20 mL) of coupling buffer, then close the bottom cap again. Block residual active sites by applying 2 mL of 50 mM cysteine solution (1:1 v/v with the gel), cap the top, mix at room temperature for 2 hours, rest 30 minutes, and drain the excess cysteine. Wash sequentially with 20 mL PBS, 20 mL 1 M NaCl, 20 mL PBS, and 20 mL 100 mM Gly-HCl, inserting the top frit during the first PBS wash. After final drainage, cap the bottom, add 10 mL storage buffer until the meniscus is  $\sim 0.8$  cm above the frit, cap the top, and store the peptide-coupled column at  $4^\circ\text{C}$ . This procedure has been improved upon the previous [5].

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