

# The Progression of the Development of Antibody pY26: Recognizing Phosphorylated Troponin I at the Tyrosine-26 Amino Acid Residue

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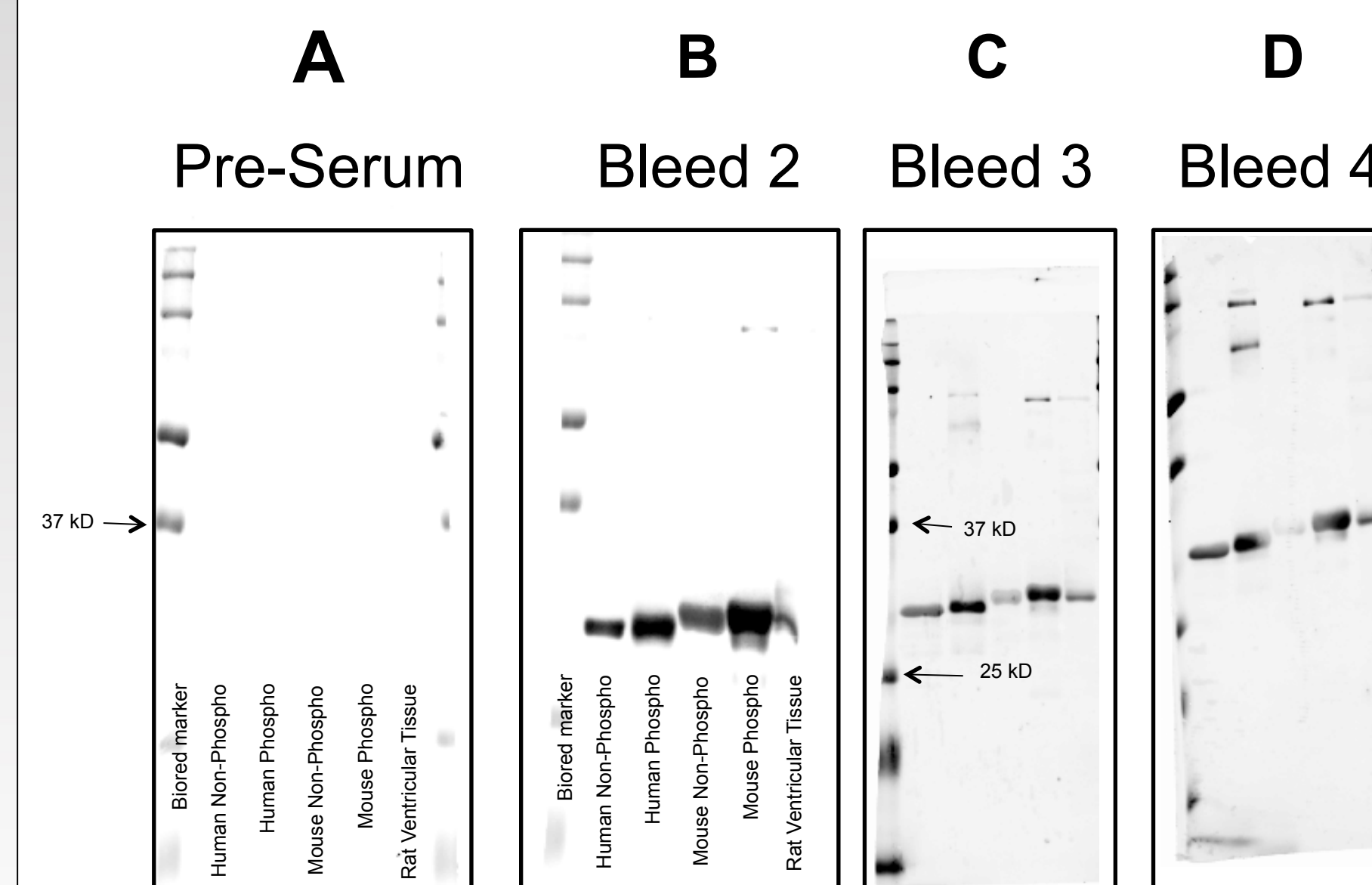
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## Introduction

Antibodies are used in the western blot technique to identify or quantify a specific protein in a sample. These antibodies cannot be synthesized in a laboratory and must be developed within an animal by employing the animal's natural immune response. Peptide antigens can be injected in a rabbit to elicit antibody production. It can take months to develop a sufficient amount of specific antibody in the rabbit blood. The antibody must be tested over time to determine its amount and specificity. The antibody of interest in this study, rabbit antibody pY26, identifies phosphorylated troponin I (TnI) at the Tyrosine-26 (Tyr-26) amino acid residue. The antibody was tested by using four known recombinant purified samples: human phosphorylated TnI, human non-phosphorylated TnI, rat phosphorylated TnI, and rat non-phosphorylated TnI. The antibody was tested at five different time intervals: Pre-bleed, and bleeds 1-4. Following injection, the antibody should be observed on the phosphorylated samples and not observed on the non-phosphorylated samples. As time progressed, more antibody was present in serum (binding to phosphorylated TnI increased) and the antibody became more specific (binding to phosphorylated TnI more than non-phosphorylated TnI). As each subsequent test bleed was used on the samples, the specificity and intensity of the antibody increased. Further work was done on the antibody in terms of determining what concentration at which it was most specific. Serum dilution of 1:60,000 generated the most specific results. With an effective antibody that identifies phosphorylated TnI Tyr-26, unknown samples can be compared for their pY26 phosphorylation levels. Trends of phosphorylation of TnI Y26 between failing and normal heart tissue can be established and ultimately treatments can be created depending on the effect of the phosphorylation.

## The Progression of Antibody Specificity Over Time

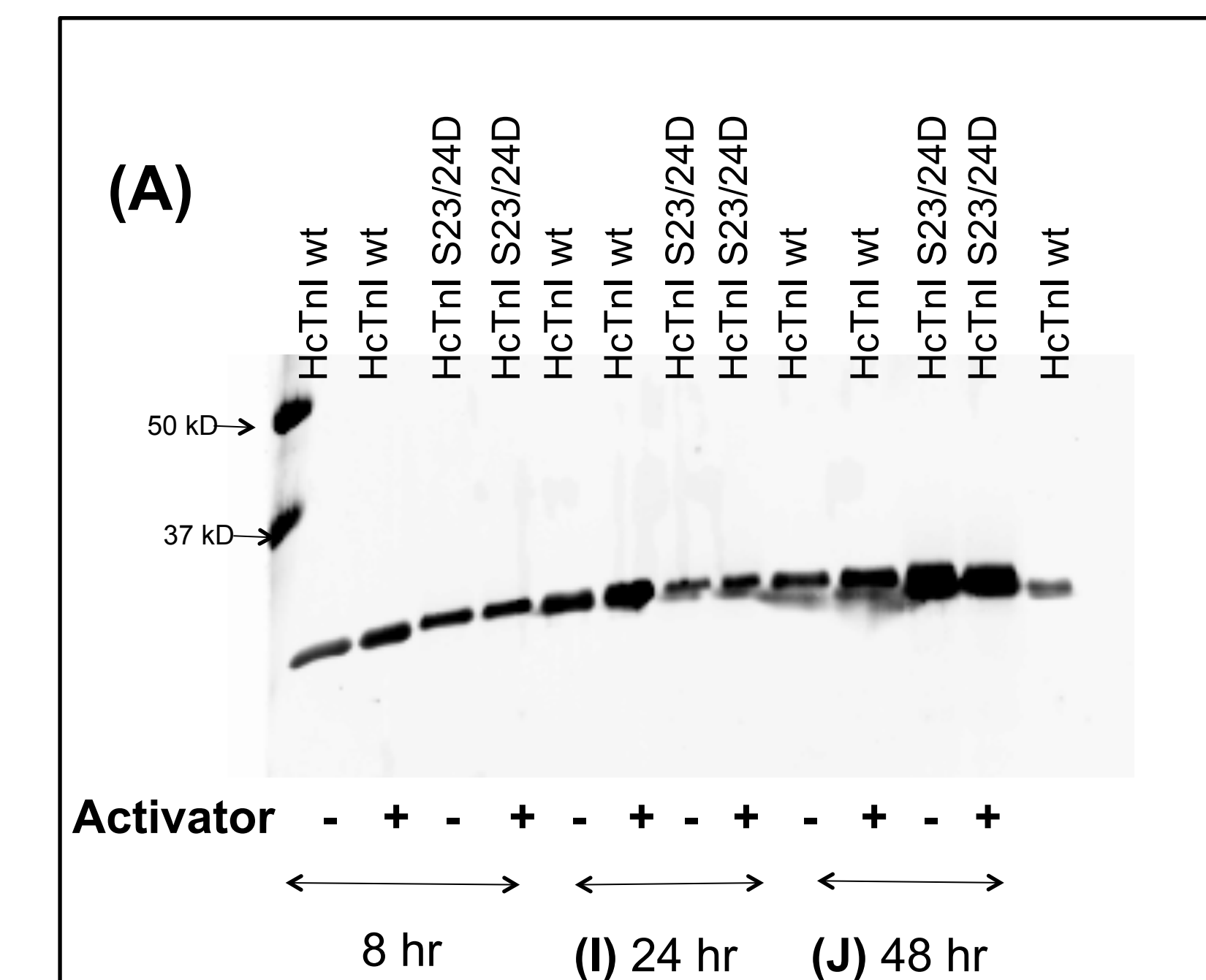


**Western blot of purified TnI.** Purified recombinant TnI samples from *E. coli* expression were subjected to Western blot with (A) pre-serum (before rabbit injections), (B) Bleed 2 (serum obtained after 2 injections), (C) Bleed 3 (serum obtained after 3 injections), (D) Bleed 4 (serum obtained after 4 injections)

**Conclusion:** There was no reactivity against TnI before injection. After injections, intensity and specificity against TnI pY26 increased over time

## Application

### TnI pY26 Western Blot



**Western blot of Time Lapse Kinase Assay.** (A) Wild type or S23/24D TnI, without (-) or with (+) a tyrosine kinase activator were incubated with Src and the reaction stopped at varied time points. Resultant samples were subjected to Western with anti-pTnI Y26 Bleed 2 at 1:60,000. (B) Quantification of pTnI Y26 phosphorylation.

**Conclusion:** pTnI Y26 phosphorylation increased over time, and both the tyrosine kinase activator and S23/24D further increase this phosphorylation.

## Background

### Phosphorylation Regulation of Cardiac Contraction

- Phosphorylation is a common post-translational modification that can modulate the function of the heart
- Phosphorylation(s) present on the inhibitory subunit of troponin (TnI) are an important mechanism to modulate cardiac contraction
- Cardiac TnI is phosphorylated at Tyr-26 (pY26) with unclear results

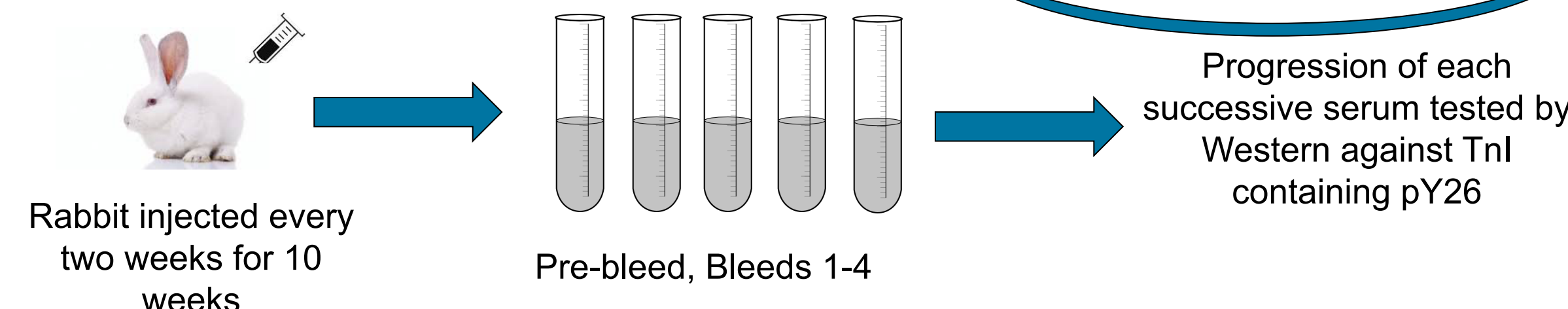
### Western Blot

- Western blot is a powerful technique involving the use of antibodies binding to specific proteins in a sample
- The quantity of a certain protein and/or specific phosphorylation is determined by antibody binding

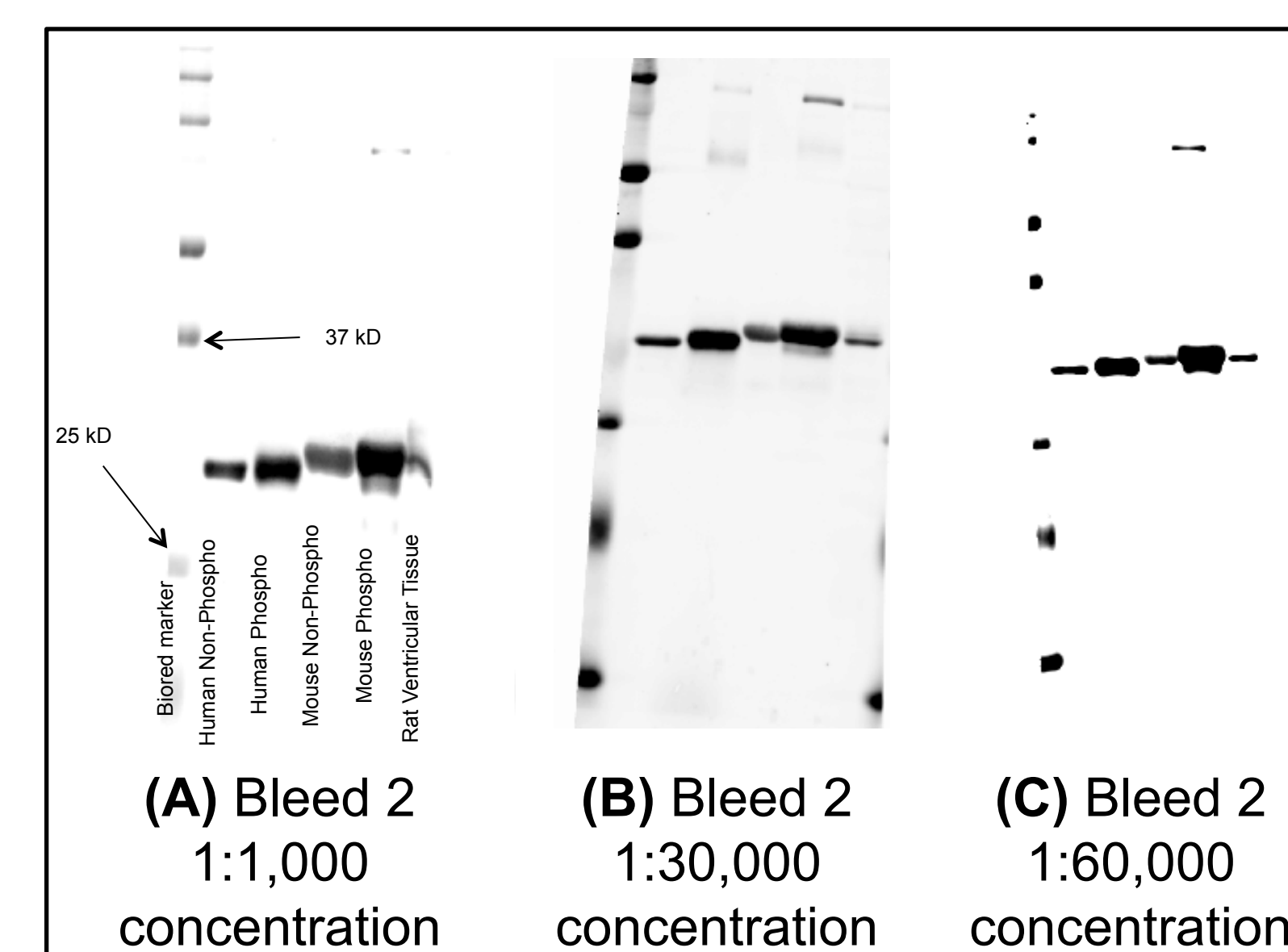
### Antibody Development

- Injected rabbit 4 times: once every 2 weeks. Collected blood (serum) every 2 weeks for analysis
- TnI amino acid sequence: N-...RRRSSANYRAYATEPHAKKK...-C

-Phosphorylated amino acid sequence: N[**pY**]RAYATEPH



## Determining the Antibody Concentration that Results in Best Specificity



**Western blot of purified TnI.** Purified recombinant TnI samples, fluorescently stained after being treated with varied concentrations of serum. (A) Bleed 2 used at a 1:1,000 concentration, (B) Bleed 2 used at a 1:30,000 concentration, (C) Bleed 2 used at a 1:60,000 concentration.

**Conclusion:** The pY26 antibody Bleed 2 serum was most specific for TnI Y26 phosphorylation when used at a 1:60,000 concentration.

## Conclusions

-The rabbit antibody TnI pY26 demonstrated increased affinity and specificity over time.

-When applying on protein samples, the pY26 antibody is most specific when used at a 1:60,000 serum.

-With a specific antibody and a known concentration that produces clear images, we can now use the antibody to monitor pY26 phosphorylation levels on many samples. Trends between physiological conditions and the pY26 phosphorylation can be better understood and established.

## Future Directions

-Purify out phospho-specific from non-phospho for Bleeds 3 and 4 by affinity purification / affinity depletion to generate the final antibody