

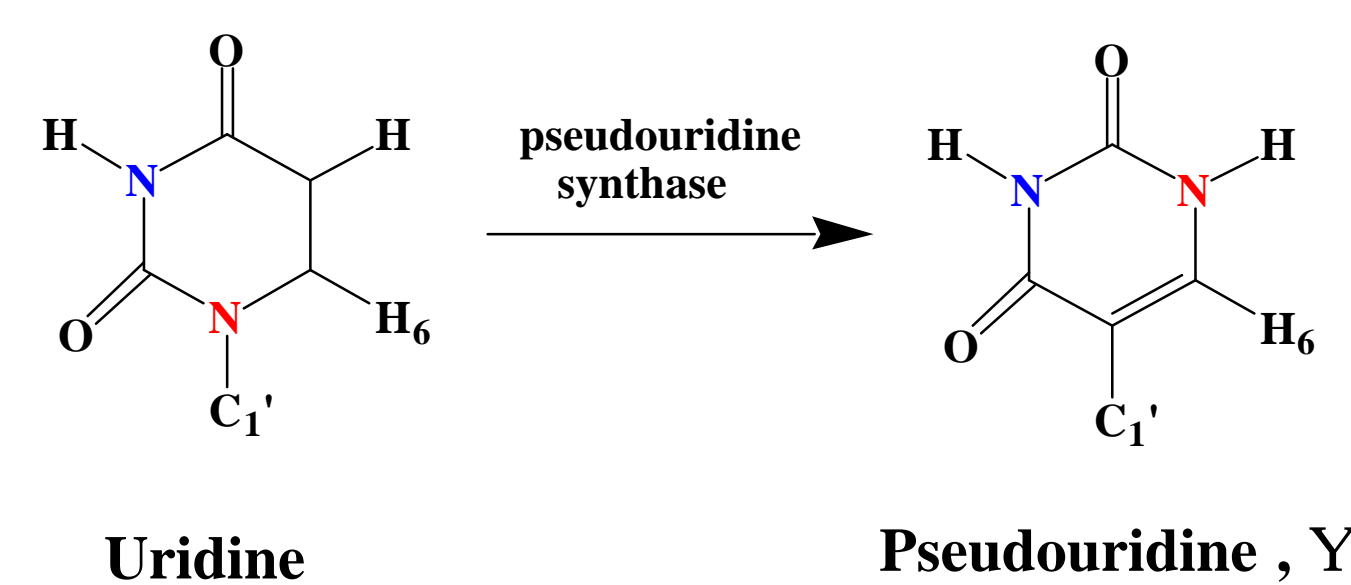
INTRODUCTION & MOTIVATION

The main purpose of our work is to understand the structural and thermodynamic effect of base modification on RNA and the cumulative effect of multiple modified bases within one RNA. The nucleotide base modification can change structure, stability and metal ion affinity of RNA. We have focused on influence of pseudouridylation on RNA structure.

Understanding the role of pseudouridine (Y) critically depends on finding the general method for incorporation of pseudouridine into RNA sequence. Chemical incorporation of Y into RNA sequence using phosphoramidite approach is possible, but does not easily permit isotope enrichment.

FUNCTIONS OF PSEUDOURIDINE :

5-(b-D-ribofuranosyl)uracil, Y, is the most common modified nucleoside found in RNA. It is formed by post-transcriptional isomerization of selected uridines in RNA by different enzymes called pseudouridine synthases.



Conserved pseudouridine play important roles in RNA stability, codon recognition and spliceosome function. Pseudouridine can stabilize helices by improving stacking interactions.

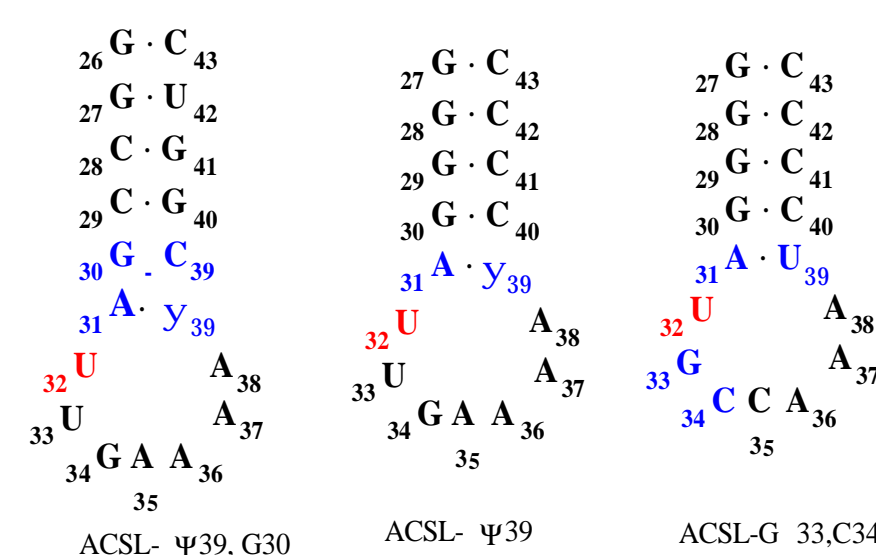
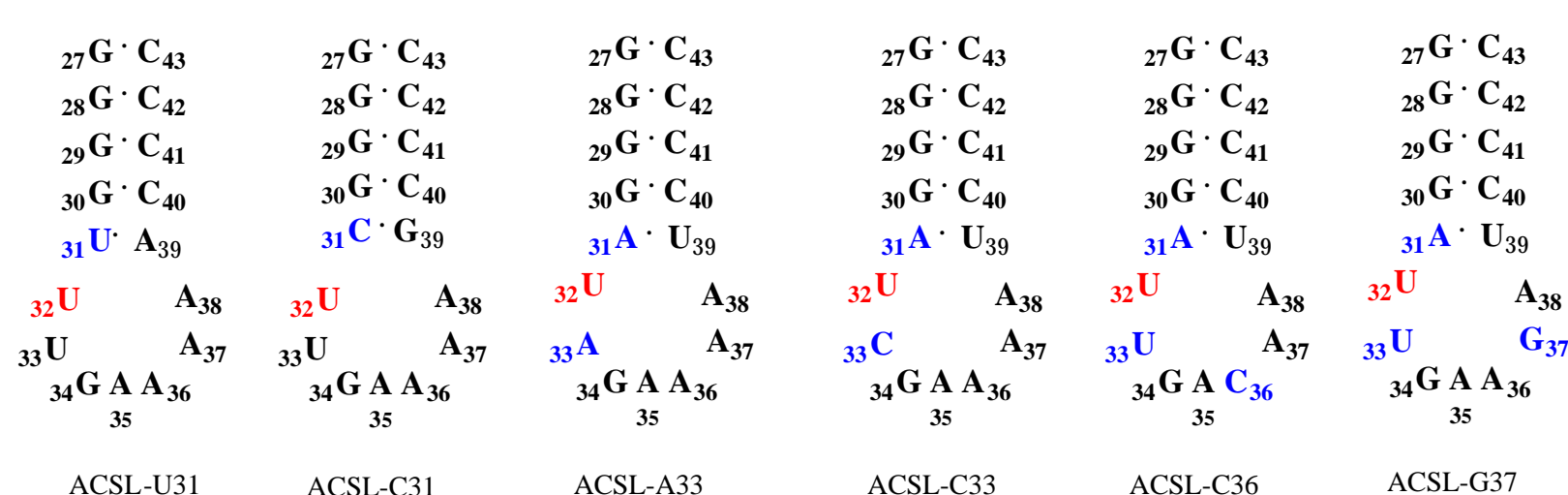
DETERMINATION

RNA SPECIFICITY OF RluA

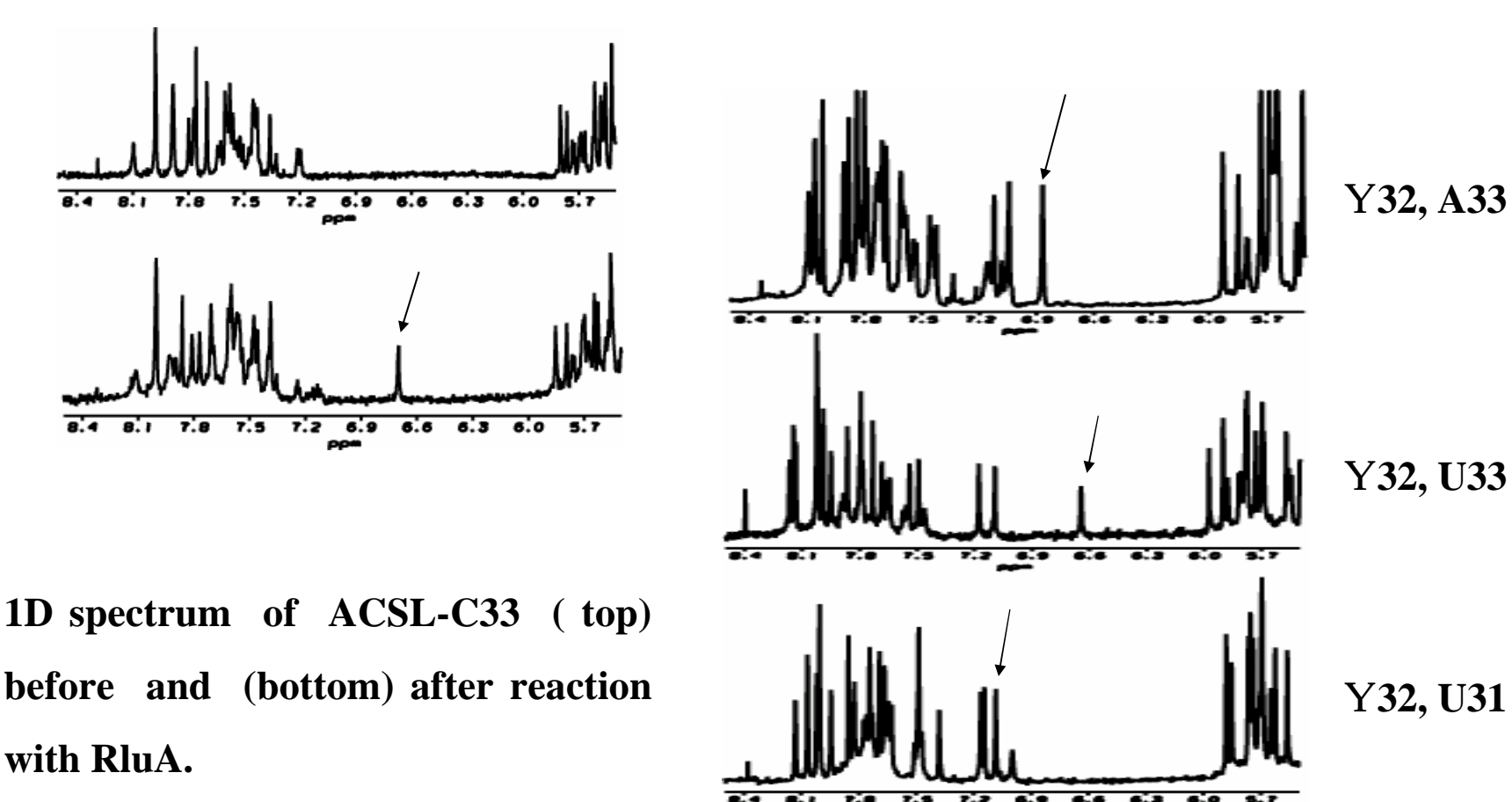
We have investigated the use of the bacterial pseudouridine synthase, RluA, as a tool to prepare isotopically enriched pseudouridine-containing RNA oligonucleotides of defined sequence.

In *E. coli*, RluA converts U to Y in the position 32 in four tRNA species and in the position 746 of helix-35 in 23S rRNA. The native RNA targets of RluA suggest that an RNA stem-loop may be sufficient for RluA recognition. In comparison to TruA and TruB, the RluA does not require the full length tRNA for activity.

We have tested several sequences of RNA and all of them are substrates for RluA.



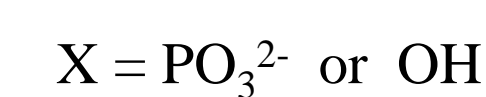
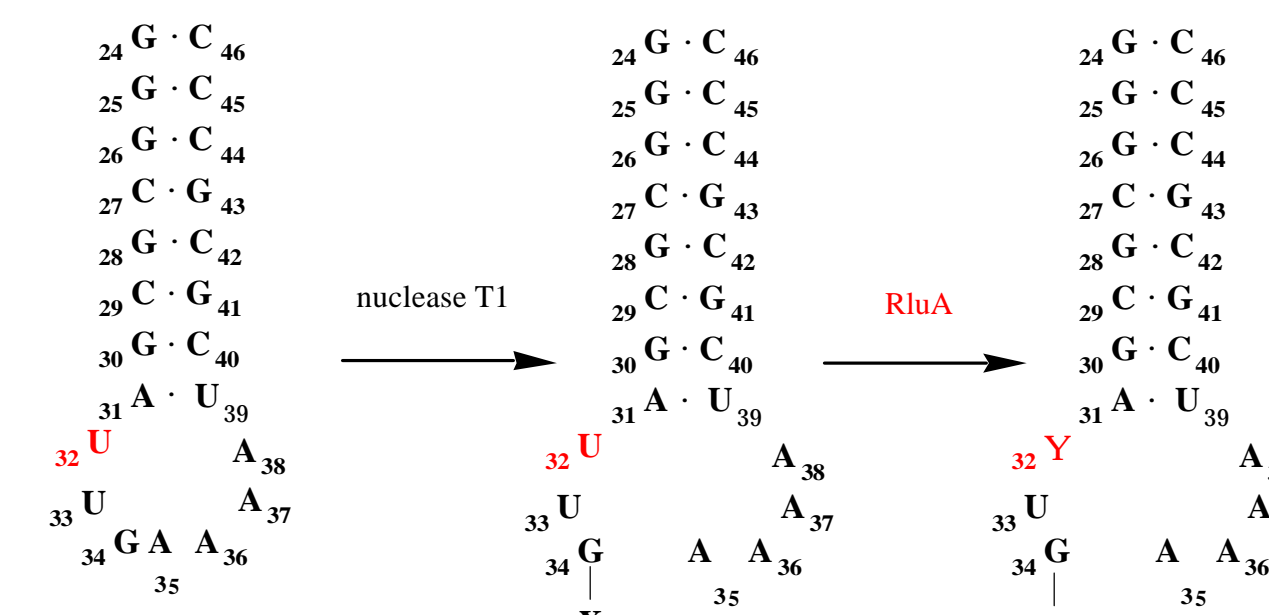
1D spectra are showing the base-1' regions for variously RluA-modified hairpins based on the native *E. coli* ACSL^{Phe}. The Y₃₂H6 (arrows) appears at ca. 6.8 ppm in most variants. Loss of H5-H6 (COSY) and H5-C5 (HSQC) peaks confirm Y32 conversion.



1D spectrum of ACSL-C33 (top) before and (bottom) after reaction with RluA. Spectra of other variants of ACSL are shown on the right.

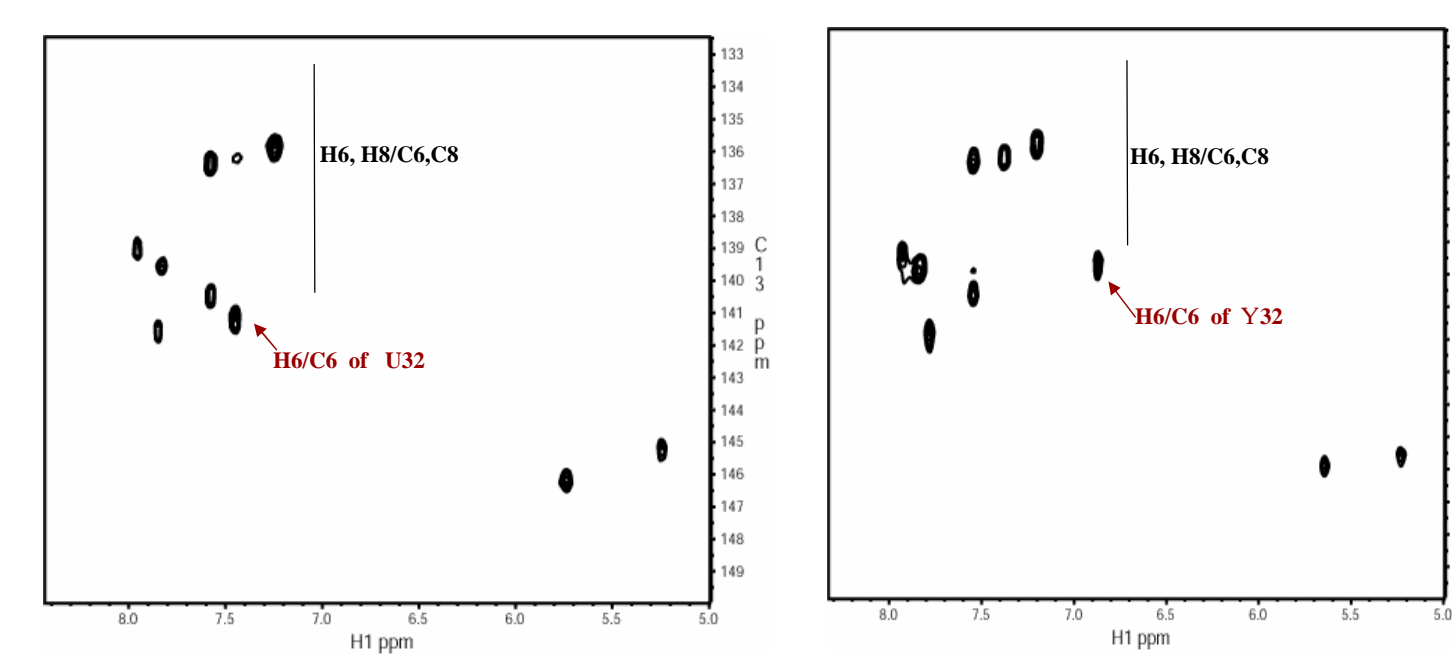
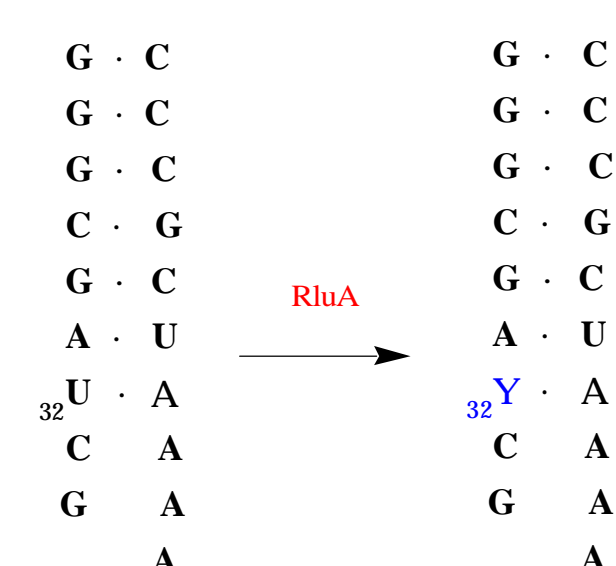
We have also tested the importance of the presence of the phosphate backbone for the RluA reaction.

The internucleotide backbone of ACSL^{Phe} was cleaved by RNase T1 and the duplex RNA was incubated with RluA. ACSL^{Phe} after digestion by using RNase T1 acts as the substrat for RluA.



¹³C labeled ACSL^{Phe}, selectively digested by using RNase T1, was modified in the pseudouridylation reaction. Comparison of the HSQC spectrum of ACSL^{Phe} before and after the reaction confirmed the formation of y32.

We have also determined that double stranded RNA which does not form stem-loop structure can be modified by RluA (scheme below).



Spectra ¹H-¹³C HSQC-CT (the region of H6,8/C6,8) of the double stranded RNA before pseudouridylation reaction (on the left) and after reaction (on the right).

Advantage of our method is based on its ability to incorporate Y in sequence of isotope labeled RNA which does not form stem-loop structure.

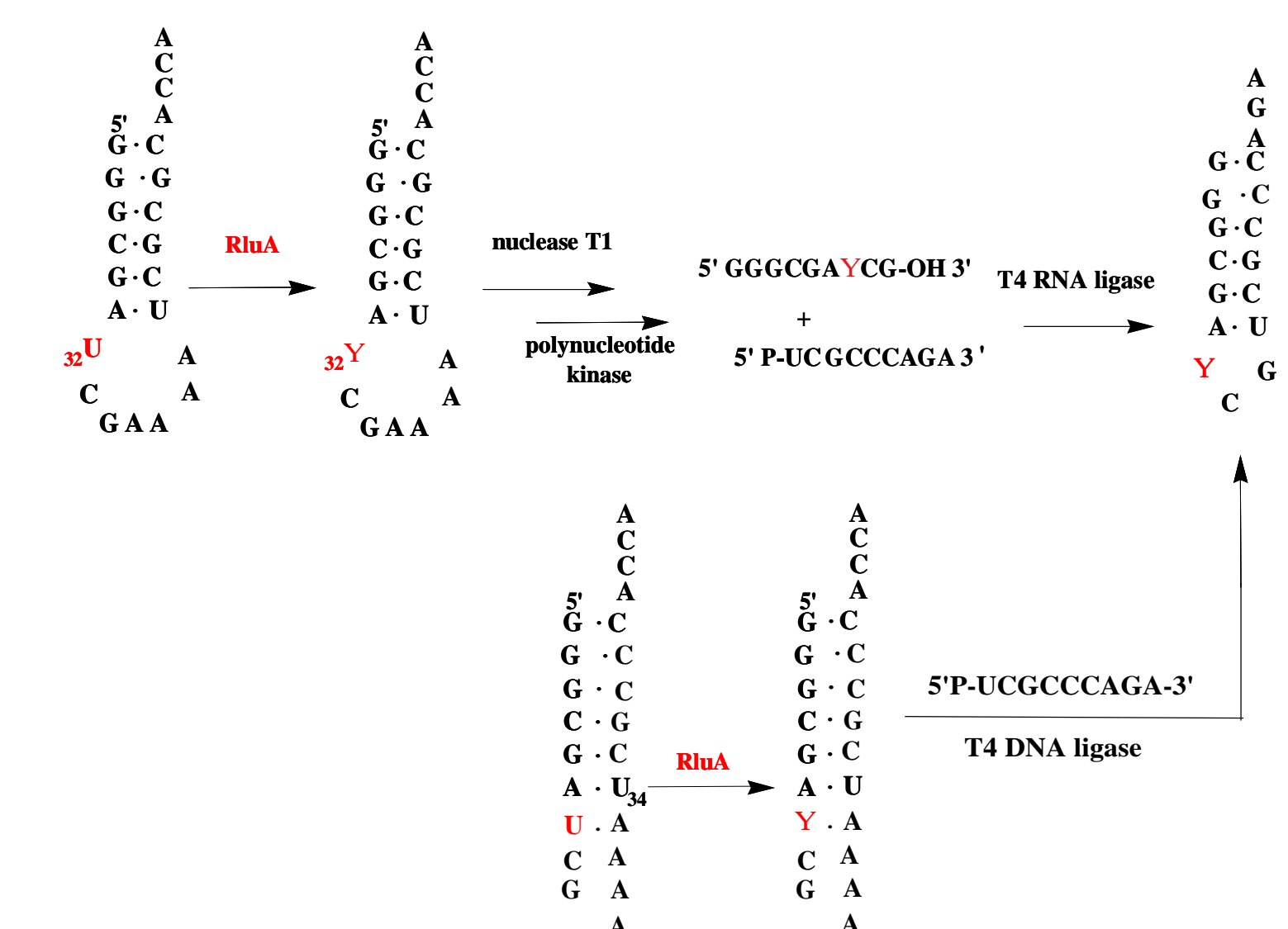
APPLICATIONS & CONCLUSIONS

We have found that the primary recognition element for RluA is the sequence:

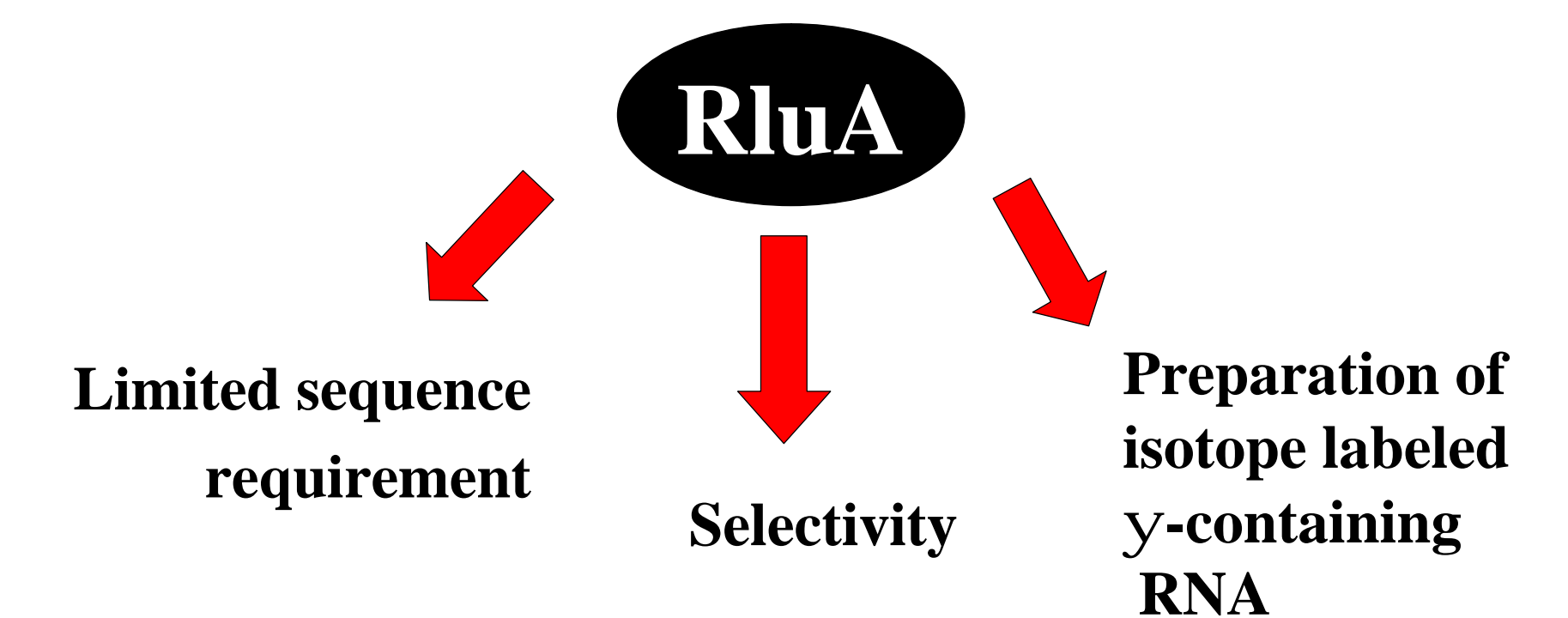


where N= C, U, G, A; X=A, C, G; Z=A, U, C; R=purine;

The properties of RluA have allowed us to develop a general method for enzymatic incorporation of pseudouridine in any site of RNA. Our strategy is based on reaction of RNA with RluA and subsequent selective digestion of internucleotide bond by using ribonuclease T1. Dephosphorylation of the modified RNA strand and ligation with the another oligonucleotide give the desired sequence of RNA.



¹³C,¹⁵N-labeled 5'-strand of RNA forms duplex with the complementary strand of RNA and reacts with RluA to generate pseudouridine. Without further purification, modified RNA is ligated to a chemically synthesized 3'-phosphorylated strand using template-directed T4 DNA ligase. Labeling of the 3' -half of the stem can be accomplished if needed.



We have determined primary sequence of RNA necessary for the recognition by RluA and have developed simple and reliable method of pseudouridylation of RNA. Our methodology is general and allows uniform isotopic enrichment of the RNA.