Ribonucleotide-crosslinked peptides can serve as direct evidence for protein-RNA interfaces [Kramer et al., Nature Methods, 2014]. Potentially, this allows for the discovery of novel domains or unstructured regions binding to RNA. Unfortunately, these peptides are notoriously hard to purify and notoriously hard to detect by MS. In fact, two recent methods use their absence in proteomic data as indirect evidence for protein-RNA interactions [Castello et al., Mol Cell, 2016; He et al., Mol Cell, 2016]. XRNAX easily scales and can thereby produce unseen amounts of ribonucleotide-crosslinked peptides, which simplifies purification and tremendously increases MS signal and detection. The following protocol describes the purification of nucleotide-crosslinked peptides starting from XRNAX extracts (refer to www.xrnax.com/theprotocol for the initial XRNAX extraction).

XRNAX

For the isolation of peptides crosslinked to nucleotides use 1000 µg of XRNAX extract produced from MCF7 cells using the extraction method described in the ‘The Protocol’ section of this website (2 confluent 245 x 245 mm² dishes MCF7 cells yield approximately 1000 µg XRNAX extract).

**Required Material**
- Tris-Cl 1 M (pH=7.5)
- DTT 1 M
- SDS 20 %
- Trypsin/LysC (Promega, V5073)
- Chloroacetamide (CAA) 1 M
- GlycoBlue (Ambion, AM9515)
- NaCl 5 M
- Quiagen RNeasy Midi Kit
- RNase A (Thermo, EN0531)
- RNase I (Ambion, AM2295)
- RNase T1 (Thermo, EN0541)
- High pH buffer 10 x (ammonium formate 200 mM, pH=10)
- Formic acid 1 %

**Complete Tryptic Digestion**
Divide the sample into two aliquots of 500 µg of XRNAX extract and combine with 50 µl tris-Cl 1 M, 10 µl DTT 1 M, 5 µl SDS 20 %. Top off with MilliQ to 950 µl total volume and mix by pipetting. Add 10 µg trypsin/LysC to achieve a final volume of 1 ml and allow digestion to occur for 1 hour at 37 °C, 700 rpm shaking. Add 20 µl CAA 1 M and allow digestion to continue for another hour.

**Silica Purification**
For the purification of peptide-crosslinked RNA from the digests use the Quiagen RNeasy Midi Kit with modified protocol (refer to kit manual for buffer descriptors). Combine 1 ml digest with 3.5 ml buffer RIL in a 15 ml falcon tube, mix by inversion and heat to 60 °C for 15 min. Let the sample reach room temperature and add 2.5 ml of 100 % ethanol. Mix by inversion and apply two times 3.5 ml sample to an RNeasy Midi column (2 digests of 1 ml are applied to 2 columns in total) by centrifugation with 3000 g for 5 minutes. Wash twice with 2.5 ml buffer RPE, do not use buffer RW1. Elute peptide-crosslinked RNA twice with 250 µl nuclease-free water by centrifugation with 3000 g for 5 minutes. All eluates should combine to approx. 900 µl, which are combined in a fresh 2 ml tube. Add 60 µl NaCl 5 M along with 1 µl glycoblue and 1 ml isopropanol. Mix by inversion and incubate for 1 hour at -20 °C. Spin down with 18000 g (or full speed) at -10 °C (or coldest setting) for 60 minutes. Discard the supernatant and wash the pellet with 2 ml of 70 % ethanol. Remove all residual ethanol and take up the pellet in 60 µl tris-Cl 10 mM.

**RNase Digestion**
Heat the sample to 85°C for 5 minutes and cool on ice before adding 1.5 µl of RNase A, RNase I and RNase T1. Allow RNA digestion to occur for 12 hours at 37 °C, 700 rpm shaking. Heat the sample again to 85 °C for 5 minutes and subsequently cool on ice. Add another 1.5 µl of RNase A, RNase I and RNase T1 to the sample and digest for another 12 hours.
RNA fragments are primarily eluting between 12-17 minutes, so that clean nucleotide-crosslinked peptides can be collected in later fractions. The peak at 37 minutes mainly contains RNase peptides. Combine fractions from 17-37 minutes and concentrate them by speedVac until dry. Take up peptides in 15 µl formic acid 1 % and analyze by HPLC-MS using the orbitrap at a resolution of 120000 or higher.

Data Analysis
An unbiased identification of peptide adducts with unknown masses can be accomplished by MSFragger [Kong et al., Nat Biotech 2017]. If it is unclear which amino acid in your protein-of-interest is crosslinked to which ribonucleotide, conventional searches taking variable modifications into account become impossible, due to the enormous multiplicity of at least four modifications (4 ribonucleotides, non-cyclic, cyclic, etc.) possibly crosslinking to all 20 amino acid.

Search your data with MSFragger in open-search mode with a mass-tolerance of +/- 1000 Da, which allows for the discovery of up to three nucleotides crosslinked to a peptide (e.g. the RNA sequence UpUpUp crosslinked to a peptide would result in an adduct mass of 936.085 Da).

Alternatively, as uridine and cyclic uridine are the most common crosslinked nucleotides identified, search your data with a conventional search engine such as MaxQuant [Cox and Mann, Nat Biotech 2008] and specify these two as variable modification.