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To: JoVE Editor

Dear Sir or Madam,

Please find the enclosed manuscript "**RiboTag immunoprecipitation in the germ cells of the male mouse**", for consideration of publication in JoVE.

The RiboTag protocol, initially published in 2009 by Sanz and colleagues, has become a landmark method for understanding the translatome of specific cell types in complex tissues. In this work, we describe a more practical method of conducting this protocol in the germ cells of the testis, a translationally complex and critical system. By employing stringent breeding schemes, verifying the efficacy of flash frozen tissue, and adding a pre-clear step to greatly abrogate nonspecific background, we mitigate a number of established issues with the exisiting protocol.

The method illustrated in this work represent improvements on the published RiboTag protocol and describe in detail the means by which to apply these methods to the murine male germ cell, providing key notes to facilitate the application of the method to other systems. As such, they should appeal to the scientifically diverse readership of JoVE.

All authors have agreed to the submission of this manuscript and declare no potential conflicts of interest. All Supplemental Materials are for final publication.

Thank you very much for your attention.

Sincerely,

Elizabeth M. Snyder, PhD

TITLE:

RiboTag Immunoprecipitation in the Germ Cells of the Male Mouse

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cell-specific, male germ cell, RNA, RiboTag, ribosome, translation, spermatogenesis

SUMMARY:

Here, we describe the immunoprecipitation of ribosomes and associated RNA from select populations of adult male mouse germ cells using the RiboTag system. Strategic breeding and careful immunoprecipitation result in clean, reproducible results that inform on the germ cell translatome and provide insight into the mechanisms of mutant phenotypes.

ABSTRACT:

Quantifying differences in mRNA abundance is a classic approach to understand the impact of a given gene mutation on cell physiology. However, characterizing differences in the translatome (the whole of translated mRNAs) provides an additional layer of information particularly useful when trying to understand the function of RNA regulating or binding proteins. A number of methods for accomplishing this have been developed, including ribosome profiling and polysome analysis. However, both methods carry significant technical challenges and cannot be applied to specific cell populations within a tissue unless combined with additional sorting methods. In contrast, the RiboTag method is a genetic-based, efficient, and technically straightforward alternative that allows the identification of ribosome associated RNAs from specific cell populations without added sorting steps, provided an applicable cell-specific *Cre* driver is available. This method consists of breeding to generate the genetic models, sample collection, immunoprecipitation, and downstream RNA analyses. Here, we outline this process in adult male mouse germ cells mutant for an RNA binding protein required for male fertility. Special attention is paid to considerations for breeding with a focus on efficient colony management and the generation of correct genetic backgrounds and immunoprecipitation in order to reduce background and optimize output. Discussion of troubleshooting options, additional confirmatory experiments, and potential downstream applications is also included. The presented genetic tools and molecular protocols represent a powerful way to describe the ribosome-associated RNAs of specific cell populations in complex tissues or in systems with aberrant mRNA storage

and translation with the goal of informing on the molecular drivers of mutant phenotypes.

INTRODUCTION:

Analysis of a cell or tissue's RNA abundance, as examined by microarray or RNA-sequencing, has proven a powerful tool to understand the molecular drivers of mutant phenotypes. However, these relatively incomplete analysis tools may not inform on the physiology or proteome of the cell, especially in systems where many mRNAs are stored prior to translation such as neural and germ cells. In these systems, defining the population of mRNAs being actively translated into protein, or the cell's translatome, may be a better indicator of the cell's actual physiological state[1,2]. For example, germ cells at various stages of developement transcribe RNAs that are stored for later translation, driven either by developmental[3] or signaling cues[4]. This process is exemplified by the mRNAs encoding protamines, wherein the mRNA transcript is detectable days before the protein is made[1,2,5,6]. Likewise, neural cells transcribe RNA in the nucleus and transport it down the axon, as is the case with β-actin[7]. In addition to these specialized cell systems, steady-state transcriptomes are unlikely to be informative in models where RNA storage, ribosome biogenesis, or mRNA translation are impacted. Multiple other factors may also impact a cell's steady-state RNA pool include mRNA decay and the activity of RNA binding proteins. In these cases, robust tools to analyze ribosome-associated RNAs or mRNAs under active translation are more likely to yield biologically relevant results.

To that end, several methods have been developed for identifying ribosome-associated or actively translated messages. Polysome profiling, which provides a snapshot of ribosome associated transcripts, has been used for many decades to isolate intact RNAs associated with either ribosomal subunits or mono-, di-, and poly-ribosome complexes[3]. Briefly, collected cell lysates are applied to a linear sucrose gradient and centrifuged as high speed, resulting in separation of the ribosome subunits, intact ribosomes, and polysomes by size. Traditionally, this technique has been applied to study one or a few mRNAs, but recently this method has been combined with RNA-seq studies to elucidate the function of potential translational regulators[8,9] While a powerful way to differentiate between actively translating and non-translating mRNAs[10], polysome profiling does require time-consuming methods (gradient fractionation and ultracentrifuge) and can require a good deal of sample making the analysis of rare cell populations challenging.

An alternative approach to examining the translatome is ribosome profiling, which identifies the portions of transcripts directly associated with the ribosome. In brief, ribosome associated RNA fragments are generated via RNase protection assay, individual ribosome complexes separated via sucrose gradient, and associated RNA fragments isolated and converted to RNA-seq tags amenable to deep sequencing[11]. One of the key benefits to ribosome profiling is the ability to determine the specific locations of the ribosomes at the time of isolation which allows identification of translation start sites, calculation of ribosomal occupancy and distribution, and identification of ribosome stalls[12]. However, this method has several inherent drawbacks, including equipment needs (gradient fractionator and ultracentrifuge), a relatively complex protocol that require extensive troubleshooting, and computational issues not easily handled by the inexperienced user[11]. Importantly, ribosome profiling is primarily applied to isolated cells in

culture and requires substantial material, making its application to mixed cell-type tissues or sorted cells from in vivo limited.

The mammalian RiboTag method, developed by Sanz et al. in 2009[13], eliminates a number of issues inherent to both polysome and ribosome profiling. In this method, ribosome-associated RNAs can be isolated from specific cell types allowing for investigation of cell-type specific translatomes in complex tissues without additional isolation techniques and specialized equipment, as is necessary in other methods[13,14]. The basis of the RiboTag method is a transgenic mouse model (RiboTag) carrying a modified locus for the 60S ribosomal subunit protein gene *Rpl22*. This locus (*Rpl22-HA*) includes a floxed terminal exon followed by an additional copy of the terminal exon amended to include a C-terminal hemagglutinin (HA) tag within the coding region. When crossed to a mouse expressing a cell-specific *Cre* Recombinase, the floxed exon is removed allowing the expression of HA-tagged RPL22 in a cell-specific manner (**Figure 1**). The HA tag can then be used to immunoprecipitate (IP) ribosomes and their associated RNAs from the selected cell type.

In addition to the initial publication that developed the technique, several other laboratories have utilized the RiboTag model. Diverse tissues such as mouse Sertoli and Leydig cells[15], microglia[16], neurons[17,18], oocytes[19], and cultured cells[20] have been profiled and studied. Though this protocol is clearly able to successfully isolate ribosomes and the associated RNAs across a diverse tissue types there are still areas needing improvement, especially when applied to mutant systems. In particular, common reliance on fresh tissue results in increased technical variation which can greatly reduce the power of the analysis. Secondly, confident identification of differentially translated targets is made more challenging when high immunoprecipitation background occurs from non-*Cre* recombined cell types as previously reported[13]. While Sanz et al. engineered the basic premise of the technique, in this manuscript the Snyder laboratory presents optimization of the protocol to reduce these concerns, rendering the method more practical and efficient.

The intent of this article is to explain the steps for breeding mice expressing cell-specific HA-tagged ribosomes, immunoprecipitating these ribosomes from flash-frozen samples, and purifying their associated RNAs for further downstream analyses. As the mammalian male germ cell and the infertility mutation studied provide unique challenges, efforts have been made to illuminate ways this technique can be adapted to other cell systems.

PROTOCOL:

All animal use and handling practices have been approved by Rutgers University's Internal Animal Care and Use Committee (IACUC).

1. Mouse breeding

1.1. Breed female mice homozygous for an RNA binding protein mutation that leads to male infertility (manuscript in preparation, referred to herein as *the gene of interest)* in trio matings to

males hemizygous for the *Stra8-iCre* allele (B6.FVB-Tg(Stra8-iCre)1Reb/LguJ) (*Cre+*), as pairing two females to every male increases breeding efficiency.

NOTE: The mutant gene of interest (*M*) was passed maternally, as females homozygous for *M* have normal fertility. This has the added benefit of allowing paternal transmission of the male germ cell *Cre* (hemizygous), as recommended[21] and optimizing the percent of offspring with the desired allelic combination. Because homozygous *Cres* exhibit germ cell toxicity[22], it is recommended the allele be maintained and passed in a heterozygous or hemizygous state. Importantly, *Cre* expression must not co-occur with the *Rpl22-HA* allele until the experimental population. Failure to isolate the *Rpl22-HA* allele from *Cre* in breeding animals will result in offspring that *globally* express *Rpl22-HA* due to germ cell *Cre* activity. This issue is specific to germ cell *Cres*. Additionally, the *Stra8*-i*Cre* is specific to the authors' cell population of interest[22], targeting cells transitioning into and very early in meiosis, including preleptotenes and leptotenes. A different *Cre* driver specific to another cell type of interest can be used instead. Common practice dictates male germ cell expressed *Cres* be transmitted on the paternal side. However, it is possible maternal transmission of the *Stra8-iCre* will result in similar transgene expression in male offspring. Regardless of breeding scheme selected, in order to generate offspring with equivalent *Cre* expression, all experimental samples should be generated via *Cre* transmission from the same parental side (either always maternal or always paternal).

1.2. Genotype resulting male pups as described in section 2.4. Select those carrying the *M* allele and positive for *Cre* (*+/M:Cre+*) for downstream breeding.

1.3. Breed female mice homozygous for the *M* allele in trio matings to males homozygous for *Rpl22-HA* (B6J.129(Cg)-*Rpl22*$^{tm1.1Psam}$/SjJ).

NOTE: The *Rpl22-HA* background is highly mixed, so care should be exercised when assessing strain-specific phenotypes.

1.4. Genotype resulting female pups to identify those carrying the *M* allele and positive for *Rpl22-HA* (*+/M:Rpl22-HA+*) (see section 2.3). Select these females for downstream breeding.

1.5. Cross *+/M:Cre+* males with *+/M:Rpl22+* females in trio matings. Genotype the resulting pups (see sections 2.3 and 2.4) to identify males that are both *Cre+* and *Rpl22+* and either wildtype or *M/M.*

NOTE: As this work focuses on a mutation resulting in complete male infertility, special considerations have been taken in the breeding scheme to most efficiently obtain desired experimental genotypes. Such considerations may be unnecessary in other experimental models. See **Figure 2** for a full breeding schematic and accompanying legend for additional discussion of breeding considerations.

2. Sample collection

2.1. Collect testes from male mice at 21 days post-partum (dpp).

2.1.1. Sacrifice mice by CO_2 inhalation followed by cervical dislocation. Make a ventral incision (3 cm) on each animal flanked by two shorter (2 cm each), connected lateral incisions. Pull the skin and peritoneum back to reveal the testes.

2.1.2. Using forceps, pull the epidymal fat pad from one side of the body cavity to reveal the epididymis and testis. With tenotomy scissors, excise the testis, taking care to trim away epididymis, surrounding fat, and any external vasculature. Place intact testes in a clean 1.7 mL tube.

2.1.3. Repeat with other side, adding the second testes to the first tube. Cap this tube and immediately submerge it in liquid nitrogen to flash-freeze.

NOTE: Samples can be preserved at -80 °C until use.

2.2. Collect additional tail tip samples for each animal (2 mm) for genotyping confirmation.

2.2.1. To extract DNA, add 100 µL of 50 mM NaOH to a 2 mm tail tip in a 1.7 mL tube and boil at 95 °C for 30 min. Add 100 µL of 50 mM HCl and 20 µL of 1 M Tris-HCl pH 8.0 and centrifuge samples for 3 min at 10,000 x *g*. Retain supernatant (containing genomic DNA) and store extracted DNA at 4 °C until use as template for the following genotyping reactions.

2.3. Perform *Rpl22-HA* genotyping following a method adapted from Sanz et al.[13] using the following primers: forward: 5' GGGAGGCTTGCTGGATATG 3'; reverse: 5' TTTCCAGACACAGGCTAAGTACAC 3'.

2.3.1. Prepare a reaction mixture consisting of 2 µL of DNA as extracted in step 2.2.1, 0.08 µL of 25 mM dNTPs, 0.1 µL of 10 mM forward primer, 0.1 µL of 10 mM reverse primer, 2 µL of a polymerase chain reaction (PCR) buffer (650 mM Tris pH = 8.8, 165 mM $(NH_4)_2SO_4$, 30 mM $MgCl_2$, and 0.1% Tween), 0.2 µL of *Taq* polymerase, and nuclease-free H_2O to a total volume of 20 µL.

2.3.2. Use the following thermocycler conditions: a 30 s primer melting step at 95 °C, 30 s anneal at 64 °C and a 30 s elongation at 72 °C, repeated 37 times with a final 5 min elongation at 72 °C.

NOTE: This yielded a product size of 260 bp for wildtype samples and 292 bp for samples that were *Rpl22-HA+*.

2.4. Perform *Cre* genotyping using the following primers: forward: 5' GTGCCAGCTGAACAACAGGA 3'; reverse: 5' AGGGACACAGCATTGGAGTC 3'. Prepare the PCR reaction as described in step 2.3.2 utilizing the *Cre* primers instead. Use the following thermocycler conditions: a 30 s primer melting step at 95 °C, 30 s anneal at 60 °C and a 15 s elongation at 72 °C, repeated 35 times with a final 5 min elongation at 72 °C.

2.5. Carry out gene of interest genotyping as is standard for the gene in question.

NOTE: The author's genotyping protocol utilizes a concentrated custom *Taq*, and as such, other users may have to modify the conditions to suit their enzymatic requirements. In order to better understand the impact of this gene of interest's loss on translation, males that were either wildtype or homozygous for the mutant allele of interest and that were also *Cre+* and *Rpl22+* were selected to be the experimental samples. Genotype selection is further discussed above in section 1.

3. Preparation of solutions

NOTE: Preparation of solutions and all subsequent steps must be done under stringently RNase free conditions.

3.1. To prepare homogenization buffer (HB), add 2.5 mL of 1 M Tris pH 7.4, 5 mL of 1 M KCl, 600 µL of 1 M $MgCl_2$, and 500 µL of NP-40 to a 50 mL tube. Bring to volume (50 mL) in diethyl pyrocarbonate (DEPC) H_2O and mix until all components are incorporated.

NOTE: The final concentrations will be as follows: 50 mM Tris pH 7.4, 100 mM KCl, 12 mM $MgCl_2$, and 1% NP-40.

3.2. To prepare high salt wash buffer (HSWB), add 2.5 mL of 1 M Tris pH 7.4, 15 mL of 1 M KCl, 600 µL of 1 M $MgCl_2$, and 500 µL of NP-40 to a 50 mL tube. Bring to volume (50 mL) in DEPC H_2O and mix until all components are incorporated.

NOTE: The final concentrations will be as follows: 50 mM Tris pH 7.4, 300 mM KCl, 12 mM $MgCl_2$, and 1% NP-40. The protocol can be paused here, and solutions can be stored at room temperature until use.

4. Preparation of tissue

4.1. Preweigh all sample tubes, recording weight, and precool in liquid nitrogen.

4.2. Precool sterile mortar, pestle, and weighing spatula on dry ice.

4.3. Grind flash-frozen tissue samples to a fine powder using a precooled, sterile mortar and pestle on dry ice.

4.3.1. Place flash frozen tissue into precooled mortar on dry ice. Gently break tissue into large pieces using pestle and grind slowly until tissue is a fine powder.

NOTE: Although fresh tissue can also be used, as per the original protocol[13], no significant difference in outcome is observed with the use of flash-frozen tissues. See Representative Results and Discussion for details.

4.3.2. Carefully transfer ground sample to precooled collection tube by scraping powder from mortar using precooled spatula. Ensure only tissue is collected and not any moisture deposited onto the cold mortar.

4.3.3. Weigh the tube with tissue, taking care to keep on dry ice whenever possible. Calculate the mass of tissue by subtracting initial weight of the tube from the weight of the tube with the sample in it. Record this value for later calculation of lysis buffer volumes.

NOTE: The protocol can be paused here, and samples can be stored at -80 °C until use.

5. Homogenization of sample

5.1. Prepare lysis buffer (10 mL HB + supplements) by adding 10 µL of 1 M dithiothreitol (DTT), 1 protease inhibitor tablet, 50 µL of RNase inhibitor (40,000 units/mL), 200 µL of 5 mg/mL cycloheximide, and 100 µL of 100 mg/mL heparin to 10 mL of HB. Mix until all components are incorporated and keep on ice until use.

NOTE: The final concentrations of the supplements in 10 mL HB will be as follows: 1 mM DTT, 200 U/mL RNase inhibitor, 100 µg/mL cycloheximide, and 1 mg/mL heparin. This solution must be used within 24 h of the addition of the supplements and can be kept at 4 °C or on ice until use.

5.2. For every 100 mg of sample, add 1 mL of lysis buffer. With the same pipette used to add the lysis buffer, carefully pipette the resulting lysate up and down to fragment cells and mix. Continue pipetting until the sample is no longer viscous, generally 25−30 strokes.

NOTE: Because the solution is very viscous at first, a large diameter pipette should be used to mix the solution. A standard 1000 µL is normally sufficient for 100 mg of tissue.

5.3. Set samples on ice for 10 min to lyse. Then, spin samples in a precooled centrifuge (4 °C) for 10 min at 10,000 x *g*. A large, loose, cloudy pellet should form in the bottom of the tube.

5.4. Being careful not to disturb the pellet, collect the lysate into a new tube and record volume for each sample. To prevent sample degredataion, ensure that samples remain cool throughout the remaining protocol, storing on ice or at 4°C whenever possible.

6. Equilibration of beads

6.1. Using the volume of lysate from step 5.2, calculate the required volume of magnetic beads coupled to the appropriate antibody binding protein (in this case, protein G). For 1 mL of lysate, use 375 µL of protein G beads (30 mg/mL).

NOTE: Choice of conjugated beads will vary with anti-HA antibody used; for example, if a rabbit-generated anti-HA antibody is selected, protein A beads will be preferable.

6.2. Using a magnetic tube rack, remove bead solvent and add an equal volume of fresh lysis buffer to the beads. Rotate 5 min at 4 °C to wash. Perform all rotation steps using a benchtop tube rotater set to 20 rpm.

6.3. Place the tube on the magnetic tube rack and remove the wash buffer.

6.4. Repeat steps 6.1−6.3 two more times.

6.5. Add lysis buffer at the original volume to equilibrated beads. Store at 4 °C or on ice until use.

7. Preclearing sample

7.1. Add 50 µL of equilibrated beads for every 1 mL of lysate to supernatant of lysed sample (lysate) and rotate at 4 °C for 1 h.

7.2. Place the tube on the magnet rack and collect lysate into a fresh tube. Discard used beads.

7.3. To the lysate add 25 µL of equilibrated beads for every 1 mL and rotate at 4 °C for 1 h. Store remaining equilibrated protein G beads at 4 °C overnight.

7.4. Place tube on the magnet rack and collect lysate into a fresh tube. Discard used beads. From the cleared lysate, retain 50 µL to act as sample input control. Store at -80 °C.

NOTE: The input sample acts as a control representing the total RNA population of the sample, including RNAs associated with other cell types in the tissue, to be used during downstream analyses to correct for gene expression changes as a function of mutation.

8. Incubation of antibody

8.1. For every 1 mL of cleared lysate, add 5 µg of anti-HA antibody. To prevent sample loss, seal the tube cap with paraffin film. Rotate samples 16−18 h at 4 °C.

NOTE: The antibody incubation time and concentration has not been optimized. The authors found an overnight incubation with 5 µg to be sufficient; however, it is possible that a shorter incubation or less antibody will be adequate, or that a longer incubation or more antibody will improve binding.

9. Incubation of beads

9.1. For every 1 mL of cleared lysate, add 300 µL of protein G beads. Reseal the tube cap with paraffin film and rotate for 2 h at 4 °C.

10. Washing of beads

10.1. Place the sample tube on the magnet rack to allow beads to separate from IPed lysate. Pipette off flow-through lysate and discard, retaining the beads.

10.2. Apply 800 µL of HSWB to beads and allow to rotate for 10 min at 4 °C. Place the sample tube on the magnet rack and allow beads to separate from wash. Remove and discard wash.

10.3. Apply another 800 µL of HSWB to beads. Close sample and allow to rotate for 5 min at 4 °C. Place the sample tube on the magnet rack and allow beads to separate from wash. Remove and discard wash.

10.4. Repeat step 10.3 once more.

11. RNA extraction

11.1. Place the sample tube on the magnet rack and allow beads to separate from wash. Pipette off and discard wash, retaining beads for RNA extraction. Add 3.5 µL of 14.2 M beta-mercaptoethanol (bME) to beads and mix by vortexing for 15 s.

11.2. Extract RNA using a commercial RNA purification kit, as per manufacturer's instructions. Elute sample in 30 µL of RNase free H_2O.

NOTE: Though the 10 µL/sample DNase treatment is optional for most kits, it is strongly encouraged. This optional cleanup step significantly reduces background, allowing for a more accurate final concentration. It is strongly recommened to use a kit that contains beta-mercaptoethanol (bME) in the lysis buffer. bME acts as an additional RNase inhibitor to prevent sample degradation during RNA isolation.

11.3. Store sample at -80 °C or analyze immediately.

12. Quantification and sample analysis

12.1. Using a UV-Vis spectrophotometer, quantify RNA concentration and preliminary quality.

12.2. Analyze quality of RNA extracted from samples via a bioanalyzer.

NOTE: The resulting RNA can then be used for RNA-sequencing or other downstream analyses such as northing blotting or quantitative reverse transcription PCR (qRT-PCR).

REPRESENTATIVE RESULTS:

Previous reports have suggested non-specific immunoprecipitation from cells lacking *Cre*[14]. In order to determine if this was the case in our modified protocol, IP efficiency was determined in samples derived from animals carrying both *Cre* and *Rpl22-HA* and animals carrying only one but not the other with the expectation that without both a *Cre*-driver and *Rpl22-HA*, immunoprecipitated RNA should be minimal. As shown in **Figure 3**, very little RNA is isolated from

samples lacking either *Cre* or *Rpl22-HA* demonstrating the effectiveness of this protocol to reduce IP background and isolate genuine HA-tagged ribosome RNAs. Further, both *Cre*-only and *Rpl22-HA*-only samples represent suitable negative controls.

Given the potential for reagent source to significantly impact the efficiency of IP, a series of antibodies and RNA isolation protocols were tested (**Figure 4**) in *Cre* and *Rpl22-HA* positive samples. These results demonstrate reagent selection can have a significant impact on IP efficiency thus any changes to reagent selection should be done so with care.

In order to test this protocol in the context of an RNA binding protein mutant, wildtype-*Cre+Rpl22-HA+* (wildtype) and *gene of interest$^{-/-}$-Cre+Rpl22-HA+ (Gene of interest$^{-/-}$)* testis were examined for the effectiveness of the RiboTag system to isolate ribosome associated RNA (**Figure 5**). When RNA concentration of wildtype input and IP was compared to *Gene of interest$^{-/-}$* input and IP, no significant difference was seen between genotypes. For both genotypes, however, the input concentration was significantly higher than the IP concentration, indicating that there was more RNA in the input sample (**Figure 5B**). This result is expected, as not all the mRNAs present in the cell are associated with the ribosome at any given time, especially in the case of germ cells where RNAs may be transcribed long before they are translated.

In order to confirm the quality of the RNA samples sent for sequencing, samples were run on a bioanalyzer. RNA integrity numbers (RINs), normally calculated as the ratio of 28S and 18S rRNA peaks, were compared across samples. In total RNA pools, RIN values are expected to be near 10 with a higher RIN correlated to higher inferred sample integrity and quality. While the IP samples had lower RIN than the inputs, the RINs were still within an acceptable range and were not dependent on sample genotype (**Figure 5C**). The reduced RIN values for IPed samples are likely a result of RNA degradation though very minor given the relatively small decrease in average nucleotide length of analyzed RNAs. Given the length of the protocol and temperatures required for the immunoprecipitation some degradation is expected. It is also possible the reduced RIN and RNA length is a function of enrichment for non-rRNA species, such as mRNAs.

FIGURE LEGENDS:

Figure 1: The RiboTag method. The premise of the method is biologically simple. A new Exon 4 is inserted into the sequence for the *Rpl22* locus downstream of the original Exon 4. In the presence of a *Cre* driver, loxP sites on either side of the original Exon 4 are cut, excising the floxed exon. The HA-tagged Exon 4 is now incorporated into *Rpl22* mRNA, generating an HA tagged RPL22 in cells expressing CRE.

Figure 2: Sample breeding scheme for RiboTag mice. The breeding scheme used to generate experimental animals is shown. A two-pronged scheme was utilized. In generation 1, two parallel sets of breeder trios were established. One side combined the allele of interest (carried maternally as this specific mutation results in male infertility) with the hemizygous *Cre* and on the other combining the allele of interest, again carried maternally, with *Rpl22-HA.* Then, in generation 2, males from the first pairing that carry the allele of interest and the *Cre* are crossed

to female offspring of the second pairing that carry the allele of interest and *Rpl22-HA.* The genotypes of the resulting offspring are determined, and experimentally relevant animals selected (in this case either wildtype or homozygous mutant carrying both the *Cre* and *Rpl22-HA* alleles). It is important to note for germ cell expressing *Cre*-drivers, the *Cre* and *Rpl22-HA* alleles should not be breed together until the experimental generation. Exposure of the *Rpl22-HA* allele to germ cell-expressed *Cre* in breeding generations will drive germ-cell *Rpl22-HA* excision. Any resulting offspring will globally express *Rpl22-HA* thus preventing cell-specific ribosome isolation. In the system described herein, an n = 4 per genotype provided sufficient statistical power for downstream analyses. Biological replicate number should be determined for each experimental system.

Figure 3: Confirmation of negative controls. Samples that are *Cre+* and *Rpl22-HA+* show higher RNA pulldown than samples that are *Cre+* or *Rpl22-HA+* alone. There is no significant difference between *Cre+* and *Rpl22-HA+* sample RNA pulldown efficacy, indicating that samples lacking either the *Cre* or the *Rpl22-HA* are suitable negative controls. A "+" indicates that the corresponding allele or transgene was present in these samples and a "-" denotes its absence. IP/input represents the ratio of RNA immunoprecipitated (IP) over total (input) RNA. Value calculated from concentration in nanograms. ** indicates $p < 0025$.

Figure 4: Reagents impact protocol success. (**A**) Flash frozen tissue results in immunoprecipitation efficiency similar to that of fresh tissue. (**B**) IP efficiency for two commercial antibodies were determined, designated as Antibody 1 and Antibody 2 (**Table of Materials**). When tested, Antibody 1 was more efficient at pulling down RNA than Antibody 2 which appeared to be unable to differentiate between negative (not expressing either *Cre* or *Rpl22-HA+)* and positive controls (expressing both *Cre* and *Rpl22-HA+*). Dots indicative of the ratio of IPed versus input RNA for individual biological replicates. (**C**) When RNA extraction kits (**Table of Materials**) were compared, Kit 2 significantly outperformed Kit 1. Though both kits IPed a similar amount of RNA from negative controls, Kit 2 resulted in a significantly higher RNA yield from positive samples. * indicates $p < 0.05$, ** $p < 0.025$, *** $p < 0.01$.

Figure 5: Application of the method to a mutant model. (**A**) Sample *Gene of interest* genotype confirmation via Western blotting using a custom in-house antibody against the *gene of interest* protein demonstrates *Gene of interest*$^{-/-}$ (*M/M*) males fail to produce the associated protein. GAPDH shown as a loading control. (**B**) Graphical bioanalyzer output from paired input and IPed samples for wildtype and mutant samples. (**C**) Comparison RNA integrity numbers (RIN) by sample type and genotype. (**D**) Average nucleotide length of RNA species analyzed by bioanalyzer by sample type and genotype. * indicates $p < 0.05$, ** $p < 0.025$, *** $p < 0.01$, N.S. not significant. N = 4 per genotype.

Figure 6: Example of *Cre* driver confirmation. Quantification of a gene translated early in germ cell development (*Stra8*) and two genes translated late in germ cell development (*Tnp1* and *Prm1*) analyzed by qRT-PCR of RNA immunoprecipitated from RPL22-HA driven by two different germ cell *Cres*, *Stra8-iCre* (expressed early in germ cell development) and *Hspa2-Cre* (expressed later in germ cell development, specifically after *Stra8* translation). Here, HA-IP of *Stra8* is

achieved with the early germ cell *Cre* driver but not with the later germ cell *Cre* driver demonstrating cell-specificity of the immunoprecipitation. In contrast, HA-IP of transcripts translated in late germ cells is achieved by both *Stra8-iCre* and *Hspa2-Cre*. This is expected as cells that express *Stra8-iCre* will generate HA-tagged RPL22 throughout the entirety of their development while those expressing *Hspa2-Cre* will only do so during the later portions of their development.

DISCUSSION:

Understanding the translatome of a particular cell type is invaluable for more accurately understanding a cell's physiology in the normal or mutant state. Special benefit is seen in systems wherein translation is uncoupled from transcription, such as in neural tissue where translation occurs very far from transcription, or in germ cells where transcription occurs long before translation. Relative to other methods of translatome analysis, the RiboTag system's biggest advantage comes from the use of the *Cre* recombinase system. This allows the freedom to target any cell population that has a relevant *Cre* driver. Secondly, the RiboTag IP as described herein is effective and much less technically challenging and time-consuming than either polysome or ribosome profiling. Lastly, RiboTag IP can be easily performed at the benchtop.

There are a number of critical steps in this protocol. Chief among them is the establishment of the RiboTag mouse line and generation of experimental animals for study. As for all genetic models, careful tracking of individuals within the mouse colony as well as careful genotyping protocols should be applied. PCR genotyping as per Sanz et al. should include primers targeting the loxP site 5' of the wildtype exon 4 to distinguish between wildtype alleles (260 bp) and mutant (290 bp)[13]. For the case of RiboTag analysis in germ cell models, very specific breeding requirements should be adhered to. First, in the case of mutants that result in infertility, thoughtful breeding strategies should include methods to optimize the number of offspring with the desired genotype in intermediate and experimental generations. Second, in the case of germ cell specific *Cres*, care should be taken regarding *Cre*-zygosity given the sensitivity of germ cells to *Cre* toxicity. In the germ cell, high levels of *Cre* protein can have deleterious effects[22], prohibiting the use of *Cre/Cre* animals in the breeding scheme. Lastly, when using germ cell *Cre*s, it is important to isolate the RiboTag allele from the *Cre* until the final generation as *Cre* expression in the germ cell of an intermediate generation will result in offspring expressing *Rpl22-HA* globally.

Regardless of cell system, a number of recommended controls are possible to verify robustness of both *Cre* expression and specificity. Proper expression of your *Cre* and expected excision of *Rpl22-HA* can be determined using multiple methods. In the first, tissue isolated from experimental animals can be stained for HA using either immunohistochemistry or immunofluorescence[15]. This method is optimal in that it requires no *a priori* knowledge of translated mRNAs in the target cell types. The second method, an example of which is demonstrated in **Figure 6**, requires some knowledge of translationally regulated messages in the target cell types. In this method, enrichment for a known translationally regulated mRNA can be confirmed from the selected *Cre*-driver using quantitative PCR of IPed versus input RNA. Likewise, robust *Rpl22-HA* expression can be confirmed by comparison of *Cre+/Rpl22+* samples (positive

controls) with either *Cre-/Rpl22+* or *Cre+/Rpl22-* samples which act as effective negative controls (see **Figure 3**). These comparisons can either by done on total IPed RNA or enrichment for a known translationally regulated mRNA assessed by qRT-PCR or some other quantitative method. Common problems in the execution of the protocol tend to only become apparent when RNA yield is unexpectedly low or high. The most common cause for these failures arise from incorrect genotyping of individuals. We recommend retaining additional tissue from collected sample to confirm genotypes of all samples in the case of unexpected RNA yields. Once confirmed, other possibilities should be considered including the possibility of RNase contamination or sample degradation. Careful sample storage and handling and maintenance of RNase free zones within the laboratory can greatly reduce or eliminate these issues. Although RNA isolation issues are another potential problem in this protocol, the use of commercial kits greatly reduces this issue though care should be taken to ensure they are maintained as RNase free and contain no expired solutions. Lastly, as with all antibody-based procedures, variations in lot, storage conditions, concentration, or even shipping have the potential to negatively impact the quality of the antibody and the subsequent success of the pulldown. As a result, following careful and repeated testing, we chose the most consistent and efficient antibody available.

This protocol contains two major modifications relative to other published RiboTag protocols that significantly enhance the likelihood of success. One is the ability to use flash frozen tissue, thereby mitigating any issues involving lot, technician, or technical run variations. Samples can be collected and stored allowing isolation of HA-ribosomes from all samples at once, lowering what may be major sources of variation. Second, the addition of the preclear step substantially reduces the sort of background reported by other users of the RiboTag system. Recently, a protocol report by Sanz et al. indicates the presence of high background due to abundance of ribosome-associated transcripts from non-*Cre*-driven cells[14]. Our protocol remedies this issue by including a preclearing step, effectively eliminating the presence of RNA in *Cre* negative IP samples.

Like all systems, the inherent limitations of the RiboTag system should be kept in mind. When using uncharacterized *Cre* drivers, analysis of expression should be performed prior to experimental sample production. From the perspective of translation, several nuances of this method should be noted. First, RiboTag does not allow differentiation between mRNAs poised to translate and those actively translating. As such, current RiboTag-based methods do not allow the quantification of translation efficiency as a function of individual mRNAs. If translation efficiency is of interest, it may be measured on a cell-specific basis if the RiboTag method is combined with other translatome analysis tools such as polysome profiling. Secondly, it is fundamental to take into account total RNA abundance changes stemming from individual or genotype dependent variance. It is for this reason that careful isolation of input RNA accompany immunoprecipitation and samples derived from each remain paired throughout any downstream analyses. Lastly, and in regards to RiboTag-based analysis, it should be remembered that association with a ribosome does not necessarily prove translation is occurring. Secondary methods of analysis should be performed to confirm translational regulation in targets of interest.

This protocol describes the isolation of ribosome-associated RNAs from the germ cells of male mice using the RiboTag model. Not accounting for mouse breeding and sample collection, the protocol takes two days, with three hours the first day, best done in the afternoon, an overnight incubation, followed by five hours of work the subsequent morning. It is strongly recommended that preparation of stock solutions (HB and HSWB) as well as tissue grinding be done in advance. The overall success of the protocol is reliant on correct genotyping and stringently RNase-free conditions. The ability to examine the translatome of specific cell types will allow future studies to better understand the relationship between transcription, translation, and the proteome in myriad cell types and mutant backgrounds.

ACKNOWLEDGMENTS:

This work was funded by NIH grant NICHD R00HD083521 to EMS and internal support from Rutgers University to EMS.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;figure1.psd

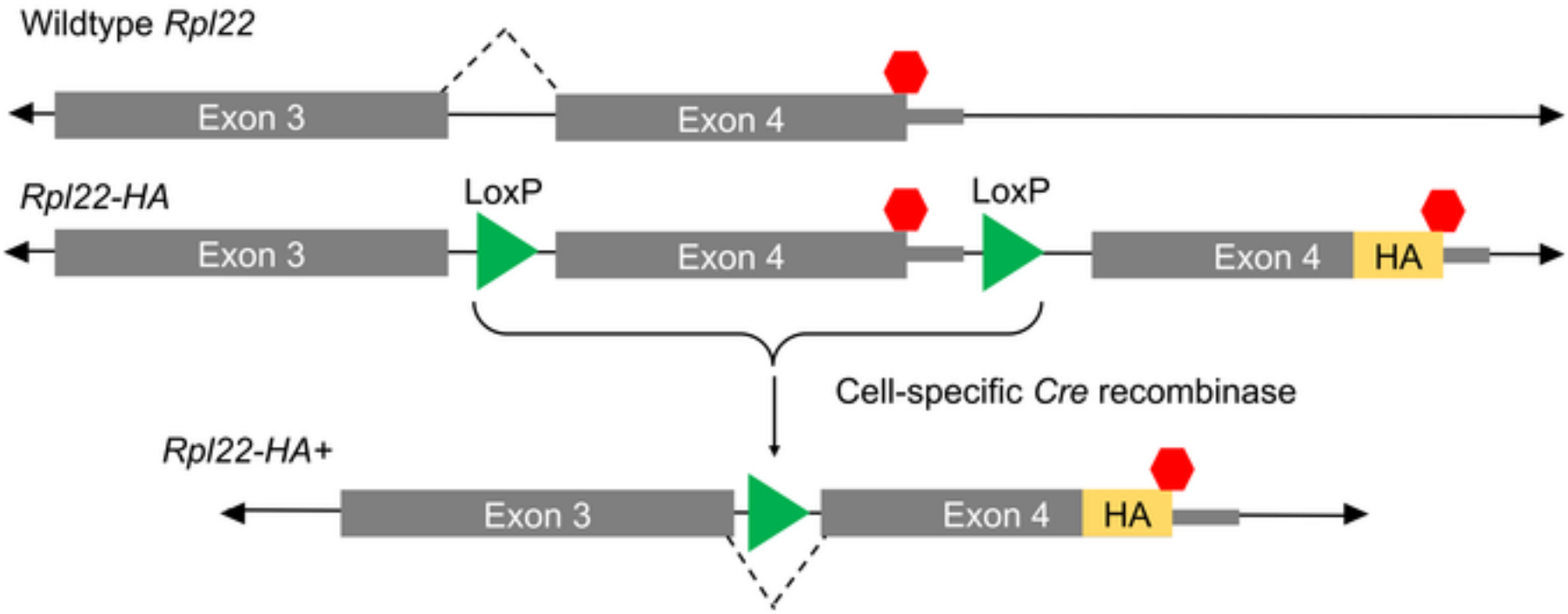


Figure 2

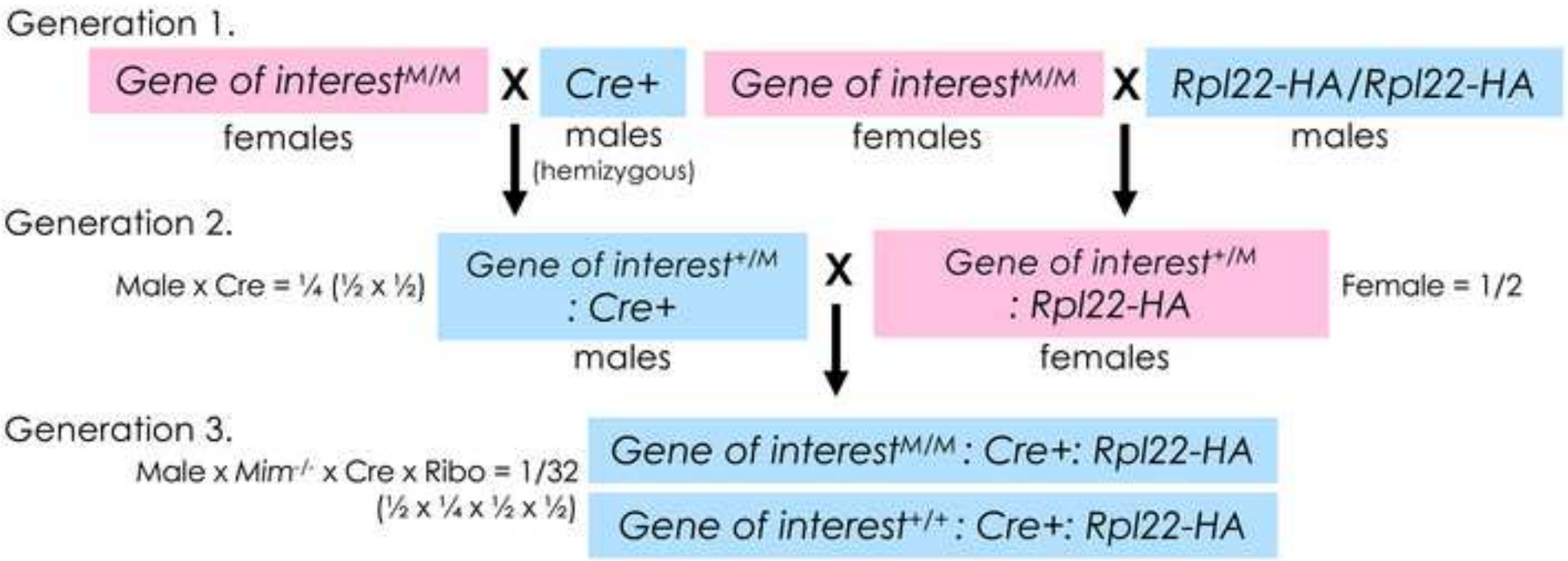


Figure 3

Click here to access/download;Figure;figure3.psd

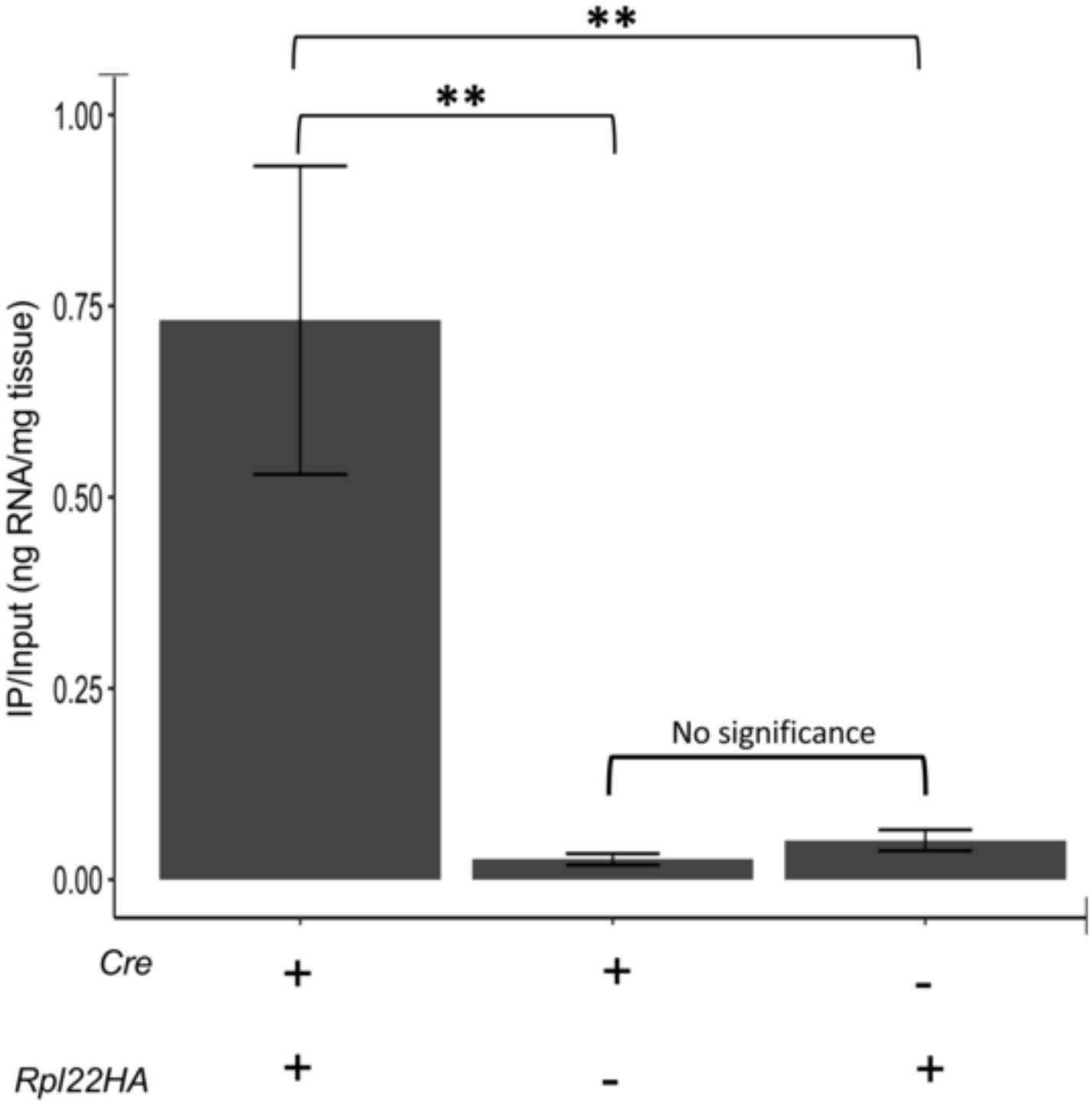


Figure 4

Click here to access/download;Figure;figure4.psd

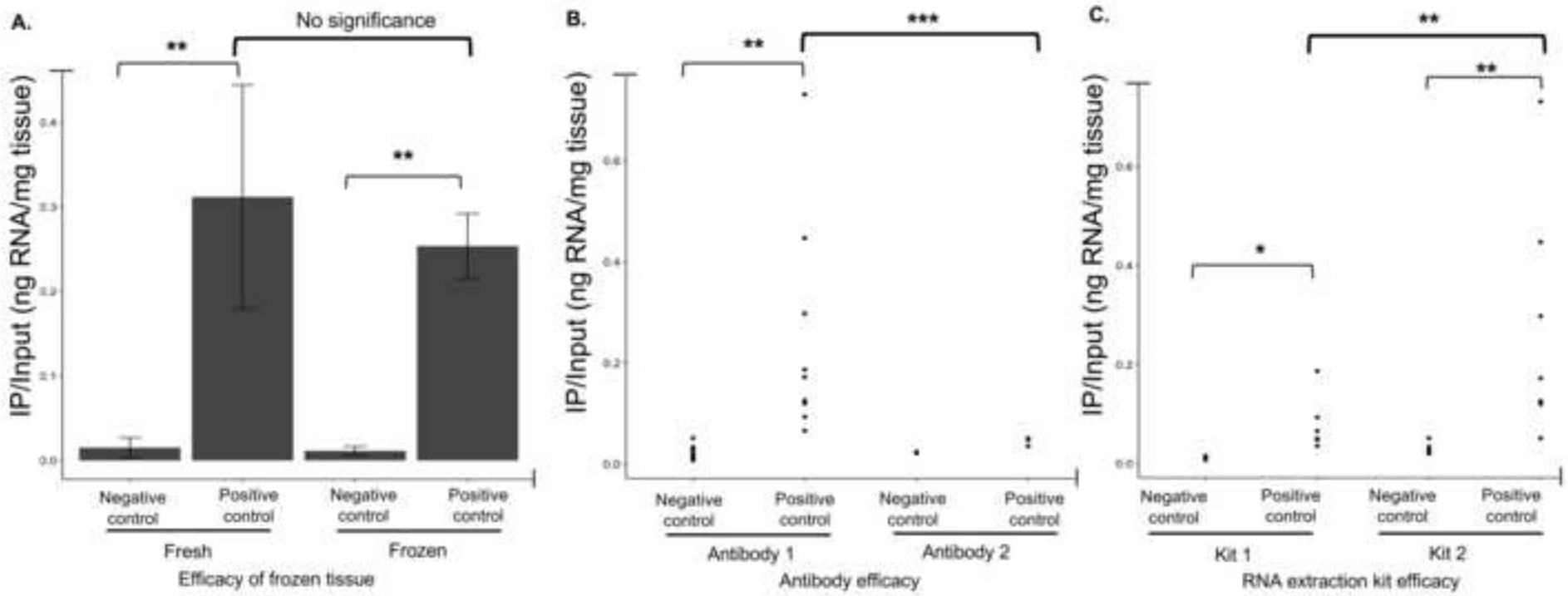


Figure 5

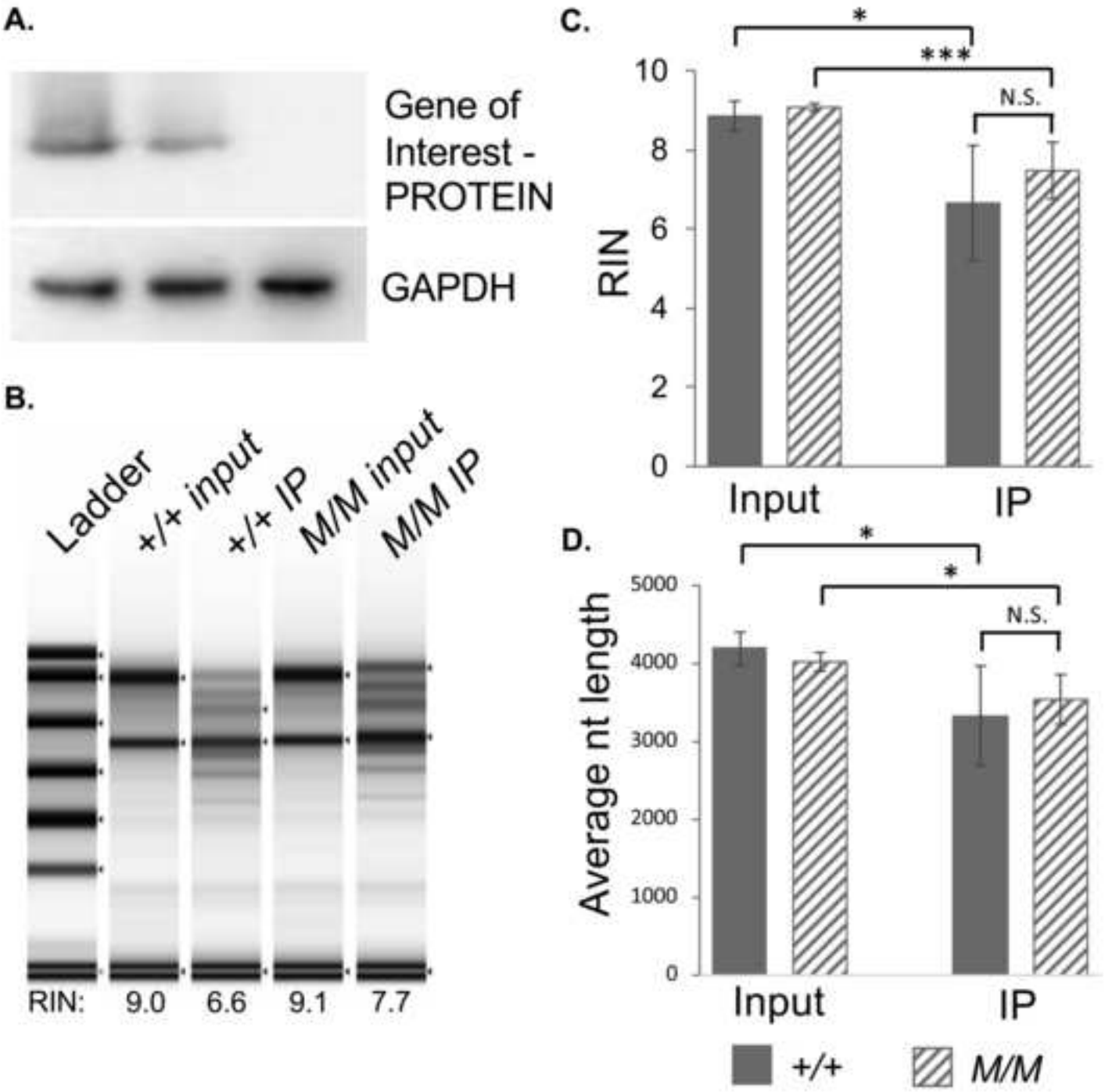
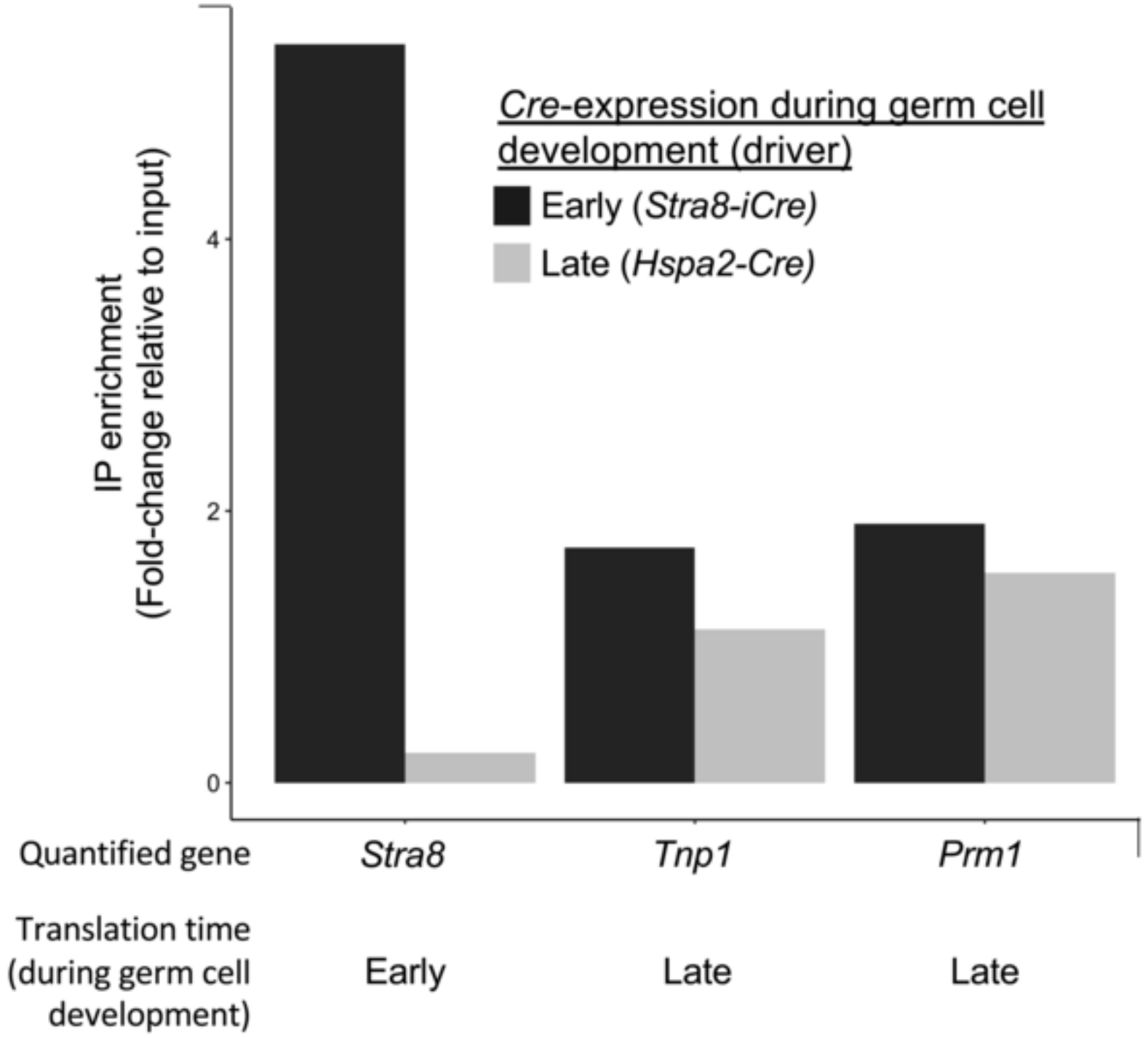


Figure 6



Name of Material/ Equipment	Company
1 mL mechanical pipette	Preference of researcher
1,4-Dithio-DL-threitol (8%	Alfa Aesar
1.7 mL or 2mL tubes	Preference of researcher
10 mL conical tubes	Preference of researcher
10 mL serological pippettes	Preference of researcher
10 uL mechanical pipette	Preference of researcher
20 uL mechanical pipette	Preference of researcher
200 uL mechanical pipette	Preference of researcher
5 mL serological pipettes	Preference of researcher
50 mL conical tubes	Preference of researcher
Anti-HA tag antibody	Abcam
Anti-HA tag antibody	Antibodies.com
B6.FVB-Tg(Stra8-icre)1Reb/LguJ Mice	The Jackson Laboratory
B6N.129-Rpl22tm1.1Psam/J Mice	The Jackson Laboratory
Benchtop centrifuge	Preference of researcher
C1000 Touch thermal cycler	BioRad
Centrifuge 5424 R	Eppendorf
Cyclohexamide	Sigma Aldrich
Dissection scissors	Preference of researcher
Dynabeads Protein G for Immunoprecipitation	Invitrogen by ThermoFisher Scientific
DynaMag-2 Magnet rack	Invitrogen by ThermoFisher Scientific
E.Z.N.A. Total RNA Kit 1	OMEGA
Heat block	Preference of researcher
Heparin	Sigma Aldrich
Magnesium Chloride (MgCl2)	Sigma Aldrich
Microdissection forceps	Preference of researcher
Microdissection scissors	Preference of researcher
MiRNeasy kit	Qiagen
NanoDrop One Microvolume UV-Vis Spectrophotometer with Wi-Fi	ThermoFisher Scientific
NP-40 Alternative - CAS 9016-45-9 - Calbiochem	Millipore Sigma
Pierce Protease Inhibitor Tablets, EDTA-free	ThermoFisher Scientific
Potassium (KCl)	Sigma Aldrich

RNase Inhibitor, Murine	New England BioLabs Inc.
SI vortex-genie 2	Scientific Industries
Tips for 1 mL mechanical pipette	Preference of researcher
Tips for 10 uL mechanical pipette	Preference of researcher
Tips for 20 uL mechanical pipette	Preference of researcher
Tips for 200 uL mechanical pipette	Preference of researcher
Tris Base (White Crystals or Crystalline Powder/Molecular Biology)	ThermoFisher Scientific
Tube Revolver / Rotator	ThermoFisher Scientific
VWR Powerpette Plus pipet controller	VWR

Catalog Number	Comments/Description
A15797	
ab18181	Antibody 1
A85278	Antibody 2
17490	Or mice carrying Cre driver of choice
11029	
184-1100	Or equivalent thermal cycler
5404000537	Or equivalent refrigerated centrifuge
C7698-1g	
10009D	
12321D	
6834-01	Kit 1
84020	
M9272-500g	
217004	Kit 2
ND-ONE-W	Or equivalent spectrophotometer
492016	
A32965	
P3911-2.5kg	

M0314	
SI-0236	Or equivalent benchtop vortex

BP152-500	
88881001	Or equivalent rotator
75856-450	Or equivalent pipette controller

To Whom It May Concern,

The authors wish to thank the reviewers for their thoughtful and thorough responses. The authors have made a best faith effort to address all listed concerns and feel they have generated a significantly improved manuscript. Responses to individual requests can be found italicized below. Changes to the manuscript are indicated as well. With the above changes, we hope the reviewers find the revised manuscript satisfactory and acceptable for publication.

Sincerely,
The Authors.

Editor's comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- *The authors have proof-read the manuscript thoroughly and have corrected any spelling or grammatical errors found.*

2. Authors and affiliations: Please provide an email address for each author in the manuscript.

- *Email addresses have been added for all authors.*

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Qiagen, Omega's kit IPed, Parafilm, etc.

- *The manuscript has been reviewed by the authors for the presence of commercial language and added references to the table of materials where necessary. Additionally, the authors would like to note that IPed is a common abbreviation for 'immunoprecipitated' and is such not commercial language. It has been defined in the manuscript in line 127.*

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

- *The manuscript has been revised to remove any personal pronouns in the text of the protocol.*

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

- *Care has been taken to move all non-imperative text to notes. Additionally, where possible, notes have been incorporated into the protocol itself.*

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

- *Additional details have been added throughout.*

7. 1.2: Please describe how genotyping is done.

- *Genotyping details for Cre and Rpl22-HA have been added to section 2 of the protocol. As the Authors wish to refrain from disclosing their gene of interest at this time, instructions have been provided for readers to genotype for their own particular gene of interest as they otherwise would. The authors have confirmed with the editors of the journal that this is acceptable.*

8. Please specify surgical tools used throughout the protocol.

- *Surgical tools are specified in Section 2 of the protocol.*

9. 2.1.2: How large are the incisions?

- *Incision size for sample collection has been specified in section 2 of the protocol.*

10. 2.3: Please describe how to extract DNA and perform PCR (including primers and conditions).

- *DNA extraction and PCR conditions have been added as relevant to section 2 of the protocol.*

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

- *Shorter protocol steps have been condensed into single, larger steps.*

12. Please include single line spacing between each numbered step or note in the protocol.

- *Spacing between notes and numbered steps has been corrected.*

13. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

- *The manuscript has been highlighted with the relevant sections for filming. For one segment (Section 3. Preparation of solutions), a note has been included in the script as it contains vital information for the success of the protocol. The highlighted note applies to ALL following steps.*

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

16. Figures 3-5: Please describe each panel of the figure in the manuscript.

- *A more detailed description of each figure panel has been added to the figure legends.*

17. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

- *Table of materials includes information on all relevant supplies, reagents and equipment utilized in the protocol and has been sorted alphabetically.*

18. References: Please do not abbreviate journal titles; use full journal name.

- *Full journal titles have been corrected.*

Reviewers' comments:
Reviewer #1:
This is a review of the written manuscript, not of the associated video.
In this manuscript, the authors Chukrallah et al. describe their method for using the RiboTag system to isolate ribosome-associated mRNAs from a specific cell type in mice, in this case spermatogonia and early spermatocytes (using the Stra8-Cre driver). The RibTag system is powerful and I expect to see it used extensively in the future. Thus, this manuscript is timely.
Overall, the manuscript is well written and easy to follow, with several very good parts (for example, I liked the overview of the RiboTag system in lines 81-92). Explanations are generally clear. Nonetheless, I have a number of questions I would like addressed before publication (see below).

Specific Comments:

1. Page 1, line 21 (Abstract) -- The Authors claim that the RiboTag system is "technically simple." I would argue that the system might seem daunting to a new researcher, especially one who has not worked before with genetically modified mice. Recommend changing to "technically straightforward."

- *The edit has been made and can be found on lines 22-23*

2. Page 3, lines 57 and 67 -- The Authors state that a gradient fractionator and ultracentrifuge are "somewhat specialized equipment." I suspect that most research facilities have several such devices on hand. Recommend changing to "somewhat time-consuming techniques," or words to that effect.

- *The edit has been made and can be found on line 77.*

3. Pages 3-4, lines 71-80 -- The discussion of FACS and its drawbacks is not germane to the current manuscript about RiboTag. Recommend shortening or deleting this paragraph.

- *The authors have removed this paragraph from the introduction.*

4. Page 4, lines 98-99 -- The sentence "compounded by issues with high immunoprecipitation background…" hints at but does not explain the issues. Please specify briefly what are the actual issues.

- *Additional language on page 4, lines 134-137, has been added to clarify the statements.*

5. Page 4, lines 100-102 -- The Authors state "the Snyder lab has optimized the protocol…." If the Snyder lab has published such optimization, please include the citation. If the work is

not yet published, please change this sentence to read "In this manuscript, the Snyder lab presents our optimizations for the protocol…," or words to that effect.

- *The edit has been made and can be found on line 138.*

6. Page 5, lines 109-110 (Ethics Statement) -- Please ensure that this statement meets the journal requirements.

- *The authors have confirmed with the editor that the statement meets the journal requirements.*

7. Page 5, line 113 -- The Authors highlight the "Male Infertility Mutation (Mim)" gene. I cannot find a gene of that name. Please list the accepted mouse gene name and abbreviation. If the Authors wish to keep the identity of this gene secret for some reason, please (1) indicate that reason in the text, and (2) discuss with the Editors how to comply with the journal's policy on proprietary information.

- *As the full phenotyping data has not yet been published, the authors wish to refrain from disclosing identifying information and have followed the editors' instructions for compliance with the journal's proprietary information policy. This reason is indicated in the text on line 158.*

8. Page 5, lines 113 and 126 -- Please describe what is meant by "trio matings." Why were these considered better than single pair matings?

- *Trio matings, a common technique wherein two females are paired with a single male to increase breeding efficiency, have been explained in the manuscript on lines 158-160.*

9. Page 5, line 117 -- The Authors cite (number 19) "Laboratory, T. J. (The Jackson Laboratory, JAX.org, 2019)." Please give a more specific citation.

- *The citation has been corrected.*

10. Page 5, line 117 -- Please describe briefly when and why it is desirable to pass the Cre driver in the male parent.

- *Common practice (and breeding recommendations from both the originator of the used Cre strain and the current commercial vendor) dictates confirmed male germ cell expressed Cres be transmitted via the male. This is likely a due to observations in other stem cell and germ cell Cres that parent of origin can impact transgene expression (EIIa-Cre, for example). As a result, to generate equivalent samples, Cre should be transmitted from either the maternal or paternal side for all experimental animals. Unfortunately, no detailed analysis of the impact of Cre transmission from the maternal side has been reported for the Cre used in this study. Text to this effect has been added on page 5, lines 173-213.*

11. Page 6, lines 131 and 135 -- The Authors state that "female pups were genotyped," and "resulting pups were screened to identify…." For the naïve reader, please specific what is meant by "genotyped" and "screened" (you can refer to your sections 2.3 and 2.4 for highlights).

- *The authors have added text to both instances referring the reader to section 2.3 for detailed genotyping instructions.*

12. Page 8, lines 197-198 -- The Authors state "With the same pipette… lysis buffer…." This line, though potentially useful in real life, is not necessary for this methods manuscript. Consider removing.

- *Based on instructions from the editor requesting additional technical detail for this portion of the protocol, the authors have left the statement in place. The authors have also found this particular step especially important to achieve efficient lysis from flash frozen and ground tissue.*

13. Page 9, line 228 -- The protocol states, "Rotate samples overnight at 4 °C." Is an overnight incubation necessary? What are the consequences of a shorter incubation (say, 2 hours)? What are the concerns for action of RNases for such a long incubation? Please give a brief justification for this incubation time.

- *The incubation time has not been optimized; it is possible that a shorter incubation may be sufficient or, similarly, that extending the incubation time will greatly improve the efficiency. The authors found an overnight incubation to be sufficient for their uses. A note to this effect has been added to page 11, lines 763-766.*

14. Page 12, lines 281-290 -- Please refer to and describe the results for Figure 6 in the Results section.

- *Figure 6 represents an example of the type of Cre-driver confirmation experiment suggested in the Discussion section. Additional text describing this experiment has been added there (lines 1037-1038) and clarification of the experiment added to the figure legend as well (lines 932-991).*

15. Figure 3 -- Please describe what is meant by "IP/Input (ng RNA)" in the figure legend.

- *IP/Input in ng RNA (the ratio of precipitated RNA to the sample's total RNA) has been explained in the legend of Figure 3.*

16. Figure 3 -- Please describe what is meant by the "+" and "-" symbols in the figure legend (possibly even indicate the mouse genotypes indicated by the symbols).

- *The mouse genotypes indicated by the symbols have been clarified in the legend for Figure 3.*

17. Figure 4 -- Please describe what is meant by "Negative control" and "Positive control" in the figure legend.

- *Negative control (samples wildtype for either Cre or Rpl22-HA) and positive control (samples carrying both) have been defined in the legend for Figure 4.*

18. Figure 4A -- How many replicates were performed for this experiment? Please indicate the number in the figure legend.

- *As some conditions were not fully balanced, the number of replicates for each condition have been indicated in the figure itself. Text to this effect has been added to the figure legend.*

19. Figure 4B -- "ABCAM" should probably be changed to "Abcam."

- *Due to the journal's requirements, commercial names cannot be included. As a result, the figure and its legends have been changed to reflect this.*

20. Figures 4B, C -- Please describe what are the dots in these figures (Individual replicate trials? Different mRNAs? What was actually tested here?)

- *Individual dots represent the ratio of IPed to input RNA for individual biological replicates. Text to this effect has been added to the figure legend.*

21. Figure 5A -- Please indicate the real name for this gene (see item 7, above).

- *As explained in item 7, above, the full phenotyping of this gene mutation has not yet been reported. As a result, the full name of this gene will not be disclosed.*

22. Figure 5A -- Is this a protein immunoblot/Western blot? Please indicate so in the figure legend.

- *The indicated panel is a Western blot, and this has been indicated in the corresponding figure legend.*

23. Figure 5A -- What antibody was used in this blot? What is its source? Please indicate so in the figure legend.

- *The antibody against the gene of interest protein is a validated custom in-house antibody, which has been noted in the figure legend. As a publication detailing the phenotype of this model is still in preparation, we are unable to provide further information. Similarly, though the GAPDH antibody is commercial, journal publication*

requirements do not allow us to provide commercial information such as source and number. For the reviewers' personal knowledge, however, the GAPDH antibody utilized is from Cell Signaling Technologies, product 2118L.

24. Figure 5C, D -- How many replicates? Please indicate the number in the figure legend.

- *Replicate number has been indicated in the figure legend.*

25. Figure 6 -- I do not understand this figure at all. Please indicate in the figure legend:

- *The author's apologize for the confusion regarding this figure, which is meant as an example of one potential way to confirm the correct Cre has been selected. Changes have been made to the primary figure as well as extensive additional experimental detail added to the figure legend, detail which can also be found below.*

a. What is meant by "Early," "Late," and "Late" (again?) translation times?

- *Translation time has been clarified as translation time as a function of germ cell development (ie. Early in germ cell development or late).*

b. What is meant by "Early germ cell Cre:Rpl22-HA" and "Late germ cell Cre:Rpl22-HA"?

- *The nature and specific names of both drivers have been indicated in the figure key.*

c. What genes were tested by qRT-PCR?

- *The names and translation times of all three genes are now indicated within the figure.*

d. What were the two different Cre-driver mice?

- *See above.*

e. Please explain what is meant by "achieved by both Cre drivers as expected…"? This sentence and an explanation should probably be added to the Results section.

- *A more in-depth explanation of these results has been in included in the figure legend.*

f. How was an experiment with "both Cre drivers" performed?

- *This statement has been removed and the experimental design more clearly explained in the figure legend.*

26. Page 15, line 379 -- With respect to Figure 4, please indicate which antibody (Abcam?) and which RNA extraction kit (Qiagen?) were the "winners."

- *Due to the journal's requirements, commercial names cannot be used in the manuscript. As a result, the figures and legends have been updated to reflect this. For the reviewer's personal knowledge, however, we found that the optimal kit was Qiagen's and the preferred antibody was Abcam's.*

Reviewer #2: Manuscript Summary:

This is a description of a protocol that I have used in my own laboratory and should be of interest to other investigators. The protocol allows for the determination of transcripts that are being actively translated in a specific cell type in a mixture of cell types. In general, the procedure is well described and should allow others to easily follow. It is well written and I have no comments that would improve the submission.

Major Concerns:
none
Minor Concerns:
none

Reviewer #3:

Manuscript Summary:
In this review, the Snyder lab does a nice job of describing the RiboTag method, and how it can be employed in different tissue- and cell-specific contexts.

Major Concerns:
The major flaw with this method, and this is not the authors' fault, is that it provides a measure of ribosome recruitment but NOT translational efficiency. It does not discriminate between 100 mRNAs having 1 ribosome each (which would likely result in very little protein, especially if the ribosomes were stalled) and 100 mRNAs with 20 ribosomes each (which would likely result in abundant protein). Polysome gradients would be able to discriminate, and if used together with RiboTag then could provide tissue specificity AND translational efficiency. RiboTag would also be unable to capture a significant change in translational efficiency, if a given condition results in a shift from 10% of an mRNA population having 10 ribosomes and 90% having 1 to 90% having 10 and 10% having 1. This significant limitation can be clearly inferred from: 1 - the original paper (Sanz et al, PNAS 2009) is rather suspect - they used protamines as their in vivo example, which MAY be the best example in the body of a regulated mRNA that goes from almost no ribosomes to lots of ribosomes, so they essentially picked the absolute best case scenario, and the differences still weren't that profound, AND, that PNAS paper was a direct submission from a fellow departmental colleague.. Why isn't that a MAJOR conflict of interest?! Was it even peer-reviewed by an unbiased reviewer(s) with any field-specific expertise? It's hard to believe that was ever even allowed...; 2 - despite being a 10 year-old technique, the authors

here state it's been used by "several other laboratories"... This reviewer is wondering, if this is such a useful technique, why isn't it being used much more widely? Please provide a rationale, because this review, if published, may encourage other labs, perhaps those naive to translational control, to perform this technique, which seems at best to identify cases of ribosome recruitment (e.g. from 0 to >1). This reviewer worries labs will mistakenly interpret differences as changes in "translational efficiency". Those conclusions would not be helpful, to anyone's field. Also, how are potential changes in mRNA abundance taken into account in tissues? If an mRNA changed in abundance but not ribosome recruitment, e.g. 10 mRNAs with 10 ribosomes each to 100 mRNAs with 10 ribosomes each would be categorized as an increase in translational efficiency if RNA-seq was performed...So, how would that be determined?

The authors would like the thank Reviewer #3 for their insightful commentary and nuanced concerns. Based on our understanding of the above, this reviewer's primary addressable concerns fall into the following four broad issues the responses to which can be found below:

- *This method "*provides a measure of ribosome recruitment but NOT translational efficiency"*. The authors fully agree with this statement and have made pains to make this clear. Throughout, RNAs isolated via the RiboTag method are referred to as ribosome-associated RNAs. Further, a statement specifically noting this protocol does not allow determination of translational efficiency has been added to the discussion (page 18, lines 1075-1090)*

- "Polysome gradients would be able to discriminate, and if used together with RiboTag then could provide tissue specificity AND translational efficiency." *The authors wholeheartedly agree the combination of polysome gradients and the RiboTag would be an extremely powerful way to determine translation efficiency in selected cell types. Ongoing efforts in our laboratory are, in fact, aimed at doing just that. For the benefit of the readers, we have included a statement suggesting this approach for questions regarding translation efficiency (page 18, lines 1076-1078).*

- "… if this is such a useful technique, why isn't it being used much more widely?" *Like many genetic-based techniques, the RiboTag system has inherent challenges to adoption. These challenges (in particular the complex breeding requirements that need to be considered) are in part the reason for this report. That being said, a number of groups have applied the RiboTag model to a diverse range of cell types, generally with good success. In order to make this clear, the above referenced text has been modified and additional primary reports describing application of RiboTag across a broader range of cell systems included (page 4, lines 130-131).*

- "how are potential changes in mRNA abundance taken into account in tissues?" *The authors fully agree this is a particularly challenging aspect of this model, especially as it relates to mutant phenotypes which may have unexpected gene expression changes. It is for this exact concern that one of the first steps of sample isolation is collection of input RNA (see Methods, 7.4). As discussed in the NOTE, this input RNA represents the*

best assessment of tissue-level RNA abundance changes. It should also be noted that for all assessed points (total RNA immunoprecipitation or specific target RNA quantification), values are reported as a fraction of the total RNA or message to correct for total RNA abundance changes. Statements to this effect have been added to the discussion (page 18, lines 1081-1093).

Minor Concerns:

Would introduce roles of RNA binding proteins and mRNA decay, which can regulate steady-state mRNA levels in addition to transcription

- *A sentence to this effect has been added to line 53-55 of the introduction.*

Translatome - is this the best term? What about "ribonome" (Mansfield and Keene, 2009)… translatome as a descriptor seems to suggest mRNAs are all translated

- *In order to stay consistent with previous publications focused on ribosome-associated RNAs, the authors have elected to use the phrase translatome (where appropriate) defined within the manuscript as 'RNAs being actively translated into protein' on page 2, lines 45-46.*

In many, many cell types, the transcriptome does not predict the proteome with great accuracy

- *We entirely agree with this statement and have stated so in the modified introduction (lines 43-45).*

Is "transcripts" the best term, as it means both unspliced pre-mRNAs AND mRNAs? Better to use mRNA or message if referring to mature spliced messages

- *Where appropriate the authors have changed the term transcript to mRNA.*

The authors are making the case that lacking rather routine instrumentation (e.g. fraction collectors, sonicators, ultracentrifuges, flow cytometers, etc) is an impediment to research… but practically speaking, don't most research institutions have at least some access to these? Realistically, would a lab be using RiboTag mice at a place that doesn't have these or access to these??

- *The authors acknowledge this and have reworded the introduction in a way that indicates that one of the benefits of the Ribotag method is that the lack of need for these instruments which makes running this protocol simpler and more widely accessible than many comparable methods.*

34 - RNA, or mRNA?

- *The edit has been made and can be found on lines 44 and 45.*

36 - what is meant by "static"? Might 'limited' or 'incomplete' be better adjectives? Most of these assays provide static measurements

- *The edit has been made and can be found on line 43.*

38 - replace "transcribed" with 'translated'

- *The edit has been made and can be found on line 45.*

40 - in some cases, yes… but not for all mRNAs or in all stages of development… would re-word

- *Modifications have made to the text to indicate that germ cells at various stages of development express select sets of mRNAs that are under translational regulation (see line 47).*

42-3 - termed local translation

- *Based on our understanding of the literature, the term local translation is normally applied to neural cells in which translation is regulated spatially, generally along an axon's length. In contrast to this, translation regulation in germ cells occurs temporally, as a function of developmental or signaling state. Given the focus of this method report is the use of the RiboTag model in male germ cells, the authors have chosen to use the more generic term "translational regulation" where appropriate. This term is commonly used by the large body of literature describing the phenomenon in male germ cells.*

52 – typo

- *The authors apologize but were unable to find the referenced typo. However, the entire manuscript has now undergone multiple rounds of grammar and spelling editing and we expect all typographical errors have been corrected.*

56 - what does "poised to translate" mean? How do polysome gradients inform about this? Couldn't mRNAs in the RNP fractions alternatively be 'poised to stay suppressed' or 'poised for degradation'?

- *This phrase has been edited to read "non-translating mRNAs".*

57 - remove "somewhat"

- *The edit has been made.*

57-8 - awkward sentence; and there are strategies to circumvent this (e.g. using reticulocyte lysates)

- *This sentence has been edited.*

71-3 - not sure what is specifically meant here… are the authors referring to making cell- or tissue-specific deletions, as in conditional KO mice?

- *This sentence has been edited to improve clarity.*

77 - "or reagents and protocols"?

- *The referenced paragraph has been removed at the request of another reviewer.*

93-5 - wondering how many publications have used the RiboTag method - it seems not that many, despite being published 10 years ago (here the authors state "several" labs have used it). Why so few, for a technique that's being touted as being so incredibly informative? Will results be informative for mRNAs other than Prm1?

- *The authors refer Reviewer #3 to the main points discussed above regarding model use in non-male germ cell systems.*

Reviewer #4:

Manuscript Summary:
The manuscript by Snyder et al, titled "RiboTag immunoprecipitation in the germ cells of the male mouse" describes a method for immunoprecipitation of HA-tagged Ribosomal Large protein 22 (Rpl22-HA) and purification of Rpl22-HA-associated mRNA from mouse male germ cells that conditionally express HA-tagged Rpl22 in the cell population of interest. The method also describes a breeding strategy that allows to create conditional Rpl22-HA knock-in mouse on the background of a desired mutant phenotype using Cre-recombination.

Major Concerns:
There are no major concerns. The manuscript is well-written and clear. The methodology is well-described and timely.

Minor Concerns:

1. The main minor concern is the lack of the positive control, where the subpopulation of the cells of interest is specifically purified (assuming the Stra8 expression, the spermatocytes subpopulation) from the testis of Rpl22-HA/Mim mutant and Rpl22-HA/Mim wt mice and the RNA moiety is shown to be the same as when immunoprecipitated from the total testis. Without this control, the question of the

specificity of the Rpl22-HA associated RNA species is still relevant. If the authors do not have the means for purification of spermatocytes or do not consider this control significant, the discussion should address this question.

- *The authors would like to thank Reviewer #4 for pointing out this shortcoming. Unfortunately, our laboratory is currently unable to isolate early spermatocytes (preleptotene and leptotene) which would represent the optimum positive control. That being said, we do find confidence in the newly presented data within Figure 6 showing dramatic enrichment of Stra8 with HA-IP from Stra8-iCre driven Rpl22-HA whole testes. Additional text describing this and other potential controls has been added to the discussion (page 17, lines 1037-1038).*

2. The figure 2 needs to be a bit clearer. Firstly, it was a bit unclear that two independent breeding trios were set up in parallel in the first round. Please use numbers (or letters) for Mim/Mim x Cre (1) and the Mim/Mim x Rpl22-HA/Rpl22-HA(2). Secondly, please make it clear on the diagram that the Cre mouse is hemizygous.

- *Numbers have been added to Figure 2 to differentiate generations and the generations have been further elaborated upon in the corresponding legend. Likewise, a note has been added both to Figure 2 and its legend indicating that the Cre mice are hemizygous.*

3. Please mention in which cell types the Stra8-iCre is active. Also, brief description to the Mim/Mim mutants (which germ cell types are affected and how) will help to understand the biology of the system better.

- *The Stra8-iCre is active in post-natal germ cells transitioning into and through very early meiosis (pre-leptotenes and leptotenes) This information has been added to lines 166-167. Unfortunately, as our gene of interest is still unpublished, we will not be disclosing detailed information about the model at this time. In brief, however, loss of this gene results in late post-meiotic germ cell loss culminating in complete male infertility. As a result, the cell population studied here develop prior to the morphological effects of the mutation. The authors would like to note that the mutant phenotype is the subject of a manuscript currently under preparation.*

4. The final step of breeding has a chance of 1/32 of producing male mice of the desired phenotype. Please give your estimate of how many trios you set up (and the average number of pups per litter) to produce enough males for statistically sound results? This practical information will help researchers to use your approach and plan their breeding strategy.

- *The authors exclude this information as it is highly system dependent. From the perspective of animal breeding, if your mutant allele has an unusual (often low) transmission rates or your selected Cre shows "leaky" behavior, aberrant allele transmission may occur. Additional factors that influence breeder numbers include*

strain background, housing conditions, time of year, and desired time frame for tissue collection. Further, to obtain statistically sound results, individual experimental power calculations are required. These calculations, by nature, require an estimate of system-specific variability. That being said, replicate numbers calculated for our system have been added to the figure legend to assist researchers in estimating variability for their system.

5. 2.2 lines 146-149: Do authors remove tunica albuginea? The removal of t. albuginea and rinsing the tubules before flash-freezing helps to minimise contamination with interstitial cells.

- *Given the highly cell-specific nature of the selected Cre-driver, the authors did not feel the need to remove the tunica albuginea. Eliminating this step has the added benefit of speeding tissue collection. Text to this effect has been added to the protocol.*

6. 4.1.1 line 184: remove "any and" (typo?)

- *The edit has been made.*

7. Line 192 and everywhere else, where buffer recipes are provided: Please provide final concentration of each constituent for all buffers. For example, in line 192 you add 10mL (!) of 1M DDT to 10mLs of HB buffer; clearly, mL referred to 1M DTT is a typo. Provision of final concentrations for all reagents, including DTT, avoids this confusion.

- *The authors thank Reviewer 4 for finding this error. It has been remedied and final concentrations have been added to each instance where reagents are generated.*

8. Line 210: is the 375µl of protein G beads referred to a 50% slurry? Please specify.

- *The concentration of the commercial protein G beads used is 30mg/mL. this information has been added to line 705.*

9. Lines 243-246: please provide information about the RNA purification kits that you used successfully. Please list the source of the DNase and the amount that was used to treat RNA purified from the 1mL of the original lysate.

- *Due to the publication requirements of the journal, commercial language such as kit names and the source of the DNase cannot be disclosed. For the reviewer's personal knowledge, however, Qiagen's miRNeasy kit was used and the DNase was also from Qiagen. The authors will be happy to provide this information using personal communications to any readers who request it. The amount of DNase (10uL/sample) has been added to line 810.*

10. Line 249: please list the concentration of bME that you used in this step.

- *The concentration (14.2M) has been added.*

11. Line 354: A brief explanation and a reference to the original article(s) of why it is important to keep the RiboTag allele away from the Cre would be beneficial to researchers who are not familiar with the Cre biology and the peculiarities of its behaviour in the male germ cells.

- *A brief explanation of Cre expression in germ cells has been added to the discussion, illustrating the importance of keeping the Cre separate from the Ribotag allele until the experimental generation. Briefly, as the Cre drives expression of Rpl22-HA in the germ cells, animals carrying both alleles will express the HA-tag in the germ cells. As a result, all subsequent generations descended from these HA-expressing germ cells will express HA globally.*