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Translating ribosome affinity purification (TRAP) for RNA isolation from endothelial cells in vivo

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Corresponding Author:	Bin Ren University of Alabama at Birmingham School of Medicine Birmingham, Alabama UNITED STATES
Corresponding Author's Institution:	University of Alabama at Birmingham School of Medicine
Corresponding Author E-Mail:	bren@uabmc.edu
Order of Authors:	Patrick Francis Moran
	Yichen Guo
	Rong Yuan
	Nicholas Barnekow
	Jordan Palmer
	Adam Beck
	Bin Ren
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1 **TITLE:**
2 **Translating Ribosome Affinity Purification (TRAP) for RNA Isolation from Endothelial Cells In**
3 **vivo**
4
5 **AUTHORS:**
6 Patrick Moran[1,2], Yichen Guo[3,4], Rong Yuan[1,3], Nicholas Barnekow[1], Jordan Palmer[2], Adam Beck[3],
7 Bin Ren[3,4,5]
8
9 [1]Blood Research Institute, Blood Center of Wisconsin, Milwaukee, Wisconsin
10 [2]Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin
11 [3]Departments of Surgery, The University of Alabama at Birmingham, Birmingham, Alabama
12 [4]Department of Biomedical Engineering, The University of Alabama at Birmingham,
13 Birmingham, Alabama
14 [5]GBS Program, Graduate School, The University of Alabama at Birmingham, Birmingham,
15 Alabama
16
17 pmoran@mcw.edu
18 yguo16@uab.edu
19 ryuan@uab.edu
20 barnekow137@gmail.com
21 jordan.palmer@marquette.edu
22 awbeck@uabmc.edu
23
24 **CORRESPONDING AUTHOR:**
25 Bin Ren
26 bren98@uab.edu
27
28 **KEYWORDS:**
29 Angiogenesis, arteriolar differentiation, vascular endothelial cells, translating ribosome affinity
30 purification, RNA extraction, real time qPCR
31
32 **SUMMARY:**
33 We present an approach to purify ribosome-bound mRNA from vascular endothelial cells (ECs)
34 directly in mouse brain, lung and heart tissues via EC-specific genetic tag of enhanced green
35 fluorescence protein (EGFP)in ribosomes in combination with RNA purification.
36
37 **ABSTRACT:**
38 Many studies have been limited to using in vitro cellular assays and whole tissues or isolating of
39 specific cell types from animals for in vitro analysis of transcriptome and gene expression by
40 qPCR and RNA sequencing. Comprehensive transcriptome and gene expression analysis of
41 specific cell types in complex tissues and organs will be critical to understand cellular and
42 molecular mechanisms by which genes are regulated and their association with tissue
43 homeostasis and organ functions. In this article, we demonstrate the methodology for isolation
44 of ribosome-bound RNA directly in vivo in the vascular endothelia of animal lungs as an

example. The specific materials and procedures for tissue processing and RNA purification will be described, including the assessment of RNA quality and yield as well as real time qPCR for arteriogenic gene assays. This approach, known as translating ribosome affinity purification (TRAP) technique, can be utilized for characterization of gene expression and transcriptome analysis of certain cell types directly in vivo in any specific type in complex tissues.

INTRODUCTION:

In complex tissues such as the mammalian brain, heart and lung, the high levels of cellular heterogeneity complicate the analysis of gene expression data derived from whole tissue samples. To observe gene expression profiles in a particular cell type in vivo, a new methodology has been developed recently, which allows the interrogation of the entire translated mRNA complement of any genetically defined cell type. This methodology is known as the translating ribosome affinity purification (TRAP) technique[1,2]. It is a useful tool to study endothelial cell biology and angiogenesis when combined with genetically manipulating other angiogenesis-associated genes in animals.

We have shown that angiogenic PKD-1 signaling and the transcription of angiogenic gene CD36 are critical for endothelial cell (EC) differentiation and functional angiogenesis[3-6]. To determine molecular mechanisms of angiogenic and metabolic signaling in gene transcription and EC transdifferentiation, we have created genetically engineered TRAP mice with specifically deleted angiogenic genes on the basis of TRAP technique[1,2]. Furthermore, in our TRAP animals, not only do they have *pkd-1 or cd36 gene* deficiency in the vascular endothelia or global deletion of *cd36* gene, but an enhanced green fluorescence protein (EGFP) is also genetically tagged onto EC's translating ribosomes. TRAP permits affinity purification of ribosome-bound mRNA directly from the vascular endothelia of targeted tissues, enabling the analysis of gene expression and identification of new transcriptomes that are associated with EC differentiation and angiogenesis directly under in vivo conditions. We have successfully isolated ribosome-bound RNA from the endothelia in these genetically engineered animals. The purified RNA can be used for further characterization of angiogenic or arteriogenic genes in the regulation of EC differentiation and functions. This protocol provides a step-by-step guide to implement the TRAP approach for the isolation of mRNA in ECs directly in vivo.

PROTOCOL

For animal experiments, all methods described here have been approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

1. **Prepare reagents**

1.1. Prepare lysis buffer to concentrations of 10 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 100 μg/mL cycloheximide, protease inhibitors, and recombinant RNase inhibitors to concentrations as described below.

89 1.1.1. Add following reagents to 500 mL of RNase-free deionized water: 1.19 g of HEPES, 5.59
90 g of KCl, 0.24 g of $MgCl_2$, 35 mg of DTT, 0.5 mL of cycloheximide, and NaOH as needed until pH
91 7.4, EDTA-free protease inhibitors (one mini tablet per 10 mL) and RNase inhibitor (10 µL/mL).
92
93 1.1.2. Store in a 4 °C fridge for up to 1 month.
94
95 1.2. Prepare a high-salt polysome wash buffer to concentrations of 10 mM HEPES, pH 7.4,
96 350 mM KCl, 5 mM $MgCl_2$, 1% vol/vol CA-630, 0.5 mM DTT, and 100 µg/mL cycloheximide.
97
98 1.2.1. Add following reagents to 500 mL of RNase-free deionized water: 1.19 g of HEPES, 13.05
99 g of KCl, 0.24 g of $MgCl_{2,}$ 5 mL of nonionic, non-denaturing detergent, 5 of 7.7 mg tubes of DTT,
100 and 0.5 mL of cycloheximide, and NaOH as needed until pH 7.4.
101
102 1.2.2. Store in 4 °C fridge for up to 1 month.
103
104 1.3. Bind anti-GFP antibody to Protein G magnetic beads prior to starting experiment.
105
106 1.3.1. Add 10 µg of anti-GFP antibody diluted in 200 µL of PBS to Protein G beads.
107
108 1.3.2. Incubate with end over end rotation for 10 minutes at room temperature.
109
110 1.3.3. Place the beads on a magnetic rack and remove the supernatant.
111
112 1.3.4. Suspend the beads in 200 µL of PBS and store in 4 °C fridge for up to 1 week.
113
114 1.4. Prepare ice-cold PBS with 100 µg/mL cycloheximide.
115
116 1.4.1. Add 1 volume of cycloheximide solution (100 mg/mL) to 99 volumes of ice-cold PBS.
117
118 2. **Isolate and lyse desired tissues**
119
120 2.1. Euthanize mice by IP injection of ketamine (500 mg/kg/body weight) and xylazine (10
121 mg/kg/body weight) and isolate desired tissues (i.e., heart, lung). Immediately proceed to next
122 step.
123
124 2.2. Place desired tissues into 500 µL of ice-cold PBS with 100 µg/mL cycloheximide.
125
126 2.3. Mince tissue into a cell suspension with a motor-driven homogenizer or a small-
127 clearance glass homogenizer. If using a motor-driven homogenizer, limit homogenization to less
128 than 1 minute at low frequency (<15,000 Hz) to avoid RNA denaturation.
129

2.4. Suspend cell pellet in 200 μL of lysis buffer by pipetting and redrawing up buffer several times. Further homogenize cell suspension with 10 strokes in a small-clearance glass homogenizer or for 15 seconds at low frequency (<15,000 Hz) in a motor driven homogenizer.

2.5. Centrifuge homogenates for 10 min at 2,000 x *g* at 4 °C to pellet nuclei and large cell debris, and keep the supernatant.

2.6. Add nonionic, non-denaturing detergent to 1% vol/vol and DHPC to 30 mM to the supernatant. Incubate on ice for 5 min.

2.7. Centrifuge lysate for 10 min at 16,000 x *g* to pellet insoluble material. Transfer and keep 15% of clear lysate as input for future steps.

3. **Isolate ribosome/mRNA complexes**

3.1. Add 50 μL of antibody-bound beads to cell-lysate supernatant and incubate mixture at 4 °C with end-over-end rotation for 30 min. This is where the anti-GFP antibodies will bind the GFP-tagged ribosomes, allowing us to further isolate the RNA from these ribosomes.

3.2. Collect beads on a magnetic rack and wash 5 times with high-salt polysome wash buffer.

3.2.1. Draw up and discard liquid once beads have collected on the side of the tube. Then pipette and redraw up 200 μL of high-salt polysome wash buffer several times. Repeat this step 5 times and discard all buffer following final repetition. Immediately proceed to next step.

4. **Isolate mRNA**

4.1. Place beads in RLT buffer. The following steps are taken directly from the RNeasy mini kit protocol and were not expanded on in any way.

CAUTION: RLT buffer contains guanidine salts; do NOT mix with bleach.

4.2. Centrifuge lysate for 3 min at full speed 13,000 rpm or 16,000 g at 4 °C. Carefully remove supernatant of 350 μL by pipetting and transfer it to a new microfuge tube. **Use only this supernatant (lysate) in subsequent steps.**

4.3. Add an equal volume of 70% ethanol into the microfuge tube.

4.4. Transfer up to 700 μL of the sample, including any precipitate that may have formed, to a spin column placed in a 2 mL collection tube. Close the lid gently and centrifuge for 15 s at ≥8,000 x *g* to wash the spin column membrane. Discard the flow-through.

172 4.5. Add 350 μL of buffer RW1 to the spin column. Close the lid gently and centrifuge for 15 s
173 at ≥8,000 x *g* to wash the spin column membrane. Discard the flow-through and reuse the
174 collection tube in next step.
175
176 CAUTION: Buffer RW1 contains guanidine salts; do NOT mix with bleach.
177
178 4.6. Add 350 μL of buffer RW1 to the spin column. Close the lid gently and centrifuge for 15 s
179 at ≥8,000 x *g.* Discard the flow-through.
180
181 4.7. Add 500 μL of buffer RPE to the spin column. Close the lid gently and centrifuge for 15 s
182 at ≥8,000 x *g* to wash the spin column membrane. Discard the flow-through.
183
184 4.8. Add 500 μL of buffer RPE to the spin column. Close the lid gently and centrifuge for 2
185 min at ≥8,000 x *g* to wash the spin column membrane. Then **carefully remove the spin column**
186 **from the collection tube, ensuring that the column does not contact the flow-through.**
187
188 4.9. Place the spin column in a new 2 mL collection tube and discard the old tube with the
189 flow-through. Close the lid gently and centrifuge at full speed for 1 min to remove residual
190 buffer.
191
192 4.10. Place the spin column in a new 1.5 mL collection tube. Add 30-50 μL of RNase-free
193 water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at
194 ≥8,000 x *g* to elute the RNA.
195
196 4.11. If expected RNA yield is >30 μg, repeat step 4.10 with another 30-50 μL of RNase-free
197 water, or using elute from Step 4.10 (if high [RNA] is required). Reuse collection tube from Step
198 4.10.
199
200 4.12. Use purified RNA for downstream analysis including RNA-sequencing or real time
201 quantitative PCR or store RNA dissolved in RNase-free H_2O at -80 °C for up to 1 year.
202
203 **REPRESENTATIVE RESULTS:**
204
205 Our previous studies[4,7] suggest that CD36 may function as a switch for arteriolar differentiation
206 and capillary arterialization via the LPA/PKD-1 signaling pathway. To study whether the
207 LPA/PKD-1-CD36 signaling axis is essential for arteriogenesis in vivo, we have established the
208 novel TRAP lines that not only have global *cd36* deficiency or endothelial-specific-*cd36-* or *pkd-*
209 *1*-deficiency but also permit selective isolation of ribosome-bound RNA from cre-marked cell
210 lineages by GFP, and are useful as a cre-activated fluorescent reporter [2].
211
212 By performing genotyping, we observed that *cd36* gene was deleted globally or in the vascular
213 endothelia for endothelial-specific *cd36* null mice (data not shown), and *pkd-1* gene was also
214 deleted in the vascular endothelia. **Figure 1** is a representative result showing the created
215 global *cd36* TRAP or endothelial-specific *pkd-1* TRAP mouse line. Using immunofluorescence

216 microscopy, we demonstrated that an enhanced GFP is genetically tagged onto the ribosomes
217 of the endothelial cells in vivo (**Figure 2**). We then isolated ribosome bound mRNA directly in
218 vivo and successfully obtained quality RNA as shown by measurement of the ratio of 260 nm
219 and 230 nm (**Figure 3**). Further analysis using real-time qPCR demonstrated that the expression
220 of certain arteriogenic genes were upregulated in the lung endothelia of *cd36* null mice (**Figure**
221 **4**), indicating that the isolated RNA directly in vivo in the vascular endothelia using the TRAP
222 technology are qualified for downstream studies. These studies include analysis of gene
223 expression at mRNA levels and identification of novel transcriptomes under physiological and
224 pathological conditions, which are essential for understanding the regulation of vascular
225 endothelial cell differentiation and functional angiogenesis.
226
227 **Figure 1: An example of genotyping for genetically engineered TRAP mice.** Representative
228 results for genotyping of global *cd36* null TRAP mice or conditional tissue-specific *pkd-1* null
229 TRAP mice. *VEC-cre* transgenic mice express Cre recombinase under the control of a *Cdh5*
230 promoter B6; 129-Tg (Cdh5-cre)1Spe/J mice were bread with B6.129S4-*Gt(ROSA)26Sor tm1*$^{(CAG-}$
231 $^{EGFP/Rpl10a,-birA)Wtp}$/J, and further with B6.129S1^{tm1Mfe}-*cd36* /J or *pkd-1*$^{loxP/loxP}$. The double mutant
232 *cd36* TRAP **(A)** and *pkd-1* TRAP **(B)** mice were obtained, in which an enhanced GFP is tagged
233 onto L_{10a} of the ribosome in vascular endothelial cells, and *cd36* gene is deleted globally and
234 *pkd-1* gene specifically in the vascular endothelia. Mouse tails were collected for DNA
235 extraction using a kit and based on the instruction from the manufacturer, and DNA in all
236 samples was amplified by polymerase chain reaction (PCR), and then evaluated by 1-2%
237 agarose-gel electrophoresis. Photographs are the agarose gel image showing the results of
238 amplification of *cd36* or *pkd-1* mutants with/without TRAP or wild type (WT) mice. Mouse
239 genotype panel **A**: lane 1, cd36$^{-/-}$;TRAP$^{+/-}$; lane 2, TRAP$^{+/+}$; lane 3, cd36$^{-/-}$;TRAP$^{+/+}$;Cdh5$^{+/-}$; lane 4,
240 TRAP$^{+/+}$;Cdh5$^{+/-}$; lane 5, cd36$^{-/-}$;TRAP$^{+/+}$;Cdh5$^{+/-}$; lane 6, cd36$^{-/-}$;TRAP$^{+/-}$;Cdh5$^{+/-}$; lane 7,
241 TRAP$^{+/+}$;Cdh5$^{+/-}$; lane 8, TRAP$^{+/-}$; lane 9, DNA ladder. Mouse genotype panel **B**: lane 1, pkd-1$^{fl/-}$;
242 TRAP$^{+/-}$; Cdh5$^{+/-}$; lane 2, pkd-1$^{fl/fl}$; TRAP$^{+/+}$; Cdh5$^{+/-}$; lane 3, pkd-1$^{fl/-}$; TRAP$^{+/+}$; lane 4, pkd-1$^{fl/fl}$;
243 TRAP$^{+/+}$; Cdh5$^{+/-}$; lane 5, pkd-1$^{fl/fl}$; TRAP$^{+/+}$; Cdh5$^{+/-}$; lane 6, pkd-1$^{fl/-}$; lane 7, DNA ladder.
244
245 **Figure 2: An example of endothelial-specific enhanced GFP expression under fluorescence**
246 **microscope.** Blood vascular endothelia in the lung tissues of *cd*36 knockout TRAP mice were
247 EGFP positive (green color, upper panel) under immunofluorescence microscope. Missing the
248 primary GFP antibody was used as a negative control (bottom panel). Mouse tissues were co-
249 stained by using GFP and CD31 antibodies with appropriate secondary fluorescence antibodies
250 (red color). Representative images acquired by using a fluorescence microscopy imaging
251 system. Bar = 200 µm.
252
253 **Figure 3: The quality and quantity of ribosomal-bound mRNA of endothelial cells purified and**
254 **directly extracted from tissues of TRAP mice.** An example for quality and concentration of
255 purified RNA from lung tissues in a *cd36* knock out TRAP mouse. A spectrophotometer was used
256 for assessment of the amount and purity of extracted RNA. As shown in this figure, the
257 concentration of RNA is 51.2 ng/µL. The ratio of absorbance at 260 nm and 280 nm is 1.87
258 whereas the ratio of 260 nm and 230 nm is 2.40, indicating the purity of the extracted RNA
259 samples.

Figure 4: An example of expression of angiogenic genes and Notch ligands in the ribosome-bound RNA of endothelial cells by real time qPCR assays. The isolated mRNA from the endothelial ribosome of the lung in the TRAP control and EC-specific *cd36* deficient TRAP mice was subjected to real-time qPCR assays, using primers purchased from a biotech company including Hey2, ephrin B2, and delta like ligand 4 (DLL4). The house keeping genes PPIA was used for normalization. The student *t*-test was used for statistical analysis. *$P < 0.05$; **$P < 0.01$.

DISCUSSION:

Angiogenesis is a complex multistep process, in which EC-specific angiogenic gene transcription and expression play an essential role in EC differentiation and angiogenic reprogramming[3,4]. To overcome the barriers from the cellular diversity and architectural complexity for better understanding the function of the mammalian vascular system at a molecular level in vivo, we have created EC-specific TRAP mice, accompanied by EC-specific *cd36*, EC-specific *pkd-1* deficiency or global *cd36* deficiency by using a versatile floxed TRAP mouse model or EGFP-TRAP generated in the Pu laboratory[2] in combination with other genetically engineered mouse lines. This will allow the examination of the entire translated mRNA complement of vascular ECs from intact tissues in vivo under EC-specific in *pkd-1* or global deficiency in *cd36* gene expression[8], which is critical for investigation into gene transcription associated with physiological and pathological angiogenesis[4,7,9,10]. Consistent to other studies[1,2], our approach to isolation of EC-specific mRNA does not need tissue fixation, dissociation of tissues, or isolation of single-cells from tissues and thus avoids the potential artifacts that result from these treatments. We were also able to perform TRAP purifications and extract quality ribosome-bound mRNA from the frozen tissues. Additionally, what was purified is the translated mRNA content of ECs directly in vivo, which will better represent the protein content compared to using the total RNA for gene expression profile. Moreover, the TRAP transgene genetically labels the ECs with EGFP, also allowing not only for extraction of ribosome-bound mRNA but also for visualization in immunohistochemical or electrophysiological studies.

However, the approach showed low RNA yields, especially with purified mRNA from heart tissues or from previously frozen tissues. We thus need optimize the conditions to increase yields. However, we observed in EC-specific *cd36* deficient mice, the levels of ephrin B2 and DLL4 were significantly increased in both lung (**Figure 4**) and heart (data not shown) endothelia when compared with the control. These results were consistent with our previous in vitro studies[3 4], which suggests that the RNA quality is sufficient for downstream analysis. The yield was low possibly due to the stringent conditions. To overcome this limitation and improve yield, it is critical to set up an RNase-free work zone and decontaminate work surfaces and equipment that may get contaminated with RNase and change gloves frequently in order to extract quality RNA. It is also critical to find suitable concentrations of GFP antibodies in the affinity matrix and use appropriate concentrations of RNase inhibitor in the tissue lysis buffer. Use of RNase-free plastic ware and reagents is beneficial for RNA extraction from endothelial ribosomes of the targeted tissues.

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310
311 **DISCLOSURES:**
312 The authors declare that they have no conflict of interest.
313
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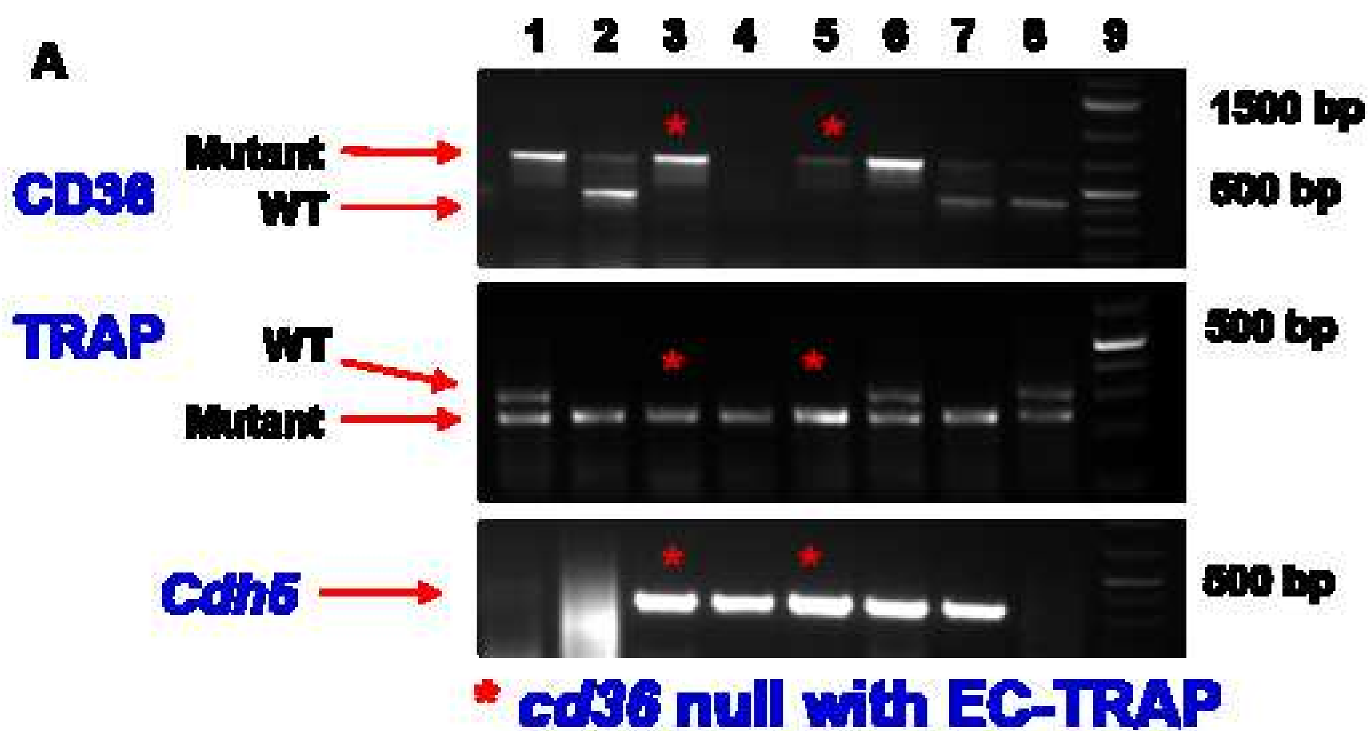

B

1 2 3 4 5 6 7

PKD-1

Mutant

WT

500 bp

TRAP

WT

Mutant

500 bp

Cdh5

500 bp

* EC-specific *pkd-1* null

** EC-specific *pkd-1* null TRAP

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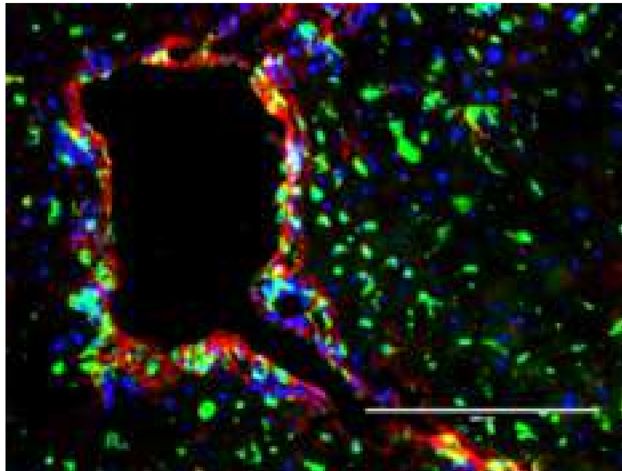

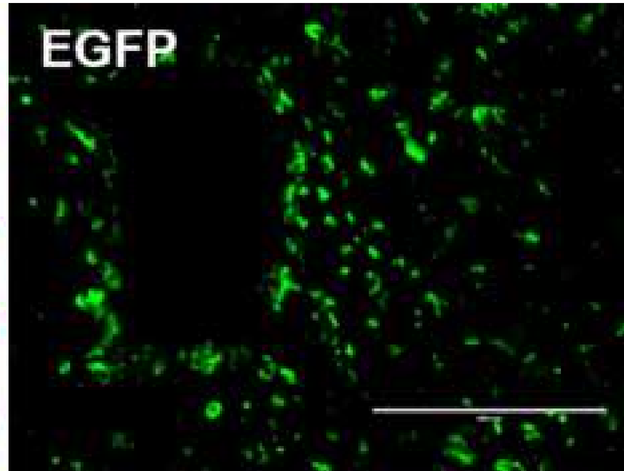

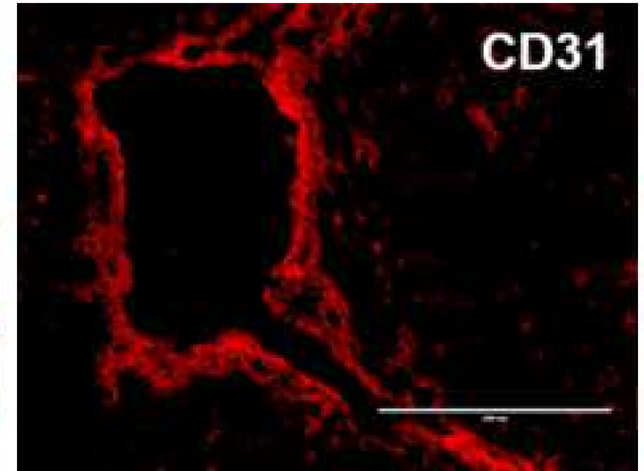

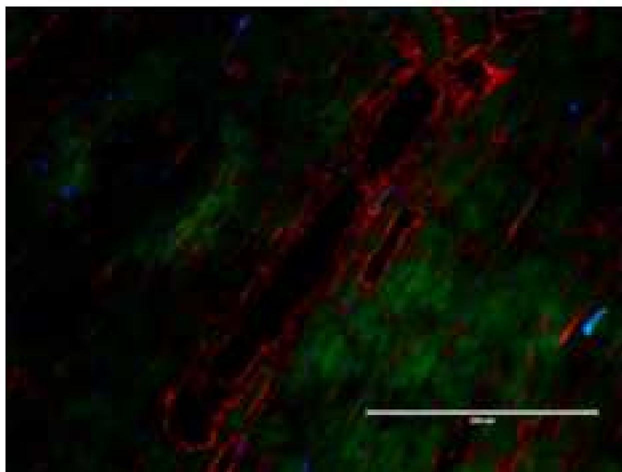

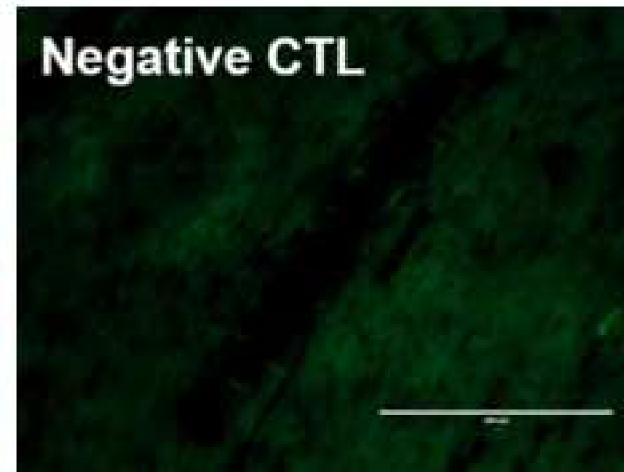

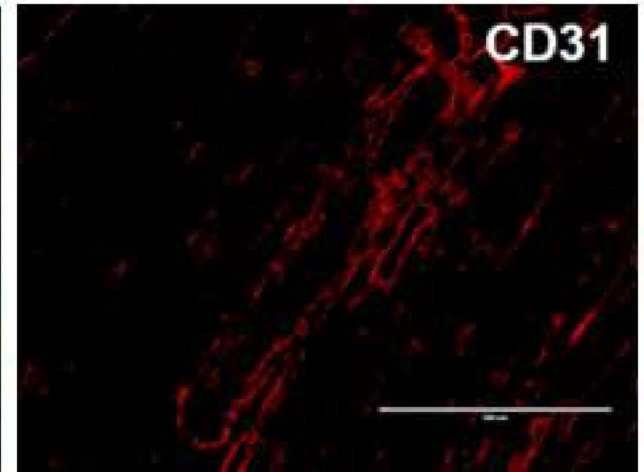

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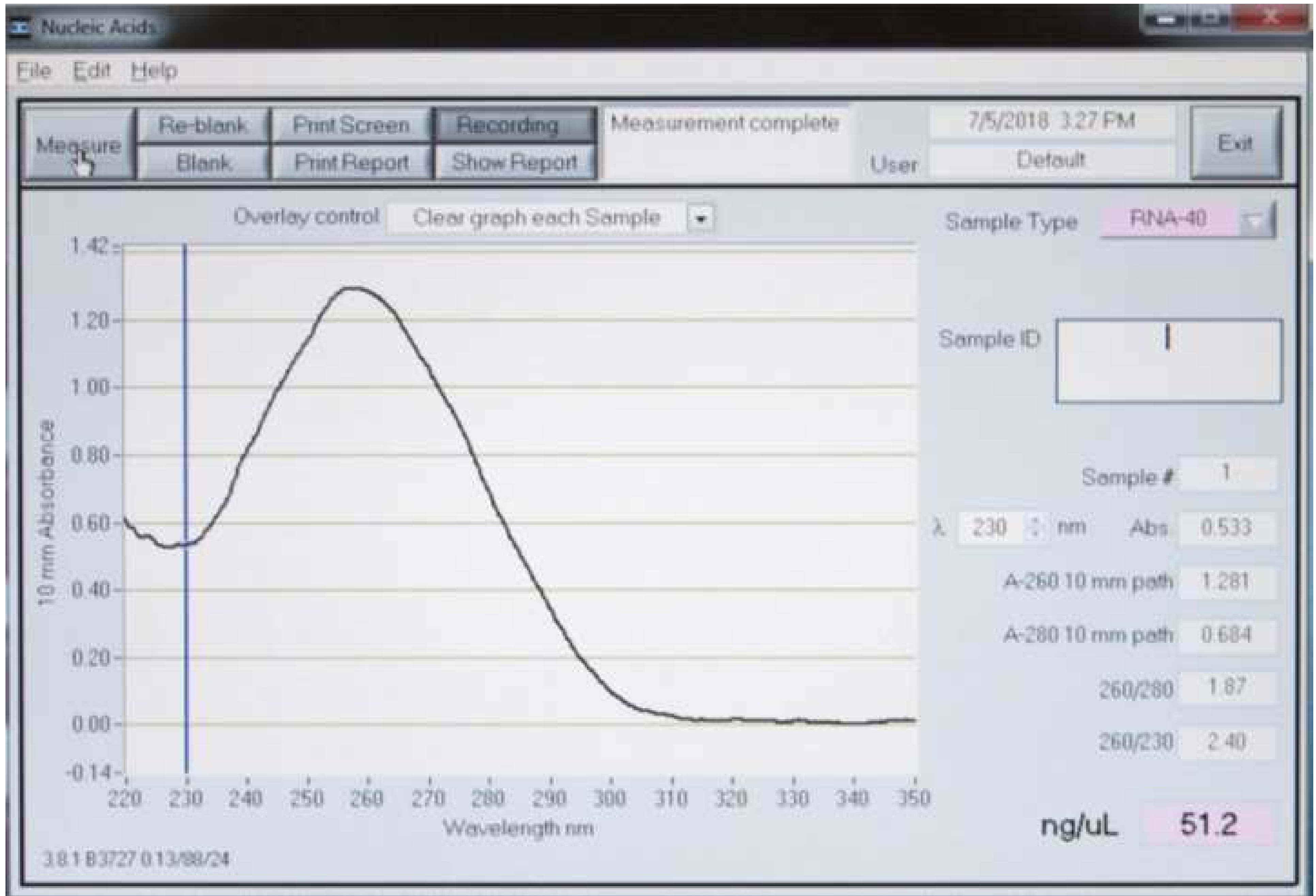

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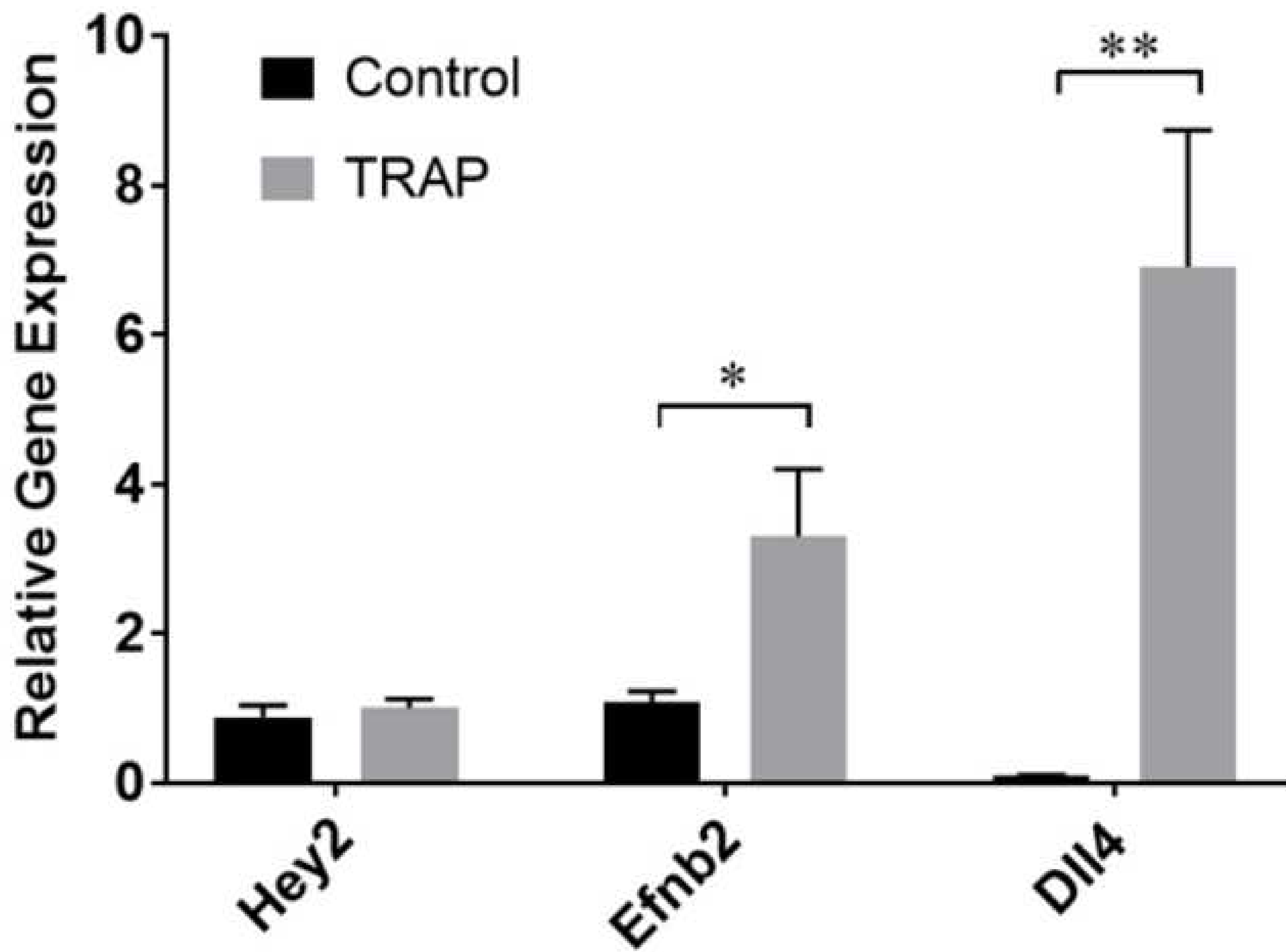

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2100 Electrophoresis Bioanalyzer with Nanochips and Picochips	Agilent	G2939AA, 5067-1511 & 5067-1513	
Cell scrapers	Sarstedt	83.1832	
Homogenizers	Fisher Scientific	K8855100020	
Magnet (Dynamag-2)	Invitrogen	123-21D	Will depend on purification scale; samples in 1.5-mL tubes can be conc
Minicentrifuge	Fisher Scientific	05-090-100	
NanoDrop 2000C spectrophotometer	Thermo Scientific	ND-2000C	
Refrigerated centrifuge	Eppendorf	5430R	with rotor for 1.5-mL microcentrifuge tubes
RNase-free 1.5mL microcentrifuge tubes	Applied Biosystem	AM12450	
Rnase-free 50-mL conical tubes	Applied Biosystem	AM12501	
RNase-free 1000-µl filter tips	Rainin	RT-1000F	
RNase-free 200-µl filter tips	Rainin	RT-200F	
RNase-free 20-µl filter tips	Rainin	RT-20F	
Rotor for homogenizers	Yamato	LT-400D	
Tube rotator, Labquake brand	Thermo Fisher	13-687-12Q	

entrated on a DynaMag-2

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CORRESPONDING AUTHOR

Name:	Patrick Moran
Department:	Medicine
Institution:	Medical College of Wisconsin
Title:	Medical Student

Signature: Date: 12/28/2018

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Division of Vascular Surgery & Endovascular Therapy
Department of Surgery, University of Alabama at Birmingham

Refer to JoVE59624R1

Phillip Steindel, Ph.D.
Review Editor
JoVE
617.674.1888

Dear Dr Steindel,

We thank your constructive comments. We have carefully revised this manuscript based on the comments from you and reviewers. As outlined at the end of this manuscript, we addressed all the concerns. Your consideration is greatly appreciated.

Sincerely,

MD, PhD, FAHA

Associate Professor

E-mail: bren98@uab.edu

Ren Lab Website: https://labs.uab.edu/bren98/

Laboratory of Molecular and Vascular Pathobiology (LMVP)
Department of Surgery & UAB Comprehensive Cancer Center
The University of Alabama at Birmingham
BDB 502 1720 2ND AVE S BIRMINGHAM AL 35294-0012

1
2 **Editorial and production comments:**
3 General:
4 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are
5 no spelling or grammar issues.
6 *Answer: Done*.
7
8 2. You have indicated 'Open' access in your Author License Agreement but 'Standard' in
9 Editorial Manager; please indicate which access you would like. If necessary, please print and
10 sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA
11 with the manuscript files to your Editorial Manager account.
12 *Answer: We'd like to choose Standard access.*
13
14 3. Please revise section 4 of the protocol to avoid overlap with previous publications.
15 *Answer: These steps of the protocol were taken from the RNeasy mini kit. We did not expand on*
16 *these particular steps of the protocol in any way, but we can add a reference if needed.*
17
18 4. Please include at least 6 key words or phrases.
19 *Answer: Done.*
20
21 5. Please reduce the length of the short abstract; it should be 10-50 words.
22 *Answer: 48 words.*
23
24 6. For in-text formatting, corresponding reference numbers should appear as numbered
25 superscripts (without parentheses) after the appropriate statement(s).
26 *Answer: done.*
27
28 7. JoVE cannot publish manuscripts containing commercial language. This includes trademark
29 symbols (™), registered symbols (®), and company names before an instrument or reagent.
30 Please limit the use of commercial language from your manuscript and use generic terms
31 instead. All commercial products should be sufficiently referenced in the Table of Materials and
32 Reagents.
33 For example: Nanodrop, Dynal, Igepal, EVOS, Qiagen, RNase-Zap
34 *Answer: Removed.*
35
36 Introduction:
37 1. Do you have a reference for the creation of the TRAP mice? It's unclear here.
38 *Answer: We have cited relevant references, and now added a phrase "on the basis of TRAP*
39 *technique" in a key place with proper citations to make it clear.*
40
41 Protocol:
42 1. Please add more details to your protocol steps. Please ensure you answer the "how"
43 question, i.e., how is the step performed? Alternatively, add references to published material
44 specifying how to perform the protocol action. If revisions cause a step to have more than 2-3

45 actions and 4 sentences per step, please split into separate steps or substeps.
46
47 Specific Protocol steps:
48 1. 1.3: Please explain further how to do this step, or include a reference.
49 2. Please rewrite the 'Materials and reagents preparation' as numbered steps in the imperative,
50 as in the rest of the protocol.
51 3. 2.1: Please provide the euthanization method. Please explain further how to isolate the
52 desired tissues or provide a reference.
53 4. 2.2: What volume of PBS?
54 5. 2.3: Please provide more details about homogenization.
55 6. 2.4: What volume of lysis buffer? How do you suspend pellet, exactly?
56 7. 3.1: What volume of beads?
57 8. 3.2: Please provide more details on washing and collection.
58 *Answers: Done*
59
60 Results:
61 1. Please provide a reference for genotyping, immunofluorescence, and qPCR.
62 *Answer: These are all original data, which are not published in any journal. The references are*
63 *thus not needed. The original figures do not need copyright permission.*
64
65 Figures:
66 1. If needed, please obtain explicit copyright permission to reuse any figures from a previous
67 publication. Explicit permission can be expressed in the form of a letter from the editor or a link
68 to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file
69 to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend,
70 i.e. "This figure has been modified from [citation]."
71
72 2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff
73 file (4 files total). Please remove 'Figure 1', etc. from the figures themselves.
74 3. Figure 1: Please label all lanes.
75 4. Figure 2: Please explain the colors in the left panel.
76 5. Figure 4: Please explain the error bars in the legend. Please also explain the stars (including
77 the statistical test used).
78 *Answer: Thanks for your suggestion. I have revised them based on your comments.*
79
80 Discussion:
81 1. As we are a methods journal, please revise the Discussion to explicitly cover the following in
82 detail in 3–6 paragraphs with citations:
83 a) Any modifications and troubleshooting of the technique
84 b) Any limitations of the technique
85 c) The significance with respect to existing methods
86 *Answer: I wrote the discussion in reference to Nature Methods and Nature Biotechnology. I edit*
87 *it a bit to emphasize the key points and make it clearer.*
88

89 References:
90 1. Please do not abbreviate journal titles.
91 *Answer: Have you changed your style? Edited.*
92
93 Table of Materials:
94 1. Please ensure the Table of Materials has information on all materials and equipment used,
95 especially those mentioned in the Protocol.
96
97 Video content:
98 1. Please ensure the protocol in the video matches with the one in the manuscript; e.g.:
99 a. 3.1: There is commentary after this step in the video that is not in the manuscript.
100 b. 4.5: This step is not mentioned in the video.
101 c. 4.8: This is done for 2 min in the video, and 15 s in the manuscript.
102 2. Only one of the figures is shown in the video; ideally, all would be mentioned.
103 *Answer: Discrepancies between manuscript and video have been addressed. No great place to*
104 *include other figures in video, unfortunately. However, other figures are used only to support*
105 *the that the sources of RNA and quality of RNA. The key of this approach is how to obtain*
106 *quality ribosome-bound mRNA directly* in vivo*. The method itself is pretty straight forward and*
107 *we emphasize several caveats. These are important, especially to those who do not often work*
108 *with RNA extraction.*
109
110 Video production:
111 1. Future submissions should include the article ID number (59624) in the video file name.
112 2. 1:59, 2:21 - The edits here are jump cuts, which tend to have a jarring effect on the viewer.
113 They should be smoothed out with crossfades instead.
114 3. 2:21-2:32 - The audio and video don't appear to be synchronized in this clip. This should be
115 corrected.
116 4. 4:44, 5:06, 7:15- The brand names Igepal, RNeasy, and Nanodrop, respectively, are
117 mentioned specifically in the narration. These references should be removed.
118 5. There is no concluding statement after the results section.
119
120 *Answer: Done. Concluding statement added.*
121
122
123 **Reviewers' comments:**
124
125 **Reviewer #1:**
126 Manuscript Summary:
127 The manuscript provides a protocol for isolation of ribosome-bound mRNAs from vascular
128 endothelial cells using engineered mouse. The paper provides a step-by-step procedure from
129 freezing ribosomes on mRNAs in isolated tissues, enriching ribosomes through
130 immunoprecipitation, and purifying the mRNAs bound on ribosomes. The manuscript is written
131 clearly and procedures are easy to follow. My specific comments are:
132

133 Major Concerns:
134 1. In order to utilize this protocol, one will need engineered mice with eGFP-tagged ribosomes. I
135 believe the protocol will be much more useful if the authors provide information on generating
136 such mice.
137
138 *Answer: This is a good suggestion. However, the flox/flox mice with e-GFP-tagged ribosomes*
139 *have been established by Dr. Pu lab and donated to Jackson lab. It is also a routine work to*
140 *produce double mutant because Jackson lab can provide different kinds of transgenic and cre*
141 *mice. Only challenge for creating this kind of mice is time-consuming and money. The paper*
142 *from Dr. Pu lab has been published and cited in our protocol. However, we found during our*
143 *pilot studies, isolation of quality ribosome-bound RNA is not so easy though the method seems*
144 *simple and straight forward. It took us a long time to optimize the conditions though the yield is*
145 *low. We think that it is useful experience for other researchers and thus contribute this video*
146 *paper.*
147
148 Minor Concerns:
149 1. Line 98: There is a typo (RNAase should be RNase). Also need a dash (-) between RNase and
150 free.
151 *Answer: Corrected.*
152
153 2. Line 127, cycloheximide should be delivered asap to freeze ribosomes. However, the protocol
154 just submerges the tissue in PBS with cycloheximide. Is there a size limit for efficient
155 cycloheximide treatment?
156 *Answer: We found that larger tissue samples (i.e >100mg) decreased the efficiency of RNA*
157 *isolation, which may have been due to inadequate Cycloheximide treatment. Shearing of tissue*
158 *samples upon placement in Cycloheximide containing PBS seemed to eliminate this problem.*
159
160 3. Line 280: Need a dash between RNase and free
161 *Answer: Corrected.*
162
163 4. To show the quality of isolated RNAs, authors should run their samples on 1% agarose gel.
164
165 *Answer: Good suggestion. Our Nonodrop assay and functional assays showed the quality of*
166 *extracted RNA is high and and we thus did not run gel analysis.*
167 5. In Figure 4, what is control?
168 *Answer: Thanks for the question. We used the ribosome-bound RNA from TRAP mice without*
169 *cd36 deficiency as the control*
170
171
172
173 **Reviewer #2:**
174 Manuscript Summary:
175 Translating ribosome affinity purification (TRAP) is a widely applied tool for purifying actively
176 translated mRNAs in specific tissue. Investigation of cell type specificity is now regarded as a

177 key part to understand cellular specificity as well as cellular vulnerability in many translational
178 researches. Overall, well described manuscript with fully detailed methods and materials were
179 provided by authors. I have no major comments for this manuscript, and have a couple of
180 questions for authors about terms and applied concentration of protease inhibitor.
181
182 Major Concerns:
183 N/A
184
185 Minor Concerns:
186 1. What is right term for purified mRNAs? Transcriptome or Translatome?
187 *Answer: Sorry for the misunderstanding. Actually, what we meant is that we purified mRNA*
188 *from endothelial ribosome in the tissues. We have edited the sentences and made this clearer.*
189
190 2. I have one question for authors. Have authors ever checked purified mRNA quality (or
191 efficiency) between concentration of protease inhibitor and different tissues? If yes, it would be
192 greatly interest for all field scientists.
193 *Answer: Good suggestion. We did not do this and we may do it in our future studies.*
194
195
196 **Reviewer #3:**
197 Manuscript Summary:
198 Well written article with good representative examples
199
200 Minor Concerns:
201 Lack of a global control. Like Actin or Gapdh to see if these are still captured and remain
202 unaffected.
203 *Answer: In our pilot experiments, we actually used wild type mice to test the isolation of tissue*
204 *RNA with the initial protocol, and found that we could extract little amount of RNA, and we later*
205 *optimize the conditions and used very stringent conditions to isolate them in TRAP mice. Though*
206 *the yield was low we get quality RNA, which may represent ribosome-bound RNA in the*
207 *endothelia* in vivo*.*
208
209