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Bio Protoc. Author manuscript; available in PMC 2017 May 30.

Published in final edited form as:

Bio Protoc. 2016 September 5; 6(17): . doi:10.21769/BioProtoc.1922.

Measurement of Transferrin- and Non-transferrin-bound Iron Uptake by Mouse Tissues

Supak Jenkitkasemwong, **Chia-Yu Wang**, and **Mitchell D. Knutson***

Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida, USA

Abstract

Iron in blood plasma is bound to its transport protein transferrin, which delivers iron to most tissues. In iron overload and certain pathological conditions, the carrying capacity of transferrin can become exceeded, giving rise to non-transferrin-bound iron, which is taken up preferentially by the liver, kidney, pancreas, and heart. The measurement of tissue transferrin- and non-transferrin-bound iron (TBI and NTBI, respectively) uptake *in vivo* can be achieved via intravenous administration of ^{59}Fe-labeled TBI or NTBI followed by gamma counting of various organs. Here we describe a detailed protocol for the measurement of TBI and NTBI uptake by mouse tissues.

Materials and Reagents

1. 1 ml TB syringe with 27-gauge needle (BD Biosciences, catalog number: 309623)
2. Plastic wrap
3. 3.5” × 3.5” weigh boat (Thermo Fisher Scientific, Fisher Scientific, catalog number: 8732113)
4. 1.5-ml microcentrifuge tubes
5. 0.45 μm filter unit (Thermo Fisher Scientific, Fisher Scientific, catalog number: 09-740-65B)
6. Amicon™ Ultra-15 centrifugal filter units (Merck Millipore, catalog number: UFC903024)
7. 5 ml tube
8. Adult mice (> 6 weeks, any strain) (*e.g.,* Balb/cJ)
9. $^{59}FeCl_3$ (Perkin Elmer, catalog number: NEZ037500UC)

   Note: The minimum specific activity of ^{59}Fe we have used to obtain decent signal from our gamma counter was 0.2 Ci/mmol at the concentration of ~3 μCi/μl, which gave whole body counts per minute of ~100,000 per each animal. Our gamma counting efficiency is about 10% for ^{59}Fe.

*For correspondence: mknutson@ufl.edu.

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10. Nitrilotriacetic acid (NTA) (Sigma-Aldrich, catalog number: N9877-100G)
11. HEPES (Thermo Fisher Scientific, Fisher Scientific, catalog number: AC172570250)
12. Tris (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP152-5)
13. Sodium bicarbonate ($NaHCO_3$) (Fisher Scientific, catalog number: S233-500)
14. Sodium citrate dihydrate (Thermo Fisher Scientific, Fisher Scientific, catalog number: S279-500)
15. Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: I88-100)
16. Hydrochloric acid (HCl) (Thermo Fisher Scientific, Fisher Scientific, catalog number: A144-212)
17. Ethanol
18. Human apo-transferrin (Sigma-Aldrich, catalog number: T2252)
19. PBS (VWR, Corning®, catalog number: 21-040-CM)
20. Sodium chloride (NaCl) (Thermo Fisher Scientific, Fisher Scientific, catalog number: S2713)
21. Fisherbrand™ absorbent underpads (Thermo Fisher Scientific, Fisher Scientific, catalog number: 14-206-64)
22. Radioactive decontaminating solution (Thermo Fisher Scientific, Fisher Scientific, catalog number: NC9633347)
23. 20 mM Fe-NTA (1:4) (see Recipes)
24. Ferric citrate solution (see Recipes)
25. ^{59}Fe-labeled ferric citrate solution (see Recipes)
26. ^{59}Fe-labeled transferrin (see Recipes)
27. 0.9% NaCl solution (see Recipes)

Equipment

1. Gamma counter (Perkin Elmer, catalog number: 2480-0010)

   The program for ^{59}Fe detection was set using the following parameters:

   a. Counting window: Dynamic-keV
   b. Peak position: 1292 keV
   c. Low boundary: 1020 keV
   d. High boundary: 1400 keV
   e. Threshold: 20%

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f. Max. assay deviation: 20%

g. Max. normalization deviation: 50%

h. Warning assay deviation: 15%

i. Significant CPM/keV: 10

j. Counting spectrum type: many peaks

2. Gamma counting tubes (5 × 28 mm) and RIA racks (Perkin Elmer, catalog number: 1480-151)

   a. Prior to gamma counting, empty gamma counting tubes are measured to ensure no ^{59}Fe contamination (*i.e.,* < 25 CPM = background).

3. Mouse restrainer (Braintree Scientific, catalog number: MTI STD)

4. Basic surgical kit for small animals

Procedure

1. For tissue NTBI uptake measurement, inject 0.1 ml of ferric citrate solution (containing 70 μg iron) to a mouse via tail vein to transiently saturate the iron-binding capacity of plasma transferrin. This step is required so that any subsequently injected iron will not bind to transferrin and hence will be non-transferrin-bound iron (NTBI). Return the mouse to the cage and wait for 10 min before going to step 2. See Figure 1 for a typical injection station setup.

   For tissue TBI uptake measurement, inject 0.1 ml of ^{59}Fe-labeled transferrin (containing 150 μg transferrin) to a mouse via tail vein. Return the mouse to the cage and wait for 2 h before going to step 3.

2. Inject 0.1 ml of ^{59}Fe-labeled ferric citrate solution (2 μCi) to the mouse via tail vein. Return the mouse to the cage.

3. Two hours after injection of ^{59}Fe-labeled ferric citrate solution (for NTBI uptake) or ^{59}Fe-labeled transferrin (for TBI uptake), euthanize the mouse with an overdose of isoflurane or other proper methods and place the mouse on an absorbent pad. Cut the mouse tail off to remove residual ^{59}Fe radioactivity that is retained in the tail (*i.e.*, radioactivity that did not enter the circulation). See Figure 1 for a typical harvesting station setup.

4. Sterilize the mouse abdomen and chest area with 70% ethanol. Open the abdominal cavity with blunt-end sterile scissors and collect tissues of interest (*e.g.,* pancreas, spleen, liver, kidney, *etc.*). Then open the chest cavity to collect tissues such as lung and heart.

5. Rinse tissue in 0.9% NaCl solution poured in a disposable weigh boat and remove excess liquid by dabbing the tissues on a stack of paper towels.

6. Use plastic wrap to wrap each tissue briefly prior to transfer into a gamma counting tube to minimize cross contamination of ^{59}Fe radioactivity between

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samples. Optionally, transfer tissue to a clean 1.5-ml microcentrifuge tube if the tissue sample can fit in it. Then measure the tissue radioactivity using a gamma counter by putting the 1.5-ml tube containing the tissue sample into a gamma counting tube rack (see Figure 2).

7. For the mouse carcass, use plastic wrap to securely wrap the whole carcass prior to transfer into a gamma counting tube (see Figure 2).

8. Measure organ and carcass ^{59}Fe radioactivity by using a gamma counter. Calculate tissue NTBI and TBI uptakes as a percentage of whole-body counts per minute (CPM) (whole-body CPM = CPM values from all tissues + CPM value from carcass) (see Figure 3).

Notes

After each experiment, use radioactive cleaning reagent to clean the surgical tools, mouse cages, and related equipment to decontaminate ^{59}Fe. Perform a radioactive swipe test on the related equipment and area where the experiment is performed after each experiment to ensure no contamination is observed. Radioactive waste products should be disposed of by following your local Environmental Health and Safety (EH&S) regulations.

Recipes

1. 20 mM Fe-NTA (1:4 molar ratio)

   20 mM HEPES/20 mM Tris buffer

   0.4 M NTA

   0.4 M $FeCl_3$

   a. Prepare 20 mM HEPES/20 mM Tris buffer

      To 450 ml ddH_2O, dissolve 2.38 g HEPES and 1.21 g Tris.

      Adjust pH to 6.0 with 10 N HCl.

      Bring final volume to 500 ml with ddH_2O.

   b. Prepare 0.4 M NTA

      In 20 ml 20 mM HEPES/20 mM Tris buffer, add 4.28 g NTA.

      Adjust pH to 6.0 with 10 N NaOH by adding ~3 ml of 10 N NaOH at the beginning then drop-wise until pH reaches 6.0 (*Caution: pH changes rapidly between pH 5–7*).

      Bring final volume to 56 ml with 20 mM HEPES/20 mM Tris buffer.

   c. Prepare 0.4 M $FeCl_3$ in 100 mM HCl

      To make 100 mM HCl, add 500 µl of 37% (equivalent to 12 M) HCl solution to 11.5 ml ddH_2O.

      Dissolve 1.08 g $FeCl_3 \cdot 6H_2O$ in 10 ml 100 mM HCl.

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d. Prepare 20 mM Fe-NTA (1:4 molar ratio)

In a 50 ml beaker with stir bar inside, add 10 ml of 20 mM HEPES/20 mM Tris buffer.

Leave the pH electrode inside the solution and track the solution pH along the rest of the procedure.

Add 2 ml of 0.4 M $FeCl_3$ prepared in step 1c. The solution pH should now be ~2.

Add 8 ml of 0.4 M NTA prepared in step 1b.

Adjust to final pH of 6.95 with 100 mM NaOH.

Bring final volume to 40 ml with 20 mM HEPES/20 mM Tris buffer.

Filter sterilized the 20 mM Fe-NTA solution through 0.45 μm filter unit.

2. Ferric citrate solution

0.1 M citrate buffer

12.5 mM $FeCl_3 \cdot 6H_2O$

a. Prepare 0.1 M citrate buffer

To 40 ml ddH_2O, dissolve 2.25 g $Na_3C_6H_5O_7 \cdot 2H_2O$ (tri-sodium citrate dihydrate).

Adjust pH to 6.6 with 10 M HCl.

Bring final volume to 50 ml with ddH_2O.

b. For 50 ml stock, add 0.169 g $FeCl_3 \cdot 6H_2O$ to 50 ml 0.1 M citrate buffer.

3. ^{59}Fe-labeled ferric citrate solution

0.1 M citrate buffer, $^{59}FeCl_3$

To 100 μl 0.1 M citrate buffer, add 2 μCi $^{59}FeCl_3$.

Mix well.

4. ^{59}Fe-labeled transferrin

10 mM $NaHCO_3$

18.75 μM apo-transferrin

20 mM Fe-NTA (1:4 molar ratio)

$^{59}FeCl_3$

a. To 50 ml PBS, add 0.042 g $NaHCO_3$.

b. Add 7.5 mg apo-transferrin to 5 ml $NaHCO_3$ in PBS buffer prepared in step 4a.

c. Add 2.5 μl $^{59}FeCl_3$ to 12.5 μl of 20 mM Fe-NTA solution.

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d. Mix solutions prepared in steps 4b and 4c. Incubate at RT for 1 h to allow ^{59}Fe to bind to transferrin.

e. Remove unbound ^{59}Fe by using Amicon™ Ultra-15 centrifugal filter unit.

i. Add 4 ml of ^{59}Fe-labeled transferrin prepared in step 4d to an Amicon™ Ultra-15 centrifugal filter unit.

ii. Centrifuge at 5,000 × *g* for 20 min at RT, discard eluate in a radioactive waste container.

iii. Add 4 ml 10 mM $NaHCO_3$ (prepared in step 4a) to the filter unit and centrifuge at 5,000 × *g* for 20 min at RT, discard eluate.

iv. Repeat step eiii for 3 additional times. In last wash, you may wish to measure ^{59}Fe radioactivity found in the eluate to ensure that the ^{59}Fe not bound to TBI has been thoroughly removed. The eluate ^{59}Fe radioactivity of greater than 30 CPM may indicate the requirement for additional washes.

v. Add 4 ml of 10 mM $NaHCO_3$ buffer on top of the filter unit and collect all the retentate (which contains the newly added 4 ml of 10 mM $NaHCO_3$ and the ^{59}Fe-labeled transferrin, making the colleting volume to ~4 ml) into a fresh 5 ml tube. Measure ^{59}Fe CPM of the ^{59}Fe-labeled transferrin solution to ensure sufficient radioactivity to be able to detect tissue-associated ^{59}Fe from each animal (desirably ~10,000 CPM/100 µl ^{59}Fe-labeled transferrin solution). Store at 4 °C until use for tail vein injection.

5. 0.9% NaCl solution, autoclaved

To every 1 L of ddH_2O, add 9 g of NaCl.

Autoclave to sterilize and store at 4 °C until use.

Acknowledgments

This work was supported by the National Institutes of Health grant DK080706 (to M.D.K.). The protocol was adapted from Craven *et al.* (1987), and a short version of the adapted protocol was published in Jenkitkasemwong *et al.* (2015).

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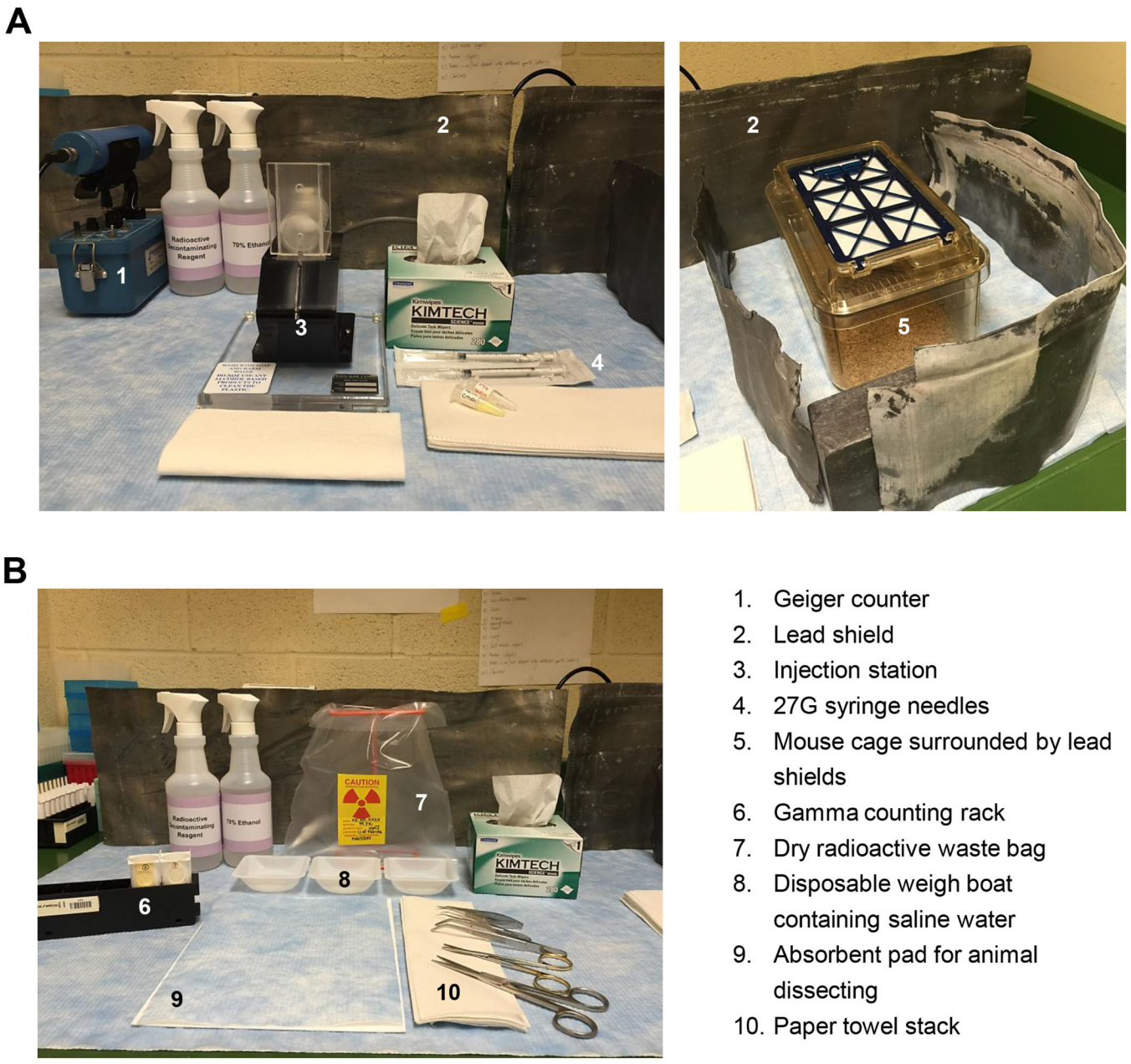

Figure 1. Typical setup for tail vein injection station (A) and tissue harvesting station (B)

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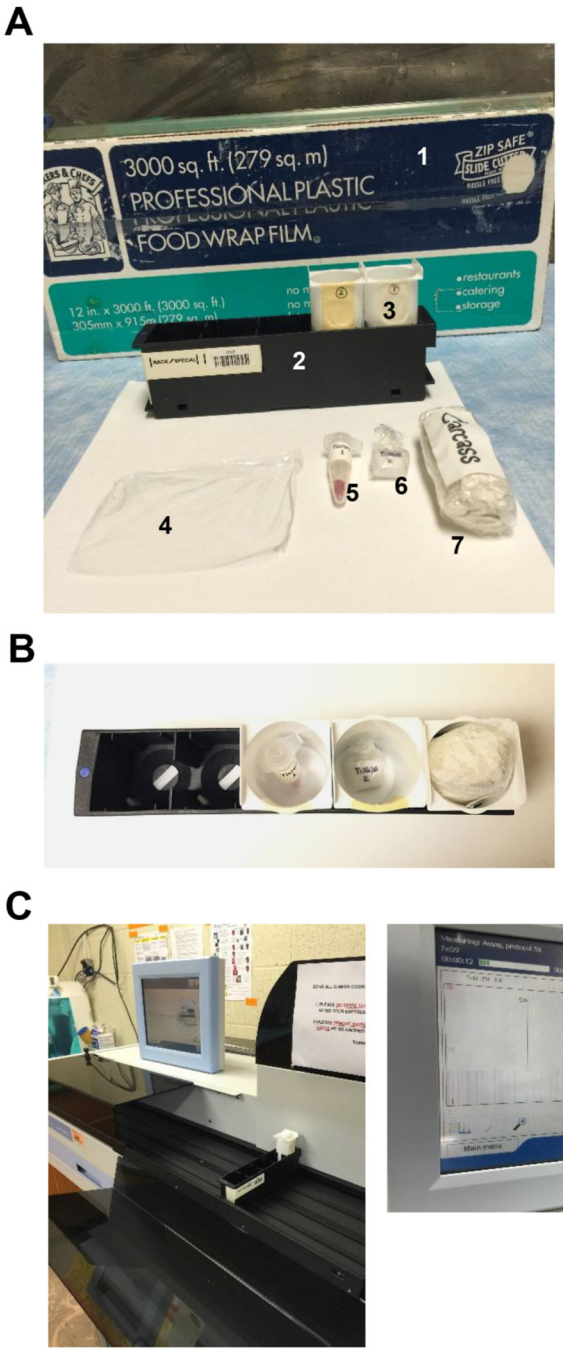

1. Plastic wrap film roll
2. Gamma counting rack
3. Gamma counting tube
4. Plastic wrap cut into small size that can wrap tissue sample securely
5. 1.5-ml microfuge tube containing tissue sample
6. Tissue sample securely wrapped in a small piece of plastic film
7. Animal carcass securely wrapped in plastic film

Figure 2. Illustration of radioactive tissue handling and loading to gamma counting tubes prior to measurement by a gamma counter

A. Materials required for tissue sample wrapping. B. Top view of the gamma counting rack with previously wrapped samples loaded in each gamma counting tube. C. The gamma counting rack containing the sample tube is placed on the rack belt of the gamma counter, ready to be loaded inside the gamma detector (left panel). The gamma counter screen showing real-time radioactivity being detected from the tissue sample (right panel).

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A

Mouse	Genotype	Counts Per Minute (CPM)						
		Liver	Pancreas	Spleen	Kidney	Heart	Carcass	Whole body
A	WT	60658.74	3667.8	2118.18	10116.52	1038.8	50830.59	128430.63
B	WT	52567.93	3335.65	5887.79	10966.39	1228.4	71993.09	145979.25
C	WT	64587.81	3874.26	904.31	6756.82	845.91	37022.37	113991.48
D	Zip14 KO	3026.36	332.59	516.25	5567.07	524.26	28995.92	38962.45
E	Zip14 KO	7252.9	629.85	1600.17	12744.32	1312.05	78224.07	101763.36
F	Zip14 KO	7922.67	752.77	1119.82	13496.2	971.14	57337.76	81600.36

B

Mouse	Genotype	Tissue ^{59}Fe accumulation (% of whole body)						
		Liver	Pancreas	Spleen	Kidney	Heart	Carcass	Whole body
A	WT	47.23	2.86	1.65	7.88	0.81	39.58	100.00
B	WT	36.01	2.29	4.03	7.51	0.84	49.32	100.00
C	WT	56.66	3.40	0.79	5.93	0.74	32.48	100.00
D	Zip14 KO	7.77	0.85	1.32	14.29	1.35	74.42	100.00
E	Zip14 KO	7.13	0.62	1.57	12.52	1.29	76.87	100.00
F	Zip14 KO	9.71	0.92	1.37	16.54	1.19	70.27	100.00

C

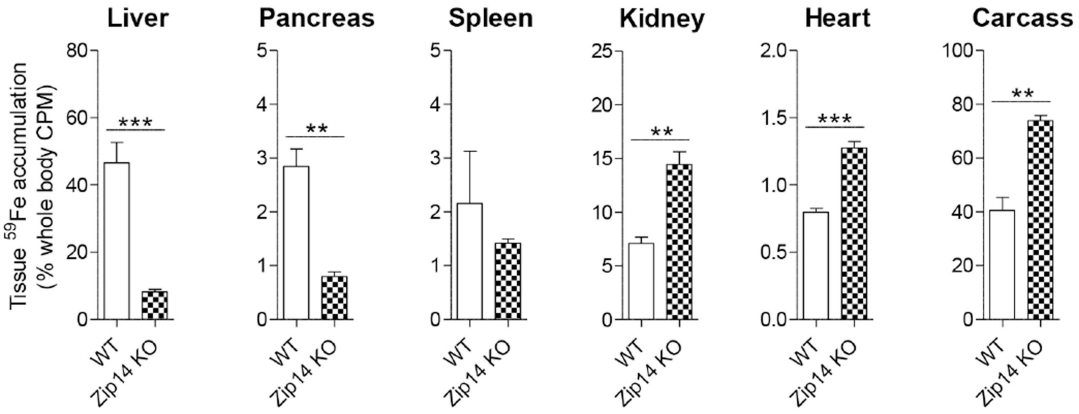

Figure 3. Example of raw data and analysis of results

Two hours after injection with ^{59}Fe-labeled ferric citrate, WT and Zip14 KO mice at 3 weeks of age were sacrificed and their tissues and carcasses were subjected to gamma counting. A. Counts per minute (CPM) values from each sample were recorded. Whole-body CPM values were calculated from the sum of all tissues and carcass CPMs. B. Tissue ^{59}Fe accumulation is expressed as % of whole body and calculated as (tissue CPM/ whole body CPM) × 100. C. Resultant figure from panel B is shown. Data were analyzed by

using unpaired student's *t*-test. Values are means ± SE (n = 3/group). ***$P < 0.001$, **$P < 0.01$.

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