Affordable Method for Hematocrit Determination in Murine Models

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Hematocrit (Hct) is a powerful tool often used in a clinical setting for the diagnosis of blood conditions such as anemia. It is also used in the research field as a hematological parameter in both human and mouse models. Measuring Hct, however, involves the use of expensive standardized equipment (such as a CritSpin™ Microhematocrit Centrifuge). Here, we describe a novel, simple, and affordable method to determine the Hct in untreated wild-type (WT) mice and phenylhydrazine (PHZ)-induced anemic mice with reasonable accuracy, using a benchtop centrifuge commonly available in laboratories. Hct of murine samples processed with a benchtop centrifuge, when compared to the standardized method CritSpin™, showed comparable results. This approach for determining Hct of murine can prove useful to research laboratories that cannot afford specialized equipment for Hct studies. © 2024 Wiley Periodicals LLC.

Basic Protocol 1: Affordable Method for Hematocrit Determination in Murine Models
Basic Protocol 2: Murine Sample Validation
Support Protocol: Phenylhydrazine-induced anemia in wild-type (WT) mice

Keywords: anemia-induced • hematocrit (Hct) • mouse models • phenylhydrazine (PHZ) • retro-orbital bleeding (REB)

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INTRODUCTION

The hematocrit (Hct), which is a measure of the percentage of whole blood occupied by red blood cells, is typically used to aid in clinical diagnostics and has been found to be useful in determining conditions such as anemia (Mondal & Lotfollahzadeh, 2022) and monitoring the state of the vasculature after treatment (Kishimoto et al., 2020). In mouse research, however, Hct is used as a hematological parameter for sickle, iron deficiency, and kidney injury (Gerkowicz & Prost, 1985; Ho & Pollock, 2014; Raabe et al., 2011). Moreover, Hct can vary greatly between young and older mice primarily because of the difference in their overall red blood cell (RBC) count and plasma volume and can also vary between transgenic mouse lines (Everds, 2007). The Hct can be determined using an automated analyzer (such as a Sysmex Analyzer) that uses spectral analysis to determine the Hct in a blood sample; this method, however, involves the use of costly equipment that requires constant maintenance and calibration. The alternative approach consists of a manual Hct determination, which involves a specialized micro-capillary rotor (such as a CritSpin™ Microhematocrit Centrifuge) that is also quite expensive. In a clinical setting,

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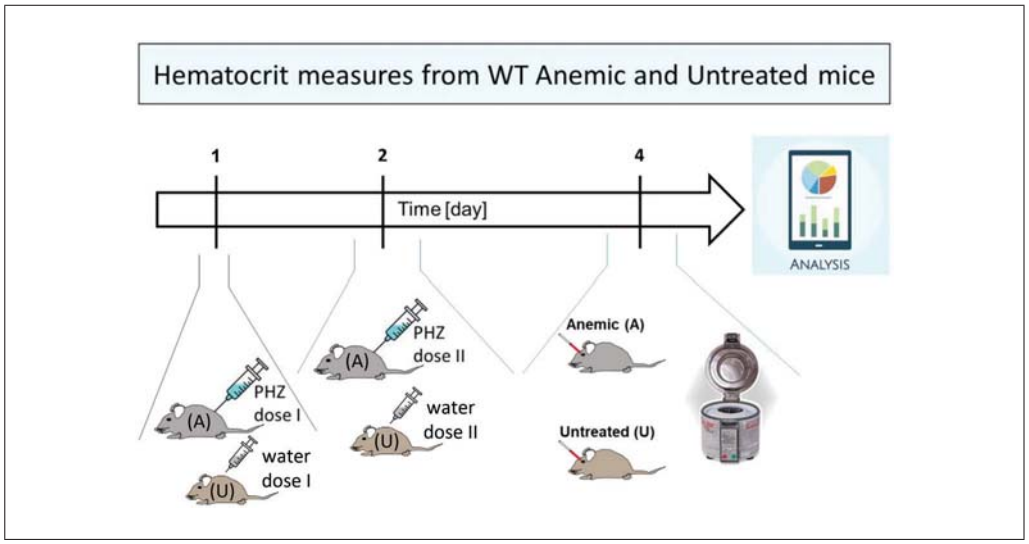

Figure 1 Schematic representation of the experimental design. Wild-type (WT) mice receive the first dose of PHZ (PHZ dose I) on the first day of the experiment (day 0), and a second dose 48 hr later (PHZ dose II). On day 4, the PHZ-treated mice have developed anemia and serve as positive controls. Both Anemic (A) and Untreated (U) WT samples can be collected and analyzed.

the quality of the health care provided to patients is dependent on the accuracy of clinical results; therefore, affordable alternatives for low-income clinics are beneficial for not just the medical personnel but also the patients receiving treatment (Audu et al., 2017). Efficient, cost-effective hematologic instrumentation is essential to minimize errors and ensure reliable results. Similarly, it is also of great importance in a research setting to obtain hematological results from animal models in an affordable manner.

Here we summarize a fast, cost-effective, manual method (we will refer to it as "benchtop") for determining the hematocrit of blood samples from wild-type (WT) mice untreated and phenylhydrazine (PHZ)-induced anemia using common laboratory equipment. Murine Hct was compared using both our suggested benchtop method and CritSpin^{TM}. We also include the PHZ-induced anemia protocol approved in our institution as a guideline for researchers interested in including a positive control.

In aggregate, this simple Hct method can prove useful in translational research and phenotypic murine studies.

NOTE: All protocols described here have been approved by the Institutional Animal Care and Use Committee (IACUC) and follow regulations for the care and use of laboratory animals.

CAUTION: *Phenylhydrazine* (PHZ) must be resuspended and injected in a suitable fume hood with efficient ventilation; safety glasses, lab coat, and reagent-impermeable protective gloves should be worn. Follow SDS guidelines for handling and waste disposal.

STRATEGIC PLANNING

We strongly recommend using a positive control to validate the experimental protocol and the equipment. Here we use a murine model of PHZ-induced anemia to generate expected low hematocrit percentages. The experimental design is quite simple, as shown in Figure 1. Briefly, samples are collected via retro-orbital bleeding (REB) in anemic and untreated WT mice on day 4 of the experiment, followed by data analysis. As per IACUC guidelines approved at Oakland University protocol 2021-1128, WT mice develop anemia on day 4 post-PHZ administration, which occurs in two doses.

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals.

AFFORDABLE METHOD FOR HEMATOCRIT DETERMINATION IN MURINE MODELS

BASIC PROTOCOL 1

This protocol offers a fast and cost-effective manual method, referred to as "benchtop," specifically designed for determining Hct in murine blood samples collected via REB. Samples used in this study include WT mice untreated, and PHZ-induced anemic subjects that served as positive controls. PHZ-induced anemia in WT mice is explained in detail in Support Protocol below.

Unlike automated analyzers that demand expensive equipment, our approach utilizes common laboratory tools, making it a practical and affordable alternative, particularly beneficial for low-income clinics and research laboratories. To validate the method proposed here, the murine Hct results obtained via manual benchtop method are compared to the Hct percentages obtained with a standard method known as CritSpin™. CritSpin™ is a specialized micro-capillary rotor that is recommended for both clinical and laboratory use that provides an accurate Hct result in 2 min (see Basic Protocol 2). To facilitate a successful execution of the protocol, we include a video to explain Basic Protocol 1 and Basic Protocol 2 step by step (see Video 1).

When conducted properly, this protocol ensures a reliable and cost-efficient means of determining Hct in murine blood samples. The results, validated through ortholog approaches, exhibit the method's efficacy in providing accurate hematological data. This simple yet impactful Hct protocol holds promise for advancing translational research and facilitating phenotypic murine studies, offering a valuable resource for research settings.

Materials

Experimental mice (C57BL/6J, WT mature mice, Jackson Laboratory)
Anesthesia: Ketamine-Xylazine or Isoflurane (according to IACUC protocol)

Appropriate PPE (lab coat, gloves)
Heat source (heat lamp or heating pad)

Video 1 Visual aid for hematocrit determination in mice. The video shows the sequential steps mentioned in the Basic Protocol 1 regarding the animal preparation, sample collection and handling, and data analysis to successfully perform Hct measurements using a bench top centrifuge. The video includes also the CritSpin™ validation, referred as Basic Protocol 2.

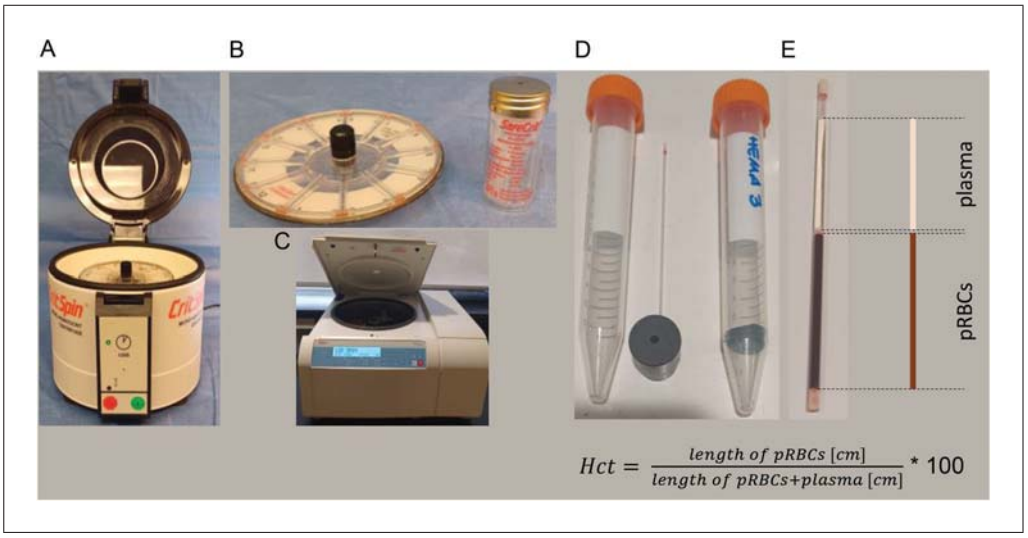

Figure 2 Equipment and materials for the validation of the Hct data. (**A**) CritSpin™ Microhematocrit Centrifuge. (**B**) Microhematocrit rotor and capillaries. (**C**) Benchtop centrifuge (Sorvall Legend XR1 Centrifuge) in use in the Washington laboratory. (**D**) Assembly of conical tube and perforated rubber stopper to support the placement of a 75-mm capillary. (**E**) Example 75-mm capillary after centrifugation using the benchtop method, and the formula used to determine the Hct.

Paper or gauze (optional)
75 mm heparinized capillary tubes (22-362-566, Fisherbrand™)
Hematocrit tube sealing compound (SKU 51007, Medical Equipment Affiliates (MEA))
Parafilm/laboratory sealing tape (PM992, Parafilm M)
Perforated stopper (524631, GSC GO size 0, 1 hole, 5-mm diameter, Fisher Scientific)
15-ml conical centrifuge tube (430790, Corning)
Sorval Legend XR1 centrifuge (75004261, Thermo Fisher)
Tweezer (long)
Hct reading card (optional)
Metric ruler, paper, and sharpened pencil (or fine tip pen)
PC and Software for statistical analysis (GraphPad, version 8.0)

Murine blood collection

1. Weigh subject for proper administration of anesthetic and administer the anesthetic.

   We are using untreated WT or anemic mice (Fig. 1). To induce anemia via PHZ administration, please refer to the Support Protocol.

2. Expose the anesthetized subject to a heat source to maintain body temperature.
3. Collect blood samples by retro-orbital bleeding (REB) using 75-mm heparinized capillaries.

   In case of abundant bleeding, dab the eye area of the subject gently with paper or gauze.

4. Seal the capillaries at both ends using the sealing compound. Assure samples are leak-proof by adding laboratory sealing tape to the ends of the capillaries.

   Incorrect placement or lack of sealing tape at the end of the capillaries results in sample loss during centrifugation. This step needs attention and meticulousness.

Tube assembly and centrifugation

5. Place the sealed 75-mm capillary into a 15-ml conical centrifuge tube vertically (Figs. 2 and 3A).

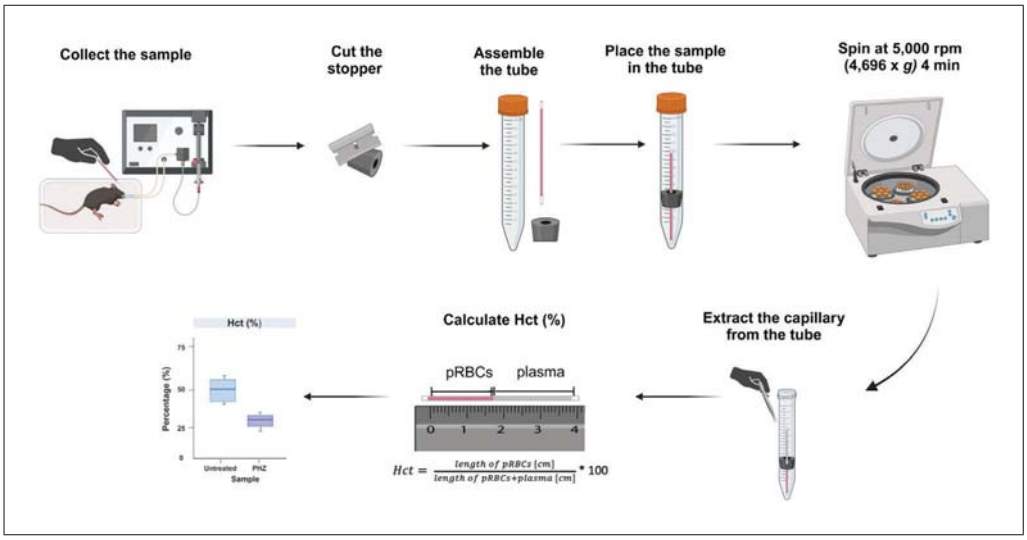

Figure 3 Schematic representation of the Hct benchtop method. Cartoon illustrating the general steps for the benchtop method starting with sample collection, assembly of the conical tube with the capillary and cutting the perforated stopper (optional), followed by centrifugation, extraction of the capillary, and measurement of pRBC and plasma to calculate Hct percentage. Created with BioRender.com.

OPTIONAL: We recommend cutting down a perforated stopper to size and placing it in a 15-ml conical tube. Place a sealed 75 mm capillary in the stopper hole to keep it vertical (this step is recommended to prevent capillary rupture).

6. Place the tube containing the capillary and an appropriate balance in a benchtop centrifuge. Spin at 5000 RPM (4696 $\times$ *g*) and room temperature for 4 min (Figs. 2 and 3C). After centrifugation, remove the capillary from the conical tube.

We suggest using a long tweezer to facilitate capillary removal.

Hct percentage assessment

7. Record the length of the packed red cells (red portion) and total volume (sum of red and clear portion) with a sharpened pencil (or fine tip pen) on a sheet of paper (Figs. 2 and 3F). Determine the Hct percentage using a metric ruler, using the following equation:

$$Hct\ [\%] = (length\ of\ pRBCs\ [cm]) / (length\ of\ pRBCs + plasma\ [cm]) * 100$$

(pRBCs: packed Red Blood Cells, cm: centimeters)

We recommend using the same metric ruler to ensure measurement consistency. Carefully remove the sealing tape and exclude the volume of the sealant compound from the calculations.

OPTIONAL: If available, you can use a Hct reading card instead of a metric ruler.

8. Input the raw data into software for statistical analysis and plot the results.

PHENYLHYDRAZINE-INDUCED ANEMIA IN WILD-TYPE (WT) MICE

SUPPORT PROTOCOL

Anemia causes a low red blood cell count, and in clinical settings it is reported as a low hematocrit percentage (Billett, 1990). In murine models, WT mice treated with PHZ develop reversible anemia in a few days, depending on the dose administered and the duration of treatment. PHZ-induced anemia is a well-accepted murine model for translational studies, developing evidence of low hemoglobin levels and low hematocrit percentages (Angulo et al., 2018). The PHZ-induced anemia model in WT animals serves here as

a positive control to assure comparable, reliable, and accurate results in the context of hematocrit validation studies.

Materials

Experimental mice (C57BL/6J, WT mature mice, Jackson Laboratory)
Anesthesia of choice and heating source, as per approved IACUC guidelines
Sterile deionized water
Phenylhydrazine hydrochloride (Sigma, 114715)

Precision laboratory scale
Laboratory fume hood
5-ml disposable tubes (Eppendorf, 0030119401)
PVDF 0.2-μm syringe filter (CELLTREAT, 229743)
Syringe (BD, 309657)
Syringes and needle (BD, 329412)
Heparinized microcapillary (Fisherbrand™, 22-362574)

Preparation of PHZ solution

1. Weigh and dissolve PHZ powder in sterile distilled water at a stock concentration of 50 mg/ml.
2. Filter-sterilize through a 0.2-μm syringe filter in a labelled disposable tube.

   The operations are conducted in a fume hood, and the operator must wear personal protection equipment. Leftover solution must be discarded according to institutional guidelines.

Administration of PHZ doses to murine subjects

3. Mice must be at least 8 weeks old.

   It is recommended to include subjects of both sexes. In this study, the untreated (vehicle water) animals included 6 males and 12 females, and the treated (PHZ) animals included 8 males and 10 females.

4. Anesthetize each subject with ketamine/xylazine via intraperitoneal (i.p.) injection or isoflurane gas anesthetic, according to approved IACUC guidelines.

   Depth of anesthesia is indicated by withdrawal response to toe pinch. The mouse will be kept warm by using a heating pad calibrated to 37°C or a heat lamp placed at an appropriate distance from the mouse.

5. *OPTIONAL* Once the subject is anesthetized, perform an eye bleeding via heparinized microcapillary to evaluate the normal hematocrit percentage.
6. The treated subject receives two doses of PHZ 60 mg/kg in 24 hr (first dose on day 1, and second dose on day 2) (Fig. 1, anemic). The untreated subject receives sterile deionized water, and serves as a negative control (Fig. 1, untreated).

   Administration should be performed with proper care for operator safety (see "preparation of PHZ solution"). In case of severe complications or casualties, the PHZ doses can be reduced to about 20% of the volume administered without significant changes in the hematocrit percentages.

7. On day 4 of the PHZ protocol, perform a hematocrit assay via REB using a heparinized microcapillary (Fig. 1).

   This hematocrit value is expected to reflect anemia as a reduction in the hematocrit percentage. Extensive data place hematocrit values in PHZ-induced anemic mice around 23%-30%, and the peak of lower hematocrit levels will be reached on days 2 through 5 post-administration (first dose on day 1).

8. Subjects can be euthanized or allowed to recover normal Hct values in about 15 to 20 days, according to IACUC approved guidelines.

MURINE SAMPLE VALIDATION

BASIC PROTOCOL 2

The CritSpin™ Microhematocrit centrifuge is a standardized centrifuge commonly used to determine Hct. To increase the robustness of our data, we collected murine blood samples in microcapillaries and processed them with the CritSpin™ centrifuge (see Video 1). The murine sample validation described here serves as a comparator for the benchtop centrifuge method (see Basic Protocol 1) to determine whether the readings are consistent.

Materials

Experimental mice (C57BL/6J, WT mature mice, Jackson laboratory)
Anesthesia: Ketamine-Xylazine or Isoflurane (according to IACUC protocol)

Appropriate PPE (lab coat, gloves)
Heat source (heat lamp or heating pad)
Paper or gauze (optional)
40-mm heparinized capillary tubes (HP8U, Safecrit)
Hematocrit tube sealing compound (SKU 51007, Medical Equipment Affiliates (MEA))
CritSpin™ (CS12, Stat spin)
Hct reading card (optional)
Metric ruler, paper, and sharpened pencil (or fine tip pen)
PC and Software for statistical analysis (GraphPad, version 8.0)

Murine blood collection

1. Weigh subject for proper administration of anesthetic and administer the anesthetic.

   We use untreated and anemic WT mice (Fig. 1). To induce anemia via PHZ administration, please refer to the Support Protocol.

2. Expose the anesthetized subject to a heat source to maintain body temperature.
3. Collect blood samples by retro-orbital bleeding (REB) using 40-mm heparinized capillaries.

   In case of abundant bleeding, gently blot the eye area of the subject with paper or gauze.

4. Seal the capillaries at both ends using the sealing compound.

Centrifugation

5. Place the sealed 40-mm capillary in the CritSpin™ Microhematocrit centrifuge (Fig. 3A-B).

   OPTIONAL: Capillaries do not need to be balanced.

6. Spin at 16,000 RPM (13,700 × *g*) for 2 min. After centrifugation, remove the capillary from the centrifuge.

Hct percentage assessment

7. Record the length of the packed red cells (red portion) and total volume (sum of red and clear portion) with a sharpened pencil (or fine tip pen) on a sheet of paper (Fig. 3F). Determine the Hct percentage using a metric ruler, using the following equation:

$$Hct\ [\%] = (length\ of\ pRBCs\ [cm]) \,/\, (length\ of\ pRBCs + plasma\ [cm]) \ * \ 100$$

(pRBCs: packed Red Blood Cells, cm: centimeters)

We recommend using the same metric ruler for measurement consistency. Exclude the volume of the sealant compound from the calculations.

OPTIONAL: If available, use a Hct reading card instead of a metric ruler.

8. Input the raw data into software for statistical analysis and plot the results.

COMMENTARY

Critical Parameters for Hematocrit Analysis

1. Sample Handling

We recommend using an anticoagulant such as sodium citrate to prevent clotting. Mix the sample by gentle inversion to ensure homogeneity.

2. Sample Preparation

To assure volume accuracy, avoid filling the whole capillary tube. This also facilitates sealing and helps prevent leaks during centrifugation. Do not vortex the sample, keep at room temperature, and perform analysis within 1 to 2 hr.

3. Conical tube and sample assembly.

When assembling the conical tube to hold a filled capillary tube in place, it is necessary to minimize the size of the stopper by cutting off the wide upper part. Once introduced in the conical tube, the stopper provides a platform to secure the capillary inside the conical tube, as shown in Figure 3E. Despite the capillary tubes not needing to remain perfectly perpendicular inside the conical tube, we recommend using this method to prevent accidental capillary rupture, which would be detrimental to the analysis and requires re-collection of the sample.

Troubleshooting Table

The proposed Hct method is reproducible, effortless, and cost-effective. However, a few issues may arise. Similarly, the CritSpin™ system used for validation, despite its proven reproducibility and cost-effectiveness, may present certain challenges during hematocrit measurements. The following table (Table 1) outlines common problems, their potential causes, and recommended solutions for maintaining the accuracy and reliability of results. It is essential to address these potential issues promptly to ensure the reliability of hematocrit measurements. Regular training, adherence to protocols, and continuous monitoring of instrument performance contribute to the successful mitigation of these challenges.

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism version 8.0. Ordinary one-way ANOVA and Tukey's multiple comparison test was used to compare statistical means between CritSpin™ and benchtop centrifuges for murine blood. For all assays, statistical significance was set to $p < 0.05$. Assays were performed in at least two

Table 1 Troubleshooting Guide for Hematocrit Measurements

Problem	Possible cause	Solution
Sample clotting	Inadequate mixing of blood samples or delays in analysis	Ensure gentle inversion of blood samples and analyze promptly. Consider re-collection if clotting is suspected.
Inaccurate volume loading	Incorrect pipetting or inaccurate sample volume	Precisely load the recommended blood volume according to the analyzer's specifications. Verify pipetting accuracy.
Incorrect parafilm sealing	Sample leaks out when centrifuging	Ensure that both ends of the capillary tube are tightly sealed with parafilm before centrifugation.
Seal failure in capillary tubes	Improper sealing or defects in sealing compound	Carefully seal capillary tubes using recommended methods. Inspect seals for integrity before analysis.
Result verification challenges	Inadequate procedures for result verification	Implement a robust system for result verification, including double-checking results and adherence to established protocols.

independent experiments, in biological triplicates, in technical replicates (TR) when possible, and data are shown as the mean ± SD. The utilization of ordinary one-way ANOVA and Tukey's multiple comparison test in our research article on mouse samples is imperative for robust statistical analysis and meaningful interpretation of experimental data (Kim 2014). The ordinary one-way ANOVA serves as a powerful tool to assess the overall differences among multiple groups, providing statistical evidence of whether there are significant variations in the means of the experimental conditions. Subsequently, Tukey's multiple comparison is crucial for post hoc analysis, enabling us to identify specific group differences when the ANOVA indicates overall significance. This method allows for a comprehensive exploration of group-wise variations. Considering the intricate nature of biological experiments involving mouse samples, the application of these tests ensures a stringent statistical approach, enhancing the reliability and validity of our findings. The combination of ordinary one-way ANOVA and Tukey's multiple comparison test, therefore, contributes to the rigor and precision of our research, facilitating a thorough examination of group disparities and providing a solid foundation for drawing meaningful conclusions from our experimental results. An alternative method to analyze this data would be the Kruskal-Wallis test, if the data did not meet the assumption of normality required for ANOVA, or if the data was non-parametric. A Dunn's test could be used as a post hoc follow-up to Kruskal-Wallis to identify specific group differences.

Understanding Results

The method described for determining hematocrit (Hct) in murine blood using common laboratory materials and equipment addresses a critical need for laboratories with limited resources. Many smaller institutions lack modern automated equipment for hematocrit measurement due to budget constraints, making affordability and reliability paramount for low-resource labs (Hoag, 2015; InterFocus, 2021; Rosetti, 2018). Our approach, utilizing readily available laboratory consumables like conical tubes, capillaries, perforated rubber stoppers, and a benchtop centrifuge, offers an accessible alternative.

The experimental design includes both a negative (untreated, water) and positive (treated, PHZ) control not only to validate the protocol, but to offer a hematocrit percentage between normal and low range. Low hematocrit levels are usually found in anemic subjects, showing fewer red blood cells than a healthy subject. In mice, a normal hematocrit should range between 40% and 50%. In anemic subjects, the hematocrit level is expected to be around 30%. The induction of anemia in murine models using PHZ is well documented and can be achieved in 4 days, as shown in Figure 1. During this period, the hemolytic PHZ causes a gradual reduction of red blood cells in the subject. The low erythrocyte count in PHZ-induced anemic mice causes a series of symptoms, including severe lethargy. It is common for the treated animals to have a lower survival rate than the untreated controls. To induce anemia without casualties, we recommend ensuring easy access to food and water in the cage, because severe lethargy may result in food deprivation.

Despite a few precautions, the procedure is simple and cost-effective as shown in the concise workflow in Figure 2. The simplicity of the protocol, and the accessibility of common laboratory consumables and equipment allow personnel to complete it in timely manner. Also, the protocol only requires basic technical skills. The Hct percentages were determined utilizing both the CritSpin™ Microhematocrit centrifuge (Fig. 3A-B) and a common benchtop centrifuge (Fig. 3C). The assembly of common laboratory consumables such as a stopper and conical tube is simple and can be recycled (Fig. 3D). In addition, the Hct percentage can be easily calculated with a reading card or a common metric ruler with a simple formula, as shown in Figure 3E. As shown in Figure 3E, the blood in the centrifuged capillary is separated into two distinctive phases: a red section of packed red blood cells (pRBCs) and a clear section containing the plasma. Between the two sections it is possible to see a buffy coat layer formed by leucocytes and platelets. Given it represents only 1% of the hematocrit, the buffy coat is usually excluded from the calculation (Figs. 2 and 3E).

The Hct measurements were determined for 18 WT untreated (U) and anemic (A) mice utilizing both the CritSpin™ and a common laboratory benchtop centrifuge. Hct percentages for both methods, after statistical analysis, showed no significant difference (Fig. 4A,B). However, as expected, the Hct percentage was significantly lower in the anemic versus the untreated mice (Fig. 4B). We also confirmed that PHZ treatment replicated the murine Hct percentages described in the literature (Angulo et al., 2018).

A

Untreated (U)	Untreated (U)	Anemic (A)	Anemic (A)
CritSpin	Benchtop	CritSpin	Benchtop
50.00	52.60	30.00	31.60
50.00	50.80	26.47	32.70
46.10	50.00	28.57	30.00
48.10	48.40	23.30	26.00
48.10	50.80	25.92	25.86
50.00	51.80	32.14	25.90
50.00	51.70	26.66	24.60
48.50	50.00	29.03	29.30
50.00	52.80	26.66	30.00
50.00	47.20	25.81	25.86
53.80	52.80	31.03	32.20
48.00	51.80	25.93	25.86
44.00	47.30	31.25	30.00
50.00	50.80	23.30	23.52
48.15	48.87	25.00	24.60
50.00	49.15	25.81	26.00
51.85	49.18	29.03	32.20
48.15	50.00	28.12	29.31

B

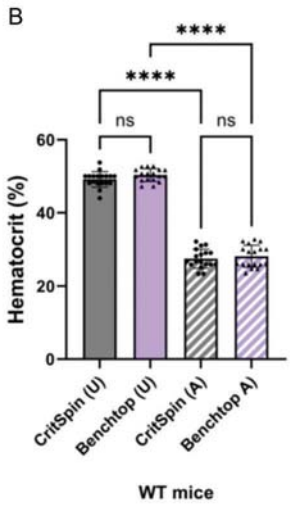

Figure 4 Murine Hct obtained with CritSpin™ and benchtop methods show no significant difference. (**A**) Raw data and (**B**) plot of Hct percentages resulting from blood samples of 18 wild type (WT) mice Untreated (U) or treated with PHZ to induce Anemia (A). Samples were processed using the CritSpin™ (CritSpin) and benchtop methods. Ordinary one-way ANOVA and Tukey's multiple comparison test. Not significant (ns), ****$p < 0.0001$.

In the context of murine models, the results revealed a statistically significant decrease in Hct percentage in anemic mice, aligning with expectations and demonstrating the sensitivity of our method to detect physiological changes. Moreover, the confirmation of murine Hct percentages consistent with the literature (Angulo et al., 2018) and successful replication of Hct alterations through PHZ treatment underscore the validity and relevance of our method in biomedical and translational research. To ensure the accuracy of our measurements, we emphasize the importance of precautions described in this protocol.

Collectively, the results obtained from murine Hct measurements support the feasibility and practicality of our proposed method. By providing an affordable alternative for laboratories lacking specialized equipment, our approach facilitates hematocrit determination in murine models, contributing to the accessibility and inclusivity of biomedical research in resource-constrained settings.

Time Considerations

Basic Protocol 1-2: The overall duration of the procedure depends on the number of subjects involved in the study. On average, depending on the expertise of the operator, the procedure (sample collection, processing, and data analysis) may take up to 15-30 min/subject.

Support Protocol: PHZ preparation, and PHZ or vehicle administration will take no more than 1 hr, during which time the subject must be monitored to assure correct temperature, level of anesthesia, and respiration. The overall procedure requires up to 4 days to successfully induce anemia in murine subjects.

Acknowledgments

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Author Contributions

Siobhan Branfield: Data curation; investigation; methodology; validation; writing—original draft; writing—review and editing. **A. Valance Washington:** Funding acquisition; project administration; resources; supervision; writing—review and editing. **Barbara Manfredi:** Conceptualization; data curation;

formal analysis; investigation; methodology; project administration; validation; visualization; writing—original draft; writing—review and editing.

Conflict of Interest

None of the contributing authors have any conflicts of interest to report

Data Availability Statement

The data, tools, and material (or their source) that support the protocol are available from the corresponding author upon reasonable request.

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