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TITLE: Genomics-, Microbiomics-, and Bioenergetics-Based Personalized Treatment for Trauma Patients at Risk for Sepsis

PRINCIPAL INVESTIGATOR: Catriona Miller, PhD (USAF PI)

CONTRACTING ORGANIZATION: RHMF/711thHPW AFRL
WRIGHT-PATTERSON AFB, OH 45433

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U.S. Army Medical Research and Development Command
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14. ABSTRACT Severely injured traumatic brain injury (TBI) patients who appear otherwise equivalent upon admission, will classically dichotomize into those who survive without life-threatening complications and those who develop organ dysfunction and/or sepsis. Some evidence indicates that variants of specific genes are associated w/poor outcomes following severe trauma; however, the relationships between gene expression & clinical consequences are unknown. Considering the evidence that the human gut microbiome plays an important role in many diseases, studies are needed to determine how changes in the gut microbiota following severe trauma may affect outcomes. This project will deliver key information regarding divergent patient responses to severe TBI, w/the potential to offer personalized approaches to therapeutics & supportive care, decreased early mortality & improved long-term functional outcome. Given the proportionately excessive incidence of TBI among members of the US military, the concepts and approaches tested by this project should have a particularly high impact on the men and women of our armed services.

15. SUBJECT TERMS
Genomics, microbiomics, DNA, traumatic brain injury, sepsis, multi-organ dysfunction, mitochondria

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Unclassified	Unclassified	Unclassified	Unclassified	**39**	

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1. **INTRODUCTION:**

Background: Severely injured traumatic brain injury (TBI) patients who appear otherwise equivalent upon admission, will classically dichotomize into those who survive without life-threatening complications and those who develop organ dysfunction and/or sepsis for unclear reasons. Some evidence indicates that variants of specific genes are associated with poor outcomes following severe trauma; however, the relationships between gene expression and clinical consequences are, at present, unknown. Considering the evidence that the human gut microbiome plays an important role in many diseases, studies are needed to determine how changes in the gut microbiota following severe trauma may affect outcomes, with the goal of eventually treating specific patients with probiotics to reduce infectious complications including sepsis or targeted therapy to reduce systemic complications such as organ failure. One target of both gut microbial metabolites and over 1000 eukaryotic gene products is the mitochondrion, which is the primary regulator of cellular and systemic energy metabolism. Based on the finding that blood cell energy metabolism reflects that of other cells and tissues, advanced cell respirometry measurements of platelets and peripheral blood mononuclear cells (PBMCs) may provide the information necessary for precision treatment of trauma patients grounded on normalization of energy metabolism.

Objectives: Short-Term - Determine the relationships between good vs. poor clinical outcomes after TBI and the temporal changes in the following unique potential biomarkers: A. Blood cell gene expression, B. Gut microbiome diversity and, C. Blood cell energy metabolism. Long-Term - Translate this information into personalized critical care treatment plans using interventions that precisely target gene expression, gut microbiome diversity, and energy metabolism, with the goal of reducing the onset of organ dysfunction and sepsis following TBI.

Aim 1. Determine the temporal relationship between the human blood cell transcriptome and the onset of organ dysfunction and/or sepsis in TBI ICU patients. Test the hypothesis that the expression of specific genes and genomic pathways are closely associated with development of organ dysfunction and/or sepsis and that these connections provide insight into both pathophysiology and more precise, personalized treatment than what is currently practiced.

Aim 2. Advance our understanding of the relationship between changes in the human gut microbiome and metabolome and the development of organ dysfunction and sepsis in TBI ICU patients. Test the hypothesis that a reduction in overall diversity of the gut microbiota contributes to the development of organ dysfunction and sepsis and that this knowledge can provide insight into gut specific personalized treatment.

Aim 3. Determine the temporal relationship between blood cell energy metabolism and the development of organ dysfunction and sepsis in TBI patients. Test the hypothesis that the relative rates of aerobic and anaerobic energy metabolism in platelets and (or) PBMCs are prognostic indicators of TBI-associated organ dysfunction and/or sepsis and that this knowledge can provide insight into future therapeutics aimed at restoring normal mitochondrial metabolism

Aim 4. Generate a repository of blood samples with associated clinical data from TBI ICU patients for future additional genomic, proteomic, and metabolomic analyses that will identify molecular biomarkers that associate with development of sepsis or organ dysfunction.

Study Design: Two hundred and forty computerized tomography-positive adult TBI patients who are admitted to the ICU will have blood samples taken on admission (Day 0) and on days 1, 2, 4, and 7. Aliquots will be used on the same day for measurements of platelet and PBMC energy metabolism consisting of rates of oxygen consumption and lactate production. RNA will be extracted from other aliquots and used for RNA-Seq measurements of gene expression. Stool samples will also be collected during days 0-7 and used for molecular biological measurements that define the gut microbial diversity (microbiome). Other aliquots of blood and stool will be stored in a repository for future exome sequencing, proteomics, and metabolomics. Bioinformatic comparisons will be made between the laboratory and clinical outcome measures to identify biomarkers associated with the onset of organ dysfunction and/or sepsis following TBI.

Impact: This project will deliver key information regarding divergent patient responses to severe TBI, with the potential to offer personalized approaches to therapeutics and supportive care, decreased early mortality, and improved long-term functional outcome. Given the proportionately excessive incidence of TBI among members of the US military, the concepts and approaches tested by this project should have a particularly high impact on

the men and women of our armed services.

2. **KEYWORDS:** *Genomics, microbiomics, DNA, traumatic brain injury, sepsis, multi-organ dysfunction, mitochondria*

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

     - *List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.*

       Major Task 1: Phase 1 Preparation and Study Initiation 25% COMPLETED

       Subtask 1: STC, UMB and DoD IRB applications submitted – Est -5 Months, Completion Date (ECD): 5/16/2019. 100% Completed.

       Subtask 2: Establish blood and stool repository organization and clinical data registry ECD: 1-3 mo. Completion Date (CD): 12/01/2019. 100% Completed.

       Subtask 3: Initiate patient recruitment and sample collection. ECD: 3 mo. 0% Completed.

       Subtask 4: Initiate blood cell bioenergetics assays ECD: 3 mo. 0% Completed.

       Subtask 5: Establish satisfactory RNA quality for assays ECD 3 Mo. 100% Completed. CD 6/15/2019.

       Local IRB Approvals ECD: -2 Mo. 100% Completed. CD: 3/18/2019.

       Milestone Achieved: DoD HRPO/ACURO Approval ECD: 0 Mo. 100% Completed. CD: 9/23/2019.

       Milestone Achieved: 70 patients enrolled and approximately 210 blood samples taken and stored ECD: 12 months. Percentage of Completion 0%

       Milestone Achieved: Blood cell bioenergetics assays performed on 75 samples from 25 patients ECD: 12 months. Percentage of Completion 0%

       Major Task 2: Phase 2 Study Continuation and Genomics Initiation

       Subtask 1: Continue patient recruitment and sample collection ECD13-24 Mo Percentage of Completion 0%

       Subtask 2: Continue blood cell bioenergetics assays ECD 13-24 Mo Percentage of Completion 0%

       Subtask 3: Initiate genomics and microbiomics assays ECD13-24 Mo Percentage of Completion 0%

       Milestone Achieved: 170 patients enrolled and approximately 510 blood samples taken and stored ECD 24 Mo Percentage of Completion 0%

       Milestone Achieved: Blood cell bioenergetics assays performed on 180 samples from 60 patients 24 Mo Percentage of Completion 0%

       Major Task 3: Phase 3 Finish enrollment, assays, and bioinformatics Percentage of Completion 0%

Subtask 1: Complete patient recruitment and sample collection ECD 24-32 Mo Percentage of Completion 0%

Subtask 2: Complete blood cell bioenergetics assays ECD 24-32 Mo Percentage of Completion 0%

Subtask 3: Complete genomics and microbiomics assays ECD 24-32 Mo Percentage of Completion 0%

Milestone Achieved: 240 patients enrolled and approximately 720 blood samples taken and stored ECD 32 Mo Percentage of Completion 0%

Milestone Achieved: Blood cell bioenergetics assays performed on 255 samples from 85 patients ECD 32 Mo Percentage of Completion 0%

Milestone Achieved: Bioinformatic analyses of laboratory and clinical data and identification of biomarkers demonstrating risk for sepsis or organ failure ECD 36 Mo Percentage of Completion 0%

Milestone Achieved: Final report submitted.

Presentations of results at national meetings and through peer-reviewed publications

- **What was accomplished under these goals?**

Regulatory approvals for Genomics, Microbiomics, and Bioenergetics-Based Personalized Treatment for Head Trauma Patients at Risk for Sepsis

This study was contracted between AFRL RQ and the University of Maryland, School of Medicine on 4/11/2019. Prior to this date, no funds were available at the University to start this study.

University of Maryland Shock Trauma Center (STC) approval and University of Maryland, Baltimore, (UMB) IRB initial applications were approved on 3/12/2019. Deferral of Headquarters-Level Review and Oversight to the Air Force Research Laboratory IRB was received from the MRMRC MEDCOM IRB on July 31, 2019. We received AFRL IRB approval - September 23, 2019. The AFRL IRB HPRO Review and Deferral letter received stated that the University of Maryland Baltimore would be the IRB of record for the study.

We submitted several modifications to the protocol adding Shock Trauma Anesthesiology Research (STAR) staff, co-investigators, lead research statistical analyst, and laboratory staff. They are hired or formally designated to work on this project. We also submitted clarifications to the study protocol based on optimized study logistics. The most recent modifications (Modification 7&8) were approved 12/18/19. A memo to the AFRL IRB indicating the changes made in both Modifications 7 and 8 was sent 12/26/19.

The current UMB IRB application will expire on 3/11/20. In anticipation, we submitted a continuing review on 12/23/19. When approved by UMB IRB, we will forward the memo and other required documentation to AFRL IRB.

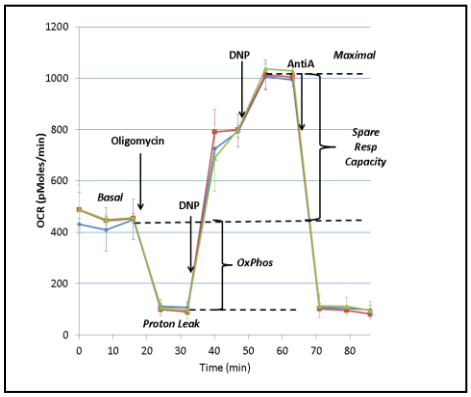
Training of STAR research staff will begin in early January and recruitment will commence as soon as staff are adequately trained. We anticipate mid-January.

Optimized Isolation of Platelets and Peripheral Blood Mononuclear Cells (PBMCs) from human blood

One of the main activities that occurred since April of 2019 was optimization of techniques for isolating purified platelets and PBMCs from a small amount of blood (two 6 ml EDTA tubes = 12 ml). Sepmate™ tubes separate PBMCs from other blood cells using a density gradient medium. The

gradient medium was loaded into the tube, and the whole blood was layered on top. Centrifugation causes the non-PBMC cells, such as platelets and red blood cells, to separate away from the PBMC-rich solution. This solution was collected and centrifuged again in order to consolidate the PBMCs and prepare them for further analysis. Although these cells could be used for bioenergetic analysis, there were a few reasons we did not adopt this protocol for PBMC isolation. First, upon inspection of the cells, we observed contamination by non-PBMC cells which could affect oxygen consumption rate. Second, this method requires a greater volume of blood to obtain both platelets and PBMCs. Finally, due to the use of the gradient medium, we would not be able to collect and store the platelet poor plasma (PPP), which could be used in future studies.

The EasySep™ method resulted in a cleaner PBMC yield (i.e. less contamination) and did not have the other drawbacks. Whole blood was centrifuged to separate platelet rich plasma (PRP) from the rest of the sample. PRP was removed and centrifuged to isolate platelets from the PPP. The remaining fraction of blood was processed using the EasySep™ cell isolation kit. An isolation cocktail containing antibodies that recognize specific non-PBMC cell-surface markers was added to the sample. Magnetic beads were then added and allowed to adhere to these labeled cells. The entire sample was placed on a specialized magnet and the PBMCs were isolated through immunomagnetic negative separation, meaning every cell other than PBMCs are drawn toward the magnet. The medium containing the PBMCs was removed and the PBMCs were pelleted through centrifugation, ready for bioenergetic analysis.



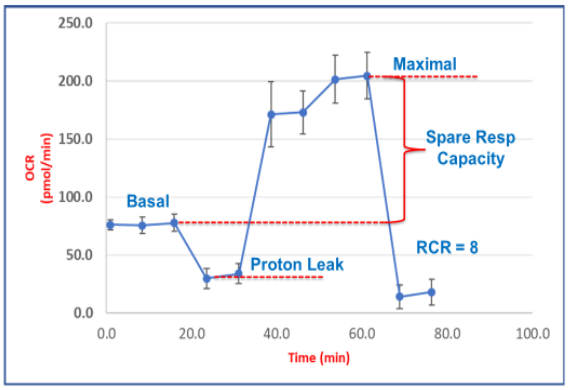


Figure 1. Measurements of oxygen consumption by human platelets (Left) and PBMCs (Right) following isolation from whole blood. Each measurement represents the mean ± standard error for n=10 wells on a 24 well plate. After numerous experiments, we found that the optimum number of cells in each well was 40 million platelets and 500 thousand PBMCs. We also found that the level of dinitrophenol (DNP) that elicits the maximum oxygen consumption rate (OCR) was 60 µM for platelets and 160 µM for PBMCs. The pattern of changes with additions of agents to the PBMCs is similar to that of fresh platelets. Specifically, the Respiratory Control Ratios (RCR) were similar (1000/100 = 10 for platelets and 200/25 = 8 for PBMCs. In general, ratios greater than 5 reflect robust efficient energy production.

Optimization of Variables Associated with Measurements of Oxygen Consumption by Isolated Platelets and PBMCs

Prior to the start of this project, we had published two articles demonstrating our ability to detect differences in platelet oxygen consumption rates (OCR) among different patient populations. These include normal pregnant and preeclamptic pregnant women and post-stroke survivors with different levels of fatigue (Klinedinst et al., 2019: Malinow et al., 2018). We had not, however, measured respiration by PBMCs isolated from human blood. Numerous experiments were performed to optimize the measurement variables for PBMCs. We eventually found that 12 ml of whole blood was sufficient for performing accurate respiration measurements with both platelets and PBMCs. Figure 1 provides an example of the raw data collected from simultaneous OCR measurements with both platelets and PBMCs. Fresh platelets and PBMCs exhibit a relatively large difference between the maximum respiration reached following the addition of the “uncoupler” molecule dinitrophenol

(DNP) and the minimum respiration reached with the addition of the ATP synthase inhibitor, oligomycin. The ratio of maximal OCR to the minimum OCR is referred to as the Respiratory Control Ratio (RCR) and is typically used as an indicator of the bioenergetic status of cells, with ratios of 5 or higher being excellent and 3 or less being abnormal. The fact that we routinely obtain ratios of >5 for both platelets and PBMCs isolated from healthy individuals indicates that our measurements have the sensitivity to detect abnormalities in severe TBI patients.

We also performed comparisons between respiration by platelets and PBMCs that were isolated within one hr of the blood draw and at 6 hr following the blood draw and found no difference. Therefore, if an early morning blood draw from the trauma genomics patients occurs at 4 am, we will still obtain accurate readings if the platelets and PBMCs are not isolated until 10 am.

Development of a Custom Nanostring Gene Expression Panel

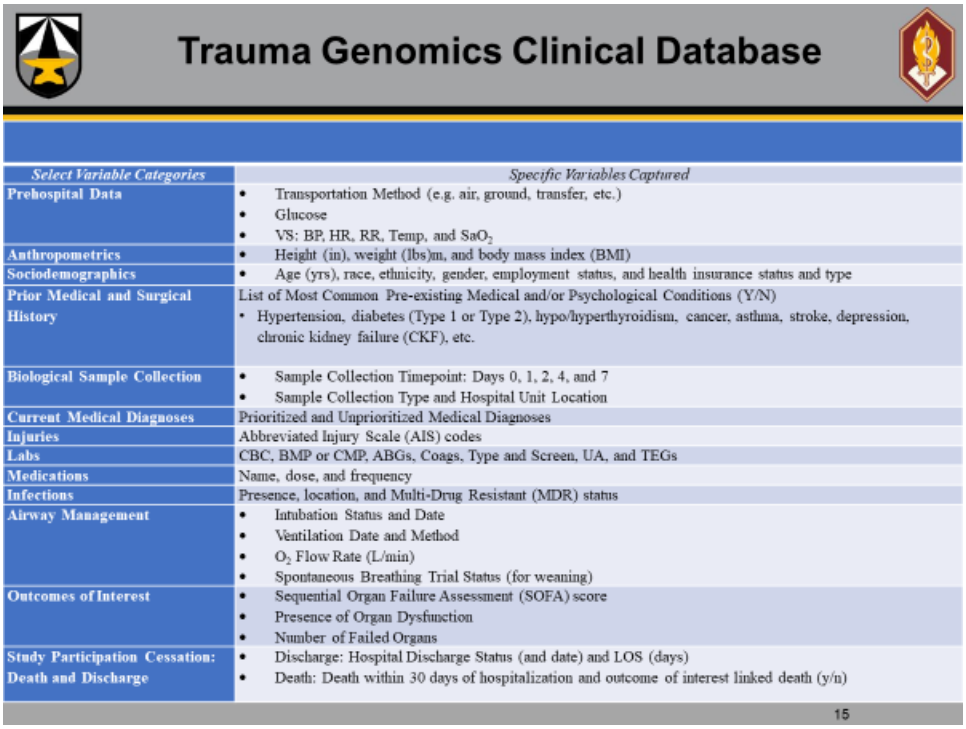
Nanostring offers several ready-made panels selective for different gene expression categories, e.g., Inflammation, Neuropathology, and Metabolic Pathways). The genes covered by these panels have their own annotations, meaning results include pathway analysis and cell typing. 50-base pair sequences are used to recognize the individual transcripts. Using a barcode method, transcripts are counted and presented as raw numbers. There is no amplification (such as with PCR), and there is relatively little need for technical replicates or qPCR validation (such as with microarrays).

Prior to developing our custom-made panel, we tested their Inflammation panel for coverage of genes and the consistency of technical replicates. We drew whole blood from two healthy donors and placed one ml from each sample in Tempus RNA tubes. Samples were processed by our University of Maryland Baltimore Center for Innovative Biomedical Resources core facility to obtain high quality, purified RNA. We also isolated PBMCs from the remaining samples and purified the PBMC RNA. In order to analyze variation within the Nanostring cassette, each RNA sample was run twice (technical replicates). There was a small variation between technical replicates based on how many transcripts were detected for each gene, which varied from <100 to >40,000. Fifty genes expressed <100 transcripts which varied between the two cassettes. In contrast, 80 genes were covered by more than 1000 transcripts, with less than 10% difference between the technical replicates. As expected the gene expression measured in isolated PBMC was different than that of whole blood. Also, there were differences between the whole blood and PBMC samples for the two donors, as expected.

Based on the successful test of the Nanostring Inflammation panel, we constructed our own custom-made panel covering 800 genes that have either been associated with trauma in general or that are very likely to be differentially expressed over one week following severe TBI. The gene expression pathways that are covered include the following: Inflammation, Cytokine Signaling, Cell Typing, Cell Stress, Hemostasis and Angiogenesis, Mitochondria, Organ Failure, and Sepsis (see Cuenca et al, 2013). The custom-made panels have been ordered and should arrive by March 2020.

Clinical Database:

The PIs and the clinical research staff for Shock Trauma Center have met to establish the database of clinical collected for each patient. A study database is under construction to house this abstracted data.



References

Cuenca AG, Gentile LF, Lopez MC, Ungaro R, Liu H, Xiao W, Seok J, Mindrinos MN, Ang D, Baslanti TO, Bihorac A, Efron PA, Cuschieri J, Warren HS, Tompkins RG, Maier RV, Baker HV, Moldawer LL; Inflammation and Host Response to Injury Collaborative Research Program. Development of a genomic metric that can be rapidly used to predict clinical outcome in severely injured trauma patients. Crit Care Med. 2013 May;41(5):1175-85. (PMID:23388514)

Klinedinst NJ, Schuh R, Kittner SJ, Regenold WT, Kehs G, Hoch C, Hackney A, Fiskum G. Post-stroke fatigue as an indicator of underlying bioenergetics alterations. J Bioenerg Biomembr. 2019 Apr;51(2):165-174. (PMID:30617735)

Malinow AM, Schuh RA, Alyamani O, Kim J, Bharadwaj S, Crimmins SD, Galey JL, Fiskum G, Polster BM. Platelets in preeclamptic pregnancies fail to exhibit the decrease in mitochondrial oxygen consumption rate seen in normal pregnancies. Biosci Rep. 2018 May 8;38(3). (PMID:29654168)

- **What opportunities for training and professional development has the project provided?**
  - Nothing to Report
- **How were the results disseminated to communities of interest?**
  - Nothing to Report
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - During the next reporting period, patient enrollment and sample collection will begin. We will begin working on Major Task 2 of the project.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
   - **What was the impact on the development of the principal discipline(s) of the project?**

- Nothing to Report.

- **What was the impact on other disciplines?**
  - Nothing to Report.
- **What was the impact on technology transfer?**
  - Nothing to Report.
- **What was the impact on society beyond science and technology?**
  - Nothing to Report.

5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
   - **Changes in approach and reasons for change**
     - Nothing to Report.
   - **Actual or anticipated problems or delays and actions or plans to resolve them**
     - IRB approval and the study initiation period has taken far longer than originally anticipated. Because MRMRC IRB has deferred to the AFRL IRB, and the AFRL IRB has deferred to the UMD IRB, all future IRB actions will be relatively expeditious in comparison. We were initially extremely conservative in our estimates of yearly patient collection rates and hope that patient recruitment will be far quicker than our original estimate, and the project will get back on track. If we have any issues with patient recruitment or consent, we are already exploring the idea of a waiver of written consent and contacting the LAR by telephone to increase patient enrollment.
   - **Changes that had a significant impact on expenditures**
     - Nothing to Report.
   - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
     - Nothing to Report.
   - **Significant changes in use or care of human subjects**
     - Nothing to Report.
   - **Significant changes in use or care of vertebrate animals.** N/A
   - **Significant changes in use of biohazards and/or select agents**
     - Nothing to Report.
6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**
     - **Journal publications.**

        - Nothing to Report.
    - **Books or other non-periodical, one-time publications.**
        - Nothing to Report.
    - **Other publications, conference papers, and presentations.**
        - Nothing to Report.
- **Website(s) or other Internet site(s)**
    - Nothing to Report.
- **Technologies or techniques**
    - Nothing to Report
- **Inventions, patent applications, and/or licenses**
    - Nothing to Report.
- **Other Products**
    - Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
    - *Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."*

Project Directors (PDs)/PIs:

- Gary Fiskum, PhD – UMD PI - 10% effort, 1 person month
    - Dr. Fiskum has been the UMD site PI coordinating the effort between all of the UMD personnel and overseeing the standardization of the collection assays.
- Catriona Miller, PhD – USAF PI 10% effort, 1 person month.
    - Dr. Miller has been the USAF site PI, coordinating the effort between UMD and the USAF. Dr Miller has done extensive work on the IRB application, interfacing between the multiple regulatory boards, and assisting with all reporting requirements.
    - USAF Civilian, not funded from study.
- Jennifer Klinedinst, PhD – 40% effort. Co-PI, 4.2 person months.

- Dr. Klinedinst has taken the lead on all of the regulatory affairs work on this study, including the drafting of the IRB protocol. She is supervising and training Dr. Georgette Cox, the research coordinator on this study.

- Deborah Stein, MD – 0% effort
  - Dr. Deborah Stein has accepted a position at UCSF and has left Shock Trauma Center. She will remain on the project as a PI and we are establishing a subcontract with UCSF for her salary support and effort. Dr. Sam Galvagno, DO, PhD has been added to the project as a PI to have a local medical doctor as part of the team.
- Claire Fraser, PhD 0% effort. Co-PI, 0.0 Study Months.
  - Dr. Fraser's effort on this study will start once sample collection begins. She has been attending study meetings and engaged in study design.
- Dr. Sam Galvagno, DO, PhD 2% effort. .02 Study months.
  - Dr. Sam Galvagno's effort on this study will ramp up once patient collection has started.
- Dr. Rosemary Kozar, MD, PHD, 5% effort, .05 study months.
  - Dr. Kozar's effort on this study will ramp up once patient collection has started.

Other Personnel:

Brian Polster – Co-PI 5% 0.5 study months.
Nupur Dutta Chowdhury – 20% effort 2 study months – USAF Research Coordinator
Ms. Chowdhury prepared the IRB application for the USAF for submission.
USAF Line Funded.
Georgette Cox – 100% effort. UMD Research Coordinator 8 Study months.
Georgette Cox is the research coordinator who is managing the study on the UMD side. She is responsible for overall study coordination. She is currently training UMD 24-7 collection staff.
Parisa Rangghran – 50% effort. Laboratory Technician, 5.3. study months.
Parisa has taken the lead on PBMC cell isolation and the seahorse bioscience measurements.
Apurva Borcar – 50% effort, POST Doc. 5.3 study months.
Apurva Borcar has taken the lead on the Nanostring assay development.
Tarek El-Maghrabi – 1 study month. Summer Student.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report.
- **What other organizations were involved as partners?**
  - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*

- *Describe partner organizations - academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) - that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed. Provide the following information for each partnership:*
    - This project has been contracted to the University of Maryland, School of Medicine to collect patient samples at the R. Adams Cowley Shock Trauma Center.
    - **Organization Name: University of Maryland, School of Medicine**
    - **Location of Organization:** *Baltimore, MD 21202*
    - **Partner's contribution to the projec**t
        - **Facilities**

8. **SPECIAL REPORTING REQUIREMENTS**
    - **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ebrap.org for each unique award..*
9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***

UNIVERSITY OF MARYLAND BALTIMORE (UMB)
TRAUMA GENOMICS STUDY
STANDARD OPERATING PROCEDURES (SOP) MANUAL

PURPOSE OF SOP MANUAL

This manual acts as a guide for all lab-based processes and procedures used to process all biological specimens collected for the UMB trauma genomics study. Additionally, this SOP provides some contextual information about the clinical research study and how other non-experimental data will be collected.

This manual may be periodically reviewed and edited by the PI and lab staff to ensure all lab processes and procedures 1) meet manufacturer guidelines and recommendations, 2) are reflective of the current technology, 3) are scientifically valid, and 4) are conducted in a safe, practical manner.

INVESTIGATOR LIST (AS OF 07/25/2019) *

Principal Investigators:
Gary Fiskum, PhD (UMB School of Medicine)
Jennifer Klinedinst, PhD, MPH, RN (UMB School of Nursing)
Rosemary Kozar, MD, PhD (UMB School of Medicine)

Clare Fraser, PhD (UMB Institute of Genomic Sciences)
Brian Polster, PhD (UMB School of Medicine)
Deborah Stein, MD (UCSF School of Medicine)
Catriona Miller, PhD (Director of Research Initiatives, US Air Force c-STARS Baltimore)

Co-Investigators (co-Is):
Sam Galvagno, DO, PhD (UMB School of Medicine)
Brian Polster, PhD (UMB School of Medicine)
**Investigator list may be subject to change*.

STUDY TEAM CONTACT LIST AND INVESTIGATOR ROLES/RESPONSIBILITIES

STUDY TEAM CONTACT LIST

TABLE 1.
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UCSF	Deborah Stein, MD		(415) 206-4623	Deborah.Stein@ucsf.edu
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UMB SOM	Rosemary Kozar, MD, PhD		(410)328 3495	rkozar@som.umaryland.edu
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	Apu Borcar, PhD	Postdoctoral Fellow	(410) 706-3418	aborcar@som.umaryland.edu
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USAF C-STARS	Nupur Chowdhury, MS	Regulatory Clinical Research Coordinator	(410)328-4181	nchowdhury@som.umaryland.edu

Shock Trauma and Anesthesiology Research (STAR) Group	Mark Scarboro, BS	Director, STAR	(410)328-8713	mscarboro@som.umaryland.edu
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	Seeta Kallam, MBBS	Regulatory Clinical Research Coordinator, STAR	(410) 328-7491	skallam@som.umaryland.edu

STUDY INVESTIGATOR ROLES AND RESPONSIBILITIES

TABLE 1a.
TRAUMA GENOMICS RESEARCH PROJECT PRINCIPAL INVESTIGATOR (PI) ROLES

Name	Department/Affiliation	Expertise	Potential Project Role
Gary Fiskum, PhD	UMB SOM	• Extensive independent research in cell energy metabolism • Interpretation of results obtained from these assays	• Regulatory affairs, safety, data management and sharing, personnel management, communication between Co-PIs, Dr. Polster (Sub-Investigator) and Statistician, progress reports, approval of public meeting abstracts and presentations, and approval of manuscripts for publication
Jennifer Klinedinst, PhD, MSN, MPH, RN	UMB SON	• Extensive research experience with clinical populations including stroke survivors, cognitively impaired older adults, and other adult populations	• Oversight of regulatory affairs, recruitment, data collection, data management, data analysis and dissemination. Communication between Co-PIs and Statistician, progress reports, approval of public meeting abstracts and presentations, and approval of manuscripts for publication. She will supervise the Lead Clinical Research Specialist dedicated to this project.
Catriona Miller, PhD	USAF	• Microbiology • Immunology	• Regulatory affairs, safety, data management and sharing, personnel management, communication between Co-PIs and Statistician, progress reports, approval of public meeting abstracts and presentations, and approval of manuscripts for publication. She will also serve as a direct line of communication to the

			Air Force Research Labs.

TABLE 1b.
TRAUMA GENOMICS SUB-INVESTIGATOR ROLES

Name	Department/Affiliation	Expertise	Potential Project Role
Rosemary Kozar, MD, PhD	UMB Medicine	• Surgery	• Provides medical expertise for patients diagnosed with severe TBI • May provide additional revisions for medical eligibility screening and defining any adverse events (AEs) • May encourage clinical staff adherence to specific research protocol areas • Responsible for overseeing staff education, patient enrollment, specimen collection, and clinical data collection and analysis.
Brian Polster, PhD		• Neuroscience • Blood Cell Bioenergetics	• Provides additional scientific expertise on bioenergetic and mitochondrial oxygen consumption • Directly trains and supervises the research assistant who will conduct these assays
Sam Galvagno, DO, PhD		• Neurocritical Care Medicine • Anesthesiology	• Provides medical expertise for patients diagnosed with severe TBI
Deb Stein, MD	UCSF Medicine	• Surgery	• Provides medical expertise for patients diagnosed with severe TBI • Responsible for overseeing staff education, patient enrollment, specimen collection, and clinical data collection and analysis • Regulatory affairs, safety, data management and sharing, personnel management, communication between Co-PIs and Statistician, progress reports, approval of public meeting abstracts and presentations, and approval of manuscripts for publication

Claire Fraser, PhD	UMB IGS	• Genomic Analysis • Pharmacology	• Provides additional scientific expertise and oversight re: genomic analyses conducted at IGS
Hegang Chen, PhD	UMB SOM	• Biostatistics	• Analyzes all clinical and laboratory data

STUDY LENGTH

The trauma genomics project was approved for a total of 3 years (2019-2021).

TARGET SAMPLE SIZE

N= 240 (target enrollment)

STUDY RATIONALE

An estimated 35% (n=85) will meet the I/E requirements specific for bioenergetic analysis.

Traumatic brain injury (TBI) is a leading cause of death, contributing up to 30% of all injury deaths in the United States. Between 2000 and 2016, 352, 612 U.S. military members have been diagnosed with TBI. Sepsis and related organ dysfunction are common after severe TBI. TBI activates a complex inflammatory response cascade whereby various processes and inflammatory mediators are released into the blood. Variability in the gut microbiome can influence this process. The net effect is that the immune system is depressed, and the ability of cells throughout the body to make cellular energy can be compromised. Our study team hypothesizes that gene transcription, gut microbiome variability, and cellular bioenergetics can serve as biomarkers during the first week following a TBI event. These biomarkers may be useful in predicting whether patients will experience sepsis or any organ dysfunction.

STUDY AIMS

- **Aim #1:** Discover if there is a temporal relationship between blood cell transcriptome and sepsis risk in severe TBI ICU patients
- **Aim #2:** Advance what we currently know about gut microbiome and sepsis risk in severe TBI ICU patients
- **Aim #3:** Discover if there is a temporal relationship between blood cell energy metabolism and sepsis risk in severe TBI ICU patients
- **Aim #4:** Generate a repository of human blood samples w/clinical data from severe TBI ICU patients

INCLUSION/EXCLUSION (I/E) CRITERIA

Inclusion:

- Adult (aged 18 y/o or older)
- Speaks English
- Admitted to One of Three UMM ICUs: Neuro ICU, Trauma Resuscitation Unit (TRU), or Multi-Trauma
- Positive CT scan for intracranial traumatic pathology

Exclusion*:

- Determination as non-survivable by neurosurgical team upon admission
- Transferred from another hospital more than 24 hours after head injury
- Hemodynamically unstable (determined by clinical team)
- Weighs less than 110 lbs.
- Prisoner
- Pregnant**

***NOTE: The exclusion criteria may be subject to change based on certain factors (i.e. feasibility, scientific validity, etc.).**

**may be confirmed with positive β HCG test via blood or urinalysis (UA) in electronic medical record (EMR)

STUDY PARTICIPANT RECRUITMENT PROCESS OVERVIEW

This study must first be approved by two Institutional Review Boards (IRBs) before any human subjects research can begin. Our study is currently approved by the UMB IRB and is pending approval from the US Air Force IRB. A total of 240 adult patients diagnosed with severe TBI will be recruited from three University of Maryland Medical Center (UMM) intensive care units (ICUs): Shock Trauma Resuscitation Unit (TRU), Multi-trauma, and Neuro Trauma.

Potential study participants will be screened for medical eligibility by the UMM Shock Trauma and Anesthesiology Research (STAR) team (**please see below for "Medical Eligibility Screening" for more information**). Any medically eligible adult patient will be approached by the STAR team to undergo informed consent (**please see below for "Informed Consent Process" for more information**). This study has a 48-hour waiver of HIPAA authorization. STAR team members can prescreen potential patients for medical eligibility, enroll them, and collect biological specimens for 48 hours without having the informed consent form (ICF) signed by either the patient or the legally authorized representative (LAR). However, the consent form needs to be signed and dated by either the patient or LAR within 48 hours of admission. If either the patient or the LAR refuse, all their samples will be properly discarded and not included for data analysis. Patients or LARs that sign the ICF will be assigned a participant ID number. Given the severity of the patient's diagnosis, it is highly likely that most ICFs will be signed by the LAR instead of the patient. Neither the patient nor the LAR will be compensated for study participation since biological sample collection is part of routine care while admitted.

YEARLY ENROLLMENT TARGET

Year 1 (2019): 70 participants
Year 2 (2020): 100 participants
Year 3 (2021): 70 participants

RESEARCH TEAM TRAINING

Before conducting medical eligibility screens or informed consent, all STAR staff, principal investigators, sub-investigators, and key clinical research staff will have received human subjects research (HSR) training. Evidence of HSR training, usually completed either through the Collaborative Institutional Training Initiative (CITI) or US National Institutes of Health (NIH), will be maintained by both the STAR team and trauma genomics research team. Certificates or completion reports of HSR related training will be maintained on the trauma genomics shared drive and updated accordingly.

MEDICAL ELIGIBILITY SCREENING

The STAR team will use the UMM Research Management System (RMS) to screen all patients admitted to the 3 ICUs (i.e. neuro ICU, multi-trauma, and TRU). RMS is computer-based system designed to interface with the UMM electronic

INFORMED CONSENT PROCESS

medical record (EMR) system. RMS will be built specifically to screen all ICU patient admits based on pre-programmed study inclusion/exclusion criteria. RMS will conduct a global screen based on surface sociodemographic characteristics. Afterwards, RMS will conduct a study specific screen using I/E criteria. Any patients meeting all inclusion criteria and having no exclusion criteria will be contacted by STAR while in the ICU. STAR will keep track of a recruitment log and send the research team weekly updates of the recruitment log as well as any associated reports.

All STAR staff and study team members interacting with potential participants will be trained to obtain informed consent. Information about the study will be provided to patients and/or LARs in a private setting, e.g. inpatient or exam rooms or office. Doors will be closed to prevent interruption or others overhearing conversations.

Patients who are eligible for this protocol are critically ill and, in many cases, not able to provide informed consent due to their medical condition. For each potential participant, the research staff member will complete an evaluation of the patient's capacity to give consent or assent using the "**Evaluation to Sign Consent**" form. If the potential participant is unable to give consent but assents to participation or is unable to assent, the LAR will be identified and approached to obtain consent. The identification of the LAR is based on information obtained from the participant and the participant's family. The LAR's ability to provide legally effective informed consent is based on the relationship of the surrogate to the research participant as outlined in the "**Legally Authorized Representative Identification Form**".

The patient or LAR, on behalf of the patient, will be asked if he/she would be interested in participating in this research study. Patients/LARs will receive a comprehensive explanation of the proposed study, including rationale, study procedures, risks and benefits, alternative therapies, and other essential elements of informed consent in accordance with applicable Federal Regulations. In addition, patients/LARs will be asked if they want to consult with others. Patients/LARs will be told that participation is voluntary, and refusal to participate or withdrawal from participation will have no bearing on the care the patient will receive. Potential participants/LARs will be allowed sufficient time to ask questions and to consider participation. The STAR staff will ask the patient if he/she would like additional time to consider participation before signing the consent. If the patient expresses understanding and willingness to participate, the consent form will be signed at that time. Participants will be given a copy of the signed consent form, and a copy will be kept on file in the research record. If the prospective participant is not certain or declines participation, the team members will thank the prospective participant and not enroll him/her.

The STAR staff will explain that the LAR should decide based on what they think is in the patient's best interests. It will be stressed during the process that participation is voluntary and that deciding not to be in the study will not impact the patient's care in any way. The IRB approved LAR Authorization Form will be used to document the appropriate LAR has been identified. If a patient is enrolled under LAR consent, the patient will be reevaluated at intervals until discharge to determine if they have regained capacity to sign consent. Prior to discharge, if the patient regains capacity to consent (after the LAR provides consent) and does not agree with participating in the study, the participant will be withdrawn from the study. All samples collected will be destroyed. If the LAR or participant withdraws from the study after consent has been obtained, all used samples and data collected up to that point will be retained and used in the study. His/her unused remaining specimens (and any extant materials derived from it, including RNA, isolated platelets/blood cells) will be destroyed. No further samples will be collected. If the LAR or participant does not give consent for samples collected pursuant to a consent waiver, all samples will be destroyed.

The patient or patient's LAR will be approached as soon as feasible. If the participant is unable to give consent and does not have an LAR with him/her on admission as is often the case, we are requesting a waiver of consent to collect blood and stool specimens for the first 48 hours after admission Day 0 and 1 samples. This will allow for the collection of a maximum of 2 blood samples (totaling 38 mLs- 19 mLs each draw), and fecal swab on admission without consent. Study procedures completed during this 48-hour time period would fit under the qualifications of minimal risk (< 50 mLs of blood and fecal swab is non-invasive and done clinically) and would allow us time to locate the LAR, explain the study, and obtain consent.

Because the blood cell bioenergetics must be analyzed within 4 hours of sample collection, bioenergetics data will be generated. No genomic or microbiome research will be performed on the sample or data of patients enrolled under a waiver until consent is obtained. If the waiver is granted, and a patient is enrolled under the waiver, we will continue to pursue identification and obtaining consent from the patient's LAR. If consent is obtained from the LAR, study procedures will continue. If the staff does not obtain consent from the LAR or the patient before 48 hours or death, the patient will be withdrawn from the research and the samples and bioenergetics data will be destroyed. If the LAR declines, then the participant is withdrawn from the study all samples collected and bioenergetics data will be destroyed. It is critical to the

outcomes of the study to have blood and stool samples from admission to be able to compare results from time of the acute injury and as time progresses. This study cannot reasonably be done without this waiver of consent. We realize that the amount of blood collected for the duration of the study will ultimately be >50 mls and that reduces consideration for minimal risk. But we believe the procedures done under the 48-hour waiver of consent would be considered minimal risk. In addition, because these patients will all be admitted to the shock trauma ICU, their hemodynamics and blood volume will be closely monitored. We will not obtain blood from any participants who are hemodynamically unstable as per clinical judgement.

If the LAR gives consent for participation, the patient will be enrolled in the study and study procedures will commence. The patient will be reevaluated at study day intervals (Day 1, 2, 4 and 7) until discharge to determine if they have regained capacity to sign consent. If they do not regain capacity, the patient will remain enrolled in the study based on the LAR consent. However, if the patient regains capacity to consent, they will go through the consent process. If the patient declines participation, all samples and bioenergetics data will be destroyed. Minimal administrative documentation supporting the patient's enrollment under waiver, efforts to consent, and sample collection dates/times will be retained to support research procedures were done per protocol and to maintain study enrollment statistics for reporting. No patient medical information will be retained. If the patient agrees, sample collection, storage and use of samples and data will proceed per protocol.

During the informed consent process, participants will be asked if they may be re-contacted in the future to provide additional information or for participation in other research. Participants who allow recontact may decline providing additional information for any reason.

NOTIFYING RESEARCH STAFF OF PARTICIPANT ENROLLMENT AND SAMPLE COLLECTION

The STAR team will notify designated trauma genomics research team members of:

- Successfully screened patients for the day
- Whether informed consent could be obtained that day (i.e. ICF signed and dated by patient or LAR)
    - If ICF couldn't be signed, when they will approach families again
- When whole blood and stool have been collected- **EXTREMELY IMPORTANT DUE TO TIME SENSITIVE NATURE OF EXPERIMENTS**
- Where specimens have been temporarily stored in the STAR lab after post AM draw- **EXTREMELY IMPORTANT DUE TO TIME SENSITIVE NATURE OF EXPERIMENTS**
- When participant screening and enrollment logs can be generated for the week
- When and if any additional reports are generated by RMS

Important report information needed: screening/enrollment logs (# Screened, # Approached, #Ineligible, # Enrolled, #Withdrew, Reasons for Ineligibility, and/or Withdrawals)

Updates on patient status up to 30 days from admission: death, hospital-based transfer (location of hospital if known), etc.

DATA COLLECTION TIMEPOINTS

Biological

Sample collection: 5 timepoints during participant's first week of hospital admission (Days 0 (Admission Day), 1, 2, 4, and 7)
Medical Record Data: Follow study participants for up to 30 days post hospital admission

DATA STORAGE AND RECORDKEEPING

All printed research documents will be stored in binders kept in a locked file cabinet. The locked cabinet is in the SOM lab research office. Additionally, while the office is not in use, the office will be locked.

Any documents containing PHI will be uploaded to an encrypted University of Maryland Baltimore SOM based shared drive only viewed by the SOM research team. If such documents are printed, they will be disposed of in the designated

Shred-It bins used to dispose of confidential documents. Printed and electronic records will be updated and maintained as needed by the SOM clinical research specialist. Medical record data collection files will have restricted access with only key SOM clinical research team provided access. Lab personnel and associated staff will not have access to the medical record data.

MEDICAL RECORD DATA ABSTRACTION

All enrolled participants will be followed for up to 30 days post-admission. From the UMM electronic medical record, various patient-level biometric and health outcome data will be extracted. These patient-level outcomes and experimental data will be compiled into a REDCap™ database. This database will be maintained on UMM encrypted shared drive.

Potential data to be collected are:
Prior to UMM Arrival (w/in 24 hours)

Clinical and Medical Lab Data (Electronic Medical Record):

- **Sociodemographic**:
  - Age, Race/Ethnicity, Gender, Employment, and Insurance Status/ Type
  - Pre-existing Medical Conditions (T2DM, Cancer, Hyper/Hypotension, Cardiovascular Disease, Coagulopathy/Clotting Disorders, etc.)
- **Anthropomorphic:** Height (ft., in.,), Weight (lbs.), and Body Mass Index (BMI)
- **Vital Signs:** blood pressure (BP), pulse (bpm), temperature (°F), respiratory rate (RR), and pain score
- **Health History:** Diagnoses, Prior Medical History, and Prior Surgeries
- **Injury Information:** Time of Injury (injury onset), Injury Type (Mechanism of Injury/Specific Injury), etc.
- **Current Infections**
- **Clinical Lab Values/Lab-based Indices:**
  - **Intracranial Pressure (ICP)**
  - **Complete Cell Blood Count (CBC) w/differential***
  - **Arterial Blood Gas Panel:** blood pH, $PaCO_2$, PaO_2, HCO_3 (bicarbonate), and O_2 saturation
  - **Sequential Organ Failure Assessment (SOFA) score** (Multiple Organ Dysfunction: SOFA Score ≥ 3)
    - *Respiratory*: Fractured Inspired Oxygen (FiO_2)- **ABG PANEL**
    - *Coagulatory*- Platelets ($x10^3$ µL)- **CBC**
    - *Liver*: Bilirubin (mg/dL) – **LIVER PANEL**
    - *Cardiovascular*: Mean Arterial Blood Pressure (**calculated from SBP/DBP**)
    - *Renal*
      - Creatine- **KIDNEY PANEL**
      - Urine Output **(optional)- NURSING NOTES (amount voided in ml/day)**
    - *Neurological*- Glasgow Coma Scale (GCS) Score**- **NEURO EXAM, NEUROSURGERY SERVICE**
    - **Toxicology:** TCAs, Opioids, etc.
    - **Current Medications and Antibiotics** (dose, frequency, and route)- **MEDICATION ADMIN. RECORD (MAR)**
      Some examples are:
      - Broad spectrum Abx (vancomycin, etc.)
      - Sedatives (propofol, etc.)
      - Anti-Emetics
      - Pain: Opioids, etc.
      - Vasoactives: Dopamine, Epinephrine, and/or Norepinephrine
    - **Procedures and Procedure Dates**
      Some examples are:
      - Surgeries
      - Wound Care Therapy
      - Line Placement and Site

- **Length of Hospital Stay** (days)
  - Hospital Admission Date
  - Hospital Discharge Date
- **Hospital Transfer Status** (Y/N)
  - Hospital/Medical Center Name (if known)
- **Airway Management**
  - **Intubation** (date/time, frequency, etc.)
  - **Ventilator Status** (hours)
    - Weening Process (y/n) (standardized on ICU floor- one weening procedure)
- **Blood Volume/Composition Management**
  - Hydration Therapy (IV saline/Lactated Ringer solution (units))
  - Transfusion
    - Composition: Whole Blood, FFP, Cryoplatelets, RBCs, etc.
    - Volume (L)
    - Time
    - Crystallization Present
      - If yes, volume present

Lab Experimental Data (SOM Lab, CIBR Biorepository, and Institute for Genomic Sciences):

a) Bioenergetics: Platelets and Peripheral Blood Mononuclear Cells (PBMCs)

- **Oxygen Consumption Rate (OCAR)**
- **Extracellular Acidification Rate (ECAR)**

b) Blood Cell Gene Expression (Biorepository)

- **DNA Isolation from Whole Blood**
- **RNA Isolation from Whole Blood**
  - **Micro RNA (miRNAs) from Exosomes**

c) Genomic Analysis

- **Stool DNA Sequencing**

***Automated differential only.**
****GCS must be calculated when patients are NOT on sedatives.**

LIST OF TESTS FOR SAMPLE ANALYSIS (as of 05/17/2019)

- **Blood Cell Gene Expression**: DNA and RNA Isolation- tests completed by UMM Center for Biomedical Innovation (CIBR) Biorepository
- **Bioenergetics:** Peripheral Blood Mononuclear Cells (PBMCs) Isolation- tests completed by UMB SOM Anesthesiology Research Lab
- **Bioenergetics:** Platelets Isolation – tests completed by UMB SOM Anesthesiology Research Lab
- **Gut Microbiome Analysis:** Genomic Analysis – tests completed by UMB Institute of Genomic Sciences (IGS)

BIOLOGICAL SAMPLE COLLECTION

Only whole blood and stool will be collected from ICU patients during their first week of hospital admission.

NOTE: Stool will be collected upon admission as part of a rectal exam. The glove should be swabbed using the swab found in the sample kit (**please see "Sample Collection Kits" below**). After the first day, fecal samples can be obtained swabbing the inside of a patient's rectal tube or fecal containment unit. The clinical care team will determine which method is most appropriate for each patient.

SCHEDULE OF BIOLOGICAL SAMPLE COLLECTION

TABLE 3.
SCHEDULE OF BLOOD AND STOOL SAMPLE COLLECTION

Day 0 (ADMISSION DAY)	Day 1	Day 2	Day 4	Day 7
-1 Tempus tube -2 EDTA tubes -1 Fecal Swab using Norgen Biotek Tube (swab glove from fecal exam)	-1 Tempus tube -2 EDTA tubes -1 Fecal Swab using Norgen Biotek Tube (only if patient spontaneously defecates- swab fecal containment unit/fecal contents)	-1 Tempus tube -2 EDTA tubes -1 Fecal Swab using Norgen Biotek Tube (only if patient spontaneously defecates- swab fecal containment unit/fecal contents)	1 Tempus tube -2 EDTA tubes -1 Fecal Swab using Norgen Biotek Tube (only if patient spontaneously defecates- swab fecal containment unit/fecal contents)	-1 Tempus tube -2 EDTA tubes -1 Fecal Swab using Norgen Biotek Tube (only if patient spontaneously defecates- swab fecal containment unit/fecal contents)

SAMPLE COLLECTION, PROCESSING, AND STORAGE

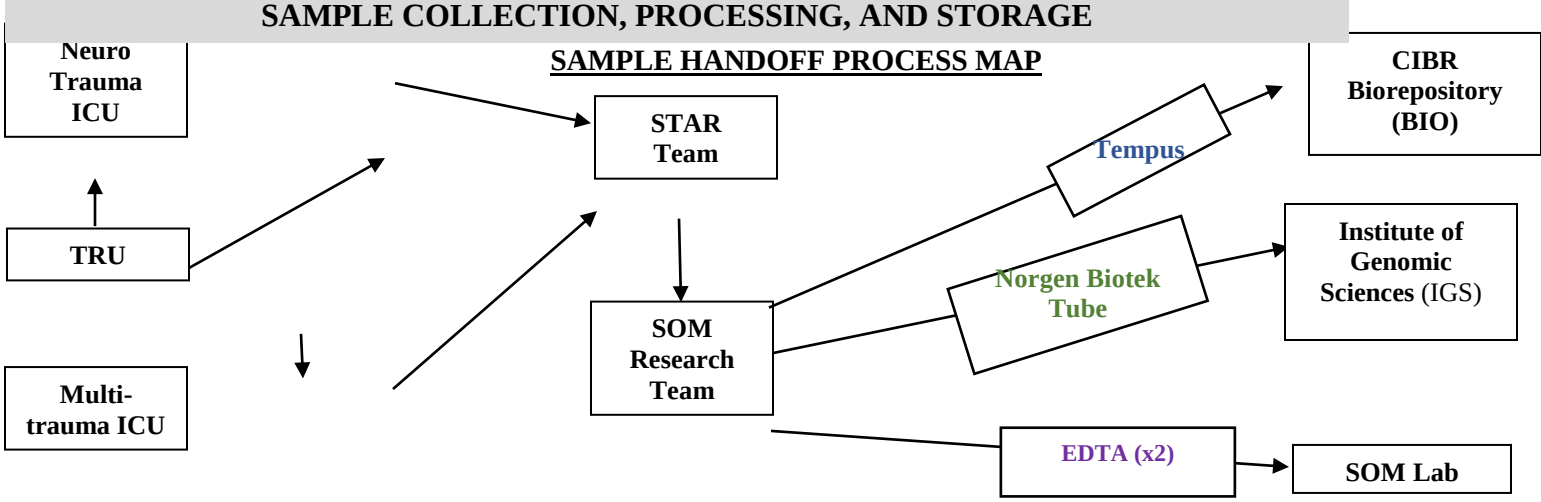


TABLE 4.
SAMPLE COLLECTION, PROCESSING, AND STORAGE

Sample Type	*Isolation Step? (Y/N)*	*Test*	*Collection Tube*	*Initial Collection*	*Processing*	*Storage Location*
Whole Blood	**YES** DNA isolated in solution	**DNA RNA**	**Tempus (Blue top)**	**On ICU Floor by Nurses* *SOM will pick up samples from STAR**	**Minor Prep in STAR/SOM w/final processing in BIO**	**BIO**
	YES- RNA isolated in solution					
	YES – Platelets (Platelet Rich Plasma- PRP)	**Bioenergetics: O_2 Consumption**	**EDTA (Purple top)**		**SOM**	
	YES- Buffy Coat (PBMCs)					

Fecal (Stool)	**No**	**Genomic Analysis**	**Fecal Swab (Norgen Biotek Tube)**		**IGS**	**BIO (temporary), then IGS**

KEY
IGS: Institute of Genomic Sciences
SOM: University of Maryland Baltimore School of Medicine, Anesthesiology Research Lab
BIO: University of Maryland Baltimore School of Medicine Biorepository

SAMPLE COLLECTION KITS

Each kit= 1 Tempus Tube + 2 EDTA tubes+ 1 Norgen Biotek Tube + Study Participant Labels+ Specimen Checklist placed in 1 Biohazard bag

Study Participant labels will include participant ID # (sequential order), date and time of sample collection, and study timepoint (e.g. Day 0, 1, 2, 4, or 7)
Specimen Checklist includes checkboxes for each tube and rationale for missing specimens

Vacutainer Attachment (1 per kit)

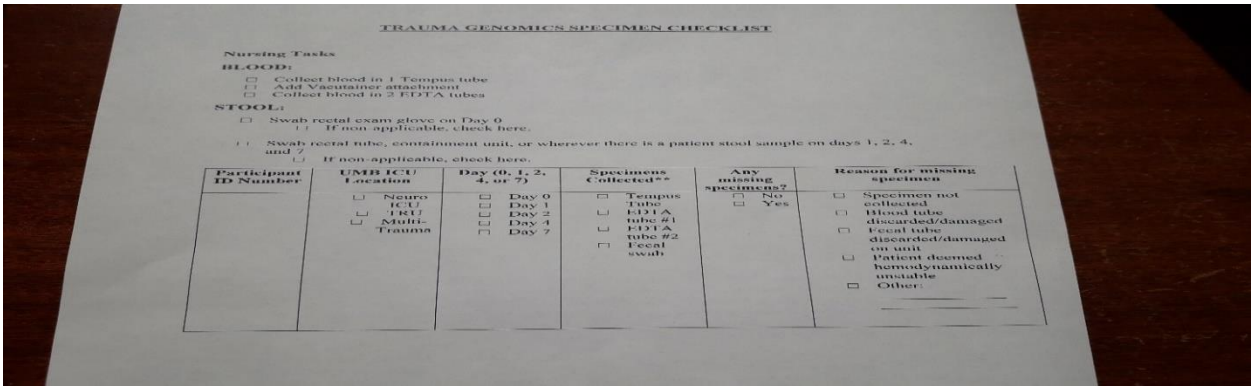
Tempus Tube (1 tube per kit)

EDTA Tubes (2 per kit)

Study Participant Labels

- Study Protocol Number/Name
- Participant ID # and Study Timepoint (up to 4 digits+ 1 digit
  - **Example: 001-4**
- Date
- Time of Draw

(4 per kit)



TRAUMA GENOMICS SPECIMEN CHECKLIST

Nursing Tasks

BLOOD:

- ☐ Collect blood in 1 Tempus tube
- ☐ Add Vacutainer attachment
- ☐ Collect blood in 2 EDTA tubes

STOOL:

- ☐ Swab rectal exam glove on Day 0
  - ☐ If non-applicable, check here.
- ☐ Swab rectal tube, containment unit, or wherever there is a patient stool sample on days 1, 2, 4, and 7
  - ☐ If non-applicable, check here.

Participant ID Number	UMB ICU Location	Day (0, 1, 2, 4, or 7)	Specimens Collected**	Any missing specimens?	Reason for missing specimen
	☐ Neuro ICU ☐ TRU ☐ Multi-Trauma	☐ Day 0 ☐ Day 1 ☐ Day 2 ☐ Day 4 ☐ Day 7	☐ Tempus Tube ☐ EDTA tube #1 ☐ EDTA tube #2 ☐ Fecal swab	☐ No ☐ Yes	☐ Specimen not collected ☐ Blood tube discarded/damaged ☐ Fecal tube discarded/damaged on unit ☐ Patient deemed hemodynamically unstable ☐ Other:

Specimen Checklist (1 per kit)

One Biohazard bag

Norgen Biotek Fecal Collection Tube (1 per kit)

Each sample collection kit includes one Tempus Tube, two EDTA tubes, one fecal swab, participant labels, specimen collection checklist, and a separate **vacutainer attachment.** **The vacutainer attachment helps prevent backflow/contamination of Tempus tube reagent into EDTA tube. This will be attached after drawing blood into the Tempus tube.**

ICU NURSES: ORDER OF BLOOD DRAW AND TUBE LABELLING

ORDER

OF DRAW: Tempus tube first, add new vacutainer attachment, then EDTA tubes last

1st step: Take out the labels in the biohazard bag.
2nd step: Write in the time of draw and date on each label.

3rd step: Draw blood into the Tempus tube 1st **(BLUE TOP)**
4th step: Shake the Tempus tube (BLUE TOP) vigorously for 10 seconds to ensure proper mixing. When you shake, make sure to invert the tube.
5th step: Unscrew the old vacutainer attachment.
6th step: Take the new vacutainer attachment out of the biohazard bag and attach it**.**
7th step: Draw blood into the EDTA tubes next **(PURPLE TOP)**
8th step: Place all labeled tubes into biohazard bag.
9th step: Fill out the specimen checklist that all tubes have been drawn. If any tubes are missing, check the reason why. Also, tell the STAR team what happened (i.e. patient hemodynamically unstable, etc.).

NOTE: Please make sure you write the blood draw time is on the tube label. The test for the EDTA tubes is time sensitive.

FECAL (STOOL) SAMPLE COLLECTION

ON DAY 0

1st step: Take the Norgen tube out of its casing.
2nd step: Use the cotton applicator tip to swab the outside of the glove used for the patient's fecal exam.
3rd step: Place the tube back into its casing.
4th step: Write the date and time of swab on the label.
5th step: Apply the label to the tube and place the labeled tube in the biohazard bag.

If you are unable to get a swab (e.g. no fecal exam done, etc.), check the reason why. Also, tell the STAR team what happened.

DURING ADMISSION WEEK (DAY 1, 2, 4, and 7)
Same as steps above, except you may not swab a glove. You may need to swab a fecal containment unit or tube depending upon how stool is collected for your patient.

STAR TEAM: TUBE LABELLING (AFTER BLOOD DRAW AND SWABBING)

1st step: Ensure that all blood tubes have specimens for the participant (i.e. 2 EDTA (pink/purple) tops and 1 Tempus (blue) tube.

If specimens are missing, please ask the nurse what happened. Write this information on the "**Specimen Collection Checklist**" (i.e. form inside the biohazard bag pouch).

2nd step: Ensure that fecal swab has been collected. Remind the ICU nurse that we are collecting stool during the 1st week of admission.

If patient did not defecate yet, write this information on the "**Specimen Collection Checklist**".

3rd step: Write the date and time of sample collection on the tube labels if this was not completed by the ICU RN. Fill in any information on the study participant label that is missing.

4th step: Verify that the patient's sticker has been affixed to the "Specimen Collection Checklist" at the upper right corner for each sample kit before you leave the ICU floor.

5th step: Notify the anesthesiology lab team that sample kit has been collected and is ready for pickup.

Try to get as much information as you can from each ICU RN that has done the sample collection before you leave the ICU floor. You can fill this information in the notes section of the specimen checklist.

STAR TEAM: LAB TEAM CONTACT FOR SAMPLE PICKUP

Please page Dr. Georgette Cox at (410) 232-8010 and send a notification email for sample pickup (specifically number of kits collected/patients). Dr. Cox is the primary designee for sample pickup. Certain study team lab members (i.e. Dr. Borcar

and Ms. Parisa Rangghran of the anesthesiology lab) may pick up blood and stool samples in Dr. Cox's absence (please see "**Study Team Contact List**", page 2). Email and calling may be the best modes of contact for other study team lab personnel. Leslie Sult, RN, STAR staff supervisor, will be notified when Dr. Cox is out of the office.

LAB STANDARD OPERATING PROCEDURES

NOTE: ONLY USE BENCHES AND MATERIALS (I.E. PIPETTES, RACKS, ETC.) SPECIFIC FOR HUMAN BLOOD. THESE BENCHES AND MATERIALS ARE LABELED "HUMAN BLOOD" IN THE LAB. WEAR GLOVES AT ALL TIMES!

This study will collect both whole blood and stool specimens. However, only the EDTA tubes will be analyzed within the SOM lab. Since only bioenergetic analysis will be done in the lab, the following SOP will pertain only to bioenergetic analysis and prepping other tubes for the SOM Biorepository or Institute of Genomic Sciences (IGS). Please review the "Sample Handoff Map" to know where DNA/RNA isolation and microbiome specimens will be dropped off.

PLATELETS AND PBMCs ISOLATION TESTS

Sample Analyzed: WHOLE BLOOD

Tube Used: EDTA Tube (Purple Top- see "Sample Collection Kits" above)

Platelets and Peripheral Blood Mononuclear Cells (PBMCs) Isolation Using EDTA Tube

DAY BEFORE EXPERIMENT (AVERAGE TIME: 15 minutes)

PURPOSE

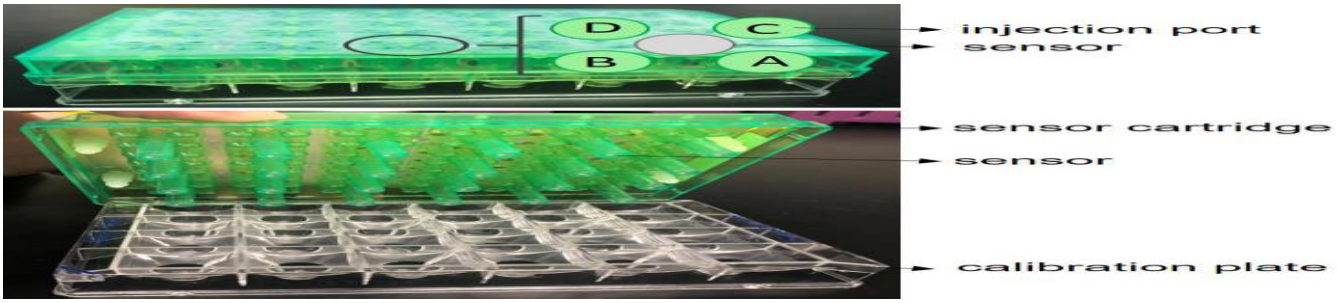
The purpose of this test is to isolate specific blood components (i.e. platelets and PBMCs) from whole blood.

MATERIALS NEEDED:

- 10 ml pipette – FAST PIPETTE
- Seahorse XF Calibrant Solution
- Seahorse Calibration Trays (green top)
- Sharpie marker
- Gloves
- 2 ml sterile tube
- Parafilm

SEAHORSE XF24 EXTRACELLULAR FLUX SENSOR CARTRIDGE (OR CALIBRATION TRAY)
Older machine

Figure 1: Seahorse XF24 and XFe24 Analyzer Calibration Trays



SEAHORSE XFE24 EXTRACELLULAR FLUX SENSOR CARTRIDGE (OR CALIBRATION TRAY)
Newer machine

Hydrating the Seahorse Tray with Calibrant Solution:

1. Figure out how many whole blood samples you are going to process for the next day. If you are going to process two, then make sure you take out two Seahorse Calibration trays (green top).
2. Remove the green topped tray from the Seahorse package.
3. Take the green top off and place it on the table with the probes side up (flat surface should touch the table). Make sure you do not touch the probes with your gloves.
4. Pipette 1 ml of Seahorse XF Calibrant solution into each tray well using the 10 mL fast pipette.
5. Place the green probe cover back onto the hydrated tray, make sure all probes are submerged.
6. Label the green probe cover with the date, time, and your initials.
7. **Wrap the hydrated tray in parafilm to prevent evaporation.**
8. Place the hydrated tray into the incubator. Check the incubator temperature and make sure it is at 37°C.
9. Leave the hydrated tray(s) in the incubator overnight.

Coating the Seahorse plate with Cell-Tak™ Solution:

10. Clean the hood in Room 5-11 or 5-23 with 70% ethanol. Use the spray near the hood.
11. Spray everything with 70% ethanol before placing it under the hood: sterile water, Cell-Tak flask and bicarbonate solution flask. Spray your gloves.
12. Open a V7 Seahorse cell culture plate package inside of the hood.
13. Check to make sure you have everything before starting your Cell-Tak™ prep.
14. Label a sterile 2 ml tube.
15. Prepare Cell-Tak™ (Corning) solution inside the hood according to the specific protocol **(consider that each Cell-Tak™ lot had a different concentration- PAY ATTENTION TO THE SOLUTION LABEL)**
16. To make the Cell-Tak™ solution:

Calculate the volume you need to use for the entire tray first. You calculate the volume based on the Cell-Tak™ solution concentration (the goal is 2.4-2.6 µg Cell-Tak per well with pH close to 8)*:*

If the Cell-Tak™ solution concentration is 1.2 mg/ml, use the following:

diH_2O: 195 µL
Cell-Tak™: 54.7 µL
Sodium Bicarbonate: 500 µL

NaOH: 27 µL

If the Cell-Tak™ solution concentration is 2.05 mg/ml, use the following:

diH_2O: 220 µL
Cell-Tak™: 30 µL
Sodium Bicarbonate: 500 µL

If the Cell-Tak™ solution concentration is 2.3 mg/ml, use the following:

diH_2O: 223.75 µL
Cell-Tak™: 26.25 µL
Sodium Bicarbonate: 500 µL

NOTE: Sodium Bicarbonate by itself is difficult to pH. You can use piece of pH paper (strips) to test the pH of the final solution. Pipette a drop of your solution onto the pH paper. Then use NaOH to increase the pH and use HCl to lower the pH. You don't want to add too much more to the volume because that will change the final Cell-Tak concentration.

- Add the reagents based on the recipe in the following order: water, Cell-Tak™, and bicarbonate
- Using the 100 or 200 µL pipette, add 30 µL of Cell-Tak™ solution in each well across the entire Seahorse plate
- Put everything else away except the plate
- Leave the plate in the hood at least 25 minutes with the lid on
- Remove the liquid from the wells with a 1 ml pipette (**do not touch the bottom of the well when you tilt the plate sideways**)
- Using a 200 µL pipette, add at least 200 µL of DPBS in each well
- Aspirate the DPBS with a 1 ml pipette (**do not touch the bottom of the well when you tilt the plate slightly sideways**)
- **Cover the plate in parafilm and put the plate in the cell culture fridge until use**

NOTE: Flasks, Cell-Tak, and sterile water are in the bottom left drawer of the cell culture fridge.

EXPERIMENT DAY (AVERAGE TIME: 4 hrs.)

MATERIALS NEEDED:

- 70% Ethanol spray for gloves
- Gloves
- DMEM **(found in the Room #5-66 fridge bottom shelf)**
- Sodium Pyruvate **(found as a powder next to scale)**
- Scale **(found in Room # 5-66)**
- Weighing Paper (**usually next to scale**)
- Microtubes (mini plastic conical tubes) (**Check purple cabinet and then the lab supply room**)
- Mini Mixing Magnet (magnetic stir bar)
- Manual Counter (**usually next to microscope in cell culture room**)
- Paper Towels (various places in lab- usually next to sinks)
- Pens
- Sharpies
- Kim Wipes (thin, paper wipes) **(check purple cabinet and then lab supply room if there aren't any)**
- Open Tube Rack
- Water Bath **(use the one behind the old Seahorse machine)**
- pH Meter **(calibrate every 2 days; use the one right next to the scale)**
- Incubator **(use the one in Room 5-11)**
- Centrifuge
- -80°C Freezer
- Hemocytometer **(found in cell culture room)**

- Cylinder
- Parafilm **(found in both Rooms 5-11 and 5-66)**
- Agilent Seahorse™ XF24 Analyzer or XFe24 Seahorse Analyzer**
- **PBMC Isolation**: EasySep Kit, EasyEight Magnetic Rack, PBS with 2% FBS and 1 mM EDTA (buffer solution; 49 ml DPBS, 1 ml FBS, 100 µl 0.5 M EDTA), round-bottom tubes, 15 ml conical tubes, vortex machine, and Trypan Blue (dye)
    - **Easy Sep Kit- found in Room 5-11 fridge #3**
    - **2% FBS- should be found in freezer (frozen aliquots) in Room #5-11**
    - **EDTA- bottles are found in the Trauma Genomics cabinet in Room #5-11**
    - **DPBS or PBS (same thing for the purposes of this experiment)- bottles are found in the Trauma Genomics cabinet in Room #5-11**
- **DRUGS**: Oligomycin, Dinitrophenol (DNP), and Antimycin A
    - **All drugs have been frozen and should be in the -20°C freezer in Room #5-66**
- **Pipettes**: 100 µL, 1mL, 1000 µL, 10 or 20 µL

****Check to make sure you choose the right calibration trays for the machine. Check the label. XFe24 calibration trays have a red Hydro Booster layer. XF24 does not have a Hydro Booster layer.**

1. Find the pre-aliquoted DMEM in a conical tube in the -20°C freezer. Take out one conical tube.
2. Use your hands to start the thawing process for the DMEM.
3. Place the DMEM into the water bath. Make sure the water bath is 37°C.
4. Once the DMEM has thawed, set aside on an open rack near the pH meter.
5. Check the pH meter calibration log to see if pH meter needs to be calibrated. pH meter needs to be calibrated every 2 days.

pH Meter Calibration:

NOTE: pH meter is set up to be calibrated from acids (pH = 4.0) to neutral (pH= 7.0) to base (pH =10). Make sure you calibrate the meter in that order.

6. Take the pH probe out of the calibration solution. Using glassware like a beaker, wash the pH probe using 70% ethanol and deionized (DI) H_2O. **Check the bottom of the probe for any mold or other growths.**
7. Wipe the pH probe with available Kim Wipes next to meter.
8. Unscrew the pH solution labeled "pH = 4.0" (should be red) and place the clean pH probe into the red solution.
9. Press the "CAL" button on the pH meter for calibration.
10. Once calibration is complete, repeat steps #6 and #7 to clean the probe.
11. Unscrew the pH solution labeled "pH = 7.0" (should be yellow) and place the clean pH probe into the yellow solution.
12. Repeat steps #9 and #10.
13. Unscrew the pH solution labeled "pH = 10.0" (should be light blue) and place the clean pH probe into the light blue solution.
14. Repeat steps #9 and #10. Place the clean probe back into the original, clear calibration solution.
15. Write down the slope generated by the pH meter in the calibration log along with the date and your initials. Press the "Save" button.

Preparing the DMEM and Pyruvate Solution:

16. Take out one sheet of weighing paper and place on the scale. Close the scale doors.
17. Press the "Tare" button on the scale.
18. Calculate 11% of the total DMEM volume. Your resulting total is the amount of sodium pyruvate powder you will use.
    - If the total volume of the DMEM is 20 ml, then you will need to add 2.2 mg of sodium pyruvate.
19. Measure the sodium pyruvate powder using the weighing paper on the scale in milligrams (mg).
20. Pour sodium pyruvate into the thawed DMEM solution.
21. Clean a mini mixing magnet with DI H_2O and dry off with paper towels.
22. Place the magnet into the DMEM + sodium pyruvate solution.
23. Put the conical tube w/ DMEM + sodium pyruvate on a mixing table. Turn on the mixing table for the desired mixing speed.
24. Place the pH meter probe into the DMEM + sodium pyruvate solution. Note the pH.

25. Adjust the pH to 7.4. You can use NaOH 0.1N to increase the pH. **Try to avoid using HCl to lower the pH. Use HCl only if necessary.**
26. Place the DMEM + sodium pyruvate solution in the incubator until use.
    NOTE: There is a large incubator in Room 5-11 in the corner. You can use this if no one is using it for experiments.

Platelet Isolation from Whole Blood:

PLATELET ISOLATION

27. Turn on the centrifuge and check the rotor (use the one for 15 mL conical tubes). Ensure temperature is set to 25°C, speed is set at 400g, and time is set for 10 minutes. **NOTE: Soft acceleration and soft deceleration should be on.**
28. Label tubes for platelet isolation and aliquots in the hood in Room 5-11. Make a mark on the blood tube to indicate original volume using a Sharpie marker.
29. If you have one tube of blood, find an empty EDTA tube and pour a similar amount of water into the tube as a balance. If you have two tubes that are close in volume, they can be used to balance each other.
30. Place the whole blood sample opposite (diagonally) from balance in the centrifuge. Close the lid.
31. Centrifuge the tubes at 400g for 10 minutes at 25°C.
32. Before using the hood, spray gloves with 70% ethanol spray. If tube rack is not in the hood, spray the tube rack with ethanol spray.
33. Using a 1 mL pipette, collect the top clear/slightly yellow liquid (platelet rich plasma- PRP) and place it into a clean 15 mL conical tube. **Be careful to not touch the buffy coat at the bottom of the PRP with your pipette. The buffy coat contains the white blood cells (WBCs) to be isolated later.**
34. Label the tube and top with "PRP", participant ID#, study timepoint (day #), and date using a Sharpie.

a) *Making aliquots of the PRP:*

35. Using a similar balance, centrifuge the PRP in the 15 ml conical tube at 1600g for 10 minutes at 25°C.
36. Get the DMEM + sodium pyruvate solution from the incubator at set aside in the hood.
37. Once centrifuged, transfer supernatant of platelet poor plasma (PPP) into a clean 15 ml conical tube. **Be careful to not touch the platelet pellet at the bottom of the original tube with your pipette.**
38. Grab about 4 1.5 ml centrifuge tubes and label as "PPP" with date, study timepoint (day #), and participant ID #.
39. Aliquot PPP from 15 ml conical (1 ml each) into each pre-labeled mini plastic conical tube. Add and label more mini plastic conical tubes if needed.
40. Place aliquots into the -80°C freezer. **There is a specimen box labeled "Optimization Experiments" in the -80°C freezer (#3) in Room 5-66.**

b) *Making the Platelet Suspension for Manual Counting:*

41. Using a 1 mL pipette, **gently resuspend** the platelet pellet in 2 mL of warm DMEM+ sodium pyruvate solution. **You can gently resuspend by going "up and down" or gently breaking up the pellet and mixing with DMEM + sodium pyruvate solution**. You mix gently so you don't activate aggregation in the platelets.
42. Measure out 990 µL of DMEM + sodium pyruvate solution and aliquot into a clean tube.
43. Using a 10 or 20 µL pipette, add 10µL of your newly mixed platelet suspension to the 990 µL of DMEM + sodium pyruvate solution. Gently mix the new suspension. This is a 100x dilution.
44. Set the solution aside near the hemocytometer.

Figure 2: Slide with Cover Slips

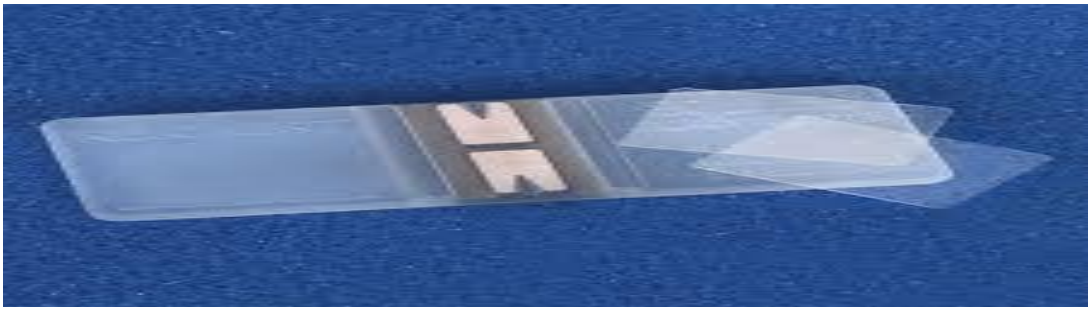
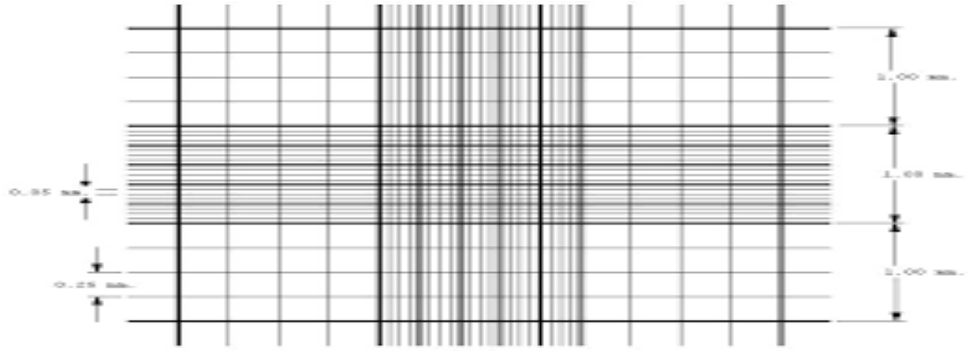


Figure 3: Hemocytometer Grid



c) Manually Counting Platelets using a Hemocytometer (in Room 5-23)

45. Wash the slide and coverslip (see **Figure 2**) with 70% ethanol. There should be ethanol near the hood in Room 5-11 or in 5-23 (cell culture room).
46. Wipe and dry the slide and coverslip using non-scratching tissue paper (Kim Wipes). Be careful to not leave small tissue paper pieces in the slide. Place coverslip onto clean slide.
47. Using a 10 or 20 µL pipette, inject 10 µL of the platelet/DMEM + sodium pyruvate solution into one side of the slide chamber.
48. Wait about 5-10 minutes for the platelet solution to settle. Do NOT place it under the microscope light or the solution will start to evaporate and the count will be inaccurate.
49. Use an inverted microscope (microscope across from the hood) to count the cells inside the 25 small squares (1 mm^2) in the center of the chamber.
    NOTE: It is possible to count only 5 squares if this is considered in the calculations. You can also count the platelets that are on the edge of square/line. The key is that your counting method is consistent across samples.
50. Count platelets in both sides of the chamber and calculate the average platelet count.

*d) Counting Methods and Calculating Platelets using the Hemocytometer (**see Figure 3**):*

Counting Platelets Using One Slide Chamber
1. Count all platelets in either a row or column. 2. Repeat count until you have covered the entire 5x5 square. 3. Multiply your new total by 100. 4. Multiply your new total by 10,000. **Your new total is the number of resuspended cells/mL of your original whole blood sample.**

e) Calculate the dilution needed to have 40 million (4×10^7) cells in 200 µL per well for the Seahorse machine:

51. Calculate the total number of cells needed according to the number of wells to be used.
52. Calculate the total volume needed based on the number of wells to be used.
53. Divide the total number of cells by the platelet concentration of the suspension. This new total will be the volume of platelet suspension.

54. Subtract this volume from the total volume needed for all the wells. This will be the volume of the DMEM +sodium pyruvate solution to add.

Calculation example:

You have 4 wells. You need 160,000,000 (40 million x4 wells) cells and 800 µL of the diluted platelet suspension (200 µL x 4 wells).

If the platelet concentration of the original whole blood sample was 1,000,000,000, then you need 160 µL (160,000,000/1,000,000,000) and 560 µL of the DMEM + sodium pyruvate solution (800 µL - 240 µL).

NOTE: Make a little more than you need to ensure there is enough suspension for all the wells you need.

55. Make the diluted platelet solution in a 15 ml conical tube and gently mix the suspension.
56. Label the 15 ml conical tube as "Platelet suspension" w/date, participant ID, study timepoint (day #).
57. Using a 200 µL pipette, add 200 µL of DMEM + sodium pyruvate solution into the background wells. **The 3 background wells are in the upper left, upper right, and lower right of the tray**.

EXAMPLE OF TRAY:

X- BACKGROUND 200 µL of DMEM + sodium pyruvate solution	Well #1	Well #2	Well #3	Well #4	**X- BACKGROUND** 200 µL of DMEM + sodium pyruvate solution
Well #5	Well #6	Well #7	Well #8	Well #9	Well #10
Well #11	Well #12	Well #13	Well #14	Well #15	Well #16
Well #17	Well #18	Well #19	Well #20	Well #21	**X- BACKGROUND** 200 µL of DMEM + sodium pyruvate solution

All background wells (upper right/left and lower left corners) are highlighted in yellow. These background wells should have no cells in them.

58. Using a 200 µL pipette, add 200 µL of platelet suspension into each of the desired wells very gently. Only use the wells in the center of the plate. Add the cells down the side of the well to avoid touching the bottom.
59. Incubate the plate with the cells at 37°C (use the incubator next to the Seahorse machine) for 30 minutes. **You want an incubator without CO_2.**
60. Keep the DMEM + sodium pyruvate solution in the incubator.
61. Add the plate shaped rotor to the centrifuge.
62. Set the centrifuge to 200g for 5 minutes at 25°C. **Make sure soft acceleration and soft deceleration are on before you centrifuge. Add a plate balance.** Centrifuge the plate.
63. After the first centrifugation, rotate the plate 180° and centrifuge the plate again at 200g for 5 minutes at 25°C. **Make sure soft acceleration and soft deceleration are on before you centrifuge.**
64. After the second centrifugation, look at the plate and see if the platelets have attached to the bottom of the well. Note if platelets are on the side of the wells. Do not consider these well (i.e. wells with platelets not in the bottom) when analyzing the data.
65. Using a 1 mL (1000 µL) pipette, **very gently** add 475 µL of warmed DMEM + sodium pyruvate solution (kept in the incubator) to each of the wells**.**
66. Incubate the plate at 37°C in the incubator without CO_2. Use the incubator next to the Seahorse machine for at least 45 min.
67. While the plate is incubating, start prepping the drugs (i.e. oligomycin, DNP, and antimycin A) to be used for the Seahorse.

Peripheral Blood Mononuclear Cell (PBMC) Isolation: PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

NOTE: Use plastic round bottom tubes for isolating the PBMCs using the EasyEight magnet. You can pre-label the tubes "PBMC isolation #1", "PBMC isolation #2", and "PBMC isolation #3" and sit these empty tubes in the magnetic rack

Using x ml of whole blood:

1. Get an aliquoted FBS solution from freezer. Leave at room temp to thaw.
2. Get a clean 50 ml tube and label "PBMC FBS/DPBS/EDTA solution"
3. Make a FBS/PBS/EDTA solution by adding the following to a clean 50 ml tube:
    - 49 ml of DPBS solution
    - 1 ml of thawed FBS
    - 100 µL 0.5 M EDTA

4. Take EDTA tube containing red blood cells and buffy coat and fill back to original volume (using previously drawn line) with PBS with 2% FBS and 1 mM EDTA (or 0.5 M EDTA). Mix together with 5 ml pipet until homogenous suspension. This should be treated as "whole blood."
5. Remove x ml "whole blood" (at most 6 ml) and put into a round-bottom tube. Add 12.5 µl of 0.5 M EDTA per 1 ml of blood to tube (for a final concentration of 6 mM). Then add 50 µl PBMC Isolation Cocktail per ml of blood to tube. Mix by pipetting. Then let sit at room temperature for 5 min.
    - EXAMPLE: If you have 4 mls of blood, you will want to add/pipette in 200 µL of PBMC Isolation Cocktail (white solution) to the blood.
6. Add x ml of PBS with 2% FBS and 1 mM EDTA to solution. Mix gently by pipetting up and down 2-3 times.
7. Vortex the RapidSpheres (beads) for 30 seconds, at which point they should be evenly dispersed.
8. Add 50 µl of the RapidSpheres per ml of blood to the solution and mix by pipetting. Place tube in EasyEights magnetic rack (designed to hold round-bottom tubes) without a lid and let sit at room temperature for 5 min.
9. Carefully pipet out free liquid by gently running a 1 ml pipet tip down the open side of the tube without disturbing the beads that have migrated to the side of the tube touching the magnet. Transfer liquid into a new round-bottom tube.
10. Repeat steps 7 , 8, and 9.
11. At this point the solution should be relatively clear (i.e. no reddish hue). You can do one more 5-minute pass with the magnet by putting the new tube with the sample liquid into the rack without adding any more RapidSpheres and repeating step 6. If there is obvious contamination (liquid is noticeably red), repeat steps 5 and 6 once more.
12. Collect entire sample and put into a 15 ml conical tube. Put a cap on the tube with sample and create a proper centrifuge balance using a new tube and water.
13. Centrifuge sample at 1200 x g for 10 minutes at room temperature. Gently remove supernatant and resuspend PBMC pellet with 1 ml DMEM + sodium pyruvate solution. Remove 10 µl and put in new 0.5 ml microtube. Add 10 µl trypan blue to this new tube and mix by gently pipetting up and down a few times (avoid creating air bubbles). Move to hemocytometer.
14. Clean your slides with ethanol and a Kim Wipe.
15. Pipette in adequate PBMC isolation (w/Trypan blue added) solution into slide chamber to view and count PBMCs.

Prepping Each Drug to Be Used for the Seahorse Probes:

NOTE: You will be pipetting microliters of a specific drug into each well on top of the green calibration tray cover. First, you will find all your drugs in the a -20°C lab freezer. Take the drugs out of the freezer, put them in a microtube holder, and leave them out at RT so they can thaw. After drugs have thawed, start aliquoting the DMEM first. Then, add the specified drug amount to the DMEM.

16. Take out four mini plastic microtubes and label accordingly by letter:
    Microtube #1*: A* *(A is for 2.5 µM oligomycin)*

    Microtube #2*: B* *(B is for DNP- 30 µM concentration)*

    Microtube #3*: C* *(C is for DNP-additional 30 µM concentration)*

    Microtube #4*: D* *(D is for10 µM Antimycin A)*

Using a 1000 µL (1 ml) pipette:

17. Aliquot 1997.8 µL (2 x 998.9 µL) of DMEM + sodium pyruvate into microtube **A**.
18. Aliquot 1780 µL (2 x 890 µL) of DMEM + sodium pyruvate into microtube **B**.
19. Aliquot 1760 µL (2 x 880 µL) of DMEM + sodium pyruvate into microtube **C**.
20. Aliquot 1980 µL (2 x 990 µL) of DMEM + sodium pyruvate into microtube **D**.
21. Find previous aliquots of oligomycin, DNP, and antimycin A in the -20°C freezer. Start thawing the oligomycin, DNP, and antimycin A with your hands. **For making aliquots of oligomycin, DNP, and antimycin A, please see additional steps at the end of this manual.**

Prepping Oligomycin

22. Using a 1 mL (or 1000 µL) pipette, add 2.08 µL of the thawed oligomycin to the 1997.8 µL of DMEM + sodium pyruvate solution.
23. Close the cap and lightly vortex the oligomycin mixture.
24. Set the oligomycin mixture aside.

NOTE: Change your pipette tips every time you are adding a new drug to the DMEM + sodium pyruvate solution.

Prepping DNP (2,4-Dinitrophenol)

Using a 1000 µL pipette:

25. Add 220 µL of thawed DNP to microtube **B.**
26. Add 240 µL of thawed DNP to microtube **C.**
27. Vortex each microtube to ensure proper mixing.
28. Set the 2 DNP mixtures aside.

Prepping Antimycin A

29. Using a 20 µL pipette, add 20 µL of antimycin A to microtube **D.**
30. Vortex microtube **D** to ensure proper mixing.
31. Set the antimycin A mixture aside.

Zig Zag Pattern (see Figure 4 below): Add drugs in the green top in the following order:

A-oligomycin

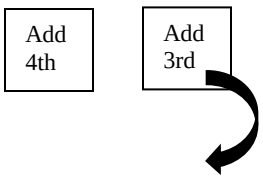
B-DNP (30 *µM* concentration*)*

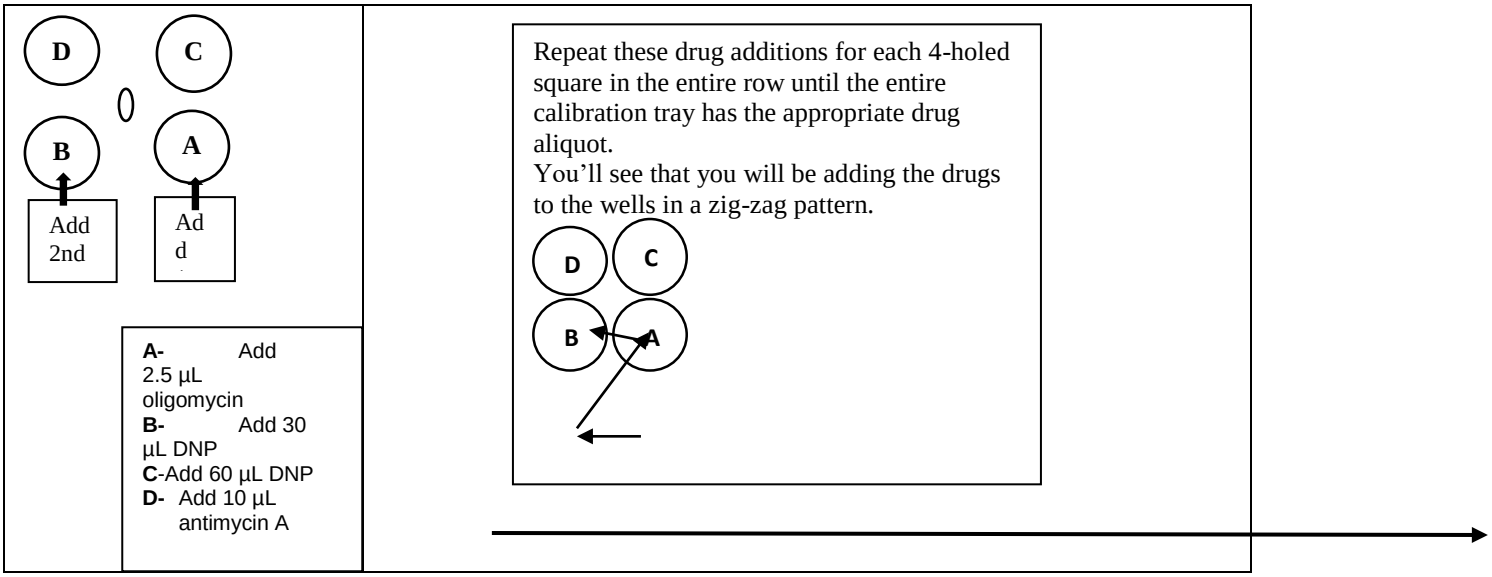
C- DNP (additional 30 *µM* concentration)

D- Antimycin A

<u>EACH SQUARE OF GREEN CALIBRATION TRAY COVER</u>

Figure 4: Drug Administration Using Green Calibration Tray





32. Remove the Seahorse calibration tray (green top) from the incubator and put it on the bench.

33. If the drugs have been sitting in the microtubes for some time, quickly vortex each one (i.e. A, B, C, and D).
34. Using a 100 µL pipette, **pipette 75 µL of oligomycin into all lower right wells** (i.e. well A) **of each square** on the green top/cover of the calibration tray.
35. Using a 100 µL pipette, **pipette 75 µL of DNP from microtube B (lower concentration) into all lower left wells** (i.e. well B) **of each square** on the green top/cover of the calibration tray.
36. Using a 100 µL pipette, **pipette 75 µL of DNP from microtube C (higher concentration) into all upper right wells** (i.e. well C) **of each square** on the green top/cover of the calibration tray.
37. Using a 100 µL pipette, **pipette 75 µL of antimycin A from microtube D into all upper left wells** (i.e. well D) **of each square** on the green top/cover of the calibration tray.

NOTE: You will notice that you are pipetting all drugs in a zig-zag pattern.

Configuring the Seahorse Machine for an Experiment

38. Set the Seahorse configuration according to the type of cell (i.e. platelets) as follows:
    - Click "Assay Wizard"
    - Fill in the information "Assay Name", "Principal Investigator" (use initials), "Instrument Operator" (use initials), and "Result File Name" (name the file and put the date)
    - Click "Next"
    - Select cell types from "Cell List"
        - If the cell type isn't on the list, click "Add Cell", then click "Cell Type Name" and type in the cell type
    - Leave "Cell Passage Number" or "Cell Seeding Number" blank
    - Click "Next"
    - Select growth media from the "Growth Media List"
        - For platelets, select "DMEM 5.5. mM, Glucose 4mM, Glutamine 6.2 mM, EDTA 1 mM, Pyruvate"
    - Click "Next"
    - Select cell type from "Available Cells" list
    - Select the wells where the cells were plated
    - Click "Assign to Wells"
        - If there is another cell type selected for the wells, select "All the Wells", click "Unassign Wells", and then proceed to select the cell and assign a new one
    - Click "Next"
    - Select running media from "Running Media List"

    - For platelets, select “DMEM 5.5. mM, Glucose 4mM, Glutamine 6.2 mM, EDTA 1 mM, Pyruvate”
- Select the wells where the cells were plates and the background wells
- Click “Assign to Wells”
    - If there is another running media selected for the wells, select “All the Wells”, click “Unassign Wells”, and then proceed to select the cell and assign a new one
- Fill the volume information with 675 µL
- Click “Next”
- Click “Next”
- Verify if all the compounds to be injected (i.e. drugs) are on the “Compound List”
    - If one is missing, click “New” and add the compound information
- Click “Next”
- Click “Next”
- Select port “A”
- Select the first compound to be injected from the list
- Select the wells where the cells were plated and the background wells
- Click “Assign to Wells”
    - If there is another running media selected for the wells, select “All the Wells”, click “Unassign Wells”, and then proceed to select the cell and assign a new one
- Repeat this procedure for ports B, C, and D
- Click “Next”
- Select “Do Background Correction”
- Select the background wells
- Click “Next”
- Label the wells according to the samples
    - Select “Well Label”
    - Type the name of the group
    - Select the wells and “Assign to Wells”
- Label the background wells as one group
- Click “Next”
- Select group number 1 from a colorful group squares
    - Select the corresponding wells
- Repeat the procedure for all the groups, including background wells
- Click “Next”
- On “Error Bar Format”, select “Standard Error of Mean”
- Click “Next”
- Set running configuration according to the type of cell; for platelets, do as follows:
    - Click “Mix” and select 3 minutes
    - Click “Wait” and select 2 minutes
    - Click “Measure” and select 2 minutes
    - Select the 3 items on the list
    - Click “Copy”
    - Click “Paste” two times
    - Click “Inject” and select **port A**
    - Click “Paste” two times
    - Click “Inject” and select **port B**
    - Click “Inject” and select **port C**
    - Click “Paste” two times
    - Click “Inject” and select **port D**
    - Click “Paste” two times
    - Click “Next”
    - Click “Save Template”

    - After saving template, click “End”
    - Start running the experiment.
- Click on “Run” on the top of the screen
- Click “Start”
- Click “Start” again
- When the drawer opens, insert the plate (make sure that it is attached and in the right position)
- After the drawer closes, the machine will start the calibration- this takes approximately 20 minutes
- After the calibration finishes, click “OK”, remove the calibration cartridge and inset the plates with the cells – make sure that it is in the right position
    - This run will take approximately 1 hour
- When the run ends, click “OK” and remove the plate
- Carefully aspirate the liquid from each well
- Using the 100 µL or 200 µL, pipette 100 µL RIPA buffer (Sigma R0278) in each well
- Shake the plate for at least 30 minutes using the shaker inside the cold room
- Label 1.5 mL tubes to collect the cells
- Collect the cells from each well by scraping the bottom with a 200 µL pipette- **change the pipette tip for each well**
- Put the content of each well in a different 1.5 mL previously labeled tube
- Keep the cells at -20°C for the protein measurement (for data normalization)

Collecting Data from the Agilent Seahorse™ Machine

39. Open data shortcut folder on the desktop.
40. Open the .xls file with the data.
41. Select “OCR” on the bottom right.
42. Click on “Well Group Mode”.
43. Select the wells where there were cells.
44. Click on the graph with the right mouse button and select “Save Graph Data”.
    - An Excel spreadsheet will appear with the data for OCR of individual wells
45. Go back to the graph sheet and click on “Mean” button in the middle of the left side.
46. Select the desired wells.
47. Click on the graph with the right mouse button and select “Save Graph Data”.
    - A different Excel spreadsheet will appear with the data for mean and standard deviation for OCR
48. Go back to the graph sheet and select “ECAR” 100.
100. Click on the graph with the right mouse button.
    - A different Excel spreadsheet will appear with the data for mean and standard deviation for ECAR
49. Go back to the graph sheet, return to individual well mode, click on the graph with the right mouse button and select “Save Graph Data”.
    - A different Excel spreadsheet will appear with the data for the ECAR of individual wells
102. Save file as Excel Workbook format (the previous file was an Excel template format).

Data Normalization

103. Calculate how many µg of protein there is in each well.
104. Open the data shortcut on the desktop of the Seahorse computer.
105. Open the Excel template file with the data.
106. Click on cell count normalization on the bottom of the screen.
107. Select each well and add the protein content in the corresponding well.
    - Select the well, add the number, click “OK” and then select the next well.
108. When finished, click the “N” button on the left side of the graph.
109. Repeat the collecting data steps above to obtain the OCR and ECAR values.
110. Save the file as an Excel Workbook format.

IF YOU HAVE ANY OUTLIERS:

Analyzing Outliers

111. Calculate 2 times the standard deviation (SD).
112. Add/subtract OCR number of each well from your calculation.
113. If the OCR value > 2*SD, it is an outlier and does not need to be included.

POSSIBLE REASONS FOR LOW OCR:

- Platelets did not fall into the center of the V7 well and were aggregated on the side of the well
- Less cells were loaded into that well compared to other wells- check this with the protein assay
- Platelets are dead or did not respond- either way, they weren't reliable and should not be used