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Rapid fractionation and isolation of whole blood components in samples obtained from a community-based setting --Manuscript Draft--

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Abstract:	Collection and processing of whole blood samples in a non-clinical setting offers a unique opportunity to evaluate community-dwelling individuals both with and without preexisting conditions. Rapid processing of these samples is essential to avoid degradation of key cellular components. Included here are methods for simultaneous peripheral blood mononuclear cell (PBMC), DNA, RNA and serum isolation from a single blood draw performed in the homes of consenting participants across a metropolitan area, with processing initiated within two hours of collection. We have used these techniques to process over 1600 blood specimens yielding consistent, high quality material, which has subsequently been used in successful DNA methylation, genotyping, gene expression and flow cytometry analyses. Some of the methods employed are standard; however, when combined in the described manner, they enable efficient processing of samples from participants of population- and/or community-based studies who would not normally be evaluated in a clinical setting. Therefore, this protocol has the potential to obtain samples (and subsequently data) that are more representative of the general population.

Author Comments:	We would like to thank Kimbra Wagner for encouraging the submission of our protocol. Please note that we would like both affiliations (University of Illinois at Urbana-Champaign (current) and Wayne State University School of Medicine (previous)) to appear in print for Amy Weckle, Monica Uddin, and Derek Wildman.
Additional Information:	
Question	Response
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MOLECULAR MEDICINE
AND GENETICS

WAYNE STATE
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SCHOOL OF MEDICINE

April 16, 2014

Dear Editor-In-Chief,

On behalf of my co-authors, I submit for consideration of publication in your journal, *JoVE*, our manuscript entitled, *Rapid fractionation and isolation of whole blood components in samples obtained from a community-based setting*, as an invited submission. The manuscript and its components are an original compilation and are not under consideration by any other journal. All participants in the described study were consenting adults. Publication permissions have been obtained from all manufacturers of the products mentioned herein. We would like to request expedited processing of our manuscript due to a pending move of the laboratory around July 1, 2014.

We outline an optimized, streamlined protocol that enables rapid, high-throughput processing of whole blood samples in a non-clinical setting. Included are methods for simultaneous peripheral blood mononuclear cell (PBMC), DNA, RNA and serum isolation from a single blood draw performed in the homes of consenting participants across a metropolitan area, with processing initiated within two hours of collection. We have used these techniques to process over 1600 blood specimens yielding consistent, high quality material which has subsequently been used in successful DNA methylation, genotyping, gene expression and flow cytometry analyses.

Thank you for your consideration.

Best Wishes,



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TITLE:

Rapid fractionation and isolation of whole blood components in samples obtained from a community-based setting

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KEYWORDS:

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non-clinical setting

SHORT ABSTRACT:

We outline a methodology for the processing of whole blood to obtain a variety of components for further analysis. We have optimized a streamlined protocol that enables rapid, high-throughput simultaneous processing of whole blood samples in a non-clinical setting.

LONG ABSTRACT:

Collection and processing of whole blood samples in a non-clinical setting offers a unique opportunity to evaluate community-dwelling individuals both with and without preexisting conditions. Rapid processing of these samples is essential to avoid degradation of key cellular components. Included here are methods for simultaneous peripheral blood mononuclear cell (PBMC), DNA, RNA and serum isolation from a single blood draw performed in the homes of consenting participants across a

metropolitan area, with processing initiated within two hours of collection. We have used these techniques to process over 1600 blood specimens yielding consistent, high quality material, which has subsequently been used in successful DNA methylation, genotyping, gene expression and flow cytometry analyses. Some of the methods employed are standard; however, when combined in the described manner, they enable efficient processing of samples from participants of population- and/or community-based studies who would not normally be evaluated in a clinical setting. Therefore, this protocol has the potential to obtain samples (and subsequently data) that are more representative of the general population.

INTRODUCTION:

Multiple studies have characterized differences in gene expression, DNA methylation and cell subset in blood among individuals with and without mental (or other) illnesses¹⁻⁴. These studies, however, have been obtained from clinical settings in which disease-associated differences may be magnified due to the generally more severe nature of the illnesses for which patients are seeking treatment. Due to advances in “omics” approaches, the past decade has seen an explosion of interest in obtaining biologic samples from community and/or epidemiologic settings⁵⁻⁷, in order to provide population-based estimates of disease prevalence and a broader picture of the environmental determinants of these mental and/or physical illnesses.

A key challenge in this regard is the requirement for rapid processing of the collected specimens. Degradation of mononuclear cells, key immune system components that are frequently used to assess the health of an individual, begins immediately upon blood draw with a significant decrease in recovery after two hours of collection⁸⁻¹⁰. To address this challenge, we present an optimized protocol in which multiple components of human whole blood are simultaneously isolated from samples obtained in homes of subjects living in a large metropolitan area. The protocol is based upon our compilation and modification of current techniques, including storage of all “extra” fractions in the event future techniques allow for further isolation/analyses. While alternative methods or kits may be employed in place of the individual methods described here, those outlined have proven to be a reliable and efficient means for processing samples in a high-throughput manner. High-quality fractions (PBMCs, DNA, serum, and RNA) of fresh blood can be produced within 2 hours of collection and all assay-ready specimens can be available within 2 days (Figure 1).

This protocol was developed to enable the efficient processing of samples collected from community-dwelling, adult residents of the city of Detroit for testing in the Detroit Neighborhood Health Study (DNHS; DA022720, RC1MH088283, DA022720-05-S1), a population-based study of the social and biological determinants of post-traumatic stress disorder (PTSD) and other mental illnesses. The prevalence of PTSD in Detroit is more than twice the national average^{11,12}. Identifying biological determinants of PTSD in this population may help to develop appropriate pharmacologic and/or cognitive-behavioral interventions to aid those suffering from the disorder, both in this urban population, and in other high-risk populations (e.g. returning military veterans). Our laboratory, previously located at Wayne State University in Detroit, Michigan, was

selected for processing based on our expertise in handling fresh tissue samples derived from a variety of sources, the necessity to begin processing the samples within two hours of collection, and our proximity to the collection sites. With this unique opportunity at hand, our goal was to optimize the processing for greatest yield of DNA, RNA, serum and peripheral blood mononuclear cells (PBMCs) from each specimen (a total of N=1639 samples over 5 waves of specimen collection). The procedures outlined here can be performed simultaneously in a non-clinical setting, thus producing starting material (see Table 1 for average yields) for a multitude of downstream applications including microarray, epigenetic, real-time RT-PCR, and flow cytometry analyses.

[Place Figure 1 here]

PROTOCOL:

The Detroit Neighborhood Health Study was reviewed and approved by the University of Michigan's Institutional Review Board. All participants provided informed consent prior to their participation in the study.

1. Overview

1.1) Properly document all stages from recruitment through analysis of endpoint data. Perform the protocols on Day 1 simultaneously, switching stages and overlapping as time permits. The sequence of stages is written to optimize performance efficiency. Figure 1 provides an overview of the entire process.

Note: The term, "participant" is used throughout to signify the de-identified blood sample drawn from a consenting individual. The times in parentheses below indicate hands-on time. Examples of the tracking sheet and specimen log can be found in supplementary Tables S1 and S2, respectively. Samples are collected by phlebotomists in homes of individuals identified as consenting participants by the study coordinator and transported to the lab by a courier (see Supplementary Information (SI) 1 for further pre-processing details).

2. Day 1 Setup

2.1) Prepare 1X Phosphate-buffered saline (PBS).

2.2) Preheat a heat block to 56 °C.

2.3) Prepare the protease (see SI 2) by aliquotting 20 µl into the appropriate number of microcentrifuge tubes for the day's deliveries (2 per participant).

2.4) Ensure Buffers AW1 and AW2 (wash buffers provided in the DNA isolation kit that increase the purity of the DNA) are ready for use, adding 100% ethanol as indicated on the label, if necessary.

2.5) Cool down refrigerated centrifuge to 4 °C.

2.6) Replace the cap on the bottle of 1X PBS with the provided rubber septum cap and pierce the rubber septum with a transfer spike, retaining the screw cap on top of the transfer spike to prevent evaporation. Repeat with the bottle of RNA stabilization solution.

2.7) Following preparation of the buffers, centrifuge and heat block, document the time of vacutainer delivery on the sample tracking sheet (see Table SI).

3. Day 1: Processing whole blood sample for DNA, PBMCs and Leukocyte RNA (2 hr)

3.1) Overview

3.1.1) Allot adequate time to minimize the delay between blood draw and processing start time. Begin processing of the ficoll containing vacutainers within two hours of blood draw to avoid an increase in red blood cell contamination and a decrease in mononuclear cell recovery.

3.1.2) Assume collection of 2 vacutainers containing ficoll gel gradient, 2 K₂ Ethylenediaminetetraacetic acid (EDTA) vacutainers, and 1 serum vacutainer per adult participant. Upon delivery of the vacutainers to the laboratory, have a technician sign the tracking sheet signifying acceptance of the samples. Begin processing immediately with the start time documented on a per vacutainer basis on the specimen log.

3.2) Serum Isolation Stage 1 (1 min)

3.2.1) Document the start time on the specimen log (see Table SI 2). Centrifuge (with brake and acceleration **OFF**) the 7.0 ml serum (red) vacutainer (1 per participant) using a swinging bucket rotor with aerosol caps (biosafety level 2; BSL2 certified) for 20 min, 1300 x g, 4 °C.

3.3) DNA Isolation Stage 1 (15 min)

Note: For further details on this isolation procedure, see manufacturer's protocol¹³.

3.3.1) Document the start time. In a BSL2 cell culture hood, invert the vacutainers containing the ficoll gel 5 times then add 200 µl whole blood from the top of one of the vacutainers into each of two 20 µl aliquots of protease (400 µl total per participant). Leaving the protease + blood microcentrifuge tubes in the hood, continue to the centrifugation of the ficoll containing vacutainers in step 3.4.1.

Note: Use the vacutainer with the largest collection volume, keeping in mind the necessity of balancing the centrifuge (i.e., it may be necessary to remove 200 µl from each of the two vacutainers) when spinning the vacutainers in step 3.4.1. Additionally, to ensure proper separation during the processing of the blue vacutainers, the level of blood remaining in the vacutainer should not be less than 2.5 inches above the ficoll layer.

231
232 **3.4) PBMC Isolation Stage 1 (1 min)**
233

234 3.4.1) Document the start time (must be within two hours of blood draw to avoid an
235 increase in red blood cell contamination and a decrease in mononuclear cell recovery).
236 Invert the ficoll containing vacutainers 8-10 times. Centrifuge (with brake and
237 acceleration **OFF**) the vacutainers (2 per participant) using a swinging bucket rotor with
238 aerosol caps (BSL2 certified) for 30 min, 1600 x g, 22 °C.
239

240 **3.5) DNA Isolation Stage 2 (20 min)**
241

242 3.5.1) Return to the hood and add 200 µl Buffer AL to each of the protease + blood
243 microcentrifuge tubes (step 3.3.1). Cap, remove from hood, vortex 15 sec and flash
244 spin.
245

246 3.5.2) Incubate 56 °C in a heat block, 10 min.
247

248 3.5.3) Remove the tubes from the heat block. Flash spin. Return to the BSL2 hood. Add
249 200 µl 100% ethanol. Cap, remove from hood, vortex 15 sec and flash spin. The
250 remaining steps can be completed outside of the hood.
251

252 3.5.4) Apply lysate (within 30 min of step 3.5.3) to a labeled spin column (in a 2 ml
253 collection tube). Close the cap to avoid cross contamination via aerosols. Centrifuge
254 6000 x g, 1 min.
255

256 3.5.5) Discard the collection tube containing the filtrate and place the spin column in a
257 new 2 ml collection tube.
258

259 3.5.6) Add 500 µl Buffer AW1 to the column without moistening the rim, close the cap,
260 and centrifuge 6000 x g, 1 min.
261

262 3.5.7) Discard the collection tube containing the filtrate and place the spin column in a
263 new 2 ml collection tube.
264

265 3.5.8) Add 500 µl Buffer AW2 to the column without moistening the rim, close the cap,
266 and centrifuge 20,000 x g, 3 min.
267

268 3.5.9) Discard the collection tube containing the filtrate and place the spin column in a
269 new 1.5 ml collection tube (not included in the kit), and centrifuge 20,000 x g, 1 min.
270

271 3.5.10) Discard the collection tube containing the filtrate and place the spin column in a
272 new 1.5 ml collection tube (not included in the kit).
273

274 3.5.11) Add 200 µl Buffer AE or water to each column and incubate at room
275 temperature for 5 min. Centrifuge 6000 x g, 1 min.
276

3.5.12) Repeat step 3.5.11 eluting into the same collection tube.

3.5.13) Pool the eluted DNA from the 2 columns per participant. Total yield ~800 µl per participant.

3.5.14) Quantify the samples using a spectrophotometer when time allows.

3.5.15) Aliquot DNA as desired into 2 ml cryovials, documenting the concentration of each on the specimen log. Transfer cryovials to a cryobox and place at -80 °C for long term storage. Document the freezer start time.

3.6) Leukocyte RNA Isolation Stage 1 (30 min)

Note: Perform in a Biosafety Level 2 certified cell culture hood. For further details on this isolation see manufacturer's instructions¹⁵.

3.6.1) Pierce the rubber septum of the K₂ EDTA vacutainer with a transfer spike. Retain the sheath and screw cap for use in step 3.6.11. Following BSL2 standard practices, take care to avoid exposure to bloodborne pathogens.

3.6.2) Attach the white slip connector to the top of the transfer spike.

3.6.3) Label the filter with the corresponding participant ID then connect the inlet (flared end) of the filter to the white slip connector.

3.6.4) Attach a sheathed 25⁵/₁₆ gauge needle to the outlet (tapered end) of the filter. Preparing the filter assembly prior to delivery expedites the process considerably and therefore, attaching the white slip connector to the transfer spike, adding the filter, then the sheathed needle and setting the assembly in a culture tube rack until use, is recommended.

3.6.5) Following assembly of the K₂ EDTA tube system, safely unsheathe the needle (use the end of a metal spatula). Stab the needle into an empty 10 ml evacuated blood collection tube (serum receiver tube) and invert the K₂ EDTA vacutainer/filter/receiver tube assembly. Following BSL2 standard practices, take care to avoid exposure to bloodborne pathogens.

3.6.6) Allow the blood to filter through until the wedge-shaped sections of the filter have cleared of blood. Filtration takes about 2 min and can be placed in a test tube rack during filtration.

3.6.7) Remove the filter from the assembly. Leave the needle in the tube containing the filtrate and discard the entire assembly in a Sharps container.

3.6.8) Attach a 5 ml syringe to the transfer spike on the bottle of 1X PBS, invert the bottle, and withdraw 3 ml.

3.6.9) Attach the syringe with PBS to the inlet of the filter and flush the filter (3-5 drops per second). Collect the flow-through in a biological waste container. Detach the syringe from the filter without retracting the plunger.

3.6.10) Following filtration and a PBS wash, withdraw 3 ml of the RNA stabilization agent using a new 5 ml syringe and the method described in step 3.6.8. Flush the filter as in step 3.6.9. The RNA stabilization agent should remain on the filter. Detach the syringe from the filter without retracting the plunger.

3.6.11) Seal the filter inlet and outlet with the sheath and screw cap retained from the transfer spike leaving the filter saturated with RNA stabilization agent. The filter can be stored at this point. Store the filter at -80 °C until time permits (~2 hr) to complete steps 5.3 to 5.4.7.

Note: Filters stored at -80 °C for up to a year after collection have been processed without a decrease in RNA quality.

3.7) PBMC Isolation Stage 2 (30 min)

3.7.1) Remove the ficoll containing vacutainers from the centrifuge (step 3.4.1) and continue in a BSL2 hood. Vacutainers should display separation as in Figure 2. If not, see Table 2.

3.7.2) Once the vacutainer is returned to the BSL2 hood, remove the stopper and withdraw 1.5 ml of the top, yellowish, plasma layer (Figure 2) using a serological pipette without getting close to the mononuclear (clear/white) layer. Transfer the plasma to a 5 ml cryovial – (Pool from 2 vacutainers – 1 participant). Log the volume collected. See step 3.7.6 for storage instructions.

Note: The best separation and greatest PBMC yields come from participants who have fasted at least 12 hours prior to blood collection.

3.7.3) Transfer the remaining plasma and the whitish, mononuclear layer (everything above the gel layer – Figure 2) using a serological pipette, to a 15 ml conical tube, pooling the mononuclear layer from each of the two ficoll containing vacutainers per participant into one conical tube.

3.7.4) Add 1X PBS to bring the total volume in the conical tube to 15 ml. Cap tube and invert 5 times. Centrifuge (with brake and acceleration **OFF**) 15 min, 300 x g, 22 °C.

3.7.5) Return to the ficoll containing vacutainers in the hood and collect the red blood cells (RBCs) by using a 5¾" Pasteur pipet to swirl around and loosen the outside of the ficoll gel layer and remove it if possible. Use a serological pipet to collect and transfer the RBCs (~4.5 ml) to a 5 ml cryovial. Log the volume collected.

3.7.6) Transfer both 5 ml cryovials (plasma in step 3.7.2 and RBCs in step 3.7.5) to a controlled rate freezing container and put at -80 °C for at least 24 hours after which time they can be transferred to a cryobox and returned to -80 °C for long term storage.

3.7.7) Return the conical tube to the hood, when the centrifugation in step 3.7.4 is complete, and aspirate all but ~500 µl of the PBS without disturbing the pellet. PBMC yield is greater if ~200 µl of PBS is left above the pellet at this stage.

3.7.8) Add fresh 1X PBS to bring the volume to 10 ml. Resuspend the pellet gently. Cap tube and invert 5 times. Centrifuge (with brake and acceleration **OFF**) 10 min, 300 x g, 22 °C.

3.8) Serum Isolation Stage 2 (10 min)

Note: Perform in a BSL2 hood.

3.8.1) Aliquot the top serum layer from the serum vacutainer, following centrifugation (step 3.2.1), into 2 ml cryovials as desired. Typical yield is 2.5 ml (Table 1). Log the volume. For example, use 4 cryovials aliquotting 200 µl into cryovial 1, 1000 µl into cryovial 2 and then divide the remaining into cryovials 3 and 4.

3.8.2) Transfer the cryovials to a cryobox and place at -80 °C for long-term storage. Document the freezer start time.

3.9) PBMC Isolation Stage 3 (15 min)

3.9.1) Return to the hood, after centrifugation (step 3.7.8), and aspirate as much supernatant/PBS as possible without disturbing the pellet. Resuspend pellet by pipetting in 2.5 ml PBMC Freezing Medium 1 (see SI 2).

3.9.2) Add 2.5 ml PBMC Freezing Medium 2 (see SI 2) to the cell/medium solution in step 3.9.1. Vortex gently.

3.9.3) Aliquot 10 µl of the cell solution into a 0.65 ml microcentrifuge tube (further dilution may be necessary). Add 10 µl of 0.4% trypan blue stain into the 0.65 ml microcentrifuge tube and mix by pipetting several times. For further details see the manufacturer's manual¹⁶.

3.9.4) Pipet 10 µl of the mixture into a cell counting chamber slide and place slide into the cell counter within 3 min of mixing. Zoom in and focus the cells. Press the "Count Cells" to obtain PBMC count.

3.9.5) Aliquot, if the viable PBMC number is above 3 million cells per milliliter (mc/ml), as desired into cryovials and continue to step 3.9.9. Store PBMCs in up to 5 cryovials at a concentration of at least 3 mc/ml each.

3.9.6) If the viable PBMC number is below 3 mc/ml, calculate the total number of cells by multiplying the viable mc/ml by 5 ml. Divide that number by 4, 3, 2 or 1 ml so that there will be at least one tube with 3 mc/ml.

3.9.7) Centrifuge the conical tube containing the cells/freezing medium solution for 5 min at 300 x g (brake and acceleration **OFF**). After centrifugation, aspirate the appropriate volume of freezing medium (supernatant) leaving the volume calculated above (4, 3, 2 or 1 ml).

3.9.8) Resuspend the pellet in the remaining supernatant and aliquot at least 3 mc/ml into the appropriate number of cryovials (1-4) at 1 ml/cryovial. Final freezing medium is 10% Dimethyl Sulfoxide (DMSO)/20% Fetal Bovine Serum (FBS)/70% Roswell Park Memorial Institute (RPMI) 1640.

3.9.9) Document the cell count per cryovial. Transfer the cryovials to a controlled rate freezing container and put at -80 °C for at least 24 hours after which time the cryovials may be transferred to a cryobox and put in a liquid nitrogen tank (vapor phase) for long term storage. Document freezer start time.

4. Day 2 Setup

4.1) Heat an aliquot (220 µl per filter) of nuclease free 0.1 mM EDTA in a nuclease-free tube to 80°C in a heat block.

4.2) Prepare wash solutions 1 and 2 (see SI 2).

5. Day 2: Long term sample storage and leukocyte filter processing (3 hr)

5.1) Overview.

5.1.1) Perform this procedure within 6 months of the application of the leukocytes to the filter.

Note: Overall, Day 2 processing takes about 3 hours and while it is labeled “Day 2,” the key requirement is that the filter processing portion should be performed on a day when there is no Day 1 processing occurring in the lab.

5.2) Long term sample storage (15 min)

5.2.1) Perform this procedure every day, prior to the delivery of new vacutainers to the lab for Day 1 processing. Transfer the cryovials that were stored overnight in controlled rate freezing containers on Day 1 into appropriately labeled cryoboxes and return them to -80 °C or liquid nitrogen (vapor phase) for long term storage. Organize the cryovials based on sample type (e.g., DNA, PBMC, RBCs, etc.) to expedite sample location tracking for endpoint assays.

5.3) Leukocyte RNA Filters Processing (45 min).

Note: For more information on this procedure see the manufacturer's instructions¹⁷.

5.3.1) Bring the filter to room temperature (thawing about 5 min).

5.3.2) Remove the sheath and screw cap from the filter. Retract the plunger of a 5 ml syringe and connect it to the inlet (flared end) of the filter, depress the plunger to expel the RNA stabilization agent from the filter ports into a biological waste container.

5.3.3) Load a new 5 ml syringe with 4 ml of a phenol and guanidine isothiocyanate solution for RNA isolation and attach it to the inlet of the filter, depress the plunger to flush the solution through the filter, collecting the lysate in a labeled 15 ml conical tube (2 per participant).

5.3.4) Disconnect the syringe from the filter, retract the plunger, re-attach it to the filter, and depress the plunger to expel residual sample trapped in the filter disk into the same 15 ml conical tube. Discard filter and syringe.

5.3.5) Add 800 µl BCP to the conical tube, close the tube tightly and vigorously shake the prep for 30 seconds.

5.3.6) Incubate at room temperature for 5 min. Centrifuge for 10 min at 2000 x g.

5.3.7) Transfer the aqueous (top) phase to a freshly labeled 15 ml conical tube (~2.5 ml).

5.3.8) Add 0.5 times the volume of the aqueous phase of nuclease-free water and mix well. Then add 1.25X the aqueous volume of 100% ethanol and mix again.

Note: For a 2.5 ml aqueous volume, add 1.25 ml of nuclease free water, mix, then add 4.7 ml of 100% ethanol ($2.5 \text{ ml} \times 0.5 = 1.25 \text{ ml}$ nuclease free water THEN $2.5 \text{ ml} + 1.25 \text{ ml} = 3.75 \text{ ml}$ new aqueous volume THEN $3.75 \text{ ml} \times 1.25 = 4.7 \text{ ml}$ 100% ethanol). This step allows for isolation of total RNA that includes the small RNA fraction. A method to omit the small RNAs from the isolation can be found in the manual¹⁷.

5.3.9) Remove the plunger from a 5 ml syringe and insert a spin cartridge in its place. Attach the cartridge/syringe assembly to a vacuum manifold. For a more secure connection to the vacuum manifold, place the cartridge/syringe assembly inside a 1.5 ml microcentrifuge tube with the bottom cut off.

5.3.10) Apply the sample from step 5.3.8 to the spin cartridge slowly with the vacuum on, carefully adding more sample as it is pulled through the cartridge.

Note: If no vacuum is present, see centrifugation method at http://www.ambion.com/techlib/misc/leuko_iso.pdf.

5.3.11) Transfer the spin cartridge to a 1.5 ml microcentrifuge tube and add 750 µl of Wash 1. Centrifuge the spin cartridge/tube assembly 5 – 10 sec at 12,000 x g.

5.3.12) Discard filtrate from the tube and return the spin cartridge to the same microcentrifuge tube.

5.3.13) Add 750 µl of Wash 2 and centrifuge the spin cartridge/tube for 5 – 10 sec at 12,000 x g. Discard filtrate as in step 5.3.12. Return the spin cartridge to the same microcentrifuge tube.

5.3.14) Repeat with another 750 µl of Wash 2 and centrifugation as in step 5.3.13. Discard filtrate. Return the spin cartridge to the same microcentrifuge tube. Centrifuge the spin cartridge/tube at maximum speed for 1 min to dry the filter.

5.3.15) Transfer the spin cartridge to a fresh, labeled 1.5 µl microcentrifuge tube.

5.3.16) Add 200 µl of nuclease-free 0.1 mM EDTA (preheated to 80 °C) to the center of the spin cartridge filter (2 per participant). Incubate at room temperature for 1 min.

5.3.17) Centrifuge for 1 min at 12,000 x g to elute the RNA. **Retain the filtrate.** Discard spin cartridge.

5.3.18) Split each 200 µl filtrate into two, 100 µl aliquots into fresh 1.5 ml microcentrifuge tubes, label and keep on ice. At this point, starting with 2 filters per participant will yield four 100 µl aliquots of RNA per participant.

5.4) DNase treatment (1 h)

Note: For further details on this treatment see the manufacturer's protocol¹⁸.

5.4.1) Create a master mix by combining 10 µl of DNase I Buffer and 2 µl rDNase I per aliquot + 1 (to account for pipetting error).

Note: For four samples, 100 µl aliquots use 50 µl DNase I Buffer + 10 µl rDNase I.

5.4.2) Aliquot 12 µl of the master mix in step 5.4.1 into each of the RNA aliquots from step 5.3.18 and mix gently. Incubate at 37 °C for 30 min in a heat block.

5.4.3) Vortex the DNase Inactivation Reagent and add 11.2 µl to each aliquot, mix well. Incubate at room temp for 2 min vortexing 2-3 times during incubation.

5.4.4) Centrifuge at 10,000 x g for 1.5 min.

5.4.5) Transfer the supernatant (RNA) to a fresh 1.5 ml tube. Aliquots from the same participant can be pooled here.

553
554 5.4.6) Run each sample on a spectrophotometer to get the concentration. A quality
555 analysis of the RNA is recommended using a Bioanalyzer (see step 5.5).
556

557 5.4.7) Aliquot the RNA into cryovials as desired. If a Bioanalyzer analysis will not be
558 performed immediately, aliquot 1.5 µl of sample into a microcentrifuge tube for later
559 analysis. Document the concentrations and the freezer start time. Store samples at -80
560 °C.
561

562 5.5) Bioanalyzer analysis (1 hr)

563

564 5.5.1) Follow the manufacturer's protocol¹⁹. When the run is complete, the data is
565 automatically stored, but save it again with a smaller, more recognizable file name.
566 Expected results (Figure 3): the ladder should have 6 peaks, samples should have 3
567 peaks (2 ribosomal peaks at 44 seconds and 50 seconds, respectively and 1 early
568 marker peak at 25 seconds).
569

570 REPRESENTATIVE RESULTS:

571 It is essential that the overall procedure produce high quality material for analysis in a
572 multitude of downstream applications including gene expression via microarray and RT-
573 PCR analysis, detection of epigenetic modifications, and cell subset variations. **Table 1**
574 indicates the average yield and quality of materials from each of the processes. **Figure**
575 **3** provides an example of the quality output of the leukocyte RNA isolation and filter
576 processing methods. The image on the upper left of Figure 3 is the gel image resulting
577 from the capillary electrophoresis. Each lane should produce two distinct bands with
578 minimal shadowing which would indicate degradation. The chromatograms below the
579 gel provide an additional look at the level and type of degradation that can be
580 determined based upon the location and size of the peaks. The RNA Integrity Number
581 (RIN) is another quality measure that ranges from 1 (low; degraded) to 10 (high; pure,
582 good quality RNA).
583

584 Such a high-throughput methodology lends itself to an occasional error, but there are
585 several checkpoints throughout the various stages of processing to ensure quality
586 control. **Figure 2** displays the proper separation following centrifugation of the ficoll
587 containing vacutainer. There are several reasons a vacutainer may not display this
588 separation including an error in centrifugation speed, low collection volume, or lack of a
589 fasting participant as indicated in **Table 2**.
590

591 Subsets (n=100) of the specimens isolated using this procedure have been analyzed
592 with success by HumanMethylation27 (HM27) DNA Analysis BeadChips including
593 validation of those results by pyrosequencing¹¹. Genotyping, targeted bisulfite
594 pyrosequencing, and real-time RT-PCR has been successfully performed in the
595 Wildman and Uddin laboratories^{20,21,22,23}. Serum, isolated in parallel with the DNA
596 isolation for both the Beadchip and genotyping analyses, was successfully analyzed for
597 IL-6 and C-reactive protein activity²⁴. T-cell subsets from the isolated PBMCs have been

successfully analyzed by flow cytometry²⁵⁻²⁸. Additionally, RNA from the modified leukocyte procedure has been subjected to Genome Wide gene expression profiling²⁹.

Figure 1. Overall work flow. The overall process depicted here includes the logistics of obtaining the blood specimens from identifying consenting participants to the blood draw itself. High-quality, fractions (peripheral blood mononuclear cells; PBMCs, DNA, serum, and RNA) of fresh whole blood can be produced within 2 hours of collection and all assay-ready specimens can be available within 2 days. Moreover, the fractions prepared through this method are suitable for long-term storage if samples are not to be tested immediately. The entire timeline outlined here could be completed in a single day (~5 hours total). However, such a day would be extremely labor intensive especially for a single technician with substantial experience with the techniques. Thus, we recommend dividing the procedures on Day 1 between at least two technicians and completing the RNA processing on Day 2.

Figure 2. Layer separation following centrifugation of ficoll containing vacutainer. A visualization of the separation of multiple blood components following centrifugation. The mononuclear layer is further purified while the other layers are stored at -80 °C.

Figure 3. Expected results from leukocyte RNA processing. Bioanalyzer results displaying two distinct bands representing 18S and 28S ribosomal RNA with RNA integrity numbers (RINs) above 8 isolated with the described leukocyte RNA isolation and filter processing methods (see protocol 3.6 and 5.3).

Table 1. Expected yields. Average quantity and quality of samples processed using the described methods.

Table 2. Troubleshooting. Common problems encountered with the described methods and potential solutions.

Supplementary Table 1. Tracking sheet. An example of tracking the vacutainers from blood collection to laboratory delivery.

Supplementary Table 2. Specimen log. An example of the document used to document the processing of the vacutainers upon delivery to the lab.

Supplementary Table 3. Cryovial Numbering System. An example of the numbering system used for each participant.

Supplementary Information 1. Preprocessing details. Details the duties of the Study Coordinator, Courier and Phlebotomist.

Supplementary Information 2. Recipes. A list of recipes for the necessary reagents.

Discussion:

We have described a streamlined protocol that has been successfully applied to process more than 1,600 whole blood samples in the Detroit Neighborhood Health Study. Although many of these techniques are available in the existing literature, our step-by-step compilation, including precisely timed alterations between each step, reflects an optimized, efficient protocol that successfully produces a variety of biologic specimens with a wide range of downstream applications, including DNA methylation, mRNA expression and immunological analysis. These specimens have already been tested in a variety of experiments, results of which have been published in peer-reviewed literature and/or presented at national meetings^{11,20,21,23,24,29}. This protocol should thus be of interest to other investigators seeking to collect biological specimens in population-based studies similar to the DNHS.

When processing samples in such a high-throughput manner, it is of utmost importance to maintain accurate records at all stages. We recommend developing a database to store all of the cryovial information at the outset. This database should include all aspects of the sample within each cryovial including the volume, concentration, quality, barcode, and storage location (storage box number and location within the box). We recommend preparing pre-labeled cryovials and storage boxes which contain a barcode. Further, we have found it convenient to have a “master” document containing the barcodes for each tube, specific to each participant (Table S3). This enables rapid input of the cryovial data into the database without excessive handling of the samples, introducing unnecessary freeze/thaw cycles to the samples and eliminates the potential for human error in entering in the barcodes manually. It is also essential that the labels adhere at extreme (e.g. vapor phase of liquid nitrogen -178 to -150 °C) temperatures. The documentation can be time consuming, and with the necessity of efficient processing upon delivery, we have found that having at least two technicians divide the processes produces the greatest efficiency.

A limitation of this method is the proximity of the laboratory to the collection site. For PBMC isolation in particular, the samples must be processed within 2 hours of collection to avoid significant increases in red blood cell contamination and decreases in viable mononuclear cells. As such, the laboratory should be no more than 30 minutes from a collection site to account for any traffic related issues that may arise. In addition, any laboratory proposing to undertake the protocol outlined here will require two technicians on hand to ensure optimal sample processing. Furthermore, each laboratory must have access to the requisite equipment for each step outlined above. Thus, laboratories with fewer staff and/or limited equipment would likely be unable to undertake this protocol.

An advantage of this protocol is the ability to collect specimens directly in the homes of consenting individuals. This allows the study to reach individuals with mental or other health issues who would not typically seek medical help, perhaps because of a lack of insurance or transportation. It also enables comparison of affected and unaffected individuals living within the same community who have experienced similar triggers, but differ in terms of their mental health symptoms. Obtaining specimens in this manner requires precise timing and coordination of phlebotomists in the field. Because our laboratory was located within the community we were studying, a phlebotomist could

typically collect samples from two-three homes and deliver the samples to the laboratory within the two-hour window of the first collection. Efficiency is key to our sample processing, and as such, we had multiple phlebotomists in the field, increasing the need for precise coordination. The laboratory received multiple blood deliveries with up to eight participants per delivery spaced two hours apart. The phlebotomists met at a designated location and combined their collections with only one phlebotomist delivering the specimens to the laboratory as a single batch. The methods described here can easily be adapted to study many other phenotypes and the biologic specimens collected can be used in a multitude of downstream assays.

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Disclosures:

The authors declare that they have no competing interests.

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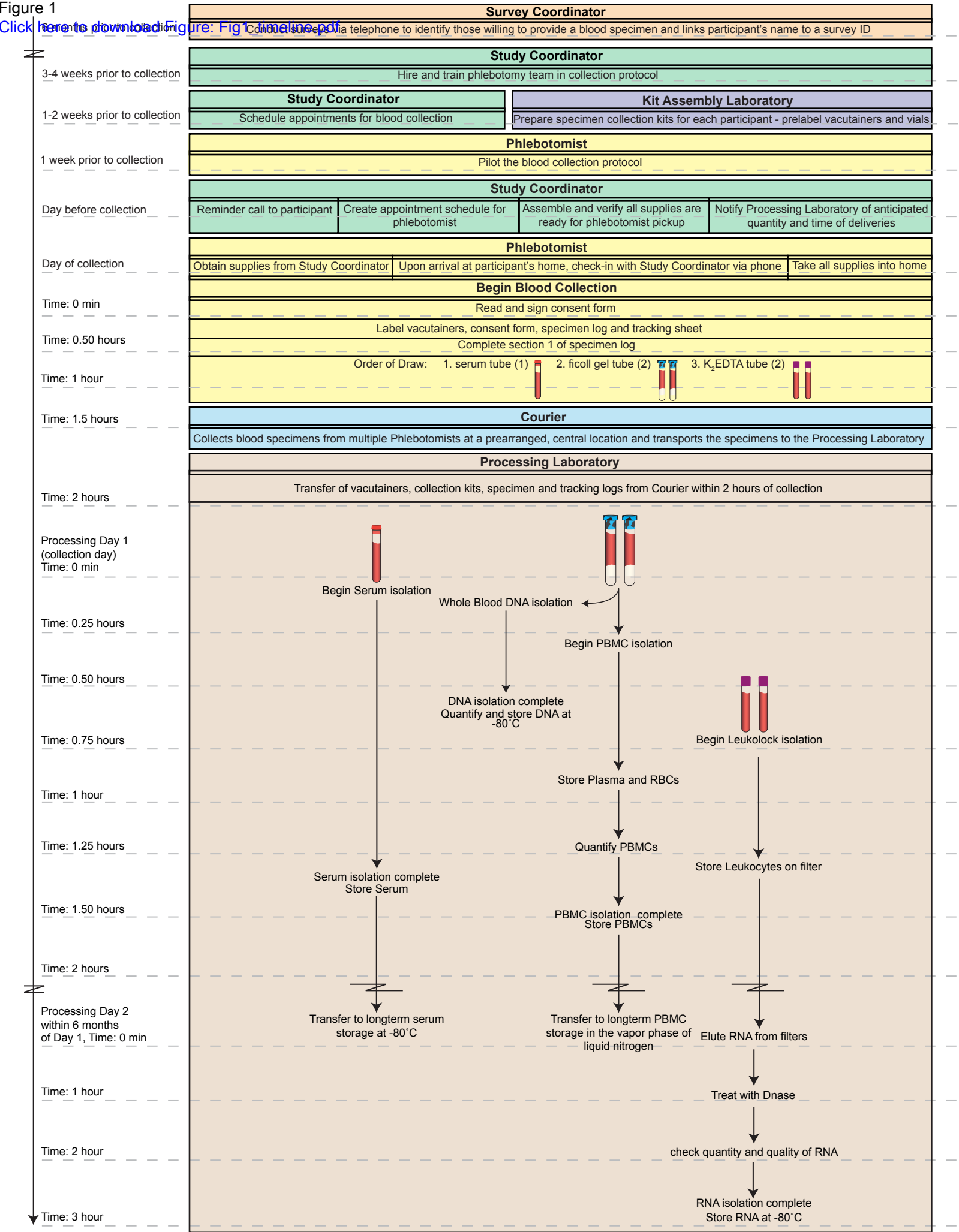


Figure 2
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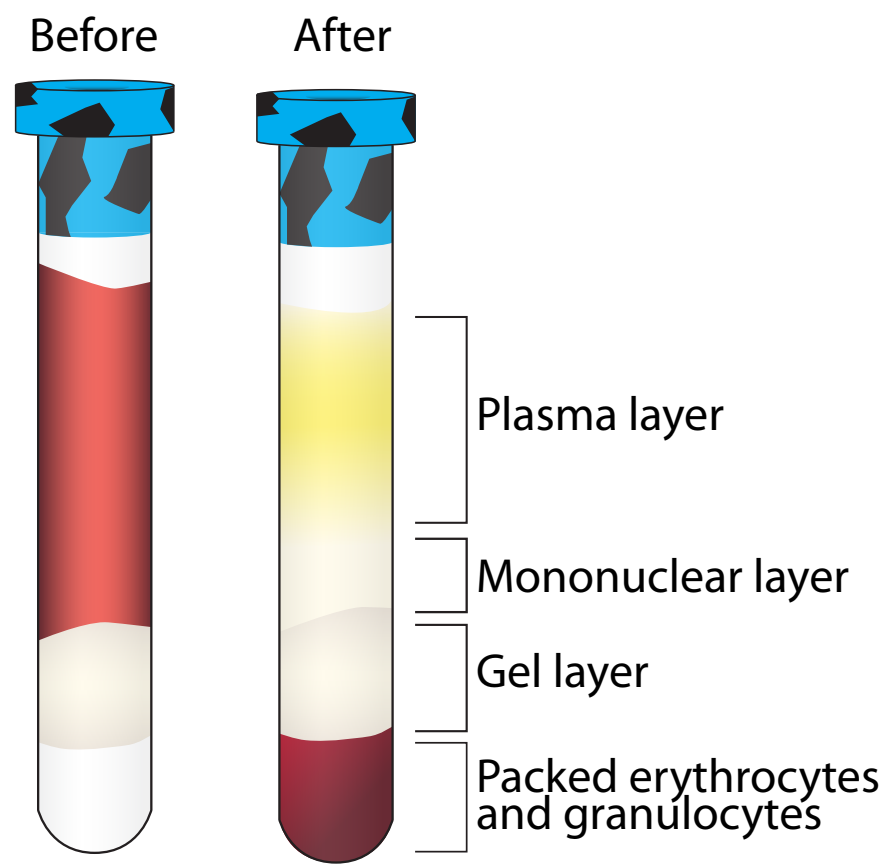


Figure 3
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Assay Class: Eukaryote Total RNA Nano
Data Path: C:\...Eukaryote Total RNA Nano_DE23101809_2010-09-22_15-55-12.xad

Created: 9/22/2010 3:55:12 PM
Modified: 9/22/2010 4:19:14 PM

Electrophoresis File Run Summary

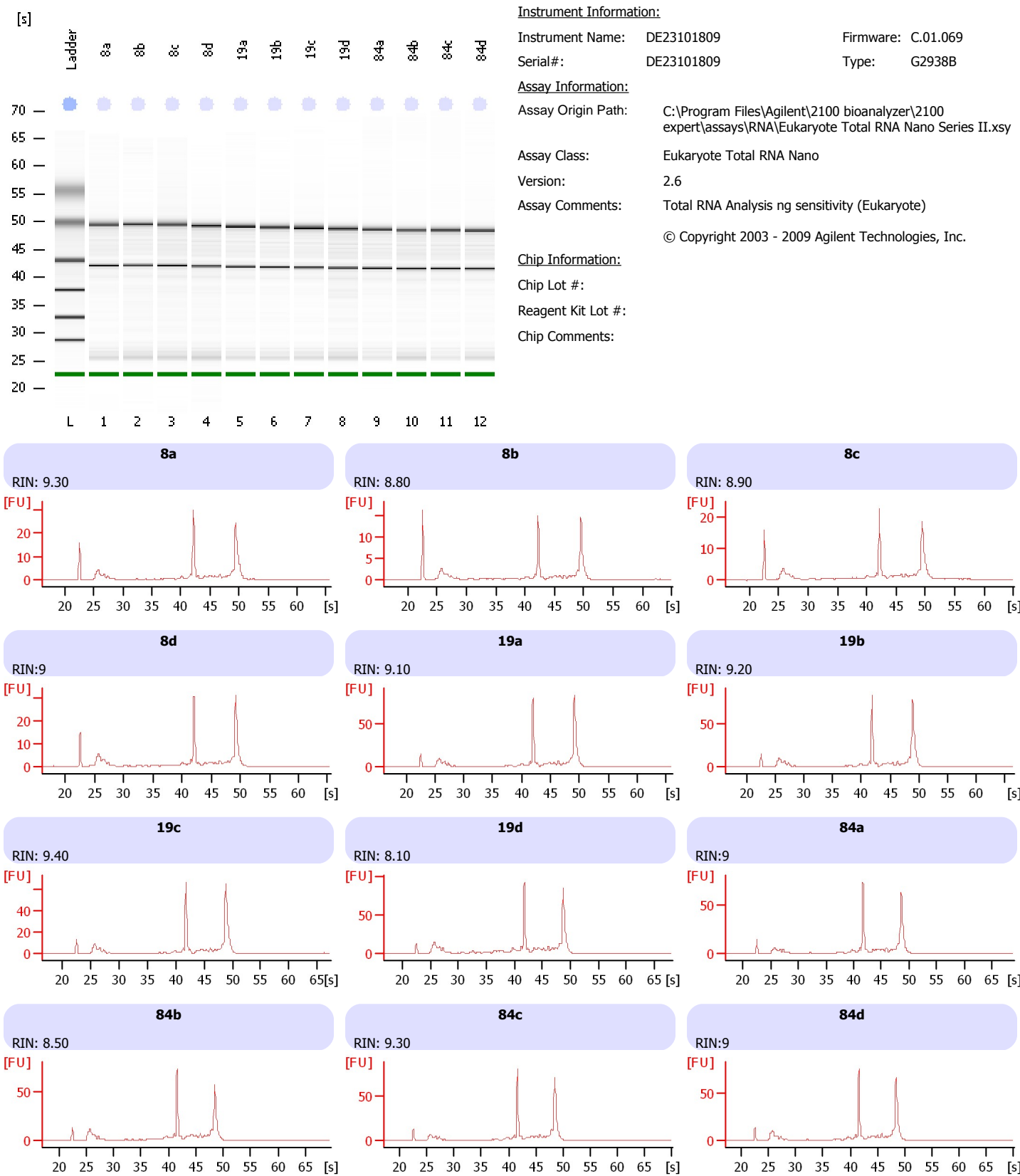


Table 1: Expected Yields		
	Total Average Yield (N≈500)	Average Quality
Serum	2.30ml	N/A
DNA from whole blood	39.97µg	Absorbance at 260/280 = 1.74
PBMC	22.25 million viable cells	At least 95% viability of overall isolation
RNA from Leukocytes	44.09µg	Absorbance at 260/280 = 2.01 RNA Integrity Number (RIN) = 6.48

<i>Problem</i>
No separation after ficoll containing vacutainer centrifugation
Low PBMC count
No separation after serum vacutainer centrifugation
Unexpected concentration using the Nanodrop 1000

Table 2: Troubleshooting

Potential Solution

Vacutainer is not filled to capacity - the minimum collection volume for adequate processing is 6ml.

Check centrifuge settings, be sure the speed is in g-force. If incorrect, set to g-force and respin.

Volume of blood draw too low.

Aspirated too close to the pellet in step 3.7.8

Non-fasting participant.

Check centrifuge settings, be sure the speed is in g-force. If incorrect, set to g-force and respin.

Make sure the measurement is for the appropriate sample type (eg. DNA or RNA).

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
QIAamp DNA Blood Mini Kit	Qiagen	51104	Day 1: DNA isolation
Phosphate-buffered saline (PBS)	Sigma	P5493-1L	Day 1: PBMC isolation
5 ¾" Pasteur pipets	Fisher	13-678-6A	Day 1: PBMC isolation
Fetal Bovine Serum (FBS), heat inactivated	Life Technologies	10082147	Day 1: PBMC isolation
Dimethyl Sulfoxide (DMSO)	Sigma	D8418-500ml	Day 1: PBMC isolation
RPMI Medium 1640, liquid	Invitrogen	11875119	Day 1: PBMC isolation
0.4% trypan blue stain	Invitrogen	T10282	Day 1: PBMC isolation
Countess Cell Counting Chamber	Invitrogen	C10283	Day 1: PBMC isolation
Countess Automated Cell Counter or cell counting device such as a microscope and hemocytometer	Invitrogen	C10281	Day 1: PBMC isolation
LeukoLOCK Fractionation & Stabilization Kit	Ambion	1933	Day 1: Leukocyte RNA isolation
25G x 5/8 in. needles	Becton Dickinson	305122	Day 1: Leukocyte RNA isolation
Syringes (5ml)	Becton Dickinson	309646	Days 1 and 2: Leukocyte RNA isolation
Denaturing Lysis Solution	Ambion	8540G	Day 2: Leukocyte RNA isolation
5M NaCl	Life Technologies	24740011	Day 2: Leukocyte RNA isolation
TRI Reagent	Ambion	9738	Day 2: Leukocyte RNA isolation
Bromo-3-chloro-propane (BCP)	Sigma	B-9673	Day 2: Leukocyte RNA isolation
spin cartridges	Ambion	10051G	Day 2: Leukocyte RNA isolation
0.1mM EDTA	Ambion	9912	Day 2: Leukocyte RNA isolation
DNA-free Kit	Ambion	AM1960	Day 2: DNase treatment
RNA 6000 Ladder	Agilent	5067-1529	Day 2: Bioanalyzer analysis
RNA 6000 Nano Series II Kit	Agilent	5067-1511	Day 2: Bioanalyzer analysis
RNaseZAP	Ambion	AM8782	Day 2: Bioanalyzer analysis
Ethanol >99%	Sigma	E7023-500ml	
Isopropanol >99%	Sigma	I9516-500ml	
Nuclease-free ultra pure water	Invitrogen	9938	
Pipette tips (nuclease-free)	Eppendorf	22491253	
Pipetter (serological)	Eppendorf	2223020-4	
Pipetters (for volumes under 1ml)	Eppendorf	3120000-054	
Pipettes (serological)	Fisher	13-678-27E	
Controlled rate freezing containers	Nalgene	5100-0001	
Cryoboxes (to hold 2ml and 5ml cryovials and 1.5ml microcentrifuge tubes)	Fisher	03-395-464	
Test tube rack	Thermo Scientific	14-804-134	
15ml polypropylene tubes	Fisher	14-959-49D	
1.5ml and 0.65 microcentrifuge tubes	Fisher	07-200-534 and 07-200-185	
2ml and 5ml cryovials	Fisher	10-500-26 and 10-269-88F	
8ml CPT vacutainer	BD Biosciences	362761	2 tubes
6ml K ₂ EDTA vacutainer	BD Biosciences	367863	2 tubes
8.5ml SST vacutainer	BD Biosciences	367988	1 tube
Vortexer	Fisher	2215365	



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CORRESPONDING AUTHOR:

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Institution: Wayne State University School of Medicine
Article Title: Rapid fractionation and isolation of whole blood components in samples obtained from a community-based setting
Signature:  Date: 04/16/14

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16 March 2015

We would like to thank the editor and the reviewers for their thorough evaluations of our manuscript and their thoughtful critique, which has improved the presentation of our work. Below are our detailed responses to each comment.

Editorial comments:

*** All of your previous revisions have been incorporated in to the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.**

Revisions were made to the document downloaded from the file inventory.

*** The total length of the highlighted text exceeds 2.75 pages. We suggest that one or more notes be unhighlighted to comply with this length limit.**

The highlighted text is better explained in the supplemental file, "Info for script," which has been approved by the Editor-in-Chief. Our compilation of multiple protocols makes trimming the highlighted sections any further very difficult. Not all of the highlighted portions need to be filmed, but they are necessary for the film crew and voice over to understand the transitions.

*** There are some scattered formatting/grammar issues which must be corrected:**

-3.6.4: Avoid use of personal pronoun "we"

This has been corrected (see lines 327-331) and now reads:

Attach a sheathed 25⁵/₈ gauge needle to the outlet (tapered end) of the filter. Preparing the filter assembly prior to delivery expedites the process considerably and therefore, attaching the white slip connector to the transfer spike, adding the filter, then the sheathed needle and setting the assembly in a culture tube rack until use, is recommended.

-5.1.1 Note: Avoid use of personal pronoun "we"

This has been corrected (see line 482) and now reads:

Note: Overall, Day 2 processing takes about 3 hours and while it is labeled "Day 2," the key requirement is that the filter processing portion should be performed on a day when there is no Day 1 processing occurring in the lab.

-5.2.1: Grammar issues make this step unclear

This has been modified (see lines 488-490) and now reads:

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Perform this procedure every day, prior to the delivery of new vacutainers to the lab for Day 1 processing. Transfer the cryovials that were stored overnight in controlled rate freezing containers on Day 1 into appropriately labeled cryoboxes and return them to -80 °C or liquid nitrogen (vapor phase) for long term storage. Organize the cryovials based on sample type (*e.g.*, DNA, PBMC, RBCs, etc.) to expedite sample location tracking for endpoint assays.

*** There is unnecessarily branding in a number of areas:**

-2.6: RNAlater

The text has been modified (see lines 210-211) and now reads:

Replace the cap on the bottle of 1X PBS with the provided rubber septum cap and pierce the rubber septum with a transfer spike, retaining the screw cap on top of the transfer spike to prevent evaporation. Repeat with the bottle of RNA stabilization solution.

-3.5.14, 5.4.6: NanoDrop 1000

This has been modified (see lines 307 and 598) and now respectively reads:

Quantify the samples using a spectrophotometer when time allows.

Run each sample on a spectrophotometer to get the concentration. A quality analysis of the RNA is recommended using a Bioanalyzer (see step 5.5).

-Section 5: LeukoLOCK, TRI Reagent

The text has been modified (see lines 475 and 513-515) and now reads:

Day 2: Long term sample storage and leukocyte filter processing (3 hr)

and

Load a new 5 ml syringe with 4 ml of a phenol and guanidine isothiocyanate solution for RNA isolation and attach it to the inlet of the filter, depress the plunger to flush the solution through the filter, collecting the lysate in a labeled 15 ml conical tube (2 per participant).

*** Please provide more description of the graphs shown in figure 3. Are there any differences in quality among the samples?**

We have added additional information (see lines 608-618) for Figure 3 and the text now reads:

Follow the manufacturer's protocol¹. When the run is complete, the data is automatically stored, but save it again with a smaller, more recognizable file name. Expected results (Figure 3): the ladder should have 6 peaks, samples should have 3 peaks (2 ribosomal peaks at 44 seconds and 50 seconds, respectively and 1 early marker peak at 25 seconds). The image on the upper left of Figure 3 is the gel image resulting from the capillary electrophoresis. Each lane should produce two distinct bands with minimal shadowing

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which would indicate degradation. The chromatograms below the gel provide an additional look at the level and type of degradation that can be determined based upon the location and size of the peaks. The RNA Integrity Number (RIN) is another quality measure that ranges from 1 (low; degraded) to 10 (high; pure, good quality RNA).

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

The manuscript has been proofread and we see no remaining errors.

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The references have been thoroughly examined and modified as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper is a great idea but it needs to be improved. I advise you to collaborate with a molecular biologist who understands the rationale behind the protocols and rewrite the manuscript.

Thank you for your thorough evaluation of our work. Our manuscript is aimed at those investigators looking to add a molecular aspect to their study. The work may be



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particularly relevant to population based scientists (epidemiologists, anthropologists) or other social scientists (psychologists, sociologists) who may not need an in-depth understanding of the molecular biology of each step in the protocol, but instead need to know what protocols to use or refer to in order to acquire and process their samples of interest. In many instances, these investigators will be collaborating with a molecular biologist with a more in-depth understanding of the protocols. The detailed molecular biology supporting the rationale behind the protocols can also be obtained through relevant manuals ^{2,3} if necessary but is, for our purposes, beyond the scope and length of this manuscript.

Major Concerns:

1. It is not clear who this article is aimed at. If it is aimed at the novice with no experience then the repetition of instructions available in the kits could perhaps be justified, however in this case other areas of technical difficulty (such as cell counting section 3.9.5) are just glossed over.

Our target audience would be those investigators looking to collect and process biospecimens from large populations in a high-throughput manner. Investigators from various fields (epidemiologists, environmental biologists, physicians, psychologists, etc) can adapt this streamlined procedure to fit their study. This procedure can add a biomolecular aspect to environmental factors or phenotypic traits already being studied. While the procedure does use many standard kits, each step is specified here to help the reader understand how each kit overlaps to produce the most efficient means of processing. Please also see our response to the summary comment above.

2. The descriptions lack depth and mostly are repetitions of instructions found in the kits and tubes that the authors recommend

What makes our procedure unique is the way we overlap the various, standard procedures to produce multiple endpoint samples in an efficient, high-throughput manner on a daily basis. The procedure is written for use by those that have likely used many of the protocols, but not necessarily, all in a single day.

3. Little attempt is made to explain why the chosen kits and methods are given instead of others available in the market

We have added the following (see lines 135-138) to the text of the introduction:

While alternative methods or kits may be employed in place of the individual methods described here, have proven to be a reliable and efficient means for processing samples in a high-throughput manner.



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4. The heading Leucocyte isolation is misleading as in effect I think they are trying to make blood RNA, also why would they not make RNA from the PBMCs? these kind of issues are not really addressed.

We have modified the headings to read, "leukocyte RNA isolation," for clarification (see lines 216, 313, 503, 625, 665, and 667). We were fortunate in that we were approved to collect two additional vials of whole blood from each participant solely for RNA isolation. This allowed us to reserve all of the PBMCs we isolated for other downstream analyses. Certainly, this is a portion of the protocol that can be modified by other investigators with more limited collections. We reference the Life Technologies LeukoLOCK kit for use during the leukocyte RNA isolation portion of the protocol. This kit allows for RNA isolation strictly from white blood cells and washes away globin mRNA that can interfere with other downstream analyses.

5. Figure 1 nor table of materials do not mention tube sizes for vacutainers , this is a very important point. The tubes mentioned come in various sizes again if this article is aimed at a novice this should be explained better. Similarly volumes of blood are not really discussed unless i missed them in all the other often unnecessary details
The table of materials has been modified to reflect the quantity and volume of the vacutainers and centrifuges necessary to perform the protocol as written.

Minor Concerns:

6. In some places they list manufacturer names for solutions e.g. AW1 buffer, AW2 buffer with no explanation or what these stand for, this is not correct for a scientific article all abbreviations need to be explained.

These are reagents contained within the kit (referenced) whose exact composition is proprietary, but are used as wash buffers to increase the purity of the DNA bound to the filter during DNA isolation. We have added the underlined portion below to the first mention of these buffers (see lines 201-202).

Ensure Buffers AW1 and AW2 (wash buffers provided in the DNA isolation kit that increase the purity of the DNA) are ready for use, adding 100% ethanol as indicated on the label, if necessary.

7. It is not made clear whether the parallel processing will require more than 1 person.

This is discussed in both the legend of Figure 1 as well as in the Discussion - please see lines 656-659 and 715-717.

Additional Comments to Authors:



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8. The authors should edit the instructions including only the relevant information not found in the kits and the rationale for why these particular kits are being recommended could be explained better.

While we appreciate that reducing the information to that not found in the kits would shorten the manuscript, we believe that the information provided is necessary to meet one of the main goals of the paper, i.e. specifying how to efficiently process multiple samples simultaneously, which requires clarification of how each protocol overlaps. It should be noted that we received permission from each of the kit manufacturers to include this level of detail in our work. Please see also our response to item 3 above.

9. A paper such as this could be of great use to inexperienced staff but that it needs much improvement.

This protocol was compiled to meet the needs of studies that required processing of multiple blood specimens in a simultaneous manner. We expect that it will be a useful resource to investigators looking to expand their research to include biospecimen collection and processing. We hope that others will be able to modify and build upon our methods to meet their own needs.

Reviewer #2:

Manuscript Summary:

This manuscript described the streamlining of a large data collection study for a population of >1600 people. The authors provide a detailed protocol for the collection of RNA, DNA, and cells for a large compendium of data necessary to characterize a large population. Overall, the manuscript was very well written and concise in description. I feel that the protocol provides a valuable tool to those in the field, including just collection of cells, or DNA, or RNA. Each of the protocols could be easily translated to various field and to the labs current equipment - of course will possible sacrifices in time or data set sizes.

Thank you for your thoughtful review. We are pleased that the idea that the protocol could be adapted to various applications was clear.

Major Concerns:

None.

Minor Concerns:

The authors provide a table of materials/equipment. It would be helpful if quantity is provided. Looking at the protocol it is clear that the impressive timeline can only be done is the laboratory has several centrifuges. The authors should (1) add

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numbers to the table and (2) acknowledge that there are equipment constraints to the descriptions provided.

The table of materials has been modified to reflect the quantity and volume of the vacutainers and centrifuges necessary to perform the protocol as written. Also, in the Discussion (see lines 725-727), we mention:

Furthermore, each laboratory must have access to the requisite equipment for each step outlined above. Thus, laboratories with fewer staff and/or limited equipment would likely be unable to undertake this protocol.

Reviewer #3:

Manuscript Summary:

This paper describes a streamlined protocol to separate nucleic acid, serum and leukocytes from blood, as well as store the sample for a long term.

Thank you for your review.

Major Concerns:

It is clearly written. No concern was raised.

Minor Concerns:

None

- 1 Technologies, A. *Agilent RNA 6000 Nano Kit Guide*, Santa Clara, California, USA, <http://www.genomics.agilent.com/files/Manual/G2938-90034_KitRNA6000Nano_ebook.pdf> (2006).
- 2 Bruce Alberts, A. J., Julian Lewis, David Morgan, Martin Raff, Keith Roberts, Peter Walter. *Molecular Biology of the Cell*. Sixth edn, (Garland Science, 2014).
- 3 Michael R. Green, J. S. *Molecular Cloning: A Laboratory Manual*. Fourth edn, (Cold Spring Harbor Laboratory Press, 2012).

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