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 25⅝ 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:

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 307and 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 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.

**\* Please make sure that your references comply with JoVE instructions for authors- your manuscript will not pass internal review unless the reference formatting is correct. In-text formatting: corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text of the manuscript. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. Volume (issue), FirstPage – LastPage, DOI, (YEAR).] JoVE follows recommended formatting for release to PubMedCentral, for specific reference questions please see the following:** [**http://www.ncbi.nlm.nih.gov/staff/beck/citations/citationtags.html**](http://www.ncbi.nlm.nih.gov/staff/beck/citations/citationtags.html)

**\* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.**

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 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 perhpas 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 innstead 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.

**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:***

**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 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).