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Use of Capillary Electrophoresis Immunoassay to Search for Potential Biomarkers of Amyotrophic Lateral Sclerosis in Human Platelets

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To

Senior Review Editor

JoVE

1 Alewife Center, Suite 200

Cambridge, MA 02140/USA

September 22, 2019

Dear Dr. Vineeta Bajaj,

Thank you for your reply regarding our manuscript # JoVE60638; entitled *Capillary electrophoresis immunoassay utilization in search of potential biomarkers for amyotrophic lateral sclerosis in human platelets.*

We are grateful for your and the reviewers comments, and the positive evaluation of our work. We have revised and modified in good faith the text and figures according to the referee's critiques. We entered our reply in colored ink in both review editor and referees' letters right after the questions. These changes have considerably improved the manuscript and we hope that it can be published without delay

Sincerely,

Abdulbaki Agbas, MSc, PhD

P.S. Please note that title of the manuscript has changed as per recommendation of the reviewer.

TITLE:

Use of Capillary Electrophoresis Immunoassay to Search for Potential Biomarkers of Amyotrophic Lateral Sclerosis in Human Platelets

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KEYWORDS:

TDP-43, phosphorylated TDP-43, capillary electrophoresis, human platelet, neurodegenerative diseases, biomarker, predictive phosphorylation value, assay optimization

SUMMARY:

Blood-based biomarkers for neurodegenerative diseases are essential for implementing large-scale clinical studies. A reliable and validated blood test should require a small sample volume as well as be a less invasive sampling method, affordable, and reproducible. This paper demonstrates that high-throughput capillary electrophoresis immunoassay satisfies criteria for potential biomarker development.

ABSTRACT:

Capillary electrophoresis immunoassay (CEI), also known as capillary western technology, is becoming a method of choice for screening disease relevant proteins and drugs in clinical trials. Reproducibility, sensitivity, small sample volume requirement, multiplexing antibodies for multiple protein labeling in the same sample, automated high-throughput ability to analyze up to 24 individual samples, and short time requirement make CEI advantageous over the classical western blot immunoassay. There are some limitations of this method, such as the inability to utilize a gradient gel (4%–20%) matrix, high background with unrefined biological samples, and

commercial unavailability of individual reagents. This paper describes an efficient method for running CEI in a multiple assay setting, optimizing protein concentration and primary antibody titration in one assay plate, and providing user-friendly templates for sample preparation. Also described are methods for measuring pan TDP-43 and phosphorylated TDP-43 derivative in platelet lysate cytosol as part of the initiative in blood-based biomarker development for neurodegenerative diseases.

INTRODUCTION:

The overall goal of CEI as described here is to provide an updated stepwise protocol for analyzing target proteins in human platelets. Assignment of a blood-based signature molecule is one of the most important tasks in the field of biomarker development in human neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), Parkinson's disease (PD), inclusion body myositis (IBM), and other protein-aggregation relevant pathologic conditions. The detection of minute amounts of such signature proteins in large volumes of blood with many interfering agents is a challenge. Therefore, the specificity, sensitivity, ability to handle large number of samples, and reproducibility of the selected method are crucial.

Human platelets can serve as a milieu to identify and assign potential biomarker proteins for neurodegenerative disease. Platelets provide the opportunity to serve as a surrogate primary cell model, which reflect some features of neuronal cells[1-3]. There are certain features that make platelets one of the preferred means to analyze biomarker candidate proteins and their chemical derivatives. First, platelets can be easily acquired using a less invasive approach by collecting blood from donors (i.e., venipuncture) or in large volumes from community blood banks. Second, platelets can be easily isolated from the whole blood with minimal preparatory work in minimally equipped laboratories[4,5]. Third, platelets do not have nuclei; therefore, they are a good model cell to study alterations in metabolism without transcriptional regulation. Fourth, the biomolecule content of platelets is encapsulated; therefore, the platelet microenvironment protects its contents from serum-interfering substances (i.e., proteases). Fifth, platelet-enriched plasma can be stored at room temperature for 7–8 days without losing metabolic activity. Therefore, platelets provide a working model in which external factors are minimized and controlled.

Traditional immunoassay techniques such as immunoblotting (e.g., western blotting) and enzyme-linked immunosorbent assay (ELISA) are more widely used in specific protein analysis. However, these two methods have several disadvantages, including multiple assay steps, requirement of hazardous chemicals and reagents, large sample size, issues with assay reproducibility, and inter-run data variabilities. These prompted the development of a method that is simpler with fewer steps and achievable in a relatively short period. Although the classical western blot technique will remain a popular laboratory method, its multi-step procedure, supplies, toxic waste (i.e., acrylamide, methanol, etc.) and assay time are becoming less desirable when performing high-throughput quantitative protein analysis.

An automated CEI approach is gradually becoming a method of choice for laboratories that conduct high-throughput protein assays[6]. CEI eliminates the need for gels, gel electrophoresis apparatuses, membranes, electrophoresis and electro-transfer devices, and more physical handling involvements. If designed well, a CEI assay should be completed within approximately 3.5 h, including quantitative data analysis, publication quality electropherogram, and graphs with statistical analysis. Another superiority of the CEI system is its requirement of 10x–20x less protein concentration, making it ideal for use in human samples used in clinical trials[7,8].

The most critical part of CEI is optimizing the assay conditions for each antibody purchased from different vendors, type of antibody (monoclonal *vs*. polyclonal), optimum protein concentrations, sample preparation, sample denaturation temperature, and electrophoresis voltage applied on the capillaries. We have developed a single-assay format optimization method for the CEI that should be implemented before any new assays, which will save time and resources. This optimization step is followed by an automated quantitative assessment of both total and phosphorylated derivative of transactivation response DNA/RNA binding protein (TARDP). Due to its size (43 kDa), the acronym TDP-43 will be used throughout this paper. Here, TDP-43 protein in human platelet lysate obtained from ALS patients are assessed to help develop predictive phosphorylation value (PPV) as a potential prognostic biomarker.

TDP-43 is a new potential disease biomarker candidate for ALS. TDP-43 is an omnipresent protein in all nucleated cells; therefore, the functions of TDP-43 during various normal cellular events and in neurodegenerative disease have been investigated[9-14]. Although TDP-43 is a nuclear protein[15], it has the ability to shuttle in and out between the nucleus and cytoplasm due to the presence of nuclear localization and nuclear export sequences[16-19]. Cytoplasmic TDP-43 is involved in various cellular events, such as mRNA stability and transport, the stress response, mitochondrial function, autophagosome[20]. However, not much is known about the role of phosphorylated derivatives of TDP-43 other than their involvement in the pathogenesis of neurodegenerative disease[21].

This protocol illustrates how to optimize the assay conditions to analyze the contents of TDP-43 and its phosphorylated derivative in platelets using the CEI approach. Since phosphorylated TDP-43 is not commercially available, it is proposed to use a predictive phosphorylation value (PPV) to assess TDP-43 profiles in ALS patients. This CEI system utilizes a small volume of sample mixture (2.5–3.0 µL per capillary). Total assay volume set-up is 8.0 µL per capillary based on the manufacturer's protocol; hence, researchers can utilize one sample mixture preparation for two separate runs. The manufacturer designed the assay protocol so that any pipetting errors are minimized, if not entirely eliminated. The 24 individual human platelet lysate sample mixtures are divided into half-volumes (i.e., 2.5–3.0 µL per sample) and consecutively analyzed those by two different antibodies within ~7 h. The CEI system described here provides a desirable high-throughput assay modality. Users need to test antibodies from different vendors and sample preparation modalities for the target protein before performing large-scale screening.

PROTOCOL:

All protocols concerning the processing of human platelets follow the guidelines of both the University of Kansas Medical Center and Kansas City University of Medicine and Biosciences IRB committees.

1. Preparation of buffers and reagents

NOTE: Prepare all samples as per the manufacturer's guidelines. Wear personal protection equipment (lab coats, gloves, and goggles) during this procedure.

1.1. Prepare citrate wash buffer by combining 0.941 g of sucrose (11 mM final concentration), 6.4 mL of 5 M NaCl (128 mM final), 5.4 mL of 0.2 M NaH_2PO_4 (4.3 mM final), 9.4 mL of 0.2 M Na_2PHO_4 (7.5 mM final), 0.352 g of sodium citrate (4.8 mM final), and 0.115 g of citric acid (2.4 mM final). Adjust the total volume to 250 mL with ddH_2O. Filter through a 0.45 µm filter disk and adjust the pH to 6.5. Store up to 1 year at 4 ˚C. Bring the solution room temperature (RT) before use[22].

1.2. Prepare the rupture buffer by combining 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-Cl (pH 7.4) in a 100 mL final volume. Store up to 1 year at 4 ˚C. Add 2 µL of phosphatase inhibitors cocktail (1:1000 final) and 1 µL of protease inhibitors cocktail (1:2000 final) into 2 mL of rupture buffer. Keep on ice until use. Discard the unused rupture buffer.

2. Platelet isolation

2.1. Collect 8–10 mL of human blood in yellow-cap blood collection tube containing acid-citrate-dextrose (ACD) solution (75 mM trisodium citrate, 124 mM dextrose, and 38 mM citric acid, pH = 7.4; ACD:blood = 1:9). Gently mix the tube content 5x–6x inverting by hand.

2.2. Centrifuge the tubes at 200 *x g* in a swinging bucket rotor for 20 min at RT.

2.3. Collect platelet-rich plasma (PRP) (~3–4 mL) into a 15 mL conical bottom tube and leave approximately 0.5 mL of the PRP from the buffy coat (hazy-looking fraction) to avoid contamination. If any red blood cell contamination occurs, repeat this step.

2.4. Centrifuge the PRP samples at 1,200 *x g* for 15 min at RT.

2.5. Wash the platelet pellets (P1) by gentle resuspension in 1 mL of citrate wash buffer and pellet by centrifugation at 1,200 *x g* for 15 min at RT.

2.6. Save the pure platelet pellet. Discard the supernatant.

2.7. Resuspend the platelet pellets in 600 µL of the rupture buffer containing inhibitor cocktails.

2.8. Sonicate the platelet suspension using a sonicator. Place the sample in a mini ice bucket. Set the sonicator at setting 3 for 20 s in continuous mode.

NOTE: Make sure to clean the probe with 10% bleach followed by distilled water.

2.9. Centrifuge the sonicated samples at 20,000 *x g* for 30 min at 4 °C to remove membranous fractions. Aliquot supernatants in 60 µL and store at -80 ˚C. Avoid repeated thawing/freezing cycles for the platelet cytosolic fractions.

3. Preparation for CEI

NOTE: 100 µL of human platelet lysate was combined from ALS patients (n = 8–10), and healthy subjects (n = 8–10) were separately pooled and used for the assay optimization.

3.1. Fill out in-house generated templates for the CEI layout (**Table 1**) and sample preparation (**Table 2**). The sample mixture preparation table is dynamic and will automatically calculate how much volume needs to be removed from source.

NOTE: Sample mixture was inserted here, which includes pooled human platelet lysate, 0.1x sample buffer, and 5x fluorescent standard.

3.2. Pre-label 25 0.2 mL PCR tubes with capillaries #1–#25 and place them in a PCR rack. Set on ice.

3.3. Pre-label 0.6 mL microcentrifuge tubes: one for each primary antibody and dilution (if needed) to be used, one for the 0.1x sample buffer, one for the luminol-S/peroxide, and one for each sample to be diluted (if needed). Place them on ice in tube rack.

3.4. Take out the sample buffer, wash buffer, one plate, and a cartridge provided in the CEI separation 12–230 kDa master kit separation module.

3.5. From the 4 °C refrigerator, take out the antibody dilution buffer, primary antibodies, secondary antibodies, luminol, hydrogen peroxide, and standard pack. Place all reagents on ice, except the standard pack, which remains at RT.

NOTE: The reagents from the standard packs are lyophilized and sealed with a foil cover. These should be spun down briefly using a mini centrifuge before opening to reduce product loss. To open, the reagent tubes can either be pierced by a pipette tip or pulled back from the corner.

3.6. To prepare the 400 mM DTT, add 40 µL of deionized water to the clear tube containing the DTT.

3.7. To prepare 40 µL of fluorescent 5x master mix, add 20 µL of the 10x sample buffer and 20 µL of the prepared 400 mM DTT solution to the pink tube provided in kit.

3.8. To prepare the biotinylated ladder, add 16 µL of deionized water, 2 µL of 10x sample buffer, and 2 µL of the prepared 400 mM DTT solution to the white tube provided in kit. Mix gently and transfer into a 0.2 mL PCR tube for denaturing.

3.9. Prepare 0.1x sample buffer by adding 1.5 µL of 10x sample buffer and 148.5 µL of deionized water to a 0.6 mL micro-centrifuge tube. Vortex to mix and place on ice.

3.10. Prepare the desired antibody dilutions. Add antibody diluent in volumes designated to each pre-labeled micro-centrifuge tube. If volumes are identical, use reverse pipetting technique[23]; if not, pre-rinse the pipette tip before dispensing.

NOTE: In this assay, a-TDP-43 pan antibody and a-p(S409/410-2) TDP-43 antibody were used. Anti-ERK antibody was used for an internal control to make sure that assay components are working.

3.11. Perform the reverse pipetting for antibody dilution as described below. Alternatively, additional information can be found in the literature[24].

NOTE: Reverse pipetting technique is preferred when dispensing small sequential volumes of solutions[23]. This technique offers some advantages: (i) providing a precise volume, (ii) eliminating the reagent foaming in the tip orifice, and (iii) ideal for small volume (<5 µL) reagents, viscous solutions, surfactant solutions, and solutions with high vapor pressure.

3.11.1. Put a proper tip in a pipette and press the plunger down to the second stop (Step-2). Immerse the pipet tip a few millimeters into the solution. Slowly release the plunger to fill up the pipet tip with the solution while the tip is still immersed in the solution. Remove the tip from the solution and gently touch against the edge of the reagent reservoir so that excess liquid remaining on the outside of the tip is removed.

3.11.2. Dispense the solution by pressing the plunger down to first stop (Step-1). Do not dispense the remaining solution in the tip.

3.11.3. Empty the remaining solution in the tip to the reagent reservoir by pressing the plunger to the second stop (Step-2). Release the plunger to the ready position for the next pipetting step.

3.11.4. Add the required antibody in volumes designated to each pre-labeled microcentrifuge tube (**Table 1**) Do not pre-rinse the pipet tip: add it directly to the diluent and flush the tip multiple times to remove antibody. Place the tubes on ice.

3.12. To prepare the CEI sample-mix, perform the steps listed below for PCR tubes labeled cap#2 through cap#25: This is in the same order as it appears in **Table 1.**

3.12.1. Open all tubes, add 1.6 µL aliquots of fluorescent 5x sample buffer to each tube using a reverse pipetting technique, then close each PCR tube upon the addition of the 5x buffer to minimize sample loss.

3.12.2. Open all tubes, add 0.1x sample buffer in volumes designated in **Table 2** to each tube, then close immediately afterward. If volumes are identical, use a reverse pipetting technique. If not, pre-rinse pipet tip before dispensing 0.1x sample buffer.

3.12.3. Open all tubes, add protein sample in volumes designated in **Table 2** to each tube, then close immediately afterward. If volumes are identical, use reverse pipetting technique. If not, pre-rinse pipet tip before dispensing 0.1x sample buffer.

3.12.4. Briefly centrifuge all PCR tubes in a benchtop centrifuge (13,000 x *g* for 30 s), flick/vortex PCR tubes to mix, then repeat the centrifugation.

3.12.5. Transfer all PCR tubes into thermocycler with a heated lid. Denature samples at defined temperature and duration (i.e., 95 ˚C for 5 min; 70 ˚C for 10 min).

NOTE: The denaturation temperature and duration need to be optimized for target protein.

3.12.6. Repeat step 3.12.4.

3.12.7. Return all PCR tubes to tube rack and place on ice.

3.12.8. During the denaturing step, prepare the development solution (1:1 luminol-S:peroxide solution), then add 200 µL of luminol-S and 200 µL of peroxide. Place on ice.

3.12.9. To load a CEI pre-filled plate with the sample prepared above, dispense reagents and samples into the assay plate shown in the assay layout (**Figure 1**). Avoid introducing air bubbles.

(Place **Figure 1** here)

NOTE: If volumes and solution are identical, use a reverse pipetting technique. If not, pre-rinse the pipet tip before dispensing and do not expel the remainder into the plate well using the second tab stop on the pipette. A 12–230 kDa separation module may contain a color-coded plate-loading guide. Place this guide under the plate while adding reagents and samples to the well, which visually helps when sample loading. The plate-loading guide can be downloaded from company website, as well.

3.12.9.1. In row D, to well D1, add 10 µL of streptavidin-HRP.

3.12.9.2. In row D, to wells D2–D25, add 10 µL of designated secondary antibody.

3.12.9.3. In row B, to each well, add 10 µL of antibody diluent.

3.12.9.4. In row C, to well C1, add 10 µL of antibody diluent.

3.12.9.5. In row C, to wells C2–C25, add 10 µL of designated primary antibody.

3.12.9.6. In row A, to well A1, add 5 µL of biotinylated ladder from PCR tube #1.

3.12.9.7. In row A, to wells A2–A25, add 3 µL of the sample, PCR tubes #2–#25 into corresponding wells #2–#25.

3.12.9.8. In row E, add 15 µL of luminol:peroxide mix to each well.

NOTE: Ideally, prepare this reagent just before use and add to each well. If this is not convenient, this mixture may be prepared no more than 30 min prior to plate loading.

3.12.9.9. Add 500 µL of wash buffer to each designated wash buffer well.

3.12.9.10. Centrifuge the plate for 5 min at 1000 *x g* at RT.

4. Performing the CEI on plate 1

4.1. First, turn on the CEI (**Table of Materials***)* analyzer, then turn on the computer. Open the software (**Table of Materials**)

4.2. Connect the analyzer to the online system (**Table of Materials**). This is a necessary step to collect the run data for trouble shooting purposes and data recovery.

4.3. Click on **Instrument** from the top-left menu, then click **Connect**. Select the instrument serial number which appears as a pop-up menu. Click **Connect.**

4.4. Select the "**Assay**" tab and select **New Assay** or select a saved template.

4.5. Input assay parameters (**Table 1**) or modify template currently. Save the file name and location.

4.6. Make sure that the blinking blue color indicator in the analyzer remains solid blue.

4.7. Touch the silver metal button on top of the orange door to open.

4.8. Carefully remove the capillary cartridge from its packaging. Insert the capillary cartridge as described by manufacturer's protocol. If correctly installed, the inside light turns to "blue".

4.9. Remove the protective seal from the assay plate. Visually observe the pre-filled wells for air bubbles. If observed, pop them with a small pipet tip (Long-shaft P10 pipet tip works well).

4.10. Load the plate holder by placing the assay plate and close the door. In computer, click on **Start** button.

4.10.1. Place ice tray containing temperature sensitive reagents and samples in the dark at 4 ˚C until ready to prepare second pre-filled plate.

4.10.2. Leave reagents/supplies out at room temperature for the second plate.

5. Performing the CEI on plate 2

NOTE: This plate is set for analyzing phosphorylated TDP-43 levels.

5.1. Remove the ice tray being stored at 4 ˚C and place it on the bench, 1 h before the estimated completion time of the first plate. Retrieve a second plate and cartridge.

5.2. Prepare any antibody dilutions needed for the second run and store them on ice. Prepare fresh 1:1 luminol-S:peroxide solution (step 3.12.8 above).

5.3. Remix and briefly re-centrifuge the sample mix and reagents needed for loading the pre-filled plate. Load the second plate according to **Figure 1**. Load a-p(S409-410-2) TDP-43 antibody solution in row C wells.

5.4. When the first run is complete, discard the first plate and cartridge. Remove the cartridge and place in a sharp's container for disposal. Keep the stickers from the plate and cartridge for reference purposes.

5.5. Close the software file and reselect the same template. The software will remember the settings from the prior run. Make any changes to the annotation as needed (i.e., changing the primary antibody).

5.6. Repeat steps 4.8–4.10. Put away all 12–230 kDa master kit separation module reagents and supplies

5.7. Discard left over CEI sample-mix, antibody dilutions, 0.1x sample buffer and luminol-S:peroxide mixes in accordance with university regulations.

6. Data analysis

6.1. Once the run is completed, ensure that the following quality checks are performed.

6.1.1. In software, select the **Show Standards** icon and **Graph View** tab. Check all 25 capillaries for the peak alignments to internal fluorescent marker sizes. Correct the misalignments by

selecting **Force Standard** or right-clicking on the incorrect peak then selecting **Not a Standard**. Perform this check for each new capillary.

6.1.2. Click on **Samples** and the **Single View** icon. Select capillary #1 (biotinylated ladder) in the experiment tab. Review the peak alignments to molecular weight markers. Click on the peak in **Graph View** and select **Remove Peak**, if an incorrect selection of the peak is done by the software.

NOTE: As an example, the 12–230 KDa biotinylated ladder will show sizing peaks at 12 kDa, 40 kDa, 66 kDa, 116 kDa, 180 kDa, and 230 kDa. The sizing of the sample peaks will be incorrect if this step is not performed and will generating inaccurate results.

6.1.3. View the electrophoretic movie and note if any abnormal migration occurred during the run.

6.1.4. Derive data (e.g., peaks table, including molecular weight, peak area, peak height, and signal-to-noise [S/N]) as needed for further calculations. There are graph annotation tools located at the upper-right corner of **Graph** window for providing more information about the graph.

REPRESENTATIVE RESULTS:

Optimization of platelet cytosolic protein concentration and primary antibody titration

It is important to establish a linear dynamic range of platelet cytosolic proteins in the assay, since changes in signal are directly proportional to changes in protein in the platelet cytosol. Use of whole platelet lysate mixture in the assay may reduce the signal intensity of the target proteins (TDP-43 and P(S409-412) TDP-43) and contribute to a high background signal. Therefore, in this assay, the clear supernatant was used (cytosolic fraction) after rupturing platelets (**Figure 2**).

(Place **Figure 2** here).

A linear dynamic range for platelet cytosol protein concentration was established at 0.2–0.8 mg/mL. An assay template was adopted so that both protein concentration and primary antibody titration were able to be performed in one assay (**Figure 3**).

(Place **Figure 3** here).

It should be noted that glycerol content in the sample preparation tube should be less than 20% (final), otherwise the high glycerol concentration will adversely affect the primary antibody binding.

Determining optimum exposure time

In the older version of the software, the optimum exposure time had to be determined by plotting peak area against protein concentration (mg/mL). The new version provides a new tool named the high dynamic range (HDR) detection profile (**Figure 4).** Using the images panel provided the option to view all exposure times (i.e., 5 s, 15 s, 30 s, 60 s, 120 s, 240 s, 480 s) together. Computer software analyzed all exposure times and automatically identified the best exposure time (HDR). HDR detection profile delivered a significantly wider dynamic range due to the greater sensitivity of CEI, which means better detection and quantitation over a larger sample concentration range. However, users still have the option to choose any exposure time that satisfies the experimental goal. Using this feature, optimum exposure time was found for TDP-43 protein. The peak represents the optimum exposure time (**Figure 4A**). A single exposure time (4 s) was defined for this antibody after reviewing all nine exposure times ranging between 1–512 s (**Figure 4B**).

(Place **Figure 4** here).

TDP-43 levels in human platelet cytosol of ALS patients

A blood base biomarker development was performed. Using optimized assay conditions, platelet lysate cytosolic fractions obtained from ALS patients were analyzed using two sets of antibodies (i.e., anti-TDP-43 [Pan] antibody, an antibody that recognizes phosphorylated derivatives of TDP-43 protein; here, a-P [S409/410-2] TDP-43 was used). In this demonstration, disease-specific TDP-43 and its phosphorylated derivative are presented (**Figure 5**).

(Place **Figure 5** here)

Total TDP-43 was quantified using the calibration curve (**Figure 6**)

(Place **Figure 6** here),

The quantification of phosphorylated TDP-43 protein was not possible due to commercially unavailability of this protein. Instead, a predicted phosphorylation value (PPV) was established that defines the percent of the phosphorylated species of TDP-43. PPV was determined from two sequential *CEI* assays for the same sample using the following equation.

$$\text{PPV} = (Fluorescence\ a.u.\ \ p(S409-410-2)TDP-43) \div (Fluorescence\ a.u.\ pan\ TDP-43)$$

Intra- and inter-run assay variability were tested in pooled human ALS platelet cytosolic fractions (**Figure 7**)

(Place **Figure 7**).

Although intra-run (capillary-to-capillary variation) coefficient variation values fell in the acceptable range (CV% = 14.9), the inter-run assay value was relatively high (CV% = 18.7). It is interpreted that this variation may be due to using capillary cartridges and sample plates from

different lots. It is recommended that reproducibility studies should be performed in CEI components that have the same lot number.

FIGURE AND TABLE LEGENDS:

Figure 1: **Assay layout**. Both primary antibody and target protein sample optimization can be performed in one assay. Capillaries 2–7, 8–13, 14–19, and 20–24 represent various protein concentration and primary antibody range. Capillary 25 represents positive control. Anti-ERK antibody was used; however, any appropriate positive control can be included.

Figure 2: **Signal clarity depends on the sample quality.** (**A**) Whole platelet lysate homogenate interferes with anti-TDP-43 antibody binding; therefore, a noisy electropherogram was observed. (**B**) Platelet cytosolic fraction was obtained from subjecting the whole lysate to centrifugation (16,000 *x g* for 30 min). Most of the membranous proteins were removed; hence, anti-TDP-43 antibody binding to TDP-43 protein was enhanced (blue line trace).

Figure 3: **Linear dynamic range for platelet cytosol protein concentration**. (**A**) Both protein concentrations and antibody titrations were optimized in one plate during the same run. (**B**) The linear working range (0.2–0.8 mg/mL) for protein was established. A 0.5 mg/mL protein load was labeled by a-ERK antibody as the positive control (capillary 25).

Figure 4: **High dynamic range (HDR) detection profile for a target protein**. (**A**) TDP-43 protein peak represents the optimum exposure time for the target protein. a-TDP-43 Ab titration was 1:300, and platelet cytosol protein concentration was 0.5 mg/mL. The software-defined blue line indicates the optimum exposure time. (**B**) This figure represents a user-defined single exposure time (4 s) after reviewing all nine exposure times ranging between 1–512 s.

Figure 5: **A representation of predictive phosphorylation value (PPV) of TDP-43**. Absolute amount of phosphorylated TDP-43 and pan TDP-43 alone did not show much difference between the ALS and control groups. However, PPV indicated a slight increase in the ALS cohort, although there was no statistical difference between the two groups due to insufficient numbers of subjects (ALS = 25, control = 27). A low Cohen's d value between the means of ALS and control group showed a low effect size between the two groups due to small sample size (control = 25, ALS = 27).

Figure 6: Standard calibration curve. Commercially purchased recombinant TDP-43 protein concentrations were used to construct a standard curve. Each data represents the average of triplicates. The protein band intensities were concentration dependent (Inset).

Figure 7: Assay variations. (**A**) Two capillaries loaded with the same sample were analyzed by CEI, and intra-run assay variation was calculated as CV% = 14.9. (**B**) The same sample was analyzed on three different assay days and in three different assay runs. Inter-run assay variation was calculated as CV% = 18.7.

Table 1: CEI assay plate loading template.

Table 2: Interactive sample mixture preparation template. After entering the stock protein concentration from unknown samples, interactive cells will automatically calculate how much volume needs to be used for preparing the sample mix.

DISCUSSION:

The capillary electrophoretic-based immunoassay is now the method of choice for high-throughput sample analysis and drug screenings[25]. Small sample volumes, well-optimized assay components, user-friendly assay platform and instrumentation, reagent expenditure, and low CV percentage are primary advantages[26,27]. Although there are several methods for separating proteins in different assay modalities, the antibody-based CEI described here can be adapted by small laboratories that are engaged in blood-based biomarker development. The CEI assay technology used here provides reliable, reproducible, and sensitive measurements for TDP-43[28] and its phosphorylated derivative[5].

The CEI system also provides a multiplexing choice of analyzing TDP-43 and its phosphorylated derivatives simultaneously and providing the direct quantification of the target protein, if purified or recombinant target protein is available. Full-length recombinant TDP-43 protein is commercially available; however, recombinant phosphorylated TDP-43 derivative is not. Since phosphorylated TDP-43 is not commercially available, a predictive phosphorylation value (PPV) was implemented to assess TDP-43 profile in ALS patients. Pan TDP-43 and phosphorylated TDP-43 amounts were permanently labelled with a fluorophore; therefore, the TDP-43 profile remains the same with or without a quantitative unit (i.e., ng/mL, pg/mL, etc.). Although determining the absolute amount of TDP-43 and its phosphorylated derivatives (i.e., P [S409-410-12] TDP-43) provides a more quantitative measurement, calculating PPV eliminates the necessity of the recombinant TDP-43 for standardization, since it is not commercially available.

CEI provides several checkpoints in the assay platform to accurately identify the problem in the case that an assay fails. This eliminates obstacles and provides better experimental design. The assay procedure is fully automated except for filling the sample plate. This is a significant feature compared to standard western blotting analysis. This feature provides consistency from run-to-run. Although every laboratory has unique standard operating procedures, it is important to adhere to practices that minimize human error. For example, it is critical to prepare the luminol-S/peroxide mixture just before plate loading, since adding peroxide into luminol starts the enzymatic reaction and consumes the luminol substrate. Loading samples and primary/secondary antibodies into plate wells without air bubbles are also critically important steps.

Additionally, since the plate wells are small in volume and there is no space between wells, users should use caution while pipetting, which is the most important step since everything else is automated. The loading order of the samples, antibodies, and other reagents is important for consistency of the assay (**Figure 1**). The process of plate preparation takes about 40–45 min.

Therefore, it is recommended to first load the plate with the required assay components and prepare the luminol-S/peroxide mixture just before pipetting. This way, there is a consistent sequence of reagent adding, and consistent luminescence signal strength will be attained. It is not recommended to use an expired luminol-S/peroxide reagent, as it primarily affects the strength of the peroxide. Recent progress in introducing the split-buffer system and including the chemical and detergent compatibility range has enhanced the assay quality and produced more reproducible and predictable results. Now, a new combo-analyzer from the same manufacturer possesses a feature for analyzing the samples labeled with chemiluminescence and fluorescence conjugated antibodies in the same run. This new feature eliminates the need to consecutively run two individual plates and eliminates run-to-run variation.

The assay plates should be stored in ambient temperature. If it is chosen to keep the assay plates in a 4 ˚C refrigerator, the plates must be taken out the night before the assay and brought to ambient temperature. Incorrectly loaded sample wells need to be extensively (4–5 times) washed with buffer provided in kit before adding the correct sample. Each primary antibody and biological samples are unique; therefore, the antibody/protein optimization should be performed before analyzing the samples for target proteins in biological fluids.

Here, the primary antibody incubation time was set for 30 min by default. If the signal is weak, users should consider increasing the primary antibody incubation time until reaching the desired signal strength without fluorescence signal burnout. For human platelets, pooled samples from patients were prepared and used for an optimization assay. The sample pooling better represents the variation among target biomolecules. It is recommended to use clear supernatants rather than total lysate or total homogenate for the CEI.

The high concentration of protein in whole platelet lysate mixture may decrease the signal-to-noise ratio **(Figure 2**). Repeated freeze-thaw cycles of samples should be avoided, as this adversely affects primary antibody binding The ingredients of the lysate buffer are important, as some reagents are not compatible with CEI[29]. It is advised to cross-check the list of compatible reagents provided on the manufacturer's website before sample preparation. This is a limitation of the system that does not tolerate high stringency conditions for sample preparation. It is recommended to optimize the assay run parameters (i.e., primary antibody dilution, protein concentration, primary antibody incubation time, etc.) using pooled samples to subsequently analyze the individual samples.

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DISCLOSURES:

The authors declare no competing financial interest except ProteinSimple, Inc. covered the publication cost of this manuscript.

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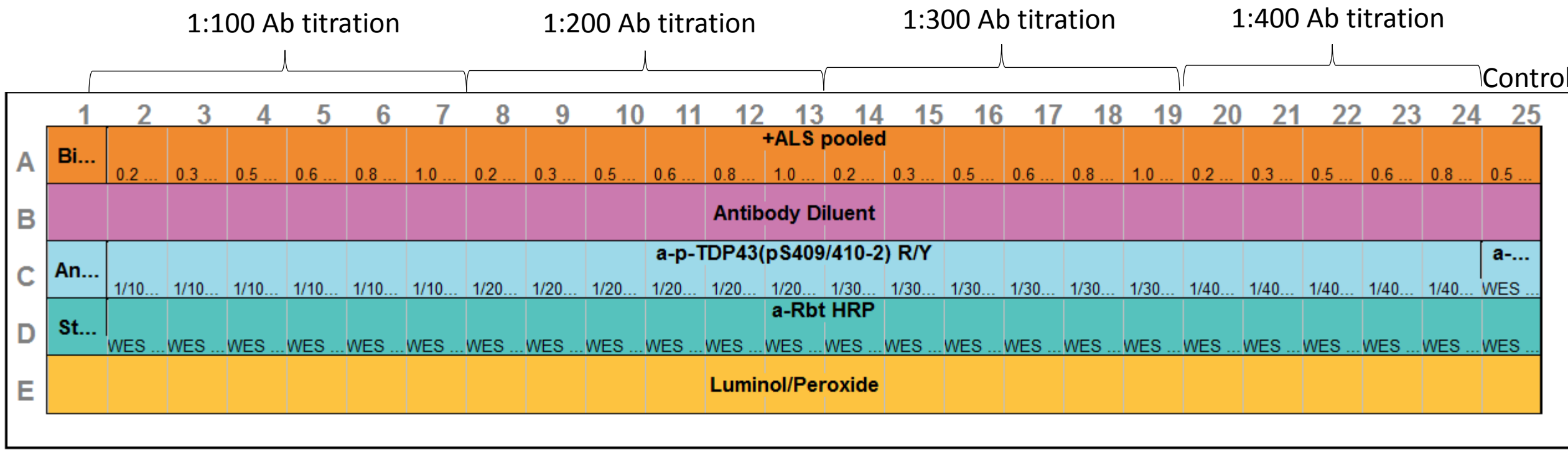
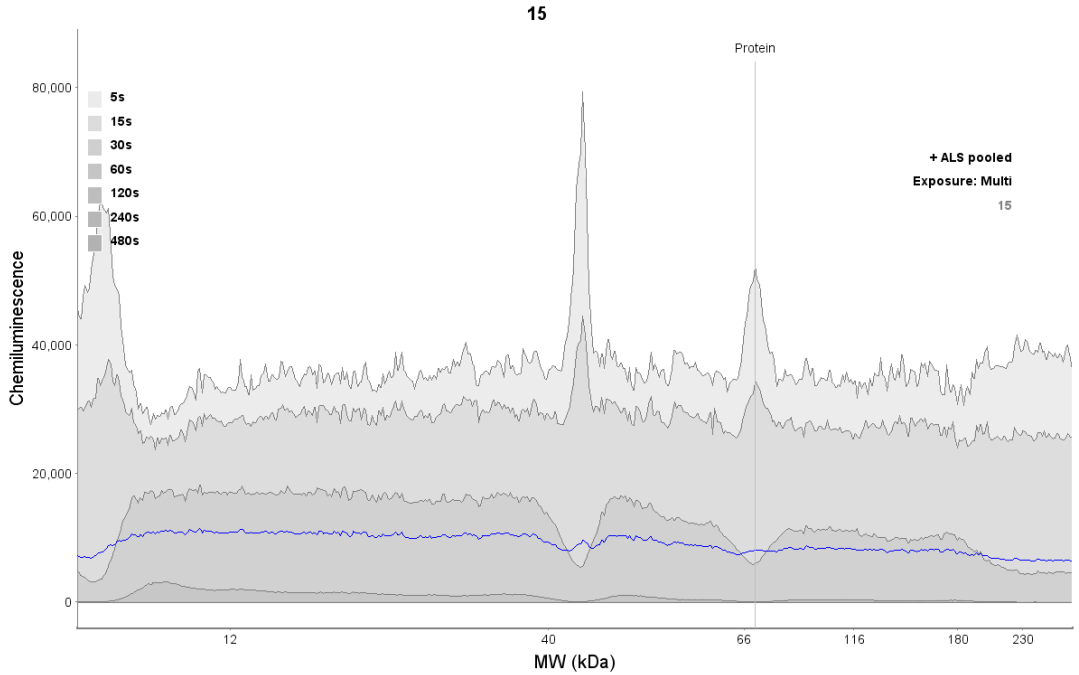


Fig.1

A

Whole platelet homogenate



B

Platelet cytosolic fraction

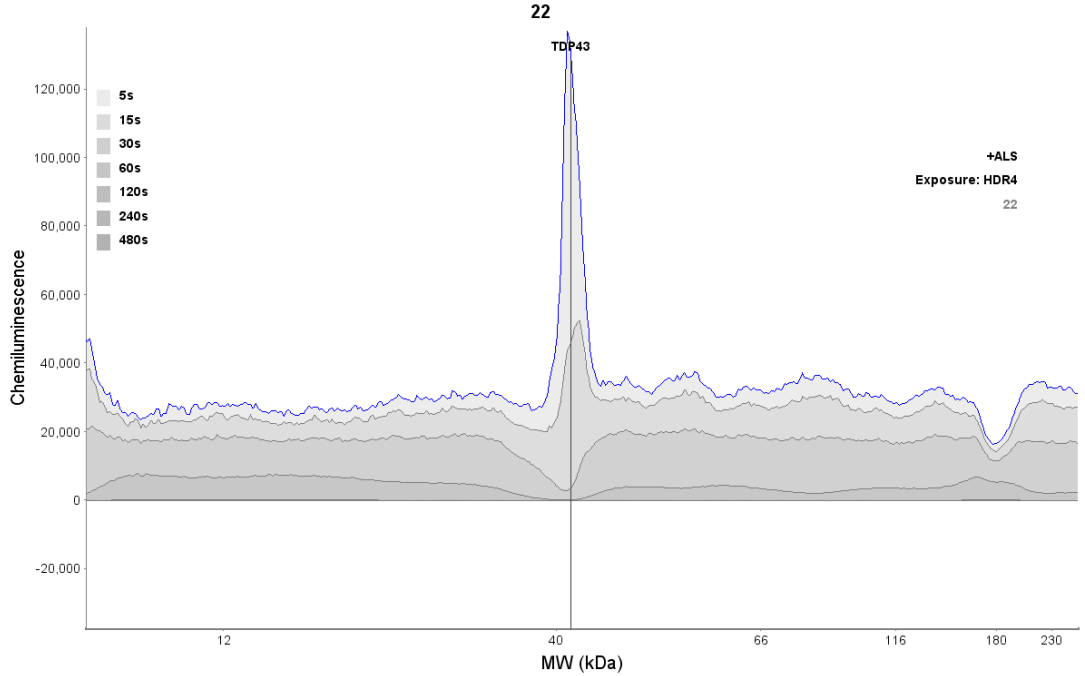


Fig.2

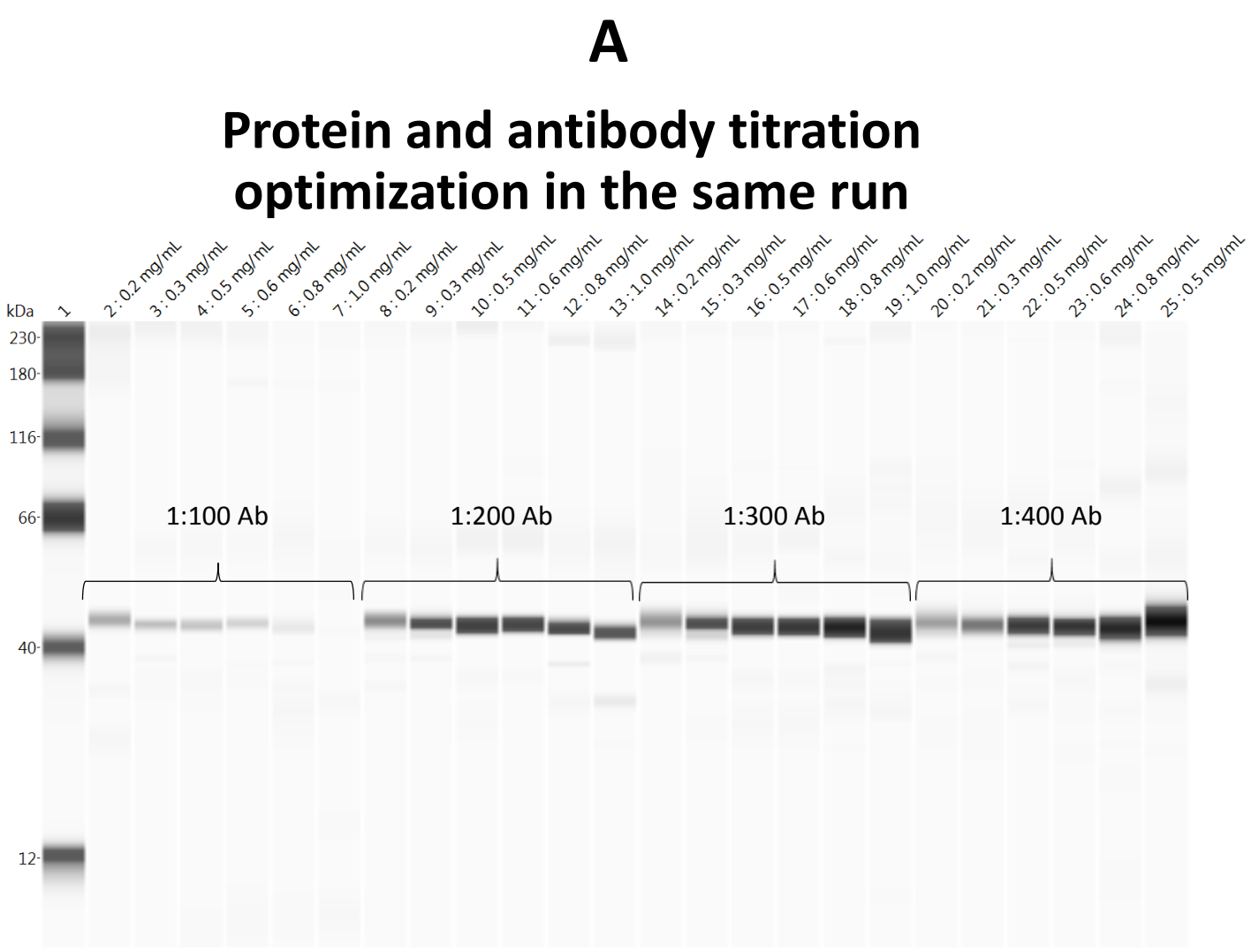
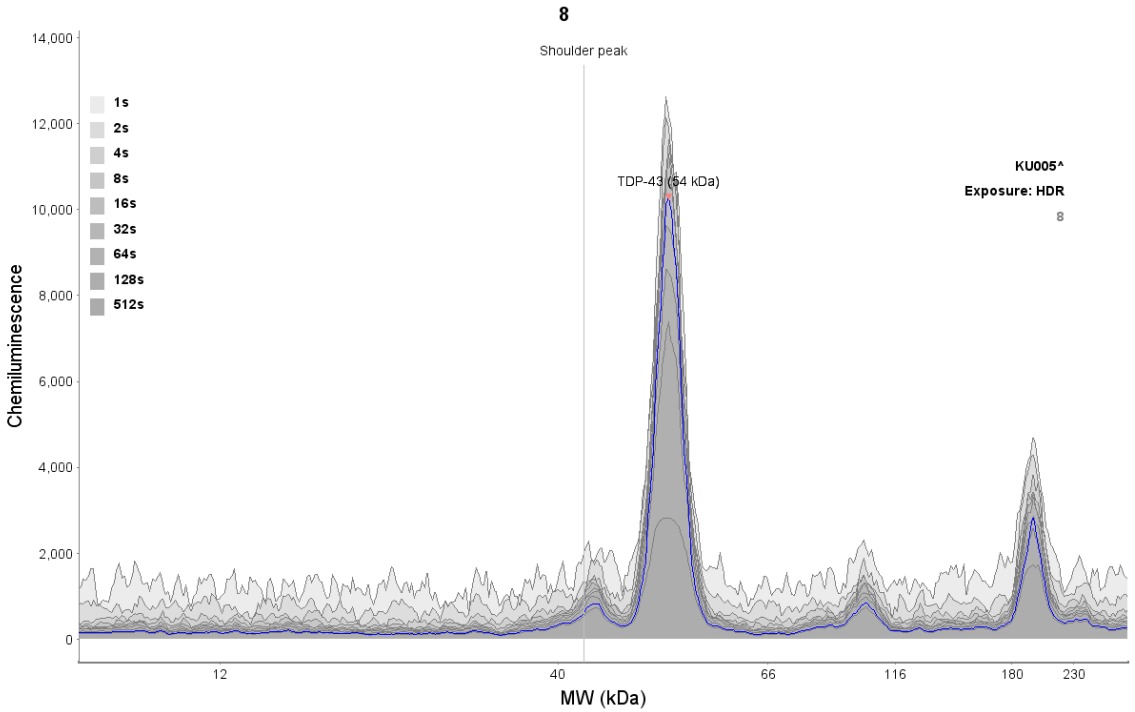


Fig.3

A

High Dynamic Range (HDR)



B

Single exposure time

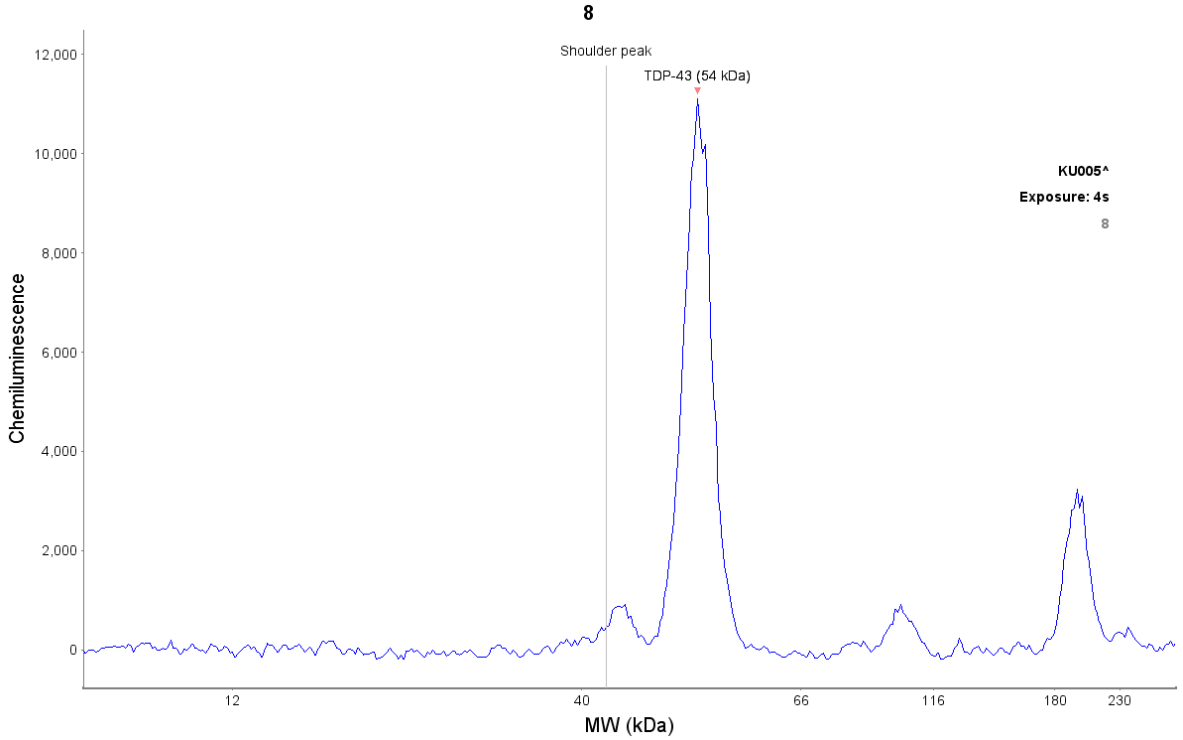


Fig.4

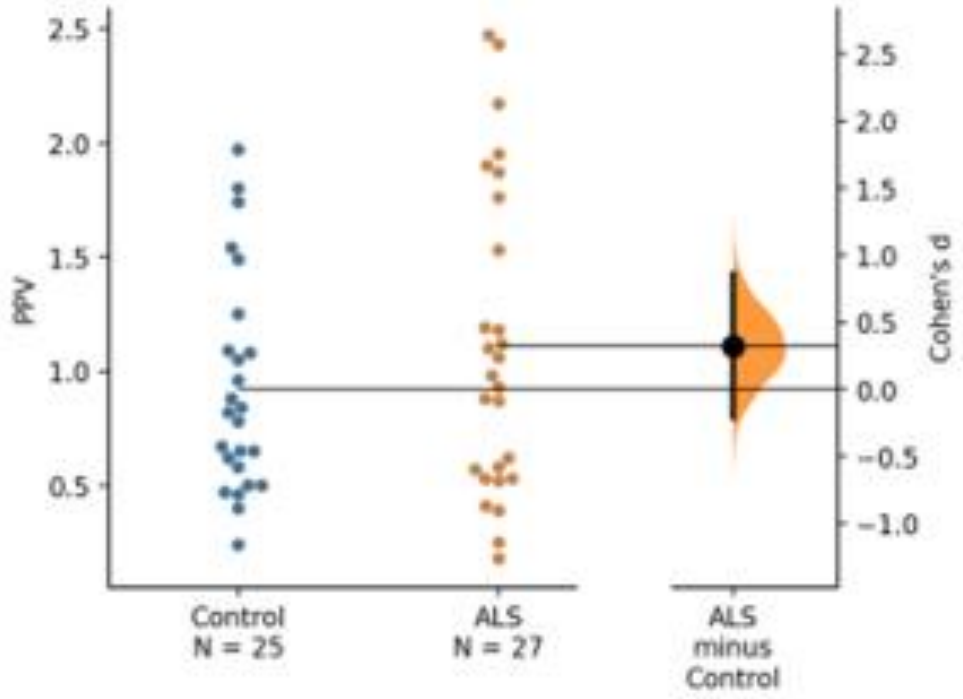


Fig.5

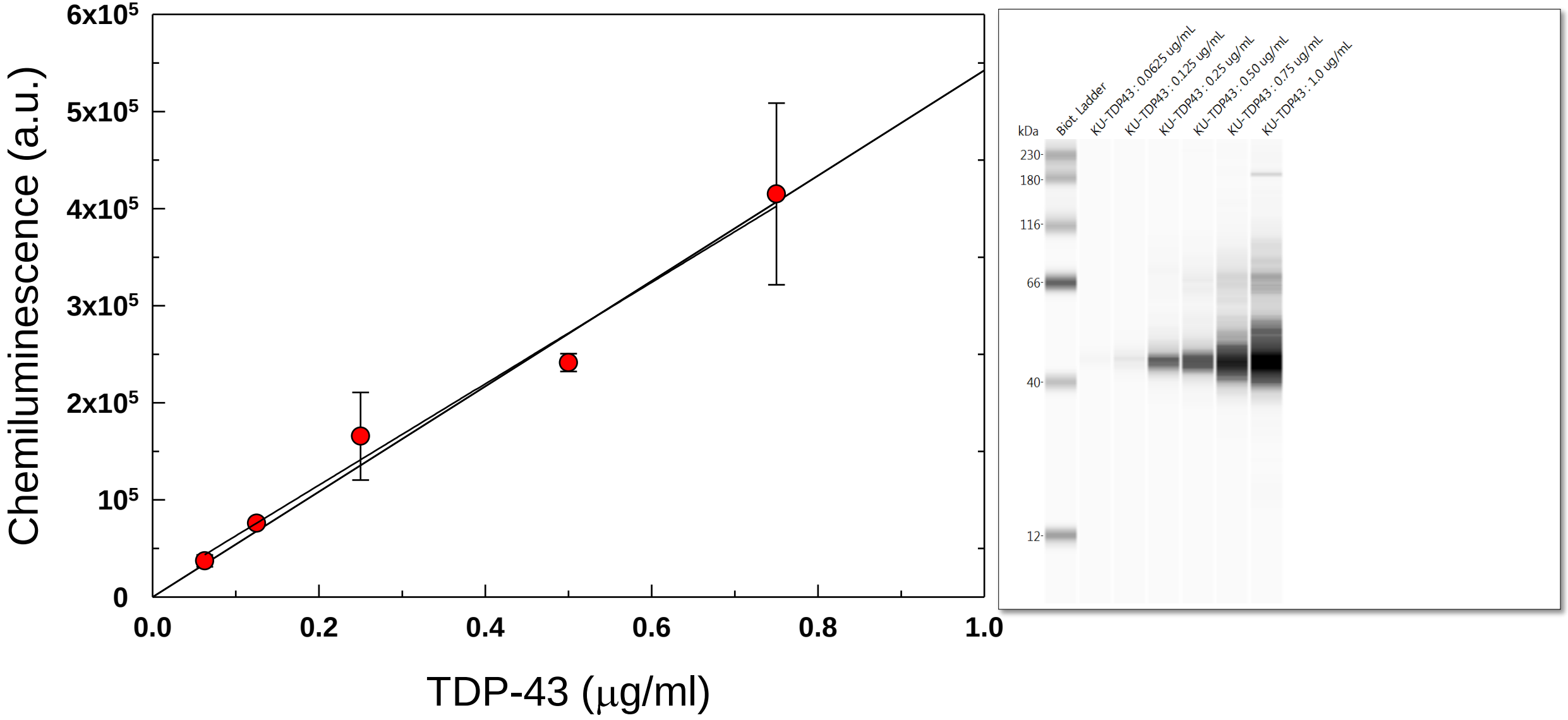
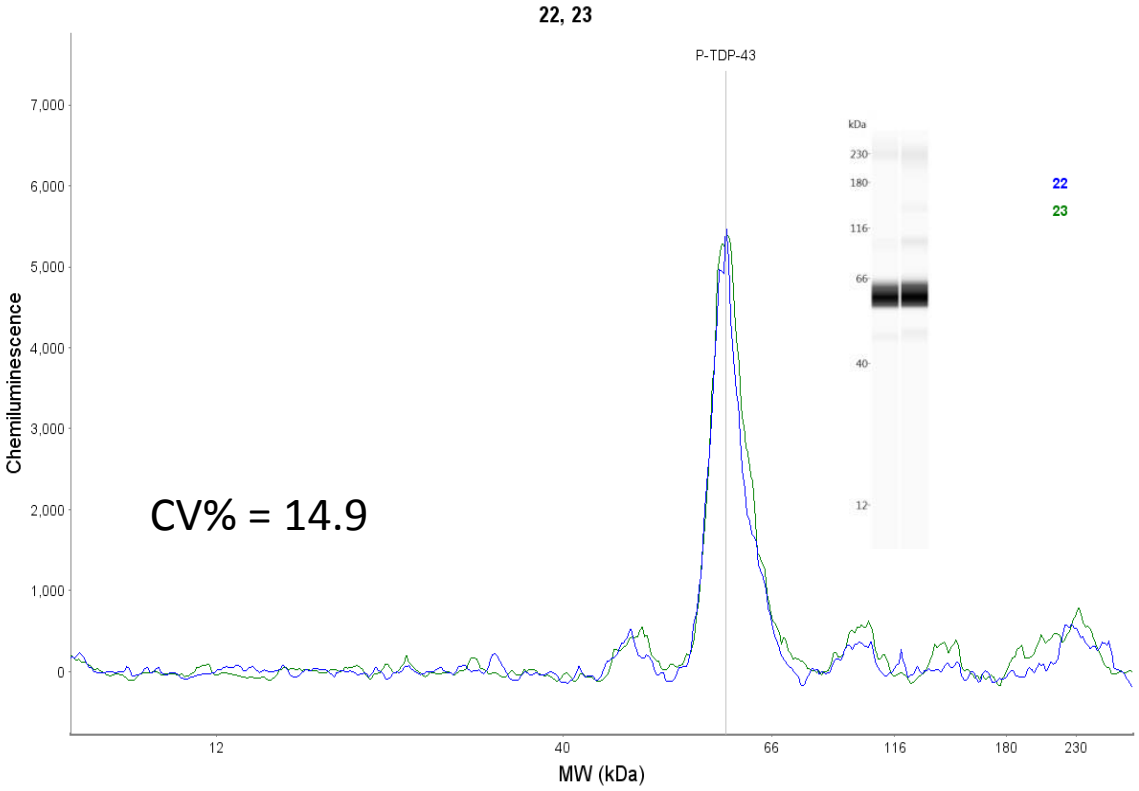


Fig.6

A
Intra-run assay variation



B
Inter-run assay variation

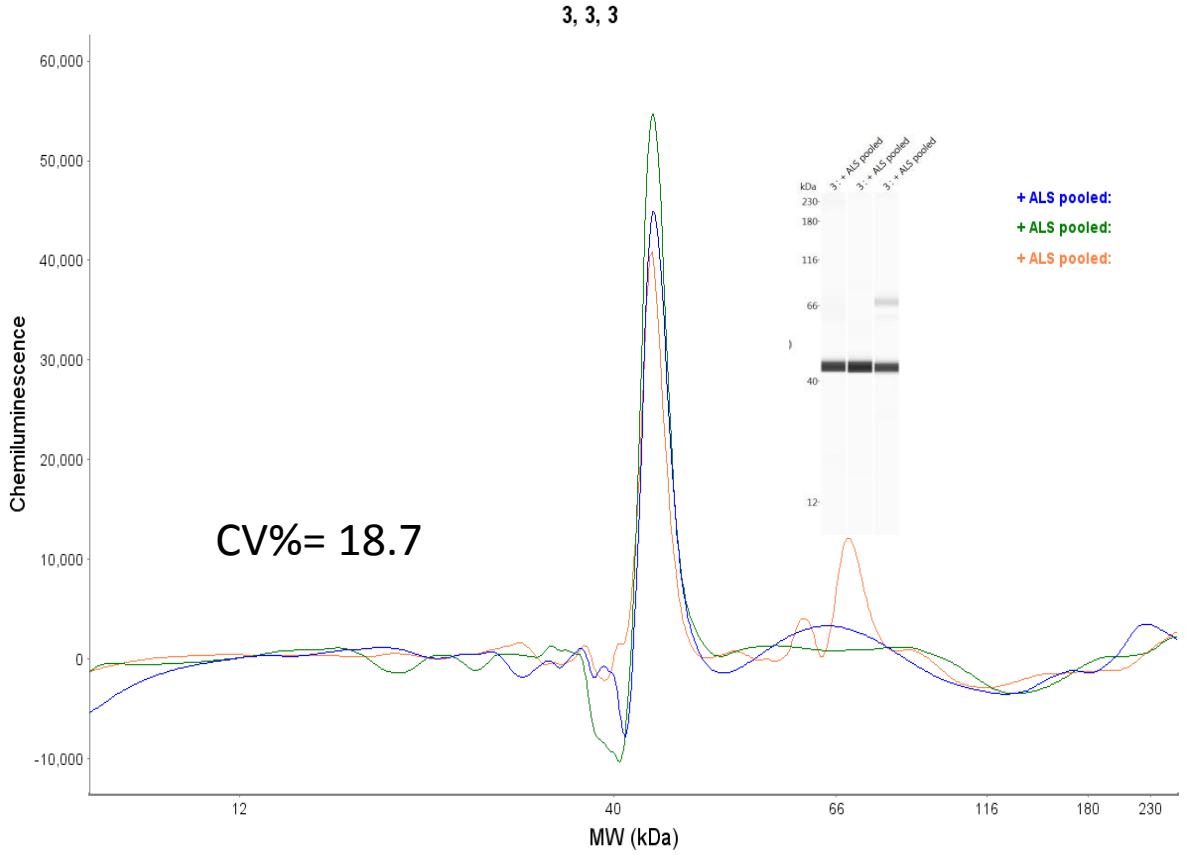


Fig.7

The updated version

Click here to access/download;Table;Table-1 (Wes Assay Layout) (single sheet).xlsx

Wes-Assay-#

Assay Title

Date:

Column	Sample ID	1° Ab	1°Ab Dilution	2° Ab	2°Ab Dilution	Vendor Information
1	MW marker	-	-	-	-	ProteinSimple
2		a-TDP43(pan)	1/400	a-Rbt WES	NEAT	ProteinTech
3		"	"	"	"	"
4		"	"	"	"	"
5		"	"	"	"	"
6		"	"	"	"	"
7		"	"	"	"	"
8		"	"	"	"	"
9		"	"	"	"	"
10		"	"	"	"	"
11		"	"	"	"	"
12		"	"	"	"	"
13		"	"	"	"	"
14		"	"	"	"	"
15		"	"	"	"	"
16		"	"	"	"	"
17		"	"	"	"	"
18		"	"	"	"	"
19		"	"	"	"	"
20		"	"	"	"	"
21		"	"	"	"	"
22		"	"	"	"	"
23		"	"	"	"	"
24		"	"	"	"	"
25		"	"	"	"	"

[Sample] **0.5 mg/ml**

NEAT = Straight from the vial. No dilution is necessary

Denaturation*= 10 min @ 70°C

** **15** ul of Luminol/Peroxide mixture per well

Begin loading plate

- Well D1: Streptavidin-HRP - 10 µL
- Well D2-25: Secondary Antibody - 10 µL
- Well C1: WES antibody diluent - 10 µL
- Row B: WES antibody diluent - 10 µL
- Well C2-25: Primary Antibody - 10 µL
- Well A1: Biotinylated Ladder - 10 µL
- Well A2-25: Prepared Samples - 3 µL
- Wash wells: WES wash buffer - 500 µL
- Row E: luminol/H_2O_2 - 15 µL

Balance plate

CF plate (1000xg = 5min @ RT)

Load plate and cartridge in WES system

Begin WES run

Estimated Completion time

Assay run parameters:

Separation time: 45 min

Separation Voltage : 250 volts

Antibody diluent time: 5 min

Primary antibody incubation time: 60 min

Secondary antibody incubation time: 30 min.

Place WES cartridge label here

Place WES plate label here

NOTES:

The updated version

Click here to access/download;Table;Table-2 (Wes Assay Sample mix preparation template) (UD).xlsx

Wes-Assay-#

Date

Required SampleVolume Calculation Template

Patient/Protein Sample								0.1x Sample Buffer	5x Flourescent Standard (C1: 5 x; C2: 1 x)
			V2		C1	C2	V1	V1	V1
Number of capillaries to load	volume of sample per capillary (µl)	Amount extra sample (µL)	Total volume of sample (µL)	sample description	Initial concentration of sample* (mg/ml)	Final concentration of sample (mg/ml)	Volume of sample to add (µL)	Volume to add (µL)	Volume to add (µL)
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
1	4.0	4.0	8.0			0.50	#DIV/0!	#DIV/0!	1.60
24.00								#DIV/0!	38.40
total # WES capillaries								total µL needed	total µL needed

Wes-Assay-#
Date

Required SampleVolume Calculation Template

Wes-Assay-#
Date

Required SampleVolume Calculation Template

Wes-Assay-#
Date

Required SampleVolume Calculation Template

Name of Material/Equipment	Company	Catalog Number
12-230 kDa Separation kit	ProteinSimple	SM-W004
3000G Thermocycler	Techne	FTC3G/02
Anti-Mouse detection kit	ProteinSimple	042-205
Anti-P(S409-410) TDP-43 antibody	ProteinTech	22309-1-AP
Anti-P(S409-412) TDP-43 antibody	CosmoBio-USA	TIP-PTD-P02
Anti-Rabbit detection kit	ProteinSimple	DM-001
Anti-TDP-43 (pan) antibody	ProteinTech	10782-2-AP
Compass for SimpleWestern (SW)	ProteinSimple	Ver.4.0.0.
Sonic Dismembrator; Model100	Fisher Scientific	
Table top centrifuge	Eppendorf	22625004
Wes analyzer	ProteinSimple	55892-WS-2203

Comments/Description
Contains pre-filled assay plate and 25-channel capillary cartridge
We used this thermocylcer for heating the sample mix
Includes HRP-conjugated secondary antibody,buffer, luminol reagent, molecular weight marker
Primary antibody that recognizes phosphorylated TDP-43
Primary antibody that recognizes phosphorylated TDP-43
Includes HRP-conjugated secondary antibody,buffer, luminol reagent, molecular weight marker
Primary antibody that recognizes whole TDP-43 protein
Compass for SW is the control and data analysis application for SimpleWestern instruments
Sonicator. Used to rupture the cell membrane. This model is discontinued (Model XL2000-350)
Model# 5810 with swinging plate bucket
Performs the capillary gel electrophoresis
Model# 5810 with swinging plate bucket

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Author(s):	Jessica M. Sage, Shareez Hall, April McVey, Richard J. Barohn, Jeffrey M. Statland, Omar Jawdat, Mazen M. Dimachkie, Abdulbaki Agbas

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CORRESPONDING AUTHOR

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Institution:	Kansas City University of Medicine and Biosciences
Title:	Professor of Biosciences

Signature:		Date:	7/30/2019

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Detailed response: We have addressed editorial comments and responded to the comments by the reviewers as follows.

Response to Reviewers

Reviewer #1

The reviewer summarizes that we described the utility of capillary electrophoresis immunoassay (CEI) to identify potential blood-based biomarkers inpatient samples. This reviewer has major and minor concerns that we address in the following. Since the reviewer did not number the concerns, we will type the concerns in bold and provide our answers

Major Concerns:

*** The authors cannot claim that TDP-43 is a "potential blood-based biomarker for ALS" in the title since Figure 4 does not show a significant difference. Instead, the title should be focused on the utility of CEI in measuring potential biomarkers in patient blood samples. TDP-43 in ALS should be mentioned only as an example, which is how the abstract and most of the paper is already written.**

We thank this reviewer for the suggestion. We agreed that our focus would be about utilization of CEI. Therefore, we have a new title as "*Capillary electrophoresis immunoassay utilization in search of potential biomarkers for amyotrophic lateral sclerosis in human platelets*."

*** The following paper needs to be referenced, and if it employs a different protocol, the differences should also be discussed: Fourier A, Escal J, Bernard E, Lachman I, Perret-Liaudet A, Leblanc P, Quadrio. Development of an automated capillary nano-immunoassay-Simple Western assay-to quantify total TDP43 protein in human platelet samples. Anal Bioanal Chem. 2019 Jan;411(1):267-275. doi: 10.1007/s00216-018-1437-4. Epub 2018 Oct 29. PubMed PMID: 30374726.**

We apologize to oversight of the manuscript written by Fourier et al. Now, we included the paper in our references. There are some differences observed and we discussed them in the following section

1. Fourier et al., paper's objective is to evaluate Simple Western technology developed on Peggy Sue analyzer to detect TDP-43 protein in platelet samples in the context of lacking validated markers for frontotemporal lobar degeneration (FTLD). Our objective is to evaluate Simple Western technology developed on Wes analyzer. Peggy Sue analyzer is based on size and charge separation of protein. Wes analyzer separates proteins based on size only. Some advantages of Wes analyzer are
    a. Affordable
    b. Suitable for small sample size (24 samples) for exploratory studies. 50 % of the reagents were pre-loaded in assay plate. Operator has to use the entire cartridge to assess few samples. Peggy Sue system uses 96 capillary cartridges
    c. Relatively short assay run time (Peggy Sue requires 5 hours while Wes requires 3 hours)
2. Fourier et al., used 4 second of exposure time. We used High Dynamic Range (HDR) for optimum exposure time that provides high signal/noise ratio without bias. We also provided a new figure to compare 4-second single point exposure time vs. HDR (Fig.4). Blue-colored trace shows HDR signal.

3. Fourier et al., used “one patient” sample for their optimization assays (Fig.2a). We have collected 27 ALS patients. One small fraction from each of 27 samples were combined. We used this “pooled platelet lysate samples for optimization purposes. We think that 27 patient samples are better represent the candidate protein profile at this development stage.
4. Fourier et al.,optimized the platelet TDP-43 assay limit based on sample dilution (i.e., 1/2, 1/3,1/5, 1/10. Fig 4a). We optimized our platelet cytosol dilution based on serial protein quantitation like 0.2,0.3,0.5,0.6,0.8, and 1 mg platelet protein/assay. This is quantitative.
5. Fourier et al., did not provide the actual electropherogram, but computer generated sudo-bands. We have provided both so that reader can see the actual peaks.
6. Fourier et al., had analyzed same sample six times within one cycle (intra-run) to demonstrate that capillary-to-capillary variation is to small coefficient variations (Fig. 4b). We included both intra-run and inter-run CV % in our work. (Fig. 7)
7. Fourier et al., used whole platelet homogenate. We have used platelet cytosolic fraction, which produces a clearer electropherogram. New figure compares whole platelet lysate to clear cytosolic fraction (Fig.2).
8. Fourier et al., analyzed 9 patients. Our subject cohort is relatively large (ALS= 27, Control= 25). We are currently designing a new study that includes 140-subject cohort from each category to achieve predicted phosphorylation value to $P<0.05$ level with 95% confidence.

*** The authors need to describe the proprietary system and kits being used up front in the manuscript, i.e. Protein Simple, WES, Simple Western assays, etc. Currently, Protein Simple is mentioned first on page 8.**

The editor has advised us that we should refrain not to mention commercial language in the manuscript. Instead, we referenced all commercial products in the Table of Material and Reagents.

<u>Minor Concerns:</u>

*** PPV is not explained in the summary or the abstract. If PPV remains in the title, then it needs to be mentioned in the abstract at least.**

We have changed the title and PPV is not mentioned in the new title.

*** Product numbers for reagents need to be mentioned whenever possible. For example, for phosphatase and protease inhibitor cocktails, luminol, 'standard pack', etc.**

This information was now provided in Table. The editor wants this information separately.

*** Line 167: Sonicator settings need to be included.**

This information was provided. Please see line 169 (no markup mode)

*** Line 178: Why the change from CEI to Wes?**

We now use only CEI throughout the text. Wes is the name of analyzer. Fourier et al. used in their paper. We thought that it is all right to use; however, this reviewer is right. We use now CEI

in entire text.

*** Lines 203 and 206: Please explain 'pink' and 'white' tubes.**

The content of “pink” and “white” tubes were explained in the first submission. We additionally added the sentence that these tubes were provided in the 12-230 kDa Master kit.

*** Lines 229 and later: Please explain the 'reverse-pipetting technique' and any associated equipment, e.g. liquid-handling robot, electronic pipet, etc.**

Definition of reverse pipetting was further explained

*** Figure 2: Please explain how the linear working range for protein and antibody titration shown resulted in selection of an optimal amount. Even if ERK antibody is used here as an example, what concentration and protein amount would be selected. Is it a common occurrence that higher amounts of antibody lead to suboptimal results?**

We used anti-ERK antibody as a positive control in a single capillary. Figure -3 was constructed based on platelet cytosolic protein. We used 0.4 mg protein/ml and 1:300 antibody dilution. This information was now added in the caption of Figure-3

*** Figure 5: Please compare to a regular western blot or ELISA to show comparable or superior calibration.**

We have used a commercial ELISA kit for analyzing TDP-43 levels; however, this kit was designed for human serum /plasma and detection levels were optimized in picogram/mL levels. We have analyzed human platelet cytosolic TDP-43 and phosphorylated TDP-43 levels. Although ELIS kit produced a nice calibration with comparable recombinant TDP-43, we cannot compare the ELISA TDP-43 calibration to ours since we have used our own recombinant TDP-43. The other issue was that the commercial ELISA TDP-43 kit used fixed concentration of anti-TDP-43 Ab and pro-coated micro-plate well. Therefore, we could only optimize the unknown protein concentrations. CEI provides more flexibility to optimize primary antibody and protein concentration in the same run that eliminates user-generated errors.

Reviewer #2:

The reviewer states that the capillary electrophoresis immunoassay method is well written and provides useful tips for researchers who are interested in this methodology to detect proteins for their interest. This reviewer has 4 major points, 3 minor points and some useful suggestions. This reviewers stated that the described method is of general interest to the JoVE readership.
We provided our response immediately after the reviewers’ comments. The reviewer’s comment appeared in **bold** text.

Major Points:

1. pTDP-43 detection using platelets

In lines 60-70, the authors indicated platelets are preferred biospecimens due to availability, convenience of isolation, and metabolic stability. They also prefer the cytosolic fraction over whole cell fraction (lines 385-388). I can understand these reasons, but there is no data shown to support the idea; is the platelet cytosolic fraction best suitable for pTDP-43 detection? Literatures suggest that TDP-43 protein in the cerebrospinal fluid (CSF) or pTDP-43 in plasma may be useful for diagnostics of frontotemporal dementia (FTD) and ALS (Majumder, et al., 2018; Suarez-Calvet, et al., 2014), but apparently pTDP-43 using platelets has not been reported. Thus the authors should show some evidence that pTDP-43 detection using platelet cytosolic fraction is as good as or even better than using CSF or plasma.

There are more through studies in CSF-based potential biomarker search. Therefore, we are unable to provide a comparable study (i.e., CSF-TDP-43 vs platelet TDP-43 levels in ALS patients). There are several reasons that why we had studies platelets as milieu. (i) To obtain platelets from peripheral blood is much less invasive than obtaining CSF. (ii) The subject recruitment and obtaining patients consent were relatively easy were for blood collection; however, we are searching for national bio-specimen repositories (i.e., Coriell Institute Biobank and Northeast ALS Consortium (NEALS)) that provide platelets and CSF samples from the same donor so that we can compare CSF vs platelets. (iii) The quantification of TDP-43 protein in CSF and plasma had produced a contradictory results due to very low concentrations of TDP-43 in extracellular fluid that has abundant immunoglobulins and albumin which interfere with anti TDP-43 Ab [1]. Also, it has not been possible to detect an immune reaction with phosphorylation dependent antibody for TDP-43 in CSF [1]. We have checked the PubMed (as of 8/28/2019) and could not come across any literature on phosphorylated TDP-43 measurements in CSF. Then, we have turned to utilize platelets as more encapsulated unit that is not influenced by extracellular fluid macromolecules. We now provide a new figure (Fig.2) in which we compared whole platelet lysate vs. platelet cytosolic fractions. As one can see that platelet cytosolic fraction provides more clean and less noisy electropherogram.

2. Reproducibility of the pTDP-43 assay

The authors suggested automated capillary electrophoresis immunoassay (CEI) for pTDP-43 detection is advantageous in assay reproducibility and inter-run data variabilities (lines ~75-80). Thus they should show evaluation of assay reproducibility such as capillary-to-capillary and run-to-run precisions. Precisions are particularly important here as the authors observed only a small difference in the pTDP-43 level between ALS and control samples (Fig. 4).

We have recognized the small difference between ALS and control due to insufficient numbers of subject. Currently, we are designing a follow up project that will include <140 samples from both control and patients cohort to satisfy $P < 0.05$ level of importance. Inter-run and run-to-run reproducibility was now shown in new graph (Fig.7)

3. Estimation of pTDP-43 level using predictive phosphorylation value (PPV)

I agree with the authors that absolute quantification of pTDP-43 is challenging without a recombinant pTDP-43 protein with known concentration (see additional comment). To address this, the authors estimated the normalized pTDP-43 level comparing to pan-TDP-

43 signal with a standard calibration curve (Fig. 5). However, PPV seems to be estimated based on different WES runs (line 416, "PPV was determined from two sequential Wes assay for the same sample"), which I think may result in a significant variability due to variable chemiluminescence signal in different experiments. I wonder why both pTDP-43 and pan-TDP-43 are determined in the same experiment. The authors should show reproducibility of PPV in repeated experiments.

We included the JESS' ability to dual label phosphorylated TDP-43 (pTDP-43) and pan TDP-43 in the same run. We could have purchase another antibody for pTDP-43 that is raised in different host than pan TDP-43 antibody (Rbt). Therefore, we probe them with two different secondary antibody for visualization. Unfortunately, anti-pTDP-43 antibody (CosmoBioUSA) is not available in another species than anti-pan TDP-43 Ab. We are in search of other vendors and test their products to identify antibodies that are more reliable. Our idea was that pan-TDP43 (Rbt) and pTDP-43 Ab (Ms) could be used in the same run; however, the bands are too close and chemiluminescence alone may not be able to distinguish the both protein bands effectively.

All reagents used in the assay are from the same batch except HRP-Luminescence mixture should be prepared freshly just before use. The only difference was the antibody solution. We recognized this reviewer's concern and we will take into consideration of reproducibility issue in our follow up study (i.e. repeating both pan TDP-43 and PTDP-43 several times(5-7) and calculating CV % for PPV)

4. Assay parameters for TDP-43 detection

For the ALS research community and JoVE readers who are interested in TDP-43 detection, it would be helpful if the suggestive pTDP-43 WES assay conditions are provided in a summary table, such as the lysis buffer recipe, protein loading amount, antibody ID and dilutions, exposure times, Compass software setting, etc.

We have provided an interactive Excel sheet (Table-2) in first submission. The user adds the unknown protein samples in initial concentration and formulated cells will automatically calculates how much 0.1X sample buffer and 5X fluorescent standard mixture need to be added. For exposure time, we choose High Dynamic Range (HDR) option. For Compass software setting, we have provided the parameters in Table-2

Additional comments/suggestions

The authors may want to mention in text that Proteinsimple has another CEI analyser called JESS which is capable of both fluorescent and chemiluminescent detection. Using JESS with dual color detection, it would be possible to assess pTDP-43 and pan-TDP-43 levels in the same capillary which would allow internal normalization of PPV.

We thank this reviewer for the suggestion on the inclusion of JESS dual-detection (fluorescent and chemiluminescent) system. We appropriately included in the text (Lines: 538-541)

Absolute quantification of pTDP-43 could be done by employing AQUA method with SRM mass spectrometer (Kettenbach, et al., 2011). Once the absolute amount of pTDP-43 in a given sample is known ("the standard"), it can be used to benchmark all the pTDP-43 experiments.

We may try this approach; however, pTDP-43 levels are expected to be well diverse due to the individual ALS patient profile and disease state. Therefore, each time we have to use AQUA method to identify the amount that is going to be costly for a clinical trial. Our focus is to develop a simple, quick, affordable, and reliable screen test.

Here the standard 12-230 kDa separation module is used. I wonder if the low MW module (2-40 kDa) allows the better separation of phosphorylated and unphosphorylated forms of TDP-43. If so, the assay can be greatly simplified by using only pan-TDP-43 antibody. A longer capillary run time may be necessary to visualize borderline size proteins.

We gave a thought at the early stage of this project; however, pTDP-43 is expected to be appear in the high MW marker range than pan TDP-43 (we have observed this in Alzheimer's disease cases [2]. Unphosphorylated form of TDP-43 migrates between 45-50 kDa under the reduced conditions. Phosphorylated form of TDP-43 could migrate to the similar range and sometimes higher MW marker levels depending on the phosphorylation status and forming the aggregates. Therefore, we used 12-230 kDa separation module to cover the expected range. We have not seen any credible low MW marker level protein species recognized by anti-PTDP-43 and anti-pan TDP-43 antibodies. We followed the separation time (25 min) as suggested by manufacturer

Minor points:

1. **Fig. 2: It is unknown what antibody was used for capillaries 2-24**

Thank you for catching the missing information. Anti-TDP-43 Ab (Rbt) was used. This information now entered in the figure legend.

2. Line 384-386: The sentence does not make sense. Check typos.

We worked on this sentence and provided better clarity (see Lines 408-411)

3. Line 417: The PPV is described as function of fluorescent a.u. Isn't it chemiluminescence?

Predictive phosphorylation value (PPV) is an index. Both p(S409-410-2) TDP-43 and pan TDP-43 readings are in arbitrary fluorescence unit (a.u); hence, in the equation of PPV = p(S409-410-2) TDP-43 (a.u.) / pan TDP-43 (a.u.) , a.u. cancel each other and PPV becomes an index without unit.

References (Reviewers provided)

Kettenbach, A.N., Rush, J. and Gerber, S.A. Absolute quantification of protein and post-translational modification abundance with stable isotope-labeled synthetic peptides. Nature protocols 2011;6(2):175-186.
Majumder, V., et al. TDP-43 as a potential biomarker for amyotrophic lateral sclerosis: a systematic review and meta-analysis. BMC Neurol 2018;18(1):90.
Suarez-Calvet, M., et al. Plasma phosphorylated TDP-43 levels are elevated in patients with frontotemporal dementia carrying a C9orf72 repeat expansion or a GRN mutation. J Neurol Neurosurg Psychiatry 2014;85(6):684-691.

REFERENCES

1. Feneberg, E., et al., *Towards a TDP-43-Based Biomarker for ALS and FTLD.* Mol Neurobiol, 2018. **55**(10): p. 7789-7801.
2. Wilhite, R., et al., *Platelet phosphorylated TDP-43: an exploratory study for a peripheral surrogate biomarker development for Alzheimer's disease.* Future Sci OA, 2017. **3**(4): p. FSO238.

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Changes to be made by the Author(s):

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Entire text was reviewed for proofreading and grammar by an independent native speaker.

2. Please provide an email address for each author.

E-mail addresses for each co-author were already provided in Editorial Manager submission tool.

3. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

The abstract is 159 word length; within the limit of 150-300 words. We believe that the abstract clearly states the goal of the protocol.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Reference numbers were reformatted (superscripts)

5. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

We formatted as much as JoVE template allowed us. I have problems with “spacing” in template. There are more than “1 line spacing” between the steps in protocol section. I do not know how to fix it. We used Calibri 12 points.

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All commercial languages and symbols were removed from the text. They were referenced in the Table of Materials and Reagents

For example: Fisher Scientific, Sonic Dismembrator, Model 100, Wes, Compass (4.0.0),

7. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a

technique, please use original language throughout the manuscript. Please see lines: 94-95, 100-107, 126-127, 199-200, 205-207, 293-295, 304-318, 341-342, 360-380.

The corresponding author did communicate with the editorial reviewer above mentioned lines. The most of them were picked up by a software identifying the same/similar sentences in published materials. Based on the e-mail conversation dated 09/06/2019 the corresponding author revised the lines 101-106, 307-309, 311-313, 360-364, 366-368, 378-380.

8. Please ensure the Introduction includes all of the following with citations:

a) A clear statement of the overall goal of this method

The clear statement of this method was mentioned in Lines: 60-62 (Track changes ON format)

b) The rationale behind the development and/or use of this technique

The rational for using human platelets was mentioned in the lines: 71-84. The rational for using CEI was mentioned in lines 86-101 (Track changes ON format)

c) The advantages over alternative techniques with applicable references to previous studies

This issue was addressed in lines : 86-94 and a new reference was added (Ref# 6) (Track changes ON format)

d) A description of the context of the technique in the wider body of literature

Context of CEI was entered in lines: 96-101 (Track changes ON format)

e) Information to help readers to determine whether the method is appropriate for their application

This information is entered in line 133-135 (Track changes ON format)

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

Protocol section was checked and revised for the inclusion of imperative sentences.

10. The Protocol should contain only action items that direct the reader to do something. Please use complete sentences throughout the protocol section.

The protocol section was checked and only action items were included

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

The protocol section was consisting of the discrete steps in general; however, some additional information will help user why they are doing certain action items. This lengthens the step. We have seen some very detailed explanation in protocol section in some previously published Jove manuscripts as well.

12. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

The protocol steps included sufficiently detailed information and additional steps. The corresponding author re-checked the protocol.

13. 3: Please bring out clarity with respect to the relationship with CEI and Wes. Also, JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. Please use generic term throughout.

The corresponding author removed the specific product name (*Wes*) and used generic term, CEI, in the entire text.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted the lines for filmable content in both (Track changes ON format) and clean format

15. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

The description was reflected in lines: 426-436

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The figures are original and non-of them are copyrighted.

17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

Critical steps were mentioned in lines: 575-588; 603-613; 614-617; 619-621

b) Any modifications and troubleshooting of the technique

Modifications and trouble shootings were mentioned in lines: 434-436; 578-582; 592-594; 603-611

c) Any limitations of the technique

The limitations were discussed in lines: 617-621

d) The significance with respect to existing methods

The significance of this method was mentioned in lines: 571-576

e) Any future applications of the technique

The future of the application of this technique was mentioned in lines: 594-600

18. Please do not abbreviate the journal titles in the references section.

The corresponding author uses APA Sixth Edition/EndNote setting. This setting is using abbreviated journal names. The corresponding author had a look at other JoVE publications and noticed that abbreviated journal names are allowed. Please advise the corresponding author on that issue.

19. Please remove the figure legends from the uploaded figures. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

Figure legends were now removed from the uploaded figures. All figures were uploaded separately with a descriptive title.

20. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns.

Table of the Materials and Reagents were updated as requested by review editor.