**MarkVCID UH3 Protocol for Plasma Exosome Endothelial Inflammation Kit**

## 1. Executive summary

The cross-validation of the Endothelial Inflammation Kit proposed by UCSF has two Aims:

**Aim 1.** To determine the technical reliability of biomarker quantifications

**Aim 2.** To demonstrate accuracy of biomarkers for measurement of VCID

**1.1. Biomarker Category**: (primary) diagnostic classification, (secondary) risk stratification

**1.2. Endothelial Inflammation Kit:** In our model of VCID pathogenesis, we posit that vascular risk factors and systemic innate immune activation are associated with endothelial injury and inflammation, with phenotypic change in endothelial cells contributing to impairment of the blood-brain barrier, followed by CNS immune activation and neurodegeneration. The two proteomic markers included in the EI kit (C3b and Bb) are activated complement factors hypothesized to represent states of innate immune activation and when measured from endothelial-derived exosomes (EDE), reflect endothelial inflammation. These two biomarkers have strong biological rationales for inclusion (Fyfe, 2017; Sartain, Turner, & Moake, 2018) and are supported by our data from UH2 phase of the MarkVCID study, as detailed below. Of note, by employing a highly innovative methodology based on quantification of these two activated complement factors from isolated endothelial-derived exosomes, we propose a biomarker kit that has increased sensitivity and specificity for VCID. This increase in accuracy, that we seek to validate, would allow capturing of preclinical disease stages, ideal for therapeutic interventions.

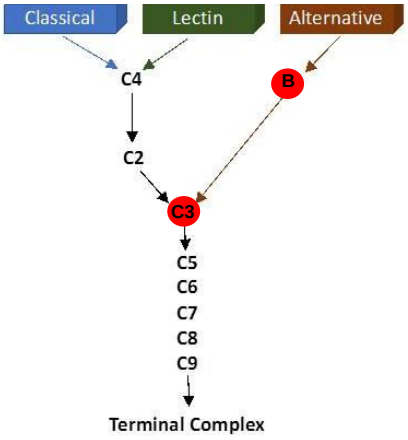
The primary outcome of this biomarker kit is diagnostic classification. This indication is based on the data we generated in the UH2 phase of the study, where we demonstrated accuracy of these biomarkers in classification of subjects with high versus low burden of T2-weighted fluid-attenuated inversion recovery (FLAIR) white matter hyperintensity (WMH) burden. The effect sizes based on group differences in levels of biomarkers were such that we had >99% accuracy in classification of functionally normal adults with and without evidence of cSVD-associated radiographic abnormalities (significant WMH). Fazekas score of 2 and above was classified as high and 0-1 as low.

Should we meet our primary outcome and proceed to collection of longitudinal data, we would also like to test this biomarker for susceptibility/risk stratification (secondary, potential utility). In UH2, despite our small sample size of 26 subjects, we also demonstrated continuous positive associations with systolic blood pressure (SBP), an important risk factor for cerebral small vessel disease (cSVD) and VCID. However, because we performed a cross-sectional study, rather than a longitudinal cohort study, we do not have the necessary evidence in support of the utility of these biomarkers for susceptibility/risk stratification in the earliest, preclinical stages of VCID. However, in light of the associations with SBP, and the demonstrated group differences in subjects with CDR=0 and absence of overt signs/symptoms of vascular disease, we suggest that at the very least a secondary use of these biomarkers could be for risk stratification within functionally intact stages of VCID, where cognitive decline is subtle and detectable with more sensitive tests of executive function. If this stage is considered preclinical for VCID and we demonstrate within a longitudinal cohort that individuals with high levels progress to mild cognitive impairment and dementia, these biomarkers would have utility in susceptibility/risk stratification. For susceptibility/risk stratification, we would also hope to see change in levels of biomarkers correlate with change in vascular risk factors or baseline levels of these biomarkers predict longitudinal trajectory of clinically significant outcomes (cognition) or established intermediate phenotypes (neuroimaging).

**1.3. Data Collected in UH2:** In the UH2 phase of MarkVCID, we performed a proof of concept study, using platelet-poor plasma to isolate EDEs and investigate their proteomic cargo. Our goal was to test the association of exosomal cargo, specifically innate immune activation and inflammation-related complement factors, with predictors and outcomes of significance for cSVD, such as SBP, WMH, and executive function, and test the accuracy of these biomarkers in classifying cases versus controls. We selected 26 neurologically normal and functionally intact older adults divided into two groups: 11

subjects with extensive WMH (modified Fazekas score ≥ 2), and 15 subjects with no WMH on visual inspection. We initially looked at 13 different complement factors covering three separate pathways (classical, alternative, and lectin) (Figure 1). The group differences provided large effect sizes with C3b demonstrating an accuracy of >99% (p<.0001) in distinguishing high WMH subjects from low WMH subjects, and Bb an accuracy of 99% (p<.0001). Overall AUC for other significant markers in the classical and alternative complement pathways were >95% (p<.0001). With linear regressions controlling for age, we also noted significant inverse associations with executive function (range p= .003 – .05), and positive associations with SBP (range p=.0008 – .05) and volumes of WMH, quantified by Dr. DeCarli's WMH pipeline (range p= .0004 – .006).

**Figure 1.** Merging of the three canonical complement pathways, and central importance of C3 and B activation into C3b and Bb respectively.




**1.4. Indication of EI kit:** We consider EDE C3b and Bb to have potential utility for **diagnostic classification**. In addition, there may be additional utility for ***susceptibility / risk stratification****,* that could be tested if the primary outcome is reached**.** Although we cannot feasibly propose testing of this biomarker kit for ***target engagement / therapeutic*** *efficacy* in absence of existing therapies, we suggest that this biomarker kit could be of utility in VCID interventions targeting innate immune activation and vascular inflammation.

**1.5. UH3 Validation Studies:** We propose cross-validation of results with relatively low number of subjects at each site: a total of 45 subjects, with 15 subjects per tertile of burden of cerebral WMH volumes based on Fazekas scoring (0-1, 2 and 3). We anticipate that these biomarkers will also demonstrate significant inverse associations with cognitive function, especially executive function, as well as positive associations with burden of WMH. Since Dr. DeCarli's WMH processing pipeline is a kit being cross-validated in UH3, we propose that each site use the visual rating of WMH based on Fazekas scale in addition to their site-specific WMH quantification algorithm for continuous measures of WMH burden. Since Fazekas scores are ordinal values, we propose ANCOVA for group-wise comparison of means, controlling for age. As for continuous measures of executive function, SBP, and WMH, linear regressions will be used, controlling for age.

There are two aims to the validation protocol: Aim1 is addressing testing of technical reliability and precision (5.2.) and Aim2 is addressing cross-validation of the accuracy of biomarkers for VCID (5.3.). We anticipate that in the short period of cross-site validation (~1 year) we may not reach a stage at which the absolute values of analytes measured are equal for a given sample across sites. Therefore, despite providing steps for testing inter-site reliability of absolute measurements, our primary **milestones are as follows:**

I. **Reliability of intra-site measurements:** demonstrated by CV below 20% for measurements of EDE C3b and Bb in ELISA assays.
II. **Test retest reliability of EDE C3b and Bb:** No significant change in levels of these biomarkers in 10 individuals across 2 weeks (measurements will be considered equivalent if they are within 1.44SD of one another.)
III. **Reliability of EDE C3b and Bb measurements from identical samples:** As demonstrated by ICC equal or higher than 0.7.
IV. **Validation of group differences in levels between Fazekas 2-3 with 0-1:** As demonstrated by a Cohen's d 1.1 or higher in levels of EDE C3b and Bb.

The 4 milestones above will define success within the initial year of UH3 and the incentive to continue to years 2-3 for additional cross-site testing of these biomarkers, and collection of longitudinal data. More time and optimization may be needed in order to reach reliability and precision of absolute measurements across sites. However, this may never be a goal if we anticipate the processing of biofluid markers by a central lab for clinical trials.

## 2. Biofluid Kit Components

### 2.1. Lab equipment, products and consumables (see Appendices)

**2.2. Clinical data:** The following clinical data will be required for cross-validation of our prelim results:

- **a. Systolic blood pressure**: defined as mean of measurements from both arms, or either arm if only one arm is used.
- **b. Executive function composite**: executive measure will be an item-response theory (IRT) generated score based on four executive tasks from the UDS 3.0: Trails B (number of correct lines per minute), backward digit span (total score), phonemic fluency (number of correct F-words in one minute), and category fluency (number of correct animal responses in one minute). Confirmatory factor analyses support good model fit for these donor scales. IRT scores will be built using baseline UDS data from 3,450 clinically normal subjects. Each measure will be entered into an item-response analysis using the R *ltm* module(Kramer et al., 2014; Mungas et al., 2003). The parameters from this analysis will be saved and applied to MarkVCID participants to generate a composite score for each individual at each time point. IRT is an advantageous approach because the composite is invariant to the specific scores that are used. Therefore, the IRT score should produce an unbiased estimate of a participant's executive ability even if different variables are used to generate the scores, making this composite particularly appropriate for longitudinal research. IRT scores will allow the consortium to maximize the sample size while also improving the reliability and robustness of the outcome measure.
- **c. White matter hyperintensity** (WMH) on FLAIR: **(a)** visual rating of WMH burden (Fazekas score), as well as **(b)** quantitative WMH measurement via each site's preferred pipeline, since the UCSF WMH pipeline is itself being tested as a kit.
- **d. Other needs:** Neuroimaging (T1, FLAIR). A lab experimentalist / operator with technical skills at the level of an SRAII or higher.

**3. Participating sites:** Prospective data collection: UK, UNM, JHU, and UCSF. Analyses of stored frozen plasma: CHARGE and Rush

**4. Protocol for data acquisition:** see Appendix 1 PPP Preparation, Appendix 2 IP of Human EDEs from PPP, Appendix 3 MarkVCID Fluid-Sample Best Practice Guidelines

**5. Step by step analytic plan:**

**Preparatory steps:**

- **a.** Technicians / experimentalists will gather at a central lab in San Francisco and observe and perform one experiment from start to finish on 4 plasma samples. These have been previously collected at UCSF and will be provided to each experimentalist. ELISAs will be run in triplicates for each as part of this training. Data will be analyzed for intra-operator reliability and precision as well as inter-operator variance.
- **b.** Technicians can be observed at their respective sites for their first experiment. This will be provided on an as need basis and most will likely not need this. All site-specific differences in equipment and settings will be reported to UCSF.

**Aim 1: To determine the technical reliability of biomarker quantifications:**

**I. Site reliability of EDE preparation:** Each site will receive 10 aliquots of frozen PPP from UCSF, from which they will: (1) isolate exosomes (see Appendix 2) and freeze 10µl of exosomal preps prior to proceeding to immuno-precipitation of EDE from the mixed population of exosomes. When EDEs are isolated, 10µl aliquot of EDE preps along with the frozen 10µl of mixed exosomal preps will be shipped to UCSF for analysis. We will analyze ratios of EDE to mixed exosomal populations. The mean per sample per operator will be the unit of comparison across sites. We will perform comparisons between each site and UCSF with paired t-test. In addition, we will test reliability using ICC. To this end, mixed effects regression models will be used to estimate the ICC by treating each value per site for each sample as repeated measures. **Power calculations:** comparison of each site to UCSF, we will have 80% power to detect a difference as small as 1SD. When comparing across sites, with 10 identical samples processed at 4 sites, we will have over 80% power to detect an ICC of 0.91 if the null hypothesis is an ICC of 0.7.

II. **Site reliability for EDE inflammatory marker quantifications:** Each operator will proceed to lysis of EDEs and quantification of: CD81, C3b and Bb (expected CV <20%). Each site will also send a frozen 50µl aliquot (per sample of EDE lysate) to UCSF for analysis. Therefore, in addition to each site running their own colorimetric ELISA assays, UCSF will be quantifying CD81 and C3b from all samples on one ELISA plate. Our expectation is that the transformed values of each of these markers within a normal distribution should not be significantly different across sites for a given sample (CV <30%). However, it is possible that the absolute values will be different. Each sample will be run in triplicates to test for outliers and gauge intra-assay variability. After outlier exclusion, the mean of remaining values will be used. We will test measurement reliability across sites with an ICC analysis. We will review results with sites in order to uncover potential sources of systematic or random error (depending on whether we get a "site/operator" effect or values are variably different). Even if there are systematic / site differences (which we expect to have), we will test the group differences across Fazekas scores and association of markers with outcomes of interest. The group differences and direction of meaningful associations we expect will be preserved. In light of the small numbers during reliability testing, we don't expect statistical significance and would simply like to see the directions of associations preserved across sites: positive correlations between biomarkers and SBP and WMH, as well as higher levels of biomarkers in higher Fazekas score groups. **Power calculations:** comparison of each site to UCSF, we will have 80% power to detect a difference as small as 1SD. When comparing across sites, with 10 identical samples processed at 4 sites, we will have over 80% power to detect an ICC of 0.91 if the null hypothesis is an ICC of 0.7.

UCSF will look into transferring the quantification of C3b and Bb onto either MesoScale Discovery (MSD) or SIMOA Quanterix platforms. These platforms diminish provide simplification in operator-dependent steps, translating into lower overall CVs. This endeavor will facilitate future studies beyond the one-year limited time for cross-validation of results.

III. **Determine need for PPP preparation (optional):** At UCSF we will compare (1) ratio of immunoprecipitated EDE to total exosomal counts, and (2) concentrations of EDE C3b from frozen aliquots of PPP versus regular frozen plasma collected from 12 subjects. This experiment will determine whether regular frozen plasma from Charge and Rush can be used. We will use an equivalence test to compare the values measured from frozen PPP and from frozen plasma. We will also estimate the correlation between the measures from the two plasma preparations. **Power calculations:** with 12 samples, we will have over 80% power to detect a coefficient of variation that is no more than 40% and will have 80% power to declare the duplicate aliquots equivalent if the means are within 0.89 standard deviations (SD) from one another.

IV. **Comparability of legacy and prospective frozen plasma (optional)**: If the differences between PPP and regular plasma is not significant as established by an equivalence test, we will test additional collection parameters. To this end, UCSF will prospectively collect and compare 12 plasma samples according to 3 protocols: Rush, Charge, and UH3 biofluid subcommittee SOP. This is important if we want to use frozen plasma aliquots from Charge and Rush. We will compute an intraclass correlation coefficients (ICC) using mixed effects models to assess the similarity in values across the 3 protocols. **Power calculations:** with 12 samples, we will have 80% power to declare the counts and C3b between plasma preparations equivalent if the means are within 0.89SD of one another. We will have 80% power to detect a correlation as small as 0.67.

V. **Fasting versus non-fasting blood collection (optional):** At a later stage, we could consider testing fasting versus non-fasting in the AM; if no difference, then AM collection (7-9AM) versus PM collection (3-5PM). These experiments can occur in parallel at UCSF. For these, we will again use an equivalence test to see if fasting and non-fasting protocols or time of day produce similar outcomes. **Power calculations:** same as above.

VI. **Testing batch effects (optional):** Divided into "plate" and "equipment/environmental" effects. In order to test "plate" effect", 10 samples will be run in duplicates on two separate CD81 ELISA plates by 3 sites. Equivalence tests within site will be conducted. Across sites, mixed effects models will be used to assess differences between plates as well as estimate the ICC. In order to test equipment / environmental effects, 10 samples will be run twice for CD81 on two distinct days by three sites. Analyses will be similar to tests of "plate effect" above. **Power calculations:** within a site we will have 80% power to declare values measured from different plates equivalent if the means are within 1SD of one another. Power will be greater for detecting ICC, since we have repeated measures across sites and across plates.

**Aim 2: To demonstrate accuracy of biomarkers for measurement of VCID:**

Each site will analyze 45 samples: 15 in each tertile of burden of disease determined by Fazekas scoring from FLAIR imaging. The following steps will be implemented in order to ensure **accuracy** of biomarker measurement for VCID.

VII. **Test-retest reproducibility:** this will be tested at UCSF. This experiment is a component of disease measurement "accuracy." The hypothesis is that VCID is a stable "slowly" progressive disease state that evolves over the time-frame of years. Consequently, levels of biomarkers that would serve to stratify subjects or provide accurate surrogate biomarkers of disease in clinical trials should remain stable or have non-significant increases over a reasonable time-frame (e.g.: under 1 month). We will therefore draw blood samples from 10 subjects on day 0, 7 (±2 days), 14 (±2 days), to measure intra-individual longitudinal change over 3 time-points by analyzing samples at UCSF. Mixed effects models will be used to model change across the 3 time points and we expect there to be no change over this two-week period. **Power calculations:** we will have 80% power to declare the means across time equivalent if they are within 1.44SD of one another.

VIII. **Cross-validation of biomarker kit for diagnostic classification:** The cross-validation of group differences in levels of biomarkers between low and high Fazekas scores will serve as our main 1-year milestone for cross-validation of our single site preliminary data completed in UH2. However, in light of the importance of clinical outcomes, we will use cognitive function (executive function) as primary outcome for the biological validation of our kit, and SBP, and measures of WMH as secondary outcomes for cross-validation of biomarkers. We will test cross-sectional group differences in levels of C3b and Bb between subjects with high versus low executive function (median split), normotensives versus subjects with hypertension (SBP >155), and among Fazekas scores (0-1, 2, and 3). We also anticipate continuous associations with SBP and site-specific automated WMH measures, and inverse associations with executive function. Analysis of variance or the non-parametric Kruskal-Wallis test will be used to compare biomarker levels across the three groups of Fazekas scores. Two-sample t-tests and AVCOVA will be used to compare levels between the groups for executive function and blood pressure. Finally, we will use linear regression to assess associations between the biomarkers and continuous measures of executive function, SBP and WMH measures, controlling for age. We will also perform logistic regressions, and discriminant functions to generate ROC curves at each site to test the accuracy of individual biomarkers for classifications of VCID subjects into different severities of WMH. In these analyses, we will obtain cut-off values. In light of the relatively small sample sizes, we also propose use of statistical methods such as bootstrapping in order to obtain confidence intervals. **Power calculations:** given the very strong effect size observed in this preliminary data (Cohen's d of 1.8 for C3b and 15 for Bb), we anticipate being highly powered in the UH3 phase for comparing means of these biomarkers between groups (e.g. minimal/moderate/severe Fazekas), even with relatively small sample sizes. In particular, we will have over 80% power to detect an effect size (Cohen's d) as small as 1.1, assuming $\alpha = .05$ and a two-sided test with n=15 per group. If we reduce the type I error rate to account for the 3 groups, we will still have over 80% power to detect an effect size as small as 1.25 ($\alpha = .017$). For regressions, with a sample size of 45 individuals, we will have over 80% power to detect an association accounting for as little as 16% of the variability in the outcome (r=0.4). Associations with clinical variables of executive function and SBP, as well as WMH were strong and highly significant with our sample size of 26 individuals (r>0.6 for all three), so we will have excellent power to replicate these findings across sites. We will have 80% power to detect an additional

contribution to the variability in the clinical outcome of 13.6-15.3% if other variables such as age and sex account for 10-20% of the variability. We are proposing larger sample sizes so as to exclude outliers and decrease the probability of winner's curse (Button et al., 2013). We observed a high classification accuracy of these biomarkers in the UH2 phase (>99%) and anticipate to obtain at least >85% accuracy in classifications with over 80% power. For the combined data, we will have even greater power than already presented for the within site analyses, even if split into derivation, test, and validation sets.

Regarding Fazekas score classification for WMH, we propose to first test a dichotomized sample of subjects with moderate-severe burden of disease (Fazekas 2-3) and none to minimal WMH burden (0-1) in order to validate what was done at our site during UH2, prior to proceeding to the comparison of 2 groups (0-1, 2, and 3).

Last, after demonstrating the merit of this kit at each site independently, we propose to combine data across participating sites in order to divide a larger dataset into derivation, test and validation sets and perform discriminant function to measure AUC for classification of subjects into low, medium, and high burden of disease (using WMH as gold standard) in a larger sample.

9. **Potential biomarker for susceptibility / risk stratification:** We will not be able to test this within the first year and will aim to test this within subsequent years with longitudinal data. We suggest that cross-validation of cross-sectional associations with vascular risk factors such as systolic blood pressure and burden of white matter hyperintensity on T2/FLAIR imaging, and inverse associations with cognitive function will provide compelling evidence motivating further investigations.

10. **Potential biomarker for target engagement / therapeutic efficacy:** this type of validation will require intervention, neuropathological investigations, as well as experiments in model systems. Should therapies targeting innate immune activation and vascular inflammation be tested in VCID such as previously done in cardiovascular disease(Ridker et al., 2017) this biomarker kit holds promise and could be investigated.

11. **Plan for longitudinal data collection:** the primary analytic methods for our cross-sectional hypotheses will be diagnostic classifications as well as testing of associations with clinically meaningful continuous measures in VCID such as SBP, WMH, and executive function. We can pursue longitudinal analyses through stored legacy data from Rush and Charge, as well as other sites if we can use regular frozen plasma or collect longitudinal data beyond the first year of UH3. We would plan to use linear mixed effects models to test the longitudinal hypothesis that baseline levels of biomarkers predict trajectories of neurodegeneration (MRI measures) and cognitive decline in VCID (McCulloch et al., 2008) and test whether change in biomarkers is associated with change in outcomes.

    Our analytical approach would be to build models that include repeated measures of diffusion tensor metrics, WMH, as well as Rush imaging kit as the dependent variables and baseline endothelial inflammation as the predictors (when the imaging kits have been validated). Baseline (i.e., time-invariant) predictors will be entered along with their interaction with time to assess their ability to predict future trajectories in the dependent variables. We will control for age and sex and education. Should the results obtained over the first year of the UH3 cross-site validation deemed satisfactory and funding provided for additional years, we would plan to collect repeated measures of EDE biomarkers in order to investigate the association of change in these biomarkers with change in the clinically meaningful outcomes detailed above.

    It should be noted that the proposed biomarker kit combines the specificity that EDEs provide for pathology affecting endothelia with the hypothesis-driven choice of C3b and Bb as complement factors implicated in cerebral degeneration. However, any other molecular markers could be quantified from EDEs. We therefore will collect more EDEs than needed for C3b and Bb quantification so as to provide the opportunity for future investigations of other promising biomarkers from EDEs.

12. **Plan for reporting outcomes and data sharing:** we will have an open line of communication with all participating sites with weekly calls throughout experiments within Aim 1 (demonstration of reliability and precision of measurements). All participating sites will have access to UCSF investigators for questions, assistance with trouble shooting, and help with analyses of data throughout the validation process. At the term of the study we will also merge and analyze data collected from all sites for analyses done with a larger overall sample size.

We will report progress at the biofluid sub-committee calls and report interim and final results to the consortium at the bi-annual meetings, in addition to national and international conferences (e.g.: ISC, AAN, and AAIC). The detailed protocols and plan for validation will be shared with all sites. We also plan to share all data with the coordinating center so that it can be shared in accordance with protocols and agreements outlined by the MarkVCID consortium.

# Appendix 1 - Platelet Poor Plasma (PPP) Preparation

## 1. PREPARATION OF PPP COLLECTION BUFFER

### Equipment & Consumables:

| Equipment Name | Manufacturer & Catalog Number | Product Link |
|---|---|---|
| 100 µl serological pipettes | Fisher Scientific 50-202-113 | Serological pipettes |
| 15 ml conical tubes | ThermoFisher Scientific 339650 | 15ml tube |
| Sterile microcentrifuge tubes | | |
| Tube rack | | |
| BD Vacutainer Glass Blood Collection Tube with ACD, 6 ml | Fisher Scientific 02-684-29 | BD Vacutainer 364816 |
| Eppendorf Research Plus Pipette: 1-10 µl,10-100 µl, 200 µl, 1000 µl (calibrated <6months) | | |
| Tube Revolver 120V/US PLUG, speed set to 10 rpm | Fisher Scientific 11676341 | Tube revolver |
| Plate/tube shaker | | |
| Benchtop cooling centrifuge | | |
| Vortex | | |
| VersaMax ELISA (Absorbance Microplate Reader): Molecular Devices | | |
| Ice Tray/ Bucket | | |
| Sterile Flasks | | |

| Product Name | Manufacturer & Catalog Number | Product Link |
|---|---|---|
| Dulbecco's Balanced Salt Solution (DPBS) no calcium or magnesium, 1,000 ml | ThermoFisher 14190-136 | Gibco DPBS |
| Prostaglandin E1 (PGE1) | Sigma-Aldrich/ MilliPore-SIGMA P5515 | PGE1 |
| EDTA (0.5M), pH 8.0, RNase-free | ThermoFisher AM9260G OR AMG9269G | EDTA |
| Acetone | Sigma-Aldrich/ MilliPore-SIGMA 650501-1L | Acetone |
| 0.1 M Phosphate Buffer Solution | Sigma-Aldrich/ MilliPore-SIGMA P5244-100mL | Phosphate Buffer |

### Reconstitution of PGE1 (5mg):

1. Spin down the vial (max spin for 30 sec in a benchtop centrifuge).
2. Reconstitute PGE1 by adding 500 µl acetone to the vial and slowly pipet up and down to dissolve the lyophilized PGE1 powder to get a 10 mg/ml solution.
3. Then add 500 µl of 0.1M phosphate buffer to get a stock solution of 5mg/ml.
4. Aliquot 90 µl of PGE1 to sterile microcentrifuge tubes (total 11) and store at -80˚C until further use.

### To prepare a PPP collection buffer solution of 600ml: DBS with 2 mM EDTA and 2 µM PGE1

1. Using a combination of the 100 ml serological pipette and smaller pipettes, aliquot 597.515ml DBS (5x 100 mL + 1x 97 ml + 1x 515 µl) into a sterile (autoclaved) flask.

2. Add 2.4 ml EDTA (0.5M) to the DBS in the flask and mix.

3. Add 85 µl of the PGE1 (5mg/ml) to the DBS in the flask and mix.

4. Aliquot 3 ml of buffer (DBS with 2 mM EDTA and 2 µM PGE1) into 15 ml conical tubes and store them at -80˚C.

## 2. PREPARATION OF PPP FROM BLOOD:

1. 6 ml of venous blood is drawn (per MarkVCID's Best Practices and Fluid Biosample Requirements) into a tube containing acid citrate dextrose (ACD) anticoagulant solution.

2. Centrifuge the tubes at 500xg for 20 min at room temperature.

3. Thaw out the required number of tubes containing PPP collection buffer.

4. Using a 1 ml pipette, gently transfer 3 ml (3 x 1 ml) of platelet-rich plasma (PRP) to the 15 ml falcon tubes containing the thawed pre-aliquoted PPP collection buffer. **When removing the PRP, it is essential not to disturb the pellet at the bottom of the ACD plasma tube.**

5. Pipet up and down to mix the PRP with the collection buffer.

6. Centrifuge the PRP and buffer solution at 2,200xg for 20 min at room temperature.

7. Then, 0.25 ml aliquots of PPP are transferred to 1.5 ml Eppendorf tubes and stored at -80$^{o}$C.

*Questions/Comments?*
*Contact kit lead Fanny Elahi (fanny.elahi@ucsf.edu) OR*
*MarkVCID Coordinating Center (hsingh6@mgh.harvard.edu)*

# Appendix 2 - IP of Human Endothelial-derived Exosomes (EDEs) from PPP

## 1. ISOLATION OF EDEs

### Equipment & Consumables:

| Equipment Name | Manufacturer & Catalog Number | Product Link |
|---|---|---|
| Eppendorf Research Plus Pipette: 1-10µl,10-100µl, 200µl, 1000µl (calibrated <6months) | | |
| 1.5 ml Eppendorf tubes | ThermoFisher Scientific 05408129 | Eppendorf Tubes |
| Tube Revolver 120V/US PLUG, speed set to 10 rpm | ThermoFisher Scientific 11676341 | Tube revolver |
| 15 ml conical tubes | ThermoFisher Scientific 339650 | 15ml tube |
| 50 ml conical tubes | ThermoFisher Scientific 339652 | 50ml tube |
| Plate/tube shaker | | |
| Benchtop cooling centrifuge | | |
| Vortex | | |
| VersaMax ELISA (Absorbance Microplate Reader): Molecular Devices | | |
| Ice Tray/ Bucket | | |
| Tube rack | | |

| Product Name | Manufacturer & Catalog Number | Product Link | Notes |
|---|---|---|---|
| Thromboplastin-D | ThermoFisher Scientific 100357 | Thromboplastin-D | Sites must request quote from ThermoFisher to purchase |
| Dulbecco's balanced salt solution | ThermoFisher Scientific 14190136 | DPBS, no calcium, no magnesium | |
| 100x Protease inhibitor cocktail | Sigma-Aldrich/ MilliPore-SIGMA P8340-1ML | Protease inhibitor | |
| 100x Halt Protease/Phosphatase inhibitor cocktail | Fisher Scientific 78441 | Phosphatase inhibitor | |
| ExoQuick solution | System Biosciences Inc. EXOQ20A-1 | ExoQuick solution | Request quote by email: Andre Lubarsky. Mention the MarkVCID to receive a 25% discount |
| 100 ml Glacial acetic acid | Sigma-Aldrich A6283-100ML | Acetic acid | |
| Distilled water | ThermoFisher Scientific 15230001 | Distilled Water | |
| Mouse anti-human CD31 biotinylated antibody (clone MEM-05) | ThermoFisher Scientific MA1-19510 | CD31 Monoclonal Antibody (MEM-05), Biotin | |
| Bovine serum albumin (BSA) | ThermoFisher Scientific 37525 | BSA (10X) in PBS | |
| Streptavidin-Plus UltraLink resin | ThermoFisher Scientific 53116 or 53117 | Streptavidin Resin 2ml OR Streptavidin Resin 5ml | |
| M-PER mammalian protein extraction reagent | ThermoFisher Scientific 78501 | M-PER Mammalian Protein Extraction Reagent | |
| Goat anti-human CD146 biotinylated antibody (Novus) | Novus Biologicals NBP2-47777B | CD146/MCAM | |
| UltraPure 1M Tris-HCl (pH 8.0) | ThermoFisher Scientific 15568025 | Tris-HCl, pH 8.0 | |
| ELISA kit CD81 | Cusabio P60033 | Human CD81 antigen(CD81) ELISA kit | |
| ELISA kit for C3b | Abcam ab195461 | Human Complement C3b ELISA Kit (ab195461) | |
| ELISA kit for Bb | Quidel-Microvue A027 | MicroVue Bb Plus EIA | |

**A. Isolation of All Exosomal subtypes from plasma**

1. Centrifuge the thawed PPP samples at 1000xg for 10 min at 4˚C.

2. Transfer 250 µl PPP supernatants to 1.5 ml Eppendorf tubes containing 75 µl of Thromboplastin-D and incubate for 60 min at room temperature.

3. Add 175 µl DBS containing 3X protease inhibitor cocktail and 3X protease/phosphatase inhibitor cocktail (164.5 µl water + 5.25 µl Protease inhibitor cocktail + 5.25 µl Halt Protease/Phosphatase inhibitor cocktail) to each tube vortex for 10 seconds and centrifuge at 3000xg for 20 min at 4°C.

4. Aliquot 126 µl ExoQuick exosome precipitation solution into fresh tubes (chilled on ice).

5. Transfer sample supernatants to tubes containing ExoQuick, mix by inversion six times, and incubate for 60 min at 4˚C. *This incubation time must be exactly 60 min.

6. After the incubation centrifuge the samples at 1500xg for 30 min at 4°C and discard the supernatants.

7. Each pellet is resuspended in 350 µl distilled water containing 1X protease and phosphatase inhibitor cocktails (343 µl water + 3.5 µl Protease inhibitor cocktail + 3.5 µl Halt Protease/Phosphatase inhibitor cocktail) for the immunochemical enrichment of endothelial exosomes. Vortex the sample for 20 s and rotate at ~10 rpm 4˚C for 2H. Vortex the sample for 30 s to further encourage pellet resuspension and then rotate at ~10 rpm overnight at 4˚C if the pellet is not resuspended. *This step is frequently performed over night to ensure maximum resuspension of the exosome pellet. (With optimal resuspension, the solution appears semi-opaque with no obvious pellet.)

**B. Immuno-precipitation of EDE**

Make a master **CD31 antibody solution**: [2 µl CD31 + 15 µl 10% BSA + 33 µl DBS] per sample. ***** Use 2 µg/µl antibody for the isolations. The current lot is 1 µg/µl, so 2 µg = 2 µl. Check the lots and recalculate the amount of antibody as needed.

8. Vortex the sample as needed to ensure that the exosomes appear resuspended followed by centrifugation at 400xg for 5 min to precipitate any insoluble material.

   **** Freeze 10 µl of total exosomal prep at -80°C to be shipped to UCSF for inter-site reliability testing**

9. Transfer the supernatants to a fresh microcentrifuge and add 50 µl CD31 antibody solution to each exosome suspension before mixing for 1H at room temperature using a rotator.

Make a master **Streptavidin solution**: [10 µl Streptavidin-Plus UltraLink resin + 12 µl 10% BSA + 18 µl DBS] per sample.

10. Add 40 µl of the Streptavidin solution* to each exosome suspension and mix on rotator for 1H at room temperature. *Pipet Streptavidin solution up and down prior to taking 40 µl to ensure that equal amount of resin gets added to each tube.

11. Centrifuge samples at 4°C at 600xg for 10 min and remove the supernatant.

Make a stock solution of **0.05 M acetic acid**: slowly add 86 µl of glacial acetic acid to 7.5 ml deionized water. Adjust the final volume of the solution to 30 ml by adding 22.414 µl of deionized water. Stock solution can be stored at 4°C.

12. Resuspend each pellet in 100 µl cold 0.05M acetic acid and vortex for 10 seconds. After a 10 min stand at 4°C, the samples are centrifuged at 4°C at 4000xg for 10 min.

Make a master of **DBS + Tri-HCl solution**: [265 µl DBS + 10 µl 1M Tris-HCl + 25 µl 10% BSA] per sample.

13. Transfer the supernatants to new, prechilled 1.5 ml Eppendorf tubes containing 300 µl of the DBS + Tri-HCl solution and vortex for 10 seconds to mix.

    **** Freeze 10 µl of EDE (intermediate prep) at -80°C to be shipped to UCSF for inter-site reliability testing**

Make a master **CD146/MCAM antibody solution**: [3.08 µl CD146/MCAM + 15 µl 10% BSA + 31.92 µl DBS] per sample. * Use 2 µg/µl antibody for the isolations. The current lot is 0.65 µg/µl, so 2 µg = 3.08 µl. Check the lots and recalculate the amount of antibody as needed.

14. Add 50 µl CD146/MCAM antibody solution to each exosome suspension and mix on rotator at room temperature for 1H

Make a master **Streptavidin solution**: [10 µl Streptavidin-Plus UltraLink resin + 12 µl 10% BSA + 18 µl DBS] per sample.

15. Add 40 µl of the Streptavidin solution* to each exosome suspension and mix for 1H at room temperature using a rotator. *Pipet Streptavidin solution up and down prior to taking 40µl to ensure that equal amount of resin gets added to each tube.

16. Centrifuge the samples at 4°C at 600xg for 10 min and remove the supernatant.

17. Resuspend each pellet in 100 µl cold 0.05 M acetic acid and vortex for 10 seconds. After a 10 min stand at 4°C, the samples are centrifuged using 4000xg for 10 min at 4°C.

Make a master BSA **+ Tris-HCl solution**: [10 µl 1M Tris-HCl + 25 µl 10% BSA] per sample.

18. Transfer the supernatants to new, prechilled 1.5 ml Eppendorf tubes containing 35 µl of the BSA + Tris-HCl solution and vortex the sample for 10 seconds to mix.

    **** Freeze 10µl of EDE (final prep) at -80°C to be shipped to UCSF for inter-site reliability testing**

Make a master **MPER solution**: [357.7 µl of M-PER + 3.65 µl protease inhibitor + 3.65 µl phosphatase inhibitor] per sample.

19. Add 365 µl of M-PER solution to each sample to lyse the exosomes and extract the EDE proteomic cargo. Perform two freeze thaw cycles, freezing at -20˚C and thawing on ice, respectively, with a 10 second vortex step in between. Aliquot out the EDE lysates and place at -80˚C for long-term storage.

    **** Freeze 100 µl of EDE lysate at -80°C to be shipped to UCSF for inter-site reliability testing**

20. EDE proteins are quantified by ELISA kits for CD81, C3b, and Bb (per manufacturer's protocol).

*Questions/Comments?*
*Contact kit lead Fanny Elahi (fanny.elahi@ucsf.edu)*
*OR the MarkVCID Coordinating Center (hsingh6@mgh.harvard.edu)*

## Appendix 3 – Biofluid collection & processing

See the MarkVCID Fluid-Sample Best Practice Guidelines for collection and processing of biofluids (MarkVCID web account required)

**References**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