SOP_002_NU_0_1_Preparing_Human_WCL_for_Qualitative_TDMS_2015_v1_CJD
Caroline J. DeHart

❖ Reagent and Materials List

Item	Part Number	Vendor
Tris Base	BP152-1	Fisher Scientific
Sodium Chloride	S9888	Sigma Aldrich
N-lauroylsarcosine	L9150-50G	Sigma Aldrich
1x HALT Protease and Phosphatase Inhibitor Cocktail (EDTA-free)	78443	Thermo Scientific
Magnesium Chloride	M8266	Sigma Aldrich
Benzonase Nuclease	E1014-25KU	Sigma Aldrich
Microplate BCA Assay (requires plate reader)	23252	Thermo Scientific
96-well plates	80086-578	VWR
ACS Grade Acetone	A18-4	Fisher Scientific
Sodium Dodecyl Sulfate	L3771-1KG	Sigma Aldrich
Dithiothreitol (DTT)	43815	Sigma Aldrich
10 % GELFrEE cartridge kit	42105	Expedeon
GELFrEE 8100	48100	Expedeon
1.5 mL Protein LoBind Microcentrifuge Tubes	13-698-794	Fisher Scientific
SDS-PAGE Running Buffer	1610732	BioRad
Precast gels (AnyKD, 15-well)	4569036	BioRad
2x Gel Sample Loading Buffer	1610737	BioRad
β-mercaptoethanol	M3148-100ML	Sigma Aldrich
Protein MW standard	1610374	BioRad
Silver Staining Kit	24612	Thermo Scientific
Optima Grade Methanol	A456-4	Fisher Scientific
HPLC Grade Chloroform	528730	Sigma Aldrich
Optima Grade Water	W6	Fisher Scientific
Optima Grade Acetonitrile	A955	Fisher Scientific
MS-Grade Formic Acid	PI-28905	Fisher Scientific

❖ Important Notes

- This protocol is designed to be performed with dry, frozen pellets; each containing 2 x 10^7 harvested human cells. Pellets should be stored at -80 °C and thawed on ice for 15 minutes prior to resuspension in lysis buffer. Human whole-cell lysate (WCL) should be kept as cold as possible until the acetone precipitation step.
- Use 1.5 mL Eppendorf LoBind microcentrifuge tubes for collecting GELFrEE fractions and performing MeOH/ $CHCl_3$/ H_2O sample cleanup. In our experience, these tubes have shown the lowest degree of plasticizer leaching and/or protein binding during use and storage.
- GELFrEE fractions can be stored at -80 °C for several weeks prior to the MeOH/$CHCl_3$/H_2O cleanup step. Once this cleanup has been performed, however, proteins will no longer be stable for long-term storage.

❖ Lysis Buffer Recipe

Component	Volume (mL)	Stock Concentration	Final Concentration
Tris Base, pH 7.5	0.2	1 M	20 mM
NaCl	0.2	5 M	100 mM
N-lauroylsarcosine	1	10% (w/v)	1% (w/v)
HALT Inhibitor Cocktail	0.1	100X	1X
H_2O	8.5	-	-
Total	**10**		

❖ Cell Lysis Protocol

- Resuspend each thawed cell pellet (2 x 10^7 cells) in 1 mL of pre-chilled **lysis buffer**.
- Pipet to mix until a marked increase in sample viscosity (due to released genomic DNA) is observed.
- Incubate lysate on **ice** for **20 min**. Pipet or invert to mix every 5 min.
- Add 1 µL of **1 M $MgCl_2$** to the lysate (to a final concentration of 1 mM).
- Add 3 µL (750 U) of **benzonase nuclease** to the lysate. Pipet to ensure complete mixing.
- Incubate lysate at **37 °C** for **20 min**. Pipet or invert to mix every 5 min.
- Chill lysates on **ice** for **5 min.**
- Pellet remaining debris by centrifugation at 13.2 krpm for **15 min.** at **4°C**.

❖ Protein Quantitation and Precipitation

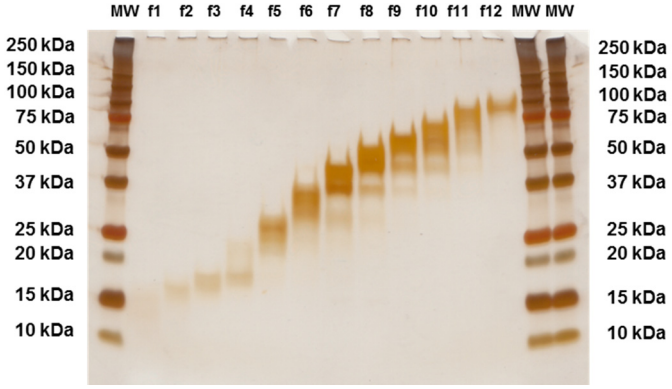
- Determine total protein concentration for each lysate by performing the **BCA Assay** according to the manufacturer's protocol. Prepare all BSA standards in lysis buffer.
- Transfer the volume of lysate containing **400 µg** of total protein to an Eppendorf LoBind tube.
- Add 6 volumes of ice-cold ACS-grade **acetone** to each tube (e.g. **600** µL to **100** µL).
- Precipitate proteins **overnight** at **-80 °C**.
- Pellet precipitated proteins by centrifugation at 13.2 krpm for **10 min.** at **4°C**.
- Remove supernatant. Wash protein pellets by adding an additional 6 volumes of ice-cold ACS-grade acetone.
- Pellet precipitated proteins by centrifugation at 13.2 krpm for **10 min.** at **4°C**.
- Remove supernatant and briefly air-dry pellets at RT.
- Resuspend each pellet in 100 µL of **1% (w/v) SDS**.

❖ MW-based separation of WCL proteins by GELFrEE

- Add 8 µL of **1M DTT** to the resuspended proteins, followed by 12 µL of **Optima-grade H_2O**.
- Add 30 µL of **5X Tris-Acetate GELFrEE sample buffer**, for a final volume of 150 µL. Completely resuspend pellet.
- Boil resuspended proteins for **10 min.** at **95 °C**.
- Pellet any remaining debris by centrifugation at 13.2 krpm for **10 min.** at **RT**.

❖ **MW-based separation of WCL proteins by GELFrEE, continued**

- Load each sample into a single lane of a new **10% GELFrEE cartridge**.
- Collect twelve fractions of human WCL proteins resolved by MW according to the manufacturer's protocol.
- To visualize quality of protein separation across the collected fractions, collect 10 µL aliquots from each fraction and combine with an equivalent volume of **2x gel sample buffer** containing 10 mM **β-mercaptoethanol**. Boil each gel sample for 10 min. at **95 °C**. Collect samples by centrifugation at 13.2 krpm for **1 min.** at **RT**. Load all samples onto a **15-well BioRad AnyKD gel**, along with two or three lanes of **MW marker**, and resolve proteins to completion via SDS-PAGE. Visualize the constituent proteins within each of the collected fractions by silver nitrate staining. An example of a typical result is shown below:



400 µg of human whole-cell lysate was resolved on a 10% GELFrEE cartridge by the method described above. Shown is the silver-stained SDS-PAGE gel containing fully-resolved 10 µL aliquots from fractions 1-12. Note low degree of overlap from protein bands between fractions; this is an indication of optimal resolution between different MW ranges.

❖ **Methanol/Chloroform/Water Cleanup of GELFrEE Fractions**

- To each 150 µL GELFrEE fraction, add 600 µL (4 volumes) of **Optima-grade methanol**. Pipet vigorously to mix.
- Add 150 µL (1 volume) of **HPLC-grade chloroform**. Pipet vigorously to mix.
- Add 450 µL (3 volumes) of **Optima-grade water**. Pipet vigorously to mix. The resulting white color is due to the immiscibility of water in chloroform.
- Centrifuge at 13.2 krpm for **10 min.** at **RT**.
- There should be distinct layers visible within each fraction: an organic (bottom) layer, an aqueous (top) layer, and an interface. The precipitated proteins should be in a single pellet floating on the interface.
- Pipet off the top layer, leaving 10-20 µL to cover the pellet. Try to not disturb the pellet or organic layer.
- Add 450 µL (3 volumes) of Optima-grade methanol to the organic layer. Mix by gentle pipetting, taking care to not break up the protein pellet.
- Wash pellet by centrifugation at 13.2 krpm for **10 min.** at **RT**.
- Remove supernatant and air-dry pellet for **2 min.** at **RT**.
- Resuspend pellet in 40 µL of **Buffer A** (95% Optima-grade water, 5 % Optima-grade acetonitrile, 0.2% MS-grade formic acid). Pipet vigorously to ensure complete pellet homogenization.
- Centrifuge at 13.2 krpm for **10 min.** at **RT** to remove any remaining debris.
- Transfer supernatant to a clean Eppendorf LoBind tube or autosampler vial. The proteins are now ready for further dilution into Buffer A and analysis by qualitative top-down mass spectrometry.