**GENERATION OF LYSATES USING PULVERIZED SAMPLES**

1) Switch on the BIOBASE THERMO-SHAKER and adjust to 97.5 oC. Switch on the Eppendorf ThermoMixer C and adjust to 37 oC.

2) Decide how many samples to lyse; 24 per day is an optimal number. The remaining steps are optimized for the lysis of 24 samples.

3) In 25 ml of “Benzonase buffer”, add 2.5 l of Benzonase (Pierce #88700), two tablets of Protease inhibitor mini tablets (EDTA-free, Thermo #A32955), 25 l of 200 mM BeSO4, 250 l of 0.5 M NaF and let it in the rocker @RT until the tablets dissolve. Add 15.4 mg of DTT into the 25 ml of 2x Laemmli buffer.

4) **Use double gloves for this step.** Remove vials containing the samples from -80 oC. **The weight of each pulverized sample must be known in advance.** Tubes must be labeled as 1-24. Use the "Benzonase buffer estimator" excel sheet to record the weight of each sample; the excel sheet will auto-calculate the volume of buffer needed. Save the excel file according to the date of sample preparation.

5) **Use double gloves for this step.** Add the calculated volume of Benzonase buffer to the tubes containing the pulverized samples as dictated by the "Benzonase buffer estimator" excel sheet. **Do not add more than 800 l of Benzonase buffer per tube, even if the excel sheet calculates a higher value!!!**

6) Transfer tubes containing the samples to the Eppendorf ThermoMixer C and incubate at 37 oC for 5 min (RPM 500).

7) Spin the tubes containing the Benzonase-treated samples at 3,500 rpm for 2.5 min to remove foam and the liquid from the caps to prevent contamination by opening the tubes.

8) **Use double gloves for this step.** Add an equal volume of the 2x Laemmli buffer (consult the auto-calculated values from the Benzonase buffer estimator excel sheet). **Do not add more than 800 l of 2x Laemmli buffer per tube, even if the excel sheet calculates a higher value!!!**

9) Transfer tubes containing the lysed samples to the BIOBASE THERMO-SHAKER and incubate at 97.5 oC for 10 min (RPM 500). **Remove the tubes and wait for 2-3 min @RT before opening the lids.**

10) Transfer the lysates to 2 ml Eppendorf tubes labeled as 1-24. Try to avoid the lipid top fraction and any insoluble material. Spin the Eppendorf tubes at 12,700 rpm @RT for 10 min.

11) Transfer the middle fraction **(avoid ALL lipid fractions and pellets)** of each tube to single bar-coded cryovials labeled as 1-24.

12) Add 10 l of each lysate to 90 l of 1-to-1 mixture of Benzonase-Laemmli buffer. Mark these as 10-fold diluted lysates, 1-24.

13) Determine the protein concentration of each 10-fold diluted lysate using the Direct Detect. Use one Card per lysate (i.e. one spot for blank using the 1-to-1 Benzonase-Laemmli buffer and three spots for determining lysate protein concentration). Use protocol “NIST BSA AM1.q3” (*1601-1701 cm-1 wavenumber absorbance integration*). **If the concentration of the *undiluted* lysate is >21 mg/ml, dilute it two- or fourfold with Benzonase-Laemmli buffer and calculate the final concentration accordingly**. Register the calculated (average measured value multiplied 10 times according to the dilution) concentration in the "Benzonase buffer estimator" excel sheet.

14) Aliquot lysates in barcoded cryovials. Use at least 200 µl per cryovial.

15) Register all aliquots of lysates in LIBRA <http://db.rppa.hu>

16) Store the registered cryovials containing the lysates at their indicated position at -80 oC.

Laemmli 2x buffer (keep at RT, make fresh every three months)

pH= 6.8

SDS 4 %

Glycerol 20 %

Tris-HCl 120 mM

DTT 4 mM (to be added on the day of use)

5 mM EDTA

5 mM EGTA

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| Laemmli 2x buffer | Cat No# | M.W. | 50 ml | 100 ml | 200 ml | 500 ml |
| SDS (4%) | L3771 | 288.38 | 2 gr | 4 gr | 8 gr | 20 gr |
| Glycerol (20%) (weigh it) | G5516 | 92.09 | 8 ml | 16 ml | 32 ml | 80 ml |
| Trizma 120 mM | T1503 | 121.14 | 0.727 gr | 1.45 gr | 2.907 gr | 7.268 gr |
| DTT 4 mM | D9163 | 154.25 | 30.85 mg | 61.7 mg | 123.4 mg | 308.5 mg |
| EDTA 5 mM | E5134 | 372.24 | 93.06 mg | 186.1 mg | 372.24 mg | 930.6 mg |
| EGTA 5 mM |  | 380.35 | 95.1 mg | 190.17 mg | 380.35 mg | 951 mg |
| pH 6.8 (HCl) | - | - |  | - | - | - |

Benzonase buffer (keep @RT, sterile filter, make fresh every three months)

pH= 7.2

MgCl2 2 mM

20 mM Tris-HCl

Benzonase 0.1 l/ml of sample, ~1-2 mg/ml

Protease inhibitors (EDTA-free)

For phosphatases inhibition: 1000-fold dilution BeSO4 0.2 mM and 100-fold dilution NaF 5 M

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| Benzonase buffer Christos | Cat No# | M.W. | 50 ml | 100 ml | 200 ml | 500 ml |
| Trizma 20 mM | T1503 | 121.14 | 121.14 mg | 242.28 mg | 484.56 mg | 1.2114 gr |
| Benzonase 0.1 l/ml Pierce #88700 |  |  | 5 ul | 10 ul | 20 ul | 50 ul |
| MgCl 2 mM | M1028 | 1 M stock | 100 ul | 200 ul | 400 ul | 1 ml |
| Protease inhibitors (EDTA-free, Thermo #A32955) |  |  | tablets | tablets | tablets | tablets |
| pH 7.2 (HCl) | - | - |  | - | - | - |