Harvest/Labelling Protocol

# General:

* + Keep 12C and 13C treatments as similar as possible!
  + If one works over a day, keep in mind, that cells still grow, HeLas double in 16-22h!, so plan media changes before actual labelling start (4h, 24h) accordingly, and count cells at reasonable times
  + HEPES buffer: HEPES 5mm, NaCl 140mm, pH 7.4, for 500 mL: 4.1 g NaCl + 0.569 g
    - Make 13C or 12C Glucose or MG stocks in plain HEPES and dilute to concentrations in the Media;  
      wash buffer contains only the C-source used to label and MG if applicable
  + *Main Factors in accuracy are: cell count, pipetting of MeOH*
  + *Practice at least steps 11&12 beforehand, or have a test run with actual solutions and empty plates, to see if everything is set up accordingly*

# Preparation

1. 24h exchange the media (total renewal)
2. Put 50% with 1 µg/mL Cinnamic Acid in freezer (can also be done earlier)
3. 4h before experiment start exchange the media, leaving 2 mL of previous media on the plate (to keep some growth factors etc there)
4. From extra plate(s) before starting a block of experiments (or shortly thereafter), count living cells (Trypan blue). ***This is the basis for normalization during data analysis! Reproducibility between plates has to be checked at a previous time point***
5. **Check list before starting**
   1. Cool 50% Methanol with 1 µg/mL Cinnamic Acid
   2. Dry ice for Methanol
   3. Heat Media and wash buffer to 37 °C
   4. Prepare Falcons with 1 mL chloroform (1 per plate), pipet precisely
   5. Prepare Eppis for Media samples (2 per plate)
   6. Unpack Scrapers
   7. Ice for cell scraping
   8. Liquid Nitrogen
   9. Tissue to dry plates
   10. Make sure to have enough tips (especially 5 mL)
   11. Waste bucket
   12. Waste for tips/scrapers and harvested plates
   13. Open all bottles, but the falcon should only be opened shortly before sample is put in (by an assistant), so the chloroform doesn’t evaporate too much
   14. Remember pipette position

# Harvest

## 2 minute Labelling, with MG (for 12C-controls perform all steps but with 12C-Glucose instead of 13C-Glucose)

1. Add 12C-Glucose Media with 25 µm MG
2. Incubate 15’
3. Take sample of Media, flash freeze *to Measure MG concentration after 15’*
4. **Take 100 µL Media** sample into 1.5 mL eppi, freeze in liquid nitrogen *(for metabolomics)*
5. Remove Media completely
6. Quickly wash with preincubated HEPES containing 7 µm MG and no Glucose *(as you would in normal cell culture, you want to remove pre-existing nutrients etc)*
7. Add **5mL of preincubated HEPES with 7 µm MG and 13C Glucose** to the plate, *start time*
8. Incubate cells in the incubator for 2 minutes (maybe keep them on your palm, so they don’t cool off completely but at 37 °C)
9. Shortly before time ends, **take 100 µL labeled Media sample (HEPES)**, freeze in liquid nitrogen *(so we can measure excreted metabolites in the media later)*
10. remove the buffer, dry on paper tissue
11. Put plate on ice, immediately ***at end of labeling time*** add ***exactly*** **5mL of ice cold MeOH**
12. Scratch the cells quantitatively from the plate, resuspend (mix) with the 5 mL pipet and transfer 5mL into the prelabeled falcons, containing 1mL chloroform **(here adding and removal of the 5 mL are the critical step, *important for quantification!*)**
13. **Shake falcon vigurously**
14. freeze in liquid nitrogen
15. store at -80 °C
16. send to Berlin ☺

## 5 minute Labelling, with MG (for 12C-controls perform all steps but with 12C-Glucose instead of 13C-Glucose)

1. Add 12C-Glucose Media with 25 µm MG
2. Incubate 15’
3. Take sample of Media, flash freeze *to Measure MG concentration after 15’*
4. **Take 100 µL Media** sample into 1.5 mL eppi, freeze in liquid nitrogen *(for metabolomics)*
5. Remove Media completely
6. Quickly wash with preincubated HEPES containing 7 µm MG and no Glucose *(as you would in normal cell culture, you want to remove pre-existing nutrients etc)*
7. Add **5mL of preincubated Media with 7 µm MG and 13C Glucose** to the plate, *start time*
8. Incubate cells in the incubator for 5 minutes
9. Shortly before time ends, **take 100 µL labeled Media sample**, freeze in liquid nitrogen *(so we can measure excreted metabolites in the media later)*
10. Shortly before time is over,remove labeled Media, dry on paper tissue and add **5mL of labeled wash buffer**
11. remove the buffer, dry on paper tissue
12. Put plate on ice, immediately ***at end of labeling time*** add ***exactly*** **5mL of ice cold MeOH**
13. Scratch the cells quantitatively from the plate, resuspend (mix) with the 5 mL pipet and transfer 5mL into the prelabeled falcons, containing 1mL chloroform **(here adding and removal of the 5 mL are the critical step, *important for quantification!*)**
14. **Shake falcon vigurously**
15. freeze in liquid nitrogen
16. store at -80 °C
17. send to Berlin ☺

Also freeze and send 500 µL aliquots of 12C/13C Hepes and Media, 50% MeOH and Chloroform (so if need be we can check these solutions later)