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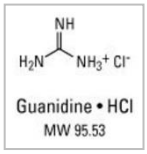
In-solution Protein Digestion with Guanidine-HCl and Trypsin Protease



Guanidine hydrochloride (Gnd-HCl) is a charged chaotropic agent and hydrogen bonds and hydrophilic interactions enabling proteins to unfold with all ionizable groups exposed to solution. Gdn-HCl does not cause obvious artifacts during high temperature (incubating samples in Gnd-HCl for up to one hour at 95 °C generally improved the overall number of protein identifications[*]). Be aware that Gnd-HCl is a trypsin inhibitor[**], and it will be necessary to reduce the concentration to about 1M. Long digestion times also increase the number of artefacts, mainly deamidation of asparagine and N-terminal glutamine cyclization of proteins/peptides. Solutions are stable over normal working pH ranges—i.e., pH 2.0 to 10.5[***]

[*] dx.doi.org/10.1021/pr300883y | J. Proteome Res. 2013, 12, 1020−1030 (Poulsen, et al. Using Guanidine-Hydrochloride for Fast and Efficient Protein Digestion and Single-step Affinity-purification Mass Spectrometry)
[**] doi.org/10.1016/j.ab.2009.05.018 (Da Ren, et al. An improved trypsin digestion method minimizes digestion-induced modifications on proteins)
[***] dx.doi.org/10.1002/0471140864.psa03as00| Curr Protoc Protein Sci. 2001 May ; APPENDIX 3: Appendix–3A (Wingfield, P.T. Use of Protein Folding Reagents)
[****] dx.doi.org/10.1021/pr500985w | J. Proteome Res. 2014, 13, 6187−6195 (Kelstrup, et al. Rapid and Deep Proteomes by Faster Sequencing on a Benchtop Quadrupole Ultra-High-Field Orbitrap Mass Spectrometer)

Sample denaturation and cysteine reduction/alkylation

Keep samples cold at all times (ice), this procedure applies for 25 µg proteins

Protein pellets: Add 100 µl boiling Gnd-HCl solution *(see right panel)* and boil for an additional 10 min.

Cells: Add 100µl boiling Gnd-HCl solution *(see right panel)*, and boil for an additional 10 min. Sonicate 3x 30s at 30% amplitude and 30 s rest between cycles.

Tissue: Add 10µl Gnd-HCl solution per mg tissue *(see right panel)* and depending on the tissue, sonicate first then boil or boil first the sonicate.

- Sonication: 3x 30s at 30-40% amplitude and 30 s rest between cycles.
- Boiling: 95°C 30-60 min.

Centrifuge sample at 4 ˚C at 13 000 rpm for 10 min, and transfer supernatant to a new Protein Lo-Bind tube. Proceed to measure the protein amount.

- *Pierce™ Coomassie Plus (Bradford) Assay Kit (Catalog number: 23236)*
- *Pierce™ BCA Protein Assay Kit - Reducing Agent Compatible (Catalog number: 23250)*

100mM TrisHCl pH 8.5: *Dissolve **6.057g Tris** (art. No. 252859, Sigma-Aldrich) in 400ml dH_2O. Correct the pH to 8.5 with HCl and adjust the volume to 500ml. Store the solution at 4 ℃.*

***Gnd-HCl solution:** 10ml 6M Gnd-HCl/100 mM Tris-HCl pH 8.5/5mM TCEP/10mM CAA:*

*Dissolve **5.732g Gnd-HCl** in 5.35 ml 100mM Tris-HCl pH 8.5 and add 100µl 0.5M TCEP and 200µl 0.5M CAA/100mM Tris-HCl pH 8.5*

***Note:** TCEP is acidic. Make sure TCEP stock solution have neutral pH or use the commercially available at pH 7 (P/N 646547-10x1ML, Sigma Aldrich)*

Sample dilution and digestion:

1. Digest the proteins with with Lys-C (FUJIFILM Wako Pure Chemical Corporation) in an enzyme/protein ratio of 1:100 (w/w) for 1 h at 37 ˚C
2. Add 25 mM Tris solution *(see right panel)* to reduce Gnd-HCl concentration to 1M (6-fold dilution)
3. Digest the proteins with with Trypsin *(see right panel)* in an enzyme/protein ratio of 1:50 (w/w) for 16 h at 37 ˚C (for 25µg protein, use 0.5µg Trypsin)
    - 25µg protein, use 0.1 µg/µl trypsin stock solution
    - 100µg protein, use 0.5 µg/µl trypsin stock solution

Note: If increased amounts of proteins need to be digested in case of PTM enrichment, use an enzyme/protein ratio of 1:200 and 1:100 for Lys-C and Trypsin respectively.

25 mM Tris/1mM $CaCl_2$:

*Add **1.1543g Tris** (art. No. 252859, Sigma-Aldrich) and **73.5mg $CaCl_2$ x $2H_2O$** (art. No. 21097, Sigma-Aldrich, stabilize trypsin) to about 400ml dH_2O. Correct the pH to 8.5 with HCl and adjust the volume to 500ml. Store the solution at 4℃.*

***0.1 µg/µl Trypsin Porcine** (Promega, art. no. V 5111);*

Dissolve each ampoule (20 µg trypsin porcine) in 200 µl 50 mM acetic acid (resuspension buffer supplied from Promega with the trypsin powder). The trypsin concentration in this stock solution is then 0.1 µg/µl

Acidification

In this final step when digestion have finished, acidify with 10% TFA (trifluoroacetic acid) to a 0.5% final concentration. Check on litmus paper that pH is 2-3. Add 0.1% TFA to dilute sample to 300 -500 µl. Proceed with desalting on OASIS C18, and dry in freezevac.