**Please cite this article as:** Satoshi and Kazutoshi, (2016). PNGase Sensitivity Assay to Study the Folding Status of Proteins, *Bio-protocol* 6 (19): e1952. DOI: 10.21769/BioProtoc.1952.

bio-protocol www.bio-protocol.org/e1952 Vol 6, Iss 19, Oct 05, 2016 DOI:10.21769/BioProtoc.1952

# PNGase Sensitivity Assay to Study the Folding Status of Proteins

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**[Abstract]** This protocol aims to evaluate folding status of proteins, utilizing peptide:N-glycanase (PNGase) sensitivity. In the cytosol, PNGase works as a deglycosylation-enzyme. N-glycans on unfolded/misfolded proteins are more susceptible to PNGase than N-glycans on folded proteins because of the preference of PNGase to non-native proteins. PNGase is endogenously expressed in various cell types, including HCT116 cells, DT40 cells and mouse embryonic fibroblast cells. Partial deglycosylation by PNGase can be detected by faster migration of band in SDS-PAGE. You can compare tightness of the folding among wild-type and mutant proteins of interest. This method can be used with regular molecular and cell biology equipment, but applied only to glycoproteins.

## Materials and Reagents

1. 6 well dish (Corning, Falcon®, catalog number: 353046) (Stored at room temperature)
2. PVDF membrane (GE Healthcare, catalog number: 10600023) (Stored at room temperature)
3. DT40 cell line (DT40 is a B cell line derived from an avian leukosis virus induced bursal lymphoma in a white leghorn chicken) (ATCC, catalog number: CRL-2111) (Endogenous expression of cytosolic PNGase)
4. Homo sapiens colon colorectal carcinoma cell line (HCT116) (ATCC, catalog number: CCL-247), HCT116 cells (ATCC, catalog number: CCL-247) (Endogenous expression of cytosolic PNGase)
5. Opti-mem (Thermo Fisher Scientific, Gibco™, catalog number: 31985-070)
6. Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen™, catalog number: 11668019)
7. Dulbecco's modified Eagle's medium (DMEM) (NACALAI TESQUE, catalog number: 08458-45) (Stored at 4 °C)
8. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10270-106)
9. 100 U/ml penicillin and 100 μg/ml streptomycin (NACALAI TESQUE) (Stored at -20 °C)
10. RPMI (NACALAI TESQUE, catalog number: 30263-95) (Stored at 4 °C)
11. Chicken serum (Thermo Fisher Scientific, Gibco™, catalog number: 16110-082)
12. 1 M dithiothreitol (DTT) (Wako Pure Chemical Industries, catalog number: 041-08976) (for reduction of proteins; in water; stored at -20 °C)
13. 20 mM carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (Z-VAD-fmk) (Promega, catalog number: G7231) (for inhibition of PNGase activity; stored at -20 °C)
14. Nonidet P-40 (NACALAI TESQUE, catalog number: 23640-94) (for HCT116; stored at room temperature)

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15. 4% digitonin (Wako Pure Chemical Industries, catalog number: 043-21371) (for DT40; in water; stored at -80 °C)
16. Anti-myc antibody (MEDICAL & BIOLOGICAL LABORATORIES, catalog number: 562) (Stored at -20 °C)
17. Anti-β-actin antibody conjugated with HRP (Wako Pure Chemical Industries, catalog number: 017-24573)
18. NaCl (Wako Pure Chemical Industries, catalog number: 191-01665)
19. $Na_2HPO_4$
20. KCl
21. $KH_2PO_4$
22. Tris/HCl, pH 8.0 (Sigma-Aldrich, catalog number: T6791) (Stored at room temperature)
23. Glycerol
24. Bromophenol blue (BPB)
25. Sodium dodecyl sulfate
26. Protease inhibitor cocktail (100x) (NACALAI TESQUE, catalog number: 25955-11) (for inhibition of various proteases' activity; in $ddH_2O$; stored at -20 °C)
27. 10 mM Z-Leu-Leu-Leu-CHO (MG132) (PEPTIDE INSTITUTE, catalog number: 3175-v) (for inhibition of proteasomal activity; in DMSO; stored at -20 °C)
28. Phosphate buffered saline (PBS) (see Recipes)
29. 2x sodium dodecyl sulfate (SDS) sample buffer (pH 6.8) (see Recipes)
30. Buffer A (Stored at 4 °C) (see Recipes)
31. Buffer B (see Recipes)

## Equipment

1. High speed refrigerated micro centrifuge (Tomy, model: MX-301)
2. mPAGE (ATTO, model: AE-6530) (Using hand-made 10% gel)
3. Transfer equipment (ATTO, model: WSE-4020)
4. Heat block (TAITEC, model: DTU-1BN)
5. Micro porator (Digital Bio, model: MP-100)

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## Software

1. ImageJ

## Procedure

1. Culture adherent HCT116 cells in Dulbecco's modified Eagle's medium (glucose 4.5 g/L) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a humidified 5% $CO_2$/95% air atmosphere. Culture suspended DT40 cells at a density of 1 x $10^5$-1 x $10^6$ cells per ml in RPMI1640 medium supplemented with 10% fetal bovine serum, 1% chicken serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 39.5 °C in a humidified 5% $CO_2$/95% air atmosphere.
2. Transfect 8.0 x $10^5$ HCT116 cells (40-60% confluent in 6-well plate) using 1 µg of plasmid DNA, 300 µl of Opti-mem and 10 µl of Lipofectamine 2000 (Invitrogen) for one well according to the manufacturers' instructions (After the transfection medium is not replaced). To obtain 1.05 x $10^7$ cells transfected cells, electroporate three times 3.5 x $10^6$ DT40 cells with 8 µg plasmid DNA with two pulses at 1,500 V for 15 msec according to the manufacturer's instructions.
3. Collect approximately 2.0 x $10^6$ HCT116 cells or 4.0 x $10^6$ DT40 cells for further analysis 24 h (HCT116 cell) or 16 h (DT40 cells) after transfection. For this the cells are washed with 1 ml PBS three times, scraped off, centrifuged at 800 *x g* for 2 min at 4 °C. After this step, samples from HCT116 cells and DT40 cells are treated in the same manner.
4. Remove supernatants of samples, suspend cell pellets in 200 µl buffer A and incubate for 20 min on ice to lyse the cells.
   *Note: The buffer A must NOT contain Z-VAD-fmk as it would inihibit PNGase activity.*
5. Clarify cell lysates by centrifugation at 17,800 *x g* for 10 min at 4 °C and transfer the supernatants to new tubes. Pellets are discarded.
6. To allow PNGase processing of misfolded proteins, incubate the cell lysates further for various periods (a good starting range would be 4 h) on ice. Remove 40-50 µl aliquots at different times, immediately mix with 40-50 µl of buffer B supplemented with 100 mM DTT (for reduction) and 2 µM Z-VAD-fmk (to inhibit the PNGase) and incubate at 100 °C for 5 min. Separate 10 µl of the samples by SDS-PAGE followed by immunoblotting using PVDF membrane and probing with an antibody against the protein of interest. Percentage of SDS-PAGE depends on the molecular weight of the protein of interest. Band intensities can be analyzed and compared to each other by ImageJ or similar programs (Figure 1).

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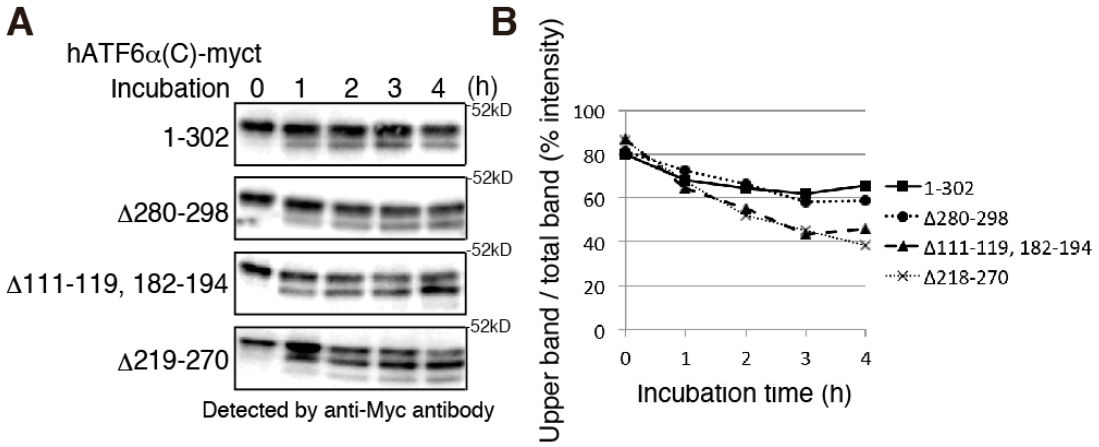


**Figure 1. Severely misfolded mutants were more sensitive to endogenous PNGase.** A. Chicken EDEM1/2/3 triple KO cells (gEDEM TKO) were described previously (Ninagawa *et al.*, 2015). The indicated myc-tagged hATF6α(C) variants (indicated on the left side of the blots) were transiently expressed in gEDEM-TKO cells and subjected to PNGase sensitivity assay. After the indicated time points the PNGase reaction was stopped and samples analyzed by a 12% SDS-PAGE followed by immunoblotting with antibodies against the myc-tag. The data shown represents a single representative experiment out of two repeats. Less folded proteins are more sensitive to PNGase, so partially deglycosylated bands were detected at earlier time points in hATF6α(C)-myct mutants, Δ111-119, 182-194 mutant and Δ219-270 mutant. See Ninagawa *et al.* (2015) for further proteins' information. B. The graph shows % intensity of upper band/total band of (A). The band intensity was analyzed by imageJ.

## Notes

1. Most cells including HCT116 cells, DT40 cells and mouse embryonic fibroblast cells express PNGase endogenously. Therefore it is not necessary to express PNGase exogenously. Other cell types have to be tested for PNGase expression. PNGase is also expressed in yeast, but we have not checked whether this assay is applicable to yeast cells.
2. This assay has been used to determine the folding state of several proteins, however, some glycoproteins are not sensitive to PNGase at all.
3. The use of strong detergents, such as SDS, is not appropriate for this method as PNGase activity can be lost. In our case, we used 1% NP-40 for mammalian cells and 1% digitonin for chicken cells. But you can use 1% NP-40 for chicken cells and 1% digitonin for mammalian cells. Not too strong detergent can be used for PNGase assay.
4. As control one can include two micromole of the PNGase inhibitor Z-vad-fmk.
5. You can normalize the sample by the concentration of proteins or cell number. Or you can use anti-β-actin antibody conjugated with HRP for SDS-PAGE to show the equal load of proteins.
6. We can provide plasmids to express hATF6α(C)-myct 1-302 and Δ280-298 for positive controls (Folded), and Δ111-119, 182-194 and Δ219-270 for negative controls (Less folded).

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7. This assay has been used for exogenously expressed proteins, but should also be applicable to endogenous proteins.
8. We recommend that when you analyze glycoproteins, you should include Z-VAD-fmk in lysis buffer in order to inhibit PNGase activity after cell lysis.
9. Recombinant PNGase is also available for PNGase sensitivity assay (Liu *et al*., 2016).

## Recipes

1. Phosphate buffered saline (PBS)
   137 mM NaCl
   8.1 mM $Na_2HPO_4$
   2.68 mM KCl
   1.47 mM $KH_2PO_4$
2. 2x sodium dodecyl sulfate (SDS) sample buffer (pH 6.8)
   100 mM Tris/HCl (pH 6.8)
   20% glycerol
   0.2% bromophenol blue (BPB)
   4% sodium dodecyl sulfate
3. Buffer A
   50 mM Tris/HCl (pH 8.0)
   1% NP-40 or 1% digitonin
   150 mM NaCl
   Protease inhibitor cocktail (Add before use)
   20 μM MG132 (Add before use)
   Stored at 4 °C
   *Note: 1% NP-40 is for mammalian cells such as HCT116 cells and 1% digitonin is for chicken cells such as DT40 cells.*
4. Buffer B
   1x SDS sample buffer (Stored at room temperature)
   100 mM dithiothreitol (Add before use)
   2 μM Z-VAD-fmk (Add before use)

## Acknowledgments

This protocol was adapted from and used in Ninagawa *et al*. (2015).

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## References

1. Liu, Y. C, Fujimori, D. G. and Weissman, J. S. (2016). Htm1p-Pdi1p is a folding-sensitive mannosidase that marks N-glycoproteins for ER-associated protein degradation. *Proc Natl Acad Sci U S A.* 113(28): E4015-4024.
2. Ninagawa, S., Okada, T., Sumitomo, Y., Horimoto, S., Sugimoto, T., Ishikawa, T., Takeda, S., Yamamoto, T., Suzuki, T., Kamiya, Y., Kato, K. and Mori, K. (2015). Forcible destruction of severely misfolded mammalian glycoproteins by the non-glycoprotein ERAD pathway. *J Cell Biol* 211(4): 775-784.