Production of NS3 helicase from Tick borne encephalitis virus.

Recombinant NS3H protein was produced in E. coli BL21-CodonPlus (DE3)-RIPL competent cells (Agilent Technologies) prior this course. Briefly, transformed competent cells were grown at 37°C in LB media supplemented with 100 µg/ml ampicillin and 35 µg/ml chloramphenicol until OD600 reached 0.5. The cells were chilled for 30 min at 4°C and the protein production was induced with 1 mM IPTG followed by incubation for 20 h at 18°C. The cells were harvested by centrifugation at 4,000 g for 30 min at 4°C and stored at -30°C prior to further use.

NS3H in pET19b

MGHHHHHHHHHHSSGHIDDDDKHMEKSRPNLPQAVVGTGWTSKGQITVLDMHPGSGKTHRVLPELIRQCIDRRLR
TLVLAPTRVVLKEMERALNGKRVRFHSPAVSDQQAGGAIVDVMCHATYVNRRLLPQGRQNWEVAIMDEAHWTDPH
SIAARGHLYTLAKENKCALVLMTATPPGKSEPFPESNGAITSEERQIPNGEWRDGFDWITEYEGRTAWFVPSIAKGGAIA
RTLRQKGKSVICLNSKTFEKDYSRVRDEKPDFVVTTDISEMGANLDVSRVIDGRTNIKPEEVDGKVELTGTRRVTTASAA
QRRGRVGRQDGRTDEYIYSGQCDDDDSGLVQWKEAQILLDNITTLRGPVATFYGPEQDKMPEVAGHFRLTEEKRKHF
RHLLTHCDFTPWLAWHVAANVSSVTDRSWTWEGPEANAVDEASGGLVTFRSPNGAERTLRPVWKDARMFKEGRDI
KEFVAYASGRR

Number of amino acids: 473

Molecular weight: 53339.97

Ext coef: 78295

Theoretical pI: 8.16

Literature: https://www.jbc.org/article/S0021-9258(22)00826-2/fulltext

Purification of recombinant NS3H:

Cell lysis:

Thaw cells on ice and suspend in 20 mL of Buffer A supplied with protease inhibitors (SIGMAFAST Protease Inhibitor Cocktail tablets, EDTA-free by Sigma-Aldrich) and DNAseI (10 µg/mL) (PanReac AppliChem).

Lyse cells using LM20 Microfluidizer® Processor (Microfludics) and incubated for 20 minutes on ice.

Clarify the cell lysate by ultracentrifugation at 4°C, 25 000 rpm for 1 hour.

Remove the supernatant and load on HisTrap column. Keep 100ul for SDS-PAGE analysis.

IMAC chromatography:

System preparation

Fit ÄKTA™ Pure M2 system (GE Healthcare) with HisTrapTM HP 5 mL column (Cytiva), wash with 3-4 column volumes (CV) of double-distilled and degassed H_2O.

Equilibrate the column with 3-4 CV of buffer A, 3-4 CV of elution buffer B and again 3-4 CV of buffer A

Sample loading

Load the sample using the sample pump with 2 mL/min flowrate.

Collect the flow through fraction upon rising of the UV signal. Take 100ul for SDS PAGE

Equilibrate the column with buffer A until the UV signal decrease to the baseline.

Perform soft-wash with 5% elution buffer B. (this wash away weakly bound proteins, collect peak fraction, use 100ul for SDS PAGE).

Elute recombinant NS3H with increasing gradient of elution buffer B for 20 min (5-100%). Monitor the UV signal and collect the peak in 3 mL fractions.

Take 100ul of each fraction for SDS PAGE.

(Measure the protein concentration on NanoDrop.)

Column maintenance:

Wash the column with 3-4CV of elution buffer B, 3-4 CV of buffer A and 3-4 CV of double-distilled and degassed H_2O. Finally wash the whole system with H_2O and 3-4CV of 20% EtOH.

SDS PAGE analysis:

Mix 100uL samples with 4xSample buffer supplemented with beta mercaptoethanol (100ul/900ul of 4x sample buffer) and boil for 5min at 95°C

HiTrap heparin purification:

Pool fractions containing recombinant NS3H together, and exchange the elution buffer B for buffer C (0.02 M sodium HEPES pH 7.0, 0.15 M NaCl) using Amicon Ultra-15 spin columns with 30 kDa cutoff (Merck). Take 100ul for SDS PAGE

System preparation

Fit ÄKTA™ Pure M2 system (GE Healthcare) with HiTrap Heparin HP 5 mL column (Cytiva), wash with 3-4 column volumes (CV) of double-distilled and degassed H_2O.

Equilibrate the column with 3-4 CV of buffer C, 3-4 CV of elution buffer D and again 3-4 CV of buffer C

Purification

Load the sample to the column and elute recombinant NS3H with increasing gradient of elution buffer D for 20 min (0-100%). Monitor the UV signal and collect the peak in 2 mL fractions.

Take 100ul of each fraction for SDS PAGE. Run the SDS PAGE as described above

(Measure the protein concentration on NanoDrop)

Column maintenance:

Wash the column with 3-4CV of elution buffer D, 3-4 CV of buffer C and 3-4 CV of double-distilled and degassed H_2O. Finally wash the whole system with H_2O and 3-4CV of 20% EtOH.

SDS PAGE analysis:

Mix 100uL samples with 4xSample buffer supplemented with beta mercaptoethanol (100ul/900ul of 4x sample buffer) and boil for 5min at 95°C

Size Exclusion Chromatography

System preparation

Polishing step will be done using Superdex200 increase 10/300 GL column (Cytiva) fitted to ÄKTA™ Pure M2 system (GE Healthcare). Wash the column by min 1-2CV of double-distilled and degassed H_2O. (start on Monday morning). Equilibrate the column with buffer C overnight.

The flow-rate has to be carefully checked and adjusted to max 0,75ml/min!

Purification

Concentrate the sample from Heparin column by Amicon Ultra-15 spin columns with 30 kDa cutoff.

Load the Superdex200 increase column and collect the peak into 0.5ml fractions.

Take 30ul for SDS PAGE, Prepare samples and run as described above

Measure the concentration via NanoDrop.

Number of amino acids: 473

Molecular weight: 53339.97

Ext coef: 78295

Theoretical pI: 8.16

Column maintenance:

Wash the column with 1 CV of buffer C and 1-2 CV of double-distilled and degassed H_2O. Finally wash the whole system with 1-2CV of 20% EtOH.

SDS PAGE analysis:

Mix 30uL of sample with 4xSample buffer supplemented with beta mercaptoethanol (100ul/900ul of 4x sample buffer) and boil for 5min at 95°C

Purification buffers:

Buffer A: 0.02 M sodium HEPES
0,5M NaCl
pH 7.0

Buffer B: 0.02 M sodium HEPES
0,5M NaCl
1M Immidazole
pH 7.0

Buffer C: 0.02 M sodium HEPES
0,15M NaCl
pH 7.0

Buffer D: 0.02 M sodium HEPES
2M NaCl
pH 7.0