# Native polyacrylamide gel electrophoresis (NAGE)

For any questions regarding this protocol, feel free to send an email to info@deep-dv.org

## Purpose

The native polyacrylamide gel electrophoresis (NAGE) can be used to investigate whether a protein forms multimeric structures (e.g. dimers). In this regard, for example, NAGE can be used to investigate the importance of certain amino acid positions for the formation of such multimeric structures by mutagenesis and comparison to the wildtype protein.

A denaturing SDS-PAGE should be performed in parallel to verify the expression of the protein. This should be done because any antibody that works well for denatured proteins may not work in NAGE because the epitope(s) may not be accessible.

Any gel caster, electrophoresis apparatus, blotting machine, and other equipment previously used for an SDS-PAGE must be thoroughly cleaned under running water with a brush to remove as much residual acrylamide and SDS as possible before being used for NAGE. Any residual acrylamide and SDS may interfere with the native structure of proteins.

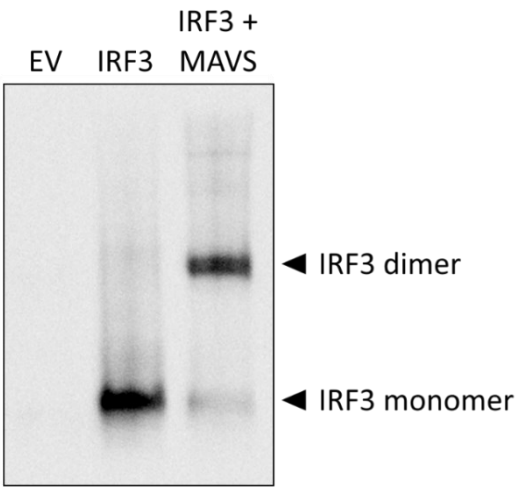
Markers/protein ladders are not used in NAGE because proteins are not solely separated according to their size but according to their folding, multimeric structure, and overall bulkiness. NAGE gel run time can be adjusted according to experience with the protein of interest from previous NAGEs.

Depending of the isoelectric point (pI) of the protein of interest, the pH values of the native running buffers may have to be adjusted. With buffers as described in this protocol, NAGEs have been performed successfully with proteins of calculated pIs of 5.17 (human IRF3), 5.92 (MCMV M35), and 6.07 (HCMV UL82/pp71).

## Example

NAGE performed with lysates of transfected HEK293-T cells. Cells were transfected with an empty vector (EV), interferon regulatory factor 3 (IRF3), or IRF3 together with the mitochondrial antiviral signalling protein (MAVS).

In a resting cell, IRF3 is present as a monomer. Upon stimulation with MAVS, IRF3 dimerises and translocates into the nucleus to activate type I IFN and ISG transcription.



# NAGE Protocol

## Protocol summary

The protein of interest will be transfected and overexpressed in HEK293-T cells and subjected to a NAGE and SDS-PAGE, followed by an immunoblot, to investigate its potential to form multimeric structures and to show its expression level, respectively.

## Seed cells

1. Seed 150.000 HEK293-T cells in 1 mL medium in a 24-well plate
2. Incubate at 37 °C and 5% $CO_2$ for ~16 h

## Transfection

*Here, FuGENE HD was used but the transfection may also be done with other transfection reagents.*

In each well, 500 ng DNA will be transfected in total. If two protein expression plasmids will be co-transfected, 250 ng each will be used. Apart from the co-transfection, 250 ng of these plasmids should also be transfected individually, alongside 250 ng of an appropriate empty vector control to reach a total transfection amount of 500 ng DNA.

The transfection can be prepared at room temperature (RT).

1. Evaluate the seeded cells; they should be adherent and evenly distributed
2. Dilute plasmids to 100 ng/µL (± 5 ng/µL)
    - › This ensures that similar amounts of plasmids will be transfected
3. Mix plasmids well by vortexing shortly, then spin down shortly
4. Mix 500 ng DNA (= 5 µL) with 30 µL OptiMEM
5. Mix well by vortexing shortly, then spin down shortly
6. Add 2 µL FuGENE HD
7. Mix 15 times by pipetting up and down
8. Incubate the DNA-FuGENE HD mixture for 15 minutes at RT
9. Add 30 µL DNA-FuGENE HD mixture dropwise to each well. Keep the pipet vertical, hovering about 1 cm above the medium surface (**do not go into the medium with the tip**), and add a drop of the mixture at different positions in the well to ensure a good distribution.
10. Carefully swirl to distribute the DNA-FuGENE HD mixture
11. Incubate for 20 h at 37 °C and 5% $CO_2$

## Preparation of NAGE and SDS-PAGE polyacrylamide gels and buffers

Gel and buffer recipes can be found in the appendix.

**Native gels must be prepared at least one day in advance** to allow complete polymerisation of the acrylamide. Free acrylamide may otherwise interfere with native protein structure. SDS-PAGE gels can be prepared on the day of the gel run. Once prepared, gels which won't be used immediately must be wrapped in a paper towel drenched in distilled water and stored in a plastic bag at 4 °C. Gels will remain usable for two weeks when stored like this. The pore size of the gels depends on the percentage of acrylamide within the gel. Here, a NAGE gel of 7.5% and an SDS gel of 10% are used which has been successfully used to show mono- and dimers of proteins whose monomers have a size of ~70 kDa.

The NAGE buffers (anode, cathode, and transfer buffer) as well as the SDS-PAGE transfer buffer must also be prepared at least a day in advance and stored at 4 °C so they will have enough time to sufficiently cool down. Gel and buffer recipes can be found at the end of the protocol.

### Harvest

Prepare cold (4 °C) 1x PBS.

1. **On ice**, prepare ~70 µL complete lysis buffer by adding 25x protease inhibitors to the cell lysis buffer (70 µL cell lysis buffer + 2.8 µL 25x protease inhibitors) per well
   - Complete lysis buffer should be prepared in excess because the cell lysis buffer is bubbly which can make pipetting difficult and because extra buffer is needed later to fill empty wells of the PAGE gels. For example, if there are 10 wells, complete lysis buffer for 15 wells should be prepared
   - **Prepare and keep the cell lysis buffer and complete lysis buffer on ice**
2. Per well, prepare one 1.5 mL Reaction tube and put it on ice to cool down
3. At 20 h post transfection, aspirate medium
4. Add cold 200 µL 1x PBS
5. Resuspend cells by pipetting up and down, washing the well in the process to resuspend as many cells as possible
   - HEK293-T cells easily detach from the plate, they can be seen as a white film that gets washed away by the 1x PBS
6. Transfer cell suspension to the prepared 1.5 mL reaction tube **on ice**
   - Handle and keep lysates always on ice
7. Pellet the cells by centrifugation at 16,000 g and 4 °C for 30 s
8. Carefully aspirate all PBS
9. Add 70 µL complete lysis buffer and resuspend the pellet by pipetting up and down
   - Keep resuspending until the pellet has been fully resuspended
10. Incubate on the rotating wheel at 4 °C for 60 min
    - This step ensures proper cell lysis
11. Centrifuge at 16,000 g and 4 °C for 10 min
    - This step pellets the cell debris
12. Transfer 55 µL supernatant into a new, cold 1.5 mL tube **on ice**
    - Make sure to not transfer the pelleted cell debris
13. Either directly continue with NAGE and/or SDS-PAGE or store the lysates at -20 °C

Gel electrophoresis

# NAGE method

The gel run must be set up and performed in a cold room since temperatures above 4 °C may disrupt protein-protein interactions.

1. Add the anode and cathode buffer to the respective compartment
2. Before sample loading, run the empty native gel at 40 mA (80 mA for two gels) for 30 min on ice/in the cold room with cold buffers.
    - **Make sure it really is only 30 minutes, or the gel and buffers will heat up substantially!** Use the timer setting on the power box if available. It is normal that the gel will become a little warm but that is no problem.
    - If you have a power box that can gate the voltage or current, turn up voltage full throttle to not gate the current. The voltage will balance itself out according to the buffer.
3. Replace cathode buffer from pre-run with new cathode buffer
4. If the lysate has been stored at -20 °C, thaw on ice
    - **Generally, lysates/samples should be kept on ice.** However, they may be taken out **briefly** for vortexing and spinning down. If they are handled at RT or warmer, protein-protein interactions may be destroyed.
5. Briefly vortex the lysate and spin down shortly, **put lysate back on ice**
6. Add 10 µL 2x native protein loading dye to a **cold** 1.5 reaction tube
7. Add 10 µL lysate, briefly vortex, and spin down shortly, **put back on ice**
8. Load 16 µL sample into each well of 12-well NAGE gel (which had been pre-run)
9. Run NAGE in the cold room:
    - Start at 6 mA per gel for 20-30 min (this is equal to 80 – 100 V – voltage will balance out by itself according to the buffer)
    - Afterwards, turn up to 10 mA per gel (this is equal to ~120 V)
10. When a particular protein is analysed via NAGE for the first time, stop the gel run once the blue dye front has run out of the gel
    - This depends on the protein of interest – the smaller the protein/complex, the faster it will run through the gel. So, the duration of the gel run can be adjusted once it is known how quickly the protein of interest runs through the gel

# SDS-PAGE method

1. If the lysate has been stored at -20 °C, thaw on ice
2. Briefly vortex the lysate and spin down shortly
3. Add 3 µL 4x SDS loading dye to a 1.5 reaction tube at RT
    - **Do not put on ice**, otherwise the SDS may precipitate
4. Add 9 µL lysate, briefly vortex, and spin down shortly
    - **Do not put on ice**, lysates can be handled at RT once mixed with the load

5. Load 11 µL sample into each well of a 12-well SDS-PAGE gel
    › Also load 5 µL marker/protein ladder into one well
6. Run SDS-PAGE at 60 V until samples have passed the stacking gel, then at 120 V, until the dye front has run out of the gel
    › When the SDS-PAGE run is stopped depends on the sizes of the proteins which should be detected. So, the duration of the run may be adjusted accordingly; for example, a longer run ensures a better separation of proteins which will be helpful if two or more proteins of similar size are to be detected.

### Western Blot

For any step involving washing or incubation, the membrane can be placed in a tray (membrane side with proteins facing upwards) or a 50 mL tube (membrane side with proteins facing inwards). Here, if indicated, washing or incubation steps will be performed while shaking or rolling, respectively.

## NAGE method

1. Soak the NAGE gel for 3 - 5 min in cold native transfer buffer
    › This allows the sodium deoxycholate to diffuse out of gel
2. Activate a polyvinylidene difluoride (PVDF) membrane by soaking it in methanol for 1 – 2 min before rinsing it with dd$H_2O$ and equilibrating it in cold native transfer buffer
    › A nitrocellulose membrane can also be used if preferred
3. Blot on PVDF membrane in a cold room (4 °C) at 350 mA for 1 h in cold native transfer buffer
4. Directly after transfer, incubate membrane in fixation solution in a closed container for 15 min while shaking/rolling
    › No prior washing step necessary
    › Handle fixation solution under the fume hood
5. Wash membrane three times with TBS-T, each for 5 minutes at RT while shaking/rolling
6. Block membrane in 5% BSA in TBS-T for 1 h at RT while shaking/rolling
7. Dilute primary antibody (protein of interest, housekeeping control, etc.) in 5% BSA in TBS-T and incubate overnight at 4 °C while shaking/rolling

## SDS-PAGE method

1. Soak the SDS-PAGE gel for 1 - 2 min in cold SDS transfer buffer for equilibration
2. Activate a polyvinylidene difluoride (PVDF) membrane by soaking it in methanol for 1 – 2 min before rinsing it with dd$H_2O$ and equilibrating it in cold native transfer buffer
    › A nitrocellulose membrane can also be used if preferred
3. Blot on PVDF membrane in a cold room (4 °C) at 350 mA for 1 h in cold native transfer buffer
4. Directly block membrane in 5% BSA in TBS-T for 1 h at RT while shaking/rolling
    › No prior washing step necessary

## Primary antibody incubation

### NAGE and SDS-PAGE membranes are handled identically from here on.

1. Dilute primary antibody (protein of interest, housekeeping control, etc.) in 5% BSA in TBS-T and incubate overnight at 4 °C while shaking/rolling

## Secondary antibody incubation

1. Wash membrane three times with TBS-T, each for 5 minutes at RT while shaking/rolling
2. Dilute secondary antibody in 5% BSA in TBS-T
    > Here, 1:10.000 dilutions were used but the dilution depends on the kind of secondary antibody used
3. Incubate at RT for 1 h while shaking/rolling
4. Wash membrane three times with TBS-T thrice, each for 5 minutes at RT while shaking/rolling
5. Develop using Lumilight ECL substrates

# RECIPES

## Gel recipes

The volumes that need to be pipetted are highlighted in blue. Each recipe makes two gels.

| | | | Separating gel (7.5%) | | Stacking gel (5%) | |
|---|---|---|---|---|---|---|
| NATIVE | | Stock | Composition | Volume | Composition | Volume |
| Acrylamide/bis-acrylamide (37.5:1) | | 30% | 7.5% | 3 mL | 5.0% | 670 µL |
| Tris-HCl | pH 8.8 | 1.5 M | 0.375 M | 3 mL | - | - |
| | pH 6.8 | 0.5 M | - | - | 0.125 M | 1 mL |
| APS | | 25% | 0.033% | 16 µL | 0.075% | 12 µL |
| TEMED | | 100% | 0.133% | 16 µL | 0.2% | 8 µL |
| $ddH_2O$ | | - | - | 5.97 mL | - | 2.31 mL |
| **Total [mL]** | | | **-** | **~ 12 mL** | **-** | **4 mL** |

| | | | Separating gel (10%) | | Stacking gel (5%) | |
|---|---|---|---|---|---|---|
| SDS | | Stock | Composition | Volume | Composition | Volume |
| Acrylamide/bis-acrylamide (37.5:1) | | 30% | 10% | 4 mL | 5.0% | 680 µL |
| Tris-HCl | pH8.8 | 1.5 M | 0.375 M | 3 mL | - | - |
| | pH6.8 | 0.5 M | - | - | 0.125 M | 1 mL |
| SDS in $ddH_2O$ | | 10% | ~0.01% | 12 µL | 0.01% | 40 µL |
| APS | | 25% | 0.033% | 16 µL | 0.075% | 12 µL |
| TEMED | | 100% | 0.133% | 16 µL | 0.2% | 8 µL |
| $ddH_2O$ | | - | - | 4.9 mL | - | 2.3 mL |
| **total [mL]** | | | **-** | **~ 12 mL** | **-** | **~ 4 mL** |

## Gel buffer recipes

**0.5 M Tris-HCl, pH 6.8**
6.1 g Tris-Base
Dissolve in 80 mL $ddH_2O$
Adjust pH to 6.8 with HCl
Adjust volume to 100 mL with $ddH_2O$
Autoclave

**1.5 M Tris-HCl, pH 8.8**
181.6 g Tris-Base
Dissolve in 800 mL $ddH_2O$
Adjust pH to 8.8 with HCl
Adjust volume to 1 L with $ddH_2O$
Autoclave

## Cell culture recipes

**HEK293-T culture medium**
500 mL DMEM
50 mL Fetal bovine serum
5 mL Penicillin/Streptomyin
Store at 4 °C, heat up to 37 °C before use.

**Cell lysis buffer**
20 mM Tris-HCl, pH 7.5
100 mM NaCl
1 mM EDTA
1% Trition X-100
0.1% SDS
0.5% Sodium deoxycholate
First, mix together Tris-HCl, EDTA and NaCl. Then add Triton X-100 and stir for 5 minutes. Add the sodium deoxycholate and stir for 5 minutes. In the end, add SDS and stir for 5 minutes before adding $ddH_2O$ to a final volume of 1 liter.
Store at 4 °C.

# NAGE buffer recipes

**2x native protein loading dye**
125 mM Tris-Cl, pH 6.8
60% Glycerol
0.2% bromophenol blue (0.02% to 0.2%) *

** Bromophenol blue is not soluble in pure water, but it does dissolve in the Tris-buffered solution. Thus, it is best to weigh in the required amount directly in the Tris-buffered solution. In the end, it does not seem to matter how much exactly is added, as long as the sample is visible with the dye.*

**10x NAGE running buffer**
30.3 g Tris-Base
144.1 g Glycine
Dissolve in 900 mL $ddH_2O$
pH 8.3 (adjusting is usually not necessary)
Adjust volume to 1000 mL with $ddH_2O$

**Native anode running buffer**
Add 100 mL 10x NAGE running buffer to 900 mL $ddH_2O$.
Store at 4°C, **must be cold before use!**

Native cathode running buffer
Add 100 mL 10x NAGE running buffer to 900 mL dd$H_2O$.
Add 0.2% sodium deoxycholate
Store at 4 °C, **must be cold before use!**

Native transfer buffer
Add 100 mL 10x NAGE running buffer to 900 mL dd$H_2O$.
Store at 4 °C, **must be cold before use!**

Fixation solution
40% Ethanol
7% Acetic acid
3% Glycerol
in dd$H_2O$
Can be recycle after use and stored for months at RT. Prepare and handle under fume hood because of the acetic acid.

## SDS-PAGE buffer recipes

4x SDS loading dye
0.25 M Tris-HCl, pH 6.8
40% Glycerol
0.04% Bromophenol blue
8% SDS
10% ß-Mercaptoethanol (ß-ME)
in dd$H_2O$
Prepare without the ß-ME and store in aliquots of 900 µL at -20 °C. When needed, thaw an aliquot and add 100 µL ß-ME (this brings the loading dye to 10% ß-ME) before use.

SDS running buffer:
25 mM Tris-base
192 mM glycine
0.1% SDS
in dd$H_2O$, pH 8.3
Prepare 2 L from 10x.
Store at RT until use.

SDS transfer buffer:
25 mM Tris-base
192 mM glycine
0.05% SDS
20% Methanol
in dd$H_2O$, pH 8.3
Store at 4 °C, **must be cold before use!** Can be reused twice.

## Miscellaneous buffers

**Tris-buffered saline with Tween 20 (TBS-T):**

2.4 g Tris
8.7 g NaCl
1 mL Tween 20
Dissolve in 900 mL $ddH_2O$
Adjust pH to 8.0 with HCl
Adjust volume to 1000 mL with $ddH_2O$
Sterile filtration