User Guide

# Experimental Procedures

## to Evaluate the Viscosity Reduction Platform

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The following protocol describes a principle procedure to prepare a low volume of a highly concentrated protein formulation to allow for viscosity measurements by a small volume viscometer. Depending on your viscometer as well as on the shear rates and iterations you intend to measure, a larger volume than described herein may be necessary. All described working steps are recommendations based on our knowledge in preparing high-concentration protein formulations, alternative procedures may be applicable.

## 1. Materials and Devices

### Materials

- Viscosity Reduction Platform Test kit*: If you have not ordered the kit please reach out to your local sales representative or feel free to order the chemicals from our e-commerce platform **sigmaaldrich.com** using the article numbers from table 1.

* Not all substances in the kit are currently available in GMP quality, this kit is not intended for pharmaceutical use.

**Table 1:** Article numbers of the Viscosity Reduction Platform substances.

| Material | Catalogue No. |
|---|---|
| L-Ornithine monohydrochloride EMPROVE® EXPERT DAB | 137118 |
| L-Phenylalanine EMPROVE® EXPERT Ph Eur, USP | 107267 |
| Benzenesulfonic acid EMPROVE® EXPERT | 103893 |
| Pyridoxine hydrochloride ≥98% (HPLC) | P9755 |
| Thiamine phosphoric acid ester chloride dihydrate EMPROVE® API | 500667 |

- 2x Basis/formulation buffer (that is standardly used for formulating your protein of interest)
- Acid and base (e.g. hydrochloric acid and sodium hydroxide solution) for pH adjustment
- Amicon® Ultra-4 Centrifugal Filter Units, PLTK Utracell-PL mebrane
  - Molecular weight cut-off (MWCO) of filters should be approx. 5 times smaller than the molecular weight of the target protein; e.g. for a 145 kDa antibody: 30 kDa MWCO (Cat. No.: UFC803008)
- Positive displacement pipettes (range: 10–250 µL)
- 2 mL microtubes
- Ultrapure water or water for injection (WFI)

### Optional

- Protein quantification assay, e.g. Bradford assay
- 96-well clear, flat bottom plates
- 50 mL conical tube

### Devices

- Centrifuge with inserts for 15 mL tubes (optional: insert for 50 mL tubes)
- pH meter
- Magnetic stirrer plates
- Photometer/spectrometer (optional: plate reader)
- Small volume viscometer
- Thermoblock (mounts for plates & tubes)

## 2. Viscosity Reducing Buffer Preparation (Exemplary for 100 mL):

1. Prepare 1 L of 2x basis/formulation buffer.

2. Dissolve viscosity reducing substances (see table 3 for weight) in 50 mL 2x formulation buffer and 40 mL ultrapure water using a 100 mL beaker and magnetic stirrer.
   - Based on your preferences, you could also alter the substance concentrations using their molecular weight provided in table 2.
   - Benzenesulfonic acid is highly hygroscopic. Keep the container open only as long as absolutely necessary.
   - If you are working in a very humid environment, consider using desiccant sachets for storage.
   - Phenylalanine takes some time to dissolve. If required, add additional ultrapure water.

3. Measure and adjust pH (using e.g. HCl and NaOH) to your respective formulation value.
   - Almost all substances will alter the pH.
   - Benzenesulfonic acid is a strong acid that might result in pH values of 1–2. Consider using a concentrated base (e.g. NaOH, 30%) to adjust pH.

4. Fill with ultrapure water to 100 mL using a volumetric flask.

5. Check pH again, adjust if necessary.

6. A control (formulation buffer, sample no. 1) is also recommended to be tested within the same data set.

**Table 2:** Abbreviations and respective molecular weight of the Viscosity Reduction Platform substances.

| Material | Abbreviation | Molecular weight [g/mol] |
|---|---|---|
| L-Ornithine monohydrochloride | OM | 168.82 |
| L-Phenylalanine | PHE | 165.19 |
| Benzenesulfonic acid | BSS | 158.18 |
| Pyridoxine hydrochloride | PYR | 205.64 |
| Thiamine phosphoric acid ester chloride dihydrate | TMP | 416.82 |

**Table 3:** Preparation of viscosity reducing buffers.

| No. | Substance 1 (cationic or uncharged) | Substance 2 (anionic or uncharged) | Weight Substance 1 [g/100 mL] | Weight Substance 2 [g/100 mL] | Dissolution | Filling |
|---|---|---|---|---|---|---|
| 1 | - | - | - | - | add 50 mL 2x buffer as well as 40 mL ultrapure water and dissolve | adjust pH and fill to 100 mL with ultrapure water |
| 2 | 150 mM OM | - | 2.53 | - | | |
| 3 | 150 mM PHE | - | 2.48 | - | | |
| 4 | - | - | - | 3.48 | | |
| 5 | - | 150 mM BSS | - | 2.37 | | |
| 6 | - | 150 mM PYR | - | 3.08 | | |
| 7 | - | 150 mM TMP | - | 6.25 | | |
| 8 | - | - | - | - | | |
| 9 | 75 mM OM | 75 mM BSS | 1.26 | 1.19 | | |
| 10 | 75 mM OM | 75 mM PYR | 1.26 | 1.54 | | |
| 11 | 75 mM OM | 75 mM TMP | 1.26 | 3.13 | | |
| 12 | - | - | - | - | | |
| 13 | 75 mM PHE | 75 mM BSS | 1.24 | 1.19 | | |
| 14 | 75 mM PHE | 75 mM PYR | 1.24 | 1.54 | | |
| 15 | 75 mM PHE | 75 mM TMP | 1.24 | 3.13 | | |

## 3. Buffer Exchange & Volume Reduction

Amicon® Ultra Centrifugal Filters are recommended to be used for efficient buffer exchange and reaching high protein concentrations.

1. Identify the correct MWCO for your protein that is approximately 5-times smaller than its molecular weight.
   - — E.g. for an antibody with approx. 145 kDa, use a 30 kDa MWCO.
   - — Use one Amicon® Ultra-4 Centrifugal Filter Unit with the respective MWCO per sample.
2. Rinse filter once with ultrapure water or basis buffer to remove potential impurities.
3. Add 4 mL ultrapure water or formulation buffer and centrifuge 2 minutes at 2000x g.
4. Discard permeate and remaining solvent from filter.
5. Calculate volume of protein solution needed to reach your selected target concentration.
   - — 250 µL of concentrated solution should be prepared.
   - — Add enough volume assuming a **filtration loss of 20%**.
6. Pipette protein amount in the Amicon® Ultra Centrifugal Filter.
   - — If more than 4.2 mL are needed, add sequentially by centrifuging 20 minutes at 2000x g between steps.
7. Repeat steps 7.1–7.3 at least **5 times** to exchange buffer (exchange minimum of 5 diavolumes).
   - 7.1. Centrifuge for 20–45 minutes at **2000x g** to reduce the volume to half
     - — **Do not use 4000x g** as specified in the Amicon® Ultra Centrifugal Filter manual as this may result in higher protein loss and may increase risk of gel formation.
   - 7.2. Discard permeate and fill again with target buffer containing the respective viscosity reducing buffers.
   - 7.3. Homogenize either by inversion of the tubes or using pipettes (carefully and without stumbling against filter membrane).
8. After last buffer addition, centrifuge until the 250 µL mark is reached by the meniscus.
   - — Homogenize every 20–40 minutes to avoid gelation of the protein at the bottom of the centrifugal filters.
   - — Total centrifugation time needed is highly variable for each protein and testing condition.
9. Homogenize and pipette each concentrate in a 2 mL microtube using **positive displacement pipettes**.
   - — **Do not use air displacement pipettes.**
10. After transfer, centrifuge the Amicon® Ultra Centrifugal Filter again for 2 minutes to collect remaining concentrate that was sticking to the filter membrane.
    - — Alternatively: invert the filter into a 50 mL tube and centrifuge at 1000x g for 2 minutes, higher protein loss might be possible.
11. Mix the protein concentrate gently but **thoroughly one more time to ensure homogeneity**.
    - — Visually check if there is no phase separation anymore.
    - — Try to avoid pipetting bubbles into your sample. These are hard to eliminate especially in very viscous samples.
    - — If there are bubbles, centrifuge the concentrate at 2000x g to remove those.

## 4. Concentration Measurement & Adjustment

The following steps are highly dependent on your protein, the concentration and the absorption spectrometer used. **The use of positive displacement pipettes is crucial with viscous liquids as use of air displacement pipettes will result in high pipetting errors.**

PYR and TMP absorb at 280 nm even at high dilutions. Thus, an alternative method than the one described below, such as HPLC or protein assay (e.g. according to Bradford or Lowry) is required to determine protein concentration.

### Dilution

Dilute samples to a suitable range to measure protein concentration.

- For analysis with absorption spectroscopy using a cuvette with a 1 cm path length, Bradford assay or HPLC usually 0.5–1.0 mg/mL is suitable.
- By using a shorter optical pathlength, a higher concentration can also be used.
- Use a positive displacement pipette to add 10 µL of your sample to the diluent (ultrapure water or 1x formulation buffer) to reach 0.5–1.0 mg/mL (e.g. for a 200 mg/mL protein concentrate a dilution factor of 300 is suitable given by adding 10 µL sample to 2990 µL diluent).
- Do not use viscosity reducing buffers! The substances may interfere with measurement, e.g. some substances absorb light at 280 nm.

1. Stir the concentrate with your pipette tip prior aspiration to check for potential inhomogeneities.
2. Visually observe that there are absolutely no bubbles in the pipette tip!
3. Clean outside of the tip with a lint-free tissue after aspiration to remove droplets.
   - Viscous solutions tend to stick to the outer wall of the pipette tip. These droplets will add to your dilution resulting in falsely too high concentration measurement.
   - Do not touch the nib of the tip!
4. Add the 10 µL protein concentrate to your diluent and mix thoroughly by pipetting and shaking, if possible vortex for several seconds.

### Protein concentration measurement

1. If PYR or TMP are present in your samples, perform a protein assay according to instructions with a standard prepared of your respective protein (or use conversion factor when calibrating with bovine serum albumin (BSA) or bovine gamma globulin (BGG)).
   - Your protein standard may be prepared using absorption measurements at 280 nm and the respective coefficient.
2. If the above-mentioned substances are not included in experiments, concentration can be measured using the corresponding extinction coefficient at 280 nm and a absorption spectrometer.

### Concentration adjustment

1. Adjust the protein concentration to its target with the corresponding viscosity reducing buffer using positive displacement pipettes.
   - The most accurate method is to withdraw a certain amount of concentrate to a new 2 mL microtube, adding the respective volume of viscosity reducing buffer (diluent) and mixing by pipetting.
   - Do not assume the volume to be 250 µL. The probability of error is high.
2. Measure concentration as described above to verify correct dilution and assess concentration range of your sample set (e.g. 150 mg/mL ±2.1%).

## 5. Viscosity Measurement Using m-VROC® Viscometer

For viscosity measurements using a m-VROC® Viscometer, approximately 100–150 µL will be needed. The following instruction is just a recommendation for a single shear rate measurement using a m-VROC® Viscometer.

1. Centrifuge your samples for 2 minutes at 2000x g to remove any bubbles formed while resuspending your samples.
   - Bubbles may result in a spontaneous pressure drop or increase in the VROC-chip strongly disturbing the measurement.

2. Put your samples in a thermoblock for pre-equilibration to your temperature preference.

3. Choose the correct chip for the estimated viscosity range of your samples.
   - At intermediate shear rates (1000–5000), the C05-chip (c-chip with 50 µm flow channel) has a broad measurement window.

4. Start software, adjust thermostat temperature and start rinsing the chip with ultrapure water until a viscosity of approx. 1 cP is reported.

5. Rinse with one syringe of your formulation/basis buffer without viscosity reducing substances.

6. In the beginning of each measurement, a priming step is required to fill the chip with your sample.
   - Duration of that step is dependent on the selected shear rate but also viscosity; 24 seconds is suitable for mid to high viscosities (approx. 100 mPas).
   - If you expect higher viscosities, extend the priming step by a few seconds.
   - For higher viscosity values than 160 cP, increase priming time to at least 30 s at 2000 $s^{-1}$ and 40 s at 1000 $s^{-1}$.

7. For measurement setup, refer to the table with typical settings.
   - A constant shear rate of 3000 $s^{-1}$ is a good starting point with the C05-chip since viscosities from 10 to 160 cP can be measured accurately.
   - For higher viscosity values than 160 cP, the shear rate needs to be reduced to 2000 or even 1000.

8. The following measurement setup can be used:

| Measurement | Shear rate [$s^{-1}$] | Measurement Time [s] | Pause time [s] |
|---|---|---|---|
| Priming | 3000 | 24 | 1 |
| 1. | 3000 | 3 | 1 |
| 2. | 3000 | 3 | 1 |
| 3. | 3000 | 3 | 1 |
| 4. | 3000 | 3 | 1 |

**Note:** When analyzing shear-thinning/thickening behavior at different shear rates, increase pause time to several seconds to allow adaptation to new shear level.

## 6. Interpretation of Results

Based on the screening conducted according to previous steps and table 3, more than one viscosity reducing buffer might have resulted in a strong viscosity reduction. The viscosity can be optimized further by adjusting the substance ratios, ideally in form of a DoE, where also other formulation components can be considered (e.g. sugars or surfactants).

For decision making, further experiments might be helpful that could include:

1. Fragmentation analysis
   - — Thermal unfolding mid-point/aggregation onset analysis
   - — Short-term stability study
   - — Monomer content after concentration process

2. Viscosity profile generation
   - — Generate a viscosity profile over a relevant protein concentration range (e.g. 100–250 mg/mL)

Besides, the most suitable substance combination might be optimizable by altering the mixing ratio. For example, a proportion of 2:1 or 1:2 could be investigated by previously mentioned methods and result in optimized:

- Viscosity
- Stability
- Osmolality

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