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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**.

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

Material	Cat. No.
L-Ornithine monohydrochloride EMPROVE® EXPERT DAB	**137118**
L-Phenylalanine EMPROVE® EXPERT Ph Eur, USP	**107267**
L-Arginine EMPROVE® EXPERT Ph Eur, ChP, JP, USP	**101587**
Benzenesulfonic acid EMPROVE® EXPERT	**103893**
Pyridoxine hydrochloride EMPROVE® EXPERT Ph Eur, BP, JP, USP, FCC	**137156**
Thiamine phosphoric acid ester chloride dihydrate EMPROVE® API	**500667**

- 2x concentrated 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: 10–500 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):

Important: It is essential to prepare the buffers as described herein, spiking the excipients in very high concentrations to a protein containing solution in the final buffer is not recommended. Ideal concentrations for VRP excipients are **75 mM for combinations** and **150 mM for single excipients**, whereas **Phenylalanine is soluble at 125 mM as a single excipient**.

1. Prepare 1 L of 2x concentrated basis/formulation buffer.
2. Dissolve viscosity reducing substances (see **table 3** for weight) in 50 mL 2x concentrated basis/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. Note that it is dependent on the properties of base buffer (Tris > Phosphate, Acetate, Histidine >> Citrate), please check after 2-3 hours. If it is not dissolved add more water (e.g. add 2 mL water to 90 mL of excipient containing buffer to yield 92 mL of total volume. If it is not dissolved after 1-2 hours, add another 2-3 mL to give rise to 95 mL of total volume). Make sure that you have below 100 mL of total volume prior to pH adjustment.
   - Thiamine phosphoric acid ester chloride dihydrate (TMP) is highly pH dependent (pH 3-6 optimal range), thus it might require pH adjustment to fully dissolve, especially at higher concentrations. Consider using 1M NaOH with few drops. If it does not dissolve
     a. add slowly a few drops (3-5) of 30% NaOH and measure the pH
     b. if it is not dissolved, add a few more drops and measure the pH.

     Repeat steps **a&b** until it is dissolved. Make sure that you have below 100 mL of total volume prior to pH adjustment. Check pH in the next step (**step 3**) and adjust to final pH in the final volume. **If you have a base formulation, which exceeds the optimal pH range for TMP (usually between pH 3-6), it is recommended to not include TMP in the platform screening.**
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. 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	Orn	168.82
L-Phenylalanine	Phe	165.19
L-Arginine	Arg	174.20
Benzenesulfonic acid	BSacid	158.18
Pyridoxine hydrochloride	Pyr	205.64
Thiamine phosphoric acid ester chloride dihydrate	TMPacid	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 Orn	-	2.53	-		
3	125 mM Phe	-	2.07	-		
4	150 mM Arg	-	2.61	-		
5	-	150 mM BSacid	-	2.37		
6	-	150 mM Pyr	-	3.08		
7	-	150 mM TMPacid	-	6.25		
8	75 mM Orn	75 mM BSacid	1.26	1.19		
9	75 mM Orn	75 mM Pyr	1.26	1.54		
10	75 mM Orn	75 mM TMPacid	1.26	3.13		
11	75 mM Phe	75 mM BSacid	1.24	1.19		
12	75 mM Phe	75 mM Pyr	1.24	1.54		
13	75 mM Phe	75 mM TMPacid	1.24	3.13		
14	75 mM Arg	75 mM BSacid	1.31	1.19		
15	75 mM Arg	75 mM Pyr	1.31	1.54		
16	75 mM Arg	75 mM TMPacid	1.31	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 trace amounts of glycerin.
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.
   - — 350 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 approximately 350 µL are 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 5 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 TMPacid 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 TMPacid 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.
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 200 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. Pre-fill the measurement chamber of the chip by pumping the sample at a shear rate of 3000 s^{-1} for 15 seconds.

7. 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 cP).
   - — 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}.

8. 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 s^{-1} or even 1000 s^{-1}.

9. The following measurement setup can be used:

Measurement	Shear rate [s^{-1}]	Measurement Time [s]	Pause time [s]
Priming	3000	20	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.

10. Rinse the chip using the formulation/basis buffer and the above mentioned setup before measurement of the next sample.

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

After final selection of the most beneficial viscosity-reducing buffer, the impact on syringeability regarding aspiration time and extraction force can be assessed at the target concentration. For further reading on the benefits of the Viscosity Reduction Platform in formulation, please see this white paper **here**.

Also, the benefit on large-scale protein concentration during drug manufacturing by tangential flow filtration (TFF) can be assessed. For further reading on the use of viscosity-reducing excipients during TFF, please see this white paper **here**.

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