PROTOCOL

AFA-Nanocrystal™ Production

Batch-scale (2mL) Felodipine Preparation Protocol

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INTENDED USE

This protocol is for the preparation of felodipine API nanocrystals on a 2mL (24.6mg) scale.

SUMMARY OF OPERATING CONDITIONS

AFA Instrument	S220x
Peak Incident Power	150 W
Duty Factor	50%
Cycles per Burst	1000
Duration	1200 seconds
Bath Temperature	10°C
Power Mode	Frequency sweeping
Degassing mode	Continuous
Volume	2mL (12x24 vessel)

Table 1: Operating Conditions

Recommended settings are subject to change without notice.

See http://covarisinc.com/resources/protocols/ for updates to this document.

SUPPLIES

Item	Description	Part Number
Focused-Ultrasonicator™	S220x with Computer and Software	S220x
Sample Vessel	Tube and Cap 12x24mm	520056
Sample Holder	Holder 12x24 Tube	500199
Sample drug	Felodipine	Sigma-Aldrich – F9677
Solvent	1-Methyl-2-pyrrolidone (99.5%, anhydrous)	Sigma-Aldrich – 328634
Stabilizer	PVP30 (polyvinylpyrrolidone) Sodium Lauryl Sulfate (SLS)	BASF Sigma-Aldrich

Table 2: Supplies

ADDITIONAL MATERIALS (SUPPLIED BY USER):

- Particle sizer and software (E.g. Malvern Mastersizer3000)
- Variable pipette and tips: 200µL and 1000µL

Values mentioned in this Quick Guide are nominal values. The tolerances are as follows:

- Temperature +/-2°C

RISK AND SAFETY INFORMATION

The following protocol uses compounds and organic solvents that according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) is considered hazardous. In the manufacturer's experience, they have no harmful effects when used and handled according to instructions.

The MSDS for the chemicals are available from the suppliers.

OPERATING CONDITIONS

1. Fill the tank with fresh deionized water to the fill line marked 15. The S220x should be equipped with a graduated water level label. If the tank lacks this label, please contact Covaris. During treatments, the tube should be partially immersed in the water to ensure a good acoustic path from the AFA transducer.
2. Degas water for the recommended 30 minutes or more. To maintain degassed water, keep the pump continuously on during operation and sample processing. Do not turn the pump off.
3. Set the chiller to the proper temperature, as listed for "Bath Temperature" in Table 1.

RECOMMENDATIONS SPECIFIC FOR NANOSUSPENSION FORMATION

The Covaris AFA process is highly reproducible, however steps should be taken to ensure the best results. The bath water is employed to couple acoustic energy to the sample vessel, thus attention must be paid to the following water treatment attributes to obtain the best results:

1. Purity: When applying acoustics in rate-limited applications, foreign materials such as algae and particulates may scatter the high frequency focused acoustic beam. Bath water should be pure distilled or DI water, changed daily or cleansed by a Covaris Water Conditioning System.
2. Degas Level: Similarly, insufficient degas levels within the bath may result in poor acoustic coupling. System degas pumps should be run in advance of and during AFA treatments, as detailed in instrument User Manuals.
3. Temperature: Warmer temperatures promote less forceful collapse of acoustic cavities within the sample fluid. Bath temperature (as reported by SonoLAB software) should therefore be closely controlled and matched run-to-run and day-to-day. Employ the temperature alert feature in SonoLAB to warn of a failure to maintain control of bath temperature.
4. Level: Attention should be paid to maintaining a consistent water level, according to published protocols. If using a Covaris Water Conditioning System, check levels daily to restore water lost to evaporation.

In summary, when employing the Covaris AFA, control and verification of treatment attributes and water quality will reduce variance and promote consistent, satisfactory results.

PROTOCOL

1. Prepare a 500mM stock solution of felodipine. To make 5mL of the stock, dissolve 960.6mg of felodipine in 5mL of N-methylpyrrolidone.
2. Prepare a 1.0%PVP30, 1.25mM SLS antisolvent stock solution. To make 100mL of the stock, dissolve 1200mg of PVP30 (Kollidon30) and 36.05mg of SLS in 100mL of deionized water.
3. Set up the Covaris S220x at the appropriate temperature following the operating conditions above.
4. Add 1800 µL of the vehicle (1.0%PVP30 and 1.25mMSLS) to the sample vessel.
5. Add 128 µL of the 500 mM stock solution of felodipine in N-methylpyrrolidone to the sample vessel and cap the tube. This will result in a 14.1:1 ratio of the vehicle to the drug solution (i.e. the drug concentration will be 33.1mM). Be careful not to introduce bubbles into the tube, which may happen due to incorrect pipetting. CAUTION: The bottom of the tube is in the acoustic field. Therefore, a bubble in the sample will deflect energy and induce variable results.
6. Quickly but carefully load the sample vessel into the appropriate holder, and insert the holder into the S220x instrument.
7. Initiate and Run process according to the operating conditions specified in Table 1.
8. Particle size measurements are immediately and 24 hours after the particles were prepared (to allow for time for equilibration to a stable particle size).

Note: Once the solvent and antisolvent have been combined, crystallization will start immediately. Without AFA treatment, crystals will grow in an uncontrolled manner. As such, load the sample and start the AFA promptly after combining the solvent and antisolvent.

DETERMINATION OF PARTICLE SIZE

A Malvern Mastersizer 3000 or a Malvern Zetasizer ZS-90 may be used to analyze the nanosuspension. The method for the Mastersizer 3000 is as follows.

1. Set up the Malvern Mastersizer according to its setup instructions.
2. Add 0.5mL of the processed sample to the machine, after aligning and measuring the background. Some dissolution may occur; add sample until the transmission is within the obscuration range.
3. Run the instrument and process the data according to the Mastersizer instructions. An average record may be created for the three readings taken.

The method for the Zetasizer is as follows.

1. Set up the Malvern Zetasizer according to its setup instructions.
2. Add 50µL of processed sample, and 950µL of deionized water to the cuvette. Mix carefully with the pipette tip without introducing air bubbles.
3. Place in Zetasizer instrument and run analysis.

Typical output readings (Mastersizer)

	D10[µm]	D50[µm]	D90[µm]	Span
1	0.178	0.322	0.541	1.13
2	0.17	0.311	0.531	1.162
3	0.17	0.31	0.525	1.146
Average	0.172666667	0.314333	0.532333	1.146
SD	0.004618802	0.006658	0.008083	0.016
CV	0.026749819	0.021182	0.015184	0.013962

Table 3: Variation in results of replicates, taken 24 hours after the particles were prepared (to allow for time for equilibration to a stable particle size)

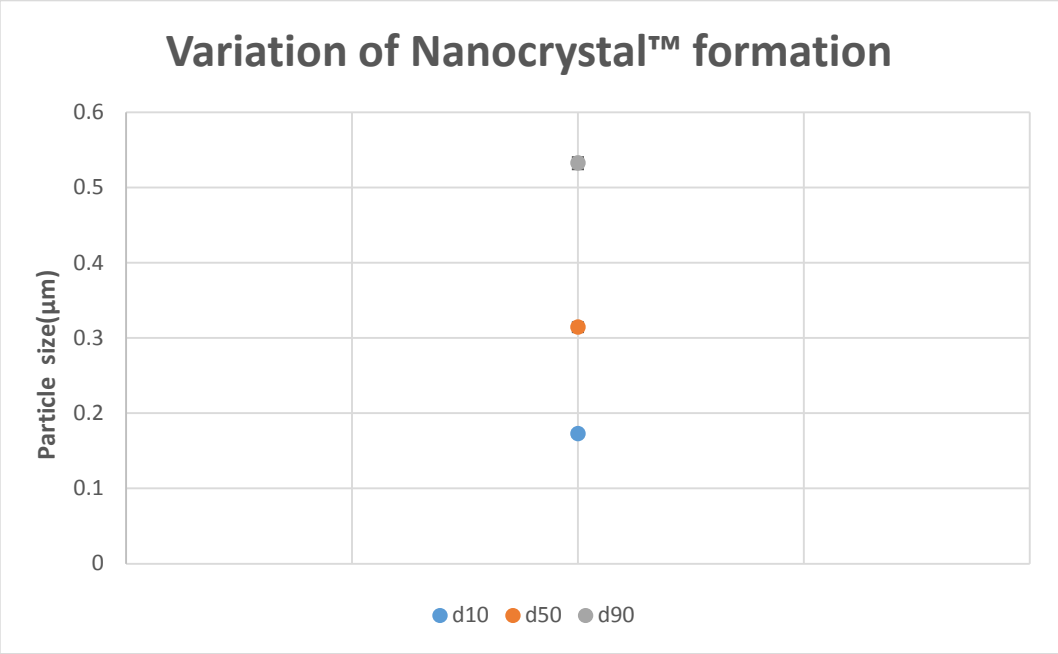

Figure 1: Graph of variation of particle sizes, taken 24 hours after the particles were prepared (to allow for time for equilibration to a stable particle size)