PROTOCOL

Covaris

AFA-Liposome™ Formation: Phospholipon 90G 2mL

Summary of Operating Conditions

Table 1. Summary of Operating Conditions

AFA Instrument	S220x
Peak Incident Power	150 W
Duty Factor	50%
Cycles per Burst	1000
Duration	180 seconds
Bath Temperature	7°C
Power Mode	frequency sweeping
Degassing Mode	continuous
Volume	2 mL

Recommended settings are subject to change without notice.

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

Supplies

Table 2. Equipment List

Item	Description	Part Number
Sample Vessel	milliTUBE 2 mL	520132
Sample Holder	Holder for 520132	500375
Focused-ultrasonicator	S220x	500218
Sample	Phospholipon 90G	Lipoid

Operating Conditions

1. Fill the tank with fresh deionized water to proper fill line. 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 achieve the specified water bath temperature, listed in Table 1.

Recommendations Specific for AFA-Liposome™ 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.
5. ***Trapped air pockets:*** **Air trapped in the vessel provides a reflective surface for the acoustical energy. When processing begins, any air pockets disperse as a multitude of small bubbles. These bubbles deflect and scatter the acoustic waveforms, effectively defocusing the energy and reducing overall effectiveness. For maximum performance, it is imperative that vessels be loaded full with aqueous sample, and air pockets minimized.**

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

Method

1. Set up the Covaris S220x at the appropriate temperature following the operating conditions above.
2. Weigh out 20 mg of Phospholipon90G and add the sample to the sample vessel.
3. Fill the vessel with water, and cap the vessel. Be careful not to introduce a bubble into the bottom of the tube. This may happen if the water is added too quickly.

   CAUTION: The bottom of the vessel is in the acoustic field. Therefore, a bubble in the sample will deflect energy and induce variable results.
4. Carefully load the sample vessel into the appropriate holder, and insert the holder into the instrument.
5. Initiate and Run process according to the operating conditions specified in Table 1.

Supplementary Data

Description

A Malvern Zetasizer Nano ZS-90 may be used to analyze the AFA-Liposome™ formation using the Covaris S220x. The sample prepared above will be utilized for analysis.

Supplies

Table 3. Supplementary Equipment List

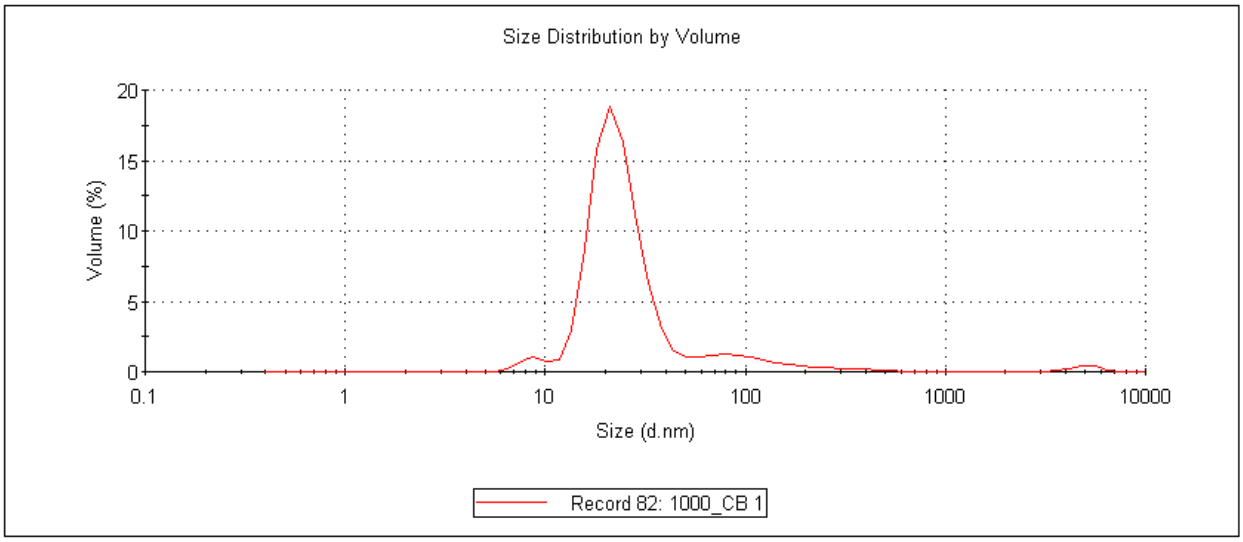
Item	Part Number
Malvern Zetasizer Nano ZS-90	ZEN3690
Sample Cuvettes	DTS0012

Method

1. Set up the Malvern Zetasizer according to its setup instructions.
2. Add 1 mL of deionized water to the cuvette.
3. Aliquot 1 mL of prepared AFA-Liposome™ sample into cuvette. The prepared sample is the sample prepared on the previous page after it has been treated with AFA. Cap the cuvette.
4. Shake the cuvette by hand until the sample is dispersed evenly in the cuvette without air bubbles.
5. Place in Zetasizer instrument and run Volume Distribution Analysis.

Typical Output Readings: Volume

Figure 1. Size Distribution by Volume



Typical Output Readings: Variation

Table 4. Variation in Results

Process	Z-Average (d.nm)	PdI
1	217.0	0.419
2	123.1	0.347
3	147.2	0.370
4	208.0	0.435
5	192.2	0.461
Average	167.6	0.403
SD	39.3	0.05
CV	23.4%	13.3%

Figure 2. Variation in AFA-Liposome™ Formation Results