PROTOCOL

AFA-Nanoemulsion™ : Rice Bran Oil in Water Continuous Process Stream 2 mL Process Vessel

Summary of Operating Conditions

Table 1. Summary of Operating Conditions

AFA Instrument	S220x
Peak Incident Power	500 W
Duty Factor	50%
Cycles per Burst	1000
Flow Rate	15 mL/min
Water Bath Temperature	10°C
Power Mode	frequency sweeping
Degassing Mode	continuous
Volume	continuous process stream

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	Flow Process Vessel SST 2mL	500387
Focused-ultrasonicator	S220x	500218
Water Conditioning	hpWCS	500370
Sample	Rice Bran Oil	Sigma Aldrich
	Tween-80	Select Origin Oils

Operating Conditions

1. Fill the tank with fresh deionized water to proper fill line. Turn on the hpWCS and check the water level. The S220x should be equipped with a graduated water level label. If the tank lacks this label, please contact Covaris. During treatment, the vessel should be fully immersed in the water to ensure a good acoustic path from the AFA transducer and appropriate cooling of the sample.

2. Place the intensifier on the transducer as instructed in the instrument's User Manual.

   CAUTION: When using SST vessels, it is imperative that the intensifier be placed on the transducer to avoid damage to the instrument.

3. Degas water for the approximately 30 minutes and ensure the oxygen level in the water bath is lower than the targeted value. To maintain degassed water, keep the vacuum pump continuously on during operation and sample processing. Do not turn the pump off. The oxygen level will be automatically maintained by data acquisition software.
4. Set the chiller 1 and chiller 2 to achieve the proper temperature, listed in Table 1.

Recommendations Specific for AFA-Nanoemulsion™ 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 the instrument's User Manual.
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. *Water 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. *Intensifier:* The intensifier should be affixed to the transducer to ensure that the intensity of the acoustic reverberation does not become too great and cause damage to the instrument.

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. Add 52.5 g Tween-80 and 436.25 g deionized water to a 500 mL beaker, and stir until dissolved (this may take up to a half hour, so gentle heating may be used to aid dissolution).
3. Assemble a continuous flow apparatus as shown in Figure 1. The two inlet flows should be calibrated such that the overall flow rate out (q_{out}) is equal to 15 mL/min, while maintaining a ratio of 22.4:1. Pump 1 was calibrated to 28.2 RPM (q_{in} = 14.36 mL/min), and Pump 2 was calibrated to 36.0 RPM (q_{in}' = 0.64 mL/min).
4. Place the flow vessel in the instrument. Begin both pumps simultaneously to add sample to vessel. When the vessel is full, begin the process according to the operating conditions specified in Table 1. To monitor the AFA-Nanoemulsion™ quality over time, collect 1 mL of sample directly from the outlet feed at intervals.

Figure 1. Schematic of Flow Apparatus
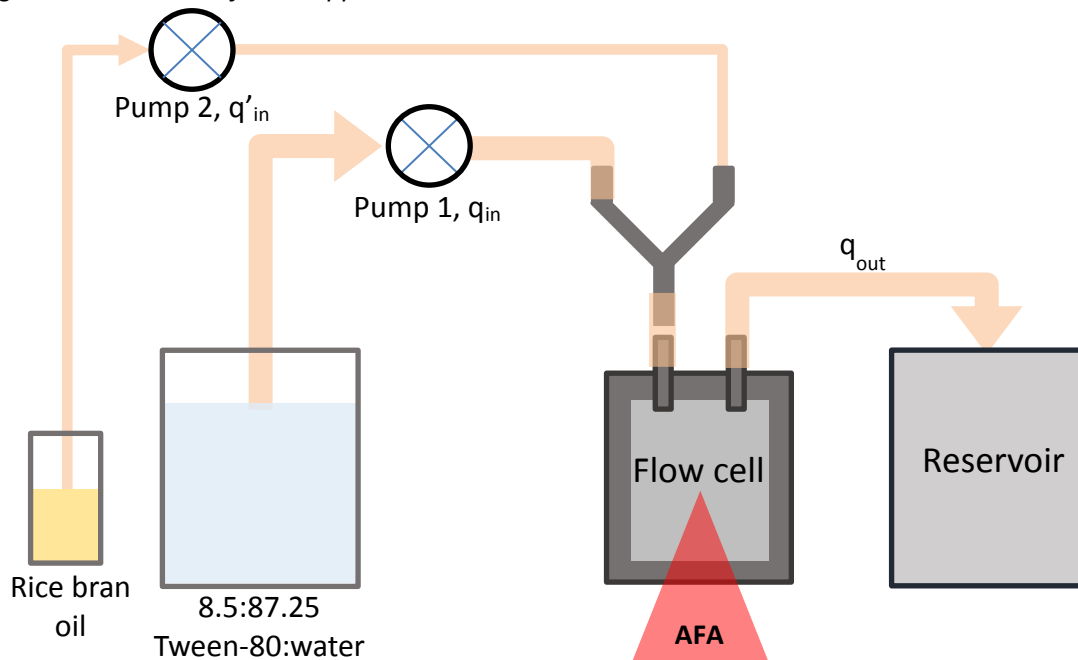
Supplementary Data

Description

A Malvern Zetasizer Nano ZS-90 may be used to analyze the AFA-Nanoemulsion™. 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 3 mL of deionized water to the cuvette.
3. Aliquot 10 µL of prepared AFA-Nanoemulsion™ 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 Intensity Distribution Analysis.

Typical Output Readings: Intensity

Figure 2. Size Distribution by Intensity

Size Distribution by Intensity
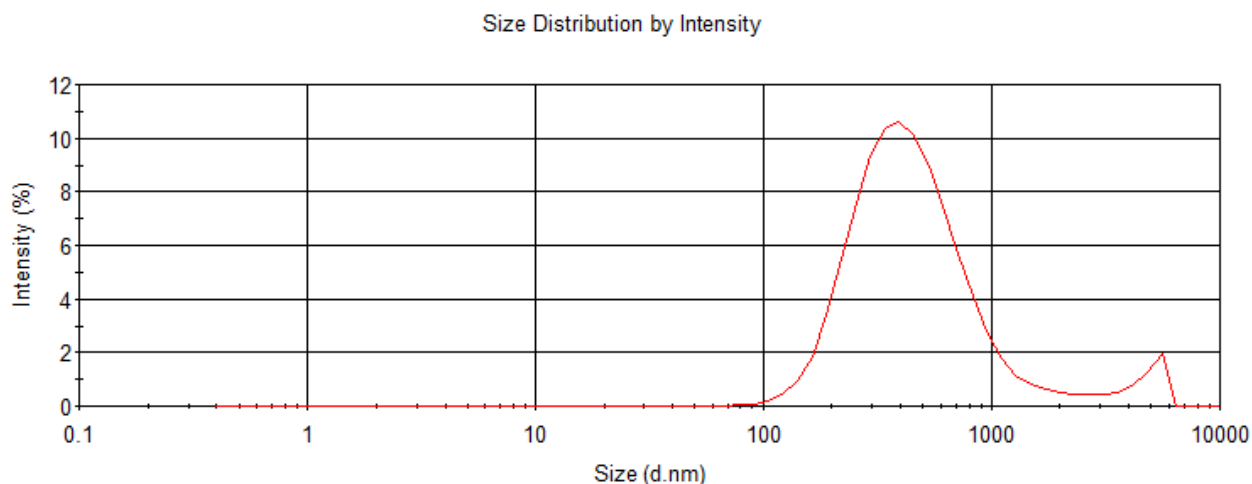
Typical Output Readings: Variation

Table 4. Variation in Results

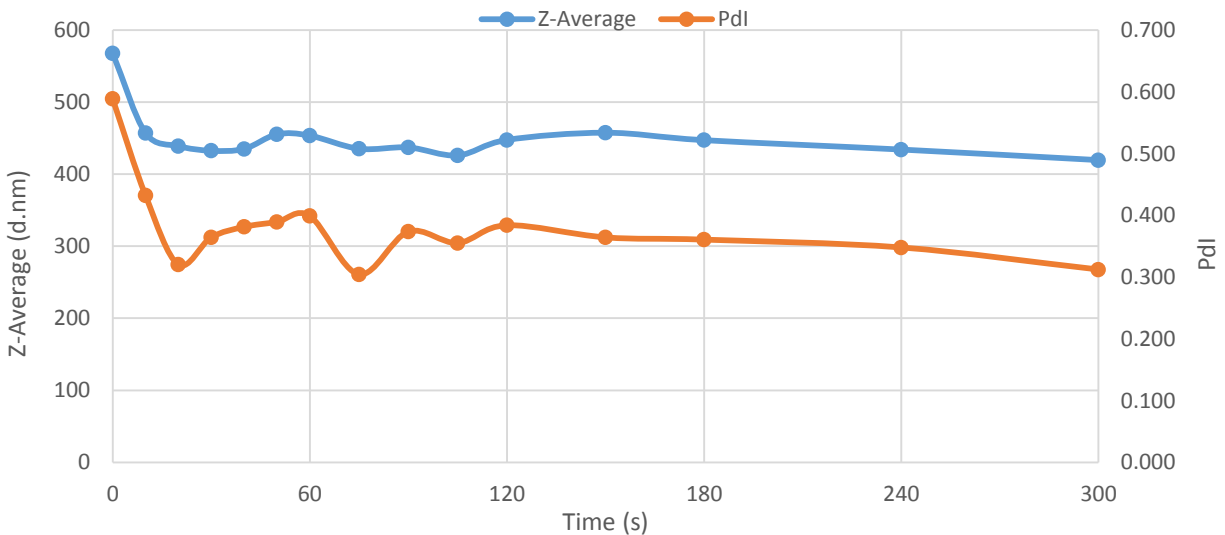| Process	Z-Average (d.nm)	PdI
0	568.0	0.589
10	457.2	0.432
20	439.0	0.320
30	432.6	0.364
40	435.1	0.381
50	455.3	0.389
60	453.6	0.399
75	435.3	0.304
90	437.1	0.374
105	426.1	0.355
120	447.6	0.384
150	457.6	0.364
180	447.2	0.361
240	434.2	0.348
300	419.6	0.312
Steady State Average	440.0	0.358
SD	14.11	0.042
CV	3.21%	11.7%

Figure 3. Equilibration of Continuous Process

Figure 4. Variation in AFA-Nanoemulsion™ Formation Results