PROTOCOL

Covaris

TITLE: mRNA and total RNA fragmentation

Summary of Operating Conditions

	mRNA	Total RNA
Duty Cycle	10%	10%
Intensity	5 (S2/E210)	5 (S2/E210)
Peak Incident Power	175 Watts (S220/E220)	175 Watts (S220/E220)
Cycles per Burst	200	200
Processing time (sec)	60-90sec	240-300sec
Fragment size range	200 bases	200 bases

Temperature (bath)	4-8°C
Power mode	Frequency Sweeping
Degassing mode	Continuous
Volume	130ul
Starting material	mRNA ≤1µg Total RNA ≤5µg
Buffer	1X TE, pH8, or 10mM Tris-HCl, pH 8.5
Water Level (Fill/RUN)	S-Series - Level 12 E-Series – Level 6
AFA Intensifier (E-Series)	**YES**

Supplies

	Description	Part Number
Sample Vessel	Snap-Cap microTube with AFA fiber and pre-split Teflon/silicone/Teflon Septa	520045
	Crimp-Cap microTube with AFA fiber and pre-split Teflon/Silicone/Teflon Septa	520052
	96 microTUBE Plate (E-Series Only)	520078
Preparation station	Snap-Cap microTube loading, and unloading station	500142
Holder for S-Series	microTUBE holder (single tube) NOTE: Snap-or Crimp-Cap	500114
Holder for E-Series	Snap-Cap microTUBE rack (24 tubes) 6mm Z	500111
	Crimp-Cap microTUBE rack (96 tubes) 7mm Z	500143
	Intensifier required for E-Series	500141

Recommended settings are subject to change without notice.
See following link http://covarisinc.com/wp-content/uploads/pn_010304.pdf for updates to this document.

Introduction

Recommendations specific for microTUBEs

The Covaris AFA process is highly reproducible; however, attention must be paid to the following treatment attributes to ensure best results:

Sample volume: For optimum performance, the volume of the sample used in the microTUBE for RNA shearing must be 130 µL. With lower sample volumes an "air-gap" may form in the sample fluid; thus, partitioning the sample which may result in a broad peak and may lead to decreased shearing efficiency.
Treatment: Since the RNA fragmentation process is rate-limited; the size of the RNA fragment (defined by mean peak nucleotide size) generated is affected by two factors; treatment duration and parameters:
1. Treatment **Duration:** This is defined as the length of period the AFA is applied (in seconds) to affect the shearing of the sample that is in the microTUBE. The value for treatment duration listed in this document is a recommended guideline. Minor adjustments in treatment duration may be made to optimize the shearing efficiency. Actual results may vary depending on the concentration, viscosity, and the type of the starting material. Covaris recommends performing a time course experiment to determine the appropriate treatment time settings.
Note: Longer treatment duration will produce smaller RNA fragments.
2. **Parameters**: As different values for acoustic parameters (intensity, duty cycle, and cycles per burst) are tested to achieve high shearing efficiency, such parameters, including well plate definitions, should be closely recorded so they can be preserved once proven effective.

Water: The bath water is employed to couple the acoustic energy of the applied beam to the sample vessel:
1. **Purity:** When applying high frequency focused acoustics in rate-limited applications, foreign materials such as algae and particulates may scatter the acoustic beam, resulting in a shift to larger mean fragment size. Thus, the water used in the bath should be pure distilled or deionized 18Ω. To prevent any future contamination, the bath water should be changed daily or cleansed by a Covaris Water Conditioning System.
2. **Degas Level**: Likewise, insufficient degassing levels within the bath water may result in poor acoustic coupling and thereby shift the resulting mean fragment size. 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, causing a shift toward larger mean fragment size. Bath temperature (as reported by SonoLAB software) should therefore be closely controlled and matched run-to-run and day-to-day to achieve reproducible shearing results. Employ the temperature alert feature in SonoLAB to alert 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 shearing results.

Materials

1. Sample Vessel: Covaris Snap-Cap or Crimp-Cap microTUBEs
2. Buffer: Tris EDTA, pH 8.0.
3. Sample Concentration: ≤ 1µg of mRNA, or ≤5µg of total RNA in 130µl
4. **E-Series** Rack and intensifier or **S-Series** holder

Operating Conditions

1. Fill the tank with fresh distilled or deionized 18 Ω water to the proper fill line (please see below). The S-Series or E-Series tank should be equipped with a graduated water level label (note: if the tank lacks this label, please contact Covaris). During treatments, the microTUBE should be partially immersed in the water to ensure a good acoustic path from the AFA transducer.
    a. For S-Series system equipped with a graduated fill line label, level = 12
    b. For E-Series system equipped with a graduated fill line label, level = 6

2. Degas water for the recommended time period mentioned below. To maintain degassed water, keep the pump continuously running during operation and sample processing. Do not turn the pump off.
    a. For S-Series system: at least 30 minutes
    b. For E-Series system: at least 60 minutes

3. Set the water chiller to the right temperature.
    a. When the temperature on the water chiller is set at 3°C, the S-Series and E-Series temperature software display should settle near 7°C.
    b. Depending on environmental conditions (for example, high relative humidity and ambient room temperature) the chiller may have to be set a little lower (or higher) to maintain the bath temperature between 6 – 8°C to offset the thermal transfer loss between the chiller and the apparatus.

Method

1. Set up the Covaris S-Series or E-Series at the appropriate temperature following the operating conditions above.

2. Place unfilled Covaris microTUBE into loading station for the S-Series system, or for the E-Series, place into the bottom portion of the 24 or 96 tube racks.

3. Keeping the cap on the tube, using a tapered pipette tip, transfer 130µl of RNA sample (in TE buffer, or 10mM Tris-HCl pH 8.5) by inserting the pipette tip through the pre-split septa. With the pipette tip approximately half way down the interior of the tube and alongside the interior wall, slowly discharge the fluid into the tube.

Note: Be careful not to introduce an air bubble into the bottom of the tube. This may happen if the sample is loaded too quickly.

CAUTION: the bottom of the tube is in the acoustic field; therefore, an air bubble in the sample will deflect energy and induce variable shearing results.

CAUTION: Do not remove the snap-cap prior to sample processing. The pre-split septa should self-seal after removal of the pipette; be careful not to pressurize the sample during loading.

4. After the microTUBEs are carefully loaded into the Covaris approved S-Series holder or E-Series rack be sure to keep the tube in a vertical orientation; again, it is important NOT to have any bubbles at the bottom of the tubes. Inspect every tube by raising the holder and check for bubbles in the tubes. The micro Tubes can be briefly and gently centrifuge to remove any bubbles.

5. Be cautious not to bounce the rack or holder and carefully insert it into the S-Series or the E-Series instrument. If using the E-Series system, double check to make sure the rack is fully inserted into the instrument platform.
Note: If the rack is not completely inserted, the sample may be exposed to variable doses of treatment.

6. Initiate and Run process according to desired base pair target peak range.

7. Following a process, remove holder or rack from apparatus. Check to see if any tubes had a bubble at the bottom; again, this bubble would introduce variable results.

8. Transfer processed sample to another vessel:

1) S-Series -Remove tube from S-Series holder and place it into prep station holder. While keeping the snap-cap on, insert a pipette tip through the pre-split septa and slowly remove fluid. Alternatively, the snap-cap may be removed with the tool supplied with the prep station

2) E-Series -It is possible to remove samples while the tubes are still in the rack through the top of the rack holder. Insert a pipette tip through the pre-split septa and slowly remove fluid.

Final Note:

Please note that there are two sources of variation in both peak value and distribution: (1) the physical process of RNA fragmentation is random and (2) the analytical process to determine fragment size has inherent variances (for example, gel electrophoresis and electropherogram). Therefore, fragment distributions and peak values, even from technical replicates, may not appear identical.
If the sheared RNA sample was to be column-purified or concentrated prior to analysis on an agarose gel or BioAnalyzer, please remember to take out an aliquot for use as control prior to that step. Column purification and concentration of the sheared DNA/RNA will generate a biased fragment distribution profile due to the inherent greater loss of the smaller DNA/RNA fragments during the process.

Supplementary Data:

The figures below show data collected using the operating conditions mentioned in the Method provided on page 1

Total RNA time course fragmentation using Covaris AFA

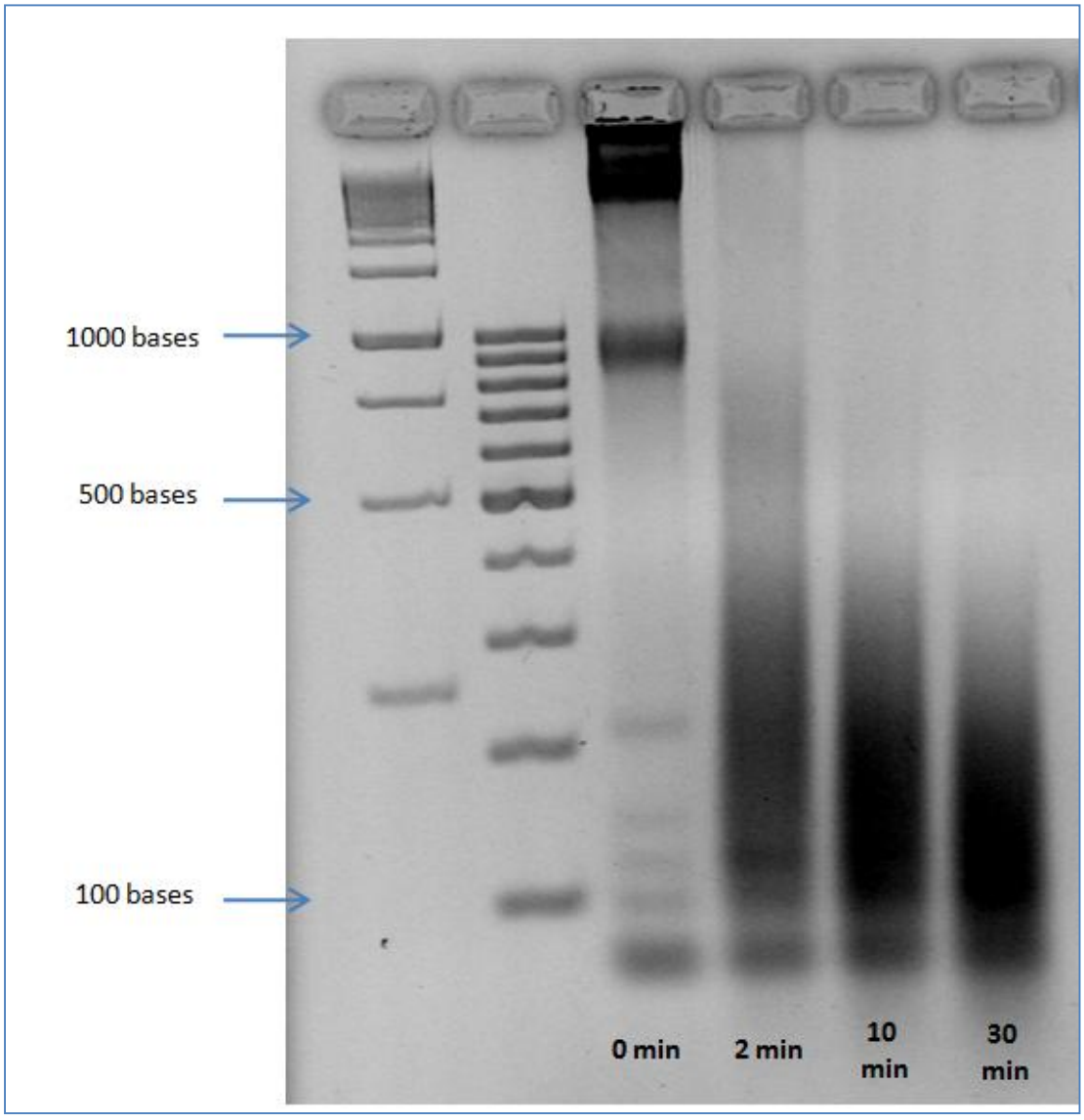

Figure 1- An image of an Agarose Gel showing results of time course processed 20ug of total RNA in 130ul of TE carried out in microTubes according to the operating conditions in page 1

mRNA Fragmentation

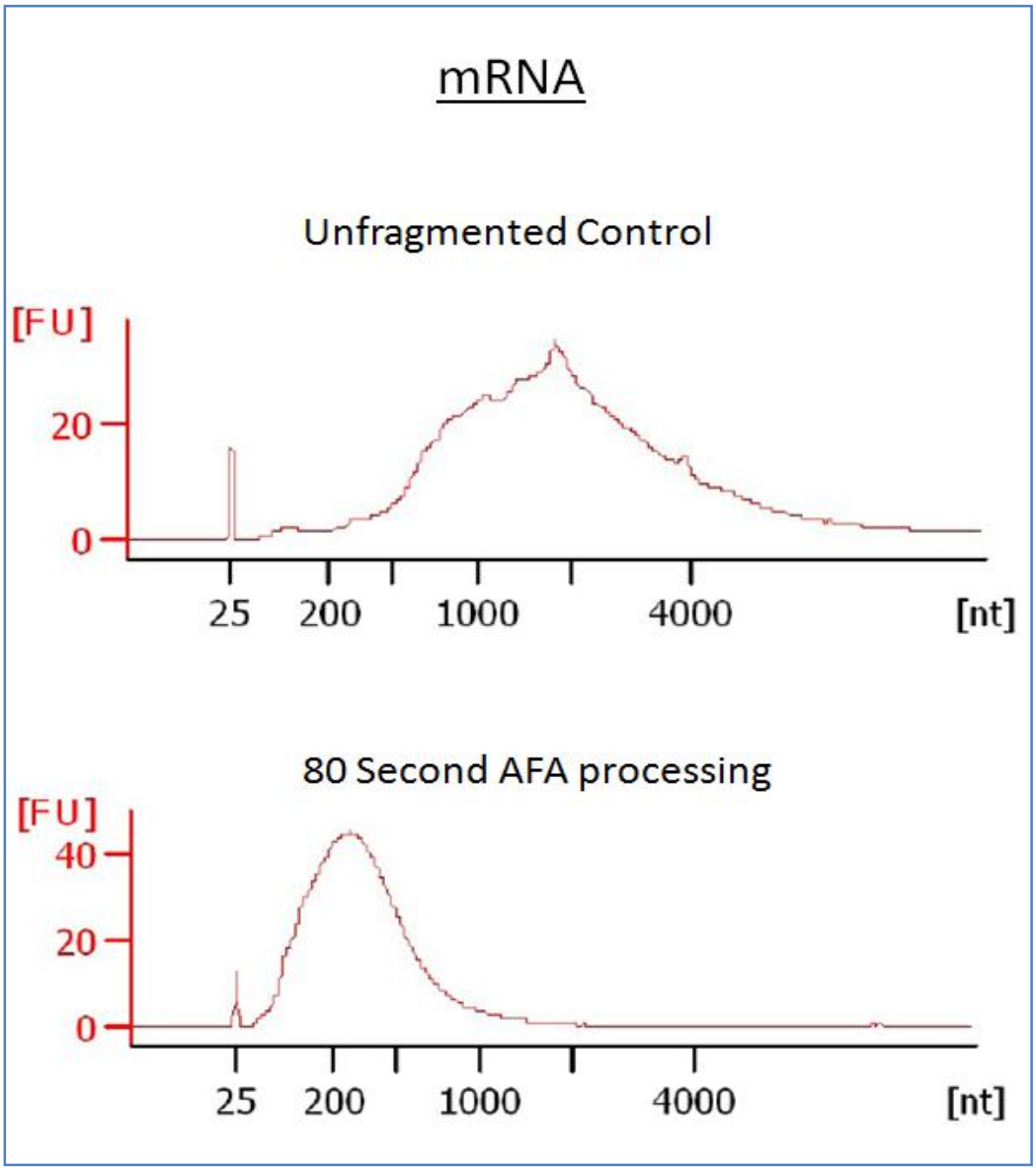

Figure 2- Agilent BioAnalyzer electropherogram of 100ng of mRNA processed in a volume of 130ul of TE in a microTUBE according to the operating conditions on page 1, showing the mean fragment size at 400 bases.

Total RNA fragmentation

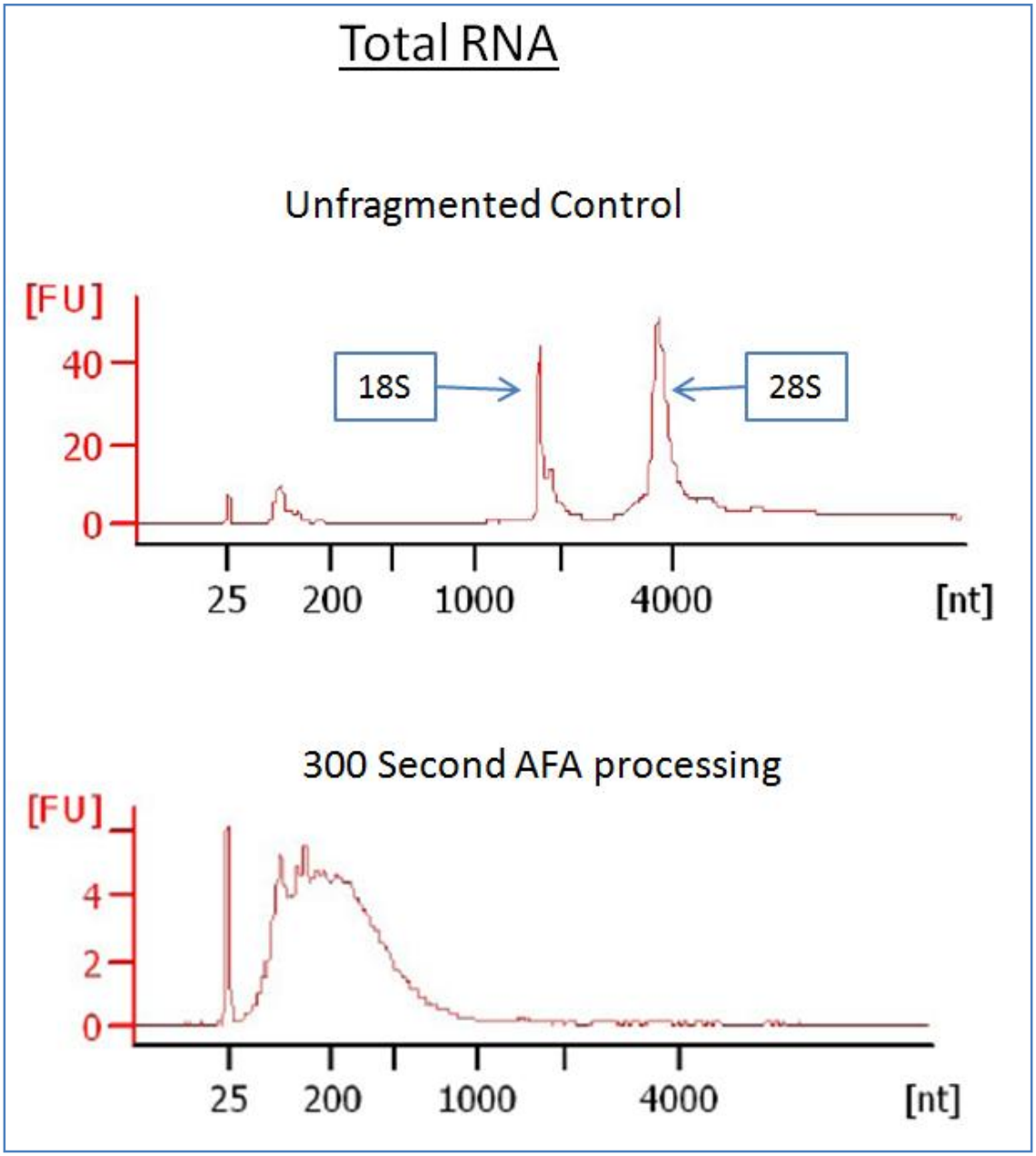

Figure 3- Agilent BioAnalyzer electropherogram of 5µg of mRNA processed in a volume of 130ul of TE in a microTUBE according to the operating conditions on page 1, showing the mean fragment size at 200 bases.