Covaris™

the sample prep advantage

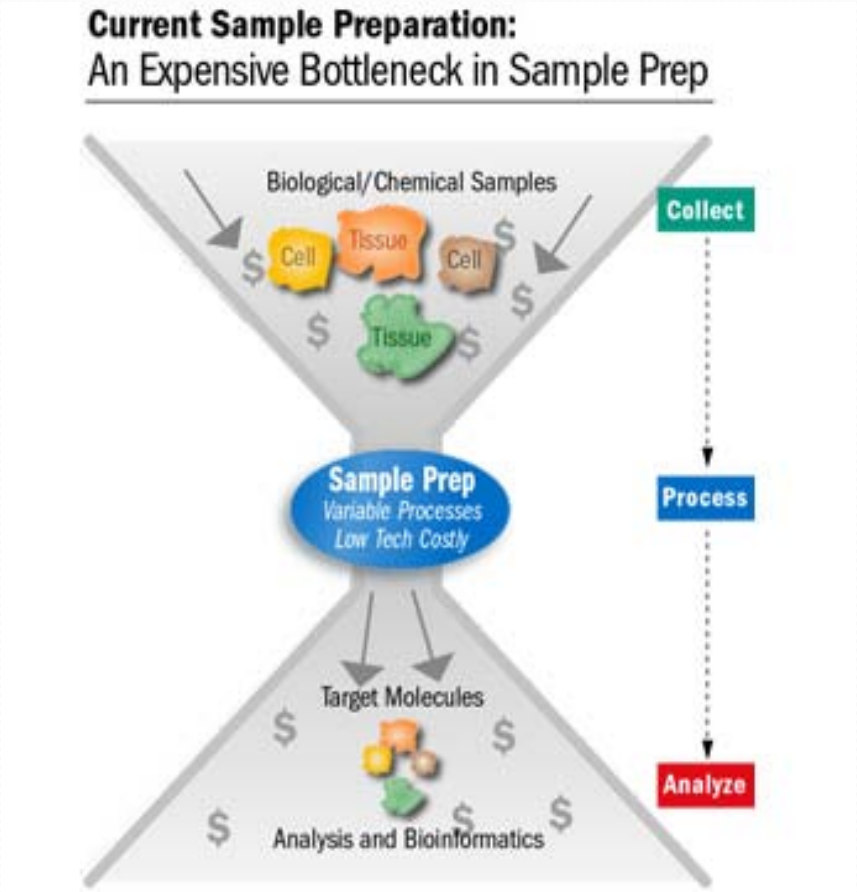
Title:	Optimizing DNA Shearing
Presented by:	Jim Laugharn
Presented at:	Sequencing, Finishing, and Analysis in the Future (SFAF)
Date:	28 May 2009

Fragmenting – 1st sequencing step

- Current options and observations
- Covaris systems – The Solution
- High-throughput directions

Sample Prep: an opportunity

- Collection - expensive
  - Starting material , storage & tracking
  - Emerging industrial approach
  - Costs - rising
- Preparation - bottleneck
  - No industrial style approach
    - Often an after-thought
  - Opportunities for substantial improvements
- Analysis - expensive
  - Developing industrial approach
  - Costs – rising



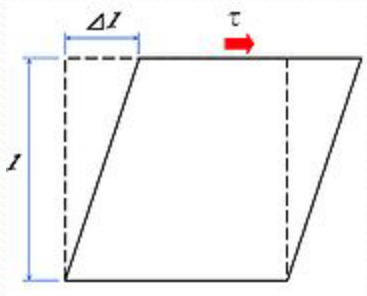
DNA shearing…random

- Enzymatic - Not random
- Thermal – Not random
- Mechanical - Random
  - Fundamental mechanism
    - Shear stress forces

…it has to be random…some techniques are not

shear stress - influences

- Mass (too high increase viscosity > increase heat)
  - Glycerol (detrimental)
- Viscosity (e.g., genomic vs. PCR products)
- Thermal energy
  - bias DNA shear patterns



A shear stress τ is applied to the top of the square while the bottom is held in place. This stress results in a strain, or deformation, changing the square into a parallelogram

CAUTION: Glycerol (not for DNA shearing)

- Dynamic viscosity mismatch - Pascal*second ($kg \cdot m^{-1} \cdot s^{-1}$)
  - Water 0.00089 Pa*sec
  - Glycerol 1.50 Pa*sec
- Attenuation 9dB/cm at 5MHz
  - Water: 0.0166
  - Glycerol (>98%): 11.1
- Shear stress and velocity gradients become non-linear
- Covaris data
  - Dramatic thermal rise >>> with glycerol addition to water
- Glycerol for DNA shearing
  - Only in low energy processes

- Temperature rise per gram (includes plate self-heating):
  - Water: 6 degree C
  - Glycerol: 28 degree C

- Conditions for both:
  - intensity=5, duty=10%, cpb=200,
  - sweep 1mm/sec

…Low power conditions

Glycerol makes process faster, but is **NOT** an appropriate adjuvant for DNA fragmentation

Shear stress techniques

- Flow boundary layer
  - HydroShear
- Wind stress boundary layer
  - Nebulizers
- Cavitation (bubble collapse)
  - Sonicators (no thermal control)
  - Covaris AFA (thermal control)

…Ideal process minimizes thermal and maximize mechanical

techniques

HydroShear

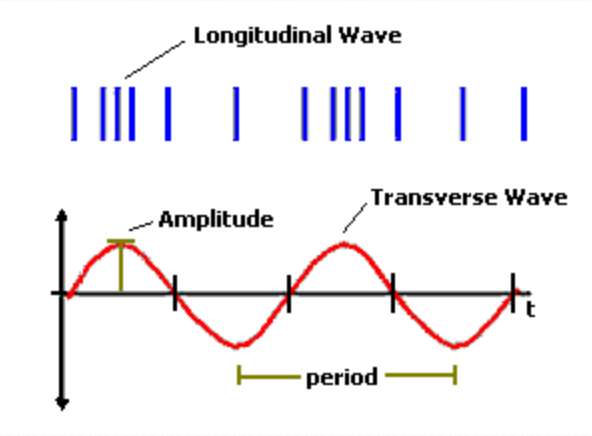
- No automation
- Not for small fragments
- Potential cross-talk

Nebulizer

- No automation
- Not for large fragments
- Poor recovery
  - Increases as target size decreases

acoustic technology

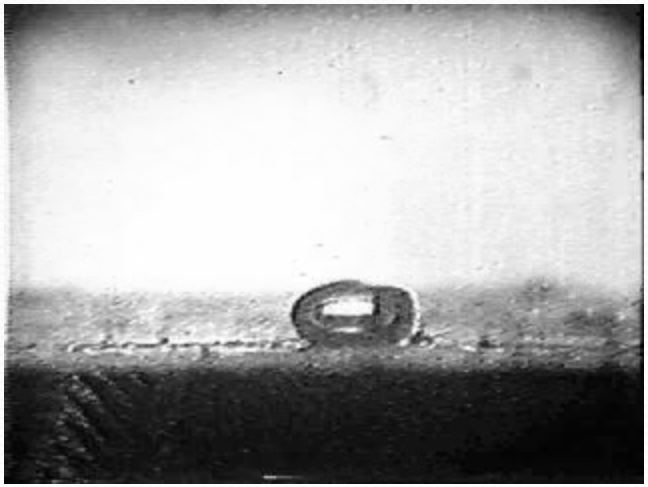
Acoustic waves and their properties have been utilized in research for decades. Operations such as sonication, ocean exploration, SONAR, apply acoustics waves.

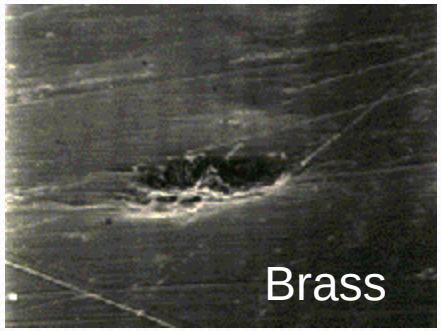


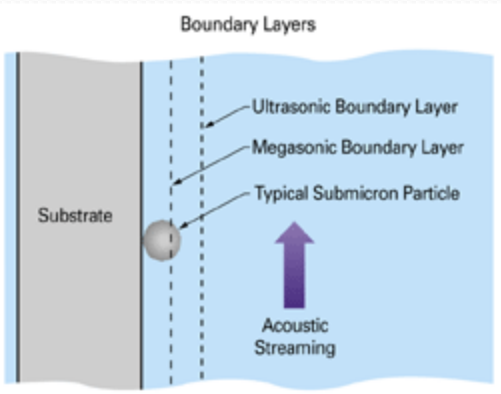
Random cavitation → Shear forces and heat → emulsify, homogenize, disrupt

cavitation-based bubble growth

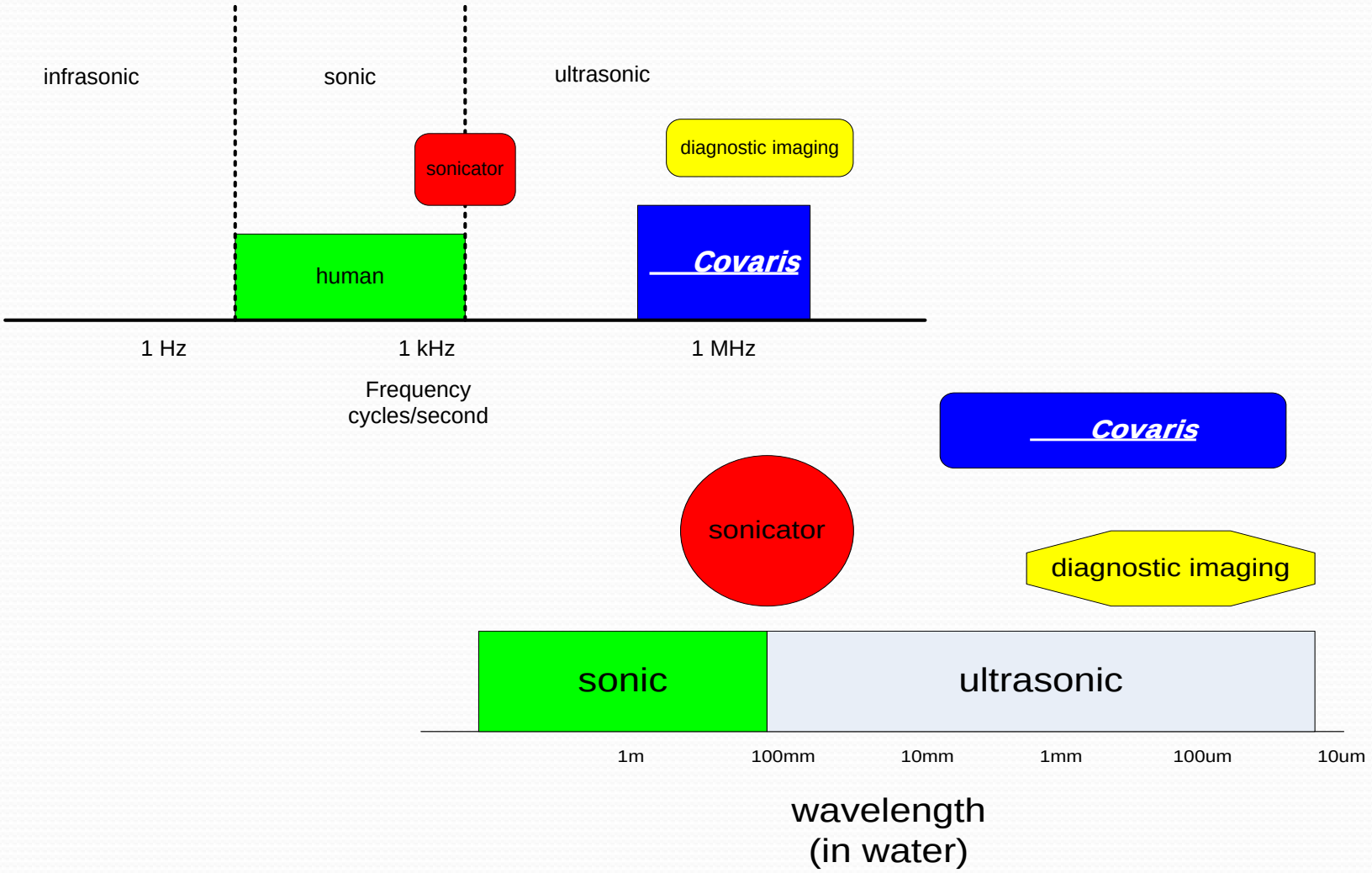
- Stroboscopic image (305,000 fps) of single collapsing 2mm bubble @ 60Hz
  - liquid jet impacts at >100m/sec
- Covaris
  - free fluid formation
- Sonicator
  - on metal tip (like video)



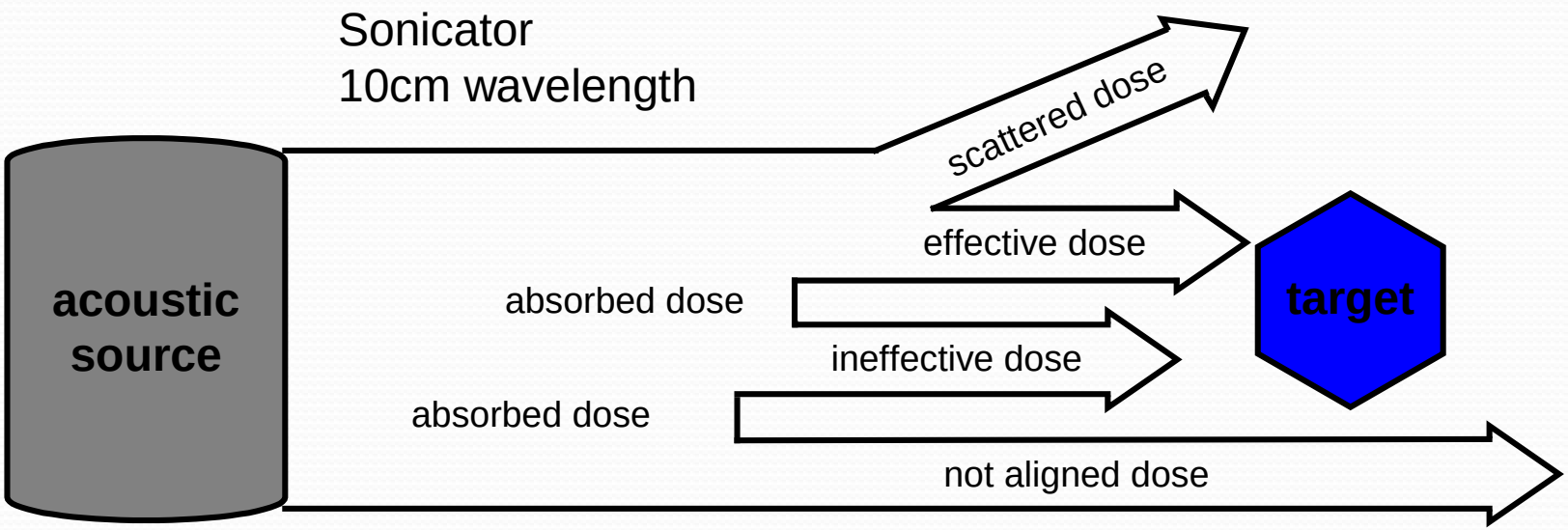




acoustic frequency > wavelength



Conventional “sonicator” Process



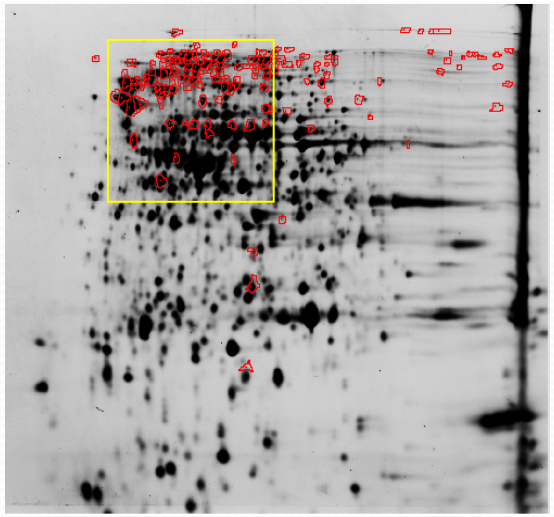
Mechanical effects > not controlled

Thermal effects > not controlled

thermal effects of sonicators

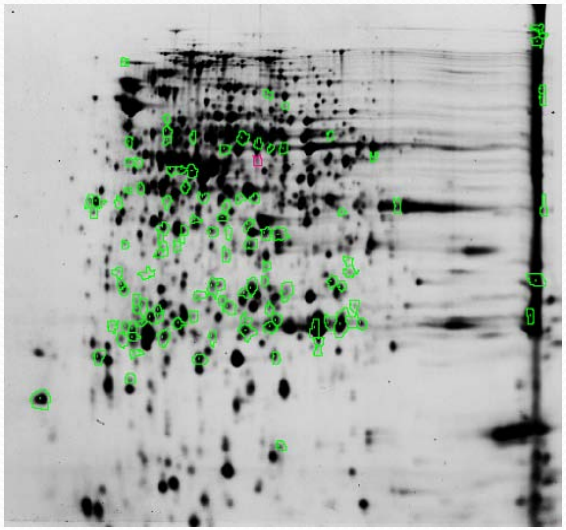
- 2D DIGE analysis
- Pooled standard from *E.coli* – *was prepared by pooling equal amounts of each lysate labeled with Cy2. Individual lysates were then labeled with either Cy3 or Cy5 respectively for each gel with reciprocal labeling carried out between the two lysates to compensate for any bias between the labeling efficiency between C3 and Cy5. Gel images show spots that were increased with the different prep process. The sonicator prepped sample indicates thermal damage*

Covaris



increase in larger MW spots relative to sonicator

sonicator



increase in smaller MW spots (breakdown products)

Fragmenting – 1st sequencing step

- Current options and observations
- Covaris systems – The Solution
- High-throughput directions

well developed technologies

Covaris – combination of:

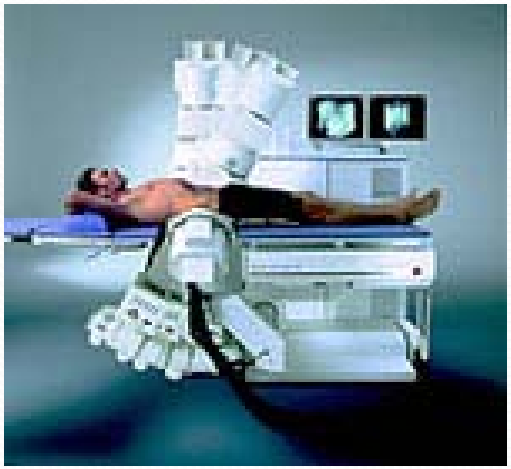
- Therapeutic Ultrasound:

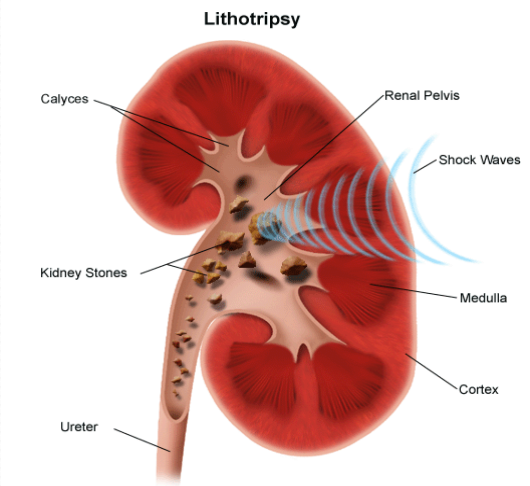
  Since1980, Extracorporeal Shockwave Lithotripsy (ESWL) treatments apply non-contact acoustic properties to break down kidney and gall stones. Also known as, High Intensity Focused Ultrasound (HIFU).

- Diagnostic Ultrasound:

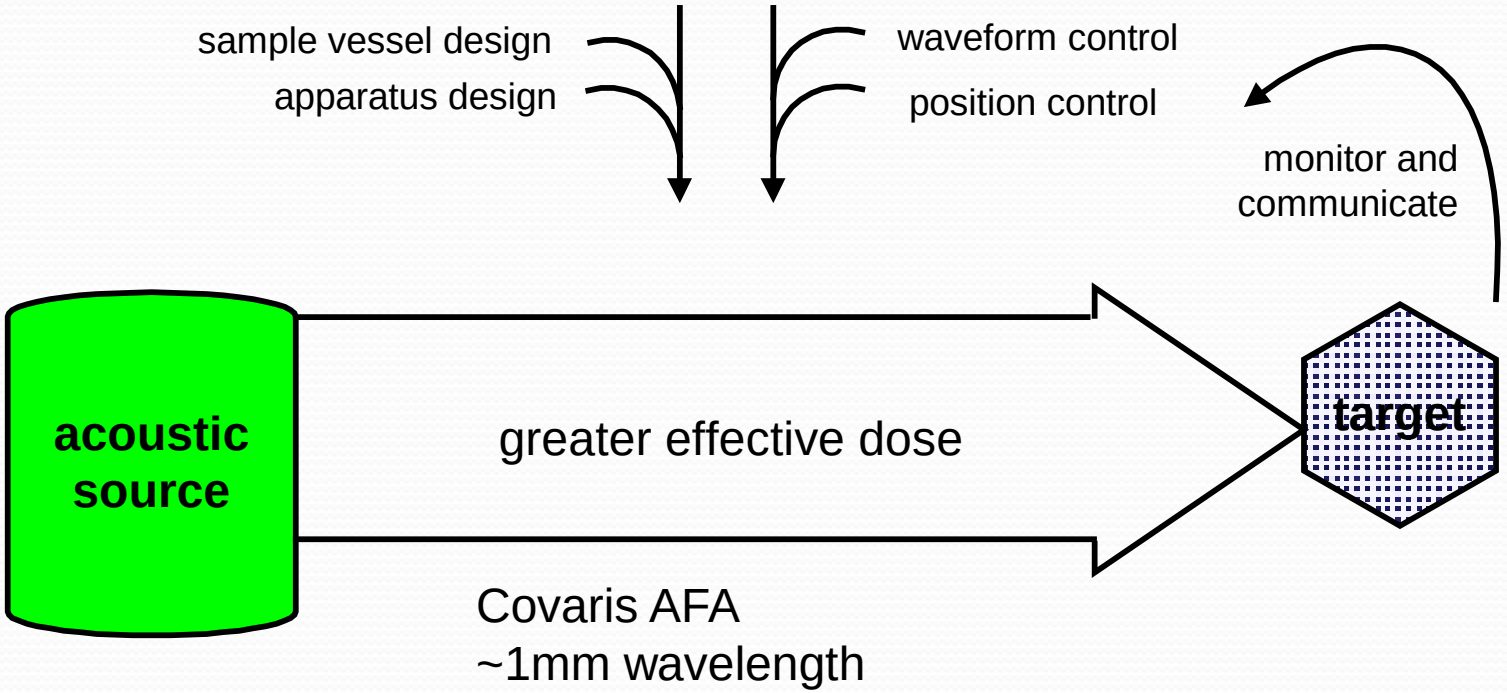
  imaging also uses related technology (low intensity focused ultrasound)

- Computer controlled





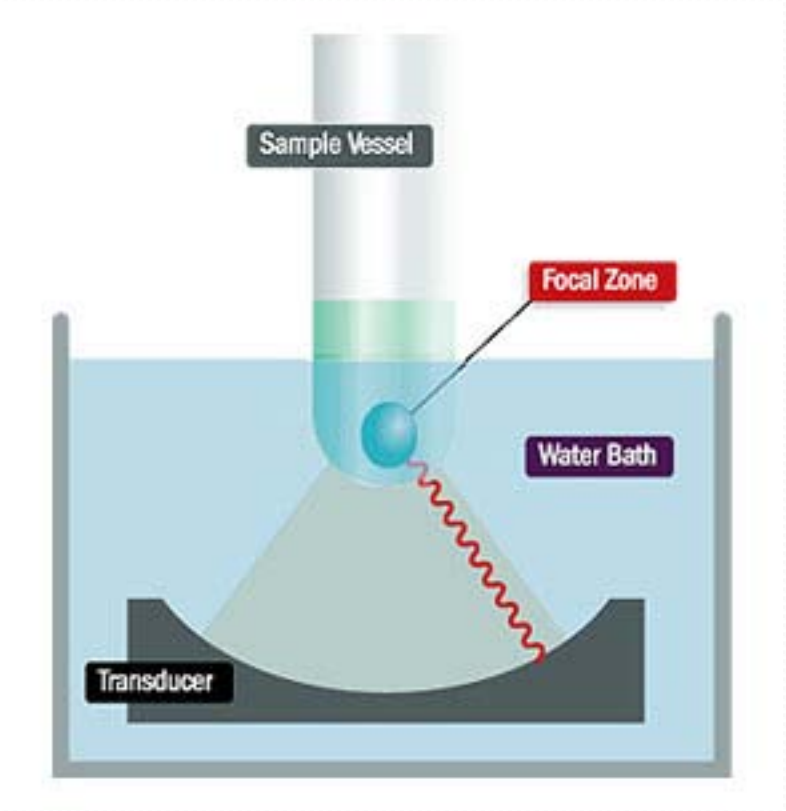
The Covaris Process



AFA – Adaptive Focused Acoustics

The Covaris Process

- Geometrically focused with spherically curved transducer
- Typically in water bath to maintain constant temperature
- Transmission
  - Power processes
- Reflection
  - Sample interrogation



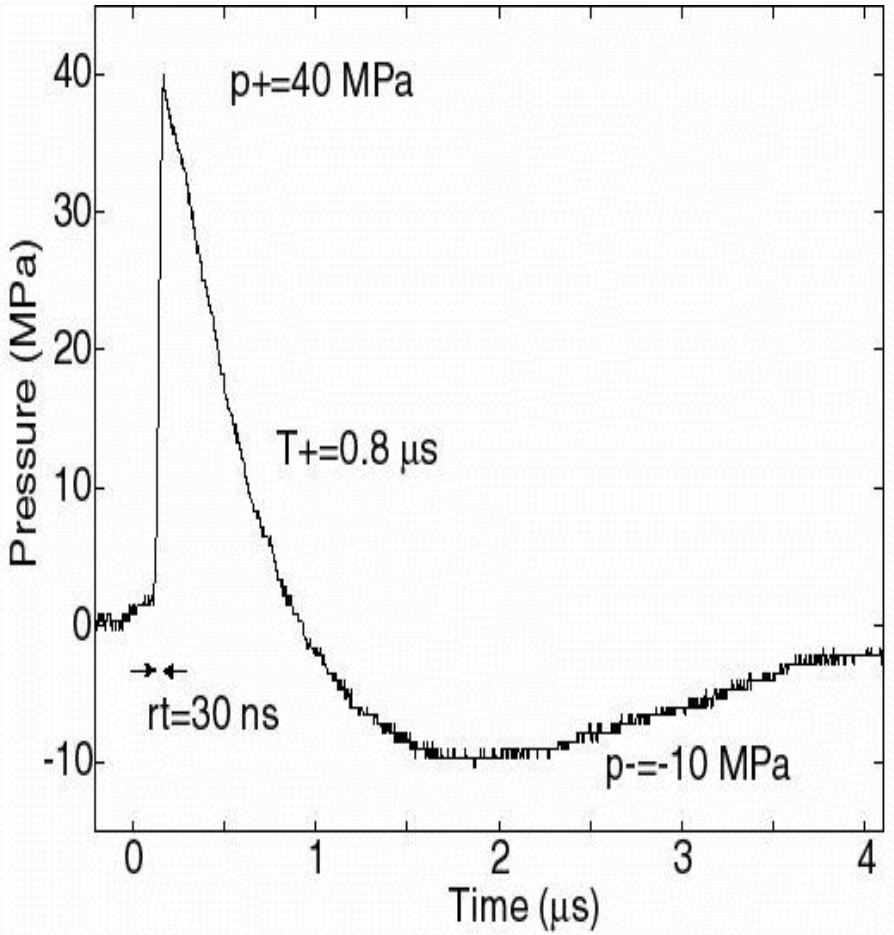
… Adaptive Focused Acoustics (AFA)

controlled energy transfer

- High-speed 1/15,000 second
- Water 2.5ml in 16x100mm
- 0.46 MHz, focused
- Tensile strength

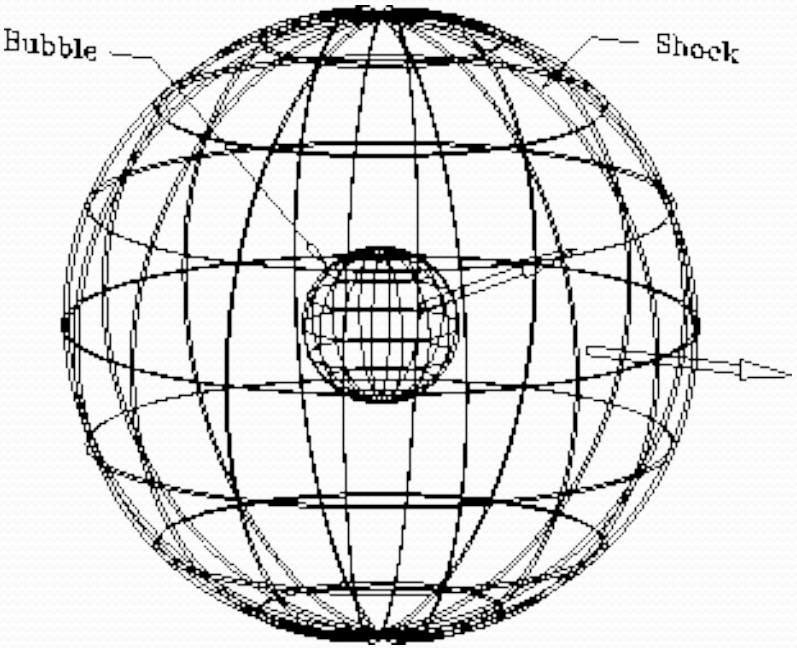
Non-linear, high intensity waveform

- recorded at the focus of a HIFU lithotripter (High Intensity Focused Ultrasound) using a PVDF membrane hydrophone
- compressive spike
  - Convergence of energy
  - Measured rise time of 30 ns is limited by the hydrophone (theoretically <1 ns)
- tensile tail
  - Rarefaction of energy
  - peak negative pressure … cavitation bubble formation



shear forces @ submicron scale

- Expanding spherical shock wave
  - >100m/sec shear force jet
- Conservation of energy the amplitude of each shock decreases 1/radius
- 1.1MHz resonant bubble radius is 3-4 microns…collapse results in submicron jets
- Area of the spherical front is proportional to r^2
  - $\Delta p \propto 1/r$
- Expanding bubble
  - 100 increase in radius > million fold increase in volume

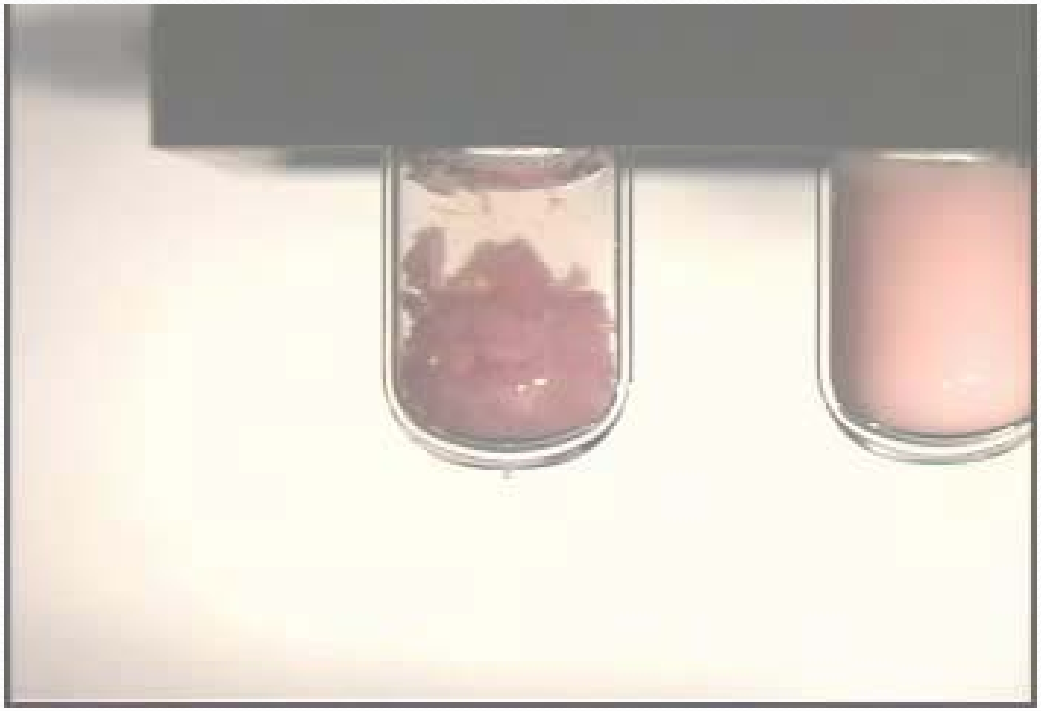


S2 – serial, single

- DNA shearing range
  - 100bp-3kb (8kb TBD)
- Stand-alone format
  - ActiveX control
  - API's for TECAN Evo
  - CE marking
  - ETL (NRTL) testing
- Covaris systems shipped
  - ~750 units

versatile tool

- **0.65 gm muscle homogenization**
- **DNA & RNA extraction from same sample**
  - **The Cancer Genome Atlas Research Network** Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature Vol 455, October, 2008



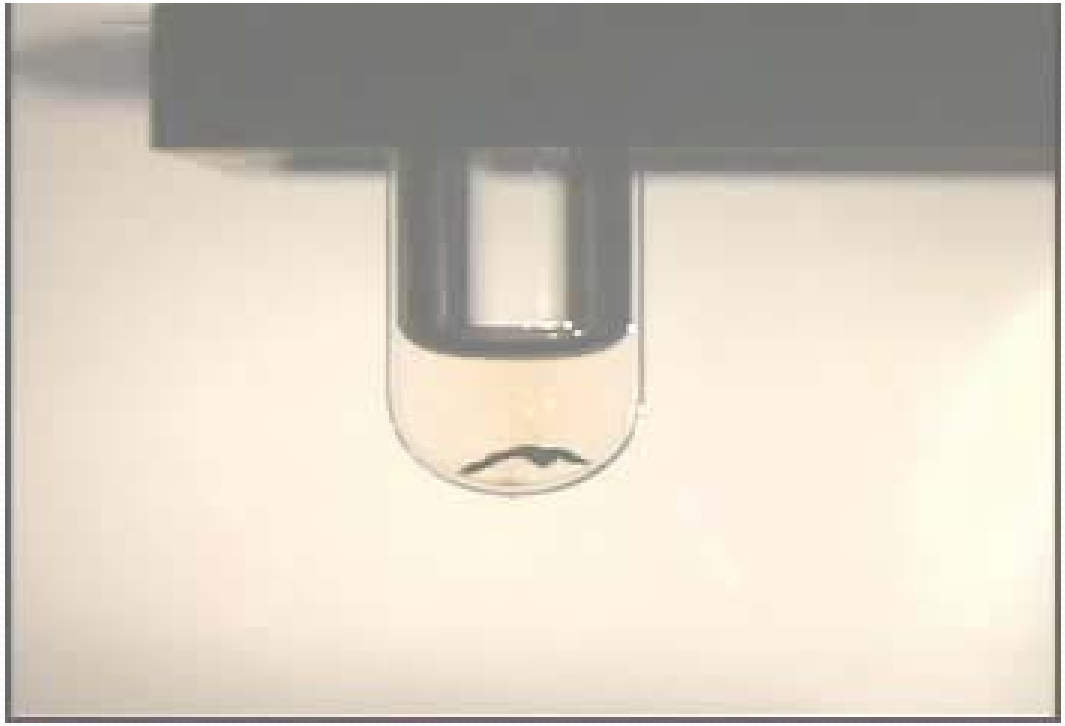
low power

- Yttrium oxide bead resuspention
  - Scintillation proximity assay

… low power

high power

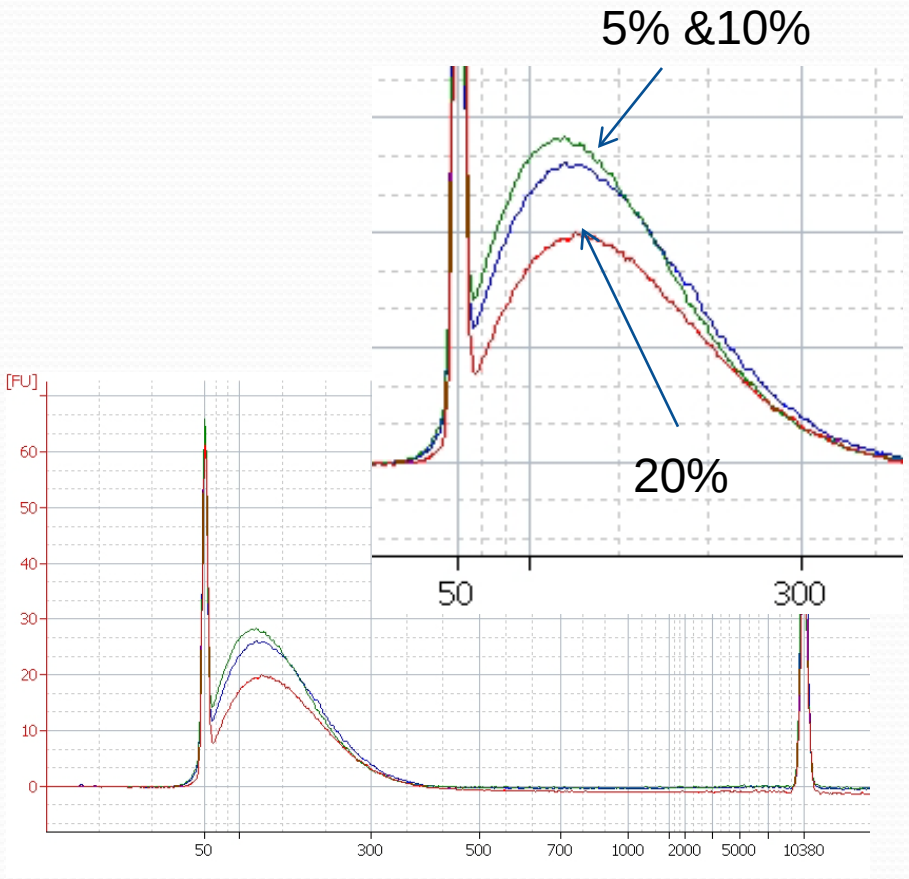
- single-walled carbon nanotubes (SWCN)
  - 1nm x ~5,000nm
- Flake >> slurry
  - 30 seconds with Covaris process
  - 3-5 hours with control process
- Controlled, no hot spots, isothermal, repeatable
- Accelerate formation of initial solvent boundary layer



…scalable for flow-through manufacturing line

reproducible process

- **Identical AFA energy dose** @ 5 intensity, 200 cpb, 0.46MHz, 8 degC,
  a) 20% - 560 seconds
  b) 10% - 1120 seconds
  c) 5% - 2240 seconds
- Result
  - all at 100bp
  - 5% & 10% - identical “mass”
  - 20% - 60% “mass”
  - High energy > lower recovery
  - Moderate energy > high recovery
- Lower dose
  - Tighter peak
  - Higher recovery

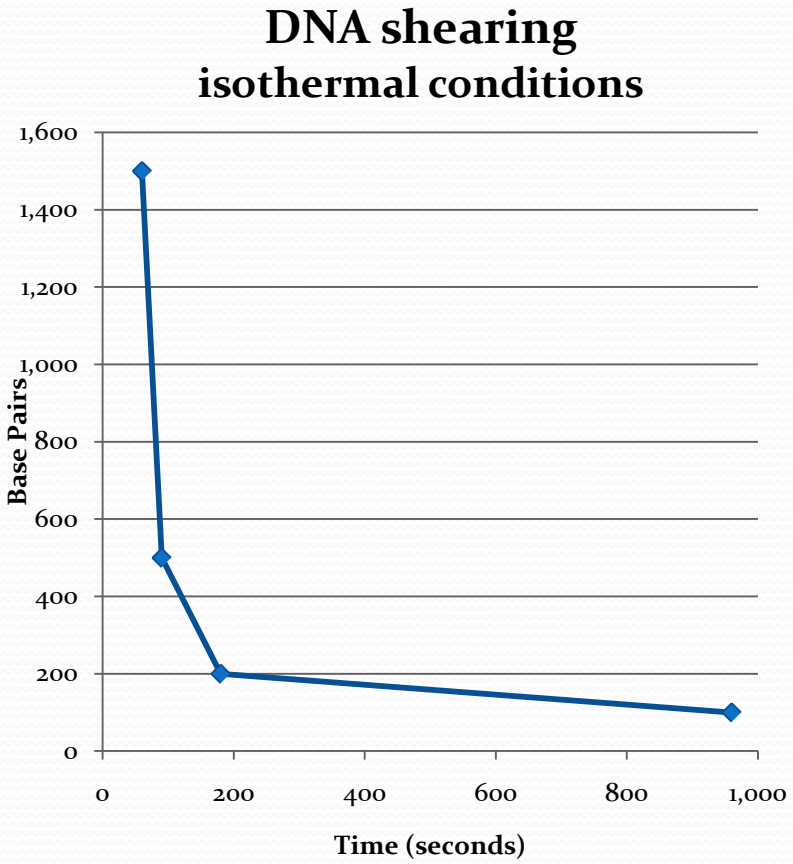


…highly repeatable process

isothermal dose – vary time

- **With a high regularity process**
- Data from Covaris protocols with symmetrical fragment peaks
  - If energy increased at target fragment size > asymmetrical (e.g., 10% dc = symmetrical and 20% asymmetrical at a given target fragment size)
- Processes that require less than 500bp maybe susceptible to operator-induced dsDNA shearing conditions…
  - Therefore, a “fast” protocol may be introducing a thermal-bias to the desired random fragmentation process

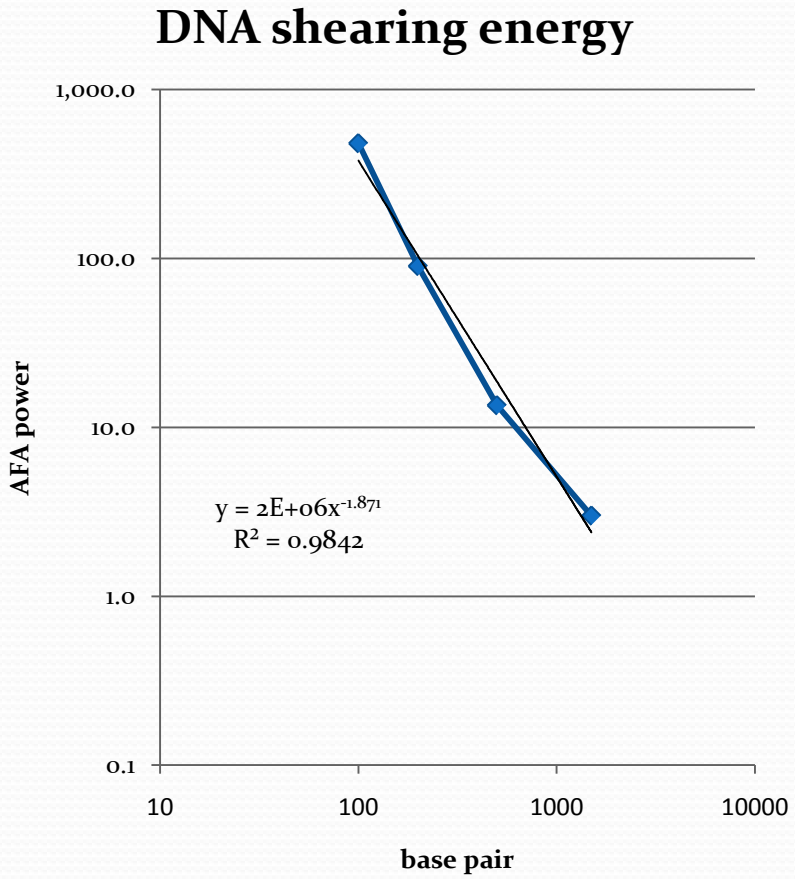
…wide dynamic range of Covaris AFA



dsDNA shearing with AFA energy

- AFA power - reproducible
  - Duty cycle, intensity, seconds
  - Data from Covaris protocols
  - lambda dsDNA in 100ul TE
- Vessels
  - microTUBE (glass)
    - <1.5kb
- Small bp constraint
  - 160x more energy required to generate 100bp than 1500bp (not 15x)

generation of small bp with a mechanical process requires high energy … potential for thermal-bias if process is overdriven



constraints

- Extremely low energy will shear genomic dsDNA
- Generation of large fragments (>1.5kb)
  - Hard to control
  - Difficult to obtain narrow fragment patterns
- Extremely high energy is required to generate small fragments (<0.5kb)
  - Concerns of thermal bias in fragment patterns
    - AT melting
  - Sample loss – single strand generation

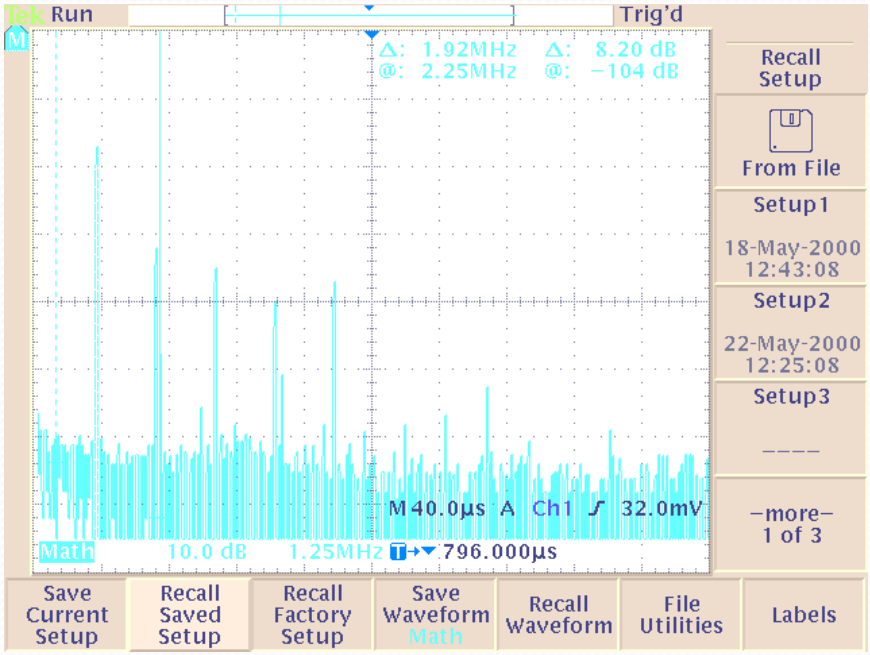
implies ~1000bp may be a natural inflection point of shear-based fragmentation

Large fragments

- Defined as >1.5kb
- constraints on achieving narrow fragment bands
  - DNA extraction process variations
  - Biological variation of starting materials
  - Very low shear stress results in ~1.5kb

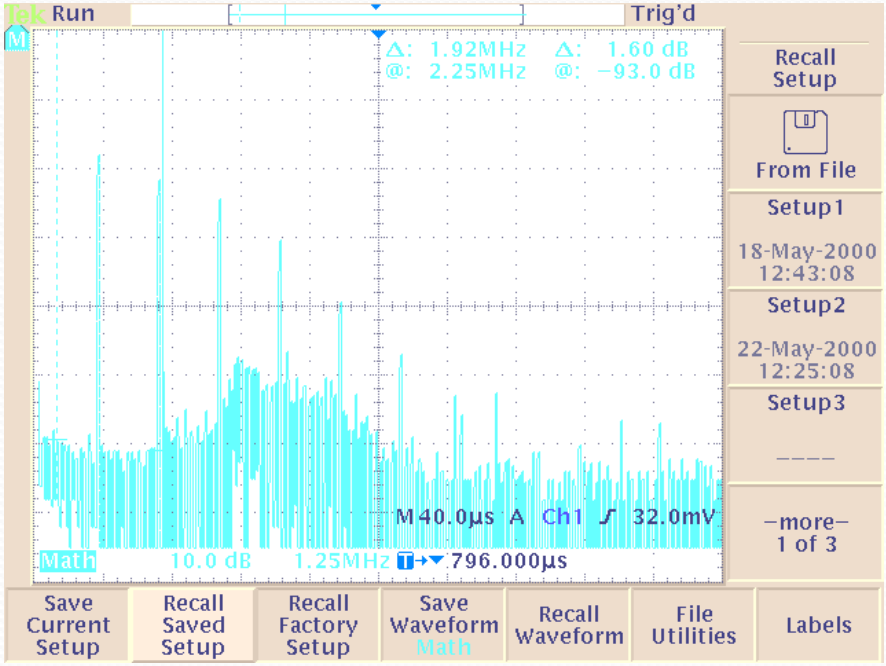
no texture

- Applied acoustic dose
- Slight cavitation
- Fast Fourier Transform of 5 MHz Passive Cavitation Detection signal



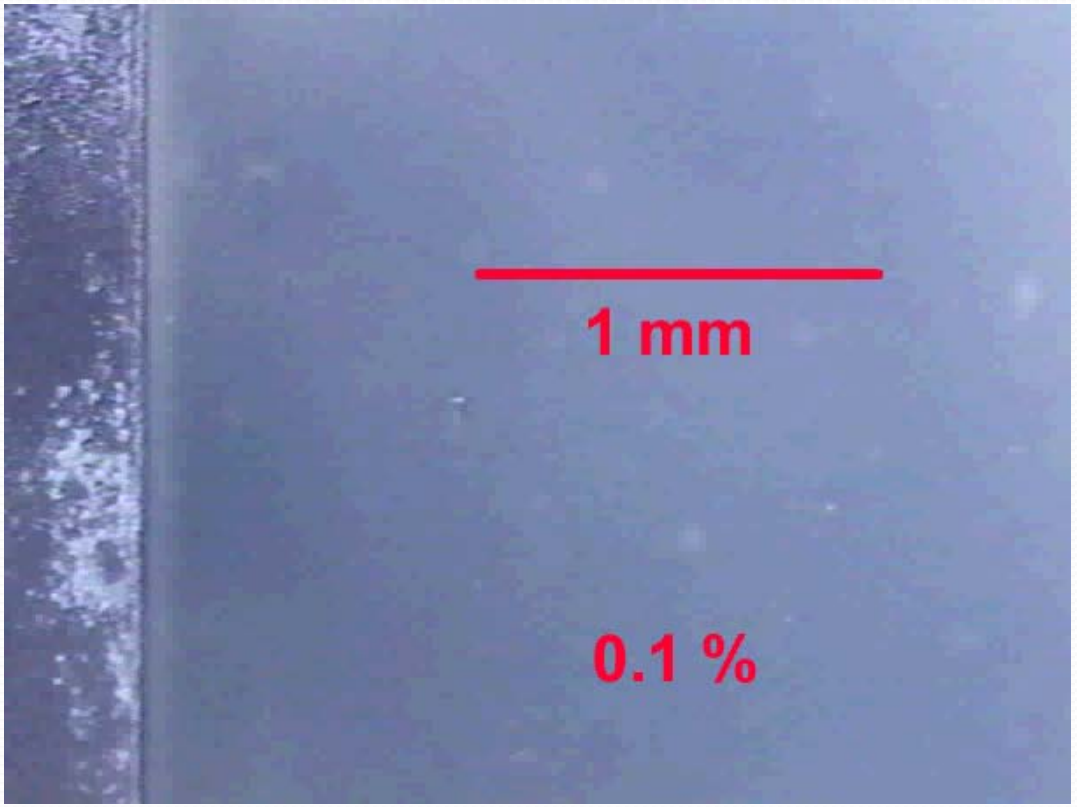
textured surface

- Applied acoustic dose
- FFT analysis of 5MHz receiving transducer - echo
  - passive cavitation detection
- Broadband signal
  - repeatable
  - reproducible



different intensity > different eddy size

- Covaris AFA control allows precise shear force generation
- Video
  - 50um gap
  - TiO2 particles
  - Real-time

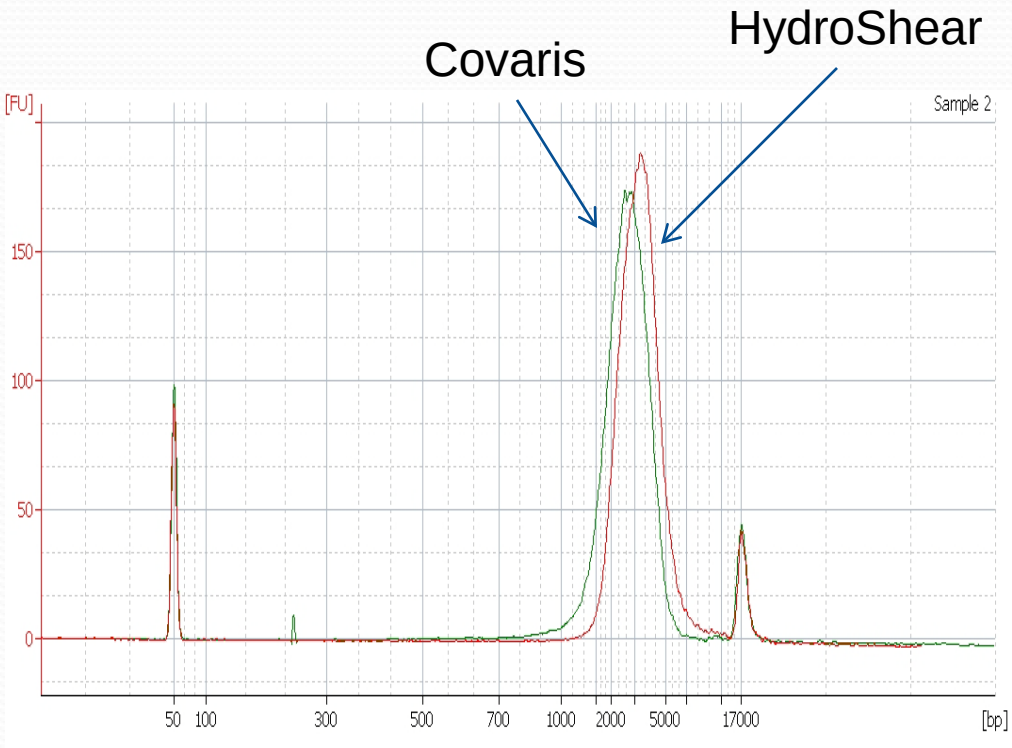


miniTUBE

- PCR tube and AFA Plug
- Active surface for Covaris AFA dose
  - Textured field of active features
  - Eddy

similar physical process

- Similar recovery with identical mass
  - Genomic DNA starting material (3-5 ug)
  - 3ug/100ul with Covaris
- Symmetrical peak shape
  - Shear forces



Small fragments <500bp

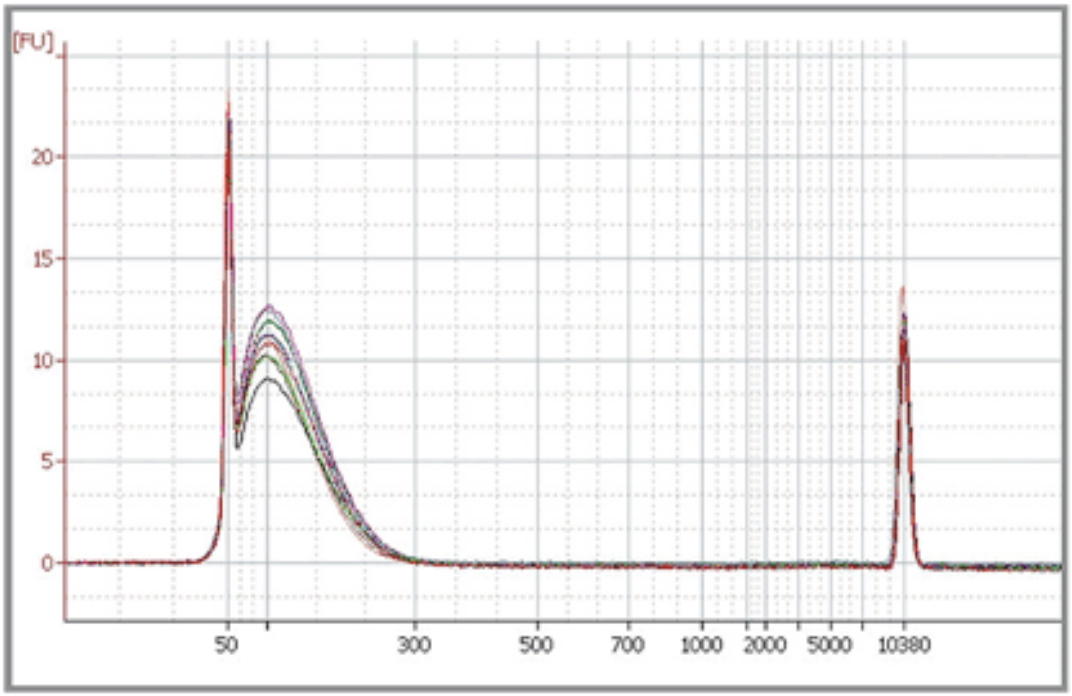
- Extremely high energy requirement
  - Easy to overheat sample
- Potential to damage shearing randomness
- Human tendency to accelerate, but detrimental effects
  - Sonicators > heat
  - Glycerol > poor buffer choice

microTUBE

- Features
  - Tube and AFA fiber designed to enable high intensity acoustic field with minimum bubble cloud formation (scattering effect)
- Benefits
  - Robust
  - Precise
  - 100 µl (may use 50 µl)
  - More efficient

microTUBE

Reproducible



N=12

Avg=100bp

CV =2.6%

S2 and E210 systems

TE buffer

100ul volume

Lambda DNA
(starting material)

	Ideal	Covaris	Genomic Solutions	Branson	Diagenode
Instrument		S2 / E210	Hydroshear	Probe Sonicator	Bioruptor
Technology		Focused acoustic beam	Pressure flow	Acoustic vibrations	Acoustic vibrations
		High Intensity Focused Ultrasound	Pressure drop	Low frequency unfocused	Low frequency unfocused
Shear Mechanism		AFA cavitation	Flow boundary layer	Cavitation on metal tip	Cavitation on metal
<100bp	Yes	Yes	No	No	No
100bp < X < 1.5bk	Yes	Yes	No	Yes	Yes
>1.5kb	Yes	Yes	Yes	No	No
Isothermal	Yes	Yes	Yes	No	No
Hands on (<1 min)	<1	Yes	No	Yes	Yes
Batch process	Yes	Yes	No	No	Yes
Random shearing	Yes	Yes	Yes	No[1]	No[1]
No clean-up	Yes	Yes	No	No	Yes/no
Closed vessel	Yes	Yes	Yes	No	Yes
Non-contact	Yes	Yes	No	No	No
Repeatable	Yes	Yes	Yes	No	No
Small volume	Yes	Yes	No	No	Yes/no

1 – concerns of biased shearing with heat generated

Fragmenting – 1st sequencing step

- Current options and observations
- Covaris systems – The Solution
- High-throughput directions

Higher-throughput

- Serial process
  - Batch process
    - 96 samples (E210 apparatus)
  - Improve acoustic circuit efficiency
    - Electronics (*i-series*)
    - Sample Vessel (microTUBE, miniTUBE)
      - 8 tube strip
- Parallel process
  - Modify L8 for DNA high power process

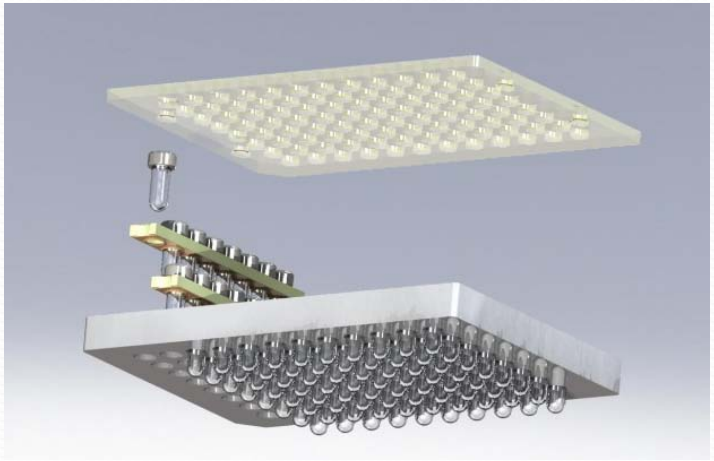
E210 – serial, batch

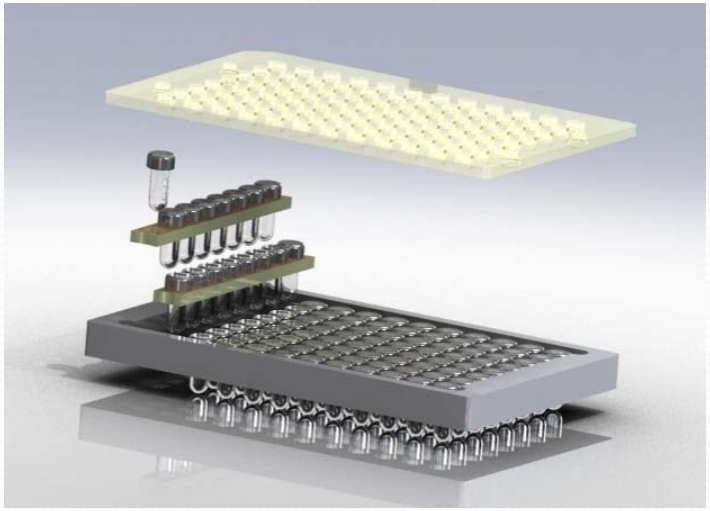
- SBS microplate format
- Unattended operation
- DNA shearing range
  - 100bp-3kb (8kb TBD)

- Stand-alone format
  - ActiveX control
  - API's for TECAN Evo
  - CE marking
  - ETL (NRTL) testing

tube strip (new)

- microTUBE
  - Designed for high intensity-power applications
    - Spore lysis,
    - 100bp fragment generation,
    - ChIP fragment generation
- miniTUBE
  - Designed for low intensity apps
    - >1.5 kb fragments
- Both sold as
  - 8 tubes/strip (for 96 format)
- Run
  - 1, 2,…12 strips/process
  - 1 tube (7 empty)

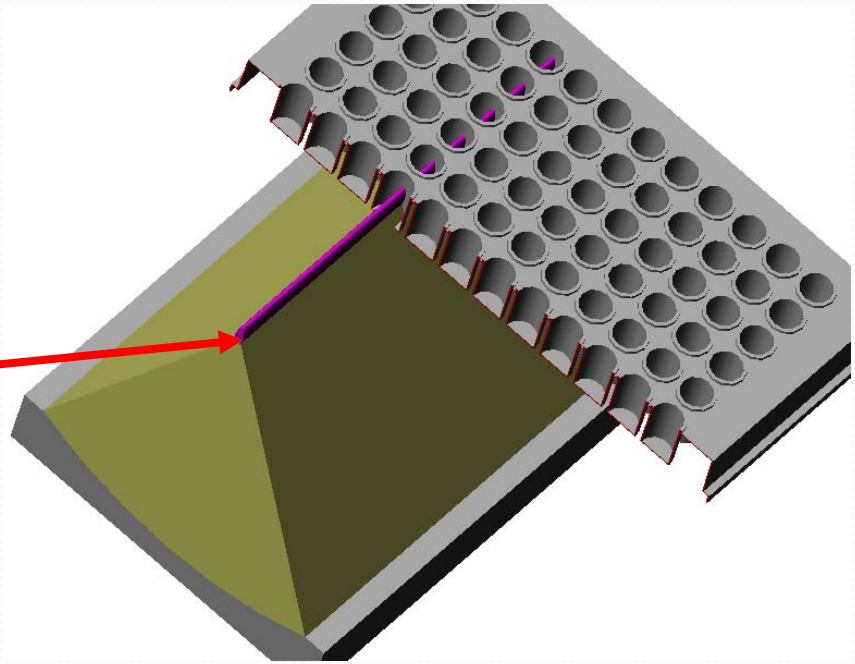




…with thermal control

parallel process

- Non-contact
- process an entire row at once
  - NOTE: acoustic side lobes not shown
- scan across plate
  - e.g., 30 seconds/plate
- link with robotics for HTS



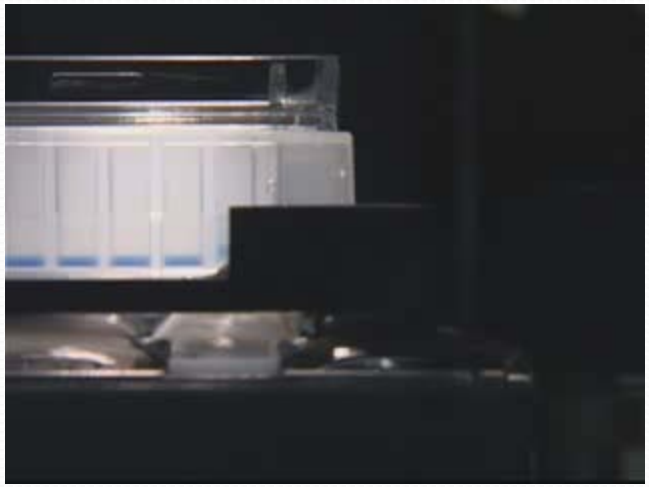
… currently for

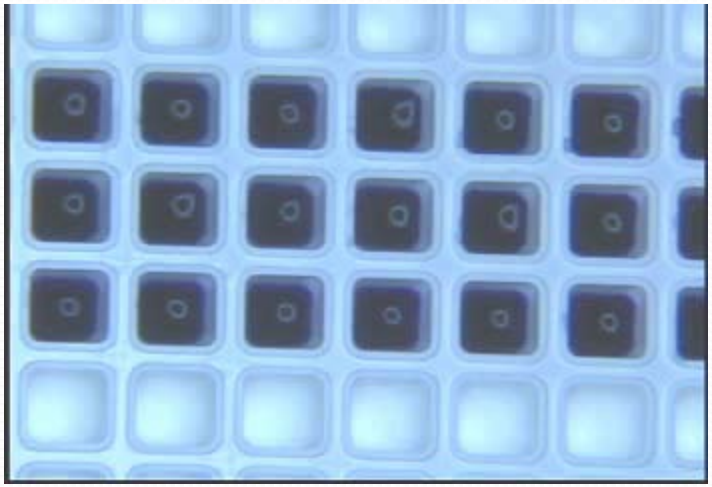
low power processes

parallel – low intensity

- “line transducer”
  - Treats entire row at once
    - e.g., 30 seconds/plate
- Video
  - 384 well Corning plate
  - 25 ul water with dye (bottom)
  - 100 ul (top)
  - Real-time, right to left
  - NOTE: air/liquid interface
  - No cover

… Non-contact

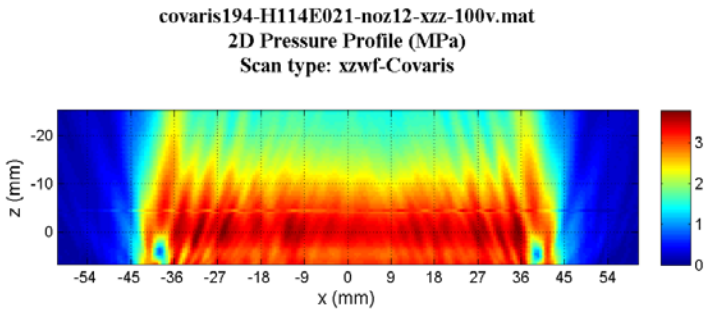


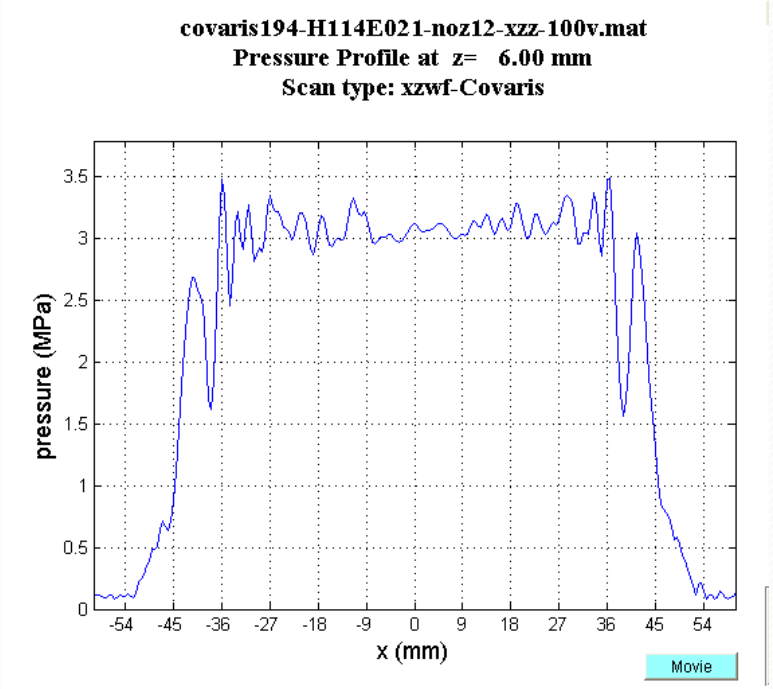


L8 – commercially available
L8i (high power) – under development

- L8i
  - Parallel 8 tubes (with 96)
    - 32 wells with 384 plate
  - All tube in thermal control
  - Higher power than L8
  - High power applications
    - 100bp
    - Spore lysis
- Controlled dynamics
  - Density (initial)
    - 96 – in 8 tube strips
  - Sample volume (96 plate)
    - 50-100ul per well in microTUBE
    - 50ul in miniTUBE

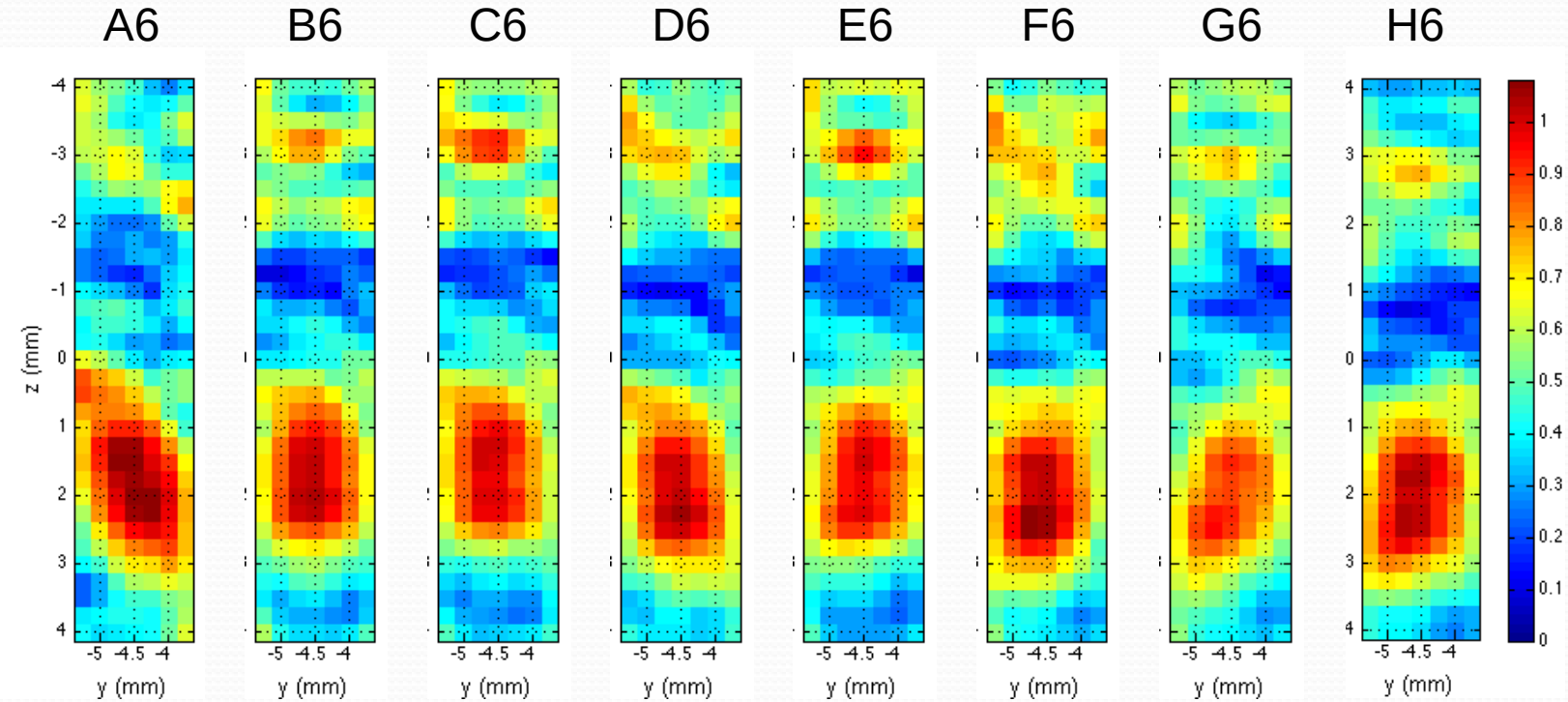
Exp1: Acoustic Map, 6x16mm glass tubes, 96 well format
XZ plot, no tube rack (XDCR w/nozzle 12)





Full scale amplitude = 3.79 MPa
Maximum pressure at Z=0, the geometric focus, which is also the well plate reference surface
Cross-section at Z=6, corresponding to the inner-bottom of the tubes

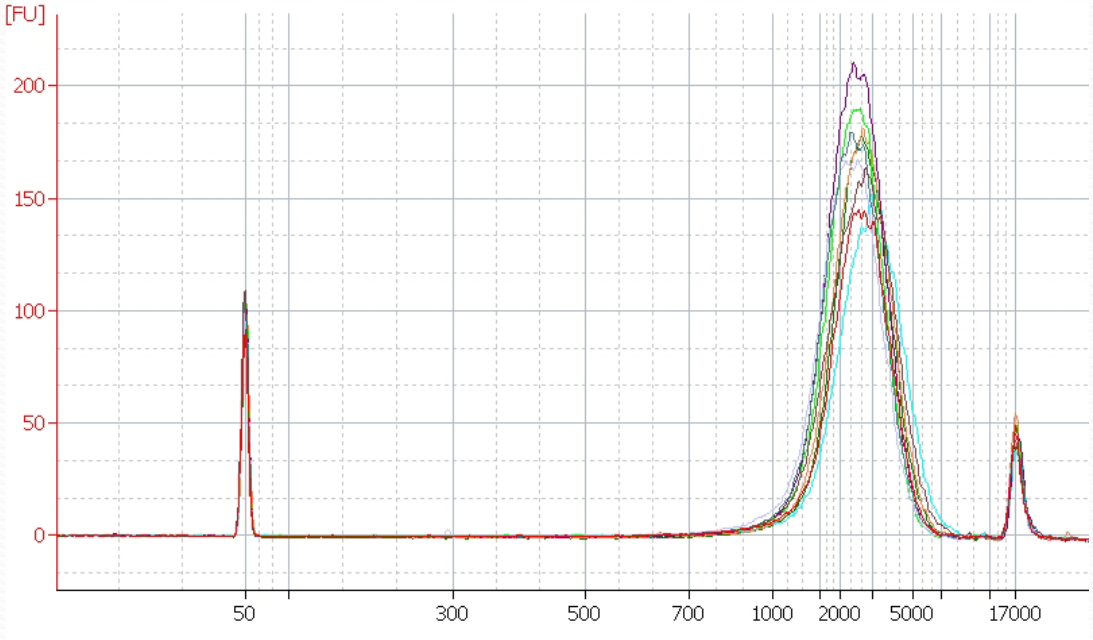
Exp1: Acoustic Map, 6x16mm glass tubes, 96 well format
YZ slice at X midline



Bottom of tubes (outside) at Z=7.0 mm
Full scale amplitude = 1.084 MPa

L8i - miniTUBE

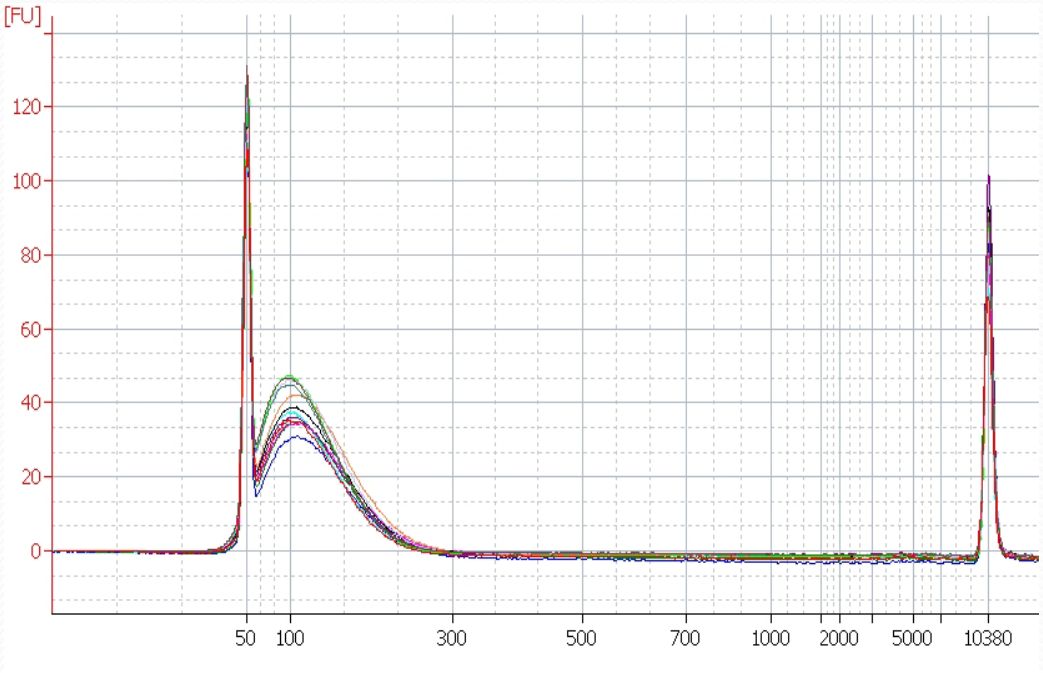
Lambda – TE
miniTUBE plug in
PCR plate
Peak 3kb
8 samples/7 minutes



L8i - microTUBE

Lambda dsDNA in TE
microTUBE
Peak 100bp
8 samples

Avg 99bp
s.d. 2bp



Systems – key points

Large Fragments

- HydroShear
  - Potential cross talk
  - No automation
- Covaris AFA
  - No cross talk
  - Automation ready

Small Fragments

- Nebulizer
  - Poor recovery
  - No automation
- Sonicator
  - Fast, heat not controlled
  - Potential cross talk
- Covaris AFA
  - Automation ready
  - Thermal control

summary

- Instrument
  - One unit for both libraries (S2/E210)
  - Highly reproducible energy
  - True thermal control
    - If not overpowered
    - If Newtonian fluids used
- Consumable
  - Fragment library
    - 100-130 bp
  - Mate-pair library
    - 1.5kb, 3kb, (8kb TBD)

The Sample Prep Advantage

Control

- Tissue/Cell Disruption
- RNA / metabolite Extraction
- Compound Dissolution
- Biomolecule separation
- Assay acceleration
- Mixing

- Standardize with
  - Identical energy for each sample
  - Non-contact
  - Closed vessel
  - Isothermal

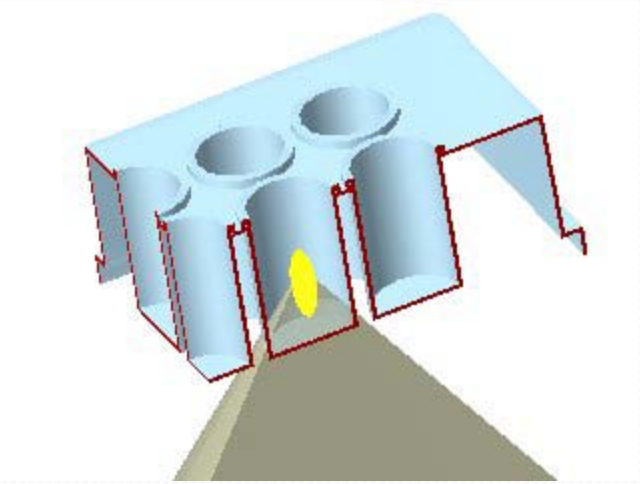
Covaris systems enable the introduction of lean/6 Sigma philosophy into many processes

Covaris™

the sample prep advantage

Covaris, Inc.
14 Gill Street, Unit H
Woburn, Massachusetts
01801-1721 USA

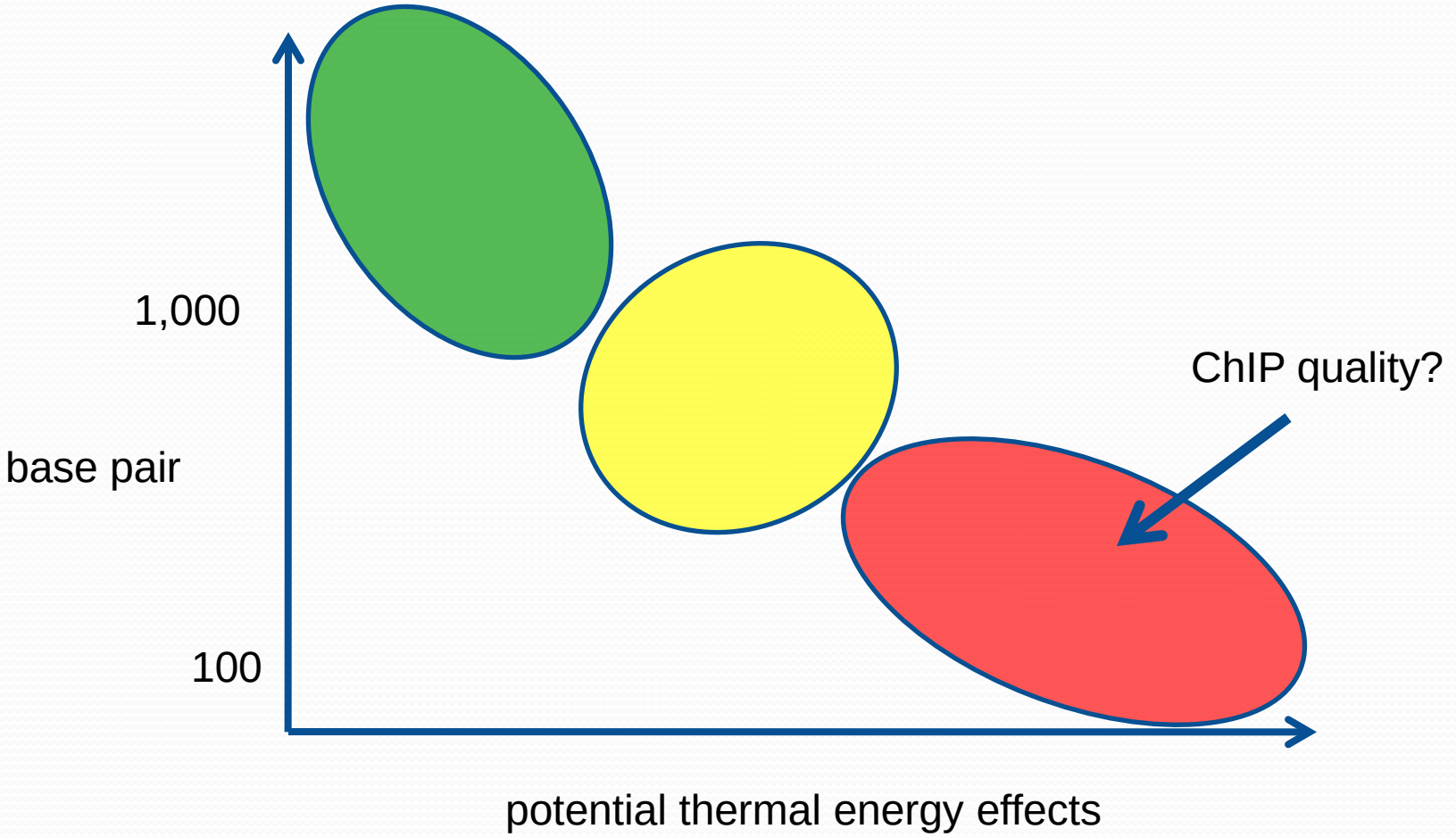
telephone: +1 781 932 3959
fax: +1 781 932 8705
e-mail: info@covarisinc.com
http:// www.covarisinc.com



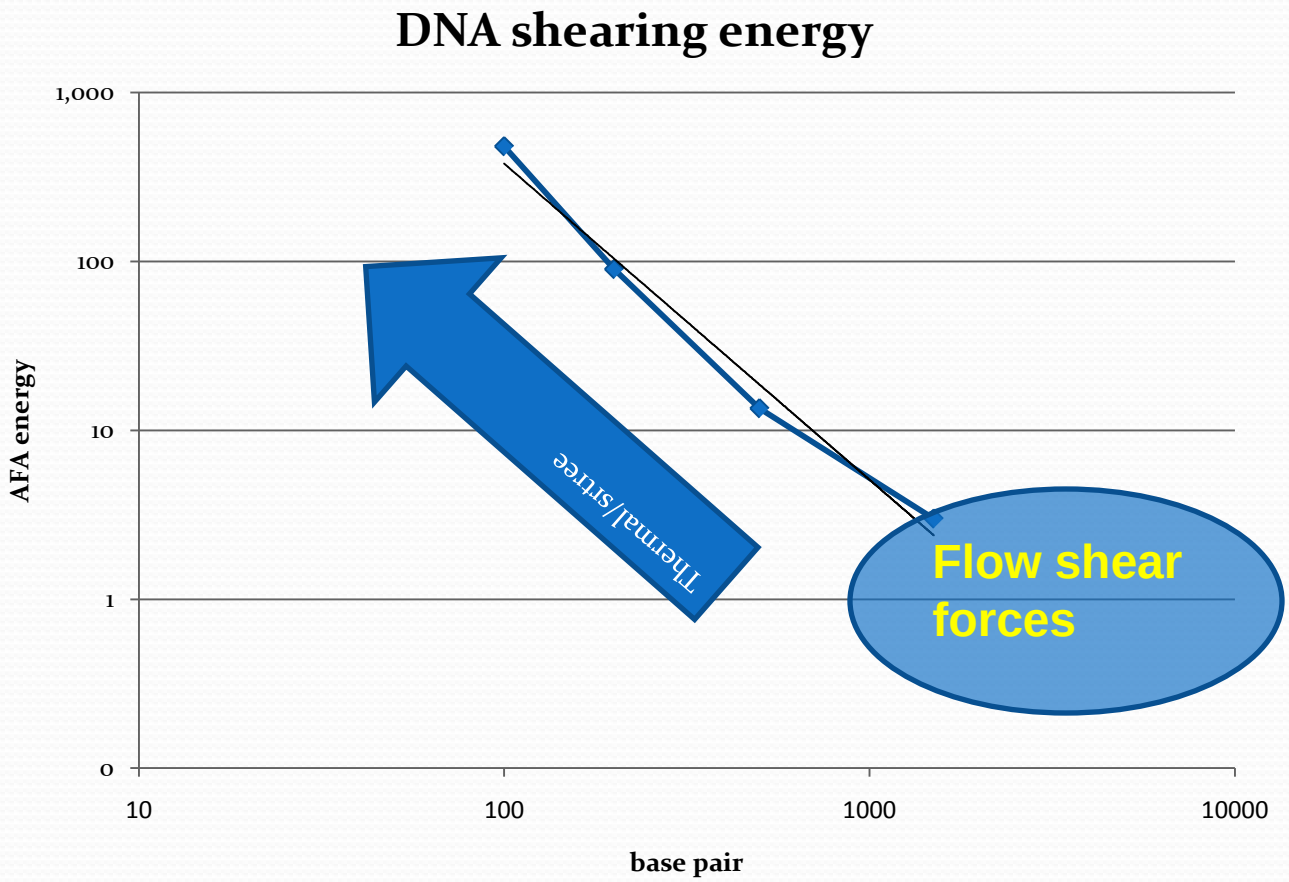
Take home message

- Potential to bias next gen sequencing
- “Fast” shearing is dangerous, not better
  - Thermal damage if sheared too fast
    - Especially small fragments as high energy is required
- AFA controlled energy delivery across broad range
  - Large fragments (miniTUBE)
  - Small fragments (microTUBE)

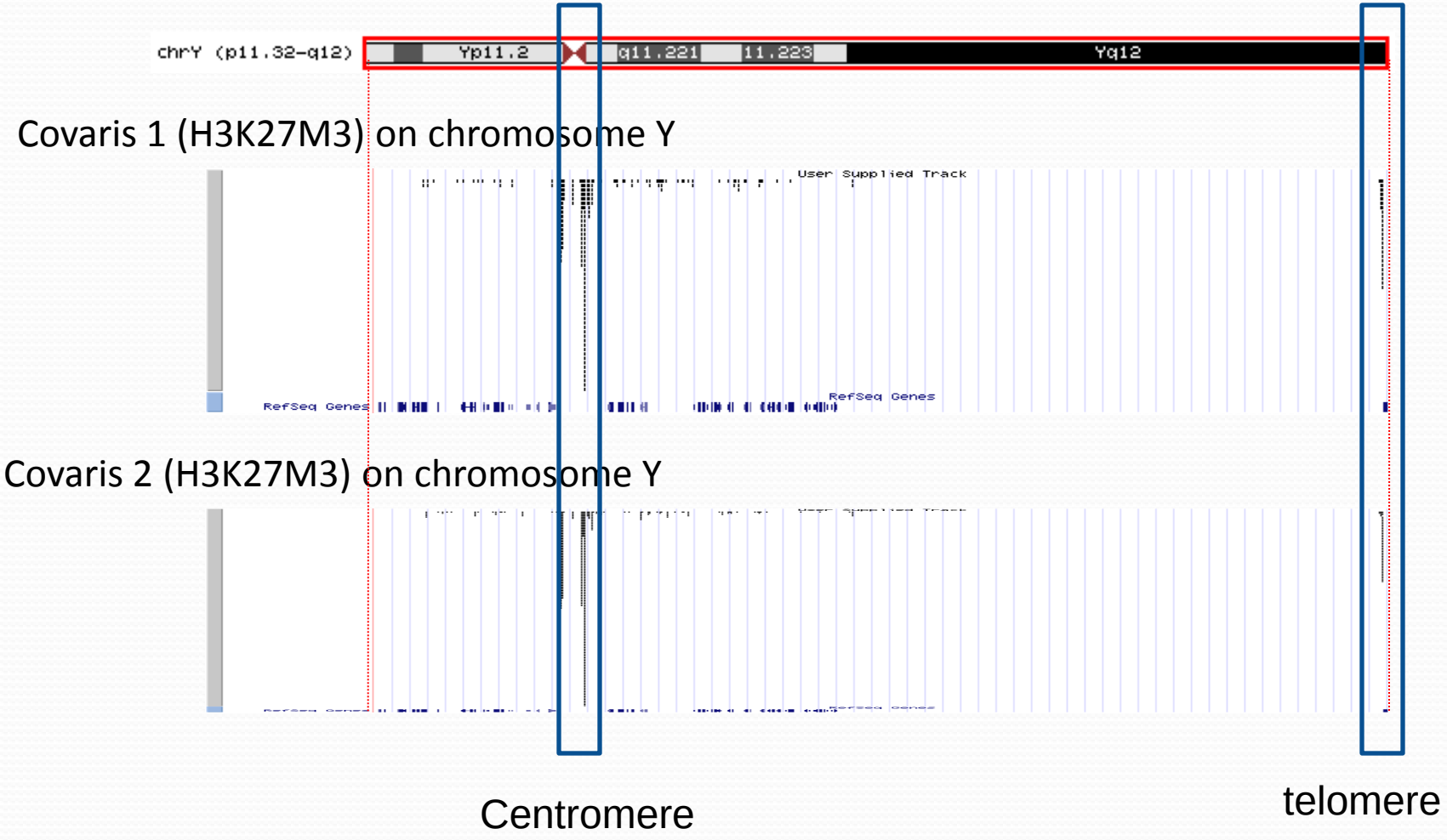
Different fragment = different energy



dsDNA shearing with AFA energy

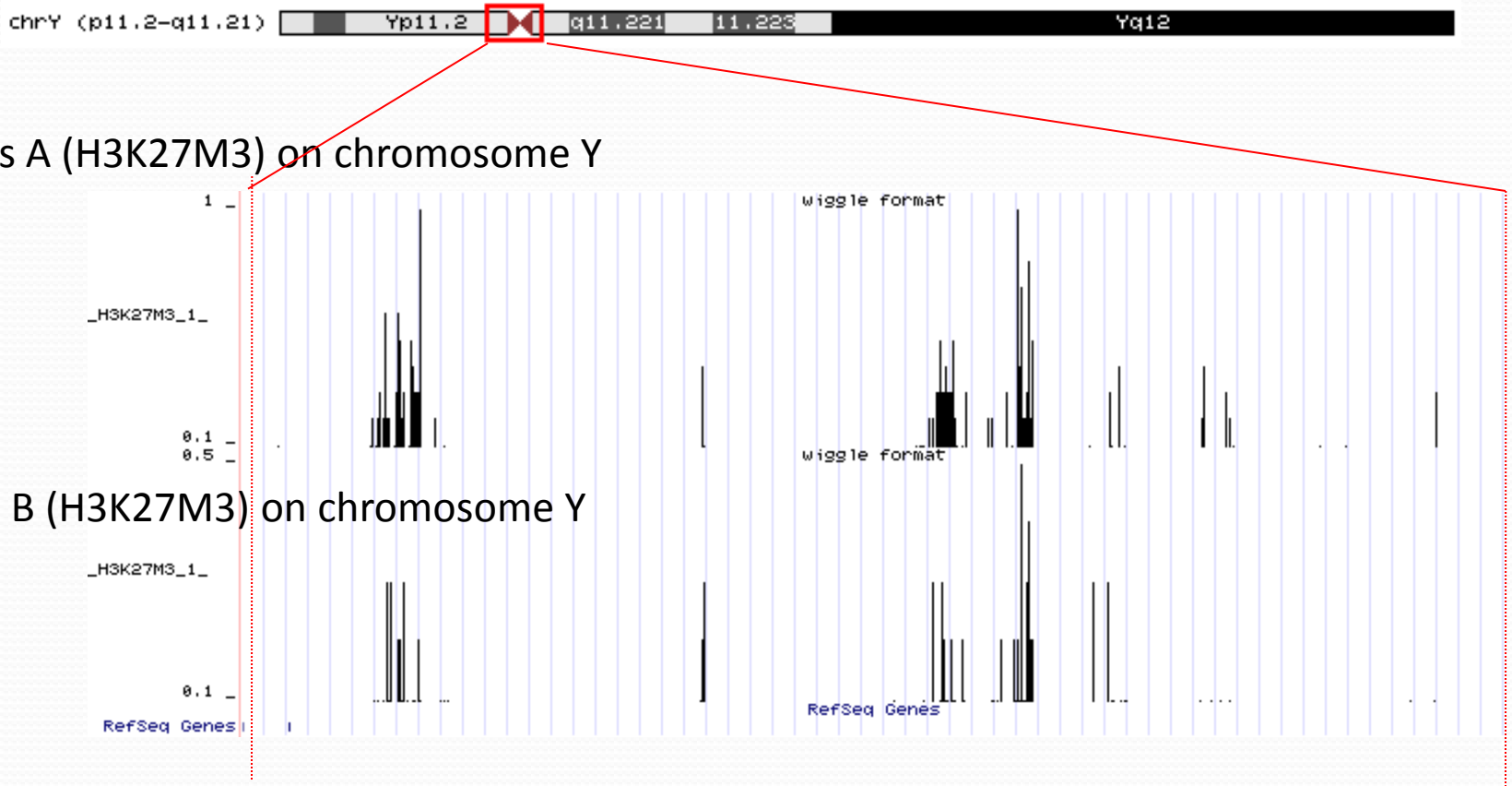


Comparing duplicate ChIP-SEQ data obtained by Covaris S2 ultrasonicator in entire chromosome Y



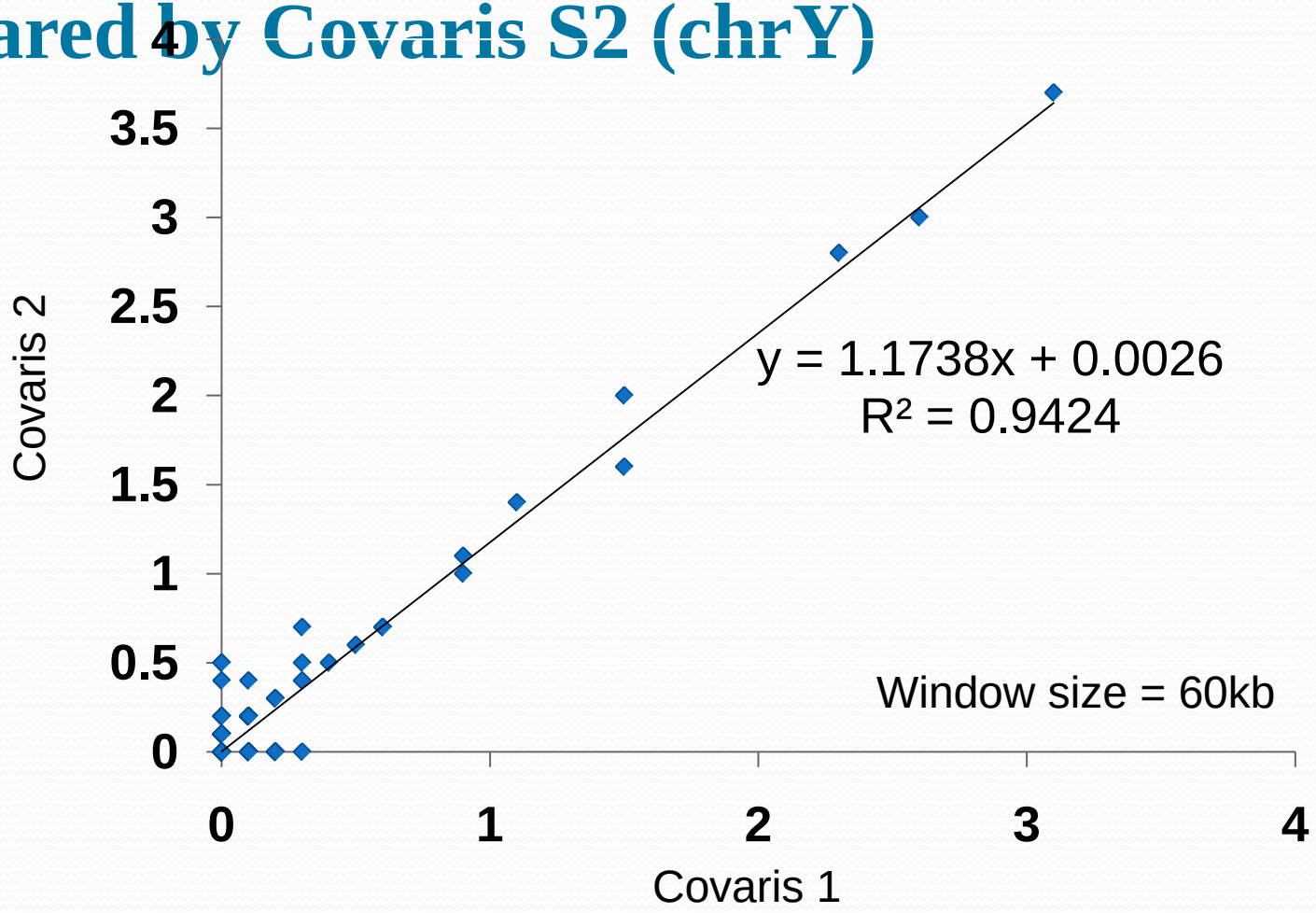
We observed high enriched patterns in both centromere and telomere from two biological independent experiments with Covaris S2

Comparing duplicate ChIP-SEQ data obtained by Covaris S2 ultrasonicator in small window



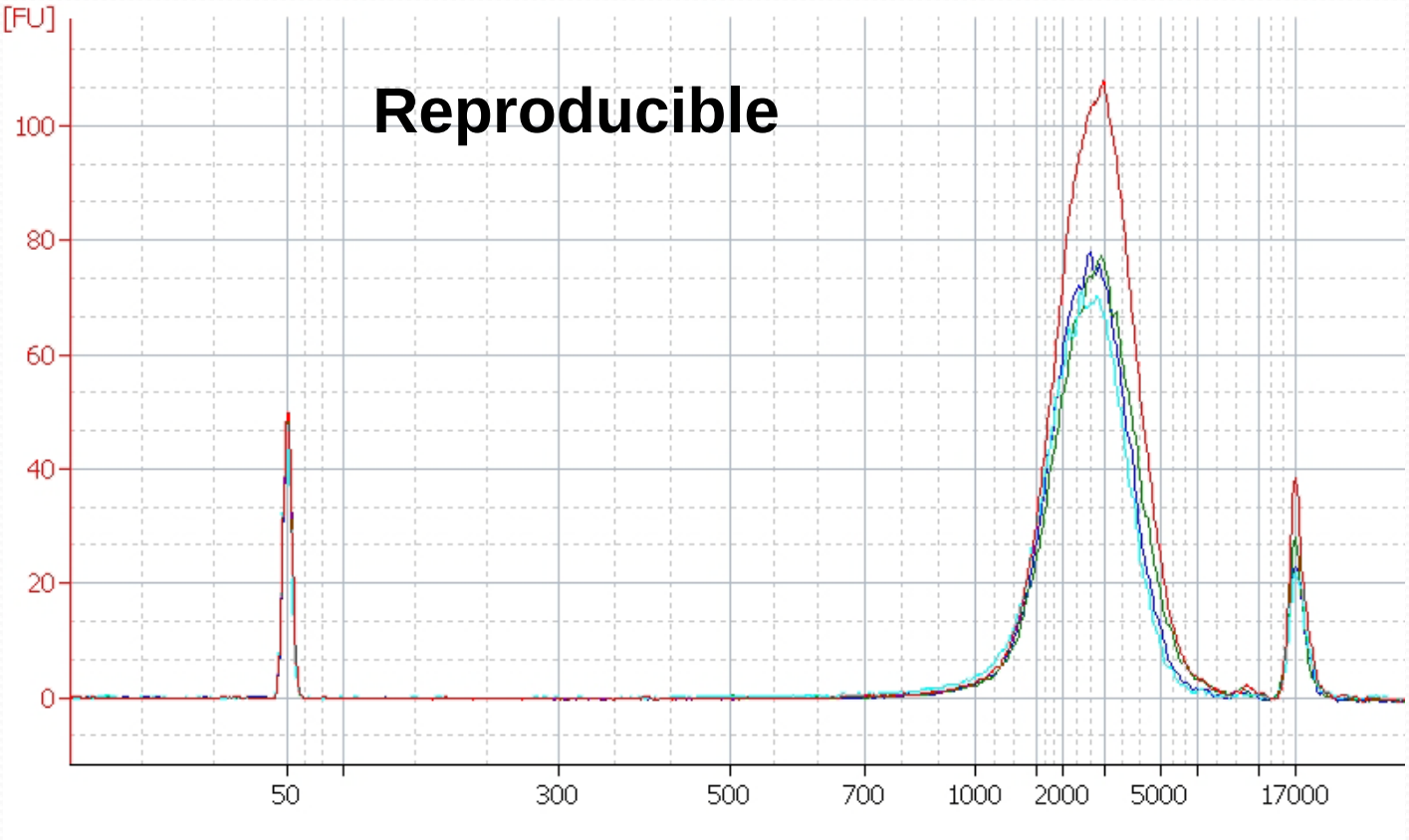
We observed high enriched patterns in both centromere and telomere from two biological independent experiments with Covaris S2

Correlation of duplicated ChIP-SEQ data sheared by Covaris S2 (chrY)



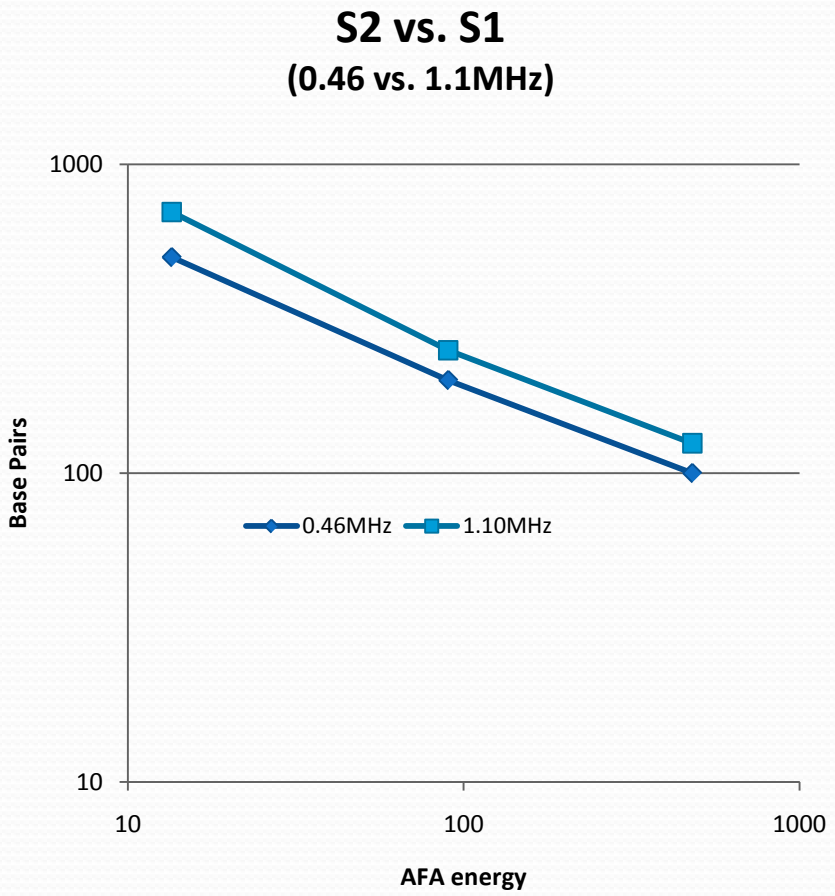
We observed high correlation of intensities from two biological independent experiments with Covaris S2

miniTUBE


[FU]
Reproducible
100
80
60
40
20
0
50
300
500
700
1000
2000
5000
17000

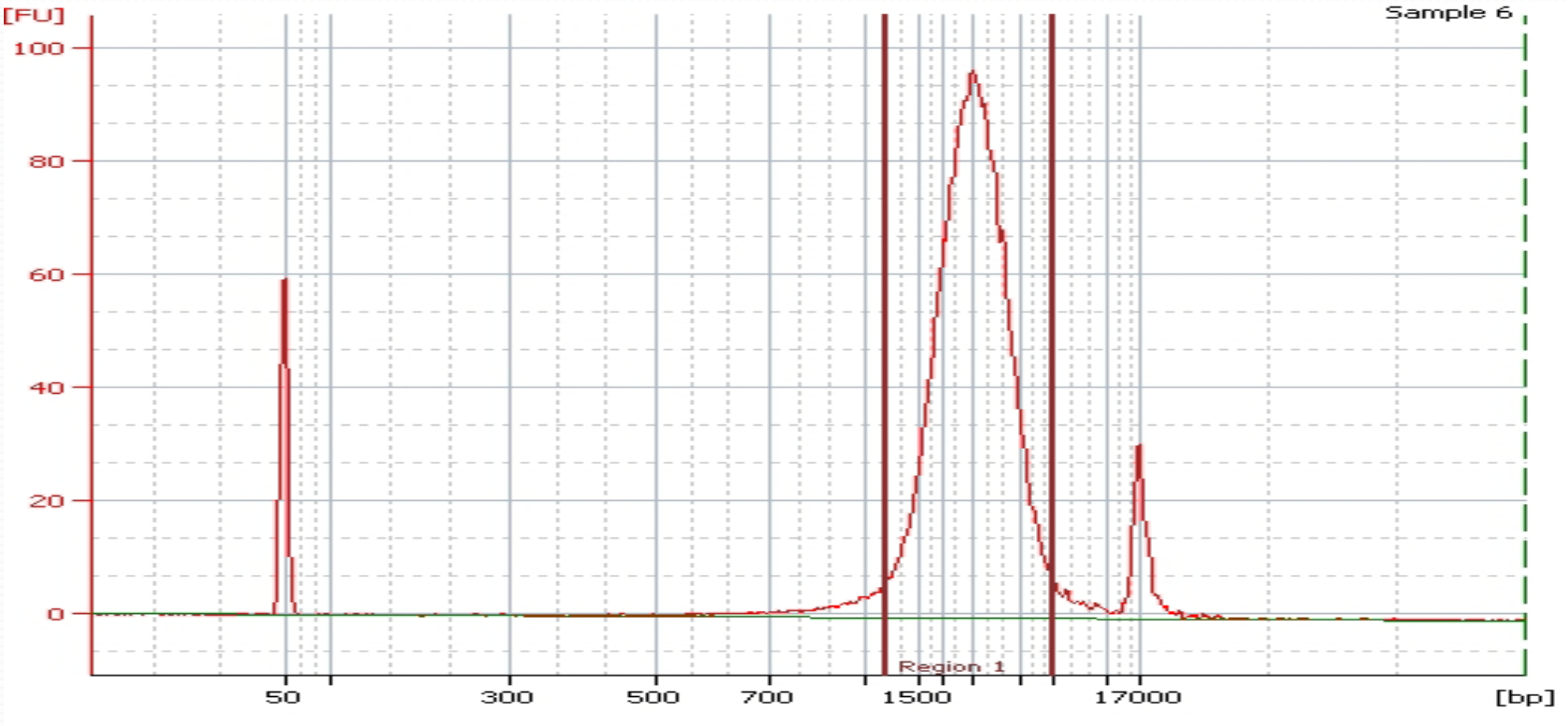
Process improvement - frequency

- microTUBE
- Identical dose (AFA energy)
- 20% less energy required with lower frequency (0.46MHz) S2 system than with higher frequency S1 (1.1MHz) system to obtain the same effect
  - AFA energy in S2 = 200bp
  - Same dose in S1 = 250bp
  - Faster process at lower frequency



3.0kb (lambda dsDNA)

- 92% between 1.2 – 7.0 kb



Potential system improvements

- Earliest potentially available
  - Higher power L8
    - L8i – summer/fall 09
  - Lower frequency
    - L4i – fall/winter 09
  - Lower volume
    - L4i + tube – winter 09/10
  - Higher density plate (384)
    - 2010

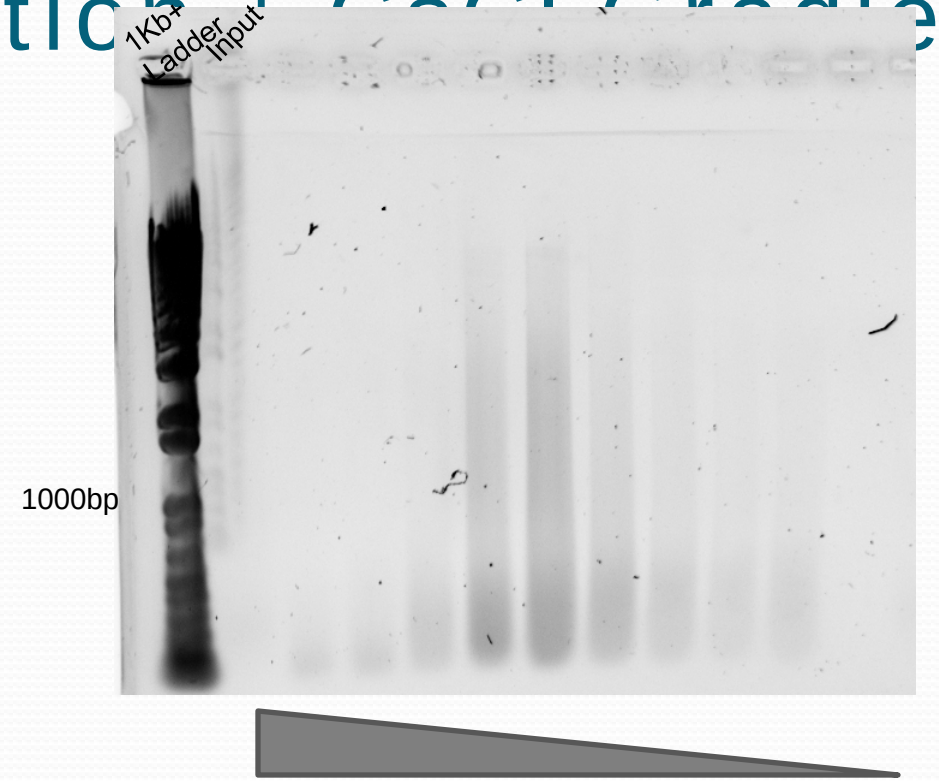
… cumulative potential to process
384 samples to 150bp in ~60 minutes

Fractionation Shear

Duty cycle:	10
Intensity:	5
Frequency:	200
Cycles:	37x15”
Volume:	500 uL
Buffer:	LB2
Total Cells:	$2x10^8$

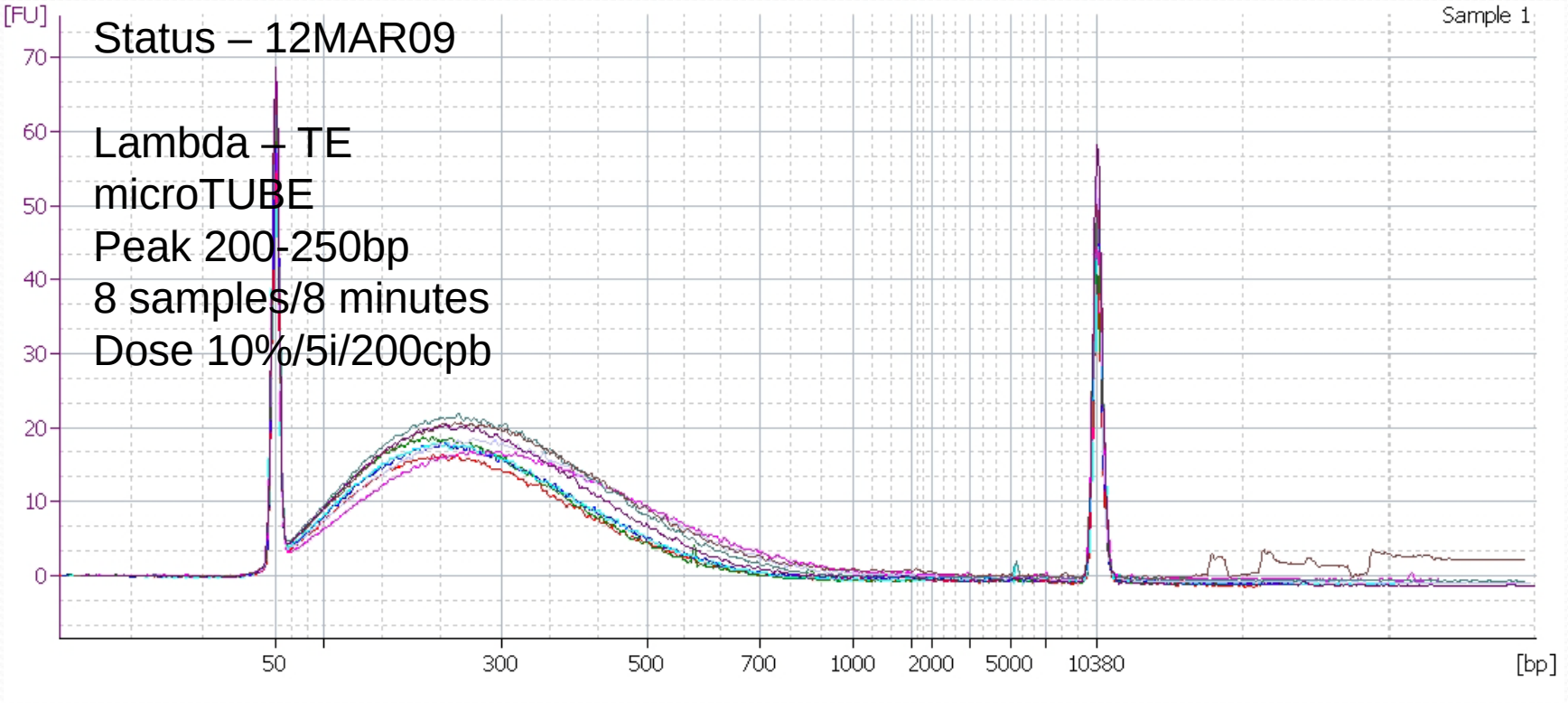
Cross-linked nuclei were subjected to the above conditions on the Covaris machine. The resulting solution was spun hard and the soluble portion applied to a density gradient. The gradient was fractionated by volume. The DNA from each fraction was isolated after RNAse and ProK treatment and run out on a pre-stained EtBr agarose gel.

Chromatin from Branson sonication + CsCl Gradient



Compared to the shearing in slide #2, the distribution of the DNA sheared using a Branson sonicator (in nearly identical conditions) has higher variance.

L8 - DNA shearing



Process improvement – 96 to 384

- Processing time is reduced as mass of sample (e.g., 100ul > 20ul) is reduced
- Width of focal zone may be increased (L4x) to process two rows simultaneously.
  - Therefore, instead of 8 samples in one “dose”, there would be 32 samples in one “dose”
- Processing time for 384 should be similar to total time for 96 samples,
  - if the volume is lower
  - if the vessel is acoustically matched to transducer

Process improvement – new circuit

- microTUBE
- Identical dose (AFA energy)
- 80% energy required with new circuit board

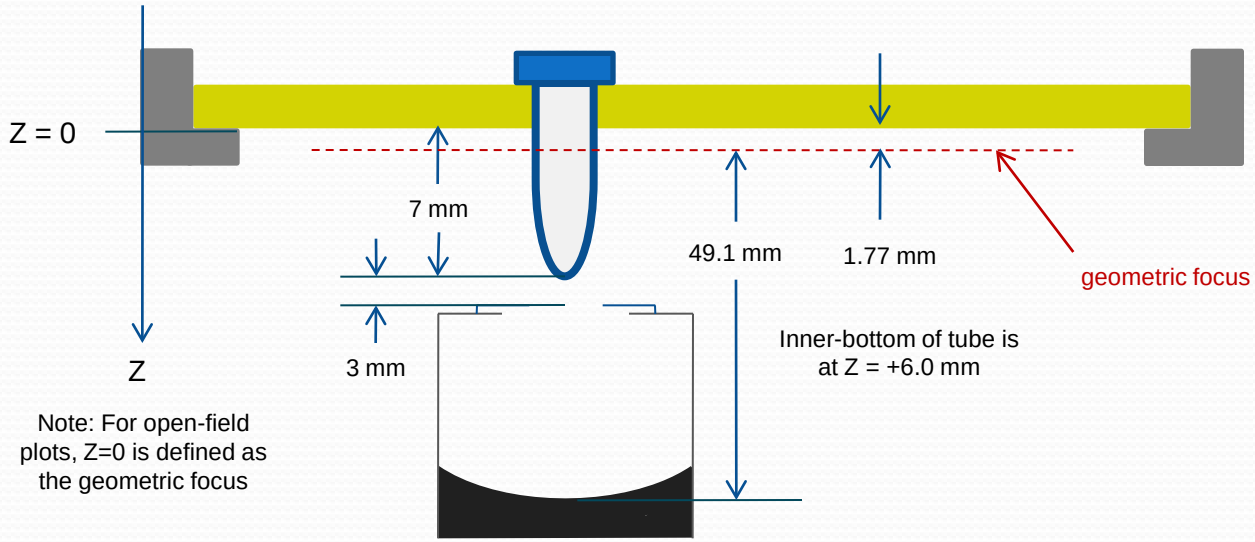
Process improvement - vessel

- Improve transmission efficiency of acoustic energy across vessel wall
- Current microTUBE – borosilicate
- Future microTUBE
  - Engineering polymers
    - High temperature
  - Ultem, Liquid Crystal Polymer, Nylon, etc.

Process improvement – volume

- Processing time is reduced as mass of sample (e.g., 100ul > 20ul) is reduced
- Same energy for 100ul and 50ul results in ~25% increase in response

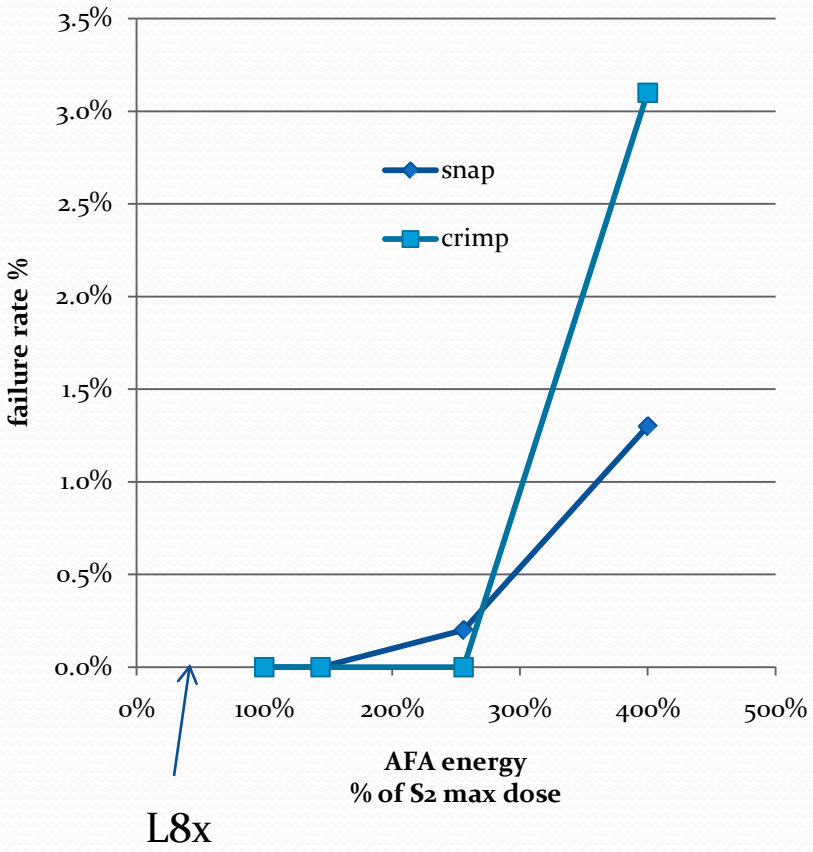
Experiment 1: Dimensions and coordinates, tube rack, 3mm gap



microTUBE - failure rate (lot 3)

- S2 – test dose (>2000 tubes tested)
  - Duty cycle = 20%
  - Time = 560 seconds
  - Cycles/burst = 200
  - Vary intensity 5, 6, 8, 10
- S2 – Covaris recommended maximum dose (100%)
  - Duty cycle = 10%
  - Intensity 5
  - 0 failure with 16k shipped

L8x max. = 30% energy/tube



Glass microTUBE: Situation

- Energy required to obtain <100bp is high
  - Serial process of S2/E210 stresses tube with point focal zone
    - Breakage 1/1000
  - Parallel process of L8/L8x requires less energy as it is spread over a 100mm line and only 44% reaches tube
    - Breakage – none to date

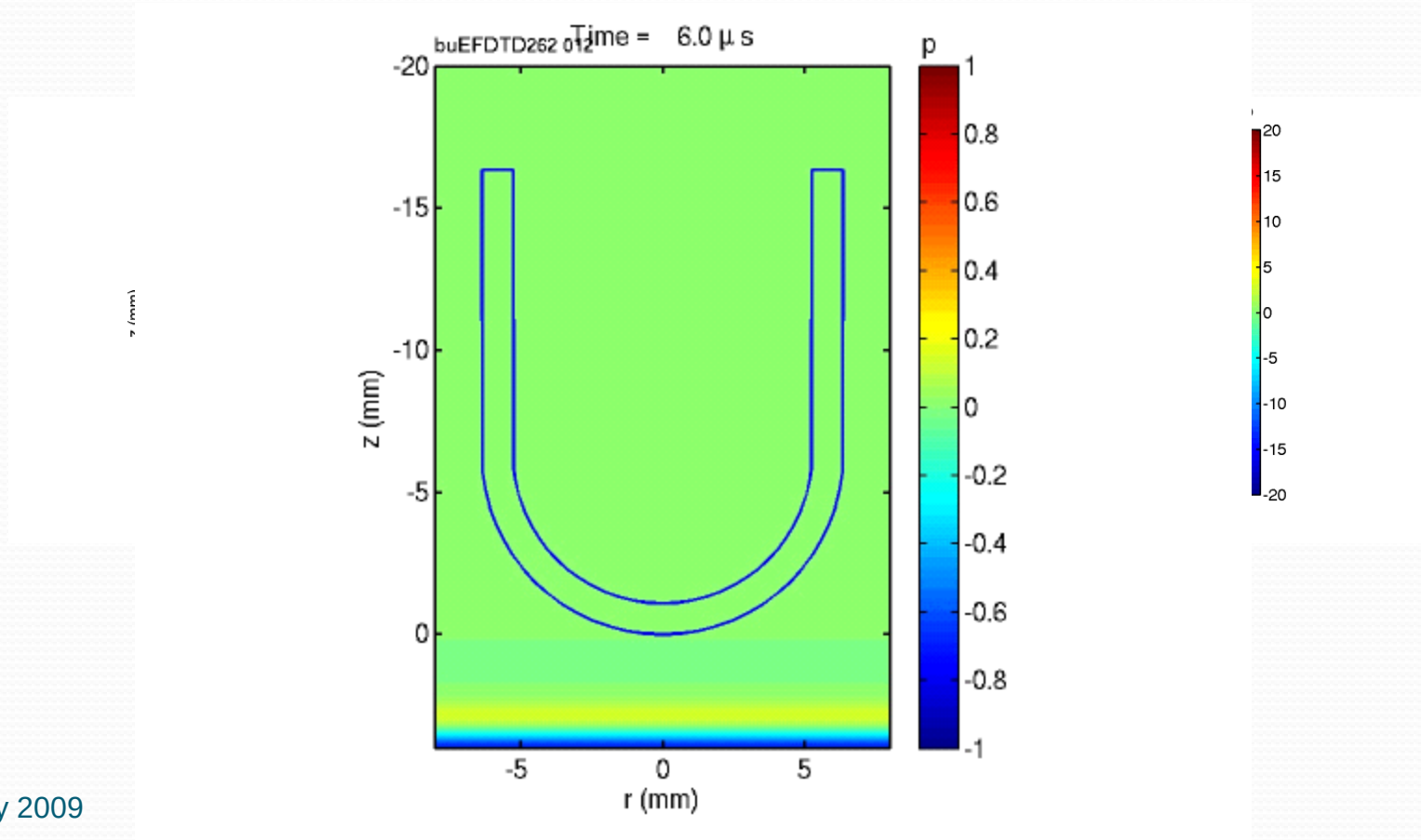
L8 less energy/tube

100bp	**S2**	**L8**	**L8x**
minutes	8	32	16
watt - peak	32	400	600
transducer length in mm		100	100
microTUBE OD in mm		5.5	5.5
number tubes		8	8
width in microTUBES		44	44
% microTUBE		44%	44%
watt - tubes		176	264
duty cycle	10%	10%	25%
watt - 8 tubes		17.6	66
tubes		8	8
watt/tube	32	2.20	8.25
	100%	7%	26%

Focusing through

tpx

glass

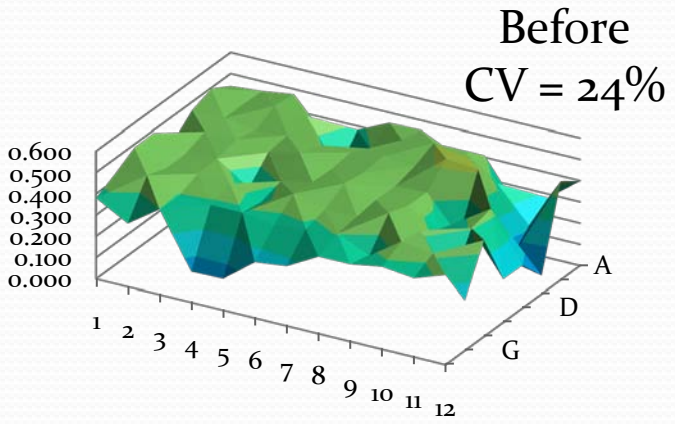


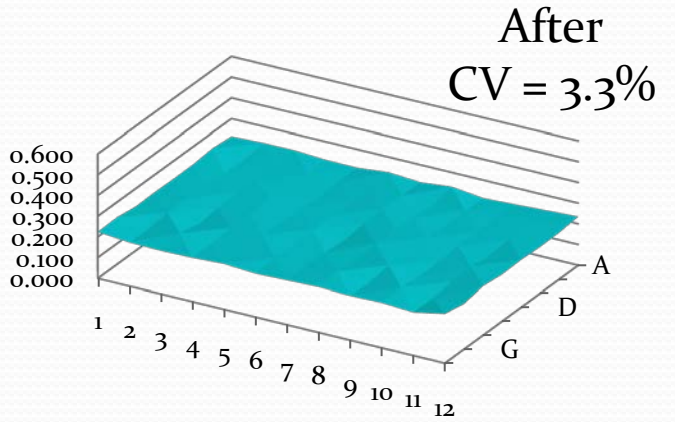
L8 – parallel AFA process

- L8
  - Parallel samples in row
    - Lower intensity than point transducers
  - General purpose
    - Enzyme, dissolution, etc.
  - API
- DNA shearing
  - microTUBES
    - >300bp – 8 samples in 2 minutes
    - **<100bp – 8 samples in __ min**
  - miniTUBES
    - >3kb – 8 samples in 7 minutes

L8 – low intensity

- 5µl of 9.6 nM p-nitrophenol was dispensed into 100µl of 0.1 N NaOH stock buffer.
- Absorbance, read soon after dispensing, showed high variance and incomplete mixing.
- Following a single 30 second Covaris AFA treatment, mixing homogeneity was strongly promoted, as illustrated by the absorbance data:





L8 – user interface

AFA high intensity

- Yeast
- TE buffer, 100ul, 6degC
- Protein assay - Bradford
- Control
  - Bead-beater
  - 120 seconds
- AFA Dose
  - 20%/5I/200cpb
  - 0, 120, 180 seconds