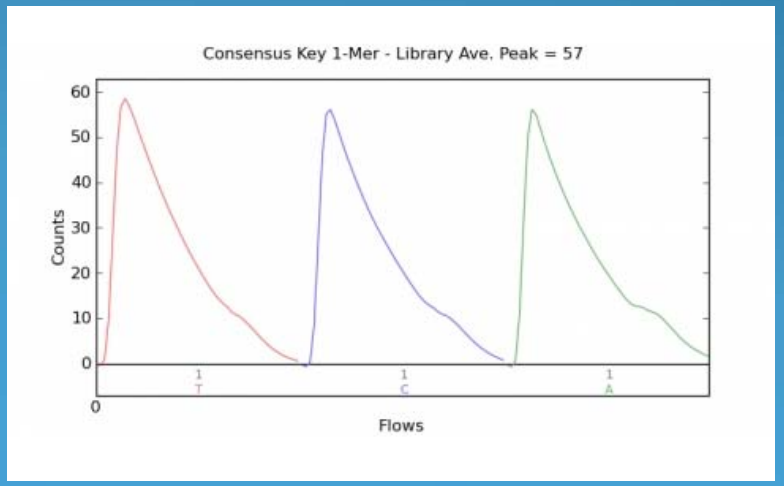
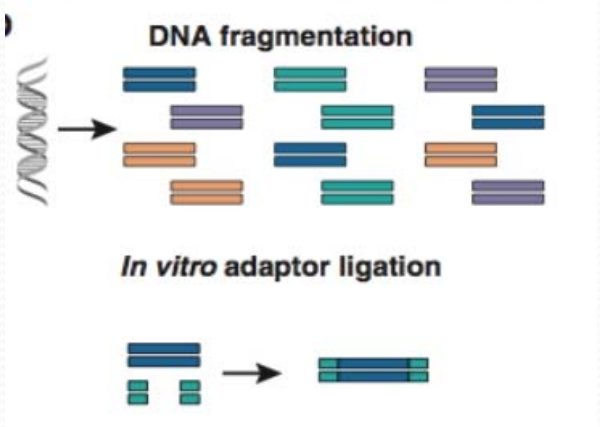
BINF 6350 ITSC 8350
Fall 2011
Biotechnology & Genomics Lab
Creating DNA libraries

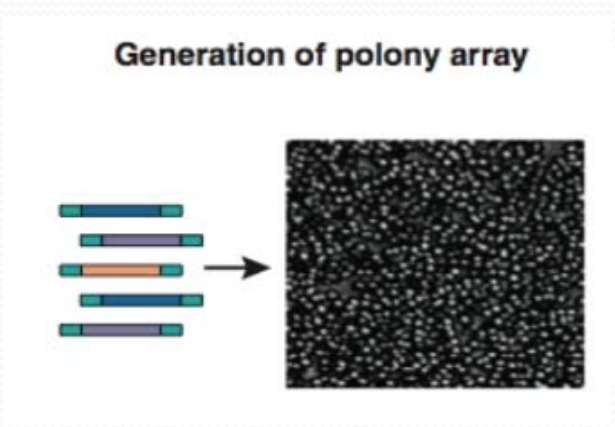
http://webpages.uncc.edu/~jweller2

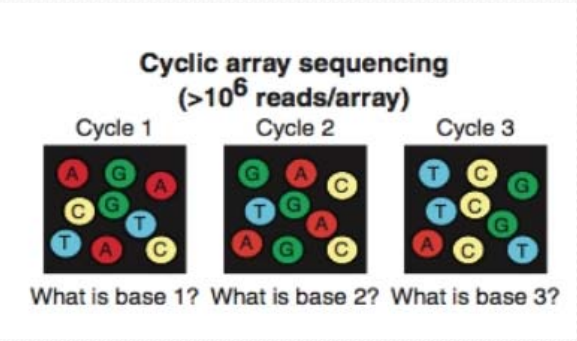


NGS data production steps

- Produce the fragments to be sequenced, clean up
- Modify fragments for processing, clean up
- Separate the modified fragments *from each other*
  - so they don't compete in the PCR process: emulsion reactors, dispersed on a surface
- Amplify each fragment to a base mass
- Sequence-by-synthesis (flowgrams, PPi with reversible terminators, etc.)
- Analyze







Shendure and Ji, Nat Biotech, 2008

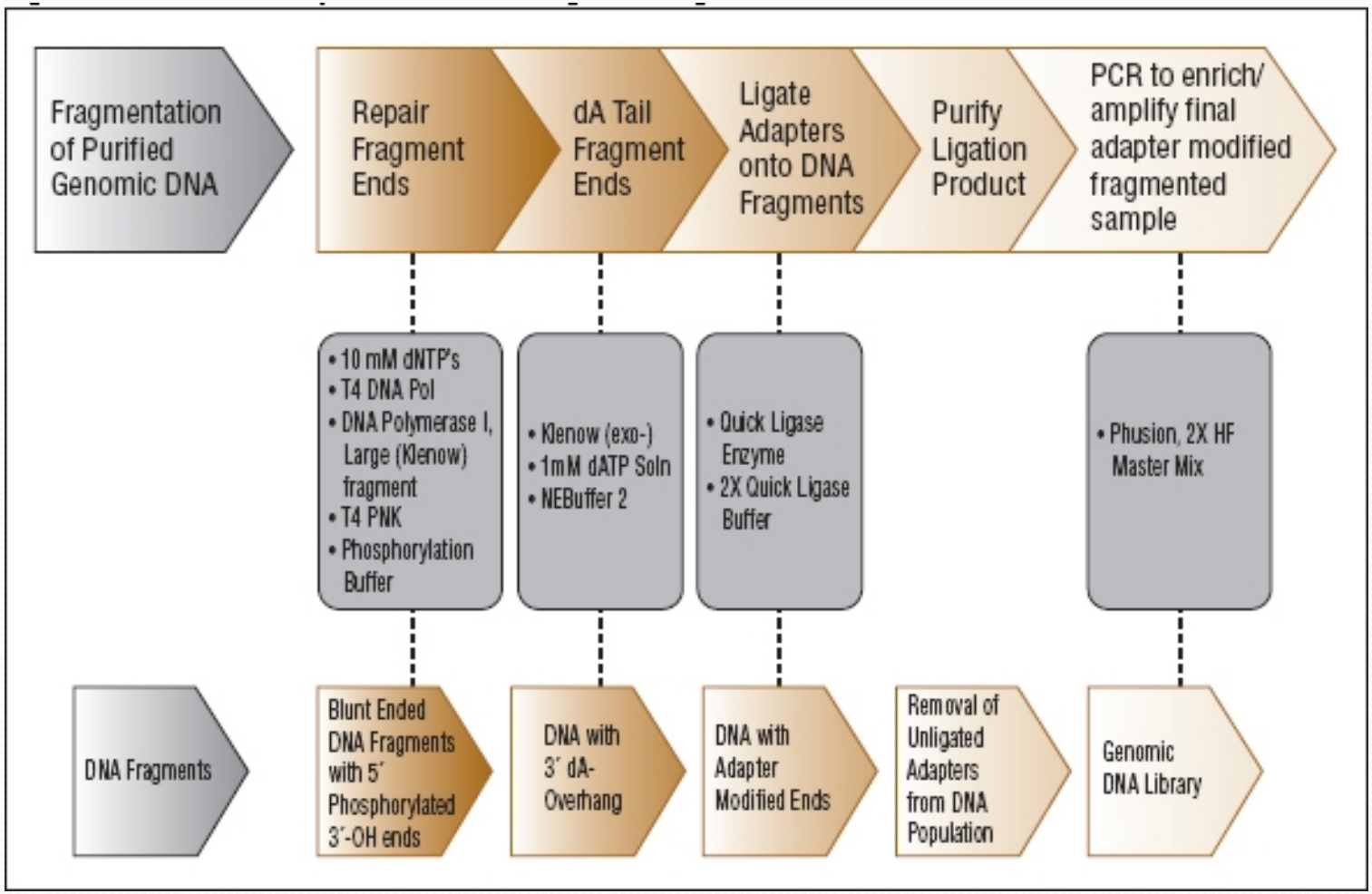
What can be sequenced?

- Genomic DNA
  - Fragmented to a size range usable by the platform
- Chromatin (DNA wrapped around nucleosomes) fragmented and selected for modifications→ ChIP-Seq
  - Chemical conversion of bases for methylated nucleotides
  - Antibody selection of modified histones
- Specific regions of the genome
  - Gene families, exons, introns, UTRs
  - Regions of specific polymorphisms, splice junctions
- RT-PCR generated products: RNA-Seq
  - Selection of specific regions of known size
  - Generic amplification of unknown regions

Fragment and Adapt: modifying NA for NGS

- Long pieces of DNA
  - Enzymatic, Chemical, Mechanical fragmentation
  - Add adaptors
    - End repair, addition of sticky bits
    - Ligation
- Genome-wide PCR products (+/- shearing)
  - Design primers and amplify
    - May need shearing
  - Add adaptors
    - Blunt-end ligation of adaptors
    - Include adaptors in primers
    - Have adaptors use an overhanging 'T' to match the 'A' that Taq adds to amplicons
- (RT)PCR products (+/-) shearing
  - Priming may be general or specific
    - An amplification step may be included
  - Adaptors will have similar rules as genomic PCR products.

Flowchart (featuring NEB mixes)

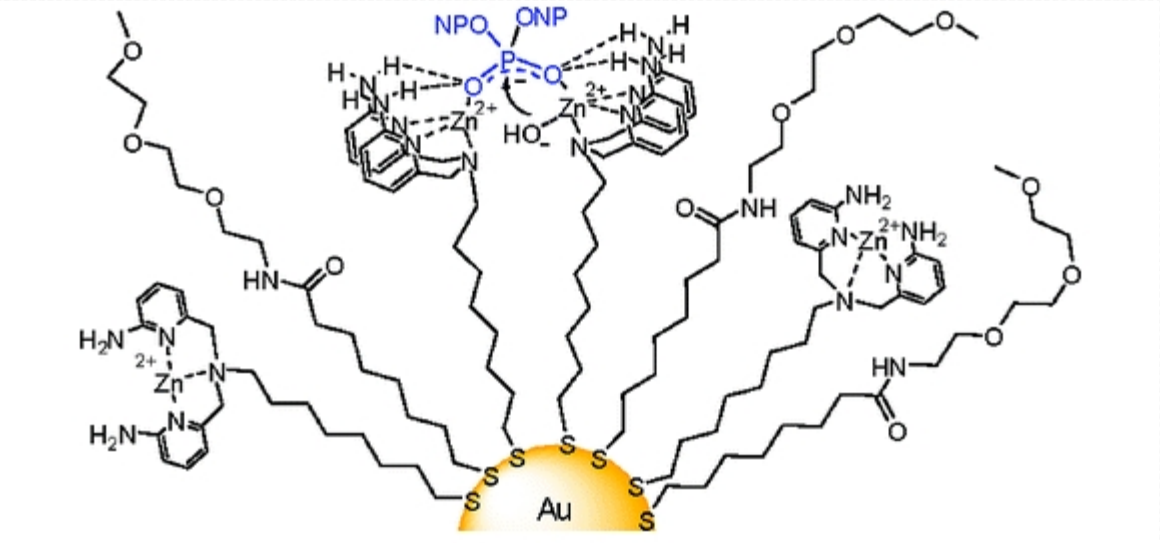


DNA fragmentation approaches depend on degree of randomness required

- Chemical and enzymatic fragmentation have sequence bias
  - Non-random distribution
  - Some material will be selectively lost.
- Mechanical shearing is strictly random
  - Nonbiased* fragment generation
  - No selective loss.
  - *Note: if the mechanical method generates heat then some selective degradation of A/T rich regions will occur.

Chemical Cleavage of Nucleic acids

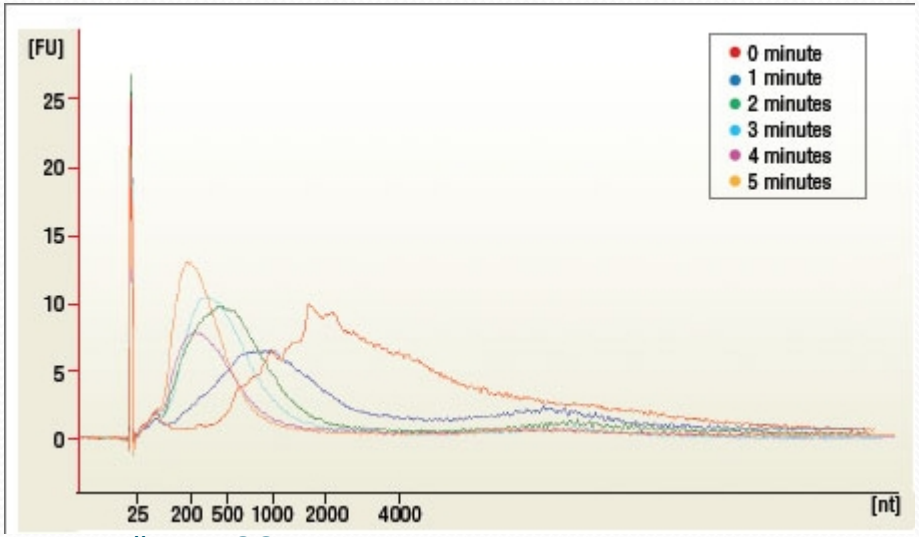
- Acid cleavage of DNA
  - 6N HCl at 110C for 18h, but the bases are separated from the sugars, as well as the phosphate backbone being cleaved
  - Metal catalysts - in the example Zn has been chelated on a nanoparticle (Bonomi et al. JACS 2008) gave ds cleavage.
    - Attachment to an oligonucleotide allows some degree of specificity
- Base cleavage of RNA
  - Performed at pH 9.0 or lower, with a temperature of 95C, for ~15 min.
  - Metal-chelator catalysts (especially with Cu or Co)



RNA example

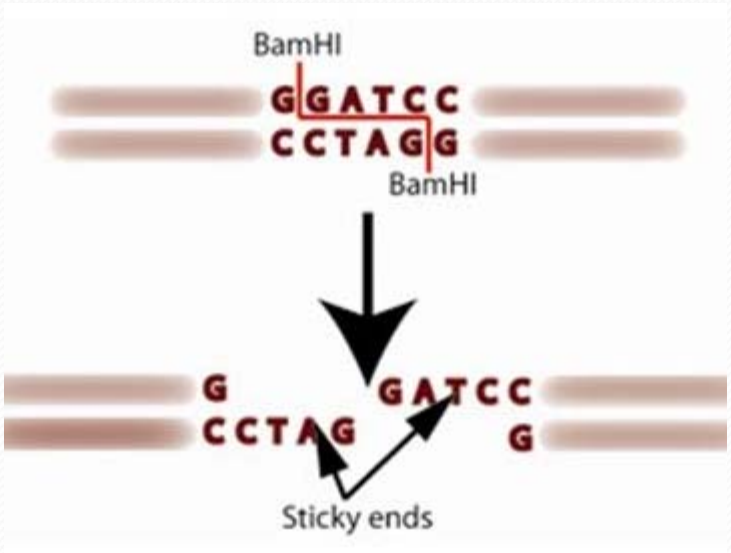
- RNA can be fragmented using divalent cations (e.g. Mg++) and elevated temperature (e.g. 95°C in NEB example)

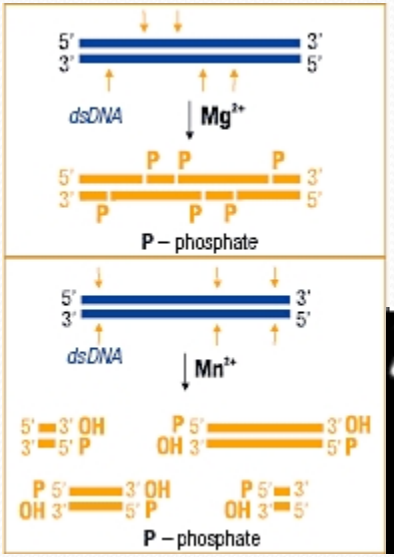
- For example, 40ug of RNA at 94°C in 50mM Mg^{++} for 1-5 min. resulted in this profile (resolved on a BioAnalyzer):

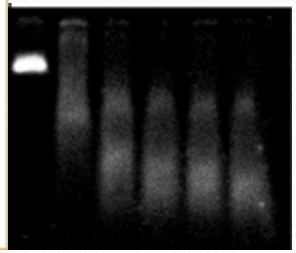


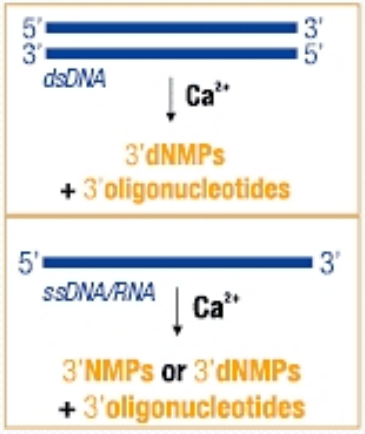
Enzymatic Shearing of Nucleic acids

- Some degree of sequence specificity results.
  - With restriction endonucleases the specificity is as precise as the recognition site – dsDNA is the required substrate
  - With DNAaseI (middle), MNase (right) there is sequence *preference* but not absolute specificity.



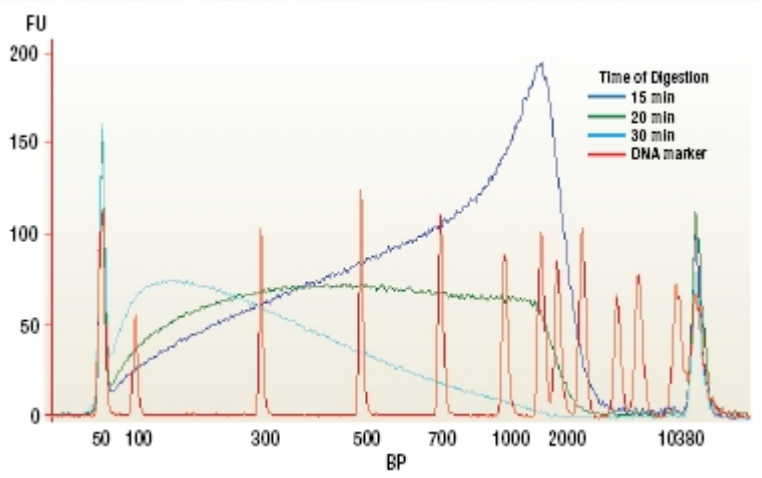


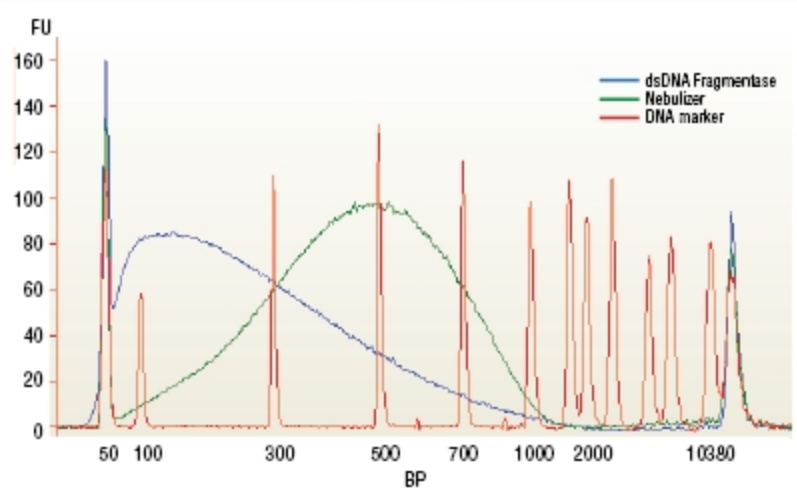




NEBnext dsDNA Fragmentase

- This enzyme mixture generates dsDNA breaks in a time-dependent manner - the yield is 100-800bt DNA fragments.
  - This is a mixture of 2 enzymes: one generated a ss nick and the other cuts the opposite strand, across from that nick.
  - The fragments have short overhangs with 5'-P and 3'-OH.
  - Comparison with sheared samples did not reveal any sequence bias.



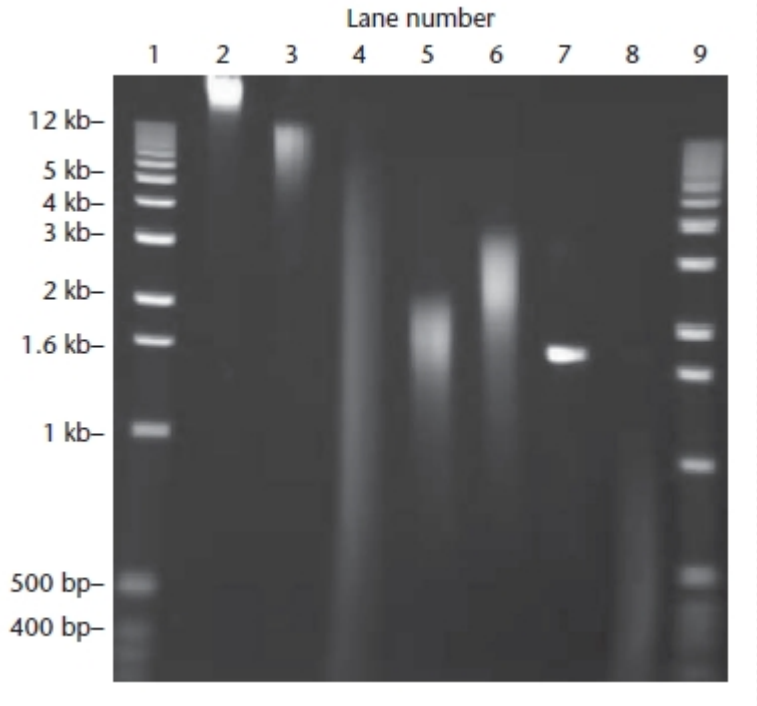


Shear-stress leads to fragmentation

- If a fluid moves along a solid boundary, shear stress occurs along the boundary.
- For viscous solutions, the ends of DNA molecules closest to the surface usually stick slightly so there is drag, while at some height from the surface the molecules have to be moving at the same rate as the solvent.

DNA shearing creates short(er) random fragments with heterogeneous ends.

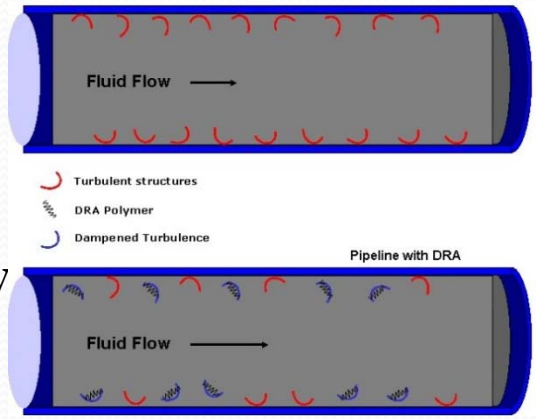
- Challenge: DNA is sticky and it is long and thin, so it is subject to shearing when opposing forces are present.
  - Example: one inversion of a solution will break Mbp DNA into 50-100kbp fragments.
  - Pipetting through micropipette tips leads to 30-70kbp fragments.

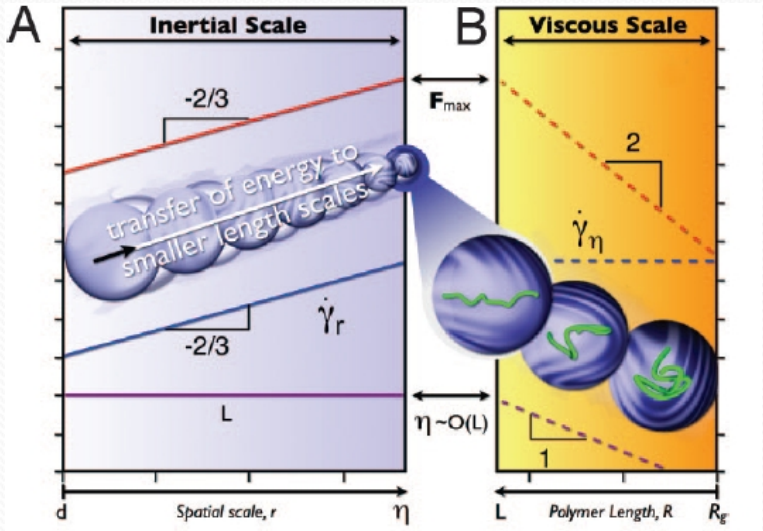


gDNA, needle-sheared, sonicated, Hydroshear, nebulized, PCR product, PCR sonicated.

Instrument types and specifications to produce mechanical shearing

- Ideal:
  - Nothing inserted into tube (potential cross-contamination);
  - Closed chamber
  - Isothermal
  - Small quantities (nanogram level) and high recovery
  - Parallel processing (96-well preferred).
- Common Platforms in Use
  - Needle shearing (hydrodynamic)
  - French Press (hydrodynamic)
  - HydroShear (point-sink; hydrodynamic)
  - Sonication
    - Ultrasonicator: Diagenode Bioruptor
    - Adaptive focused acoustics: Covaris
  - Nebulization (jet, acoustic or mesh)
    - Invitrogen





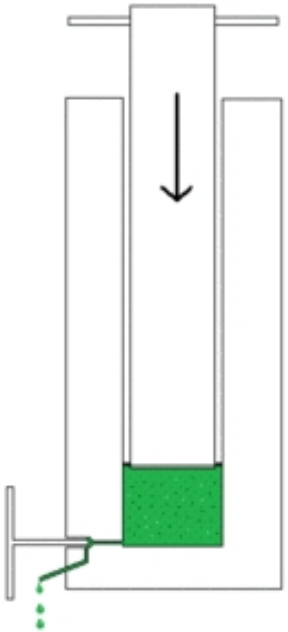
Vanapalli et al. PNAS 2006

Needle shearing is very low-tech.

- If you force a long sticky molecule through a narrow opening it undergo scission (break up).
  - The size of the aperture, the force, the viscosity, all affect the average length and distribution of the final fragment set.
  - Example: gDNA>10kbp at 10ug/200ul is loaded into a 1ml syringe with a 30-ga needle and repeatedly forced through (>10X passages).
- Results: large distribution and avg. length ~5kbp.
  - Disposable, cheap.

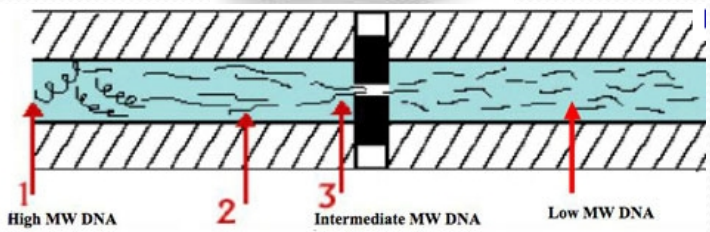
French press method.

- A solution is pushed through a small aperture using an hydraulic ram (pressure will be 20k-50k psi).
  - The DNA has a wide length distribution and avg. length of ~450bp (@ higher psi).
  - For more control a lower pressure setting with a manual setting is used: 250-1000psi
    - Results in 1-4kbp fragments
- Not disposable - the device must be disassembled/cleaned after each use.

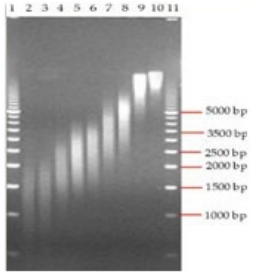


HydroShear is an example of point-sink shearing: is uses a high-pressure LC pump to shove DNA through narrow tubing.

- Fluid accelerates to maintain the flow rate of the volume through the constriction
  - The DNA stretches, then snaps.
- Pump speed, tubing diameter determine the length of the final product.
  - Good:
    - 90% of the product falls in a 2X length range.
    - Volume is ~60ul
  - Bad:
    - No high-throughput mode, decontamination is troublesome.



Lane	Pump Speed
1	500 Bp MWM
2	3
3	5
4	7
5	9
6	11
7	13
8	15
9	17
10	19
11	500 Bp MWM



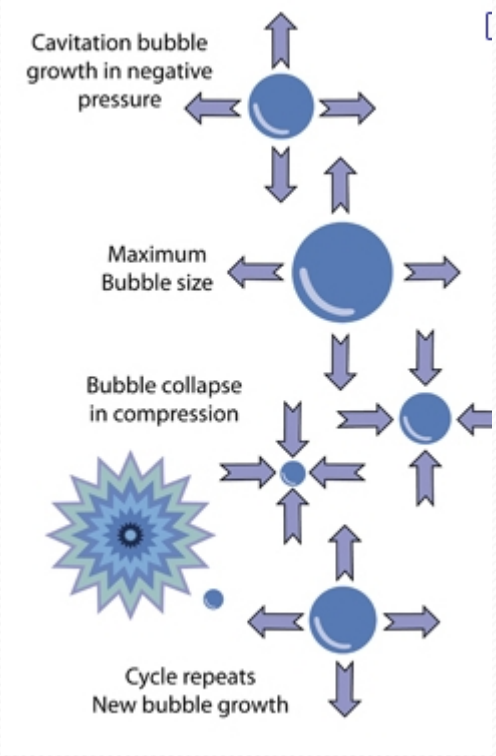
Sonicators create forces by producing acoustic waves.

- Sound waves travel through the solution: the ends of the DNA move in opposite directions
  - the middle of the molecule experiences the most force, so that is where breakage occurs.
  - Heating of the solution will occur, so samples are kept on ice during processing.

- Variations
  - In some models the probe is inserted into the sample- direct sonication, fast energy transfer, focus depends on probe diameter
  - Cup-horn models have the probe adjacent and exterior to the sample tube – indirect, can chill, isolate. Less energy, longer time.

In the ultrasonic range the relevant force is cavitation.

- Electrical energy is converted into oscillation, giving vibrational energy to the fluid.
- The rapid vibration causes cavitation – formation and violent collapse of microscopic bubbles that release energy into the cavitation field.
  - The liquid is being rapidly pushed and pulled – microscopic shockwaves result.
  - The liquid cannot keep up, so during contraction small vacuum cavities are formed, which then implode when hit in the other direction – occurs in microseconds.
  - Size matters – the particles need to be less than 100um in size at ultrasonic levels.
  - Heating occurs very rapidly so energy bursts are very short.



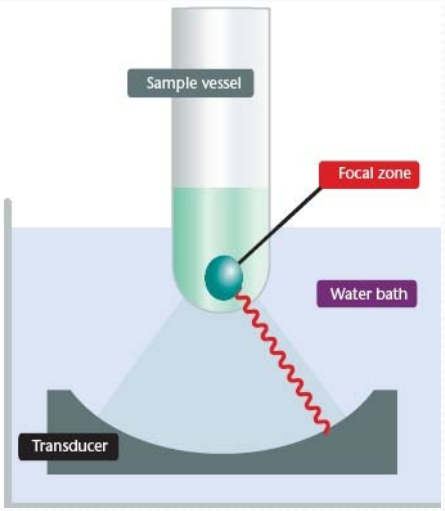
In this lab are using a tunable ultra-sonicator called the Bioruptor.

- Ultrasonicators: 20kHz and higher, with λ = 10cm.
- The abrupt alteration of pressure (on/off of ultrasonic waves) causes small bubbles of dissolved gases to form – they have intense vibrational energy rapid motion causes mixing, which produces mechanical stress

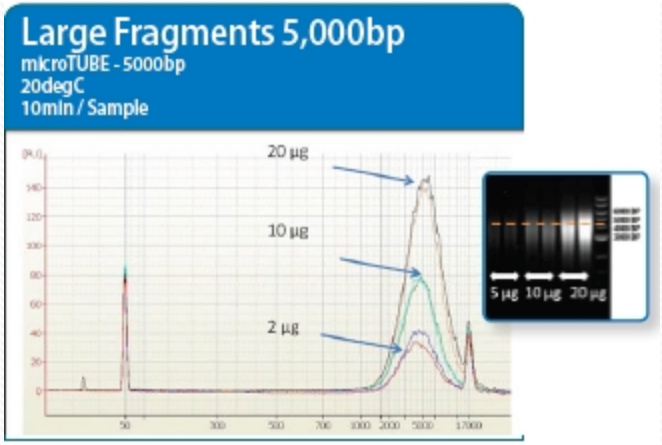
From Diagenode Web site – note that we care more about what happened inside the tubes.

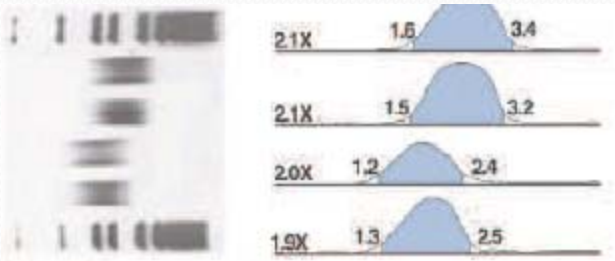
AFA is adaptive focused acoustics, used by the Covaris platform.

- High-frequency acoustic waves are focused on the sample using a lens-shaped transducer.
  - 500kHz with λ = 1mm, and tunable
- Cavitation results – the rapid mixing breaks the polymer backbone.
  - Covaris devices are optimized for both tubes and plates.



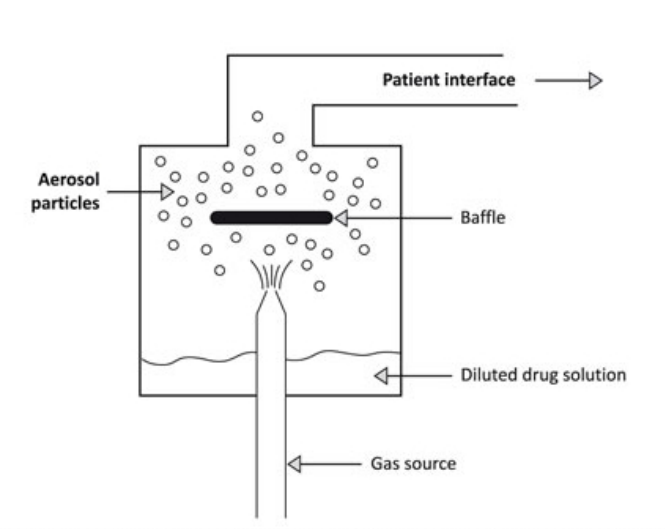
Covaris (top) Vs Hydroshear (bottom) best results

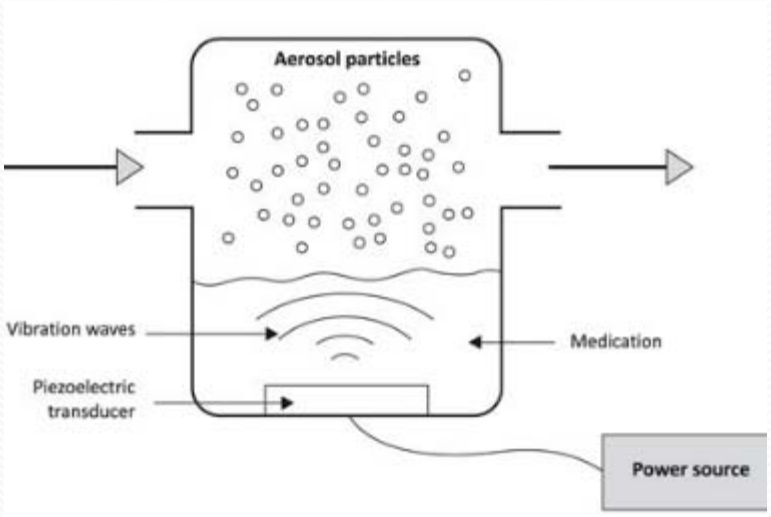




Nebulization

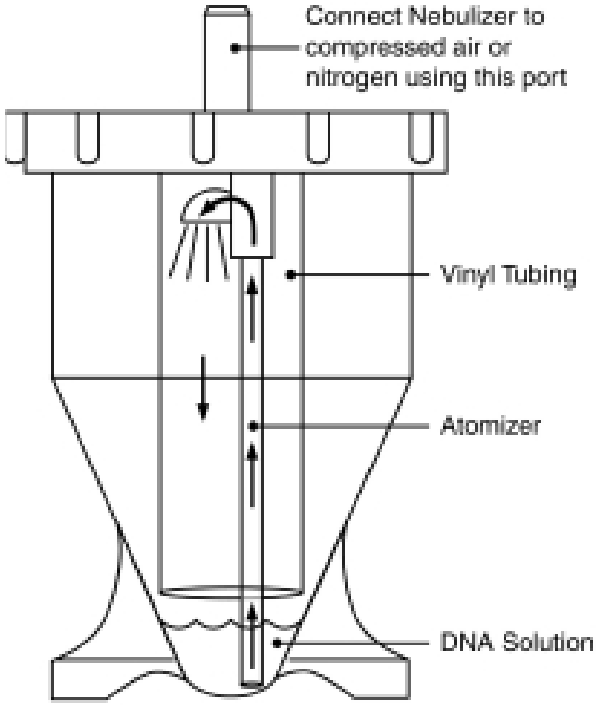
- A nebulizer creates a mist of a liquid
  - compressed gas or ultrasonic energy produces microdroplets, with d=1-5um.
- Jet nebulizers
  - attached to a compressor that flows gas along the surface of a liquid at high rate
  - Cooling occurs
- Ultrasonic wave nebulizers
  - electronic oscillator generates an ultrasonic wave – the resulting vibrations through the liquid produces a mist at the air surface.
  - Heating occurs.
- Vibrating mesh nebulizers
  - um-sized holes in a vibrating membrane held at the top of the liquid – the pressure changes generate a mist
  - Less heating of the sample occurs.





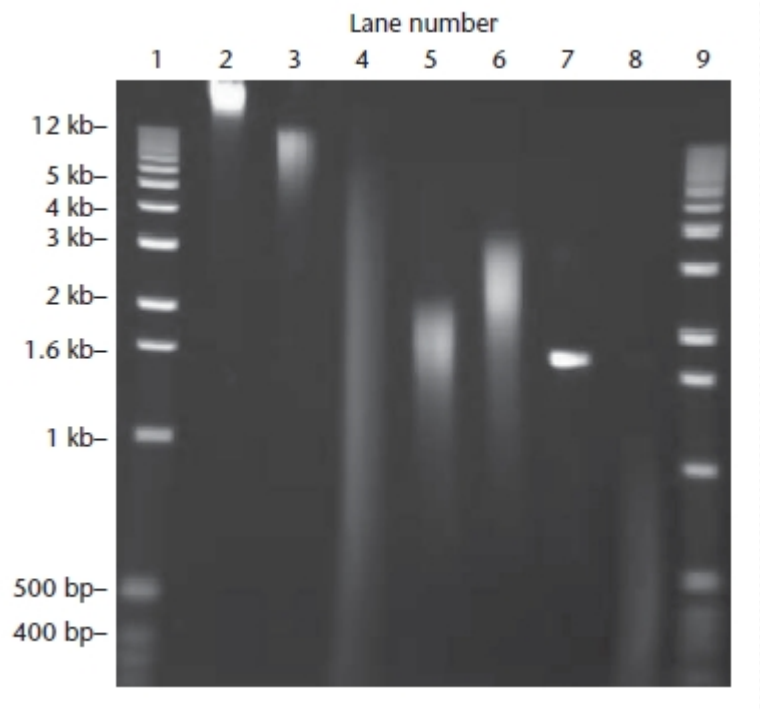
Nebulizers create fine aerosols – formation of the droplets shears dissolved polymers.

- Invitrogen example is shown (produces 1-6kbp fragments)
  - The port size is selected for the polymer
  - The port is connected to N2(Gas) tank with regulator.
- A plastic cone is used to provide a vaporization surface
  - The rim is cut off and the cone is inverted
  - 0.5-10ug DNA, 2ml buffer with 25% glycerol is added to the cone
  - Gas is bubbled through the solution.
- Beckman makes SPRIworks, which gives 200-2000bp fragments.



Concentration of polymer in un-aerosolized solution increases, not all solution can be aerosolized (ie there is some sample waste).

Revisiting results



gDNA, needle-sheared, sonicated, Hydroshear, nebulized, PCR product, PCR sonicated.

Sample protocols for each method, and discussions, can be found at the sites below.

- http://seq.molbiol.ru/g_dna_shear.html has a protocol for each device.
- http://seqanswers.com/forums/showthread.php?t=164

Project suggestions

- How could you improve the ligation process (blunt-end ligation is not very efficient) for sheared DNA products
- How might you make asymmetric strands, to select just one fragment in one orientation?
- How would you do paired-end sequencing on the Ion Torrent?
- How might you improve the bead enrichment step so more beads have a fragment?
- How might you select against beads with more than one fragment?
- How might you select for beads having a sufficient loading of PCR product to give a usable signal?
- How might you determine what proportion of ends from a mechanical shearing process were not amenable to polishing?
- What conditions could you modify to disrupt structure of the template without denaturing the enzyme, for the Ion Torrent?
- Other projects relevant to your own interests are allowed.
- How might you determine whether structures interfere with the sequencing of a target?
- What conditions would you change to generate longer reads on the Ion Torrent?

Project requirements

- You may modify an approach that is used with another platform (like the Illumina or 454 platforms) that has not yet been demonstrated for the Ion Torrent. Methods for RNA-Seq, ChIP-Seq, etc. are examples. Or you can take an existing method and try to improve it – identify a problem and suggest ways to overcome it, or test for a potential problem, and explain how to determine if it is a real problemSee the Ion Community Web page for existing protocols and discussion.
  - Cite the original method if there is one.
  - How must it be modified for the Ion Torrent
  - Give detailed and specific protocols , including quality control for reagents and preparative steps.
  - Explain what controls will be needed to determine whether the approach is successful
  - Explain how many sample replicates will be produced and the expected data redundancy.

References

- The Ion Commuity Web page is here http://lifetech-it.hosted.jivesoftware.com/index.jspa
- MA Quail "DNA: Mechanical Breakage" Encyclopedia of Life Sciences 2010, John Wiley and Sons.
- Oefner PJ, Hunicke-Smith SP, Chiang L et al. (1996) Efficient random subcloning of DNA sheared in a recirculating pointsink flow system. Nucleic Acids Research 24: 3879–3886.
- Covaris. Products: S, L and E series AFA shearing platforms- Details of products, applications, publications and videos. http://www.covarisinc.com/index.html
- Diagenode. Products: Bioruptor–Diagram and recommended protocol for shearing prior to next gen sequencing http://www.diagenode.com/en/topics/sonication/sonication.ph p
- GeneMachines. Products: HydroShear–a description and video of the apparatus http://www.genemachines.com/HydroShear.html
- Matrical. Products: Sonicman- Product details and application note for DNA shearing http://www.matrical.com/SonicMan_High_Throughput_Sonication.php