

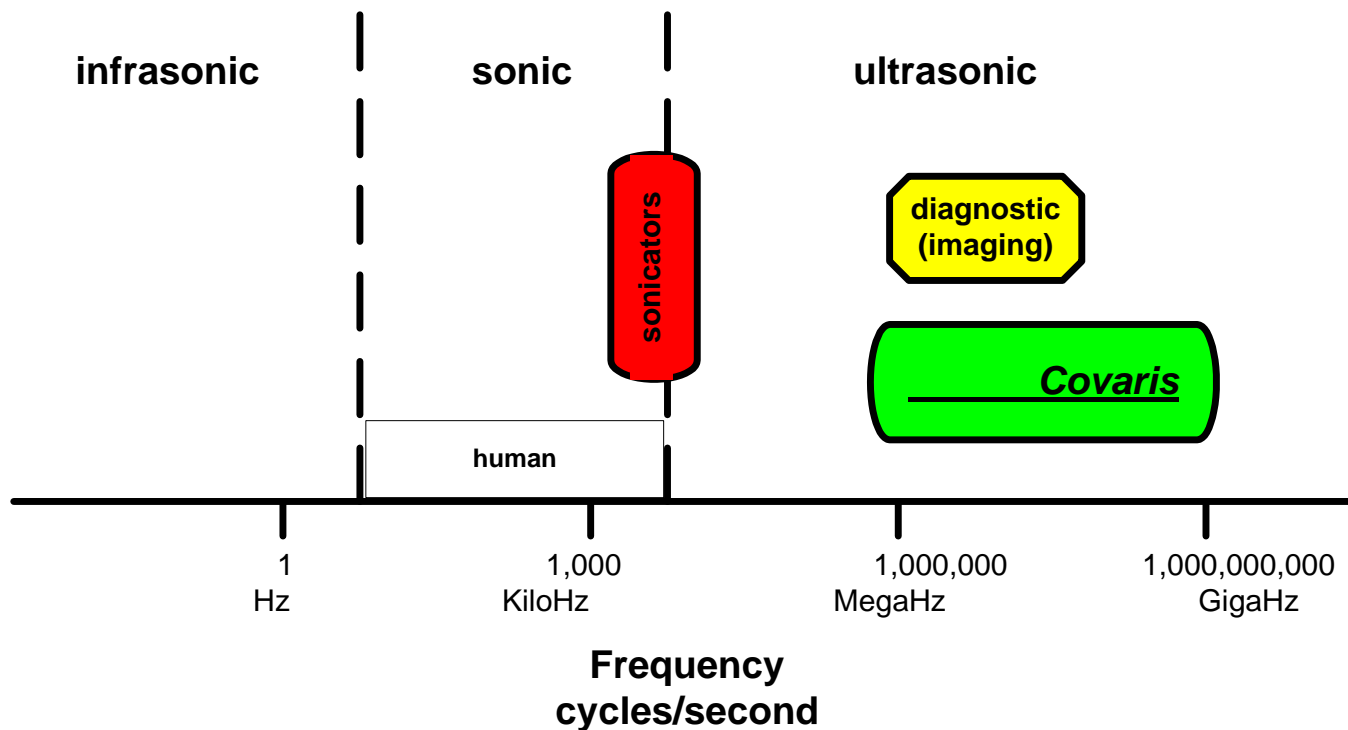
An Investigation into the Utility of Adaptive Focused Acoustics in Bacterial Cell Lysis

Martin Hibbs GSK Harlow

Adaptive Focussed Acoustics and the Covaris

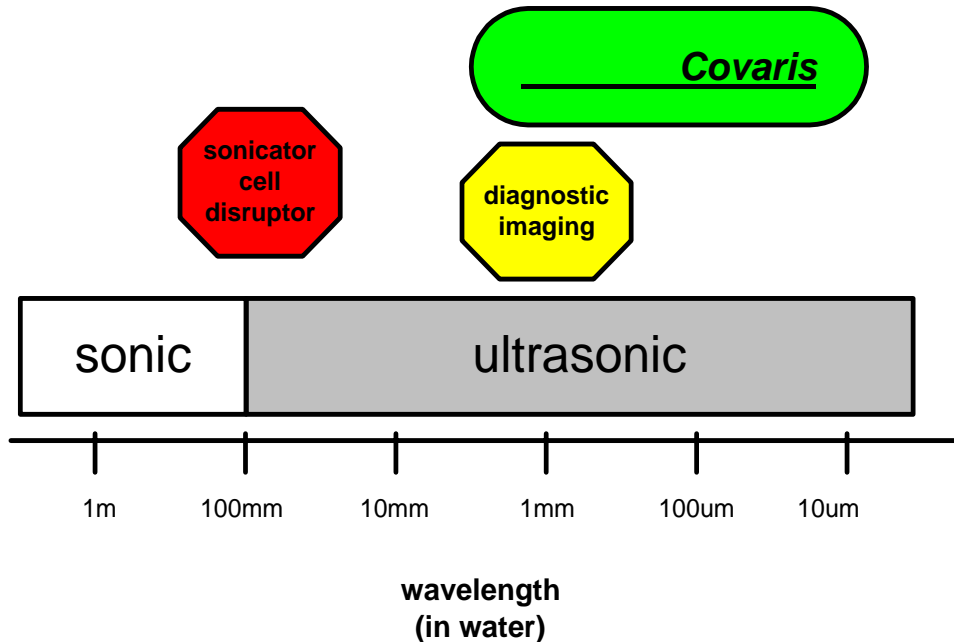
- Utilises High Frequency Adaptive Focused Acoustics (AFA).
- Evolved from kidney stone disruption and ultrasound imaging industries.
- Applies powerful acoustic energy to sample through temperature controlled water bath.
- High frequency acoustic waves sent from dish-shaped transducer – waves converge to single point.
- Acoustic energy directed into sample in a non-contact and isothermal mode.

acoustic frequency

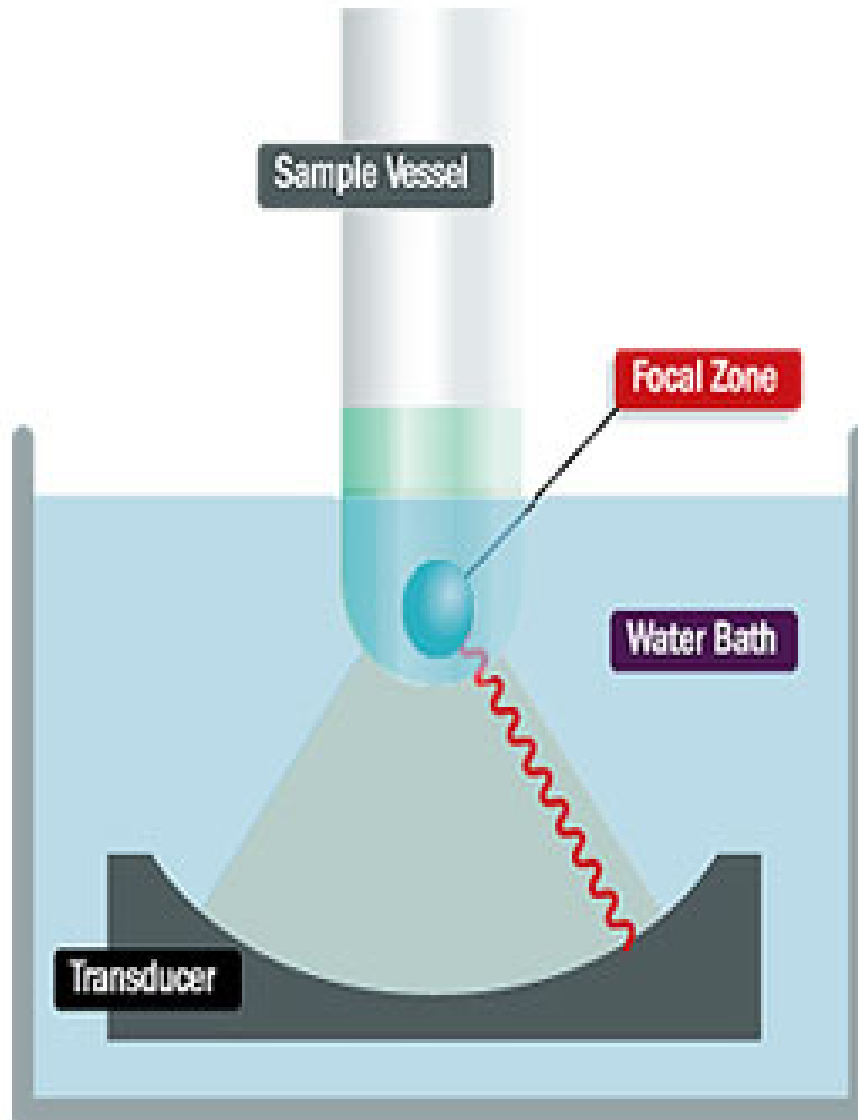
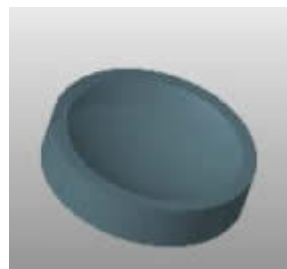


Acoustic Wavelength

Acoustic transducer operates at 500kHz with wavelength of ~1mm, unlike conventional sonics with wavelength of ~100mm.

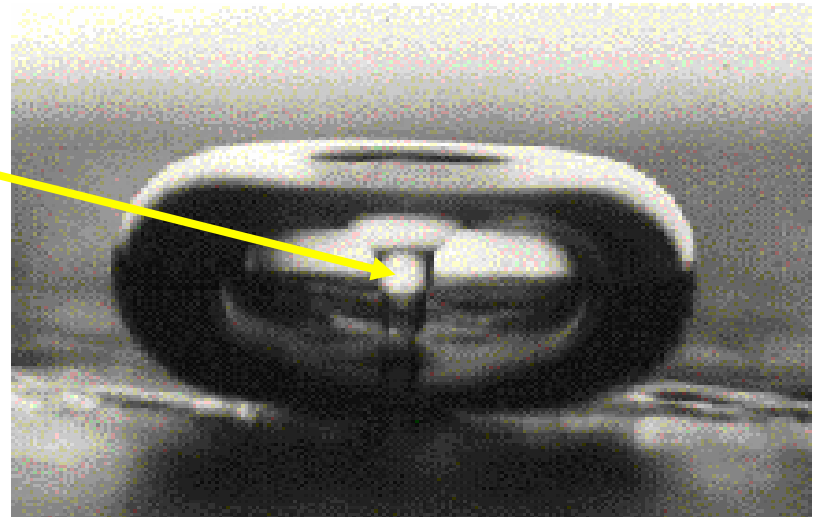


Covaris :: focused mechanical energy



AFA causes cavitation effects

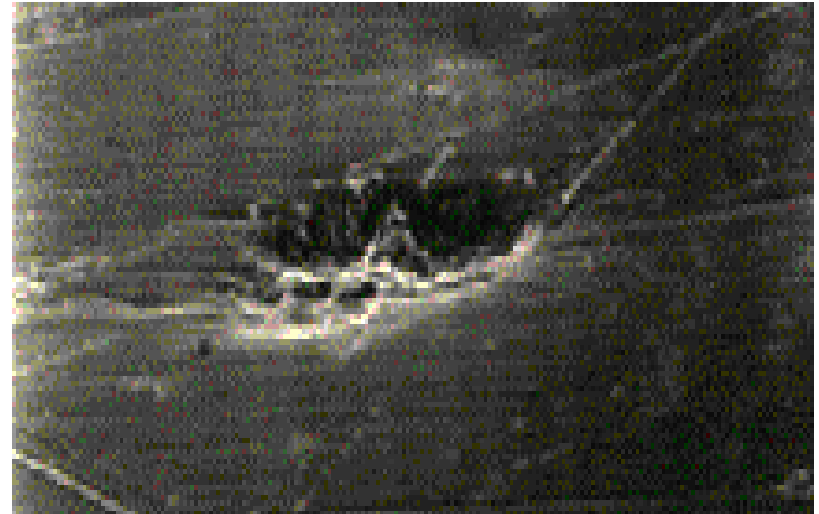
- Photograph of a liquid jet produced by a collapsing cavitation bubble
- liquid jet impacts at $>100\text{m/sec}$
- Width of the bubble is approximately one mm.



1 mm

Bubble Collapse

- scanning electron micrograph of a brass plate after a few shocks
- craters formed are indicative of damage by high speed jets caused by collapsing cavitation bubbles.



S-series – single sample

- Small footprint
 - 25cm wide
 - 30cm deep
 - 20 cm high
- Stand-alone format
 - Predefined protocols
- No robotics
 - Lower cost
- Chiller not shown



Covaris tube types/ various models



Relatively easy to customise

Covaris Device Settings

- **Power Tracking** - more powerful, energy focused at one point.
- **Frequency Sweeping** – frequency changes, energy more dispersed
- **Duty Cycle (0-20%)** – percentage of time the acoustic energy is ‘on’.
- **Intensity (0-10)** – amplitude of the ‘on’ time.
- **Cycles/ Burst** – number of bursts of full power within the ‘on’ time.



Covaris

SonoLAB Single Sample Preparation System

S2

Version: 2.2.0
Modified: 7/18/2005

Date: 20/04/2006
Time: 4:30:37 PM

Process Name:

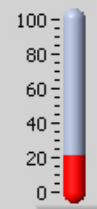
Treatment

Duty Cycle	Intensity	Cycles/Burst
20% -	10 -	1000 -
10% -	8 -	500 -
5% -	6 -	200 -
2% -	4 -	100 -
1% -	2 -	50 -
1% -	1 -	50 -

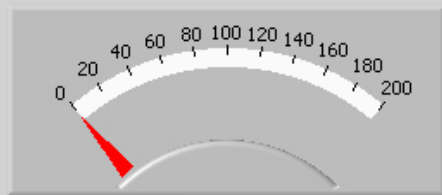
Time: s

Unsaved Changes: ●

-
-
-
-




Bath Temperature: 21.0 C



Acoustic Power: ● W

DEGAS ●

Estimated Time to Completion:

Progress (%) 

0 20 40 60 80 100

-
-
-

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Process Configuration

Process Name:

Mode:

Number of Cycles:

Bath Temperature Limit: °C

Total Process Time:

Unsaved Changes: ●

Treatment 1

Duty Cycle **Intensity** **Cycles/Burst**

Time s

Treatment 2

Duty Cycle **Intensity** **Cycles/Burst**

Time s

Treatment 3

Duty Cycle **Intensity** **Cycles/Burst**

Time s

Treatment 4

Duty Cycle **Intensity** **Cycles/Burst**

Time s

Open...

Save

Save As...

Cancel

Return to Main Panel

Load process from: [?] [X]

Save in: [Navigation icons]

- My Recent Documents
- Desktop
- My Documents
- My Computer
- My Network Places

- 1sec
- Bacterial Lysis 1g
- large tube test
- pharmdev
- S3 test large tube**
- tdec chem

File name: [Save] [Cancel]

Save as type: [Save] [Cancel]

Total Process Time:

Unsaved Changes: ●

Treatment 4

<p>Time <input type="text" value="30"/> s</p>	<p>Time <input type="text" value="0"/> s</p>	<p>Time <input type="text" value="0"/> s</p>
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s/Burst	Duty Cycle	Intensity	Cycles/Burst
<input type="text" value="20"/>	<input type="text" value="10%"/>	<input type="text" value="10"/>	<input type="text" value="1000"/>
<input type="text" value="10"/>	<input type="text" value="5%"/>	<input type="text" value="5"/>	<input type="text" value="500"/>
<input type="text" value="5"/>	<input type="text" value="2%"/>	<input type="text" value="2"/>	<input type="text" value="100"/>
<input type="text" value="1"/>	<input type="text" value="1%"/>	<input type="text" value="1"/>	<input type="text" value="50"/>

Time s

Open...

Save

Save As...

Cancel

Return to Main Panel

Covaris Pilot Studies Objectives

- Conduct mini studies to establish utility
- Evaluate effectiveness of Covaris in *E.coli* lysis
- Determine optimum lysis times.
- Establish key parameters.
- Establish robustness of equipment
- Probe limitations of S2 device
- Identify areas of research for more in-depth investigation.

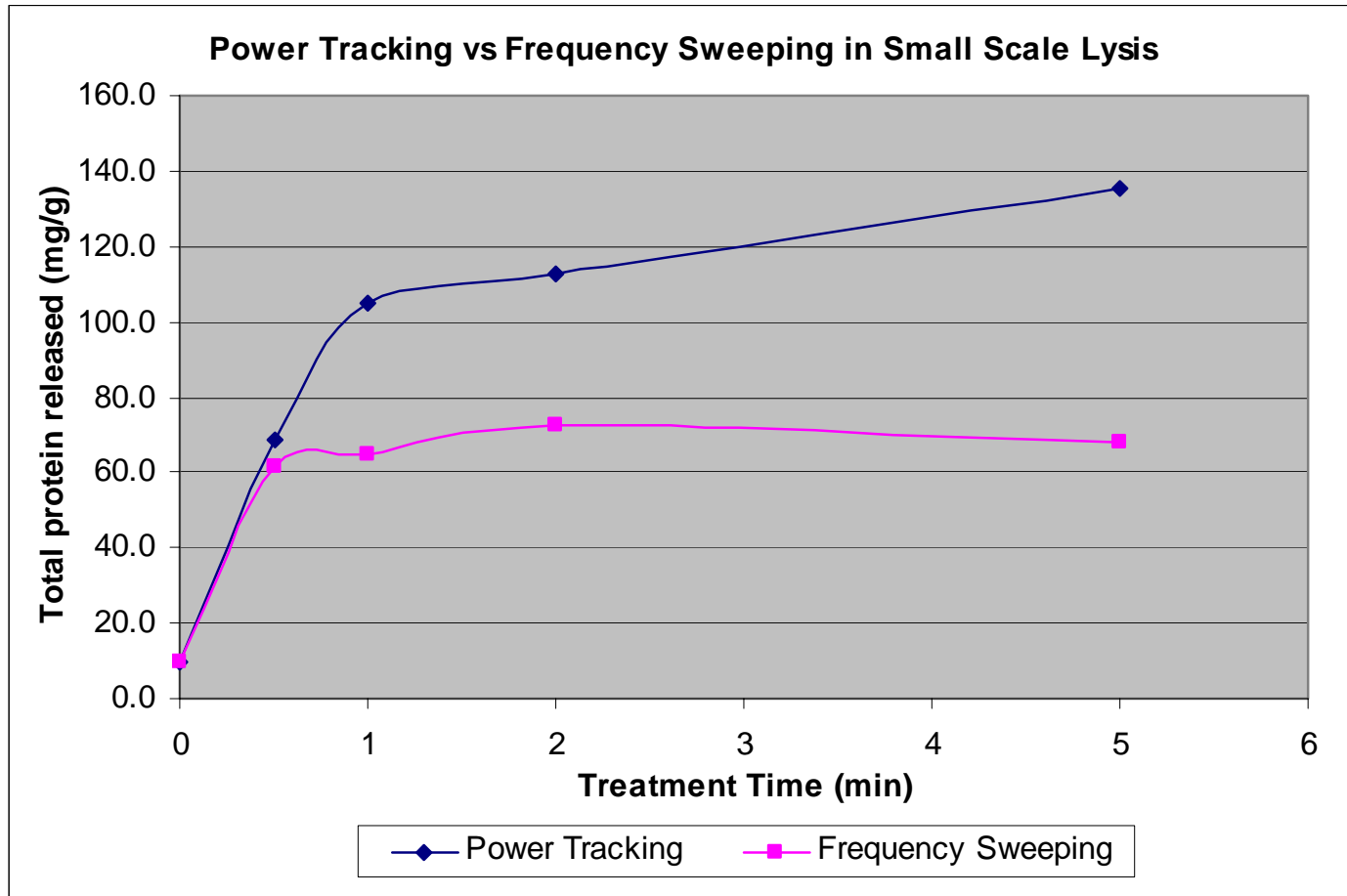
Pilot study conclusions

- **Lysis time**
 - 1g of E.coli cells fully lysed in < 5mins
- **De-gassing**
 - No advantage
- **Parameters**
 - Duty cycle 20%
 - Intensity 10
 - Cycles/burst 500
- **Robustness**
 - No major issues over 4 weeks
 - Occasional issues with temperature settings
- **Limitations**
 - 1g of cells maximum with small tube set up
 - Flow cell system trialled but abandoned
 - Probably need higher power system for > 50g lysis
- **Further work**
 - Very encouraging pilot
 - More in depth study required

Phase 1 studies

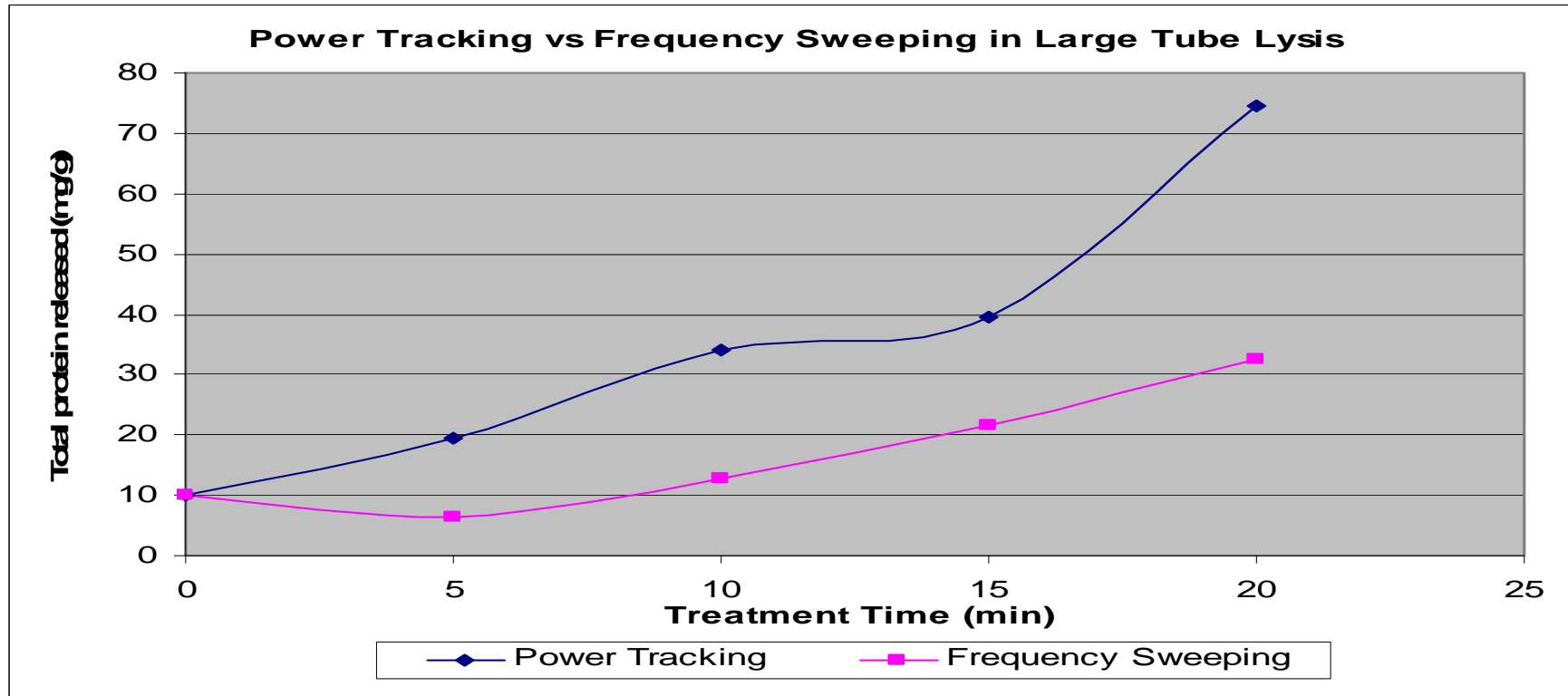
- E.coli cell lysis
- Instrument parameters from pilot adopted
- More rigorous examination
- Additional parameters also investigated
 - Power tracking and frequency sweeping
- Extended study to 10g of cells
- Comparison with other lysis methods
- Degradation concerns investigated
 - SDS/PAGE
 - Western Blotting

Power Tracking versus Frequency sweeping 1g/3mls



- Lysis was rapid, >70% after 1 min
- Power tracking generated higher total protein release
- Protein release close to theoretical maximum of 80-100 mgs/g

PT vs FS large tube lysis 10g/40mls

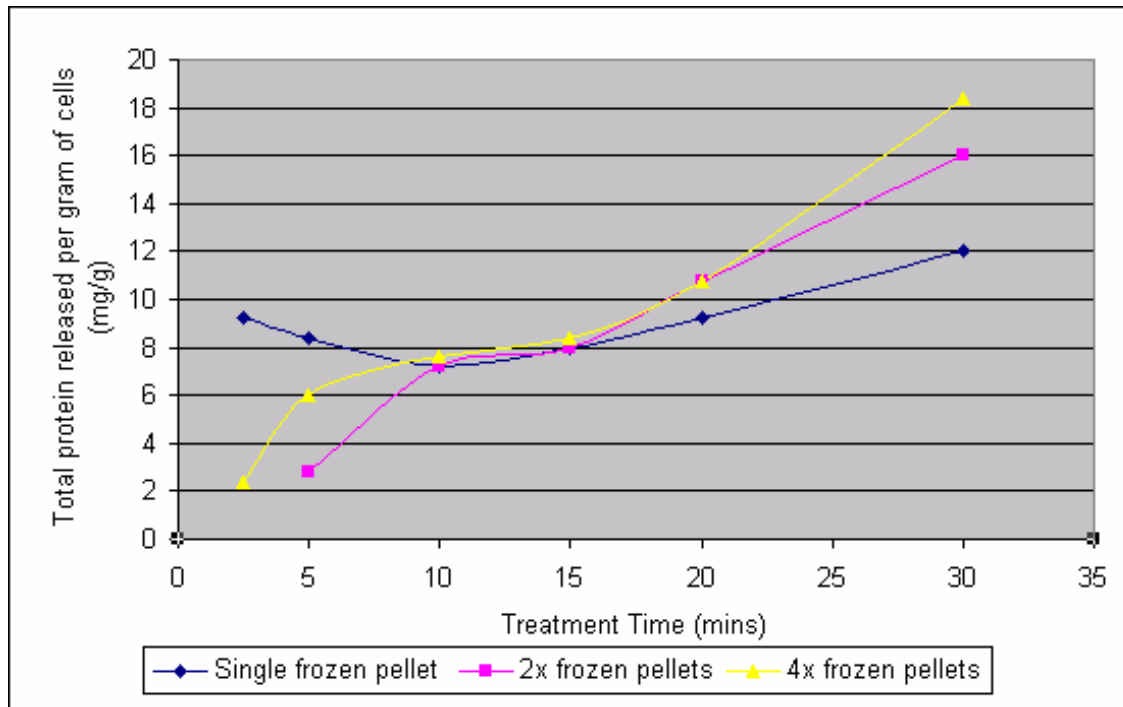


- Power Tracking most effective
- Speed of lysis reduced , however mg/g figures acceptable for PT
- Maximum time probably 20-30 mins

Optimisation of large tube Lysis

- 1) **Different sized frozen pellets** – single pellet, 2x pellets and 4x pellets.
 - Carried out in Power Tracking mode for total treatment time of 30mins.

Graph to show the comparison of large tube lysis of different sized frozen pellets



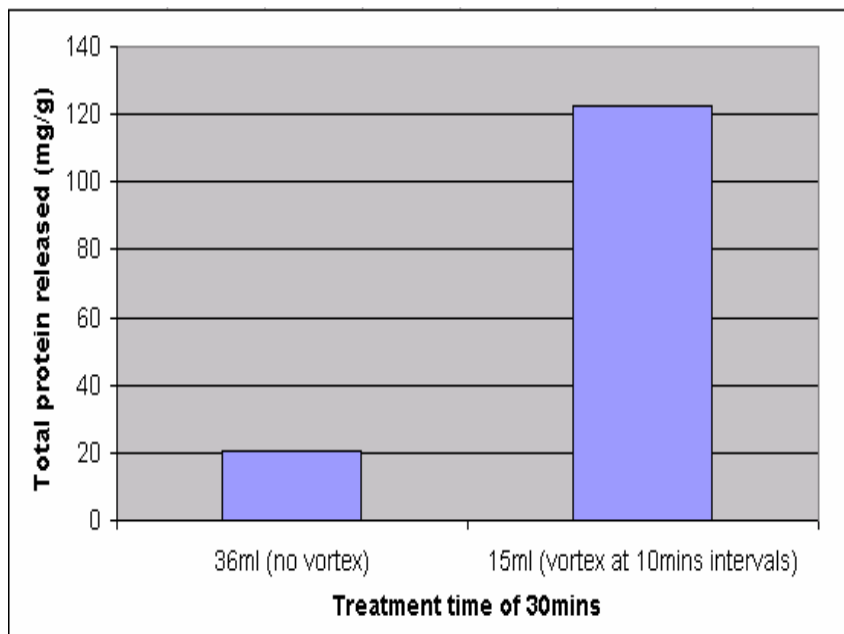
Conclusion: 4x frozen pellets gave better recovery of protein after 30mins

At the initial time points little difference between the different sized pellets.

Optimisation of Large Tube Lysis

- 2) **Reduction of sample volume** - lysis buffer reduced to 15ml (typically 36ml) for 9g of cells.
 - The acoustic energy is more concentrated in a smaller volume.
- 3) **Vortexing / mixing sample** at 5mins & 10mins intervals.
 - To remove thawed out sample stuck at bottom of tube.
 - To ensure sample is more homogeneous, hence acoustic energy is evenly absorbed throughout sample.

Graph to show the comparison of lysis of frozen pellets in 36ml lysis buffer (no vortexing) with 15ml lysis buffer plus vortexing at 10mins intervals



Conclusion: There is a 6 times increase in total protein released when sample volume was reduced and vortexing introduced.

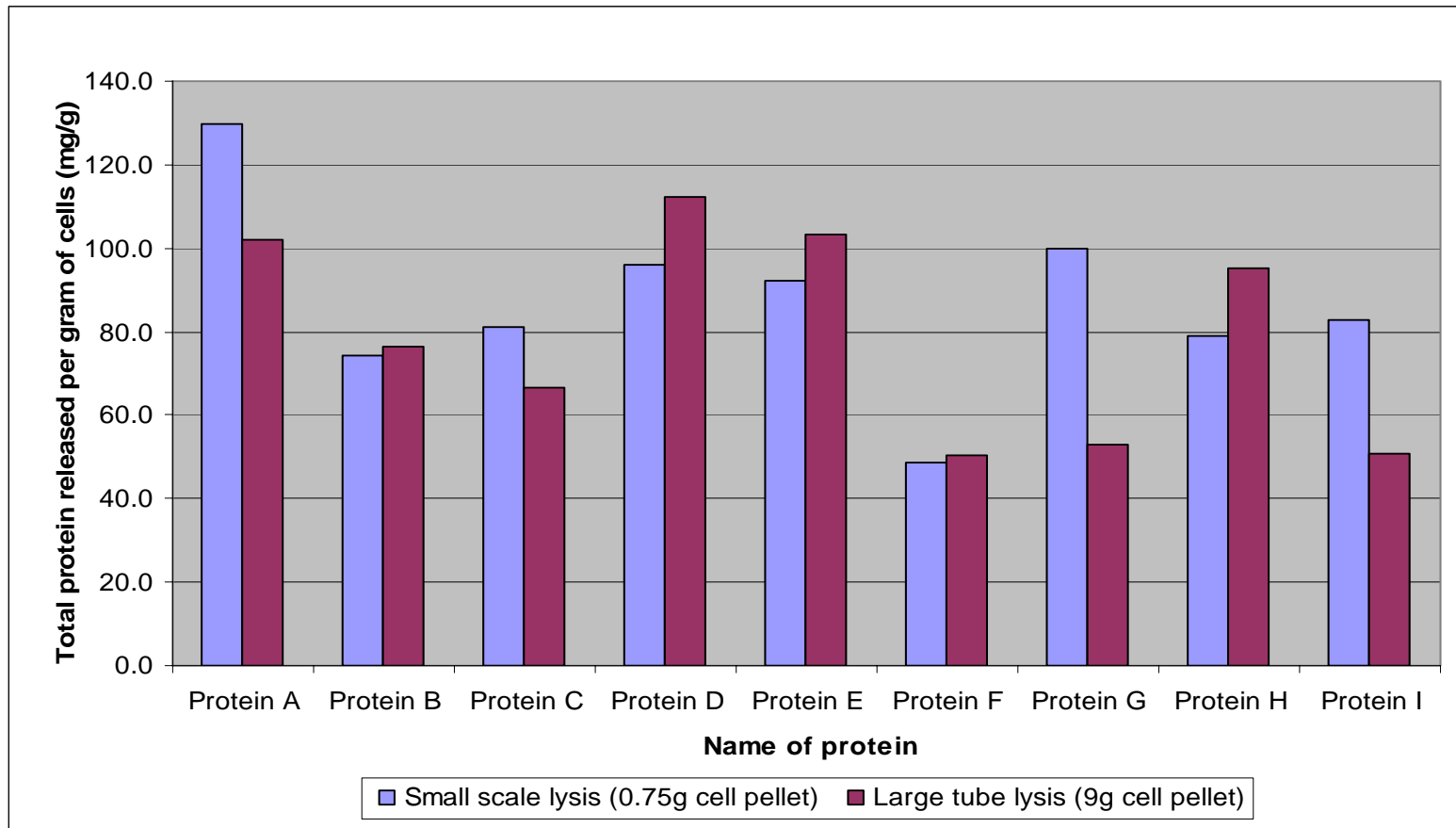
Preliminary Large Tube Protocol

Optimised Conditions:

1. **Acoustic mode: Power tracking**
2. **Covaris settings: Duty cycle 20%; Intensity 10; Cycles/ Burst 500**
3. **Sample volume: ~15ml (9g cells in 15ml lysis buffer)**
4. **Sample: 4x frozen pellets**
5. **Vortex every 10mins for total treatment time of 30mins**

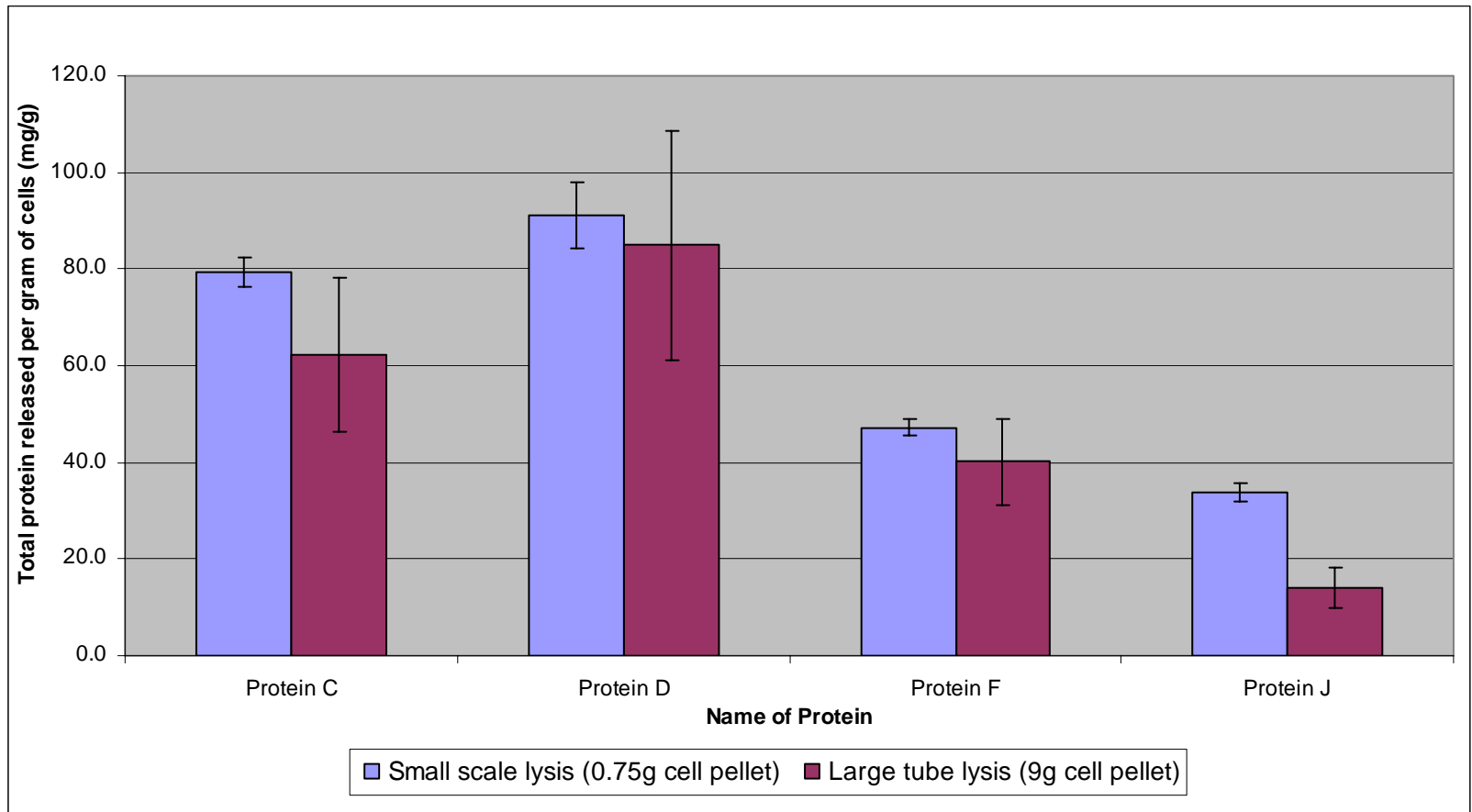
Validation of 10g Lysis Protocol

E.coli cell lysis using 9 recombinant proteins



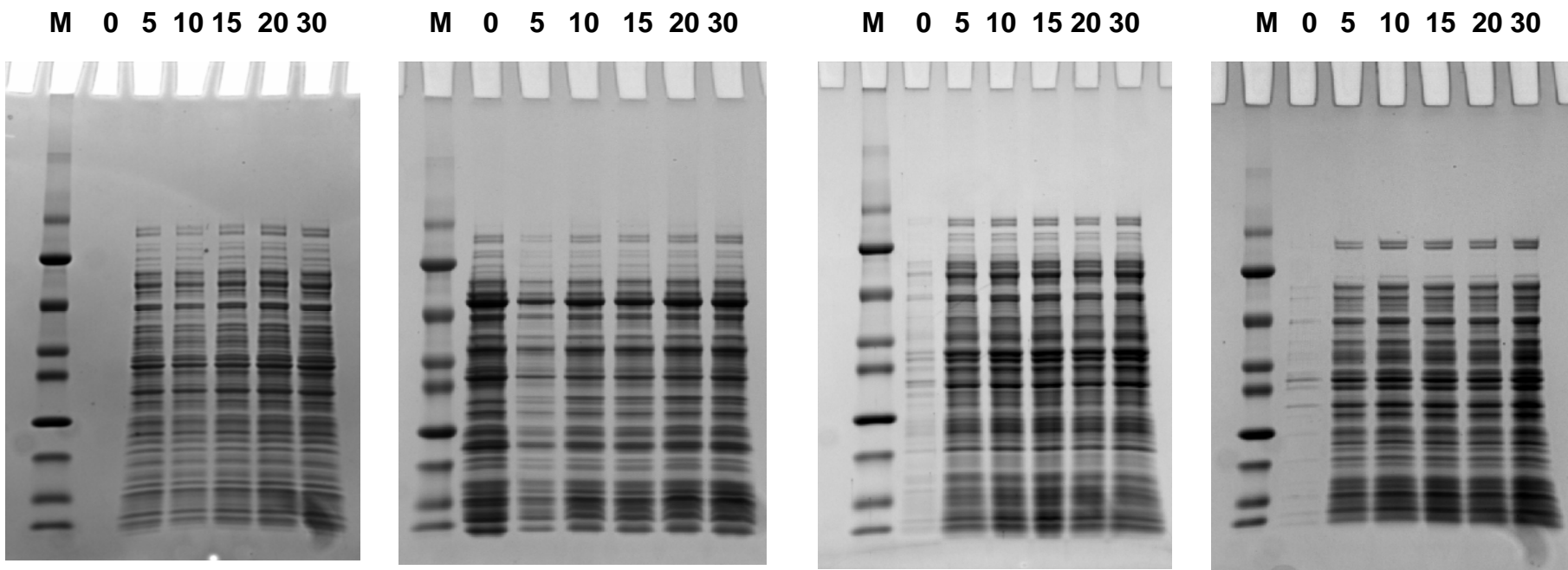
- Generally good correlation between 1 and 10g lysis
- Differences in mg/g figures probably due to cell density of pellets
- Protein release was acceptable in all cases

E.coli cell lysis



- error bars are SDs,
- no significant difference for 3 out of 4.
- Good protein release

Degradation Profile of *E.coli* proteins over 30mins during 15ml Lysis



Protein C

Protein D

Protein F

Protein J

Conclusion: No protein degradation can be seen on the SDS-PAGE gel as treatment time progresses for a total time of 30mins. All protein concentrations have been diluted to 1mg/ml for loading into gel.

Covaris vs other lysis methods

Mechanical

- Covaris
Utilises High Frequency Adaptive Focused Acoustics (AFA)
Acoustic energy (500kHz)
- Sonication
Applies ultrasound (20-40kHz)

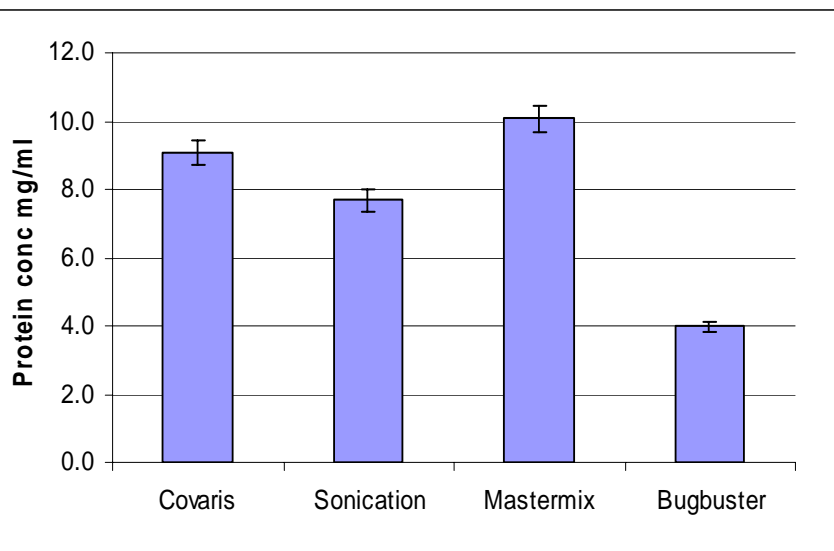
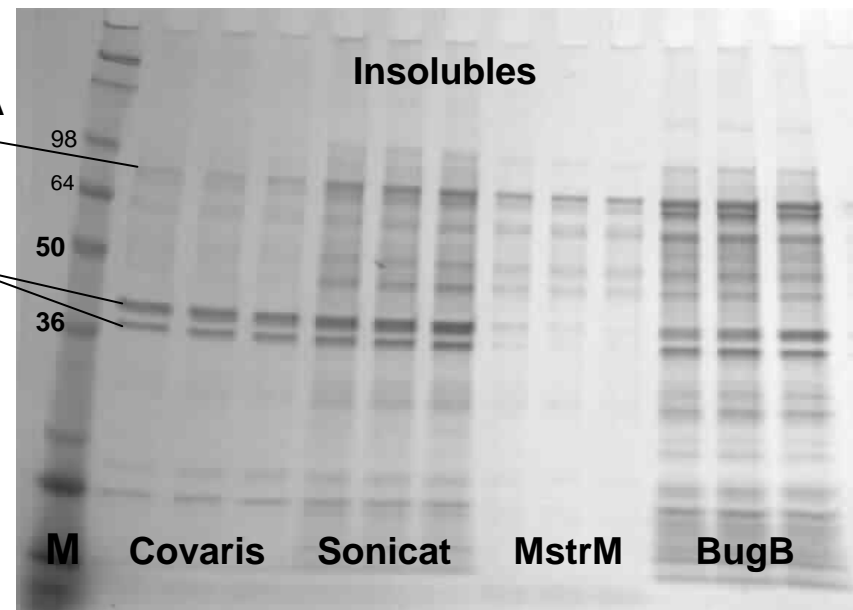
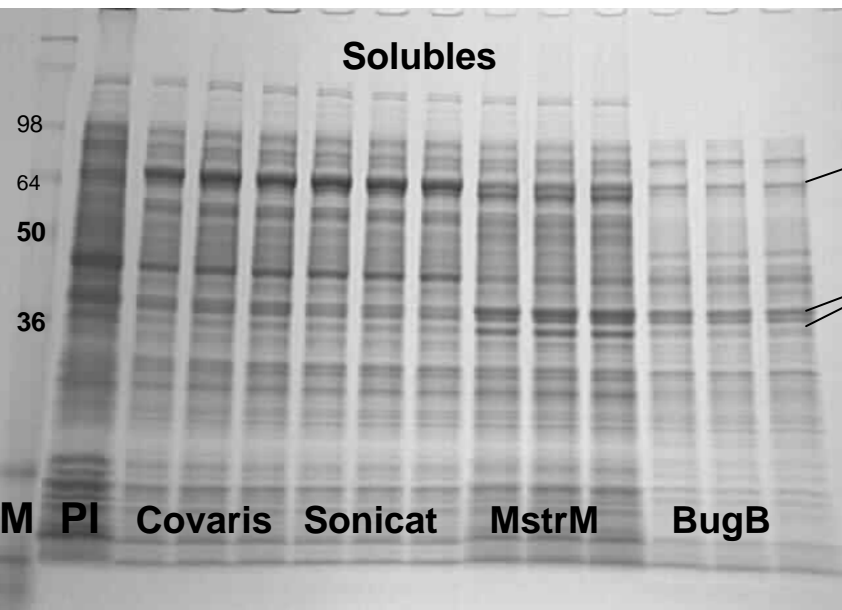
Chemical

- Bugbuster
Utilises detergent mix capable of cell wall perforation
- Mastermix
Bugbuster + rLysozyme (hydrolyses NAM linkages in the cell wall)

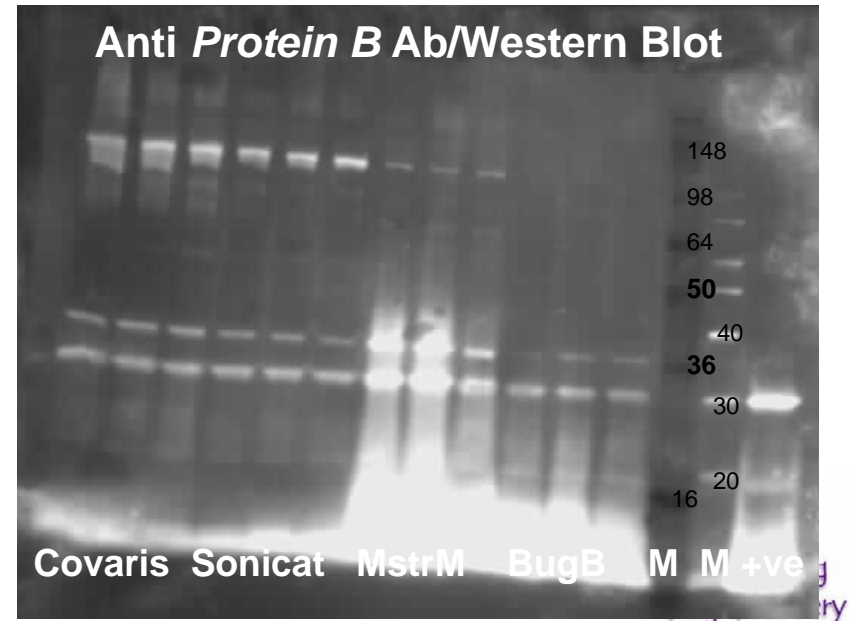
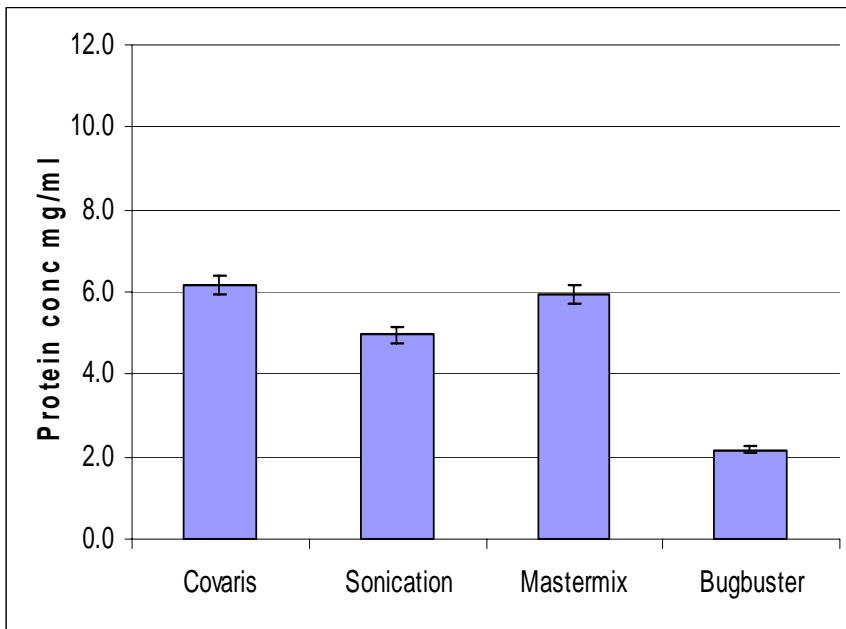
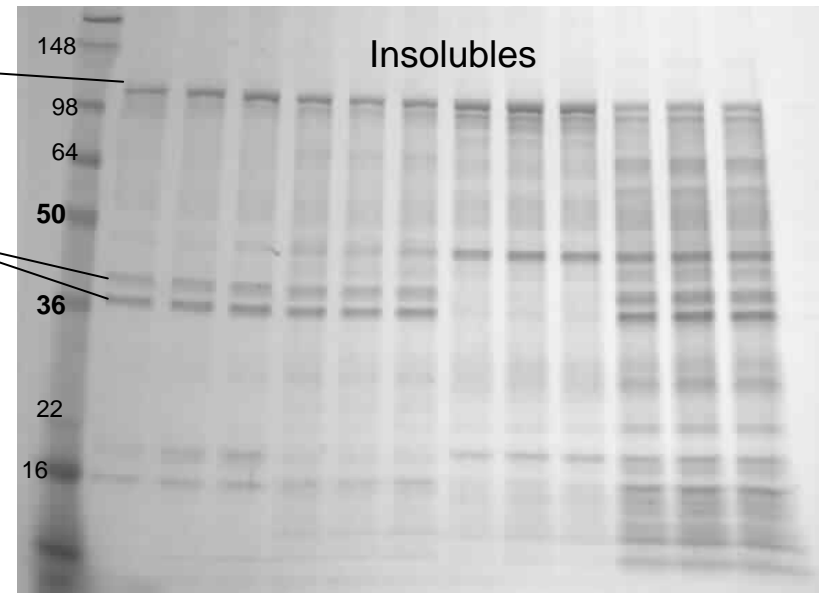
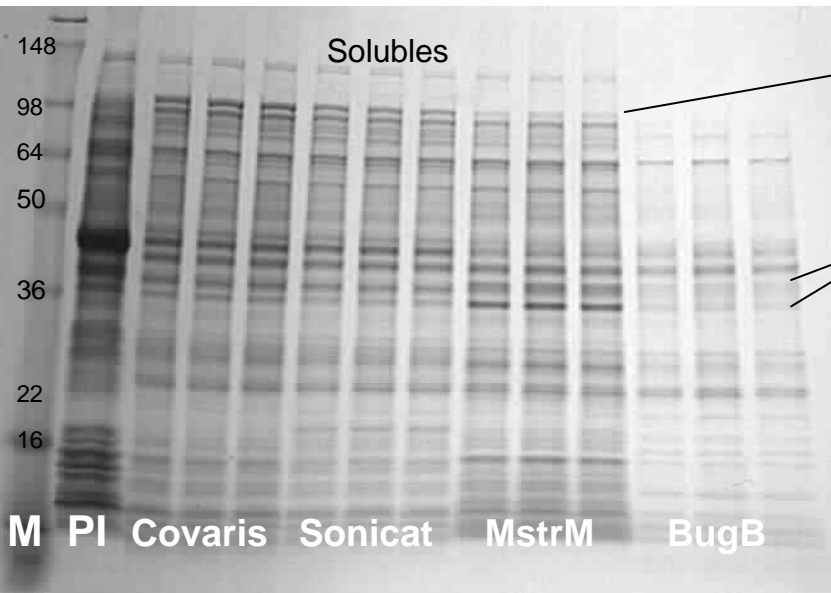
Protocol

- **Standard lysis buffer for covaris & sonication**
(25mM Tris, 1mM EDTA, 5mM Dithiothreitol, pH 8.0)
- **1gm cell pellet resuspended in 10ml**
(Bugbuster (DNAse)/Mastermix/Std lysis buffer)
- **Bugbuster and Mastermix** 1ml aliquots of samples incubated for ~30 minutes
- **Sonication samples** resuspended in standard lysis buffer and then 3ml aliquots sonicated for 4 min (5sec on/5 sec off)
- **Covaris samples** resuspended in standard lysis buffer and then 3ml acoustic treated for 5 min
- 1ml of each sample centrifuged for 30min at 4°C, supernatant (lysate) decanted
- Samples in Triplicate

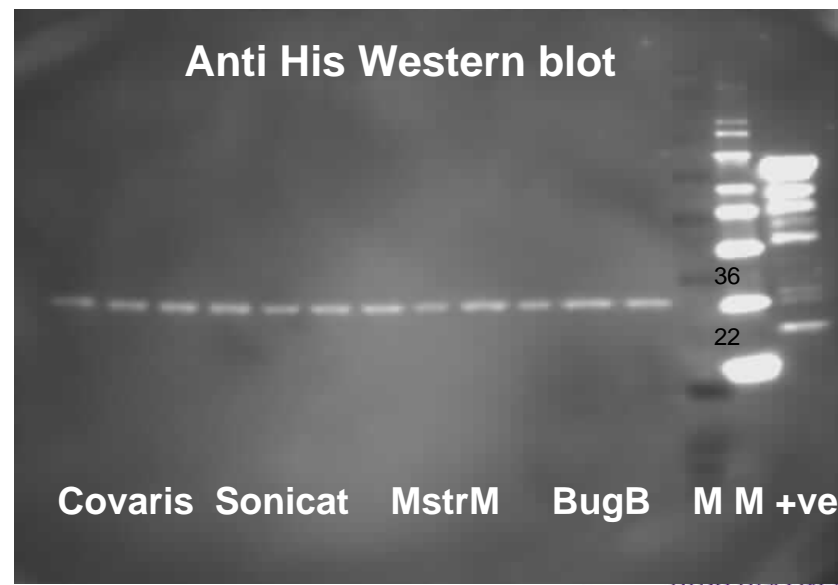
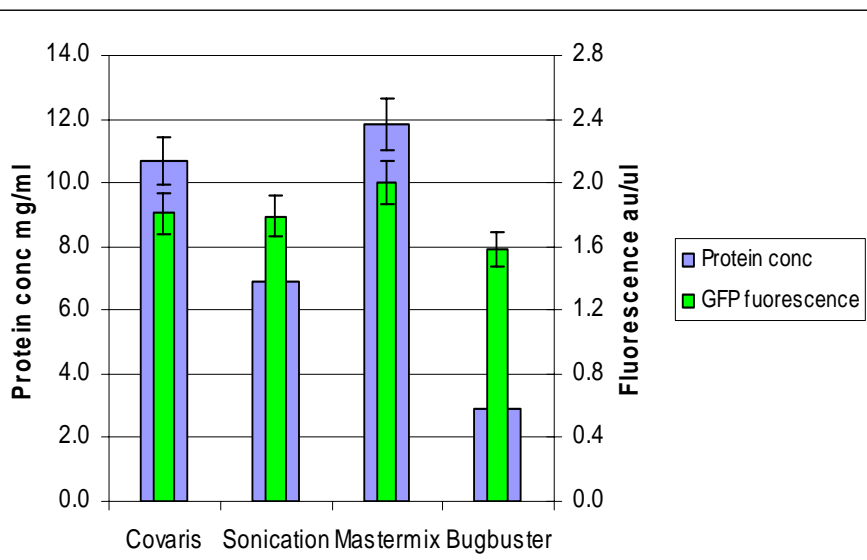
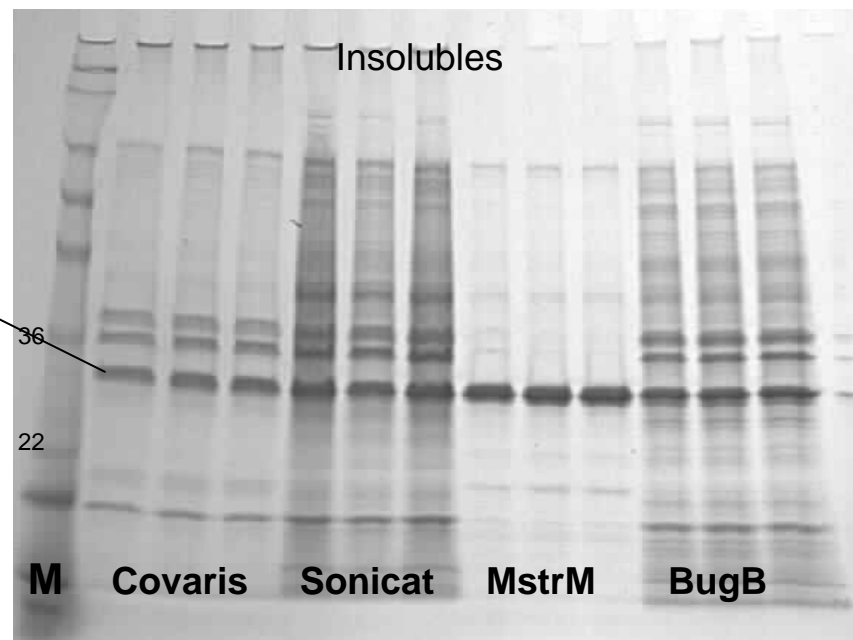
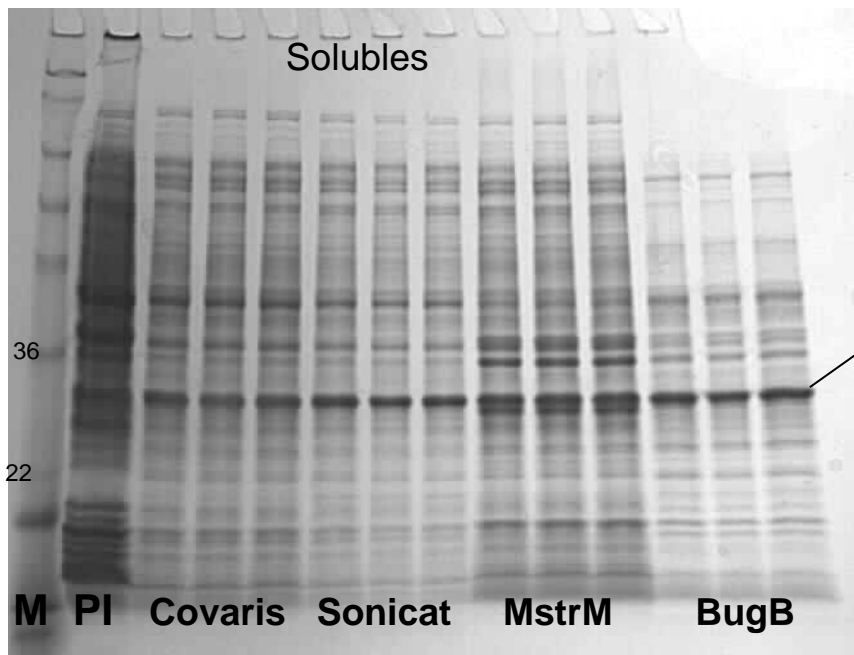
Protein A (78kDa)



Protein B (111kDa)



Green Fluorescent Protein (27kDa)



Covaris Conclusions

- Highly effective at lysing 1g *E.coli* pellets
- Effective at lysing 10g pellets, but intervention required.
 - vortexing improves lysis
 - reducing buffer/cell ratio improves lysis
 - Longer lysis time needed
- Protein degradation probably not an issue
 - Functional activity studies required
- Lysis is comparable to sonication
- Temperature control using chiller was effective
- Covaris was easy to use and robust

Other Applications

- Membrane bound proteins
 - Preliminary data indicates advantages
- Transfection/Transformation
 - Will need low energy controllable power source
- Vesicle preps
 - Significant advantages over existing manual methods
- Compound dissolution
 - Offers promise, evaluation ongoing
- Accelerated enzymic lysis
 - Maybe useful in generating tryptic peptides

Covaris S2 Summary

- **PROs**

- As effective as sonication
- Sealed system (eliminates contamination)
- Easy to use (programs stored, intuitive software)
- Highly configurable (easy to change from Eppendorf to large tubes)
- Isothermal (no ice buckets, cardice etc.)
- Eliminates requirements for cabinets and dedicated labs (silent use, can be used on open bench)
- Potential to provide a single methodology across groups.
- Potential single multifunctional tool for a variety of tasks

- **CONs**

- High cost ca. £25K for S2
- Processing 10g samples requires manual intervention
- Processing large samples >50mls unproven

The Future

- Modified S3 under evaluation
 - Potential x10 increase in power
 - 100g lysis maybe possible
- A single highly flexible machine desirable
 - Microtitre to 500 mls
 - Consistent methodology
- HTP approaches,
 - Automated lysis of multiple samples

Acknowledgements

- Praveen Singh (staff GEPB)
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