# DNA extraction from Banana Slug and library preparation for sequencing

# DNA extraction for Sequencing

- Collection and examination of biological samples (Banana Slug)
- **DNA extraction(Overview of different techniques)**
- **Quantification of isolated DNA**
  - Gel Electrophoresis
  - Bioanalyzer
- **Sample preparation**
  - DNA shearing
  - End polishing
  - Adaptor ligation
  - Quantification
- DNA amplification (ePCR or bridge-PCR)
- Enrichment
- Sequencing

# DNA source

- Tissue (i.e. muscle, liver, brain)
- Saliva
- Blood
- Bone marrow
- Hair
- Sperm

# DNA extraction

Goals:

2. isolation of nucleic acid from the cell
3. purification of isolated DNA

**Yield/quality ratio should be as high as possible**

# DNA extraction methods:

- Organic
- Ion-exchange Resin
- Silica Matrix
- High Salt
- Magnetic Affinity Resin

# Organic

- Lysis of the cells:

  alkaline buffer or chaotropic agents, proteinase K, incubation in 56ºC

- Purification:

phenol:chloroform

- Extraction:

  ethanol or isopropanol

http://en.wikipedia.org/wiki/Chaotropic_agents

# Ion-exchange Resin

- Lysis of the cells:

  sample is heated in the presence of resin to 100ºC

- Purification:

  resin binds cellular components other than DNA

- Extraction:

  centrifugation removes resin leaving DNA in the supernatant

# Silica matrix

- Lysis of the cells:

chaotropic agents, proteinase K, incubation in 56ºC

- Purification:

DNA binds to the silica matrix

contaminantsare eliminated by washing steps

- Extraction:

DNA iseluted with water from the matrix

# Why to use a commercial kits?

- Non-toxic methods
- Short incubation and centrifugation times
- Easy procedure steps
- Able to get rid of inhibitors that are solvable in the aqueous phase
- Ready for automatisation

# DNA measurement

- DNA concentration was measured fluorometrically using PicoGreendsDNAQuantitation Reagent (Invitrogen - Molecular Probes)
- Agilent Bioanalyzer, which is a microfluidics-based platform for sizing, quantification and quality control of DNA, RNA, proteins and cells on a chip using gel.

# Material

- A Banana Slug from UCSC campus was captured by David B.  and used as source for DNA extraction and sequencing
- The Slug was rinsed with water, ethanol,  dried and carefully dissected to remove intestines and skins, and stored in -80C
- For DNA extraction ~250 mg of “muscle” was used for DNA extraction using Invitrogen DNA

# **PureLink™ Pro Genomic DNA Kit** (Invitrogen)

- The PureLink™Genomic DNA Kit is based on the selective binding of DNA to silica-based membrane in the presence of chaotropic salts.

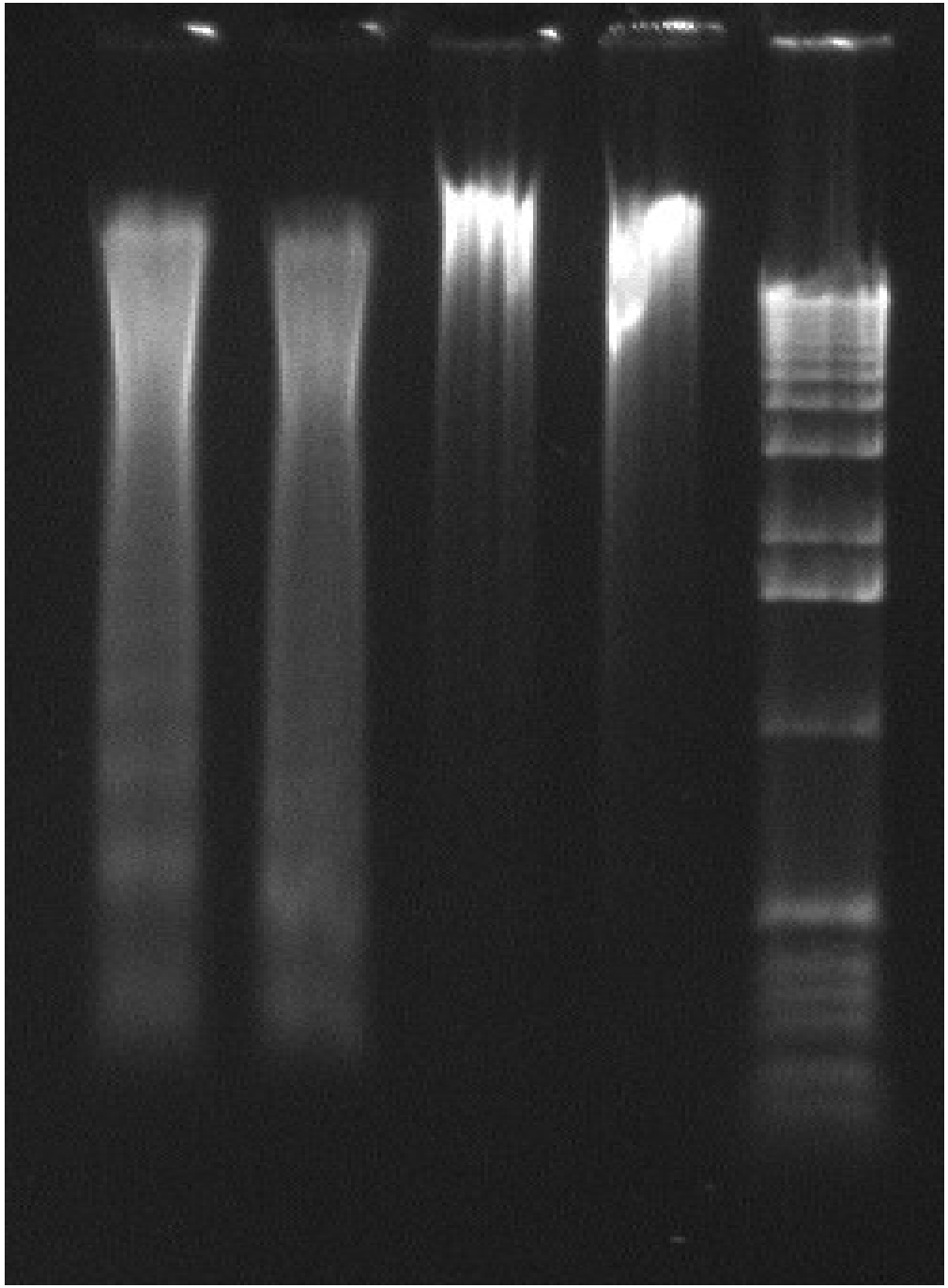
- The lysate is prepared from “muscle” tissues by incubation for 2hrs in the presence of ProteinaseK at 55°C using an optimized digestion buffer formulation that aids in protein denaturationand enhances Proteinase K activity.

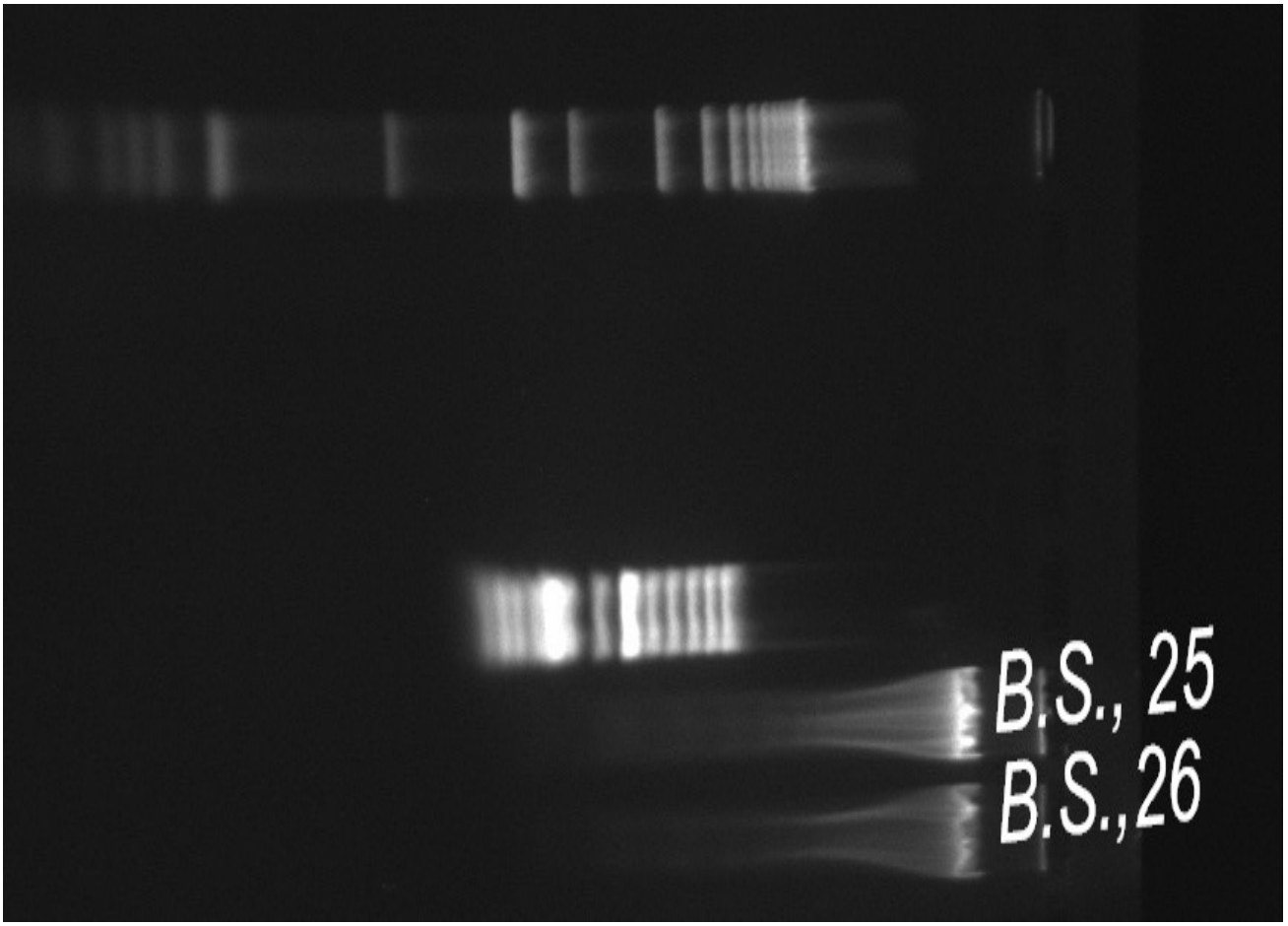
- Any residual RNA is removed by digestion with Rnase A prior to binding samples to the silica membrane.

- The lysate is mixed with ethanol and PureLink™ Genomic Binding Buffer that allows genomic DNA to bind the PureLink™ gDNAFilter.

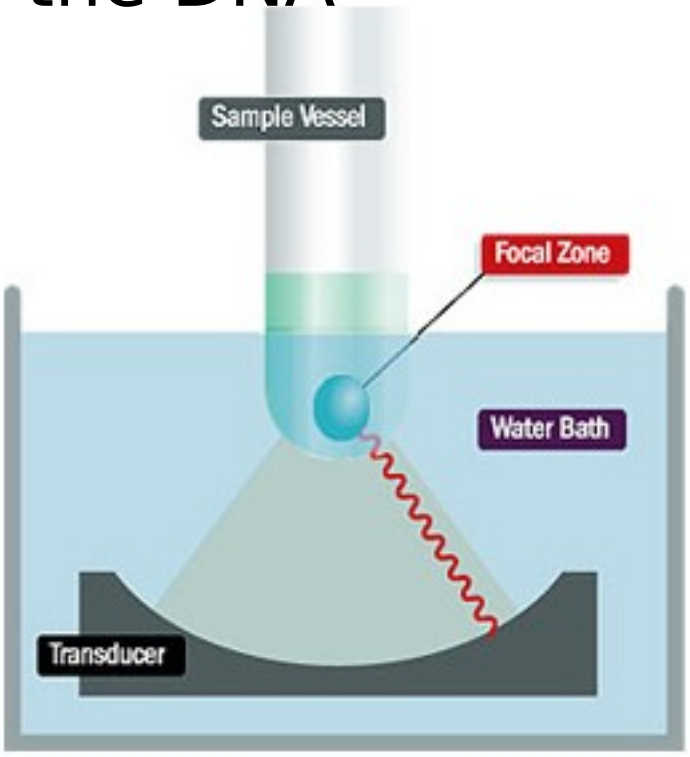
- The DNA binds to the membrane in plate and impurities are removed by thorough washing with Wash Buffers.

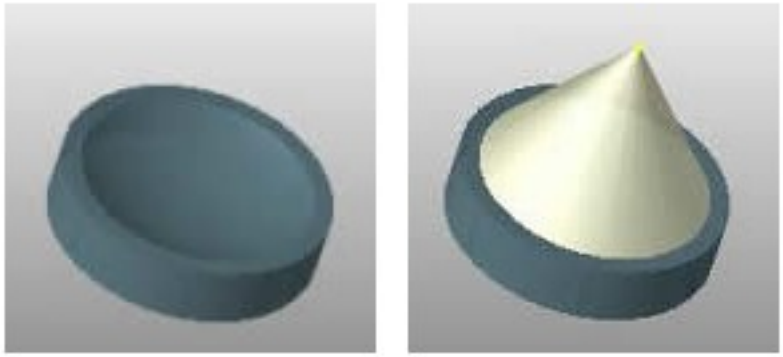
- The genomic DNA is then eluted in low salt Elution Buffer or just warm DI-water.




B.S., 25
B.S.,26

# Covaris- to shear the DNA



Transducer | Transducer + beam

http://www.covarisinc.com/products_services.htm

# Covaris- to shear the DNA

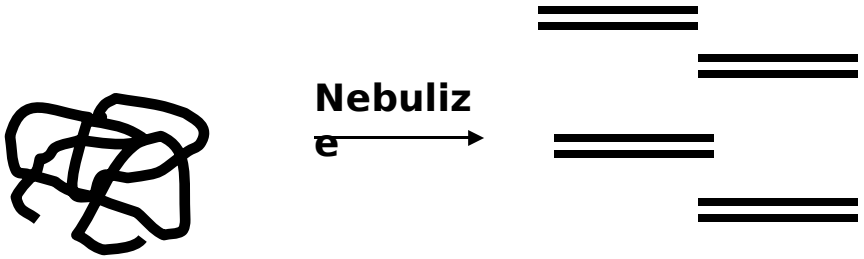
# Covaris- to shear the DNA

Adaptive Focused Acoustics is a form of mechanical energy. As acoustic / mechanical energy transfers through the sample, the material undergoes compression and rarefaction (expansion). At high intensity with fluid samples, this is typically embodied as cavitation events.

As the number of bubbles is extremely high and the time interval is short (micro seconds), the mixing and/or disruption power capability of the process is significant.

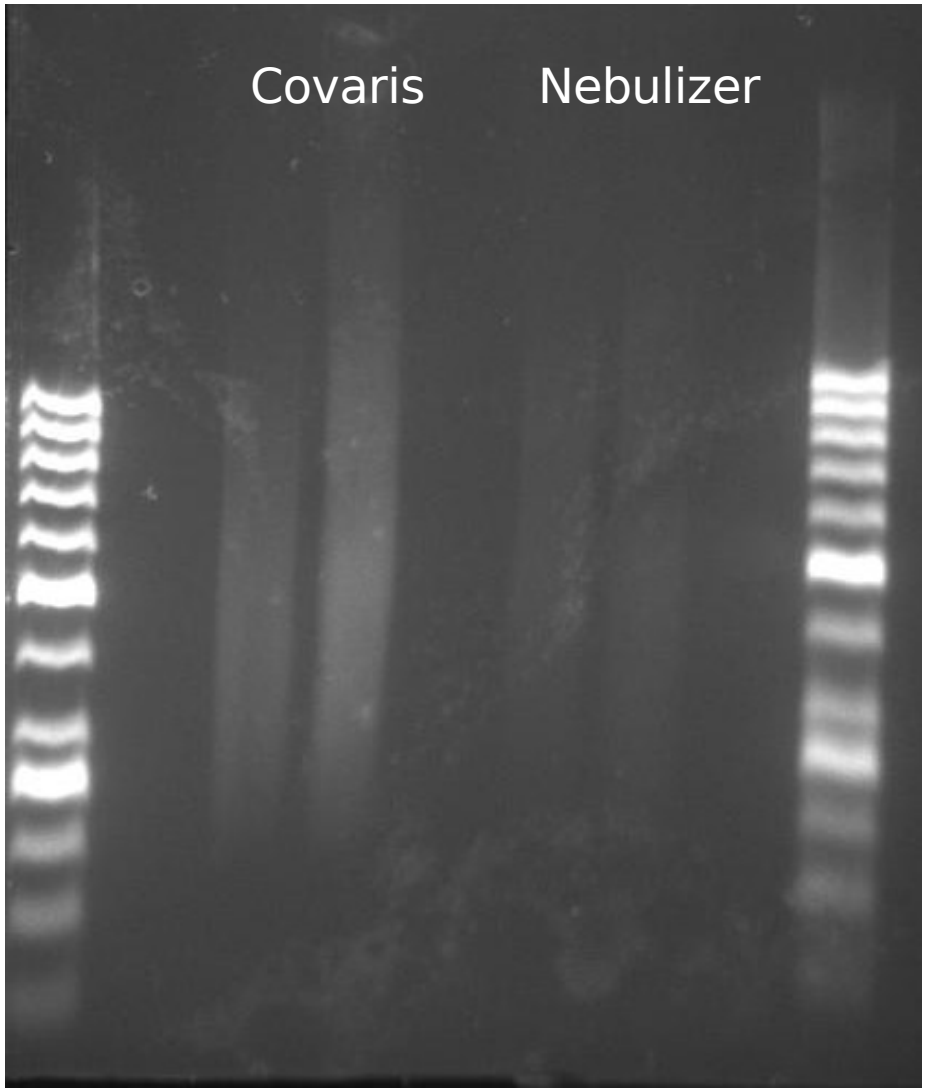
A key point is the precise, reproducible control that is obtainable with the Covaris instrument systems utilizing AFA.

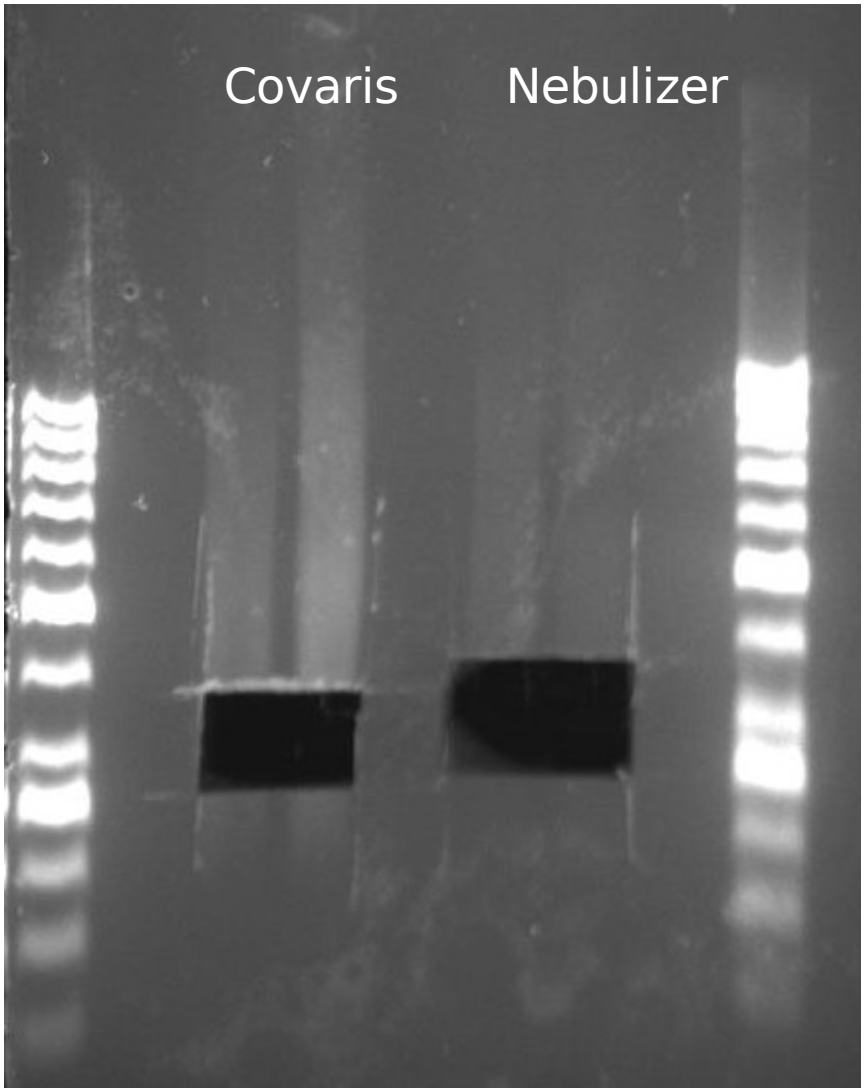
# Nebulizer

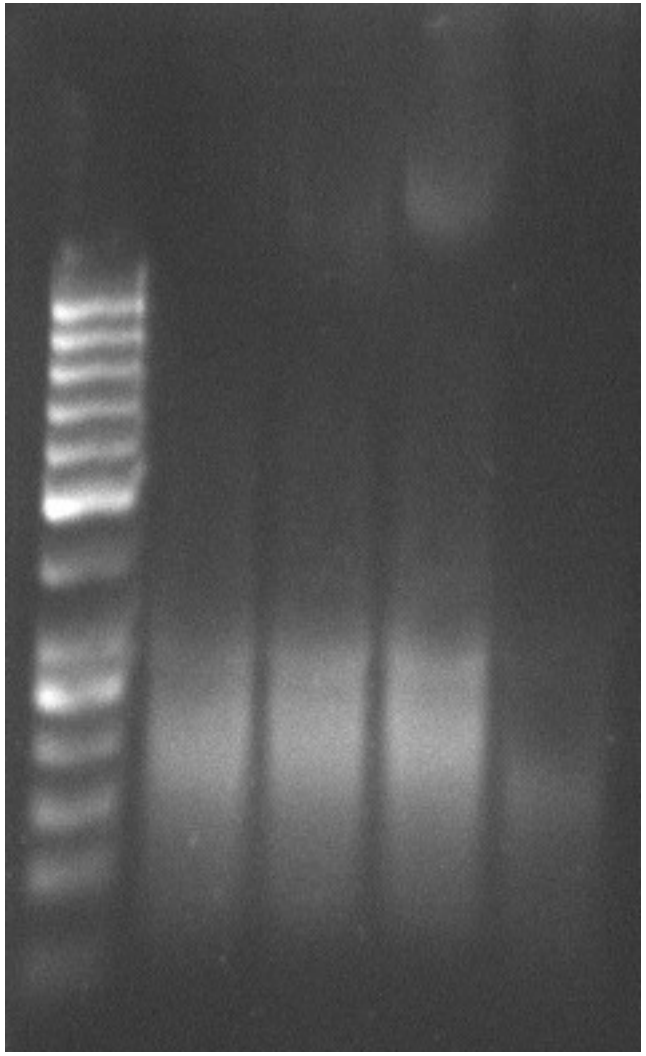


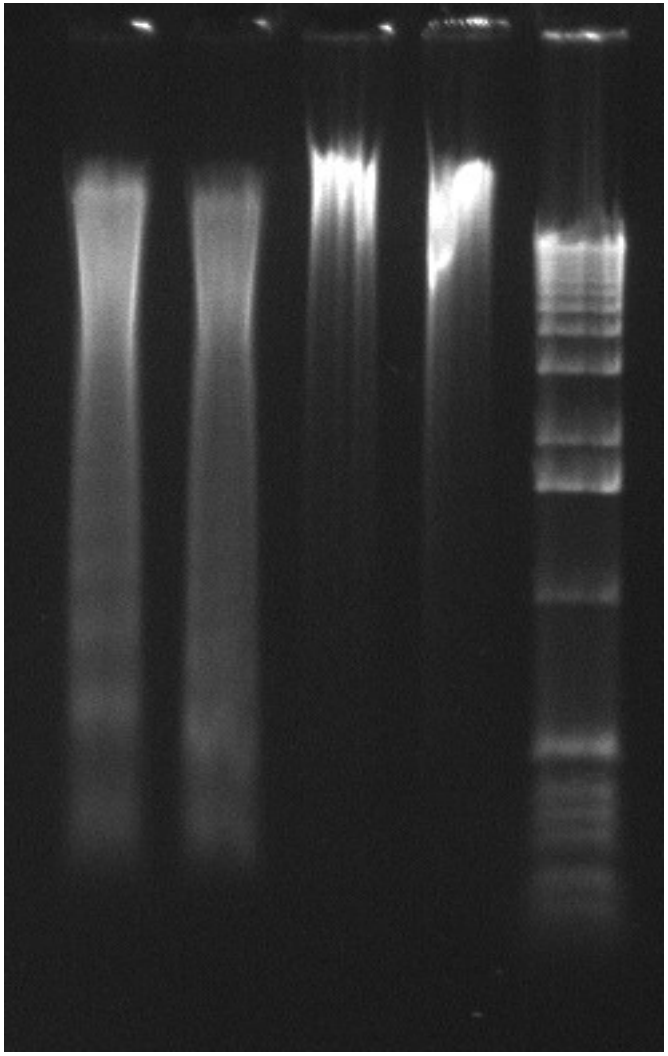

***Nebulization***: 500 ng of DNA is nebulized at 30 psi (2.1 bar) with vented cap nebulizer. Vented cap now supplied with nebulizer kit.

***Fragment Purification***: nebulized fragments using one Qiagen Minelute column


Covaris
Nebulizer


Covaris
Nebulizer

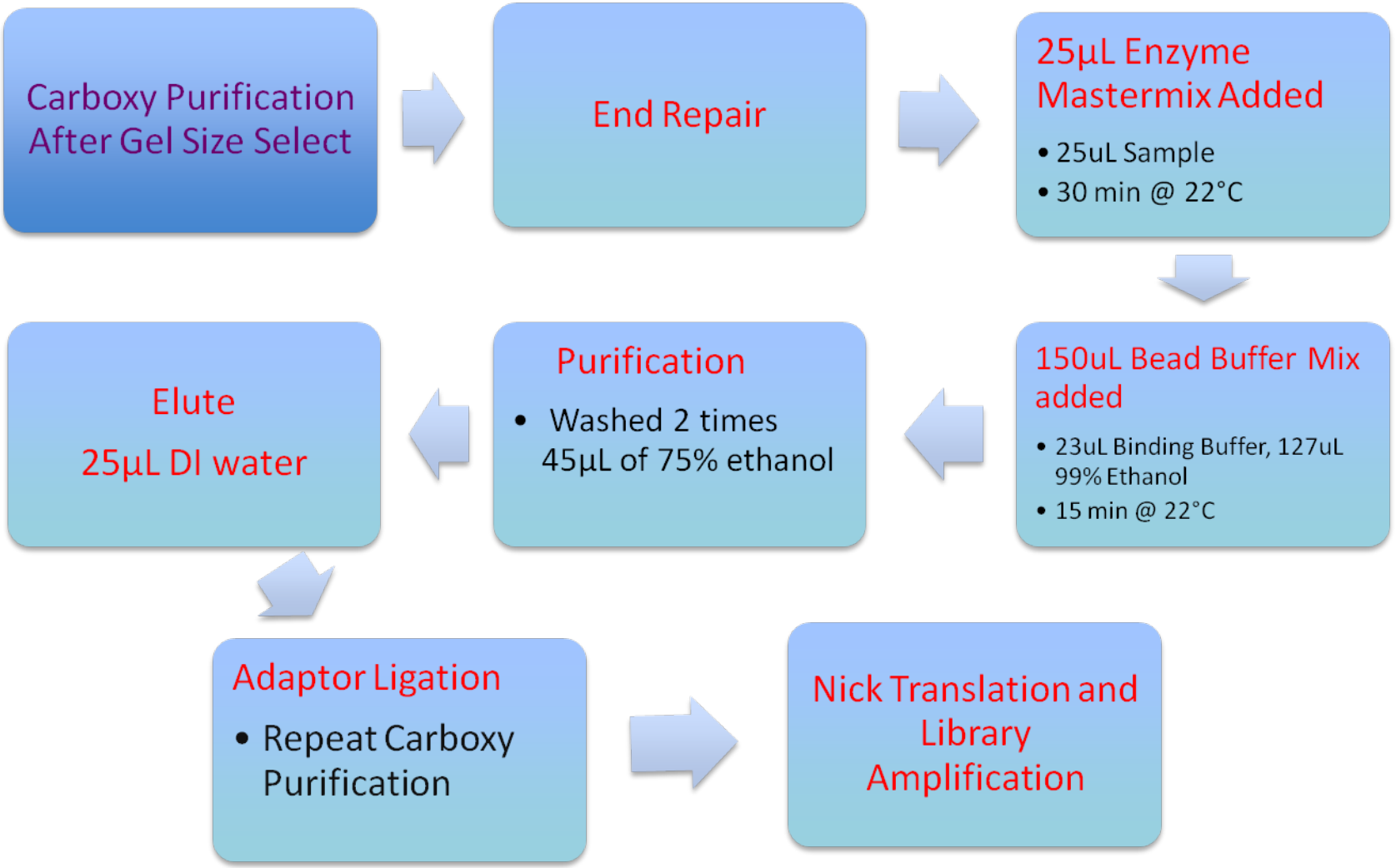




# Sample Preparation

1. Shear DNA(50-200 bp or 300-800 bp ) – Covaris
2. Size Select 2-4% Gel
3. Carboxyl Magnetic Purification
4. End Repair
5. Carboxyl Magnetic Purification
6. Adaptor Ligation
7. Carboxy Purification
8. Nick Translation and Large Scale PCR
9. Lonza Gel-Check Library Amplication
10. Carboxyl Magnetic Purification
11. Bioanalyzer (to quantify the library)
12. Gel-Purify Size Select (remove adaptors)

# Protocol optimization



# Rapid Library Preparation

**Standard Library Preparation**

AmPure Bead Calibration
Input Amount **3,000 ng**
Nebulize 3,000 ng
Column Purification
Fragment Isolation
DNA 7500 Chip
Fragment Polishing
Column Purification
Adaptor Ligation
Column Purification
Small Fragment Removal
Fragment Immobilization
Fill-In Reaction
Single Stranded Library Isolation
Column Purification
RiboGreen Assay
Agilent RNA 6000 Pico Chip
Calculate Molecules/ul based on Size and Mass

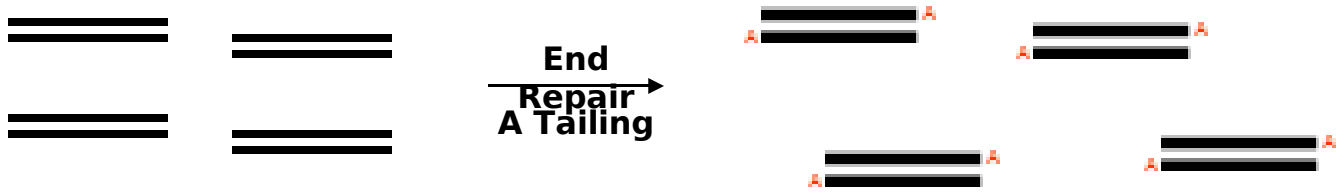
**Rapid Library Preparation**

**No**AmPure Bead Calibration Needed
Input Amount **500 ng**
Column Purification
Fragment Polishing and A Tailing
Adaptor Ligation
AmPure Bead with Sizing Solution
Quantify with FAM Standard

Fewer steps = faster preparation
500ng DNA input amount into library preparation
17 step process condensed to 6 steps
One **single** column purification in place of 4 column purifications

# Rapid Library Preparation (Feb. 2010)

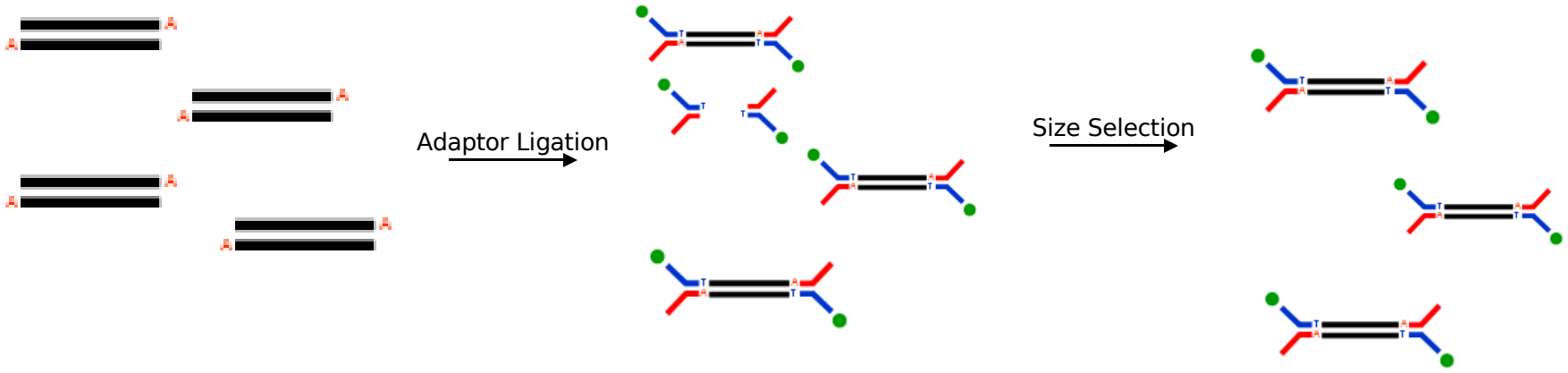
*Polishing and A Tailing*




- ***Polishingthe ends***: fragments are polished to create blunt ends for adaptor ligation
- ***A Tailing***:  A tails are added to fragments to allow for TA ligation of the library adaptor

# Rapid Library Preparation

*Adaptor Ligation and Size Selection*

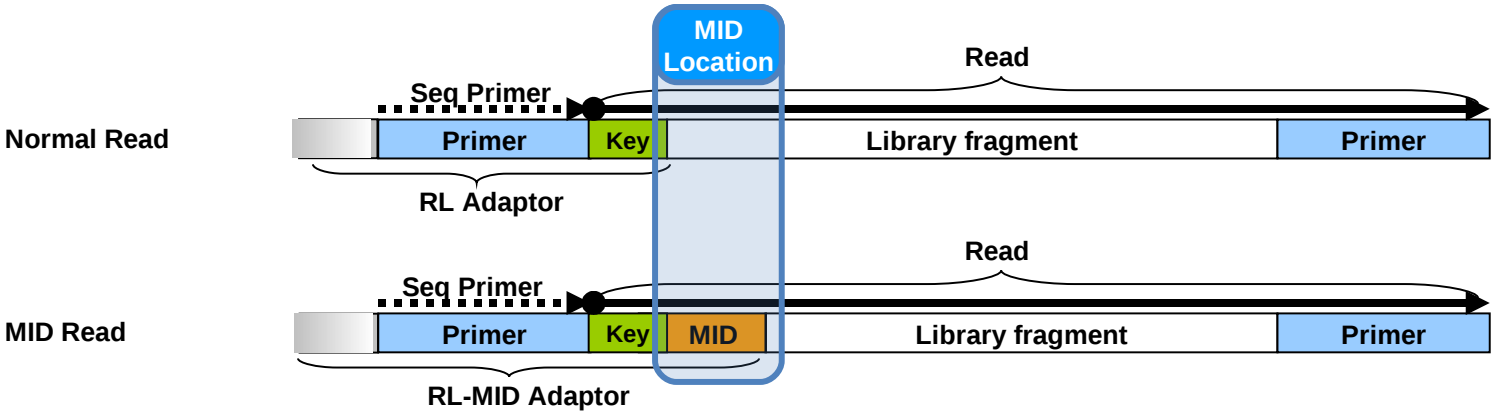



- ***Ligation***: Adaptors are ligated onto polished fragments. Adaptors contain flourscent molecule for direct quantitaiton of the library. Each double stranded adaptor contains both A and B sequences. New key sequence of GATC.
- ***Size Selection***: AMPure bead size exclusion of fragments. No calibration of AmPure Beads required. Wash steps during size selection are critical. Complete removal of buffer during washing steps will ensure removal of residual adaptor molecules

# GS Rapid Library Preparation

*Rapid Library MID Adaptors*


- **Kit Containing 12 MID Adaptors**
- **Replaces MID protocol released with Phase B (for Basic Set)**
- **Works with Rapid Library Prep Kit ONLY**

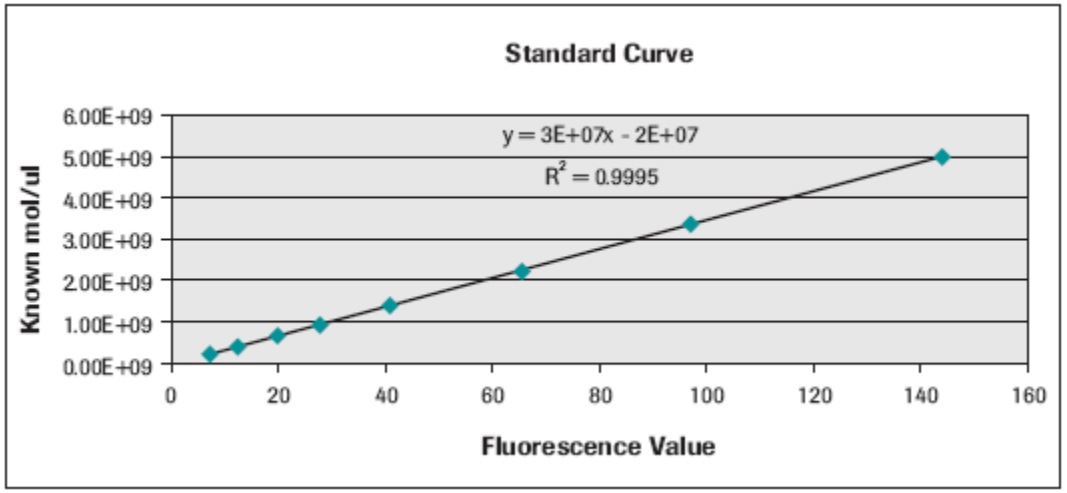



- RL-MID Adaptor = Rapid Library Adaptor + specially encoded 10-base sequence after key

# GS Rapid Library Preparation Overview

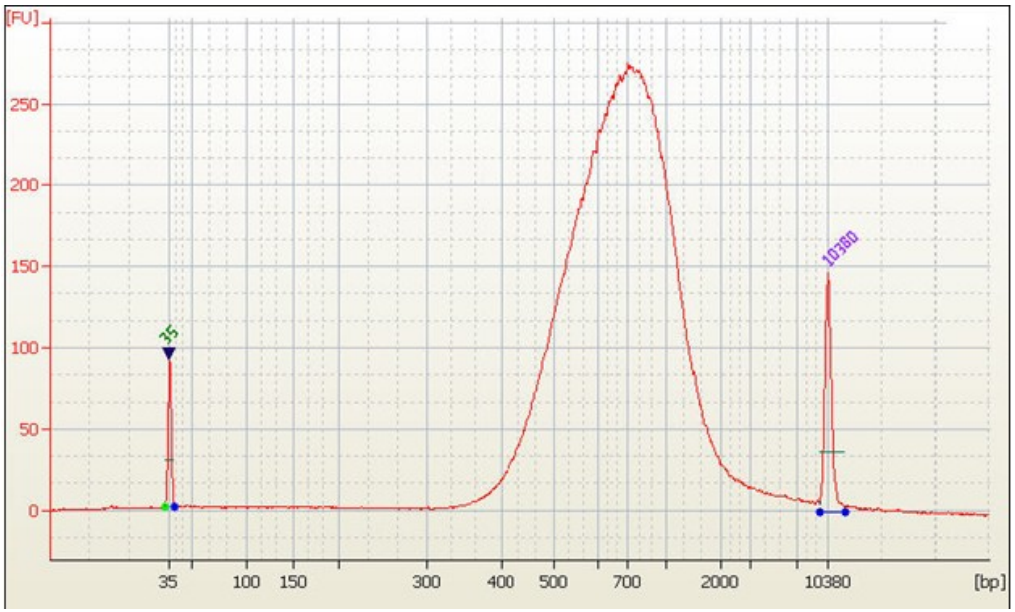
## *Quantization*

| Fluorescence | Concentration (mol/ul) |
|---|---|
| 143 | 5.00E+09 |
| 96.62 | 3.33E+09 |
| 65.43 | 2.22E+09 |
| 41.61 | 1.48E+09 |
| 28.6 | 9.88E+08 |
| 21.02 | 6.58E+08 |
| 12.76 | 4.39E+08 |
| 8.511 | 2.93E+08 |




- Direct quantitation of the library using fluorescent tag on adaptor
- Fluorescence standard included as part of kit (RL Standard)
- ***Rapid Library Calculator:*** (link to be provided)
  - provided for use through the my454 customer access site
  - plots the standard curve
  - assess quantitation results
  - calculate dilution volumes.

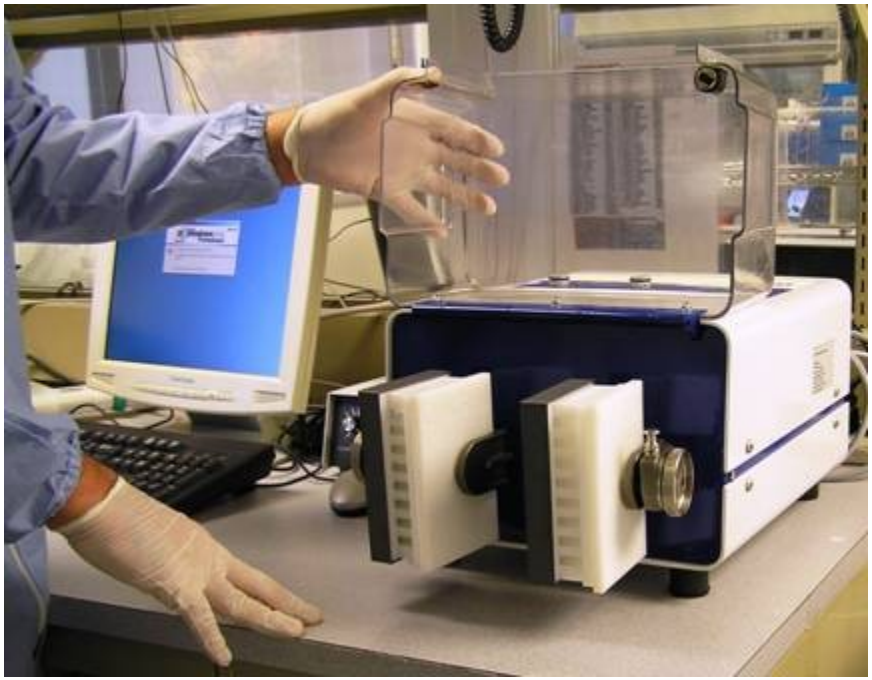
# Rapid Library Preparation Overview
# *QC*

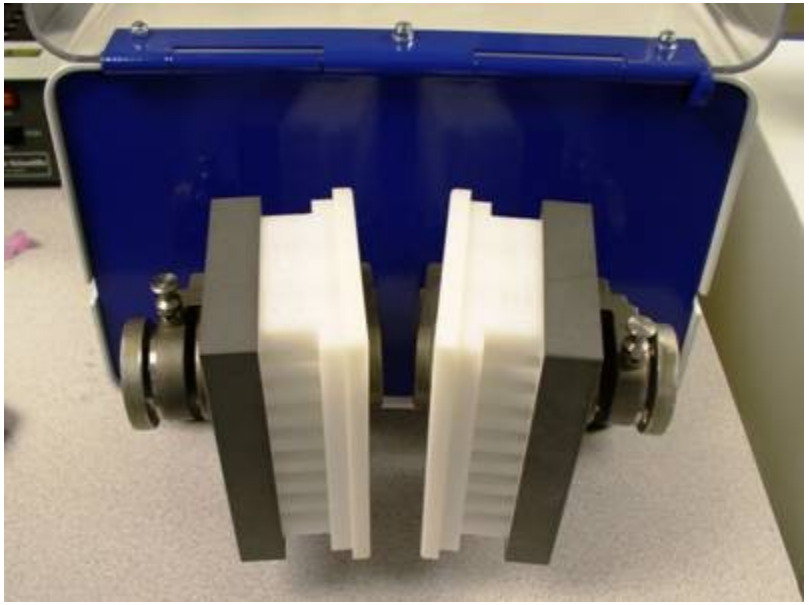



- ***QC of the library***: Average peak size of 600bp to 900bp with less than 10 % of fragments below 350bp. Bioanalyzer High Sensitivity DNA chip used to visualize trace.

# GS FLX Titanium Chemistry

## *Emulsion Formation*

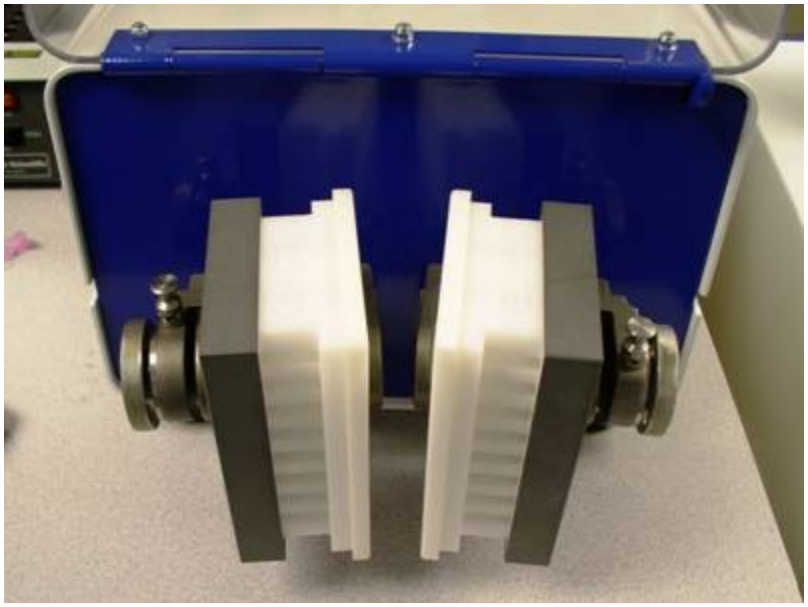




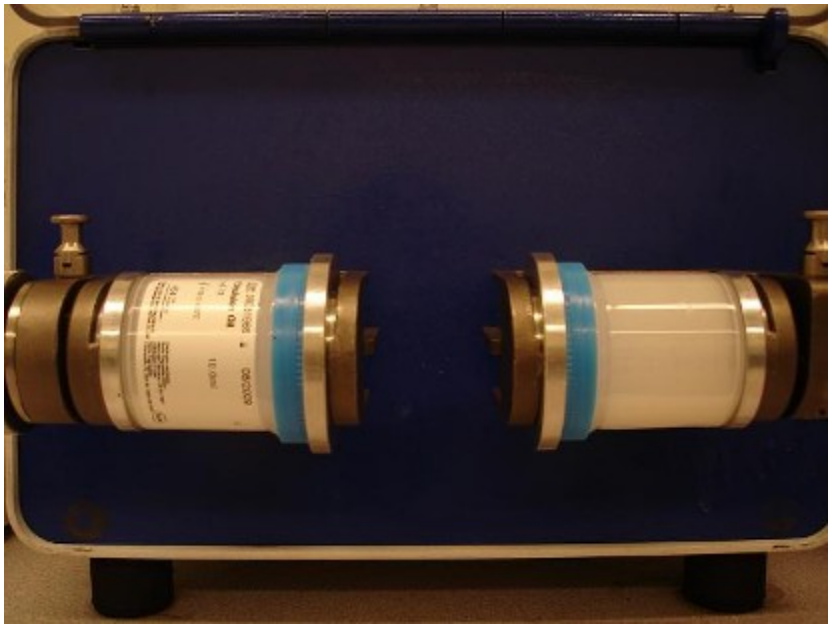
Emulsion Oil and PCR mix containing Capture Beads are mixed using a QiagenTissueLyser as a high speed shaker

# GS FLX Titanium Chemistry

## *Emulsion Formation*



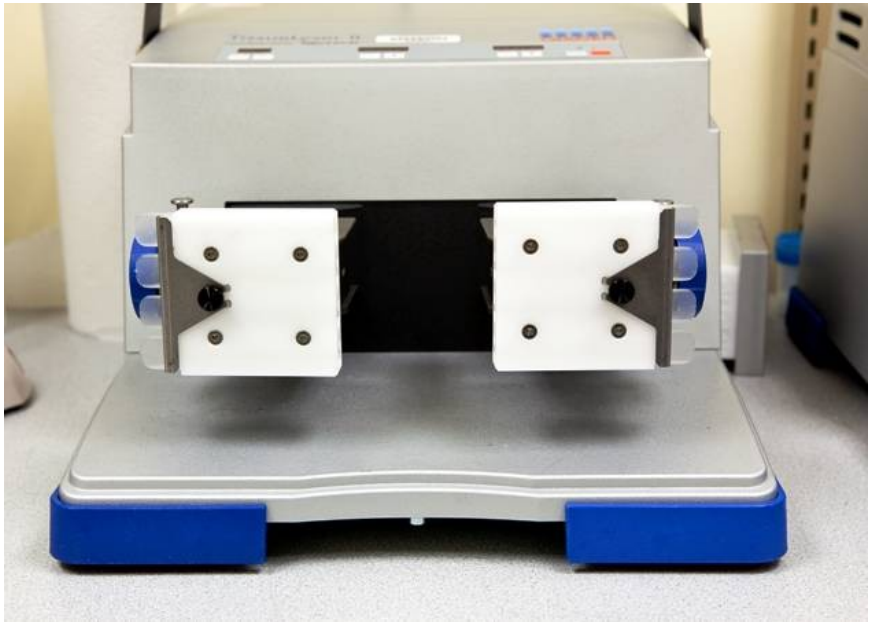
Small Volume Emulsions



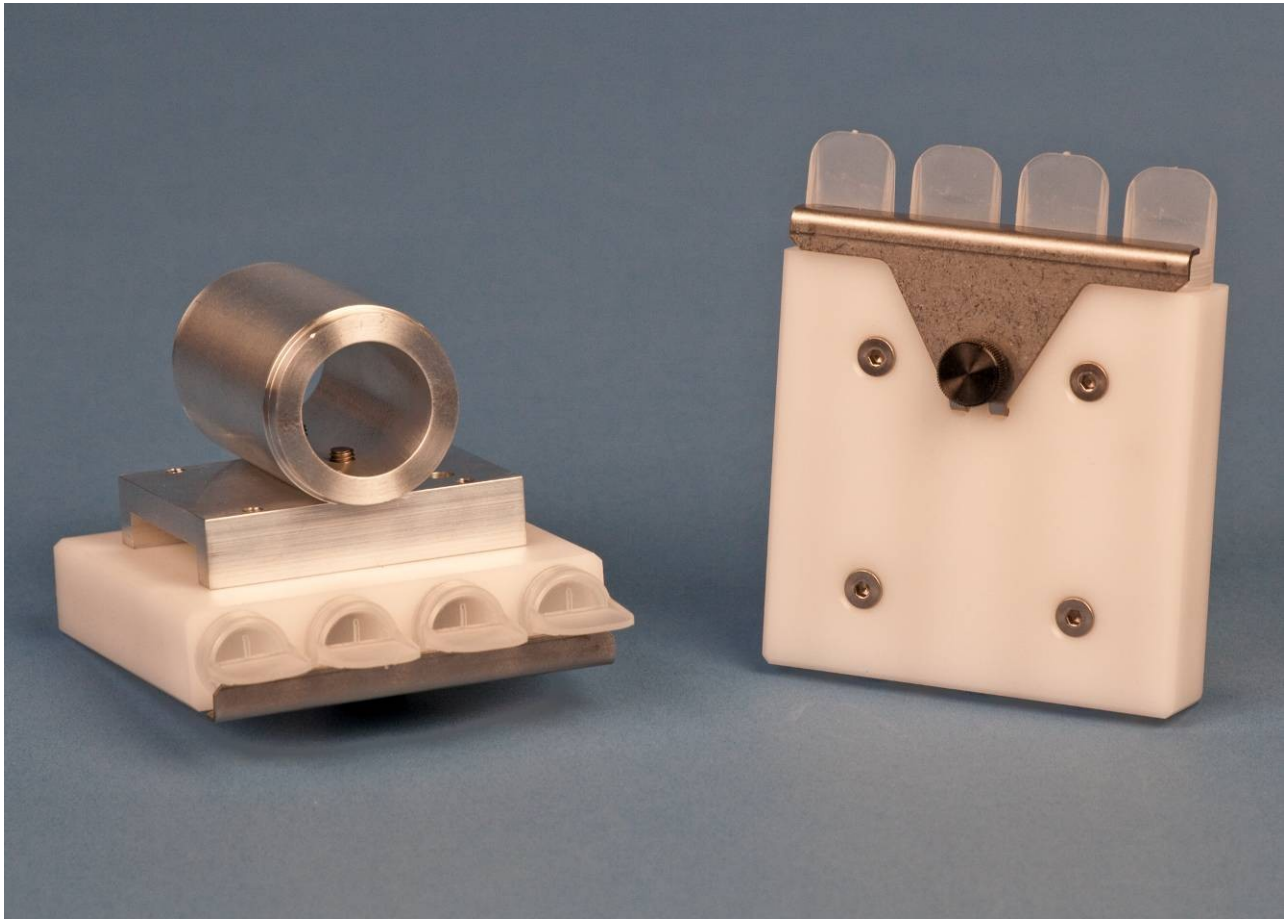
Large Volume Emulsions

Emulsions are formed in individual tubes for Small Volume Emulsions (SVE) or in cups for Large Volume Emulsions (LVE) or ‘bulk’ reactions.

# Medium Volume Emulsions

- Medium Volume ideal for use in 4 and 8 region run preparation
- GS Titanium emPCR Shaker Adapters MV Kit (05618487001) required for use of MV Oil format



# Emulsion Changes

# GS FLX Titanium Chemistry

## *Emulsion PCR*



- Type of emulsion prepared (SVE, MVE, or LVE) depends on type of sample and coverage required
- 1 LV Kit (2 cups) required for 2 region PTP
- Use SVE or MVE for other gasket options
  - Appropriate for multiple samples requiring little coverage, titrations, etc.

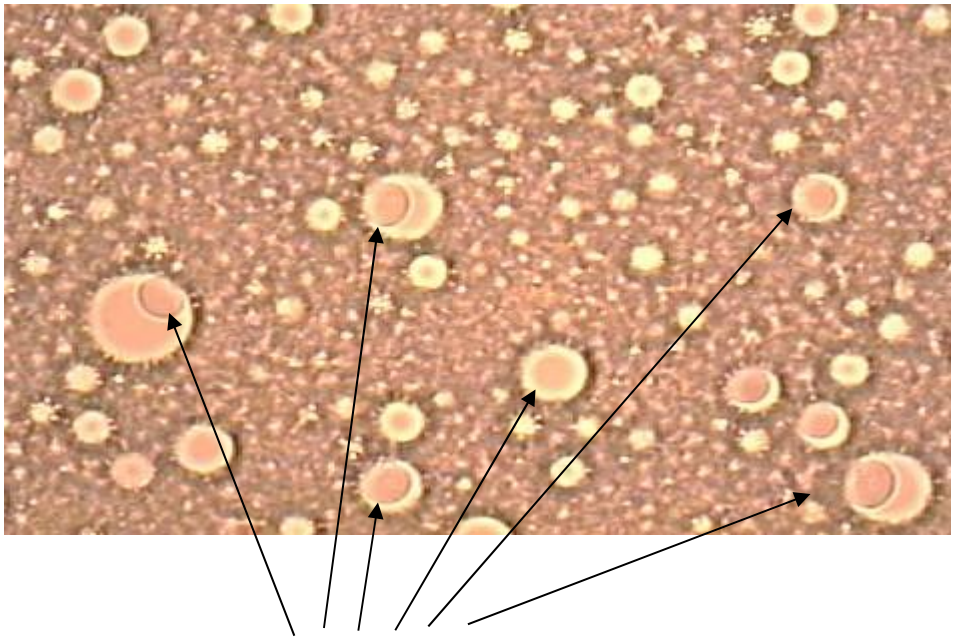
# GS FLX Titanium Chemistry

## *Emulsion PCR*

- Emulsions are prepared on TissueLyser
- Emulsions must then be aliquoted into PCR plates for amplification
- Up to 4 PCR plates required per sequencing run
- PCR program takes approximately 6 hours

# GS FLX Titanium Chemistry

*Emulsion PCR*

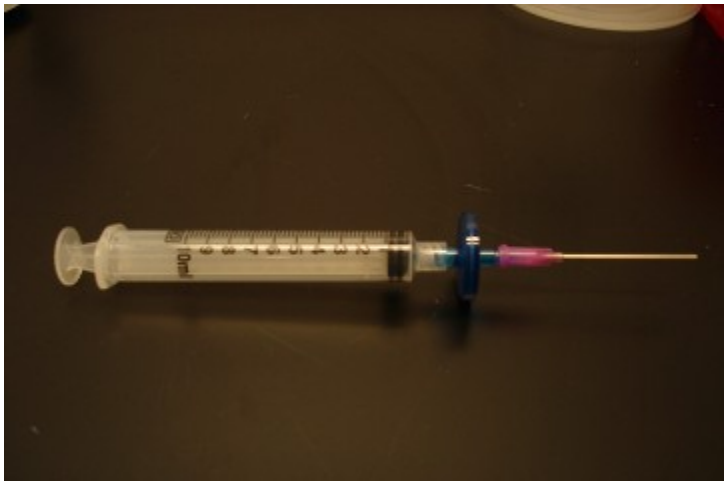


**DNA Capture Beads**

- All samples processed in parallel
- “B” attached to capture bead.
- “A” primer is in solution
- Microreactors are amplified simultaneously.
- After PCR, each bead contains millions of clonal copies

# GS FLX Titanium Chemistry

## *Breaking the Emulsion*



### LVE and MVE Breaking Procedure

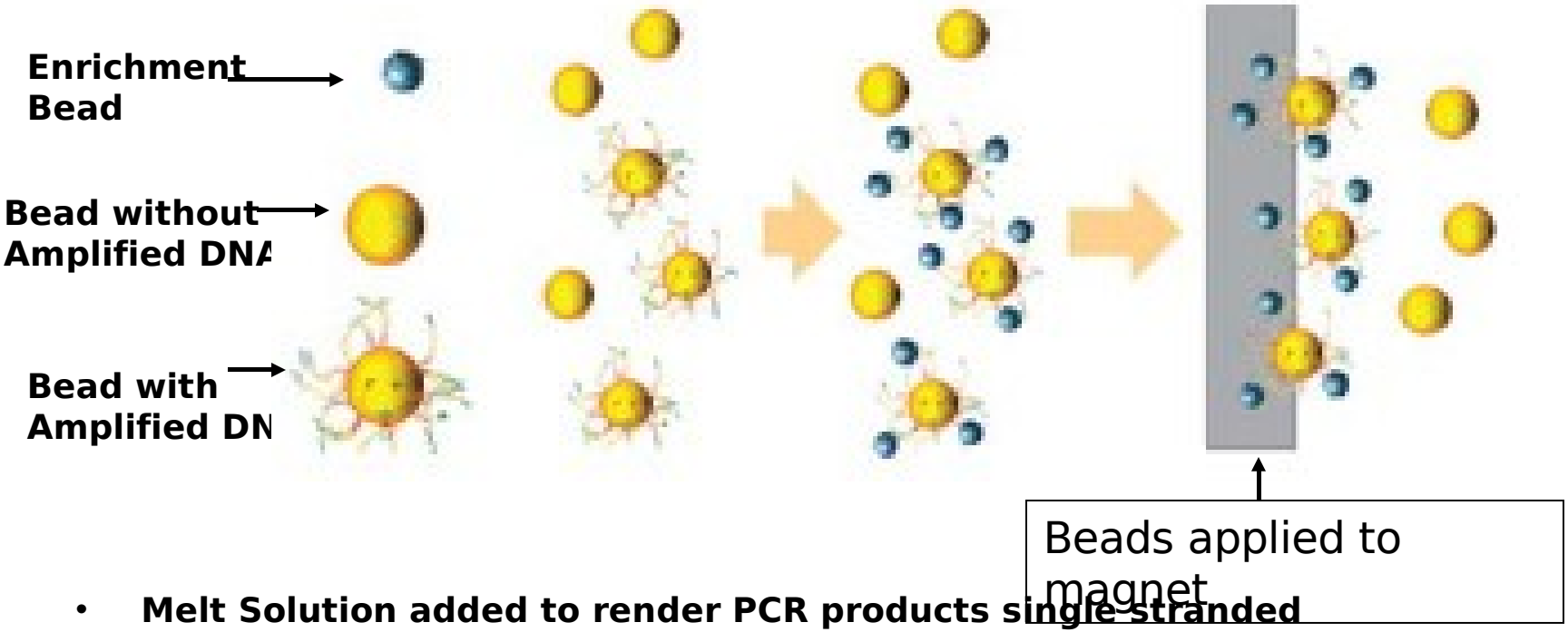
- Use Vacuum-assisted Emulsion Breaking Apparatus, supplied in LV breaking kit
- Connected to a vacuum source (supplied by customer)
- Emulsion is aspirated from plate into 50 ml tubes
- Plate is washed using isopropanol
- Wash beads using centrifugation to complete breaking procedure

### SVE Breaking Procedure

- Pull emulsions into 10 ml syringe with blunt needle
- Pass Emulsion through Millipore filter, supplied in SV breaking kit (beads are retained)
- Wash Beads using filter
- Recover beads from filter

# GS FLX Titanium Chemistry

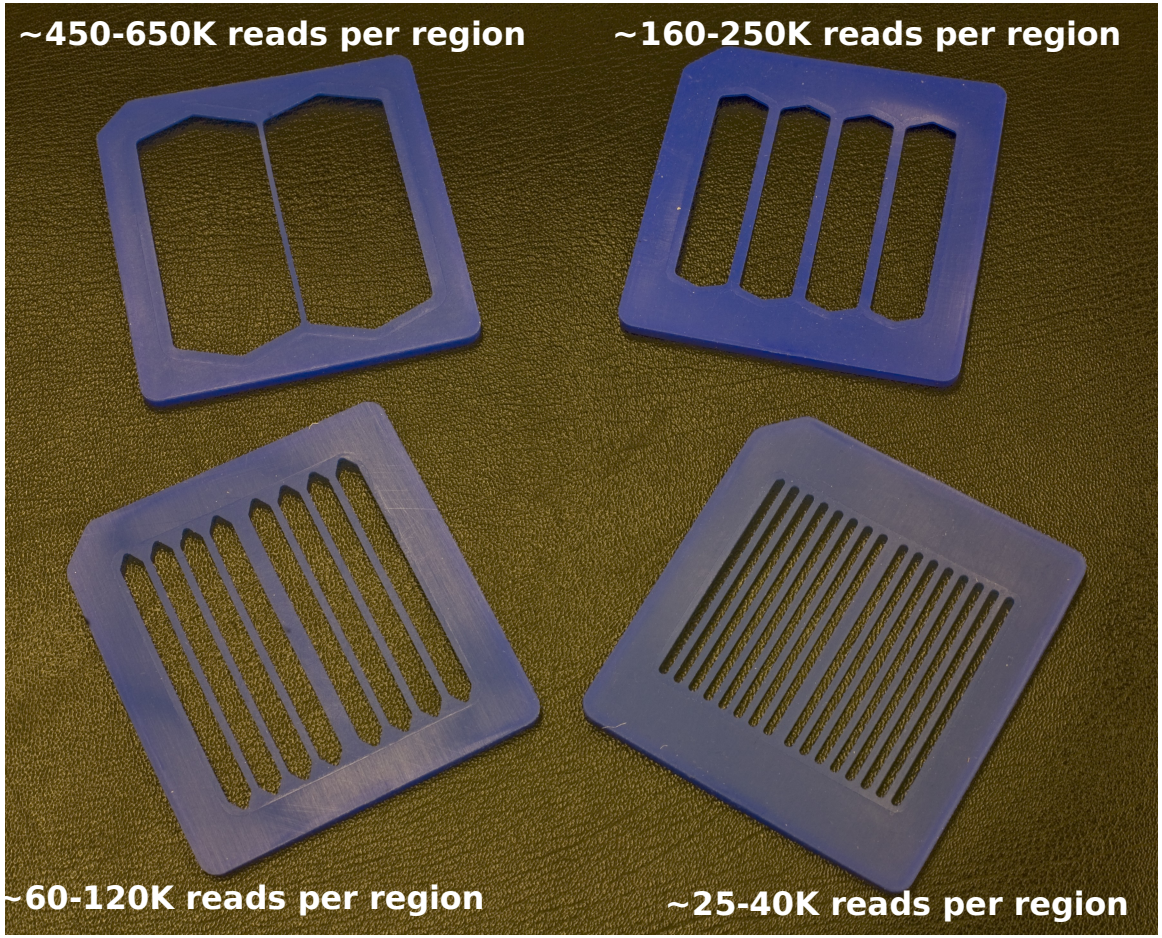
## *Enrichment*




- **Melt Solution added to render PCR products single stranded**
- **Biotinylated Enrichment primer is annealed to fragments on capture beads**
- **Streptavidin coated, magnetic Enrichment beads are added and bind to biotin**
- **Beads with DNA product are extracted using magnet**
- **DNA positive beads recovered by denaturing enrichment primer and collecting non-magnetic DNA beads**
- **Ideally, approximately 10% of beads are recovered**

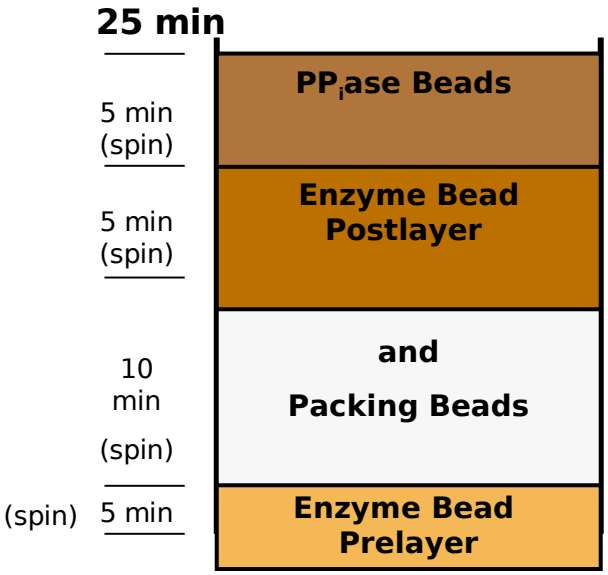
# GS FLX Titanium Chemistry

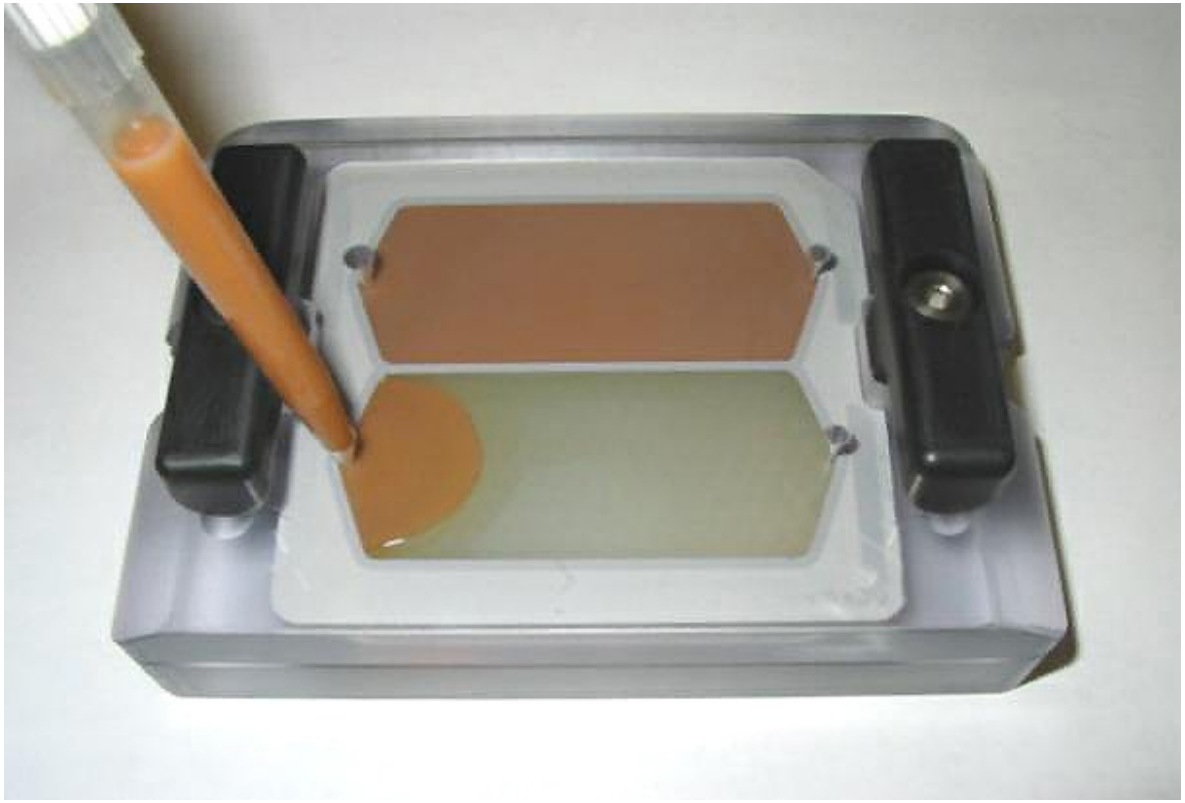
*Loading Gaskets for 70X75 PTP*



# GS FLX Titanium Chemistry

## *Bead Deposition Procedure*



- **Each chamber is filled with DNA beads, packing beads, enzyme beads, and PPiase beads in 4 separate layers by centrifugation**

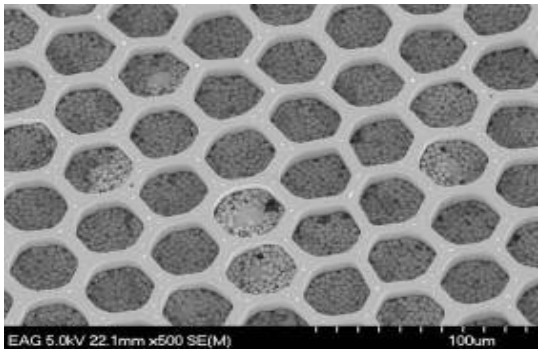
# GS FLX Performance

| GS FLX Standard Chemistry | GS FLX Titanium Chemistry |
| --- | --- |
| ✓ ~100 million bases<br>✓ >200 bp reads<br>✓ ~ 400,000 HQ reads<br>✓ Single 7.5-hour run | ✓ > 300 million bases<br>✓ > 300 bp reads ( with 500 bp mode)<br>✓ ~ 1 Million HQ reads<br>✓ Single 9-hour run |

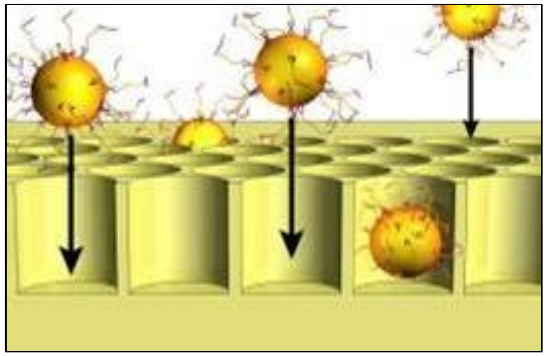
- Developed complete system starting from library preparation through to *de novo* assembly
- GS20 launched in Oct 2005 and GS FLX became available in Jan 2007. The Titanium chemistry for the GS FLX became available September 2008.
- Peer reviewed articles in Nature, Science, PNAS, etc
  - >454publications to date
- Global technology acceptance with sequences submitted to NCBI in the SFF format
- Roche Applied Science acquired 454 Life Sciences in April 2007

# GS FLX Titanium Chemistry

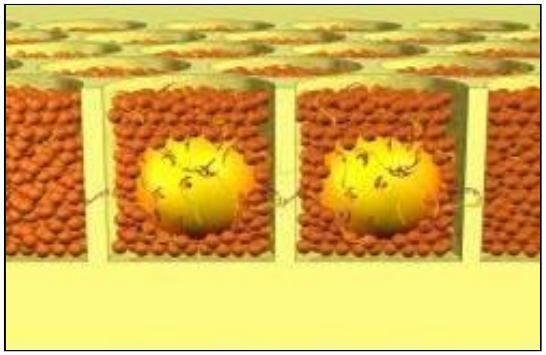
*Bead Deposition*




PicoTiterPlate ™



DNA beads are loaded into the wells of the PTP.



DNA beads packed into wells with surrounding beads and sequencing enzymes.

*A well diameter of 29 µm allows for a single **20 µm**bead per well*