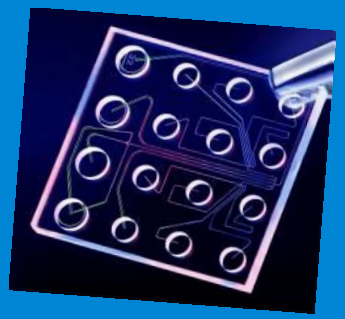
The Importance of Reliable Sample Quality Control in Multiple Workflows

2100 Bioanalyzer System



Rev. 2 [March 9, 2016]

Outline

Importance of Quality Control

Sample QC in Next Generation Sequencing

Sample QC in Gene Expression

Sample QC in Proteomics

… and even more!

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Importance of Quality Control

Sample QC in Next Generation Sequencing

Sample QC in Gene Expression

Sample QC in Proteomics

… and even more!

Quality of Samples is Critical for Experimental Success

Garbage in, garbage out!

A. Impact on sequencing:

Good DNA library quality helps ensure good quality reads and maximizing sequencing output

B. Impact on gene expression arrays and generation of RNAseq libraries:

Quality of input RNA will determine success of library preparation and reproducibility of microarrays

C. Impact on data analysis:

Integrity of RNA helps assure accurate determination of relative gene expression levels, support for existence of variants, transcripts and splice forms

- across different genes

- across alternatively spliced variants

Outline

Importance of Quality Control

Sample QC in Next Generation Sequencing

Sample QC in Gene Expression

Sample QC in Proteomics

… and even more!

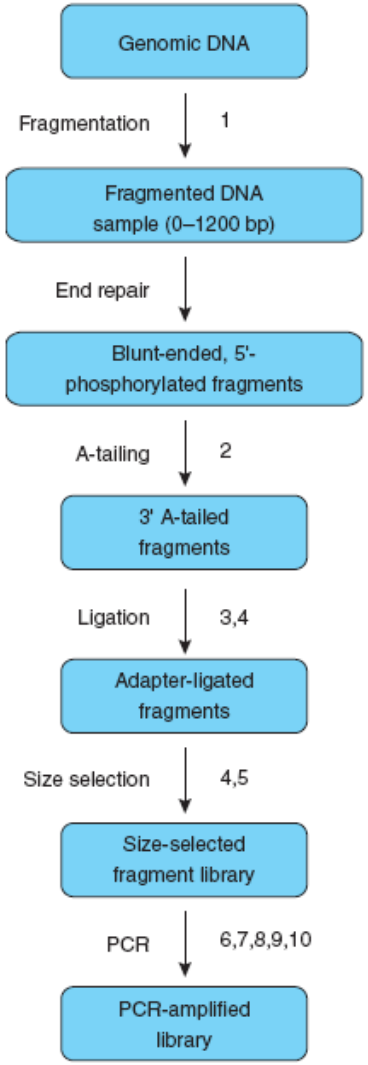
Quality of DNA Libraries is Critical for Sequencing Success

Garbage in, garbage out!

For every application of next-gen sequencing, eg. genome sequencing, transcriptome sequencing (RNA-seq), chromatin immunoprecipitation sequencing (ChIP-seq) or targeted resequencing, there is a specific protocol to convert the source nucleic acid to standard DNA libraries.

The aim of recent developments in library preparation methods is to produce a high-quality representative, non-biased DNA library from small amounts of starting material.

Quality Control during Library Preparation



Integrity of starting material

Size distribution after fragmentation

Size distribution of adapter-ligated fragment library

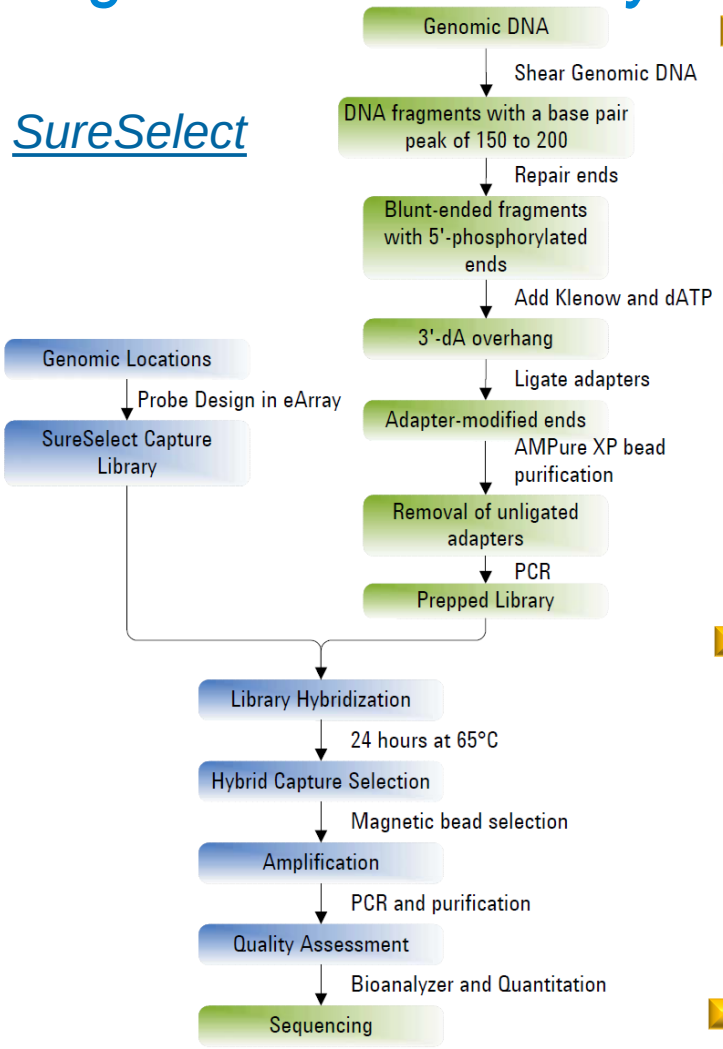
Quality control and quantification after PCR

Adapted from Quail et al (2008) Nature Methods)

Quality Control during Library Preparation

Target Enrichment Systems

SureSelect



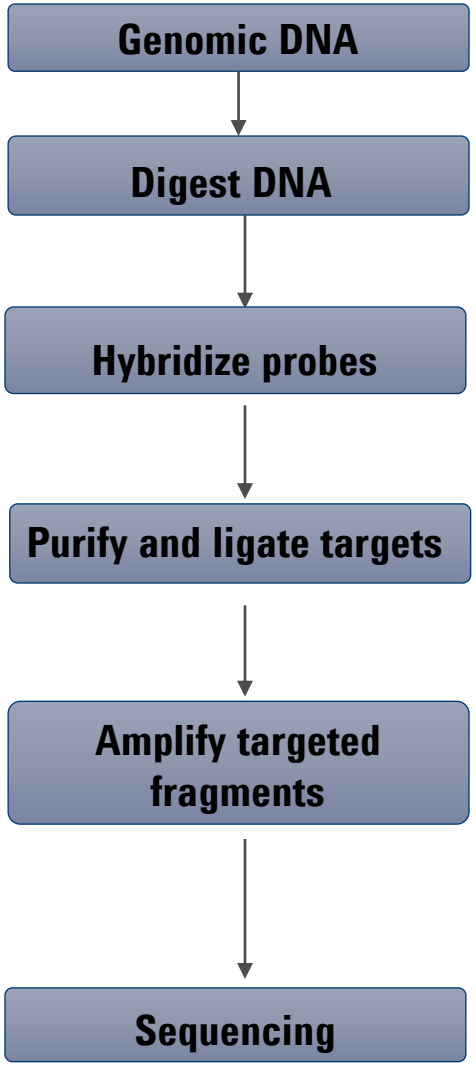
Integrity of starting material

Size distribution after fragmentation

Size distribution of adapter-ligated fragment library

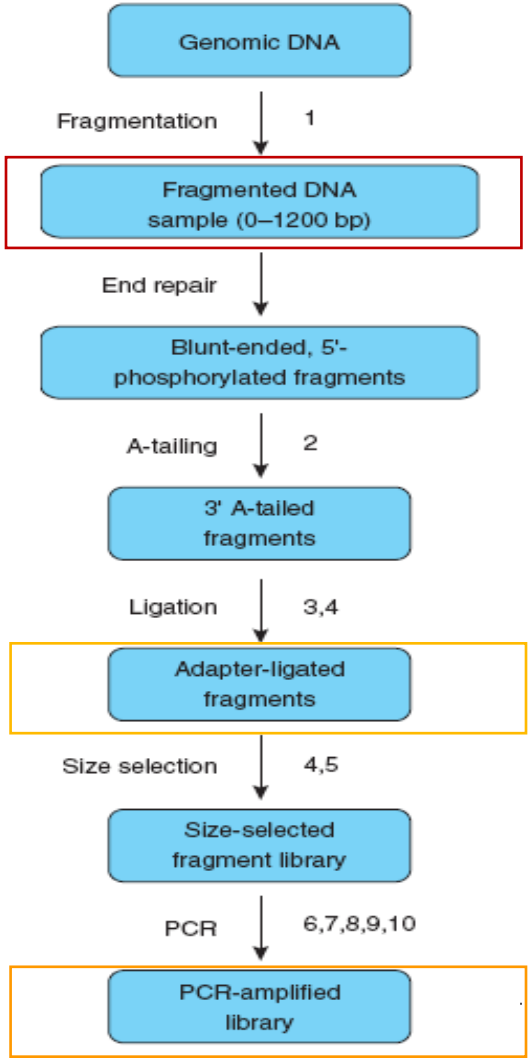
Quality control and quantification after PCR

HaloPlex

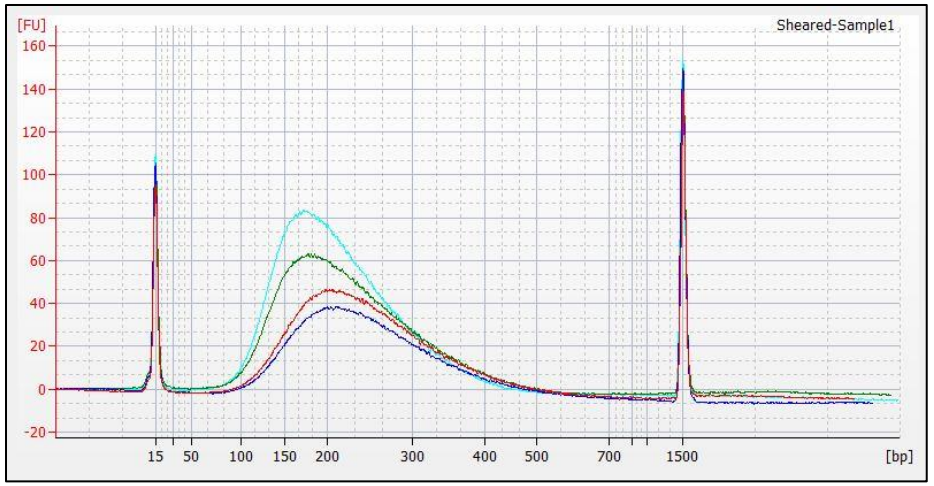


Next Generation Sequencing

- Steps where QC assessment is critical and Bioanalyzer analysis is recommended

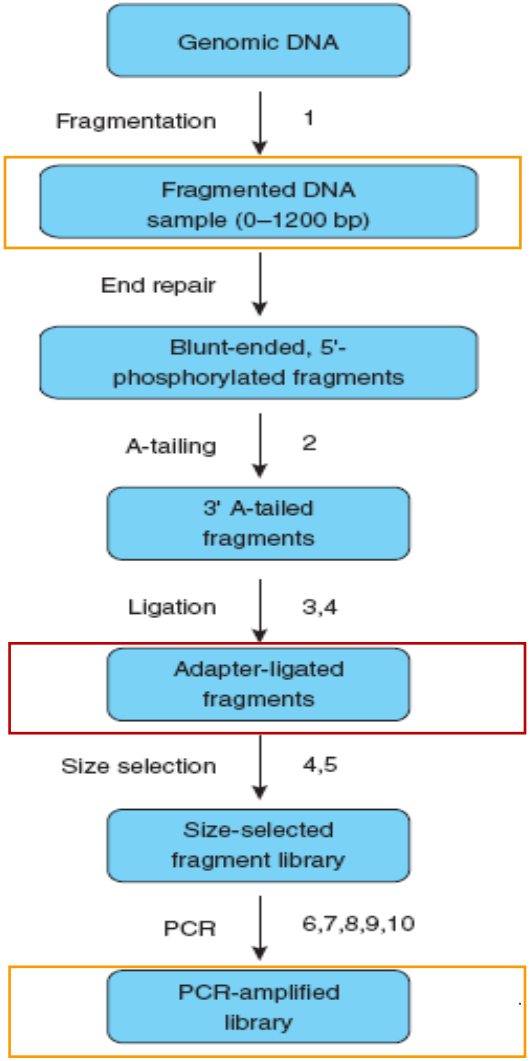


Samples sheared on Covaris and run on the Bioanalyzer DNA 1000 Kit:

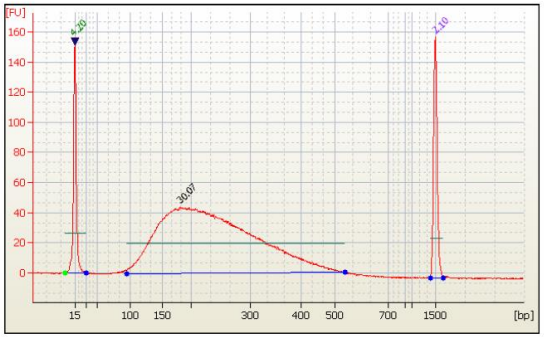


Next Generation Sequencing

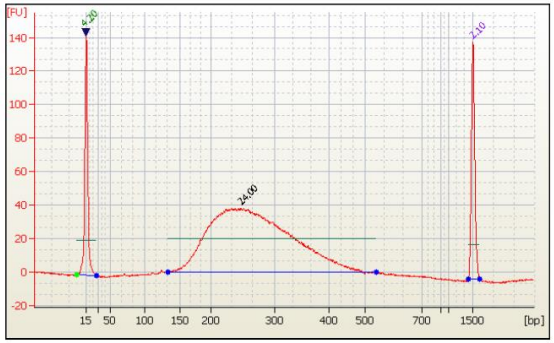
- Steps where QC assessment is critical and Bioanalyzer analysis is recommended



Post – shearing – peak size of 190bp



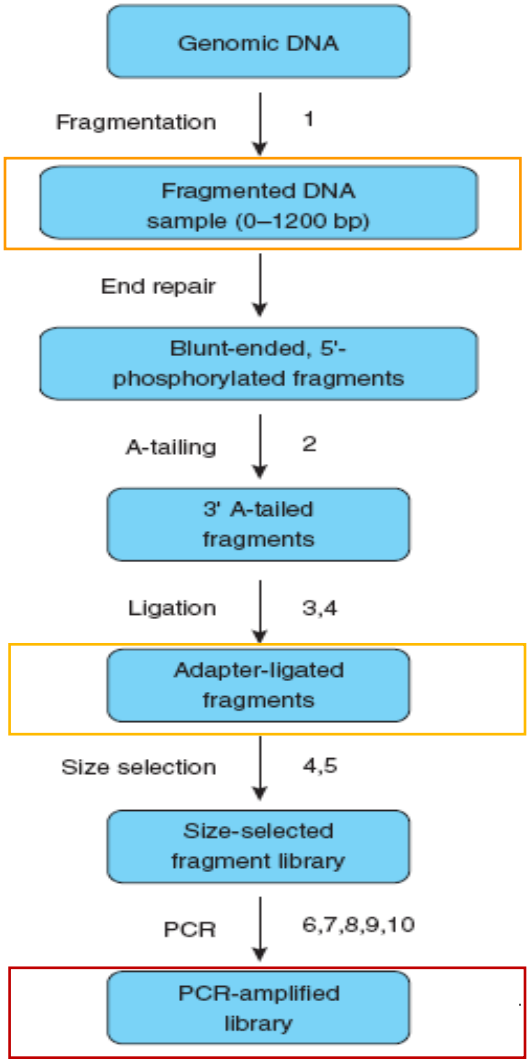
After adaptor ligation – peak size of 250± 10bp



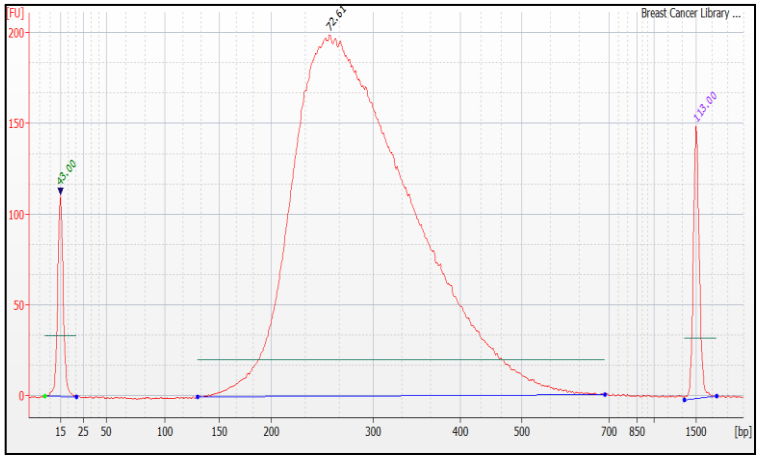
Inefficient adapter ligation will result in reduced library complexity after PCR.

Next Generation Sequencing

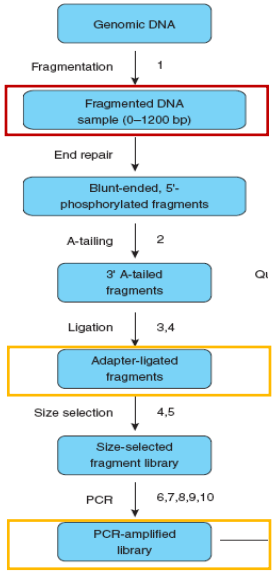
- Steps where QC assessment is critical and Bioanalyzer analysis is recommended



Amplified library run on the Bioanalyzer DNA 1000 Kit:



The Importance of Monitoring Size Distribution



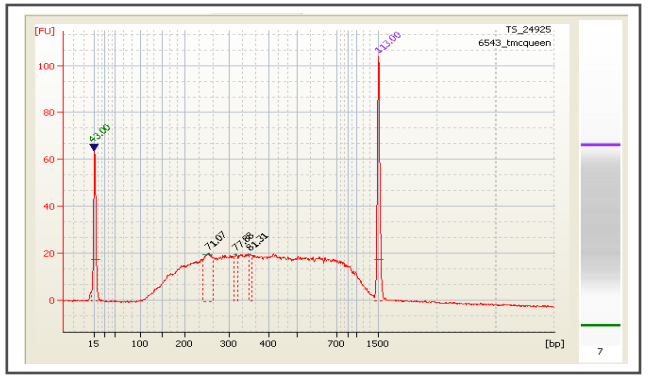
- Choice of fragmentation can significantly affect the recovery of desired fragments and hence the amount of starting material required.

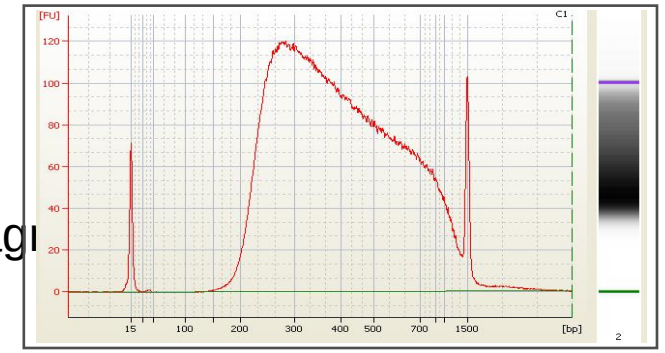
- Sequencing fragments that do not fall within the recommended size distribution may lead to low read depth or even a lack of read coverage for specific portions of the sequence.

- When performing SureSelect target enrichment, fragment size distribution can affect final % on-target capture.

- Probe-based sonication methods routinely introduce sample-to-sample variability and are not recommended.

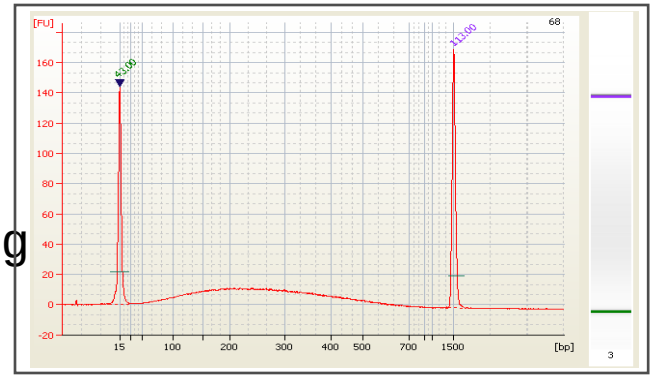
Shearing - Common issues





Uneven shearing

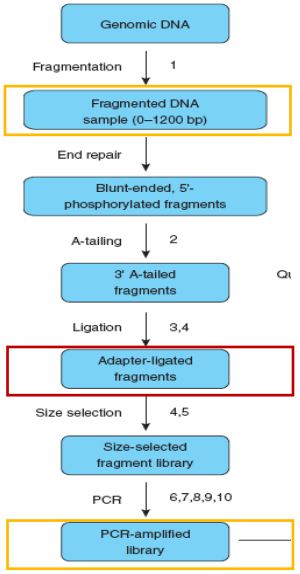
- Poor DNA quality
  - DNA eluted in incorrect buffer (not TE).
- Covaris issues
  - Bubbles in microtube will cause inconsistent frag
  - Water level is too high/low.
  - Temperature of water bath is not between 6-8ºC.
  - Insufficient degassing



Extended size range

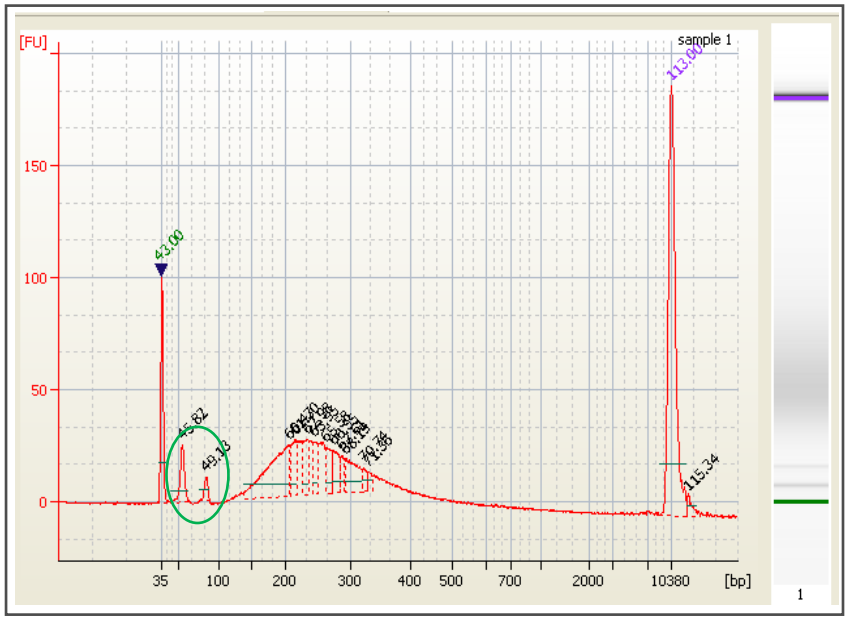
- Covaris issues. Perform a control experiment using as commercially available lambda DNA.

Adapter Ligation - Common issue

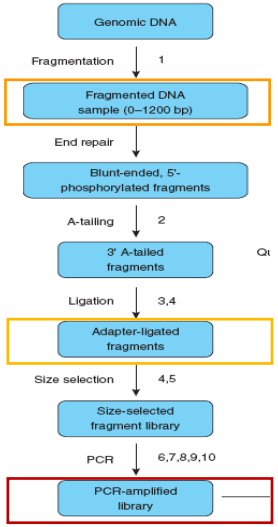


Excess Adapters

- Inefficient ligation due to too much input DNA or the use of incorrect ligation temperature (ligation is performed at 20-25ºC. When using a PCR machine, make sure the lid is not heated).



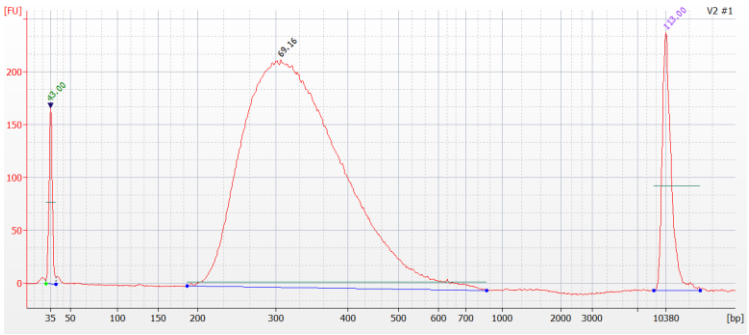
Quality Control after PCR Amplification



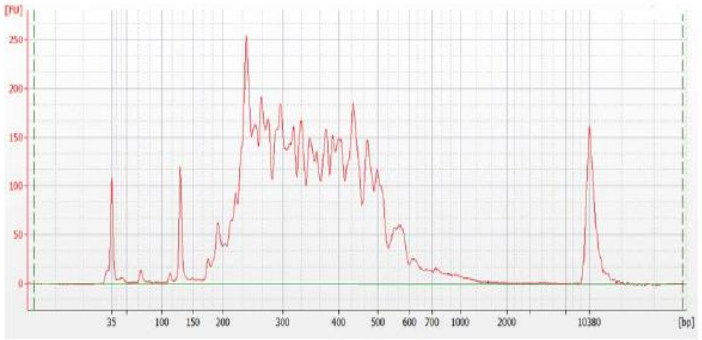
- PCR amplification is required for enrichment of adapter-ligated fragments, as well as to add indexes.

- In the SureSelect and HaloPlex protocols, PCR is also used to amplify captured DNA for final QC and quantification. *Libraries are analyzed on the Bioanalyzer High Sensitivity DNA assay*

SureSelect Libraries



HaloPlex Libraries

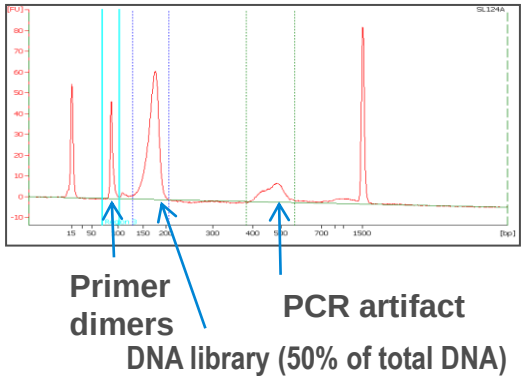


- PCR can create bias. PCR artifacts caused by overamplification or primer dimers can also affect sequencing coverage and accuracy.

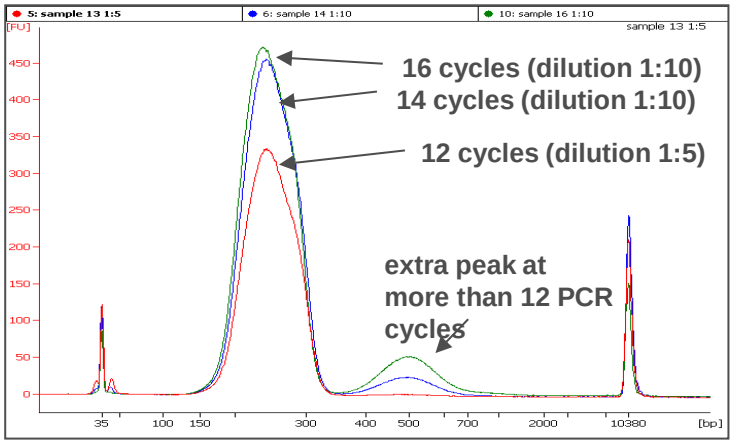
PCR Amplification – Common issues

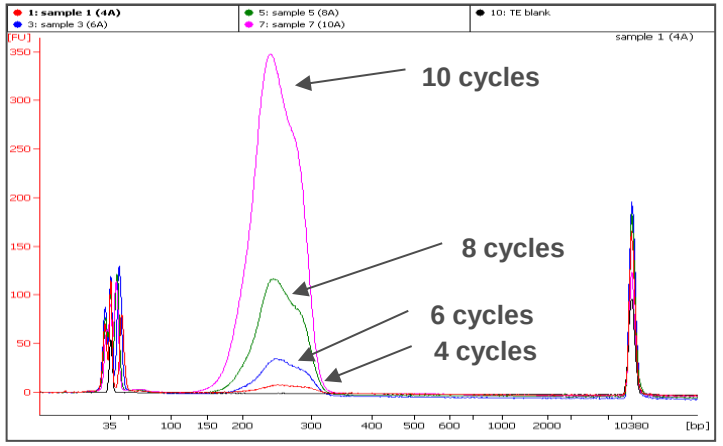
Primer dimers and artifacts

- Primer dimers present in the library will be sequenced.
  - Primer dimers may be removed by performing additional bead clean-up steps.
  - Gel-based size selection can also remove primer dimers.



- Repeat PCR with fewer cycles to prevent formation of artifacts using remaining library.

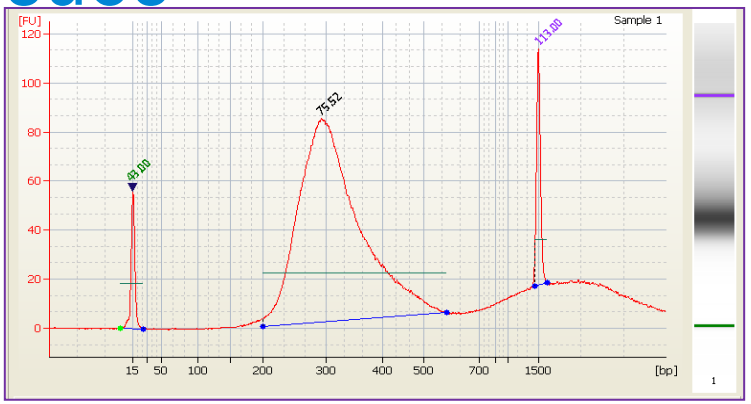




PCR Amplification – Common issues

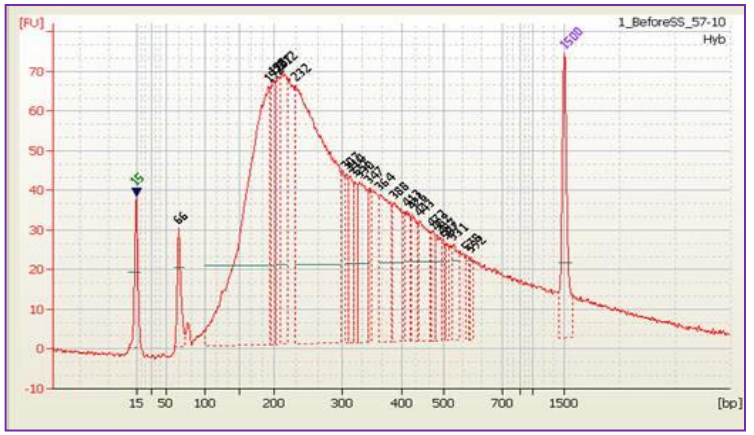
Bead carryover –

- SPRI bead carryover from post-PCR clean-up step.
- Use a strong magnet for bead separation and pipette carefully during elution to avoid disturbing beads.



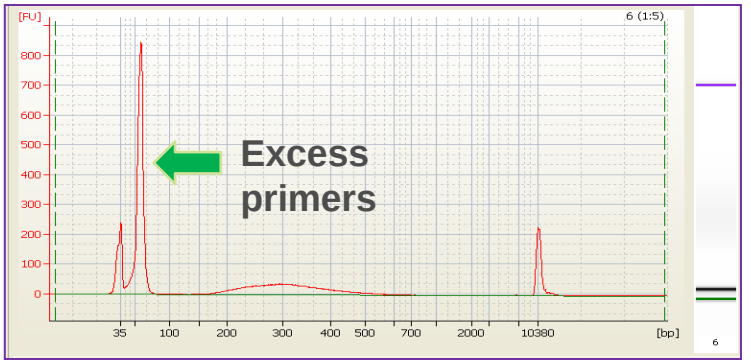
Residual buffer –

- Likely due to buffer carryover from post-PCR clean-up using columns.



Low PCR yield –

- Inefficient PCR cycling results in low yield and an excess of primers.
- Can be a result of poor adapter ligation, low DNA quality, inefficient bead clean-up, use of too few cycles or PCR instrument is not well-calibrated.
- When performing target capture, low yields after post-capture PCR can also indicate suboptimal hybridization.



Outline

Importance of Quality Control

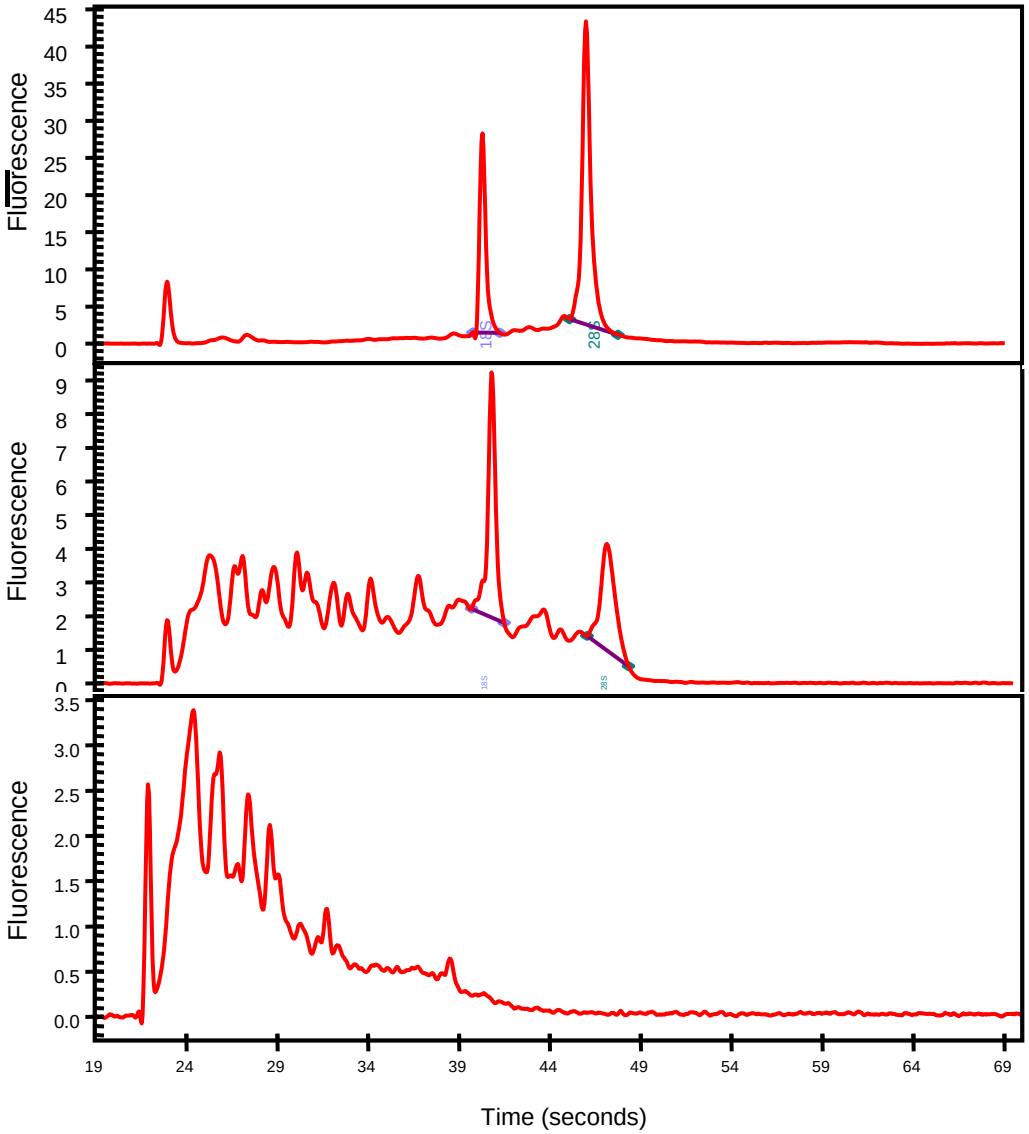
Sample QC in Next Generation Sequencing

Sample QC in Gene Expression

Sample QC in Proteomics

… and even more!

RIN – Standardized Assessment of RNA Integrity



Intact RNA: RIN 10

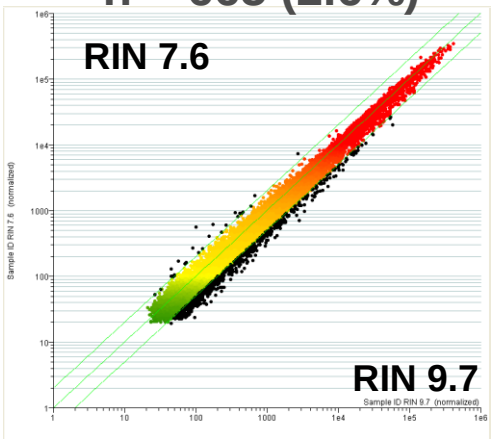
Partially degraded RNA: RIN 5

Strongly Degraded RNA: RIN 3

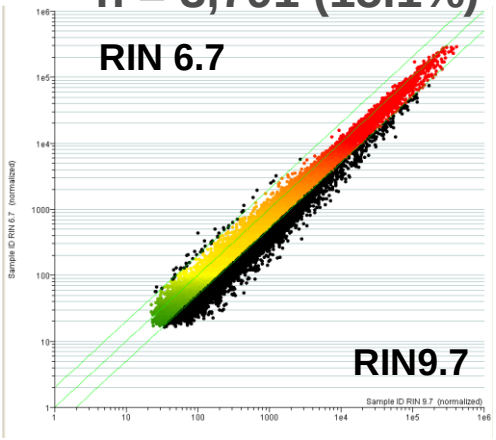
RNA QC is Critical for Microarray Success

Garbage in, garbage out!

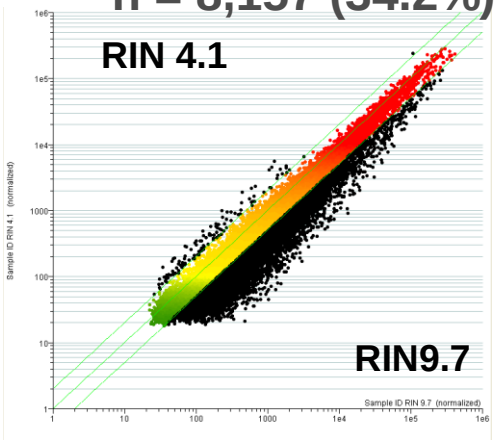
n = 665 (2.6%)



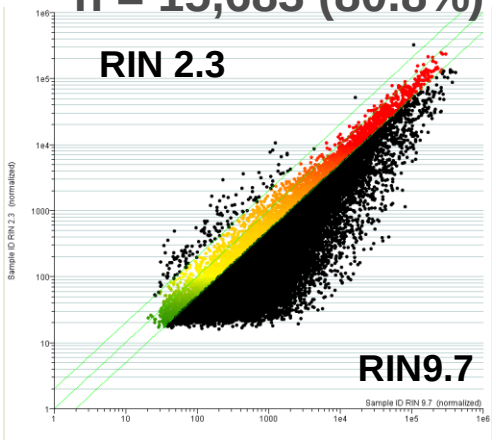
n = 3,791 (15.1%)



n = 8,157 (34.2%)



n = 15,683 (80.8%)



QC is Important for RNA-seq, too!

RNA Integrity (RIN score)

Input RNA → fragmented mRNA → ds cDNA → 5′-P fragments → 3′-dA overhang → Adapter-ligated library → Prepped RNASeq library

Library yield & distribution

Library yield & distribution

- Total RNA *OR* mRNA
- purification
- fragmentation

- RT/ 1st strand synthesis
- 2nd strand cDNA synthesis
- digest template RNA

- End repair
- A-tailing
- Adapter ligation
- Optional size selection

Library yield & distribution

- PCR enrichment

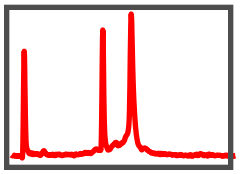
Tutorial: "2100 Bioanalyzer: The Gold Standard for RNA QC – Focus on RNASeq" by Maria Celeste Ramirez, Ph.D. Application Scientist Agilent Technologies, Inc.

"Preparation of High Quality RNA-Seq Libraries for Next-Generation Sequencing" by Michael H. Farkas, Ph.D. Ocular Genomics Institute Massachusetts Eye and Ear Infirmary Harvard Medical School

RNA QC in Routine Gene Expression Workflow

Cells / Culture

RNA isolation



Total RNA

RNA QC via Bioanalyzer System

RIN

Software feature RIN as independent and standarized measure of sample quality

RIN above threshold

Start again with sample isolation

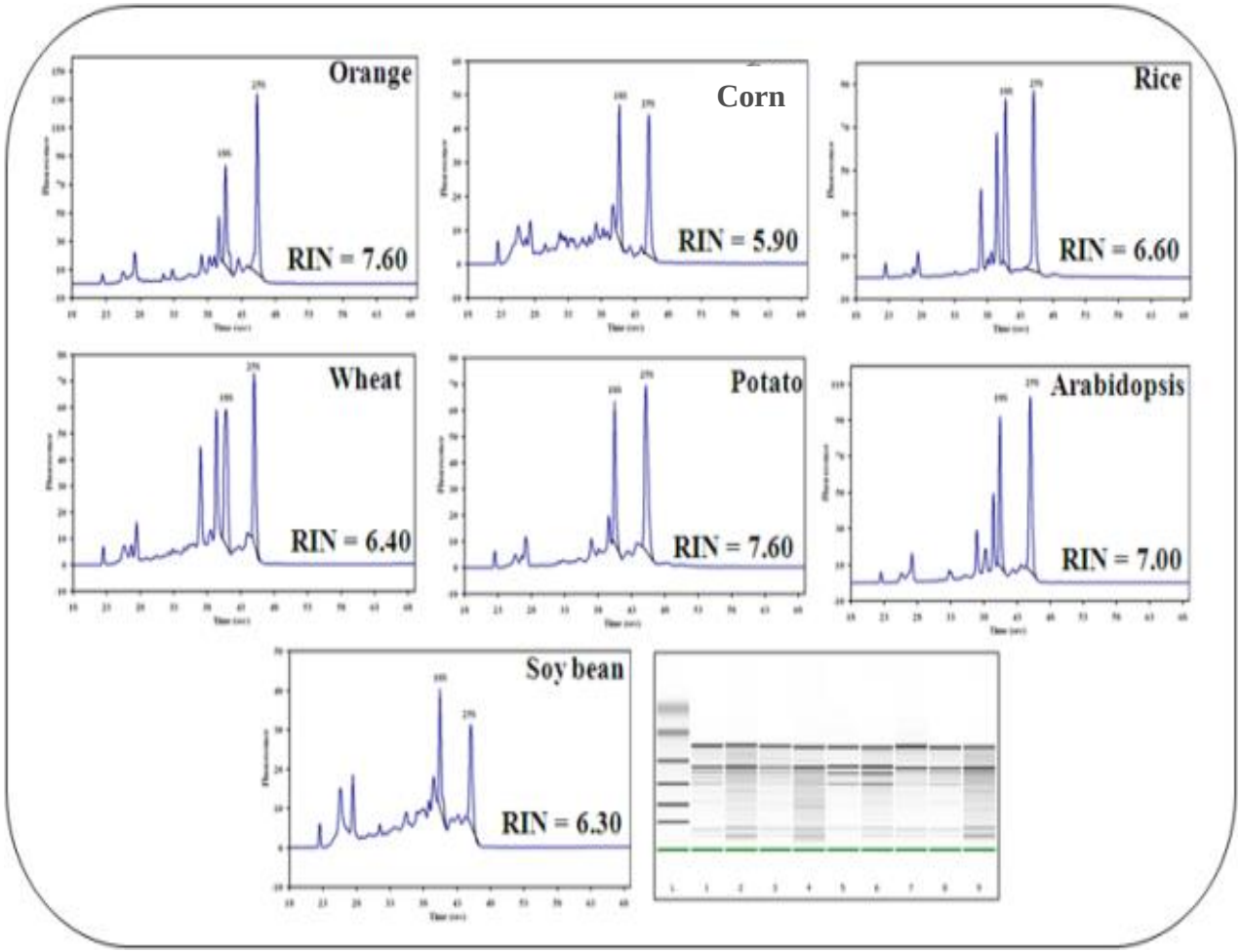
Continue with downstream Experiment (Microarray, real-time PCR, etc.)

MIAME Guidelines for GEx Microarrays:
http://compbio.dfci.harvard.edu/pubs/MIAME.pdf

MIQE Guidelines for QPCR:
http://www.clinchem.org/cgi/reprint/55/4/611

Application Plant RNA

Application Note: 5990-8850EN

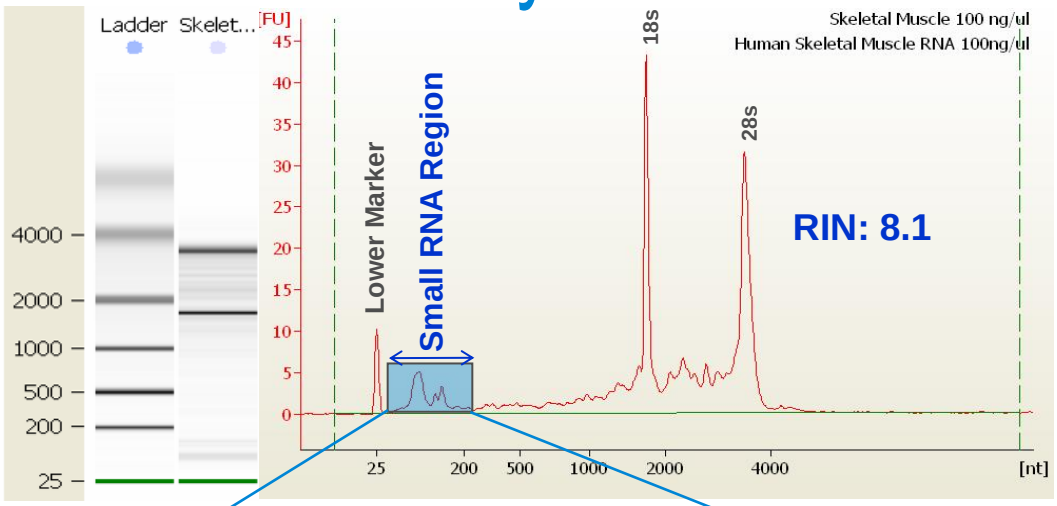


Plant RNA

RNA from green tissue contains additional rRNA units (e.g. 25S) in contrast to other cells.

- new software feature (assay) as of B.02.07
- now the 2100 can analyze different tissue plant RNA samples to assess integrity of plant RNA from a variety of sources

Small RNA Assay versus RNA Nano Assay



RNA 6000Nano kit

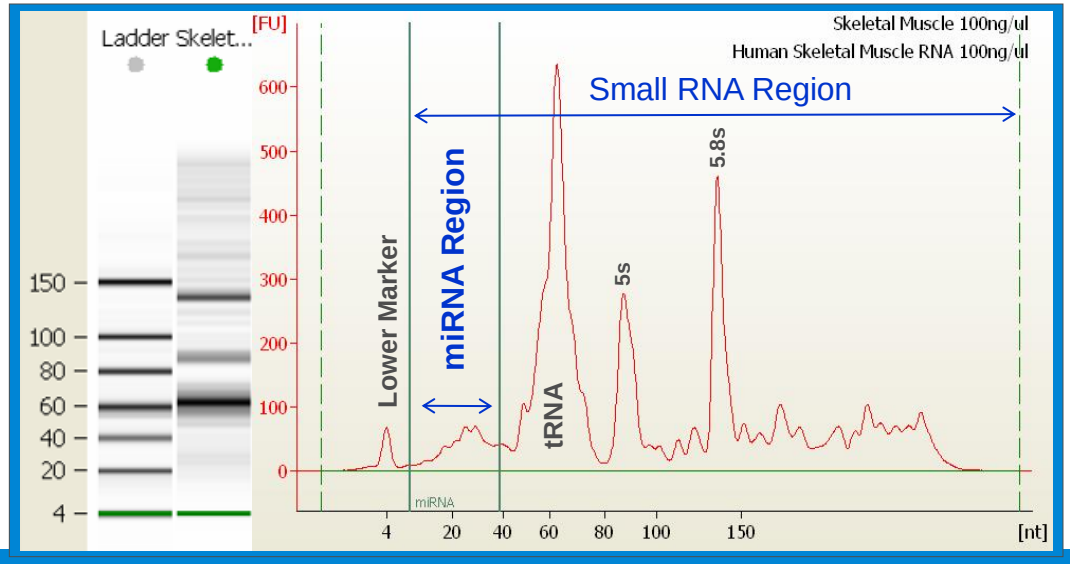
RNA 6000 Nano

Size range: 25-6000nt

Results: Integrity, Total RNA amount, gDNA contamination

Changed multiple parameters:
-Dye composition
-Gel formulation
- Electronic Script
- Analysis setpoints in SW

Small RNA Kit



Small RNA

Size range: 6-150nt

Results: miRNA amount, Ratio and amount of other Small RNA

No RIN for small RNA!

Establishing Individual QC Criteria Analysis of Small RNAs from *Drosophila* Schneider Cells

Analysis of small RNAs from Drosophila Schneider cells

Small RNA electropherogram:

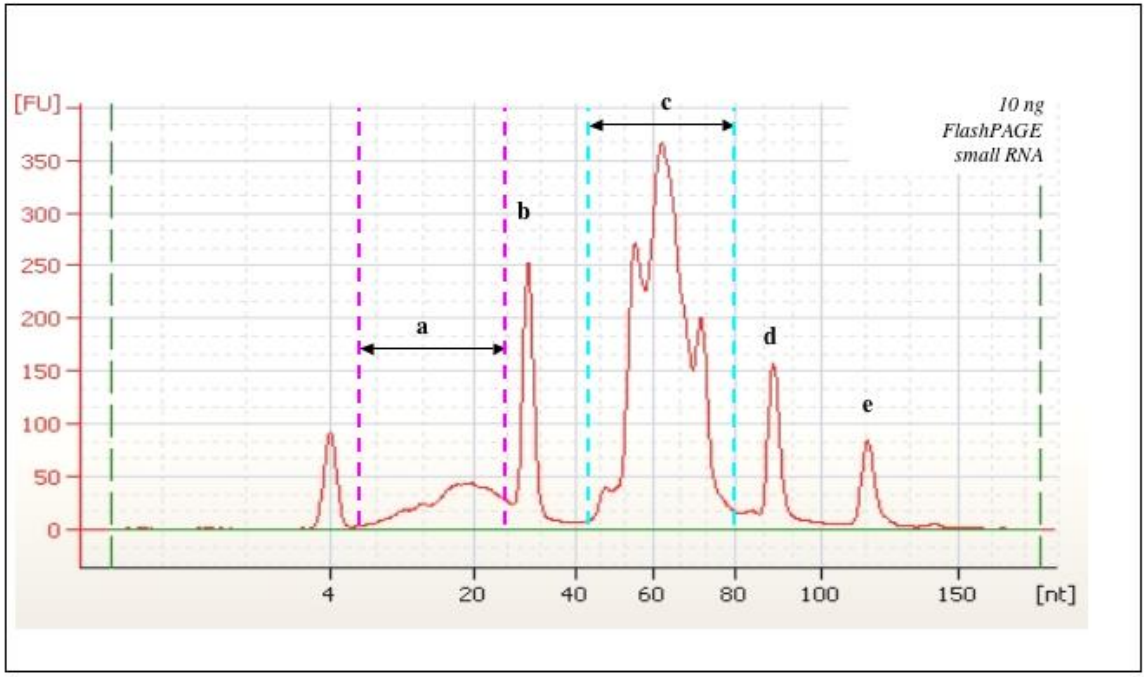
a) miRNA region

b) 2S RNA region

c) transfer RNA

d) 5S rRNA

e) 5.8S rRNA



The presence of sharp individualized 5S and 2S rRNA peaks can be used to directly evaluate the quality of small RNA samples.

Odile Sismeiro, Institut Pasteur, Paris, France

Outline

Importance of Quality Control

Sample QC in Next Generation Sequencing

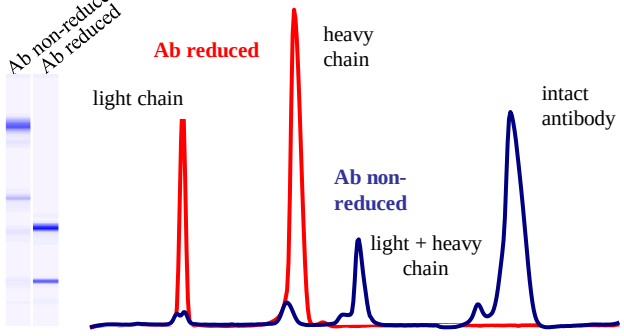
Sample QC in Gene Expression

Sample QC in Proteomics

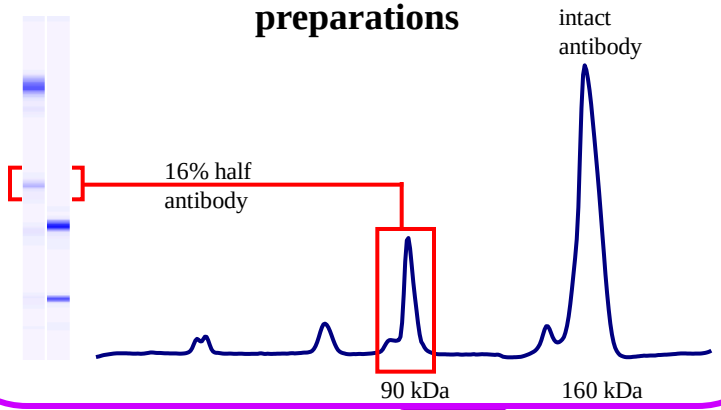
… and even more!

Quality Control of Antibodies

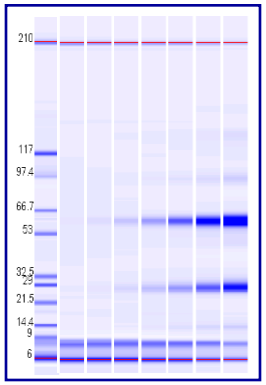
Antibody analysis under reducing and non-reducing conditions

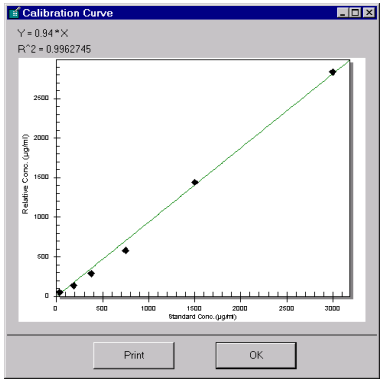


Determine the half antibody content in IgG preparations



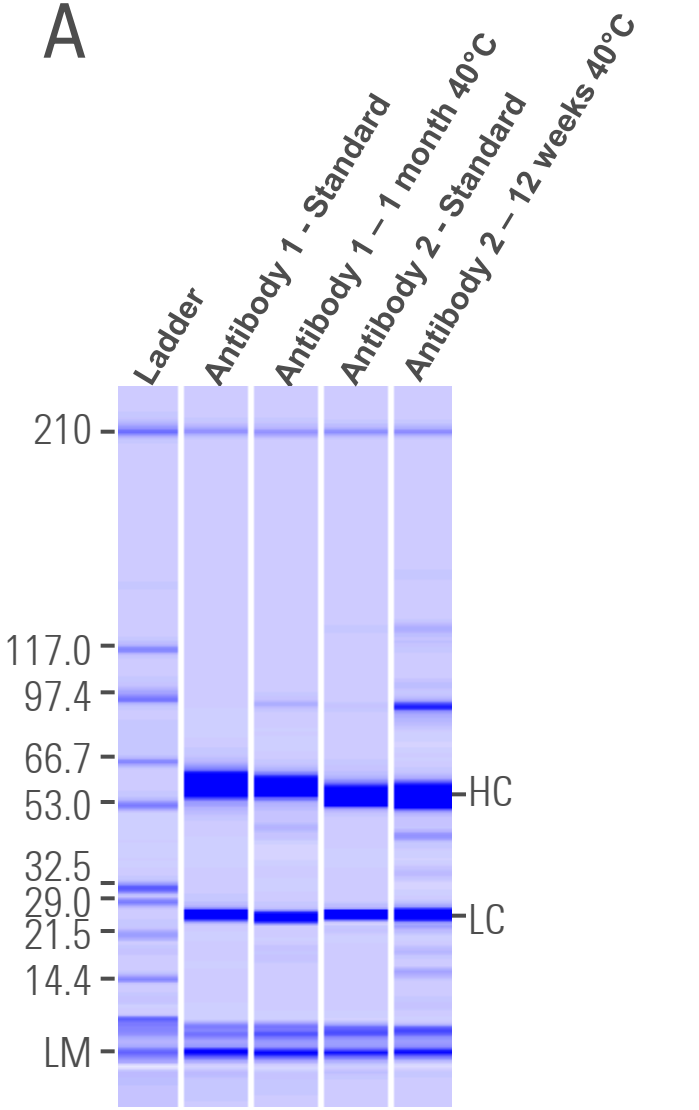
Absolute Quantitation of IgG samples



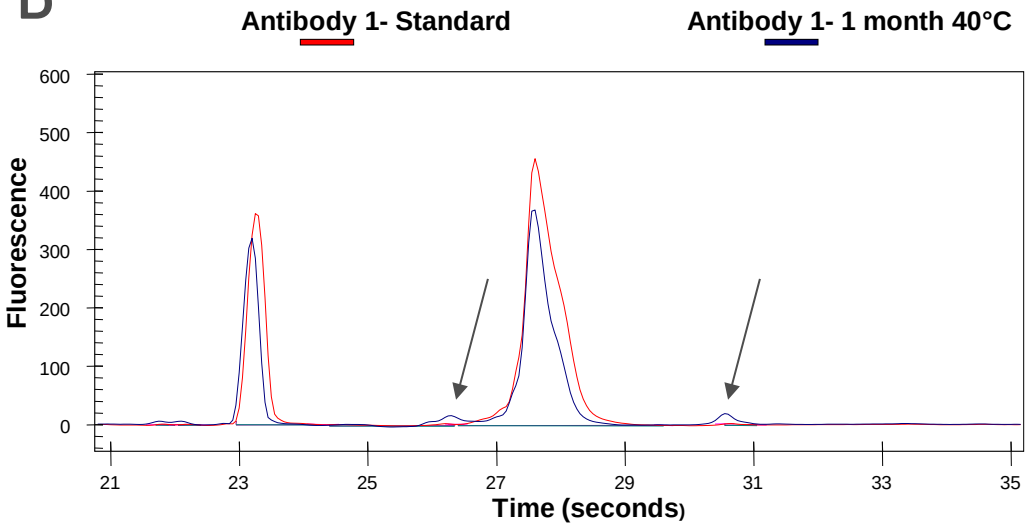


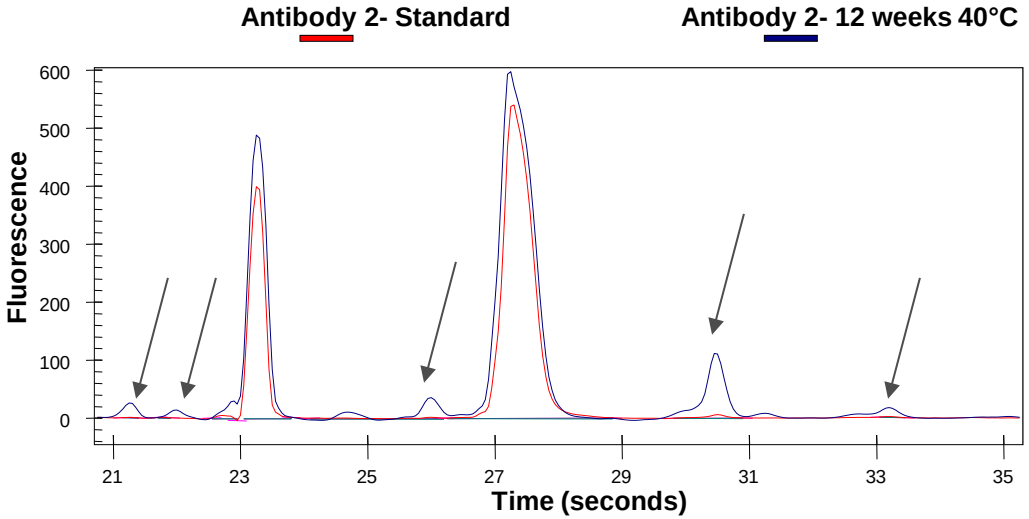
Analysis of Antibody Stability – Stress Test

A

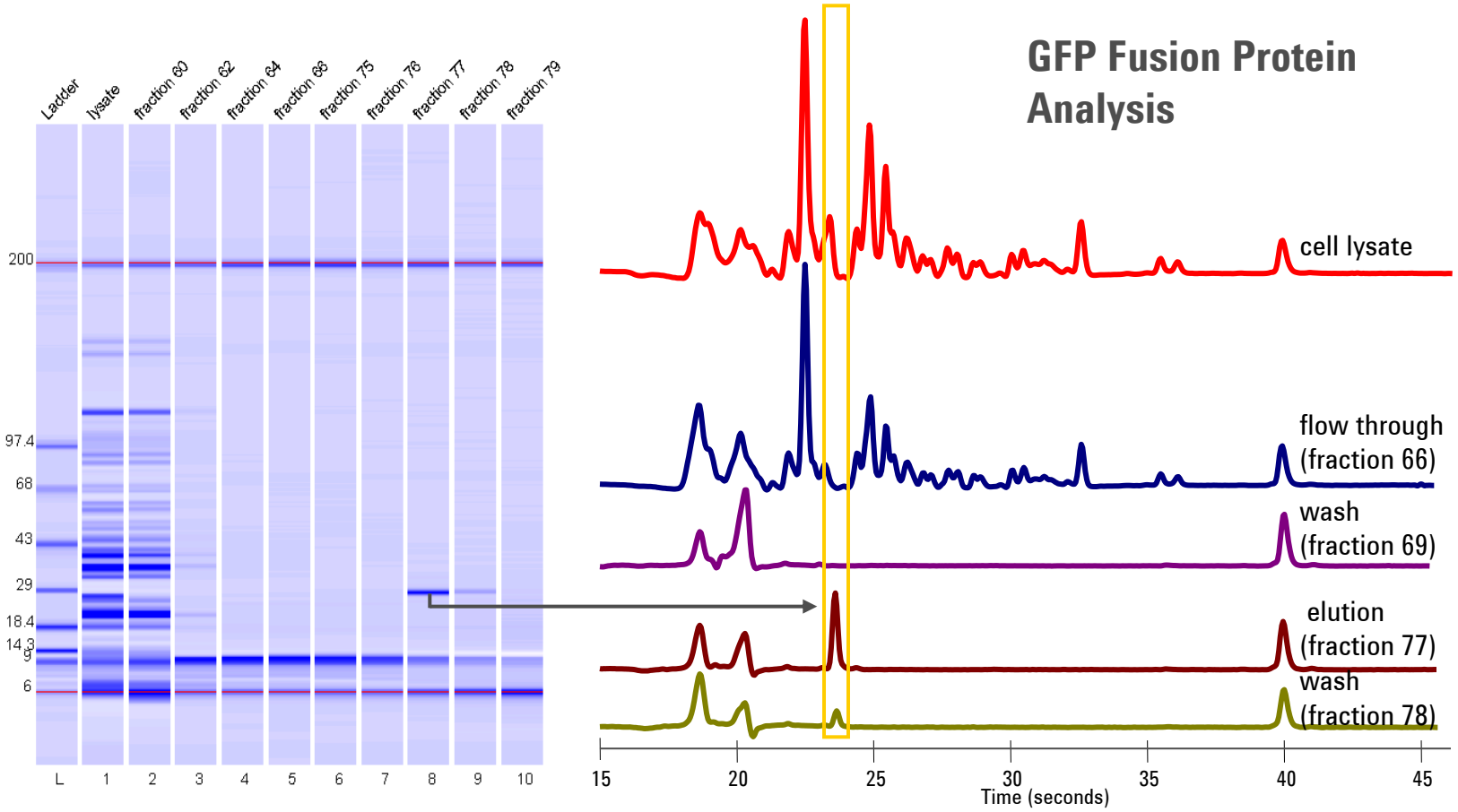


B





Monitoring of Protein Purification Process

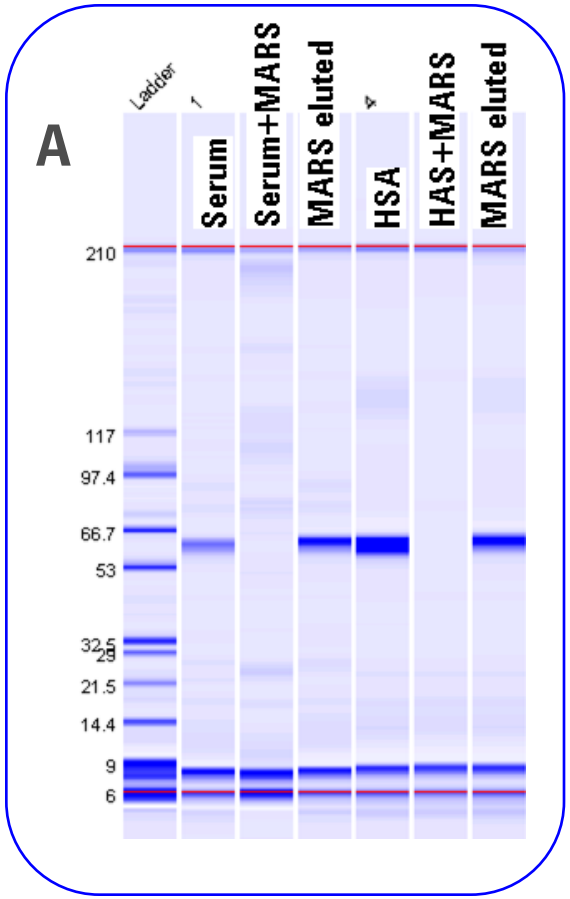


2100 Bioanalyzer gel-like image

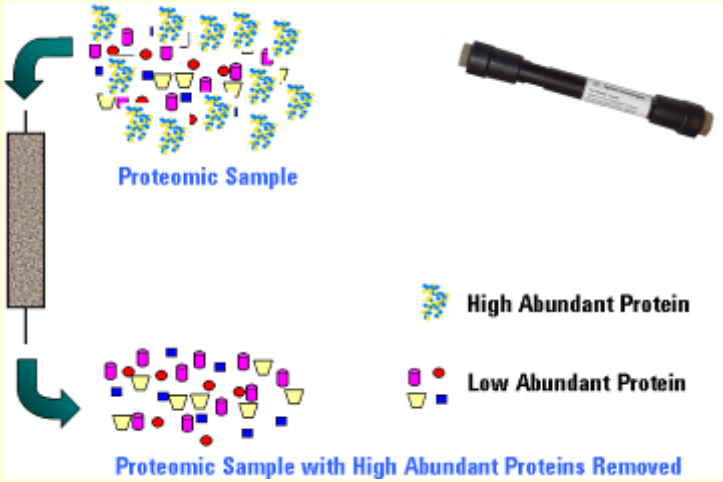
2100 Bioanalyzer electropherogram

Courtesy of P. Sebastian and S.R. Schmidt
GPC-Biotech AG, Martinsried, Germany

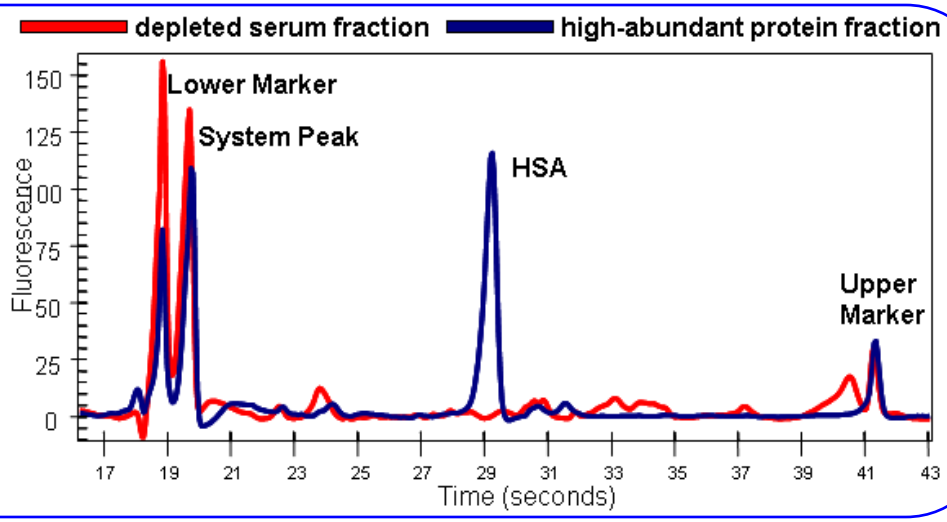
Depletion of High Abundance Proteins in Human Serum



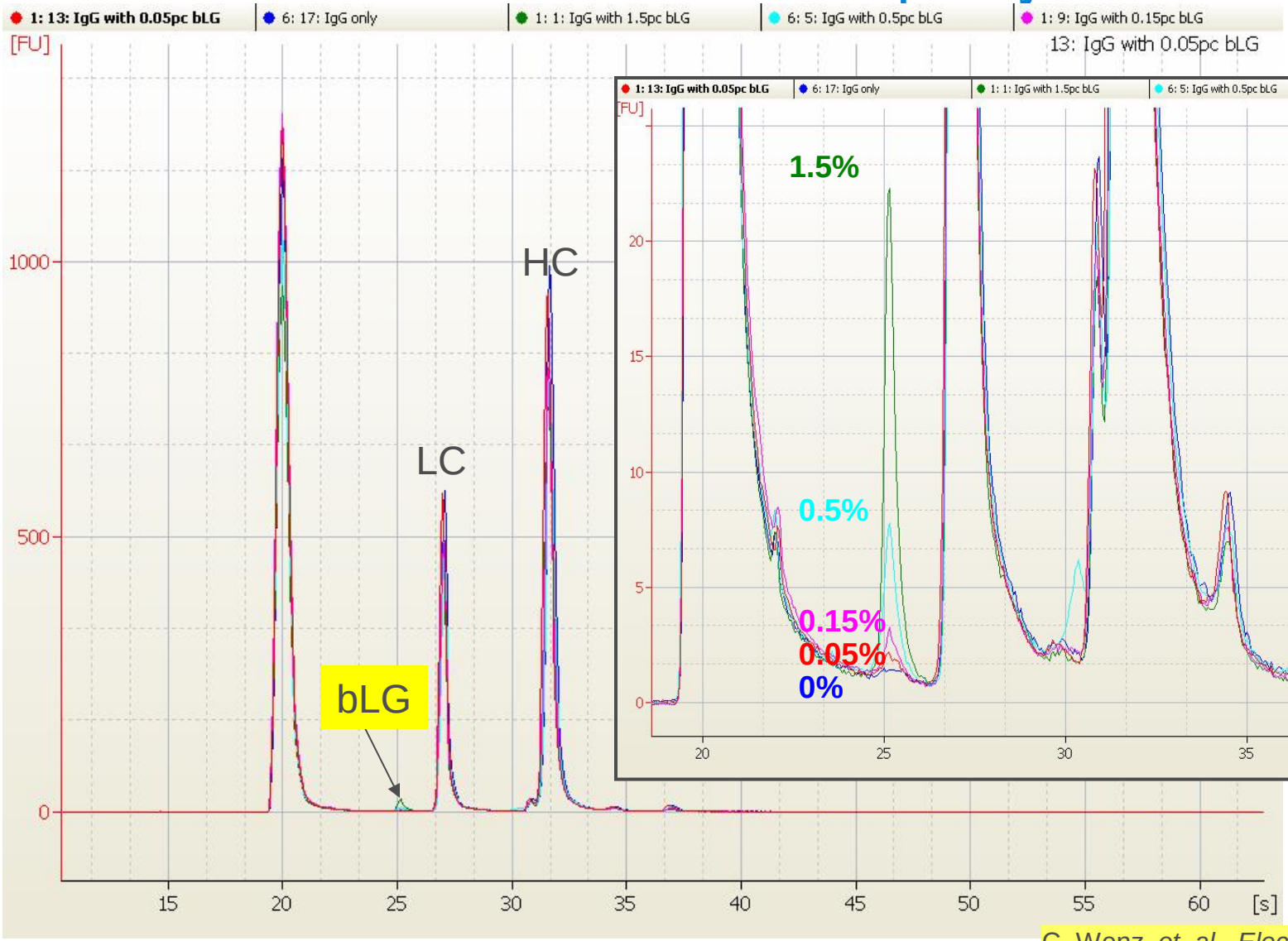
Depletion of High Abundance Proteins by Agilent MARS HPLC columns



Fractions checked by 2100



ICH Guidelines: Simulation of Impurity Detection



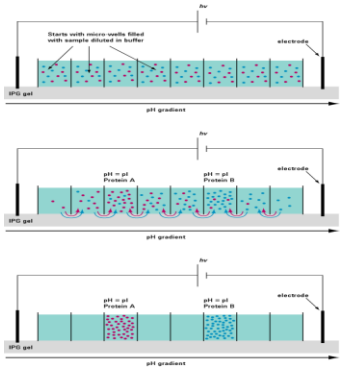
C. Wenz, *et. al., Electrophoresis 31,* 2010

2-DG Equivalent: HSP250 + OFFGEL Fractionation (IEF)

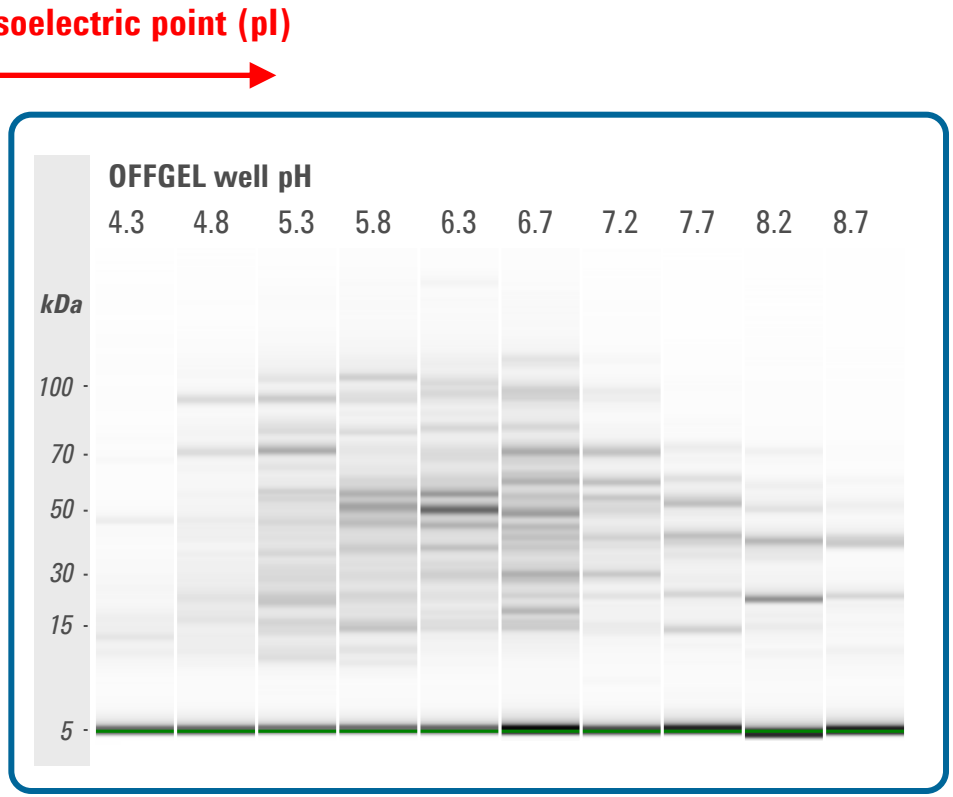
E. coli lysate
(50 µg)

Protein clean up and labeling

OFFGEL Electrophoresis



2100 Bioanalyzer
HSP-250 Assay



Application Note: 5989-8419EN

Outline

Importance of Quality Control

Sample QC in Next Generation Sequencing

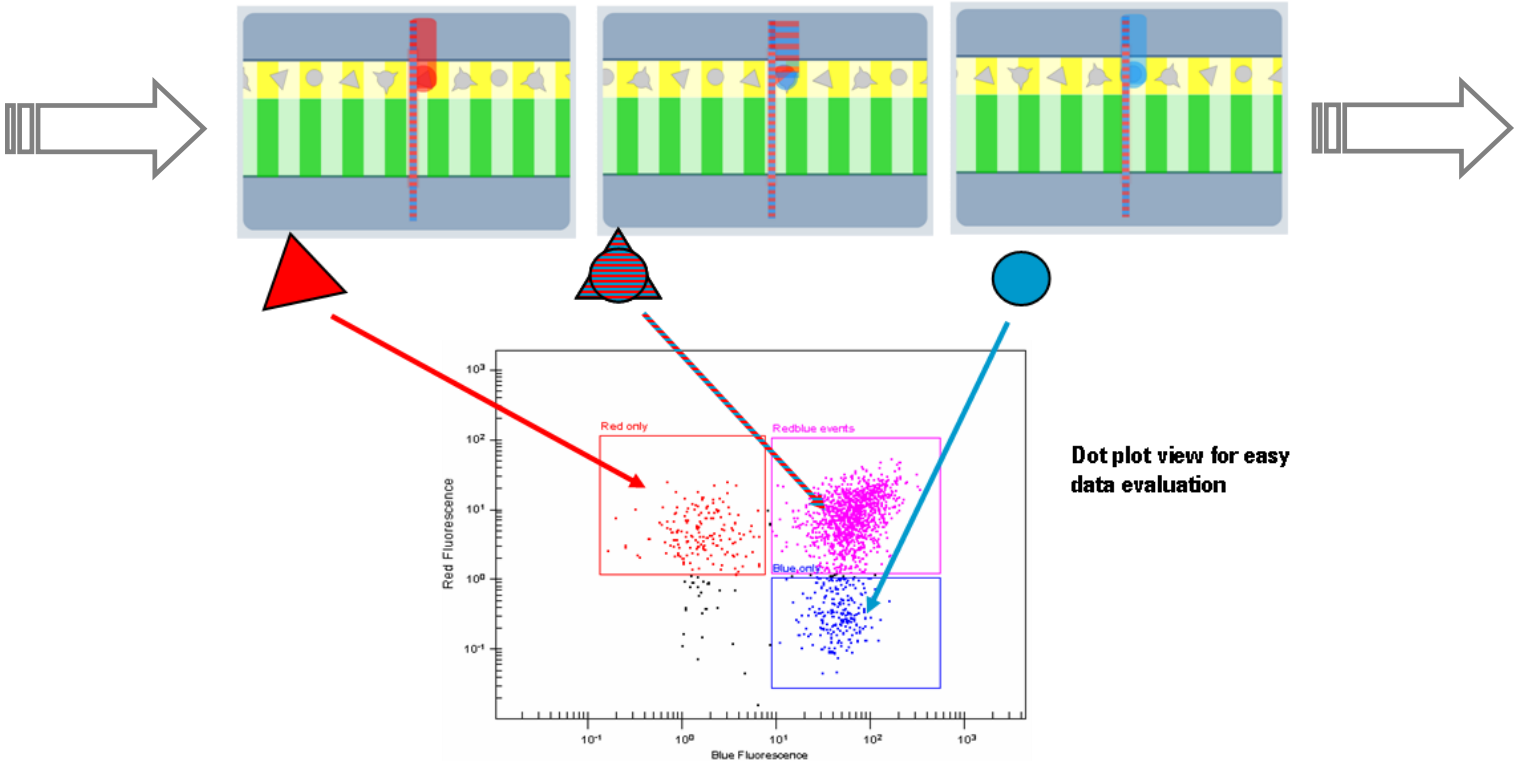
Sample QC in Gene Expression

Sample QC in Proteomics

… and even more!

Cell Applications

Protein Expression | Apoptosis Detection | Gene Silencing | Transfection Monitoring | Cell Staining



Flow Cytometry on a Chip - Optics & Detection

2100 Bioanalyzer

Red detection channel:

- 620-645 nm excitation with Laser (Maximum 630 nm)
- 674-696 nm detection range (Maximum 680 nm)

Blue detection channel:

- 458-482 nm excitation with LED (Maximum 470 nm)
- 510-540 nm detection range (Maximum 525 nm)

SYNTHETIC BIOLOGY

DNA& RNA Applications in Genome Editing

PCR products

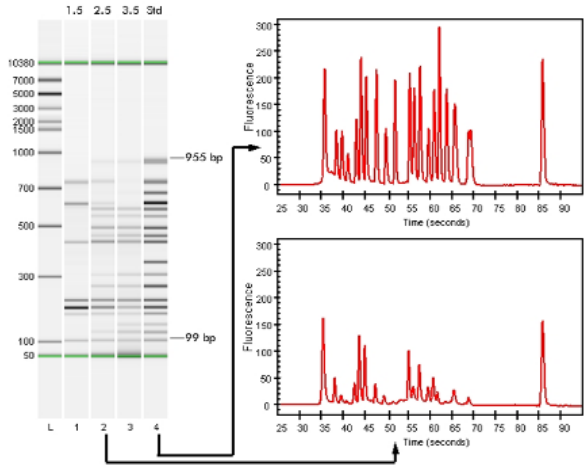
gDNA integrity

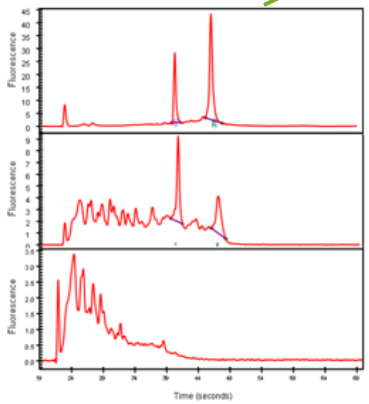
Restriction Digest Analysis

Quality Control in NGS

Total RNA integrity

Quality Control of Oligos



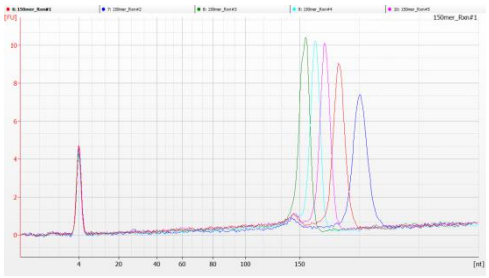


Intact RNA: RIN 10

Partially degraded RNA: RIN 5

Strongly Degraded RNA: RIN 3

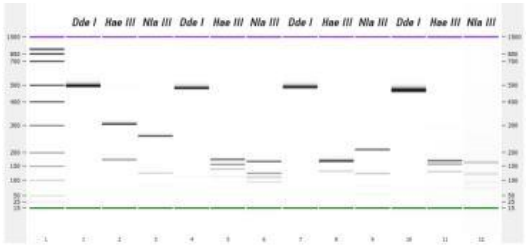
Oligo tailing reaction



Examples of Electrophoresis in Synthetic Biology workflows

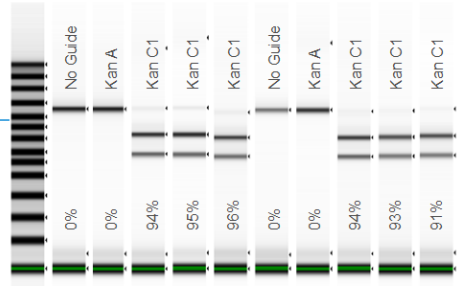
Vector Assembly

- ❑ Starting material QC: Gene of interest after PCR amplification
- ❑ Verifying vector composition with restriction analysis
- ❑ Vector analysis by NGS (including multiple QC steps)



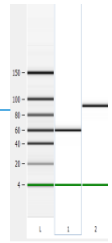
CRISPR/ Cas9

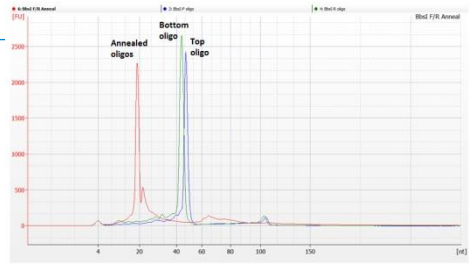
- ❑ DNA target after PCR amplification
- ❑ Cleavage efficiency of digestion with Cas9 Nuclease (Detection and Quantitation)



guide RNA synthesis

- ❑ Starting material QC: oligo templates*
- ❑ Monitoring oligo annealing reactions
- ❑ Quantitation and QC of gRNA product*

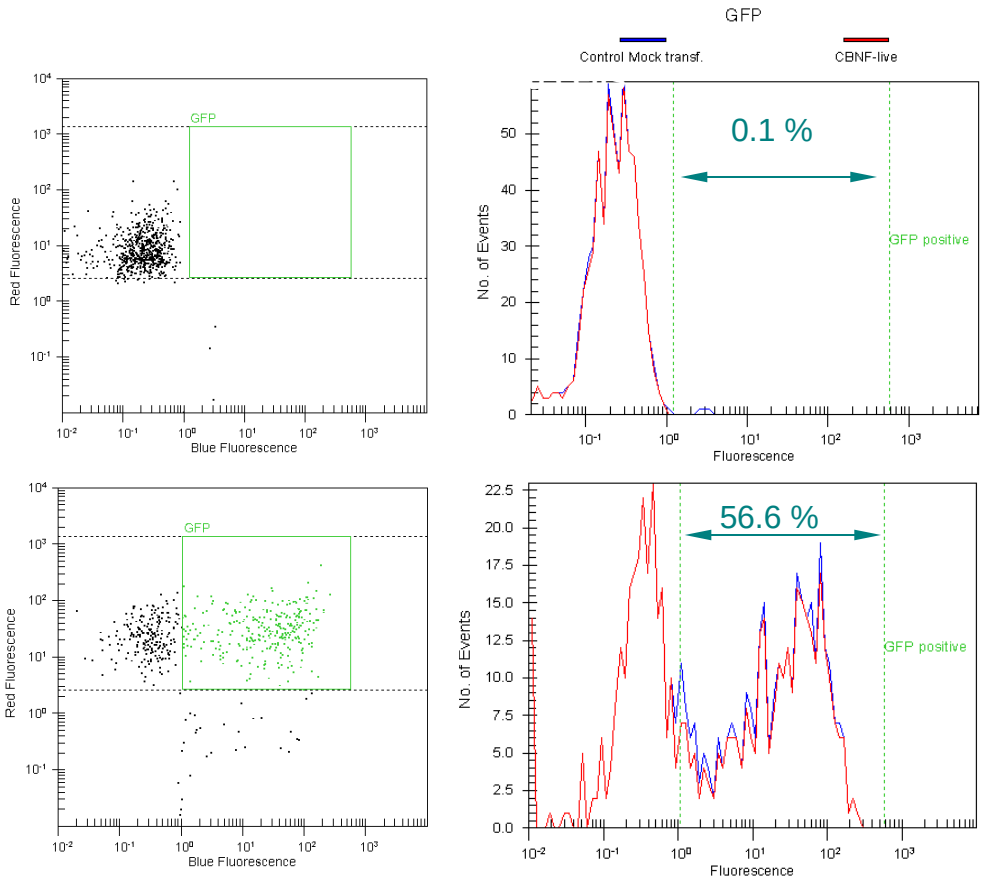




* Utilizes Small RNA Assay on the 2100 Bioanalyzer System

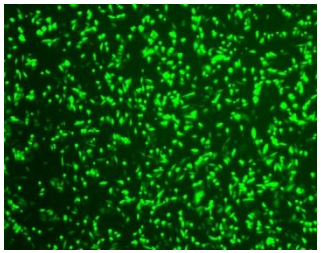
Applications: Protein Expression Analysis

GFP Transfection Efficiency Control



CHO-K1 cells were transfected with EGFP DNA and Lipofectamine.

Control



EGFP transfected

More Information

www.agilent.com/genomics/bioanalyzer

- Application Notes
- Publications
- Data Sheets
- Videos
- Brochures
- Special Offers
- Free Software Downloads