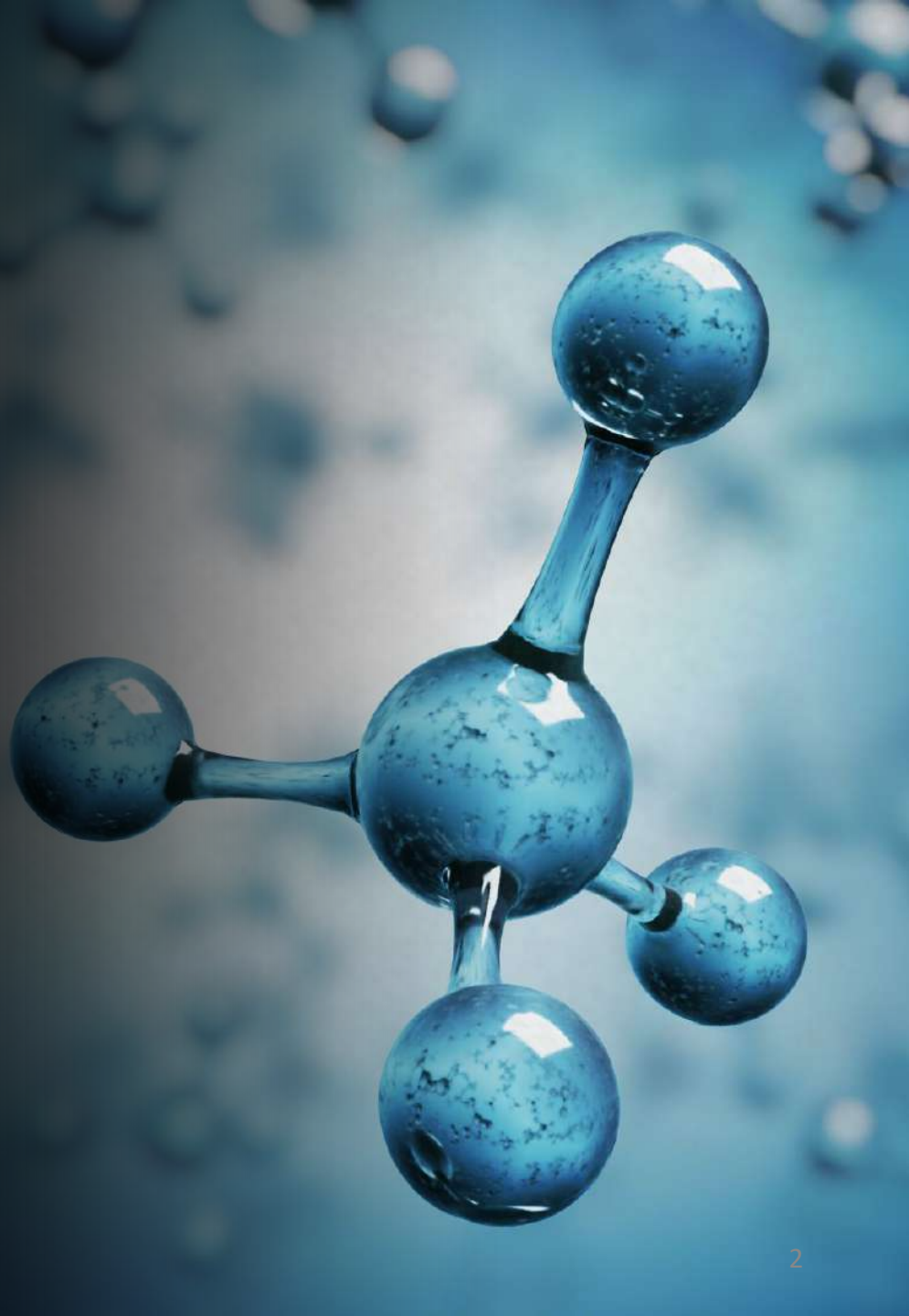
Quality and Quantity Assessment of Nucleic Acids and Proteins

Assoc. Prof. Dr. Ilker BUYUK

- Quantification of nucleic acids
- Quality assessment of nucleic acids
- Quantification of proteins



The amount and quality of molecules (nucleic acids or proteins):

Reproducibility

Accuracy

Efficiency

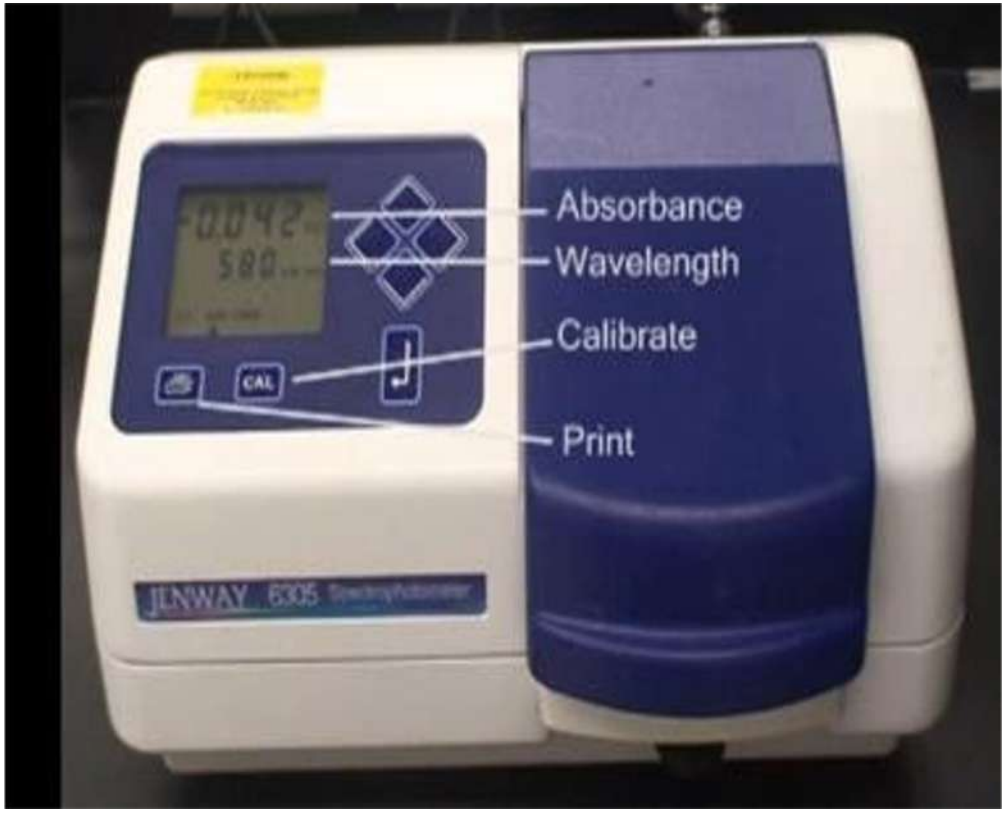
Quantification of Nucleic Acids

Three quantification methods in common use:

- Spectrophotometric measurement (UV spectrometry)
- Fluorescent dye (Fluorometry) based measurement
- Real-time amplification (Absolute quantification)

Spectrophotometric Quantification

- Measurement of light intensity at different wavelenghts
- *Transmittance*: the amount of light that passes completely through the sample
- *Absorbance*: measurement of light that is absorbed by the sample



Spectrophotometric Quantification

- Bases in RNA/DNA absorb UV at 250-265nm
- Heterocyclic rings
- Measurements at A260nm, A280nm, A230nm
- Concentration estimation

Spectrophotometric Quantification

- A260*nm:*
- Lambert-Beer Law: $C_{\mu g/\mu l} = A$ x dilution factor x ε
- ε: molar extinction coefficient
  - physical constant
  - Unique
  - Amount of absorbance at 260nm of 1M nucleic acid solution measured in a 1cm path-length cuvette.

At a wavelength of 260 nm, the average **extinction coefficient** for double-stranded **DNA** is 0.020 $(\mu g/ml)^{-1}cm^{-1}$, for single-stranded **DNA** it is 0.027 $(\mu g/ml)^{-1}$ cm^{-1}, for single-stranded RNA it is 0.025 $(\mu g/ml)^{-1}$ cm^{-1} and for short single-stranded oligonucleotides it is dependent on the length and base composition.

Spectrophotometric Quantification

- A230 and A280 readings
  - A260/A280
  - A260/A230

- A260: DNA/RNA, Guanidine isothiocyanate
- A270: Phenol, TRIzol
- A280: Proteins
- A230: Phenol, TRIzol, Guanidine HCL

Spectrophotometric Quantification

A260/A280: ~1.8 for DNA, ~2.0 for RNA

Low A260/A280 ratio

- Residual phenol or other reagent associated with the extraction protocol
- A very low concentration (> 10 ng/ul) of nucleic acid

High 260/280 ratio

- RNA/DNA contamination

Spectrophotometric Quantification

- A260/A230*nm* ratio 2.0-2.2

Low A260/A230 ratio

- Carbohydrate carryover (often a problem with plants).
- Residual phenol from nucleic acid extraction.
- Residual guanidine

(often used in column based kits)

- Glycogen used for precipitation.

High A260/A230 ratio

- Making a Blank measurement

on a dirty pedestal

- Using an inappropriate solution for the Blank measurement.

Factors Affecting Absorbance

- A260/A280 ratio:
  - pH
  - ionic strength
- Water often has an acidic pH
- Buffered solution (Tris-EDTA at pH 8.0)

Conventional Spectrophotometers

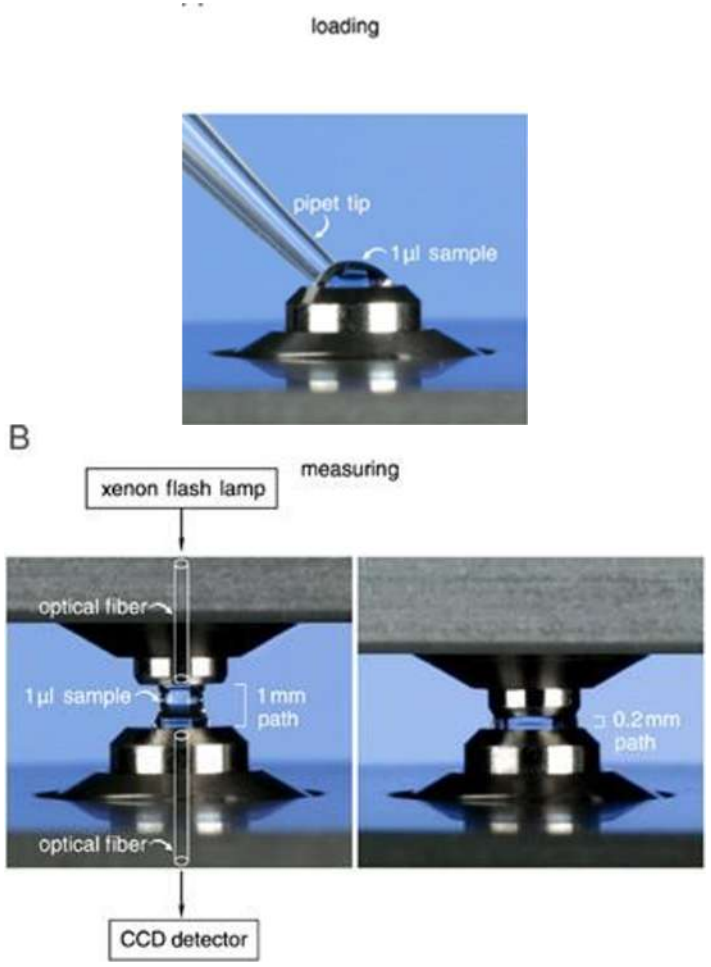
- Conventional spectrophotometers:
  - Requires sample dilution
  - Low sensitivity (lower limit 0.5-1µg nucleic acid)

NANOSPECTROPHOTOMETRY

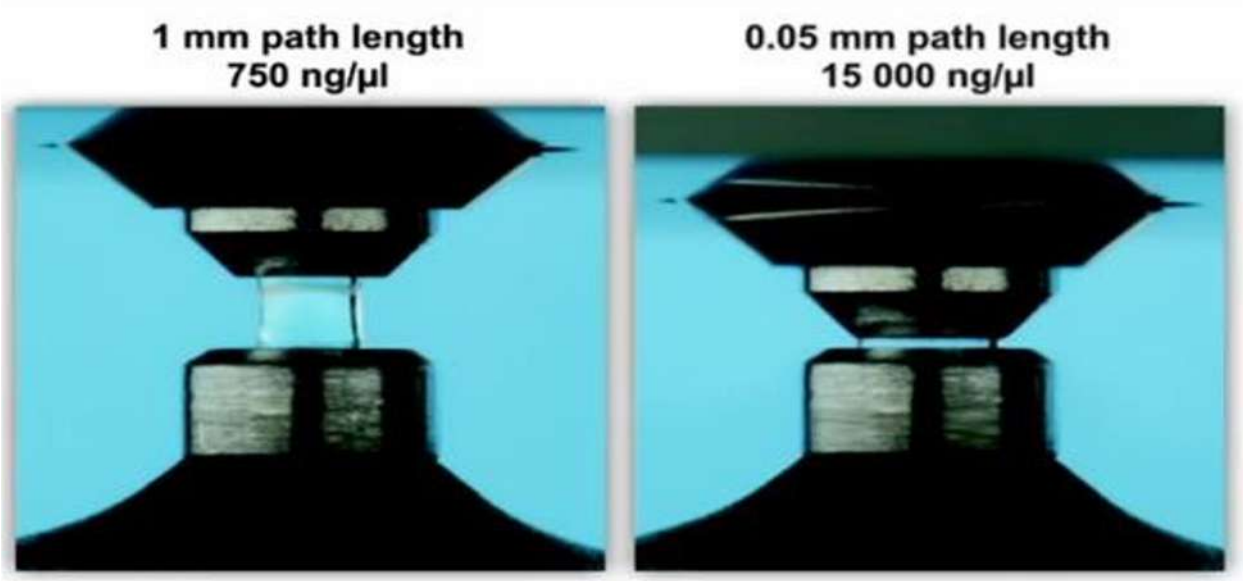
- Miniaturization of UV spectrophotometers:
  - Rapid
  - Direct quantification of nucleic acids in microvolumes

NANOSPECTROPHOTOMETRY-NanoDrop

- Sample retension system
- Inherent surface tension of liquids
- Microvolume samples (0.5-2µl)
- Liquid column → Vertical optical path



NANOSPECTROPHOTOMETRY

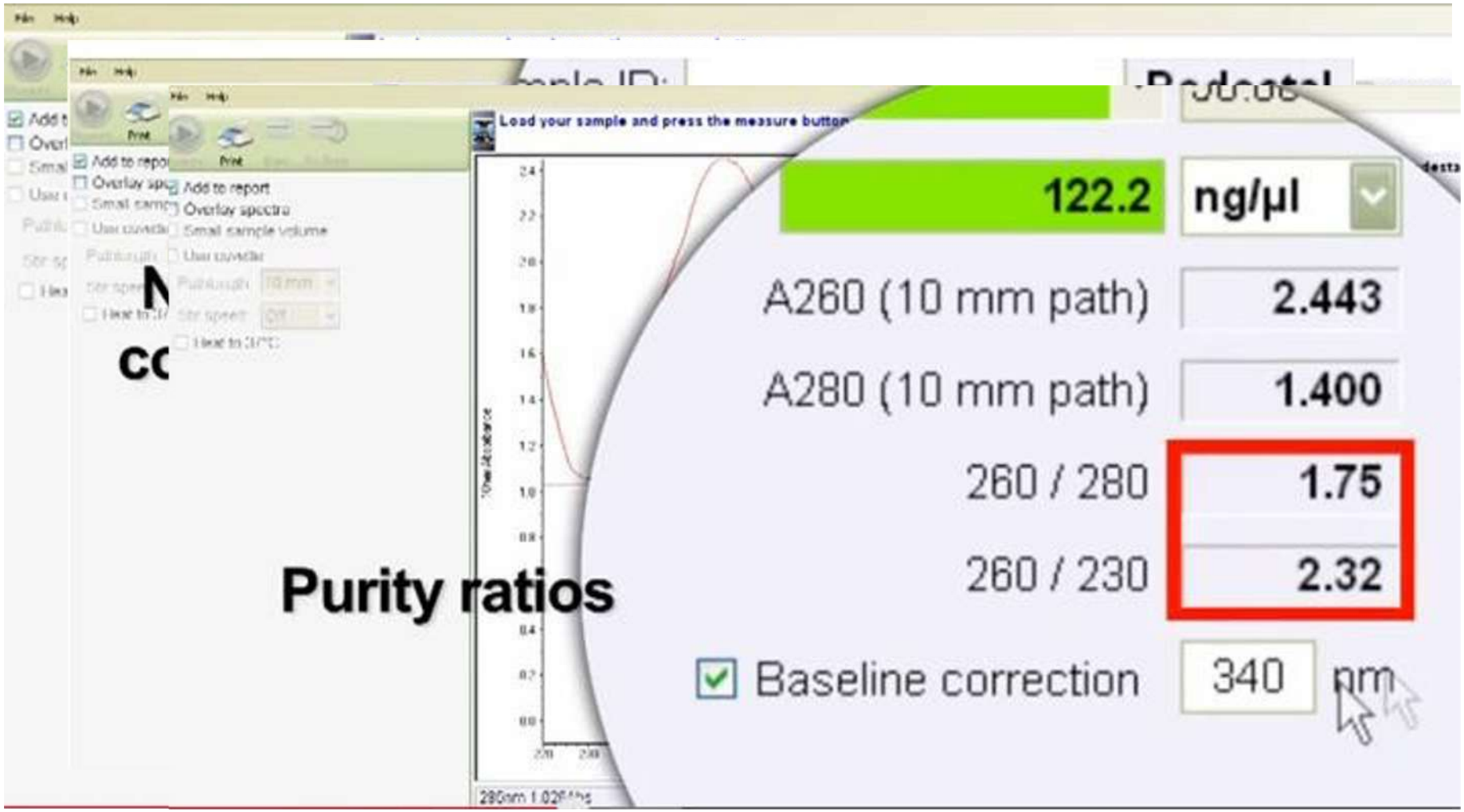


- Vertical path length
  - Automatically changed
  - Shorter path length → higher concentration of sample

NANOSPECTROPHOTOMETRY

- Benefits:
  1. Small sample volume at 0.5-2µl
  2. Large dynamic range (2ng/µl-3700ng/µl)
  3. Cuvette free operation
  4. Short measurement time
  5. High accuracy and good reproducibility.

NANOSPECTROPHOTOMETRY



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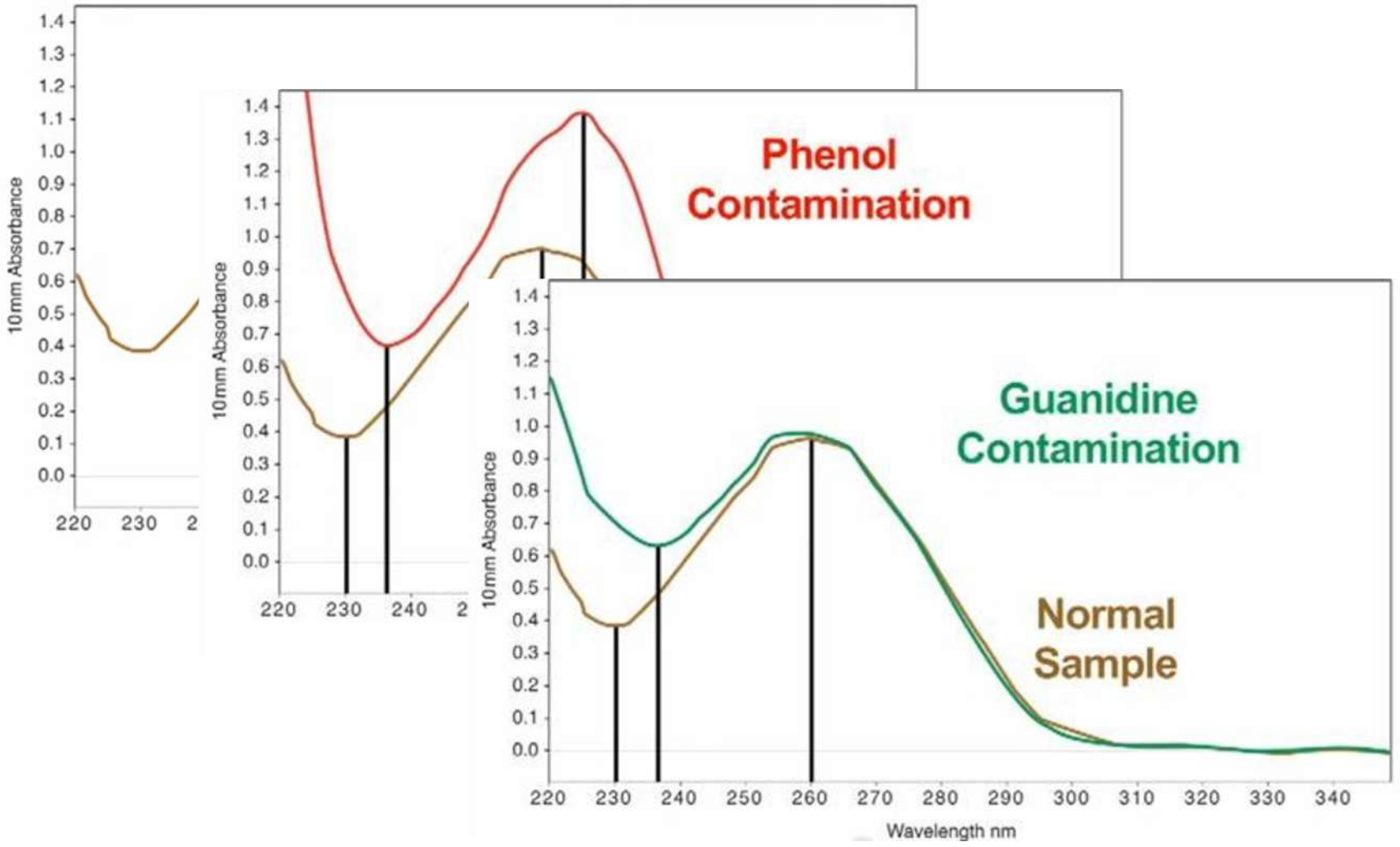


Quantification with a Fluorescent Dye

- DNA/RNA intercalating dyes
- Measurement of fluorescence

- ~1000 times more sensitive than UV absorbance

Quantification with a Fluorescent Dye

- Ethidium Bromide (EtBr)
- SYBR Green
- Hoechst 33258
- PicoGreen
- RiboGreen

Quantification with a Fluorescent Dye

EtBr:

- PCR products, gDNA
- Band intensity calculation
- Comparison to known reference
- Agarose and PAGE
- Not precise, relative

SYBR Green I:

- Highly sensitive
  - 25-100 times more than EtBr
- ssDNA, dsDNA
- Agarose and PAGE
- Less mutagenic

Quantification with a Fluorescent Dye

- Fluorometer

- Hoechst 33258 (DNA):
  - Binds to A-T bp in dsDNA
  - Emission Max. At 460nm

  } Emission max. at 530nm

- PicoGreen (DNA)
- RiboGreen (RNA)

Quantification with a Fluorescent Dye

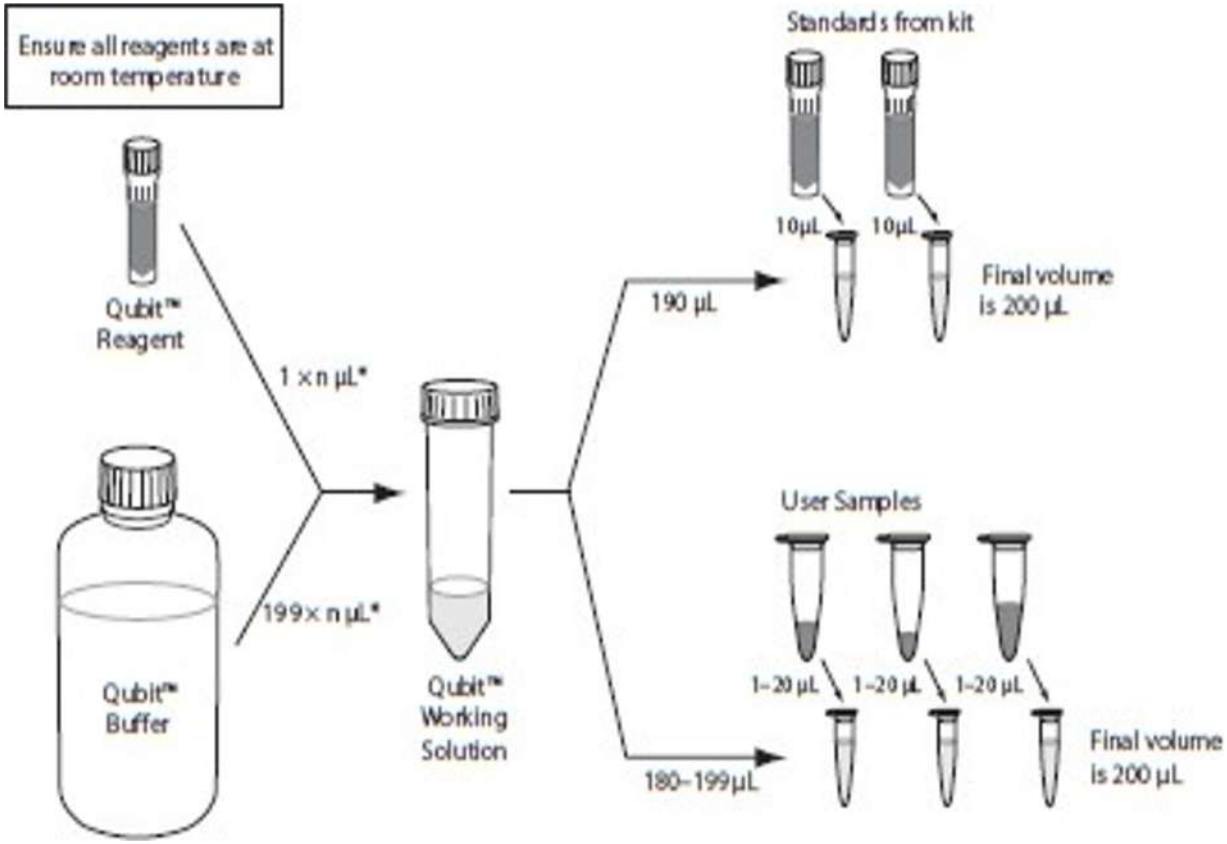
TBS-380 Fluorometer 3800: 2mL or 50µl with minicell adapter

Aquaflour: 2mL

NanoDrop3300 Fluorospectrometer:

Qubit: 2ooµl

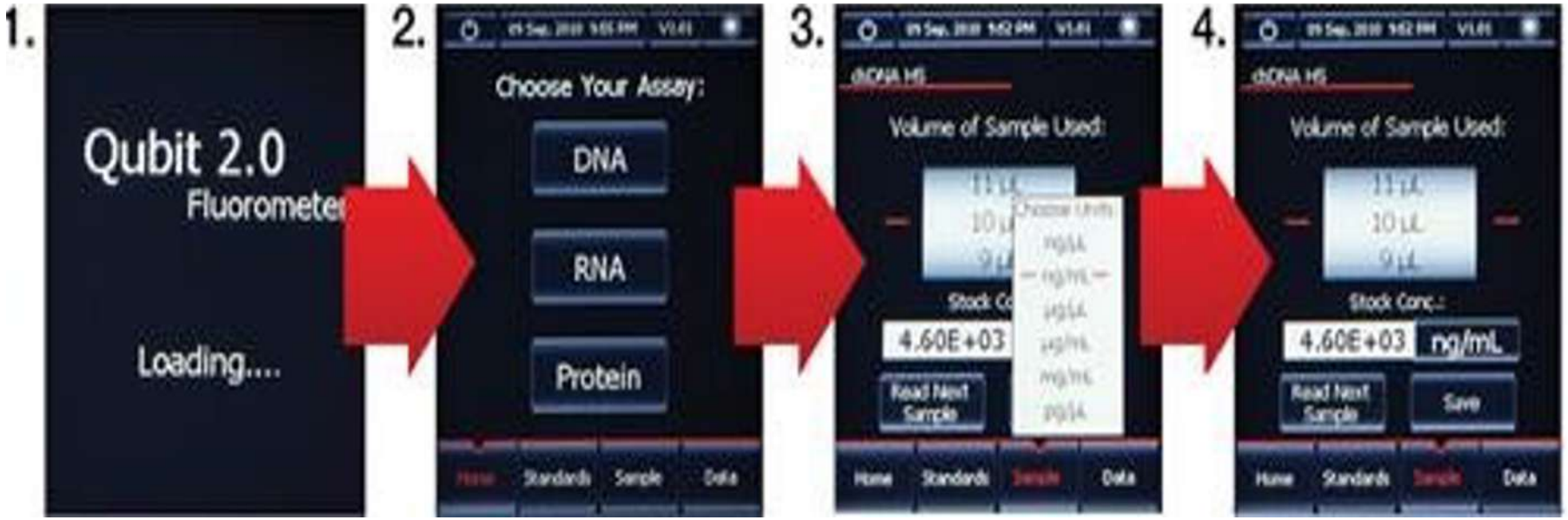
Quantification with a Fluorescent Dye: Qubit



Quantification with a Fluorescent Dye

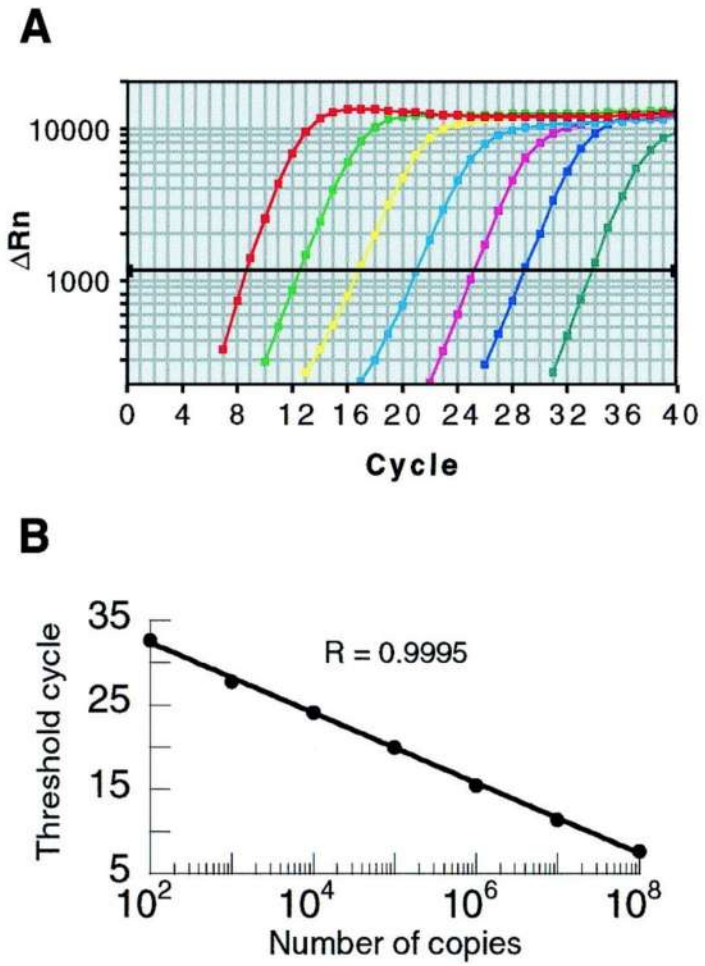
The Qubit® 2.0 Fluorometer displays the standard curve after completion of the calibration.

Quantification with a Fluorescent Dye



Quantification by Real-Time PCR

- DNA, RNA (cDNA)
- Absolute quantification
- Serially diluted standards
  → Standard curve
- Determination of concentration of unknowns based on Ct (Threshold cycle) values



Quality Assessment of Nucleic Acids

Quality Assessment of Nucleic Acids

- Level of degradation
- Assay amenability (FFPE tissues)
- Method: nature of nucleic acid

Methods of Quality Assessment of Nucleic Acids

DNA

1. Agarose gel electrophores is
2. PCR amplification of fragments with increasing length

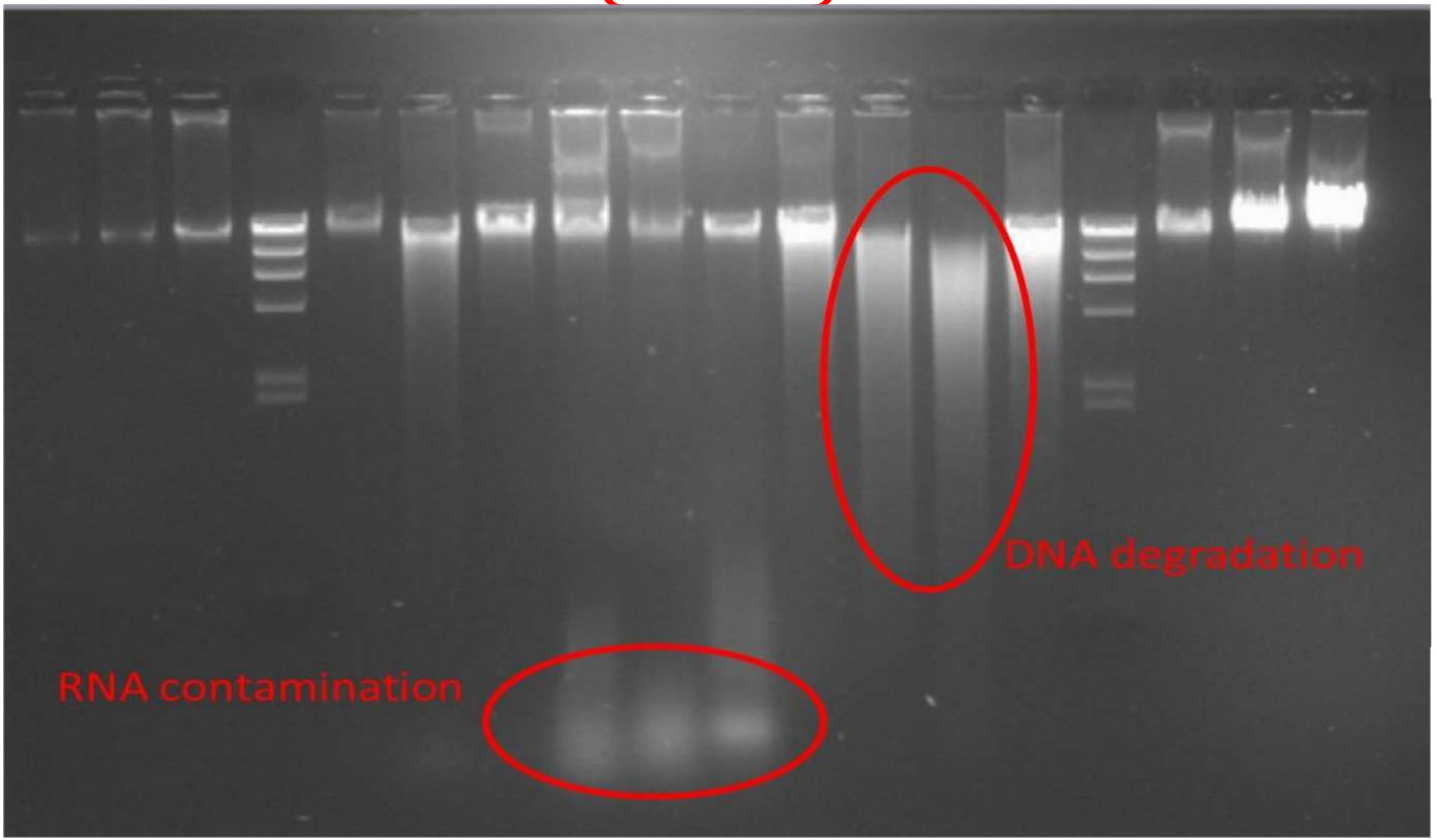
RNA

1. Denaturing gel electrophores is
2. RT-PCR amplification of mRNA fragments of increasing length

DNA and RNA

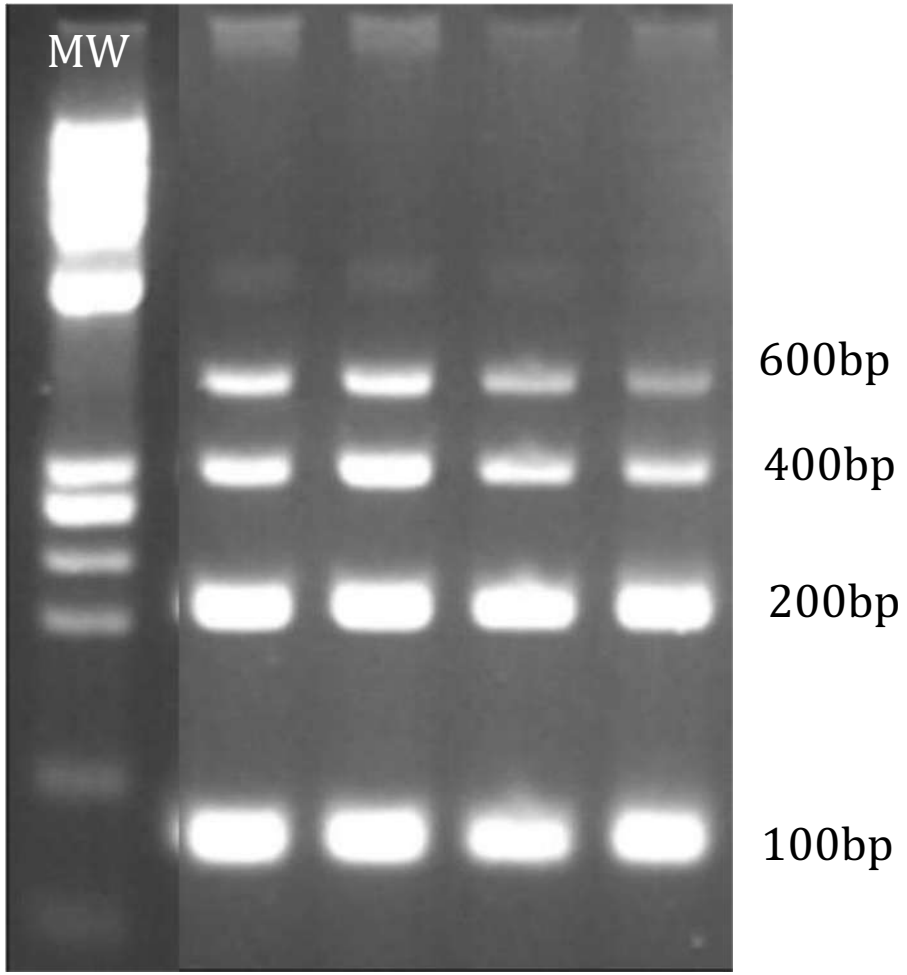
1. Agilent 2100 Bioanalyzer

Agarose gel electrophoresis (DNA)



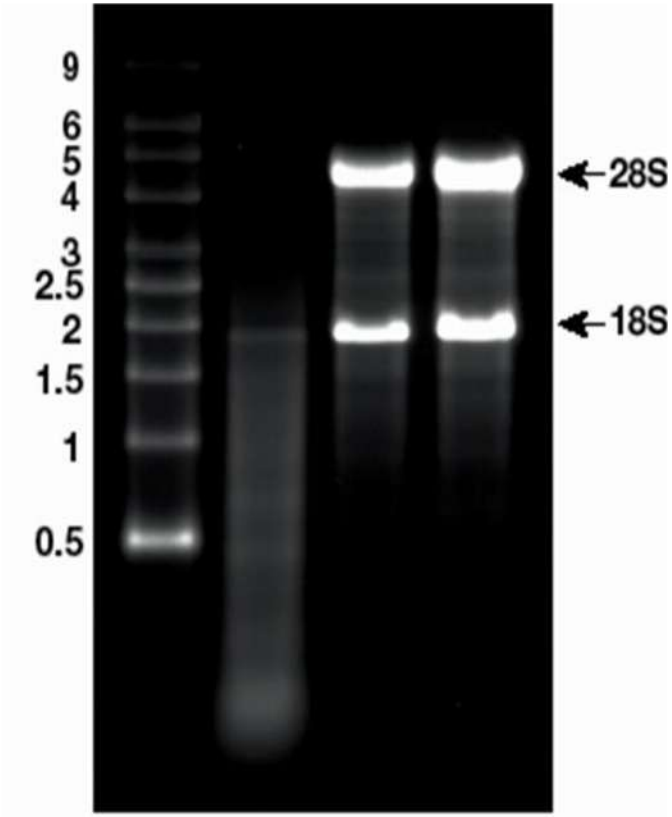
PCR Amplification of Fragments with Increasing Length (DNA)

- Archival tissues
- Spesmen control size ladder
- Multiplex PCR



Denaturing Agarose Gel Electrophoresis (RNA)

- Most common method of integrity assessment :
- Secondary structure of RNA → altered migration pattern
  - Electrophoresis buffer :Formaldehyde and MOPs (3-[N-Morpholino]-propanesulfonic acid)
  - Loading buffer: Glyoxal



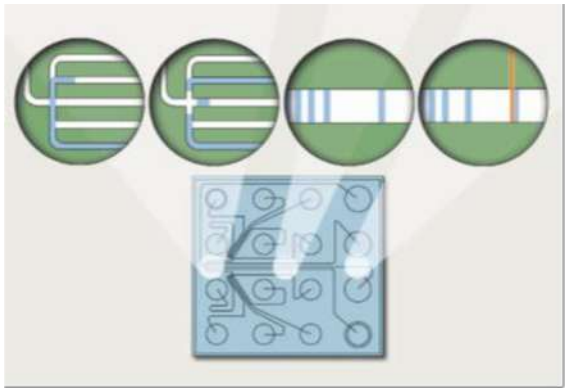
RT-PCR Amplification of mRNA Fragments of Increasing Length

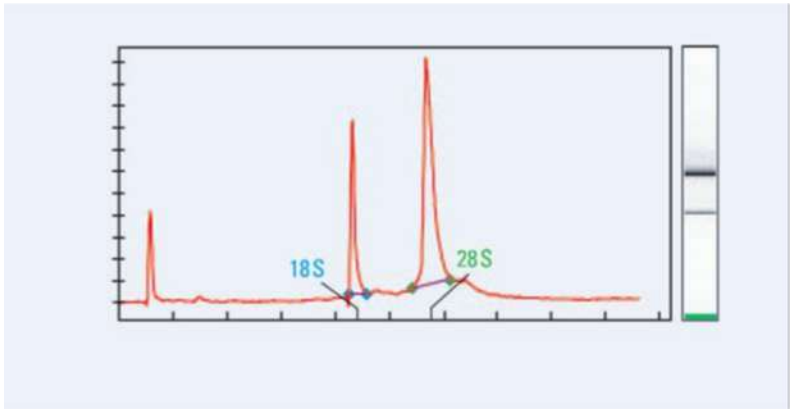
- mRNA integrity assessment
- Housekeeping gene
- cDNA subjected to amplification
- Fragments with increasing length
- Independent from rRNA integrity

Quality Assessment with Capillary Microchip Electrophoresis:

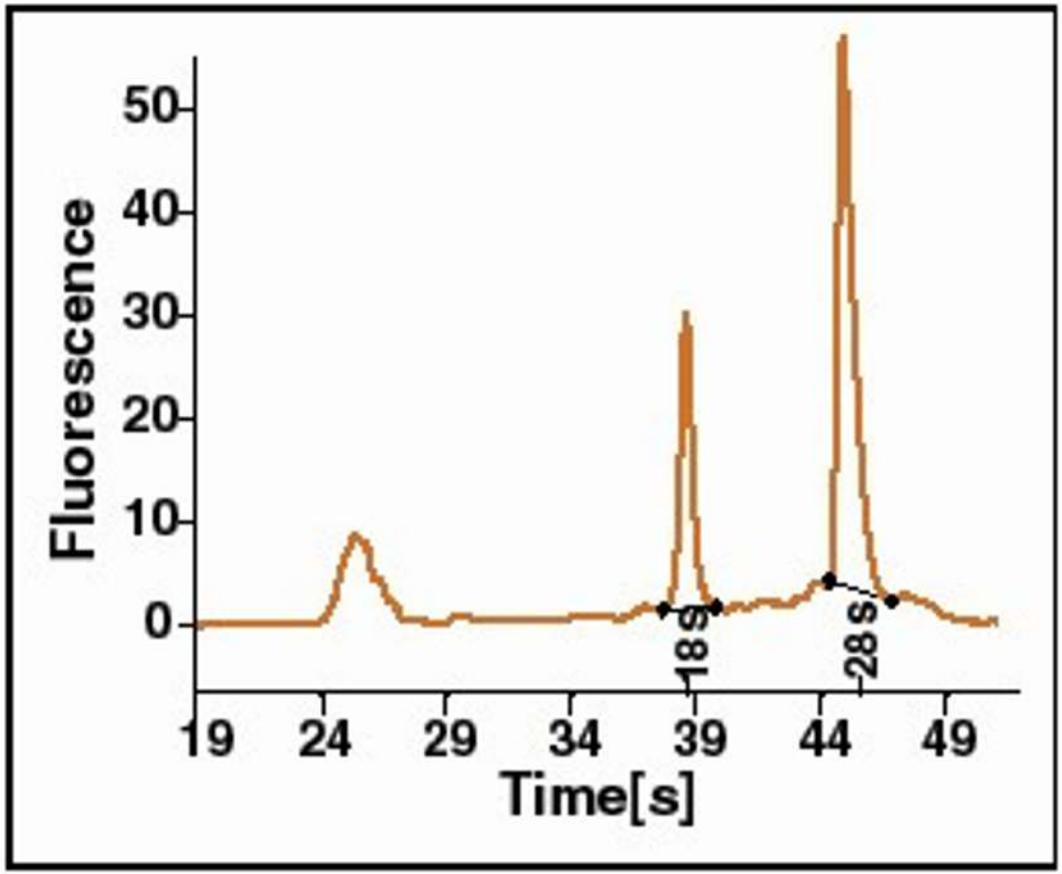
- LabChip systems (Caliper)
- MCE-202 MultiNA microchip electrophoresis system (Shimadzu)
- P/ACE MDQ (Beckman Coulter)
- 2100 BioAnalyzer (Agilent): microfluidics, capillary electrophoresis, fluorescent detection

Agilent 2100 BioAnalyzer





Agilent 2100 BioAnalyzer

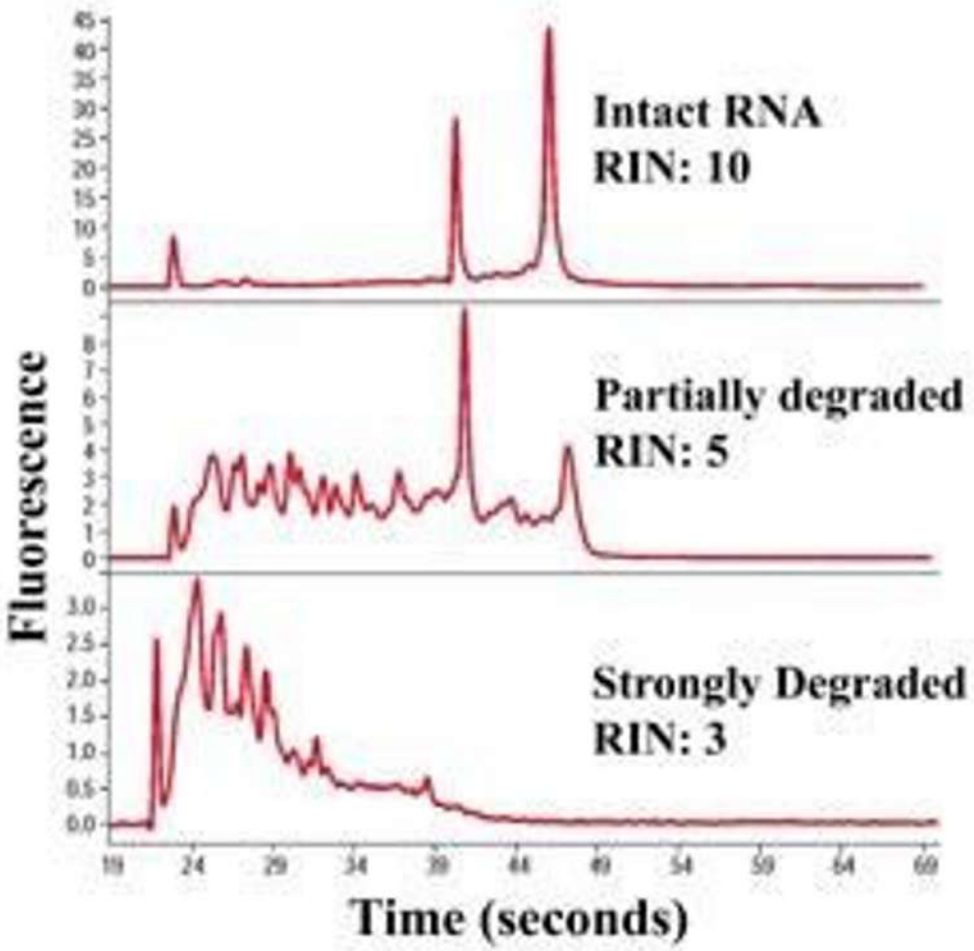


28S/18S ratios ~ 2

The RNA Integrity Number (RIN)

- 28S/18S rRNA ratio is reasonable but not ideal!!!
- Nondenaturing conditions
- Software algorithm for the the entire electrophoretic trace
- Estimation of the integrity of total RNA samples.
- Numbering system 1-10

RIN



RIN 1 : most degraded RIN 10: most intact

RIN

- Downstream application
  - RT-PCR vs Microarray

- RINs > 7-8 work well for most experiments.
- RINs < 7 require extra validation studies

Quantification of Proteins

- Different methods:
  - Accuracy required
  - Amount and purity of protein

- Spectrophotometric assays:
  - UV Absorbance methods
  - Colorimetric and fluorescent-based detection

Quantification of Proteins

- Assay selection criteria:
  1. Sample volume (microplate assay vs cuvette-based)
  2. Sample recovery (UV spectroscopy)
  3. Throughput (microplate compatible rapid assay)
  4. Robustness (repeatability)
  5. Chemical modifications (Covalent modifications!)
  6. Protein aggregation (solubility of the protein)

Quantification of Proteins

- UV absorbance: quantitation of purified protein
  - Proteins that contain Trp, Tyr residues
  - Cys-Cys disulphide bonds

- Colorimetric assays: uncharacterized protein solutions and cell lysate
  - Bradford
  - BCA
  - Lowry

Quantification ofProteins: UV Absorption Spectroscopy

- UV Absorption at 280nm
  - Range 20-3000µg
  - Aromatic aa (tyrosine and tryptophan)
  - Molar extinction coefficient
    - Beer-Lambert Law: $A = a_m \times C \times l$
  - Protein standard

Quantification ofProteins: UV Absorption Spectroscopy

- UV Absorption at 205nm
  - Range 1-100µg
  - Arsorption of photons by peptide bonds
    - Molar extinction coefficient at A205
    - Protein Standard

UV Absorption Spectroscopy

- NanoDrop 1000, NanoDrop 8000 A280 modules
- Concentration of purified protein samples
- 1µl sample

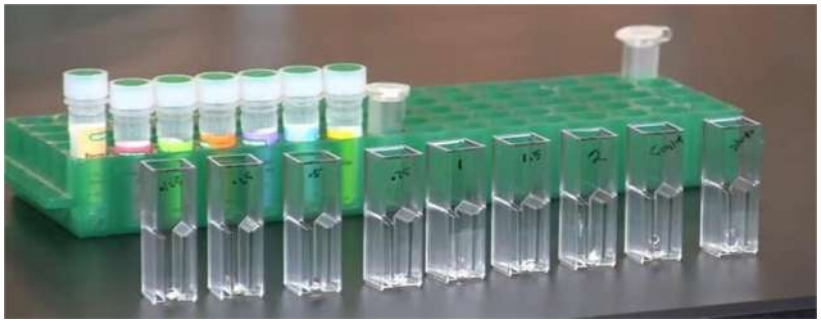
Dye-Based Assays: Bradford Assay

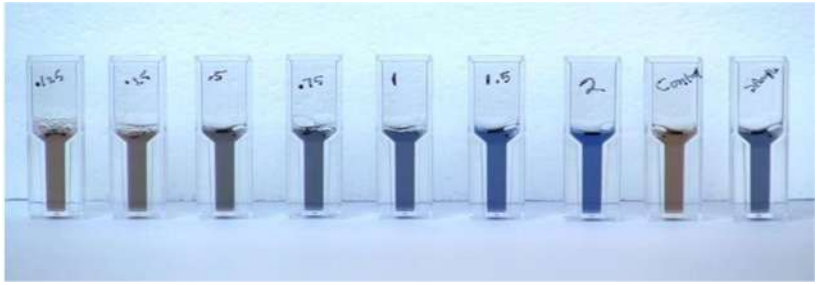
Bradford (Coomassie Blue) Assay (1-50µg):

- Binding of Coomassie Brillant Blue mainly to Tryptophan and tyrosine residues at acidic pH

- Shift in the absorbance of acidic CB solution from 465nm to 595nm

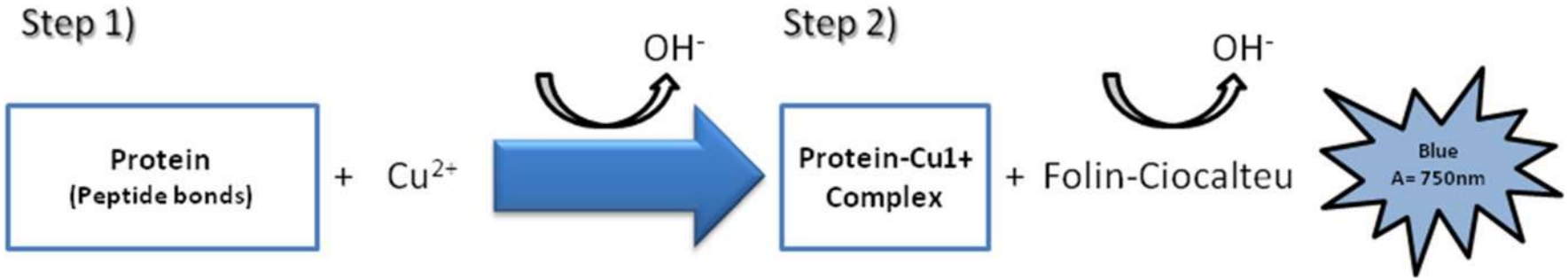
Bradford Assay





Dye-Based Assays: Lowry Assay

- Lowry (Alkaline Copper Reduction) Assay (5-100µg):
  - Two-step procedure
  - Reduction of Cu by proteins in alkaline solutions
  - Reduction of Folin reagent (a mixture of phosphotungstic acid and phosphomolybdic acid)
  - A blue color is formed with absorbance max. at 750nm



Dye-Based Assays: Bicinchoninic Assay

Bicinchoninic (BCA) Assay (0.2-50µg)

- Bicinchoninic acid (replacement of Folin's reagent)
- Improved sensitivity
- Tolerance to interfering substances
- Intense purple complex (562nm)

Protein analysis with the Agilent 2100 Bioanalyzer

- Microvolume analysis
- Different assays
  - For protein analysis in the low molecular weight range
  - General protein analysis up to 230 kDa
  - Picogram sensitivity

Quality Assessment of Proteins

1. Composition-Based and activity-based analysis
2. Electrophoretic methods (SDS gel electrophoresis)
3. Chromatographic methods
    I. Gel filtration Chromatography
    II. Reversed phase HPLC
4. Sedimentation velocity methods
5. Mass spectrometry methods
6. Light scattering methods