Genomic DNA isolation and Quantification Workshop

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Table 1.1 Composition of living cells

Component	Percent of total cell weight	
	***E. coli* cells**	**HeLa (human) cells**
Water	70.0	70.0
Inorganic ions	1.0	1.0
Amino acids	0.4	0.4
Nucleotides	0.4	0.4
Lipids	2.2	2.8
Proteins	15.0	22.3
RNA	6.0	1.7
DNA	1.0	0.85

Specimen Collection

- When collecting a specimen for molecular testing, it is critical to protect the specimen from contamination from exfoliated skin cells as well as to protect the individual collecting the specimen from potentially infectious materials and infectious agents.

- Gloves should be worn throughout the entire collection process and gloves should be changed frequently.

- There are numerous specimens from which nucleic acid can be obtained.

- **Whole blood**, plasma, and serum are common sources of samples for nucleic acid testing.
- Whole blood often yields a very high quality and ample quantity of nucleic acid for testing.
- The most common anticoagulants are EDTA or acid citrate dextrose (ACD). Both chelate calcium as their mode of action.
- ACD is useful when prolonged shipping may be involved as it will help maintain the integrity of the nucleated cells.

- The DNA in plasma or serum is most likely from dead cells. It is a good source but it may not be of high molecular size. (ccfDNA)

- **Anatomic samples**, either fresh, frozen, fixed, and/or archived, are very useful in molecular diagnostic testing.
- In routine histopathology, most tissues are fixed in formalin and embedded in paraffin for long-term preservation. DNA can be extracted from these tissues for subsequent molecular analysis by amplification methods.
- In brief, microtome tissue sections (5-20µm) are placed into microfuge tubes, then deparaffinized with xylene.

- The xylene is removed with ethanol washes, and the tissue is treated with proteinase K to make DNA available for amplification.

- **Freshly excised tissues** should be dropped immediately into liquid nitrogen.
- Large organs should be cut into smaller, more manageable pieces (1 cm3) as this will ease freezing, storage, and subsequent manipulations.

- Any organ can be taken, but should liver be required, a 24-h starvation period prior to sacrifice will improve DNA quality.

- Tissues harvested in this way can be stored at -70°C for several years prior to use

- **Swabs** are a common collection method for nucleic acid testing, and there are mainly two paradigms.
- The first is a cheek swab, and it is useful when the samples are not being collected at the institution but are collected off-site at clinics.
- There are many types of fibers available, and some even have course bristles.

- The concept is to brush or swipe the inside of the cheek to remove cells containing nucleic acids. Care needs to be taken in how hard the cheek is “scrubbed” as bleeding may ensue at which point there will be erythrocytes that may lead to complication during the isolation process.

- It is also a good idea to have the client rinse prior to the process to remove debris that may contribute to sample mass collected.

- A second approach is a swab of a lesion for submission, most often to identify a pathogen's nucleic acid. Viral, bacterial, and fungal targets are commonly found using a swab from a lesion.

Cell Breakage

- The best procedure for opening cells and obtaining intact DNA is through application of chemical (detergents) and/or enzymatic procedures.
- Detergents can solubilize lipids in cell membranes resulting in gentle cell lysis.
- In addition, detergents have an inhibitory effect on all cellular DNases and can denature proteins, thereby aiding in the removal of proteins from the solution.
- The lysis of animal cells is usually performed using anionic detergents such as SDS (sodium deodecyl sulfate) or Sarcosyl (sodium deodecyl sarcosinate).

Removal of Protein

- The second step in purification involves removing a major contaminant ,namely protein, from the cell lysate.
- This procedure is called deproteinization.
- **Deproteinization using organic solvents**
- The most frequently used methods for removing proteins explore the solubility differences between proteins and nucleic acids in organic solvents.

- The organic solvents commonly used are phenol and chloroform containing 1% isoamyl alcohol.
- Proteins can be removed from DNA preparations using a protease that can digest all proteins, i.e. a general-purpose protease.
- Two such enzymes are in use, proteinase K and pronase. Commercial preparations of these enzymes are inexpensive.

- Proteinase K and pronase are usually used in DNA purification procedures at final concentrations of 0.1–0.8mg/ml.

- The characteristics of enzymatic removal of proteins make enzymatic deproteinization an ideal and indispensable first step in nucleic acid purification.

Removal of RNA

- The removal of RNA from DNA preparations is usually carried out using an enzymatic procedure.
- Two ribonucleases that can be easily and cheaply used are namely:
- Ribonuclease A (RNase A)
- Ribonuclease T1 (RNase T1).

Precipitating and concentrating the DNA

- Precipitating with alcohol is usually performed for concentration of DNA from the aqueous phase of the deproteinization step.

- Two alcohols are used for DNA precipitation: ethanol and isopropanol.

- This can be accomplished by the addition of salts to DNA solutions.

DNA Purification Methods

1- Phenol/chloroform extraction method

2-Silica membrane spin columns purification method

3- Anion-Exchange chromatography method

4- Salting out Method

Phenol/chloroform extraction

- The DNA solution is first extracted with a phenol/chloroform/isoamyl alcohol mixture to remove protein contaminants, and then precipitated with 100% ethanol.

- The DNA is pelleted after the precipitation step, washed with 70% ethanol to remove salts and small organic molecules, and resuspended in buffer.

Silica membrane spin columns purification method

- DNA is bound to the silica membrane spin column in the presence of a high concentration of chaotropic salt, contaminants are washed away, and the DNA is then eluted from the silica membrane in water.

- This method of DNA purification is quick and convenient, and can produce a high yield of pure DNA.

- Use of optimized buffers in the lysis procedure ensures that only DNA is adsorbed while cellular proteins, and metabolites remain in solution and are subsequently washed away.

- This is simpler and more effective than other methods where precipitation or extraction is required.

- Ready-to-use DNA is then eluted from the silica-gel membrane using a low-salt buffer.

- No alcohol precipitation is required.

Salting out Method

- Starting with a crude lysate, ”salting-out” is technique where proteins and other contaminants are precipitated from the cell lysate using high concentrations of salt such as potassium acetate or ammonium acetate.
- The precipitates are removed by centrifugation, and the DNA is recovered by alcohol precipitation. Removal of proteins and other contaminants using this method may be inefficient, and RNase treatment, dialysis, and/or repeated alcohol precipitation are often necessary before the DNA can be used in downstream applications

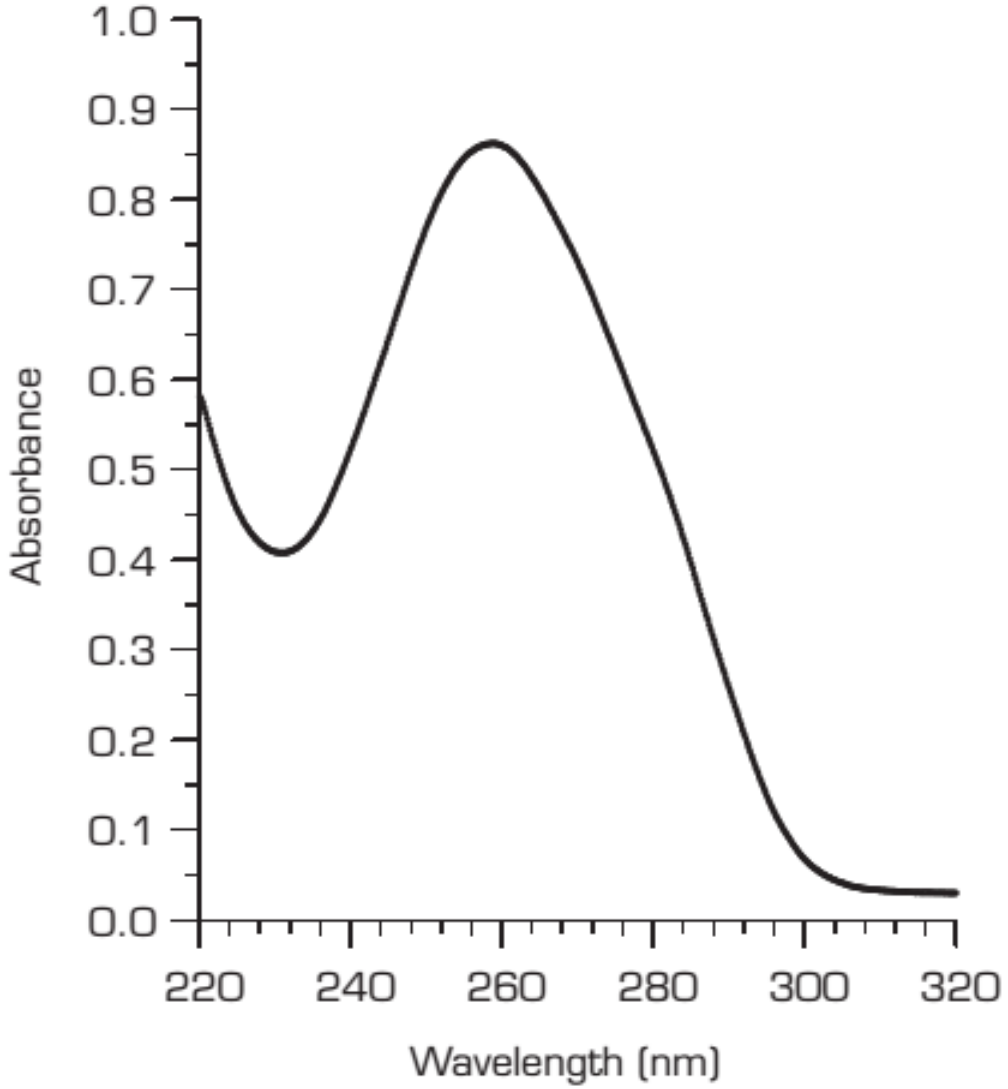
Determination of the Purity, Quantity and quality of purified DNA

Determination of the Quantity of purified DNA

- UV spectrophotometry is used for the determination of DNA concentration.
- The DNA has maximum A at 260 nm.

- However, these are strongly affected by the degree of base ionization and, hence, pH of the measuring medium.

- Measurements of the OD at 260 and 280 nm are less variable and more reliable at pH 8.0



Absorption spectrum of high purified DNA. The DNA was diluted 20 times in PBS and scanned using a UV spectrophotometer. The A260/A280 =1.7 and the A260/A234=1.8.

Table 2.2.6 Spectrophotometric Measurements of Purified DNA[a,b]

Wavelength (nm)	Absorbance[c]
325	0.01
280	0.28
260	0.56
230	0.30

[a]Typical absorbance readings of highly purified calf thymus DNA suspended in 1× TNE buffer.

DNA Quantitation by Spectrophotometry

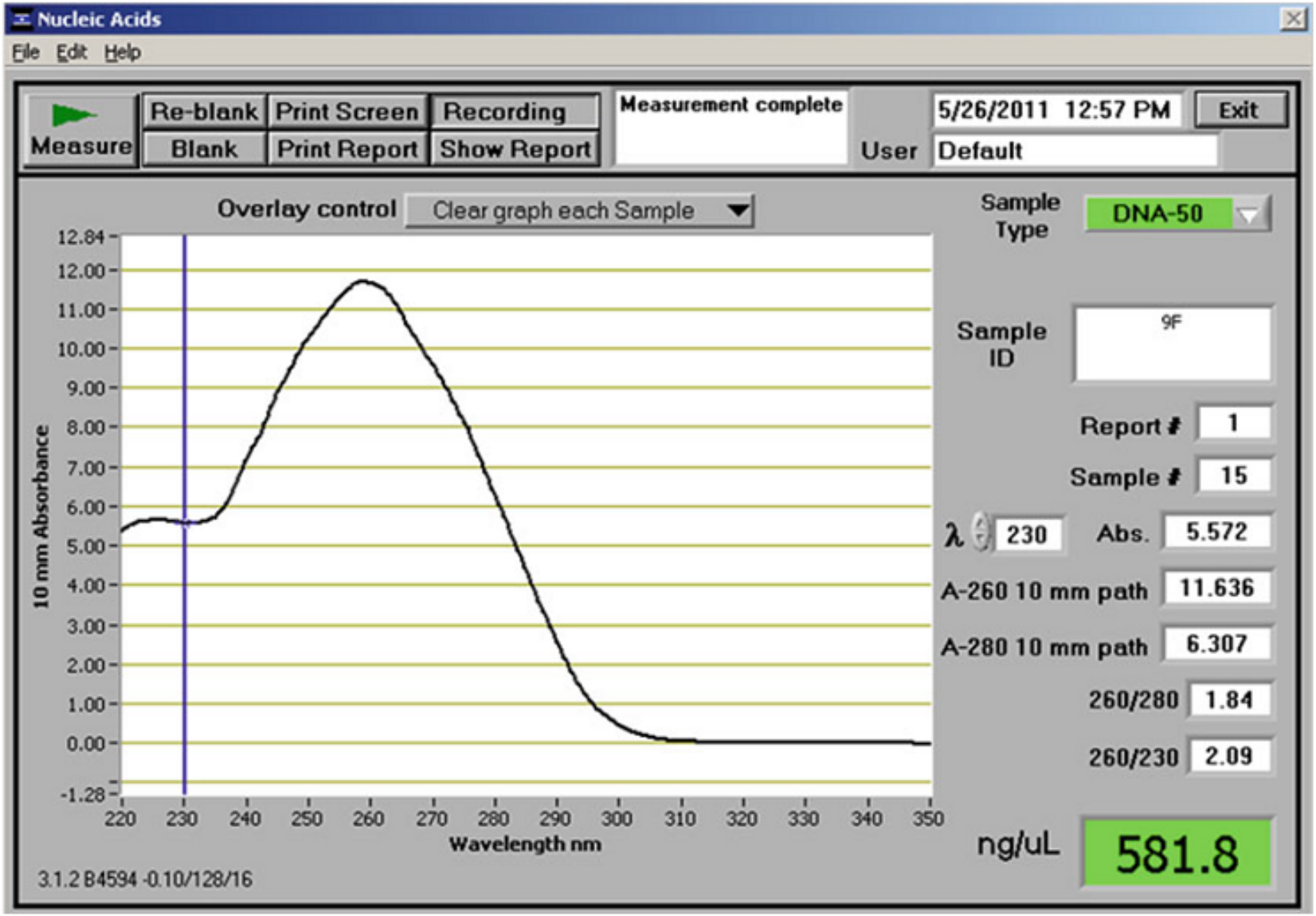
- 1. Mix the DNA sample by gentle vortexing and inversion.

- 2. Add 5 µL of the DNA sample to 495 µL of sterile water and mix well.

- 3. Place the diluted sample in a quartz microcuvet and measure the absorbance at 260 and 280 nm against a water blank. (Nucleic acids absorb light maximally at 260 nm whereas proteins absorb strongly at 280 nm.)

- 4. Compute the DNA concentration

- DNA con.(µg/ml)=A260x 50 x dilution factor (100)


Nucleic Acids
File Edit Help
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DNA-50
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Sample #
15
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230
Abs.
5.572
A-260 10 mm path
11.636
A-280 10 mm path
6.307
260/280
1.84
260/230
2.09
ng/uL
581.8
10 mm Absorbance
12.84
12.00
11.00
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7.00
6.00
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4.00
3.00
2.00
1.00
0.00
-1.28
220 230 240 250 260 270 280 290 300 310 320 330 340 350
Wavelength nm
3.1.2 B4594 -0.10/128/16

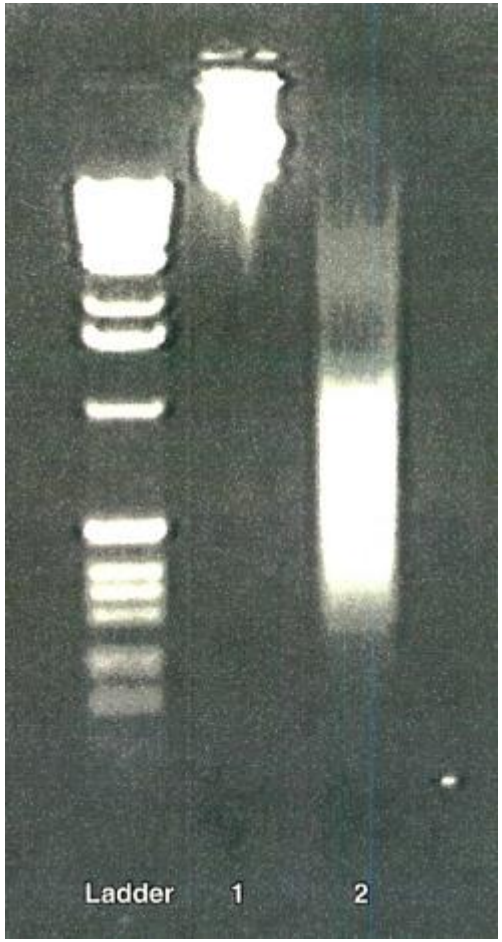
Measurement Concentration Range

The NanoDrop 1000 Spectrophotometer will accurately measure dsDNA samples up to 3700 ng/ul without dilution. To do this, the instrument automatically detects the high concentration and utilizes the 0.2mm pathlength to calculate the absorbance.

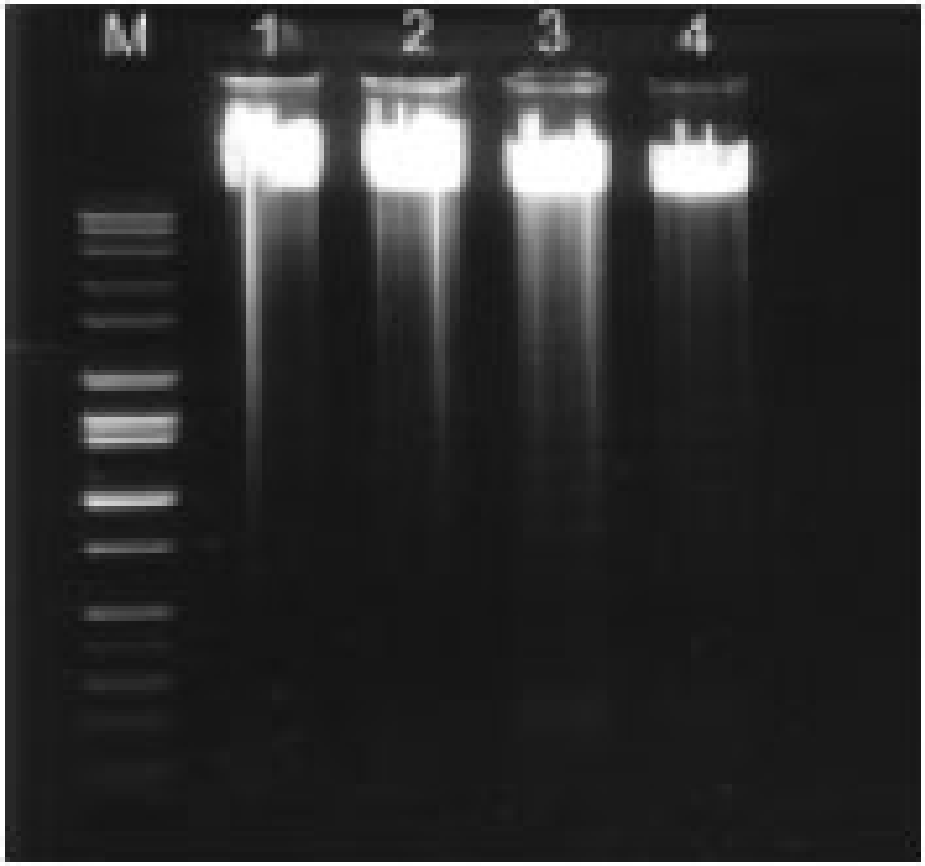
Detection Limit (ng/ul)	Approx. Upper Limit (ng/ul)	Typical Reproducibility (minimum 5 replicates) (SD= ng/ul; CV= %)
2	3700 ng/ul (dsDNA) 3000 (RNA) 2400 (ssDNA)	sample range 2-100 ng/ul: ± 2 ng/ul sample range >100 ng/ul: ± 2%

Gel Electrophoresis to Analyze DNA Quality

- Agarose gel electrophoresis can be used to assess the intactness of purified DNA.
- 1. Prepare a 0.7% agarose gel in 1X TAE buffer
- Mix an 5-10 µl of the extracted DNA sample with loading buffer, and load into well. Control samples representing intact and degraded DNA should be loaded into adjacent wells.
- 3. Electrophorese in 1X TAE buffer with 0.5 µg/mL ethidium bromide at 2 V/cm, until the dye front reaches the end of the gel.
- 4. View the gel under UV light. High molecular weight DNA is too large to migrate well under these conditions, whereas degraded DNA contains a spectrum of smaller fragment sizes that appear as a smear across the lane.



Assessment of DNA quality by gel electrophoresis. DNA extracted from two whole blood specimens was resolved on 1%agarose gel and compared with a molecular weight size marker (ladder). High quality DNA extractions are associated with high molecular weight fragments, as shown in lane I. Lane 2 illustrates DNA extracted from a degraded whole blood sample wherein the molecular weights of DNA fragments are much reduced.



Purified genomic DNA (~200 ng) was electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Observe high quality genomic DNA that ranges from ~30 to 50 kb in size. Lane M contains molecular weight markers from 10 kb to 50 bp in length. Lanes 1-4 are genomic DNA samples obtained from blood samples of 4 different individuals