Isolation and Purification of Nucleic Acids

Nucleic Acid Purification

There are many DNA purification methods. All must:

1. Effectively disrupt cells or tissues
   (usually using detergent)
2. Denature proteins and nucleoprotein complexes
   (a protease/denaturant)
3. Inactivate endogenous nucleases
   (chelating agents)
4. Purify nucleic acid target away from other nucleic acids and protein
   (could involve RNases, proteases, selective matrix and alcohol precipitations)

Disruption of Cells/Tissues

Most purification methods disrupt cells using lysis buffer containing:

- **Detergent** to disrupt the lipid bilayer of the cell membrane
- **Denaturants** to release chromosomal DNA and denature proteins

Additional enzymes are required for lysis of some cell types:

- Gram-positive bacteria require lysozyme to disrupt the bacterial cell wall.
- Yeasts require addition of zymolase to disrupt the cell wall.
- Plant cells may require cellulase pre-treatment.

Disruption of Cells: Membrane Disruption

- Detergents are used to disrupt the lipid:lipid and lipid:protein interactions in the cell membrane, causing solubilization of the membrane.
- Ionic detergents (such as sodium dodecyl sulfate; SDS) also denature proteins by binding to charged residues, leading to local changes in conformation.

Protein Denaturation

Denaturation = Modification of conformation to unfold protein, disrupting secondary structure but not breaking the peptide bonds between amino acid residues.

Denaturation results in:

- Decreased protein solubility
- Loss of biological activity
- Improved digestion by proteases
- Release of chromosomal DNA from nucleoprotein complexes ("unwinding" of DNA and release from associated histones)

Protein Denaturing Agents

- Ionic detergents, such as SDS, disrupt hydrophobic interactions and hydrogen bonds.
- Chaotropic agents such as urea and guanidine disrupt hydrogen bonds.
- Reducing agents break disulfide bonds.
- Salts associate with charged groups and at low or moderate concentrations increase protein solubility.
- Heat disrupts hydrogen bonds and nonpolar interactions.

- Some DNA purification methods incorporate proteases such as proteinase K to digest proteins.

Inactivation of Nucleases

- Chelating agents, such as EDTA, sequester Mg^{2+} required for nuclease activity.
- Proteinase K digests and destroys all proteins, including nucleases.

- Some commercial purification systems provide a single solution for cell lysis, protein digestion/denaturation and nuclease inactivation.

Removal of RNA

- Some procedures incorporate RNase digestion during cell lysate preparation.
- In other procedures, RNase digestion is incorporated during wash steps.

Isolated DNA could be used in:

- Amplification methods (PCR)
- Cloning
- Sequencing
- Transfection
- *Invitro* transcription

Nucleic Acid Preparation
Choosing an Isolation Method

- Important factors are:
  - Processing speed
  - Ease of use
  - Yield of DNA or RNA
  - Quality of DNA and RNA prepared (amplification performance)
  - Shelf life/storage conditions
  - Quality assurance criteria
  - Cost of preparation

Basic Steps in Isolating DNA from Clinical Specimens

Separate WBCs from RBCs, if necessary

↓

Lyse WBCs or other nucleated cells

↓

Denature/digest proteins

↓

Separate contaminants (*e.g.*, proteins) from DNA

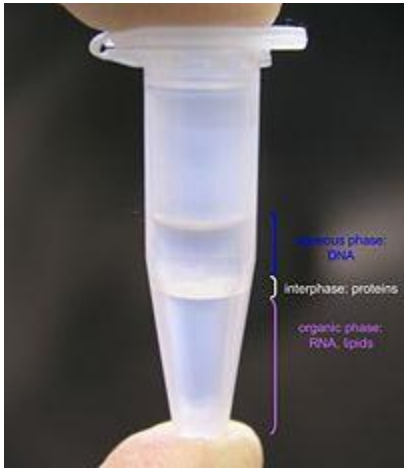
↓

Precipitate DNA if necessary

↓

Resuspend DNA in final buffer

DNA Isolation Methods
Liquid Phase Organic Extraction



- Phenol chloroform/isoamyl alcohol
  - Two layers are formed.
  - Proteins remain at interface.
  - DNA is removed with top aqueous layer.
  - DNA is precipitated with ethanol and rehydrated.
- Disadvantages:
  - Slow, labor-intensive, toxic (phenol, chloroform)
  - Fume hood required, disposal of hazardous materials required

DNA Isolation Methods: Liquid Phase Nonorganic Salt Precipitation

- Cell membranes are lysed and proteins are denatured by detergent (such as SDS).
  - RNA is removed with RNase.
  - Proteins are precipitated with salt solution.
  - DNA is precipitated with alcohol and rehydrated.
- Advantages:
  - Fast and easy method
  - Uses nontoxic materials, no fume hood required, no hazardous materials disposal issues
  - Produces high-quality DNA

DNA Isolation Methods
Solid Phase Procedures

- Uses solid support columns, magnetic beads.
- Solid support columns: Fibrous or silica matrices bind DNA allowing separation from other contaminants.
- Magnetic beads: DNA binds to beads; beads are separated from other contaminants with magnet.
- Advantages:
  - Fast and easy, no precipitation required

DNA Purification Method Comparison

Liquid Phase

(Lyse RBCs)

Lyse cells

(Protein digestion-ProK)

Separate proteins from DNA

Precipitate DNA-alcohol

Rehydrate DNA

Solid Phase

(Pre-lyse cells)

Apply sample

Wash

Elute DNA

Basic Steps in Isolating RNA

Separate WBCs from RBCs, if necessary

↓

Lyse WBCs or other nucleated cells in presence of protein denaturants, RNase inhibitors

↓

Denature/digest proteins

↓

Separate proteins, DNA, and contaminants from RNA

↓

Precipitate RNA if necessary

↓

Resuspend RNA in final buffer

Precautions for Working with RNA

- **RNA is not a stable molecule!**
  - It is easily degraded by RNase enzymes.
- Use sterile, disposable plastic ware (tubes, filter tips) marked "**For RNA Use Only**".
- Always wear gloves and work in a hood whenever possible/practical.

RNA Isolation Methods
Guanidinium-based Organic Isolation

- Phenol/guanidinium solution disrupts cells, solubilizes cell components, but maintains integrity of RNA.
- Add chloroform, mix, and centrifuge.
- Proteins/DNA remain at interface.
- RNA is removed with aqueous top layer.
- RNA is precipitated with alcohol and rehydrated.
- Disadvantages: fume hood required, hazardous waste disposal issues

RNA Isolation Methods
Nonorganic Salt Precipitation

- Cell membranes are lysed and proteins are denatured by detergent (such as SDS) in the presence of EDTA or other RNase inhibitors.
- Proteins/DNA are precipitated with a high concentration salt solution.
- RNA is precipitated with alcohol and rehydrated.
- Advantages:
  - Fast and easy, nontoxic
  - Produces high quality RNA

Resuspending Final Nucleic Acid Samples

- Have some idea of expected nucleic acid yield.
  - Choose diluent volume according to desired concentration.
- Resuspend DNA in TE buffer or ultra pure DNAse-free water.
- Resuspend RNA in ultra pure RNase-free water.
- Nucleic acid concentration could be measured by Abs at 260 nm.

Nucleic Acid Analysis

- DNA or RNA is characterized using several different methods for assessing quantity, quality, and molecular size.
  - UV spectrophotometry
  - Agarose gel electrophoresis

Quantity from UV Spectrophotometry

- DNA and RNA absorb maximally at 260 nm.
- Proteins absorb at 280 nm.
- Background scatter absorbs at 320 nm.

Quantification Methods

Spectrophotometry: Use of light absorbance to measure concentration. Many biological substances absorb light. The spectrophometer measures absorbance of light at specific wavelengths

- Most commonly used method
- DNA concentration can be calculated based on a
  nm:
  $A = \varepsilon \times c \times l$ (Beer-Lambert Law)
  A = absorbance
  ε = extinction coefficient
  c = concentration
  l = path length

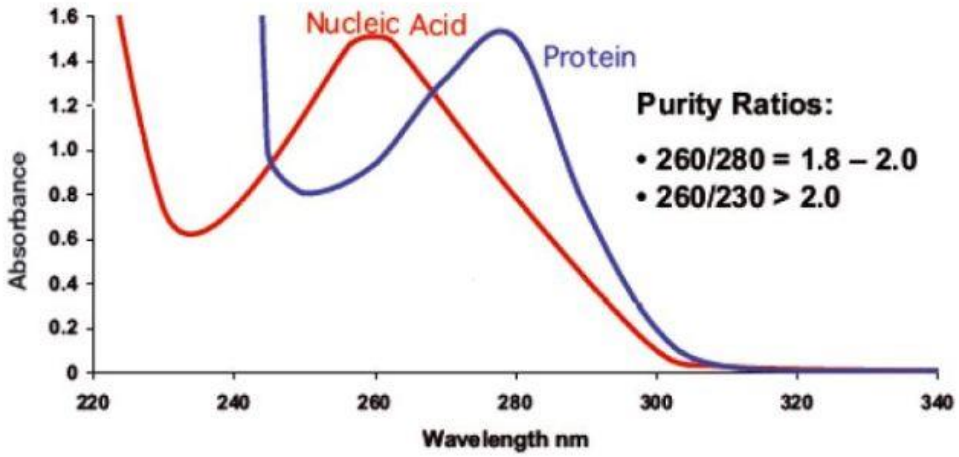
Quality from UV Spectrophotometry

A_{260}/A_{280} = measure of purity

$(A_{260} - A_{320})/(A_{280} - A_{320})$

1.8 – 2.0 = good DNA or RNA

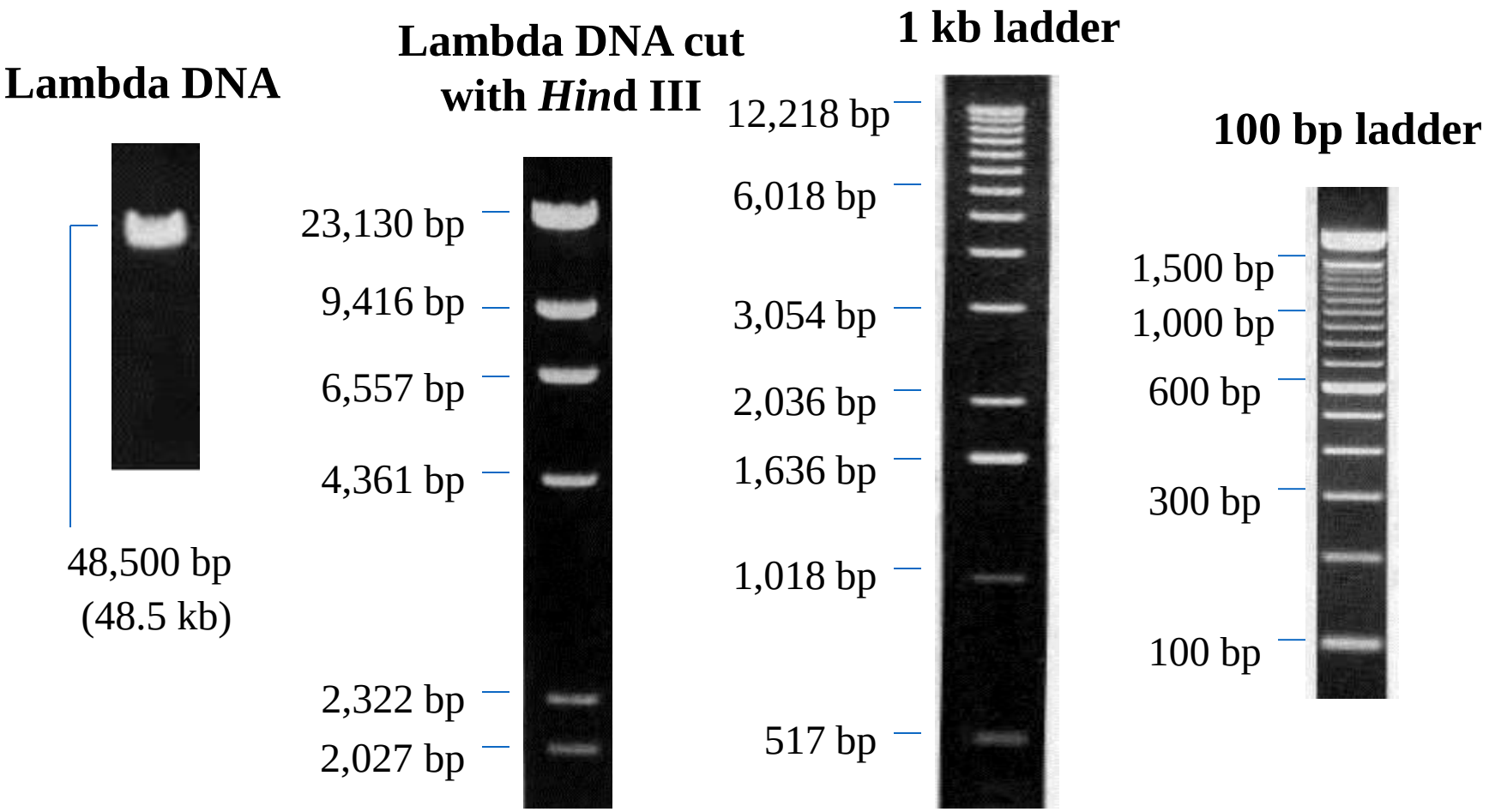
<1.8 = too much protein or other contaminant (salt, EDTA or organic solvents)



Quality from Agarose Gel Electrophoresis

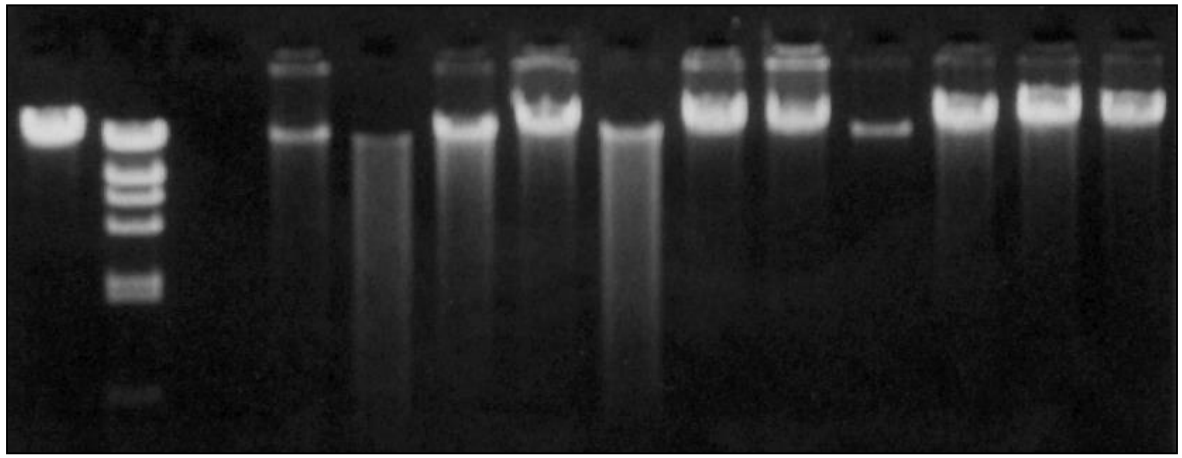
- Genomic DNA:
  - 0.6% to 1% gel, stain with ethidium bromide.
  - Electrophorese at 70–80 volts, 45–90 minutes.
- Total RNA:
  - 1% to 2% gel, stain with ethidium bromide.
  - Electrophorese at 80–100 volts, 20–40 minutes.

DNA Size from Agarose Gel Electrophoresis: Compares unknown DNA to known size standards



DNA Quality from Agarose Gel Electrophoresis

- Smearing indicates DNA degradation (or too much DNA loaded).



Lambda DNA marker

Lambda DNA cut with *Hin*d III marker

Whole blood genomic DNA

Storage Conditions

- Store DNA in TE buffer at 4 °C for weeks or at –20 °C to –80 °C for long term.
- Store RNA in RNase-free ultra pure water at –80 °C.

Troubleshooting Nucleic Acid Preparation Methods

- Problem: No or low nucleic acid yield.
  - Make sure that ample time was allowed for resuspension or rehydration of sample.
  - Repeat isolation from any remaining original sample (adjust procedure for possible low cell number or poorly handled starting material).
  - Concentrate dilute nucleic acid using ethanol precipitation.
- Problem: Poor nucleic acid quality
  - If sample is degraded, repeat isolation from remaining original sample, if possible.
  - If sample is contaminated with proteins or other substances, clean it up by re-isolating (improvement depends on the extraction procedure used).

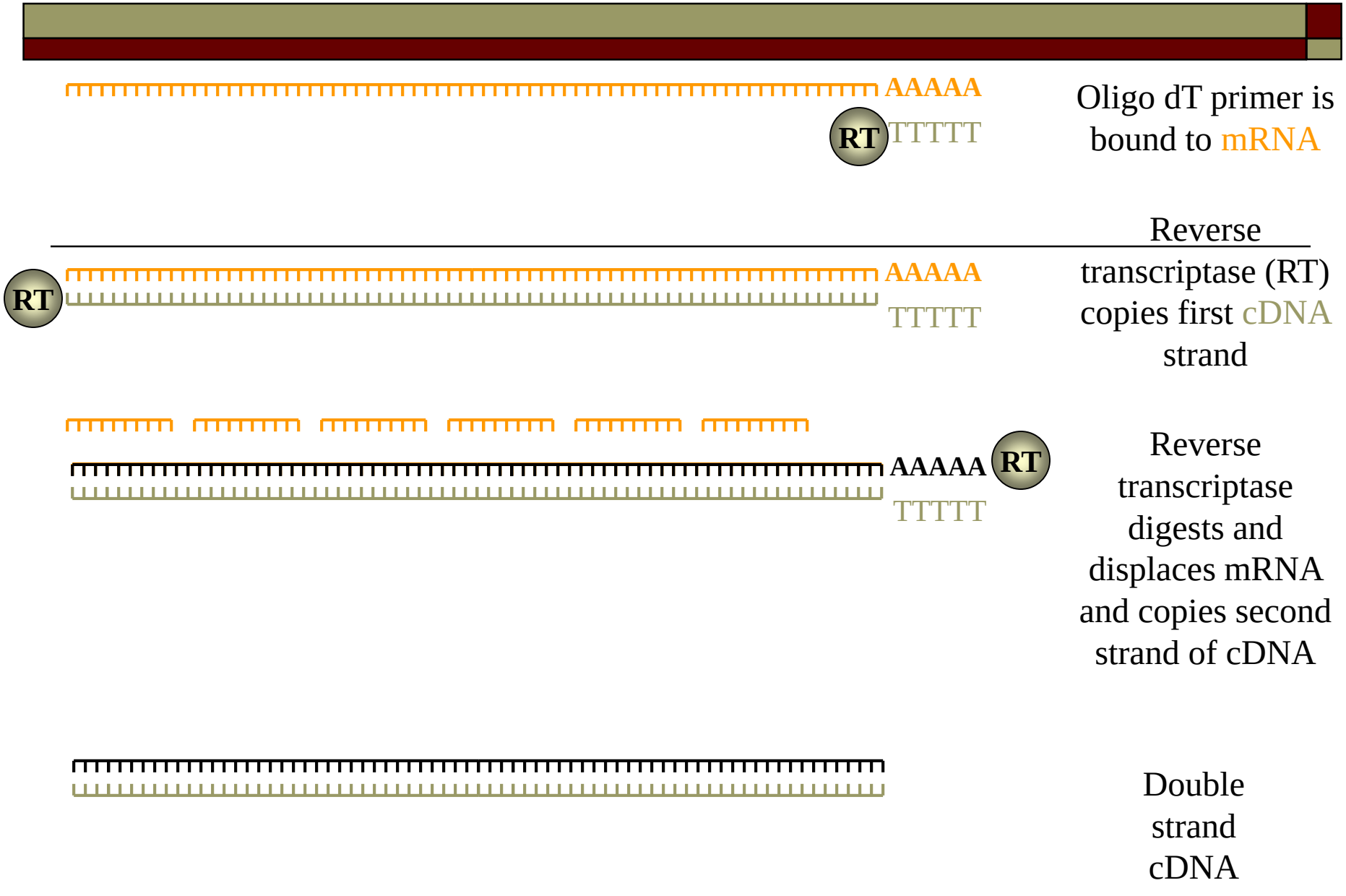
Extraction and purification of plasmids

- Alkaline lysis of bacterial cells followed by adsorption of DNA onto silica membrane in the presence of high salt.
- The procedure consists of three basic steps:

1. Preparation and clearing of a bacterial lysate
2. Adsorption of DNA onto the silica membrane
3. Washing and elution of plasmid DNA

- All steps are performed without the use of phenol, chloroform, and without alcohol precipitation.
- For further reading:

http://vlab.amrita.edu/?sub=3&brch=77&sim=314&cnt=1



Conversion of mRNA to cDNA by Reverse Transcription

There are commercially available kits to isolate DNA easily from any source

From plasmid to genomic DNA and from DNA clean-up to automation, Invitrogen™ products bring flexible, innovative solutions to meet virtually every researcher's needs.

Explore the Invitrogen portfolio of leading solutions, kits and benchtop devices for reliable results.

For further reading:
http://bitesizebio.com/1660/plasmid-v-genomic-dna-extractionthe-difference/