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Chapter 3

Molecular Techniques
Separation and Detection, Part 1

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Molecular Techniques

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- Electrophoresis – Gel and Capillary
- Blotting and probing procedures
- Probe hybridization
- In-situ hybridization
- Nucleic acid purification

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Objectives

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- Discuss the principles of electrophoresis as it applies to nucleic acids.
- List the similarities and differences between agarose and polyacrylamide gels and uses of each.
- Explain the use of buffers and additives in electrophoresis.
- Discuss capillary electrophoresis.

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Objectives

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- Describe the types of equipment used for electrophoresis and how samples are handled for the procedure.
- Discuss differences and similarities between pulse field gel electrophoresis and regular electrophoresis regarding method and applications.
- Discuss detection systems used in nucleic acid applications.

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Electrophoresis

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- http://www.cytologystuff.org – Electrophoresis lab
- Principle – movement of molecules by an electric current. Usually done in a gel matrix so that the variously sized molecules of nucleic acid spread out and can be differentiated. Since the DNA has a phosphate group, it is negatively charged and moves towards the positive pole (anode).

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Why do the molecules spread out?

- The variously sized molecules travel at different speeds through the gel.
- Smaller sized molecules can go faster, and thus travel farther in a given time than larger molecules.
- At the end of the run, the smallest pieces will have gone the farthest and the largest pieces will be closest to the beginning.

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Reptation

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- This word describes the wriggling motion of the molecules as they make their way through the gel. Think of a “snake-like” movement.
- In addition to the size of the molecule, other factors affecting the distance of travel are the strength of the electric field, buffer, and the density of the agarose gel.

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Results of gel electrophoresis

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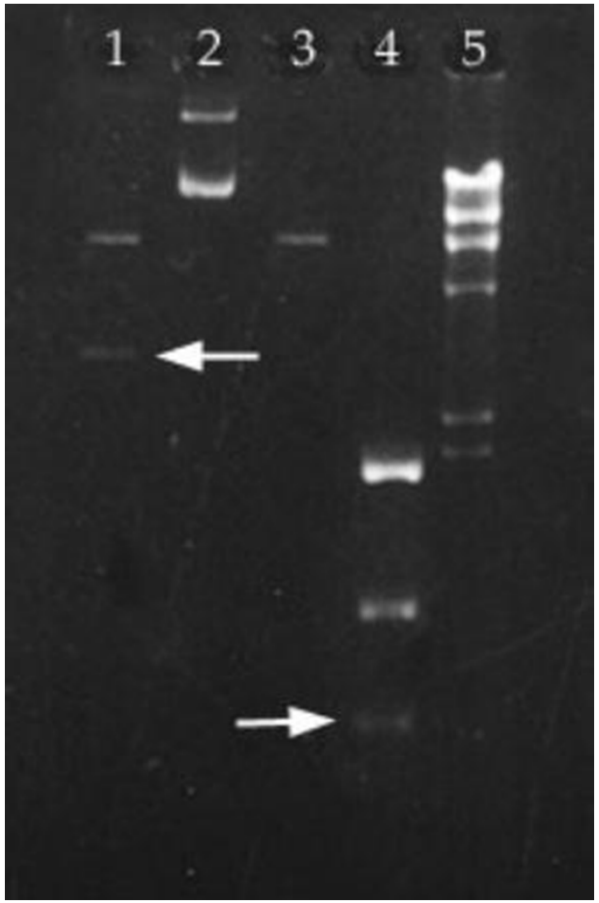

Note the 5 lanes in this autoradiograph of a gel. Lanes 1-3 are the sample DNA. Lanes 4 and 5 represent the molecular weight controls or DNA ladder. Comparing the location of the bands in the sample with the known weights of the controls gives an estimation of the molecular weight of the bands. The arrows are pointing to some faint bands of DNA which might be overlooked. Where are the smallest molecules in this gel, at the top or at the bottom?
Since the wells are at the top, the smallest molecules are at the bottom of this gel.
http://www.life.illinois.edu/molbio/geldigest/photo.html

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Gel Electrophoresis Virtual Lab

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- http://learn.genetics.utah.edu/content/labs/gel/
- Roche Diagnostics CD, - Molecular Technology, Electrophoresis

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Agarose vs. Polyacrylamide Gels

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- **Agarose** is purified from seaweed, boiled, buffered, and poured into a mold.
- Small pieces of DNA (50-500 bp) are run on more concentrated gels, 2-3%.
- Larger pieces (2000-50,000 bp) are run on lower concentrations, 0.5-1%.
- **Polyacrylamide** gels are used for very small DNA fragments and ssDNA
- Can be used to detect a variation in one base pair in a 1-kb molecule (0.1% difference)

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Running the Gel

- Bromophenol Blue is added to the samples prior to loading to provide a color indication of the performance of the gel.
- Glycerol is also added to weigh down the sample of DNA so it will sink to the bottom of the well in the gel and run correctly.
- The inclusion of a known mixture of variously sized DNA fragments is also run as a standard to compare against samples. This is called a DNA ladder.

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Visualizing the DNA

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- **Ethidium bromide** binds to the DNA and fluoresces under UV light. Can be added to the gel before or after the run.
- **CAUTION!** Ethidium bromide is a powerful mutagen (cancer causing agent) and is moderately toxic. Gloves should be worn at all times.
- **SyBr green** can also be used to visualize the DNA.
- **Silver stain** can also be used but is somewhat more complex to stain.

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Quantitation and Quality by Electrophoresis/Fluorescence

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- Intensity of fluorescence is proportional to the total mass of DNA
- Compare to DNA of known concentration to estimate concentration of the sample
- High quality nucleic acid appears as a tight band but degraded DNA shows as a long smear on the gel.
- Thus one should determine if DNA is intact by electrophoresis prior to spectrophotometry to make sure the DNA is intact. Degraded DNA would absorb as strongly at 260nm as a non-degraded sample.

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Pulsed Field Gel Electrophoresis (PFGE)

- For very large pieces of DNA (50,000 – 250,000 bp) pulses of current applied to the gel in alternating dimensions enhance migration
- These changes in direction resolve the large fragments but the process takes longer than usual gels (24 hours or more).
- Can resolve chromosome sized fragments such as are used in epidemiologic bacterial typing. Small mutational differences can detect source differences in the same organism.

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Capillary Electrophoresis

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- Uses thin glass tubes (capillaries) with an internal diameter of 25-100 microns.
- Fluorescent light is shown through a window in the tube to detect the fragments as they pass.
- A negative charge along the walls of the tube is due to the silicone molecules of the glass. When a current is introduced along the length of the capillary, an electro-osmotic flow is established.
- Fragments migrate according to size (small = faster) and charge (negative = faster).

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Capillary Electrophoresis

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- Can be run at higher charge than gels so run time is reduced.
- Used in forensics and paternity testing by analyzing short tandem repeat polymorphisms. Also used for clonality testing, microsatellite instability detection, and bone marrow engraftment analysis.
- Increased sensitivity, smaller amounts of DNA can be used, and immediate detection of bands. Controls, standards, and test samples can be run together, reducing lane to lane variations in gels.

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Blotting and Probing Techniques

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- Southern blot
- Northern blot
- Western blot
- In situ hybridization (ISH)
- Dot blots
- Microarrays

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Nucleic acid hybridization

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- dsDNA can be denatured by heat (or extremes of pH) by breaking the hydrogen bonds between the strands, creating two single stranded DNA segments.
- When cooled, the hydrogen bonds can reform and the DNA renatures.
- In the ssDNA state, DNA from another source (the probe) can hybridize to sample if it is complementary.

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DNA/RNA hybrid

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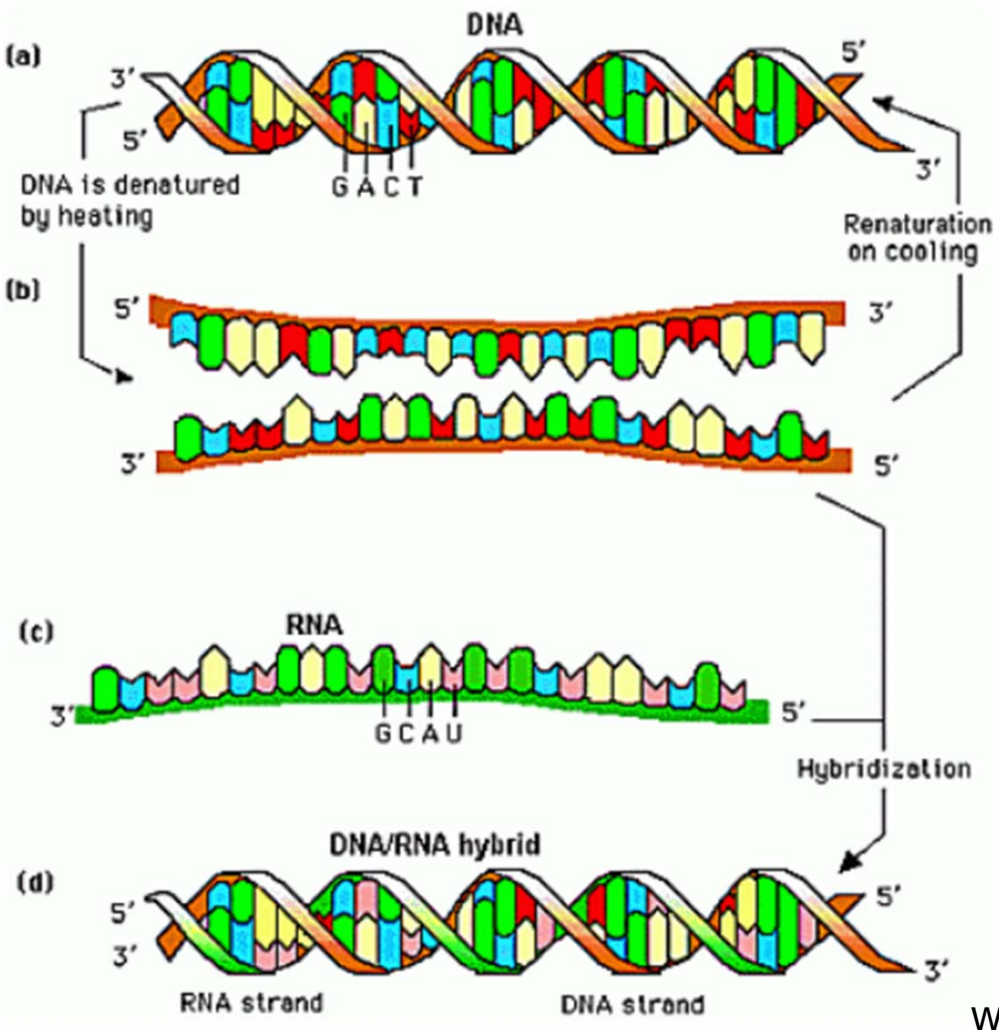

dsDNA will separate (denature) by heating. If cooled it will renature (recombine).

A complementary strand of RNA can hybridize with the ssDNA.

This creates a DNA/RNA hybrid.

www.biochem.arizona.edu/.../HYBRID.JPG

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Hybridization

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- **Annealing** (base-specific hydrogen bonding) of a probe to a target nucleic acid to form a double stranded molecule
- **Probe** – a labeled single stranded DNA (or PNA – peptide nucleic acid) molecule, about 20 – 20,000 bp in length
- **Target** – Single stranded DNA or RNA molecule

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Many applications of hybridization

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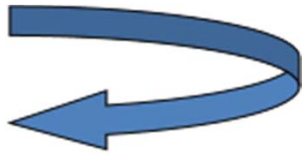
Can detect sample's sequence since probe is of a known sequence.

ATTTGGCA Probe (known)
? ? ? ? ? ? ? ? Target (unknown)



If they anneal, target must be TAAACCGT (surmised sequence of Target)

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Types of applications

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- Southern blot (DNA is the target)
- Northern blot (RNA is the target)
- In situ hybridization
- Dot blots
- Microarrays

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Principle of Hybridization

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- Add labeled probe to target DNA
- Denature to separate dsDNA of target
- Allow to reanneal (bonds reform between complementary base pairs)
- Some target DNA will reanneal to itself and some probe will reanneal to itself
- But some heteroduplexes of probe and target will form and be detected by the label

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Factors affecting annealing

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- DNA concentration
- Strand Length
- Base Composition
- Mismatch
- Stringency

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Renaturation kinetics

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1. DNA concentration – the higher the concentration of complementary DNAs, the faster the rate of annealing
2. Probe length – the longer the probe, the slower the rate
- the longer the probe, the more stably bound to target

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Renaturation kinetics

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1. Base composition – G/C base pairs have more hydrogen bonds than A/T (3 vs. 2), so more G/C pairs:

-the faster the rate

-the more stable the binding

1. Mismatch between probe and target – can be tolerated but:

- hybridization rate is inversely proportional to % mismatch (above 10%)
- mismatch decreases stability of binding

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Renaturation kinetics

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5. **Stringency** – tolerance for mismatch between probe and target in hybridization experiment: the higher the stringency, the lower the tolerance for mismatch

Three factors affecting stringency:

A. Temperature

B. Na ion concentration

C. Chemical denaturants: formamide, urea

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Stringency - temperature

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- Increasing temperature destabilizes hydrogen bonds
- **Tm** = temperature at which half the DNA is denatured (melted)=**melting temperature**
- The closer the hybridization temperature is to the Tm, the higher the stringency
- Rule of thumb – hybridize at 25 degrees C below the probe Tm

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Effects if stringency too high or too low

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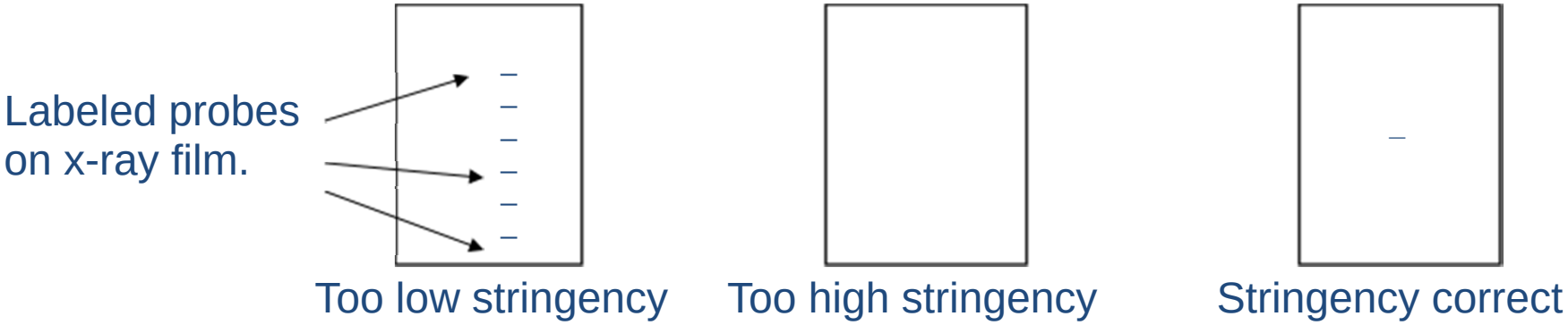
- If too low, too much mismatch – background is staining is high
- If too high, nothing binds
- If correct, only binding to complementary DNA



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Probes

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- Single stranded DNA, RNA, or PNA about 20-2000 bases in length
- Labeled in some way
  - Radioactive
  - Non-radioactive
  - Short probes can be manufactured containing labeled nucleotides (20 bp)

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Labeling

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- **Radioactive isotopes** 32P, 33P, 35S, 3H
- Can be detected using film by autoradiography (x-ray film)
- Dangerous – various emission intensities and half-lives
- **Non-radioactive labeling** – **Fluorophore**: fluorescein, rhodamine (incorporated into nucelotides) – detect by fluorescence in UV
- **Ligand**: Biotin, digoxigenin – non-fluorescent detected by avidin for biotin or anti-Dig antibody

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Southern Blot
RNA probe, DNA target

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1. Digest target DNA with restriction endonuclease (cutting it into fragments)
2. Run on agarose gel
3. Denature in alkali
4. Apply gel to a nitrocellulose or nylon membrane and transfer DNA to membrane*

*(See methods of transfer)

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*Methods of Transfer to Membrane

- **Capillary transfer** – layer of wet paper, gel, membrane, covered by dry paper – As fluid transfers up into dry paper, DNA transfers from gel to membrane
- **Electrophoresis transfer** – current is used to move the DNA from the gel onto the membrane
- **Vacuum transfer** – vacuum is used to move DNA from gel down onto membrane

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Southern Blot, cont.

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1. For probe, add label (radioactive, for example)
2. Denature by heat
3. Hybridize to immobilized target DNA on membrane
4. Wash off excess probe
5. Expose x-ray film to membrane with labeled probe
6. Develop film (radioactive labels will expose film in bands)

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Southern blot

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- Roche CD, Molecular Technology, Southern blot
- http://highered.mcgraw-hill.com/olcweb/cgi/pluginpop.cgi?it=swf::535::535::/sites/dl/free/0072437316/120078/bio_g.swf::Southern%20Blot
- To detect restriction fragment length polymorphisms (RFLPs)
- http://highered.mcgraw-hill.com/olcweb/cgi/pluginpop.cgi?it=swf::535::535::/sites/dl/free/0072437316/120078/bio20.swf::Restriction%20Fragment%20Length%20Polymorphisms

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Northern blot

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- Northern blot differs from Southern blot in that DNA is the probe and RNA the target for Northern
- Usually in Northern blot mRNA is targeted to examine gene expression levels
- http://www.gene-quantification.de/movie.html
- Important to include an internal control to correct for errors in processing. The amount of expression is then determined relative to the internal control.
- Roche Diagnostics CD, Molecular Technology, Northern blot

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Western blot

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- The target for Western blot is protein
- Serum, cell lysate, or extract is separated on SDS-polyacrylamide gels (SDS-PAGE) or isoelectric focusing gels (IEF)
- SDS-PAGE resolves by molecular weight
- IEF resolves by charge
- Molecular weight standards are run to determine the sizes
- Proteins can then be blotted to a membrane for analysis
- Used for HIV and Hepatitis C

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Western blot

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- Roche Diagnostics CD, Molecular Technology, Western blot, Troubleshooting

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