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Pearls of Laboratory Medicine
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TITLE: Nucleic Acid Preparation

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Slide 1:
Welcome to this Pearl of Laboratory Medicine on "**Nucleic Acid Preparation**" authored by Stephanie Thatcher and Linnea Baudhuin. The purpose of this talk is to present basic techniques used to prepare DNA and RNA for molecular analyses. With many procedures and products available today, a good understanding of the basic principles of nucleic acid preparation can help guide selection of the best procedure for your application.

Slide 2:
Four basic steps define most procedures for nucleic acid preparation: First, release of nucleic acids from the cell or organism via lysis is performed. Second, the nucleic acids are usually separated from other components of the cells, organisms, or other sample background material such as proteins or debris. Third, the nucleic acid can be purified further by washing away all potentially inhibitory compounds. And fourth, the nucleic acids can be concentrated by precipitation or binding and releasing in a smaller volume for analysis. Using the right combination of steps leads to a final solution of nucleic acids that is the appropriate purity, concentration, and volume for a particular application.

Slide 3:
The main factors to consider when selecting a procedure are listed here. They include input considerations, such as: what is the sample type, what volume is available, and what are the target cell types? They also include output considerations such as: What sensitivity is required (meaning what input volume and concentration should be used), is DNA or RNA being isolated, how pure does the nucleic acid need to be, what is the batch size, procedure complexity, and time to result requirements? As you can see, some of these factors are dependent on each other.

Slide 4:
Sample factors to consider include the sample background (or sample type), volume, and how the sample type will fit into the procedure. The sample type can impact how effective lysis is. For example, a viscous sample may need pre-treatment for lysis to be effective. More difficult samples like sputum or stool may require more extensive purification steps to remove inhibitors to downstream analysis, like proteins and debris. The sample input volume can impact sensitivity. A larger input volume that allows for concentration of the nucleic acids into a smaller output volume can lead to better sensitivity. Sample

flexibility is mentioned here because it is often good to use the same or similar procedures for a wide variety of samples to minimize on complexity in the lab. This can usually be achieved with some pre-processing modifications.

Slide 5:
Some solid samples may require pre-processing. As mentioned in the previous slide, more difficult samples like stool may require more extensive purification steps to remove inhibitors to downstream analysis, like removal of proteins and debris. These types of samples can literally clog or interfere with DNA or RNA procedures if not properly handled. Additional pre-processing steps such as filtration, digestion, dilution, freeze-thaw cycles, or physical manipulations can be useful steps. Formalin fixed paraffin embedded tissue is a common solid sample type used for nucleic acid analysis. Pre-processing of the tissue with xylene and ethanol washing is often used to remove paraffin prior to nucleic acid extraction.

Slide 6:
Nucleic acids are released through one of several lysis methods that are differentially effective depending on the cell/organism
Chemical lysis (using salts, including chaotropic salts, or detergents) is very common for nucleic acid extraction. It is very effective to use in easy to lyse cells. In the picture shown here, you can see that detergent, with its hydrophilic head and hydrophobic tail properties, can penetrate the cell membrane phospholipid bilayer, resulting in mixed micelles of fragmented cell membrane and detergent.
Enzymatic methods, using lysozyme or proteases, are also an effective way to lyse cells. Some sample types may require mechanical or physical methods in order to be lysed.

Slide 7:
Proteins in samples can interfere with nucleic acid isolation, or with downstream analyses. Proteases, chaotropic salts, or detergents are common additives in a nucleic acid preparation procedure that will effectively denature or degrade proteins in the sample. Further isolation of nucleic acids through washing or concentration steps will further remove proteins and other potential inhibitors. While most procedures will easily remove most inhibitors, specific extraction procedures may be required to remove special inhibitors if an analysis is sensitive. To test for the presence of inhibitors, dilute a sample and re-analyze. If results improve with dilution, it is likely that inhibitors are present.

Slide 8:
There are many different methods for nucleic acid extraction, and here we have listed some of the most critical factors in order to help choose a method. The most important factor is what downstream molecular tests will be run with the nucleic acid, since some tests require higher purity nucleic acid. The desired sensitivity is also important, so that the volume and method used give results in the desired range. To select a final procedure, also factor in the batch size, turn-around time, cost, complexity of procedures, and whether hazardous reagents will be used.

Slide 9:
There are many different procedures available for nucleic acid isolation, including liquid phase and solid phase procedures. In the next few slides, we will explore some of these methods.

Slide 10:
Liquid phase inorganic extraction is advantageous because it is a fast, easy method, utilizes non-hazardous reagents, and produces high-quality DNA. The first step of liquid phase inorganic extraction is lysis of cell membranes with SDS. Next, proteins are precipitated with a salt solution. Finally, DNA is precipitated with alcohol, and then rehydrated.

Slide 11:
Liquid phase organic extraction is a common method used for nucleic acid isolation that leads to a very pure product. Nucleic acids can be isolated from other molecules because of their differential solubility in immiscible liquids. Phenol mixed with choloroform and isoamyl alcohol is the most common reagent for liquid phase organic extraction. Phenol denatures proteins, whereas the chloroform and isoamyl alcohol help separate the phases and prevent foaming. Two layers are formed, with the top aqueous layer containing the DNA, and the bottom organic layer containing proteins and lipids. The top layer is transferred to another tube and the DNA can be precipitated with alcohol, followed by rehydration.

The disadvantages to liquid phase organic extraction are that it is a manual process, must be performed in a fume hood, creates hazardous waste, and is not amenable to high-throughput needs

Slide 12:
Most extraction procedures used today are solid phase methods, in which the nucleic selectively binds to a surface or a bead under one condition, and comes off (after being washed) in another condition. Solid phase extraction methods are advantageous because they utilize minimal hazardous reagents, require fewer and easier manipulations, are automatable, and can be utilized for high throughput needs. The three major types of solid phase extraction will be discussed in the next slides.

Slide 13:

In gel filtration, nucleic acid molecules can be separated by size through a gel matrix. Gel filtration usually takes place in a spin filter or column. Sephadex, made up of cross-linked porous agarose particles, is most commonly used as a gel matrix due to its high chemical and physical stability. The gel matrix has a specific pore size which will hold smaller molecules, while larger ones can pass through. Nucleic acid elution from the gel matrix is achieved by washing with the same buffer used during the filtration process. One of the drawbacks to gel filtration is the lack of specificity, in that molecules of the same size as the nucleic acids will also be captured in the gel matrix.

Slide 14:
In ion exchange chromatography, nucleic acid molecules can bind to surfaces with charged groups. In the top panel of the figure, you can see that a common anion exchange resin is DEAE-C (diethylaminoethyl cellulose), which the negatively charged nucleic acid will bind to. Addition of high-salt buffer initially allows for unbound substances to be washed away. The lower panel of the figure shows that increasing the concentration of the salt in the solution eventually enables the nucleic acid to be released as salt ions exchange with the nucleic acid for binding to the resin.

Slide 15:
Affinity chromatography has 4 basic steps: lysis, binding, washing, and elution. Similar chemicals, e.g. chaotropic salts, can be used for lysis and surface-binding. Any binding surface that has a negative charge and nucleic acid binding properties can be utilized. The most common binding surface utilized with this method is silica. Hydrogen bonding allows for the linear nucleic acids to bind lengthwise to the silica surface. Binding of nucleic acids to silica is optimized by the presence of high concentrations of chaotropic salts or alcohol plus a low pH. Washing of the silica surface is usually achieved with ethanol.

Elution occurs when the nucleic acid binding to silica is reversed with water. Removal of salt and alcohol and hydration of the silica surface are all important conditions which allow for nucleic acid release from the silica surface.

Slide 16:
After you have extracted your nucleic acids, it is important to determine the quantity and quality of nucleic acid. A common method for doing this is by the use of spectrophotometry. Nucleic acids absorb light at a wavelength of 260 nm, whereas proteins absorb light at 280 nm. In order to calculate nucleic acid quantity, the Beer-Lambert law is used with a factor of 50 for DNA and a factor of 40 for RNA, and takes into consideration the measurement at A260 as well as the sample dilution factor. The purity of DNA should have an A260/280 ratio of 1.6-2.0. If this ratio is <1.6, then that indicates protein contamination; whereas a ratio of >2.0 indicates RNA contamination. RNA is a little more forgiving and can have a higher A260/280 ratio (in the range of 2.0-2.4), with a ratio <1.7 indicating protein contamination.

Slide 17:
A specific nucleic acid procedure may not work well for everything. In some cases, low or no nucleic acid will be produced. In these cases, nucleic acid isolation can be repeated, or consider whether ample time was allowed for sample re-suspension, or consider using nucleic acid concentration methods. It is common for a procedure to work very well for some targets, but not as well for others if the lysis is inadequate for some organisms or cells. The nucleic acid is just not released and cannot be isolated, so a better lysis technique should be used. As discussed previously, inhibitors can be problematic, so inhibition controls can be used to ensure that the procedure is effective. Also, it should be noted that large batch sizes can be advantageous in the right setting, but do lead to longer pre-processing and wait times.

Slide 18:
Once procedures are determined, here are a few suggestions for optimization. Determine the best input volume, as big as possible if lower sensitivity is desired. Set the output (or elution) volume as low as possible to increase the concentration of nucleic acids while leaving enough sample for all the work required and possible repeat testing. Make sure the appropriate sample portion is being prepared. Get all information and pre-processing requirements for your particular sample. Test for inhibition using a positive control, and text for extraction occasionally with an extraction control. Test your workflow for cross-contamination by mixing positive and negative samples to ensure there is no sample contamination. Automated systems and manual procedures are both prone to cross contamination if workflow is not carefully set up.

Slide 19:

So many procedures are available today to prepare DNA and RNA from a wide variety of sample types. The procedures can be done fast, or in large batches. The resulting nucleic acid can be very pure, or just pure enough. With so many molecular methods out there today, nucleic acids can be easily isolated from a variety of sources for downstream detection. Thank you for your attention to this talk on nucleic acid preparation.

Slide 20: References

Slide 21: Disclosures

Slide 22: Thank You from www.TraineeCouncil.org
Thank you for joining me on this Pearl of Laboratory Medicine on "**Nucleic Acid Preparation**."