Clinical Chemistry 61:1
89–99 (2015)

DNA/RNA Preparation for Molecular Detection

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BACKGROUND: Effective upstream preparation of nucleic acid (NA) is important for molecular techniques that detect unique DNA or RNA sequences. The isolated NA should be extracted efficiently and purified away from inhibitors of a downstream molecular assay.

CONTENT: Many NA sample preparation techniques and commercial kits are available. Techniques for cell lysis and isolation or purification of NA were discovered in early NA characterization studies, evolved in the 20th century with molecular techniques, and still serve as the foundation for current methods. Advances in solid phase extraction methods with nonhazardous chemicals and automated systems have changed the way NA is prepared. Factors to consider when selecting NA preparation methods for molecular detection include lysis (from sources as diverse as human cells, viruses, bacterial spores, or protozoan oocysts), DNA vs RNA, sample background, appropriate preparation chemicals, and required detection limits. Methods are also selected on the basis of requirements for a particular application, such as sample volume or removal of inhibitors. Sometimes tradeoffs are made.

SUMMARY: Good automated and manual methods are available to effectively prepare NA for molecular detection in under an hour. Numerous systems are available for various applications, including techniques that are flexible for multiple sample types, are capable of processing large batches, can be performed in <10 min, or that can yield high-purity NA. When methods are selected using the most applicable combination of lysis isolation efficiency and concentration, NA preparation can be very effective, even for molecular detection of multiple targets from the same sample.

Effective preparation of nucleic acid (NA)[2] is important for molecular techniques that detect unique DNA or RNA sequences. Many NA sample preparation techniques and commercial kits are now available. Techniques for cell lysis and isolation or purification of NA were discovered in early NA characterization studies, and still serve as the foundation for current methods.

A History of Nucleic Acid Preparation Tools

The function of NAs was unknown when initially isolated from eukaryotic nuclei in the 1860s by chemist Friederich Miescher, and remained controversial for over 80 years *(1)*. Early isolation of NA, originally called nuclein, was performed using tedious methods to isolate pure material. Chemists worked for decades with NA prepared by these chemical techniques to determine the NA molecular structure and identify the nuclear material that carried hereditary information. Most early methods included an alkaline lysis followed by acid and alcohol precipitation to extract pure NA from cells *(2)*. The 4 bases of DNA were initially identified in 1891 by Kossel *(3)*. After the 1950s, it was accepted that DNA carried genetic material, and it became important to isolate NA from larger numbers of samples to investigate unique sequences, especially after sequencing became more common. In the process of discovering the role of NA, advances were made in plasmid DNA isolation, but techniques specific to plasmids will not be discussed here.

Preparation of NA is no longer just the realm of research, it is an important part of many standard diagnostic procedures that use DNA or RNA to identify genetic variants or the presence of particular sequences in human genes or a variety of pathogens *(4)*. NA is routinely prepared from large numbers of samples for whole genome sequencing, mutation identification in small or large fragments by PCR or single-nucleotide polymorphism genotyping on arrays, and pathogen detection. Current NA preparation procedures have become faster and less hazardous and have been developed primarily by companies, sometimes linking them to downstream testing.

Future NA preparation methods should be faster, more flexible, automated, and smaller. Other reviews have pointed out the need for faster methods, especially for rapid diagnostics to be used outside of the laboratory *(5–7)*. Flexibility is defined by the ability to isolate DNA or RNA from multiple cell or organism types in many potential sample backgrounds. Some pathogen genomes are RNA and some targets for disease diagnosis are RNA

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Received June 2, 2014; accepted October 14, 2014.
Previously published online at DOI: 10.1373/clinchem.2014.221374

[2] Nonstandard abbreviations: NA, nucleic acid; FTA, fast technology for analysis of nucleic acids; SCODA, synchronous coefficient of drag alteration.

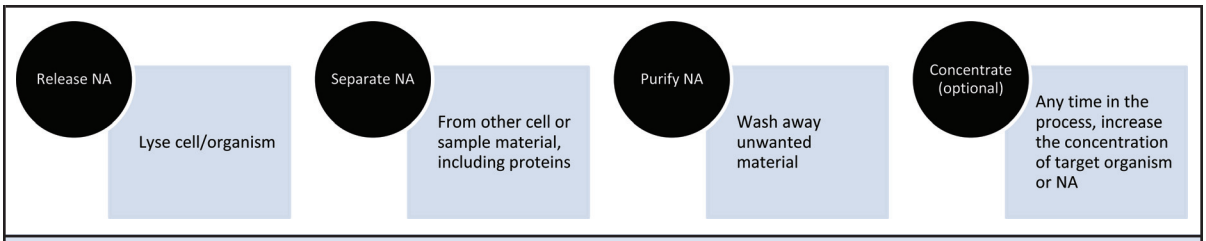

Fig. 1. Basic NA preparation steps utilized in most procedures (not all steps are used for a given method).

transcripts. Thus, it can be important to separate both DNA and RNA from a sample, or to consider which is important for a particular application. Consider the source of the desired NA, i.e., human cell, virus, gram-positive bacteria, bacterial spore, fungal cell, or protozoan oocyst, and the sample background. Each sample presents unique challenges, possibly interfering with NA preparation or downstream analyses *(8)*.

NA preparation can include isolation, extraction, purification, or separation. These terms are used interchangeably in the literature to describe preparations of NA. For the purposes of this review, the preferred term is NA preparation because the type and combination of methods used may vary. Three steps are used in most procedures (Fig. 1): (*a*) extracting or releasing the NA from the sample and the cell or organism by lysis; (*b*) separating or isolating NA from other cellular or sample material; and (*c*) purification by removal of inhibitory substances from the NA. A fourth optional step, concentrating, is important for detection of low-concentration analytes. There are options that can be used for all of these steps, and some steps can be skipped depending on the analysis used. Many basic methods of NA preparations are described in this review, ways to select the best combination of methods for a specific application are also discussed.

Release of NA from cells, nuclei, or organisms is important for preparation. For many cells, cell lysis is simple and can be performed with a salt solution *(9)*. Early methods used alkaline lysis of cells to separate NA from proteins, using chemicals such as sodium hydroxide. Detergents can also help with lysis by breaking down membranes. It is interesting that several tools used for lysis were observed long ago to have bactericidal effects, notably sodium hydroxide, detergents, heat, and lytic enzymes *(10)*. Lytic enzymes were discovered that are bacteriolytic or yeast lysing. Lysozymes digest the extensive peptidoglycan layer of some gram-positive bacteria *(11)*. They can also help lyse gram-negative bacteria, but detergents are used first to remove the outer membrane.

Lysis is a bigger challenge when working with complex sample types or a wider variety of pathogens such as bacterial spores or oocysts. Although human cells and viral capsids are easily lysed by salts or detergents, these organisms require more aggressive chemical, enzymatic, or physical methods. Gram-positive bacteria have a much thicker peptidoglycan layer that is difficult to digest and requires more extensive lysis. Bacterial spores *(12)*, fungi, yeasts, and oocysts *(13)* have complex coats or walls containing proteins and other complex molecules cross-linked to make them resistant to many environmental factors, including chemicals or organism defenses. Given that these organisms have very different molecules that make up their structure, it is difficult to design an enzymatic or chemical approach that works for all. Mechanical lysis, using external mechanical forces, is a method for all cell types discussed below. Other physical manipulations such as sonication, or temperature changes like boiling *(14)* or freeze–thaw cycles *(15)*, can lyse cells.

Proteins in samples and cells can be detrimental to either the preparation process or downstream analyses. Enzymes such as nucleases can break down the target NA during the preparation method if not removed, proteases can interfere with downstream enzymatic procedures, and large amounts of proteins can lead to nonspecific binding that interferes with specific binding of NAs in some systems. Some sample backgrounds also contain large amounts of RNases, and RNA is particularly unstable in the presence of RNases. Therefore, it is often important to denature or remove proteins from a sample. Usually this needs to happen immediately, in the lysis reaction, because NA degradation can be very rapid. Chemical or enzymatic techniques can be used to eliminate proteins by degradation or precipitation. Chaotropic acids such as guanidine hydrochloride or guanidinium thiocyanate were discovered to protect NA from nucleases because of their potent protein denaturing properties *(16)*. These chemicals are now used in many NA preparation methods, because they also help to lyse bacterial and yeast cells in blood *(17)*.

Detergents are added in many NA preparation methods to dissociate or remove proteins from NA preparations. SDS was an early detergent used in the preparation of NA *(18)* to help separate NA from nuclear proteins and all other sources of protein, including membrane proteins. Its use evolved from the observation that SDS and other surfactants disintegrated bacterial and vi-

ral structures by solubilizing the proteins. Another common detergent used is Triton X-100 *(19)*.

Unwanted proteins and enzymes can also be digested by the addition of proteases in the method. Proteases are naturally occurring enzymes that are found in plants, animals, and microorganisms and serve many purposes, including protection from infection *(20)*. Some proteases are used in NA preparations to reduce protein background and aid in lysis by digesting membrane or capsid proteins. Chemicals and detergents can only denature proteins, whereas proteases will actually break them into smaller molecules by cleaving peptide bonds. The primary protease used for NA preparations is the serine protease, proteinase K, originally isolated from *Engyodontium album (21)*. Its broad lysis specificity and protein-degrading properties are very useful for NA preparation. Other proteases, such as the temperature-stable proteinase EA1, may also be useful in NA preparations *(22)*. Most proteases are too specific in their cleavage sites and not useful for NA preparations or are too difficult to produce in production quantities. Proteases may require an incubation step, sometimes at increased temperatures (37 °C for example), and they need to be removed or inactivated or they may interfere with analysis.

Once cell lysis is complete, NA can be isolated from other cellular or sample materials by separation methods. Proteins, polysaccharides, metals, salts, organic compounds, and dyes are examples of molecules that may need to be removed. Isolation is not required if molecules are tolerated, or a sample is relatively clean. Several techniques have been used to isolate NA from other components of the cell or sample background. The following paragraphs discuss 2 types of methods: (*a*) liquid–liquid extraction (liquid phase separation and precipitation) and (*b*) liquid–solid extraction (by solid size exclusion or affinity separation). The process of isolation can also concentrate the NA, increasing detection capabilities.

Liquid phase extraction is a common method used for NA isolation that leads to a very pure product. NA can be isolated from other molecules by differential solubility in immiscible liquids. The primary solvent used is phenol *(23)*, usually mixed with chloroform and isoamyl alcohol *(24)*. Phenol denatures proteins, which stay in the organic phase, whereas the NA is in the aqueous phase. The addition of chloroform and isoamyl alcohol helps to separate the phases and prevent foaming. NA in the aqueous phase is precipitated by ethanol to remove residual phenol for a clean, concentrated product. Initially designed to purify relatively unstable RNA vulnerable to degradation by RNases, it is very effective, but also manually tedious, must be performed in a fume hood, and creates hazardous waste. Although not well suited for high-throughput needs, microfluidics researchers still consider its use because liquids can be easy to manipulate *(25)*.

Precipitation of NA is a liquid phase method used to achieve a very clean product and concentrate NA. NA precipitates in the presence of alcohols such as ethanol or isopropanol and a high concentration of salt (0.1–0.5 mol/L), such as ammonium acetate, sodium acetate, or sodium chloride *(26)*. Other liquids that can precipitate NA are acetone and lithium chloride. Centrifugation concentrates an NA pellet from the rest of the liquid and manual manipulation must be used to dry and resuspend the pellet. The resulting NA is very clean, although some molecules can coprecipitate with the NA. Because manipulation of the pellet is required, this method is tedious.

Solid phase extraction methods have become the most common method of NA isolation for several reasons. They include minimal hazardous chemicals, fewer and easier manual manipulations, automation capability, and increased throughput. Solid phase methods used for NA separation include 3 principle techniques: size exclusion by gel filtration, ion exchange chromatography by charge-based reversible adsorption, and affinity chromatography by reversible surface adsorption. Any of these methods can be incorporated into a spin filter or column, or the latter 2 onto other surfaces such as beads.

In gel filtration, NA molecules can be separated from smaller molecules by size through a gel matrix. With the use of this method, the gel matrix in a spin filter or column, with a specific pore size, allows larger molecules to pass through while smaller molecules are held in the pores. This is useful for separating NA from smaller molecules, but molecules similar in size will separate with the NA. Sephadex *(27)* or a derivative is the most common matrix used.

In ion exchange chromatography, NA molecules can bind selectively to surfaces with charged groups surrounded by free counterions. Charged NAs exchange places with the ions, thus binding to the surface by charge. Unbound substances are washed away. NA is released by displacing it with a flood of free ions that replace the NA molecules *(28)*. For example, DEAE-C (diethylaminoethyl cellulose) is a common anion exchange resin that negatively charged NA will bind to. NA is released when other ions in a high-salt buffer are present to exchange with the NA. Ion exchange techniques can also be used in reverse to specifically bind and separate unwanted molecules. For example, Chelex resin is used to separate metallic compounds and inhibitors of PCR away from NA *(29)*.

Using affinity chromatography, reversible surface adsorption of NA to surfaces like silica is the separation method of choice for many NA preparation procedures. This technique is very common in automated methods. All NA will bind to silica surfaces under specific binding conditions, especially in the presence of chaotropic salts *(30)*. NA binding to silica was discovered rather than

designed. Binding occurs when linear NA adsorbs lengthwise to silica surfaces due to complex hydrogen bond formation between the silica and NA surfaces in the presence of chaotropic salts or alcohols at high concentration and low pH (below pH 7) *(31)*. Because both silica and NA surfaces are negatively charged, the binding is due to adsorption in high ionic strength conditions and hydrogen bonding that occurs as water is removed from the surfaces. The NA is released when the salt or alcohol is removed and the surfaces are hydrated. Any surface with similar NA binding properties can be used in this way, such as diatomaceous earth. Affinity preparation is often described by 4 basic steps: lysis, binding, washing, and eluting. Serendipitously, similar chemicals can be used for lysis and surface-binding. Chaotropic salts can be used for cell lysis and binding to a silica surface *(32)*. In the 1990s, NA preparation became simplified using this method. Washing of the silica surface is often accomplished with alcohol. Elution occurs when binding to silica is reversed with water. Elution with small volumes can help increase the concentration of target. No specific chemical is required for elution, like it is with ion exchange.

Solid affinity isolation is flexible in that the binding surface can be anywhere. A binding filter or column is common. A liquid sample passes through by centrifugation (spin filter), pressure (syringe filter), or vacuum. Kits with silica spin filters are fast to perform and do not require hazardous chemicals. Qiagen kits remain a gold standard for NA preparation and have been optimized to work with a wide variety of sample types. A drawback is that filters can clog with thick sample types and steps are necessary to load the binding, wash, and elution solutions. Alternatively, the binding surface can also be on beads or particles that mix freely with a sample to collect free NA. Glass beads or particles are the simplest silica-based surfaces. Because the beads move through the sample, clogging is not a concern. Beads or particles can then be collected by filtration of particles, centrifugation, or a magnet. Surface binding capacity is determined by the area available for binding.

Surface binding methods have been improved extensively for automation or simplicity. Paramagnetic beads coated with NA binding surfaces such as silica are used widely on automated platforms *(33, 34)*. Paramagnetic beads respond to a magnetic force, which is used to move the beads through solutions, but do not generate a magnetic field themselves. Many silica paramagnetic systems are commercially available and are more easily automated, and some are amenable to the use of aqueous chemistry *(35)*.

Paper surface binding methods, in which NA bind to cellulose *(36)*, are a convenient and fast approach to NA preparation. Chemically treated paper contains lysis and binding reagents that combine with the sample when added. Lysis and binding occur in the paper, followed by washing and elution off the paper, usually in a small volume *(37)*.

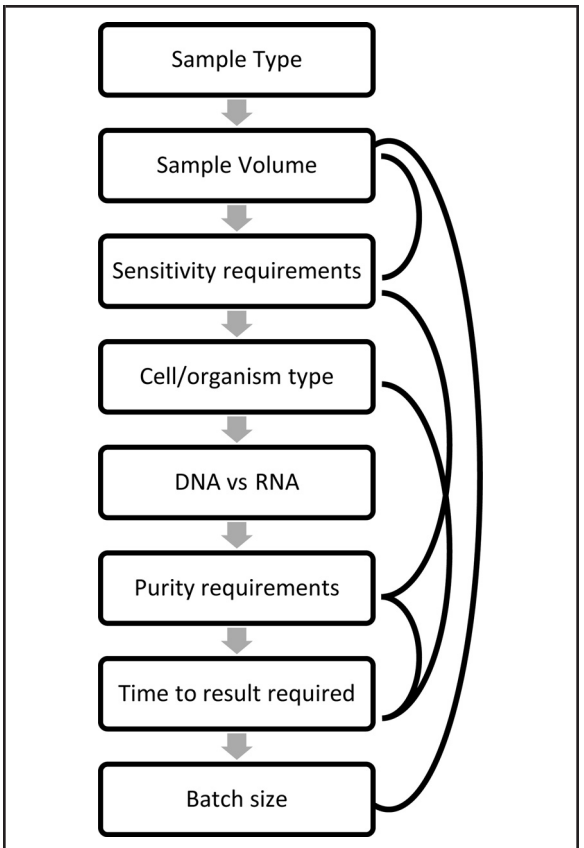

Fig. 2. Decisions to make when selecting an NA preparation method.

Connecting lines refer to relationships between decisions, for example, when sensitivity requirements are determined the sample volume may need to be reconsidered.

How to Select an NA Preparation Method

A real challenge for NA preparation in molecular diagnostics or research is deciding which methods or products to use. No single method is optimal for every application, but methods can be binned by applicability. Many factors should be considered by assessing the goals (Fig. 2). These factors include how clean and concentrated the NA needs to be, the source from which the NA is isolated (sample type and organism), and the downstream detection method and its analytical sensitivity, batch size, and time. These factors are also listed in Table 1, and several are discussed in the following section. It is also important to consider what the preparation itself is adding. Chemicals or enzymes used in extraction, such as chaotropic or

Table 1. Factors to consider when selecting NA preparation method(s).

Important factor	Potential tradeoffs	Considerations
Sample flexibility	Yield will vary by sample	Select for most difficult sample
	Longer procedure	Input for liquid and solid samples
Small sample volume	Limited sensitivity	Fast methods
	Limited concentration options	
High sensitivity	Larger sample volume	Concentration methods
	Limited sample flexibility	Good yield
Cell/organism flexibility	Specific equipment for lysis	RNA and DNA
		Broad lysis, i.e., mechanical
Long-term stability of NA	Extensive procedure, more time	High-purity methods
High purity	Extensive procedure, more time	Good separation method, i.e. precipitation
Fast	Limited sensitivity	Minimal chemicals or enzymes
	Not as pure	Automated systems
Large batch size	More time to collect samples, setup	Automated systems
	Larger equipment	Methods with minimal user input
Miniaturization	Limited sensitivity	Limiting sample types
	Difficulty introducing solid samples	

other salts, alcohols, or proteases, should be removed or inactivated before downstream analysis.

The ultimate downstream analysis to be performed dictates the extent of NA purity required. Common molecular methods such as PCR tests or loop-mediated isothermal amplification require NAs of varying purity *(38)* or concentration. Molecules never removed, or copurified with NA, can inhibit enzymatic and/or chemical reactions like PCR *(39)*, or interfere with visual real-time detection by blocking light or changing background fluorescence. Molecules that may need to be removed are nucleases/proteins, polysaccharides, salts, solvents such as alcohols introduced, pigments such as heme, or humic acids. Research into molecules that inhibit a particular analysis is difficult, especially because samples are so variable. What matters is effective detection of NA in the desired system. If long-term storage is desired, a more extensive purification procedure is important.

A separate approach is to make the analysis method less sensitive to interference so that the preparation procedure is less important. Some approaches for PCR include using forms of *Taq* DNA polymerase that are more resistant to inhibitors from complex sample backgrounds *(40)* or changing reaction parameters.

LYSIS

Which lysis method to use is a critical decision that can limit, expand, or simplify NA preparation. Sometimes lysis is all that is required, and once the NA is released, it can be analyzed *(41)*. Most human cells and other pathogens require only simple chemical lysis (alkaline lysis, salt, detergents, or chaotropic agents), especially viruses and gram-negative bacteria. Enzymatic lysis, with enzymes like proteinase K, lysozyme, or mutanolysin *(42)* can increase lysis efficiency by degrading membrane or capsid proteins, or attacking the peptidoglycan layer. Heat *(43)* or freeze–thaw cycles can also increase lysis efficiency. Gram-positive bacteria (with a thicker peptidoglycan layer or proteinaceous spore coat), tissues, fungal cells, and protozoan oocysts are particularly resistant to some lysis techniques because of their complex cell walls. More efficient techniques should be use if sensitivity is a concern *(44)*.

Lysis by physical means is the best option for many hard-to-open pathogen cells because it is a nonspecific method that will work in many sample environments. Mechanical lysis is being widely adopted, although it requires special instrumentation, because of its usefulness in multiplexed applications, and because it is fast *(45)*. Mechanical lysis systems require a large input of energy and are often loud, but they do not require the addition of chemicals or enzymes that need to be removed later. NA shearing can be a concern, so care should be taken to minimize lysis time, but most shearing does not impact detection because the NA fragments are larger than what is required for analysis *(46)*. Bead milling occurs by rapid motion of beads or sonication with sound waves *(47, 48)*. Small beads are moved rapidly within a sample and their collision with cells physically breaks open cells. The Roche and Qiagen automated systems have added optional external mechanical lysis systems *(49, 50)*. Pressure is another mechanism to lyse cells *(51)*. If an analysis

system detects NA from many types of cells or organisms, a broad approach like mechanical lysis is desired *(52)*.

DNA VS RNA

Equally important is the desired NA target material, RNA and/or DNA. In multiplex detection systems, all NA from an unknown sample may be desired because target molecules can be DNA or RNA. Because RNA is more susceptible to degradation, it is important to consider if RNA should be copurified with DNA. Many methods will prepare both DNA and RNA from the same sample, although special effort must go into protecting RNA from degradation, especially from RNases. Chaotropic agents are effective for removing nucleases, including RNases.

Some methods are designed for only DNA or RNA *(53)* and contain steps to remove the other NA, possibly by adding a nuclease specific for the undesired NA. Specific NA can also be separated by size exclusion, or with liquid phase separation techniques such as TRIzol.

SAMPLE TYPES

Sample types can contain different inhibitors of downstream analysis *(54)* and extraction efficiency may vary. Preparation can be designed for a specific sample background, or be flexible to handle many sample backgrounds *(55)*. When procedures are specific, they may be laborious for optimal recovery from a complex sample matrix. The ability of a system to purify RNA from stool is a good measure of its sample flexibility, and several systems are able to purify RNA from stool *(56)*. A few method comparison reports are referenced in this review. In one stool comparison, Qiagen, MagNA Pure, KingFisher, and easyMAG systems were used to isolate rotavirus RNA from stool. The PCR crossing threshold results were similar to each other. Recovery differences of NA often vary by small amounts if the methods are comparable. Many comparison reports show negligible difference between similar systems. Note that coisolation of inhibitors is a concern with complex sample matrices like stool, and a process control is good to determine if the preparation was effective *(57)*.

Clinical samples range from relatively clean fluids like urine to thick fluids like blood to solid material like stool and sputum. The latter are some of the most challenging samples and contain solid materials that may sequester NA-containing cells and make it difficult to mix with liquid NA preparation solutions. Notably, solid samples can clog filters, even with centrifugation, or small channels for liquid to flow through. Sample flexibility can be helped by avoiding filters or small channels. Some procedures use blood plasma or serum to avoid complications of whole blood, but pathogen may be lost when blood is separated. Archived tissue is a complex solid sample that needs careful consideration, especially because sample material is limited *(58)*.

Analytical Sensitivity

A good NA preparation should match the needs of the analysis. Because target NA may be present in low concentrations of pathogens or target DNA *(59)*, analytical sensitivity requirements must be considered, as well as the limit of detection of the downstream analysis. Factors that impact NA concentration are: (*a*) starting sample volume, (*b*) final volume needed for testing, (*c*) efficiency of NA recovery (yield), and (*d*) upstream concentration steps that increase the starting concentration of the target material. Large sample volumes are often not available. Yield, or extraction efficiency, is dependent on the sample background, the cell or organism, the concentration of analyte, and the method of measurement. Most silica binding isolation systems have very similar recovery when compared side by side, even when automated *(60)*.

Various techniques can be used for increasing the target NA concentration in a preparation. Some pathogens can be grown to higher quantities for detection by culture, but this requires significant time. Selective recovery of cells by centrifugation or filtration is sometimes possible, but in complex samples too much unwanted material can clog a filter or overwhelm the system. Centrifugation methods are often used for blood. For example, red blood cells can be specifically lysed to allow recovery of other cells, red blood cells containing malaria can be selectively recovered by density gradient centrifugation *(61)*, or white blood cells can be concentrated in a buffy coat layer. Selection by binding is another concentration method. Some paramagnetic beads used for NA isolation also claim to bind bacterial cells nonspecifically *(62)*. Beads with specific antibodies are used in immunomagnetic separation techniques, designed to bind specific bacteria or cells as a concentration method *(63)*. New methods are being developed to selectively concentrate circulating cancer cells *(64)*. It may be difficult to bind specific cells in dirty sample backgrounds because of nonspecific binding by other materials in the mix. The end NA product can also be concentrated after extraction if a large volume of material is produced, for example by ethanol precipitation.

Selecting from Current Methods

There are many good automated NA preparation systems available. They are well designed to maximize sample flexibility and minimize time. The systems listed in Table 2 have combined lysis and isolation techniques for DNA and RNA that are relatively free from impurities and can handle many sample types. Several reports have been referenced that compare these systems. The systems re-

Table 2. Available automated methods for processing several types of samples.

Automated method	Sample flexibility	Lysis	Optional external mechanical lysis	NA	Isolation method	Sample size	Batch size	Time (min)	References
Small lab automation, good options for sample flexibility, ranked according to sample versatility, and performance									
NucliSens easyMAG (bioMérieux)	Many, including blood, swabs, and sputum	Chemical	Yes	DNA RNA	Silica paramagnetic bead binding	<1 mL (≅10-fold)	24	40–60	Jeddi *(44)*; Loens et al. *(55)*; Esona et al. *(56)*; Shulman et al. *(57)*; Huijsmans et al. *(58)*; Schuurman et al. *(60)*; Dundas et al. *(65)*; Verheyen et al. *(66)*
MagNA Pure Systems (Roche)	Many, including blood and tissue	Chemical enzymatic	MagNA Lyser	DNA RNA	Silica paramagnetic bead binding	≤1 mL (≅10-fold)	8, 32 or 96	20–180	de Boer et al. *(50)*; Esona et al. *(56)*; Shulman et al. *(57)*; Schuurman et al. *(60)*; Verheyen et al. *(66)*
QIAcube (Qiagen)	Many, using several Qiagen kits. Flexible	Chemical enzymatic	Tissue Lyser	DNA RNA	Silica spin-column binding (centrifugation)	Kit dependent	12		van Tongeren et al. *(43)*; de Boer et al. *(49)*; Esona et al. *(56)*; Shulman et al. *(57)*; Huijsmans et al. *(58)*; Shipley et al. *(67)*; Chandler et al. *(68)*
EZ1 (Qiagen)	Many, including blood, swabs, tissue, and forensic samples	Chemical	Tissue Lyser	DNA RNA	Silica paramagnetic bead binding No alcohols	0.35 mL (2-3-fold)	6 or 14	Varies	Dundas et al. *(65)*
Maxwell (Promega)	Blood, cells, and tissue	Chemical enzymatic		DNA RNA	Cellulose paramagnetic bead binding Ethanol wash	≤0.3 mL (≅6-fold)	16	20–40	
Large lab automation, good option for large batch sizes									
QIAsymphony (Qiagen)	Many, including swabs and tissue	Chemical	Tissue Lyser	DNA RNA	Paramagnetic bead binding	≤1 mL (5–10-fold)	96		Halstead et al. *(48)*; Verheyen et al. *(66)*
Wizard (Promega)	Cells and Tissue	Enzymatic		DNA	Column plate Vacuum	Cells 0.5 mL	96	20	
QIAxtractor (Qiagen)	Many, including blood and tissue	Chemical	Tissue Lyser	DNA RNA	Glass fiber plate binding Vacuum	Unknown	96	96	
Open platform									
KingFisher (Thermo Sci.)	Flexible	User determined		DNA RNA	Paramagnetic bead binding	User	15, 24, or 96	User	Esona et al. *(56)*; Shulman et al. *(57)*
Resource-limited settings									
QuickGene (Kurabo)	Blood, Tissue	Enzymatic		DNA RNA	Porous membrane binding Pressure	0.2 mL	6 or 8	6–20	Shipley et al. *(67)*

ported to perform well with multiple sample types are the bioMerieux easyMAG and the Roche MagNA Pure. The system held as a gold standard by its many specific kit options is the Qiagen QIAcube, which automates most of their spin filter kits for many applications. These systems use <1 mL of sample, typically lyse with chemicals and enzymes, and isolate NA by washing away impurities. Some of these systems recognize the importance of mechanical lysis and have included the option to include it as part of preprocessing. They utilize magnetic bead surface separation with paramagnetic bead binding, or silica spin filters in the case of the QIAcube. The end sample is well suited for most molecular methods with a decent sensitivity. Automated methods are generally just as effective as manual methods *(65)*. Many valuable comparisons of methods have been published *(66, 67)* but are usually limited to only a few methods and a single sample target combination. Most comparisons demonstrate that there is not a big difference between preparation methods.

Batch size is a consideration for many laboratories. It is desirable to process many samples at a time in a large laboratory to save time. Some systems, such as the QIAsymphony, are designed to process larger batch sizes using plate systems. These systems are similar to the other automated systems but are much larger and take up more room in a laboratory. High-throughput methods often take longer in actual use because of the time required to build up enough samples for a full run (96 samples or more) and preparation time. Factors to consider are batch size, time, measures implemented to prevent sample cross-contamination, lysis technique, and sample applicability. When purchasing an automated system, consider the setup time as well as the run time. Some systems require the addition of buffers and also have extensive preprocessing steps. Others have cartridges with buffers, enzymes, and beads required for the process ready to go.

There are 3 other categories of methods worth mentioning (Table 3): fast single-step or simple methods, methods that yield very pure NA, and automated methods that are inline with detection.

Some applications require fast NA preparation, when time to result must be minimized, or simple techniques for resource-limited settings. Some of these methods are or could be incorporated with downstream analysis and are generally fast (30 min or less). The simplest methods are sample dilution and lysis only. These are very quick for clean samples but do not improve sensitivity. Simple enzymatic methods using lysozyme or proteases may be adequate for bacterial cultures. Sometimes Chelex inhibitor removal is all that is needed, performed by adding the Chelex resin to the sample and removing the supernatant. Simple manual methods include kits that utilize paramagnetic bead binding, like ChargeSwitch, MagaZorb, and QuickPick. These small kits use minimal equipment and work with many sample types, especially because they will not clog the way spin filters can. The fast technology for analysis of nucleic acids (FTA) paper procedure described above is very fast and useful when sample archiving is needed. The TruTip pipette tip method is very fast and incorporates the binding matrix in a tip used to manipulate the sample *(68)*. There is also a small automated system for resource-limited settings, the QuickGene, for research purposes.

Some NA preparation methods can lead to very clean NA. Phenol methods were previously discussed and are still useful when a very pure product is desired. TRIzol products can be used for this purpose and are especially useful for RNA. A new technology can purify large amounts of NA from almost any sample. In a spin on gel purification, a method called synchronous coefficient of drag alteration (SCODA) is used for clean concentrated NA preparation from any sample *(69)*. A rotating electric field is used to focus NA in a spot that can be removed and purified. Advantages are that a large volume of almost any sample can be added and concentrated into a small volume, and NA is easily separated from any background in its movement through the gel matrix. A disadvantage is that it takes about 4 h to run. A similar idea not currently in a product is that NA-bound beads could be moved through an immiscible liquid wash phase to remove contaminants *(70)*.

Some detection methods contain sample NA preparation inline as part of the test. The benefits are that the full sample can be analyzed and the user skips sample handling steps that can lead to sample cross-contamination *(71, 72)*. The 2 systems mentioned here are PCR systems with upstream NA preparation steps. The FilmArray system uses mechanical lysis for cell type flexibility and paramagnetic bead binding with aqueous chemistry. The NA is washed and then removed from the beads for downstream analysis within the pouch cartridge. The GeneXpert system uses sonication for cell flexibility, and uses surface binding in the cartridge in a similar way. Both of these systems were designed to utilize mechanical lysis for pathogen detection versatility because they can detect multiple targets in a single sample.

Inline NA preparation is talked about frequently as part of microfluidic systems. Many research publications on microfluidic techniques for NA preparation demonstrate novel and promising technologies that could be implemented in such devices *(73)*. Challenges to these techniques include accepting raw complex samples, analytical sensitivity limitations, and effective lysis for hard-to-lyse organisms. Some effort is going into developing these systems for larger volumes because if the input volume is too small, the method may not be sensitive enough for some applications. Very few microfluidic NA preparation methods are actually on the market because

Table 3. Specialized manual or in-line NA preparation methods.

Other methods	Description, benefits, tradeoffs	Sample flexibility	Lysis	NA	Isolation method	Sample size	Time (min)	References
Simple methods for lysis or inhibitor removal, ranked by time								
Sample dilution	Not sensitive		No	DNA	None	Not applicable	<5	Not applicable
Lysis only [PCT (Pressure BioSci.)]	Mechanical lysis	Clean samples only	Mechanical	DNA	None	None	<10	Folgueira et al. *(41)*; Gross et al. *(51)*
QuickExtract (Illumina)	Enzymatic lysis	Cells, tissue, food, bacteria	Lysozyme and heat	DNA	None		3-15	Salazar and Asenjo *(11)*
prepGEM (ZyGEM)	Enzymatic treatment with heat stable Bacillus EA1 proteinase	Unknown	Enzymatic digestion	DNA	None	Unknown	22	Lounsbury et al. *(22)*
InstaGene Matrix (Bio-Rad)	Method to remove inhibitors	Small volume Blood cells	Boiling	RNA DNA	Bind inhibitors with Chelex	Dilution	30	Polski et al. *(29)*
Rapid manual methods with minimal steps for NA isolation, ranked by time								
TruTip (Akonni)	Tip based, automatable	Flexible	Chemical	DNA	Porous binding matrix in tip	5-10-fold concentration	<5	Chandler et al. *(68)*
FTA cards (GE)	SimplePreserves NA	Many; blood, cells, and tissue	Chemical	RNA DNA	Washes	Spot Concentration by drying	<30	Li et al. *(37)*; van Tongeren et al. *(43)*
ChargeSwitch (Life Technologies) MagaZorb (Promega) QuickPick (Bio-Nobile)	Simple, aqueous reagents	Blood, cells Forensic samples Many sample types	Varies Protease Heat	DNA RNA	Ion exchange ligand coated on beads or surfaces Paramagnetic Particles	<1 mL	60	
Methods that yield very clean NA								
Aurora (Boreal Genomics)	Yield clean NA concentrated by gel purification, slow	Any sample type	Pre-lyse	DNA RNA	SCODA-gel isolation by rotating field to focused spot	<10-fold concentration	240	Broemeling et al. *(69)*
TRIzol Plus (Life Technologies)	For clean RNA, complex, hazardous chemicals	Cells, tissue, bacteria, yeast	Chemical	RNA	Chloroform separation, Ethanol precipitation, Silica cartridge	Precipitation, pure product	60	
Automated NA preparation methods linked to downstream analysis								
GeneXpert (Cepheid)	Cartridge linked to detection	Many	MechanicalLysis by sonication	DNA RNA	Surface	Entire sample to detection	Unknown	Niemz et al. *(4)*; Raja et al. *(71)*
FilmArray (BioFire)	Pouch linked to detection	Many	Mechanical Lysis by bead milling	DNA RNA	Silica Paramagnetic Beads	Entire sample to detection	12	Poritz et al. *(72)*

of the extensive cost associated with developing a complex automated product *(74)*.

Future Improvements

As the world moves to rapid NA identification, the need to speed up and simplify NA preparation increases *(75)*. This includes (*a*) reduced need for hazardous chemicals, (*b*) reduced need for cold storage, (*c*) fewer preparation steps, (*d*) faster or more effective automated lysis, and (*e*) automation. Hard-to-lyse cells and low concentrations of NA will continue to be a challenge.

Some analytes are present at very low concentrations in samples. More methods are needed that can increase target concentrations in the sample before NA preparation, especially methods not specific to a certain organism. This requires a larger volume of sample to be processed to increase the amount of material added. For example, if an analyte is present at 1 U/mL of blood and the sensitivity of analysis requires 10 U, at least 10 mL of blood must be concentrated. This can be difficult because larger samples require large volumes of material from a patient. However, it is something that is being actively pursued because low-concentration analytes sometimes need to be detected.

Conclusions

NA preparation methods are necessary for good molecular detection. Target NA can come from many human cell types or free NA, tissues, and pathogens as diverse as viruses, bacteria, protozoans, and fungi. Many techniques are available, so thought must go into which product, or combination of techniques, should be used for a given application. Several suggestions have been made for some of these applications. Many NA preparation techniques are designed by companies who specialize in making NA preparation simpler for the user, sometimes automating the process for ease of use. Solutions are currently evolving to the complex needs of an evolving diagnostic world. When methods or new technologies consider the best combination of lysis, isolation efficiency, and target concentration, NA preparation can be simple, reproducible, and effective.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: S.A. Thatcher, BioFire Diagnostics.
Consultant or Advisory Role: None declared.
Stock Ownership: S.A. Thatcher, BioFire Diagnostics.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Patents: S.A. Thatcher, patent number WO2013074391 A1.

Acknowledgments: Thanks to the following for help editing this document: Deepika de Silva, Kendall Rasband, Nick Garrone, Jeremy Green, and Carl Wittwer.

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