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Routine Challenges in Molecular Biology

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Executive Summary

Molecular techniques involving nucleic acids are manifold, and they are employed in a wide variety of areas. Even though these techniques may be rather complex in part, they are based on similar principles, and they are therefore subject to the same conditions. In this White Paper, the requirements pertaining to the quantity and quality of sample material, as well as the efficient use of resources, will be evaluated more closely, and approaches to solutions will be presented.

Introduction

Research in the field of molecular biology encompasses studies on the structure and function of nucleic acids and proteins, as well as their biosynthesis and their interactions with each other. One important goal is to understand the function of genes. The field of molecular biology intersects to a large degree with the fields of genetics and biochemistry. Moreover, the respective laboratory techniques are strongly interlinked with the methods employed in the fields of cell biology and microbiology.
In order to focus the wide-ranging topic of methods employed in molecular biology, the scope of this White Paper will be limited to those applications that pertain to nucleic acids – the methods of molecular genetics.

Further to academic research, these methods are nowadays employed in a variety of other areas on a routine basis. These include food analytics for the purpose of detecting genetically modified organisms (GMOs) and pathogens. In contrast, GMOs are employed in biotechnological processes for the purpose of producing goods for the food sector, pharmaceuticals or biofuel.
In the area of medical applications, these techniques are utilized in the diagnostics of genetic and infectious diseases as well as for gene therapy, and they play a role in the development of pharmaceuticals. The famous “genetic fingerprint” represents a further application which is nowadays indispensable in the realm of criminal and forensic investigation.

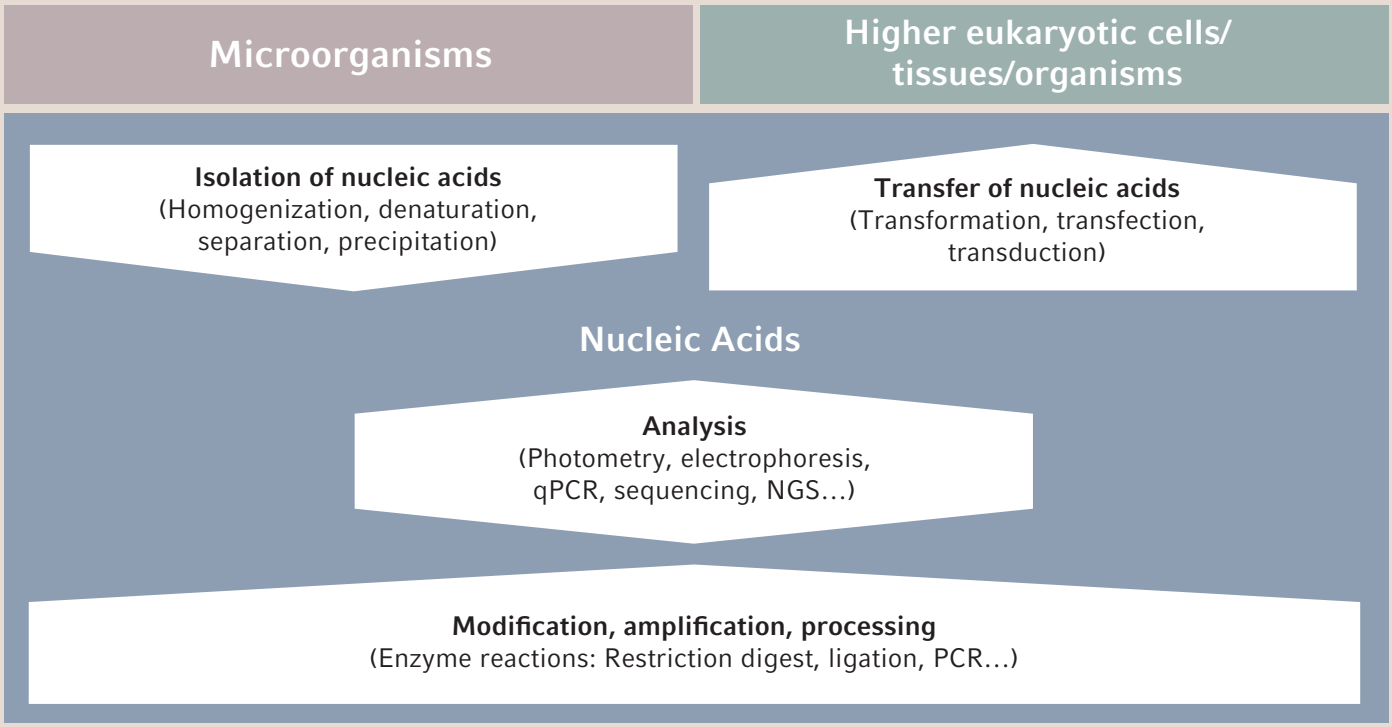


Figure 1: Workflow of molecular biology standard techniques for nucleic acids.

A basic simplified workflow outlining the sequence of events of standard molecular applications is shown in figure 1. Nucleic acids originating from microorganisms or from higher eukaryotic organisms, isolated from the respective original material, serve as the starting point. In addition, the purification of nucleic acids following enzymatic reactions is included in this step. Analysis is the centerpiece of all methods: techniques like electrophoresis and photometry are considered an intermediate step within ongoing quality control, while at the same time other methods deliver the results of an experiment. These include, for example, qPCR and sequencing, including NGS (next generation sequencing). Manifold processing steps exist which may serve the alteration or amplification of nucleic acids. These methods are for the most part based on enzymatic reactions and include the classic restriction digest as well as PCR. It is further possible to introduce nucleic acids into organisms via techniques such as transformation or transfection, where the nucleic acids are either amplified or their effect on cells is investigated with the help of molecular cloning.

Many molecular biology applications, among them current methods such as CRISPR/Cas and NGS, are rather complex in nature; however, they are nevertheless based on similar basic techniques, resulting in common essential requirements that must be fulfilled in order to achieve reliable and reproducible results.

This White Paper will provide information regarding these challenges, and it will describe possible approaches to solutions.

Identification of challenges

Essentially, experiments have two general goals. On the one hand, reliable results are to be obtained, which includes accuracy, precision and reproducibility. On the other hand, the time and material expended should be minimized, and the experiments themselves should be relatively easy to carry out.
Prerequisites include the availability of sufficient amounts of sample material as well as error-free, efficient performance of the procedures involved. The resulting challenges faced by molecular laboratories when working with nucleic acids will be examined more closely as follows.

1. Quantity and quality of the sample

For the most part, nucleic acids are isolated from source materials such as cells or microorganisms prior to being employed in subsequent procedures or analyses. It is therefore crucial that DNA and RNA are available in sufficient quantities as well as suitable concentrations. In addition to the integrity of the nucleic acid sample, it is important that it is as pure as possible, i.e. without critical impurities. Contamination of samples with other substances poses the danger of compromising downstream reactions, resulting in incorrect or irreproducible results.
What could be the reasons for insufficient quantities of sample material? One cause is limited source material, as is often the case in the field of forensics. Furthermore, the nucleic acids of interest may be present in small amounts, such as in the case of low-copy plasmids. In addition, the purification method may not be ideal, or simply unsuitable, leading to sample loss. This includes the phenomenon that nucleic acids may be bound to the surface of the tube, as is frequently the case under high-salt conditions that are encountered in nucleic acid purification kits [1], thus rendering these nucleic acids unavailable for subsequent reactions.
Correct sample processing and storage also play an important role, as degraded nucleic acids cannot, or not entirely, partake in subsequent experiments. Degradation of DNA and RNA is strongly dependent on environmental conditions, where temperature and nucleases constitute major factors. Strand breaks may also be triggered by exposure to UV light and mechanical shearing. Further impacting the amount of available sample, the necessary quality control steps place an additional demand on precious materials.
Characteristic impurities found in nucleic acid preparations include residuals from the purification process, such as organic solvents, proteins or salts, but also nucleases and nucleic acids. The latter two may originate from previous experiments (carry-over), and they may have been transmitted via equipment, air or human contact. Cross-contaminations (from one sample to another) are also problematic. Contaminations may cause reactions to fail entirely (for example, PCR, through residuals which exert an inhibitory effect) or to lower the efficiency, which can be expressed in reduced length of the readout of sequencing reactions. At the same time, impurities may lead to the acquisition of false data, for example, if the values obtained from quantification of nucleic acids by absorption measurements are either too high or too low, or if false positive data result from cross-contamination or carry-over.

2. Use of resources (time, consumables, equipment and space)

The complexity of molecular applications originates from the fact that nucleic acids are isolated from a wide variety of different source materials and that, depending on the objective, these materials may be used for several vastly different experiments. Most experiments comprise a number of small but time-consuming incremental steps, and there exists a considerable diversity with regards to the numbers of samples, the volume or the type of vessel used. The scaling of methods to the required format, but also sample-specific techniques such as PCR, require optimization. Furthermore, the consumables must withstand a variety of demands with respect to resistance to chemicals, robustness during centrifugation, heat conductivity and tightness of seal.
Low efficiency or the lack of successful completion of experiments may be a source of great expenditure and effort.

While user error is one reason, instrument malfunction as well as the use of unsuitable or sub-optimal laboratory equipment, including consumables, contribute to the problem. As a result, an experimental step may have to be repeated multiple times, thus constituting a drawback, particularly if the amount of source material is limited.
Further challenges in the field of molecular biology are presented by the fact that many users may work in the same laboratory, processing large numbers of samples. This may lead to bottlenecks and wait times where certain instruments are concerned. Large sample numbers also translate to high costs of consumables as well as the need for sufficient storage space. Many (different) instruments place an additional demand on laboratory space.

Solutions & Benefits

1. Quantity and quality of the sample

A) Sufficient sample quantity for downstream applications

The following strategies contribute to the availability of sufficient sample material for downstream processing and analysis steps: if on hand, the amount of sample material employed in subsequent processing steps should be as high as possible, and the efficiency of processing methods should be enhanced wherever possible, thus preventing loss. Alternatively, downstream applications may be miniaturized, thus requiring less sample material.

Use of larger amounts of source material: Utilization of Eppendorf Tubes® 5.0 mL (figure 2a) allows the use of larger quantities of source material without the need to prepare several reactions in small tubes simultaneously. In Application Note 262 [2], the example of isolation of a low copy plasmid using the Eppendorf 5.0 mL system demonstrates that this method may even contribute to increasing yields (figure 2b).

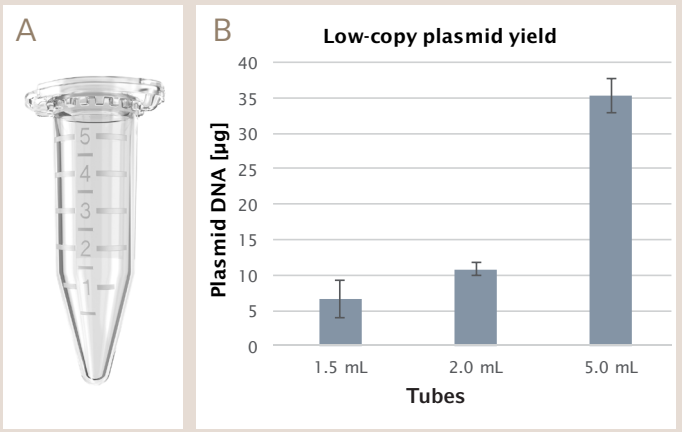


Figure 2a: Eppendorf Tubes 5.0 mL
Figure 2b: Yield of a low copy plasmid after processing in different vessel formats.

Increase of yield: The appropriate method of isolation is dependent on the source material as well as on the nucleic acid to be isolated, and it is crucial in order to obtain a satisfactory yield. Commercial kits and manually prepared reagents alike are capable of delivering good results; however, sample loss may occur at every processing step.

Certain steps lend themselves to optimization of the recovery rate, for example, centrifugation, where centrifugation speed may be adapted. As described in Application Note 234 [3], the recovery rate of plasmid DNA after alcohol precipitation will increase with increasing g-force (figure 3a). The "30,000 x *g* system" by Eppendorf was used to demonstrate this method (figure 3b).

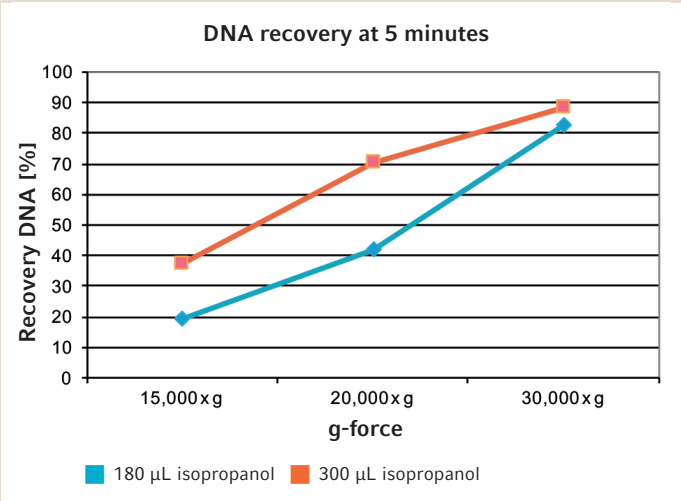


Figure 3a: Yield of plasmid DNA in percent, following precipitation using isopropanol and centrifugation at different g-force values for 5 minutes.

Figure 3b: Components of the Eppendorf "30,000 x g System" with Safe-Lock tubes (A) and high speed rotor (B) of Eppendorf Centrifuge 5430/R (C).

Prevention of loss: Especially when working with nucleic acid solutions of low concentrations, a relatively larger proportion may bind to the vessel, consequently becoming unavailable for the actual experiment. The use of vessels or plates made from Eppendorf LoBind® material is capable of increasing the recovery rate of nucleic acids considerably.

As described in White Paper 34 [4], the Eppendorf twin.tec® PCR Plates 96 LoBind (figure 4a) afford a recovery rate that is considerably higher than that achieved with plates made from standard materials following storage of DNA at -20 °C (figure 4b).

Figure 4a: Eppendorf twin.tec PCR Plates 96 LoBind

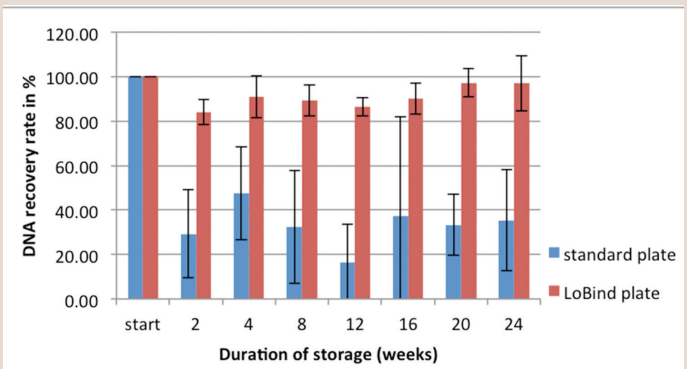


Figure 4b: Recovery rate following storage of DNA at -20 °C in plates made from standard materials and in twin.tec PCR Plates 96 LoBind.

Miniaturization of downstream applications: If downstream processing and analysis steps were to be carried out in smaller volumes, thus requiring less sample material, the additional benefit of potentially cost saving reductions in consumables emerges (please also refer to chapter 2B). Following isolation, a proportion of sample material must be sacrificed in order to satisfy the needs of essential quality control steps. Analyses performed on the microvolume scale, such as the measurements facilitated by the Eppendorf µCuvette® G1.0 in combination with an Eppendorf BioPhotometer® or Eppendorf BioSpectrometer (figure 5A) accommodate samples as small as 1.5 µL [5]. Alternatively, it is possible to quantify nucleic acid solutions via fluorescence measurements. Since this method is far more sensitive (1000-fold) than absorbance measurements, even very small amounts can be detected accurately. This method further allows the distinction between different types of nucleic acids (e.g. RNA and DNA). The Eppendorf BioSpectrometer fluorescence, featuring pre-programmable methods, is ideally suited for such applications (figure 5b) [6, 7].

Figure 5a: Eppendorf µCuvette G1.0 with Eppendorf BioSpectrometer

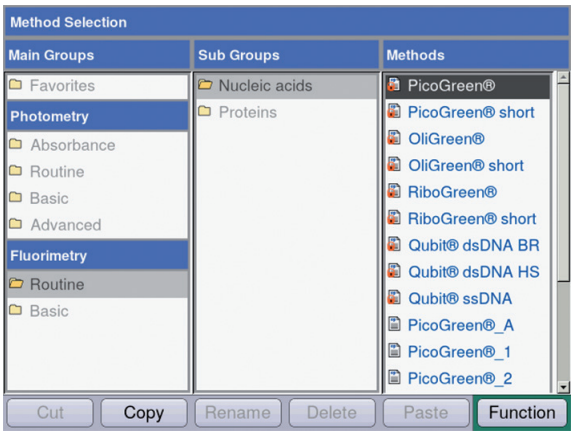


Figure 5b: Display of the Eppendorf BioSpectrometer fluorescence with an overview of pre-programmed methods for fluorescence-based nucleic acid quantification.

B) High sample quality

High sample quality is ensured if the sample is intact and if no contaminations are present that could compromise the sample or subsequent downstream reactions. To this end, correct and careful handling of the sample, including storage, as well as maintaining a clean work environment, including surfaces and equipment, are paramount. Nucleic acids can be further protected from contamination by selecting suitable consumables.

Correct and meticulous work: It is critical that experimental protocols be implemented accurately in order to prevent any carry-over of contaminations originating from the purification process or from the source material. Especially when performing experiments involving RNA, it is important to work quickly and to inactivate the ubiquitous nucleases through appropriate measures. Diligent work includes the wearing of lab coats and gloves, as well as regular cleaning of all work areas and equipment. Autoclavability represents an important feature of laboratory tools. The two manual pipettes Eppendorf Research® plus and Eppendorf Reference® 2 (figure 6) are easy to clean, and they are completely autoclavable. Most Eppendorf Centrifuge rotors are also autoclavable, while at the same time featuring high resistance to different cleaning agents [8].

Figure 6: Autoclavable Eppendorf pipettes Eppendorf Reference 2 and Eppendorf Research® plus.

Proper storage: Storage of biological sample material is a complex and wide-ranging subject. Suitable conditions are dependent on the duration of storage and the type of sample to be stored. Freezing is one common method, where the general rule applies that the lower the temperature, the fewer biochemical processes will take place that may lead to the degradation of the nucleic acids [9]. In the case of deep-frozen samples, it is important to ensure that they not be thawed and re-frozen multiple times, as this may also compromise the integrity of nucleic acids [9]. In order to avoid this situation, it is advisable to generate aliquots, and it is imperative that samples can be identified quickly. The Eppendorf Freezer Boxes with their highly transparent lids allow easy and quick location of samples (figure 7a). Maximum sample safety is ensured if the nominal temperature of the freezer can be restored quickly following door opening and if the instrument is capable of maintaining its temperature for extended periods of time, for instance in the case of a power outage (figure 7b).

Figure 7a: Eppendorf Freezer Boxes: with clear, transparent lid for quick identification of samples.

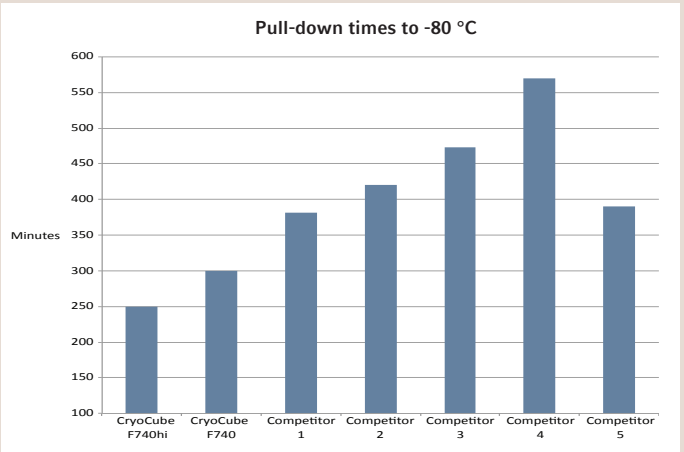


Figure 7b: Pull-down times of the CryoCube® Freezer in comparison with instruments by competitors [10].

Pure consumables: The purity of consumables that are in direct contact with the sample is an important consideration. While autoclaving, a standard laboratory technique, is efficient at inactivating DNases, DNA itself will only be partially destroyed [11], and this method is mostly ineffective against RNases. Single-use items, which are certified free from contaminating DNA and nucleases, offer a high degree of security. Small packages will further minimize the risk of contamination. The purity grades Eppendorf PCR clean (figure 8a) and Eppendorf Forensic DNA grade (figure 8b) are recommended specifically for PCR-based techniques. The latter purity grade was conceived with the more stringent safety standard in mind that applies to forensic applications, and it is compliant with ISO 18385. Individually packaged products such as those available for the purity grade Eppendorf Biopur® (figure 8c), as well as for certain products of Eppendorf Forensic DNA grade, are advantageous when handling delicate samples. All three purity grades have in common that they are subject to lot specific testing, and they are certified free from human DNA, DNase and RNase.

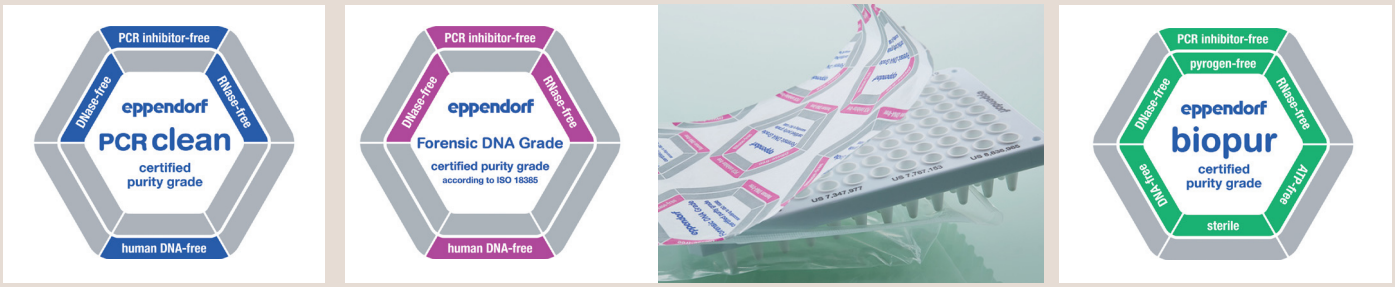


Figure 8a: Eppendorf PCR clean logo; **8b:** Eppendorf Forensic DNA Grade logo and single-blistered PCR Plate; **8c:** Biopur® logo

Protection from aerosols: In the laboratory, aerosols harbor the risk of carrying contaminations, which, in the case of sensitive techniques such as PCR, may prove to be detrimental. In order to prevent carry-over of DNA, and thus prevent the risk of false positive results, pre- and post-PCR processes should be physically separated from one another, and separate equipment should be available for each of the two areas. The release of aerosols, too, may be minimized with the use of appropriate equipment. Pipetting poses the highest risk; aerosols that make their way into the interior of the pipette may be released into a subsequent sample during the dispensing step. The ep Dualfilter T.I.P.S.® (figure 9a) offer a two-phase filter as protection from contaminations originating from aerosols. Moreover, the ep Dualfilter T.I.P.S. SealMax (figure 9b) offer additional protection against accidental over-pipetting; their self-sealing barrier will prevent liquid from entering the pipette. A further alternative for the protection from aerosols is provided by positive displacement systems, such as the Multipette® in combination with Combitips advanced®.

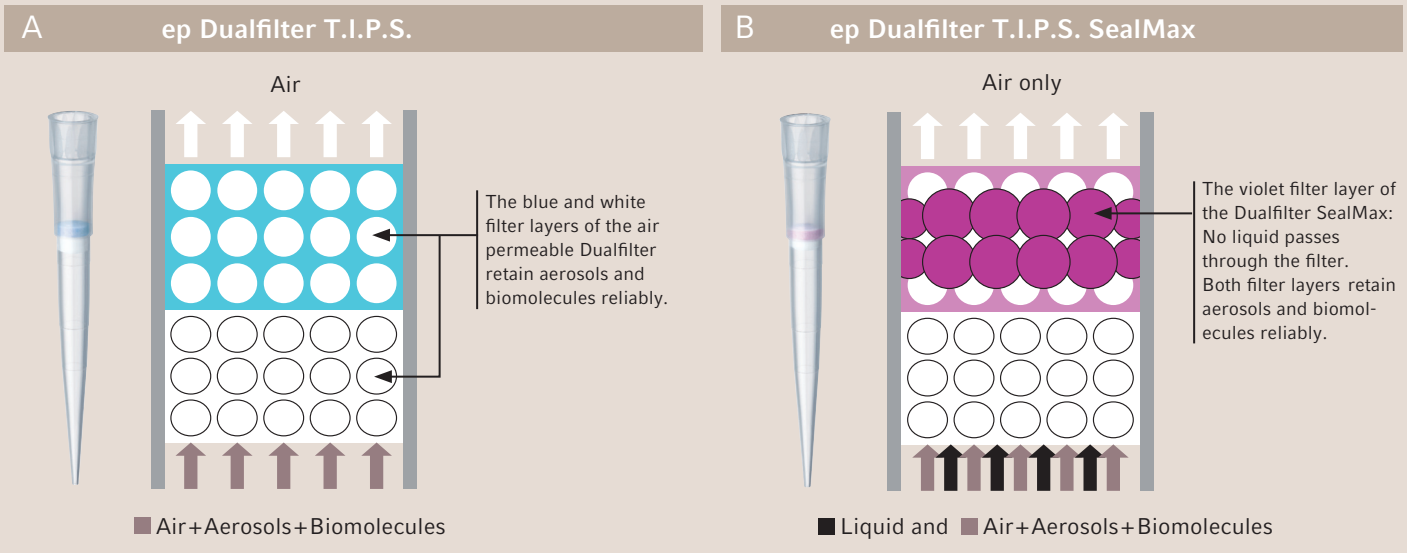


Figure 9: a) ep Dualfilter T.I.P.S., b) ep Dualfilter T.I.P.S. SealMax

2. Use of resources

This chapter will focus on achieving the goal of obtaining reliable and reproducible results through efficient allocation of work time and optimal utilization of instruments, consumables and the available space.

A) Effective allocation of work time

The following strategies may contribute to reducing the time required to carry out experiments: labor-intensive manual steps can be avoided or, whenever possible, processing steps and reactions can be performed more quickly. High optimization expenditures are to be reduced, and sources of errors are to be minimized.

Automation: Automation of processing steps, especially the dispensing of liquid, helps reduce labor-intensive manuals steps and prevents mistakes and deviations caused by human error. Particularly in cases where a large number of samples require processing and when methods are employed which comprise many steps with a high proportion of "Liquid Handling", automation may afford considerable time savings. The automated workstation ep*Motion*® (figure 10), for example, can process a wide variety of kit-based methods for nucleic acid purification in the 96-well format within 1 – 2 h, whereas manual processing would require closer to 5 – 6 h hands-on time [12]. Library preparation for the purpose of NGS, too, can be accelerated considerably if crucial processing steps are automated.

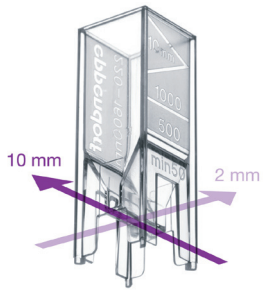
Figure 10: The workstation epMotion 5073

Acceleration of work steps: Standard PCR presents an excellent example for how reactions may be accelerated. While this type of experiment previously took up the better part of the day, these days modern thermocyclers featuring highly efficient temperature regulation require less than one hour (table 1) [13]. During the process of quantification of nucleic acids, the generation of dilutions tends to be time-consuming. Absorbance measurements in a cuvette with a standard path length of 10 mm are only useful for a limited concentration range. The UVette®, which combines two different path lengths, 2 mm and 10 mm, as well as the Eppendorf µCuvette G1.0 with a path length of 1 mm, significantly widen the range of concentrations suitable for measurement (table 2).

Table 1: Run times for a standard PCR protocol and ramp rates of different cyclers [13].

Thermal cycler	Run time [hh:mm:ss]	Ramp rate accord. to techn. Data [°C/s]
Mastercycler® X50s	00:39:29	10
Mastercycler X50l	00:45:02	5
TAdvanced 96S	00:47:05	8
PeqSTAR 96X	00:47:10	5
Mastercycler nexus gradient	00:51:15	3
Veriti® Dx Fast	00:56:06	5

Table 2: Measurable dsDNA concentrations for the path lengths of the respective cuvettes within the optimum measurement range (0.05 - 2 A) of the Eppendorf BioSpectrometer.

	Eppendorf Uvette		Eppendorf µCuvette G1.0
			
path length	10 mm	2 mm	1 mm
dsDNA [µg/mL]	2.5 - 100	12.5 - 500	25 - 1000

Minimization of the optimization effort: The optimization effort may be reduced if several reaction conditions can be tested simultaneously. PCR represents one application with a high need for optimization; therefore, the gradient technology of thermocyclers is proving to be especially advantageous [14]. When establishing a new PCR system, it is common practice to determine the optimal annealing temperature via gradient PCR. In addition, determining the suitable denaturation temperature may be useful, for example, in the case of GC-rich templates. The block of the Eppendorf Mastercycler X50 allows the creation of gradients in two directions. Harnessing this new 2D gradient technology, optimization of annealing temperature as well as denaturation temperature can be carried out in a single run (figure 11) [15].

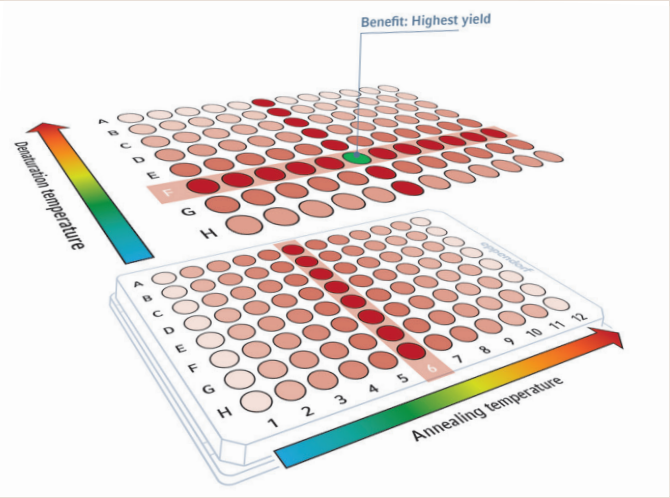


Figure 11: The 2D gradient technology of the Mastercycler X50.

Error prevention: Errors can be avoided through correct techniques and careful and diligent work, assisted by suitable laboratory equipment. Dispensing of liquids is the fundamental technique behind all experiments, and the smaller the volumes used, the higher the likelihood of error. Generally, electronic systems achieve higher reproducibility [16], but the associated consumables will also influence the result. According to ISO 8655, pipette and tip are to be regarded as a system, necessitating renewed calibration whenever a different type of tip is used. Application Note 354 covers this topic, showing comprehensive comparisons between pipette tips made by different manufacturers (figure 12) [17].

Error prevention includes the avoidance of unintended alterations of instrument settings. To this end, password protection options, such as those offered in the user management section of the Mastercycler were designed to help protect the integrity of settings programmed by the user. Inadvertent mix-up of samples constitutes a further source of error, the likelihood of which increases considerably if tube labelling is limited to the lid. These lids frequently break off when small columns are centrifuged in 1.5/2.0 mL tubes. The kit rotors available for Eppendorf Centrifuges stabilize the open tube lids during centrifugation. Lid and tube will remain connected, thus preventing mix-up (figure 13).

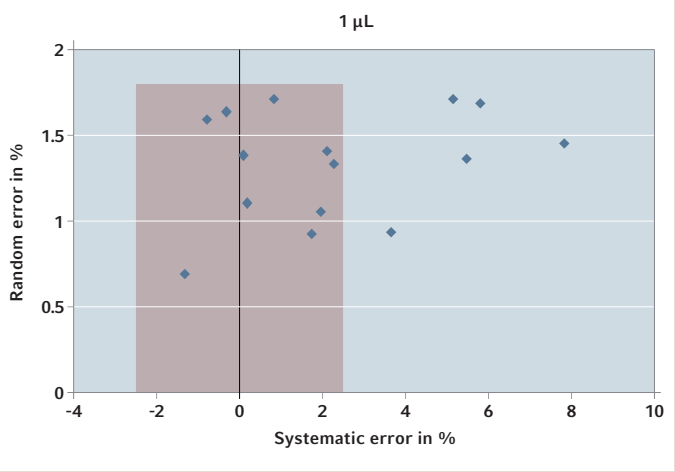


Figure 12: Systematic error and random error achieved with the use of the Eppendorf Xplorer® in combination with tips made by different manufacturers. 5 tips are outside the manufacturer's specifications (colored area).

Figure 13: Rotor FA-45-24-11-Kit of the Eppendorf Centrifuge 5427 R.

B) Efficient use of equipment, consumables and space

It makes sense to simplify processes if a number of researchers use the same equipment and/or many samples require processing at the same time. This is generally organized by laboratory management, with the support of the following approaches to solutions: the efficiency of many reactions may be enhanced by specific consumables, enabling either a reduction in the number of experiments required or a reduction in scale. Automation and the choice of suitable liquid handling consumables can further minimize the costs for reagents. Instruments that are suitable for several different applications or which allow the use of a variety of vessel formats increase flexibility. A better reproducibility is achieved by ensuring homogeneous experimental conditions.

Less effort through efficient reactions: Application Note 182 shows that the sensitivity of a real-time PCR increases if the fluorescence signals are enhanced by using plates with white wells [18]. The use of Eppendorf LoBind® tubes and plates can further contribute to a more efficient process. To this end, the Application Note 226 [19] demonstrates an increase in amplification efficiency within qPCR when Eppendorf DNA LoBind Tubes are used for the preparation and storage of standards.
Moreover, improved results were obtained for NGS library preparation. An improvement of up to 23% more reads and up to 30% more transcript species was achieved, respectively, compared with results obtained with plates made from standard material (figure 14) [20].

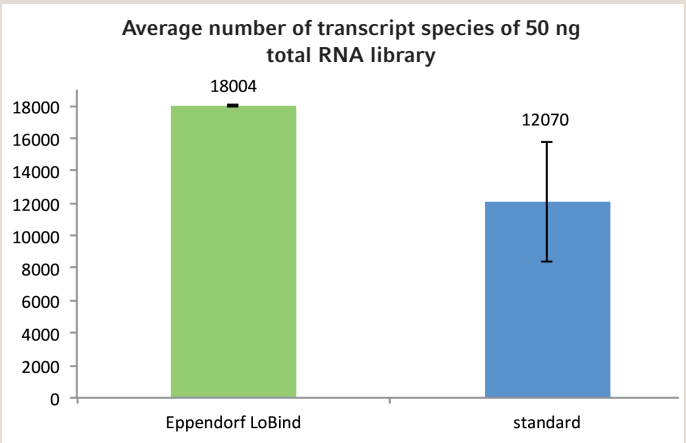


Abbildung 14: RNA transcript-species following library preparation in twin.tec PCR Plates LoBind in comparison with plates made from standard material.

Miniaturization made possible by exact dispensing: Particularly when many samples are to be processed or analyzed, a smaller scale will help save reagents and therefore money. Many enzyme solutions contain stabilizers such as glycerol and detergents, hindering exact dispensing using air-cushion pipettes due to residues that tend to remain inside the tip. This situation can be helped by positive displacement systems such as the Eppendorf Multipettes in combination with the Combitips advanced or by extremely hydrophobic tips. The epT.I.P.S.® LoRetention (figure 15a) will achieve high pipetting accuracy, while at the same time saving reagent cost (figure 15b) [21]. Automation of PCR and qPCR preparation using the epMotion obtained excellent, reproducible results with reaction volumes as small as 3 µL [22] or 5 µL [23], respectively.

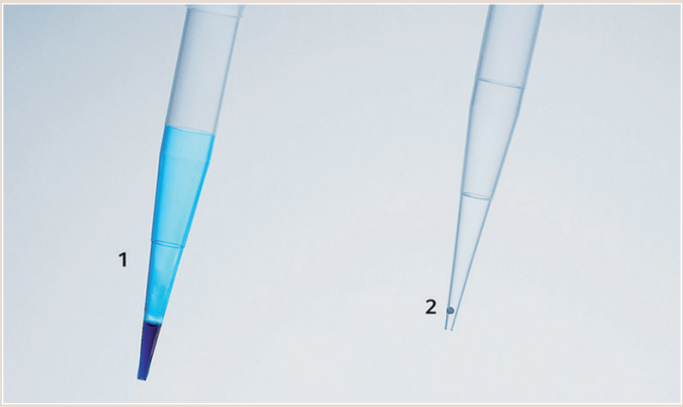


Figure 15a: Wetting of standard tips (1) and epT.I.P.S. LoRetention (2) with detergent solution.

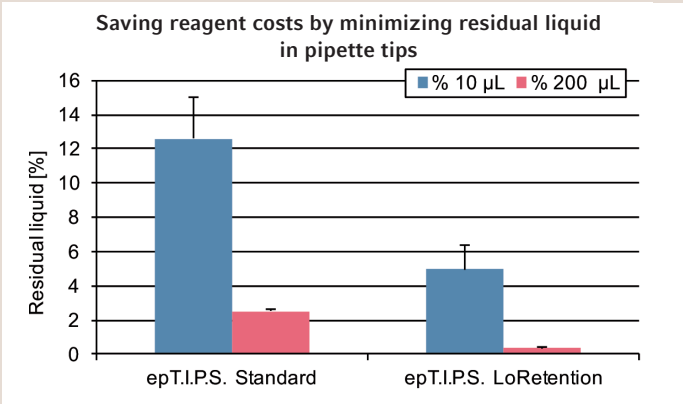


Figure 15b: Comparison of standard tips with epT.I.P.S. LoRetention while dispensing PCR master mix.

Versatile equipment: Versatile instruments are advantageous whenever there is a need for simultaneous operation by different users; for example, the Mastercycler nexus X2 is equipped with two asymmetrical blocks featuring 64 and 32 wells, respectively, which can be programmed and operated independently of one another. This opens up the option of running two PCR protocols in parallel and therefore avoiding bottlenecks in the laboratory (figure 16) [24]. Standard laboratory instruments such as centrifuges and thermomixers are needed for a variety of different applications and must therefore accommodate different types of vessels. Instead of purchasing a different device for each purpose, an inexpensive and space-saving solution is at hand when selecting instruments that, when equipped with the appropriate accessories, will accommodate different applications/vessels. A comprehensive selection of accessories is available for the Eppendorf Centrifuges and Eppendorf ThermoMixer®.

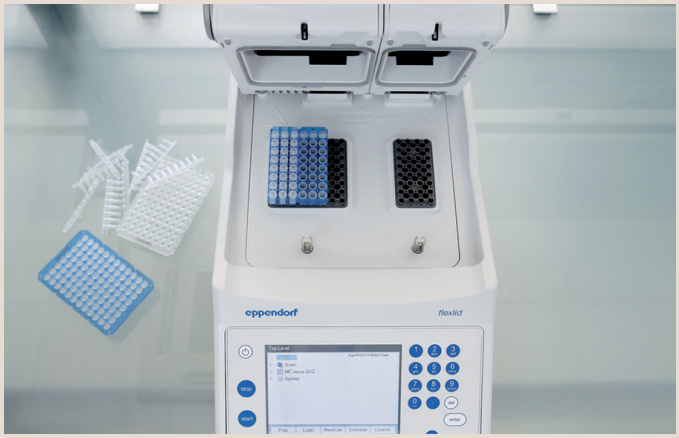


Figure 16: Eppendorf Mastercycler nexus X2 with asymmetrical dual block.

Robust systems through homogeneous reaction conditions: Temperature homogeneity of cycler blocks is an important prerequisite for reproducible PCR results across the entire block. The Mastercycler nexus achieves minimal well-to-well deviations, as shown in Application Note 244 [25]. While all PCR instruments are equipped with a heated lid by today's standard, other reactions besides PCR such as, for example, the enzymatic restriction digest, also require samples to be incubated at temperatures high enough to generate condensation effects. Especially in the case of small volumes, the water droplets that form inside the lid will lead to an increase in the concentrations of all reaction components in the bottom of the tube, preventing the reaction from proceeding smoothly and efficiently. A heated lid, such as the Eppendorf Thermotop® for the Eppendorf ThermoMixer, effectively counteracts the formation of condensation, thus helping safeguard the reproducibility of experiments (figure 17a + b) [26].

Figure 17a: Eppendorf ThermoMixer C with Eppendorf Thermotop.

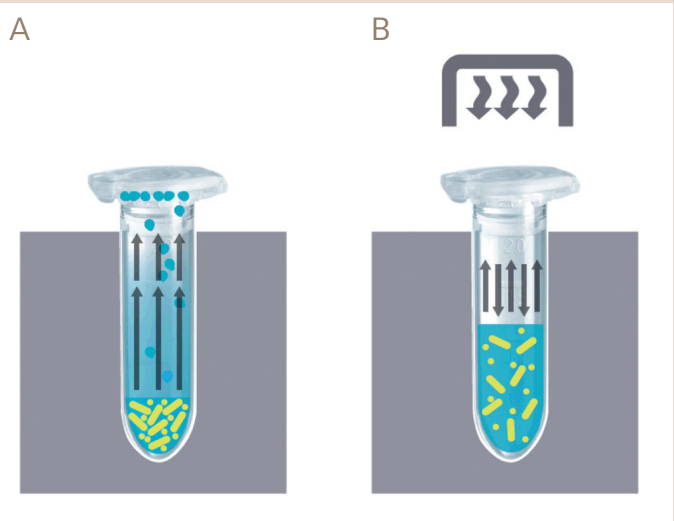


Figure 17b: Heating of liquid sample without (A) and with heating lid (B) of the Eppendorf ThermoMixer

References

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