



Molecular biological tests

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10.1 Introduction

Molecular biological testing has become a mainstay in the repertoire of infectious disease diagnostics like in no other field of medicine. The major ambassador of this technology is the polymerase chain reaction (PCR). Its popularity arose from the speed and sensitivity with which PCR made it possible to ascertain the etiology of an infection. Classic microbiological diagnostics usually takes at least 36–48 hours before the first results from culturing and resistance testing are available. A long delay can be fatal for the patient, so starting empirical antibiotic therapy is generally the preferred strategy. Depending on the severity of the infection, broad-spectrum antibiotics are chosen, without confirmation of the causative pathogen or its antimicrobial resistance. The development of resistance is an undesired consequence of this antibiotic strategy [4].

Direct pathogen detection, not requiring a bacterial culture and only needing time for pure analysis, offers rapid and targeted diagnostics. In recent years, two main methods have established themselves for this direct detection, one of which is PCR. Besides PCR, immunochromatography is the other method used – often in the form of test strips (lateral flow assays) or test cards (▶ Chapter 9). A significant advantage of test strips is their great ease of use and speed coupled with moderate cost. Notwithstanding these selling points, the performance capability of older systems is only moderate. Moreover, specific antibody responses must generally take place (for antibody detection) or specific antibodies (for antigen detection) need to be available in the target organisms before immunological rapid tests can be established. In challenging situations, where the pathogen density is likely to be low, PCR is often a better choice because of its higher sensitivity [4]. PCR has the advantage that, in addition to its main pathogen detection function, resistance or virulence factors can be determined simultaneously. In the age of multidrug-resistant (nosocomial) pathogens, rapid and reliable molecular biological differentiation is becoming increasingly impor-

tant given the urgency indicated to effectively isolate affected patients at the earliest possible chance.

10.2 Integrated and miniaturized systems

For a long time, PCR was seen purely as a laboratory method requiring lots of manual input. In the meantime, this has undergone a paradigm shift, with the list of molecular biological methods capable of point-of-care use expanding constantly. This expanded range of the POCT applications was made possible by the development of **single-use systems with integrated cartridges**, where analysis occurs in a closed plastic test carrier. With the reagents pre-packaged and ready-to-use in these test cartridges (unit use), hands-on work for the user of such systems is limited to loading the sample and starting the PCR run. First, the sample is mixed with lyophilized reagents, triggering digestion of the sample. Fluid movements cause the reagent mixture to then move through cast plastic arrays or channels into the reaction chambers where the next PCR process steps (DNA amplification and signal detection) occur. By varying the reagents, the test cartridges can easily be adapted to detect the various pathogens.

The more novel systems not only feature single-use cartridges, but are also distinguished by increasing **miniaturization**. This trend is ongoing for both test cartridges as well as for thermocouples and control electronics. Bench-top devices are now available that are no larger than a small coffee machine. If this trend continues, the next generation will be characterized by fully portable hand-held devices that are completely independent of all stationary technology. However, in the context of infectious diagnostics, the trend toward increasing miniaturization is not always seen as progress. Methodologically, tests using smaller aliquots tend to suffer from limits to the pathogen detection sensitivity achievable because of the mostly heterogeneous sample materials col-

lected (swabs, aspirates, biopsy tissue or the likes) and the often very inhomogeneous distribution of the individual pathogens in the sample. This aspect should always be considered when miniaturizing microbiological diagnostic devices [5].

10.3 Selection criteria for POCT systems

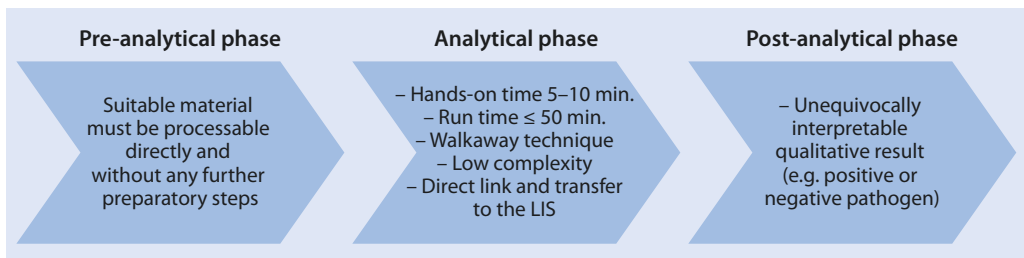
In order to achieve significant improvements in medical care, a point-of-care diagnostic system must be integrated as smoothly possible into clinical processes. The main challenge is often that staff without any laboratory-specific training (e.g. in emergency departments or intensive care units) are tasked with carrying out these diagnostic tests. Under these circumstances, the danger is that a lack of time and (laboratory) experience can lead to unintended sample contamination and operational or diagnostic errors. These inherent risks should not be further exacerbated by making POCT technology too complex. Careful selection of diagnostic systems not only protects the patients but also reduces stress and responsibility for staff.

Note

The careful, coordinated selection of POCT systems for molecular biological diagnostics – adapted to the situation at the place of use – not only protects against diagnostic errors but also reduces responsibility and stress for staff.

It is easier to understand the relevance of certain selection criteria if the diagnostic approach is seen as a three-step sequence (pre-analysis, analysis and post-analysis) A positive aggregate result is only achieved if the characteristics of the chosen technology for each step of the process are relevant to the situation at the point of use (■ Fig. 10.1). With regard to pre-analysis, this means that the type and volume of the sample to be analyzed must conform to the test specification and that the sample can be used directly, without prior preparation. This prerequisite can only be achieved by PCR systems designed with integrated sample preparation; other systems are excluded from the outset (► Section 10.2). In medical microbiology settings, practical experience explicitly dictates that nucleic acid-based pathogen detection is only feasible in sample material that is very likely to contain traceable amounts of the target pathogen [5].

From an analytical viewpoint, technical complexity and hands-on time are critical factors alongside test speed (► Section 10.4). In the hectic and stressful routine of an ICU or central emergency room, only straightforward and robust technologies are feasible. The best solutions that minimize hands-on time are those systems where manual input is reduced to loading the sample and starting the reaction, which is otherwise fully automated. This walk-away function frees up staff to do other jobs. The output, reading and interpreting of results (post-analysis) should be clear and straight forward, not requiring further interpretation, e.g. “pathogen detected/not detected” or “mutation present/not present”. More complex interpreta-



■ Fig. 10.1 Selection criteria for point-of-care molecular biological diagnostic systems

tion of results (e.g. results from multiplex PCR) should be the responsibility of a physician specialized in microbiology. The result of a PCR analysis not performed in a central laboratory should be transferred immediately and automatically to the laboratory information system to allow cumulative result interpretation (e.g. in microbiology and infectious diseases).

In **Tab. 10.1**, some examples of current molecular diagnostic systems are shown that largely meet the above-mentioned selection criteria and are suitable for point-of-care use by virtue of their test speed (**Chapter 10.4**). There are also other new pipeline products from smaller companies with innovative reagents and/or device designs that are poised to prove their merits on the market. A prime example is the PDQeX 2400 by ZyGEM (Hamilton, New Zealand).

10.4 System concepts to shorten analysis time

Test speed is one of the most important criteria that qualifies a molecular diagnostics system for POCT. PCR, which involves three temperature steps, has been the methodological standard in nucleic acid amplification for many years. GeneXpert by Cepheid was the first point-of-care PCR system to reach market maturity and, since its launch, has been the standard among the molecular systems [12, 13]. The range of available GeneXpert test cartridges has steadily increased in recent years to over twenty (**Tab. 10.1**, **Fig. 10.2**). PCR tests take at least 45–60 minutes (even with the GeneXpert system), which makes them slower than test strips in terms of providing POCT results. For that reason, test manufacturers have worked intensively to develop even faster technologies. By cleverly optimizing the processes, the test speed of the conventional PCR has been ramped up even further.

The LIAT system (LIAT, lab in a tube) by Roche is one example of this type of acceleration (**Fig. 10.3**) [8, 9]. The test cartridge is shaped like a small tube. This tube contains all



Fig. 10.2 GeneXpert Omni. (Courtesy of Cepheid, Sunnyvale, CA, USA)



Fig. 10.3 LIAT. (Courtesy of Roche Diagnostics, Mannheim, Germany)

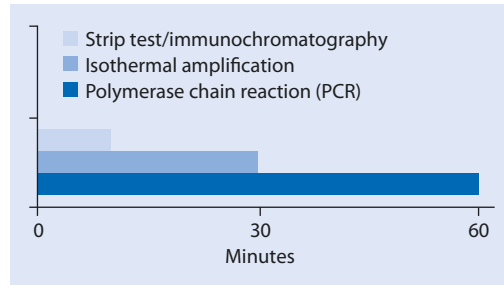
necessary reagents stored within small chambers of a proprietary plastic compartmentalized system, arranged in rows. After insertion, the sample passes through these chambers step by step. The fluid column of the reagent mix only

moves up and down in the analyzer, allowing it to reach the different temperature zones of the PCR. Thanks to this simple principle of up-and-down movement, the PCR requires only a small space, making the reaction significantly faster [8, 9]. Depending on the pathogen-specific application, the LIAT system delivers definitive results within 20 minutes. It therefore more suitably fulfills the above-mentioned POC criteria and assists with rapid treatment decisions on site (■ Tab. 10.1).

The io system by Atlas Genetics also reflects a PCR process optimization that accelerates the reaction process. Amplification products are measured in the io cartridge by a specific (electrochemical) detection reaction, which is so fast that it produces a PCR result after just 30 minutes [10, 11]. At present, the io system is mainly available for screening of sexually transmitted and nosocomial infections (■ Tab. 10.1).

In addition to conventional PCR, a number of alternative **nucleic acid amplification techniques** are now on the market [1–3]. Historically, such processes were developed by diagnostics manufacturers who wanted to bypass PCR patent protection in the field of nucleic acid diagnostics in an era of extremely restrictive PCR licensing practices. Of the many PCR-alternative technologies, e.g. branched DNA signal amplification (bDNA), loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), only a few have reached full market maturity to date and only of the assays that have been developed only address some selected niche applications.

The most successful and advanced PCR-alternative technology launched thus far is **isothermal amplification** [3]. Isothermal (Latin: equal heat) means that continuous amplification reactions occur at a constant temperature; this contrasts with conventional PCR, which relies on thermal cycles (three temperature steps). Not only are the whole process and test run times significantly shorter, but the instrumentation is also simpler, since the method only aims at reaching one constant temperature without the need for repeated thermal cycles.



■ Fig. 10.4 Comparison of the test run times for conventional PCR vs. isothermal amplification

Compared to PCR, isothermal reactions inherently lose some specificity in the hybridization events. This is compensated, however, by optimizing conditions and through the addition of different enzymatic and biochemical reaction components – a modification which has proven diagnostically satisfactory across a broad range of applications [3]. The first commercial assays for use in POCT settings have now appeared on the diagnostics market. Influenza A and B as well as group A Streptococcus, for example, can be detected with the i system by Alere [6, 7]. Test run times are surprisingly short (less than 20 minutes) because the reaction occurs at a constant temperature (■ Fig. 10.4).

10.5 Spectrum of molecular biological assays

The recent assays of newer systems are setting a clear trend towards the amplification of multiple pathogens in a single assay (**multiplex PCR**). The big advantage of such a broad approach is the expeditious and sensitive etiological diagnosis of patients' symptoms, which could be caused by various pathogens (e.g. patients with a cough or diarrhea). Further details on this topic are presented in (► Chapter 20). The FilmArray system by bioMérieux is a prime example of testing for "syndromic diagnosis". The panels allow the detection of numerous pathogens, which can cause respiratory, gastrointestinal illnesses and infections alongside the simultaneous detection of resistance markers

Tab. 10.1 Overview of point-of-care molecular biological diagnostic systems (numerical values represent analysis times in minutes)

System/ manufacturer	Method	Single test/duplex test/multiplex test	Sexually transmitted infections								Respiratory infections			
			Human immunodeficiency virus (HIV) ^a	Hepatitis B virus (HBV)	Hepatitis C virus (HCV)	Human papillomavirus (HPV)	Group B streptococci	Chlamydia trachomatis	Neisseria gonorrhoeae	Trichomonas vaginalis	Mycoplasma genitalium	Influenza A and B	Respiratory Syncytial Virus (RSV)	Group A-Streptococci
Alere i	ITA	ST, DT										15		8
Roche Cobas LIAT	PCR	ET										20		20
		MP										20	20	
Atlas io	PCR	ST						30	30	30	30			
		MP						30	30	30	30			
Cepheid GeneXpert	PCR	ST, DT	90		105	60	52	90	90	40		75		
		MP										40	40	
Spartan RX	ARR													
bioMérieux FilmArray	ARR		Meningitis/encephalitis panel: E. coli K1, Haemophilus influenzae, Listeria monocytogenes, Neisseria meningitidis, Streptococcus agalactia											
			Respiratory panel: Adenovirus, Coronavirus (229E, HKU1, OC43, NL63), human Metapneumovirus, human Rhinovirus/Enterovirus,											
			Gastrointestinal panel: Campylobacter (jejuni, coli & upsaliensis), Clostridium difficile (toxin A/B), Plesiomonas shigelloides, Salmonella, Yersinia enterocolitica, Vibrio (parahaemolyticus, vulnificus & cholerae), E. coli O157, enteroaggregative E. coli (EAEC), enteropathogenic E. coli (EPEC),											

ITA Isothermal amplification; PCR Polymerase chain reaction; ARR Microarray with upstream PCR; ST Single test; DT Duplex test; MP Multiplex test.

^a Also quantitative, as IVD-compliant virus load assessment

Life-threatening infections			Nosocomial infections						Non-pathogen-specific detection			References
Ebola virus	Enterovirus in cerebrospinal fluid	M. tuberculosis/rifampicin-resistance	Norovirus	Clostridium difficile	Methicillin-resistant S. aureus (MRSA)	Vancomycin-resistant enterococci (VRE)	Carbapenem-resistant Enterobacteriaceae	Factor II and factor V mutation	BCR-ABL transcription products	Cytochrome P450 allele 2C19		
											[6, 7]	
											[8, 9]	
			30	30	30						[10, 11]	
98	150	117	60	45	60	45	50	30	140		[12, 13]	
										60	[14]	
and pneumoniae, CMV, Enterovirus, HSV 1 and 2, HHV6, human Parechovirus, VZV, Cryptococcus neoformans											[15, 16]	
Influenza A (A/H1, A/H1–2009, A/H3), Influenza B, Parainfluenza 1–4, RSV, Bordetella pertussis, Chlamydia pneumoniae, Mycoplasma pneumoniae												
enterotoxigenic E. coli (ETEC) It/st, shiga-like toxin-producing E. coli (STEC) stx1/stx2 E. coli O157, Shigella/enteroinvasive E. coli (EIEC), Adenovirus F 40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, Sapovirus (I,II, IV, and V), Cryptosporidium, Cyclospora cayetanensis, Entamoeba histolytica, Giardia lamblia												

(■ Tab. 10.1) [15, 16]. This parallel detection, however, leads to the greater need for interpretation of results and, for now, is still best suited for laboratory use.

Simpler systems more suitable for deployment near the patient are still dominated by PCR assays for single pathogens or two pathogen variants (e.g. influenza A and B or vancomycin-resistant *vanA* and *vanB* with *Enterococci* genes); such duplex PCR approaches can be advantageous to solve some microbiological problems (■ Tab. 10.1). Linking the current assays to medical specialties, it is striking that it is the field of molecular biology that has opened up such numerous new areas of application. Five years ago, the assays were used almost exclusively to detect sexually transmitted, nosocomial and respiratory infections. More recently, they have expanded to include infection diagnostics (e.g. detection of hepatitis B and C, some as quantitative and IVD-compliant virus load assessment as well) alongside the development of very promising applications outside of microbiology (■ Tab. 10.1).

Beyond infectious disease medicine, among the specialties currently benefiting from new-generation products are oncology (BCR-ABL transcription), coagulation centers (detection of factor II and factor V mutations) and cardiology (cytochrome P450 allele 2C19) (■ Tab. 10.1). The latter-named assay is the first that combines PCR and array hybridization (microarray) for point-of-care use. A cytochrome-P450 2C19 variant allele, which genetically determines the response to clopidogrel treatment, can be detected within 60 minutes [14]. Given its the highly complex nature (the assay was categorized by the FDA as “high complexity” in the American approval), it remains to be seen whether testing for CYP2C19 will become established for direct point-of-care applications.

10.6 Synopsis for infectiology

Molecular biological diagnostic systems for point-of-care testing are now being used for a diverse range of infectious diseases. The opera-

tion of devices is becoming increasingly user friendly, the technical workflows more reliable, while devices continue to become more compact. Molecular biological testing is now even evolving into point-of-care applications for non-pathogen-related problems. Isothermal amplification technologies and process optimization in conventional PCR have significantly shortened test run times further yet, where the newest generation of tests can deliver results after just 15–30 minutes. Research and development departments in the diagnostics industry have focused intensively on this dynamic market segment: There is no doubt that new test concepts can be anticipated in the foreseeable future.

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