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PERSPECTIVE

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Ten years of R&D and full automation in molecular diagnosis

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A 10-year experience of our automated molecular diagnostic platform that carries out 91 different real-time PCR is described. Progresses and future perspectives in molecular diagnostic microbiology are reviewed: why automation is important; how our platform was implemented; how homemade PCRs were developed; the advantages/disadvantages of homemade PCRs, including the critical aspects of troubleshooting and the need to further reduce the turnaround time for specific samples, at least for defined clinical settings such as emergencies. The future of molecular diagnosis depends on automation, and in a novel perspective, it is time now to fully acknowledge the true contribution of molecular diagnostic and to reconsider the indication for PCR, by also using these tests as first-line assays.

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KEYWORDS
• assay development • automation • flexibility • high-throughput tests • intracellular bacteria • molecular diagnosis • quality assessment • real-time PCR

Background

During the last 10 years, molecular diagnosis has become increasingly important in microbiology. Indeed, when micro-organisms do not grow or grow poorly on axenic media, the current trend is to use molecular methods to detect their nucleic acids (NAs) directly from the sample, most often by real-time PCR. This is the case for most viruses, many intracellular and fastidious bacteria, as well as for some fungi and parasites. The use of highly sensitive and specific NA amplification techniques, for the diagnosis of infectious diseases such as pneumonia, endocarditis, pericarditis, meningitidis and urogenital infections, has also been associated with a reduced rate of cases with unknown etiology [1–10]. Moreover, the evolution of molecular techniques during the last 20 years contributed to increase the impact of these tests on patients' care, by also reducing the turnaround time (TAT) from sample collection to definitive diagnosis.

Compact and easy-to-use instruments allowed rapid automation of the molecular tests and many ready-to-use kits arrived on the market making molecular diagnostics widely available. Thanks to the introduction of real-time PCR, the risk of contamination due to amplicons (leading to false-positive results) dramatically decreased. Similarly, the sensitivity of the new molecular techniques is so high that direct examination or even cultures show their limits in terms of sensitivity, and their use as gold standards is now challenged. Thus, bacterial culture of valvular samples was shown to exhibit a sensitivity of only 10%, which makes culture simply useless, especially considering the very low positive predictive value of the few positive results, due to a concomitant lack of specificity [2]. Even the use of PCR diagnostics for tuberculosis showed an added value compared with conventional microscopy in the evaluation of the risk of tuberculosis transmission in a low-endemic country [11,12].

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Until recently, molecular diagnostic methods were considered as too expensive, too slow, being associated with high contamination rates, too complicated and/or poorly reproducible. To demonstrate that these ideas belong to the past, we share in this article our 10 years' experience with our automated molecular diagnostic platform. We also review progresses in molecular diagnostic microbiology essentially discussing: why automation is important, how our platform was implemented, how our homemade PCRs were developed as well as the advantages and disadvantages of homemade PCRs, including the critical aspects of troubleshooting and quality assessment and the need to further reduce the TAT for specific samples, at least for defined clinical settings such as emergencies. Finally, we conclude with the positive impact of automation on technician workload and we discuss the future of molecular diagnosis as envisioned in our laboratory.

The molecular diagnostic laboratory of the Institute of Microbiology at the University Hospital of Lausanne (Switzerland) was created to provide a comprehensive panel of NA-based tests, especially dedicated to fastidious microorganisms. It was mainly based on in-house tests performed manually by laboratory technicians. In 2004, we realized that the constantly increasing number of tests to detect fastidious intracellular bacteria as well as the foreseen transfer of the whole field of virology necessitated higher automation levels to improve its efficiency and flexibility. This new laboratory became operational in 2005 upon automation of NA extraction as well as that of real-time PCR setup. It now carries out 91 different real-time PCR targeting 69 different pathogens among bacteria, viruses, parasites as well as fungi **(Table 1)**. The use of a single, fast amplification profile allows the simultaneous detection of any pathogen in a single 384-well plate, four-times a day. These plates are assembled in a semi-quantitative or quantitative format, and organized as species or as syndrome panels.

Consistent with the continuously increasing diversity of targeted pathogens **(Table 2 & Supplementary Table 1)**, the number of samples and PCR per year has increased twice and five-fold, respectively, during the last 10 years. Thus in 2014, we tested almost 20,000 samples by performing more than 50,000 PCR tests **(Figure 1)**. As mentioned above, our approach is based on the use of homemade Taqman real-time PCR assays with a common PCR amplification profile. Thus, a large R&D effort was necessary to adapt published assays to this prerequisite amplification profile or to design entirely new assays. Although we are fully aware that such an automated platform based on homemade tests may not be reproduced in some countries due to strict regulatory issues, we think that this model is efficient enough to drive the necessary lobbying needed to keep the autonomy of clinical microbiologists by avoiding over-regulation, which may threaten the flexibility provided by homemade tests. Please note that such homemade tests are performed in Switzerland according to local regulations and strict accreditation as well as national and international quality programs.

Importance of automation

During the last decade, there has been a huge increase in the number of molecular tests requested in our institution because of the added value of molecular diagnosis in a large variety of clinical situations, such as blood-negative endocarditis, meningitidis and sexually transmitted diseases due to *C. trachomatis* and *N. gonorrheae* [2,3,5,13,14]. We also progressively moved from pathogen-oriented diagnosis to a syndrome-oriented diagnosis, which is facilitated by the versatility of molecular diagnostics, hence upgrading our system and further increasing the number of tests performed annually. For example, from 2005 to 2014, we faced an increase of approximately 96% in the number of samples received for molecular diagnosis, 365% in the number of PCR tests performed, whereas,

Table 1. PCR currently available in our routine diagnostic laboratory.

Pathogens	PCR	In-house (%)	Adapted	Target gene	Genus	Species	Bs-PCR
Bacteria	42	20 (48)	22	37	21	26	2
Virus	32	5 (16)	27	25	21	30†	
Fungi	9	4 (44)	5	4	4	8	1
Parasites	8	7 (88)	1	3	4	5	1
All	91	36 (40)	55	69	50	69	4

†From 11 different virus families.
Bs-PCR: Broad spectrum PCR.

Table 2. Assays and targets prepared in our platform.

Pathogens	Target
Acanthamoeba	18S rRNA gene
Adenovirus	Hexon
Anaplasma phagocytophilum	*msp*2 gene
Bacillus anthracis	*pag* gene
	slp1 gene
	cap gene
Aspergillus fumigatus	*cyt*B gene
Bartonnella henselae	*htr*A gene
Bartonnella quintana	*htr*A gene
BK virus	Large T antigen
Bordetella parapertussis	IS1001
Bordetella pertussis	IS481
Brucella sp.	IS711
Candida albicans	ITS2
Candida dubliniensis	ITS2
Candida glabrata	ITS1
Candida kruseii	ITS2
Candida parapsilosis	ITS2
Candida tropicalis	ITS2
Chlamydiales broad range	16S rRNA gene
Chlamydia psittaci/abortus	Intergenic spacer16S-23S rRNA
Chlamydia psittaci	Coding DNA sequence CPSIT_0607
Chlamydia trachomatis	Cryptic plasmid
Chlamydia pneumoniae	Pst-1 fragment
Coronavirus C43	Polymerase
Coronavirus E229	Nucleocapside
Coronavirus NL63	Nucleocapside
Coronavirus HKU1	Polyprotein
Coxiella burnetii	*ompA* gene
Cytomegalovirus	Phosphoprotein 65
Enterovirus[†]	NTR
Epstein–Barr virus	BALF5 (polymerase)
Francisella spp	*Fop*A gene
Francisella tularensis	*Fop*A gene
Fungal broad range	18S rRNA gene
Haemophilus influenzae b/c	*bex*A gene
Haemophilus influenzae	*frd*B gene
Hepatitis D virus	Ribozyme region
Hepatitis E virus	Capsid protein
HSV-1	Glycoprotein B
HSV-2	Glycoprotein B
HHV-6	U65–U66
HHV-8	ORF26
Influenza A[†]	Matrix
Influenza B[†]	Hemaglutin
Influenza H1N1[†]	Hemaglutin
Influenza H5N1	Hemaglutin
JC virus	Large antigen T
Legionella pneumophila	*mip* gene

[†]Currently performed in Lausanne using the GeneXpert system (Cepheid).
HSV-1: Herpes simplex virus type 1; HSV-2: Herpes simplex virus type 2; HHV-6: Human herpes virus 6; HHV-8: Human herpes virus 8.

Table 2. Assays and targets prepared in our platform (cont.).

Pathogens	Target
Leishmania sp.	Kinetoplast DNA
Listeria monocytogenes	*hly*O
Mycobacterium tuberculosis complex	IS6110
Human metapneumovirus	Major nucleocapsid
Mycoplasma hominis	16S rRNA gene
Mycoplasma pneumoniae	*P1* gene
Neisseria gonorrhoeae	*por*A pseudo gene
	opa gene
Neisseria meningitidis	*ctr*A gene
	*ctr*A gene modified
Norovirus 1[†]	*orf1–orf2*
Norovirus 2[†]	*orf1–orf4–6*
Parainfluenza 1	Hematoglutinin neuraminase
Parainfluenza 2	Hematoglutinin neuraminase
Parainfluenza 3	Hematoglutinin neuraminase
Parainfluenza 4	Phosphoprotein Para4A
Parvovirus B19	Capsid protein VP1
Picornavirus	*UTR*
Plasmodium broad range	18S rRNA gene
Plasmodium falciparum	18S rRNA gene
Plasmodium malariae	18S rRNA gene
Plasmodium ovale	18S rRNA gene
Plasmodium vivax	18S rRNA gene
Pneumocystis jirovecii	26S rRNA gene
Rickettsia gr. Typhus	*glt*A gene
Rickettsia sp. broad range	16S rRNA gene
Respiratory syncytial virus A[†]	Major nucleocapside protein
Respiratory syncytial virus B[†]	Major nucleocapside protein
Staphylococcus aureus	*femA* gene
*Staphylococcus mec*A	*mec*A gene
*Staphylococcus mec*C	*mec*C gene
Staphylococcus aureus PVL	*lukS-PV* gene
Streptococcus pneumoniae	Autolysin (*lyt*A gene)
Toxoplasma gondii	*B1* gene
Tropheryma whipplei	Repetitive sequence 1
	Repetitive sequence 2
Ureaplasma urealyticum	16S rRNA gene
Varicella-zoster virus	Thymidine kinase
Yersinia pestis	*pla* pPCP1
	cafl pMT1
	pCD1 plasmid
	Chromosomal *(entF3)*

[†]Currently performed in Lausanne using the GeneXpert system (Cepheid).
HSV-1: Herpes simplex virus type 1; HSV-2: Herpes simplex virus type 2; HHV-6: Human herpes virus 6; HHV-8: Human herpes virus 8.

during the same period, the laboratory staff only increased by 35.9% **(Figure 1)**. We estimated that we have saved four technicians' full-time equivalent (FTE), corresponding to salary costs of approximately US$ 400,000/year thanks to automation; these four FTEs have been used to face increasing activity still requiring hands-on time, and to perform value-added tasks such as data validation, quality control and R&D. Apart from increasing efficiency and reducing costs, other reasons to move toward automation were to increase the sensitivity, specificity and reproducibility of pathogen detection, and to reduce the TAT.

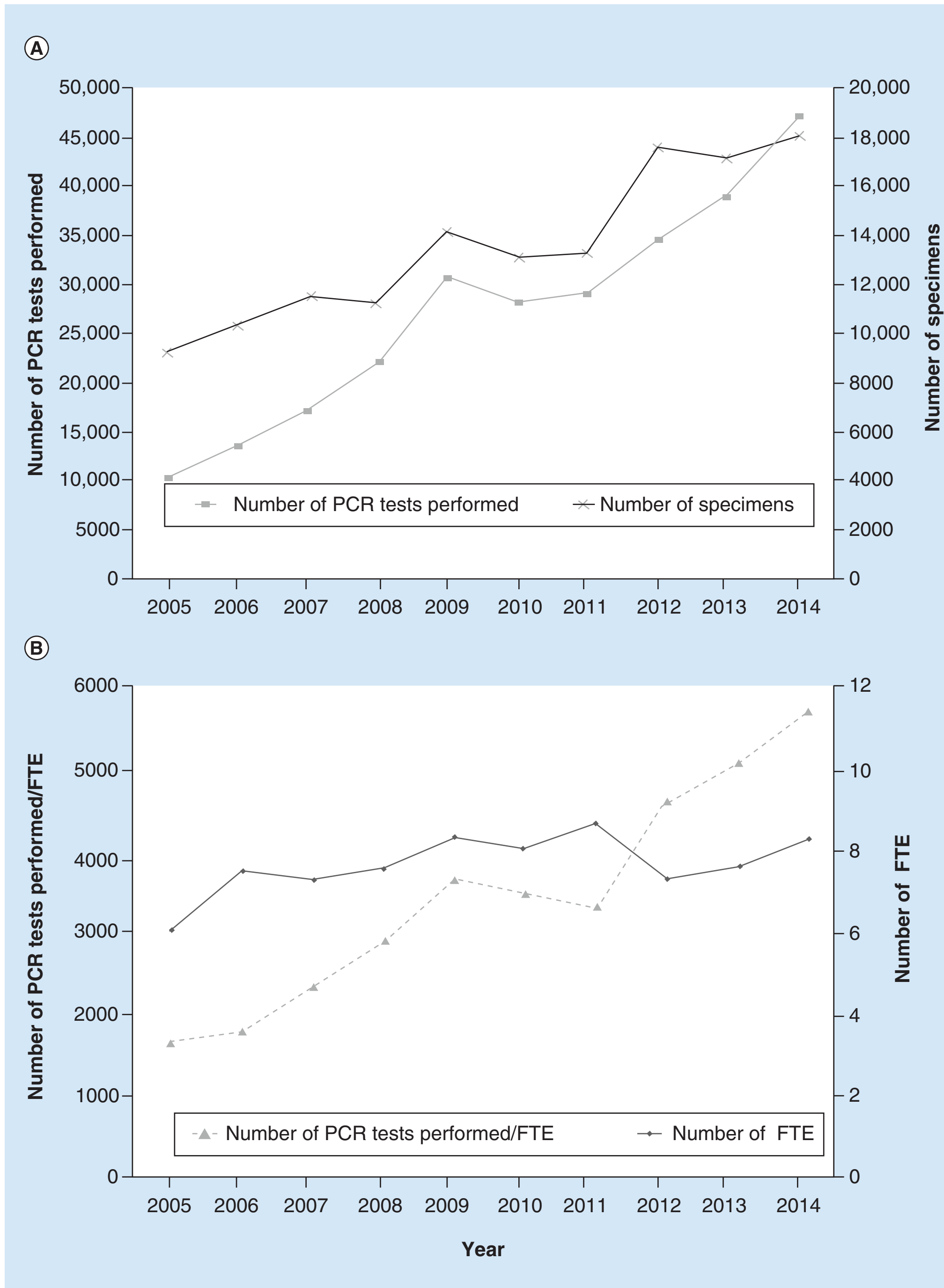

Figure 1. Evolution of the number of specimens received and of tests performed at the Molecular Diagnostic Laboratory of the Institute of Microbiology of Lausanne in relation to the number of full-time equivalent. The period ranges from 2005 (initial implementation of robotics) to 2014. Despite a large increase in the number of samples and tests over this period **(A)**, automation allowed a large increase of number of samples processed/FTE during these 10 years **(B)**. Please note that the number of PCR tests done per sample increased from 1.1 to 2.6 during the same period.
FTE: Full-time equivalent.

Implementation of automation

Automation of molecular diagnosis was based on the availability of real-time PCR in the late 1990s [15–17]. With little fundamental changes, this allowed to move from the low- or medium-throughput, contamination-prone and long TAT of classical PCR, which involved amplicons detection by agarose gel electrophoresis, to the high-throughput amplification and detection in a closed tube offered by real-time PCR. Not only was the risk of vertical contamination due to amplicons generated during previous amplification considerably reduced, but very importantly the TAT was significantly diminished. We also used automated DNA extraction platforms to reduce intersample contaminations during NA extraction and to further reduce both TAT and workload.

• Real-time PCR

The prerequisite of a real-time PCR automated setup used in diagnostics is that the assays must exhibit adequate analytical sensitivity and specificity under a single amplification profile. All assays, therefore, were either established *de novo* or adapted from the literature to conform to the following specifications: identical master mix and $MgCl_2$ concentration, primers and probes adapted to 60°C annealing/synthesis temperature, initial 95°C enzyme activation and template denaturation time (see the 'R&D process & validation' section). For the sake of simplicity, a single manufacturer was selected for the master mix based on price and robustness of reagents (Life Technologies, CA, USA), while the technologies and costs prevailed for oligonucleotides that were sometimes modified with manufacturer's exclusive reagents (primers and probes containing locked nucleic acids [LNA] by Eurogentec, Seraing, Belgium; Minor Groove Binding [MGB] probes by Applied Biosystems; any standard primers and probes, by Microsynth, Balgach, Switzerland, Eurogentec or Applied Biosystems). Thus, our in-house molecular platform currently runs 91 different real-time PCRs that correspond to 69 pathogens (26 bacteria, 30 viruses, 8 fungi and 7 parasites). In addition, the PCRs compatible with our platform also include two resistance-encoding genes (*mec*A and *mec*C of *Staphyloccocus aureus*) and one virulent encoding gene (PVL of *S. aureus*), as well as four broad-spectrum PCR (Fungal, Plasmodium, Rickettsiaceae and Chlamydiales; **Tables 1 & 2**) (for details, see **Supplementary Table 1**). They all have been developed or adapted during the last 10 years through a similar R&D approach. Practically, until December 2011, the PCRs were performed using the Universal master mix (Applied Biosystems) on the ABI 7900HT instrument, with the following cycling conditions: 2 min at 50°C, 10 min at 95°C followed by 45 cycles of 15 sec at 95°C and 1 min at 60°C. Then, in order to accelerate the TAT, since January 2012, we use the Fast advanced Master mix (Applied Biosystems) on the ABI 7900HT instrument (Applied Biosystems) with the following cycling conditions: 2 min at 50°C, 10 min at 95°C followed by 45 cycles of 1 sec at 95°C and 20 sec at 60°C. This reduced the time of the PCR from 2 h and 20 min to 1 h. Fast cycling also improved amplification of structurally complex targets, often G+C rich, like that of *M. tuberculosis* and hepatitis D virus. While the initial 95°C denaturation time seems proportionally too long for the fast procedure, it was necessary for the correct assessment of viruses with short circular genomes in a supercoiled structure that are more difficult to quantitatively convert to a PCR template. Similarly, the extended 10-min denaturation time was needed for adequate plasmid denaturation.

Pipetting of 384-well plates on a regular basis could only be achieved using a liquid handling system. Our first robot was from TECAN (Freedom EVO 150, TECAN, Männedorf, Switzerland) equipped with an 8-channel LiHa arm. This robot was initially selected because aspirated and dispensed volumes were determined by a water column. It was felt that in any contamination event, the whole system could be cleaned if necessary. This robot was very reliable, however, its maintenance was expensive and the liquid displacement technology was more prone to errors that led to invalid runs in particular early after setup **(Table 3)**. This rate decreased considerably (from 3 to 1.1%) after exchange of the LiHA arm to a newer version. The Tecan system has recently been replaced in our laboratory by two robots from Hamilton (Microlab Star, Hamilton, Bonaduz, Switzerland) equipped with air displacement channels. Based on our previous experience with the Tecan robot, we were confident that the robot itself did not contribute to contaminations **(Table 3)**, and that the liquid displacement system was not necessary other than to provide excellent pipetting performances. We could therefore confidently select robots with an air displacement system, reducing considerably the daily maintenance that was otherwise necessary with the liquid displacement arm. Invalid runs associated with robotic errors have now virtually disappeared, reflecting either a technological

Table 3. Evaluation of the reliability of our automated molecular diagnostic setup: in 2009 among a total of 7056 runs, 521 (7.4%) were invalid, whereas in 2014 among a total of 8719 runs, 394 (4.5%) were invalid.

Type of troubleshootings	2009		2014	
	n	%	n	%
Liquid handling system problems	217	3	97	1.1
Master mix issues	18	0.2	7	0.08
Procedural problems (human errors)	81	1.1	100	1.15
Negativity of the 10 copies positive control	59	0.08	135	1.5
Primers and probes issues	19	0.26	6	0.07
Contaminations investigated	127	1.8	49†	0.56
Contaminations	na	na	15†	0.17

†15 were finally considered as contamination. The 34 others were not representing a contamination event but corresponded to a weak transient fluorescent signal, slightly above the cut-off.

improvement of the robots themselves or accumulated experience of the users. Other causes of real-time PCR invalid runs included human procedural errors (1.1%), defective primers and probes (0.26%), use of a defective real-time PCR master mix (0.2%) and progressive degradation of the positive control (0.08%). The relatively high rate of invalid runs due to a defective master mix (0.2%) observed in 2009, was solved by changing manufacturer and adopting the master mixes from Life Technologies (still used now), which was very reliable with very low lot-to-lot variation. Standard curves with tenfold dilution of positive control plasmids were used to provide quantitative results (see the section 'Positive & inhibition controls: the plasmid strategy'). For long-term storage, these control plasmids were adjusted at 10^8 copies/µl in a background of 10 ng/ml of human DNA and were aliquoted in low binding vials to increase stability of plasmidic DNA. As an example of the robustness of the PCR reactions, **Figure 2** shows the reproducibility of the *C. trachomatis* PCR positive controls containing tenfold dilution of *Chlamydia* plasmids corresponding to 1000, 100 and 10 copies per reaction that were tested from 2009 to 2014, respectively. Precise copy number estimate is possible through the prolonged initial denaturation time in the PCR reactions, which by creating nicks in the DNA renders all DNA molecules structurally similar (loss of supercoiling of the positive controls or that of small viral genomes and bacterial plasmids).

With a run being defined as a set of reactions regarding a single pathogen within a plate, contamination events (i.e., false-positive reaction or false-positive negative control) were detected in 127 out of 7056 runs (1.8%) in 2009 and decreased to 49 out of 8719 runs (0.56%) in 2014 **(Table 3)**. Out of the 49 events recorded in 2014, 15 corresponded to samples suspected of being contaminated because there was only a single weak positive PCR reaction out of 3. In order to differentiate between a very weak positive result and a contamination, the DNA was extracted from another aliquot of the sample and ten PCRs were performed on the newly extracted DNA together with ten PCRs on the original DNA. If the 20 reactions were all found negative, the original weakly positive signal was considered as corresponding to a false-positive result likely due to a contamination **(Box 1A)**. For 31 events, the DNA extraction negative control was positive, suggesting a pre-analytical contamination issue **(Box 1B)**. The remaining three events concerned three no template negative controls associated with two different probes that were greater than 2 years old, and were proven defective on troubleshooting **(Box 1C)**. These probes were immediately reordered. Our local experience shows that, currently, contamination issues are no longer due to amplicons (vertical contamination), but rather associated mainly with suboptimal handling of highly positive samples, exposing simultaneously processed negative samples to horizontal contamination.

• Nucleic acid extraction

Regarding DNA extraction, we initially used different standalone automated systems such as Magnapure LC (Roche) or EasyMag (bioMérieux) together with manual extraction with QIAgen kits. To further increase our throughput, a fully automated NA extraction capable of processing 96 samples in an hour was recently implemented. This system is composed of two robots: a STARlet liquid handling (Hamilton®) to distribute samples using barcodes into the NA extraction plate coupled to a MagNAPure 96®

(Roche) instrument in which NA extraction actually takes place. NAs are extracted from 200 µl of specimen, such as flocked swabs, EDTA blood, grounded biopsies and liquefied sputa. These instruments were validated in order to guarantee correct and consistent extraction of DNA or RNA from a variety of pathogens [18]. The absence of cross-contamination with this new extraction system was tested with samples exhibiting a very high viral load (10^9 to 10^{12} DNA copies/ml) of BK virus, VZV, HSV and Parvovirus B19. The NA extraction efficiency of the MagNAPure 96® system (Roche) was compared with that of the MagNAPure® LC (Roche) and that of the EasyMag® (BioMérieux) for DNA and RNA respectively on 108 specimens. After extraction, NA were amplified by home-developed real-time PCR on the ABI 7900® (Applied Biosystems). Efficiency was evaluated by comparing the cycle threshold (Ct) values according to the extraction method. For 66% of specimens (71/108), the agreement between the M96 and the other extraction instruments (difference of copies/ml) was <0.5 log, and for 90% of specimens (97/108) <1 log. Major discrepancies were found at the LOD and can be attributed to the stochastic distribution of the NAs in the tube. Other high-throughput extraction systems are currently available such as the AutoMate Express™ Nucleic Acid Extraction System (Applied Biosystems) and the QIAsymphony® (Qiagen). The authors think that each automated system needs to be thoroughly tested before being adopted.

- **Laboratory information technology**

Information technology (IT) is key for automation. We are using the commercially available Laboratory Information System (LIS), MOLIS (CompuGroup Medical, AG), which is used by most of the clinical laboratories of our hospital, including the hematology, the clinical chemistry and the immunology laboratory. Practically, we had to adapt automation to MOLIS rather than the converse. Multiple layers of middlewares were sometimes needed for managing the connections between different instruments. However, some unexpected IT incompatibilities remain which explains that this expanded platform is not yet fully automated and that we still manually prepare PCR master mixes on a daily basis.

Nevertheless all tasks could be progressively automated. As an example, until the 30 June 2014, PCR template files were created for the barcoded microamp optical 384-well reaction plates (Applied Biosystems), using the 2.4 SDS software (Applied Biosystems). The template files were exported in text formats (.txt)

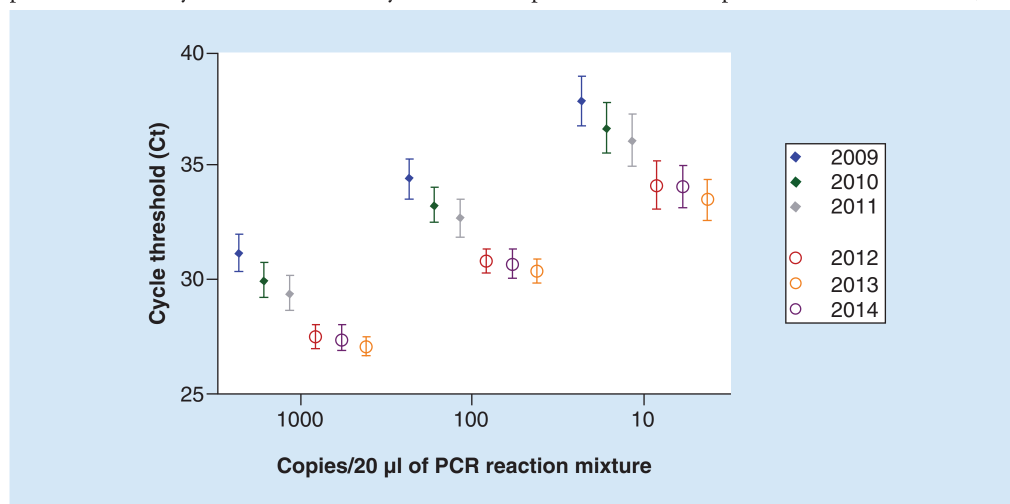

Figure 2. Analytical sensitivity and reproducibility of real-time PCR. As an example, the in-house *Chlamydia trachomatis* assay, which was done from 2009 to 2011 (785 runs) using the Universal master mix from Applied Biosystems (diamond symbol), and from 2012 to 2014 (809 runs) using the Fast Advanced master mix (also from Applied Biosystems; circle symbol). Please note that both kits exhibited a good analytical sensitivity since they could both detect ten copies of DNA; however, the Fast advanced master mix exhibited a significantly lower Ct value, of about two to three cycles less, representing an improved sensitivity of about 1 log DNA copies. The exact data are provided in a table in the **Supplementary Material**.

Box 1. Algorithms used in the presence of a possible contamination.

(A) Algorithm performed to assess whether a putative contamination is a weak positive sample or if there is a contamination during the pre-analytical or analytic processes (for example: 1 reaction positive out of 3 performed reactions)

- Check if there is a strong positive sample in the same run of the pre-analytical process, in the extraction run or in the amplification run that could explain an horizontal contamination
- Extract another aliquot of the native sample
- Perform in parallel ten PCR with the 'old' extracted DNA and ten additional PCR with the 'newly' extracted DNA in order to check for any additional positive reaction
- If at least one additional PCR reaction with the newly extracted DNA is positive, this signs the (weak) positivity of the sample
- If only the 'old' extracted DNA is positive, this likely represents a contamination during the preanalytical step or during the extraction step
- If no reaction is positive, this likely represents a contamination

(B) Algorithm performed when a contamination in the extraction process is suspected (for example: negative extraction control exhibits a positive signal)

- Perform five negative extraction controls (i.e., PBS instead of sample) and then test them in triplicate with the incriminated PCR
- If at least one PCR reaction is positive, transiently stop using this PCR for diagnostic purposes and throw away all the reagents of the extraction procedure
- Once new reagents are ready, check again using the same procedure
- If the contamination persists with new reagents, sequence the PCR products in order to check whether or not the contaminating DNA exhibits the four nucleotides that were added in all plasmid-positive controls (this helps to differentiate a plasmidic contamination from a chromosomic contamination)

(C) Algorithm performed when a contamination in the reagent of the PCR and/or in the DNA-free laboratory is suspected (for example: negative PCR control exhibits a positive signal)

- Perform 50 negative PCR controls of the incriminated PCR (i.e., reactions containing all the reagents of the PCR reaction with water instead of DNA)
- If at least one PCR reaction is positive, stop the PCR, through away all the reagents and decontaminate the laboratory again
- Once new reagents are ready, check again using the same procedure
- If the low positive result persists with new reagents, sequence the PCR product in order to check whether or not it exhibits the four nucleotides that were added in all our plasmid-positive controls

to the Gemini software (Tecan, Männedorf, Switzerland). The master mixes and the DNA (or cDNA for RNA viruses) were then distributed into the 384-well plate by the liquid handling Tecan Freedom Evo 150 8 channels, according to the text files exported to Gemini software. Since we changed robotic system on 1 July 2014, PCR template files are now created using the 2.4 SDS software but are then exported in text file format to the Venus three software (Hamilton, Bonaduz, Switzerland). The master mixes and the DNA (or cDNA for RNA viruses) are then distributed into the 384-well plate by the air handling system Microlab STAR®, 8 channels (Hamilton), according to the text files exported to Venus three software. The flexibility of our system allowed us to change robotic system and provider without any problem in this evolving field.

Thanks to barcoding, samples can be traced through all steps of the procedure, in other words, from the NA extraction to the distribution of the PCR mix in the 384-well plate and finally during the amplification **(Figure 3)**. Once introduced into the system, no sample inversion can occur.

• Reducing PCR contamination

The risk of contamination has been substantially reduced by using real-time PCR. Nevertheless, to further prevent PCR contamination, we use the following different rooms for corresponding procedures: 'DNA-free room', in other words, for master mixes preparation, 'nucleic acid extraction room' as well as 'amplification room' and 'postamplification room'. Moreover, the sample reception and processing unit is considered as one of the most important step of the procedure **(Figure 3A)**. Strict precautions such as wearing gloves, wearing laboratory coats as well as using separated and dedicated flowhoods (for the physiologically sterile samples vs other samples) have been implemented to prevent contamination between different samples during pre-analytical steps. When a contamination is suspected, a procedure is initiated and notified (see **Box 1** for detailed procedure). Practically, the idea is to be able to quickly distinguish between a true contamination (where the 20 additional PCR tests will all be negative) versus a low positive sample, positive in a single reaction well due to stochastic distribution of the microbial DNA.

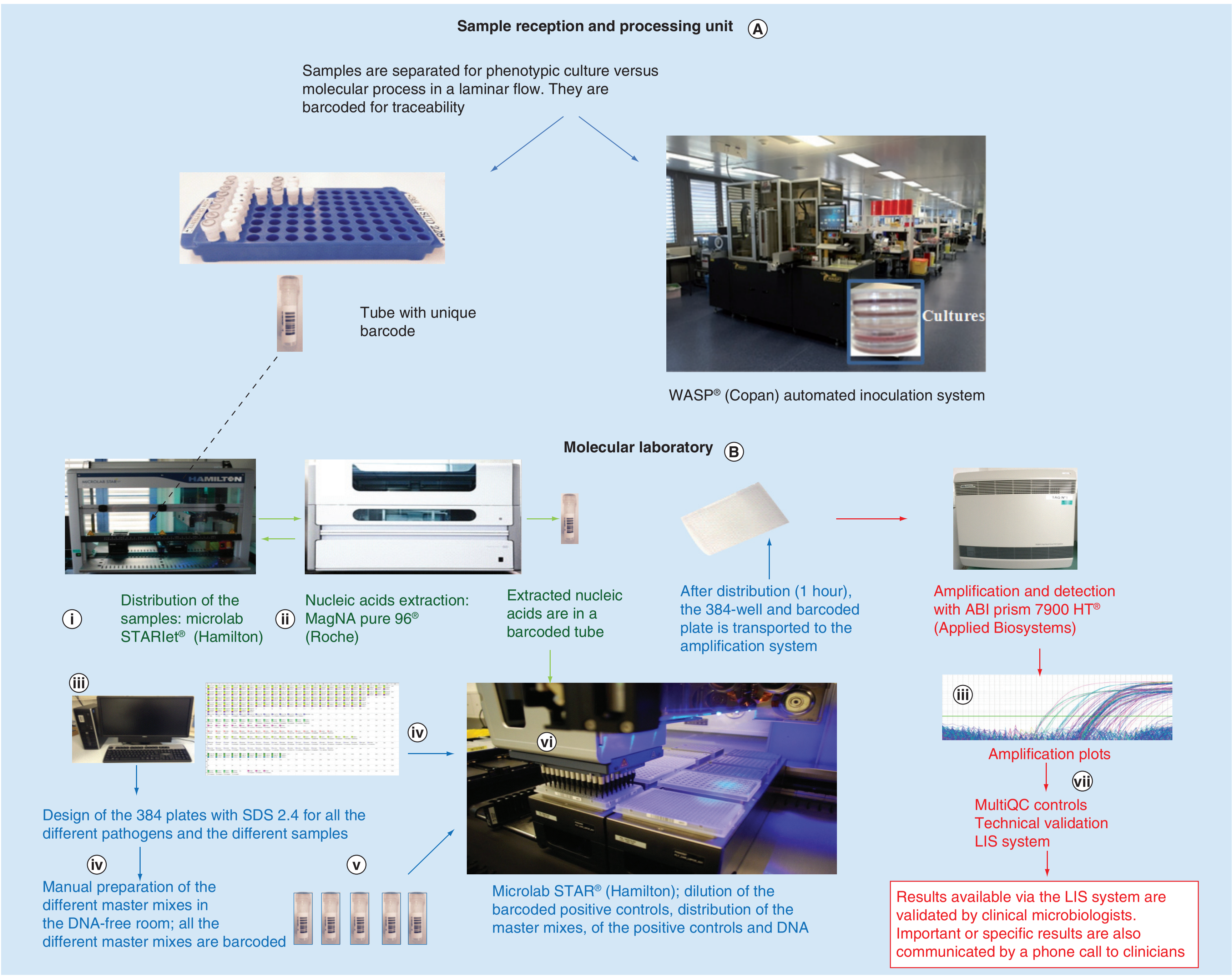
Sample reception and processing unit (A)
Samples are separated for phenotypic culture versus molecular process in a laminar flow. They are barcoded for traceability
Tube with unique barcode
Cultures
WASP® (Copan) automated inoculation system
Molecular laboratory (B)
(i) Distribution of the samples: microlab STARlet® (Hamilton)
(ii) Nucleic acids extraction: MagNA pure 96® (Roche)
Extracted nucleic acids are in a barcoded tube
After distribution (1 hour), the 384-well and barcoded plate is transported to the amplification system
Amplification and detection with ABI prism 7900 HT® (Applied Biosystems)
(iii)
(iv)
Design of the 384 plates with SDS 2.4 for all the different pathogens and the different samples
(iv) Manual preparation of the different master mixes in the DNA-free room; all the different master mixes are barcoded
(v)
(vi) Microlab STAR® (Hamilton); dilution of the barcoded positive controls, distribution of the master mixes, of the positive controls and DNA
(iii) Amplification plots
(vii) MultiQC controls
Technical validation
LIS system
Results available via the LIS system are validated by clinical microbiologists. Important or specific results are also communicated by a phone call to clinicians

Figure 3. Workflow of the Lausanne automated platform (see facing page). Samples are barcoded already at the sample reception unit **(A)** and then delivered to the molecular diagnostic laboratory **(B)**. Step 1 (green arrows and bullets): nucleic acid extraction. Each barcoded tube containing the sample is scanned automatically by the Microlab STARlet® (Hamilton) controlled by a PSH-Roche software **(i)** that will create an xml file (sample list) **(ii)** for the preparation of the extraction plate. This file is then imported by the MagNA Pure® software for the extraction procedure. After the end of extraction, the xml file will be imported back to the Microlab STARlet® in order to transfer the DNA extracted in the individual tubes with the 'same unique barcode'. Step 2 (blue arrows and bullets): template creation for the 384-well plate for ABI 7900HT® (Applied Biosystems). The SDS 2.4.1 software (Applied Biosystems) **(iii)** is used to create the template for the 384-well plate assembly and is then exported as a text file **(iv)** to an access database to create a worksheet **(v)** in order to prepare the different master mixes (one for each target). The master mixes are prepared manually in barcoded tubes, each of these corresponding to a specific PCR. Then the same text file is also exported to the Venus three software (Hamilton, Bonaduz, Switzerland) **(vi)** that drives the Hamilton robot. Finally the master mixes and the DNA (or cDNA for RNA viruses) are distributed into the 384-well plate by the robot according to the exported text file **(iv)** corresponding to the 384-well template. The SDS 2.4.1 software (Applied Biosystems) **(iii)** is used by the technicians to interprete the amplifications. The results (qualitative and quantitative) are then checked and introduced into the LIS system. At the end of the process the final results are validated by a clinical microbiologist. Regarding the monitoring of positive controls, the SDS 2.4.1 file is exported through an Excel file **(vii)** to the multiQC software.
LIS: Laboratory information system.

Practically in 2014, we investigated 49 out of 8719 runs for putative contaminations **(Table 3)**.

• Positive & inhibition controls: the plasmid strategy

In order to gauge the accuracy and reproducibility of all PCRs, positive, inhibition and negative controls are tested in each run. The dilution of the positive controls as well as the DNA spiking for the inhibition controls are done by our automated handling system in order to avoid contamination and to reduce hands-on time.

To avoid contamination issues that could be associated with in-house production, the positive control plasmids are synthesized *in vitro* by a manufacturer. Each plasmid carries the corresponding PCR target with modification of four nucleotides that distinguish it from its natural counterpart (see the ' R&D process and validation' section). This allows evaluating by DNA sequencing if contaminants are from this plasmid or from the samples themselves, when necessary. Then, sets of corresponding tenfold dilutions of plasmid-positive controls are added in each run. They correspond to 1000, 100, 10 copies/reaction that translates to 100,000, 10,000 and 1000 copies/ml of the original clinical sample. They not only allow semi-quantification of the pathogen in clinical samples, but also allow the continuous evaluation of the sensitivity and efficacy of each assay, as well as intrarun and inter-run reproducibility.

The plasmid-positive controls strategy also provides quantitative results for different important clinical situations such as systemic viral infections, that helps in distinguishing symptomatic from asymptomatic infection, providing important information about the disease progression and/or helps in assessing the efficacy of antiviral therapy [19]. Identically, in many situations, the therapeutic response of treated patients could be monitored with quantitative PCR for pathogens such as *Plasmodium*, *Leishmania* and many others [20–22]. Even for bacterial pathogens present in lower respiratory tract samples, semi-quantification by real-time PCR helps predicting disease progression, as recently described [23].

Samples may be false negative due to inhibitory molecules co-purifying with the NAs [24]. Presence of these inhibitors is monitored by spiking 200 copies of the corresponding target plasmid DNA (in 1 μl) to DNA of each specimen (4 μl). Inhibition is considered when the copy numbers was below 50 copies per reaction. Under this condition, a single freeze–thaw cycle of the original sample NAs was often sufficient to revert the inhibition. If this is not enough to remove inhibitors, NAs are re-extracted and the whole PCR is repeated.

Regarding negative control, each run of NA extraction included a control that contained PBS instead of NAs and that closely mimics the entire NA extraction process. One such control is used per batch of extractions and is systematically subjected to each target PCR. In addition to this extraction control, a no template water control is used to detect any reagent contamination at the time of PCR setup.

After PCR, the ABI 7900 file is evaluated by the technicians and the results table is exported as a result's text file. This file is

converted automatically via an in-house Excel Visual Basic for Applications (VBA) to a temporary file that can be interpreted by MultiQC (developed by Philippe Marquis, France), a quality monitoring program. For each target, the Ct values of controls as well as the standard curve slope are thus imported and interpreted statistically by MultiQC as a function of time. Technicians can then analyze the displayed results to check the analytical parameters of each PCR as well as on a long-term basis to monitor drifts in performance that may justify preventive measures such as implementing a new batch of reagents ([25,26].

R&D process & validation

There are numerous homemade PCR that have been developed for the detection of individual microbial agents and proper validation and standardization are mandatory. Validation has to be performed at several levels such as extraction procedure, amplification and detection on clinical specimens and according to the accreditation rules, and internationally recognized recommendations [27]. For quantitative PCR in particular, one may refer to the MIQE guideline [28].

In this context, each parameter that was developed was first evaluated through an R&D process and validated by our R&D committee. Over the years, we developed a 'to-do' list that helps completion of the R&D process **(Box 2)**.

To select and prioritize the PCR to be developed, the project leader of each new proposal had to convince the R&D committee that the pathogen is important enough in terms of prevalence and/or critical importance to deserve a development and that analytical and clinical validation may be performed adequately, according to internationally recognized guidelines as mentioned above. This necessitated a collaborative effort, local expertise and/or networking to collect enough clinical samples with clinical information for proper validation of the test. For instance, to develop our *Chlamydia psittaci*/*Chlamydia abortus* duplex PCR, we obtained clinical samples from partners from Belgium, south Australia and the UK [32]. The choice of the developed PCRs was largely dependent on the clinical needs, but was also influenced by the available PCR that were published at that time and by the field of expertise of the different project leaders. Thus in Lausanne, we mainly developed in-house PCRs for bacterial agents (n = 20; 48% of all bacterial PCR), for parasites (n = 7; 88% of parasitic PCRs) and fungi (n = 4; 44% of all fungal PCR). For viruses, we mainly adapted PCRs (27; 84% of all viral PCRs) and developed 16% of all 32 PCRs targeting viral NAs, due mainly to the fact that real-time PCRs were developed earlier in virology than in the other fields **(Table 1)**.

The second step was to search in the literature if primers and probes targeting the pathogen were already described and fulfilled our requirements in terms of sensitivity and specificity, as well as compatibility with the prerequisites of our platform (i.e., Tm at 60°C, short amplicon and small reaction volume of 20 µl). If all these points were satisfied, it was then possible to implement this PCR. Even when the PCR was adapted from a previous publication, we *in silico* checked the primers and probe sequences with sequences available on the NCBI website. Known positive clinical specimens were used for the initial evaluation of a newly developed or implemented PCR assay. For instance, cardiac valves were used for *Coxiella burnetti* [26], positive urine for *C. trachomatis* [5] or a pleural fluid for *Mycoplasma hominis* [34]. Then, in order to have a sustainable amount of positive controls and to allow quantification, plasmid containing the specific target sequence was synthesized *in silico* or by cloning as previously described [5,35]. To avoid contamination, the production of plasmids was outsourced to R&D Biotech (Besançon, France). The intrarun and inter-run reproducibility were assessed once for each plasmid as described [5,26,35]. Briefly, tenfold dilutions (10^5 copies to 1 copy/µl) were used to check the reproducibility and sensitivity of detection. Then a Bland–Altman graph was used to control the reproducibility of the test. Moreover, the sensitivity of detection was for each pathogen evaluated on 20 replicates for 50, 20, 5, 1 and 0.5 DNA copies per reaction [26,35]. Usually the PCR showed 100% detection for 50 and 20 DNA copies, and then lower percentage for 5, 1 and 0.5 DNA copies per reaction. Positive controls were used to derive a standard curve, and samples were all analyzed in duplicate.

Several published PCR that appeared (at least *in silico*) specific and sensitive by amplifying all strains of a given species at the time of publication displayed reduced specificity or sensitivity after a few years, since some yet unexplained

biodiversity of pathogens are discovered with corresponding sequences appearing in databases such as NCBI [29].

On the other hand, if (as it was often the case) no PCR was developed for a given pathogen, or if the method described did not convince us, we developed ourselves the PCR. In this latter case, the crucial choice of the target gene was done by our expert in the field. The choice of the target gene was also based (among others) on the number of copies to enhance the sensitivity, the polymorphism of the gene and the number of sequences on database such as NCBI. The primers and probes were chosen mainly with the Primer Express Software (Applied Biosystems) and their expected behavior visualized during hybridization via software such as Meltcalc [30] in order to have a first idea on specificity of the target gene (check *in silico*). Steps that we follow are listed in **Box 2**.

The average cost for the development of a given new real-time PCR was estimated at 1600 Euro for reagents (i.e., primers, probes, plasmids, master mix) and 2240 Euro for technician work corresponding approximately to 1 day per week for 1 month's work (32 h at 70 Euro/h). The academic work, in other words, literature, primers, probes, plasmids design is not included in this calculation.

Current limits of real-time PCR: some specific samples

Despite the high sensitivity and specificity of the real-time PCR, the molecular diagnosis still exhibits some limitations either due to the low number of pathogen present in some samples or due to PCR inhibition. To illustrate this we shortly address four specific situations: the blood as a sample, the molecular diagnosis of sepsis directly from blood, the fungal infections and the mycobacterial infections.

• Blood sample

Cytomegalovirus (CMV) and EBV DNAmia in transplant patients are regularly tested to prevent post-transplant disease. DNA extraction is done directly from 200 µl EDTA blood. A large number of leukocytes can either inhibit the PCR reaction or their DNA can disturb the process of DNA extraction by saturating the magnetic beads. Consequently, the amount of leukocytes in the concomitant blood of the patient (measured by the hematology laboratory) is checked for each sample and if it exceeds 10 g/l, the DNA extraction and the PCR is repeated on fivefold diluted blood. If the leukocyte count is not available, we warn clinicians of possible lower sensitivity by a sentence accompanying the results on the report form.

• Molecular diagnosis of sepsis directly from blood

There is a need to accelerate the microbial diagnosis of sepsis [24]. Blood cultures are able to test a large volume of blood, 10 ml per bottle and remain the gold standard reference method even so they have their own limitations [36]. Many molecular tests, applicable to blood have been recently developed, but so far none can fully replace the traditional blood culture method [37]. Indeed, the major problem is the low number of bacteria that circulate in the blood in most cases, in other words, 1–10 CFU/ml, which makes the probability of target bacterial DNA to be in the PCR reaction very low when starting from 200 µl of blood. To overcome this problem, blood should be concentrated prior to DNA extraction (see review by Opota *et al.* [24]). However, in Lausanne, PCR on blood is only validated for a few bacteria species such as *Rickettsia*, *Coxiella*, *Bartonella* and *Tropheryma whipplei*.

• Fungal infections

The fungal DNA extraction is difficult and we use the MagNA Lyser instrument (Roche) based on a bead-beating technology before extracting the DNA. The MagNA Lyser instrument is a small benchtop instrument with disposable 2 ml tubes containing ceramic beads. Once the sample is added to the ceramic beads tube, they are vigorously shaken in the instrument in order to disrupt the tissues and fungi. Then the samples are transferred to the MagNA Pure 96 instrument for DNA isolation. For blood, we also use the Red Blood Cell Lysis Buffer® (Roche) in order to lyse the red blood cells before bead-based lysis and extraction. But for patients with suspected invasive aspergillosis, with usually low DNA burden present in most clinical samples, the sensitivity is an issue and we therefore also inform the clinicians of the low negative predictive value of the PCR in such situations [38].

• Mycobacterial infections

Similar to *Aspergillus* infection, *Mycobacterium tuberculosis* infection is often paucibacillary

Box 2. To do list for the R&D process of a new diagnostic PCR (as used in our laboratory in Lausanne for all new PCR developments).

Define the objectives of the test
- What will be the added value of the new test, is another test or an alternative test available?

Literature search on the topic
- Does the test already exist? Are the primers and the probes described? Does the PCR fit our pre-requirement? Is the analysis of the specificity ok? Is the sensitivity of the PCR ok?

If yes to all those points
- Check the *in silico* specificity of the primers and the probe on NCBI, to take into account the new data as published PCR that appeared specific at the time of publication came out to be no more specific after a few years, since some yet unexplained biodiversity of sequences appear in databases such as NCBI in the meanwhile [29]
- Check also the primers and probe behavior during hybridization via Melticalc for example [30]
- Then decide to use the describe PCR or to develop a new one

If nothing exists in the literature
- Identify the target gene that could better suit the project according to the specificity, number of copies and so on. This may be done by literature search and/or by comparative genomics [97].

Primers & probe choice

In silico specificity
- Select the target gene by searching on GeneBank the sequences available from American Type Culture Collection (ATCC) strains that are representative of the species that was defined to be targeted
- Align the sequences with a good software; select the target region according to the expected specificity (for example, primer express), select primers and probe
- Visualize the behavior of the probe during hybridization (Melticalc for example)
- Use the BLAST software on Genbank to ensure the specificity of the primers and probe
- According to the project (monoplex, multiplex), choose the fluorochromes and quenchers
- Order primers and probe
- Order the plasmids corresponding to the targeted sequence with a four-base modification (as described in the main text)

NA extraction procedure
- Automated system will be preferred
- Check whether a prelysis step is required
- Assessment of the type of sample to be extracted
- If available, use a reference strain or a clinical specimen for the first tests. Quantify the extracted DNA
- Then dilute the extracted NA to reach the limit of sensitivity

Analytical performance of the test

Specificity
- Optimization of the primers and probe concentration using low quantity of DNA with decreasing concentration of primers and probe, in other words, 0.1/0.1, 0.05/0.1; 0.2/0.4 μM
- Select concentrations of primers and probe with maximal analytical sensitivity. With the chosen concentrations of primers and probe, check the specificity on DNA from ATCC strains, clinical strain and clinically positive specimens. The amount of DNA to be tested ranged from 1 to 10 ng per strain

Sensitivity
- Test the developed PCR on tenfold dilutions of a positive control (plasmid, ATCC strain, etc.)
- Test a decreasing concentration of plasmidic DNA ranging from 10,000 to 0.1 copies per well in order to obtain the sensitivity limit of the method
- Repeat this test five-times to ensure reproducibility
- Define inter and intrarun variability by testing a 1000, 100 and 10 copies positive control in ten independent runs. Then test 20 duplicates of a 100, 50, 20, 10, 5, 2, 1 and 0.5 copies positive control

Box 2. To do list for the R&D process of a new diagnostic PCR (as used in our laboratory in Lausanne for all new PCR developments).

Clinical performance of the test
- Run the developed test on positive and negative specimen already available for the targeted pathogens (retrospective analysis)
- Run the developed test prospectively during the time necessary to have enough positive and negative specimen for a robust validation (prospective analysis)
- Compare the developed test to another intensively tested molecular commercial assay if available [5] or to other reference tests [1,31]
- Run the developed test on external quality controls
- Ask for specimen to international collaborators if needed to have enough positive and negative specimens and to check that the PCR also detect possible variant prevalent in another country [26,32,33]

After starting routine use of any new PCR, analyze the results, especially focusing on clinically discordant results

and the result of PCR do not replace the mycobacterial culture that should be run in parallel and still represents the gold standard even so PCR greatly accelerates the microbial diagnosis [39,40].

The syndromic approach & the help of broad-spectrum PCR

Using real-time PCR routinely for years on various samples with excellent accuracy let us shift to a molecular syndromic approach. Thus, a panel of PCR targeting different micro-organisms frequently involved in a given clinical situation can be tested on a single specimen **(Table 4)**, during the same day. Since all the PCRs are developed for a single amplification profile, it is easy to be flexible and to react quickly when facing a problem. Thus, for example we moved to a duplex *Coxiella–Legionella* PCR to a monoplex *Coxiella* PCR during the Q fever outbreak that occurred in 2012 in Lavaux near Lausanne in order to test the large number of samples received at that time (>2000 were tested in 4 months for *Coxiella* [26]).

On the other hand, when cultures are remaining dramatically negative, especially if intracellular or difficult to grow bacteria are responsible of the disease [14] or when antibiotics were administered before sampling, we used in a second step broad-range PCRs to identify unexpected pathogens [14,33,34,79,84,85].

Time to results

One of the major goals of molecular diagnosis automation was to shorten the time to result to less than 1 day after reception of the sample, in order to positively impact patients' management. This was achieved by performing at least two runs of NA extraction per day on the MagnaPure 96 system **(Figure 4)**; the first one at 8:00 for the samples arriving late in the former afternoon or overnight (**Figure 4**, E1), the second one at noon for the sample arriving in the morning (**Figure 4**, E2). Then, the extracted NAs targeting the different pathogens were amplified on four different runs of real-time PCR using the 384 plates: early in the morning for pathogens with high added value in order to get the results rapidly before 12:00 (*M. tuberculosis*, *N. meningitidis*, HSV in cerebral fluids, among others) (**Figure 4**, Run 1), then the second run with results available for clinician around 12:00 (*C. trachomatis, N. gonorrhoeae*, CMV, EBV, HSV, VZV and so on) (**Figure 4**, Run 2), during the afternoon for RNA viruses (retro-transcription was performed in the meanwhile) to get the results at 17:00 (RNA viruses) (**Figure 4**, Run 3) and when needed, the last run with the important late specimen to get the results also the same day (**Figure 4**, Run 4). This algorithm was made possible by using two robots performing the PCR setup in less than 1 h, and with amplification time reduced to an hour thanks to the use of the fast advanced master mix. Thus, a minimum of 4 h are needed from the time of the reception of the sample to biomedical validation. However, as we batch specimens' analyses in four runs, the time to result will mainly depend on the time of arrival of the sample in the laboratory. Thus, for some critical situations for which very rapid results impact decision for hospitalization, isolation or antibiotic treatment of the patients [86], more rapid molecular diagnostic systems such as GeneXpert (Cepheid, CA, USA), may be used as a complementary approach. In Lausanne, the enterovirus, *M. tuberculosis,* norovirus*,* MRSA tests are now proposed 7 days/7 from 07:00 to 22:00. Indeed, the enterovirus test allows to reduce the duration of hospitalization from a median of 4 days to 0.5 day (11 h), with a cost decrease of fivefold, from 3691 euro to 580 euro for the hospital [41], whereas the other

Table 4. Nonexhaustive list of PCR considered adequate for a given clinical situation: automation and standardization allow the syndromic approach of molecular diagnostics.

Clinical syndrome	PCR targets	Comments (ref.)
Meningitis	*Haemophilus influenzae*	[3]
	Listeria monocytogenes	
	Neisseria meningitidis	[3]
	Streptococcus pneumoniae	
	Enterovirus[†]	Perform GeneXpert Enterovirus first; if the result is positive other PCR are generally not indicated [41–45]
	HSV 1–2	
	VZV	
Meningo-encephalitis	*Listeria monocytogenes*	
	Enterovirus[†]	Perform GeneXpert Enterovirus first; if the result is positive other PCR are generally not indicated [41,42]
	HSV1–2	[42–45]
	VZV	[43–45]
	HHV-6	[46]
	JC virus	Immunocompromised patients [45–49]
	EBV	Immunocompromised patients [45–49]
	Toxoplasma	Immunocompromised patients [50]
Respiratory syndrome	Bacteria (mostly pneumonia):	
	– *Chlamydia pneumoniae*	[51,52]
	– *Chlamydia psittaci*	[32]
	– *Coxiella burnetii*	Serology is recommended in paralell since PCR generally becomes negative at the time of seroconversion [26]
	– *Legionella pneumophila*	[1]
	– *Mycoplasma pneumoniae*	[1]
	– *Bordetella pertussis*	[53,54]
	– *Bordetella parapertussis*	[6]
	Virus (mostly bronchitis):	
	– Adenovirus	
	– Coronavirus	
	– Influenzae virus (A+B)[†]	Perform GeneXpert Inf/RSVfirst; if the result is positive other PCR are generally not indicated (except for ICU or immunocompromised patients) [55, 56]
	– Respiratory syncytial virus[†]	Perform GeneXpert Inf/RSVfirst; if the result is positive other PCR are generally not indicated (except for ICU or immunocompromised patients) [55,56]
	– Metapneumovirus	Severly ill, immunocompromised, ICU patients
	– Parainfluenzae virus	Severly ill, immunocompromised, ICU patients
	– Picornavirus	Severly ill, immunocompromised, ICU patients
	– CMV	Immunocompromised patient [57,58]
Suspected tuberculosis	*M. tuberculosis* complex[†]	Perform GeneXpert if urgent. A culture should always be performed (to get a strain for AB susceptibility testing) [11,12,59,60]
Sexually transmitted infection	*Chlamydia trachomatis*	Respiratory specimen only for newborn <3 months [5,61]
	Neisseria gonorrhoeae	Respiratory specimen only for newborn <3 months
	Mycoplasma hominis	

[†]Performed in Lausanne using the GeneXpert system (Cepheid).
BK virus: BK polyomavirus; HHV: Human herpes virus.

Table 4. Nonexhaustive list of PCR considered adequate for a given clinical situation: automation and standardization allow the syndromic approach of molecular diagnostics (cont.).

Clinical syndrome	PCR targets	Comments (ref.)
Sexually transmitted infection (cont.)	*Ureaplasma hominis*	
	HSV1–2	[62–64]
Skin infection	HSV1–2	HSV always performed with VZV [65]
	VZV	
	Rickettsiae sp./group typhus	[33,66]
	Broad-range mycobacterial PCR	[9]
Adenopathies	*Bartonella henselae*	[67]
	Francisella tularensis	[68,69]
	M. tuberculosis complex	[70]
	Toxoplasma gondii	Perform also a serology if infectious mononucleosis is suspected
	EBV	
Systemic (immunosuppressed patients)	Adenovirus	[71]
	BK virus	[72]
	CMV	[72]
	EBV	[73,74]
	HHV-6	[75]
	HHV-8	[76]
	Parvovirus B19	
Endocarditis/systemic infection	*Bartonella henselae/quintana*	[77]
	Coxiella burnetii	May also be performed on sera or EDTA blood. Serology is recommended since PCR is generally becoming negative at the time of seroconversion [26,78]
	Mycoplasma hominis	[79]
	Rickettsiae sp./group typhus	[33]
	Tropheryma whipplei	May also be performed on sera or EDTA blood as well on saliva or stools [80]
Zoonotic infections	*Bacillus anthracis*	Specimens according to the clinical situation [81]
	Bartonella henselae/quintana	[67]
	Brucella sp	[82]
	Chlamydia psitacci	[32]
	Coxiella burnetii	[26]
	Francisella tularensis	[68,69]
	Rickettsiae sp/group typhus	[33]
	Pan-*Chlamydiales* PCR	[83]
	Hepatitis E virus	

[†]Performed in Lausanne using the GeneXpert system (Cepheid).
BK virus: BK polyomavirus; HHV: Human herpes virus.

rapid PCR have a direct impact on decision to isolate a patient or not, to prevent intrahospital spread of these infections. To help the management at the emergency ward, we also proposed the influenza/RSV GeneXpert on a 7 days/7 basis, from 07:00 to 22:00 during the outbreak period. Noteworthy, the use of GeneXpert tests is associated with much higher reagents costs than those of our automated platform. Nevertheless, such higher costs are acceptable for specific situations given the huge impact of these 'rapid miniaturized' PCR for logistic and patient care.

The advantages & disadvantages of homemade PCR

The main advantages and disadvantages of homemade PCR are summarized in **Table 5**. The high throughput is one of the main advantage of our molecular diagnosis platform, which

allows us to run any PCR every day even for one specimen, and to quickly respond to specific needs for which no readily available tests are commercially available, for instance during outbreaks [26,87] or when new pathogens are discovered [88–90]. Moreover, due to the low costs of one PCR reaction (1.5 Euro) we could adapt the PCR format to specific pathogens and/or template to biological specificities. For example, duplicates are normally performed for each specimen, but for pauci-bacillary pathogens such as *M. tuberculosis*, triplicates are performed to increase the tested volume. Currently, there are several automated systems on the market such as Cobas 4800® (Roche), Anyplex® System (Seegene), BD Max® System (Beckton Dickinson) [91–93]. Most of these systems are proposed as fully automated and integrated PCR platform but either exhibit a very low flexibility (i.e., only tests proposed by the manufacturer can be performed using the Cobas 4800 system for example), or exhibit not a complete automation (BD-Max System is still time consuming but has the advantage of being an open platform). These systems use different approaches, target different pathogens without flexibility. Since some requested tests with a high added value are missing in many closed systems, they need then ideally to be developed to fill the gap.

Conclusion & future perspective

In conclusion, the automated platform that we developed in Lausanne **(Figure 3)** allowed us to significantly increase the number of samples and number of tests processed per year with a minimal increase in the number of FTEs. This flexible high-throughput automated system has the advantage to allow testing on a single 384-well format microplate, various combinations of the 69 different pathogens for which a PCR has been adapted or developed. This flexibility in terms of pathogen diversity (bacteria, viruses, parasites and fungi) is mainly provided by the same annealing/synthesis temperature used for all PCRs. Moreover, as compared with most commercial PCRs, the format that we use can provide quantitative results, is cheap and also provide a high level of flexibility. Altogether, this automated platform has been pivotal to help us facing several outbreaks by allowing high-throughput samples testing, but also provided

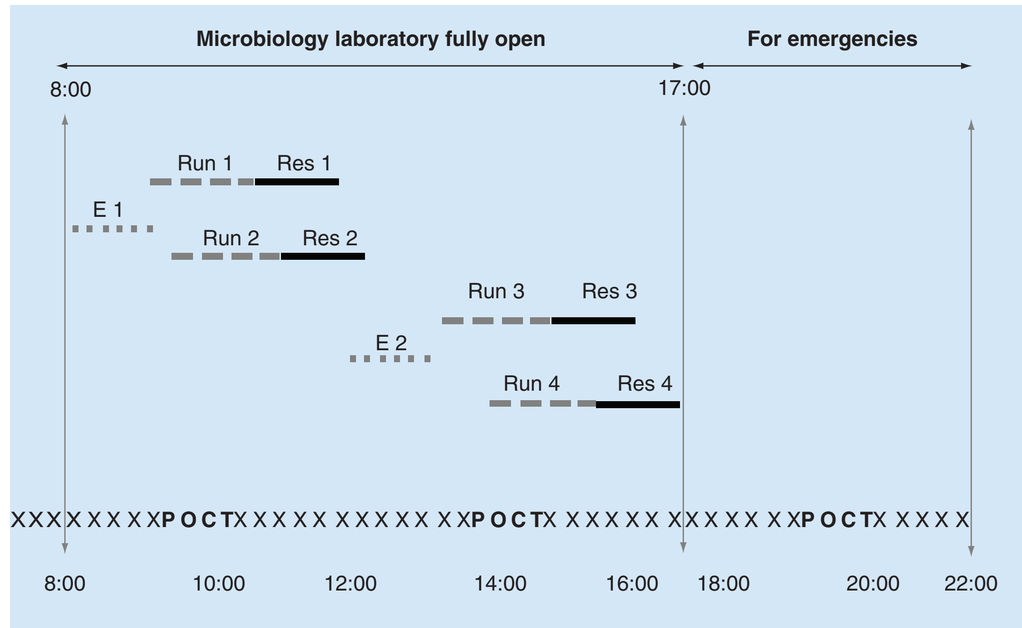

Figure 4. Ideal automated workflow: from the pre-analytical step to the molecular result.
E 1,2: First and second run of extraction, respectively; Run 1,2,3,4: first, second, third and fourth run of assembly and PCR; Res 1,2,3,4: first, second, third and fourth run of results given to the clinicians. Laboratory official opening hours: 08:00 to 17:00 for real-time PCR and 07:00 to 22:00 for 'POCT' PCR tests.
POCT: GeneXpert PCR for enterovirus, influenza, RSV, norovirus, *M. tuberculosis*, MRSA, VRE and *Clostridium difficile* performed at the core laboratory (and not at bedside).

Table 5. Advantages/disadvantages of automated real-time PCR platform.

	Advantages	Disadvantages
Higher throughput	Short TAT	Need to add single very rapid test (POCT) for emergency in order to shorten the turnaround time for important specimen or specific pathogens
	Every day tests if needed	
	Bacterial, virus, fungal, parasite molecular diagnosis performed on the same platform	
	Possibility to adapt to exceptional events (for example, *Coxiella burnetii* blood screening during an outbreak [26])	
	Possibility to collaborate with research team, as it was the case for instance to test large number of ticks for the presence of *Anaplasma*, *Coxiella* and *Chlamydia*-related bacteria [68]	
Quantification	Viral infections (asymptomatic or not), disease progression information	Need a continuous monitoring of positive controls
	Monitoring of therapeutic response [20,22,23]	
Reduced costs	Syndromic flexible approach	Many internal and external controls have to be done.
	Possibility to collaborate with epidemiologists for large studies (for example: *Chlamydia trachomatis* in urogenital infections [61])	Expensive to develop (about 2000 to 4000 Euro to develop a new PCR), but then 5× less expensive than commercial systems
	Thanks to the syndrome-oriented tests, possibility to identify pathogens that would otherwise not have been tested for (for example: *Chlamydophila pneumoniae* in asthma [51])	
Flexibility	Possibility to adapt the performed tests for local need (including outbreaks) and to local expertise	Need a continuous monitoring of the scientific literature and a control of new sequences available
	Possibility to adapt to changing knowledge (for example, *C. trachomatis* Swedish mutant [5], polymorphism in *Neisseria meningitidis ctr*A gene [94], MRSA *mec*A gene [95], *Neisseria gonorrhoeae* polymorphism [96]	Help from experts is needed at least during the R&D process
Full automation	Single platform	Not FDA/CE approved
	More expertise at each step	
Expertise in R&D	Interpretation of the test results more efficient	Not every laboratory is able to develop its own PCRs
	Know-how for teaching and training	Skilled and trained staff is needed
		Troubleshootings have to be solved by the technicians and the local molecular diagnosis experts (no commercial support)

POCT: GeneXpert PCR for enterovirus, influenza, RSV, norovirus, M. tuberculosis, MRSA, VRE and Clostridium difficile performed at the core laboratory (and not at bedside)
TAT: Turnaround time.

significant costs savings, which could be invested in the development of additional homemade PCRs, for the benefit of patients' care.

Furthermore, automated molecular diagnosis allowed (at least in our institution) to change how molecular diagnosis is perceived both by the clinicians and by the microbiologists themselves. During the late 1990s, there were many concerns that molecular tests were difficult to interpret and prone to contamination. Nowadays, these tests can be standardized, are easier to perform, are automatized and have a real added value as compared with culture considering TAT, sensitivity and specificity. In addition, the availability of molecular tests demonstrated that very tiny concentration of DNA may be retrieved from clinical samples interrogating on the appropriateness of still considering culture as the ultimate gold standard. Therefore, it is time to move beyond these old concepts, to fully acknowledge the true contribution of molecular diagnostic and to stop first considering a positive molecular result as a possible contamination when facing an unexpected result not congruent with culture results and/or out of the established dogma. Nevertheless, these unexpected results must be checked and challenged, as it is the rule for other laboratory results, including cultures. Thus, to fully use the full pattern of automated molecular tools in clinics, we need to reconsider the position of PCR in our diagnostic algorithm by more commonly including these tests as first-line assays.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb.15.152

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EXECUTIVE SUMMARY

- Molecular diagnosis is entering as a common first line diagnostic test for many different microbes due to improved quality and efficiency provided by automation and modern real-time technologies.
- When automatized, the molecular tests are easy to perform and have a real added value as compared to culture regarding time to results, sensitivity and specificity.
- Contaminations are no more a problem thanks to real-time PCR (avoiding tube opening) and thanks to automation.
- Quantification provides key informations useful to interpret clinical significance as well as to monitor treatment impact and/or disease progression
- Home-developed PCRs allow flexibility and adaptation to local needs (including novel outbreaks), but a thoughtful R&D process is necessary, based on specific expertise and know-how.
- Automation improves throughput and reduce costs allowing syndrome-oriented tests, enabling detection of unusual pathogens that would otherwise not have been tested.
- An automated molecular platform using the same annealing and DNA elongation temperature for all PCRs allows flexibility in terms of pathogen diversity (bacteria, viruses, parasites and fungi).

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