Executive Summary

At project end, ROUTINE has delivered **a fully functional reader** capable of communicating remotely assay results obtained in an **injection molded chip** containing all necessary reagents using a consortium developed **software interface with diagnostic decision making algorithms** included.

The ultimate goal of ROUTINE was to provide and to validate the developed ROUTINE system performance with UTI patient samples and compare with gold standard diagnostic methods. The comparison to gold standards, combined with cost estimates for the reader and the injection molded single-use chips, will be used to provide input into a go/ no go commercialization decision.

In the extended work after the project, the members are at the stage of optimizing reader/chip settings using bacteria spiked samples in preparation for these UTI patient tests and a commercialization go/no go decision will be taken within half a year after the ROUTINE project's end date.

ROUTINE has been very important to all consortium members: Finbiosoft has developed an array reader software with a complete user interface and remote communications capabilities combined with a decision supporting algorithm. This is now a commercially available product offer, and this would not have been possible without the work performed in ROUTINE with partners Q-Linea and University of Antwerp.

Q-Linea has developed a compact and cost optimized complete reader system with integrated software which could directly be commercialized post-project.

microfluidic ChipShop has developed and refined previous injection molded chip-designs to accommodate the highest density of functions to date and produced them in the 500 device range, which could easily be extended to several thousand post project.

Royal Institute of Technology-KTH has benchmarked its proprietary OSTE+ platform with injection molded Topaz plastic chips and has learned invaluable lessons. The know-how has been transferred to the KTH spin-off Mercene Labs and formulations partially based on the knowledge gained is now commercially available for universities and companies alike.

University of Antwerp has gained an invaluable tool for their research into resistant bacteria and combined with their expertise and links to clinics worldwide will, through knowledge gained into spread and mutations of resistant bacteria, serve the community well to counter one of the largest threats to human health; bacterial infections.

The project has been disseminated broadly, in international conferences, trade show and conventions, and to date, two peer reviewed publications have been published with more to come using results from preclinical and clinical testing of the ROUTINE system.

The majority of the impact on society will be in the extension of the project, where a diagnostic device for UTI based on the ROUTINE reader, chip, software and chemistry will be made available on a commercial basis, if the preclinical and clinical testing give the expected rapid, precise and multiplexed results.

Project context and Objectives

A key barrier towards improving the management of infectious diseases is the absence of rapid and accurate diagnostic information to direct treatment decisions at the point-of-care. Urinary tract infections (UTI) are among the most common bacterial infections and pose a significant healthcare burden. Almost 50% of the global population will experience a UTI at some point in their lives, and those who suffer from recurrent, complicated UTI can have more than three episodes of infection per year. As the most common healthcare-associated infection, UTI accounts for more than 30% of infections reported by acute-care hospitals, where Gram-negative bacteria (GNB) are the most common causative pathogens of UTI. Among GNB, *Escherichia coli* is the most common cause of UTI and accounts for approximately 75 to 95 percent of all infections, while other GNB such as *Klebsiella*, *Proteus, Acinetobacter* and *Pseudomonas* are less prevalent. Treatment options however for UTI due to GNB are severely limited because of the huge explosion of antimicrobial resistance that has been globally witnessed in GNB during the last decade, attributed to extensive promiscuity and horizontal transfer of resistance genes among different bacterial species.

Currently, treatment of UTI is initiated empirically because conventional diagnosis of UTI depends on culturing the clinical sample in a centralized diagnostic laboratory, which has a typical delay of two to three days from sample acquisition to delivery of the bacterial identification and antibiotic susceptibility results. Even worse, some of the carbapenemases, like OXA-48, are commonly missed on phenotypic screens and can only be detected by genotypic techniques typically available only at reference laboratories. These inherent complexities of resistance gene expression make it quite clear that any rapid diagnostic test for detection of MDR- GNB would have to be based on genotypic detection. Furthermore, for a test to be able to address the imminent problems in primary care (empirical therapy of UTI patients), hospitals (guided therapy, early detection and patient isolation), nursing homes (increased burden of UTI in aging population due to urinary incontinence and extensive catheter use) and from a public health perspective (decrease inappropriate antibiotic use and hence, resistance), this test would need to be robust, standalone, cheap, and technically non-demanding.

State of the art: Clinical approach today of UTI diagnostics

In primary care, empirical treatment of UTI, without additional testing, is the norm, which is very unsatisfactory due to the unavoidable overprescription of antibiotics.

Commercially available metabolic activity tests

Current strategies to predict bacteriological UTI and reduce inappropriate antibiotic use include clinical diagnostic algorithms and urine dipsticks. These tests improve the positive predictive value, but suffer from limited value in increasing diagnostic prediction and a poor negative predictive value in ruling out infection, respectively. For instance, a dipstick urinalysis positive for leukocyte esterase and/or nitrites in a midstream-void specimen is used to reinforce the clinical diagnosis of UTI. Leukocyte esterase is specific (94 to 98 percent) and reliably sensitive (75 to 96 percent) for detecting uropathogens but only at very

high bacterial loads (equivalent to 100,000 colony forming units (CFU) per mL of urine). Nitrite tests may be negative if the causative organism is not nitrate reducing (e.g., *Acinetobacter*). Therefore, the sensitivity of nitrite tests ranges from 35 to 85 percent, but the specificity is 95 percent. However, none of these tests can predict bacterial resistance patterns that, as is evident from the current scenario, is the primary reason for failure of empirical treatment and has also exacerbated a vicious cycle, with newer, broadspectrum agents being increasingly prescribed.

Culture based phenotypic tests

In a healthcare setting, detection of uropathogens and associated antibiotic resistance is primarily culture-based with a total assay time of 48-72 hours. The introduction of the 'rapid' chromogenic media (ChromID ESBL, biomerieux, France; CHROMagar KPC, CHROMagar Microbiology, France; Brilliance UTI Clarity, Oxoid, United Kingdom) that allow a colour based presumptive identification of the bacterial pathogens within 20 hours has cut short the total assay time till drug susceptibility results are available to 48 hours. However, these delays still result in initiation of empiric treatment of UTI patients, which is necessary to prevent much worsening conditions possibly even death. Furthermore, a phenotypic drug susceptibility testing cannot detect all antibiotic resistances and require downstream genotypic methods for further detection of such genes.

Culture based genotypic tests

Currently available genotypic methods include a PCR amplification either followed by double-strand sequencing or characterization by a microarray-based system. However, it needs to be underscored that the currently commercially available PCR-microarray-based systems (e.g., Check-Points MDR CT103 and others, Check-Points, The Netherlands; Identibac AMR -ve Genotyping, Alere, Germany) for detection of ESBLs and carbapenemases are not only laborious and technically demanding, and hence can only be set-up in reference laboratories but also very importantly, these systems cannot be utilized directly on clinical samples like urine but only on pure bacteria cultured conventionally from urine or other samples. Hence despite being 'rapid' genotypic identification systems, these are heavily reliant on conventional culture and also impeded by the same delays in results.

Project Objectives

The ROUTINE project was initiated based on a gap analysis of the UTI diagnostic market. Given the performance of today's UTI-tests, the following main objectives (test features) that would ensure ROUTINE relevance and competitive edge, were identified:

1) direct bacterial lysis from raw urine sample to minimize sample preparation time and complexity;

2) PCR instead of phenotypic testing, negating the need for culture and allowing definitive detection and characterization of resistance genes;

3) microscale PCR, speeding up the thermal cycling time significantly;

4) fluorescent, instead of colorimetric readout, being 5x faster due to obviating the need for lengthy colour generating reactions after binding of the complementary DNA strand;

5) integration of PCR and an on-cartridge array with automated sample handling, minimising hands-on time, and

6) development of a software algorithm for interpreting possible treatment options based on the presence/absence of ESBL and/or carbapenemase combinations in the infecting pathogen.

Main S/T results/foreground

To summarize at project end, ROUTINE has delivered a fully functional reader capable of communicating remotely assay results obtained in an injection molded chip containing all necessary reagents using a consortium developed software interface with diagnostic decision making algorithms included.

The ultimate goal of ROUTINE was to provide the above and to validate the system performance with UTI patient samples and compare with gold standard diagnostic methods. This, combined with cost estimates for the reader and the injection molded single-use chips provide input into a go/ no go commercialization decision.

Currently, the extended project is at the stage of optimizing reader/chip settings using bacteria spiked samples in preparation for these UTI patient tests and a go/no go decision will be taken within half a year after the ROUTINE project's end date.

The details of the S/T results are provided below divided into four categories: **Chip, Reader, Software** and **Assay.**

Chip

An integrated chip has been developed comprising various individual functionalities on one single cartridge enabling a simple sample-in-result-out procedure.

Injection molds for the final ROUTINE chip was fabricated and a batch of 500 identical Topas (highly transparent thermoplastic material) chips were produced. The system has been evaluated on different levels and is currently placed at UA where the biological evaluation of patient samples is taking place.

In ROUTINE It was one of the overarching aims to design an integrated chip that combines all individual components and functionalities on one integrated microfluidic cartridge sufficiently miniaturized to easily fit into a bench top reader unit.

Starting with an injected molded chip on microscopic slide format, the PCR reaction on chip including the mixing steps and the lyophilization of required reagents for DNA amplification was tested and successfully established (Fig. 1).

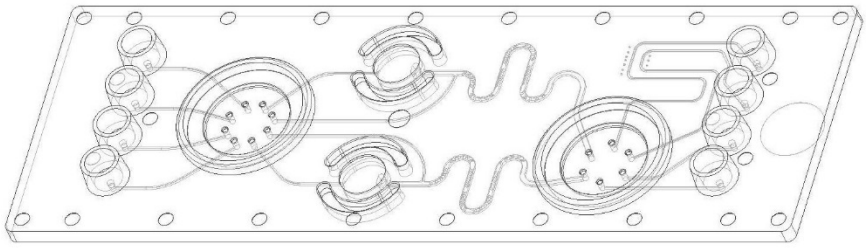

Figure 1: ROUTINE 01 design with PCR reaction chambers and Herringbone mixing structures

Then all single functionalities, reagent storage and connection interfaces were combined on one integrated chip going through several design iterations that were produced by milling in small quantities. Here, the material of choice was PMMA – a polymer that can be easily milled and surface functionalized to ensure minimal bubble creation and predictable flow rates. After several iterations between MFCS and QL (responsible for the reader unit) with different designs of valves and ports, as well as positioning of channels and blister packs, a final design was approved and released for production in MFCS main injection molding line.

Metal injection molds, capable of tens of thousands of molded parts, based on the final released design were produced. 500 chips of the final design were fabricated in Topas, a thermoplastic material with low autofluorescence and therefore suitable for PCR detection on chip (Fig. 2).

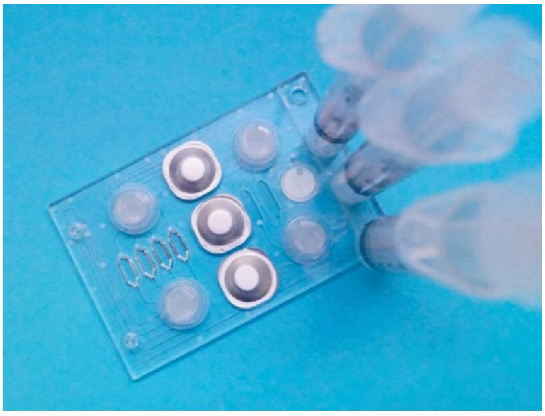

Figure 2: (left) Injection molding machine at microfluidic ChipShop, (right) Fully processed, closed ROUTINE 02 chip with blisters, turning valves and syringes. The chip measures 75x50mm, i.e. double glass slide format, which is an industry standard.

A construction drawing of the final integrated ROUTINE chip is shown in Figure 3 and includes:

- a sample intake interface
- a membrane component required for urine debris removal and bacterial up concentration prior to lysis
- mixing chambers
- four PCR reaction chambers including a negative control, a positive control and 2 sample chambers
- On-the-chip storage of additional reagents by blisters
- A waste connection interface
- and four integrated turning valves to navigate the flow

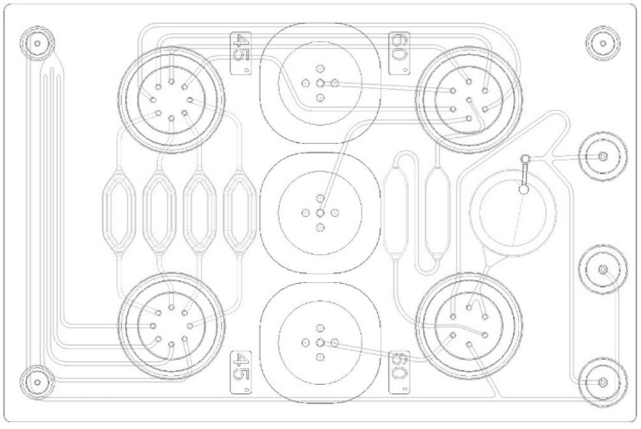

Figure 3: (left) illustrated final design of the integrated chip, (right) scheme of the final ROUTINE chip

Based on the decision for the end-point PCR detection instead of the originally planned DNA array, the optical detection zone is located in the area of the 4 PCR reaction chambers.

The final ROUTINE design was successfully evaluated using colored liquids, liquid PCR reagents and finally lyophilized (freeze dried) PCR reagents directly stored in the PCR reaction chambers.

System performance evaluation

Characterization of the various interfaces between cartridge and instrument has successfully taken place with the help of colored liquids while the instrument was still at Qlinea. Once this was finished, the final instrument was transferred to UA where the evaluation of patient samples is taking place.

The final ROUTINE system including the final instrument, the adapted software as well as integrated cartridges contained lyophilized reagents for the detection of *E. coli* and KPC is currently evaluated at UA.

Reader

A compact and low-cost readout machine that provides the necessary fluidic, thermal and optical interface to the cartridge, as well as a logical and easy user interface has been produced for consortium use. The reader is ready for commercialization.

Design input

Final design of the prototype reader is based on a prototype System Requirement Specification as well as information provided by MicroFluidic Chip Shop (MFCS) on the proposed physical properties of the cartridge, and a description how the cartridge is to be operated (Fig. 4).

Figure 4: Sequence of operating routine cartridge where turning valves integrated on the chip are controlled via the reader to activate fluid streams as required. On chip blisters with stored reagents are punched in a precise sequence to ensure delivery of reagents as needed in the carefully timed on-chip reactions (MFCS, example from early-generation chip concept.)

Design

A critical part of the chip operation is the thermal cycling. The thermocycler was designed from the ground up. Figure 5 shows a graph obtained during a performance test of the cycler, which indicates that the performance fulfilled the requirements for ramp time.

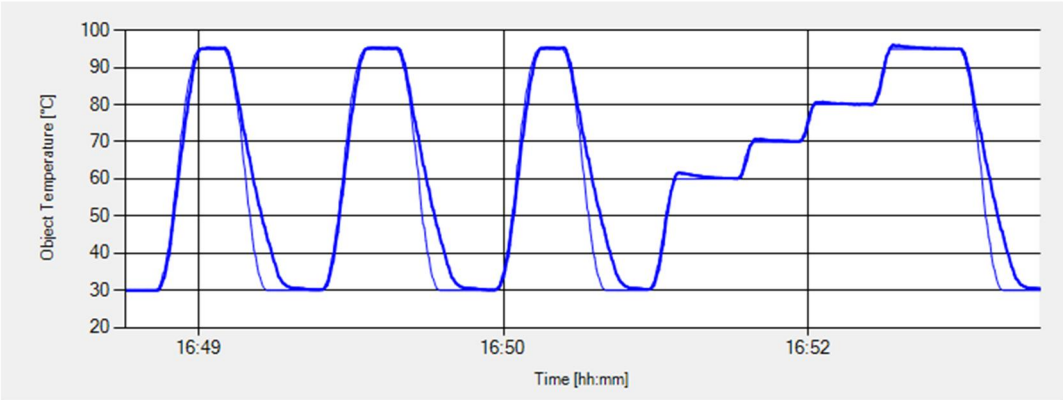

Figure 5: Temp vs time graph of thermocycler performance during part of the ROUTINE thermal protocol.

The final prototype is quite complex, due to the fact that there are a number of mechanical axes required to move the cartridge valves, the valve motors, the reagent syringes, and the array reader. In addition, the positioning precision needed for proper cartridge operation

placed relatively high requirements on the ship holding structure. In a commercial POCT instrument, a revisit of the design can be done (of both cartridge and instrument) in order to arrive at a design that is more cost effective at production volumes.

Figure 6 shows a view of the submodules comprising the ROUTINE instrument.

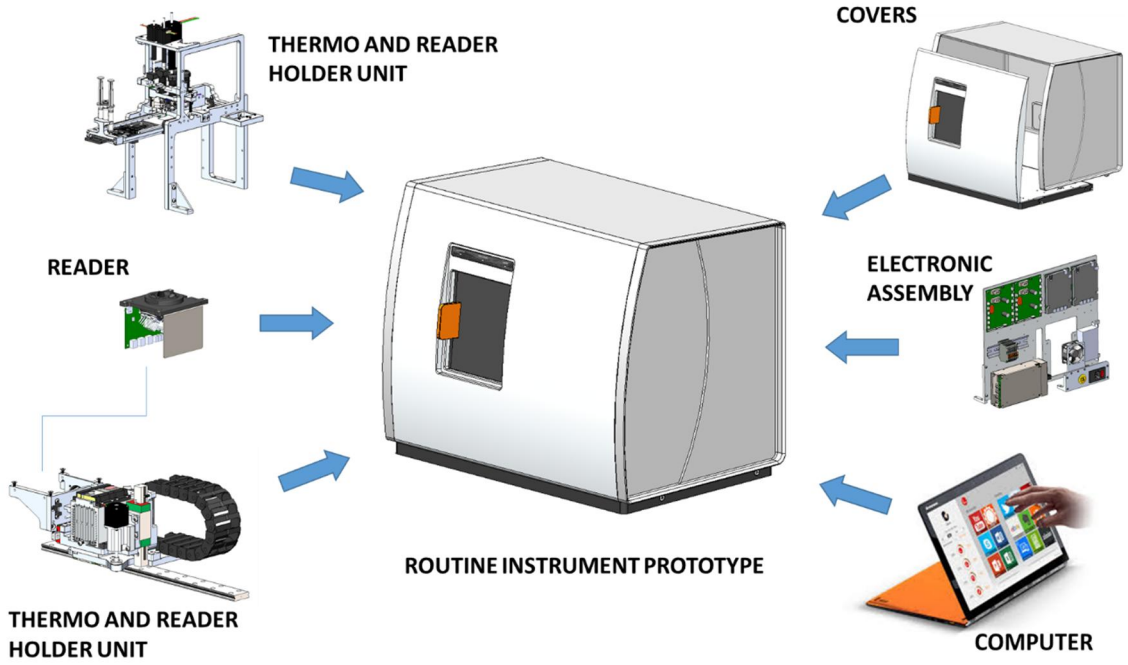

Figure 6: ROUTINE instrument components.

Array reader

The array reader is of course the most expensive part of the system. A project decision has been taken to remove the array from the chip, reading instead intensity levels directly from the PCR chambers. While this in principle can reduce the performance specifications and hence the cost of the reader significantly, the decision was taken at a time in the project timeline when the reader design was locked, and parts had been ordered. Therefore, the present prototype reader is still in principle capable of reading fluorescent microarrays, at a future time, while optimizing the reader for the present requirements could lead to cost savings in a serial production setting.

Technical tests

The final prototype reader (Fig. 7) has been developed, assembled, and has undergone technical tests at Q-linea.

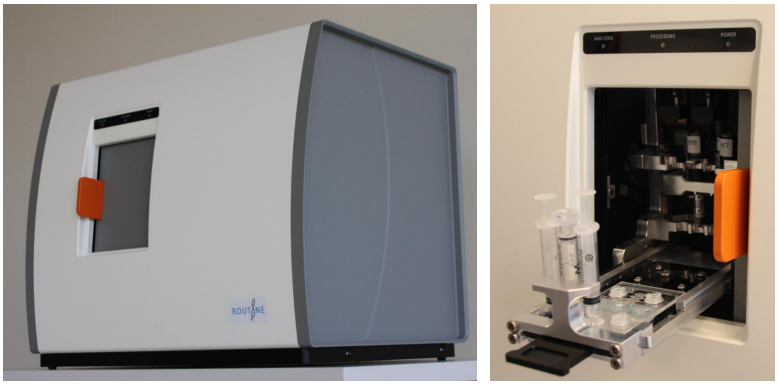

Figure 7: Left: Routine prototype reader. Right: Load hatch open; chip in position.

All technical tests were performed successfully (Fig. 8), and in january 2016 the system was sent to the University of Antwerpen (UA) for biological tests, to be commenced as soon as the final microfluidic cartridge was available. The system has been upgraded to the final system GUI version while in place at UA.

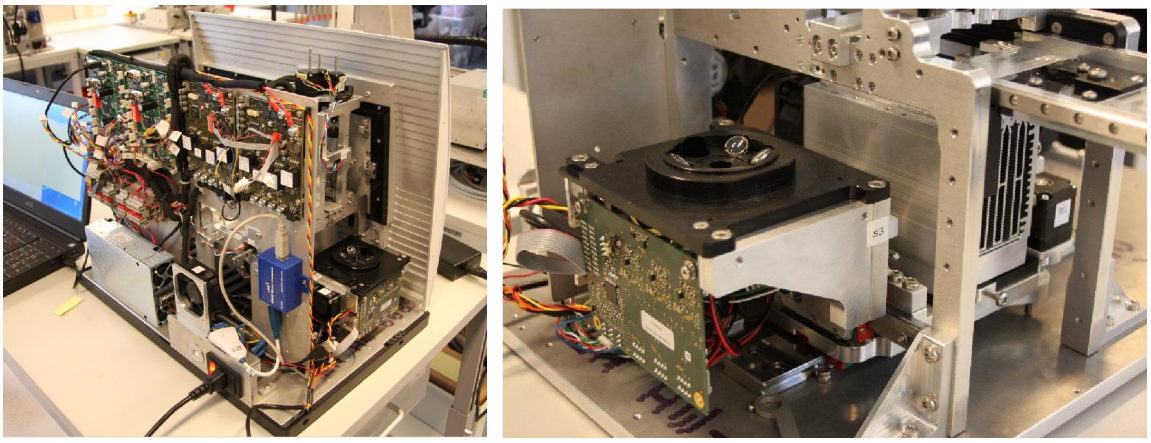

Figure 8: ROUTINE instrument under test. Left: system without covers. Right: Close-up of array reader module.

As part of handing over the system to UA, a user manual has also been written. The manual provides an overview of the instrument, installation instructions, and instructions on how to load and run the instrument.

Control system development

The control system development has proceeded in sync with HW development, and for the GUI in collaboration with Finbiosoft OY. Based on operating sequences a workflow for the instrument was defined, which served as underpinnings for both Q-linea and Finbiosoft SW development. The ROUTINE Analyzer SW as experienced by the user is described in the User manual.

Software development

All planned tools are completed and published for consortium use. Furthermore, both microarray analysis software ("Advisor Endpoint") and validation tool ("Validation Manager") have been productized and are now marketed for diagnostic companies and clinical laboratories. Both pieces of software have raised significant interest in IVD industry and they are anticipated to be notable exploitable products after ROUTINE project.

The main goal for the software development was to construct a user friendly interface that

automatically provides quantitative assay results from fluorescence intensity measurements. Furthermore, the software should include algorithms that give the treating physician clinical decision support.

Creating a novel diagnostic end-point analysis software

Earlier in the project, FBS modified its colorimetric microarray analysis software Advisor to be compatible with the microarray chips printed by MFCS and with the fluorescence readout technology to-be-developed by Q-Linea.

At project end, a new user interface was built for ROUTINE reader instrument and it was connected to Q-Linea instrument control layer and FBS analysis software. This reader control UI is now capable of running the final instrument workflow and taking images of the PCR chambers for result analysis.

Consortium's decision to change detection technology from endpoint PCR + microarray to endpoint qPCR, made a late change also to analysis software specifications. Current implementation of the analysis software is capable of both automatic analysis of microarray images as well as manual analysis of endpoint qPCR images.

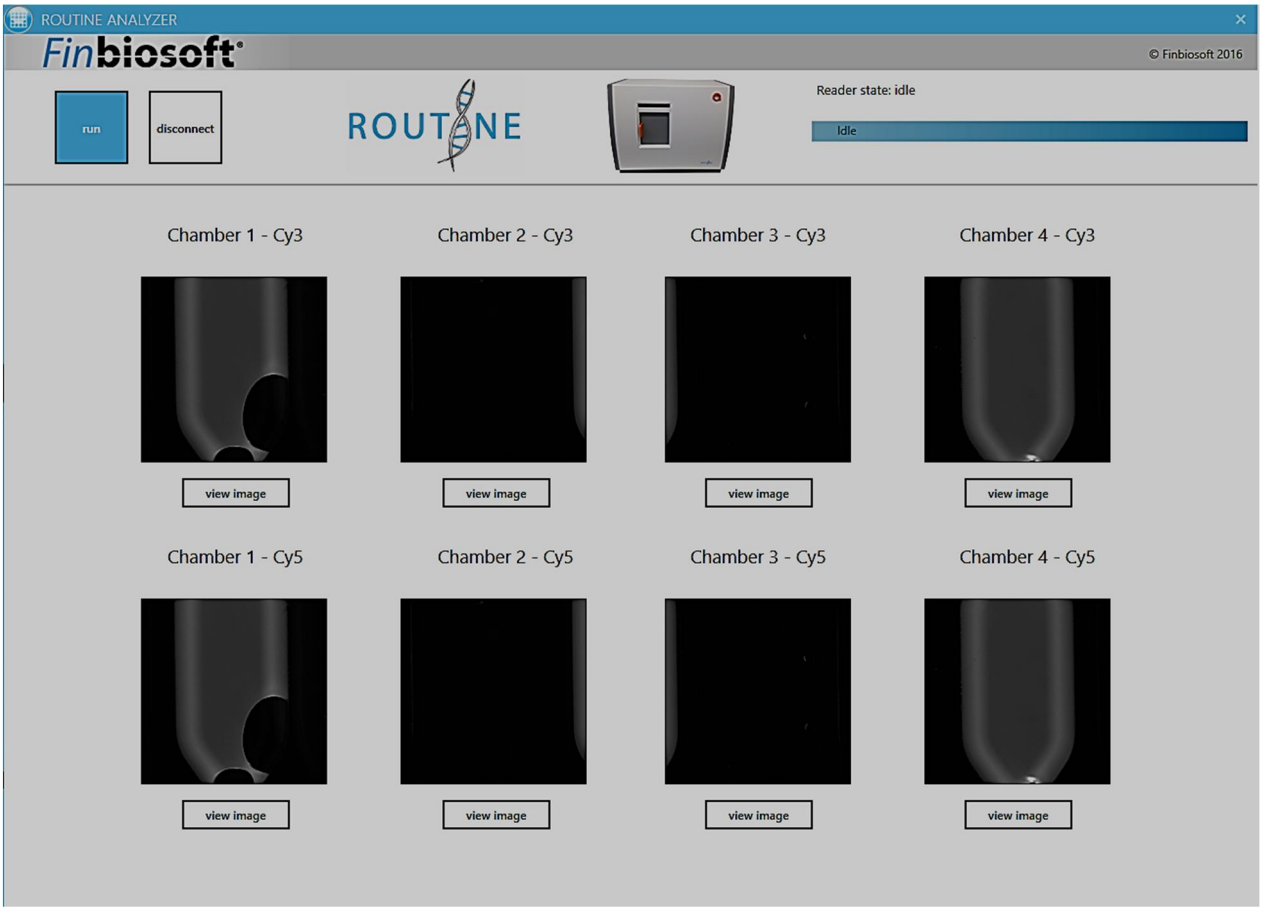

Figure 9: ROUTINE analyzer software executes instrument workflow and acquires endpoint signals from 2 fluorescence channels

Design and implementation of remotely accessible data management system

Validation Manager is now a released, online service capable of managing both analytical validations and clinical evaluations of diagnostic MDx tests.

In its current state, Validation Manager standardizes the way how laboratories and diagnostic companies plan, execute and report the validations and verifications. It enables easy data sharing between users, departments and laboratories through a web-based user interface. Validation reporting is achieved with automated validation results calculation according to CLSI guidelines and a fully digitalized, one-click validation project reporting. The central data repository provides an easy manner for all authenticated users to access any previously conducted validations with a trace from the achieved validation reports all the way to the underlying instruments' raw data and other associated data.

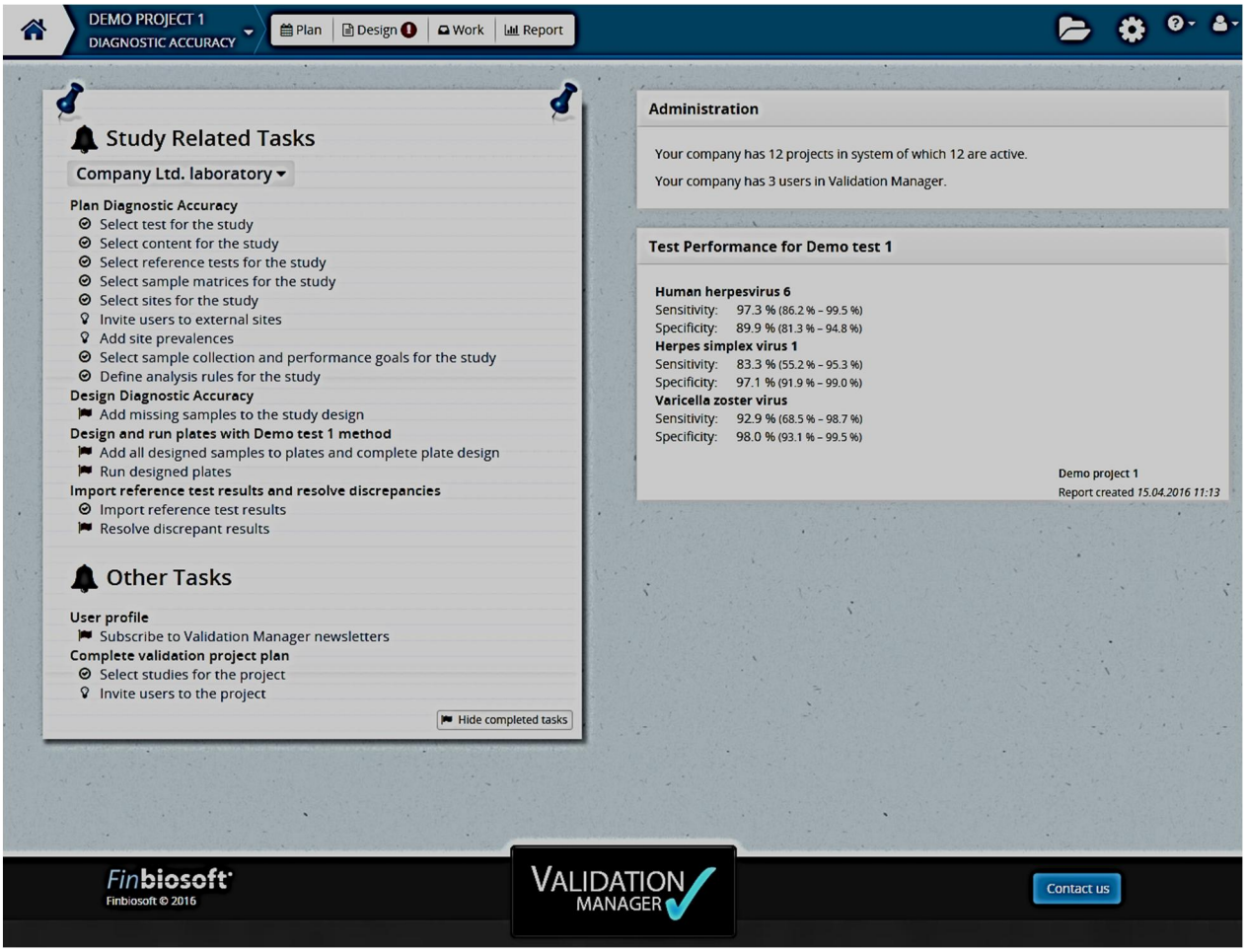

Figure 10: Validation Manager standardizes the way an organization plans and executes verifications and validations

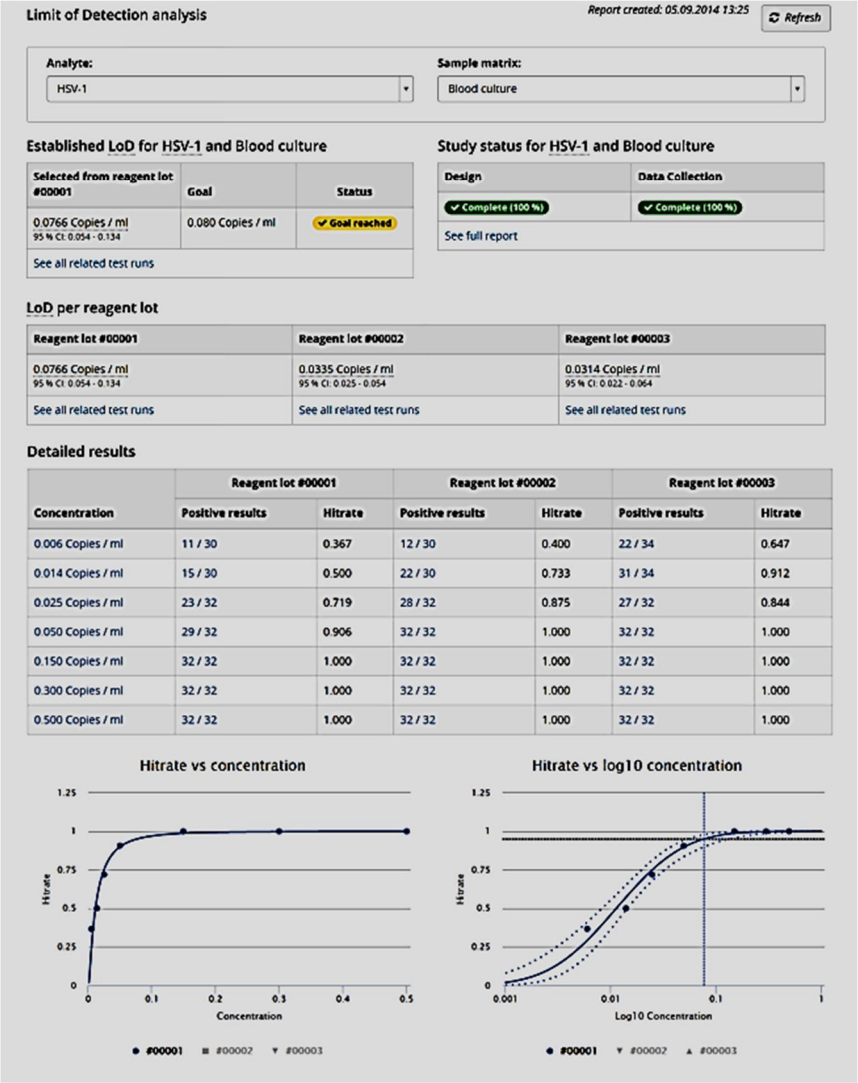

Figure 11: Validation Manager supports latest CLSI validation protocols, e.g. for measuring LoD with Probit analysis

Validation Manager was set up and trained to UA for final assay verification and validation activities.

Treatment algorithms

The treatment algorithms developed allow a precise interpretation of the diagnostic test results and intend to take therapeutic decision-making to the era of personalized medicine. In contrast to the existing clinical algorithms that are based on patient characteristics and that primary care physicians employ to decide on empiric treatment of UTI, the software algorithms developed are focused on the type of infecting organism and, more importantly, on the kind of antibiotic resistance genes present in these organisms (microbialized/antimicrobialized medicine).

Assay

Optimal primer/probe combinations that could be efficiently lyophilized on chip and showed good fluorescent signals were developed. One probe for each of the *E. coli* and KPC PCRs were included on chip and this configuration is currently undergoing patient sample tests at UA (May 2016).

The main tasks addressed in assay development were: to select which bacterial species are to be detected, based on information gathered from real patient samples, and which antibiotic resistance genes are to be included, based on current literature and assessments of the severity and spread, i.e. the threat they pose, of these genes; and design probes capable of close to 100% specificity. In this manner, the treating physician will have actionable knowledge of which antibiotics to select to combat the UTI infection.

Summary Assay

The consortium successfully established an off-chip TaqMan based real-time PCR protocol for species identification (*uidA* for *E. coli*) and detection of antimicrobial resistance (KPC). A total of 9 primer-probe combinations (4 probes for *uidA* and 5 probes for KPC) were tested on panels of target-positive and negative strains and showed a specificity of 100% on the strains included in the panel. Based on gradient temperature PCR results, a range of possible annealing temperatures that allowed the simultaneous detection of the targets was selected and taken forward to the on-chip protocol. Although in the off-chip protocol, some primer-probe combinations worked slightly better than others, none performed inadequately so all were taken forward to the on-chip stage as it is known that off-chip and on-chip specifications might vary due to the microscale level of the reactions. Further experiments at MFCS resulted in optimal primer/probe combinations that could be efficiently lyophilized on chip and showed good fluorescent signals. One probe for each of the *E. coli* and KPC PCRs was included on chip.

Compliance with ethical screening report

ROUTINE used 'discarded' samples that come for routine workup from UTI patients to the microbiology laboratory, including screening for presence of ESBL-harboring pathogens. After the routine analysis was performed, these samples were frozen for us and shipped to Antwerp for testing on the ROUTINE diagnostic platform. Ethical board approvals for ongoing clinical studies as part of the SATURN project in Antwerp primary care centres and in University of Geneva hospitals were already in place before ROUTINE started.

Routine samples collected from hospitals were not linked to any personal patient information and were transported to UA in a blinded fashion.

UA already had frozen urine samples collected during the FP7-EU project SATURN, however here as well these were not linked to the patient's personal information. Samples were randomized and blinded by an adminstrator before use on the ROUTINE diagnostic platform.

Testing, optimization and verification of final result interpretation software

Integrated reader software was first released for testing to QL. After protocol optimizations and software improvements, the reader software was delivered together with the QL-developed reader instrument to UA for final testing and verification. Based on feedback gathered, multiple fine-tunings were made to instrument workflows and software functionalities, resulting in a number of software updates and a final, integrated software + reader combination.

A TaqMan PCR was established instead of the end-point PCR with fluorescent DNA microarray detection. In this type of qPCR, a specific region will be amplified using two primers. A separate probe, containing a fluorophore and a quencher binds the central region of this amplified product. During the amplification process, the probe will be removed from the DNA by the progressing polymerase, resulting in its cleavage and thus separation of quencher and the fluorophore, leading to increasing fluorescence that correlates with the amount of product.

- *E. coli* detection
    - Confirmation of 2 primers targeting the *uidA* gene. This gene allows the detection of over 97% of E. coli strains, including EHEC strains which are phenotypically glucuronidase negative (McDaniels et al., 1996). Other *Escherichia* species will not be detected. Genetically closely related *Shigella* species will, however, be similarly identified
    - Selection of 1 of 4 TaqMan probes that have been designed by UA (more details in D2.2)
    - Investigation of specificity of the TaqMan PCR. Temperature gradient study of the various primer/probes combinations on a set of strains (*E. coli*, *E. hermannii*, EHEC, *Shigella sonnei* and *Citrobacter freundii*) to establish the optimal temperature for specific detection of *uidA* positive strains. Higher temperatures drive the specificity of the PCR, while lower temperatures favour binding of the probe
- KPC detection
    - Confirmation of 2 primers targeting all currently known variants of the KPC gene
    - Selection of 1 of 5 TaqMan probes that have been designed by UA (more details in D2.2)
    - Except for KPC-7, all currently known KPC variants yield an identical PCR product using those 2 primers
    - Investigation of specificity of the probes. Temperature gradient study of the primer probe combinations on a set of strains (KPC-2, KPC-3). KPC-2 and 3 represent the majority of strains. KPC-7 is not included, however, a probe covering this nucleotide difference in the amplified region is also included in the panel
    - Given the greater stringency requirements of the *E. coli* qPCR, selection of temperature was mainly driven by that reaction

All PCR reactions were run on the BioRad® CFX96 Touch™ Real-Time PCR Detection System with SensiFAST™ Probe No-ROX Kit from Bioline®.

The PCR composition is summarized in table 1.

	concentration	Volume (µL)	Final concentration
No-ROX mix	2x	10	1x
Primer FW	10µM	0,8	400nM
Primer Rev	10µM	0,8	400nM
Probe	10µM	0,2	100nM
Water		4,2	
Template		4	
	Total volume	20	

Table 1. Components and concentrations of the real-time TaqMan PCR.

For the temperature gradient PCR the obtained results were similar for all the tested probes. In general, lower annealing temperatures resulted in steeper amplification curves, although plateauing (or even slightly decreasing) near the lowest values. An example results are presented in Figure 12.

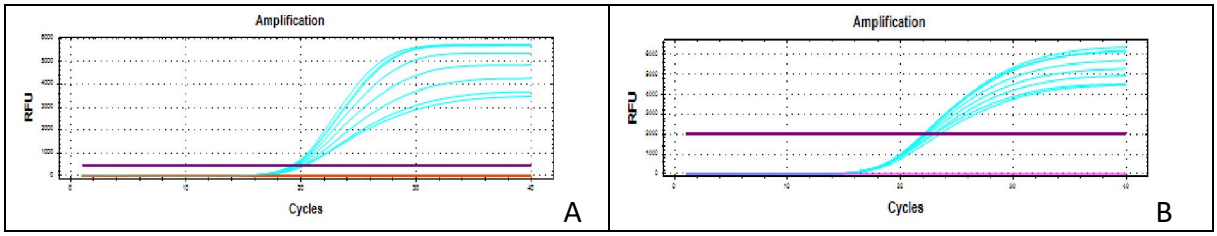

Figure 12: Amplification curves in relation to the annealing temperature (gradient: 53,6°C, 54,3°C, 55,6°C, 57,5°C, 60,0°C, 61,9°C, 63,1°C, 63,6°C). **A.** ECPROBE2 on *E. coli*. **B.** KPCPROBE2 on KPC positive *K. pneumoniae*.

Some differences were observed when comparing performance of different probes in detection of the same target (Fig. 13) with some probes performing slightly better than others. Similar results were obtained for both *uidA* and KPC targets. However, since the off-chip results cannot be directly transferred on chip, all the probes and a range of temperatures were included in the on-chip evaluation.

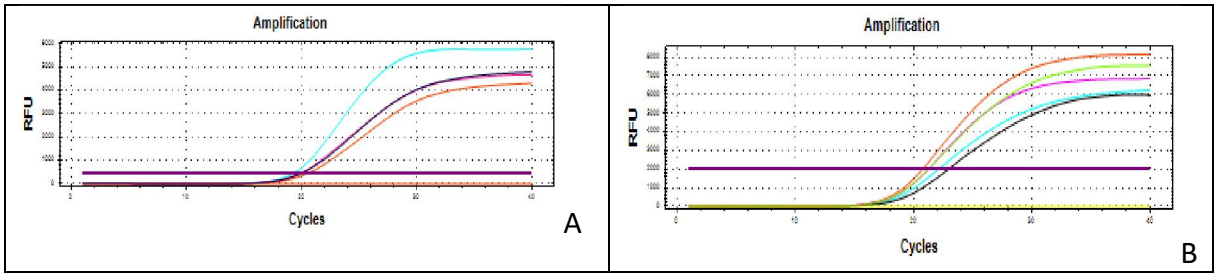

Figure 13: Different probes detecting the same target in an example annealing temperature of 55.6°C. **A.** ECPROBEs (2, 3, 4, 5) on *E. coli*. **B.** KPCPROBEs (2, 3g, 4, 5, 7) on KPC positive *K. pneumoniae*.

When tested a panel of targets positive and negative strains, a specificity of 100% was found for both *uidA* and KPC (Fig. 14).

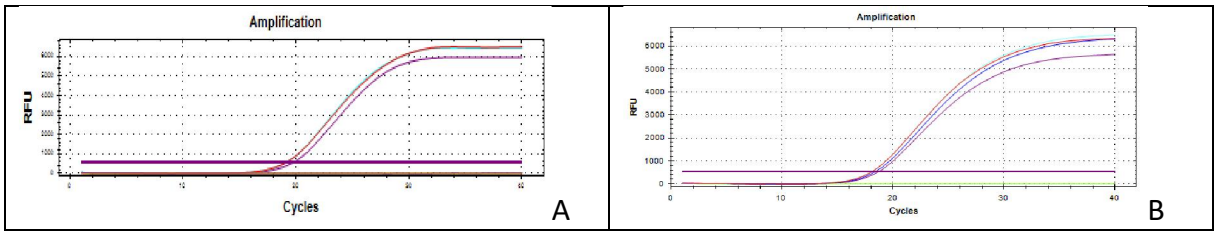

Figure 14: Specificity results in an example annealing temperature of 55.6°C. **A.** ECPROBE2 on *E. coli*, *E. hermannii*, *EHEC*, *Shigella Sonnei*, *C. freundii*. **B.** KPCPROBE2 on unknown KPC, KPC neg., KPC-2, KPC-3, unknown KPC.

Multiplex PCR optimization

To demonstrate proof of principle, two targets were selected for final PCR on-chip. The *uidA* gene, which encodes for beta-D-glucuronidase, was selected for the identification of *E. coli*, the main causative pathogen for UTIs. It allows the detection of over 97% of *E. coli* strains, including EHEC (Enterohaemorrhagic *Escherichia coli*) strains, which are phenotypically glucuronidase negative (McDaniels et al., 1996), while not detecting other *Escherichia* species. Genetically closely related *Shigella* species will, however, be similarly identified. The KPC gene (*Klebsiella pneumoniae* carbapenemase) was included as an example of a resistance marker.

In the final chip the targets are being detected in separate chambers, each allowing for the detection of a single target. However, as all chambers should run under the same conditions, a single PCR program, common for both targets was optimized to allow synchronized detection.

Fluorescent DNA microarray optimization

Due to the lower sensitivity of a strategy using end-point PCR coupled to a fluorescent DNA microarray in comparison to a real-time PCR strategy using fluorescently labelled TaqMan probes, the consortium decided to use the latter strategy. The use of TaqMan probes instead of intercalating dyes served to increase the specificity of the overall reaction and to limit the background fluorescence. That specificity was investigated by temperature gradient PCR of the various primer/probes combinations on a set of strains (*E. coli*, *E. hermannii*, EHEC, *Shigella sonnei* and *Citrobacter freundii*) to establish the optimal temperature for specific detection of *uidA* positive strains. A similar investigation was conducted for KPC running, by a temperature gradient PCR on strains harbouring KPC-2 or KPC-3 and including KPC negative strains. Except for KPC-7, all currently known KPC variants yield an identical PCR product using those 2 primers, hence a probe compatible with KPC-7 (has one point mutation in comparison to the PCR product amplified on other KPC variants) was included. However, all the probes are labelled with the same fluorescent dye, as there is no clinical need to differentiate the KPC-7 variant. As it is known that higher temperatures drive the specificity of the PCR, while lower temperatures favour binding of the probe, a range of temperatures that yielded high signal levels, while having good specificity was selected to be taken toward the on-chip optimisation.

First, evaluation of primers and probes for *E.coli* and KPC were tested in a standard qPCR cycler compared to a simple reaction chamber test chip. When the functionality of the PCR reaction on chip was proven, several lyophilization rounds with different additive concentrations were performed. The mixture with the highest sensitivity of each primer-probe pair was chosen to be lyophilized on the final chip. In the first set of 100 chips, all 4 reaction chambers were filled with both primer-probe combinations for both targets.

On-chip multiplex microPCR for amplification of bacterial species markers and antibiotic resistance markers

Although the PCR protocol itself is established, a final optimisation of the fully integrated workflow from sample in to result out is currently ongoing. In iterative rounds between UA, MFCS and QL, the protocol to run the chip on the device undergoes further fine-tuning to allow for bacterial lysis, DNA purification, migration to the chambers, amplification of the targets and finally fluorescence imaging.

On- chip fluorescent array readout

The software as developed by partner Finbiosoft contains a feature to record the images in a fully automated fashion. As the instrument and chips were brought together at UA in April 2016, optimal positioning of the fluorescence detector compared to the various PCR chambers and fine-tuning of the intensity of the signal will be performed while running the preclinical validation experiments at UA.

Impact

ROUTINE has been a successful project for the participating partners, and as such has had a large impact on the operations of, primarily, the three SMEs.

Finbiosoft has developed an array reader software with a complete user interface and remote communications capabilities combined with a decision supporting algorithm. This is now a commercially available product offer, and this would not have been possible without the work performed in ROUTINE with partners Q-Linea and University of Antwerp.

Q-Linea has developed a compact and cost optimized complete reader system with integrated software which could easily be commercialized post-project.

microfluidic ChipShop has developed and refined previous injection molded chip-designs to accommodate the highest density of functions to date and produced them in the 500 device range, which could easily be extended to several thousand post project.

Royal Institute of Technology-KTH has benchmarked its proprietary OSTE+ platform with injection molded Topaz plastic chips and has learned invaluable lessons. The knowledge has been transferred to the KTH spin-off Mercene Labs and formulations partially based on the knowledge gained is now commercially available for universities and companies alike.

University of Antwerp has gained an invaluable tool for their research into resistant bacteria and combined with their expertise and links to clinics worldwide will, through knowledge gained into spread and mutations of resistant bacteria, serve the community well to counter one of the largest threats to human health; bacterial infections.

The ROUTINE POCT is fully aligned with the current strategy of clinicians, researchers and the pharmaceutical industry to a better understanding of how to make more informed antimicrobial choices, to improve antibiotic stewardship, to be able to make correct, early antimicrobial choices, to reduce spread of MDR-GNB, to focus on translational science/medicine, to identify companion diagnostics, and to support development of new drugs against MDR-GNB.

Specifically, the impacts on key areas and key players in the society are:

(1) On patients: Targeted antibiotic treatment reduces mortality and reduces usage of highly toxic last-line (colistin) and less efficient (tigecycline) drugs.

(2) On health care facilities: Reduced transmission of MDR-GNB due to improved infection control.

(3) On society: Reduction in treatment costs due to less treatment failures and use of laborious diagnostic tests. Also reduction in doctors' visits as effective antibiotics is prescribed at the first visit.

(4) On policy makers: The algorithms developed for the ROUTINE readout have a wider use and can be used in outpatients and hospitals to give tailor-made treatment depending on the
type of ESBL/carbapenemase.

(5) On the pharmaceutical industry: companion diagnostics for new drugs as early as possible in the development phase and to reduce the cost of clinical trials by enriching the number of subjects infected with MDR-GNB.

Project internal dissemination through staff exchange: Project internal dissemination has been performed through several short durations (1-2 days) and 2 extended stay visits (2-3 weeks) by scientists and engineers at partner's sites. This had a tremendous impact on the quality of technology integration. In fact, at least cursive knowledge of the capabilities and limitations inherent in all ROUTINE's technical fields was paramount for project success, and staff-exchange were therefore promoted and budgeted for. The trust built among the partners during InTopSens and RAPP-ID combined with the clear allocation of tasks and minimal risk of competition between partners were fundamental factors to enable such exchanges.

Dissemination to other related national and international partners: ROUTINE partners are involved in other related projects, e.g. IMI RAPP-ID, and cross-project dissemination of results were performed. In fact, the device focus, and indeed device solutions, of the small

ROUTINE consortium was used in parts of IMI RAPP-ID to generate quicker and better results in one of the less complex point of care applications targeted in RAPP-ID.

Project public website and contact details.

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