Executive summary:

The objective of PINVIALEG (Portable microfluidic-based device for in situ detection of viable legionella) was to develop a portable microfludic device composed of a filtration and bacteria collection system, a disposable card and a user-friendly readout system.

The work was organized in four different fields:
- Filtration: Water samples filtration, the water should be converted from 1 litre to ten milliliter.
- Legionella detection (NASBA): The developed technology will be integrated in the final device.
- Read system by NASBA-NALFIA technology: A readout platform.
- Microfluidic chips and integration: Legionella detection on chips and once it is reached, the technologies integration.

The results of the project have been:
- It was difficult to determine the recovery efficiency from Legionella cells from the microsieves to be injected in the microfluidic cartridges. Several experiments were performed, however the results were non conclusive. Therefore, it was decided to use the wash-off procedure as described in deliverable 2.4. A good correlation was found beween Legionella cell retention using the microsieve and the results from plating according to ISO protocols, both qualitative (95%) and quantitative (72%).
- A microfludic card has been designed and manufactured. This card is able to: (i) collect and wash the magnetic beads-RNA complexes and elute the RNA from the beads, (ii) perform specific NASBA amplification and (iii) dilute the amplified sample with NALFIA buffer and addition of NALFIA probes. The microfluidic card design contains inlets to sample and to connect external peristaltic pumps and valves to control the liquid flows and to add the reagents, and one outlet to the NALFIA strip. The design also contains several optical detection points to monitor the liquid flows on the card and to achieve timed mixing of reagents and probes with the main sample flow on the card.
- A miniaturized magnetic-based RNA extraction and capture protocol from environmental samples has been optimized and set up to fulfill the microfluidic card requirements. It has been tested in pure culture of L. pneumophila and real samples (hot water systems and refrigeration towers).
- A specific NASBA design and protocol, including primers, has been optimized for L. pneumophila reaction. Specificity has been proved (negative reference strains: Legionella non-pneumophila and non-Legionela). The limit of detection is RNA corresponding to 50-100 genome equivalents (GE) of L. pneumophila.
- The NASBA assay has been optimized (reaction mix, time, final volume) taking into account the biocompatibility of the reagents with the material of the card and the complexity of the microfludic itself as surface/volume ratio and card design. NASBA assays performed onto the microfluidic card have shown the same yield, in terms of NASBA product, as controls carried out on tube.
- Tag-labelled probes to sandwich NASBA amplicons in NALFIA have been designed successfully. Moreover, a lateral flow-based method for the detection of Legionella was developed successfully. The NASBA-NALFIA procedure has been evaluated but an extensive validation would be recommended. Furthermore, a multiplex PCR-NALFIA detection system has been developed. This procedure has been briefly evaluated, but an extensive validation would be recommended as well.

- A special card holder has been designed to interface the polymeric microfluidic card with the fluidic components in the unit. The fluidic connections are located in the bottom plate of the holder and permanent magnets are placed in the top plate of the holder.
- A software to control the system has been developed. This software includes a user interface and controls the timed sequence execution including the control of all pumps, valves and the incubation heater.

- A laboratory scale integrated system has been designed and developed. The PINVIALEG analysis system consists of a microfluidic card holder, NALFIA strip with its housing, external pumps and valves, vials for reagent storage and waste, and required electronics. The whole integrated analysis system fits on an optical breadbord of 300 mm x 450 mm in size. The height of the whole system is 250 mm and the weight 11 kg. In addition to this integrated analysis system a reader device for NALFIA strips and a laptop are needed.

As an impact of the project, two SMEs (Innosieve and Q-Bioanalytics) have join forces in applying for a follow-up of certain products derived from the project. A proposal is applied for FP7-SME-2013, Activity 2.3: Demonstration activity.

Moreover, the results and knowhow can be exploited in further jointly funded and commercial projects for developing miniaturized NASBA/PCR based amplification systems for different analytical needs.

Project Context and Objectives:

PINVIALEG project aimed to achieve an innovative tool to rapidly detect Legionella from sanitary warm and cold water for human consumption, and thereby, improving European citizen quality of life and safety. For this purpose, a compact device for the rapid detection of Legionella viable cells, according to EU regulations requirements was tried to be designed and developed.

Nowadays this analysis detect only the viable and culturable cells, has to be performed in the facilities of analytical laboratories and the result takes more than 10 days. The main profit for the SMEs members of this consortium was to obtain, by providing a new portable tool to identify Legionella on site in environmental samples, decreasing dramatically time to result (from 10 days to 3 hours) and improving the sensitivity in comparison to techniques/devices currently available in the market.

Our main goal was to develop a portable device integrating (i) a filtration and bacteria collection system, (ii) a microfluidic disposable card (able to perform both nucleic acids concentration and RNA amplification through NASBA) and (iii) a readout system based on a NALFIA strip. An essential part of PINVIALEG is the portability related to sample filtration, extraction and amplification. Thus, the whole process of Legionella detection (from sample collection to results) in environmental samples can be performed on site. NASBA is an isothermal process, facilitating portable equipment production. A transportable device to identify Legionella contamination on site could prevent from the unavoidable sample pre-treatment prior to analysis in the laboratory, normally far away from the source of contamination. Moreover, the development of a NALFIA strip as a reading system make the device very simple to use by non-qualified personnel. On the other hand, fast result acquisition could allow implementing the appropriate plan of action to eradicate the bacteria in the minimum possible time.

Therefore, the development of this new portable, disposable, microfluidic based system able to detect Legionella on site in less than 3 hours and used by non-qualified users could imply a great advantage to the industrial maintenance market.

OBJECTIVES

A new rapid system to detect Legionella spp consisting of a miniaturised microfluidic card based device able to capture target bacteria from water samples, isolate and concentrate nucleic acids and subsequently to detect on-site the presence of viable bacteria based on a genetic control was tried to be developed. The use of microfluidic chambers could allow miniaturisation of the device, and therefore make the system able to be used onsite. The proposed biosensor aimed to be portable, disposable and able to identify Legionella spp in less than 3h, using the innovative NASBA technique, that can amplify RNA as an indicator of viable bacteria presence; hence, bacteria able to grow and spread through the environment. Since NASBA is based on molecular techniques, the sensitivity of the new microdevice would be higher than routinely used methods (culture). NASBA associated with a nucleic acid lateral flow immuno-assay (NALFIA) using colloidal carbon nanoparticles as the final reading part of the device, should make final detection sensible, fast and easy, thus suited to be used by non-qualified personnel.

Scientific objectives:
- To develop a system for sample filtration and bacteria collection and its interface with the device.
- To acquire knowledge to miniaturize a laboratory inside a disposable card in order to detect molecular markers.
- To adapt NASBA reaction to on card requirements, achieving at least the same sensitivity as in bulky devices.
- To develop a NASBA-nucleic acid lateral flow immuno-assay (NALFIA) using colloidal carbon nanoparticles as a sensitive, as a fast and simple detection method.

Technological objectives:
- The main short term technological objective would tried to develop a portable biosensor to detect on-site a specific molecular marker for Legionella spp, through a NASBA-NALFIA technology. For this purpose, genomic databases will be used for marker design.
- To provide different type of users (hospitals, healthcare units, individual citizens, food production units) with a portable and disposable biosensor based on NASBA-NALFIA technology for rapid identification of a bacterial pathogen infection.
- To fulfil the whole procedure from sample preparation till NALFIA detection in less than 3 hours and to match the requirements of the EWGLI (=1000 cfu Legionella /L)
- To design and develop a simplified system for sample preparation in order to get an on-site detection system. Filtration elements need to be included in the sample collection container and reduce the filtrated sample volume to less than 5 millilitres.

Social and economic objectives:
- To manufacture the disposable microfluidic cartridge at less than 5 euros in volumes (10,000's tests). The portable unit will cost less than2000 euros (in volumes 100's /year).
- To allow end-users to perform assays for detecting viable Legionella at less than40 euros/test and long term target to push the cost down to 10 euros/test, in comparison to greater than100 euros/test (not including cost of sample transport logistics) from standard procedures (culture). To fulfil the whole procedure from sample preparation till NALFIA detection in less than 3 hours.
- To develop the microfluidic-based system taking into account future applications in other areas, besides the one described in the project, as health and food sector.
- To match the European standard requirements for Legionella detection in water systems.
- To increase the profitability of the SMEs due to the commercialization of the products, which produce a high profit margin and can be exported.
- To allow sample recollection and assays performance more frequently than legal duty. This is a way to act faster, if necessary, avoiding people contamination.
- To improve maintenance tasks.

Industrial objectives:
- To obtain a novel and short-term exploitable product, with high add value that allow all partners to reach advantageously a new and emerging market.
- To expand the current market for the SMEs involved in this project with the manufacture of this product, diversifying their business. The future success of this product can lead to the manufacturing of other similar diagnostic products for the environmental sector.

- The alliance of different SMEs and technological centres in the framework of this project will let a narrower collaboration in other projects with reciprocal benefit.
- Benefits for each partner:
- Ondoan was seeking to improve tasks of maintenance of water installations in order to be more competitive.
- Innosieve aimed to develop their existing expertise in filtration systems and adapted it to a new system that can reach other market fields
- Ayuntamiento Bilbao was looking to offer a fast, accurate and sensitive tool to detect Legionella, improving the features of the current techniques available in the market.
- Benefit of Q-BA was to further develop their existing portfolio of Real-Time and conventional PCR kits to the NASBA and NALFIA based approaches as standalone solution and in combination with the microfluidic developments.
- MKFluidics expected to get tested product prototypes for Legionella detection and in future develop new products for Legionella and other bacterial detection applications. And thus gain significant markets in Legionella and other bacterial testing business.
- Gaiker improve its knowledge in the miniaturization of biological reactions in microfluidic devices, being able to apply it to other fields as health and food sectors.
- For DLO-FBR: develop of a diagnostic procedure and device that can be used for other applications as well such as food pathogens and veterinary / zoonotic microorganisms.
- VTT apply its previous expertise and knowhow in microfluidics (card design and fabrication as well as the measurement interface development) for a new analysis application. Co-operation with SMEs provides an opportunity to commercialize the results of this research.

Project Results:

The main S&T results/foreground are explained below by work package:

WP2 – DESIGN AND DEVELOPMENT OF A PORTABLE FILTRATION DEVICE FOR SAMPLE COLLECTION

Task2.1:Definition of sample collection and manipulation

After a meeting in Bilbao among Bilbao Council, Health Department, Ondoan and Gaiker, it has been established that:
- Cooling towers are the samples to be analysed. According the complexity level, it's necessary to differentiate between acclimatization and industrial systems.
- Volume of sample: The requirements on an integrated device able to be portable demands a procedure as easy and quick as technology makes possible, using the less volume of sample. But on the other hand, we need to have a compromise between process simplicity and global device requirements in terms of sensitivity and selectivity (as European regulations are demanding), for an accurate Legionella detection and identification from real samples. Thus, 250 ml has been decided to be the minimum volume of sample to analyse. In this way we can use one microsieve per sample, according to microsieve specifications.

Task 2.2: Procedure for sample collection and manipulation and sample filtration.
The goal was to set up an optimized procedure for sample collection and manipulation. In close collaboration with Innosieve Diagnostics a filtration system based on vacuum filtration that could be used for microsieves (spintubes) was built. This vacuum filtration unit is described in D2.2 and used to perform several experiments to filtrate different types of waters: tap water, surface water, and cooling tower water. Furthermore, the prototype filtration unit that has been designed and produced in this workpackage was based on this vacuum filtration unit.

Tap water
Two different approaches were used to filtrate tap water through a 0.45 µm microsieve. The first approach was a variation on the conventional ISO method: 500 ml tapwater was filtrated through a 0.4 µm polycarbonate filter. The retentate on the filter was resuspended in 5 ml tap water and this 5 ml was totally filtrated through a 0.45 µm microsieve. In the conventional method 250 mL is filtrated and 100 µl of the 5 ml retentate is plated on agar plates and counted, which means 1/50 of the starting amount. In the second approach a 0.45 µm microsieve was used to filtrate the tap waters directly.

The first approach was used for 3 Dutch tap waters (from Wageningen). In all three cases, the 5 mL retentate could be filtrated easily through the microsieve, so in total 500 mL of tap water could be filtrated through a 0.45 µM microsieve.

Initially, for the second approach, 5 Dutch tap waters (Wageningen) were filtrated. The lowest volume that could be filtrated was 33 mL whereas the highest volume was 36,5 mL. On average 35 mL could be filtrated. It can be seen that after 10 minutes the flow is very low and almost no tap water could be filtrated after 10 minutes. Furthermore, the second approach was used to filtrate 11 tap waters from different

locations. Each tap water was measured in triplicate. The amount of tap water that could be filtrated was at least 35 ml and maximal 112 ml. 35 ml is 1/7 of 250 ml, so the minimal amount that could be filtrated directly through a microsieve was 7 times higher than the plated amount in the conventional method.

Conclusion tap water
The second approach is easier to handle than the first approach, because in the first approach the retentate of the polycarbonate filter has to be resuspended in water, which is laborious. As 25 ml tapwater is still 5 times more than the tested amount in the conventional method, it has been decided that for the sample collection system for the PINVIALEG project 25 ml tap water will be filtrated directly through a 0.45 µm microsieve.

Different vacuum strengths
In the described experiments a vacuum pump with a fixed strength was used. In addition, an experiment with Wagenings tap water was performed to study different strengths of vacuum. Here for a vacuum pump with adjustable vacuum strengths was used (PV 100 Red-Evac ™, Hoefer scientific instruments) for the lower vacuum strengths (450, 600 and 800 mBar), for the middle vacuum strenths a pump with 2 possible vacuum strengths (185 and 330 mBar) was used (KNF, Neuberger, N035.1.2.AN.18) and a vacuum pump with fixed vacuum (60 mBAR) (Diaphragh pump, IKA, Germany) was used for the strongest vacuum strenght. The experiment was performed in triplicate. Surprisingly, the highest volume could not be filtrated with the highest vacuum strength, but has an optimum between 185 mBar and 450 mBar.

Surface water and cooling tower water
Furthermore a surface water sample and cooling tower samples have been studied in this vacuum based filtration system. Via direct filtration on average 1.9 mL of surface water could be filtrated and 5.1 mL of cooling tower water could be filtrated (1 sample, in triplicate). In another experiment 4 cooling tower waters were analyzed. In this experiment a pre-filtration with a 2 µM filter (in a spintube) was performed followed by filtration through the 0.45µm pores microsieve. Very low amounts of cooling tower water could be filtrated with this approach (on average less than 1 ml, or no water). The protocol that was used for tap water (first approach) resulted in an average volume of 4.9 mL with a start volume of 100 mL.

Conclusion surface water and cooling tower water
For surface water and cooling tower waters, the volumes that could be filtrated with our system were very low (5 ml), so therefore within the consortium it was decided that the vacuum filtration system would be used for filtration of tap water and not for more dirtier waters such as cooling tower waters.

Task T2.3 Prototype design of sample collection and filtration device
During the project it was decided that it was not possible to integrate the filtration unit in the same system with the microfluidic card. This was due to a fact that the required volume of the water sample is large (25-100 ml), RNA extraction should be performed and it is not possible to perform filtration and nucleic acid extraction in a microfluidic environment in a short time. Thus, a separate filter unit was designed and fabricated such that it fits to a commercial vacuum filtration system (Millipore) and the Innosieve spintube filter device.

VTT designed a special filter holder that accepts the spintube filter and that can be placed on top of the Millipore vacuum filtration system. The water sample is introduced in the funnel on top of the filter tube and sucked by the vacuum pump through the Innosieve filter.

The evaluation of the filter unit operation was done by Innosieve. The filter units were tested for filtration efficiency and vacuum leakage. With the first pre-prototypes a pressure lock was introduced, resulting in no filtration of water samples. Increment of the filtration pore (increase diameter) did not result in a better performance. Without the funnel-part the adapter however, worked from the beginning. VTT and Innosieve decided the adapter was successful, but the funnelpart needed to change. To enable that, Innosieve provided spintubes and a commercially available funnelpart. VTT developed an intermediate part to connect the funnel to the spintube. This combination worked properly and after finetuning of the connectors, the prototype was functionally accepted. Next, Innosieve performed many filtration tests to study the applicability for large volume filtration. Within 1 minute at least 25 ml was filtrated. No unacceptable stress was observed on the microsieve.

In total three evaluation and design optimization rounds were required to end up to the final well functional filter unit concept. After the final evaluation by Innosieve, VTT fabricated six copies of the filter holder out of stainless steel and delivered them to Innosieve.

For the funnel part (sample reservoir) VTT supplied a single prototype. Injection moulding companies were contacted for a possible small scale series production of the funnel parts after the project.

Task T2.4 Connection of the filtration device with the microfluidic cartridge

It was decided during the project that lysis and binding of RNA to magnetic beads is done outside the microfluidic cartridge. The sample injection to the microfluidic cartridge was solved in such a way that both the RNA sample bound to the magnetic beads and the eluted RNA containing sample can be introduced to the microfluidic cartridge. The sample transfer is done with a pipette and the pipette tip functions as a sample reservoir in the PINVIALEG analysis unit. The sample transfer to the microfluidic cartridge was realized in WP5.

Task 2.5: Protocol for Legionella collection

Recovery of Legionella cells from the filtration device is very important for direct application in the chip. Therefore, experiments have been performed to determine the recovery efficiency from Legionella cells from the microsieve to be injected in the microfluidic cartridge. One of the experiments is described below.

Typical experiment determination recovery efficiency

Legionella pneumophila type 1 (16.2) was cultivated on BCYE agar plates and incubated for 4 days at 37°C. Cells were dissolved in PBS and serial dilutions of 100x were made. The amount of Legionella cells were determined by plating and RNA/DNA isolation. Five hundred µL of cells were loaded on the microsieve and centrifuged to collect the cells on the membrane. The eluate was used to determine the amount of cells that was filtrated through the microsieve (flow-through). To recover the cells from the microsieve, 500 µL of PBS was added on the microsieve, vortexed for 10 seconds and pipetted to a new eppendorf tube (wash off). The

amount of cells in this fraction was determined with plating as well with DNA/RNA isolation.

The amount of DNA in the different fractions (start, eluate and wash off) was measured by the nanodrop method, whereas the amount of RNA was determined by the nanodrop method as well as on a Bioanalyzer (Agilent). The amount of RNA/DNA in the samples appeared to be in the low-end of the standard curves of Nanodrop and Bioanalyzer. Furthermore, inconclusive data were achieved. In the nanodrop the added amounts of eluate and wash off were far above 100% in most cases, indicating that the mass balance was completely wrong. In table 1 concentrations are depicted from the DNA concentrations (Nanodrop) of the different samples and fractions (not all DNA and RNA concentrations of the different fractions can be shown).

The results of the plating method were inconclusive as well. The dilution series of 100x could not be seen in these results. Plates were incubated for 7 days. However, some of the plates were completely overgrown with other microorganisms, most probably due to the long incubation time of 7 days. In other cases the results were quite inconclusive due to complete absence of growing colonies.

After recovering the cells, the microsieve was stained with a fluorescent anti-Legionella antibody (provided by Innosieve Diagnostics) and analysed on the muScan (Innosieve Diagnostics) to check whether cells were still attached to the microsieve. Unfortunately, the results showed images with quite a lot of nonspecific background staining, so no definite conclusions could be drawn from this experiment.

Taken together, in this experiment several problems were encountered. It appeared difficult to count the exact amount of bacteria. The dilution series were not good, because the bacteria sticked together. Furthermore, more bacteria were counted in the different fractions together than in the start fraction and also many bacteria were counted in the eluate. Finally, the concentrations of the isolated DNA and RNA fractions were low. In general low amounts of RNA were isolated by the protocol for RNA isolation optimized in this project.

To solve this, different lysis buffers were evaluated to improve this amount (see workpackage 3). To be able to quantify the amount of cells also the crystal violet staining was tried. However, the background was too high to be able to measure low amounts. To solve the problem with the bad dilution series for the Legionella pneumophila cells, FBR-DLO successfully used peptone physiological salt as proposed by Gaiker. Using the plating method, many cells were found in the eluate and more cells were counted in the different fractions together than in the start fraction. Innosieve performed experiments on this as well and they concluded that, most probably, cell chains are disrupted during resuspending (vortexing) from the microsieve resulting in more colony forming units (CFUs) (1 chain = 1 CFU; disrupting 1 chain in two chains will give 2 CFU instead of 1). As 1 cell can have dimensions of 0.2x0.2µm these small cells will be able to pass the membrane (pores of 0.45µm).

Innosieve performed a comparison between number of cells retained on the membrane with plating by using 100 different tap waters. For plating the conventional plating method was used according to ISO. From this, a clear correlation was found between Legionella cell retention using the microsieve, and plating. A qualitative correlation was found of 95%, while a quantitative correlation was observed of 72% between the two

methods. These results suggest that the direct filtration using a microsieve followed by detection can be an efficient alternative for standard plating.

Conclusion

It was difficult to determine the recovery efficiency from Legionella cells from the microsieves to be injected in the microfluidic cartridges. Several experiments were performed; however it was not possible to count the amount of cells in the different fractions. Therefore, it was decided to use the wash-off procedure as described in deliverable 2.4. A good correlation was found between Legionella cell retention using the microsieve and results from plating according to ISO protocols, both qualitative (95%) and quantitative (72%).

WP3 - PROTOCOL FOR RNA ISOLATION BY BACTERIA LYSIS AND MAGNETIC BEADS CAPTURE

The main goal of the WP3 is to develop a "microfluidic labcard" where the biological procedures of RNA extraction from environmental samples and the subsequent specific NASBA reaction can be carried out. The microfluidic cartridge will be able to perform both magnetic particles-based nucleic acid (NA) concentration and specific Legionella detection by the amplification of the RNA marker by NASBA.

During the kick off meeting of the project the whole consortium changed the target of interest for the project, from Legionella spp. to Legionella pneumophila due to the highest prevalence of this strain (90% of reported legionellosis outbreaks are caused by L. pneumophila). On the other hand, two years ago, most of commercial kits for environmental samples were focused in Legionella spp. The SMEs of the consortium considered that the specific identification of L. pneumophila provided an added value to the goal of the project, increasing the innovation of the foreseen device. Thus, the partnership would obtain a product more attractive and competitive to the market.

The interface between filtration (WP2) and microfluidic-based device (WP3): Capture of Legionella cells onto the sieve surface. Evaluation of lysis cell onto the sieve. (Gaiker and DLO)

The goal was to use the microsieve as interface between filtration unit (WP2) and the microfluidic labcard (WP3). Once the Legionella cells were retained onto the sieve surface, lysis buffer plus magnetic beads would be added directly to the sieve and after lysis time, the mixture (containing fragments of lysed cells and RNA-magnetic particles complexes) would be collected manually and introduced into the microfluidic labcard to concentrate and wash the complexes, and finally elute the RNA to be used as template for NASBA.

The general performance of the sieves for Legionella capture has been tested by Gaiker and DLO-FBR as an activity between WP2 and WP3. For that, pure culture samples were grown and collected onto the sieves according to manufacturer instructions, and the recovery of retained cells and RNA extracted was measured. Bacterial dilutions (1 ml) of pure cultures of Legionella pneumophila (DSMZ 7513) have been used as samples.

Assays related to the retention of cells on the microsieve membrane have been non-conclusive in the two labs (DLO results in WP2). Testing three parts (cells recovered from membrane, cells retained on membrane and cells passed through membrane) have resulted non sense data. Therefore no

clear data could be obtained about cell retention. So further experimentation related to the retention of the microsieve membrane for PINVIALEG application needs to be carried out.

Improvement of cell lysis for nucleic acids (RNA and DNA) release (Gaiker and DLO)

In order to get the maximum sensitivity of our device for L. pneumophila detection is crucial to get the greatest cell recovery from environmental samples (WP2), the maximum yield of release of RNA from sample and the maximum efficiency of NASBA reaction (WP3) and the highest limit of detection of NALFIA strip tests (WP4).

Cell lyses is a vital step for the yield of RNA release from cells captured by the filtration unit. A review was carried out by Gaiker in order to define the current state of art of Legionella lysis procedures in bibliography and market. We selected guanidine thiocyanate for that. In an attempt to get the highest efficiency of cell lyses, a commercial reagent (TriReagent®) was tested by DLO. Although it performed properly, it needs a step with phenol and it cannot be used in the PINVIALEG analysis system due to requirements of safety and material specifications. On the other hand, Gaiker tested several concentrations of guanidine thiocyanate but it was not observed a proportional increment of RNA yielded in comparison to the extraction protocol optimized during WP3.

In order to develop the PCR-NALFIA (WP4), a protocol for DNA release was optimized by DLO-FBR. Several lysis buffers have been used to develop a protocol for DNA isolation for PCR-NALFIA. TriReagent®, containing phenol and guanidine thiocyanate, with and without bead beating, CTAB, containing hexadecyl trimethylammoniumbromide and a protocol provided by Q-Bioanalytic using thermally breaking up (boiling at 100 °C) have been used. The thermally breaking up protocol was the easiest protocol and could be successfully implemented. Therefore it was decided that this protocol would be used for the validation studies for PCR-NALFIA (WP4).

Development of a magnetic beads-based protocol for bacterial RNA isolation for NASBA amplification (Gaiker)

A magnetic beads-based RNA extraction protocol (from now on named as PVL extraction protocol) to isolate bacterial RNA from environmental samples has been optimized and adapted to microfluidic requirements. For the optimization the main goal has been to get the maximum miniaturization, simplicity, economy and rapidity of the whole process, considering the compatibility with the labcard material. To achieve these requirements, time recommended by the manufacturer for lysis and binding was decreased from 30 to 15 minutes, as well as the volume of lysis and washing buffers. The whole procedure is completed in 50 minutes.

The general performance of PVL protocol in terms of yield and limit of detection has been compared with the reference procedure. PVL extraction protocol is able to isolate RNA down to 102 cfu of L. pneumophila. These data are similar to the results obtained with the reference protocol (Qiagen) (table 1).

The reproducibility and repetitivity of PVL extraction protocol has been confirmed in more than 60 assays performed in different days, by different technicians and with several replicates each day (data not shown).

Table 1. Comparison of RNA extraction performance from Legionella, PVL and the reference protocol (Qiagen). Sample refers to 10 fold dilutions of pure culture L. pneumophila .Cqs have been obtained from Legionella spp. real-time NASBA amplification.

	Reference (Qiagen)			PVL protocol		
SAMPLE (cfu)	Cq	Slope	R2	Cq	Slope	R2
107	4.87	-2.953	0.851	7.90	-2.466	0.98
107	4.65	6.58				
105	12.44	11.65				
105	11.36	12.5				
103	16.59	17.32				
103	20.36	16.83				
102	17.8	20.65				
102	18.29	18.48				
NTC	N/A	N/A				

Design and test NASBA reaction for Legionella pneumophila (Gaiker and Ayuntamiento Bilbao)

A specific NASBA protocol for Legionella pneumophila detection has been set up and optimized (including specific set of primers) for microfluidic requirements, in terms of yield, volume of reagents and duration. NASBA reaction has been tested in more than 200 assays, several days, different batches of reagents and technicians.

Our NASBA reaction is able to detect specifically RNA corresponding to 100-50 genome equivalents (GE) of L. pneumophila. The assay has been performed using 10 fold dilutions of a stock RNA, extracted with the standard protocol (Qiagen)from a pure culture of 107 Legionella pneumophila .

The specificity of the NASBA reaction for L. pneumophila has been checked in 5 different species of Legionella and 9 other "negative" strains, which confirmed the specificity of the assay. The selection of strains for inclusivity and exclusivity assays has been supervised by Ayuntamiento of Bilbao (Departamento de Sanidad Ambiental).

NASBA and real samples (Gaiker and Ondoan)
NASBA reaction for L. pneumophila detection has been evaluated with 8 real samples delivered to Gaiker by Ondoan: 6 from hot water systems and 2 from refrigeration towers (table 3). RNA from samples 1 to 3 was extracted with the reference protocol, whereas RNA from samples 4 to 8 was extracted with the magnetic beads-based PVL protocol.

NASBA confirmed the presence of L. pneumophila in samples 2 (Hot water system) and 7 (refrigeration tower), while the analysis of these samples by conventional methods (bacterial culture (LD= 2,5 x 101 ufc/L)), provided by ONDOAN, didn't detect the presence of Legionella spp. or L. pneumophila in any of them. These data could be explained by the higher sensitivity of NASBA reaction in comparison to microbiological method.

Development of a packaged microfluidic card for both RNA concentration and NASBA reaction (VTT)
The disposable microfluidic card intended for use in the NASBA amplification has been designed by VTT. The design is based on a serpentine type channel structure. Two different test designs were made and fabricated in PMMA before the final system was defined. The first

amplification trials on these test designs at Gaiker were used to define the required liquid volumes and thereof the volume of the microfluidic channels on the final microfluidic card design. A more complex design was needed to realize the required functional procedures for amplification, hybridization and transfer of the product to the NALFIA strip.

The channel width in this final design is 400µm, the channel depth 100µm and the channel length in total 2217mm. The overall size of the microfluidic card is 80 mm x 85 mm.

The microfluidic card contains the following functions: collection of the magnetic beads with the bound RNA, washing of the beads and elution of the RNA from the beads, two-step NASBA amplification and dilution of the amplified sample with NALFIA buffer and addition of NALFIA probes. The microfluidic card design contains one inlet to inject the sample, ten inlets to connect external peristaltic pumps and valves to control the liquid flows and to add the reagents, and one outlet to the NALFIA strip. The design also contains several optical detection points to monitor the liquid flows on the card and to achieve timed mixing of reagents and probes with the main sample flow on the card.

All the reagents and buffers are located outside the card. The sample is brought into the card in a pipette tip and the sample is then pumped through the polymer card step by step according to the analysis procedure. The sample flow is stopped for the incubation. There are basically four zones for the incubation: 1) sample elution from magnetic beads, 2) incubation during the first NASBA step, 3) incubation during the second NASBA step, and 4) incubation for the hybridization of the NALFIA probes. At the end of the process the amplified sample is flushed to a lateral flow stick for the NALFIA analysis readout.

Microfluidic cards were fabricated by hot embossing on 500 µm thick thermoplastic polymethyl methacrylate (PMMA) foil (Plexiglas). Before the hot-embossing step the inlet and outlet holes were drilled with a CNC machine in the designed positions. The 500 µm thick card with hot-embossed channel structure was lidded with a 125 µm thick PMMA (Plexiglas) sheet. The lidding was done by solvent bonding. After lidding the outer edges of the microfluidic cards were cut with a laser to achieve the final shape and required edge quality.

Well over 100 microfluidic cards were fabricated during the project. 100 microfluidic cards were sterilized with a gamma sterilization before sending them to DLO for the system evaluation tests.

RNA capture onto the microfluidic on card (Gaiker)
The general procedure for the capture of RNA onto the card is planned as follows (as previously described in The interface between filtration (WP2) and microfluidic-based device (WP3). Once cells from environmental sample are captured onto the microsieve (WP2), lysis buffer containing magnetic particles is added to the membrane of the sieve, and lysis and coating of particles by nucleic acids is performed. Then this mixture is injected into the card. The magnetic beads are retained into the card due to the magnetic field created by magnets, while the rest of the sample flows to the waste. Then, washings are performed and finally RNA is eluted from beads with elution buffer and moved to zone 2, for the incubation with reagents during the first NASBA step. During all this procedure magnets are on.

This protocol was tested into the microfluididc card by Gaiker. Once the sample was mixed with the lysis buffer and the beads, it was introduced into the card to capture the RNA-beads complexes with magnets. After washing steps, RNA was eluted from beads inside the card and recovered with a pipette. These assays showed a low reproducibility and a high variability.

Regarding to the capture of the RNA onto the microfluidic card several issues were observed.

A compatibility issue was observed between the card material and the washing solvents used in the extraction procedure. The PMMA polymer used in the cartridge did not tolerate the solvents used in extraction causing leakage. It was solved increasing to 500 µm the ceiling of the cards. Another alternative polymer material, e.g. Cyclic Olefin Copolymer (COC), could be successfully tested for the further development phases of the microfluidic card, according to their chemical nature.

In addition, filling and emptying of the liquid wells was not repeatable. In the final device (developed in WP5) the protocol will be fully automatized, including the filling and emptying of the card.

The shape and dimensions of the microfluidic channel favored the clogging of magnetic beads. Although this last issue was avoided by dilution of washing buffers, the efficiency of RNA recovery decreased and the variability increased among replicates.

Due to the structure and the timeframe of the project, these problems were noticed at the very late phase of the project and the time and resources available at that moment did not permit changes. To optimize the recourses available it was decided to focus the effort in the development of the NASBA reaction on card.

NASBA protocol on microfluidic card (Gaiker)

Regarding the miniaturization requirements for the microfluidic card, different volumes of NASBA reaction (20, 15, 10 and 5µl) have been tested on card in order to know the minimum volume possible to use without affecting the NASBA reaction yield . It was concluded that the NASBA reaction volume can be reduced down to 10 µl without affecting the efficiency of the reaction (data not shown).

By other hand, one of the goals of the PINVIALEG device is to provide a faster procedure for Legionella detection that allows having results in a short period of time. In this way, several assays have been performed to reduce the duration of the NASBA reaction on card. Different times (90, 60, 45 and 30 minutes) for the incubation step at 41°C have been tested, concluding that the NASBA reaction can be reduced down to 60 minutes without affecting the yield (data not shown).

With the aim of testing the biocompatibility of material and NASBA reagents, NASBA was carried out in the microfluidic cards. RNA from L. pneumophila was used as template for the reaction. All the reagents were mixed on tube and then introduced into the card and heated at 41°C for 90 minutes. The results showed that from 7 assays, we could collect NASBA products from five samples, four were properly diagnosed (3 positives and 1 negative (NTC)) and one was a false positive (lane 9).

It was noticed that it was absolutely necessary to define and control a pretreatment in order to avoid cross contamination. These results were very promising to go on with NASBA experimentation in the final device (WP5).

Structural interface between cartridge and readout system (VTT and DLO)
In collaboration with VTT, system requirements for the microfluidic card were defined by DLO. Here for, the requirements for probe hybridization and requirements for the mixing of NASBA products with hybridized probes together with NALFIA running buffer were characterized. To facilitate the mixing procedure in the microfluidic card, volumes to be mixed were made equal. In NALFIA 2 µL of NASBA product will be mixed with 2 µL hybridization buffer (32x SSC, pH 7). After that, this mixture of 4 µL will be mixed with 4 µL of probes. In the end this mixture of 8 µL will be added to 90 µL of running buffer (in WP4, this buffer will be specified in detail).

WP4 – DEVELOPMENT OF A NASBA-NALFIA-BASED READOUT SYSTEM
Purpose and scope
The main goal of work package 4 is to develop a NASBA product detection method using a lateral flow-strip-based readout platform that can be integrated into the final Legionella pneumophila diagnostic system. On the one hand the lateral flow assay should be suited to be coupled to the device in which the NASBA protocol will be performed and on the other hand the lateral flow strip should be suited for direct signal reading by the user or for digitisation of the signal by flatbed scanning or digital imaging. During the project a new task was defined to develop a Legionella multiplex PCR-NALFIA to create a marketable product.

Task T4.1- Design and development of tag-labelled probes to sandwich NASBA amplicons in NALFIA
Detection of NASBA amplicons in NALFIA requires the immobilization of anti-tag antibodies on nitrocellulose membranes. This is done via a sandwich of an immobilised antibody on nitrocellulose, a NASBA product with two hybridised tagged-probes, and carbon nanoparticles bound to neutravidin. After NASBA amplification two tagged-probes should be added to the final solution. These probes are complementary to the anti-sense single-strand RNA-amplicon produced. Specific hybridisation yields a partly double-strand product having two tags that can be sandwiched in the lateral flow assay. The carbon nanoparticles are used as a signal label in this assay.

An important aspect of probe development is a resulting hybridised product that can be sandwiched in the NALFIA. Since single strand RNA amplicons can adopt complex tertiary configurations it is essential to use probes that counteracts this tendency and yields hybridised products with an extended configuration. Therefore, several probes were designed to be tested in the NASBA-NALFIA procedure from the gene sequence available. This process was preceded by the design of suitable primers to amplify the RNA strand of choice (work package 3). The design and choice of primers and probes is based on the Mip gene that is Legionella pneumophila specific. In total 3 series of probe sets were designed.
Probeset 1:
ACCGAACAGCAAATGAAAGACGTTCTTAACAAGTTTCAGAAAGATTTGATGGCAAAACGTACTGCTGAATTCA
ATAAGAAAGCGGATGAAAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGCGTT

Probeset 2:

ACCGAACAGCAAATGAAAGACGTTCTTAACAAGTTTCAGAAAGATTTGATGGCAAAACGTACTGCTGAATTCA
ATAAGAAAGCGGATGAAAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGCGTT

Probeset 3:
ACCGAACAGCAAATGAAAGACGTTCTTAACAAGTTTCAGAAAGATTTGATGGCAAAACGTACTGCTGAATTCA
ATAAGAAAGCGGATGAAAATAAAGTAAAAGGGGAAGCCTTTTTAACTGAAAACAAAAACAAGCCAGGCGTT

Probe set 1 contains two long probes with a GC% as high as possible for a strong binding of the probes to the amplicon. However, these two probes appeared to form probe dimers. To avoid this, shorter probes were designed (probe set 2). These probes gave a positive result, however this procedure appeared not to be reproducible in later experiments. Therefore, probe set 3 was designed with longer spacers between the probe sequence and the tag to avoid steric hindrance. In probe sets 1 and 2 "A-nucleotide" spacers were used. As the amplicon contains many U-nucleotides and the spacers were made longer in probe set 3, in this probe set "T-nucleotide" spacers were used to avoid nonspecific binding of the spacers to the amplicon. In table 1 a summary can be found of the probes developed.

Task T4.2- Design and test a lateral flow-based detection system
Detection of NASBA amplicons in NALFIA requires the hybridisation of two tagged-probes to the NASBA product. Therefore, the optimal conditions for the NASBA-NALFIA probe hybridisation were determined. The probes hybridising to the amplicon were tested in many combinations but no results were seen on NALFIA. When hybridisation of only one probe to the NASBA product, we were able to detect a small increase in size on a 4% agarose gel. Only for a small number of developed probes the increase was detected. First of all, the pH of the running buffer and the buffer composition were optimized. Based on the results from the agarose gels, a new selection of probes was made and subsequently these probes were tested in pairs in different types of buffer and different pH ranges. It was found that a PBS running buffer with pH 7.4 showed the strongest signal with a clear background. Furthermore, it was found that adding increasing amounts of saline-sodium citrate (SSC) in the hybridisation buffer was essential for the hybridisation. Different amounts of SSC were tested in the hybridisation buffer and 8x SSC appeared to be the optimal concentration which resulted in stronger lines on NALFIA. Various hybridisation temperatures and incubation times were tested, whereas 2 minutes at 41°C showed the best results for hybridisation. After testing all different probes, buffers, pH values and SSC concentrations, only one combination of probes, probe 1 and probe 3 appeared to be successful for NASBA-NALFIA detection.

The developed NASBA-NALFIA procedure was performed as follows: NASBA product (2 µL) was mixed with 2 µL probe number 1 (labelled with digoxigenin, concentration 0.5 µM) and 2 µL probe number 3 (labelled with biotin, concentration 0.5 µM). Hybridisation was performed in 8x SSC buffer (2 µL) at 41°C in a heating block for two minutes. Subsequently, samples were transferred to a running buffer (PBS pH 7.4, 1% BSA, 0.05% Tween-20) and mixed with a conjugate of carbon nanoparticles and immobilised neutravidin. The combined solution (100 µL) was added to a NALFIA nitrocellulose membrane onto which a goat-anti-digoxigenin antibody had been immobilised (1 µL per 0.5 cm of a 100 ng/uL solution). Dependent on the amount of amplicon the strip could be read between 5 and 15 minutes. The appearance of a black/grey line indicated the presence of the specific amplicon product.

Task T4.3- Validation of the NALFIA readout system

Due to the time consuming development of the NASBA-NALFIA procedure, during the 18 month meeting, it was decided within the consortium that two minor evaluations would be performed instead of a validation. The RNA isolation and NASBA amplification were performed at Gaiker, whereas DLO-FBR performed the further probe hybridisation and NALFIA detection. Specificity and sensitivity of the NASBA-NALFIA procedure were evaluated. For the specificity a collection of 29 culture samples from different Legionella subspecies and other bacterial species were evaluated according to the developed NASBA-NALFIA procedure. This evaluation showed positive lines for L. pneumophila samples, whereas all other Legionella subspecies were negative, except for one of the two L. anisa samples that indicated a faint line. All other bacterial species and nuclease-free water controls were negative as expected.

To evaluate the sensitivity of NASBA-NALFIA, a culture sample of Legionella pneumophila was diluted in 1/10 dilution series and RNA was extracted. These RNA samples were evaluated in the developed NASBA-NALFIA procedure, resulting in a detection limit of around 100 Legionella pneumophila cells in the RNA extraction.

Task 4.4: Development of a L. pneumophila PCR-NALFIA detection system

This newly defined task within WP4 was requested by Q-Bioanalytic. A L. pneumophila multiplex PCR-NALFIA was developed to create a marketable product. For this purpose, DLO-FBR received primer sequences (listed in table 2) for L. pneumophila based on the Mip gene and for Legionella subspecies from Q-Bioanalytic. These primers have been tested without a template for primer-dimers (no false-positive reactions seen) and analysed on agarose gels. Also a variation of annealing temperatures were tested to optimize the PCR protocol. For L. pneumophila the combination of primer 2 and 4 appeared to be successful with the result of an amplicon with a size of 141 nucleotides. The proper combination for Legionella subspecies was the combination of primer 6 and 7 with the result of an amplicon with a size of approximately 230 nucleotides. The selected primers for the multiplex PCR are highlighted in yellow.

Primer set L. pneumophila greater than L. pneumophila serogroup 1 Mip gene

GCAACCGATGCCACATCATTAGCTACAGACAAGGATAAGTTGTCTTATAGCATTGGTGCCGATTTGGGGAAGA
ATTTTAAAAATCAAGGCATAGATGTTAATCCGGAAGCAATGGCTAAAGGCATGCAAGACGCTATGAGT

Primer set Legionella subspecies greater than L.anisa strain

CTAATTGGCTGATTGTCTTGACCATATAATCTGAGTTACTTCAGATTGTGAATGCGAATACAAGATGTAGGTT
GGGCCAAGGCTCAACCTACGCAGAACTACTTGAAACAAAGTGTGAACTTCTTTATTTACCTAATGCTTGATTG
AGGTATAATGCCTTACAATCAATGCAAAACCAGTTTTCCTGGCGACCATAGCGGTTTGGAACCACCTGAATCC
ATCTCGAACTCAGAAGTGAAACGAACCCGCGCCAATGATAGTGTGAG

The PCR primers were labelled with a tag so after amplification of the potential amplicon, the sample can directly be sandwiched in the lateral flow assay. The L. pneumophila¬ primers have a biotin and dinitrophenyl tag, whereas the Legionella¬ subspecies primers have a biotin and digoxigenin tag. Both parameters can be detected in one assay.

DNA (1 µL) was mixed with 0.5 µL off each 4 primers (labelled with respectively dinitrophenyl, digoxigenin or biotin and a concentration of 20 µM), 4 µL dNTP's (concentration 10 µM), 4 µL Phire Hot Start DNA Polymerase Buffer (concentration 5x), 0.4 µL Phire Hot Start DNA Polymerase II (concentration 5 U/µL) and 8.6 µL nuclease-free water. PCR amplification was performed in a Piko Thermal Cycler (Thermo Scientific)

with an incubation sequence of 30 sec. 98°C; 30 cycles of 5 sec. 98°C, 5 sec. 60°C, 5 sec. 72°C and finally 60 sec. 72°C. Subsequently, samples were transferred to a running buffer (Borate Buffer pH 8.8, 1% BSA, 0.05% Tween-20) and mixed with a conjugate of carbon nanoparticles and immobilised neutravidin. The combined solution (100 µL) was added to a NALFIA nitrocellulose membrane onto which two antibodies had been immobilised. One anti-dinitrophenyl-KLH antibody (1 µL per 0.5 cm of a 100 ng/uL solution) which is specific for L. pneumophila and one goat-anti-digoxigenin antibody (1 µL per 0.5 cm of a 100 ng/uL solution) which is specific for Legionella subspecies. Dependent on the amount of amplicon the strip could be read between 5 and 15 minutes. The appearance of a black/grey line indicated the presence of the specific amplicon product.

A minor evaluation was performed to determine the specificity of the multiplex PCR-NALFIA procedure. A collection of 44 culture samples from different L. pneumophila subspecies, Legionella subspecies and other bacterial species were evaluated according to the developed PCR-NALFIA procedure. This evaluation showed two positive lines for all L. pneumophila samples, except for L. pneumophila type 9 which indicated no lines. One line was detected for the Legionella non-pneumophila subspecies and all other bacterial species and nuclease-free water controls were negative as expected. The analysis of the PCR products on agarose gel showed the same results (not shown).

Conclusions
Within this work package tag-labelled probes to sandwich NASBA amplicons in NALFIA were designed successfully. Moreover, a lateral flow-based method for the detection of Legionella pneumophila was developed successfully. The NASBA-NALFIA procedure was briefly evaluated, but an extensive validation would be recommended. Furthermore, a multiplex PCR-NALFIA detection system was developed. This procedure was briefly evaluated, but an extensive validation would be recommended as well.

WP5 – SYSTEM INTEGRATION

Task T5.1- System specifications
The initial system specifications reported in D5.1 have been adjusted according to the progress of WPs 2, 3, and 4. Based on the results of WP2 and WP3, the Legionella cell lysis and the capture of the RNA are to be done outside of the PINVIALEG analysis device. The analysis procedure of the NASBA-NALFIA on chip was fixed based on the results of WP3 and WP4. Testing of the NASBA steps on chip in WP3 provided information of the required sample and reagent volumes and incubation times to be used in the analysis. The same information for the NALFIA part of the analysis was received from the method development in WP4.

The final system specifications were set in M32 and these specifications were used in finalising the PINVIALEG analysis system. The final analysis procedure and the final hardware specification of the PINVIALEG analysis system are presented in tables 1 and 2, respectively.

The system is a plug-in device. There is no battery operation available even though the components have been selected to have as low power consumption as possible. The pumps are small size peristaltic pumps and all valves are external to the chip. The incubation heater is an aluminium plate with heating resistors and a thermocouple for monitoring the temperature of the plate. The tubing is mainly of PEEK (poly ethyl

ether ketone). C-FLEX tubing is used in the peristaltic pumps. All reagents are stored in sterile vials and replaced between each analysis run.

Stability of the NASBA reagents

In the PINVIALEG system the reagents are not stored in the chip. They are brought in from external vials as the analysis procedure proceeds. Therefore, Gaiker studied the reagent stability in order to assess how long the reagents stay valid in the room temperature.

NASBA reagents are supplied in pellets (each pellet for 8 reactions) by Biomeireux. Those pellets need to be resuspended and then can be stored at specific temperatures, 4-8 and -20°C, depending on their chemical nature, for 15 days maximum according to manufacturer. The PINVIALEG project aimed to develop a prototype to perform analyses on site. Our goal was to perform the maximum number of reactions once the pellets are resuspended. In order to get a simple prototype, the PINVIALEG aimed to perform analyses at room temperature. Thus, an important point to take into account for the detection of Legionella on-site with the portable device was to know the stability of the NASBA reagents at room temperature, so several assays have been performed in this way.

Once they were resuspended, which is considered time 0, the reagents were let at room temperature for x (8-168 hours) and they were used for the NASBA assays. Gaiker established different periods of time: 0, 8, 24, 48 (2 days), 96 (4 days), 120 (5 days), 144 (6 days) and 168 h (7 days). In total, 4 assays in different weeks and by different technicians, performing NASBA reactions at these different times with the same reagents (three replicates each time). The coefficient of variation was always less than 15% except for longer times, 5, 6 and 7 days.

The results showed minor variations between data obtained after 24 hours, in comparison to time 0. Taking into account the format NASBA reagents are supplied by the company, 24 hours could be "the" period of time for the PINVIALEG analyses with the same reagents (8 samples per day).

Task T5.2- Realization of the integrated system

The integrated system is used to perform the NASBA-NALFIA analysis on a polymeric microfluidic cartridge or chip. The microfluidic card was designed and developed in WP3 based on the analysis procedure defined in Task T5.1. Realization of the integrated system was done in WP5 in parallel with the card development in WP3.

Card holder

A special card holder was designed to interface the polymeric microfluidic card with the fluidic components in the unit. The fluidic connections are located in the bottom plate of the holder and permanent magnets are placed in the top plate of the holder. The polymeric microfluidic card is inserted between the top and bottom plates and aligned with the fluidic connections.

VTT has fabricated three different card holder prototypes during the project. The first two prototypes were made to test NASBA reagent and enzyme mixing and NASBA amplification on the card. These holders were built to fit with the test designs of the polymer card. The first prototype was fabricated from PMMA (polymethyl metacrylate) and delivered to Gaiker for testing NASBA on chip. Later a duplicate was fabricated of PEEK (polyether ether ketone) and sent also to Gaiker for testing. The first prototype had 16 fluidic access ports but it was without integrated

fluid flow control. The sample and the reagents could be injected to the chip and aspirated from it by ordinary syringes. The ports not used were sealed with tape.

The second card holder prototype fabricated of PEEK had also 16 fluidic access ports but now it was equipped with integrated fluidic control. Three small peristaltic pumps were connected to the fluidic access ports using silicone tubing. Each pump could be connected to any of the 16 fluidic access ports to test different fluidic channel lengths/volumes on the polymer card or different mixing configurations. The calibrated pumping speeds could be adjusted with potentiometers. A PC control option for setting the pumping speeds was also available.

The third and final card holder was made to fit the final polymer microfluidic card design and it was used in the final integrated analysis system. Heater and thermistor elements were placed in the bottom plate of the card holder to achieve the required temperature control. The number of fluidic access ports was 10. In addition there was one fluid access port in the top plate designed for pipette tip access for sample injection. Optical detection was used to monitor the liquid front end propagation in the microfluidic card to set the triggering for the steps. Five permanent magnets were embedded in the holder lid for magnetic bead capture. The NALFIA strip was inserted in its own housing at the edge of the card holder.

PINVIALEG analysis system

The laboratory scale integrated NASBA-NALFIA analysis system built in the PINVIALEG project consists of a microfluidic card holder, NALFIA strip with its housing, external pumps and valves, vials for reagent storage and waste, and required electronics (I/O modules, power supply unit, electrical interconnections, optical detector amplifiers). The whole integrated analysis system was fitted on an optical breadbord of 300 mm x 450 mm in size. The height of the whole system is 250 mm and the weight 11 kg. In addition to this integrated analysis system a reader device for NALFIA strips and a laptop are needed.

For reading the resulted lines in the NALFIA strip VTT modified its existing POCTER reader technology. The existing POCTER reader was fit to be used with the stick type and size used in the PINVIALEG project. Modifications were done both to the mechanics (stick adapter) and the software (number and location of lines on the stick).

Testing of the fluidic functionalities at VTT

Once the system was built the basic operation and fluidic functionalities of the PINVIALEG analysis system were tested at VTT. Real reagents were used in testing. The first tests were performed on shorter entities of the whole procedure. These were to be combined when the parameters for flow control were defined during these sub-procedure tests.

The total analysis time is about 2 hours 15 min. This includes filling and changing the vials manually, changing the polymeric chip and performing the cleaning procedure between adjacent analyses.

The sample injection (volume 5 µl) and the capture of the magnetic beads worked well and repeatedly. Unfortunately some leakage problems occurred when the magnetic beads were washed on the chip. The PMMA chip material (microfluidic card) could not tolerate the alcohols that were present in the washing liquids. This problem had been seen earlier but the

preventive measures taken to overcome the problem were not enough in the final system. Thus, it was decided that the lysis and elution of the sample RNA was done outside the polymeric chip in the final analysis procedure.

After RNA amplification and NALFIA hybridisation the sample is flushed to the NALFIA strip from the chip edge. For this purpose a special tip structure was designed in the microfluidic chip and optimized based on the test results.

For cleaning of the integrated system a special cleaning procedure was developed. All tubings in the system and the chip holder are washed with a cleaning liquid and then rinsed with pure water before replacing a new microfluidic card into the system and starting the next measurement.

Task T5.3- Control software
A separate laptop is used to run the control software. This software includes a user interface and controls the timed sequence execution including the control of all pumps, valves and the incubation heater.

The software to control the system was developed using National Instruments Lab View graphical programming. The program frame is an event driven state machine in which the code proceeds from state to state based on the recorded events or triggering. The events can be timed, sensor signal dependent or manual (user input necessary). The LabView control software runs according to a set sequence that is the guideline for the program to make the state transitions. The sequence consists of numbered steps, each step contains a trigger condition to advance to the next step in the sequence.

The main user interface of the LabView control software shows all measured signals and temperatures and the progress of the program execution. It is also used to control the program execution (starting, pausing, stopping, and continuing).

As only a laboratory scale prototype was realised and the analysis protocol could not be fully fixed, no embedded software control for the system was realized during the project.

Task T5.4: System evaluation.
The integrated analysis system was delivered to DLO for application testing in month 24. About 100 sterilized polymer microfluidic cards were sent to DLO with the system. The fluidic parts of the PINVIALEG analysis system were autoclaved at DLO before taking the system into use.

The main goal at DLO was to assess the prototype device together with the microfluidic card that both have been developed at VTT in Finland. Therefore, researchers of VTT and FBR-DLO collaborated in the laboratory of FBR-DLO to perform the first experiments with the device. Both researchers had expertises that were useful for these first tests. The VTT researcher explained how to work with the system (also a manual was made) and the FBR-DLO researcher performed the handling with the RNA, the enzyme and the buffers.

The NASBA-NALFIA test to detect Legionella pneumophila had been developed as an assay in tube using a thermal cycler (see D4.2: "Document describing the design of the lateral flow-strip-based readout platform"). Tests with the device were compared with that method. A first test showed

that it was not possible to run a complete positive test in the device. Therefore the procedure of the device was divided in subsequent steps and the individual steps were tested:
1. NASBA performed in chip followed by the hybridization of the probes and running of the NALFIA outside the device (in tube in a thermal cycler). The NASBA reagents and RNA template were mixed in a tube and incubated at 65°C for 2 minutes in a thermal cycler (PIKO thermal cycler, Thermo Scientific) before adding to the device (= NASBA mixture). After that:
a. The NASBA mixture was directly injected in the chip (by pipetting) and incubated at 41°C for 60 minutes in the chip. The NALFIA of this NASBA product was successful and of the same intensity as the NASBA-NALFIA that was performed in a tube in a thermal cycler.
b. The NASBA mixture was injected via the pump system of the device and incubated at 41°C for 60 minutes in the chip. Also this NASBA-NALFIA was successful and of the same intensity as the NASBA-NALFIA that was performed in a tube in a thermal cycler.
2. Injection of RNA, NASBA enzyme, and NASBA reagents into the device via the pump system of the device followed by performing the NASBA reaction in the device. The hybridization of the probes was performed in tube in the thermal cycler. No positive NALFIA line was found using this NASBA reaction whereas a line was found after analysing the NASBA performed in tube (positive control).
3. Using the same method and conditions as in point 2, also higher RNA concentrations (up to 10 times higher) have been used. These higher RNA concentrations did not result in a NALFIA line using the NASBA reaction produced in the device. The positive controls performed in tube resulted in positive NALFIA lines.
4. Injection of NASBA product in the device followed by hybridization of the probes to the NASBA product in the device. The NALFIA readout was performed in the device as well and resulted repeatedly in a positive line. The analysis unit was run in automatic mode during these tests.

Taken together these different experiments, it can be concluded that most probably the mixing of the NASBA components is a problem. In contrary to the NASBA reaction components, mixing of the NASBA product with probes and the hybridization buffer for NALFIA, occurs with equal volumes. Most probably these equal volumes facilitate the mixing.

Knowing these results, it was not possible to perform the planned validation sensitivity study and the validation study with real water samples at FBR-DLO and Gaiker. Furthermore, in the final meeting of the project (month 26), it was decided not to send the device to Ayuntamiento of Bilbao in Spain as it would not be possible to test real samples. The PINVIALEG-device has been send back to VTT.

Task 5.5: Evaluation of the portable and integrated device
Based on the results of Task 5.4 this task could not be started.

Potential Impact:

The strategic impact of this project was to provide a new robust device for the front-line of Legionella detection, applying a more rapid and sensitive technology based on recent advances in molecular biology.

Once finished the project, the impact of the project with the results achieved are:

- Improving water safety in the European Union by making rapid multi-analyze tests available for water testing labs

- Strengthening the expertise of the involved SMEs in product of molecular tests

- Enhancing the competitiveness of the involved SMEs by adding new products to their portfolio

Legionella testing in drinking water is one of the most performed surveillance test in Europe. The numbers of cases differ considerably high in the European Union. In Germany in November 2011 a new guideline for drinking water was set into force that requires regular legionella testing in water heating units above 400l. In the Netherland and France similar regulations exist. Background is the rising legionellosis in Europe. In Germany a rise of 360% in the last 10 years was observed. 15% of the cases are lethal.

Currently the official surveillance testing is based on classical culture methods, which take up to 14 days to deliver results. The tests to be transformed into validated commercial kits in combination with the large filtration unit can deliver results within a few hours. This can lead to significant better workflow of testing saving time and energy and allowing rapid reactions upon cases of high contamination.

The primary customers include water testing service laboratories across Europe, estimated at a total number of 8,000. The laboratories run an approximate annual volume of Legionella tests 10,000 and this number is growing at a rate of 5%. Currently, a share of 98% of this volume is accounted to conventional cultural tests, namely 78,4 Million tests If the cost for one test is 100 EUROS the market size would be 392 Mio. EUROS per year. It is expected that the availability of new diagnostic tools will enable to acquire a significant fraction of this market. If there is a market share of 5% an annual turnover of 20 Mio. EUROS can be expected. It might take three more years to reach this market share. However, the market penetration will be costly in the first phase in terms of personnel, As highly skilled personnel are needed for this, the marketing activities will also lead to the strengthening of the job market in the knowledge based economy the SMEs are part of.

This business expectation of (parts of) the rapid methods developed in this project is based on the fact that there is a clear regulatory and/or economic demand for rapid diagnostic tools to assess the Legionella status. Such validated test kits and filtration units are lacking at the moment, and thereby, further underpin the high importance of the outcome of the PINVIALEG project.

Legionella outbreaks have great sanitary, economic and social implications. Currently the only accredited method for detection and

quantification of Legionella is bacterial culturing. Despite the presence of different alternative methods, these have not yet found equal to the standard ISO method (culturing) by means of living /dead discrimination, quantification and/or sensitivity. In the FP7 project, PINVIALEG, a new approach for rapid, preferably on site detection of Legionella was developed.

The prototypes derived from this PINVIALEG project were successful as separate modules of the desired integrated system for on-site diagnostic analysis of Legionella in water systems, being
1) an innovative filtration unit for large volume handling and
2) a rapid and specific molecular amplification procedure followed by
3) a novel nucleic acid lateral flow immuno assay (NALFIA).

The prototypes have great market potential separately, but especially as combined tool for rapid, same day diagnostics for water pathogens like Legionella. The combination of the filtration unit prototype, rapid nucleic acid amplification of solely viable RNA and the prototype lateral flow detection strip (NALFIA technology), allow for easy and simple to use on site diagnosis of presence of Legionella. Application of the combined system will lead to dramatic reduction in time and costs, and gives rise to increased human safety and a higher level of Legionella prevention strategies.

Connecting the prototype modules, a rapid methodology will be available that generates rapid results and will support decision making in Legionella prevention strategies in the European Union and worldwide.

Moreover, results will broaden the application areas of hot-embossed, disposable polymeric cards.

Dissemination strategies
The dissemination of these results will be facilitated through conversion of the developments into commercial products. A subsequently to this project necessary intense validation of the tests will be required to reach market acceptance. The process of product development and is already ongoing.

The commercialization of the products will be done by own sales forces and by distributors. QBA is actively seeking for distributors. For this purpose expositions at trade fairs took place and will be further done.

List of Websites:
http://www.pinvialeg.eu