Microchip device with dry-stored reagents for Loop mediated isothermal amplification

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Abstract. Storage of key reagents for amplification in a microfluidic device is decisive for point-of-care diagnostics. A simple approach of drying the reagents without special stabilizers by dehydration in a glass microchip is described. For loop mediated isothermal amplification of nucleic acids, a microchip device with two reactors was approbated. It was shown that the microchip device is suitable for detecting nucleic acids template of the Sacbrood virus. The experimental results obtained in microchip reactors with dry-stored primers were comparable to the results with non-dried primers. The shelf life of dehydrated primers without declined its reactivity turned out less than three months.

1. Introduction
In recent years, there has been a growing interest in developing integrated, portable microfluidic devices for nucleic acid-based diagnostic for many applications, including clinical diagnostics, food quality control, and environmental monitoring [1, 2]. The advantages of microfluidic devices include: 1) small reaction volumes that reduce sample and reagents consumption; 2) small thermal mass what ensures rapid heat transfer with low thermal inertia; 3) portability with low power consumption; 4) the possibility to combine a sample preparation, purification and extraction of nucleic acids, its amplification and detection on a microfluidic platform. If the microfluidic device contains dry or liquid reagents, simple pipetting operations are required to start the processes. This prevents contamination of samples and facilitates rapid diagnostic by minimally trained personnel for point-of-care applications especially under conditions of resource constraints.

Polymerase chain reaction (PCR) is a widely used amplification technique for molecular diagnostics, which enabling to amplification of target nucleic acids templates containing just a few molecules to detectable levels. Typically, the reaction mixture is prepared immediately before the PCR by mixing all the components, some of which are stored at low temperature (-20°C). Long-term storage, as well as repeated freezing and thawing can lead to loss of reactivity of the reagents. To preserve the reactivity of PCR reagents and increase their shelf life at temperatures above 0°C, freeze-drying is used. Addition of special stabilizers allows maintaining the properties of the dried PCR mixture for more than a year when stored in a wide temperature range [3]. Successful on-chip PCR amplifications
with dried reagents stored were reported [4-6]. However, the PCR requires a special temperature mode with rapid temperature transitions and precise temperature control for efficient and selective amplification of nucleic acids. Isothermal amplification techniques, such as loop mediated isothermal amplification (LAMP), may resolve the challenges associated with the accuracy of the amplification temperature mode without compromising analytical sensitivity and specificity. LAMP has a moderate incubation temperature leading to simplified heating and low power consumption, and allows direct genetic amplification from bacterial cells due to tolerance to substances that typically inhibit PCR, in addition, the LAMP result can be detected either visually or by simple detectors [10, 11]. The amplification results obtained with the storage of the LAMP reagents were also presented [7-9]. These advantages make it especially interesting at developing of microfluidic devices for point-of-care applications.

The point-of-care diagnostics include tests at home, at the bedside or in the field when resources are limited. It is relevant not only for diagnosing of human diseases, but also animals and insects. The Säcbrood virus (SBV) is deadly to broods of honey bees. A vector of the virus is adult bee, for which the disease can be asymptomatic, while a single diseased larva can infect up to 3000 healthy larvae [12]. Therefore portable detection methods are necessary to control and eradicate SBV in the field. A one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed for the rapid identification of SBV [13]. Isothermal amplification was carried out in a simple water bath for 30 min at 65°C, and a positive amplification reaction was visible to the naked eye due to addition of intercalating fluorescence dye (SYBR Green I). The authors concluded that color observation method (using fluorescence dye) was ten times more sensitive than the white turbidity observation.

The purpose of our research was to choose simple techniques for implementation a prototype microfluidic chip for SBV detecting with dehydrated reagents inside. We believe that developing of miniature device containing dry-stored reagents for LAMP would simplify even more this assay for application in the field diagnostics. We used the same primer system [13] and LAMP reaction mixture with an intercalating fluorescence dye to amplify the SBV target templates. Primers were chosen as reagents for storage because of they relate to the key reagents for amplification and determine the specificity and final yield of the reaction. Dry-stored primers for different target templates in consequently different microfluidic channels allow a multiplex LAMP assay, for example, for rapid analysis of several genes [8]. At the same time, primers are much less sensitive to drying than other main components of the reaction such as polymerase enzymes.

An overview of current methods for integration various classes of reagents inside microfluidic devices are considered in [14]. There are two main approaches: 1) the immobilization of reagents on carriers (beads, hydrogels and paraffin capsules) followed by an integration of these carriers onto a microfluidic device and 2) the direct pattering of reagents onto surfaces in microstructures. The latter one includes contact deposition methods (micro and nanocontact printing, high-resolution patterns using Dip-pen nanolithography and FluidFM) and non-contact deposition methods (pipetting robots, sprayers and piezoelectric inkjet printers). Their advantages are reproducibility, high accuracy of dosing of small volumes down to femtoliters, and the possibility of industrial application with high throughput of deposition and low dead volumes. However, most of these techniques require complex technology equipments to treatment of surface at the intermediate stage of microchip manufacturing, when access to the microstructures is free. In this case at the next stage of device manufacturing it will be necessary to protect the received layers of reagents from affecting of the applied technology of bonding for hermetization of microstructures. A method of drying reagents by dehydration in microstructures was chosen as the most simple and affordable method of introducing reagents into microchip reactors. This enabled the integration of the reagents in ready-made microchip devices.

2. Experimental technique

Microfluidic chip consisted of two glass plates that were bonding together with photo-curing adhesive. There were two reaction cells with a depth of 100 µm connected to four wells for liquid input and
output on the microchip (Fig.1a). These reaction cells (reactors) were fabricated by photolithography methods and acid etching. The total volume of each reactor was 2 µl, and, in addition, the same volume was required for the input/output system.

Four microliters of primers solution (F3/B3 0.3 µM; FIP/BIP 1.2 µM; LF/LB 0.6 µM) was injected into one of the reactors via the inlet well; the other reactor was used as positive control of the amplification process. Since the glass surface was hydrophilic, the solution filled the entire reactor evenly and then evaporated completely, leaving the primers in the microchannel. The microfluidic chips with dried reagents were stored at room temperature for 24 hours, 1 week and 3 months without refrigeration and with inlet and outlet open. Sample preparation with extraction and reverse transcription of SBV nucleic acids was carried out on the base of the All-Russia Research Institute of Agricultural Biotechnology. Reagents for LAMP with intercalating Eva Green dye and SD polymerase were provided by SYNTOL (Moscow, Russia). Nine microliters of reagent solution (with or without primers) and one microliter of DNA-template solution were premixed. As the target template the cDNA fragment of SBV was used with concentrations 10⁴ and 10⁵ copies/µl. Four microliters of the mixture were injected into a reaction cell. To avoid evaporation during heating, all microchip wells were sealed with mineral oil. Then the microfluidic chip was heated by a thermocycler in an isothermal temperature mode for about 45 minutes. The final result of DNA amplification was determined based on the fluorescence induced by intercalating dye. Prototype model of the scanner device (IAI RAS, Russia) was used to get result images. In parallel with the experiments on microfluidic chip, control tests were carried out in polypropylene tubes with 10 µl mixture by the analyser ANK–32 (IAI RAS, Russia). There were tubes with negative control (without DNA template), positive control (solution contained all reaction components, as in the control reactor on a microchip), control of primers lack (solution without primers, as in the reactor with dry-stored primers). Also, tubes with dehydrated primers were used, which had been prepared and stored under the same conditions as the microchip with dried reagents.

3. Result and discussion
Since any intercalating fluorescence dye is non-specific and can have a fluorescent signal from non-specific DNA targets, it was important to evaluate the level of the background fluorescent signal under our conditions. Images obtained after isothermal amplification without target templates (negative control) in one microchip cell and with the addition of 104 copies of the target DNA (positive control) in another cell are presented in Fig.1b.

The heterogeneity of the fluorescence signals within a cell area requires more careful analysis. Simulation of informative signals from the images of positive and negative control allows us to draw the following conclusions.
1. If informative signals is considered as random quantities, then the distribution of these random variables has the following general integral characteristics: the asymmetry coefficient $A$ is small (absolute value is not more than 0.1), the excess coefficient $\epsilon$ for these distributions has the value 2.1±0.3. These parameters are approximate to the integral characteristics of a uniform distribution. However, the probability for hypothesis of a uniform distribution with parameters found from a sample of 10 measurements estimated based on a $\chi^2$ criterion (Pearson-criterion) as less than 1%. Even the refinement of the parameters increases this probability to 4.5%, which also does not allow it to be accepted.
2. Such a pattern of distributions is more adequately modeled either by a mixture of normal and uniform distributions, or by a sum of 2-3 normal distributions with spaced mathematical expectations and comparable weights.
In particular, the informative signal with positive control (see Fig.1b) is well approximated by the sum of three Gaussian distributions with equal weights and variances. In this case, the mathematical expectations are shifted relative to each other by approximately 2.5 standard deviations. The value of the selective correlation coefficient between the experimental and model distributions estimated at 12 points exceeds 0.91, which indicates the adequacy of such an approximation.
This distribution law corresponds to the following scheme for the formation of an information signal: an optical signal is measured from a part of the region with a transfer function of the Gaussian type, and then similar informative signals are added from the other two parts. In this case, the field of view overlaps slightly.

The parameters considered earlier - the coefficients of asymmetry and excess - refer to the normalized characteristics and do not allow estimating the quantitative coincidence of the signals. Eventually, a comparative estimate can be given for two parameters: amplitude (maximum of the signal after compensation of the baseline) – 6.7 and 8.9 relative units; area of the peak (area under the curve) - 23 and 32 relative units. The level of fluorescence signal obtained in positive control is more than the same in negative control. Thus, our glass microchip is suitable for detecting of Sacbrood virus by loop mediated isothermal amplification method with intercalating dye.

The results obtained on microchip with dry reagents stored for 24 hours are presented in Fig. 1c. In this case concentration of $10^5$ copies/µl of the target DNA-template was used. The amplitudes of signals resulted were 11.6 and 10.7 relative units, consequently. Areas of the peak (area under the curve) were 46.2 and 42.5 relative units. There was a good quantitative coincidence of the estimates. Consequently, the primers stored in one of the microchip cells were successfully hydrated by injected mixture and the LAMP reaction began to progress without deterioration the efficiency.

Further experimental results showed that the activity of dehydrated primers was persistent after storage for a week, but decreased after three months of storage both in the microchip reactor and in polypropylene tubes. To determine the more accurate lifetime of dry primers research should be continued.
4. Conclusion
A simple approach to the integration of primers into the glass microchip device by dehydration
technique was applied, and the results obtained after loop mediated isothermal amplification in a
reactor with dehydrated primers were comparable to the results with non-dried primers. The storage
life of dehydrated primers turned out less than three months. It is attractive that all the technologies
used were simple and commonly available.

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