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METHODS AND PROTOCOLS

PCR-tips for rapid diagnosis of bacterial pathogens

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Abstract

Micropipette tips are currently among the most used disposable devices in bioresearch and development laboratories. Their main application is the fractionation of solutions. New functionalities have recently been added to this device, widening their applications. This paper analyzed disposable micropipette tips as reagent holders of PCR reagents. PCR has become a prevalent and often indispensable technique in biological laboratories for various applications, such as the detection of coronavirus and other infectious diseases. A functional micropipette tip was implemented to simplify PCR analysis and reduce the contamination chances of deoxynucleotides and specific primers. This disposable device is prepared by tip coating processes of reagents, using polyvinyl alcohol polymer and additives. The coated layer is optimized to load and release PCR reagents efficiently. As a proof of concept, we show that the detection of *Bordetella pertussis*, the etiological agent of whooping cough whose diagnostic relies on PCR, can be quickly done using practical-functional tips. This device is an excellent example of testing the functionality and contribution of molecular diagnostic PCR tips.

Key points

- *Functional micropipette tips are prepared by coating with dNTPs and primers.*
- *Functional tips are used to replace dNTPs and primers in the PCR master mix.*
- *PCR diagnostic of Bordetella pertussis is performed using functional tips.*

Keywords Reagent fractionation · Disposable tips · PCR · *B. pertussis* detection

Introduction

Rapid diagnostic tests (RDTs) are point-of-care diagnostics intended to provide diagnostic results in a short time to the patient with little to no laboratory equipment or medical training. Also, RDTs should be simple, sensitive, specific, and low cost. Current RDT platforms are lateral flow tests (Mak et al. 2016), latex agglutination tests (Ortega-Vinuesa and Bastos-González 2001), vertical flow devices (Chen et al. 2019), and microfluidic or lab-on-a-chip devices (Pires et al. 2014). PCR test is currently not included in these platforms. However, PCR has become a prevalent and often indispensable technique in biological laboratories for a wide variety of applications, including biomedical research and molecular diagnostics (Valasek and Repa 2005), criminal forensics (Morling 2009), and, more recently, as a gold standard for detection of Coronavirus and other infectious diseases (Shen et al. 2020; Adachi et al. 2004).

PCR assays can be done with fewer than 2 h of turnaround time. An optimized form of this technique is a promising tool for the dissemination control of infectious diseases. Recently, it was described a novel nanoPCR device that can detect SARS-CoV-2 RNA in 17 min, integrating reverse transcription, fast thermocycling via plasmonic heating through magneto-plasmonic nanoparticles, and in situ fluorescence detection (Cheong et al. 2020).

PCR test requires the preparation of different reagents, mixtures, and finally incubation under repeated heating and cooling cycles to perform the exponential amplification of

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the target sequence. A critical limitation of this technique is that even the smallest amount of contaminating DNA can be amplified. Therefore, to minimize the chance of contamination, reagents should be dispensed into single-use aliquots.

PCR analysis can be simplified and reduce the contamination chances. This paper analyzed disposable micropipette tips as reagent holders of PCR reagents. The goal behind developing novel, intelligent, and accurate, disposable devices is to simplify and optimize the diagnostic time.

The use of common elements, such as tips for innovative applications, in terms of profitability, is increasing. These everyday items are mass-produced, widely used in most laboratories, and fit for out-of-the-box applications. For example, the tips of micropipettes began to be used for different purposes in addition to dispensing liquids, their primary function. These novel applications include a full electrochemical biosystem in a pipette tip applied towards the detection of glucose in commercial soft drinks (Cinti et al. 2020) and miniaturization of solid-phase extraction onto pipette-tip (SPE-tip) for rapid screening of benzodiazepines in dietary supplements (Sun et al. 2020); SPE-tip for non-steroidal anti-inflammatory drugs from water samples (Ganesan et al. 2020), and SPE-tip of vanadium species from water and food samples (Tuzen and Kazi 2018).

Our research group recently designed a novel platform for nucleic acid purification using disposable tips (Sánchez et al. 2020). This platform was based on coated micropipette tips capable of adsorbing nucleic acids onto the internal layer of the tip. The coating layer was embedded with silica nanoparticles (Sánchez et al. 2020).

Moving further in this direction, in this work, we show the preparation of disposable tips with new functionality for PCR assays. Micropipette tips are a platform for dispensing PCR reagents to detect pathogens, such as *Bordetella pertussis* rapidly.

Whooping cough is a highly contagious reemerging infectious disease caused by *B. pertussis*. Historically considered a children´s disease, it also infects adolescents and adults, which are the reservoirs and sources of infection (Oh et al. 2021). Although the recovery of *B. pertussis* from the clinical sample is the golden standard, the isolation of the bacteria is difficult, time-consuming, and renders too many false negatives. For that reason, some years ago, the PCR, which proved very specific and more sensitive than the culture, was introduced as the test for *B. pertussis* detection. Until the PCR assay introduction, the high *pertussis* insistence worldwide was adequately assessed and acknowledged (van der Zee et al. 2015). Molecular diagnoses are considered one of the most specific and sensitive methods that exist, becoming an established laboratory tool for detecting and identifying many infectious pathogens such as *B. pertussis*. Improvements in PCR diagnosis could mean a significant step forward for many disease diagnoses by this method.

This work shows how PCR reagents can be included in a polymeric coating consolidated into a pipette tip, which serves as the platform to hold the components needed for PCR assay. Commercially available micropipette tips can be configured to contain the reagents to perform a PCR diagnostic test of *B. pertussis*.

Materials and methods

Materials

Polyvinyl alcohol (PVA) of 64 kDa and fluorescein were purchased from Sigma Chemical Co. (USA). DIAMOND D300 micropipette tips of 300 µL in volume were purchased from Gilson Co. (USA). dNTPs were purchased from Promega (Buenos Aires, Argentina), glycerol was purchased from Biopack (Buenos Aires, Argentina), and D(+)-trehalose dihydrate from Anedra (Buenos Aires, Argentina). All chemicals were reagent grade and used without further purification.

Preparation of coated micropipette tips

A PVA 10% solution is prepared by dissolution of PVA in distilled water at 80 ºC with constant stirring. Subsequently, fractions of the PVA solution are separated, and different amounts of trehalose are added until reaching the following concentrations: 5, 10, 20, and 30% or glycerol 25% with slow stirring until total dissolution. Finally, fluorescein is added at a concentration of 5 µM (1/200 dilution of a 0.1 mM stock solution). The concentration of fluorescein is determined by measuring the fluorescence at 515 nm using a Nanodrop 3300 fluorospectrometer, excited with blue light.

In the case of dNTPs, an amount is dissolved so that the concentration is 1 mM. The final concentration value of dNTPs is adjusted by measuring the Abs at 260 nm of the final solution. The final solution was used during the same working day.

The preparation of coated micropipette tips was performed in two steps: (i) a coating process on the inner surface of the tips using a PVA solution containing and (ii) a dry step of the tip.

Coating procedure Each tip is placed in a P200 micropipette, the volume is adjusted to 20 µL, and it is slowly loaded with PVA solutions containing additives. It was kept in this condition for 30 s and then released the solution. This process is repeated 3 times. The hole of the tip is blocked with a needle, placed in a Petri dish, and dried in the oven at 55 ºC for 12 h. A set of 20 tips are weighed before and after coating. Considering the coating of dNTPs, a set of three tips were individually weighed previous to and after coating

to determine the loaded dNTPs amounts with higher accuracy. The amount of dNTPs was calculated considering the increment of the weight of each tip and the mass fraction of dNTPs of the coating solution.

The tips modified with the solution of PVA with fluorescein are called Fluo-tips and those containing dNTPs are called dN-tips.

Pr-tips were prepared using a PVA/trehalose solution containing the specific primers at 1 µM concentration and PCR-tips were prepared as dN-tips with the addition of *B. pertussis* primers at 1 µM concentration.

Release of coated micropipette tips

The release of reagents from the coated micropipette tips was done using 25 µL water for Fluo-tips, 30 µL for dN-tips, and 20 µL buffer in case of PCR-tips. A new solution was used for each release step. Fluorescence was measured in Nanodrop 3300 fluorospectrometer.

One release cycle is performed by placing the studied tip in a P200 micropipette. Volume is adjusted, and the tip is loaded with the liquid retained for 30 s. This procedure is repeated 3 times for each release cycle. Release experiments using Fluo-tips were performed in quintuplicate and experiments using dN-tips in triplicate. Errors in measurements were determined as one standard deviation.

Spectroscopic characterization

PVA films were prepared onto Petri dishes and dried in the oven at 55 ºC for 12 h. Dried samples were measured directly using an FTIR IR-Affinity instrument (Shimadzu Corporation, Japan), equipped with an attenuated total reflectance accessory (ATR), GladiATR diamond single reflection accessory (PIKE Technologies, USA). Spectra were acquired by 64 scans in the wavenumber range 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1}. ATR and atmosphere correction were applied for all spectra.

UV–Vis spectra were measured using the Nanodrop 1100 spectrophotometer. In the case of films, a drop of water was added to the pedestal before putting two layers of the dried film. The spectrum acquisition was performed immediately.

Detection of *B. pertussis* by PCR

The coding region of insertion sequence elements IS481 of *B. pertussis* was amplified from genomic DNA as previously described (Houard et al. 1989) with minor modifications. Briefly, PCR was performed using GoTaq TM DNA polymerase (Promega, Madison, WI, USA). Mix-BP contains GoTaqTM buffer ($MgCl_2$ 1.5 mM), primers forward and reverse (1 µM), GoTaq DNA Polymerase (1.25u), Nucleotide Mix (0.2 mM each dNTP), and nuclease-free water. In the case of functional tips experiments, complementary "mastermixes" were prepared according to the following: Mix-A: GoTaqTM buffer ($MgCl_2$ 1.5 mM), primers forwards and reverse (1 µM), GoTaq DNA polymerase (1.25u), and nuclease-free water. Mix-B: GoTaqTM buffer ($MgCl_2$ 1.5 mM), GoTaq DNA polymerase (1.25u), nucleotide mix (0.2 mM each dNTP), and nuclease-free water. Mix-C: GoTaqTM buffer ($MgCl_2$ 1.5 mM), GoTaq DNA polymerase (1.25u), and nuclease-free water. The positive DNA sample is from *B. pertussis* strain B213, a streptomycin-resistant derivative of Tohama strain (King et al. 2001). DNA from *B. parapertussis* Bpp 12,822 was used to determine the specificity of the test.

The PCR reaction was carried out using the following conditions. Initial denaturation cycle at 94 ºC for 5 min, followed by 35 cycles of 30 s at 94 °C, 20 s at 53 °C, and 30 s at 72 °C; and finally, a 5-min hold at 72 °C. The primers were as follows BP1 5-GGGGTCACCGCGCCGACTGT-3; BP4 5-GGGCCTGATGCTCGTGTAGCGC-3, amplification site 288pb (Riffelmann et al. 2005). The PCR products were detected by 0.8% agarose gel electrophoresis, and the bands were visualized by staining with ethidium bromide. PCR reactions were performed in triplicate.

Results

A PCR mixture is characterized by many components such as primers (at least forward and reverse), dNTPs (dATP, dTTP, dCTP, and dGTP), Taq polymerase, buffer, and the DNA sample. Usually, this mixture, excluding the sample, called "master mix", should be prepared very carefully to avoid contamination.

This work aims to develop disposable devices which can simplify this critical step. We prepare micropipette tips that contain some of the master mix components. Tips are coated on the inner surface to minimize the potential mistakes, which the incorporation of multiple reagents can do. Figure 1 schematizes the general idea of practical tips containing one or two PCR reagents. The selected ones were dNTPs (dN-tip), primers (Pr-tip), and PCR-tip containing both reagents. Since there are different DNA polymerase enzymes and their residual activity at room temperature, DNA polymerases and buffers were not considered target reagents to be trapped. Therefore, they should be added later at the time of the assay.

Coating tips

Initially, the feasibility of coating and eluting a reagent loaded into the micropipette tip was evaluated using fluorescein dye as a solute model to be delivered. Considering the

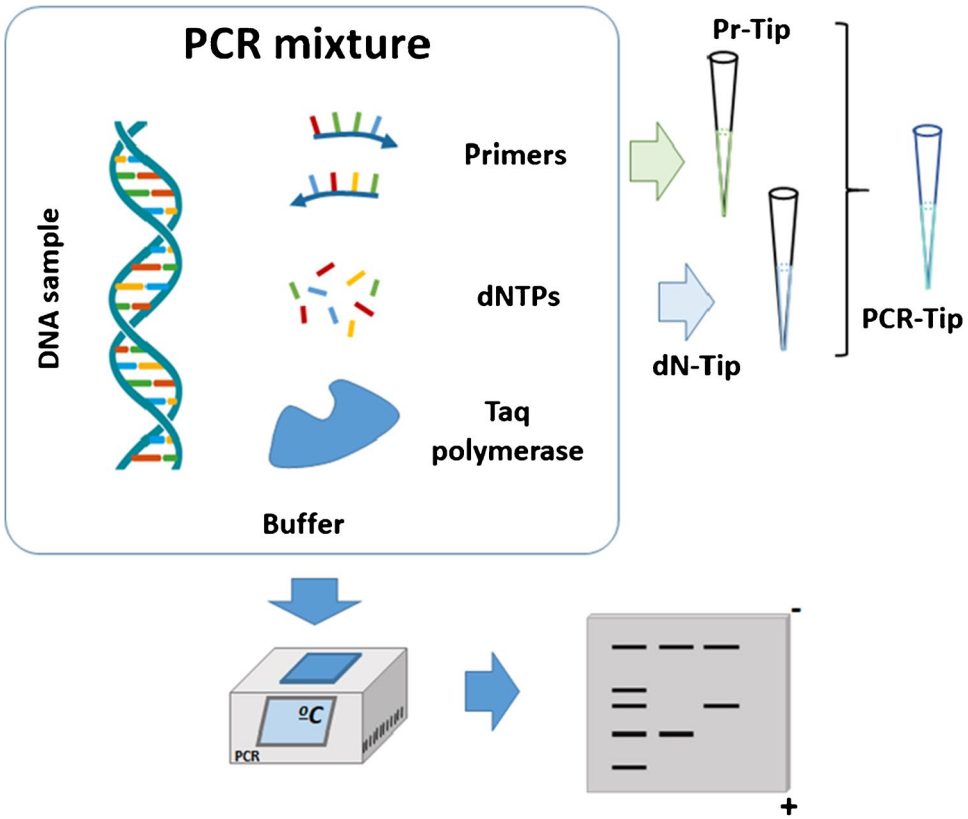

Fig. 1 Scheme of PCR analysis and the functional tips prepared with the components of PCR mixture

low amount of reagents, a water-soluble and neutral polymer was used as a carrier. PVA is a polymer that contains hydroxyl moieties as the unique functional groups, and it has been widely used in manufactures, such as paintings because it is an excellent film-forming material and also can be used as a stabilizer of biological and chemical reagents (Van Veldhoven and Mannaerts 1987; Seok et al. 2017).

A simple release process is a critical factor for the development of RDT. The tips were coated with a PVA mixture containing fluorescein and later dried until constant weight. Fluorescein release was evaluated by elution in water up to five times using a micropipette and the protocol described in the M&M section, and quantification was made by fluorescence measurements in the consecutive releases. Figure 2 shows how the fluorescein molecules are entrapped into the polymeric coating and release of molecules from it by water or buffer loading. The exact mechanism is proposed to occur for dNTPs and the specific primers used for a *Bordetella* diagnostic test.

Preliminary coated tips using PVA solutions containing fluorescein showed a poor solute recovery using water (Table 1). Therefore, additives such as glycerol and trehalose have been included in the coating formulation. Table 1 shows that the addition of glycerol and trehalose improves the expected function of the tip. However, the latter allows the load and release of higher amounts of fluorescein with higher efficiency.

PVA films containing trehalose as an additive show the best performance, and it was selected for further studies. It was previously reported that this molecule is one kind of non-reducing sugar possessing a special freeze-drying protective effect on biological systems from injury caused by ice forming or the damage of cell membrane under extreme circumstances (Crowe 2002; Izutsu et al. 2011; Mazur et al. 1972). The cryoprotective effect of trehalose is highly related to its interaction with water (Weng et al. 2016). Therefore, it is expected to influence the solubilization of PVA-coated layers.

Fluorescein was added to the PVA solution containing different concentrations of trehalose (5, 10, 20, and 30%). These solutions were used to prepare coated tips (Fluo-tips) trapping fluorescein. The amount of fluorescein coated and released is measured to analyze its influence on the functionality of Fluo-tips.

Release yields for the different Fluo-tips are plotted in Fig. 3 for five release cycles each. The number of optimal release steps was also evaluated because it is an essential parameter for the RTD performance.

The Fluo-tip elution process was achieved successfully in more than 45% in one release step for all trehalose concentrations. Anyhow, a trehalose concentration-dependent behavior was observed in the release yield. For all studied cases, carrying out three release steps was a compromise between the total eluted amount and each elution step's

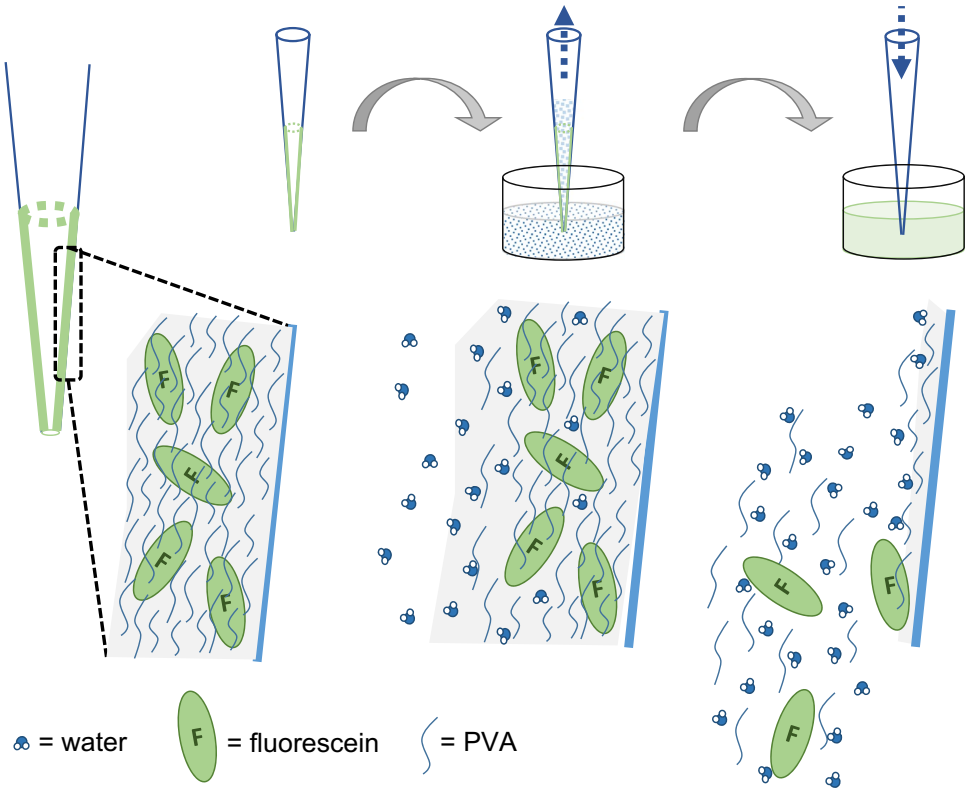

Fig. 2 Scheme of the coated tip with a dry internal PVA layer containing the solute (fluorescein) and the effect of water during the releasing step

Table 1 Maximum fluorescein release (after five release cycles) and total release yield of tips coated with different solutions

Coating solution	Fluorescein release* (pmols)	Yield %
PVA 10% + fluorescein	2.5 ± 0.5	51
PVA 10% + glycerol 25% + fluorescein	5.6 ± 0.2	68
PVA 10% + trehalose 10% + fluorescein	14.1 ± 0.4	86

*Average of five eluted tips

efficiency. For 5% and 10%, the elution process between the 3rd and 5th elution steps was more significant than 20% and 30%. Up to 10%, a bimodal release behavior is found; meanwhile, higher trehalose concentrations show a burst effect. It was decided to make 3 elution steps for further experiments. No differences between the 20% and 30% range were found, so trehalose 20% as an additive was selected to reduce the saccharide concentration.

Tips containing dNTPs (dN-tips)

The following goal was to test the preparation of functional tips with a fundamental component of a PCR: dNTPs. Every molecule will interact with the coated polymer based on its tridimensional structure and size, which directly impacts the solubility and diffusivity into the polymeric matrix; therefore, coated tips containing dNTPs were tested to evaluate the behavior of these molecules inside the entrapment matrix. Table 2 shows the amount (μg) of dNTPs trapped in each dN-tip.

These results show that the incorporation of trehalose diminishes the amount of dNTPs entrapment compared with PVA-coated tips. As well, as was expected, dN-tips increased their coating layer when more amount of trehalose was added to the coating solutions. However, coating solutions containing trehalose 5%, 10%, and 20% reach dN-tips and are comparable in the amount of dNTPs entrapped into the coated layer. Trehalose 30% showed discouraging results in terms of entrapped capacity.

Coating characterization

Coating layer composition was checked by FT-IR and UV–Vis spectroscopy. Figure 4 shows the ATR FT-IR spectra of films prepared with the different reagents.

Fourier transform infrared spectroscopic (FTIR) analysis examined raw materials and composite films. PVA and trehalose spectra share most of the IR bands due to their similar composition of functional groups. Two central dominant absorption regions appeared in all the FTIR spectra (Fig. 4); one was from 3700 to 2600 cm^{-1} in the high-wavenumber region, and the other was from 1730 to 800 cm^{-1} in the low-wave-number region. Peaks around 3300 cm^{-1},

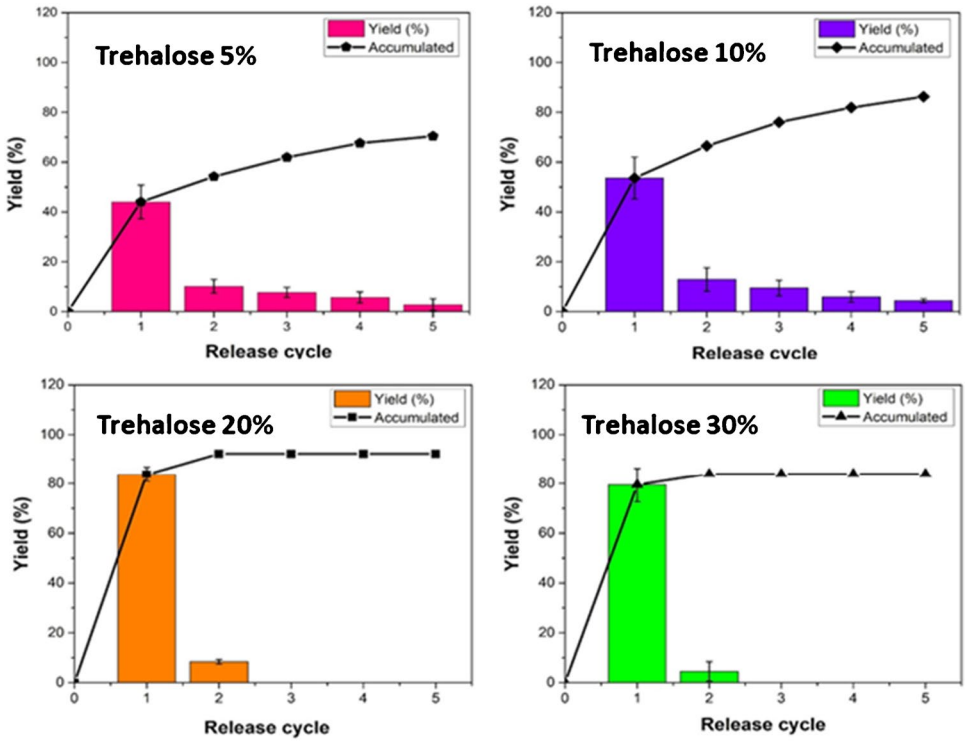

Fig. 3 Yield of fluorescein released after one to five release steps, considering coated tips prepared with coating mixtures containing different trehalose proportions

Table 2 Preparation of dN-tips using different trehalose concentrations in the coating solution

Condition	Δ weight (mg)	dNTPs fraction*	dNTP/tip (μg)**
PVA 10% + dNTPs	0.5 ± 0.06	0.0073	3.6 ± 0.4
PVA 10% + trehalose 5% + dNTPs	0.6 ± 0.04	0.0017	1.05 ± 0.07
PVA 10% + trehalose 10% + dNTPs	0.7 ± 0.06	0.0017	1.16 ± 0.10
PVA 10% + trehalose 20% + dNTPs	0.8 ± 0.04	0.0014	1.13 ± 0.06
PVA 10% + trehalose 30% + dNTPs	0.9 ± 0.05	0.0010	0.87 ± 0.05

*Mass fraction of dNTPs in the PVA solution

**Calculated from the dNTP mass fraction

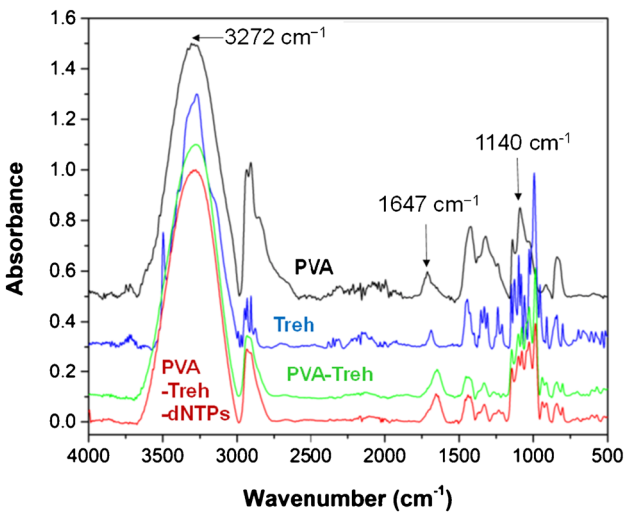

Fig. 4 FTIR-ATR spectra of PVA film, solid trehalose, PVA/trehalose (10%/10%) film, and the latter containing dNTPs

2900 cm^{-1}, and 1050 cm^{-1} in the spectra of all the samples corresponded to stretching vibrations of O–H, C–H, and C–O groups, respectively. The peaks at 1421 cm^{-1} (CH_2 scissoring), 1321 cm^{-1} (CH_2 deformation), 1140 cm^{-1} (C–O stretching, crystalline form), 1090 cm^{-1} (C–O stretching), and 840 cm^{-1} (CH_2 rocking) were the characteristic peaks of PVA (Lee et al. 2008). The peak at 1140 cm^{-1}, corresponding to the infrared 'crystallinity sensitive' band, becomes weaker in PVA/trehalose films, suggesting the suppression of PVA crystallization. This result might be ascribed to the inter-molecular complexes formed through hydrogen bonding between trehalose and PVA. The FTIR results suggest that PVA and trehalose are tightly integrated with the film.

Another exciting peak to analyze is the corresponding 1688 cm^{-1} in the trehalose (dihydrate) spectrum. It has been assigned to water molecules' H–O–H deformation modes (Márquez et al. 2018). This peak shifts to lower wavenumbers (1647 cm^{-1}) in the PVA/trehalose films, which can be

attributed to the increment of the hydrogen bond network of the remnant water in the films. This entrapped water remains in the film due to the low temperature of the dryness process. In the same way, –OH stretch peaks of the PVA/trehalose films spectra (3272 cm^{-1}) are shifted to lower wavenumbers concerning the PVA film spectrum (3306 cm^{-1}). More H bonds would explain this feature between molecules and the remnant water.

Considering the meager mass fraction of dNTPs in the PVA/trehalose/dNTPs film (see Table 2), it is not expected to find IR signals corresponding to dNTPs in this sample. Meanwhile, by UV–Vis spectroscopy (Fig. 5), the content of dNTPs (maximum absorption peak at 260 nm) is identified in the PVA/trehalose/dNTPs film.

The dN-tip functionality is analyzed by two main parameters, entrapment amount, and release yield of a particular solute. A suitable dN-tip device would be the one that could trap the maximum amount of dNTPs and ultimately release them. A device that could trap a molecule but not release it would not be helpful as a delivery platform. According to Table 2, trehalose addition in 5% to 20% reaches a similar entrapment capacity.

The results of the release process corresponding to one and three release steps are shown in Fig. 6 and Table 3.

Results showed a higher amount of released dNTPs at trehalose 10%, for one or the sum of three release steps. However, no burst effect was found at this trehalose concentration, similarly to Fluo-tips. Using dN-tips, the burst effect was noticed just for trehalose at 30%; meanwhile, it was measured from trehalose at 20% using fluorescein.

The data in Table 2, trehalose diminished the amount of reagent entrapment. Nevertheless, the trehalose addition had essential advantages in the released product (Fig. 3).

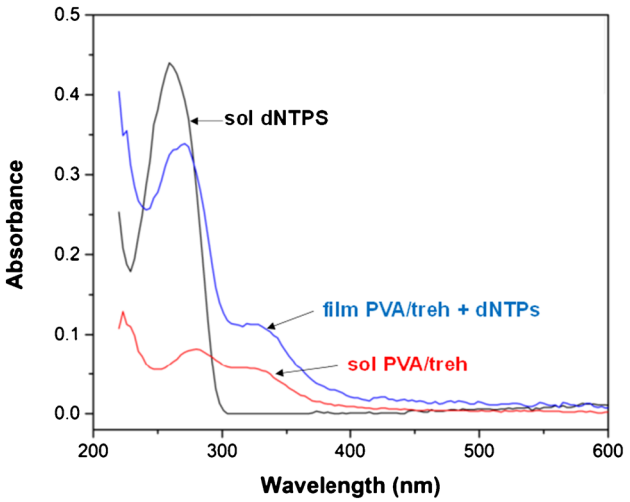

Fig. 5 UV–Vis spectra of the dNTPs solution, PVA/trehalose (10%/10%) solution and PVA/trehalose film containing dNTPs

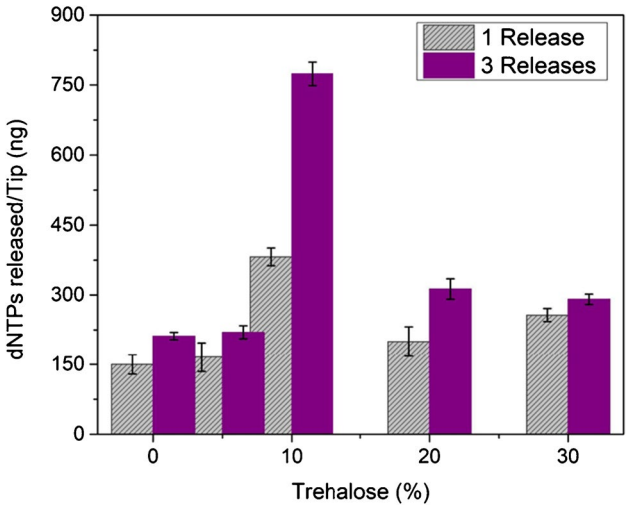

Fig. 6 Amount of dNTPs eluted after one or three release cycles of dN-tips prepared with different percentages of trehalose

In addition to the total amount of released product, it is essential from the economic point of view the completeness of release of the coated product. Both entrapment and release data let us understand the behavior of a particular solute inside the matrix. The goal was to determine the best coating solution for a successful dNTP dispensation step. Considering that the best approach was trehalose 10% and this one was not in a burst effect condition, it was necessary to test more than an elution cycle. A remarkable result is obtained for the device prepared with a polymer coating with trehalose 10%, with the maximum amount of released dNTPs. Table 3 reports the yield % of the recovered dNTPs after the 1st and 3rd elution steps.

According to Table 3, low trehalose concentrations (0% and 5%) reach a yield increment between the elution steps between 30 and 50%; meanwhile, for higher trehalose concentrations, the increment is between 10 and 56%.

Table 3 Release yield (Y%) after one and three release cycles using dN-tips prepared with different coating solutions

Coating solution	Y% (1 step)*	Y% (3 steps)**	% increment*
PVA 10% + dNTPs	4	6	50
PVA 10% + trehalose 5% + dNTPs	16	21	31
PVA 10% + trehalose 10% + dNTPs	33	67	103
PVA 10% + trehalose 20% + dNTPs	18	28	56
PVA 10% + trehalose 30% + dNTPs	30	33	10

*Percentage increment of yield = $(Y\%_{3step} - Y\%_{1step}) / Y\%_{1step}$

Functional tips prepared with trehalose 10% reach more than 100% of increment, showing that this formulation offers the best composition for the entrapment-dispensation of this PCR reagent. Unfortunately, a burst effect is not found with this coating condition, and three release steps are required. It is also essential to notice that still a remnant of 33% of dNTPs are trapped in the device.

When conditions of PVA coating and PVA/trehalose 10% are compared, it is remarkably seen, the benefit of trehalose incorporation in the coating formulation. The release yield of dNTPs increased ten times (67% vs. 6%) for the latter condition (see Table 3).

The stability of the reagents in functional tips was studied by measuring the amount of fluorescein released by Fluo-tips prepared some time before. Fig. S1 shows a plot of relative fluorescein elution (RFU eluted from Fluo-tip prepared × time previously / RFU eluted from Fluo-tip prepared the same day) from Fluo-tips after storage in dark and room temperature for different periods. Each time is an average of the elution of three Fluo-tips. The fluorescence of fluorescein solutions, stored at room temperature and at room temperature and darkness (same condition as Fluo-tips), is plotted for reference. Fluo-tips lost 50% of the initial fluorescein loading after 9 months, against 3 and more than a year for fluorescein solutions stored at room temperature in daily light and dark conditions, respectively. The stability of dNTPs' containing tips is difficult to measure by PCR because an excess substrate is added to the reaction. Furthermore, the hydrolysis of dNTP's cannot be followed by UV–Vis spectroscopy at 260 nm. Therefore, it is not possible to determine the amount of dNTPs by this assay.

Proof of concept

The *B. pertussis* PCR diagnosis, for specificity, high sensitivity, and speed, meant a significant advance for the disease's epidemiology and has become a critical method for detecting a considerable number of pathogens (Kilgore et al. 2016).

To investigate the functionality and contribution to molecular diagnosis of functional tips, molecular detection of *B. pertussis* was used as proof of concept. PCR test for *B. pertussis* detection was performed by preparation with functional tips. Three different functional tips were prepared:

1. dN-tips (prepared with trehalose 10%) containing the required dNTPs.
2. Pr-tips containing the primers against the most common PCR target for detection of *B. pertussis*, the insertion sequence elements IS481 (present in 50–250 copies per genome).
3. PCR-tips containing both reagents.

In addition to the master-mix (Mix-BP), complementary "master-mixes" were prepared to complete the required reagents for PCR assays: master-mix A without dNTPs (Mix-A), the master-mix B without forward and reverse primers (Mix-B), and master-mix C without dNTPs and primers (Mix-C).

Figure 7 shows the conditions assayed. The Mix-BP with *B. pertussis* DNA sample was used as a positive control (line 1), and as the negative control, the Mix-BP without DNA sample loading (line 2). PVA 10% (line 3) and glycerol 20% (line 4) were used to evaluate the possible interference of the PVA or glycerol to the Mix-BP. Then, Mix-A allowed us to evaluate the addition of dNTPs released by the dN-tip (line 6) and the respective control without the dN-tip (line 5). Mix-B allowed to evaluate the addition of primers forward and reverse release by Pr-tip (line 8) and the respective control without Pr-tip (line 7). Finally, Mix-C allowed to evaluate the reaction with the addition of dNTPs and primers forward and reverse release by PCR-tip (line 10) and the respective negative control (line 9).

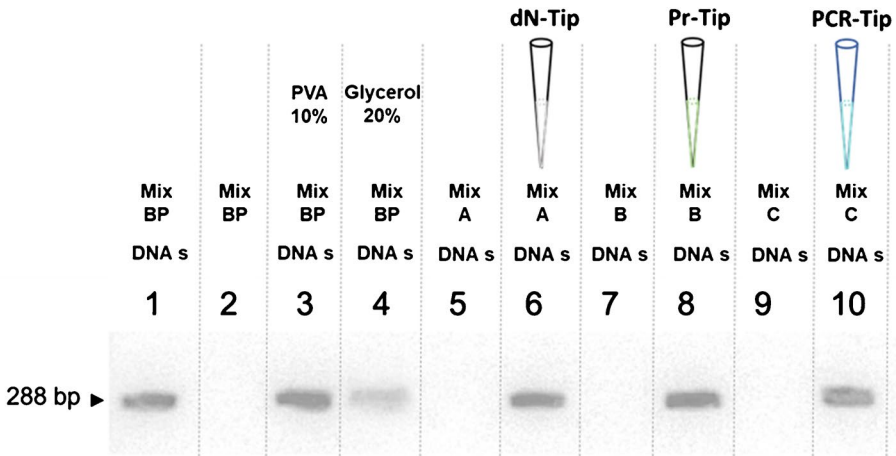

Fig. 7 PCR analysis using different PCR mixtures and functional tips showing the result of ADN amplification in Agarose gel

As it can be seen, all PCR reactions for *B. pertussis* detection were positive by the amplification product of 288 bp when the master mix was supplemented with the addition of remnant reagents by a functional tip. In addition, it checked the inertness of PVA 10% in the PCR reaction by adding this polymer into the master mix (line 3). In line 6, the dN-tip completes the reaction with the addition of dNTPs. In line 8, Pr-tip adds forward and reverse primers, and in line 10, PCR-tip includes both reagents simultaneously. The reduction of the band's intensity corresponding to line 4 shows the interference of glycerol.

Considering the results from Fig. 6, dN-tips prepared with 10% trehalose release 0.9 µg dNTPs against 20 µg loaded in the PCR-positive control. According to the results shown in Fig. 7, this lower amount of dNTPs is still enough to reach a positive result.

The PCR specificity using PCR-tip (containing the *B. pertussis* primers) was tested by running a PCR analysis using PCR-tip with master-mix C and *B. parapertussis* DNA sample as a negative control. Agarose gel shows a negative result of PCR analysis (see Fig. S2).

Considering the storage stability of PCR-tips at room temperature and darkness, they yield positive results up to 8 months of storage time (maximum time-analyzed).

Discussion

A simple method was implemented to fractionate PCR reagents into common and affordable consumable materials such as micropipette tips. Functional micropipette tips (dN-tips, Pr-tips, and PCR-tips) can act as delivery platform reagents for quicker diagnostics assays.

Functional tips are prepared by a coating process performed using a solution of PVA, a film-forming polymer, containing the reagents. PVA films containing various additives, such as trehalose, sucrose, and propylene glycol, have been used to prepare films for intradermal drug delivery systems (Engelke et al. 2018). Considering that in the proposed application DNA polymerase could be inhibited by additives, these were minimized, and trehalose and glycerol were studied as possible additives. According to the data described in Table 1, the former showed an improved dissolution property and did not interfere with the PCR reaction (see Fig. 7).

Trehalose is a kosmotropic molecule that promotes hydrogen bonding, and therefore, it will interfere with the intra- and inter-molecular hydrogen bonding network of PVA due to the absence of internal hydrogen bonds of trehalose (Wang 2000). Other interesting technological features of trehalose include its low hygroscopic property and very low chemical reactivity (Wang 2000).

Figure 3 shows the amount of trehalose incorporated. Trehalose has an impact on the solute release process of a model molecule such as fluorescein. Indeed, incorporating this kosmotropic molecule offers a better environment for the entrapment of molecules because it will interfere with PVA molecules' cooperative hydrogen bond formation. In addition, the osmotic effect will enhance the initial film dissolution (Engelke et al. 2018). Comparison between the Fluo-tips without trehalose and with lets us conclude that there is a positive effect of incorporating this component into the coating polymer and the burst effect during the release step.

High proportions of trehalose have also been used to prepare PVA cryogel because this typical kosmotropic agent promotes hydrogen bonds (Kolosova and Lozinsky 2019). It was observed that high proportions of trehalose improve the cryogel properties (such as rigidity); however, it is worthy to remark that there is no linear relationship between them. It was observed that cryogel prepared with PVA 10%/trehalose 10% composition has lower rigidity than PVA cryogels and other PVA/trehalose ratio cryogels (Kolosova and Lozinsky 2019). Each molecule's potential hydrogen bonding forming at this mass ratio is similar (6.8 equivalents/L for PVA against 8.8 equivalents/L for trehalose). This characteristic is compatible with reducing the infrared "crystallinity sensitive" band (Fig. 4), suggesting a PVA amorphous structure (Lee et al. 2008).

In the case of tip coating, the combination of a polymeric coating of PVA and trehalose allows retained reagents in the coating layer under an aqueous condition to be quickly released. However, the optimum trehalose concentration is not equal for every cosolute. The burst effect for fluorescein was found in the film prepared with trehalose 20% (Fig. 3); meanwhile, dNTP entrapment needs 30% (Fig. 6), where the triphosphate moiety is the most relevant chemical difference between both molecules. It has been demonstrated that the hydrogen bond of trehalose and phosphate has higher strength than trehalose-trehalose or phosphate-phosphate hydrogen bonds (Weng and Elliott 2015). Therefore, a higher proportion of trehalose will be required to interact with dNTPs.

Comparing the overall performance, including the amount of reagent immobilization, reagent release, and release yield, PVA 10%/trehalose 10% solution showed the best performance as the coating formulation of dN-tips.

Taking into account the loading of other reagents on the tips, the trehalose concentration must be optimized for each solute. However, the best concentration would be in the range of 10 to 20%.

In a further step, prepared functional tips: dN-tips, Pr-tips, and PCR-tips were successfully used to analyze a *B. pertussis* sample using the PCR technique.

This configuration offers an innovative perspective for designing diagnostic devices useful for simplifying PCR diagnostic kits, minimizing the source of errors and contaminations.

The ready-to-use functional tip would be useful for massive and accurate analyses. Also, standardization of a particular PCR assay will be easier.

As proof of concept, prototypes were used to detect the bacteria *Bordetella pertussis*, which causes a disease with cyclic epidemic outbreaks that often need a rapid and massive molecular diagnostic (Guiso and Wirsing von König 2016). Results were promising, and the PCR tip represents an essential contribution to standardization and promotes a quick detection of *B. pertussis* for routine diagnosis.

This development also set the basis for a new type of functionalized materials that could be employed in different massive molecular diagnostics. PCR-tips could be a simple solution to replace the in-house aliquoting to avoid freeze–thaw cycles of the master mix and to insulate against reagent contamination.

In 2020, with the COVID outbreak, people started to become familiar with the PCR concept. The sanitary situation forced the scientific community to offer a quick response in terms of diagnosis, pushing in this way the development of intelligent devices for RDTs. Functional micropipette tips (dN-tips, Pr-tips, and PCR-tips) are presented as a novel solution to improve the settled procedures for this necessity.

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Author contribution CG, MLS and MG conceived and designed the research.
CG conducted the experiments.
MLS and HAV conducted the PCR diagnosis experiments.
HAV and MER contributed new reagents or analytical tools.
CG and MG analyzed the data.
MLS, HAV, and MER analyzed the PCR experiments.
MLS and MG wrote the manuscript.
MG supervised and reviewed the manuscript.
All the authors read and approved the manuscript.

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Data availability All the data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare they have no financial interests.

This article does not contain any studies with human participants or animals performed by any of the authors.

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