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Basic evaluation of the CRISPR/Cas system stability for application to paper-based analytical devices†

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Despite the promising features of the CRISPR/Cas system for application to point-of-care nucleic acid tests, there are only a few reports on its integration into paper-based analytical devices (PADs) for the purpose of assay simplification. In most cases, paper platforms have only been used for the final signal readout in an assay otherwise performed in a test tube. Therefore, there is very limited information on the suitability of the CRISPR/Cas system for on-device reagent storage. To fill this gap, the current work primarily investigated the influence of various factors, including the type of paper, reagent drying method, effect of stabilizers, and storage condition on the storage stability of reagents necessary for CRISPR-based assays on paper substrates, by comparing the fluorescence signal emitted by the *trans*-cleavage of the dsDNA-activated Cas12a complex. The results obtained in the form of fluorescence signals emitted after *trans*-cleavage of a ssDNA probe through a dsDNA-activated Cas12a complex on paper substrates showed that CRISPR-related reagents spontaneously dried at room temperature on BSA blocked paper retained over 70% of their initial activity when stored at −20 °C for 28 days, independent of the type of paper substrates, which was improved by the addition of sucrose as a stabilizer. In addition, reagents dried on paper substrates under the optimized conditions exhibited stronger heat tolerance at temperatures above 65 °C compared to their corresponding solutions. This work is expected to contribute to the future development of fully integrated PADs relying on CRISPR/Cas systems for point-of-care applications requiring no additional reagent handling.

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Introduction

Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) systems have become widely used in nucleic acid detection assays for their high sensitivity, single-base specificity, and simplicity.[1,2] For analytical assay purposes, Cas12 and Cas13 in combination with a CRISPR RNA (crRNA) designed to recognize a specific target nucleic acid are of particular interest. After binding target DNA (Cas 12) or RNA (Cas 13) complementary to the crRNA sequence, the property of indiscriminately cleaving surrounding ssDNA (Cas 12) or RNA (Cas 13), referred to as *trans*-cleavage, is activated.[1] This activity exists even at room temperature, making CRISPR-based assays applicable to point-of-care testing (POCT).[3,4]

On the other hand, due to their low cost, ease of operation, disposability, simplicity and mass-producibility, paper-based analytical devices (PADs) are drawing great attention as point-of-care platforms,[5,6] meeting the ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid, Robust, Equipment free, and Deliverable to end-users) proposed by the World Health Organization.[7,8] Furthermore, designing microfluidic channels on PADs enables simultaneous multiplex target detection,[9,10] control of multi-step reactions,[10,11] and liquid flow manipulation.[12,13] Therefore, PADs are regarded as a promising fundamental platform to realize POCT.

Since the first report of a nucleic acid detection method using a CRISPR/Cas system,[14,15] a large variety of CRISPR-based assays has been realized. Not surprisingly, this also includes examples of CRISPR-based assays in combination with paper platforms. However, in most cases reported so far, PADs have been used as a component of an assay generally performed in a test tube, for example for the final signal readout.[16–18] To the best of our knowledge, CRISPR-related reagents have rarely been pre-deposited and dried on paper platforms for preservation and later use without additional reagent handling. Gootenberg *et al.* and Nguyen *et al.* reported assays with freeze-drying of the CRISPR-related reagents on paper-based substrates;[15,19] however, neither of the two studies investigated the storage stability, one of the most important factors when it

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† Electronic supplementary information (ESI) available: Sequences of nucleic acids, characteristics of used filter papers, the designed wax printing pattern, an illustration of the assay mechanism, dependence on types of stabilizers, statistical comparison of data obtained with different stabilizers, long term storage stability, the target dsDNA concentration response range, the target dsDNA concentration response with reagents stored in the frozen state in microtubes, heating tolerance in the solution state, and heating tolerance in the absence of BSA blocking. See DOI: https://doi.org/10.1039/d4ay00848k

comes to POCT devices. Rybnicky *et al.* looked at the storage stability of CRISPR assay-related reagents freeze-dried in a microtube and reported that a combination of Cas12a, crRNA, and a ssDNA reporter including a fluorescent dye and quencher pair (FQ reporter) retained approximately 75% of the initial activity after 2 months of storage at room temperature.[20] Furthermore, others confirmed that freeze-dried mixtures containing reagents for both nucleic acid amplification and the CRISPR assay stored at 4 °C and −20 °C are stable for at least 1 month.[21] While these studies indicate a high storage stability of CRISPR assay-related reagents after freeze drying, they neither looked at methods other than freeze drying, nor investigated the storage stability of paper substrates.

Given the advantages of CRISPR-based assays as well as PADs in general, we believe that the combination of these techniques holds great promise for the development of fully integrated paper-based assay tools in the context of POCT, with all necessary reagents pre-deposited and stored on-device for enhanced user-friendliness with minimal reagent handling. Therefore, we regard it as highly important to evaluate the stability of involved reagents on these specific platforms. The current work investigates the storage stability of CRISPR assay-related reagents on the example of the CRISPR/Cas system on paper platforms depending on various experimental conditions including the type of paper substrates, drying methods, stabilizers, and storage conditions.

Materials and methods

Materials

All nucleic acids used in this work including target dsDNA (tgDNA), crRNA, and FQ reporter were purchased from Integrated DNA Technologies (Singapore). The corresponding sequences are listed in Table S1.† NEbuffer r2.1 and Lba Cas12a were bought from New England Biolabs (Ipswich, MA, USA). Filter papers (Whatman Grade 1 (WF1) and Grade 541 (WF541)) were purchased from GE Healthcare Life Sciences (Marlborough, MA, USA), while Advantec 5C (A5C) was obtained from Toyo Roshi, Co., Ltd (Tokyo, Japan). Bovine serum albumin (BSA), trehalose dihydrate, sucrose, glycine, and dextran (MW: 70 000) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Nuclease-free water was purchased from Nacalai Tesque (Kyoto, Japan).

Device preparation

The micro-spot pattern (Fig. S1†) designed using Adobe Illustrator CC software was printed on filter papers by means of a solid ink "wax-printer" (ColorQube 8580, Xerox, Norwalk, USA). Subsequently, the wax printed papers were heated on a hot plate (NHS-450 ND, Nissin Rika, Chiba, Japan) at 150 °C for 2.5 min (WF1 and WF541) or 3 min (A5C), respectively. For the blocking of paper substrates, 10 μL of 1% BSA in nuclease-free water was dropped on each micro-spot and dried at 37 °C for 60 min before the deposition of other reagents.

In preparation for drying of CRISPR-related reagents on paper substrates, a mixture containing 100 nM Cas12a, 200 nM crRNA, 4 μM FQ reporter and 0–10% (weight ratios) stabilizer (trehalose, sucrose, dextran, or glycine) in 2× NEBuffer r2.1 was pre-incubated for 15–30 min. 4 μL of this mixture was then dropped on each paper micro-spot and dried for 60 min, either at room temperature in a laboratory with temperature and relative humidity maintained in the 23–24 °C and 30% to 40% range, at 37 °C in an oven, or by freeze drying (snap freezing by using liquid nitrogen followed by applying vacuum). Finally, the modified papers were stored until further use at either room temperature, 4 °C, or −20 °C in aluminium-coated pouches containing a silica gel desiccant in a way preventing physical contact with the reagent-modified surfaces.

CRISPR assay on paper micro-spots

8 μL of tgDNA sample solution in nuclease-free water was dropped on each micro-spot of the above prepared paper substrates, followed by incubation for 60 min in a humid environment by placing the devices in a closed container lined with a wetted kitchen paper towel. Subsequently, the paper-based substrate was removed from the container and dried at 37 °C in an oven for 30 min. Lastly, fluorescence emission was measured using a ChemiDoc MP (Bio-Rad, Hercules, CA, USA) imaging system (exposure time: 0.020 s, λ_{ex} = 460–490 nm, and $\lambda_{em} = 532 \pm 14$ nm). The results were analysed using the Image Lab software, by calculating the mean intensity value in each micro-spot.

Reference experiments performed in microtubes

A mixture containing 100 nM Cas12a, 200 nM crRNA, 4 μM FQ reporter and 1% sucrose in 2× NEBuffer r2.1 was pre-incubated for 15–30 min. 4 μL of this mixture was then added into tubes and stored at −20 °C for further use. For the CRISPR assay, prepared reagent liquids were thawed at room temperature, before adding 4 μL of tgDNA sample solution in nuclease-free water, followed by incubation for 60 min. Fluorescence emission intensity was measured and analysed as described above by placing the tubes inside the ChemiDoc imaging system.

Investigation of heat tolerance

The prepared paper micro-spots or microtubes with 4 μL of pre-incubated CRISPR-related reagents were left at room temperature or placed in an oven at 37, 65, or 95 °C for 60 min. The content of microtubes was shortly spun down by centrifugation to collect the mixture from the bottom of the tube. Subsequently, the CRISPR assay was performed as described above.

Results and discussion

Selection of drying and storage methods

At first, the drying and storage methods for CRISPR-related reagents on paper substrates were optimized. It is well known that the stability of proteins in solution is significantly affected by structural changes according to solution properties and the structure of water itself.[22] Therefore, the application of proteins for biosensors, especially enzymes, requires protection of their 3D-structures around the active sites by means of drying or

adding stabilizers. Among three major drying methods including heating, vacuuming, and freeze-drying, the latter is the most widely used for the preparation of protein pharmaceutical compositions,[23–25] although this method suffers from some drawbacks such as the equipment setup and drying stress. When it comes to drying reagents on PADs, the reagent liquid volumes involved are generally much lower compared to those in the case of pharmaceutical preparations, and thus drying of enzymes on paper by spontaneous drying at room temperature is a possible alternative.[10,12] In addition to drying methods, storage temperature is also an essential factor affecting the stability of proteins in the solid state.[25] Based on these facts, the storage stability of the CRISPR-related reagents was compared using the following three drying methods in combination with storage at various temperatures (25, 4, and −20 °C): spontaneous drying at room temperature, drying at 37 °C, and freeze-drying. The activity retained after drying and storage was monitored in terms of the fluorescence emission intensity recorded as a consequence of the *trans*-cleavage activity of CRISPR/Cas12a in the presence of tgDNA, resulting in a fluorescence signal turn-on upon spatial separation of the FAM fluorophore and the quencher of the ssDNA FQ reporter (Fig. S2†). As shown by the results in Fig. 1, the CRISPR-related reagents dried and stored on a WF1 paper substrate retained over 70% of their original activity after 28 days, with the exception of samples prepared by spontaneous drying combined with storage at 4 °C (Fig. 1A). This is a strong indication that the storage of CRISPR-related reagents on paper substrates over extended time periods is possible, even at room temperature. When focusing on the drying methods, no significant difference was observed between them. Given that spontaneous air-drying under ambient conditions is the simplest and most energy efficient approach, it was selected as the optimal drying method. In terms of storage temperature, the signal was most stable over the investigated 28 day period when paper substrates were stored at −20 °C (Fig. 1A–C). Thus, that storage condition was selected for further investigations.

Influence of paper substrates

For most paper-based analytical devices, filter paper substrates are used due to their high purity and large surface area available for reagent immobilization, in addition to sample liquid transport capability driven by capillary forces. Differences among the various types of commercially available laboratory filter papers are mostly in their porosity, thickness and the type of cellulose fibres used. To investigate the influence of the nature of paper substrates on the storage stability of the CRISPR-related reagents, three types of filter papers were evaluated in this study, the characteristics of which are shown in Table S2.† As shown by the data in Fig. 2A, the dried state CRISPR-related reagents retained over 70% of their original activity after storage for 28 days (−20 °C) for all paper substrates, suggesting that there is no significant difference among the three types of filter papers from a storage stability perspective. In contrast, when it comes to the absolute fluorescence signal intensities and the signal to noise ratio (S/N), the WF1 platform provided the highest values throughout the 28 day storage period. The fluorescence intensity observed on paper substrates depends on many factors including the paper density, thickness, and material.[26] It is assumed that

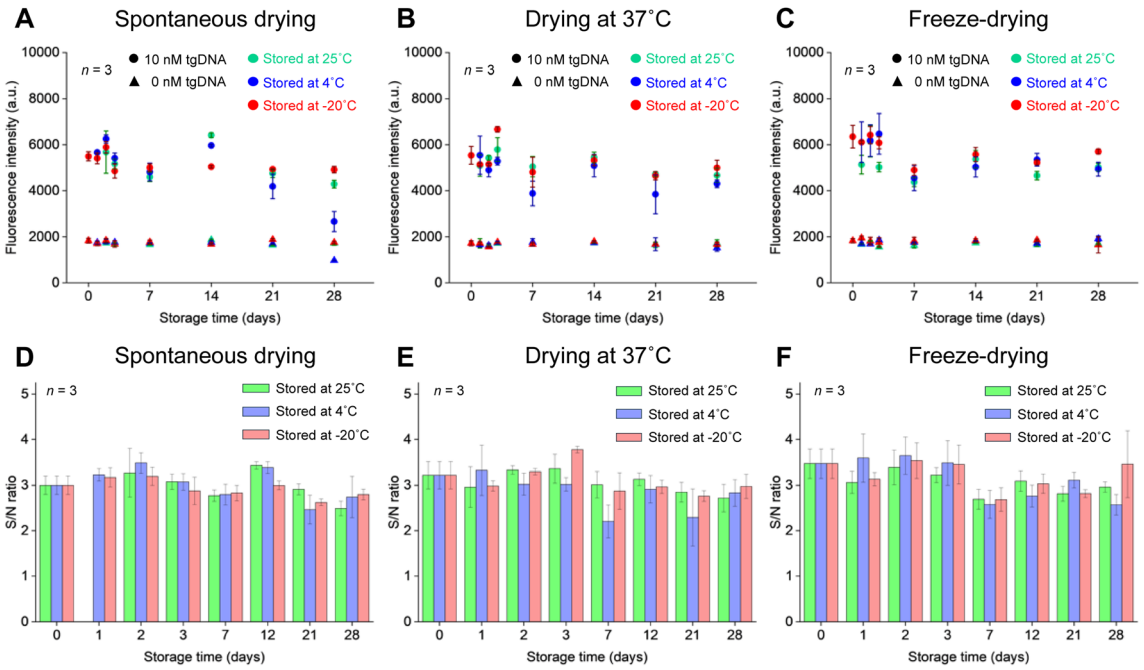

Fig. 1 Influence of drying and storage conditions on the stability of CRISPR-related reagents on a WF1 paper substrate (all dried in the presence of 10% trehalose as the stabilizer): (A)–(C) absolute fluorescence signal intensities in the presence (10 nM) or absence of target dsDNA, and (D)–(F) the corresponding signal (10 nM tgDNA) to noise (blank sample) ratio; error bars represent mean values ±1σ (n = 3).

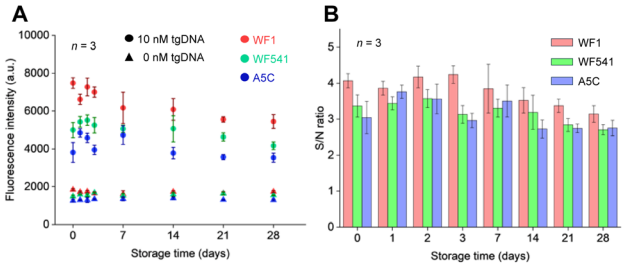

Fig. 2 Influence of the type of filter paper substrate on stability of CRISPR-related reagents spontaneously dried at room temperature (in the presence of 10% trehalose as the stabilizer) and stored at −20 °C: (A) absolute fluorescence emission intensities and the (B) signal to noise ratio; error bars represent mean values ±1σ (n = 3).

fluorophores are equally distributed throughout the entire thickness of the paper. This results in a higher fluorophore concentration per unit area for thinner filter papers. Moreover, a higher density of cellulose fibres leads to stronger scattering of both excitation and emitted light, reducing the light penetration into and out of the depth of the paper. These facts account for the lowest observed fluorescence signals in the case of the A5C paper with its very narrow pore size and hence, the highest fibre density in addition to the largest thickness. The situation is less clear for the comparison of the WF1 and WF541 signal intensities. But according to Luongo *et al.*[26] fluorescence emission signal intensities tend to increase with the paper substrate thickness, due to the strong scattering of light from fibres behind the fluorescent dye molecule. For the reason of providing the highest signal to noise ratio, the WF1 substrate was selected for further experiments.

Selection and optimization of stabilizers

As mentioned above, the presence of stabilizers in the reagent solution is also an important factor to extend the storage stability of proteins. According to prior studies investigating the effect of freeze-drying on the performance of CRISPR-related reagents, the following five substances are major candidates for such purposes: sucrose, trehalose, dextran, BSA and glycine.[20,27] Since in the current work BSA is already present on the paper substrates as a blocking agent, the remaining four candidates were investigated for their stabilizing effect. As Fig. S3† indicates, CRISPR-related reagents spontaneously dried in the presence of disaccharides including trehalose and sucrose mostly resulted in larger signal intensities and generally higher S/N ratios compared to those of other stabilizer candidates. Since the mechanism behind stabilizing effects on the 3D-structure varies between proteins, drying methods and other experimental conditions,[28] the observations are not further discussed, and sucrose and trehalose were selected for further investigation of the storage stability depending on stabilizers. The corresponding results are shown in Fig. 3, indicating that fluorescence signal intensities of over 70% were maintained over 28 days of storage at −20 °C. The statistical significance of the signal intensities related to the addition of stabilizers (Fig. 3A and B) was evaluated, and the corresponding results are shown in Fig. S4.† Although up to 3 days of storage no statistically significant decrease in signal intensity was observed for devices prepared in the absence of sucrose, the situation was different after storage for 7 days or longer (Fig. S4A†), where the presence of sucrose contributes to storage stability in a statistically significant manner. But when adding more than 5% of sucrose, blank signals were significantly increased (Fig. S4B†), negatively affecting the S/N ratio (Fig. 3C). In the case of evaluating trehalose as a stabilizer, the addition of this sugar did not significantly affect signal intensities for up to 12 days of storage, while addition of 5% of trehalose enhanced the storage stability after day 21 (Fig. S4C†). As far as the influence of trehalose on the blank signal is concerned, a statistically significant increase was observed when using 10% (Fig. S4D†), which resulted in decreased S/N ratios (Fig. 3D). On the other hand, all of these results together suggest that the storage stability is not substantially affected by the presence or absence of stabilizers. We explain this as a consequence of the presence of BSA used for paper blocking, which also contributes to preservation of the CRISPR-related reagents. Looking at the S/N ratios (Fig. 3C and D) and taking into account the lower cost of sucrose compared to trehalose, 1% of sucrose was selected as the optimal stabilizer for the spontaneous room temperature drying of CRISPR-related reagents. After continued storage at −20 °C for 84 days, the original fluorescence intensity was maintained (Fig. S5†). Considering the results so far, the optimized conditions are summarized as follows: drying at room temperature in the presence of 1% of sucrose on a BSA blocked WF1 paper substrate and storing at −20 °C.

Target dsDNA concentration response depending on the storage time

Lastly, the target dsDNA (tgDNA) concentration response obtained with micro-spots on paper substrates with CRISPR-

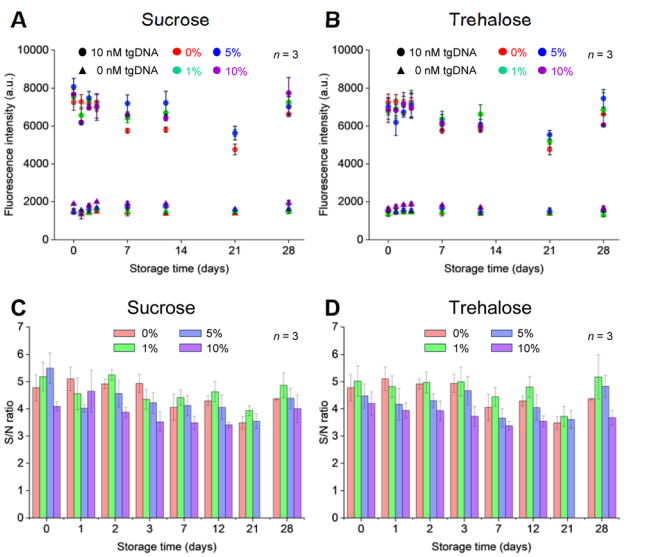

Fig. 3 Influence of the type and concentration of disaccharide on the storage stability of CRISPR-related reagents spontaneously dried at room temperature on WF1 paper substrates stored at −20 °C: (A and B) absolute fluorescence emission intensities and the (C and D) signal to noise ratio; error bars represent mean values ±1σ (n = 3).

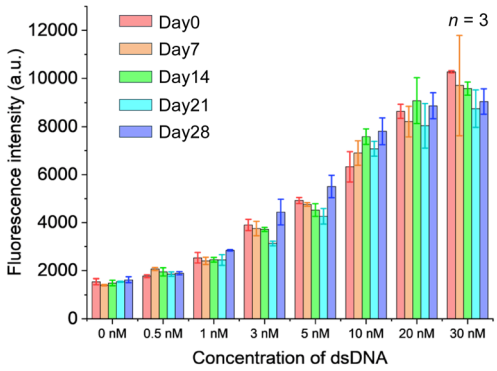

Fig. 4 Target DNA concentration-dependent fluorescence intensities after various periods of storage at −20 °C observed with CRISPR-related reagents (1% sucrose) spontaneously dried at room temperature on WF1 paper substrates; error bars represent mean values ±1σ (n = 3).

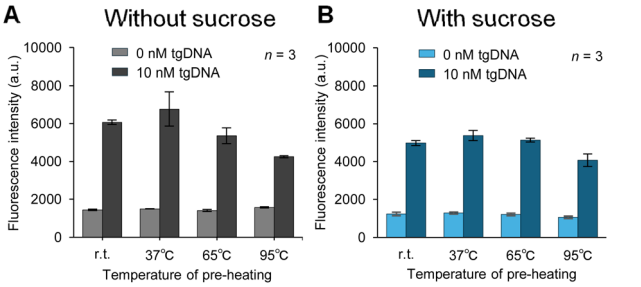

Fig. 5 Fluorescence intensities obtained with assays performed after pre-heating at various temperatures involving CRISPR-related reagents dried at room temperature spontaneously on WF1 paper substrates (A) without sucrose and (B) with 1% of sucrose; error bars represent mean values ±1σ (n = 3).

related reagents pre-deposited and dried according to the above selected conditions was investigated as a function of storage time. As a comparison experiment, tgDNA concentration-dependent response behaviour for the CRISPR-related reagent solution stored in the frozen state at −20 °C was also investigated. The response range of the CRISPR assay was found to be from 0.5 to 30 nM of tgDNA for both the paper- and microtube-based assays (Fig. S6†). Thus, 8 concentrations of tgDNA (0, 0.5, 1, 3, 5, 10, 20, and 30 nM) were used to investigate the concentration response behaviour over different periods of storage. According to Fig. 4A, the CRISPR-related reagents stored in the dry state on paper substrates resulted in the tgDNA concentration response maintaining over 70% of the initial activity over the 28 day storage range, suggesting a sufficiently high storage stability to preserve the quantitative assay function. In contrast, as expected, the response obtained with frozen solution state stored CRISPR-related reagents showed a significant drop in signal intensity after only 7 days, followed by further gradual signal loss (Fig. S7†). Finally, the activity after 28 days of storage decreased down to 30% of its initial value (at 10 nM tgDNA). These results clearly demonstrate the enhanced storage stability of dried state CRISPR-related reagents on paper substrates, providing support for the successful application of CRISPR/Cas-based PADs for POCT applications requiring no reagent handling by the assay user.

Investigation of heating tolerance of CRISPR-related reagents

Considering the application of CRISPR-related reagents for practical POCT use, most assays involve a nucleic acid pre-amplification step to achieve the required sensitivity.[29–31] Regardless of the pre-amplification method selected (*e.g.*, PCR or isothermal), heating is generally involved. For the previously reported solution phase assays performed in microtubes, the processes of target amplification and CRISPR/Cas-based detection can be readily separated. However, this is not the case when attempting to combine nucleic acid pre-amplification and a CRISPR/Cas reaction into a single user-friendly PAD. On-device amplification of nucleic acids combined with using CRISPR-based reagents to facilitate the signal readout in a single platform has, for example, been achieved by P. Q. Nguyen and coworkers.[19] They combined recombinase polymerase amplification (RPA) in a first paper layer with a CRISPR/Cas reaction in a second paper layer into a single fully integrated paper-based device. However, RPA only requires heating to a maximum of 37 °C. To further expand the possibilities for integrated devices, it is of significant importance to investigate the heat tolerance of the CRISPR-related reagents at higher temperatures, since other common pre-amplification methods like PCR and loop-mediated amplification (LAMP) involve heating up to around 95 and 65 °C, respectively. Thus, the heating tolerance at these temperatures was investigated using paper dried CRISPR-related reagents. As Fig. 5 indicates, the paper micro-spots with dried reagents remained functional at all temperatures regardless of the presence of sucrose, while the reagent activity in the microtube solution state was lost upon heating at 65 and 95 °C (Fig. S8†), as expected from its instability on interaction with water as mentioned earlier. These results demonstrate the significantly enhanced heating tolerance of dried state CRISPR-related reagents on paper substrates. To investigate the contribution of BSA used for paper blocking to the heating tolerance, the same experiment was performed with non BSA-blocked paper substrates (Fig. S9†). Reagent activity was not retained at any pre-heating temperature, which indicates the role of BSA in stabilizing the dried-state CRISPR-related reagents, as already mentioned above. Taking into account that there already exist multiple reports on paper-based LAMP amplification,[32–34] the heating tolerance of CRISPR-related reagents experimentally demonstrated in this work emphasizes the possibility of the development of a fully integrated PAD combining target nucleic acid pre-amplification and CRISPR/Cas reactions.

Conclusions

The current work focused on investigating the storage stability of reagents necessary for CRISPR/Cas-based assays on paper substrates depending on various conditions. Different from all previous studies reporting the storage stability after freeze-drying in microtubes, CRISPR-related reagents spontaneously

dried at room temperature on BSA blocked paper substrates showed preserved activity when stored at −20 °C for 28 days, which was improved by the addition of sucrose as a stabilizer, although not to a degree that might be regarded as substantial for practical applications. This storage stability observed for CRISPR-related reagents on filter paper substrates achieved through a simple drying method strongly supports the applicability of the CRISPR/Cas system to fully integrated PADs with all required reagents pre-deposited on devices to reach the goal of the highest possible user-friendliness. Finally, the heat-tolerance of CRISPR-related reagents dried under optimized conditions on PADs suggests the possibility of combining nucleic acid pre-amplification methods with CRISPR/Cas assays into a single fully integrated paper-based analytical device for highly sensitive nucleic acid POCT.

Author contributions

Yohei Tanifuji: conceptualization, methodology, investigation, data curation, formal analysis, writing – original draft. Hikaru Suzuki: conceptualization, methodology, investigation, supervision. Guodong Tong: conceptualization, methodology, investigation, supervision. Yuki Hiruta: validation, writing – review & editing, supervision, funding acquisition. Daniel Citterio: conceptualization, methodology, validation, writing – review & editing, supervision, project administration, funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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