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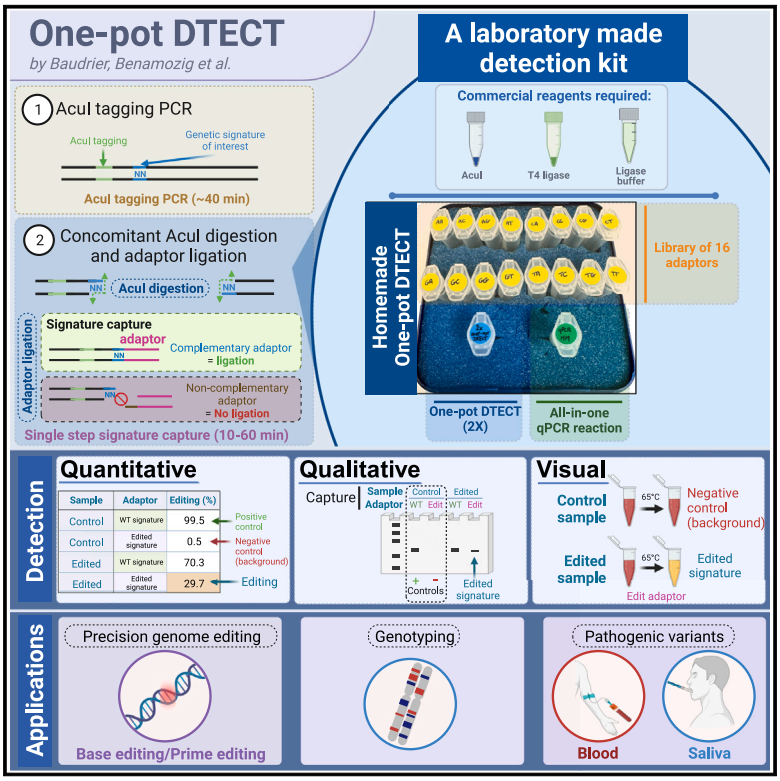
One-pot DTECT enables rapid and efficient capture of genetic signatures for precision genome editing and clinical diagnostics

Graphical abstract



Highlights

- One-pot DTECT enables all-in-one genetic signature exposure and capture
- Enhanced one-pot reaction with optimized buffers and controlled digestion
- One-pot DTECT-LAMP enables real-time visual detection of genetic signatures
- One-pot DTECT captures pathogenic variants in saliva and blood samples

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In brief

Baudrier et al. introduce One-pot DTECT, an efficient and versatile platform for detecting genetic signatures with different detection modalities, including qualitative, quantitative, and visual outputs, using an optimized homemade one-pot reaction. One-pot DTECT streamlines the detection of variants for precision genome editing and clinical applications.

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One-pot DTECT enables rapid and efficient capture of genetic signatures for precision genome editing and clinical diagnostics

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MOTIVATION The rapid and accurate detection of genetic signatures of interest is fundamental for the advancement of both basic research and clinical diagnostics. However, conventional methods typically rely on external genomic services, incurring elevated costs and extended turnover time. To address this, we developed One-pot DTECT, a homemade detection method formulated with off-the-shelf reagents that streamlines the capture of genetic signatures of interest by combining multiple enzymatic activities into an efficient one-pot reaction and a simple library of common adaptors. This approach accelerates and simplifies detection workflows and expands the scope of detection capabilities of genetic signatures through qualitative, quantitative, or visual detection.

SUMMARY

The detection of genomic sequences and their alterations is crucial for basic research and clinical diagnostics. However, current methodologies are costly and time-consuming and require outsourcing sample preparation, processing, and analysis to genomic companies. Here, we establish One-pot DTECT, a platform that expedites the detection of genetic signatures, only requiring a short incubation of a PCR product in an optimized one-pot mixture. One-pot DTECT enables qualitative, quantitative, and visual detection of biologically relevant variants, such as cancer mutations, and nucleotide changes introduced by prime editing and base editing into cancer cells and human primary T cells. Notably, One-pot DTECT achieves quantification accuracy for targeted genetic signatures comparable with Sanger and next-generation sequencing. Furthermore, its effectiveness as a diagnostic platform is demonstrated by successfully detecting sickle cell variants in blood and saliva samples. Altogether, One-pot DTECT offers an efficient, versatile, adaptable, and cost-effective alternative to traditional methods for detecting genomic signatures.

INTRODUCTION

Over the past few decades, our capacity to read and edit genetic sequences has been transformed, bolstered by advances in sequencing and genome editing technologies.[1–3] For instance, recent developments in CRISPR-based precision genome editing, including base editing[4–8] and prime editing,[9] have fundamentally changed our ability to manipulate genomic sequences.[10–12] This genomic revolution has deepened our understanding of how genetic information orchestrates cellular functions and influences susceptibility to diseases and disorders, fostering the day-to-day exploration of genetic signatures and variations in basic research and clinical laboratories. However, despite these advancements, the efficient, rapid, and cost-effective detection of genetic signatures in routine laboratory practices remain challenging and typically relies on external parties.

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Current approaches rely primarily on sequencing technologies, such as Sanger sequencing or next-generation sequencing (NGS).[13–18] Although NGS provides massively parallel sequencing of DNA molecules, its high sensitivity and throughput often exceed the needs of routine laboratory applications.[19,20] Sanger sequencing, on the other hand, has comparatively lower precision and detection limit due to variabilities in sequencing resolution.[21,22] Moreover, both methodologies generally require outsourcing sample preparation and analysis to specialized genomic companies or core facilities, resulting in increased costs, extended turnaround times, and impedes laboratory autonomy.

In this study, we introduce One-pot DTECT, a detection method that leverages two enzymatic activities to simultaneously expose and capture genetic signatures of interest in an easy to assemble one-pot mixture. Our results demonstrate that One-pot DTECT yields visual, qualitative, and quantitative data, and is capable of highly precise quantification of targeted mutations in a manner comparable with Sanger sequencing or NGS for the detection of nucleotide changes introduced with base editing and prime editing in cancer cells and human primary T cells. Furthermore, One-pot DTECT has significant potential clinical applications in diagnosing genetic diseases as we demonstrated it can readily detect disease-associated genetic signatures in clinical samples, including blood samples or saliva swabs, for mutation carriers and sickle cell patients. One-pot DTECT will challenge the traditional practice of outsourcing the detection of genomic sequences of interest to genomic companies by promoting detection through a user-friendly homemade kit for routine laboratory procedures.

RESULTS

Workflow of DTECTv1

The general concept and workflow of DTECTv1 are depicted in Figure 1. This approach harnesses two enzymatic activities: the type IIS restriction endonuclease AcuI, which exposes specific genetic signatures, and a DNA ligase that attaches DNA adaptors to enable signature capture (Figure 1).[23,24]

The locus of interest is amplified using an AcuI-tagging primer that embeds the 5′-CTGAAG-3′ AcuI cognate sequence motif into the PCR amplicon (Figure 1A, step 1).[25] The AcuI-tagged amplicon is then subjected to digestion by AcuI (Figure 1B, step 2) to generate 3′ dinucleotide overhangs by cleaving DNA at a fixed distance of 14 and 16 nucleotides from its motif. The smaller fragment is then isolated using solid-phase reversible immobilization beads (Figure 1B, step 3) to retain only one signature to prevent interference with adaptors at the subsequent step. Next, the isolated DNA fragment is captured by the ligation of adaptors that have complementary (Figure 1B, step 4 in blue) or noncomplementary (Figure 1B, step 4 in brown) dinucleotides to the newly created overhang signatures. A library composed of only 16 unique adaptors with each dinucleotide overhang is necessary and sufficient to capture every possible dinucleotide signature. The ligated material is then detected either by quantitative PCR using primers that bind to the 5′ end of the AcuI-tagging oligos and 3′ end of the adaptors (Figure 1C, step 5) to quantify the relative proportion of variants in a population or by analytical PCR (Figure 1C, step 5) for rapid determination of the presence or absence of specific variants.

To test the high programmability and versatility of this method, we captured genetic signatures of various SARS-CoV-2 variants (Figure S1A).[26] By programming AcuI to expose dinucleotide signatures of interest, we found that DTECTv1 effectively detects and discriminates different signatures from either the SARS-CoV-2 reference genome or variants using signature-specific adaptors (Figures S1B and S1C). These experiments demonstrate the ability of DTECTv1 to capture a variety of specific variants while maintaining high specificity and low background (0.08%, n = 9) (Figure S1B). The use of variant-specific adaptors in control samples enabled precise quantification of background levels, thereby enhancing the accuracy of the capture and eliminating the risk of false positive results.

AcuI digestion and adaptor ligation are crucial, while bead isolation is dispensable

Despite the robust and relatively rapid execution (~5 h) of DTECTv1, its multistep nature not only adds a layer of complexity but also requires substantial experimenter interventions. This increases the potential for technical variability and sample cross-contamination, thus impacting its reliability, reproducibility, and general adoption.

To evaluate whether the distinct steps of the capture (AcuI digestion, bead isolation, and adaptor ligation) are essential, we conducted DTECTv1 but omitted each individual step/enzyme: (1) AcuI digestion, (2) small fragment isolation using beads, and (3) and adaptor ligation using DNA ligase (Figure 2A). We assessed the performance of DTECT by quantifying ligation efficiency (hereafter referred to as *capture efficiency*) (Figure S2A) and ligation specificity (referred to as *capture specificity*) (Figure S2B). As expected, DTECTv1 resulted in robust capture efficiency (Figure 2B) and specificity (Figure 2C, in light yellow) for the capture of the SARS-CoV-2 E484K variant (specific adaptor) compared with the E484 Wuhan reference signature (nonspecific adaptor). Moreover, omission of AcuI or T4 ligase abolished signature capture to the same extent as the nonspecific adaptor (Figures 2B and 2C) confirming that AcuI activity is crucial for exposing overhang signatures and that ligation of the adaptors is required for efficient signature capture.

Next, we were interested in the small fragment isolation step, which serves to separate the DNA fragments generated by digestion, ensuring that adaptors do not compete with the larger DNA fragment that shares the same complementary dinucleotide. Surprisingly, omitting the bead isolation step had no impact on capture efficiency (Figure 2B) or specificity (Figure 2C), as also confirmed by analytical detection (Figure S2C) and sequencing of the ligation product that identified the expected ligation product (Figure S2D). These experiments reveal that the bead isolation step is dispensable, simplifying DTECT to only require off-the-shelf enzymes (AcuI and T4 ligase) and demonstrating the potential for enhancing DTECTv1.

Development of an accelerated and simplified DTECTv2 assay

These findings prompted us to reassess each step of DTECTv1 to develop a more efficient and streamlined capture strategy. First, we investigated how various parameters influence AcuI digestion. Time-course experiments were conducted, to

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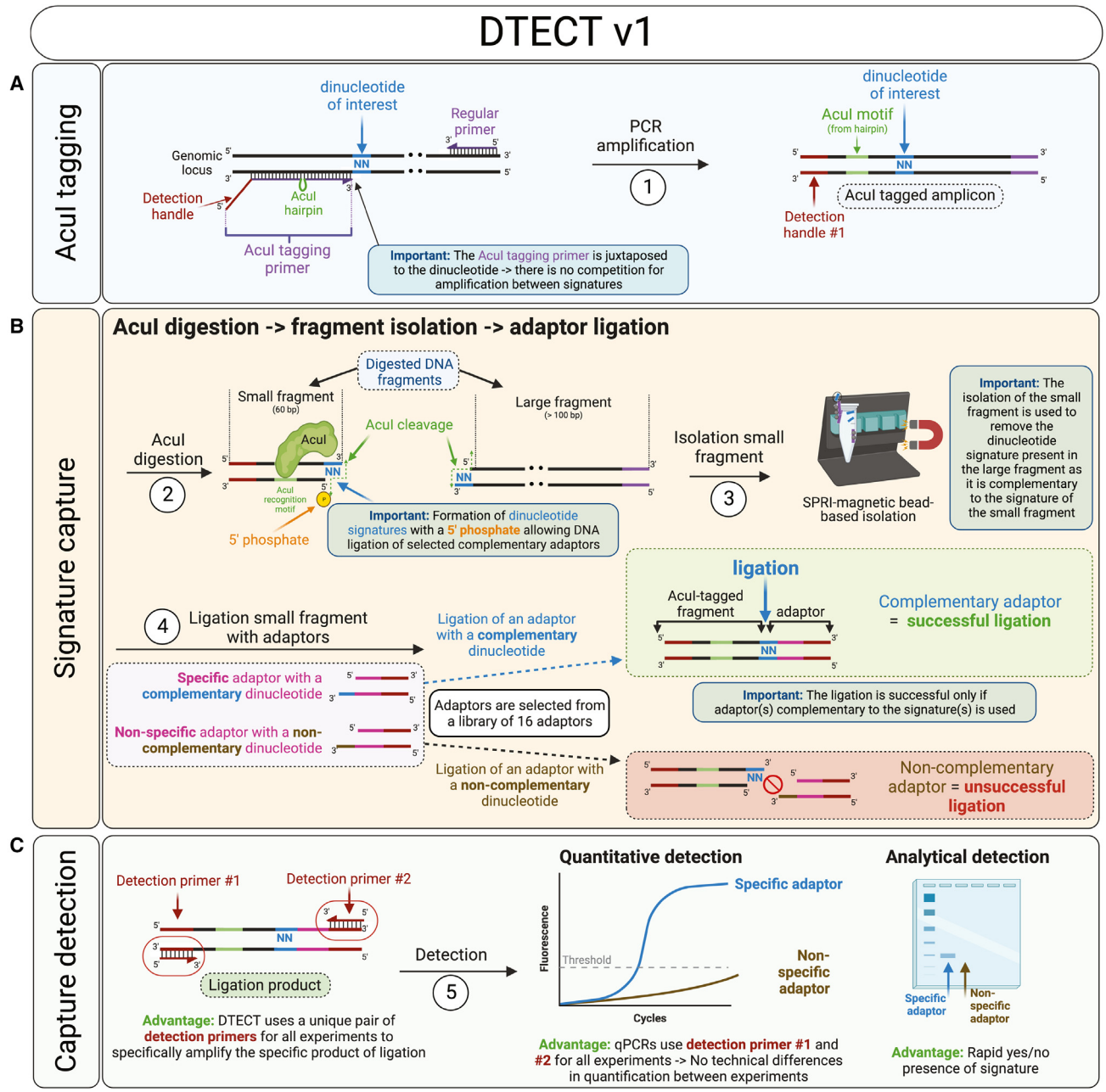

Figure 1. General workflow of DTECTv1

(A) Schematic representation of the Acul-tagging step.

(B) Illustration of the signature capture steps. Signature capture consists of three independent steps: Acul digestion, small fragment isolation, and adaptor ligation.

(C) Schematic representation of the capture detection step. Detection handles no. 1 and no. 2 (red) are used to detect the presence of ligated products using a unique pair of detection primers: no. 1 and no. 2 (red arrows). These primers allow specific amplification of the specific ligation product between the small fragment and the adaptors for quantitative or qualitative PCR (step 5).

investigate the kinetics of Acul digestion. Notably, our results demonstrated that a short 5-s incubation yielded capture efficiency and specificity comparable with a 60-min digestion (Figures 2D and S2E). Control reactions without Acul or using a nonspecific adaptor showed similar low background capture (Figure 2D, in green at t = 60 min, and red, respectively). These findings indicate that Acul-mediated digestion of tagged DNA amplicons can expose sufficient dinucleotide signatures for maximal capture in a few seconds.

Next, we evaluated the potential to accelerate Acul heat inactivation, which is typically performed at 65°C for 20 min according to supplier recommendations. By first inactivating

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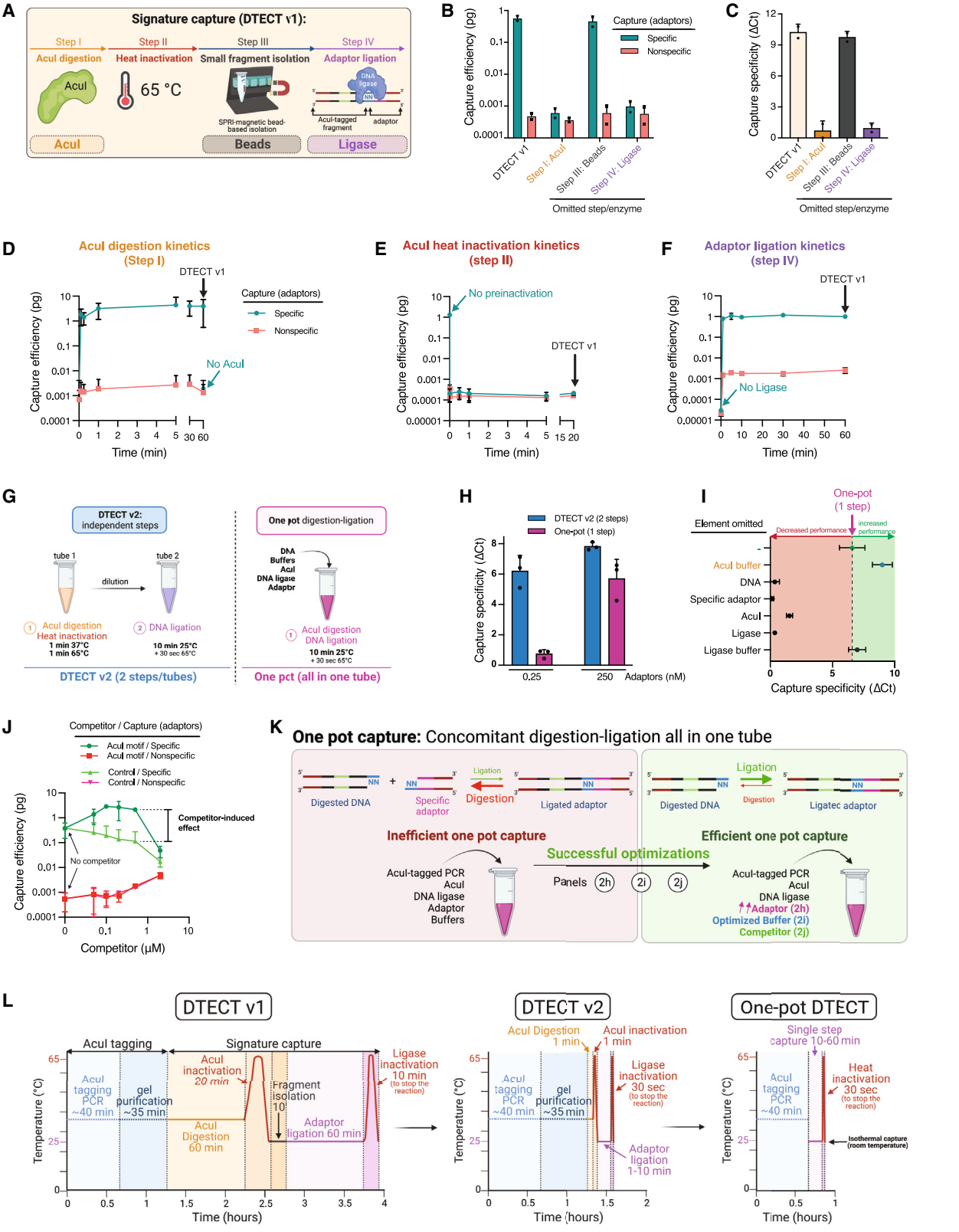

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AcuI for durations ranging from 30 s to 20 min, and subsequently adding the AcuI-tagged amplicon to the reaction, we assessed the degree of AcuI inactivation based on the capture levels. As expected, a control reaction without AcuI preinactivation resulted in robust capture (Figures 2E and S2F). Intriguingly, a 30-s preincubation of AcuI at 65°C effectively prevented capture to the same extent as the 20-min reference in DTECTv1 (Figures 2E and S2F), demonstrating that AcuI denaturation can be completed in just 30 s, which is consistent with the irreversible impact of heat on protein stability and function.[27]

In addition, we explored the possibility of optimizing the ligation reaction to enhance signature capture. A titration of DNA ligase revealed that the original conditions provided the highest capture efficiency and specificity (Figure S2G). We also assessed the impact of ligation time on capture efficiency. These experiments demonstrated that a 1-min ligation at 25°C was sufficient to achieve maximal capture, comparable with the 1-h ligation used in DTECTv1 (Figure 2F). These findings indicate that the ligation step can be reduced to 1 min without compromising efficiency or specificity.

Collectively, these experiments demonstrate that DTECTv1 can be significantly improved by eliminating the bead isolation step and accelerating AcuI digestion (from 60 to 1 min), AcuI heat inactivation (from 20 min to 30 s), and adaptor ligation (from 60 to 1 min). To examine whether these modifications can be combined effectively, we compared the original DTECTv1 method (capture: 3 steps; ~2.5 h) with the accelerated DTECTv2 protocol (capture: 2 steps; ~5 min) (Figure S2H). Using both DTECTv1 and DTECTv2, we efficiently captured the SARS-CoV-2 E484K variant signature although with a slightly lower efficiency for both specific and nonspecific adaptors (Figure S2I). We independently validated these results by capturing the commonly found oncogenic signature within the *PIK3R1* gene (PIK3R1-R348*), or its reference signature (PIK3R1-WT) (Figure S2J), confirming that the improvements can be successfully combined without negatively impacting DTECT performance.

These pivotal enhancements led to the development of DTECTv2, achieving a remarkable 97% reduction in capture duration, facilitating rapid (~5 min) and sensitive signature capture.

Development of an efficient one-pot capture

Determining that the bead isolation step is not necessary was an important advance because it enabled to envision a more streamlined approach that combines AcuI digestion and DNA ligation into a single reaction tube within an optimized buffer that accommodates both enzymatic activities to work simultaneously.

To evaluate the compatibility of AcuI and DNA ligase in a one-pot reaction, we performed DTECTv2, in which the two enzymatic reactions are physically separated into two independent steps/tubes with their respective optimal buffers (Figure 2G, left), and compared it with a one-pot assay that merges the two enzymes, adaptors, and buffers for a 10-min incubation at 25°C (Figure 2G, right). Due to the rapidity of the ligation (1 min) and to prevent technical variabilities due to the time required for the pipetting between samples (which may exceed 1 min) we extended the adaptor ligation to 10 min. As expected, DTECTv2 successfully captured the signatures with high specificity with 0.25 nM adaptors (Figure 2H, blue). However, the one-pot reaction did not yield any capture at this concentration of adaptors (Figure 2H, pink). One possibility is that concomitant digestion/ligation is inefficient because ligation is disfavored over a highly efficient AcuI digestion, resulting in the digestion of the captured product by AcuI. We speculated that increasing the concentration of adaptors could displace the enzymatic reaction equilibrium in favor of ligation, even in the presence of active AcuI. While DTECTv2 maintained consistent capture efficiency regardless of adaptor concentration, increasing the concentration of adaptors at 250 nM restored capture in a one-pot reaction (Figure 2H, pink). These experiments reveal that merging AcuI and ligase activities under optimized conditions to expose and capture signatures of interest concomitantly is feasible in a one-pot mixture.

Enhancement of the one-pot reaction through optimized buffer composition and controlled AcuI digestion

To further refine the one-pot capture reaction, we tested the potential for enhancing the buffer composition. By analyzing the impact of removing each buffer component on capture efficiency, we aimed to identify a minimal buffer composition. Interestingly, we observed that eliminating the commercial AcuI

Figure 2. Development and validation of One-pot DTECT

(A) Schematic representation of the four independent steps required to capture signatures with DTECTv1.

(B) Measure of the capture efficiency of the SARS-CoV-2 E484K signature using a variant-specific adaptor or a nonspecific adaptor as a control using DTECTv1, or with the omission of AcuI, bead isolation, or ligase, as indicated. Error bars represent SD of two independent experiments. Individual data points are shown.

(C) Measure of the capture specificity of the SARS-CoV-2 E484 or E484K signatures. DTECTv1 was used as a control (yellow). Experiments were conducted with or without AcuI, bead isolation, or DNA ligase, as indicated. Error bars represent SD of two independent experiments. Individual data points are shown.

(D) Time-course experiment of AcuI digestion in DTECTv1. Error bars represent SD of two independent experiments.

(E) Time-course experiment of heat inactivation of AcuI in DTECTv1. Error bars represent SD of two independent experiments.

(F) Time-course experiment of adaptor ligation in DTECTv1. Error bars represent SD of two independent experiments.

(G) Schematic representation of the experiment presented in (H).

(H) Comparison of the capture specificity between DTECTv2 and One-pot DTECT using the indicated adaptor concentration. Error bars represent SD of three independent experiments. Individual data points are shown.

(I) Modification of buffer composition in One-pot DTECT. Error bars represent SD of two independent experiments.

(J) Capture efficiency of One-pot DTECT containing a DNA competitor fragment (dark green and dark red) or a control DNA fragment (light green and light red). Error bars represent SD of three independent experiments.

(K) Schematic representation of the different modifications applied to DTECTv2 to allow the development of One-pot DTECT.

(L) Schematic representation of the different steps in DTECTv1, DTECTv2, and One-pot DTECT. The approximate time required for each step is also indicated.

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buffer from the reaction improved capture (Figure 2I), an effect on catalysis likely attributable to the additive Mg^{2+} present in both AcuI and ligase buffers. Addition of known ligation enhancers did not enhance capture efficiency at any concentration tested (Figure S3A).[28–30]

Next, we systematically evaluated the activity of various commercial DNA ligases to identify the most effective enzyme for capturing signatures in the one-pot assay. Among the ligases tested, T4 ligase exhibited the most robust capture activity, followed by T3 ligase (Figure S3B), consistent with their preference for sticky ends. The high performance of T4 ligase prompted us to examine multiple commercial T4 ligases and variants, including heat-resistant (Hi) and highly concentrated (H.C) T4 ligases. Each T4 ligase demonstrated robust activity in capturing the dinucleotide signature with high sensitivity and specificity (Figure S3B). Notably, the one-pot capture utilizing classical T4 ligase from two distinct suppliers (Figure S3B, no. 1 and no. 2) yielded identical robust and specific capture, reaffirming the reliability and efficiency of the one-pot digestion-ligation capture using off-the-shelf reagents. As a control, T7, 9°N, and Taq ligases did not effectively capture the signature, which is consistent with their preference to ligate nicks of adjacent DNA strands.

A critical characteristic of type IIS enzymes is the dissociation between their binding motif and cleavage position, as they cleave DNA at a shifted distance from their recognition motif.[31] As a result, AcuI can remain bound to digested DNA substrates potentially cleaving newly ligated adaptors, leading to the digestion of ligation products and a decrease in ligation efficiency. We hypothesized that incorporating uncleavable competitor DNA fragments containing an AcuI motif would limit AcuI's capacity to digest newly ligated adaptors, thus protecting ligation products from degradation by AcuI. To examine this, we prepared a double-stranded DNA fragment containing an AcuI motif sequence surrounded by 12 nucleotides, ensuring that the competitor fragment is bound but not cleaved by AcuI. The addition of 0.05–0.5 μM competitor enhanced the capture efficiency (Figures 2J and S3C) compared with the addition of a DNA control lacking the AcuI motif. Increasing the amount of competitor DNA prevented capture, likely because the excess competitor inhibits AcuI, thereby preventing digestion (Figure S3C). These experiments demonstrate that uncleavable competing DNA fragments can be used to protect newly ligated adaptors from the digestion by AcuI, leading to increase in capture efficiency.

In addition, we demonstrated that one-pot capture works similarly at 16°C, 25°C, or 37°C (Figure S3D), enabling its isothermal use at room temperature without requiring incubation or specialized instrumentation. Finally, given that One-pot DTECT requires only 2.5 fmol of AcuI-tagged amplicon, we demonstrated that purification of the AcuI-tagged amplicon is dispensable, as all types of purifications (gel, column, or bead purification) or direct dilution of the PCR product yielded comparable capture levels (Figure S3E).

In summary, we achieved concomitant amplicon digestion and adaptor ligation in a one-pot reaction, despite the presence of active AcuI, by (1) optimizing the concentration of adaptors, (2) enhancing buffer conditions, and (3) adding an AcuI competitor DNA fragment (Figure 2K). These findings establish an advanced method for the highly sensitive one-pot capture of genomic signatures. This approach, which we named One-pot DTECT, can be completed in approximately 1–2 h from DNA extraction to capture with minimal experimenter interventions (Figure 2L).

Integration of a visual detection method with One-pot DTECT

Loop-mediated isothermal amplification (LAMP) is a highly sensitive real-time colorimetric assay for targeted nucleic acid detection.[32] LAMP uses multiple oligonucleotides (labeled as F1-F3 and B1-B3 in Figure 3A) to amplify targeted sequences, but its extensive primer design requirements and inability to efficiently discriminate single-nucleotide polymorphisms have limited its applications for the detection of targeted variants.[33] A significant advantage of One-pot DTECT lies in the customizability of the adaptors, allowing for its potential coupling with LAMP by integrating LAMP-specific sequences on each side of the ligated products, thereby creating a system in which loop amplification depends on successful ligation of adaptors (i.e., the presence of the variant of interest) but is independent on the targeted sequences or variants.

To test this approach, we replaced the detection handles of One-pot DTECT with validated LAMP sequences that have been used for detecting the SARS-CoV-2 *ORF1a* locus (Figure 3A). To do this, we integrated the F3 and F2 LAMP sequences into the 5′ end of the AcuI-tagging primers and the F1, B1, B2, and B3 LAMP sequences into the adaptors (Figure 3A). We tested this novel method, which we named One-pot DTECT-LAMP, to detect SARS-CoV-2 E484K or E484 signatures with the respective adaptors. Real-time color change was observed in reactions with the respective specific adaptors (variant or WT), and completed within 30 min of incubation at 65°C (Figure 3B). These results were confirmed by quantifying colorimetric change by measuring the optical density of the reaction over time (Figure 3C), and with independent adaptors integrating a different set of validated LAMP sequences within the adaptors and AcuI-tagging primers (Figure S4A).

The integration of One-pot DTECT with LAMP presents a simple and efficient approach for real-time visual detection of variants of interest. Importantly, detection with One-pot DTECT-LAMP is independent of the targeted sequences and variants as it requires the integration of published and validated LAMP sequences in the 5′ end of the AcuI-tagging oligo and 3′ end of the adaptors. Moreover, this highlights the high versatility of the One-pot DTECT platform, which enables the development of multiple detection modalities through simple modifications in the adaptor sequences. The successful development of a visual One-pot DTECT-LAMP assay holds great potential for real-time detection of targeted genetic signatures.

One-pot DTECT is accurate and specific

Next, we sought to assess the accuracy and specificity of One-pot DTECT for the quantitative detection of dinucleotide signatures. First, we examined the capture efficiency of all 16 dinucleotides using their respective complementary adaptors. Strikingly, One-pot DTECT exhibited highly consistent capture across all 16 possible dinucleotides (Figure 4A). Ligation efficiency remained unaffected by the number of A/T or G/C

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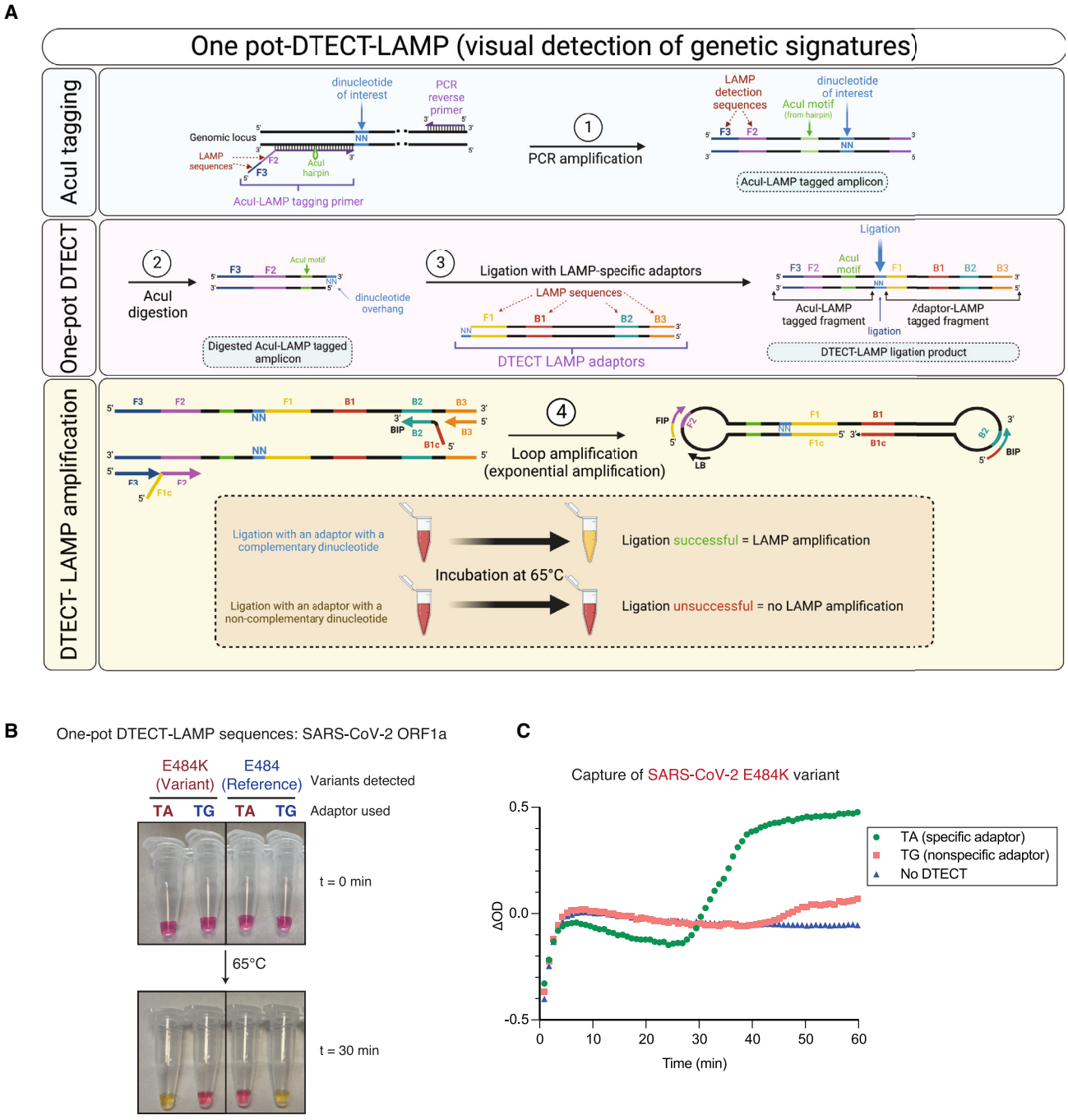

Figure 3. Development and validation of One-pot DTECT-LAMP

(A) Schematic representation of One-pot DTECT-LAMP. One-pot DTECT-LAMP follows the same amplification and capture procedure as One-pot DTECT with only small modification variations in the AcuI-tagging primer and adaptor sequences. AcuI-tagging primers contain F2 and F3 LAMP sequences at their 5′ end, and adaptors contain F1, B1, B2, and B3 LAMP sequences.

(B) One-pot DTECT-LAMP reactions were conducted to capture the SARS-CoV-2 E484 or E484K signatures.

(C) Capture of the SARS-CoV-2 E484K variant using One-pot DTECT-LAMP with real-time quantification of changes in absorbance.

nucleotides within the dinucleotide (Figure 4B), and no difference in capture efficiency was observed, regardless of C/G or A/T nucleotides at the 5′ or 3′ position of the dinucleotide (Figure 4C). Second, we assessed the accuracy of DTECT in quantifying the relative amounts of a mixture of genetic signatures. For this purpose, known ratios of WT and variant signatures (100:0, 75:25, 50:50, 25:75, and 0:100) were mixed, and the respective WT and variant signatures were captured using One-pot DTECT.

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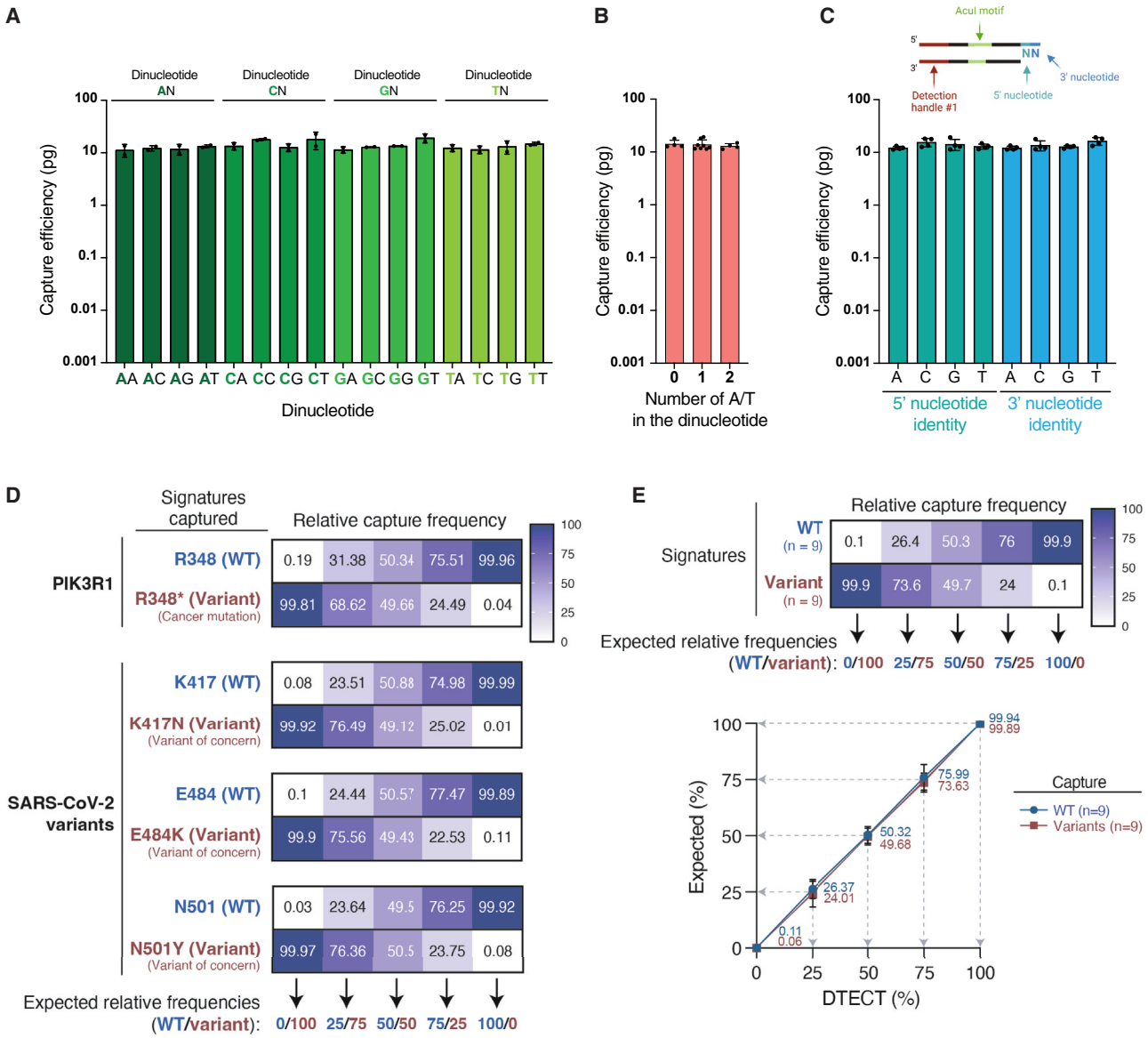

Figure 4. One-pot DTECT quantifies signatures with high accuracy

(A) Measure of the capture efficiency for each of the 16 dinucleotide signatures using their respective specific adaptors. Error bars represent the SD values of two independent experiments. Individual data points are shown.

(B) Comparison of the capture efficiency between dinucleotide signatures that contain 2A/T, 1 A/T + 1 C/G, or 2 C/G bases. Error bars represent SD values of the independent dinucleotides. Individual data points are shown.

(C) Comparison of the capture efficiency between dinucleotide signatures according to the identity of the nucleotide located either in the 5′ or 3′ end of the dinucleotide signature. Error bars represent SD values of the independent dinucleotides. Individual data points are shown.

(D) Known relative amounts of WT and variant signatures were mixed and captured using One-pot DTECT to assess its accuracy at determining expected frequencies. Heatmap representing the relative abundances of PIK3R1 R348 or R348*, SARS-CoV-2 K417 or K417N, E484 or E484K, and N501 or N501Y signatures measured by One-pot DTECT. Numbers show the average frequencies detected by One-pot DTECT from two or three independent experiments. Expected relative frequencies of the WT and variants are indicated by the arrow.

(E) Quantification of the relative abundance of predefined amounts of WT and variants of PIK3R1 and SARS-CoV-2 variants from (D). Error bars represent SD values of the capture.

The relative capture frequency was highly quantitative for the detection of SARS-CoV-2 variants of concern and PIK3R1 oncogenic signatures (Figure 4D), with the detected average percentage of capture aligning with the expected values (i.e., 0.1%, 26.4%, 50.3%, 76%, and 99.9% for the WT signatures, and 0.1%, 24%, 49.7%, 73.6%, and 99.9% for the variant signatures when 0%, 25%, 50%, 75%, and 100% were expected, respectively) (Figures 4E and S4B).

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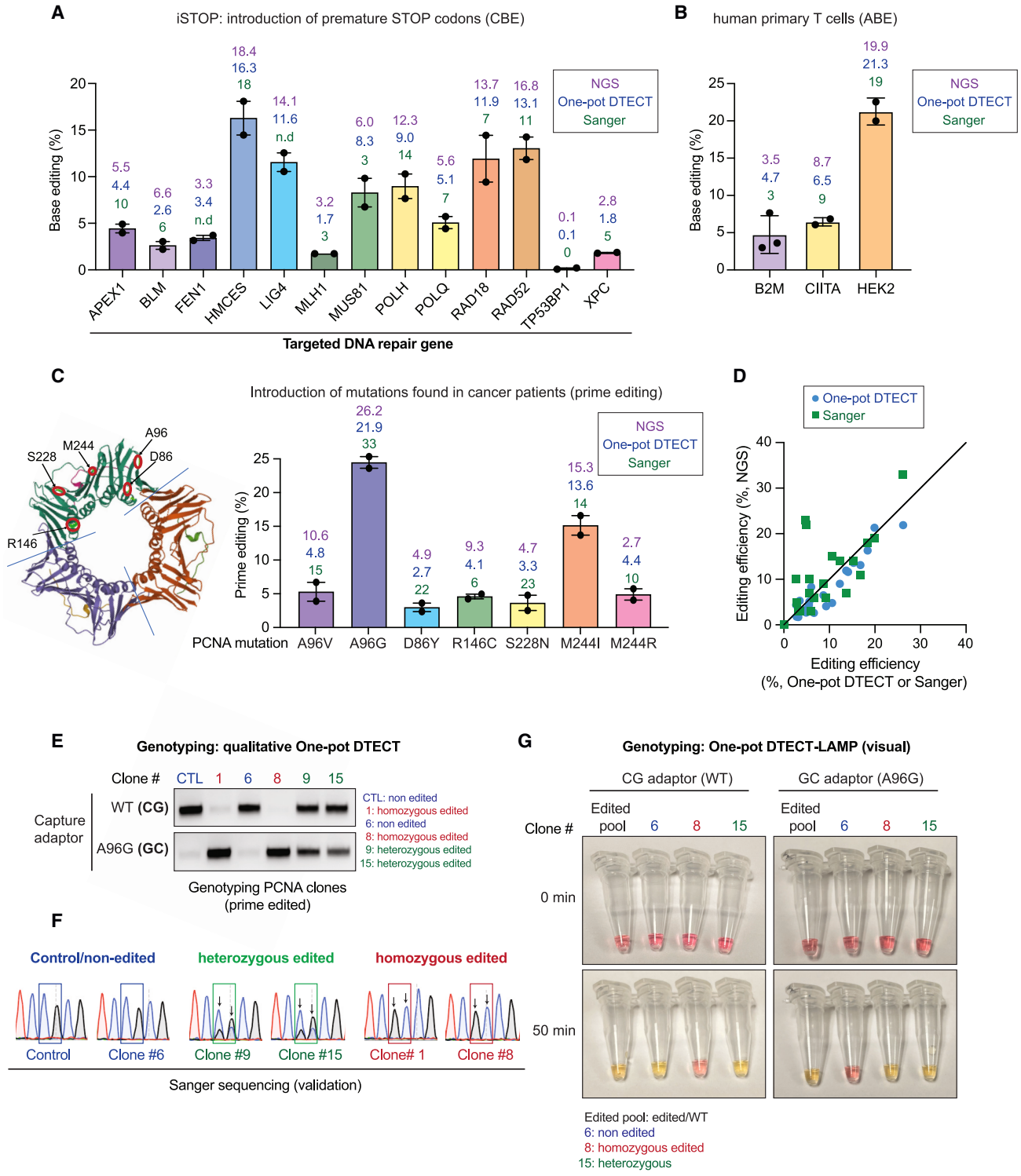

Figure 5. Detection of precision genome editing using One-pot DTECT

(A) Quantification of precision cytosine base editing levels for the inactivation of the 13 indicated DNA repair genes in HEK293T cells using NGS (purple), One-pot DTECT (blue), or Sanger sequencing (green). Error bars indicate SD values of two independent One-pot DTECT replicates. Individual data points are shown.

(B) Quantification of precision adenine base editing levels at the three indicated genomic loci in human primary T cells using NGS (purple), One-pot DTECT (blue), or Sanger sequencing (green). Error bars indicate SD values of two or three independent One-pot DTECT replicates. Individual data points are shown.

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In summary, these experiments establish that the quantification of variants with One-pot DTECT is highly accurate and is capable of precisely determining the relative frequency of signatures.

One-pot DTECT enables rapid quantification of precise modifications introduced by base editing and prime editing in cancer cell lines and human primary T cells

We subsequently evaluated the efficacy of One-pot DTECT in detecting newly generated signatures induced by cytosine base editing (CBE),[6] adenine base editing (ABE),[5] and prime editing[9] (Figures 5A–5C). In particular, 13 DNA repair genes were inactivated using CBE into HEK293T cells by introducing a premature stop codon[8] (Figure 5A), 3 genomic loci were edited with ABE in human primary T cells (Figure 5B), and prime editing was used to introduce a panel of 7 cancer-associated mutations in the critical DNA replication and repair gene *PCNA*[34] (Figure 5C). These experiments targeting 17 independent genes and genomic loci for the insertion of 23 different mutations using base and prime editing provide evidence that One-pot DTECT can accurately detect newly created signatures in a manner similar to NGS and Sanger sequencing (Figures 5D, S5A, and S5B) in only 1–2 h for a fraction of the cost (Figure S6). Moreover, the quantification of edited signatures in unedited control samples validated the low nonspecific capture background of One-pot DTECT (mean background capture = 0.71%, median = 0.51%) (Figure S5C). The comparison of capture levels between edited and control samples is important to confirm specific editing levels and prevents false positives and negatives.

Moreover, One-pot DTECT enabled the accurate genotyping of individual clones harboring *PCNA* mutations introduced with prime editing (Figure 5E), as confirmed by Sanger sequencing (Figure 5F). We also successfully used One-pot DTECT-LAMP to visually detect the presence of prime-edited signatures in cellular pools and clones (Figure 5G).

Collectively, these experiments demonstrate that One-pot DTECT is an effective and accurate tool for the quantification of precision genome editing using base and prime editing and to facilitate rapid genotyping of cellular clones.

Development of a rapid clinical diagnostic platform

Sickle cell disease is a life-threatening genetic disorder characterized by the presence of abnormal hemoglobin due to mutations in the hemoglobin subunit beta (*HBB*) gene. Early and accurate diagnosis is therefore crucial for initiating appropriate management and treatment strategies. To test whether One-pot DTECT can be used as a rapid point-of-care assay for the detection of disease-associated variants in clinical samples, a total of 21 patients and control individuals were enrolled, and venous peripheral blood samples (both blood extracts and dried spots) and saliva swabs were collected. The HgB A, S, or C alleles were captured using One-pot DTECT with independent adaptors complementary to the signatures (Figure 6A). Experimenters were blinded to subject genotypes. We extracted genomic DNA, amplified the *HBB* locus by AcuI-tagging PCRs, and conducted One-pot DTECT. Our streamlined protocol readily identified the various signatures and unambiguously distinguished affected individuals from carriers and controls with 100% accuracy (57/57 samples, 21/21 patients) and 0% false positives/negatives (0/57, 0/21) (Figure 6B). Moreover, we used One-pot DTECT-LAMP for real-time detection of the presence of each of the five different genotypes (AA, AC, AS, SC, and SS) visually (Figure 6C). Overall, One-pot DTECT holds significant potential as a rapid point-of-care technique for streamlining the diagnosis of genetic diseases in clinical samples, providing real-time and accurate results that can guide patient management and contribute to reducing the burden of genetic diseases.

DISCUSSION

Here, we present One-pot DTECT, a method designed for the rapid and facile detection of targeted dinucleotide signatures through various detection modalities, including qualitative, quantitative, and real-time visual detection. We demonstrate the high versatility and accuracy of One-pot DTECT across multiple applications, such as quantification of precision genome editing in cancer and primary cells, the genotyping of edited cell lines, and the detection of clinically relevant mutations from patient samples.

AcuI tagging allows for programmable signature exposure

One-pot DTECT harnesses the particular characteristic of AcuI to cleave DNA at a shifted but precise distance from its recognition motif[31] to create 3′ overhang dinucleotide signatures of unspecified sequences.[23] The disconnect between AcuI binding and activity has allowed us to tightly control its binding to DNA using uncleavable competitor DNA molecules (Figure 2J). We showed that, by inserting the short AcuI motif into AcuI-tagging oligonucleotides, it is possible to amplify loci of interest while programming the exposure and capture of any dinucleotides of interest using adaptors (Figure 1). While any type IIS endonuclease could theoretically be used in One-pot DTECT, only AcuI possesses high cleavage activity and specificity,[23,24] as AcuI is notable for its low slippage activity.[25] Moreover, AcuI's short and single recognition motif can be readily incorporated into the AcuI-tagging primers. Its capacity to generate short 3′ overhang signatures at a significant distance from the recognition motif (i.e., 14/16 nucleotides) allows locus amplification and AcuI tagging (Figure 1). In contrast, other type IIS

(C) Quantification of precision prime editing levels for the insertion of the seven indicated PCNA cancer mutations in HEK293T cells using NGS (purple), One-pot DTECT (blue), or Sanger sequencing (green). Error bars indicate SD values of two independent One-pot DTECT replicates. Individual data points are shown.
(D) Correlation between NGS (y axis), One-pot DTECT (x axis, blue), or Sanger sequencing (x axis, green) for the quantification of precision genome editing introduced by CBE, ABE, and PE (n = 23).
(E) Genotyping using One-pot DTECT-based analytical PCR of single clones carrying WT PCNA, or heterozygous or homozygous PCNA A96G.
(F) Sanger sequencing of WT, heterozygous, and homozygous PCNA mutants from (E).
(G) Real-time detection of WT PCNA alleles or A96G variant alleles introduced with prime editing using One-pot DTECT-LAMP.

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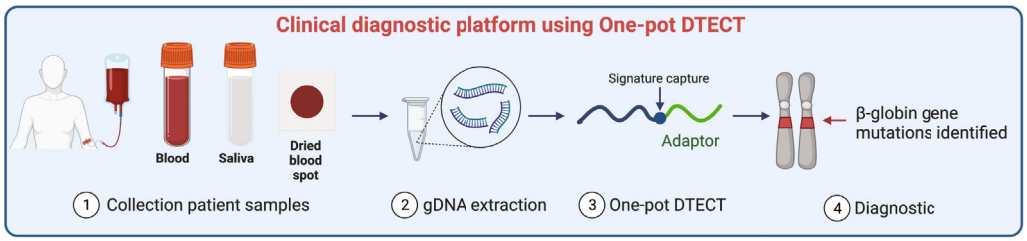

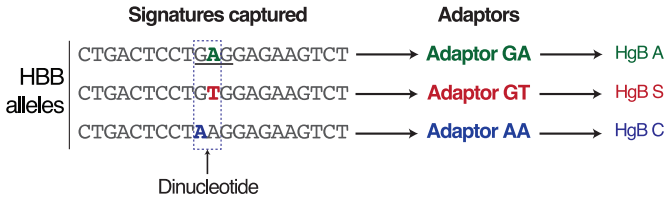

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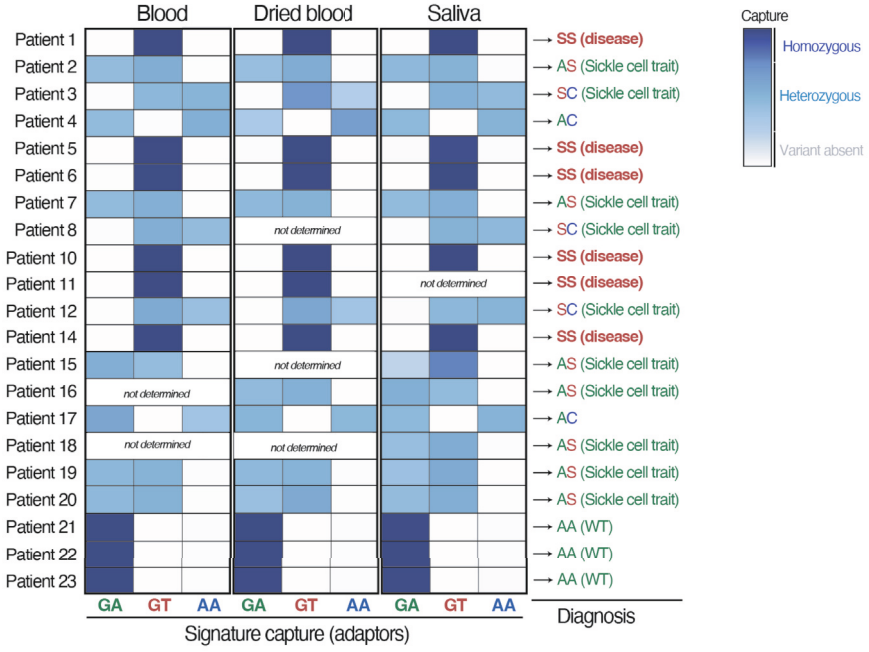

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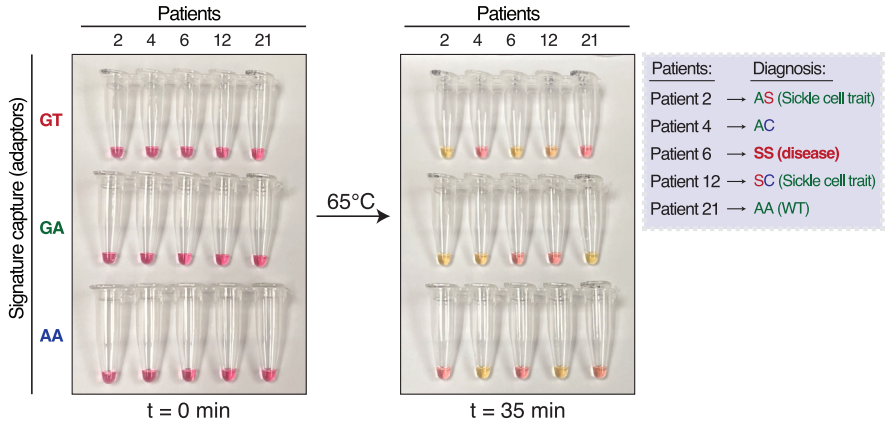

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endonucleases have low cleavage specificity and cleave DNA in close proximity to their recognition site(s),[24] making them difficult to harness for One-pot DTECT.

Strengths and comparison of One-pot DTECT

The strengths of One-pot DTECT enhance its reliability and specificity while simultaneously reducing technical variabilities both within and between experiments ensuring efficiency and reproducibility.

First, the quantification of ligated products is robust, as it only requires a unique pair of detection primers across all experiments, as illustrated in Figures 1C and S6. We demonstrate that specific amplification of the captured product is obtained by positioning the detection primer sequences within the adaptors and AcuI-tagging primers. This feature is a crucial advantage of One-pot DTECT, as it eliminates biases and technical variabilities due to variations in PCR efficiencies and locus or mutation specificities, which is typical of primer- and probe-based detection methods. This unique pair of qPCR primers maintains consistent efficiencies between experiments and captured signatures to help ensure maximal reproducibility and accuracy.

Second, One-pot DTECT is a ligation-based approach that generates a covalent phosphodiester bond between signatures and adaptors to generate a stable ligation product. This is in stark contrast to primer/probe-based approaches that rely exclusively on weak and transient nucleic acid interactions to produce the detection signal, as they inherently have significant variable efficiencies across genomic loci and mutations. Unlike One-pot DTECT, detection methodologies employing variant-specific primers or probes exhibit limited specificity in distinguishing the reference from the variants, as their specificity is typically conferred by a single-nucleotide change over the probe length of approximately 20 nucleotides (1/20 = 5% difference between reference and variant). Even Sanger sequencing relies on locus-specific primers to generate detection signals, which is frequently causing low specificity (high background) due to inefficient amplification or poor annealing.

Third, an inherent strength of One-pot DTECT is that it has built-in positive and negative controls in every experiment. Specific and nonspecific adaptors used with control samples provide positive (confirms One-pot DTECT worked) and negative (provides background) controls, respectively. The inclusion of these controls is key to validating the accuracy of the results of each experiment, including mitigating false positives and negatives.

Finally, a simple library with only 16 adaptors is sufficient for capturing every possible dinucleotide signature (Figure S6). Dinucleotides provide high-specificity capture as a single-nucleotide change provides a specificity of 50% (1/2 change per dinucleotide). In contrast, detecting 4- or 6-nucleotide overhangs require exponentially larger libraries (256 and 1,296 unique adaptors, respectively) and offer lower specificity with only 25% or 16.7% specificity (1/4 or 1/6 change, respectively). Importantly, we show that adaptors exhibit similar capture efficiencies, making One-pot DTECT a robust and accurate capture-based detection approach (Figure 4). Moreover, the library of adaptors is highly adaptable as demonstrated by the integration of validated LAMP sequences to accommodate novel detection modalities. We envision that future modifications to the adaptors will broaden the landscape of the applications for One-pot DTECT. For instance, incorporating fluorophores and quenchers could potentially pave the way for the development of real-time-sensitive fluorescence-based detection via ligation-mediated signal quenching. However, our preliminary attempts have not yet been successful. Future refinement will require optimizations in signal detection sensitivity and lengths of the adaptors.

Compared with traditional detection methods, One-pot DTECT provides several advantages. Its rapidity, cost-effectiveness, and simplicity stand in stark contrast to sequencing-based strategies, which can be expensive and rely on external entities for sample processing and analysis. Our direct comparisons between One-pot DTECT and both NGS and Sanger sequencing revealed striking accuracy in the quantification of targeted signatures (Figures 5D and S5B). Moreover, One-pot DTECT readily detects the genotypes of cell clones (Figures 5E and 5G), which shows its versatility for routine laboratory applications. CRISPR-based diagnostic tools offer simple, rapid, and highly sensitive methods for nucleic acid detection.[35–38] However, the specificity and sensitivity of CRISPR-based diagnostic tools are impacted by the target sequences,[39] introducing significant complexity to the diagnostic process. Moreover, these approaches are limited to qualitative capabilities. In contrast, One-pot DTECT combines quantitative, qualitative, and visual detection capabilities, thereby offering a broader diagnostic scope, as exemplified by the successful detection of multiple pathogenic variants and accurately determined their ploidy (Figure 6).

Limitations of One-pot DTECT

The limitations of One-pot DTECT are primarily related to its restriction to dinucleotides and the juxtaposition of the AcuI-tagging primer with the dinucleotide of interest.

First, the capture is confined to a dinucleotide window, which can hinder the capture of multiple independent variants occurring at a locus. This limitation potentially impacts detection of base editing for which multiple nucleotides can be edited in a window larger than 2 nucleotides. Nevertheless, most relevant pathogenic mutations identified in humans are single-nucleotide changes, which can be efficiently captured using the two-nucleotide window of One-pot DTECT. Moreover, One-pot DTECT can capture complex signatures within the dinucleotide, as

Figure 6. One-pot DTECT, a clinical diagnostic platform for the detection of genetic variants

(A) Schematic representation of the experiments conducted on sickle cell patient samples. Blood, saliva, and dried blood samples were collected from patients, carriers, and control individuals. Genomic DNA was prepared blindly and tested for the presence of the HgB, HgB S, and HgB C alleles using One-pot DTECT using the GA, GT, or AA adaptors, respectively.

(B) Heatmap showing the detection of sickle cell traits and disease alleles from patient samples using One-pot DTECT.

(C) Real-time detection of the respective sickle cell alleles using One-pot DTECT-LAMP.

exemplified by the detection of sickle cell disease signatures (Figure 6A).

A second limitation is due to the proximity of the AcuI-tagging oligonucleotide to the dinucleotide signature of interest. Indeed, the potential occurrence of bystander editing in the context of base editing can impact the measured efficiency of One-pot DTECT as they may interfere with the binding of the AcuI-tagging primer. However, bystander editing outside the activity window is generally marginal, and recent advancements with base editors exhibiting narrower activity windows[1] have lessened the occurrence of bystander editing.

Finally, similar to Sanger sequencing, One-pot DTECT is unable to detect low-frequency editing byproducts (below 1%), a background determined by ligating variant-specific adaptors to control samples (Figure S5C), thus necessitating the use of NGS when low-frequency byproducts need to be identified. Nevertheless, one of the strengths of One-pot DTECT is that the detection background is always determined in each experiment by capturing the variant signature in control samples (WT/nonedited), preventing false positives and negatives. Despite this caveat, One-pot DTECT can be used as a complementary approach for identifying desired mutations prior to more comprehensive NGS analysis for the detection of lower-frequency edits and off-window edits.

Potential clinical applications of One-pot DTECT

Screening for sickle cell disease typically requires initial high-performance liquid chromatography on dried blood spot samples to detect potential pathogenic variants and subsequent molecular validation through venipuncture in newborns,[40] which incurs additional costs and extends turnaround time that often spans weeks, if not months. Unlike these approaches, One-pot DTECT has demonstrated its high accuracy in detecting multiple pathogenic signatures across diverse, non-invasive, inexpensive, and easily collectible clinical samples, such as blood spots and saliva swabs, in the same day. Notably, we showed that One-pot DTECT can be performed on the initial newborn screen blood spot rather than requiring venipuncture for confirmatory testing. Moreover, One-pot DTECT distinguished between homozygous (pathogenic) and heterozygous (carrier) sickle cell states while also detecting the HgbC variant[41] using three different adaptors from a unique AcuI PCR. This suggests that One-pot DTECT can streamline clinical diagnostics for genetic diseases associated with founder mutations and has the potential to be expanded to mutations in *CD3δ*, *ADA*, or *ZAP70*, which are known to harbor highly prevalent pathogenic mutations in specific populations. The robustness and demonstrated efficiency of One-pot DTECT enhance its potential as a valuable tool for routine clinical diagnostics of genetic diseases, especially in resource-limited settings, despite the need for specialized equipment, such as a thermocycler and qPCR, and basic molecular biology skills.

In summary, One-pot DTECT emerges as a robust and versatile method addressing the current lack of homemade, reliable, and user-friendly assays for the detection of genetic variants for routine laboratory experiments, including precision genome editing, genotyping, and clinical diagnostics. This platform provides a rapid and cost-effective methodology for the accurate detection of genetic variants of interest. The efficiency, simplicity, and unparalleled autonomy offered by One-pot DTECT, coupled with the use of a simple predesigned adaptor library and a unique set of detection primers, promise to expedite the detection of genetic variants of interest. Consequently, the development of One-pot DTECT promotes replacing the traditional practice of outsourcing the detection of genetic variants of interest to genomic companies/platforms with laboratory-based detection through a homemade detection kit (Figure S6).

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2024.100698.

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AUTHOR CONTRIBUTIONS

L.B., O.B., and P.B. conceived One-pot DTECT and designed the experiments. L.B., O.B., J.L., and S.C. performed the DTECT optimizations,

detection experiments, and genome-editing experiments under the supervision of P.B. S.B. conducted editing in T cells under the supervision of D.J.M. T.K. and G.S. recruited SCD patients and collected blood and saliva samples under the supervision of N.A.M.W. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

L.B., O.B., and P.B. filed patents related to the development of One-pot DTECT.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Peripheral blood samples	This paper	N/A
Dried blood spots	This paper	N/A
Saliva samples	This paper	N/A
Chemicals, peptides, and recombinant proteins		
T4 DNA ligase	Invitrogen	Cat#15224025
AcuI	NEB	Cat#R0641
Power SYBR Green PCR Master Mix	Applied Biosystems	Cat#4367659
Q5 High-Fidelity DNA Polymerase	NEB	Cat#M0491
LAMP	NEB	Cat#M1800
TransIT-293	Mirus Bio	Cat#MIR 2700
Quick extract DNA extraction solution	LGC Biosearch Technologies	Cat#QE09050
JetPRIME	Polyplus	Cat#101000046
PEG-it	System BioSciences	Cat#LV825A-1
TransAct beads	Miltenyi Biotec	Cat#130-128-758
IL-7	Biolegend	Cat#581904
IL-5	Biolegend	Cat#570304
Fetal bovine growth serum	RMBIO	Cat#FGR-BBT
RetroNectin	Takara	Cat#T100 A/B
SYBR Gold Nucleic Acid Gel Stain	Invitrogen	Cat#S11494
Critical commercial assays		
pCR-Blunt II TOPO vector	Life technologies	Cat#450245
DNeasy Blood and Tissue	Qiagen	Cat#69504
Agencourt AMPure XP magnetic beads	Beckman Coulter	Cat#A63881
Zymoclean gel DNA recovery kit	Zymo Research	Cat#D4008
Deposited data		
Next-generation sequencing	This paper	PRJNA992096 (NCBI)
Unprocessed images of gels	This paper	https://doi.org/10.17632/dwp2nnfgtf.1
Raw qPCR data and quantification	This paper	https://doi.org/10.17632/dwp2nnfgft.1
Experimental models: Cell lines		
HEK293T cells	ATCC	CRL-11268
Human primary T-cells	This paper	N/A
Lenti-X 293 cell line	Takara	Cat#632180
Oligonucleotides		
Primers for PCR	This paper	https://doi.org/10.17632/dwp2nnfgft.1
Oligonucleotides for gRNA/pegRNA cloning	This paper	https://doi.org/10.17632/dwp2nnfgft.1
Oligonucleotides for One-pot DTECT and One-pot DTECT-LAMP	This paper	https://doi.org/10.17632/dwp2nnfgft.1
Other		
QuantStudio 6 Flex Real-Time PCR System	Applied Biosystems	Cat#4484642
ProFlex 3x32-well PCR Sysstem	Applied Biosystems	Cat#4484073
ChemiDoc Touch Gel Imaging System	Bio-Rad	Cat#1708370
12-Tube Magnetic Separation Rack	NEB	Cat#S1509

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pierre Billon (pierre.billon@ucalgary.ca).

Materials availability

All materials will be made available by lead contact, Pierre Billon (pierre.billon@ucalgary.ca) upon request.

Data and code availability

Raw qPCR data and list of oligonucleotides/primers are available in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1). A point-by-point protocol to produce homemade One-pot DTECT is available on Mendeley (https://doi.org/10.17632/dwp2nnfgft.1). Sequencing data have been deposited at the Single Read Archive (SRA) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human cell culture

T-cells were isolated from a healthy 36-year-old male donor. Isolation was approved by the Health Research Ethics Board of Alberta (HREBA.CC-16-0762). Human T cells were expanded and cultured in ImmunoCult-XF medium (Stemcell; 10981), supplemented with 10 mg/mL IL-7 (Biolegend; 581904), and 10 mg/mL IL-15 (Biolegend; 570304). T cells were activated with TransAct beads (Miltenyi Biotec; 130-128-758). HEK293T cells were obtained from ATCC and cultured in DMEM (Thermo Fisher) containing 10% Fetal bovine growth serum (RMBIO) and antibiotics.

Clinical samples

Peripheral blood, dried blood spots and saliva samples were collected from subjects recruited through the Alberta Children's Hospital Hemoglobinopathy Clinic, Calgary, Alberta. Siblings and parents of patients with sickle cell disease were recruited as heterozygous sickle cell disease carriers. Informed consent, and assent if applicable, was obtained. The study was approved by the research ethics board (REB21-0375) of the University of Calgary.

Genomic DNA extraction of clinical samples was conducted blinded using the Qiagen DNeasy Blood and Tissue kit according to the manufacturer's protocol.

To recover dried blood, Whatman papers were placed in 1.5 mL microcentrifuge tubes, and 180 μL of ATL buffer was added and incubated at 90°C for 15 min. After incubation, 20 μL of proteinase K solution was added and incubated for 1 h at 56°C. The solution was then collected, and 200 μL of buffer AL was added and incubated for 10 min at 56°C. Finally, genomic DNA was extracted by ethanol precipitation and column purification following the manufacturer's recommendations.

Extraction of genomic DNA from saliva was performed by mixing 1 mL of saliva with 1 mL of PBS and centrifuged at 1,800 x g for 5 min. The cell pellet was resuspended in 180 μL of PBS. Then, 25 μL of proteinase K and 200 μL of AL buffer were added, and the mixture was vortexed and incubated at 56°C for 10 min. Finally, genomic DNA was extracted by column purification according to the manufacturer's recommendations.

Extraction of genomic DNA from anticoagulated blood was performed by mixing 10 μL of anticoagulated blood with 20 μL of proteinase K solution in 190 μL of PBS and incubated at 56°C for 10 min. Then, 220 μL of AL buffer was added and incubated at 56°C for 10 min. Finally, genomic DNA was extracted by column purification according to the manufacturer's recommendations.

All recovered genomic DNA was quantified by nanodrop and stored at −20°C.

METHOD DETAILS

Tissue culture and single clone isolation

T cell isolation from healthy donors was approved by the Health Research Ethics Board of Alberta (HREBA.CC-16-0762). Human T cells were expanded and cultured in ImmunoCult-XF medium (Stemcell; 10981), supplemented with 10 mg/mL IL-7 (Biolegend; 581904), and 10 mg/mL IL-15 (Biolegend; 570304). T cells were activated with TransAct beads (Miltenyi Biotec; 130-128-758). HEK293T cells were obtained from ATCC and cultured in DMEM (Thermo Fisher) containing 10% Fetal bovine growth serum (RMBIO) and antibiotics.

Single clones were isolated by trypsinization of the initial cell population to separate cells into individual cells. Individualization of cells was closely monitored under the microscope. The cell density was subsequently measured by a cell counter, and the cells were diluted to a concentration of approximately 0.13 cells/μl which is equivalent to 20 cells per 150 μL. 5-fold serial dilutions were

prepared, and 150 μL of the diluted cell mixture was seeded into 96-well plates. Single clones were expanded and genotyped using One-pot DTECT, and Sanger sequencing was used to confirm.

Synthetic DNA, molecular cloning, and plasmids

Synthetic DNA molecules were designed to include specific portions of the SARS-CoV-2 genome, both with or without designated mutations. These were purchased as gBLOCK DNA fragments (IDT). The DNA fragments were resuspended in TE (10 mM Tris and 0.5 mM EDTA) buffer, TOPO-cloned into the pCR-Blunt II-TOPO vector (ThermoFisher Scientific), and transformed into DH5α. Successful cloning and SARS-CoV-2 sequences were confirmed using Sanger sequencing.

For the sequencing of the ligation product (Figure S2D), the ligation product was cloned into the pCR-Blunt II-TOPO vector (Thermo Fisher Scientific), transformed into DH5α and sequenced by Sanger sequencing.

Previously published plasmids were used in this work: pCMV-PEmax was a gift from David Liu (Addgene plasmid # 174820; http://n2t.net/addgene:174820; RRID:Addgene_174820), SpCas9 TadCBEd was a gift from David Liu (Addgene plasmid # 193835; http://n2t.net/addgene:193835; RRID:Addgene_193835), pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454; http://n2t.net/addgene:8454; RRID:Addgene_8454), psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260; http://n2t.net/addgene:12260; RRID:Addgene_12260), pBS-CMV-gagpol was a gift from Patrick Salmon (Addgene plasmid # 35614; http://n2t.net/addgene:35614; RRID:Addgene_35614), pCAG-CBE4max-SpRY-P2A-EGFP (RTW5133) was a gift from Benjamin Kleinstiver (Addgene plasmid # 139999; http://n2t.net/addgene:139999; RRID:Addgene_139999), DTECT - Plasmid for Standard Curve was a gift from Alberto Ciccia (Addgene plasmid # 139333; http://n2t.net/addgene:139333; RRID:Addgene_139333), and pCMV-MMLVgag-3xNES-ABE8e was a gift from David Liu (Addgene plasmid # 181751; http://n2t.net/addgene:181751; RRID:Addgene_181751).

gRNA/pegRNA cloning, precision genome editing and genomic DNA extraction

The cloning of gRNAs and pegRNAs was conducted as previously described[8,24] using golden gate cloning of annealed oligonucleotide and digested plasmids. Oligonucleotides used to clone gRNAs/pegRNAs are available in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1).

Editing was performed by seeding 55,000 HEK293T cells into 48-well plates 24 h prior to transfection. Cells were transfected using TransIT-293 (Mirus) according to the supplier's recommendations. In brief, 500 ng of editor-expressing plasmid and 100 ng of gRNA-expressing plasmid were mixed with 1 μL of transIT-293. The mixture was incubated for 20 min at room temperature before being added dropwise to the cells. Editing levels were measured 72 h-post transfection using One-pot DTECT, NGS or Sanger sequencing.

The genomic DNA of cell populations and individual clones was recovered by resuspending the cell pellets in Quick Extract DNA Extraction Solution (Lucigen), and incubated at 65°C for 6 min followed by a 95°C incubation for 5 min. The isolated gDNAs were diluted in water, quantified using the nanodrop and used directly in PCR reactions, or stored at −20°C.

Viral-like particle production and T cell transduction

Engineered viral-like particles were produced by transfecting producer lenti-X 293T cells, as recently described.[42] Lenti-X 293T cells were seeded in 75 cm flasks at a density of 5 million cells per flask 24 h before transfection. Cells were transfected using jetPRIME (Polyplus) by mixing 400 ng of VSV-G, 3.375 μg MLVgag-pro-pol, 1.125 μg MLVgag-ABE8e and 4.4 μg of gRNA-expressing plasmid. Media was changed 12 h post transfection. Ninety-two hours post-transfection, the supernatant of producer cells was harvested and centrifuged at 500 g for 5 min to remove cellular debris, and then filtered through a 0.45 μm PVDF filter. The filtered supernatant was then concentrated 100x using PEG-it according to the manufacturer's recommendations. To recover more particles, producer cells were replated into 2 × 75 cm flasks, and particles were harvested two days later.

T cells were transduced at a density of 50,000 cells in 50 μL T cell medium by adding 5 μL of concentrated ABE-VLP. Cells were transduced three times separated by 24 h as follows: T cells were plated before each transduction cycle in a 96-well plate precoated with RetroNectin (Takara; T100 A/B) at 10 μg/cm^2. Activation beads were removed after completion of the second infection. For each transduction, cells were spinoculated at 800 x g for 90 min at 32°C. T cells were harvested 6 days after the last transduction, and genomic DNA was isolated as described above.

Preparation of the library of adaptors

A unique library of 16 adaptors was used for the capture of all 16 dinucleotide signatures with One-pot DTECT. The library comprises 16 double-stranded DNA adaptors generated from 17 individual oligonucleotides (sequences available in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1)), which consist of 1 constant oligonucleotide and 16 individual oligonucleotides for each dinucleotide signature. The constant oligonucleotide contains the sequence 5′-GCAATTCCTCACGAGACCCGTCCTG-3′, which enables the detection of the ligated products. The 16 individual oligonucleotides consist of the sequence complementary to the constant oligonucleotide and one of the 16 different dinucleotides at their 3′ end (available in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1)).

Each oligonucleotide was resuspended at a concentration of 100 μM in TE buffer. To generate the double-stranded adaptors, oligonucleotides were annealed. Annealing reactions consisted of 2.5 μL of the constant oligonucleotide and 2.5 μL of each unique dinucleotide oligonucleotide in 1X ligase buffer in a 20 μL reaction. Reactions were incubated for 5 min at 95°C to remove any

potential secondary structures, followed by a gradual temperature decrease from 95°C to 15 °C at a ramp rate of 0.5 °C/s. Then, 100 μL H_2O was added to dilute the adaptors, which were stored at −20°C.

Design of AcuI-tagging primers and PCR amplification

AcuI-tagging PCR utilizes a pair of primers named "AcuI-tagging primer" and "reverse primer" (sequences available in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1)). AcuI-tagging PCRs serve to amplify the locus of interest, insert the motif recognized by AcuI 14 bp from a targeted dinucleotide, and introduce a handle that is used for detection (Figure 1A).

The AcuI-tagging primers are 60 nt long with an AcuI motif (5′-CTGAAG-3′) inserted 14 bp from the 3′ end of the primer. The 5′ end contains a detection handle sequence (5′-GCAATTCCTCACGAGACCCGTCCTG-3′) that is used for detection. Therefore, the AcuI-tagging primer has the following architecture: 5′- GCAATTCCTCACGAGACCCGTCCTG N(15)CTGAAGN(14)-3′ with "N" corresponding to A, T, G, or C bases complementary to the targeted locus.

The reverse primer is a standard locus-specific oligonucleotide that is designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) with the following parameters: length of "min = 25, Opt = 27, Max = 30" and a Tm of "min = 57.0°C, opt = 60.0°C, max = 63.0°C"

The AcuI-tagging PCR was conducted in a 25 μL with 1 unit of Q5 polymerase, 1X Q5 buffer, 1 μM of each primer, 10 ng of plasmid or 1–100 ng of genomic DNA, 0.1 mM dNTP in a thermocycler: 95°C for 30 s; 40 cycles of 95°C for 10 s, 58°C for 10 s, 72°C for 45 s and a final amplification at 72°C for 1 min. The PCR mixture was then loaded on a 2% agarose gel in TAE buffer, and the amplicon was extracted from the gel and column purified (Zymo Research #D4008). The purified AcuI-tagged amplicon was quantified with a nanodrop and stored at −20°C.

DTECTv1 protocol

The DTECTv1 protocol was conducted as detailed previously.[23,24] Briefly, the genomic locus of interest was amplified using an AcuI-tagging primer and a reverse primer. The purified AcuI-tagged amplicon was digested by AcuI in a 20 μL reaction as follows: 0.2 pmol AcuI-tagged amplicon and 1.25 units AcuI (NEB #R0641) in 1X CutSmart buffer. The digestion was incubated at 37°C for 1 h followed by heat inactivation at 65°C for 20 min. 10 μL of the digestion reaction was mixed with 18 μL of SPRI beads by pipetting up and down ten times and incubated at room temperature for 5 min. The tubes were then placed on a magnetic rack for 5 min, and the supernatant was recovered and diluted in 40 μL H_2O. Next, ligation of the adaptors was performed in the following reaction: 6.5 μL of H_2O, 2 μL of 5X ligase buffer, 0.5 μL of T4 ligase, 0.5 μL of adaptor, and 0.5 μL of the isolated digested product. The ligation reaction was incubated for 1 h at 25°C in a thermocycler and stopped by incubating the reaction at 65°C for 10 min to denature the ligase. The captured material was detected using either quantitative PCR or analytical PCR, as described below.

DTECTv2 protocol

The DTECTv2 protocol relies on DTECTv1 but merges multiple optimizations. The durations of digestion and heat inactivation have been shortened to 1 min at 37°C and 1 min at 65°C, respectively. The digested reaction was diluted by the addition of 100 μL H_2O and used directly for ligation. The adaptor ligation was conducted in a volume of 10 μL by mixing 2 μL of ligase buffer, 0.5 μL of T4 ligase (Invitrogen), 0.5 μL of the selected adaptor, and 0.5 μL of diluted digestion. Reactions were incubated for 10 min at 25°C and stopped by incubating for 1 min at 65°C. Finally, analytical or quantitative PCRs was performed as detailed below.

One-pot DTECT

One-pot DTECT requires AcuI, T4 ligase as described in our point-by-point protocol and a list of oligonucleotides available in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1) to prepare the library of adaptors and competitor DNA fragments. The recipe to prepare 100 μL of 2X One-pot DTECT master mix is as follows: 40 μL ligase buffer, 10 μL T4 ligase, 1 μL AcuI, and 20 μL competitor DNA (from 1 μM stock) completed with H_2O. To perform One-pot DTECT, a 5 μL reaction was prepared by mixing 2.5 μL of 2X One-pot DTECT master mix, 0.25 μL of selected adaptor, and either 1 μL of 1/100th-diluted AcuI tagged PCR or 2.5 fmol of purified AcuI tagged PCR. The One-pot DTECT reaction was incubated for 10 min to 1 h at 25°C and 1 min at 65°C before analysis by qPCR, analytical PCR or LAMP.

The double-stranded competitor DNA fragments were generated by the annealing of complementary oligonucleotides. The two complementary oligonucleotides are 30 nt long with an AcuI motif containing 12 randomly-selected nucleotides at the 5′ and 3′ ends of the oligonucleotides. The structure of the oligonucleotides is 5′-N(12)CTGAAGN(12)-3′, with "N" corresponding to randomly selected A, T, G, or C bases. The sequences of the competitor DNA used in this paper are 5′-AGCCTGTGGTTCCTGAAGATCG CGTCCGAT-3′ and 5′-ATCGGACGCGATCTTCAGGAACCACAGGCT-3'. The oligonucleotides were resuspended at a concentration of 100 μM in TE buffer or water. The annealing reactions consisted of 1 μL of each oligonucleotide in 1X ligase buffer. The reactions were incubated for 5 min at 95°C to remove any potential secondary structures, followed by a gradual temperature decrease from 95°C to 15 °C at a ramp rate of 0.5 °C/s. Then, the competitor stock was diluted to 1 μM before being added to the 2X One-pot DTECT master mix.

To quantify the amount of captured products in v1, v2 and One-pot DTECT, qPCRs were conducted using QuantStudio 6 (Applied Biosystems). qPCRs were performed as follows: 5 μL of 2X SYBR Green master mix, 0.1 μL of each detection primers (100 μM) and

1 μL of ligated products in a 10 μL reaction. The qPCR program was as follows: 1) a hold stage of 1 cycle at 50.0°C for 2 min and 95.0°C for 10 min 2) A PCR stage of 40 cycles at 95°C for 10 s and 60°C for 30 s. 3) A melt curve stage of 1 cycle of incubations at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s.

Analytical PCR detection was performed by standard PCR in a 12.5 μL containing 0.1 μL Q5 polymerase, 1X Q5 buffer, 0.25 μL of each detection oligonucleotides, 0.05 mM dNTP, and 1 μL ligation products. The PCR program for the analytical reaction was as follows: 95°C for 1 min and 22 cycles of 95°C for 10 s, 65°C for 5 s and 72°C for 7 s. PCRs were incubated with SYBR Gold, loading dye, and loaded on a 2% agarose gel with TAE buffer.

To determine the accuracy of the One-pot DTECT capture, each adaptor was ligated to annealed oligonucleotides containing complementary dinucleotides. To mimic the 5′ phosphorylation induced by AcuI in One-pot DTECT experiments, the reverse oligonucleotide was initially phosphorylated by PNK (NEB) in a 20 μL reaction as follows: 5 μL of reverse oligonucleotide, 4 μL of 5X ligase buffer, and 0.5 μL of PNK. The reaction was incubated for 1 h at 37°C, followed by PNK inactivation for 20 min at 65°C. Upon completion of the phosphorylation reaction, 40 μL of 5X ligase buffer and 130 μL of H_2O were added to the reaction to dilute the oligonucleotide. Then, the diluted and phosphorylated oligonucleotide was annealed to 16 complementary oligonucleotides in a 10 μL reaction, as follows: 9.5 μL of the diluted-phosphorylated oligonucleotide and 0.5 μL of each of the 16 oligonucleotides (50 μM). Annealing was conducted by incubating for 5 min at 95°C to remove any potential secondary structures followed by a gradual temperature decrease from 95°C to 15 °C at a ramp rate of 0.5 °C/s. This resulted in the generation of double-stranded DNA with an overhang of 2 nucleotides, mimicking the product of AcuI digestion. The ligation between the adaptors and the phosphorylated products was performed as follows: 1 μL of annealed oligonucleotides, 1 μL CutSmart buffer, 1 μL competitor DNA (1μM), 0.5 μL of T4 ligase and 0.5 μL of the complementary adaptor in a 10 μL reaction. The one-pot reaction was incubated for 1 h at 25°C and 1 min at 65°C. Quantification was performed using qPCR as described above.

One-pot DTECT-LAMP

The library of adaptors for DTECT-LAMP is composed of 16 double-stranded DNA adaptors generated from 17 individual oligonucleotides, which consist of 1 constant oligonucleotide and 16 individual oligonucleotides for each dinucleotide signature. Two versions of these adaptors have been designed that contain the geneN or ORF1 LAMP sequences that were used for SARS-CoV-2 detection, as listed in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1). The 16 individual oligonucleotides are composed of the sequence complementary to the constant oligonucleotide and one of the 16 different dinucleotides at their 3′ end.

Each oligonucleotide was resuspended at a concentration of 10 μM in TE buffer. Annealing reactions consisted of 1 μL of the constant oligonucleotide and 1 μL of each unique dinucleotide oligonucleotide in a 5 μL reaction with H_2O. The reactions were incubated for 5 min at 95°C to remove any potential secondary structures, followed by a gradual temperature decrease from 95°C to 15 °C at a ramp rate of 0.5 °C/s. Then, annealed LAMP adaptors were diluted 1/50th in H_2O and stored at −20°C.

A pool composed of the F3, FIP, B3, BIP and LB LAMP detection primers (100 μM stock) was prepared by mixing 4 μL F3, 32 μL FIP, 4 μL B3, 32 μL BIP and 8 μL LB in a 100 μL tube, brought up to volume with H_2O.

One-pot DTECT-LAMP was conducted by an initial One-pot DTECT using One-pot DTECT-LAMP-specific adaptors to capture signatures of interest, as described above, followed by a LAMP reaction. LAMP reactions were set up in a volume of 10 μL by mixing 1.6 μL betaine (5 M stock), 5 μL 2X LAMP (NEB), 2.5 μL DTECT (diluted 1/1,000th) and 0.5 μL of oligo pool. The LAMP reaction was incubated at 65°C, and pictures were taken at the indicated times.

Measurement of the absorbance was conducted using the Spectra Max iD3 plate reader with 384-well plates with the following parameters: standard opaque 17.5 mm height, wavelengths 415 nm and 560 nm. The absorbance was measured every 30 s for a total duration of 2 h.

Sanger and next-generation sequencing

Samples for Sanger sequencing were prepared by amplification of the loci using regular PCR primers designed using Primer3 (https://primer3.ut.ee/). An aliquot of the PCR was run on an agarose gel to confirm the specificity and the size of the product. Once confirmed, PCR products were purified by commercial column extraction.

Samples for NGS were prepared by dual PCR. PCR1 was used to amplify the genomic loci of interest using locus-specific oligonucleotides, and PCR2 added the indexes and Illumina sequences. A total of 1–200 ng of isolated genomic DNA was used as a template for PCR1 with a total of 12 cycles. 1 μL of PCR1 was used as a template for PCR2 run for 25 cycles. Successful amplification was monitored on a 2% agarose gel, and samples were purified from the gel. Samples were sequenced at the Donnelly Sequencing Center (Toronto) on the MiSeq instrument (paired end 2x250 reads) or by Amplicon-EZ by Genewiz. The results were analyzed using a published homemade R-based script.[24] Oligonucleotides used for PCR amplification with indexes and barcodes are listed in Mendeley (https://doi.org/10.17632/dwp2nnfgft.1).

QUANTIFICATION AND STATISTICAL ANALYSIS

A standard curve to determine the efficiency of the qPCR amplification and the linearity of the amplification was generated with a plasmid that contains a DTECT ligation product (Addgene #139333) using detection primers. The linearity of the standard curve has the mathematical formula y = −3.3245x + 7.5504, as previously determined.[24]

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Each sample analyzed by qPCR was tested in technical duplicates, and the mean Ct for each sample was calculated. The capture is defined as the concentration of the captured material (in picogram of ligated products) for each sample multiplied by 10^6. It is measured as follows: Capture efficiency = 10^((Mean Ct – 7.5504)/-3.3245).

We assessed the performance of DTECT by quantifying ligation efficiency (referred to as *capture efficiency*), which corresponds to the amount (in picogram) of ligated DNA (Figure S2A), and ligation specificity (referred to as *capture specificity*), which corresponds to the difference in cycle threshold (Ct) between specific dinucleotide signature capture and background capture using a nonspecific adaptor (Figure S2B). The capture specificity was calculated using the formula: ''ABS(Ct specific adaptor - Ct nonspecific adaptor)''.

Sample sizes are described in the figures and figure legends.