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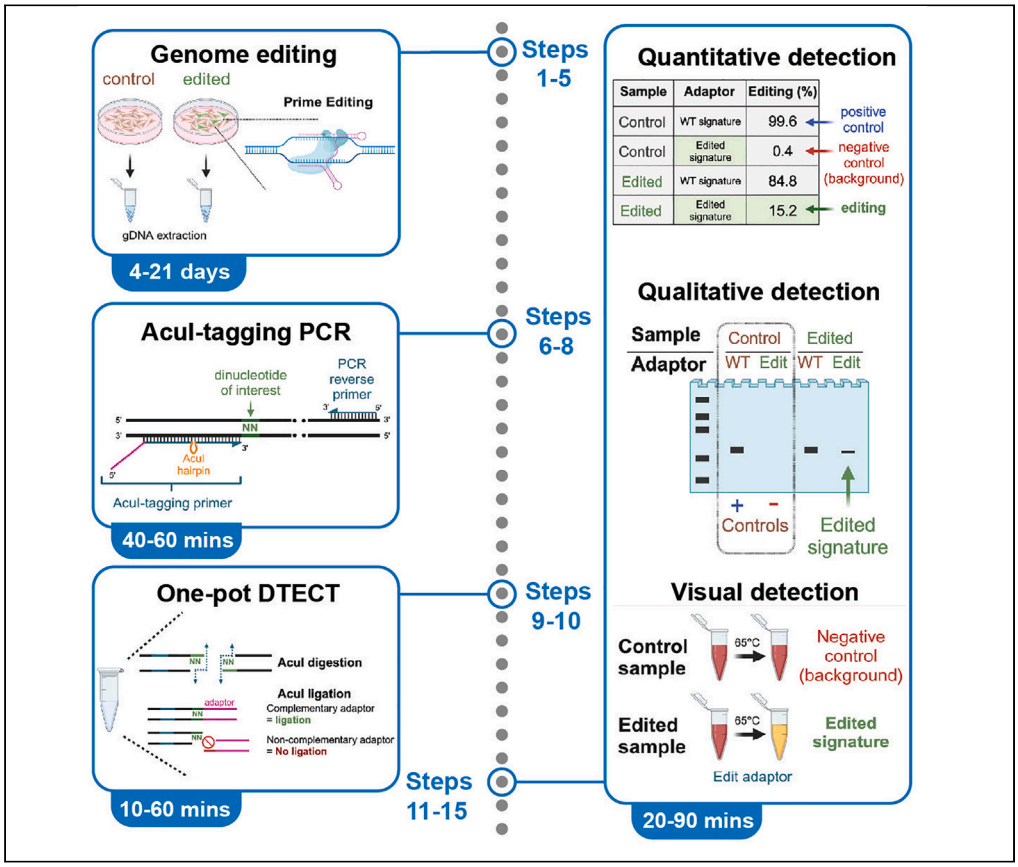
A protocol for the detection of precision genome editing in human cells using One-pot DTECT



Prime editing is a highly versatile CRISPR-based genome editing technology that allows for the precise installation of desired genetic variants. This protocol describes how to use One-pot DTECT to assess prime editing efficiency in human cells. Key steps include conducting prime editing, extracting genomic DNA, performing Acul-tagging PCR, capturing genetic signatures, and detecting captured signatures through qualitative, quantitative, and visual methods. One-pot DTECT enables same-day detection of targeted genetic signatures introduced by precision genome editing technologies using off-the-shelf reagents.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Perform prime editing in human cells

Prepare a homemade One-pot DTECT kit combining multiple enzymes

Conduct Acul-tagging PCRs to amplify and tag targeted genetic signatures

Detect prime editing signatures using quantitative, qualitative, and visual detection

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A protocol for the detection of precision genome editing in human cells using One-pot DTECT

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SUMMARY

Prime editing is a highly versatile CRISPR-based genome editing technology that allows for the precise installation of desired genetic variants. This protocol describes how to use One-pot DTECT to assess prime editing efficiency in human cells. Key steps include conducting prime editing, extracting genomic DNA, performing AcuI-tagging PCR, capturing genetic signatures, and detecting captured signatures through qualitative, quantitative, and visual methods. One-pot DTECT enables same-day detection of targeted genetic signatures introduced by precision genome editing technologies using off-the-shelf reagents.

For complete details on the use and execution of this protocol, please refer to Baudrier et al.[1]

BEFORE YOU BEGIN

Prime editing is a CRISPR-Cas9-based system that employs a reverse transcriptase and a prime editing guide RNA (pegRNA) to install precise genomic changes. Prime editing is a highly versatile tool for basic research and a promising therapeutic tool for the correction of genetic diseases.[2] Traditional methods for assessing precision genome editing outcomes or genotyping samples typically rely on external genomic services, leading to delayed turnaround times and increased costs.

To address these challenges, we developed One-pot DTECT,[1] a homemade detection method that uses readily available reagents. One-pot DTECT offers an efficient and versatile method for capturing targeted genetic signatures through a one-pot reaction and a simple library of universal adaptors. This approach reduces dependency on specialized genomic companies, thereby accelerating detection workflows, and reducing costs.

In this protocol, we describe the design and implementation of a homemade One-pot DTECT detection kit in the laboratory. This detection kit combines a restriction enzyme, AcuI, that exposes targeted dinucleotide signatures, and a DNA ligase that ligates universal DNA adaptors to the digested amplicons to enable detection. We present three independent detection strategies of the genetic variants: quantitative, qualitative, or visual detection. Here, we provide a step-by-step One-pot DTECT protocol for detecting precise edits introduced in human cells using prime editing (Figure 1).

Although this protocol details the specific steps for detecting genetic variants introduced by prime editing in the *PCNA* gene in HEK293T cells, it is also applicable to other genes, mutation types, cell

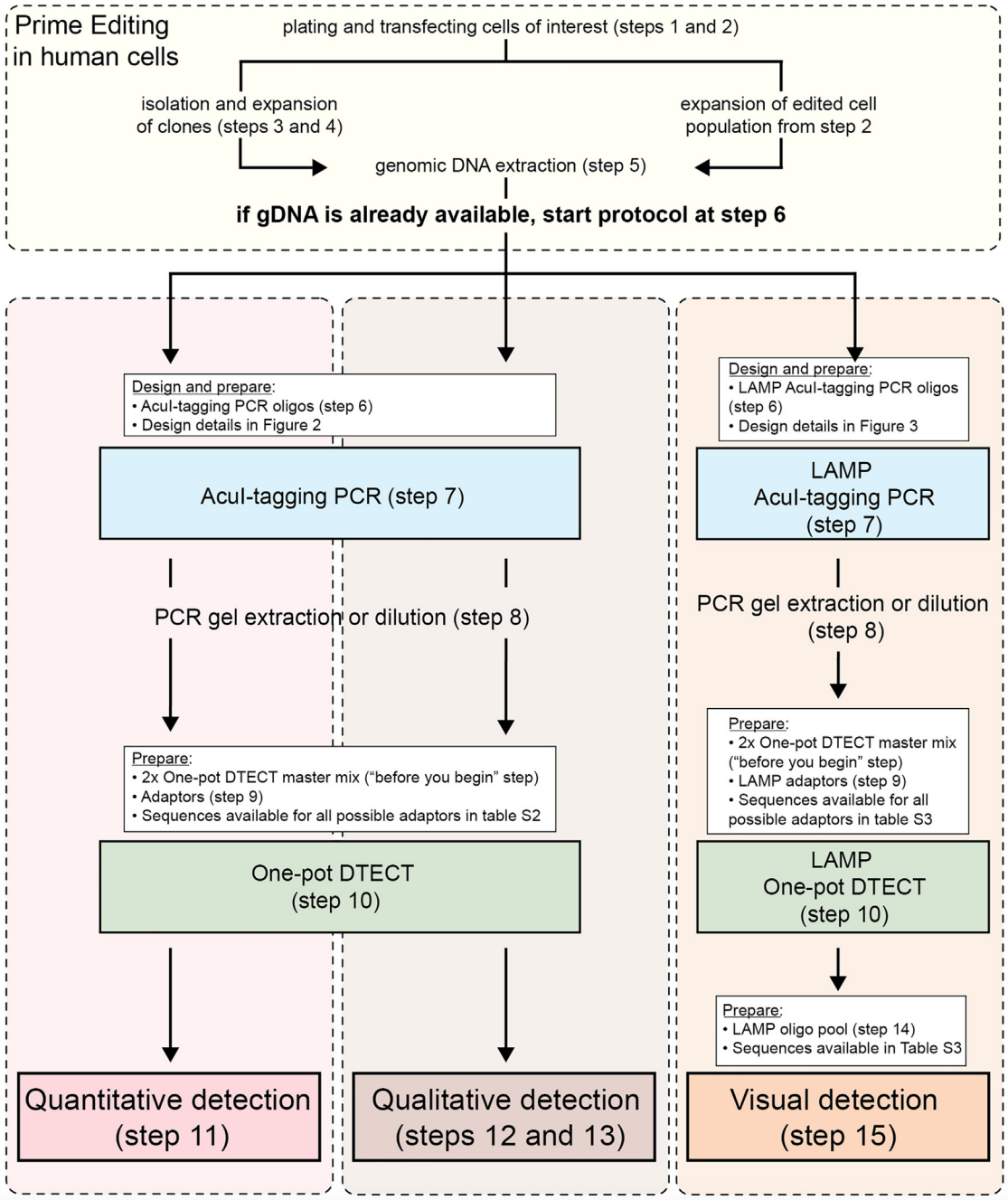

Figure 1. A schematic workflow of the One-pot DTECT options

A comprehensive flowchart detailing the step-by-step protocol for One-pot DTECT. The protocol is divided into two main processes following cell plating: expansion of the edited cell population (step 2) and isolation of single clones (steps 3 and 4). Next, the cells are harvested and genomic DNA is extracted (step 5). Targeted dinucleotides of interest are amplified by AcuI-tagging PCR (step 7). The amplified PCR products are purified (step 8), and the PCR amplicons are subjected to the One-pot DTECT process to capture the genetic signatures (step 10). Finally, detection of the captured signatures can be conducted using one of three approaches: quantitative (step 11), qualitative (Steps 12 and 13) or visual detection (step 15).

lines and precision genome editing tools, such as base editing. For instance, we have successfully applied this protocol to multiple other genes, cancer-derived cell lines, human primary T cells, as well as from patient blood and saliva samples in our accompanying publication.[1]

Preparation of a 2X one-pot DTECT master mix

Timing: 40 min

Timing: 30 min (for step 1)

Timing: 10 min (for step 2)

The One-pot DTECT master mix is prepared by mixing off-the-shelf reagents: AcuI, T4 DNA ligase, and uncleavable competitor DNA fragments. The AcuI enzyme is responsible for exposing the targeted dinucleotide signature on the PCR amplicon, enabling their ligation to the selected adaptors. The T4 DNA ligase catalyzes the ligation of DNA adaptors to the digested amplicons, thereby capturing the target genetic signatures. The uncleavable competitor DNA fragments ensure high capture efficiency by maintaining the integrity of the ligation products. These fragments contain an AcuI motif that restrains AcuI from cleaving newly ligated adaptors. Importantly, the uncleavable competitor DNA fragments are universal and do not need to be designed for specific genes (refer to Table S2 for sequences). The 2X One-pot DTECT master mix is used in step 10 to capture multiple genetic signatures introduced by prime editing in human cells.

1. Preparation of the double-stranded competitor DNA by annealing two complementary oligonucleotides.
   a. Combine 6 μL nuclease-free water (ddH_2O), 2 μL ligase buffer (5X), 1 μL of AcuI-competitor forward oligo (100 μM), and 1 μL of AcuI-competitor reverse oligo (100 μM) in a microcentrifuge tube (refer to Table S2 for oligo sequences).
   b. Place the mixture in a thermocycler and incubate at 95°C for 5 min, followed by a gradual temperature decrease to 15°C at a rate of 0.5°C/s.
   c. Add 90 μL ddH_2O.
   d. Measure the concentration of the competitor DNA fragment with a nanodrop (the expected value is approximately 100 ng/μL) and adjust the concentration to achieve a final concentration of 1 μM.

   Note: The length of the AcuI-competitor DNA is 30 bp.

   e. Aliquot and store the competitor DNA at −20°C for long-term preservation.
2. Preparation of 100 μL 2X One-pot DTECT master mix (40 reactions).
   a. On ice, combine 29 μL ddH_2O, 40 μL ligase buffer (5X) and 20 μL of prepared competitor DNA (1 μM) in a microcentrifuge tube.
   b. Add 1 μL AcuI restriction enzyme (5 units) and 10 μL T4 DNA ligase (10 units) to the master mix.
   c. Mix to homogenize the solution and centrifuge.
   d. Store the 2X One-pot DTECT master mix at −20°C for long-term preservation.

   Note: The master mix can be stored for at least 1 year under these conditions. We recommend preparing aliquots of the 2X One-pot DTECT master mix to avoid multiple freeze-thaw cycles to maintain its integrity and efficacy.

   Note: Equivalent reagents can be purchased from other suppliers to prepare the 2X One-pot DTECT master mix.

   ⚠ CRITICAL: Before starting, make sure to review steps 6 and 9 as they contain important information for designing oligonucleotides.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
T4 DNA ligase (1 unit/μL)	Invitrogen	Cat#15224025
AcuI (5,000 units/mL)	New England Biolabs	Cat#R0641
Power SYBR Green PCR Master Mix	Applied Biosystems	Cat#4368708
Q5 High-fidelity DNA polymerase	New England Biolabs	Cat#0491
WarmStart Colorimetric LAMP 2X Master Mix	New England Biolabs	Cat#M1800
TransIT-293 Transfection Reagent	Mirus Bio	Cat#MIR 2700
QuickExtract DNA extraction solution	LGC Biosearch Technologies	Cat#QE09050
Fetalgro bovine growth serum	Rocky Mountain Biologicals	Cat#FGR-BBT
Betaine	Sigma	Cat#61962
Gel loading dye, purple (6X)	New England Biolabs	Cat#B7024S
SYBR gold nucleic acid stain (10,000X concentrate in DMSO)	Invitrogen	Cat#S11494
Critical commercial assays		
GenepHlow Gel/PCR Kit	Geneaid	Cat#DFH300
ZymoPURE II Plasmid Midiprep Kit	Zymo Research	Cat#4201
Experimental models: Cell lines		
293T/17 (HEK 293T/17)	ATCC	Cat#CRL-11268
Oligonucleotides		
See Tables S1–S3 for list of oligonucleotides		
Recombinant DNA		
pCMV-PE2-SpRY	Kweon et al.[3]	Addgene plasmid#159979
pCMV-PEmax-P2A-hMLH1dn	Chen et al.[4]	Addgene plasmid#174828
epegRNA PCNA Ala96Gly	*Baudrier* et al.[1]	N/A
epegRNA PCNA Met244Ile	*Baudrier* et al.[1]	N/A
epegRNA PCNA Asp86Tyr	*Baudrier* et al.[1]	N/A
epegRNA PCNA Arg146Cys	*Baudrier* et al.[1]	N/A
epegRNA cloning backbone	*Baudrier* et al.[1]	N/A
Software and algorithms		
QuantStudio Real-Time PCR Software v1.7.2	Applied Biosystems	https://www.thermofisher.com/us/en/home/global/forms/life-science/quantstudio-6-7-flex-software.html
Other		
QuantStudio 6 Flex real-time PCR system	Applied Biosystems	Cat#4485692
ProFlex 3 × 32-well PCR system	Applied Biosystems	Cat#4484073
ChemiDoc Touch gel imaging system	Bio-Rad	Cat#1708370

MATERIALS AND EQUIPMENT

- **Preparation of a 500 mM EDTA stock solution:** Dissolve 186 g of EDTA in approximately 800 mL of ddH_2O. Adjust the pH to 8.0 with NaOH. Bring the final volume to 1 L with ddH_2O. Store at 25°C for <1 year.
- **Preparation of a 1 M Tris-HCl solution:** Dissolve 121.1 g Tris base in 800 mL of ddH_2O. Adjust the pH to 8.0 with HCl. Complete the volume to 1 L with ddH_2O. Store at 25°C for <1 year.
- **Preparation of TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0):** Combine 1 mL of 1 M Tris-HCl and 200 μL of 500 mM EDTA (pH 8.0) with 98.8 mL of ddH_2O. Store at 25°C for <1 year.
- **Preparation of 50X TAE solution:** Dissolve 242 g of Tris base in approximately 700 mL of ddH_2O. Add 57.1 mL of glacial acetic acid and 100 mL of 500 mM EDTA (pH 8.0). Adjust the volume to 1 L with ddH_2O. Store at 25°C for 1–3 years.
- **Preparation of a diluted SYBR gold solution for DNA staining:** Dilute 10 μL of SYBR gold (10,000X) in 990 μL DMSO in a microcentrifuge tube. Store at 25°C for 6 months to 1 year, protected from light.

- **Preparation of the 5 M betaine solution:** Dissolve 5.86 g of betaine in 8 mL of ddH_2O. Bring the final volume to 10 mL and filter sterilize. Store at −20°C for 2 years.

STEP-BY-STEP METHOD DETAILS

Experimental outline

Introduction of multiple cancer-associated PCNA *variants in HEK293T cells using prime editing*

Timing: 4 days

Timing: 10 min (for step 1)

Timing: 72 h (for step 2)

In this protocol, we utilize prime editing to introduce specific *PCNA* variants that have been mapped in cancer genomes, including Ala96Gly (c.287C>G),[5] Asp86Tyr (c.256G>T),[6] Arg146Cys (c.436C>T),[7] and Met244Ile (c.732G>C).[8] Engineered pegRNAs (epegRNAs) were designed and cloned following published protocols.[1,9] Notably, Asp86Tyr and Arg146Cys variants have been introduced using a non-canonical PAM-recognizing prime editor, PE2-SpRY,[3] and Ala96Gly and Met244Ile with the canonical prime editor, PEmax.[4]

1. Plating of cells prior to transfection.
   a. Seed approximately 5.5 × 10^4 HEK293T cells into each of two independent wells on a tissue culture treated 48-well plate for adherent cells that will serve as the edited and control samples.
   b. Add complete DMEM to each well to reach a final volume of 250 μL.
   c. Incubate the cells at 37°C with 5% CO_2 for approximately 16 h.

   Note: Ensure the cells are evenly distributed in the wells for optimal growth.

2. Transfection of cells with prime editor and epegRNA constructs for editing.
   a. On the next day, assess the confluency of cells under the microscope to ensure that they have reached an optimal confluency of ~80% prior to transfection.
   b. Dispense 25 μL of pre-warmed serum-free Opti-MEM into two sterile microcentrifuge tubes.
   c. Add 500 ng of the respective plasmids at a 1:1 molar ratio as indicated in the tables below.

Tube 1 (Ala96Gly edited sample)	Concentration (ng/μL)	Size (bp)	Mass required (ng)	Volume (μL)
pCMV-Pemax-P2A-hMLH1dn	322	12,117	419	1.30
epegRNA PCNA Ala96Gly	81.9	2,347	81	0.99

Tube 2 (unedited control sample)	Concentration (ng/μL)	Size (bp)	Mass required (ng)	Volume (μL)
pCMV-PEmax-P2A-hMLH1dn	322	12,117	400	1.24
epegRNA cloning backbone	121	3,046	100	0.83

   d. Add 1 μL of *Trans*IT-293 to each tube and homogenize by gentle pipetting followed by a quick spin.
   e. Incubate the transfection mixtures at 25°C for 15 min.
   f. Add the transfection mixtures to the cells in a drop-wise manner.
   g. Gently mix by rocking the plate.
   h. Incubate the cells at 37°C with 5% CO_2 for 72 h.

   Note: Alternative transfection reagents can be utilized according to supplier guidelines.

Note: If high cellular death post-transfection is observed, see troubleshooting problem 1.

Isolation of clones containing the *PCNA* Ala96Gly variant

Timing: variable, up to 21 days

Timing: 30 min (for step 3)

Timing: variable, up to 21 days (for step 4)

The purpose of this step is to isolate and expand individual clones from the PCNA Ala96Gly edited cell population to obtain heterozygous and homozygous clones of cells with the PCNA Ala96Gly variant.

3. Isolation of individual Ala96Gly edited clones.
   a. Wash the Ala96Gly edited HEK293T cell population with PBS.
   b. Individualize and detach HEK293T cells by adding pre-warmed 0.05% trypsin-EDTA.
   c. Incubate for 5 min and monitor the cells under the microscope to ensure that they are detached from the plate and successfully individualized.
   d. Add at least 3 volumes of complete DMEM to neutralize the trypsin and resuspend the cells by pipetting up and down.
   e. Count cells using an automated cell counter or a hemocytometer.
   f. Dilute cells in complete DMEM to achieve ~25 cells/mL, which is equivalent to 5 cells/200 μL.
   g. Prepare 2-fold serial dilutions by mixing 10 mL of complete medium and 10 mL of diluted cells to obtain dilutions of 2.5 cells/200 μL, 1.25 cells/200 μL and 0.75 cells/200 μL.
   h. Distribute 200 μL of the serial dilutions into a 96-well plate.

⚠ CRITICAL: Conserve the leftover edited and non-edited HEK293T cell population for gDNA extraction.

Note: It is recommended to freeze the edited cell population.

4. Expansion of Ala96Gly clones.
   a. Expand isolated clones until sufficient growth is observed.
   b. Passage the expanded clones into 48-well plates.
   c. Upon reaching confluency in larger plates (e.g., 12-well plates), keep half in culture or freeze, and collect the other half for gDNA extraction.

Genomic DNA extraction

Timing: 30 min

The genomic DNA from the unedited and edited cell populations, as well as the clones, is isolated using the QuickExtract DNA Extraction Solution protocol. Alternative gDNA isolation procedures can also be used.

5. Collection of cells and extraction of genomic DNA.
   a. Harvest cells from step 4c in a microcentrifuge tube.
   b. Centrifuge at 300 × *g* for 3 min.
   c. Carefully remove the medium to preserve the cell pellet.
   d. Wash cells by resuspending the pellet in 500 μL of PBS.
   e. Centrifuge at 300 × *g* for 3 min.
   f. Carefully aspirate the PBS without disrupting the cell pellet.

Pause point: Cell pellets can be stored at −80°C for long-term storage.

g. Resuspend the cell pellet in 25 μL of QuickExtract solution.
h. Vortex for 15 s and incubate at 65°C for 6 min.
i. Vortex again for 15 s and incubate at 98°C for 2 min.
j. Vortex and quick spin.
k. Measure the concentration of gDNA with a nanodrop.

Note: If the gDNA solution is viscous, add 50 μL of ddH_2O, vortex for 5 s, and incubate the solution at 50°C for 1 min. Repeat the process, if necessary.

l. Adjust the concentration of the gDNA to ~100 ng/μL with ddH_2O.

Pause point: gDNA samples can be stored at −20°C for long-term storage.

AcuI-tagging PCR

Timing: variable, 55 min to 2 h

Timing: 15 min (for step 6)

Timing: 40–60 min (for step 7)

Timing: 30 min (for step 8)

The AcuI-tagging PCR requires an AcuI-tagging oligonucleotide and a reverse oligonucleotide which are the only gene-specific element of the One-pot DTECT protocol. The purpose of the AcuI-tagging PCR is to amplify the targeted genomic locus while simultaneously introducing the detection handles and tagging the targeted dinucleotide with the AcuI motif. This step requires designing a pair of oligonucleotides named "AcuI-tagging primer" and "reverse primer". Purification of the AcuI-tagging PCR on gel is optional but generally recommended.

Note: We illustrate the One-pot DTECT for the capture of the *PCNA* Ala96Gly signature. Primer sequences for the other cancer-associated *PCNA* mutations are available in Table S1. The general methodology used to design AcuI-tagging and reverse primers for the detection of any genomic sites of interest is explained in steps 6a and 6b.

6. Design of oligonucleotides for AcuI-tagging PCRs.
   a. Composition and design of the AcuI-tagging oligonucleotide.
      i. The AcuI-tagging oligonucleotide is composed of the detection handle at the 5′-end and the AcuI recognition motif (5′-CTGAAG-3′) as illustrated in Figure 2. The structure of the AcuI-tagging oligonucleotide is as follows: 5′-**GCAATTCCTCACGAGACCCGTCC TG**N(15)**CTGAAG**N(14)-3′ with "N" corresponding to A, T, G, or C bases complementary to the targeted locus (Figure 2).

Note: In this protocol, the AcuI-tagging PCNA Ala96Gly oligonucleotide sequence is (5′ → 3′): **GCAATTCCTCACGAGACCCGTCCTG**TATCATTACACTAAG**CTGAAG**GGCCGAAGATA ACG, with the detection handle and AcuI motif highlighted in bold.

Note: Four independent AcuI-tagging PCR oligonucleotides can be designed per dinucleotides of interest, as detailed in our original publication[10] and Figure S1.

   b. Composition and design of the reverse oligonucleotide.

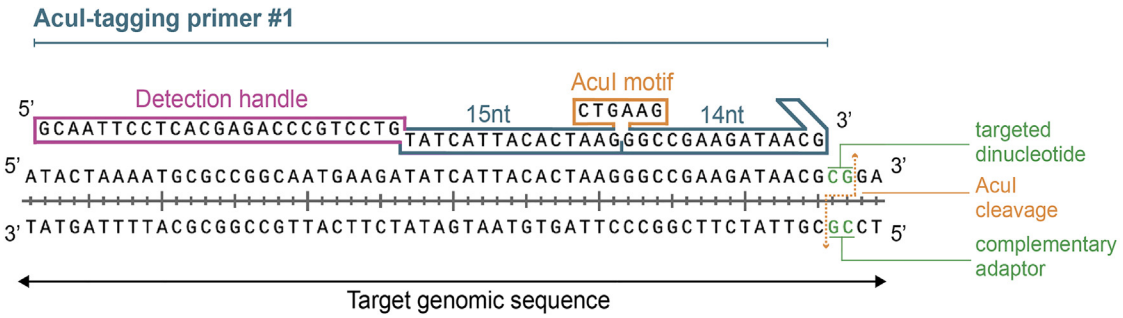

Figure 2. Schematic of AcuI-tagging primer design and target genomic sequence for detection with One-pot DTECT

The AcuI-tagging primer is composed on three main components: a detection handle (in pink), a target-specific complementary sequence (in dark blue) consisting of 15 and 14 nucleotides upstream of the targeted dinucleotide, and an AcuI recognition motif (in orange) located between the two genomic sections. The AcuI cleavage pattern is indicated by a dashed orange arrow around the targeted dinucleotide (CG) of the *PCNA* A96 codon (green). The adaptor dinucleotide, which is complementary to the captured dinucleotide, is also shown.

i. The reverse oligo is complementary to the targeted genomic locus to facilitate amplification by PCR. For optimal design, use Primer 3 (https://bioinfo.ut.ee/primer3-0.4.0) with the following parameters:

Mispriming library = "model organism".
Primer size "min = 25, Opt = 27, Max = 30"
Primer Tm "Min = 57.0C, Opt = 60.0C, Max = 63.0C"
All other parameters as defaults.

Note: In this protocol, the Ala96Gly reverse oligonucleotide sequence is (5′ → 3′): CTATGA CATAACTATCGTGCCTGTG

⚠ CRITICAL: The specificity of the AcuI-tagging primers and reverse primers is crucial to ensure accuracy of the amplification and to avoid cross-amplification with unspecific amplicon. This is especially important when detecting genetic signatures in paralogs with low sequence diversity.

Note: For colorimetric detection with One-pot DTECT-LAMP, AcuI-tagging primers are designed similarly but contain the 5′ detection handle sequence CTGCACCTCATGGTCAT GTTATGGTTGAGCTGGTAGCAGA[11] that enable LAMP detection (as illustrated in Figure 3). Oligonucleotide sequences for the LAMP adaptors are available in Table S3.

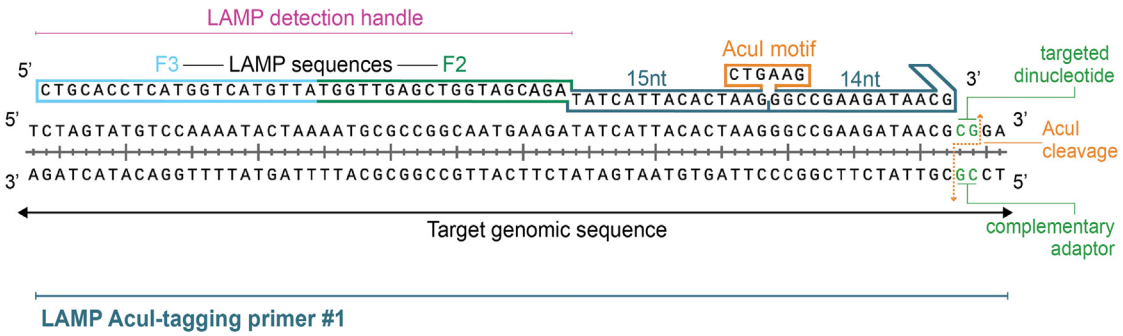

Figure 3. Schematic of LAMP AcuI-tagging primer design for detection with One-pot DTECT-LAMP

The AcuI-tagging primer for One-pot DTECT-LAMP includes the LAMP sequences F3 (in light blue) and F2 (in dark green), a target-specific complementary sequence (in dark blue) of 15 and 14 nucleotides upstream of the targeted dinucleotide, and an AcuI recognition motif (in orange) located between the two genomic sections. The AcuI cleavage pattern is denoted with the dashed orange arrow around the targeted dinucleotide (CG) of the *PCNA* A96 codon (in light green).

7. Amplification of the targeted genomic locus by AcuI-tagging PCR.
   a. Prepare a PCR master mix to amplify the genomic locus in the cellular pools of edited and non-edited cell populations, as well as the isolated clones.

Note: Here, we illustrate the detection of homozygous and heterozygous clones for Ala96Gly (clones 6, 8, and 15) (sequences are available in Table S1).

Reagent	Final concentration	Volume for single reaction (µL)	Volume for 5.5x reactions (µL)
Q5 Reaction buffer (5X)	1X	5	27.5
dNTP mix (10 mM)	100 µM	0.25	1.375
AcuI-tagging PCNA Ala96Gly primer (100 µM)	1 µM	0.25	1.375
PCNA Ala96Gly reverse primer (100 µM)	1 µM	0.25	1.375
Q5 High-Fidelity DNA polymerase (2 units/µL)	0.4 unit	0.2	1.1
ddH_2O	N/A	18.05	99.275

Alternatives: The master mix recipe is based on the NEB Q5 High-Fidelity DNA polymerase protocol from the supplier; alternative DNA polymerases can be used.

   b. Aliquot 24 µL of the prepared master mix into separate PCR tubes.
   c. Add 1 µL of the corresponding gDNA (100 ng) to each tube.
   d. Homogenize the reactions by inversion and perform a quick spin.
   e. Program the thermocycler according to the following conditions.

Steps	Temperature (°C)	Time (sec)	Cycles
Initial Denaturation	95	30	1
Denaturation	95	10	40
Annealing	62	10	
Extension	72	30	
Final extension	72	60	1
Hold	4	infinite	

⚠ CRITICAL: Annealing temperature and extension time need to be optimized for each set of AcuI-tagging primers.

Note: If no amplification of the targeted genomic locus occurs, refer to troubleshooting problem 2.

Pause point: AcuI-tagging PCR samples can be stored at −20°C for long-term storage.

8. Gel extraction of AcuI-tagging PCR products (optional but recommended).
   a. Prepare a 2% (w/v) agarose gel by combining 1 g of agarose in 50 mL of 1X TAE.
   b. Melt the agarose mixture using a microwave until agarose is fully dissolved.
   c. While melting, gently swirl the mixture periodically (~every 30 s) to ensure complete dissolution and homogenization of the agarose and to prevent buffer evaporation.
   d. Pour the agarose into a small gel electrophoresis system.

Optional: As an alternative to gel purification, prepare a 1/100th dilution of the PCR product by adding 1 µL of the PCR product to 99 µL of ddH_2O in a new microcentrifuge tube (see troubleshooting problem 4).

   e. Add 0.5 µL of diluted SYBR gold and 4 µL of loading dye to each PCR tube.

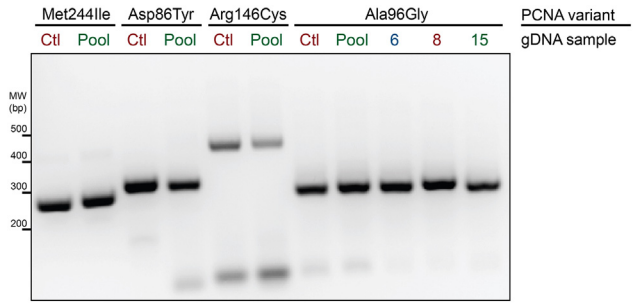

Figure 4. AcuI-tagging PCR amplification of the *PCNA* gene loci from genomic DNA

PCR amplification of the *PCNA* genomic loci from unedited (control) and edited genomic DNA. The PCR products were loaded on a 2% agarose gel and stained with SYBR gold. Lanes 1 and 2 represent to the AcuI-tagging PCRs from the unedited and edited samples for Met244Ile, respectively. Lanes 3 and 4 represent the AcuI-tagging PCRs from the unedited and edited samples for Asp86Tyr, respectively. Lanes 5 and 6 represent the AcuI-tagging PCRs from the unedited and edited samples for Arg146Cys, respectively. Lanes 7 to 11 represent the AcuI-tagging PCRs from unedited, edited samples and clones for Ala96Gly.

f. Homogenize the samples by inversion.
g. Once the gel has solidified, load PCR samples into the wells, alongside a 100 bp DNA ladder.
h. Run the gel at 100 V in 1X TAE for a minimum of 30 min (see Figure 4).
i. Excise the expected 281 bp DNA fragments from the gel under blue light using a scalpel.
j. Wash the scalpel with 70% ethanol and ddH_2O between each sample to prevent cross-contamination.
k. Dissolve the agarose fragments and purify AcuI-tagged amplicon by using Geneaid Gel Extraction DNA purification kit, according to manufacturer's recommendations.

Pause point: Purified PCR products can be stored at −20°C for long-term storage.

Optional: If AcuI-tagging PCR appears as a clear single DNA fragment on the gel at the expected size, the 1/100th dilution can be directly used in the next step for capture with One-pot DTECT (see troubleshooting problem 4).

Capture of genetic signatures using one-pot DTECT

Timing: variable, 10 min to 2 h

Timing: 90 min (for step 9)

Timing: 10–60 min (for step 10)

Here, the prepared 2X One-pot DTECT master mix is used with the appropriate adaptors for the capture of the Ala96Gly signature.

9. Preparation of the unique One-pot DTECT adaptors for the capture of dinucleotide signatures.
   a. Order the constant and variable oligonucleotides containing the complementary dinucleotides to the WT and edited signatures.
      i. For the Ala96Gly signature, the CG dinucleotide adaptor is used to capture the WT and the GC dinucleotide adaptor is used to capture the edit.
      ii. For the Met244Ile signature, the TG dinucleotide adaptor is used to capture the WT and the TC dinucleotide adaptor is used to capture the edit.
      iii. For the Asp86Tyr signature, the TC dinucleotide adaptor is used to capture the WT and the TA dinucleotide adaptor is used to capture the edit.

iv. For the Arg146Cys signature, the CG dinucleotide adaptor is used to capture the WT and the CA dinucleotide adaptor is used to capture the edit.

Note: For a complete list of all adaptors see Table S2.

b. Upon receiving, briefly spin down oligonucleotides and resuspend them at 100 μM in TE buffer.
c. Anneal the complementary oligonucleotides to create double-stranded DNA adaptors by combining 2.5 μL of the variable oligonucleotide (CG or GC for Ala96Gly), 2.5 μL of the constant oligonucleotide, 4 μL ligase buffer and 11 μL ddH_2O to a final volume of 20 μL in a PCR tube.
d. Homogenize the reactions by inversion and perform a quick spin.
e. Place the mixture in a thermocycler at 95°C for 5 min, followed by a gradual temperature decrease to 15°C at a rate of 0.5°C/s.
f. Add 100 μL ddH_2O.
g. Aliquot and store the adaptors at −20°C for long-term preservation.

Note: Adaptor stocks should be prepared in advance and stored at −20°C for long-term storage.

⚠ CRITICAL: If LAMP is the chosen method of detection, prepare the 1a LAMP adaptors as follow: upon receiving, resuspend 1a LAMP oligonucleotides at 10 μM in TE buffer. Combine 1 μL of the LAMP 1a NN-variable adaptor (where 'NN' is the dinucleotide of interest, e.g., CG or GC), 1 μL of constant LAMP 1a oligonucleotides, and 3 μL of ddH_2O to a final volume of 5 μL in a PCR tube. Follow steps 9d and 9e and dilute DNA adaptors 1/25th with ddH_2O before aliquoting and storing at −20°C for long-term preservation.

10. Capture of PCNA Ala96Gly signatures using One-pot DTECT.
    a. Quantify the purified AcuI-tagging PCR products generated in step 8 using a nanodrop. Dilute the samples to 2.5 nM.

Alternatives: Use the 1/100th dilution as an alternative to the 2.5 nM (see troubleshooting problem 4).

b. On ice, prepare two One-pot DTECT master mixes.
   i. One for the capture of the Ala96 WT signature using the CG adaptor.
   ii. Another to capture the Ala96Gly edited signature using the GC adaptor.

Reagent	Final concentration	Volume per reaction (μL)	Volume for 5.5x reactions (μL)
DTECT master mix (2X)	1X	2.5	13.75
Diluted CG/GC adaptor (4 μM)	0.2 μM	0.25	1.375
ddH_2O	N/A	1.25	6.875

Note: If LAMP is the chosen method of detection, the LAMP adaptors (specifically the LAMP 1a-CG and LAMP 1a-GC adaptors) should be used instead of the regular adaptors. These adaptors should be diluted 1/25th, rather than 1/100th.

c. In a PCR tube, assemble 4 μL of the prepared One-pot DTECT master mix with 1 μL of the diluted AcuI-tagging PCR (2.5 nM or diluted AcuI-tagging PCR 1/100th).
d. Mix and briefly centrifuge.
e. Incubate the reaction in a thermocycler at 25°C for 10 min- 1 h.
f. Inactivate the reaction by heating the samples at 65°C for 1 min.

Note: The captured material can be analyzed by qPCR (quantitative), analytical PCR (qualitative), or LAMP (visual) as detailed below. Select the option that best suits the desired results. Refer to Figure 1 for additional details and guidance.

Pause point: DTECT samples can be stored at −20°C for long-term storage.

Quantitative detection of the captured signatures

Timing: 90 min

The relative amount of the captured signatures in the edited or control samples is determined by qPCR using a universal pair of detection primers (sequences available in Table S2) that should be used for all quantitative and qualitative One-pot DTECT detection reactions.

11. Run a qPCR to determine the relative frequency of signature captured by the respective adaptors to determine editing frequency.
    a. On ice, prepare a qPCR master mix by following the recipe below. In this example, both edited and unedited *PCNA* Met244Ile samples are used.

Reagent	Final concentration	Volume per reaction (μL)	Volume for 8.5x reactions (μL)
SYBR green master mix (2X)	1X	5	42.5
Detection primer #1 (100 μM)	1 μM	0.1	0.85
Detection primer #2 (100 μM)	1 μM	0.1	0.85
ddH_2O	N/A	3.8	32.3

Note: Two independent technical replicates of qPCR are run in parallel for each sample.

Note: This pair of detection primers is universally applicable for all detections using One-pot DTECT, as they amplify the ligated product.

    b. Dispense 9 μL of SYBR green master mix into 8 wells of a 384 qPCR-well plate. Keep the plate cold.
    c. Add 1 μL of each of the One-pot DTECT sample into two corresponding wells. Each sample is quantified by two qPCR technical duplicates.
    d. Seal the qPCR plate with transparent adhesive film to prevent sample evaporation.
    e. Centrifuge the qPCR plate at 300 × *g* for 1 min.
    f. Place the qPCR plate into the QuantStudio 6 qPCR system and set the following system parameters (QuantStudio Real-Time PCR Software v1.7.2):
        QuantStudio 6.
        SYBR Green.
        384-well.
        Standard curve.
        Leave the other parameters as default and select wells corresponding to samples.
    g. Program the thermocycler according to the following conditions:

Steps	Temperature (°C)	Time (sec)	Cycles
Hold	50	120	1
	95	600	
PCR stage	95	10	40
	60	30	
Melting	95	15	1
	60	60	
	95	15	

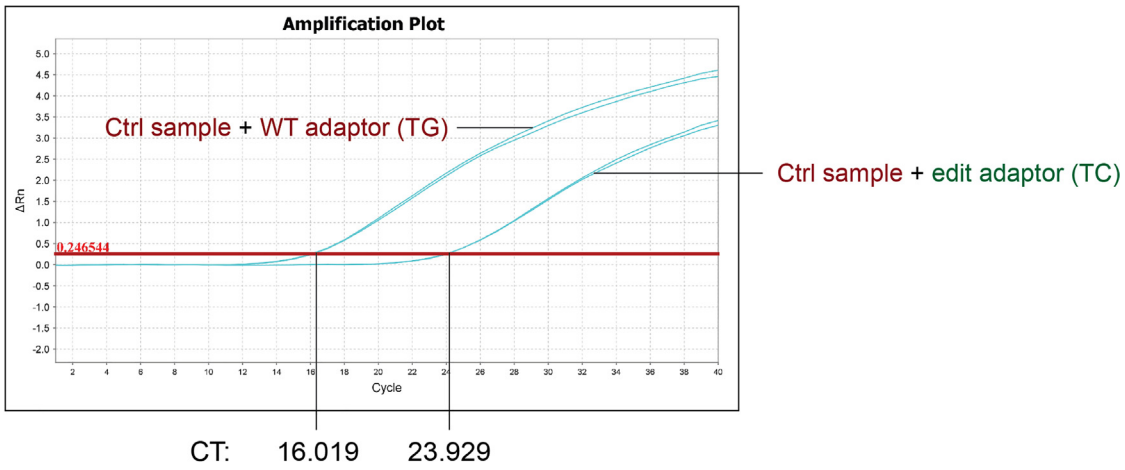

Figure 5. Quantitative PCR amplification curves

qPCR amplification plots obtained using the QuantStudio Real-Time PCR Software v1.7.2 for a control sample of PCNA Met244Ile. Two distinct amplification curves are shown for the captured products from the control samples using either the WT (TG) or edit (TC) adaptors. Each sample is represented by two curves corresponding to each technical replicate. Ct values for each sample are indicated.

Alternatives: Any other qPCR systems or well-plates can be used.

h. Retrieve the Ct values for each well (Figure 5).

Note: See quantification and statistical analysis section to calculate editing frequency from Ct values (Figure 6).

Qualitative detection of the one-pot DTECT capture

Timing: 55 min

Timing: 25 min (for step 12)

Timing: 30 min (for step 13)

This alternative detection method allows for a quick assessment of the presence or absence of variants on an agarose gel. The One-pot DTECT products are amplified using the same unique pair of detection primers as those used in qPCR.

qPCR duplicates (input) =(C2+C3)/2 =10^((D2-I2)/I1)

	Sample	Adaptor used	CT values	Mean Ct	Concentration	Frequency (%)	
1							
2	PCNA Met244Ile control	TG (WT)	15.978	16.019	0.00283	99.58	=(F2/(F2+F4))*100 Positive control (confirms experiment worked)
3			16.061				
4		TC (edit)	23.903	23.929	0.00001	0.42	=(F4/(F2+F4))*100 Negative control (background capture)
5			23.955				
6	PCNA Met244Ile edited pool	TG (WT)	16.775	16.667	0.00181	84.75	=(F6/(F6+F8))*100 Capture of WT signature in edited sample
7			16.559				
8		TC (edit)	19.187	19.143	0.00033	15.25	=(F8/(F6+F8))*100 Capture of edit signature (% editing)
9			19.099				
	A	B	C	D	E	F	

Equation standard curve:	slope a	-3.3245	1
	b	7.5504	2
	G	H	I

Figure 6. Quantitative PCR data analysis spreadsheet

Screenshot of the spreadsheet used for calculating the relative capture frequencies in control and edited samples using both WT and edit adaptors. The formulas utilized to quantify the relative frequency of WT and edited signatures are shown.

12. Detection of the signatures in One-pot DTECT samples by PCR.
    a. On ice, assemble a PCR master mix to amplify the 120 bp One-pot DTECT ligated products.

Note: In this example, edited and unedited *PCNA* Ala96Gly samples and clones 6, 8 and 15 are used.

Reagent	Final concentration	Volume for single reaction (μL)	Volume for 10.5x master mix (μL)
Q5 Reaction buffer (5X)	1X	2.5	26.25
dNTP mix, 10 mM	160 μM	0.2	2.1
Detection primer #1 (100 μM)	0.5 μM	0.0625	0.656
Detection primer #2 (100 μM)	0.5 μM	0.0625	0.656
Q5 High-Fidelity DNA polymerase (2 units/μL)	0.2 unit	0.1	1.05
ddH_2O	N/A	9.075	95.29

    b. In a PCR tube, assemble 12 μL of the prepared PCR mix and add 0.5 μL of the One-pot DTECT sample.
    c. Mix and briefly centrifuge.
    d. Program the thermocycler according to the following conditions.

Steps	Temperature (°C)	Time (sec)	Cycles
Initial Denaturation	95	60	1
Denaturation	95	10	18
Annealing	65	5	
Extension	72	7	
Final extension	72	60	1
Hold	4	infinite	

Note: The Q5 polymerase is recommended for AcuI-tagging PCR because, in our experience, it provides robust amplification of DNA from genomic DNA. However, alternative DNA polymerases can be used, but modifications in the PCR conditions may be necessary.

⚠ CRITICAL: Adjust the number of cycles depending on the expected efficiency of the capture. Use 23–25 for lower abundance and 15–22 for greater amounts.

13. Visualization of the 120 bp captured products on a 2% agarose gel.
    a. Prepare a 2% (w/v) agarose gel in 1X TAE buffer.
    b. Add 0.5 μL of diluted SYBR Gold and 2.5 μL of loading dye to the 12.5 μL PCR reactions.
    c. Load 5 μL of the mixture into the gel, alongside a 100 bp DNA ladder, and run the gel in 1X TAE at 100 V for at least 30 min.
    d. Develop the gel using a gel imaging system, such as the Bio-Rad Chemidoc (Figure 8).

Visual detection of prime editing using one-pot DTECT LAMP

Timing: variable, 20–55 min

Timing: 5 min (for step 14)

Timing: variable, 20–50 min (for step 15)

This alternative detection allows for the colorimetric detection of the targeted signature. When the oligo pool for LAMP amplification is added, the successful ligation of the LAMP adaptors to the AcuI-

tagging products (from step 10) can initiate LAMP-mediated amplification, resulting in a color change of the solution from pink to yellow.

⚠ CRITICAL: This step is an alternative to the quantitative or qualitative detection methods and requires an AcuI-tagging PCR that includes specific AcuI-tagging LAMP sequences (see Note in step 6). Additionally, a One-pot DTECT reaction must be performed using LAMP-specific adaptors (CRITICAL in step 9). Oligonucleotide sequences are available in Table S3 and mechanistic details can be found in our original publication.[1]

14. Prepare the 1a LAMP oligo pool stock for using One-pot DTECT-LAMP as detection.

Reagent	Final concentration (μM)	Volume (μL)
F3-1a (100 μM)	4	4
FIP-1a (100 μM)	32	32
B3-1a (100 μM)	4	4
BIP-1a (100 μM)	32	32
LB-1a (100 μM)	8	8
ddH_2O	N/A	20

Note: The LAMP oligo pool is universal and does not need to be modified for specific genes (refer to Table S3 for sequences).

15. Visual detection by LAMP.
    a. Dilute One-pot DTECT-LAMP samples from step 10 1/2,000th (refer to troubleshooting problem 3).
    b. Prepare a LAMP reaction master mix on ice.

Reagent	Final concentration	Volume for single reaction (μL)	Volume for 10.5x reactions (μL)
WarmStart Colorimetric LAMP 2X Master Mix (2X)	1X	5	52.5
Betaine (5 M)	0.8 M	1.6	16.8
Oligo pool 1a	N/A	0.5	5.25
ddH_2O	N/A	0.4	4.2

⚠ CRITICAL: Always include an unedited pool as a negative control to monitor LAMP reaction.

    c. On ice, combine 7.5 μL of the prepared LAMP mix and add 2.5 μL of the diluted One-pot DTECT-LAMP sample. Keep samples on ice throughout the process.
    d. Mix the reactions thoroughly and briefly centrifuge.
    e. Incubate all samples, including the negative control, in a thermocycler at 65°C.
    f. Monitor the color change closely every ~5 min. A color change from pink to yellow typically occurs between 25 and 35 min for positive controls (Figure 9).

Note: The negative control should remain pink for at least 90 min.

Alternatives: Follow colorimetry using a plate reader over time, like the Spectra Max iD3. Change of color in samples has successfully been recorded with the following settings:

Measurement: mode Absorbance (ABS), type kinetics.
Plate: 384-wells, standard opaque 17.5 mm height, with lid on.

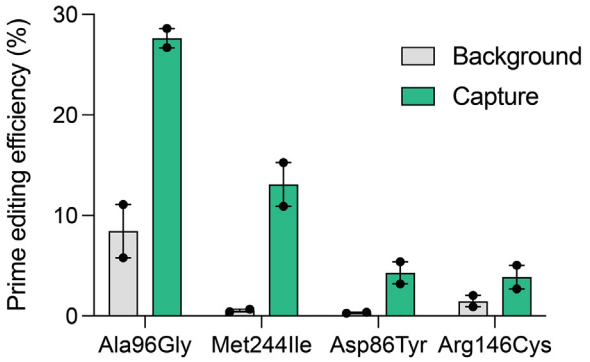

Figure 7. Prime editing efficiency for cancer-associated PCNA variants

Prime editing efficiencies determined using quantitative One-pot DTECT for *PCNA* Ala96Gly, Met244Ile, Asp86Tyr, and Arg146Cys variants. The green and gray bars represent the capture frequencies for the edited and control samples, respectively.

Wavelength: Luminescence Mode 1 (LM1) 415 nm; Luminescence Mode 2 (LM2) 560 nm.

Detection: method precise.

Kinetic: interval 30 s; 241 reads; time 2 h.

Temperature: 65°C.

EXPECTED OUTCOMES

In this protocol, we introduce multiple cancer-associated mutations in *PCNA* in HEK293T cells using prime editing. We used One-pot DTECT and One-pot DTECT-LAMP to detect the presence of these mutations and to quantify the editing frequencies. The protocol successfully utilizes three independent detection strategies, such as quantitative (Figure 7, step 11), qualitative (Figure 8, steps 12 and 13), and visual (Figure 9, steps 14 and 15).

QUANTIFICATION AND STATISTICAL ANALYSIS

To determine the editing frequency, begin by retrieving the cycle threshold (Ct) values obtained from the qPCR analysis in step 11. First, calculate the mean Ct values from qPCR technical duplicates for each sample. Second, calculate the capture efficiency using the equation derived from the standard curve associated with the universal One-pot DTECT detection primers: $y = -3.3245x + 7.5504$.[10] Once the capture efficiency for each adaptor is obtained, determine the relative abundance of each genetic signature (see Figure 6).

Note: The detection primers are universal for all One-pot DTECT reactions, so this formula can be applied to the detection of any genetic signatures.

LIMITATIONS

While One-pot DTECT is an efficient method for detecting precise changes introduced by CRISPR-based precision genome editing technologies, like prime editing or base editing, it has certain limitations. First, One-pot DTECT is unable to detect unpredictable insertions and deletions that may occur as editing byproducts, which are rare events in base and prime editing. However, One-pot DTECT remains highly effective at detecting predictable small insertions and deletions through the capture of the dinucleotide at the junction of the insertion/deletion. Second, One-pot DTECT is restricted to capturing dinucleotide signatures, which limits its ability to detect multiple edits or complex edits simultaneously. Finally, the presence of AcuI motifs within the genomic sequence amplified during the AcuI-tagging PCR step can generate bystander signatures. These bystander signatures can potentially interfere with the specific detection of the targeted dinucleotide

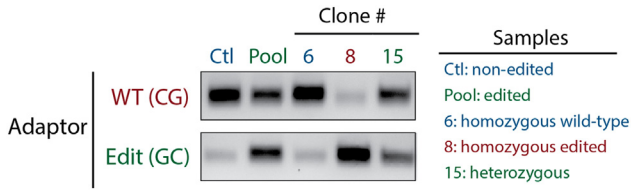

Figure 8. Analytical detection of One-pot DTECT for PCNA Ala96Gly clones

PCR amplification of Ala96 and Ala96Gly signatures captured using One-pot DTECT in the specified samples. Samples include an unedited control (WT), edited cell population, and clones 6 (homozygous wild-type), 8 (homozygous edited), and 15 (heterozygous).

signatures. Finally, when the relative capture frequency of the wild-type and edited signatures are around 50%, such as in diploid heterozygous cells, the quantitative capture results become highly sensitive to the quality of the qPCR replicates. In this context, minor differences in the Ct values due to pipetting errors can lead to variations in the calculated editing frequency. Running multiple qPCR replicates of the samples will mitigate this variability.

TROUBLESHOOTING

Problem 1

Low cell viability post-transfection (related to Step 2).

Potential solution

- To mitigate cellular death upon transfection, use endotoxin-free midiprep kits (e.g., ZymoPURE II Plasmid Midiprep kit) for plasmid preparation.
- Ensure cells are at optimal confluency (around 80%) at the time of transfection. Refer to TransIT-293 Transfection Reagent supplier protocol for detailed guidance on transfection conditions.

Problem 2

No amplification of the targeted genomic locus during AcuI-tagging PCR (related to Step 7).

Potential solution

- Optimize the PCR conditions by adjusting the temperature and extending the duration of elongation time.

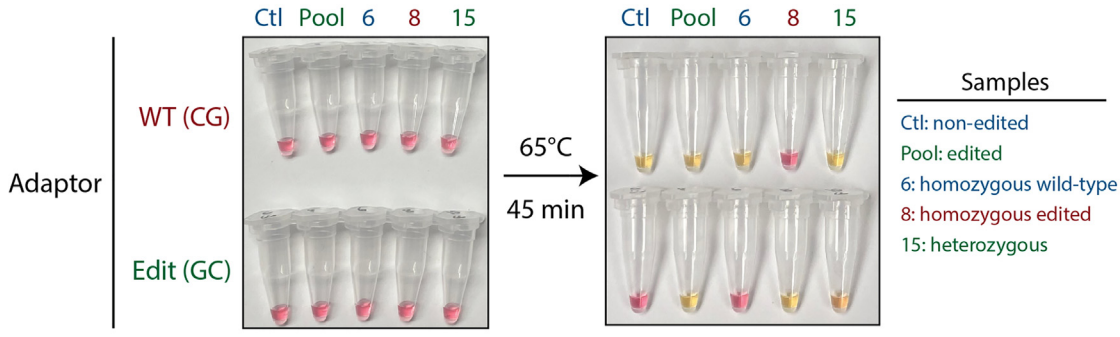

Figure 9. Analysis of PCNA Ala96Gly using One-pot DTECT-LAMP

Qualitative analysis of the capture of *PCNA* Ala96 and Ala96Gly signatures using One-pot DTECT-LAMP. The reaction was incubated at 65°C with pictures taken before incubation (left) or 45 min after incubation (right). Adaptors specific for CG (wild-type) and GC (edit) signatures were used. Samples include unedited control (wild-type), edited cell population, and clones 6 (homozygous wild-type), 8 (homozygous edited), and 15 (heterozygous).

- Use buffers designated for difficult-to-amplify loci (e.g., GC enhancer buffers) to facilitate amplification of complex genomic regions.
- If PCR inhibitors are suspected, dilute the gDNA samples 1:10 to reduce their impact.
- Perform a conventional PCR to amplify the target locus first, then use this amplicon as a template for the AcuI-tagging PCR.
- Design an alternative AcuI-tagging primer to capture a different dinucleotide signature of the same variant (refer to Figure S1).

Problem 3

LAMP negative control shows color change from pink to yellow (related to step 15).

Potential solution

- The LAMP detection assay is highly sensitive and can sometimes result in false positive in the negative control. Optimize the dilution of the One-pot DTECT samples (e.g., 1/500th, 1/1000th, 1/2000th, and 1/4000th) to reduce background noise.

Problem 4

High non-specific capture in the negative control (related to expected outcomes).

Potential solution

This protocol relies on both positive and negative controls to confirm the specificity of the assay. High background capture in control samples can arise from several factors, such as the sensitivity of the qPCR, the quality of the AcuI-tagging PCR, or the activity of AcuI. DTECT is a highly sensitive assay and can capture low-frequency signatures in AcuI-tagging PCR samples. Here are potential solutions to address high background capture.

- First, since qPCR is a sensitive technique, it is crucial to ensure that the difference between technical duplicates does not exceed 0.5. If the technical differences are high, repeat the technical duplicates to improve reliability.
- The most likely cause of high background capture is the low specificity of the AcuI-tagging PCR. Ensure the quality of the AcuI-tagging PCR before running the One-pot DTECT reaction, and consider optimizing the melting temperature to increase the specificity of the amplification.
- If diluting the AcuI-tagging PCR results in high background capture, purify the amplicon on a gel. This step removes potential contaminants and ensures the purity of the AcuI-tagging PCR product.
- Finally, high background capture can occur if a genomic AcuI site that is amplified during the AcuI-tagging PCR, interfering with the specific signatures and impacting capture specificity. To address this, redesign the primers to avoid amplifying the bystander AcuI motif.

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).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Pierre Billon (pierre.billon@ucalgary.ca).

Materials availability

Plasmids generated in this study are available upon request.

Data and code availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

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Figures were created with BioRender.com.

AUTHOR CONTRIBUTIONS

J.L. and L.B. performed the experiments. J.L., L.B., and P.B. wrote the manuscript. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

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

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103307.

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