Supplementary Information

for

CATCH: high specific transcriptome-focused fusion gene variants discrimination

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Methods

Material and regents

RNase Inhibitor, Murine and HiScribe™ T7 High Yield RNA Synthesis Kit were supplied by New England Biolabs (Beverly, USA). DEPC-Treated Water (DEPC), Isopropyl-1-thio-b-D-galactopyranoside (IPTG), PrimeSTAR® Max DNA Polymerase, Recombinant DNase I, Coomassie Blue staining, Gold-view and DL20 DNA Marker were purchased from TaKaRa (Dalian, China). Benzonase, protease inhibitors and SUMO protease were obtained from HaiGene Biotech Co., Ltd. (Harbin, China). The plasmid of pC013-Twinstrep-SUMO-huLwCas13a was obtained from the Addgene platform by signing material transfer agreement (MTA). The plasmids of EML4-ALK variant 1 (GenBank accession no. AB274722.1), EML4-ALK variant 2 (GenBank accession no. AB275889.1), EML4-ALK variant 3 splicing isoform a (GenBank accession no. AB374361.1), EML4-ALK variant 3 splicing isoform b (GenBank accession no. AB374362.1), EML4-ALK variant 4 (GenBank accession no. AB374363.1) EML4-ALK variant 5 splicing isoform a (GenBank accession no. AB374364.1) and EML4-ALK variant 5 splicing isoform b (GenBank accession no. AB374365.1), EML4 (GenBank accession no. NM_019063.4), ALK (GenBank accession no. NM_004304.4), KIF5B-ALK variant 1 (GenBank accession no. AB462413.1), TFG-ALK (GenBank accession no. AF125093.1), KLC1-ALK (GenBank accession no. AB781674.1), HIP1-ALK (GenBank accession no. AF360988.1), STRN-ALK (GenBank accession no. KJ162576.1) and all oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China), which were designed by the software (https://sg.idtdna.com/calc/analyzer). The specificity of primers for the EML4-ALK variants and other ALK relative fusion genes were confirmed in the Basic Local Alignment Search Tool (BLAST), and their corresponding sequences were included in Table S1. The buffer solutions used in this study were as follows: nuclease buffer (40 mM Tris-HCl, 60 mM NaCl, 6 mM $MgCl_2$, pH 7.3), Tris-Borate-EDTA (TBE) buffer (445 mM Tris, 445 mM boric acid, 10 mM EDTA, pH 8.3). Lysis buffer (20mM Tris-HCl, 500 mM NaCl, 1 mM DTT, pH 8.0), Digestion buffer (30mM Tris-HCl, 500mM NaCl, 1mM DTT, 0.15% NP-40, pH 8.0), S200 buffer (10mM HEPES, 1M NaCl, 5mM $MgCl_2$, 2mM DTT, PH 7.0).

LwCas13a protein expression and purification

The LwCas13a protein expression and purification were carried out as follows. Briefly, the *Escherichia coli* BL21 was transformed with pC013-Twinstrep-SUMO-huLwCas13a plasmid and screened using ampicillin resistance plates. The positive colonies are picked and inoculated in 8 mL TB medium with ampicillin at 37℃ and 220 rpm/s overnight. When the OD600 of the bacterial solution reached 0.6, 500 μM IPTG was added in to induce bacterial protein expression at 18℃ overnight. Cells were harvested by centrifugation at 5200 g for 15 min in 4℃ and lysed by sonication in lysis buffer and collected the supernatant. After the collected supernatant was filtered, the supernatant was incubated with streptavidin resin for 1 h. Then, the protein-bound streptavidin resin was lysed and resuspended in digestion buffer along with 1000 Units of SUMO protease at 4°C overnight. Following, a 10K MWCO centrifugal concentrator was applied to concentrate the supernatant and made a final volume concentrated to 1~2 mL in S200 buffer. Finally, the gained production was subjected to gel filtration chromatography by ÄKTA pure chromatography and confirmed by SDS-PAGE and Coomassie blue staining.

Target mRNAs and crRNA preparation

Target RNAs were obtained by PCR amplification of the synthesized plasmids with 50 μL of reaction mixture comprised 50 ng of plasmids, 750 nM forward primer, 750 nM reverse primer, and 1 × PrimeSTAR® Max DNA Polymerase. The reactions were performed on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Massachusetts, US). Thermocycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 32 cycles of amplification at 95°C for 30 s, 56°C for 20 s and 72°C for 20 s, a final extension was carried out at 72°C for 5 min and a 12°C hold. The amplified dsDNA products were purified with HiPure Gel Pure DNA Mini Kit (Magentec, Guangzhou, China), and analyzed by 3500 Series Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, US). Purified dsDNA was incubated with T7 RNA polymerase using the HiScribe T7 Quick High Yield RNA Synthesis Kit.

For preparation of crRNAs, a T7 promoter region was previous design in the dsDNA template for *in vitro* crRNA transcription, via gradient annealing (95°C for 5 min followed by cooling to room temperature at a ramp rate of 0.1°C/s).

The transcription reactions were performed with 100 nM dsDNA template, 2 μL T7 RNA

polymerase Mix, 10 mM ATP, 10 mM GTP, 10 mM UTP and 10 mM CTP in 1 × Reaction Buffer (40 mM Tris-HCl, 2 mM spermidine, 1 mM DTT, 6 mM $MgCl_2$, pH 7.9) at 37°C for 16 h. After digesting with DNase I at 0.2 U/μL to remove the DNA template, all transcription products were purified with HiPure RNA Pure Micro Kit and analyzed by native polyacrylamide gel electrophoresis (Native-PAGE), quantified by Nanodrop 200 (Thermo Fisher Scientific, Massachusetts, US) and stored at -80°C for further use.

Sample collection and processing

All NSCLC patient histological specimens were collected from Chongqing Cancer Hospital (Table S2). The study was approved by the Ethics Committee of Chongqing Cancer Hospital (No. CZLS2022096-A). The isolation of total RNA was achieved with an ADx® FFPE DNA/RNA Nucleic Acid Isolation Kit (AmoyDx, Xiamen, China) according to the manufacturer's protocol. Subsequently, the resulting total RNAs were qualified by Nanodrop 2000 (Thermo Fisher Scientific, Massachusetts, US) and stored at -80°C until use.

Total RNA extraction from patient samples

ADx® FFPE DNA/RNA Nucleic Acid Isolation Kit

1. Sample dewaxing

(i) Add 1mL xylene and 2 μL Tissue Tracer to the sample in a 1.5 ml centrifuge tube, vortex 10 s; centrifuge at 13,000 g for 2 min; discard the supernatant.

(ii) Add 1mL absolute ethanol and 2 μL Tissue Tracer to the tube, vortex 10 s. Centrifuge at 13,000 g for 2 min and discard the supernatant.

(iii) Place the tube in a heater at 56°C for 1-10 min, until the surface of the sample was not reflective.

2. Cell digestion

(i) Add 200 μL Buffer RTL and 25 μL Proteinase K to the sample, vortex and incubate at 500 rpm at 56°C for 30 min.

(ii) Centrifuge at 13,000 g for 2 min. Transfer 180 μL of supernatant to a new 1.5 mL centrifuge tube for next RNA extraction.

(iii) Add 140 μL Buffer DTL and 15 μL Proteinase K to the solution and pellet obtained from step (ii) vortex and incubate at 500 rpm at 56°C for 30 min digestion.

3. RNA isolation

(i) Transfer the solution obtained from step 2 to 80°C and incubate at 500 rpm for 30 min, and then cool to room temperature abruptly.

(ii) Add 30 μL DNase I mixture to the centrifuge tube, gentle pipetting and mixing, and digest at 37°C for 15 min.

(iii) Add 340 μL Buffer RTB and 750 μL absolute ethanol to above mixture, and vortex 5 s.

(iv) Transfer the solution obtained from step (3) to a column (RNA Absorption Column) with a collection tube; centrifuge at 13,000 g for 30 s; discard the waste in the collection tube; and place the column back in the collection tube.

(v) Add 600 μL Wash Buffer A to the column; centrifuge at 13,000 g for 30 s; discard the waste in the collection tube; and place the column back in the collection tube.

(vi) Add 600 μL Wash Buffer B to the column; centrifuge at 13,000 g for 30 s; discard the waste in the collection tube; and place the column back in the collection tube.

(vii) Repeat the step (vi), and discard the tube.

(viii) Place the column in a new 1.5 ml centrifuge tube, and incubate at 56°C for 3 min.

(ix) Add 80-100 μL of Buffer RTE in the middle of the membrane, and incubate at 56°C for 2 min with cover closed. Then, centrifuge at 13,000 rpm for 1 minute, and collect the RNA solution. If it is not used immediately, store at -70°C.

For the detailed ADx® FFPE DNA/RNA Nucleic Acid Isolation Kit protocol, please visit http://www.amoydx.com/productDetail_29.html.

Native-PAGE

For 12% native polyacrylamide gel electrophoresis (native PAGE), 8 mL 30% w/v acrylamide/methylene bisacrylamide (29:1) stock solution (Sangon Biotech, Shanghai, China), 4 mL 5 × TBE, 20 μL of N,N,N',N'-tetramethylethylenediamine (TEMED), 200 μL of 10% w/v freshly dissolved ammonium persulfate (APS) and distilled water to a final volume of 20 mL were mixed together. After solidification, the analytes were incubated in 1 × TBE at 90 mV for 100 min. Gels were stained with 4S Red Plus Nucleic Acid Stain (Sangon Biotech, Shanghai, China) and visualized by a ChemDoc™ MP Imaging System (Bio-Rad, California, US).

Sanger sequencing

Purified EML4-ALK V1 dsDNA amplified products (2 ng/μL) were used for sequencing PCR with the aid of reverse primer (500 pM), Big Dye (2 μL) and Big Dye Sequencing buffer (3 μL). They were brought to 20 μL in double-distilled water (ddH_2O) and sequenced as follows: 1 min at 96°C, then 25 cycles of 96°C for 30 s, 56°C for 20 s and 72°C for 20 s, and a final hold at 4°C. Sequencing products were purified with ethanol/EDTA/NaAc precipitates according to Chen. et al (sequencing products: EDTA (125 mM): NaAc (3M, pH 5.2) = 10: 1: 1). After the products were air-dried, 10 μL Hi-Di formamide was added, vortexed and centrifuged to dissolve DNA. Then, the supernatant was transferred into a plate and covered with a rubber pad. Sequencing was performed by capillary electrophoresis on an ABI3500 Genetic Analyzer (Applied Biosystems, Foster City, US) with Seq_Std_BDTV3.1_ASSY_POP7 automatically. Finally, the results were aligned and examined by sequencing analysis.

LwCas13a-triggered recognition

Recognition assays were performed with 100 nM purified LwCas13a, 100 ng/μL crRNA, 160 nM mediator RNA, 1 U/μL RNase inhibitor, 40 ng/μL of background total human RNA, and 2 μL varying amounts of input EML4-ALK V1 RNA target in nuclease buffer at 37°C for 40 min.

Collateral cleavage assisted signal amplification

The reaction combining CHA signal amplification and Cas13a-triggered collateral cleavage conversion of target RNAs to CHA was performed by integrating the reaction conditions above with CHA mix. Briefly, a 50 μL mixture consisted of 75 nM H1, 75 nM H2, 10 mM KCl solution in recognition reaction at 37°C for 30 min.

Statistical analysis

All experiments and assays were repeated three times. The statistical tests were analyzed with GraphPad Prism version 8.0 and SPSS version 20.0. One-way analysis of variance (ANOVA) was adopted for the comparison of multiple groups with a suitable post hoc test. *p* values less than 0.05 were considered statistically significant. Cut-off values were selected with the average value minus 1.96SD (95% confidence level) of negative samples (in the absence of target fusion genes).

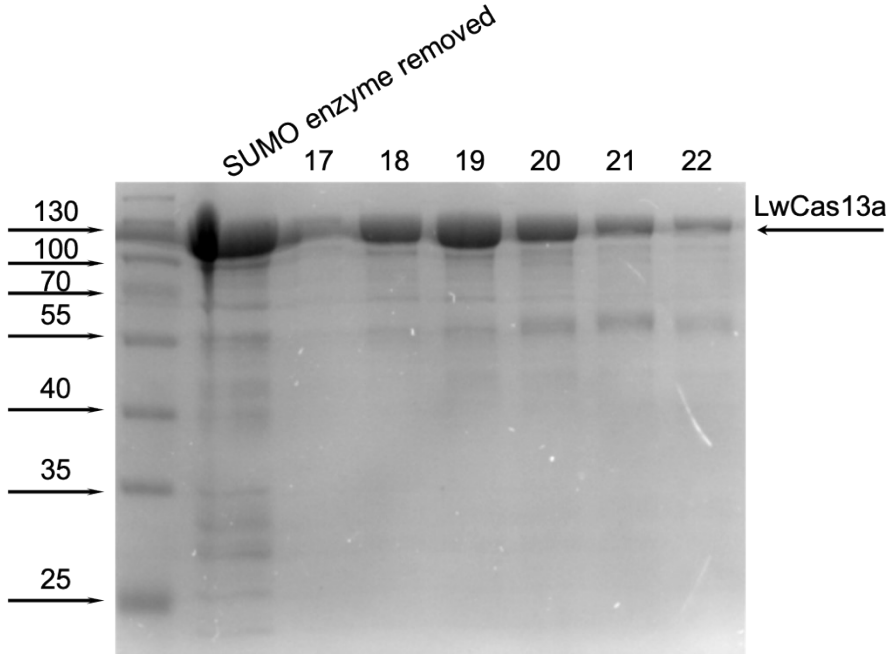

Fig. S1 Coomassie blue stained acrylamide gel of fraction size in 17-22.

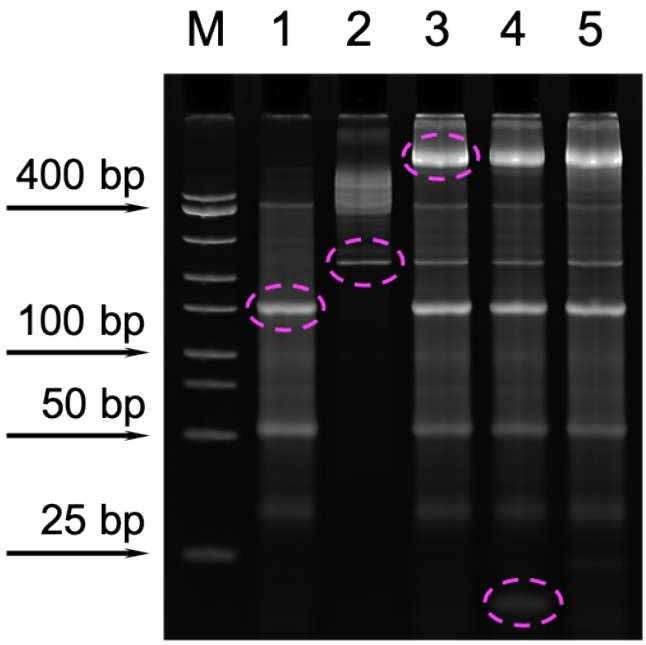

Fig. S2 Native-PAGE analysis for the Cas13a-induced recognition and cleavage assay. Line 1 shows the EML4-ALK RNA of amplification and transcription, line 2 shows the transcript crRNA, line3 shows the complex of Cas13a/crRNA, line 4 and 5 show the cleaved products without or with Cas13a/crRNA cleavage process, respectively.

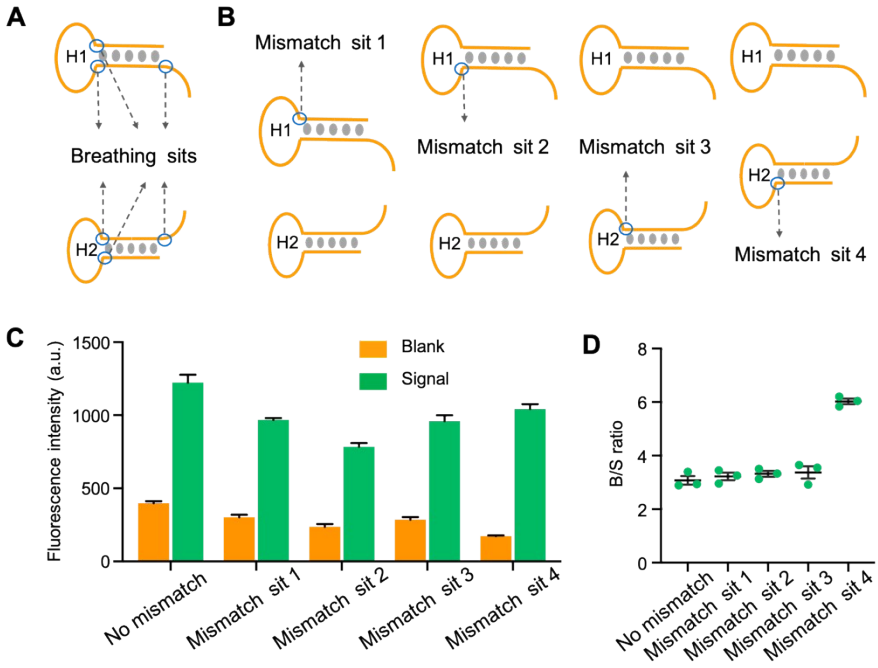

Fig. S3 Mechanism of breathing sits of CHA. (A) Schematic overview of breathing sits of H1 and H2 in conventional CHA reaction. (B) The four mismatch positions correspond to the revealed interactions between H1 and H2. (C) The peak of fluorescence intensities and (D) B/S ratio corresponding to the four different mismatches, respectively. Error bars represent the mean ± SEM, where n = 3 independent replicates.

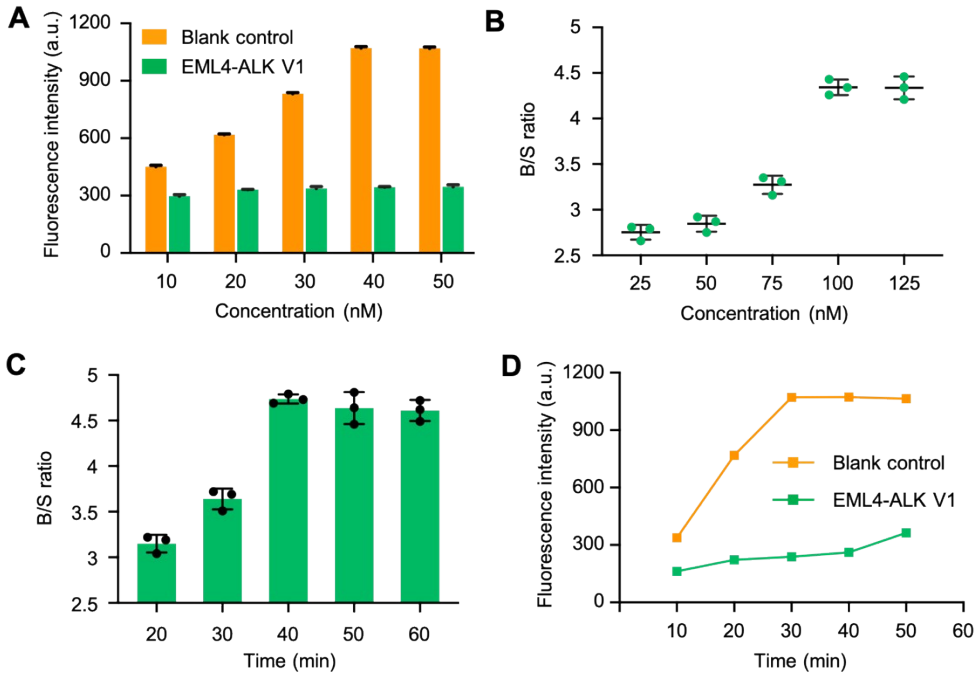

Fig. S4 Optimization of various parameters. The concentration of (A) mediator RNA and (B) Cas13a. The incubation time of (C) Cas13a-induced recognition and (D) collateral cleavage assisted signal amplification.

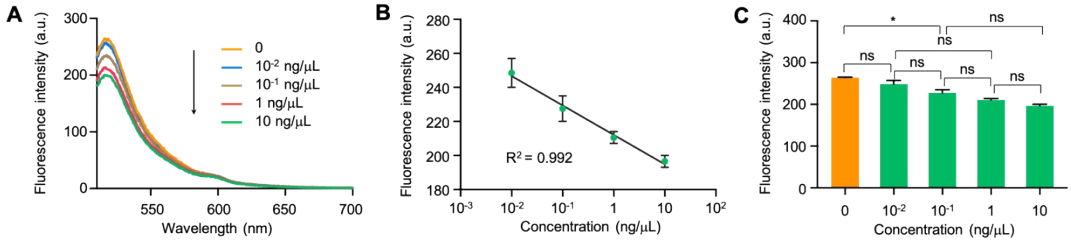

Fig. S5 Quantitation performance of the Cas13a-triggered recognition and collateral detection. (A) Fluorescence spectra and (B) Correlation of the logarithm of the target EML4-ALK V1 RNA at various concentrations. (C) Quantitation of the target EML4-ALK V1 RNA concentration with detected fluorescence. Error bars represent the mean ± SEM, where n = 3 independent replicates.

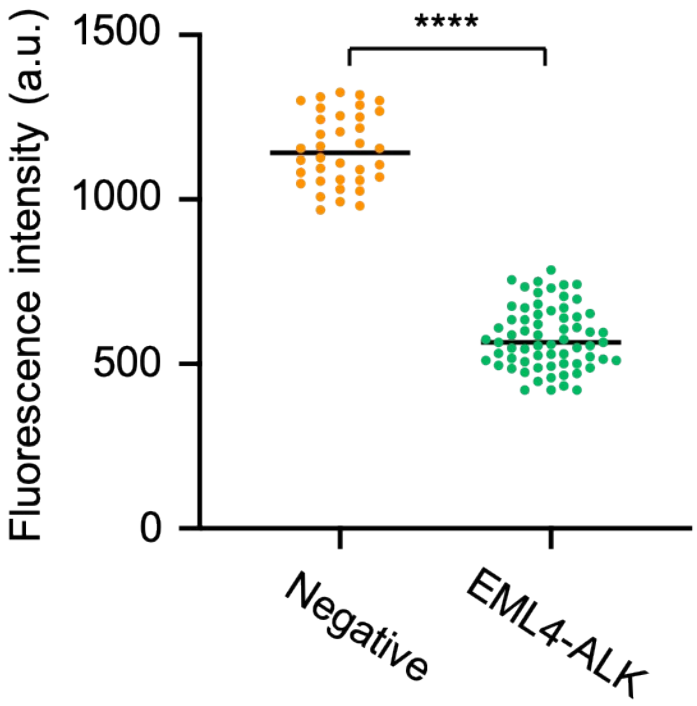

Fig. S6 The fluorescence intensities for the negative and EML4-ALK (+) in the clinical samples from NSCLC patients (****, $p < 0.0001$).

Table S1. Oligonucleotide sequences used in this study; sequence orientation is presented 5'-3'.

Name	Sequence (5'-3')
V1-F	AATCTAATACGACTCACTATAGGAATGGAGATGTTCTTACT
V1-R	CATGATGGTCGAGGTGCGGAGC
V2-F	AATCTAATACGACTCACTATAGCACTGGACATTCCAGCTAC
V2-R	CATGATGGTCGAGGTGCGGAGC
V3a-F	AATCTAATACGACTCACTATAGTAATAAATTGTCGAAAATA
V3a-R	CATGATGGTCGAGGTGCGGAGC
V3b-F	AATCTAATACGACTCACTATAAATTAATACCAAAAGTTACC
V3b-R	CATGATGGTCGAGGTGCGGAGC
V4-F	AATCTAATACGACTCACTATACAGATGAGAAATGGGATGTT
V4-R	GTCTTGCCAGCAAAGCAGTAGT
V5a-F	AATCTAATACGACTCACTATACTGTGCTAAAGGCGGCTTTG
V5a-R	CATGATGGTCGAGGTGCGGAGC
V5b-F	AATCTAATACGACTCACTATACTGTGCTAAAGGCGGCTTTG
V5b-R	AACATGGCCTGGCAGCCTGGCC
KIF5B-ALK-F	AATCTAATACGACTCACTATACAGAAATAGGAATTGCTGTG
KIF5B-ALK-R	CAGTAGTTGGGGTTGTAGTCGG
TFG-ALK-F	AATCTAATACGACTCACTATACGCTTAACAGATGATCAGGT
TFG-ALK-R	CAGCTTGTACTCAGGGCTCGCA
KLC1-ALK-F	AATCTAATACGACTCACTATACAACCTGGCATCCTGCTATT
KLC1-ALK-R	TGGTCGAGGTGCGGAGCTTGC
HIP1-ALK-F	AATCTAATACGACTCACTATACCTGCCTCAGAGCCCCACCT
HIP1-ALK-R	CAGCTTGTACTCAGGGCTCGCA
STRN-ALK-F	AATCTAATACGACTCACTATACAAGGATCTTGTGAGGAGGA
STRN-ALK-R	TCTTGCCAGCAAAGCAGTAGTT
EML4-F	AATCTAATACGACTCACTATAGGAATGGAGATGTTCTTACT
EML4-R	CCCTCCTCCAGTTAATAACATCCCAT
ALK-F	AATCTAATACGACTCACTATAGGAGCCACACCTGCCACTC
ALK-R	CATGATGGTCGAGGTGCGGAGC
V1 crRNA template	GAAAGGACCTAAAGTGTACCGCCGGAAGGTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAGCTT
V2 crRNA template	TGAAATATTGTACTTGTACCGCCGGAAGGTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAGCTT
V3a crRNA template	CATCATCAACCAAGTGTACCGCCGGAAGGTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAGCTT

V3b crRNA template	AAAAAACAGCCAAGTGTACCGCCGGAAGGTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAGCTT
V4 crRNA template	AAAGAGAAATAGAGATATGCTGGATGAGGTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAGCTT
V5a crRNA template	AGTCTCAAGTAAAGTGTACCGCCGGAAGGTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAGCTT
V5b crRNA template	AGTCTCAAGTAAAGGTTCAGAGCTCAGGGTTTTAGTCCCCTTCGTTTTTGGGGTAGTCTAAATCCCCTATAGTGAGTCGTATTAGCTT
T7 promoter	AAGCTAATACGACTCACTATA
Trigger RNA	CCAUUUUUAACCGGUCUCUUUUUC
H1	GAAAAAG/i6FAMdT/GACCGGTTAAAAATGGCCATGTGTAGACCATTTTTAACCGGTC-Dabcyl
Mismatch sit 1	GAAAAAG/i6FAMdT/GACCGGTTAAAAATGGCCATGTGTA*C* *G*CCATTTTTAACCGGTC-Dabcyl
Mismatch sit 2	GAAAAAG/i6FAMdT/GACCGGTTAAAAATGG*AA*ATGTGTAGACCATTTTTAACCGGTC-Dabcyl
H2	AAAAATGGTCTACACATGGCCATTTTTAACCGGTCTCTT
Mismatch sit 3	AAAAATGGTCTACACA*AC*GCCATTTTTAACCGGTCTCTT
Mismatch sit 4	AAAAATGGT*GA*ACACATGGCCATTTTTAACCGGTCTCTT
Reporter	FAM-UUUUUU-Dabcyl

The underline and italic portion represent the mismatched bases in hairpin substrates.

Table S2. Characteristics of the clinical samples.

Sample ID	Specimen	Histology	Concentration of extracted RNA (ng/μL)
1	Tissue	Adenocarcinoma	129.3
2	Tissue	Adenocarcinoma	34.1
3	Tissue	Adenocarcinoma	161.6
4	Tissue	Adenocarcinoma	69.3
5	Tissue	Adenocarcinoma	137.5
6	Tissue	Adenocarcinoma	60.2
7	Tissue	Adenocarcinoma	35
8	Tissue	Adenocarcinoma	75.8
9	Tissue	Adenocarcinoma	156.6
10	Tissue	Adenocarcinoma	155.4
11	Tissue	Adenocarcinoma	208.6
12	Tissue	Adenocarcinoma	64.6
13	Tissue	Adenocarcinoma	38.2
14	Tissue	Adenocarcinoma	43.12
15	Tissue	Adenocarcinoma	90.2
16	Tissue	Adenocarcinoma	58.7
17	Tissue	Adenocarcinoma	31.42
18	Tissue	Adenocarcinoma	174.3
19	Tissue	Adenocarcinoma	198.8
20	Tissue	Adenocarcinoma	157.8
21	Tissue	Adenocarcinoma	114.8
22	Tissue	Adenocarcinoma	160.2
23	Tissue	Adenocarcinoma	184.9
24	Tissue	Adenocarcinoma	125.7
25	Tissue	Adenocarcinoma	226.7
26	Tissue	Adenocarcinoma	28.4
27	Tissue	Adenocarcinoma	35.9
28	Tissue	Adenocarcinoma	138.8
29	Tissue	Adenocarcinoma	13.3
30	Tissue	Adenocarcinoma	10.1
31	Tissue	Adenocarcinoma	387.4
32	Tissue	Adenocarcinoma	379.7
33	Tissue	Adenocarcinoma	56.23
34	Tissue	Adenocarcinoma	9.98

Table S3. Comparison of fusion gene detection methods.

Signal amplification[a]	Analytical method[b]	Target	Variants	Linear range	Detection limits	Time (h)	Ref.
RF-RCA	PCR	RNA	Yes	10^{-19} ~ 5×10^{-13} M	10^{-19} M	2.5	14
DNA track	Electrochemiluminescent	DNA	No	10^{-15} ~ 10^{-10} M	1.8×10^{-16} M	1	21
Nanofluidic-enhanced solid-phase isothermal amplification	Chronoamperometric Electrochemical	RNA	No	50～1000 copies	50 copies	0.5	22
Locker probe enrichment and magneto-bioelectrocatalytic cycling	Electrochemical	RNA	Yes	10~10^5 copies	10 copies	1	23
DDPCR™	Fluorescence	DNA	No	1~5 ng/μL	425 pg/μL	1	24
RNA/probe hybridization	NanoString nCounter	RNA	Yes	10 ~ 30 ng/μL	10 ng/μL	36	25
PEI-Lum and DNAzyme motor	Electrochemiluminescent	DNA	No	10^{-14} ~ 10^{-8} M	3.75×10^{-15} M	1	26
ET-HC	Fluorescence	RNA	Yes	2×10^{-18} ~ 2×10^{-13} M	5×10^{-19} M	2	27
RT-LAMP	Fluorescence and Colorimetric	RNA	Yes	5×10^{-3} ~ 5 ng/μL	1.25×10^{-2} ng/μL	1	28
CATCH	Fluorescence	RNA	Yes	10^{-4}~10 ng/μL	4.9×10^{-5} ng/μL	1	This work

[a] RF-RCA, RNA fusion-triggered rolling circle amplification, RT-LAMP, Reverse-transcriptase loop-mediated isothermal amplification, ET-HC, Exponential transcription-triggered hemin catalysis strategy, PEI-Lum, self-enhanced polyethyleneimine-luminol, LIEXA, Ligation-triggered isothermal exponential amplification, POLARA, polymerase-amplified release of ATP, DDPCR™, droplet digital PCR

Table S4. Performance of CATCH compared to NGS.

Method	Mutation	Positive	Negative	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV[b] (%)	Concordance (%)
NGS	EML4-ALK	22	0	/	/	/	/	/
	Not detected	0	22					
CATCH	EML4-ALK	22	0	100	100	100	100	100
	Not detected	0	22					

[a]PPV: Positive predictive value; [b]NPV: Negative predictive value.

Table S5. Definitions of true positives (TPs), false positives (FPs), true negatives (TNs), and false negatives (FNs).

	NGS	CATCH
TP	Related fusion gene detection	Weak fluorescence intensity
FP	Unrelated fusion gene detection	Strong fluorescence intensity
TN	Unrelated fusion gene detection	Strong fluorescence intensity
FN	Related fusion gene detection	Weak fluorescence intensity

Several analytic performance factors of the proposed strategy were calculated with the following equations:

$$Sensitivity = \frac{TP}{TP + FN}$$

$$Specificity = \frac{TN}{TN + FP}$$

$$PPV = \frac{TP}{TP + FP}$$

$$NPV = \frac{TN}{TN + FN}$$

$$Concordance = \frac{TP + TN}{TP + FP + TN + FN} \times 100$$