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Circularized blocker-displacement amplification for multiplex detection of rare DNA variants

Wei Lai,[a] Mingshu Xiao,[a] Haihong Yang,[a] Li Li,[a] Chunhai Fan[b] and Hao Pei*[a]

[a.] *Shanghai Key Laboratory of Green Chemistry and Chemical Processes, School of Chemistry and Molecular Engineering, East China Normal University, 500 Dongchuan Road, Shanghai, 200241, P. R. China*

[b.] *School of Chemistry and Chemical Engineering, and Institute of Molecular Medicine, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200240, China.*

Experimental Section

Reagents. Enzymes used in this work were purchased from NEB (New England Biolabs), unless otherwise stated. Ampligase thermostable DNA ligase was provided by Epicentre Technologies. dNTPs, SYBR Green I, SYBR Green II RNA gel stain, Marker I DNA Ladder, TEMED, APS, 40% Acr/Bis (29: 1) were bought from Solarbio (Beijing), QIAamp DNA Mini Kit (50) from Qiagen (Germany). Other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd, unless otherwise stated.

DNA oligonucleotides. All DNA oligonucleotides including primer 1, primer 2, non-modified blocker, padlock probes, and synthetic DNA template oligonucleotides were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China) with standard desalting, purified with HPLC, and used without further purification. Cancer mutation reference gDNA samples were bought from Horizon Discovery, and the samples with different proportions of a specific allele were produced by mixing the two gDNA samples at various ratios. DNA oligonucleotides were resuspended in 1× TE buffer and stored at 4 °C for further use.

Circularization of padlock probes. To prepare circularized probes, 50 μL of 1× ampligase reaction buffer (20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM

NAD and 0.01 % Triton X-100) containing synthetic DNA templates/genomic DNA targets (100 nM), padlock probes (100 nM), dNTP (200 μM), blocker 1 (300 nM), Taq DNA polymerase (0.25 U/μL), and Ampligase DNA ligase (0.5 U/μL) were incubated for 5 min at 95 °C and 2 h at 60 °C for hybridization of padlock probes with target DNA, gap filling, and probe ligation. Following, exonuclease I (0.5 U/μL) and exonuclease III (10 U/μL) were added into the mixture at 37 °C for 20 min, at 95 °C for 5 min and at 37 °C for 5 min to digest linear DNA (the unreacted padlock probes and the excess oligonucleotides) and inactivate enzyme activity. Then the yielded products were purified with electrophoresis and stored at 4 °C for further use.

CirBDA assay. CirBDA assays were conducted in 20 μL ThermoPol buffer (20 mM Tris•HCl (pH 8.8), 10 mM KCl, 2.7 mM $MgSO_4$, 5% v/v DMSO, 0.1% Triton X-100), containing the synthesized circularized probes (10 nM), blocker 2 (1.5 μM), primer 1 (1 μM), primer 2 (0.5 μM), dNTP (200 μM), and Vent (exo-) DNA polymerase (0.2 U/μL). Additionally, Phage T4 gene-32 protein (50 $ng \cdot \mu L^{-1}$) was added. Thereafter, the mixture was incubated at 92 ºC for 3 min, followed by at 60 ºC for 60 min. Finally, the CirBDA products were stored at 4 ºC for further use.

Extraction of KRAS gDNA. KRAS gDNA was extracted from human colon cancer cell line (SW48 and SW620, provided by Fuheng Biology) according to the following procedure. In typical, SW48 (WT)/SW620 (VT, G12V) cells were cultured in Dulbecco's Modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Sijiqing Corporation, Hangzhou) and 100 U/mL penicillin-streptomycin. Then, the harvested cells (5×10^6) were lysed using the QIAamp DNA Mini Kit (50) on the basis of the standard handbook protocols. The KRAS gDNA was purified by AGE and quantified with UV-visible absorption spectrum (Agilent Technologies, Cary 60).

Sanger sequencing. Circularization of padlock probes and CirBDA reactions were carried out as mentioned in ‘Circularization of padlock probes and CirBDA assay’. The yielded CirBDA products were purified and sequenced by Sangon Biotech (Shanghai) Co., Ltd.

Electrophoretic analysis. The circularized probes were analyzed by precast 8% PAGE with 1× TBE buffer containing 8 M urea. The PAGE gels were run at 200 V for 80 min, which were stained with SYBR Green II after electrophoresis and imaged by a UV transilluminator of the UVP GelDoc-It 310 Imaging System with an excitation wavelength of 365 nm. CirBDA products were then analyzed using 3% AGE running at 100 V for 3 h. AGE gels were stained with GelRed after electrophoresis and visualized under UV light.

Fluorometric analysis. 20×SYBR green I was added to the CirBDA products to enable the fluorescence signal generation. Fluorescence spectra were recorded by F-7000 spectrometer (Hitachi, Japan), and the measurement settings were fixed at 497 nm with a recording emission range from 500 to 620 nm.

The discrimination factor is defined as the VT-to-WT fluorescent intensity ratio with the same experimental conditions (DF = F_{VT}/F_{WT}, F denotes the fluorescence intensity), where F_{VT} and F_{WT} are fluorescence intensity of VT and WT, respectively.

CirBDA technique for SNV analysis. Given its superselective amplification capability, we utilized this CirBDA technique for SNV analysis under optimal experimental conditions (primer 2 concentration: 0.5 mM (Fig. S3, ESI†), dNTP concentration: 200 μM (Fig. S4, ESI†)). To test the detection performance of our CirBDA technique, we prepared gDNA samples with different VAFs (from 0% to 100%) by mixing WT and VT samples at specific ratios with total gDNA concentration of 10 pM for fluorescence measurement of G12S C>T SNV. As shown in Fig. S5 (ESI†), the fluorescence signal was found to increase with increasing VAF since the samples with higher VAF can produce more CirBDA products. The discrimination factor (DF, defined as F_{VT}/F_{WT}) is used to evaluate the selectivity capability between VT and WT. A high DF value is essential to ensure the detection of low-abundance DNA variants. We discovered that 1398-fold signal was produced for the gDNA sample with 100% VAFs and 41.4-fold signal for the gDNA sample with 0.1% VAFs (Fig. S1e, ESI†). Moreover, a linear relationship between DF and VAFs ranging from 0.1% to 100% was observed (Fig. S1e, ESI†), indicating that our method can even distinguish as low as a 0.1% SNV, which is superior to or

comparable to other amplification techniques for SNV detection.[1] The high sensitivity of our CirBDA technique can be attributed to the superselective amplification of DNA variants signal and efficient suppression of WT signal. Given its capability of efficient selection, maximized enrichment, and amplified signal output of DNA variants, our CirBDA technique can be exploited for rare mutations detection.

Figures and Table

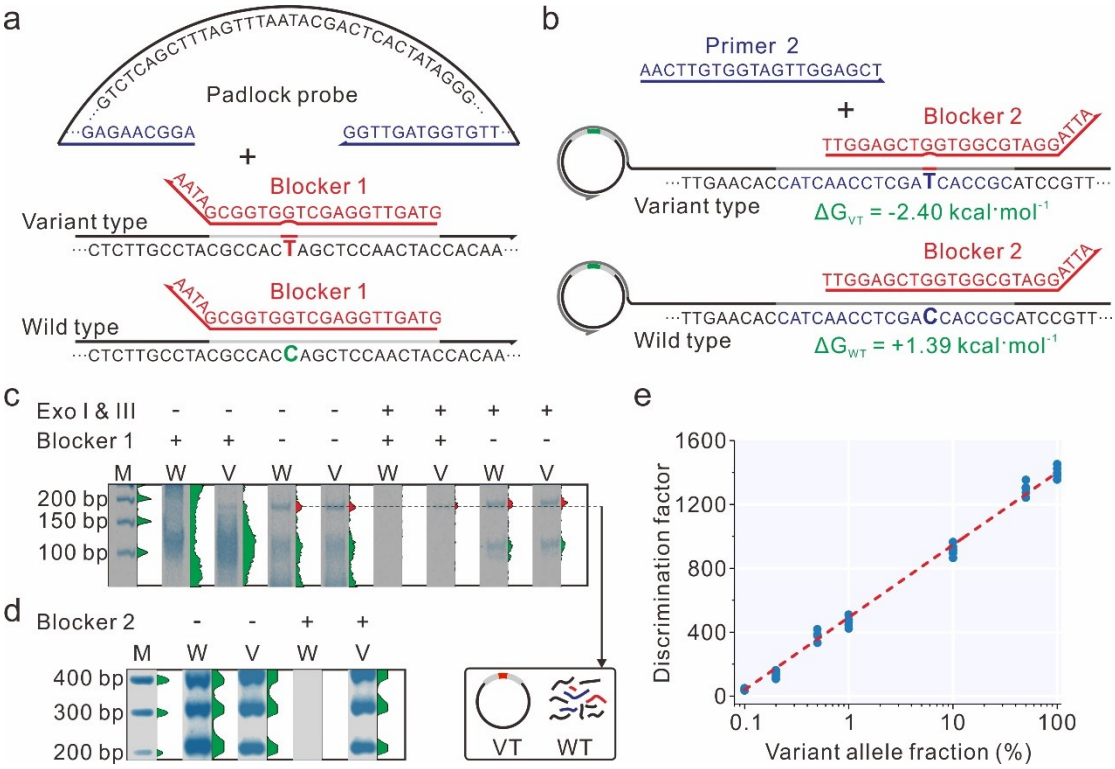

Fig. S1 CirBDA technique for detecting KRAS SNV. Scheme of CirBDA for selective enrichment of KRAS mutations, including (a) selective displacement of blocker 1 hybridizing to VT by padlock probe through intramolecular SDR and (b) selective displacement of blocker 2 hybridizing to VT by primer 2 through intermolecular SDR. (c) PAGE analysis of the formation of circularized probe. (d) Electrophoretic analysis of CirBDA products of allele gDNA in the presence or absence of blocker 2 and VT template. (e) DF as a function of different VAFs. The error bars represent the standard deviations calculated from five independent experiments.

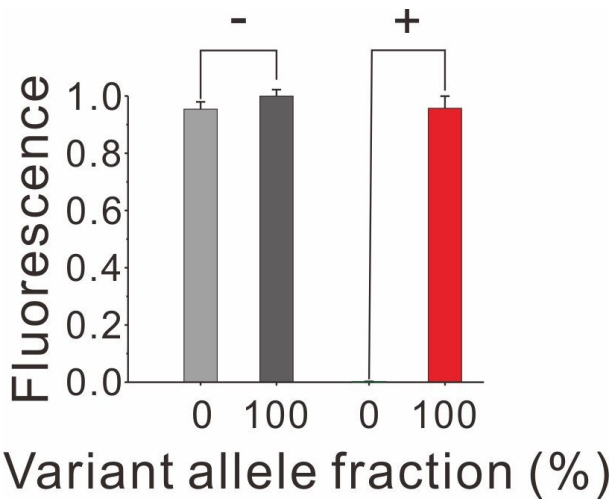

Fig. S2 Fluorescence measurement of CirBDA products of allele gDNA in the absence (–) or presence (+) of blocker 2 for both WT and VT templates.

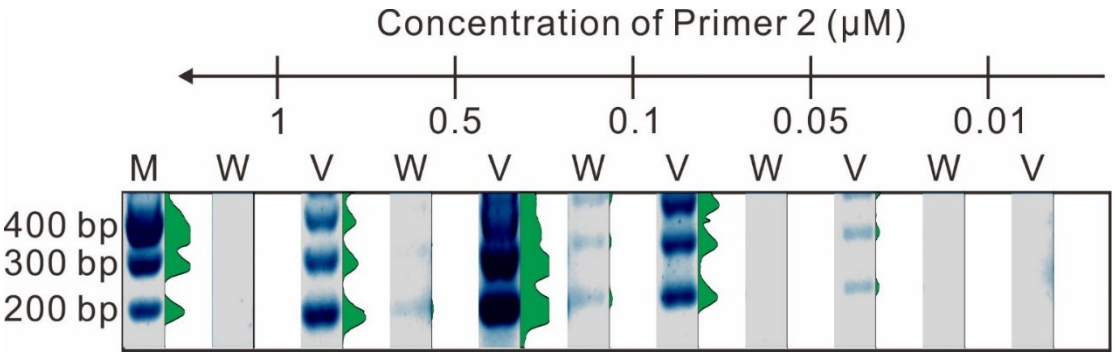

Fig. S3 Optimization of primer 2 concentration. Electrophoretic identification of the CirBDA products by 3% AGE and SYBR Green I-staining. Lanes represent the DNA ladder marker, HRCA products from 1 μM primer 2, 0.5 μM primer 2, 0.1 μM primer 2, 0.05 μM primer 2, and 0.01 μM primer 2 from left to right, respectively.

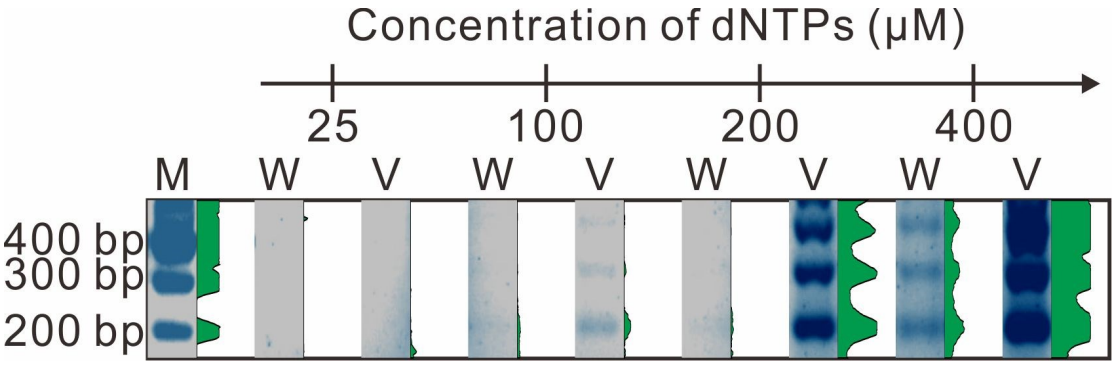

Fig. S4 Optimization of dNTP concentration. Electrophoretic identification of the CirBDA products by 3% AGE and SYBR Green I-staining. Lanes represent the DNA ladder marker, HRCA products from 25 μM dNTP, 100 μM dNTP, 200 μM dNTP, and 400 μM dNTP from left to right, respectively.

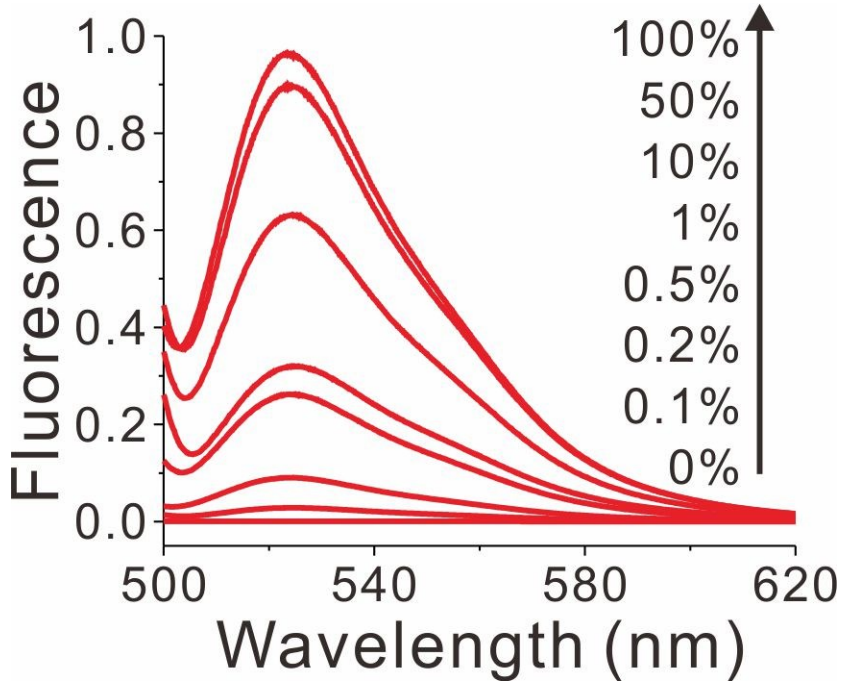

Fig. S5 Fluorescence measurement of CirBDA products with different VAFs (from 0% to 100% V/W).

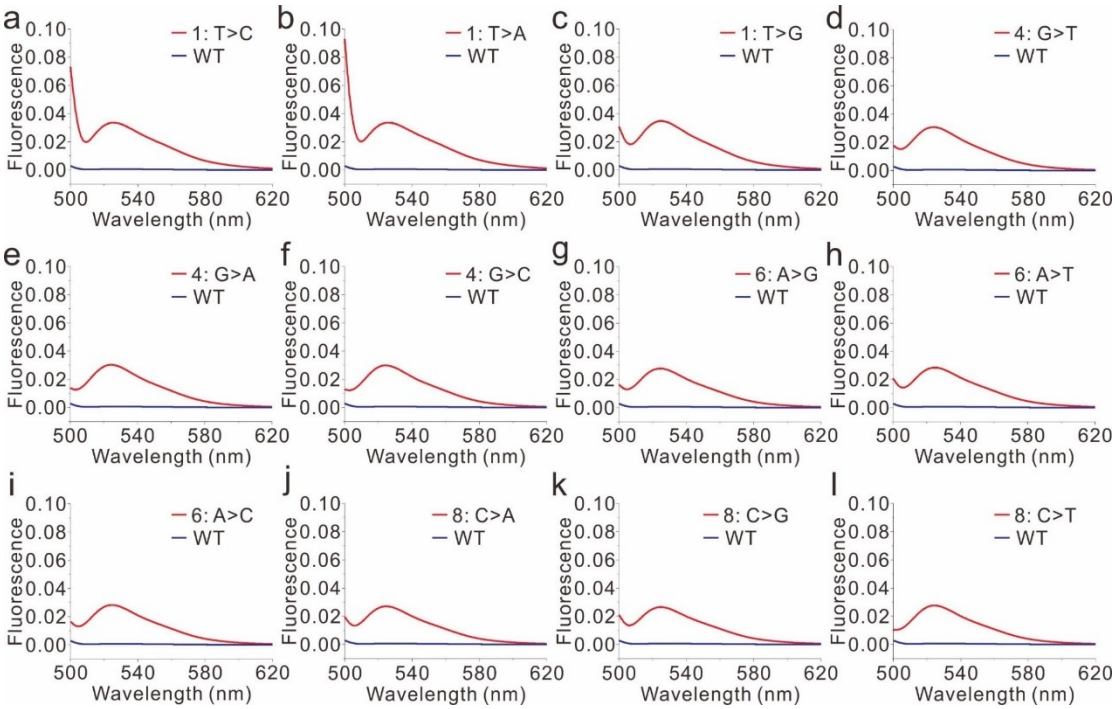

Fig. S6 Fluorescence spectra of CirBDA products of different hotspot mutants of KRAS (G12S).

Table S1. DNA oligonucleotides used in this work

Experiment	DNA name	DNA sequence (5'-3')
Blocker-mediated HRCA assay	Primer 1	CTAAAGCTGAGACATGACGAGTC
	Primer 2	AACTTGTGGTAGTTGGAGCT
	Blocker 1	GTAGTTGGAGCTGGTGGCGATAA
	Blocker 2	TTGGAGCTGGTGGCGTAGGATTA
	Padlock probe	AGGCAAGAGTGCCTTGACGATACAGCATTCTGACTCGTCATGTC TCAGCTTTAGTTTAATACGACTCACTATAGGG TATAAACTTGTGGTAGTTGG
	WT template	TTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCAC**C**AGCTCCAA CTACCACAAGTTTATATTC
	VT template	TTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCAC**T**AGCTCCAA CTACCACAAGTTTATATTC
Hotspot detection of BRAF rs3789806	Primer 1	CTAAAGCTGAGACATGACGAGTC
	Primer 2	CTTGTATATAGACGGTAAAATAAACACCA
	Blocker 1	AATAAACACCAAGACGTGGTAAATAAAAT
	Blocker 2	AAACACCAAGACGTGGTAAATATTTACCTGGAAAA
	Padlock probe	AATATTTACCTGGTCCCTGTTGTTGATGTTCTGACTCG

Detection of clinical sample	WT template	TTTACCTGGTCCCTGTTGTTGATGTTCTGACTCGTCATGTCTCAG CTTTAGTTTAATACGACTCACTATAGGGACTTGTATATAGACGGT AAAATAAACA AAACATCAACAACAGGGACCAGGTAAATATTTACCACGTCTTG GTGTTTATTTTACCGTCTATATACAAGGCT
	Variant-1A	AAACATCAACAACAGGGACCAGGTAAATATTTACCACGTCATGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-1G	AAACATCAACAACAGGGACCAGGTAAATATTTACCACGTCGTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-1C	AAACATCAACAACAGGGACCAGGTAAATATTTACCACGTCCTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-4A	AAACATCAACAACAGGGACCAGGTAAATATTTACCACATCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-4C	AAACATCAACAACAGGGACCAGGTAAATATTTACCACCTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-4T	AAACATCAACAACAGGGACCAGGTAAATATTTACCACTTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-6G	AAACATCAACAACAGGGACCAGGTAAATATTTACCGCGTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-6C	AAACATCAACAACAGGGACCAGGTAAATATTTACCCCGTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-6T	AAACATCAACAACAGGGACCAGGTAAATATTTACCTCGTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-8A	AAACATCAACAACAGGGACCAGGTAAATATTTAACACGTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-8G	AAACATCAACAACAGGGACCAGGTAAATATTTAGCACGTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Variant-8T	AAACATCAACAACAGGGACCAGGTAAATATTTATCACGTCTTGG TGTTTATTTTACCGTCTATATACAAGGCT
	Primer 1	CTAAAGCTGAGACATGACGAGTC
	Primer 2	CACTCTTGCCTACGCCA
	Blocker 1	GCCTACGCCACCAGCTCATTA
	Blocker 2	GCCTACGCCACCAGCTCATTA
	Padlock probe	CCAACTACCACAAGTTTATAATTCTGACTCGTCATGTCTCAGCTTT AGTTTAATACGACTCACTATAGGGGCTGTATCGTCAAGGCACTC TT

Table S2. Comparison of different strategies for multiplex detection of rare DNA variants

Strategies for DNA variant detection	Mutation limit of detection (%)	Discrimination factor	Ref.
Circularized blocker-displacement amplification	0.1	Range: 37.7 to 49.5	This work
Simulation-guided DNA probe	0.1	Range: ~200 to 2720	2
Modified DNA structure in combination with Lambda exonuclease	0.5%	Range: 4.2 to 320	3
Energy driven cascade recognition	NA	Range: 9.5 to 25.9	4
Sequestration-assisted molecular beacons	0.5%	Range: 12 to 1144	5
Strand displacement and selective digestion	0.2%	Range: 50.2 to 970	6
A dynamic sandwich assay	0.1%	393	7
Hairpin masking with fluorescent nanoparticle counting	0.05%	Range: 204 to 1176	8
Toehold-mediated strand displacement triggered isothermal DNA amplification	NA	Range: 7.4 to 12.5	9
Rolling circle amplification combined with gold nanoparticles-aptamer labeling	NA	27	10
Gold nanoparticle-conducted DNA bioarrays	NA	Range: 6.8 to 19.7	11
Spontaneous cascade DNA branch migration	NA	Range: 7.8 to 17.9	12

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