INSELGRUPPE

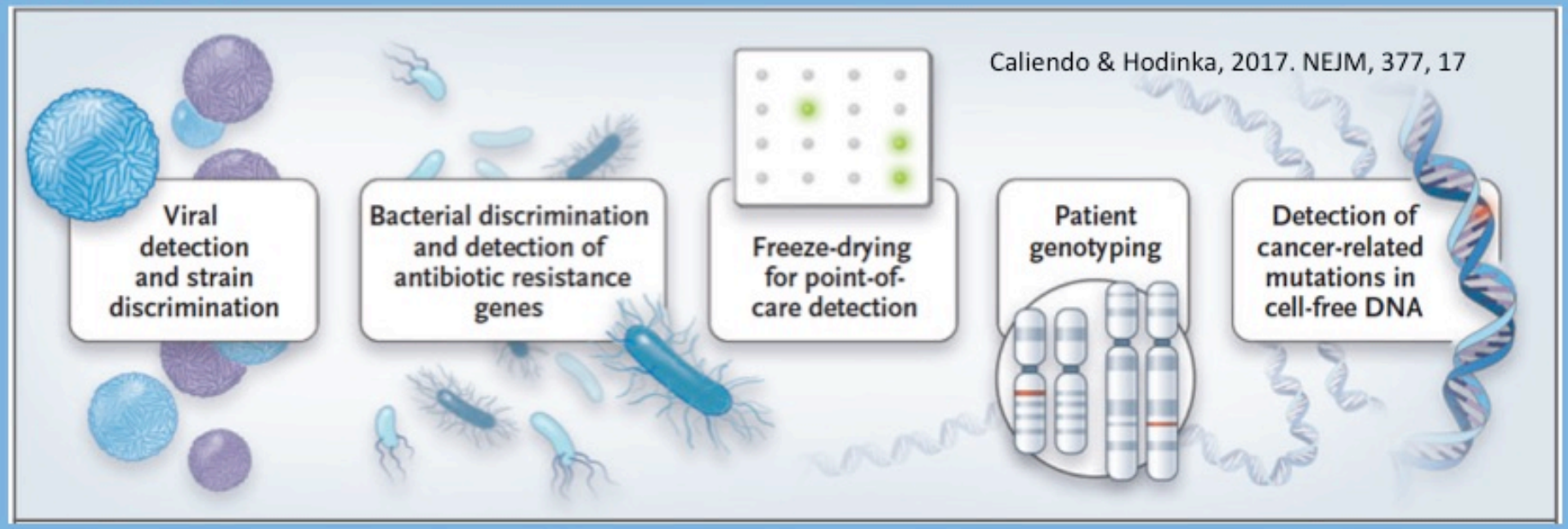
UNIVERSITÄT BERN

zlm zentrum für labor medizin bern

CRISPR-Based Nucleid Acid Tests: Basic Principles and Future Potential

Molecular Diagnostics 2018 Symposium

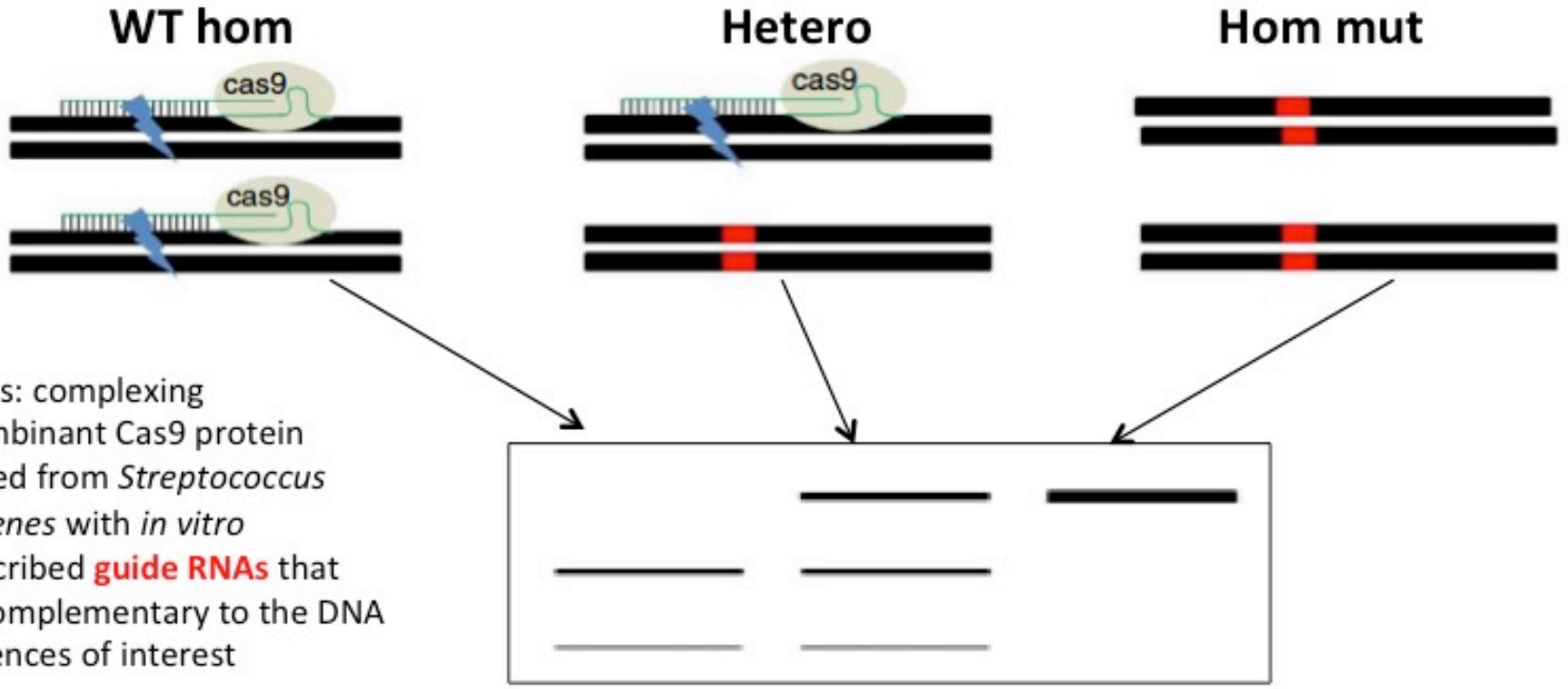
Carlo Largiadèr, Center of Laboratory Medicine – University Institute of Clinical Chemistry, Inselspital, University Hospital Bern



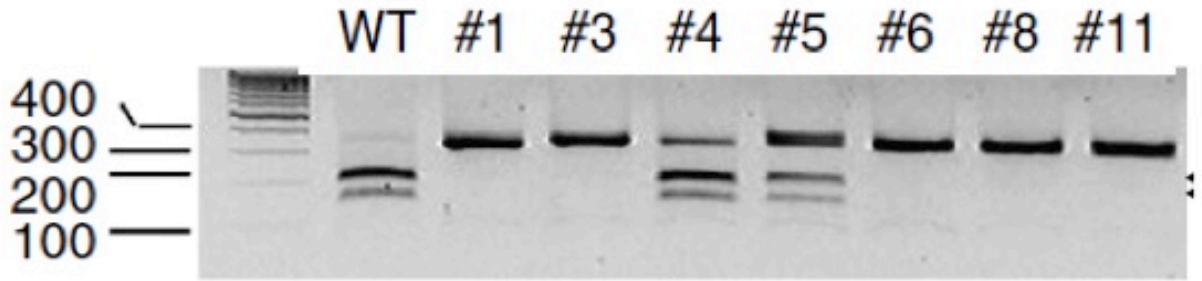
March 8; Credit Swiss Forum, St. Peter, Zurich

CRISPR/Cas-derived RNA-guided engineered nucleases (RGENs) in RFLP analysis

Kim et. al. 2014. Genotyping with CRISPR-Cas-derived RNA-guided Endonucleases NATURE COMMUNICATIONS, 5:3157

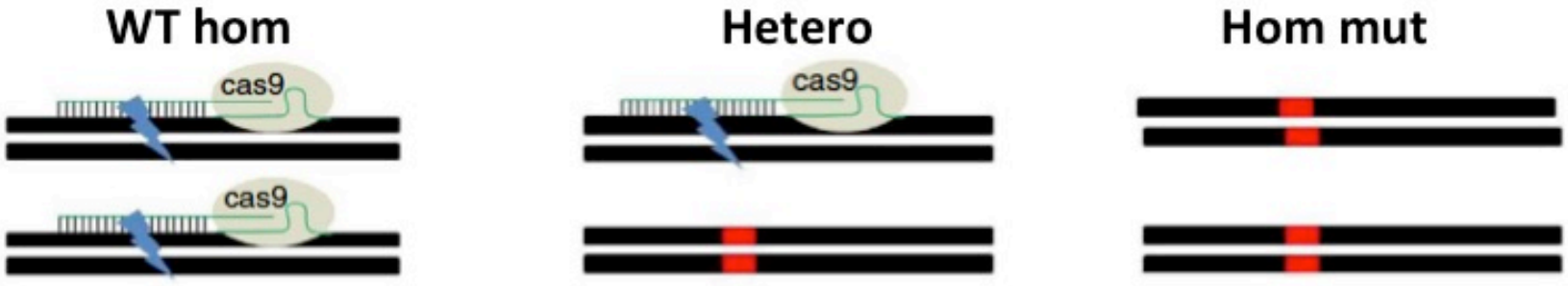


RGENs: complexing recombinant Cas9 protein derived from *Streptococcus pyogenes* with *in vitro* transcribed **guide RNAs** that are complementary to the DNA sequences of interest



CRISPR/Cas-derived RNA-guided engineered nucleases (RGENs) in RFLP analysis

Kim et. al. 2014. Genotyping with CRISPR-Cas-derived RNA-guided Endonucleases NATURE COMMUNICATIONS, 5:3157



May be useful in cases where Restriction fragment length polymorphism (RFLP) analysis is limited by the availability of restriction endonuclease sites.

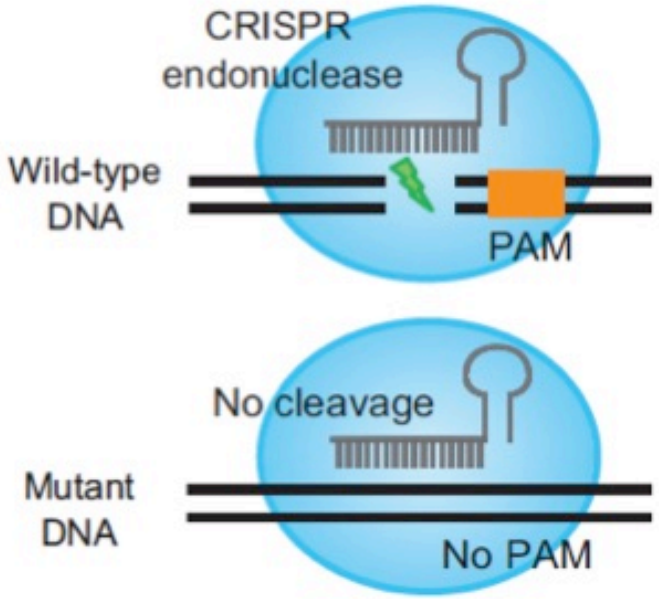
In the case of substitutions, however, CRISPR endonucleases can typically cleave mutant DNAs as well as wild-type DNAs in vitro because of their ability to recognize sites that vary by one or a few nucleotides from the gRNA sequence (**off-target effects**).

Solution: guide RNAs that contain a single-base mismatch rather than perfectly matched guide RNAs to distinguish the wild-type sequences from the mutant sequences with substitutions

CUT-PCR: CRISPR-mediated, Ultrasensitive detection of Target DNA using PCR

SH Lee et al. Oncogene (2017) 36, 6823–6829

Principle: CRISPR endonucleases are used **to enrich and detect the extremely small amounts of tumor DNA** fragments among the much more abundant wild-type DNA fragments by specifically eliminating the wild-type sequences followed by **deep sequencing**.



PAM (Protospacer **A**djacent **M**otif)**:** short motif directly adjacent to the target sequence (also called protospacers)

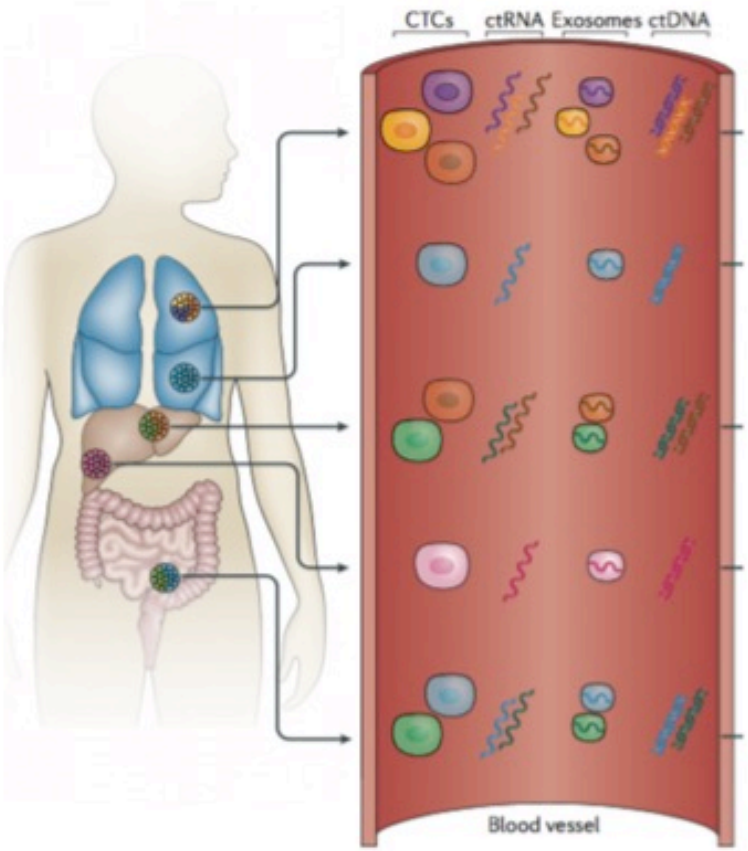
Original function of PAM: self/ nonself discrimination

Less off-target effects: PAM recognition by CRISPR endonucleases is much stricter than for gRNA

CUT-PCR: Potential application in „Liquid Biopsy“

ctDNA: circulating Tumor-DNA

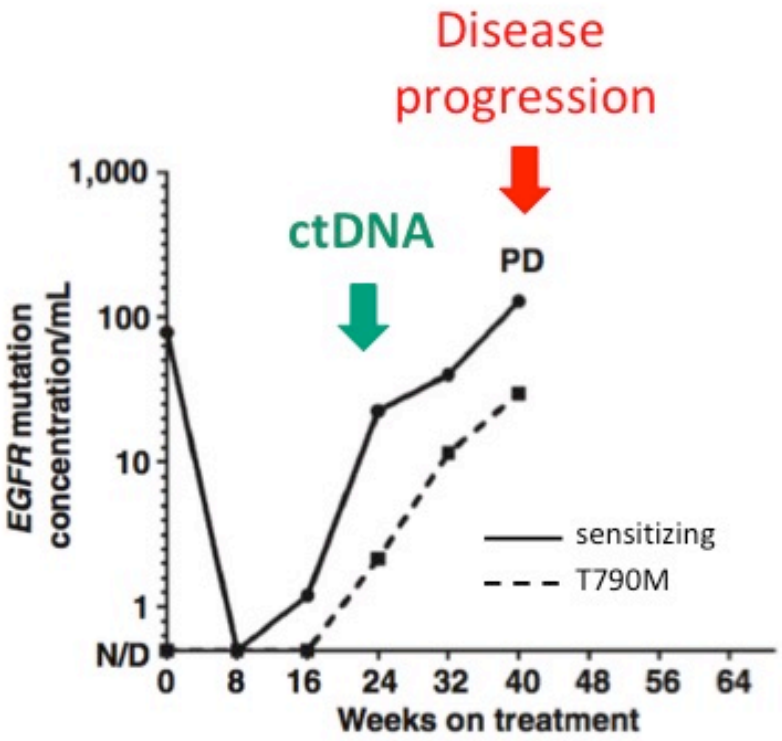
Very low amounts of DNA and only small fraction of the cell free DNA (=cfDNA) in the blood)



Siravegna et al. *Nat Rev Clin Oncol* (2017)

Monitoring of tumor mutations in plasma:

Early detection of progression

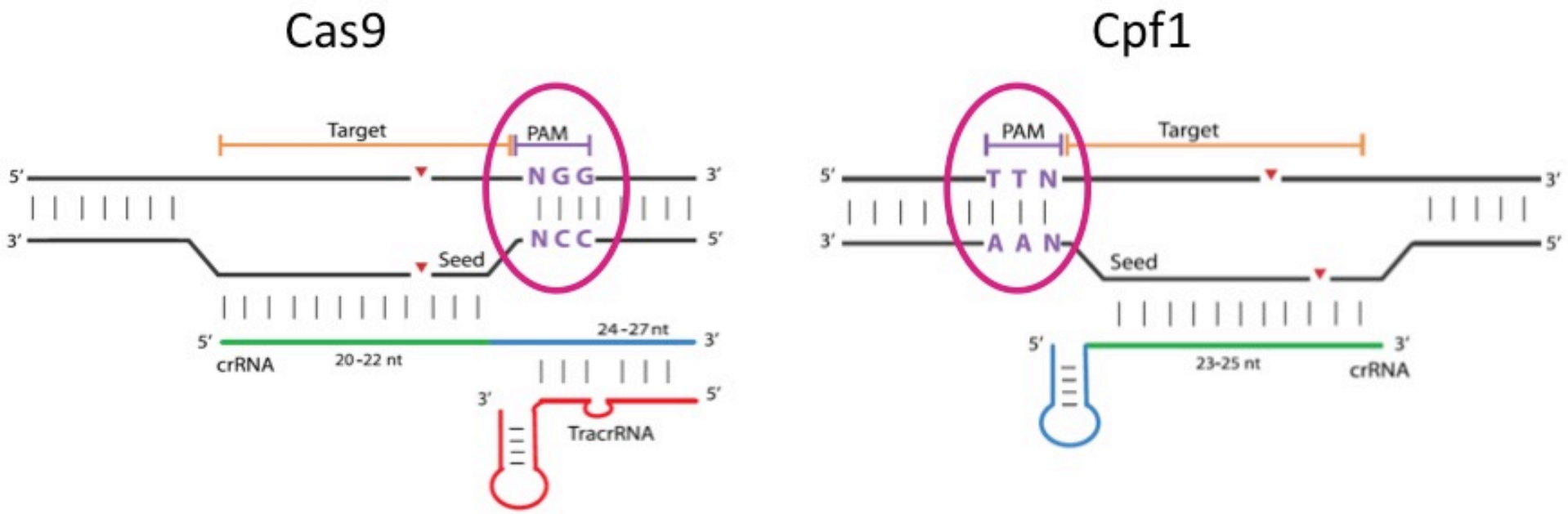


Oxnard et al. *Clin Cancer Res* (2014)

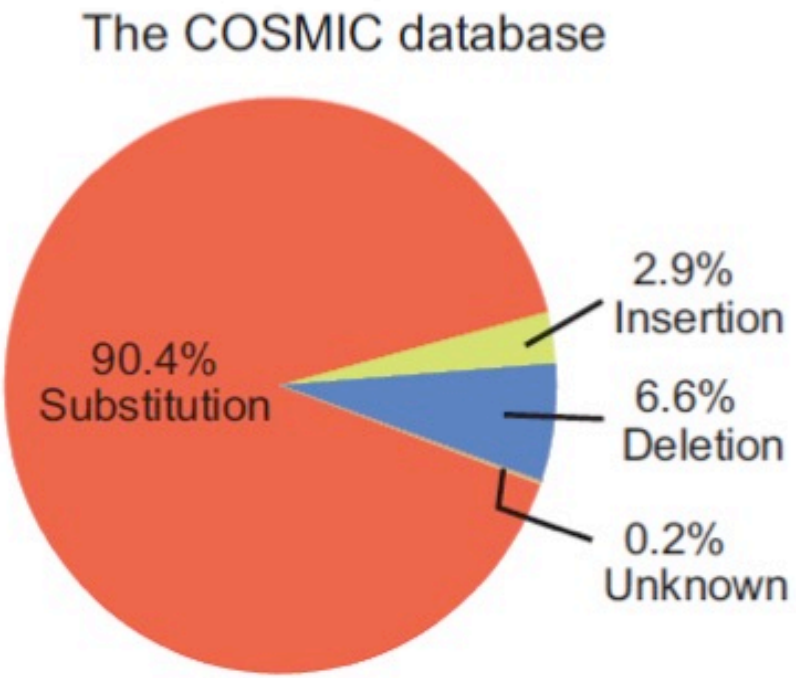
CUT-PCR: CRISPR-mediated, Ultrasensitive detection of Target DNA using PCR

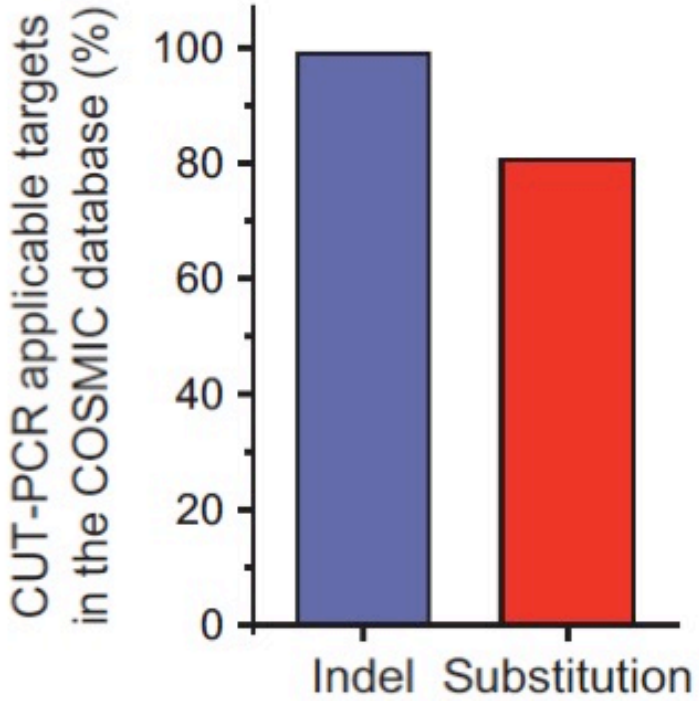
SH Lee et al. Oncogene (2017) 36, 6823–6829

A wide range of mutations can be targeted by using various orthologonal CRISPR endonucleases such as SpCas9 and FnCpf1 => different PAM sequences



CUT-PCR method potentially applicable to 80% of known cancer-linked substitution mutations registered in the COSMIC database



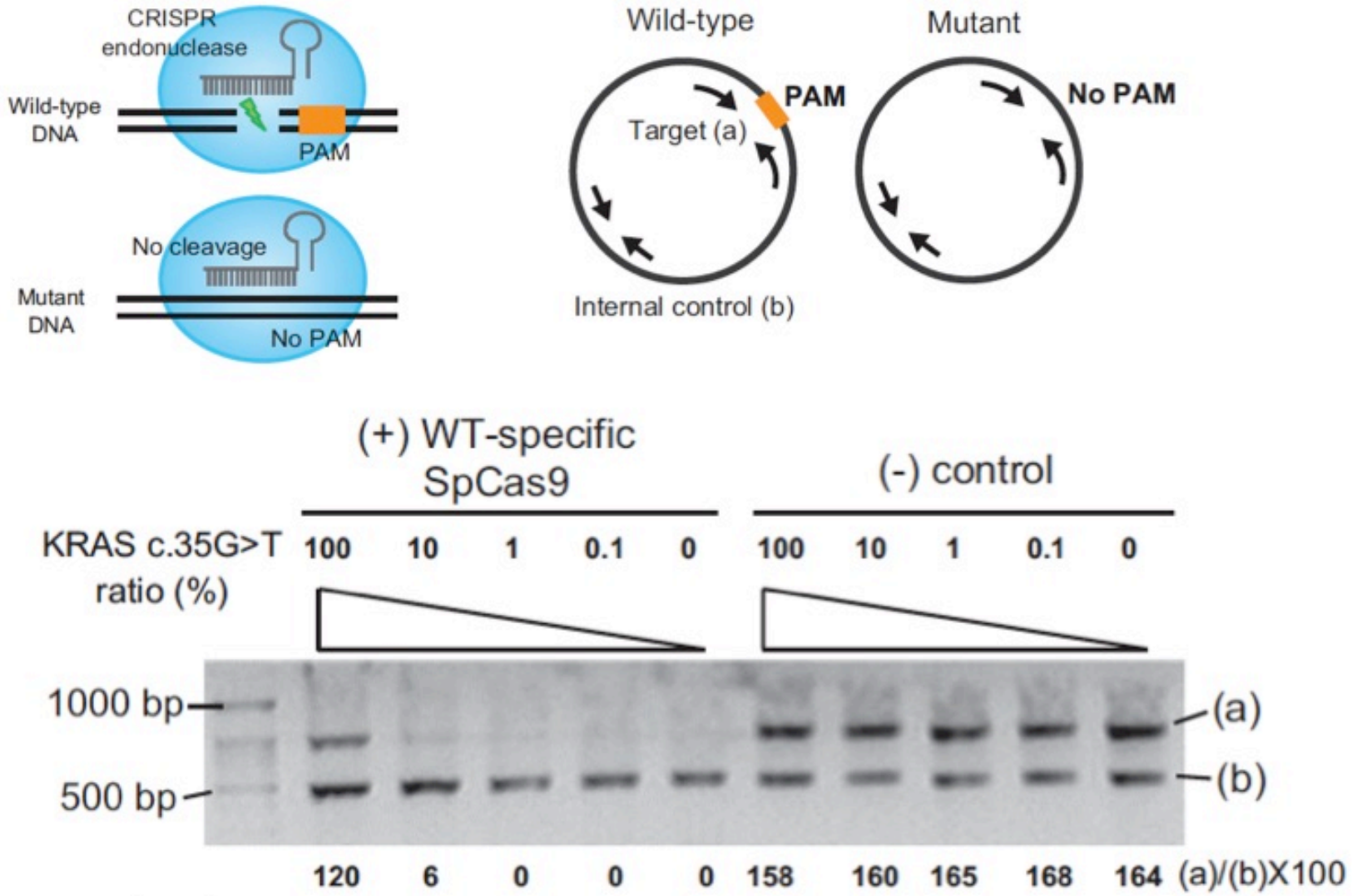


H Lee et al. Oncogene (2017) 36, 6823–6829

The COSMIC (Catalogue of Somatic Mutations in Cancer) database includes hundreds of thousands of human cancer-associated somatic mutations that are classified by tumor type and disease.

http://cancer.sanger.ac.uk/cosmic

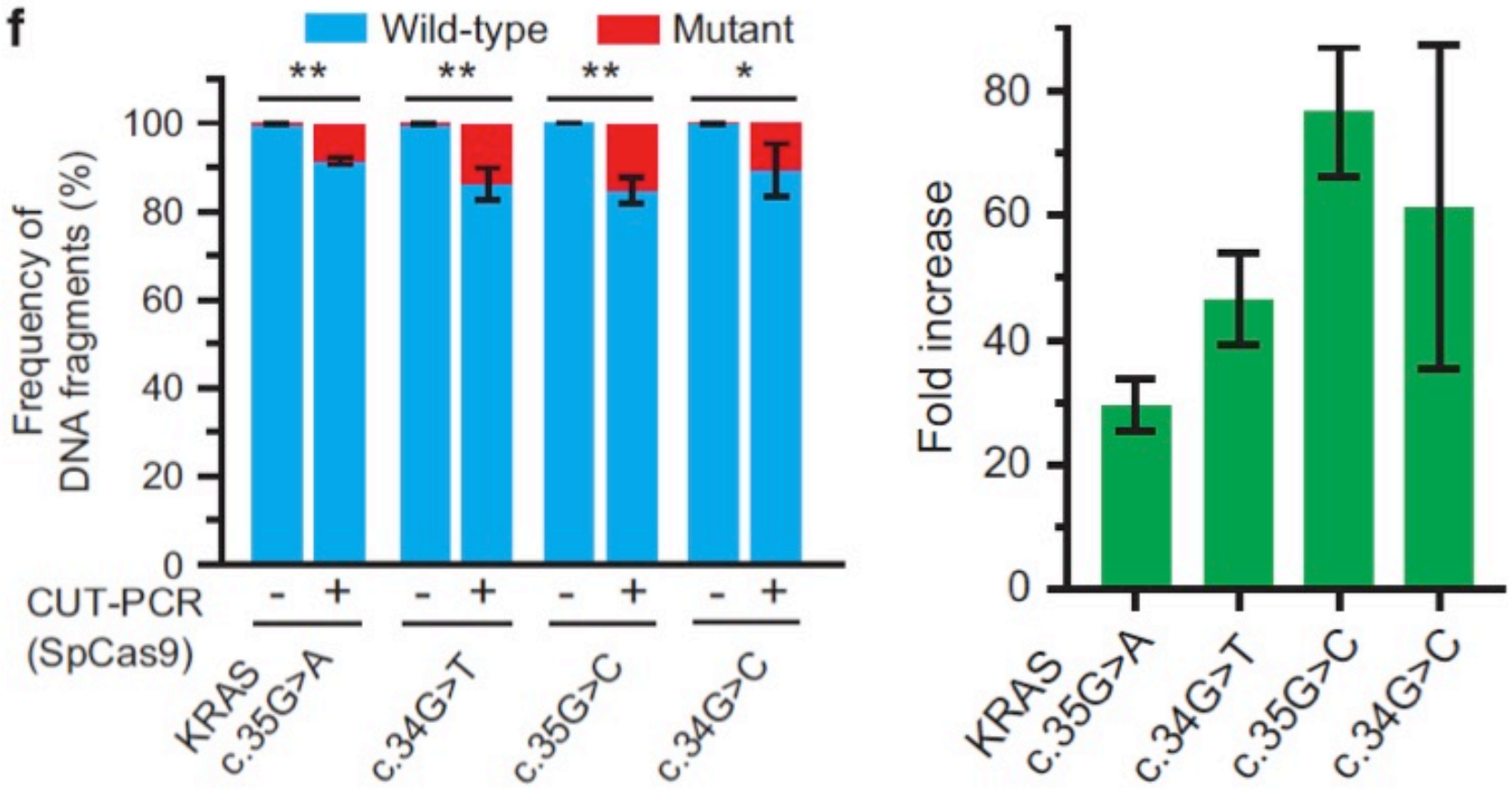
CUT-PCR-based enrichment of plasmid-borne sequences containing missense mutation



H Lee et al. Oncogene (2017) 36, 6823–6829

CUT-PCR-based enrichment of recurrent *KRAS*-mutations

KRAS mutant plasmids originally mixed with wild-type plasmids at a ratio of 0.1%

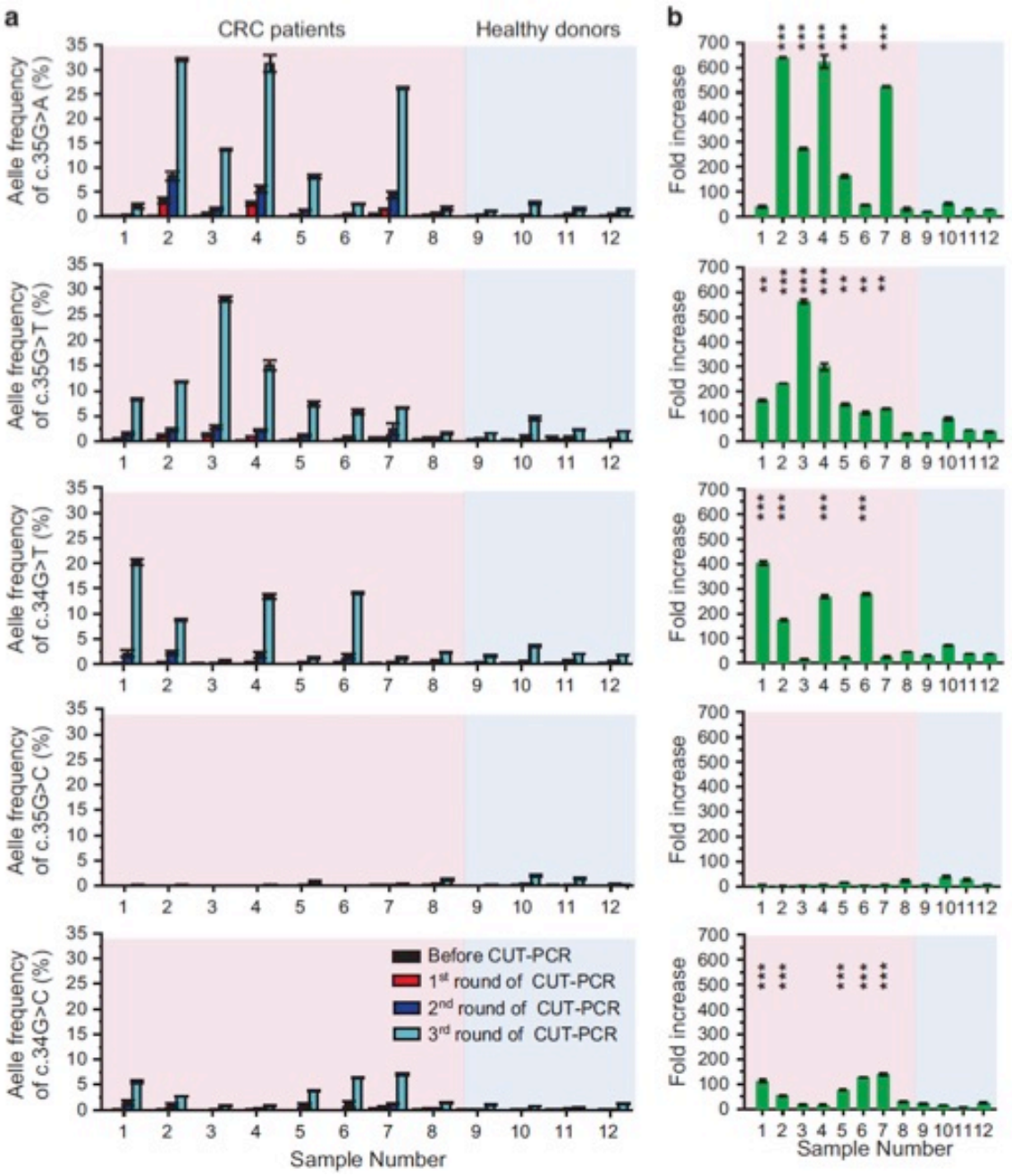


H Lee et al. Oncogene (2017) 36, 6823–6829

CUT-PCR summary

- High sensitivity: 0.01% (NGS & dPCR: 0.1-0.05%)
- Can be applied to a majority of the known cancer mutations

- Open questions:
  - little real data
  - amplification artefacts
  - quantification



H Lee et al. Oncogene (2017) 36, 6823–6829

SHERLOCK: Specific High-sensitivity Enzymatic Reporter unLOCKing

RESEARCH

REPORT Gootenberg *et al.*, *Science* **356**, 438–442 (2017) 28 April 2017

CRISPR TECHNOLOGY

Nucleic acid detection with CRISPR-Cas13a/C2c2

Jonathan S. Gootenberg,[1,2,3,4,5*] Omar O. Abudayyeh,[1,2,3,4,6*] Jeong Wook Lee,[7] Patrick Essletzbichler,[1,2,3,4] Aaron J. Dy,[1,4,8] Julia Joung,[1,2,3,4] Vanessa Verdine,[1,2,3,4] Nina Donghia,[7] Nichole M. Daringer,[8] Catherine A. Freije,[1,9] Cameron Myhrvold,[1,9] Roby P. Bhattacharyya,[1] Jonathan Livny,[1] Aviv Regev,[1,10] Eugene V. Koonin,[11] Deborah T. Hung,[1] Pardis C. Sabeti,[1,9,12,13] James J. Collins,[1,4,6,7,8†] Feng Zhang[1,2,3,4†]

Science

REPORTS

Cite as: J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

Based on the specific behavior of the Cas13a endonuclease called "**promiscuous RNAase activity**" combined with isothermal pre-amplification to detect single molecules of RNA or DNA

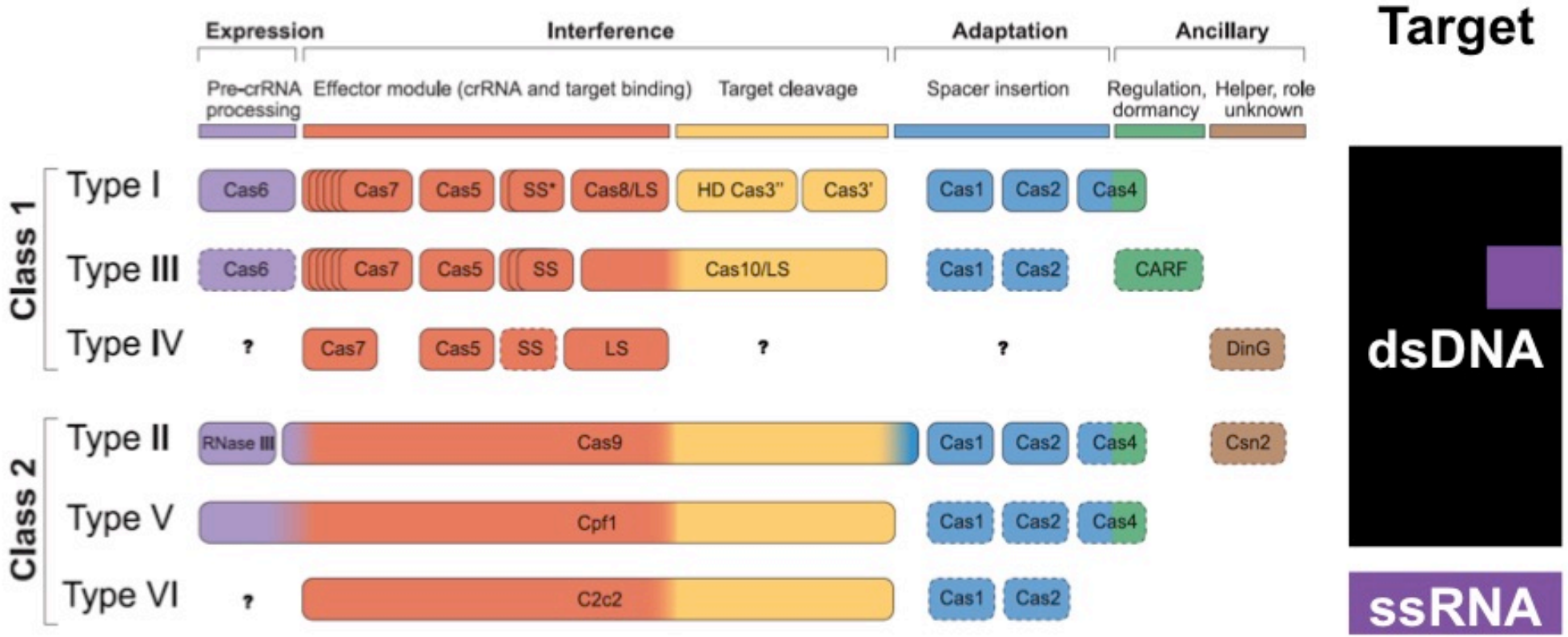
Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6

Jonathan S. Gootenberg,[1,2,3,4,7*] Omar O. Abudayyeh,[1,2,3,4,5*] Max J. Kellner,[1] Julia Joung,[1,2,3,4] James J. Collins,[1,4,5,6,8] Feng Zhang[1,2,3,4†]

[1]Broad Institute of MIT and Harvard Cambridge, MA 02142, USA. [2]McGovern Institute for Brain Research at MIT, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [3]Department of Brain and Cognitive Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [4]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [5]Department of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [6]Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [7]Department of Systems Biology, Harvard University, Boston, MA 02115, USA. [8]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA.

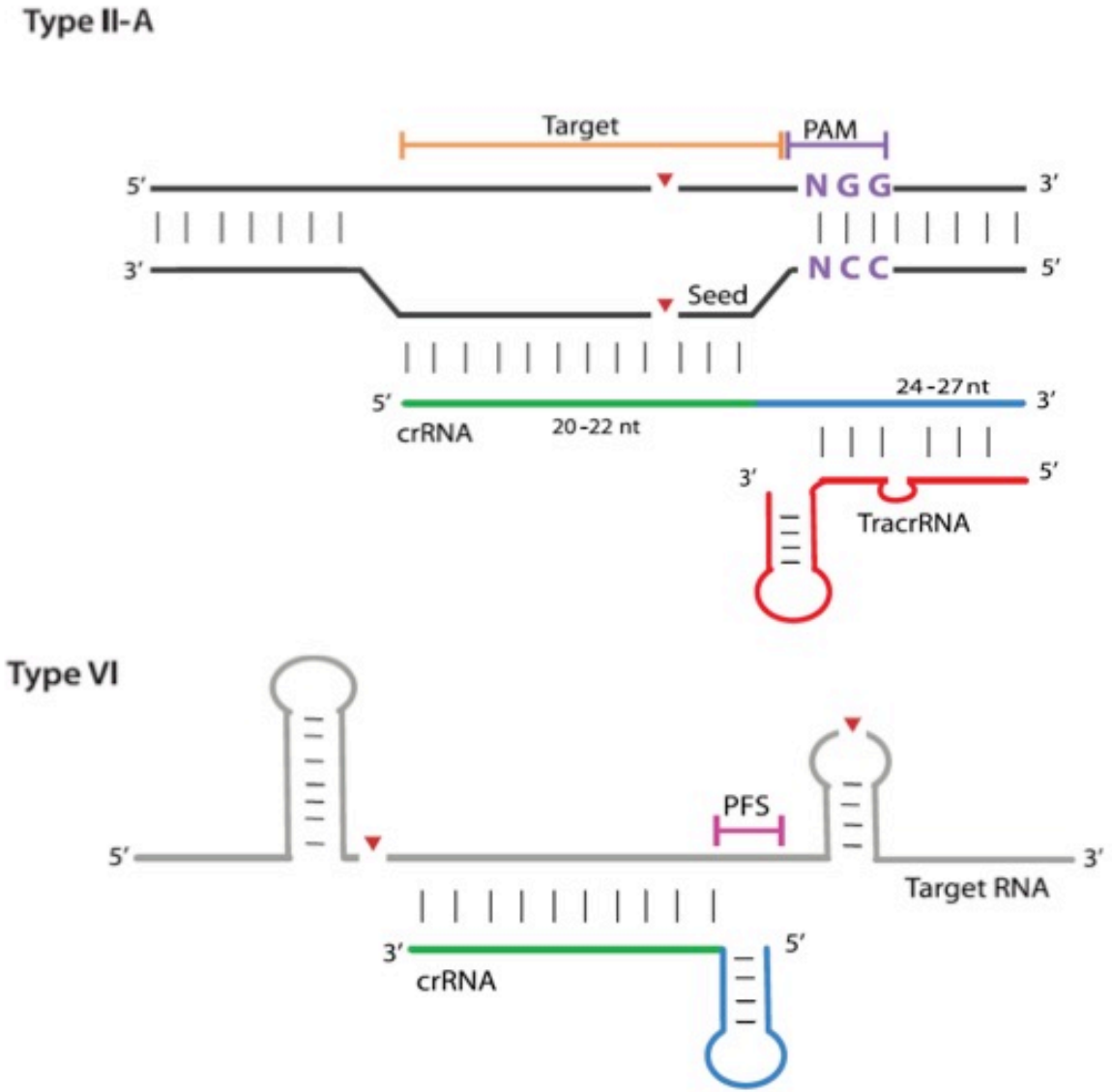
*These authors contributed equally to this work. †Corresponding author. Email: zhang@broadinstitute.org

CRISPR diversity and evolution



Mohanraju et al. 2016. SCIENCE ,VOL 353 ISSUE 6299

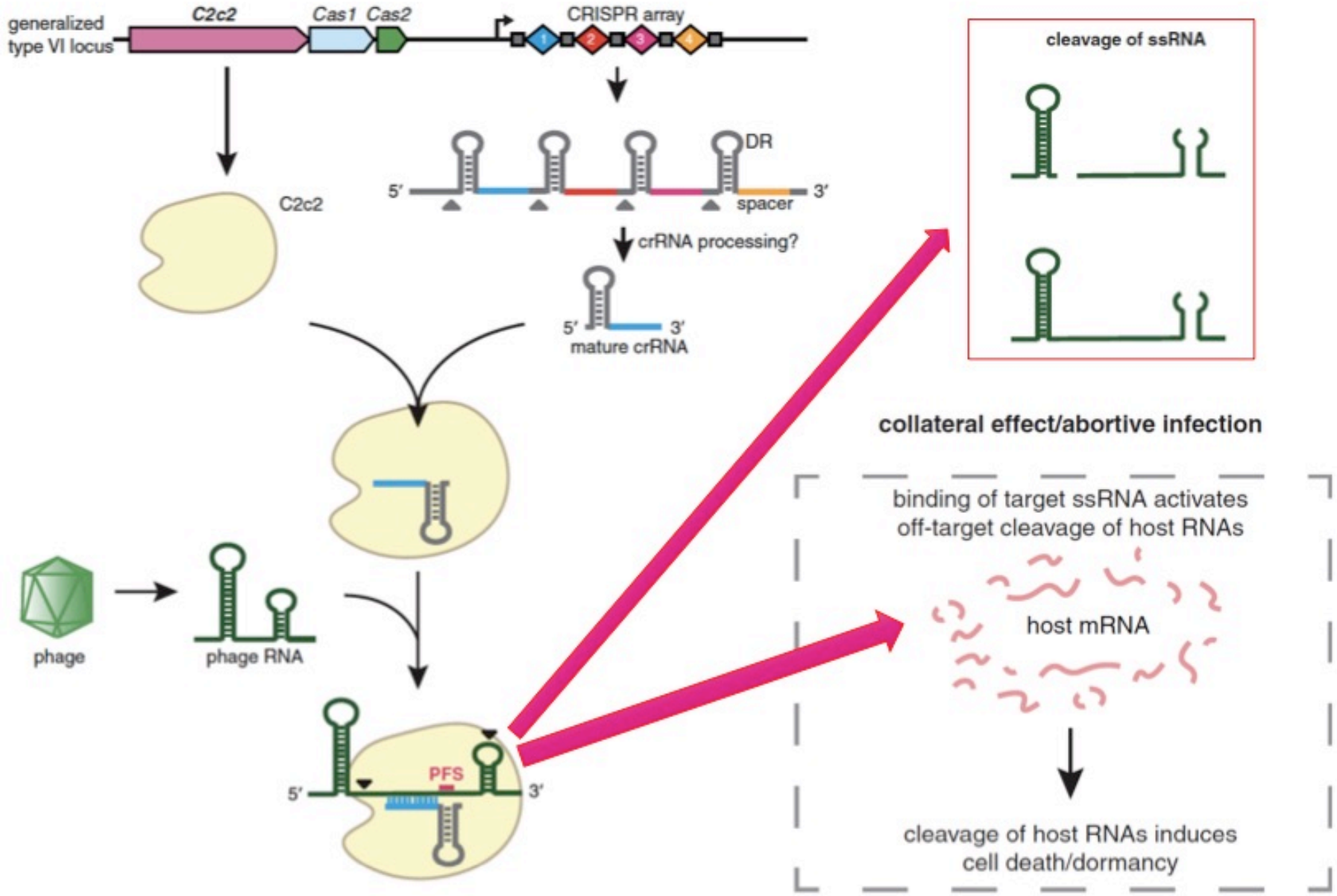
Schematic representation of target interference for the subtypes II-A (Cas9) and VI (Cas13a)



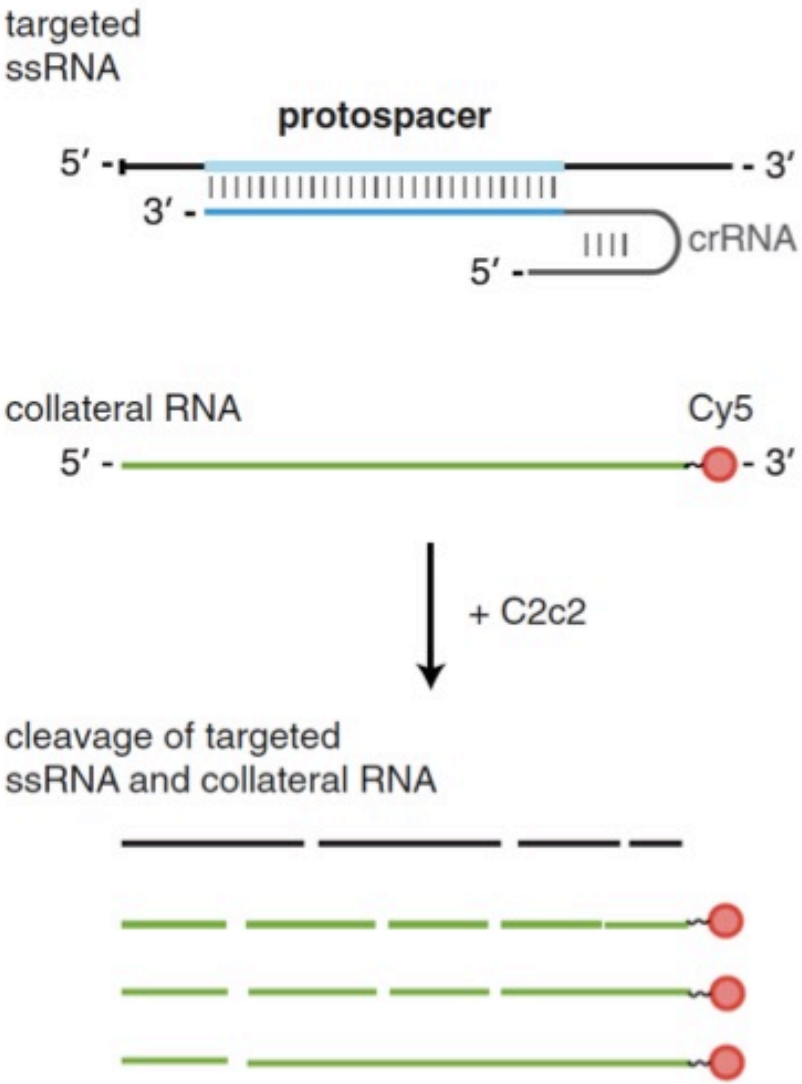
PFS: Protospacer Flanking Site => affects efficacy of C2c2 mediated targeting

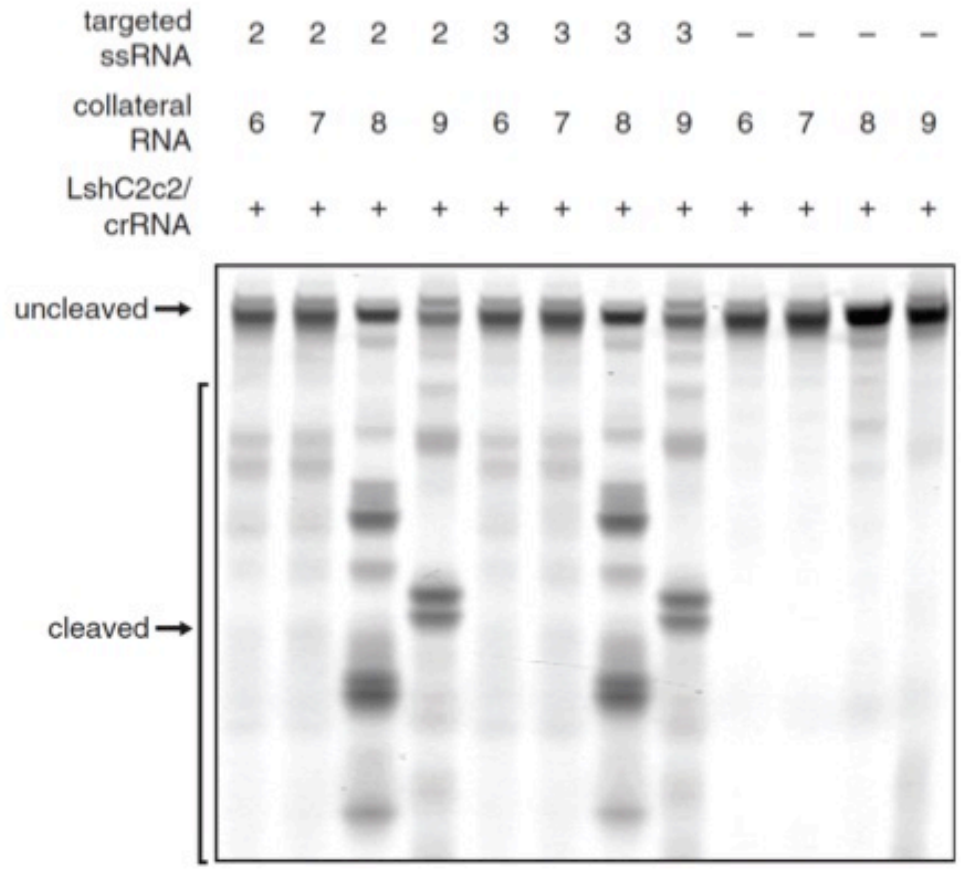
Discovery of crRNA-programmed collateral cleavage activity

Abudayyeh et al., 2016. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. **Science** 353(6299): aaf5573



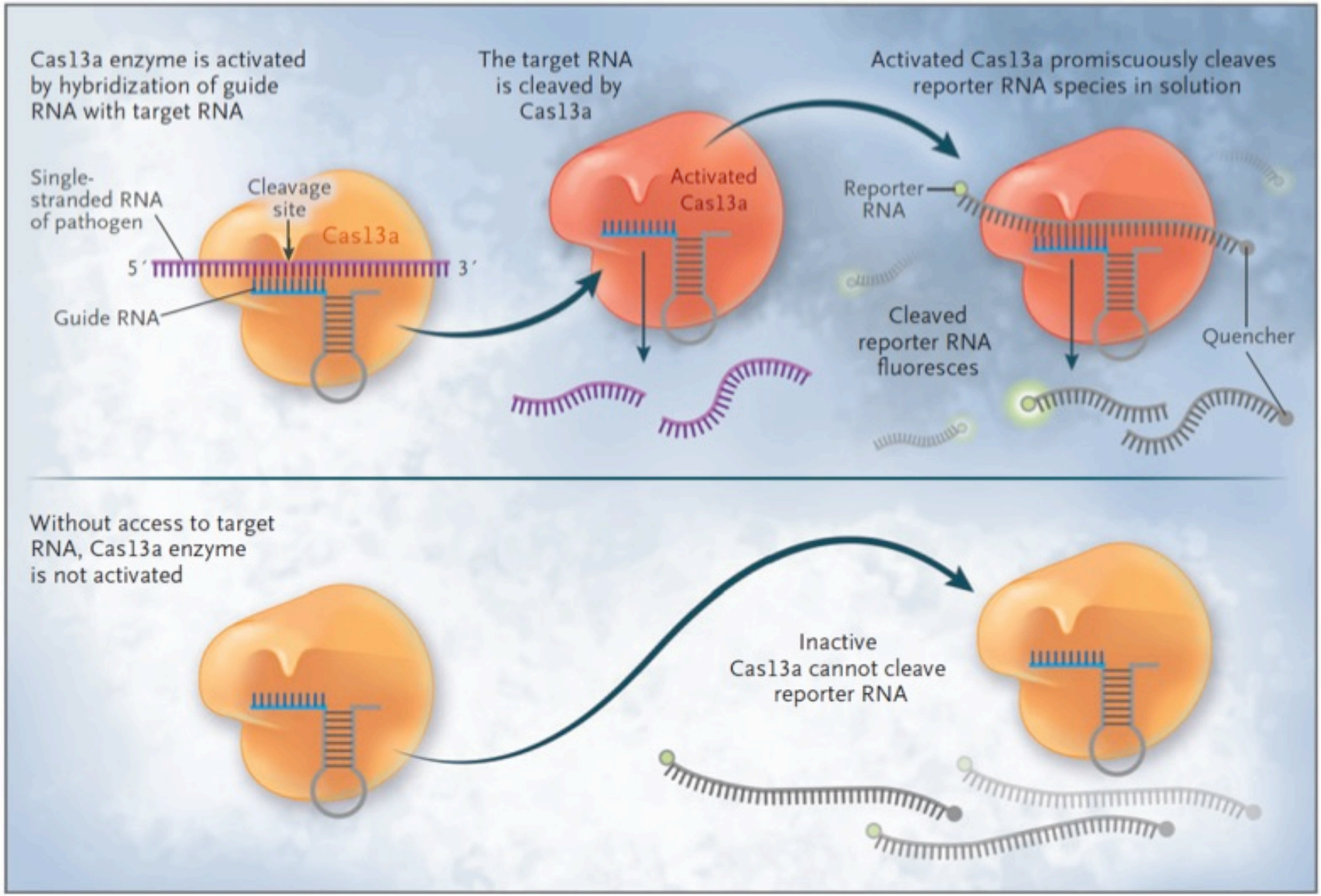
crRNA-guided ssRNA cleavage activates nonspecific RNase activity of LshC2c2





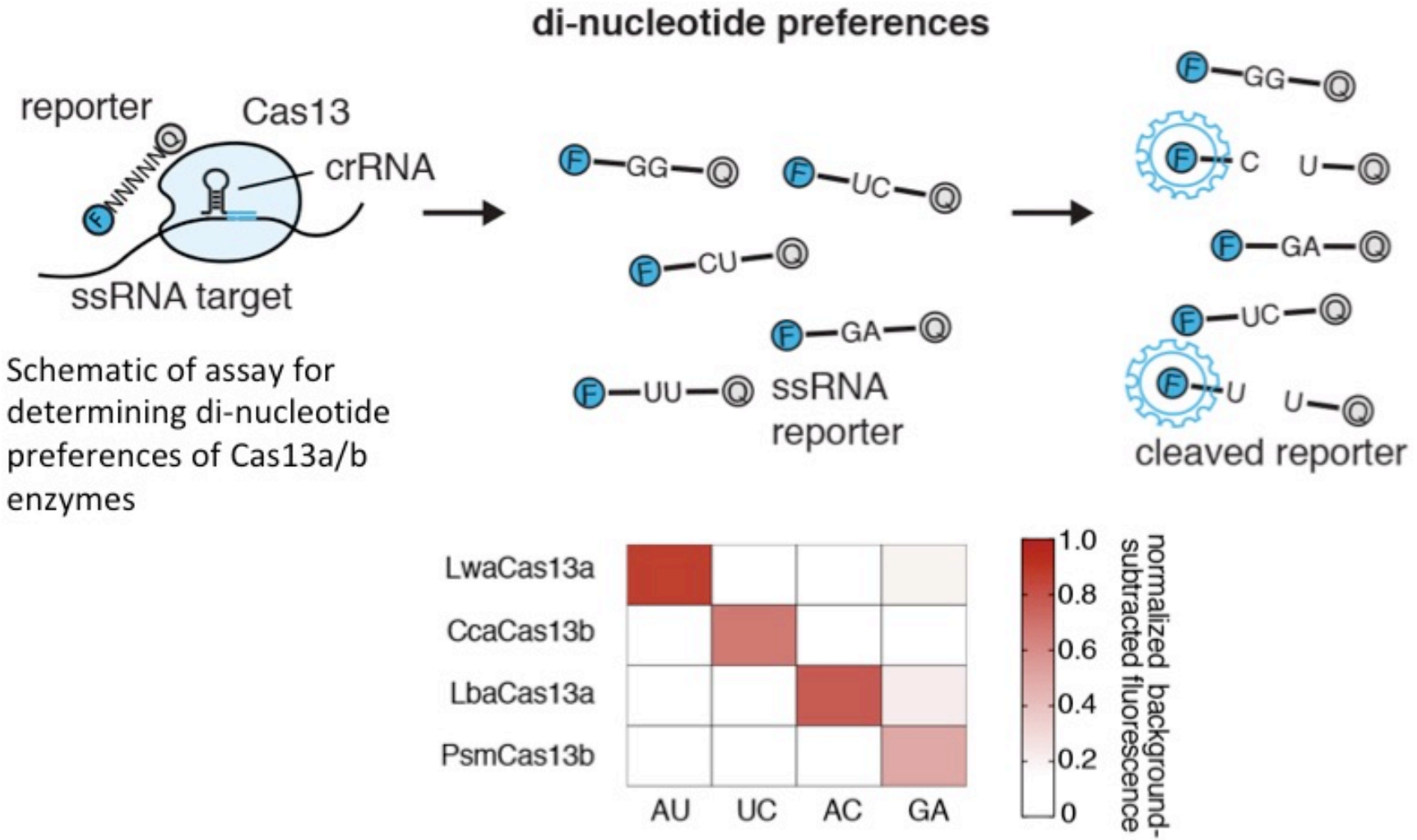
Abudayyeh et al., 2016.**Science** 353(6299): aaf5573

Cas13a-directed collateral detection with SHERLOCK



Caliendo & Hodinka, 2017. NEJM, 377, 17

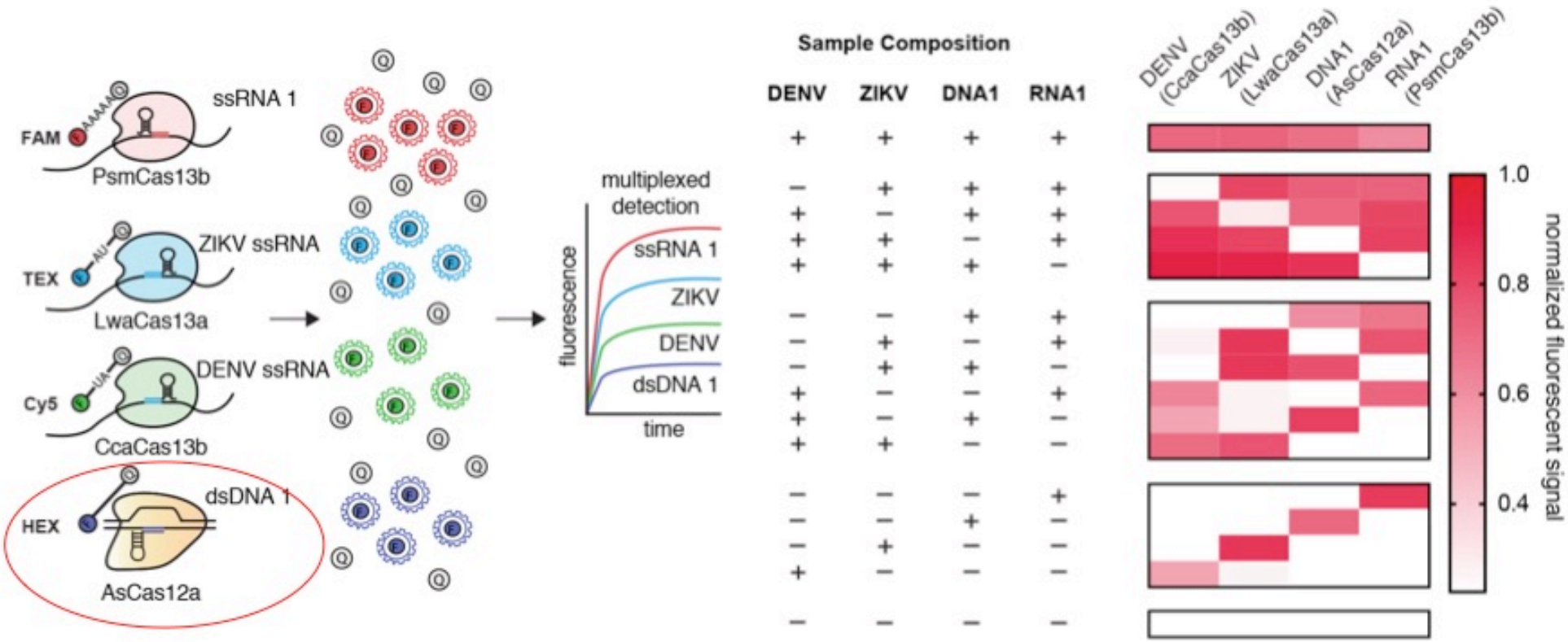
Multiplexed SHERLOCK detection with orthogonal collateral activity of Class 2 enzymes



Schematic of assay for determining di-nucleotide preferences of Cas13a/b enzymes

J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

In-sample multiplexing of four targets



CRISPR-Cas12a (Cpf1) proteins are type V RNA-guided DNA targeting enzymes belonging to Class 2 systems.

J. S. Chen, E. Ma, L. B. Harrington, X. Tian, J. A. Doudna, CRISPR-Cas12a target binding unleashes single-stranded DNase activity. *bioRxiv* [preprint]. 29 November 2017.

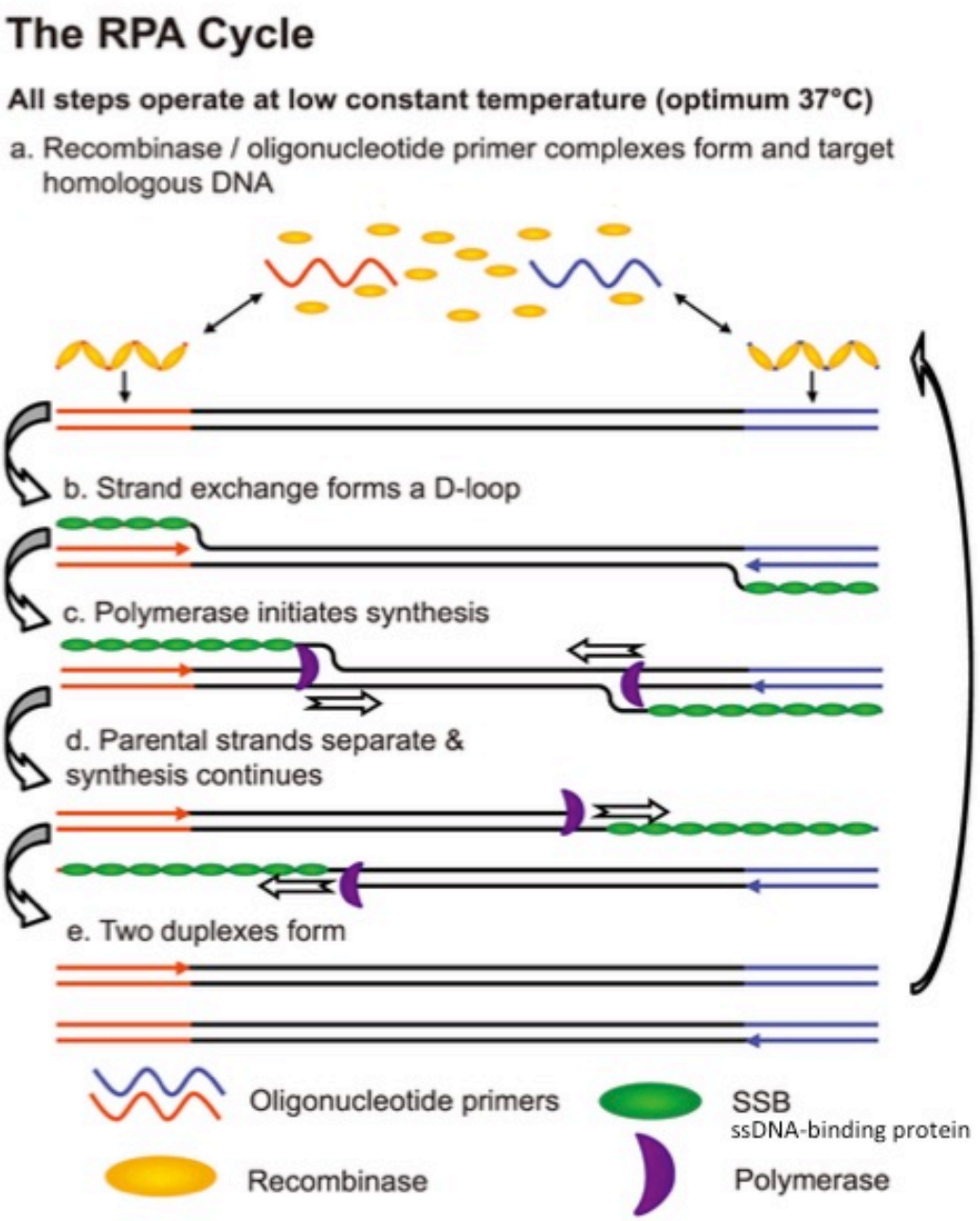
J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

SHERLOCK: Increased sensitivity by isothermal amplification

A) Depending on the target, pre-amplification of DNA with **recombinase polymerase amplification (RPA)** or a pre-amplification of RNA with reverse-transcription RPA.

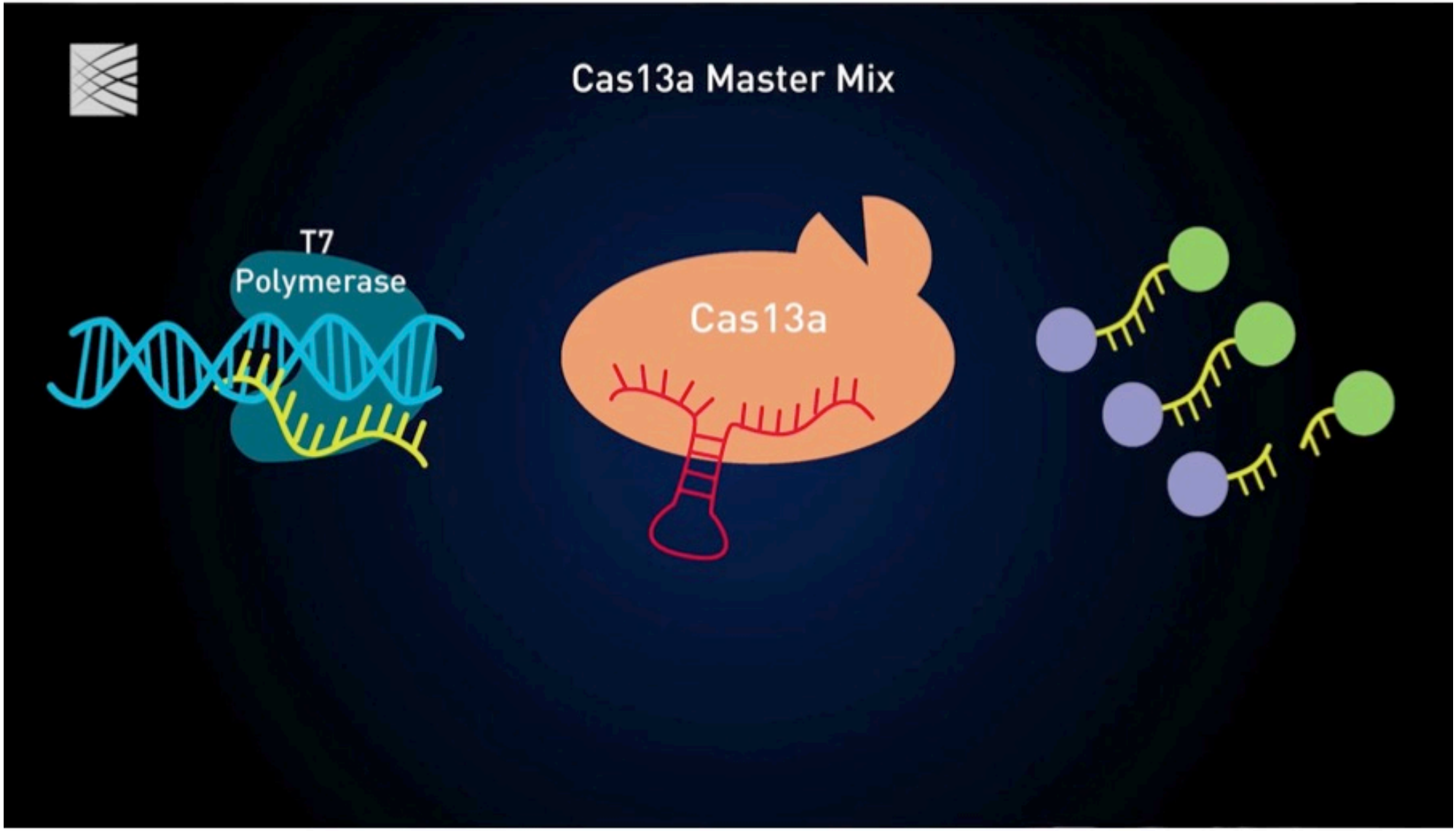
B) During pre-amplification, T7 RNA polymerase promoters are added to allow the transcription of amplified DNA to RNA.

C) In general,SHERLOCK reactions that involve a pre-amplification are performed in two steps with the **RPA reaction** being directly **added into the Cas13 assay without** any **purification step**.



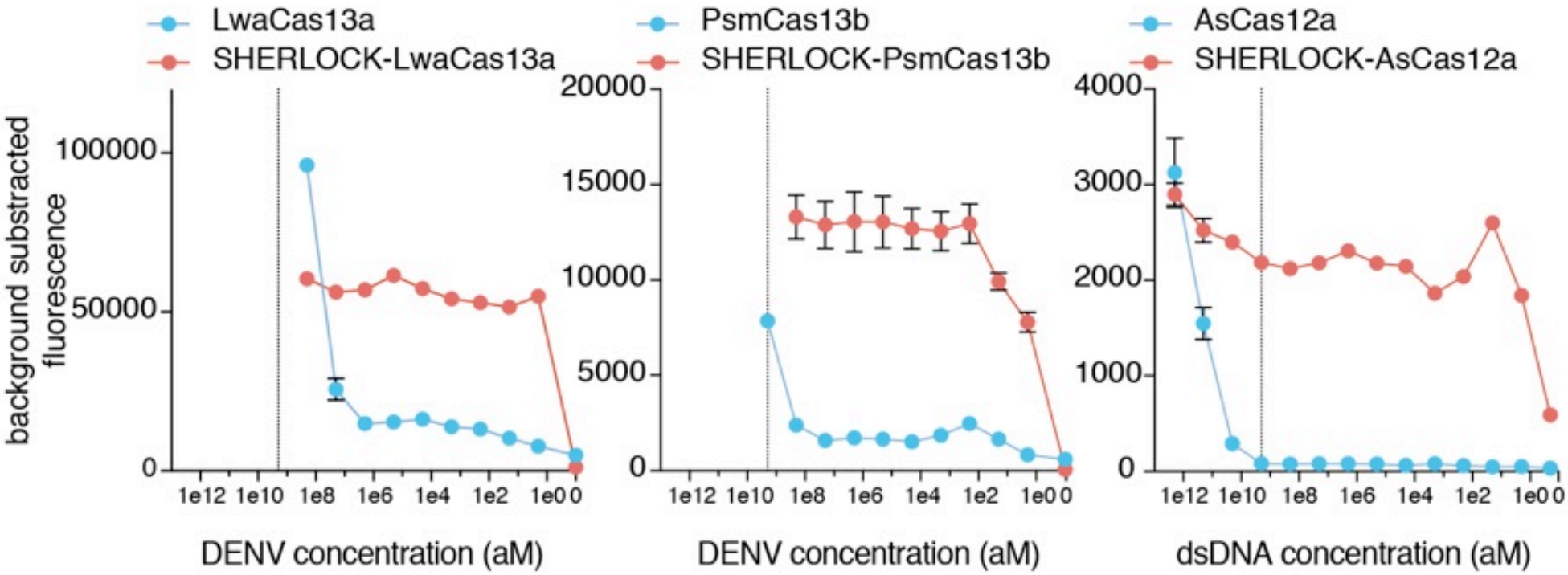
Boyle et al. (2014. PLOS ONE 9(8): e103

https://www.youtube.com/watch?time_continue=58&v=dtPyvtFyes8



SHERLOCK: Increased sensitivity by isothermal amplification

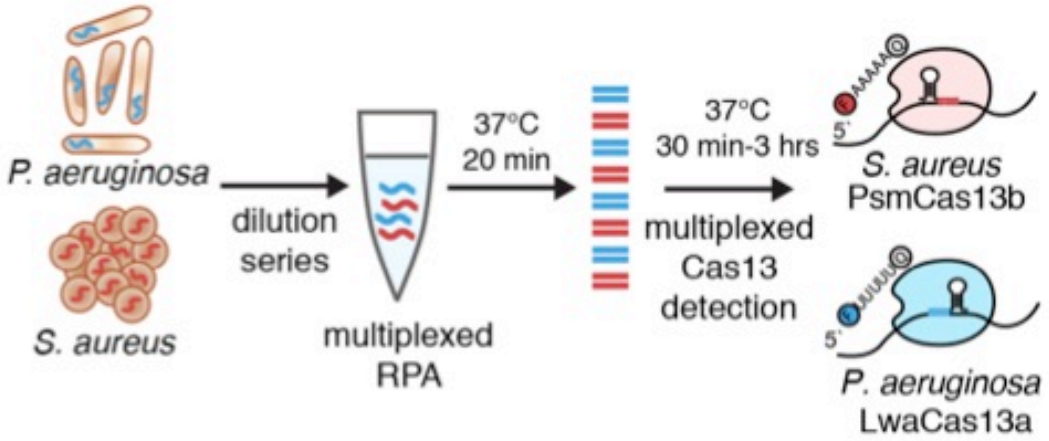
J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

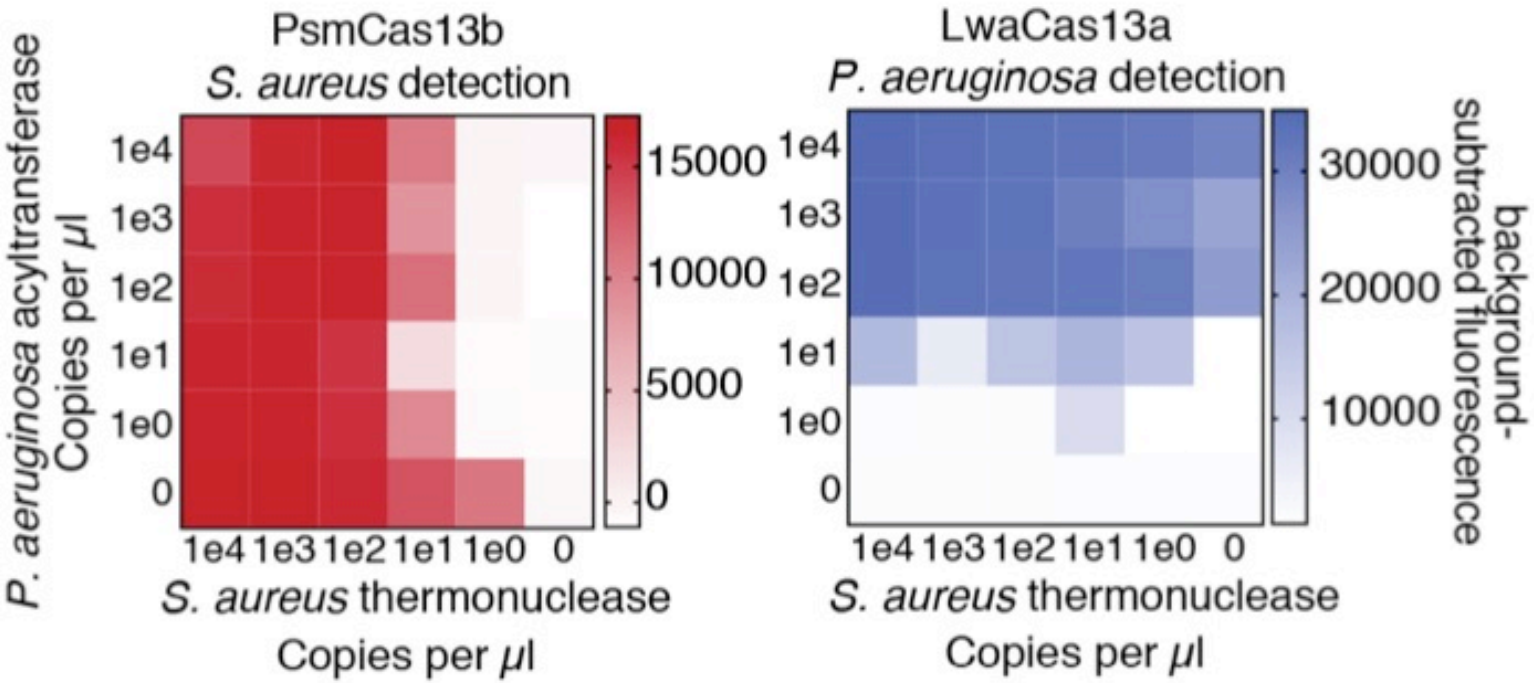


The dotted line denotes 2e9 = 2‘000‘000‘000 (aM), the limit of AsCas12a sensitivity without pre-amplification

0.000 001	**10^{-6}**	**micro**	**µ**
0.000 000 001	**10^{-9}**	**nano**	**n**
0.000 000 000 001	**10^{-12}**	**pico**	**p**
0.000 000 000 000 001	**10^{-15}**	**femto**	**f**
0.000 000 000 000 000 001	**10^{-18}**	**atto**	**a**
0.000 000 000 000 000 000 001	**10^{-21}**	**zepto**	**z**

In-sample multiplexed RPA and collateral detection at decreasing concentrations of *S. aureus* thermonuclease and *P. aeruoginosa* acyltransferase synthetic targets with LwaCas13a and PsmCas13b

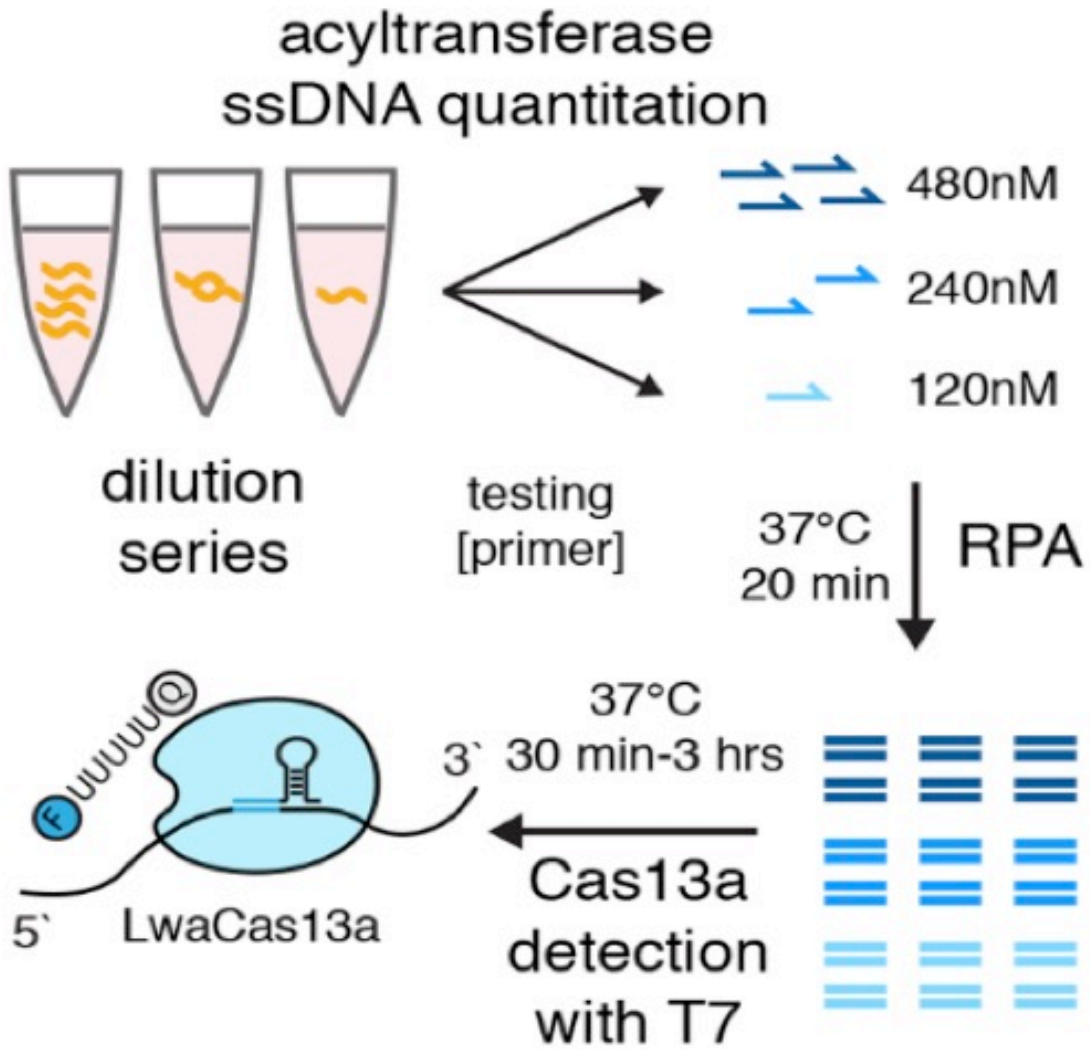




J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

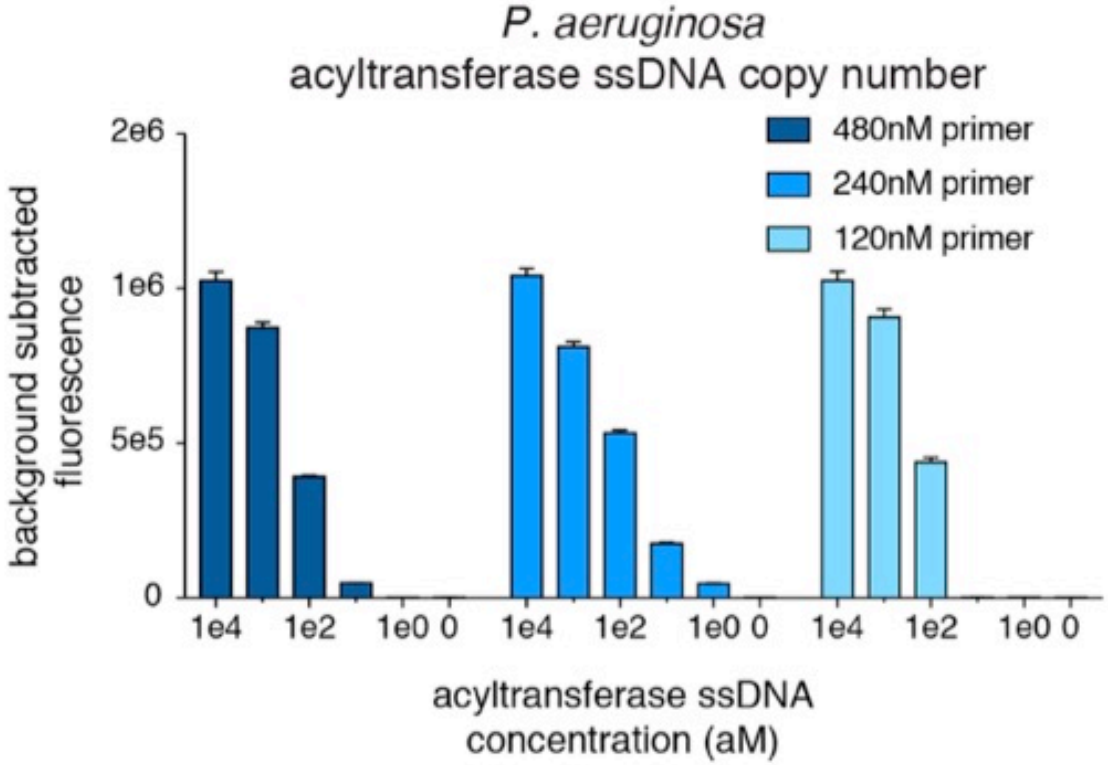
Single molecule quantitation SHERLOCK

Schematic of DNA reaction scheme for quantitation of *P. aeroginosa* synthetic DNA

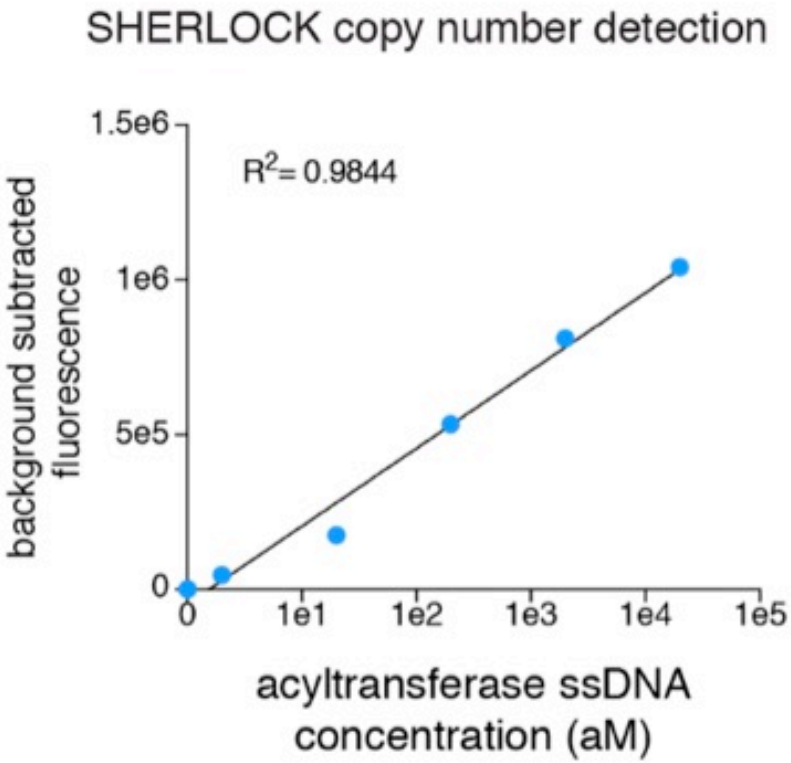


J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

Single molecule quantitation with SHERLOCK



Quantitation of *P. aeroginosa* synthetic DNA at various RPA primer concentrations



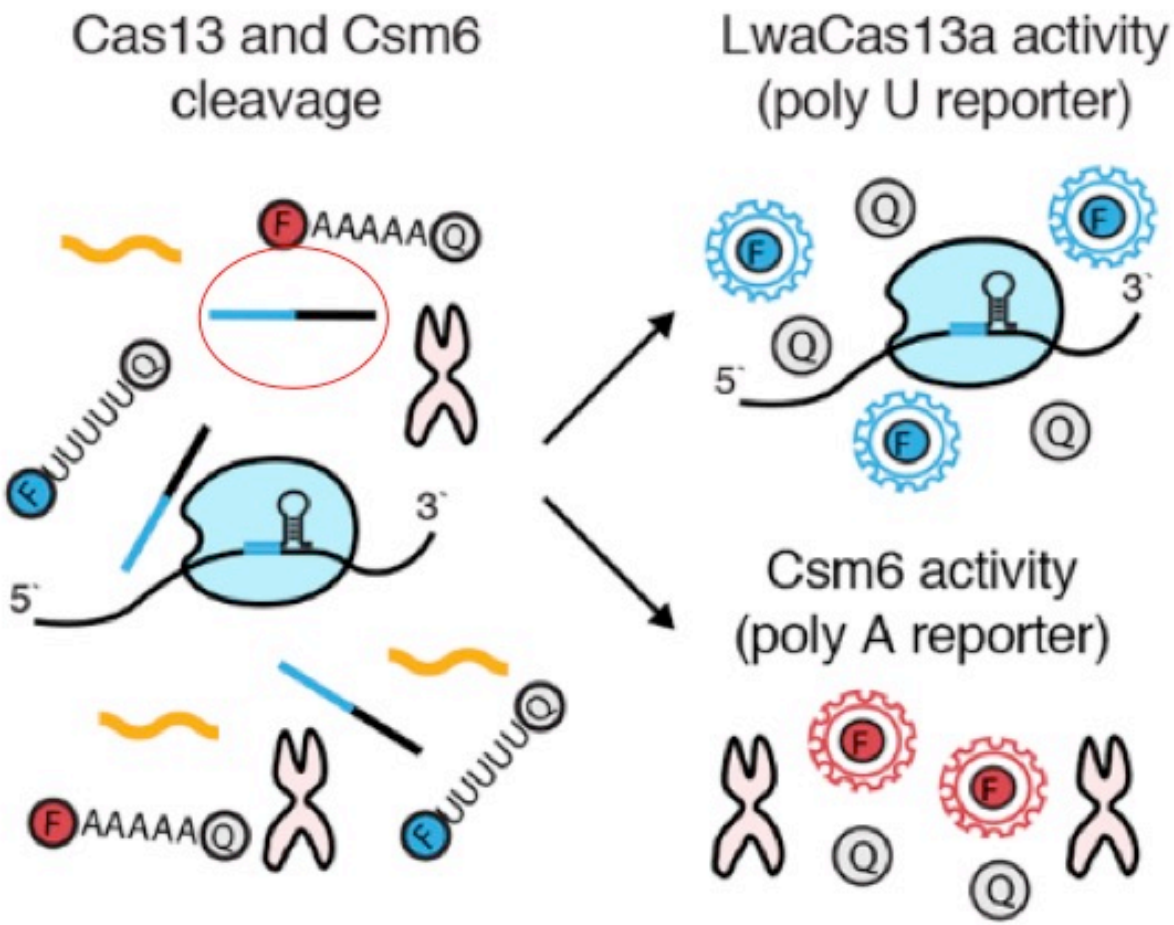
Correlation of *P. aeroginosa* synthetic DNA concentration with detected fluorescence

J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

Enhanced signal with SHERLOCK and Csm6

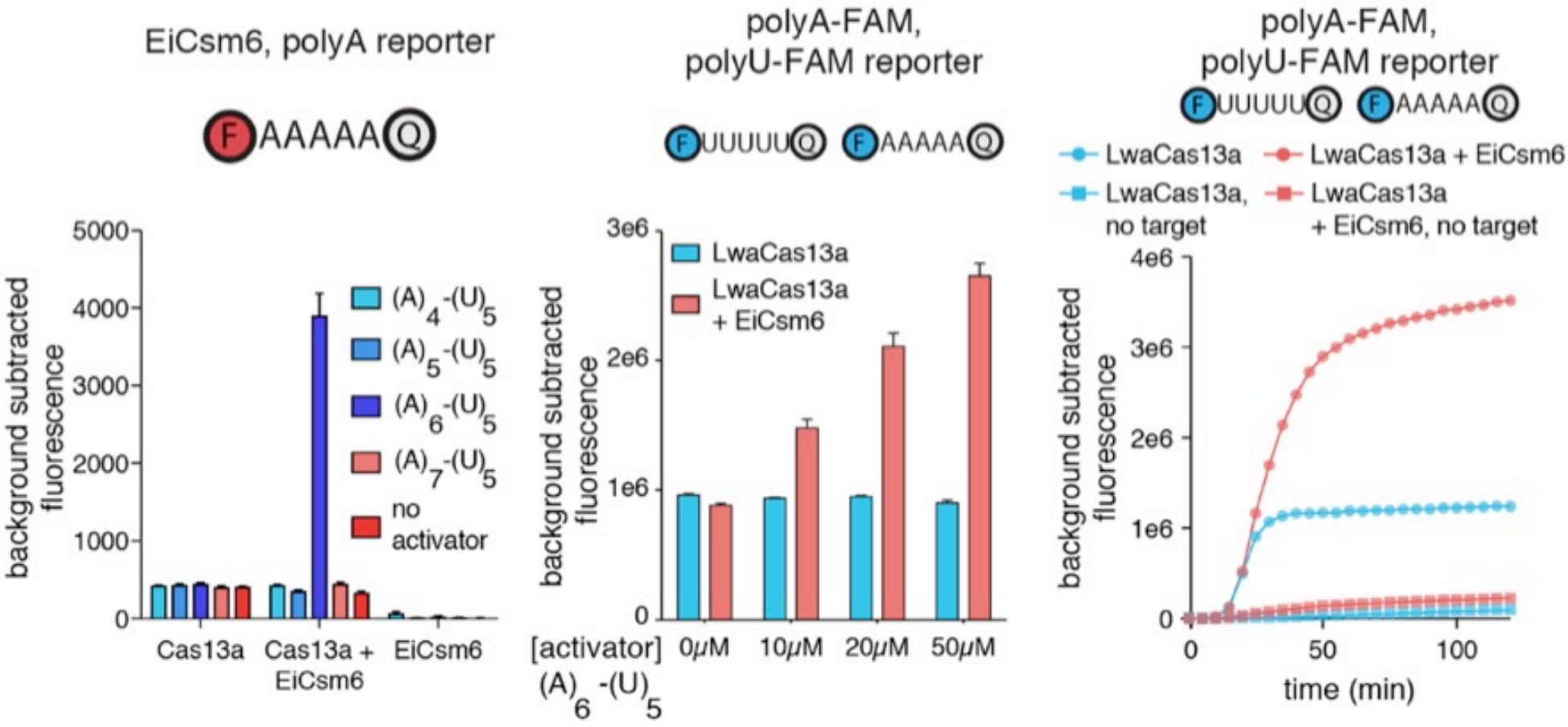
A) CRISPR type-III effector nuclease Csm6 is activated by linear adenine homopolymers terminated with a 2′, 3′-cyclic phosphate

B)protected RNA activators: poly-A stretch followed by a protecting poly-U stretch that could be cleaved by a uracil preferring Cas13 enzyme



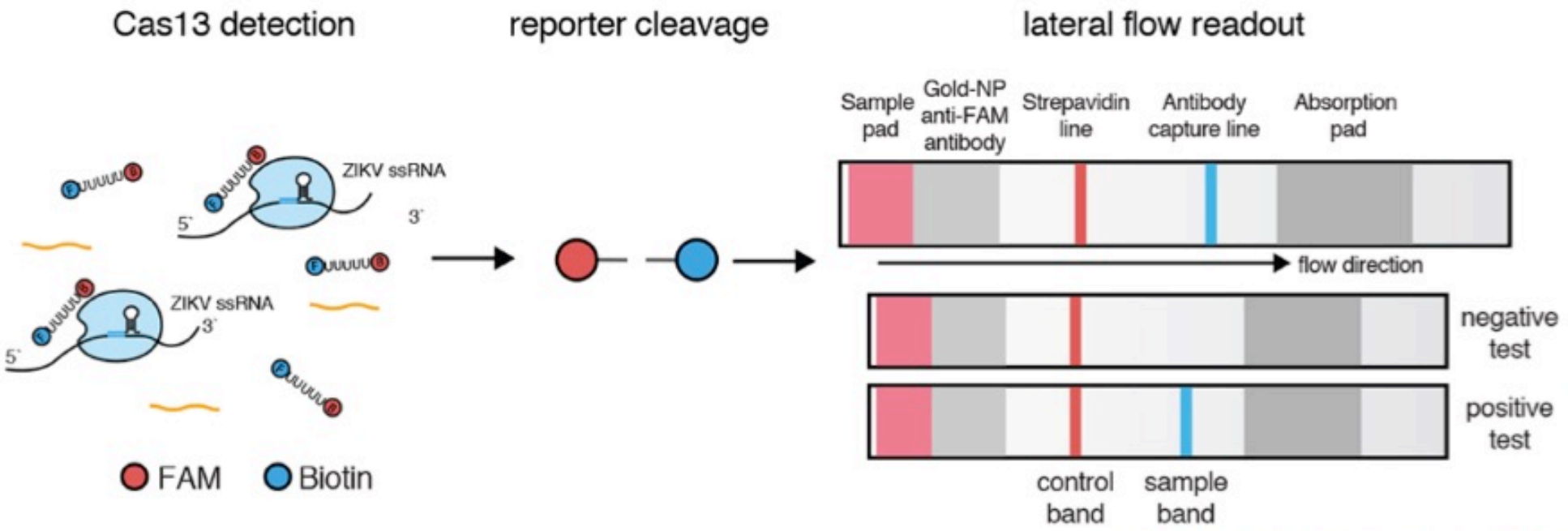
Schematic of independent readout of LwaCas13a and Csm6 cleavage activity with orthogonal reporters

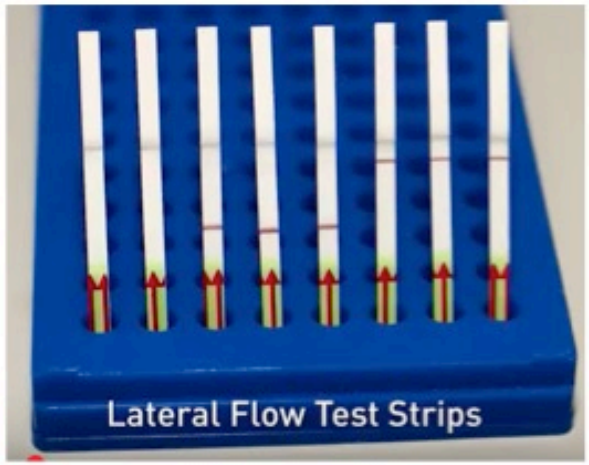
Enhanced signal with SHERLOCK and Csm6



J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

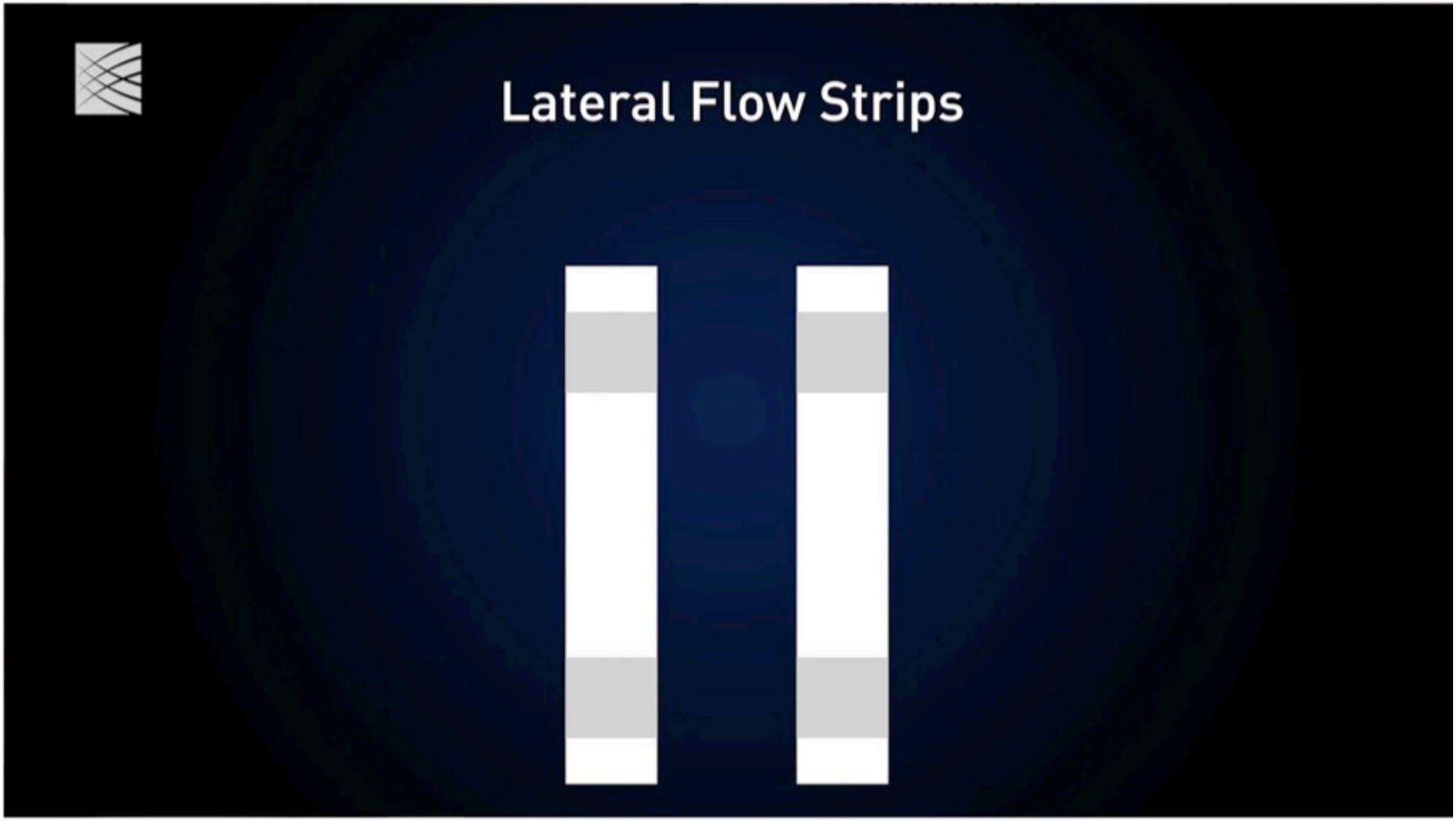
Schematic of lateral flow detection with SHERLOCK



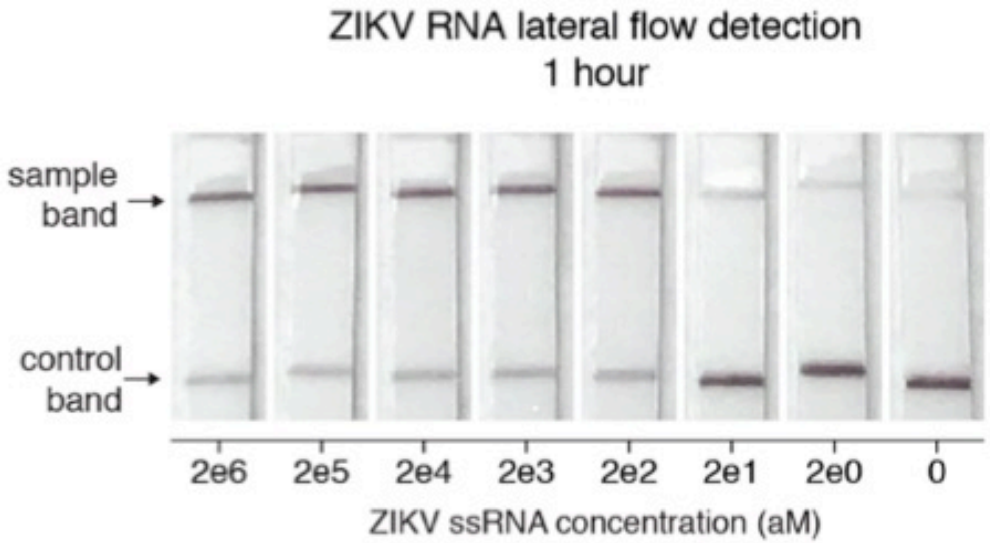


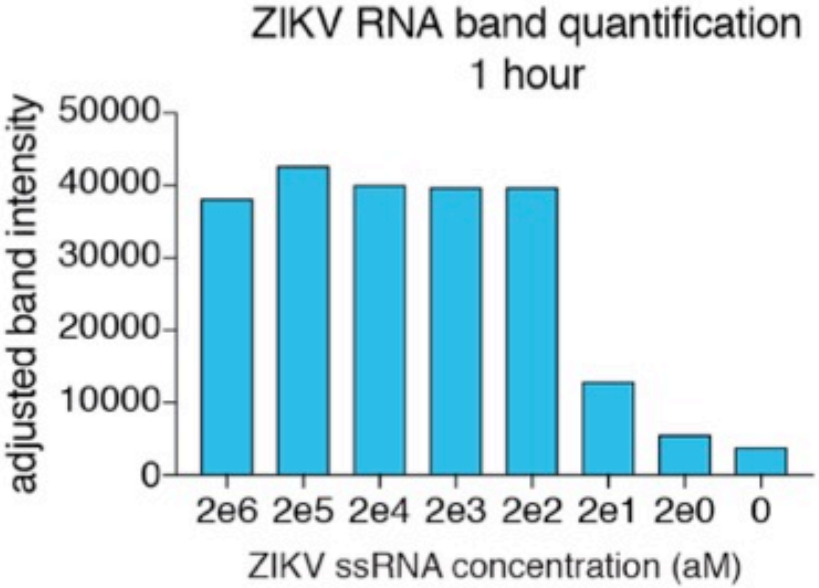
J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

https://www.youtube.com/watch?time_continue=58&v=dtPyvtFyes8



Detection of synthetic ZIKV ssRNA using lateral flow SHERLOCK with 1 hour of LwaCas13a reaction

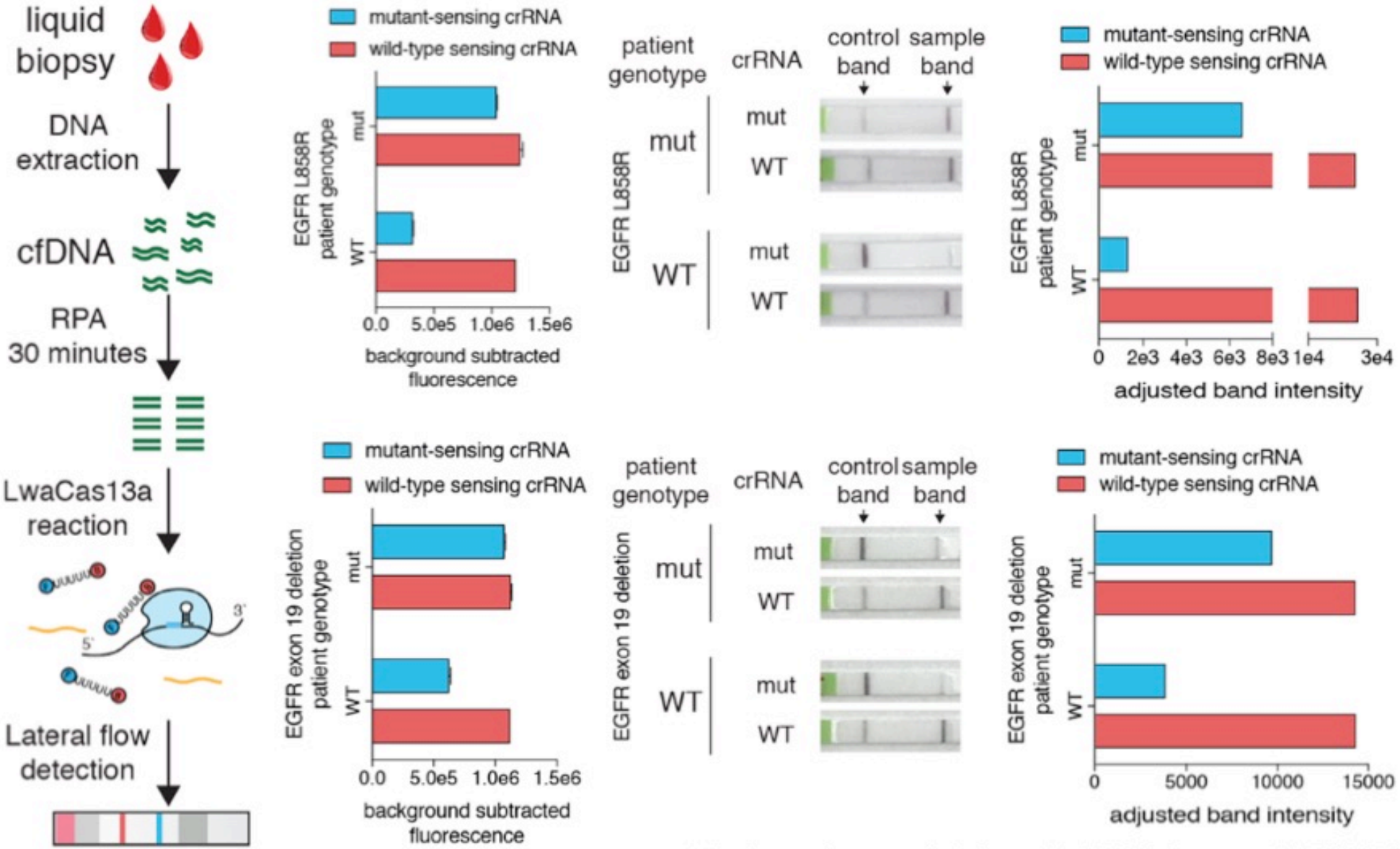




0.000 001	10^{-6}	micro	μ
0.000 000 001	10^{-9}	nano	n
0.000 000 000 001	10^{-12}	pico	p
0.000 000 000 000 001	10^{-18}	atto	a
0.000 000 000 000 000 000 001	10^{-21}	zepto	z

J. S. Gootenberg *et al*., *Science* 10.1126/science.aaq0179 (2018).

Lateral flow detection of therapeutically relevant EGFR mutations from patient liquid biopsy samples



J. S. Gootenberg *et al.*, *Science* 10.1126/science.aaq0179 (2018).

SHERLOCK Summary

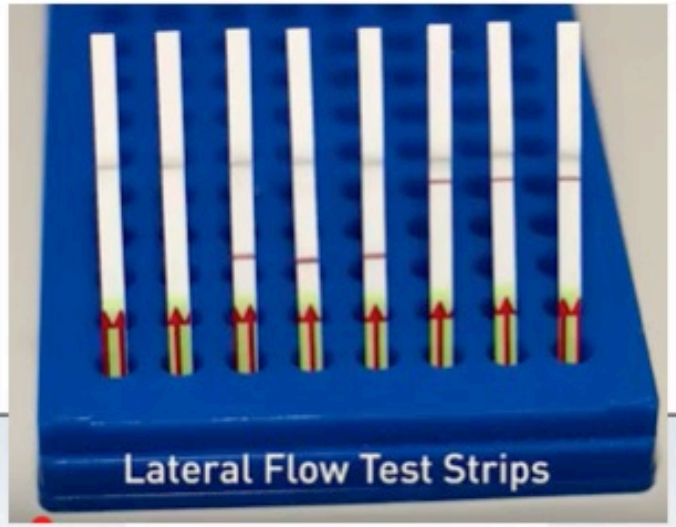
Rapid improvement by leveraging on specific properties of further CRISPR-Cas systems (Cas13a => Cas13b , Cas12a and Csm6)

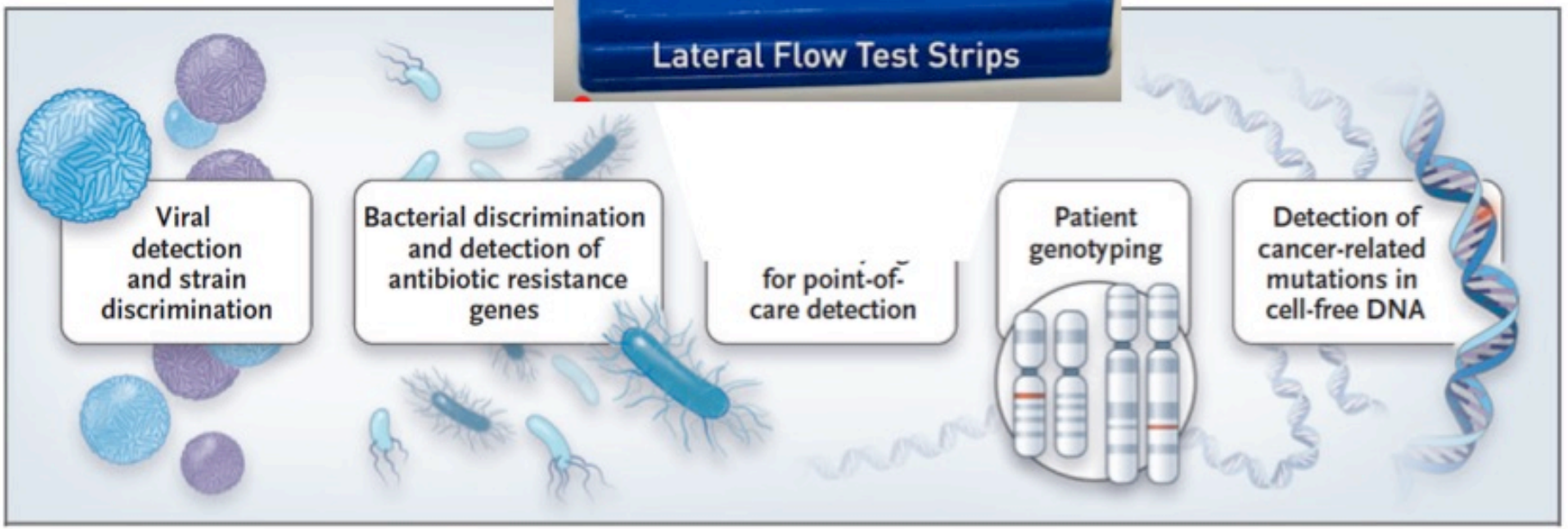
Table S9: Comparison of SHERLOCKv1 and SHERLOCKv2

Characteristic	SHERLOCKv1	SHERLOCKv2
Sensitivity	2aM	8zM
Specificity	Single-nucleotide	Single-nucleotide
In-sample multiplexing	Single	Up to four targets
Spatial multiplexing	Unlimited	Unlimited
Speed	2 hours	30 minutes (from crude sample to detection)
Readouts	Fluorescence	Fluorescence, visual by lateral flow
Signal amplification	None	Csm6 enhancement

Costs < $0.60cts

SHERLOCK Summary





Caliendo & Hodinka, 2017. NEJM, 377, 17

Little instrumentation required
Low Costs (< $0.60)

YouTube CH https://www.youtube.com/watch?time_continue=58&v=dtPyvtFyes8



SHERLOCKv2: Detection results on a paper strip

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Broad Institute
Published on Feb 15, 2018

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Jonathan Gootenberg and Omar Abudayyeh, of the Zhang lab at the Broad Institute, explain how a strip of paper can now indicate presence of pathogens, tumor DNA, or any genetic signature of interest. 100-fold greater sensitivity, the ability to detect multiple targets at once,

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