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Recent advances in CRISPR-based single-nucleotide fidelity diagnostics

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K. A. V. Kohabir[1,2,3], E. A. Sistermans[1,2] & R. M. F. Wolthuis[1,4]

Accurate point-of-care (PoC) detection of single nucleotide variants (SNVs) can support rapid and cost-effective clinical decision-making in tasks such as diagnosing pathogenic genetic variants, identifying pathogen resistance, or tracing viral lineage differentiation. Traditional nucleic acid diagnostics involving PCR and sequencing lack PoC applicability. CRISPR-based diagnostics (CRISPRdx) offer the necessary operational simplicity and ability to integrate specific nucleic acid sequence detection with isothermal amplification. However, achieving single-nucleotide fidelity is not self-evident and often requires empirical optimization. This Review explores recent strategics aimed at refining CRISPRdx specificity for SNV detection including various ways of tactical guide RNA (gRNA) design, fine-tuned effector selection, and improved reaction conditions. While the approaches described here are functional and can be occasionally combined, they often require optimizations to support specific clinical aims. Looking ahead, leveraging computational and AI tools for gRNA design, and harnessing newly discovered CRISPR systems, will broaden applicability and improve precision detection of CRISPRdx in diverse clinical settings.

Detecting clinically relevant nucleic acids with single-nucleotide specificity is often vital for medical decision-making, for instance when testing for human single nucleotide variants (SNVs), identifying resistance-acquiring mutations in pathogens, or distinguishing between viral lineages during infections. With the growing demand for rapid and precise DNA tests, genetic diagnostics are expected to play an increasingly critical role in tailoring treatments and improving prognostics.

Most of the current nucleic acid detection assays are based on polymerase chain reaction (PCR) amplification or next generation sequencing, which, although robust, are labor-intensive, require specialized equipment and are not always cost-efficient[1]. Furthermore, some diagnostic scenarios necessitate quick tests on-site or in the field. These quick tests are valuable in epidemic outbreak management and for testing in regionally remote or economically underdeveloped areas. On-site testing in the form of a *point-of-care* (PoC) test that can be undertaken at a bedside allows for rapid diagnostics to be performed closer to the patient, providing lower diagnostic turnaround times and use during surgery or time-restricted scenarios. However, current genetic tests are not suitable for affordable PoC applications as they require complex laboratory machinery. Instead, samples are sent to centralized labs which increases diagnostic turnaround times and costs.

To bridge these gaps, there is a need for innovative, rapid, cost-effective and user-friendly tests that retain the required accuracy and are PoC-amenable. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) proteins have revolutionized genomic engineering and manipulation[2]. In this Review we discuss the recent developments in CRISPR-based diagnostics (CRISPRdx), focusing on the ongoing approaches to enable robust, point mutation-specific diagnostics.

CRISPR-assisted diagnostics

Originally discovered in prokaryotes, CRISPR-Cas systems provide adaptive immunity against phages. Upon primary infection, parts of the phage genome are incorporated into the CRISPR locus of the bacterial genome. Transcription of the CRISPR locus results in guide RNAs (gRNAs) with complementarity to the target phage genome. These gRNAs can form ribonucleoprotein complexes with Cas proteins and direct them to cleave target nucleic acids of the same phage clade upon future infections. CRISPR-Cas systems have been successfully repurposed as reprogrammable RNA-guided endonucleases to facilitate precision genome editing and manipulation and increasingly are being applied in in vitro nucleic acid diagnostics.

The concept of CRISPR-assisted diagnostics was first introduced in 2016 as a Cas9-based test[3]. Soon after, the CRISPR toolbox expanded to include Cas proteins that exhibit indiscriminate *trans*-cleavage activity after RNA-dependent recognition of a target *in cis*, which can be utilized for

[1]Department of Human Genetics, Amsterdam UMC—Locatie Vrije Universiteit, Amsterdam, The Netherlands. [2]Amsterdam Reproduction and Development research institute, Amsterdam, The Netherlands. [3]Imaging and Biomarkers, Cancer Center Amsterdam, Amsterdam, The Netherlands. [4]Cancer Biology and Immunology, Cancer Center Amsterdam, Amsterdam, The Netherlands. e-mail: r.wolthuis@amsterdamumc.nl

CRISPR-based diagnostics (CRISPRdx; Fig. 1)[4,5]. This collateral activity can degrade single stranded nucleic acids, including synthetic fluorophore-bound reporters, a process which can be measured in various ways. Cas12 and Cas13 respectively recognize DNA and RNA *in cis* followed by non-specific single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA) degradation in trans. This isothermal reaction does not require thermocycling machinery[6], is competitive in cost compared to (quantitative) PCR-based or sequencing-based tests[4], and can give results within one hour[4,5]. By leveraging these unique properties, CRISPRdx enables diagnostics in diverse settings, including low-resource environments and bedside applications, where traditional methods like PCR or sequencing are impractical.

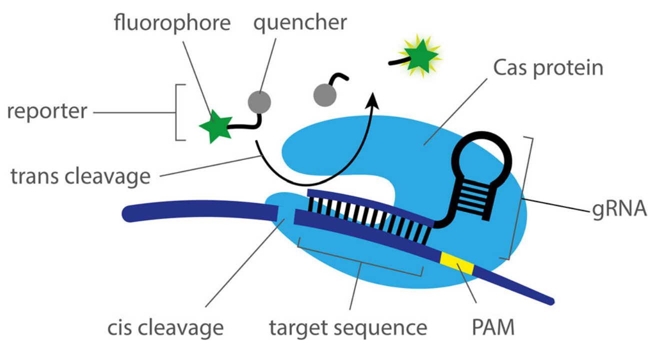


Fig. 1 | Schematic overview of trans cleavage-based CRISPRdx using a fluorescent reporter. Cas12 or Cas13 can pair with a gRNA to form a ribonucleoprotein complex that recognizes a target sequence. Most Cas12 and some Cas13 proteins respectively require a protospacer adjacent motif (PAM) or protospacer flanking sequence (PFS). The complex initiates cis-cleavage of the matched target and proceeds to trans-cleave single stranded nucleic acids without specific sequence requirements. Cas12 and Cas13 can trans-cleave ssDNA and ssRNA reporters respectively, which can be visualized using a quenched fluorophore reporter.

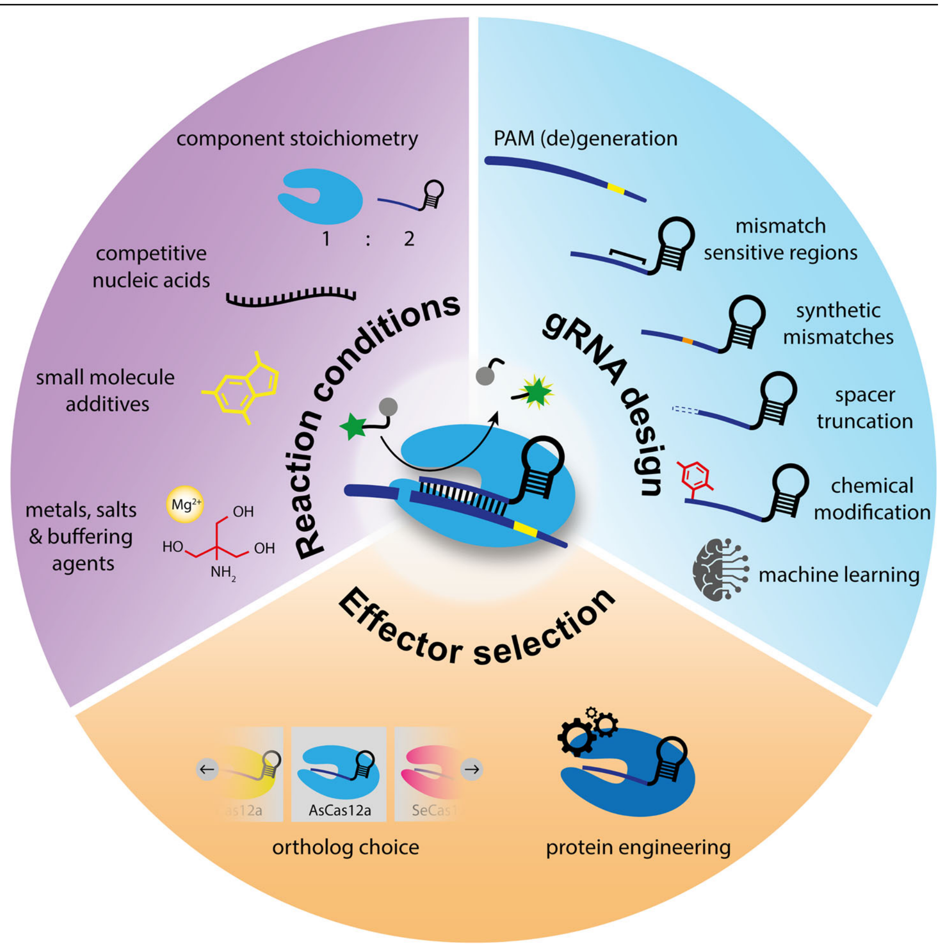
Although CRISPRdx in essence offers promising potential for PoC settings, several limitations still need to be addressed before implementation as a diagnostic test becomes feasible. A key issue is the limited sensitivity, reaching only picomolar (10^{-12}) detection limits, while relevant clinical target levels are often in the attomolar (10^{-18}) range in clinical samples[7,8]. Coupling the detection reaction to an isothermal nucleic acid amplification step can bridge this gap without reducing PoC-suitability. Cas12 and Cas13 have been used in assays such as DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR)[5], one-HOur Low-cost Multipurpose highly Efficient System (HOLMES)[9,10] and Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK)[4], respectively, to achieve attomolar sensitivity by incorporating isothermal amplification.

Another issue is that Cas proteins can tolerate mismatches, which consequently leads to false-positive test results. This occurs even though the target recognition is dependent on significant sequence complementarity between the gRNA and the target. Increasing efforts aim to resolve this specificity issue by introducing various strategies to achieve single-nucleotide specificity in detecting variants of interest. These can be divided into three main types of strategy: (i) gRNA design, (ii) Cas protein choice and (iii) biochemical reaction conditions (Fig. 2). We explain and compare these different reported strategies and comment on upcoming innovative approaches to reach high fidelity CRISPRdx.

gRNA design

A gRNA consists of a direct repeat, which serves as a structural element for the Cas protein to recruit the gRNA, and a spacer sequence that

Fig. 2 | Schematic overview of strategies for optimizing single-nucleotide CRISPRdx.



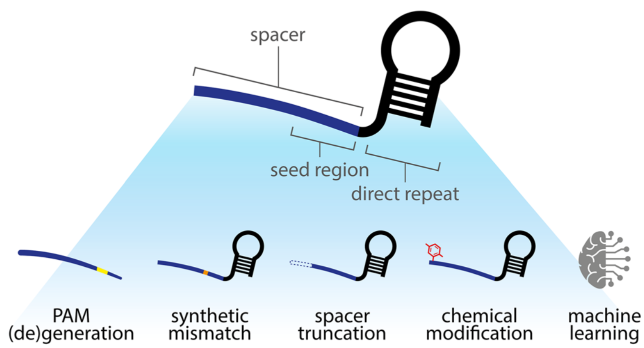


Fig. 3 | Schematic overview of gRNA design approaches to improve single-nucleotide specificity. Most Cas12 and Cas13 gRNAs consist of a spacer and a direct repeat (Cas9 requires an additional trans-activating crRNA [tracrRNA]). For some Cas proteins, the spacer contains a defined window, referred to as the seed region, in which mismatches are poorly tolerated. gRNAs can be designed strategically by making use of SNV-dependent PAM (de)generation. Spacer engineering for improved specificity can involve introduction of synthetic mismatches, truncation or chemical modification. Applied machine learning models can be adopted for generation of high-fitness gRNAs with low off-target predictions.

recognizes the target site (Fig. 3). In some cases, such as Cas9[11], Cas12c[12], and Cas12d[12], the direct repeat can bind a secondary structural RNA that is recognized by the Cas protein. For Cas9, fusing these two domains yields a functional chimeric single gRNA[11]. Since the spacer sequence directly interrogates target nucleic acids, rational gRNA design and engineering can influence the detection specificity, allowing for precision at single nucleotide level.

PAM (de)generation

Before gRNA-target duplex formation takes place, most DNA-targeting Cas proteins scan candidate targets for the presence of preferred protospacer adjacent motif (PAM) sequences. Biologically, PAM sequences are vital for the prokaryotic defense system to discriminate between the chromosomal CRISPR locus and viral DNA, thereby avoiding autoimmunity[13]. The first application of CRISPRdx used SNV-related (de)generation of PAMs to discriminate between single nucleotide differences. PAM generation occurs when an SNV results in the introduction of a PAM sequence, enabling CRISPR-based detection only when the target sequence harbors that specific mutation. Conversely, PAM degeneration occurs when an SNV disrupts an existing PAM, preventing CRISPR binding and cleavage at the mutated site. By designing assays that selectively activate or inhibit CRISPR function based on PAM presence, users can achieve single-nucleotide specificity in detection.

The first CRISPRdx application involved Cas9 to detect specific Zika virus strains[3]. As Zika is an RNA virus, and Cas9 detects dsDNA, the authors used isothermal nucleic acid sequence based amplification (NASBA[14]), in which the target RNA is repeatedly reverse transcribed to DNA and subsequently transcribed to RNA. For Zika lineage discrimination, the authors directed Cas9 to a strain-specific point mutation that resulted in a PAM sequence, enabling digestion of the corresponding DNA intermediates. If DNA intermediates were not digested, these would lead to NASBA-mediated build-up of RNA copies. The authors designed an RNA sensor (toehold switch) that, upon binding such RNA copies, activated in vitro expression of a chromogenic enzyme, enabling detection with minimal instrumentation in low-resource settings.

Similarly, Cas12 gRNA designs to yield SNV-dependent PAMs achieved specific detection of SARS-Cov-2 lineages[15–17] or of variety differentiation in plants[18]. As PAM interaction is crucial to target binding, designing gRNAs to detect SNVs that (de)generate PAMs has repeatedly shown to be a successful method. However, not all SNVs of interest (de) generate PAMs, which prevents a wide application of this method. Moreover, Cas13-based systems do not use PAMs, interacting instead with a more flexible protospacer flanking sequence (PFS) or have no preferences for the sequences flanking the target.

Mismatch-sensitive positions

CRISPR off-target prediction models are in silico data-driven algorithms that predict gRNA performance scores[19]. Mismatches along the gRNA spacer contribute to a penalty score that negatively impacts cleavage initiation[20,21]. A mismatch in the gRNA-target heteroduplex can be tolerated as long as the remainder of the spacer sequence binding kinetics to the target sufficiently exceeds the threshold for activating cleavage. Penalty scores vary with mismatch positions along the spacer, which weigh differently depending on the CRISPR effector[4,5,11,22]. Spacer positions where mismatches incur the highest penalty score together form a defined seed region[11,23,24] (Fig. 3). Therefore, designing gRNAs to detect SNVs in such mismatch-sensitive positions can be a practical approach[25]. If the Cas protein requires a PAM and the SNV is outside the seed region, the SNV may be relocated for detection within the seed by introducing a PAM through mutagenic primers[9,26]. However, although mismatches in the seed region are the least tolerated, for CRISPRdx they do not necessarily completely disrupt the binding of the rest of the spacer, nor prevent it from reaching the cleavage threshold[27,28]. Furthermore, strategic gRNA design should take into account that potential Wobble base pairing (G·U) can reduce penalty scores and thereby lead to false positives[29,30].

Synthetic mismatches

A gRNA's penalty score can also be increased by incorporating intentional mismatches. Introducing so-called synthetic mismatches can aid in making the gRNA more specific for SNV detection. The first reported use of synthetic mismatches was to enable the single-nucleotide fidelity of SHERLOCK[4]. This strategy was soon adapted in other Cas13a-CRISPRdx[29,31–37] and assays based on Cas9[38], Cas12a[39,40], Cas12b[41], Cas12f[42] and Cas10-Csm[43]. Depending on the changed nucleobase and the relative position to the SNV of interest, this strategy has varying success rates[28,44], is context-dependent and may occasionally not work[45]. Use of synthetic mismatches has also been reported to reduce overall gRNA activity, even for on-target detection[30]. Recently, a computational algorithm for tARgeting paThogEnic Mutations In the Seed region (ARTEMIS) demonstrated effective genome-wide identification of clinically relevant target candidate SNVs within the Cas12a seed region that could be detected with high specificity using a synthetic mismatch elsewhere within the seed region[28].

gRNA spacer truncation

Compared to using a full-length spacer, a mismatch within a shortened spacer yields a relatively increased penalty score and can be sufficient to abort the cleavage reaction. This can reduce mismatch tolerance, making the reaction more specific for targeting SNVs, which resonates with earlier findings on Cas9 fidelity in genome editing[46]. Early Cas12 and Cas13 CRISPRdx studies reported that using gRNA spacer truncation did improve single-nucleotide fidelity[4,9]. This approach was soon adopted by others, for example to detect anthrax SNPs[26,47] or SARS-CoV-2 variants[42,48,49]. While allowing for improved specificity, a slight decrease in activity due sub-optimal kinetics has been reported[37].

Chemical modifications

Other approaches to improve gRNA specificity involve chemical modifications such as incorporated 2'-O-Methyl[50], phosphorothioate bonds[51], non-canonical bases[52], DNA/RNA extensions/substitutions[53,54] and insertions/deletions[55]. Such modifications are commonly explored to enhance genome editing fidelity and efficiency[56–59], and have also been shown to improve both CRISPRdx detection rates[60] and specificity[50]. While these chemical modifications maintain gRNA:target complementarity, they can alter the melting temperature and binding kinetics of gRNAs, thereby influencing the specificity and gRNA activity[57]. Additionally, they can be

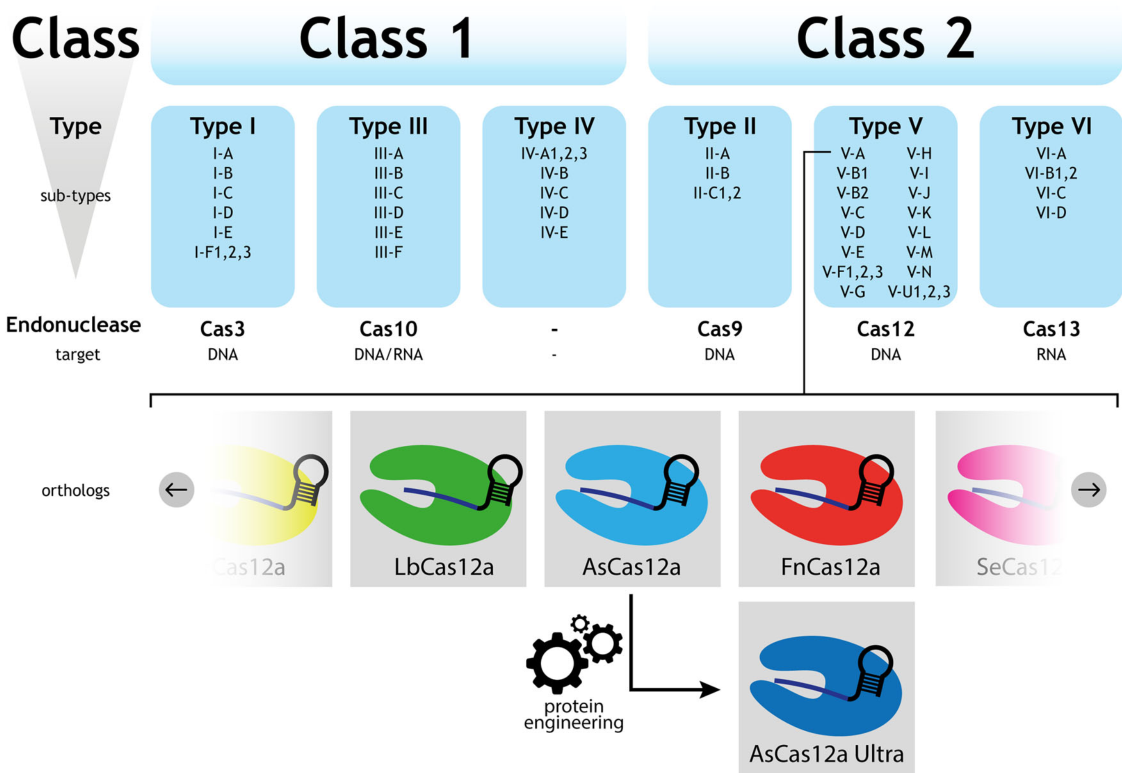


Fig. 4 | Overview of CRISPR classification and diversification. Protein engineering of CRISPR orthologs can result in Cas proteins (such as AsCas12a Ultra), with altered activity, specificity or PAM preference. This overview does not include the newly proposed Class 1 Type VII system, with Cas14-based RNase activity[178,179]. The proposed Cas14 in these newly identified systems is different from the previously published Type V-F Cas14 proteins[22], which we refer to as Cas12f throughout this Review. Exceptionally, although classified as a Type V effector, Cas12a2 was shown to detect RNA *in cis*, and collaterally cleave ssRNA, ssDNA and dsDNA in trans[125,126].

applied to protect a gRNA against degradation by ribonucleases, and to promote tertiary RNA structure stability[58].

Machine learning

Recent advances in computational methods have leveraged machine learning for predicting gRNA activity and specificity. Machine learning can correlate specific gRNA features with their on-target fitness, which can be applied when designing gRNAs for detecting other targets with predicted performance. This can significantly reduce the need for empirical optimization, as there is no requirement to fully understand the gRNA design rules rationally. An exemplary study performed massively multiplexed CRISPRdx with over 19,000 gRNA:target pairs, yielding a large dataset to train a deep learning model for designing competent gRNAs[61]. This method includes predictions for single-nucleotide mismatches, which can be used for gRNA design to detect specific SNVs. CRISPRdx methods such as microfluidic Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (mCARMEN)[62], Streamlined Highlighting of Infections to Navigate Epidemics (SHINE) v1[63], and SHINEv2[6] use such machine learning.

Effector selection

Variant/ortholog choice

CRISPR effectors can be divided into two classes based on whether the system requires a protein effector complex (Class 1) or a single effector protein (Class 2) for target recognition and interference. These classes encompass Type I, Type III & Type IV nucleases and Type II, Type V & Type VI nucleases respectively, based on the characteristics of the effector proteins. There are recognized subtypes based on dissimilarities between systems among the same Type (Fig. 4). The discovery of collateral activity in Type V (Cas12) and Type VI (Cas13) CRISPR systems simplified CRISPR-based detection assays, since a single CRISPR protein was now capable of catalyzing multiple turnover reporter degradation upon RNA-guided recognition of a target. Type V and Type VI systems are remarkably diverse, with many Cas proteins only discovered recently[64,65]. This includes subtype variants (e.g., Cas12a, Cas12b, Cas12c) and further diversification in the form of orthologs (e.g., AsCas12a from *Acidaminococcus sp.*, LbCas12a from *Lachnospiraceae bacterium*, FnCas12a from *Francisella novicida*) with differences in activity, mismatch-sensitive regions, PAM preferences and temperature tolerances. Therefore, strategic variant/ortholog choice is important when striving for optimized specificity.

Cas12a is most commonly used among the reported Type V-based SNV diagnostics (Table 1). AsCas12a, LbCas12a and FnCas12a exhibit varying tolerance to single-nucleotide mismatches, depending on the mismatched base and position[30,66]. CasDx1, also a Type V-A effector, was demonstrated to outperform AsCas12a and LbCas12a when discriminating SNVs[67]. Among Type II effectors, FnCas9 was found be highly sensitive to single mismatches compared to the canonically used SpCas9[68], enabling SNV-specific discrimination between SARS-CoV-2 variants[69].

Strategic choice of Type V CRISPR proteins can not only improve specificity but may also expand the conditions under which CRISPRdx can operate. This is particularly important for one-pot reactions integrating isothermal amplification and detection, which often operate at temperatures exceeding physiological range. Thermotolerant Cas12a variants are rare but do exist[70,71]. In contrast, most Cas12b orthologs originate from extremophiles[72,73], and are suitable effectors for high-fidelity SNV detection at high temperatures, enabling one-pot assays[10]. Cas12c orthologs, on the other hand, offer wider targeting possibilities with a minimal 5'TG PAM preference[74]. As opposed to Cas12a, Cas12c has been shown to be extremely sensitive to PAM-distal mismatches[74], allowing for PAM-distal single-nucleotide specificity. Cas12f is a Type V effector that naturally detects

Table 1 | Overview of effectors used in single nucleotide-specific CRISPRdx, including the lowest reported limit of detection (LOD)

Class	Type	Sub-type	Effector	PAM/PFS/rPAM[a]	Target	LOD	Reference
1	I	E	EcoCascade +EcoCas3	AAG	dsDNA	1.7 aM	140
	III	B	TtCmr+TTHB144	None[b]	ssRNA	800 aM	141
2	II	A	SpCas9	NGG	dsDNA	1.67 aM	3,142–145
			MgaCas9	NNGAD	dsDNA	6.3 fM	142
		B	FnCas9	NGG	dsDNA	10 copies	38,69
		C	CjeCas9	NNNNRYAC	dsDNA[c]	1.7 aM	114
	V	A	AsCas12a	TTTV	dsDNA	10 aM	146,147
			ErCas12a	YTTN	dsDNA	50 pM	146
			FnCas12a	YTN/TTN	dsDNA	9 fM	148,149
			LbCas12a	TTTV	dsDNA	0.5 aM	9,15–18,26,30,39,40,44,47,48,50,150–169
			SeCas12a[d]	TCCA	dsDNA	1 aM	166
			CasDx1	YYN	dsDNA	332 aM	67
		B	AaCas12b (AapCas12b)	TTN	dsDNA	1 aM	41,49
			AacCas12b	TTN	dsDNA	10 aM	10
		C	Cas12c1	TG	dsDNA	230 aM	74
		F	Cas12f1	None[e]	ssDNA	1.66-166 aM	22,27,42
		J	Cas12j (CasΦ)	TBN	dsDNA	63 pM	87
	VI	A	LbuCas13a	None	ssRNA	166 aM	35,37
			LtrCas13a	?[f]	ssRNA	2.4 aM	170
			LwaCas13a	H/none	ssRNA	1 aM	4,6,29,31–34,36,45,62,168,170–175
		B	PsmCas13b	none	ssRNA	1 aM	31
		D	EsCas13d	none	ssRNA	65 pM	176
			RspCas13d	none	ssRNA	69 pM	176

Regarding the LOD, the lowest achievable detection level reported across the listed references for each effector range from low attomolar to low femtomolar, depending on whether the study used pre-amplification, what read-out was used or what the assay setup was (one-/dual-pot). Better sensitivity levels may be reached upon further analysis. Therefore, the LOD here serves as a practical indication of what sensitivity levels have been reached so far with the listed effectors.

[a]PAM applies to Type I, Type II and Type V systems; PFS applies to Type VI systems; rPAM applies to Type III systems.

[b]Type III-B systems require absence of complementarity between the 5' handle of the gRNA and the target RNA in order to produce second messengers that activate RNase activity of CRISPR-associated Rossmann fold (CARF) proteins that degrade reporter RNAs[141].

[c]CjeCas9 was used to detect RNA using tracrRNAs that pair with an RNA target of interest. As a result, the dual guide system allows Cas9 to degrade a DNA reporter.

[d]Engineered proteins.

[e]In case of ssDNA detection. Cas12f1-based dsDNA recognition uses T or C rich PAMs[177].

[f]Not reported, likely none.

ssDNA and can also discriminate SNVs with high fidelity at PAM-distal sites[22]. In a study where Cas12a could not detect an SNV of interest switching to Cas12f-based detection resolved the issue as the SNV could be detected with a mismatch-sensitive window[27].

Although not as diverse as Type V effectors, there is a multitude of Type VI systems that have been explored for single-nucleotide fidelity detection (Table 1). Since Cas13 proteins are RNA-targeting effectors, they do not require PAM sequences, which are typically needed in DNA-targeting systems to avoid autoimmune activity due to self-targeting spacers[75]. Instead, some Cas13a and Cas13b proteins can display preferences for a 1-3nt PFS[24,76]. To date, most other characterized Cas13 proteins are less strict or show no PFS preference. Selection of such effectors widens the targetable landscape, enabling detection of many more SNVs. Furthermore, apart from sequence preferences *in cis*, different Cas13 variants exhibit sequence preference in trans. For example, LwaCas13a prefers reporters containing an AU dinucleotide motif, whereas CcaCas13b, LbaCas13a and PsmCas13b prefer UC, AC and GA motifs, respectively[31]. Using ssRNA reporters tailored to this preference allows these systems to be exploited for multiplexed detection of different targets[31].

Protein engineering

If none of the naturally occurring characterized effectors meet the proposed criteria, desired characteristics can still be accomplished through protein engineering. Directed evolution, a method that mimics natural selection by introducing random mutations and selecting for improved traits, has produced multiple effectors with non-canonical PAMs[77–80], increased activity[81–83], and increased specificity[84]. Whereas directed evolution often relies on random mutagenesis and selective pressure for a desired trait, rational engineering uses detailed structural knowledge, either from crystal structures or in silico modeling, to introduce targeted mutations for achieving enhanced traits. This approach often involves understanding a protein's molecular and structural biology, complemented by in silico modeling to predict the effects of candidate mutations. Some rationally engineered effectors have been used for improved CRISPRdx sensitivity[85] and specificity[37,86,87]. However, most CRISPR protein engineering aims to improve the performance for genome editing/manipulation inside live cells, which is a different environment compared to in vitro CRISPRdx. It is important to note that, compared to the ancestral non-engineered proteins, enhanced genome editors have been found to perform with reduced single-nucleotide fidelity in CRISPRdx[30].

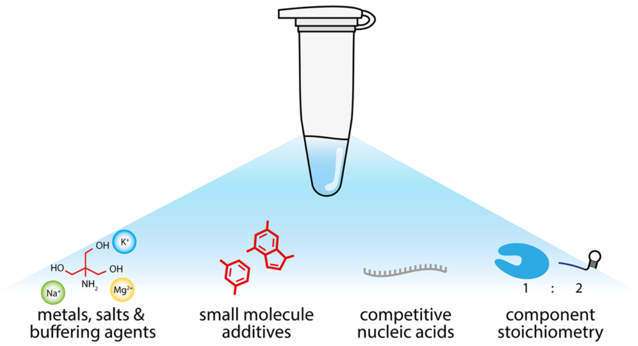


Fig. 5 | Schematic overview of reaction conditions that impact CRISPRdx specificity. Divalent metals, salts and buffering agents, as well as small molecule additives can be titrated to enhance single-nucleotide specificity. Competitive nucleic acids can be used as additives to disfavor mismatches, yielding higher specificity. Stoichiometry between different components in the CRISPRdx reaction can also reduce off-target activity (graphic shows stoichiometry between Cas protein and gRNA as an example).

Reaction conditions

Apart from strategic design of the CRISPRdx ribonucleoprotein complex, considering reaction conditions can also improve detection fidelity. Several factors influence the performance of CRISPR effectors, including buffer composition and component stoichiometries (Fig. 5).

Buffer composition

Divalent metal ion availability, mostly in the form of magnesium (Mg^{2+}) ions, is crucial for cleavage activity[11,88,89] and gRNA tertiary structure[90,91]. Recent findings elucidated that magnesium is essential for collateral activity and that titration thereof can enhance Cas12a's single-nucleotide specificity[92–94]. Cas12a cannot use all divalent metals[53], and some, such as manganese (Mn^{2+}), can promote off-target or even gRNA-independent cleavage activity[95,96]. Metal chelators such as ethylenediaminetetraacetic acid (EDTA) can reduce the ion availability and inhibit Cas protein activity and are sometimes used to quench assays[95,97]. Apart from optimizing Mg^{2+} concentrations, optimization of salts and buffering agents (such as NaCl, KCl, HEPES and TRIS) can positively impact the activity of Cas12[41,98,99] and Cas13[31,63].

Additives

Chemical additives can improve the performance of PCR or isothermal amplification. Several studies have explored the use of such small molecule additives to further enhance the in vitro performance of Cas proteins, often in the context of one-pot assays. Among others, dithiothreitol (DTT)[60], sucrose[86], taurine[100], glycine[100], glycerol[101,102], betaine[101,103], Dimethyl Sulfoxide (DMSO)[103], L-proline[103] and dNTPs[104,105] have been shown to affect activity and detection sensitivity. Exact mechanisms for how these additives play a role in boosting the detection performance remain to be resolved, but hypotheses often lean towards protein stabilization and altered solvation states. Glycerol can boost one-pot RPA-Cas12 assays by forming a viscous layer that maintains separated RPA and CRISPR phases, resulting in enhanced amplification and detection efficiency[101]. Titration of agents that lower melting temperature, such as DMSO and glycerol, was recently shown to make Cas12a-based detection more single-nucleotide specific[102]. PAM-independent, SNV-specific activation is claimed at 48 °C, based on reduced target dsDNA stability, facilitating strand invasion by Cas12a. This effect was further enhanced by use of agents that reduced the melting temperature. Adjusting the incubation temperature has been shown to enhance the activity of the effector[106]. However, there are no reports on the effect of temperature on single-nucleotide fidelity of CRISPR effectors[107].

Recent studies have explored innovative approaches involving competitive nucleic acids to bypass PAM requirements, while still achieving single-nucleotide detection resolution. Toehold-mediated strand displacement, for example, activates Cas12a based purely on target:gRNA binding kinetics, strongly disfavoring single mismatches[94,108]. One report mentions the use of short DNA or RNA sequences named occluders, which hybridize with the target sequence, selectively disfavoring single-nucleotide mismatches to achieve high-fidelity detection[109]. These studies demonstrate promising emerging PAM-independent different-angle strategies for SNV-specific detection.

Component stoichiometries

The ratios between gRNA, CRISPR effector and target can influence off-target activity. Optimizing these component stoichiometries can significantly reduce false positives and background noise. For instance, Cas12a can non-specifically degrade ssDNA in the absence of gRNA, especially in the presence of Mn^{2+} ions. Maintaining a surplus of gRNA relative to Cas12a can mitigate this undesired effect[95,110]. Another study investigated the influence of amplification rate on the single-nucleotide specificity of Cas12a, as the extent of amplification directly impacts the amount of target available in the CRISPRdx reaction. Reducing amplification levels increased detection specificity, although this came with the trade-off of longer assay duration due to the resulting reduced target:Cas12a ratio[30].

Other CRISPR-based approaches

CRISPR technology has revolutionized genome editing and manipulation, and now opens new avenues for rapid, PoC nucleic acid detection without reliance on complex or expensive instrumentation. CRISPRdx offers operational simplicity and flexibility, with material costs under $1 per test[3,4], positioning it as a promising alternative for settings where traditional methods such as PCR or sequencing are impractical or too slow. Despite this potential, achieving consistent single-nucleotide fidelity remains a challenge, and often requires additional, mostly empirical or less self-evident, measures to refine specificity. In this Review, we outlined various strategies that aim at optimizing CRISPRdx with single-nucleotide fidelity.

While this Review focuses on direct CRISPR-based SNV detection optimization strategies, alternative approaches use a selective pre-amplification step such as blocker displacement amplification (BDA)[27] and improved allele-specific PCR (iARMS-PCR)[111] to enhance SNV discrimination before the CRISPRdx reaction. BDA and iARMS-PCR use primers that exhibit highly different binding thermodynamics upon a single-nucleotide mismatch, which consequentially determines whether the amplification reaction succeeds or not. Although these methods do not retain the simplicity of one-step CRISPR assays, they provide an additional strategy for improving specificity in cases where direct CRISPRdx detection or optimization remains challenging. In this Review, we identified approaches related to gRNA design, effector choice, and reaction conditions. Although many of these approaches resolved low specificity issues, their implementation still requires empirical optimization to some extent. Machine learning offers the potential to reduce optimization in gRNA design, as demonstrated by mCARMEN[62] and SHINE[6,63]. Exploiting AI-based gRNA design algorithms through integration in accessible online design tools may further simplify the gRNA design process[112]. Machine learning could also offer potential for aiding rational protein engineering, to anticipate and optimize structural changes in engineered Cas proteins. For example, this has been applied to specifically change a Cas9 PAM sequence from NGG to KKH[113]. This can be beneficial for CRISPRdx, as PAM or PFS dependency still limits the number of candidate targets. Alternatively, reprogramming the anti-repeat of the tracrRNA to hybridize with ssRNA targets of interest enabled PAM-independent SNV detection with Cas9[114] and Cas12[115].

As expected, we found that well-characterized and commercially available effectors are most frequently used in CRISPRdx. However, the variety of CRISPR effectors, especially among the Type V nucleases, is vast and allows for opportunities to expand CRISPRdx tools. Cas12d (also known as CasY) has collateral activity but has only been explored for tolerance to pairs of adjacent mismatches (not single mismatches)[12], making it suitable for detection with synthetic mismatches. Some Type V effectors, such as Cas12e[116] (also known as CasX), Cas12k[117] and Cas12m[118] exhibit minimal collateral activity, making them less interesting for *trans* cleavage-based CRISPRdx. Cas12g[119] and Cas12l[120] (also known as Casπ[121]) showed remarkable *trans*-ssRNase activity in addition to collateral ssDNA cleavage. This enables use of RNA-based reporters for Type V CRISPRdx. Cas12i[119,122] and Cas12n[123] both exhibit collateral activity and can *cis*-cleave with single-nucleotide precision, but have not been harnessed for CRISPRdx yet. Miniature CRISPR systems from phages can also harbor *trans*-cleaving effectors. Cas12j[87] (also known as CasΦ) and Casλ[124] are both examples that have been examined for mismatch-sensitive regions. Furthermore, Cas12a2 was previously found to be an RNA-guided RNA detector, collaterally degrading ssRNA, ssDNA and dsDNA, using the same gRNA as Cas12a[125,126]. The property of gRNA sharing between orthologues could be exploited to simultaneously detect RNA and DNA species of the same sequence. Recently published patents report newly engineered Type V systems (Cas12p & Cas12q), which seemingly also exhibit both DNase and RNase activity in trans[127]. Though not all Type V systems are well characterized yet, the vast diversity offers promising options for tailored and alternative detection approaches. The diversity of Type VI systems, though smaller in magnitude, has also been explored for RNA targeting. Cas13a, Cas13d, Cas13e, Cas13X and Cas13Y exhibit highly varying collateral activity[65,128]. Among the few other subtypes, Cas13h was shown to exhibit trans-RNase activity, with a mismatch-sensitive internal seed region[129]. For therapeutic use, Cas13 preferably has minimal collateral activity to reduce bystander effects in trans. For instance, Cas13X was engineered to eliminate its *trans*-activity, while enhancing the on-target specificity, for targeted knock-down in cells[130]. With this vast landscape of CRISPR effectors, effective comparison and standardization can be challenging. Definition of trans-units has been shown to be effective for comparing the efficacy of optimization steps in CRISPRdx[131].

Alternatives to CRISPR

There are emerging alternatives for RNA-guided nucleic acid diagnostics that do not use CRISPR effectors. Recent work identified TnpB, IscB and IsrB as transposon-encoded RNA-guided nucleases that are ancestral to CRISPR nucleases[132]. These obligate mobile element–guided activity systems use ωRNAs to guide nucleases for nucleic acid interference in a target adjacent motif dependent manner. TnpB, ancestral to Type V CRISPR effectors, exhibits collateral activity that has yet to be explored for nucleic acid detection. Even when excluding eukaryotic descendant Fanzor proteins[133,134], over one million diverse prokaryotic TnpB loci have been identified[132]. This delivers a considerable opportunity for discovering and characterizing efficient and high-fidelity nucleases. Effectors do not have to exhibit collateral nucleic acid interference to be of use in diagnostics. Short prokaryotic Argonaute and the associated TIR-APAZ (SPARTA) proteins have recently been discovered to initiate collateral $NAD(P)^+$ depletion upon RNA-guided detection of a target DNA sequence[135]. DNA sequences of interest were detected by converting the $NAD(P)^+$ reduction to a fluorescence signal. Argonaute proteins operate isothermally, are RNA-guided, do not require PAMs and rely fully on guide-target complementarity[136], making them interesting candidates for precision nucleic acid detection[137].

Concluding remarks

CRISPRdx has the potential to move genetic testing out of the lab, with operational ease and without the requirement of complex instruments and the associated costs. The COVID-19 pandemic highlighted the critical need for fast, accessible testing for timely decision-making at a moment that resources and turnaround times were constrained. Although not widely deployed clinically during the pandemic, the programmability and flexibility of CRISPRdx make it ideal for quickly developing new tests for emerging targets. As it is focused on achieving single nucleotide-fidelity CRISPRdx, this Review does not discuss approaches to improve testing sensitivity. Although some of the discussed approaches contribute to both, sensitivity can additionally be improved by strategic choices of nucleic acid amplification and readout techniques. Electric CRISPRdx biosensors[138], for instance, offer highly sensitive and semi-quantitative detection options compared to lateral flow or colorimetric-based approaches. A different approach for increased sensitivity can be explored through volume reduction, effectively raising the in-reaction target concentration. Digital droplet CRISPRdx assays using (sub-)nanoliter droplet reaction volumes have demonstrated amplification-free detection on microfluidic devices[139].

Moving forward, the key step to bringing CRISPRdx into practice will likely involve enhancing standardization to achieve robust, reproducible high-fidelity detection across diverse sample types. A possible approach towards standardization includes integration of amplification and detection into a one-pot assay, reducing risk of cross-contamination, while making tests more user-friendly to execute. Some of the strategies discussed in this Review have already contributed to optimizing fidelity of such assays[6,29,63]. These advancements do not only hold significant clinical and real-world potential for rapid identification of viral lineages but can also be effective in detection of other SNVs of interest in PoC and resource-limited settings.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

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Correspondence and requests for materials should be addressed to R. M. F. Wolthuis.

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