nature portfolio

Peer Review File

CRISPR Anti-tag-Mediated Room-Temperature RNA Detection Using CRISPR/Cas13a

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Parts of this Peer Review File have been redacted as indicated to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study presents a novel Cas13a-based assay for RNA detection, incorporating an innovative cascade signal amplification mechanism. The authors introduce an "anti-tag" sequence paired with a complementary DNA strand, effectively masking the target sequence within a CRISPR anti-tag hairpin. Upon target recognition, Cas13a's collateral activity cleaves the hairpin, releasing additional target sequences, thereby amplifying the signal. This work addresses an important challenge related to the accessibility of HIV testing via Cas13a-based nucleic acid detection, which holds significant clinical implications. The assay's overall performance is well characterized, demonstrating higher detection sensitivity compared to conventional Cas13a/crRNA detection approaches. While the study is well-executed, addressing the following points will further strengthen the manuscript before publication:

1. Consideration of practical implementation in low-resource settings: given the potential clinical application in resource-limited areas, the fluorescence-based readout may present practical constraints due to the need for a fluorescence reader. The authors could explore alternative readout strategies, such as paper-based detection or portable reader designs, to enhance the assay's accessibility.

2. Clarification of potential leakage in DNA blocker mechanism: in line 211, the authors state that "even with shorter DNA blockers, the HIV target RNA within HP does not activate Cas13a/HIV crRNA." However, Supplementary Figure 2 suggests that 14D HP and 16D HP exhibit cleavage even in the absence of the target, indicating possible leakage with shorter blockers. A similar inconsistency appears in line 224 and Figure 3d (Step 2), where the claim that "no cleavage occurred in the absence of target M" is contradicted by a visible band. If this reflects potential blocker leakage, a discussion on minimizing leakage would be valuable for ensuring the robustness of the technology.

3. Strengthening data interpretation in Figures 6c and 6d: including a correlation between Cq values and fluorescence intensity would enhance the validity of the results. Some positive samples exhibit RFU values close to negative controls, which might complicate differentiation. Additional data representation or statistical analysis could bolster confidence in the assay's performance.

4. Cost comparison for field applications: since the assay is designed for point-of-care and resource-limited environments, providing a cost comparison between CRISPR-based detection and gold-standard qPCR would contextualize its practical feasibility. A brief discussion on the assay's potential cost advantages could strengthen its impact for real-world deployment.

Reviewer #2

(Remarks to the Author)
This manuscript, CRISPR Anti-tag-Mediated Room-Temperature RNA Detection Using CRISPR/Cas13a, employs the trans-cleavage activity of LwaCas13a in conjunction with a tag:anti-tag hairpin RNA mechanism to enhance the sensitivity of

LwaCas13a for nucleic acid detection. The design principle is well-conceived and demonstrates significant potential for amplification-free detection. However, the quality of the data presented could not reach publication requirements.
Major comments:
1. In the first paragraph of "Development and optimization of the CARRD assay," the authors claim that Cas13a is more effective at 25°C than at 37°C. However, Figures 2b and 2c suggest that LwaCas13a reaches end-point fluorescence at 37°C when the experiment starts. The lack of fold change in fluorescence could be the result of the completion of the reaction at 37°C before the measurement starts. One should assume that the no-target control and target samples start at the same or similar fluorescence level. Additionally, all four conditions reach a similar end-point fluorescence level, which further supports the idea that the reaction is already complete at 37°C. The authors may consider lowering the target ssRNA concentration to prevent the experiment from reaching completion before measurements begin. Furthermore, given this observation, it may be beneficial to review all fluorescence experiments throughout the paper to ensure they are appropriately designed and executed.
2. The core functionality of this design appears to rely on the RNA-hairpin-DNA mechanism, where the RNA serves as the target sequence, and the DNA binds to the RNA to inhibit recognition by Cas13a. Cas13a trans-cleaves the hairpin to release the RNA from DNA. Furthermore, the authors demonstrate substantial trans-cleavage activity in the presence of an anti-tag. This suggests that a broader selection of hairpin sequences may be feasible beyond what is currently proposed in the paper. While testing additional hairpin sequences may be outside the immediate scope of this study, the paper should acknowledge the potential for alternative sequences to expand the applicability of this approach.
3. When the anti-tag is cleaved in trans, a mixture of products is expected depending on the cleavage position. Given that LwaCas13a preferentially cleaves at uracil (U) sites, it is likely to generate incomplete anti-tag fragments rather than the anticipated 8-nt anti-tag. By mutating each nucleotide individually, the authors may identify the most efficient cleavage position or context. Additionally, the position of cleavage might be inferred from the gel image presented in Figure 3d. This observation aligns with point 2, suggesting that the anti-tag sequence itself may not be the crucial factor. Instead, the general design principle appears to be effective, and further variations in the anti-tag sequences could have been explored to optimize performance.
4. The data shown in Figures 2a and 2d are confusing:
a. 5'-DNA tag HP has a very high fluorescence level. It is understandable that the anti-tag is at the 5' end so it should not inhibit the Cas13 cleavage after the anti-tag is cleaved. However, to have Cas13 bind to the RNA region, the RNA-DNA duplex has to either dissociate or have its anti-tag cleaved. If dissociation between the DNA-RNA happens, 5, 6, and 7 should have similar RFU. If the anti-tag has to be cleaved, but without initial ssRNA as a target, it suggests a very high background level of trans-cleavage to initiate this process. This is also discussed in point 6. Can the authors explain the reasons behind this observation and possible ways to decipher this?
b. If we assume the anti-tag is being cleaved in trans without the initial ssRNA target, then it is reasonable to say the anti-tag and rU8 are also cleaved in trans. The authors show that anti-tag targets do not necessarily inhibit trans-cleavage. Then why do 5'-RNA tag HP and 5'-RNA rU8 HP not achieve an RFU that is much higher than the background in Figure 2d?
5. Why do the authors choose 5'-RNA tag-HP with complementary DNA instead of complementary RNA? Cas13a should not cleave double-stranded RNA in trans either.
6. We also have major concerns regarding the high levels of background activities in the HP groups:
a. Figure 3d, Supplementary Figure 2, 4, and 6 show a very high background when HP is added. In fact, in Figures 4 and 6, the background level is already higher or similar to the LOD of the conventional assay. This is quite concerning when comparing the two assays because the baseline in the CARRD is very high. Has there been any attempt to reduce this background activity?
b. Related to minor point 2, maybe it is more reasonable to compare the fold change between different concentrations instead of absolute values. For example, the end-point fluorescence at 1 fM in CARRD compared to CARRD control is 10x whereas the end-point fluorescence at 1 fM in conventional assay to its control is 2x (These numbers are arbitrary. I did not do actual calculations). This helps readers gauge the level of amplifications since the plots mostly use CARRD scales for both assays and it is impossible to see the actual values in the conventional assay.
c. Related to point 6b, in the supplementary figures, the authors can also show the conventional assay in the correct scale to better see the conventional assay activities in the lower concentrations.
d. Taking supplementary figure 6 as an example, the control in DP is already generating a signal similar to 1 pM of target RNA in the conventional assay. This suggests that HP is equivalent to having 1 pM of target RNA in the assay. We can also see that 10 aM in the conventional assay generates little to no signal, suggesting very little trans-cleavage activities. When 10 aM of initial target RNA is present in CARRD, we can think of it as control + 10 aM, or 1 pM + 10 aM. Then how does it generate a much higher signal than the background, especially when there does not seem to be a lag phage in the control where 10 aM might have been able to jump-start the anti-tag cleavage? The same question also applies to supplementary figure 4.
Minor points:
1. In Figure 3d, it is hard to tell if the 14D HP was cleaved or not. A better control could be an annealed product of the two separately synthesized fragments representing the two strands after cleavage or the authors can use denaturing gel so that the hairpin shows up as a single band and the cleaved 14D HP shows up as 2 or more bands.
2. We know that PCR amplifies signals exponentially by 2n and conventional Cas13a reaction is a first-order reaction. Is it possible to estimate the level of amplification achieved with this method to better understand the kinetics behind it?
3. Can the authors estimate the viral RNA concentrations in the clinical samples? The range of sample concentrations seems to be very narrow based on the Ct values.

Reviewer #3

(Remarks to the Author)

This manuscript presents a new nucleic acid detection method that incorporates an anti-tag hairpin, offering a promising approach to advancing current molecular diagnostics. However, there are several points that have not been fully addressed.

Comments
1. The authors observed that lwCas13a exhibited the highest trans-cleavage activity at 25°C. Did the authors investigate whether the length of the spacer:target pairing duplex influences the trans-cleavage activity? Additionally, did lwCas13a consistently show the highest trans-cleavage activity at 25°C, regardless of the length of the spacer:target pairing duplex?"
2.The authors found that the CARRD assay showed higher sensitivity compared to the conventional Cas13a/crRNA reaction without the CRISPR anti-tag HP. In the conventional Cas13a assays, such as SHERLOCK, the spacer length is typically 28 nt. Did the CARRD assay also performed higher sensitivity when using a crRNA with a 28 nt spacer?
3. The authors observed that the anti-tag hairpin inhibits trans-cleavage activity. Upon cleavage of the anti-tag, the target region is released from the hairpin structure, leading to the activation of Cas13a. Based on this mechanism, the anti-tag may reduce Cas13a activity if it is not fully cleaved, as any remaining anti-tag at the 3' end of the target could partially inhibit Cas13a. Could optimizing the sequence of the loop region in the hairpin improve the trans-cleavage efficiency of the loop region and, as a result, enhance the overall detection sensitivity?
4. Why did the authors design the anti-tag hairpin using a chimeric DNA-RNA structure rather than an all-RNA hairpin? Could you elaborate on the design principle behind this choice? Specifically, what advantages does the chimeric DNA-RNA structure offer in terms of stability, cleavage efficiency, or overall performance compared to an all-RNA hairpin?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have thoroughly addressed all technical questions raised in the previous review. Methodological clarifications have been appropriately incorporated, and the revisions have notably improved the overall clarity and rigor of the manuscript.

Reviewer #2

(Remarks to the Author)

Reviewer #3

(Remarks to the Author)
The authors have thoroughly addressed the reviewer's comments.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
My comments are based on the authors' revision in response to reviewer 2's questions. Generally, all comments from Reviewer 2 have been well addressed. The fact that the Cas13a activity is minimal at 37 C is indeed intriguing, as many studies revealed the best temperature for Cas13a is 37 C. Therefore, authors should explain and discuss in the manuscript (e.g., at line 137 or in the discussion section) possible reasons that lead to such a discrepancy.

Response to Reviewer 1's Comments:

We appreciate to reviewer 1 for taking his/her valuable time to comment. Below, we have presented reviewer 1's comment in italics, and the corresponding responses are marked in blue font.

Reviewer #1 (Remarks to the Author):

This study presents a novel Cas13a-based assay for RNA detection, incorporating an innovative cascade signal amplification mechanism. The authors introduce an "anti-tag" sequence paired with a complementary DNA strand, effectively masking the target sequence within a CRISPR anti-tag hairpin. Upon target recognition, Cas13a's collateral activity cleaves the hairpin, releasing additional target sequences, thereby amplifying the signal. This work addresses an important challenge related to the accessibility of HIV testing via Cas13a-based nucleic acid detection, which holds significant clinical implications. The assay's overall performance is well characterized, demonstrating higher detection sensitivity compared to conventional Cas13a/crRNA detection approaches. While the study is well-executed, addressing the following points will further strengthen the manuscript before publication:

Response: We thank the reviewer for highlighting that our manuscript addresses an important challenge with significant clinical implications.

Comments 1. *Consideration of practical implementation in low-resource settings: given the potential clinical application in resource-limited areas, the fluorescence-based readout may present practical constraints due to the need for a fluorescence reader. The authors could explore alternative readout strategies, such as paper-based detection or portable reader designs, to enhance the assay's accessibility.*

Response: Thank you for your insightful comment regarding the practical implementation of our assay in low-resource settings. We fully recognize the importance of developing diagnostic tools that are accessible and easy to use where advanced laboratory infrastructure is limited. While fluorescence detection remains one of the most sensitive and reliable methods, we agree that exploring more accessible readout strategies—such as paper-based detection and low-cost, portable reader technologies—will greatly enhance the assay's usability and support point-of-care applications without the need for specialized equipment. In this study, we focus on investigating the cascade signal amplification mechanism of the CARRD assay and validating its performance. We appreciate your suggestion and will commit to advancing these efforts. In the Discussion section of our revised manuscript (**lines 359-362, page 13**), we added literature and discussion on adapting these technologies to our assay for use in resource-limited settings.

Comments 2. *Clarification of potential leakage in DNA blocker mechanism: in line 211, the authors state that "even with shorter DNA blockers, the HIV target RNA within HP does not activate Cas13a/HIV crRNA." However, Supplementary Figure 2 suggests that 14D HP and 16D HP exhibit cleavage even in the absence of the target, indicating possible leakage with shorter blockers. A similar inconsistency appears in line 224 and Figure 3d (Step 2), where the claim that "no cleavage occurred in the absence of target M" is contradicted by a visible band. If this reflects potential blocker leakage, a discussion on minimizing leakage would be valuable for ensuring the robustness of the technology.*

Response: Thank you for your suggestion. In this study, we developed an approach that employs DNA blockers embedded within a hairpin structure to modulate Cas13a activation. While this strategy enhances sensitivity under specific conditions, our results indicate that shorter DNA blockers (14D HP and 16D HP) can lead to unintended activation, resulting in nonspecific cleavage even in the absence of target RNA. As suggested by the reviewer, we

have thoroughly discussed this in the Discussion section of the revised manuscript (**lines 363-371, page 13**).

Additionally, we have revised the manuscript to reflect the results and implications of these observations more accurately as follows:

In **lines 208-218**, **page 8**,

"However, when hairpins containing 14, 16, or 18 bp DNA blockers were used, a notable increase in fluorescence signal was detected in the presence of target M. Unlike the 20D HP, when the DNA blocker complementary to the target RNA was shorter, the HIV target RNA within HP could activate Cas13a/HIV crRNA in step 2 after the loop (anti-tag sequence) was cleaved by the activated Cas13a/M crRNA in step 1. In the absence of target M, a weak fluorescence signal was generated, which indicates the background signals resulting from the interactions between HIV target RNA within the HP and Cas13a/HIV crRNA; However, these results demonstrated that signals undetectable in the absence of the hairpin were amplified to observable levels when the hairpin is used (**Figure 3c** and **Supplementary Figure 2**). In other words, initially undetectable signals from low concentrations of target M were amplified to observable levels using HP."

In **lines 226-228**, **page 8**,

"Additionally, FQ reporter cleavage was predominantly observed in the step 1 reactant containing target M and 14D HP; however, the cleavage reaction diminished in the absence of target M (**Figure 3d, step 2, lanes 1** and **2**)".

Comments 3*. Strengthening data interpretation in Figures 6c and 6d: including a correlation between Cq values and fluorescence intensity would enhance the validity of the results. Some positive samples exhibit RFU values close to negative controls, which might complicate differentiation. Additional data representation or statistical analysis could bolster confidence in the assay's performance.*

Response: We appreciate the reviewer's suggestion regarding the correlation between Cq values and fluorescence intensity (RFU). As part of our analysis, we examined the relationship between Cq values obtained from RT-qPCR and RFU values from the CARRD assay. However, no strong correlation was observed, likely due to fundamental differences in their signal amplification mechanisms. Specifically, while Cq values reflect the cycle threshold of amplified DNA in a logarithmic manner, RFU values are influenced by Cas13a *trans*-cleavage activity and cascade signal amplification.

In addition, to ensure accurate classification, we established the fluorescence threshold using a background-subtracted fluorescence method. The threshold was defined as the mean fluorescence of negative samples plus three standard deviations (mean + 3σ), corresponding to the blue line in **Figure 6d**, following methodologies reported in previous literature[1, 2, 3]. This statistical thresholding method effectively distinguishes true positive samples from background noise, minimizing the risk of misclassification. Additionally, the ROC analysis in **Figure 6f** (AUC = 0.9709) further supports the assay's ability to accurately differentiate between positive and negative samples. Given these statistical methods, we believe our current approach provides sufficient confidence in the assay's performance.

To further support the performance of the CARRD assay, we calculated key diagnostic metrics based on the confusion matrix (**Figure 6b**). The assay achieved a sensitivity of 77.8%, specificity of 100%, and an overall accuracy of 93.3%. The positive predictive value (PPV) and negative predictive value (NPV) were 100% and 91.3%, respectively. These results demonstrate high precision and specificity of the assay, with robust differentiation between true positives and true negatives.

We have revised the manuscript as follows in **lines 314-319, page 11**:

"Based on the confusion matrix in **Figure 6b**, the assay demonstrated a sensitivity of 77.8%, a specificity of 100%, and an overall accuracy of 93.3%. The positive predictive value (PPV) and negative predictive value (NPV) were calculated to be 100% and 91.3%, respectively. These results confirm the assay's high precision and specificity, demonstrating its robust ability to differentiate true positives from true negatives in clinical plasma samples."

Comments 4. *Cost comparison for field applications: since the assay is designed for point-of-care and resource-limited environments, providing a cost comparison between CRISPR-based detection and gold-standard qPCR would contextualize its practical feasibility. A brief discussion on the assay's potential cost advantages could strengthen its impact for real-world deployment.*

Response: As suggested by the reviewer, we have included a cost comparison table between the CARRD assay and qPCR in the manuscript (**Supplementary Table 2**). Accordingly, we have revised the manuscript and added some discussion (**lines 346-347, page 12**).

Supplementary Table 2. Cost comparison between CARRD assay and qPCR

CARRD assay:

Reagent	Price per kit	Volume per kit	Cost per reaction
LwCas13a (active)	$170	100 µL	$0.044
crRNA	$169	100 µL	$0.003
Fluorescence-quencher probe	$704	750 µL	$0.141
CRISPR Anti-tag Hairpin Mediator	$286	150 µL	$0.0038
Total			$0.192

qPCR:

Reagent	Price per kit	Volume per kit	Cost per reaction
Reliance One-Step Supermix	$549.00	1 mL (200 rxns)	$2.75
Forward Primer (10 µM)	$25.00	250 µL	$0.05
Reverse Primer (10 µM)	$25.00	250 µL	$0.05
Probe (TaqMan, 10 µM)	$588.00	610 µL	$0.48
Total			$3.33

Response to Reviewer 2's Comments:

We appreciate reviewer 2 for taking his/her valuable time to comment. Below, we have presented reviewer 2's comment in italics, and the corresponding responses are marked in blue font.

Reviewer #2 (Remarks to the Author):

This manuscript, CRISPR Anti-tag-Mediated Room-Temperature RNA Detection Using CRISPR/Cas13a, employs the trans-cleavage activity of LwaCas13a in conjunction with a tag:anti-tag hairpin RNA mechanism to enhance the sensitivity of LwaCas13a for nucleic acid detection. The design principle is well-conceived and demonstrates significant potential for amplification-free detection. However, the quality of the data presented could not reach publication requirements.

Response: We appreciate the reviewer for highlighting that our manuscript demonstrated significant potential for amplification-free detection.

Major comments:

Comments *1. In the first paragraph of "Development and optimization of the CARRD assay," the authors claim that Cas13a is more effective at 25°C than at 37°C. However, Figures 2b and 2c suggest that LwaCas13a reaches end-point fluorescence at 37°C when the experiment starts. The lack of fold change in fluorescence could be the result of the completion of the reaction at 37°C before the measurement starts. One should assume that the no-target control and target samples start at the same or similar fluorescence level. Additionally, all four conditions reach a similar end-point fluorescence level, which further supports the idea that the reaction is already complete at 37°C. The authors may consider lowering the target ssRNA concentration to prevent the experiment from reaching completion before measurements begin. Furthermore, given this observation, it may be beneficial to review all fluorescence experiments throughout the paper to ensure they are appropriately designed and executed.*

Response: We thank the reviewer for the insightful comment. As suggested by the reviewer, we performed additional experiments using a lower concentration of target ssRNA. As shown in **Figure R1**, at lower target levels, the initial fluorescence signal was comparable to the no-target control, suggesting negligible background activity before measurement. As the reaction progressed, a clear increase in fluorescence was observed in the presence of target ssRNA, with the magnitude of the signal dependent on the reaction temperature. Notably, the strongest fluorescence signal occurred at 25 °C, supporting our initial conclusion that LwaCas13a exhibits optimal reaction efficiency at this temperature.

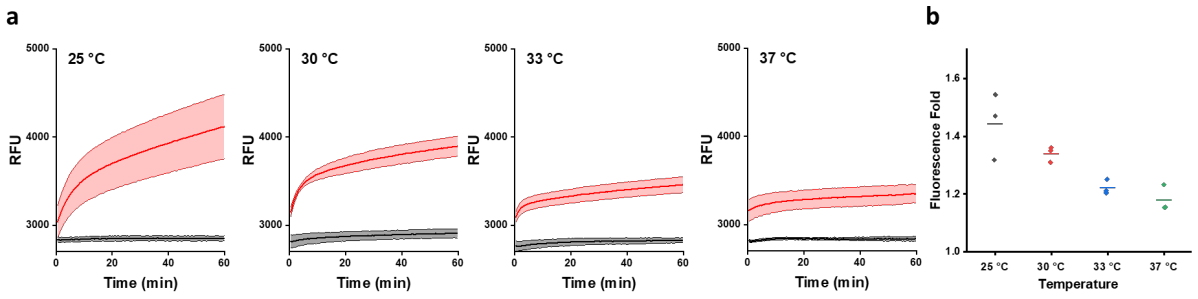


Figure R1. (a) *The trans-cleavage reaction of LwaCas13a under different temperature conditions (25°C, 30°C, 33°C, and 37°C) in real-time and* ***(b)*** *fluorescence fold (F/F_{NTC}). [Target ssRNA] = 10 pM. (n = 3 and data represent mean ± s.d. of three technical replicates).*

Comments *2. The core functionality of this design appears to rely on the RNA-hairpin-DNA mechanism, where the RNA serves as the target sequence, and the DNA binds to the RNA to*

inhibit recognition by Cas13a. Cas13a trans-cleaves the hairpin to release the RNA from DNA. Furthermore, the authors demonstrate substantial trans-cleavage activity in the presence of an anti-tag. This suggests that a broader selection of hairpin sequences may be feasible beyond what is currently proposed in the paper. While testing additional hairpin sequences may be outside the immediate scope of this study, the paper should acknowledge the potential for alternative sequences to expand the applicability of this approach.

Response: We appreciate the reviewer's comment regarding the potential applicability of alternative hairpin sequences. In this manuscript, we have demonstrated that the anti-tag hairpin structure enables effective signal amplification across diverse RNA targets, as evidenced by successful detection of both HIV and HCV (**Figure 4**). This indicates that our approach is not limited to a specific target sequence but is adaptable to different RNA sequences while maintaining robust *trans*-cleavage activity. We acknowledged that further exploration of diverse hairpin designs could expand the utility of this system and we have added some discussions in the revised manuscript (**lines 355-356, 363-371, page 13**).

Comments *3. When the anti-tag is cleaved in trans, a mixture of products is expected depending on the cleavage position. Given that LwaCas13a preferentially cleaves at uracil (U) sites, it is likely to generate incomplete anti-tag fragments rather than the anticipated 8-nt anti-tag. By mutating each nucleotide individually, the authors may identify the most efficient cleavage position or context. Additionally, the position of cleavage might be inferred from the gel image presented in Figure 3d. This observation aligns with point 2, suggesting that the anti-tag sequence itself may not be the crucial factor. Instead, the general design principle appears to be effective, and further variations in the anti-tag sequences could have been explored to optimize performance.*

Response: Thank you for your comments. As the reviewer mentioned, LwaCas13a prefers to cleave at the uracil (U) position. There are four consecutive uracils in the 8nt anti-tag, and depending on which position is cleaved by Cas13a, various mixtures can be generated. However, since it is known that the anti-tag shows the most effective inhibition when it is 8nt, the inhibition effect disappears once it is cleaved.[4] In other words, it is important to note that once any cleavage occurs, regardless of the specific uracil position, the inhibitory effect of the anti-tag on Cas13a-mediated recognition of target RNA within the hairpin is diminished. This indicates that the presence of any cleavage significantly reduces the suppressive effect of the anti-tag.

Furthermore, as demonstrated in **Figure 2**, statistical analysis has revealed a significant difference in Cas13a recognition of target RNA sequences when the loop is formed by the anti-tag sequence, compared to when it consists solely of uracil (U) residues. This finding indicates that the anti-tag sequence itself plays a crucial role in regulating Cas13a activity, underscoring its importance beyond merely serving as a cleavage site. While further exploration of anti-tag variations may enhance performance, the primary focus of this study is to demonstrate the effectiveness of the current anti-tag sequence in detecting target RNA. We have discussed the implications of these findings and potential future directions in the Discussion section of the revised manuscript (**lines 363-371, page 13**).

Comments *4. The data shown in Figures 2a and 2d are confusing:*
a. *5'-DNA tag HP has a very high fluorescence level. It is understandable that the anti-tag is at the 5' end so it should not inhibit the Cas13 cleavage after the anti-tag is cleaved. However, to have Cas13 bind to the RNA region, the RNA-DNA duplex has to either dissociate or have its anti-tag cleaved. If dissociation between the DNA-RNA happens, 5, 6, and 7 should have similar RFU. If the anti-tag has to be cleaved, but without initial ssRNA as a target, it suggests a very high background level of trans-cleavage to initiate this process. This is also discussed in point 6. Can the authors explain the reasons behind this observation and possible ways to decipher this?*

b. *If we assume the anti-tag is being cleaved in trans without the initial ssRNA target, then it is reasonable to say the anti-tag and rU8 are also cleaved in trans. The authors show that anti-tag targets do not necessarily inhibit trans-cleavage. Then why do 5'-RNA tag HP and 5'-RNA rU8 HP not achieve an RFU that is much higher than the background in Figure 2d?*

Response to both points a and b: We apologize for any confusion. To clarify, in the experiment shown in **Figures 2a** and **2d**, no target ssRNA was added separately. Consequently, Cas13a activation and subsequent *trans*-cleavage do not occur initially, making the assumption that the anti-tag is cleaved first inaccurate. Instead, the experiment focuses on how Cas13a recognizes the target RNA based on the anti-tag's position and the target's structural context.

In the case of 5'-DNA-tag HP **(condition 5** in **Figures 2a** and **2d)**, the anti-tag is positioned at the **5' end** of target RNA. Therefore, when Cas13a recognizes the target RNA sequence within the hairpin, the presence of 'the anti-tag' and 'the loop structure' does not interfere with this recognition, leading to a relatively high fluorescence signal. This is comparable to the RNA:DNA hybrid (**condition 3** in **Figures 2a** and **2d**) and suggests that recognition of RNA within the hybrid is somewhat feasible.

In contrast, in **conditions 6** in **Figures 2a** and **2d**, the anti-tag sequence is positioned at the **3' end** of the target RNA within the hairpin. As a result, when Cas13a attempts to recognize the target sequence, the presence of the anti-tag directly interferes with this recognition, leading to a lower fluorescence signal. In addition, compared to **condition 4**, **condition 6** exhibits a lower fluorescence signal, indicating that the loop structure also provides steric hindrance, further complicating Cas13a's recognition of the target RNA within the hairpin. This suggests that both the anti-tag's location and the structure play a critical role in modulating Cas13a's ability to access the target RNA.

When comparing **conditions 6** and **7** in **Figures 2a** and **2d**, **condition 6** contains the anti-tag sequence within the loop, whereas condition 7 contains only uracil (U) residues in the loop. The fact that **condition 6** exhibits significantly lower fluorescence than **condition 7** indicates that the anti-tag sequence itself interferes with Cas13a's ability to recognize the target RNA within the hairpin structure. This finding suggests that in the absence of target ssRNA, it serves as an effective blocking mechanism, reducing background signal more efficiently than a hairpin loop composed solely of uracil residues. Therefore, the results demonstrate that the anti-tag sequence actively suppresses unintended Cas13a activation, further supporting its role in regulating trans-cleavage activity. In the revised manuscript, we have further emphasized these explanations and provided additional clarifications (**lines 146-151, 158-163, page 6**).

Comments *5. Why do the authors choose 5'-RNA tag-HP with complementary DNA instead of complementary RNA? Cas13a should not cleave double-stranded RNA in trans either.*

Response: Thank you for your question. We selected a 5'-RNA tag-HP with complementary DNA instead of complementary RNA primarily for stability and cost reasons. According to a recent study[5], comparing the activation of Cas13a by various duplex structures, dsRNA can unexpectedly activate Cas13a effectively, whereas RNA/DNA hybrids show limited activation. Therefore, in this study, we determined that RNA/DNA hybrids are more suitable than dsRNA for reducing background noise. Additionally, the synthesis of DNA oligonucleotides is significantly less expensive than that of RNA oligonucleotides, making the overall assay more cost-effective and practical for widespread application.

Comments *6. We also have major concerns regarding the high levels of background activities in the HP groups:*

a. *Figure 3d, Supplementary Figure 2, 4, and 6 show a very high background when HP is added. In fact, in Figures 4 and 6, the background level is already higher or similar to the LOD*

of the conventional assay. This is quite concerning when comparing the two assays because the baseline in the CARRD is very high. Has there been any attempt to reduce this background activity?

Response: Thank you for your comments. We have explored various strategies to mitigate this issue, including adjusting the length of the DNA blocker and concentration of the hairpin as detailed in the manuscript. Additionally, although not discussed in the manuscript, we experimented with changes to the buffer composition, such as the addition of dithiothreitol (DTT), and the incorporation of chemical functionalities like phosphorothioate into the hairpin design. However, these efforts did not result in significant improvements in reducing background activity. We will continue to explore different strategies to further reduce the background.

b. *Related to minor point 2, maybe it is more reasonable to compare the fold change between different concentrations instead of absolute values. For example, the end-point fluorescence at 1 fM in CARRD compared to CARRD control is 10x whereas the end-point fluorescence at 1 fM in conventional assay to its control is 2x (These numbers are arbitrary. I did not do actual calculations). This helps readers gauge the level of amplifications since the plots mostly use CARRD scales for both assays and it is impossible to see the actual values in the conventional assay.*

c. *Related to point 6b, in the supplementary figures, the authors can also show the conventional assay in the correct scale to better see the conventional assay activities in the lower concentrations.*

Response to both points b and c: We appreciate the reviewer's suggestion regarding the use of fold change instead of absolute values for comparison. However, we intentionally present absolute fluorescence values to emphasize that our signal amplification strategy allows for the detection of target molecules at lower concentrations.

Absolute fluorescence values provide a direct measure of the detection range and sensitivity, which are critical for evaluating the practical applicability of the assay. In contrast, fold change represents only a relative variation between conditions and does not directly reflect the actual detection sensitivity or limit of detection (LOD) of the assay.

Moreover, fold change comparisons can sometimes be misleading, especially when fluorescence signals are already low in conventional assays. For instance, a 2x fold change in a low-intensity signal may not be meaningful in terms of detection capability, whereas absolute values allow for a clearer interpretation of how much lower the detectable concentration is in our system.

For these reasons, we believe that presenting absolute fluorescence values is the most appropriate approach to demonstrate the advantages of our detection system in terms of sensitivity and signal amplification.

d. *Taking supplementary figure 6 as an example, the control in DP is already generating a signal similar to 1 pM of target RNA in the conventional assay. This suggests that HP is equivalent to having 1 pM of target RNA in the assay. We can also see that 10 aM in the conventional assay generates little to no signal, suggesting very little trans-cleavage activities. When 10 aM of initial target RNA is present in CARRD, we can think of it as control + 10 aM, or 1 pM + 10 aM. Then how does it generate a much higher signal than the background, especially when there does not seem to be a lag phage in the control where 10 aM might have been able to jump-start the anti-tag cleavage? The same question also applies to supplementary figure 4.*

Response: Thank you for your questions. Our CARRD assay may involve a step where small amounts of target RNA initiate a cascade signal amplification, significantly enhancing the

detectable signal even at very low initial concentrations. Unlike conventional assays, the CARRD's design allows for the continuous activation of Cas13, leading to an accelerated cleavage cycle.

Specifically, even if only a very small amount of Cas13 is activated by 10 aM of target RNA, the relatively high concentration of hairpin probes (HP) in the system provides abundant substrates. Once Cas13 cleaves a portion of these hairpins (anti-tag loop cleavage), additional Cas13 activation occurs, triggering a cascade reaction. This self-sustained amplification loop explains why CARRD can generate a much higher signal than the background, even when the initial target concentration is extremely low.

Minor points:

Comments *1. In Figure 3d, it is hard to tell if the 14D HP was cleaved or not. A better control could be an annealed product of the two separately synthesized fragments representing the two strands after cleavage or the authors can use denaturing gel so that the hairpin shows up as a single band and the cleaved 14D HP shows up as 2 or more bands.*

Response: Since the stem region remains hybrid due to its complementary base-pairing, it does not separate into distinct fragments even after loop cleavage. Therefore, the expectation of multiple distinct bands is not feasible. In addition, annealing two separately synthesized fragments is impractical due to the uncertainty in confirming precise cut sites. To address this, we conducted an additional experiment using a 5'-fluorescent-labeled hairpin, which allowed for a more distinct visualization of the cleavage events (**Figure R2**). The results confirm the cleavage of 14D HP, consistent with what is depicted in **Figure 3d** of the manuscript.

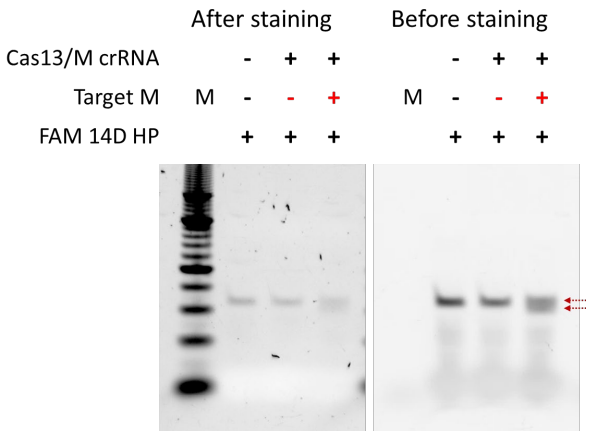


Figure R2. *Analysis of the trans-cleavage effect of Cas13a/M crRNA on FAM 14D HP in the presence and the absence of target M. Gel image after SYBR gold staining (left), before staining (right).*

Comments *2. We know that PCR amplifies signals exponentially by 2n and conventional Cas13a reaction is a first-order reaction. Is it possible to estimate the level of amplification achieved with this method to better understand the kinetics behind it?*

Response: Thank you for your question. Unlike well-established PCR amplification mechanism, the CARRD assay system involves both sequential and simultaneous reactions, initiated by the target and followed by subsequent interactions. This complexity makes it difficult to precisely determine the number of hairpins involved and to track the dynamic interaction between Cas13a and the target within each hairpin. As a result, it is challenging to estimate the level of amplification in CARRD assays.

Comments *3. Can the authors estimate the viral RNA concentrations in the clinical samples? The range of sample concentrations seems to be very narrow based on the Ct values.*

Response: Thank you for your comments. Owing to the recent advances in antiretroviral

therapy, the HIV viral load in acquired immunodeficiency syndrome (AIDS) patients can now be effectively suppressed to relatively low levels (e.g., ~10,000 copies/mL) or even to undetectable levels. For instance, according to the Centers for Disease Control and Prevention (CDC), 82.8% of individuals diagnosed with HIV in the United States were connected to medical care within one month of their diagnosis. It is challenging to obtain HIV clinical samples with high viral loads (e.g., ≥100,000 copies/mL).

Response to Reviewer 3's Comments:

We appreciate to reviewer 3 for taking his/her valuable time to comment. Below, we have presented reviewer 3's comment in italics, and the corresponding responses are marked in blue font.

Reviewer #3 (Remarks to the Author):

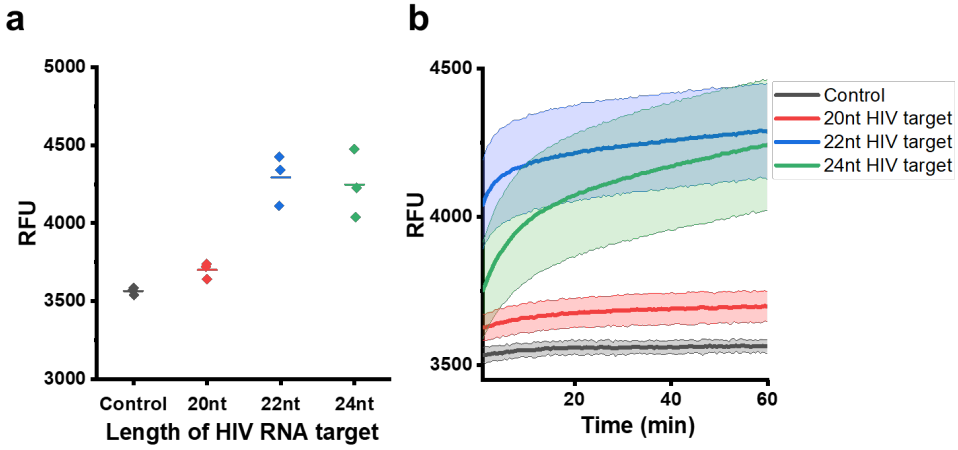
This manuscript presents a new nucleic acid detection method that incorporates an anti-tag hairpin, offering a promising approach to advancing current molecular diagnostics. However, there are several points that have not been fully addressed.

Response: We appreciate the reviewer for highlighting that our manuscript offered a promising approach to advancing current molecular diagnostics.

Comments

***Comments** 1. The authors observed that lwCas13a exhibited the highest trans-cleavage activity at 25°C. Did the authors investigate whether the length of the spacer:target pairing duplex influences the trans-cleavage activity? Additionally, did lwCas13a consistently show the highest trans-cleavage activity at 25°C, regardless of the length of the spacer:target pairing duplex?"*

Response: Thank you for your comments. We investigated whether the length of the spacer:target duplex affects LwaCas13a trans-cleavage activity by testing target RNAs of various lengths (20 nt, 22 nt, and 24 nt) at 25 °C. As shown in **Supplementary Figure 1,** the results indicate that a duplex length of at least 22 nt is required to initiate detectable Cas13a activity when using a crRNA with a 24-nt spacer. These findings suggest that the duplex length plays a critical role in the activation of the Cas13a system.



***Supplementary Figure 1.** Cas13a/HIV crRNA trans-cleavage reaction depending on the length of the HIV RNA target (20, 22, and 24 nt). **(a)** Final fluorescence intensity at 60 min and **(b)** real-time fluorescence signal. (n = 3 and data represent mean ± s.d. of three technical replicates).*

To further evaluate whether the highest *trans*-cleavage activity at 25 °C is consistent across different spacer:target duplex lengths, we additionally tested 20nt and 22nt target RNAs under the various temperature conditions (25 °C, 30 °C, and 37 °C). As shown in **Figures R3** and **R4**, the results demonstrate that, for both target lengths, LwaCas13a

consistently exhibited the strongest trans-cleavage activity at 25 °C, confirming that this temperature is optimal regardless of duplex length.

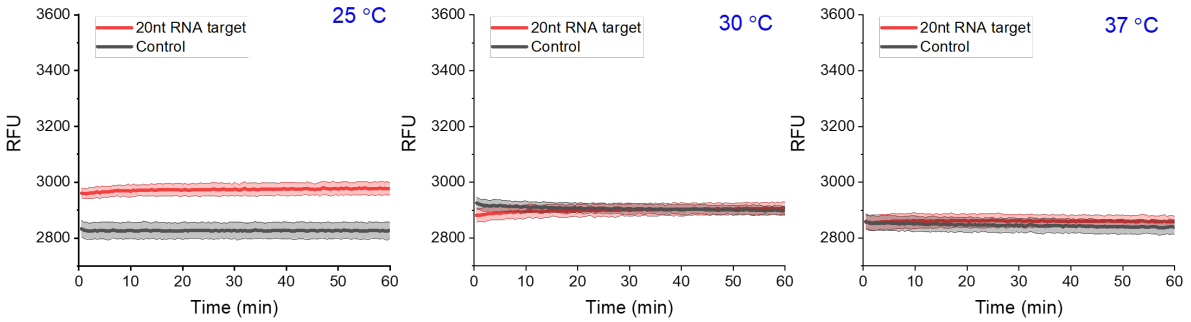


Figure R3. *Real-time trans-cleavage activity of LwaCas13a using a 20-nt RNA target at different temperatures (25°C, 30°C, and 37°C). [Target ssRNA] = 10 pM. (n = 3 and data represent mean ± s.d. of three technical replicates).*

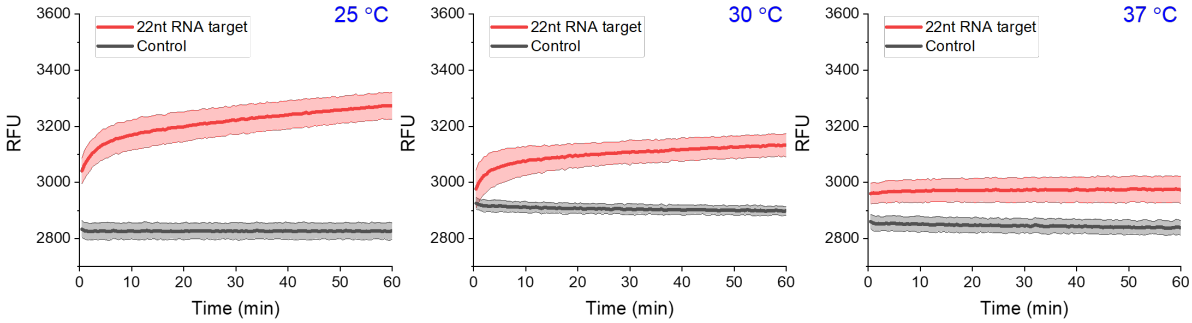


Figure R4. *Real-time trans-cleavage activity of LwaCas13a using a 22-nt RNA target at different temperatures (25°C, 30°C, and 37°C). [Target ssRNA] = 10 pM. (n = 3 and data represent mean ± s.d. of three technical replicates).*

Comments *2. The authors found that the CARRD assay showed higher sensitivity compared to the conventional Cas13a/crRNA reaction without the CRISPR anti-tag HP. In the conventional Cas13a assays, such as SHERLOCK, the spacer length is typically 28 nt. Did the CARRD assay also performed higher sensitivity when using a crRNA with a 28 nt spacer?*

Response: Thank you for your questions. At the beginning of our experiments, we evaluated the trans-cleavage activity of 24-nt and 28-nt crRNAs in conventional Cas13a assays across different target RNA lengths. We observed generally similar activities for both crRNA lengths, although the 24-nt crRNA showed slightly better efficiency with shorter target RNAs (**Figure R5**). Based on these observations, we expect no significant differences in performance between these crRNA lengths in the CARRD assay. It is reasonable to suggest that the 24-nt crRNA is more appropriate for the CARRD assay, as it uses a target RNA within the hairpin that is approximately 22-nt in length.

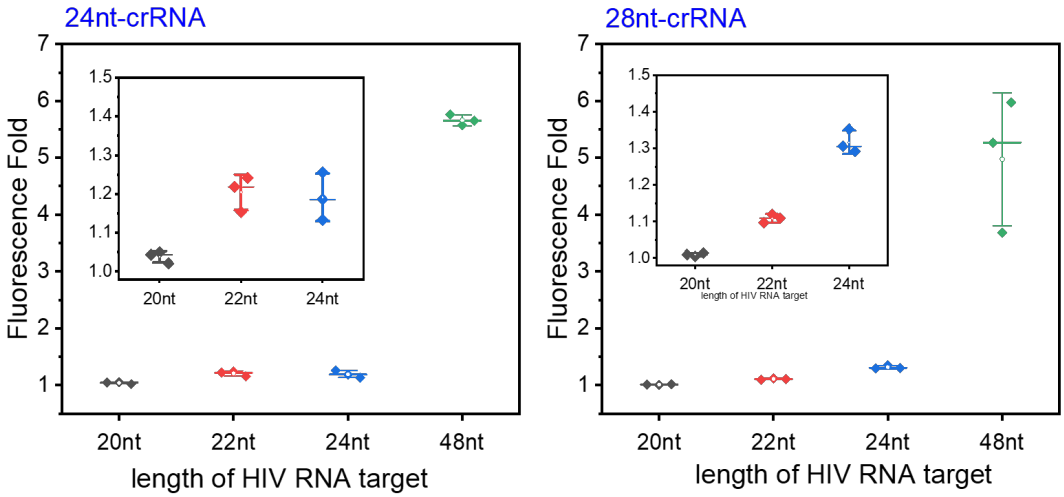


Figure R5. *Comparison of trans-cleavage activity of Cas13a/HIV crRNAs with different crRNA length (24-nt and 28-nt) on different lengths of target HIV RNA (20-, 22-, 24-, 48-nt).*

Comments *3. The authors observed that the anti-tag hairpin inhibits trans-cleavage activity. Upon cleavage of the anti-tag, the target region is released from the hairpin structure, leading to the activation of Cas13a. Based on this mechanism, the anti-tag may reduce Cas13a activity if it is not fully cleaved, as any remaining anti-tag at the 3' end of the target could partially inhibit Cas13a. Could optimizing the sequence of the loop region in the hairpin improve the trans-cleavage efficiency of the loop region and, as a result, enhance the overall detection sensitivity?*

Response: Thank you for your comments. Previous studies have shown that an 8nt anti-tag sequence offers the most effective inhibition of Cas13a activity.[4] When the anti-tag length is shorter than 8nt, its inhibitory effect significantly decreases. These findings indicate that the Cas13a activity is affected by the anti-tag length. Additionally, the anti-tag sequence has been fixed and is not subject to further modification in this study. Consequently, even if part of the anti-tag remains at the 3' end of the target RNA after *trans*-cleavage, the inhibitory effect is expected to weaken. This suggests that partial cleavage may still allow sufficient Cas13a activation, contributing to the overall detection efficiency.

Comments *4. Why did the authors design the anti-tag hairpin using a chimeric DNA-RNA structure rather than an all-RNA hairpin? Could you elaborate on the design principle behind this choice? Specifically, what advantages does the chimeric DNA-RNA structure offer in terms of stability, cleavage efficiency, or overall performance compared to an all-RNA hairpin?*

Response: Thank you for your question. We selected a 5'-RNA tag-HP with complementary DNA instead of complementary RNA primarily for stability and cost reasons. According to a recent study[5], comparing the activation of Cas13a by various duplex structures, dsRNA can unexpectedly activate Cas13a effectively, whereas RNA/DNA hybrids show limited activation. Therefore, in this study, we determined that RNA/DNA hybrids are more suitable than dsRNA for reducing background noise. Additionally, the synthesis of DNA oligonucleotides is significantly less expensive than that of RNA oligonucleotides, making the overall assay more cost-effective and practical for widespread applications.

References

1. Cai S*, et al.* Single-molecule amplification-free multiplexed detection of circulating microRNA cancer biomarkers from serum. *Nat Commun* **12**, 3515 (2021).

2. Gupta R*, et al.* Ultrasensitive lateral-flow assays via plasmonically active antibody-conjugated fluorescent nanoparticles. *Nat Biomed Eng* **7**, 1556-1570 (2023).

3. He Y*, et al.* Lab-in-a-Tip: a multiplex immunoassay platform based on a self-assembled barcoded protein array. *Nat Commun* **16**, 3990 (2025).

4. Wang B*, et al.* Structural basis for self-cleavage prevention by tag:anti-tag pairing complementarity in type VI Cas13 CRISPR systems. *Mol Cell* **81**, 1100-1115 e1105 (2021).

5. Deng F, Gulati S, Sang R, Li Y, Goldys EM. Modulating Cas13a trans-cleavage by double strand RNA: Application to the development of an autocatalytic sensor. *bioRxiv* 583472 (2024).

Response to Reviewer 1's Comments:

We appreciate reviewer 1 for taking his/her valuable time to comment. Below, we have presented reviewer 1's comment in italics, and the corresponding responses are marked in blue font.

Reviewer #1 (Remarks to the Author):

The authors have thoroughly addressed all technical questions raised in the previous review. Methodological clarifications have been appropriately incorporated, and the revisions have notably improved the overall clarity and rigor of the manuscript.

Response: Thank you for your positive comments!

Response to Reviewer 2's Comments:

We appreciate reviewer 2 for taking his/her valuable time to comment. Below, we have presented reviewer 2's comment in italics, and the corresponding responses are marked in blue font.

Reviewer #2 (Remarks to the Author):

I appreciate the authors' efforts in addressing our previous comments and providing additional data. However, despite the new experiments, I still have several major concerns. While the design principle aims to enhance Cas13a activity, many of the mechanistic interpretations remain open to debate. My primary concern lies with the fundamental aspects of the experimental design, particularly the adequacy of controls. In their current form, the controls may substantially influence or even overestimate the reported enhancement associated with the tag:anti-tag HP strategy.

Comments 1. *The additional data provided are appreciated. However, there appears to be a misunderstanding regarding the original comment. The primary concern is not that Cas13a performs better at 25°C than at 37°C, as CRISPR-Cas assays can exhibit variable activities depending on multiple factors, including crRNA spacer sequence, target RNA sequence, RNA reporter sequence, tag:anti-tag hairpin design, and others. The major concern relates to the observation that Cas13a activity at 37°C is markedly different from what has been consistently reported in the literature for in vitro RNA detection. While some variation with temperature is expected, the shift from 25°C to 37°C should not result in a complete loss of activity. In both Figure 2b and the newly submitted data, Cas13a activity at 37°C remains essentially flat, indicating minimal activity. By contrast, numerous published studies demonstrate that Cas13a typically displays robust activity at 37°C, producing characteristic hyperbolic kinetic curves and achieving significantly higher fold changes in signal amplification than the 1- to 3-fold increases shown here. The absence of this expected kinetic behavior raises concerns, as these basic activity measurements serve as the experimental foundation for all subsequent fluorescence assays in the study, prior to the incorporation of the tag:anti-tag HP design. While the recommended experiments have been conducted, the new results do not address the core issue. A more comprehensive experimental assessment may help resolve this discrepancy. For example, incorporating crRNA and target RNA sequences from published studies and demonstrating that Cas13a produces comparable activity at 37°C would help validate the assay system. Running these tests side-by-side with the conditions presented in Figure 2b, using both literature-based and study-specific components, would strengthen confidence in the assay workflow and help distinguish whether the observed differences arise from protein activity, crRNA design, target RNA sequence, or other assay components. As for the aligning the starting points of control and sample groups, the authors have shown they can achieve that in supplementary figure 2, 4 and 6, so it should also be achievable in figure 2b.*

Comments 2. *Upon closer examination of the raw data for Supplementary Figures 4b and 6b, the results show very stable, low background noise and high levels of signal amplification. These data, replotted here based on the authors' raw data, appear more consistent with previously published results for conventional Cas13a assays. Although a clear hyperbolic curve is not observed—likely due to low levels of activated LwaCas13a—the overall signal levels and background stability are more representative of expected Cas13a kinetics. In comparison, Figure 2b would be expected to exhibit similar background noise levels and comparable amplification levels, provided that appropriate concentrations are selected to capture the hyperbolic response curve. Importantly, there is no apparent reason why the background signal should differ between Figure 2b and the replotted data, as the negative control components are identical across these experiments. While fluorescence signal values are reported in arbitrary units, experiments conducted on the same instrument, using identical settings, protocols, and reaction components, should yield comparable baseline fluorescence levels between datasets.*

Response to Comments 1 and 2: We thank the reviewer for their thoughtful and constructive comments regarding Cas13a's activity at 37°C and the interpretation of baseline fluorescence.

To address the concern regarding the low Cas13a activity observed at 37°C in **Figure 2b**, we would like to highlight a recent study by Feng et al. (*Angew. Chem. Int. Ed.*, **2024**, 63, e202404069) [1], which investigated Cas13a activity across different temperatures and has been cited and discussed in our manuscript. For ease of comparison, we have reproduced their **Figure S5B** and **D** as **Figure R1** below. As shown in **Figure R1**, their experimental results clearly demonstrate that Cas13a can exhibit enhanced activity at lower temperatures under specific conditions. Notably, *their data also show that Cas13a activity at 37°C remains largely flat, with only a modest 1- to 3-fold increase in signal amplification—findings that are consistent with our observations in **Figure 2b***. To improve clarity, we have highlighted the existing reference (Reference 24; **Lines 535–537, Page 19**) and the corresponding discussion in the main manuscript (**Lines 134-137, Page 5**).

As described in the manuscript, our assay platform was systematically optimized and validated at 25°C, a temperature at which Cas13a exhibited reliable and reproducible activity across all experimental settings. Consequently, the data obtained at 37°C, while of scientific interest, do not undermine our conclusions, which are derived from a rigorously controlled and internally consistent dataset under the optimized assay conditions.

[Figure Redacted]

Figure R1 (reproduced **Figure S5B** and **D** [1]**). The consistent effect of temperature on the trans-cleavage kinetics of LwaCas13a**. **(B)** and **(D)** Trans-cleavage kinetics of Cas13a at 6 different temperatures. *These experimental results demonstrate that Cas13a exhibits enhanced signal amplification at lower reaction temperatures compared to 37°C under specific assay conditions. This temperature-dependent variation in activity aligns with our findings in **Figure 2b**.*

Regarding the reviewer's comment on the differences in baseline fluorescence between **Figure 2b** and the other figures, we would like to clarify that **Figure 2b** was measured using a real-time PCR instrument, while the remaining figures were generated using a microplate reader, as described in the Method section. These differences in instrumentation can introduce variations in baseline signal levels,

even when identical assay components are used. Therefore, the observed discrepancies are technical in nature and do not impact the interpretation of the results, which are based on relative fluorescence changes rather than absolute signal intensities.

We hope that the explanation, together with the supporting literature [1], adequately addresses the reviewer's concerns and reinforces the validity of our system-specific optimization and results.

Comments 3. *It is understandable that many factors can contribute to background noise in Cas13a fluorescence assays. However, the primary concern is that the elevated background signal may result from leakiness of the 14D HP construct, as suggested by the data presented in Figure 2d. This issue could significantly affect the interpretation of amplification levels when comparing HP-based assays to conventional Cas13a assays, particularly if the two conditions exhibit substantially different baseline fluorescence levels. While Figure 2d indicates that the background noise for the 14D HP and the no-HP control are comparable, mitigating this concern in that instance, however, the same consistency is not observed in Figure 3d, Supplementary Figures 4, and 6, where notable differences in background levels between the conventional and HP conditions are apparent. This concern is further compounded by the absence of explicit background or control values in Figures 4a–4d, 5a–5c, and 6d. Without these data, it is difficult to fully assess whether the comparisons between conventional and HP conditions reflect true differences in amplification or are confounded by variations in baseline signal.*

Response to comment 3: We appreciate the reviewer's careful assessment regarding the potential influence of background signal caused by 14D HP. In the original manuscript, we acknowledged this issue and discussed strategies to mitigate its impact (**Lines 363-371, Page 13**).

In **Figure 2d**, we employed a relatively lower concentration of the 14D HP compared to other experiments, which resulted in fluorescence signals that were comparable to the control condition (absence of 14D HP). In contrast, the higher background signals observed in the other figures are attributed to the use of a higher concentration of the 14D HP, which was optimized to improve the analytical sensitivity of the assay.

In **Figures 4a-c** and **5a-c**, we presented the differences in fluorescence values ($\Delta F = F_{Target} - F_{Control}$) to effectively illustrate the enhancement in signal due to the target under both HP and no-HP conditions. This approach inherently accounts for the background signal contributed by the HP. The raw fluorescence data corresponding to **Figures 4a-c** are already available in Supplementary **Figures 4** and **6**, while the raw data for **Figure 5b-c** have been additionally included in the updated Resource Data file to facilitate the reviewer's evaluation.

For **Figures 5d** and **6d**, absolute fluorescence values were directly shown. In **Figure 5d**, the control group for the black bars represents "Cas13a + HIV crRNA + 14D HP", while the HIV and HCV groups refer to the addition of 1 pM HIV or 10 pM HCV RNA, respectively. Similarly, for the orange bars, the control is "Cas13a + HCV crRNA + 14D HP", and the HIV and HCV groups include either 10 pM HIV or 1 pM HCV RNA, respectively. These control values accurately represent the baseline fluorescence associated with the 14D HP. Likewise, in **Figure 6d**, both sample types (HIV-negative and -positive) were tested under identical assay conditions, including Cas13a, HIV crRNA, and the 14D HP. Therefore, the negative samples reflect the baseline fluorescence signal associated with the assay components, including the 14D HP, and serve as the appropriate background reference for signal interpretation.

We hope that our explanations, together with the provided data, appropriately address the reviewer's concern about background signal variability.

New comment 1. *The reaction components in control in Figure 4d are confusing. My understanding is that the crRNA, target RNA, and 14D HP are always present in all six conditions. For example, for the black bars, HIV refers to Cas13a + HIV crRNA + 10 pM HIV RNA + 1 pM HCV RNA + HIV 14D*

HP, HCV refers to Cas13a + HIV crRNA + 1 pM HIV RNA + 10 pM HCV RNA + HIV 14D HP (or HCV 14D HP?). For the control conditions, it is unclear whether the absence refers to the Cas13a protein or the HIV RNA target. If the protein is absent, the observed background signal appears unusually high, given that no reporter cleavage should occur in the absence of Cas13a activity. Alternatively, if the HIV RNA target is absent, the control group would be expected to exhibit a significantly lower signal compared to the HCV black bar sample, which contains 1 pM HIV RNA target. Clarification on the specific design of the control groups is necessary to properly interpret the reported background levels.

Response to New comment 1: We appreciate the reviewer's comment and the opportunity to clarify the control conditions used in **Figure 4d**.

We confirm that Cas13a, crRNA, and the 14D HP are consistently included in all conditions, including controls. The only variable component across these groups is the presence or absence of the target RNA (HIV or HCV). In the black bars, the control condition refers to "Cas13a + HIV crRNA + HIV 14D HP" without target RNA. The HIV and HCV groups contain either 1 pM HIV or 10 pM HCV RNA, respectively. Similarly, in the orange bars, the control is "Cas13a + HCV crRNA + HCV 14D HP" without target RNA, while the HIV and HCV conditions include 10 pM HIV RNA or 1 pM HCV RNA, respectively. Each control group, therefore, reflects the background fluorescence signal of the corresponding 14D HP construct in the absence of the target RNA. To improve clarity, we have highlighted this information in the caption of **Figure 4d** (**Lines 649-654, Page 28**). Additionally, we have re-labeled **Figure 4d** in the updated version of **Figure 4** in the revised manuscript.

Reference

1. Feng W, Peng H, Zhang H, Weinfeld M, Le XC. A Sensitive Technique Unravels the Kinetics of Activation and Trans-Cleavage of CRISPR-Cas Systems. Angew Chem Int Ed Engl **63**, e202404069 (2024).

Response to Reviewer 3's Comments:

We appreciate reviewer 3 for taking his/her valuable time to comment. Below, we have presented reviewer 3's comment in italics, and the corresponding responses are marked in blue font.

Reviewer #3 (Remarks to the Author):

The authors have thoroughly addressed the reviewer's comments.

Response: Thank you for your positive comments!

Response to Reviewer 4's Comments:

We appreciate reviewer 4 for taking his/her valuable time to comment. Below, we have presented reviewer 4's comment in italics, and the corresponding responses are marked in blue font.

Reviewer #4 (Remarks to the Author):

My comments are based on the authors' revision in response to reviewer 2's questions. Generally, all comments from Reviewer 2 have been well addressed. The fact that the Cas13a activity is minimal at 37 C is indeed intriguing, as many studies revealed the best temperature for Cas13a is 37 C. Therefore, authors should explain and discuss in the manuscript (e.g., at line 137 or in the discussion section) possible reasons that lead to such a discrepancy.

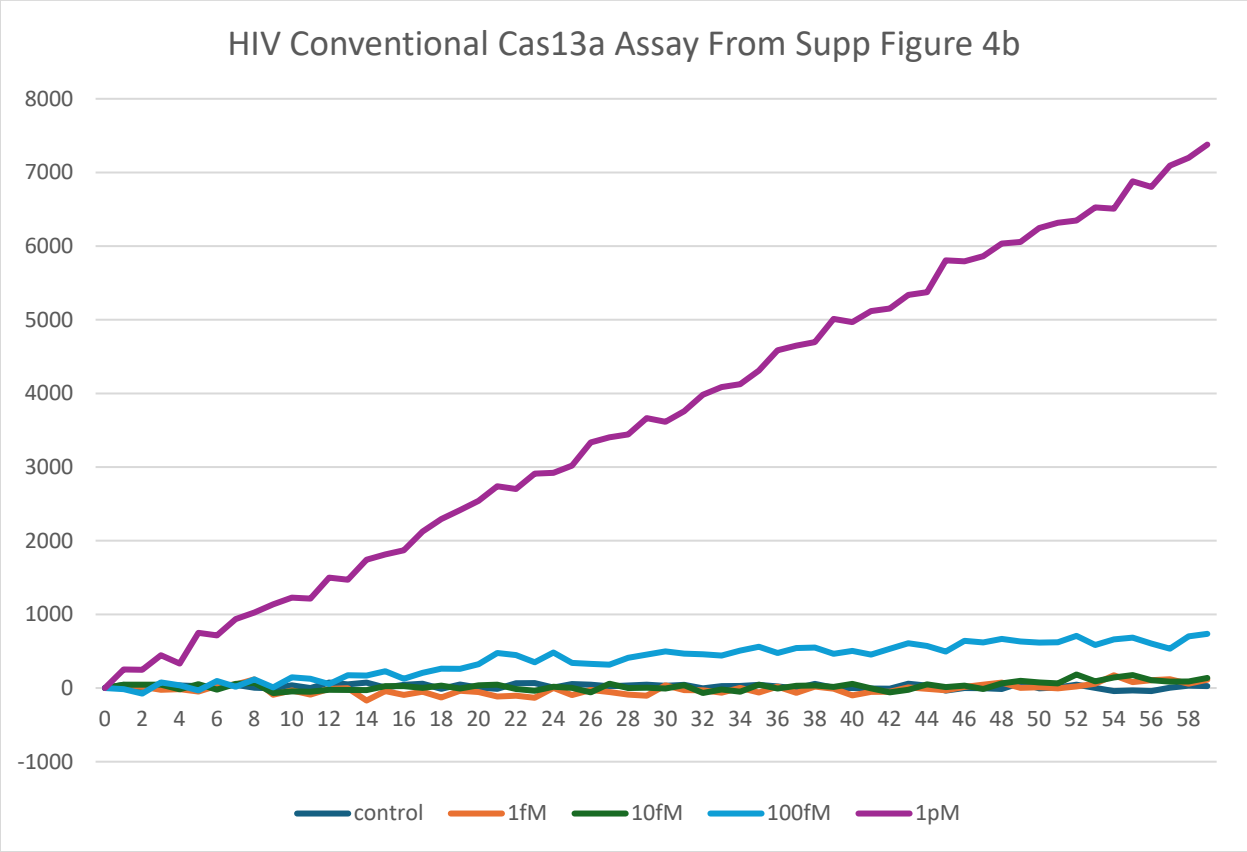
Response: Thank you for your positive comments and suggestions. As suggested, we have discussed the potential reasons for this discrepancy in the revised manuscript as follows:

"This discrepancy from earlier reports suggesting 37 °C as the optimal temperature may result from differences in RNA secondary structure, which influence the efficiency of ternary complex formation. Efficient formation of the Cas13a ternary complex at lower temperatures has been identified as a key factor enabling robust cleavage activity even at room temperature". (**Lines 134-138, Page 5**)

I appreciate the authors' efforts in addressing our previous comments and providing additional data. However, despite the new experiments, I still have several major concerns. While the design principle aims to enhance Cas13a activity, many of the mechanistic interpretations remain open to debate. My primary concern lies with the fundamental aspects of the experimental design, particularly the adequacy of controls. In their current form, the controls may substantially influence or even overestimate the reported enhancement associated with the tag:anti-tag HP strategy.

1) The additional data provided are appreciated. However, there appears to be a misunderstanding regarding the original comment. The primary concern is not that Cas13a performs better at 25°C than at 37°C, as CRISPR-Cas assays can exhibit variable activities depending on multiple factors, including crRNA spacer sequence, target RNA sequence, RNA reporter sequence, tag:anti-tag hairpin design, and others. The major concern relates to the observation that Cas13a activity at 37°C is markedly different from what has been consistently reported in the literature for in vitro RNA detection. While some variation with temperature is expected, the shift from 25°C to 37°C should not result in a complete loss of activity. In both Figure 2b and the newly submitted data, Cas13a activity at 37°C remains essentially flat, indicating minimal activity. By contrast, numerous published studies demonstrate that Cas13a typically displays robust activity at 37°C, producing characteristic hyperbolic kinetic curves and achieving significantly higher fold changes in signal amplification than the 1- to 3-fold increases shown here. The absence of this expected kinetic behavior raises concerns, as these basic activity measurements serve as the experimental foundation for all subsequent fluorescence assays in the study, prior to the incorporation of the tag:anti-tag HP design. While the recommended experiments have been conducted, the new results do not address the core issue. A more comprehensive experimental assessment may help resolve this discrepancy. For example, incorporating crRNA and target RNA sequences from published studies and demonstrating that Cas13a produces comparable activity at 37°C would help validate the assay system. Running these tests side-by-side with the conditions presented in Figure 2b, using both literature-based and study-specific components, would strengthen confidence in the assay workflow and help distinguish whether the observed differences arise from protein activity, crRNA design, target RNA sequence, or other assay components.As for the aligning the starting points of control and sample groups, the authors have shown they can achieve that in supplementary figure 2, 4 and 6, so it should also be achievable in figure 2b.
2) Upon closer examination of the raw data for Supplementary Figures 4b and 6b, the results show very stable, low background noise and high levels of signal amplification.

These data, replotted here based on the authors' raw data, appear more consistent with previously published results for conventional Cas13a assays. Although a clear hyperbolic curve is not observed—likely due to low levels of activated LwaCas13a—the overall signal levels and background stability are more representative of expected Cas13a kinetics. In comparison, Figure 2b would be expected to exhibit similar background noise levels and comparable amplification levels, provided that appropriate concentrations are selected to capture the hyperbolic response curve. Importantly, there is no apparent reason why the background signal should differ between Figure 2b and the replotted data, as the negative control components are identical across these experiments. While fluorescence signal values are reported in arbitrary units, experiments conducted on the same instrument, using identical settings, protocols, and reaction components, should yield comparable baseline fluorescence levels between datasets.



3) It is understandable that many factors can contribute to background noise in Cas13a fluorescence assays. However, the primary concern is that the elevated background signal may result from leakiness of the 14D HP construct, as suggested by the data presented in Figure 2d. This issue could significantly affect the interpretation of amplification levels when comparing HP-based assays to conventional Cas13a assays, particularly if the two conditions exhibit substantially different baseline fluorescence levels. While Figure 2d indicates that the background noise for the 14D HP and the no-HP control are comparable, mitigating this concern in that instance,

however, the same consistency is not observed in Figure 3d, Supplementary Figures 4, and 6, where notable differences in background levels between the conventional and HP conditions are apparent. This concern is further compounded by the absence of explicit background or control values in Figures 4a–4d, 5a–5c, and 6d. Without these data, it is difficult to fully assess whether the comparisons between conventional and HP conditions reflect true differences in amplification or are confounded by variations in baseline signal.

New comment:

1. The reaction components in control in Figure 4d are confusing. My understanding is that the crRNA, target RNA, and 14D HP are always present in all six conditions. For example, for the black bars, HIV refers to Cas13a + HIV crRNA + 10 pM HIV RNA + 1 pM HCV RNA + HIV 14D HP, HCV refers to Cas13a + HIV crRNA + 1 pM HIV RNA + 10 pM HCV RNA + HIV 14D HP (or HCV 14D HP?). For the control conditions, it is unclear whether the absence refers to the Cas13a protein or the HIV RNA target. If the protein is absent, the observed background signal appears unusually high, given that no reporter cleavage should occur in the absence of Cas13a activity. Alternatively, if the HIV RNA target is absent, the control group would be expected to exhibit a significantly lower signal compared to the HCV black bar sample, which contains 1 pM HIV RNA target. Clarification on the specific design of the control groups is necessary to properly interpret the reported background levels.