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CRISPR-based rapid molecular diagnostic tests for fusion-driven leukemias

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Abstract:
Fusion oncogenes can be cancer-defining molecular alterations that are essential for diagnosis and therapy selection.1,2 Rapid and accessible molecular diagnostics for fusion-driven leukemias such as acute promyelocytic leukemia (APL), Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL), and chronic myeloid leukemia (CML) are unavailable, creating a barrier to timely diagnosis and effective targeted therapy in many healthcare settings, including community hospitals and low-resource environments. We developed CRISPR-based RNA-fusion transcript detection assays using SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) for the diagnosis of fusion-driven leukemias. We validated these assays using diagnostic APL and CML patient samples from academic centers and dried blood spots from low-resource environments, demonstrating 100% sensitivity and specificity. We identified assay optimizations to enable the use of these tests outside of tertiary cancer centers and clinical laboratories, enhancing the potential impact of this technology. Rapid point-of-care diagnostics can improve outcomes in cancer patients by expanding access to therapies for highly treatable diseases that would otherwise lead to serious adverse outcomes due to delayed or missed diagnoses.

Conflict of interest: COI declared - see note

COI notes: J.K., O.A., J.G., F.Z., R.S.V, and R.C.L are inventors on patent PCT/US2020/049257, which covers the application of SHERLOCK for cancer diagnostics. R.C.L reports consultancy for Takeda Pharmaceuticals, Bluebird Bio, Qiagen, Sarepta Therapeutics, Verve Therapeutics, Jazz Pharmaceuticals and Vertex Pharmaceuticals and research funding from Bluebird Bio. J.R. reports consultancy for Cepheid. All other authors declare no competing financial interests.

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Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: All data and methods required to replicate the study are included in the main and supplemental text, figures, tables as well as cited references. Please contact corresponding author (coleman_lindsley@dfci.harvard.edu) for details regarding any original data.

Clinical trial registration information (if any):

Figure 1

Figure 1

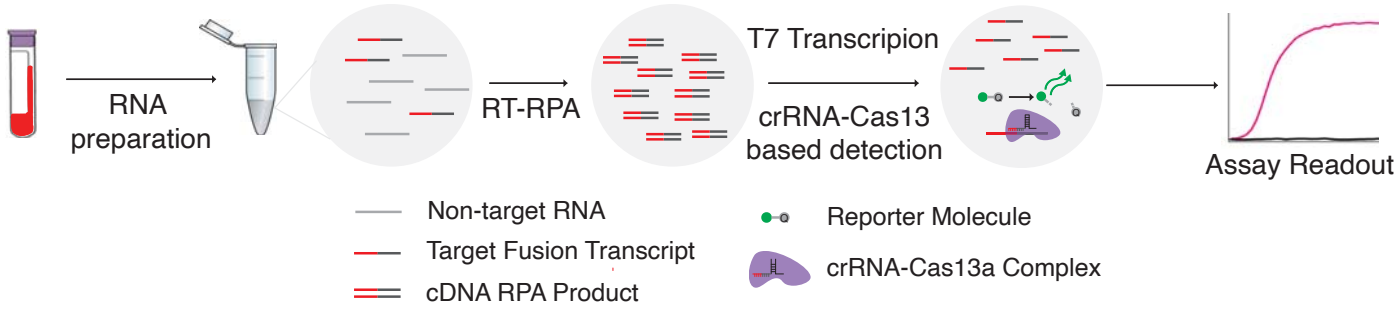


Figure 2

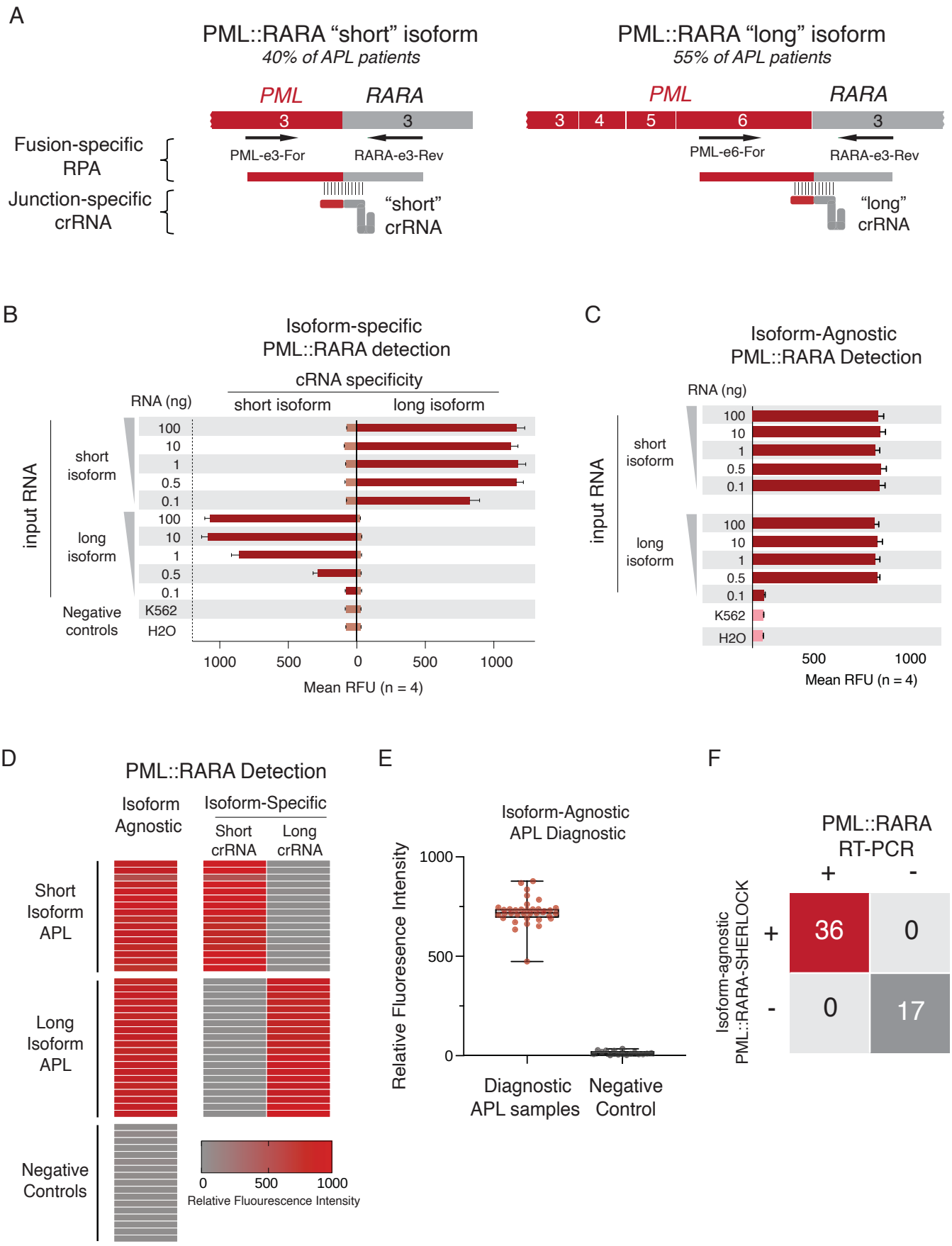
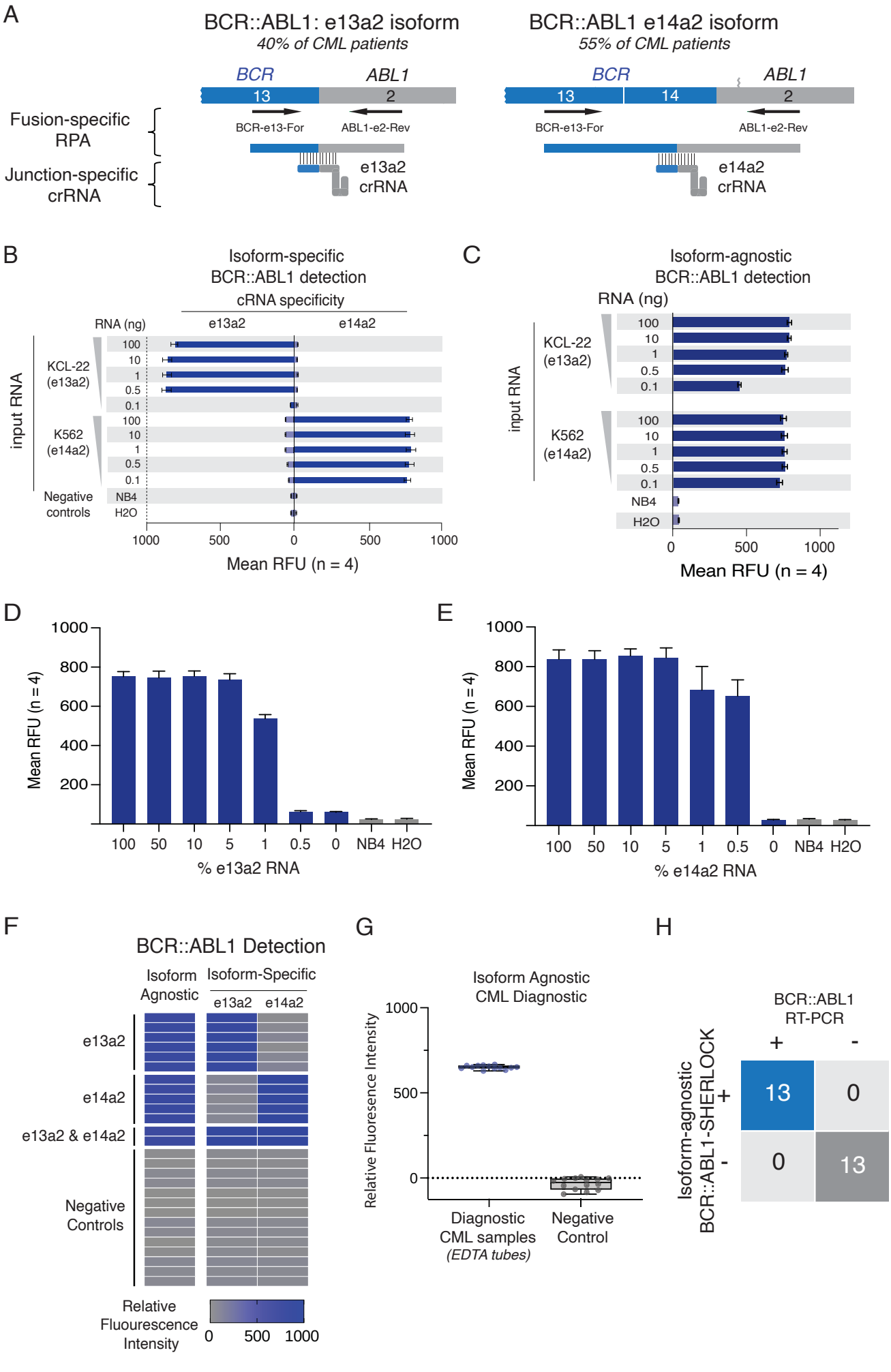
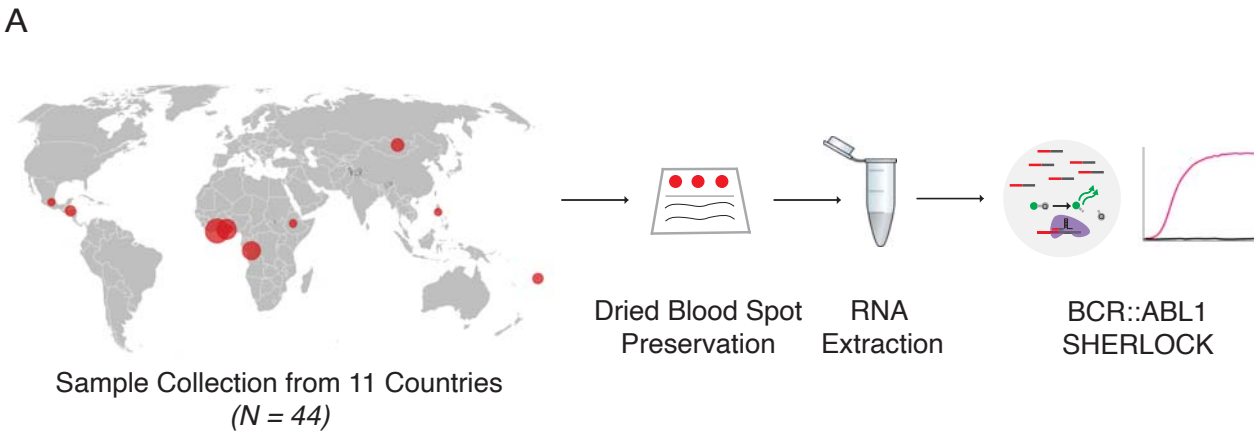
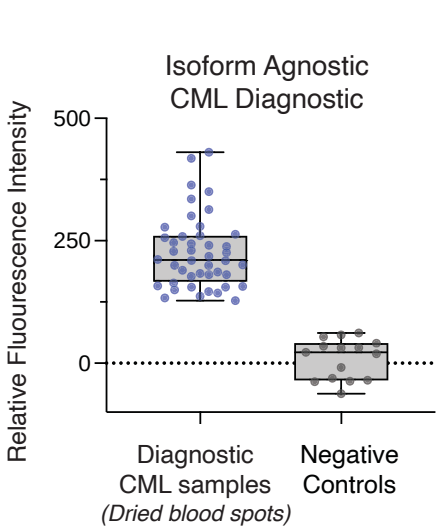
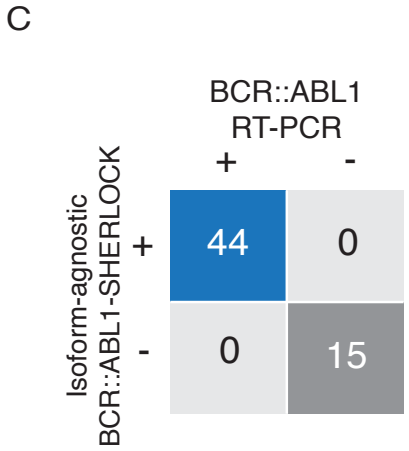

A
PML::RARA "short" isoform
40% of APL patients
PML::RARA "long" isoform
55% of APL patients
PML
RARA
Fusion-specific RPA
Junction-specific crRNA
PML-e3-For
RARA-e3-Rev
"short" crRNA
PML-e6-For
"long" crRNA
B
Isoform-specific PML::RARA detection
cRNA specificity
short isoform
long isoform
RNA (ng)
input RNA
Negative controls
K562
H2O
Mean RFU (n = 4)
C
Isoform-Agnostic PML::RARA Detection
D
PML::RARA Detection
Isoform Agnostic
Isoform-Specific
Short crRNA
Long crRNA
Short Isoform APL
Long Isoform APL
Negative Controls
Relative Fluouresence Intensity
E
Isoform-Agnostic APL Diagnostic
Relative Fluoresence Intensity
Diagnostic APL samples
Negative Control
F
PML::RARA RT-PCR
Isoform-agnostic PML::RARA-SHERLOCK
36
0
0
17

Figure 3


A
BCR::ABL1: e13a2 isoform
40% of CML patients
BCR::ABL1 e14a2 isoform
55% of CML patients
BCR
ABL1
13
2
14
Fusion-specific RPA
Junction-specific crRNA
BCR-e13-For
ABL1-e2-Rev
e13a2 crRNA
e14a2 crRNA
B
Isoform-specific BCR::ABL1 detection
cRNA specificity
RNA (ng)
e13a2
e14a2
input RNA
KCL-22 (e13a2)
K562 (e14a2)
Negative controls
NB4
H2O
Mean RFU (n = 4)
C
Isoform-agnostic BCR::ABL1 detection
D
% e13a2 RNA
E
% e14a2 RNA
F
BCR::ABL1 Detection
Isoform Agnostic
Isoform-Specific
Negative Controls
Relative Fluorescence Intensity
G
Isoform Agnostic CML Diagnostic
Relative Fluorescence Intensity
Diagnostic CML samples (EDTA tubes)
Negative Control
H
BCR::ABL1 RT-PCR
Isoform-agnostic BCR::ABL1-SHERLOCK


A
Sample Collection from 11 Countries
(N = 44)
Dried Blood Spot
Preservation
RNA
Extraction
BCR::ABL1
SHERLOCK


B
Isoform Agnostic
CML Diagnostic
Relative Fluorescence Intensity
500
250
0
Diagnostic
CML samples
(Dried blood spots)
Negative
Controls


C
BCR::ABL1
RT-PCR
+
-
Isoform-agnostic
BCR::ABL1-SHERLOCK
+
-
44
0
0
15

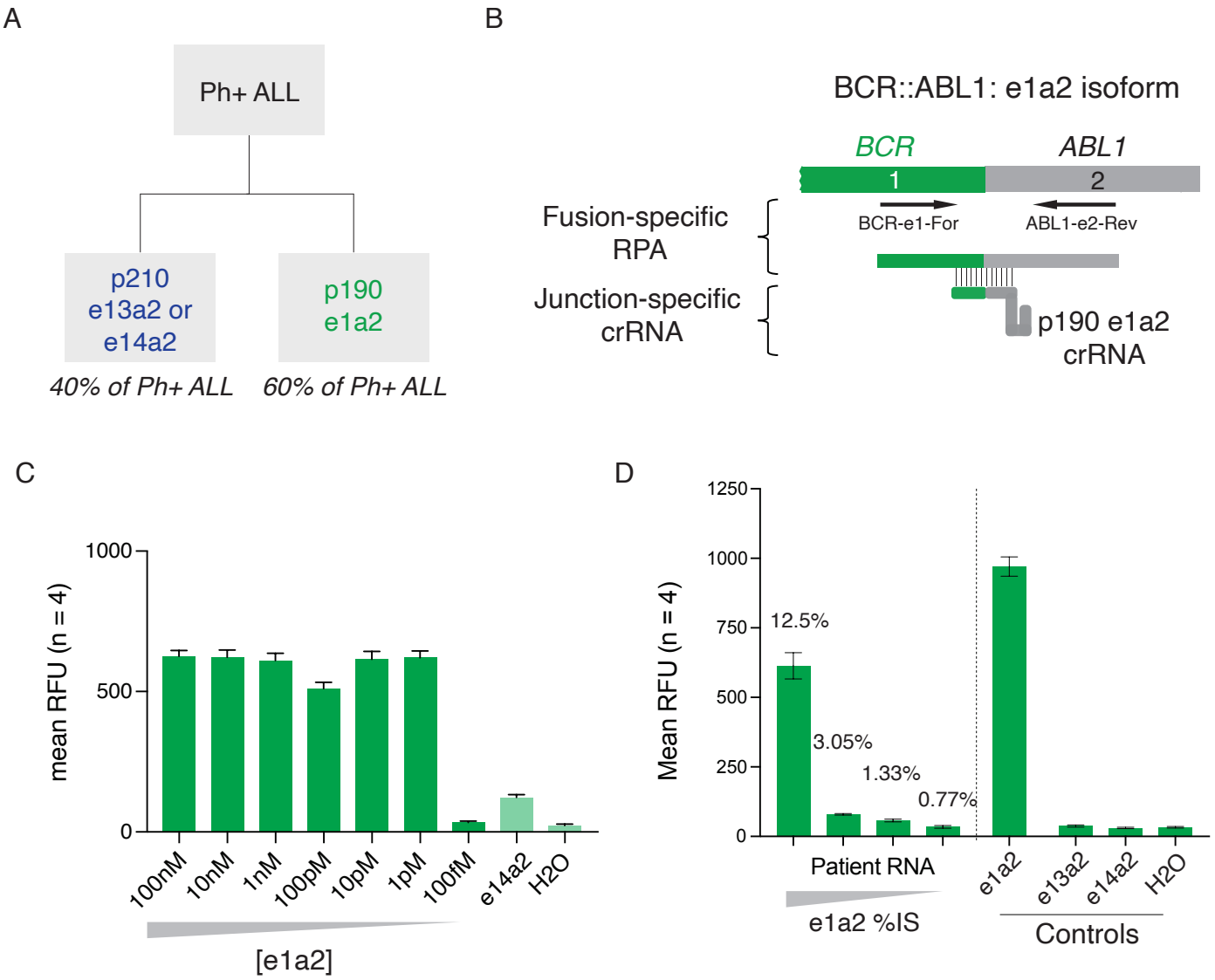
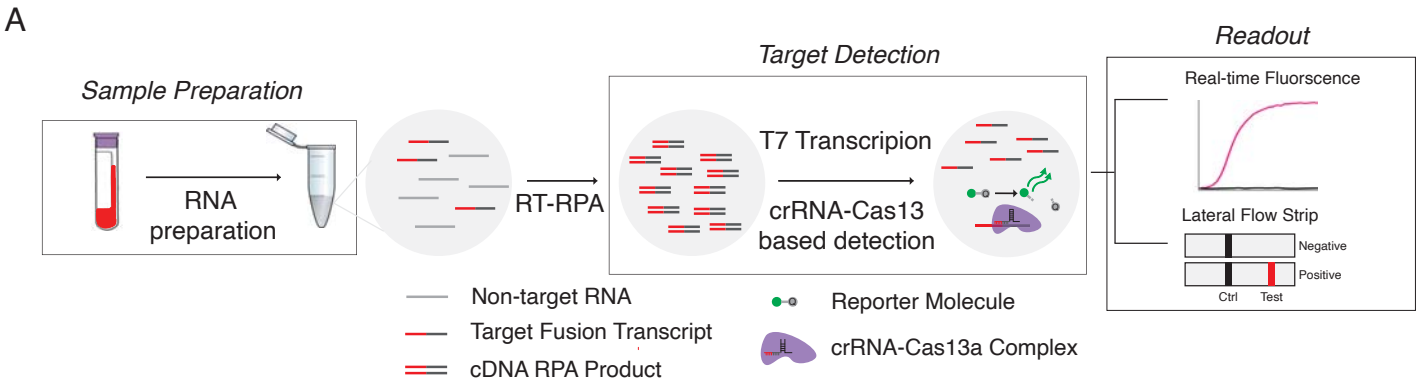
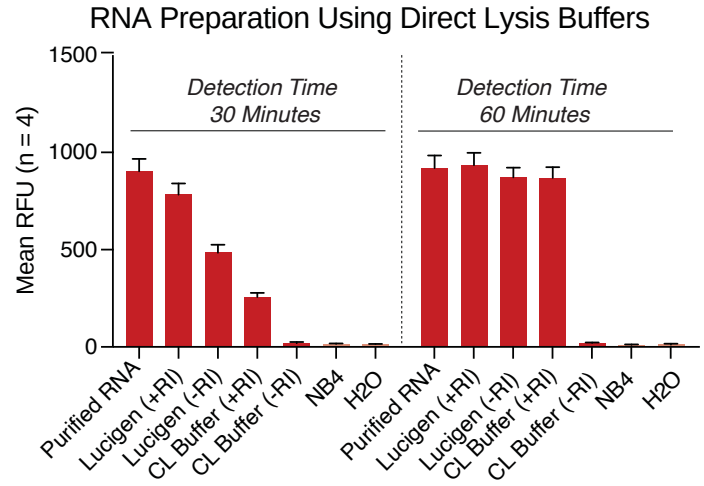
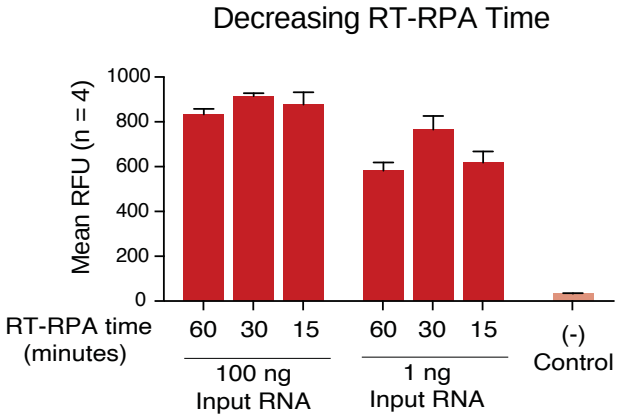
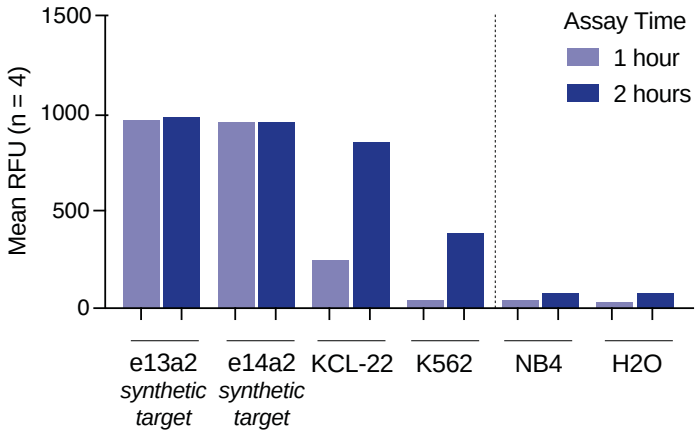

A
Ph+ ALL
p210
e13a2 or
e14a2
p190
e1a2
40% of Ph+ ALL
60% of Ph+ ALL
B
BCR::ABL1: e1a2 isoform
BCR
ABL1
1
2
Fusion-specific
RPA
BCR-e1-For
ABL1-e2-Rev
Junction-specific
crRNA
p190 e1a2
crRNA
C
mean RFU (n = 4)
1000
500
0
100nM
10nM
1nM
100pM
10pM
1pM
100fM
e14a2
H2O
[e1a2]
D
Mean RFU (n = 4)
1250
1000
750
500
250
0
12.5%
3.05%
1.33%
0.77%
Patient RNA
e1a2 %IS
e1a2
e13a2
e14a2
H2O
Controls

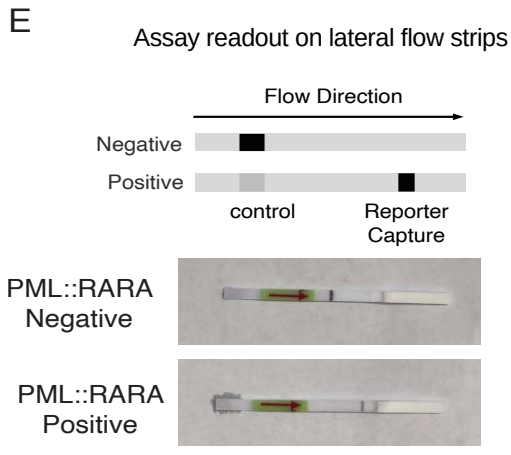
Figure 6


A
Sample Preparation
RNA preparation
RT-RPA
Target Detection
T7 Transcripion
crRNA-Cas13 based detection
Readout
Real-time Fluorscence
Lateral Flow Strip
Negative
Positive
Ctrl
Test
Non-target RNA
Target Fusion Transcript
cDNA RPA Product
Reporter Molecule
crRNA-Cas13a Complex


B
RNA Preparation Using Direct Lysis Buffers
Mean RFU (n = 4)
1500
1000
500
0
Detection Time 30 Minutes
Detection Time 60 Minutes
Purified RNA
Lucigen (+RI)
Lucigen (-RI)
CL Buffer (+RI)
CL Buffer (-RI)
NB4
H2O


C
Decreasing RT-RPA Time
Mean RFU (n = 4)
1000
800
600
400
200
0
RT-RPA time (minutes)
60 30 15
60 30 15
(-) Control
100 ng Input RNA
1 ng Input RNA


D
Combined RT-RPA/Cas13 Detection
"One-Pot" Assay
Mean RFU (n = 4)
1500
1000
500
0
Assay Time
1 hour
2 hours
e13a2 synthetic target
e14a2 synthetic target
KCL-22
K562
NB4
H2O


E
Assay readout on lateral flow strips
Flow Direction
Negative
Positive
control
Reporter Capture
PML::RARA Negative
PML::RARA Positive

SHERLOCK: CRISPR-Based Rapid Molecular Diagnostics for Fusion-Driven Leukemias

SHERLOCK WORKFLOW

Blood/RNA | Fusion-Specific Amplification | Junction-Specific Detection | Fluorescence or Lateral Flow



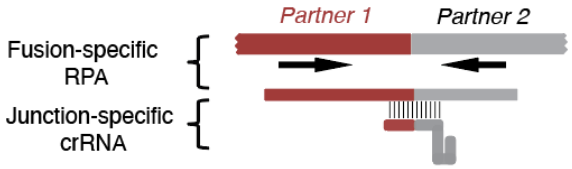
APPLICATIONS

Prevent Early Mortality
Acute promyelocytic leukemia
PML::RARA

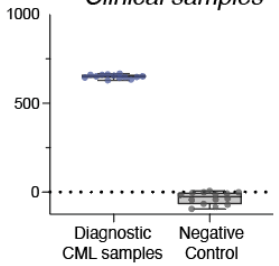
Enable Access to Treatment
CML and Ph+ B-ALL
BCR::ABL1

Design and validation

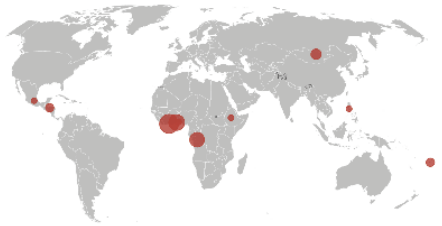
Transcript Specific Programmability | 100% Sensitive and Specific | Global Validation



Clinical samples



Dried blood spots



Conclusions: SHERLOCK is 100% sensitive and specific for the diagnosis of acute promyelocytic leukemia (APL) and chronic myeloid leukemia (CML) in patient samples. SHERLOCK has the potential to improve access to life-saving therapies for clinically actionable fusion-driven leukemias.

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blood Visual Abstract

CRISPR-based rapid molecular diagnostic tests for fusion-driven leukemias

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Data Sharing Statement:

All data and methods required to replicate the study are included in the main and supplemental text, figures, tables as well as cited references. Please contact the corresponding author (coleman_lindsley@dfci.harvard.edu) for details regarding any original data.

Running Title: *CRISPR Leukemia Diagnostics*
Word count (abstract): 169
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Figures: 6
Tables: 0
References: 29
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Key Points:

- SHERLOCK is 100% sensitive and specific for the diagnosis of APL and CML in patient samples.
- SHERLOCK has the potential to improve access to life-saving therapies for clinically actionable fusion-driven leukemias.

Abstract:

Fusion oncogenes can be cancer-defining molecular alterations that are essential for diagnosis and therapy selection.[1,2] Rapid and accessible molecular diagnostics for fusion-driven leukemias such as acute promyelocytic leukemia (APL), Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL), and chronic myeloid leukemia (CML) are unavailable, creating a barrier to timely diagnosis and effective targeted therapy in many healthcare settings, including community hospitals and low-resource environments. We developed CRISPR-based RNA-fusion transcript detection assays using SHERLOCK (*S*pecific *H*igh-sensitivity *E*nzymatic *R*eporter un*LOCK*ing) for the diagnosis of fusion-driven leukemias. We validated these assays using diagnostic APL and CML patient samples from academic centers and dried blood spots from low-resource environments, demonstrating 100% sensitivity and specificity. We identified assay optimizations to enable the use of these tests outside of tertiary cancer centers and clinical laboratories, enhancing the potential impact of this technology. Rapid point-of-care diagnostics can improve outcomes in cancer patients by expanding access to therapies for highly treatable diseases that would otherwise lead to serious adverse outcomes due to delayed or missed diagnoses.

Introduction:

PML::RARA and *BCR::ABL1* are essential, disease-defining oncogenic fusions in acute promyelocytic leukemia (APL) and in chronic myeloid leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL), respectively. Currently, laboratory-based tests such as metaphase cytogenetics, fluorescence *in situ* hybridization (FISH), or reverse transcription-polymerase chain reaction (RT-PCR) are required to establish these diagnoses and thereby inform treatment decisions. However, these tests are not available in most clinical laboratories and healthcare settings, requiring samples to be sent to central testing laboratories or transfer of patients to tertiary care centers. As a result, reliance on these methodologies can introduce clinically significant delays to diagnosis and initiation of treatment.

More than 90% of patients with APL can be cured without chemotherapy using a combination of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO).[3] Despite this high curative potential, the initial clinical presentation of APL remains an oncologic emergency because of the well-defined risk of catastrophic hemorrhage. Treatment with ATRA/ATO rapidly reverses the APL-associated coagulopathy, but treatment delays measured in hours or days continue to drive a high risk of early mortality, as highlighted in real-world data that shows the incidence of early death reaching at least 30%.[4–8] In order to fully realize the transformative potential of current APL therapy demonstrated in clinical trial cohorts, where the rates of early death are low and long-term cure are high, the clear imperative is to shrink the gaps between presentation, diagnosis, and initiation of guideline-concordant treatment.

Similarly, tyrosine kinase inhibitors (TKIs) targeting the BCR::ABL1 fusion oncogene have revolutionized the treatment of Ph+ ALL and CML. Prior to the TKI era, the prognosis of patients with Ph+ ALL was dismal, with poor response to intensive chemotherapy, and a limited number of patients eligible for curative potential allogeneic stem cell transplant. TKIs are now the

backbone of all Ph+ ALL treatment regimens and have dramatically improved outcomes in both younger and older patients.[9] For patients with CML, TKIs have normalized life expectancy and imatinib is widely available across the world.[10–12] However, eligibility for TKI therapy is dependent on access to molecular diagnostic testing, which represents a barrier to effective treatment for patients with CML in low-resource environments.

Cancer outcome disparities widen when effective diagnostics and therapeutics are available but limited to tertiary care and academic centers.[13] Rapid, point-of-care molecular diagnostics for fusion-driven hematologic malignancies have the potential to address this discrepancy and transform patient care based on two paradigms: one highlighted by application in APL and Ph+ ALL in which rapid turnaround at the point-of-care enables early initiation of targeted therapy in acute leukemia and another highlighted by application in CML in which widespread access to point-of-care molecular diagnostics can link patients to life-expectancy normalizing drugs.

To address these challenges, we developed a CRISPR-based RNA-fusion transcript detection platform using SHERLOCK (*S*pecific *H*igh-sensitivity *E*nzymatic *R*eporter un*LOCK*ing) for APL, CML, and Ph+ ALL. SHERLOCK combines isothermal reverse-transcription and recombinase polymerase amplification (RT-RPA) of target RNA with programmable Cas13-mediated detection by cleavage of a collateral reporter for assay readout (Figure 1).[14–16] We utilized a systematic approach to validating the assay using synthesized RNA (pure target), cell lines (target in a mixture of total RNA), and patient samples, including from dried blood spots obtained in low-resource clinical settings from 11 countries across 4 continents (Africa, Asia, Oceania, North America). We further demonstrated the potential to streamline these assays for the point-of-care in low-resource environments through multiple assay optimizations.

Methods

Nucleic acid reagents and SHERLOCK assay

We synthesized RNA targets and CRISPR guide-RNAs (crRNA) by *in vitro* transcription from Ultramer ssDNA templates (Integrated DNA Technologies [IDT]) using the HiScribe T7 Quick High Yield RNA Synthesis kit (NEB) and purified using RNAXP clean beads (Beckman Coulter). Primers for RPA reactions were ordered from IDT. The SHERLOCK assay was performed as previously described.[16] Briefly, RT-RPA was performed by combining 1uL of synthetic target RNA, cell line (K562, NB4, and KCL-22) RNA, or patient sample-derived RNA (purified or direct lysis preparation) combined with fusion-specific RPA primers, AMV reverse transcriptase, and TwistAmp RPA reaction mix (TwistDx) at 37C from 15 to 60 minutes. RT-RPA product (1uL) was then incubated in Cas13 detection mix (20mM HEPES, 9mM MgCl2) with T7 RNA polymerase, rRNTPs, junction-specific crRNAs (IDT), RNase inhibitor, LwaCas13a protein, and RNaseAlert v2 reporter (IDT) at 37C. Readouts of Cas13 detection were performed in real time using a SpectraMax plate reader for up to 3 hours. For combined RT-RPA and Cas13 detection experiments ("one-pot" assay), purified RNA and all of the above reagents were combined and then incubated in the plate reader at 37C with real-time measurement over 3 hours.[16] For end-point colorimetric readout, Cas13 detection mix (using an alternative 3' biotinylated reporter molecule: 6-FAM/rUrUrUrUrU-Biotin) was incubated with 1 uL of RT-RPA product for 60 minutes, and then a lateral flow strip (Milenia Biotec-Hybridetect) was dipped in this reaction mixture. Sequences of all synthetic targets, RPA primers, and crRNAs are listed in Supplemental Table 1.

Patient samples

Diagnostic blood or bone marrow samples from patients with confirmed or suspected APL, CML, and Ph+ ALL were obtained from Johns Hopkins Medical Institute, Dana-Farber Cancer Institute, and Brigham & Women's Hospital. RNA was purified using column-based extraction (Qiagen) or direct lysis of cells was performed using a Tris/Igepal based buffer[17] or RNA

QuickExtract Buffer (Lucigen). CML patient samples collected by the Fred Hutchinson Cancer Center RNA were preserved as dried blood spots on paper cards (Whatman 903 Protein Saver) and RNA was extracted using Trizol reagent and column-based extraction (Qiagen).

The study was conducted with approval of the DFCI, BWH, JHMI, and FHCC Institutional Review Boards.

Results

Design and Validation of APL PML::RARA SHERLOCK Assay

We developed an APL PML::RARA SHERLOCK assay to detect the two common transcript isoforms of PML::RARA in 95% of patients with APL- PML exon 3: RARA exon 3 and PML exon 6: RARA exon 3, referred to as *short* and *long*, respectively. We incorporated two design features to maximize detection sensitivity and specificity: (1) Fusion-specific RT-RPA: for each target, RT-RPA primer pairs bind junction-flanking exons from both fusion partners, thereby supporting selective amplification of the fusion transcript without off-target amplification, (2) Junction-specific detection: for each isoform of each target, we designed crRNAs that span the fusion junction sequence, thereby activating reporter cleavage by the crRNA-Cas13a complex only in samples that contain the unique, tumor-specific complementary fusion RNA sequence (Figure 2A). Using synthesized PML::RARA short and long transcript RNA, we determined the limit of detection (LOD) to be 10fM with isoform-specific RT-RPA primers and crRNAs (Supplemental Figure 1A-B). To validate the detection of target in a complex RNA mixture, we next determined the isoform-specific LOD using the NB4 cell line, which expresses the PML::RARA *long* transcript, and using APL patient-derived RNA expressing the *short* isoform, since there is no cell line expressing the PML::RARA *short* transcript. PML::RARA isoform-specific SHERLOCK demonstrated a LOD of 100-500pg of input RNA with 100% specificity (Figure 2B). Since isoform-specificity is not required for APL diagnosis or therapeutic decision-

making, we developed an isoform-agnostic assay by combining the PML e3 and e6 forward primers and RARA e3 reverse primer into a single RT-RPA reaction and combining the *short* and *long* isoform crRNAs into a single Cas13a detection reaction. This single, isoform-agnostic PML::RARA assay maintained similar LOD without non-specific detection of off-target RNA (Figure 1C).

To validate APL PML::RARA SHERLOCK, we defined the test characteristics in diagnostic clinical samples from 36 patients with PML::RARA positive APL (*short* n = 16 and *long* n = 20) and 17 samples confirmed by clinical RT-PCR to be *PML::RARA* negative. We observed 100% concordance between APL PML::RARA SHERLOCK and clinical PML::RARA RT-PCR results, including positive results with both the isoform agnostic and isoform specific assays in all 36 diagnostic APL samples, and no false positives in PML::RARA negative samples (Figure 2D-E).

Design and Validation of CML BCR::ABL1 SHERLOCK Assay

We developed a CML BCR::ABL1 SHERLOCK assay to detect the two common transcript isoforms of BCR::ABL1 p210 found in ~95% of patients with CML (referred to as *e13a2* and *e14a2*) (Figure 3A). Similar to the PML::RARA assay, we found the synthetic target LOD to be 10fM for both e13a2 and e14a2 (Supplemental Figure 1C-D). We then used KCL-22 (e13a2) and K562 (e14a2) cell line RNA to validate both an isoform-specific and isoform-agnostic BCR::ABL1 assay. For all RT-RPA reactions, the BCR e13 forward primer and ABL1 e2 reverse primer were used (Figure 3A). Both assays demonstrated a LOD in the 100-500pg range (Figure 3B-C). To simulate decreasing target transcript content in a fixed amount of cellular RNA, we performed a dilution series of KCL-22 RNA with K562 RNA (and vice versa) with a fixed 10ng of total input RNA. e13a2 RNA was detectable to 1% of total RNA (100pg) and e14a2 RNA was detectable to 0.5% of total RNA (50pg) (Figure 3D-E). We next validated the sensitivity and specificity of CML BCR::ABL1 SHERLOCK in 13 diagnostic CML samples

(e13a2 n = 6, e14a2 n = 5, both transcripts n =2) and 13 negative control samples confirmed by clinical RT-PCR. All BCR::ABL1 positive samples had transcript IS% > 10%. The CML BCR::ABL1 SHERLOCK assay was 100% sensitive and specific (Figure 3F-H).

Finally, to test the performance of SHERLOCK on samples acquired from non-academic healthcare settings, we obtained 44 CML dried blood spot samples from regions with limited diagnostic resources in 11 countries where local BCR::ABL1 molecular testing was not available (Figure 4A). Samples were collected as dried blood spots on paper cards and preserved from 139-579 days (median 260 days) prior to RNA extraction and testing by isoform agnostic CML BCR::ABL1 SHERLOCK and concurrent gold-standard BCR::ABL1 RT-PCR. Even using such samples with non-standardized collection, shipment, and storage protocols, we found that the CML BCR::ABL1 SHERLOCK assay was 100% sensitive and specific, demonstrating the capability of SHERLOCK to diagnose CML from samples acquired in globally diverse non-academic contexts (Figure 4B-C).

Ph+ ALL BCR::ABL1 p190 SHERLOCK

Approximately 20-30% of cases of B-cell ALL harbor the *BCR::ABL1* fusion oncogene (Ph+ ALL). In addition to the BCR::ABL1 p210 transcript which defines CML, approximately 60% of patients with Ph+ ALL harbor the p190 transcript (e1a2). Therefore, detection of both BCR::ABL1 p210 (e13-a2, e14a2) and p190 (e1a2) is essential to diagnose Ph+ ALL (Figure 5A). Using a similar strategy to that of APL PML::RARA and CML BCR::ABL1 SHERLOCK, we designed fusion specific RT-RPA primers and junction-specific crRNAs for the BCR::ABL1 e1a2 transcript (Figure 5B). Using synthetically generated e1a2 target RNA, we determined the LOD of this assay to be 1pM of target RNA (Figure 5C). We confirmed the ability of this assay to detect the p190 transcript in serial samples obtained on 4 consecutive days from a patient treated with TKI. The p190 transcript was easily detected compared to background and control

in the pre-treatment sample which corresponded to a peripheral blood transcript IS% of 12.5%, but was indistinguishable from background in subsequent post-treatment samples, corresponding to ~ < 3% IS (Figure 5D). This demonstrates the capability of SHERLOCK to detect the BCR::ABL1 p190 transcript at the time of diagnosis of Ph+ ALL in peripheral blood.

Fusion Transcript SHERLOCK Assay Optimizations

We next sought to decrease the turnaround time and simplify the execution of the assay by making improvements to different phases of the SHERLOCK workflow: (1) sample preparation, (2) target detection, and (3) readout (Figure 6A). Validation of fusion transcript SHERLOCK assays was performed using silica column-based RNA isolation kits, which require a microcentrifuge and multiple handling steps. To circumvent this process, we tested a Tris/Igepal-based lysis buffer and a commercial lysis buffer (Lucigen RNA QuickExtract) with and without RNase Inhibitor (RI) for rapid RNA preparation. Direct lysis of NB4 cells harboring the PML::RARA *long* isoform using the Lucigen buffer +/- RI and the Tris/Igepal buffer + RI yielded a positive signal compared to the background by 30 minutes of Cas13 detection (Figure 6B). At 60 minutes of detection, these lysis conditions resulted in a maximum signal that was comparable to column-purified RNA.

We took two separate approaches to streamline target detection for the PML::RARA and BCR::ABL1 SHERLOCK assays. First, we determined that RT-RPA time could be decreased from 60 minutes to 15 minutes without compromising assay performance (Figure 6C). Second, to eliminate the need for two distinct sample handling steps, we consolidated the RT-RPA and Cas13 detection steps into an isoform-agnostic “one-pot” reaction. BCR::ABL1 e13a2 and e14a2 synthetic targets were efficiently detected by 1 hour of total assay time, while cell line-derived e13a2 (KCL-22) and e14a2 (K562) were reliably detected above background by 2 hours of assay time (Figure 6D).

Finally, to demonstrate the capability of fusion transcript SHERLOCK assays to provide results in field settings, we performed the isoform agnostic APL PML::RARA assay with patient-derived RNA from an APL and control sample using an alternative reporter RNA suitable for lateral flow detection, with a 5' 6-FAM and 3' biotin, in the Cas13-detection reaction step.[14] After 60 minutes of incubation in the Cas13 reaction, we dipped a lateral flow strip with a control band coated with streptavidin and a reporter band coated with an antibody specific to 6-FAM into the reaction mix. In the absence of PML::RARA transcript, the intact biotinylated reporter is trapped at the control band, while in the presence of PML::RARA transcript, crRNA-Cas13 cleaved 6-FAM-linked reporter fragments migrate to the reporter band, indicating a positive test for APL (Figure 6E and Supplemental Figure 2).

Discussion:

Here we demonstrate 100% accurate CRISPR-based assays using SHERLOCK to diagnose the fusion-driven leukemias APL, CML, and Ph+ ALL. The combination of isothermal fusion-specific RT-RPA and junction-specific crRNA-guided Cas13 detection achieves high target sensitivity and specificity while eliminating the need for resource and time-intensive processes such as thermocycling PCR and next-generation sequencing required by traditional leukemia molecular diagnostics. We validated the performance of our assays using diagnostic patient samples from blood and bone marrow showing 100% concordance with clinical gold standard RT-PCR assays. We further demonstrated the performance of the assay in diagnosing CML from RNA derived from dried blood spots obtained in diagnostic resource-limited settings around the world. Based on the application and available resources at the point of use, we show that the SHERLOCK workflow can be streamlined by simplifying sample preparation through direct cell lysis from fresh samples and consolidating the RT-RPA and Cas13 detection steps into a "one-pot" reaction. We demonstrate two readout methods: one using fluorescence in a

plate format amenable to higher throughput and another using lateral flow strips applicable to lower resource environments. SHERLOCK has the potential to transform molecular diagnostics for oncology, highlighted here for immediately clinically actionable, disease-defining targets in leukemia.

The current model for leukemia molecular diagnostics largely relies on centralized testing, where testing involves either the transfer of a patient to a tertiary cancer center or shipment of a blood sample to a high-volume reference laboratory. Turnaround time for the centralized testing model reflects an aggregate of sample collection, shipment, processing, and hands-on testing time. For patients with APL, a long turnaround time can impose a barrier to diagnosis which has been linked to delayed initiation of guideline-concordant therapy and a higher risk of catastrophic hemorrhage and death.[18] In clinical trials, the incidence of early death is low, ranging from 0 to 8%,[3,19] but in registry-level studies that reflect real-world outcomes, early death has been reported to occur in up to 30% of patients with newly diagnosed APL.[6,7] The rate of very early death occurring in patients with APL prior to diagnosis and initiation of treatment is more difficult to quantify and is likely even higher.[20] Thus, despite the low overall incidence of APL, the profound consequences of under- or delayed diagnosis and the transformative impact of timely disease-specific treatment together mandate a testing model built on expanded access and rapid turnaround with a high true-negative testing rate. Therefore, the development of highly accurate, low-cost, rapid testing for APL that can be deployed in community-based settings that identify most patients with suspicious cytopenias and coagulopathies could eliminate missed and delayed diagnoses and improve outcomes. The capacity to easily diagnose APL in this manner may also prompt emergency department pharmacies to stock limited doses of ATRA when they otherwise may not.

One approach to disrupt the centralized testing model for patients with a suspected CML diagnosis has been a global initiative to collect and ship dried blood spots from low and middle-income countries for BCR::ABL1 testing at molecular diagnostics laboratories[21]. For example, the Spot on CML Initiative, a partnership between the Max Foundation and the Fred Hutchinson Cancer Research Center, has improved access to BCR::ABL1 TKIs by providing confirmatory RT-PCR testing to patients around the world. However, to fully transform CML diagnosis and TKI access worldwide, molecular testing should be readily available at the point of care.

CRISPR-based nucleic acid detection assays enable the development of highly sensitive molecular diagnostics by exploiting the sequence-specific activity of Cas enzymes. SHERLOCK combines reverse transcription and RPA for isothermal fusion target enrichment, followed by Cas13-mediated target detection and assay reporting. The single nucleotide specificity of RNA-guided Cas13 enzymatic activity enables the use of pre-defined target transcript-specific crRNAs for targets of interest. We show that crRNAs can be pooled to perform isoform-agnostic disease diagnostic tests enabling discrete, positive/negative readouts. Another application of Cas enzymes involves the use of Cas9-directed adapter ligation for target enrichment and library preparation followed by targeted nanopore long-read DNA sequencing in a flow cell.[22,23] This method was recently applied to the detection of oncogenic DNA fusions in leukemias.[22] Given the use of DNA sequencing, tiling of several guide RNAs may be required to cover potentially large expanses of intron regions where breakpoints in fusion partner genes may occur.

Additional advances are required to fully enable SHERLOCK diagnostic tests for fusion-driven leukemias to be carried out at the point of care. For example, we showed that direct cell lysis methods simplify the sample preparation step and do not interfere with RT-RPA or Cas13-based detection, but removal of red blood cells by hypotonic lysis or density gradient centrifugation is

still required for optimal assay performance. Current methods for preparing nucleic acid directly from whole blood have technical constraints that limit rapid RNA-based molecular diagnostics, and future improvements would expand access by decreasing cost and hands-on time. Additional modifications that would enhance field deployability include reagent lyophilization (to eliminate cold storage needs) and optimization of lateral flow strip readout. To this end, the performance of SHERLOCK assays using lyophilized reagents has been demonstrated previously.[24] Additionally, we showed that the plate-based fluorescent readout that maximizes sensitivity and specificity is readily deployable in laboratory settings and confirmed the feasibility using lateral flow strips for assay readout, consistent with performance of other SHERLOCK applications, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) diagnostics.[25] At scale, SHERLOCK diagnostic tests are estimated to cost $0.03/test for plate-based testing (not including capital equipment) and $0.61/test for lateral flow strip-based testing.[15]

Rapid, point-of-care molecular diagnostics are increasingly utilized for infectious diseases such as Mycobacterium tuberculosis and SARS-CoV-2, where access and speed are essential to treat patients and limit the spread of infection.[26] Likewise, SHERLOCK has primarily been developed for infectious disease applications, including SARS- CoV- 2, Ebola virus, and Lassa virus. Oncologic applications have been limited to proof of concept detection of *EGFR* alterations in cell-free DNA and micro-RNA for research applications in medulloblastoma and breast cancer cell lines.[27–29] We demonstrate the potential for SHERLOCK to be translated to highly sensitive, rapid diagnostic tests that improve access to life-saving therapies for clinically actionable fusion-driven leukemias.

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Authorship Contributions

Contribution: R.S.V., R.C.L., F.Z., O.A., and J.K. designed the study; R.S.V., H.Q.K., and F.L. performed the experiments and data analysis; E.S.W., J.S.G., M.R.L., G.G., L.W.B, O.S-T., J.R., and A.S.K. enabled patient sample validation; R.S.V. and R.C.L. wrote the manuscript; All authors reviewed the manuscript during its preparation and approved the submission.

Conflict of Interest Disclosures

J.K., O.A., J.G., F.Z., R.S.V, and R.C.L are inventors on patent PCT/US2020/049257, which covers the application of SHERLOCK for cancer diagnostics. R.C.L reports consultancy for Takeda Pharmaceuticals, Bluebird Bio, Qiagen, Sarepta Therapeutics, Verve Therapeutics, Jazz Pharmaceuticals and Vertex Pharmaceuticals and research funding from Bluebird Bio. J.R. reports consultancy for Cepheid. All other authors declare no competing financial interests.

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Figure Legends

Figure 1: PML::RARA and BCR::ABL1 SHERLOCK. Schematic diagram of SHERLOCK assay from sample preparation to readout.

Figure 2: Design and Validation of PML::RARA SHERLOCK Assay for APL. (A) Illustration of PML::RARA target specific assay design strategy with “fusion-specific” RT-RPA primers and “junction-specific” crRNA guides. (B) Limit of detection for isoform specific PML::RARA RNA. (C) Limit of detection for isoform agnostic PML::RARA RNA. (D) Heatmap illustrating background subtracted fluorescence intensity for isoform specific and isoform agnostic APL PML::RARA SHERLOCK using RNA extracted from patient samples. (E) Background-subtracted fluorescence from isoform-agnostic APL PML::RARA SHERLOCK performed on diagnostic PML::RARA RT-PCR-positive samples (n=36) and control samples (n=17). (F) Summary concordance of isoform-agnostic APL PML::RARA SHERLOCK and PML::RARA RT-PCR.

Figure 3: Design and Validation of BCR::ABL1 p210 SHERLOCK Assay for CML. (A) Illustration of BCR::ABL1 p210 target-specific assay design strategy with “fusion-specific” RT-RPA primers and “junction-specific” crRNA guides. (B) Limit of detection for isoform-specific BCR::ABL1 p210 RNA. (C) Limit of detection for isoform agnostic BCR::ABL1 RNA. Dilution series of K562 and KCL-22 RNA measuring limit of detection for (D) e13a2 RNA and (E) e14a2 RNA as a percentage of a fixed 10ng of total RNA input. (F) Heatmap illustrating background-subtracted fluorescence intensity for isoform-specific and isoform agnostic CML BCR::ABL1 SHERLOCK using RNA extracted from patient samples. (G) Background-subtracted fluorescence from isoform-agnostic CML BCR::ABL1 SHERLOCK performed on diagnostic BCR::ABL1 RT-PCR-positive samples (n=13) and control samples (n=13). (H) Summary concordance of isoform-agnostic CML BCR::ABL1 SHERLOCK and BCR::ABL1 RT-PCR.

Figure 4: Validation of CML BCR::ABL1 SHERLOCK on dried blood spot samples. (A) Schematic diagram illustrating the sources of dried blood spot samples and approach to testing by isoform-agnostic CML BCR::ABL1 SHERLOCK. Red points indicate sample country of origin and size of point reflects number of samples from country. (B) Background-subtracted fluorescence from isoform-agnostic CML BCR::ABL1 SHERLOCK performed on diagnostic BCR::ABL1 RT-PCR-positive CML dried blood spots samples (n=44) and BCR::ABL1 RT-PCR-negative controls (n=15). (C) Summary concordance of isoform-agnostic CML BCR::ABL1 SHERLOCK results and BCR::ABL1 RT-PCR in field-based dried blood spot CML and control samples.

Figure 5: BCR::ABL1 p190 SHERLOCK Assay for Ph+ ALL. (A) Prevalence of BCR::ABL1 p210 and p190 transcripts in Ph+ ALL. (B) Illustration of BCR::ABL1 p190 e1a2 assay design strategy with “fusion-specific” RT-RPA primers and “junction-specific” crRNA guides. (C) Limit of detection for BCR::ABL1 p190 synthetic target, (D) Background subtracted mean fluorescence intensity for serial samples from Ph+ ALL patient and control RNA. Corresponding RT-qPCR transcript %IS noted for each SHERLOCK measurement.

Figure 6: SHERLOCK Optimization Strategies. (A) Schematic of SHERLOCK workflow with areas of workflow optimization highlighted. (B) Comparison of direct cell lysis methods compared to column-based RNA purification from patient cells harboring PML::RARA short isoform at 30 minutes and 60 minutes of Cas13 detection. (C) Effect of decreasing RT-RPA time with fixed Cas13 detection time at 100ng and 1ng of input PML::RARA RNA, (D) Background subtracted fluorescence intensity for detection of synthetic and cell-line derived BCR::ABL1 p210 isoforms using a “one-pot” assay combining RT-RPA and Cas13a detection, (E) Demonstration of SHERLOCK assay readout in APL diagnostic sample using lateral flow strips.