Development of a Panel of Recombinase Polymerase Amplification Assays for Detection of Biothreat Agents

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Syndromic panels for infectious disease have been suggested to be of value in point-of-care diagnostics for developing countries and for biodefense. To test the performance of isothermal recombinase polymerase amplification (RPA) assays, we developed a panel of 10 RPAs for biothreat agents. The panel included RPAs for *Francisella tularensis, Yersinia pestis, Bacillus anthracis*, variola virus, and reverse transcriptase RPA (RT-RPA) assays for Rift Valley fever virus, Ebola virus, Sudan virus, and Marburg virus. Their analytical sensitivities ranged from 16 to 21 molecules detected (probit analysis) for the majority of RPA and RT-RPA assays. A magnetic bead-based total nucleic acid extraction method was combined with the RPAs and tested using inactivated whole organisms spiked into plasma. The RPA showed comparable sensitivities to real-time RCR assays in these extracts. The run times of the assays at 42°C ranged from 6 to 10 min, and they showed no cross-detection of any of the target genomes of the panel nor of the human genome. The RPAs therefore seem suitable for the implementation of syndromic panels onto microfluidic platforms.

S yndromic panels for infectious and emerging infectious diseases have been suggested to be of value in point-of-care (POC) diagnostics for developing countries and for biodefense (1). Since the introduction of molecular diagnostics and in particular real-time PCR, ample proof of its sensitivity and specificity has been generated. Indeed, molecular diagnostics are deemed superior to bacterial culture techniques or serological diagnostics (2–4). It has even been suggested to entirely eliminate the old methods in order to streamline centralized laboratories for molecular diagnostics (5–7).

In recent years alternative isothermal amplification methods which can be categorized into (i) T7 promoter-driven amplifications (transcription-mediated amplification [TMA], nucleic acid sequence-based amplification [NASBA], and single primer isothermal amplification [SPIA]), (ii) strand displacement methods (strand displacement amplification [SDA], loop-mediated isothermal amplification [LAMP], and smart amplification [SmartAmp]), (iii) helicase-dependent amplification (HDA), (iv) recombinase polymerase amplification (RPA), and (v) rolling-circle amplification (RCA) methods (8–12) have been developed. Some were purposely designed for isothermal amplification starting from RNA (TMA, NASBA, and SPIA), whereas others initially targeted DNA (SDA, LAMP, HDA, RPA, and RCA) and were only later adapted for RNA targets. Nonspecific intercalating fluorophores or fluorescent primers have been used for real-time detection in LAMP, SDA, HDA, and RCA, and specific detection probe formats have been developed for NASBA, RCA, HDA, and RPA (13–17).

In isothermal and exponential RPA, the phage recombinase UvsX and its cofactor UvsY form a nucleoprotein complex with oligonucleotide primers to scan for homologous sequences in a DNA template. Recognition of a specific homologous sequence leads to the initiation of strand invasion of the complex and the opposing oligonucleotides are then extended by isothermal (42°C) strand displacement amplification via Sau polymerase (*Staphylococcus aureus*), yielding double-stranded DNA (dsDNA)

amplificates very much like in PCR. Real-time amplificate detection can be performed by using TwistAmp exo probes. Exo probes carry internal fluorophore and quencher linked to thymine bases and separated by an abasic site mimic (tetrahydrofuran) localized approximately 15 nucleotides (nt) upstream from the 3′ end of the probe (45 to 55 nt). Once the probe hybridizes to its target sequence, the abasic site is recognized and cleaved by exonuclease III. The smaller downstream probe section carrying the quencher is released and fluorescence develops proportionally to the RPA-mediated amplification (15).

The second probe type for real-time fluorescent detection is the TwistAmp fpg probe, a 30-nt oligonucleotide, which carries a quencher at the 5′ end and the fluorophore at an internal position 4 to 5 nt downstream from the quencher via a C-O-C linker (or dR group). During hybridization of the probe the linker is cleaved by the DNA glycosylase FPG (*Escherichia coli*), thus causing separation of fluorophore and quencher and subsequently the proportional increase of fluorescence.

The purpose of the present study was to develop a panel of RPAs for a POC microfluidic platform. We describe the development of highly sensitive and specific fluorescent real-time RPA and RT-RPA assays for the detection of relevant category A bioterrorism agents, including Gram-positive and Gram-negative

bacteria, and DNA and RNA viruses on a mobile ESEquant Tub-escanner device. This mobile small-footprint device collects fluo-rescence signals over time, allowing for simultaneous real-time documentation of increasing fluorescence signals in an eight-tube strip (18, 19).

MATERIALS AND METHODS

Quantitative molecular standards. For bacteria, quantitative pCRII backbone plasmid standards were generated for the *pagA* gene (*Bacillus anthracis* plasmid pX01) and the *pla* gene (*Yersinia pestis*) as described previously (20). A *capC* gene carrying plasmid (*B. anthracis*, plasmid pX02) was provided by the Robert-Koch-Institut (21). For DNA virus, the variola virus (VARV) HA gene was synthesized and ligated into pMA-RQ by Geneart, Regensburg, Germany, and the vaccinia virus (VACV) plas-mid carrying the LE gene was provided by the Robert-Koch-Institut (22). For RNA viruses, quantitative Ebola virus (EBOV), Sudan virus (SUDV), and Marburg virus (MARV) NP-gene RNA standards were used as de-scribed previously (23, 24). A new quantitative sigma virus (SIGV) G gene based RNA standard was generated and transcribed as described previ-ously (24).

Viral and bacterial material. Genomic DNA of orthopoxviruses (VACV [Elstree 5], camelpox virus [CP19], monkeypox virus [MP4], and orthopoxvirus [OPV 90/3]) was provided by Hermann Meyer, Institute of Microbiology, German Armed Forces. Inactivated and gamma-irradiated bacteria and viruses were provided by the following institutes: *B. anthracis* spores (ATCC 14578); *Yersinia pestis* (03-1501) and *Francisella tularensis* (Ft 12) by the Center for Biosecurity 2, Robert Koch Institute, Berlin; VACV NYCBH strain (VR-1536) and Rift Valley fever virus (ZH548) by the Center for Biosecurity 1, Robert Koch Institute; MARV (Musoke strain) and EBOV (Zaire strain) by the Bernhard-Nocht Institute, Ham-burg, Germany; and SIGV by the Institute of Virology, Göttingen, Ger-many. The organisms were cultured in the donating institutions at bio-safety 3 or 4 levels.

Real-time PCR. The quantitative standards for *B. anthracis capC* and *pagA*, *Y. pestis pla*, *F. tularensis tul-4*, VACV, RVFV, EBOV, SUDV, and MARV were tested using published real-time PCR protocols (22–24). A new real-time PCR amplicon was designed for the SIGV G gene and for the VARV HA gene. Real-time PCR assays for DNA and RNA targets were performed using the LightCycler Fast-Start DNA Master HybProbe kit and the LightCycler 480 RNA master hydrolysis probes, respectively, on a Light Cycler 2.0 (Roche, Mannheim, Germany) using the second derivative method for analysis. All real-time PCR assays showed the sensitivities reported in the original publications. The SIGV and the VARV assays showed analytical sensitivities of 10 molecules detected per reaction.

RPA conditions. RPA was performed in a 50-µl volume using a TwistAmp Exo kit (TwistDX, Cambridge, United Kingdom) 420 nM RPA primers and 120 nM RPA probe, 14 mM magnesium acetate, and TwistAmp rehydration buffer. All reagents except for the template or sample DNA and the magnesium acetate were prepared in a mastermix, which was distributed into 0.2-ml reaction tubes, each containing a dried enzyme pellet. Magnesium acetate was pipetted into the tube lids. Subse-quently, 1 µl of standard DNA or genomic DNA was added to the tubes. The lids were closed, the magnesium acetate was centrifuged into the tubes using a Minispin centrifuge, and the tubes were immediately placed into an ESEquant Tubescanner device (Qiagen Lake Constance, Stockach, Germany).

For RT-RPA 10 U of Transcriptor (Roche), 20 U of RNaseOUT, 2 mM dithiothreitol (DTT), and 22.4 mM magnesium acetate were added to the DNA-RPA mixture described above. The same amount of primers and TwistAmp fpg probe were used with the rehydration buffer and the en-zyme pellets of the TwistAmp fpg kit. Subsequently, 1 µl of cDNA was added to the tubes. Fluorescence measurements (excitation, 470 nm; de-tection, 520 nm [FAM channel]) were performed at 42°C for 20 min. This reaction temperature was determined as optimal in terms of sensitivity

from a temperature range of 39 to 42°C. The Tubescanner software per-mits evaluation of the increase of fluorescence above three standard devi-ations over the background determined in minute 1 (adaptable), i.e., threshold validation. Additionally, the slope of the curve as the mV/time can be used (slope adaptable), i.e., slope validation. For confirmation, the calculation of the second derivative of the turning point of the upward fluorescence development can be applied to individual fluorescence curves with a very low slope (18, 19).

Determination of sensitivity and specificity. All quantitative DNA and RNA standards were tested by RPA in eight replicates, the threshold time (in minutes) was plotted against molecules detected, and a semilog regression was calculated. For exact determination, probit regression (25) was performed, and the sensitivity at 95% was calculated using Statistica software (StatSoft, Hamburg, Germany).

In order to assay the sensitivity of extraction and detection in samples containing representative whole organisms of each category in the panel, inactivated *Bacillus anthracis* spores (Gram positive), *Y. pestis* (Gram neg-ative), VACV (DNA virus), and RVFV (RNA virus) were diluted in 10-fold steps in phosphate-buffered saline and spiked into plasma to achieve a final concentration of 10^1 to 10^4 genomic copies/ml. In addition, 2 µl of SIGV in a concentration of 10^5 genomic copies/ml was added to the pre-pared spiked plasma dilutions to monitor the performance of the extrac-tion procedure.

Total nucleic acids from all bacterial and viral pathogens were pre-pared as spiked plasma samples using a single innuPREP MP basic kit A (Jena Analytik, Jena, Germany) and a magnetic bead separation rack com-bined with proteinase K treatment according to the manufacturer's in-structions. The nucleic acids were eluted in 100 µl of nuclease-free dis-tilled H_2O, and 5 µl was subjected to PCR or RPA.

RESULTS

Amplicon design. The design of the RPA primers differs from PCR primers, since the minimum length of 30 to 35 nt rather than the TM guides design. Since it is not clear which features of the 5′ end sequence of the primer actually supports the initiation of strand invasion, typically several primer pairs have to be tested. On average three, and at maximum eight, primer pairs were tested, and the final amplicon lengths in general ranged from 107 to 164 nt (Tables 1 and 2). Due to the high homology among the orthopoxvirus sequences. the most challenging design was that for VARV (Fig. 1). In the final design, the RPA probe overlaps the upstream primer sequences by 4 nt and covers a gap in the variola virus sequence, which is not present in the other orthopoxvirus sequences. Additionally, the downstream primer mismatched all other orthopoxvirus sequences at position 3 down from the 3′ end to specifically select for the VARV sequences according to the ARMS principle (26). An RPA for VACV was designed for the same region for use in the extraction experiments.

Assay development steps for RT-RPA. The detailed develop-ment of DNA-RPA and RT-RPA was described for the assays for *F. tularensis* and RVFV, respectively, elsewhere (27, 28).

We also compare here the performance of the fluorescent TwistAmp exo probe and the TwistAmp fpg probe in RT-RPA. We designed exo and fpg probes for the same target regions for EBOV, MARV, and SIGV and tested them on the respective quan-titative RNA standards. The sensitivities for the TwistAmp fpg probe assays were, respectively, 3-, 6-, and 3-$\log_{10}$ steps lower than the sensitivities of the TwistAmp exo probe assays. The results for EBOV are shown in Fig. 2A.

RPA sensitivity. Quantitative molecular plasmid and RNA standards were generated and verified by real-time PCR (data not shown) and used to test the analytical sensitivities of all final RPAs

TABLE 1 Details of RPA amplicon design

Infectious agent	Target gene	Reference sequence, position(s) of RPA amplicon	RPA amplicon length (nt)	Sequences used in the design
Y. pestis	*pla*	AF053945, 7267–7420	153	AF528089, AF053945, AL1009969, NC_003132, NC_004837, AE017046, NC_005816, CP001592, CP001596, NC_014027
B. anthracis	*pagA*	CP001216, 144299–144424	125	AF306778-83, AE011190, NC_003980, AE017336, CP001216, NC_012579, CP001599, NC_012656
B. anthracis	*capC*	AF188935, 55735- 55885	150	AF188935, AE01191, NC_003981, AE017335, NC_007323, CP001214, NC0125771, CP001597, NC_012655
Variola virus	HA	X69168, 151606- 151732	126	X69168, Y16780, DQ441416, DQ441418-48, DQ437500, DQ437581-91
Vaccinia virus	HA	DQ121394, 165441- 165584	143	M35027, U94848, AY243312, AY313847-48, AY603355, NC_006998, DQ121394
Ebola virus	NP	AY142960, 1779–1943	164	Jo44337, L11365, AF086833, AY142960, AF499101, AF272001, EU22440, AY354458, Y09358, AY054908, AY058895, EU051640-50
Sudan virus	NP	AF173836, 1783- 1868	130	AF173836, AY729654, NC_006432, EU338380
Marburg virus	NP	FJ750959, 1121–1256	135	Z12132, Z29337, NC_0016081, DQ217792, AY430365-66
Marburg virus	NP	FJ750953, 1121–1260	107	X68495, M72714, DQ447649-61, AY358025, FJ750953-59
Sigma virus	G	X06171, 84–960	119	X00171

(Fig. 3 and Table 3). The analytical sensitivity of the DNA-RPA assays was approximately 10 molecules detected (md) or as determined by probit analysis 16 to 19 md per reaction. Only the assay for the *capC* gene of *B. anthracis* plasmid pX02 showed a lower sensitivity of 100 to 1,000 md or as determined by probit analysis

778 md per reaction (Fig. 3A and Table 3). The standard deviation of the standard curve threshold time values ranged from 0.1 at 10^7 molecules to 2.6 at 10 md. The slopes of the semilog standard regression lines (SRLs) ranged from -0.33 to -0.96, indicating an efficiency of 1,072 to 11 if using the formula $E = 10^{1/slope}$ used for

TABLE 2 Primers and probes

Assay type and primer or exo probe[a]	Sequence (5′–3′)[b]
DNA assays	
BA1 RPA FP	TACAGGGGATTTATCTATTCCTAGTTCTGAG
BA1 RPA RP	GTAGCAAATGTATATTCATCACTCTTCTTAAC
BA1 RPA P	GAAAATATTCCATCGGAAAACCAATATTTTCA-BTF-GCTATTTGGTCAGGAT-P
BA2 RPA FP	CTGGAACAATAACTCCAATACCACGGAATTCA
BA2 RPA RP	GGTGTTTCAAGATTCATGATTTTATATGGCCG
BA2 RPA P	TGGCATAACAGGATAACAATAATCAAATAAAAGT-BTF-AAACAAATACCTGTAATTAGC-P
YP RPA FP	CCAGTATCGCATTAATGATTTTGAGTTAAATGC
YP RPA RP	TCCAGCGTTAATTACGGTACCATAATAACGTGAG
YP RPA P	CGACTGGGTTCGGGCACATGATAATGATGAGCACTA-BTF-GAGAGATCTTACTT-P
VARV RPA FP	GAGAATCCACAACMGACAAGACKTCSGGAC
VARV RPA RP	TTGGCGGTTGATTTAGTAGTGACAATTTCA
VARV RPA P	TGTATGAGACAGTGTCTGTGACTGTATGA-BTF-TCTTTATTAGTAATTGGTCC-P
VACV RPA FP	ACATACACTAGTGATAGCATTAATACAGTAAG
VACV RPA RP	AGATGATGTACTTACTGTAGTGTATGAGACAGT
VACV RPA P	TCTTCTTATCAGTAATTGGTTCCGGAGTCTCG-BTF-TGTGGATTCTCCA-P
RNA assays	
EBO RPA FP	GACGACAATCCTGGCCATCAAGATGATGATCC
EBO RPA RP	CGTCCTCGTCTAGATCGAATAGGACCAAGTC
EBO RPA P	GATGATGGAAGCTACGGCGAATACCAGAG-BTF-CGGAAAACGGCATG-P
SUD RPA FP	CAACTATYCCAGGTGGTGTTGTTGACCCGT
SUD RPA RP	GGCTGTCRTCATCGTCGTCGTCCAAATTGAAGA
SUD RPA P	CTCCTGTGGTGCCTTCAGCCGAATCCTCG-BTF-CAGGATAATTATTACT-P
MAR1 RPA FP	CATGAACATCAGGAAATTCAAGCTATTGCMGARG
MAR1 RPA RP	CTAATTTTTCTCGTTTCTGGCTGAGGACGGC
MAR1 RPA P	TGTGTGTGATTTCAGTTTTYTGAAGGTGGAAY-BTF-TCTAATATCTTCC-P
MAR2 RPA FP	CGACATGAACACCAGGAAATTCAGGCCATCGCC
MAR2 RPA RP	CGAGCTAGTTTCTCTCGTTTCTGGCTGAGGAC
MAR2 RPA P	AATCTCAGTCTTCTGGAGATGGAACTGTTCTAA-BTF-TTTTCTCTCTTCGTC-P
SIGV RPA FP	TGACCATCCTAACTCTGTGACATTCCAAGT
SIGV RPA RP	GTTGACAGTGAGCTCTTGAATCTCTGGGTT
SIGV RPA P	ACTGATTTCCCTCCGTGTCCTCCCGGTACCAC-BTF-CCAAACTGCCGTTGTG-P
MARV FPG P	(dR-FAM)GCCGT-B-CTCAGCCAGAAACGAGARAAAYTAGC(C3-Spacer)
ZEBO FPG P	(dR-FAM)GGCG-B-ATACCAGAGTTACTCGGAAAACGGCATGAA-P
SIG FPG P	(dR-FAM)TGTC-B-TCCCGGTACCACTATCCAAACTGCCGTTGT-P

[a] FP or RP, forward or reverse primer; P, probe.
[b] BTF, thymidine nucleotide carrying Blackhole Quencher 1, tetrahydrofuran spacer, and thymidine nucleotide carrying fluorescein; P, 3′phosphate to block elongation.

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Cowpox      .............A.G....TC...A..........G.........AGAAGAAGAAGAAGA...........A.............................................---...G.......................A..G.
Camelpox    .............A.G....TC...A......C...G.......AGA------------..............................................................G.......................A..G.
Monkeypox   .............A.G....TC...A..........G.......AGA------------...............A.............................................G.......................A..G.
Vaccinia    .............A.G....TC...A..........G.......AGA------------..............................................................G.......................A..G
Variola     GAGAATCCACAACAGTCAAGACGTCGGGACCAATTACTAATAAAGAAGA--------------TCATACAGTCACAGACACTGTCTCATACACTACAGTAAGTACATCATCTGAAATTGTCACTACTAAATCAACCGCCAA
VARV RPA FP  GAGAATCCACAACMGACAAGACKTCSGGAC
VARV RPA P                              GGACCAATTACTAATAAAGAAAA               TCATACAGTCACAGACACTGTCTCATACA
VARV RPA RP                                                                                                                          TGAAATTGTCACTACTAAATCAACCGCCAA
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FIG 1 Details of the RPA amplicon for VARV. All nucleotides in the alignment matching in the VARV sequence are presented as dots. Primer sequences are presented as full sequences. Gaps are presented as hyphens. VARV RPA FP is presented in sense, and VARV RPA P and RP are presented as reverse complement sequences. Gray fields: VARF RPA FP, degenerated IUB code positions; VARV RPA P, the TTT triplet used for the attachment of BTF (Table 2); VARF RPA RP, nucleotide at position 3 of the 3′ end mismatching all other orthopoxviruses. Sequences: cowpox virus, AY902252; camelpox virus, AF438165; monkeypox virus, AF380138; vaccinia virus, M35027; variola virus, X69198.

real-time PCR assays, which at an idealized exponential efficiency have an efficiency of 1.

RT-RPA was performed, by adding Transcriptor RT enzyme (Roche) to the RPA mix. Optimal performance was observed at 22.4 mM magnesium acetate and 2 mM DTT, and the analytical sensitivities of the RPAs ranged from 10 to 100 md or as determined by probit analysis from 16 to 21 md per reaction (Fig. 3B and Table 3). The standard deviations of the standard curve threshold time values ranged from 0.2 at 10^7 md to 2.6 at 10 md. SRL slopes ranged from 0.9×10^{-6} to 0.56, indicating an efficiency ($E = 10^{1/slope}$) of 10^8 to 61.

Sensitivity of RPAs in whole-organism nucleic acid extracts. The extraction efficiency of an innuPREP MP basic kit was tested with plasma spiked with whole organisms of each organism category encountered in the biothreat panel using real-time PCR assays. The results of the performance of the innuPREP MP basic kit tested by real-time PCR analysis are illustrated in Fig. 4. Pathogens spiked in plasma at a range of 10^4 to 10^1 md per reaction were efficiently extracted by the innuPREP MP basic kit and were detected at high sensitivity by real-time PCR methods. In addition, the internal control was efficiently detected in extracts of all of the spiked samples as determined by real-time PCR (threshold cycle, 22.56 ± 0.51; $n = 64$). The results here demonstrate that this total nucleic acid extraction method is robust and highly reproducible. The same extracts were used for detection by the respective RPAs. The results of RPA performance are summarized in Table 4. The RT-RPA assays for *F. tularensis* and RVFV each specifically detected strains of respective strain panels as described elsewhere (35a, 35b).

Specificity. The specificity of all RPAs was determined by cross-testing human genome DNA and the nucleic acids of all of the other biothreat agents in the panel, i.e., genomic DNA of *Y. pestis* (strain 03-1501), *F. tularensis* subsp. *holarctica* (strain LVS), *B. anthracis* (strain 3007), VACV (VR-1536), the VARV plasmid, and the genomic RNAs of EBOV, SUDV, MARV, and SIGV. Only specific detection was observed. Additionally, the RPA for VARV did not detect the genomic DNA of tested orthopoxviruses. These were all detected by the VACV RPA, which did not detect the VARV plasmid. The RPAs for the detection of bacteria were tested against a panel of bacterial genomes as described elsewhere (35a; see also Table S1 in the supplemental material) and showed exclusively specific detection.

To assay the influence of human genomic DNA on the RPAs, we determined the concentrations of human DNA in the eluates of negative sera extracted with the RNeasy kit (Qiagen). We then added the determined average amount of 70 ng of human genomic DNA/µl to RPA and RT-RPA reactions. The added background DNA delayed threshold time points negligibly for RPA and up to 3 min for one-step RT-RPA. It had no effect on the sensitivity (Fig. 2B and C).

DISCUSSION

In order to develop a panel of isothermal detection assays for category A bioterrorism agents, we assessed recombinase polymerase amplification (RPA) for the following reasons: (i) it is an exponential amplification, with specific amplificate confirmation using a fluorescent probe; (ii) it contains GP32, a single-strand binding protein and a good enhancer for the amplification of RNA molecules with complicated secondary structures (36); (iii) it needs only three conserved regions for oligonucleotide design; and (iv) available dried pellet reagents facilitate field use or point of care applications.

With regard to analytical sensitivity and specificity, the RPAs developed showed a performance equal to PCR (Table 3) and showed no cross-detection among their respective targets. Compared to PCR, however, the RPA reaction time was much shorter and, surprisingly, one-step-RT-RPA assays were quicker (limit of detection [LOD] reached at 4 to 8 min) than RPAs (LOD reached at 7 to 10 min).

The SRLs of the RT-RPA assays showed even lower slope values, indicating very fast reaction kinetics. We assume that this might be due to an additive effect of the fluorogenic detection of (i) RNA templates, (ii) the initially generated cDNA (ssDNA; generically detected in T7 promoter-driven isothermal assays such as TMA or NASBA), and (iii) the RPA products (dsDNA). Alternatively, the initiation of RPA may be facilitated by single-stranded cDNA.

The published K_m values for exonuclease III ($K_m = 6.3 \times 10^{-9}$ M [nicks/min]) (37) and FPG ($K_m = 7 \times 10^{-9}$ M [excisions/min]) (38) range in the same order of magnitude, implying comparable activity levels. Nevertheless, the assays using cleavage of fpg probes showed a significantly reduced sensitivity compared to the exo probe assays (Fig. 2A), suggesting that in RPA the FPG enzyme kinetics are not as favorable to real-time detection as those of exonuclease III.

The results of the whole-organism extraction experiments indicate that the magnetic bead-based total nucleic acid extraction kit used showed efficient extraction of DNA and RNA for all tested organism categories. Moreover, it was demonstrated that real-time PCR and RPA show comparable detection sensitivities in these extracts (Table 4).
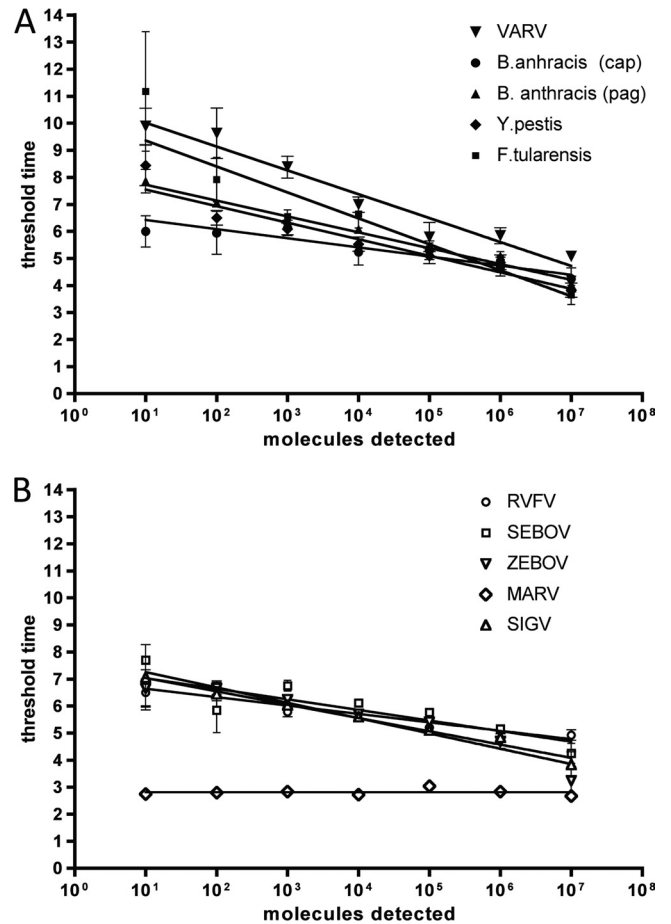
LAMP assays may also be considered a good option for isothermal detection and miniaturization (9). In general, LAMP assays need four to six primers, leading to longer amplicons and possibly more difficult design in the case of highly variable RNA viruses, whereas the RPA design with three oligonucleotides offers almost the same flexibility as real-time PCR. However, the longer Twist-Amp exo probes can be a design obstacle, which can be partly circumvented by allowing probe and primer to overlap. The use of
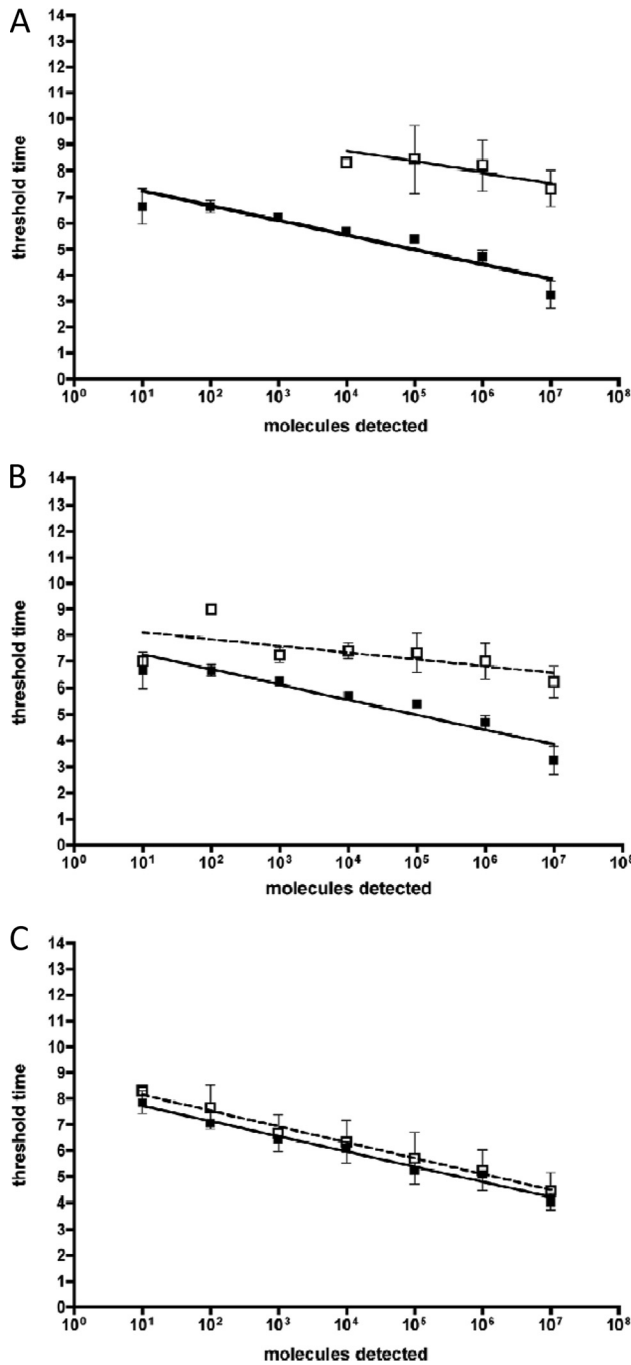
FIG 2 Real time RT-RPA assay performance. (A) Comparison of exo and fpg probe performance in RT-RPA. Standard regression lines (SRLs) for EBOV one-step-RT-RPA were generated from eight data sets (exo probe, black squares) and three data sets (fpg probe, white squares). (B) Influence of background DNA on EBOV one-step-RT-RPA. Black squares, SRL as described above; white squares, SRL of the same assay with 70 ng of human genome DNA background. (C) Influence of background DNA on RPA. Black squares, SRL derived from eight data sets of *B. anthracis* RPA; white squares, SRL of the same assay with 70 ng of human genome DNA background.

FIG 3 SRLs of all developed assays, including assays for *F. tularensis* and Rift Valley fever virus (described elsewhere [35b]). The SRLs were derived from eight data sets each. (A) DNA-RPA assays; (B) one-step-RT-RPA assays.

LNA nucleotides might help to reduce probe length, as has been shown for TaqMan probes (39, 40).

In comparison, published LAMP assays for *B. anthracis*, mon-keypox virus, RVFV, MARV, and EBOV (Table 3) have longer run times (18 to 60 min) at 60 to 63°C than the RPAs but show about the same sensitivity (Table 3). However, not all LAMP assays have been adapted for real-time fluorescence since some of them use the turbidity index for readout.

The current advantage of RPA is that the reaction mixtures containing enzymes, nucleotides, and buffer are provided in dried pellets, which is very amenable to POC or field use. This is now also possible for RT-RPA (35b). The only ingredients that need to be added are primers, probe, and sample.

With a small footprint of 17.4 by 18.8 cm and a weight of 1 kg (including the laptop), the ESEquant Tubescanner system is sig-nificantly lighter and smaller than all other available state of the art mobile PCR cyclers such as the SmartCycler, Rapid, and Razor (5 to 35 kg) cyclers or the Loopamp real-time Turbidimeter 2.0 for LAMP assays (5 kg). At 4,000 euros, the ESEquant Tubescanner is also considerably less expensive than any of the mobile PCR de-vices. In combination with the ESEquant Tubescanner, RPA is therefore a very attractive nucleic acid detection method that could easily be installed in hospitals or laboratories that cannot afford real-time PCR cyclers.

The only constraints of isothermal amplification methods are enzyme activity rates since there is no dependency on rapid tem-perature ramping as in PCR. This feature makes them more ame-nable to engineer microfluidic lab-on-chip devices than PCR. A

TABLE 3 Sensitivity of RPAs

Infectious agent (target gene)	Real-time PCR sensitivity (three runs) (reference)	RPA sensitivity (eight runs) (reference)	RPA probit 95% sensitivity (eight runs)	RPA threshold time to sensitivity limit (min)	Sensitivity LAMP (reference)
DNA assays					
B. anthracis (_pagA_)	10^1 to 10^2 (21)	10^1 to 10^2	16	8	10^3 (27)
B. anthracis (_capC_)	10^2 to 10^3 (21)	10^2 to 10^3	778	7	10^3 (27)
F. tularensis (_tul-4_)	10^{2a} (28)	10^1 to 10^2 (35a)	19	10	ND[c]
Y. pestis (_pla_)	2^a (29)	10^1 to 10^2	16	8	ND
Variola virus	10^1 to 10^2 (30)	10^1 to 10^2	16	10	10^{2b} (31)
RNA assays					
Rift Valley fever virus (N)	10^2	10^1 to 10^2 (35b)	19	7	10^2 (32, 33)
Ebola virus (NP)	10^2	10^1 to 10^2	21	7	10^1 (34)
Sudan virus (NP)	10^1	10^1 to 10^2	17	8	ND
Marburg virus (NP)	10^1	10^1 to 10^2	21	8	10^2 (35)
Sigma virus (G)	10^1	10^1 to 10^2	16	4	ND

[a] Calculated from the fg value given in the source reference publication.
[b] As determined from the monkeypox LAMP assay.
[c] ND, not determined.

recent review on miniaturization efforts for NASBA, LAMP, HDA, SDA, RCA, and RPA pointed out that low-temperature isothermal methods such as SDA, NASBA, RCA, and RPA show an advantage for miniaturization since they need much less energy input and are therefore better candidates for battery driven hand-held devices than high-temperature isothermal reactions (LAMP, SmartAmp, and HDA) (9).

The implementation of RPA on centrifugational LabDisks was

recently described (41). This type of cartridge could fulfill the requirements for simple benchtop devices if sample preparation were included. It would come closest to a lab on a cartridge, in contrast to the majority of current miniaturized molecular assay systems, which have aptly been described as "chip in lab" rather than "lab on chip" platforms (9).

In summary we have developed a panel of very rapid and highly sensitive isothermal real-time RPAs for the detection of category A
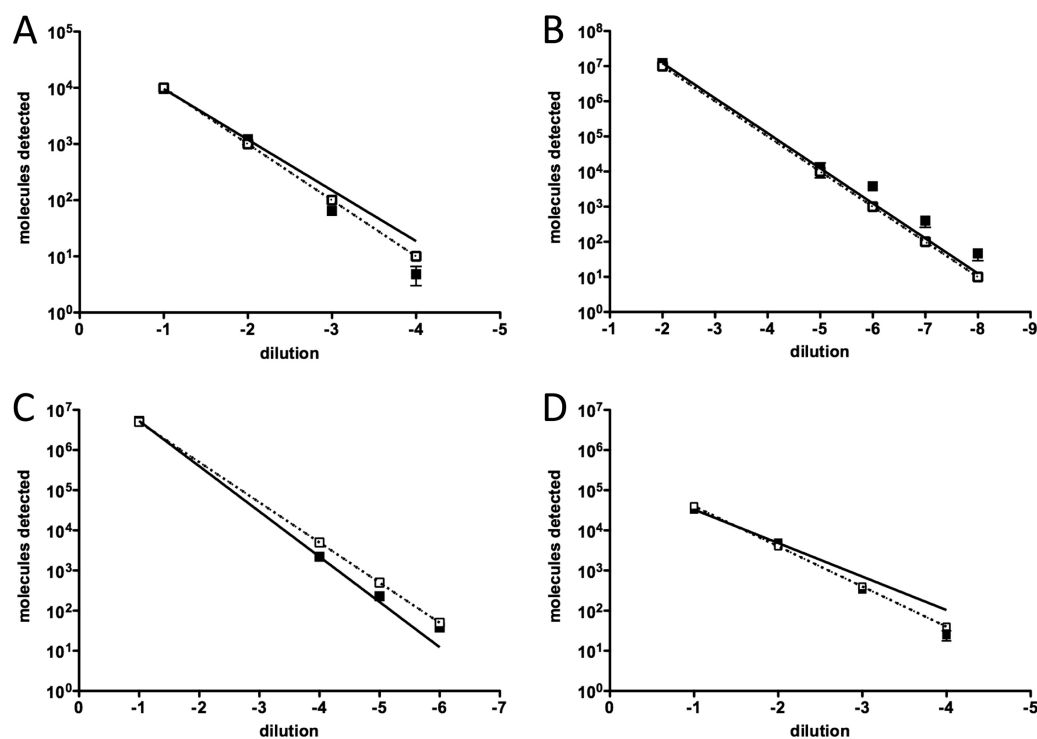

FIG 4 Extraction efficiency of the innuPREP MP basic kit. Plasma was spiked with whole organisms (range, 10^1 to 10^4 genomic copies/ml), and nucleic acids were extracted. Eluates were tested by respective quantitative real-time PCR assays in triplicate. The amount of measured molecules detected (md) per reaction is plotted against the 10-fold serial dilution of pathogens in plasma. The dotted line represents the calculated 100% efficiency of extraction. (A) _B. anthracis_ (Gram-positive) extracts tested with pag-PCR; (B) _Y. pestis_ (Gram negative) tested with pla-PCR; (C) VACV tested with LE-PCR; (D) MARV tested with NP-PCR.

TABLE 4 Comparison of assay sensitivity in nuclear extracts

Infectious agent	Real-time sensitivity in extracts (md)	
	PCR	RPA
B. anthracis[a] (Gram positive)	10	10
Y. pestis (Gram negative)	10	10
Vaccinia virus (DNA virus)	10	10
RVFV (RNA virus)	100	100
SIGV (RNA virus IPC)	100	1,000

[a] *pagA* was used for detection.

bioterrorism agents covering Gram-negative and Gram-positive bacteria, DNA viruses, and RNA viruses. We also showed that a commercially available magnetic bead-based total nucleic extraction kit, which could be used in resource-poor settings, can be efficiently combined with RPA. We now seek to integrate all assays onto a microfluidic POC device and test this syndromic panel of RPAs on clinical samples.

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