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Development of a panel of Recombinase Polymerase Amplification assays for the detection of biothreat agents

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ABSTRAC

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 ten RPAs for biothreat agents. The panel included RPA assays for *Francisella tularensis*, *Yersinia pestis*, *Bacillus anthracis*, variola virus, and Recerse Transcriptase Recombinase Polymerase Amplification (RT-RPA) assays for Rift Valley fever virus, Ebola virus, Sudan virus and Marburg virus. Their analytical sensitivities ranged from 16-21 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 RPA assays 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 6-10 minutes and they showed no cross detection of any of target genomes of the panel nor of the human genome. The RPA assays therefore seem suitable for implementation of syndromic panels onto microfluidic platforms.

Introduction

Syndromic 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 (29). 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 (6, 34, 44). It has even been suggested to entirely eliminate the old methods in order to streamline centralised laboratories for molecular diagnostics (5, 13, 14).

In recent years alternative isothermal amplification methods which can be categorized into (i) T7 promotor driven amplifications (transcription mediated amplification (TMA), Nucleic Acid Sequence-Based Amplification (NASBA), Single primer isothermal amplification (SPIA)), (ii) strand displacement methods (Strand Displacement Amplification (SDA), Loop-mediated isothermal amplification (LAMP), Smart amplification (SmartAmp)), (iii) helicase dependent amplification (HDA), (iv) recombinase polymerase amplification (RPA), and (v) rolling circle amplification (RCA) methods (1, 3, 12, 17, 36) have been developed. Some were purposely designed for isothermal amplification starting from RNA (TMA, NASBA, SPIA), whereas others initially targeted DNA (SDA, LAMP, HDA, RPA, 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 (24, 28, 31, 37, 38).

In isothermal and exponential recombinase polymerase amplification (RPA) the phage recombinase UvsX and its co-factor 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 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 upstream from the 3'end of the probe (45-55 nucleotides (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 (31).

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

The purpose of the study to develop a panel of RPA assays 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+, gram- bacteria, and DNA and RNA viruses on the mobile ESEquant Tubescanner device. This mobile small footprint device collects fluorescence signals over time allowing for simultaneous real time documentation of increasing fluorescence signals in a 8 tube strip (15, 33).

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Materials and Methods

Quantitative molecular standards

Bacteria: Quantitative pCRII backbone plasmid standards were generated for the pagA gene (*Bacillus anthracis* plasmid pX01), pla gene (*Yersinia pestis*) as described in (40). A capC gene carrying plasmid (*B.anthracis,* plasmid pX02) was provided by the Robert-Koch-Institut (7). DNA virus: The Variola virus (VARV) HA gene was synthesized and ligated into pMA-RQ by Geneart, Regensburg, Germany, the Vaccinia virus (VACV) plasmid carrying the LE gene was provided by the Robert-Koch-Institut (19). RNA Viruses: A quantitative Ebola virus (EBOV), Sudan virus (SUDV), Marburg virus (MARV) NP-gene RNA standards were used as described (41, 43). A new quantitative Sigma virus (SIGV) G gene based RNA standard was generated and transcribed as described (43).

Viral and bacterial material

Genomic DNA of Orthopox viruses (Vaccinia virus (Elstree 5), Camelpox virus (CP19), Monkeypox virus (MP4) and Orthopox virus (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), *Y. pestis* (03-1501) and *F. tularensis* (Ft 12) by Centre for Biosecurity 2, Robert Koch Institute, Berlin; Vaccinia virus NYCBH strain (VR-1536), and Rift Valley fever virus (ZH548) by Centre for Biosecurity 1, Robert Koch Institute, Berlin; Marburg virus (Musoke strain) and Ebola virus (Zaire strain) by Bernhard-Nocht Institute, Hamburg; and Sigma virus by Institute of Virology, Göttingen. The organisms were cultured in the donating institutions at biosafety 3 or 4 levels.

Real time PCR

The quantitative standards for *B. anthracis*, capC and pagA, *Y. pestis* pla, *F. tularensis* tul4, VACV, RVFV, EBOV, SUDV and MARV were tested using published real time PCR protocols (19, 41, 43). 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 2nd 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 50µl volume using the TwistAmp™ exo kit (TwistDX, Cambridge, UK) 420nM RPA primers and 120nM RPA-probe, 14mM Mg acetate and TwistAmp^{TM} rehydration buffer. All reagents except for the template or sample DNA and Mg-acetate were prepared in a mastermix, which was distributed into 0.2 ml reaction tubes each containing a dried enzyme pellet. Mg–acetate was pipetted into the tube lids. Subsequently 1µl standard DNA or genomic DNA was added to the tubes. The lids were closed and the Mg-acetate centrifuged into the tubes using a minispin centrifuge and the tubes were immediately placed into a ESEquant tubescanner device (Qiagen Lake Constance, Stockach, Germany).

For RT-RPA 10U Transcriptor (Roche, Mannheim, Germany), 20U RNaseOut, 2mM DTT and 22.4 mM Mg acetate were added to the DNA-RPA mix described above. The same amount of primers and TwistAmp^{TM} fpg probe were used with the rehydration buffer and the enzyme pellets of the TwistAmpTM fpg kit. Subsequently

1µl cDNA was added to the tubes. Fluorescence measurements (Excitation 470nm, Detection 520nm (FAM channel)) were performed at 42°C for 20 minutes. This reaction temperature was determined as optimal in terms of sensitivity from a temperature range of 39°C to 42°C. The Tubescanner software permits evaluation of the increase of fluorescence above three standard deviations over the background determined in minute one (adaptable) i.e. threshold validation. Additionally the slope of the curve as mV/time can be used (slope adaptable) i.e. slope validation. For confirmation the calculation of the 2nd derivative of the turning point of the upward fluorescence development can be applied to individual fluorescence curves with a very low slope (15, 33).

Determination of sensitivity and specificity

All quantitative DNA and RNA standards were tested by RPA in 8 replicates, the threshold time (in minutes) was plotted against molecules detected and a semi-log regression was calculated. For exact determination probit regression (35) was performed and the sensitivity at 95% calculated using the 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, *B. anthracis* spores (gram positive), *Y. pestis* (gram negative), VACV (DNA virus), and RVFV (RNA virus) were diluted in 10-fold steps in PBS and spiked into plasma to achieve a final concentration of 10^1 - 10^4 genomic copies / ml. Additionally, 2 µl of Sigma virus in a concentration of 10^5 genome genomic copies / ml were added to the prepared spiked plasma dilutions to monitor the performance of the extraction procedure.

Total nucleic acids from all bacterial and viral pathogens were prepared spiked plasma samples using the single innuPREP MP Basic Kit A (Jena Analytik, Jena, Germany) a magnetic bead separation rack combined with proteinase K treatment according to the manufacturer´s instructions. The nucleic acids were eluted in 100µl of nuclease-free distilled H_2O and 5µl were subjected to PCR or RPA.

Results

Amplicon design

The design of the RPA primers differs from PCR primers, as the minimum length of 30-35 nt rather than the TM guides design. Since it is not clear which features of the 5-prime end sequence of the primer actually supports the initiation of strand invasion typically several primer pairs have to be tested. On average 3 and at maximum 8 primer pairs were tested and the final amplicon lengths in general ranged from 107nt to 164nt (table 1). Due to the high homology among the Orthopoxvirus sequences the most challenging design was that for VARV. In the final design the RPA probe overlaps the upstream primer sequences by four nucleotides and covers a gap in the Variola 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-prime end to specifically select for the VARV sequences according to the ARMS principle (27). A RPA assay for VACV was designed for the same region for use in the extraction experiments.

Assay development steps for RT-RPA

The detailed development of DNA-RPA and RT-RPA was described for the assays for *F. tularensis* assay and RVFV respectively elsewhere (9, 10).

We here additionally describe comparison of 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 quantitative 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 sensitivity of all final RPA assays (Figure 3AB, table 3). The analytical sensitivity of the DNA-RPA assays was about 10 molecules detected (md) or as per probit analysis 16-19 md per reaction. Only the assay for the *capC* gene of *B. anthracis* plasmid pX02 showed a lower sensitivity of 100-1000 md or as per probit analysis 778 md per reaction (Figure 3A, 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 semi-log standard regression lines (SRL) ranged from -0.33 to -0.96 indicating an efficiency of 1072 to 11 if using the formula $E=10^{1/slope}$ used for real time PCR assays which at an idealised exponential efficiency have an efficiency of 1.

RT-RPA was performed, by adding Transcriptor RT enzyme (Roche, Mannheim) to the RPA mix. Optimal performance was observed at 22.4mM Mg acetate and 2mMDTT and the analytical sensitivities of the RPA assays ranged from 10-100 md or as per probit analysis from 16-21 md per reaction (Figure 3B, Table 3). The standard deviation of the standard curve threshold time values ranged from 0.2 at 10^7

244 molecules to 2.6 at 10 md. SRL slopes ranged from 0.9 $x10^{-6}$ to 0.56 indicating an
245 efficiency ($E=10^{1/slope}$) of 10^{8} to 61.
246
247 **Sensitivity of RPA assays in whole organism nucleic acid extracts**
248 Extraction efficiency of the innuPREP MP basic kit was tested with plasma spiked
249 with whole organisms of each organism category encountered in the biothreat panel
250 using real time PCR assays. Results of the performance of the innuPREP MP basic
251 kit tested by real time PCR analysis are illustrated in Fig. 4. Pathogens spiked in
252 plasma at a range of 10^{4}-10^{1} md per reaction were efficiently extracted by the
253 innuPREP MP basic kit and were detected at high sensitivity by real-time PCR
254 methods. Additionally, the internal control was efficiently detected in extracts of all
255 the spiked samples as determined by real time PCR (CT 22.56 ± 0.51, n= 64). The
256 results here demonstrate that this total nucleic acid extraction method is robust and
257 highly reproducible. The same extracts were used for detection by the respective
258 RPA assays. The results of RPA performance are summarized in table 4. The (RT)-
259 RPA assays for *F. tularensis* and RVFV each specifically detected strains of
260 respective strain panels as described in (9, 10).
261
262 **Specificity**
263 The specificity of all RPA assays was determined by cross testing human genome
264 DNA, and the nucleic acids of all the other biothreat agents in the panel i.e. genomic
265 DNA of *Y. pestis* (strain 03-1501), *F. tularensis ssp. holarctica* (strain LVS), *B.*
266 *anthracis* (strain 3007), Vaccinia virus (VR-1536), the VARV plasmid, the genomic
267 RNA of Ebola virus, Sudan virus, Marburg virus and Sigma virus. Only specific
268 detection was observed. Additionally the RPA assay for VARV did not detect the
269 genomic DNA of tested Orthopox viruses. These were all detected by the VACV RPA

assay, which did not detect the VARV plasmid. The RPA assays for the detection of bacteria were tested against a panel of bacterial genomes as described earlier (Table 1 in (9), table S1) and showed exclusively specific detection.

To assay the influence of human genomic DNA on the RPA assays, we determined the concentration 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/µl human genomic DNA 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. 2 B, C).

5. 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 (42), (iii) it needs only three conserved regions for olignucleotide design, (iv) available dried pellet reagents facilitate field use or point of care applications.

As to analytical sensitivity and specificity, the RPA assays developed showed a performance equal to PCR (table 3) and showed no cross detection amongst their respective targets. Compared to PCR, RPA reaction time however was much shorter and surprisingly one-step-RT-RPA assays were quicker (LOD reached at 4-8 minutes) than RPA assays (LOD reached 7 -10 minutes).

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) as is generic in T7 promotor 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 x 10^{-9} M (nicks/minute), (18), and FPG (K_m= 7 x 10^{-9} M (excisions/min), (4)) range in the same order of magnitude implying comparable activity levels. Nevertheless, the assays using cleavage of fpg-probes showed a significantly reduced sensitivity than the exo-probe assays (Fig. 2A), suggesting that in RPA the FPG enzyme kinetics are not as favourable 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 when in these extracts (table 4).

LAMP assays may also be considered as a good option for isothermal detection and miniaturisation (2). In general LAMP assays need 4-6 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 TwistAmp™ exo-probes can be a design obstacle, which can be partly circumvented by allowing probe and primer to overlap. The use of LNA nucleotides might help to reduce probe length as has been shown for TaqMan probes (11, 39).

In comparison published LAMP assays for *B. anthracis*, Monkeypox, RVFV, MARV and EBOV (table 3) have longer run times (18 – 60 minutes) at 60-63°C than the

322 RPA assays but show about the same sensitivity (Table 3). However, not all LAMP
323 assays have been adapted for real time fluorescence as some of them use turbidity
324 index for readout.
325 The current advantage of RPA is that the reaction mixture containing enzymes,
326 nucleotides and buffer are provided in dried pellets, which is very well amenable to
327 POC or field use. This is now also possible for RT-RPA (10). The only ingredients
328 that need to be added are primers, probe and sample.
329 With a small footprint of 17.4 x 18.8 cm and a weight of 1kg (including the labptop)
330 the ESEquant tubescanner system is significantler lighter and smaller than all other
331 available state of the art mobile PCR cyclers such as SmartCycler, R.A.P.I.D.,
332 RAZOR (5-35kg), or the Loopamp® Realtime Turbidimeter 2.0 for LAMP assays
333 (5kg). At 4000 Euro the ESEquant tubescanner is also considerably cheaper than
334 any of the mobile PCR devices. In combination with the ESEquant tubescanner RPA
335 is therefore a very attractive nucleic acid detection method that could easily be
336 installed in hospitals or laboratories, which cannot afford real time PCR cyclers.
337 The only constraints of isothermal amplification methods are enzyme activity rates
338 since there is no dependency on rapid temperature ramping as in PCR. This feature
339 makes them more amenable to engineer microfluidic lab-on-chip devices than PCR.
340 A recent review on miniaturisation efforts for NASBA, LAMP, HDA, SDA, RCA and
341 RPA pointed out that low temperature isothermal methods such as SDA, NASBA,
342 RCA and RPA show an advantage for minaturisation as they need much less energy
343 input and are therefore better candidates for battery driven handheld devices than
344 high temperature isothermal reactions (LAMP, SmartAmp, HDA) (2).
345 The implementation of RPA on centrifugational LabDisks was recently described
346 (25). This type of cartridge could fullfill to the requirements for simple benchtop
347 devices if sample preparation were included. It would come closest to a lab on a

cartridge in contrast to the majority of systems for miniaturized molecular assays currently developed which have aptly been described as ‘chip in lab’ rather than ‘lab on chip’ platforms (2).

In summary we have developed a panel of very rapid and highly sensitive isothermal real time RPA assays for the detection of Category A Bioterrorism Agents covering gram negative, 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 aim at integrating all assays onto a microfluidic POC device and testing this syndromic panel of RPA assays on clinical samples.

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509 **Figure Legends**

510

511 Figure 1. Details of the RPA amplicon for VARV. All nucleotides in the alignment
512 matching in the VARV sequence are presented as dots. Primer sequences are
513 presented as full sequences. Gaps are presented as hyphens. VARV RPA FP is
514 presented in sense and VARV RPA P and RP are presented as reverse complement
515 sequences. Grey fields: VARF RPA FP: Degenerated IUB code positions, VARV
516 RPA P: The TTT triplet used for the attachment of **BTF** (table 2), VARF RPA RP:
517 nucleotide at positon 3 of the 3-prime end mismatching all other orthopoxviruses.
518 Sequences: cowpox AY902252, camelpox AF438165, monkeypox AF380138,
519 vaccinia virus M35027, variola virus X69198.

520

521 Figure 2. Real time (RT-)RPA assay performance. A: Comparison of exo-probe and
522 fpg-probe performance in RT-RPA. Standard regression lines (SRLs) for EBOV one-
523 step-RT-RPA were generated from eight data sets (exo-probe, black squares) and 3
524 data sets (fpg-probe, white squares). B: Influence of background DNA on EBOV one-
525 step-RT-RPA, black squares: SRL as above, white squares: SRL of the same assay
526 with 70ng human genome DNA background. C: Influence of background DNA on
527 RPA, black squares: SRL derived of 8 data sets of *B. anthracis* RPA, white squares:
528 SRL of the same assay with 70ng human genome DNA background.

529

530 Figure 3. Standard regression lines (SRL) of all developed assays including assays
531 for *Francisella tularensis* and Rift Valley fever virus already decribed in (9, 10). SRLs
532 were derived from 8 data sets each. A: DNA-RPA assays. B: one-step-RT-RPA
533 assays.

534

Figure 4:

Extraction efficiency of the innuPREP MP basic kit. Plasma was spiked with whole organisms (range: 10^x -10^x/ml) and nucleic acids were extracted. Eluates were tested by respective quantitative real time PCR assays in triplicate. The amount of measured md per reaction is plotted against the 10-fold serial dilution of pathogens in plasma. The dotted line represents calczulated 100% efficiency of extraction. A: *B. anthracis* (gram positive) extracts tested with pag-PCR, B: *Y.pestis* (gram negative) tested with pla-PCR, C Vaccinia virus tested with LE-PCR, D Marburg virus tested with NP-PCR.

546
547
548 Table 1. Details of RPA amplicon design
549

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

550
551

Table 2. Primers and probes

name	RPA primers and exo-probes
	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**

FP / RP: forward and reverse primer, P probe, BTF: B: thymidine nucleotide carrying Blackhole quencher 1, T: tetrahydrofuran spacer, F: thymidine nucleotide carrying Fluorescein, Phosphate: 3'phosphate to block elongation.

Table 3 Sensitivity of RPA assays

Infectious agent and (target gene)	Sensitivity Real-time-PCR 3 Runs (source)		Sensitivity RPA 8 Runs		Sensitivity RPA Probit 95% 8 Runs	RPA threshold time to sensitivity limit (min)	Sensitivity LAMP (source)	
DNA assays								
B. anthracis (pagA)	10^1 - 10^2	(7)	10^1 - 10^2		16	8	10^3	(21)
B. anthracis (capC)	10^2 - 10^3	(7)	10^2 - 10^3		778	7	10^3	(21)
F. tularensis (*tul4*)	10^2 *	(8)	10^1 - 10^2	(9)	19	10	n.d.	
Y. pestis (*pla*)	2*	(26)	10^1 - 10^2		16	8	n.d.	
Variola virus	10^1 - 10^2	(32)	10^1 - 10^2		16	10	10^2	(16)
RNA assays								
Rift Valley fever virus (N)	10^2		10^1 - 10^2	(10)	19	7	10^2	(23, 30)
Ebola virus (NP)	10^2		10^1 - 10^2		21	7	10^1	(22)
Sudan virus (NP)	10^1		10^1 - 10^2		17	8	n.d.	
Marburg virus (NP)	10^1		10^1 - 10^2		21	8	10^2	(20)
Sigma virus (G)	10^1		10^1 - 10^2		16	4	n.d.	

*** calculated from fg given in original publication, **monkeypox LAMP assay**

571 Table 4 Comparison of assay sensitivity in nuclear extracts
572

Infectious agent	Real time PCR sensitivity in extracts (molecules detected)	Real time RPA sensitivity in extracts (molecules detected)
*B. anthracis** (gram+)	10	10
Y. pestis (gram-)	10	10
Vaccinia virus (DNA virus)	10	10
RVFV (RNA virus)	100	100
SIGV (RNA virus IPC)	100	1000

573

574 • pagA assays used for detection
575
576
577

Figure 1

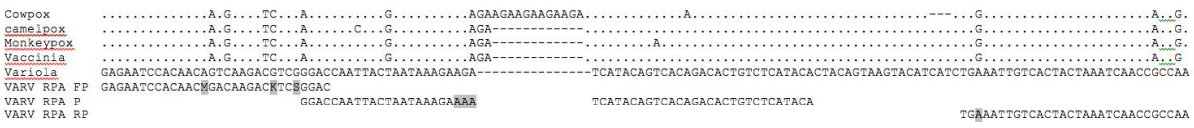
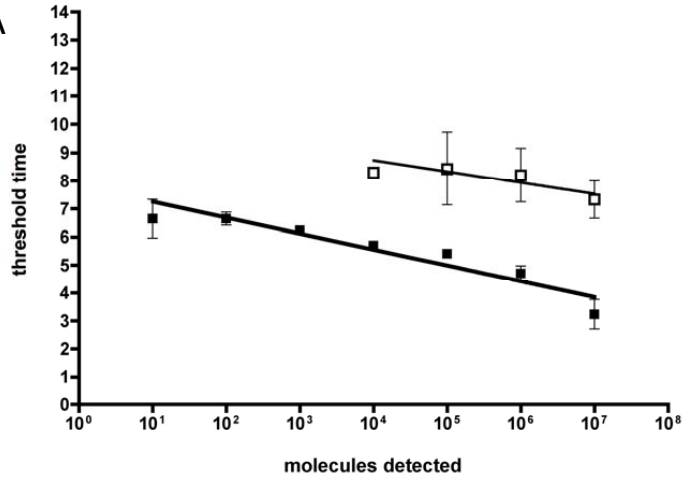
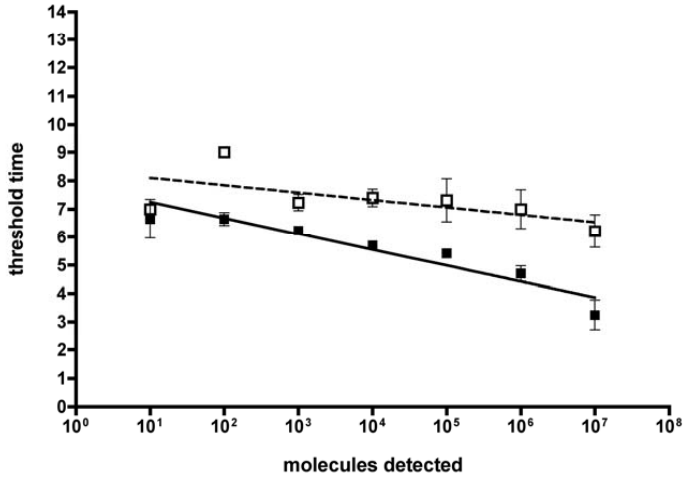

Cowpox .....A.G....TC...A.....G.....AGAAGAAGAAGAAGA.....A.....---...G.....A..G.
camelpox .....A.G....TC...A.....C...G.....AGA----------.....G.....A..G.
Monkeypox .....A.G....TC...A.....G.....AGA----------.....A.....G.....A..G.
Vaccinia .....A.G....TC...A.....G.....AGA----------.....G.....A..G
Variola GAGAATCCACAACAGTCAAGACGTCGGGACCAATTACTAATAAAGAAGA----------TCATACAGTCACAGACACTGTCTCATACACTACAGTAAGTACATCATCTGAAATTGTCACTACTAAATCAACCGCCAA
VARV RPA FP GAGAATCCACAACMGACAAGACKTCSGGAC
VARV RPA P GGACCAATTACTAATAAAGAAA TCATACAGTCACAGACACTGTCTCATACA
VARV RPA RP TGAAATTGTCACTACTAAATCAACCGCCAA

Figure 2

A


threshold time
molecules detected

B


threshold time
molecules detected

C

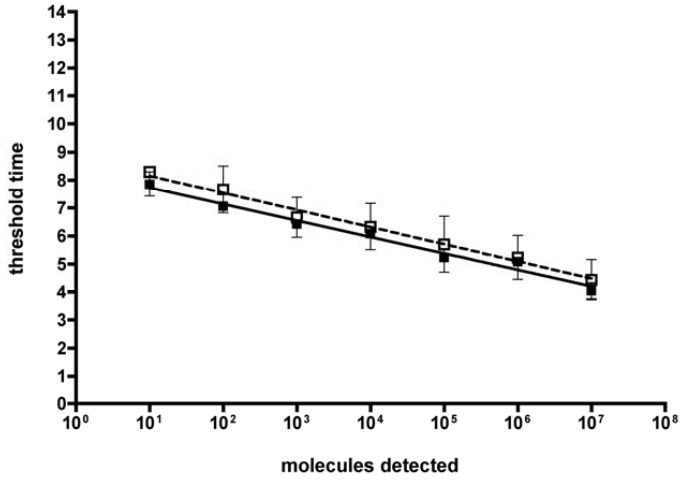
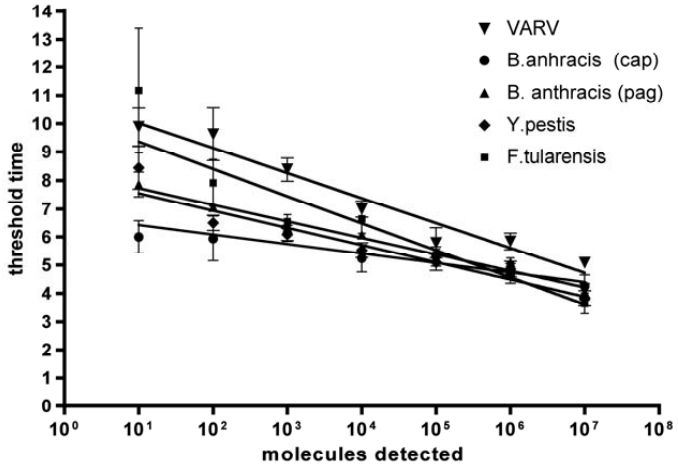

threshold time
molecules detected

Figure 3

A


VARV
B.anhracis (cap)
B. anthracis (pag)
Y.pestis
F.tularensis
threshold time
molecules detected

B

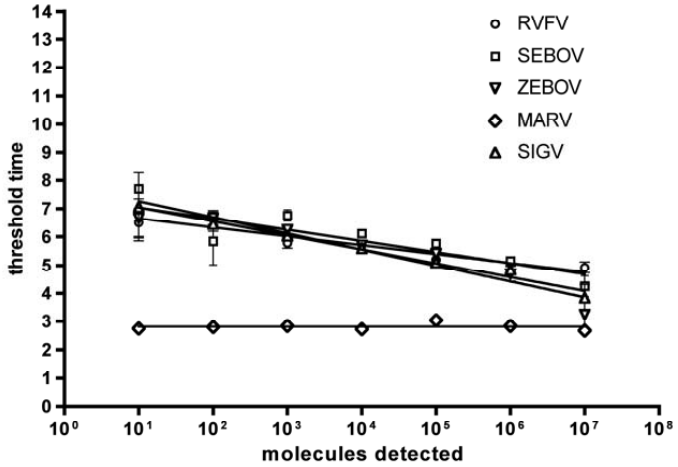

RVFV
SEBOV
ZEBOV
MARV
SIGV
threshold time
molecules detected

Figure 4