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A Fast and Accessible Method for the Isolation of RNA, DNA, and Protein to Facilitate the Detection of SARS-CoV-2

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Abstract

Management of the COVID-19 pandemic requires widespread SARS-CoV-2 testing. A main limitation for widespread SARS-CoV-2 testing is the global shortage of essential supplies, among these, RNA extraction kits. The need for commercial RNA extraction kits places a bottleneck on tests that detect SARS-CoV-2 genetic material, including PCR-based reference tests. Here we propose an alternative method we call PEARL (Precipitation Enhanced Analyte RetrievaL) that addresses this limitation. PEARL uses a lysis solution that disrupts cell membranes and viral envelopes while simultaneously providing conditions suitable for alcohol-based precipitation of RNA, DNA, and proteins. PEARL is a fast, low-cost, and simple method that uses common laboratory reagents and offers comparable performance to commercial RNA extraction kits. PEARL offers an alternative method to isolate host and pathogen nucleic acids and proteins to streamline the detection of DNA and RNA viruses, including SARS-CoV-2.

Introduction

The COVID-19 pandemic has had a devastating social and economic impact worldwide. As the disease continues to spread, global SARS-CoV-2 testing is more urgent than ever. Reference tests for SARS-CoV-2 rely on the isolation of viral genetic material followed by PCR-based amplification[1]. The rate-limiting step for this approach is the extraction of viral RNA from human samples. Commercial solid-phase RNA extraction kits that isolate viral RNA are the starting point for PCR-based SARS-CoV-2 reference tests. These kits use silica-based columns to purify viral RNA after disruption of cells and viral particles with proprietary reagents. The global demand for these kits has made them a limiting resource for SARS-CoV-2 testing, fueling the development of alternative SARS-CoV-2 RNA isolation methods and protocols that bypass the RNA extraction step altogether. These alternative approaches include organic solvent-based RNA extraction, and the use of chaotropic agents and proprietary buffer formulations.

TRIzol, a phenol- and guanidine-based reagent routinely used for isolation of RNA, DNA, and protein, has been used to isolate SARS-CoV-2 RNA[2–4]. However, TRIzol extraction is labor intensive, which makes it difficult to scale-up to meet testing demands. Moreover, it requires special considerations for the disposal of organic solvents. A 5-minute RNA preparation method has been recently reported, but depends on expensive proprietary lysis solutions originally developed for genomic DNA isolation[5]. Recently, direct detection of SARS-CoV-2 in nasopharyngeal swab samples without RNA extraction was reported, indicating that the initial RNA isolation step could be omitted[6–8]. Despite encouraging results, this approach results in reduced sensitivity of downstream quantitative PCR (qPCR)-based detection. On average, this method required an additional 5-7 PCR cycles to reach the detection threshold when compared to reactions templated on

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purified RNA. Because detection of low viral loads is critical for minimizing false negative results, it is essential that new approaches do not compromise sensitivity. In a more recent report, guanidium chloride was used for sample lysis in nasal swabs obtained from COVID-19 positive patients[9]. Total RNA was subsequently precipitated with isopropanol. This approach conveniently concentrates the RNA, which can increase detection sensitivity in downstream analyses. However, the use of the toxic chaotropic agent guanidium chloride requires special disposal guidelines.

To address the aforementioned shortcomings, we developed a simple technique to isolate nucleic acids and proteins from cells and viruses we call PEARL (Precipitation Enhanced Analyte RetrievaL). PEARL is fast, easy to perform, and uses common laboratory reagents. Moreover, PEARL allows the detection of specific SARS-CoV-2 viral sequences with comparable sensitivity to that afforded by commercial RNA extraction kits. PEARL can be used to isolate nucleic acids and proteins, and it can be coupled to nucleic acid amplification or immunodetection methods to detect host and viral RNA, DNA, and proteins from multiple sources. PEARL does not require specialized equipment or highly trained personnel, and offers a low-cost straightforward alternative to facilitate virus detection.

Results

We designed PEARL to provide a low-cost, column-free approach for the isolation of nucleic acids and proteins that uses common laboratory reagents (Fig. 1A, Supplemental Table 1). PEARL uses a non-ionic detergent-based lysis solution (see Materials and Methods) to disrupt cell membranes and viral envelopes, while simultaneously providing conditions suitable for alcohol-based precipitation and recovery of RNA, DNA, and proteins. To benchmark our method, we extracted RNA from de-identified SARS-CoV-2 positive samples using PEARL or a dedicated RNA extraction kit (QIAamp Mini Elute Virus Spin Kit, Qiagen). Next, we used the isolated RNA to examine the levels of the SARS-CoV-2 nucleoprotein (N) gene as well as the host RNaseP mRNA in the samples using the 1-step reverse transcription qPCR reference test for COVID-19 recommended by the United States Centers for Disease Control and Prevention (CDC) (TaqMan RNA-to-Ct 1-Step Kit, ThermoFisher). In these experiments, we detected the SARS-CoV-2 N1 site using the qPCR primers and probes recommended by the CDC.

To maximize SARS-CoV-2 detection sensitivity, we tested various sample to PEARL-lysis-solution ratios. In these experiments, we observed that 250 µl of initial swab sample input and 500 µl of PEARL lysis solution resulted in the lowest RT-qPCR Cq values (Fig. S1). PEARL required a modest increase in initial sample input (1.25-fold) to achieve similar sensitivity to that of the commercial RNA extraction kit we used (Fig. 1B, note that the sample input for PEARL was 250 µL while the sample input for the QIAamp Kit was 200 µl). Together, these results indicate that PEARL can be used as an alternative to commercial RNA extraction kits without significant loss in sensitivity.

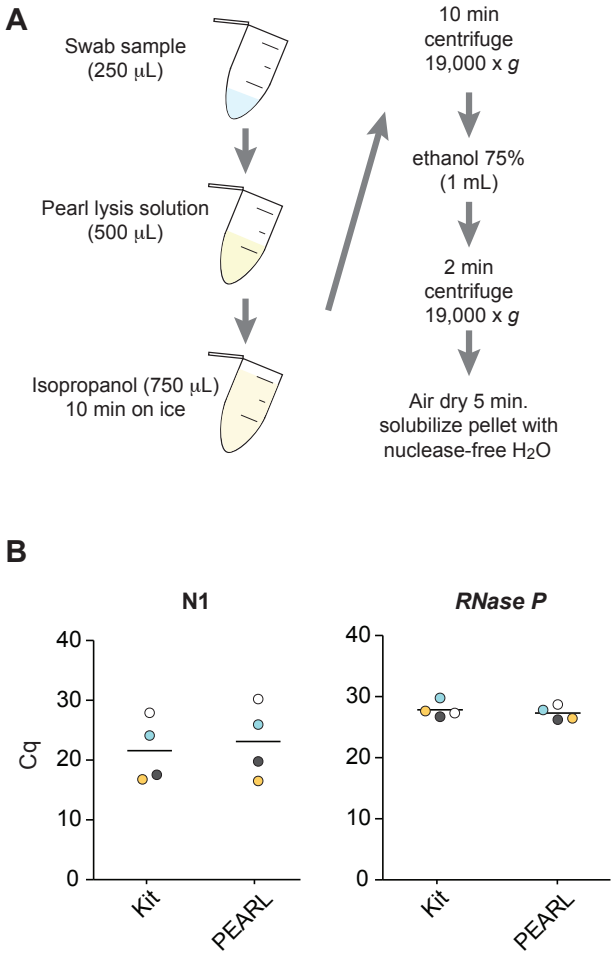

Figure 1. A) Overview of PEARL. **B)** Comparative RT-qPCR analysis of the levels of SARS-CoV-2 nucleocapsid (N1) and *RNaseP* RNA sequences in four de-identified SARS-CoV-2 positive samples after RNA extraction using PEARL or a dedicated RNA extraction kit (QIAamp Mini Elute Virus Spin Kit). Corresponding samples are color-coded. The black lines indicate the median.
P = 0.72 (N1), P = 0.56 (*RNaseP*), t-test.

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We reasoned that, because DNA and protein co-precipitate with RNA upon addition of isopropanol during extraction[10], PEARL could be used for the streamlined retrieval of RNA, DNA, and proteins from different viruses. To test whether PEARL can be used to detect different types of viruses, we infected cells with Kaposi's Sarcoma Associated Herpesvirus (KSHV), which contains a DNA genome, or with Zika virus (ZIKV), a flavivirus that contains an RNA genome and no DNA replication intermediates in its life cycle[11]. In these experiments, we used iSLK-219 cells, which are latently infected with a GFP-expressing recombinant KSHV[12], or HeLa cells infected with the ZIKV strain isolated in Puerto Rico in 2015 (PRVABC59) at a multiplicity of infection (MOI) of 1. We collected 100,000 cells, which corresponds to the estimated cellular yield of a typical buccal swab[13], and prepared 10-fold dilutions to determine the detection limit for RNA, DNA, and protein. Next, we prepared PEARL extracts and probed for viral and host nucleic acids and proteins using qPCR- and immunodetection-based assays, respectively. To ensure the specificity of RNA or DNA detection, we treated the PEARL extracts with DNase I (to detect RNA) or RNase A (to detect DNA). For protein immunodetection, we treated the PEARL extracts with RNase and DNase before SDS-PAGE and western blotting to ensure undisturbed migration of the proteins during electrophoresis, or left them untreated for dot-blot detection.

To detect host and viral transcripts, we synthesized first-strand complementary DNA from the DNase I treated samples, and used it for qPCR detection of the host β-actin mRNA (*ACTB*), as well as viral transcripts. These viral mRNAs included the KSHV latency-associated nuclear antigen (*LANA*) and KHSV-encoded *GFP* (Fig. 2A), as well as ZIKV RNA regions encoding the non-structural proteins NS1 and NS5 (Fig. 3A). In these experiments, we detected viral transcripts in PEARL extracts obtained from as few as 1,000 infected cells, and we did not observe significant differences in sensitivity between the detection of KSHV or ZIKV transcripts. Thus, PEARL can facilitate the detection of mRNAs from DNA and RNA viruses.

To detect host and KSHV genomic sequences, we used PEARL extracts treated with RNase A. Our target sequences for DNA detection corresponded to the genes for the host and viral transcripts aforementioned (Fig. 2A, 3A). In agreement with our observations for KSHV transcripts, we detected the viral genome in as low as 1,000 latently infected cells (Fig 2B). We also detected the host DNA β-Actin locus) in all samples, regardless of the infection status (Fig 2B, 3B). In these experiments, we used the same pair of PCR primers for detection of the β-Actin mRNA and genomic DNA sequences, which eliminates an additional source of variability stemming from dissimilar amplification efficiencies of different primer pairs. The primers target sequences in different β-Actin exons (Fig. S2A), distinguishing mRNA amplicons from genomic DNA amplicons by molecular size. As a control, and to corroborate that the amplification products in Figs. 2B and 3B were not templated on contaminant RNA, we used PCR primers that amplify the non-transcribed promoter region of the host gene HSPA5 (Fig. S2B). As expected, we detected an amplification product only in the PEARL extracts treated with RNase A but not in those treated with DNase I (Figs. S2C, S2D), verifying the specificity of the amplification reaction.

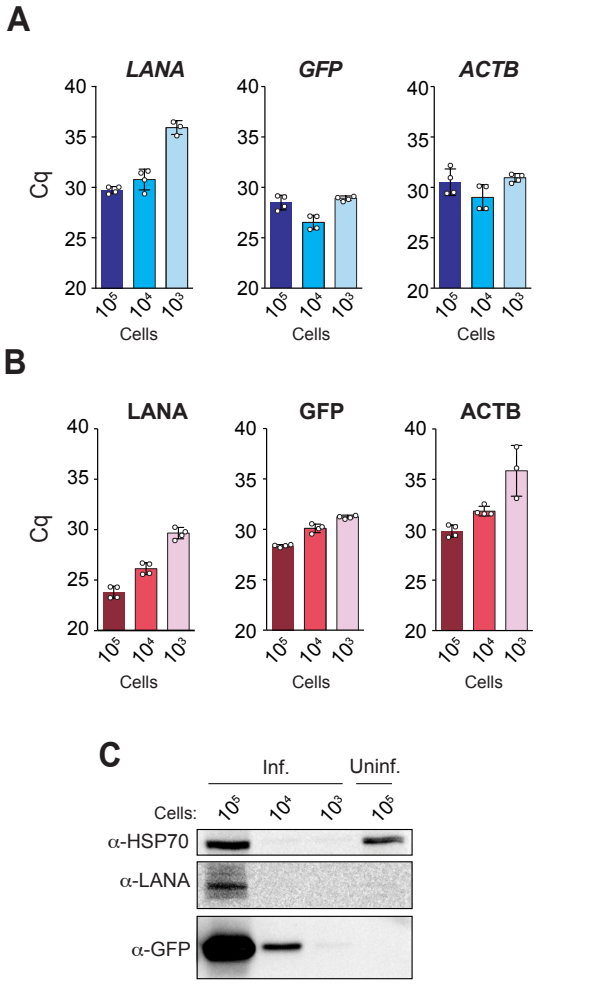

Figure 2. A) RT-qPCR analysis of the levels of herpesvirus (LANA, GFP) and host β-actin (ACTB) mRNAs, and **B)** their corresponding genomic sequences. **C)** Western blot analysis of the expression of herpesvirus (LANA, GFP) and host (HSP70) proteins. Inf., infected; Uninf., uninfected.

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An additional benefit of PEARL over column-based commercially available RNA extraction methods is that it allows the recovery of proteins in addition to nucleic acids. To confirm the presence of host and viral proteins in PEARL extracts, we carried out western blot and dot-blot assays using antibodies against the ubiquitous host chaperone HSP70, the viral proteins KHSV-LANA, KSHV-encoded GFP, and ZIKV-NS2B. In these experiments, we detected the host chaperone HSP70 in PEARL extracts obtained from 100,000 cells (HeLa and iSLK-219) by western blot (Fig. 2C and 3C) and in as few as 12,500 cells (HeLa and iSLK-219) by dot-blot (Fig. S3A, and S3B). Detection of KSHV-encoded GFP was achievable with approximately 1,000 iSLK-219 cells (Figs. 2C and S3B). Detection of KSHV-LANA was significantly less sensitive by western blot than by dot-blot, requiring 100,000 and 1,250 iSLK-219 cells, respectively (Figs. 2C and S3B). Taken together, our results indicate that PEARL can be used as a reliable and efficient method to extract host and virus nucleic acids and proteins from a wide range of viral infections.

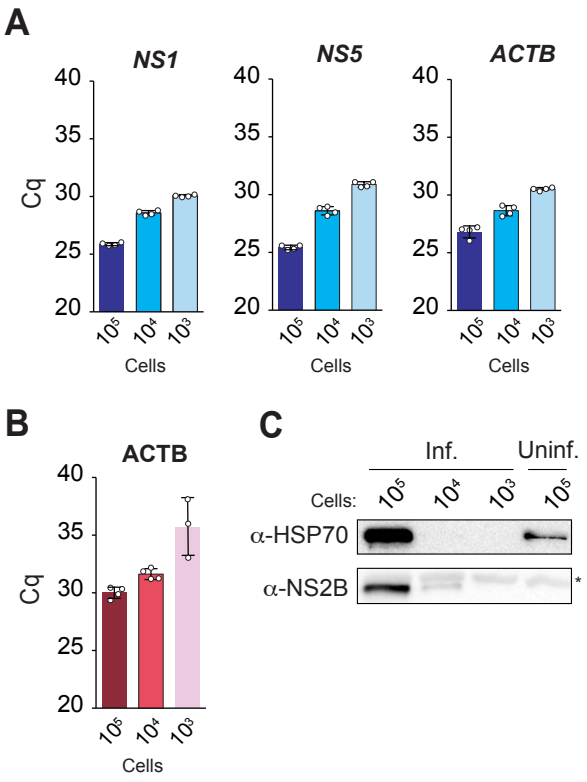

Figure 3. A) RT-qPCR analysis of the levels of ZIKV non-structural proteins NS1 and NS5 and host β-actin (ACTB) mRNAs **B)** qPCR analysis of the expression host ACTB genomic DNA sequences in ZIKV-infected samples. **C)** Western blot analysis of expression of ZIKV (NS2B) and host (HSP70) proteins. *non-specific band.

Discussion

The primary tool to combat the COVID-19 pandemic is widespread and accessible testing to monitor SARS-CoV-2 prevalence and spread, which informs deployment of containment and mitigation measures. Globally scaled testing remains an unmet public health need, as attempts to meet this demand have resulted in shortages of the reagents and supplies necessary for RNA extraction and sample processing. Here we present data to support PEARL as a cost effective, simple, and less-toxic alternative for the isolation of RNA, DNA and proteins. Our results indicate that PEARL facilitates the detection of SARS-CoV-2 transcripts in COVID-19 positive swab samples with sensitivity comparable to that afforded by commercially available RNA extraction kits. This outcome highlights the validity of using PEARL as a viable alternative to facilitate the sensitive detection of SARS-CoV-2 in respiratory samples.

Our data also show that PEARL extracts can be used to efficiently detect host and viral transcripts, genomic DNA, and proteins regardless of the nature of the infection—PEARL was equally useful in detecting DNA and RNA viruses with different tropism. Coupling PEARL to different downstream analyses for detection of nucleic acids and proteins can provide a powerful tool for detection of diverse viruses. Moreover, because RNA, DNA, and proteins are extracted at once, PEARL reduces sample handling time, allowing streamlining diagnostic procedures. Thus, it may enable both nucleic acid and antigen-based SARS-CoV-2 testing. PEARL's minimal handling requirements also make it scalable, which is desirable for high-volume testing operations, as is needed for SARS-CoV-2 testing.

It is possible that the collection medium used to store samples before processing may influence the performance of PEARL. For example, the viral transport media recommended by the CDC to store and inactivate samples for SARS-CoV-2 testing (2% Fetal Bovine Serum, 100 µg/mL Gentamicin, 0.5 µg/mL Amphotericin B, and various salts)[14] has components that could co-precipitate with target analytes. Isopropanol is less polar than ethanol, and therefore, it has a higher propensity to precipitate salts and antibiotics[15]. Regardless, co-precipitation of salts and antibiotics does not appear to compromise downstream RT-qPCR or immunodetection assays. Concerns regarding the efficiency of downstream detection assays could be addressed by using ethanol instead of isopropanol.

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It is also possible that PEARL may introduce extraction bias, as short RNAs including tRNAs, snoRNAs and miRNAs, are more difficult to precipitate than longer RNA and DNA molecules[15]. Though we have not directly tested whether small RNAs are underrepresented in PEARL extracts, we have designed PEARL to enhance the precipitation of all RNAs by using linear polyacrylamide as a carrier. Additionally, longer and faster centrifugation speeds can be used to enhance small RNA recovery, if needed[10]. Further improvements may be required to implement PEARL as mainstream nucleic acid and protein isolation tool in the detection of other viruses obtained from sources different than those described here, as the sample type may dictate overall performance. Future work outside the scope of this study will be required to address whether this is the case.

Finally, since PEARL uses common reagents and it does not require expensive equipment or highly trained personnel, it can provide an accessible alternative for streamlining diagnostics in geographic areas that lack access to capital, specialized reagents, and professional laboratories. In view of these considerations, coupling PEARL to our recently developed CREST protocol for detection of SARS-CoV-2 genetic material[16] could allow streamlined, affordable, widespread testing, lowering the barrier of "luxury testing" in many regions of the world.

Materials and Methods

PEARL

Samples are mixed in a 1:2 (v/v) sample:lysis solution (0.5% IGEPAL CA-630, 450 mM sodium acetate, 20 % glycerol, 20 mM TCEP, 50 µg/ml linear polyacrylamide, and 20 mM HEPES-KOH pH 7.2) ratio and incubated for 5-minutes at room temperature. Next, nucleic acids and proteins are precipitated on ice, for 10 minutes, using one volume of cold isopropanol. The precipitated material was collected by centrifugation at 19,000 RCF for 10 minutes, washed once with 75% ethanol, air-dried for 5 minutes at room temperature and solubilized in 20 µl of nuclease-free water for amplification-based detection of nucleic acids or immunodetection of proteins.

Cell culture and infections

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS), L-Glutamine, and antibiotics (penicillin/streptomycin, 100 units/mL), and were maintained in a humidified incubator at 37°C and 5% CO_2. iSLK-219 cells are latently infected with KSHV.219[12]. This recombinant virus is maintained in cells as an episome. GFP is constitutively expressed from the episome, under the control of the human EF1 promoter. iSLK-219 cells also harbor the gene for a doxycycline-inducible KSHV RTA transcription activator. Uninfected iSLK and KSHV-infected iSLK-219 cells were grown to 80% confluence, were collected by trypsinization after two washes with 1X PBS (GenClone), were counted, and were suspended at the desired density in 250 µL of PBS for PEARL extraction. For ZIKV infections, HeLa cells were grown to 60% confluency and then infected with ZIKV at a multiplicity of infection (MOI) of 1. 48 hours post-infection, the cells were collected by trypsinization after two washes with 1X PBS (GenClone), and were counted. Cells were resuspended at the desired concentrations in a final volume of 250 µL in PBS, and then subjected to PEARL.

qPCR

PEARL extracts were obtained from de-identified human samples or cultured cells. SARS-CoV-2 positive human samples were heat-inactivated by incubation at 56°C for 30 minutes before RNA extraction. RNA from these samples was obtained using the QIAamp Mini Elute Virus Spin Kit (Qiagen), using 200 µL of sample input, following the manufacturer's protocol, and eluted in 50 µL. PEARL extracts were prepared using 250 µL of SARS-CoV-2 positive human samples or a fixed number of cultured infected cells suspended in 250 µL of PBS. PEARL extracts from cultured cells were treated with either DNase I (1 unit per every 8 µL of PEARL extract, New England BioLabs) or with RNase A (0.1 mg per every 8 µL of PEARL extract, ThermoFisher) in a final volume of 10 µL for 30 minutes at 37°C. 5 µL of DNase-treated samples were reverse transcribed in 10 µl using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol and diluted 5-fold in nuclease-free water before qPCR. Target detection by qPCR was carried out using the SYBR Select Master Mix (Applied Biosystems) with 2 µL of diluted cDNA as template, and following the manufacturer's protocol. The whole 10 µL from RNase-treated samples (genomic DNA) was diluted 5-fold with nuclease-free water. Detection of specific genes was carried out using the SYBR Select Master Mix (Applied Biosystems) with 2 µL of diluted genomic DNA as template, and following the manufacturer's protocol. Detection of SARS-CoV-2 N1 gene sequences and host RNase P mRNA from de-

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identified SARS-CoV-2 positive samples was carried out with the one-step TaqMan RNA-to-Ct 1-Step Kit (ThermoFisher), using 2 µL of undiluted PEARL extract, and following the manufacturer's protocol. All qPCR data were collected using a CFX96 touch real-time PCR instrument (BioRad), and analyzed with the CFX maestro 1.1 software (BioRad). Cq values were determined by regression. Data analysis and statistical tests were performed using the Graph Pad Prism 6.0 software.

Immunodetection

Nuclease-treated PEARL extracts were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad) for western blot analysis. The membranes were blocked in 0.5% BSA-TBST for 30 minutes. Primary antibodies were diluted in 0.5% BSA-TBST as follows: α-HSP-70 (Cell Signaling Technology 4872) 1:1,000; α-LANA/ORF73 (Advanced Biotechnologies 13-210-100) 1:3,000; α-GFP (Invitrogen A11122) 1:3,000; α-NS2B (GeneTex GTX133308) 1:1,000. The membranes were incubated with primary antibodies for 30 minutes at room temperature. Following primary antibody incubation, the membranes were washed with 1X TBST 3 times prior to the addition of HRP-conjugated secondary antibodies. The membranes were incubated for 30 minutes with secondary antibody diluted 1:3,000 in 0.5% BSA-TBST. Immunoreactivity was detected using the Radiance Plus HRP Substrate (Azure Biosystems). All images were captured with an Azure Biosystems C300 gel imaging system. Image post-processing was carried out in Photoshop CC (Adobe) using automatic contrast. For dot-blot-based immunodetection, nitrocellulose membranes (Bio-Rad) were spotted with 1 µl of PEARL extract and allowed to dry completely at room temperature for 30 minutes. For the remainder of the procedure, the membranes were treated identically as described for western blotting and images were captured and processed in the same way.

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