Routine use of the BioRobot® EZ1 in detection of veterinary and zoonotic pathogens*

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The BioRobot EZ1 workstation was used for nucleic acid purification from a variety of veterinary samples for detection of pathogens and genotyping for resistance to scrapie. The BioRobot EZ1 system provides a flexible solution for automated nucleic acid purification, reducing the time from samples to reliable results.

Culture is the method generally used for detection of animal pathogens. However, some pathogens can be difficult or even impossible to culture (e.g., chlamydia or viruses). For others, the time required for culture is too long or incompatible with optimal sanitary management (e.g., mycobacterium for tuberculosis or Johne's disease). In addition, some pathogens are either extremely infectious or deadly (e.g., coxiella, anthrax, and filoviruses such as Ebola or Marburg), requiring facilities with high biosafety levels.

For detection of pathogens, PCR provides a rapid method to drastically reduce the time required while serology can subsequently complement PCR results. Genotyping by PCR can also provide effective screening for genetic susceptibility to protein pathogens, such as scrapie in sheep. Accurate PCR analysis relies on efficient nucleic acid purification from a variety of sample types ranging from blood and tissue biopsies to milk and feces. A variety of different PCR assays are required for various types of bacterial, viral, and parasitic pathogens. Optimization is often required depending on the sample type, the presence of potential inhibitors, and the type of pathogen.

This study describes the use of the BioRobot EZ1 workstation for purification of pathogen nucleic acids from a variety of veterinary samples for subsequent PCR testing.

Materials and methods

Q fever

Fresh or frozen milk from cows was centrifuged at 5000 x *g* for 10 minutes. The pellet was resuspended in 190 µl Buffer G2 plus 10 µl proteinase K. The samples were then incubated at 56°C for 20 minutes. Bacterial DNA was purified on the BioRobot EZ1 workstation using the EZ1 DNA Tissue Kit and the EZ1 DNA Bacteria Card.

Biopsies of bovine placenta were disrupted using the TissueLyser and then processed as described for milk. Bovine vaginal swabs stored in PBS were incubated at 37°C for 15–30 minutes in a shaking water bath. The liquid was pressed out of the swab, and the suspension was centrifuged. The pellet was then resuspended and treated as described for milk.

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Fresh bovine vaginal swabs were added directly to the lysis buffer and incubated with proteinase K. The liquid was pressed out of the swab, and bacterial DNA was purified from the suspension on the BioRobot EZ1 workstation using the EZ1 DNA Tissue Kit and the EZ1 DNA Bacteria Card.

A 2 µl aliquot of each eluate was used in an in-house real-time PCR assay for Coxiella burnetii DNA based on the QuantiTect® Probe PCR Kit on the LightCycler® System using FRET probes targeting the gene for insertion sequence element IS1111a.

Bovine tuberculosis and Johne's disease

Pulmonary lymphatic nodes from cattle were spiked with a serial dilution of a *Mycobacterium bovis* stock and processed using a modification of a previous, manual purification method (1). Nodes were disrupted in Buffer ASL (with antifoaming Reagent DX, QIAGEN) using the TissueLyser and a mixture of 5 mm, 3 mm, and 1.5 mm diameter glass beads. After disruption, the samples were incubated at 95°C for 10 minutes. For complete disruption of mycobacteria, micro glass beads (250 µm diameter) were then added and the samples were disrupted again on the TissueLyser. The samples were then briefly centrifuged, and 180 µl of the supernatant was transferred to a new sample tube. Proteinase K (20 µl) was added, and the samples were incubated at 70°C for 10 minutes. Bacterial DNA was purified on the BioRobot EZ1 using the EZ1 DNA Tissue Kit and the EZ1 DNA Bacteria Card.

A 2 µl aliquot of each eluate was used in a 20 µl, in-house, real-time PCR assay for *Mycobacterium bovis* based on the QuantiTect SYBR® Green PCR Kit on the LightCycler System.

Stool samples were spiked with a serial dilution of a *Mycobacterium* ssp. *paratuberculosis stock* and processed using a modification of a previous, manual purification method (1). Samples (1 g) were weighed and vortexed with 5 ml Buffer ASL (with antifoaming Reagent DX, QIAGEN). The samples were then incubated at 95°C for 10 minutes, centrifuged, and 500 µl of supernatant was transferred to a sample tube containing three 5 mm glass beads and 250 mg of 250 µm glass beads. Samples were then disrupted on the TissueLyser at 30 Hz for 2 x 5 minutes. Following

Table 1. Direct and Indirect Diagnosis of Q Fever in an Infected Bovine Herd

		C. burnetii DNA detection by PCR			
Cow ID number	Antibody detection	Placenta	Vaginal swab	Milk	Interpretation and comments
605	–	n.d.	–	–	Uninfected
740	+	n.d.	+	–	Infected; with signs of infection in the genital area
1191	+	n.d.	–	++	Infected; with signs of bacteria in the milk
4081	++	++++	++	+++	Infected and ill
6316	–	n.d.	–	–	A priori uninfected
2703	–	n.d.	–	+	Retest
1741	–	n.d.	–	–	Uninfected
739	–	n.d.	–	–	Uninfected
6069	–	n.d.	Ambiguous	–	Apparently uninfected
5794	–	n.d.	–	–	Uninfected

n.d.: not determined.

a brief centrifugation, 180 µl of supernatant was transferred to a new tube, and 20 µl proteinase K was added. The sample was incubated at 70°C for 10 minutes, and bacterial DNA was purified on the BioRobot EZ1 using the EZ1 DNA Tissue Kit and the EZ1 DNA Bacteria Card.

A 2 µl aliquot of each eluate was used in a 20 µl, in-house, real-time PCR assay for *Mycobacterium avium* ssp. *paratuberculosis* based on the QuantiTect SYBR Green PCR Kit on the LightCycler System.

Bovine viral diarrhea

Viral nucleic acids were purified from bovine serum, spiked with a serial dilution of a bovine viral diarrhea virus (BVDV) stock. Samples were processed manually using the QIAamp® UltraSens® Virus Kit or on the BioRobot EZ1 using the EZ1 Virus Kit and the EZ1 Virus Card. A 2 µl aliquot of the eluate was used in a 20 µl, in-house, real-time RT-PCR assay for bovine viral diarrhea virus based on the QuantiTect SYBR Green RT-PCR Kit on the LightCycler System.

Scrapie

Fresh and frozen sheep blood was diluted with an equal volume of physiological serum. Genomic DNA was purified from a 200 µl aliquot on the BioRobot EZ1 using the EZ1 DNA Blood 200 µl Kit and the EZ1 DNA Blood Card. A 2 µl aliquot of the eluate was used in a 20 µl real-time PCR assay (Roche), following recommendations of TIB MOLBIOL GmbH (Berlin, Germany).

Results and discussion

Q fever

Q fever is a zoonosis (2) that can cause severe symptoms in humans, including high temperature, abdominal pain, nausea, endocarditis, and abortion in pregnant women. The disease is caused by the highly infectious bacterium *Coxiella burnetii*. The bacterium is fairly resistant to heat and drying and can survive in pasteurized milk from infected animals (2). Infection of humans can occur from ingestion of infected milk, contact with animal feces, and during animal abortion, mainly by aerosol. Since the bacterium is very infectious and hardy, it is also a potential agent for bioterrorism.

Table 1 shows the results of molecular testing for presence of *C. burnetii* DNA by real-time PCR (Figure 1). The molecular tests corresponded well with serological testing, providing a fast and accurate means to diagnose *C. burnetii*.

Detection of *Coxiella burnetii* DNA by Real-Time PCR

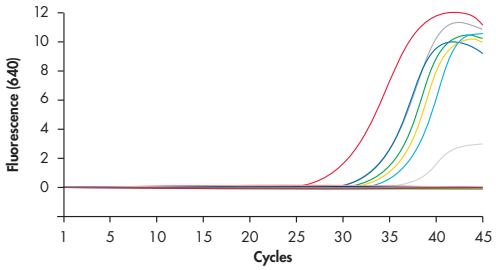

Figure 1 DNA was purified from various sample types on the BioRobot EZ1 workstation, using the EZ1 DNA Tissue Kit and the EZ1 DNA Bacteria Card, as described in "Materials and methods". Real-time PCR analysis of *C. burnetii* DNA was carried out on the LightCycler System. Results are summarized in Table 1.

Sensitive Detection of *Mycobacterium bovis* DNA in Bovine Lymphatic Nodes

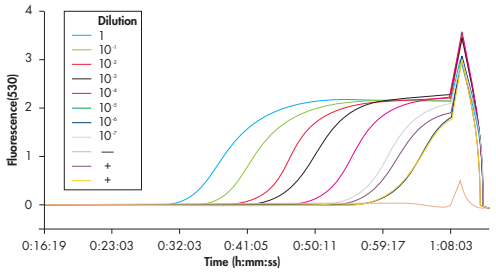

Figure 2 Bovine lymphatic nodes spiked with the indicated dilutions of a *M. bovis* stock were disrupted using the TissueLyser, and bacterial DNA was purified on the BioRobot EZ1 workstation, using the EZ1 DNA Tissue Kit and the EZ1 DNA Bacteria Card, as described in "Materials and methods". Real-time PCR analysis of *M. bovis* DNA was carried out on the LightCycler System.

Removal of PCR Inhibitors for Detection of *M. avium* ssp. *paratuberculosis* DNA in Bovine Stool Samples

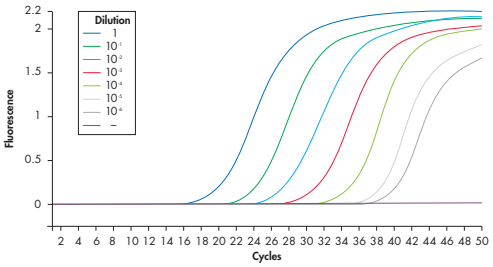

Figure 3 Bovine stool samples spiked with the indicated dilutions of a *M. avium* ssp. *paratuberculosis* stock were disrupted using the TissueLyser, and bacterial DNA was purified on the BioRobot EZ1 workstation, using the EZ1 DNA Tissue Kit and the EZ1 DNA Bacteria Card, as described in "Materials and methods". Real-time PCR analysis of *M. avium* ssp. *paratuberculosis* DNA was carried out on the LightCycler System.

Sensitive Detection of BVDV RNA with Melting-Curve Analysis

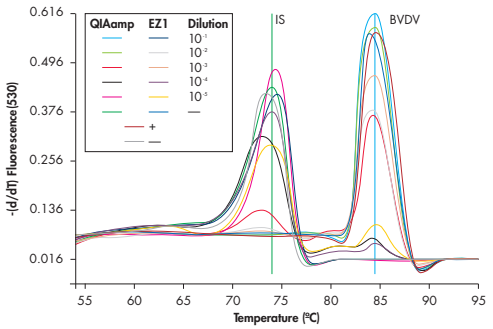

Figure 4 A BVDV stock was diluted in bovine serum as indicated, and viral nucleic acids were purified using the QIAamp UltraSens Virus Kit (**QIAamp**) or on the BioRobot EZ1 workstation, using the EZ1 Virus Mini Kit and the EZ1 Virus Card (**EZ1**). Real-time RT-PCR of BVDV RNA was carried out on the LightCycler System, followed by melting-curve analysis of the BVDV amplicon (**BVDV**) and the amplified internal standard (**IS**).

Bovine tuberculosis and Johne's disease

Bovine tuberculosis and Johne's disease are caused by different species of mycobacteria: *Mycobacterium bovis* and *Mycobacterium avium* ssp. *paratuberculosis*, respectively. Both mycobacteria are potentially zoonotic (3), especially hardy, small, Gram-positive bacteria. Since it takes approximately 3 months to assess a negative result by culture, a fast and accurate method for testing animals showing symptoms of bovine tuberculosis is required. In addition, screening for *M. avium* ssp. *paratuberculosis* in stool samples allows identification of animals excreting the mycobacterium, which represents an increased risk for spreading the disease (4).

Molecular testing for *M. bovis* in lymph node biopsies enabled successful detection and quantification of bacterial DNA (Figure 2). *M. avium* ssp. *paratuberculosis* DNA was successfully detected in stool samples, with complete removal of potential PCR inhibitors (Figure 3).

Rapid automated DNA purification on the BioRobot EZ1 workstation, followed by real-time PCR, provides substantial time savings over culture methods. Results can be assessed in only 3–4 hours instead of 3 months, greatly reducing the time from sample collection to results.

Bovine viral diarrhea

Bovine viral diarrhea virus (BVDV) is a small RNA virus that can cause a variety of symptoms, including, in some cases, a hemorrhagic syndrome. Since there are no reliable clinical signs for identifying BVDV infection, laboratory diagnostic investigations are required to identify the virus (5).

Screening animal serum by PCR provides a rapid means to determine the presence of BVDV. Bovine serum samples were processed using both a manual spin-column procedure, with the QIAamp UltraSens Virus Kit, and the automated protocol on the EZ1 Virus Card with the BioRobot EZ1 workstation. Detection by real-time RT-PCR on the LightCycler System, followed by melting-curve analysis, showed comparable sensitivity using either method (Figure 4). Rapid viral nucleic acid purification on the BioRobot EZ1 workstation, combined with real-time RT-PCR analysis, provided a quick and sensitive method for detection of BVDV.

Scrapie

Scrapie is a transmissible spongiform encephalopathy (TSE) of sheep, one of a group of fatal, infectious diseases, including Creutzfeldt-Jacob disease in humans and bovine spongiform encephalopathy (BSE) in cattle. Scrapie is caused by an abnormal form of the normally occurring cellular prion protein (PrP^C). In sheep, scrapie resistance is associated with polymorphisms at codons 136, 154, and 171 of the PRP^C gene. Genotyping programs are underway in the EU, the US, and elsewhere,

to identify and breed scrapie-resistant sheep. Genetic selection may be helpful to control and possible eliminate the disease in flocks (6).

Although sequencing is the gold standard for detecting polymorphism, it is extremely time-consuming. Alternatively, real-time PCR, followed by melting-curve analysis, clearly distinguished different polymorphisms in the PRP^C gene (Figure 5). Genotyping results were available in less than 2 hours, with only 20 minutes required for DNA purification, representing a much faster time from samples to results.

Conclusions

- Fully automated DNA and viral nucleic acid purification on the BioRobot EZ1 workstation provides rapid processing, with faster times from samples to reliable results.
- Purification on the BioRobot EZ1 workstation provides high-quality DNA and viral nucleic acids from a variety of different veterinary sample types, including samples rich in inhibitors, (e.g., stool) and hardy, Gram-positive bacteria (e.g., *Mycobacterium avium* ssp. *paratuberculosis*).
- The high-quality DNA performs well in sensitive downstream pathogen detection assays, such as real-time PCR and melting-curve analysis.
- The flexibility of the BioRobot EZ1 system makes it well-suited for the wide range of samples and pathogens encountered in veterinary diagnostics laboratories.

Genotype Analysis for Scrapie Susceptibility in Sheep

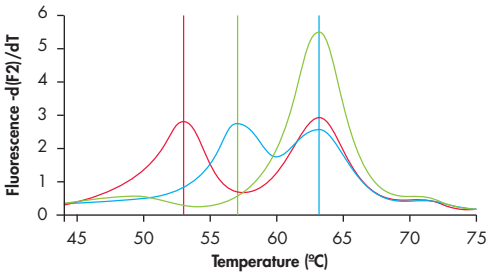

Figure 5 Genomic DNA was purified from sheep blood, diluted 1:1 with physiological serum, on the BioRobot EZ1 workstation, using the EZ1 DNA Blood 200 µl Kit and the EZ1 DNA Blood Card. Following real-time PCR, melting-curve analysis distinguishes 3 different polymorphisms of the PRP^C gene.

References

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Ordering Information

Product	Contents	Cat. no.
BioRobot EZ1	Robotic workstation for automated purification of nucleic acids using EZ1 kits, installation, 1-year warranty on parts and labor*	9000705
EZ1 DNA Tissue Kit (48)	48 Reagent Cartridges (Tissue), 50 Disposable Tip Holders, 50 Disposable Filter-Tips, 50 Sample Tubes (2.0 ml), 50 Elution Tubes (1.5 ml), Buffer G2, Proteinase K	953034
EZ1 DNA Bacteria Card	Preprogrammed card for EZ1 bacterial DNA purification protocols	9016362
EZ1 Virus Mini Kit (48)	For 48 virus nucleic acid preps: Reagent Cartridges, Disposable Tips, Disposable Tip-Holders, Sample Tubes, Elution Tubes, Buffers	955338
EZ1 Virus Card	Preprogrammed card for BioRobot EZ1 virus protocols	9016386
EZ1 DNA Blood 200 µl Kit (48)	For 48 x 200 µl preps: Reagent Cartridges, Disposable Tip Holders, Disposable Filter-Tips, Sample Tubes, Elution Tubes	951034
EZ1 DNA Blood Card	Preprogrammed card for BioRobot EZ1 DNA Blood 200 µl and 350 µl Protocols	9015585
QIAamp UltraSens Virus Kit (50)†	For 50 viral nucleic acid preps: 50 QIAamp Mini Spin Columns, Proteinase K, Carrier RNA, Collection Tubes (2 ml), Buffers	53704
TissueLyser	Universal laboratory mixer-mill disruptor	85210‡ 85200§ 85220¶
QuantiTect Probe PCR Kit (200)†	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe PCR Master Mix, 2 x 2.0 ml RNase-free water	204343
QuantiTect SYBR Green PCR Kit (200)†	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green PCR Master Mix, 2 x 2.0 ml RNase-free water	204143 203203
QuantiTect SYBR Green RT-PCR Kit (200)†	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green RT-PCR Master Mix, 100 µl QuantiTect RT Mix, 2 x 2.0 ml RNase-free water	204243

* Warranty PLUS 2 (cat. no. 9237720) recommended: 3-year warranty, 1 preventive maintenance visit per year, 48-hour priority response, all labor, travel, and parts.

† Larger kit sizes available; please inquire.

‡ US and Canada.

§ Japan.

¶ Rest of the world.

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