Automated extraction of viral RNA from blood samples for veterinary diagnostics using the JANUS Automated Workstation

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Abstract

The performance of the JANUS® Automated Workstation as a high throughput nucleic acid extraction platform was analyzed using bluetongue virus (BTV)-positive blood samples. The sensitivity of the automated extraction protocol was assessed using a tenfold dilution series and was nearly identical to the manual extraction protocol (mean difference: 0.88; 95% CI 0,65-1,12; P<0,0001). The variability of the automated protocol was very low with a total coefficient of variation ranging from 1.47 to 8.96%. A comparative analysis of 42 field samples indicated that the manual and automated extraction protocol can be used interchangeably. No cross contamination was observed when extracting 48 negative samples interspersed with 48 strongly positive samples. The automated extraction protocol is very fast allowing the extraction of 92 samples in approximately 2 hours. The results described in this application note therefore clearly demonstrate that the JANUS Automated Workstation offers a user friendly and versatile platform for the high throughput extraction of nucleic acids from whole blood samples.

Introduction

Bluetongue (BT) is an insect-borne disease of domestic as well as wild ruminants that induces variable clinical signs depending on the species and the breed (MacLachlan, 1994; Verwoerd, 2004). The disease is caused by the bluetongue virus (BTV) which is the type species of the genus *Orbiviruses* within the family *Reoviridae* (Mertens et al., 2004). In the summer of 2006, BTV emerged for the first time in North-Western Europe and quickly became disseminated over the Netherlands, Belgium, Germany, Luxembourg, Switzerland, half of France, and the South-East of the UK (Toussaint et al., 2006; Wilson et al, 2007).

Since the introduction of BTV is associated with substantial economic losses, the disease has to be strictly monitored by sensitive, reproducible and robust methods. Although the World Organization for Animal Health (Office international des epizooties, OIE) acknowledges various tests, conventional or real-time RT-PCR (RT-qPCR) analysis has become the method of choice to determine the presence of viruses. Prior to PCR analysis, the viral nucleic acids need to be extracted from the sample. Due to their speed, efficiency and ease of automation most laboratories nowadays use silica-based purification methods that are commercially available from a variety of sources.

This application note describes a high throughput silica-based protocol for the extraction of nucleic acids from whole blood samples using a JANUS Automated Workstation (PerkinElmer). The research reported in this application note was conducted at the Veterinary and Agrochemical Research Centre (VAR) in Belgium. The VAR is recognized by the European Union as the Belgian Federal Reference Laboratory for the identification of the former list A pathogens of the World Organization for Animal Health (Office international des epizooties, OIE).

Materials and Methods

Instrumentation

Nucleic acids were extracted on a JANUS Automated Workstation equipped with an eight-channel pipetting arm and a gripper arm. VersaTip® PLUS adapters were installed to allow the use of washable and disposable tips in the same protocol without any user-intervention. To allow vacuum filtration steps, the PerkinElmer Vacuum Manifold (PVM) was installed which is an automation friendly manifold plus gasket that automatically self-seals and self-vents during filtration steps. It is compatible with multiple labware types, and also allows accurate positioning of filter plates. Additional hardware included a vacuum pump (VacuuBrand) and a reagent/plate heater (Inheco) which are both controlled by the WinPREP® software. The duration/intensity of the vacuum and the temperature of the heater can be easily adapted by the user (Table 2). To prevent the spread of aerosols throughout the laboratory, the complete workstation was enclosed in a biosafety cabinet (Bigneat).

Molecular analysis

Extraction Automated extractions were performed with the NucleoSpin® 8/96 Virus Core Kit (Macherey-Nagel) according to the manufacturer's instructions with slight modifications. The deck layout of the automated extraction protocol is presented in Figure 1. The various pipetting and vacuum steps are summarized in Tables 1 and 2.

Manual extractions were performed with the NucleoSpin RNA Virus kit according to the manufacturer's instructions.

RT-qPCR analysis RNA samples were analyzed with a BTV-specific RT-qPCR on the 7900HT Fast Real-Time PCR System (Applied Biosystems) according to Vandenbussche et al, (2008).

Table 1: VersaTip, Gripper and remote vacuum control make the RNA extraction fully automated with JANUS.
DT: Disposable Tips FT: Fixed Tips

Procedure	Note
Add RAV1	FT
Add Proteinase	FT
Mixing	DT
Move samples for 90°C incubation	Gripper
Add Ethanol	FT
Transfer Lysates 1	DT
Vacuum for binding	See Table 2
Transfer Lysates 2	DT
Vacuum for binding	See Table 2
Change waste plate in manifold	Gripper
Wash 1 with buffer RAW	FT
Vacuum for Wash 1	See Table 2
Wash 2 with buffer RAW	FT
Vacuum for Wash 2	See Table 2
Change waste plate in manifold	Gripper
Wash 3 with buffer RAV3	FT
Vacuum for Wash 3	See Table 2
Wash 4 with buffer RAV3	FT
Vacuum for Wash 4	See Table 2
Vacuum for drying	See Table 2
Place elution plate in manifold	Gripper
Add elution buffer	FT
Vacuum for elution 1	See Table 2
Vacuum for elution 2	See Table 2

Table 2: Vacuum settings pre-defined in the WinPREP template for the viral RNA extraction.

Vacuum Procedure	Pressure (mbar)	Time (secs)
for binding	600	300
for wash 1 – buffer RAW	600	180
for wash 2 – buffer RAW	600	180
for wash 3 – buffer RAV3	600	60
for wash 4 – buffer RAV3	600	60
for drying	400	360
for elution 1 – elution buffer	600	120
for elution 2 – elution buffer	600	60

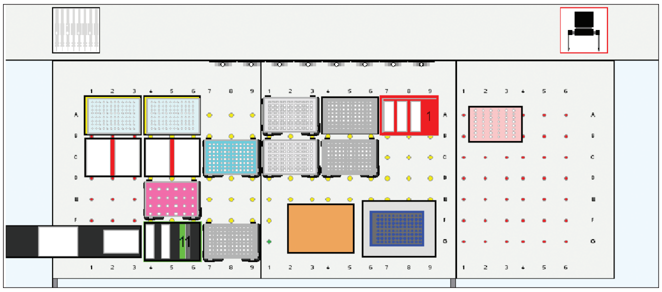

Figure 1: Graphical deck layout from the WinPREP template used for automated RNA extraction.

Samples

In order to determine the sensitivity of the automated extraction protocol, bovine blood samples were spiked with BTV-8 (strain Bel2006/01) in 8 different concentrations ranging from 0.05 to 7.05 $\log_{10}$ $TCID_{50}$ ml^{-1}. Forty-two genuine field samples from the 2007 BTV epizootic were included in the study to compare the manual and automated extraction protocols. The risk of cross contamination was assessed by extracting 48 strongly positive and 48 negative samples that were arranged in a checkerboard pattern.

Statistical analyses

All statistical analyses were carried out using the MedCalc 9.0 software. To allow statistical analysis, all negative samples were given a Ct value (i.e. the concentration of DNA molecules multiplied by time) of 45.1. Prior to analysis, the assumption of normality was assessed using the Kolmogorov-Smirnov test. A paired t-test was performed to determine whether the differences between the manual and automated extraction protocols are statistically significant. Bland-Altman plots (Bland and Altman, 1995), Passing-Bablok regression analysis (Passing and Bablok, 1983) and Pearson's correlation coefficient were used to assess the agreement and correlation between both extraction protocols. A p-value of less then 0.05 was considered statistically significant.

Results and Discussion

Sensitivity

The sensitivity was determined by analyzing a tenfold dilution series and comparing the Ct values from the manual and automated extraction protocol. As shown in Figure 2, the manual extraction method performed slightly better than the automated extraction method (mean difference: 0,88; 95% CI 0,65-1,12; P<0,0001). Although statistically significant, the observed difference is diagnostically irrelevant as it does not influence the limit of detection.

Repeatability and Reproducibility

Repeatability (intrarun variability) and reproducibility (interrun variability) were assessed by extracting 8 virus dilutions ranging from 0.05 to 7.05 $\log_{10}$ $TCID_{50}$ ml^{-1}. All dilutions were tested 6 times in each of 3 independent runs. The mean results and the variations for each dilution are summarized in Table 3. The coefficient of variation (CV) ranged from 1.47 to 8.96% indicating low variation between different repetitions and different runs. As expected, the CV increased towards the limit of detection (1.05 and 0.05 $\log_{10}$ $TCID_{50}$ ml^{-1}).

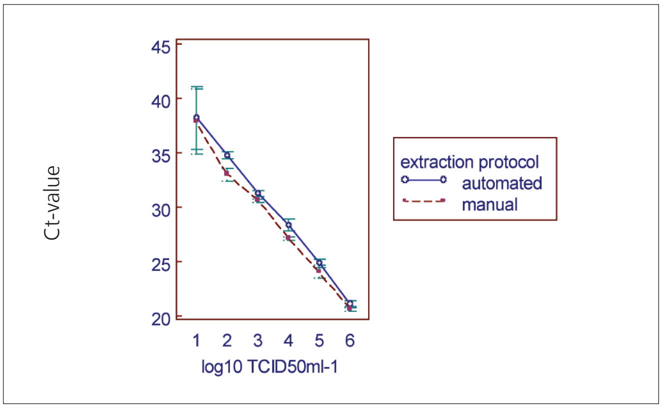

Figure 2: Ct-values obtained after manual (red) and automated (blue) extraction of blood samples spiked with different virus dilutions. The manual and automated Ct-values are the average of 3 and 6 repetitions, respectively.

Comparative analysis

A total of 42 field samples from the 2007 BTV epizootic were extracted both manually and automatically and analyzed by RT-qPCR. Based on these results a comparison was made between both extraction protocols. Pearson's coefficient showed a significant correlation between both protocols (r=0,9884; 95% CI 0,9784-0,9938; P<0,0001). Bland-Altman analysis (Figure 3A) indicated that there is no systematic bias between the differences and averages of the paired results. The differences between both extraction protocols were homogeneously distributed over the range of Ct values and 92.8% of them fell within the 95% confidence interval (±1,96 standard deviation). The mean difference between both extraction protocols was -0.3702 (95% CI: 0.6374 - -0.1031). Passing-Bablok regression analysis (Figure 3B) showed a slope of 1.0000 (95% CI: 0.9549 to 1.0477) and an intercept of 0.4200 (95% CI: 0.8377 to 1.7520) indicating an excellent agreement between both extraction protocols.

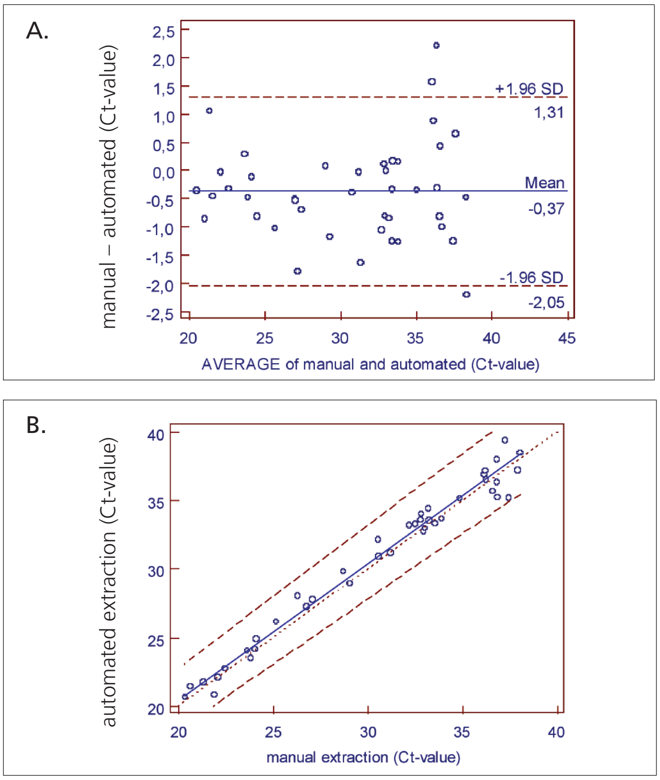

Figure 3: (A) Bland-Altman analysis of the Ct-values obtained after the manual and automated extraction protocol on 42 field samples. For each sample, the difference between the Ct values is plotted against the average of the manual and automated Ct-values of each paired result.

(B) Passing-Bablock regression analysis of the Ct-values obtained after the manual and automated extraction protocol. The solid line indicates the regression line with the confidence interval marked by the dashed lines. The dotted line represents the identity line (x=y).

Cross contamination

A low risk of cross contamination is of the utmost importance to diagnostic laboratories which handle samples with both high and low viral loads. To validate the absence of cross contamination, 48 strongly positive and 48 negative samples were arranged in a checkerboard pattern. After automated extraction, no viral RNA could be detected in any of the 48 negative samples.

Throughput

The number of blood samples that can be purified manually is limited to 24 due to the capacity of our microcentrifuge. As the manual procedure requires several centrifugation steps, the purification of a single batch of 24 samples takes 2 hours. The automated purification of nucleic acids from 92 blood samples takes approximately 2 hours. The estimated set-up time for the JANUS Automated Workstation varies between 15 and 30 minutes depending on the format of the received blood samples (individual tubes versus micronic plate).

The JANUS Automated Workstation allows the automation of nucleic acid extractions from whole blood samples for BTV diagnosis. The automated extraction protocol described in this application note exhibits nearly the same sensitivity as the manual extraction protocol but enables the user to process 92 samples in approximately 2 hours. The automated protocol is highly repeatable/reproducible and not prone to cross-contamination. Finally, a comparative analysis of the manual and automated extraction protocol indicates that both protocols give essentially the same result and can therefore be used interchangeably.

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			Results (Ct-value)		
Samples ($\log_{10}$ $TCID_{50}$ ml^{-1})	**Mean**	**Intrarun SD**	**interrun SD**	**Total SD**	**Total C V (%)**
7.05	17.89	0.53	0.73	0.80	4.47
6.05	21.66	0.53	0.62	0.76	3.50
5.05	24.96	0.40	0.74	0.73	2.94
4.05	28.18	0.46	0.42	0.56	1.99
3.05	31.47	0.40	0.33	0.46	1.47
2.05	34.97	0.50	0.77	0.81	2.31
1.05	39.82	3.53	1.36	3.57	8.96
0.05	43.46	2.06	1.93	3.17	7.29

Table 3: Intra- and interrun variation of the automated extraction protocol.

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