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TITLE: Ribosomal RNA depletion or exclusion has negligible effect on the detection of viruses in a pan viral microarray

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1 The effect of ribosomal RNA depletion or exclusion on the detection of viruses in a pan
2 viral microarray
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**Abstract**

Pan viral DNA microarrays, which can detect known, novel and multiple viral infections, are major laboratory assets contributing to the control of infectious diseases. The large quantity of ribosomal RNA (rRNA) found in tissue samples is thought to be a major factor contributing to the comparatively lower sensitivity of detecting RNA viruses, as a sequence-independent PCR is used to amplify unknown samples for microarray analysis. This study aimed to determine whether depletion or exclusion of rRNA can improve microarray detection and simplify its analysis. The use of two different rRNA depletion and exclusion protocols, RiboMinus™ technology and non-rRNA binding hexanucleotides, was compared with the standard global nucleic acid amplification protocol. This study concludes that the two procedures, described to deplete or exclude rRNA, have little effect on the microarrays detection and analysis and might only in combination with further techniques result in a significant enhancement of sensitivity. Currently, existing protocols of random amplification and background adjustment are pertinent for the purpose of sample processing for microarray analysis.

**Keywords**

Microarray, RiboMinus™, Hexanucleotide, Ribosomal RNA depletion, virus discovery

## 1.0 Introduction

The control of viral diseases is reliant on identifying the causative agent in order to help devise and implement appropriate control measures. Virus identification is less challenging when probing known viral diseases, in particular, those with characteristic clinical signs. However, some viral diseases are not pathognomonic and therefore challenging to diagnose as is the case when unknown or new viruses are involved. Most detection assays are dependent on the availability of antibodies, antigens or sequence information, requirements, which are often lacking when investigating novel or divergent pathogens.

Infectious diseases are continuously emerging in new species and geographical locations due to factors such as pathogen mutations, genetic reassortment, animal and human movement and climate change. In this regard, RNA viruses are of particular concern, as they mutate at a higher rate than DNA viruses and lack proofreading enzymes to prevent errors during RNA replication (Holland et al., 1982). The constant threat of new infectious diseases reiterates the need for rapid and multiplex detection assays such as microarrays, which can probe thousands of viruses simultaneously. In addition, these assays have the potential to detect viruses closely related to known viral pathogens and viral co-infections. Microarrays, however, do not come without challenges.

Ribosomal RNA (rRNA) is estimated to make up 80 % of total cellular RNA, being comprised mostly of 28S and 18S rRNA species in mammals (de Vries et al., 2011). The necessary use of sequence independent amplification to process tissues from unknown viral diseases results in co-amplification of host along with viral nucleic acids. This non-specific amplification is also thought to complicate the interpretation of readout values due to cross hybridisation. The rRNA may also compete with viral RNA amplification and instigate lower detection sensitivity. Ribosomal RNA depletion methods, such as RiboMinus™ technology (Life Technologies), were stated to improve microarray analysis (Gilbert et al., 2010, Kang et al., 2011). In the RiboMinus™ protocol, rRNA molecules are depleted from total RNA using biotin labelled oligonucleotide probes, which hybridize to 18S, 28S, 5.8S and 5S rRNA before being removed with streptavidin-coated magnetic beads. The procedure has been found to reduce large rRNA by 80 % (Gilbert et al., 2010).

Endoh and colleagues (2005) have used a mix of 96 non rRNA binding hexamers, screened from 4096 hexamers, to exclude rRNA molecules from amplification. The procedure was claimed to decrease the amount of non specific amplification and enhance the sensitivity

of a virus discovery assay. The 96 hexamers were also shown to reduce rRNA amplification by >90% and to improve sensitivity of high throughput sequencing (de Vries et al., 2011). The hexamers also increased microarray specificity and simplified its analytical process (Kang et al., 2011). These studies, however, only looked at viruses isolated in cell culture and nasal swabs.

The analytical process of microarrays is also complex and the interpretation of the output files is likely to be challenging (Kang et al., 2011) although bioinformatic tools have already been developed to improve and simplify data analysis, such as the DetectiV software (Watson et al., 2007). Some studies have looked at optimising sample processing, such as, reducing genomic DNA and optimising hybridisation conditions (Kang et al., 2011, Han et al., 2006). Although these steps have improved microarray detection rate significantly, cross hybridisation and a comparatively low sensitivity still remain problematic when testing tissue samples. This study therefore compared the application of rRNA depletion or exclusion methods using tissue samples to investigate if either method can improve microarray detection of RNA viruses and simplify microarray analysis in comparison with the standard random priming protocol.

96 **2.0 Materials and methods**

97

98 *2.1 Samples and nucleic acid extraction*

99 A selection of virus positive tissue samples were used in this study (Table 1).
100 Tissue samples were homogenised and RNA was extracted using QIAamp® Viral RNA
101 Mini kit (Qiagen) for tonsil samples or TRIzol (Life Technologies) for brain samples
102 following the manufacturers' protocols. Nucleic acid was quantified using Nanodrop 2000
103 spectrometer (Agilent Technologies) and diluted to a concentration of 4 μg in 32 μl of
104 nuclease free water, from which three aliquots of 8 μl were subjected to DNase digest
105 using amplification grade DNase I (Life Technologies). Briefly, 1 μl of 10x DNase buffer
106 and 1 μl of DNase I enzyme (1 units/μl) were added to each 8 μl nucleic acid extract and
107 incubated at 37 °C for 30 minutes. 1 μl of 25 mM EDTA was then added to the mix and
108 incubated at 65 °C for 10 minutes to inactivate the DNase I enzyme.

109

110 *2.2 Depletion of rRNA using RiboMinus™ Technology*

111

112 Ribosomal RNA was removed from one of the DNase digested aliquots of nucleic
113 acid using the Ribominus™ Eukaryote Kit for RNA-Seq (Life Technologies) according to
114 the manufacturer's protocol. Briefly, for each viral extract, nucleic acid was added to 10
115 μl of RiboMinus™ probe (15 pmol/μl) and 100 μl of hybridization buffer and incubated
116 at 70-75 °C for 5 minutes. The sample was transferred to prepared RiboMinus™ Magnetic
117 beads and incubated at 37 °C for 15 minutes. The beads were separated using a magnet
118 leaving RiboMinus™ RNA (target RNA) in the supernatant, which was transferred into
119 fresh RiboMinus™ beads. The sample was incubated at 15 °C for 15 minutes and the beads
120 were separated. The nucleic acid was finally precipitated with ethanol and re-suspended
121 in 11 μl of water before being converted into cDNA using the random priming protocol
122 (section 2.3.1).

123

124 *2.3 cDNA synthesis*

125

126 *2.3.1 Using random primer A (random priming)*

127 The tailed primer A, 5' GTT TCC CAG TCA CGA TAN NNN NNN NN 3', referred to
128 hereafter as primer A, was used to generate random cDNA from the rRNA depleted nucleic
129 acid, from the Ribominus™ protocol, and the second aliquot of nucleic acid as described
130 by Wang et al., (2002). Briefly, 1 μl of 12.5 mM dNTP (Promega) and 1 μl of 40 μM primer
131 A were added to each of the two aliquots. The mixtures were then heated at 95 $^{o}$C for 5

132 minutes and chilled on ice immediately. 4 μl 5 x RT buffer (Life Technologies), 1 μl 0.1M
133 DTT, 1 μl RNasin Ribonuclease inhibitor (20-40 u/μl, Promega) and 1 μl Superscript III
134 (200 u/μl) (Life Technologies) were then added and the mix was incubated at 25 °C for 5
135 minutes, 42 °C for 60 minutes and 70 °C for 15 minutes to stop the reaction.
136
137 *2.3.2 Using the 50 hexamers*
138 The third aliquot of nucleic acid was converted into cDNA using the 50 hexamers
139 (section 3.1) and following the random priming protocol, but replacing the primer A with
140 the 50 hexamers at a concentration of 80 μM.
141
142 2.4 *Second strand DNA synthesis and nucleic acid amplification*
143 This was carried out as described by Wang et al., 2002 with minor modification
144 for the cDNA generated using the 50 hexamers. Briefly, cDNA was denatured at 94 °C for
145 2 minutes and cooled to 10 °C before adding Sequenase enzyme mix [2 μl 5x Sequenase
146 buffer (Affymetrix), 0.3 μl Sequenase DNA polymerase and 7.7 μl water]. This mix for the
147 cDNA generated with the 50 hexamers had an additional 1 μl of 40 μM primer A. The
148 reactions were heated from 10 °C to 37 °C over an 8 minute period using a Veriti
149 thermocycler (Life Technologies) and held at 37 °C for another 8 minutes before being
150 terminated at 94 °C for 2 minutes. Amplification of the double-stranded DNA (dsDNA)
151 was performed using a mix containing 5 μl 10x KlenTaq PCR buffer (Sigma Aldrich), 1 μl
152 12.5 mM dNTP mix, 1 μl 100 μM primer amino-B (amino-C6 5' GTT TCC CAG TCA CGA TA
153 3'), 0.5 μl KlenTaq® LA DNA polymerase (5 units/μl), 5 μl of template and water to a total
154 volume of 50 μl. The thermal profile used was 94 °C for 4 minutes, 68 °C for 5 min then
155 35 cycles of 94 °C for 30 seconds, 50 °C for 1 min, 68 °C for 1 min and a final extension of
156 68 °C for 2 minutes. The amplified PCR products were run on a 2% agarose gel with SYBR®
157 safe DNA gel stain (Life Technologies) and visualised on a trans UV illuminator (Bio Rad),
158 which should show a smear between 200 and 1000 bp (Chen et al., 2011).
159
160 *2.5 Labelling DNA with fluorescent dye*
161 Indirect labelling of the amplified DNA templates (5 μl) was performed using 15-
162 20 cycles of PCR which incorporates amino allyl dUTP (Life Technologies) into the
163 reaction (Gurrala et al., 2009). The labelled products were purified using the MinElute
164 PCR purification Kit (Qiagen) following the manufacturer's protocol, substituting the
165 wash buffer with 75 % ethanol and eluting the sample in 13 μl of water. The fluorescent
166 dye was coupled to the amino allyl labelled PCR product by adding 6 μl of Sodium

167 Bicarbonate (25 mg in 1 ml of water) and 4 µl of Alexa Fluor® 647 Reactive Dye (Life
168 Technologies), reconstituted in 18 µl of DMSO, to the eluted DNA, vortexing and
169 incubating at room temperature in the dark for up to two hours. The unincorporated dye
170 was removed using the illustra™ AutoSeq™ G-50 Dye terminator removal Kit (GE
171 Healthcare), according to the manufacturer's protocol. The labelled DNA was quantified
172 on the Nanodrop 2000 spectrophotometer (Agilent Technologies).
173
174 *2.6 Microarray hybridization, slide washing, scanning and data analysis*
175 The pan-viral microarray chip used in this study contained 47,000 probes (60
176 mers) to around 2,500 virus species. The chip was printed by Agilent Technologies in an
177 8 x 60K format so that 8 samples could be processed simultaneously.
178 The hybridization mix was composed of 2.5 µl Cot-1 DNA (1.0 mg/ml, Kreatech
179 Diagnostics), 5 µl Agilent 10X blocking agent, 25 µl Agilent 2X hi-RPM buffer and 17.5 µl
180 of the labelled product. The mix was heated on a thermocycler at 95 °C for 3 minutes and
181 37 °C for 30 minutes before being applied onto the gasket slide. A microarray slide was
182 then lowered onto the gasket slide and secured inside an Agilent hybridization chamber.
183 The chamber was placed into a pre-heated rotating hybridization oven (Agilent
184 Technologies) at 65 °C and set to rotate at 10 rpm overnight. The slide, whilst attached to
185 the gasket slide, was submerged into room temperature Agilent Oligo aCGH/Chip-on-chip
186 wash buffer 1 (Agilent Technologies) to remove the gasket slide. The microarray slide was
187 then transferred into a fresh jar of the buffer and stirred using a magnetic stirrer for 5
188 minutes. The slide was subsequently transferred into pre-warmed 37 °C buffer 2 and
189 stirred for another 1 minute before being scanned. The slide was scanned on a microarray
190 Agilent C scanner with 2 micron resolution as instructed by the manufacturer. The output
191 file from the Feature Extraction software of the scanner was analysed using DetectiV
192 software in R (http://www.R-project.org) (Watson et al., 2007), using data from an
193 unrelated experiment to correct for the background noise. Results were compared based
194 on whether the correct virus was identified within the top virus hits when using the p-
195 value and/or average of normalised signal intensities.
196
197 *2.7 Quantitative PCR (qPCR)*
198 The virus specific real time PCR mix for all viruses except rabies virus (RV) was
199 composed of 1 X QuantiTect Virus + ROX Vial Kit (Qiagen), forward and reverse primers
200 at a final concentration of 0.4 mM and virus specific TaqMan probe at a final concentration
201 of 0.2 mM, 1X ROX, 3 µl of template DNA and water to total a volume of 20 µl ( McGoldrick
202 et al., 1998; Lanciotti et al., 2000; Marriott et al., 2006; Bilk et al., 2012) The thermal profile

203 used was 95 °C for 5 minutes and 45 cycles of 95 °C for 15 seconds, 60 °C for 45 seconds.
204 The 18S rRNA real time PCR was performed using 0.6 μl 18S rRNA primers/probe mix
205 (Life Technologies), the QuantiTect Virus + ROX Vial Kit as described above and 2 μl
206 template DNA. For RV, 10 μl Brilliant® II SYBR® Green QPCR with low ROX master mix
207 (Agilent Technologies) was used with JW12 & N165-146 primers, each totalling a final
208 concentration of 1 mM, 3 μl template DNA and water to a final volume of 20 μl (Wakeley
209 et al., 2005). The thermal profile used was 94 °C for 2 minutes, 45 cycles of 95 °C for 1
210 minute, 55 °C for 30 seconds and 72 °C for 20 seconds. Each sample was tested in
211 duplicate and a no-template control (NTC) was also included in each run to check for cross
212 contamination and background noise.
213
214

## 3.0 Results

### *3.1 Selection and assessment of the non-rRNA binding hexanucleotides*

The 50 hexamers used in this study (Supplement 1) were selected from a list of 96 hexamers described by Endoh et al., 2005 using a mathematical script to blast the 96 hexamers against additional sequences, equine 18S rRNA, porcine 18S rRNA, bovine 18S & 28S rRNA, and human 18S & 28S rRNA sequences (accession numbers AJ311673, AY265350.1, DQ222453 and U13369 respectively). This was to exclude further hexamers which share identical DNA sequences with these rRNAs. The ensuing 50 hexamers were then mapped to genome sequences of several viruses of human and animal importance (Table 2). This was to assess the number of binding sites of the 50 hexamers on the viral genomes and also to measure the nucleotide distance between the binding sites. Among the viruses used, Louping Ill virus (LIV) genome had the lowest binding sites and also the largest distance between the binding sites, prompting us to include this virus in the study to evaluate the efficiency of the 50 hexamers in generating cDNA.

Furthermore, in order to assess whether the 50 hexamers performance could be influenced by low viral load, serial 10-fold dilutions of a Classical Swine Fever virus (CSFV) positive nucleic acid were made in nucleic acid derived from a virus negative tissue. The nucleic acid dilutions were then subjected to cDNA synthesis using the 50 hexamers or random priming followed by CSFV qPCR to quantify the virus-specific amplicons. The Ct values, obtained by the qPCR for each of the CSFV nucleic acid dilutions using either of the two protocols, were comparable with only minor differences. The average CSFV Ct values for random priming were 25.3, 24.2, 27.9, 31.2 compared to 26.6, 24.1, 27.1 and 30.8 for the 50 hexamers using neat, $10^{-1}$, $10^{-2}$ and $10^{-3}$ CSFV dilutions respectively.

### *3.2 Visual comparison of gel electrophoresis images of amplicons*

The three methods, the 50 hexamers, RiboMinus™, and random priming, produced PCR amplicons of the expected size range, with smears of 200 to 1000 bp, on agarose gel electrophoresis (data not shown) using CSFV, LIV, West Nile virus (WNV) and RV positive nucleic acids as targets. The 50 hexamers were found to be just as efficient as the random priming in producing cDNA, even for viruses with few binding sites for the hexamers, such as LIV. In addition, the use of hexamers resulted in the most evenly spread DNA smears, indicating an arbitrary amplification of total nucleic acid.

250 *3.3 Relative qPCR of cDNA and PCR amplicons for virus specific product and rRNA*
251 Virus specific qPCRs were carried out to quantify the amount of virus amplicons
252 obtained for each virus using the 50 hexamer, RiboMinus™ and random priming
253 protocols (Table 3). Considering the Ct values, there appears to be no pattern to suggest
254 that any of the three protocols are contributing to a higher sensitivity in amplifying viral
255 nucleic acid extracted from tissues samples. The removal or exclusion of rRNA from virus
256 positive tissues was also quantified by an 18S rRNA qPCR using cDNA, generated with the
257 50 hexamers, RiboMinus™ and random priming protocols, as template (Table 4). The
258 cDNAs generated with the 50 hexamers showed marginal effect whilst the RiboMinus™
259 protocol demonstrated a clear decrease in rRNA, compared to those cDNAs generated
260 with random priming protocol.
261
262 *3.4 Analysis of virus amplicons generated from the three protocols by microarray*
263 The effect of removing rRNA on microarray specificity, sensitivity and ease of data
264 interpretation was assessed by analysing microarray outputs from the three protocols
265 and seven known positive virus samples. Averages and p-values of probes' fluorescent
266 intensity from each virus, calculated by the DetectiV software, were considered in the
267 interpretation of microarray outputs. The 50 hexamers, RiboMinus™ and random
268 priming had 86 %, 71 % and 86 % detection rates respectively if p values of probes signal
269 intensity were considered for virus identification. The only difference was in the detection
270 of low CSFV where the RiboMinus™ protocol used for sample processing. The detection
271 rate was; however, 100 % for the three protocols when the averages of signal intensity of
272 virus probes were interrogated to identify the target virus.
273 To investigate whether depletion or exclusion of rRNA could reduce cross
274 hybridization of the target virus nucleic acid with unrelated probes on the microarray, the
275 proportion of variance between average of probes fluorescent intensity for the top virus
276 hit and those of 19 unrelated viruses was visualised using Scree plots (Fig. 1). Visually,
277 there was no difference in the reduction of cross hybridisation frequency among the three
278 methods; however this is subject to an individual's interpretation of results. The three
279 sample processing protocols showed a large difference in the average frequency from the
280 target virus to the unrelated virus hits enabling a clear identification of target virus.
281

282 **4.0 Discussion**
283
284 Microarray has proven to be a successful tool in detecting novel viruses and viral
285 co-infections establishing itself as a front-line diagnostic tool for investigation of
286 emerging infectious diseases. Enhancing assay's performance and thereby simplifying
287 interpretation of its output is therefore critical for its use in routine diagnostic testing.
288 Improvements have already been made in the analytical process by using statistical
289 software, such as DetectiV (Watson et al., 2007), to enable an easier analysis and
290 interrogation of microarray outputs. Many groups have also attempted to improve sample
291 preparation (Han et al., 2006, Nicholson et al., 2011, Kang et al., 2011) whilst others
292 worked on depleting rRNA from extracted nucleic acid for the same purpose (Kang et al.,
293 2011, Gilbert et al., 2010, Endoh et al., 2005). This study looked at implementing two
294 different rRNA depletion or exclusion methods to assess whether an improvement to
295 microarray detection of RNA viruses from tissue samples could be made. RiboMinus™
296 technology and non-rRNA binding hexamers were the two methods used in this study and
297 compared with the in-use random priming method. The 50 hexamers were selected from
298 the originally described 96 hexamers (Endoh et al., 2005) to further reduce rRNA binding
299 hexamers in order to increase their selectivity towards viral RNAs.
300 The 50 hexamers did not hamper amplification of virus nucleic acid from tissue
301 samples, even from those samples with low viral load. However, only a small decrease in
302 18S rRNA load could be achieved by the hexamers with no repercussion on microarray
303 detection. On the contrary, de Vries at al (2011) found non rRNA binding hexamers
304 reducing 28S rRNA amplification by up to 100 fold depending on the region of the rRNA
305 genome used for quantification. The work, however, was carried out using only
306 nasopharyngeal swabs which have trivial amounts of cell contamination in comparison to
307 tissue samples. Furthermore, as a commercial primers-probe mix was used in the study,
308 it was not possible to verify which region of 28S or 18S rRNA genome was targeted by the
309 PCR for comparison.
310 RiboMinus™ technology was successful in removing rRNA to a large extent, as
311 also indicated by other researchers (Gilbert et al., 2010). However, no difference in
312 microarray detection was observed compared to the random priming protocol, especially
313 when applied to deplete rRNA from samples with a low viral load. This may be due to the
314 length of nucleic acid handling time and several steps of separations and washes in the
315 RiboMinus™ protocol, causing degradation and poor recovery of viral nucleic acid.
316 Therefore, the protocol may have removed rRNA effectively, but in the mean time
317 adversely affected viral nucleic acid integrity. In addition, RiboMinus™ Technology is

318 comparatively expensive and time consuming, which restricts its application where a high
319 throughput testing is sought. For these reasons, it would be unrealistic to justify
320 implementing RiboMinus™ Technology, unless it was highly effective at improving a test,
321 which we cannot confirm for microarray.
322 On the whole, the microarray results for all three methods showed no difference
323 in the overall detection rates and the amount of cross hybridisation seen. Kang at al,
324 (2011) found that non-ribosomal hexanucleotides had improved the microarrays
325 specificity. However, all the samples used in the evaluation were virus isolates, which
326 often contain a small amount of cellular contamination and therefore cannot represent
327 tissue samples used for microarray analysis in its intended clinical application. In
328 addition, no parallel comparison was made, using random priming, to verify their
329 findings. The script used to run the DetectiV software may also have been a major
330 contributing factor for equal performance of the three protocols seen in this study,
331 especially when analysing cross hybridisation. The software employs a script with
332 instructions to subtract florescence data of an unrelated sample from those of an in-test
333 sample. This background adjustment has already minimised the effect of cross
334 hybridisation signals and may have contributed to the comparable outcome for the three
335 protocols. The importance of using signal averages in addition to p-values in microarray
336 analysis was also identified, as the detection rate was found to be lower when using the
337 p-value only. The original work describing the DetectiV software (Watson et al, 2007) has
338 relied only on the p values to analyse microarray outputs. Finally, tissue samples from
339 experimental conditions, which harbour a higher load of rRNA, compared to cell culture
340 isolates, may have rendered a lower efficiency for the rRNA exclusion of the 50 hexamers.
341 The short length of hexamers and consequently non specific binding may also contribute
342 to the findings of this study, therefore longer oligonucleotides e.g. nonamers may be more
343 applicable.
344 In conclusion, this study identified that the two rRNA depletion or exclusion
345 protocols have no significant effect on microarray detection or reduction in cross
346 hybridisation. Accordingly, the current random amplification and background adjustment
347 protocols are pertinent for the purpose of investigating novel and emerging diseases via
348 microarray analysis whilst the findings also emphasize the importance of selecting the
349 most appropriate samples for analysis. Protocols suggesting improvement for nucleic acid
350 preparation should also include tissue samples on validation if intended for diagnostic
351 purposes.
352

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**Table 1**
List of viruses used in this study

| Virus | Genus | Strain | Original host | Country of origin | Tissue type |
|---|---|---|---|---|---|
| CSFV | Pestivirus | CBR/93 | Porcine | Thailand | Porcine tonsil |
| SBV | Orthobunyavirus | NA | Ovine | England | Ovine brain |
| RV | Lyssavirus | CVS 11 | Laboratory adapted | | Murine brain |
| RV | Lyssavirus | 404 | Mongoose | South Africa | Murine brain |
| WNV | Flavivirus | DAKAR | unknown | Africa | Murine brain |
| WNV | Flavivirus | NY99 | unknown | USA | Murine brain |
| LIV | Flavivirus | LI 3/1-Arb 126 | Ovine | Scotland | Murine brain |

CSFV, Classical Swine Fever Virus; SBV, Schmallenberg Virus; RV, Classical Rabies Virus; WNV, West Nile Virus; LIV, Louping ill Virus.

**Supplement 1**
The 50 non-rRNA binding hexanucleotides used in this study

| | | | | |
|---|---|---|---|---|
| GATATC | CGGTTA | TATAGC | GTACTA | GCGATA |
| TAGTAT | ATTACG | CTTGTA | TCGATA | CAATAT |
| TATAGT | AGTATC | CTATAG | GTACCA | GTGCTA |
| TATATA | TGTTAA | TAGCTA | GTATCA | CGACAT |
| ATATAT | ACTATT | TACTAG | ACATTA | GCTATA |
| ACTATA | TAACCG | AGTAGT | ATATTG | ATGTTA |
| CGTAAT | CGATAT | GTCTAC | CGTCTA | TGGTAT |
| CTATAC | GTATAC | TACAAG | CTTACA | GGATAT |
| TATGCG | TAGCAC | TACCAG | CGCTTA | TTACTA |
| GATACT | ATATCG | ATAGTA | CTCATA | ACTCGT |

**Table 2**
The 50 hexamers binding capabilities towards several viral genome sequences

| Virus name | Accession No. | No. of binding sites in the genome | Max. distance between binding sites (nucleotides) |
|---|---|---|---|
| Louping ill virus | Y07863.1 | 38 | 1553 |
| Rabies virus, strain CVS 11 | GQ918139.1 | 111 | 949 |
| West Nile virus, strain NY99 | NC_009942 | 60 | 825 |
| Schmallenberg virus | HE649912, HE649913, HE649914 | 243 | 506 |
| Bovine respiratory coronavirus | FJ938066.1 | 255 | 430 |
| Classical swine fever virus, strain Eystrup | AF326963.1 | 149 | 442 |
| Border disease virus, strain X818 | AF037405.1 | 165 | 523 |
| Bovine viral diarrhoea virus, strain 1-NADL | M31182.1 | 163 | 522 |
| Equine arteritis virus | X53459.3 | 105 | 696 |
| Porcine reproductive and respiratory syndrome virus | AF046869.1 | 79 | 911 |

**Table 3**
Virus specific qPCR of the PCR amplicons generated using the 50 hexamers, RiboMinus™ and random priming protocols.

| Sample | Average cycle threshold (Ct) value | | |
|---|---|---|---|
| | The 50 hexamers | RiboMinus™ technology | Random priming |
| CSFV (low virus load) | 23.33 | 36.75 | 23.57 |
| CSFV (high virus load) | 10.59 | 11.32 | 16.49 |
| RV (CVS 11 strain) | 26.46 | 26.12 | 26.81 |
| RV (RV404 strain) | 26.38 | 27.33 | 25.26 |
| WNV (DAKAR strain) | 11.81 | 12.09 | 11.52 |
| WNV (NY99 strain) | 13.38 | 12.62 | 12.83 |
| LIV | 24.09 | 23.37 | 22.8 |
| NTC | No Ct | No Ct | No Ct |

CSFV, Classical swine fever virus; RV, Classical rabies virus; SBV, Schmallenberg virus; WNV, West Nile virus; LIV, Louping ill virus; NTC, no template control

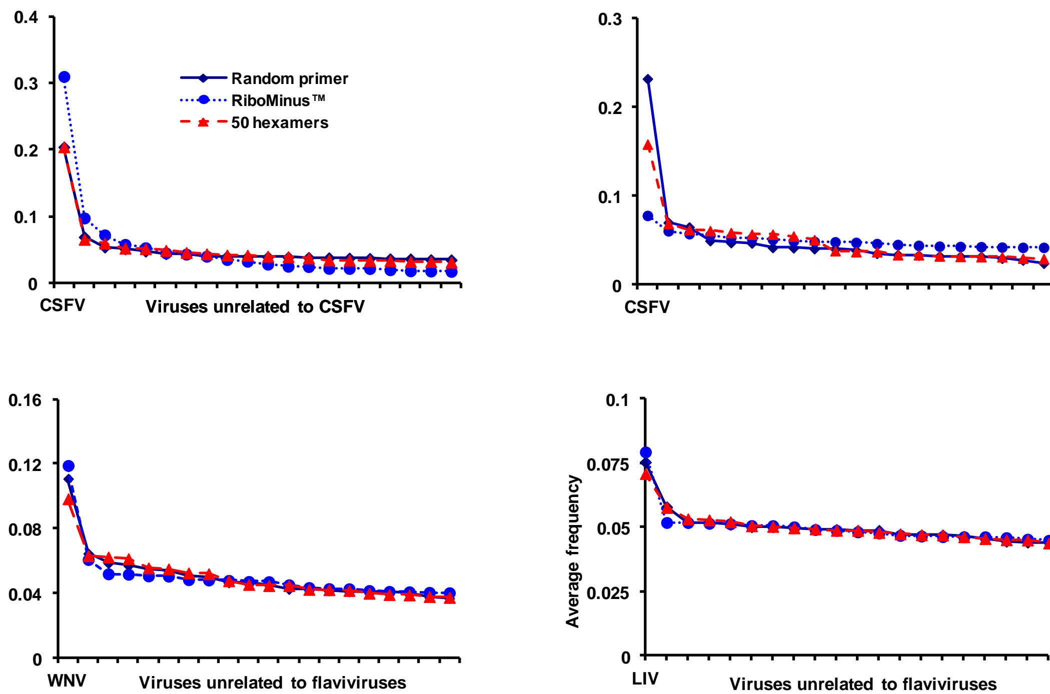
**Table 4**
Quantitative PCR of 18S rRNA in cDNAs generated using the 50 hexamers, RiboMinus™ and random priming protocols.

| Sample | Cycle threshold (Ct) value | | |
|---|---|---|---|
| | 50 hexamers | RiboMinus™ technology | Random priming |
| CSFV (low virus load) | 19.41 | 24.96 | 19.99 |
| CSFV (high virus load) | 19.73 | 22.9 | 20.54 |
| RV (CVS 11 strain) | 14.77 | 20.16 | 13.75 |
| RV (RV404 strain) | 14.53 | 19.27 | 12.05 |
| WNV (DAKAR strain) | 13.53 | 19.7 | 12.75 |
| WNV (NY99 strain) | 14.12 | 19.84 | 12.03 |
| LIV | 13.49 | 17.66 | 12.94 |
| NTC | No Ct | No Ct | No Ct |

CSFV, Classical swine fever virus; RV, Classical rabies virus; SBV, Schmallenberg virus; WNV, West Nile virus; LIV, Louping ill virus; NTC, no template control

**Fig 1**

Microarray analysis of PCR amplicons generated using the 50 hexamers, RiboMinus™ and random priming protocols.




The top 20 viruses with the highest normalised average of probe fluorescent intensities were considered for analysis. The frequency of the average for each of the top 20 viruses was calculated by dividing each average by the sum of all averages.