

1 Comparison of different extraction kits to isolate 2 microRNA from *Galleria mellonella* (wax moth) 3 larvae infected with *Metarhizium brunneum* 4 (ARSEF4556).

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10 **ABSTRACT:** MicroRNAs (miRNAs) play an important role in regulating gene expression and are
11 involved in developmental processes in animals, plants and fungi. To understand the role of
12 miRNAs in a biological system, it is important to optimise the extraction procedures to obtain high
13 quality and quantity nucleic acid that enable high throughput sequencing and expression analysis.
14 Numerous kit-based miRNA extraction protocols have been optimised generally to single cell or
15 tissue cultures. Fungi, however, often occupy physically and chemically complex environments
16 which miRNA make extraction challenging, such as fungal pathogens interacting within plant or
17 animal host tissue. We used a *Galleria mellonella* (wax moth) larvae and entomopathogenic fungus
18 *Metarhizium brunneum* ARSEF 4556 host/pathogen model to compare commercially available
19 miRNA extraction kits (Invitrogen PureLink™ miRNA Isolation Kit, Ambion mirVana™ miRNA
20 Isolation Kit and Norgen microRNA purification Kit). Our results showed reproducible and
21 significant differences in miRNAs extraction between the kits, with the Invitrogen PureLink™
22 miRNA Isolation protocol demonstrating the best performance in terms of miRNA quantity,
23 quality and integrity isolated from fungus-infected insect tissue.

24 **Keywords:** *Galleria mellonella*; *Metarhizium brunneum*; miRNA extraction; Invitrogen; Ambion;
25 Norgen

26 Introduction

27 Small RNA (sRNA) molecules have been increasingly recognised as significant factors
28 regulating gene expression [1]. MicroRNAs (miRNAs) are an endogenous, 22-nucleotide,
29 noncoding, single stranded RNA species that form a group of gene regulators involved in
30 developmental processes in animals, plants and fungi [1, 2]. Ensuring the isolation of good quality
31 miRNA samples is essential for downstream analysis, i.e. high throughput sequencing, with
32 challenges associated with sample handling and miRNA extraction needing to be addressed [3].
33 Errors during sample handling (such as accidental contamination during the extraction process) and
34 poor storage conditions can compound RNA quality-loss [4, 5]. As total RNA and miRNA are
35 extracted in the same way, degraded total RNA will mean low miRNA concentration in a sample [6,
36 7]. Furthermore, low concentration of total RNA in a sample makes the estimation of miRNA
37 abundance particularly difficult [8].

38 Extraction of miRNAs from samples can be technically challenging because of their small size
39 and their attachment to cellular lipids and proteins [9-11]. Earlier studies on relatively low complex
40 samples (e.g. single cell lines) have identified differences in quantity and quality of miRNA
41 extracted with different commercial kits, with some highlighting the need for protocol optimisation
42 [12, 13]. The success of commercial miRNA extraction kits on more complex systems consisting of a
43 range of tissue types and/or multiple organisms are not well described, particularly comparing
44 between treatments where samples change and deteriorate over time, e.g. host-pathogen

45 interactions. In order to obtain miRNA from fungal pathogen, both the host tissue and fungal cells
46 need to be homogenised and disrupted to release the nucleic acids.

47 *Metarhizium brunneum* ARSEF 4556 (previous name *M. anisopliae*) is a broad host range
48 entomopathogenic fungus used as a biocontrol agent that undergoes morphogenic and
49 physiological change during the infection process [14, 15]. A reproducible extraction protocol is
50 required to investigate the potential regulation by miRNAs during pathogenesis. We tested three
51 commonly used miRNA extraction protocols using a complex mixed system of *M. brunneum* against
52 the insect host *Galleria mellonella* using both healthy and infected host tissue. *G. mellonella* is
53 increasingly used as a model system to test microbial pathogenesis [16-19]. In this study, the
54 interaction of *G. mellonella* larvae with *M. brunneum* provides a general fungal pathogen system with
55 which to assess molecular protocols aimed at assessing fungi differentiating within living tissues.

56 The three protocols tested were PureLink™ miRNA Isolation Kit (Invitrogen),
57 mirVana™ miRNA Isolation Kit (Ambion) and microRNA purification Kit (Norgen). To the best of our
58 knowledge these kits have not been previously compared, and not for complex samples. We report
59 that the quantity and quality of miRNA extracted varied significantly between the different
60 extraction protocols. While extraction quality between *G. mellonella* healthy and *M.*
61 *brunneum*-invaded tissue remained constant for any given protocol, the Invitrogen PureLink™
62 provided the greatest miRNA yield and quality from our samples.

63 METHODS

64 Fungal culture

65 *M. brunneum* (ARSEF 4556) was obtained from the Swansea University culture collection and
66 grown on Sabourand dextrose agar (SDA, 40 gL⁻¹ D- glucose, 10 gL⁻¹ mycological peptone, 5 gL⁻¹
67 technical agar (Sigma,UK), 0.5 gL⁻¹ chloramphenicol) at 28 °C in the dark for 14 days to obtain the
68 conidia. The conidia were harvested by using sterile distilled water containing 0.03% v/v Tween 80
69 and the concentration determined using a haemocytometer. Conidial viability was determined over
70 a 122 hr time course using a plate count technique on SDA [20].

71 Preparation and Inoculation of *G. mellonella*

72 *G. mellonella* (Lepidoptera) were maintained at 28°C in an artificial nutrition medium (15% (v/w)
73 bee honey, 15% (w/w) wax, 15% (w/w) glycerol, 15% (w/w) fat free dry milk, and 40% (w/w) corn
74 and wheat flour. Four *G. mellonella* larvae at 5-6th stage [21] were submerged in 40 ml *M. brunneum*
75 conidia suspension (1x10⁸ conidia ml⁻¹) for 35 seconds, placed into Petri plates with moist filter paper
76 and then sealed with Parafilm® and incubated at 28°C. Control larvae were dipped into 0.03% (v/v)
77 Tween 80 for 35 seconds and all treatments were repeated in triplicate. After incubation the larvae
78 were frozen under liquid nitrogen and stored at -80 °C.

79 MicroRNA and RNA Extraction

80 Invitrogen PureLink™ miRNA Isolation Kit, Ambion mirVana™ miRNA Isolation Kit and
81 Norgen microRNA kits were used to isolate miRNA and total RNA from *G. mellonella* larvae 72 hr
82 post-infection with *M. brunneum*, uninfected *G. mellonella* larvae and *M. brunneum* SDA-grown
83 conidia (see Table 1 for kit overviews). Samples were prepared following manufacturer's guidelines.
84 Tissue, 100 mg, was used for Invitrogen PureLink™ miRNA Isolation and Ambion
85 mirVana™ miRNA Isolation kits (the whole *G. mellonella* larvae were used for both kits) and 50 mg
86 tissue was used (half larva was used) for the Norgen microRNA purification kit. All samples were
87 ground with a micropestle under liquid nitrogen and the standard protocol (frozen tissue extraction)
88 was followed for each kit. The RNA was eluted in 100 µl RNase-free water for the Invitrogen
89 PureLink™ miRNA Isolation kit, 100 µl elution buffer for the Ambion mirVana™ miRNA Isolation
90 and 50 µl for the Norgen microRNA purification kit, and stored at -80°C.

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Table 1. Overview of the miRNA and RNA isolation kits used in this study.

	Invitrogen	Ambion	Norgen
	PureLink	mirVana	MicroRNA
Molecules isolated	Total RNA inc small RNA [#]	Total RNA inc small RNA	Total RNA inc small RNA
Quantity of biomass required	100 mg	100 mg	50 mg
Isolation chemistry	Guanidine isothiocyanate Ethanol precipitation	Phenol:Chloroform Ethanol precipitation	Guanidine salt Ethanol precipitation
Column details	Silica-based membrane, 2 columns (total & smRNA extraction)	Glass fibre filter, 3 columns (total & smRNA extraction)	Resin-based membrane, 2 columns (total & smRNA extraction)
Cost per sample*	3.36 GBP	12.75 GBP	10.8 GBP
Steps in protocol	6	7	6
Protocol time per sample	15 minutes	30 minutes	30 minutes

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[#] Small RNA = microRNA, small interfering RNA, tRNA and 5S RNA.

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* Purchased 2016 in Pounds Sterling (GBP) inc. taxes.

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RNA analysis

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miRNAs and total RNA were quantified and integrity analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the Agilent Small RNA chip and RNA pico-chip kits respectively. RNA concentration and purity was also measured at 260nm and 280nm absorbance using the Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Data processing and analysis were conducted using GraphPad prism V5.0d software to compare the quantity and quality of microRNA. Molecular data sets were analyzed using two-way Analysis of Variance (ANOVA) with Tukey HSD post-test. Statistical analysis of the data was carried out in SPSS [22].

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Results

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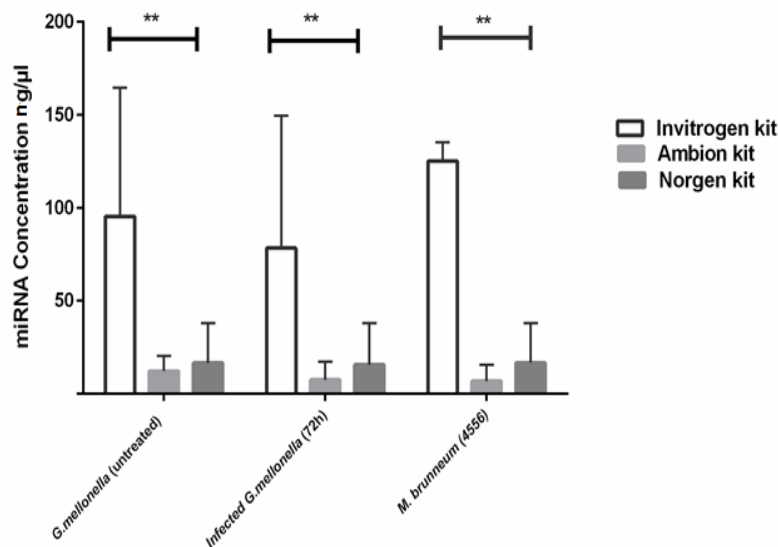
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Invitrogen, Ambion and Norgen miRNA extraction kits were successfully used to isolate total RNA including miRNA from infected *G. mellonella* larvae with *M. brunneum* (72 hrs post- incubation), uninfected larvae (72 hrs) and *M. brunneum* culture. The quantity of miRNA isolated from the most complex sample (infected *G. mellonella*) showed significantly greater yield obtained from the Invitrogen PureLink kit (146.9 ng/μl, +/-5.1) measured by the Agilent Bioanalyzer compared with the Norgen MicroRNA (2.29 ng/μl, +/-0.434) or Ambion mirVana (0.773 ng/μl, +/-0.159) kits (Table 2). Similar results were obtained from uninfected *G. mellonella* and *M. brunneum* pure culture (Figure 1). In addition, the 260:280 and 260/230 absorbance ratios obtained using the Nanodrop showed that miRNA A260:A280 purity obtained with Invitrogen kit was better at 1.97 than that obtained with Norgen (1.77) or Ambion (1.51) kits, and A260:A230 values of 0.94 Norgen, 0.89 Ambion and 1.97 Invitrogen.

116 **Table 2.** Assessment of small RNA extraction quality obtained from Invitrogen PureLink, Ambion
 117 mirVana and Norgen MicroRNA extraction kits from a complex sample of two interacting species:
 118 *Galleria mellonella* infected with *M. brunneum* for 72 hr were prepared in triplicate and values are
 119 presented as mean SD (range).

	Invitrogen kit	Ambion kit	Norgen kit
Small RNA concentration [ng/μl]	117.1 (+/-18.2)	1.6 (+/-0.0036)	8.0 (+/-3.37)
miRNA concentration [ng/μl]	146.9 (+/- 5.1)	0.773 (+/-0.159)	2.29 (+/-0.434)
miRNA range (ng/μl)	(141.8-151.2)	(0.574-0.892)	(1.856-2.724)
miRNA:small RNA ratio	80:1	48:1	29:1

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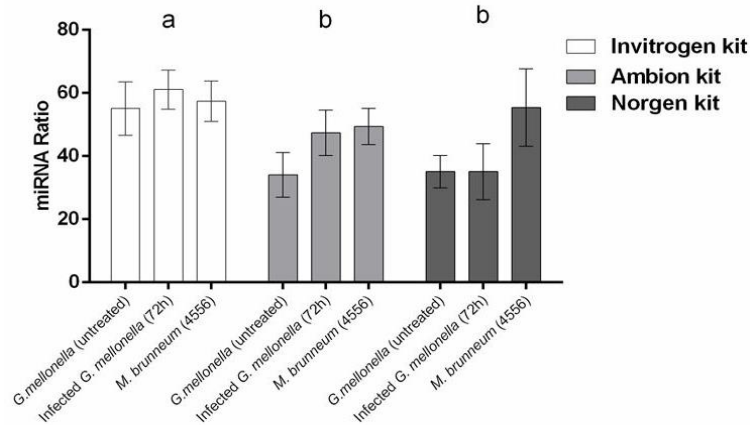


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122 **Figure 1.** miRNA concentration extracted from *G. mellonella* and *M. brunneum* samples using the
 123 Aglient Bioanalyzer. The miRNA concentrations represent mean of three repeat extractions using
 124 the Invitrogen PureLink, Ambion mirVana, Norgen MicroRNA purification kits. Error bars shows
 125 significant differences (**) in the miRNA concentration (two way ANOVA, $p < 0.01$) between samples
 126 processed by each of the kits.

127 The quality of RNA obtained with the Invitrogen isolation kit (as indicated by the miRNAs and
 128 small RNA ratio of 80:1 using Bioanalyzer) was significantly higher than the 48:1 and 29:1 obtained
 129 by the Ambion kit and the Norgen kit respectively (Figure 2, ANOVA, $p < 0.005$). Greater quality of
 130 miRNA was also obtained from the Invitrogen kit across all samples used, i.e. infected / uninfected
 131 *G. mellonella* samples and *M. brunneum* cultures. The miRNA fraction with sizes of approximately
 132 18 nt and 30 nt measured by Bioanalyzer were of a higher purity for the Invitrogen kit than the other
 133 kits (Figure 3A). The Invitrogen kit appeared to yield miRNA with greater integrity when
 134 comparing the sizes of miRNA from the Bioanalyser-derived electropherograms (Figure 3A, B, and
 135 C), suggesting that the greater miRNA quantity obtained was in part due to lower degradation of the
 136 sample. The miRNA obtained using the Intvitrogen extraction protocol met the criteria (the
 137 percentage of miRNA in small RNA to assess the RNA quality) for further processing and high
 138 throughput Illumina sequencing of the miRNAs present. The performance of the Ambion and
 139 Norgen kits appeared similar to one another with regards to the integrity of the miRNA obtained,
 140 i.e. evidence of degraded RNA in most samples (low RNA yield would result in failure of detection
 141 of miRNA present in low abundance). Figure 4 provides a representative example of the gel images
 142 obtained for samples extracted via each of the kits and shows the quality of miRNA verification. The

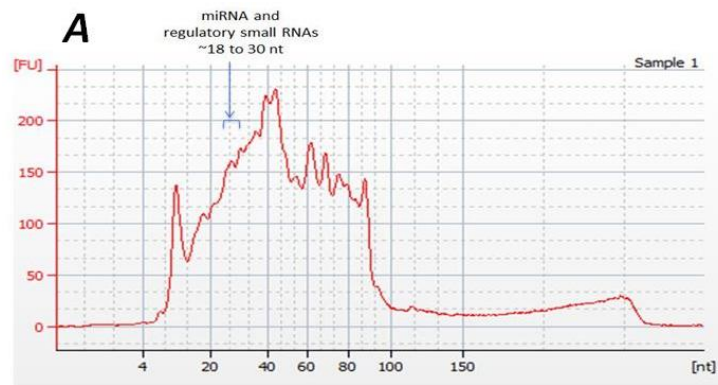
143 relative quantity and quality of miRNA obtained from the more complex sample of *G. mellonella*
144 larvae infected with *M. brunneum* for 72 hrs was comparable to that obtained from the non-infected
145 larvae and *M. brunneum* culture controls (Figure 4C).
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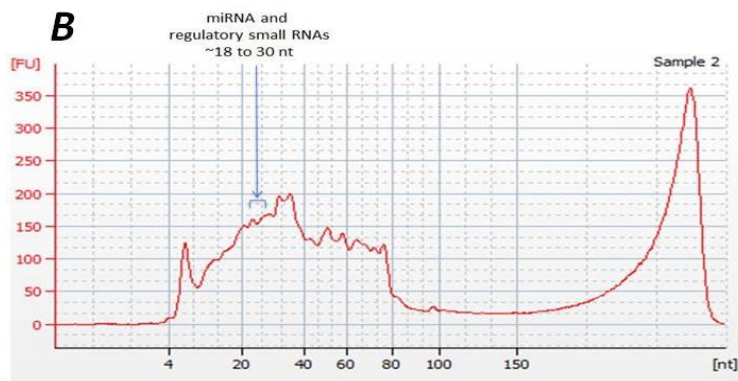
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148 **Figure 2.** miRNA to small RNA ratio quantification from each sample obtained using the different
149 extraction kits. Error bars indicate \pm SEM, different letters above bars indicate significant differences
150 ($p < 0.05$, ANOVA with Tukey HSD) in the miRNA ratio between kits.

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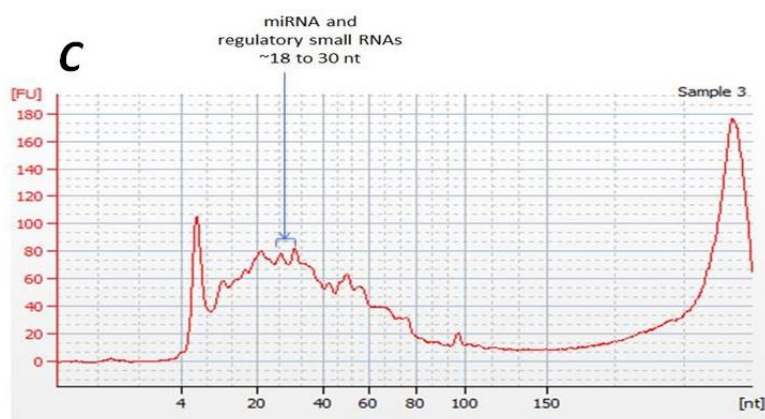
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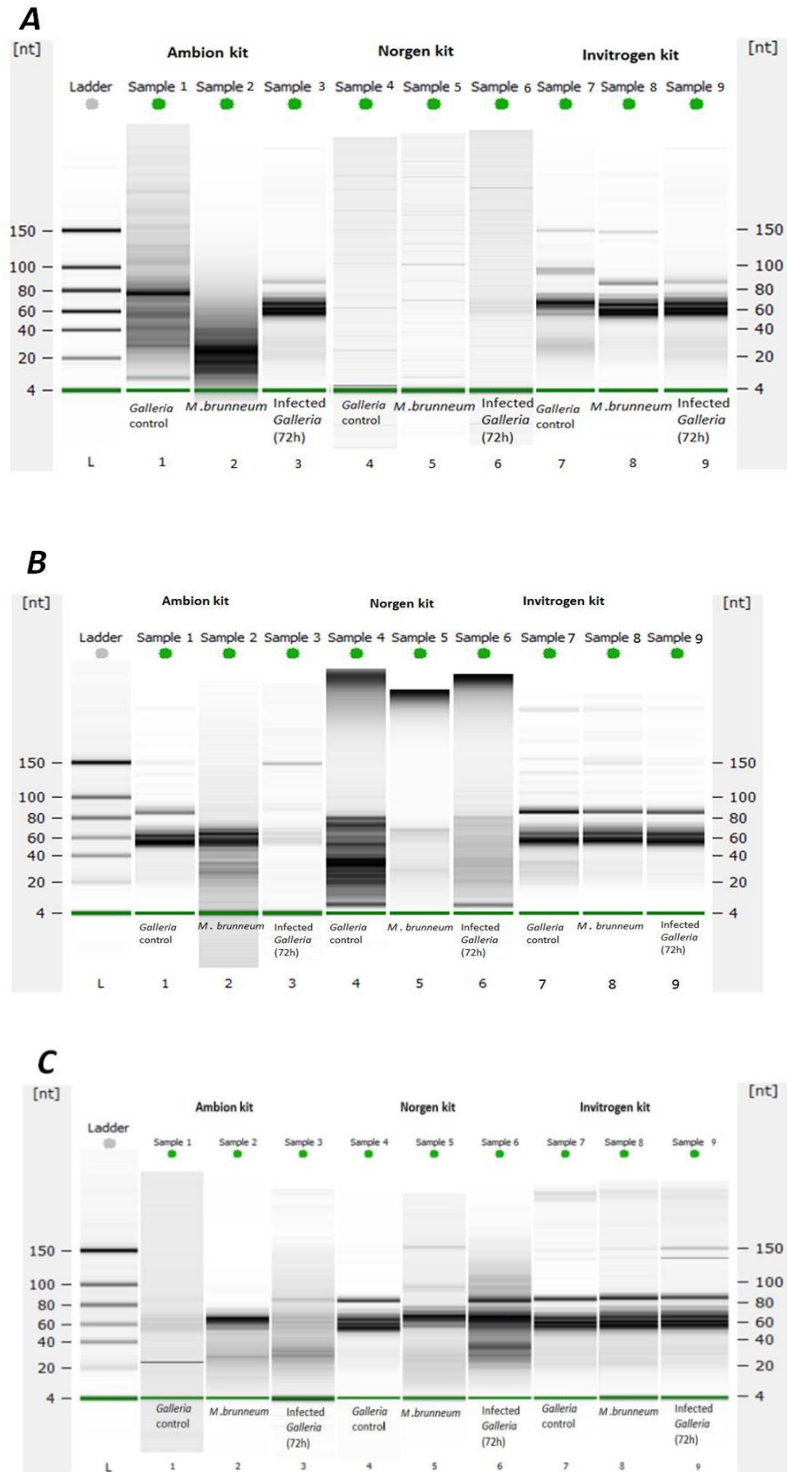


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Figure 3. Image of a typical electropherograms for miRNAs analysis performed with the Small RNA Assay on the 2100 Bioanalyzer. Data presented from infected *G. mellonella* with *M. brunneum* for miRNA isolations using Invitrogen (A), Ambion (B) and Norgen (C) kits.



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Figure 4. Bioanalyzer electronic gel image showing miRNA extracts using commercially available miRNA extraction kits from. A, B and C represent replicate densitometry plots for each extraction kit for the treatments *M. brunneum* (ARSEF 4556, contol), *G. mellonella* (control) and infected *G. mellonella* (72hrs).

180 Discussion

181 High throughput molecular investigations of complex biological systems are dependant on the
182 quality of material extracted from samples where mistakes or poor sample quality can be expensive.
183 Sample preparation and subsequent downstream processing and analysis have been made almost
184 routine by proprietary kit-based protocols that offer reliability and consistency. While the rationale
185 for the selection of a company's kit method is not always presented by researchers, previous work
186 on pure cultures and cell lines have shown the importance of comparing technologies when isolating
187 microRNAs [23, 24]. The improvement in yield using a column-based protocol over
188 non-column-based approaches (e.g. Trizol) are documented [25]. A fungal pathogen interacting
189 within host tissues will provide specific challenges to miRNA extraction that less complex cell
190 culture samples will not, e.g. disrupting fungal cells to obtain intact RNA, elevated presence of
191 nucleases, diverse biochemistry. Our work emphasises the importance of correct kit selection when
192 considering a more complex system for which high quantity, undegraded RNA is required for
193 downstream high throughput sequencing and miRNA analysis.

194 While using a comparison of miRNAs from *M. brunneum*-infected *G. mellonella* larvae, *M.*
195 *brunneum* culture and uninfected *G. mellonella* samples, we have shown that the selection of RNA
196 extraction kit could have important consequences for subsequent miRNA sequencing and analysis.
197 Such considerations should be relevant to any plant or animal pathogen study. The kits we selected
198 (Invitrogen PureLink, Ambion mirVana and Norgen microRNA extraction protocols) were evaluated
199 using the protocols prescribed by the manufacturers to obtain the best results, and no modifications
200 were made to optimize or otherwise alter the protocols. This comparison allowed us to identify a kit
201 that not only provided the highest miRNA yield, but also had good quality miRNA and total RNA
202 from infected *G. mellonella* larvae, consistent with non-infection controls. The Invitrogen kit was
203 selected for our experiments because it had the highest small RNA yield and it was the easiest to use.
204 We have shown that the Invitrogen kit produced the highest yield of microRNA (e.g. 117 ng/ μ l from
205 *M. brunneum*-infected *G. mellonella* larvae) and better $A_{260}:A_{280}$ ratios (>1.9) compared to the Norgen
206 (1.6 ng/ μ l) and Ambion (8.0 ng/ μ l) kits. While the low ratios can result from low concentration of
207 extracted RNA [26], other studies also recorded that both Ambion and Norgen protocols yielded a
208 similar miRNA quantity (sample extracted from pure human cell lines) in line with our findings on
209 whole organisms and pathogen-infected cultures [4, 12].

210 The Invitrogen PureLink protocol combined silica column-based extraction protocol with
211 ethanol RNA precipitation and guanidine isothiocyanate protection from degradation from RNases.
212 A similar process is described for the Norgen MicroRNA extraction kit, except a proprietary resin
213 replaced silica in the column. In addition the Norgen kit is more limited in the amount of tissue that
214 can be processed per sample (50 mg) and required more sample handling, e.g. passing the
215 supernatant through a filter cartridge via centrifugation. The Ambion mirVana protocol is
216 fundamentally different employing a phenol:chloroform extraction and ethanol precipitation and use
217 of glass fibre-based filtration. Phenol use and disposal places an additional consideration for some
218 laboratories. While it is not clear whether the differences in extraction chemistry resulted in the
219 different extraction values between the kits, the lower level of RNA degradation observed for the
220 Invitrogen PureLink kit suggests that the reduced handling time of 15 minutes per sample could be a
221 key factor (NB samples were extracted at the same time to increase efficiency so each individual
222 extracted sample was less than 15 or 30 minutes as recorded). Improved yield and quality may have
223 been obtained for each extraction kit following in depth optimisation, but in conclusion our findings
224 showed that the Invitrogen PureLink™ miRNA Isolation Kit offers more precision in extracting
225 sequencing quality miRNA from insect and fungal tissues without the need for further optimisation.

226 Conclusion

227 By trialing different commercially available miRNA extraction kits, we have shown variation in
228 terms of isolated miRNA quality, quantity and reproducibility between protocols when extracting
229 from complex tissues, namely insect larvae parasitised by a pathogenic fungus. We demonstrated
230 that, for our experiments, the Invitrogen PureLink™ miRNA Isolation Kit provided the highest

231 quality and quantity of miRNA to allow high throughput sequencing of the sample. Also the
232 miRNA obtained via Ambion and Norgen kits showed a greater amount of degradation. In addition
233 the Invitrogen protocol was technically simpler with fewer steps and did not use phenol. Therefore,
234 while we recommend that researchers extracting miRNA from complex / environmental samples
235 should consider testing different commercial protocols when optimising their methodology, in our
236 hands the Invitrogen PureLink™ miRNA Isolation Kit worked well with a mixed insect-fungal
237 pathogen system.

238 **Declarations**

239 **Ethics approval and consent to participate:** Not applicable

240 **Consent for publication:** We confirm that this work is original and has not published elsewhere, nor it is currently
241 under consideration for publication elsewhere. All authors have agreed to the submitted version of the
242 manuscript. There are not any financial supports or relationships that may pose conflict of interest.

243 **Availability of data and material:** All data generated or analysed during the current study are included in this
244 published article study

245 **Competing interests:** The authors declare that they have no competing interests.

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247 scholarship.

248 **Authors' contributions:** MAA, TMB, DCE conceived and designed the experiments. MAA Performed the
249 experiments and performed the statistical analysis. MAA and DCE analyzed the data. Contributed reagents and
250 materials MAA, TMB and DCE. MAA and DCE wrote the paper.

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