MicroRNA preparations from individual monogenean *Gyrodactylus salaris*-a comparison of six commercially available totalRNA extraction kits

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**Abstract**

**Background:** Describing and evaluating miRNA inventories with Next Generation Sequencing is a goal of scientists from a wide range of fields. It requires high purity, high quality, and high yield RNA extractions that do not only contain abundant ribosomal RNAs but are also enriched in miRNAs. Here we compare 6 disparate and commercially available totalRNA extraction kits for their suitability for miRNA-preparations from *Gyrodactylus salaris*, an important but small (500 μm in length) monogenean pathogen of Norwegian Atlantic salmon (*Salmo salar*).

**Findings:** We evaluated 1 salt precipitation method (MasterPure™ Complete RNA Purification Kit, Epicentre), 2 Phenol based extraction methods (mirVana Kit, Ambion, and Trizol Plus Kit, Invitrogen), 1 paramagnetic bead extraction method (RNA Tissue kit, GeneMole) and 2 purification methods based on spin column chromatography using a proprietary resin as separation matrix (Phenol-free Total RNA Purification Kit, Amresco, and ZR MicroPrep Kit, Zymo Research). The quality of the extractions from 1, 10 and 100 individuals, respectively, was assessed in terms of totalRNA yield, RNA integrity, and smallRNA and miRNA yield. The 6 RNA extraction methods yielded considerably different total RNA extracts, with striking differences in low molecular weight RNA yield. The Phenol-free Total RNA Purification Kit (Amresco) showed the highest totalRNA yield, but the best miRNA/totalRNA ratio was obtained with the ZR MicroPrep Kit (Zymo Research). It was not possible to extract electrophoretically detectable miRNAs from *Gyrodactylus salaris* with the RNA Tissue Kit (GeneMole) or the Trizol Plus Kit (Invitrogen).

**Conclusions:** We present an optimized extraction protocol for single and small numbers of *Gyrodactylus salaris* from infected Atlantic salmon that delivers a totalRNA yield suitable for downstream next generation sequencing analyses of miRNA. Two of the six tested totalRNA kits/methods were not suitable for the extraction of miRNAs from *Gyrodactylus salaris*.

**Background**

MicroRNAs (miRNAs) are key regulators of many biological processes in eukaryotes [1]. Besides their investigation through bioinformatical analyses of whole genomes, research focuses on analyses of miRNAs from whole organisms, specific tissues, and/or developmental stages without a fully sequenced and annotated genome at hand [2].

MiRNAs are single-stranded, 22 nucleotide long, non-coding transcripts derived from different genome-encoded hairpin precursors, and regulate gene expression by various mechanisms [3]. First described from *Caenorhabditis elegans* [4] they represent the most recently discovered gene regulators, involved in a broad variety of biological processes including cell proliferation and metabolism [5], developmental timing [6], cell death [7], haematopoiesis [8], neuron development [9], tumorigenesis [10], DNA methylation and chromatin modification [11], and as immune defense against viruses [12]. In evolutionary terms miRNAs are unusual in that they are continuously added to, highly conserved, and rarely lost from metazoan genomes [13,14]. Clearly they are under strong selection, and may therefore represent candidate phylogenetic markers. It may even be possible to reconstruct the miRNA complement of the last common ancestor of all Metazoa [15-17].

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Several methods for isolation of totalRNA have been
developed, with the focus primarily on high molecular
weight RNAs [18]. Commercially available totalRNA kits
are affordable, fast, and suitable for RNA extraction
from a broad spectrum of samples. Most manufacturers
promote their products as suitable for extraction of
totalRNA, but frequently it is unclear whether they are
equally suitable for smallRNA species, and especially for
miRNAs. For extractions of these molecules not only is
overall RNA quality and integrity an issue, but also due
to their low abundance, yield is of high importance.
This is a particular issue if only limited material is avail-
able, as in the case of, for example, micro-dissected tis-
sue samples or small invertebrates.

The current study focuses on the ectoparasitic platyhel-
minth Gyrodactylus salaris (Monogenea, Gyrodactylidae).
This parasite is responsible for a major epidemic disease
of wild salmon in Norway and Russia [19], but gyrodacty-
lids are widespread on teleost fishes and several cause
culture disease in aquaculture [19,20]. Apart from this applied
importance, an understanding of the miRNA comple-
mits respectively. Particular emphasis was placed on
assessing (i) total RNA yield (ii) RNA integrity (iii)
smallRNA yield, and (iv) miRNA yield.

Methods
Gyrodactylus salaris culture
Gyrodactylus salaris were maintained on Atlantic salmon
(Salmo salar) parr in 500 liter tanks in charcoal-filtered
and dechlorinated, continuously running Oslo tap water
at 5-6°C. Fish were fed daily on pellet feed (Ewos) and
maintained under continuous dim illumination [21].

Sampling of Gyrodactylus salaris specimen for totalRNA
analyses
Single infected fish were anaesthetised using 0.1% chlor-
butanol and killed by pithing, taking care to avoid con-
tamination with blood. Fins were cut and stored in -20°C
ethanol in 2 ml RNAse-free DNA LoBind tubes (Eppen-
dorf) until further processing. Using a binocular micro-
scope Gyrodactylus were removed from fins using a
mounted needle, taking care to avoid contamination with
fish tissue. Individual parasites were rinsed with ethanol
at -20°C to further remove contaminating fish mucus or
epithelial cells. For RNA extraction groups of 1, 10, and
100 G. salaris individuals were pooled in 10 μl of -20°C
ethanol and immediately processed.

Ribonucleic acid extraction
Six commercially available kits were compared: Master-
Pure™ Complete RNA Purification Kit (Epicentre, EPI),
Molestrips totalRNA basic for the GeneMole extraction
robot (GeneMole, GM), Phenol-free Total RNA Purification
Kit (Amresco, AMR), ZR RNA MicroPrep kit (Zymo
Research, ZR), mirVana Kit (Ambion, AMB), and the
Trizol Plus Kit (Invitrogen, INV).

The selected kits represent four different extraction
strategies for total RNA: rapid desalting and precipitation
(EPI), solid phase extraction on silica-coated magnetic
beads (GM), solid phase extraction on filter membranes
(ZR, AMR) and sequential organic and solid phase
extractions (AMB and INV). EPI utilises gentle lysis in a
SDS-containing buffer with proteinase K whereas GM,
AMR, ZR, and AMB kits all employ guanidinium thiocy-
nate-(GTC) containing chaotropic buffers for the lysis of
the tissue/cells and the simultaneous inactivation of
RNAses. The INV kit uses Trizol, a GTC-containing
chaotropic lysis-buffer premixed with phenol. The EPI
kit consecutively precipitates proteins and nucleic acids
from the sample, and uses RNAse free DNAse for the
subsequent RNA purification. Following the protocols of
the GM, AMR, and ZR kits the samples in the lysis buffer
(LB) are directly loaded onto the respective binding
matrix, whereas the AMB and the INV kits include an
acid-phenol-chloroform extraction step prior to the bind-
ing of the RNA to a silica-based glass-fiber filter mem-
brane in a spin cartridge. Under high concentrations of
salt the RNA binds to the silica-matrices whereas DNA
and Proteins flow through the column. The bound RNA
is eventually washed from the filters/beads and collected
(Table 1).

For all extractions we followed kit protocols with the
minor modification that all extracted RNA was eluted or
resuspended in 100 μl RNAse-free Water (Ambion). The
AMB kit protocol offers also a microRNAs only option
but this was not followed as the totalRNA was also in the
focus of this study and would have been lost. For the ZR
Kit the quantitative smallRNA recovery option was used
which should deliver smallRNA enriched total RNA with
reasonable high mass RNA as well.

All kits were assessed for extractions from 1, 10, and
100 G. salaris individuals, respectively. The obtained
RNA extracts were heat-denatured for 2 min at 70°C and
immediately stored in aliquots in 0.5 μl RNAse-free DNA
loBind tubes (Eppendorf) at -80°C until further use.

Determination of RNA concentrations and RNA integrity
Quantification of totalRNA yield and assessment of
integrity was done with the Experion (Bio-Rad labora-
tories) and 2100 Bioanalyzer (Agilent systems using the
Experion RNA HighSense Analysis Kit and the Agilent
RNA 6000 Pico Kit respectively. Both systems make use
of the micro-fluidic electrophoresis technology for the analysis of biological samples. The Small RNA Kit (Agilent) was used to analyse smallRNA and miRNA content with the 2100 Bioanalyzer (Agilent). The Agilent Small RNA kit offers a fast detection of small RNA with a sensitivity higher than agarose or polyacrylamide gels (for further details see: http://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Product&SubPageType=ProductDetail&PageID=1647). RNA Quality Indicators (RQI) and RNA integrity numbers (RIN, 1 = degraded, 10 = intact), were derived using the system software. All kits were used following the manufacturers protocols, and quantifications were done in duplicate.

Verification of ribosomal RNA
To verify that totalRNA preparations contained *Gyrodactylus salaris* RNA all preparations and *Salmo salar* RNA controls were reverse transcribed into cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad) and a temperature profile of 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. The cDNA was probed for 5S, 18S, and 28S rRNA *Gyrodactylus salaris* using the specific primer pairs 5Sforward: 5'-TCACTCGGCTCACGTGACGA-3', 5Sreverse: 5'-GCCCTTAGCCGCCATTTGCG-3'; 18Sforward: 5'-TGGTTAAACCGCAAACGGCT-3', 18Sreverse: 5'-GTGTCCTGGCAACGGTCCAT-3'; 28Sforward: 5'-CCCGAGCACGGAAGCCTACGC-3', 28Sreverse: 5'-AAACCGCTTCGCCCTCCACC-3'. PCR primers were designed from GenBank entries Z72477, Z26942, and AJ542394. The PCR amplification was done using the AmpliTaq Gold® Fast PCR Master Mix (Applied Biosystems) and a protocol consisting of 30 cycles with 94°C for 30s, 60°C for 40s, and 72°C for 60s. Only *Gyrodactylus salaris* preparations yielded positive PCR products that were verified by DNA sequencing, for control preparations from uninfected fish no PCR products were obtained (not shown).

Results and discussion
Total RNA yield and integrity
The totalRNA yield and integrity of the extractions were compared. The AMR kit was best for extracting high quality totalRNA (RQI, RIN = 10) from single *Gyrodactylus salaris* individuals with a yield of 15.4 ng (Figure 1); the other kits yielded less totalRNA and/or total RNA of lower quality (RQI, RIN < 10). The totalRNA yields ranged between 2.8 ng and 25.8 ng per individual. The respective yields for 10 individuals ranged between 19.0 ng and 158.4 ng, and between 212.8 ng and 190.8 μg for 100 individuals (Table 2 and Figure 2). The overall average yield was 9.7 ng ± 6/individual.

The yield differences between the kits were highest for extractions from just 1 individual. This may reflect different developmental stages of the respective animals, but may also be due to uncertain estimates due to the sensitivity limitations of the electrophoretic systems. RNA concentrations of less than 50 pg/μl and 100 pg/μl (corresponding to 5 ng and 10 ng in 100 μl) are below the sensitivity limit of the 2100 Bioanalyzer and Experion systems, respectively. Such effects are expected to be less important for the extractions of 10 and 100 individuals.

In general, the quality of totalRNA extractions from single worms was low (RQI/RIN 5.3 ± 3.1) but was much better for 10 (7.5 ± 3.4) and best for 100 (8.1 ± 2.4) individuals. All preparations with the ZR and the EPI kits...
had relatively low quality indices (2.3-6.2), an indication of increased levels of degradation. The rather poor baseline in the electropherogram shows that, particularly in the EPI preparation, the RNA integrity had suffered from a relatively long extraction time including DNase treatment. For the ZR kit, however, the low RQI/RIN values seem to have a different explanation. The protocol efficiently extracts small RNAs and the high molecular weight RNAs contribute comparatively less to the totalRNA. Although there is no noise indicating RNA degradation (Figure 3), the distribution of RNA seems to have been misinterpreted by the analysis algorithm as an indication of poor quality.

Interestingly the assessed RNA extraction kits did not only show differences in yield per individual, they also differed significantly in yield per individual between the extractions using 1, 10, and 100 individuals, respectively. This is unlikely to be due to exceeding the extraction limits of the respective kits, and it is also unlikely - at least for the EPI kit, as it is a precipitation kit only - to be a consequence of the binding capacity of the corresponding binding matrix.

The electrophoretic profile of all *Gyrodactylus* preparations with a major 18S rRNA peak as the most

| Table 2 totalRNA yield in ng | 1 | 10 | 100 | average per individual |
|-----------------------------|---|----|-----|-----------------------|
| totalRNA Extraction Kit     | yield SD | Quality SD | yield SD | quality SD | yield SD | quality SD | yield SD | quality SD | yield SD | quality SD |
| Trizol Plus                  | 2.8 ± 0.8 | 2.6 ± 1.1 | 190 ± 2.2 | 96 ± 0.4 | 2128 ± 506 | 99 ± 0.1 | 2.3 ± 0.4 | 7.4 ± 4.1 |
| Molestrips totalRNA basic   | 5.0 ± 0.9 | 8.3 ± 0.2 | 757 ± 14.2 | 100 ± 0.1 | 5627 ± 2452 | 94 ± 1.3 | 6.1 ± 1.4 | 92 ± 0.9  |
| mirVana                     | 7.8 ± 0.6 | 6.7 ± 1.3 | 911 ± 11.6 | 94 ± 0.9  | 4109 ± 53.8 | 97 ± 0.5 | 7.0 ± 2.6 | 86 ± 1.6  |
| ZymoResearch RNA miniprep   | 20.7 ± 2.4 | 2.7 ± 0.3 | 740 ± 5.2  | 30 ± 0.1  | 5763 ± 1249 | 40 ± 0.5 | 113 ± 8.2 | 32 ± 0.7  |
| Phenol-free Total RNA       | 15.4 ± 6.1 | 9.2 ± 1.2 | 1420 ± 27.8 | 99 ± 0.2  | 19085 ± 5006 | 95 ± 0.9 | 162 ± 2.5 | 95 ± 0.4  |
| Purification                | 258 ± 43  | 2.3 ± 0.2 | 1584 ± 25.9 | 32 ± 0.5  | 5050 ± 59.7  | 62 ± 0.7 | 15.6 ± 10.4 | 39 ± 2.0  |

TotalRNA yield and quality of all assessed totalRNA extraction kits for 1, 10 and 100 *Gyrodactylus salaris* specimens. RNA-quality is expressed as RQI/RIN values determined by the software following the Experion and 2100 Bioanalyzer electrophoresis systems respectively. The global average over all methods and sample sizes is depicted in bold.

SD-standard deviation
prominent signal was different from the “usual”
totalRNA preparation profile with a high 28S rRNA signal,
a less prominent 18S rRNA signal, and a rather low
signal from the smallRNAs. A very similar pattern has
been reported from insect RNA preparations. For sev-
eral insects it has been shown to be a result of heat-
and chemical-dissociable 28S rRNA species, which auto-
catalytically degrade into two 18S rRNA pieces [22,23].
Similar processes may also affect the 28S rRNA of Gyro-
dactylus. In our study this peculiarity of Gyrodactylus
28S rRNA allows us to detect sample contamination
with fish RNA. As the Atlantic salmon 28S rRNA does
not show this property (Figure 4), a very prominent 28S
rRNA peak would point to sample contamination. No
such contamination has been observed in any Gyrodac-
tyulus preparation.

Small and microRNA yield
As expected the yield of smallRNA of the assessed kits
varied as the totalRNA yield: for 1 individual the
values ranged between 1.3 ng and 22.3 ng, between 8.2
ng and 103.4 ng for 10, and between 13.6 ng and 389.3
ng for 100 individuals (see Table 3). The overall aver-
age yield of smallRNAs was 5.3 ng/individual. The
smallRNA fraction includes the microRNAs, and there-
fore it was no surprise that the microRNA yield dif-
f ered almost proportionally to the smallRNA yield. The
respective values ranged between 0.2 ng and 9.6 ng for
1, between 2.5 ng and 26.6 ng for 10, and between 0.9
ng and 51.6 ng for 100 individuals (see Figure 2). The
overall average yield of microRNAs was 1.7 ng/indi-
vidual. The best microRNA to smallRNA ratio (with the
highest absolute microRNA value of 51.6 ng) was
achieved with the ZR kit for 100 individuals. In con-
trast, only very small amounts of either smallRNAs or
microRNAs could be detected in extracts from the GM
and the INV kits.

Meaningful assessment of the yield of smallRNAs
depended on the quality of the sample, as an electro-
pherogram of degraded RNA may resemble that of sam-
ples with a high smallRNA content. Given the low RQI/
RIN values we consider the relatively large smallRNA
fraction obtained with the EPI kit as mainly consisting
of degraded high molecular weight RNA.

Summarizing the data gathered in this study an aver-
age Gyrodactylus salaris individual yields 9.7 ng
totalRNA out of which 5.3 ng are small RNAs, including
1.7 ng microRNAs.

Conclusions
We have demonstrated successful extraction of high
quality RNA from a single Gyrodactylus salaris indivi-
dual, a small monogenean ectoparasite with a body
length of 500 μm only. Careful choice of totalRNA
extraction kit is crucial, and in our comparison, the best
results for totalRNA containing smallRNAs were
obtained with the Phenol-free Total RNA Purification
Kit (Amresco). However the highest amount of miRNAs
with a yield of 9.6 ng for one individual could be
extracted using the ZR RNA MicroPrep kit (Zymo
Research), which is also the only kit that allows elution
with a volume as little as 6 μl, one order of magnitude
smaller than for the other kits and recommended as
input for some downstream NGS applications (8 μl,
ScriptMiner™ Small RNA-Seq Library Preparation Kit,
Lit. # 316 · 11/2010, Cambio).

The amount of totalRNA extracted in this study sug-
gests that a sample size of 100 Gyrodactylus salaris indivi-
duals is sufficient for a microRNAs sequencing run
with an Illumina Genome Analyzer 2 (1-5 μg, ScriptMi-
ner™ Small RNA-Seq Library Preparation Kit, Lit. # 316 · 11/2010, Cambio), making such genomic and tran-
scriptomic analyses feasible with manageable numbers
of these pathogenic fish parasites.

Other totalRNA extractions kits such as the Moles-
strips totalRNA basic for the GeneMole extraction robot
(GeneMole) and the Trizol Plus Kit (Invitrogen) were
found to be inappropriate for extracting small RNA and
microRNA from rather small samples. This may be of
particular interest as kits from the Trizol (Invitrogen)
family have frequently been used as the standard extrac-
tion method in recent microRNAs studies (e.g., [17]).

The successful extraction of suitable amounts of RNA,
especially miRNAs of reasonable quality offers an inter-
esting novel tool for assessing the miRNA complement
of single small sized parasites including those of differ-
ent developmental stages.
**Table 3: small/miRNA yield in ng**

| Number of Gyrodactylus salaris individuals | 1 | 10 | 100 | average per individual |
|-------------------------------------------|---|----|-----|------------------------|
| totalRNA Extraction Kit | smallRNA SD | miRNA SD | smallRNA SD | miRNA SD | smallRNA SD | miRNA SD | smallRNA SD | miRNA SD | average per individual |
| Trizol Plus | 4.3 ± 4.5 | 1.1 ± 0.9 | 8.2 ± 11.2 | 2.5 ± 3.6 | 22.9 ± 29.6 | 3.5 ± 5.0 | 18 ± 2.2 | 0.5 ± 0.6 |
| Molestrips totalRNA basic | 4.2 ± 4.7 | 0.5 ± 0.7 | 10.8 ± 15.1 | 2.5 ± 3.5 | 13.6 ± 8.8 | 0.9 ± 0.7 | 18 ± 2.1 | 0.2 ± 0.2 |
| mirVana | 1.3 ± n.a. | 0.4 ± n.a. | 44.4 ± 399 | 6.1 ± 56 | 2142 ± 817 | 224 ± 96 | 24.1 ± 13.6 | 0.4 ± 13.6 |
| ZymoResearch RNA miniprep | 22.3 ± 13.8 | 96 ± 48 | 700 ± 28.5 | 266 ± 94 | 3334 ± 1120 | 516 ± 164 | 109 ± 10.1 | 4.3 ± 47 |
| Phenol-free Total RNA Purification | 1.8 ± n.a. | 0.2 ± n.a. | 56.9 ± 56.6 | 7.8 ± 76 | 389.3 ± 2610 | 337.2 ± 220 | 3.8 ± 20 | 0.04 ± 0.3 |
| MasterPure Complete RNA Purification | 19.7 ± 100 | 9.2 ± 3.6 | 103.4 ± 36.5 | 25.9 ± 90 | 2077 ± 471 | 41.1 ± 8.7 | 10.7 ± 8.8 | 4.1 ± 46 |
| average absolute | 8.9 ± 9.5 | 3.5 ± 46 | 490 ± 364 | 11.9 ± 113 | 169.9 ± 1549 | 256 ± 205 | 5.3 ± 4.3 | 1.7 ± 2.0 |
| average per individual | 9.7 ± 9.2 | 3.9 ± 44 | 4.9 ± 36 | 1.2 ± 11 | 20 ± 1.5 | 0.3 ± 0.2 |

SmallRNA and microRNA yield of all assessed totalRNA extraction kits for 1, 10 and 100 Gyrodactylus salaris specimen. The global average over all methods and sample sizes is depicted in bold.

**SD-standard deviation**

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**Authors’ contributions**

BF conceived the study, designed and carried out all the experiments and drafted the manuscript. All authors have read and approved the final version of the manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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