Running Title (45 char max): Metabarcoding with fusion primer sets

Title: Scaling up DNA metabarcoding for freshwater macrozoobenthos monitoring

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Abstract:

The viability of DNA metabarcoding for assessment of freshwater macrozoobenthos has been demonstrated over the past years. It matured to a stage where it can be applied to monitoring at a large scale, keeping pace with increased high throughput sequencing (HTS) capacity. However, workflows and sample tagging need to be optimized to accommodate for hundreds of samples within a single sequencing run. We here conceptualize a streamlined metabarcoding workflow, in which samples are processed in 96-well plates. Each sample is replicated starting with tissue extraction. Negative and positive controls are included to ensure data reliability. With our newly developed fusion primer sets for the BF2+BR2 primer pair up to three 96-well plates (288 wells) can be uniquely tagged for a single Illumina sequencing run. By including Illumina indices, tagging can be extended to thousands of samples. We hope that our metabarcoding workflow will be used as a practical guide for future large-scale biodiversity assessments involving freshwater invertebrates. However, we also want to point out that this is just one possible metabarcoding approach, and that we hope this article will stimulate discussion and publication of alternatives and extensions.

Key words: Biomonitoring, High throughput sequencing, Macrozoobenthos, Multiplexing, Fusion Primer, Metabarcoding workflow, Replication

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Introduction

Reliable monitoring of freshwater macroinvertebrate diversity is a key component in the assessment and management of stream ecosystems (Dudgeon *et al.* 2006; Vörösmarty *et al.* 2010). DNA-based identification methods such as metabarcoding are promising alternatives (Baird & Hajibabaei 2012) to morphological identification, which is often limited in resolution and dependent on taxonomic experience (Sweeney *et al.* 2011). In addition to reducing human bias, DNA based identifications can also lead to improved stream assessment (Stein *et al.* 2013). Over the past few years several studies demonstrated the feasibility of metabarcoding-based monitoring of freshwater macroinvertebrates (Hajibabaei *et al.* 2011; Carew *et al.* 2013; Gibson *et al.* 2015; Andújar *et al.* 2017). Despite some methodological limitations (presence/absence data, primer bias (Piñol *et al.* 2014; Elbrecht & Leese 2015)), assessment results are at least comparable if not superior to conventional morphology-based stream monitoring approaches (Gibson *et al.* 2015; Elbrecht *et al.* 2017b; Emilson *et al.* 2017). Some macroinvertebrate reference databases are already fairly comprehensive especially for common taxa (Carew *et al.* 2017; Curry *et al.* 2018). Furthermore, many aspects of the metabarcoding approach have been thoroughly validated recently (Hajibabaei *et al.* 2012; Carew *et al.* 2013; Elbrecht & Leese 2015; Gibson *et al.* 2015; Elbrecht & Leese 2017; Elbrecht, Peinert & Leese 2017a; Emilson *et al.* 2017; Andújar *et al.* 2017). Consequently, many countries are now actively working towards the use of DNA metabarcoding for routine monitoring of macroinvertebrates (Leese et al. 2018).

Routine stream monitoring requires the collection and identification of thousands of kick samples (Buss *et al.* 2015), however, current metabarcoding studies are typically limited to a few dozen samples. If DNA metabarcoding is to be used in routine large scale monitoring projects, a substantial scale up of laboratory protocols is needed in a way that ensures a high level of reliability and quality of data.

We propose a streamlined metabarcoding approach that runs up to 288 individual samples on a single Illumina sequencing run (Fig. 1), using the BF2+BR2 fusion primer system (Elbrecht & Leese 2017) which has been shown to work well with macroinvertebrate monitoring samples (Elbrecht *et al.* 2017b). The extended primer set allows for flexible multiplexing of samples in up to three 96-well plates thereby simplifying sample handling and reducing the risk of cross-contamination. By incorporating replicates already at the tissue homogenization stage, as well as including positive and negative controls, samples affected by laboratory issues can be reliably detected and if necessary excluded from subsequent analysis. Furthermore, by minimizing the number of validation steps throughout the protocol and the use of streamlined fusion primer tags in a 96-well format, we ensure practicality of the protocol. Rather than continuously validating or replicating every step of the workflow, we recommend utilizing controls and replicates in a manner that highlights samples affected by errors.

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Sample collection, homogenization and DNA extraction

After samples are collected using a standardized protocol (Fig. 1A, (Buss et al. 2015)), invertebrate specimens are usually separated from any debris such as substrate and non-target organic matter as part of the morphological identification process (Fig. 1B). While this increases the chance that some taxa and specimens will be overlooked (Haase *et al.* 2010), most metabarcoding studies of freshwater macroinvertebrates do separate specimens from debris (Carew *et al.* 2013; Gibson *et al.* 2015; Elbrecht *et al.* 2017b; Emilson *et al.* 2017). This is often done as part of preceding morphological identifications, or out of the concern that homogenizing an entire sample might introduce PCR inhibitors and complicates standardization. Although work intensive methods like specimen flotation are currently being explored (Andújar et al. 2017), there is not enough evidence yet to decide if homogenization of full kick samples is also feasible without separating invertebrate specimens from collected substrate. Once specimens are separated from debris they can be dried (Fig. 1C) and homogenized (Fig. 1D). Alternatively, DNA of bulk samples has also been extracted directly from the preservation ethanol (Hajibabaei *et al.* 2012), through homogenization of the wet sample (Hajibabaei *et al.* 2011), or by lysing the complete sample (Braukmann *et al.* in prep). To ensure complete homogenization, it is recommended to grind dried bulk samples using e.g. bead mills, as it allows DNA extraction of the entire community using just a small quantity of tissue powder (10-15 mg, (Elbrecht & Leese 2015; Elbrecht *et al.* 2017b)). Two replicates per sample should be used for DNA extraction (Fig. 1E), both of which are metabarcoded to facilitate the detection of insufficient tissue homogenization. If homogenization was incomplete taxon composition between replicates will vary substantially. Any DNA extraction method yielding high quality DNA can be used (e.g. Silica based spin columns, Fig. 1F). However, as tissue powder is easily electrically charged, direct transfer of powder into the 96-well plate should be avoided. Rather the powder should be incubated in individual 1.5 ml reaction tubes which already contain lysis buffer to reduce electric charging. The tissue can then be incubated according to extraction protocol and the lysate safely transferred into the 96 well plate, to reduce the risk of cross-contamination. A strong adhesive plate sealing tape (if necessary detergent resistant) should be used throughout the entire workflow to prevent spilling of samples. Additionally, plates should always be centrifuged before opening and sealed with fresh sealing tape (ideally tightened with a plastic squeegee). To facilitate detection of cross-contamination each row and each column needs to contain an extraction blank that will be included in PCR and sequencing (Fig. 1). Tissue powder from a previous project or a mock sample can be used as a positive control throughout the metabarcoding workflow. It's recommended to homogenize the positive control sample with liquid nitrogen to ensure it's homogeneity if used across several experiments (Elbrecht & Leese 2015). To increase PCR success and for easier troubleshooting we recommend normalizing all DNA

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extracts to identical concentrations. DNA can be quantified by fluorometric methods (Qubit, Life Technologies, USA) or by using a chromatogram-based approach (e.g. Fragment analyzer, Advanced Analytical, USA), which measures DNA quality at the same time.

Amplification and tagging: Two step PCR protocol

After the DNA is extracted and normalized, the barcode marker can be amplified. For freshwater macrozoobenthos, the cytochrome oxidase subunit I (COI) gene is usually used, but some authors also recommended ribosomal markers (Deagle *et al.* 2014). We think ribosomal markers do not offer any advantages over well-designed degenerated COI primer sets (Elbrecht & Leese 2017). Additionally, ribosomal markers often lack adequate reference data (Elbrecht *et al.* 2016). The use of highly degenerated primer sets is recommended, e.g. the BF2+BR2 primer set, as it was specifically designed for freshwater macrozoobenthos and has been already evaluated using both mock and kick samples (Elbrecht *et al.* 2017b; Elbrecht & Leese 2017). Further PCR and primer modifications are dependent on the strategy used to multiplex several uniquely tagged samples for a sequencing run. We recommend the use of a two-step PCR protocol, in which the first PCR amplifies the target fragment utilizing universal primers, while the second PCR uses fusion primer versions of the same primer sets, which include an inline tag and Illumina sequencing tails (Fig. 2). Fusion primers can be used directly in a single PCR approach, but a two-step PCR setup is less susceptible to PCR inhibition (Schnell, Bohmann & Gilbert 2015). Additionally, fusion primers greatly reduce the chance for tag switching (Elbrecht *et al.* 2017b), which can become an issue with other more modular tagging approaches (Esling, Lejzerowicz & Pawlowski 2015; Schnell *et al.* 2015). Furthermore, inline tags of different length and parallel sequencing in forward and reverse direction can substantially increase sequence diversity which in turn leads to better results on Illumina machines and allows for a reduced spike-in of ~5% PhiX (Wu *et al.* 2015; Elbrecht & Leese 2015). That being said, fusion primers can be quite costly, as many versions with different in-line tags are needed. They also need to be developed for each new primer set (thus using commercial indexing kits for small projects might be more cost effective). However, if the same fusion primer set is used more frequently, it can become highly cost effective. One primer costs around $50 and yields over 100 µl with a 100 pmol/µl concentration, of which 25 pmol are used per 50 µl PCR reaction. Such a set (forward + reverse primer) can be used to tag 400 samples at a cost of $100 ($0.25 per reaction).

Previously developed BF2+BR2 fusion primer sets were limited to tagging a maximum of 72 samples (Elbrecht & Leese 2017), which will quickly become insufficient for large-scale metabarcoding projects. Therefore, we developed new fusion primer sets that allow unique tagging and multiplexing of up to 288 samples on three 96-well microplates within the same

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run (Fig. 3, see Fig S1 for full primer sequences and Tab S1 for plate layouts). These new tags use a 7 bp sequence for both forward and reverse primers, while avoiding inline tags of 0 - 1 bp length which are easily affected by insertions or deletions caused by sequencing errors (Faircloth & Glenn 2012). Because the manual development of large numbers of different tags is difficult, we employed an R script that we used to randomly generate 100.000 tagging sets (Script S1). Seven previously developed primer pairs were incorporated into the design process, but the overall base composition was kept similar where possible (Fig S2). The similarity between tags of each generated set was subsequently visualized (Fig S3), and the primer set with most divergent tags was chosen in order to reduce potential tag switching through sequencing errors. Tags in the selected set differed by at least 3 bp, with the exception of four fusion primers that had only a 2 bp insert. We also calculated the Levenshtein distance utilizing the R package stringdist v0.9.4.6 (Van der Loo 2014) to ensure single insertions or deletions (indels) won't lead to tag switching (Figure S4, Faircloth and Glenn 2012). The Levenshtein distance was always 2 or higher, which should be sufficient given that Illumina sequencers are relatively unaffected by indels (Salipante et al. 2014). For PCR we recommend using a reaction volume of 50 µl with a high quality standard *Taq*. It is our experience that proof reading *Taq's* often struggle with degeneracy and long primer tails. For the first PCR (Fig. 2), a master mix using the standard BF2+BR2 primers is added to each 96-well plate. As the extracted DNA (including negative/positive controls) is already present in a 96-well format, ~25 ng DNA can be easily transferred to the PCR plate (Fig. 1H). After the initial PCR 1 µl amplicon is used as template for the second PCR that individually tags each sample (Fig. 1I). The number of cycles needed in each PCR might have to be optimised depending on how strongly samples are inhibited. While the cycle number should be kept as low as possible, studies on barcoding data show that a high number of cycles is not necessarily compromising data quality (Vierna *et al.* 2017; Krehenwinkel *et al.* 2017). PCR success of the first and second PCR can be verified by electrophoresis, however, bands might only be visible after the second PCR depending on cycle number. PCR reactions that failed or showed only weak amplification should be excluded from sequencing.

Library Preparation and Pooling

Amplicons of the second PCR can be directly used for sequencing after chromatographic quantification (Fig. 1J) and cleanup (to remove residual primers and other PCR components). As long as it is possible to measure the concentration of amplicons independently from primer dimers, samples can be pooled first and then subjected to cleanup. Otherwise, each individual sample will need to be cleaned separately before quantification. Usually, all samples are pooled with identical amplicon concentration to ensure similar sequencing depth across all of them. However, in some cases sample concentrations can be adjusted, e.g. if amplicons of different length are sequenced on the same run (Elbrecht & Leese 2017)

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or if the number of specimens across samples is highly variable (Theissinger et al. 2018). Beerman et al. in review). It should also be stressed that both the quantification and pooling step are absolutely essential for the desired sequencing depth across samples, and the accuracy of any used quantification method should be verified prior to any experiments (Elbrecht *et al.* 2017b). As negative controls are difficult to quantify due to low concentration any adjustment to the concentration of other samples would lead to a strong overrepresentation. We therefore recommend adding each negative control to the library in volumes equal to the average volume of the samples used for pooling.

An effective solution for cleanup is magnetic bead purification as it also allows for removal of amplicons that do not match the targeted marker length (Fig. 1L). Usually a left-sided size selection is sufficient as long as no strong double bands are present. Alternative cleanup methods (e.g. spin column based) will be needed if BSA was included as a PCR enhancer, as it can prevent re-suspension of magnetic beads (Elbrecht *et al.* 2017a). The clean library can then be directly loaded onto an Illumina sequencer. As only inline barcodes are used for sample tagging, both Illumina indexing read steps can be skipped (Fig. 1M). Following sequencing, reads are demultiplexed using the first 7 bp of read one and two (e.g. implemented in the R package "JAMP", http://github.com/VascoElbrecht/JAMP).

Sequencing depth

The number of samples (or plates) that can be sequenced on the same run depends on the number of sequences a platform produces as well as on the desired sequencing depth for each sample. The lower the sequencing depth the more taxa will remain undetected, especially those with low abundance, low biomass, and those strongly affected by primer bias (Alberdi *et al.* 2017; Elbrecht *et al.* 2017a). For macrozoobenthos bulk samples we recommend a sequencing depth of at least 100.000 sequences per replicate, but can be more or less dependent on sample biomass. As the BF2+BR2 primer set amplifies a 421 bp region, paired end sequencing with at least 250 bp sequence length is necessary. Table 1 shows an overview of currently available Illumina sequencers that meet these criteria (end of 2017) and the expected sequencing depth they can produce per well. A library can be easily re-sequenced when sequencing depth turns out to be insufficient. Additionally, sequencing depth between samples might vary depending on quantification accuracy for individual samples. Samples with insufficient sequencing depth can be recovered, e.g. by adding additional PCR product to the affected samples in a library for a re-run (alternatively respective samples can be excluded from the dataset if only a few are affected).

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Bioinformatics processing and troubleshooting

The choice of bioinformatics pipelines and clustering settings can drastically affect the resulting taxon list, especially when it comes to rare taxa (Fig. 1N, (Kopylova *et al.* 2016)). However, as long as data is strictly filtered (removal of singletons, abundance based filtering of Operational taxonomic units (OTUs)) and an appropriate OTU clustering algorithm is used for the pool of all samples, results should be reliable (see e.g. (Elbrecht *et al.* 2017b)). However, only samples with sufficient sequencing depth should be used in such analysis, and if samples vary strongly in sequencing depth, rarefaction should be applied across all samples to ensure equal sequencing depth. If a single replicate is of insufficient sequencing depth, the sample should be removed from the dataset. Both replicates for each sample should be very similar in OTU composition. Any discrepancies could indicate problems e.g. caused by insufficient tissue homogenization, cross-contamination or PCR and sequencing errors (Lange *et al.* 2015; Zepeda-Mendoza *et al.* 2016). Low abundance OTUs that are not shared among replicates should be removed, or the complete sample should be discarded (Fig. 1O). However, these samples and OTUs should still be included and highlighted when reporting the raw data, ideally in form of an OTU table. Strong cross-contamination can also be detected by discrepancies between the replicates, especially if the contamination is patchy and not systematic (Kelly *et al.* 2005). The positive control can be used to confirm consistency of the metabarcoding protocol between plates and sequencing runs. Additionally, the 12 negative controls should be inspected for potential cross-contamination and severe tag switching. Some tag switching might be observed, but usually only at a very low abundance and therefore it is not of concern (Elbrecht *et al.* 2017b). The sum of the abundance of each OTU in the negative controls can be subtracted from all other samples in order to reduce the effects of low abundance tag switching on the data set. However, if severe tag switching or cross-contamination is detected, the entire metabarcoding run might have to be repeated (ideally starting from the DNA extraction stage).

Discussion and conclusions

Our proposed metabarcoding strategy is based on sufficiently validated laboratory methods, while still keeping the workflow simple and scalable. By working with 96-well microplates high sample throughput can be easily achieved while at the same time reducing the risk of cross-contamination. By running two replicates starting at the DNA extraction stage, together with negative and positive controls, we ensure that errors are still detectable despite the reduced need to validate each individual laboratory step. The BF2+BR2 fusion primer sets which are extended here (Fig S1) are well tested for macroinvertebrate communities (Elbrecht *et al.* 2017b; Elbrecht & Leese 2017), enabling the tagging and sequencing of up

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to 288 wells in a single sequencing run. We are confident that this metabarcoding workflow will produce reliable results for up to 123 replicated samples per sequencing run (Fig. 1) utilizing a simplified fusion primer based sample tagging process. The number of samples that can be multiplexed with our tagging system is optimized for the currently available Illumina platforms. However, the throughput of sequencers continues to increase with new sequencers and kits being introduced frequently. Already today a shorter COI fragment could be used to amplify DNA from macrozoobenthos bulk samples (Meusnier *et al.* 2008), which would allow for sequencing at ~50x increased throughput (e.g. HiSeq vs. NovaSeq). Such an approach would require thousands of samples being uniquely tagged and multiplexed for a single sequencing run. Although our inline tags are only able to tag 288 wells, they could be extended to several thousand tagging combinations by incorporating Illumina indexing into the fusion primers.

While we are convinced that our metabarcoding approach is efficient and reliable it needs to be validated in practice and thoroughly compared to other protocols. We hope that this manuscript will encourage discussion and helps to find better approaches for the scale-up of metabarcoding for biodiversity assessment. Variations of our proposed workflow as well as comparisons to alternative metabarcoding protocols are explicitly encouraged.

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Table 1: Sequencing depth per well with different Illumina sequencing platforms suitable for the BF2+BR2 fusion primers (k = 1.000 spots).

Sequencer	MiSeq			HiSeq (1 of 2 lanes)
Sequencing Kit	250 PE v2 Nano*	250 PE v2	300 PE v3	250 PE v2 rapid run
Throughput (max)	1 000k	15 000k	25 000k	150 000k
Number of plates sequenced:				
One (96 wells)	10.4k	156.2k	260.4k	1 562.5k
Two (192 wells)	5.2k	78.1k	130.2k	781.3k
Three (288 wells)	3.5k	52.1k	86.8k	520.8k

* ~2/3 the cost of the 250 PE v2 kit, too expensive

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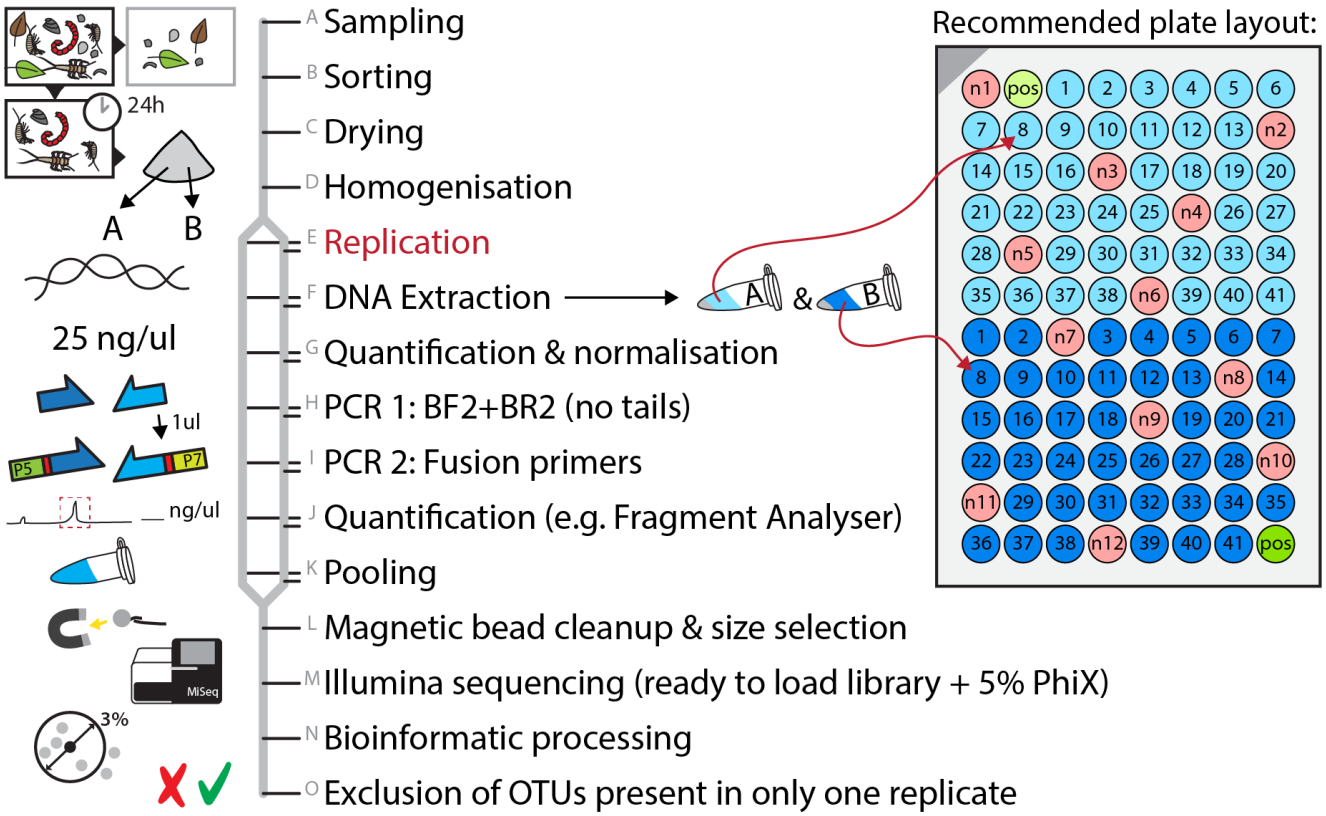

Figure 1: Overview of the proposed metabarcoding work flow for macroinvertebrates using a 96-well plate format and replication for each sample (in light and dark blue). Twelve negative controls are included (n1 - n12) at the DNA extraction stage (F) to detect potential cross-contamination as well as tag switching. One positive control in replication (pos) can be used to estimate the overall performance of the metabarcoding run. Failed extractions or PCRs can be excluded from the sequencing run, and repeated on a new plate. With the newly designed BF2+BR2 fusion primers developed in this publication up to three 96-well plates can be multiplexed for a single run.

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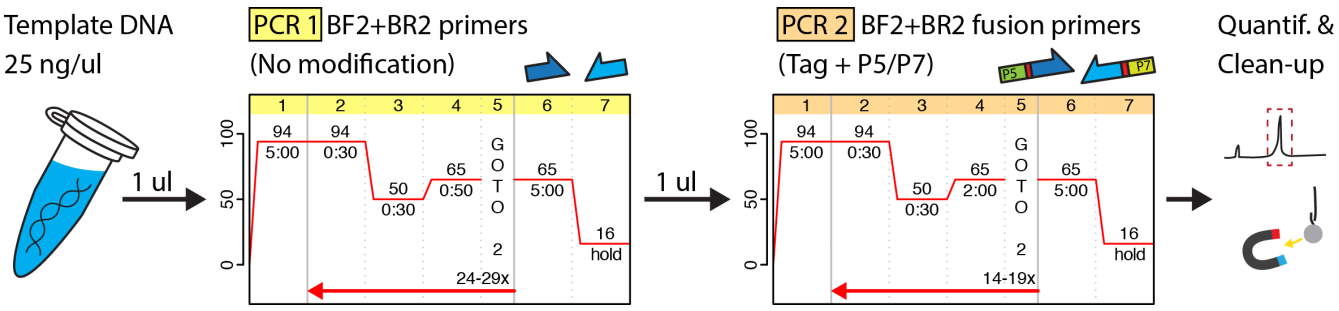

Figure 2: Overview of the two-step metabarcoding PCR protocol (using HotMaster *Taq*, QuantaBio, USA in this example). The first PCR uses the standard BF2+BR2 primers without modifications, thereby increasing amplification efficiency. Subsequently, 1 µl of amplicon product from the first PCR is used (without cleanup) as template for the second PCR step utilizing fusion primers, which adds inline tags as well as Illumina sequencing adaptors. Note that the extension time is increased for the second PCR in order to ensure the entire fusion primer gets amplified. After the second PCR the product can be prepared for sequencing (quantification, pooling with other amplicons and clean-up).

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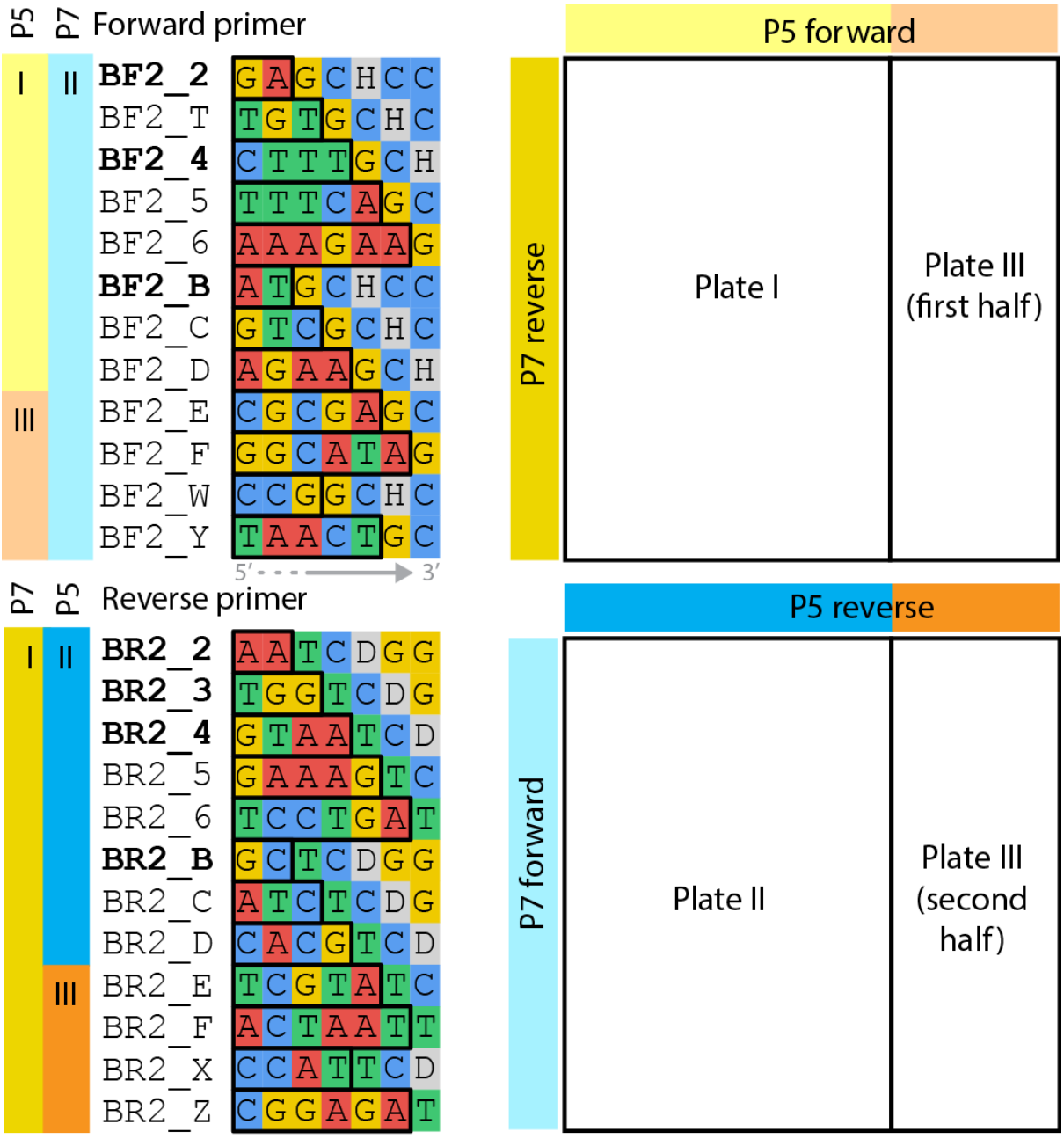

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239 **Figure 3:** Overview of the newly developed inline tags for the BF2+BR2 primer set. Names of previously published
240 primers are highlighted in bold (Elbrecht & Leese 2017) and the inline tag for each primer is indicated by a black box (the
241 full 7 bp sequence has to be used for demultiplexing). The pipetting schema for three 96-well plates is shown on the right.
242 All three plates can be pooled and used for the same sequencing run, or just plate I + II if two plates are sufficient, or only
243 plate III if tagging for only one plate is desired.

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Author contributions

V.E. developed the laboratory workflow, fusion primer set, and wrote the manuscript. D.S. revised the manuscript.

Supporting information

Fig. S1: Newly developed fusion primer sets (BF2+BR2), suitable for tagging 288 individual wells.

Fig. S2: Base composition of the inline tagging region.

Fig. S3: Hamming distance between tags for all fusion primers.

Fig. S4: Levenshtein distance between tags for all fusion primers.

Script S1: R script used to randomly generate inline barcodes for the given primer sets (includes visualization, as shown in Fig. S3 and Fig. 3).

Table S1: Table providing an overview of proposed tagging combinations (as shown in Fig. 3).

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