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RESOURCE ARTICLE

Express barcoding with NextGenPCR and MinION for species-level sorting of ecological samples

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Abstract

The use of DNA barcoding is well established for specimen identification and large-scale biodiversity discovery, but remains underutilized for time-sensitive applications such as rapid species discovery in field stations, identifying pests, citizen science projects, and authenticating food. The main reason is that existing express barcoding workflows are either too expensive or can only be used in very well-equipped laboratories by highly-trained staff. We here show an alternative workflow combining rapid DNA extraction with HotSHOT, amplicon production with NextGenPCR thermocyclers, and sequencing with low-cost MinION sequencers. We demonstrate the power of the approach by generating 250 barcodes for 285 specimens within 6 h including specimen identification through BLAST. The workflow required only the following major equipment that easily fits onto a lab bench: Thermocycler, NextGenPCR, microplate sealer, Qubit, and MinION. Based on our results, we argue that simplified barcoding workflows for species-level sorting are now faster, more accurate, and sufficiently cost-effective to replace traditional morpho-species sorting in many projects.

KEYWORDS
express barcoding, nanopore sequencing, ONT sequencer, rapid identification, species-level sorting

1 | INTRODUCTION

In a perfect world, all biologists should be able to go from a specimen sample to specimen identifications and an estimate of species richness within hours without having to deal with the accuracy problems of morpho-species sorting by parataxonomists or complicated DNA extraction protocols, time-consuming PCR protocols, and access to a capital-extensive sequencing facility (Grant et al., 2021). One method that could deliver such fast species-level sorting is a sufficiently simple express barcoding protocol suitable for time-sensitive ecological projects, pest identification, food authentication, Bioblitzes, and university courses. Express barcoding would also be useful for field stations because researchers could optimize sample collecting based on test samples obtained within hours of placing traps and/or initiate the search for immature stages of important species initially only collected as adults.

Developing express barcoding techniques has thus been pursued for almost 15 years. Ivanova et al. (2009) first achieved the goal for minibarcodes using an optimized workflow that required approximately 2 h and consisted of the following steps: 5 min DNA extraction

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of 55 samples (suitable samples: insect fragments, FTA cards and blood), 25 min PCR, 25 min cycle-sequencing, 10 min clean-up, 45 min capillary sequencing, and 5 min trace file analysis. However, the workflow failed to become popular which we surmise was due to the high capital and manpower cost of a laboratory capable of in situ high-throughput Sanger sequencing. Such a lab would have to own its own AIT capillary sequencer and pipetting robots run by well-trained lab technicians, because Sanger sequencing requires many repetitive tasks that have to be executed for each specimen. They are DNA extraction, PCR, amplicon clean-up, cycle-sequencing, cycle-sequencing reaction clean-up, capillary sequencing, and trace file bioinformatics.

More recently, alternatives to express barcoding sensu Ivanova et al. (2009) have become feasible with the advent of nanopore sequencing developed by Oxford Nanopore Technologies (ONT). ONT sequencers not only have low capital costs, but also require the execution of fewer repetitive tasks, because amplicons can be pooled directly after PCR; that is, no pipetting robot is needed for tasks such as cycle-sequencing, clean-up, and capillary sequencing. In addition, MinION devices are low-cost and suitable for barcoding specimens under field conditions (Johnson et al., 2017; Parker et al., 2017; Pomerantz et al., 2018, 2022). However, the published methods are not particularly cost-effective because they often utilize whole ONT flowcells for barcoding few specimens (Maestri et al., 2019; Menegon et al., 2017; Pomerantz et al., 2018), involve expensive and time-consuming molecular methods, and/or require complicated bioinformatics procedures. For example, the DNA is extracted using kits requiring tissue digestion (Pomerantz et al., 2022), the amplicons are tagged using two PCR reactions (Seah et al., 2020), or the DNA for each sample is quantified before pooling (Maestri et al., 2019). These workflows are particularly impractical when hundreds of specimens need to be processed quickly, which is often the case in ecological studies.

Here, we show that these issues can be overcome by combining DNA 'leaching' with HotSHOT, amplicon pooling, MinION sequencing, and the use of fast NextGenPCR® thermocyclers. The latter are still underutilized in molecular ecology although they have been widely used in COVID-19 detection because very short DNA fragments can be amplified in 30 PCR cycles within 2 min. Such fast cycling is achieved through a special polymerase and the compression of microplate wells that allow for fast heating/cooling of reagents in three heating zones preset to optimal denaturing, annealing, and extension temperatures. We here show that by combining the fast cycling of NextGenPCR® with MinION sequencing, one can generate and analyse 250 barcodes in 6 h. All the processes are so robust that they can be learned within days and are suitable for use in university classes. We thus suggest that it is time that many projects replace species-level sorting based on morphology with species-level sorting with DNA barcodes (Wang et al., 2018).

2 | MATERIALS AND METHODS

Two lab members carried out the core experiment that followed Srivathsan et al. (2021) unless mentioned otherwise (see here for the video of the experimental procedures: https://www.youtube.com/watch?v=0fwd9g0hkR8). In the core experiment, we used 10 μL of alkaline lysis buffer to leach DNA from specimens belonging to Phoridae (Diptera) in three 96-well plates (=285 specimens and 3 control wells). DNA were obtained within 20 min (18 min: 65°C; 2 min: 98°C) and the specimens were recovered intact. Afterwards, 10 μL of neutralizing buffer was added to achieve a suitable pH (pH 8.1) for PCR as well as for DNA storage. For the pipetting of lysis and neutralizing buffers manual multichannel pipettes were used. The PCR recipe was as follows: 3 μL of template, 1 μL each of primers (10 μM), 5 μL of the Arctic Fox HF Chemistry-2x (#50050, MBS) polymerase mix. We amplified a 313 bp cytochrome c oxidase subunit I (COI) minibarcode using tagged primers: m1COIintF (Leray et al., 2013) and modified jgHCO2198 (Geller et al., 2013). The PCR plates (#33602, MBS) were sealed with EZtrieve™ aluminium seals (#30110, MBS) using a NextGenPCR™ Semiautomatic Heat Sealer (#10101, MBS) that operates on high temperatures (170°C for 2 s at pressure level 8 with a copper plate). A resealing step was performed to ensure proper sealing and avoid pre-PCR cross contamination. The PCR reaction was performed on a NextGenPCR® Thermal Cycler (#10001, MBS) and took 22 min with the following conditions: Initial denaturation for 30s at 98°C followed by 5 cycles at 98°C for 10s, 45°C for 30s, 68°C for 10s, followed by 30 cycles at 98°C for 5 s, 45°C for 15 s and 68°C for 10s. In follow-up experiments, we established that the same PCR settings also successfully amplified the (1) full-length DNA barcode (658 bp) and (2) worked for a wide range of taxa. Note that not even the elongation time had to be lengthened. This implies that even faster cycling is feasible for minibarcodes.

For the assessment of PCR success rates in the core experiment, agarose gel electrophoresis was performed for 8 samples of Plate 1 and Plate 2 (including the negative controls) while this step was skipped for Plate 3 to save time. Afterwards, the PCR products were pooled (4 μL each) and 100 μL of mixed amplicons was cleaned using 0.8X AMPure XP magnetic beads in a 30 min wash protocol. All access to the PCR products involved piercing the sealing film with pipette tips mounted on a multichannel pipette. The DNA library was prepared with the Nanopore ligation kit (SQK-LSK114) and NEBNext Companion Module kit (E7180S). Nanopore sequencing (Run 1) of the tagged amplicons employed a flowcell FLO-MIN114 with Oxford Nanopore MinION in an Mk1B and base-calling was conducted using a local computer using Guppy v. 6.3.7 + 532d62603 and super-accuracy model. Sequencing was stopped after 30 min. We resequenced (Run 2) the same pool again using a flowcell dongle 'Flongle' FLO-FLG114 because this low-cost flowcell is particularly interesting to ecological labs that only occasionally need a few hundred barcodes. The library preparation used the same procedures, but with the use of less consumables as recommended by the official Flongle library protocol of ONT. Reads were base-called using the super-accuracy model (Guppy v. 6.4.2 + 97a7f0659). Demultiplexing and barcode calling used ONTbarcoder (2021) under default settings. The filtered barcodes were blasted using SequenceID in GBIF; 95% Query coverage, >89% Identity. Sequences were clustered with

Objective Clustering (Meier et al., 2006) at two thresholds; 2% and 4% (Srivathsan et al., 2019).

In addition to the core experiment, we carried out three sets of additional experiments. Firstly, we tested whether the same materials and methods are also suitable for other taxa by applying them to 95 specimens belonging to Coleoptera, Hemiptera, Hymenoptera, and different Diptera families. Secondly, we tested whether the same techniques can be used for obtaining full-length barcodes (658 bp). Thirdly, we established whether the cost of express barcoding can be lowered by using less Arctic Fox polymerase. This was done by diluting the Arctic Fox master mix used in each PCR reaction with PCR-grade water (half dilution: 2.5 μL master mix with 2.5 μL of water and one-fifth dilution: 1 μL master mix with 4 μL of water). Lastly, we optimized the plate sealing settings because we had observed improperly sealed wells in several experiments. The sealing experiments used food colouring instead of PCR reagents while the cycling program of NextGenPCR® thermocycler remained the same.

3 | RESULTS

We went from 285 specimens to 250 identified barcodes within 6 h (Figure 1: start: 9:00 am, completion: 3:00 pm) although only one regular thermocycler was used for the HotSHOT DNA extractions and one NextGenPCR® machine for generating amplicons. Agarose gel electrophoresis of the subsamples (8 per plate) from Plates 1 & 2 showed 100% PCR success rates but we only obtained 250 barcodes because of sealing issues with some PCR reactions in Plate 3 (see above). All amplicons were then sequenced twice. The first time using a fast method by obtaining 507,230 raw reads from a MinION flow cell that only sequenced for 30 min (Table 1). The second time, we used a more cost-effective method by employing a Flongle flow cell that produced 321,462 reads in 24 h, generating a total of 241 barcodes (fast method: 250 barcodes). Even with the slow sequencing method, >75% of the barcodes required only the reads obtained within 1 h of Flongle sequencing (see Table 1 for results after 1–24 h).

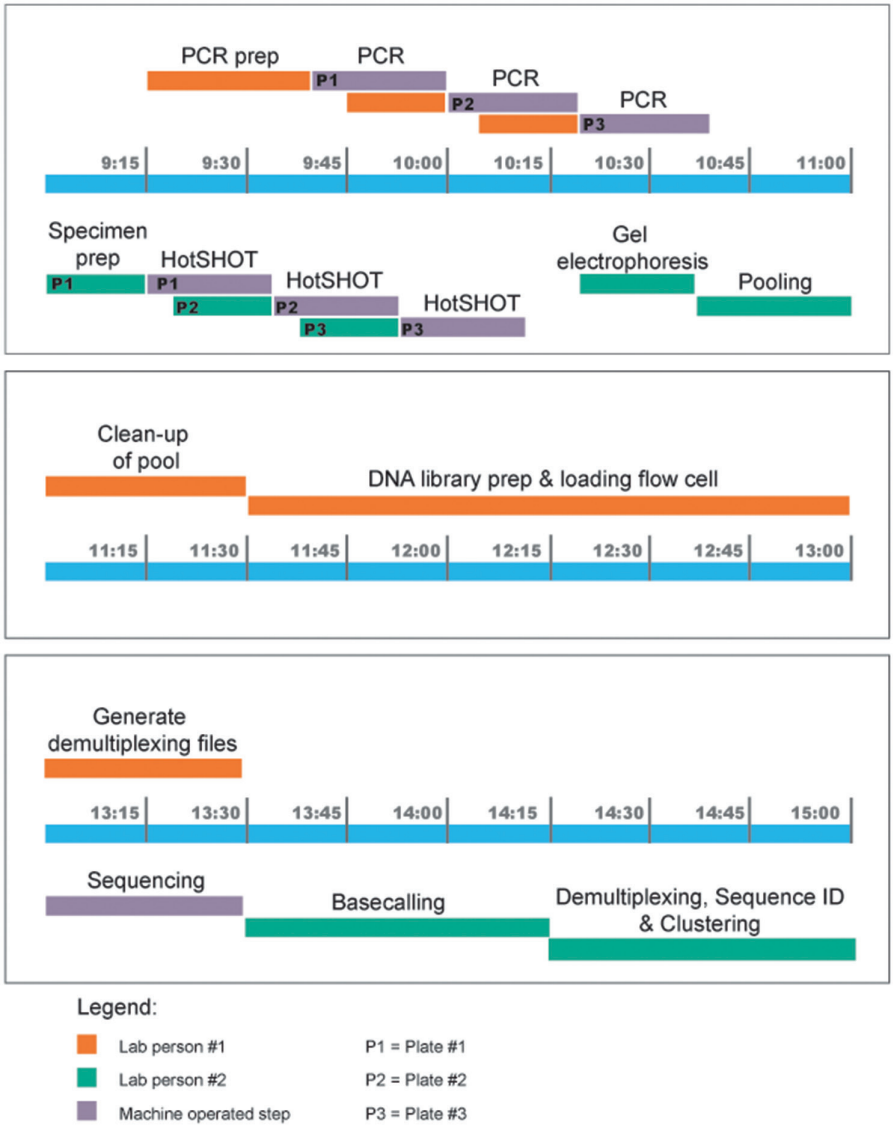

FIGURE 1 Experiment timeline in blocks of 2 h (9.00 am – 15.00 pm).

TABLE 1 Barcoding success rates using MinION and Flongle flow cells.

	Number of reads	Plate 1	Plate 2	Plate 3	Overall
MinION flow cell					
0.5 h of sequencing	507,230	97.89%	91.58%	73.68%	87.72%
Flongle flow cell					
24 h of sequencing	321,462	95.79%	89.47%	68.42%	84.56%
3 h of sequencing	139,963	94.74%	85.26%	67.37%	82.46%
2 h of sequencing	98,885	93.68%	82.11%	63.16%	79.65%
1 h of sequencing	53,440	90.53%	78.95%	60.00%	76.49%

All barcodes were then identified by blasting them via the user-friendly 'SequenceID' that is implemented on GBIF (https://www.gbif.org/tools/sequence-id: accessed 04 Nov 2022), 249 of the 250 sequences were Phoridae (species belonging to *Anevrina*, *Megaselia*, *Phora*, and *Triphleba*). The only non-phorid barcode belonged to *Apis mellifera*, which is known to be parasitized by *Megaselia rufipes* (Dutto & Ferrazzi, 2014). Query coverage was over 95% in all sequences; 19 sequences retrieved an exact match, 207 sequences had a close match (90%–98.7%) and 24 sequences had a weak match (89.4%–89.7%). Objective clustering at multiple thresholds (2%–4%) yielded a species richness estimate of 27–30 mOTUs.

The additional experiments confirmed that the same methods used for the core experiment can also yield full-length DNA barcodes across many insect taxa (Coleoptera, Hemiptera, Hymenoptera, and other Diptera, see also Lee et al., 2022; Yeo et al., 2018; Yeo et al., 2021; Ho et al., 2020; Srivathsan et al., 2022). We furthermore found that the following settings had a 100% success rate in sealing all wells of five plates: 170°C for 6 seconds at pressure level 8 using a copper plate that should be flat and smooth.

When only considering the properly sealed wells, the overall PCR success rates were 96% for 313 bp and 92% for 658 bp barcodes (Tables S1 and S2) while the experiments with diluted Arctic Fox master mix yielded lower success rates: 71% and 70%. However, a BLAST of all sequences in the specimen-specific read bins revealed more barcodes in the read sets obtained with diluted mastermix. In total 2, 4, 17, and 17 barcodes could be retrieved for each of the plates: 313, 658 (full reaction), 658 (half reaction), and 658 (one-fifth reaction) thus yielding even higher success rates of 99%, 97%, 88%, and 88%, respectively. All data are available from https://figshare.com/s/5a4c6a9615fe71ad8284.

4 | DISCUSSION

Fast species-level sorting of samples consisting of hundreds of specimens is a bread-and-butter task in ecology, university classes, Bioblitzes, etc. Currently, it involves unpleasant choices because none of the available techniques are fast, cheap, and accurate. In many projects, species-level sorting is still delegated to parataxonomists who can be quick and cost-effective, but also produce results of unpredictable accuracy because the taxonomic training varies between parataxonomists and the complexity of samples varies between projects and sites (Baraloto et al., 2007; Derraik et al., 2010; Egli et al., 2020; Krell, 2004; Oliver & Beattie, 1993). At the other end of the scale in terms of speed, cost, and accuracy is the identification of specimens by taxonomic experts. In 2006, Marshall et al. provided a cost and time estimate for species-level expert identification, which was inflation-adjusted USD 3.30 per specimen. On average, the identification took 10.65 min per specimen , but the 10 taxonomic experts employed by the project could identify only approximately half of the specimens to species-level. Presumably, this was due to the lack of taxonomic expertise or identification tools for the remaining specimens.

We here show that species-level sorting with express barcodes can be achieved within hours with a modest amount of equipment, manpower and training. This is achieved by fast cycling using a NextGenPCR® thermal cycler with cost-effective third-generation sequencing with MinION. Barcoding required only 6 h using techniques that can be learned within days. This distinguishes the new workflow from other express barcoding methods that are rarely used due to high complexity, capital, manpower, and/or consumable cost. Express barcoding only requires one NextGenPCR® thermal cycler (including plate sealer) in addition to standard equipment found in most molecular laboratories (pipettes, Qubit, regular thermocycler, MinION). Like all other express barcoding protocols, short PCR cycling times are in part achieved by using special enzymes that are responsible for much of the cost of express barcoding. Ivanova et al. (2009) recommended TaKaRa Z-Taq, or KAPA 2G Fast, while we here used NextGenPCR® Arctic Fox HF which currently costs ca. EUR 1.70 c.q. USD 1.85 per specimen.

The high enzyme costs could affect the attractiveness of express barcoding and we thus carried out experiments with less enzyme. We overall found lower success rates that were likely due to more variable and lower read numbers (Table S1). However, after analysing all sequences in the specimen-specific read bins, we recovered overall very similar numbers of barcodes as with the recommended amount of Arctic Fox polymerase. This suggests that increasing sequencing depth would allow for the use of less enzyme. This reduces the enzyme cost per specimen to USD 0.83 (½ enzyme volume) or even USD 0.37 (⅛ enzyme

volume); that is, barcoding can already be cheaper than expert identification (Marshall et al., 2006) and further cost reductions can be achieved if the results are not needed within 24 h (see discussions below).

We here generated 250 barcodes for 285 specimens in 6 h (success rate: 87.72%) using a partial MinION flow cell and two staff members (ca. 2.5 minutes/specimen); that is, express barcoding does not result in lower barcoding success rates than what is obtained with regular protocols. Furthermore, we estimate that the use of three cyclers would have reduced the processing time by another hour if a third person had helped with the initial step of placing specimens into wells. However, we wanted to report realistic times for those users (e.g. field station, citizen science project) that would like to implement express barcoding with a minimal amount of equipment. We find that such realistic estimates are lacking for other express barcoding protocols because they are vague about the time needed for DNA extraction, tagging amplicons (often done using two PCR reactions), and specimen handling. Note that the times discussed here are not only valid for 313 bp minibarcodes obtained for phorid flies, but also for full-length barcodes obtained for Coleoptera, Hymenoptera, Hemiptera, and several other families of Diptera. This is mentioned here, because full-length barcodes are often preferred over mini-barcodes, although both yield similar identification success rates (Yeo et al., 2020).

Two protocol variations are attractive to users who prioritize low cost over speed. The first is the use of a Flongle instead of a MinION flow cell for sequencing. This adds several hours to the protocol because Flongle flow cells have fewer pores and thus sequence more slowly. The additional waiting time depends on the targeted success rate and the number of amplicons in the pool (Table 1). If only 200 specimens have to be barcoded, we show that 3 h of Flongle sequencing will yield similar success rates as 15–20 min of sequencing using a standard MinION flow cell. The attractive aspect of using a Flongle is the low consumable cost of only ca. USD 120 (including library prep). Obtaining the same data with a standard MinION flow cell would be more expensive, but the precise cost is hard to estimate because regular MinION flow cells can be washed and reused. A second cost-saving modification of the express barcoding workflow presented here involves the use of cheaper polymerases and several traditional thermocyclers. For example, when using three thermocyclers simultaneously, the DNA in the three microplates could be amplified using conventional enzymes (cost ranges from USD 0.10 to USD 0.60 per specimen for a regular high-performance enzyme). Assuming a cycling protocol that requires 3 h, this would add 2 h to the 1 h needed with a single NextGenPCR® cycler. However, the use of more equipment is unattractive for field stations, as space and electrical outlets are at a premium. Longer waiting times are also unattractive when members of the public in citizen science projects or students in university classes are waiting for results although the waiting times can be used productively by using the recently described real-time barcoding (Srivathsan et al., 2023) that allows for analysing barcodes while sequencing is still ongoing. One way or another, the use of a Flongle and conventional thermocyclers means that the cost per barcode drops well below USD 1 per specimen and specimen sorting with barcodes is considerably cheaper than expert sorting/identification (Marshall et al., 2006). Note, however, that the latter can yield more species names, because barcode databases still lack identified sequences for many species and specimens can only be assigned to mOTUs.

AUTHOR CONTRIBUTIONS

Cristina Vasilita and Rudolf Meier conceived and designed the project; Cristina Vasilita, Vivian Feng and Amrita Srivathsan collected the data; Cristina Vasilita, Vivian Feng, Aslak Kappel Hansen and Amrita Srivathsan analysed the data; Emily Hartop and Aslak Kappel Hansen contributed valuable discussion of the obtained results; Robin Struijk optimized barcoding protocols for NextGenPCR® thermocyclers and coordinated access. All authors contributed to the manuscript and gave final approval for submission.

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CONFLICT OF INTEREST STATEMENT

Co-author Robin Struijk is an employee of Molecular Biology Systems, which developed and sells NextGenPCR® thermocyclers and reagents. MBS also provided the consumables for the experiment.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [[insert provided URL from Open Research Disclosure Form]].

DATA AVAILABILITY STATEMENT

The data are available from https://figshare.com/s/5a4c6a9615fe71ad8284 (Vasilita et al., 2023).

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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