Advances in *Flavivirus* research applications: new techniques using the FastPrep-24 5G™ sample preparation system

The FastPrep-24 5G is the most innovative bead-beating sample-preparation instrument and produces quantitative, thorough and rapid grinding, lysis and homogenization of routine and resistant samples.

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The FastPrep-24 5G (**Fig. 1**) is a high-speed, benchtop reciprocating instrument that is intended for the optimal lysis of challenging and routine sample types. Its use of bead-beating technology makes it suitable in all applications that require grinding, lysing or homogenization of various solid sample materials. Mechanical sample lysis offers rapid sample preparation for the isolation of DNA, RNA, proteins, metabolites and other small molecules while eliminating the need for chemicals, enzymes and detergents, which minimizes the introduction of potential inhibitors to downstream processes. Sample types include but are not limited to the following: all types of human, animal and plant tissues, including cultured cells; bacterial, yeast and fungal cells, including spores and oocytes; and environmental and metagenomic samples, including soil and fecal samples. The FastPrep-24 5G instrument uses a unique, optimized motion to disrupt cells through the multidirectional, simultaneous beating of specialized Lysing Matrix beads on the sample material. Developed for difficult and resistant samples, the FastPrep-24 5G instrument thoroughly and quickly lyses any tissues and cells and thus allows easy and reproducible isolation of stable RNA, active proteins and full-length genomic DNA. The FastPrep-24 5G is the only available homogenizer with nine optional adaptors, which allows for unique versatility in sample number, with tube sizes of 2, 4.5, 15 and 50 ml available for processing samples under ambient or cryogenic temperature conditions. Samples and buffers are added to a Lysing Matrix tube containing specialized Lysing Matrix beads. A wide range of Lysing Matrix tubes are available and contain beads of different materials, sizes and shapes that have been optimized by sample type and guarantee thorough homogenization every time.

Figure 1 | Examples from the FastPrep family of sample preparation instruments, Lysing Matrix tubes and purification kits.

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Virus, vector, versatility

Katz[1] used molecular techniques previously used for mosquito phylogenetic studies to analyze both interspecific and intraspecific variation. Mitochondrial DNA (mtDNA) is isolated rather than nuclear DNA because it is more sensitive to genetic drift differentiation and has higher mutation rates, thereby speeding the evolutionary process. The protein-coding gene NADH dehydrogenase 5 subunit (*MT-ND5*) is especially variable, and as such it is an excellent marker for characterizing the genetic structures of mosquitoes. Species and populations of mosquitoes can be distinguished with PCR using a primer pair that amplifies a region of mtDNA in the *MT-ND5* gene, followed by sequence analysis. Each sample was homogenized using a FastPrep-24 instrument. DNA from the specimens was extracted using the FastDNA® Spin Kit for Soil. Samples were diluted into 5 ng/μl solutions and prepared for amplification by PCR. PCR products were electrophoresed (**Fig. 2**), and bands were then excised, purified and Sanger sequenced. Sequences were edited and then BLAST-analyzed to determine percent similarity.

Japanese encephalitis virus (JEV) is a type of *Flavivirus* that is mosquito-borne throughout much of Asia. It is known to be transmitted by the *Culex tritaeniorhynchus* species of mosquito. Kim *et al.*[2] collected mosquitos in five regions in Korea where people were known

to have contracted JEV between 2007 and 2011. For virus detection, female mosquitos ($n \leq 50$ individuals) were homogenized in a FastPrep tube with six 2.5-mm glass beads installed. Tubes were processed on a FastPrep-24 for two cycles of 20 s each at a speed of 5.0 m/s. Viral RNA was extracted from the homogenates and analyzed by RT-PCR using four flavivirus-specific primers detecting the NS5 partial gene. Melt-curve analysis of RT-PCR products verified samples positive for JEV, but the results also included other *Flavivirus* spp. including dengue virus, yellow fever virus and West Nile virus. Positive products were extracted from the gel and PCR direct-sequenced with flavivirus primers FL-F1, FL-R3 and FL-R4. Then five primers were designed to obtain the complete JEV envelope gene. RNA from the mosquito homogenate was reverse transcribed, and the resulting cDNA was amplified in both directions with the five primers. PCR products were sequenced as above, and after compilation the resulting 1,500-bp envelope genes were compared to known JEV genotypes in GenBank. Resulting data analysis not only showed that all positive JEV mosquito pools in Korea were of the same viral genotype, but also confirmed JEV genotype in two other mosquito spp., *Culex orientalis* and *Culex pipiens*, suggesting that they may also be transmitters.

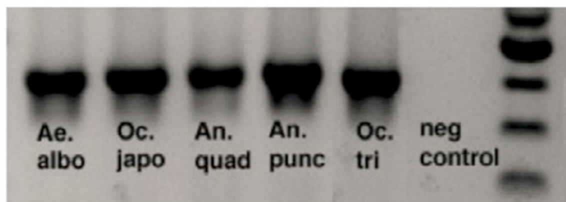

Figure 2 | The target region of mtDNA was successfully amplified in the specimens of the five species used in ref. 1 (Ae. albo, *Aedes albopictus*; Oc. japo, *Ochlerotatus japonicus*; An. quad, *Anopheles quadrimaculatus*; An. punc, *Anopheles punctipennis*; Oc. tri, *Ochlereotatus triseriatus*). Each band visualized on the 1% agarose gel represents the PCR product of 450 bp. Reproduced with permission from ref. 1, Katz, M.B.

Tools for mosquito species identification are being modified as well as improved. Dieme *et al.*[3] developed a proteomic method for species identification at the aquatic larval stage of the life cycle that uses matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF-MS) profiling instead of the usual molecular biology techniques. Vector control measures aimed at reducing adult biting mosquito populations have advantages when aimed at aquatic breeding sites. Mosquitos in their larval or pupal stages not only are nonbiting and thus not active transmitters, but also are confined to the aquatic breeding site, which makes eradication measures at those sites more effective. Because the possibility exists for multiple species to be present at a single breeding site, correct taxonomic identification is crucial in terms of species and population densities in order to direct the specific vector control treatment. However, in the larval stage, morphological-based species identification is more problematic. Entomological expertise is necessary, and the process is time consuming and prone to examiner-specific misidentifications. PCR and other molecular-based identification methods rely on multiple genes analyzed to differentiate between species, resulting in more sequences analyzed, adding to the time and cost necessary to validate field efforts. This proteomic taxonomic method using MALDI-TOF-MS was developed as a rapid, economical tool for the identification of mosquito species in the aquatic development stage, and it was optimized for variables including sample preparation, sample storage conditions, diet and body parts used. Each whole larval or pupal mosquito sample was homogenized in 70% HCOOH by one of two methods: manual grinding in a microfuge tube using a micro-pestle, or FastPrep-24 processing in the presence of glass beads. The resulting homogenates were mixed with 50% acetonitrile and centrifuged for clearance. One microliter of the supernatant was deposited onto the steel target plate, and 1 μl of CHCA was directly overlaid and dried on the target plate, after which the plate was loaded into the MALDI-TOF-MS instrument. The resulting protein spectra profiles were used to create a database that correctly distinguished individuals from the *Anopheles*, *Aedes* and *Culex* genuses in their juvenile aquatic stages. FastPrep sample preparation yielded MS profiles (**Fig. 3**) with quality equal to that of profiles from manually prepared samples, leading the study authors to recommend automated sample prep for high-throughput workflows.

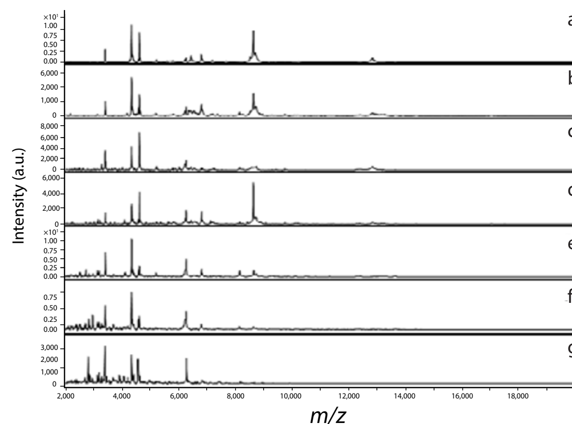

Figure 3 | Effect of sample preparation methods and storage conditions on spectra quality. Comparison of the MALDI-TOF-MS spectra of whole A. gambiae at the L3 stage homogenized manually with a pestle (a); homogenized automatically with a FastPrep apparatus (b); homogenized manually in deionized water and then subjected to acidic extraction (c); stored at −20 °C for 60 d and homogenized manually with a pestle (d); and stored in 70% ethanol for 7 (e), 14 (f) and 60 (g) d. a.u., arbitrary units; m/z, mass-to-charge ratio. Adapted with permission from ref. 3.

Mosquito densoviruses (DNVs) have been investigated as possible infectious agents for paratransgenic-vector control methods. DNVs possess genes that encode for proteins that prevent vector transmission of pathogenic viruses. These DNV genes can then be inserted into a bacterial symbiont, and that symbiont can be introduced into the vector mosquito. The anti-transmission genes then express that protein in the host vector, rendering the vector incapable of virulent transmission. Infected host mosquitos are then released into the wild, and their progeny will not be transmitters. It is known that the *Anopheles gambiae* DNV (AgDNV)

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is nonpathogenic to the larval mosquito, but the cost of infection to the adult (transmitting) mosquito is not known. Ren *et al.*[4] used microarray analysis of AgDNV-infected adult mosquitos to determine survivability after infection as well as to analyze the adult transcriptome. Pools of 20 AgDNV-infected adult mosquitos were processed in a Lysing Matrix D tube with 1 ml of Trizol in a FastPrep 120 machine for two cycles of 45 s each at a speed setting of 6.0 m/s with ice incubation between cycles. Extracted RNA was purified, loaded onto a *Plasmodium/Anopheles* microarray, hybridized, scanned and analyzed. Global gene expression analysis showed that AgDNV infection of *A. gambiae* resulted in only four genes being modestly differentially upregulated, with zero genes downregulated. The results were validated by qPCR analysis of the four suspect genes. Of those four genes, two were identified as associated with mild stress response, one partially regulates hydrolysis during digestion, and one is a component of the mating plug. These modest expression differentiations are contrasted by other pathogenic viruses infected into *A. gambiae* that were found to affect the expression of hundreds of genes, including many responsible for innate immune response and antiviral pathways. This suggests that AgDNV has minimal impact on survivability and gene expression in its infected host and is therefore a good candidate for a paratransgenesis approach to vector control.

Buruli ulcer (BU) is an infectious disease that occurs primarily in sub-Saharan Africa and is caused by the polyketide toxin mycolactone produced by *Mycobacterium ulcerans*, a ubiquitous environmental pathogen. BU cases typically involve children who have been swimming in African rivers, and the disease is believed to be transmitted by the bite of a water bug that harbors *M. ulcerans* in its mouth. BU infection can result in severe scarring ulcerations that can cause disability if not properly treated. A BU outbreak in an arid Australian city prompted Johnson *et al.*[5] to suspect a new mode of transmission. Pools of ≤23 mosquitos were homogenized in Lysing Matrix A tubes on a FastPrep instrument, and DNA was purified using the FastDNA Kit per the manufacturer's protocol. Resulting pure DNA was used in a fluorescence-based RT-PCR assay that was primed and probed for three high-copy sequences. Two were insertion sequences, IS*2404* and IS*2606*, taken from the previously sequenced *M. ulcerans* genome, and the third was the ketoreductase B domain from the *p*MUM001 plasmid, previously designed to express the genes that encode for mycolactone production. Pools that were positive for all three sequences were subjected to amplification and sequencing of variable number tandem repeats (VNTR) locus 9 with nested PCR. PCR products that met the expected fragment length were sequenced and analyzed using CDC software to determine the maximum likelihood that a given mosquito was infected with *M. ulcerans*. Maximum-likelihood estimates for five different species of mosquitos trapped in the BU-outbreak region were higher than those for species trapped in regions with no human BU cases, suggesting that the mosquitos were the transmission vector.

Conclusions

The FastPrep-24 5G is a novel, ultra-high-performance sample preparation system that allows for the extraction of fully intact, biologically functional macromolecules from routine and highly resistant samples and that is well suited for virus-research applications. MP Biomedicals offers solutions for other research applications from low- to high-throughput scale with a selection of sample preparation instruments, Lysing Matrix tubes and complete purification kits. Learn more at http://www.mpbio.com/fastprep5g.

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