Next Generation Sequencing of Microbiomes

New Solutions for Nucleic Acid Extraction and PCR Amplification

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Key words: microbiome, removal of DNA from dead microbes and human cells, universal lysis of microbes, DNA-free extraction reagents, DNA-free amplification reagents, Universal Microbe Detection

The Human Microbiome Project and other approaches are dedicated to the exploration of the vast complexity of microbial communities living in association with the human body, including the skin, oral cavity, upper respiratory tract, stomach, intestine, and genitourinary tract. The aim of these projects is to understand host-microbe interactions with regard to human health and disease. Next Generation Sequencing is a powerful analytical tool capable of resolving the microbiota in respect to community structure, dynamics, and activity, i.e., gene expression. This overview discusses pre-analytical problems and solutions contributed by new tools.

While several sophisticated Next Generation Sequencing (NGS) platforms have been developed for sequencing several hundreds Mb per run, important constraints have been realized to be associated with the processes upstream of the analytics. In their recent review Rogers and Bruce [1] flagged pre-analytic parameters that can dramatically influence the quality of NGS results. Among the error-prone steps are sample processing, nucleic acid extraction and purification, and PCR amplification (Fig. 1). In the field of molecular diagnostics of infectious diseases, much experience has been gained with highly sensitive detection and identification of microorganisms in human specimens. Table 1 summarizes a conspicuous problem, i.e., contamination of reagents and consumables. Cutting-edge commercial solutions have been developed that are worth considering for NGS. Among them is Molzym’s Universal Microbe Detection which comprises a collection of diagnostic research tools dedicated to the removal of DNA from human cells and dead microbial cells, universal lysis of Gram-negative, Gram-positive and fungal organisms and provision of DNA-free extraction and PCR reagents. In the following sections we will pinpoint the critical pre-analytical processes and discuss Universal Microbe Detection as a potential solution that may contribute to more accurate results in NGS approaches of microbiome research.

Sample processing

The problem: Major aims of NGS are the characterization of the viable microbiota and the investigation of its dynamics in a certain community. However, PCR-based analysis does not discriminate between DNA originating from viable and dead microbes. Rogers and Bruce [1] emphasize that persisting DNA from dead cells can contribute greatly to an incorrect picture of the inferred microbiome and mask changes in the community structure. Another less obvious, but highly significant parameter is the co-extraction of human DNA which, in particular with universal 16S rRNA gene PCR approaches will be co-amplified [2]. The generation of nonspecific human sequences during amplification will thus increase the number of sequencing runs and hence increase the cost of analysis. An illustrative example of the problem is given by the approach of Auburn et al. [3] who analyzed by NGS the diversity of

![Fig. 1: Steps in the processing of samples for Next Generation Sequencing analysis (adapted from [1]) and pre-analytic parameters negatively influencing the results. Thereby, the inferred microbial community structure and function may be significantly divergent from the real microbial community. Indicated by the bracket are the steps (Universal Microbe Detection) where new pre-analytic solutions may contribute to the accuracy of the results.](image-url)
### Table 1: DNA contamination of commercial reagents and consumables

<table>
<thead>
<tr>
<th>Material</th>
<th>% False-positives (found/tested) *</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Collection of samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood collection tubes</td>
<td>17 (31/185)</td>
<td>Aspergillus spp.</td>
<td>[9]</td>
</tr>
<tr>
<td>Blood serum tubes</td>
<td>10 (16/160)</td>
<td>Aspergillus spp.</td>
<td>[9]</td>
</tr>
<tr>
<td>Urine collection tubes</td>
<td>8 (2/25)</td>
<td>Aspergillus spp.</td>
<td>[9]</td>
</tr>
<tr>
<td>Blood culture medium</td>
<td>n.d.</td>
<td><em>Lactococcus lactis; Bacillus coagulans</em></td>
<td>[12]</td>
</tr>
<tr>
<td><strong>B) Nucleic acid extraction and processing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zymolase</td>
<td>n.d.</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>[13]</td>
</tr>
<tr>
<td>DNA extraction</td>
<td>100 (20/20)</td>
<td><em>Burkholderia</em> spp., <em>Pseudomonas saccharophilia</em>, <em>Ralstonia</em> spp., <em>Acaligenes</em> spp.</td>
<td>[14]</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
<td><em>Brucella</em> spp.</td>
<td>[18]</td>
</tr>
<tr>
<td>Nucleic acid precipitation (glycogen)</td>
<td>22 (2/9)</td>
<td><em>Acinetobacter</em> <em>twwolfii</em></td>
<td>[19]</td>
</tr>
<tr>
<td>RNA stabilization reagent</td>
<td>5 (1/20)</td>
<td><em>Aspergillus</em> spp.</td>
<td>[9]</td>
</tr>
<tr>
<td><strong>C) PCR reagents</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq polymerases and master mixes</td>
<td>100 (4/4)</td>
<td>bacteria</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>100 (4/4)</td>
<td><em>Pseudomonas</em> spp.</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>8 (2/24)</td>
<td><em>Sphingomonas</em> spp., <em>Moraxella</em> spp.</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>2 (1/41)</td>
<td><em>Acinetobacter</em> <em>junii</em></td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>10-30 (n.a.)</td>
<td><em>Coxiella burnetii</em></td>
<td>[23]</td>
</tr>
<tr>
<td><strong>D) Plastic consumables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipette tips</td>
<td>18 (6/32)</td>
<td>bacteria</td>
<td>[24]</td>
</tr>
</tbody>
</table>

* Tested samples of the same or different lots or samples from different manufacturers; signals were observed in negative PCR controls using molecular grade water; species were identified by sequencing of the amplicons and BLAST search.

Plasmodium falciparum extracted directly from human blood samples. By using various human DNA depletion methods (≥70% reduction) the Plasmodium sequence yield could be increased to obtain approx. 40-fold coverage of the genome per lane.

**The solution:** The Universal Microbe Detection methodological approach provides a tool, MolYsis™, which by selective lysis of human cells and DNase treatment eliminates false human signals generated during amplification [2]. Similarly, human DNA was shown to be removed at 90-100% efficacy from clinical caries and periodontal specimens [4]. As a consequence of MolYsis™ pre-treatment of blood samples, a 20-fold increase of MRSA diagnostic sensitivity as compared to total DNA extraction was observed [5]. Last, by using MolYsis™ sample pre-treatment to remove DNA from dead microbial cells, Sakka et al. [6] were able to monitor by Real-Time PCR the decline of a *Staphylococcus epi-dermidis* bacteremia as a response to successful antibiotic treatment. In recent studies, MolYsis™ pre-sample treatment contributed greatly to the culture-independent identification of etiologies of sep-sis and endocarditis, including mixed strain infections [7, 8]. In conclusion, by removing DNA from human and dead microbial cells, MolYsis™ may contribute also to the accuracy of NGS analysis of community structure and dynamics.

**Nucleic acid extraction and purification**

The problem: A crucial step in the preparation of samples for analysis is nucleic acid extraction and purification. In order to reflect representative members of the community by NGS the ideal extraction procedure could lyse any microbe present in a sample and efficiently recover microbial nucleic acids, including removal of amplification inhibitors. The maximum diversity and the accuracy of relative abundance of species depend on the efficacy of the nucleic acid preparation. Rogers and Bruce [1] emphasize careful consideration of the particular characteristics of the sample type and the selection of suit-
able methods for processing. Another important constraint is the presence of microbial DNA contaminating extraction chemicals and consumables (see Table 1, B).

The solution: MolYsis™ addresses the aforementioned limits of nucleic acid extraction and purification. Experience again comes from molecular diagnostics. The kit contains a reagent, BugLysis, which includes a blend of enzymes lysing Gram-positive and Gram-negative bacteria as well as fungi. In a multitude of finalized and running clinical evaluations using specimen types as diverse as blood, cerebrospinal fluid, joint aspirates, sputum, BAL, heart valves, lung, and skin biopsies, 168 species from 89 genera within the major eubacterial clades (Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes) and 24 species from 13 fungal genera have been identified by universal PCR plus sequencing so far. The broad range of organisms identified shows the great potential of BugLysis reagent as a means of supplying DNA from diverse microorganisms to amplification for more accurate analysis of complex microbiota of the human body.

DNA purification reagents and columns delivered with MolYsis™ are quality-controlled for the absence of contaminating microbial DNA (≤3% false positive results). Using the clinical material mentioned above, PCR inhibitors have been found to be efficiently removed as controlled by PCR internal control reactions.

Amplification

The problem: Because NGS is a high resolution approach, contamination of PCR reagents by microbial DNA (see Table 1, C) has a significant impact on the data generated. Also, plastic consumables may be a source of microbial DNA (Table 1, D). Contamination becomes even more a problem when universal amplification like 16S rRNA gene PCR is employed.

The solution: Within the Universal Microbe Detection concept, Molzym provides DNA-free PCR reagents, including a highly active Taq polymerase or ready-to-use mastermixes with or without primers for 16S rRNA gene PCR. Manufacturing is strictly controlled in order to guarantee DNA-free (≤3% false positives) PCR reagents with high polymerizing activity.

Universal Microbe Detection

All-in-one: Besides single components addressing particular problems on the track of sample preparation, a complete system is available for NGS analysis of microbiota associated with the human body. Universal Microbe Detection provides protocols for the processing of a variety of samples, nucleic acid extraction of living microbial cells, and PCR amplification of taxonomically resolving sections of the 16S and 18S rRNA genes of bacteria and fungi, respectively. All components are DNA-free thereby excluding reagent and consumables-borne DNA contamination from NGS analysis.

References


* The complete list of species is available on request


[24] Own results. Among three manufacturers, one was showing severe contamination of the tips. The other products (PCR tubes, pipette tips) were continuously free of any DNA contamination as analysed by 16S/18S rDNA PCR (n=32 to 320; different lots tested).

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