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Internal Control DNA for PCR Assays Introduced into Lambda Phage Particles Exhibits Nuclease Resistance, Markus Stöcher and Jörg Berg* (Institute of Laboratory Medicine, General Hospital Linz, Krankenhausstrasse 9, A-4020 Linz, Austria; * author for correspondence: fax 43-732-7806-1815, e-mail joerg.berg@akh.linz.at)

Real-time PCR assays are widely used for the detection and quantification of pathogen-derived nucleic acids in clinical samples (1–3). Because clinical specimens contain PCR-inhibitory moieties that are not always reliably removed during nucleic acid extraction, real-time PCR assays are furnished with internal amplification controls (IACs) to identify those samples in which the PCR is inhibited (4–9). Thus, false-negative test results or falsely diminished quantification results can be avoided.

For pathogen-specific real-time PCR assays, the IAC is usually added to clinical samples, recovered in the process of nucleic acid extraction, and amplified in either a multiplex or competitive fashion with detection usually by discrimination of hybridization probes (4–9). Plasmid-derived DNA is commonly used as IAC. In this instance, the procedure does not provide entire control of PCR assays because the lysis of pathogens during the nucleic acid purification procedure is not monitored. Plasmid-derived IACs consist of bare, unprotected DNA; therefore, the IAC may become degraded within the clinical specimen before nucleic acid extraction or during storage in the working stock, which could lead to unreliable amplification of IAC DNA, necessitating repetition of testing.

In this report, we use a traditional lambda phage cloning procedure to generate phage particles containing target-specific IAC DNA, i.e., phage IAC. As IAC DNA we used the previously described multiple IAC that was generated for a panel of virus-specific real-time PCR assays with competitive IACs (10). We show that the obtained phage IAC contains one IAC DNA fragment, is resistant to DNase I digestion, and exhibits improved storage and handling properties as well as reliable amplification in the respective competitive real-time PCR assays.

The IAC DNA was excised from the respective plasmid (pCR-II-TOPO) by EcoRI restriction under standard conditions (37 °C for 4 h). The DNA fragments were separated on a 2.5% agarose gel. After excision, the IAC DNA was recovered by use of the QiAquick PCR Purification Kit (Qiagen), according to the manufacturer’s instructions. The purified IAC DNA fragment was inserted into the EcoRI cloning site of lambda phage DNA with use of the Lambda gt11/EcoRI/CIAP-Treated Vector Kit (Stratagene). Phages were generated according to the manufacturer’s instructions with minor modifications. Briefly, the ligation reaction was carried out at 16 °C for 16 h in a 5-μL volume containing 1 ng of IAC DNA, 1000 ng of EcoRI-digested lambda DNA, and 10 U of Escherichia coli DNA Ligase (Invitrogen). A 2-μL portion of this ligation mixture was then subjected to in vitro phage packaging. Transfection was performed under standard conditions with E. coli host strain Y1088 (200 μL of cell suspension and 1 μL of in vitro-packaged phage particles). Plating was performed according to the manufacturer’s instructions except that 10-cm standard sterile plastic Petri dishes (Greiner Bio-One) were used. After incubation at 37 °C for ~8 h, single plaques were recovered in 400 μL of SM buffer (100 mmol/L NaCl, 50 mmol/L Tris, 8 mmol/L MgSO4·0.1 g/L gelatin, pH 7.5). Each phage clone that contained a single IAC DNA insert was amplified in E. coli host strain Y1088 in a separate 10-cm Petri dish, according to the manufacturer’s instructions. Thereafter, the plates were overlaid with 6 mL of SM buffer, and phages were allowed to diffuse into the SM buffer at 4 °C overnight. The obtained phage IAC particles were stored in SM buffer at 4 °C, and two 1-mL aliquots were stored frozen at −80 °C in SM buffer containing 70 mL/L dimethyl sulfoxide.

Phage IAC DNA was purified from suspensions in SM buffer (200 μL) or from clinical specimens, either manually with use of the High Pure Viral Nucleic Acid Kit (Roche, Applied Science), or by an automated process using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche) on the MagNA Pure LC instrument (software version 2.1). Both methods were performed according to the manufacturer’s instructions, and the elution volume was 50 μL.

Phage clones were tested for the presence of IAC DNA by real-time PCR on the LightCycler instrument 1.0 (Roche; software version 3.5.3) with use of Fast Start DNA Master Hybridization Probes (Roche) added to 4 mM MgCl2, 0.2 μM each of the phage-specific primers (gt11-for, 5′-CGACTCCTGGAGCCCG-3′; gt11-rev, 5′-TGA-CACCAAGCACAAGTGTTAG-3′), and 0.2 μM each of the IAC-specific fluorescence resonance energy transfer hybridization probes (5′-TGACAGGGCTGATC-CGCT-fluorescein-3′ and 5′-LC-Red705-CTGCCCATTC-GACACGAG-phosphate-3′; see Fig. 1A); PCR conditions were exactly as described by Stöcher et al. (10). In

References

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addition, the PCR products were also analyzed by agarose gel electrophoresis to test for concatemers and the correct product size of 425 bp. Of 20 phage IAC clones picked and analyzed, 4 clones contained a single IAC DNA insert, 6 clones appeared to contain concatemers of IAC DNA, and 10 plaques showed no DNA inserts. The results of analyses of two clones are presented in Fig. 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue11/. DNA from one phage IAC clone containing a single IAC DNA insert was assessed by ultraviolet spectrophotometry and subjected to automated sequence analysis, which confirmed the expected DNA sequence.

To examine whether the obtained phage IAC exhibits nuclease resistance, we incubated phage IAC (10^4 copies) for 1 h at 37 °C in 20 μL containing 4 U of DNase I...
(Ambion). Equal amounts of purified phage IAC DNA and plasmid IAC DNA were examined in parallel. After DNase I treatment, sample volumes were brought to 200 µL, and samples were subjected to automated DNA purification and analyzed by real-time PCR with the outermost primers for the IAC DNA [for PCR conditions and primer sequences, see Stöcher et al. (10)]. As shown in Fig. 1B, the phage IAC was not degraded by DNase I, whereas the IAC DNAs were completely degraded. Notably, there was no shift in crossing points between DNase I-treated and mock-treated phage IAC samples. This suggests that the generated phage IAC preparation contained intact phage particles, which protected the IAC DNA from nuclease degradation.

A storage stock of phage IAC was prepared as a 1:10⁷ dilution, corresponding to ~5 × 10⁶ copies/mL, in SM buffer and was kept at 4 °C. The stability of this stock was tested over the following 6 months. At the end of each month, eight 20-µL aliquots were removed and brought to 200 µL. DNA was prepared from each aliquot by the automated process and tested by real-time PCR with the outermost primers for the IAC DNA. The results are presented in Table 1 and show only small variations in the crossing points over the 6-month period. This suggests that the prepared phage stock was stable over at least 6 months at 4 °C (Table 1). This is not surprising because lambda phage cloning procedures describe stable storage of those phages in SM buffer for several years (11).

To determine whether the phage IAC would complement our panel of real-time PCR assays, we prepared a working stock by diluting the storage stock 1:50 in SM buffer. When 20 µL (corresponding to 2000 copies of phage IAC) of this working stock was added to 1 mL of plasma sample, the assay detection limits were the same as reported recently (9) and the phage IAC was reliably amplified. Examples of the amplification results obtained from our routine clinical testing of patient samples to which phage IAC had been added are shown in Fig. 1C. When the negative controls, i.e., normal plasma to which phage IAC had been added, were analyzed in the respective PCR assays, the intra- and interassay CVs were 0.9–1.6% and 1–2.6%, respectively. These results agreed with or were better than those obtained when freshly isolated plasmid-derived IAC DNA was used (9). However, when the plasmid-derived IACs were stored over an extended period of time at 4 °C or at −20 °C or were subjected to more than one freeze/thaw cycle, we observed much more reliable amplification of the phage IAC than the plasmid-derived IAC.

The described procedure to generate phage particles containing IAC DNA should also be applicable for other plasmid-derived IACs. Thus, either an IAC DNA fragment or a whole plasmid could be cloned into the lambda vector used because this type of vector allows the insertion of DNA fragments ranging from a few base pairs up to ~7000 bp (12, 13). The cloning procedure involves EcoRI restriction sites; therefore, the IAC DNA fragment should ideally not contain an EcoRI restriction site. This is only a partial requirement, however, because there are other lambda phage vectors available with cloning sites other than EcoRI (13). For ligation of the IAC DNA into the lambda DNA, it is important that the lambda DNA is used in considerable excess of the IAC DNA to avoid gross generation of concatemeric inserts. After cloning and selection of the respective phage IACs, one round of phage amplification yielded ~10¹⁴ phage IAC copies. This should theoretically suffice for the testing of more than 10¹⁴ patient samples when phage particles equivalent to ~2000 IAC copies/mL are added before DNA purification.

When handling phage IACs from storage or working stocks, we noted that careful multiple up-and-down pipetting is crucial for the preparation of homogeneous suspensions and dilutions of the phage IAC. This ensures that working stocks are prepared with reliable phage concentrations and that each clinical sample receives the same amount of phage IAC. When the phage IAC was used in our panel of real-time PCR assays, IAC amplification was very reliable in our clinical laboratory routine. One reason for this may relate to the fact that the IAC DNA is packed into phage protein and thus is protected from nuclease attack, as shown by our experiments with DNase I. A 7-day short-term stability test in plasma and cerebrospinal fluid revealed that the phage IAC is stable in plasma and cerebrospinal fluid for 3 days at room temperature or at 4 °C at the concentrations added to patient samples before PCR analysis. After 3 days, we observed various degrees of degradation of the phage IAC. In contrast, we observed degradation of the plasmid-derived IAC after 24 h at room temperature or after 48 h at 4 °C (for results, see Table 1 in the online Data Supplement).

The generation of phage particles has already been successfully applied to the generation of RNA IACs (Armored RNA) for reverse transcription-PCR assays (6, 14). The cloning of our IAC DNA fragment into lambda phage and the subsequent application to our PCR assays provide advantages similar to those described for Armored RNA IACs (14). Collectively, these are the resistance to nuclease, much lower degradation in clinical samples, and the possibility of storage of low-titer stocks at 4 °C over extended periods of time. These characteristics allow convenient use of the presented phage IAC in the routine clinical laboratory. The presented lambda phage IAC bears infectivity but with a strong bacterial resistance to nuclease.

**Table 1. Stability of phage IAC stored at 4 °C.**

<table>
<thead>
<tr>
<th>Storage time, months</th>
<th>Mean (SD) crossing point, cycle no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33.09 (0.12)</td>
</tr>
<tr>
<td>1</td>
<td>33.11 (0.71)</td>
</tr>
<tr>
<td>2</td>
<td>33.38 (0.49)</td>
</tr>
<tr>
<td>3</td>
<td>33.36 (0.46)</td>
</tr>
<tr>
<td>4</td>
<td>33.15 (0.48)</td>
</tr>
<tr>
<td>5</td>
<td>32.97 (0.64)</td>
</tr>
<tr>
<td>6</td>
<td>32.24 (0.51)</td>
</tr>
</tbody>
</table>

*Phage IAC was stored at 1:10⁷ dilution in SM buffer.*
host specificity. There thus is little danger that a laboratory worker could be infected (15). However, proper clinical laboratory safety precautions should be followed because the phage IACs represent genetically modified organisms. The presented protocol to generate phage IAC could be applied to any clinical PCR assay in which DNA from pathogens is detected. When phage IACs are added to clinical specimens before DNA extraction, all steps of the PCR assay, including lysis of pathogen particles, can be controlled.

In addition, the presented approach might also be used for the generation of positive-control DNAs or DNA quantification standards for PCR assays. The resulting phage standards should be more stable than purified DNA and should exhibit improved handling and storage conditions compared with purified DNA standards for the clinical laboratory. For pathogen-specific PCR assays, the use of phage DNA standards could avoid handling of infectious pathogenic standards possible.

In conclusion, the generated phage IAC is one example of the application of lambda phage cloning for the generation of IACs for clinical PCR assays. The presented phage IAC, with its improved storage and handling properties, gave good results when used in our panel of real-time PCR assays and appears suitable for routine use; it also allows complete monitoring of PCR assays for sample adequacy.

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References


Simultaneous Measurement of Kynurenine and Tryptophan in Human Plasma and Supernatants of Cultured Human Cells by HPLC with Coulometric Detection, Benjamin Maneglier,1,2* Christine Roegz-Kreuz,2,3 Paulette Cordonnier,1 Patrice Theron,1 Charles Advenier,1 Dominique Dormont,2 Pascal Clayette,4 and Odile Spreux-Varoquaux1 (1 Département de Biologie, Pharmacologie, Centre Hospitalier de Versailles, Faculté de Medecine Paris Ile de France Ouest, Le Chesnay, France; 2 Service de Neurovirologie, Commissariat à l’Energie Atomique (CEA), Centre de Recherches du Service de Santé des Armées, Université Paris XI, Ecole Pratique des Hautes Etudes, Institut Paris Sud sur les Cytokinés, Fontenay-aux-Roses, France; 3 Unité de Biologie du Développement et de la Reproduction, Département de Physiologie Animale, Institut National de Recherche Agronomique, Jouy-en-Josas, France; 4 SPI-BIO, c/o Service de Neurovirologie, CEA, Fontenay-aux-Roses, France; * address correspondence to this author at: Département de Biologie, Pharmacologie, Centre Hospitalier de Versailles–Hôpital Andre Mignot, 177 rue de Versailles, 78157 Le Chesnay, France; fax 33-1-3963-9598, e-mail bmaneglier@ch-versailles.fr; † deceased)

L-Tryptophan (Trp) is an essential amino acid and an abundant protein component. Trp is metabolized in mammals via two pathways: biosynthesis of the neurotransmitter serotonin and the kynurenine (Kyn) pathway (1, 2). Trp is the substrate for the first step in both of these pathways. In the Kyn pathway, the indole ring of Trp can be opened by the enzymes tryptophan pyrrolase [tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase (IDO)] (3). The reactions catalyzed by both of these enzymes constitute the rate-limiting step in the Kyn metabolic pathway (4). The organ distribution of the two enzymes differs, allowing them to be clearly distinguished (5, 6). Tryptophan 2,3-dioxygenase is located primarily in the liver, and its activity is up-regulated in response to Trp and metabolic steroids. IDO can be found in various cells, but it is not active in healthy humans and its activity is induced only during immune responses mediated by proinflammatory cytokines such interferon-γ (7, 8).

The enzymatic reactions of the Kyn pathway produce NAD and other intermediates, including Kyn and quinolinic acid (5, 9). The removal of Trp from the microenvironment via this pathway protects the organism by limiting the growth of intracellular pathogens and malignant