MethodsX 2 (2015) 159–164

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Improved internal control for molecular diagnosis assays

T. Vinayagamoorthy *, Danielle Maryanski, Dilanthi Vinayagamoorthy, Katie S.L. Hay, Jacob Yo, Mark Carter, Joseph Wiegel

MultiGEN Diagnostics LLC, 854 Paragon Way, Rock Hill, SC 29730, United States

A B S T R A C T

The two principal determining steps in molecular diagnosis are the amplification and the identification steps. Accuracy of DNA amplification is primarily determined by the annealing sequence of the PCR primer to the analyte DNA. Accuracy for identification is determined either by the annealing region of a labelled probe for the real time PCR analysis, or the annealing of a sequencing primer for DNA sequencing analysis, that binds to the respective analyte (amplicon). Presently, housekeeping genes (Beta globin, GAPDH) are used in molecular diagnosis to verify that the PCR conditions are optimum, and are thus known as amplification controls [1–4]. Although these genes have been useful as amplification controls, they lack the true definition of an internal control because the primers and annealing conditions are not identical to the analyte being assayed. This may result in a false negative report [5]. The IC-Code platform technology described here provides a true internal control where the internal control and analyte share identical PCR primers annealing sequences for the amplification step and identical sequencing primer annealing sequence for the identification step.

- The analyte and internal control have the same PCR and sequencing annealing sequences.
- This method assures for little or no false negatives and false positives due to the method's design of using identical annealing conditions for the internal control and analyte, and by using DNA sequencing analysis for the identification step of the analyte, respectively.
- This method also allows for a set lower limit of detection to be used by varying the amount of internal control used in the assay.

A R T I C L E I N F O

Method name: True negative control for PCR amplification and DNA sequencing coupled analysis

Keywords: Multiplex PCR, Nucleic acid amplification test, Sanger sequencing, Analyte, Pb2a, IC-Code

Article history: Received 19 January 2015; Accepted 6 March 2015; Available online 12 March 2015

* Corresponding author.
E-mail address: moorthy@multigen-diagnostics.com (T. Vinayagamoorthy).

http://dx.doi.org/10.1016/j.mex.2015.03.002

Method

Primer design

IC-Code™ is a DNA sequencing platform technology to develop nucleic acid amplification test (NAAT) with true internal control. The scientific concept of IC-Code™ is based on using a synthetic DNA construct that has the same PCR and sequencing primer annealing sequences as that on the analyte (Fig. 1). The analyte and synthetic DNA construct will have identical sequencing primer annealing sites located approximately 25 bp internal of either the downstream or forward primer, with sequencing being targeted toward that primer. Therefore, the sequence generated will represent only that of 25 bp region. To distinguish the internal control amplicon from the analyte amplicon, the nucleotide sequence between the 3′end of the PCR primer and 3′ end of the sequencing primer will carry a homo-nucleotide sequence (dTTP in this report) to tag the internal control. PCR primers used were Pb2a UP: ATGATATAAACCACCCAATTTGTCTGCCAGTTTCTCCTTG and Pb2a LP: TCAATCTATAGCG-CATTAGAAAATAATGGCAATATTAACGCACCTC. Sequencing primer used was Pb2a SP: TTATGCAAACT-TAATTGGCAAATCCGGTAC.

Sequencing analysis

The synthetic DNA is either added externally before the sample is processed or will be in the sample collection tube itself. In the absence of the analyte (true negative), the PCR primers will generate a single amplicon using the synthetic DNA of the IC-Code™ as its template (Fig. 1). During sequencing of this amplicon, the sequencing primer will bind to its annealing site on the synthetic DNA internal control and generate a unique poly-T sequence. However, in the presence of an analyte (true positive), two amplicons will be generated with one from the analyte and the other from the synthetic internal control, and both will be sequenced simultaneously (Fig. 1). Therefore, the electropherogram will show mixed bases from both amplicons. In order to visualize the analyte nucleotide sequence, T signals are masked from the sequences generated by the two amplicons using a feature in the analysis

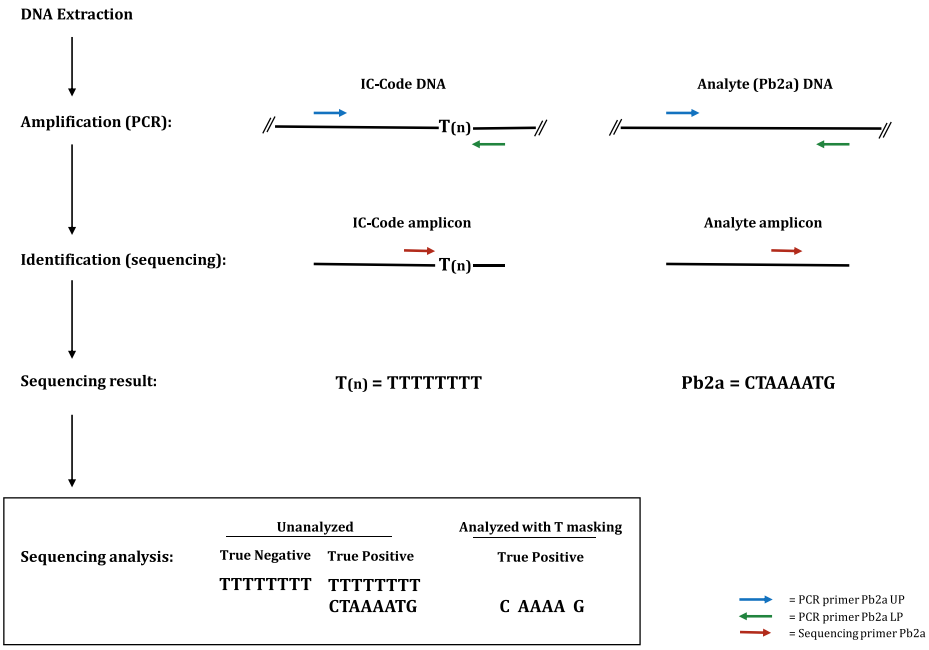

Fig. 1. Schematic flowchart and expected data for the application of IC-Code™ for molecular diagnostic assays.