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A set of external reference controls/probes that enable quality assurance between different microarray platforms

Hideo Akiyama [a,*], Yoji Ueda [a], Hitoshi Nobumasa [a], Hiroyuki Ooshima [b], Yohei Ishizawa [c], Koji Kitahiro [d], Isao Miyagawa [d], Kazufumi Watanabe [e], Takazumi Nakamura [f], Ritsuka Tanaka [g], Nobuko Yamamoto [h], Hiroki Nakae [h], Mitsuo Kawase [i], Nobuhiro Gemma [j], Yuji Sekiguchi [k], Wataru Fujibuchi [l], Ryo Matoba [c,*]

[a] New Projects Development Division, Toray Industries, Inc., Kamakura, Kanagawa 248-8555, Japan
[b] Yokohama Research Laboratories, Mitsubishi Rayon Co. Ltd., Yokohama, Kanagawa 230-0053, Japan
[c] DNA Chip Research Inc., Yokohama, Kanagawa 230-0045, Japan
[d] Technical Research Laboratory, Kurabo Industries Ltd., Neyagawa, Osaka 572-0823, Japan
[e] Hokkaido System Science Co. Ltd., Sapporo, Hokkaido 001-0932, Japan
[f] S-BIO Business Division, Sumitomo Bakelite Co. Ltd., Amagasaki, Hyogo 661-8588, Japan
[g] Department of Bio Research, Kamakura Techno-Science, Inc., Kamakura, Kanagawa 248-0036, Japan
[h] Japan Multiplex bio-Analysis Consortium (JMAC), Chiyoda-ku, Tokyo 102-0083, Japan
[i] Graduate School of Biomedical Engineering, Tohoku University, Sendai, Miyagi 980-8579, Japan
[j] Ricoh Institute of Technology, Ricoh Company, Ltd., Yokohama, Kanagawa 224-0035, Japan
[k] Bio-Measurement Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, Ibaraki 305-8566, Japan
[l] Computational Biology Research Center, Advanced Industrial Science and Technology (AIST), Koto-ku, Tokyo 135-0064, Japan

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ABSTRACT

RNA external standards, although important to ensure equivalence across many microarray platforms, have yet to be fully implemented in the research community. In this article, a set of unique RNA external standards (or RNA standards) and probe pairs that were added to total RNA in the samples before amplification and labeling are described. Concentration–response curves of RNA external standards were used across multiple commercial DNA microarray platforms and/or quantitative real-time polymerase chain reaction (RT–PCR) and next-generation sequencing to identify problematic assays and potential sources of variation in the analytical process. A variety of standards can be added in a range of concentrations spanning high and low abundances, thereby enabling the evaluation of assay performance across the expected range of concentrations found in a clinical sample. Using this approach, we show that we are able to confirm the dynamic range and the limit of detection for each DNA microarray platform, RT–PCR protocol, and next-generation sequencer. In addition, the combination of a series of standards and their probes was investigated on each platform, demonstrating that multiplatform calibration and validation is possible.

Recent advances in DNA microarray technology have opened up new applications in both basic and clinical research [1–4]. Consequently, new tests in many areas of biomedical science, including clinical pharmacogenetics, cancer genotyping, and cancer prognosis, have been developed [5–7].

Clinical applications of DNA microarray technology include gene expression analysis for early disease detection, disease classification and diagnosis, selection of treatment protocol, determination of changes in disease status, and the monitoring of therapeutic effects and side effects. A clinical application in which DNA microarray gene expression analysis has already been applied is the "MammaPrint," developed in the United States and Europe, used to select the optimal breast cancer treatment [5]. In addition, OncoType DX, a product based on quantitative real-time polymerase chain reaction (RT–PCR)[1], has also been used for analyzing the expression of multiple RNA targets as an indicator in the selection of optimal breast cancer treatment [6].

* Corresponding authors.
E-mail addresses: hideo_akiyama@nts.toray.co.jp (H. Akiyama), matoba@dna-chip.co.jp (R. Matoba).

[1] *Abbreviations used:* RT–PCR, real-time polymerase chain reaction; HURR, human universal reference total RNA; HBRR, human brain reference total RNA; JMAC, Japan Multiplex bio-Analysis Consortium; cDNA, complementary DNA; 3D, three-dimensional; aRNA, antisense amplified RNA; SSC, sodium saline citrate; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; mRNA, messenger RNA.

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However, if DNA microarray data are to be routinely used for clinical applications, it is vital that the data are both reliable and reproducible and that errors or ambiguities in the interpretation of results are eliminated [8–10]. In particular, because gene expression is highly variable, quality assurance in the handling of specimens—storage conditions, transport conditions, and pretreatment protocols—must be robust (Fig. 1).

We report here the development of a set of unique RNA external standards (or RNA standards) and probe pairs that may be spiked into test samples to ensure equivalence across many microarray platforms. This suite of synthetic nucleotides is derived from unique non-mammalian sequences and designed to minimize cross-hybridization with common transcripts from humans, mice, and rats. Six microarray platforms were evaluated using this set of standards: 3D-Gene (Toray Industries, Tokyo), Agilent SurePrint (Agilent Technologies, Santa Clara, CA, USA), Genopal (Mitsubishi Rayon, Tokyo), GeneSQUARE (Kurabo Industries, Osaka, Japan), S-Bio (Sumitomo Bakelite, Tokyo), and NimbleGen (Roche NimbleGen, Basel, Switzerland). An RT–PCR protocol (Life Technologies, Foster City, CA, USA) and a next-generation sequencer GAII (Illumina, San Diego, CA, USA) were also tested. We compared performance across DNA microarray platforms and/or RT–PCR and next-generation sequencing by spiking a set of our standards into a commonly available commercial total RNA sample. A variety of standards can be added in a range of concentrations spanning high and low abundances, thereby enabling the evaluation of assay performance across the expected range of concentrations found in a clinical sample.

Using this approach, we show that we are able to confirm the dynamic range and the limit of detection for each DNA microarray platform, RT–PCR protocol, and next-generation sequencer. In addition, the combination of a series of standards and their probes was investigated on each platform, demonstrating that multiplatform calibration and validation is possible (Fig. 2).

Materials and methods

RNA external standard transcripts

Ten candidate external RNA standard clones (in pUC19 plasmid) were synthesized from artificial sequences designed to have the following characteristics: (i) a unique sequence that exhibits low similarity with any eukaryotic genome and EST sequence known to date, (ii) no nucleic acid homopolymer longer than three bases, (iii) a G+C content in the range of 40 to 60%, (iv) no repeated sequences such as a motif, and (v) no strong secondary structure within the sequence. The standard sequences were designed by using our original program software. Inserts for the clones are 500 to 1000 bp with a 30-bp polyadenylated tail and T7 promoter sequence. All candidate standards were prepared by in vitro transcription of linearized plasmids using a T7 RNA polymerase (MEGAScript Kit, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Ten transcripts corresponding to the RNA external standards were purified using TURBO DNase (Life Technologies) and further purified by phenol–chloroform extraction and ethanol precipitation. The 10 standard transcripts were dissolved in RNase-free water and then quantified using a Quant-iT RNA Assay Kit (Life Technologies). The sequences of the external standards (R001-500 to R010-1000) have been deposited in the DDBJ/GenBank/EMBL databases under the accession numbers AB610939 to AB610950.

RNA external standard spiked total RNA cocktail

Human universal reference total RNA (HURR, Agilent Technologies) and human brain reference total RNA (HBRR, Agilent Technologies) controls were used. Ten external RNA standards were diluted using HURR or HBRR RNA solution at 50 ng/ml. The standard spiked total RNA cocktail (see Supplementary Tables S1 and S2 in online supplementary material) was prepared at the Japan Multiplex bio-Analysis Consortium (JMAC) central laboratory and delivered to each test site.

Design of probe for RNA external standards

For probe design, each external standard was divided into two regions as follows: 1- to 300-nt and 301- to 500-nt regions for 500-nt RNA and 1- to 500-nt and 501- to 1000-nt regions for 1000-nt RNA, numbering from their 3′ ends. All candidate sequences from the sense strand were extracted by moving 60-nt windows in each region.

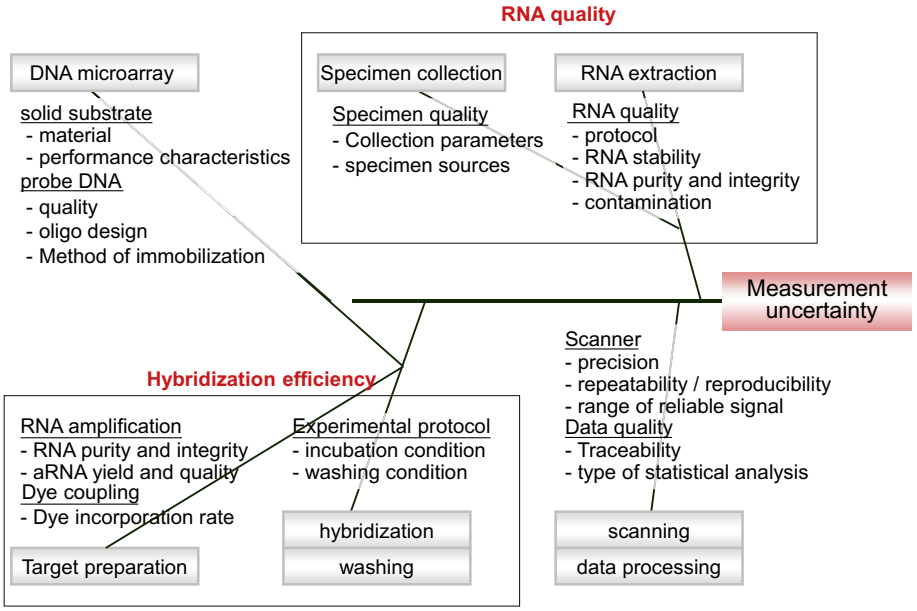

Fig.1. Measurement uncertainty of DNA microarray analysis. Unless the uncertainties of a measurement are being evaluated and stated, the fitness for the purpose of measurement cannot be judged properly. The uncertainties of a measurement using microarray are complicated and intertwined. The sources of uncertainties come from mainly the platform material, RNA quality, and hybridization efficiency and during data acquisition and processing.