SPOTLIGHT feature

Biotechnology, Immunology & Pharmaceuticals

Ultrapure Water as a Component of Multi-Parametric Assays Used in Drug Discovery

Sven P. Wichert[1], Elmar Herbig[2], Michael C. Wehr[1]
Correspondence: Michael C. Wehr, Systasy Bioscience GmbH, Adams-Lehmann-Str. 56, 80797 Munich, Germany; e-mail: wehr@systasy.de

Applications for ultrapure water are widespread in biotechnology as this water is used for cell cultivation, biochemical procedures and molecular biology techniques, such as polymerase chain reactions (PCR). This article describes the specific use of ultrapure water, generated by the arium® pro VF laboratory water system, as an integral component of cell-based multi-parametric profiling assays. Such assays, termed EXTassays, are employed at early stages of drug discovery. In these assays, cellular signalling events are captured by molecularly barcoded reporters, resulting in the acquisition of large data sets, which are obtained using next-generation sequencing (NGS). In a sample experimental setup, differential signalling properties were addressed, which were either caused by a specific stimulus (EGF-like domain) or a broad stimulus (PMA and serum). EGF-like domain-treated cells displayed an immediate early gene response, whereas the addition of PMA and serum caused activation of immune response pathways. Addition of lapatinib inhibited virtually all responses induced by EGF-like domain, whereas PMA/serum-mediated responses were only partially reverted. Notably, ultrapure water was effectively used in various processing steps, which included, inter alia, the isolation of molecular barcode reporters, their amplification using various PCR strategies and the NGS run. The ultrapure water applied meets all the criteria for use in sensitive applications of molecular biology.

In biomedical research and pharmaceutical drug discovery, cell-based assays are increasingly combined with multi-parametric assay techniques to better understand highly complex disease mechanisms and the effects of compounds targeting diseases. Such assays require the implementation of methods based on molecular and cell biology, which themselves use ultrapure water as basis for performing many individual techniques, such as polymerase chain reactions, PCR, or for cultivating cells.

Cell-based assays are applied to identify differences of defined cellular signalling actions caused, for example, by the addition of a given compound. These differences in activity can be determined by relative changes of defined classes of reporter molecules (e.g., messenger RNA [mRNA], micro-RNA, proteins, etc.). Investigating the effects of biologically relevant molecules, such as chemical compounds and antibodies, on defined cellular activities is commonly very time-consuming, costly, and requires considerable quantities of consumables. In addition, it is important to ensure the highest purity of the solutions employed, e.g. of water. For these reasons, multi-parametric assays are becoming increasingly important.

Description of the Assay Procedure

Cell-based multi-parametric assays allow analysis of compound actions on many cellular target molecules, called targets, and profiling of cellular signal pathways in parallel. These comprehensive analyses of complex cellular signalling activities are required to show the desired medicinal effect on targets (ON target effects) and pathways (ON pathway effects) and to simultaneously identify any undesirable side effects (OFF target/pathway effects). Such assays may also be used for uncovering new uses of approved medical drugs in so-called drug repurposing studies. Furthermore, the acquisition of large data sets from multi-parametric assays provides remarkable cost benefits, which can be obtained, for instance, using EXTassay technology [1].

This technology is based on molecular barcode reporters, enabling highly paralleled assays. EXT stands for *Expressed Sequence Tag*: EXTs are short synthetic RNA molecules with a specifically encoded sequence that has a length of 49 bp. Thus, EXTassay technology permits acquisition of several millions of data sets within one measurement as individual cellular signalling events captured by molecularly encoded reporters can be analysed by sequencing [1] (*Figure 1a*). Standard reporter gene assays allow measurements of only one or two data points per unit. Therefore, instrument-based solutions are used to acquire large data sets. By contrast, the specific assay system is based on reporter molecules, so-called EXT reporters, also termed EXT barcodes. Each EXT reporter, which is invariably linked to a defined individual signalling event, has a molecular address that enables precise analysis of the reporter's spatial and temporal expression. Using these barcodes, many different cellular signalling activities can be simultaneously monitored, such as the activity of receptors and downstream cellular signalling events (*Figure 1b*).

In the multi-parametric assays discussed in this paper, next-generation sequencing (NGS) is used as a readout for functional and quantitative analysis of the various cellular activities. NGS was initially employed to sequence genomes [2]. The high-paced advancement of NGS resulted in its present use involving medical aspects, such as therapeutic diagnostics and the identification of disease risk genes. The following describes the possibilities for using ultrapure water in the various stages of this multifaceted method.

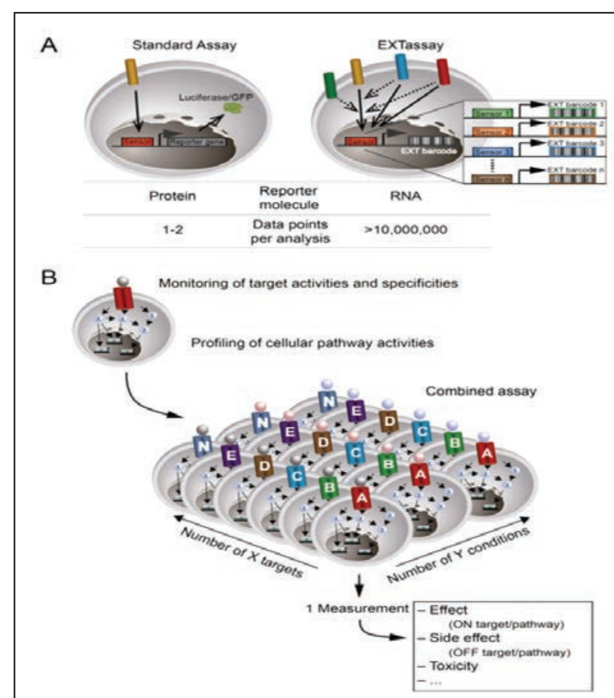

Figure 1: A) The specific assays allow simultaneous analysis of several cellular signalling activities within one measurement. Unlike standard reporter gene assays, specific assays provide more than 10 million data sets per experiment. B) The assays profile compound actions on target specificities and cellular signalling pathways. Signalling profiles of X-different targets under Y-different conditions are simultaneously captured in one measurement (source: all figures provided by the authors unless otherwise specified).

Materials and Methods

Uses of Ultrapure Water for Multi-parametric Assays

Ultrapure water plays a critical role in the various steps within one experiment using specific reporters. In our studies autoclaved ultrapure water was used, for instance, to humidify cell culture incubators to ensure microbe- and spore-free incubation of cells. Ultrapure water was also employed to determine molecular biological parameters (e.g., concentrations of DNA and RNA).

Furthermore, ultrapure water was used to prepare samples for NGS; i.e., purification of EXT reporters using an RNA isolation kit, reverse-transcribing into cDNA sequences and subsequent performance of PCR steps needed to amplify the reporter material for high-throughput sequencing. Ultrapure water was also utilised in the final NGS runs performed using an *Ion Torrent Personal Genome Machine®* (Ion PGM™ sequencer) supplied by Life Technologies (see below).

The ultrapure water was produced using arium® pro VF (*Figure 2*) as described in Nitzki and Herbig [3].

Cell Culture

PC12-tet-OFF (PC12-OFF) cells (Clontech) were cultivated in DMEM medium (glucose concentration of 1 g/L, Lonza) on poly-L-lysine-coated plates at 37°C in an incubator with an air atmosphere humidified by ultrapure water and containing 5% CO_2. The medium was supplemented with 10% Fetal Bovine Serum (FBS), 5% Horse Serum (HS), 1% penicillin streptomycin and 1% GlutaMAX (Invitrogen).

Transfection of Assay Reporter Vectors

PC12-OFF cells were transfected in suspension with assay reporter vectors. For this purpose, the cells were trypsinized, pelleted and resuspended in DMEM (supplemented with only 1% FBS) with a density of 10^6 cells per mL. DNA-Lipofectamine complexes were prepared with 1 µg of DNA and 4 µL of Lipofectamine 2000 (Invitrogen) in aliquots of 100 µL OptiMEM Medium per 1.5 x 10^6 cells. After 20-minute incubation at room temperature, the mixture was combined with the cell suspension and incubated at 37°C for 4 hours. To remove the transfection reagents, the cells were washed once with DMEM medium (1% FCS). Before analysis, the cells were cultivated under identical conditions. For the assays, 5 ng per plasmid DNA and per EXT exporter construct were used. Three to five reporter constructs were cloned with different EXTs for each cis-regulatory element.

Figure 2: Current arium® pro VF ultrapure water system (source: Sartorius Lab Instruments)

Purification of Sequencing Probes

RNA Extraction

RNA was purified using a Qiagen RNeasy kit along with on-column DNase-I digestion according to the manufacturer's protocol. After RNA purification, the probes were precipitated by one-half volume 7.5 M ammonium acetate (prepared using ultrapure water) and three-fold volume of 100% ethanol. For cDNA synthesis, 1 µg of extracted RNA, Superscript III Reverse Transcriptase (Invitrogen) and a 120 pmol random nanomer primer were used. The samples were tested for their RNA purity, and possible DNA contamination was excluded by running controls that did not contain any reverse transcriptase (-RT).

Amplification of EXT-cDNA Products by PCR for Decoding

Decoding PCR runs were performed using a HotStarTaq Plus DNA polymerase, dNTPs (both supplied by Qiagen) and ultrapure water. Thirty 3-step cycles (30 sec. at 95°C, 30 sec. at 59°C and 30 sec. at 72°C) were run (*Figure 3*). Reverse-transcribed cDNA served as the starting material. No cDNA was used in the negative control (last lane).

Coding PCR runs were carried out also using HotStarTaq Plus DNA polymerase and ultrapure water; however, only ten 3-step cycles were run (cycle steps: 30 sec. at 95°C; 20 sec. at 58°C; 20 sec. at 72°C) (*Figure 3*). For each Coding PCR, 1 µL of a 1:10 diluted Decoding PCR sample was utilised as the starting material; no cDNA was used in the negative control. All PCR products were visualised and tested by agarose gel electrophoresis.

Sequencing According to the Semiconductor Method

EXT reporter molecules were sequenced by NGS using an Ion PGM™ machine (*Figure 4*). The sequencing technology of this machine is based on sequential detection of protons that are released during the polymerisation process of DNA. Depending on the base to be polymerised (adenine, guanine, cytosine or thymine) on the complementary strand, the corresponding dNTP is incorporated and a proton is released, which is detected by ion sensors [4].

Sequence Analysis

Individual UNIX-based tools from the FastX Toolkit [5] were used to analyse the sequencing data (FASTQ-to-FASTA conversion program and FASTX barcode splitter). The presence and identity of each EXT was determined by BLAST (Basic Local Alignment Search Tool) analysis [6] against a self-prepared reference library of EXTs, and differentially expressed EXTs were identified and quantified using R scripts [7].

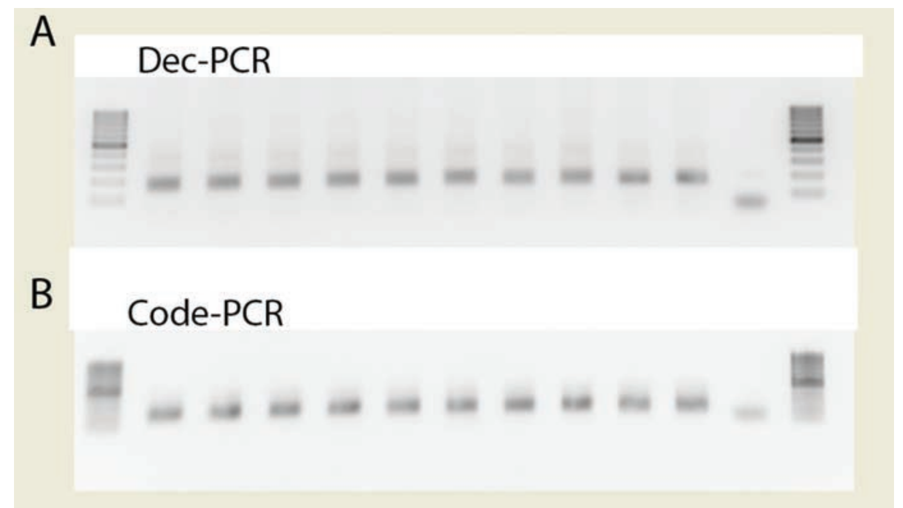

Figure 3: Quality control of EXT-amplifying PCR steps. Agarose gel runs of A) Decoding PCR and B) Coding PCR. The last lane of each run indicates the negative control with ultrapure water; primer dimers are visible in the last lanes.

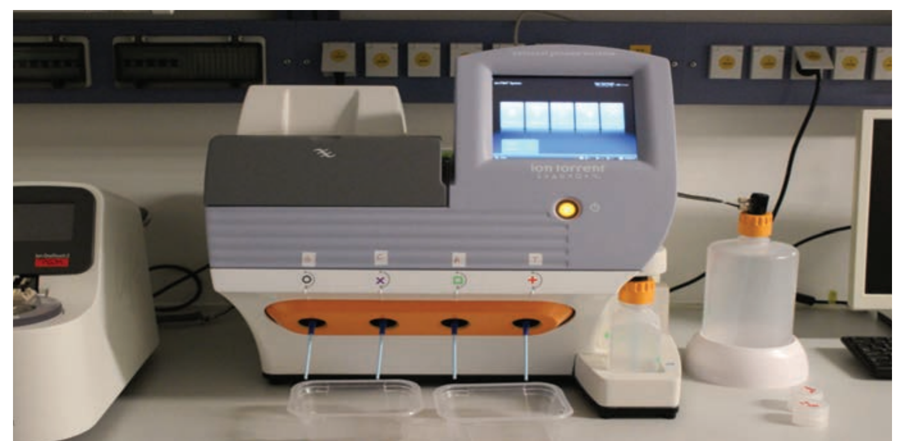

Figure 4: Ion Torrent Personal Genome Machine® (Ion PGM™ sequencer) (source: laboratory at Systasy Bioscience GmbH, Munich, Germany).

Results

The multi-parametric assay platform was used in combination with NGS and ultrapure water to measure a large number of downstream cell signalling responses that occur after a) a specific stimulus, b) a broad stimulus or c) after the addition of a specific inhibitor. In this case, PC12 cells were transfected with 33 different EXT reporters, which represent the activities of eleven cellular signalling pathways. To apply a specific stimulus (a), the ERBB4 receptor was transfected. This receptor plays a role in many different biological processes, including heart development and differentiation of neurons, as well as in associated human diseases, such as cancer and psychiatric disorders, including schizophrenia [8,9]. The ERBB4 receptor can be activated by its ligand Neuregulin 1, or by the biologically active and soluble domain of Neuregulin 1, EGF-like domain (EGFld), and multi-parametric assays were previously used to monitor the activities of the ERBB4 receptor [1,10]. Further, ERBB4 signalling activity mediated by EGFld was specifically blocked by the ERBB kinase inhibitor lapatinib (c), which is also used as a cancer therapy drug. A combinatorial stimulus consisting of phorbol-12-myristate-13-acetate (PMA) and serum was selected as a broad stimulus (b).

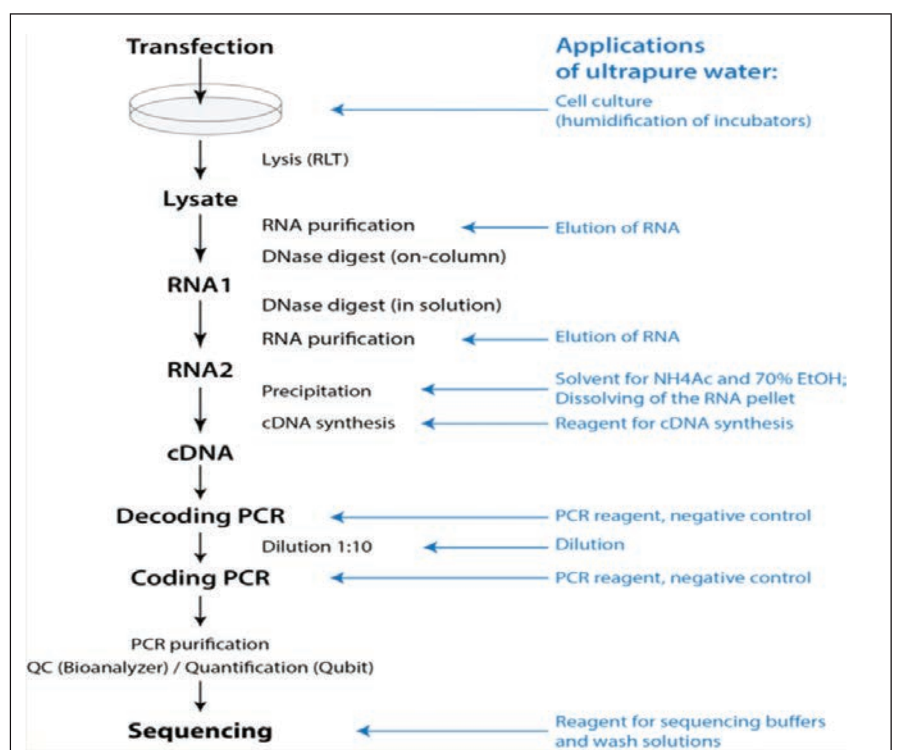

Figure 5: Workflow for purification of sequencing samples and the use of ultrapure water for the individual steps

Ultrapure Water as an Integral Component of Sample Preparation for Sequencing

Ultrapure water was successfully used in several steps for sample preparation (*Figure 5*). In particular, it was employed in molecular biological procedures, such as for PCR runs for sample preparation (Decoding and Coding PCRs, *Figure 3*) and for sequencing reactions, which were performed using the Ion PGM^{TM} sequencer (*Figure 6*). High water quality with a resistivity of 18 MΩ x cm was required for sequencing with this instrument according to the instruction manual (see User Guide [11]), and reliable sequencing would otherwise not be guaranteed. The ultrapure water used for the experiments described in this study was free of detectable quantities of RNase and DNase (concentrated RNases and DNases below the detection limits of 20 ng/mL and 80 ng/mL, resp.) and was delivered in consistent, stable quality required for the experiments, with a very low TOC concentration (≤ 2 ppb) and a resistivity of 18.2 MΩ x cm that was compensated to 25°C.

Quantitative and Kinetic Analysis of Cellular Signalling Activities

A quantitative and kinetic analysis of downstream signalling activities shows a differential activation pattern, which is specific for each particular stimulus (*Figure 7*). Thus, in EGFld-treated cells, the immediate early gene (IEG) response was strongly activated (IEGs are genes that are activated transiently and rapidly in response to cellular stimuli via MAP kinase signalling cascades; exemplary IEGs are EGR1, EGR2, FOS, FOSB and JUN [12]), whereas signalling pathways related to immune stress responses (IL6, IL8 and NFkB) were not activated (*Figure 7*, columns 1–4). Cells treated with both EGFld and the inhibitor lapatinib showed a nearly complete inactivation of all signalling pathways measured (*Figure 7*, columns 5–8). In cells stimulated with PMA/serum, immune stress responses were markedly induced (*Figure 7*, columns 9–12), which, in contrast, were only partially reverted after treatment with lapatinib (*Figure 7*, columns 13–16) (cf. data sets for 2 hours after addition of lapatinib).

Ultrapure water was effectively used within multi-parametric assays and subsequent NGS analyses. In a sample experimental setup, it was demonstrated that cellular effects of compounds could be examined using the EXTassay multi-parametric profiling platform by monitoring the activities of various signalling pathways. This will enable researchers to better understand the mechanisms of ON and OFF target effects of compounds within cells already during the early stages of drug development. Further, this profiling approach may thus substantially lower attrition rates and costs in medical drug discovery.

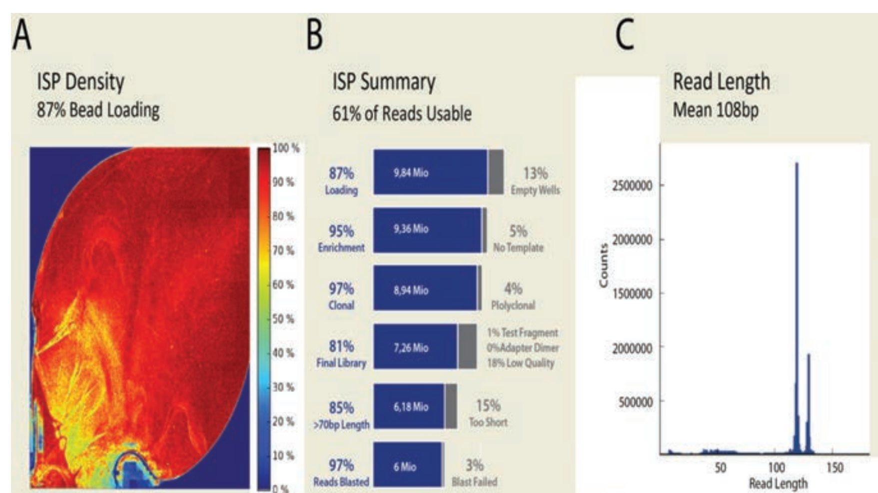

Figure 6: Quality control of sequencing using the Ion Torrent Personal Genome Machine® (PGM^{TM} sequencer). A) The ion sphere particle (ISP) density shows the loading density of the bead-loaded IPSs according to a colour scale. B) The ISP summary contains information on the loading density (loading 87%, 9.84 million wells), the enrichment of sequence-loaded ISPs (95% enrichment) before sequencing, the number of clonal ISPs (97% clonal), the sum of all sequences obtained (7.26 million reads) and the number of the sequences that could be used for analysis (6 million reads blasted). C) Information on the average length of the sequences individually obtained (read length).

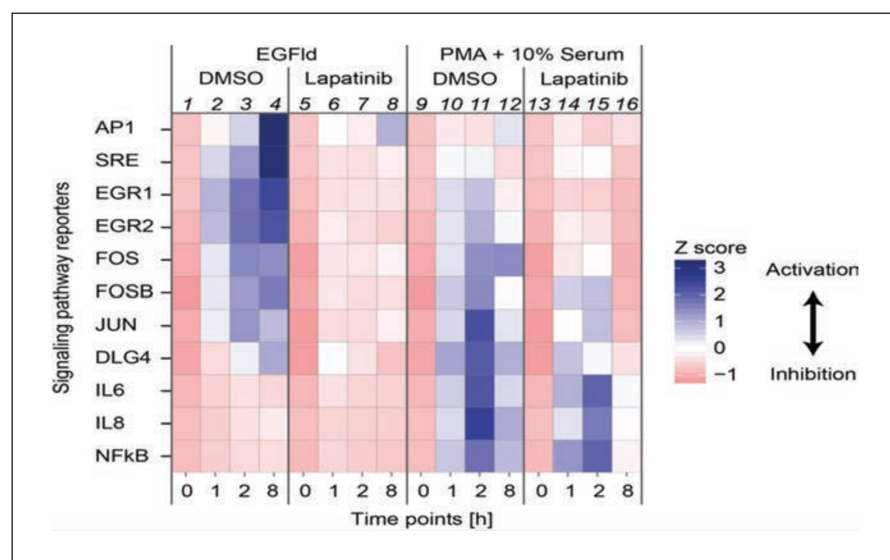

Figure 7: Quantitative and kinetic analysis of cellular signalling activities using the specific assay technology (the binding sites for the transcription factors are designated AP1, SRE and DLG4; see text for explanations of the other signalling pathway reporters). Signalling activities induced by the specific stimulus EGF-like domain (EGFld) or by the broad stimulus phorbol-12myristate-13-acetate (PMA)/10% serum are inhibited to varying degrees by the inhibitor lapatinib. The column numbers are given in italics.

Discussion

The ultrapure water employed in the experiments described here can be readily used for the biochemical, molecular biological and cell biological applications described above, leading to meaningful results. This water was utilised, inter alia, for the critical steps of NGS sample preparation using PCR strategies and subsequent high-throughput sequencing runs (*Figures 3 and 6*). Using multi-parametric assays, robust responses of cellular signalling that were differentiated between a specific and a broad stimulus could be identified (*Figure 7*).

Sequencing reactions in particular require high ultrapure water standards, which the Type 1 water used in these experiments meets with its exceptionally low TOC concentration and consistent quality.

In addition, ultrapure water obtained from the lab water system was free of detectable nuclease activity – this feature is important as just a trace of nuclease activity may substantially interfere with sensitive applications in molecular biology, such as PCR runs – and constantly provides a conductivity of 18.2 MΩ x cm.

Accurate sequencing with the Ion PGM^{TM} machine can therefore be performed on samples prepared with the ultrapure water generated by the lab water system used. Such ion torrent sequencing requires constant water purity with a resistivity of 18.2 MΩ x cm. Unpublished data show that purified reagent-grade water with a resistivity of less than 18.2 MΩ x cm is not suitable for biochemical and molecular biological applications, such as Western blot analyses and PCR runs. All quality parameters listed for ultrapure water must be fulfilled in order to obtain reproducible results.

By contrast, use of purified water with poorer quality parameters or slight impurities will lead to unsatisfactory results. Tap water should be completely avoided from the start as it contains many constituents including salts, organic compounds, nucleases, microorganisms and unbound DNA molecules. Moreover, tap water quality may considerably vary from region to region.

Summary

Multi-parametric assays allow comprehensive profiling of cellular signalling activities and are increasingly used in the early phases of drug discovery to rapidly and cost-effectively discriminate between desirable ON target and undesirable OFF target effects of compounds. The assays described in this study are based on genetic sensors coupled to molecular barcode reporters, and require that all materials utilised meet the highest quality standards. This was exemplarily demonstrated for ultrapure water that was successfully used for various experimental steps within multi-parametric assays.

Acknowledgements

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[1]Max Planck Institute for Experimental Medicine (Max-Planck-Institut für Experimentelle Medizin), Goettingen, Germany; now Systasy Bioscience GmbH, Munich, Germany

[2]Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany