Original Article

A daily quality assurance routine for ultrasounds *in vitro* experiments

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Background: In few decades, the technical steps forward accomplished into the acoustic signal transduction and control fields, have brought to the safe release of a large amount of acoustic energy in the body. Today, the high intensity focused ultrasound (HIFU) machines are able to induce tissue's necrotic thermocoagulation in the region of interest (ROI) without the need of any invasive procedure, simply setting the physical parameters modulating the acoustic beam, like the phase of the elements of the transduction array. This technology found its natural employment into the treatment of solid tumors, where thermal therapies were already well established, but is now emerging for his non-thermal effects. These are the mechanical ones, first of all cavitation, for years held up as a side effect to be avoided and now seen as a possible way to enhance drug delivery or realize malignant tissues disruption through histotripsy. While thermal effects are clearly known, high repeatable and daily employed into the clinical field; mechanical effects of ultrasounds are still under investigation and the way to the oncological treatment seems to be long. For these reason, but not only, HIFU *in vitro* experimentations are still an open field in which a non accurate control, could lead to inconclusive or discordant results.

Methods: We approached the question suggested by the clinical practice, where a daily quality assurance (DQA) is required in order to execute a treatment. The InSightec ExAblate 2100 (InSightec Ltd, Tirat Carmel, Israel) MRgFUS equipment available in our institute (LaTO srl, Cefalù (PA), Italy) has been employed, assessing the power delivered at the hypersonic focus with a series of radiation force measurements, 3D modeling and geometrical tests.

Results: A linear relation between the electrical power and the real acoustic power, at the spot elevation, has been obtained. A simple software with a simpler graphical user interface to plan and automatically execute ultrasounds *in vitro* experiments has been specifically designed. A clear protein denaturation in target phantoms has been achieved with a high spatial precision.

Conclusions: In the following paper we report the practice we established in order to declare our system efficiency before *in vitro* experiments can start. This routine is not perfect, and it's not an universal formula, but it satisfies our repeatability requirements. Our intent is to reinforce the self-controlling attitude that has to be part of every research.

Keywords: *In vitro*; quality control; ultrasound; radiation force

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Introduction

Ultrasounds (US) are extensively used in medicine both for diagnostic imaging and for noninvasive therapies. Their high-intensity and focused version are recently emerging as a promising weapon for solid tumors treatment (1). Therapeutic strategies are based on the capability of acoustic waves to interact with biological soft tissues through thermal and non-thermal physical mechanisms, to produce a wide range of bioeffects. As thermal bioeffects are referred changes in living cells and tissues resulting from temperature increase due to the conversion of acoustic energy into heat. In relation with the total acoustic power delivered and the duration of the exposure, different temperatures could be reached. In the low-power and low-duration acoustic applications, formerly that one used in the diagnostic medical sonography applications, energy absorption is limited and temperature rise is well below 2-3 ℃ (2). This rise cannot be neglected for pre-natal ecography where the temperature of the fetus should not safely rise more than 0.5 ℃ above its normal temperature (3). Even though there is no experimentation that can prove a causal link between birth defects and in utero ultrasound exposure, the relation between brain malformations and US exposure longer than 30 minutes in mice is been demonstrated (4). Increasing energies beyond the range of diagnostic ultrasound, the range of the hyperthermia (to 40-43 ℃) is found, in which the ultrasound are used to heal or to enhance others therapies (5). Over, with temperatures higher than 43 ℃, it possible to reach the thermal ablation of tissues, with an efficacy that depends on the duration of the exposure and on the cellular types (6,7). Nowadays the clinical application of the High Intensity Focused Ultrasound (HIFU) systems is limited to few procedures, every one based on the employment of the thermal effects. The most established of these is the treatment of uterine fibroids (8), for which, the Magnetic Resonance guided Focused Ultrasound Surgery (MRgFUS) ExAblate 2000 (InSightec Ltd, Tirat Carmel, Israel) received in 2004 the FDA approval. In the meanwhile, HIFU systems guided by both magnetic resonance and US, underwent clinical trials for fields in which have proven them reliability: palliative treatment of bone metastasis (9), breast (10) and prostate (11) cancer but also in treatment of the essential tremor (12). Some of these systems are daily employed in Europe thanks to the CE mark approval and time seems to be ripe for liver treatment too (13). Much more work is required to push on the clinical employment of non-thermal US for therapy applications. Large part of non-thermal mechanisms could be referred to mechanical effects, such as radiation force, radiation torque and acoustic streaming which act as physical forces applied on tissues (2). One of the most relevant non-thermal effects is the cavitation, described as the formation, growth, oscillation and collapse of gaseous microbubbles within tissues (14). The negative pressure peak makes water and volatile substances pass from the liquid to the gas state at a very small scale [depending on US amplitude and frequency (15)]. Bubbles of gas begin oscillate around a neutral position, expanding and rarefacting their volume in a harmonic way. In the low intensity regime, the phenomenon is almost stable, generating some micro-streaming that induces small sized pores into the cell's plasma membrane (16). Increasing energy can turn cavitation into unstable, making bubbles collapse with the generation of a powerful micro-jet (17) that can damage permanently the lipid membrane. At the highest energies, cavitation can lead to a complete tissue disruption, useful feature for an application like histotripsy (18). It is impossible to totally avoid cavitation during an ablation treatment; for this reason this phenomenon is often used as an advantage in solid tumor treatment, but it's the most widely investigated non-thermal bioeffects for its non-disruptive potential. Ultrasound mediated Targeted Drug Delivery (UmTDD) (19) and SonoDynamic Therapy (SDT) (20) are two of the novel promising therapeutic applications of Focused UltraSound (FUS) employing mechanical effects. Extensive physical and biological studies have been carried out and there are encouraging evidences to suggest that FUS exposure is a powerful stimulus to induce stress response and apoptosis with minimal lysis in several cancer cell lines (21-24); hence, in non-surgical cancer treatment, US apoptotic anticancer therapy is beginning to emerge as a preferred approach to achieving tumor cell killing. Furthermore, US irradiation of cancer cell lines in conjunction with hyperthermia (25), photodynamic therapy (26), radiotherapy (27,28) and chemotherapy (29) produces a synergistic effect to cell death *in vitro*, described as the overall decrease in number of viable cells and inhibition of cancer cell proliferation. In order to investigate the potential of US in these applications, *in vitro* researches are essential, to identify and better understand molecular mechanisms induced within cells and tissues. In a few *in vitro* experiments, clinical systems are also used to guide and monitor the beam, some examples are the magnetic resonance imaging (19,30) and the US (23). In other experiments authors

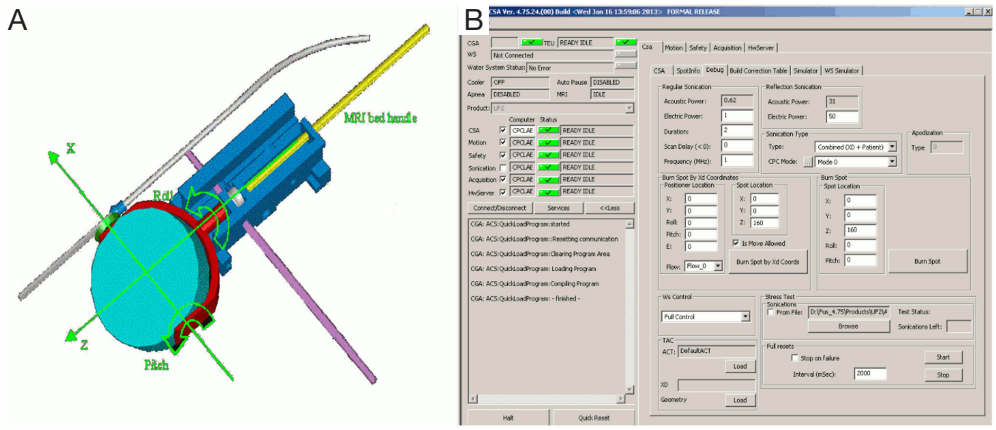

Figure 1 The transducer (A) can be moved acting on CGA-CSA. 4.75.24.[00] software interface (B).

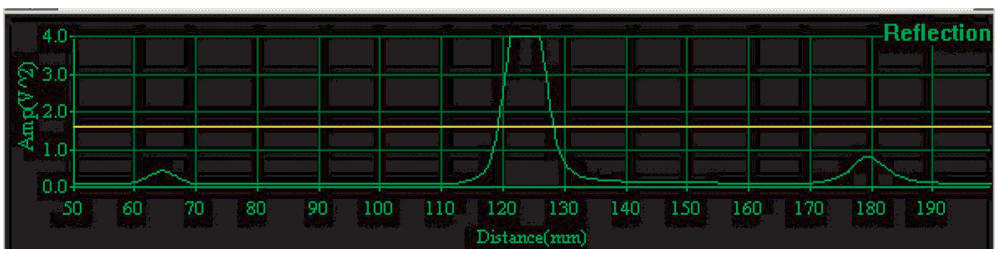

Figure 2 Test of the plate's distance from the transducer by energy reflection.

reserve a part of the study to the acoustic characterization, employing professional instruments such as US power meter (14,21,22,27,31) or hydrophones (24,25,32-35) to determine the averaged (in space, time and both of them) intensity (W/cm^2) or the pressure field (MPa). In these works, cells are grown onto supports of various kind and nature, like flasks (36), tubes (25,37), and Petri dishes (38,39), and exposed to the protocol in study into water basins (40) in which the transducer is housed. This is an easy way to ensure a strong acoustic coupling of the whole apparatus, but at the same time carry with it a lot of uncertainty: to avoid stationary wave arise, beams are often addressed to cells with a non-perpendicular path, but that can cause unpredicted reflections and cells exposition to an high spatial variability (17). For focused clinical systems, the use of cultures plates is preferred (41,42). Plates are housed in a fixed geometry setup, coupled with the transducers bath. Flasks, plates, tubes or Petri dishes, if not specifically designed (34,43), are made of plastic materials (polyethylene, polystyrene), which are not impermeable to ultrasound and could potentially absorb a large part of the energy delivered (25,37). In order to obtain repeatable and precise results an accurate ultrasound characterization and control is needed, like it is done in the clinical field, where a daily quality assurance (DQA) is a routine for the HIFU applications (44,45). Our belief is that, if the role of a physical variable of interest is investigated, the capability of the apparatus to control it should be checked before every session. In the following we will show the tests we have carried out in order to obtain useful data from our *in vitro* researches, considering that our initial interest is on the power delivered and the focus position in the space.

Materials and methods

Focused ultrasound system

The source of US, is the InSightec ExAblate 2100 (InSightec Ltd, Tirat Carmel, Israel) MRgFUS equipment available in our institute (LaTO srl, Cefalù (PA), Italy) (46). It incorporates a 208 elements phased array transducer which can operate with a frequency that varies from 0.9 to 1.3 MHz and an energy from 100 to 6,000 J. This transducer has five degrees of freedom in the space: it can

be elevated (Z), moved in the horizontal plane (XY) and rotated around the two axes that form this plane (roll and pitch angles). The transducer can be moved through his axis thanks to CGA-CSA. 4.75.24.[00] software (*Figure 1*). It also is possible to modify power, frequency and duration of the signal. A continuous impulse with 1 MHz frequency, 20 s duration and a variable intensity (<10 W/cm^2) have been preferred for the purpose of assess the ranges commonly used in "low intensity" *in vitro* experiments (47). Below, we have characterized the radiation force. The software used (*Figure 1*), gives the possibility to monitor the reflection (*Figure 2*) in the hypersonic field giving a single pulse of desired power before the requested insonation. This feature reduces the probability of damaging the transducer, and gives the possibility to monitor the distance of the objects receiving the acoustic energy. Spot position in the space is one of the relevant variables that has been investigated too.

Radiation force and weighing system

US are the result of a mechanical perturbation of a medium, like air or water, which is elastic and continuous. This perturbation generates the travel of a pressure wave that runs from the emitter to the receiver. Real bodies are not perfect emitter and their vibration produces not just a single traveling plane wave, but a field to which, every single object within is subjected. This pressure is said "radiating" (P_{Rad} N/cm^2) and the integration of its value all across a surface is the Radiation Force F_{Rad}. It is difficult to obtain a complete understanding of this pressure field, P_{Rad}(S), but it is possible to apply a simplification when the total acoustic power output W_{ac} and the speed of the sound c, in the medium in which is submerged, are known:

$$F_{Rad} = W_{ac} / c \ \text{[N]} \qquad [1]$$

This expression is reliable for high absorbing ($\alpha \approx 1$), low reflecting bodies (2). A correct estimation of the radiation force means a correct evaluation of transducer power and then of the intensity brought to the target spot. An easy and cheap way to measure radiation force indirectly is made by the acquisition of the apparent weight reduction of a high absorbing target. This reduction is the result of the difference between the real weight force of the target and the radiation force of the hypersonic beam, supposed to act in opposition into the vertical direction. For this purpose we employed a high precision (0.01 g) digital scale for generic purpose. The scale was placed over the top of a water phantom, with its weighing plate firmly linked to the guides of the acoustic target, as shown in *Figure 3*. An external

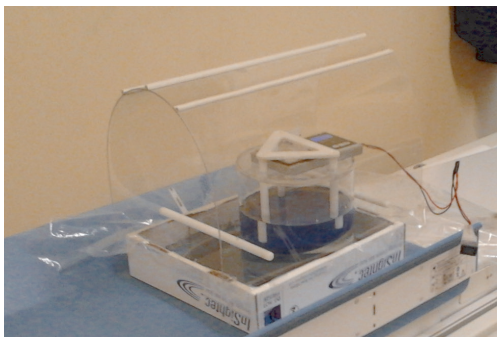

Figure 3 Weighing system with shield and external commando.

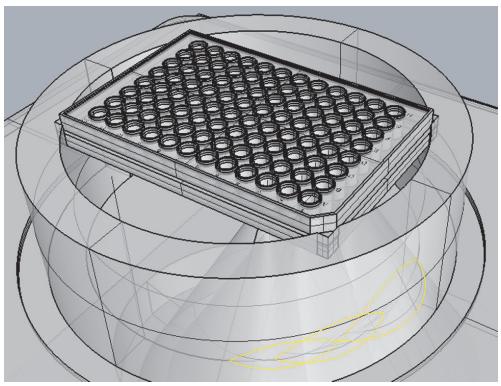

Figure 4 3D model of the InSightec support for culture plates. In yellow the hypothetic interceptions of the hypersonic cones with the support.

command was added to avoid the perturbations of the system caused by the movement induced by cyclic tare procedures. Then an acrylic-glass support for the MR pelvic coil was put around the weighing system to shield it (*Figure 3*) from the environmental perturbations. Those evidence were averaged and transcribed into a spreadsheet with a resolution of 1 record for second.

3D modeling

To overcome the deficit of an US guide and predict beams path, we have developed the 3D model shown in *Figure 4*. The model reproduces the essential parts of the ExAblate 2100 in the *in vitro* configuration. The coupling and positioning system for culture plates, supplied by InSightec, are also represented. It has a water phantom with two plastic rings specially carved to house the standards culture plates and keep them in a fixed position in the space. These plates, used in *in vitro* studies (26,41,48), have standard dimension.

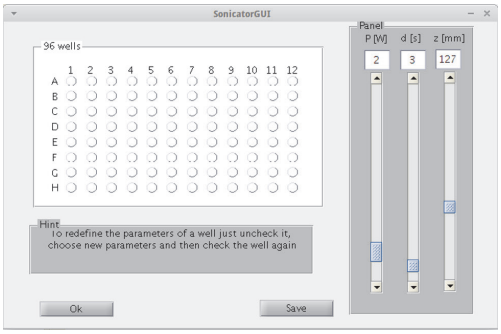

Figure 5 The planning interface of the custom code "Sonicator".

Gel phantom targets

Following the procedure described in (49), a transparent polyacrylamide hydrogel, supplemented with bovine serum albumin (BSA) has been realized. This phantom has the feature to be optically transparent at room temperature, becoming opaque once overheated, thanks to protein denaturation of BSA. We filled with this gel some of the 96 wells of a special plate with a membrane at the bottom (Gas Permeable Plates. Coy Laboratory Products, Grass Lake, MI, United States), in order to verify the geometrical accuracy of our automatic system.

Custom software

Geometrical calibration is an iterative process. The CGA-CSA has the capability to execute multiple insonations with different features reading the informations from an *.ini file (stress test panel, *Figure 1B*); but the coordinates have to be known. In order to automatically execute these operations, a MatLab® (MathWorks) custom code was been wrote (*Figure 5*). This reduces the preparation phase and gives to the user the possibility to plan the parameters of each well before the calibration phase. This consists in the process to establish a relationship between the inner reference system of the FUS apparatus and the system of the code. Output of the MatLab® (MathWorks) code is an *.ini file that can be read by the machine as described above.

Results

Radiation force tests

A total of 213 tests to evaluate the radiation force have been performed. The data are listed in *Table 1*. During each insonation, CGA-CSA software displays the real electrical power $P_{el,\mathrm{Real}}$ emitted by the transducer, this data are reported in *Table 1*. Trials are made with a mean duration d of 20 seconds and an average of 11 repetitions for each power. F_{rad} is obtained multiplying the apparent weight reduction w, read from the scale, for the gravitational acceleration g. For every value of radiation force, the type

Table 1 Radiation force tests overview

P_{el} (W)	$P_{el,Real}$ (W)	$W_{ac,sf}$ (W)	F_{rad} (mN)	U(Pel) (%)
1.0	0.9	0.50	0.37	4.42
1.5	1.6	0.93	0.49	2.58
2	2.1	1.24	0.68	2.07
2.5	2.6	1.55	0.82	1.77
3.0	3.3	1.86	1.05	1.34
3.5	3.2	2.17	1.11	2.60
4.0	4.0	2.48	1.37	0.75
4.5	4.4	2.79	1.52	1.69
5.0	5.2	3.10	1.79	1.25
5.5	5.6	3.41	1.85	0.66
6.0	6.0	3.72	2.01	0.67
6.5	6.5	4.03	2.16	1.76
7.0	7.5	4.34	2.57	1.74
7.5	8.1	4.65	2.64	0.92
8.0	8.6	4.96	2.87	0.71
8.5	8.6	5.27	3.11	1.45
8.5	9.1	5.58	2.95	0.98
9.0	9.8	5.89	3.27	0.47
9.5	10.3	6.20	3.17	3.35

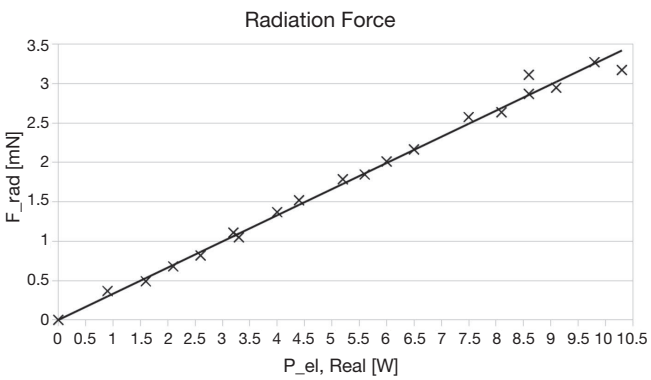

Figure 6 Linear relation between the electric power absorbed by the transducer Pel, Real and the relative radiation force perceived by the digital scale Frad. Straight line: linear interpolation; Crosses: experimental mean values.

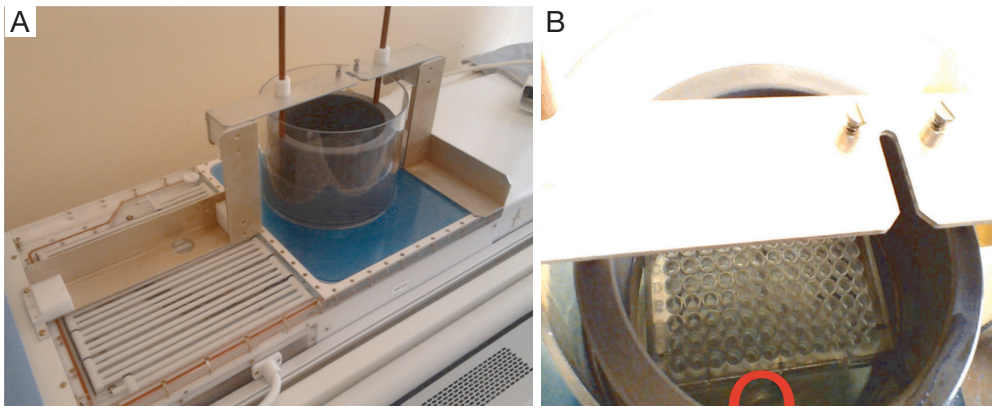

Figure 7 (A) The stand provided by InSightec Ltd, (Tirat Carmel, Israel) to employ the ExAblate 2100 into *in vitro* researches; (B) targeting system calibration. Highlighted in red the radiation stream caused by one insonation.

A uncertainty *U*% has been calculated with a confidence interval of the 95 percentile. There's no value with an uncertainty over 5%. The maximum type A uncertainty recorded is 4.42% for the lower power of 1 W which produces an apparent weight reduction *w* of 0.037 g: too close to the sensitivity of the scale. Those medium values were fitted with a straight line forced to the origin of the axes trough an ordinary least squares regression:

$$F_{Rad} = 0.332154 P_{el,\mathrm{Real}} \ [\mathrm{mN}] \qquad [2]$$

The data and the fit are shown in the *Figure 6*, the coefficient of determination, R^2, is 0.9981.

Geometrical tests

An area of the plate, that is shadowed by the ring of the coupling system, was been identified thanks to the 3D model (*Figure 4*). This kind of interference is reasonable that would not have been recognized without any kind of imaging. To verify the correspondence between wells coordinates in the 3D model and in the real system, a test has been done looking the insonation effect on the water surface (acoustic streaming) in some well of the plate (see *Figure* 7). The 96-wells plate position was been not always the same, so a calibration of coordinates is needed to guarantee the correct position of each insonations. Usually a 5 mm correction of coordinates was been enough. The software stores the last values used and suggests these to the user for the next calibration. Calibration in the *z* direction is done comparing reflection graph in the acquisition part of the CGA-CSA software, with the predicted *z* into the 3D model. In *Figure 2*, the reflection from the water table at the head

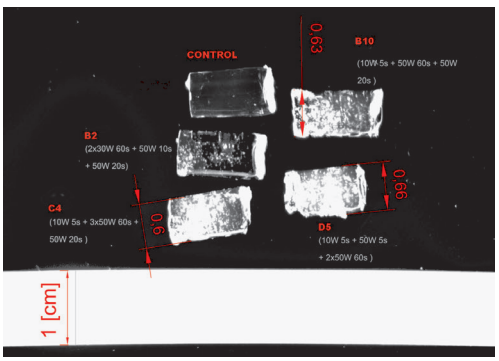

Figure 8 Polyacrylamide Gel-Phantoms used to test our system. In red the name of the well sonicated; under that, repetitions, power and duration of the exposition. Grey scale images contrast enhanced with GIMP.

of the well ($z \approx 125$ mm) is visible. The software reliability was tested with the phantoms described above. All the prescribed wells were insonated with an adequate spatial precision, as shown by the evident proteins denaturation in *Figure 8*.

Discussion

Agreeing with the Buldakov *et al.*'s statement (14), it is very difficult to derive solid theories from the *in vitro* US experimentations accomplished, and this is due to a lack of systematic works that study the influence of changing each and all of the parameters involved. Going beyond this, a series of controls to ensure the repeatability and the accuracy of our *in vitro* experiments was been established. The spots

Table 2 Apparent weight (w) reduction for daily quality assurance with the relative uncertainty accepted for our experiments

P_{el} (W)	w (g)
1	0.0373±0.005
4	0.1393±0.002
9	0.333±0.002

position in the space was assessed employing custom made software based on a 3D model of the system. Its reliability was first verified visually, and then established with the protein denaturation of some specifically made gel phantoms placed inside a 96 well plate. The acoustic power delivered was assessed with a series of test, obtaining a linear relation between the electric power requested by the transducer and the radiation force perceived by the target (Eq. [2]). The experience gained allowed us to write two separate sessions of controls that fit our needs in an easy and cheap manner, making our results almost operator independent; everyone who materially executes the tests has to:

Power check

(I) Make a low power (1 W) insonation, without moving the transducer, to obtain the z coordinate of the free surface z_{fs} (mm) into the water phantom;
(II) Prepare the ExAblate 2100 with the InSightec *in vitro* system, without any culture plate. Fill it of degassed water to the height of the housing of the plate in the lower ring;
(III) Put on the weighing system described above (see *Figure 3*);
(IV) Prepare the insonation with $z=z_{fs}$ –10 (mm) and do tree different tests to verify the correspondence between what is get and *Table 2*.

Spot position check

(I) Prepare the ExAblate 2100 system with the *in vitro* support (*Figure 6*) and the drilled plate, ensuring that water reaches the bottom of the wells;
(II) Start Sonicator, and retrieve the default H6 well's coordinates from it;
(III) Perform a low power (≤5 W) insonation with these coordinates;
(IV) Visually verify *x* and *y* position, if *z* is correct, a little cloud of vapor (not hot!) should come from the surface. If the location is not correct try subtracting/adding 1 mm on *x* and *y* coordinates and search the reflection plot for free surface distance. Iterate this process till the bottom of the H6 well is targeted with precision;
(V) Put the right H6 coordinates in Sonicator and repeat steps from (II) to (V) for the A7 well;
(VI) Ask *Sonicator* to plan the treatment of 3 different wells and visually verify their correct execution;

These are just examples of how an *in vitro* DQA can be articulated. Our intent is to promote such a kind of behavior, and suggest everyone who is involved into these researches the adoption of his own procedure.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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