

Fast response temperature measurement and highly reproducible heating methods for 96-well plates

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Hyperthermia, the procedure of exposing cells to a temperature between 42° and 49°C, has been shown to be a promising approach for cancer treatment. To understand the underlying mechanisms of hyperthermic killing of cancer cells, it is critical to have an accurate temperature measurement technique and a heating method with high reproducibility. To this end, we have developed a method using fine thermocouples with fast response time to measure the temperatures in multiple wells of a 96-well plate. The accuracy of temperature measurement was $\pm 0.2^\circ\text{C}$. Such a capability allows a complete record of the time and temperature of the treatment procedure and helps define an accurate thermal dose. We have also compared several methods for heating 96-well plates and found that use of copper blocks in contact with the lower surface of the 96-well plate in an incubator provides a highly reproducible heating method. The common method of using water bath to heat cells in vitro resulted in a decrease of cell viability even at the control temperature of 37°C and a decrease in the reproducibility of certain biological assays. In summary, using these improved techniques, proposed thermal dose can be defined more precisely, and highly reproducible heating in vitro can be achieved.

INTRODUCTION

Hyperthermia, the procedure of exposing cancerous tissue to a temperature between 42° and 49°C, can kill a tumor with limited toxicity to normal tissue (1–5). Significant improvements have been shown in clinical outcome when hyperthermia treatment was used as an adjunct to radiotherapy or chemotherapy in treating numerous cancers (1–5).

Studies have indicated that hyperthermia might kill various cancer cells through the following possible mechanisms: (i) by inducing p53-dependent and p53-independent apoptosis in some human cancer cells (6–10); (ii) by inducing tumor necrosis factor α (TNF- α)-dependent apoptosis in human leukemia cells (11); and (iii) by damaging tumor vasculature and thus inhibiting tumor growth (12). In addition, some researchers speculate that other possible mechanisms might involve indirect regulation of the host immune system (13). More studies are warranted to understand more

precisely how hyperthermia kills cancer cells and whether the thermal dose affects which mechanisms hyperthermia acts through. Furthermore, hyperthermia may also have a new role in the development of gene therapy strategies for cancer; for instance regulated expression of a therapeutic protein can be achieved in hyperthermia treatment with the use of a heat-inducible promoter (14).

A 96-well plate format is ideal for examining multiple cell types and performing multiple assays at the same time using very small amount of materials. However, there are no easy methods available to measure temperature in wells of 96-well plates for in vitro studies. It is important to have an accurate measurement since studies have shown that a temperature difference of 0.5°C for a long treatment time could cause different cytotoxicity in some situations (15,16).

Most laboratories have used water baths as a way to heat cells in vitro. However, to prevent contamination of

the cultures, plates are normally sealed using Parafilm®. We note that the time required to perform the tasks of wrapping the plates with Parafilm can vary dramatically between experiments and laboratory technicians, leading to variations between experiments. Some studies have incubated cells in water baths for up to 3–6 h (17,18). The effects of lower CO₂ levels in the media accompanying such long incubation time might be very significant on cell viability in some cell types. Other problems with water bath treatment are that the 96-well plates treated in a water bath tend to have condensation on the lid. As a result, the volume of the media of each well might not be consistent after treatment. This decreases the reproducibility of certain biological assays, such as the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) cell viability assay (19).

Another alternative is to use a standard incubator. However, the bottom of a commercially available 96-well plate is slightly recessed from the edges of the plate. This prevents the plate from making direct contact with the rack when it is placed in the incubator. The heat exchange takes place mostly from the air in the incubator, and the air temperature in the incubator is not stable when the door is opened to move the plates in or out. This can cause temperature variations in hyperthermia experiments.

To overcome the shortcomings discussed above, both in accurate and reproducible measurement of thermal dose, we present in this paper a method to measure continuously the temperature of the media in a 96-well plate. We then compare several methods of heating or cooling 96-well plates using our measuring method.

MATERIALS AND METHODS

Cell Line and Normal Culture Condition

A human melanoma cell line, A375, was obtained from ATCC (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum

(Gemini Bio-Products, Woodland, CA, USA) and grown as a monolayer culture in an incubator at 37°C with 5% CO₂.

Temperature Measurement Equipment

The temperature measurement system is composed of T-type thermocouples, a signal conditioning system (SCXI), and a computer with a data acquisition card. The thermocouples were fabricated using copper and constantan wires (TT-T-36-SLE; Omega, Stamford, CT, USA). The accuracy of these thermocouples in the range of temperature measurement in our experiment is ±0.2°C. All other components were from National Instruments (Austin, TX, USA). The SCXI system consisted of a shielded chassis SCXI 1000 that housed a combination of the multiplexer amplifier module SCXI 1100 and the terminal box SCXI 1303. Each thermocouple was connected to the SCXI 1303. Data acquisition was performed using a PCI-MIO-16E-4 card, which in turn was connected to the SCXI system by a SH68-68-EP shielded cable.

The thermocouple readings were processed by the signal conditioning system and multiplexed via a data acquisition card in the computer. The temperature measurements were recorded using a computer program, LabVIEW 6i. The cold junction voltage was compensated by the specified junction temperature,

and the measurement system was calibrated prior to each experiment.

Temperature Measurement

The monitor plates were 96-well plates (Costar, Corning, NY, USA). Five temperature probes were placed into five wells without touching the bottom of the wells (Figure 1). These wells were four peripheral wells (positions B2, B11, G2, and G11) and a center well (position E6). All 96 wells were filled with 100 µL media and placed in a 37°C incubator overnight before treatment. Since the duration of the treatment lasts up to approximately 3 h, there is a likelihood of drift in temperature measurement. To account for this, two additional thermocouples (Control 1 and 2) were put into a water bath at a known stable temperature, 45.7°C, as internal controls. The water bath temperature was recorded every 10 min with a regular-mercury and a digital thermometer. The variation of the temperature in the water bath was found to be within the accuracy of the thermocouples. When using thermocouples identical to those used in the cell plate, a small amount of drift in the temperature reading was detected. To compensate this drifting effect, the average value of control temperatures was calculated using readings of Control 1 and 2, and the difference between this average value and the actual water bath

temperature was taken as the “drift correction.” This correction was then applied to the readings of all the probes as follows:

$$\text{adjusted temperature} = \text{actual reading} - \text{drift correction.}$$

The temperatures reported in the section of Results and Discussion are the adjusted temperatures.

Procedure for Simultaneously Treating the Cells and Monitoring the Temperature

Cells were grown in 96-well plates with 100 µL media overnight prior to treatment. Plates containing cells were treated as indicated in each experiment. Twenty-four hour posttreatment, MTS assays, and examination of cells under an Eclipse TS100 microscope (Nikon, Melville, NY, USA) were performed. A monitor plate with probes was filled with 100 µL media in each well and was treated at the same time in the same way as the plates with cells. A sampling rate of 15 s was determined based on the calculated response time of the thermocouples. For the thermocouples used, the bead diameter is about 0.5 mm in diameter. Following the standard procedure (20), the time-constant for a thermocouple is determined as $\tau = \rho c V / h A$, where ρ is the density, c is the specific heat, V is the volume, h is the convection heat transfer coefficient, and A is the area of the bead. The time for the bead to reach the 95% of the difference between the temperature variations is about $t = 3\tau = 0.3$ s (21). The selected sampling rate of 15 s ensures that the thermocouple can follow the temperature change of the liquid inside the cells.

Water Bath Treatment

The plates were taken out of a 37°C incubator and put onto a laboratory bench (at room temperature or approximately 24°C) wrapped with Parafilm around the sides. Before submerging the plates into a water bath at 37°C, three rubber bands were applied. The plates were then placed on a preheated plastic rack in the water bath to avoid direct contact with the metal bottom of the water bath upon immersion. Water bottles, preheated in the same water

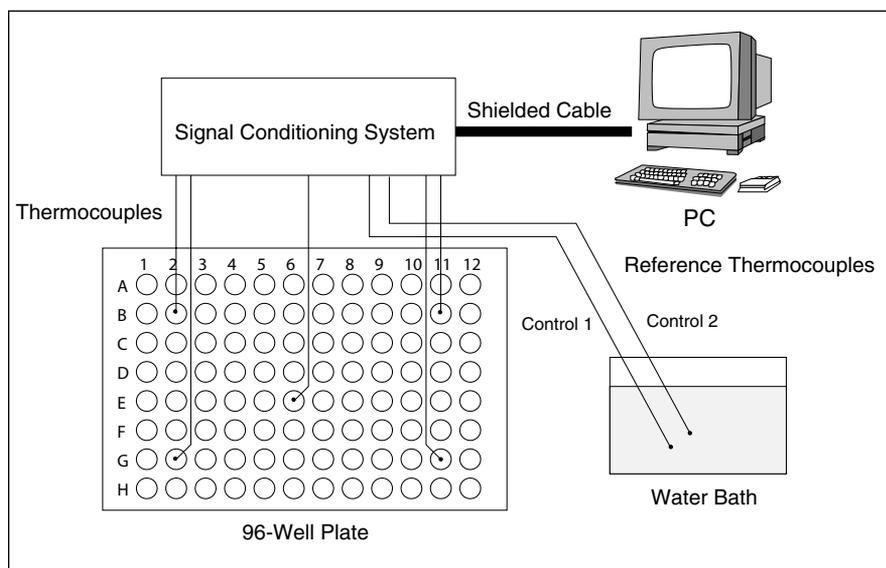


Figure 1. The equipment setup for temperature measurement of media in a 96-well plate.

bath, were put on top of the plates to prevent them from floating. After the indicated treatment time in each experiment, the plates were taken out of the water bath. The wrapping materials were removed and the outside of the plate dried with Kimwipes® before the plates were transferred back to the 37°C incubator.

Incubator Treatment with or without Copper Blocks for 96-Well Plates

All incubators mentioned in this manuscript were maintained at 5% CO₂. Copper blocks were custom-made with the following dimensions: 7.3 × 10.8 × 2.5 cm by Olympic Metal (Commerce City, CO, USA). These blocks were slightly smaller than the bottom of the 96-well plate allowing the bottom of the 96-well plates to have direct contact with the surface of the block. Copper blocks were put into the incubator for at least 3 days before we performed any experiments. After calibrating the recording device, the 96-well plate was quickly moved to the indicated incubator either on the rack directly (rack) or on a prewarmed copper block (block) in the incubator.

Thermal Cycler Treatment for a 96-Well Plate

The Express™ HBPX 05 thermal cycler was purchased from Thermo Hybrid (Middlesex, UK). The heating program was set as 37°C for 10 min, 48°C for 59 min, and then hold at 37°C.

Cell Proliferation Assay

Cell viability was quantified using the MTS assay (19,22). Cell Titer 96® Aqueous One reagents were obtained from Promega (Madison, WI, USA), and procedures were followed according to the manufacturer's instructions. Briefly, assays were performed by adding 20 µL of the reagent to each well containing cells with 100 µL media for 1–4 h, and absorbance (A) readings at 490 nm were recorded with a Model EL312e biokinetics microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The A reading for the media alone was used as background reading.

RESULTS AND DISCUSSION

For simultaneous measurement of media temperature in multiple wells of a 96-well plate, we developed a system for automated temperature acquisition. The equipment setup is shown in Figure 1. The accuracy of this method is ±0.2°C. The technique can provide a complete record of time and temperature of the treatment procedure and can be easily adapted for other tissue culture plates.

Figure 2 shows one example of a temperature measurement; in this case a record of media temperature during a water bath incubation. The plot shows a substantial temperature drop to 26° from 37°C just before the temperature rose to 48.6°C. This corresponds to the time when the plate was moved from the 37°C incubator to the lab bench for wrapping the plate with Parafilm and rubber bands. As expected, the center well (E6) temperature was lower than of peripheral wells.

We also performed similar experiments with cells seeded in the wells and found little difference in the temperature measurements compared to those obtained using media alone (data not shown). This is not unexpected given very small thermal masses of cells in each well.

We then examined the viability of cells incubated using the water bath method. Many laboratories have used water baths to perform hyperthermia treatments with 37°C commonly being used as the control temperature. Here, we used an MTS assay to compare the viability/proliferation of cells incubated at 37°C in an incubator or immersed in a water bath for 10, 20, and 40 min (Figure 3A). We used a paired 2-tailed Student's *t*-test to compare cell viabilities of each water bath treatment versus the incubator control at each cell density (statistical significance at $P < 0.05$). There were statistically significant differences in cell viability between the water bath and the incubator treatment at all cell densities other than 6×10^3 cells/well. Forty minutes of water bath treatment had the most dramatic inhibitory effects on cell viability among all the treatments tested. Visualization of these cells under a microscope confirmed the results from the MTS assay that there were fewer cells following the 40-min water bath treatment when compared with the control sample (Figure 3B). This is probably due to a combination of the sudden cold shock and deprivation of CO₂ for longer treatment times. These data suggest that the proper controls are very important for perform-

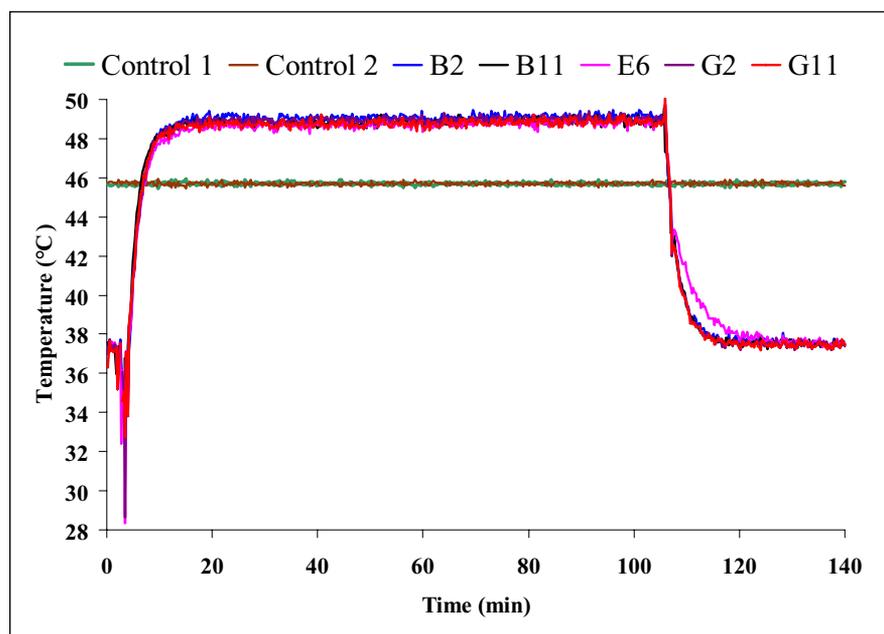


Figure 2. The temperature measurement of media in a 96-well plate. The plate was wrapped and transferred from the 37°C incubator to a 48.6°C water bath for 103 min, then transferred back to the 37°C incubator.

ing hyperthermia experiments in a water bath. These results also provide further evidence that water bath treatment is not the best choice for hyperthermia experiments using 96-well plates.

Due to the limitations of water bath treatment, we investigated whether hyperthermia studies could be performed using a standard incubator. Because of concerns about efficiency of heat exchange in air, we made a custom copper block that would allow direct contact between the metal and the base of the 96-well plate. We then compared hyperthermic treatment in a water bath, in a standard incubator, or in a standard incubator with a copper block (Figure 4A). Both the incubator and the water bath were set at 48°C. The cells heated

in the water bath were the first to reach the set temperature. In comparison, the incubator could not heat the plates to the set temperature within 2 h with or without a copper block. The time to reach a stable temperature was 20 min for the water bath, 22 min for the copper block heating, and 50 min for direct rack heating (Figure 4A). The average stable temperature was 48°C for the water bath (time interval 20–70 min) and 47.2°C for both the block heating (time interval 20–70 min) and for the rack heating (time interval 50–70 min).

We also compared cooling methods for 96-well plates in the incubator, with and without a block (Figure 4B). In these studies, we performed the experiments with two different blocks. As

before, the use of a copper block helped to decrease the time needed to change the temperature and, in this case, reduced the time to cool 96-well plates in the incubator from >35 min for the rack to <15 min from 48° to 37.5°C.

It is worth noting that the temperatures between Block 1 and 2 were very consistent. The differences were to within $\pm 0.2^\circ\text{C}$ (Figure 4B). This variation is similar to that between the two probes in the same water bath (Figure 2, Control 1 and 2). This suggests that reproducibility between the plates within the experiment is very good.

To evaluate reproducibility from experiment to experiment, we then compared two experiments using copper blocks for heating and cooling performed at different times. The temperature profiles were only 30 s apart (Figure 4C). Paired 2-tailed Student's *t*-test showed that there was no statistically difference between these two experiments ($P = 0.16$).

We also compared the temperatures between the wells of the same plate. Figure 4D shows one example. The center well (E6) heated and cooled more slowly than the peripheral wells (Figure 4D). However, the differences were quite small.

Our results have shown that heating the cells with copper blocks in the incubator reached 0.8°C short of the set temperature. This problem can be easily overcome by increasing the temperature setting in the incubator appropriately. However, it points out to the need for in situ temperature measurement using a reference plate.

Finally, we tested one thermal cycler to determine if this system could be used for reproducible hyperthermic treatment of cells in 96-well plates. We found that there was about a 2°C difference between the center well and the peripheral wells throughout the entire experiment when the plate was heated to 48°C (Figure 4E). We note that the thermal cycler method will also have similar CO₂ issues, as in the water bath treatment, and one machine can only heat one plate at a time. Therefore, we conclude that the use of the thermal cycler might not be appropriate for hyperthermia experiments.

In summary, we have developed a method to measure temperature

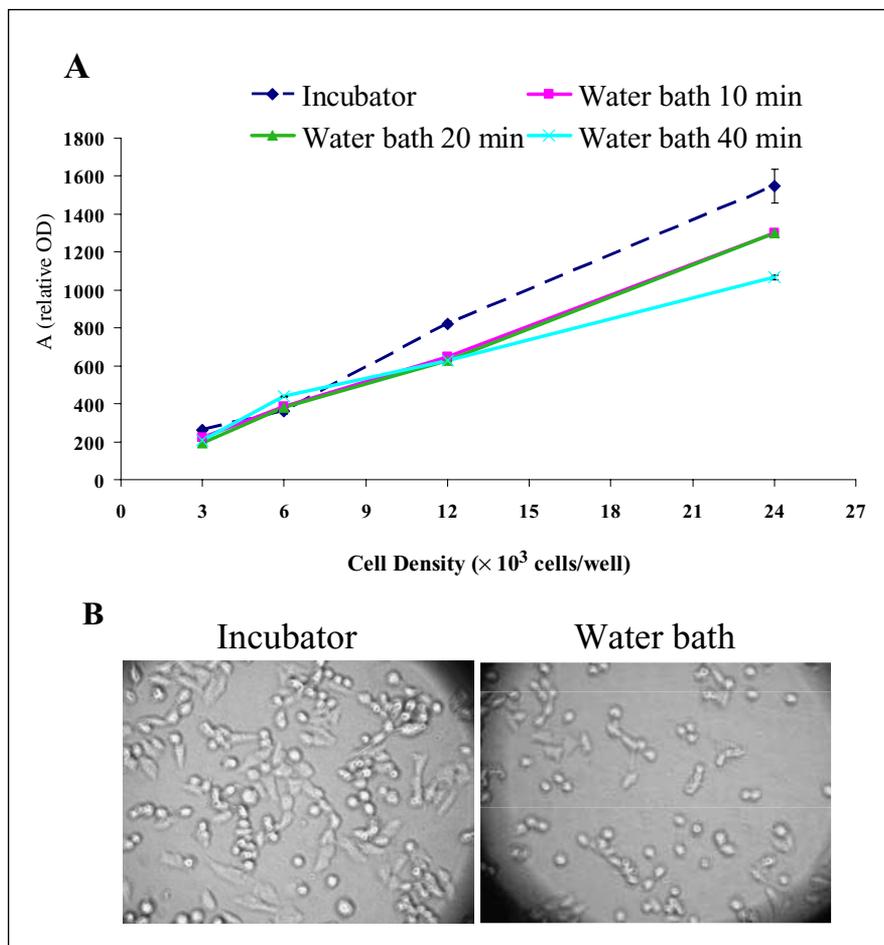


Figure 3. The effects of water bath treatment on cell viability. Cells were seeded at indicated cell densities in 96-well plates overnight in 37°C incubator prior to treatment. Twenty-four hours posttreatment, the cells were analyzed as indicated. (A) MTS assays. Data points represent mean (\pm SEM) of 10 samples per data point. (B) Micrographs of cells viewed under Nikon Eclipse TS100 microscope. Pictures were taken with a Nikon COOLPIX digital camera (magnification 100 \times). These cells were seeded at 12×10^3 cells/well and were given a 40-min treatment at 37°C. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; A, absorbance; OD, optical density.

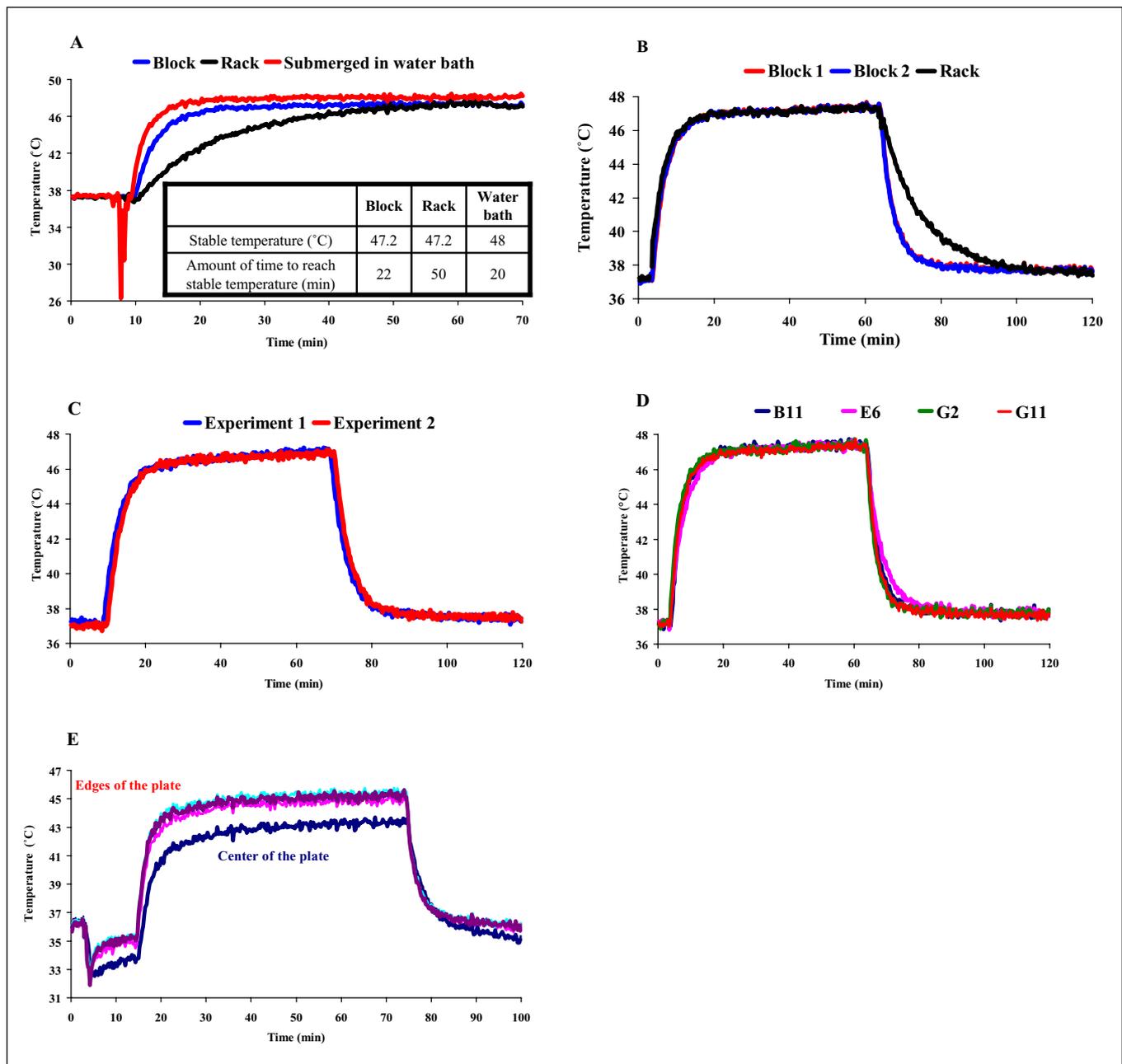


Figure 4. Comparison of the temperature measurements of media in a 96-well plate among different heating or cooling methods. The temperature measurements of each plate were the average temperatures of five probes in each plate at each time points. (A) Heating methods. (B) Cooling methods. All three plates were heated using copper blocks in 48°C incubator described above. Then two plates were cooled either by using copper blocks (Block 1 and 2) or on the rack in a 37°C incubator (Rack). (C) Reproducibility between experiments. Average temperatures of each plate were plotted. (D) Reproducibility between wells. (E) Thermal cyclers heating.

in multiple wells of tissue culture plates. This is an improvement over the current techniques and permits us to define in vitro thermal dose more precisely. In addition, we have also demonstrated an easy to perform and highly reproducible heating method using copper blocks in a standard tissue culture incubator. The result is a

lower probability of contamination and increased reproducibility. This improved method eliminates several limitations of water bath or thermal cyclers and will help advance research studying the kinetics of hyperthermia treatment and the relationship between the thermal dose and cell death pathways.

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Western blot analysis of Src kinase assays using peptide substrates ligated to a carrier protein

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We have applied intein-mediated peptide ligation (IPL) to the use of peptide substrates for kinase assays and subsequent Western blot analysis. IPL allows for the efficient ligation of a synthetic peptide with an N-terminal cysteine residue to an intein-generated carrier protein containing a cysteine reactive C-terminal thioester through a native peptide bond. A distinct advantage of this procedure is that each carrier protein molecule ligates only one peptide, ensuring that the ligation product forms a sharp band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We demonstrate the effectiveness of this approach by mutational analysis of peptide substrates derived from human cyclin-dependent kinase, Cdc2, which contains a phosphorylation site of human c-Src protein tyrosine kinase.

INTRODUCTION

Protein phosphorylation plays a pivotal role in many signal transduction pathways (1,2). Yet the dissection of kinase signaling pathways presents a challenge to existing techniques (3). The use of synthetic peptide substrates has become a powerful tool to determine kinase specificities, allowing mutational analysis and investigation into optimal phosphorylation sites (4). One extensively used technique for kinase activity assessment is the measurement of the incorporation of radiolabeled γ -phosphate from ATP (5). An alternate approach for the detection of protein phosphorylation is to determine the immunoreactivity of a protein to a phosphor-specific antibody that recognizes the phosphorylated epitopes within proteins (6). Two common methods for the determination of im-

munoreactivity are enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Because of the small size of synthetic peptides, typically in the range of 10–20 amino acid residues, they are not suitable for Western immunoblotting analysis.

In this report, we describe an easy and effective method for producing carrier protein-peptide ligation products for kinase assays and subsequent Western blot analysis. A peptide possessing a phosphorylation site of interest is first synthesized with an amino-terminal cysteine residue. The peptide is then ligated to the cysteine-reactive carboxyl terminus of a carrier protein via a peptide bond by intein-mediated protein ligation (Figure 1) (7–9). This results in the ligation product migrating as a single band on a sodium dodecyl sulfate (SDS) polyacrylamide gel.

MATERIALS AND METHODS

Generation of Peptides and Carrier Protein

All peptides were synthesized with an N-terminal cysteine and purified by high-performance liquid chromatography (HPLC) (New England Biolabs, Beverly, MA, USA) (Table 1; References 10 and 11). For ligation to the peptides, the paramyosin Δ Sal fragment from *Dirofilaria immitis* (12) was expressed as a paramyosin-intein-chitin binding domain fusion protein (PXB) and purified on chitin resin. Intein-mediated cleavage was carried out at 4°C for 16 h by incubation of the chitin resin in column buffer containing 20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, and 50 mM 2-mercaptoethanesulfonic acid (MESNA; Sigma, St. Louis, MO, USA). Protein elutions were collected in 5-mL fractions from the column, and protein concentrations were determined by Bradford assay (13).

Ligation of Carrier Protein and Peptide

For the experiment shown in Figure 2, the ligation reactions were performed overnight at 4°C in a 100- μ L reaction volume with 500 μ M of peptide and 20 μ M of paramyosin carrier protein in the presence of 100 mM Tris-HCl, pH 8.5, and 10 mM MESNA. For the experiment shown in Figure 3, the ligation reactions were carried out as described above except for a shorter ligation time (4 h at 4°C) and an increase in peptide concentration to 1 mM final concentration.

Kinase Assays

The ligated samples were dialyzed against 5 mM Tris-HCl, pH 7.5, 50 mM NaCl to remove the unligated peptide using a 0.025 μ m filter (Millipore, Bedford, MA, USA). Kinase assays were carried out at 30°C for 60 min in a 10- μ L reaction with each sample containing 10 μ M (or 0.30 mg/mL) of the carrier protein and 12.5 U Src kinase (Upstate, Lake Placid, NY, USA) in the presence of 100 μ M ATP and 25 mM Tris-HCl, pH 7.2, 31.25 mM MgCl₂, 25 mM MnCl₂, 0.5 mM ethylene glycol-bis(2-