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# Towards a personalized approach to aromatase inhibitor therapy: a digital microfluidic platform for rapid analysis of estradiol in core-needle-biopsies

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Despite advances in breast cancer prevention and treatment, variability in patient-response has revealed the need for a more "personalized" approach to medicine, in which treatments are tailored to each patient's biology. Motivated by this idea, we introduce a technique that allows for quantification of small-molecule analytes directly from core needle biopsy (CNB) tissue samples on a miniaturized platform. The new technique, powered by digital microfluidics, integrates tissue-liquid extraction and magnetic bead-based competitive immunoassay for quantification of estradiol in milligram-sized CNB samples. Each measurement (from start to finish) requires ~40 minutes, a duration consistent with a visit to a doctor's office. The performance of the new technique was validated by the gold-standard analysis method (high performance liquid chromatography coupled to tandem mass spectrometry), and was applied to evaluate human patient samples before and after a course of treatment with aromatase inhibitor therapy. We propose that the new technique has great potential for eventual use for fast, automated, and quantitative analysis of biomarkers in tissue samples, towards a personalized medicine approach.

## Introduction

The classical approach to treating cancer and other systemic illnesses employs a "standard of care" regime, in which all patients receive similar interventions. Although the efficacy of treatments has improved over the decades, the variability in outcomes has revealed the need for a "personalized" approach to treatment, in which therapies are tailored to each patient's specific response. Personalized medicine has the potential to maximize the impact of therapeutic measures and minimize adverse effects caused by unnecessary intervention. A key barrier to realizing the potential benefits of personal-

ized medicine is a lack of rapid, accessible diagnostic tools that can be used in the clinician's office.

Motivated by this need, this study introduces a diagnostic tool that may prove useful for personalized treatment of estrogen receptor (ER)-positive breast cancer. The traditional approach to treating this disease is the prescription of selective estrogen receptor modulators (SERMs), which inhibit ER activity in tumor tissue. Briefly, when estradiol (E2) binds ER in normal or tumor cells, a biochemical cascade is initiated that eventually activates transcription of proteins responsible for cell proliferation.[1] SERMs are thus used to inhibit this effect, which limits tumour cell proliferation. More recently, an alternative treatment known as aromatase inhibitor therapy (AIT) has become popular for the treatment of post-menopausal ER-positive breast cancer patients. AIT, which acts by inhibiting the biosynthesis of E2, is believed to be more effective than SERMs in some patients because in some cases, SERMs act as ER-agonists (rather than antagonists).[2] In addition, there are a number of catechol-estrogens and other estrogen metabolites that are believed to be mutagenic; the formation of these metabolites is inhibited by AIT but not by SERMs.[3] Further, there is evidence of cross-talk between the steroid receptor pathway and growth-factor-receptors (co-expressed by breast cancer carcinomas) that

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may lead to SERM-resistance.[4] Finally, postmenopausal women with early or advanced ER-positive breast cancer have reported fewer side-effects for AIT relative to SERMs.[5]

In addition to the recent emergence of AIT as a front-line treatment for post-menopausal patients with ER-positive breast cancer, AIT has also long been the standard-of-care adjuvant therapy prescribed to healthy patients that were previously diagnosed with contralateral breast cancer.[6] While this treatment can avert the development of 70–80% of ER-positive breast cancers,[7] AIT has little effect on outcome for the remainder of patients, likely caused by inter-individual variations in aromatase activity.[8] Patient-specific variations in drug resistance are also likely contributors to this effect, which may include intrinsic resistance (for tumors that are inherently non-responsive to endocrine treatment) and acquired resistance, in which tumors initially respond to the endocrine agent, but then become resistant during the course of treatment. In the latter case, the resistance is often agent-selective, and can be overcome by changing the type of AIT that is prescribed.[9,10]

Given the variability in patient response to AIT, we propose that regular monitoring of E2 levels in breast tissue during the course of treatment would be useful to determine the effectiveness of the therapy. As a step towards personalized treatment, we recently reported a digital microfluidic (DMF) strategy for quantifying E2 and other hormones in core needle biopsy (CNB) samples of breast tissue.[11] CNBs are ideal for personalized medicine, as the ~milligram-sized samples can be collected in the doctor's office, without general anesthesia or risk of scarring. But our original technique[11] relied on analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS); this type of instrument is not readily available in clinics or small labs, making it inappropriate for the rapid turn-around needed to guide personalized care.

Here we report a technique for the rapid determination of patient response to AIT: an integrated, portable microfluidic platform that allows quantification of E2 in CNB samples. The instrument is small (shoe-box size) and can be easily operated outside of the laboratory, making it potentially useful for on-site decision-making. The new technique, powered by digital microfluidics, integrates tissue-liquid extraction and a magnetic bead-based competitive immunoassay in a miniaturized format, facilitating the quantification of E2 from milligram-sized CNB samples. We propose that this system may eventually be useful in aiding physicians as they select and dose aromatase inhibitors in the management of breast cancer. If successful, this system will join the rising tide of microfluidic techniques that are paving the way for a personalized medicine approach to healthcare.[12–14]

## Experimental

### Reagents and materials

Unless otherwise specified, reagents were purchased from Sigma Chemical (Oakville, ON), including 1,3,5-estratriene-3,17β-diol (estradiol, E2) and 1,3,5-estratriene-3,17β-diol-16,16,17-d3 (estradiol-d3, E2d3). Deionized (DI) water had a resistivity of 18 MΩ cm$^{-1}$ at 25 °C. Stock solutions (1 mg mL$^{-1}$ each) of E2 and E2d3 were prepared in neat methanol, and working solutions of each standard (10 μg mL$^{-1}$ each) were formed in methanol by serial dilution. All stock and working solutions were stored at −20 °C until use. Standard solutions (100 ng mL$^{-1}$ each) of dansylated E2 and dansylated E2d3 were prepared by evaporating 10 μL samples of working solutions of E2 or E2d3 in 1.5 mL polypropylene vials to dryness at room temperature for ~10 min, and then reconstituting in 1000 μL of a 1:1 mixture of 1 mg mL$^{-1}$ dansyl chloride in acetone and aqueous 100 mM sodium bicarbonate (pH 10.7). In each case, the cap was affixed and the tube was transferred to a water bath and incubated at 60 °C for 5 min.

Rabbit monoclonal anti-E2 coated paramagnetic microparticles, and E2 assay diluent (containing surfactant in citrate buffer to prevent non-specific adsorption) were adapted from ARCHITECT immunoanalyzer reagent kits obtained from Abbott Laboratories (Abbott Park, IL). Estradiol conjugated with horse radish peroxidase (E2-HRP, conjugated via 6-CMO), was purchased from BiosPacific (Emeryville, CA). SuperSignal enzyme-linked immunosorbent assay (ELISA) femto chemiluminescent substrate, comprising separate solutions of stabilized hydrogen peroxide ($H_2O_2$) and luminol with phenolic enhancer, was purchased from Thermo Fisher Scientific (Rockford, IL). SuperBlock™ (a proprietary mixture of proteins in phosphate buffered saline), used as both particle and conjugate diluent, was purchased from Thermo Fisher Scientific. Prior to use, all reagents were supplemented with Tetronic 90R4 (0.1% v/v) obtained from BASF Corp (Ludwigshafen, Germany), which serves to facilitate droplet movement and prevent biofouling. A DMF-compatible Tris wash buffer was formed from Tris-base (0.35 g L$^{-1}$), Tris-HCl (1.10 g L$^{-1}$), NaCl (8.367 g L$^{-1}$), and Tetronic 90R4 (0.1% v/v), an immunoassay reconstitution buffer was formed from bovine serum albumin (4% w/v) in Dulbecco's phosphate buffered saline (DPBS) with Tetronic 90R4 (0.1% v/v), and LC-MS/MS reconstitution buffer was formed from a 1:1 mixture of 1 mg mL$^{-1}$ dansyl chloride in acetone and aqueous 100 mM sodium bicarbonate (pH 10.7).

### DMF device fabrication, assembly, and operation

Two-plate DMF devices were fabricated in the Nanofabrication Center (TNFC) at the University of Toronto. Top plates were formed from indium tin oxide (ITO)-coated glass substrates (RhyScientific, ON, Canada), spin-coated with 50 nm Teflon-AF as described previously.[11] Unique to this work, each top-plate was patterned to include one 3 mm-diameter circular region of exposed ITO (known as the "hydrophilic anchor"), formed by lift-off of a circular piece of dicing tape (applied and removed before and after spin-coating, respectively). Bottom plates were also formed as described previously[11] from chromium-coated glass substrates (Telic, Valencia, CA) coated with 7 μm Parylene-C and 50 nm Teflon-

AF. Each bottom plate featured an array of 80 actuation electrodes (2.2 × 2.2 mm each) connected to 8 reservoir electrodes (16.4 × 6.7 mm each). The actuation electrodes were roughly square with interdigitated borders (140 μm peak-to-peak sinusoids), with inter-electrode gaps of 30–80 μm. Devices were assembled by joining a top and bottom plate with a spacer formed from two pieces of 3 M Scotch double-sided tape (St. Paul, MN) with a total spacer thickness of 180 μm. Unit droplets (covering one actuation electrode) in this system were thus ~0.8 μL.

Droplet movement, extraction, reaction, and detection was implemented using an integrated, home-made instrument[15] that allows for control of droplet position, control of a magnet mounted on a step-motor that can be moved to enable separation of magnetic particles, as well as a photomultiplier tube (PMT) to measure chemiluminescence. Aliquots of reagents were loaded onto a DMF device by pipetting a droplet onto the bottom plate at the edge of the top plate, and simultaneously applying sine wave voltages (~100 $V_{rms}$, 10 kHz) between the top plate electrode and successive electrodes on the bottom plate via a custom pogo-pin connector, to draw the fluid into the reservoir and further manipulate droplet movement. The automation system (including the magnet) was programmed and managed by Microdrop, an open-source software package for the manipulation of droplets on DMF devices.[16]

## On-chip tissue extraction

E2 was extracted from tissue samples on DMF devices in a six-step procedure. (1) A tissue homogenate (preparation described below) was collected from a 1.5 mL polypropylene vial and then loaded onto the bottom plate of a device. A top plate was affixed onto the device, and 20 μL extraction solvent (neat methanol for immunoassays or 10 ng mL$^{-1}$ E2d3 in methanol for LC-MS/MS experiments) was loaded into a reservoir. (2) A 4.8 μL droplet of extraction solvent was dispensed onto the array and delivered to the CNB sample, and E2 was extracted into the droplet by moving in a circular pattern around the tissue sample for 5 min. (3) The sample-extract droplet was driven away from the tissue and delivered to the hydrophilic anchor on the top plate for solvent evaporation (5 min at room temperature). (4–5) Steps 2 and 3 were repeated. (6) The analyte dried on the hydrophilic anchor was redissolved in an appropriate solvent for analysis. For immunoassay experiments, step (6) comprised dispensing a 4.8 μL droplet of immunoassay reconstitution buffer onto the anchor and actuating the droplet for 30 s around the spot, followed by moving the droplet onto the working area of the device for on-chip immunoassay processing (see below). For LC-MS/MS, in step (6) the top plate was removed and a 25 μL aliquot of LC-MS/MS reconstitution buffer was pipetted onto the anchor, where it was manually agitated for 30 s. This solution was collected into a 1.5 mL polypropylene vial, incubated in a 60 °C water bath for 5 min, mixed with 25 μL of 50% methanol/DI water and then transferred into polypropylene vials for analysis by LC-MS/MS.

## On-chip immunoassay

E2 was measured in standards and in tissue extracts (generated on-chip as above) in a sixteen-step DMF immunoassay. (1) A 1.6 μL droplet containing paramagnetic particles was dispensed from a reservoir and separated from the diluent by engaging the magnet. (2) A 4.8 μL droplet of E2 standard or reconstituted sample extract was delivered to the immobilized particles for resuspension. (3) The droplet was actively mixed with the particles for 6 min before engaging the magnet and driving the supernatant to waste. (4) A 1.6 μL droplet of wash-buffer was dispensed onto the array, driven to the particles, and the particles were resuspended and mixed for 10 s. The magnet was engaged and the supernatant was driven to waste. (5–7) Step (4) was repeated three times. (8) A 1.6 μL droplet of E2-HRP conjugate solution was dispensed and delivered to the immobilized particles, which were resuspended. (9) The droplet was actively mixed for 2 min. (10–13) Step (4) was repeated four times. (14) A 0.8 μL droplet of H$_2$O$_2$ was dispensed and delivered to the immobilized particles. (15) A 0.8 μL droplet of luminol/enhancer solution was dispensed and delivered to the immobilized particles, which were resuspended in the combined (H$_2$O$_2$ and luminol/enhancer) droplet. (16) The droplet was actively mixed for 10 min, and chemiluminescence was measured using the integrated H10682-110 PMT (Hamamatsu Photonics K.K., Hamamatsu, Japan).

For quantification of E2 in tissue extract, a standard calibration curve was generated, which consisted of signal measured from ten different concentrations of E2 (0, 10, 50, 100, 500, 1000, 2500, 5000, 10 000 and 20 000 pg mL$^{-1}$) that were formed by serial dilutions of E2 stock solution in assay diluent. The chemiluminescent signal was plotted as a function of concentration and fit with a four-parameter logistic curve. The chemiluminescent signals observed from each tissue extract were compared to the curve to determine the concentration measured in the sample, which was reported as a ratio of absolute amount of analyte detected relative to sample mass. Note that no internal standard was used in this procedure.

## HPLC-MS/MS

E2 was measured from standards or tissue extracts (generated on-chip, as above). 30 μL samples were loaded into 250 μL polypropylene vials (Agilent Technologies, Santa Clara, CA) positioned in a 54-vial plate via a CTC Analytics Leap HTS PAL Autosampler (Alexandria, Virginia). Chromatographic separations were performed using an Agilent Technologies 1100 series HPLC system (Santa Clara, CA), with an Agilent Zorbax Eclipse Plus C18 column (2.1 mm i.d. × 100 mm long, 1.8 μm particle dia.) protected by a C18 Zorbax guard column (2.1 mm i.d. × 12.5 mm long, 5 μm particle dia., Agilent) and an in-line filter (2.1 mm dia., 0.2 μm pore

dia., Agilent). The HPLC was operated at ambient temperature in gradient elution mode at a flow rate of 0.1 mL min$^{-1}$. The gradient started with 50% mobile phase A (0.1% formic acid in DI water) and 50% mobile phase B (0.1% formic acid in methanol), changed linearly to 80% mobile phase B over 2 min, and then changed linearly again to 100% mobile phase B over the next 2 min. Mobile phase B was then held at 100% for 5 min before decreasing linearly back to 50% over 2 min and holding at 50% for 11 min, for a total runtime of 22 min. HPLC eluent was interfaced into an API4000 triple stage quadrupole mass spectrometer (ABSciex, Foster City, CA, USA) *via* an electrospray ionization (ESI) source. The source voltage was 5.5 kV, the declustering potential was 45 V, the entrance potential was 10 V, the collision energy was 35 eV, the collision cell exit potential was 6 V, the source temperature was 50 °C, and the multiple reaction monitoring (MRM) $m/z$ transitions of dansylated E2 and dansylated E2d3 were 506/171 and 509/171, respectively.

For quantification of E2 in tissue extract, standard calibration curves were generated, which consisted of signal measured from seven different concentrations of dansylated E2 (0, 20, 50, 100, 500, 2000 and 5000 pg mL$^{-1}$), each containing 2000 pg mL$^{-1}$ of internal standard (dansylated E2d3), that were formed by serial dilutions in 50:50 methanol:DI water. The ratios of the areas under the curve (AUC) in MRM chromatograms for dansylated E2 product ions relative to those of dansylated E2d3 were plotted as a function of concentration and fit with a linear regression. A new calibration curve was generated before and after each set of tissue extracts were analyzed, and E2 concentration in the extracts were calculated from the average regression lines from the two plots. Measured E2 was then reported as a ratio of absolute amount of analyte detected relative to sample mass.

## Rat tissue samples

Rat breast tissue samples were obtained from lactating and non-lactating female rats at the animal facility in the Donnelly Centre for Cellular and Biomolecular Research. Rats were euthanized without anesthesia using $CO_2$ for ~4 min; ~5 mg tissue specimens were collected from the breast region with scissors and were instantly frozen under liquid nitrogen before storing at −80 °C until use. Prior to analysis, specimens were thawed, sectioned, transferred to 1.5 mL polypropylene vials and weighed, followed by homogenization by manual grinding with a disposable polypropylene pestle (VWR, ON, Canada), while on dry ice. Samples were then kept on dry ice until analysis. In some experiments, exogenous E2 was spiked into samples prior to homogenization (as described below).

Rat tissue samples were used for three types of experiments. (1) To evaluate HPLC-MS/MS analytical performance, tissue samples from lactating rats were spiked with aliquots of E2 to give exogenous amounts of 0, 3.1, 3.6, 24.0, 26.1, 54.8, 61.2, 64.0, 100.1, 117.8 and 124.5 fmol mg$^{-1}$. These samples were extracted on-chip and analyzed by HPLC-MS/

MS (as above) to determine the amount of measured (or "found") E2 in each sample. After subtracting the amount of found endogenous E2 (measured in the sample with no spiked analyte), the data was plotted against the spiked amounts, fitted with a linear regression, and the % recovery was defined as the average ratio of found/spiked E2 for the 10 spiked samples (expressed as a percentage). (2) To evaluate on-chip immunoassay performance and compare it to that of HPLC-MS/MS, two sets of tissue samples from lactating rats were spiked with aliquots of E2 to give exogenous amounts of 0, 8.2, 8.5, 12.3, 19.1, 30.4, 30.8, 62.0, 69.8 and 116.9 fmol mg$^{-1}$ (each). One set of samples was measured by HPLC-MS/MS while the other set was measured by on-chip immunoassay (as above). After subtracting the amount of found endogenous E2 from both types of measurements, the found immunoassay measurements were plotted against those from the HPLC-MS/MS, and fitted with a linear regression. In addition, on-chip immunoassay % recovery was defined as the average ratio of found to spiked E2 for the 10 spiked samples (expressed as a percentage). (3) To evaluate the difference between native E2 in lactating and non-lactating rats, samples from each type of rat were measured by on-chip immunoassay. These raw values were multiplied by the reciprocal of the immunoassay extraction efficiency to determine the corrected amounts.

## Human samples

Ethical approval was granted by the Research Ethics Board of Mount Sinai Hospital (reference number 07-0015A). Written informed consent was obtained from each participant before enrolment. Paired core needle biopsy breast tissue samples were collected from each patient, including: (1) one sample prior to treatment, (2) a second sample after AIT and hormone replacement therapy (HRT) for seven days [*i.e.*, 2.5 mg Letrozole (Novartis Canada, Dorval, Quebec) every other day and 1 mg per day Estrace (Warner Chilcott, Rockaway, NJ USA)]. Each sample was collected using a Quick-Core Biopsy Needle (QC-16-6.0-20 T, Cook Medical, USA) after local intradermal injection of 0.5 mL of 2% Xylocaine (AstraZeneca, Wilmington, DE). All samples were taken from the right breast at the 10 o'clock position, 5 cm from the border of the areola. The samples were stored in 1.5 mL polypropylene vials at −80 °C until use. Prior to analysis, each sample was transferred to a fresh 1.5 mL polypropylene vial where it was weighed and then homogenized by grinding with a disposable polypropylene pestle (VWR, ON, Canada), while kept on dry ice. Samples were further stored on dry ice until analysis by on-chip immunoassay (as above). Raw and corrected amounts of E2 in each sample were determined as in rat-tissue experiment (3). Note that using % recovery determined from rat tissue samples to correct for measurements in human samples was deemed necessary, as the human samples were too precious to "waste" for spike-recovery assays.

# Results and discussion

## Integrated method development

The primary goal of this work was to develop a portable platform that allows quantification of E2 in CNB samples in 40 min, a duration chosen to allow for acquisition of results during a visit to the physician. As described in the introduction, this goal was motivated by the need to evaluate the tissue-specific efficacy of AIT, which is prescribed to post-menopausal patients as both a front-line treatment and as an adjuvant therapy for ER-positive breast cancer. We recently demonstrated that DMF can be used to evaluate E2 and other steroids in CNB samples;[11] unfortunately, that method relies on analysis by HPLC-MS/MS, which is not compatible with the primary objective of this work (above).

Here, we describe E2 quantification in CNB samples by means of DMF immunoassays, using an instrument the size of a large shoebox[15] (18 × 23 × 30 cm). The system includes an integrated photomultiplier and step-motor-mounted magnet, and was originally developed for DMF immunoassays in liquid samples[15] (e.g., serum or buffer). Here, we have extended the DMF immunoassay workflow to accommodate solid CNB samples, through a complex two-stage procedure comprising: (1) a six-step analyte extraction stage, and (2) a sixteen-step analysis stage. Preliminary versions of these two stages have been described previously (i.e., on-chip CNB-extraction[11] and on-chip immunoassays[15]), but they have never before been joined together.

In initial work, the system was optimized for stage (1), which is depicted in Fig. 1A and B. Briefly, in each experiment, a ~5 mg tissue sample is loaded onto the device, where it is extracted into 4.8 μL droplets of methanol, which are driven to a hydrophilic anchor to dry. The extractate is then reconstituted in buffer for analysis, either by on-chip ELISA or off-chip HPLC-MS/MS. The hydrophilic anchor represents an improvement on our original DMF CNB-extraction technique, reducing the drying time by 6-fold (from 30 min previously[11] to 5 min). As reported previously[17] for unrelated applications, a hydrophilic anchor on a DMF device allows for reproducible concentration of analytes for subsequent recovery and analysis. In addition, by defining a sacrificial area for analyte-adsorption, the remainder of the hydrophobic device surface is protected from fouling,[18] prolonging device lifetime.

In initial tests, tissue samples from lactating rats (which are known to have low endogenous E2 levels) were spiked with exogenous E2, extracted by DMF, and analyzed by HPLC-MS/MS (Fig. 1C). The average percent recovery for this
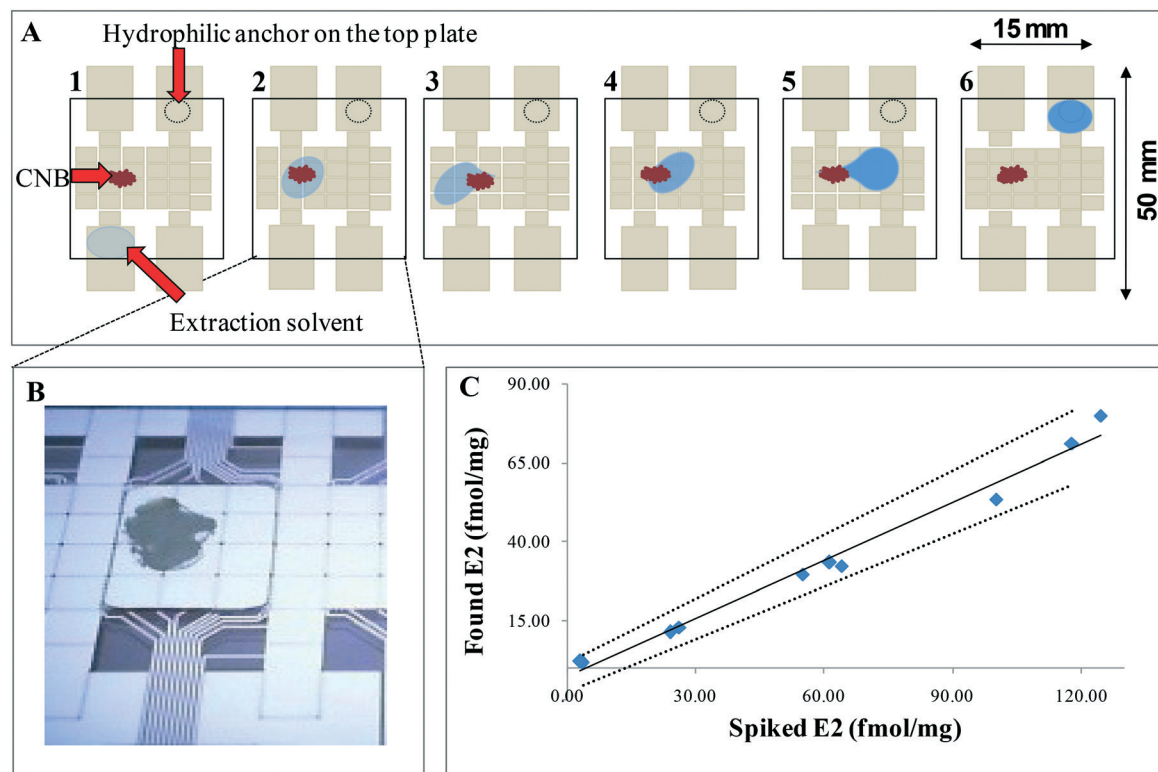


**Fig. 1** Digital microfluidic (DMF) extraction of estradiol (E2) from core needle biopsy (CNB) samples. (A) Cartoon depicting E2 extraction. (1) CNB sample and extraction solvent are loaded onto the device. (2–6) E2 is extracted into droplet of extraction solvent by actively moving the droplet around the sample, before drying onto a hydrophilic anchor on the top plate (this process is repeated twice for each tissue-sample). (B) Picture of device and CNB sample during step (2). (C) Quantification of E2 recovered from lactating rat breast tissue samples extracted on-chip followed by analysis by LC-MS/MS (as found E2 vs. spiked E2, blue diamonds, n = 10). The line of regression (solid black line; $y = 0.615x - 2.724$) has $R^2 = 0.984$; the dashed black lines indicate the 95% confidence interval region for these data.

HPLC-MS/MS method was found to be 56 ± 13% (mean ± std. dev. for $n$ = 10), which is consistent with our previous study of CNB extraction on DMF, which reported 65% recovery.[11] As shown in Fig. 1C, the amount of E2 determined by HPLC-MS/MS were found to be linear ($R^2$ = 0.984) and reproducible (95% confidence interval for the slope = 0.553–0.677). Note that % recovery can be improved with longer extraction times or larger extraction volumes; in this work, it was determined that 2 × 5 min extraction into 4.8 μL droplets was an acceptable compromise between extraction time and extraction efficiency (in recognition of the goal of rapid analysis for this application). Overall, the extraction and reconstitution process (stage 1) requires ~15 min from start to finish.

After optimization of on-chip extraction of CNB samples (stage 1), we turned our attention to on-chip quantification of E2 in CNB extract (stage 2). As shown in Fig. 2, an automated sixteen-step DMF-ELISA procedure was developed, relying on anti-E2-modified magnetic particles and chemiluminescence detection. Briefly, reconstituted CNB extract was exposed to the immunosorbent particles (steps 1–3), which were then

washed (steps 4–7), mixed with E2–enzyme conjugate (steps 8–9), washed again (steps 10–13), and mixed with reporters and analyzed (steps 14–16). This process requires ~25 min, bringing the full duration (for stage 1 + stage 2) to ~40 min, making it suitable for point-of-care (POC) applications.

The immunoassay technique described here builds from a DMF E2-assay described in previous work,[19] which was implemented on a manually operated platform and was applied to analyzing standards dissolved in buffer. In developing the new method, it was found that a number of improvements were required, addressing challenges related to (1) assay performance and (2) sample compatibility. For the former improvement (1), the complex matrix evaluated here (concentrated tissue extract) was found to be incompatible with the previous technique,[19] which relied on the droplet-additive Pluronic L64 to enhance droplet movement and prevent biofouling. In this work, the much more potent droplet-additive, Tetronic 90R4 (recently reported[20] to be useful for manipulating undiluted serum) was included at 0.1% (v/v) in all reagent and sample solutions, which allowed for robust
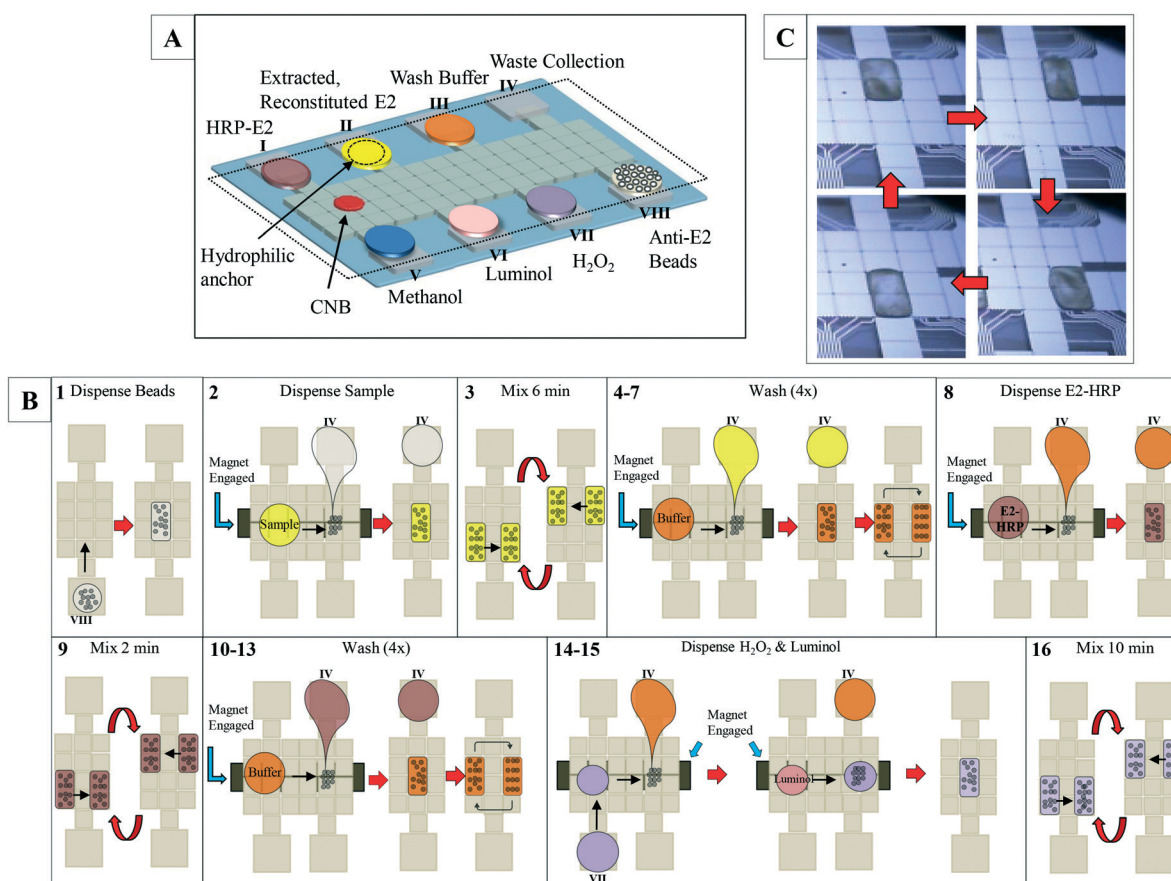


Fig. 2 Quantification of E2 by on-chip DMF immunoassay. (A) Schematic of DMF device bearing eight reservoirs (I–VIII) – in this view, E2 has already been extracted (as in Fig. 1) from the CNB sample (red) into extraction solvent (blue), dried onto the hydrophilic anchor, and reconstituted in sample-buffer (yellow). The other reagents include wash buffer (orange), estradiol conjugated to horse radish peroxidase (E2-HRP) (burgundy), a suspension of anti-E2 antibody-modified paramagnetic particles (white circles), a solution of hydrogen peroxide ($H_2O_2$) (purple), and a solution of luminol (pink). (B) Schematic of the 16-step immunoassay process, comprising (1–7) magnetic bead and sample delivery, incubation, and bead washing, (8–13) conjugate delivery, incubation, and bead washing, and (14–16) reporter/reagent delivery, incubation, and analysis by chemiluminescence. (C) Four frames from a video depicting magnetic bead mixing during step 16.

droplet movement for the duration of the assay. For the latter improvement (2), the sample and wash-buffer incubation durations as well as the number of incubation steps were optimized to allow for analyte-quantification over a wide dynamic range (*i.e.*, 0–20 ng mL$^{-1}$ here relative to 0–1 ng mL$^{-1}$ reported previously[19]).

As far as we are aware, the immunoassay technique described here (Fig. 2) is the first (in any format) to be designed for direct quantification of small molecule-analytes from core-needle-biopsy samples. Thus, a test was devised to evaluate the extraction efficiency of the on-chip immunoassay and to compare its performance relative to that of HPLC-MS/MS. Breast tissue samples from lactating rats were spiked with exogenous E2 and then measured by HPLC-MS/MS and the on-chip immunoassay. As shown in Fig. 3, there was a strong positive correlation between the two sets of measurements (*r* = 0.996), and the relationship was reproducible across the concentration range tested (95% CI for the slope = 0.423–0.488). The slope <1 for this analysis indicates that the immunoassay has reduced % recovery relative to HPLC-MS/MS. This was confirmed by comparing found to spiked values obtained using the immunoassay, which had recovery of 20.2 ± 4.6% (mean ± std. dev. for *n* = 10). This immunoassay-specific recovery (determined in rat tissue samples) was used to correct raw measurements in specimens (from rat and human samples) collected for individual analysis, described below.

The reduced recovery for the on-chip immunoassay relative to that of HPLC-MS/MS is not surprising, as an internal standard is used for the latter technique that compensates for sample loss during the extraction procedure (*e.g.*, from small volumes of solvent that do not separate from the tissue). Further, it is widely understood that LC-MS/MS[21] and GC-MS/MS[22,23] are "gold standard" laboratory techniques for steroid hormone analysis, with advantages (relative to immunoassays) in detection limits, specificity, and multiplexing capabilities. But the integrated immunoassay described here is useful because it is portable, and the precision and correlation of the new technique to HPLC-MS/MS gave us confidence to apply the new technique to precious CNB samples, as described below.

### Application to measurements of endogenous E2

Armed with the first integrated technique that allows for hormone quantification in tissue samples by immunoassay, we turned our attention to evaluating individual specimens. As a first test, endogenous E2 levels were measured in breast tissue samples collected from lactating and non-lactating rats (Fig. 4). The hormone amounts measured using the new technique (5.7 ± 1.9 and 23.8 ± 11.9 fmol mg$^{-1}$; mean ± std. dev. for *n* = 4 lactating and *n* = 7 non-lactating animals, respectively) are consistent with what is known from previous studies,[24,25] and the differences between the two groups are (as expected) significant (*p* = 0.0454, Student's *t*-test). These results gave us added confidence to apply the new method to precious human CNB samples.

Thirty CNB samples collected from 15 patients were evaluated using the new DMF-immunoassay system – two samples from each patient, one prior to treatment, and a second after one week of AIT (Table 1). A first observation from the data is that the E2 levels vary substantially, ranging from 7.3 to 437.4 fmol mg$^{-1}$. This is consistent with our previous observations[11] (by HPLC-MS/MS), and underscores the importance (for this application) in evaluating paired samples from the same patient. A second observation is that the patients seem to fall into three "groups" (groups 1, 2, and 3: these labels were arbitrarily assigned on the basis of the measurements). Group 1 includes six patients whose breast tissue E2 levels decreased after therapy, group 2 includes three patients
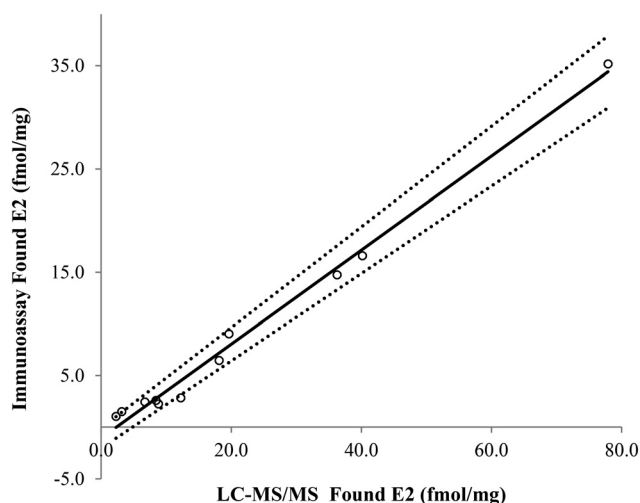


**Fig. 3** Comparison between on-chip DMF immunoassay and off-chip HPLC-MS/MS for E2 quantification. Spiked rat breast tissue samples were extracted on-chip and then analyzed using either the DMF immunoassay (*Y*-axis) or HPLC-MS/MS (*X*-axis) (open circles, *n* = 10). The line of regression (solid black line; *y* = 0.455*x* − 1.074) has $R^2$ = 0.991; the dashed black lines indicate the 95% confidence interval region for these data.
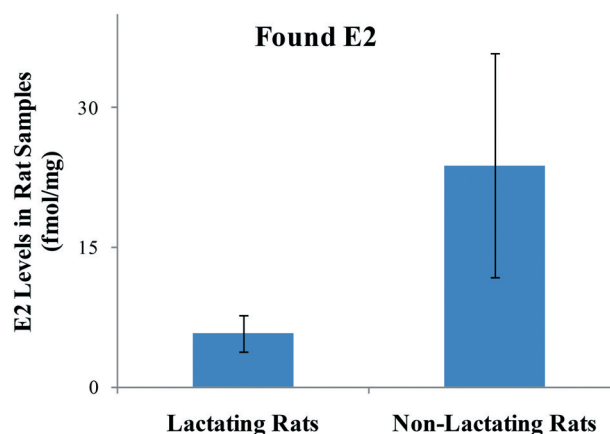


**Fig. 4** Evaluation of rat breast tissue samples extracted and analyzed using the integrated DMF immunoassay technique. Endogenous levels of E2 were measured in ~5 mg samples collected from lactating (*n* = 4) and non-lactating (*n* = 7) rats. Error bars represent ±1 S.D.

**Table 1** Estradiol values measured using the integrated DMF method with on-chip immunoassay in core needle biopsy (CNB) tissue samples collected from fifteen post-menopausal subjects before and after treatment with AIT

| Patient group (determined arbitrarily from results) | E2 level in breast tissue CNB sample (fmol mg$^{-1}$) before AIT | E2 level in breast tissue CNB sample (fmol mg$^{-1}$) after AIT |
|---|---|---|
| 1 | 35.3 | 30.0 |
|   | 17.5 | 14.8 |
|   | 69.8 | 18.5 |
|   | 62.1 | 27.9 |
|   | 40.9 | 16.7 |
|   | 437.4 | 23.9 |
| 2 | 44.5 | 47.9 |
|   | 32.7 | 32.9 |
|   | 26.9 | 30.3 |
| 3 | 7.30 | 44.1 |
|   | 35.7 | 50.5 |
|   | 30.8 | 156.5 |
|   | 18.7 | 112.5 |
|   | 15.6 | 67.7 |
|   | 21.4 | 31.0 |

whose levels remained approximately the same, and group 3 includes six patients whose levels increased.

The groups of patient results in Table 1 suggest several interesting hypotheses. For example, one hypothesis is that the variations in E2 levels measured here represent natural fluctuations with time or heterogeneous E2 distribution in breast tissue (unfortunately, control samples serially collected one week apart from patients not undergoing AIT were not available in this study). Alternatively, a second hypothesis is that physicians who treat patients in group 1 should feel confident that a suitable drug and dose are prescribed, while patients in group 2 might benefit from a larger dose or more frequent administration of drug, and patients in group 3 might be candidates to move to a different aromatase inhibitor (or a SERM, or other form of therapy). In a third hypothesis, administration of AIT for more than one week (or without combination with HRT) may be required for suppression of E2 in patients in groups 2–3. Either way, additional study seems merited, given the potential benefit to patients if the hypotheses 2–3 (or something like them) are borne out. In the latter case, a method similar to the one described here, that allows for rapid analysis (compatible with implementation during a visit to a physician's office) on a portable instrument that can be employed at the POC would prove to be an invaluable tool in the march towards a personalized regime for disease assessment and treatment. Many more samples should be tested (perhaps with multiplexed detection of E2 and other relevant analytes utilizing multivariate statistical analysis tools[26]) to assess the potential predictive value of steroid measurements in breast-tissue on AIT effectiveness.

## Conclusion

The new method reported here, which relies on digital microfluidics for sample extraction and magnetic particle-based immunoassay for analysis, is the first technique capable of quantification of small-molecule biomarkers in core needle biopsy samples on a miniaturized platform. The results described here are preliminary, but if follow-up testing supports the utility of this approach, a wide range of applications is possible, including a personalized regime for managing aromatase inhibitor therapy in post-menopausal breast cancer patients.

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## References

1 M. Marino, P. Galluzzo and P. Ascenzi, *Curr. Genomics*, 2006, **7**, 497–508.

2 D. P. McDonnell, C. E. Connor, A. Wijayaratne, C. Y. Chang and J. D. Norris, *Recent Prog. Horm. Res.*, 2002, **57**, 295–316.

3 W. Yue, J. P. Wang, Y. Li, W. P. Bocchinfuso, K. S. Korach, P. D. Devanesan, E. Rogan, E. Cavalieri and R. J. Santen, *Clin. Cancer Res.*, 2005, **11**, 925s–930s.

4 J. Shou, S. Massarweh, C. K. Osborne, A. E. Wakeling, S. Ali, H. Weiss and R. Schiff, *J. Natl. Cancer Inst.*, 2004, **96**, 926–935.

5 S. R. D. Johnston and M. Dowsett, *Nat. Rev. Cancer*, 2003, **3**, 821–831.

6 I. E. Smith and M. Dowsett, *N. Engl. J. Med.*, 2003, **348**, 2431–2442.

7 J. Cuzick, *J. Clin. Oncol.*, 2005, **23**, 1636–1643.

8 W. R. Miller and J. O'Neill, *Steroids*, 1987, **50**, 537–548.

9 M. Ellis, *Oncologist*, 2004, **9**, 20–26.

10 C. X. Ma, T. Reinert, I. Chmielewska and M. J. Ellis, *Nat. Rev. Cancer*, 2015, **15**, 261–275.

11 J. Kim, S. Abdulwahab, K. Choi, N. M. Lafrenière, J. M. Mudrik, H. Gomaa, H. Ahmado, L.-A. Behan, R. F. Casper and A. R. Wheeler, *Anal. Chem.*, 2015, **87**, 4688–4695.

12 A. Khamenehfar and P. C. H. Li, *Curr. Pharm. Biotechnol.*, 2016, **17**, 810–821.

13 M. Astolfi, B. Peant, M. A. Lateef, N. Rousset, J. Kendall-Dupont, E. Carmona, F. Monet, F. Saad, D. Provencher, A. M. Mes-Masson and T. Gervais, *Lab Chip*, 2016, **16**, 312–325.

14 K. Plevniak, M. Campbell, T. Myers, A. Hodges and M. He, *Biomicrofluidics*, 2016, **10**, 054113.

15 K. Choi, A. H. Ng, R. Fobel, D. A. Chang-Yen, L. E. Yarnell, E. L. Pearson, C. M. Oleksak, A. T. Fischer, R. P. Luoma, J. M. Robinson, J. Audet and A. R. Wheeler, *Anal. Chem.*, 2013, **85**, 9638–9646.

16 R. Fobel, C. Fobel and A. R. Wheeler, *Appl. Phys. Lett.*, 2013, **102**, 193513.

17  A. E. Kirby, N. M. Lafreniere, B. Seale, P. I. Hendricks, R. G. Cooks and A. R. Wheeler, *Anal. Chem.*, 2014, **86**, 6121–6129.

18  S. H. Au, P. Kumar and A. R. Wheeler, *Langmuir*, 2011, **27**, 8586–8594.

19  A. H. Ng, K. Choi, R. P. Luoma, J. M. Robinson and A. R. Wheeler, *Anal. Chem.*, 2012, **84**, 8805–8812.

20  B. Seale, C. Lam, D. G. Rackus, M. D. Chamberlain, C. Liu and A. R. Wheeler, *Anal. Chem.*, 2016, **88**, 10223–10230.

21  R. M. Büttler, F. Martens, F. Fanelli, H. T. Pham, M. M. Kushnir, M. J. W. Janssen, L. Owen, A. E. Taylor, T. Soeborg, M. A. Blankenstein and A. C. Heijboer, *Clin. Chem.*, 2015, **61**, 1475.

22  N. Krone, B. A. Hughes, G. G. Lavery, P. M. Stewart, W. Arlt and C. H. L. Shackleton, *J. Steroid Biochem. Mol. Biol.*, 2010, **121**, 496–504.

23  R. J. Santen, L. Demers, S. Ohorodnik, J. Settlage, P. Langecker, D. Blanchett, P. E. Goss and S. Wang, *Steroids*, 2007, **72**, 666–671.

24  D. A. Battin, R. P. Marrs, P. M. Fleiss and D. R. J. Mishell, *Obstet. Gynecol.*, 1985, **65**, 785–788.

25  N. L. Petrakis, M. R. Wrensch, V. L. Ernster, R. Miike, J. Murai, N. Simberg and P. K. Siiteri, *Int. J. Cancer*, 1987, **40**, 587–591.

26  B. K. Kim, J. W. Lee, P. J. Park, Y. S. Shin, W. Y. Lee, K. A. Lee, S. Ye, H. Hyun, K. N. Kang, D. Yeo, Y. Kim, S. Y. Ohn, D. Y. Noh and C. W. Kim, *Breast Cancer Res.*, 2009, **11**, R22.