DEVICES

Droplet-Scale Estrogen Assays in Breast Tissue, Blood, and Serum

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Estrogen is a key hormone in human reproductive physiology, controlling ovulation and secondary sexual characteristics. In addition, it plays an important role in the pathogenesis of breast cancer. Indeed, estrogen receptor antagonists and aromatase inhibitors (which block estrogen biosynthesis) are primary drugs used for treatment and prevention in at-risk populations. Despite its importance, tissue concentrations of estrogen are not routinely measured because conventional techniques require large samples of biopsies for analysis. In response to this need, we have developed a digital microfluidic method and applied it to the extraction and quantification of estrogen in 1-microliter samples of breast tissue homogenate (as would be collected with fine-needle aspiration), as well as in whole blood and serum. This method may be broadly applicable to conditions requiring frequent analysis of hormones in clinical samples (for example, infertility and cancer).

INTRODUCTION

In addition to its well-known role as a blood-borne hormone, estrogen is an important intracrine and paracrine messenger in many tissues, including the breast (*1*–*4*). Indeed, in situ biosynthesis of estrogen contributes up to 75% of the total estrogen produced in the breasts of premenopausal women (menstruating women of reproductive age) and almost 100% in menopausal women (*5*). There is a need for measuring estrogen concentrations in breast tissue to identify women at risk for developing breast cancer or to monitor the effect of antiestrogen breast cancer therapies, such as aromatase inhibitors (*6*, *7*). Local breast tissue estrogen concentrations are not routinely measured because existing methods require invasive biopsies of hundreds to thousands of milligrams of tissue (*8*, *9*). Such procedures are not performed, in part, because they require local anesthesia and carry the risk of scarring or deformity. Moreover, before analysis, large tissue samples must be processed (by lysis, homogenization, extraction, purification, and resolubilization), which requires many hours of laboratory time (*10*, *11*). These procedures are ill suited for routine testing. Although most problematic for tissue samples, many of these same limitations apply to blood and serum samples [for example, in applications related to monitoring low concentrations of hormones (*12*–*15*) or in management of infertility].

In response to these challenges, we have developed a miniaturized, automated, and integrated method for hormone analysis in 1-μl samples. The method relies on digital microfluidics (DMF), a technique in which sample and reagent droplets are moved across an open surface by applying electrical potentials to an array of electrodes (*16*). This technique is particularly well suited to multistep sample processing, and, in this paper, we describe the application of DMF to sample cleanup and extraction of estradiol (the most biologically active form of estrogen) in breast tissue from postmenopausal breast cancer patients, as well as from samples of whole blood and serum.

RESULTS

Figure 1A depicts the device designed to adapt conventional techniques for estrogen extraction from tissue samples to the DMF format. As shown, an array of electrodes connects a series of reservoirs containing the sample and reagents. The process of estrogen extraction from a sample of human blood is depicted in Fig. 1B. In typical assays, samples were lysed, the estradiol was extracted into a polar solvent (methanol), unwanted constituents were extracted into a nonpolar solvent (isooctane), and the extract was delivered to a collection reservoir. The device allows easy circulation of the methanolic phase within the isooctane phase (Fig. 1B, frame 6, and movie S1) and separation of the two phases after liquid-liquid extraction (Fig. 1B, frame 7). It can be applied to breast tissue homogenate, whole blood, serum, and standard solutions.

Mass spectrometry (MS) was used to confirm that estradiol was extracted by the DMF method. We tested whole samples and DMF-extracted samples of blood and serum obtained from a female volunteer at two different days of one reproductive cycle. The dominant estradiol fragment [mass/charge ratio (*m*/*z*) 183] was not detected in the spectra from the whole samples but was the peak of highest intensity in spectra from DMF-extracted samples (Fig. 2). In addition, the peaks of potential interfering compounds, tentatively identified as fragments of tyrosine (*17*) (*m*/*z* 178), DNA helicase (*18*) (*m*/*z* 677), and porphyrin (*19*) (*m*/*z* 715), were suppressed in the spectra of

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extracted samples, indicating that their concentrations had been substantially reduced relative to that of estradiol (Fig. 2). These data highlight the importance of sample processing for this application. Estradiol can only be ionized (and thus detected by MS) after the many interfering compounds are removed (*20*).

To test whether estradiol can be quantified in samples extracted by the DMF method, liquid chromatography and tandem mass spectrometry (LC-MS/MS) with selected reaction monitoring (SRM) (*21*) was used to evaluate estradiol extracted by DMF from standard solutions (Fig. 3A), from breast tissue homogenate from a postmenopausal breast cancer patient (Fig. 3B), and from whole blood from a female volunteer (Fig. 3C). As shown, estradiol was detected with high signal-to-noise ratio at a retention time of ~3.2 min for all cases.

A commercial enzyme-linked immunosorbent assay (ELISA) test was used to evaluate the percent recovery of the DMF technique and to evaluate the variance in the measurements. The DMF-based recoveries were high, ranging from 86 to 119% (*22*), and the coefficients of variation (CVs) were low, ranging from 7 to 10% (Fig. 4). A similar method was then applied to analyzing extracts from breast tissue homogenate from a postmenopausal breast cancer patient. Replicate analyses of 1-μl samples of breast tissue homogenate solution (requiring ~20 min of processing by DMF) yielded 522 pg estradiol per milligram tissue (with a CV of 1%). These data verify that this method is capable of quantitative analyses of estrogen in tiny amounts of breast tissue and other clinical samples.

DISCUSSION

Estrogen and other steroid hormones are fundamental for growth and reproduction, and disturbances in their physiological levels can be associated with a multitude of clinical disorders, including hormone-sensitive cancers (for example, breast, endometrial, and prostate cancers), infertility, and pregnancy complications such as intrauterine growth restriction (*23*–*27*). Moreover, hormonal therapeutics have been used for decades as anticancer medications, contraceptives, hormone replacement, and fertility drugs (*28*–*33*). Thus, a wide range of clinical conditions require frequent monitoring of these hormones in tissue or blood for accurate diagnosis and treatment.

Here, we report the extraction and quantification of the sex steroid estrogen in 1-μl volume samples. This sample size is 1000 to 4000 times smaller than that required for conventional methods of extrac-

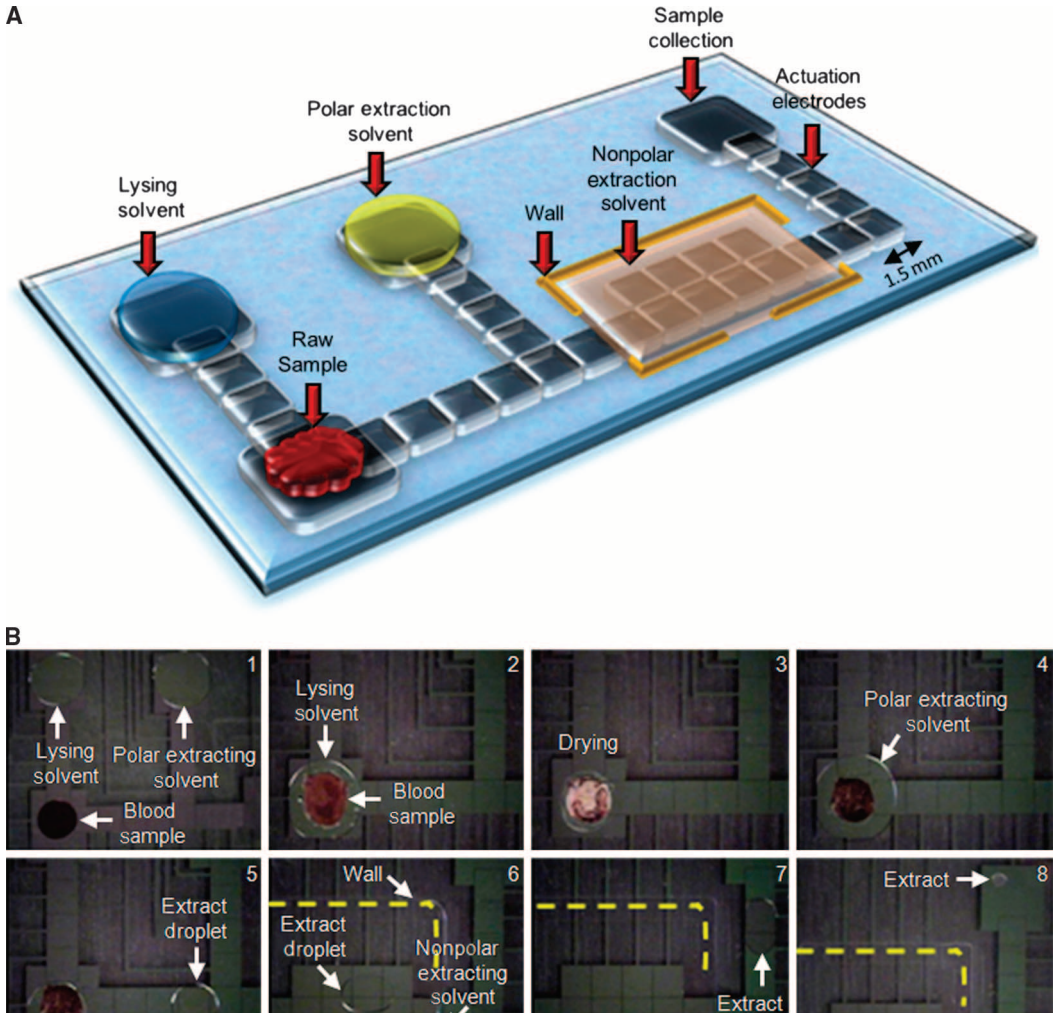

Fig. 1. DMF device design and operation. (**A**) Schematic of the DMF device, which includes sample and solvent reservoirs and the liquid-liquid extraction zone (bounded by a photoresist "wall"). (**B**) A series of frames from a movie (1 to 8) illustrating the key steps in the DMF-based extraction of estrogen from a 1-μl droplet of human blood.

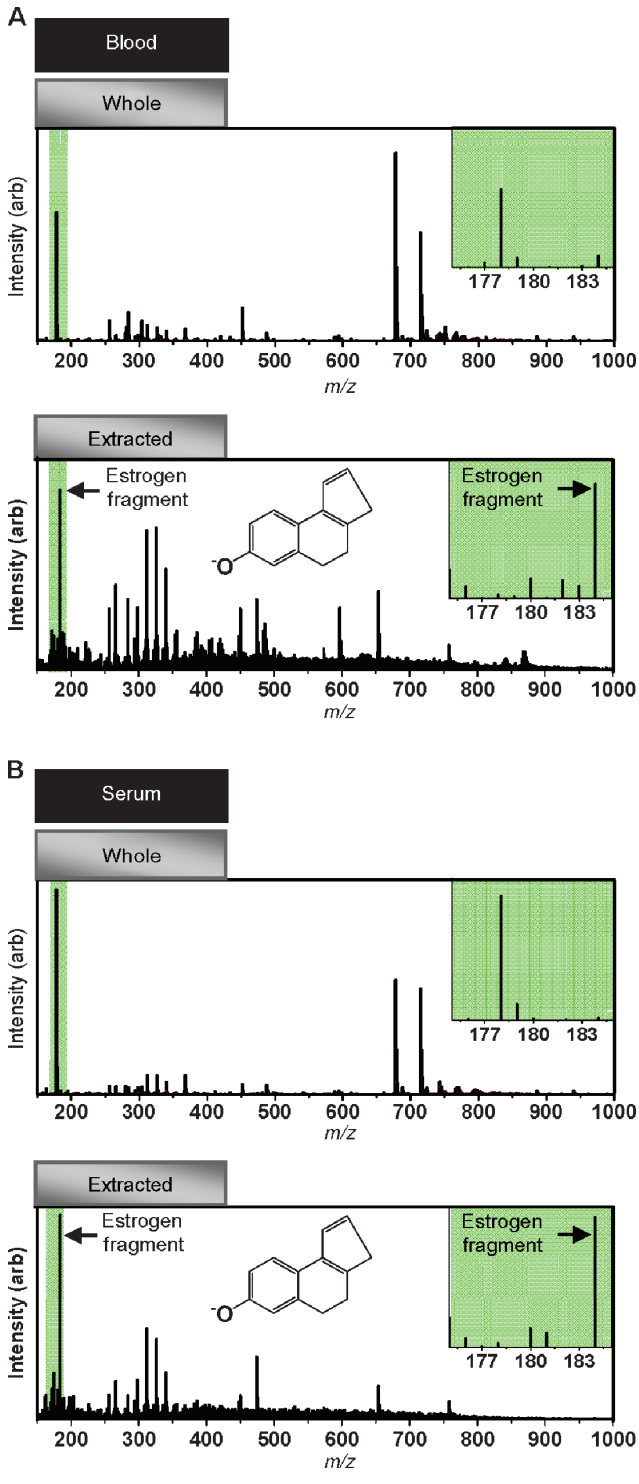

Fig. 2. Mass spectra of whole and DMF-extracted samples. Representative spectra generated from (**A**) blood and (**B**) serum obtained from a female volunteer at different days of the menstrual cycle. The insets show that the estrogen fragment at *m/z* 183 is detected in extracted samples but not in whole samples. In addition, several peaks of potential interferants, tentatively identified as fragments of tyrosine (*m/z* 178), DNA helicase (*m/z* 677), and porphyrin (*m/z* 715), are absent in the extracted samples.

tion and quantification of steroids, including extraction followed by immunoassays or MS (*8*, *9*, *34*–*37*) (fig. S1). The method could be applied to routine screening of breast estrogen concentrations in microaspirates as a potential marker of cancer risk or of blood estrogen in finger pricks to monitor hormone concentrations in infertility patients. In addition to the advantages that come with smaller samples, automation of the DMF method allows considerably less time- and labor-intensive assays relative to conventional processing techniques. Specifically, conventional 5- to 6-hour hormone extraction techniques (including various liquid-liquid extraction and solid-phase extraction–based protocols), which require extensive pipetting, centrifugation, and drying, could be replaced with the 10- to 20-min DMF process described here.

DMF (*16*, *38*, *39*) is a technology similar to but distinct from microchannel-based fluidics. Although microchannels are well suited for many applications (for example, electrophoresis, in vitro culture, and analysis of cells), microchannel-based fluidics would likely perform poorly in the application described here. Indeed, in studies (*40*–*42*) of microchannel-powered methods for liquid-liquid extraction (representing only one of the series of steps required for estrogen processing from clinical samples), the techniques have been inherently limited by the challenge of separating and collecting one liquid phase from the other after they have come into contact. In contrast, this step is straightforward in the method we have reported here (Fig. 1B, frame 7). The precise control over different reagents (*43*), phases (*44*), and volumes (*45*) afforded by DMF makes it a good match for this application.

Finally, we note that sample cleanup, extraction, and recovery are necessary steps for estrogen measurement in tissue, whole blood, and plasma; however, there are some immunoassay kits intended for detection of estrogen and other steroids in nonextracted samples of serum. The utility of these tests has been questioned because of cross-reactivity with other steroid hormones, and consensus is building that sample cleanup is a prerequisite for accurate quantification of steroids, even in serum (*12*, *15*, *46*–*48*). Thus, we anticipate that integrated sample cleanup methods, such as the one described here, may prove useful for a wide range of clinically relevant applications in many different sample types.

METHODS

Study subjects

Breast tissue was obtained from apparently normal areas adjacent to breast cancer tumors during surgery in two postmenopausal breast cancer patients and kept at –80°C until analysis. Blood and serum samples were collected from a healthy female volunteer during five different reproductive cycles (midluteal phase) and kept at –20°C until analysis. Human ethics approvals were obtained from Mount Sinai Hospital and the Ontario Tumor Bank Research Ethics Boards.

Chemicals and reagents

Dichloromethane (DCM) and 2,2,4-trimethylpentane (isooctane, 99.8%) and high-performance liquid chromatography (HPLC)–grade water were purchased from Sigma. Methyl alcohol (methanol, HPLC grade) was from Fisher Scientific. Estradiol (17-β) was purchased from Steraloids, Inc. Estradiol ELISA kits were from ALPCO Diagnostics.

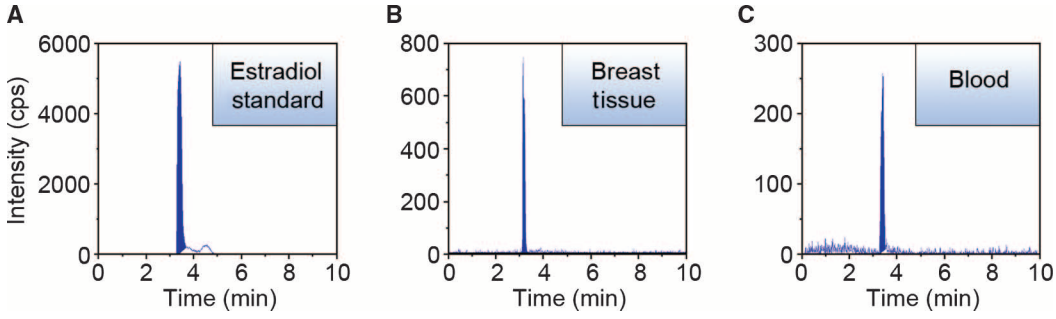

Fig. 3. LC-MS/MS analysis of DMF-extracted samples. Chromatograms generated by LC-MS/MS with SRM from 1-μl samples of (**A**) estradiol standard solution (2 mg/ml), (**B**) breast tissue homogenate from a postmenopausal patient with breast cancer, and (**C**) whole blood. The estradiol-specific ion pair evaluated for SRM was *m/z* 271/145.

DMF device fabrication and operation

Details relating to device fabrication and operation (including a description of droplet-reservoir volumes) can be found in the Supplementary Material.

DMF estrogen extraction

Two DMF-driven estrogen extraction techniques were developed; method 1, used in most experiments, comprised four steps. First, an aliquot of whole blood, serum, breast tissue homogenate, or estradiol standard solution was positioned in the sample reservoir of a device. Standard solutions were used immediately, and blood, serum, or tissue homogenate samples were allowed to dry on the surface. The top plate was then affixed and the solvents [DCM/acetone, 80:20 (v/v) as lysing solvent] were loaded. Second, a series of reservoir volumes (9 × 1.1 μl or 5 × 2.2 μl) of DCM/acetone was dispensed and driven by DMF dropwise (*49*) to the sample, each of which was allowed to incubate at room temperature until dry (~1 min per reservoir volume). Third, a reservoir volume of methanol (polar extraction solvent) (1.1 or 2.2 μl) was dispensed and driven by DMF to the dried lysate to dissolve the steroids. A unit droplet of the dissolved sample (200 or 400 nl) was dispensed and delivered by DMF to the isooctane (nonpolar extracting solvent) reservoir and circulated within the pool for ~20 s (movie S1) before the droplet was driven out of the isooctane and toward the collection reservoir. This process was repeated until the sample reservoir was empty of the methanol. Fourth, step three was repeated with successive reservoir volumes of methanol (for a total of 9 × 1.1 μl or 5 × 2.2 μl) to ensure the extraction of all of the free estradiol. Finally, all extract droplets were pooled in the output reservoir and allowed to dry.

In method 2, used to analyze percent recovery and experimental precision by ELISA, a standard solution of estradiol in methanol (1 μl) was positioned in the sample reservoir and the top plate was affixed (the lysis and polar solvent reservoirs remained empty). In each experiment, a single unit droplet (200 nl) of sample was dispensed, translated (and circulated) through isooctane, and delivered to the collection reservoir, all by DMF. The sample reservoir was then washed (manually) with methanol three times, and a fresh sample was positioned in the reservoir and the process was repeated (twice), such that three extract droplets from replicate samples (~600 nl total volume) were pooled in the collection reservoir and allowed to dry.

For all experiments, after the extract was collected and dried, the devices were stored at −20°C. Immediately before analysis, each extract was resolubilized in an aliquot (30 μl) of methanol/DCM (2:1, v/v), which was then dispensed into a small centrifuge tube. The solvent was then evaporated and the dry extract was reconstituted in a medium specific for the desired analysis.

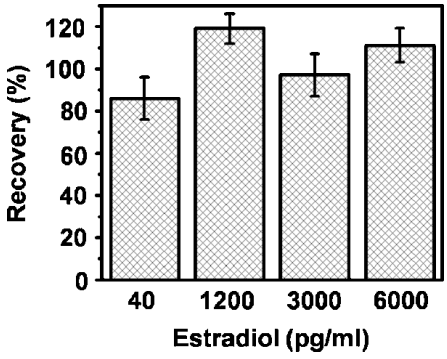

Fig. 4. Extraction efficiency analysis by ELISA. Estradiol standards before and after extraction by DMF were evaluated by ELISA. As shown, extraction efficiencies from standard solutions ranged from 86 to 119%, with CVs ranging from 7 to 10%.

Mass spectrometry

MS was used to evaluate the performance of the DMF cleanup process in samples of whole blood or serum (obtained at two different days of the reproductive cycle). In each experiment, a 5-μl sample was dried and extracted by DMF (method 1, as above), and the extract was reconstituted in 50 μl of methanol containing formic acid (0.1%, v/v). Control (nonextracted) samples were prepared by drying and reconstituting 5-μl aliquots of blood or serum in 50 μl of methanol/formic acid (0.1%, v/v), sonicating (10 min), and passing through a syringe filter (nylon membrane, 0.2-μm pore diameter). Samples were injected by nanoelectrospray into an LTQ Mass Spectrometer (Thermo Scientific) operating in the negative mode at 250°C with a flow rate of 0.5 μl/min. Under these conditions, the highest-magnitude peak observed for estradiol standards alone was *m/z* 183, which we attribute to the retrocyclization structure shown in Fig. 2. Replicate spectra were obtained for DMF-extracted and control samples of both blood and serum.

LC-MS/MS with SRM (*21*) was used to evaluate estradiol in extracts from standard solutions, breast tissue homogenate, and blood. Standard solutions (1 μl, 2 mg/ml in methanol) and blood (dried from 1 μl) were extracted by DMF (method 1, as above) with no previous processing, whereas breast tissue (400 mg) was manually homogenized in DCM (1 ml), from which 1 μl of samples was taken, dried, and processed similarly. In all cases, after extraction, samples were resuspended in 100 μl of methanol/water (80:20, v/v), 10 μl of which was injected onto an HPLC system (HP-Agilent 1100 series LC) interfaced by electrospray to a QTRAP LC-MS/MS system (Applied Biosystems). The samples

were analyzed in negative mode with SRM, evaluating an ion transition of *m*/*z* 271/145 to identify and determine the abundance of estradiol (*50*). Operating parameters included 300 μl/min flow rate, 4200 V spray potential, 60 V collision energy, and 400°C nebulizing temperature. A microbore (2.1-mm inner diameter × 50 mm) Thermo Gold C18 (2.2 μm) column with isocratic elution through a mobile phase of methanol/water (80:20, v/v) was used for LC separation.

ELISA

An estradiol-specific ELISA (ALPCO Diagnostics) was used (i) to evaluate the recovery and precision of the DMF method and (ii) to quantify estradiol in breast tissue samples. In these experiments, at least three replicate samples were evaluated for each condition by absorbance, measured at 450 nm with a μQuant microplate spectrophotometer (Bio-Tek Instruments). The optical densities of samples and controls were compared with those of ELISA calibrator solutions using a standard curve to calculate estradiol concentrations per the manufacturer's instructions.

For application (i), analysis of recovery and precision, the samples were composed of serial dilutions of estradiol standard in methanol. DMF extracts of three 1-μl samples were prepared with method 2 (as above), and for comparison, (nonextracted) control samples were prepared with the same procedure but in devices lacking isooctane. After extraction, samples were resuspended in a 50-μl mixture of estrogen-free serum (ALPCO Diagnostics) and methanol (4:1, v/v) and evaluated by ELISA (final concentrations, 40, 1200, 3000, and 6000 pg/ml). Recovery percentages were calculated as the concentrations of extracted samples divided by those from nonextracted controls. For application (ii), 1-μl samples of breast tissue homogenate (60 mg) in DCM (0.15 ml) from a breast cancer patient (a different patient than the one evaluated by MS) were processed manually (as above) and extracted by DMF with method 1. The extract was reconstituted in a 50-μl mixture of estrogen-free serum/methanol and was evaluated by ELISA.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Reduction in sample volume.
References
Movie S1

REFERENCES AND NOTES

1. H. Sasano, T. Suzuki, N. Harada, From endocrinology to intracrinology. *Endocr. Pathol.* **9**, 9–20 (1998).
2. E. R. Simpson, M. Misso, K. N. Hewitt, R. A. Hill, W. C. Boon, M. E. Jones, A. Kovacic, J. Zhou, C. D. Clyne, Estrogen—the good, the bad, and the unexpected. *Endocr. Rev.* **26**, 322–330 (2005).
3. S. E. Bulun, L. S. Noble, K. Takayama, M. D. Michael, V. Agarwal, C. Fisher, Y. Zhao, M. M. Hinshelwood, Y. Ito, E. R. Simpson, Endocrine disorders associated with inappropriately high aromatase expression. *J. Steroid Biochem. Mol. Biol.* **61**, 133–139 (1997).
4. E. R. Simpson, Sources of estrogen and their importance. *J. Steroid Biochem. Mol. Biol.* **86**, 225–230 (2003).
5. F. Labrie, Intracrinology. *Mol. Cell. Endocrinol.* **78**, C113–C118 (1991).
6. C. A. Lamar, J. F. Dorgan, C. Longcope, F. Z. Stanczyk, R. T. Falk, H. E. Stephenson Jr., Serum sex hormones and breast cancer risk factors in postmenopausal women. *Cancer Epidemiol. Biomarkers Prev.* **12**, 380–383 (2003).
7. M. S. Beattie, J. P. Costantino, S. R. Cummings, D. L. Wickerham, V. G. Vogel, M. Dowsett, E. J. Folkerd, W. C. Willett, N. Wolmark, S. E. Hankinson, Endogenous sex hormones, breast cancer risk, and tamoxifen response: An ancillary study in the NSABP Breast Cancer Prevention Trial (P-1). *J. Natl. Cancer Inst.* **98**, 110–115 (2006).
8. J. Szymczak, A. Milewicz, J. H. H. Thijssen, M. A. Blankenstein, J. Daroszewski, Concentration of sex steroids in adipose tissue after menopause. *Steroids* **63**, 319–321 (1998).
9. M. A. Blankenstein, J. van de Ven, I. Maitimu-Smeele, G. H. Donker, P. C. de Jong, J. Daroszewski, J. Szymczak, A. Milewicz, J. H. Thijssen, Intratumoral levels of estrogens in breast cancer. *J. Steroid Biochem. Mol. Biol.* **69**, 293–297 (1999).
10. C. Bélanger, F.-S. Hould, S. Lebel, S. Biron, G. Brochu, A. Tchernof, Omental and subcutaneous adipose tissue steroid levels in obese men. *Steroids* **71**, 674–682 (2006).
11. F. Labrie, A. Bélanger, P. Bélanger, R. Bérubé, C. Martel, L. Cusan, J. Gomez, B. Candas, I. Castiel, V. Chaussade, C. Deloche, J. Leclaire, Androgen glucuronides, instead of testosterone, as the new markers of androgenic activity in women. *J. Steroid Biochem. Mol. Biol.* **99**, 182–188 (2006).
12. F. Z. Stanczyk, J. S. Lee, R. J. Santen, Standardization of steroid hormone assays: Why, how, and when? *Cancer Epidemiol. Biomarkers Prev.* **16**, 1713–1719 (2007).
13. L. Albrecht, D. Styne, Laboratory testing of gonadal steroids in children. *Pediatr. Endocrinol. Rev.* **5**, 599–607 (2007).
14. S. N. Rahhal, J. S. Fuqua, P. A. Lee, The impact of assay sensitivity in the assessment of diseases and disorders in children. *Steroids* **73**, 1322–1327 (2008).
15. C. Ankarberg-Lindgren, E. Norjavaara, A purification step prior to commercial sensitive immunoassay is necessary to achieve clinical usefulness when quantifying serum 17β-estradiol in prepubertal children. *Eur. J. Endocrinol.* **158**, 117–124 (2008).
16. A. R. Wheeler, Chemistry. Putting electrowetting to work. *Science* **322**, 539–540 (2008).
17. C. Deng, N. Li, X. Zhang, Rapid determination of amino acids in neonatal blood samples based on derivatization with isobutyl chloroformate followed by solid-phase microextraction and gas chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **18**, 2558–2564 (2004).
18. The Global Proteome Machine Database, http://gpmdb.rockefeller.edu/ (accessed 9 February 2009).
19. M. Danton, C. K. Lim, Porphyrin profiles in blood, urine and faeces by HPLC/electrospray ionization tandem mass spectrometry. *Biomed. Chromatogr.* **20**, 612–621 (2006).
20. L. L. Jessome, D. A. Volmer, Ion suppression: A major concern in mass spectrometry. *LCGC North Am.* **24**, 498–510 (2006).
21. SRM is a highly selective method used to quantify a known analyte by tandem mass spectrometry. Two sequential mass selections are carried out: the first to isolate the analyte parent ion and the second to isolate a fragment ion after reaction with a carrier gas. The intensity of the fragment ion is recorded.
22. The percent recovery is the ratio of the amount of analyte measured before and after extraction, reported as a percentage. The values generated here are comparable to those that are typically generated in ELISA experiments, which often exhibit some bias in measurement accuracy (for example, with values >100%).
23. D. A. Ehrmann, Polycystic ovary syndrome. *N. Engl. J. Med.* **352**, 1223–1236 (2005).
24. S. Smith, S. M. Pfeifer, J. A. Collins, Diagnosis and management of female infertility. *JAMA* **290**, 1767–1770 (2003).
25. B. E. Henderson, R. Ross, L. Bernstein, Estrogens as a cause of human cancer: The Richard and Hinda Rosenthal Foundation award lecture. *Cancer Res.* **48**, 246–253 (1988).
26. R. Kaaks, S. Rinaldi, T. J. Key, F. Berrino, P. H. Peeters, C. Biessy, L. Dossus, A. Lukanova, S. Bingham, K. T. Khaw, N. E. Allen, H. B. Bueno-de-Mesquita, C. H. van Gils, D. Grobbee, H. Boeing, P. H. Lahmann, G. Nagel, J. Chang-Claude, F. Clavel-Chapelon, A. Fournier, A. Thiébaut, C. A. González, J. R. Quirós, M. J. Tormo, E. Ardanaz, P. Amiano, V. Krogh, D. Palli, S. Panico, R. Tumino, P. Vineis, A. Trichopoulou, V. Kalapothaki, D. Trichopoulos, P. Ferrari, T. Norat, R. Saracci, E. Riboli, Postmenopausal serum androgens, oestrogens and breast cancer risk: The European prospective investigation into cancer and nutrition. *Endocr. Relat. Cancer* **12**, 1071–1082 (2005).
27. K. Allvin, C. Ankarberg-Lindgren, H. Fors, J. Dahlgren, Elevated serum levels of estradiol, dihydrotestosterone, and inhibin B in adult males born small for gestational age. *J. Clin. Endocrinol. Metab.* **93**, 1464–1469 (2008).
28. F. A. Pitt, J. Brazier, Hormone replacement therapy for osteoporosis. *Lancet* **335**, 978 (1990).
29. A. Szarewski, J. Guillebaud, Contraception. *BMJ* **302**, 1224–1226 (1991).
30. C. Djerassi, Chemical birth of the pill. *Am. J. Obstet. Gynecol.* **194**, 290–298 (2006).
31. R. Hertz, J. C. Bailar III, Estrogen-progestogen combinations for contraception. *JAMA* **198**, 1000–1006 (1966).
32. G. R. Cunningham, Testosterone replacement therapy for late-onset hypogonadism. *Nat. Clin. Pract. Urol.* **3**, 260–267 (2006).
33. J. Geisler, B. Haynes, G. Anker, H. Helle, D. Ekse, M. Dowsett, P. E. Lønning, Treatment with high-dose estrogen (diethylstilbestrol) significantly decreases plasma estrogen and androgen levels but does not influence in vivo aromatization in postmenopausal breast cancer patients. *J. Steroid Biochem. Mol. Biol.* **96**, 415–422 (2005).

34. R. T. Falk, E. Gentzschein, F. Z. Stanczyk, L. A. Brinton, M. Garcia-Closas, O. B. Ioffe, M. E. Sherman, Measurement of sex steroid hormones in breast adipocytes: Methods and implications. *Cancer Epidemiol. Biomarkers Prev.* **17**, 1891–1895 (2008).
35. R. J. Santen, L. Demers, S. Ohorodnik, J. Settlage, P. Langecker, D. Blanchett, P. E. Goss, S. Wang, Superiority of gas chromatography/tandem mass spectrometry assay (GC/MS/MS) for estradiol for monitoring of aromatase inhibitor therapy. *Steroids* **72**, 666–671 (2007).
36. T. Yasui, M. Yamada, H. Kinoshita, H. Uemura, N. Yoneda, M. Irahara, T. Aono, S. Sunahara, Y. Mito, F. Kurimoto, K. Hata, Combination of automatic HPLC-RIA method for determination of estrone and estradiol in serum. *J. Clin. Lab. Anal.* **13**, 266–272 (1999).
37. K. Kurecková, B. Maraliková, K. Ventura, Supercritical fluid extraction of steroids from biological samples and first experience with solid-phase microextraction-liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **770**, 83–89 (2002).
38. M. G. Pollack, R. B. Fair, A. D. Shenderov, Electrowetting-based actuation of liquid droplets for microfluidic applications. *Appl. Phys. Lett.* **77**, 1725–1726 (2000).
39. J. Lee, H. Moon, J. Fowler, T. Schoellhammer, C.-J. Kim, Electrowetting and electrowetting-on-dielectric for microscale liquid handling. *Sens. Actuators A Phys.* **95**, 259–268 (2002).
40. Y. Okubo, T. Maki, N. Aoki, T. Hong Khoo, Y. Ohmukai, K. Mae, Liquid-liquid extraction for efficient synthesis and separation by utilizing micro spaces. *Chem. Eng. Sci.* **63**, 4070–4077 (2008).
41. J. G. Kralj, H. R. Sahoo, K. F. Jensen, Integrated continuous microfluidic liquid-liquid extraction. *Lab Chip* **7**, 256–263 (2007).
42. P. Znidarsic-Plazl, I. Plazl, Steroid extraction in a microchannel system—Mathematical modelling and experiments. *Lab Chip* **7**, 883–889 (2007).
43. D. Chatterjee, B. Hetayothin, A. R. Wheeler, D. J. King, R. L. Garrell, Droplet-based microfluidics with nonaqueous solvents and solutions. *Lab Chip* **6**, 199–206 (2006).
44. M. J. Jebrail, A. R. Wheeler, Digital microfluidic method for protein extraction by precipitation. *Anal. Chem.* **81**, 330–335 (2009).
45. M. Abdelgawad, S. L. S. Freire, H. Yang, A. R. Wheeler, All-terrain droplet actuation. *Lab Chip* **8**, 672–677 (2008).
46. L. Dibbelt, G. Von Postel, R. Knuppen, Determination of natural and synthetic estrogens by radioimmunoassay. I. Comparison of direct and extraction methods for quantification of estrone in human serum. *Clin. Lab.* **44**, 137–143 (1998).
47. M. J. Diver, J. A. Nisbet, Warning on plasma oestradiol measurement. *Lancet* **2**, 1097 (1987).
48. Report of a workshop on Hormone Standardization held by the Division of Laboratory Sciences at the Centers for Disease Control and Prevention, March 17-18, 2008, http://www.cdc.gov/nceh/dls/hormone_standardization.htm (accessed 26 July 2009).
49. In digital microfluidics, small unit droplets are routinely dispensed from larger reservoir droplets. We use the term "dropwise" to mean sequential dispensing of multiple unit droplets to be merged to form a large combined volume. For example, nine 1.1-μl unit droplets of DCM/acetone were dispensed, dropwise, to form a larger volume of 9.9 μl of lysis solution on the sample.
50. A. Gentili, D. Perret, S. Marchese, R. Mastropasqua, R. Curini, A. Di Corcia, Analysis of free estrogens and their conjugates in sewage and river waters by solid-phase extraction then liquid chromatography-electrospray-tandem mass spectrometry. *Chromatographia* **56**, 25–32 (2002).
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