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An Electrochemical Clamp Assay for Direct, Rapid Analysis of Circulating Nucleic Acids in Serum

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The analysis of cell-free nucleic acids (cfNA), present at significant levels in the blood of cancer patients, can reveal the mutational spectrum of a tumor without the need for invasive sampling of tissue; however, this requires the differentiation of nucleic acids that originate from healthy cells from the mutated sequences shed by tumor cells. Here we report an electrochemical clamp assay that directly detects mutated sequences in patient serum. This is the first successful detection of cfNA without the need for enzymatic amplification, a step that normally requires extensive sample processing and is prone to interference. The new chip-based assay reads out the presence of mutations within 15 minutes using a collection of oligonucleotides that sequester closely-related sequences in solution, thus allowing only the mutated sequence to bind to a chip-based sensor. We demonstrate excellent levels of sensitivity and specificity and show that the clamp assay accurately detects mutated sequences in a collection of samples collected from lung cancer and melanoma patients.

The discovery that cell-free nucleic acids (cfNAs) released from tumors are present in the blood of patients with cancer has focused attention on their potential as a new marker for cancer diagnosis and management.¹⁻³ Detection of cfNAs in plasma or serum could serve as a liquid biopsy, potentially replacing tumor tissue biopsies in certain diagnostic applications. Although cancer patients often have higher levels of cfNAs than do healthy controls, the levels of overall cfNAs vary considerably in plasma or serum samples in each group.⁴ For the analysis of cfNAs to be clinically meaningful it must enable the specific detection of cancer-related sequences.

Detection of mutated sequences (e.g. *KRAS* and *BRAF*) linked to cancer in cfNAs could allow specific monitoring of tumor-related sequences^{5,6}; however, this would require a very sensitive and specific approach to detect successfully low amounts of mutant genes in the presence of high levels of wild-type sequences in patient samples.⁷ The existing approaches that are able to monitor cfNAs rely on the polymeric chain reaction (PCR)⁸ or DNA sequencing⁹. DNA sequencing is an excellent approach for research studies seeking to profile large regions of DNA, but its implementation is prohibitively expensive for routine clinical use, and the slow turnaround time (2-3 weeks) is not ideal for optimal treatment outcomes⁵. PCR is not typically effective for the detection of point mutations, but the introduction of peptide nucleic acid (PNA) clamps boosts the accuracy of this approach.¹⁰⁻¹² The PNAs serve as sequence-selective clamps that prevent amplification of wild-type DNA during PCR, and the mutated sequence is then selectively amplified. Unfortunately, because PCR is prone to interference from the components of biological samples, clamp PCR is not able to detect cfNA mutations in blood or serum samples directly. As well, it can introduce bias based on the amplification efficiency of different sequences⁴, and requires preprocessing of samples and purification of nucleic acids from large-volume samples (e.g. > 5 ml).¹³ A method that is more accurate, and able to detect cfNA mutations directly in serum or blood, is thus urgently required.

Chip-based methods leveraging electronic or electrochemical readout represent attractive alternatives for clinical sample analysis because they are amenable to automation and the development of cost-effective testing devices¹⁴. Electrochemical methods in particular have received significant attention because of their low cost and potential for high levels of multiplexing and sensitivity¹⁵. This type of testing approach has been applied successfully to the analysis of a subset of cancer biomarkers¹⁶⁻²⁰, as well as a variety of infectious pathogens²¹⁻²⁴, but the feasibility of analyzing cell-free nucleic acids for cancer-related mutations in clinical samples has not been established. Previous efforts to achieve point mutation detection based on electrochemical methods²⁵⁻²⁶ have relied on stringent control of assay conditions or mismatch-sensitive enzymes²⁷, but these types of approaches would not yield significant selectivity in heterogeneous patient samples where a mutated sequence may be outnumbered by a high level of the wild-type sequence.

Here we report an electrochemical approach that is the first to enable direct analysis of cfNAs from patient serum samples. Electrochemical sensors are functionalized with molecules that render them specific for a nucleic acids sequence of interest, and a series of clamp molecules are used to eliminate cross-reactivity with wild-type DNA and with deliberately-selected mutants. Electrochemical analysis has been used previously with a variety of clinically-relevant analytes²⁸⁻³², but this is the first study demonstrating the specific detection of cancer-related mutations in cfNAs. We show that the approach is highly specific, rapid and sensitive, reading sequences of interest in five femtograms of isolated RNA. We further show that it can be used with unprocessed, banked serum from cancer patients and produces results that are consistent with the gold-standard method. The approach reported enables much broader analysis of cfNA in patient samples.

Results and discussion

Design of the electrochemical clamp assay

The design of the electrochemical clamp assay for detection of cfNA mutations is depicted in Figure 1. The first detection target is the *KRAS* gene, which has 7 mutations at codons 12 and 13 of 2 exons, which are 135A, 135C, 135T, 134A, 134C, 134T, and 138A. Mutated *KRAS* (Kirsten rat sarcoma-2 virus) genes are associated with lung cancer, colorectal cancer, and ovarian cancer,^{3,5,8,33-34} and the efficacies of several therapies are affected by mutations in this gene. A given patient sample may contain one of the 7 mutant alleles and a large amount of wild type nucleic acids (Figure 1A). To limit binding of all sequences except the exact one of interest, clamps are designed to target each of the mutants and wild type NAs.

We illustrate the approach with the case of detecting the presence of a sequence containing the 134A mutation. A mixture of six clamps corresponding to the six other *KRAS* mutations, plus a single clamp binding the wild-type sequence, is added to the target-containing solution. The clamps hybridize to the six non-target mutants and the wild-type sequence, leaving the target mutant of interest unhybridized. We then introduce this solution mixture onto a sensor chip that has been functionalized with a probe corresponding to 134A. Only the mutant 134A hybridizes to the probe; all other mutants including wild type are blocked by their clamps and simply remain in solution and are washed away (5 of Figure 1A). After 15 minutes, we read out the electrochemical signal and determine thereby the identity of the sequence.

Fabrication of the electrochemical clamp assay chip

Using photolithographic patterning, we defined an array of forty sensors to form a bioelectronic integrated circuit (Figure 1). Beginning with a SiO₂-coated silicon wafer, we formed contact pads and electrical leads, after which we deposited a layer of Si₃N₄ to passivate the top surface of the IC. To provide a template for the growth of electrodeposited sensors, photolithography was used next to open 5

μm apertures in the top passivation layer. We carried out Au electrodeposition at locations determined by the opened apertures to grow three-dimensional microstructures for subsequent biosensing. The microstructured sensors protrude from the surface and reach into solution^{16,21,35,36}, with their size and morphology programmed by deposition time, applied potential, Au concentration, supporting electrolyte, and overcoating protocol. Since nanostructures increase the sensitivity of the assay significantly^{22,37-40}, we coated the Au structures with a thin layer of Pd to form finely nanostructured microelectrodes (NMEs) (Figure 1E). The micron-size scale of the three-dimensional electrodes increases the cross-section for interaction with analyte molecules, while the nanostructuring maximizes sensitivity by enhancing hybridization efficiency between tethered probe and the analyte in solution⁴¹.

We functionalized the NMEs with PNA probes specific to the mutant target of interest (Figure 1). After target binding and washing, we used an electrocatalytic reporter pair comprised of $\text{Ru}(\text{NH}_3)_6^{3+}$ and $\text{Fe}(\text{CN})_6^{3-}$ to read out the presence of specific nucleic acids sequences.⁴² $\text{Ru}(\text{NH}_3)_6^{3+}$ is electrostatically attracted to the negatively-charged phosphate backbone of nucleic acids that bind to the probes immobilized on the surface of electrodes and is reduced to $\text{Ru}(\text{NH}_3)_6^{2+}$ when the electrode is biased at the reduction potential. The $\text{Fe}(\text{CN})_6^{3-}$ present in solution chemically oxidizes $\text{Ru}(\text{NH}_3)_6^{2+}$ back to $\text{Ru}(\text{NH}_3)_6^{3+}$ allowing for multiple turnovers of $\text{Ru}(\text{NH}_3)_6^{3+}$, which generates an high electrocatalytic current. The difference between pre-hybridization and post-hybridization currents is used as a metric to determine target binding (typical differential pulse voltammograms (DPVs) before and after 100 fg/ μL target mutant NA (134A) binding shown in Figure 1C).

Specific mutant detection with the clamp chip

The chip was designed to genotype seven distinct point mutation alleles of the *KRAS* gene that are associated with lung cancer. A set of PNA probes and clamps was designed, tested, and validated for sensitivity and specificity using both electrochemical (Figure 2A) and PCR-based methods (Figure S1). A sample

containing a complex mixture including complementary mutant target, non-complementary mutants, wild type sequence, total human RNA, and the clamp cocktail was used to measure the positive signal at sensors functionalized with probes corresponding to each mutant. A mixture that was identical except that it lacked the complementary mutant was tested as a negative control. The negative controls did not produce a positive signal change in any of the sensors tested; in contrast, the positive samples produced current changes ranging from 7 to 12 nA (Figure 2A), well above background. These results clearly demonstrate that the clamp assay can specifically detect each of mutant alleles of *KRAS* genes. It is noteworthy that if a complementary sequence is not included in a sample, a slightly negative change in the electrochemical signal is often observed. This is a systematic effect that arises because a small amount of the probe monolayer dissociates from the sensor during incubation, which lessens the background signal.

To investigate whether the clamps were necessary for accurate point mutation detection, we challenged a mutant sensor with purified nucleic acids from a wild-type patient sample, a mutant-positive patient sample, and a healthy donor in presence and absence of the clamp for the wild-type sequence. Although hybridization and washing were performed at an elevated temperature, we observed a signal increase for all three samples if the clamp for the wild-type sequence was not present in solution (Figure 2B). However, in the presence of the clamp for the wild-type sequence, we did not observe any positive signal change for mutant negative and healthy donor samples. A significant signal change was observed for the mutant-positive sample in the presence of the clamp. The change of current in the presence of clamp is slightly lower than in the absence of clamp because clamp minimizes interference from wild-type nucleic acids. These results clearly show that for sensitive and specific detection of mutations within cfNAs, the presence of the clamp sequence is essential.

To evaluate the sensitivity of our clamp assay, we investigated the dependence of the electrochemical signal on concentration when the sensors were challenged

with RNA isolated from A549 cells that carry the 134A mutation. RNA containing the wild-type sequence was isolated from U733v3 cells and used to evaluate specificity. To identify the detection limit of our assay, we tested concentrations of RNA ranging from 1 fg/ μ L to 100 pg/ μ L (Figure 3A). The signal change increased with increasing concentration of the target over six orders of magnitude. Moreover, to observe specificity of the sensor further we performed three additional control experiments: testing of a blank, a noncomplementary RNA target (NCT of Figure 3A), and using a noncomplementary probe. For all three controls, we obtained a limit of detection of 1 fg/ μ L. To evaluate the detection speed of the clamp assay, we investigated the time-dependent signal change by varying hybridization time of 10 fg/ μ L target RNA (Figure 3B). The electrochemical clamp assay is capable to delivering results very rapidly: statistically-significant signals were obtained within five minutes.

To establish that the clamp method is generally applicable to other sequences, we investigated the detection of mutations within *BRAF*, which are associated with melanoma, the deadliest form of skin cancer.⁶ After we validated a set of BRAF-specific probes, we checked specificity and sensitivity of the clamp assay for RNA from the MW9 cell line (Figure 4A and B). In this case we also obtained a similar sensitivity, specificity, and speed of the clamp assay.

Patient sample analysis

The ultimate goal of our effort to develop a mutation-discriminating chip was to enable the direct analysis of mutated sequences in patient samples. We challenged our electrochemical clamp assay with the task of analyzing cfNA in serum samples from lung cancer patients (*KRAS*) and melanoma cancer patients (*BRAF*) (Table 1 and 2). In the trials analyzing samples for *KRAS* mutations, a universal probe mixture (Figure S2 and Table 3) was used that allowed us to screen for all of the possible types of mutations in cfNAs using a single experiment. This approach leverages a mixture of sequences that are perfect complements to all known mutants of the sequences of interest. By using the

universal probe approach, it is possible to use a single reaction to look for all possible mutations.

cfNAs were isolated and purified from serum using commercially available kits from 14 lung cancer and 9 melanoma patients. Serum from a healthy donor was also processed the same way and analyzed. Three of the fourteen lung cancer patient samples were positive for *KRAS* mutations and four of the nine melanoma cancer patient samples were positive for *BRAF* mutations. For each sample analyzed using the electrochemical method, the mean signal (plus three standard deviations) for a sample from a healthy donor was used as a cutoff value. If the current level observed with a patient sample is higher than the cutoff value, the sample is determined to be positive for the mutation, and if it is lower, the sample is negative for the mutation. In each case, a previously-validated clamp PCR⁴³ method was used to confirm the presence or absence of the *KRAS* and *BRAF* mutations, and the results were in agreement with the electrochemical clamp assay results.

We also challenged the clamp assay with undiluted serum from the same lung cancer and melanoma patients (Table 1 and 2). The signal changes observed in undiluted serum are lower than for purified samples, which is expected because the concentrations of cfNA are much lower than in the purified samples. Nonetheless, the mutated cfNAs are correctly identified in each of the patient samples.

The clamp assay thus successfully detects cfNA *KRAS* and *BRAF* mutations directly in undiluted serum of lung cancer and melanoma patients. We note that clamp PCR was not able to detect *KRAS* and *BRAF* directly in undiluted patient serum. We investigated the PCR-based method with undiluted serum, but did not observe any detectable levels of enzymatic amplification (Figure S3). We also explored the application of literature protocols⁴⁴ that have been used successfully to amplify viral targets in unpurified serum. We diluted same patient serum 5-fold in PBS and tested these samples along with those that had undergone heat

treatment. PCR amplification of mutated sequences was not successful despite the application of these protocols (Figure S3). The fact that this treatment of serum was successful for viral targets versus the cfNAs studied here likely relates to the very low levels of the mutated sequences under analysis here relative to the higher abundance of viral sequences. This set of studies clearly indicates that while the chip-based clamp assay can successfully be applied to unpurified serum samples, PCR is not amenable to the same type of direct sample analysis. Direct analysis of patient samples, without the requirement of purification, is a significant advantage, as it eliminates any issues related to bias in the pool of sequences that are isolated. In addition, very small volumes (50 μ L) of serum can be analyzed with this approach, whereas 5 ml of serum is required to yield enough purified cfNA for analysis via PCR.

While the experiments described above establish the sensitivity and specificity of cfNA analysis with the electrochemical clamp assay, they did not, on their own, distinguish between DNA or RNA analytes. To identify whether the assay was interrogating genomic DNA or transcribed mRNA, we challenged our clamp assay with cfNAs, cfNAs digested with DNase I, and cfNAs digested with RNase A. The change for total cfNAs and cfRNAs are similar, whereas no signal change was observed for cfDNA (Figure 5). We conclude that the analytes detected are predominantly cfRNAs.⁴ Indeed, the assay relies on no steps to denature or fragment the cfNAs, and the sensitivity to RNA is therefore to be expected.

Conclusion

We have analysed cfNAs (both cfDNA and cfRNA) of lung cancer and melanoma patients using a highly specific and sensitive electrochemical clamp assay. The clamp chip accurately detects mutated sequences of cfNA directly in patient serum. The clamp assay requires a collection of sequences that effectively compete with the sequence of interest and only allow the specific target to bind to the chip. Minimally invasive serum analyses of cfNA offer a simpler alternative to cancer tissue biopsies. They also suggest a means to monitor drug responses

and treatment effectiveness. Furthermore, the chip-based approach we describe has a number of advantages over existing methods that have been used to analyze cell-free nucleic acids. The clamp assay produces the same accuracy as PCR, but is functional when unpurified serum is used as the sample type. This leads to a much simpler assay workflow, minimizes sample loss, and permits the analysis of small samples. The sensitivity of the clamp assay chip is extremely high, and allows for the use of samples that can be collected non-invasively to profile the mutational spectrum of a tumor. The analysis time required, which can be as short as five minutes, is significantly more rapid than the 2-3 hours required for PCR, and the days required for sequencing. Finally, the use of a chip-based format for the assay will allow for straightforward automation of the approach and a low cost per test.

Materials and Methods

Nucleic acids. All of the PNA probes and PNA clamps were obtained from PNA Bio, USA. PCR primers, synthetic DNA targets, and DNA clamps were obtained from ACGT, Canada. See supporting information for detail.

Fabrication of NMEs. Fabrication of the chips and growth of the NMEs was performed as described previously.³⁷ The control of sensor surface area has been characterized extensively⁴⁵ and in this study, the average surface area was $4.75 \pm 0.3 \times 10^{-4} \text{ cm}^2$ as determined by electrochemical Pd oxide stripping.

Clamp chip protocol. A 2 μM probe solution in water was prepared from a 20% acetonitrile solution containing 100 μM PNA probe. Probe solutions were then heated to 65 °C for 5 min and chilled on ice for 5 min before deposition. 50 μL of the probe solution was dropped onto the chips and incubated for overnight in a dark humidity chamber at room temperature for immobilization of probe. The effect of deposition conditions on probe surface coverage has been studied extensively,³⁸ and the conditions used here lead to a surface coverage of 2×10^{13} molecules/ cm^2 . The chip was then washed for 10 min with PBS at 60 °C followed by washing for 10 min at room temperature. After initial electrochemical scanning the chips were then treated with different targets at 60°C. Optimal hybridization time was determined to be 15 min. After washing for 10 min with PBS at 55°C followed by washing for 10 min at room temperature of the chip final electrochemical scan was performed.

cfNAs isolation from exosomes. MW9 and U373v3 exosomes were isolated by ultracentrifugation method and RNA was extracted by Trizol (Invitrogen). A549 exosomal RNA (mutant *KRAS* 134A) and exosomal RNA from patient serums was extracted, using Norgen biotek kit catalog number 51000. Isolated RNA had a A260/A280 ratio > 2, indicating a high level of purity.

cDNA synthesis and clamp PCR. A volume of 2 μL purified cfNA (30-754 ng) was used for cDNA synthesis, in 20 μL reaction, with random hexamer primers

and Superscript III reverse transcriptase, Invitrogen kit. A volume of 2 μ L cDNA was used in 50 μ L not-competitive clamp PCR reaction with 2 μ M final concentration of gene specific primers, or in a 20 μ L of real-time clamp PCR reaction, Panagene kit.

Mutant *BRAF* and mutant *KRAS* clamp optimization. To validate that 60°C was an appropriate specific temperature for the sensor assay, clamp PNA was tested in a qualitative PCR assay. The PCR program was as follows: template denaturing at 95°C for 3 minutes followed by 35 cycles of template denaturing at 95°C for 30 seconds, primer annealing and DNA chain extension at 60°C for one minute. The PCR products were visualized using agarose gel electrophoresis.

Real-time competitive clamp PCR. Mutant *BRAF* and mutant *KRAS* real-time competitive clamp PCR were performed using a Panagene kit (mutant *BRAF* product number PNAC-2001 and mutant *KRAS* product number PNAC-1002). The real time clamp PCR was performed on ABI 7500 thermocycler and the SYBR Green reading was set at 72°C. The PCR program was as follows: template denaturing at 94°C for 5 min followed by 40 cycles of template denaturing at 94°C for 30 sec, PNA clamp at 70°C for 20 sec, primer annealing at 63°C for 30 sec and DNA chain extension at 72°C for 30 sec.

***KRAS* and *BRAF* detection in whole serum.** For mutation detection in whole serum we co-deposited 6-mercaptohexanol (MCH) with the probe to minimize nonspecific binding. For *KRAS* mutation detection, we used a universal probe for *KRAS* point mutations that was a combination of all of the possible mutant probes. An aqueous solution containing 2 μ M of PNA probes was heated to 65°C for 5 min and after annealing 18 μ M of MCH was mixed with this probe solution. After that the solution was dropped onto chip for overnight following by washing as described above. Serum sample was prepared by adding 12.5 μ L of lysis buffer (1 X PBS containing 10% NP40 and 10% Triton X100), 1 μ L of 10 μ M clamps for wild-type, 3 μ L of RNAase inhibitor (Ambion, Am 2694) to 50 μ L of patients' serum. After initial electrochemical scanning above serum sample was

dropped onto chip and incubated at 60°C for 15 min. After washing, a final electrochemical scan was performed.

Electrochemical analysis. Signal changes corresponding to specific target were calculated with background-subtracted currents: change in currents = ($I_{\text{after}} - I_{\text{before}}$) (where I_{after} = current after target binding, I_{before} = current before target binding). See supporting information for detail.

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Author contributions

J.D., I.I., J.R., E.H.S., and S.O.K. conceived the experiments. J.D. and I.I. designed the experiments. L.M. contributed critical materials. J.D., I.I., E.H.S. and S.O.K. co-wrote the paper. All authors reviewed and improved the paper.

Additional information

Supplementary information is available in the online version of the paper. Correspondence and requests for materials should be addressed to S.O.K. (shana.kelley@utoronto.ca).

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Figure 1. The clamp chip for electrochemical analysis of mutated cfNAs. (A) *Clamp strategy.* Schematic representation of clamp approach to detecting mutations of *KRAS*. The sample (1) is mixed with clamp sequences (2) that will sequester the wild-type sequence and all mutants except the one that is the detection target. All of the mutants and the wild-type sequence hybridize to the respective complementary clamps in solution (3), except the target of interest, e.g. 134 A (green). **(B) *Sensor-based detection.*** The sample is applied to the PNA probe-modified microsensor, and only the target mutant NAs hybridizes to an immobilized PNA probe and the other six mutants and wild type NAs are prevented from binding, and eventually are washed away. **(C) *Electrochemical readout.*** After target hybridization and washing, sensors were interrogated using a electrocatalytic reporter system (left). Differential pulse voltammetry (DPV, right) is used to monitor whether a signal increase is observed in the presence of cfNA. **(D) *Chip layout.*** Schematic of a microfabricated chip that possesses sensors that are electroplated into apertures patterned at the end of gold leads. **(E) *Scanning electron microscopy image*** of a nanostructured microelectrode.

Figure 2. Proof of principle and validation of probes. (A) Validation of seven probes (P135 A, P135 C, P135 T, P134 A, P134 C, P134 T, and P138 A) of seven mutant (MT) alleles of *KRAS*. Sensors were challenged with mixtures of nucleic acids with (positive control) and without (negative control) mutant target of interest. Positive control contains all of the seven mutant oligonucleotides with 1 nM concentration of each, 100 nM of wild-type (WT) synthetic oligonucleotides, 50 pg/ μ L NAs from healthy donors, and seven clamps except one that is complementary for target of interest. Negative control contains all of the above except target of interest and its clamp. **(B)** Current change for wild type NAs in presence and absence of clamps at a mutant probe-modified sensor. A sensor modified with a probe specific for a mutated sequence was challenged with a mutant negative patient sample (mutant -) in presence (grey) and absence of clamp (black) for WT, a mutant positive patient sample (mutant +) in presence and absence of clamp for WT, and a sample for healthy donor in presence and absence of clamp for WT. Error bars represent standard error and data represents ≥ 5 trials.

Figure 3. Limit of detection studies and time dependence for *KRAS* cfNAs. (A) Concentration dependent signal change for A549 exosomal RNAs containing seven clamps except clamp for 134 A at 134 A mutant sensor. NCT represents negative target i.e., U733v3 exosomal RNA was used instead of A549 exosomal RNA and NCP represents noncomplementary probe i.e., A549 exosomal RNA was tested with 135 A sensor. The sensor is able to detect 1 fg/ μ L A549 exosomal RNA. **(B)** Hybridization time-dependent signal change for the challenge of 10 fg/ μ L of A549 exosomal RNA. 10 fg/ μ L of A549 exosomal RNA is detectable in 15 minutes. Error bars represent standard error and data represents ≥ 5 trials.

Figure 4. Limit of detection studies and time dependence for *BRAF* cfNAs. The minimal amount of RNA and time needed to generate a detectable signal was determined. (a) Concentration-dependent signal change for MW9 exosomal RNA containing clamp for wildtype. This analysis indicates that as little as 1 fg/ μ L of RNA can be detected. NCT represents noncomplementary target. (b) Time-dependent signal change obtained when sensors were challenged with 50 fg/ μ L of MW9 exosomal RNA. In as few as five minutes, a detectable signal is generated. Error bars represent standard error and data represents ≥ 5 trials.

Figure 5. Determination of target analyte. Both cell-free RNA and DNA have been found in patient samples, and the sensing approach described cannot directly discriminate which type of nucleic acid is present. By introducing enzymes that can digest either RNA or DNA specifically, the type of cfNA can be determined. Three different samples were tested: a) cfNAs only, b) DNase I digested cfNAs, c) RNase A digested cfNAs. The loss of signal for c) indicates that RNA is the material being detected in the assay described. Error bars represent standard error and data represents ≥ 5 trials.

Table 1. Analysis of lung cancer patient samples. (A) Lung cancer patient samples analysis. Left. Sensor was challenged with total cfNAs isolated from lung cancer patients by using commercially available kit. A threshold for this sensor is 1.25 nA. Middle. Wildtype *KRAS* clamp PCR with total cfNAs. For clamp PCR, $\Delta\text{Ct}-1 \geq 2$ is considered to be mutated and $\Delta\text{Ct}-1 < 0$ is considered to be non mutated. If $0 < \Delta\text{Ct}-1 < 2$, it needs to consider another parameter $\Delta\text{Ct}-2$ and if $\Delta\text{Ct}-2 > 6$ is considered to be non mutated. Right. Sensor was challenged with undiluted lung cancer patient serum. A threshold for this sensor is 0.42 nA. We use mean current plus 3 times of standard deviation (mean + 3SD) of healthy samples as a threshold value for electrochemical clamp assay. If current change for any sample is higher than the threshold, the samples is considered as *KRAS* mutated. The error values represent standard error.

Table 2. Analysis of melanoma cancer patient samples. Melanoma patient samples analysis. Left. Sensor was challenged with total cfNAs isolated from melanoma patients by using commercially available kit. A threshold for this sensor is 2.46 nA. Middle. Wild type *BRAF* clamp PCR with total cfNAs. For clamp PCR, according to manufacturer, $\Delta\text{Ct}-1 \geq 2$ is considered to be mutated and $\Delta\text{Ct}-1 < 0$ is considered to be non mutated. Right. Sensor was challenged with undiluted melanoma patient serum. A threshold for this sensor is 0.69 nA. We use mean current plus 3 times of standard deviation (mean + 3SD) of healthy samples as a threshold value for electrochemical clamp assay. If current change for any sample is higher than the threshold, the samples is considered as *BRAF* mutated. The error values represent standard error.

Supporting information for:

An Electrochemical Clamp Assay for Direct, Rapid Analysis of Circulating Nucleic Acids in Serum

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Supplementary Figure 1: Validation of specific clamping of KRAS and BRAF

Supplementary Figure 2: The clamp chip for electrochemical cfNAs detection with a universal probe

Supplementary Figure 3: Raw data illustrating lack of PCR amplification of cfNA in serum

Supplementary Figure 4: Concentration dependence of electrochemical clamp assay.

Supplementary Table 1: Sequences used in this work

Materials. HAuCl_4 , potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), and hexamine ruthenium (III) chloride ($\text{Ru}(\text{NH}_3)_6\text{Cl}_3$) were obtained from Sigma-Aldrich. ACS-grade acetone, isopropyl alcohol (IPA), and perchloric acid were obtained from EMD; hydrochloric acid was purchased from VWR. Phosphate-buffered saline (PBS, pH 7.4, 1×) was obtained from Invitrogen. The A549 lung cancer cell line (catalog number CCL-185) and cultured medium (F-12K medium, catalog number 30-2004 supplemented with 10% (v/v) FBS and 5% CO_2 atmosphere) were obtained from ATCC, Canada. Lung cancer and melanoma patient serums were obtained from Bioreclamation Inc., USA. PCR primers for BRAF (95bp PCR product): Forward primer: FPBRAF3 (5'-cct-cac-agt-aaa-aat-agg-tga-ttt-tgg-3'), Reverse primer: RPBRAF3 (5'-cac-aaa-atg-gat-cca-gac-aac-tgt-tc-3'). PCR primers for KRAS (80bp PCR product): Forward primer: FPKRAS (5'-gcc-tgc-tga-aaa-tga-ctg-aat-ata-3'), Reverse primer: RPKRAS (5'-tta-gct-gta-tcg-tca-agg-cac-tc-3'). All other sequences used in this work are shown in Table S1.

Electrochemical analysis and scanning electron microscopy (SEM). All electrochemical experiments were carried out using a Bioanalytical Systems Epsilon potentiostat with a three-electrode system featuring a Ag/AgCl reference electrode and a platinum wire auxiliary electrode. Electrochemical signals were measured in a 0.1 x PBS containing 10 mM $[\text{Ru}(\text{NH}_3)_6]\text{Cl}_3$, and 4 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$. Differential pulse voltammetry (DPV) signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width 50 ms, and a pulse period of 100 ms. SEM images were obtained using an Aspek 3025 SEM.

Supplementary Figure 1. Validation of specific clamping of *KRAS* and *BRAF*.(A) Validation of specific clamping of the 134A *KRAS* and WT *KRAS* amplification at 60 °C, by non competitive clamp PCR. Clamp PCR using A549 and U373v3 exosomal RNA. A459 cells are 134A *KRAS* homozygous and U373v3 are WT homozygous (134G *KRAS*). (D) Non competitive PNA clamp RT-PCR- specific clamping of the WT *BRAF* amplification at 60 °C.

Supplementary Figure 2. The clamp chip for electrochemical of point mutant NAs detection with a *KRAS* universal probe.

(A) Schematic representation of point mutation detection of seven alleles of *KRAS*. (1) Heterogeneous solution containing seven mutants (135 A, 135 C, 135 T, 134 A, 134 C, 134 T, 138 A) and wild type nucleic acids. (2) Clamp for wild type NA are added to this solution. (3) Clamps hybridize to the wild type nucleic acids and mutant targets remain un-hybridized. (4) Only the mutant nucleic acid hybridizes to an immobilized PNA probe and wild type nucleic acids are prevented from binding. (5) After target hybridization for 15 min and washing, sensors were interrogated using an electrocatalytic reporter system.

Supplementary Figure 3. Raw data illustrating lack of clamp PCR amplification of cfNA in serum. The plot shown illustrates the rise in fluorescence observed as a function of PCR cycle number. While positive results were obtained when purified cell-free nucleic acids were amplified (a), the use of undiluted serum (b) did not produce data with a clear exponential rise in signal. Diluted serum (c) and diluted and heated serum (d) were also used in an attempt to apply literature protocols to the use of clamp PCR for this application, but negative results were obtained. All patient samples tested using this approach did not produce detectable amplification.

Supplementary Figure 4. Concentration dependence of electrochemical clamp assay. (A) *KRAS* mutant sensor. Concentration-dependent signal change for A549 exosomal NAs containing seven clamps except clamp for 134 A at 134 A mutant sensor. **(B)** *BRAF* mutant sensor. Concentration-dependent signal change for MW9 exosomal NA containing clamp for wild type.

Name	Type	Sequence ^{a,b}
KRAS		
Probe for 135A allele	PNA	Cys-Gly-CTA CGC CAT <u>CAG</u> CTC CAA C
Probe for 135C allele	PNA	Cys-Gly-CTA CGC CAG <u>CAG</u> CTC CAA C
Probe for 135T allele	PNA	Cys-Gly-CTA CGC CAA <u>CAG</u> CTC CAA C
Probe for 134A allele	PNA	Cys-Gly-CTA CGC CAC TAG CTC CAA C
Probe for 134C allele	PNA	Cys-Gly-CTA CGC CAC <u>GAG</u> CTC CAA C
Probe for 134T allele	PNA	Cys-Gly-CTA CGC CAC AAG CTC CAA C
Probe for 138A allele	PNA	Cys-Gly-CTA CGT <u>CAC</u> CAG CTC CAA C
Universal probe for <i>KRAS</i> point mutants	PNA	Cys-Gly-CTA CGX <u>CAX</u> <u>XAG</u> CTC CAA C Where, X = mixture of A, T, and G with unimolar ratio
Clamp for 135A allele	PNA	ACG CCA TCA GCT C
Clamp for 135C allele	PNA	ACG CCA GCA GCT C
Clamp for 135T allele	PNA	ACG CCA ACA GCT C
Clamp for 134A allele	PNA	ACG CCA CTA GCT C
Clamp for 134C allele	PNA	ACG CCA CGA GCT C
Clamp for 134T allele	PNA	ACG CCA CAA GCT C
Clamp for 138A allele	PNA	CCT ACG TCA CCA G
Clamp for wild type	PNA	ACG CCA CCA GCT C
Target for 135A allele	DNA	GTT GGA GCT GAT GGC GTA G
Target for 135C allele	DNA	GTT GGA GCT GCT GGC GTA G
Target for 135T allele	DNA	GTT GGA GCT GTT GGC GTA G
Target for 134A allele	DNA	GTT GGA GCT AGT GGC GTA G
Target for 134C allele	DNA	GTT GGA GCT CGT GGC GTA G
Target for 134T allele	DNA	GTT GGA GCT TGT GGC GTA G
Target for 138A allele	DNA	GTT GGA GCT GGT GAC GTA G
Target for wild type (135G)	DNA	GTT GGA GCT GGT GGC GTA G
BRAF		
Probe for <i>BRAF</i> mutant (1799 T>A)	PNA	Cys-Gly-GAT TTC TCT GTA GCT A
Clamp for <i>BRAF</i> wild type	PNA	GAT TTC ACT GTA G
Target for <i>BRAF</i> mutant	DNA	TAG CTA CAG <u>AGA</u> AAT C
Target for <i>BRAF</i> wild type	DNA	TAG CTA CAG TGA AAT C

^a 5' to 3' for DNA sequences, N to C terminus for PNA sequences.

^b Underlined portions denote point mutations.

Supplementary Table 1. Sequences used in this work.

Figure 1. The clamp chip for electrochemical analysis of mutated cfNAs. (A) *Clamp strategy*. Schematic representation of clamp approach to detecting mutations of *KRAS*. The sample (1) is mixed with clamp sequences (2) that will sequester the wild-type sequence and all mutants except the one that is the detection target. All of the mutants and the wild-type sequence hybridize to the respective complementary clamps in solution (3), except the target of interest, e.g. 134 A (green). (B) *Sensor-based detection*. The sample is applied to the PNA probe-modified microsensor, and only the target mutant NAs hybridizes to an immobilized PNA probe and the other six mutants and wild type NAs are prevented from binding, and eventually are washed away. (C) *Electrochemical readout*. After target hybridization and washing, sensors were interrogated using an electrocatalytic reporter system (left). Differential pulse voltammetry (DPV, right) is used to monitor whether a signal increase is observed in the presence of cfNA. (D) *Chip layout*. Schematic of a microfabricated chip that possesses sensors that are electroplated into apertures patterned at the end of gold leads. (E) *Scanning electron microscopy image* of a nanostructured microelectrode.

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Figure 3. Limit of detection studies and time dependence for *KRAS* cfNAs. (A) Concentration-dependent signal change for A549 exosomal RNAs containing seven clamps except clamp for 134 A at 134 A mutant sensor. NCT represents negative target i.e., U733v3 exosomal RNA was used instead of A549 exosomal RNA and NCP represents noncomplementary probe i.e., A549 exosomal RNA was tested with 135 A sensor. The sensor is able to detect 1 fg/μL A549 exosomal RNA. (B) Hybridization time-dependent signal change for the challenge of 10 fg/μl of A549 exosomal RNA. 10 fg/μl of A549 exosomal RNA is detectable in 15 minutes. Error bars represent standard error and data represents ≥ 5 trials.

Figure 4. Limit of detection studies and time dependence for *BRAF* cfNAs. The minimal amount of RNA and time needed to generate a detectable signal was determined. (a) Concentration-dependent signal change for MW9 exosomal RNA containing clamp for wild type. This analysis indicates that as little as 1 fg/μL of RNA can be detected. NCT represents noncomplementary target. (b) Time-dependent signal change obtained when sensors were challenged with 50 fg/μl of MW9 exosomal RNA. In as few as five minutes, a detectable signal is generated. Error bars represent standard error and data represents ≥ 5 trials.

Figure 5. Determination of target analyte. Both cell-free RNA and DNA have been found in patient samples, and the sensing approach described cannot directly discriminate which type of nucleic acid is present. By introducing enzymes that can digest either RNA or DNA specifically, the type of cfNA can be determined. Three different samples were tested: a) cfNAs only, b) DNase I digested cfNAs, c) RNase A digested cfNAs. The loss of signal for c) indicates that RNA is the material being detected in the assay described. Error bars represent standard error and data represents ≥ 5 trials.

	Purified nucleic acids					Serum	
	Clamp chip		Clamp PCR			Clamp chip	
<i>Samples</i>	Δi (nA)	<i>Assessment</i>	$\Delta Ct-1$	$\Delta Ct-2$	<i>Assessment</i>	Δi (nA)	<i>Assessment</i>
1	- 2.3 $\pm$ 1.0	Wild type	1	16	Wild type	0 $\pm$ 0.1	Wild type
2	- 1.5 $\pm$ 0.5	Wild type	-1	18	Wild type	- 0.1 $\pm$ 0.1	Wild type
3	8.3 $\pm$ 1.3	KRAS mutated	6	8	KRAS mutated	2.1 $\pm$ 0.9	KRAS mutated
4	- 0.6 $\pm$ 0.7	Wild type	0	16	Wild type	- 0.1 $\pm$ 0.1	Wild type
5	- 1.9 $\pm$ 0.5	Wild type	-2	16	Wild type	- 0.4 $\pm$ 0.2	Wild type
6	- 2.5 $\pm$ 0.9	Wild type	1	14	Wild type	- 0.1 $\pm$ 0.1	Wild type
7	- 1.3 $\pm$ 0.6	Wild type	- 4	14	Wild type	- 0.4 $\pm$ 0.1	Wild type
8	- 1.2 $\pm$ 0.5	Wild type	-4	18	Wild type	- 0.8 $\pm$ 0.2	Wild type
9	4.8 $\pm$ 0.7	KRAS mutated	4	6	KRAS mutated	1.3 $\pm$ 0.2	KRAS mutated
10	- 1.5 $\pm$ 0.2	Wild type	-3	18	Wild type	- 0.8 $\pm$ 0.2	Wild type
11	- 0.6 $\pm$ 0.4	Wild type	-	-	Wild type	- 0.1 $\pm$ 0.2	Wild type
12	4.5 $\pm$ 1.4	KRAS mutated	3	16	KRAS mutated	1.4 $\pm$ 0.3	KRAS mutated
13	- 2.2 $\pm$ 0.5	Wild type	-2	16	Wild type	- 1.2 $\pm$ 0.1	Wild type
14	- 1.5 $\pm$ 0.3	Wild type	-2	15	Wild type	0.4 $\pm$ 0.1	Wild type
HD	- 1.0 $\pm$ 0.3	Wild type	0	16	Wild type	- 0.5 $\pm$ 0.1	Wild type

Table 1. Analysis of lung cancer patient samples. (A) Lung cancer patient samples analysis. Left. Sensor was challenged with total cfNAs isolated from lung cancer patients by using commercially available kit. A threshold for this sensor is 1.25 nA. Middle. Wild type *KRAS* clamp PCR with total cfNAs. For clamp PCR, $\Delta Ct-1 \geq 2$ is considered to be mutated and $\Delta Ct-1 < 0$ is considered to be non mutated. If $0 < \Delta Ct-1 < 2$, it needs to consider another parameter $\Delta Ct-2$ and if $\Delta Ct-2 > 6$ is considered to be non mutated. Right. Sensor was challenged with undiluted lung cancer patient serum. A threshold for this sensor is 0.42 nA. We use mean current plus 3 times of standard deviation (mean + 3SD) of healthy samples as a threshold value for electrochemical clamp assay. If current change for any sample is higher than the threshold, the samples is considered as *KRAS* mutated. The error values represent standard error.

	Purified nucleic acids				Serum	
	Clamp chip		Clamp PCR		Clamp chip	
<i>Samples</i>	Δi (nA)	<i>Assessment</i>	$\Delta Ct-1$	<i>Assessment</i>	Δi (nA)	<i>Assessment</i>
1	5.3 ± 0.5	<i>BRAF</i> mutated	4.9	<i>BRAF</i> mutated	1.7 ± 0.3	<i>BRAF</i> mutated
2	7.9 ± 0.8	<i>BRAF</i> mutated	5.3	<i>BRAF</i> mutated	1.2 ± 0.6	<i>BRAF</i> mutated
3	0.8 ± 0.3	Wild type	-1.4	Wild type	- 0.5 ± 0.1	Wild type
4	0.6 ± 0.8	Wild type	-3.1	Wild type	0.3 ± 0.4	Wild type
5	1.2 ± 0.7	Wild type	-2.1	Wild type	- 1.4 ± 0.2	Wild type
6	- 1.1 ± 1.0	Wild type	-1	Wild type	- 0.1 ± 0.2	Wild type
7	21.9 ± 1.5	<i>BRAF</i> mutated	2	<i>BRAF</i> mutated	2.1 ± 0.3	<i>BRAF</i> mutated
HD	0.05 ± 0.6	Wild type	-1.2	Wild type	0.3 ± 0.1	Wild type

Table 2. Analysis of melanoma cancer patient samples. Melanoma patient samples analysis. Left. Sensor was challenged with total cfNAs isolated from melanoma patients by using commercially available kit. A threshold for this sensor is 2.46 nA. Middle. Wild type *BRAF* clamp PCR with total cfNAs. For clamp PCR, according to manufacturer, $\Delta Ct-1 \geq 2$ is considered to be mutated and $\Delta Ct-1 < 0$ is considered to be non mutated. Right. Sensor was challenged with undiluted melanoma patient serum. A threshold for this sensor is 0.69 nA. We use mean current plus 3 times of standard deviation (mean + 3SD) of healthy samples as a threshold value for electrochemical clamp assay. If current change for any sample is higher than the threshold, the samples is considered as *BRAF* mutated. The error values represent standard error.