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An ELISA-based high throughput protein truncation test for inherited breast cancer

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Abstract

Introduction: Breast cancer is the most diagnosed and second leading cause of cancer deaths in the U.S. female population. An estimated 5 to 10 percent of all breast cancers are inherited, caused by mutations in the breast cancer susceptibility genes (*BRCA1/2*). As many as 90% of all mutations are nonsense mutations, causing a truncated polypeptide product. A popular and low cost method of mutation detection has been the protein truncation test (PTT), where target regions of *BRCA1/2* are PCR amplified, transcribed/translated in a cell-free protein synthesis system and analyzed for truncated polypeptides by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. We previously reported a novel High Throughput Solid-Phase PTT (HTS-PTT) based on an enzyme-linked immunosorbent assay (ELISA) format that eliminates the need for radioactivity, SDS-PAGE and subjective interpretation of the results. Here, we report the next generation HTS-PTT using triple-epitope-tagged proteins and demonstrate, for the first time, its efficacy on clinical genomic DNA samples for *BRCA1/2* analysis.

Methods: Segments of exons 11 of *BRCA1/2* open reading frames were PCR amplified from either blood derived genomic DNA or cell line mRNA. PCR primers incorporate elements for cell-free transcription/translation and epitope tagging. Cell-free expressed nascent proteins are then antibody-captured onto the wells of a microtiter plate and the relative amount of truncated polypeptide measured using antibodies against the N- and C-terminal epitope tags in an ELISA format.

Results: 100% diagnostic sensitivity and 96% specificity for truncating mutations in exons 11 of *BRCA1/2* was achieved on one hundred blood-derived clinical genomic DNA samples which were previously assayed using the conventional gel based PTT. Feasibility of full gene coverage for *BRCA1/2* using mRNA source material is also demonstrated.

Conclusions: Overall, the HTS-PTT provides a simple, quantitative, objective, low cost and high throughput method for analysis of truncating mutations as an alternative to gel based PTT for *BRCA* analysis. The technology is readily accessible to virtually any laboratory, with the only major instrumentation required being a PCR thermocycler and a basic micro-well plate reader. When compared to conventional gel based PTT, the HTS-PTT provides excellent concordance.

Introduction

Breast cancer is the most diagnosed and second leading cause of cancer deaths in the U.S. female population, with ~200,000 new cases and ~40,000 deaths reported annually [1]. Ovarian cancer is the second ranking gynecological cancer, with ~22,000 cases and ~15,000 deaths annually [1]. ~3-5% of all breast and ~10% of ovarian cancers are due to inherited mutations in the breast and ovarian cancer susceptibility 1 or 2 genes (*BRCA1* or *BRCA2*) [2-4]. Lifetime risks of *BRCA1/2* mutation carriers have been estimated at ~80% for breast cancer and ~40% and ~20% for ovarian cancer (BRCA1 and 2, respectively) [5].

Nonsense or frameshift mutations, which result in the truncated gene product (and presumably non-functional or dysfunctional BRCA protein), account for ~90% of the clinically important *BRCA1/2* entries in the Breast Cancer Information Core (BIC) database and ~50% fall within the large exon 11 (of BRCA1 and 2) alone [6]. Mutation specific techniques are rendered impractical due to the sheer number of mutations (>850 clinically important mutations can be found in *BRCA1* and >750 in *BRCA2* [6]).

In addition to inherited breast and ovarian cancers, a variety of other inherited diseases are caused by chain truncations as the primary mode, including familial adenomatous polyposis (*APC*) [7], hereditary non-polyposis colon cancer (*MSH2/MLH1*) [8], polycystic kidney disease (*PKD1*) [9], neurofibromatosis (*NF1* and *NF2*) [10, 11] and Duchenne muscular dystrophy (*DMD*) [12].

Direct automated intra-exonic DNA sequencing is the “gold standard” scanning method for detection of *BRCA1/2* mutations (Myriad Genetics, Salt Lake City, UT). A variety of other approaches are possible, including several electrophoretically based assays that essentially resolve the mutant DNA or protein by mobility differences. These include the protein truncation test (PTT), conformation-sensitive gel electrophoresis (CSGE) and two-dimensional gene scanning (TDGS), as well as variants of these basic methods. Analogous to the electrophoretic methods, denaturing high performance liquid chromatography (DHPLC) is a highly sensitive method to detect mutant-WT DNA heteroduplexes. High throughput or multiplexed mutation-specific methods (i.e. requiring *a priori* knowledge of possible mutations), such as DNA microarrays and RT-PCR are also possible. A recent review of *BRCA1/2* mutation detection methods [2] found that while the specificity was essentially 100% for most methods, the sensitivity varied significantly, with the different electrophoretic mobility based DNA assays ranging from 50-100%, the PTT at 75% and DHPLC as the top performer at 100% in all cases.

However, methods such as DNA sequencing, DNA microarrays and DHPLC are expensive to perform and/or require expensive and specialized instrumentation, thus, despite its imperfect sensitivity, the PTT assay is popular for its simplicity, cost-effectiveness and general accessibility to virtually any laboratory [5]. Conventional PTT begins with PCR amplification of target gene segments from the patient source material (e.g. genomic DNA or mRNA from blood). The PCR primer pair incorporates additional sequences into the amplicons required for subsequent protein production, including an RNA polymerase promoter, a Kozak (ribosome binding) site as well as start and stop

codons. The amplified DNA is then added to a cell-free transcription-translation extract along with radioactive amino acids (^{35}S-methione or ^{14}C-leucine) and the expressed protein is then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Chain truncation mutations are detected by the presence of a lower molecular weight (increased mobility) species relative to the wild-type protein band. More recent variants of the SDS-PAGE based PTT include non-radioactive versions based on Western blotting [13] or as reported by us, using tRNA mediated protein engineering to fluorescently label the expressed protein [14]. Additional benefits of the PTT include: *i*) the potential to be one of the highest throughput mutation scanning methods, since 1-3 kb of DNA sequence can be scanned from a single PCR reaction, compared to 200-300 bp in many other methods [15, 16]; *ii*) ability to detect large genomic deletions and insertions as well as mRNA splicing errors that can be missed by methods such as direct DNA sequencing and DHPLC [16, 17].

However, as with all the electrophoretic based methods, PTT is inherently low throughput, difficult to automate and results, which are based on visual inspection of the gel, can be difficult to interpret thereby making them subject to human error [2]. Furthermore, mutations in the 5' end of the *BRCA* gene resulting in small truncation fragments are missed (not resolved on gel) [16], affecting the diagnostic sensitivity.

To overcome these limitations, we previously developed [14] the first High Throughput Solid-Phase Protein Truncation Test (HTS-PTT) which uses an industry-standard microtiter plate enzyme-linked immunosorbent assay (ELISA) format, which can be

implemented into any clinical laboratory with minimal, inexpensive and widely-used instrumentation. This assay, which we previously applied to the detection of Familial Adenomatous Polyposis (FAP) [14], used a triple tag system comprised of N-terminal and C-terminal epitope tags for normalization and mutation detection respectively, as well as directly incorporated biotin labels as the capture tag for protein immobilization on the ELISA plate. Unfortunately, biotin incorporation during translation using tRNA mediated protein engineering occurs at very low efficiency, thereby adversely affecting mutation detection capabilities and diagnostic sensitivity (data not shown). Du et. al [18] later introduced a 2-tag variant of our assay as applied to the genetic disorder ataxia-telangiectasia, however, it lacks the ability to normalize for the amount of total nascent protein that is expressed and captured on the ELISA plate, critical for the reproducibility and accuracy required in a clinical and/or diagnostic setting.

The next generation HTS-PTT reported here uses three different epitope tags, two in tandem at the N-terminus (one detection and one capture tag) and one at the C-terminus (detection tag), added by using specially designed PCR primers which amplify target gene segments from patient blood. The amplified DNA is cell-free expressed and simultaneously captured and purified by epitope capture onto the ELISA plate, and the remaining two tags enable N- and C-terminal detection (Figure 1). Using this improved assay, we have applied it for the first time to the detection of truncation mutations in exons 11 of the breast cancer susceptibility genes *BRCA1/2* and have evaluated it using 100 clinical genomic DNA test samples.

Materials and methods

Human samples were obtained through the Women's College Research Institute (Reference No. 2007-0036-B) for these experiments and for the collaboration with AmberGen, Inc. All study subjects provided informed written consent. For HTS-PTT analysis, purified genomic DNA samples were provided to AmberGen in de-identified form, marked only with non-descriptive alphanumeric codes along with only the BRCA1/2 mutation status/designation, and no identifying information or clinical annotation was provided to AmberGen.

DNA and PCR

One hundred human genomic clinical DNA samples isolated from blood and previously characterized by the conventional gel based PTT (method as described in [19]) were used for HTS-PTT. The genomic DNA was extracted from peripheral blood leukocytes using the Gentra Puregene Blood Kit (Gentra Systems, Minneapolis, USA). mRNA samples for HTS-PTT were extracted from several BRCA mutant cell lines (B-lymphocytes from blood) obtained from the Coriell Cell Repository (Camden, NJ) using the Qiagen Rneasy Mini Kit (Germantown, MD) and converted to cDNA using the SMART™ PCR cDNA Synthesis Kit (Clontech, Mountain View, CA). PCR amplification was carried out using 50 ng of genomic DNA or cDNA, 0.245 µM each of the forward and reverse primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (each), and the Phusion Hot Start DNA Polymerase with the HF Buffer (New England Biolabs, Ipswitch, MA). Amplification was performed as follows: An initial denaturation at 95ºC for 2.5 min, forty

cycles of denaturation at 95ºC for 30 s, annealing at 67.5ºC for 45 s, extension at 72ºC for 3.5 min and a final extension step at 72ºC for 10 min. The gene-specific PCR primers used on genomic DNA or cDNA templates were as follows:

Forward Primer:

5'ggATCC*TAATACgACTCACTATA*gggAgACCACCATg**TACACCgACATCgAgATgAA CCgCCTgggCAAg**ggAggA**CAgCCTgAACTCgCTCCAgAggATCCggAAgAT** [Gene-Specific Hybridization Region]3'

Forward Primer Key: The italicized nucleotides correspond to the T7 promoter, the underlined ATg is the initiation codon, the boldface nucleotide region codes for the N-terminal detection tag (VSV-G; YTDIEMNRLGK), the underlined boldface nucleotide region codes for the N-terminal capture tag (HSV; QPELAPEDPED), the bracketed nucleotide sequence codes for the BRCA-specific complementary region (see later below) and the remaining nucleotide sequences correspond to the Kozak (ribosome binding) and spacer regions.

Reverse Primer: 5'TTATTA**CAgCAgCTTgTgCAggTCgCTgAAggT** [Gene-Specific Hybridization Region]3'

Reverse Primer Key: The boldface nucleotides code for the C-terminal detection tag (p53-derived tag; TFSDLHKLL), the underlined TTATTA is two successive stop codons

and the bracketed nucleotide sequence codes for the BRCA-specific complementary region (see later below).

Gene-Specific Hybridization Regions and sequence of the primers are provided in Table 1. After amplification, the quality and quantity of the PCR products was analyzed by agarose gel electrophoresis.

Refer to Figure 2 for an example of agarose gel analysis of the five different PCR segments and refer to Figure 3 for the names of the aforementioned cell lines used for the mRNA samples.

Cell-Free Protein Synthesis

The rabbit reticulocyte cell-free reaction mixture contained 8.5 µL of TNT T7 Quick Rabbit Reticulocyte Lysate for PCR DNA (Promega, Madison, WI), 0.5 µL of a complete amino acid mix (50 µM final for each) and 1 µL of PCR amplified DNA (approximately 100-200 ng). The coupled transcription-translation reaction was allowed to proceed for 45 min at 30°C.

HTS-PTT

MicroLite 2+ 96-well microtiter plates (Thermo Fisher Scientific, Waltham, MA) were pre-coated with 0.5 ng/µL of an anti-HSV tag monoclonal antibody (EMD Biosciences, La Jolla, CA) solution in 50 mM sodium carbonate at a pH of 9.5. The plates were then washed 4X with TBS containing 0.05% (v/v) Tween-20 (TBS-T) and blocked for 30 min

with TBS-T containing 1% (w/v) BSA. After protein synthesis, the reaction mixture was diluted 100-fold with TBS-T further supplemented with 0.1% Triton X-100, 1% (w/v) BSA, and one of either the N- or C-terminal detection antibodies (anti-VSV-G-HRP at 40 ng/mL, Clone P5D4, Roche Applied Science, Indianapolis, IN; or anti-p53-HRP at 80 ng/mL, Clone BP53-12, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; HRP = horseradish peroxidase). Alternatively, both the anti-VSV-G-HRP antibody and an anti-p53-AP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; AP = alkaline phosphatase) could be used in the same solution (same concentrations as above) to ultimately allow both signals to be detected in the same well using this dual-reporter system [14]. In either case, 100 µL of the diluted reaction mixture was then added to each well of the aforementioned antibody-coated microtiter plates and incubated for 45 min on an orbital shaker. The plate was washed 4X with TBS-T and developed using a chemiluminescent HRP substrate (Super Signal Femto, Pierce Chemicals, Rockford, IL). If the dual-reporter system was used, a chemiluminescent alkaline-phosphatase substrate was added first (Roche Applied Science, Indianapolis, IN) and following signal readout, the plates washed 4X with TBS-T and developed using the HRP substrate described above. All signals were read using a LumiCount luminescent plate reader (Packard Biosciences, Meriden, CT). The background levels correspond to cell-free protein synthesis reactions that lacked only the added DNA in the reaction mixture.

Results and Discussion

The Basic HTS-PTT

A schematic of the HTS-PTT assay is shown in Figure 1 (PCR and expression not shown). *BRCA1/2* Open Reading Frames (ORFs) are amplified and divided into "working" segments using PCR (corresponding to roughly 50-75 kDa overlapping protein segments within exons 11 of BRCA1 and BRCA2, see also Table 1). As detailed in the Materials and Methods, the PCR reaction also incorporates all the needed expression sequences (e.g. promoter) and epitope tag sequences, as required by the HTS-PTT method. Following cell-free coupled transcription/translation of the PCR products, the nascent proteins are analyzed by HTS-PTT for truncation mutations. The N-terminal capture tag (HSV epitope) is used for concurrent immobilization/purification of the cell-free expressed protein onto antibody coated microtiter well ELISA plates. The N- and C-terminal detection tags (VSV-G and p53 epitopes, respectively) are subsequently used for measuring the relative level of shortened protein produced by the chain truncation mutation (Figure 1). Detection is achieved using horseradish peroxidase (HRP) labeled epitope tag antibodies and a highly sensitive chemiluminescent readout in separate replicate wells of the ELISA plate. Alternatively, if tighter control is desired, a per well normalization is possible using N- and C-terminal epitope tag antibodies in the same wells, whereby each antibody carries a different enzyme reporter [14].

PCR Optimizations on BRCA1/2 for HTS-PTT

Despite the lengthy sequences needing to be added by the PCR primers in the HTS-PTT process, we have fully optimized the PCR conditions so that this can be achieved in a single reaction (1-step PCR) with a single primer pair (~130-mer forward and ~60-

mer reverse), as opposed two sequential PCR reactions commonly used to add sequences of this length to an amplicon. This is important since two sequential PCRs are not compatible with standard clinical PCR clean-room practices (i.e. separate pre- and post-PCR rooms). A significant problem we encountered is that the lengthy 1-step PCR primers are prone to forming non-specific and/or primer dimer extension products which produce substantial background in the HTS-PTT, since both can be expressed into protein and can contain the in-frame epitope tags. Optimizing factors such as primer concentration, annealing temperature, magnesium concentration and the use of a heat-activated “hot start” DNA polymerase were found to be critical in avoiding this problem (data not shown). Extreme care should be taken to avoid the primer dimer extension products in both the 1-step and even the 2-step PCR methodologies, as these products are ideal expression templates and can interfere with HTS-PTT even when present at levels near or below the detection limit of conventional ethidium bromide agarose gel electrophoresis. For instance, the quantity and quality of the genomic DNA input into the PCR reaction should closely monitored (discussed later), as in general, less cognate template means more primer dimer extension products formed. All experiments described in this manuscript utilize this optimized 1-step PCR method (see Figure 2 for example of agarose gel analysis of five different PCR segments).

Linearity of the HTS-PTT Assay

Following the HTS-PTT assay (Figure 1), the C-terminal signal arising from full-length protein in a given sample is normalized against its N-terminal signal representing total nascent protein produced and captured on the ELISA plate. These C-terminal to N-

terminal signal ratios (C/N ratios) of the test samples are calculated as a percent of the ratio arising from known *BRCA* wild-type (WT) control samples (% C/N). Thus, in theory, *BRCA* WT samples would have a 100% C/N ratio and heterozygous BRCA mutants a 50% ratio.

In practice however, the % C/N ratios of the BRCA heterozygous mutants deviate from 50% in either direction (e.g. see Figure 3a and b; red bars for various segments), a phenomenon that can be explained by several possible factors. All of these factors essentially derive from the co-existence of 2 protein species in the heterozygous mutant samples (full-length and truncated), as opposed to a single protein species in the WT samples (full-length). One likely factor is skewed ratios of the actual mutant and WT proteins within a heterozygous sample, either in the expressed sample itself or ultimately what is captured on the ELISA plate, which could occur through various mechanisms. For example, the enhanced expression or plate-binding kinetics of the mutant protein, due to its decreased size compared to the WT [14], could increase the ratio of mutant protein actually bound to the ELISA plate to >50% and therefore skew the % C/N ratio to <50%. Related to this point, nucleotide composition differences between the mutant and WT can ultimately alter the mRNA secondary structure and hence result in differences in expression efficiency between the mutant and WT, which may be particularly applicable to larger deletions or insertions and could skew the % C/N in either direction. Differences in the physical accessibility (i.e. steric hindrance) of the N-terminal epitope tag between the mutant and WT proteins could be caused by

different secondary and tertiary folding structures between the two protein species and could thereby skew the ratio of mutant and WT protein captured on the ELISA plate.

Importantly, these effects could vary among the different BRCA test segments as well as from sample to sample (depending on the position of the mutation). As seen in Figure 3a and b (red bars; mutant samples), the former is the overriding factor, as each *BRCA* segment tends to have a unique % C/N ratio for the mutants. For example, BRCA2 Exon 11 Segment 1 mutants fall in the range of 30±6% C/N, while BRCA2 Exon 11 Segment 2 mutants fall in the range of 61±5% C/N (Figure 3b). However, intra-segment variation in the % C/N ratio for the mutants is also apparent.

Figure 3c shows an example of linearity of the HTS-PTT assay as a function of different amounts of translated protein input into the ELISA assay. The standard curves in Figure 3c are for the N- and C-terminal detection tags of the wild-type reference sample for BRCA2 exon 11 segment 1 (n = 7). Importantly, although this verified excellent linearity of the assay over an approximate 10-fold range (R^2 = 0.99), the y-intercepts of these N- and C-terminal signal curves do not pass precisely though zero. A critical consequence of this is that in order to correctly compare the % C/N ratio of the wild-type reference sample to that of the test samples, the input amount of translated protein (for the ELISA) must be standardized among all the samples. This is easily and typically achieved by normalizing the input amount of PCR product into the cell-free protein expression reaction and can be quality controlled by monitoring the raw N-terminal signals (total expressed and captured protein).

Clinical Validation of a Genomic DNA Based HTS-PTT on BRCA1/2 using a 100-Member Training Sample Set

To evaluate the HTS-PTT for BRCA mutation analysis on clinical samples, we designed primer pairs to fully cover the large exons 11 of both *BRCA1* and *BRCA2*, dividing them into 2 and 3 overlapping segments respectively, each of roughly 2 kb in size. One hundred clinical genomic DNA samples collected from blood were analyzed using HTS-PTT (25 *BRCA1* mutants, 25 *BRCA2* mutants and 50 normal controls). The mutation status of the samples had been previously determined by conventional gel based PTT. HTS-PTT results are shown in Figure 3a and b. The range of mutations covered is detailed in Supplemental table S1 in Additional file 1. This sample cohort contained deletions as large as 40 bases, all of which were detected by our HTS-PTT method.

Despite the aforementioned segment-specific % C/N ratios for heterozygous mutants in HTS-PTT, a fixed method, based on the variability within the WT cohort, was still able to be employed for setting the diagnostic scoring cutoffs. HTS-PTT scoring cutoffs for a positive protein truncation were fixed at three standard deviations below the mean of the 50 normal (wild-type) samples (on a segment-by-segment basis), for a ~99% confidence interval. Using these cutoffs (Figure 3a and b; green bar and green line), the sensitivity and specificity of the HTS-PTT (as compared to conventional PTT) was 100% and 96% respectively. Note that the % C/N ratios for the only two false positive calls (Figure 3a and b; black bars) were just slightly below the cutoff, and were likely due to assay variance (approximately 1/100 positive false calls are statistically expected with

the aforementioned 99% confidence interval used). Finally, it is worth noting that in the HTS-PTT assay, raw signal-to-noise ratios of the N- and C-terminal detection were never less than 20:1 and in most cases, were better than 100:1.

An mRNA Based HTS-PTT for BRCA1/2

In order to obtain full *BRCA1/2* (open reading frame) coverage using a genomic DNA test, roughly fifty HTS-PTT segments per patient would be required due to the large number of small exons in both genes, outside of the large exons 11. The high throughput ELISA based HTS-PTT is certainly more amenable to such large segment numbers than the conventional gel-based PTT. However, one way to further alleviate this issue is to design an mRNA based assay, which would allow the *BRCA1/2* transcripts to be divided evenly into approximately ten HTS-PTT segments of roughly 1-2 kb in size (an effective size for the HTS-PTT). To demonstrate feasibility of this approach, we performed experiments on cDNA created from cell-lines obtained from the Coriell Cell Repository having known *BRCA1/2* truncating mutations (Coriell Institute for Medical Research, Camden, New Jersey). Figure 4 shows *BRCA1* results for one gene segment on 13 cell-lines (1 mutant) and again demonstrates 100% accuracy. Importantly, nonsense mediated decay of the mutant transcripts was not found to be a problem in this small set, but should the problem occur, mutation detection sensitivity as low as 25% is possible with HTS-PTT [14].

Conclusions

The HTS-PTT has the potential to cover the vast majority of clinically important *BRCA1/2* mutations (~90% of BIC entries) using a rapid and facile microtiter plate ELISA assay. The assay avoids radioactivity as well as very low throughput SDS-PAGE analyses, and unlike SDS-PAGE based PTT, uses concrete mathematical thresholds to distinguish mutant and WT samples. Here, in *BRCA1/2* exons 11, clear thresholds could be established to provide 100% diagnostic sensitivity and 96% specificity for truncating mutations in these one hundred clinical genomic DNA samples. Furthermore, an mRNA based HTS-PTT shows feasibility of covering all of *BRCA1/2* and would require about ten segments. Overall, the assay affords a simple, low cost and high throughput method of BRCA analysis that would be readily accessible to virtually any laboratory with rudimentary instrumentation (PCR machine and basic ELISA plate reader).

Abbreviations

BRCA1/BRCA2, breast and ovarian cancer susceptibility gene 1/2; ELISA, enzyme-linked immunosorbent assay; PTT, protein truncation test; HTS-PTT, high-throughput solid-phase protein truncation test; HRP, horseradish peroxidase; AP, alkaline phosphatase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CSGE, conformation-sensitive gel electrophoresis; TDGS, two-dimensional gene scanning; DHPLC, denaturing HPLC.

Competing interests

The authors declare that multiple authors are current or past employees of AmberGen Incorporated, a developer of commercial diagnostic assays (Drs. Mark J. Lim and Heather P. Ostendorff and Mr. Gabriel J. Foster are current employees and Dr. Sadanand Gite is a former employee within the last 5 years; Dr. Kenneth J. Rothschild is a co-founder of the company). This project was financed by AmberGen, in part using SBIR grant funds from the National Institutes of Health awarded to AmberGen (see Acknowledgements). AmberGen is the assignee on issued patents related to the commercial use of the HTS-PTT technologies described in this manuscript. However, these patents of course do not preclude the use of the HTS-PTT by the general research community. Furthermore, we disclose in the manuscript, in full detail, the methodology required for researchers to perform HTS-PTT, which needs no specialized reagents or instrumentation (and requires nothing to be purchased from AmberGen).

Authors' contributions

Drs. Mark J. Lim, Sadanand Gite and Kenneth J. Rothschild conceived of the HTS-PTT assay, and its application to BRCA analysis, as well as participated in the design and coordination of all studies in this manuscript. Drs. Mark J. Lim, Heather P. Ostendorff, Steven Narod and Kenneth J. Rothschild contributed significantly to the drafting of the manuscript. Dr. Steven Narod supervised and coordinated the isolation and gel-PTT analysis of all clinical genomic DNA samples used in this manuscript. Mr. Gabriel J.

Foster was responsible for performing and designing PCR amplifications, cell-free protein expressions and HTS-PTT assays for optimization purposes and for the final analyses of the clinical samples. Dr. Heather P. Ostendorff performed advanced PCR assays which contributed to a better understanding of the effects of primer dimers and non-specific PCR extension products on the HTS-PTT assay for BRCA analysis. All authors read and approved the final manuscript.

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Figure legends

Figure 1: Schematic Representation of the High Throughput Solid-Phase Protein Truncation Test (HTS-PTT). The next generation HTS-PTT uses three different epitope tags, two in tandem at the N-terminus (one capture and one detection tag) and one at the C-terminus (detection tag), added using specially designed primers (not shown here). The chain truncation mutations are calculated using % C/N (the C- to N-terminal signal ratio of a given sample normalized against known wild-type controls).

Figure 2: Example of Agarose Gel Analysis of Five Different PCR Segments Spanning Exons 11 of *BRCA1* and *BRCA2*. The segment sizes correspond to the values in Table 1, demonstrating the quality and quantity of the PCR fragments.

Figure 3: Clinical Validation of HTS-PTT on *BRCA1/2* Exons 11 for Inherited Breast Cancer Susceptibility. One hundred blood-derived clinical genomic DNA samples were analyzed by HTS-PTT. **(a)** *BRCA1* covered in two overlapping segments. **(b)** *BRCA2* covered in three overlapping segments. **(c)** Standard curve of different protein inputs into the HTS-PTT ELISA assay using serial dilutions of known wild-type samples. % C/N is the C- to N-terminal signal ratio of a given sample normalized against known wild-type controls. The green bars and lines indicate the designated threshold (3

standard deviations below the mean for the wild-type cohort), above which samples are scored as wild-type (blue bars) and below, mutant (red bars). Yellow bars indicate the predicted mutant % C/N based on the known wild-type standard curves (see (c)). Black bars denote false positives. The tick mark on the x-axis divides the 50 patients with known BRCA1/2 truncation mutations (left of tick mark) from the 50 wild-type patients (right of tick mark).

Figure 4: Example of *BRCA1* mRNA Based HTS-PTT. mRNA extracted from thirteen cell-line samples was analyzed using the HTS-PTT. % C/N is the C- to N-terminal signal ratio of a given sample normalized against known WT controls. The mutant sample is designated by the red bar.

Table 1. BRCA1/2 Exon 11 Primers and Segments for HTS-PTT

Segment	Nucleotides	Segment Size (bp)	Primer Sequence (5' to 3') F = Forward; R = Reverse; Subscript "I" = Intronic Primer
***BRCA1**			
1	123,126-125,033	1,908	F: gCTTgTgAATTTTCTgAgACggAT R: TgTATTCTgCAAATACTgAgCATCAAg
2	124,617-126,578	1,962	F: gTCAATCCTAgCCTTCCAAgAgAA R_I: gggCAAACACAAAAACCTggTTCC
***BRCA2**			
1	25,758-27,458	1,701	F_I: TTTTgTCACTTTgTgTTTTTATgTTTAgg R: gCCAgCAAACTTCCgTTTAATTTC
2	27,174-29,330	2,157	F: AACCATAATTTAACACCTAgCCAAAAg R: TgAAgAgCTAgTCACAAgTTCCTC
3	29,181-30,743	1,563	F: gATTCTggTATTgAgCCAgTATTgAAg R_I: CACgAAAggTAAAAATgAACACTTACC

*NCBI Reference Sequence: Ng_005905.2 for *BRCA1* and Ng_012772.1 for *BRCA2*, both numbered from whole sequence entry.

Additional files

Additional file 1

Title: Supplemental Table 1 – BRCA1/2 Mutations Covered by the HTS-PTT.

Description: This table lists the mutation designations for the 50 patient genomic DNA samples tested which were positive for BRCA1/2 truncation mutations. HTS-PTT segments containing the mutation and the measured % C/N ratios are also listed.

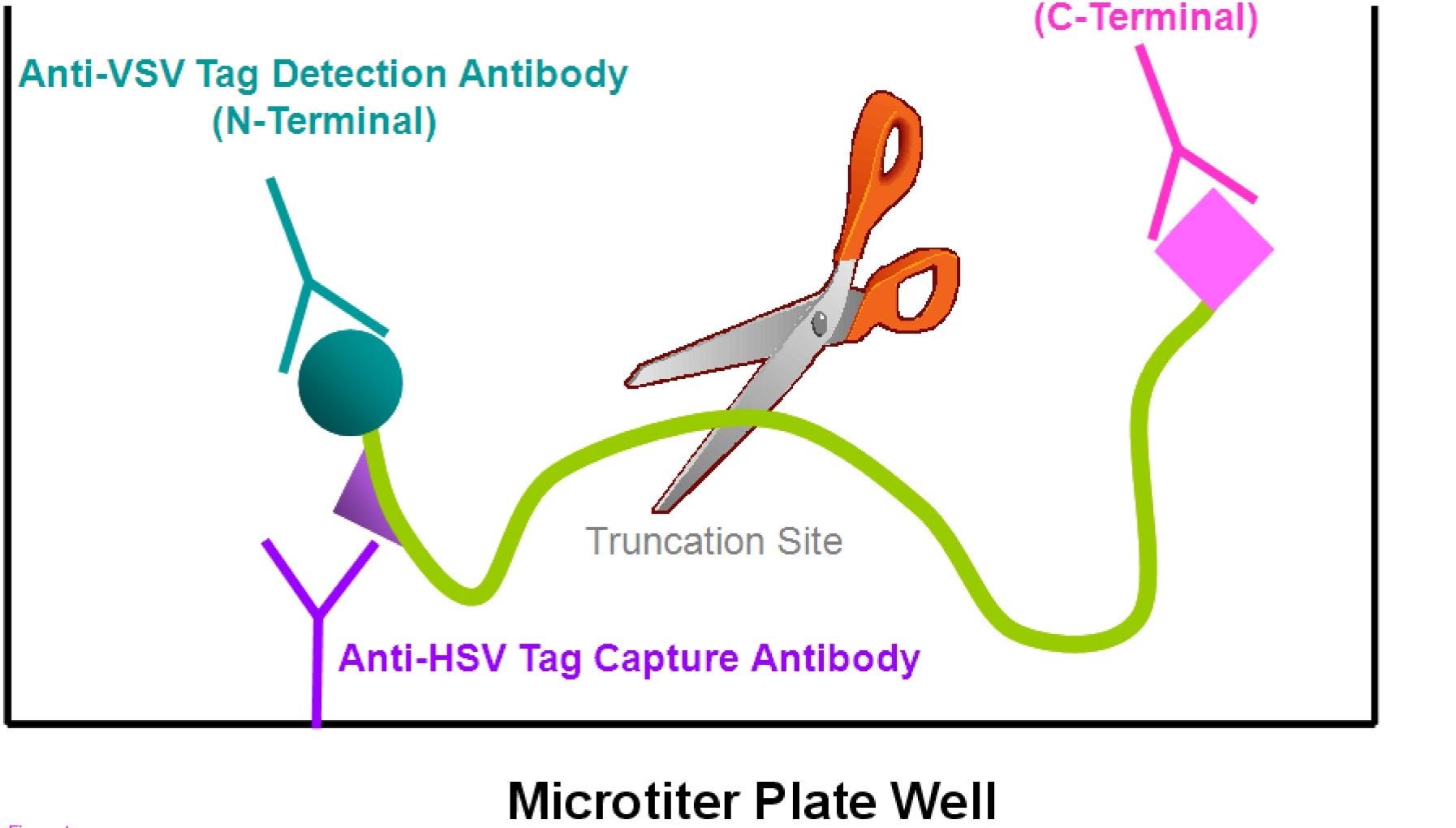
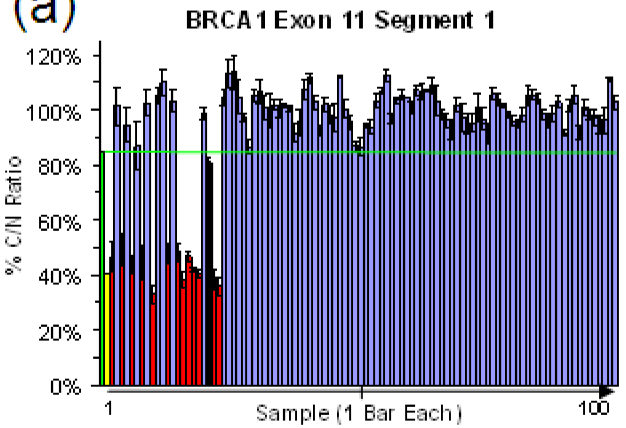

Anti-P53 Tag Detection Antibody
(C-Terminal)
Anti-VSV Tag Detection Antibody
(N-Terminal)
Truncation Site
Anti-HSV Tag Capture Antibody
Microtiter Plate Well

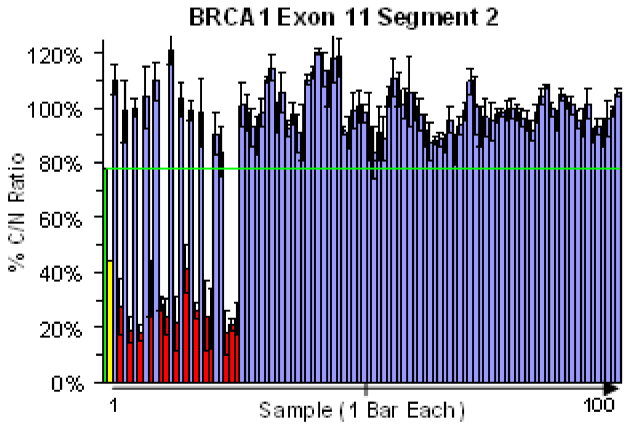
Figure 1

BRCA1 Exon 11
BRCA2 Exon 11
Segment 1
Segment 2
Segment 1
Segment 2
Segment 3
Marker
kb
3.0
2.0
1.5
1.2
1.0
0.5

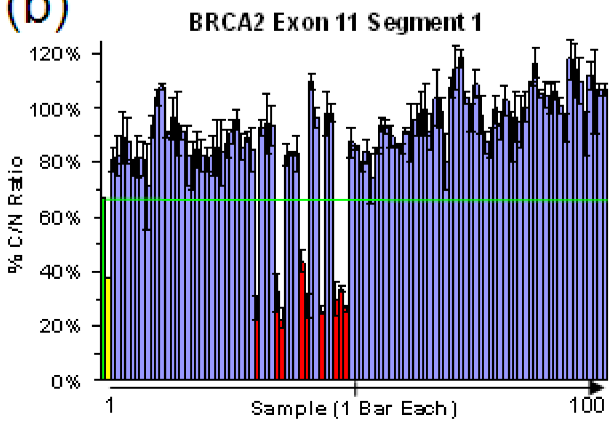
Figure 2

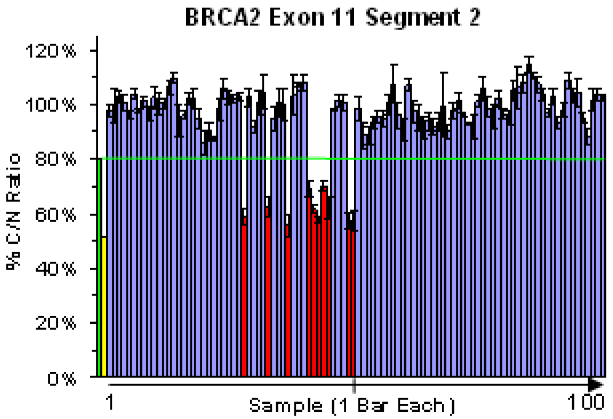
(a)

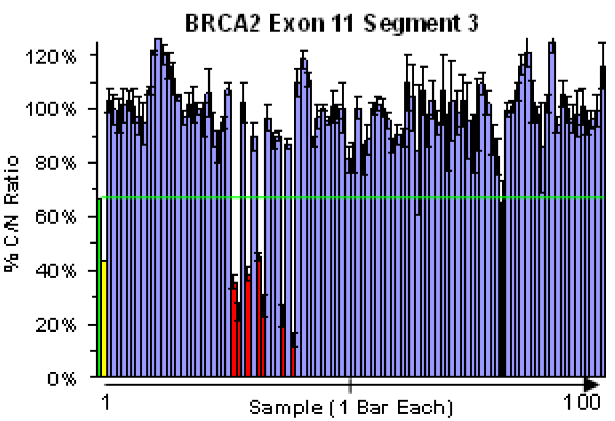

BRCA1 Exon 11 Segment 1
% C/N Ratio
120%
100%
80%
60%
40%
20%
0%
1
Sample (1 Bar Each)
100


BRCA1 Exon 11 Segment 2
% C/N Ratio
120%
100%
80%
60%
40%
20%
0%
1
Sample (1 Bar Each)
100

(b)


BRCA2 Exon 11 Segment 1
% C/N Ratio
120%
100%
80%
60%
40%
20%
0%
1
Sample (1 Bar Each)
100


BRCA2 Exon 11 Segment 2
% C/N Ratio
120%
100%
80%
60%
40%
20%
0%
1
Sample (1 Bar Each)
100


BRCA2 Exon 11 Segment 3
% C/N Ratio
120%
100%
80%
60%
40%
20%
0%
1
Sample (1 Bar Each)
100

(c)

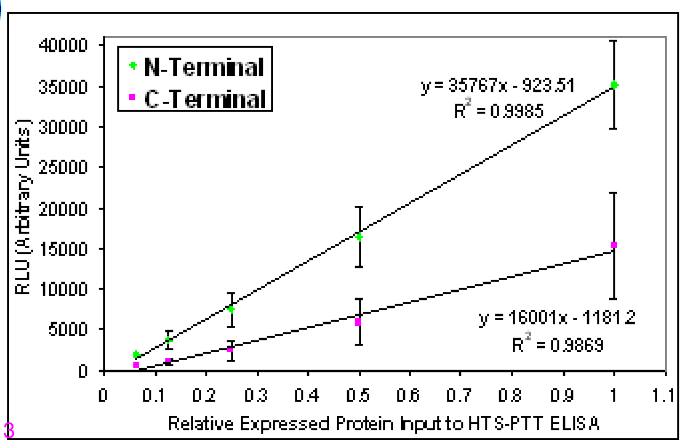

N-Terminal
C-Terminal
y = 35767x - 923.51
R² = 0.9985
y = 16001x - 1181.2
R² = 0.9869
RLU (Arbitrary Units)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1.1
Relative Expressed Protein Input to HTS-PTT ELISA

Figure 3

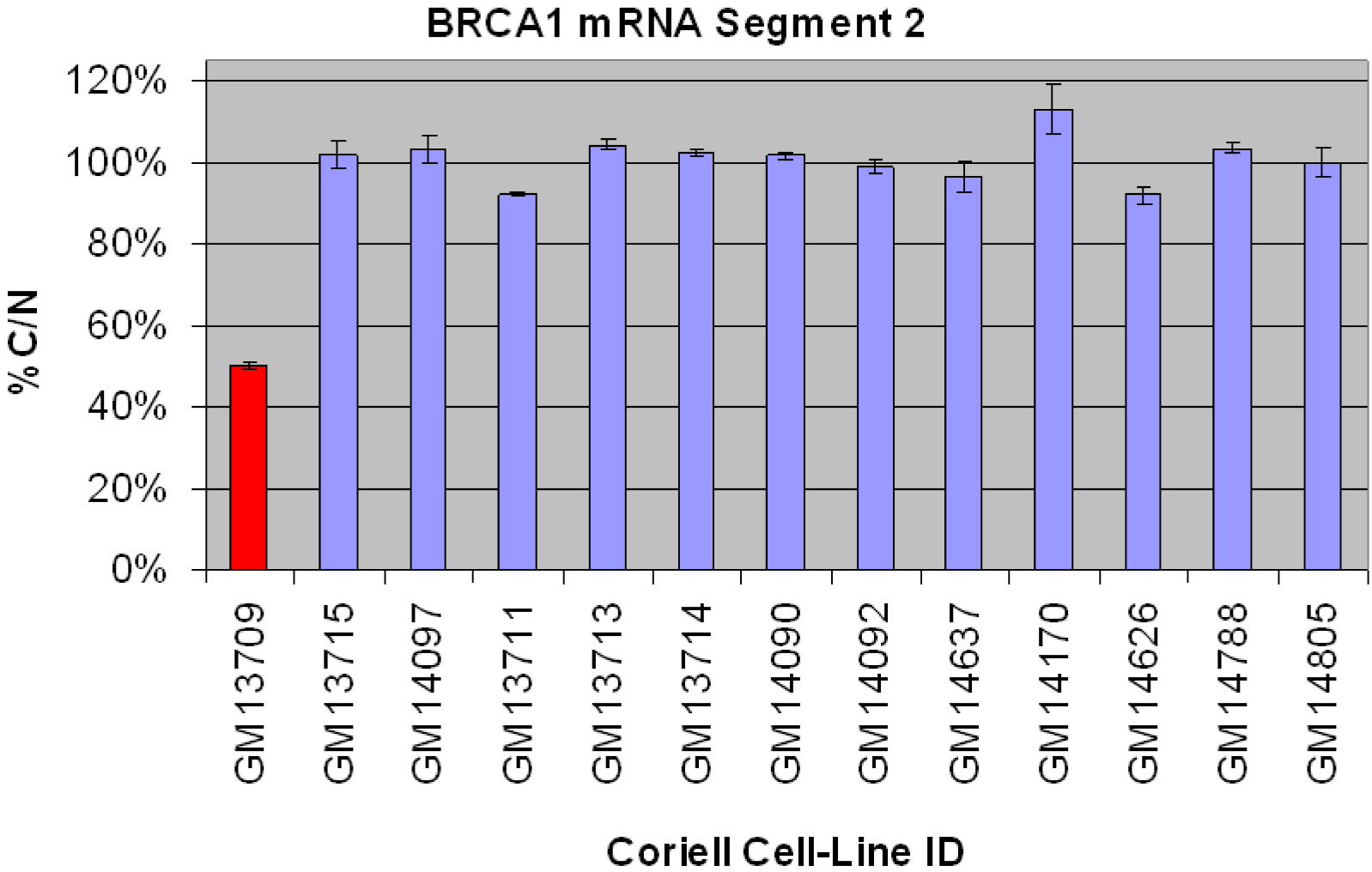

BRCA1 mRNA Segment 2
120%
100%
80%
60%
40%
20%
0%
% C/N
GM13709
GM13715
GM14097
GM13711
GM13713
GM13714
GM14090
GM14092
GM14637
GM14170
GM14626
GM14788
GM14805
Coriell Cell-Line ID

Figure 4

Additional files provided with this submission:

Additional file 1: Suppl. Table 1_Revision 1_Lim et al._BRCA HTS-PTT_BCR v02.doc, 75K
http://breast-cancer-research.com/imedia/1197010343455294/supp1.doc