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Systematic optimization of multiplex zymography protocol to detect active cathepsins K, L, S, and V in healthy and diseased tissue: compromise between limits of detection, reduced time, and resources

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Abstract

Cysteine cathepsins are a family of proteases identified in cancer, atherosclerosis, osteoporosis, arthritis and a number of other diseases. As this number continues to rise, so does the need for low cost, broad use quantitative assays to detect their activity and can be translated to the clinic in the hospital or in low resource settings. Multiplex cathepsin zymography is one such assay that detects subnanomolar levels of active cathepsins K, L, S, and V in cell or tissue preparations observed as cleared bands of proteolytic activity after gelatin substrate SDS-PAGE with conditions optimal for cathepsin renaturing and activity. Densitometric analysis of the zymogram provides quantitative information from this low cost assay. After systematic modifications to optimize cathepsin zymography, we describe reduced electrophoresis time from 2 hours to 10 minutes, incubation assay time from overnight to 4 hours, and reduced minimal tissue protein necessary while maintaining sensitive detection limits; an evaluation of the pros and cons of each modification is also included. We further describe image acquisition by smartphone camera, export to Matlab, and densitometric analysis code to quantify and report cathepsin activity, adding portability and replacing large scale, darkbox imaging equipment that could be cost prohibitive in limited resource settings.

Keywords

cathepsins; zymography; detection; cysteine protease; cancer

Introduction

Cysteine cathepsins are a potent class of proteases that have been shown to be upregulated in a number of tissue-destructive diseases such as cancer, arthritis, osteoporosis, and atherosclerosis [1–7]. They are produced by several cell types as well that all contribute to the pathological tissue remodeling associated with these diseases. Endothelial cells [5,8], smooth muscle cells [9–11], macrophages [12–14], fibroblasts [15], epithelial cells [16,17], and several other cell types have all been shown to upregulate cathepsin activity under inflammatory or disease conditions which has led to great interest in inhibiting them pharmacologically as a therapy [18–21]. The challenges for identifying and quantifying their activity in cells and tissue have been an important limiting factor hindering their larger scale study broader applications. Development of activity-based probes that only bind to the active form and are tagged with

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radioactive or fluorescent tags have enabled great strides in the field [22,23], and of course real-time PCR, genetic knockouts, and other synthetic substrates have been used in cooperation to tease apart their activity in concert or antagonistically. These methods all require some level of complexity or expense and must still address the promiscuity and cross-reactivity of substrates and inhibitors for several members of the 11-member cathepsin family making it difficult to find that “specific” reagent.

Previously, our group detailed the development of multiplex cathepsin zymography, an assay based on SDS-PAGE, to quantify and distinguish the levels of active cathepsins K, L, S, and V in cells and tissue [24–26]. Briefly, multiplex cathepsin zymography is modified from gelatin zymography, also referred to as substrate SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) due to polymerizing a gelatin substrate in the polyacrylamide gel meshwork. Recombinant enzymes, cells, or tissue are loaded into the polyacrylamide gel and non-reducing electrophoresis in a standard Bio-Rad Mini-Protean chamber is used to separate the proteins by their molecular weight and their hydrodynamic radius. Since there is no beta-mercaptoethanol, the disulfide bridges in the cathepsin native conformation are not broken preventing full linearization of the molecule. Only renatured cathepsins are capable of degrading the gelatin substrate leaving behind cleared white bands of proteolytic activity at the electrophoretic migration distance to which the enzymes are separated. Only cathepsins that have renatured will provide a signal, removing the confounding influence of the pro-forms of the enzymes that will be detected by ELISA, Western blot, and other antibody based methods, but not by zymography.

Multiplex cathepsin zymography has great potential to impact clinical diagnoses as well as research methodology and findings in a broader way due to its many benefits: 1) it does not require antibodies making it relatively inexpensive and species-independent, 2) separation of proteins by nonreducing electrophoretic migration through polyacrylamide gels visually confirms cathepsin identity after staining of the gel, 3) densitometry can be used for quantitative analysis, and 4) multiplexed detection allows distinction of active cathepsins K, L, S, and V in one cell or tissue extract. Inexpensive technologies that accurately detect and quantify cathepsin activity in short timeframes can be attractive for translating bench top knowledge of disease-induced upregulation of cathepsin activity to earlier clinical diagnosis of such diseases.

We then went on to apply this inexpensive technique to studies of human breast, cervical, and lung cancer tissue, and quantified an increase of as much as 30-fold over normal, patient-matched tissue loading only 10 µg of protein [25]. This has been useful to study cathepsin activity as upregulated by the inflammatory conditions of several other diseases (ongoing studies in lab), suggesting a broader applicability for this technology and cathepsin K, L, S, and V roles in a wider gamut of diseases.

As previously published, this technique required a total of three hours of attention and preparation prior to an overnight incubation and usually started with 10–15 µg of protein material to load.

1)	2 hours	Non-reducing SDS-PAGE at 110 V
2)	0.5 hours	Cathepsin renaturation for three washes at 10 minutes each
3)	0.5 hours	Equilibration of the gel to acidic pH in assay buffer, pH6
4)	15–18 hours	Overnight assay incubation
5)	1 hour	Staining and destaining with Coomassie blue
6)	Image acquisition (ImageQuant) and densitometric analysis (NIH ImageJ)	

Our goal is to optimize this protocol for two settings: the clinic and field work in resource limited settings such as university and hospital laboratories in the developing world. These two settings require different design goals. First, the clinic has access to larger amounts of tissue, electricity, image analysis equipment, and trained staff; due to health insurance regulations and staff shift changes, it would be optimal for this entire assay to be completed in one 8-hour work day. In the field, integrity of samples and expense of large scale image analysis equipment may be prohibitive, so the limits of detection for this assay and appropriate conditions that support those limits are necessary to know to analyze samples, but perhaps with a longer assay time to reduce cost and waste of protein material. Since zymography uses the same equipment as SDS-PAGE, the additive cost for a lab trained to run Western blots is negligible.

Here, we describe efforts to improve this protocol, by minimizing total time required to generate a detectable signal that still differentiates normal from tumor specimens moving towards its use as a rapid diagnosis platform. Using a systematic approach, the key timed steps of electrophoresis, enzyme renaturing, and proteolytic activity requirements were analyzed either computationally or empirically to identify steps that could save time and reduce starting material (tissue) needed for a quantifiable signal. Subsequently, solutions were engineered to address them, while considering the biochemical mechanisms occurring at each stage affecting enzyme activity. Secondly, to optimize it for use in field applications abroad, a process was developed to streamline image acquisition with a smartphone camera and quantitative analysis with Matlab code to provide final output of cathepsin activity in separate cell and tissue samples.

Materials and Methods

Reagents

Recombinant human cathepsin K isolated from insect cells (Enzo); human cathepsin L isolated from human liver (Enzo); recombinant human cathepsin S from insect cells (Enzo); recombinant human cathepsin V from NSO cells (Enzo); ProtoGel (National Diagnostics); N,N,N',N'-tetramethylethylenediamine (TEMED) (GE Healthcare).

Human tissue

Normal and cancer lung tissue lysates were purchased from Protein Technologies, Inc., San Diego, CA. Tissue specimens were collected during the surgery process and immediately snap frozen with liquid nitrogen. Tumor samples were staged and graded by pathologists based on the American Joint Committee on Cancer (AJCC) Staging Manual [27]. Frozen tissues were minced and homogenized in cold modified RIPA buffer, and clarified by centrifugation. Protein concentrations of the lysates were normalized to 1 mg/mL.

Multiplex cathepsin zymography

Cathepsin zymography was performed as described previously [24]. Non-reducing loading buffer (5× – 0.05% bromophenol blue, 10% SDS, 1.5 M Tris, 50% glycerol) was added to all samples prior to loading. Protogel, an optimized mix of acrylamide and bisacrylamide solution, was added with appropriate buffers, SDS, gelatin solution (5 mg/mL), and ammonium persulfate (APS) (a radical initiator). TEMED, a catalyst of the formation of free radicals produced with APS, was added and the solution was mixed. Subsequently, acrylamide and bisacrylamide were polymerized between glass plates to form a gel matrix, incorporating 3.3% gelatin, which was used for sieving proteins. Running buffer comprising of Tris-base, glycine, and SDS was added to the gel during the electrophoresis process as a majority of the current is carried by buffer ions, controlling the conductivity of the gel. Mini gels were prepared from shortened glass plates, and a mini-Protean 3 multi-casting chamber (Bio-Rad) was used to prepare standard size gels approximately 92 mm × 68 mm (width × height). Mini gels 92 mm

× 29 mm (width × height) were prepared from shortened glass plates. Standard zymograms were rinsed three times for 10 minutes each in renaturing buffer (20% glycerol, 65 mM Tris buffer, pH 7.4), equilibrated in assay buffer (phosphate buffer, pH 6, 2 mM DTT, and 1 mM EDTA) for 30 minutes prior to overnight incubation at 37 °C in fresh assay buffer. Mini-zymo gels were washed three times for 5 minutes each in renaturing buffer, equilibrated in assay buffer, prior to assay buffer incubation to activate enzymes.

Densitometry

An ImageQuant scanner (GE Healthcare) was used to image gels in the standard densitometry protocol. The images were adjusted in Adobe Photoshop, and densitometry was completed with ImageJ.

Smartphone and Matlab image acquisition and analysis

Images were acquired with an 8.0 MP HD video/camera phone and imported into Matlab as a jpeg file. A Matlab code was developed to automatically contrast the gel image, as requested by the user. The user would input the number of bands to be analyzed and draw line segments between lanes at the top of each band. Subsequently, `improfile`, a built-in Matlab function, calculated the average pixel values across the user defined line segments. An intensity plot was then displayed with the quantified band intensities.

Results and Discussion

Incubation assay time

First steps were taken to reduce total time of the assay and the longest incubation period was for the overnight assay period during which the refolded enzymes were degrading the gelatin substrate at the site of their electrophoretic migration and separation. In previous experiments, there was a range of variability in the length of time required for the enzymes to generate a detectable signal depending on the cell type, tissue type, and disease state [25,28,29]. However, in other studies by our group, incubating recombinant cathepsins in their soluble, native conformation, with fluorogenic substrates to quantify proteolytic activity, there would be a loss of cathepsin activity after about two hours [30]. Zymography is different in that the cathepsins are renatured around polyacrylamide fibers and are in the stationary phase compared to the fluid phase of the soluble experiments, but we decided to test the hypothesis that reducing the incubation time would still produce a detectable zymogram signal after staining with Coomassie blue.

To test this, recombinant cathepsin K (1 ng), L (20ng), S (5 ng), and V (5 ng) were loaded for multiplex cathepsin zymography, electrophoresed, and renatured as described, but separate gels were incubated for either overnight as usual, or stopped and stained after only four hours. Representative zymograms are shown in figure 1A, and as expected, there is a reduction in the intensity of the signals. The zymograms shown were imaged together, side by side, in the ImageQuant dark box from GE Healthcare, and the images were not independently adjusted. Cathepsins L and V signals were not visible after only 4 hours but cathepsins K and S maintained some level of signal. It is important to note that the different amounts of cathepsins loaded into the zymograms had been determined previously by us to produce detectable signals with the overnight incubation, as they do in figure 1A, but these results at four hours show that there are cathepsin family member specific differences to being detected with shorter incubation times.

With recombinant enzyme generating a signal at the reduced incubation time, the next step was to determine if cathepsin activity could be detected in cells with reduced incubation time. RAW264.7 macrophages produce a large amount of cathepsins and serve as excellent positive

controls and test cells for these studies. Since amount of cathepsin loaded could have influenced the recombinant enzyme experiment (Fig 1A), increasing amounts of total RAW cell lysate were loaded for zymography and again, separate gels with the same samples were incubated for either overnight or just 4 hours (Fig 1B). The shorter incubation still produced detectable cathepsin activity signals for cathepsins K, L, S, and V in RAW macrophages but the signal is dependent on the cathepsin family member and the amount of starting material loaded with a differential visible at the 2.5 μ g of RAW cell lysate protein producing different signals from different cathepsins that could be misinterpreted as no cathepsin K in the system and that the assay worked since there are cathepsin S and L bands but that would be misleading.

Electrophoresis time

A shorter assay time already reduced the assay time by 8–16 hours, and the next longest time period was the actual electrophoresis of the proteins through a 12.5% polyacrylamide gel polymerized with 1 mg/ml of gelatin. Under the previously published protocol, this time period was two hours at 110 V. In Tris-glycine buffer systems, applying a voltage across the gel generates a current that pushes the electrically charged protein through the pores of the polyacrylamide matrix [31]. The polyacrylamide gel serves as the resistive element and completes the circuit by connecting the buffer and ions in the upper and lower chambers of the electrophoresis apparatus; here, the commonly used Mini-Protean from Bio-Rad (Figure 2A). From Ohm's law ($V=IR$), voltage and current are directly proportional; the most direct way to increase current and accelerate protein migration was to increase the voltage from 100 V to 200 V, which reduced electrophoresis running time from 2 hours to 50 minutes for full separation. There was a concern about increasing the voltage further and generating excessive heat that could irreversibly denature the cathepsins, preventing thermodynamically favorable renaturation necessary to refold the cathepsins into their native, active conformation to degrade the gelatin substrate during assay incubation.

Resistive elements generate heat in circuits, so opportunities to lower resistance, even with elevated voltage, were the next component optimized. Again, the polyacrylamide gel is the major resistor in the circuit (Fig 2A). Lowering the percentage of polyacrylamide in the gel would reduce resistance, but would also sacrifice the resolution of protein band separation. Cathepsins identified by multiplex cathepsin zymography generally are between 20 and 37 kDa, and are best separated by 12.5% polyacrylamide. Alternatively, a reduction in gel height should decrease the linear distance of the major resistive element in the electrophoresis circuit, and thereby reduce the total resistance and heat generated by higher voltages. Custom short plates that fit the mini-Protean gel casting system were fabricated by cutting the front, short glass plate in half to produce a gel of only 34 mm height compared to the standard 68 mm height (Fig 2B).

Zymogram gels were prepared as usual with same chemical composition as before, and used to measure drops in electrical resistance due to decreased gel height. Voltage was set at 2000 V, current was recorded during electrophoresis as indicated on the voltage box, and resistance was calculated with Ohm's law. A current of 0.04 A was measured for the standard size gels while a higher current of 0.1 A was measured for mini-gels at 200 V, and the overall electrophoresis time was reduced from 2 hours down to only 10 minutes; the resistance was reduced from 5 M in the standard gel down to 2 M (Table 1). The ratio of heights between the standard gel and mini gel was 0.43 and ratio of resistances was 0.4, closely correlating gel height with resistance in the circuit.

Cathepsins K, L, S, and V active bands were still distinguishable in the final zymogram with this accelerated electrophoretic migration, producing bands of cleared proteolytic activity at distinct electrophoretic migration distances (Fig 2C). It is important to note that in order to achieve current of 0.1 A through a standard sized gel, 500 V would have to be applied. Voltages

this high would generate excessive heat, and are out of the range of the Bio-Rad voltage box rendering this possible solution prohibitive. Taken together, this supports the importance of shortening the gel height to minimize resistance, maintaining low heat, while still accelerating protein migration, to cathepsin distinct distances.

Limits of detection of cathepsins K, L, S, and V with accelerated mini-zymo protocol

As determined in the earlier study of incubation time, there are cathepsin specific signal differences depending on the assay incubation times, so it was important to assess the effect, if any, of this accelerated electrophoretic migration on the multiplex zymography cathepsin activity signals and resolution. Limits of detection for cathepsins K, L, S, and V were determined using this accelerated protocol by loading increasing amounts of each cathepsin, 10 minute electrophoresis through mini-gels at 200 V, renaturation for 15 minutes, and incubation overnight. After Coomassie staining and destaining, cathepsin band intensity imaged with the ImageQuant darkbox from GE Healthcare, and band density was quantified with ImageJ (Fig 3A–D). Cathepsins K, S, and V were in the subnanogram range (0.05 ng), comparable to the femtomole quantities of mature cathepsin K detected in our initial study [28]; cathepsin L required a much greater amount, with a limit of detection at 10 ng, an order of magnitude higher than the other cathepsins (Fig 3B).

Limits of detection were also determined for the accelerated protocol with 10 minutes electrophoresis, and 4 hour assay time. As expected, the shortened assay incubation time led to reduced sensitivity and higher limits of detection (more enzyme required to generate a detectable signal) (Fig 4A–D). Limits of detection were as follows: cathepsin K – 1 ng, cathepsin S – 2.5 ng, cathepsin V – 0.05 ng, and cathepsin L – 10 ng. It is important to note that the mini-zymo detection limits of cathepsins L and V for the 4 hour assay time (Fig. 4) were greater than the limit for the standard gel for the same assay time (Fig 1A), which did not produce a signal for cathepsin L at 10 ng and cathepsin V a 1 ng. An explanation is that mini-zymo gels are smaller than standard gels, which helps concentrate the cathepsins during electrophoresis, enhancing the sensitivity of the assay when the same amount of protein is focused in a smaller gel volume.

Application of mini-zymo to human cancer specimens

Recombinant enzymes and cells were used to optimize the protocol, but the levels of cathepsins in tissues can vary and we hope that this accelerated, optimized protocol would still be applicable for use by the broader research community and for clinical translation. In a previous study, we showed that normal and cancerous lung tissue had higher levels of cathepsin activity compared to breast and cervical tissue, with the tumor lung tissue having the highest. With evidence and appreciation for quantity and cathepsin-specific differences in activity based on modifications to the standard protocol from the earlier results, there may be tissue specific differences in tailoring the accelerated protocol with compromises between speed and detectable cathepsin activity signal.

Accelerated mini-zymo protocol was used to assay human lung normal and tumor tissue. Ten µg of protein were loaded into mini-zymo gels, electrophoresed for 10 minutes at 200 V, renatured for 15 minutes with three washes, and incubated overnight. Parallel samples were incubated for four hours to test if high cathepsin activity lung cancer specimens provided sufficiently detectable and distinguishable signal with the most stringent and rapid protocol. Representative zymograms for two patients are shown in figure 5A. Cathepsin bands were observed in mini-zymos incubated for both overnight and 4 hours, indicating that proteolytic activity of cathepsins in 10 µg lung tissue was sufficient to degrade enough gelatin to produce

a mini-zymo band within 4 hours. Although lung cancer specimens produce less signal when incubated at 4 hours compared to overnight (Fig 5B), there were still marked increases in the tumor specimen compared to normal when quantifying total cathepsin activity of each specimen supporting the potential application of the mini, accelerated protocol for lung cancer detection.

Application of smartphone camera and Matlab code for image acquisition and analysis

Previously, the ImageQuant dark box with a 16-bit dynamic range (4 orders of magnitude) available from GE Healthcare was used to capture zymogram images in our lab, adjustments to improve contrast between background and signal was performed in Adobe Photoshop, saved as a tiff file to be opened in NIH ImageJ for densitometry of the bands, and graphs of cathepsin activity plotted in Excel. For portability and practicality in clinical labs, and for labs in the developing world that could benefit from this low cost zymography assay, a cheaper imaging modality is required. To simplify image acquisition and analysis, a smartphone camera was used to acquire the gel image and a Matlab code was written to analyze the gel jpeg for densitometry with basic input (Fig 6) from the user according to the following code:

```
Gel = rgb2gray(Gel0);
Gel = histeq(Gel); %as needed by user
[Xx,Yy,Bck] = improfile; %User selects a background band
for i = 1:n
h = 1;
[Jx(h), Jy(h), J(h)] = improfile;
while Jy(h) < Yy
[Jx(h+1), Jy(h+1), J(h+1)] = improfile;(Gel, Jx(h), Jy(h) + step);
J(h) = J(h) - Bck;
if J(h) < 0
J(h) = 0
end
```

To summarize the code's actions, first the zymogram picture is imported from the smartphone and automatically converted to a grayscale image using the `rgb2gray` function. The user then defines the number of samples to be quantified and an image of the zymogram is displayed. The lower boundary of the cathepsin bands is defined by drawing a line at the base completely below the bands, which also defines the background, `Bck`, and the upper bound is demarcated with a line at the top of the highest cathepsin band. The code then measures the sum of all pixels between those user-defined boundaries and subtracts the background intensity value from each measurement; any values below `Bck` are set to zero. The final intensity value (`J`) represents total cathepsin activity in each sample, which is automatically plotted for each lane in the gel.

When comparing normal lung vs. tumor cathepsin activity, results obtained with the semi-automated Matlab analysis of smartphone images were comparable, at least ratiometrically, to those obtained by the original ImageQuant/Photoshop/ImageJ/Excel combination (Fig 6B). Differences in fold changes of tumor/normal cathepsin activity as determined by ImageJ densitometry vs. Matlab densitometry are plotted in Figure 6B, both detect higher cathepsin activity in tumor tissue compared to normal tissue by a minimum of two-fold and the fold changes are not significantly different when calculated with Matlab or ImageJ ($n=4-6$). Assay time reduced to four hours from overnight also had no statistically significant difference in

fold change, especially when quantified with Smartphone and Matlab, supporting its utility as an efficient, cost effective alternative to large scale imaging devices while still yielding comparable values of cathepsin activity after accelerated mini-zymography.

With the minimized and accelerated protocol, the last part of the protocol to optimize was the starting material needed to generate a detectable signal. Reducing the starting material by 70% (down from 10 μ g to 3 μ g) still produced distinguishable cathepsin activity signal with differences between normal and tumor lung lysates imaged with a Smartphone and analyzed by the Matlab code (Fig 7), but only when incubated overnight; 3 μ g of lung tissue lysate did not yield reliable, distinguishable signals after only 4 hours assay incubation. Interestingly, a four-fold increase was observed for the 3 μ g paired samples compared to the 1.8 fold-change observed in the 10 μ g paired samples. This suggests that a reduction of input lysate loaded in mini-zymos may actually enhance the signal to noise ratio of the assay.

In conclusion, by taking a systematic and empirical approach to optimize multiplex cathepsin zymography, we were able to reduce the total assay time by 80% (from 22 hours to 4.5 hours) and for certain tissues (lung cancer) were able to reduce the starting tissue sample by 70%, from 10 μ g down to just 3 μ g. It is important to note, however, that this worked for lung tissue, but breast tissue did not have sufficient tumor activity to be detectable and distinguishable from normal specimens with only 3 μ g of tissue (Fig 7). Perhaps most importantly, however, these studies verify the utility of capturing zymo images with a smartphone that can be sent to a computer for simplified Matlab image analysis. This opens the possibility for use in low resource settings and in laboratories in the developing world that may be able to afford electrophoresis equipment but not the large scale imaging equipment that costs 20–40 fold the price. Portability afforded by such technology increases the access to quantitative measures of cathepsin activity across normal and healthy cells and tissues.

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Figure 1. Reduced assay incubation time of recombinant cathepsins and RAW264.7 cell lysates yields detectable, though reduced, signal intensity in a cathepsin specific manner
(A) Recombinant cathepsin K (1 ng), L (20ng), S (5 ng), and V (5 ng) were loaded for multiplex cathepsin zymography as described but incubated in assay buffer for either 4 hours or overnight. (B) Different amounts of protein from RAW 264.7 macrophage cell lysates were loaded as well. 1.25, 2.5, and 5 μ g were loaded and again, incubated in assay buffer for either 4 hours or overnight.

Figure 2. Minimizing gel height reduces electrophoresis time from two hours to 10 minutes while maintaining distinct identification of cathepsins K, L, S, and V

The resistance of the completed circuit is related to the size of the gel, standard size and miniature gel. Miniature multiplex zymography detects mature cathepsin K, L, S, and V at distinct migration distances with a 10 minute electrophoresis process. Human recombinant cathepsin K (1 ng), L (20 ng), S (5 ng), and V (5 ng) were loaded in the standard zymography gel. Human recombinant cathepsin K (10 ng), L (50 ng), S (10 ng), and V (10 ng) were loaded into the mini-zymo.

Figure 3. Limits of detection for cathepsins K, L, S, and V by mini-zymo with overnight assay incubation

Different amounts of recombinant human cathepsins K (A), L (B), S (C), and V (D) were loaded into mini-zymo gels to determine limits of detection. Minimum detection of 0.05 ng for cathepsins K, S, and V, and minimum detection of 10 ng for cathepsin L. Densitometry plots are shown indicating a linear relationship between cathepsin amounts and signal intensity.

Figure 4. Limits of detection for cathepsins K, L, S, and V by mini-zymo with four hour assay incubation

Different protein amounts of cathepsins K (A), L (B), S (C), and V (D) were loaded into mini-zymo gels to determine limits of detection. Minimum detection of 1 ng for cathepsin K, 10 ng for cathepsin L, for 2.5 ng for cathepsin S, and .05 ng for cathepsin V. Densitometry plots are shown indicating a linear relationship between cathepsin amounts and signal intensity.

Figure 5. Mini-zymo distinguishes normal from tumor lung tissue and maintains separation of distinct active cathepsins

Representative zymogram of normal (N) and tumor (T) lung tissue lysates showing two out of the five patients tested. 10 μ g of protein were loaded for mini-zymo and incubated for either overnight or just 4 hours. Zymograms are shown and there is reduced signal intensity at 4 hours compared to overnight (A). However, even with reduced signal, densitometric analysis indicates that total cathepsin activity is greater in tumor samples compared to patient-matched normal tissue when incubated overnight or for four hours (B).

Figure 6. Smartphone camera image acquisition with Matlab image analysis provides faster, cheaper, and comparable quantitative cathepsin activity data

Smartphone camera was used to acquire the zymogram image as a jpeg file. This acquired jpeg was then imported into Matlab to quantify band intensity and graph the results. A schematic of this process is shown (A). The quantified densitometry signal from Matlab was plotted as fold-change of tumor cathepsin activity over that of the normal patient-matched tissue ($n = 4-5$) and plotted on the same graph as the signal quantified with NIH ImageJ (B). Fold increases of cathepsin activity from normal to tumor lysates indicate that Matlab quantification is comparable to quantification using ImageJ across several patients and there are no significant differences between fold changes using either the overnight or the four hour assay incubation times.

Figure 7. Mini-zymo distinguishes normal from tumor for lung tissue with less starting material, but is below limit for breast tissue

Either 3 or 10 μ g of normal or tumor lung or breast tissue lysates were analyzed by the mini-zymo with overnight incubation. Image acquisition and cathepsin activity quantification was done by Smartphone camera/Matlab method. Cathepsin K (1 ng) was loaded in the first lane as a positive control and reference signal. This is from one breast cancer patient and one lung cancer patient.

Table 1

Electrophoresis parameters for standard gel and accelerated mini-zymo.

	Height (mm)	Current (A)	Resistance (Ω)	Time (min)
Standard	68	0.04	5000	120
Mini	29	0.1	2000	10
Ratio (Mini/Standard)	0.43	2.5	0.4	0.2