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Target Discovery and Validation in Pancreatic Cancer

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1. Introduction to Pancreatic Cancer

Infiltrating ductal adenocarcinoma of the pancreas, more commonly known as “pancreatic cancer” is the fourth leading cause of cancer deaths in the United States ¹. It is estimated that in 2005, approximately 213,000 individuals worldwide will be diagnosed with pancreatic cancer and approximately 213,000 will die from it ². It is an almost uniformly fatal disease, with an overall 5-year survival rate of less than 5%. The overwhelming majority (~80%) of patients present with locally advanced or with metastatic malignancies, rendering the cancers surgically unresectable. Unfortunately, even patients who undergo surgical resection eventually succumb to metastatic disease, including those cases where the primary lesion was extremely small at diagnosis ³. The implication is that pancreatic cancer must metastasize distantly, prior to clinical presentation, in virtually all cases. A small number of cytotoxic drugs and radiation therapy are reported to have modest clinical benefit, measured as time to progression or clinical improvement, in the treatment of pancreatic cancer. The most common current regimen is gemcitabine, used as either as a single agent, or increasingly, in combination with a platinum compound (e.g., cisplatin, oxaliplatin) ⁴. Despite these interventions, however, pancreatic cancer continues to be, in essence, a disease of near-uniform mortality.

The last 15 years have seen an exponential increase in our understanding of the pathogenesis of pancreatic cancer, and it is now fairly evident that pancreas cancer is a disorder of the genome, caused by progressive accumulation of inherited and acquired mutations ⁵. These alterations include activating point mutations in the K-ras gene, overexpression of *HER-2/neu*, and inactivation of the *p16*, *p53*, *DPC4* and *BRCA2* tumor suppressor genes ^{1, 3, 6–11}. Other mechanisms may contribute to carcinogenesis of the pancreas, such as overexpression of growth factors and their receptors or changes in activity of signal transduction pathways ^{12, 13}. While these gene-by-gene approaches have contributed towards a better understanding of pancreatic cancer pathogenesis, the advent of global profiling technologies have led to quantum advances in identifying molecular targets that can form the substrate for developing rational early detection and therapeutic strategies for pancreatic cancer ^{14–22}. Unraveling the transcriptome and proteome of human pancreatic cancers using large-scale approaches such as DNA microarrays ²³, serial analysis of gene expression (SAGE) ²⁴ and mass spectrometry ²⁵ have led to an unbiased elucidation of cancer biomarkers, many of which have are being translated to direct patient care while others have the potential to do so in the near future.

In this review, we will discuss three approaches – the first two, cDNA microarrays and SAGE analyzing the pancreatic cancer transcriptome and the third, liquid chromatography and tandem mass spectrometry (LC/MS/MS), its proteome; all three of these global approaches have been used with considerable success for identification of molecular targets in human pancreatic cancer. The last portion of this review will focus on candidate molecular targets identified through such large scale profiling studies and how these relate to care of patients with pancreatic cancer.

2. Introduction to DNA microarrays

DNA microarrays consist of hundreds or thousands of PCR-amplified cDNAs or synthetic oligonucleotides spotted onto a glass microscope slide, in a high-density pattern of rows and columns²³. DNA microarrays were first used widely to quantify gene expression across hundreds or thousands of genes simultaneously^{26, 27}. To measure gene expression, mRNAs from two different samples are differentially fluorescently labeled and co-hybridized to a DNA microarray, which is then scanned in dual-wavelengths. For each DNA element on the microarray, the ratio of fluorescence intensities reflects the relative abundance for that mRNA between the two samples.

The two-color fluorescence format provides robust measurements of gene-expression ratios, effectively mollifying variations in amounts of spotted DNA and of hybridization kinetics. In some experimental designs, the selection of the second-color “reference” specimen is natural, for example the zero time-point in a time course, or the untreated sample in a pharmacological treatment. In other cases, an arbitrary “universal” RNA reference, comprising a pool of mRNA from cell lines or tissues, is more appropriate²⁸. Since each sample is hybridized against the same reference, samples can be readily compared to each other. In such cases, gene-expression ratios are typically “mean-centered”, i.e. reported in relation to the average of the specimens assayed (rather than to the arbitrary reference).

For small specimens (e.g. tissue biopsies or microdissected samples), insufficient input RNA may be available for the labeling protocol detailed below. In such cases, the reader is directed to linear RNA amplification protocols based on T7 RNA polymerase mediated *in vitro* transcription²⁹.

2.1. Materials

2.1.1. Processing arrays

1. 20X SSC solution
2. 10% SDS solution
3. Bovine Serum Albumin (BSA) (Sigma, #A7888); Prepare a 10mg/ml BSA stock solution, filtered.
4. Ethanol, 95% (Gold Shield Chemical Co.)
5. Diamond scribe (VWR, #52865-005)
6. Slide humidifying chamber (Sigma, #H6644)
7. Heating block (e.g. VWR, #13259-050), invert so solid metal surface faces up
8. Ultraviolet source (Stratagene Stratalinker 2400 or equivalent)
9. Pyrex dish for denaturing spotted cDNA on arrays (Fisher, #08-741F)
10. Slide rack and glass staining dish (Wheaton)

11. Desktop centrifuge (Beckman Allegra 6R, or equivalent), with microtiter plate carriers

2.1.2. Labeling mRNA

1. RNase-free H₂O (Ambion #9935, or equivalent)
2. Anchored oligo-dT RT primer (T₂₀VN; custom primer from Operon or equivalent; HPLC purify); prepare 2.5 ug/ul stock in TE_{8.0}
3. Superscript II reverse transcriptase (Invitrogen, #18064-014)
4. 5X First Strand Buffer (Invitrogen, #Y00146)
5. 0.1M DTT (Invitrogen, #Y00147)
6. RNasin (Promega, #N2511)
7. 50X dNTP mix (10 mM dTTP; 25 mM each dGTP, dATP, dCTP, in TE_{8.0}); prepare from ultrapure dNTP solutions; Amersham Biosciences)
8. Cy5-dUTP and Cy3-dUTP (Amersham, #PA55022 and #PA53022)
9. 0.5M EDTA
10. Water bath(s) (70°C, 42°C, and 37°C)

2.1.3. Hybridization of labeled sample

1. RNase-free H₂O (Ambion #9935, or equivalent)
2. PolyA RNA (Sigma, #P9403). Prepare 10 ug/ul stock solution in TE_{8.0}.
3. Yeast tRNA (Invitrogen, #15401-011). Prepare 5 ug/ul stock solution in TE_{8.0}.
4. Human Cot-1 DNA (Invitrogen, #15279-011). Supplied as 1 ug/ul stock solution.
5. 20X SSC solution
6. 10% SDS solution
7. Microcon 30 Filters (Amicon)
8. Hybridization chambers (Corning; Monterey Industries: <http://www.montereyindustries.com>, or equivalent)
9. Glass microscope slide cover slips, 22×60 mm (Fisher)
10. Slide rack and three glass staining dishes (Wheaton)
11. Desktop centrifuge (Beckman Allegra 6R, or equivalent), with microtiter plate carriers
12. Water bath (65°C)

2.1.4. Data reduction and analysis

1. GenePix 4000B scanner (Axon), or equivalent, for imaging microarrays following hybridization
2. GenePix software (Axon), or equivalent, for data extraction (i.e. matching pixels to DNA spots; calculating fluorescence ratios)
3. Microsoft Excel, or more specialized microarray databases (e.g. AMAD; <http://www.microarrays.org/software.html>), for data storage, retrieval and analysis

2.2. Methods

Many universities, medical centers, and companies have established “core facilities” dedicated to printing DNA microarrays. Detailed protocols for printing spotted DNA microarrays have been published³⁰. DNA microarrays can also be purchased from various commercial vendors (e.g. Agilent Technologies). The protocols that follow are geared specifically to spotted cDNA microarrays. Where distinct methods are required for spotted oligonucleotide arrays, alternative protocols are detailed in the notes.

2.2.1. Processing and prehybridization of DNA microarrays—Prior to use, and unless already processed by the manufacturer, DNA microarrays should be processed and pre-hybridized to enhance specific hybridization to DNA spots and block non-specific hybridization to the glass surface. The following protocol applies to microarrays of cDNAs spotted onto amino-silane coated slides (e.g. Corning GAPSII), a widely-used surface for nucleic acid immobilization. Using a diamond scribe, etch the underside (non-printed) surface of the array to demark the boundaries of the printed area (see **note 1**). Optionally, if arrayed DNA spots appear small, re-hydrate arrays over ddH₂O preheated to 50°C using a slide humidifying chamber (printed surface facing down) for 20 seconds. Then quickly snap-dry each array by placing on a pre-heated 75°C heating block (printed surface facing up) for 5 seconds (see **note 2**). UV crosslink printed DNA onto glass substrate with 60 mJ energy using Stratalinker (place slides printed side up; use Energy mode: 600 × 100μJ), then transfer arrays to a slide rack. Denature arrayed cDNA spots by immersing arrays in near-boiling (see **note 3**) ddH₂O in Pyrex dish and agitate for 2 minutes. Pre-hybridize arrays in 3X SSC, 0.1mg/ml BSA, 0.1% SDS in a glass staining dish (in a waterbath) at 50°C for 60 minutes. Then, rinse arrays by gentle shaking in ddH₂O at room temperature for 5 minutes; repeat twice. Subsequently, plunge arrays into 95% ethanol and agitate for 2 minutes, spin-dry arrays in a table-top centrifuge at 500 rpm at room temperature for 5 minutes and use arrays the same day (for oligonucleotide arrays, see **note 4**).

2.2.2. Preparation of mRNA—Both total RNA and mRNA are suitable substrates for fluorescence labeling and expression profiling. Total RNA can be isolated from specimens using Trizol reagent (Invitrogen) or by anion-exchange methods (e.g. Qiagen RNeasy). mRNA can be isolated by oligo-dT affinity purification (e.g. Invitrogen FastTrack 2.0).

2.2.3. Labeling of mRNA—For each test or reference sample, in a 1.5 ml eppendorf tube on ice add 50μg of total RNA (or 2 ug mRNA), 5 ug RT primer, and RNase-free H₂O to a total volume of 15μl. Heat samples at 70°C for 10min, then cool on ice 3 min, quick spin at 4°C and hold on ice. On ice, add 3μl of Cy-5 dUTP or Cy-3 dUTP to test and reference samples, respectively (see **note 5**). Tap gently to mix then quick spin. On ice, add 12.1 ul RT master mix (6 ul 5X First-Strand buffer; 3 ul 0.1M DTT; 0.6 ul 50x dNTPs; 0.5 ul RNasin; 2 ul Superscript II). Tap gently to mix, then quick spin, and incubate 42°C for 1 hr. Thereafter, add an additional 1ul Superscript II, mix gently, quick-spin and incubate at 42°C an additional 1 hr. Cool on ice and add 3μl 0.5M EDTA stop solution, mix gently and quick spin.

2.2.4. Hybridization of labeled sample—Combine Cy5-labeled test and Cy3-labeled reference samples, together with 450 ul TE_{7.4}, into a microcon 30 filter, and spin 12,000 g 9–12 min at room temperature in a microcentrifuge; retained volume should be approximately 20 ul (see **note 6**); discard the flow-through. Add an additional 500 ul TE_{7.4}, spin 12,000 g 9–12 min, and discard the flow-through (see **note 7**). Add an additional 450 ul TE_{7.4}, 2 ul polyA RNA stock solution, 2 ul yeast tRNA stock solution, and 20 ul human Cot-1 stock solution (see **note 8**). Spin 12,000 g 9–12 min and discard the flow-through. The retained volume should be less than 32ul. Invert microcon 30 filter into a new eppendorf tube, and spin 12,000 g 1 min to recover labeled cDNA sample. Transfer the labeled cDNA mixture to a screw-top

microcentrifuge tube, determine the sample volume using a pipette, and increase the volume to 32 μ l by adding ddH₂O. Add 6.8 μ l 20X SSC, and mix. Add 1.2 μ l 10X SDS, mix gently to avoid forming bubbles. Boil the hybridization mixture for 2 min, incubate 37°C for 20 min, then quick spin. Carefully pipette hybridization mixture onto DNA microarray, and overlay with a 22×60 mm glass coverslip (see **note 9**). Overlay coverslip with several small droplets of 3X SSC (totaling 20 μ l) to provide hydration, then enclose and seal slide in hybridization chamber. Incubate at 65° C for 16–18 hrs (see **note 10**).

2.2.5. Washing microarrays after hybridization—Following hybridization, wash the DNA microarrays to remove unbound labeled sample. The following three sequential wash steps should be performed using a slide rack, transferring among three separate glass staining dishes, each containing volumes of 350–400 ml, with gentle agitation: Wash 1: 2X SSC/0.03% SDS (see **note 11**), at 65°C (see **note 12**), 5 min; Wash 2: 1X SSC, room temperature, 5 min; Wash 3: 0.2X SSC, room temperature, 5 min. Following the third wash, DNA microarrays should be spun-dry using a desktop centrifuge, at 500 rpm at room temperature for 5 minutes (see **note 13**).

2.2.6. Microarray imaging, data reduction and analysis—Following hybridization, DNA microarrays should be scanned in dual-wavelengths using a GenePix 4000B (Axon) scanner, or equivalent. GenePix software, or equivalent, should be used to extract fluorescence intensity ratios for each DNA element on the DNA microarray. To compensate for variable labeling efficiencies, fluorescence ratios can be normalized such that the average fluorescence ratio for all DNA elements on the array is set to 1 (i.e. “global” normalization). Microsoft Excel, or any of several more sophisticated commercial or academic microarray databases (see for example ³¹) can be used to manipulate and analyze microarray data.

2.2.7. Notes—**1.** Following processing, the array spots will no longer be visible, so demarking the boundaries is important for later positioning the labeled sample and coverslip correctly. **2.** Rehydration should produce spot sizes that are enlarged by approximately 20%, with the DNA distributed more uniformly within spots. **3.** Bring ddH₂O to a boil in the Pyrex dish, and then remove from heat. Add microarrays in slide rack immediately after the boiling (bubbling) has subsided. **4.** For processing oligonucleotide arrays, UV-crosslink using 70 mJ, wash arrays in 0.2% SDS at room temperature for 10min, wash three times in ddH₂O at room temperature for 5 min each, rinse in 95% EtOH for 1min, then spin dry in centrifuge at 500 rpm. **5.** Often by convention, the test sample is labeled with Cy5 while the reference sample is labeled with Cy3. **6.** Centrifuge times are estimates. If necessary, here and in subsequent microcon steps, spin in additional 1 min increments until volume of retained solution is approximately 20 μ l. **7.** This additional wash step serves to further remove unincorporated fluorescent nucleotides. If labeling is successful, the retained labeled cDNA mixture should appear light blue. **8.** PolyA and human Cot-1 pre-annealing serves to block non-desirable hybridization to polyA tails and highly-repetitive DNA, respectively, contained in a subset of cDNA microarray elements. Yeast tRNA functions to block non-specific hybridization. **9.** A total volume of 40 μ l for the hybridization solution is appropriate when using a 22×60 mm cover slip. If using a different-sized cDNA microarray and cover slip, adjust the total volume of hybridization solution accordingly, while maintaining final SSC and SDS concentrations. **10.** The described hybridization protocol is optimized for cDNA microarrays. For oligonucleotide microarrays, improved hybridization sensitivity and specificity can be achieved by performing the hybridization with 35% formamide (with the same concentration of SSC/SDS) and at 42°C for at least 24 hrs. **11.** Add SDS last to avoid precipitation. **12.** Performing the first wash above room temperature (65°C) provides higher stringency to increase specific to non-specific hybridization signal. For oligonucleotide arrays, perform the first wash at 42°C. **13.** Work quickly to avoid air-drying (which will leave salt residue) between last wash and spin-dry steps.

3. Introduction to Serial Analysis of Gene Expression (SAGE)

Serial Analysis of Gene Expression (SAGE) is a comprehensive expression profiling technology for quantitative gene expression in samples that does not depend on the prior availability of transcript information²⁴. In this aspect, it varies from other expression profiling technologies such as cDNA microarrays (*described above*) where analyses is limited to a known repertoire of arrayed sequences. The premise of SAGE rests on the concept that a short sequence of nucleotides (~11bp in length, known as a “tag”) is sufficient for uniquely identifying a transcript^{24, 32}. The “serial” aspect of this platform comes from the generation of “concatemers” by ligating large numbers of SAGE tags within a “library” that are then serially analyzed by sequencing for gauging relative transcript levels.

The sequential steps involved in generation of a SAGE library are illustrated in Figure 1 (*reproduced with permission from American Society of Clinical Oncology*)³². Briefly, double-stranded cDNA is generated from mRNA isolated from a sample of interest and immobilized on magnetic beads. The cDNA is then digested with a frequent cutting restriction enzyme such as *Nla*III, and ligated to a linker with a restriction recognition site and a PCR primer site. Subsequent digestion by another restriction enzyme (e.g., *Bsm*I) generates “tags” that contain the linker and a short nucleotide sequence (~11bp) downstream of *Bsm*I recognition site that is specific for the transcript from which it was generated. Pairs of tags are then ligated to generate so-called “ditags” which are PCR amplified, digested by *Nla*III to remove the linkers and concatenated. The concatemers are subcloned into an appropriate vector, and the colonies resulting thereof are sequenced. The sequence data from a given SAGE library can be analyzed using a variety of online publicly as well as commercially available resources (*see below*), and any two or more such libraries (say pancreatic cancer and normal pancreas) compared for differential gene expression. Since its original description, numerous modifications have been made to the SAGE protocol^{33, 34}, including a “microSAGE”³⁵ (separately described as SAGE-lite³⁶) technique that permits generation of libraries from minute quantities of starting template (nanograms of mRNA), such as from microdissected material.

3.1. Materials for SAGE

The materials required for SAGE can be divided into 13 discrete steps and these will be listed stepwise. Note that some reagents are required in multiple steps.

Step 1

1. Linker Sequences

Linker 1 A (obtain gel-purified)

5' TTT GGA TTT GCT GGT GCA GTA CAA CTA GGC TTA ATA GGG ACA TG
3'

Linker 1 B (obtain gel-purified)

5' TCC CTA TTA AGC CTA GTT GTA CTG CAC CAG CAA ATC C[amino mod.
C7] 3'

Linker 2 A (obtain gel-purified)

5' TTT CTG CTC GAA TTC AAG CTT CTA ACG ATG TAC GGG GAC ATG 3'

Linker 2 B (obtain gel-purified)

5' TCC CCG TAC ATC GTT AGA AGC TTG AAT TCG AGC AG[amino mod. C7]
3'

2. NEB T4 Polynucleotide Kinase (10U/μl; NEB #201S)

3. The 12% PAGE-TAE gel may be made from the following:
40% Polyacrylamide (19:1 acrylamide:bis) (Bio-Rad #161-0144)
50X Tris-Acetate-EDTA (TAE) buffer Quality Biological #330-008-161) (See **note 1**)

Step 2

1. Dynabeads oligo (dT)25 mRNA direct kit (Dyna, #610.11).
2. Dynal Magnetic Particle Concentrators (DynaL MPC-S #120.20)
3. Superscript Choice System cDNA Synthesis Kit (Gibco BRL #18090-019)
4. 0.5M EDTA Sigma # E7889
5. Sodium dodecyl sulfate (SDS) (Sigma L4390)

Step 3

1. *Nla* III (NEB, #125S)

Step 4

1. T4 ligase (high concentration = 5U/μl; Life Technologies #15224-041)
2. BSA (Bovine Serum Albumin) (NEB #B9001S)

Step 5

1. *Bmsf*I (NEB#572S)
2. Phenol-Chloroform
3. 0.5M EDTA. Sigma #P2069
4. LoTE = 3mM Tris-HCl (pH 7.5) + 0.2mM EDTA (pH 7.5)

Step 6

1. Klenow 2U/μl (Pharmacia #27-0929-01)
2. 7.5M ammonium acetate (Sigma #A2706)

Step 7

1. T4 Ligase (High Concentration 5U/μl; Life Technologies #15224-041)

Step 8

1. Primer 1
5' GGA TTT GCT GGT GCA GTA CA 3'
2. Primer 2
5' CTG CTC GAA TTC AAG CTT CT 3'
3. 40% Polyacrylamide (19:1 acrylamide:bis) (Bio-Rad Cat No. 161-0144) for making 12% PAGE-TAE gel
4. 96 well plates for PCR (Sigma #Z37,490-3)
5. Taq polymerase (BRL #10996-034)

Step 9

1. SYBR Green I (Molecular Probes S-7567)
2. SpinX filtration microcentrifuge tubes (Costar Corp, Cambridge, MA)

Step 10

1. TE (10mM Tris-HCl (pH 7.5), 1mM EDTA (pH 8.0))

Step 11

1. 40% polyacrylamide (37.5:1 acrylamide:bis) (Bio-Rad #161-0148)

Step 12

1. SphI restriction enzyme (NEB #182S)
2. PZero plasmid (Invitrogen #K2500-01)

Step 13

1. ElectroMAX DH10B competent cells
2. Zeocin (Invitrogen # R250-01)
3. Glass beads
4. Agarose (Gibco BRL Select Agar #30391-023)

3.2. Methods

3.2.1. Kinasing reaction for linkers—The linkers need to be “kinased” as described in the NEB T4 polynucleotide kinase instructions. Briefly, dilute Linker 1B, 2B, 1A and 2A to 350ng/μl. Incubate at 37°C for 30 minutes, and heat inactivate at 65°C for 10 minutes. Mix 9μl Linker 1A (350ng/ml) to the 20μl kinased Linker 1B prepared above (final conc. 200ng/μl) to generate “1AB”. Then, mix 9μl Linker 2A (350ng/μl) to the 20μl kinased Linker 2B prepared above (final conc. 200ng/μl) to generate “2AB”. Anneal both tubes of linkers with the following incubations: 1) 95°C for 2 min., 2) 65°C for 10 min., 3) 37°C for 10 min., 4) Room temp for 20 min., 5) Store at −20 °C.

Now test the anneal mixes. Kinasing should be tested by self-ligating both mixes and incubating at 37°C for 30 minutes. Analyze on a 1.5mm 12% PAGE-TAE gel. The ligated dimers should run at 80bp, and the monomers at 40bp. Load all of the self-ligated linkers, and 1μl of the annealed mixes as negative controls. Kinased linkers should allow linker-linker dimers (80–100 bp) to form after ligation, while unkinased linkers will prevent self-ligation. Only linker pairs that self-ligate greater than 70% should be used in further steps.

3.2.2. First and Second Strand cDNA synthesis—Use Dynal Dynabeads oligo (dT)25 mRNA direct kit and magnets with the Superscript Choice System cDNA Synthesis Kit to accomplish first and second strand synthesis. Thoroughly resuspend Dynabeads oligo (dT)25 beads. Transfer 100–120μl to an RNase-free, 1.5ml eppendorf tube, and place it on the magnetic eppendorf tube holder. After the mixture of beads and buffer has cleared, remove the supernatant. Then wash the beads in 500μl of lysis/binding buffer, and mix 10ug of total RNA with 1ml lysis/binding buffer (see **note 2**). Remove the 500μl lysis/binding buffer supernatant from the Dynabeads., and add the RNA/buffer mixture to these prewashed Dynabeads. Shake at room temperature for 5 minutes by hand, place the tube on the magnet for 2 minutes, and remove supernatant. Wash the beads twice with 1ml of Buffer A/washing buffer containing LiDS. Then, wash the beads once with 1ml of Buffer B/washing buffer containing 20μg/ml

glycogen. Then wash the beads four times with 100µl of 1X 1st strand buffer. Remove the supernatant and resuspend beads in the 1st strand synthesis mix from the cDNA kit. Place the tube at 42°C for 2 minutes, then add 3µl of SuperScript II RT. Incubate at 42°C for 1 hr. Mix the beads every 10 min. by vortexing, or tapping the tube. After incubation, place the tube on ice to terminate the reaction. Directly to the first strand reaction, and on ice, add the second strand synthesis components for a total of 500µl. Incubate at 16°C for 2 hr., mix the beads every 10 minutes. After incubation, place the tubes on ice, and terminate the reaction by adding 40µl of 0.5M EDTA, pH 8.0. Place the beads on the magnet and remove the supernatant. Wash the beads once with 500µl of 1X BW containing 1% SDS. Resuspend the beads in 200µl of 1X BW/1% SDS and heat at 75°C for 20 minutes. Wash four times with 200µl of 1X BW containing 2XBSA. Wash twice with 200µl of 1x NEB Buffer 4 containing 2XBSA.

3.2.3. Cleavage of cDNA with anchoring enzyme (*NlaIII*)—Resuspend the beads by setting up a restriction digest with *NlaIII*. Mix and incubate at 37°C for 1 hour (mix every 15 minutes).

3.2.4. Ligating linkers to cDNA—After the *NlaIII* restriction digest, remove the supernatant, and wash the beads twice with 200µl of 1X BW/0.1% SDS containing 2X BSA. Wash four times with 200µl of 1X BW containing 2X BSA (freshly made). Wash twice with 200µl of 1X ligase buffer. Divide the beads into two tubes, and place them on a magnet. Remove the last wash and set up a 10µl ligation with linker 1AB (1µl) and linker 2AB (1µl) in separate tubes. The amount of linkers used needs to be adjusted according to the number of cells (see **note 3**). Two different linkers are used to prevent looping of the template in the PCR reaction. The linkers encode for the *BsmfI* restriction enzyme site. Heat tubes at 50°C for 2 minutes, then let sit at room temp for 15 minutes. Add 1µl of T4 high concentration ligase (5U/µl) to each tube (10µl reaction). Incubate at 16°C for 2 hr, and mix beads intermittently by tapping the tube.

3.2. 5. Release tags using Tagging Enzyme (*Bsmf I*) of cDNA—After ligation, wash each sample twice with 200µl of 1X BW/0.1% SDS containing 2X BSA (freshly made), by adding the 200µl of buffer to the 10µl ligation reaction. Pool tube 1 and tube 2 together after the first wash in order to minimize losses in subsequent steps. Wash four times with 200µl of 1X BW/2X BSA (freshly made). Wash twice with 200µl of 1X NEB Buffer 4 with 2X BSA (freshly made). Set up a restriction digest with *BmsfI*. *BsmfI* produces a 5' sticky end of 4 bases (10bp + 4bp into unknown sequence). Incubate at 65°C for 1 hr., mix intermittently, then centrifuge at 14,000 for 2 min. at 4°C. Transfer supernatant to new tube (see **note 4**). Wash beads once with 40µl of LoTE, thereafter pool the 200µl supernatant, and the 40µl wash together (240µl final volume). Perform phenol:chloroform extraction by adding 240µl of phenol:chloroform, pH8 ("PC8"). Vortex, then centrifuge for 5 minutes at 10,000rpm at 4°C, and transfer the upper aqueous phase to a new tube. Then precipitate with 100% ETOH at -80°C. Centrifuge for 30 minutes at 14,000 rpm at 4°C. Wash twice with 200µl of 75% EtOH. Resuspend pellet in 10µl LoTE by pipeting up and down.

3.2.6. Blunt ending released tags—The tags now have a protruding end which needs to be filled in for blunt end ligation to form the ditags. Set up blunt end ligation using Klenow (2U/µl). Incubate at 37°C for 30 minutes, and add 190µl of LoTE (240µl final vol.). Phenol:chloroform extract with 240µl of PC8 (480µl total volume). Vortex, centrifuge for 5 minutes at 14,000 rpm at 4°C. Remove 200µl of the upper aqueous phase containing the nucleic acids, and aliquot into the "ligase-plus" tube, and aliquot the rest of the aqueous upper phase into "ligase-minus" tube. Equalize the volume of the plus and minus ligase samples by adding 160µl LoTE to the ligase-minus sample. ETOH precipitate both the plus and minus tubes with 7.5M ammonium acetate. Centrifuge for 30 minutes at 10,000rpm at 4°C. Remove the

supernant, and wash twice with 200µl of 75% EtOH. Resuspend the ligase-plus reaction in 5µl of LoTE. Resuspend the ligase minus reaction in 3µl of LoTE.

3.2.7. Ligate to form ditags—Using high concentration T4 ligase (5U/µl), set up a ligation to form ditags. Add 5µl of 2x ligase-plus mix to the ligase-plus tube. Add 3µl of 2x ligase-minus mix to the ligase-minus tube. Therefore, the total volume of ligase-plus is 10µl, and the ligase-minus is 6µl. Incubate overnight at 16°C. Add 10µl of LoTE to the ligase-plus tube (20µl total volume). Add 14µl of LoTE to the ligase-minus tube (20µl total volume).

3.2.8. PCR amplification of ditags—Use the P1 and P2 primers (350ng/µl each) to amplify ditags for a test PCR to determine the optimal conditions for the large-scale PCR that follows. Optimize amplification by using different dilutions of the ditag template (see **note 5**). Cycle conditions are as follows: A) 1 cycle: 94°C for 1 min., B) 27 cycles (ligase-plus) or 35 cycles (ligase-minus): 94°C for 30sec.; 55°C for 1min.; 70°C for 30sec. C) 1 cycle: 70°C for 5 minutes. Use 27 cycles for the ligase-plus reactions, and 35 cycles for the ligase-minus reactions (see **note 6**). Remove 10µl from each reaction, and mix with 1µl of loading dye. Electrophorese the samples on a 1mm 12% polyacrylamide gel. Use 10bp and 100bp ladder as a marker. Amplified ditags should be 102bp in size. A background band of equal or lower intensity occurs around 80bp. All other background bands should be of substantially lower intensity. The ligase-minus samples should not contain any amplified product of the size of the ditags even at 35 cycles.

After the PCR test to determine the appropriate dilution, perform a large-scale PCR. About 300 reactions of 50µl each of the optimal dilution is needed for the pooled PCR products. Three 96 well plates with a 50µl reaction per well is adequate. Use **one less cycle for the large scale than** for the small scale PCR.

3.2.9. Isolation of ditags—After the large-scale PCR is complete, centrifuge the 96 well microplates in a swinging bucket centrifuge for 10 minutes to spin down the condensation. Collect the PCR products into a 50ml plastic conical tube, and perform a phenol:chloroform extraction with an equal volume of PC8, and then centrifuge in a swinging bucket rotor for 10 minutes.. Transfer the upper aqueous phase to a new 50ml tube and EtOH precipitate with 100% ETOH and 7.5M ammonium acetate at -80°C. Centrifuge at 4°C for 30 minutes. Wash with 5ml 75% EtOH, and centrifuge for 5 minutes at 4°C, followed by air drying the pellet. Resuspend each pellet in 200µl of LoTE, and pool the two tubes into one 400µl sample, and then place it on ice. Add 40µl of 10X loading dye. Apply 110µl to each of four 1.5mm 12% acrylamide TAE gels using one large well comb. Electrophorese the gel for approximately 1.5 hr. at 160V until the blue xylene band is near the bottom of the gel and the purple bromophenol blue band has run off the gel. Stain the gel using SYBR Green I stain, at a 1:10,000 dilution. Let the gel soak in the stain for 15 min. on a shaker. Visualize the gel on a UV box using the yellow SYBR Green filter. Protect the DNA by putting an ethanol cleaned glass plate on the UV box, then put the gel on the glass plate. Cut out only the amplified ditag 102bp band from the gel. Be sure to remove the markers, and do not take the 80bp background band. Place one third of each band in a 0.5ml microcentrifuge tube (twelve tubes total) whose bottom has been pierced 2 times with a 21 gauge needle to form small holes of about 0.5mm diameter. Pierce the tubes with a syringe needle from the inside out for safety. Place the 0.5ml microcentrifuge tubes in 2ml round bottom microcentrifuge tubes, and centrifuge in microfuge at 10,000rpm for 5 minutes at room temp. This serves to break up the cut-out-bands into small fragments at the bottom of the 2ml microcentrifuge tubes. If most of the gel is not contained in the 2ml microfuge tube, then centrifuge for 5 more minutes. Discard 0.5ml tubes add 250µl LoTE and 50µl 7.5M ammonium acetate to each 2ml tube. Tubes can remain at 4°C overnight at this point (see **note 7**). Vortex each tube, place at 65°C for 15 minutes, and pulse centrifuge at 6,000 rpm to collect the condensation at the bottom of the tube. Mix the contents well, and the transfer the contents of each tube to a SpinX filtration tube. Transfer as much of the gel

remnants, and viscous solution as possible. Centrifuge each SpinX tube for 5 minutes at 10,000 rpm at room temperature. ETOH precipitate the eluates in new 1.5ml tubes with 7.5M ammonium acetate by centrifuging at 10,000 rpm for 30 minutes at 4°C. Wash twice with 200µl of 75% EtOH for 5 minutes at 4°C for each wash. Resuspend DNA in 10µl of LoTE in each tube. Pool samples into one tube, 120µl total. Remove 1µl for quantitation of DNA concentration. The total amount of DNA at this stage should be 10 to 20µg. If the concentration is not this high, ETOH precipitate and redo the large scale prep, and then combine the two 102bp band samples. If the DNA concentration is too high, then the sample can be split in half, and the two digestion reactions can be electrophoresed. If the sample is split in half, then the reaction should be brought to volume with LoTE. Digest the 102bp DNA with *Nla*III by incubating 1 hour at 37°C (see **note 8**).

3.2.10. Purification of ditags—Extract with an equal volume PC8 (400µl). Vortex, then centrifuge for 5 minutes at 10,000 rpm at 4°C. **Be very careful** in the following steps because the small 20bp DNA segments are unstable. A low temperature and high salt concentrations are needed. Transfer the upper aqueous phase into 2 tubes (200µl each), and then ETOH precipitate at –80°C. Then centrifuge the tubes at 4°C for 30 minutes at 10,000 rpm. Remove the supernatant, and wash once with 200µl cold 75% EOH. The ETOH is cold to protect the 26bp ditags from denaturation. The melting point of 26bp DNA is below room temperature. Remove ETOH traces by air-drying on ice. Resuspend the pellet in each tube in 10µl of cold TE. This higher concentration Tris buffer (as compared to LoTE) is needed to protect the 26bp DNA ditags. Pool the resuspended DNA into 1 tube (20µl total). On ice, add 2µl of 10X loading dye (22µl total volume). Load 5.5µl of this sample into 4 lanes of a 1.5mm 12% polyacrylamide TAE gel (10-well), and electrophorese at 100V to 140V (see **note 9**). Load markers on both sides of the 4 lanes of sample leaving one open lane between the sample lanes and the marker lanes. Stain the gel using SYBR Green I stain, at a 1:10,000 dilution.

Cut out the 24–26bp band from the 4 lanes using a glass plate, pre-cleaned with ETOH, on top on the UV box. Place 2 cut-out bands in each 0.5ml microcentrifuge tube (2 tubes total). More 0.5ml microcentrifuge tubes can be used if more lanes are loaded with 26bp ditags, or if the bands are large. As before, pierce the bottom of 0.5ml tube 2 times with a 21 gauge needle, and place the tubes in 2.0ml round bottom microcentrifuge tubes, and centrifuge in the microfuge at 10,000rpm for 5 minutes at room temperature. Continue to spin the tube until all of the gel is collected in the 2.0ml tube. Discard the 0.5ml tubes, and add 50µl 7.5M ammonium acetate and 250µl LoTE (in this order) to the 2.0ml tubes. Vortex the tubes, and place at 37°C for 20 minutes. Use 4 SpinX tubes to isolate the eluate. Spin for 5 minutes at 10,000rpm at room temperature. ETOH precipitate in 3 tubes at –80°C. Centrifuge at 4°C at 10,000rpm for 30 minutes. Wash twice with 200µl of cold 75% ETOH. Air-dry to remove all residual ETOH. Resuspend each DNA sample in 2.5µl of cold LoTE, making sure that the total volume is 8µl after resuspending the pellet. Remove 1µl of the purified ditags for quantification.

3.2.11. Ligation of ditags to form concatemers—Length of ligation time depends on quantity and purity of ditags. Typically, several hundred nanograms of ditags are isolated and produce large concatemers when the ligation reaction is carried for 1 to 3 hr. at 16°C. Lower quantities, or less pure ditags, will require longer ligations. Set up a ligation using the pooled purified ditags (7µl), 5X ligation buffer (2µl), and the high concentration (5U/µl) T4 ligase (1µl). If the volume of pooled purified ditags is high, the reaction volume can be increased. Incubate for 1 hr and 10 minutes at 16°C if you started with 10µg of total RNA; fewer ditags require a shorter incubation. Heat the sample at 65°C for 5 minutes. Place it on ice for 10 minutes, and then add loading dye to the ligation reactions. Use a 1mm 8% polyacrylamide TAE gel. Load the concatemers in the center well, skip a lane on either side of the concatemers, and then use a 100bp and a 1kb ladders for molecular markers. Samples are electrophoresed at 130V until the purple bromophenol blue dye reaches the bottom of the gel. Stain the gel with

SYBR Green I 1:10,000 dilution (see **note 10**). Visualize on UV box using the yellow SYBR Green filter. Concatemers will form a smear with a range from about one hundred base pairs to several kilobases. Isolate the 800–1500bp region, and the 1500bp to 3000bp region. As before, place each of these gel pieces into a 0.5ml microcentrifuge tube with a needle pierced bottom (2 tubes total). Place the tubes in a 2.0ml round bottom microcentrifuge tube, and centrifuge at 10,000rpm for 5 minutes at room temp. Discard the 0.5ml tubes, add 300µl of LoTE to the 2.0ml tubes. Vortex the tubes, and place at 65°C for 20 minutes. Transfer the contents of each tube into 1 SpinX microcentrifuge tube, and centrifuge for 5 minutes at 10,000 rpm at 4°C. ETOH precipitate both the high and low weight concatemers, and place at –80°C overnight.

3.2.12. Ligating concatemers into pZero—Prepare the pZero cloning vector by digesting with the *SpHI* restriction enzyme, by incubating for 20 minutes at 37°C. Remove 1µl of the digestion to electrophorese on a 1% agarose TAE gel with markers, and uncut pZero. pZero should migrate in the gel at approx. 2.5kbp. Add 30µl LoTE to the rest of the sample, and heat it at 70°C for 10 minutes to inactivate the enzyme. The concentration is now 25ng/µl, and 1µl can be used per ligation. Centrifuge the concatemers and pZero at 10,000rpm for 30 minutes at 4°C. Wash the concatemers and pZero twice with 200µl of 75% EtOH. Resuspend each tube of the purified concatemer DNA in 6µl of LoTE. Resuspend the pZero in 30µl of LoTE (approximately at a concentration of 30ng/µl).

The following volumes are in microliters.

Fraction	High Mol. Wt. Fraction	Low Mol. Wt. Fraction	Vector only	No ligase added
Vector	1	1	1	1
5X ligase buffer	2	2	2	2
T4 ligase (5U/µl)	1	1	1	0
dH2O	0	0	6	7
Concatemers	6	6	0	0

Incubate overnight at 16°C in 1.5ml eppendorf tubes. Before putting the ligation in, be sure that you can continue the next day.

3.2.13. Electroporation of ligation products and colony PCR—Add 190µl LoTE to the ligation mix to bring the sample volume to 200µl. Phenol:chloroform extract, and precipitate at –80°C, and then centrifuge. Transfer the upper aqueous upper layer to a new tube. ETOH precipitate the aqueous phase in 1.5ml tubes at –80°C. Centrifuge at 10,000rpm at 4°C for 30 minutes. Wash four times with 200µl of 70% ETOH. Centrifuge at 10,000 rpm for 5 minutes at 4°C. Remove ETOH, and air-dry the pellets. Resuspend each pellet in 10µl LoTE: **these are your ligation mixtures.**

Place ligation mixtures on ice. Electroporate the 1µl of ligation into 25µl of ElectroMAX DH10Bs cells. Plate the transformation mixture onto fresh LB-Zeocin plates (100µg/ml Zeocin). 100µl of liquid must be dispersed on each plate. Various amounts of both the high and low concatemer fractions are plated separately. The cells are spread by using glass beads with sterile technique. Two dilutions of each fraction are used: A) 100µl of undiluted electroporated competent cell culture, B) 1:10 dilution of electroporated competent cell culture (diluted with LB media), C) 100µl of the two negative controls are also plated appropriately.. Incubate overnight at 37°C. Keep the rest of the transformation mixtures at 4°C. Plasmids with

insert should have hundreds to thousands of colonies while control plates should have zero to tens of colonies.

Check the insert sizes by performing colony-PCR. Check at least 36 colonies of each ligation in order to calculate the average insert size. Set up 25µl PCR reactions using the M13-forward and M13-reverse primers. Use a sterile pipette tip, or a toothpick, to pick colonies. Suggested parameters for PCR are as follows: A) 1 cycle: 95C for 2 min; B) 30 cycles: 95C for 30 sec., 56C. for 1 min., 70C for 1min., C) 1 cycle: 70C for 5 min. Electrophorese 5µl of sample on a 1.5% agarose TAE gel at 100V until the purple bromophenol blue band is near the bottom of the gel. Load a 1kb and 100bp ladder to determine the insert size. Place 1µl of 10x loading buffer in the wells of a 96-well PCR plate. Transfer 5µl of the PCR reactions to the dye using the multi-channel pipette, and load into the gel.

3.2.14. Bioinformatics analyses of SAGE Tags—The very last step of SAGE is to sequence the colonies and perform appropriate bioinformatics analyses on the sequenced products in order to elucidate SAGE tag sequences and calculate relative expression levels of various tags in the sample (i.e., creation of a SAGE “library”). The frequency of each SAGE tag in the SAGE library directly correlates with transcript abundance. Sequencing of concatemers can be done either in-house in a core facility or at any commercial sequencing laboratory. The bioinformatics associated with analysis of SAGE tags is outside the scope of this chapter but the reader is referred to excellent reviews on this subject^{37–39}, as well as to relevant websites (SAGENet: <http://www.sagenet.org/>; SAGEMap: <http://www.ncbi.nlm.nih.gov/projects/SAGE/>; TagMapper: http://tagmapper.ibioinformatics.org/index_html; and SAGE Genie: <http://cgap.nci.nih.gov/SAGE>). Of note, many of the databases house publicly available SAGE libraries of a variety of human tissues, cell types and diseased specimens, including pancreatic cancer, and form a readily available resource for generating differentially expressed transcripts in an entity of interest.

3.2.15. Notes—**1.** High quality linkers are crucial to several steps in the SAGE method. Linkers 1A, 1B, 2A, 2B, should be obtained gel-purified from the oligo synthesis company. These oligonucleotides can be ordered from Integrated DNA Technologies (tel. 800-328-2661), or other oligonucleotide synthesis companies. **2.** The RNA volume should not exceed 100µl. **3.** A 1:10 dilution is often a good start. **4. Keep the supernatant** – this contains your actual tags! **5.** Use 1µl of 1/50, 1/100, and 1/200 dilutions of ligation product per PCR reaction. This step will indicate the best dilution for the reactions. If the starting RNA is poor quality, start out with a 1/25 dilution. **6.** The appropriate cycle number is critical for isolating an adequate amount of ditag DNA for SAGE. Too few cycles will result in a low yield, and may cause problems with subsequent steps. Too many cycles will give erratic results, and can also result in low yields. Therefore, trying various cycle numbers (e.g. 26, 28, 30) to determine the optimal number is recommended. **7.** The DNA seeps out of the gel overnight. Do not leave for more than one night, or the acrylamide can break down too much. Do not put at –80°C. **8.** Freeze/thaw cycles decrease the effectiveness of *Nla*III. Freeze/thaw *Nla*III only once to insure optimal activity. **9.** Do not run the voltage too high or your 26bp product could melt. **10.** This gel is thin and prone to rip. Be careful!

4. Introduction to mass spectrometric analysis of proteins

Initial sample collection and preparation is crucial for obtaining optimal results for most analytical techniques including mass spectrometry analysis of proteins. Despite the high sensitivity of current mass spectrometers (routine analysis at femto-mole) great care should be taken to avoid degradation of the sample due to the high abundance of naturally occurring proteases present in pancreatic juice and pancreatic tissue. General guidelines and protocols

regarding sample handling, initial preparation and fractionation by 1D electrophoresis, staining, tryptic digestion, nanoLC-MS/MS analysis, database searches and validation of data are provided below. It should be emphasized that these are general protocols that should be modified if more specific analysis is needed. It is *very important* not to contaminate the sample with e.g. keratin (hair and skin). In addition, universal precautions must be followed during handling of the specimens.

4.1 Materials

4.1.1. Preparation of pancreatic juice and pancreatic tissue

1. Protease inhibitor tablets (Roche Diagnostics, #1697498)
2. DC Protein Assay Kit I (Biorad, #500-0111)
3. Scalpel handle (Sigma, #S7772-1EA)
4. Scalpel blade (Sigma, #S2646-100EA)
5. Phosphate buffered saline (PBS) tablets (Sigma, #P4417-100TAB)
6. Modified RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1 % NP-40, 0.25 % Sodium deoxycholate, EDTA 1 mM)
7. Sodium chloride (Sigma, #S9888)
8. Deoxycholic acid (sodium deoxycholate) (Sigma, #D6750)
9. NP-40 (Calbiochem, #492015)
10. Trizma base (Sigma, #T6066)
10. Ethylenediaminetetraacetic acid (EDTA) (Sigma, #E4884)
11. Sonicator (Branson, Sonifier 250 or similar)

4.1.2. Fractionation by 1D gel-electrophoresis

1. XCell SureLock (Invitrogen, #EI0001)
2. NuPAGE 10%, Bis-Tris gel, 1.5mm, 10 well (Invitrogen, #NP0315BOX)
3. NuPAGE 4–12%, Bis-Tris gel, 1.5mm, 10 well (Invitrogen, #NP0335BOX)
4. NuPAGE sample reducing agent (10X) (Invitrogen, #NP0009)
5. NuPAGE LDS sample buffer (4X) (Invitrogen, #NP0007)
6. NuPAGE antioxidant (Invitrogen, #NP0005)

4.1.3. Protein staining (silver staining and coomassie staining)

1. Colloidal blue staining Kit (Invitrogen, #LC6025)
2. Acetic acid, glacial (Fisher Chemicals, #A38-500)
3. Methanol (EMD Chemicals Inc, #MX0475P-4)
4. Sodium carbonate (Sigma, #S-2127)
5. Silver nitrate (EMD Chemicals Inc Catalog, #SX0205-5)
6. Sodium thiosulfate (EMD Chemicals Inc, #SX0820-1)
7. Formaldehyde (JT Baker, # 2106-01)

4.1.3. Tryptic digestion

1. Scalpel handle (Sigma, #S7772-1EA)
2. Scalpel blade (Sigma, #S2646-100EA)
3. 10 mM dithiothreitol, DTT, (Sigma, #43815)
4. 55 mM iodoacetamide (Sigma, #I-6125)
5. 50 mM and 100 mM NH_4HCO_3 (Sigma, #09830)
6. Acetonitrile (JT Baker, #9017-03)
7. 12.5 ng/ μl modified trypsin, sequencing grade (Promega, # V5111)
8. 5% Formic acid (Formic acid (Sigma, #06450)

4.1.4. Reverse phase columns

1. Fused-silica capillary tubing for liquid chromatography 75 μm ID/365 μm OD, (Polymicro Technologies, #TSP075375)
2. Kasil 1TM (Potassium/Silicate solution): PQ Corporation (ASTRO product code: 25110)
3. Formamide (super pure grade), (Fisher Scientific, #BP228-100)
4. Reversed-phase material for liquid chromatography SB-C₁₈, 5–15 μm (Zorbax)
5. Reversed-phase material for liquid chromatography MS218, 5 μm (Vydac)
6. Methanol (EMD Chemicals Inc, #MX0475P-4)
7. High-pressure vessel for nano-LC column packing, (Proxeon A/S, #SP035)

4.1.5. LC-MS/MS

1. Emitters for liquid chromatography FS360-20-10-N-20, tip:10 $\pm$ 1 μm (New Objective, #FS3602010N20)
2. Mobile phase A (H_2O with 0.4% acetic acid and 0.005% heptafluorobutyric v/v)
3. Mobile phase B (90% acetonitrile, 0.4% acetic acid, 0.005% heptafluorobutyric acid in water)
4. Acetonitrile (JT Baker, #9017-03)
5. Heptafluorobutyric acid (HFBA) (Sigma, #H-7133)
6. Acetic acid, glacial (Fisher Chemicals, #A38-500)

4.2. Methods

4.2.1. Sample collection and preparation of pancreatic juice—Human pancreatic juice is normally collected during surgery from the pancreatic duct of patients undergoing pancreatectomy. A total volume of 20–500 μl is usually collected and immediately stored at -80°C with or without any protease inhibitors. The sample should be kept at on ice (4°C) all time when not stored at -80°C . To remove potential debris the pancreatic juice is centrifuged at 4°C for 30 min at 16,000g. The protein concentration should be determined using a protein assay kit. Several different protein assay kits can be used including e.g. Modified Lowry and Bradford. Modified Lowry (absorbance at 750 nm) is very sensitive but is a two step procedure and requires an incubation time (approximately 15–20 min). In addition, strong acids and ammonium sulfate can interfere with the measurement in Modified Lowry. The Bradford

(absorbance at 590 nm) method is even more sensitive and can be read within 5 min. However, proteins with low arginine content will be underestimated when using Bradford. The protein concentration in pancreatic juice can vary from approximately 2–15 mg/ml depending on the specific sample.

4.2.2. Sample collection and preparation pancreatic tissue—The amount of tissue needed depends on the type of analysis one wish to perform. This protocol is based on approximately 50 mg of starting material (tissue) which translates into approximately 10–25 mg of protein after extraction. Note these numbers are only rough numbers and can fluctuate from sample to sample. After surgical removal the pancreatic tissue is snap-frozen and stored at -80°C . During handling (cutting) the tissue is kept on a clean glass plate placed on top on an ice-bucket to minimize degradation of the proteins. The tissue is first cut into small pieces by a sterile scalpel and transferred to a 1.5 ml tube. The tissue is then gently washed (by inverting the tube) in ice-cold PBS-buffer to remove excess blood from the sample. The PBS-buffer is removed and replaced by 1 ml ice-cold modified RIPA buffer containing protease inhibitors (150 mM NaCl, 50 mM Tris, pH 7.4, 1 % NP-40, 0.25 % Sodium deoxycholate, EDTA 1 mM, one protease inhibitor tablet per 50 ml). The tissue is subsequently sonicated (output control 3–4, duty-cycle 30–40%, time: 30 seconds per cycle) 3–4 times. If possible the sample should be kept on ice during sonication but otherwise placed on ice for 5 min after every cycle of sonication. Alternatively a polytron homogenizer can be used followed by sonication for protein extraction (on ice). After protein extraction the sample is centrifuged for 30 min at 4°C and the supernatant is transferred to a new 1.5 ml tube.

4.2.3. Fractionation of pancreatic juice and tissue proteins by 1D gel-electrophoresis—Due to the relatively high complexity of pancreatic juice and tissue it is recommended that the sample is fractionated by e.g. 1D or 2D gel-electrophoresis prior nanoLC-MS/MS analysis. Alternatively, one can perform in-solution digestion combined with 2D LC-MS/MS (not discussed in this chapter)^{40, 41}. For automated nanoLC-MS/MS analysis a total of 20–30 μg of sample should be loaded on the gel (higher/lower amounts of sample can be loaded on the gel depending on the specific analysis). Approximately 20–30 μg of protein is transferred to a 1.5 ml tube and mixed (1:5) with 6x loading buffer containing 10% beta-mercaptoethanol. Vortex sample shortly (5 sec) and boil for 5–8 min to denature the proteins before loading on 1D-gel. For most purposes a 10% gel is recommended but alternatively a gradient gel can be used (4–12%) for higher resolution in a broad molecular mass range. The parameter for running the gel depends on the gel apparatus system and gel size and has to be done according to the individual manufacturer's instructions.

4.2.4. Staining of proteins after 1D gel-electrophoresis—After resolving the proteins by 1D gel-electrophoresis the proteins are visualized by either silver staining⁴² or colloidal coomassie⁴³. Silver staining can sensitize proteins down to 1–2 ng of protein⁴⁴, whereas colloidal coomassie staining can detect levels down to approximately 10–20 ng of protein. Both methods are equally compatible with mass spectrometry analysis.

4.2.5. Visualizing of proteins by silver-staining—After resolving the proteins by 1D gel-electrophoresis the gel is fixed in destaining solution (50% methanol, 5% acetic acid, 45% water (Milli-Q), v/v) for 20–30 min at room temperature (on shaker). The gel is rinsed in Milli-Q water for 30–60 min to remove acid (change water 3–4 times). The gel can be left overnight in water to reduce background (more transparent in areas with no protein staining). The gel is then sensitized for 1–2 min with 0.02% sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) and subsequently washed two times with Milli-Q water (1 min each time). The gel is incubated in cold (4°C) 0.1% silver-nitrate (AgNO_3) solution at 4°C for 20–40 min (on shaker). The gel is then washed two times in Milli-Q water as previously described (2×1 minute) and finally developed in developing

solution (0.04% formaldehyde/formalin, 2% Na_2CO_3). The development is quenched by discharging the developing solution and replacing it with 1% acetic acid (**See Note 1**). After staining (silver or colloidal coomassie) the gel is stored at 4°C in 1% acetic acid (container with tight lid). The gel can be stored for several months under these conditions.

4.2.6. Visualizing of proteins by colloidal coomassie staining—After gel-electrophoresis the gel is fixed in destaining solution (50% methanol, 5% acetic acid, 45% water (Milli-Q), v/v) for 10 min at room temperature (on shaker). The destaining solution is discharged and replaced by staining solution without stainer B (55 ml Milli-Q water, 20 ml methanol, 20 ml stainer A) for 10 min at room temperature (on shaker). Add 5 ml of stainer B to the existing solution and leave for 3–12 h. When protein bands become visible decant staining-solution and replace by 200–300 ml of Milli-Q water. Shake the gel for at least 7 h or until the background becomes clear. The water should be changed several times during destaining (**See Note 2**).

4.2.7. In-gel tryptic digestion of proteins resolved by 1D gel-electrophoresis—After visualization of the proteins by either silver staining or colloidal coomassie staining, the resolved proteins are digested by trypsin prior to mass spectrometry analysis¹⁷. The gel lane is excised into approximately 20–30 bands (depending on the size of the gel) and each band is further cut into smaller pieces (approximately 2×2 mm). Gel pieces from each band are washed with Milli-Q water and water/acetonitrile 1:1 (v/v) two times for 15 min at room temperature. Solvent volumes in the washing step roughly equaled five times the gel volume. After washing, the liquid is removed and the gel pieces are covered in 100% acetonitrile to shrink the gel pieces. After approximately 5 min, the acetonitrile is removed and replaced by 10 mM dithiothreitol (DDT) in 100mM ammonium bicarbonate (NH_4HCO_3) for 45 min at 56 °C to reduce the cysteines. The liquid is removed and replaced by 55mM iodoacetamide in 100mM ammonium bicarbonate and subsequently incubated for 30 min at room temperature in the dark to alkylate the cysteines. The iodoacetamide solution surplus is removed and the gel pieces are washed two times in water and acetonitrile as described above and subsequently dehydrated by adding 100% acetonitrile. The gel pieces are rehydrated in a digestion buffer containing 12.5 ng/μl of trypsin (Promega, modified sequencing grade) in 50 mM ammonium bicarbonate and incubated for 45 min on ice. Enough digestion-buffer is added to cover the gel pieces. After 45 min the digestion buffer is replaced by approximately the same volume 50mM ammonium bicarbonate but without trypsin to keep the gel pieces wet during enzymatic digestion. The samples are incubated at 37°C overnight. After digestion the remaining supernatant is removed and saved in a 1.5 ml tube. The remaining peptides are extracted from the gel pieces by incubating in 5% formic acid (enough to cover the pieces) for 15 min and then adding the same volume of 100% acetonitrile for further 15 min incubation. The extraction is repeated twice and all three fractions are pooled and dried down in a vacuum centrifuge and resuspended in 10–20μl of 5% formic acid.

4.2.8. Liquid chromatography tandem mass spectrometry analysis (LC-MS/MS)—Depending on the type of sample analyzed and available hardware (e.g. HPLC-system and mass spectrometer) the LC-MS/MS setup can be modified in many possible ways. However, two different strategies are frequently used for separation of peptides. One setup utilizes two columns in tandem (a pre-column followed by an analytical column) whereas the second setup only uses a single analytical column. The tandem column setup is preferred when large volumes (10–40 μl) have to be analyzed and/or if the samples need to be desalted/washed extensively. This setup is very robust and can be used for most types of samples. One drawback is that one might lose sensitivity due to broader chromatographic peaks. Several column materials can be used for pre-columns (e.g. YMC ODS-A, 5–15 μm beads; Kanamatzu USA Inc., New York, NY, USA) and analytical columns (e.g. Vydac MS218, 5μm beads; Vydac,

Columbia, MD, USA) and it is therefore strongly suggested that several trial runs including different reverse phase materials are tested for obtaining optimal separation and resolution during the LC-MS/MS analysis.

The following sections will describe a general strategy for building and packing reverse-phase columns used for nano-flow LC-MS/MS analysis in addition to some general chromatographic guidelines (LC-program, length of gradient and context of mobile phase).

4.2.9. Preparation of reverse phase column—The column is packed in a fused silica capillary tubing (375µm OD and 75µm ID) ⁴⁵. A “frit” restrictor has to be generated inside the fused silica to block the reverse phase material during packing (the silica tubing is open in both ends). Mix 44 µl of Kvasil 1™ with 8 µl of formamide and vortex for 1–3 min (the solution becomes viscous). One end of a 20–25 cm fused silica is dipped in the solution for 1–2 seconds (the solution will move upward into the fused silica by capillary action). Excess solution is wiped off and the fused silica is left at room temperature o.n. to polymerize. The frit is checked under a microscope and should be approx 2–5 mm in lengths and has to present a V-shaped cone pointing outward. The frit can be cut if it is too long. Insert the fused silica into a pressure vessel and wash it with 100% methanol. This assures that solvent can flow through the frit and when pressure is applied the frit is still affixed to the capillary. The reverse phase material (approx 4 mg) is placed in a glass micro-vial and resuspended in 500 µl methanol. To keep the column material in suspension a small magnetic stirring rod is added to the glass micro-vial before placing it in the pressure vessel. Place the pressure vessel on a stir plate and turn the magnetic stirrer at low speed. Turn the gas (He) pressure to 50 bar (800 psi) which initiate the packing of the column. The column should be 5–10 cm for pre-columns and 15–20 cm for analytical columns. The fused silica is connected to the LC-system and flushed with mobile phase B (90% acetonitrile, 0.4% acetic acid, 0.005% heptafluorobutyric acid in water) for 30 min and subsequently 30 min with mobile phase A (H₂O with 0.4% acetic acid and 0.005% heptafluorobutyric v/v) to equilibrate the column. The performance of the generated reverse phase column is tested with several trial runs by a tryptic digest of a standard protein (e.g. 100–200 fmol BSA). Elution time for the individual peaks in the LC-chromatogram should be approx 15–20 seconds with 100 fmol of BSA and produce sequence coverage between 50–60% with a Mascot protein score of approx 1500 (see section about database search later).

4.2.10. Sample analysis (LC-MS/MS)—The sample is automatically loaded onto the column by the auto-sampler in the HPLC-system. The peptides are loaded onto the reverse phase column using a linear gradient of 5–10% mobile phase B (90% acetonitrile, 0.4% acetic acid, 0.005% heptafluorobutyric acid in water) during 6–8 min. No peptides will elute during this time interval. If needed the loading time can be extended for additional washing/desalting of the sample. The peptides are subsequently separated and eluted from the column by 10–45% mobile phase B during 35–40 min, followed by 45–90% mobile phase B for 3 min. The column is finally rinsed flushed with 90% mobile phase B for 1–2 min and equilibrated in 5% mobile phase B for 3 min. The flow rate delivered from the pumps to the column(s) depends on the LC-setup. In a tandem column setup the flow rate should be 4–5 µl/min during loading and 1–1.5 µl/min for the single column setup. During peptide separation/elution the flow rate is decreased to 250–300 nl/min for both setups. During equilibration of the column the flow is again increased to 4–5 µl/min for tandem column setup and 1–1.5 µl/min for single column setup. Note that this program is only a rough guide and should be modified as required.

4.2.11. Database search and validation—The final step of the analysis is searching the generated LC-MS/MS data using a search engine. Several search engines are available (e.g. Mascot, SEQUEST, XTandem, Spectrum Mill) which uses their own individual search algorithm and scoring system. In this section Mascot search engine (version 2.0) will be used as an example. Before the search can be performed peak-list files have to be generated. Peak-

list files are made from the files initially recorded during the LC-MS/MS analysis (named e.g. RAW-files on a Micromass instrument) and contain information about the mass of the precursor selected for MS/MS fragmentation and the masses and relative intensity of the fragment ions. Depending on the type of instrument used for the LC-MS/MS analysis the search parameters has to be adjusted accordingly. The following Mascot search parameters can be used for high resolution type instruments (e.g. Micromass QTOF, Sciex Q-STAR). Several search parameters have to be specified on the Mascot MS/MS ion-search including peptide tolerance (set at 0.2 Da), MS/MS tolerance (set at 0.2 Da), enzyme (used in digestion, trypsin), missed cleavages (set at 1 or 2), fixed modifications (set carbamidomethylation on cysteins), variable modifications (set oxidation of methionine), peptide charge (set +1, +2, +3) and mono-isotopic mass. One also has to specify the type of database (e.g. NCBIInr, SwissProt, RefSeq) and taxonomy (e.g. human, mouse, bacteria, fungi) the peak list files has to be search in. A widely used non-redundant database is RefSeq 46 (<http://www.ncbi.nlm.nih.gov/RefSeq/>) from NCBI which contains 1,310,800 entries from 2780 organisms. One very important aspect of the final analysis is verification of the data obtained from the database searches. Far from all proteins retrieved from the database search are correct. Peptides identified by the Mascot search engine with a peptide score under 30–40 are usually false positives and has to be discarded from the data set. In cases where protein identification is based on a single peptide special caution has to be taken. The amino acid sequence identified by the Mascot search engine has to be verified manually to make sure that the MS/MS spectrum truly corresponds to the peptide sequence predicted by Mascot. Manual validation is therefore essential to avoid a large number of false positives in the list of identified proteins.

4.2.12. Notes—1. Developing of the gel should take approximately 2–3 min and care should be taken not to overexpose the gel (have the 1% acetic acid solution ready for quenching the reaction). **2.** Stainer A and B are provided in the colloidal Coomassie kit provided by Invitrogen (Invitrogen, Carlsbad, CA).

5. Molecular Targets Identified by Large Scale Transcriptomic and Proteomic Analysis of Human Pancreatic Cancers

The large scale analyses of human pancreatic cancers using techniques described above has led to the identification of nearly 200 differentially overexpressed transcripts and a similar number of differentially overexpressed proteins^{15–18}. These molecules have immediate translational relevance in terms of patient care, harboring the potential for application in imaging studies, targeted therapies and early detection. As the ensuing discussion highlights, such translational work is actively underway. For example, the transcripts prostate stem cell antigen (PSCA) and mesothelin were identified by Argani et al as being differentially overexpressed in human pancreatic cancers by SAGE analysis^{19, 20}. Since both PSCA and mesothelin are membranous proteins, they can be used for specific targeting of imaging agents to pancreatic cancers *in vivo*⁴⁷, or as a target for monoclonal antibody therapy using humanized antibodies (see <http://www.agensys.com>). Mesothelin has also been utilized as a target for immunotoxin therapy using a conjugated anti-mesothelin antibody⁴⁸. Most recently, it has also been reported that mesothelin is a target antigen against which a host immunologic reaction is mounted by the cytotoxic T-cell repertoire in human pancreatic cancer patients⁴⁹, and thus, it is currently being utilized in the preparation of pancreatic cancer vaccines. In addition to therapeutic applications for pancreatic cancer, PSCA and mesothelin are also secreted proteins, and therefore, one can envision quantitative assessment of these molecules in pancreatic juice samples for early detection of pancreatic cancer in at-risk patients⁵⁰. Claudin 4 is an overexpressed cell surface protein in human pancreatic cancers identified using an oligonucleotide microarray approach⁵¹. This tight junction molecule has also been used for targeting immunotoxins to pancreatic cancer cells^{52, 53}, as well for imaging purposes⁴⁷. On

the lines of transcriptomic studies, proteomic analysis of pancreatic juice samples has led to the identification of regenerating islet-derived 3 alpha (REG3A), also known as pancreatitis-associated protein (PAP), as overexpressed in cancer samples compared to non-neoplastic pancreatic juice ²¹. The selective overexpression of REG3A in neoplastic juice samples implies that this protein can be utilized as an early detection biomarker for pancreatic cancer.

In summary, pancreatic cancer is a lethal disease and rational strategies for early detection and targeted therapies are urgently required in order to alleviate the dismal prognosis of this neoplasm. The use of global RNA and protein expression profiling technologies, such as the ones described above, have led to identification of cellular targets with considerable potential for clinical application and patient care. These studies underscore the importance of pursuing large scale profiling of human cancers not only to further our understanding of the pathogenesis of these malignancies, but also for developing strategies to improve patient outcomes.

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Figure 1. Principle of SAGE Analysis (reproduced with permission from Polyak and Riggins, J Clin Oncol. 2001; 19(11):2948–58, copyright American Society for Clinical Oncology).

Figure 2.

Preparation of a reverse phase column for nanoLC-MS/MS analysis. A “frit” restrictor composed of a mix of Kvasil 1[™] and formamide is generated in one end of the fused silica capillary tubing (375 μm OD and 75 μm ID) to block the reverse phase material during packing. The “frit” restrictor should present a V-shape cone and be 2–5 mm in length (panel A). The fused silica tubing with the “frit” restrictor is subsequently placed in small pressure vessel (“frit” restrictor pointing outwards of the pressure vessel) containing a slurry of reverse phase material (panel B). The column is packed by applying a pressure (He) of 50 bar (800 psi) in the pressure vessel. The column should be approximately 15–20 cm in lengths.