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(54) **Title:** DETECTION OF BIOMARKERS FOR NON-SMALT, CELL LUNG CANCER

Figure 2
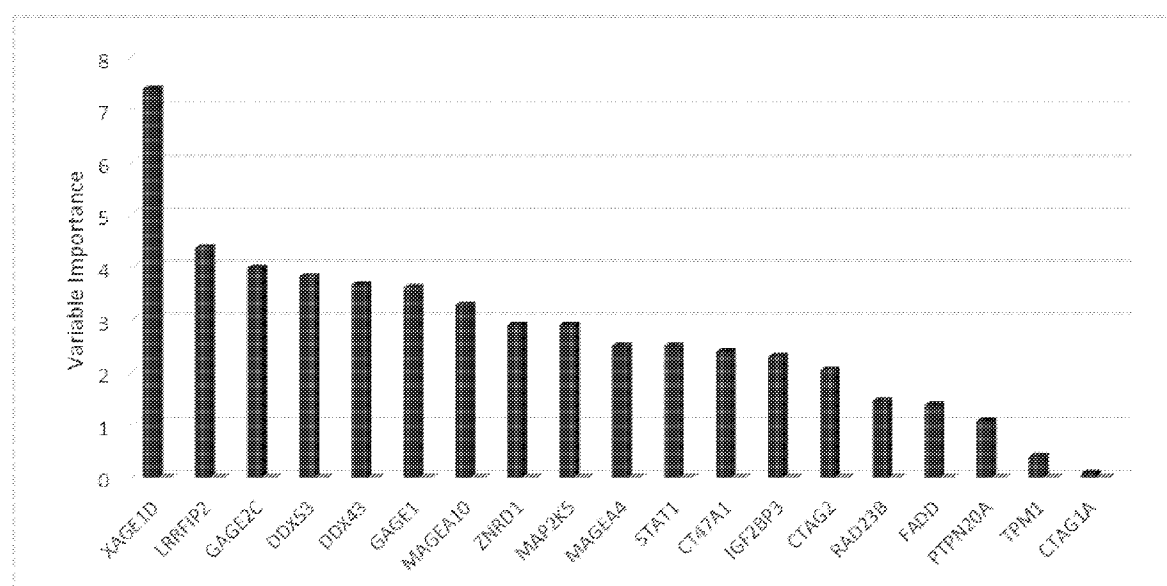
(57) **Abstract:** A method for diagnosing Non-Small Cell Lung Cancer (NSCLC) from a sample extracted from a subject by testing the sample for the presence of biomarkers, the biomarkers being autoantibodies against XAGE1D, LRRFIP2 and GAGE2C. Also claimed are a method of manufacturing a kit, and compositions comprising a panel of said antigens or exosomal autoantibodies.

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KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
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SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
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DETECTION OF BIOMARKERS FOR NON-SMALL CELL LUNG CANCER

Field of Invention

The invention relates to the detection of biomarkers for Non-Small Cell Lung Cancer
(NSCLC).

Background

Despite technological advances in the area of proteomics research, there are only a
handful of biomarkers that have entered the clinic, and 90% of the biomarkers are protein
biomarkers. Autoantibody biomarkers as described herein are autoantibodies to antigens,
autoantibodies being antibodies which are produced by an individual which are directed
against one or more of the individual's own proteins ('self antigens). Some of the main
reasons for failure of biomarkers to make it into clinical practice are:

  1) Low sensitivity and specificity of diagnosis of cancerous diseases

  2) Low prognostic/predictive value

  3) Not important for clinical decision making

  4) Original claims fail validation (false discoveries)

For Non-small cell Lung Cancer (NSCLC), although many individual proteins have been
reported to aid diagnosis and prognosis, very few have demonstrated sufficient value to
be introduced into clinical use. Furthermore, many protein biomarkers discovered in the
serum/plasma samples seem to overlap with other diseases, especially other cancers and
inflammatory diseases.

An aim of the invention therefore is to provide an improved panel of autoantibody
biomarkers for the detection of Non-Small Cell Lung Cancer.

Summary of Invention

In one aspect of the invention, there is provided a method for diagnosing Non-Small Cell
Lung Cancer from a sample extracted from a subject, comprising the steps of:

  (i) testing the sample for the presence of autoantibody biomarkers specific for
       Non-Small Cell Lung Cancer;

(ii)   determining whether the subject has Non-Small Cell Lung Cancer based on the detection of said autoantibody biomarkers;

characterised in that the biomarkers are autoantibodies to antigens comprising XAGE1D, LRRFIP2 and GAGE2C.

Advantageously the autoantibody biomarkers can be used in the diagnosis of non-small cell lung cancer.

In one embodiment the sample is tested using a panel of antigens that correspond to the autoantibody biomarkers. Typically the antigens are biotinylated proteins. Advantageously the biotinylation ensures that the antigens are folded in their correct form to ensure accuracy of detection by the autoantibody biomarkers.

In one embodiment the antigens further comprise one or more of DDX53, DDX43, GAGE1, MAGEA10, ZNRD1, MAP2K5, MAGEA4, STAT1, CT47A1, IGF2BP3, CTAG2, RAD23B, FADD, PTPN20A, TPM1, CTAG1A.

It should be noted that not all antigens generate an autoantibody response and it is not possible to predict *a priori* which antigens will do so in a given cancer patient cohort - of more than 1600 antigens tested, only autoantibodies against the 19 antigens described above are suitable as biomarkers in NSCLC. Advantageously some of the 19 antigens are recognised by autoantibody biomarkers even when the well-known EGFR test for NSCLC is negative.

In one embodiment each biotinylated protein is formed from a Biotin Carboxyl Carrier Protein (BCCP) folding marker which is fused in-frame with the protein.

In one embodiment the biotinylated proteins are bound to a streptavidin-coated substrate.

Advantageously full-length proteins are expressed as fusions to the BCCP folding marker which itself becomes biotinylated *in vivo* when the fusion partner is correctly folded. By comparison misfolded fusion partners cause the BCCP to remain in the 'apo' (i.e. non-biotinylated) form such that it cannot attach to a streptavidin substrate. Thus only

correctly folded fusion proteins become attached to the streptavidin substrate via the biotin moiety appended to the BCCP tag.

In one embodiment the substrate comprises a glass slide, biochip, strip, slide, bead, microtitre plate well, surface plasmon resonance support, microfluidic device, thin film polymer base layer, hydrogel-forming polymer base layer, or any other device or technology suitable for detection of antibody-antigen binding.

In one embodiment the substrate is exposed to a sample extracted from a person, such that autoantibody biomarkers from the sample may bind to the antigens.

Typically the sample comprises any or any combination of exosomes, blood, serum, plasma, urine, saliva, amniotic fluid, cerebrospinal fluid, breast milk, semen or bile.

Advantageously as exosomes contain membrane-bound proteins that reflect their originating cell, and in cancer have been shown to be implicated in the crosstalk between tumour cells and normal cells thereby facilitating the malignant process, exosomes have been found to be promising as enriched sources of diagnostic and prognostic markers. The exosomal autoantibody biomarkers detected using the BCCP folding marker technology are therefore potentially superior compared to the majority of serological biomarkers identified using conventional approaches.

In one embodiment following exposure to the sample, the substrate is exposed to a fluorescently-tagged secondary antibody to allow the amount of any autoantibodies from the sample bound to the antigens on the panel to be determined. Typically the secondary antibody is anti-human IgG, but it will be appreciated that other secondary antibodies could be used, such as anti-IgM, anti-IgGl, anti-IgG2, anti-IgG3, anti-IgG4 or anti-IgA.

In one embodiment the presence of non-small cell lung cancer corresponds to the relative or absolute amount of autoantibodies from the sample specifically binding to the antigens.

In one embodiment the method is performed *in vitro.*

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In a further aspect of the invention, there is provided a method for manufacturing a kit for diagnosing Non-Small Cell Lung Cancer from a sample extracted from a subject, comprising the steps of:

 for each antigen in a panel, cloning a biotin carboxyl carrier protein folding marker in-frame with a gene encoding the antigen and expressing the resulting biotinylated antigen;

 binding the biotinylated antigens to addressable locations on one or more streptavidin-coated substrates, thereby forming an antigen array;

 such that the amount of autoantibodies from the sample binding to the antigens on the panel can be determined by exposing the substrate to the sample and measuring the response;

 characterised in that the antigens comprise XAGE1D, LRRFIP2 and GAGE2C.

In one embodiment the antigens further comprise one or more of DDX53, DDX43, GAGE1, MAGEA10, ZNRD1, MAP2K5, MAGEA4, STAT1, CT47A1, IGF2BP3, CTAG2, RAD23B, FADD, PTPN20A, TPM1, CTAG1A.

In one embodiment the method comprises detecting upregulation/downregulation of one or more autoantibody biomarkers. Thus the method can be used for monitoring the response of a subject undergoing chemo/targeted/immuno-therapy for lung cancer and stratifying the subjects based on their autoantibody profile.

In a further aspect of the invention there is provided a method for detecting non-small cell lung cancer by exposing a composition comprising a panel of antigens as herein described to a sample extracted from a person, and determining the level of autoantibodies from the sample binding to the antigens.

In a yet further aspect of the invention there is provided a method for diagnosing non-small cell lung cancer by exposing a composition comprising a panel of antigens as herein described to a sample extracted from a person *in vitro,* and determining the level of autoantibodies from the sample binding to the antigens.

In further aspect of the invention, there is provided a composition comprising a panel of antigens for detecting non-small cell lung cancer, characterised in that the antigens comprise XAGE1D, LRRFIP2 and GAGE2C

5   In one embodiment the antigens further comprise one or more of DDX53, DDX43, GAGE1, MAGEA10, ZNRD1, MAP2K5, MAGEA4, STAT1, CT47A1, IGF2BP3, CTAG2, RAD23B, FADD, PTPN20A, TPM1, CTAG1A.

In one embodiment the antigens are biotinylated proteins

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In one embodiment the amount of one or more exosomal autoantibody biomarkers binding *in vitro* to the antigens in a sample from a patient can be measured to determine the presence of non-small cell lung cancer.

15   In yet further aspect of the invention, there is provided a composition comprising a panel of exosomal autoantibody biomarkers for detecting non-small cell lung cancer;
        wherein the levels of exosomal autoantibody biomarkers are measured in a sample from a NSCLC patient;
        characterised in that the exosomal autoantibody biomarkers are selected from
20   autoantibodies specific for at least X Antigen Family Member ID (XAGE1D), LRR Binding FLII Interacting Protein 2 (LRRFIP2) and G Antigen 2C (GAGE2C).

    Brief Description of Drawings
25   It will be convenient to further describe the present invention with respect to the accompanying drawings that illustrate possible arrangements of the invention. Other arrangements of the invention are possible, and consequently the particularity of the accompanying drawings is not to be understood as superseding the generality of the preceding description of the invention.

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Figure 1 illustrates the structure of the *E. coli* Biotin Carboxyl Carrier Protein domain.

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Figure 2 is a graph illustrating variable importance scores across 19 biomarkers identified from the NSCLC study.

Figure 3 illustrates ROC curves for 19 biomarkers.

Figure 4 is a graph illustrating levels of the autoantibody biomarkers at different stages of NSCLC.

Figure 5 illustrates a comparison of the levels of the core biomarker panel (XAGE1D, LRRFIP2 and GAGE2C) with respect to EGFR status in patients.

Figure 6 illustrates the overall autoantibody profiles of the core set of biomarkers in three different stages of NSCLC.

Figure 7 illustrates the distinct molecular signatures for the 19 shortlisted biomarkers for NSCLC patients.

Figure 8 illustrates the pPR09 plasmid used as a vector.

Figure 9 illustrates the ROC curve for the 19 shortlisted biomarkers for NSCLC (XAGE1D, DDX53, GAGE2C, LRRFIP2, GAGE1, DDX43, MAGEA10, ZNRD1, STAT1, MAP2K5, MAGEA4, IGF2BP3, FADD, RAD23B, CT47A1, CTAG2, PTPN20A, TPM1, CTAG1A).

Figure 10 illustrates the ROC curves for the best panel of 7 biomarkers in the validation study.

Figure 11 illustrates the ROC curves for the 19 biomarkers in the validation study.

Detailed Description

Materials and Methods

Gene synthesis and cloning. The pPR09 plasmid (see Figure 8 below) was constructed by standard techniques and consists of a c-myc tag and BCCP protein domain, preceded by a multi-cloning site. A synthetic gene insert was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into pPR09 using Spel and Ncol cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%. 5μg of the plasmid preparation was lyophilized for storage.

The recombinant baculoviruses are generated via co-transfection of a bacmid carrying the strong viral polyhedrin promoter together with a transfer vector carrying the coding sequences of protein of interest, into the Sf9 cell line which is a clonal isolate derived from the parental *Spodoptera frugiperda* cell line IPLB-Sf-21-AE. Homologous recombination initiated by the viral system causes the transfected cells to show signs of viral cytopathic effect (CPE) within few days of culture incubation. The most common CPE observed was the significantly enlargement of average cell size, a consequences of viral progeny propagation. These baculoviruses known as P0 were then released into the culture medium, and viral amplification were done to generate a higher titre of P I viruses.

Protein Expression. Expressions were carried out in 24 well blocks using 3ml cultures containing 6×10^6 Sf9 cells per well. High titre, low passage, viral stocks of recombinant baculovirus ($>10^7$ pfu/ml) were used to infect sf9 insect cells. The infected cells were then cultured for 72 hours to allow them to produce the recombinant protein of interest. The cells were washed with PBS, resuspended in buffer, and were frozen in aliquots at -80°C ready for lysis as required. Depending on the transfer vector construct and the nature of the protein itself, recombinant protein lysate can be pelleted either from the cultured cell or the cultured medium. Positive recombinant proteins were then analyzed via SDS-PAGE and Western blot against Streptavidin-HRP antibody. In total, 1630 human antigens were cloned and expressed using this methodology.

Array fabrication. HS (hydrogel-streptavidin) slides were purchased from Schott and used to print the biotinylated proteins. A total of 9 nanoliters of crude protein lysate was printed on a HS slide in quadruplicate using non-contact piezo printing technology. Print buffer that have a pH between 7.0 and 7.5 were used. The slides were dried by centrifugation (200 x g for 5 min) before starting the washing and blocking. The printed arrays were blocked with solutions containing BSA or casein (concentration: 0.1 mg/ml) in a phosphate buffer. The pH was adjusted to be between 7.0 and 7.5 and cold solutions were used (4 °C - 20 °C). Slides were not allowed to dry between washes, and were protected from light. In total, each resultant 'Immunome array' comprised 1630 antigens, each printed in quadruplicate.

Experimental Procedure. Each critical experimental step of running the Immunome array required a second trained person to thoroughly check, precisely record and cross-check all steps in the protocol, in order to reduce operator bias. Samples were picked, randomised and assigned to assay racks accordingly. These samples were then stored at -20°C until the experimental setup was complete.

1. Study cohort

A cohort comprising of 209 participants between the age of 29 and 85 was recruited for the study. The subjects were selected across more than 6 ethnicities diagnosed with different types of lung cancer including adenocarcinoma, squamous cell carcinoma, non-small cell lung carcinoma, large cell carcinoma and other types of lung malignancies. A total of 31 patients were diagnosed with early stage lung cancer while 78 patients were diagnosed with late stage lung cancer. A total of 33 subjects were smoker and 44 subjects were non-smoker. EGFR was tested positive in 30 subjects and negative in 39 subjects. A total of 100 samples from age and gender-matched healthy subjects were also collected.

2. Sample preparation

A total of 209 plasma samples were collected from the above cohort and exosomes were isolated from each sample using an Invitrogen Total Exosome Isolation kit (Thermo Fisher cat. no. 4484450). Exosomal preparations were frozen at -20°C until use.

Exosome samples were placed in a shaking incubator set at +20°C and allowed to thaw for 30 minutes. When completely thawed, each sample was vortexed vigorously three times at full speed and spun down for 3 minutes at 13,000 rpm using a microcentrifuge. 22.5 μL of the sample was pipetted into 4.5 mL of Serum Assay Buffer (SAB) containing 0.1% v/v Triton, 0.1% w/v BSA in PBS (20°C) and vortexed to mix three times. The tube was tilted during aspiration to ensure that the plasma was sampled from below the lipid layer at the top but does not touch the bottom of the tube in case of presence of any sediment. This exosome dilution process was carried out in a class II biological safety cabinet. Batch records were marked accordingly to ensure that the correct samples were added to the correct tubes.

Other types of samples, such as serum, plasma, blood, urine, saliva, amniotic fluid, cerebrospinal fluid, breast milk, semen or bile were diluted as per the above protocol before assay.

3. Biomarker Assay

Each protein microarray was removed from the storage buffer using forceps, placed in the slide box and rack containing 200 mL cold SAB and shaken on an orbital shaker at 50 rpm, for 5 minutes. After washing, each protein microarray was placed, array side up, in a slide hybridization chamber with individual plasma which had been diluted earlier. All slides were scanned using the barcode scanner into the relevant batch record and incubated on a horizontal shaker at 50 rpm for 2 hours at 20°C.

4. Array Washing After Plasma Binding

The protein microarray slide was then rinsed twice in individual "Pap jars" with 30 mL SAB, followed by 200 mL of SAB buffer in the slide staining box for 20 minutes on the shaker at 50 rpm at room temperature. All slides were transferred sequentially and in the same orientation.

5. Incubation with Cy3-anti IgG

Binding of autoantibodies to the arrayed antigens on replica Immunome arrays was detected by incubation with Cy3-rabbit anti-human IgG (Dako Cytomation) labelled according to the manufacturer's recommended protocols (GE Healthcare). Arrays were

immersed in hybridization solution containing a mixture of Cy3- rabbit antihuman IgG solution (diluted 1:1000 in SAB buffer) and shaken for 2 hours at 50 rpm at 20°C.

6. Washing After Incubation with Cy3-anti IgG

After incubation, each slide was washed in 200 mL of SAB buffer, 3 times for 5 minutes, with shaking at 50 rpm at room temperature. Excess buffer was removed by immersing the slide in 200 mL of pure water for a few minutes. Slides were then dried for 2 min at 240g at room temperature. Slides were then stored at room temperature until scanning (preferably the same day). Hybridization signals were measured with a microarray laser scanner (Agilent Scanner) at 10μm resolution. Fluorescence intensities were detected according to the manufacturer's instructions, whereby each spot is plotted using Agilent Feature Extraction software.

Spot segmentation Semi-automatic QC process was carried out in order to produce a viable result. The output from the microarray scanner is a raw .tiff format image file. Extraction and quantification of each spot on the array were performed using the GenePix Pro 7 software (Molecular Devices). A GAL (GenePix Array List) file for the array was generated to aid with image analysis. GenePix Pro 7 allows for automatic spot gridding and alignment of each spot on the array for data extraction. Following data extraction, a GenePix Results (.GPR) file was generated for each slide which contains numerical information for each spot; Protein ID, protein name, foreground intensities, background intensities etc.

Bioinformatics analysis.

Image Analysis: Raw Data Extraction
The aim of an image analysis is to evaluate the amount of autoantibody present in the plasma sample by measuring the median intensities of all the pixels within each probed spot. A raw .tiff format image file is generated for each slide, i.e. each sample. Automatic extraction and quantification of each spot on the array are performed using the GenePix Pro 7 software *(Molecular Devices)* which outputs the statistics for each probed spot on the array. This includes the mean and median of the pixel intensities within a spot along with its local background. A GAL (GenePix Array List) file for the array is generated to

aid with image analysis. This file contains the information of all probed spots and their positions on the array. Following data extraction, a GenePix Results (.GPR) file is generated for each slide which contains the information for each spot; Protein ID, protein name, foreground intensities, background intensities etc. In the data sheet generated from the experiment, both foreground and background intensities of each spot are represented in relative fluorescence units (RFUs).

Data Handling and Pre-processing

For each slide, proteins and control probes are spotted in quadruplicate - 4 arrays on each slide. The following steps were performed to verify the quality of the protein array data before proceeding with data analysis:

Step 1:

Calculate net intensities for each spot by subtracting background signal intensities from the foreground signal intensities of each spot. For each spot, the background signal intensity was calculated using a circular region with three times the diameter of the spot, centered on the spot.

Step 2:

Remove replica spots with RFU ≤ 0.

Step 3:

Zero net intensities if only 1 replica spot remaining.

Step 4:

Calculating percentage of coefficient of variant (CV%) of to determine the variations between the replica spots on each slide.

$$CV\% = \frac{S.D.}{Mean} \times 100\%$$ **Equation 1**

Flag a set of replica spots with only 2 or less replica/s remaining and CV% > 20% as "High CV". The mean RFU of these replica spots (i.e. proteins) will be excluded from the downstream analysis.

5 For proteins/controls with a CV% > 20% and with 3 or more replica spots remaining, the replica spots which result in this high CV% value were filtered out. This was done by calculating the standard deviation between the median value of the net intensities and individual net intensities for each set of replica spots. The spot with the highest standard deviation was removed. CV% values were re-calculated and the process repeated.

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Step 5:

Calculating the mean of the net intensities for the remaining replica spots.

Step 6:

15 Inspecting signal intensities of two positive controls: IgG and Cy3-BSA.

Step 7:

Composite normalisation of data using both quantile-based and total intensity-based modules. This method assumes that different samples share a common underlying

20 distribution of their control probes while taking into account the potential existence of flagged spots within them. The Immunome array uses Cy3-labelled biotinylated BSA (Cy3-BSA) replicates as the positive control spots across slides. Hence it is considered as a housekeeping probe for normalisation of signal intensities for any given study.

25 The quantile module adopts the algorithm described by Bolstad *et al.*, 2003. This reorganisation enables the detection and handling of outliers or flagged spots in any of the Cy3BSA control probes. A total intensity-based module was then implemented to obtain a scaling factor for each sample. This method assumes that post-normalisation, the positive controls should have a common total intensity value across all samples. This

30 composite method aims to normalise the protein array data from variations in their measurements whilst preserving the targeted biological activity across samples. The steps are as follows:

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Quantile-Based Normalisation of all cy3BSA across all samples

(i = *spot number and j = sample number*)

1. Load all Cy3-BSA across all samples, *j*, into an *i* X*j* matrix X
2. Sort spot intensities in each column *j* of X to get X_{sort}
3. Take the mean across each row *i* of X_{sort} to get $< Xi >$

Intensity-Based Normalisation

1. Calculate sum of the mean across each row i, $\sum < Xi >$
2. For each sample, *k*, calculate the sum of all Cy3-BSA controls, $\sum Xk$
3. For each sample, *k*,

$$\text{Scaling factor (k)} = \frac{\sum < Xi >}{\sum Xk} \qquad \textbf{Equation 2}$$

Data Analysis

High concentrations of an arrayed protein may occasionally give a "false" positive signal in serology assays because of concentration-driven, non-selective binding of an immunoglobulin to the target. This can arise theoretically due to an avidity effect: weak, non-specific immunoglobulin binding sites on a specific protein becoming coupled across multiple neighbouring protein molecules via an antibody as a result of the high density of immobilized protein, thus making the protein appear to be highly antigenic. Whenever this phenomenon occurs, it would be expected to be observed in the healthy control samples and will give rise to high intensity signals and/or signals that are close to saturation on the arrays. In Sengenics Immunome, proteins such as RBPJ and IGHG1 show consistently high signal intensities across all samples.

For this reason, given a large sample number (i.e. 100 - 200 samples) and availability of sample cohort, a penetrance-based fold change (pFC) analysis method is implemented for the identification of highly expressed proteins in each case sample. This method will remove any false positive signals from the data by setting a protein-specific threshold (i.e. background threshold). This defined per-protein background threshold is calculated based on the signal intensities for each specific protein measured for a given cohort of healthy control samples. A step-by-step description of this method is as follows:

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Step 1:

Individual fold changes for both case and control are calculated by dividing the RFU value for each protein in each sample, H, by the mean of the RFU values of each protein across all the control samples (i.e. background threshold).

$$\text{Individual FC} = \frac{\text{H}_{\text{Case or Control}}}{\mu(\text{H}_{\text{Control}})} \qquad \textbf{Equation 3}$$

Step 2:

For proteins with individual fold change of less than 2 fold above the background threshold, their signal intensities (RFU) are replaced with zeroes.

Step 3:

Penetrance frequency (number of case and control samples with individual fold changes ≥ 2 fold) for both case ($Frequency_{Case}$) and control ($Frequency_{Control}$) are determined for each protein along with their difference.

$$\text{Frequency}_{\text{Case}} = \text{n}(\text{Individual FC (Case)}) \geq 2) \qquad \textbf{Equation 4}$$

$$\text{Frequency}_{\text{Control}} = \text{n}(\text{Individual FC (Control)}) \geq 2) \qquad \textbf{Equation 5}$$

$$\text{Frequency}_{diff} = \text{Frequency}_{\text{Case}} - \text{Frequency}_{\text{Control}} \qquad \textbf{Equation 6}$$

Step 4:

Penetrance Fold Changes for both case and control groups are calculated for each protein.

$$Penetrance\ Fold\ Change_{case} = \frac{\mu(H_{Case}[i])}{\mu(\text{H}_{\text{Control}})} \qquad \textbf{Equation 7}$$

$$Penetrance\ Fold\ Change_{control} = \frac{\mu(H_{Control}[i])}{\mu(\text{H}_{\text{Control}})} \qquad \textbf{Equation 8}$$

$$H_{Case}[i] = H_{Case}\ with\ FC\ Case\ \geq 2\ fold$$

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$$H_{Control}[i] = H_{Control} \; with \; FC \; Control \; \geq 2 \; fold$$

Putative biomarkers are identified and ranked according to the following criteria:

1. *Penetrance Fold Change $_{Case}$* $\geqq 2$.

2. % Frequency $_{Case}$ $\geq 10\%$

% Frequency differential $\geqq 10\%$

The invention utilises the Biotin Carboxyl Carrier Protein (BCCP) folding marker which is cloned in-frame with the gene encoding the protein of interest, as described above and in EP1470229. The structure of the *E. coli* BCCP domain is illustrated in Figure 1, wherein residues 77-156 are drawn (coordinate file lbdo) showing the N- and C- termini and the single biotin moiety that is attached to lysine 122 *in vivo* by biotin ligase.

BCCP acts not only as a protein folding marker but also as a protein solubility enhancer. BCCP can be fused to either the N- or C-terminal of a protein of interest. Full-length proteins are expressed as fusions to the BCCP folding marker which becomes biotinylated *in vivo*, but only when the protein is correctly folded. Conversely, misfolded proteins drive the misfolding of BCCP such that it is unable to become biotinylated by host biotin ligases. Hence, misfolded proteins are unable to specifically attach to a streptavidin-coated solid support. Therefore only correctly folded proteins become attached to a solid support via the BCCP tag.

The surface chemistry of the support is designed carefully and may use a three-dimensional thin film polymer base layer (polyethylene glycol; PEG), which retains protein spot morphologies and ensures consistent spot sizes across the array. The PEG layer inhibits non-specific binding, therefore reducing the high background observed using other platforms. The solid support used to immobilize the selected biomarkers is thus designed to resist non-specific macromolecule adsorption and give excellent signal-to-noise ratios and low limits of detection (i.e. improved sensitivity) by minimising non-specific background binding. In addition the PEG layer also preserves the folded structure and functionality of arrayed proteins and protein complexes post-immobilisation. This is critical for the accurate diagnosis because human serum antibodies are known in general

to bind non-specifically to exposed hydrophobic surfaces on unfolded proteins, thus giving rise to false positives in serological assays on arrays of unfolded proteins, moreover, human autoantibodies typically bind to discontinuous epitopes, so serological assays on arrays of unfolded proteins or mis-folded proteins will also give rise to false negatives in autoantibody binding assays.

As biotinylated proteins bound to a streptavidin-coated surface show negligible dissociation, this interaction therefore provides a superior means for tethering proteins to a planar surface and is ideal for applications such as protein arrays, SPR and bead-based assays. The use of a compact, folded, biotinylated, 80 residue domain BCCP affords two significant advantages over for example the AviTag and intein-based tag. First, the BCCP domain is cross-recognised by eukaryotic biotin ligases enabling it to be biotinylated efficiently in yeast, insect, and mammalian cells without the need to co-express the *E. coli* biotin ligase. Second, the N- and C-termini of BCCP are physically separated from the site of biotinylation by 50A (as shown in Figure 1), so the BCCP domain can be thought of as a stalk which presents the recombinant proteins away from the solid support surface, thus minimising any deleterious effects due to immobilisation.

The success rate of BCCP folding marker mediated expression of even the most complex proteins is in excess of 98%. The technology can therefore be applied in a highly parallelised pipeline resulting in high-throughput, highly consistent production of functionally validated proteins..

The addition of BCCP permits the monitoring of fusion protein folding by measuring the extent of *in vivo* biotinylation. This can be measured by standard blotting procedures, using SDS-PAGE or *in situ* colony lysis and transfer of samples to a membrane, followed by detection of biotinylated proteins using a streptavidin conjugate such as streptavidin-horseradish peroxidase. Additionally, the fact that the BCCP domain is biotinylated *in vivo* is particularly useful when multiplexing protein purification for fabrication of protein arrays since the proteins can be simultaneously purified from cellular lysates and immobilised in a single step via the high affinity and specificity exhibited by a streptavidin surface.

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The biomarkers of the present invention can be used in early diagnosis of NSCLC, patient stratification and treatment monitoring. This includes any distinguishable manifestation of the condition, including not having NSCLC. The test can determine the presence or absence of NSCLC in a patient, the risk of developing NSCLC, the stage or severity of NSCLC and the effectiveness or response to treatment of NSCLC. Based on this status, further medical procedures may be indicated, including additional diagnostic tests or therapeutic procedures or regimens.

The microarray prototype and final product can be multiplexed far beyond the technical capability of other immunoassay systems and will enable exquisitely sensitive and specific testing of patients and high-risk population for NSCLC.

The power of a diagnostic test to correctly predict status is commonly measured as the sensitivity and specificity of the assay or the area under a receiver operated characteristic ("ROC") curve. Sensitivity is the percentage of true positives that are correctly predicted to be positive, while specificity is the percentage of true negatives that are correctly predicted to be negative. The greater the area under the ROC curve, the higher the prediction power of the test.

Autoantibody biomarkers were determined by Penetrance fold change method where age matched controls are considered as baseline to observe the elevated frequencies (≥ 2 Foldchange) of individual biomarkers in lung cancer patients. The list of the biomarkers identified here for diagnosis of NSCLC is shown in Table 1.

Table 1

Protein	Penetrance Frequency (NSCLC)	Penetrance Frequency % (NSCLC)	Mean Penetrance (NSCLC)	Penetrance Fold Change (NSCLC)	Penetrance Frequency (Control)	Penetrance Frequency % (Control)	Mean Penetrance (Control)	Penetrance Fold Change (Control)	Frequency Differential	Frequency % Differential	Penetrance Fold Change Difference	Mean (Control)
XAGE1D	26	23.85321	24654.94	9.678011	4	4	10338.27	4.058168	22	19.85321	5.619843	2547.522
PTPN20A	17	15.59633	12505.05	3.883074	4	4	12011.96	3.729957	13	11.59633	0.153117	3220.401
TPM1	15	13.76147	16940.47	2.845931	6	6	25399.96	4.267092	9	7.761468	-1.42116	5952.523
CTAG1A	14	12.84404	23341.53	10.43956	4	4	10141.22	4.535687	10	8.844037	5.903878	2235.872
RAD23B	14	12.84404	8193.595	3.231442	5	5	9467.406	3.733816	9	7.844037	-0.50237	2535.585
ZNRD1	14	12.84404	12647.16	2.988961	4	4	12658.28	2.991589	10	8.844037	-0.00263	4231.29
CTAG2	13	11.92661	20739.05	8.484654	5	5	10221.57	4.181798	8	6.926606	4.302857	2444.301
LRRFIP2	13	11.92661	16271.97	3.374414	9	9	11729.15	2.432342	4	2.926606	0.942072	4822.161
MAGEA10	13	11.92661	7393.198	3.156872	5	5	7423.496	3.169809	8	6.926606	-0.01294	2341.938
STAT1	13	11.92661	21774.47	5.037478	9	9	11512.56	2.663407	4	2.926606	2.374071	4322.495
DDX43	12	11.00917	10360.57	4.337159	2	2	8368.541	3.503251	10	9.009174	0.833908	2388.793
GAGE1	12	11.00917	12540.95	5.515388	2	2	7578.361	3.33289	10	9.009174	2.182498	2273.81
GAGE2C	12	11.00917	11572.43	5.342098	3	3	7604.188	3.510266	9	8.009174	1.831832	2166.271
MAGEA4	12	11.00917	9790.552	4.88818	3	3	7559.253	3.774148	9	8.009174	1.114032	2002.903
MAP2K5	11	10.09174	10511.43	3.676394	2	2	6913.192	2.417902	9	8.091743	1.258492	2859.17
FADD	10	9.174312	18237.53	5.423004	6	6	11120.35	3.306683	4	3.174312	2.116321	3362.994
IGF2BP3	9	8.256881	14223.89	6.351528	2	2	6903.872	3.082851	7	6.256881	3.268677	2239.444
CT47A1	8	7.33945	15453.54	6.885753	6	6	9374.437	4.177039	2	1.33945	2.708714	2244.278
DDX53	7	6.422018	30847.36	11.16542	4	4	17135.53	6.202328	3	2.422018	4.963095	2762.758

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To evaluate the sensitivity of individual biomarkers towards lung cancer, ROC and area under the curve (AUC), 95% confidence intervals and also likelihood ratios were calculated, as set out in Table 2. Variable ranking was performed by using all combination of 19 biomarkers as separate panels and each panel was subjected to recursive feature elimination by generating random forests. The biomarkers were ranked based on random forest estimated variable importance measure derived from each panel (see Figure 2). Mean variable importance scores determine three core set of biomarkers which are common across all biomarker panels. i.e. XAGE1D, LRRFTP2 and GAGE2C.

Table 2

Protein	AUC	Confidence Interval (95% CI)	Likelihood Ratio		Variable Importance	Rank based on Importance
			LR-Positive	LR-Negative		
XAGE1D	0.696	[0.622-0.77]	7.75	0.593	7.39	1
LRRFIP2	0.56	[0.48-0.641]	3.068	0.735	4.37	2
GAGE2C	0.612	[0.534-0.691]	13.321	0.713	3.98	3
DDX53	0.635	[0.557-0.714]	3.229	0.614	3.81	4
DDX43	0.641	[0.564-0.719]	2.601	0.588	3.67	5
GAGE1	0.638	[0.562-0.715]	4.239	0.676	3.61	6
MAGEA10	0.651	[0.574-0.728]	3.817	0.598	3.28	7
ZNRD1	0.573	[0.493-0.653]	1.462	0.665	2.9	8
MAP2K5	0.65	[0.573-0.726]	4.037	0.654	2.9	9
MAGEA4	0.654	[0.577-0.73]	3.176	0.553	2.5	10
STAT1	0.554	[0.472-0.635]	2.745	0.776	2.5	11
CT47A1	0.623	[0.544-0.702]	2.382	0.594	2.39	12
IGF2BP3	0.621	[0.542-0.699]	2.295	0.644	2.3	13
CTAG2	0.619	[0.54-0.698]	2.637	0.664	2.04	14
RAD23B	0.59	[0.51-0.67]	2.758	0.722	1.45	15
FADD	0.602	[0.522-0.681]	3.633	0.737	1.38	16
PTPN20A	0.605	[0.525-0.684]	2.327	0.682	1.08	17
TPM1	0.607	[0.527-0.686]	2.22	0.641	0.4	18
CTAG1A	0.625	[0.547-0.703]	2.653	0.687	0.08	19

A total of 19 potential autoantibody biomarkers have been identified for diagnosis of NSCLC; namely XAGE1D, PTPN20A, TPM1, CTAG1A, RAD23B, ZNRDl, LRRFIP2, STAT1, MAGEA10, CTAG2, GAGE1, GAGE2C, DDX43, MAGEA4, MAP2K5,

FADD, IGF2BP3, CT47A1, DDX53. The Uniprot IDs, description, nucleotide sequence and protein sequence are set out in Table 5 below.

The Immunome array contains >1630 antigens, presented on the array surface in a folded, functional form, as described above. Notably, it would not have been obvious *a priori* which specific 19 antigens out of the collection of >1630 antigens that were tested would give rise to a measurable autoantibody response that is diagnostic for NSCLC.

In this panel of 19 antigens that correspond to the 19 autoantibody biomarkers, CTAG2 is observed in 25-50% of tumor samples of melanomas, non-small-cell lung carcinomas, bladder, prostate and head and neck cancers. CTAG1A is a tumor cell antigen found in various types of cancers, which makes it a good candidate for a cancer vaccine.

ZNRD1 contains two potential zinc-binding motifs and may play a role in regulation of cell proliferation. The encoded protein may be involved in cancer and human immunodeficiency virus progression.

XAGE1D and MAGEA4 RNA markers have been considered for use in screening of lung neoplasia for detecting presence of lung cancer. In normal tissues, XAGE1D is highly expressed in testis, highly expressed in breast cancer, prostate cancer and many types of lung cancers, including squamous cell carcinoma, small cell carcinoma, non-small cell carcinoma, and adenocarcinoma, as well as in Ewings cell lines, in some Ewings sarcoma patient samples, and in one of one alveolar rhabdomyosarcoma patient sample. MAGEA4 is expressed in many tumors of several types, such as melanoma, head and neck squamous cell carcinoma, lung carcinoma and breast carcinoma, but not in normal tissues except for testes and placenta.

LRRFIP2 is involved in the Wnt signalling pathway and aberrant Wnt signalling underlies a wide range of pathologies in humans. It has been suggested that the Wnt signalling pathway has important functions in stem cell biology, cardiac development and differentiation, angiogenesis, cardiac hypertrophy, cardiac failure and ageing (Rao & Kuhl, 2010). GAGE2C belongs to a family of genes that are expressed in a variety of tumors but not in normal tissues, except for the testis.

PTPN20A is present in many cell lines (at protein level) and is widely expressed. TPM1 is detected in primary breast cancer tissues but undetectable in normal breast tissues in Sudanese patients. Isoform 1 is expressed in adult and fetal skeletal muscle and cardiac tissues, with higher expression levels in the cardiac tissues. Isoform 10 is expressed in adult and fetal cardiac tissues, but not in skeletal muscle.

DDX53, STAT1 and FADD expression levels were elevated in late stage group. DDX53 is a cancer-testis antigen that shows wide expression in many tumours. DDX53 has been reported to interact with EGFR and bind to the promoter sequences of EGFR. Signal transducer and activator of transcription (STAT) 1 is part of the (JAK)/STAT signalling cascade and is best known for its essential role in mediating responses to all types of interferons (IFN). A correlation of STAT1 protein expression levels with poor prognosis, increased invasive and metastatic potential has been reported in three breast cancer studies (Meissl *et al.*, 2017). It was concluded that STAT1 can promote tumour progression, and therefore, it can be a potential marker or indicator of cancer progression (Meissl *et al.*, 2017). Phosphorylation of FADD promotes KRAS induced lung cancer (Bowman *et al.*, 2015). Fas-associated death domain protein (FADD) is the key adaptor molecule transmitting the apoptotic signal delivered by death receptors. It was also reported that the release of FADD by human NSCLC correlates positively with both tumour progression and aggressiveness and could be a new marker of poor prognosis (Cimino *et al.*, 2012).

Cancer-testis antigens are a family of >1000 highly developmentally restricted fetal proteins (Wang *et al.*, 2016; Silva *et al.*, 2017) that are silenced in all somatic tissues except the testes and occasionally placenta, but which can be aberrantly expressed in cancerous tissues, thereby driving an autoantibody response. The Immunome array contains 202 cancer-testis antigens, presented on the array surface in a folded, functional form as described above. Notably, the 19 autoantibody biomarkers of non-small cell lung cancer identified here are significantly enriched for cancer-testis antigens, yet it would not have been obvious *a priori* which specific 10 cancer-testis antigens out of the collection of 202 that were tested would give rise to a measurable autoantibody response that is diagnostic for NSCLC.

The best method to verify a lung cancer diagnosis involves a multiple biomarker approach rather than a single biomarker approach.

5   As illustrated in Figure 3, Receiver operating characteristic (ROC) curves were calculated based on individual fold changes for 19 biomarkers to show sensitivity of biomarkers towards lung cancer patients. Area under curve (AUC), 95% confidence intervals (Cl) and Optimal cutoff of individual fold change (Cutoff (IFC) for each were calculated based on the method described in Lopez-Raton *et al.*, (2014) using "OptimalCutpoints" R

10   package. Cl and Optimal cut-off values help to determine diagnostic ability of the biomarkers by showing positive or negative test results with lung cancer patients. The ROC curve for the panel of 19 biomarkers is illustrated in Figure 9.

Figure 4 shows the autoantibody biomarkers determined by Penetrance fold change (pFC)

15   method where age matched controls are considered as baseline to observe the elevated frequencies (≥ 2 Foldchange) of individual biomarkers in lung cancer patients. The data was generated from profiling of Normalised RFU values of 19 biomarkers (Table 6) identified by pFC method across healthy controls (Ctrl), Early stage lung cancer patients (Early) and Late stage lung cancer patients (Late).

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Figure 5 shows a comparison of the core autoantibody biomarker panel (autoantibodies against antigens XAGE1D, LRRFIP2 and GAGE2C) levels with respect to EGFR status in patients. The data was generated from comparing normalised RFU values of the three core set of biomarkers (identified based on variable ranking using random forest) across

25   healthy controls (Control), Early stage lung cancer patients (Earlystg) and Late stage lung cancer patients (Latestg). Patient cohorts were sub-divided based on EGFR mutation status i.e. Positive (patients with EGFR mutation), negative (patients without EGFR mutation), unknown (patients with unknown EGFR mutation status). An overall elevation of the antigen-specific autoantibody levels was observed in late stage NSCLC compared

30   to early stage NSCLC and control.

A similar observation is seen in Figure 6, wherein the data was generated from comparing normalised RFU values of the three core set of biomarkers (identified based on variable

ranking using random forest) against healthy controls (Ctrl), Early stage lung cancer patients (Early) and Late stage lung cancer patients (Late).

Furthermore, the elevation of antigen-specific autoantibody levels in late stage NSCLC is independent of EGFR status of patients. In addition, with further reference to Figure 7, distinct differences in autoantibodies against XAGE1D, CTAG1A, CTAG2, GAGE1 and GAGE2C were observed in Late and Early stage NSCLC compared to the control. Unsupervised clustering of individual fold changes across all healthy controls (Control), Early stage lung cancer patients (EarlyStg) and Late stage lung cancer patients (LateStg) for 19 biomarkers identified by pFC method. Clustering was performed for biomarkers based on Ward's method and distance calculated based on Euclidean distance. The shaded bar on the top of the heatmap represents patient cohorts i.e. Control, Early stage and Late Stage samples.

Validation study using a Custom Array containing 19 antigens identified from the Phase 1 Study

Protein expression:

Nineteen BCCP-tagged antigens (XAGE1D; CTAG2; CTAG1A; STAT1; DDX53; MAGEA4; IGF2BP3; MAGEA10; LRRFIP2; ZNRD1; PTPN20A; RAD23B; CT47A1; MAP2K5; FADD; GAGE1; DDX43; GAGE2C; & TPM1) identified from the Phase 1 discovery study (see above) were expressed in insect cell cultures as previously described (see above). Cells were harvested and lysed as described above.

Custom array fabrication:

Crude insect cell lysates for each of the 19 BCCP-tagged antigens were aliquoted into separate wells of a source plate and robotically printed on to streptavidin-coated hydrogel slides (Schott HS slides) to form a protein microarray. Each of the 19 antigens were printed in triplicate on one array. Sixteen replica arrays were printed in discrete areas of a 7.5 x 2.5cm HS slide. Following printing, arrays were wash as stored as described above.

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Study cohort:

Plasma samples from an independent cohort of 126 late stage NSCLC patients, 30 early stage NSCLC patients and 83 age-matched healthy controls were used to validate the 19 shortlisted antigens from the Phase 1 study, using the custom array fabricated as described above.

Sample preparation, Data handling and QC:

For each plasma sample, 22.5 μL of the sample was pipetted into 4.5 mL of Serum Assay Buffer (SAB) containing 0.1% v/v Triton X-100, 0.1% w/vBSA in PBS (20°C) and vortexed to mix three times. Diluted plasma were then assayed on custom protein microarrays, essentially as described above. Briefly, each custom protein microarray was removed from storage buffer using forceps, placed in a slide box containing 200 mL cold SAB and shaken on an orbital shaker at 50 rpm, for 5 minutes. The slides were then placed, array side up, in a slide hybridization chamber with individual plasma which had been diluted as above. All slides were scanned using a barcode scanner and incubated on a horizontal shaker at 50 rpm for 2 hours at 20°C. Each protein microarray slide was then rinsed twice with 30 mL SAB, followed by 200 mL of SAB buffer for 20 minutes on the shaker at 50 rpm at room temperature. All slides were transferred sequentially and in the same orientation. Arrays were then immersed in hybridization solution containing Cy3-rabbit anti-human IgG (diluted 1:1000 in SAB buffer) for 2 hours, with shaking at 50 rpm at 20°C.

After incubation, the slide was washed in 200 mL of SAB buffer, 3 times for 5 minutes with shaking at 50 rpm at room temperature. Excess buffer was removed by immersing the slide in 200 mL of pure water for a few minutes. Slides were then dried for 2 min at 240g at room temperature and stored at room temperature until scanning. Hybridization signals were measured with a microarray laser scanner (Agilent Scanner) at 10μm resolution. Fluorescence intensities were detected according to the manufacturer's instructions, whereby each spot is plotted using Agilent Feature Extraction software.

Slide scanning, raw data handling and QC were carried out as described above for the Phase 1 study.

Data analysis:

A Penetrance Fold Change analysis was performed for each of the 19 antigens, comparing NSCLC patients and healthy controls, using the method described for the Phase 1 study data analysis. This demonstrated that all 19 antigens had an individual penetrance frequency > 10% and a penetrance fold change > 2 fold.

The results are summarised below. Table 3 shows the penetrance fold change analysis results for Late Stage NSCLC versus Healthy controls across all 19 antigens from the validation study. Table 4 shows the penetrance fold change analysis results for Early Stage NSCLC versus Healthy controls across all 19 antigens from the validation study

Table 3

Protein	Penetrance Frequency (Late Stage)	Penetrance Frequency % (Late Stage)	Mean Penetrance (Late Stage)	Penetrance Fold Change (Late Stage)	Mean (Healthy Control)
XAGE1D	33	26.19	2103.27	4.32	486.84
CTAG2	26	20.63	1896.52	3.93	482.11
CTAG1A	25	19.84	3649.61	8.28	440.94
STAT1	21	16.67	1219.31	2.70	451.38
DDX53	20	15.87	7340.65	13.67	536.89
MAGEA4	20	15.87	2722.11	6.17	440.83
IGF2BP3	20	15.87	1428.20	3.13	455.58
MAGEA10	20	15.87	1274.75	2.72	468.93
LRRFIP2	20	15.87	1366.38	2.65	516.39
ZNRD1	19	15.08	1440.51	2.96	486.98
PTPN20A	19	15.08	1244.62	2.67	466.38
RAD23B	18	14.29	1211.94	2.88	421.08
CT47A1	18	14.29	1330.74	2.74	486.50
MAP2K5	17	13.49	1227.44	2.86	429.29
FADD	16	12.70	1362.23	2.79	487.64
GAGE1	16	12.70	1741.70	2.75	633.18
DDX43	16	12.70	1274.21	2.70	471.32
GAGE2C	14	11.11	1441.22	2.93	491.95
TPM1	13	10.32	3066.49	2.81	1090.13

Table 4

Protein	Penetrance Frequency (EarlyStage)	Penetrance Frequency % (EarlyStage)	Mean Penetrance (EarlyStage)	Penetrance Fold Change (EarlyStage)	Mean (Control)
RAD23B	8	26.67	992.75	2.36	421.08
GAGE1	5	16.67	1498.35	2.37	633.18
DDX43	5	16.67	1095.95	2.33	471.32
XAGE1D	4	13.33	3219.83	6.61	486.84
LRRFIP2	4	13.33	1343.66	2.60	516.39
MAGEA4	4	13.33	1141.63	2.59	440.83
MAGEA10	4	13.33	1121.54	2.39	468.93
GAGE2C	4	13.33	1160.49	2.36	491.95
CT47A1	4	13.33	1129.55	2.32	486.50
PTPN20A	4	13.33	1075.17	2.31	466.38
MAP2K5	4	13.33	984.14	2.29	429.29
STAT1	4	13.33	1033.02	2.29	451.38
ZNRD1	4	13.33	1109.92	2.28	486.98
TPM1	3	10.00	3102.74	2.85	1090.13
CTAG1A	3	10.00	1088.11	2.47	440.94
IGF2BP3	3	10.00	1123.99	2.47	455.58
CTAG2	3	10.00	1145.82	2.38	482.11
DDX53	2	6.67	1260.99	2.35	536.89
FADD	2	6.67	1095.78	2.25	487.64

The performances of biomarker panels were validated by a Random Forest - Recursive feature elimination (RF-RFE) algorithm which is a backwards selection, iterative process used to select the best subset of biomarkers for the classification of NSCLC. Validation of the selected biomarkers involves using a training and testing set for model generation and performance evaluation before using an independent validation set to validate the final performance of the models.

During model generation, all possible combinations of 19 biomarkers were generated and the individual fold change values based on the biomarker combinations were used as inputs for model generation. Data from the phase I (209 samples) were separated into training (2/3rds) and test (1/3rd) datasets. Training using the RF-RFE was done using default parameters with 5-fold cross validation and panel size being fixed to the number of biomarkers in each model. The generated models were used to predict both the testing and validation sets to evaluate the performance of the panels in the stratification of NSCLC. All recursive feature elimination and Random Forest analyses were performed

using the caret (Kuhn, 2008 (https://www.jstatsoft.org/article/view/v028i05)) package in R.

The performance of the RF-RFE models on the training dataset are summarized in Table 7 which includes results for the performance of the core biomarkers, 19 biomarkers and the top 20 panels based on descending AUC values.

Results have demonstrated that the panel of 7 biomarkers which includes the core biomarkers (XAGE1D, LRRFIP2, GAGE2C) outperform a panel of 19 biomarkers with Sensitivity and Specificity of 0.753 and 0.721 compared to 0.680 and 0.652 respectively. Figures 10 and 11 show the ROC curves for the best panel of 7 biomarkers (XAGE1D, LRRFIP2, MAGEA10, GAGE2C, STAT1, ZNRDl, RAD23B; with an AUC of 0.818), and the 19 biomarkers (with an AUC of 0.702) respectively.

Extracellular vesicles can be divided into three main categories, namely apoptotic bodies, microvesicles, and exosomes. Exosomes are the smallest extracellular vesicles which are naturally secreted by almost every cell type and can be found in almost all biological fluids including blood, serum, plasma, urine, saliva, amniotic fluid, cerebrospinal fluid, breast milk, semen and bile. In general, cells release exosomes via two mechanisms. The classic pathway involves the formation of intraluminal vesicles within multivesicular endosomes. In turn, the membrane of multivesicular endosomes fuses with the plasma membrane, resulting in the release of intraluminal vesicles. When secreted, intraluminal vesicles are called exosomes. Alternatively, the direct pathway involves the release of vesicles, indistinguishable from exosomes, directly from the plasma membrane (van der Pol *et al.* 2012). Interestingly, exosomes from cancer cells have been shown to promote angiogenesis, modulate the immune system and remodel the surrounding parenchymal tissue, all factors supporting tumor progression (Hessvik and Llorente, 2018).

Exosome samples from patients with NSCLC and healthy controls were collected and isolated using Invitrogen Total Exosome Isolation *(from plasma)* kit (Thermo Fisher Scientific.) based on the established protocol from the manufacturer.

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The discovery of these autoantibody biomarkers using exosome samples can be more disease-specific and meaningful as they contain membrane-bound proteins that reflect their originating cell. In cancer, exosomes have been shown to be implicated in the crosstalk between tumour cells and normal cells thereby facilitating the malignant process. Several studies have found exosomes to be promising as diagnostic and prognostic markers (Sanfeld-Paulsen *et al,* 2016).

It will be appreciated by persons skilled in the art that the present invention may also include further additional modifications made to the system which does not affect the overall functioning of the system.

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Table 5

Protein Name	UniprotID	Description
XAGE1 D	Q9HD64	>P003055_Q21 1_Q21 1_tube_XAGE1_9503_0_NM_02041 1.2_0_Q9HD64_0_ Insert sequence is gene optimized by GeneArt_0_0_0

Nucleotide Sequence **(Seq ID No. 1):**

ATGGAATCCCCCAAGAAGAAGAACCAGCAGCTGAAGGTCGGAATCCTGCACCTGGGTTCCCGTCAGAAGAAGA
TCCGTATCCAGCTGCGTTCCCAGTGCGCTACCTGGAAGGTCATCTGCAAGTCCTGCATCTCCCAGACCCCCGG
TATCAACCTGGACCTGGGCTCCGGTGTCAAGGTCAAGATCATCCCCAAGGAAGAACACTGCAAGATGCCCGAG
GCTGGCGAGGAACAGCCCCAGGTG

Protein Sequence **(Seq ID No. 20):**

MESPKKKNQQLKVGILHLGSRQKKIRIQLRSQCATWKVICKSCISQTPGINLDLGSGVKVKIIPKEEHCKMPEAGEEQP
QV

| **LRRFIP2** | Q9Y608 | >P001 894_Q305_Q305p2_LRRFIP2_9209_Homo sapiens leucine rich repeat (in FLU) interacting protein 2_BC053668.1 _AAH53668. 1_Q9Y608_0_0_1 203_0_1 200 |

Nucleotide Sequence **(Seq ID No. 2):**

ATGGGGACTCCTGCTTCTGGAAGGAAAAGAACACCTGTGAAAGACCGATTTTCTGCAGAAGATGAAGCTTTGAG
TAACATTGCCAGAGAGGCAGAGGCAAGGCTGGCAGCAAAACGGGCTGCCCGGGCAGAAGCAAGAGATATACG
CATGAGAGAACTGGAACGACAACAAAAAGAGTTGGATGAAAAATCTGACAAACAGTATGCTGAAAATTATACAA
GACCTTCATCTCGAAATTCTGCCTCAGCAACAACCCCTCTAAGTGGAAACTCATCCAGACGAGGAAGTGGGGAC
ACCAGCAGCTTAATAGATCCAGACACTTCATTAAGTGAATTGCGGGAATCTTTGTCTGAAGTGGAAGAAAAATAC
AAGAAAGCCATGGTTTCCAATGCACAGTTAGACAATGAGAAGAACAATTTGATCTACCAAGTAGACACACTCAAG
GATGTTATTGAAGAGCAGGAGGAACAGATGGCAGAATTTTATAGAGAAAATGAAGAAAAATCAAAGGAGTTAGA
AAGGCAGAAACATATGTGTAGTGTGCTGCAGCATAAGATGGAAGAACTTAAAGAAGGCCTGCGGCAAAGAGAT
GAGCTTATTGAGAAACATGGCTTAGTTATAATCCCCGATGGCACTCCCAATGGTGATGTCAGTCATGAACCAGT
GGCTGGAGCCATCACTGTTGTGTCTCAGGAAGCTGCTCAGGTCTTGGAGTCAGCAGGAGAAGGGCCATTAGAT
GTAAGGCTACGAAAACTTGCTGGAGAGAAGGAAGAACTACTGTCACAGATTAGAAAACTGAAGCTTCAGTTAGA
GGAGGAACGACAGAAATGCTCCAGGAATGATGGCACAGTGGGTGACCTGGCAGGACTGCAGAATGGCTCAGA
CTTGCAGTTCATCGAAATGCAGAGAGATGCCAATAGACAAATTAGCGAATACAAATTTAAGCTTTCAAAAGCAGA
ACAGGATATAACTACCTTGGAGCAAAGTATTAGCCGGCTTGAGGGACAGGTTCTGAGATATAAAACTGCTGCTG
AGAATGCTGAGAAAGTTGAAGATGAATTGAAAGCAGAAAAACGGAAGCTACAACGAGAGTTACAACAGCACTG
GACAAGATTGAGGAGATGGAGATGACCAACAGCCACCTGGCCAAGCGGCTGGAGAAGATGAAGGCCAATAGG
ACAGCACTTCTGGCCCAGCAG

Protein Sequence **(Seq ID No. 21):**

MGTPASGRKRTPVKDRFSAEDEALSNIAREAEARLAAKRAARAEARDIRMRELERQQKELDEKSDKQYAENYTRPS
SRNSASATTPLSGNSSRRGSGDTSSLIDPDTSLSELRESLSEVEEKYKKAMVSNAQLDNEKNNLIYQVDTLKDVIEEQ
EEQMAEFYRENEEKSKELERQKHMCSVLQHKMEELKEGLRQRDELIEKHGLVIIPDGTPNGDVSHEPVAGAITVVSQ
EAAQVLESAGEGPLDVRLRKLAGEKEELLSQIRKLKLQLEEERQKCSRNDGTVGDLAGLQNGSDLQFIEMQRDANR
QISEYKFKLSKAEQDITTLEQSISRLEGQVLRYKTAAENAEKVEDELKAEKRKLQRELRTALDKIEEMEMTNSHLAKRL
EKMKANRTALLAQQ

| GAGE2C | Q 13066 | >P003037_Q21 1_Q21 1_tube_GAGE2C_2574_0_NM_001 472.2_0_Q1 3066_0 |
| | | _Insert sequence is gene optimized by GeneArt_0_0_0 |

Nucleotide Sequence **(Seq ID No. 3):**

ATGTCCTGGCGTGGTCGTTCCACCTACCGTCCCCGTCCTCGTCGnACGTCGAGCCCCCCGAGATGATCGGTC
CCATGCGTCCCGAGCAGTTCTCCGACGAGGTCGAGCCCGCTACCCCCGAGGAAGGCGAGCCTGCTACTCAGC
GTCAGGACCCCGCTGCTGCTCAAGAGGGCGAGGACGAGGGCGCTTCCGCTGGCCAGGGTCCTAAGCCCGAG
GCTCACTCCCAAGAGCAGGGTCACCCCCAGACCGGTTGCGAGTGCGAGGACGGTCCCGACGGTCAAGAGATG
GACCCCCCCAACCCTGAGGAAGTCAAGACCCCCGAAGAGGGCGAAAGCAGTCCCAGTGC

Protein Sequence (*Seq* ID **No. 22):**

MSWRGRSTYRPRPRRYVEPPEMIGPMRPEQFSDEVEPATPEEGEPATQRQDPAAAQEGEDEGASAGQGPKPEAH
SQEQGHPQTGCECEDGPDGQEMDPPNPEEVKTPEEGEKQSQC

| DDX53 | Q86TM3 | >P003022_Q21 1_Q21 1_tube_DDX53_1 68400_0_NM_1 82699.2_0_Q86TM3_ |
| | | OJnsert sequence is gene optimized by GeneArt_0_0_0 |

Nucleotide Sequence **(Seq ID No. 4):**

ATGTCCCACTGGGCTCCCGAGTGGAAGCGTGCTGAGGCTAACCCCCGTGACCTGGGCGCTTCTTGGGACGTG
CGCGGTTCCCGTGGTAGCGGTTGGAGCGGTCCCTTCGGTCACCAAGGTCCCCGTGCTGCTGGTTCCCGCGAG
CCCCCCCTGTGCTTCAAGATCAAGAACAACATGGTCGGAGTGGTCATCGGTTACTCCGGTTCCAAGATCAAGG
ACCTGCAGCACTCCACCAACACCAAGATCCAGATCATCAACGGCGAGTCCGAGGCTAAGGTCCGCATCTTCGG
CAACCGCGAGATGAAGGCTAAGGCCAAGGCTGCTATCGAGACTCTGATCCGCAAGCAAGAGTCCTACAACTCC
GAGTCCTCCGTGGACAACGCTGCTTCCCAGACCCCCATCGGTCGTAACCTGGGTCGTAACGACATCGTGGGC
GAGGCTGAGCCCCTGTCCAACTGGGACCGTATCCGCGCTGCTGTGGTCGAGTGCGAGAAGCGCAAGTGGGCT
GACCTGCCCCCCGTGAAGAAGAACTTCTACATCGAGTCCAAGGCTACCTCCTGCATGTCCGAGATGCAAGTGA
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GCCTACCTGCCGTTTCAAGGACGCTTTCCAGCAGTACCCCGACCTGCTGAAGTCCATCATCCGTGTGGGTATC
GTGAAGCCCACCCCCATCCAGTCCCAGGCTTGGCCAATCATCCTGCAGGGTATCGACCTGATCGTGGTGGCTC
AGACCGGCACCGGCAAGACCCTGTCCTACCTGATGCCCGGTTTCATCCACCTGGACTCCCAGCCCATCTCCCG
CGAGCAGCGTAACGGTCCCGGCATGCTGGTGCTGACCCCTACCCGTGAACTGGCTCTGCACGTCGAGGCTGA
GTGCTCCAAGTACTCCTACAAGGGCCTGAAGTCTATCTGCATCTACGGTGGTCGTAACCGTAACGGCCAGATC
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CCGTGAACCTGCGTTCCATCACCTACCTGGTCATCGACGAGGCTGACAAGATGCTGGACATGGAGTTCGAGCC
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TCGAGAACATGTCCCCCAACGACAAGGTCATCATGTTCGTGTCCCAGAAGCACATTGCTGACGACCTGTCCTCC
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ATCAAGATCTTGGACCGTGCTAACCAGTCCGTGCCCGAGGACCTGGTGGTCATGGCTGAGCAGTACAAGCTGA
ACCAGCAGAAGCGTCACCGCGAGACTCGTTCCCGCAAGCCCGGACAGCGTCGCAAGGAGTTCTACTTCCTGTC
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Protein Sequence **(Seq ID No. 23):**

MSHWAPEWKRAEANPRDLGASWDVRGSRGSGWSGPFGHQGPRAAGSREPPLCFKIKNNMVGVVIGYSGSKIKDL
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DRIRAAVVECEKRKWADLPPVKKNFYIESKATSCMSEMQVINWRKENFNITCDDLKSGEKRLIPKPTCRFKDAFQQY
PDLLKSIIRVGIVKPTPIQSQAWPIILQGIDLIVVAQTGTGKTLSYLMPGFIHLDSQPISREQRNGPGMLVLTPTRELALH
VEAECSKYSYKGLKSICIYGGRNRNGQIEDISKGVDIIIATPGRLNDLQMNNSVNLRSITYLVIDEADKMLDMEFEPQIR
KILLDVRPDRQTVMTSATWPDTVRQLALSYLKDPMIVYVGNLNLVAVNTVKQNIIVTTEKEKRALTQEFVENMSPNDK
VIMFVSQKHIADDLSSDFNIQGISAESLHGNSEQSDQERAVEDFKSGNIKILITTDIVSRGLDLNDVTHVYNYDFPRNID
VYVHRVGYIGRTGKTGTSVTLITQRDSKMAGELIKILDRANQSVPEDLVVMAEQYKLNQQKRHRETRSRKPGQRRKE
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DDX43	Q9NXZ2	>P003043_Q21 1_Q21 1_tube_DDX43_5551 0_0_NM_01 8665.2_0_Q9NXZ2_0 _Insert sequence is gene optimized by GeneArt_0_0_0

Nucleotide Sequence (Seq ID No. 5):

ATGTCCCACCACGGTGGTGCTCCCAAGGCTTCCACCTGGGTGGTGGCTTCCCGTCGTTCCTCCACCGTGTCCC
GTGCTCCCGAGCGTCGTCCCGCTGAGGAACTGAACCGTACCGGTCCCGAGGGCTACTCCGTGGGTCGTGGTG
GTCGTTGGCGTGGCACCTCTCGTCCCCCTGAGGCTGTGGCTGCTGGTCACGAGGAACTGCCCCTGTGCTTCG
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CAACACCACCATCCAGATCATCCAAGAGCAGCCCGAGTCCCTGGTCAAGATCTTCGGTTCCAAGGCTATGCAG
ACCAAGGCTAAGGCTGTGATCGACAACTTCGTGAAGAAGCTGGAAGAGAACTACAACTCCGAGTGCGGTATCG
ACACCGCTTTCCAGCCCTCCGTGGGCAAGGACGGTTCCACCGACAACAACGTGGTGGCTGGCGACCGTCCCC
TGATCGACTGGGACCAGATCCGTGAAGAGGGCCTGAAGTGGCAAAAGACCAAGTGGGCTGACCTGCCCCCCA
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AGATCCTGCTGGACGTGCGTCCCGACCGTCAGACCGTGATGACCTCCGCTACCTGGCCCCACTCCGTGCACC
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CTCGAGCGTGCTAACCAGTCCATCCCCGAGGAACTGGTGTCTATGGCTGAGCGTTTCAAGGCTCACCAGCAAA
AGCGCGAGATGGAACGCAAGATGGAACGTCCCCAGGGTCGTCCCAAGAAGTTCCAC

Protein Sequence (Seq ID No. 24):

MSHHGGAPKASTWVVASRRSSTVSRAPERRPAEELNRTGPEGYSVGRGGRWRGTSRPPEAVAAGHEELPLCFAL
KSHFVGAVIGRGGSKIKNIQSTTNTTIQIIQEQPESLVKIFGSKAMQTKAKAVIDNFVKKLEENYNSECGIDTAFQPSVG
KDGSTDNNVVAGDRPLIDWDQIREEGLKWQKTKWADLPPIKKNFYKESTATSAMSKVEADSWRKENFNITWDDLKD
GEKRPIPNPTCTFDDAFQCYPEVMENIKKAGFQKPTPIQSQAWPIVLQGIDLIGVAQTGTGKTLCYLMPGFIHLVLQPS
LKGQRNRPGMLVLTPTRELALQVEGECCKYSYKGLRSVCVYGGGNRDEQIEELKKGVDIIIATPGRLNDLQMSNFVN
LKNITYLVLDEADKMLDMGFEPQIMKILLDVRPDRQTVMTSATWPHSVHRLAQSYLKEPMIVYVGTLDLVAVSSVKQN
IIVTTEEEKWSHMQTFLQSMSSTDKVIVFVSRKAVADHLSSDLILGNISVESLHGDREQRDREKALENFKTGKVRILIAT
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Nucleotide Sequence (Seq ID No. 6):

ATGTCCTGGCGTGGTCGTTCCACCTACTACTGGCCCCGTCCCCGTCGTTACGTGCAGCCCCCCGAGATGATCG
GTCCCATGCGTCCCGAGCAGTTCTCCGACGAGGTCGAGCCCGCTACCCCCGAGGAAGGCGAGCCTGCTACTC
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GAGGCTGACTCCCAAGAGCAGGGTCACCCCCAGACCGGTTGCGAGTGCGAGGACGGTCCCGACGGTCAAGA
GATGGACCCCCCCAACCCTGAGGAAGTCAAGACCCCCGAAGAGGGCGAAGGCCAGTCCCAGTGC

Protein Sequence (Seq ID No. 25):

MSWRGRSTYYWPRPRRYVQPPEMIGPMRPEQFSDEVEPATPEEGEPATQRQDPAAAQEGEDEGASAGQGPKPE
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MAGEA10	P43363	>P003053_Q21 1_Q21 1_tube_MAGEA1 0_41 09_0_NM_001 0 11543.1 _0_P433 63_0_lnsert sequence is gene optimized by GeneArt_0_0_0

Nucleotide Sequence (Seq ID No. 7):
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CCAGCAGCTTCCCATCCTCCAGCTCCTCTAGCTCCTCCTCCTGCTACCCCCTGATCCCCTCCACCCCCGAGGA
AGTGTCCGCTGACGACGAGACTCCCAACCCCCCCCAGTCCGCTCAGATCGCTTGCTCCTCCCCCTCCGTGGTG
GCTTCCCTGCCTCTGGACCAGTCCGACGAGGGTTCCAGCTCCCAGAAGGAAGAGTCCCCCAGCACCCTGCAG
GTCCTGCCCGACTCCGAGTCCCTGCCCCGTTCCGAGATCGACGAGAAGGTTACAGACCTGGTGCAGTTCCTGC
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GACCCTACCGGTCACTCCTTCGTGCTGGTCACCTCCCTGGGCCTGACCTACGACGGCATGCTGTCCGACGTGC
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GGGCTCAGGACCGTATCGCTACCACCGACGACACCACCGCTATGGCTTCCGCTTCCTCTAGCGCTACCGGTTC
CTTCAGCTACCCCGAG

Protein Sequence (*Seq* ID No. 26):
MPRAPKRQRCMPEEDLQSQSETQGLEGAQAPLAVEEDASSSTSTSSSFPSSFPSSSSSSSSSCYPLIPSTPEEVSA
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PITKAEILESVIRNYEDHFPLLFSEASECMLLVFGIDVKEVDPTGHSFVLVTSLGLTYDGMLSDVQSMPKTGILILLSIVF
IEGYCTPEEVIWEALNMMGLYDGMEHLIYGEPRKLLTQDWVQENYLEYRQVPGSDPARYEFLWGPRAHAEIRKMSL
LKFLAKVNGSDPRSFPLWYEEALKDEEERAQDRIAnDDnAMASASSSATGSFSYPE

ZNRD1	Q9P1 UO	>P000875_TRN_TRNp2_ZNRD1_30834_Homo sapiens zinc ribbon domain containing 1 transcript variant b_BC01 0898.1 _AAH1 0898.1 _Q9P1 U0_0_0_381 _0_378

Nucleotide Sequence (Seq ID No. 8):
ATGTCTGTCATGGACCTCGCCAATACTTGCTCCAGCTTTCAGTCGGACCTGGATTTCTGTTCAGATTGCGGCTC
GGTCCTGCCTCTGCCCGGGGCTCAGGATACGGTCACCTGTATTCGCTGTGGCTTCAACATCAACGTTCGGGAC
TTTGAGGGGAAGGTTGTGAAGACTTCGGTTGTGTTCCACCAACTGGGGACAGCCATGCCTATGTCGGTGGAGG
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GAAGGAAGACTCT

Protein Sequence (Seq ID No. 27):
MSVMDLANTCSSFQSDLDFCSDCGSVLPLPGAQDTVTCIRCGFNINVRDFEGKVVKTSVVFHQLGTAMPMSVEEGP
ECQGPVVDRRCPRCGHEGMAYHTRQMRSADEGQTVFYTCTNCKFQEKEDS

MAP2K5	Q13163	>P000081_KIN96_KIN_MAP2K5_5607_Homo sapiens mitogen-activated protein kinase kinase 5, transcript variant A_BC008838.2_AAH08838. 1_Q13163_41 11679_0_1 347_0_1 344

Nucleotide Sequence (Seq ID No. 9):
ATGCTGTGGCTAGCCCTTGGCCCCTTTCCTGCCATGGAGAACCAGGTGCTGGTAATTCGCATCAAGATCCCAAA
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Protein Sequence (Seq ID No. 28):
MLWLALGPFPAMENQVLVIRIKIPNSGAVDWTVHSGPQLLFRDVLDVIGQVLPEATTTAFEYEDEDGDRITVRSDEEM
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ISICTEFMDGGSLDVYRKMPEHVLGRIAVAVVKGLTYLWSLKILHRDVKPSNMLVNTRGQVKLCDFGVSTQLVNSIAK
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MAGEA4	P43358	>P001216_CAG_CAGp1_MAGEA4_4103_Homo sapiens melanoma antigen family A4_BC017723.1_AAH17723.1_P43358_0_0_954_0_951

Nucleotide Sequence (Seq ID No. 10):
ATGTCTTCTGAGCAGAAGAGTCAGCACTGCAAGCCTGAGGAAGGCGTTGAGGCCCAAGAAGAGGCCCTGGGC
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GCACCCTGGAGGAAGTGCCTGCTGCTGAGTCAGCAGGTCCTCCCCAGAGTCCTCAGGGAGCCTCTGCCTTAC
CCACTACCATCAGCTTCACTTGCTGGAGGCAACCCAATGAGGGTTCCAGCAGCCAAGAAGAGGAGGGGCCAA
GCACCTCGCCTGACGCAGAGTCCTTGTTCCGAGAAGCACTCAGTAACAAGGTGGATGAGTTGGCTCATTTTCTG
CTCCGCAAGTATCGAGCCAAGGAGCTGGTCACAAAGGCAGAAATGCTGGAGAGAGTCATCAAAAATTACAAGC
GCTGCTTTCCTGTGATCTTCGGCAAAGCCTCCGAGTCCCTGAAGATGATCTTTGGCATTGACGTGAAGGAAGTG
GACCCCACCAGCAACACCTACACCCTTGTCACCTGCCTGGGCCTTTCCTATGATGGCCTGCTGGGTAATAATCA
GATCTTTCCCAAGACAGGCCTTCTGATAATCGTCCTGGGCACAATTGCAATGGAGGGCGACAGCGCCTCTGAG
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Protein Sequence (Seq ID No. 29):
MSSEQKSQHCKPEEGVEAQEEALGLVGAQAPTTEEQEAAVSSSSPLVPGTLEEVPAAESAGPPQSPQGASALPTTI
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KASESLKMIFGIDVKEVDPASNTYTLVTCLGLSYDGLLGNNQIFPKTGLLIIVLGTIAMEGDSASEEEIWEELGVMGVYD
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STAT1	P42224	>P000068_KIN96_KIN_STAT1_6772_Homo sapiens signal transducer and activator of transcription 1 9 1kDa transcript varian_BC002704.2_AAH02704.1_P42224_93992.1 5_0_21 39_0_21 36

Nucleotide Sequence (**Seq ID No. 11**):

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CCTCCATCCTTTGGTACAACATGCTGGTGGCGGAACCCAGGAATCTGTCCTTCTTCCTGACTCCACCATGTGCA
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GCTGAACATGTTGGGAGAGAAGCTTCTTGGTCCTAACGCCAGCCCCGATGGTCTCATTCCGTGGACGAGGTTT
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GGACCAGCAGCCGGGGACCTTCCTGCTGCGGTTCAGTGAGAGCTCCCGGGAAGGGGCCATCACATTCACATG
GGTGGAGCGGTCCCAGAACGGAGGCGAACCTGACTTCCATGCGGTTGAACCCTACACGAAGAAGAACTTTCT
GCTGTTACTTTCCCTGACATCATTCGCAATTACAAAGTCATGGCTGCTGAGAATATTCCTGAGAATCCCCTGAAG
TATCTGTATCCAAATATTGACAAGACCATGCCTTTGGAAAGTATTACTCCAGGCCAAAGGAAGCACCAGAGCC
AATGGAACTTGATGGCCCTAAAGGAACTGGATATATCAAGACTGAGTTGATTTCTGTGTCTGAAGTG

Protein Sequence (**Seq ID No. 30**):

MSQWYELQQLDSKFLEQVHQLYDDSFPMEIRQYLAQWLEKQDWEHAANDVSFATIRFHDLLSQLDDQYSRFSLEN
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MCIEHEIKSLEDLQDEYDFKCKTLQNREHETNGVAKSDQKQEQLLLKKMYLMLDNKRKEVVHKIIELLNVTELTQNALI
NDELVEWKRRQQSACIGGPPNACLDQLQNWFTIVAESLQQVRQQLKKLEELEQKYTYEHDPITKNQVLWDRTFSL
FQQLIQSSFVVERQPCMPTHPQRPLVLKTGVQFTVKLRLLVKLQELNYNLKVKVLFDKDVNERNTVKGFRKFNILGTH
TKVMNMEESTNGSLAAEFRHLQLKEQKNAGTRTNEGPLIVTEELHSLSFETQLCQPGLVIDLETTSLPVVVISNVSQL
PSGWASILWYNMLVAEPRNLSFFLTPPCARWAQLSEVLSWQFSSVTKRGLNVDQLNMLGEKLLGPNASPDGLIPWT
RFCKENINDKNFPFWLWIESILELIKKHLLPLWNDGCIMGFISKERERALLKDQQPGTFLLRFSESSREGAITFTWVER
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Nucleotide Sequence (Seq ID No. 12):
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GGCTGCTGTGCCTCGTGGTGGTTCCGCTGAAGAGGACTCCGACATCGGTCCCGCTACCGAGGAAGAGGAAGA
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Protein Sequence (Seq ID No. 31):
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VGPHAAARNLWGNLPPLLLPQRLGAGAAARAGEGLGLIQEAASVPEPAVPADLAEMAREPAEEAAEEKLSEEATEE
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| IGF2BP3 | 000425 | >P003224_Q31 1_Q31 1_tube_IGF2BP3_1 0643_0_NM_006547.2_0_000425_ 0_Insert sequence is gene optimized by GeneArt_0_0_0 |

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GGCTCTGAAGGCTATCGAGGCTCTGTCCGGCAAGATCGAGCTGCACGGCAAGCCCATCGAGGTCGAGCACTC
CGTGCCCAAGCGTCAGCGTATCCGCAAGCTGCAGATCCGTAACATCCCCCCACACCTCCAGTGGGAGGTGCT
GGACTCCCTGCTGGTGCAGTACGGTGTCGTCGAGTCCTGCGAGCAAGTGAACACCGACTCCGAGACTGCTGT
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TGCGACCTGCCCCTGCGTCTGCTGGTGCCTACCCAGTTCGTGGGTGCTATCATCGGCAAGGAAGGTGCTACCA
TCCGCAACATCACCAAGCAGACCCAGTCCAAGATCGACGTCCACCGCAAGGAAACGCTGGCGCTGCTGAGAA
GTCCATCACCATCCTGTCCACCCCCGAGGGCACCTCCGCTGCTTGCAAGTCCATCCTCGAGATCATGCACAAG
GAAGCCCAGGACATCAAGTTCACCGAGGAAATCCCCCTGAAGATCCTGGCTCACAACAACTTCGTGGGTCGTC
TGATCGGAAAGGAAGGCCGTAACCTGAAGAAGATCGAGCAGGACACCGACACCAAGATCACCATCTCCCCACT
GCAAGAGCTGACCCTGTACAACCCCGAGCGTACCATCACCGTGAAGGGCAACGTGGAAACCTGCGCTAAGGC
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CTGATCCCCGGCCTGAACCTGAACGCTCTGGGCCTGTTCCCCCCTACCTCCGGCATGCCTCCTCCCACCTCTG
GTCCCCCCTCCGCTATGACCCCCCCCATACCCCCCAGTTCGAGCAGTCCGAGACTGAGACTGTGCACCTGTTCAT
CCCCGCTCTGTCCGTCGGTGCCATCATCGGAAAGCAGGGCCAGCACATCAAGCAGCTGTCCCGTTTCGCTGGT
GCTTCCATCAAGATCGCTCCCGCTGAGGCTCCCGACGCTAAGGTCCGCATGGTCATCATCACCGGTCCCCCCG
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AGTGAAGCTCGAGGCTCACATCCGTGTGCCATCCTTCGCTGCTGGTCGTGTTATCGGCAAGGGTGGCAAGACC
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Protein Sequence (Seq ID No. 32):
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YPQFEQSETETVHLFIPALSVGAIIGKQGQHIKQLSRFAGASIKIAPAEAPDAKVRMVIITGPPEAQFKAQGRIYGKIKEE
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Nucleotide Sequence **(Seq ID No. 14):**
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CGCAGGATGGAAGGTGCCCCTGCGGGGCCAGGAGGCCGGACAGCCGCCTGCTTGAGTTGCACATCACGATG
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CCAGGGGCGGTTCTGAAGGACTTCACCGTGTCCGGCAACCTACTGTTTATGTCAGTTCGGGACCAGGACAGGG
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GATCTCAGAACACCCAAACACAAGGTCTCAGAACAGAGACCTGGTACACCAGGCCCGCCGCCACCCGAGGGA
GCCCAGGGAGATGGGTGCAGAGGTGTCGCCTTTAATGTGATGTTCTCTGCCCCTCACATT

Protein Sequence **(Seq ID No. 33):**
MQAEGQGTGGSTGDADGPGGPGIPDGPGGNAGGPGEAGATGGRGPRGAGAARASGPRGGAPRGPHGGAASAQ
DGRCPCGARRPDSRLLQLHITMPFSSPMEAELVRRILSRDAAPLPRPGAVLKDFTVSGNLLFMSVRDQDREGAGRM
RVVGWGLGSASPEGQKARDLRTPKHKVSEQRPGTPGPPPPEGAQGDGCRGVAFNVMFSAPHI

RAD23B	P54727	>P000309_SIG_SIG1 -1_RAD23B_5887_Homo sapiens RAD23 homolog B (S. cerevisiae)_BC020973.2_AAH20973.1_P54727_541 73_0_1 230_0_1 227

Nucleotide Sequence **(Seq ID No. 15):**
ATGCAGGTCACCCTGAAGACCCTCCAGCAGCAGACCTTCAAGATAGACATTGACCCCGAGGAGACGGTGAAAG
CACTGAAAGAGAAGATTGAATCTGAAAAGGGGAAAGATGCCTTTCCAGTAGCAGGTCAAAAATTAATTTATGCA
GGCAAAATCCTCAATGATGATACTGCTCTCAAAGAATATAAAATTGATGAGAAAAACTTTGTGGTGGTTATGGTG
ACCAAACCCAAAGCAGTGTCCACACCAGCACCAGCTACAACTCAGCAGTCAGCTCCTGCCAGCACTACAGCAG
TTACTTCCTCCACCACCACAACTGTGGCTCAGGCTCCAACCCCTGTCCCTGCCTTGGCCCCCACTTCCACACCT
GCATCCATCACTCCAGCATCAGCGACAGCATCTTCTGAACCTGCACCTGCTAGTGCAGCTAAACAAGAGAAGCC
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CATGTCAATGGGCTATGAACGAGAGCAAGTAATTGCAGCCCTGAGAGCCAGTTTCAACAACCCTGACAGAGCA
GTGGAGTATCTTTTAATGGGAATCCCTGGAGATAGAGAAAGTCAGGCTGTGGTTGACCCCCCTCAAGCAGCTA
GTACTGGGGTTCCTCAGTCTTCAGCAGTGGCTGCAGCTGCAGCAACTACGACAGCAACAACTACAACAACAAG
TTCTGGAGGACATCCCCTTGAATTTTTACGGAATCAGCCTCAGTTTCAACAGATGAGACAAATTATTCAGCAGAA
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Protein Sequence **(Seq ID No. 34):**
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TSPTATDSTSGDSSRSNLFEDATSALVTGQSYENMVTEIMSMGYEREQVIAALRASFNNPDRAVEYLLMGIPGDRES
QAVVDPPQAASTGAPQSSAVAAAAATTTATTTTTSSGGHPLEFLRNQPQFQQMRQIIQQNPSLLPALLQQIGRENPQ
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CEKNENLAANFLLQQNFDED

| FADD | Q13158 | >P00041 7_SIG_SIG1 -2_FADD_8772_Homo sapiens Fas (TNFRSF6)-associated via death domain_BC000334.2_AAH00334.1_Q131 58_34265.45_0_627_0_624 |

Nucleotide Sequence **(Seq ID No. 16):**
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CTGCTGGAGCAGAACGACCTGGAGCCCGGGCACACCGAGCTCCTGCGCGAGCTGCTCGCCTCCCTGCGGCG
CCACGACCTGCTGCGGCGCGTCGACGACTTCGAGGCGGGGGCGGCGGCCGGGGCCGCGCCTGGGGAAGAA
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AGTCTCAGACACCAAGATCGACAGCATCGAGGACAGATACCCCCGCAACCTGACAGAGCGTGTGCGGGAGTC
ACTGAGAATCTGGAAGAACACAGAGAAGGAGAACGCAACAGTGGCCCACCTGGTGGGGGCTCTCAGGTCCTG
CCAGATGAACCTGGTGGCTGACCTGGTACAAGAGGTTCAGCAGGCCCGTGACCTCCAGAACAGGAGTGGGGC
CATGTCCCCGATGTCATGGAACTCAGACGCATCTACCTCCGAAGCGTCC

Protein Sequence **(Seq ID No. 35):**
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VDDFEAGAAAGAAPGEEDLCAAFNVICDNVGKDWRRLARQLKVSDTKIDSIEDRYPRNLTERVRESLRIWKNTEKEN
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Nucleotide Sequence **(Seq ID No. 17):**
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TCAACTCCGAGAAGGTCAAGCTGTCCCTGCGCAACTTCCCCCACAACGATTACGAGGACGTGTTCGAGGAACC
CTCCGAGTCCGGTTCCGACCCCTCCATGTGGACCGCTCGTGGTCCCTTCCGTCGTGACCGTTGGTCCTCCGAG
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GGCGAGCTGGACCAGCTGGCTCAGATCCGTCCCCTGATCTTCAACTTCCACGAGCAGACCGCTATCAAGGACT
GCCTGAAGATCCTCGAGGAAAAGACCGCTGCTTACGACATCATGCAAGAGTTCATGGCTCTCGAGCTGAAGAA
CCTGCCCGGCGAGTTCAACTCCGGCAACCAGCCCTCCAACCGCGAGAAGAACCGTTACCGTGACATCCTGCCT
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CCCTGCTGCTGCGCTACTGCGCT

Protein Sequence **(Seq ID No. 36):**
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STIDDFWQMVLENNSNVIAMITREIEGGIIKCYHYWPISLKKPLELKHFRVFLENYQILQYFIIRMFQVVEKSTGTSHSVK
QLQFTKWPDHGTPASADSFIKYIRYARKSHLTGPMVVHCSAGIGRTGVFLCVDVVFCAIVKNCSFNIMDIVAQMREQ
RSGMVQTKEQYHFCYDIVLEVLRKLLTLD

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Nucleotide Sequence (Seq ID No. 18):

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GAAGAACAATTAAGAATAATGGATCAGACCTTGAAAGCATTAATGGCTGCAGAGGATAAGTACTCGCAGAAGGA
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Protein Sequence (Seq ID No. 37):

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EDADRKYEEVARKLVIIESDLERAEERAELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEEIKVLSDKLKE
AETRAEFAERSVTKLEKSIDDLEDELYAQKLKYKAISEELDHALNDMTSI

CTAG1A	P78358	>P003024_Q21 1_Q21 1_tube_CTAG1 A_2461 00_0_NM_1 39250.1 _0_P78358 _0_lnsert sequence is gene optimized by GeneArt_0_0_0

Nucleotide Sequence (Seq ID No. 19):

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AACGGTTGCTGCCGTTGCGGTGCTCGCGGTCCCGAGTCCCGTCTGCTCGAGTTCTACCTGGCTATGCCCTTCG
CTACCCCTATGGAAGCTGAGCTGGCTCGTCGTTCCCTGGCTCAGGACGCTCCTCCTCTGCCCGTGCCCGGTGT
CCTGCTGAAGGAGTTCACTGTCTCCGGCAACATCCTGACCATCCGTCTGACCGCTGCTGACCACCGTCAGCTC
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Protein Sequence (Seq ID No. 38):

MQAEGRGTGGSTGDADGPGGPGIPDGPGGNAGGPGEAGATGGRGPRGAGAARASGPGGGAPRGPHGGAASGL
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SCLQQLSLLMWITQCFLPVFLAQPPSGQRR

Table 6: Normalised RFU values for the 19 biomarkers

Protein	CT47A1	CTAG1A	CTAG2	DDX43	DDX53	FADD	GAGE1	GAGE2C	IGF2BP3	LRRFIP2	MAGEA10	MAGEA4	MAP2K5	PTPN20A	RAD23B	STAT1	TPM1	XAGE1D	ZNRD1
Control1_021608	1509.358	1665.573	1625.151	2051.253	2063.69	2342.416	1936.961	2921.207	1864.379	10256.51	1707.204	1270.237	2062.856	3315.828	2081.126	2617.833	4049.264	1506.695	2626.203
Control1_021611	744.8538	840.2482	689.2973	1011.785	1008.013	1106.472	1000.557	905.3507	984.5742	1642.046	949.5056	717.2795	980.4752	1986.406	905.2051	977.5851	1796.781	863.7998	1532.123
Control1_021630	2308.622	2320.635	3698.177	2686.878	2394.465	4457.038	2656.298	1767.186	2495.178	3443.966	2016.665	1715.477	2661.723	5203.608	1990.034	3163.671	6107.433	1897.377	3821.562
Control1_021631	1648.979	1653.373	2014.062	1947.228	1745.636	3154.698	1963.353	2408.739	1769.07	2760.66	2086.983	2381.255	2711.591	2212.801	2347.861	2764.548	4874.361	1577.602	2855.739
Control1_021642	1908.076	2463.134	2466.247	2669.508	2731.679	3765.574	2711.856	2790.404	2347.117	5726.215	1819.513		2590.68	5952.694	2026.351	6089.905	4518.586	1985.198	4152.455
Control1_021643	903.8178	1280.729	1094.733	1480.856	1737.577	1901.206	1653.898	1536.486	1577.693	2475.029	1392.899	1095.408	1734.76	1687.801	1234.18	2229.621	2248.575	1155.519	2530.437
Control1_021650	2268.797	2064.734	3027.739	1922.742	1862.127	6656.127	1702.062	1538.051	1712.624	3158.91	2166.367	3076.028	3988.011	4640.239	2266.734	9483.072	4720.325	2449.356	5897.559
Control1_021660	1646.455	1641.704	2635.412	2104.055	2228.304	2471.875	2434.567	2913.973	2025.719	10491.47	1699.251	1931.718	2483.095	2033.607	1338.988	3689.077	4882.12	1281.009	3234.591
Control1_021661	1813.748	2232.638	1907.396	3502.799	3281.538	2910.518	3433.232	2851.365	2947.112	4964.015	2260.505	1715.278	2982.163	2494.186	2421.713	3949.958	6032.581	1948.014	3304.639
Control1_021663	7361.666	1747.892	1808.937	3356.196	2994.698	2869.315	2993.111	2762.496	2921.823	3766.776	2208.297	1894.731	2643.505	2964.772	4486.685	3322.445	3789.802	2135.498	4907.796
Control1_021674	1065.267	1391.603	1352.962	2290.515	2156.563	2278.898	2439.543	1912.377	1885.233	5666.105	1571.638	1156.283	2244.439	2457.223	1833.585	2449.947	8639.902	1360.602	4240.082
Control1_021679	1154.795	956.4944	1118.479	1632.494	1530.023	2318.788	1627.435	1436.882	1483.428	2188.354	1499.469	1066.008	2001.052	1444.111	1802.526	1950.754	2527.683	1278.041	2860.415
Control1_021680	3498.123	1930.224	1855.749	2352.687	2406.267	2802.602	2489.483	2315.313	2291.881	7936.889	2166.929	1679.373	2362.385	3149.52	2543.393	15907.4	4196.795	1774.904	3386.469
Control1_021681	8854.106	8411.776	8780.085	4507.529	18451.28	9194.071	4543.1	3534.392	3826.813	10582.86	6322.936	4911.227	5544.572	13939.81	4156.378	9128.958	19809.27	6150.266	9369.373
Control1_021682	1968.383	1557.664	1865.677	2790.045	2426.774	2398.539	2494.837	2171.401	2371.601	4526.223	1991.898	1569.11	4169.351	2907.473	2309.431	2437.564	3893.909	1728.037	3301.781
Control2_021005	19062.75	18286.29	21721.06	7277.07	7509.16	21723.39	8024.274	7810.164	6199.772	11076.7	11112.01	9611.882	6652.302	19237.07	5670.086	4960.157	22784.55	18345.03	20955.56
Control2_021007	3700.532	4218.712	3151.603	4712.313	3869.997	4623.076	2852.438	2929.432	3216.338	5692.778	3194.603	2842.006	4446.303	3910.378	17546.81	7313.667	7748.537	3598.932	4526.242
Control2_021016	2798.772	1907.347	2099.19	2252.848	2179.747	3351.127	2478.487	1937.812	1894.693	3015.911	2324.273	1948.089	2766.241	2710.309	2141.714	3366.335	5555.07	2589.406	3272.007
Control2_021017	1753.571	1402.848	1540.14	1802.829	1780.391	2588.142	1590.338	1678.784	1714.025	3137.241	1811.556	1476.747	2095.534	2572.001	1747.83	3847.813	4092.125	2194.708	2639.944
Control2_021025	1559	2078.736		2155.411	2566.253	2697.4	2064.229	1783.645	2301.794	5997.156	1928.071	1764.336	2214.804	2567.013	1995.162	3132.801	4412.822	2386.34	2807.273
Control2_021037	1640.906	1349.049	1760.375	1917.732	2594.986	2383.889	1833.83	1568.244	1786.695	11870.33	1835.236	1526.275	2408.522	3380.793	1679.377	3354.587	4200.855	2184.704	3093.381
Control2_021038	1296.442	1068.401	1352.522	1510.341	1483.363	1730.292	1498.059	1215.978	1468.671	2967.612	1523.531	1207.042	2009.926	1666.915	1384.307	2710.608	3518.954	1880.842	2744.047

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Control2_021045	1755.101	1821.33	1657.713	2107.653	2190.138	2525.574	2252.285	1949.365	1833.289	2309.808	2015.164	1736.922	2395.172	2511.068	1973.987	1904.59	3795.172	2411.016	2970.695
Control2_021046	2544.023	3285.646	3830.951	3493.117	4036.784	3754.021	2887.819	2281.996	3026.465	6848.673	2894.598	2433.767	3258.239	5672.938	2410.957	3980.489	5714.518	4737.667	3939.826
Control2_021401	1790.79	2078.056	1554.37	2146.542	2368.271	2731.86	2223.912	2136.282	2153.839	3751.081	2099.173	2967.183	2414.103	2164.446	5811.137	3107.932	7447.278	2357.483	3127.564
Control2_021405	1633.525	1266.149	1722.513	1767.528	2204.463	1972.807	1695.027	1588.648	1577.527	2708.492	1709.801	1549.398	2091.493	2295.796	1536.453	2455.628	3139.008	1753.436	2103.498
Control2_021406	1231.18	1052.587	1221.755	1492.635	1617.316	1516.808	1419.205	1242.477	1377.186	3379.66	1510.359	1138.178	1824.205	1522.514	2339.278	3625.717	3868.642	1370.279	1894.139
Control2_021419	1161.514	969.2148	1340.876	1298.33	1271.956	1791.68	1169.562	1080.648	1327.409	2950.186	1288.873	1206.732	1920.516	3093.032	1303.955	3515.891	2877.05	1374.645	1776.012
Control2_021420	1134.712	1149.875	1308.413	1473.83	1407.462	1798.174	1488.179	1345.406	1505.299	3191.296	1258.148	1800.616	1843.103	1192.939	3347.262	3224.197	1403.629	1900.957	
Control2_021423	2775.048	2484.158	3319.35	3998.832	3444.215	5014.536	2747.573	2749.234	3570.623	6620.853	3579.061	2918.362	4426.904	4727.215	3432.053	4868.726	11813.17	4171.47	5555.068
Control2_021426	1832.683	3149.643	3941.681	3331.141	3193.393	8540.402	2683.709	2773.037	2939.304	8472.987	5489.914	2853.974	2939.504	2777.067	3783.557	6349.507	6183.704	3560.529	7570.511
Control2_021430	1740.691	1262.074	1439.203	1784.948	1981.945	1970.871	1632.47	1614.165	1712.129	3839.469	1920.69	1343.425	2271.696	2344.028	1525.482	2578.638	70438.51	1758.205	2714.357
Control2_021436	1238.38	1622.209	1339.236	1766.58	1570.416	1970.871	1839.317	1492.531	2310.743	1607.355	1279.09	1830.218	1714.501	1358.224	2193.62	2708.096	1785.438	2455.876	
Control2_021451	2114.425	1537.93	2366.879	2916.57	2079.048	3845.347	2548.36	2707.548	2780.555	3572.501	2861.352	2283.854	3366.02	3448.814	3060.993	4270.824	6303.973	2949.225	8755.411
Control2_021453	1618.877	1772.711	1704.868	2010.795	1923.692	1949.649	1938.773	1955.682	1756.808	6044.063	2175.041	1564.877	2295.219	2062.084	2084.262	3021.721	6835.281	1951.169	3240.717
Control2_021454	1238.661	1215.827	1245.483	1523.407	1445.537	1606.954	1606.287	1577.288	2848.697	1455.822	1306.672	1894.117	1759.484	2367.539	2441.304	4791.178	1549.041	2289.409	
Control2_021455	2118.806	2056.799	2135.804	2460.721	2409.344	3278.959	2368.928	2564.917	4994.603	2369.251	2530.726	3277.945	2818.599	2358.646	4807.185	6212.127	2661.667	3698.437	
Control2_021463	3277.456	2954.683	6329.681	4028.11	3885.942	8013.651	3643.477	3340.58	10964.05	4476.259	3557.854	4176.659	5050.335	3590.858	7434.23	9568.385	4946.078	5981.495	
Control2_021470	1404.597	1243.895	1509.513	1683.949	1605.746	2041.225	1716.442	1589.304	5710.754	1632.83	1402.099	2130.032	2535.66	1462.485	2516.297	1017.116	1667.289	2791.111	
Control2_021477	3228.131	2485.288	2693.976	3065.942	3048.999	4125.412	2669.848	3498.464	6047.108	2975.548	2798.397	3279.928	6853.659	2609.898	4227.737	7400.694	3379.937	3876.3	
Control2_021478	2702.577	2203.357	3179.785	2097.886	1878.866	3515.833	1763.884	1975.51	4209.962	3187.603	1924.962	2382.097	3422.74	1919.419	6160.237	4680.497	3213.554	3278.552	
Control2_021484	1867.657	1752.908	1825.902	2021.439	1897.227	4221.542	1997.38	2030.785	4996.788	2260.441	1635.343	2426.476	2468.278	1856.142	3234.766	4323.386	2336.339	2965.36	
Control2_021494	1226.113	1187.936	1371.995	1461.011	1289.884	1730.896	1411.166	1358.159	6289.746	1428.486	1155.8	1760.796	3294.846	1606.667	3721.188	2527.812	1406.064	2014.624	
Control2_021495	7510.731	6547.905	8569.185	9460.011	24887.84	11979	7132.448	7607.972	10760.02	8813.236	8154.649	4858.083	8017.285	10114.45	9327.442	13563.37	10985.96	11552.79	
Control2_021497	2103.323	2325.289	2423.39	2140.501	1881.936	2612.635	1828.612	2022.71	2381.643	2071.199	1836.147	2880.86	2694.962	8194.55	4910.062	4861.554	2291.933	3220.736	
Control2_021801	1684.077	1614.954	1535.561	2231.253	1987.477	1996.131	1799.308	2131.438	2938.354	1934.592	1637.284	2040.849	2076.451	2481.315	2854.822	3918.524	2763.663	2642.432	

Control2_021802	1196.061	1161.766	1299.801	1585.175	1516.791	1907.229	1876.651	1209.1	1582.688	1721.553	1333.201	1159.959	2289.404	5436.568	1385.581	3183.812	2829.928	1533.262	3402.505
Control2_021804	1813.897	1627.858	1980.221	2420.979	2252.938	2496.24	2142.618	1772.867	2513.429	4985.019	1938.967	1565.209	2898.718	2946.264	2550.711	2801.768	4235.614	2523.631	5712.794
Control2_021805	1823.253	1975.388	1764.159	1876.648	2049.388	2313.581	1586.62	1558.38	1813.176	4710.699	1744.263	1476.581	3112.112	2164.224	2154.696	3056.303	4473.074	1850.957	5102.744
Control2_021806	3227.12	2526.679	3146.424	3098.552	2464.066	3832.353	2866.158	2476.056	2214.155	3592.359	3411.004	2731.398	4373.876	4083.713	2606.994	5057.475	5208.636	2913.279	7700.55
Control2_021809	7768.215	1240.79	1653.211	1662.744	1754.941	2201.225	1664.758	1504.47	1756.475	3069.143	1770.113	1568.689	2315.789	1870.303	3831.109	2834.949	3286.348	1695.697	2539.116
Control2_021810	2441.267	2074.878	2048.36	2517.456	2467.174	2497.116	2520.784	2145.236	2409.725	4595.2	2483.45	2014.932	3161.544	2782.631	2098.649	3753.405	4439.834	2398.99	3046.975
Control2_021811	2362.442	1943.874	2300.76	1417.942	1302.352	2023.503	1786.874	2726.91	1230.763	1702.497	1990.884	1592.71	2056.904	2890.082	1837.785	2695.945	3664.561	1835.566	1736.29
Control2_021812	3601.507	3159.063	3554.258	4238.192	3735.361	4730.045	3681.06	3574.706	3736.236	6074.945	5099.298	3351.37	5101.794	4984.095	3646.176	6592.742	7476.003	4860.687	5299.261
Control2_021818	1212.13	1505.043	1698.016	2248.258	3775.838	17693.85	1669.632	1509.442	1723.422	3067.722	1692.577	2609.891	2401.301	1806.957	1766.938	13912.53	2448.699	1935.279	5124.145
Control2_021822	1783.632	3716.23	1896.898	2328.595	2554.369	4283.265	2294.682	2177.366	2258.871	5106.237	2454.382	1692.475	2726.053	2685.046	1965.292	3332.935	3820.505	2458.486	4966.497
Control2_021823	1099.564	2210.086	1106.176	1579.562	2073.087	2047.938	1886.623	1732.564	1782.433	5978.676	1373.312	1059.723	2141.575	2758.369	1343.488	2322.024	2924.873	1400.678	3526.075
Control2_021824	5689.149	3643.473	5707.856	2441.248	2073.087	7271.611	1596.983	1735.312	1929.38	3387.352	5770.001	3186.82	2379.998	6112.298	1756.883	6932.623	1206.942	5871.825	7241.109
Control2_021825	1927.505	1370.127	1906.145	2845.125	2542.267	3858.186	2619.755	2365.264	2894.506	12133.5	3019.027	2065.98	3470.955	3126.288	2659.892	3660.737	4434.643	3017.324	6072.551
Control2_021826	2170.275	2212.367	2186.7	2585.956	3076.474	2347.794	2926.708	2668.387	2975.528	3619.189	2449.931	1765.577	2665.075	4058.315	1588.61	3918.005	5187.333	2401.518	3481.845
Control2_021829	727.9997	821.4169	966.9506	1252.757	1200.859	1308.101	1360.473	991.0163	1078.297	2541.436	1017.431	829.9832	1492.023	1211.206	974.2268	1802.962	2828.033	1128.866	2941.595
Control2_021831	1082.576	1191.876	1338.483	1541.967	1694.252	1659.618	1599.125	1224.445	1388.789	17426.87	1383.007	1163.831	1803.056	2659.547	1234.548	2592.773	3259.55	1438.736	2054.688
Control2_021834	2115.083	2133.577	2971.31	2478.802	2751.062	4492.736	2664.171	2371.209	2731.65	3946.079	2371.834	2448.149	3712.545	3132.718	2709.399	4566.653	5304.744	2605.529	7395.801
Control2_021835	1800.552	4143.1	2827.079	2165.51	1599.139	3511.991	1954.184	1569.674	1425.636	4211.808	2462.035	1860.317	2387.911	3136.524	1843.991	4033.994	3249.438	2702.522	3695.011
Control2_021836	1245.328	3282.619	1606.868	2021.895	2213.233	2169.312	1826.482	1525.452	2048.427	3871.687	1729.768	1484.16	2294.109	1869.645	2803.469	3491.743	3627.928	1791.521	2439.615
Control2_021837	1232.15	1843.519	1565.995	1737.465	1662.996	3514.607	1634.608	1499.075	1745.759	2669.79	1544.561	1699.246	2605.019	2069.25	1769.233	3720.748	3504.717	1782.507	4207.138
Control2_021839	1520.202	1957.838	1960.711	2279.169	2273.681	3209.642	2471.854	2103.487	2341.041	3813.47	2127.806	1811.276	2915.447	2172.454	2231.778	3304.446	3685.084	2180.862	5415.791
Control2_021840	1006.846	1466.336	1352.943	1436.497	1554.106	2073.867	1482.862	2043.637	1466.736	2785.596	1425.364	1161.345	1872.754	1764.8	1310.607	2112.964	2436.706	1458.616	3747.051
Control2_021844	1527.763	2143.553	2124.143	2583.149	2526.574	3471.964	2444.126	2209.262	2580.429	5380.09	2196.979	1899.78	3372.265	2346.553	2283.847	3591.101	5118.613	2345.115	5396.899
Control2_021845	1708.471	2597.062	3056.738	2764.51	2509.027	3627.698	2486.593	2214.397	2724.959	4277.382	2346.401	1932.341	3312.14	2775.452	2272.65	4054.93	6627.734	2873.661	6528.291

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Control2_021848	1511.619	1337.722	1397.268	1647.631	1456.605	2385.061	1408.622	1224.608	1456.744	2689.313	1499.23	1191.049	2159.648	6106.434	1689.162	11687.5	2823.277	1698.773	4285.151
Control2_021849	1866.328	2832.416	4456.51	2224.86	2056.209	3537.863	2201.666	1893.812	1987.449	3206.646	2118.117	2061.679	4011.126	3273.238	3212.107	4313.128	4844.187	1940.267	5146.62
Control2_021850	2220.246	1058.777	1760.467	2172.435	1948.469	2902.667	2243.549	1943.425	2179.94	2594.357	2051.556	1817.563	2913.705	2595.519	1710.077	2985.272	10907.08	2022.729	4563.897
Control2_021851	3033.04	2883.127	3632.569	3669.286	3355.756	4199.051	3220.745	3010.425	3358.091	5593.778	3204.729	3319.564	7174.081	5437.286	2906.247	5124.673	6914.633	3478.561	5951.68
Control2_021852	1652.118	1463.437	1920.526	2158.32	1879.306	2640.924	2086.825	1879.865	1963.275	2311.181	1711.839	1746.169	2653.722	2219.421	1947.486	3140.212	3194.615	2367.34	5520.482
Control2_021853	891.6693	1094.034	1197.545	1478.783	1579.288	1847.533	1463.02	1369.585	1542.191	2405.584	1254.529	2225.92	1840.036	1395.407	1300.163	3414.311	2726.988	1324.848	4586.414
Control2_021855	1430.95	1762.848	1859.345	2189.602	2208.481	2695.143	2471.515	1924.412	2092.828	5193.978	1821.72	1955.116	3112.859	2274.963	4717.033	3128.178	4070.028	2258.982	6357.409
Control2_021861	1597.079	1676.007	1582.567	1887.047	1917.212	2577.912	2051.175	1694.966	1766.345	2249.844	1485.959	1441.116	2357.153	1741.011	1846.351	3192.134	3243.591	1926.237	3090.701
Control2_021862	1113.862	1470.806	1490.145	1769.074	1888.085	2226.298	1665.863	1505.797	1758.152	3119.105	1388.473	1208.254	2264.852	1470.605	1345.939	2384.382	2742.069	1728.213	3670.252
Control2_021864	1668.539	1449.478	1434.442	1329.608	1308.309	1871.11	1584.434	1139.643	1228.498	1617.374	1600.64	1097.203	1773.925	1405.638	1423.723	2487.983	2064.618	1366.074	2795.789
Control2_021866	2106.947	3537.804	2356.46	3515.985	3030.057	4083.057	3325.615	2975.695	3340.706	3692.312	3083.481	2305.219	4003.694	3172.705	3376.394	5048.73	4946.702	3457.549	6293.909
Control2_021869	3801.609	4345.063	4445.315	2569.386	2154.123	5376.007	1874.028	1627.357	2041.283	5513.359	4128.772	3249.421	2908.544	5007.78	2079.015	5426.798	5380.724	4940.874	4144.626
Control2_021870	1405.225	1733.58	1940.332	2278.261	2483.545	3020.657	2064.894	1919.133	2274.004	5381.599	3390.585	1763.338	3202.194	2078.389	2362.266	4841.695	4649.188	2360.201	5403.713
Control2_021872	1286.815	7318.886	2611.127	2152.928	2157.179	2850.372	2268.582	2110.246	2353.007	7394.805	1774.071	1518.602	2707.483	2249.85	1797.54	3300.761	4053.954	2168.691	4660.637
Control2_021874	1724.234	2365.071	2544.429	2717.166	2505.942	3590.685	2480.078	2400.147	2754.599	5223.595	2337.521	1887.844	3652.429	2367.014	2208.745	3585.306	5739.748	2704.132	5517.491
Control2_021875	1417.375	1366.168	1691.543	2298.582	2233.337	2976.039	2081.084	2989.963	2835.615	6109.194	1990.811	1606.504	2701.638	2239.367	1994.916	3145.048	3000.913	2236.848	5457.432
Control2_021876	1537.92	1520.497	1819.945	2074.254	2079.594	2127.166	1807.031	1649.114	2026.153	5614.535	1726.306	1692.19	2859.931	2391.009	3490.562	2938.155	4877.939	1748.16	2883.442
Control2_021877	2169.568	2520.335	2805.955	2901.827	2795.744	3439.995	2824.899	2605.987	2683.601	4044.607	2816.4	2392.499	3991.897	3151.645	2617.009	6545.128	13734.66	3055.266	4792.461
Control2_021882	1853.488	1463.824	2709.683	2630.134	2613.521	4889.535	2481.181	2151.083	2432.832	5635.096	2318.964	3012.98	3779.524	3017.525	2502.86	9005.231	6358.341	2580.803	4540.223
Control2_021884	1966.939	1262.075	1971.59	2222.155	2054.503	2464.76	2088.945	1878.722	2162.308	4175.698	1896.357	1930.513	2802.656	2045.35	2109.724	3084.8	5077.241	2120.757	3006.417
Control2_021885	2316.15	1641.508	2386.34	2210.695	2094.819	2590.088	2073.195	1878.869	2322.551	2360.523	2020.608	2019.363	2912.89	3519.932	3397.924	3343.496	4043.697	2422.662	3089.727
Control2_021887	1531.977	1308.395	1976.089	1951.533	1830.006	2269.807	1804.872	1627.947	1907.668	8062.939	1698.869	1543.445	1760.354	2107.129	2829.737	6973.591		1813.83	2448.871
Control2_021888	1816.136	1727.809	2177.399	2355.914	2248.237	3075.7	2270.959	2016.192	2362.468	6705.703	2263.509	2009.607	3116.192	2917.681	2180.773	10092.09	6037.169	2303.328	3242.51
Control2_021889	1191.999	1224.372	1456.478	1671.608	1769.808	1729.559	1738.464	1479.342	1724.205	3193.269	1365.617	1264.229	2102.197	4013.068	1402.45	2661.556	4983.02	1468.28	2082.255

Sample	Values
Control2_021893	961.8649, 1231.316, 1339.618, 1244.098, 1399.984, 1578.939, 1009.998, 1266.354, 1532.566, 1124.324, 1133.104, 1673.636, 1486.05, 1290.029, 2445.214, 2708.811, 1151.813, 2311.744
Control2_021896	1175.956, 1869.874, 1767.732, 1848.923, 2319.642, 1848.402, 1313.737, 1786.878, 3973.447, 1376.932, 1401.714, 2109.678, 1795.883, 1920, 2577.86, 5293.315, 1487.054, 2189.525
Control2_021898	1421.408, 1274.498, 1793.884, 2191.324, 2017.862, 2054.023, 1780.855, 2316.975, 4514.717, 2123.111, 1578.816, 2815.736, 2178.103, 1781.437, 15068.83, 4513.11, 1985.627, 2620.897
Control2_021899	1169.115, 1018.919, 1380.195, 1747.41, 1623.652, 1759.251, 1608.307, 2066.902, 2999.335, 1525.146, 1254.638, 2230.895, 2104.384, 1492.37, 2394.735, 3072.453, 1643.78, 2270.203
Control2_021900	1805.134, 2272.669, 2339.801, 2877.322, 2870.634, 3154.306, 2943.415, 3650.904, 3837.566, 3456.82, 2574.759, 3255.242, 3745.962, 2716.952, 4014.755, 5178.692, 2951.938, 7874.77
Control2_021962	1299.376, 1469.512, 1752.46, 2072.822, 2154.644, 1847.226, 1801.589, 3048.746, 5116.496, 1706.608, 1628.084, 2814.555, 2084.987, 2067.581, 5010.089, 4188.351, 1975.819, 3179.895
EarlyStg1_021633	888.7379, 1083.956, 1139.705, 1745.988, 1624.386, 1864.5, 1599.96, 1465.2, 3890.38, 1037.369, 1451.583, 1668.078, 10190.59, 2556.25, 3108.472, 2287.157, 2088.815
EarlyStg1_021651	1718.134, 1869.381, 2002.153, 2829.701, 2301.549, 3393.112, 2191.697, 2351.123, 6750.972, 1771.912, 2854.07, 2561.211, 2358.632, 2967.15, 3584.864, 1801.215, 6011.781
EarlyStg1_021654	1414.691, 2009.296, 1875.053, 3674.002, 2959.501, 3266.428, 2816.123, 2713.403, 3945.555, 1859.56, 2002.649, 2533.283, 3189.184, 2382.979, 3204.194, 1758.753, 5859.363
EarlyStg1_021655	2360.272, 2071.366, 2048.578, 4079.875, 3856.399, 3060.038, 2967.291, 3210.863, 6902.838, 3250.834, 2143.019, 3295.438, 3250.834, 2346.357, 2888.672, 5218.852, 2056.624, 4117.299
EarlyStg1_021662	1046.279, 1038.759, 1247.205, 1608.22, 1934.638, 1675.178, 1536.208, 1641.528, 2295.528, 1315.652, 1296.914, 2313.41, 1630.089, 1478.589, 2377.041, 1865.311, 1784.796
EarlyStg1_021675	2008.526, 2211.421, 2262.93, 3836.686, 3311.173, 4348.88, 3216.422, 3272.398, 5877.409, 2722.891, 3145.656, 3171.789, 2286.916, 3358.434, 5073.502, 2378.691, 6228.465
EarlyStg1_021678	809.411, 1196.975, 1171.397, 1416.018, 1513.61, 1406.448, 1243.303, 849.612, 5240.118, 1195.363, 4169.675, 1276.357, 1124.136, 1342.022, 2590.081, 2450.418, 2513.441
EarlyStg2_021024	1666.611, 2348.44, 1861.542, 1900.167, 2175.75, 2635.829, 1641.05, 1784.729, 2599.311, 1926.939, 1678.941, 2452.743, 1825.564, 3177.2, 3875.282, 2259.64, 2789.643
EarlyStg2_021403	864.2702, 908.2656, 966.5297, 1152.405, 1152.189, 2138.762, 1193.16, 2495.09, 1988.684, 8670.834, 1149.056, 1061.351, 826.697, 1508.257, 3280.338, 1056.332, 1462.285
EarlyStg2_021435	818.7927, 946.9137, 862.6614, 1138.72, 1062.796, 1208.682, 1132.829, 999.4889, 1300.412, 812.8782, 1992.811, 3039.989, 1482.107, 1708.522, 1111.212, 2970.026
EarlyStg2_021440	1115.634, 1256.625, 1676.983, 1456.852, 1876.273, 1804.083, 1597.008, 1495.375, 6394.945, 1536.416, 1741.883, 1220.193, 1759.137, 1274.902, 2116.541, 1489.374, 2207.21
EarlyStg2_021443	2209.834, 2323.86, 2268.553, 4227.051, 2746.796, 3529.186, 2838.406, 2627.111, 3346.902, 2821.284, 2716.052, 4253.328, 3857.54, 6291.922, 3782.145, 2910.532, 4271.11
EarlyStg2_021458	2764.343, 2321.842, 3096.833, 2692.237, 2468.356, 3620.752, 2475.345, 2253.773, 5134.364, 2902.898, 2492.874, 3200.766, 7027.378, 2891.019, 3304.315, 5084.472, 3052.856, 3851.58
EarlyStg2_021462	1168.597, 1095.781, 1259.984, 1473.306, 1355.856, 1573.764, 1327.889, 1447.512, 1962.651, 1353.171, 1190.796, 1692.075, 2083.382, 1220.256, 1848.675, 3257.003, 1419.641, 1844.949
EarlyStg2_021466	2917.855, 2471.582, 2442.846, 3334.242, 3444.158, 2739.576, 2864.376, 3539.669, 3898.782, 3162.375, 2048.981, 7417.409, 5946.867, 2380.558, 3931.357, 4237.54, 5538.389, 4529.409
EarlyStg2_021489	3814.376, 64378.83, 54002.38, 1724.073, 1619.1, 2201.599, 1538.798, 1833.571, 2224.745, 1882.389, 1828.498, 1361.955, 2600.137, 1708.257, 2492.632, 3881.379, 2120.155, 2455.012
EarlyStg2_021496	2140.219, 2044.7, 2620.907, 2860.899, 2714.917, 3523.238, 2716.084, 2532.979, 8436.43, 2620.774, 2268.858, 4002.257, 3331.943, 3119.467, 4494.802, 6993.668, 2825.078, 3852.443
EarlyStg2_021814	1434.233, 1436.983, 1273.89, 1802.932, 1544.033, 1577.473, 1621.802, 1248.348, 1184.509, 2266.998, 1189.78, 1175.504, 1631.434, 1240.605, 2024.513, 2875.169, 1276.932, 1868.431

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
EarlyStg2_021815	6157.471	3813.734	3852.406	8214.276	10197.87	7104.856	5543.301	6291.762	14434.92	7528.605	5889.771	5140.262	11153.38	23529.81	4652.537	8918.846	13542.18	10916.19	29827.34
EarlyStg2_021820	1272.23	1459.923	1404.584	2228.886	1796.769	2841.497	3885.665	4033.956	1791.473	2298.301	1780.983	1591.671	2375.364	2462.813	1846.98	2687.747	3147.172	1999.272	4358.43
EarlyStg2_021827	616.5434	786.2398	791.7878	977.2698	979.6347	1131.393	1511.574	838.4045	862.3957	1000.234	745.288	700.6462	1164.85	862.7676	735.5688	1349.104	1553.147	807.8251	2005.31
EarlyStg2_021830	1350.571	1322.662	1347.479	1637.868	1574.216	1864.733	1802.094	1318.946	1509.441	2078.313	1378.075	1235.311	1996.329	1743.144	1373.751	2402.751	2745.201	1520.204	1758.649
EarlyStg2_021832	685.3966	768.7034	771.6687	907.4298	959.8832	881.5877	1484.217	940.8926	955.0221	1441.072	749.3359	680.3148	1144.174	877.5483	693.6878	1215.148	1877.743	821.4908	989.1862
EarlyStg2_021842	559.1504	728.8939	773.4969	886.8648	874.0025	1128.443	1246.985	812.1934	787.061	1720.34	740.0612	1044.132	1072.525	843.0966	732.0325	1241.119	1438.012	792.5935	1880.585
EarlyStg2_021843	998.7775	1042.343	1037.048	1316.874	1545.713	1488.783	1464.504	1162.424	1227.167	1199.951	1252.088	932.2355	1420.142	1299.8	1218.456	1565.295	1760.286	1234.466	2293.205
EarlyStg2_021847	847.6904	834.0404	1065.834	1241.691	984.5962	1537.282	1765.055	1053.372	988.6661	1455.479	1061.089	976.3165	2364.797	1223.133	916.262	1675.788	1953.103	1106.615	2237.163
EarlyStg2_021858	2884.206	3845.013	4219.314	3940.725	3779.591	4932.521	3850.321	3779.076	3654.264	5782.754	6309.748	4035.687	5097.5	4892.059	3918.399	5739.398	7182.912	4633.46	11607.87
EarlyStg2_021867	1027.782	3742.673	1421.976	1480.335	1415.318	1834.294	1702.586	1363.897	1471.776	2148.901	1221.715	1187.602	1867.458	2958.356	1508.762	2039.943	2430.543	1407.597	3551.464
EarlyStg2_021873	1991.314	64387.9	68263.43	2576.418	2450.933	3204.567	6102.298	61141.06	2437.692	3159.13	6431.019	2019.762	3573.626	2866.673	2360.751	3891.723	6348.432	2662.724	6903.598
EarlyStg2_021895	1309.615	1142.613	1833.176	1552.477	1668.209	2018.585	1748.757	1350.093	1657.107	3322.74	1374.092	1347.937	2303.925	2011.179	1259.669	2291.862	2859.402	1732.711	1940.729
EarlyStg2_021960	2893.513	3014.18	3981.726	31577.67	4138.143	6326.917	4832.018	7208.038	4473.527	6067.125	2660.867	3715.26	5565.572	4084.967	5344.483	7626.605	11731.3	4567.441	6935.041
LateStg1_021607	2233.818	33710.4	27509.89	2683.862	2431.354	5415.112	2263.816	2008.929	2639.71	4167.536	21571.06	20747.58	2873.485	7195.459	2548.429	3653.705	5542.575	2238.342	9084.524
LateStg1_021612	1077.574	1286.721	1463.395	1459.115	1434.265	1880.945	1247.632	1032.569	1283.638	1777.645	1451.691	1189.345	1739.08	1653.534	1226.084	4710.854	27371.76	24821.54	2435.91
LateStg1_021632	3286.639	1480.678	1291.98	1680.948	1599.405	1828.069	1593.324	1280.285	1799.823	3101.039	2060.273	1602.773	5195.655	1645.745	2239.28	1861.048	3672.684	11641.46	2496.863
LateStg1_021683	1332.092	920.1105	1136.96	1548.33	1181.713	1415.653	1581.494	1335.489	1143.095	2445.638	1851.534	4298.669	8690.543	1780.728	1150.62	2402.067	3062.754	1141.802	2028.778
LateStg1_021691	3844.224	3683.971	6199.383	3335.339	3994.33	8536.385	2753.94	2924.456	2503.818	8177.706	4079.96	2844.118	2816.506	6253.56	2437.998	7010.066	4723.274	4053.18	6519.304
LateStg1_021692	1632.176	7969.794	4384.263	2698.668	2282.849	2693.694	2417.603	2147.67	8336.307	2067.293	2052.88	3534.455	2314.055	2435.415	11902.46	6559.493	12545.8	1963.734	11183.95
LateStg1_021696	6599.412	5823.924	6186.081	5783.523	4699.548	7686.687	5192.874	4561.238	5254.942	7300.942	5486.125	3686.07	10444.32	10842.8	3874.061	6828.621	7978.743	5397.418	7341.834
LateStg1_021699	908.5583	943.1396	1345.248	1535.726	1361.538	2004.212	1679.826	1162.804	1463.845	1553.582	1393.758	1065.26	1777.222	1628.989	1197.207	12961.49	3163.428	1263.17	2505.567
LateStg2_021004	3221.85	4463.517	3620.611	3510.513	3849.087	5065.177	3227.483	3134.377	3447.883	6800.578	3186.653	2627.675	3490.451	4227.487	2755.789	2133.374	7471.008	15774.18	4833.175
LateStg2_021006	2339.927	2435.977	2422.967	3627.886	4482.165	3510.889	2848.815	2488.987	3785.679	12021	3061.898	3122.281	3726.921	3191.783	3055.728	5022.605	13003.68	3081.717	4111.345
LateStg2_021028	2707.792	4469.606	3026.737	4088.437	4885.905	4633.554	4514.939	5517.361	4012.385	4351.325	3588.626	3046.919	4898.403	3893.103	4476.661	3874.278	6939.209	15318.87	4612.729

Identifier																			
LateStg2_021029	4950.915	4573.694	5365.522	5212.313	5258.751	7136.894	4720.304	4089.176	4817.28	7556.265	5566.21	4689.437	4079.114	8339.843	3731.415	3904.665	18153.11	5939.745	9798.426
LateStg2_021039	3522.816	3382.284	4025.145	4416.734	5475.508	4942.533	4098.692	4279.457	4220.891	10757.38	5050.079	3931.166	3348.218	7727.377	3605.788	4050.392	11286.33	31136.4	10124.7
LateStg2_021040	2002.684	1739.248	2184.697	2733.813	2964.582	2841.992	2364.913	2064.116	2472.303	12349.52	3101.401	2486.638	2116.17	3078.854	2011.853	2894.685	6237.084	12377.03	3488.47
LateStg2_021402	1361.212	1273.69	1436.17	30543.01	1661.542	2105.953	1792.681	1556.671	1665.538	10941.54	2091.446	2164.561	3789.045	23209.96	3864.334	2131.699	1684.28	1855.743	2460.073
LateStg2_021404	1825.759	1561.872	1859.06	2102.176	1937.207	2621.452	1884.312	1886.038	2103.374	10730.8	2619.036	2301.383	1848.497	2114.464	2806.652	3262.728	5923.29	2109.56	2837.274
LateStg2_021409	1821.798	1622.475	1888.661	2005.027	2042.316	2466.845	2172.961	1965.279	2287.658	2560.275	2258.806	2146.635	1698.201	2988.973	6123.263	45207.66	3710.094	2240.805	2672.31
LateStg2_021411	12831.44	2658.677	2981.266	3455.966	66480.3	3872.695	2669.909	2881	3275.688	5937.628	3631.025	3641.948	2783.715	3842.071	2780.356	9740.508	8277.123	86593.27	4647.849
LateStg2_021412	1375.199	1239.54	1325.421	1639.334	3807.727	1852.533	1571.277	1348.274	1421.224	1966.427	1777.86	1540.838	1344.967	1717.123	1367.526	2384.968	2527.974	4301.716	2051.114
LateStg2_021413	1771.981	1487.26	2007.343	1924.228	2049.404	2192.016	1768.964	1748.733	1909.862	8649.381	2701.412	1902.606	1753.411	2172.419	2608.381	3445.016	26526.73	2274.128	2877.102
LateStg2_021418	1312.756	1172.529	1327.484	1582.212	1511.383	2946.084	1518.947	1320.067	1400.382	1756.549	1712.24	6483.009	12480.7	1907.998	1232.774	2193.431	2718.157	1707.805	2045.315
LateStg2_021421	3372.893	1793.388	1865.367	1997.554	2003.966	2556.354	2058.865	1860.537	2016.597	3387.465	2362.854	1986.933	1592.037	2431.391	1757.695	7521.871	6986.991	2146.607	2784.716
LateStg2_021422	2876.725	2164.961	2960.132	2820.349	4247.47	3985.439	2012.289	1996.894	2534.09	9135.84	2880.509	3163.03	2608.115	3781.505	2337.046	6197.789	5642.467	51156.32	3961.097
LateStg2_021424	1510.005	1436.097	1764.94	2312.824	2095.89	2981.925	2034.561	2011.813	2096.701	5080.653	1669.388	2221.863	2880.509	2746.375	2017.996	3606.207	4320.564	81892.36	3396.019
LateStg2_021425	4155.837	3164.881	3549.289	4399.627	3495.884	69262.64	3726.524	3610.125	3821.13	5625.683	10796.37	5187.55	33888.13	22975.41	17817.6	8637.526	10970.58	4581.09	7241.283
LateStg2_021427	6471.712	5562.278	5414.813	2359.317	1931.492	5062.902	2487.975	2015.596	2006.091	4911.945	2693.277	5642.549	3571.163	6688.38	2318.45	6676.587	9097.011	5138.452	3666.584
LateStg2_021428	1315.405	1923.432	1325.323	1540.169	1567.625	1995.128	1862.058	1623.103	1391.924	1660.351	9092.653	1548.41	1357.469	1680.029	1365.524	3474.558	2805.974	1418.446	3555.568
LateStg2_021429	2123.084	2559.612	2236.494	3001.687	2607.627	3412.53	2292.985	2415.871	2844.512	4992.574	3387.576	2967.538	2066.671	2708.596	2401.982	3614.203	5568.374	3001.216	4089.902
LateStg2_021431	1761.144	1820.044	2118.511	2601.028	2288.129	3056.034	2245.269	3071.717	2317.118	4144.749	3046.623	2499.042	2225.565	2595.132	2159.14	4110.286	5855.135	2399.371	4049.696
LateStg2_021432	2360.041	2259.147	2011.696	2624.354	2347.857	3114.266	2267.014	2232.296	2551.755	4449.926	2791.503	2553.865	1790.208	2231.627	2113.099	3429.028	4533.118	2518.522	3669.681
LateStg2_021433	2535.536	2996.462	2903.991	3686.228	3635.17	44392.76	3044.162	3183.385	3632.759	7242.118	4657.339	3571.259	2868.022	4197.165	3288.039	17338.42	7431.575	3735.118	5408.98
LateStg2_021437	2480.932	2964.578	2577.313	3395.92	3330.783	3915.391	2871.597	3034.91	3358.309	36422.07	5346.232	3309.372	2486.033	4058.445	5720.515	51111.89	6558.229	73076.88	5622.161
LateStg2_021441	2178.753	3019.759	2484.399	3276.622	3424.187	4497.703	4627.948	5516.538	3184.235	23989.57	3894.148	3092.49	2360.327	4081.13	7333.667	5608.731	12014.06	3198.293	5385.842
LateStg2_021442	1402.243	1687.07	1595.617	2190.64	2035.534	2949.132	1854.978	1630.612	1953.693	3097.947	1844.334	2175.145	1574.884	2440.885	3378.815	2767.79	3709.88	1951.919	4524.983
LateStg2_021450	72225	4925.92	5586.278	7038.924	6264.657	9305.774	5407.448	6106.828	62465.09	6436.29	9856.237	7744.264	5774.264	8325.764	10278.63	9800.541	11756.66	8106.801	13587.36

Probe	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17
LateStg2_021452	2351.882	2117.25	2515.784	2714.059	2709.585	3236.665	2628.204	7171.939	2770.725	2191.751	3443.649	2911.636	2955.094	3955.263	26551.05	3107.26	4242.407
LateStg2_021456	2281.465	1624.104	2069.136	2084.323	1906.072	2652.478	2295.275	2391.687	1978.448	1735.68	3055.247	2944.797	7126.781	3647.837	4024.424	2143.845	3360.468
LateStg2_021457	7111.74	5438.97	6081.31	7192.698	6515.937	8094.362	6740.558	13294.24	7297.811	7370.232	26310.33	10478.36	6932.051	9832.866	19790.06	7344.632	13282.43
LateStg2_021459	1662.911	1488.942	1709.95	5439.639	1756.361	2467.384	1685.922	1945.565	1814.398	1634.251	2973.406	2348.097	1709.713	2914.202	3567.059	1904.825	2874.884
LateStg2_021467	2871.091	2477.123	2697.42	3051.383	2669.106	4019.018	2514.498	3031.402	3030.005	3013.527	5899.702	3568.804	2425.005	5735.723	5177.811	3191.262	4545.997
LateStg2_021468	2239.595	2067.363	2455.974	2399.43	2260.627	3172.673	2192.644	5898.635	2555.232	2403.385	3099.336	3734.919	2250.546	3828.926	9905.679	2752.943	3839.993
LateStg2_021469	1134.769	1041.707	1124.96	1394.012	1423.355	1609.735	1274.357	2334.31	1346.583	1284.269	1683.193	1550.628	1463.695	2068.044	2449.744	1328.585	2466.248
LateStg2_021471	1883.788	1395.216	2140.146	2416.612	2918.033	2983.943	1685.225	2138.6	2029.309	2197.31	3165.057	2605.741	2245.675	3627.445	14696.91	2467.378	3167.771
LateStg2_021476	3258.855	2759.33	2993.915	3391.916	2972.729	4209.934	2853.457	6284.123	2345.696	2583.433	3163.012	14982.35	2189.458	4731.425	5321.727	21050.91	5567.529
LateStg2_021479	2880.929	2591.085	2935.565	3095.02	2751.518	3877.997	2929.471	2761.362	2663.487	2786.301	3713.563	16733.18	2874.51	4326.346	7389.014	3331.391	4580.993
LateStg2_021485	3236.978	2840.985	3298.95	3514.251	3336.928	4831.199	4070.197	5153.553	3019.581	3824.449	4860.078	5718.838	5384.661	6508.286	11009.77	3845.124	6364.518
LateStg2_021490	2055.398	2328.332	2304.917	2529.798	2578.518	2827.262	2270.409	2361.421	2608.352	2090.352	3147.381	3369.221	3426.581	3655.384	9930.515	2640.637	3786.199
LateStg2_021493	2089.283	1969.834	2252.854	2541.756	2554.018	3403.82	2142.646	2208.38	2510.315	2021.454	2975.269	2943.559	2136.006	54080.8	4759.46	2612.632	3790.428
LateStg2_021498	3994.694	3345.87	3519.226	4221.659	4350.851	4858.934	4285.45	3779.842	4261.771	3229.588	5198.682	4788.694	3818.572	5490.723	12841.46	5246.952	6432.803
LateStg2_021499	2712.5	2477.536	2535.785	3120.787	3083.258	4619.17	3050.456	3503.044	3132.654	2428.364	3670.777	4016.163	2945.576	8863.933	5309.148	3196.865	4388.622
LateStg2_021803	2568.571	2524.476	2509.832	2820.704	2609.458	2990.457	2279.461	2550.41	2471.884	2004.119	3726.039	3126.381	2455.32	3660.465	5247.491	2842.318	5724.04
LateStg2_021817	2184.182	2720.577	2811.529	4809.278	63866.5	4006.276	2172.796	3489.376	2882.902	2628.133	4423.612	3248.255	5146.921	6176.601	6944.245	3376.811	9550.769
LateStg2_021819	2690.661	2528.864	2868.553	3699.345	4265.994	3856.545	2969.606	3685.966	3108.269	2545.667	4701.069	3748.161	3673.165	5193.176	6400.219	3449.647	7443.841
LateStg2_021821	3862.728	5570.37	2950.673	3903.449	3465.224	5141.088	2991.057	3855.53	3294.47	2903.67	5139.618	3552.171	3290.416	5084.206	6759.796	3728.741	8677.661
LateStg2_021828	3612.2	32668.33	13719.52	3451.276	4548.635	5624.391	3771.425	3411.682	3897.674	3308.141	5759.796	4285.685	3254.309	7498.517	9501.756	4700.525	9935.705
LateStg2_021833	988.1922	1768.821	1152.149	1436.283	1414.762	1567.374	1619.189	1172.344	1216.094	974.8655	1689.938	1283.639	1469.673	2038.617	2763.421	1149.49	1673.634
LateStg2_021838	1659.286	2605.402	1884.594	2379.837	2659.232	2909.178	2613.869	2226.825	2289.099	1881.045	2790.638	2913.932	1876.231	3387.326	4041.425	4749.024	5877.246
LateStg2_021841	7280.658	8393.207	6239.35	5096.393	4927.956	7779.298	4618.61	4848.952	7852.988	5259.368	6938.347	7712.883	4960.22	9289.899	9842.119	12962.39	12360.34
LateStg2_021846	1017.125	1024.531	1290.688	1716.599	2259.379	2301.196	1865.429	2159.849	1474.7	1227.702	2012.191	1497.391	1289.456	2194.223	3448.56	1486.978	3482.839

ID																		
LateStg2_021854	1631.081	2256.517	1994.206	2089.055	2115.195	2810.059	2315.125	1818.905	2089.664	2749.929	2461.663	2074.198	2346.469	1614.691	2547.062	15433.09	2182.438	2600.972
LateStg2_021856	1757.762	1863.022	1772.417	2268.092	2477.31	2647.954	2176.189	1961.227	2287.684	4127.567	2147.37	1690.916	2545.96	1702.953	2811.449	6186.714	2217.469	4138.507
LateStg2_021857	3901.552	3670.615	3626.944	4188.205	4158.46	4751.309	3987.096	3730.004	3967.139	5754.583	3596.755	3645.516	5079.897	4070.815	12466.72	8308.568	4216.411	8964.893
LateStg2_021859	1470.294	1885.057	1935.032	2271.902	2294.143	3048.345	2117.187	1776.545	2313.868	3634.111	1929.03	1781.592	2948.952	1991.047	3284.598	3241.526	2186.996	4791.754
LateStg2_021860	1377.228	1912.527	1545.966	2029.096	3843.036	2294.62	2148.919	1831.696	1964.879	2275.137	1656.999	1510.77	2197.393	2036.264	2753.512	3284.302	4857.857	3644.717
LateStg2_021863	1389.546	1225.311	1220.319	1592.922	1506.453	1743.039	37436.57	20785.09	1770.846	2499.974	1226.013	1140.628	1928.769	1697.19	2135.424	5495.126	77817.12	3516.442
LateStg2_021865	847.9526	72243.9	58963.65	1584.339	1561.829	1890.786	1446.563	1301.552	1523.649	2533.475	1172.621	1082.984	2098.228	1465.648	2245.101	2560.516	15878.09	2716.509
LateStg2_021868	841.1623	1730.123	1106.992	1520.119	1326.74	1839.049	1526.568	1216.93	1359.645	2195.805	1229.105	1027.971	1746.367	1267.317	2054.09	2606.964	1274.319	3338.825
LateStg2_021871	4293.53	11133.89	6076.056	6914.307	6755.31	13075.63	5942.146	5916.069	7184.004	15378.62	6834.217	5741.78	10556.95	7260.707	32976.48	12429.49	8715.363	19074.28
LateStg2_021880	1790.208	1980.132	2614.285	2057.024	2096.017	3959.8	1832.259	1487.634	2133.393	4583.188	1977.947	1747.046	2667.728	1632.411	3510.768	4512.184	2456.451	3226.212
LateStg2_021881	2384.826	1999.357	2666.056	2852.15	3033.592	3377.481	4271.626	3901.21	2895.45	7062.843	2719.433	2410.467	3115.291	4520.945	4258.419	12393.42	2759.778	4259.74
LateStg2_021883	2065.41	1622.946	2569.527	2671.253	4642.282	3220.608	2297.504	2061.914	2484.5	16564.51	2198.001	2264.344	3763.834	17526.91	4550.17	6416.215	2650.601	3773.043
LateStg2_021890	3022.65	1988.88	2282.66	2251.566	2248.979	3052.289	2415.519	2128.133	2178.214	16641.9	2523.614	2173.581	3724.101	2897.678	7377.704	5154.293	2388.609	3284.202
LateStg2_021891	1124.762	961.2568	1354.482	1361.077	1241.26	1576.201	1462.741	1096.385	1186.718	1491.571	1603.177	1328.941	3133.506	1392.966	3067.306	2590.952	1235.433	1900.166
LateStg2_021892	2474.483	4105.839	4501.21	2967.051	2868.365	4078.879	2353.423	2167.423	2937.444	5562.712	3328.625	2585.967	4427.688	3443.103	4587.612	6758.511	3181.595	4032.283
LateStg2_021894	2221.702	1767.34	2488.434	1923.35	2652.345	5760.332	4985.451	1684.064	1890.908	3334.574	2396.437	2163.834	2887.473	3018.096	4094.685	4474.155	5663.077	2795.796
LateStg2_021897	2094.173	1872.113	2564.54	3134.878	3177.1	3170.251	2812.481	2291.9	3112.535	6338.442	2545.71	2285.715	3852.078	3186.883	6763.967	6534.485	2598.865	3976.887
LateStg2_021959	1944.575	1075.842	1370.23	6504.857	55850.97	2370.069	1624.237	1465.625	1477.145	6609.375	1137.616	1249.313	4849.732	1808.159	2373.879	3499.995	1359.307	2181.833
LateStg2_021961	2126.008	2601.519	2931.139	3868.475	3742.739	5313.381	3308.218	3188.797	3932.856	8437.228	3262.102	2669.569	4774.873	3491.703	5256.041	6366.071	3609.642	5268.444
LateStg2_021499	2712.5	2477.536	2535.785	3120.787	3083.258	4619.17	3050.456	3503.044	3477.192	5287.922	3132.654	2428.364	3670.777	2945.576	8863.933	5309.148	3196.865	4388.622

Table 7

	ROC	Variables	Sens	Spec	ROCSD	SensSD	SpecSD	biomarker.panel	AUC
1	0.832	7	0.753	0.721	0.051	0.046	0.216	XAGE1D,LRRFIP2,MAGEA10,GAGE2C,STAT1,ZNRD1,RAD23B	0.818
2	0.819	5	0.723	0.752	0.044	0.123	0.091	XAGE1D,LRRFIP2,STAT1,FADD,RAD23B	0.812
3	0.821	6	0.737	0.752	0.089	0.126	0.127	XAGE1D,LRRFIP2,STAT1,GAGE1,FADD,RAD23B	0.812
4	0.821	11	0.770	0.623	0.081	0.126	0.059	XAGE1D,LRRFIP2,GAGE2C,DDX43,STAT1,CT47A1,GAGE1,MAP2K5,CTAG2,FADD,RAD23B	0.812
5	0.814	8	0.782	0.738	0.063	0.051	0.121	XAGE1D,LRRFIP2,GAGE1,MAGEA4,STAT1,ZNRD1,CTAG2,CTAG1A	0.811
6	0.816	7	0.756	0.723	0.057	0.106	0.086	XAGE1D,DDX43,LRRFIP2,GAGE1,GAGE2C,STAT1,PTPN20A	0.809
7	0.818	8	0.754	0.681	0.083	0.119	0.107	XAGE1D,LRRFIP2,DDX43,MAGEA10,GAGE2C,STAT1,CTAG2,ZNRD1	0.809
8	0.819	6	0.740	0.799	0.117	0.138	0.091	XAGE1D,LRRFIP2,MAGEA10,STAT1,RAD23B,CTAG2	0.808
9	0.828	7	0.737	0.754	0.067	0.126	0.107	XAGE1D,CT47A1,LRRFIP2,GAGE1,STAT1,ZNRD1,RAD23B	0.808
10	0.815	11	0.768	0.753	0.031	0.117	0.085	XAGE1D,LRRFIP2,GAGE2C,CT47A1,STAT1,GAGE1,MAGEA4,ZNRD1,DDX53,MAP2K5,RAD23B	0.808
11	0.807	8	0.695	0.738	0.058	0.141	0.044	XAGE1D,LRRFIP2,DDX43,STAT1,GAGE2C,MAGEA10,GAGE1,FADD	0.807
12	0.813	5	0.742	0.738	0.093	0.146	0.131	XAGE1D,LRRFIP2,STAT1,FADD,CTAG2	0.807
13	0.809	9	0.766	0.769	0.081	0.135	0.115	XAGE1D,LRRFIP2,DDX43,GAGE1,STAT1,MAGEA4,CTAG2,RAD23B,FADD	0.807
14	0.826	8	0.724	0.740	0.086	0.179	0.138	XAGE1D,LRRFIP2,DDX43,GAGE2C,CT47A1,STAT1,CTAG2,RAD23B	0.807

15	0.808	9	0.737	0.751	0.049	0.103	0.123	XAGE1D,LRRFIP2,GAGE2C,STAT1,MAGEA4,MAGEA10,MAP2K5,CTAG2,TPM1	0.806
16	0.805	8	0.767	0.765	0.050	0.097	0.141	XAGE1D,LRRFIP2,GAGE2C,GAGE1,MAGEA4,STAT1,ZNRD1,TPM1	0.806
17	0.814	7	0.740	0.724	0.028	0.107	0.080	XAGE1D,LRRFIP2,GAGE1,DDX53,MAGEA4,STAT1,MAP2K5	0.806
18	0.816	7	0.712	0.738	0.067	0.097	0.084	XAGE1D,LRRFIP2,CT47A1,GAGE2C,GAGE1,STAT1,ZNRD1	0.806
19	0.817	10	0.770	0.622	0.115	0.162	0.132	XAGE1D,LRRFIP2,DDX43,STAT1,CT47A1,GAGE1,GAGE2C,MAP2K5,ZNRD1,CTAG2	0.805
20	0.812	6	0.740	0.741	0.085	0.118	0.137	XAGE1D,DDX43,LRRFIP2,GAGE1,STAT1,CTAG2	0.805
21	0.707	19	0.680	0.652	0.096	0.142	0.139	XAGE1D,LRRFIP2,DDX43,DDX53,GAGE1,GAGE2C,STAT1,CT47A1,MAP2K5,DDX53,ZNRD1,MAGEA10,CTAG2,MAGEA4,IGF2BP3,TPM1,FADD,RAD23B,CTAG1A,PTPN20A	0.702
22	0.731	3	0.710	0.649	0.095	0.088	0.218	XAGE1D,LRRFIP2,GAGE2C,	0.727

Claims

1. A method for diagnosing Non-Small Cell Lung Cancer from a sample extracted from a subject, comprising the steps of:

    (i)   testing the sample for the presence of biomarkers specific for Non-Small Cell Lung Cancer;

    (ii)   determining whether the subject has Non-Small Cell Lung Cancer based on the detection of said biomarkers;

    characterised in that the biomarkers are autoantibodies to antigens comprising XAGE1D, LRRFIP2 and GAGE2C.

2. The method according to claim 1 wherein the antigens further comprise one or more of DDX53, DDX43, GAGE1, MAGEA10, ZNRDl, MAP2K5, MAGEA4, STAT1, CT47A1, IGF2BP3, CTAG2, RAD23B, FADD, PTPN20A, TPM1, CTAG1A.

3. The method according to claim 1 or 2 wherein the antigens are biotinylated proteins.

4. The method according to claim 3 wherein each biotinylated protein is formed from a Biotin Carboxyl Carrier Protein folding marker which is fused in-frame with a protein.

5. The method according to claim 3 or 4 wherein the biotinylated proteins are bound to a streptavidin-coated substrate.

6.   The method according to claim 5 wherein the substrate comprises a hydrogel-
forming polymer base layer.

7.   The method according to any preceding claim wherein the antigens are exposed to
a sample extracted from a person, such that autoantibody biomarkers from the
sample may bind to the antigens.

8.   The method according to claim 7 wherein the antigens are subsequently exposed to
a fluorescently-tagged secondary antibody to allow the amount of any
autoantibodies from the sample bound to the antigens to be determined.

9.   The method according to claim 8 wherein the presence of non-small cell lung
cancer corresponds to the relative or absolute amount of autoantibodies from the
sample specifically binding to the antigens.

10.   The method according to any preceding claim wherein the sample comprises any
or any combination of exosomes, blood, serum, plasma, urine, saliva, amniotic
fluid, cerebrospinal fluid, breast milk, semen or bile.

11.   The method according to any preceding claim wherein the steps are performed *in
vitro*.

12.   The method according to any preceding claim wherein the method comprises detecting upregulation/downregulation of one or more of said biomarkers.

13.   A method for manufacturing a kit for diagnosing Non-Small Cell Lung Cancer from a sample extracted from a subject, comprising the steps of:

for each antigen in a panel, cloning a biotin carboxyl carrier protein folding marker in-frame with a gene encoding the antigen and expressing the resulting biotinylated antigen;

binding the biotinylated proteins to addressable locations on one or more streptavidin-coated substrates, thereby forming an antigen array ;

such that   the amount of autoantibodies from the sample binding to the antigens on the panel can be determined by exposing the substrate to the sample and measuring the response;

characterised in that the antigens comprise XAGE1D, LRRFIP2 and GAGE2C.

14.   The method according to claim 13 wherein the antigens further comprise one or more of DDX53, DDX43, GAGE1, MAGEA10, ZNRD1, MAP2K5, MAGEA4, STAT1, CT47A1, IGF2BP3, CTAG2, RAD23B, FADD, PTPN20A, TPM1, CTAG1A.

15.   A composition comprising a panel of antigens for detecting non-small cell lung cancer, characterised in that the antigens comprise XAGE1D, LRRFIP2 and GAGE2C.

16.   A composition according to claim 15 wherein the antigens further comprise one or more of DDX53, DDX43, GAGE1, MAGEA10, ZNRD1, MAP2K5, MAGEA4, STAT1, CT47A1, IGF2BP3, CTAG2, RAD23B, FADD, PTPN20A, TPM1, CTAG1A.

17.   A composition according to claim 15 or 16 wherein the antigens are biotinylated proteins.

18.   A composition according to any of claims 15-17 wherein the amount of one or more exosomal autoantibody biomarkers binding *in vitro* to the antigens in a sample from a patient can be measured to determine the presence of non-small cell lung cancer.

19.   A composition comprising a panel of exosomal autoantibody biomarkers for detecting non-small cell lung cancer:

wherein the levels of exosomal autoantibody biomarkers are measured in a sample from a NSCLC patient;

characterised in that the exosomal autoantibody biomarkers are selected from autoantibodies specific for at least X Antigen Family Member ID (XAGE1D), LRR Binding FLU Interacting Protein 2 (LRRFIP2) and G Antigen 2C (GAGE2C).

Figure 1
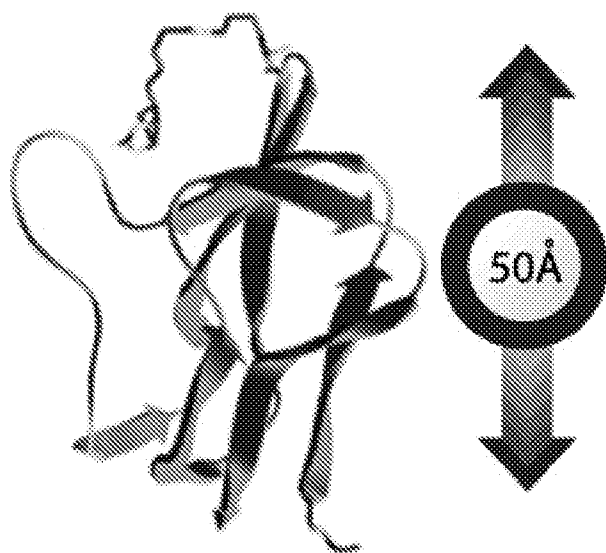
Figure 2
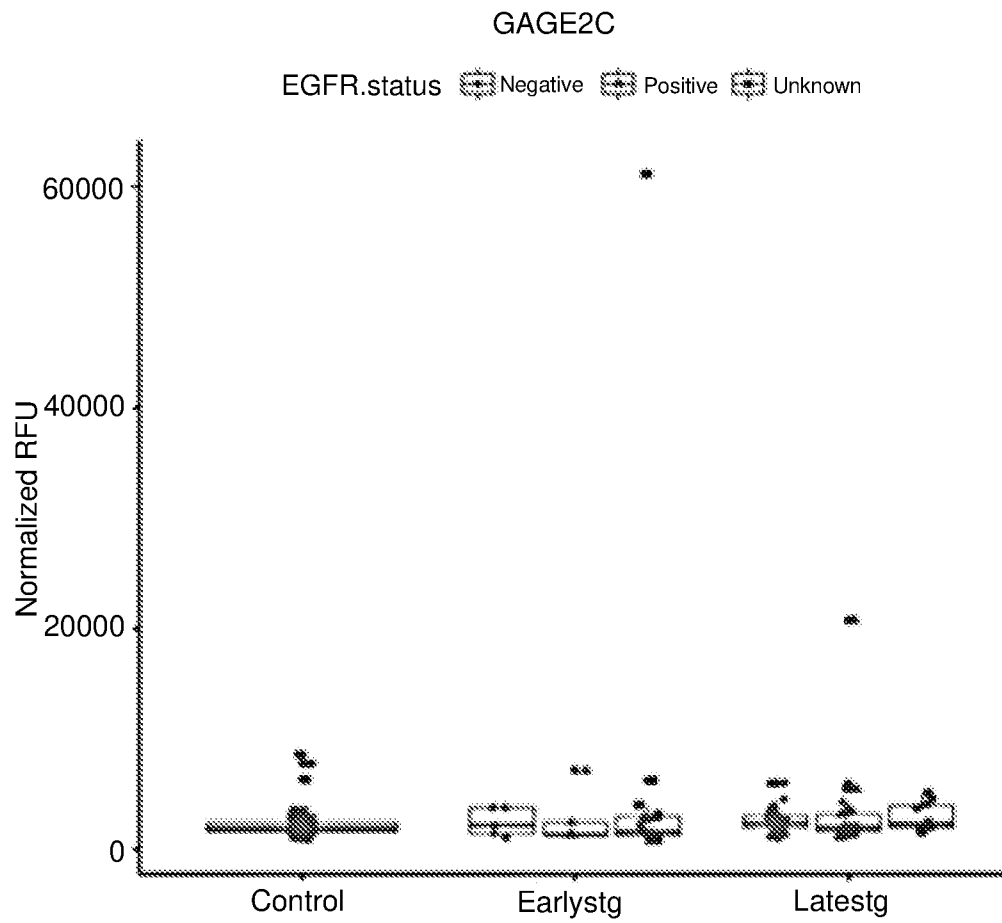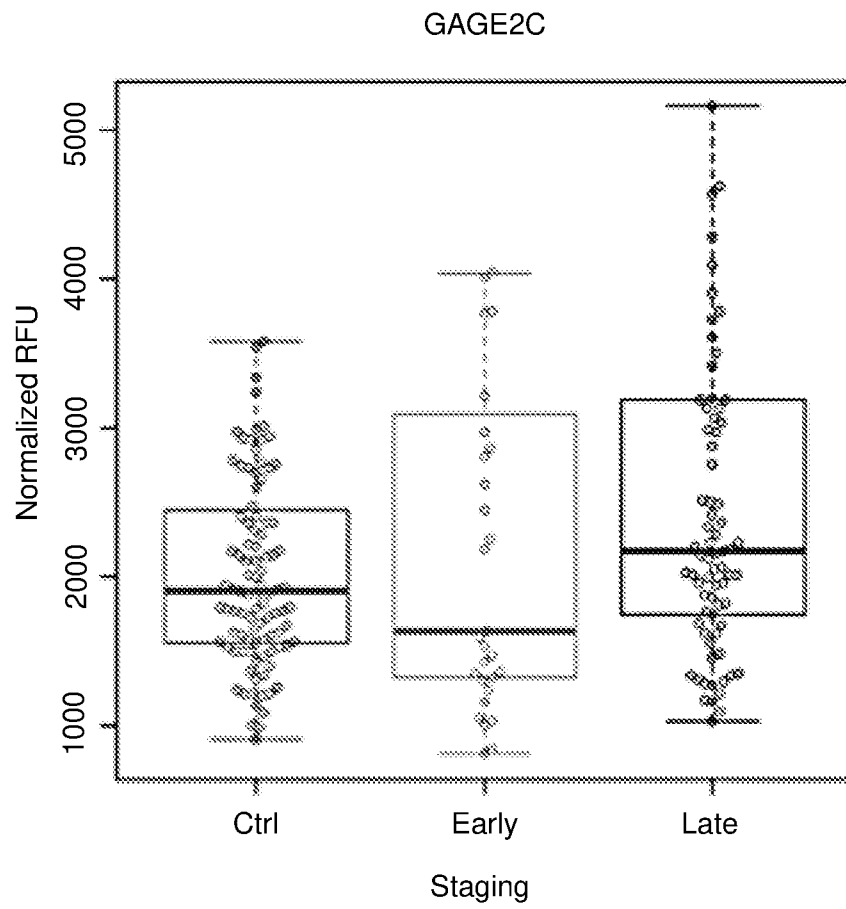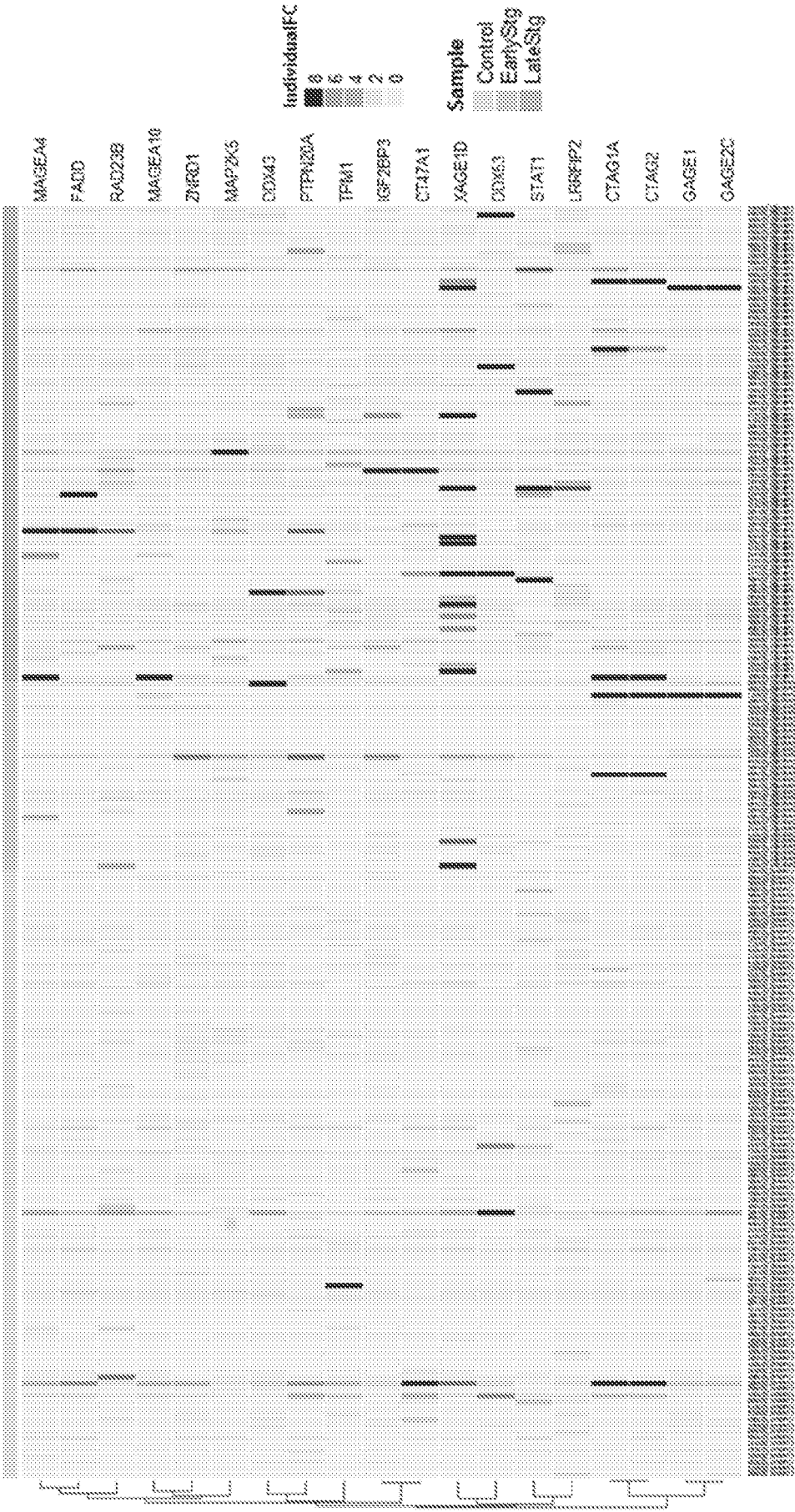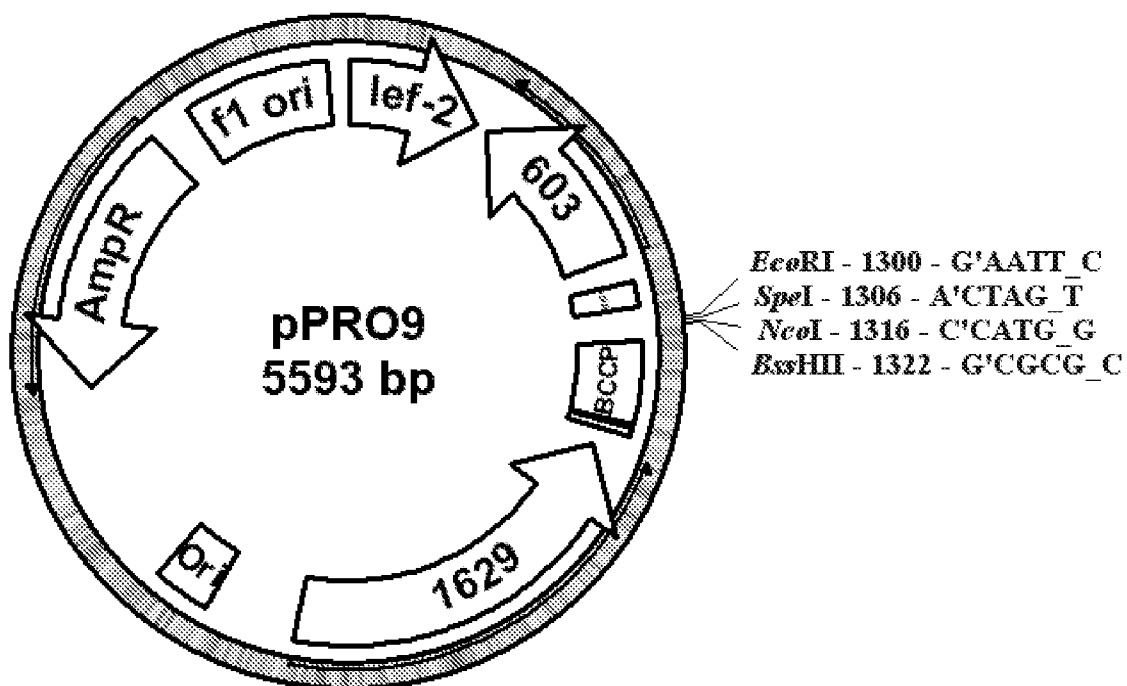
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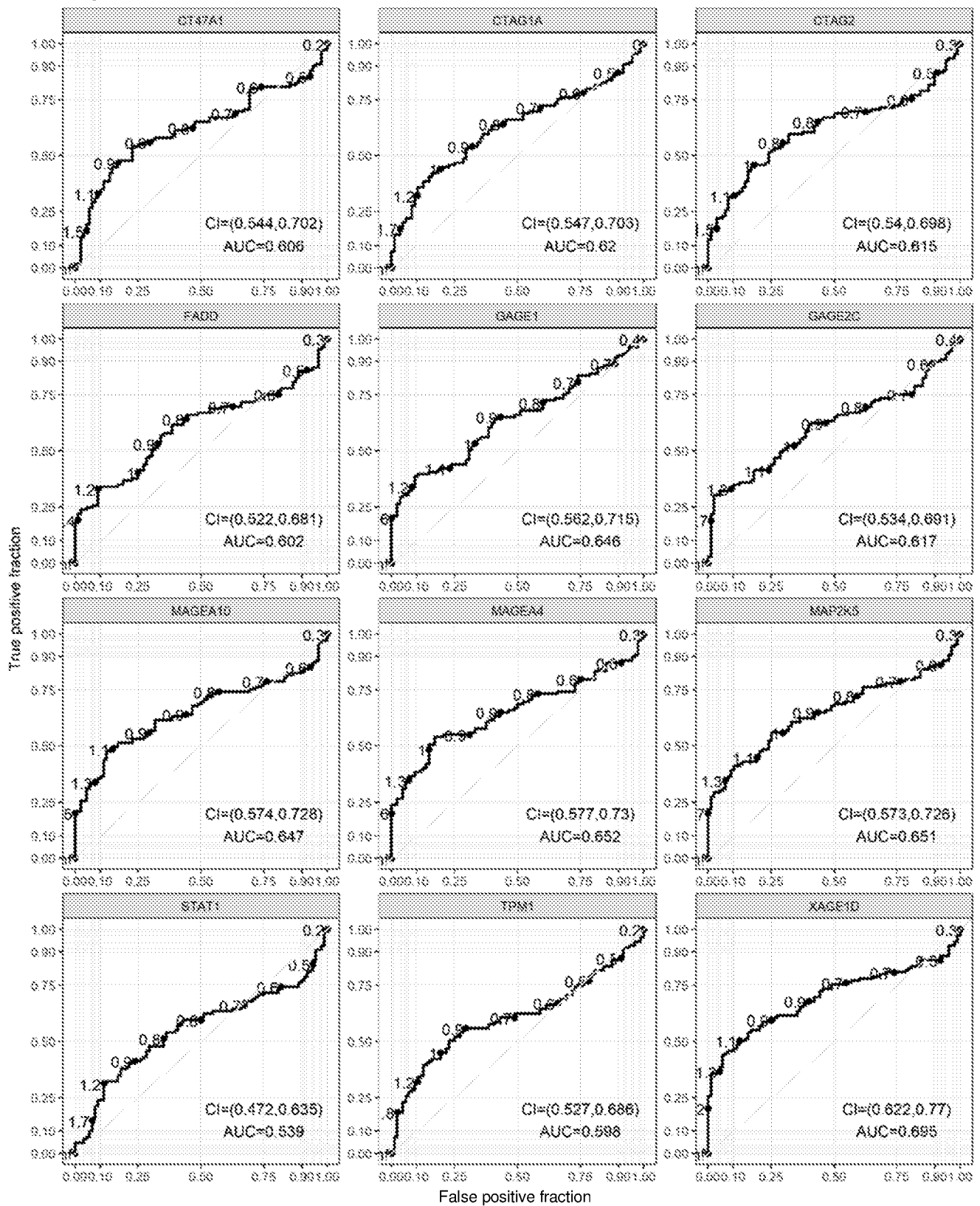
Figure 3 (continued)

Figure 4

Figure 5

XAGE1D

EGFR.status   Negative   Positive   Unknown

LRRFIP2

EGFR.status   Negative   Positive   Unknown
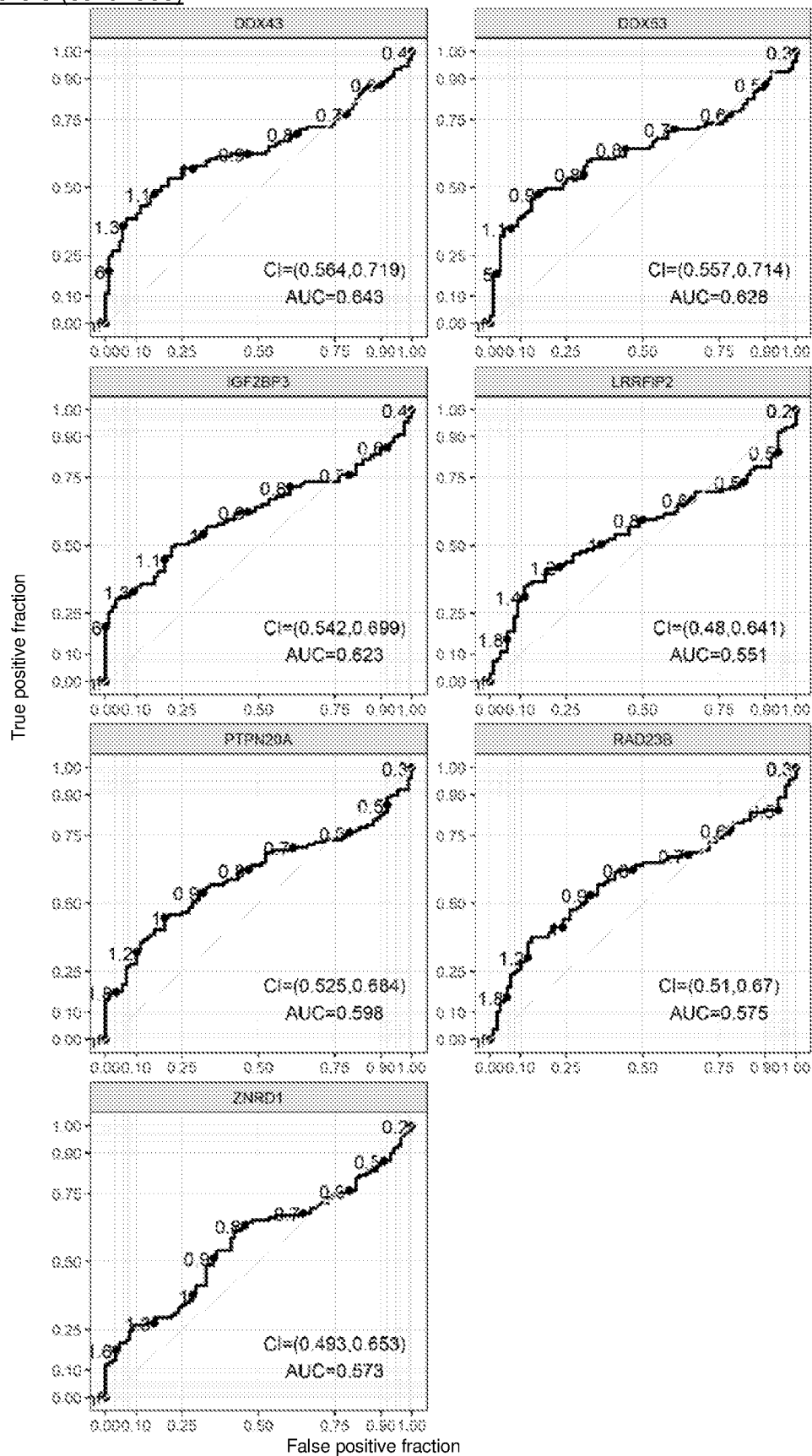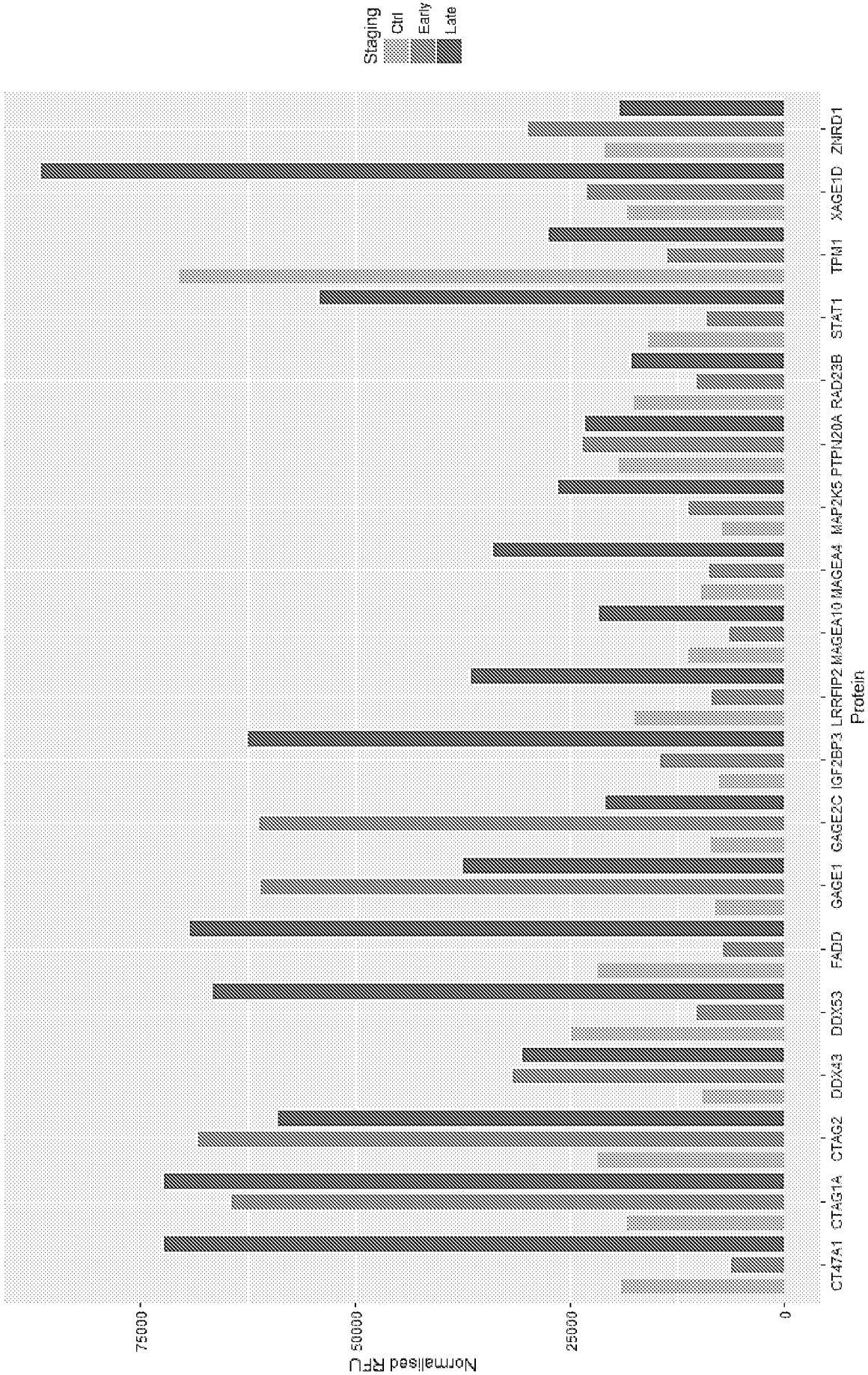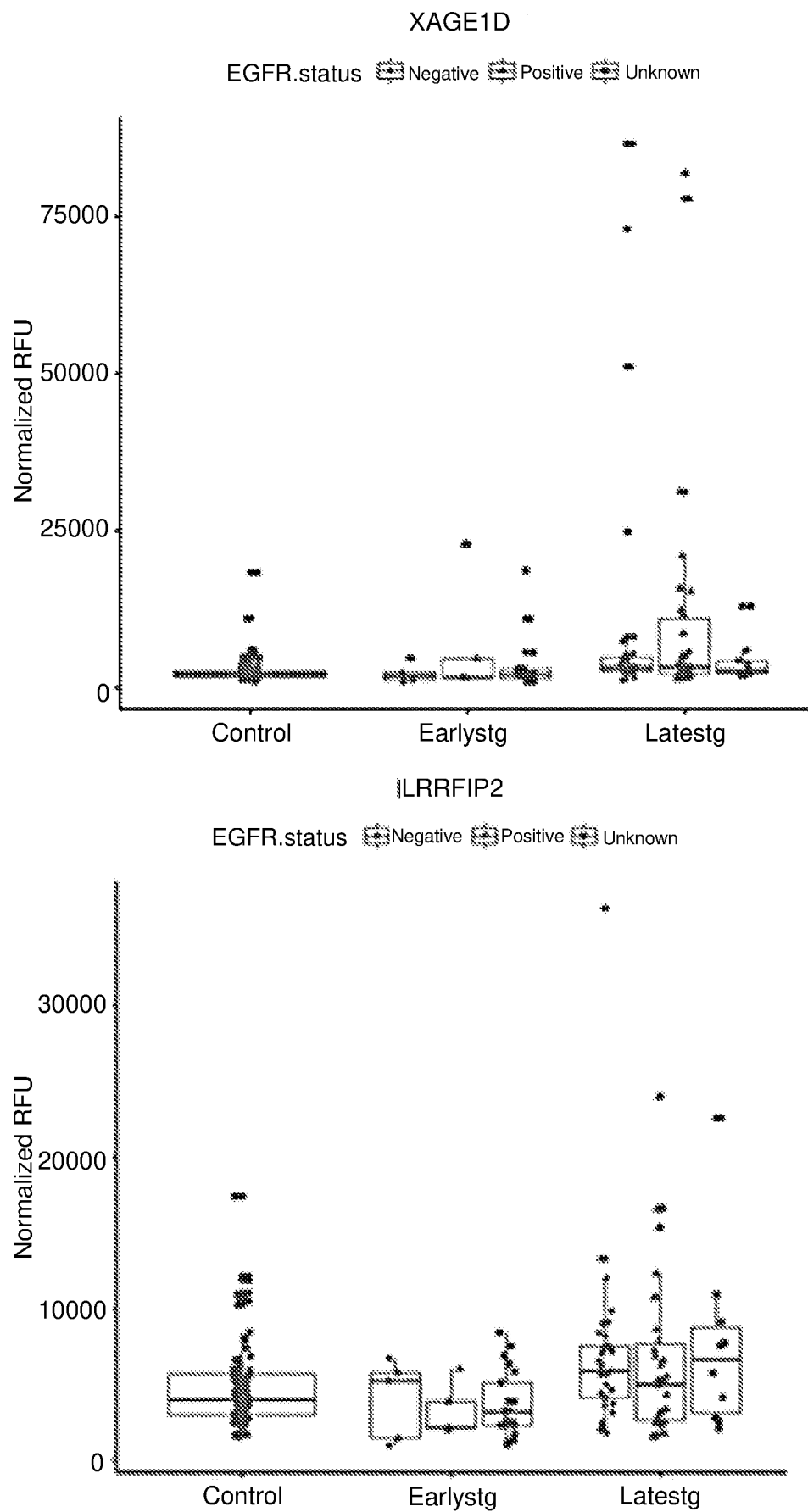
Figure 5 (continued)

GAGE2C
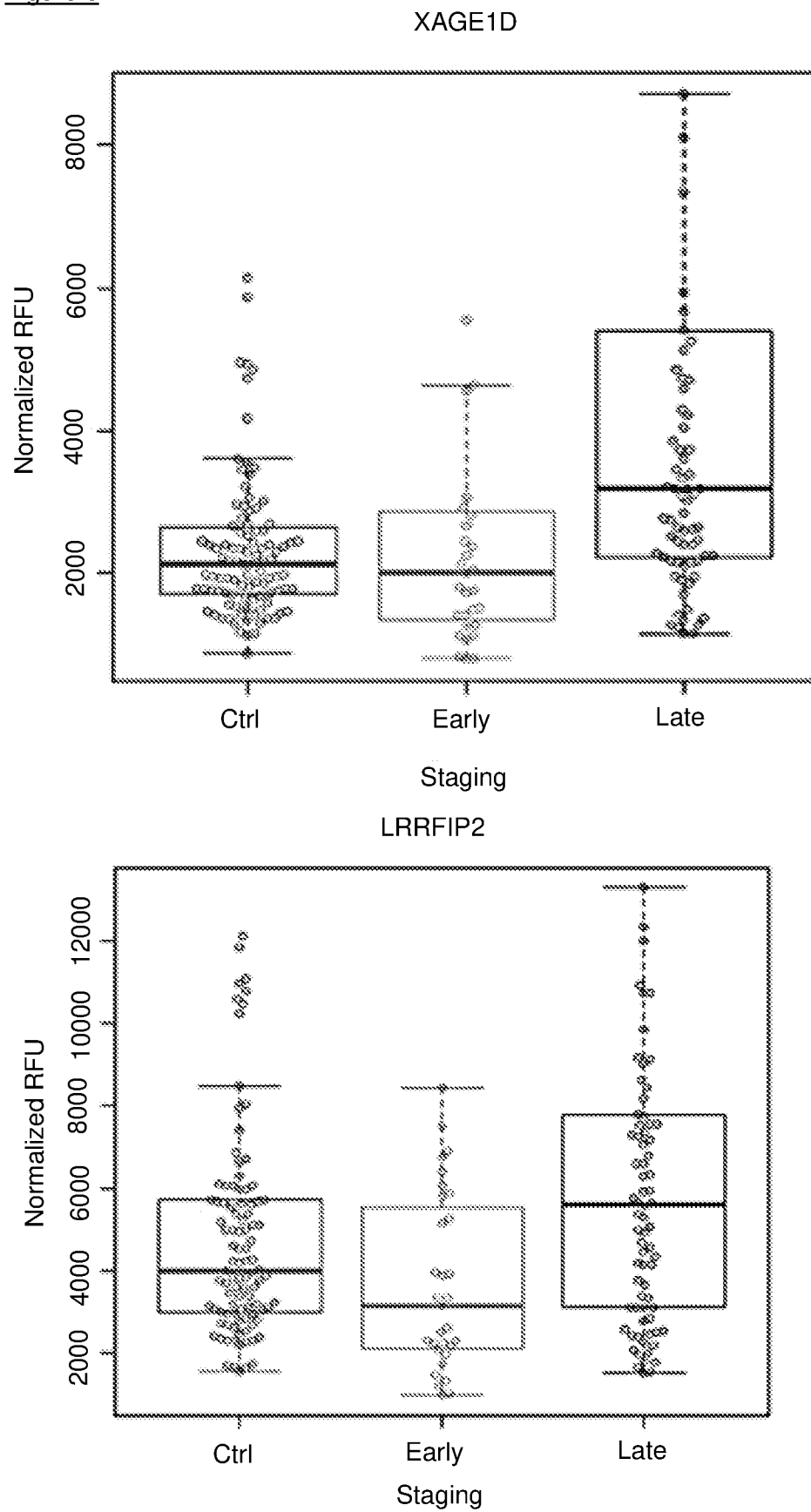
Figure 6

Figure 6 (continued)

GAGE2C

Figure 7

Figure 8

Figure 9

ROC Curve of Biomarker Panels

Panels
— panel-524268

Panel-524268(AUC) =0.6948

Figure 10

Figure 11