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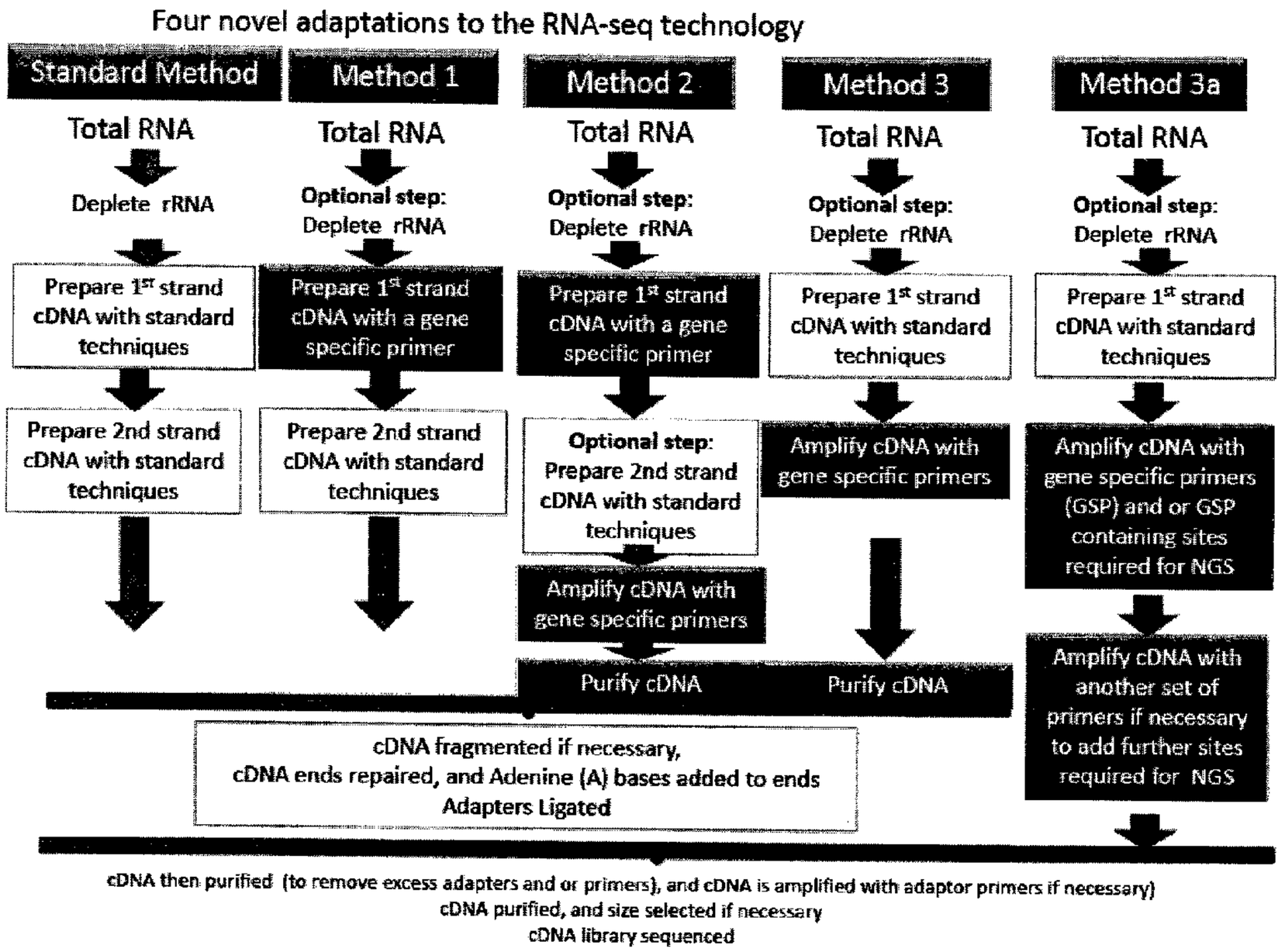
(54) **Titre : METHODES CIBLEES CONNUES SOUS LE NOM D'ARN-SEQ ET MATERIAUX POUR LE DIAGNOSTIC DU CANCER DE LA PROSTATE**
(54) **Title: TARGETED RNA-SEQ METHODS AND MATERIALS FOR THE DIAGNOSIS OF PROSTATE CANCER**



(57) **Abrégé/Abstract:**
Methods for diagnosing the presence of a disorder, such as prostate cancer, in a subject are provided, such methods including detecting the relative frequency of expression of RNA biomarkers in a biological sample obtained from the subject using RNA-Seq technology and comparing the relative levels of expression with predetermined threshold levels. Levels of expression of at least two of the RNA biomarkers that are above the predetermined threshold levels are indicative of the presence of prostate cancer in the subject. The methods involve the use of at least one gene-specific primer in at least one point of the RNA-Seq protocol for targeted sequencing of relevant transcripts.

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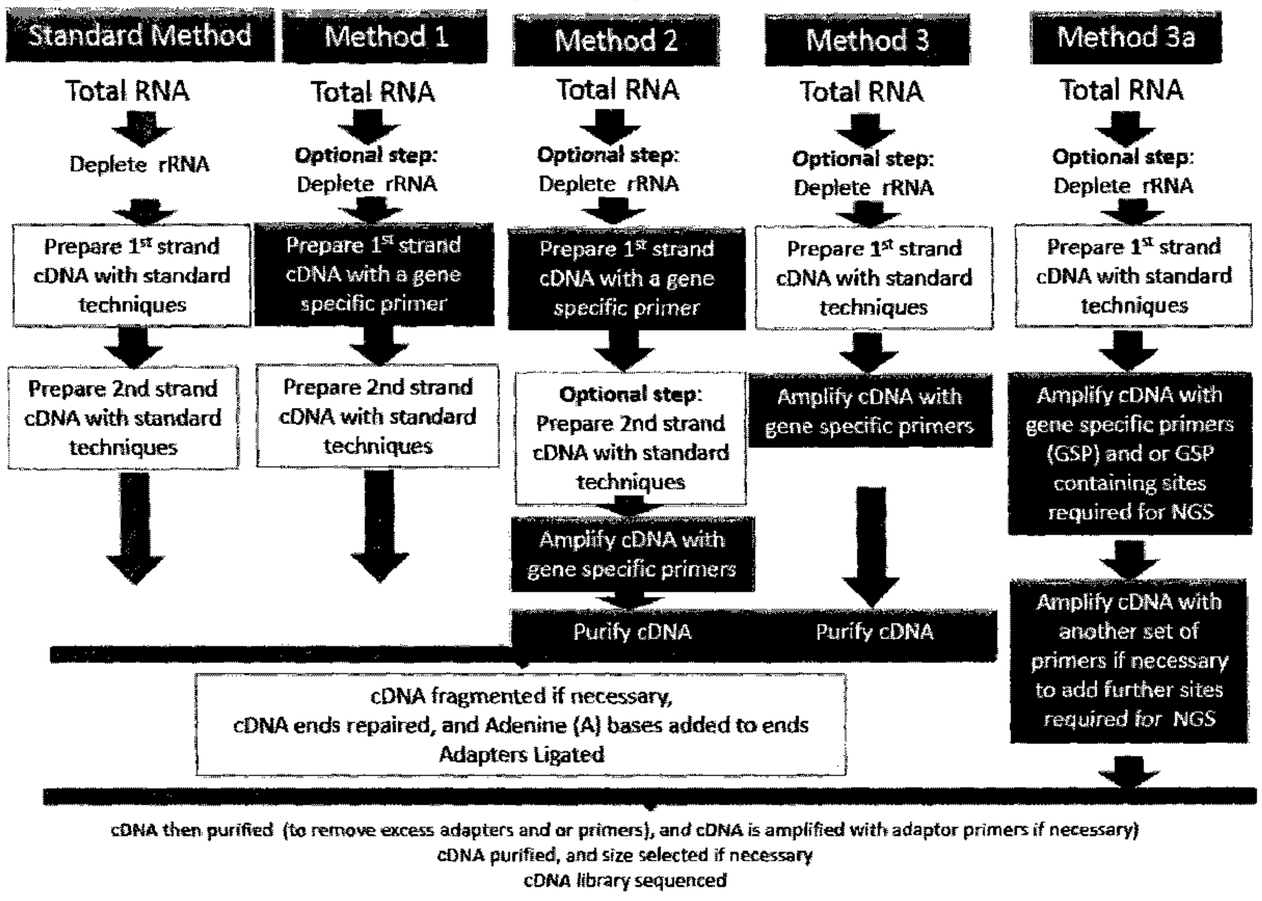
Figure 1: Four novel adaptations to the RNA-seq technology



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TARGETED RNA-SEQ METHODS AND MATERIALS FOR THE DIAGNOSIS OF PROSTATE CANCER

RELATED APPLICATIONS

This application claims the benefit of US Provisional Application No. 61/665,849, filed 28 June 2012, US Provisional Application No. 61/691,743, filed 21 August 2012, and US Provisional Application No. 61/709,517, filed 4 October 2012.

TECHNICAL FIELD

The present disclosure relates to methods and compositions for diagnosing and defining the staging or progress of disorders such as prostate cancer.

BACKGROUND

The use of prostate specific antigen (PSA) as a diagnostic biomarker for prostate cancer was approved by the US Federal Drug Agency in 1994. In the nearly two decades since this approval, the PSA test has remained the primary tool for use in prostate cancer diagnosis, in monitoring for recurrence of prostate cancer, and in following the efficacy of treatments. However the PSA test has multiple shortcomings and, despite its widespread use, has resulted in only small changes in the death rate from advanced prostate cancers. To reduce the death rate and the negative impacts on quality of life caused by prostate cancer, new tools are required not only for more accurate primary diagnosis, but also for assessing the risk of spread of primary prostate cancers, and for monitoring responses to therapeutic interventions.

Today, a blood serum level of around 4 ng per ml of PSA is considered indicative of prostate cancer, while a PSA level of 10 ng per ml or higher is considered highly suggestive of prostate cancer. The PSA blood test is not used in isolation when checking for prostate cancer; a digital rectal examination (DRE) is usually also performed. If the results of the PSA test or the DRE are abnormal, a biopsy is generally performed in which small samples of tissue are removed from the prostate and examined. If the results are positive for prostate cancer, further tests may be needed to determine the stage of progression of the cancer, such as a bone scan, a computed tomography (CT) scan or a pelvic lymph node dissection.

While the PSA test has a good sensitivity (80%), it suffers from a false positive rate that approaches 75%. For example, it has been estimated that for PSA values of 4-10 ng/ml, only one true diagnosis of prostate cancer was found in approximately four biopsies

performed (Catalona et al. *J. Urol.* 151(5):1283-90, 1994). Tests that measure the ratio of free to total (i.e., free plus bound) PSA do not have significantly greater specificity or sensitivity than the standard PSA test.

Higher PSA levels often lead to biopsies to determine the presence or absence of cancer cells in the prostate, and may lead to the surgical removal of the localized prostate gland. While surgery removes the localized cancer and often improves prostate cancer-specific mortality, it also masks the fact that many patients with prostate cancer, even in the absence of surgery, do not experience disease progression to metastasis or death.

The high false positive rate associated with the PSA test leads to many unnecessary biopsies. In addition to the physical discomfort and psychological distress associated with biopsies, it has been suggested that performing a biopsy may promote inflammation of cancerous tissue and increase the risk of cancer metastasis.

Currently, the established prognostic factors of histological grade and cancer stage from biopsy results, and prostate-specific antigen level in blood at diagnosis are insufficient to separate prostate cancer patients who are at high risk for cancer progression from those who are likely to die of another cause.

Once high risk or virulent forms of prostate cancer have been diagnosed, control strategies may involve surgery to remove the prostate gland if identified before metastasis, radiation to destroy cancer cells within the prostate and drug-based testosterone repression, generally referred to as androgen depletion therapy. These various treatments may bring about cures in some instances, or slow the time to death. However, for those with the most virulent forms of prostate cancer, the cancer will usually recur after surgery or radiation therapy and progress to resistance to androgen depletion therapy, with death a frequent outcome.

Early detection of virulent forms of prostate cancer is critical but the conclusion of specialist physicians is that the PSA test alone is inadequate for distinguishing patients whose cancers will become virulent and progress to threaten life expectancy from those with indolent cancers.

The following are some key reasons why the PSA test does not meet the needs of men's health:

i) The type of cancer

There are at least two basic cell types involved in prostate cancer. Adenocarcinoma is a cancer of epithelial cells in the prostate gland and accounts for approximately 95% of

prostate cancers. Neuroendocrine cancers may arise from cells of the endocrine (hormonal) and nervous systems of the prostate gland and account for approximately 5% of prostate cancers. Neuroendocrine cells have common features such as special secretory granules, produce biogenic amines and polypeptide hormones, and are most common in the intestine, lung, salivary gland, pituitary gland, pancreas, liver, breast and prostate. Neuroendocrine cells co-proliferate with malignant adenocarcinomas and secrete factors which appear to stimulate adenocarcinoma cell growth. Neuroendocrine cancers are rarer, and are considered non-PSA secreting and androgen-independent for their growth.

ii) Asymptomatic men

Some 15 to 17% of men with prostate cancer have cancers that grow but do not produce increasing or high blood levels of PSA. In these patients, who are termed asymptomatic, the PSA test often returns false negative test results as the cancer grows.

iii) BPH, prostatitis and PIN

Benign prostate hypertrophy (BPH), a non-malignant growth of epithelial cells, and prostatitis are diseases of the prostate that are usually caused by an infection of the prostate gland. Both BPH and prostatitis are common in men over 50 and can result in increased PSA levels. Incidence rates increase from 3 cases per 1000 man-years at age 45–49 years, to 38 cases per 1000 man-years by the age of 75–79 years. Whereas the prevalence rate is 2.7% for men aged 45–49, it increases to at least 24% by the age of 80 years. While prostate cancer results from the deregulated proliferation of epithelial cells, BPH commonly results from proliferation of normal epithelial cells and frequently does not lead to malignancy (Ziada et al. (1999) *Urology* 53(3 Suppl 3D):1-6). Bacterial infection of the prostate can be demonstrated in only about 10% of men with symptoms of chronic prostatitis/chronic pelvic pain syndrome. Bacteria able to be cultured from patients suffering chronic bacterial prostatitis are mainly Gram-negative uropathogens. The role of Gram-positives, such as staphylococci and enterococci, and atypicals, such as chlamydia, ureaplasmas, mycoplasmas, are still debatable.

Another condition, known as prostate intraepithelial neoplasia (PIN), may precede prostate cancer by five to ten years. Currently there are no specific diagnostic tests for PIN, although the ability to detect and monitor this potentially pre-cancerous condition would contribute to early detection and enhanced survival rates for prostate cancer.

iv) The phenotype of the prostate cancer

The phenotype of prostate cancer varies from one patient to another. More specifically, in different individuals prostate cancers display heterogeneous cellular morphologies, growth rates, responsiveness to androgens and pharmacological blocking agents for androgens, and varying metastatic potential. Each prostate cancer has its own unique progression involving multiple steps, including progression from localized carcinoma to invasive carcinoma to metastasis. The progression of prostate cancer likely proceeds, as seen for other cancers, via events that include the loss of function of cell regulators such as cancer suppressors, cell cycle and apoptosis regulators, proteins involved in metabolism and stress response, and metastasis related molecules (Abate-Shen et al. *Polypeptides Dev.* 14(19):2410-34, 2000; Ciocca et al. *Cell Stress Chaperones* 10(2):86-103, 2005).

At present health authorities do not universally recommend widespread screening for prostate cancer with the PSA test. There are concerns that many men may be diagnosed and treated unnecessarily as a result of being screened, at high cost to health systems as well as risking the patient's quality of life, such as through incontinence or impotence. Despite these concerns, prostate cancer is the most prevalent form of cancer and the second most common cause of cancer death in New Zealand, Australian and North American males (Jemal et al. *CA Cancer J. Clin.*, 57(1):43-66, 2007). In reality, at least some of the men incubating life threatening forms of prostate cancer are being missed until their cancer is too advanced, due to the economic costs of national screening, the need to avoid unnecessary over-treatment, and/or the presence of progressive cancers producing only low or background levels of PSA. The need for a better diagnostic test could not be clearer.

The lack of a diagnostic test that distinguishes a non-life threatening from a potentially life-threatening cancer raises the important clinical question as to how aggressively to treat patients with localized prostate cancer. Treatment options for more aggressive cancers are invasive and include radical prostatectomy and/or radiation therapy. Androgen-depletion therapy, for example using gonadotropin-releasing hormone agonists (e.g., leuprolide, goserelin, etc.), is designed to reduce the amount of testosterone that enters the prostate gland and is used in patients with metastatic disease, some patients who have a rising PSA and choose not to have surgery or radiation, and some patients with a rising PSA after surgery or radiation. Treatment options usually depend on the stage of the prostate cancer. Men with a 10-year life expectancy or less, who have a low Gleason score from a biopsy and whose cancer has not spread beyond the prostate are often not treated. Younger men with a low Gleason score and a prostate-restricted cancer may enter a phase of "watchful

waiting" in which treatment is withheld until signs of progression are identified. However, these prognostic indicators do not accurately predict clinical outcome for individual patients.

Unlike many cancer types, specific patterns of gene expression have not been consistently identified in prostate cancer progression, although a number of candidate genes and pathways likely to be important in individual cases have been identified (Tomlins et al., *Annu. Rev. Pathol.* 1:243-71, 2006). Several groups have attempted to examine prostate cancer progression by comparing gene expression of primary carcinomas to normal prostate tissue. Because of differences in technique, the integrity of the tissue samples used as well as the biological heterogeneity of prostate cancers, these studies have reported thousands of candidate genes that share only moderate consensus. Also sample type differences could contribute to the lack of consensus seen from these studies. For example formalin fixed paraffin embedded (FFPE) tissues allow a convenient comparison of tumor and adjacent tissues but many of the cDNA microarray studies have used snap frozen tissues (Bibikova et al., *Genomics* 89:666-72, 2007; van't Veer et al., *Nature* 415:530-6, 2002). In addition, some studies have included accident victim donors as controls to overcome potential field effects (Aryee et al. *Sci Transl Med* 5, 169ra10 2013; Chandran et al. *BMC Cancer*, 5:45 doi:10.1186/1471-2407-5-45, 2005). However, a few genes have emerged including hepsin (HPN; Rhodes et al., *Cancer Res.* 62:4427-33, 2002), alpha-methylacyl-CoA racemase (AMACR; Rubin et al., *JAMA* 287:1662-70, 2002, Lin et al. Biosensors 2:377-387, 2012), enhancer of Zeste homolog 2 (EZH2; Varambally et al. *Nature,* 419:624-9, 2002), L-dopa decarboxylase (DDC; Koutalellis et al. *BJU International*, 110:E267-E273, 2012) and anterior-gradient 2 (AGR2; Hu et al. *Carcinogenesis* 33:1178-1186, 2012) which have been shown experimentally to have probable roles in prostate carcinogenesis.

More recently, bioinformatic approaches employing data from gene expression profiling using both microarray and RNA-seq have generated lists of dysregulated genes in prostate cancer. RNA-seq is a technique based on enumeration of RNA transcripts using next-generation sequencing methodologies. However, because of their different experimental approaches, these studies have also shown few consensus genes, (Aryee et al. *Sci Transl Med* 5, 169ra10, 2013; Chandran et al. *BMC Cancer*, 5:1471-2407 2005; Pflueger et al. *Genome Res.* 21:56-67, 2011; Prensner et al. *Nature Biotechnology* 29:742–749, 2011; Shancheng Ren et al. *Cell Research* 22:806–821, 2012).

A number of studies have also shown distinct classes of prostate cancers separable by their gene expression profiles (Glinsky et al., *J. Clin. Invest.* 113:913-23, 2004; Hsieh et al., *Nature* doi:10.1038/nature.10912, 2012; Lapointe et al., *Proc. Natl. Acad. Sci. USA* 101:811-

6, 2004; LaTulippe et al., *Cancer Res.* 62:4499-506, 2002; Markert et al., *Proc. Natl. Acad. Sci.* doi:10.1073/pnas.1117029108, 2012; Rhodes et al., *Cancer Res.* 62:4427-33, 2002; Singh et al., *Cancer Cell* 1:203-9, 2002; Yu et al., *J. Clin. Oncol.* 22:2790-9, 2004; Varambally et al., *Nature* 419:624-9, 2002). Additionally, these approaches have been used to identify the genomic fusion of androgen-regulated genes including transmembrane protease, serine 2 (TMPRSS2) with members of the erythroblast transformation specific (ETS) DNA transcription factor family (Tomlins et al., *Science* 310:644-8, 2005, Tomlins, *Nature* 448: 595–599, 2007). These fusions appear commonly in prostate cancers and have been shown to be prevalent in more aggressive cancers (Attard et al., *Oncogene* 27:253-63, 2008; Barwick et al. *Br. J. Cancer* 102:570-576, 2010; Demichelis et al., *Oncogene* 26:4596-9, 2007; Nam et al., *Br. J. Cancer* 97:1690-5, 2007). Transcriptional modulation of TMPRSS2-ERG fusions has been shown to be associated with prostate cancer biomarkers and TGF-beta signalling (Brase et al., *BMC Cancer* 11:507 doi: 10.1186/1471, 2011). In addition to specific gene fusions, a vast array of mutational changes, including copy number variants, have been associated with prostate cancer tumours (Berger et al., *Nature* 470:214-220, 2011; Demichellis et al., *Proc. Natl. Acad. Sci.* doi:10.1073/pnas.117405109, 2012; Kumar et al., *Proc. Natl. Acad. Sci.* 108:17087-17092, 2011). Intratumor heterogeneity has also been found which has been suggested to result in underestimation of the degree of tumor heterogeneity (Gerlinger et al., *New Eng, J. Med.* 66:883-892, 2012). In particular mutations involving the substrate binding cleft of SPOP, which was found in 6-15% of prostate tumors, lacked ETS family gene rearrangements suggesting that tumors with SPOP mutations define a new class of prostate tumors. Also tumors with SPOP mutations lacked PTEN deletions in primary tumors but not in metastatic tumors (Barbieri et al., *Nature Gen.* 44:685-689, 2012).

Gene expression is the transcription of DNA into messenger RNA by RNA polymerase. Up-regulation describes a gene which has been observed to have higher expression (higher RNA levels) in one sample (for example, from cancer tissue) compared to another (usually healthy tissue from a control sample). Down-regulation describes a gene which has been observed to have lower expression (lower RNA levels) in one sample (for example, from cancer tissue) compared to another (usually healthy tissue from a control sample).

A common technology used for measuring RNA abundance is RT-qPCR where reverse transcription (RT) is followed by real-time quantitative PCR (qPCR). Reverse transcription first generates a DNA template from the RNA. This single-stranded template is called cDNA. The cDNA template is then amplified in the quantitative step, during which the

fluorescence emitted by labeled hybridization probes or intercalating dyes changes as the DNA amplification process progresses. Quantitative PCR produces a measurement of an increase or decrease in copies of the original RNA and has been used to attempt to define changes of gene expression in cancer tissue as compared to comparable healthy tissues (Nolan T, et al. *Nat Protoc* 1:1559-1582, 2006; Paik S. *The Oncologist* 12:631-635, 2007; Costa C, et al. *Transl Lung Cancer Research* 2:87-91, 2013).

Massive parallel sequencing made possible by next generation sequencing (NGS) technologies is another way to approach the enumeration of RNA transcripts in a tissue sample and RNA-seq is a method that utilizes this. It is currently the most powerful analytical tool used for transcriptome analyses, including gene expression level difference between different physiological conditions, or changes that occur during development or over the course of disease progression. Specifically, RNA-seq can be used to study phenomena such as gene expression changes, alternative splicing events, allele-specific gene expression, and chimeric transcripts, including gene fusion events, novel transcripts and RNA editing. However, there are currently no methods that allow the use of RNA-seq for the accurate and reproducible quantification of multiple specific RNAs for reliable applications in the field of diagnostics.

Why is it important to detect multiple biomarkers?

Using multiple biomarkers in a diagnostic or prognostic test is preferable to using a single biomarker because of the following:

Each individual tumor is heterogeneous with respect to all of the different aspects of their genome, transcriptome and proteome;

Multiple tumor foci are commonly found in tissues;

A single biomarker does not allow tumors of different lethality, aggressiveness or specificity to be differentiated;

A single biomarker may be affected by a treatment regime or other environmental influence;

A single biomarker may be affected by a field effect either as part of the progression of the disease or due to the tumor itself; and

A single biomarker may be less effective in particular ethnic groups.

Why does RT-qPCR not allow the accurate detection of multiple biomarkers?

RT-qPCR is a time consuming technique as expression differences are determined for

a single gene at a time, which does not allow multiple biomarkers to be compared/assessed at one time.

Comparing expression levels for genes across different experiments is often difficult, and can require complicated normalization methods that may not be suitable for integration into a diagnostic.

RT-qPCR does not allow the accurate detection of down-regulated genes because it is limited in its fluorescence detection range, compared to NGS based methods. This causes genes that are at a low and/or high abundance to be problematic. Very often these transcripts, for which differential expression is difficult to measure, are the ones with the most diagnostic and/or progonostic value. RT-PCR does not allow multiplexing which causes a rise in cost per RNA biomarker, and hence the overall cost of the diagnostic test.

There thus remains a need in the art for an accurate test for prostate cancer.

SUMMARY

The present invention provides methods for determining the presence and progression of a disorder in a subject. Such methods employ modified RNA-seq techniques to determine the relative frequency of one or more RNA biomarkers (also referred to as gene transcript biomarkers) specific for the disorder in the subject compared to that in healthy controls. Determination of the relative frequency of expression levels of specific combinations of RNA biomarkers using the methods disclosed herein can also be used to determine the type and/or stage of a disorder, and to monitor the progression of a disorder and/or the effectiveness of treatment. Disorders that can be diagnosed and monitored using the methods disclosed herein include, but are not limited to, cancers, such as prostate and breast cancers.

The methods disclosed herein allow the determination of the frequency of multiple RNA biomarkers simultaneously using a process known as multiplexing. Multiplexing is a process wherein oligonucleotides specific for multiple biomarkers are amplified together to produce a pool of amplicons. The advantages of multiplexing are that it allows simultaneous testing of multiple RNA biomarkers in one or a small number of tubes, which in turn:

Reduces cost;

Reduces the amount of tissue required;

Increases the level of reproducibility due to less hands-on manipulation;

Reduces time involved in set-up; and

Increases throughput.

More specifically, the disclosed methods employ oligonucleotides specific for RNA biomarkers known to be associated with the presence and/or progression of a disorder, such as prostate cancer, at specific steps of a RNA-seq protocol to selectively identify cDNAs for the RNA biomarkers, and compare their relative frequency of expression between prostate cancer donors and healthy donors, as well as defining differences in expression between different stages of the disorder.

In conventional RNA-seq methodologies, the actual frequency of expression of each transcript is determined for the whole genome. These frequencies can be biased by differences in the efficiency of the cDNA production and subsequent PCR amplification steps for each transcript. The inventors believe that the methods disclosed herein avoid these biases by determining the relative, rather than actual, frequency of expression of RNA biomarkers. The biases are not relevant as long as they are neutral with respect to the comparisons made. The relative changes in frequency of expression of RNA biomarkers specific for prostate cancer allows detection of prostate cancers, distinguishing prostate cancers from benign prostate hypertrophy (BPH) and prostatitis, and detection of prostate cancers in asymptomatic men whose prostate cancer may produce low levels of PSA with high sensitivity and specificity. In certain embodiments, the disclosed methods determine changes in frequency of expression of RNA biomarkers in order to distinguish between indolent cancers, which have a low likelihood of progressing to a lethal disease, and more aggressive forms of prostate cancer which are life threatening and require treatment.

In one aspect, the present disclosure provides methods for detecting the presence of a disorder in a subject, comprising: (a) determining the relative frequency of expression of at least one RNA biomarker in a biological sample obtained from the subject using RNA sequencing; and (b) comparing the relative frequency of expression of the at least one RNA biomarker in the biological sample with a predetermined threshold value, wherein increased or decreased relative frequency of expression of the at least one RNA biomarker in the biological sample indicates the presence of the disorder in the subject. In related aspects, the disclosed methods comprise: (a) determining the relative frequency of expression of a plurality of RNA biomarkers in the biological sample; and (b) comparing the relative frequency of expression of the plurality of RNA biomarkers in the biological sample with predetermined threshold values, wherein increased or decreased relative frequency of expression of at least two or more of the RNA biomarkers in the biological sample indicates the presence of the disorder in the subject.

In one embodiment, the relative frequency of expression of at least one RNA biomarker is determined by: (a) isolating total RNA from the biological sample; (b) generating first strand cDNA from the total RNA using a first oligonucleotide primer specific for the at least one RNA biomarker; (c) synthesizing second strand cDNA to provide double-stranded cDNA (dsDNA); (d) addingat least one sequencing adapter to the double-stranded cDNA; (e) amplifying the double-stranded cDNA to provide a cDNA library from the double-stranded cDNA; and (f) sequencing the cDNA library and determining the relative frequency of expression of the at least one RNA biomarker. Optionally, such methods also comprise: (i) removing rRNA from the total RNA prior to step (b); (ii) end repairing the double stranded cDNA and adding an overhanging adenine (A) base to the 3' end of the double stranded cDNA after step (c) and prior to step (d); and/or (iii) purifying and, optionally, size selecting the cDNA in the cDNA library after step (e) and prior to step (f).

In a related embodiment, such methods further comprise the option of synthesizing cDNA by polymerase chain reaction (PCR) using an oligonucleotide primer pair specific for the at least RNA biomarker after step (b) and prior to step (d) or by the standard methods. In certain embodiments, one of the oligonucleotides in the primer pair will be the same as the oligonucleotide primer used in the generation of the first strand cDNA.

In a further embodiment, the relative frequency of expression of the at least one RNA biomarker is determined by: (a) isolating total RNA from a biological sample; (b) generating first strand cDNA from the total RNA; (c) amplifying cDNA by polymerase chain reaction using an oligonucleotide primer pair specific for the at least one RNA biomarker to provide amplified double-stranded cDNA; (d) adding at least one sequencing adapter to the amplified double-stranded cDNA; (e) further amplifying the amplified double-stranded cDNA using primers specific for the at least one sequencing adapter to provide a cDNA library; and (f) sequencing the cDNA library and determining the relative frequency of expression of the at least one RNA biomarker. Optionally, such methods also comprise: (i) removing rRNA from the total RNA prior to step (b); (ii) end repairing the double stranded cDNA and adding an overhanging adenine (A) base to the 3' end of the double stranded cDNA after step (c) and prior to step (d); and/or (iii) purifying and, optionally, size selecting the cDNA in the cDNA library after step (e) and prior to step (f).

In certain embodiments, the disclosed methods comprise determining the expression level of multiple RNA biomarkers corresponding to polynucleotide biomarkers selected from the group consisting of those listed in Tables 1, 2 and 3. Oligonucleotide primers that can be employed in the methods disclosed herein include, but are not limited to, those provided in

SEQ ID NO: 76-232 and 293-326. In certain embodiments, the methods disclosed herein include detecting the relative frequency of expression of a RNA biomarker comprising an RNA sequence that corresponds to a DNA sequence of SEQ ID NO: 1-75 and 235-287 or a variant thereof, as defined herein. Those of skill in the art will appreciate that the RNA sequences for the disclosed RNA biomarkers are identical to the cDNA sequences disclosed herein except for the substitution of thymine (T) residues with uracil (U) residues.

In a further aspect, the present disclosure provides an oligonucleotide primer comprising, or consisting of, a sequence selected from the group consisting of SEQ ID NO: 76-232 and 293-326, and variants thereof. In certain embodiments, such oligonucleotide primers have a length equal to or less than 30 nucleotides. The disclosed oligonucleotide primers can be effectively employed in methods for diagnosing the presence of, and/or monitoring the progression of, prostate cancer using methods well known to those of skill in the art, including quantitative real time PCR or small scale oligonucleotide microarrays.

Biological samples that can be effectively employed in the disclosed methods include, but are not limited to, urine, blood, serum, cell lines, peripheral blood mononuclear cells (PBMCs), biopsy tissue and prostatectomy tissue.

In accordance with one embodiment of the invention, a method for detecting the presence of a disorder and/or monitoring the progression of the disorder in a subject, comprises: (a) determining the relative frequency of expression of a plurality of RNA biomarkers simultaneously in a biological sample obtained from the subject, wherein the relative frequency of expression is determined using next generation sequencing of an amplicon cDNA library prepared using a plurality of oligonucleotide primers specific for the plurality of RNA biomarkers; and (b) comparing the relative frequency of expression of the plurality of RNA biomarkers in the biological sample with a predetermined threshold value, wherein increased or decreased relative frequency of expression of the plurality of RNA biomarkers in the biological sample relative to the predetermined threshold value indicates the presence of the disorder and/or progression of the disorder in the subject.

In another embodiment, a method for monitoring progression of a disorder in a subject, comprises: (a) determining the relative frequency of expression of a plurality of RNA biomarkers simultaneously in a biological sample obtained from the subject at a first time point, and determining the relative frequency of expression of the plurality of RNA biomarkers

simultaneously in a biological sample obtained from the subject at a second, subsequent, time point, wherein the relative frequency of expression is determined using next generation sequencing of an amplicon cDNA library prepared using a plurality of oligonucleotide primers specific for the plurality of RNA biomarkers; and (b) comparing the relative frequency of expression of the plurality of RNA biomarkers in the biological sample with a predetermined threshold value, wherein an increase or decrease in the relative frequency of expression of the plurality of RNA biomarkers in the biological sample at the second time point compared to the relative frequency of expression of the plurality of RNA biomarkers at the first time point indicates the progression of the disorder in the subject.

In still another embodiment, the amplicon cDNA library is prepared by: (a) isolating total RNA from the biological sample; (b) generating first strand cDNA from the total RNA using a plurality of first oligonucleotide primers specific for the plurality of RNA biomarkers; (c) synthesizing second strand cDNA to provide double-stranded cDNA; (d) adding at least one sequencing adapter to the double-stranded cDNA; and (e) amplifying the double-stranded cDNA to provide the amplicon cDNA library.

In still yet another embodiment, the first oligonucleotide primer is selected from the group consisting of: SEQ ID NO: 76-223 and 293-326.

In a further embodiment, the amplicon cDNA library is prepared by further comprising amplifying the double-stranded cDNA by polymerase chain reaction using a plurality of oligonucleotide primer pairs specific for the plurality of RNA biomarkers after step (c) and prior to step (d).

In still a further embodiment, the plurality of oligonucleotide primer pairs is selected from the group consisting of: SEQ ID No: 76-223 and 293-326.

In still yet a further embodiment, the amplicon cDNA library is prepared by: (a) isolating total RNA from the biological sample; (b) preparing first strand cDNA to provide single-stranded cDNA; (c) amplifying the singled-stranded cDNA by polymerase chain reaction using a plurality of oligonucleotide primer pairs specific for the plurality of RNA biomarkers to provide amplified double-stranded cDNA; (d) adding at least one sequencing adapter to the amplified double-stranded cDNA; and (e) further amplifying the amplified double-stranded cDNA using primers specific for the at least one sequencing adapter to provide the amplicon cDNA library.

In another embodiment, the amplicon cDNA library is prepared by: (a) isolating total

RNA from the biological sample; (b) preparing first strand cDNA to provide single-stranded cDNA; (c) amplifying the single-stranded cDNA by polymerase chain reaction using a plurality of oligonucleotide primer pairs specific for the plurality of RNA biomarkers to provide amplified double-stranded cDNA; (d) adding at least one sequencing adapter to the double-stranded cDNA; and (e) amplifying the double-stranded cDNA using primers specific for the at least one sequencing adapter to provide the amplicon cDNA library.

In yet another embodiment, at least one member of the plurality of oligonucleotide primer pairs is selected from the group consisting of SEQ ID NO: 76-223 and 293-326.

In still yet another embodiment, the disorder is a cancer.

In a further embodiment, the disorder is prostate cancer and the plurality of RNA biomarkers comprises a RNA sequence corresponding to a DNA sequence selected from the group consisting of: SEQ ID NO: 1-71 and 235-287.

In yet a further embodiment, the biological sample is selected from the group consisting of: urine, blood, serum, cell lines, PBMCS, biopsy tissue, and prostatectomy tissue.

In still yet a further embodiment, the plurality of RNA biomarkers comprises at least ten RNA biomarkers.

In another embodiment, the frequency of expression of the plurality of RNA biomarkers is normalized to at least one expression standard.

In yet another embodiment, at least one expression standard is selected from the group consisting of SEQ ID NO: 72-75 and 288-292.

In still yet another embodiment, the amplicons in the amplicon cDNA library are 60-300 bp in size.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows four adaptations to conventional RNA-seq technology that are employed in the disclosed methods.

DEFINITIONS

As used herein, the term "biomarker" refers to a molecule that is associated either quantitatively or qualitatively with a biological change. Examples of biomarkers include

polypeptides, proteins, fragments of a polypeptide or protein; polynucleotides, such as a gene product, RNA or RNA fragment; and other body metabolites.

As used herein, the term "RNA biomarker" or "gene transcript biomarker" refers to an RNA molecule produced by transcription of a gene that is associated either quantitatively or qualitatively with a biological change.

As used herein the term "RNA sequence corresponding to a DNA sequence" refers to a sequence that is identical to the DNA sequence except for the substitution of all thymine (T) residues with uracil (U) residues.

As used herein, the term "oligonucleotide specific for a biomarker" refers to an oligonucleotide that specifically hybridizes to a polynucleotide biomarker or a polynucleotide encoding a polypeptide biomarker, and that does not significantly hybridize to unrelated polynucleotides. In certain embodiments, the oligonucleotide hybridizes to a gene, a gene fragment or a gene transcript. In specific embodiments, the oligonucleotide hybridizes to the polynucleotide of interest under stringent conditions, such as, but not limited to, prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65°C.

As used herein the term "oligonucleotide primer pair" refers to a pair of oligonucleotide primers that span an intron in the cognate RNA biomarker.

As used, herein the term "polynucleotide(s)," refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases and includes DNA and corresponding RNA molecules, including hnRNA, mRNA, and non-coding RNA, molecules, both sense and anti-sense strands, and includes cDNA, genomic DNA and recombinant DNA, as well as wholly or partially synthesized polynucleotides. An hnRNA molecule contains introns and corresponds to a DNA molecule in a generally one-to-one manner. An mRNA molecule corresponds to an hnRNA and DNA molecule from which the introns have been excised. A non-coding RNA is a functional RNA molecule that is not translated into a protein, although in some circumstances non-coding RNA can be coding and vice a versa.

As used herein, the term "subject" refers to a mammal, preferably a human, who may or may not have a disorder, such as prostate cancer. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

As used herein, the term "healthy subject" refers to a subject who is not inflicted with a disorder of interest.

As used herein in connection with prostate cancer, the term "healthy male" refers to a male who has an undetectable PSA level in serum or non–rising PSA levels up to 1ng/ml, no evidence of prostate gland abnormality following a DRE and no clinical symptoms of prostatic disorders.

As used herein in connection with prostate cancer, the term "asymptomatic male" refers to a male who has a PSA level in serum of greater than 4ng/ml, which is considered indicative of prostate cancer, but whose DRE is inconclusive and who has no symptoms of clinical disease.

The term "benign prostate hypertrophy" (BPH) refers to a prostatic disease with a non-malignant growth of epithelial cells in the prostate gland and the term "prostatitis" refers to another prostatic disease of the prostate, usually due to a microbial infection of the prostate gland. Both BPH and prostatitis can result in increased PSA levels.

As used herein, the term "metastatic prostate cancer" refers to prostate cancer which has spread beyond the prostate gland to a distant site, such as lymph nodes or bone.

As used herein, the term "biopsy tissue" refers to a sample of tissue (e.g., prostate tissue) that is removed from a subject for the purpose of determining if the sample contains cancerous tissue. The biopsy tissue is then examined (e.g., by microscopy) for the presence or absence of cancer.

As used herein, the term "prostatectomy" refers to the surgical removal of the prostate gland.

As used herein, the term "sample" is used herein in its broadest sense to include a sample, specimen or culture obtained from any source. Biological samples include blood products (such as plasma, serum and whole blood), urine, saliva and the like. Biological samples also include tissue samples, such as biopsy tissues or pathological tissues, that have previously been fixed (e.g., formalin, snap frozen, cytological processing, etc.).

As used herein, the term "predetermined threshold value of expression" of a RNA biomarker refers to the level of expression of the same RNA biomarker in a corresponding control/normal sample or group of control/normal samples obtained from normal, or healthy, subjects, e.g. from males who do not have prostate cancer.

As used herein, the term "altered frequency of expression" of a RNA biomarker in a test biological sample refers to a frequency that is either below or above the predetermined threshold

value of expression for the same RNA biomarker in a control sample and thus encompasses either high (increased) or low (decreased) expression levels.

As used herein, the term "relative frequency of expression" refers to the frequency of expression of a RNA biomarker in a test biological sample relative to the frequency of expression of the same RNA biomarker in a corresponding control/normal sample or group of control/normal samples obtained from normal, or healthy, subjects, (e.g., from males who do not have prostate cancer). In preferred embodiments, the frequency of expression of the RNA biomarker is also normalized to the frequency of an internal reference transcript.

As used herein, the term "prognosis" or "providing a prognosis" for a disorder, such as prostate cancer, refers to providing information regarding the likely impact of the presence of prostate cancer (e.g., as determined by the diagnostic methods) on a subject's future health (e.g., the risk of metastasis).

DETAILED DESCRIPTION

As outlined above, the present disclosure provides methods for detecting the presence or absence of a disorder, such as prostate cancer, in a subject, determining the stage of the disorder and/or the phenotype of the disorder, monitoring progression of the disorder, and/or monitoring treatment of the disorder by determining the frequency of expression of specific RNA biomarkers in a biological sample obtained from the subject. The methods disclosed herein employ one or more modifications of standard RNA-seq protocols. RNA-seq is a relatively new technology that has been employed for mass sequencing of whole transcriptomes, and that offers significant advantages over other methods employed for transcriptome sequencing, such as microarrays, including low levels of background noise, the ability to detect low levels of expression, the ability to detect novel mutations and transcripts, and the ability to use relatively small amounts of RNA (for a review of RNA-seq, see Wang et al., *Nat. Rev. Genet.* (2009) 10:57-63).

The disclosed methods employ oligonucleotides specific for one or more RNA biomarker in combination with RNA-seq technology to perform directed sequencing and thereby determine the relative frequency of expression of the RNA biomarker(s). Such methods have significant advantages over other technologies typically employed to determine expression levels of polynucleotide biomarkers, including improved accuracy, reproducibility and speed, the ability to easily determine the frequency of expression of a multitude of RNA biomarkers in a large number of samples at a relatively low cost, and the ability to identify novel mutations and transcripts.

In specific embodiments, such methods use oligonucleotides specific for one or more biomarkers selected from those shown in Tables 1, 2 and 3.

In one embodiment, the disclosed methods comprise determining the relative frequency of expression levels of at least two, three, four, five, six, seven, eight, nine, ten or more RNA biomarkers selected from the group consisting of: SEQ ID NO: 76-223 and 293-326 in a biological sample taken from a subject, and comparing the relative frequency of expression levels with predetermined threshold values.

The disclosed methods can be employed to diagnose the presence of prostate cancer in subjects with early stage prostate cancer; subjects who have had surgery to remove the prostate (radical prostatectomy); subjects who have had radiation treatment for prostate cancer; subjects who are undergoing, or have completed, androgen ablation therapy; subjects who have become resistant to hormone ablation therapy; and/or subjects who are undergoing, or have had, chemotherapy.

In certain embodiments, the RNA biomarkers disclosed herein appear in subjects with prostate cancer at levels that are at least two-fold higher or lower than, or at least two standard deviations above or below, the mean level in normal, healthy individuals, or are at least two-fold higher or lower than, or at least two standard deviations above or below, a predetermined threshold of expression.

All of the biomarkers and oligonucleotides disclosed herein are isolated and purified, as those terms are commonly used in the art. Preferably, the biomarkers and oligonucleotides are at least about 80% pure, more preferably at least about 90% pure, and most preferably at least about 99% pure.

In certain embodiments, the oligonucleotides employed in the disclosed methods specifically hybridize to a variant of a polynucleotide biomarker disclosed herein. As used herein, the term "variant" comprehends nucleotide or amino acid sequences different from the specifically identified sequences, wherein one or more nucleotides or amino acid residues is deleted, substituted, or added. Variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variant sequences (polynucleotide or polypeptide) preferably exhibit at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to a sequence disclosed herein. The percentage identity is determined by aligning the two sequences to be compared as described below, determining the number of identical residues in the aligned portion, dividing that number by the total number of residues in the inventive (queried) sequence, and multiplying the result by 100.

In addition to exhibiting the recited level of sequence identity, variants of the disclosed biomarkers are preferably themselves expressed in subjects with prostate cancer at a frequency that are higher or lower than the levels of expression in normal, healthy individuals.

Polypeptide and polynucleotide sequences may be aligned, and percentages of identical amino acids or nucleotides in a specified region may be determined against another polypeptide or polynucleotide sequence, using computer algorithms that are publicly available. The percentage identity of a polynucleotide or polypeptide sequence is determined by aligning polynucleotide and polypeptide sequences using appropriate algorithms, such as BLASTN or BLASTP, respectively, set to default parameters; identifying the number of identical nucleic or amino acids over the aligned portions; dividing the number of identical nucleic or amino acids by the total number of nucleic or amino acids of the polynucleotide or polypeptide of the present invention; and then multiplying by 100 to determine the percentage identity.

Two exemplary algorithms for aligning and identifying the identity of polynucleotide sequences are the BLASTN and FASTA algorithms. The alignment and identity of polypeptide sequences may be examined using the BLASTP algorithm. BLASTX and FASTX algorithms compare nucleotide query sequences translated in all reading frames against polypeptide sequences. The FASTA and FASTX algorithms are described in Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444-2448, 1988; and in Pearson, *Methods in Enzymol.* 183:63-98, 1990. The FASTA software package is available from the University of Virginia, Charlottesville, VA 22906-9025. The FASTA algorithm, set to the default parameters described in the documentation and distributed with the algorithm, may be used in the determination of polynucleotide variants. The readme files for FASTA and FASTX Version 2.0x that are distributed with the algorithms describe the use of the algorithms and describe the default parameters.

The BLASTN software is available on the NCBI anonymous FTP server and is available from the National Center for Biotechnology Information (NCBI), National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894. The BLASTN algorithm Version 2.0.6 [Sep-10-1998] and Version 2.0.11 [Jan-20-2000] set to the default parameters described in the documentation and distributed with the algorithm, is preferred for use in the determination of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN, is described at NCBI's website and in the publication of Altschul, *et al.*, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Res.* 25:3389-3402, 1997.

Variant sequences generally differ from the specifically identified sequence only by conservative substitutions, deletions or modifications. As used herein with regards to amino acid sequences, a "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. Variants may also, or alternatively, contain other modifications, including the deletion or addition of amino acids that have minimal influence on the antigenic properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a

linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

In another embodiment, variant polypeptides are encoded by polynucleotide sequences that hybridize to a disclosed polynucleotide under stringent conditions. Stringent hybridization conditions for determining complementarity include salt conditions of less than about 1 M, more usually less than about 500 mM, and preferably less than about 200 mM. Hybridization temperatures can be as low as 5°C, but are generally greater than about 22°C, more preferably greater than about 30°C, and most preferably greater than about 37°C. Longer DNA fragments may require higher hybridization temperatures for specific hybridization. Since the stringency of hybridization may be affected by other factors such as probe composition, presence of organic solvents and extent of base mismatching, the combination of parameters is more important than the absolute measure of any one alone. An example of "stringent conditions" is prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65°C.

The expression levels of one or more RNA biomarkers in a biological sample can be determined, for example, using one or more oligonucleotides that are specific for the RNA biomarker. In one method, the expression level of one or more RNA biomarkers disclosed herein is determined by first collecting urine from a subject following DRE or prostate massage via a bicycle or exocycle. RNA is isolated from the urine sample, and the frequency of expression of the RNA biomarker is determined as described below using modified RNA-seq technology in combination with oligonucleotides specific for the RNA biomarker of interest.

In other embodiments, the levels of mRNA corresponding to a prostate cancer biomarker disclosed herein can be detected using oligonucleotides in Southern hybridizations, *in situ* hybridizations, or quantitative real-time PCR amplification (qRT-PCR). Solid phase substrates, or carriers, that can be effectively employed in such assays are well known to those of skill in the art and include, but are not limited to, microporous membranes constructed, for example, of nitrocellulose, nylon, polyvinylidene difluoride, polyester, cellulose acetate, mixed cellulose esters and polycarbonate. Suitable microporous membranes include, for example, those described in US Patent Application Publication no.

US2010/0093557A1. Methods for performing such assays are well known to those of skill in the art.

The oligonucleotides employed in the disclosed methods are generally single-stranded molecules, such as synthetic antisense molecules or cDNA fragments, and are, for example, 6-60 nt, 15-30 or 20-25 nt in length.

Oligonucleotides specific for a polynucleotide, or RNA, biomarker disclosed herein are prepared using techniques well known to those of skill in the art. For example, oligonucleotides can be designed using known computer algorithms to identify oligonucleotides of a defined length that are unique to the polynucleotide, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that may interfere with hybridization. Oligonucleotides can be synthesized using methods well known to those in the art. In specific embodiments, the oligonucleotides employed in the disclosed methods and compositions are selected from the group consisting of: SEQ ID NO: 76-223 and 293-326.

For tests involving alterations in RNA expression levels, it is important to ensure adequate standardization. Accordingly, in tests such as the adapted RNA-seq technology disclosed herein, quantitative real time PCR or small scale oligonucleotide microarrays, at least one expression standard is employed. Expression standards that can be employed in such methods include, but are not limited to, those listed in Table 3 below.

The present disclosure further provides methods employing a plurality of oligonucleotides that are specific for a plurality of the prostate cancer RNA biomarkers disclosed herein.

The following examples are intended to illustrate, but not limit, this disclosure.

EXAMPLES

MATERIALS AND METHODS

RNA extraction

a) Cell lines

RNA was isolated from LNCaP and A549 cell lines that had been harvested from cell culture and stored in Trizol using a ZYMO Direct-zol™ kit (Ngaio Diagnostics Ltd.) following the manufacturer's instructions. RNA quality was assessed using the Agilent BioAnalyser and the Agilent RNA 6000 nano assay protocol. The LNCaP and A549 RNA had a RIN value of 9.5 and 9.8 respectively. The RNA was also checked on the NanoDrop 2000

spectrophotometer, (Thermo Scientific), and its concentration ascertained by the Qubit® 2.0 Fluorometer (Life Technologies).

b) FFPE prostatectomy tissue

Histological blocks from subjects were reviewed by a clinical histopathologist, and tumor and histologically adjacent regions deemed "normal" were identified. These sections were then excised and reset in paraffin. Approximately fifteen freshly cut sections at a thickness of ten microns were then processed using a Qiagen RNeasy FFPE kit (Cat No: 74404, 73504). The method used in all extractions for deparaffinization step was the original method from the Cat no: 74404 kit, and the remainder of the protocol was performed following the manufacturer's instructions. The RNA was checked on the NanoDrop, and its concentration ascertained by the Qubit® 2.0 Fluorometer (Life Technologies).

c) Urine

RNA was isolated from one or more separate fresh urine samples from donors by sedimentation of the cellular material using centrifgation at 1000g for five minutes at 4°C. The urine was decanted and the cell pellet resuspended in 1.8 ml of ice cold 1X PBS containing 2.5% Fetal Bovine Serum (Invitrogen). The cell suspension was transferred to a 2ml Eppendorf® tube and the cellular material collected by centrifugation at 400g for 5 minutes at 4°C. The supernatant was removed (leaving around 50µl) and the cell pellet resuspended in 1.8 ml of ice cold 1X PBS containing 2.5% Fetal Bovine Serum (Invitrogen). The cells were again collected by centrifugation at 400g for 5 minutes at 4°C. The supernatant was removed (leaving around 50µl) and the cell pellet resuspended in 1.8 ml of ice cold 1X PBS containing 2.5% Fetal Bovine Serum (Invitrogen). The cells were collected by centrifµgation at 400g for 5 minutes at 4°C and all but 100 µl of the supernatant removed. The cells were resuspended in the remaining 100 µl of supernatant, and 8µl was taken for microscopic analysis. A total of 300 µl of Trizol LS (Invitrogen) and 5µg of E. coli 5S rRNA was added and the cell suspension was stored at -80°C. RNA was extracted as described by ZYMO using the Direct-zol™ kit, or as described by Invitrogen and further purified using Qiagen RNeasy™ spin columns. RNA was stored at -80°C prior to use.

cDNA preparation

cDNA was produced from approximately 1-1.5ug of total RNA from either cell lines, biopsy tissue or urine extracts using random primers for the production of the first strand cDNA

using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies) or RNA biomarker-specific primers.The cDNA preparations were stored at -80°C prior to use and then diluted 1/5 in sterile water prior to qRT-PCR.

qRT-PCR methods

RNA biomarker specific primers were used to perform real time SYBR green PCR quantification from cell line-, biopsy- or urine-derived cDNA using the Roche Lightcycler 480 using standard protocols for determining the specificity and efficiency of the amplification. The relative amount of the marker gene in each of the samples tested was determined by comparing the cycle threshold (Ct value: number of PCR cycles required for the SYBR green fluorescent signal to cross the threshold exceeding background level within the exponential growth phase of the amplification curve). Following 30 cycle RT-PCR reactions, the amplicons were electrophoresed on a 2% agarose gel and sequenced with standard Sanger chemistry using an Applied Biosystems 3130xl DNA sequencer.

RNA biomarker amplicon production

The relative frequency of expression of specific RNA biomarkers was determined using the isolated RNA in one or more of the four methods described below. Each of these methods includes at least one modification of conventional RNA-seq technologies. Conventional RNA-seq technologies are well known to those of skill in the art and are described, for example, in Wang et al. (*Nat. Rev. Genet.* (2009) 10:57-63), and Marguerat and Bahler (*Cell. Mol. Life Sci.* (2010) 67:569-579).

Method 1

In a first method, sequence specific priming is employed during the generation of first strand cDNA. An optional first step in this method is to deplete the total RNA of rRNA using an industry-provided kit, if necessary. An industry-provided first strand cDNA kit is used to combine total RNA or rRNA-depleted total RNA with at least one strand specific oligonucleotide primer (i.e. an oligonucleotide primer specific for the RNA biomarker of interest) and generate first strand cDNA according to the manufacturer's protocol. Second strand cDNA is then synthesized in an unbiased manner using standard techniques. The resulting double-stranded cDNA is fragmented if necessary using standard methods, and the cDNA ends are repaired using standard methods in which any overhangs at the cDNA ends are converted into blunt ends using T4 DNA polymerase. An overhanging adenine (A) base

is added to the 3' end of the blunt DNA fragments by the use of Klenow fragment to assist with ligation of adapters required for the sequencing process. The adapters are ligated to the ends of the cDNA fragments using standard procedures, and then the cDNA fragments are run on a gel for purification and removal of excess adapters. The cDNA is amplified using adapter primers, purified, denatured and further diluted for cluster generation and sequencing, for example on a HiSeq2000 according to Illumina Corporation's standard protocols (208 cycles sequencing program, paired-end with indexing). The cDNA library is sequenced, and the relative frequency of expression of the specific RNA biomarkers in cancer patients and healthy controls is determined.

Method 2

As in method 1, sequence specific priming is employed during the generation of first strand cDNA. This is achieved using an industry provided first strand cDNA kit and at least one strand specific oligonucleotide primer to generate first strand cDNA from total RNA (or rRNA depleted total RNA if necessary) according to the manufacturer's protocol. The second strand cDNA can either be prepared in an unbiased manner using standard techniques, or it can be directly amplified using a set of specific oligonucleotide primers (i.e. oligonucleotide primers specific for the RNA biomarkers of interest) to amplify a specific set of PCR amplicons by either primer limited or cycle limited PCR. In preferred embodiments, the oligonucleotide primer employed to generate the first strand cDNA can be the same as one of the pair of oligonucleotide primers used to amplify the double-stranded cDNA. The cDNA is then purified via a cleanup procedure to remove excess PCR reagents. The cDNA is fragmented if necessary using standard methods, and the cDNA ends are repaired using standard methods in which any overhangs at the cDNA ends are converted into blunt ends using T4 DNA polymerase. An overhanging adenine (A) base is added to the 3' end of the blunt DNA fragments by the use of Klenow fragment to assist with ligation of adapters required for the sequencing process. The adapters are ligated to the ends of the cDNA fragments using standard procedures, and the cDNA fragments are then purified to remove excess adapters. The cDNA is amplified using adapter primers, purified, denatured and further diluted for cluster generation and sequencing, for example on a HiSeq2000 according to Illumina Corporation's standard protocols (208 cycles sequencing program, paired-end with indexing). The cDNA library is sequenced and the relative frequency of expression of the specific RNA biomarkers in cancer patients and healthy controls is determined.

Method 3

This method employs total RNA or rRNA-depleted RNA if necessary. The first strand cDNA is synthesized using standard methods. The first strand cDNA is then directly amplified using a set of specific oligonucleotide primers (i.e. oligonucleotide primers specific for the RNA biomarkers of interest) to amplify a specific set of PCR amplicons using either primer limited or cycle limited PCR. The cDNA is purified via a cleanup procedure to remove excess PCR reagents. The cDNA is fragmented if necessary using standard methods, and the cDNA ends are repaired using standard methods, in which any overhangs at the cDNA ends are converted into blunt ends using T4 DNA polymerase. An overhanging adenine (A) base is added to the 3'end of the blunt DNA fragments by the use of Klenow fragment to assist with ligation of adapters required for the sequencing process. Adapters are ligated to the ends of the cDNA fragments using standard procedures, and the cDNA is purified to remove excess adapters. The cDNA is then amplified using adapter primers and purified. The cDNA can be size selected via gel electrophoresis using standard methods if necessary. The cDNA library is sequenced, and the relative frequency of expression of the specific RNA biomarkers in cancer patients and healthy controls is determined.

Method 4

Method 4 differs from Method 3 in that all sequences necessary for next generation sequencing are incorporated via either a one or two step PCR amplification.

An optional first step in this method is to deplete the total RNA of rRNA using an industry-provided kit, if necessary. The first strand cDNA is then synthesized using standard methods. The first strand cDNA is directly amplified using a set of specific oligonucleotide primers (i.e. oligonucleotide primers specific for the RNA biomarkers of interest) also containing Next Generation Sequencing (NGS) primer sites, using either primer limited or cycle limited PCR. The cDNA is then purified via a cleanup procedure to remove excess PCR reagents, and re-amplified with another set of primers, if necessary, in order to add further sites required for NGS using either primer limited or cycle limited PCR. The cDNA is then purified to remove excess PCR reagents and, if necessary, is again amplified using adaptor primers and purified. The cDNA is amplified using adapter primers, purified, denatured and further diluted for cluster generation and sequencing, for example on a HiSeq2000 according to Illumina Corporation's standard protocols (208 cycles sequencing program, paired-end with indexing). The cDNA library is sequenced, and the relative frequency of expression of the specific RNA biomarkers in cancer patients and healthy controls is determined.

Identification of Prostate Cancer Biomarkers

RNA biomarkers were selected using annotation and analysis of publicly available RNA expression profile data in the NCBI databases GSE6919 and GSE38241 as these data-sets include data from cancer free donors. The biomarkers shown in Table 1 below is a unique set identified as being over-expressed in subjects with prostate cancer. Similarly, the biomarkers shown in Table 2 is a second unique combination of RNA biomarkers identified as being under-expressed in subjects with prostate cancer.

The NCBI database GSE6919, which was developed at the University of Pittsburgh, contains data from three Affymetrix® GeneChip® products (U95A, U95B and U95C), representing more than 36,000 gene reporters. The database, which has been analyzed by Chandran et al. (*BMC Cancer* 2005, 5:45; BMC Cancer 2007, 9:64), and Yu et al. (*J Clin Oncol* 2004, 22:2790-2799), contains RNA profiles from more than 200 individual prostate tumor samples, combined with adjacent "normal" or "healthy" tissues, or prostate tissues from individuals believed to be free of prostate cancer.

Table 1: RNA Biomarkers with Elevated Expression Levels in Prostate Cancer Patients

REPORTER	GENBANK ACCESSION	GENE DESCRIPTION	GENE SYMBOL	SEQ ID NO:	PRIMER SEQ ID NOS:	PRIMER IDS
34777_at	D14874	Adrenomedullin	ADM	1	76, 77	ND654, ND655
38827_at	AF038451	Anterior gradient 2 homolog	AGR2	2	78, 79	ND543, ND544
37399_at	D17793	Aldo-keto reductase family 1, member C3	AKR1C3	3	80, 81	ND498, ND499
41764_at	AA976838	Apolipoprotein C-I	ApoC1	4	82, 83	ND414, ND599
608_at	M12529	Apolipoprotein E	ApoE	5	84, 85	CH350, CH351
1577_at	M23263	Androgen receptor	AR	6	86, 87 88, 89	ND460, ND461, ND532, ND533
56999_at	AI625959	Chromosome 15 open reading frame 48	C15ORF48	7	90, 91	CH075, CH076
36464_at	X94323	cysteine-rich secretory protein 3	CRISP3	8	92, 93	ND536, ND537
40201_at	M76180	Dopa decarboxylase	DDC	9	94, 95	CH127, CH128
37156_at	AF070641	ets variant gene 1	ETV1	10	96, 97	ND440, ND441

REPORTER	GENBANK ACCESSION	GENE DESCRIPTION	GENE SYMBOL	SEQ ID NO:	PRIMER SEQ ID NOS:	PRIMER IDS
2084_s_at	D12765	ets variant gene 4 (E1A enhancer binding protein, E1AF)	ETV4	11	98, 99	ND410, ND411
35245_at	M16967	F5, Coagulation factor V	F5	12	100, 101	ND714, ND715
36622_at	AI989422	Fibrinogen	FGG	13	102, 103	ND442, ND443
36201_at	D13315	Glycoxalase 1	GLO1	14	104, 105	CH186, CH187
39135_at	AB018310	GRAM domain containing 4	GRAMD4	15	106, 107	ND484, ND589
48885_at	R61847	Glutamate receptor, ionotropic N-methyl-D-aspartate 3A	GRIN3A	16	108, 109	CH328, CH329
1039_s_at	U22431	Hypoxia inducible factor 1, alpha subunit	HIF-1A	17	110, 111	ND700, ND701
37851_at	AF055019	Homeodomain interacting protein kinase: TF kinase	HIPK2	18	112, 113	ND612, ND613
32480_at	X07495	Homeobox C4	HOXC4	19	114, 115	ND422, ND423
56429_at	AI525822	Homo sapiens hematological and neurological expressed 1	HN1	20	116, 117	ND490, ND491
32570_at	L76465	Hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	21	118, 119	ND528, ND529
37639_at	X07732	hepsin (transmembrane protease, serine 1)	HPN	22	120, 121	ND595, ND596
63673_at	AI635057	HSBP1 - Heat shock protein 27A	HSBP1	23	122, 123	ND702, 703
1232_s_at	M74587	Insulin like growth factor binding protein 1 precursor	IGFBP1	24	124, 125	ND608, 609
1804_at	X07730	kallikrein-related peptidase 3	KLK3	25	126, 127 128, 129	ND438, ND439 ND470, ND471
217_at, 41721_at	S39329	kallikrein-related peptidase 2	KLK2	26	130, 131	ND418, ND419
62175_at	AI50156	Homo sapiens laminin, alpha 1	LAMA1	27	132, 133	ND662, ND663
60019_at, 56912_at	AA947309.1	Leucine rich repeat neuronal 1 - Homo sapiens leucine-rich repeats and calponin homology (CH) domain containing 4 (LRCH4)	LRRN1	28	134, 135	ND428, ND429
1083_s_at, 927_at	M35093	Mucin1 cell surface associated protein	MUC1	29	136, 137	CH284, CH285
52116_at	AI697679	Myelin expression factor 2	MYEF2	30	138, 139	ND396, ND397
35024_at	L37362	OPRK1 receptor	OPRK1	31	140, 141	ND404, ND405
------	--------	*Homo sapiens* SET domain and mariner transposase fusion gene (SETMAR) transcript	PCAT1	32	142, 143	ND492, ND493

REPORTER	GENBANK ACCESSION	GENE DESCRIPTION	GENE SYMBOL	SEQ ID NO:	PRIMER SEQ ID NOS:	PRIMER IDS
		variant 3, non coding RNA				
------	--------	*Homo sapiens* uncharacterized LOC100506990, transcript variant 2 non-coding RNA	PCAT14	33	144, 145	ND488, ND489
51776_s_at	AI749525	PDZK1 interacting protein 1	PDZK1IP1	34	146, 147	ND500, ND501
31610_at	U21049					
59794_g_at	AA872415					
41281_s_at	AF060502	Peroxisomal biogenesis factor 10	PEX10	35	148, 149	CH139, CH140
40116_at	X16911	Homo sapiens phosphofructokinase, liver (PFKL)	PFKL	36	150, 151	ND708, ND709
39175_at	D25328	Homo sapiens phosphofructokinase, platelet (PFKP) gene	PFKP	37	152, 153	ND696, ND697
41094_at	Y10179	Prolactin Induced Protein	PIP	38	154, 155	ND502, ND503
37068_at	U24577	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	PLA2G7	39	156, 157	CH212, CH213
63958_at	AI583077	prostate stem cell antigen	PSCA	40	158, 159	ND380, ND381
1739_at, 1740_g_at	M99487	**Prostate**-specific membrane antigen	PSMA	41	160, 161	ND402, ND403
33272_at	AA829286	Serum amyloid A2	SAA2	42	162, 163	CH320, CH321
36781_at	X01683	Serpin peptidase inhibitor clade A	SERPINA1	43	164, 165	ND446, ND447
54293_at	N30034	Solute carrier family 10, member 7	SLC10A7	44	166, 167	ND734, ND735
39926_at	U59913	Homo sapiens SMAD family member 5 (SMAD5)	SMAD5	45	168, 169	ND710, ND711
52576_s_at	AW007426	Spondin 2 extracellular matrix protein	SPON2	46	170, 171	ND358, ND359
34342_s_at	AF052124	Osteopontin:secreted phophoprotein	SPP1	47	172, 173	ND472, ND473
1938_at	K03218	Homo sapiens v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog	SRC	48	174, 175	ND704, ND705
----	-----	Homo sapiens tudor domain containing 1 (TDRD1)	TDRD1	49	176, 177	ND726, ND727
32154_at	M36711	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	TFAP2A	50	178, 179	ND494, ND495
47890_at	AI921465	Homo sapiens transmembrane channel-like 5 (TMC5)	TMC5	51	180, 181	ND670, ND671

REPORTER	GENBANK ACCESSION	GENE DESCRIPTION	GENE SYMBOL	SEQ ID NO:	PRIMER SEQ ID NOS:	PRIMER IDS
45574_g_at	AA534688	TPX2-microtubule associated	TPX2	52	182, 183	ND436, ND437
57239_at	AI439109	Homo sapiens isolate TRIB1-VI-T tribbles-like protein 1	TRIB1	53	184, 185	ND718,719
56508_at	W22687	Tetraspanin 13	TSPAN13	54	186, 187	ND386, ND387
6315_f_at	T50788	UDP glucuronosyltransferase 2 family polypeptide B15	UGT2B15	55	188, 189	ND452, ND453
33279_at	X80062	acyl-CoA synthetase medium-chain family member 3	ACSM3	235	293, 294	
	NM_001106.3		ACVR2B	236	-	
41706_at	AJ130733	alpha-methylacyl-CoA racemase	AMACR	237	-	
	NM_000479.3		AMH	238	-	
36106_at	X01388	Apolipoprotein C-III	ApoCIII	239	-	
31355_at	U77629.1	Achaete-scute complex homolog 2	ASCL2	240	-	
56999_at	AI625959	Chromosome 15 open reading frame 48	C15ORF48	241	-	
	NM_178840.2		C1orf64	242	295, 296	
	NM_033150.2		COL2A1	243	-	
39925_at	M95610	collagen, type IX, alpha 2	COL9A2	244	-	
40162_s_at	AC003107	Cartilage Oligomeric Matrix protein precursor	COMP	245	-	
45399_at	T77033	Cysteine-rich secretory protein LCCL domain containing 1	CRISPLD1	246	297, 298	
37020_at	X56692	C-reactive protein	CRP	247	-	
35506_s_at	J03870	Cystatin S	CST4	248	299, 300	
34623_at	M97925	Defensin alpha 5, Paneth cell specific	DEFA5	249	-	
52138_at	AI351043, AI351043	v-ets erythroblastosis virus E26 oncogene like (avian)	ERG	250	-	
45394_s_at	AA563933	Family with sequence similarity 3, member D	FAM3D	251	301-304	
31685_at	Y08976	FEV (ETS oncogene family)	FEV	252	-	
	NM_002046.4		GAPDH	253	-	
	NM_001098518.1		GPR116	254	305, 306	
32430_at	M73481	Gastrin releasing peptide receptor	GRPR	255	-	
40327_at	U57052	homeo box B13	HOXB13	256	-	

REPORTER	GENBANK ACCESSION	GENE DESCRIPTION	GENE SYMBOL	SEQ ID NO:	PRIMER SEQ ID NOS:	PRIMER IDS
36227_at	AF043129	Interleukin 7 receptor	IL7R	257	-	
46958_at	AI868421	Potassium voltage gated channel, Shaw-related subfamily, member 2	KCNC2	258	-	
33606_g_at	AF019415	NK2 homeobox	NKX2-2	259	-	
		NM_001136157.1	OTUD5	260	-	
		NR_015342.1	PCA3	261	307, 308	
33703_f_at, 33702_f_at	L05144	Phophoenol pyruvate carboxy kinase I	PCK1	262	-	
39696_at	AB028974	Paternally expressed 10	PEG10	263	-	
58941_at	AI765967	Phospholipase A1	PLA1A	264	-	
62240_at	AI096692	Proline rich 16	PRR16	265	-	
33259_at	M81652	Semenogelin II	SEMG2	266	309, 310	
928_at	L02785	Solute carrier 26, member 3	SLC26A3	267	-	
51847_at	AA001450	Solute carrier family 44, member 5	SLC44A5	268	311, 312	
35716_at	AB008164	Sulfotransferase	SULT1C2	269	313, 314	
	NM_003226.3		TFF3	270	-	
40328_at	X99268	TWIST homolog 1	TWIST1	271	-	
1651_at	U73379	Ubiquitin-conjugating enzyme E2C	UBE2C	272	-	
44403_at	AI873501	Clone HH0011_E05 mRNA sequence		273	-	

Table 2: RNA Biomarkers Showing Reduced Expression Levels in Prostate Cancer Patients

REPORTER	GENBANK ACCESSION	GENE DESCRIPTION	GENE SYMBOL	SEQ ID NO:	PRIMER SEQ ID NOS:	PRIMER ID'S
32200_at	M24902	acid phosphatase, prostate	ACPP	56	190, 191	ND496, ND497
35834_at	X59766	Alpha-2-glycoprotein 1, zinc-binding	AZGP1	57	192, 193	CH161, CH162
36780_at	M25915	Clusterin	CLU	58	194, 195	ND698, ND699
38700_at	M33146	Cysteine and glycine-rich protein 1	CSRP1	59	196, 197, 198, 199	DR583, DR584, ND690, ND691
65988_at	W19285	Early b-cell factor 3	EBF3	60	200, 201	ND730, ND731
38422_s_at	U29332	4.5 LIM domains	FHL2	61	202, 203	DR569, DR570
32749_s_at	AL050396	filamin A	FLNA	62	204, 205	ND624, ND625

53270_s_at	AW021867	Homo sapiens mitogen-activated protein kinase kinase kinase 7	MAP3K7	63	206, 207	ND682, ND683
32149_at	AA532495	microseminoprotein, beta-	MSMB	64	208, 209	CH143, CH144
32847_at	U48959	Myosin kinase	MYLK	65	210, 211	DR567, DR568
33505_at, 1042_at, 62940_f_at	AI887421 U27185 AI669229	Retinoic acid responder	RARRES1	66	212, 213	DR575, DR576
64449_at	AI810399	Selenoprotein M	SELM1	67	214, 215	DR559, DR560
32521_at	AF056087	Secreted frizzled-related protein 1	SFRP1	68	216, 217	DR555, DR556
39544_at	AB002351	Synemin	SYNM	69	218, 219	DR579, DR580
48039_at	AI634580	Synaptopodin 2	SYNPO2	70	220, 221	DR737,738
32314_g_at	M75165	Tropomyosin 2	TPM2	71	222, 223	DR565, DR566
32755_at	X13839	Actin SM	ACTA2	274	-	
1197_at	D00654	Actin gamma2	ACTG2	275	-	
32527_at	AI381790	Unknown	C10orf116	276	315, 316	
34203_at	D17408	Calponin 1, basic, smooth muscle	CNN1	277	317, 318	
57241_at	AI928870	Dystrobrevin binding protein 1	DBNDD2	278	-	
38183_at	U13219	Forkhead box F1	FOXF1	279	319, 320	
33396_at	U12472	glutathione S-transferase P1	GSTP1	280	-	
53796_at	AI819282	Potassium channel	KCNMA1	281	321, 322	
49502_i_at	AI379607	Mutated in CRC	MCC	282	323, 324	
767_at 37407_s_at 773_at 774_g_at 32582_at	AF001548 AF013570 D10667 D10667 X69292	Myosin, heavy chain 11, smooth muscle	MYH11	283, 284	-	
37576_at	U52969	Purkinje cell protein 4	PCP4	285	-	
63827_at	AI479999	Solute carrier family 22, member 17	SLC22A17	286	325, 326	
	NM_016950.2		SPOCK3	287		

For tests measuring the changes in frequency of RNA expression levels, it is essential to ensure adequate standardization. For this reason we have analyzed the NCBI database to identify reporters with the least variation between gene expression profiles, as shown in Table 3 below, in prostate cancer and healthy donor tissues. These reporters form a robust set of RNA expression standards that can be used where appropriate in tests involving

quantification of RNA expression, such as in the modified RNA-seq technology described herein.

Table 3: Reporters with Least Variation between Gene Expression Profiles

REPORTER	PROBE	GENE SYMBOL	GENE DESCRIPTION	SEQ ID NO:	PRIMER SEQ ID NOS:	PRIMER ID'S
35184_at	AB011118	ZFC3H1	zinc finger, C3H1-type containing CCDC131	72	224, 225	ND514, ND515
31826_at	AB014574	FKBP15	FK506 binding protein 15, 133kDa	73	226, 227	ND468, ND469
39811_at	AA402538	C19orf50	chromosome 19 open reading frame 50	74	228, 229, 230, 231	CH035, CH036, ND505
33397_at	AL050383	CDIPT	CDP-diacylglycerol--inositol 3-phosphatidyltransferase	75	231, 232	CH103, CH104
36003_at	AJ005698	PARN	poly(A)-specific ribonuclease (deadenylation nuclease)	288	-	
35337_at	AL050254	FBXO7	F-box protein 7	289	-	
F39020_at	U82938	SIVA	CD27-binding (Siva) protein polymerase	290	-	
36027_at	AA418779	POLR2F	PDGFA associated protein 1	291	-	
38703_at	AF005050	DNPEP	Aspartyl aminopeptidase	292	-	

Primers for the production of an RNA biomarker specific amplicon were created using a multistep primer design strategy. Specific intron-spanning primers were created to amplify an amplicon of a specific size (60-300bp) that can be used for Next Generation Sequencing (NGS).

The primers were designed using Primer3 (v. 0.4.0) software and the primers were checked to ensure that certain criteria were met:

- No more than three C's or G's in the last five base pairs;
- No runs (more than three) of G's in either primer;
- No or limited self-complementarity, or hairpin formation; and
- Primer BLAST of the primer set hits the cognate RNA target of the expected size.

In order to use these RNA specific amplicon primer sets for the RNA Biomarker Amplicon Sequencing (RBAS), nucleotides incorporating sequencing primers were added to the 5' end of the primers in the first round PCR as described in Table 4 below, and a second set of primers used for a second round of PCR were used to add further sequences containing an index and adaptor sequence.

Table 4: Specification of the added sequence to the RNA biomarker specific primer use for the first round PCR for biomarker specific amplicon

1st round PCR

Sequence added to forward primer 5' end	ACGACGCTCTTCCGATCT (SEQ ID NO: 233)
Sequence added to reverse primer 5' end	CGTGTGCTCTTCCGATCT (SEQ ID NO: 234)

All primers used in the studies described herein were designed by the inventors and supplied by Invitrogen or IDT, except for a set of primers for PSA (KLK3) which are taught by Hessels et al. (*European Urology* 44: 8-16, 2003.

EXAMPLE 1

Use of RNA Biomarker Amplicon Sequencing to compare RNA biomarker expression profiles in a prostate adenocarcinoma cell line (LNCaP) and a lung adenocarcinoma cell line (A549)

The ability of RNA Biomarker Amplicon Sequencing (RBAS) to be used for the accurate detection and relative quantification of multiple RNA biomarkers was demonstrated by:

a) producing a selected set of 25 specific RNA biomarker amplicons from LNCaP cells (epithelial cell line derived from androgen-sensitive human prostate adenocarcinoma lymph node metastasis) and A549 cells (epithelial cell line derived from lung alveolar basal tissue); and

b) detecting and measuring the relative abundance of the LNCaP- and A549-derived RNA biomarker specific amplicons by massive parallel sequencing.

1) Amplicon production

An amplicon is defined as the specific amplification product obtained by PCR using a pair of oligonucleotide primers targeted to a specific RNA biomarker. The template used for the amplicon production was the single strand DNA complementary to the RNA extracted from LNCaP and A549 cells (see method section above). The cDNA was produced using

random primers in this example but biomarker specific primers can also be used to initiate the reverse transcription from the extracted RNA.

DNA amplicons compatible with Illumina Corporation's Next Generation Sequencing technology were produced in this example. Amplicons compatible for sequencing using other NGS technology can also be prepared using the same rationale. The 25 specific primer pairs were targeted to 21 prostate cancer RNA biomarkers and 4 reference RNA biomarkers and contained added sequences for adaptor introduction to the 5' and 3' ends of the amplicons according to Illumina's specification (the RNA biomarker selection and primer design strategies are presented in the method section above).

Technical triplicates for each individual RNA biomarker were produced during a first round of PCR. The same cDNAs produced from RNA of LNCaP or A549 cells were used as a template for each of the three separate first round PCR amplifications. Six amplicon pools were then prepared by combining equal volumes of each of the 25 biomarker specific amplicons produced individually during the first round PCRs. These six amplicon pools, technical triplicates for each of the two cell types, were purified to remove residual primers and dNTPs using Agencourt AMPureXP system (Beckman Coulter, Inc.), and then analyzed with the 2100 Bioanalyser (Agilent Technologies Inc.) and Qubit® 2.0 Fluorometer (Life Technologies) to ascertain quality, average size distribution and the concentration of amplicons in each pool.

2) Preparation of amplicon libraries

After dilution, the six cleaned amplicon pools were used as individual templates for the second round PCR performed with sequencing primers specific for the adaptor added during the first round PCR. The sequencing primers also contained a barcode sequence for indexing and a tag sequence for clustering. The amplicon libraries produced during the second round PCR were analyzed and the concentration determined using the 2100 Bioanalyser (Agilent Technologies, Inc.) and Qubit® (Life Technologies – Invitrogen). Residual primers and dNTPs were removed using Agencourt AMPureXP system (Beckman Coulter, Inc.) and then pooled together at equimolar concentration to produce a single amplicon library sequencing pool. The sequencing pool was denatured and further diluted for cluster generation and sequenced on a HiSeq2000 according to Illumina Corporation's standard protocols (208 cycles sequencing program, paired-end with indexing).

3) Amplicons relative quantification

Illumina bcl2fastq conversion software (version 1.8.3) was used for the de-multiplexing of the sequence reads acquired during the sequencing program and base call conversion to fastq paired end read data. Quality statistics for percentage of bases > Q30 and mean QScore for all reads showed that all amplicon libraries sequenced and de-multiplexed very well. This data set was used to generate the read counts per amplicon (Read counts (Rc) Tables 5 and 6). This is the number of sequencing reads of at least 50bp in length that map to the corresponding amplicon. This number is directly proportional to the amount of the amplicon in the library, and is also proportional to the specific RNA biomarker abundance from which the amplicon was derived.

By using the read count obtained for each amplicon it is thus possible to establish a precise assessment of the relative abundance of the corresponding RNA biomarkers in each sample studied.

Different methods can be used for the normalization of the read count to minimize biases generated by the acquisition of wide count distribution by massive parallel sequencing. The average of the read counts obtained from the four reference amplicons were used to normalize the raw read counts of the amplicons produced from the LNCaP and A549 RNA using the 21 primer pairs specific for the prostate cancer RNA biomarkers. The reference amplicons were made with specific primers targeted to four different RNA biomarkers selected due to their low level of expression variation between different prostate cancer and healthy donor control tissues. The raw counts obtained for the four reference amplicons derived from A549 and LNCaP RNA were consistent between replicates and between the two cell types compared (Table 5). The data confirms the low level of differential expression of these reference RNAs and validates the selection of these RNA biomarkers as reference amplicons.

Table 5: Read counts obtained in triplicate (Rep. 1, 2, 3) for the four Reference Amplicons (Ref)

a) Reference read Counts from A549 amplicons

Ref.	Rep.1	Rep.2	Rep.2	Avr.	StDev
CDIPT	520,522	513,026	531,305	242,890	13,173
C19orf50	209,037	211,595	210,174	210,268	1,282
ZFC3HI.	207,606	222,590	311,090	247,095	55,925
FKBP15	11,112	40,746	23,749	25,202	14,870
Avr. Ref.	**237,069**	**246,989**	**269,079**	**160,855**	

b) Reference read Counts from LNCaP amplicons

Ref.	Rep.1	Rep.2	Rep.2	Avr.	StDev
CDIPT	473,707	590,290	533,300	267,674	44,723
C19orf50	236,952	283,338	380,160	300,150	73,069
ZFC3HI.	96,551	201,322	160,785	152,886	52,830
FKBP15	37,939	80,900	39,426	52,755	24,386
Avr. Ref.	**211,287**	**288,962**	**278,418**	**168,597**	

In Table 6, the normalization of the read count for each of the non-reference RNA biomarker specific amplicons derived from LNCaP and A549 RNA (termed target amplicons) was calculated by dividing each target read count by the average read count calculated from the mean of the four reference amplicons either from LNCaP or A549 RNA. This normalization was performed for each replicate (Table 6: target amplicon read counts/ average references read counts).

The assessment of the RNA biomarker differential expression fold change (FC) between the LNCaP and A549 cells was performed by comparing the normalized read counts per amplicon converted to a $\log_2$ number. The $\log_2$ FC was calculated for the read counts before (raw read counts) and after normalization (Normalised read counts) and was compared in order to assess the effect of the amplicon library count distributions on the evaluation of the differential expression (Table 6). The data in Table 6 compares the expression of 21 target RNA biomarkers in LNCaP and A549 cells. A negative $\log_2$ number indicates a decrease, or down regulation of RNA biomarkers while a positive $\log_2$ number indicates an increase, or up regulation of RNA biomarkers.

Table 6: Read counts and relative quantification (Log_2 FC) of RNA biomarker specific amplicons derived from LNCaP RNA compared with A549 RNA

		Fold change (FC) calculated with the raw read count (Rc)					**FC calculated with the normalized count normalised read count (Rc)**				
		Rc		**Log_2**		**Log_2 FC LNCaP/ A549**	**Rc**		**Log_2**		**Log_2 FC LNCaP/ A549**
		A549	**LNCaP**	**A549**	**LNCaP**		**A549**	**LNCaP**	**A549**	**LNCaP**	
ACPP	Rep.1	108	52,877	6.8	15.7	**8.9**	0.0005	0.2503	-11.1	-2	9.1
	Rep.2	145	51,052	7.2	15.6	**8.9**	0.0006	0.1767	-10.7	-2.5	8.2
	Rep.3	143	63,492	7.2	16	**9.2**	0.0005	0.2280	-10.9	-2.1	8.7
	Avr.	**132**	**55,807**	7	**15.8**	**9**	**0.0005**	**0.2183**	**-10.9**	**-2.2**	**8.7**
	Stdev	**21**	**6,718**	**0.2**	**0.2**	**-0.2**	**0.0001**	**0.0377**	**0.2**	**0.3**	**0.4**
AGR2	Rep.1	676,547	48,098	19.4	15.6	**-3.8**	2.8538	0.2276	1.5	-2.1	-3.6
	Rep.2	703,769	63,188	19.4	15.9	**-3.4**	2.8494	0.2187	1.5	-2.2	-3.7
	Rep.3	712,083	71,317	19.4	16.1	**-3.2**	2.6464	0.2562	1.4	-2	-3.4
	Avr.	**697,466**	**60,868**	**19.4**	**15.9**	**-3.5**	**2.7832**	**0.2342**	**1.5**	**-2.1**	**-3.6**
	Stdev	**18,587**	**11,782**	**0**	**0.3**	**0.3**	**0.1185**	**0.0196**	**0.1**	**0.1**	**0.2**
AKRI C3	Rep.1	773,556	10,121	19.6	13.3	**-6.3**	3.2630	0.0479	1.7	-4.4	-6.1
	Rep.2	763,968	12,768	19.5	13.6	**-5.9**	3.0931	0.0442	1.6	-4.5	-6.1
	Rep.3	721,042	16,204	19.5	14	**-5.6**	2.6797	0.0582	1.4	-4.1	-5.5
	Avr.	**752,855**	**13,031**	**19.5**	**13.6**	**-5.9**	**3.0119**	**0.0501**	**1.6**	**-4.3**	**-5.9**
	Stdev	**27,965**	**3,050**	**0.1**	**0.3**	**0.3**	**0.3000**	**0.0073**	**0.1**	**0.2**	**0.3**
AR460	Rep.1	147,236	257,216	17.2	18	0.8	0.6211	1.2174	-0.7	0.3	1
	Rep.2	145,185	272,469	17.1	18.1	0.9	0.5878	0.9429	-0.8	-0.1	0.7
	Rep.3	146,121	237,525	17.2	17.9	0.7	0.5430	0.8531	-0.9	-0.2	0.7
	Avr.	**146,181**	**255,737**	**17.2**	**18**	**0.8**	**0.5840**	**1.0045**	**-0.8**	**0**	**0.8**
	Stdev	**1,027**	**17,519**	**0**	**0.1**	**0.1**	**0.0392**	**0.1898**	**0.1**	**0.3**	**0.2**
AR532	Rep.1	267,160	1,062,230	18	20	2	1.1269	5.0274	0.2	2.3	2.2
	Rep.2	267,201	431,144	18	18.7	0.7	1.0818	1.4920	0.1	0.6	0.5
	Rep.3	295,910	448,932	18.2	18.8	0.7	1.0997	1.6124	0.1	0.7	0.6
	Avr.	**276,757**	**647,435**	**18.1**	**19.2**	**1.1**	**1.1028**	**2.7106**	**0.1**	**1.2**	**1.1**
	Stdev	**16,587**	**359,333**	**0.1**	**0.7**	**-0.7**	**0.0227**	**2.0073**	**0**	**1**	**1**
AZGP 1	Rep.1	324	129,118	8.3	17	**8.6**	0.0014	0.6111	-9.5	-0.7	8.8
	Rep.2	240	104,903	7.9	16.7	**8.3**	0.0010	0.3630	-10	-1.5	8.5
	Rep.3	308	79,348	8.3	16.3	**7.9**	0.0011	0.2850	-9.8	-1.8	8
	Avr.	**291**	**104,456**	**8.2**	**16.6**	**8.3**	**0.0012**	**0.4197**	**-9.8**	**-1.3**	**8.4**
	Stdev	**45**	**24,888**	**0.2**	**0.4**	**0.4**	**0.0002**	**0.1703**	**0.2**	**0.6**	**0.4**
CRISP 3	Rep.1	74	9,068	6.2	13.1	**6.9**	0.0003	0.0429	-11.6	-4.5	7.1
	Rep.2	131	6,967	7	12.8	**6.6**	0.0005	0.0241	-10.9	-5.4	5.5
	Rep.3	302	7,297	8.2	12.8	**6.6**	0.0011	0.0262	-9.8	-5.3	4.5
	Avr.	**169**	**7,777**	**7.2**	**12.9**	**6.7**	**0.0007**	**0.0311**	**-10.8**	**-5.1**	**5.7**
	Stdev	**119**	**1,130**	**1**	**0.2**	**0.2**	**0.0004**	**0.0103**	**0.9**	**0.4**	**1.3**
DDC	Rep.1	11,844	403,659	13.5	18.6	**5.1**	0.0500	1.9105	-4.3	0.9	5.3
	Rep.2	13,632	448,386	13.7	18.8	**5.2**	0.0552	1.5517	-4.2	0.6	4.8
	Rep.3	47,271	404,380	15.5	18.6	**5.1**	0.1757	1.4524	-2.5	0.5	3
	Avr.	**24,249**	**418,808**	**14.3**	**18.7**	**5.1**	**0.0936**	**1.6382**	**-3.7**	**0.7**	**4.4**
	Stdev	**19,958**	**25,618**	**1.1**	**0.1**	**0.1**	**0.0711**	**0.2410**	**1**	**0.2**	**1.2**

	Fold change (FC) calculated with the raw read count (Rc)					FC calculated with the normalized count normalised read count (Rc)				
		Rc		Log_2		Log_2 FC LNCaP/ A549	Rc		Log_2		Log_2 FC LNCaP/ A549
		A549	LNCaP	A549	LNCaP		A549	LNCaP	A549	LNCaP	
ETV1	Rep.1	80,571	574,119	16.3	19.1	**2.8**	0.3399	2.7172	-1.6	1.4	3.0
	Rep.2	65,909	594,479	16	19.2	**2.9**	0.2668	2.0573	-1.9	1	2.9
	Rep.3	76,805	645,353	16.2	19.3	**3**	0.2854	2.3179	-1.8	1.2	3.0
	Avr.	**74,428**	**604,650**	**16.2**	**19.2**	**2.9**	**0.2974**	**2.3642**	**-1.8**	**1.2**	**2.9**
	Stdev	**7,614**	**36,690**	**0.2**	**0.1**	**0.1**	**0.0379**	**0.3324**	**0.2**	**0.2**	**0**
ETV4	Rep.1	222,417	1,426	17.8	10.5	-7.3	0.9382	0.0067	-0.1	-7.2	-7.1
	Rep.2	197,816	2,018	17.6	11	-6.8	0.8009	0.0070	-0.3	-7.2	-6.8
	Rep.3	187,812	2,698	17.5	11.4	-6.4	0.6980	0.0097	-0.5	-6.7	-6.2
	Avr.	**202,682**	**2,047**	**17.6**	**11**	**-6.8**	**0.8124**	**0.0078**	**-0.3**	**-7**	**-6.7**
	Stdev	**17,808**	**637**	**0.1**	**0.5**	**-0.5**	**0.1205**	**0.0016**	**0.2**	**0.3**	**0.5**
HN1	Rep.1	292,321	311,090	18.2	18.2	0.1	1.2331	1.4724	0.3	0.6	0.3
	Rep.2	257,665	362,158	18	18.5	0.3	1.0432	1.2533	0.1	0.3	0.3
	Rep.3	246,021	348,395	17.9	18.4	0.3	0.9143	1.2513	-0.1	0.3	0.5
	Avr.	**265,336**	**340,548**	**18**	**18.4**	**0.2**	**1.0635**	**1.3257**	**0.1**	**0.4**	**0.3**
	Stdev	**24,084**	**26,423**	**0.1**	**0.1**	**-0.1**	**0.1603**	**0.1270**	**0.2**	**0.1**	**0.1**
MUC1	Rep.1	13,230	924	13.7	9.9	-3.8	0.0558	0.0044	-4.2	-7.8	-3.7
	Rep.2	13,647	902	13.7	9.8	-3.9	0.0553	0.0031	-4.2	-8.3	-4.1
	Rep.3	17,202	941	14.1	9.9	-3.8	0.0639	0.0034	-4	-8.2	-4.2
	Avr.	**14,693**	**922**	**13.8**	**9.8**	**-3.8**	**0.0583**	**0.0036**	**-4.1**	**-8.1**	**-4.3**
	Stdev	**2,183**	**20**	**0.2**	**0**	**0.1**	**0.0049**	**0.0007**	**0.1**	**0.3**	**0.3**
MYLK	Rep.1	293,518	24,448	18.2	14.6	-3.6	1.2381	0.1157	0.3	-3.1	-3.4
	Rep.2	276,460	31,241	18.1	14.9	-3.2	1.1193	0.1081	0.2	-3.2	-3.4
	Rep.3	251,537	22,665	17.9	14.5	-3.7	0.9348	0.0814	-0.1	-3.6	-3.5
	Avr.	**273,838**	**26,118**	**18.1**	**14.7**	**-3.5**	**1.0974**	**0.1017**	**0.1**	**-3.3**	**-3.4**
	Stdev	**21,113**	**4,525**	**0.1**	**0.2**	**0.2**	**0.1528**	**0.0180**	**0.2**	**0.3**	**0.1**
PCAT1	Rep.1	114,546	386,617	16.8	18.6	1.8	0.4832	1.8298	-1	0.9	1.9
	Rep.2	124,881	385,426	16.9	18.6	1.8	0.5056	1.3338	-1	0.4	1.4
	Rep.3	208,422	413,859	17.7	18.7	1.9	0.7746	1.4865	-0.4	0.6	0.9
	Avr.	**149,283**	**395,301**	**17.1**	**18.6**	**1.8**	**0.5878**	**1.5500**	**-0.8**	**0.6**	**1.4**
	Stdev	**51,476**	**16,083**	**0.5**	**0.1**	**0.1**	**0.1622**	**0.2540**	**0.4**	**0.2**	**0.5**
PDZK1IP1	Rep.1	125,239	4,428	16.9	12.1	-4.8	0.5283	0.0210	-0.9	-5.6	-4.7
	Rep.2	118,631	11,141	16.9	13.4	-3.5	0.4803	0.0386	-1.1	-4.7	-3.6
	Rep.3	111,850	8,550	16.8	13.1	-3.9	0.4157	0.0307	-1.3	-5	-3.8
	Avr.	**118,573**	**8,040**	**16.9**	**12.9**	**-4.1**	**0.4748**	**0.0301**	**-1.1**	**-5.1**	**-4.3**
	Stdev	**6,695**	**3,385**	**0.1**	**0.7**	**0.7**	**0.0565**	**0.0088**	**0.2**	**0.4**	**0.6**
PEX10	Rep.1	115,769	308,004	16.8	18.2	1.4	0.4883	1.4578	-1	0.5	1.6
	Rep.2	137,943	378,401	17.1	18.5	1.7	0.5585	1.3095	-0.8	0.4	1.2
	Rep.3	231,140	344,061	17.8	18.4	1.6	0.8590	1.2358	-0.2	0.3	0.5
	Avr.	**161,617**	**343,489**	**17.2**	**18.4**	**1.6**	**0.6353**	**1.3343**	**-0.7**	**0.4**	**1.1**
	Stdev	**61,221**	**35,202**	**0.5**	**0.1**	**0.1**	**0.1969**	**0.1131**	**0.4**	**0.1**	**0.5**
PSCA	Rep.1	4,960	24,551	12.3	14.6	2.3	0.0209	0.1162	-5.6	-3.1	2.5
	Rep.2	2,638	27,668	11.4	14.8	2.5	0.0107	0.0957	-6.5	-3.4	3.2
	Rep.3	2,396	23,267	11.2	14.5	2.2	0.0089	0.0836	-6.8	-3.6	3.2
	Avr.	**3,331**	**25,162**	**11.6**	**14.6**	**2.3**	**0.0135**	**0.0985**	**-6.3**	**-3.4**	**2.9**
	Stdev	**1,416**	**2,263**	**0.6**	**0.1**	**0.1**	**0.0065**	**0.0165**	**0.6**	**0.2**	**0.4**

		Fold change (FC) calculated with the raw read count (Rc)					FC calculated with the normalized count normalised read count (Rc)				
		Rc		Log_2		Log_2 FC LNCaP/ A549	Rc		Log_2		Log_2 FC LNCaP/ A549
		A549	LNCaP	A549	LNCaP		A549	LNCaP	A549	LNCaP	
SYNM	Rep.1	177,946	14,501	17.4	13.8	-3.6	0.7506	0.0686	-0.4	-3.9	-3.5
	Rep.2	164,377	16,199	17.3	14	-3.5	0.6655	0.0561	-0.6	-4.2	-3.6
	Rep.3	154,079	14,466	17.2	13.8	-3.6	0.5726	0.0520	-0.8	-4.3	-3.5
	Avr.	**165,467**	**15,055**	**17.3**	**13.9**	**-3.6**	**0.6629**	**0.0589**	**-0.6**	**-4.1**	**-3.5**
	Stdev	**11,971**	**991**	**0.1**	**0.1**	**0.1**	**0.0890**	**0.0087**	**0.2**	**0.2**	**0.1**
TFAP 2A	Rep.1	94,299	27,021	16.5	14.7	-1.8	0.3978	0.1279	-1.3	-3	-1.6
	Rep.2	106,592	25,883	16.7	14.7	-1.9	0.4316	0.0896	-1.2	-3.5	-2.3
	Rep.3	127,323	28,986	17	14.8	-1.7	0.4732	0.1041	-1.1	-3.3	-2.2
	Avr.	**109,405**	**27,297**	**16.7**	**14.7**	**-1.8**	**0.4342**	**0.1072**	**-1.2**	**-3.2**	**-2.0**
	Stdev	**16,691**	**1,570**	**0.2**	**0.1**	**0.1**	**0.0378**	**0.0193**	**0.1**	**0.3**	**0.3**
TPM2	Rep.1	647,658	18,974	19.3	14.2	-5.1	2.7319	0.0898	1.4	-3.5	-4.9
	Rep.2	571,092	21,325	19.1	14.4	-4.9	2.3122	0.0738	1.2	-3.8	-5
	Rep.3	570,539	27,813	19.1	14.8	-4.5	2.1203	0.0999	1.1	-3.3	-4.4
	Avr.	**596,430**	**22,704**	**19.2**	**14.5**	**-4.9**	**2.3882**	**0.0878**	**1.2**	**-3.5**	**-4.8**
	Stdev	**44,366**	**4,578**	**0.1**	**0.3**	**0.3**	**0.3128**	**0.0132**	**0.2**	**0.2**	**0.3**
UGT2 B15	Rep.1	524	317,083	9	18.3	9.2	0.0022	1.5007	-8.8	0.6	9.4
	Rep.2	535	154,557	9.1	17.2	8.2	0.0022	0.5349	-8.9	-0.9	7.9
	Rep.3	2,478	294,434	11.3	18.2	9.1	0.0092	1.0575	-6.8	0.1	6.8
	Avr.	**1,179**	**255,358**	**9.8**	**17.9**	**8.9**	**0.0045**	**1.0310**	**-8.1**	**-0.1**	**8.1**
	Stdev	**1,125**	**88,028**	**1.3**	**0.6**	**0.6**	**0.0041**	**0.4835**	**1.2**	**0.8**	**1.3**

The data shows that the difference between FC values calculated either using the log_2 value for raw counts or the log_2 value for the normalized counts is not large. However, the normalization process allows a more accurate detection of the relative difference in expression of RNA biomarkers in A549 and LNCaP cells.

For the data in Table 7 we have accepted Log_2 FC values greater than 2 are significant and grouped the expression levels of the 21 prostate cancer specific RNA biomarkers tested using LNCaP and A549 RNA in two groups: $Log_2FC > 2$; and $Log_2FC < 2$.

Table 7: Comparison of Log_2 FC expression levels of RNA biomarkers in LNCaP and A549 RNA

Log_2 Fc	**Elevated expression in LNCaP RNA**	**Elevated expression in A549 RNA**
Log_2 Fc>2	ACPP, AZGP1, CRISP3, DDC, UGT2B15, ETV1 PSCA	AKRIC3, ETV4 , MUC1,PDZK1IP1, TPM2, AGR2, MYLK, , SYNM
Log_2 Fc<2	AR460, AR532, HN1, PCAT1, PEX10	TFAP2A

The data reveals an even split of RNA biomarkers with Log_2 FC >2 between the two RNAs.

The data contained in Table 8 are basic statistical analyses of the Log_2 FC differences between the 21 RNA biomarkers expressed in LNCaP and A549 RNA calculated by dividing the normalized Log_2 FC of each RNA biomarker from LNCaP RNA by the corresponding Log_2 FC from A549 RNA. The level of differential expression calculated by the limma-based linear model fit analysis (T= limma moderated t- statistic) highlights some significant levels of differential expression of the RNA biomarker between the LNCaP and A549 cell types (T value) with correlating P value.

Table 8: Significance levels comparing the differential expression of each RNA biomarker between LNCaP and A549 cells

Target	Log_2 FC difference	t	P.Value	adj.P.Val
ACPP	8.7	30	9.E-14	2.E-12
AZGP1	8.4	24	3.E-12	6.E-11
UGT2B15	8.1	15	1.E-09	2.E-08
ETV4	-6.7	-24	2.E-12	6.E-11
AKRIC3	-5.9	-22	6.E-12	1.E-10
CRISP3	5.7	13	4.E-09	7.E-08
TPM2	-4.9	-17	1.E-10	3.E-09
DDC	4.4	--10	2.E-07	2.E-06
MUC1	-4.3	-15	9.E-10	2.E-08
PDZKIP1	-4.3	-14	2.E-09	3.E-08
AGR2	-3.6	-14	2.E-09	3.E-08
SYNM	-3.5	-13	5.E-09	9.E-08
MYLK	-3.4	-13	7.E-09	1.E-07
PSCA	2.9	7	5.E-06	5.E-05
ETV1	2.9	9	3.E-07	4.E-06
TFAP2A	-2.0	-8	2.E-06	2.E-05
PCAT1	1.4	4	2.E-03	2.E-02
AR532	1.1	2	8.E-02	5.E-01
AR460	0.8	2	9.E-02	5.E-01
PEX10	1.1	3	1.E-02	9.E-02
HN1	0.3	0	8.E-01	1.E+00

These two cell lines, LNCAP and A549, were chosen for this example to demonstrate a proof of concept by comparing RNA biomarker expression in two cell lines; one (LNCaP cells) of prostate origin and the other (A549 cells) of lung origin. As might be expected, there is significant differential expression between these two cell lines of the RNA biomarkers chosen on the basis of their possible involvement in prostate cancer.

The data provided in the above example shows that it is possible to detect the change in expression of specific RNA biomarkers through quantitative amplicon synthesis followed by enumeration using a Next Generation DNA sequencing methodology.

EXAMPLE 2

RNA amplicon biomarker sequencing (RBAS) in the analysis of differential gene expression profile using prostate cancer tissue from formalin-fixed paraffin embedded (FFPE) human prostatectomy tissue

This example demonstrates that the RNA amplicon biomarker sequencing (RBAS) method is diagnostically and prognostically relevant by quantifying the relative expression of 79 RNA biomarkers using amplicon production and NGS to establish their RNA expression profile in prostate cancer tissues.

Stored formalin-fixed paraffin embedded (FFPE) prostatectomy tissue blocks were reviewed by a clinical histopathologist to select tissues for analysis. Prostatectomy tissue from two subjects was selected.

Subject 1 is a 63 year old male who underwent a prostate biopsy in 2007 and was diagnosed with prostate cancer with a Gleason score of 4+5. The subject underwent a radical prostatectomy at the age of 58. A stored FFPE block containing the original prostatectomy tissue was re-examined and a tumor region was identified with a Gleason score of 4+5. The region identified was reset in paraffin and then sectioned. Three tissue samples were selected from Subject 1 for RNA extraction: Tumor tissue 4+5 (T); adjacent glandular tissue (Adj.G); and adjacent muscle tissue (Adj.M) deemed histologically normal.

Subject 2 is a 67 year old male who underwent a prostate biopsy in 2012 and was diagnosed with prostate cancer with a Gleason score of 3+4. The subject underwent a radical prostatectomy at the age of 66. A stored FFPE block containing the prostatectomy tissue was re-examined. Three tumors were identified with different Gleason scores, 4+5 (T1), 3+4 (T2) and 3+3 (T3) respectively. The different regions from the blocks were reset, and then sectioned. Tissue samples were selected from each of the three tumor regions as well as an adjacent glandular tissue (Adj.G) deemed histologically normal. No Adj.M region was identified in Subject 2 tissue samples.

Total RNA was extracted separately from the seven selected tissue samples from Subject 1 and 2 using a Qiagen FFPE RNeasy extraction kit (Cat No: 74404, 73504). The RNA was then used to generate cDNA for each tissue sample as described above in the

methods section. This cDNA was used for amplicon production in triplicate, using a total of 79 RNA biomarker primer pairs that included five reference amplicons from four RNA biomarkers. The second round PCR sequencing of the 79 RNA biomarker specific amplicons produced in the first round PCR was done in two separate runs. During the second round PCR, the barcode sequence for indexing and a tag sequence were added and the amplicon libraries were pooled together for clustering and sequencing on the Illumina Hiseq2500 instrument as described in Example 1.

As described in Example 1, Illumina bcl2fastq conversion software (version 1.8.3) was used to obtain the number of sequence reads per amplicon (read counts).

The raw counts of the five reference amplicons from each of the sequencing runs (Run1, Run2) is presented in Table 9. The sequence counts for all the reference amplicons were lower in run 1 than the run 2. However, the ratio of the individual reference RNA biomarkers to each other was very similar in the two runs.

Table 9A: Subject 1 - Average of raw counts for the triplicates for reference amplicons tested in triplicates from Tumor (T) and adjacent glandular (AdjG) or adjacent muscular (AdjM) RNA samples

	T		Adj.G		Adj.M	
Run 1	**Avr.**	**StDev**	**Avr**	**StDev**	**Avr.**	**StDev**
CDIPT	181,602	108,375	69,387	25,776	109,665	22,597
FKBP15	26,420	14,819	14,726	5,349	19,283	9,148
ZFC3H1	26,996	13,809	11,019	4,804	10,355	5,742
C19orf50.35/36	11,518	5,887	4,873	1,696	7,909	3,387
C19orf50.35/505	11,484	5,941	4,892	1,738	8,029	3,384
Avr.	51,604	28,926	20,979	6,330	31,048	7,989
	T		**Adj.G**		**Adj.M**	
Run 2	**Avr.**	**StDev**	**Avr**	**StDev**	**Avr.**	**StDev**
CDIPT	579,696	428,581	392,492	26,856	312,658	28,339
FKBP15	107,916	67,181	91,199	4,604	52,760	10,832
ZFC3H1	164,089	104,640	75,341	2,445	82,436	13,887
C19orf50.35/36	39,019	27,178	33,147	6,143	23,112	5,425
C19orf50 .35/505	39,049	26,955	32,880	6,194	23,372	5,712
Avr.	185,954	130,620	125,012	5,966	98,868	6,648

Table 9B: Subject 2 - Average raw counts for the triplicate reference amplicons from Tumors (T1,T2 and T3) and adjacent glandular (Adj.G) RNA samples

	T1		Adj.G		T2	
Run 1	Avr.	**StDev**	**Avr**	**StDev**	**Avr.**	**StDev**
CDIPT	141,808	57,175	108,540	13,054	157,843	84,787
FKBP15	32,004	1,364	11,053	9,047	11,090	2,664
ZFC3H1	25,860	7,845	21,315	10,432	21,694	9,172
C19orf50 35/36	5,514	368	3,478	699	4,377	2,372
C19orf50 35/505	5,578	246	3,418	792	4,278	2,306
Avr.	42,153	13,400	29,561	1,977	39,856	19,405
	T3		**Adj.G**		**T2**	
Run 2	Avr.	StDev	Avr	StDev	Avr.	StDev
CDIPT	453,616	163,307	482,506	80,991	444,554	19,270
FKBP15	82,124	40,266	69,754	10,656	90,864	19,203
ZFC3H1	124,362	54,650	99,653	31,461	138,021	19,628
C19orf50 (35/36)	14,934	5,048	11,097	4,414	20,073	8,693
C19orf50 (35/505)	14,997	5,010	11,129	4,241	20,223	8,519
Avr.	138,007	50,711	134,828	20,977	142,747	10,484

The raw counts obtained for the reference amplicons presented in Table 9 were generally consistent between replicates across the prostatectomy–derived RNA samples and the data supports the selection of these RNA biomarkers as reference amplicons.

The average of the read counts from the five reference amplicons was used to normalize the raw read counts of the amplicons produced from the appropriate tumor and adjacent glandular and muscular tissue pairings.

Subject 1 RNA Biomarker Analysis

For the analysis of Subject 1, the data compared the relative expression of the RNA biomarkers between tumor tissue and both adjacent glandular and adjacent muscular tissue. The raw counts of triplicate samples from tumor tissue and both adjacent glandular and adjacent muscular tissue is given followed by the $\log_2$ normalized counts. The $\log_2$ FC expression of each RNA biomarker from the tumor region of the prostatectomy tissue RNA samples is given relative to the adjacent glandular and muscular adjacent muscular tissue RNA. Finally the $\log_2$ FC of the adjacent glandular relative to the muscular adjacent muscular tissue RNA is presented (Table 10).

Those RNA biomarkers with a differential amplicon count (Log_2 FC >2) from Subject 1 were selected from the tumor, adjacent glandular and adjacent muscular samples with the data being presented in Table 11.

Table 10: Subject 1 - Raw read counts, Log_2 normalization of the read counts and relative quantification (Log_2 FC) of RNA biomarker specific amplicons

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
		T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
ACPP	Rep.1	218,083	640,127	31,967	2.94	5.24	0.51	-2.30	2.43	4.734
	Rep.2	163,669	656,380	30,575	1.95	5.21	-0.33	-3.26	2.27	5.534
	Rep.3	700,788	883,399	31,581	3.06	4.97	-0.04	-1.91	3.10	5.001
	Avr.	360,847	726,635	31,374	2.65	5.14	0.05	-2.49	2.60	5.09
	StDv	295,652	136,004	719	0.61	0.15	0.42	0.70	0.44	0.407
AGR2	Rep.1	131,239	31,120	6,276	2.21	0.88	-1.84	1.33	4.05	2.72
	Rep.2	162,340	35,938	4,981	1.94	1.02	-2.94	0.92	4.88	3.961
	Rep.3	476,179	49,861	3,389	2.50	0.82	-3.26	1.68	5.76	4.074
	Avr.	256,586	38,973	4,882	2.22	0.91	-2.68	1.31	4.90	3.585
	StDv	190,808	9,732	1,446	0.28	0.10	0.74	0.38	0.85	0.751
AKR1C3	Rep.1	7,565	7,573	11,688	-1.91	-1.16	-0.94	-0.75	-0.97	-0.22
	Rep.2	11,053	8,093	27,577	-1.94	-1.13	-0.47	-0.81	-1.47	-0.66
	Rep.3	25,510	11,563	19,632	-1.72	-1.29	-0.72	-0.43	-1.00	-0.57
	Avr.	14,709	9,076	19,632	-1.86	-1.19	-0.71	-0.66	-1.14	-0.48
	StDv	9,515	2,169	7,945	0.12	0.08	0.24	0.20	0.28	0.234
ADM	Rep.1	383	177	45	-6.21	-6.58	-8.96	0.37	2.75	2.386
	Rep.2	6,725	794	2,117	-2.66	-4.48	-4.18	1.83	1.52	-0.31
	Rep.3	3,618	497	34	-4.54	-5.83	-9.89	1.29	5.36	4.064
	Avr.	3,575	489	732	-4.47	-5.63	-7.68	1.16	3.21	2.049
	StDv	3,171	309	1,199	1.78	1.06	3.07	0.74	1.96	2.204
AR(460)	Rep.1	87,414	63,945	64,627	1.62	1.92	1.52	-0.30	0.10	0.395
	Rep.2	106,349	75,612	98,985	1.33	2.09	1.37	-0.76	-0.04	0.721
	Rep.3	201,173	84,483	62,643	1.26	1.58	0.95	-0.32	0.31	0.626
	Avr.	131,645	74,680	75,418	1.40	1.86	1.28	-0.46	0.12	0.581
	StDv	60,952	10,301	20,433	0.19	0.26	0.30	0.26	0.18	0.168
AR(532)	Rep.1	42,868	43,461	22,464	0.59	1.36	0.00	-0.77	0.59	1.363
	Rep.2	67,215	21,630	28,560	0.67	0.29	-0.42	0.38	1.09	0.709
	Rep.3	111,816	60,319	43,444	0.41	1.09	0.43	-0.68	-0.01	0.668
	Avr.	73,966	41,803	31,489	0.56	0.91	0.00	-0.36	0.56	0.913
	StDv	34,966	19,398	10,792	0.13	0.56	0.42	0.64	0.55	0.39
AZGP1	Rep.1	198,131	545,971	35,292	2.80	5.01	0.65	-2.21	2.15	4.362
	Rep.2	104,449	650,870	23,844	1.30	5.20	-0.68	-3.90	1.98	5.88
	Rep.3	672,265	871,798	40,138	3.00	4.95	0.31	-1.95	2.69	4.636
	Avr.	324,948	689,546	33,091	2.37	5.05	0.09	-2.68	2.28	4.959
	StDv	304,410	166,321	8,367	0.93	0.13	0.69	1.06	0.37	0.809
CLU	Rep.1	26,673	24,462	48,500	-0.09	0.53	1.11	-0.62	-1.20	-0.58
	Rep.2	36,616	30,951	103,633	-0.21	0.80	1.44	-1.01	-1.65	-0.63
	Rep.3	92,251	52,909	71,777	0.13	0.90	1.15	-0.77	-1.01	-0.25
	Avr.	51,847	36,107	74,637	-0.06	0.75	1.23	-0.80	-1.29	-0.49
	StDv	35,343	14,908	27,678	0.18	0.19	0.18	0.20	0.33	0.21
CRISP3	Rep.1	13,110	984	266	-1.12	-4.10	-6.40	2.99	5.29	2.298
	Rep.2	17,388	4	10	-1.29	-12.12	-11.90	10.83	10.62	-0.21
	Rep.3	17,838	143	36	-2.24	-7.63	-9.81	5.39	7.58	2.185
	Avr.	16,112	377	104	-1.55	-7.95	-9.37	6.40	7.83	1.423
	StDv	2,610	530	141	0.60	4.02	2.78	4.02	2.67	1.418
DDC	Rep.1	49	1	2	-9.18	-14.05	-13.46	4.87	4.28	-0.59
	Rep.2	1	1	1	-15.37	-14.12	-15.23	-1.26	-0.15	1.11
	Rep.3	199	601	670	-8.72	-5.56	-5.59	-3.17	-3.13	*0.038*
	Avr.	83	201	224	-11.09	-11.24	-11.43	0.15	0.33	0.186

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
		T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
	StDv	103	346	386	3.71	4.92	5.13	4.20	3.73	0.859
ETV1	Rep.1	323,226	19,968	28,271	3.51	0.24	0.33	3.27	3.18	-0.09
	Rep.2	470,090	16,096	42,166	3.47	-0.14	0.14	3.61	3.33	-0.28
	Rep.3	697,535	24,370	28,491	3.05	-0.21	-0.18	3.27	3.24	-0.03
	Avr.	496,950	20,145	32,976	3.34	-0.04	0.10	3.38	3.25	-0.13
	StDv	188,595	4,140	7,960	0.25	0.24	0.26	0.20	0.08	0.13
ETV4	Rep.1	501	1,011	829	-5.83	-4.06	-4.76	-1.76	-1.06	0.697
	Rep.2	2	871	2	-14.37	-4.35	-14.23	-10.02	-0.15	9.876
	Rep.3	1,636	571	10	-5.68	-5.63	-11.66	-0.05	5.98	6.03
	Avr.	713	818	280	-8.63	-4.68	-10.22	-3.95	1.59	5.534
	StDv	837	225	475	4.98	0.83	4.89	5.33	3.83	4.61
FLNA	Rep.1	427,572	338,722	869,661	3.91	4.32	5.27	-0.41	-1.36	-0.95
	Rep.2	374,615	451,638	1,877,290	3.14	4.67	5.62	-1.53	-2.47	-0.95
	Rep.3	1,169,697	462,865	1,064,855	3.80	4.03	5.04	-0.23	-1.24	-1.01
	Avr.	657,295	417,742	1,270,602	3.62	4.34	5.31	-0.72	-1.69	-0.97
	StDv	444,543	68,663	534,395	0.41	0.32	0.29	0.70	0.68	0.034
GLOI	Rep.1	215272	35,392	28,114	0.62	1.33	1.78	2.42	2.40	0.46
	Rep.2	132276	53,092	31,252	0.65	1.00	1.58	1.96	2.23	0.31
	Rep.3	487668	76,360	29,474	0.55	0.65	1.85	1.20	2.40	0.00
	Avr.	278405	54948	29613	0.61	0.99	1.74	1.86	2.34	0.26
	StDv	185917	20547	1574	0.05	0.34	0.14	0.62	0.10	0.23
HN1	Rep.1	3,784	1,871	147	-2.91	-3.18	-7.26	0.27	4.35	4.08
	Rep.2	2,614	2,796	4,995	-4.02	-2.67	-2.94	-1.35	-1.08	0.273
	Rep.3	6,432	4,393	1,246	-3.71	-2.69	-4.70	-1.02	0.99	2.013
	Avr.	4,277	3,020	2,129	-3.55	-2.84	-4.96	-0.70	1.42	2.122
	StDv	1,956	1,276	2,542	0.57	0.29	2.17	0.86	2.74	1.906
HPGD	Rep.1	10,885	6,589	11,129	-1.38	-1.36	-1.01	-0.02	-0.37	-0.35
	Rep.2	22,378	12,952	13,946	-0.92	-0.45	-1.46	-0.47	0.5 4	1.003
	Rep.3	47,146	20,066	12,168	-0.83	-0.49	-1.41	-0.34	0.58	0.916
	Avr.	26,803	13,202	12,414	-1.05	-0.77	-1.29	-0.28	0.25	0.525
	StDv	18,531	6,742	1,425	0.30	0.51	0.24	0.23	0.54	0.755
KLK2	Rep.1	300,931	494,877	34,461	3.40	4.87	0.62	-1.47	2.79	4.254
	Rep.2	496,385	636,865	25,665	3.55	5.17	-0.58	-1.62	4.13	5.743
	Rep.3	858,522	630,712	27,354	3.35	4.48	-0.24	-1.13	3.60	4.722
	Avr.	551,946	587,485	29,160	3.44	4.84	-0.07	-1.40	3.50	4.906
	StDv	282,917	80,260	4,668	0.10	0.34	0.62	0.25	0.67	0.761
KLK3	Rep.1	1,201,462	1,510,521	121,070	5.40	6.48	2.43	-1.08	2.97	4.052
	Rep.2	1,715,345	1,465,004	121,869	5.34	6.37	1.67	-1.03	3.67	4.697
	Rep.3	2,869,519	1,541,639	87,096	5.09	5.77	1.43	-0.67	3.67	4.34
	Avr.	1,928,775	1,505,721	110,012	5.28	6.21	1.84	-0.93	3.44	4.363
	StDv	854,265	38,542	19,850	0.16	0.38	0.52	0.22	0.40	0.323
LAMA1	**Rep.1**	38	1	2	-9.55	-14.05	-13.46	4.50	3.91	-0.59
	Rep.2	2	2	1,480	-14.37	-13.12	-4.69	-1.26	-9.68	-8.42
	Rep.3	526	1	1	-7.32	-14.79	-14.98	7.47	7.66	0.195
	Avr.	189	1	494	-10.41	-13.98	-11.04	3.57	0.63	-2.94
	StDv	293	1	854	3.60	0. 84	5.55	4.44	9.12	4.764
MSMB	Rep.1	671,389	929,667	51,400	4.56	5.78	1.19	-1.22	3.37	4.587
	Rep.2	910,538	848,857	18,772	4.43	5.58	-1.03	-1.15	5.45	6.609
	Rep.3	1,628,017	11,765	15,852	4.28	-1.26	-1.03	5.54	5.31	-0.24
	Avr.	1,069,981	596,763	28,675	4.42	3.37	-0.29	1.06	4.71	3.654
	StDv	497,846	508,232	19,735	0.14	4.01	1.28	3.88	1.16	3.516
MUC1A	Rep.1	262	1	5	-6.76	-14.05	-12.13	7.29	5.37	-1.91

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
		T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
	Rep.2	1	1	1	-15.37	-14.12	-15.23	-1.26	-0.15	1.11
	Rep.3	73	2	1	-10.17	-13.79	-14.98	3.62	4.81	1.195
	Avr.	112	1	2	-10.77	-13.98	-14.11	3.22	3.35	0.131
	StDv	135	1	2	4.34	0.17	1.72	4.28	3.04	1.769
MYLK	Rep.1	715,065	617,785	1,953,630	4.65	5.19	6.44	-0.54	-1.79	-1.25
	Rep.2	610,439	657,898	2,799,061	3.85	5.21	6.19	-1.36	-2.34	-0.98
	Rep.3	1,951,162	943,798	1,861,415	4.54	5.06	5.85	-0.52	-1.31	-0.79
	Avr.	1,092,222	739,827	2,204,702	4.35	5.15	6.16	-0.81	-1.81	-1
	StDv	745,701	177,779	516,791	0.44	0.08	0.30	0.48	0.52	0.234
PCAT1	Rep.1	46,874	32,022	49,088	0.72	0.92	1.13	-0.20	-0.40	-0.21
	Rep.2	32,297	32,088	42,375	-0.39	0.85	0.15	-1.25	-0.54	0.709
	Rep.3	108,603	34,684	44,589	0.37	0.29	0.46	0.08	-0.09	-0.17
	Avr.	62,591	32,931	45,351	0.23	0.69	0.58	-0.46	-0.34	0.112
	StDv	40,508	1,518	3,421	0.57	0.34	0.50	0.70	0.23	0.517
PDZK1IP1	Rep.1	3,534	279	81	-3.01	-5.92	-8.12	2.92	5.11	2.195
	Rep.2	7,452	763	25	-2.51	-4.54	-10.58	2.03	8.07	6.041
	Rep.3	14,745	941	32	-2.51	-4.91	-9.98	2.40	7.47	5.073
	Avr.	8,577	661	46	-2.68	-5.12	-9.56	2.45	6.88	4.436
	StDv	5,690	343	31	0.29	0.72	1.29	0.44	1.57	2.001
PEX10	Rep.1	4,988	2,592	142	-2.51	-2.71	-7.31	0.20	4.80	4.601
	Rep.2	11,488	2,484	18	-1.88	-2.84	-11.06	0.95	9.17	8.218
	Rep.3	15,027	2,866	1,354	-2.48	-3.30	-4.58	0.82	2.10	1.277
	Avr.	10,501	2,647	505	-2.29	-2.95	-7.65	0.66	5.35	4.698
	StDv	5,092	197	738	0.35	0.31	3.25	0.40	3.57	3.472
PIP	Rep.1	54	20	3	-9.04	-9.72	-12.87	0.69	3.83	3.147
	Rep.2	1	1	1	-15.37	-14.12	-15.23	-1.26	-0.15	1.11
	Rep.3	214	6	2	-8.62	-12.20	-13.98	3.59	5.36	1.78
	Avr.	90	9	2	-11.01	-12.01	-14.03	1.00	3.02	2.012
	StDv	111	10	1	3.78	2.20	1.18	2.44	2.84	1.039
PSCA	Rep.1	5,241	1,893	584	-2.44	-3.16	-5.27	0.72	2.83	2.107
	Rep.2	1,732	2,623	64	-4.61	-2.76	-9.23	-1.85	4.61	6.467
	Rep.3	21,332	1,448	64	-1.98	-4.29	-8.98	2.31	7.00	4.695
	Avr.	9,435	1,988	237	-3.01	-3.40	-7.82	0.39	4.81	4.423
	StDv	10,451	593	300	1.41	0.79	2.22	2.10	2.10	2.193
RARRES1	Rep.1	32,243	22,582	13,675	0.18	0.42	-0.72	-0.23	0.90	1.134
	Rep.2	60,617	19,969	49,942	0.52	0.17	0.38	0.35	0.13	-0.21
	Rep.3	95,938	25,022	22,595	0.19	-0.18	-0.52	0.37	0.71	0.342
	Avr.	62,933	22,524	28,737	0.30	0.14	-0.28	0.16	0.58	0.421
	StDv	31,911	2,527	18,898	0.19	0.30	0.59	0.34	0.40	0.677
SELM1	Rep.1	45,074	60,198	56,679	0.67	1.83	1.33	-1.17	-0.67	0.497
	Rep.2	81,299	74,988	256,748	0.94	2.08	2.74	-1.14	-1.81	-0.67
	Rep.3	187,357	85,734	154,857	1.16	1.60	2.26	-0.44	-1.10	-0.66
	Avr.	104,577	73,640	156,095	0.92	1.84	2.11	-0.92	-1.19	-0.28
	StDv	73,943	12,821	100,040	0.25	0.24	0.72	0.41	0.57	0.669
SFRP1	Rep.1	20,200	13,851	10,177	-0.49	-0.29	-1.14	-0.20	0.65	0.855
	Rep.2	38,279	14,458	25,213	-0.15	-0.30	-0.60	0.15	0.46	0.307
	Rep.3	67,428	13,976	22,144	-0.32	-1.02	-0.55	0.70	0.23	-0.47
	Avr.	41,969	14,095	19,178	-0.32	-0.53	-0.76	0.21	0.45	0.231
	StDv	23,829	321	7,945	0.17	0.42	0.33	0.45	0.21	0.665
SPP1	Rep.1	17,123	8,549	5,130	-0.73	-0.98	-2.13	0.25	1.40	1.147
	Rep.2	33,838	5,495	9,376	-0.32	-1.69	-2.03	1.37	1.71	0.339
	Rep.3	47,407	5,307	7,799	-0.83	-2.41	-2.05	1.59	1.23	-0.36
	Avr.	32,789	6,450	7,435	-0.63	-1.70	-2.07	1.07	1.44	0.375

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
		T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
	StDv	15,169	1,820	2,146	0.27	0.71	0.05	0.71	0.24	0.755
SYNM	Rep.1	38,214	38,025	108,172	0.43	1.17	2.27	-0.74	-1.84	-1.1
	Rep.2	24,320	27,575	136,330	-0.80	0.64	1.83	-1.44	-2.63	-1.2
	Rep.3	128,472	65,055	113,498	0.61	1.20	1.81	-0.59	-1.20	-0.61
	Avr.	63,669	43,552	119,333	0.08	1.00	1.97	-0.92	-1.89	-0.97
	StDv	56,550	19,342	14,958	0.77	0.32	0.26	0.45	0.72	0.315
TFAP2	Rep.1	4,593	4,894	921	-2.63	-1.79	-4.61	-0.84	1.98	2.82
	Rep.2	12,213	5,609	12	-1.80	-1.66	-11.64	-0.13	9.85	9.978
	Rep.3	17,866	7,267	409	-2.23	-1.96	-6.31	-0.27	4.07	4.346
	Avr.	11,557	5,923	447	-2.22	-1.80	-7.52	-0.42	5.30	5.715
	StDv	6,661	1,217	456	0.42	0.15	3.67	0.37	4.07	3.77
TMC5	Rep.1	42,344	5,080	1,449	0.58	-1.74	-3.96	2.31	4.53	2.22
	Rep.2	156,493	9,681	4,101	1.88	-0.87	-3.22	2.76	5.11	2.349
	Rep.3	184,408	12,510	344	1.13	-1.18	-6.56	2.31	7.69	5.379
	Avr.	127,748	9,090	1,965	1.20	-1.26	-4.58	2.46	5.78	3.316
	StDv	75,268	3,750	1,931	0.66	0.44	1.75	0.26	1.68	1.788
TPM2	Rep.1	349,025	360,643	697,757	3.62	4.41	4.96	-0.80	-1.34	-0.54
	Rep.2	258,123	394,476	1,498,424	2.61	4.47	5.29	-1.87	-2.68	-0.82
	Rep.3	1,091,972	518,778	1,081,988	3.70	4.20	5.06	-0.50	-1.36	-0.87
	Avr.	566,373	424,632	1,092,723	3.31	4.36	5.10	-1.05	-1.79	-0.74
	StDv	457,445	83,269	400,441	0.61	0.15	0.17	0.72	0.77	0.174
TPX2	Rep.1	148	19	1,930	-7.58	-9.80	-3.54	2.21	-4.04	-6.26
	Rep.2	2	39	1,802	-14.37	-8.83	-4.41	-5.54	-9.96	-4.42
	Rep.3	648	4	4	-7.02	-12.79	-12.98	5.77	5.96	0.195
	Avr.	266	21	1,245	-9.66	-10.47	-6.98	0.81	-2.68	-3.49
	StDv	339	18	1,077	4.09	2.06	5.22	5.78	8.05	3.324
UGT2B15	Rep.1	1,427	8	26	-4.32	-11.05	-9.76	6.73	5.44	-1.29
	Rep.2	174	4	3	-7.93	-12.12	-13.64	4.19	5.71	1.525
	Rep.3	1,210	1,234	24	-6.12	-4.52	-10.40	-1.60	4.28	5.879
	Avr.	937	415	18	-6.12	-9.23	-11.26	3.11	5.14	2.038
	StDv	670	709	13	1.81	4.11	2.08	4.27	0.76	3.612
ApoC1	Rep.1	174,984	60,571	15,853	0.32	-1.02	-2.61	1.34	2.93	1.586
	Rep.2	109,280	61,628	16,719	0.37	-1.10	-2.48	1.47	2.86	1.388
	Rep.3	287,167	63,189	16,083	-0.21	-0.93	-2.72	0.71	2.51	1.797
	Avr.	190,477	61,796	16,218	0.16	-1.02	-2.61	1.17	2.77	1.59
	StDv	89,950	1,317	449	0.33	0.08	0.12	0.41	0.22	0.205
ApoE	Rep.1	291,532	162,851	193,580	1.06	0.40	1.00	0.65	0.06	-0.6
	Rep.2	176,541	148,789	166,165	1.06	0.18	0.83	0.89	0.23	-0.65
	Rep.3	598,834	164,006	168,695	0.85	0.45	0.67	0.40	0.18	-0.22
	Avr.	355,636	158,549	176,147	0.99	0.34	0.83	0.65	0.16	-0.49
	StDv	218,323	8,472	15,151	0.12	0.15	0.17	0.25	0.09	0.237
C15orf48	Rep.1	91,710	72,158	11,140	-0.61	-0.77	-3.12	0.16	2.51	2.348
	Rep.2	24,923	90,805	13,560	-1.76	-0.54	-2.79	-1.22	1.02	2.249
	Rep.3	335,481	73,301	9,586	0.01	-0.71	-3.47	0.72	3.48	2.758
	Avr.	150,705	78,755	11,429	-0.79	-0.67	-3.13	-0.11	2.34	2.452
	StDv	163,468	10,452	2,003	0.90	0.12	0.34	1.00	1.24	0.27
CSRP1.583	Rep.1	501,452	720,127	1,040,681	1.84	2.55	3.43	-0.71	-1.59	-0.88
	Rep.2	211,188	999,386	1,129,536	1.32	2.92	3.60	-1.60	-2.27	-0.67
	Rep.3	1,187,574	454,677	685,654	1.83	1.92	2.69	-0.09	-0.86	-0.77
	Avr.	633,405	724,730	951,957	1.67	2.46	3.24	-0.80	-1.57	-0.77
	StDv	501,389	272,384	234,865	0.30	0.51	0.48	0.76	0.71	0.104
CSRP1.690	Rep.1	428,472	677,330	878,261	1.61	2.46	3.18	-0.85	-1.57	-0.72
	Rep.2	135,826	860,624	776,997	0.69	2.71	3.06	-2.02	-2.37	-0.35

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
		T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
	Rep.3	939,564	682,836	907,654	1.50	2.51	3.09	-1.01	-1.60	-0.59
	Avr.	501,287	740,263	854,304	1.26	2.56	3.11	-1.29	-1.85	-0.55
	StDv	406,786	104,272	68,544	0.50	0.13	0.06	0.64	0.45	0.19
EBF3	Rep.1	3,600	7,994	7,110	-5.28	-3.95	-3.77	-1.34	-1.51	-0.18
	Rep.2	2,129	4,120	5,084	-5.31	-5.00	-4.20	-0.31	-1.11	-0.8
	Rep.3	11,296	3,972	4,659	-4.88	-4.92	-4.51	0.04	-0.37	-0.41
	Avr.	5,675	5,362	5,618	-5.16	-4.62	-4.16	-0.54	-1.00	-0.46
	StDv	4,923	2,281	1,310	0.24	0.59	0.37	0.71	0.58	0.313
F5	Rep.1	358,681	9,657	4,497	1.36	-3.67	-4.43	5.03	5.78	0.755
	Rep.2	185,570	5,448	115	1.14	-4.60	-9.67	5.73	10.80	5.072
	Rep.3	282,916	3,853	2,263	-0.24	-4.96	-5.55	4.73	5.32	0.591
	Avr.	275,722	6,319	2,292	0.75	-4.41	-6.55	5.16	7.30	2.139
	StDv	86,779	2,999	2,191	0.86	0.66	2.76	0.52	3.04	2.541
FGG	Rep.1	67	6	321	-11.03	-14.33	-8.24	3.30	-2.79	-6.09
	Rep.2	1	1	2	-16.37	-17.01	-15.51	0.64	-0.85	-1.49
	Rep.3	341	4	3	-9.93	-14.87	-15.11	4.94	5.18	0.238
	Avr.	136	4	109	-12.44	-15.40	-12.95	2.96	0.51	-2.45
	StDv	180	3	184	3.44	1.42	4.09	2.17	4.16	3.27
FHL2	Rep.1	58,230	70,377	35,517	-1.27	-0.81	-1.45	-0.46	0.18	0.639
	Rep.2	38,348	76,367	39,815	-1.14	-0.79	-1.23	-0.35	0.09	0.445
	Rep.3	116,597	69,405	35,387	-1.52	-0.79	-1.59	-0.72	0.07	0.795
	Avr.	71,058	72,050	36,906	-1.31	-0.80	-1.42	-0.51	0.11	0.626
	StDv	40,671	3,770	2,520	0.19	0.01	0.18	0.19	0.06	0.175
GLOI	Rep.1	58,230	70,377	35,517	-1.27	-0.81	-1.45	-0.46	0.18	0.639
	Rep.2	38,348	76,367	39,815	-1.14	-0.79	-1.23	-0.35	0.09	0.445
	Rep.3	116,597	69,405	35,387	-1.52	-0.79	-1.59	-0.72	0.07	0.795
	Avr.	71,058	72,050	36,906	-1.31	-0.80	-1.42	-0.51	0.11	0.626
	StDv	40,671	3,770	2,520	0.19	0.01	0.18	0.19	0.06	0.175
GRAMD4	Rep.1	40,612	35,025	14,160	-1.79	-1.81	-2.77	0.03	0.99	0.959
	Rep.2	15,180	47,756	17,947	-2.48	-1.46	-2.38	-1.01	-0.09	0.918
	Rep.3	85,337	44,607	31,849	-1.97	-1.43	-1.74	-0.54	-0.23	0.309
	Avr.	47,043	42,463	21,319	-2.08	-1.57	-2.30	-0.51	0.22	0.729
	StDv	35,518	6,631	9,314	0.36	0.21	0.52	0.52	0.67	0.364
HIF1A	Rep.1	391,182	387,463	283,585	1.48	1.65	1.55	-0.17	-0.07	0.103
	Rep.2	185,075	532,691	278,680	1.13	2.02	1.58	-0.88	-0.44	0.44
	Rep.3	905,548	469,050	235,023	1.44	1.96	1.14	-0.52	0.30	0.82
	Avr.	493,935	463,068	265,763	1.35	1.88	1.42	-0.53	-0.07	0.454
	StDv	371,065	72,799	26,734	0.19	0.20	0.24	0.36	0.37	0.359
HIPK2	Rep.1	166,274	152,208	52,407	0.25	0.30	-0.89	-0.06	1.13	1.191
	Rep.2	121,045	186,578	58,276	0.52	0.50	-0.68	0.02	1.20	1.184
	Rep.3	387,919	143,266	74,611	0.22	0.25	-0.51	-0.03	0.73	0.764
	Avr.	225,079	160,684	61,765	0.33	0.35	-0.69	-0.03	1.02	1.047
	StDv	142,825	22,866	11,506	0.17	0.13	0.19	0.04	0.25	0.244
HOXC4	Rep.1	2,026	151	3,808	-6.11	-9.67	-4.67	3.56	-1.44	-5
	Rep.2	12,598	2,903	5,307	-2.74	-5.50	-4.14	2.76	1.39	-1.36
	Rep.3	22,809	57	3,547	-3.87	-11.04	-4.91	7.17	1.04	-6.14
	Avr.	12,478	1,037	4,221	-4.24	-8.74	-4.57	4.50	0.33	-4.17
	StDv	10,392	1,617	950	1.71	2.88	0.39	2.35	1.55	2.493
HPN	Rep.1	148,315	10,413	4,335	0.08	-3.56	-4.48	3.65	4.56	0.917
	Rep.2	171,935	9,123	4,240	1.03	-3.85	-4.46	4.88	5.49	0.611
	Rep.3	266,748	9,548	9,841	-0.32	-3.65	-3.43	3.33	3.11	-0.22
	Avr.	195,666	9,695	6,139	0.26	-3.69	-4.13	3.95	4.39	0.436
	StDv	62,681	657	3,207	0.69	0.15	0.60	0.82	1.20	0.589

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
		T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
HSBP1	Rep.1	739,041	741,668	736,840	2.40	2.59	2.93	-0.19	-0.53	-0.34
	Rep.2	310,328	857,558	664,962	1.88	2.70	2.83	-0.83	-0.95	-0.13
	Rep.3	1,413,987	743,511	811,331	2.08	2.63	2.93	-0.54	-0.85	-0.3
	Avr.	821,119	780,912	737,711	2.12	2.64	2.90	-0.52	-0.78	-0.26
	StDv	556,389	66,383	73,188	0.26	0.06	0.06	0.32	0.22	0.113
IGFBP1	Rep.1	391	18	33	-8.49	-12.74	-11.52	4.26	3.03	-1.22
	Rep.2	5	4	3	-14.04	-15.01	-14.93	0.96	0.88	-0.08
	Rep.3	1,724	4	6	-7.59	-14.87	-14.11	7.28	6.52	-0.76
	Avr.	707	9	14	-10.04	-14.21	-13.52	4.17	3.48	-0.69
	StDv	902	8	17	3.49	1.27	1.78	3.16	2.84	0.575
KLK3.470	Rep.1	371,338	339,916	49,813	1.41	1.46	-0.96	-0.06	2.37	2.423
	Rep.2	123,291	234,580	77,137	0.55	0.83	-0.28	-0.29	0.82	1.11
	Rep.3	673,083	288,995	47,031	1.01	1.27	-1.18	-0.25	2.19	2.443
	Avr.	389,237	287,830	57,994	0.99	1.19	-0.80	-0.20	1.79	1.992
	StDv	275,333	52,678	16,637	0.43	0.32	0.47	0.12	0.84	0.764
LRRN1	Rep.1	2,400	1,967	3,990	-5.87	-5.97	-4.60	0.10	-1.27	-1.37
	Rep.2	1,538	4,512	3,130	-5.78	-4.87	-4.90	-0.91	-0.88	0.033
	Rep.3	4,314	2,719	3,327	-6.27	-5.47	-5.00	-0.81	-1.27	-0.47
	Avr.	2,751	3,066	3,482	-5.97	-5.43	-4.83	-0.54	-1.14	-0.6
	StDv	1,421	1,308	451	0.26	0.55	0.21	0.56	0.23	0.71
MAP3K7	Rep.1	285,317	268,102	197,273	1.03	1.12	1.03	-0.10	0.00	0.095
	Rep.2	159,676	327,841	224,968	0.92	1.32	1.27	-0.40	-0.35	0.049
	Rep.3	736,305	343,367	243,733	1.14	1.51	1.20	-0.37	-0.05	0.318
	Avr.	393,766	313,103	221,991	1.03	1.32	1.16	-0.29	-0.13	0.154
	StDv	303,226	39,738	23,373	0.11	0.20	0.12	0.17	0.19	0.144
MYEF2	Rep.1	46,838	35,016	26,471	-1.58	-1.82	-1.87	0.23	0.29	0.056
	Rep.2	37,413	47,082	29,873	-1.17	-1.48	-1.65	0.31	0.47	0.162
	Rep.3	107,994	36,896	29,425	-1.63	-1.70	-1.85	0.08	0.23	0.15
	Avr.	64,082	39,665	28,590	-1.46	-1.67	-1.79	0.21	0.33	0.123
	StDv	38,320	6,492	1,848	0.25	0.17	0.13	0.12	0.13	0.058
OPRK1	Rep.1	5,217	2,718	36	-4.75	-5.50	-11.39	0.76	6.65	5.891
	Rep.2	1,995	1,118	792	-5.40	-6.88	-6.88	1.48	1.48	0.003
	Rep.3	3,156	2,030	25	-6.72	-5.89	-12.05	-0.84	5.33	6.167
	Avr.	3,456	1,955	284	-5.62	-6.09	-10.11	0.47	4.49	4.02
	StDv	1,632	803	440	1.01	0.71	2.81	1.18	2.68	3.482
PCAT14	Rep.1	21,748	32,046	33,751	-2.69	-1.94	-1.52	-0.74	-1.17	-0.42
	Rep.2	7,029	32,465	23,679	-3.59	-2.02	-1.98	-1.57	-1.61	-0.04
	Rep.3	51,291	28,036	24,567	-2.70	-2.10	-2.11	-0.60	-0.59	0.014
	Avr.	26,689	30,849	27,332	-2.99	-2.02	-1.87	-0.97	-1.12	-0.15
	StDv	22,541	2,445	5,576	0.52	0.08	0.31	0.52	0.51	0.238
PFKP	Rep.1	128,373	126,959	148,613	-0.13	0.04	0.62	-0.17	-0.74	-0.57
	Rep.2	79,892	161,519	164,803	-0.08	0.29	0.82	-0.37	-0.90	-0.52
	Rep.3	337,725	109,308	143,071	0.02	-0.14	0.43	0.16	-0.41	-0.57
	Avr.	181,997	132,595	152,162	-0.06	0.07	0.62	-0.13	-0.68	-0.55
	StDv	137,026	26,558	11,292	0.07	0.22	0.19	0.27	0.25	0.027
PFKL	Rep.1	84,518	86,343	53,852	-0.73	-0.51	-0.85	-0.22	0.12	0.334
	Rep.2	57,137	116,264	56,622	-0.56	-0.18	-0.72	-0.38	0.16	0.544
	Rep.3	177,580	71,945	53,309	-0.91	-0.74	-1.00	-0.17	0.09	0.256
	Avr.	106,412	91,517	54,594	-0.73	-0.48	-0.86	-0.26	0.12	0.378
	StDv	63,136	22,608	1,777	0.17	0.28	0.14	0.11	0.04	0.149
PLA2G7	Rep.1	35,242	9,098	2,481	-1.99	-3.76	-5.29	1.77	3.30	1.527
	Rep.2	18,511	17,773	2,808	-2.19	-2.89	-5.06	0.70	2.87	2.168
	Rep.3	26,899	7,983	3,493	-3.63	-3.91	-4.93	0.28	1.30	1.016

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
		T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
	Avr.	26,884	11,618	2,927	-2.60	-3.52	-5.09	0.92	2.49	1.57
	StDv	8,366	5,359	516	0.90	0.55	0.18	0.77	1.05	0.577
PSMA	Rep.1	325,305	29,181	3,040	1.22	-2.08	-4.99	3.29	6.21	2.915
	Rep.2	291,538	31,302	4,664	1.79	-2.07	-4.32	3.86	6.11	2.252
	Rep.3	267,804	13,383	3,813	-0.32	-3.17	-4.80	2.85	4.49	1.635
	Avr.	294,882	24,622	3,839	0.90	-2.44	-4.71	3.33	5.60	2.267
	StDv	28,896	9,791	812	1.09	0.63	0.34	0.51	0.97	0.641
SAA2	Rep.1	18,550	47,657	453	-2.92	-1.37	-7.74	-1.55	4.82	6.37
	Rep.2	3,824	38,395	27	-4.46	-1.78	-11.76	-2.69	7.29	9.979
	Rep.3	38,531	49,483	787	-3.11	-1.28	-7.08	-1.83	3.96	5.798
	Avr.	20,302	45,178	422	-3.50	-1.48	-8.86	-2.02	5.36	7.382
	StDv	17,420	5,945	381	0.84	0.27	2.53	0.59	1.73	2.267
SERPINA1	Rep.1	71,980	74,165	22,531	-0.96	-0.73	-2.10	-0.23	1.14	1.371
	Rep.2	25,468	46,560	7,792	-1.73	-1.50	-3.58	-0.23	1.86	2.085
	Rep.3	128,858	61,216	17,040	-1.37	-0.97	-2.64	-0.40	1.27	1.668
	Avr.	75,435	60,647	15,788	-1.35	-1.07	-2.78	-0.29	1.42	1.708
	StDv	51,782	13,811	7,449	0.38	0.39	0.75	0.10	0.38	0.358
SLC10A7	Rep.1	40,424	11,602	8,678	-1.79	-3.41	-3.48	1.62	1.69	0.071
	Rep.2	3,727	2,626	2,051	-4.50	-5.65	-5.51	1.15	1.01	-0.14
	Rep.3	45,902	6,739	4,999	-2.86	-4.16	-4.41	1.30	1.55	0.254
	Avr.	30,018	6,989	5,243	-3.05	-4.40	-4.47	1.35	1.42	0.063
	StDv	22,933	4,493	3,320	1.36	1.14	1.02	0.24	0.36	0.196
SMAD5	Rep.1	284,815	312,813	262,701	1.02	1.34	1.44	-0.32	-0.42	-0.1
	Rep.2	131,876	336,415	220,795	0.64	1.35	1.24	-0.71	-0.60	0.113
	Rep.3	589,034	310,738	276,986	0.82	1.37	1.38	-0.55	-0.56	-0.01
	Avr.	335,242	319,989	253,494	0.83	1.36	1.35	-0.53	-0.52	0.002
	StDv	232,713	14,263	29,205	0.19	0.01	0.10	0.20	0.10	0.105
SPON2	Rep.1	213,150	368,410	72,098	0.61	1.58	-0.43	-0.98	1.03	2.006
	Rep.2	123,703	514,190	67,857	0.55	1.97	-0.46	-1.41	1.01	2.427
	Rep.3	373,228	376,107	57,551	0.16	1.65	-0.89	-1.48	1.05	2.531
	Avr.	236,694	419,569	65,835	0.44	1.73	-0.59	-1.29	1.03	2.322
	StDv	126,418	82,035	7,481	0.24	0.21	0.26	0.28	0.02	0.278
SRC	Rep.1	27,107	46,057	35,875	-2.37	-1.42	-1.43	-0.95	-0.94	0.013
	Rep.2	25,281	63,357	33,209	-1.74	-1.06	-1.49	-0.68	-0.25	0.438
	Rep.3	57,395	50,210	21,905	-2.54	-1.26	-2.28	-1.28	-0.26	1.02
	Avr.	36,594	53,208	30,330	-2.22	-1.24	-1.73	-0.97	-0.48	0.49
	StDv	18,037	9,031	7,417	0.42	0.18	0.47	0.30	0.40	0.506
SYNPO2	Rep.1	702,319	1,004,740	976,835	2.33	3.03	3.33	-0.70	-1.01	-0.31
	Rep.2	261,029	1,022,928	1,169,889	1.63	2.96	3.65	-1.33	-2.02	-0.69
	Rep.3	1,912,903	984,079	1,293,086	2.52	3.03	3.60	-0.51	-1.08	-0.57
	Avr.	958,750	1,003,916	1,146,603	2.16	3.01	3.53	-0.85	-1.37	-0.52
	StDv	855,272	19,438	159,406	0.47	0.04	0.17	0.43	0.56	0.195
TDRD1	Rep.1	415	153	1,634	-8.40	-9.65	-5.89	1.25	-2.51	-3.76
	Rep.2	3	4	39	-14.78	-15.01	-11.23	0.23	-3.55	-3.78
	Rep.3	1,886	5	24	-7.47	-14.55	-12.11	7.09	4.65	-2.44
	Avr.	768	54	566	-10.22	-13.07	-9.74	2.86	-0.47	-3.33
	StDv	990	86	925	3.98	2.97	3.37	3.70	4.46	0.769
TRIB1	Rep.1	221,374	165,506	56,123	0.66	0.43	-0.79	0.23	1.45	1.213
	Rep.2	134,990	182,298	54,023	0.68	0.47	-0.79	0.21	1.47	1.26
	Rep.3	321,378	153,222	57,415	-0.05	0.35	-0.89	-0.40	0.84	1.239
	Avr.	225,914	167,009	55,854	0.43	0.42	-0.82	0.01	1.25	1.237
	StDv	93,277	14,596	1,712	0.42	0.06	0.06	0.36	0.36	0.024
TSPAN13	Rep.1	157,778	49,173	13,875	0.17	-1.33	-2.80	1.50	2.97	1.478

	Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)		
	T	Adj.G	Adj.M	T	Adj.G	Adj.M	T/ Adj.G	T/ Adj.M	Adj.G/ Adj.M
Rep.2	84,561	53,576	15,083	0.00	-1.30	-2.63	1.30	2.63	1.334
Rep.3	221,110	47,740	19,395	-0.59	-1.33	-2.45	0.74	1.86	1.123
Avr.	154,483	50,163	16,118	-0.14	-1.32	-2.63	1.18	2.49	1.312
StDv	68,334	3,041	2,902	0.40	0.02	0.17	0.39	0.57	0.179

Table 11: Subject 1 - RNA biomarkers with differential expression (Log2 FC>2) in Tumor and adjacent tissues

	T/Adj.G		T/Adj.M		Adj.G/Adj.M	
Marker	Avr.	StDv	Avr.	StDv	Avr.	StDv
ETV1	3.38	0.20	3.25	0.08	-0.13	0.13
HPN	3.95	0.82	4.39	1.20	0.44	0.59
F5	5.16	0.52	7.30	3.04	2.14	2.54
PSMA	3.33	0.51	5.60	0.97	2.27	0.64
UGT2B15	3.11	4.27	5.14	0.76	2.04	3.61
CRISP3	6.40	4.02	7.83	2.67	1.42	1.42
TMC5	2.46	0.26	5.78	1.68	3.32	1.79
PDZK1IP1	2.45	0.44	6.88	1.57	4.44	2.00
MSMB	1.06	3.88	4.71	1.16	3.65	3.52
PSCA	0.39	2.10	4.81	2.10	4.42	2.19
TFAP2	-0.42	0.37	5.30	4.07	5.71	3.77
KLK3 438	-0.93	0.22	3.44	0.40	4.36	0.32
KLK2	-1.40	0.25	3.50	0.67	4.91	0.76
OPRK1	0.47	1.18	4.49	2.68	4.02	3.48
PEX10	0.66	0.40	5.35	3.57	4.70	3.47
C15orf48	-0.11	1.00	2.34	1.24	2.45	0.27
AGR2	1.31	0.38	4.90	0.85	3.58	0.75
ADM	1.16	0.74	3.21	1.96	2.05	2.20
KLK3 470	-0.20	0.12	1.79	0.84	1.99	0.76
PLA2G7	0.92	0.77	2.49	1.05	1.57	0.58
SPON2	-1.29	0.28	1.03	0.02	2.32	0.28
HN1	-0.70	0.86	1.42	2.74	2.12	1.91
ACPP	-2.49	0.70	2.60	0.44	5.09	0.41
AZGP1	-2.68	1.06	2.28	0.37	4.96	0.81
SAA2	-2.02	0.59	5.36	1.73	7.38	2.27

A number of biomarkers are found to be differentially expressed in either the tumor samples or the adjacent glandular or muscular tissues and these have been grouped in Table 12 below.

Table 12: Subject 1 - Comparison of the tumor, adjacent glandular and adjacent muscule tissue expression of select RNA biomarkers

Tumor vs adjacent glandular and muscle tissue differential expression with log2FC >2	**RNA biomarkers**
Up regulated in tumor compared with adjacent glandular and muscle tissues and no difference between the adjacent glandular and muscle tissues.	ETV1, HPN, F5, PMSA, UGT2BI5, CRISP3
Up regulated in the tumor and the glandular adjacent tissue compared with the adjacent muscle tissue, with higher up regulation in the tumor than in the glandular adjacent tissue.	TMC5, PDZK1IP1, MSMB, PSCA
No difference between the tumor and the adjacent glandular tissus and up regulated compared with adjacent muscule tissue.	TFAP2, KLK3 438, KLK2, OPRK1, PEX10, C15orf48, AGR2, KLK3 470, PLA2G7, SPON2,
Higher in the glandular tissue compared with the tumor tissue compared with the adjacent muscle tissue.	ACPP, AZGP1, SAA2

It is common practice in this area of cancer research, particularly when using archival FFPE blocks as the source of tumor tissue, to use tissue adjacent to the tumor as control healthy tissue when studying differential expression. However, studies that have compared gene expression profiles or the chromatin status of prostate tumor tissue with adjacent tissue and benign prostate tissue from brain dead organ donors with no evidence of prostate cancer have suggested that the adjacent tissue has a genome and transcriptome that is more similar to the tumor than to the donor control tissues, suggesting that field effects exist (Chandran et al. 2005, Aryee et al. 2013).

The RBAS analysis using Subject 1 tissue shows that the glandular adjacent tissue has an RNA expression profile more similar to the tumor which is very likely due to field effects as described for prostate cancer tissues by Chandran et al (2005), Rizzi et al. (*PLoS One* 3(10):e3617, 2008) and reviewed in Trujillo et al. (*Prostate Cancer*, 2012).

Subject 2 RNA Biomarker Analysis

The analysis of Subject 2 used prostatectomy tissue and the data compares the relative expression of the RNA biomarkers between three tumor tissues with different Gleason scores (termed T1, T2, and T3) to the adjacent glandular tissue only. The raw counts of triplicate samples from T1, T2 and T3 tumor tissues and adjacent glandular tissue is given followed by the $\log_2$ normalised counts. Finally the $\log_2$ FC expression of each RNA biomarker from the tumor region of the prostatectomy tissue RNA samples is given relative to the adjacent glandular tissue RNA.

The raw counts acquired for each amplicon from Subject 2 samples is presented in Table 13 with the calculation of the normalized count and FC.

Table 13: Subject 2 - Raw read counts, Log_2 normalization and relative quantification (Log_2 FC) of RNA biomarker specific amplicons

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T2	Adj.G	T1	T2	Adj.G	T1/T2	T1/ Adj.G
ACPP	Rep.1	1,115,466	578,078	212,966	4.43	4.28	2.92	0.16	1.51
	Rep.2	4	381,347	138,256	1.74	3.79	2.26	-2.06	-0.53
	Rep.3	478,421	707,704	171,359	3.87	3.51	2.43	0.36	1.44
	Avr.	531,297	555,710	174,194	3.35	3.86	2.54	-0.51	0.81
	StDv	559,608	164,324	37,436	1.42	0.39	0.34	1.34	1.16
AGR2	Rep.1	967,584	227,247	305,013	4.23	2.93	3.44	1.30	0.79
	Rep.2	4	285,242	416,971	1.74	3.37	3.86	-1.64	-2.12
	Rep.3	551,975	408,212	508,593	4.08	2.71	4.00	1.36	0.08
	Avr.	506,521	306,900	410,192	3.35	3.01	3.77	0.34	-0.42
	StDv	485,389	92,406	101,959	1.40	0.34	0.29	1.71	1.51
AKR1C3	Rep.1	29,847	6,708	18,883	-0.79	-2.15	-0.57	1.36	-0.22
	Rep.2	1	12,909	25,412	-0.26	-1.09	-0.18	0.83	-0.08
	Rep.3	15,224	15,973	4,578	-1.10	-1.96	-2.80	0.86	1.69
	Avr.	15,024	11,863	16,291	-0.72	-1.74	-1.18	1.02	0.46
	StDv	14,924	4,720	10,656	0.42	0.57	1.41	0.30	1.07
ADM	Rep.1	10	454	3	-12.33	-6.04	-13.19	-6.30	0.86
	Rep.2	1	4	1,210	-0.26	-12.75	-4.57	12.48	4.31
	Rep.3	1,165	1,647	6	-4.81	-5.24	-12.37	0.43	7.56
	Avr.	392	702	406	-5.80	-8.01	-10.05	2.21	4.24
	StDv	669	849	696	6.10	4.12	4.76	9.52	3.35
AR(532)	Rep.1	156,637	62,951	26,553	1.60	1.08	-0.08	0.52	1.68
	Rep.2	2	55,735	76,486	0.74	1.02	1.41	-0.28	-0.67
	Rep.3	69,267	101,758	90,656	1.08	0.71	1.51	0.37	-0.43
	Avr.	75,302	73,481	64,565	1.14	0.94	0.95	0.21	0.19
	StDv	78,492	24,753	33,673	0.43	0.20	0.89	0.43	1.29
AR(460)	Rep.1	90,088	37,428	54,162	0.80	0.33	0.95	0.48	-0.14
	Rep.2	2	28,087	20,226	0.74	0.03	-0.51	0.71	1.25
	Rep.3	33,627	62,350	42,563	0.04	0.00	0.42	0.04	-0.38
	Avr.	41,239	42,622	38,984	0.53	0.12	0.29	0.41	0.24
	StDv	45,523	17,712	17,249	0.42	0.18	0.74	0.34	0.88
AZGP1	Rep.1	1,205,621	257,386	176,572	4.55	3.11	2.65	1.44	1.89
	Rep.2	4	488,064	484,084	1.74	4.15	4.07	-2.41	-2.33
	Rep.3	577,755	953,508	474,743	4.14	3.94	3.90	0.21	0.24
	Avr.	594,460	566,319	378,466	3.48	3.73	3.54	-0.26	-0.07
	StDv	602,982	354,597	174,908	1.52	0.55	0.77	1.97	2.13
CLU	Rep.1	31,199	29,463	27,065	-0.73	-0.02	-0.05	-0.71	-0.67
	Rep.2	1	25,901	45,362	-0.26	-0.09	0.66	-0.18	-0.92
	Rep.3	19,033	65,755	59,009	-0.78	0.08	0.89	-0.86	-1.67
	Avr.	16,744	40,373	43,812	-0.59	-0.01	0.50	-0.58	-1.09
	StDv	15,724	22,053	16,028	0.28	0.08	0.49	0.36	0.52
CRISP3	Rep.1	49	16	4	-10.04	-10.86	-12.78	0.82	2.74
	Rep.2	1	6	7	-0.26	-12.16	-12.01	11.90	11.74
	Rep.3	8	10	12	-12.00	-12.60	-11.37	0.61	-0.62
	Avr.	19	11	8	-7.43	-11.88	-12.05	4.44	4.62
	StDv	26	5	4	6.29	0.90	0.70	6.46	6.40
DDC	Rep.1	2	1,199	1	-14.66	-4.64	-14.78	-10.02	0.12
	Rep.2	1	1	2	-0.26	-14.75	-13.81	14.48	13.55
	Rep.3	1	1	2	-15.00	-15.93	-13.96	0.93	-1.04
	Avr.	1	400	2	-9.97	-11.77	-14.18	1.80	4.21
	StDv	1	692	1	8.41	6.21	0.52	12.27	8.11
ETV1	Rep.1	55,213	19,124	17,021	0.10	-0.64	-0.72	0.74	0.82
	Rep.2	1	7,861	12,058	-0.26	-1.81	-1.26	1.54	0.99
	Rep.3	19,210	23,675	21,091	-0.77	-1.39	-0.59	0.63	-0.17
	Avr.	24,808	16,887	16,723	-0.31	-1.28	-0.86	0.97	0.55
	StDv	28,028	8,141	4,524	0.43	0.59	0.35	0.50	0.63
ETV4	Rep.1	1,075	1	4	-5.59	-14.86	-12.78	9.28	7.19
	Rep.2	1	2	3	-0.26	-13.75	-13.23	13.48	12.97

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T2	Adj.G	T1	T2	Adj.G	T1/T2	T1/ Adj.G
	Rep.3	1	1,466	148	-15.00	-5.41	-7.75	-9.59	-7.25
	Avr.	359	490	52	-6.95	-11.34	-11.25	4.39	4.30
	StDv	620	846	83	7.46	5.17	3.04	12.29	10.41
FLNA	Rep.1	642,702	419,884	592,030	3.64	3.81	4.40	-0.18	-0.76
	Rep.2	10	350,713	643,645	3.06	3.67	4.48	-0.61	-1.42
	Rep.3	288,460	679,776	656,776	3.14	3.45	4.37	-0.31	-1.23
	Avr.	310,391	483,458	630,817	3.28	3.65	4.42	-0.37	-1.14
	StDv	321,907	173,499	34,226	0.31	0.18	0.06	0.22	0.34
GLO1	Rep.1	66,877	106,272	53,755	-1.21	-0.54	-1.33	-0.067	0.12
	Rep.2	80,576	105,012	56,706	-1.14	-0.36	-1.00	-0.78	-0.14
	Rep.3	66160	119,919	99018	-0.29	-0.21	-0.65	-0.08	0.36
	Avr.	71,204	110,401	6,9826	-0.88	-0.37	-0.99	-0.31	0.11
	StDv	8,124	8,267	2,5324	0.51	0.17	0.34	0.41	0.25
HN1	Rep.1	5,906	3,391	610	-3.13	-3.14	-5.52	0.01	2.40
	Rep.2	1	1,965	1,360	-0.26	-3.81	-4.40	3.54	4.14
	Rep.3	1,485	2,475	123	-4.46	-4.65	-8.01	0.19	3.55
	Avr.	2,464	2,610	698	-2.62	-3.87	-5.98	1.25	3.36
	StDv	3,072	723	623	2.14	0.76	1.85	1.99	0.89
HPGD	Rep.1	51,645	15,143	24,758	0.00	-0.98	-0.18	0.98	0.18
	Rep.2	1	42,608	21,512	-0.26	0.63	-0.42	-0.89	0.16
	Rep.3	36,518	45,268	33,203	0.16	-0.46	0.06	0.62	0.10
	Avr.	29,388	34,340	26,491	-0.03	-0.27	-0.18	0.23	0.15
	StDv	26,550	16,678	6,035	0.21	0.82	0.24	0.99	0.04
KLK2	Rep.1	821,034	397,634	319,495	3.99	3.74	3.51	0.26	0.48
	Rep.2	5	327,028	295,541	2.06	3.57	3.36	-1.51	-1.30
	Rep.3	282,724	504,677	269,503	3.11	3.02	3.08	0.09	0.03
	Avr.	367,921	409,780	294,846	3.05	3.44	3.32	-0.39	-0.26
	StDv	417,092	89,445	25,003	0.97	0.38	0.22	0.98	0.93
KLK3 438	Rep.1	3,461,933	1,020,587	715,738	6.07	5.10	4.67	0.97	1.40
	Rep.2	6	1,013,939	821,767	2.32	5.20	4.83	-2.88	-2.51
	Rep.3	726,379	1,380,170	888,446	4.47	4.47	4.80	0.00	-0.33
	Avr.	1,396,106	1,138,232	808,650	4.29	4.92	4.77	-0.64	-0.48
	StDv	1,825,551	209,551	87,098	1.88	0.40	0.09	2.00	1.96
LAMA1	Rep.1	1	3	1	-15.66	-13.28	-14.78	-2.38	-0.88
	Rep.2	1	1	1	-0.26	-14.75	-14.81	14.48	14.55
	Rep.3	1	1	1	-15.00	-15.93	-14.96	0.93	-0.04
	Avr.	1	2	1	-10.30	-14.65	-14.85	4.35	4.54
	StDv	0	1	0	8.70	1.33	0.10	8.93	8.68
MSMB	Rep.1	2,227,552	502,180	575,321	5.43	4.07	4.36	1.36	1.07
	Rep.2	14	521,847	606,686	3.54	4.25	4.40	-0.70	-0.85
	Rep.3	829,160	1,035,285	539,522	4.67	4.06	4.08	0.61	0.58
	Avr.	1,018,909	686,437	573,843	4.55	4.13	4.28	0.42	0.27
	StDv	1,125,826	302,271	33,606	0.95	0.10	0.17	1.04	1.00
MUC1A	**Rep.1**	1	1	1	-15.66	-14.86	-14.78	-0.79	-0.88
	Rep.2	1	2	1	-0.26	-13.75	-14.81	13.48	14.55
	Rep.3	1	1	1	-15.00	-15.93	-14.96	0.93	-0.04
	Avr.	1	1	1	-10.30	-14.85	-14.85	4.54	4.54
	StDv	0	1	0	8.70	1.09	0.10	7.79	8.68
MYLK	Rep.1	1,530,334	910,551	908,063	4.89	4.93	5.02	-0.04	-0.13
	Rep.2	4	690,874	1,217,163	1.74	4.65	5.40	-2.91	-3.66
	Rep.3	584,868	$1.1\ 10^6$	1.4	4.16	4.22	5.44	-0.05	-1.28
	Avr.	705,069	919,376	1,169,326	3.60	4.60	5.29	-1.00	-1.69
	StDv	772,213	233,040	240,933	1.65	0.36	0.24	1.65	1.80
PCAT1	Rep.1	176,018	51,153	60,175	1.77	0.78	1.10	0.99	0.67

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T2	Adj.G	T1	T2	Adj.G	T1/T2	T1/ Adj.G
	Rep.2	1	23,697	37,342	-0.26	-0.22	0.37	-0.05	-0.64
	Rep.3	56,838	50,071	47,687	0.80	-0.31	0.58	1.11	0.21
	Avr.	77,619	41,640	48,401	0.77	0.08	0.69	0.69	0.08
	StDv	89,830	15,549	11,433	1.02	0.60	0.37	0.64	0.66
PDZK1 IP1	Rep.1	8,995	2,067	1,865	-2.52	-3.85	-3.91	1.33	1.39
	Rep.2	1	3,536	4,238	-0.26	-2.96	-2.76	2.70	2.50
	Rep.3	7,861	17,707	2,509	-2.06	-1.81	-3.66	-0.24	1.61
	Avr.	5,619	7,770	2,871	-1.61	-2.87	-3.45	1.26	1.83
	StDv	4,898	8,637	1,227	1.19	1.02	0.60	1.47	0.59
PEX10	Rep.1	7,719	9	1,944	-2.74	-11.70	-3.85	8.95	1.11
	Rep.2	1	784	1,078	-0.26	-5.13	-4.74	4.87	4.48
	Rep.3	8,785	3,000	3,908	-1.90	-4.37	-3.02	2.48	1.13
	Avr.	5,502	1,264	2,310	-1.63	-7.07	-3.87	5.43	2.24
	StDv	4,793	1,552	1,450	1.26	4.03	0.86	3.27	1.94
PIP	Rep.1	2,284	1	1	-4.50	-14.86	-14.78	10.37	10.28
	Rep.2	1	1	1	-0.26	-14.75	-14.81	14.48	14.55
	Rep.3	2	898	1	-14.00	-6.11	-14.96	-7.88	0.96
	Avr.	762	300	1	-6.25	-11.91	-14.85	5.66	8.60
	StDv	1,318	518	0	7.03	5.02	0.10	11.90	6.95
PSCA	Rep.1	12,535	8,670	61,407	-2.04	-1.78	1.13	-0.26	-3.17
	Rep.2	1	3,413	52,009	-0.26	-3.01	0.85	2.75	-1.12
	Rep.3	8,366	2,537	68,425	-1.97	-4.62	1.11	2.65	-3.07
	Avr.	6,967	4,873	60,614	-1.42	-3.14	1.03	1.71	-2.45
	StDv	6,383	3,317	8,237	1.01	1.42	0.15	1.71	1.16
RARRES1	Rep.1	170,826	64,937	19,653	1.73	1.12	-0.51	0.60	2.24
	Rep.2	1	66,464	22,545	-0.26	1.27	-0.35	-1.54	0.09
	Rep.3	63,506	84,074	23,140	0.96	0.43	-0.46	0.52	1.42
	Avr.	78,111	71,825	21,779	0.81	0.94	-0.44	-0.14	1.25
	StDv	86,344	10,635	1,865	1.00	0.45	0.08	1.21	1.08
SELM 1	Rep.1	168,631	61,687	37,098	1.71	1.05	0.40	0.66	1.31
	Rep.2	2	64,482	80,173	0.74	1.23	1.48	-0.49	-0.74
	Rep.3	69,773	84,097	59,539	1.09	0.43	0.90	0.66	0.19
	Avr.	79,469	70,089	58,937	1.18	0.90	0.93	0.28	0.25
	StDv	84,732	12,212	21,544	0.49	0.42	0.54	0.67	1.02
SFRP1	Rep.1	54,883	43,772	8,160	0.09	0.55	-1.78	-0.46	1.87
	Rep.2	1	46,965	28,240	-0.26	0.77	-0.03	-1.03	-0.23
	Rep.3	44,799	57,534	37,830	0.46	-0.11	0.25	0.57	0.20
	Avr.	33,228	49,424	24,743	0.09	0.40	-0.52	-0.31	0.61
	StDv	29,214	7,203	15,141	0.36	0.46	1.10	0.81	1.11
SPP1	Rep.1	88,187	20,998	5,469	0.77	-0.51	-2.36	1.28	3.13
	Rep.2	1	23,950	6,577	-0.26	-0.20	-2.13	-0.06	1.87
	Rep.3	42,213	27,737	4,804	0.37	-1.17	-2.73	1.54	3.10
	Avr.	43,467	24,228	5,617	0.29	-0.62	-2.41	0.92	2.70
	StDv	44,106	3,378	896	0.52	0.49	0.30	0.86	0.72
SYNM	Rep.1	113,741	48,343	49,560	1.14	0.70	0.82	0.44	0.32
	Rep.2	1	50,482	69,718	-0.26	0.88	1.28	-1.14	-1.54
	Rep.3	32,666	84,942	104,171	0.00	0.45	1.71	-0.45	-1.71
	Avr.	48,803	61,256	74,483	0.29	0.67	1.27	-0.38	-0.98
	StDv	58,562	20,541	27,616	0.75	0.21	0.45	0.79	1.13
TFAP2 A	Rep.1	4,633	4,198	7,283	-3.48	-2.83	-1.95	-0.65	-1.53
	Rep.2	1	4,808	2,263	-0.26	-2.52	-3.67	2.25	3.41
	Rep.3	2,925	6,336	2,544	-3.48	-3.30	-3.64	-0.19	0.16
	Avr.	2,520	5,114	4,030	-2.41	-2.88	-3.09	0.47	0.68
	StDv	2,342	1,101	2,821	1.86	0.39	0.99	1.56	2.51

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T2	Adj.G	T1	T2	Adj.G	T1/T2	T1/ Adj.G
TMC5	Rep.1	99,782	31,783	10,280	0.95	0.09	-1.45	0.86	2.40
	Rep.2	1	46,113	18,809	-0.26	0.75	-0.61	-1.01	0.35
	Rep.3	129,750	78,656	17,485	1.99	0.34	-0.86	1.65	2.85
	Avr.	76,511	52,184	15,525	0.89	0.39	-0.98	0.50	1.87
	StDv	67,933	24,019	4,590	1.13	0.33	0.43	1.37	1.33
TPM2	Rep.1	533,651	370,786	430,778	3.37	3.64	3.94	-0.27	-0.57
	Rep.2	2	286,949	595,345	0.74	3.38	4.37	-2.65	-3.63
	Rep.3	266,214	529,695	678,024	3.03	3.09	4.41	-0.06	-1.39
	Avr.	266,622	395,810	568,049	2.38	3.37	4.24	-0.99	-1.86
	StDv	266,825	123,293	125,863	1.43	0.27	0.26	1.44	1.59
TPX2	Rep.1	2,010	5	2	-4.68	-12.54	-13.78	7.86	9.09
	Rep.2	1	2	3	-0.26	-13.75	-13.23	13.48	12.97
	Rep.3	1,433	2	3	-4.51	-14.93	-13.37	10.41	8.86
	Avr.	1,148	3	3	-3.15	-13.74	-13.46	10.59	10.31
	StDv	1,034	2	1	2.50	1.19	0.28	2.82	2.31
UGT2 B15	Rep.1	13	786	1,209	-11.96	-5.25	-4.54	-6.71	-7.42
	Rep.2	1	137	148	-0.26	-7.65	-7.60	7.39	7.34
	Rep.3	3,199	6	4,002	-3.35	-13.34	-2.99	9.99	-0.36
	Avr.	1,071	310	1,786	-5.19	-8.75	-5.04	3.56	-0.15
	StDv	1,843	418	1,991	6.06	4.16	2.35	8.98	7.38

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T3	Adj.G	T1	T3	Adj.G	T1/T3	T1/ Adj.G
ApoC1	Rep.1	98,101	68,822	23,748	-0.66	-1.17	-2.51	0.51	1.85
	Rep.2	134,903	52,205	17,831	-0.40	-1.37	-2.67	0.97	2.27
	Rep.3	50,743	49,348	5,790	-0.67	-1.49	-4.75	0.82	4.07
	Avr.	94,582	56,792	15,790	-0.58	-1.34	-3.31	0.76	2.73
	StDv	42,190	10,516	9,151	0.16	0.16	1.25	0.24	1.18
ApoE	Rep.1	113,238	92,674	50,929	-0.45	-0.74	-1.41	0.28	0.96
	Rep.2	120,951	97,766	26,427	-0.56	-0.46	-2.10	-0.10	1.55
	Rep.3	53,870	80,438	36,240	-0.59	-0.79	-2.10	0.20	1.51
	Avr.	96,020	90,293	37,865	-0.53	-0.66	-1.87	0.13	1.34
	StDv	36,706	8,906	12,332	0.07	0.18	0.40	0.20	0.33
C15orf48	Rep.1	462,524	760,825	23,822	1.58	2.30	-2.51	-0.72	4.08
	Rep.2	635,716	641,300	22,420	1.84	2.25	-2.34	-0.41	4.18
	Rep.3	321,882	563,978	7,408	1.99	2.02	-4.39	-0.03	6.38
	Avr.	473,374	655,368	17,883	1.80	2.19	-3.08	-0.39	4.88
	StDv	157,198	99,175	9,099	0.21	0.15	1.14	0.35	1.30
CSRP1.583	Rep.1	921,105	514,866	939,933	2.57	1.74	2.79	0.83	-0.22
	Rep.2	1,361,542	570,555	989,617	2.94	2.08	3.12	0.85	-0.19
	Rep.3	390,734	242,180	690,001	2.27	0.80	2.15	1.47	0.12
	Avr.	891,127	442,534	873,184	2.59	1.54	2.69	1.05	-0.10
	StDv	486,098	175,731	160,574	0.33	0.66	0.50	0.36	0.19
CSRP1.690	Rep.1	610,121	317,158	490,682	1.97	1.04	1.86	0.94	0.12
	Rep.2	789,293	344,428	517,589	2.15	1.36	2.19	0.79	-0.04
	Rep.3	404,039	122,907	423,777	2.32	-0.18	1.45	2.50	0.87
	Avr.	601,151	261,498	477,349	2.15	0.74	1.83	1.41	0.32
	StDv	192,784	120,795	48,306	0.17	0.81	0.37	0.94	0.49
EBF3	Rep.1	11,409	6,191	8,760	-3.77	-4.64	-3.95	0.88	0.19
	Rep.2	12,494	583	8,772	-3.83	-7.85	-3.69	4.02	-0.14
	Rep.3	2,412	750	294	-5.07	-7.53	-9.05	2.47	3.98
	Avr.	8,772	2,508	5,942	-4.22	-6.68	-5.56	2.45	1.34

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T3	Adj.G	T1	T3	Adj.G	T1/T3	T1/ Adj.G
	StDv	5,534	3,191	4,891	0.73	1.77	3.02	1.57	2.29
F5	Rep.1	19,321	17,161	6,991	-3.01	-3.17	-4.28	0.17	1.27
	Rep.2	21,147	13,841	90	-3.07	-3.28	-10.30	0.21	7.23
	Rep.3	2,486	20,749	9,499	-5.02	-2.74	-4.03	-2.28	-0.99
	Avr.	14,318	17,250	5,527	-3.70	-3.07	-6.20	-0.64	2.50
	StDv	10,287	3,455	4,872	1.15	0.28	3.55	1.42	4.25
FGG	Rep.1	5	2	2	-14.92	-16.24	-16.05	1.32	1.13
	Rep.2	4,110	1	1	-5.44	-17.04	-16.79	11.60	11.36
	Rep.3	1	2	1	-16.30	-16.09	-17.25	-0.22	0.94
	Avr.	1,372	2	1	-12.22	-16.45	-16.70	4.23	4.47
	StDv	2,371	1	1	5.92	0.51	0.60	6.43	5.96
FHL2	Rep.1	102,579	47,546	62,719	-0.60	-1.70	-1.11	1.10	0.51
	Rep.2	109,719	57,142	51,134	-0.70	-1.24	-1.15	0.54	0.45
	Rep.3	41,940	14,593	24,991	-0.95	-3.25	-2.64	2.30	1.69
	Avr.	84,746	39,760	46,281	-0.75	-2.06	-1.63	1.32	0.89
	StDv	37,243	22,317	19,326	0.18	1.06	0.87	0.90	0.70
GRAMD 4	Rep.1	31,350	25,907	28,223	-2.31	-2.58	-2.26	0.27	-0.04
	Rep.2	37,363	24,238	29,679	-2.25	-2.47	-1.93	0.22	-0.32
	Rep.3	20,118	35,834	16,514	-2.01	-1.96	-3.23	-0.05	1.23
	Avr.	29,610	28,660	24,805	-2.19	-2.34	-2.48	0.15	0.29
	StDv	8,753	6,269	7,217	0.16	0.33	0.68	0.17	0.82
HIF1A	Rep.1	398,064	419,595	340,458	1.36	1.44	1.33	-0.08	0.03
	Rep.2	771,120	404,282	369,458	2.12	1.59	1.70	0.53	0.41
	Rep.3	297,843	557,606	438,692	1.88	2.00	1.50	-0.12	0.38
	Avr.	489,009	460,494	382,869	1.78	1.68	1.51	0.11	0.28
	StDv	249,401	84,449	50,472	0.39	0.29	0.19	0.37	0.21
HIPK2	Rep.1	109,550	170,523	42,729	-0.50	0.14	-1.67	-0.64	1.16
	Rep.2	149,913	143,176	70,970	-0.25	0.09	-0.68	-0.34	0.43
	Rep.3	75,965	201,996	72,517	-0.09	0.54	-1.10	-0.63	1.01
	Avr.	111,809	171,898	62,072	-0.28	0.26	-1.15	-0.54	0.87
	StDv	37,026	29,434	16,769	0.21	0.25	0.50	0.17	0.39
HOXC4	Rep.1	1,626	4,220	22	-6.58	-5.19	-12.59	-1.38	6.01
	Rep.2	25	10,154	13	-12.80	-3.73	-13.09	-9.07	0.29
	Rep.3	6,815	14,781	12	-3.57	-3.23	-13.66	-0.34	10.09
	Avr.	2,822	9,718	16	-7.65	-4.05	-13.11	-3.60	5.47
	StDv	3,549	5,294	6	4.71	1.02	0.54	4.77	4.92
HPN	Rep.1	27,181	61,191	2,616	-2.51	-1.34	-5.70	-1.18	3.18
	Rep.2	45,014	56,079	2,152	-1.98	-1.26	-5.72	-0.72	3.74
	Rep.3	24,434	44,764	1,615	-1.73	-1.64	-6.59	-0.09	4.86
	Avr.	32,210	54,011	2,128	-2.07	-1.41	-6.00	-0.66	3.93
	StDv	11,174	8,406	501	0.40	0.20	0.51	0.54	0.85
HSBP1	Rep.1	715,949	515,099	585,263	2.21	1.74	2.11	0.47	0.10
	Rep.2	936,366	390,235	488,172	2.40	1.54	2.11	0.86	0.29
	Rep.3	434,201	353,606	747,508	2.42	1.35	2.27	1.08	0.16
	Avr.	695,505	419,647	606,981	2.34	1.54	2.16	0.80	0.18
	StDv	251,706	84,669	131,025	0.12	0.19	0.09	0.31	0.10
IGFBP1	Rep.1	4,956	3	3,852	-4.97	-15.65	-5.14	10.68	0.17
	Rep.2	2,768	3	9,424	-6.01	-15.45	-3.59	9.45	-2.42
	Rep.3	3	3	5	-14.72	-15.50	-14.92	0.78	0.20
	Avr.	2,576	3	4,427	-8.56	-15.54	-7.88	6.97	-0.68
	StDv	2,482	0	4,736	5.36	0.10	6.15	5.40	1.50
KLK3.470	Rep.1	152,238	296,395	38,574	-0.03	0.94	-1.81	-0.97	1.79
	Rep.2	118,440	150,567	19,551	-0.59	0.16	-2.54	-0.75	1.95
	Rep.3	92,387	178,823	40,080	0.19	0.36	-1.96	-0.17	2.15

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T3	Adj.G	T1	T3	Adj.G	T1/T3	T1/ Adj.G
	Avr.	121,022	208,595	32,735	-0.14	0.49	-2.10	-0.63	1.96
	StDv	30,009	77,338	11,442	0.40	0.40	0.38	0.41	0.18
LRRN1	Rep.1	370	78	7,572	-8.71	-10.95	-4.16	2.24	-4.55
	Rep.2	4,313	397	5	-5.37	-8.41	-14.47	3.04	9.10
	Rep.3	1,651	843	1,282	-5.62	-7.37	-6.92	1.75	1.31
	Avr.	2,111	439	2,953	-6.56	-8.91	-8.52	2.34	1.95
	StDv	2,011	384	4,051	1.86	1.85	5.34	0.65	6.85
MAP3K7	Rep.1	313,649	286,012	327,741	1.01	0.89	1.27	0.13	-0.26
	Rep.2	481,330	323,184	393,629	1.44	1.26	1.79	0.17	-0.36
	Rep.3	305,428	340,706	532,702	1.92	1.29	1.78	0.62	0.14
	Avr.	366,802	316,634	418,024	1.46	1.15	1.61	0.31	-0.16
	StDv	99,269	27,929	104,636	0.45	0.23	0.30	0.27	0.26
MYEF2	Rep.1	22,256	26,221	17,459	-2.80	-2.56	-2.96	-0.24	0.16
	Rep.2	43,512	50,275	11,295	-2.03	-1.42	-3.33	-0.61	1.30
	Rep.3	18,439	33,731	24,686	-2.13	-2.04	-2.65	-0.09	0.52
	Avr.	28,069	36,742	17,813	-2.32	-2.01	-2.98	-0.31	0.66
	StDv	13,510	12,306	6,703	0.42	0.57	0.34	0.27	0.58
OPRK1	Rep.1	17	7	2,208	-13.16	-14.43	-5.94	1.27	-7.22
	Rep.2	2,902	248	3,210	-5.94	-9.08	-5.14	3.15	-0.79
	Rep.3	71	3	4,485	-10.15	-15.50	-5.11	5.35	-5.04
	Avr.	997	86	3,301	-9.75	-13.01	-5.40	3.26	-4.35
	StDv	1,650	140	1,141	3.63	3.44	0.47	2.04	3.27
PCAT14	Rep.1	9,159	11,924	19,837	-4.08	-3.70	-2.77	-0.39	-1.31
	Rep.2	16,009	8,041	9,785	-3.47	-4.07	-3.54	0.59	0.06
	Rep.3	7,083	5,460	24,145	-3.51	-4.67	-2.69	1.16	-0.83
	Avr.	10,750	8,475	17,922	-3.69	-4.14	-3.00	0.45	-0.69
	StDv	4,671	3,254	7,369	0.34	0.49	0.47	0.78	0.70
PFKP	Rep.1	144,614	98,784	122,550	-0.10	-0.65	-0.15	0.54	0.04
	Rep.2	171,077	139,353	117,508	-0.06	0.05	0.05	-0.11	-0.11
	Rep.3	99,055	83,294	108,599	0.29	-0.74	-0.52	1.03	0.81
	Avr.	138,249	107,144	116,219	0.04	-0.45	-0.20	0.49	0.25
	StDv	36,430	28,949	7,064	0.22	0.43	0.29	0.57	0.49
PFKL	Rep.1	43,313	33,493	41,348	-1.84	-2.21	-1.71	0.37	-0.13
	Rep.2	65,474	71,324	55,748	-1.44	-0.92	-1.03	-0.53	-0.42
	Rep.3	44,011	41,829	66,882	-0.88	-1.73	-1.22	0.85	0.34
	Avr.	50,933	48,882	54,659	-1.39	-1.62	-1.32	0.23	-0.07
	StDv	12,598	19,877	12,802	0.48	0.65	0.36	0.70	0.38
PLA2G7	Rep.1	2,638	7,777	698	-5.88	-4.31	-7.60	-1.57	1.72
	Rep.2	15,312	7,533	28	-3.54	-4.16	-11.98	0.62	8.45
	Rep.3	1,237	9,543	2,435	-6.03	-3.86	-6.00	-2.17	-0.04
	Avr.	6,396	8,284	1,054	-5.15	-4.11	-8.53	-1.04	3.38
	StDv	7,753	1,097	1,242	1.40	0.23	3.10	1.47	4.48
PSMA	Rep.1	48,780	219,535	13,959	-1.67	0.51	-3.28	-2.18	1.61
	Rep.2	39,582	266,004	162	-2.17	0.98	-9.45	-3.15	7.28
	Rep.3	12,045	155,230	3,076	-2.75	0.16	-5.66	-2.91	2.91
	Avr.	33,469	213,590	5,732	-2.20	0.55	-6.13	-2.74	3.94
	StDv	19,115	55,626	7,272	0.54	0.41	3.11	0.51	2.97
SAA2	Rep.1	32,915	23,385	5,206	-2.24	-2.72	-4.70	0.49	2.47
	Rep.2	16,951	10,526	334	-3.39	-3.68	-8.41	0.29	5.02
	Rep.3	11,263	12,714	3,183	-2.85	-3.45	-5.61	0.61	2.76
	Avr.	20,376	15,542	2,908	-2.82	-3.28	-6.24	0.46	3.42
	StDv	11,225	6,880	2,448	0.58	0.50	1.93	0.16	1.39
SERPIN A1	Rep.1	123,407	96,522	39,550	-0.33	-0.68	-1.78	0.35	1.45
	Rep.2	94,620	28,318	12,562	-0.91	-2.25	-3.18	1.34	2.26

		Raw read counts (Rc)			Log_2 Normalised Rc			Differential Expression (Log_2 FC)	
		T1	T3	Adj.G	T1	T3	Adj.G	T1/T3	T1/ Adj.G
	Rep.3	48,679	53,221	41,185	-0.73	-1.39	-1.92	0.65	1.18
	Avr.	88,902	59,354	31,099	-0.66	-1.44	-2.29	0.78	1.63
	StDv	37,691	34,513	16,074	0.30	0.79	0.77	0.51	0.56
SLC10A7	Rep.1	16,866	34,875	7,675	-3.20	-2.15	-4.14	-1.05	0.94
	Rep.2	3,660	5,356	3,205	-5.60	-4.65	-5.15	-0.95	-0.46
	Rep.3	7,367	13,761	1,632	-3.46	-3.34	-6.57	-0.12	3.12
	Avr.	9,298	17,997	4,171	-4.09	-3.38	-5.29	-0.71	1.20
	StDv	6,811	15,209	3,135	1.32	1.25	1.22	0.51	1.80
SMAD5	Rep.1	369,739	350,017	407,427	1.25	1.18	1.59	0.07	-0.33
	Rep.2	290,176	196,854	221,405	0.71	0.55	0.96	0.16	-0.26
	Rep.3	196,008	163,982	204,033	1.28	0.24	0.39	1.04	0.88
	Avr.	285,308	236,951	277,622	1.08	0.66	0.98	0.42	0.10
	StDv	86,968	99,288	112,750	0.32	0.48	0.60	0.53	0.68
SPON2	Rep.1	120,585	152,859	71,489	-0.36	-0.02	-0.92	-0.35	0.56
	Rep.2	177,482	137,573	49,919	0.00	0.03	-1.18	-0.03	1.18
	Rep.3	87,791	85,642	68,463	0.12	-0.70	-1.18	0.82	1.30
	Avr.	128,619	125,358	63,290	-0.08	-0.23	-1.10	0.14	1.01
	StDv	45,382	35,234	11,678	0.25	0.41	0.15	0.60	0.40
SRC	Rep.1	22,920	29,855	12,967	-2.76	-2.37	-3.39	-0.39	0.63
	Rep.2	20,332	26,195	16,253	-3.13	-2.36	-2.80	-0.77	-0.33
	Rep.3	13,691	17,857	33,398	-2.56	-2.96	-2.22	0.40	-0.35
	Avr.	18,981	24,636	20,873	-2.82	-2.56	-2.80	-0.25	-0.01
	StDv	4,761	6,149	10,971	0.29	0.34	0.58	0.59	0.56
SYNPO2	Rep.1	1,269,282	764,162	1,271,402	3.03	2.31	3.23	0.73	-0.20
	Rep.2	1,854,291	663,642	1,094,823	3.38	2.30	3.27	1.08	0.11
	Rep.3	1,054,005	725,221	1,560,688	3.70	2.38	3.33	1.32	0.37
	Avr.	1,392,526	717,675	1,308,971	3.37	2.33	3.28	1.04	0.10
	StDv	414,133	50,683	235,194	0.34	0.05	0.05	0.30	0.29
TDRD1	Rep.1	9,108	2,685	847	-4.09	-5.85	-7.32	1.76	3.23
	Rep.2	3,369	1,050	1,123	-5.72	-7.00	-6.66	1.28	0.94
	Rep.3	1,790	176	5	-5.50	-9.63	-14.92	4.13	9.43
	Avr.	4,756	1,304	658	-5.10	-7.49	-9.64	2.39	4.53
	StDv	3,851	1,274	582	0.88	1.94	4.59	1.52	4.39
TRIB1	Rep.1	41,926	46,385	34,225	-1.89	-1.74	-1.99	-0.15	0.10
	Rep.2	47,764	35,288	23,641	-1.90	-1.93	-2.26	0.03	0.37
	Rep.3	22,768	15,896	12,646	-1.83	-3.13	-3.62	1.30	1.79
	Avr.	37,486	32,523	23,504	-1.87	-2.27	-2.62	0.39	0.75
	StDv	13,076	15,431	10,790	0.04	0.75	0.87	0.79	0.91
TSPAN13	Rep.1	126,805	135,413	60,500	-0.29	-0.19	-1.16	-0.10	0.87
	Rep.2	127,130	153,934	66,513	-0.48	0.19	-0.77	-0.68	0.29
	Rep.3	99,522	52,802	42,203	0.30	-1.40	-1.88	1.70	2.18
	Avr.	117,819	114,050	56,405	-0.16	-0.46	-1.27	0.31	1.11
	StDv	15,847	53,844	12,662	0.41	0.83	0.56	1.24	0.97

In Table 14, the data represents those RNA biomarkers with a Log_2 FC >2 in the differential expression in the tumour compare to the adjacent gland. Most of these RNA biomarkers are up regulated in the tumor compared with the adjacent glandular tissue. Only two biomarkers were detected in a higher amount in the adjacent glandular tissue compared

with all tumors. Some distinctions between the different grades of tumors can be made, for example with the OPRK1 and PSMA RNA biomarkers.

Table 14: RNA biomarker with differential expression (Log_2 FC) in Tumor and adjacent tissues of Subject 2

Differential expression (>2Log_2FC) in Subject 2 tumors* compared with adjacent glandular tissue		**RNA Biomarkers**
Up regulated in:	T1	TPX2, SPP1, PIP
	T2	HOXC4, HPN, KLK3.470, C15orf48, PSMA, PLA2G7, SAA2, HN1
	T3	HPN, C15orf48, KLK3.470, ApoC1, SAA2
Down regulated in:	T1	PSCA
	T2	PSCA, OPRK1, IGFBP1
	T3	OPRK1

*T1(Gleason score 4+5), T2 (3+4), and T3 (3+3))

Comments on RNA biomarker expression in subject 1 and subject 2

Before proceeding with the amplicon production for RBAS analysis, the efficiency of all the RNA specific primers was tested by real time PCR or by visualization of the produced amplicon of the expected size. Therefore, the lower sequence counts observed for certain amplicons produced from prostatectomy tissues RNA cannot be attributed to the inefficiency of the amplicon production. As seen in Example 1, raw sequence counts of 900 and 13,000 were obtained from the MUC1 amplicon produced from LNCaP and A549 cell RNA respectively (Table 6).

The process used to select RNA biomarkers disclosed herein is by selecting those that are up-regulated or down-regulated in a small number of prostate tumors, rather than in all prostate tumors. For this reason it is not expected that differential expression of all the RNA biomarkers would be seen in all prostate tumors or their adjacent tissues. The data indicate that tumors examined from Subjects 1 and 2 are likely not to have some of the RNA dysregulated within their tissue. The analysis of tumors from a range of subjects will will likely reveal differences in the expression of these and other RNA biomarkers. That is the major reason why, for diagnostic and prognostic use, RNA biomarker panels are selected from a large RNA biomarker pool. RBAS methodology has been developed to allow rapid screening of tumor samples for a large number of RNA biomarkers simultaneously.

In conclusion, these observations highlight the issue with staging prostate cancers and illustrate reasons for developing multi-RNA biomarker diagnostics, as it is unlikely that a single RNA biomarker can diagnose and stage prostate cancers, or distinguish prostate cancer from begnin prostate hyperplasia or prostatitis.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, method, method step or steps, for use in practicing the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

SEQ ID NO: 1-326 are set out in the attached Sequence Listing. The codes for nucleotide sequences used in the attached Sequence Listing, including the symbol "n," conform to WIPO Standard ST.25 (1998), Appendix 2, Table 1.

Claims

1. A method for detecting the presence of a disorder and/or monitoring the progression of the disorder in a subject, comprising:

(a) determining the relative frequency of expression of a plurality of RNA biomarkers simultaneously in a biological sample obtained from the subject, wherein the relative frequency of expression is determined using next generation sequencing of an amplicon cDNA library prepared using a plurality of oligonucleotide primers specific for the plurality of RNA biomarkers; and

(b) comparing the relative frequency of expression of the plurality of RNA biomarkers in the biological sample with a predetermined threshold value, wherein increased or decreased relative frequency of expression of the plurality of RNA biomarkers in the biological sample relative to the predetermined threshold value indicates the presence of the disorder and/or progression of the disorder in the subject.

2. The method of claim 1, wherein the amplicon cDNA library is prepared by:

(a) isolating total RNA from the biological sample;

(b) generating first strand cDNA from the total RNA using a plurality of first oligonucleotide primers specific for the plurality of RNA biomarkers;

(c) synthesizing second strand cDNA to provide double-stranded cDNA;

(d) adding at least one sequencing adapter to the double-stranded cDNA; and

(e) amplifying the double-stranded cDNA to provide the amplicon cDNA library.

3. The method of claim 2, wherein the first oligonucleotide primer is selected from the group consisting of: SEQ ID NO: 76-223 and 293-326.

4. The method of claim 2, further comprising amplifying the double-stranded cDNA by polymerase chain reaction using a plurality of oligonucleotide primer pairs specific for the plurality of RNA biomarkers after step (c) and prior to step (d).

5. The method of claim 4, wherein the plurality of oligonucleotide primer pairs is selected from the group consisting of: SEQ ID NO: 76-223 and 293-326.

6. The method of claim 1, wherein the amplicon cDNA library is prepared by:

(a) isolating total RNA from the biological sample;

(b) preparing first strand cDNA to provide single-stranded cDNA;

(c) amplifying the single-stranded cDNA by polymerase chain reaction using a plurality of oligonucleotide primer pairs specific for the plurality of RNA biomarkers to provide amplified double-stranded cDNA;

(d) adding at least one sequencing adapter to the amplified double-stranded cDNA; and

(e) further amplifying the amplified double-stranded cDNA using primers specific for the at least one sequencing adapter to provide the amplicon cDNA library.

7. The method of claim 6, wherein at least one member of the plurality of oligonucleotide primer pairs is selected from the group consisting of SEQ ID NO: 76-223 and 293-326.

8. The method of any one of claims 1 to 7, wherein the disorder is a cancer.

9. The method of any one of claims 1 to 8, wherein the disorder is prostate cancer and the plurality of RNA biomarkers comprises a RNA sequence corresponding to a DNA sequence selected from the group consisting of: SEQ ID NO: 1-71 and 235-287.

10. The method of any one of claims 1 to 9, wherein the biological sample is selected from the group consisting of: urine, blood, serum, cell lines, PBMCS, biopsy tissue, and prostatectomy tissue.

11. A method for monitoring progression of a disorder in a subject, comprising:

(a) determining the relative frequency of expression of a plurality of RNA biomarkers simultaneously in a biological sample obtained from the subject at a first time point, and determining the relative frequency of expression of the plurality of RNA biomarkers simultaneously in a biological sample obtained from the subject at a second, subsequent, time point, wherein the relative frequency of expression is determined using next generation sequencing of an amplicon cDNA library prepared using a plurality of oligonucleotide primers specific for the plurality of RNA biomarkers; and

(b) comparing the relative frequency of expression of the plurality of RNA biomarkers in the biological sample with a predetermined threshold value, wherein an increase or decrease in the relative frequency of expression of the plurality of RNA biomarkers in the biological sample at the second time point compared to the relative frequency of expression of the plurality of RNA biomarkers at the first time point indicates the progression of the disorder in the subject.

12. The method of claim 11, wherein the amplicon cDNA library is prepared by:

(a) isolating total RNA from the biological sample;

(b) generating first strand cDNA from the total RNA using a plurality of first oligonucleotide primers specific for the plurality of RNA biomarkers;

(c) synthesizing second strand cDNA to provide double-stranded cDNA;

(d) adding at least one sequencing adapter to the double-stranded cDNA; and

(e) amplifying the double-stranded cDNA to provide the amplicon cDNA library.

13. The method of claim 12, wherein the first oligonucleotide primer is selected from the group consisting of SEQ ID NO: 76-223 and 293-326.

14. The method of claim 12, further comprising amplifying the double-stranded cDNA by polymerase chain reaction using a plurality of oligonucleotide primer pairs specific for the plurality of RNA biomarkers after step (c) and prior to step (d).

15. The method of claim 11, wherein the amplicon cDNA library is prepared by:

(a) isolating total RNA from the biological sample;

(b) preparing first strand cDNA to provide single-stranded cDNA;

(c) amplifying the single-stranded cDNA by polymerase chain reaction using a plurality of oligonucleotide primer pairs specific for the plurality of RNA biomarkers to provide amplified double-stranded cDNA;

(d) adding at least one sequencing adapter to the double-stranded cDNA; and

(e) amplifying the double-stranded cDNA using primers specific for the at least one sequencing adapter to provide the amplicon cDNA library.

16. The method of claim 15, wherein at least one member of the plurality of oligonucleotide primer pairs is selected from the group consisting of SEQ ID NO: 76-223 and 293-326.

17. The method of any one of claims 11 to 16, wherein the disorder is a cancer.

18. The method of any one of claims 11 to 17, wherein the disorder is prostate cancer and the plurality of RNA biomarkers comprises a RNA sequence corresponding to a DNA sequence selected from the group consisting of: SEQ ID NO: 1-71 and 235-287.

19. The method of any one of claims 11 to 18, wherein the biological sample is selected from the group consisting of: urine, blood, serum, cell lines, PBMCS, biopsy tissue, and prostatectomy tissue.

20. The method of any one of claims 1 to 10, wherein the plurality of RNA biomarkers comprises at least ten RNA biomarkers.

21. The method of any one of claims 1 to 10 and claim 20, wherein the frequency of expression of the plurality of RNA biomarkers is normalized to at least one expression standard.

22. The method of claim 21, wherein the at least one expression standard is selected from the group consisting of SEQ ID NO: 72-75 and 288-292.

23. The method of any one of claims 1 to 10 and claims 20 to 22, wherein amplicons in the amplicon cDNA library are 60-300 bp in size.

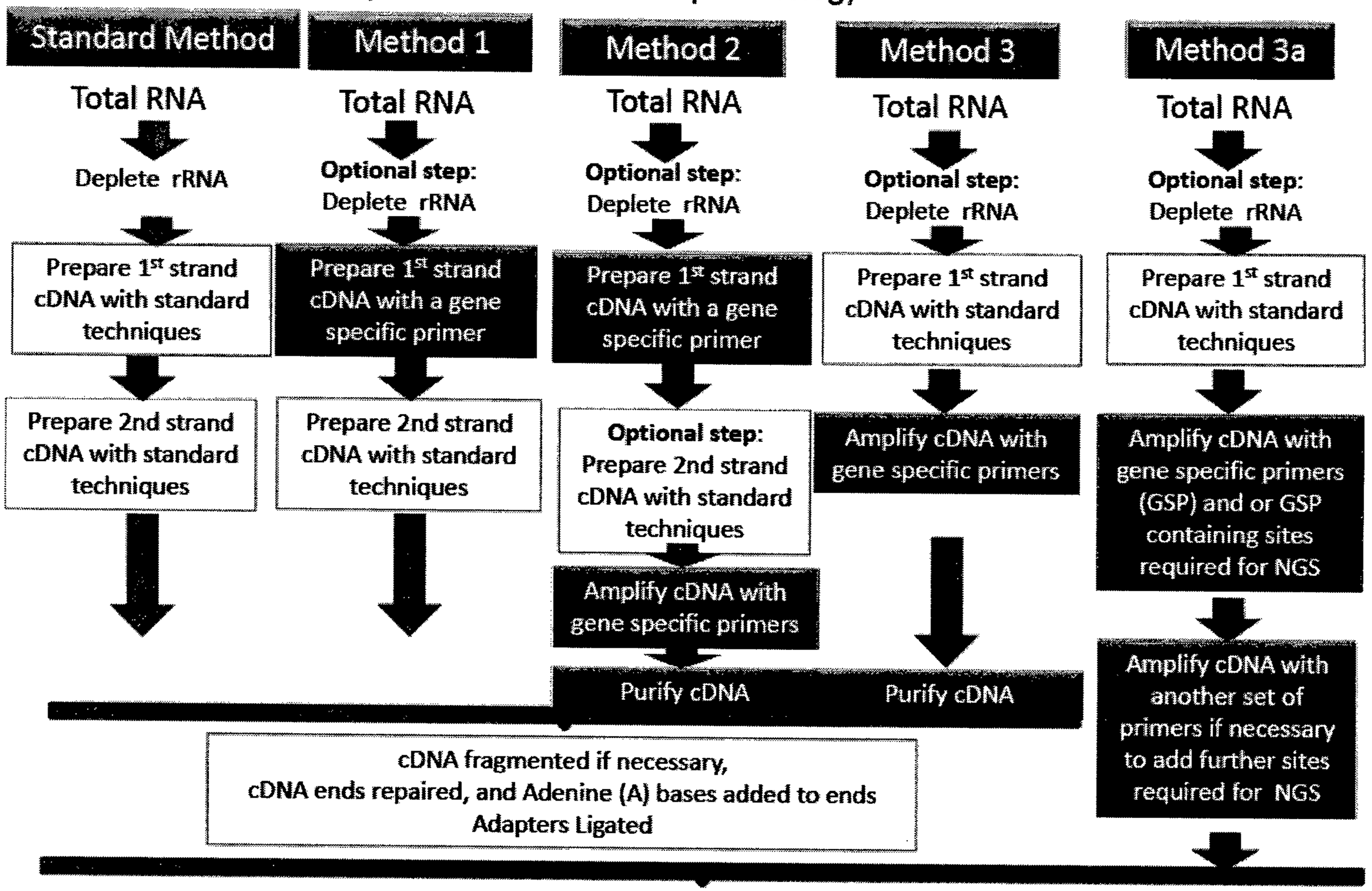
24. The method of any one of claims 11 to 19, wherein the plurality of RNA biomarkers comprises at least ten RNA biomarkers.

25. The method of any one of claims 11 to 19 and claim 24, wherein the frequency of expression of the plurality of RNA biomarkers is normalized to at least one expression standard.

26. The method of claim 25, wherein the at least one expression standard is selected from the group consisting of SEQ ID NO: 72-75 and 288-292.

27. The method of any one of claims 11 to 19 and claims 24 to 26, wherein amplicons in the amplicon cDNA library are 60-300 bp in size.

Figure 1: Four novel adaptations to the RNA-seq technology



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Four novel adaptations to the RNA-seq technology

Standard Method	Method 1	Method 2	Method 3	Method 3a
Total RNA	Total RNA	Total RNA	Total RNA	Total RNA
Deplete rRNA	**Optional step:** Deplete rRNA	**Optional step:** Deplete rRNA	**Optional step:** Deplete rRNA	**Optional step:** Deplete rRNA
Prepare 1st strand cDNA with standard techniques	Prepare 1st strand cDNA with a gene specific primer	Prepare 1st strand cDNA with a gene specific primer	Prepare 1st strand cDNA with standard techniques	Prepare 1st strand cDNA with standard techniques
Prepare 2nd strand cDNA with standard techniques	Prepare 2nd strand cDNA with standard techniques	**Optional step:** Prepare 2nd strand cDNA with standard techniques	Amplify cDNA with gene specific primers	Amplify cDNA with gene specific primers (GSP) and or GSP containing sites required for NGS
		Amplify cDNA with gene specific primers		Amplify cDNA with another set of primers if necessary to add further sites required for NGS
Purify cDNA		Purify cDNA	Purify cDNA	

cDNA fragmented if necessary,
cDNA ends repaired, and Adenine (A) bases added to ends
Adapters Ligated

cDNA then purified (to remove excess adapters and or primers), and cDNA is amplified with adaptor primers if necessary)
cDNA purified, and size selected if necessary
cDNA library sequenced