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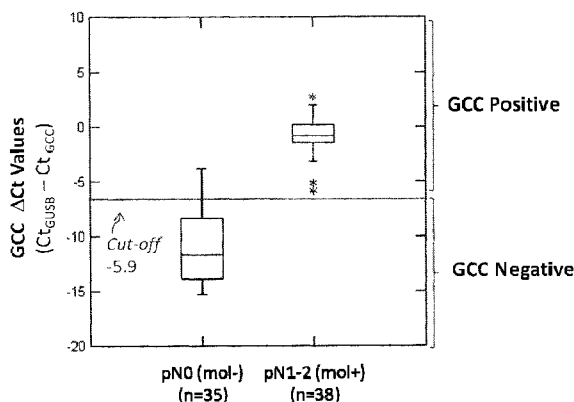(54) Title: METHOD FOR DETECTING METASTASIS OF GI CANCER

Figure 19. GCC relative expression levels in histopathology-negative and GCC/ACTB-negative (pN0(mol-)) and stage III GCC/ACTB-Positive (pN1-2(mol+)) LNs tested with the TaqMan GCC/GUSB assay. A receiver operating characteristic (ROC) analysis of the relative quantification using delta-Ct ($Ct_{GUSB} - Ct_{GCC}$) was used to determine a cut-off value for the GCC/GUSB duplex assay.

(57) Abstract: The present invention provides a novel method for diagnosing, monitoring, prognosing and staging Lymph Node (LN) status in colorectal cancer (CRC)that is more sensitive and accurate than conventional detection technologies such as histopathology. The Guanylyl Cyclase C (GCC) gene is specifically expressed in apical epithelial cells of the GI tract from the duodenum to the rectum and the detection of GCC mRNA in LNs is indicative of the presence of metastases. Quantitative RT-PCR (RT-qPCR) detection of GCC mRNA to identify the presence of colorectal cancer (CRC) cells in LNs has the potential to aid in CRC staging. When used in combination with glucuronidase B (GUSB), accurate quantification of GCC can be achieved with less than a 2-fold variation between intact and highly degraded RNA specimens. The invention also relates to a newly designed GCC/GUSB assay that uses relative quantification having improved prognostic value for time to recurrence and relapse-free survival in Stage I or II colon cancer patients. The GCC/GUSB assay also improves the statistical power of prognosis stratification for relative risk of recurrence and relapse-free survival.

METHOD FOR DETECTING METASTASIS OF GI CANCER

[000]   This application claims priority from US provisional applications 61/155,172 filed on Feb. 25, 2009 and 61/246,197 filed on Sept. 28, 2009, the content of which is herein incorporated by reference in their entirety.

5   **Field of the Invention**

[001]   The present invention relates generally to a method for detecting a biomarker target in a sample obtained from a patient. In particular, the present invention provides a method for detecting the presence of Guanylyl cyclase C (GCC or GUCY2C) expressing cells in human tissues or biological fluids where GCC is not normally expressed. More

10   particularly, the present invention provides a method for detecting the presence of metastatic cancer cells originating from cancerous lesions of the gastro-intestinal (GI) tract, particularly of colorectal cancer type, in a lymph node, blood, or another tissue sample obtained from a patient. Also, the invention relates to the use of RT-qPCR methods for the quantification of GCC mRNA for the staging of cancer patients or to

15   predict the likelihood that colorectal cancer patients will relapse after curative surgery.

Background of the invention

[002]   The following discussion of the background of the invention is simply provided to aid the reader in understanding the invention.

[003]   Colorectal cancer (CRC) is the second most common cause of death in developed

20   countries despite significant changes in the understanding of the disease and treatment. Nevertheless, most treatment options for early stage colorectal cancer are inefficient and the current treatment paradigm is unclear. CRC is one of many diseases for which there is a tight connection between staging and outcome. Once a patient is diagnosed with CRC, the likelihood of a recurrence is related to the degree of tumor penetration through the

25   bowel wall and the presence or absence of nodal involvement. These characteristics are the basis of the CRC staging system defined by the American Joint Committee on Cancer (AJCC). Pathologists, oncologists and surgeons show a great deal of interest in detecting metastases in lymph nodes, as lymph node involvement is a strong prognostic factor in many solid tumors. While histopathology (HP) remains the mainstay of CRC staging,

30   lymph node (LN) examination can be difficult in the presence of single or even small

clumps of tumor cells from other cell types. The current method to identify the presence of CRC metastases in LNs is histopathological examination of tissue sections stained with Hematoxylin and Eosin (H&E). This technique is limited by the fact that only a small proportion, typically one or two tissue sections of 4-5 µm, of each LN is usually assessed

5   for the presence of CRC metastases, leaving most volume (typically > 99%) of each node unexamined. As a result, predicting outcome for CRC patients considered free of lymph node metastases by HP examination remains challenging as approximately 20% of these patients will develop disease recurrence. There is clearly a need to uncover better techniques to identify presence of metastatic cancer cells of the GI tract.

10   [004] To improve the postoperative outcome of CRC patients, both guanylyl cyclase C (aka GUCY2C, more commonly GCC, also known as ST receptor) and its alternative transcript, CRCA-1, have been demonstrated to be good indicators of the presence of metastatic colorectal cancer cells in extra-intestinal/colorectal tissues or bodily fluids. The transcription product of the GCC gene is uniquely expressed by intestinal epithelium and

15   is endogenous downstream target of the transcription factor CDX2. The expression of GCC is preserved throughout the transition from adenoma to carcinoma in colorectal tissues. More recently, screening and diagnostic methods based on guanylyl cyclase C or on CRCA-1 for primary and/or metastatic stomach or esophageal cancer have been disclosed. See particularly: US 5,601,990, US 5,731,159, US 5,928,873; US 6,060,037;

20   US 6,120,995; US 6,602,659; US6,767,704; US 7,135,333; US 7,316,902 and US 7,402,401.

[005] Clinical studies have demonstrated that the detection of GCC by reverse transcription and real-time quantitative polymerase chain reaction (RT-qPCR) is indicative of the presence of metastatic cancer cells in lymph nodes (LN) that were

25   histopathologically negative in patients with colorectal cancer (CRC). These studies, including one described by Schulz (Clin Cancer Res. 2006 Aug 1;12(15):4545-52) and one recently published by Waldman (JAMA, 2009; 301(7):745-752), are based on the detection of the GCC biomarker ("target") in frozen specimens of partial (e.g. approximately half) lymph nodes.

30   [006] The use of GCC and other targets in the diagnosis, staging and monitoring of cancer requires the development of effective and efficient systems, processes and methods which utilize fresh or archived tissue samples. Currently, the majority of

pathology samples are preserved in fixed, paraffin-embedded (FPE) tissue blocks as a standard archival method that allows tissue morphology preservation for years at ambient temperature. In order for a test using GCC as a biomarker target to have an impact on clinical management, such test needs to be compatible with FPE tissue preservation.

5   However, such samples represent a major technical issue because the fixation process is known to degrade nucleic acids through protein cross-linking or oxidation over time due to addition of mono-methyl groups to nucleic acid bases. Consequently, methods for detecting metastatic cancer cells must be migrated to more robust clinical platforms such as those enabling reverse transcription and real-time quantitative polymerase chain

10   reaction (RT-qPCR). RNA degradation and chemical modifications that occur in FPE clinical samples can affect target detection accuracy in RT-qPCR.

[007]   The applicant has previously developed a clinical test for colorectal cancer staging that uses the ability of RT-qPCR to detect very small fragments of GCC mRNA to accommodate specimens with degraded RNA. The GCC duplex assay, using the

15   Scorpions™ technology for monitoring the qPCR reaction, with beta-actin (β-actin or ACTB) as an internal control RNA, circumvents most of the frequent problems associated with absolute quantification by taking into account the efficiency of the reverse transcriptase and PCR reactions and monitoring for the presence of reverse transcriptase and PCR inhibitors. However, because of its high abundance, ACTB needs to be

20   adjusted to prevent competition with GCC in the duplex assay. Therefore, use of ACTB as a reference gene to monitor RNA degradation or RNA input is currently limiting because the assay is insensitive to variations such as time-dependent degradation and harsh fixation conditions. In this context, only highly degraded samples and/or highly inhibited samples and/or samples with extremely low RNA concentrations produce ACTB

25   Ct values below an established limit of detection (LOD). Because GCC is more affected than ACTB by all these stress conditions, the current absolute quantification requires that all samples be free of any inhibitors, have similar degradation status and RNA input, thus lowering sensitivity and accuracy of the assay.

[008]   There is therefore a need for a sensitive and/or accurate and/or repeatable and/or

30   cost-effective method and system for detecting a target, such as GCC, in a sample obtained from a patient.

[009]   Unfortunately, simply measuring transcript levels of one or more prognostic RNA transcripts does not account to produce a diagnostic test of sufficient sensitivity and specificity to determine a clinical outcome associated with molecular markers. Raw data obtained from real-time PCR must be processed to obtain the relative quantity of target

5   mRNA. Absolute quantification requires a standard curve in each experiment in order to determine by interpolation the number of mRNA copies in a given sample relative to known amounts of a synthetic DNA or RNA transcript. Relative quantification (delta Ct (ΔCt)) may be used to determine the changes in mRNA level of a target gene (i.e. GCC) across samples by expressing this change in relation to the levels of an endogenous

10   reference gene (RG; sometimes referred to as housekeeping gene), used as an internal control RNA. One such well known and often-used reference gene is ACTB. Alternatively, the difference (delta-delta Ct (ΔΔCt)) between the average delta Ct (ΔCt) value of a target sample and the average delta-Ct (ΔCt) for the corresponding control sample is used to calculate expression fold change value.

15   [010]   Evaluation of stable endogenous reference genes in clinical samples is a prerequisite to precise and accurate normalization of relative gene expression using RT-qPCR platform or other related amplification methods. The use of a single so-called "universal" reference gene may lead to misinterpretation of the expression of the GCC gene.

20   [011]   There is also a need for the identification of a reference gene to be used in conjunction with GCC for the detection of CRC cells in a patient's sample, this reference gene having an expression not affected by the presence of cancer cells in a lymph node, and a behavior similar to GCC in samples degraded because of long storage periods, poor storage conditions or other stress factors. Many such reference genes have been

25   investigated and found to be useful in different contexts. However, there is presently a consensus that the optimal, universal reference gene does not exist (Green *et al.* 2009, Diagn Mol Pathol 18 (4), 243-249 and ref 11 therein).

Summary of the invention

[012]   It has now been found that beta-glucuronidase (GUSB) is particularly useful as a

30   reference gene for normalization of PCR data. In the particular context of evaluating GCC for GI tract cancers diagnosis and/or prognosis, GUSB provides unforeseen advantages

such as allowing one to measure much lower limits of detection (LOD) than could be obtained with other reference genes.

[013]   The invention provides a method for the detection of cancer cells or GCC expressing cells in a sample, which method detects and/or measures/quantifies GCC from processed harvested tissue or biological fluid, in combination with the detection and/or quantification of one or more reference genes in the same sample. GUSB (beta-glucuronidase) was found to be a superior reference gene. Particularly, GUSB was found to be a superior reference gene to be used in complement to GCC in the detection of CRC cells in lymph nodes. RT and PCR reactions were designed to obtain an efficient duplex test simultaneously amplifying GCC and GUSB mRNAs. The analytical performance of this test was verified, showing better strength compared to the GCC/ACTB test. These better analytical characteristics (higher informative rate, higher analytical sensitivity and relative quantification) of the GCC/GUSB test can lead to a more accurate stratification of the recurrence risk (RR) when tested with a population of patients diagnosed with Stage II CRC.

[014]   The invention also provides a diagnostic method for detection of GCC in a sample collected from a patient, comprising the following steps: detecting GCC in the sample; detecting (either simultaneously or sequentially) beta-glucuronidase (GUSB) in the same sample, and establishing relative quantification of GCC versus GUSB wherein presence of GCC versus GUSB above a fixed threshold is indicative of the presence of GCC positive cells in the sample.

[015]   According to a general aspect, there is provided a method of measuring GCC in a sample collected from a patient, comprising the following steps: measuring GCC in the sample by RT-qPCR to establish a Ct_{GCC}; measuring GUSB in the same sample RT-qPCR to establish a Ct_{GUSB}; and establishing relative quantification (delta-Ct) of Ct_{GUSB} minus Ct_{GCC} wherein a delta-Ct of above about -12 is indicative of the presence of GCC positive cells in the sample.

[016]   The invention also provides a diagnostic method for the detection of GCC that uses the expression fold change (delta-delta-Ct) to determine the changes in mRNA level of GCC across samples by expressing this change in relation to the RNA levels of GUSB.

[017]   According to another aspect of the invention, there is provided a method of staging or monitoring a patient already diagnosed with GI tract cancer, comprising the steps of: detecting GCC in the sample (consisting of human tissues or biological fluids in which GCC is not normally expressed, such as particularly extra-colorectal/intestinal tissue);

5   detecting GUSB in the same sample; and establishing a relative quantification (delta-Ct) of $Ct_{GUSB} - Ct_{GCC}$, wherein a delta-Ct of above -12 is indicative of the presence of GCC positive cells in the sample, wherein the presence of GCC positive cells is indicative of metastasized colorectal, stomach, small intestinal, pancreatic or esophageal cancer.

[018]   According to another aspect of the invention, there is provided a method of

10   diagnosing a patient suspected of having a primary stomach, esophageal or pancreatic cancer, comprising the steps of: measuring GCC in an extra-intestinal/colorectal sample; measuring GUSB in the same sample; and establishing a relative quantification (delta-Ct) of $Ct_{GUSB} - Ct_{GCC}$, wherein a delta-Ct of above -12 is indicative of the presence of GCC positive cells in the sample, wherein the presence of GCC positive cells is indicative of a

15   primary stomach, esophageal or pancreatic cancer.

[019]   The method of the present invention allows detection of the presence or absence of GCC in lymph nodes harvested following a stomach, small intestine, esophageal, pancreatic or colorectal resection, thereby allowing molecular staging of a cancer. According to another aspect of the invention, there is provided a method, comprising the

20   steps described above, to discriminate between cancer patients with histopathologically negative and histopathologically positive lymph nodes.

[020]   According to this method, cancer patients with GCC positive cells in one or several of their lymph nodes have a risk of recurrence and survival comparable to those of patients considered as having a higher risk by histopathology, thereby indicating that

25   these patients might benefit from treatment with adjuvant chemotherapy. According to this method, cancer patients with all LNs negative for GCC are at a lower risk of disease recurrence and would not require adjuvant chemotherapy, consequently avoiding the negative side effects of these treatments.

[021]   The method of the present invention detects the presence of GCC in blood from

30   patient previously diagnosed with cancer patients to predict the risk of recurrence, monitor the recurrence and the response to cancer therapy of the cancer. According to another aspect of the invention, there is provided a method of prognosticating a patient with

cancer, comprising the steps as defined above, wherein the presence of GCC positive cells is indicative of a poor prognosis.

[022] In accordance with another aspect of the present invention, there is provided a method of determining if a patient already diagnosed with GI tract cancer will benefit from a treatment, comprising the steps of: measuring the expression level of GCC in an extra-intestinal/colorectal sample collected from the patient; measuring the expression level of GUSB in the same extra-intestinal/colorectal sample; and determining the quantity of GCC relative to the quantity of GUSB in the extra-intestinal/colorectal sample; wherein if the quantity of GCC in the extra-intestinal/colorectal sample is above a given level when compared to GUSB, the patient will benefit from the treatment.

[023] Particularly, the invention provides a method of determining the quantity of GCC in an extra-intestinal/colorectal sample collected from a patient already diagnosed with GI tract cancer, comprising the steps of : measuring the expression level of GCC mRNA in the sample ; measuring the expression level of GUSB mRNA in the same sample; and using a mathematical calculation to normalize the expression level of GCC mRNA to the expression level of GUSB mRNA to next establish a relative GCC expression (GUSB level – GCC level); wherein if the relative GCC expression is higher than the limit of detection of an external standard, the absolute quantity of GCC in the sample is calculated and expressed in number of GCC copies.

[024] Particularly, the invention provides a method of predicting the likelihood of cancer recurrence in a patient already diagnosed with GI tract cancer, comprising the steps of: determining the quantity of GCC as defined above in one or more lymph nodes collected from the patient; classifying each of the one or more lymph nodes as GCC-negative or GCC-positive; and establishing a lymph node ratio, wherein the lymph node ratio is the number of GCC-positive nodes over the total of GCC-negative and GCC-positive lymph nodes; whereby the larger the lymph node ratio means the greater the likelihood of cancer recurrence.

[025] Particularly, the invention provides a method of predicting the likelihood of cancer recurrence in a patient already diagnosed with GI tract cancer, comprising the steps of: measuring the expression level of GCC in one or more lymph nodes collected from the patient; measuring the expression level of GUSB in the same one or more lymph nodes; and determining the quantity of GCC relative to the quantity of GUSB in each individual

lymph nodes; wherein a relative quantity of GCC above a pre-established cut-off is indicative of the presence of GCC in a lymph node, whereby if GCC is present in one lymph node or more, the patient has an increased likelihood of cancer recurrence.

[026]   Particularly, the pre-established cut-off level is between about -6 and -3. More particularly, the cut-off level is selected from the group consisting of: -5.9, -5.5, -5.0; -4.5; -4.0; -3.5; and -3.0.

[027]   Particularly, the invention provides a method of predicting the likelihood of cancer recurrence in a patient already diagnosed with GI tract cancer, comprising the steps of: quantifying by RT-qPCR RNA levels of GCC in an extra-intestinal/colorectal sample collected from the patient to establish a cycle threshold for GCC (Ct_{GCC}); quantifying by RT-qPCR RNA levels of GUSB in the same sample to establish a cycle threshold for GUSB (Ct_{GUSB}); and calculating a relative quantification of Ct_{GUSB} minus Ct_{GCC} (delta-Ct); wherein a delta-Ct equal or higher than about -6 is indicative of the presence of GCC positive cells in the sample, whereby the presence of GCC positive cells is indicative that the patient has increased risk of recurrence of cancer.

[028]   Particularly, the invention provides a method of determining if a patient already diagnosed with cancer has GCC nodal involvement, comprising the steps of: measuring the expression level of GCC in a lymph node collected from the patient to establish a cycle threshold for GCC (Ct_{GCC}); measuring the expression level of GUSB in the same lymph node collected from the patient to establish a cycle threshold for GUSB (Ct_{GUSB}); and determining a relative quantification of Ct_{GUSB} minus Ct_{GCC} (delta-Ct); wherein if delta-Ct is equal or higher than about -6, the lymph node is GCC-positive.

[029]   Particularly, the invention provides a method of determining the GCC burden of a patient already diagnosed with cancer, comprising the steps of: measuring the expression level of GCC mRNA in an extra-intestinal/colorectal sample collected from the patient to establish a cycle threshold for GCC (Ct_{GCC}); measuring the expression level of GUSB in same extra-intestinal/colorectal sample collected from the patient to establish a cycle threshold for GUSB (Ct_{GUSB}); and calculating a relative quantification of Ct_{GUSB} minus Ct_{GCC} (delta-Ct); wherein if delta-Ct is equal or higher than about -12, the quantity of GCC mRNA may be calculated in terms of number of copies, whereby the GCC burden is expressed in number of GCC copies in the sample.

[030]   Particularly, the invention provides a method of staging or monitoring a patient already diagnosed with colorectal cancer, comprising the steps: obtaining an extra-intestinal/colorectal sample taken from the patient; measuring GCC in the sample to establish a cycle threshold for GCC (Ct_{GCC}); measuring GUSB in the same sample to
5   establish a cycle threshold for GUSB (Ct_{GUSB}); and establishing relative quantification (delta-Ct) of Ct_{GUSB} minus Ct_{GCC}, wherein a delta-Ct of higher than about -6 is indicative of the presence of GCC positive cells in the sample, wherein the presence of GCC positive cells is indicative of metastasized colorectal cancer.

[031]   According to another aspect of the invention, the detection of the presence of GCC
10   positive cells in tissues harboring metastases of an unknown origin (CUP) is a confirmation that the primary cancer is a colorectal, a stomach, an intestinal, a pancreatic or an esophageal cancer.

[032]   In yet another aspect of the invention, the materials for use in the methods of the present invention are suited for preparation of a kit. According to another aspect of the
15   invention, there is provided a kit for the detection, diagnosis, prognosis and/or staging of a cancer in a patient, wherein the kit comprises: PCR reagents for detecting GCC in the sample, PCR reagents for detecting GUSB in the same sample, and instructions on how to calculate (delta-Ct) or (delta-delta-Ct) between GCC and GUSB.

[033]   The invention also provides kits comprising reagents, which may include gene-
20   specific probes and/or primers, for quantifying the expression of the disclosed genes for predicting the likelihood of developing disease recurrence. Such kits may optionally contain reagents for the extraction of RNA from a sample, in particular fixed paraffin-embedded tissue samples and/or reagents for RNA amplification.

Detailed description of the invention

25   Brief description of the figures

[034]   The accompanying figures, which are incorporated in and constitute a part of this specification, illustrate various embodiments of the present invention, and, together with the description, serve to explain the principles of the invention. In the figures:

[035]   **Figure 1** represents the expression levels of putative reference genes, presented
30   as average Ct values in matched fresh frozen (FF) and FFPE colon cancer LNs;

[036]   **Figure 2** represents the expression levels of putative reference genes, presented as average Ct values in GCC negative and positive FFPE LNs. Targeted Ct range was delimited by the two dotted lines;

[037]   **Figure 3** represents the average expression stability values of control genes;

5   [038]   **Figure 4** represents the determination of the optimal number of control genes for normalization;

[039]   **Figure 5** represents the expression profile of selected reference gene in GCC positive and negative LNs. Ct value for GCC, GUSB, HPRT1, PGK1 and TBP in FFPE samples tested in simplex reaction with 312.5 ng of cDNA. In minus RT experiments, no

10   RT enzyme was added during the cDNA synthesis step;

[040]   **Figure 6** represents the effect of NaOH treatment on RNA quality and gene expression measures. The extent of RNA degradation following NaOH treatment was determined by capillary electrophoresis using the Agilent 2100 Bioanalyzer. Panel A) show a representative RNA fragmentation profile compared to intact RNA isolated from

15   fresh frozen colon tissues and matched FFPE material. B) Ct values for GCC and 5 reference gene assays including ACTB Scorpions™ were determined at each time point of hydrolysis;

[041]   **Figure 7** represents the GCC expression variation (delta-delta-Ct) during NaOH degradation using five reference genes (GUSB, HPRT1, PGK1, TBP and ACTB) for

20   normalization. Variations in delta Ct values (delta-delta-Ct = $[(Ct_{GCC}-Ct_{RG})_{tx}-(Ct_{GCC}-Ct_{RG})_{t0}]$ were determined at each time point of hydrolysis. Error bar represent SD from 3 independent RNA pools. To perform comparison between three experiments, the threshold for each gene was manually fixed to 0.10;

[042]   **Figure 8** represents the effect of carbonate buffer alkaline treatment on RNA

25   quality and gene expression pattern. The quality of RNA isolated at indicated time points was measured by capillary electrophoresis using the Agilent 2100 Bioanalyzer. A) Representative RNA fragmentation profile compared to intact RNA isolated from fresh frozen colon tissues. B) Electropherograms of RNA sample. Each time point contained an equal amount of material. C) Ct values for GCC and 5 reference gene assays, including

30   the GCC/ACTB duplex assay;

[043]   **Figure 9** represents the GCC expression variation (delta-delta-Ct) following controlled RNA degradation in carbonate buffer alkaline conditions. Variation in delta Ct values (delta-delta-Ct = $[(Ct_{GCC}-Ct_{RG})_{tx}-(Ct_{GCC}-Ct_{RG})_{t0}]$ were determined at each time point of hydrolysis;

5   [044]   **Figure 10** represents the gene expression profiling in frozen and fixed, paraffin-embedded (FPE) tissues using TaqMan simplex assays and the GCC/ACTB duplex assay. The quality of RNA isolated from each condition was measured by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Condition A and E are TRIzol™ extract, B: Neutral buffered formalin 10%, C: Non-buffered formalin 10%; D: Bouin's

10   solution). Expression (Ct values) of GCC and 4 reference genes (GUSB, HPRT1, PGK1 and TBP) in TaqMan simplex reactions was compared to the GCC/ACTB duplex assay;

[045]   **Figure 11** shows the comparison of GCC expression levels (delta-Ct) in cryo-sections of colon cancer tissue samples fixed or not. Variation in delta Ct values (delta-Ct = $(Ct_{GCC}-Ct_{RG})$) were determined for each RNA extract. The reverse transcription reactions

15   were performed with the Superscript™ III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's recommendation, using gene-specific primers (2 μM) and 500 ng of total RNA based on Quant-IT data. The real-time PCR were carried out in a 20-μl reaction volume with the Applied Biosystems 7900HT Fast Real-Time PCR Systems using either TaqMan simplex or the GCC/ACTB duplex assay. Primers and probes

20   concentration for each simplex assays were 900 nM and 250 nM respectively. All reactions were performed in duplicate;

[046]   **Figure 12** shows the comparison of GCC Taqman simplex and duplex amplifications in RNA extracted from a FFPE colon tissue. Upper panel shows GCC and various RG PCR amplifications in FFPE samples tested in simplex and duplex reactions

25   using gene-specific reverse primers at 2 μM and 1.25 μg of RNA. Lower panel shows a comparison of minus RT-PCR amplification for GCC and various reference genes in simplex and duplex reactions;

[047]   **Figure 13** shows the comparison of GCC and GUSB Ct values between duplex and simplex reactions. The real-time PCR were carried out in duplex or simplex reactions

30   of 20 μl with the Applied Biosystems 7900HT Fast Real-Time PCR Systems using TaqMan Fast Universal PCR Master Mix. Synthesis of cDNA was performed with 250 ng/μl (A) or 25 ng/μl (B) of uRNA spiked or not with 1x106 GCC IVT. For real-time PCR

reaction in simplex, primers concentration was 900 nM and FAM or VIC-labeled probes concentration was 250 nM. For duplex reactions, 4 concentrations of reverse and forward primers were tested while both FAM and VIC-labeled probes were fixed at 200 nM in a 20 µl PCR reaction;

5   [048]   **Figure 14** represents the GCC and RG delta-Ct variation in duplex and simplex assays during NaOH degradation. Expression of GCC was normalized with either GUSB or HPRT1. Variations in GCC relative quantification (delta-delta-Ct = $[(Ct_{GCC}-Ct_{RG})_{tx}-(Ct_{GCC}-Ct_{RG})_{t0}]$ were determined at indicated time points during NaOH hydrolysis. Variation between non-degraded and degraded samples should be lower than 1 delta-Ct to be

10 considered not significant;

[049]   **Figure 15** represents the comparison of FFPE colon cancer LNs tested with TaqMan and Scorpions™ duplex assays in minus RT condition. Panel A and C show Ct values for ACTB, GUSB, HPRT1 and GCC in 8 FFPE samples tested with 312.5 ng of cDNA in duplex reaction. In minus RT experiments (B and D), no RT enzyme was added

15 during the cDNA synthesis step;

[050]   **Figure 16** shows the comparison of GCC expression levels (delta-Ct) in cryo-sections of colon cancer tissue samples fixed or not. Delta Ct values $\Delta Ct = (Ct_{GCC}-Ct_{RG})$ were determined for each RNA extract using either GUSB (A and B) or HPRT1 (C and D). The reverse transcription reactions were performed in simplex using gene-specific primers

20 at 2 µM and in duplex with indicated primers concentration. The real-time PCR were carried out in a 20µl reaction volume with the Applied Biosystems 7900HT Fast Real-Time PCR Systems using either TaqMan® simplex or duplex assay were compared to Scorpions™ duplex reaction. All reactions were performed in triplicate. Variation of less than 1 delta-Ct between frozen and fixed samples was considered not significant;

25 [051]   **Figure 17** represents the amplification of reference genes in RNA extracted from 55 FPE pericolonic lymph node tissues with different archiving times from 1 month to 22 years. A) Capillary electrophoresis profiles of RNA extracted from archival FPE tissues. One µl of each RNA extract (150ng/µL) was analysed using the Agilent 2100 Bioanalyser and RNA Nano Chips. B) Ct values for ACTB and GUSB in FPE colon lymph node

30 tissues. The real-time PCR were carried out in simplex reactions of 20 µl using the Applied Biosystems 7900HT Fast Real-Time PCR Systems and TaqMan Fast Universal PCR Master Mix;

[052]   **Figure 18** shows the comparison of ACTB and GUSB Ct value observed in group of blocks with different archiving time. Box-and-Whisker plots of the ACTB (A) and GUSB (B) mRNA expression (Ct value) in FPE colon lymph node tissues. For multiple comparisons, one-way ANOVA and post-hoc Turkey's test were used and $P < 0.05$ was considered statistically significant;

[053]   **Figure 19** represents the GCC relative expression levels in histopathology-negative and GCC/ACTB-negative (pN0(mol-)) and stage III GCC/ACTB-Positive (pN1-2(mol+)) LNs tested with the TaqMan GCC/GUSB assay. A receiver operating characteristic (ROC) analysis of the relative quantification using delta-Ct ($Ct_{GUSB} - Ct_{GCC}$) was used to determine a cut-off value for the GCC/GUSB duplex assay;

[054]   **Figure 20** represents the GCC relative expression levels evaluated with the TaqMan GCC/GUSB duplex assay. GCC relative expression ΔCt ($Ct_{GUSB} - Ct_{GCC}$) in colon cancer stage I, II and III histopathology negative (HP-) and stage III positive (HP+) LNs. GCC mRNA positive status was based on the analytical cut-off value of -5.9;

[055]   **Figure 21** shows the average expression stability values of control genes in blood samples using geNorm;

[056]   **Figure 22** shows the determination of the optimal number of reference genes for normalization using geNorm;

[057]   **Figure 23** represents the GCC expression level in blood samples. GCC mRNA positive status was based on the analytical cut-off value of 75 GCC units/mL;

[058]   **Figure 24** shows the distribution of A) ACTB Ct values and B) Glucuronidase GUSB Ct values for two different patient cohorts. Wilcoxon rank test p-value: 0.0001;

[059]   **Figure 25** shows the combination of HDQ and UMass cohorts (n=73) ROC curve analysis for both GCC/ACTB and GCC/GUSB assays taking the highest GCC copies of any given LN of a case as the continuous variable. Sensitivity is defined as the detection rate of recurrent cases (after 36 months) while specificity is defined as the proportion of negative cases without recurrence;

[060]   **Figure 26** illustrates the relation between risk of recurrence for patients with a GCC positive test result and the GCC expression level used to determine test positivity;

[061]   **Figure 27** is a Kaplan-Meier graphical analysis of time to recurrence based on GCC positivity. A) GCC/ACTB test with 100 copies cut-off; B) GCC/GUSB test with 100 copies cut-off; C) GCC/ACTB test with 25 copies cut-off; D) GCC/GUSB test with 25 copies. The GCC Negative cases are represented by the straight black line and the GCC

5   Positive cases by the gray dashed line. Patients lost to follow-up were censored-out and are represented by straight up marks;

[062]   **Figure 28** is a Kaplan-Meier graphical analysis of RFS based on the GCC positivity for the GCC/GUSB test with -5.9 ΔCt for cut-off. The GCC Negative cases are represented by the straight line and the GCC Positive cases by the dashed line.

10   Censored-out cases are represented by straight up marks;

[063]   **Figure 29** is a Kaplan-Meier graphical analysis of time to recurrence with 2 levels of stratification for GCC positive patients. A) GCC/ACTB test with 100 copies cut-off; B) GCC/GUSB test with 100 copies cut-off; C) GCC/ACTB test with 25 copies cut-off; D) GCC/GUSB test with 25 copies. Number of patients at risk is also indicated for each

15   group;

[064]   **Figure 30** is a Kaplan-Meier graphical analysis of time to recurrence with 2 levels of stratification for GCC positive patients. A) GCC/GUSB test with -5.9 delta-Ct for cut-off. Number of patients at risk is also indicated for each group. B) Comparison of total recurrence rate between GCC/GUSB (delta-Ct = -5.9) and GCC/ACTB (GCC copies =

20   25); and

[065]   **Figure 31** is a Kaplan-Meier graphical analysis of time to recurrence with stratification for the number of GCC positive LNs per patients. A) GCC/ACTB test with 25 copies cut-off; B) GCC/GUSB test with 25 copies cut-off. Number of patients at risk is also indicated for each group.

25   <u>Definitions</u>

[066]   To facilitate an understanding of the invention, a number of terms are defined below.

[067]   The term "about" as used hereinbelow refers to a margin of + or – 5% of the number indicated. For sake of precision, the term "about" when used in conjunction with,

30   for example, the integer 10, means 10 +/- 5% i.e. from 9.5 to 10.5.

[068]   As used herein, the term "GCC" is meant to refer to the gene transcription product (RNA) expressing the cellular protein guanylate cyclase 2C (GUCY2C also referred to as the heat stable enterotoxin receptor or ST receptor), which is expressed by normal colorectal cells, as well as primary and metastasized colorectal, intestinal, stomach and

5   esophageal cancer cells. In normal individuals, GCC is found exclusively in cells of intestine, in particular in cells in the duodenum, small intestine (jejunum and ileum), colon (caecum, ascending colon, transverse colon, descending colon and sigmoid colon) and rectum. The term "GCC" also includes fragments of a GCC gene transcript which are functional with respect to nucleic acid molecules with full length sequence, such as a

10   functional fragment which may be useful as an oligonucleotide or nucleic acid probe, a primer, an antisense oligonucleotide or nucleic acid molecule or a coding sequence. The term "GCC" also comprises the CRCA-1 alternative transcript.

[069]   As used herein, the term "colorectal cancer" is meant to include the well-accepted medical definition that defines colorectal cancer as a medical condition characterized by

15   presence of cancer cells in the intestinal tract below the small intestine (i.e. the large intestine (colon), including the caecum, ascending colon, transverse colon, descending colon, and sigmoid colon, and rectum). Additionally, as used herein, the term "colorectal cancer" is meant to further include medical conditions which are characterized by presence of cancer cells in the duodenum and small intestine (jejunum and ileum). The

20   definition of colorectal cancer used herein is more expansive than the common medical definition but is provided as such since the cells of the duodenum and small intestine also contain GCC.

[070]   As used herein, the term "GI tract cancer" or "gastro-intestinal cancer" is meant to include the medical conditions which are characterized by presence of cancer cells in the

25   esophagus, the stomach, the pancreas, the small intestine as well as in colon and rectum. Additionally, as used herein, the term "GI tract cancer" in meant to further include medical conditions which are characterized by presence of cancer cells in the pancreas, which like liver and gallbladder is an accessory organ of the GI tract. The definition of GI tract cancer used herein is more expansive that the common medical definition but is provided as such

30   since pancreatic cancer cells are known to express GCC.

[071]   As used herein, the terms "upper GI tract" consists of the mouth cavity, salivary glands, pharynx, esophagus, diaphragm, stomach, gall bladder, bile duct, liver, and

duodenum. The term "upper GI tract cancer" as used herein particularly refers to the esophagus, stomach and pancreas.

[072]   As used herein, the terms "lower GI tract" means of the bowel or intestines and the rectum and comprises the small intestine including duodenum, jejunum, ileum; and the large intestine or colon including caecum (and appendix); colon (ascending, transverse and descending) and the rectum (anus).

[073]   As used herein, the term "stomach cancer" is meant to include the well-accepted medical definition that defines stomach cancer as a medical condition characterized by presence of cancer cells in the stomach.

[074]   As used herein, the term "esophageal cancer" is meant to include the well-accepted medical definition that defines esophageal cancer as a medical condition characterized by presence of cancer cells in the esophagus.

[075]   As used herein, the term "pancreatic cancer" is meant to include the well-accepted medical definition that defines pancreatic cancer as a medical condition characterized by presence of cancer cells in the pancreas.

[076]   As used herein, the term "metastasis" is meant to refer to the process in which cancer cells originating in one organ or part of the body, with or without transit by a body fluid, and relocate to another part of the body and continue to replicate. Metastasized cells can subsequently form tumors which may further metastasize. Metastasis thus refers to the spread of cancer, from the part of the body where it originally occurred, to other parts of the body.

[077]   As used herein, the term "metastasized colorectal cancer cells" is meant to refer to colorectal cancer cells which have metastasized. Metastasized colorectal cancer cells are localized in a part of the body or body fluid other than the duodenum, small intestine (jejunum and ileum), large intestine (colon), including the caecum, ascending colon, transverse colon, descending colon, and sigmoid colon, and rectum.

[078]   As used herein, the term "metastasized stomach cancer cells" is meant to refer to stomach cancer cells which have metastasized. Metastasized stomach cancer cells are localized in a part of the body other than the stomach.

[079] As used herein, the term "metastasized esophageal cancer cells" is meant to refer to esophageal cancer cells which have metastasized. Metastasized esophageal cancer cells are localized in a part of the body other than the esophagus.

[080] As used herein, the term "metastasized pancreatic cancer cells" is meant to refer to pancreatic cancer cells which have metastasized. Metastasized pancreatic cancer cells are localized in a part of the body other than the pancreas.

[081] As used herein, the terms "non-intestinal/rectal" and "extra-intestinal/colorectal" are used herein interchangeably and are meant to refer to a sample of tissue or body fluid from a source other than intestinal (small intestine and colon) and rectal tissue. In some preferred embodiments, the extra-intestinal/colorectal sample is a sample of tissue such as lymph nodes. In some preferred embodiments, the non-intestinal/rectal sample is a sample of extra-intestinal/colorectal tissue which is an adenocarcinoma of unconfirmed origin. In some preferred embodiments, the non-intestinal/rectal tissue is a biopsy of a suspected stomach, pancreatic or esophagus cancer. In some preferred embodiments, the non-intestinal/rectal sample is a blood sample.

[082] As used herein, "an individual suffering from an adenocarcinoma of unconfirmed origin" or "cancer of unknown primary origin" (CUP) is meant to refer to an individual who has a tumor in which the origin has not been definitively identified.

[083] As used herein, the terms "subject" and "patient" refer to any animal, such as a mammal like livestock, pets, and preferably a human. Specific examples of "subjects" and "patients" include, but are not limited, to individuals requiring medical assistance, and in particular, patients with cancer.

[084] As used herein, the term "target" or "target marker" or "biomarker target" refers to any molecule that can be derived from a eukaryotic cell. Targets include but are not limited to proteins or nucleic acid molecules. In the present invention, the level of a messenger RNA that is specifically expressed in cells of gastrointestinal origin is measured. Alternatively, a tissue specific protein or DNA alteration (e.g. methylation or mutation) could be an equivalent target. In preferred embodiments of the present invention, single targets such as mRNA are detected individually. In alternative embodiments of the present invention, multiple targets are detected in combination.

[085]   As used herein, the terms "reference gene" or "reference marker" or "reference target" or "control" or "control marker" or "control target" refers to a reference molecule that controls and/or can be used to control for potential process interfering factors and/or provides one or more indications about the sample quality, the effective sample

5   preparation and/or assembly of the RT-PCR reaction in the sample. A control may either be co-detected or detected separately from targets.

[086]   As used herein, the term "sample" refers to a biological material containing cells or other material retrieved from the patient. Sample material includes but is not limited to: tissue such as lymph node tissue; biopsy material; exhaled breath; or fluids such as blood

10   (including serum or plasma); urine; semen; sputum, saliva; and combinations of these. To practice the methods of the present invention, the sample is processed (e.g. a lymph node is separated from other tissue and/or cut in multiple sections or cores, exposed or not to a chemical reaction, subjected to a separation process or blood is enriched in tumor circulating cells). Each process may result in a portion of the sample remaining,

15   hereinafter referred to as "remaining sample" or simply "sample". The portions of the sample may be sized randomly or according to a predetermined scheme or mathematical formulaic determination. Sample may be defined as a single tissue sample, such as a single lymph node, or sample may define multiple samples, such as multiple lymph nodes or lymph node chain. In preferred embodiments of the present invention, single samples

20   such as single lymph nodes are processed individually. In alternative embodiments of the present invention, multiple samples are "pooled" or processed together. In another preferred embodiment, the sample includes at least one entire lymph node.

[087]   The term "external standard" as used herein means a synthetic DNA or RNA transcript in known amount(s) or concentration(s) that is tested separately from the test

25   sample, i.e. through interpolation or extrapolation to a standard curve.

[088]   As used herein the term "parameters", also known as "process parameters", include one or more variables used in the method and system of the present invention to detect one or more targets. Parameters include but are not limited to: primer type; probe type; amplicon type; concentration of a substance; mass or weight of a substance; time for

30   a process; temperature for a process; cycle threshold (Ct); activity during a process such as centrifugation, rotating, shaking, cutting, grinding, liquefying, precipitating, dissolving, electrically modifying, chemically modifying, mechanically modifying, heating, cooling,

preserving (e.g. for days, weeks, months and even years) and maintaining in a still (unagitated) state. Parameters may further include a variable in one or more mathematical formulas used in the method of the present invention. Parameters may include a threshold used to determine the value of one or more parameters in a

5   subsequent step of the method of the present invention. In a preferred embodiment, the threshold is a cycle count threshold.

[089] As used herein, the term "cycle threshold" (Ct) refers to the threshold in qPCR at which the fluorescence generated within a reaction well exceeds an established threshold or cutoff level. The cycle threshold refers to the same value than the terms "crossing point'

10   (Cp) and "take-off point" (TOP) used by competing manufacturers of real-time PCR instruments for reasons of product differentiation. For standardization purposes, the MIQE Guidelines (Bustin et al., Clinical Chemistry, 55:4, pp. 611-622 (2009)) have proposed that the use of the term "quantification cycle" (Cq) be preferred over all those alternatives.

[090] The term "hybridization" is to be understood as a bond of an oligonucleotide to a

15   complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure.

[091] "Stringent hybridization conditions," as defined herein, involve hybridizing at 68°C in 5X SSC/ 5X Denhardt's solution/ 1.0% SDS, and washing in 0.2X SSC/ 0.1% SDS at room temperature, or involve the art-recognized equivalent thereof (e.g., conditions in

20   which a hybridization is carried out at 60°C in 2.5X SSC buffer, followed by several washing steps at 37°C in a low buffer concentration, and remains stable). Moderately stringent conditions, as defined herein, involve including washing in 3X SSC at 42°C, or the art-recognized equivalent thereof. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and

25   the target nucleic acid. Guidance regarding such conditions is available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N. Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N. Y.) at Unit 2.10.

[092] As used herein, the expression "clinical assessment" is meant to include a

30   potential range or continuous or discrete values used for the screening, diagnosis, staging, prognosis, treatment planning, monitoring and surveillance of a cancer patient.

[093]   As used herein, the expression "clinical outcome" or "outcome" is meant to be expressed in terms of different endpoints such as Disease-Free Survival (DFS), Relapse-Free Survival (RFS), Time-to-Recurrence (TR), Cancer-Specific Survival (CSS) or Overall Survival (OS), in accordance with the recommendations of Punt CJ *et al.*, J. Natl. Cancer
5   Inst. (2007) 99 (13): 998-1003.

[094]   As used herein, the "Time-to-Recurrence" (TR) is defined as the time to any event related to the same cancer. All same cancer recurrences and deaths from the same cancer are events. Second primary same cancers and other primary cancers are ignored. Deaths from other cancers, non-cancer-related deaths, treatment-related deaths, and loss
10   to follow-up are censored observations.

[095]   As used herein, the expression "Relapse-Free Survival" or "Recurrence-Free Survival" (RFS) is defined as the time to any event, irrespective of the cause of this event, except for any second primary cancer. Recurrence of or death from the same cancer and all treatment-related deaths or deaths from other causes are events. Second primary from
15   the same cancers and other primary cancers are ignored, and loss to follow-up is censored.

[096]   As used herein, the "Cancer-Specific Survival" (CSS) is defined as the time to death caused by the same cancer, whether the death is caused by the primary tumor or a second primary same cancer. Locoregional recurrence, distant metastases, second
20   primary same cancers, and second other primary cancers are ignored. Deaths from other cancers, non-cancer-related deaths, treatment-related deaths, and loss to follow-up are censored.

[097]   As used herein, the expression "Disease-Free Survival" (DFS) is defined as the time to any event, irrespective of the cause of this event. All events are included, except
25   loss to follow-up which is censored.

[098]   As used herein, the "Overall Survival" (OS) is defined as the time to death, irrespective of cause, whether or not the death was due to cancer. Locoregional recurrence, distant metastases, second primary colorectal cancers, and second other primary cancers are ignored. Loss to follow-up is censored.

30   [099]   As used herein, the "staging" or "stage" of a cancer refers to the TNM (for tumors/nodes/metastases) system, from the American Joint Committee on Cancer (AJCC)

(Greene *et al.* (eds.), AJCC Cancer Staging Manual, 6th edition, New York, NY: Springer; 2002), which depends on the extent of local invasion, the degree of lymph node involvement and whether there is distant metastasis. Staging is done after surgery has been performed and pathology reports reviewed. In the TNM system, "T" denotes the degree of invasion of the intestinal wall, "N" the degree of lymphatic node involvement, and "M" the degree of metastasis. The broader stage of a cancer is usually quoted as a number I, II, III, IV derived from the TNM value grouped by prognosis; a higher number indicates a more advanced cancer and likely a worse outcome. Details of this system for colorectal cancer are the following:

AJCC stage	TNM stage	TNM stage criteria for colorectal cancer	Dukes	Astler-Coller
Stage 0	Tis N0 M0	Tis: Tumor confined to mucosa; cancer-*in-situ*	-	-
Stage I	T1 N0 M0	T1: Tumor invades *submucosa*	A	A
Stage I	T2 N0 M0	T2: Tumor invades *muscularis propria*	A	B1
Stage II-A	T3 N0 M0	T3: Tumor invades *subserosa* or beyond (without other organs involved)	B	B2
Stage II-B	T4 N0 M0	T4: Tumor invades adjacent organs or perforates the visceral peritoneum	B	B3
Stage III-A	T1-2 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.	C	C1
Stage III-B	T3-4 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.	C	C2, C3
Stage III-C	any T, N2 M0	N2: Metastasis to 4 or more regional lymph nodes. Any T.	C	C1, C2, C3
Stage IV	any T, any N, M1	M1: Distant metastases present. Any T, any N.	-	D

[0100] The stage can also be reported in letters rather than numbers, according to the Dukes and Astler-Coller staging systems, which often combine different AJCC stage groupings and are not as precise, as shown in the above table.

[0101] As used herein, the survival rates for colon cancer are from a study of the National Cancer Institute's SEER database, looking at nearly 120,000 people diagnosed with colon cancer between 1991 and 2000. In this study, survival was better for stage IIIA than for stage IIB.

Stage	5-year Survival Rate
I	93%
IIA	85%
IIB	72%
IIIA	83%
IIIB	64%
IIIC	44%
IV	8%

5 [0102] As used herein, the term "lymph node involvement" refers to a qualitative notion about the presence of metastases in lymph nodes as determined visually through a histopathology procedure. A patient harboring no involved nodes is designated "N0" or pN0. When metastases are detected in 1 to 3 lymph nodes, the lymph node involvement is designated "N1" or "pN1". "N2" or "pN2" is used to designate a lymph node involvement, 10 or presence of metastases, in 4 or more regional lymph nodes. The lymph node involvement is a criteria used by clinicians to determine whether or not a patient should receive adjuvant chemotherapy.

[0103] As used herein, the term "GCC nodal involvement" refer to a qualitative notion about the presence or absence of Guanylyl cyclase C (GCC or GUCY2C) mRNA in an 15 individual lymph node, which is indicative of the presence or absence of nodal metastases including occult metastases i.e. metastases or a cluster of cancer cells that cannot be detected by histopathology. When a lymph node is invaded by GCC expressing cells, i.e. exhibits a detectable quantity of GCC mRNA, it is called "node positive". When no GCC can be detected, the lymph node is "negative". A patient harboring at least one GCC 20 positive node is called "GCC positive" while a patient or case with no GCC positive nodes is called "GCC negative".

[0104] As used herein, the terms "GCC burden" or "GCC load" refer to a quantification of the amount of GCC expressing cells found in a particular lymph node or to a total amount

of GCC mRNA in a group of lymph nodes of a patient. The GCC burden is not significant or not clinically significant when the detectable quantity of GCC mRNA in a given node or in all the lymph nodes collectively is below a given level. However, when the quantity of GCC mRNA detected in a given lymph node or in a group or all of the lymph nodes of a

5   patient is above a given level, the GCC burden is significant or clinically significant and can be used to discriminate between patients with a lower risk of recurrence from those with a higher risk. The level of GCC mRNA in a given lymph node or in a group of lymph nodes can be expressed in many ways, such as in terms of copies or copies per lymph node mass (absolute quantification) or in terms of delta Ct (ΔCt), delta-delta Ct ($\Delta\Delta$Ct), or

10   fold change ($2^{-\Delta\Delta Ct}$) (2 exponent minus delta-delta Ct), these last three parameters being based on the expression level of GCC relative to the expression level of a reference gene (relative quantification), such as, but not limited to, GUSB, in the same lymph node.

[0105] As used herein, the term "lymph node ratio" or "LNR" refers to the number of GCC-positive lymph nodes over the total number of measurable lymph nodes tested for a given

15   patient.

Description of particular embodiments

[0106] Particularly, the invention provides a method selected from the ones as defined herein and more particularly as defined above, wherein one or more reference genes is normally expressed in normal cells of the extra-intestinal/colorectal sample. Particularly,

20   the reference gene is beta-glucuronidase (GUSB). More particularly, the measuring of expression levels is carried out using RT-qPCR.

Detecting

[0107] According to a general aspect, there is provided a method of detecting GCC in a sample collected from a patient, comprising the following sequential steps:

25   • obtaining the sample from the patient;
            • homogenizing the sample;
            • extracting nucleic acid from the sample;
            • detecting GCC mRNA in the sample for example by measuring its Ct level;
            • detecting beta-glucuronidase (GUSB) mRNA in the same sample, for example by

30   measuring its Ct level; and

- establishing relative quantification (delta-Ct) of Ct_{GUSB} minus Ct_{GCC};

wherein a delta-Ct of above a predetermined threshold is indicative of the presence of GCC positive cells in the sample.

[0108] According to a particular aspect, there is provided a method for the detection of GCC in an extra-intestinal/colorectal sample collected from a subject, comprising the steps of:

- detecting Guanylyl Cyclase C (GCC) in said sample;
- detecting beta-glucuronidase (GUSB) in the same said sample; and
- calculating an amount of GCC in relation to an amount of GUSB.

Measuring

[0109]   According to a particular aspect, there is provided a method for the measurement of GCC in a sample, comprising the steps of :

- measuring expression level of GCC mRNA in said sample;
- measuring expression level of GUSB mRNA in the same said sample; and
- using a mathematical calculation to normalize the expression level of GCC mRNA to the expression level of GUSB to establish a relative GCC expression (GUSB level minus GCC level) or (GCC level minus GUSB level).

[0110]   According to a particular aspect, there is provided a method for the measurement of GCC in a sample, comprising the following steps:

- measuring the expression level of GCC in the sample by RT-qPCR to determine a cycle threshold for GCC (Ct_{GCC});
- measuring beta-glucuronidase (GUSB) in the same sample by RT-qPCR to determine cycle threshold for GUSB (Ct_{GUSB}); and

wherein the detection of GCC uses relative quantification (delta-Ct) to determine the changes in mRNA level of GCC in a sample and expresses it relative to the mRNA levels of beta-glucuronidase (GUSB) (delta-Ct= Ct_{GUSB} minus Ct_{GCC}).

[0111] Particularly, the method as defined above uses the expression fold change (delta-delta-Ct) to determine the changes in mRNA level of GCC in said sample and expresses it relative to the mRNA level of beta-glucuronidase (GUSB) in the same sample.

[0112] Still particularly, there is provided a method of determining the GCC burden of a patient diagnosed with cancer, comprising carrying the steps of the method as defined herein, wherein if delta-Ct is equal or higher than about -12, the quantity of GCC mRNA is calculated in terms of number of copies in relation to an external standard, whereby the GCC burden is expressed in number of GCC copies in the sample

Diagnosing

[0113] Particularly, there is provided a method of diagnosing cancer in a patient suspected of having cancer, comprising the steps of quantifying GCC in an extra-intestinal/colorectal sample of said patient in accordance with the method of the invention; and determining whether said sample harbors GCC positive cells, whereby the presence of GCC positive cells is indicative of colorectal, stomach, small intestine, esophageal or pancreatic cancer.

Staging

[0114] Still, particularly, there is provided a method of staging a human patient already diagnosed with cancer, comprising the steps of:

a) detecting or measuring GCC in accordance with the method of the invention; and

b) establishing a disease-stage based on the results of step a.

Monitoring

[0115] Still, particularly, there is provided a method of monitoring, or diagnosing metastasis in, a human already diagnosed with cancer, comprising the steps:

- measuring GCC in said sample by RT-qPCR to determine a cycle threshold for GCC (Ct_{GCC})) in an extra-intestinal/colorectal sample from said patient;
- measuring beta-glucuronidase (GUSB) in said sample by RT-qPCR to determine a cycle threshold for GUSB (Ct_{GUSB}) in said sample; and
- establishing relative quantification (delta-Ct) of $Ct_{GUSB} - Ct_{GCC}$,

wherein a delta-Ct of above about -12 is indicative of the presence of GCC positive cells in the sample, wherein the presence of GCC positive cells is indicative of metastasized colorectal, stomach, small intestine, pancreatic or esophageal cancer.

Selecting a cancer patient who can benefit from treatment

[0116] Still, there is particularly provided a method to select among cancer patients having histopathologically negative lymph nodes those who can benefit from a course of treatment, comprising:

5   • carrying out the steps according to the method of the invention; and

           • prescribing a course of treatment;

whereby cancer patients with GCC positive cells in at least one lymph node have a risk of recurrence and survival rate comparable to that of patients considered of a higher risk by histopathology, thereby indicating that these patients might benefit from treatment with

10   adjuvant chemotherapy, and whereby cancer patients with GCC negative lymph nodes are at a lower risk of disease recurrence and can avoid said treatment.

Predicting risk of recurrence

[0117] Particularly, there is also provided a method of predicting the risk of cancer recurrence for a patient already diagnosed with cancer, comprising carrying the steps

15   according to the method of the invention, wherein a delta-delta-Ct between -6 and -3 is indicative of the presence of GCC positive cells in the sample, whereby the presence of GCC positive cells is indicative that the patient has increased risk of recurrence of cancer.

Establishing tumor burden

[0118] There is further provided a method of determining the GCC burden of a patient

20   diagnosed with cancer, comprising carrying the steps of the methods as defined herein, wherein if delta-Ct is equal or higher than about -12, the quantity of GCC mRNA is calculated in terms of number of copies in relation to an external standard, whereby the GCC burden is expressed in number of GCC copies in the sample.

Threshold and cut-off

25   [0119] Particularly, the threshold or cut-off for positive identification of GCC positive cells is a delta-Ct above about -12. More particularly, the threshold for positive identification of GCC positive cells is a delta-Ct above about -10. More particularly, the threshold for positive identification of GCC positive cells is a delta-Ct above about -8. Still, more particularly, the threshold for positive identification of GCC positive cells is a delta-Ct

30   above about -6. Even more particularly, the threshold for positive identification of GCC

positive cells is a delta-Ct above about -4. Most particularly, the threshold for positive identification of GCC positive cells is a delta-Ct above -2.

[0120] Particularly, the method as defined herein may include one or more analyses or algorithms used to detect a target or perform an analysis based on the detection of the at least two targets (GCC and GUSB). Such analysis or algorithm may have a bias, such as a false-positive or false-negative bias. For example, the analysis or algorithm may take into account a combination of disease factors or clinical factors such as: age, race, an existing patient condition, use of adjuvant therapy, heredity; and so on.

[0121] More particularly, the method may comprise the inclusion of multiple parameters used to perform a step of a procedure or used by an algorithm of the procedure such as multiple reference genes detected and measured in addition to GUSB.

Cancer and Patient

[0122] It should be appreciated that the invention is applicable to be performed with a sample from a patient with cancer, particularly GI tract cancer of a wide variety of stages, level of aggressiveness, level of illness, symptomatic or asymptomatic, or other adverse conditions. In a particular embodiment, the patient has a GI tract cancer from the upper or lower GI tract. More particularly, the cancer may be selected from a colorectal cancer, a small intestine, a stomach cancer, a pancreatic cancer, or an esophageal cancer.

[0123] Particularly, the patient has a stage I or stage II cancer. More particularly, the cancer is colorectal cancer.

[0124] Throughout the application, the patient may be referred to as a cancer patient such as a colorectal cancer patient. This terminology shall include patients in whom the presence of cancer has been confirmed, currently or historically, as well as patients that may, for any reason, be suspected of having cancer or otherwise receive a cancer diagnostic test of the present invention. Positive detection of the target may correlate to the presence of cancer; a specific prognosis or diagnosis of the cancer; or other clinical assessment or recommendation.

Sample

[0125] It should be appreciated that the method of the invention can be carried out on numerous forms of samples such as extra-intestinal/colorectal sample including but not

limited to tissue or biological fluid. Particularly, the sample is taken from an organ that does not normally express GCC. In particular, a sample can be a tissue which has been preserved or otherwise archived. The sample may be one or more lymph nodes collected from a single patient, particularly during a resection procedure. More particularly, the

5   lymph nodes are collected during a colorectal, esophagus, stomach or pancreatic resection.

[0126] Currently, the histopathologic evaluation of lymph nodes is performed using typically one to three Hematoxylin and Eosin (H&E) slides. There is a high, demonstrated risk of "missing" metastatic cells due to sampling issues, visual inspection shortcomings,

10   human error and other complexities. The method of the present invention avoids and/or reduces these issues, and can detect one or more targets indicative of numerous patient adverse conditions including but not limited to the presence of: metastases; micrometastases; occult metastases; isolated tumor cells; clusters of tumor cells and combinations of these. The molecular evaluation of the current invention provides more

15   systematic, repeatable, automatable tests that can be performed with high accuracy, sensitivity and repeatability.

[0127] The sample is particularly an archived lymph node (e.g. a fixed, formalin-embedded sample including one or more lymph nodes), but may be fresh or frozen tissue. The sample may include tissue from one or more of the following anatomical

20   locations/organ: breast, prostate, stomach, esophagus, pancreas, kidney, spleen, cervix, vagina, ovary, bladder, thyroid, colon, rectum, small intestine, brain, skin, liver and lung.

[0128] In another particular embodiment, the sample includes multiple nodes which are "pooled" or processed together. The number of copies detected is correlated to a specific assessment of patient condition including but not limited to cancer stage or therapy

25   outcome.

[0129] While a majority of the applications has been described in reference to samples including a peri-colonic lymph node, alternatively or additionally, other lymph nodes, other tissue and other samples may be processed by the method described herein.

[0130] The method of the present invention may provide an analysis of a sample that is a

30   cancer of unknown origin. In a particular embodiment, a cancer sample such as a brain, lung or liver tumor, is processed to detect GCC to determine that the origin of the cancer

as the colon or rectum or stomach, esophagus, small intestine or pancreas (e.g. vs. the lung, liver, brain or other location).

[0131] The method of present invention produces results from one or more molecular tests, such as a molecular test for GCC in a lymph node harvested in a surgical procedure
5   removing a portion of a patient's colon. In a particular embodiment, the lymph nodes or other tissues are also histologically analyzed and the results of both the molecular test(s) and histological test(s) are combined to perform a subsequent assessment.

[0132] In a particular embodiment, the number of GCC copies is correlated with the number of cells identified as cancerous in the histological analysis. The correlation can be
10 made on a first patient, or a first set of patients. Subsequently, the number of copies detected can be determined via molecular testing, and correlated to a predicted number of cells that would be identified in histological tests. This predicted number, combined with or without a histological examination for cells, is used to produce a more specific assessment of patient condition including but not limited to cancer stage or therapy
15 outcome.

[0133] Particularly, the sample may include other body tissues, or biological fluids such as exhaled breath, blood, urine, sputum, saliva and/or semen. Particularly, the sample is blood.

[0134] In a particular embodiment, precautions are taken throughout each step to avoid
20 cross-contamination of tissue, such as contamination between tissue samples received from the patient (e.g. two lymph nodes), or contamination from a first patient to a second patient. In another particular embodiment, the sample is retrieved from the patient in a clinical setting such as a hospital, and one or more further processing steps are also performed at that or an additional clinical setting. The sample is then transferred to a
25 clinical or medical laboratory, such as a CLIA laboratory, for further processing. Results of the further processing may be analyzed, at the laboratory and/or a clinical setting (e.g. by a clinician of the patient). In a particular embodiment, the sample consists of multiple patient lymph nodes collected in a colorectal resection procedure, typically consisting of 12 lymph nodes but optionally from 1 up to 100 or more, including sentinel nodes.

30 [0135] In the various steps of the method of the present invention, the sample is processed (e.g. physically divided such as a lymph node separated from other tissue

and/or a lymph node cut in multiple sections or cores with a scalpel; exposed to a physico-chemical reaction such as a deparaffinization and/or a precipitation procedure; exposed to a separation process such as separation in a centrifuge; exposed to a washing procedure; and the like). Each process may result in a portion of the sample remaining, hereinafter

5   referred to as "remaining sample" or simply "sample". The portions of the sample may be sized randomly, or according to a predetermined scheme or mathematical formulaic determination. Sample may be defined as a single tissue sample, such as a single lymph node, or sample may define multiple samples, such as multiple lymph nodes. In preferred embodiments of the present invention, single samples such as single lymph nodes are

10   processed individually. In alternative embodiments of the present invention, multiple samples are processed in combination. In another preferred embodiment, the sample includes at least one entire lymph node, such as to avoid testing a first portion of a lymph node that does not include the target wherein a second portion does include the target. Samples may be preserved (an "archived sample") such as to prevent degradation over

15   time. Preservation methods include but are not limited to: refrigeration such as freezing; use of a preservative tissue solution; dehydration; and combinations of these. Particular tissue preservative solutions include but are not limited to: commercial products such as formalin (a buffered or non-buffered aqueous solution of formaldehyde); Bouin's solution (consisting of a mixture of picric acid and formaldehyde); PAXgene Tissue Fix, PAX gene

20   Tissue Stabilizer, RNARetain™ solution; RNALater™ solution; nonaqueous solutions such as that described in US7,138,226; and combinations of these. Paraffin-embedding and/or other similar material-embedding may or may not be performed after tissue fixation, such as to assist in the creation of sections such as slide sections, to facilitate transport and non-detrimental storage.

25   [0136] Particularly, in reference to the method of the invention, the extra-intestinal/colorectal sample is a lymph node or blood. Particularly, the extra-intestinal/colorectal sample is a lymph node; more particularly, one single lymph node, most particularly two or more lymph nodes, still most particularly at least four lymph nodes, and even most particularly at least twelve lymph nodes.

30   [0137] Particularly, in reference to the method of the invention, when the positive results are found in 1 to 3 lymph nodes of the same patient, the relative risk of recurrence for this patient according to the GCC/GUSB test is intermediate. More particularly, when the

positive results are found in 4 or more lymph nodes of the same patient, the relative risk of recurrence for this patient according to the GCC/GUSB test is high. Still more particularly, the method allows to discriminate between cancer patients having histopathologically negative lymph nodes, wherein cancer patients with GCC positive cells in at least one

5   lymph node have a risk of recurrence and survival rate comparable to that of patients considered at higher risk by histopathology, thereby indicating that these patients might benefit from treatment with adjuvant chemotherapy. In contrast, cancer patients with all lymph nodes being GCC negative are at a lower risk of disease recurrence and can avoid negative side effects of treatment with adjuvant chemotherapy. Most particularly, the

10   presence of GCC positive cells is indicative of a poor prognosis.

[0138] Particularly, in reference to the method of the invention, the quantity of GCC detected is calculated for each individual lymph node. More particularly, the quantity of GCC is the sum of the individual quantities of GCC in all lymph nodes of the patient.

[0139] Particularly, in reference to the method of the invention, the GCC burden is

15   established for each individual lymph node. More particularly, the GCC burden is established on the basis of the total amount of GCC in all lymph nodes of the patient. Still, more particularly, the GCC burden is determined and a GCC burden above a given number of GCC copies is indicative of an increased likelihood of cancer recurrence.

PCR methodology

20   [0140] According to one aspect of the invention, a GCC RT-qPCR test is used to detect the presence of GCC in lymph nodes, tissues or biological fluids obtained from a colon-, rectum-, esophagus-, small intestine-, pancreas-, or stomach-cancer patient, the detection correlating to one or more clinical assessment related to that patient's cancer.

[0141] In a particular embodiment, formalin-fixed paraffin-embedded (FFPE) lymph nodes

25   are processed and a RT-qPCR assay is used to quantitatively detect GCC. The processing includes homogenization of the lymph node tissue followed by nucleic acid (e.g. RNA) extraction. The RT-qPCR assay may use a non-specific (e.g. SYBR green) or specific (e.g. Scorpions™ , Molecular Beacons, Locked Nucleic Acid (LNA) Fluorescent Probes, Amplifluor, or Taqman) detection chemistries.

30   [0142] In a particular embodiment, GCC is quantified relative to the average expression of beta-glucuronidase (GUSB) as a reference or control gene. In an alternative embodiment,

PCR efficiency correction is used. In a particular embodiment, the assay is a duplex assay detecting GCC and GUSB as reference gene. In an alternative embodiment, GCC and GUSB are detected from simplex assays. In an alternative embodiment, the assay is a triplex assay detecting GCC, GUSB and another reference marker, such as: GAPDH, HPRT1, PGK1 and TBP and/or a spiked internal control. Particular examples of endogenous genes that can be used as reference genes are those associated with SEQ ID NOs: 2-16 and listed in Table 1. Despite the degradation caused by the fixation (e.g. formalin fixation) and embedding the lymph nodes in a supporting medium, a high accuracy and/or sensitivity and/or repeatability is achieved. Typical supporting material is paraffin wax. However other materials can be used such as certain inert plastics or epoxies, or other supportive material lacking reactivity with the sample.

Table 1. *Endogenous reference genes evaluated in this study.*

SEQ ID No.	Accession number	Gene Name	Official Symbol	Function	Amplicon length	Exons Boundary	PCR eff'y
2	NM_000181	Beta glucuronidase	GUSB	Carbohydrate metabolic process	81	11-12	99.7
3	NM_001101	Beta Actin	ACTB	Structural constituent of cytoskeleton	69	3-4	102.5
4	NM_004048	Beta-2-microglobulin	B2M	MHC class I protein complex	75	2-3	99.5
5	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Glucose metabolic process	73	3-4	106.8
6	NM_000194	Hypoxanthine phosphoribosyltransferase 1	HPRT1	Nucleoside metabolic process	62	7-8	101.9
7	NM_000291	Phosphoglycerate kinase 1	PGK1	Glycolysis, Metabolism of carbohydrates	75	4-5	93.3
8	NM_002676	Phosphomannomutase 1	PMM1	Mannose biosynthetic process	60	1-2	79.4
9	NM_021128	DNA directed RNA polymerase II polypeptide L	POLR2L	Regulation of transcription from RNA polymerase I promoter	74	1-2	99.3
10	NM_021130	Peptidylprolyl isomerase A (cyclophilin A)	PPIA	Protein folding	68	3-4	101.4
11	NM_002798	Proteasome subunit beta 6	PSMB6	Ubiquitin-dependent protein catabolic process	55	5-6	102.9
12	NM_001002	Ribosomal protein, large, P0	RPLP0	Ribosome biogenesis and assembly	61	5-6	101.5

SEQ ID No.	Accession number	Gene Name	Official Symbol	Function	Amplicon length	Exons Boundary	PCR eff'y
13	NM_003194	TATA box binding protein	TBP	Transcription initiation from RNA polymerase II promoter	62	4-5	100.4
14	NM_003234	Transferrin receptor	TFRC	Endocytosis	79	13-14	99.5
15	NM_003295	Tumor protein, translationally-controlled 1	TPT1	Calcium ion transport	68	2-3	103.9
16	NM_021009	Ubiquitin C	UBC	Protein modification process	71	1-2	101.5

[0143] Although PCR methods, and more specifically RT-qPCR, are preferred, other similarly reliable, sensitive and specific amplification and detection methods such as Rolling Circle Amplification methods (RCA), Branched-Chain DNA Amplification (BCA), Ligase Chain Reaction methods (LCR), Strand Displacement Amplification methods (SDA), Nucleic Acid Sequence Based Amplification methods (NASBA), Transcription-Mediated Amplification methods (TMA) and others can also be used. Detection technologies, which may or may not follow nucleic acid amplification, may include MALDI-TOF mass spectrometry, capillary electrophoresis, and similar detection methods.

Reverse transcriptase primers

[0144] In another embodiment, GCC is quantified relative to the average expression GUSB as a reference or control gene. According to another aspect of the invention, the primers for GCC and GUSB are RT primers and are added in the same assay at a predetermined ratio in order to optimize the detection of each marker by itself and in relation to the other marker. Particularly, in an assay comprising an amount of 1.25 μg of total extracted RNA, the RT primers for GCC are selected from polynucleotides capable of hybridizing to: NM_004963 (**SEQ ID No.1**) and are added to the assay in a quantity of about 10μM to 30μM. More particularly, the primers for GUSB are selected from polynucleotides capable of hybridizing to: NM_000181 (**SEQ ID No.2**) and are added to the assay in a quantity of about 1μM to 3 μM. Particularly, such primers are capable of hybridizing to a location on the GCC or GUSB coding regions, preferably such primers are spanning two exons. More particularly, such primers are free from single nucleotide polymorphism (SNP).

[0145] Particularly, primers and probes of the useful for this method comprise polynucleotides having 90% identity to SEQ ID NOs: 17-43. More particularly, primers

comprising polynucleotides having 90% identity to SEQ ID NOs: 17, 18, 20, 21, 23, 24, 26, 26, 29, 30, 32, 33, 35, 36, 38, 39, 41 and 42 can be useful for this method. Particular examples of polynucleotide primers and probes of the invention are shown as SEQ ID NOs: 17-43 and are listed in Table **2**. More particularly, primers and probes of SEQ ID

5   NOs. 20, 21, 22, 38, 39 and 40 are useful for the present method.

Table 2 Selected designs for GCC and endogenous reference genes evaluated

Gene	Accession	Exons	Reagent Type	Reagent Name	Sequence	SEQ ID No
GCC	NM_004963	2-3	Forward Primer	GCC_F1	GCGACTGCCGGAGTAGCA	17
			Reverse Primer	GCC_R1	CCGTTGTGCATTTGAAATTT TC	18
			Probe	GCC_Tq1	CTGTGAAGGCCTCG	19
		3-4	Forward Primer	GCC_F2	CCACCTTCCAGATGTACCTT GAC	20
			Reverse Primer	GCC_R2	CCAAAACTTCCAGCTGAGA TCA	21
			Probe	GCC_Tq2	CAGAATTGAGCTACCCC	22
		6-7	Forward Primer	GCC_F3	AGTGGCTGAAGACATTGTC ATTATTC	23
			Reverse Primer	GCC_R3	GGCTGTGACATTGTCCTCC AA	24
			Probe	GCC_Tq3	AGTGGATCTTTTCAATGACC AG	25
		12-13	Forward Primer	GCC_F4	ATGTTAGCCTCAAGATCGAT GATG	26
			Reverse Primer	GCC_R4	TCGTATTTGCACTGTCGTAG TCTCT	27
			Probe	GCC_Tq4	CAAAAGACGAGATACAATC	28
		15-16	Forward Primer	GCC_F5	CCCTCCGGGAAGTTTTAAAT G	29
			Reverse Primer	GCC_R5	TCTTAAACTCCCAATCCATG AATG	30
			Probe	GCC_Tq5	CACAATTTCCTACCCTGATG	31
		19-20	Forward Primer	GCC_F6	GAAACCCTTCCGCCCAGAT	32
			Reverse Primer	GCC_R6	ATCTTCCTCCCAACAGTTTT TTACA	33
			Probe	GCC_Tq6	AAAAAGAGCTAGAAGTGTA CCTAC	34
		21-22	Forward Primer	GCC_F7	GCTTCCAAGGCTAGTGGTA AAGTC	35
			Reverse Primer	GCC_R7	TCATATAGTTCCGGCTCCAC AA	36
			Probe	GCC_Tq7	CTGAAGGAGAAAGGC	37
GUSB	NM_000181	11-12	Forward Primer	GUSB_F1	TGGTTGGAGAGCTCATTTG GA	38
			Reverse Primer	GUSB_R1	ACTCTCGTCGGTGACTGTT CAG	39
			Probe	GUSB_Tq1	TTTTGCCGATTTCATG	40
		10-11	Forward Primer	GUSB_F2	AAGCCCATTATTCAGAGCG AGTA	41
			Reverse Primer	GUSB_R2	CAGAGGTGGATCCTGGTGA AA	42
			Probe	GUSB_Tq2	AGCAGAAACGATTGCAG	43
GAPDH	NM_002046	2-3	Forward Primer	GAPDH_F2	CCACATCGCTCAGACACCA T	44
			Reverse Primer	GAPDH_R2	GTGACCAGGCGCCCAAT	45
			Probe	GAPDH_Tq2	AGTCAACGGATTTGGTC	46

Gene	Accession	Exons	Reagent Type	Reagent Name	Sequence	SEQ ID No
		1-2	Forward Primer	GAPDH_F4	CTGTTCGACAGTCAGCCGC	47
			Reverse Primer	GAPDH_R4	CCCCATGGTGTCTGAGCG	48
			Probe	GAPDH_Tq4	TCGCCAGCCGAGCC	49
HPRT1	NM_000194	6-7	Forward Primer	HPRT1_F1	CCTTGGTCAGGCAGTATAA TCCA	50
			Reverse Primer	HPRT1_R1	GGTCCTTTTCACCAGCAAG CT	51
			Probe	HPRT1_Tq1	AGATGGTCAAGGTCG	52
		2-3	Forward Primer	HPRT1_F2	TTATGGACAGGACTGAACG TCTTG	53
			Reverse Primer	HPRT1_R2	GCACACAGAGGGCTACAAT GTG	54
			Probe	HPRT1_Tq2	AAGGAGATGGGAGGCCA	55
PGK1	NM_000291	4-5	Forward Primer	PGK1_F2	TGGAGAACCTCCGCTTTCA T	56
			Reverse Primer	PGK1_R2	TGGCTCGGCTTTAACCTTGT T	57
			Probe	PGK1_Tq2	AAGGGAAAAGATGCTTCT	58
		1-3	Forward Primer	PGK1_F5	GATCGACTTCAATGTTCCTA TGAAGA	59
			Reverse Primer	PGK1_R5	GCTTGGGACAGCAGCCTTA A	60
			Probe	PGK1_Tq5	CAACCAGATAACAAACAA	61
TBP	NM_003194	4-5	Forward Primer	TBP_F1	CGAATATAATCCCAAGCGG TTT	62
			Reverse Primer	TBP_R1	CCGTGGTTCGTGGCTCTCT	63
			Probe	TBP_Tq1	CTGCGGTAATCATG	64
		5-6	Forward Primer	TBP_F2	CAGGAGCCAAGAGTGAAGA ACA	65
			Reverse Primer	TBP_R2	TGGAAAACCCAACTTCTGTA CAAC	66
			Probe	TBP_Tq2	AGACTGGCAGCAAGAA	67

[0146] Particularly, the RT primers for GCC are present at about 10-20µM and the primers for GUSB are added to the assay at about 1-4µM.

[0147] More particularly, the RT primers for GCC:GUSB are present in the assay at a ratio of about 10:1.

Probes

[0148] According to another aspect of the invention, probes specific for GCC or GUSB are selected from the group consisting of: polynucleotides capable of hybridizing to GCC short coding sequences or GUSB short coding sequences under stringent conditions. Particular examples of these probes are listed in Table 2.

[0149] Particularly, probes specific for GCC are added to the assay in an amount of about 200 nM.

[0150] Particularly, probes specific for GUSB are added to the assay in an amount of about 200 nM.

Amplicons

[0151] According to another aspect of the invention, a short GCC amplicon length and a short GUSB amplicon length are used to detect GCC expressing cells. In a particular embodiment, a test of the present invention analyzes RNA of less than 100 nucleotides in
5   a sample. In another particular embodiment, a test of the present invention analyzes RNA of less than 80 nucleotides. In yet another particular embodiment, a test of the present invention analyzes RNA of less than 70 nucleotides.

Table 3. Comparison of recommended conditions for the GCC/GUSB TaqMan assay and GCC/ACTB Scorpions™.

	GCC/GUSB TaqMan	**GCC/ACTB Scorpions™**
Amplicon size	GCC (64 bp) /GUSB (61 bp)	GCC (66bp) / ACTB (69bp)
RT Reverse primers		
GCC	10-20 µM (GCC_R2)	20 µM (GCC_R2)
reference Gene	1-4 µM (GUSB_R1)	0.02 µM (ACTB_R3)
qPCR		
Number of Cycles	40	40
GCC primers	400-900 nM	350 nM
Reference Gene primers	200-600 nM	350 nM
Probes (GCC and RG)	200 nM	n/a

10

Detection

[0152] According to a particular embodiment, the present method provides the detection of the GCC gene transcription product in a sample. The term detection particularly refers to identifying, locating, obtaining a positive signal, measuring, or quantifying.

15   [0153] Positive detection of the target may include the detection of one or more targets. Positive detection of the target may require detection of the target above a threshold. Positive detection may be a direct measure of finding cancer cells (e.g. target is cancer

cell), and/or a direct measure that provides a diagnosis or prognosis of cancer. Alternatively, positive detection of the marker may be associated with a surrogate to an assessment, the surrogate being the measure of finding cancer cells, and/or surrogate measure that provides a diagnosis or prognosis of cancer.

5   Delta-Ct

[0154] The present invention provides a method for detecting GCC in a sample collected from a patient. Detection of GCC includes a quantification of GCC relative to the quantification of GUSB found in the same sample, and may be used to diagnose, stage, prognosticate, monitor and/or manage the treatment of an adverse patient condition such

10   as the presence of cancer.

[0155] The method of the present invention includes one or more analyses or algorithms used to detect a target or perform an analysis based on the detection of the at least two targets (GCC and GUSB). The cycle threshold (Ct) or cycle number in qPCR is the threshold at which the fluorescence generated within a reaction exceeds an established

15   threshold or cutoff level. Positive and negative signals are respectively defined as being beyond and below an established cutoff level (threshold). The cutoff level is established by testing two populations of samples with known conditions, one collected from donors having the condition (positive) and the second one collected from donors not having the condition (negative). For example, the population of positive samples may be lymph

20   nodes collected from colorectal cancer patients and having been identified as pN1 or pN2 by histopathology; the population of negative samples may be lymph nodes collected from patients having other conditions than colorectal cancer, such as breast cancer, lung cancer, gastrointestinal inflammatory conditions, etc. Alternatively, the population of positive samples may be lymph nodes collected from colorectal cancer patients having

25   recurred from the disease during the 5 years following the resection of the primary tumor and having been identified as pN1 or pN2 by histopathology; the population of negative samples may be lymph nodes collected from colorectal cancer patients having not recurred or died from the disease during the 5 years following the resection of the primary tumor and having been identified as pN0 by histopathology. Alternatively, the population

30   of positive samples may be blood samples collected from colorectal cancer patients having recurred or died from the disease during the 5 years following the resection of the primary tumor; the population of negative samples may be blood samples collected from

colorectal cancer patients having not recurred from the disease during the 5 years following the resection of the primary tumor.

[0156] An analysis or algorithm may have a bias, such as the false-positive or false-negative bias. An analysis or algorithm may be modified by an existing patient condition,

5   such as has been described hereinabove.

[0157] The method of the present invention includes multiple parameters used to perform a step of a procedure or used by an algorithm of the procedure. The parameters may be established and/or modified through testing of various types of tissue not from the patient of the present invention, such as lymph nodes or other tissue harvested from other

10   humans, pigs and cows.

[0158] Particularly, in reference to the method of the invention, a delta-Ct equal or higher than -6 (if $Ct_{GUSB}-Ct_{GCC}$) or equal or lower than +6 (if Ct_{GCC} minus Ct_{GUSB}) is indicative of the presence of GCC positive cells in the sample, whereby the presence of GCC positive cells is indicative that the patient has increased risk of recurrence of cancer. More

15   particularly, a delta-Ct equal or higher than -5.9 represents a GCC positive result and a delta-Ct lower than -5.9 represents a GCC negative result, whereby said result allow discrimination for risk of recurrence and relapse-free survival (RFS) between GCC-negative and GCC-positive results.

[0159] Particularly, in reference to the method of the invention, a delta-Ct equal or higher

20   than about -6 represents a GCC positive result and a delta-Ct lower than about -6 represents a GCC negative result, where the result allow discrimination for risk of recurrence and relapse-free survival (RFS) between GCC-negative and GCC-positive results. More particularly, in reference to the method of the invention, a delta-Ct equal or higher than -5.9 represents a GCC positive result and a delta-Ct lower than -5.9

25   represents a GCC negative result, where the result allows discrimination for risk of recurrence and relapse-free survival (RFS) between GCC-negative and GCC-positive results.

[0160] Particularly, a pre-established cut-off level for delta-Ct of between about -6 and -3 is suitable to determine the status of GCC positive or GCC-negative cells. More

30   particularly, the cut-off level is selected from the group consisting of: -5.9, -5.5, -5.0; -4.5; -4.0; -3.5; and -3.0.

Kit

[0161] Numerous kit configurations are also to be considered within the scope of this application. A kit may include one or more components, supports, vials, substances or reagents as well as instructions booklet, as is described in detail herein.

5   [0162] Particularly, the kit for the detection, diagnosis, prognosis, monitoring and/or staging of a cancer in a patient, comprises reagents for detecting GCC in an extra-intestinal/colorectal sample from the patient; reagents for detecting GUSB in the same sample; and instructions on how to quantify GCC in relation to GUSB.

[0163] More particularly, the kit for the detection, diagnosis, prognosis, monitoring and/or
10   staging of a cancer in a patient, wherein the kit comprises: PCR reagents for detecting GCC in an extra-intestinal/colorectal sample from the patient; instructions on how to determine a cycle threshold for GCC (Ct_{GCC}); PCR reagents for detecting GUSB in the same sample; instructions on how to determine a cycle threshold for GUSB (Ct_{GUSB}); and instructions on how to calculate (delta-Ct) or (delta-delta-Ct) between Ct_{GCC} and Ct_{GUSB}.

15   [0164] It should be understood that numerous other configurations of the method and kit described herein can be employed without departing from the spirit or scope of this application. Portions of the method described above may individually be considered a unique invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed
20   herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims. In addition, where this application has listed the steps of a method or procedure in a specific order, it may be possible, or even expedient in certain circumstances, to change the order in which some steps are performed and/or combine one or more steps, and it is
25   intended that the particular steps of the method or procedure claim set forth herein below not be construed as being order-specific unless such order specificity is expressly stated (for example as "sequentially").

Informative Rate

[0165] One of the interesting features of the GCC/GUSB test observed is the possibility to
30   increase the informative rate with formalin-fixed paraffin-embedded (FFPE) tissue samples. Using a cut-off Ct value of 35 for GUSB, the GCC/GUSB test outperformed the

informative rate obtained with the reference GCC/ACTB test with only five inadequate LNs (1264/1269 or 99.6%, CI95% 99.3-100 vs. 1253/1269 or 98.7% CI95% 98.1-99.4, respectively), an overall gain of 11 adequate LNs (Table 4). This increase of informative rate detected with 1269 FFPE LNs tissues from two independent cohorts confirmed our
5   initial observations.

Table 4: Informative rate of GCC/ACTB and GCC/GUSB tests with 1269 LNs.

	Informative Rate	
GCC/GUSB (≤35)	1264/1269	99,6%
GCC/ACTB (≤29)	1253/1269	98,7%

[0166] Another reason for changing the reference gene (RG) from ACTB to GUSB was to
10 take advantage of the relative quantification to determine the level of GCC mRNA in each LN. We had shown previously that a longer archival time in FFPE block could impair the quantification of gene expression. Because GCC is more affected than ACTB in these conditions, we were unable to use ACTB as a reference gene to monitor RNA degradation since the assay was insensitive to small variations. The age of the blocks (number of
15 years from surgery through nucleic acid extraction) was analysed per site. The samples from the University of Massachusetts (UMass) patient cohort were much older than those of Hôtel-Dieu de Québec (HDQ) (12 vs. 6 years in average). The distribution of ACTB and GUSB Ct values is presented in **Figure 24A**. When compared to HDQ, a significant increase in the GUSB Ct value was observed (p <0.0001) for the UMass cases (**Figure**
20 **24B**), suggesting a time-dependent degradation of GUSB mRNA. Ultimately, when relative expression of GCC was measured using GUSB as the reference gene, there was no difference between the relative level of GCC in positive cases from HDQ and UMass (mean delta-Ct: -2.7 vs. -3.0; p-value: 0.5682). Conversely, when a delta-Ct was calculated with the 100 copies external standard ($Ct_{Std100} - Ct_{sample}$) for the GCC/ACTB
25 assay, a greater decrease in the GCC delta-Ct per specimen was observed for the UMass cases compared to HDQ samples (mean delta-Ct: -4 vs. -2). This difference did not however reach a statistical significance (p=0.072), probably due to an insufficient number of samples tested.

Examples

[0167] Evaluation of stable endogenous reference genes in clinical samples is a prerequisite to precise normalization of relative gene expression using quantitative real-time reverse transcription-PCR. The use of a single so-called "universal" reference gene

5   may lead to misinterpretation of the expression of the GCC gene. We set out to: a) identify a reference gene that is less abundant than ACTB in FPE LNs and confirm a stable expression not affected by the LN GCC status; b) if necessary, develop custom assays with amplicons compatible with FPE samples; and c) select the 5 most stable genes to further develop duplex assays.

10   1.   Evaluation of putative reference genes in matched frozen (FF) and formalin-fixed paraffin-embedded (FFPE) pericolonic lymph nodes.

[0168] In real-time quantitative reverse transcription PCR (RT-qPCR), relative quantification using reference genes is a common approach but the determination of a suitable reference gene should be first assessed in the tissues under investigation. The

15   use of "universal" reference genes may lead to misinterpretation of the expression of the target genes. The aim of this study was to identify 5 stably expressed reference genes suitable for normalization in the GCC Lymph Node Colon Cancer staging test. Fifteen reference genes with different abundance and functional classes (Table 3) were evaluated for stable expression. Fundamentally, a suitable normalization gene candidate has to be

20   stably expressed in the tissues of interest and have expression levels above background. In the GCC assay format of the invention, the suited normalization gene should not be differentially expressed between GCC positive and GCC negative LNs, have expression values (Ct) 4-8 cycles higher than ACTB and have PCR efficiencies between 90-110%.

[0169] Several parameters have been standardized in order to obtain reliable quantitative

25   expression measures for reference genes analysis including initial sample amount, RNA integrity and efficiency of cDNA synthesis. The reverse transcription reactions were performed with the Superscript™ III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's recommendation, using random hexamers (50 ng/μl) and 1 μg total RNA. The real-time PCR were carried out in a 20-μl reaction volume with the

30   Applied Biosystems 7900 HT Fast Real-Time PCR Systems using TaqMan Fast Universal PCR Master Mix and 50 ng cDNA. Primers and FAM-labeled probes concentration for

each PCR assay was 900 nM and 250 nM respectively. All reactions were performed in duplicate, and results were averaged. PCR efficiencies in individual samples were evaluated for each gene using the LinReg software.

[0170] In a first experiment, a subset of endogenous reference genes (ACTB, B2M,
5   GUSB, UBC, POLR2L, PGK1 and TRFC) were evaluated in two matched fresh frozen (FF) and formalin-fixed paraffin-embedded (FFPE) colon cancer LNs. For these 7 endogenous reference genes, the amplicon lengths of the pre-designed TaqMan gene expression assays available from Applied Biosystems were between 69-81 bp (**Table 1**). All genes were amplified with Ct values <35 (Figure 1) and PCR efficiencies were
10   between 90-110% in both FFPE and FF samples. FFPE samples showed less variation than FF tissues with similar PCR efficiencies (**Figure 1**). In a second experiment, we designed custom Taqman assays for GAPDH, HPRT1, PMM1, PPIA, PSMB6, RPLP0, TBP, TPT1 with amplicons compatible with FFPE samples. The amplicon lengths of the custom designed assays were restricted to 75 bp and covered one exon-exon junction
15   (**Table 1**). The evaluation of these custom TaqMan® assays was performed on the same data set. The expression levels of the selected reference genes using these custom assays were between Ct values of 19 to 35. Similarly, we observed less variation between FFPE samples than FF tissues. Seven of the eight custom TaqMan assays met our initial criterion and had PCR efficiencies between 90-110%. Only the PMM1 assay had PCR
20   efficiency below 90% in both FF and FFPE samples. This is most likely due to the low level of expression of PMM1 gene in LN (mean Ct of 34.5).

[0171] Based on geNorm criteria, all of the genes tested had a stable expression between GCC negative and GCC positive LN. The most stable reference genes identified were GAPDH, PGK1, HPRT1, TBP and GUSB. ACTB was ranked 11th. The most stable
25   reference genes had PCR efficiencies between 90-110% and expression levels 2-6 cycles lower than ACTB.

[0172] Our results confirm that there is no single universal reference gene for any tissue type or condition and underline the importance of specific evaluation of potential reference genes for any experimental condition. The stable reference genes identified are putative
30   candidates to replace ACTB in the assay format described by Beaulieu et al. (Diagnostic Molecular Pathology, 2010) (i.e. the GCC/ACTB Scorpions™ duplex assay) to establish a relative quantification approach independent of the GCC IVT-based standard curve.

2.   Confirmation of reference genes suitable for RT-qPCR normalization.

[0173] To identify endogenous reference genes with stable expression in colon cancer LNs, expression of all 15 candidate endogenous reference genes was determined in 32 GCC negative and 31 GCC positive FFPE colon LNs. The RT-qPCR was performed as described above in example 1 and each plate included a control reaction using commercial human universal RNA. The GCC levels in positive LN from stage II-III CRC patients ranged from 200 to over 35 000 GCC copies/well while negative LN had GCC level below the predetermined cut-off (100 GCC copies/well) The average values of the expression levels for each selected reference gene and the standard errors are shown in **Figure 2** for GCC negative and GCC positive FFPE colon LNs. The relative expression levels observed corresponded well with the preliminary results presented in **Figure 1**. An ideal reference gene should maintain constant expression in both GCC negative and GCC positive FFPE LN tested. From all the genes tested using 63 individual specimens, we observed that the average expression in both populations fluctuated by less than 1 cycle **(Figure 2)**. Additionally, we used commercial human universal RNA to monitor plate-to-plate variation. For each endogenous reference gene tested, Ct values in the universal RNA were constant with an intra-plate standard deviation of $Ct < 0.2$ and inter-plate coefficient of variation (CV) < 4%.

[0174] Expression stability was analyzed using the geNorm software. GeNorm uses a pair-wise comparison model to select the gene pair showing the least variation in expression ratio across samples. The software computes a measure of gene stability (M) for each endogenous reference gene. **Figure 3** shows the M values for all tested genes. Our analysis of the expression level of the 15 reference genes revealed that all genes demonstrated M values lower than the geNorm default threshold of 1.5, confirming that this selection of genes from the literature corresponds to adequate stable reference genes. Although all reference genes selected have stable expression, they are not equivalently stable. GAPDH and PGK1 were identified as the most stable gene pair followed by HPRT1, TBP and GUSB **(Figure 3)**. ACTB, on which is based the GCC/ACTB Scorpions™ duplex assay is in eleventh position and PP1A showed the highest variability in expression in the FFPE colon LNs.

[0175] It has been a standard practice in quantitative PCR to use a single reference gene for RNA expression normalization. However, our preliminary study have documented that

reference gene expression can vary considerably, which suggests that the use of multiple reference genes may improve accuracy in relative quantification studies. Therefore, it is important to identify the appropriate combination of reference genes used for the tissue being tested. To determine the optimal number of reference genes required for

5   quantitative PCR normalization, the geNorm software calculates a pairwise variation V for each sequentially increasing number of reference genes added. **Figure 4** shows a graph of the pairwise variation calculated by the geNorm software. The geNorm default V value of 0.15 was used as a cutoff to determine the optimal number of genes. This analysis reveals that the optimal number of reference genes is three (GAPDH, PGK1 and HPRT1)

10   when using RNA extracted from FFPE colon cancer LNs (**Figure 4**).

[0176] The optimal reference genes among those tested for GCC relative quantification analysis using FFPE colon cancer LNs were GAPDH, PGK1, HPRT1, TBP and GUSB. Besides, these five endogenous reference genes are less abundant than ACTB in FPE LNs and are not as affected by the presence of GCC mRNA in the LN. Since these

15   reference genes have expression levels 3-6 cycles lower than ACTB, they are less likely to compete with GCC in the reverse transcription reaction. This suggests that in contrast to ACTB, the primer concentration in the RT reaction should not be limited. Due to its high expression, ACTB is not optimal for relative quantification, although we found its expression to be stable in pericolonic LNs. For that reason, the combination of three

20   genes rather than ACTB alone was considered for the development of a test format using relative quantification as opposed to a standard curve-based quantification.

3.   Development and evaluation of two TaqMan® simplex assays for each selected reference gene.

[0177] For each of the 5 reference genes previously identified (GAPDH, PGK1, HPRT1

25   TBP and GUSB), we developed two designs with amplicon length similar to GCC (65-75 bp) using PrimerExpress (**Table 2**). The assays developed for each reference gene spanned two different exons and their probes and primers were free from single nucleotide polymorphism (SNP) (**Table 2**). Serial dilutions of a commercial human Universal RNA (uRNA) allowed us to perform an initial qualification of the PCR efficiency

30   of the assay designs. The reverse transcription reactions were performed with the Superscript™ III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's recommendation, using gene-specific reverse primers (2 µM) and RNA

input ranged from 1250 ng to 0.125 ng. The real-time PCR were carried out in simplex reactions of 20 µl with the Applied Biosystems 7900HT Fast Real-Time PCR Systems using TaqMan® Fast Universal PCR Master Mix. Primers and FAM or VIC-labeled probes concentration for each PCR assay was 900 nM and 250 nM respectively as

5   recommended. Apart for 1 HPRT1 design, all other assays produced PCR efficiencies between 90-110% **(Table 1)**. Expression levels (Ct values) of GUSB, HPRT1, PGK1 and TBP ranged between 21-23 with 1250 ng and 34-36 with 0.125 ng. Both GAPDH designs had Ct values lower than our primary specification (Ct values between 23-36).

4.   Reference gene expression in pericolonic lymph nodes samples

10   [0178] The 5 selected reference genes were evaluated in FFPE RNA extracted from GCC positive and negative LN in order to select a reference gene with the lowest Ct variation and standard deviation. First, expression levels of the reference genes were determined in a serial dilution of RNA from GCC positive LNs using 10 different TaqMan MGB assays **(Table 2)**. The Ct values for all 10 reference gene assays ranged from 18 to 34 **(Table 5)**.

15   GAPDH assays gave Ct values lower than our specification and both GUSB_Tq2 and HPRT1_Tq2 had PCR efficiencies outside the 90-110% range. Minus RT reactions were also performed in parallel and we consistently obtained Ct values below 35 for TBP_Tq1 and Tq2, GAPDH_Tq1 and HPRT1_Tq2 **(Table 5)**, suggesting a non-specific amplification of genomic DNA or PCR byproducts by these assays. Stable relative expression levels

20   independent of RNA input between GCC and each reference gene were obtained in RNA sequentially diluted. Finally, the smaller amplicons tended to have less Ct variation than the larger ones **(Table 5)**.

Table 5. Selected TaqMan® simplex assays for each reference genes

Specifications	GAPDH		GUSB		HPRT1		PGK1		TBP	
	Tq_2	Tq_4	Tq_1	Tq_2	Tq_1	Tq_2	Tq_2	Tq_5	Tq_1	Tq_2
TaqMan assay design										
Amplicon size between 65-75 bp	74	68	61	66	62	78	76	74	62	77
Covered exon-exon junction	2-3	1-2	11-12	10-11	6-7	2-3	4-5	1-3	4-5	5-6
Serial dilution of universal RNA (250 ng/µl to 0.025 ng/µl)										
Efficiency between 90-110%	100,5%	103,1%	102,9%	107,8%	106,7%	215,4%	102,3%	108,8%	98,1%	102,0%
R² > 0.98 on the serial dilution	0,9995	0,9996	0,9995	0,9998	0,9975	0,9275	0,999	0,9992	0,9987	0,9989
The minus RT have Ct > 35	40	40	40	40	40	40	40	40	40	40
The No Template Control (NTC) should have Ct = 40	40	40	40	40	40	40	40	40	40	40
Ct ranging between 23-36	18-31	17-30	23-36	23-35	21-34	23-31	21-34	23-36	22-36	23-36
Serial dilution of FFPE RNA from GCC positive LN (250 ng/µl to 2.5 ng/µl)										
Efficiency between 90-110%	92,9%	101,8%	94,7%	111,6%	96,3%	137,0%	95,0%	109,8%	93,8%	105,6%
R² > 0.98 on the serial dilution	0,9975	0,9990	0,9995	0,9964	0,9996	0,9879	0,9984	0,9966	0,9995	0,9697
The minus RT should have Ct > 35	32,8	40,0	37,1	36,3	40,0	32,6	40,0	30,7	34,4	32,2
The No Template Control (NTC) Ct should be 40	40	40	40	40	40	40	40	40	40	40
Ct ranging between 23-30	18-25	20-26	23-30	23-29	25-32	25-30	23-30	22-29	24-31	26-33
Ct variation < 0,5 between 250 ng/µl and 2.5 ng/µl	0,40	0,18	0,30	0,24	0,16	1,19	0,28	0,23	0,26	0,27
Status:	Failed	Failed	Passed	Failed	Passed	Failed	Passed	Failed	Failed	Failed

[0179] We used the results obtained in the previous experiments with uRNA and FFPE RNA to select one design per reference gene. We also concluded that the relative abundance of GAPDH was too high and decided not to characterize further the 2 GAPDH Taqman assay designs.

[0180] Expression levels of the four remaining reference gene assays was then confirmed in RNA extracted from FFPE LNs of colon cancer patients and used to measure relative expression of GCC mRNA. RNA from 3 GCC positive (Cybrdi) and 3 GCC negative (ABS, McGill and Tristar) LNs were tested. The nucleic acid remains from 3 GCC negative lymph nodes were selected from a previous experiment in which ACTB expression could be detected in minus RT controls. Minus RT reactions were also performed in parallel. The Ct values measured for each reference gene were between 22 and 26 cycles compared to 20 for ACTB using the Scorpions™ duplex assay (**Figure 5**). The GCC Ct values obtained with TaqMan® simplex assay were comparable to those obtained with the GCC/ACTB Scorpions™ duplex reaction. No GCC, HPRT1 or PGK1 amplification was detected in the minus RT conditions tested (**Figure 1**). However, TBP and GUSB had measurable Ct values in all minus RT controls.

5.   Quantitative RT-PCR in partially hydrolyzed RNA

[0181] Two degradation models were developed to simulate RNA fragmentation in FFPE samples. GUSB, HPRT1, PGK1 and TBP were evaluated to identify the reference gene having a loss of signal similar to GCC in degraded samples. To develop the proposed models, degradation experiments were optimized to obtain GCC levels that cover the dynamic range of the GCC/ACTB Scorpions™ duplex assay, i.e. with at least 5 degradation points with measurable GCC levels.

a. Sodium hydroxide degradation

[0182] An aliquot of twenty micrograms of TRIzol™ -extracted RNA from a fresh frozen colon tissue sample was treated with 0.1N NaOH at 60°C. At various time points (30 min, 1 h, 2h 3h, 4h and 5h) an equal volume of ice-cold 0.1M HCl was added to the sample to neutralize NaOH and stop RNA degradation. The resulting degraded RNA was characterized on the Agilent 2100 Bioanalyzer. Controlled nucleic acid degradation at elevated pH generated RNA fragments from 50 to 300 nucleotides depending on the time of hydrolysis. Substantial RNA degradation with more than 50% of RNA fragments below

150 nucleotides was observed after 3 h of treatment (**Figure 6A**). RNA from each time point was amplified using the GCC TaqMan® assay and the four selected reference gene designs (GUSB, HPRT1, PGK1 and TBP). For comparison, the GCC Scorpions™ duplex with ACTB was also tested. Because we designed GCC and RG assays with short

5   amplicons, measurable expression could be detected even after 5 hours of treatment. As expected, a constant increase of GCC values was observed throughout treatment while only a slight increase can be observed for ACTB Ct values (**Figure 6B**). The increase of the 4 other reference gene Ct values was very similar to GCC mRNA in this model (**Figure 6B**). The resulting GCC relative expression levels (Ct of GCC minus Ct of the

10 reference gene candidate) calculated with GUSB, HPRT1 and TBP mostly compensated for fragmentation-induced increase of GCC Ct values with less than a 2-fold delta-Ct variation between intact and highly degraded RNA specimens (**Figure 7**).

b. Carbonate degradation

[0183] In this second degradation model, RNA from a TRIzol™ -extracted fresh frozen

15 colon tissue sample was dissolved in an equal volume of NaHCO3/Na$_2$CO$_3$ buffer (pH10) and incubated at 60°C for various time points (30 min, 1h, 2h, 3h and 4 h). To stop the hydrolysis, 1/10 volume of 3M CH$_3$COONa pH 5.2 was added on ice followed by precipitation with 3 volumes of 99% ethanol. Small fragmented RNAs were recovered in 50 μl and purified on mini Quick Spin RNA Columns (Roche Applied Science, IN). Prior to

20 Agilent 2100 Bioanalyzer and RT-PCR analyses, RNA was DNase-treated using the TURBO DNA free kit (Ambion).

[0184] Intact RNA was partially hydrolyzed by incubation at elevated pH and temperature (**Figure 8A**). After 30 minutes, electropherograms of RNA samples show fragments of about 500 nucleotides and after 120 minutes an average size of less than 200 nucleotides

25 was observed (**Figure 8B**). RNA samples were then subjected to RT-qPCR and Ct values for GCC and five reference genes are shown for a representative experiment (**Figure 8C**). In this model, GCC Ct values increased by more than 6 cycles after 4h of treatment. Most of the reference genes tested, including ACTB, behave similarly to GCC in this model (**Figure 8C**). However, when we compared delta-Ct value variations (delta-delta-Ct)

30 throughout the treatment, only GUSB, HPRT1 and TBP gave stable expression levels with less than 2-fold variation between intact and carbonate-degraded RNA samples (**Figure 9**). This result strongly suggests that the increase of GCC Ct values in controlled RNA

degradation can be compensated by the selection of a reference gene with similar behaviors allowing robust relative quantification.

c. Comparison of fixation methods from OCT-compound (Optimal Cutting Temperature) embedded tissues

5   [0185] It is known that pre-fixation parameters are critical for RNA quality and that once the tissue is fixed, dehydrated and embedded in paraffin, further degradation is limited. In order to select reference genes that are minimally affected by pre-fixation variables, one OCT-embedded colon tumor tissue was sectioned in 40 slices of 20 μm. Eight non-continuous sections were pooled and either fixed or extracted with TRIzol™ reagent.

10   Effects of tissue fixative on RNA quality were measured in cryo-sections that were fixed for 16 hrs with either neutral buffered formalin, non-buffered formalin or Bouin's solution by using the Agilent 2100 Bioanalyzer (**Figure 10**). As expected, each condition generated a unique RNA profile. Both RNA extracted with TRIzol™ reagent showed the typical 18S and 28S fragments. RNA extracted from sections fixed with neutral buffered

15   formalin appeared to be smaller than 400 bp, while non-buffered formalin seemed to have retained genomic DNA along with RNA molecules with overall less fragmentation. Bouin's solution was the most destructive fixative with a majority of RNA molecules smaller than 150 bp.

[0186] We then measured GCC, ACTB and reference gene mRNA levels from intact and

20   fixation-degraded RNA using RT-qPCR assays. **Figure 6** shows Ct values for GCC and 4 reference genes detected with TaqMan® simplex assay compared to the GCC/ACTB Scorpions™ duplex assay. As there is no initial biological variation in RNA before freezing or fixing samples, the GCC Ct increase was specifically due to RNA quality. As expected from the Bioanalyzer results, Bouin's solution produced the largest Ct increase

25   for all genes tested. Because the selected reference gene behave similarly to GCC, delta-Ct was rather stable with less than 0.5 delta-Ct variation between fresh frozen and neutral buffered formalin fixed samples when normalized with GUSB, HPRT1 and TBP (**Figure 11**). This suggests that variations in Ct values caused by pre-analytical stress factors can be compensated with a suitable normalization procedure.

30   [0187] Our results confirm that relative quantification of GCC using newly characterized reference genes can be rendered less sensitive to tissue fixation and RNA degradation. Based on RNA degradation models that mimic FPE degradation, we were able to select

three reference genes (GUSB, HPRT1 and TBP) that showed less than a 2-fold variation between RNA from fresh frozen samples and highly degraded RNA from the same sample. Effect of tissue fixatives on reference gene expression was also measured in cryo-sections that were fixed in buffered formalin, non-buffered formalin and Bouin. RNA

5   extracted from FPE samples presented less than 1 delta-Ct variation compared to intact RNA isolated from fresh frozen sections of the same tissue. A duplex assay simultaneously amplifying GCC and either GUSB, HPRT1 or TBP would allow robust relative quantification (delta-Ct) in clinical specimens previously processed under variable pre-analytical conditions and independent of RNA input.

10   6.   Evaluation of the GCC TaqMan® duplex assay with selected reference genes

[0188] Based on tissue fixation and RNA degradation models, we identified 3 reference genes (GUSB HPRT1 and TBP) that behave similarly to GCC in highly degraded and non-degraded RNA samples. Duplex designs of GCC with each of these reference genes were tested with the TaqMan assay using human uRNA and FFPE colon material (Figure 12).

15   The reverse transcription reactions were performed with the Superscript™ III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's recommendation, using both GCC and reference gene reverse primer at 2 µM and 1.25 µg of total RNA. Three concentrations (900 nM, 600 nM and 300 nM) of primers and one concentration of TaqMan® MGB probes (200 nM) were tested by real-time PCR. The GCC probe was

20   labelled with FAM while VIC was used to detect the reference gene. After initial duplex testing, we obtained GCC duplex signal in FFPE colon samples with less than 0.5 Ct variation compared to the simplex reaction for all tested genes (GCC, GUSB, HPRT1 and TBP) (Figure 12A). Only GUSB and HPRT1 had no minus RT amplification signal (Ct=40) in duplex reactions for any of the conditions tested. Conversely, amplification of TBP at

25   Cts below 35 cycles was observed in both simplex and duplex conditions (Figure 12B).

7. Comparison of HPRT1 and GUSB and selection of the best reference gene

a.   Development of two TaqMan® duplex assays for GCC LN relative quantification.

[0189] In a first set of experiments, the concentration of primers in the qPCR reaction was adjusted in the duplex assay to minimize the competition between GCC primers and

30   reference gene primers. Primer titration was performed in human universal RNA (uRNA) spiked or not with 1×10^6 in vitro transcribed (IVT) GCC molecules. Two uRNA inputs (250

ng/μl and 25 ng/μl) were used in order to obtain data with respectively high and low reference gene expression. The reverse transcription reactions were performed in duplex reactions using the Superscript™ III First-Strand Synthesis SuperMix (Invitrogen) with both GCC and reference gene specific reverse primers at 2 μM. This approach, combined with spiking experiments, allowed us to evaluate the mutual impact of GCC and reference gene expression on each other in conditions that closely related to testing GCC-positive and GCC-negative LNs. The concentration of primers tested for each gene varied from 150 to 900 nM while TaqMan® MGB probes were fixed to 200 nM.

[0190] Analysis of qPCR primers titration allowed us to determine the most favorable conditions for duplex amplification. The selected conditions were the same for both GUSB and HPRT1 duplex assays: GCC forward and reverse primers at 900 nM and reference gene forward and reverse primer at 300 nM with both TaqMan® MGB probes at 200 nM (**Figures 13-14**). Using these newly established conditions, RT primers were adjusted in a duplex assay to provide a saturated RT reaction for each gene. RT primers titration was performed in a duplex reaction using fresh frozen colon RNA treated with NaOH. One goal was also to reduce the ΔCt variation due to RNA degradation. For that reason, we tested increasing RT primer concentrations from 2 μM up to 20 μM for each gene reverse primer. At their best, the performance of GCC and reference gene in duplex should be similar to simplex (variation<1Ct). Among the conditions tested, the best performance was achieved with GCC reverse primer at 20 μM and reference gene reverse primer at 2 μM (**Figure 14**).

b.   Monitoring the amplification in minus RT reactions

[0191] To compare TaqMan® and Scorpions™ duplex assays in minus RT conditions, 8 samples previously found to yield a detectable ACTB signal in minus RT were tested with the new assays. Importantly, no amplification was detected in minus RT neither for GCC nor for the selected reference gene while ACTB amplification continued to be observed in the same samples tested with the reference Scorpions™ assay (**Figure 15**). Moreover, in two GCC-positive samples tested, the GCC Ct values obtained with the new assays had less than 1 Ct difference compared to the GCC CT values of the Scorpions™ assay, confirming that the selected conditions were matching the performance of the GCC/ACTB Scorpions™ assay. Together, these results suggest that both TaqMan® duplex assays are specific to their target gene.

c.   *Effect of tissue fixatives on GCC relative quantification*

[0192] It is known that pre-fixation parameters are critical for RNA quality and that once a tissue is fixed and paraffin-embedded, the RNA degradation process is reduced. Experiments were performed to assess the impact of the fixative type on GCC mRNA

5   results. Effect of tissue fixatives on reference gene expression was determined in cryo-sections that were fixed in buffered formalin, non-buffered formalin or Bouin's fixative. The RNA extracted from FPE samples was compared to intact RNA isolated from fresh frozen sections of the same tissue using both GCC/GUSB and GCC/HPRT1 duplex assays. The new duplex assay generated a stable delta-Ct quantification between fresh frozen and

10   fixed tissues compared to the GCC/ACTB Scorpions™ duplex (**Figure 16**). With less than 1 delta-Ct variations between all tissues fixatives, the GCC/GUSB duplex combination was the most effective one to reduce variations due to the tissue fixative.

d. *Effect of Archival Time on Quantitation of Fragmented RNA*

[0193] To compare TaqMan® and Scorpions™ assays in fixed and paraffin-embedded

15   (FPE) blocks, we selected 55 FPE pericolonic lymph node tissues with different archiving times ranging from 1 month to 22 years. Past 1 year of archival, samples had similar RNA fragmentation profiles (**Figure 17**) and the degradation process seemed to slow down considerably. Therefore, we observed less variation in GUSB Ct value from samples stored for more than one year old. We also observed that Ct values for GUSB were higher

20   than ACTB irrespective of the age of the specimen. Interestingly, the ACTB Ct values did not increase significantly between the different groups of block (**Figure 18A**). Because GCC was more affected than ACTB by these conditions, we were unable to use ACTB as a reference gene to monitor RNA degradation since the assay was insensitive to small variations. In contrast, we observed a direct relationship between GUSB Ct values and

25   archival time (**Figure 18B**). As a result, the GUSB Ct values in specimens older than 10 years was significantly higher than in those with less than 6 months (26.4 [95%CI: 26.0-26.8] vs 24.5 [95%CI: 23.8-25.3]). Therefore, using a reference gene which behaves like the target amplicon in archival material, it is possible to test decades old specimens and still obtain informative results.

8.   GUSB is a superior reference gene for the GCC assay

a. Lower Limit of Detection (LOD)

[0194] To determine the limit of detection (LOD), each gene-specific primers/probes set was tested for sensitivity using a serial dilution. The duplex reaction was performed in commercial LN RNA (250 ng/µl) spiked with 1×10^5 GCC IVTs and serially diluted until a theoretical GCC copy number of less than 1 should be reached. These experiments were performed using 5 replicates per dilution point. Quantification of GCC copies was obtained by interpolation of the Ct value using a GCC standard curve. The performance characteristics of the calibration curves obtained for these assays are presented in **Table 6**. Each log dilution of GCC IVT was detected by additional three cycles of qPCR which corresponds to an efficiency of 102% for GCC/GUSB duplex and 97% for GCC/HPRT1 duplex. Usually, an efficiency number greater than 100% indicates a saturation of the RT, which was precisely the condition selected above.

Table 6. Comparison of lower limits of detection and other parameters between GCC/GUSB TaqMan® , GCC/HPRT1 TaqMan® and GCC/ACTB Scorpions™ assays

	TaqMan®		Scorpions™
	GCC/GUSB (20;2)	GCC/HPRT1 (20;2)	GCC/ACTB (20; 0.02)
Limit of detection (LOD) [4/5 replicates different from 0 (Ct = 40)]			
reference gene			
LN RNA Spiked with 1x10⁵ GCC IVT	0,01 ng/µl	0,05 ng/µl	0.0025 ng/µl
reference gene Ct	36,4	35,1	36,1
GCC			
RNA input	0,025 ng/µl	0,025 ng/µl	0,01 ng/µl
GCC Ct	32,9	35,2	37,45
GCC copies	9,3 ± 2,3	5.9 ± 4.1	1.9 ± 1.1
Limit of Quantification			
LN RNA Spiked with 1x10⁵ GCC IVT	250 - 0,025 ng/µl	250 - 0,25 ng/µl	250 - 0,25 ng/µl
Dymanic range	**4 log**	**3 log**	**3 log**
Efficiency	102%	97%	90,3%
R2	0,9997	0,9992	0,9975
Data at LOQ	0.025 ng/µl	0.25 ng/µl	0. 25 ng/µl
reference gene Ct	33,3	31,7	29,0
CV (%)	1,47	0,69	0,32

	TaqMan®		Scorpions™
	GCC/GUSB (20;2)	GCC/HPRT1 (20;2)	GCC/ACTB (20; 0.02)
GCC Ct	32,9	30,5	31,5
CV (%)	1,09	0,61	0,61
GCC copies	9,3 ± 2,3	82.0 ± 11.1	95.0 ± 13.2
Max ΔCt	0,13 (250 ng/µl)	-0,75 (250 ng/µl)	3,15
Min ΔCt	-0,63 (0.05 ng/µl)	-1,18 (0.25 ng/µl)	2,44
max-min	0,76	0,43	0,71

[0195] The LOD was defined as the lowest dilution point giving a signal different from the background on at least 4 out of 5 replicates, whereas limit of quantitation (LOQ) was defined as the lowest concentration at which fluorescence could be detected consistently in all specimens. A summary is presented in **Table 6**. The LOD was used to determine a cut-off Ct value for adequate RNA samples. The Ct values of GUSB and HPRT1 at LOD were respectively 36.4 and 35.1. Accordingly, any sample with a reference gene Ct value higher than 35 was considered non-informative. Using the GCC/GUSB duplex assay, the Ct value of GUSB at LOQ (0.025 ng/µl RNA input) was 33.3 whereas the Ct value of GCC was 32.9 corresponding to 9 GCC copies. Both GCC and GUSB Ct values had CV < 1.5 % at LOQ. The LOQ determined with the GCC/HPRT1 was higher at 0.25 ng/µl of RNA input and a GCC Ct value of 30.5 was reached, which corresponds to 82 GCC copies. The relative expression of GCC calculated with either GUSB or HPRT1 was found to be stable with less than 1 delta-Ct within LOQ range. Unexpectedly, when compared to the GCC/ACTB Scorpions™ assay, the GCC/GUSB duplex gave a better analytical performance **as it could bring down the LOQ to approximately 10 GCC copies per reaction** (vs. 95 copies for the GCC/ACTB duplex assay).

9.   Analytical performance of the selected GCC/GUSB duplex assay

[0196] Additional experiments were performed to confirm the analytical performance of the GCC/GUSB duplex assay. Determination of its lower limit of quantification and stable expression in RNA degradation and fixation models can provide robust Relative Quantification (RQ) measurements in FPE LN from patients with colorectal cancer. The main objective of this study was to reproduce the analytical sensitivity and specificity

obtained with the reference GCC/ACTB Scorpions™ assay. Nucleic acids were extracted from 172 FFPE colon LNs collected from patients with stage I (12), stage II (65) and stage III (95) colon cancer disease. Of those samples, 14 were found inadequate with the GCC/ACTB reference assay (ACTB Ct > 29) and 37 had GCC levels below reportable range (< 100 copies/reaction) while 17 histology-positive LN were negative for GCC. GCC and GUSB mRNA expression (Ct values) was first evaluated in FFPE LNs from 35 stage I-II histology- and GCC-negative samples (pN0(mol-) and 38 stage III histology- and GCC-positive samples (pN1-2(mol+) to determine an analytical cut-off for the new duplex assay. A receiver operating characteristic (ROC) analysis was used to determine the optimal cut-off (delta-Ct of -5.9). **Figure 19** shows a Box-and-Whisker plot of the signals expressed as GCC delta-Ct ($Ct_{GUSB} - Ct_{GCC}$) using -5.9 delta-Ct value as the selected cut-off. The estimated detection rate of that assay for the 38 GCC-ACTB-positive samples (sensitivity) was 97% (37/38; 95%CI: 86.1-99.6) while that for the 35 GCC/ACTB-negative samples was 14% (5/35; 95% CI: 6.6-33.7).

[0197] Relative GCC mRNA expression was next evaluated in the remaining samples. The informative rate of samples tested with the GCC/GUSB assay increased by 2%, as overall, only 6% of LNs had inadequate GUSB amplification versus 8% for the GCC/ACTB assay (**Table 7**). Comparison of GCC/GUSB and GCC/ACTB assays revealed that qPCR relative quantification with GUSB achieved concordant results in all the 49 true-positive (pN1-2(mol+)) samples tested. The relative quantification of GCC mRNA to GUSB mRNA increased correct identification of Stage III LN to 66% compared to 52% using absolute quantification with the Scorpions™ assay (**Table 7 and Figure 20**). When testing LNs from Stage I and patients, thus showing no LN metastases by HP, 39 % of the nodes were GCC mRNA-positive with the GCC/GUSB assay compared to 19 % using the Scorpions™ assay. One way to achieve a comparable performance with the GCC/ACTB Scorpions™ assay is to lower its reportable range to 25 copies per reaction ($\Delta Ct < -2$). Even when compared to a GCC/ACTB assay using this lower cutoff, the TaqMan® GCC/GUSB assay still detected 6 stage I and II patients that were negative with the GCC/ACTB assay, although the difference was not statistically significant. Also, lowering the cut-off value of the GCC/ACTB assay had no effect on its informative rate as the GCC Ct value is independent of the ACTB Ct value used for acceptance of the result.

Table 7. Performance of GCC/ACTB and GCC/GUSB duplex assays in selected LNs from colon cancer patients.

Scorpions™ duplex assay

Stage	Nb LNs	HP Neg	%	HP Pos	%
I	12	12	100%	0	0%
II	65	65	100%	0	0%
III	95	37	39%	58	61%
Total	172	114	66%	58	34%

Cut-off ΔCt (Std 100 -GCC) < 0

	Scorpions™ duplex GCC/ACTB					
	Inad.	%	Neg	%	Pos	%
I	1	8%	10	83%	1	9%
II	6	9%	45	69%	14	22%
III	7	7%	39	41%	49	52%
Total	14	8%	94	55%	64	37%

Cut-off ΔCt (Std 100 -GCC) < -2

Stage	Nb LNs	Scorpions™ duplex GCC/ACTB					
		Inad.	%	Neg	%	Pos	%
I	12	1	8%	9	75%	2	17%
II	65	6	9%	37	57%	22	34%
III	95	7	7%	25	26%	63	66%
Total	172	14	8%	71	41%	87	51%

TaqMan duplex assay

Cut-off ΔCt (GUSB -GCC) < -5,8516

	TaqMan® duplex GCC/GUSB					
	Inad.	%	Neg	%	Pos	%
I	1	8%	8	67%	3	25%
II	5	8%	33	51%	27	42%
III	5	5%	27	28%	63	66%
Total	11	6%	68	40%	93	54%

[0198] The GCC/GUSB duplex assay was next tested with nucleic acid extracts from 43 rectal cancer LNs including 24 LNs harvested from patients treated with neo-adjuvant radio and/or chemotherapy. Once again, the informative rate in this selected population was higher when samples were tested with the GCC/GUSB duplex assay (**Table 8**; 81% (8/43) vs 70% (13/43)). Only 8 RNA extracts were considered inadequate with the GCC/GUSB assay compared to 13 with the GCC/ACTB Scorpions™ assay, corresponding to a 38% reduction. Moreover, the number of LNs found positive for both histopathology and GCC (with the GCC/GUSB assay) was consistently higher (6 to 12%) and was not affected by the fact that some patients had received neo-adjuvant therapy.

Table 8. *Performance of Scorpions™ and TaqMan® duplex assays with LNs from rectal cancer patients treated or not with neo-adjuvant therapy.*

	Nb LNs	Scorpions™ duplex assay Cut-off ΔCt (Std 100 -GCC) < 0						TaqMan® duplex assay Cut-off ΔCt (GUSB -GCC) < -5,8516					
		Scorpions™ duplex GCC/ACTB						TaqMan® duplex GCC/GUSB					
		Inad.	%	Neg	%	Pos	%	Inad.	%	Neg	%	Pos	%
Non-treated													
Total	**19**	**10**	**53%**	**3**	**33%**	**6**	**67%**	**6**	**32%**	**2**	**15%**	**11**	**85%**
HP-	3	1	33%	2	100%	0	0%	2	67%	1	100%	0	0%
HP+	16	9	56%	1	14%	6	86%	4	25%	1	8%	11	92%
Neo-Adjuvant													
Total	**24**	**3**	**13%**	**16**	**76%**	**5**	**24%**	**2**	**8%**	**15**	**68%**	**7**	**32%**
HP-	16	3	19%	13	100%	0	0%	2	13%	13	93%	1	7%
HP+	8	0	0%	3	38%	5	63%	0	0%	2	25%	6	75%

Study Conclusions

[0199] We successfully identified GUSB as a reference gene not affected by the presence of GCC expressing cells in LN and with an analytical behavior similar to GCC irrespective of the pre-analytical conditions affecting RNA integrity. RT and PCR reactions were optimized to obtain an efficient duplex assay simultaneously amplifying GCC and GUSB mRNAs. Selected specimens previously processed under various pre-fixation conditions were tested with this new assay and the results demonstrated that robust relative quantification (delta-Ct) could be achieved. The sensitivity and the linear dynamic range of the GCC/GUSB assay were improved when compared to the GCC/ACTB assay using

delta-Ct of 0 as the cut-off value. Our preliminary results strongly suggest that the GCC/GUSB assay, combined with an optimized nucleic acid extraction process, increases the informative rate.

10.   Detection of circulating GCC positive cells in blood

5   [0200] Circulating tumor cells (CTCs) are known to exist at ultra-low concentrations in peripheral blood of patients with carcinomas. Effective detection of those cells is most useful for monitoring response to therapy and detecting early relapse. Clinical outcomes in patients with colon cancer could be substantially improved using such a test.

[0201] Using nested RT-PCR, Carrithers *et al.* (Proc. Natl. Acad. Sci. U.S.A. 1996;
10   93(25): 14827-32) and Fava *et al.* (J. Clin. Oncol., 2001; 19 (19): 3951-59). demonstrated that GCC may be useful for detecting circulating colorectal cancer cells in blood from Dukes D (Stage IV) patients. On the other hand, Fava *et al.* also demonstrated that CD34+ cells were a source of ectopically expressed epithelial cell-specific markers potentially contributing to the high false-positive rate generally observed when markers
15   such as GCC are used to detect rare CTCs by RT-PCR. The low level of ectopic transcription of GCC by CD34+ progenitor cells in healthy donors was reduced to undetectable levels by using a limiting quantity of mononuclear cell total RNA ($\leq 0.8\mu g$) in the nested RT-PCR.

[0202] The PAXgene Blood RNA System (Qiagen, cat# 762164) consists in (1) a blood
20   collection tube, intended for blood collection, storage and RNA stabilization, and (2) a nucleic acid purification kit for extraction and purification of intracellular RNA from whole blood for subsequent testing with RT-PCR.

[0203]   PAXgene blood System allows extraction of good quality RNA ($2.7 \pm 1.1\mu g$ of RNA/mL of blood) as demonstrated by the RNA integrity Number (RIN) of 8.9 and high
25   260/280 ratios observed in frozen specimens. No significant genomic DNA (gDNA) contamination was observed. Furthermore, no significant variation in the yield (overall p=0.194) and the RT-qPCR results (p=0.300 for the GCC copies/mL of blood) was observed neither between fresh and frozen blood specimens nor between 2 specimens of blood collected at different days from the same donor (p=0.066).

[0204] GUSB and GAPDH were found to be the most stable reference or reference genes (RG) in blood among the 7 genes tested (HPRT1, ACTB, B2M, PGK1, RPLPO, TBP, GAPDH and GUSB) (**Figure 21**).

[0205] The geNorm pairwise variation V was used to calculate the optimal number of reference genes required for the quantitative PCR normalization (**Figure 22**), Using the geNorm default V value cutoff of 0.15, the analysis reveals that the optimal number of reference genes is 2 (GUSB and GAPDH) when using RNA extracted from blood samples.

Clinical blood specimens

[0206] A background GCC level ranging from 34 to 36 Cts was observed with all 14 healthy donors. GCC Ct values observed with colon cancer patients also felt within that range showing that the difference of mRNA GCC copies between healthy donors and colon cancer patients is very small. Those differences are better visualized when the GCC Ct titer is converted into GCC units/mL of blood (GCC copies per per µg/mL of blood). All specimens were found adequate using GUSB simplex assay.

[0207] Using a GCC arbitrary cut-off >75 units/mL, the % GCC positivity (sensitivity) observed with colon cancer patients was:

- 78% (7/9) for stage IV;
- 80% (4/5) for stage III;
- 33% (3/9) for stage II;
- 0% (0/2) for stage I.

[0208] Using that same cut-off, the specificity observed with healthy donors was 93% (13/14). As shown in **Figure 23**, a significant difference was obtained between healthy donors and stage IV colon cancer patients (p=0.001 using the GCC units/mL of blood).

[0209] Circulating tumor cells (CTCs) being at very low concentrations in peripheral blood, the difference in the GCC level of expression between healthy donors and metastatic colon cancer patients is very low. Nevertheless, our results confirm the potential of the GCC RT-qPCR assay to detect metastatic circulating cancer cells in colon cancer patients using the PAXgene Blood System (Qiagen) for RNA stabilization and purification.

11.   *GCC is expressed in other tissues of the GI tract*

[0210] To ascertain the specificity of the GCC mRNA marker to the GI tract, we tested 91 FFPE normal and tumor-matched specimens from 18 organs. FFPE sections of these specimens were subjected to the GCC/ACTB duplex assay. Among the non-tumor tissues tested, 1 of 2 gastric tissues and all the large intestine (8), rectal (2) and small intestine (1) tissues tested were positive (**Table 9**). All other non-tumor containing organs tested were GCC mRNA-negative, including liver (1), esophagus (2), lung (4), pancreas (1), adrenal gland (2), thyroid (2), brain (1), breast (1), spleen (1), skeletal muscle (1), skin (2), prostate (2), ovary (2), bladder (2), and kidney (2). For each of the organs listed above, matching tumor tissues were also tested. As expected, strong GCC mRNA signals were detected in all of the colon and rectal tumor tissues as well as from metastatic liver specimens tested, which were clearly identified as originating from a primary colon cancer (**Table 4**). Three of 4 pancreatic cancer tissues tested gave significant GCC signals. One type of lung cancer (squamous cell carcinoma) and 2 gastric cancers tissues tested also produced low to moderate GCC mRNA signals. The biological implications of these observations are indicative that the detection of GCC in organs other colon and rectum may be indicative of a diagnosis of cancer, particularly in organs such as pancreatic or gastric cancer. Particularly, the presence of GCC mRNA in pancreatic and gastric cancer cells has been previously reported (Birbe R *et al.*: Hum Pathol 2005, 36: 170-179; and Kloeters O, *et al.* Scand J Gastroenterol 2008, 43: 447-455).

Table 9. *GCC mRNA expression in FFPE specimens from different human tissues and matched tumors tested with the RT-qPCR GCC mRNA assay.*

Tissue Source	Pathology Diagnosis	GCC copies ± SEM*	Tissue Source	Pathology Diagnosis	GCC copies ± SEM*
Esophagus	Normal	15 ± 8 5 ± 2	Adrenal Gland**	Normal	22 ± 22 26 ± 4
	Tumor	37 ± 9 57 ± 18		Tumor	3 ± 3 4 ± 4
Stomach	Normal	17 ± 3 2156 ± 589	Thyroid Gland	Normal	1 ± 1 13 ± 13
	Tumor	38295 ± 775 109106 ± 20920		Tumor	3 ± 3 13 ± 13
Small Intestine	Normal	11971 ± 906	Lung **	Normal	0 ± 0
	Tumor	30990 ± 3114			2 ± 2

Tissue Source	Pathology Diagnosis	GCC copies ± SEM*	Tissue Source	Pathology Diagnosis	GCC copies ± SEM*
Large Intestine, Caecum	Normal	71529 ± 3468 99670 ± 11795			0 ± 0 14 ± 2
	Tumor	54807 ± 5850 82154 ± 1260			0 ± 0 0 ± 0
Large Intestine, Colon	Normal	37977 ± 3755 14367 ± 1339 4463 ± 113 1313 ± 67		Tumor	19 ± 1 41 ± 4 45 ± 6 0 ± 0
	Tumor	88394 ± 21137 214281 ± 8863 72472 ± 689 7632 ± 1210			138 ± 28
			Pancreas Gland	Normal	9 ± 1
Large Intestine, Sigmoid colon	Normal	36992 ± 2313 26207 ± 135		Tumor	18 ± 3 282182 ± 12560 953 ± 247 144 ± 19
	Tumor	38743 ± 253 75420 ± 3247	Prostate Gland	Normal	15 ± 7 19 ± 4
Large Intestine, Rectum	Normal	56748 ± 5921 81827 ± 3212		Tumor	0 ± 0 17 ± 1
	Tumor	169925 ± 4703 111293 ± 3372	Skeletal Muscle	Normal	26 ± 1
				Tumor	82 ± 11
Liver **	Cirrhosis	0 ± 0	Skin	Normal	7 ± 1 23 ± 2
	Tumor	0 ± 0 84 ± 12 53 ± 14		Tumor	57 ± 15 80 ± 36
	Metastatic Neoplasm	14853 ± 869 32769 ± 1461 8826 ± 29	Ovary	Normal	0 ± 0 22 ± 0
Spleen **	Normal	0 ± 0		Tumor	20 ± 10 41 ± 6
	Tumor	0 ± 0 0 ± 0	Bladder	Normal	0 ± 0 21 ± 5
Brain	Normal	1 ± 1		Tumor	0 ± 0 75 ± 16
	Tumor	0 ± 0	Kidney	Normal	0 ± 0 10 ± 10
	Ependymoma	0 ± 0		Tumor	21 ± 6 51 ± 5
Breast	Normal	2 ± 0			
	Tumor	2 ± 2			

*Indicates standard error of the mean (SEM) of 2 independent triplicate analyses.
**Indicates specimens that were not paired.

12. Assessment of the prognostic potential of GCC when measured in combination with GUSB

[0211] Two independent cohorts of patients diagnosed with node-negative (pN0) colon cancer were tested for GCC mRNA expression analysis using a RT-qPCR method. The first set of FFPE (formalin-fixed paraffin-embedded) LN tissues was collected from 98 patients with Stage IIA (T3/N0/M0) colon cancer, all of them having undergone curative surgical resection between 1991 and 1998. FFPE LN tissues from these patients were obtained from the archives of the Pathology Department of the University of Massachusetts Medical Hospital (UMass, Worcester, MA, USA). A second cohort of 25 patients diagnosed with Stage I and II colon cancer between 1999 and 2005 was obtained from the archives of the Pathology Department of the Hôtel-Dieu de Québec Hospital (HDQ, Québec, Qc, Canada). Following surgical resection of the tumor, none of the 123 patients from these two cohorts received adjuvant therapy. Of them, a selection of 73 cases (1283 LNs) was constituted in order to include only cases with at least (1) distal or proximal recurrence or 36 months of follow-up for the non-recurrent cases and (2) a minimum of 10 LNs tested by qRT-qPCR.

[0212] The method used to measure the GCC mRNA expression levels is RT-qPCR. The first step, which includes the gene-specific duplex cDNA synthesis with GCC reverse primer and either human ACTB or human beta-glucuronidase (GUSB) reverse primers, was performed using nucleic acid extract and the SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA) in 20 µL reaction volumes as recommended by the manufacturer. The cDNA product (including those from samples, external standards and controls) were next used in triplicate to conduct duplex real-time PCR and establish a cycle threshold (Ct) value for GCC, ACTB and GUSB. GCC Ct values could then be converted into GCC copies by interpolation using a standard curve.

[0213] The RT-qPCR plate setup was designed to include at least but not exclusively: control materials made with three different concentration of GCC *in vitro* transcripts (IVTs) added to human lymph node total RNA, a calibration curve build from a serial dilution of purified GCC IVTs diluted in yeast RNA and a no template control. Control materials used in each plate served to validate each run and to monitor variability.

13. Detection rate and correlation with outcome

[0214] The relationship between GGC mRNA detection rates and the likelihood of developing disease recurrence for a patient with a GCC-positive lymph node involvement was compared between the GCC/GUSB and the GCC/ACTB tests. To allow this comparison, all the GCC Ct measurements obtained by RT-qPCR were converted into GCC copies by interpolation on the standard curves. A receiver operating characteristic (ROC) curve analysis was performed to establish sensitivity, specificity and cut-off values used to determine a GCC positive test result (**Figure 25**), sensitivity being defined in this example, but not restricted to, as the detection rate of recurrent cases after 36 months of follow-up by the test. As shown in **Figure 25**, the GCC/GUSB assay increased the area under the curve (AUC) index by 11% compared to the reference GCC/ACTB test (0.692 vs. 0.623), which correlates with a better sensitivity for a given cut-off. For example, applying a cut-off of 25 GCC copies, the sensitivity with the GCC/GUSB assay would be 82% (95% CI: 57%-96%) compared to 65% (95%CI: 38%-86%) for the GCC/ACTB reference test.

[0215] **Table 10** shows the association between detection rates at pre-selected cut-offs and the proportion of patients at risk of developing recurrent disease. Subsequent analyses were done with two different cut-off values: (1) 100 GCC copies and (2) 25 GCC copies. Using a cut-off value of 100 GCC copies, 32 patients (44%) were GCC-positive with GCC/GUSB compared to 12 (16 %) for the GCC/ACTB test. A similar difference was observed with the cut-off value of 25 GCC copies: 43 cases (59%) were GCC positive with the GCC/GUSB assay as opposed to 27 (37%) with the GCC/ACTB assay (**Table 10**). These results show a clear increase of GCC-detection rate with the GCC/GUSB test. Moreover, the superior sensitivity of the GCC/GUSB test allows identifying GCC mRNA level in LNs at a threshold that is still predictive of disease recurrence as illustrated in **Figure 26**. A strong correlation ($r^2 > 0.95$) between the risk of recurrence for a patient with a GCC-positive test and the GCC mRNA levels used to determine what a GCC-positive test result is could only be obtained with the GCC/GUSB assay (**Figure 26**).

Table 10: Risk of recurrence for patients with a GCC-positive or -negative test result as a function of cut-off selected for either the GCC/GUSB or the GCC/ACTB assays.

GUSB

GCC-Positive

Cutoff (ΔCt)	Total Nb	Total %	Relapse Nb	Relapse %	Not Relapse Nb	Not Relapse %	RFS	CI 95%
2	1	1%	1	100%	0	0%	0%	0% - 0%
1	2	3%	1	50%	1	50%	50%	39% - 61%
-3	26	36%	10	38%	16	62%	62%	50% - 73%
-5	40	55%	14	35%	26	65%	65%	54% - 76%
-6	44	60%	14	32%	30	68%	68%	57% - 79%
-8	50	68%	15	30%	35	70%	70%	59% - 81%
-11	63	86%	16	25%	47	75%	75%	65% - 85%

GCC-Negative

Cutoff (ΔCt)	Total Nb	Total %	Relapse Nb	Relapse %	Not Relapse Nb	Not Relapse %	RFS	CI 95%
2	72	99%	16	22%	56	78%	78%	68% - 87%
1	71	97%	16	23%	55	77%	77%	68% - 87%
-3	47	64%	7	15%	40	85%	85%	77% - 93%
-5	33	45%	3	9%	30	91%	91%	84% - 98%
-6	29	40%	3	10%	26	90%	90%	83% - 97%
-8	23	32%	2	9%	21	91%	91%	85% - 98%
-11	10	14%	1	10%	9	90%	90%	83% - 97%

GCC-Positive

Cutoff (Copies)	Total Nb	Total %	Relapse Nb	Relapse %	Not Relapse Nb	Not Relapse %	RFS	CI 95%
600 Copies	10	14%	5	50%	5	50%	50%	39% - 61%
200 Copies	22	30%	9	41%	13	59%	59%	48% - 70%
100 Copies	32	44%	12	38%	20	63%	63%	51% - 74%
25 Copies	43	59%	14	33%	29	67%	67%	57% - 78%
6 Copies	55	75%	14	25%	41	75%	75%	65% - 85%
4 Copies	59	81%	15	25%	44	75%	75%	65% - 85%
0.4 Copies	65	89%	16	25%	49	75%	75%	66% - 85%

GCC-Negative

Cutoff (Copies)	Total Nb	Total %	Relapse Nb	Relapse %	Not Relapse Nb	Not Relapse %	RFS	CI 95%
600 Copies	63	86%	12	19%	51	81%	81%	72% - 90%
200 Copies	51	70%	8	16%	43	84%	84%	76% - 93%
100 Copies	41	56%	5	12%	36	88%	88%	80% - 95%
25 Copies	30	41%	3	10%	27	90%	90%	83% - 97%
6 Copies	18	25%	3	17%	15	83%	83%	75% - 92%
4 Copies	14	19%	1	7%	13	93%	93%	87% - 99%
0.4 Copies	8	11%	1	13%	7	88%	88%	80% - 95%

ACTB

GCC-Positive

Cutoff (Copies)	Total Nb	Total %	Relapse Nb	Relapse %	Not Relapse Nb	Not Relapse %	RFS	CI 95%
400 Copies	6	8%	1	17%	5	83%	83%	75% - 92%
200 Copies	10	14%	3	30%	7	70%	70%	59% - 81%
100 Copies	12	16%	5	42%	7	58%	58%	47% - 70%
25 Copies	27	37%	10	37%	17	63%	63%	52% - 74%
6 Copies	35	48%	11	31%	24	69%	69%	58% - 79%
4 Copies	43	59%	11	26%	32	74%	74%	64% - 84%
0.4 Copies	67	92%	16	24%	51	76%	76%	66% - 86%

GCC-Negative

Cutoff (Copies)	Total Nb	Total %	Relapse Nb	Relapse %	Not Relapse Nb	Not Relapse %	RFS	CI 95%
400 Copies	67	92%	16	24%	51	76%	76%	66% - 86%
200 Copies	63	86%	14	22%	49	78%	78%	68% - 87%
100 Copies	61	84%	12	20%	49	80%	80%	71% - 89%
25 Copies	46	63%	7	15%	39	85%	85%	77% - 93%
6 Copies	38	52%	6	16%	32	84%	84%	76% - 93%
4 Copies	30	41%	6	20%	24	80%	80%	71% - 89%
0.4 Copies	6	8%	1	17%	5	83%	83%	75% - 92%

13. GCC status and Recurrence-Free Survival Analysis

[0216] The stratification of relative risk of recurrence according to GCC status was assessed for different cut-off values (**Table 11**). All analyses showed discrimination for risk of recurrence and recurrence- or relapse-free survival (RFS) between GCC-negative

5   and GCC-positive results tested with both RT-qPCR assays. The performance of the GCC/GUSB test was compared to the GCC/ACTB reference test using the 100 GCC copies and 25 GCC copies cut-off values. **Table 11** shows that at cutoff values of 100 and 25 copies respectively, GCC-negative patients tested with the GCC/GUSB test have higher RFS (88% and 90%) than if the reference GCC/ACTB test was used (80% and

10   85%). This example illustrates how the GCC/GUSB test could be beneficial to determine the negative predictive value (i.e. the proportion of patients with a GCC-negative test result who will not develop disease) compared to the reference test.

[0217] Kaplan-Meier analyses were realized with the 73 patients evaluated by both RT-qPCR assays (**Figure 27**). As seen for the RFS, the main effect of the GCC/GUSB test is

15   to reduce the rate of recurrence in the GCC negative group [12% (5/41) vs. 20% (12/61) at 100 copies and 10% (3/30) vs. 15% (7/46) at 25 copies].

[0218] Another improvement of the GCC/GUSB test is the possibility to take advantage of a relative quantification based on a reliable reference gene that compensates for variations observed with different amounts and quality of RNA input in the reaction, a

20   feature that is not available with ACTB because its high expression rendered this reference gene stable towards various stress factors (age, type of fixative, temperature, etc) creating a bias when tested in a duplex assay with GCC. Using a method based on relative quantification, risks of recurrence were calculated (**Table 11**) and a Kaplan-Meier analysis was performed (**Figure 28**) using a cutoff corresponding to a ΔCt of -5.9 (Ct_{GUSB} -

25   Ct_{GCC}). The rate of GCC-positive results (55%; 40/73) obtained with that cutoff was very similar to the one obtained with the 25 copies cut-off (59%; 43/73), the only difference being three specimens without recurrence that were barely positive with the 25 copies cut-off but were negative in ΔCt because of their high GUSB level. As a result, the hazard ratio (HR) according to GCC status using delta-Ct calculation was also higher than for any

30   other method used to segregate GCC positive status (**Table 12**). Overall, these results show that the GCC/GUSB test can not only identify more GCC positive samples but can also increase the statistical power of the discrimination between GCC-positive and -

negative groups. It can be seen that GCC-negative Stage II patients classified using a cutoff of delta-Ct at -5.9 have a recurrence rate (RR) of 9%, very close to the one observed with standard Stage I patients (7 %) in the SEER database while the GCC-positive patients rather show a recurrence rate (35%) closer to Stage IIIB patients (36%).

5   [0219] By analyzing the specimens from the two sites of this cohort individually (Table 13), it can clearly be demonstrated that the GUSB and GCC delta-Ct cutoff points could be adjusted to increase the accuracy of the recurrence prediction as well as the negative predictive value (NPV) or positive predictive value (PPV)."

10   Table 11: Risk of recurrence stratified for GCC positivity using two cut-offs (100 Copies or 25 Copies) for both tests and the pre-selected -5.9 delta-Ct cut-off for the GCC/GUSB test.

		73 Specimens of the Cohort						Log Rank Test
		Total	Recurrence	Risk of Recurrence		RFS		
		(Nb)	(Nb)	%	95% CI	%	95% CI	p value
Absolute Quantification	**100 Copies Cut-off** *GCC/ACTB*							
	Negative	61	12	20%	(11% - 29%)	80% (71% - 89%)		0,0098
	Positive	12	5	42%	(30% - 53%)	58% (47% - 70%)		
	GCC/GUSB							
	Negative	41	5	12%	(5% - 20%)	88% (80% - 95%)		0,0091
	Positive	32	12	38%	(26% - 49%)	63% (51% - 74%)		
	25 Copies Cut-off *GCC/ACTB*							
	Negative	46	7	15%	(7% - 23%)	85% (77% - 93%)		0,0027
	Positive	27	10	37%	(26% - 48%)	63% (52% - 74%)		
	GCC/GUSB							
	Negative	30	3	10%	(3% - 17%)	90% (83% - 97%)		0,0221
	Positive	43	14	33%	(22% - 43%)	67% (57% - 78%)		
Relative Quantification	**-5.9 ΔCt Cut-off** *GCC/GUSB*							
	Negative	33	3	9%	(2% - 16%)	91% (84% - 98%)		0,0049
	Positive	40	14	35%	(24% - 46%)	65% (54% - 76%)		

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Table 12: Analysis of hazard ratio for time to recurrence using Cox's proportional hazards models.

		GCC/ACTB			GCC/GUSB		
		Hazard Ratio	95% CI	P value	Hazard Ratio	95% CI	P value
Absolute	100 Copies	3,45	1.27 - 9.38	0,0254	3,66	1.29 - 10.34	0,0099
Quantification	25 Copies	4,12	1.52 - 11.18	0,0044	3,87	1.12 - 13.40	0,0161
Relative Quantification	-5.9 dCt	N/A			5,07	1.46 - 17.64	0,0035

Table 13: Effect of cutoffs on the results obtained from 2 different cohorts

HDQ Cohort

Cutoff (ΔCt=GUSB-GCC)	GCC-Positive								GCC-Negative							
	Total		Recurr-Pos		Recurr-Neg		RFS	CI 95%	Total		Recurr-Pos		Recurr-Neg		RFS	CI 95%
	Nb	%	Nb	%	Nb	%			Nb	%	Nb	%	Nb	%		
-12.0	19	95%	5	26%	14	74%	74%	54% - 93%	1	5%	0	0%	1	100%	100%	100% - 100%
-8.0	17	85%	5	29%	12	71%	71%	51% - 91%	3	15%	0	0%	3	100%	100%	100% - 100%
-5.9	14	70%	4	29%	10	71%	71%	52% - 91%	6	30%	1	17%	5	83%	83%	67% - 100%
-5.0	11	55%	3	27%	8	73%	73%	53% - 92%	9	45%	2	22%	7	78%	78%	60% - 96%
-4.0	10	50%	3	30%	7	70%	70%	50% - 90%	10	50%	2	20%	8	80%	80%	62% - 98%
-3.0	5	25%	3	60%	2	40%	40%	19% - 61%	15	75%	2	13%	13	87%	87%	72% - 102%

UMass Cohort

Cutoff (ΔCt=GUSB-GCC)	GCC-Positive								GCC-Negative							
	Total		Recurr-Pos		Recurr-Neg		RFS	CI 95%	Total		Recurr-Pos		Recurr-Neg		RFS	CI 95%
	Nb	%	Nb	%	Nb	%			Nb	%	Nb	%	Nb	%		
-12.0	44	83%	9	20%	35	80%	77%	66% - 89%	9	17%	3	33%	6	67%	56%	42% - 69%
-8.0	32	60%	10	31%	22	69%	69%	56% - 81%	21	40%	2	10%	19	90%	81%	70% - 92%
-5.9	24	45%	10	42%	14	58%	58%	45% - 72%	29	55%	2	7%	27	93%	86%	77% - 95%
-5.0	21	40%	10	48%	11	52%	52%	39% - 66%	32	60%	2	6%	30	94%	88%	79% - 96%
-4.0	16	30%	7	44%	9	56%	56%	43% - 70%	37	70%	5	14%	32	86%	81%	71% - 92%
-3.0	9	17%	2	22%	7	78%	78%	67% - 89%	44	83%	10	23%	34	77%	73%	61% - 85%

14. Evaluation of Recurrence Risk in discordant and concordant cases

[0220] We compared the detection rate observed with both assays using different cut-off levels. The primary objective of this comparison was to determine whether there is a significant risk of recurrence associated with patients identified as GCC-positive with the GCC/GUSB assay but previously found GCC-negative using the GCC/ACTB assay. **Table 14** shows detection rates and associated risks of recurrence with combined results from the GCC/ACTB and the GCC/GUSB tests. Interestingly, at a cut-off value of 25 GCC copies, the risk of recurrence among discordant patients (i.e. considered positive for GCC mRNA with the GCC/GUSB test but negative with the GCC/ACTB 19%; 95%CI: 10%-28%) is two times higher than the rate observed with patients having a concordant GCC-

negative result using both tests (10%; 95%CI: 3%-17%) suggesting that the increased detection rate observed with the GCC/GUSB assay is also associated with an increased risk of disease recurrence for colon cancer patients. Conversely, the risk of recurrence associated with a positive test response with the GCC/ACTB assay but a negative

5   response with the GCC/GUSB is null using the same cutoff value of 25 GCC copies.

Table 14 Detection rate and risk of recurrence with combined result from GCC/ACTB and GCC/GUSB test.

		Cases (Nb)	Recurence (Nb)	Risk of Recurrence (%)	95% CI
Cut-off 25 copies					
GCC/ACTB	**GCC/GUSB**				
Positive	Positive	27	11	41%	(29% - 52%)
Positive	Negative	1	0	0%	(0% - 0%)
Negative	Positive	16	3	19%	(10% - 28%)
Negative	Negative	29	3	10%	(3% - 17%)
Cut-off 100 copies					
GCC/ACTB	**GCC/GUSB**				
Positive	Positive	8	4	50%	(39% - 61%)
Positive	Negative	4	1	25%	(15% - 35%)
Negative	Positive	24	8	33%	(23% - 44%)
Negative	Negative	37	4	11%	(4% - 18%)
Cut-off dCt -5,9 *					
GCC/ACTB	**GCC/GUSB**				
Positive	Positive	26	11	42%	(31% - 54%)
Positive	Negative	2	0	0%	(0% - 0%)
Negative	Positive	14	3	21%	(12% - 31%)
Negative	Negative	31	3	10%	(3% - 16%)

* A cut-off of 25 copies was used for the GCC/ACTB test

15. Correlation of Recurrence Risk with the number of GCC-positive lymph nodes

10   [0221] To determine if the GCC/GUSB test could be used to stratify the risk of recurrence based on the number of GCC-positive LNs per case we performed a Kaplan-Meier analysis with GCC-positive patients dichotomized in having a single GCC-positive LN or ≥ 2 GCC-positive LNs (**Figure 29**). Comparison with GCC/ACTB was also considered with cut-off values of 100 GCC copies and 25 GCC copies. Using a cut-off value of 100 GCC

copies, the number of patients with at least 2 GCC-positive LNs is 4 times higher with the GCC/GUSB assay, indicating an increase of not only the number of GCC-positive patients but also of the number of GCC-positive LNs for a given patient. Even if the difference in mean time to recurrence was more important between GCC-positive and GCC-negative

5   groups with the GCC/GUSB assay (p=0.0063 vs. p=0.0326 with GCC/ACTB assay), the overall recurrence rate for GCC-positive patients is still lower due to a fairly increased number of positive cases that will not develop recurrent disease. Again, when the GCC-positive status was determined according to relative quantification (ΔCt value), total recurrence rates observed in each group were similar to those observed with the

10   GCC/ACTB assay with a cut-off value of 25 GCC copies (**Figure 30**). Finally, applying the AJCC standard definition of pN1 and pN2 for regional LN involvement revealed that patients having ≥ 4 LNs positive with the GCC/ACTB assay exhibited a prognostic risk similar to patients with 1 to 3 positive LNs (43% vs 40%, respectively) (**Table 15**). **Figure 31** shows that the relative risk of recurrence for patients with 1 to 3 positive LNs according

15   to the GCC/GUSB test is clearly lower than for patients having ≥ 4 LNs positive (26% vs 44%). Together, these results demonstrate that the GCC/GUSB test can improve the prognostic risk stratification by integrating the number of involved LNs.

Table 15: Risk of recurrence with stratification based on the number of GCC- positive LNs for GCC/ACTB tests (cut-off of 25 GCC copies) and the GCC/GUSB test (cut-off of ΔCt =

20   -5.9).

	73 Specimens of the Cohort						Log Rank Test
	Total	Recurrence	Risk of Recurrence		RFS		
	(Nb)	(Nb)	%	95% CI	%	95% CI	p value
25 Copies Cut-off GCC/ACTB							0,0123
Negative	46	6	13%	(5% - 21%)	87% (79% - 95%)		
GCC Pos 1-3 LNs	20	8	40%	(29% - 51%)	60% (49% - 71%)		
GCC Pos≥4LNs	7	3	43%	(32% - 54%)	57% (46% - 68%)		
ΔCt = -5.9 Cut-off GCC/GUSB							0,0161
Negative	30	3	10%	(3% - 17%)	90% (83% - 97%)		
GCC Pos 1-3 LNs	27	7	26%	(16% - 36%)	74% (64% - 84%)		
GCC Pos≥4LNs	16	7	44%	(32% - 55%)	56% (45% - 68%)		

Study conclusion

[0222] In a previous study, the prognostic value of a GCC/ACTB test for time to recurrence and relapse-free survival was demonstrated in fresh frozen LNs collected from Stage I and II colon cancer patients (Waldman *et al.*, JAMA, 301(7), pp.745-752 (2009)).

5   In that context, we intended to validate these findings with the newly designed GCC/GUSB assay that uses relative quantification instead of absolute quantification obtained by extrapolation to a standard curve. At first, we did confirm that the GCC/GUSB increased the informative rate of clinical samples that are 10 to 15 years old. The detection rate of recurrent cases could also be increased partly because the relative

10   quantification was established with a reliably stable reference gene. Although a higher proportion of node-negative patients were classified as GCC-positive with the novel assay, the overall prognosis stratification was also better since less than 10% of GCC-negative patients actually relapsed, a rate close to those reported for CRC Stage I patients (5-8%). Beyond the better informative rate and better detection rate obtained with

15   the GCC/GUSB assay, results presented here also demonstrate that the GCC/GUSB assay can improve the statistical power of prognosis stratification for relative risk of recurrence and relapse-free survival. Finally, when the number of involved LNs is taken into account, there is a better stratification by molecular staging using the GCC/GUSB assay than the reference test.

We Claim:

1.   A method for the detection of GCC in a sample collected from a subject, comprising the steps of:

- detecting Guanylyl Cyclase C (GCC) in said sample;

5   - detecting beta-glucuronidase (GUSB) in the same said sample; and

- calculating an amount of GCC in relation to an amount of GUSB.

2.   The method according to claim 1, comprising the steps of:

- measuring expression level of GCC mRNA in said sample;

- measuring expression level of GUSB mRNA in the same said sample; and

10   - using a mathematical calculation to normalize the expression level of GCC mRNA to the expression level of GUSB to establish a relative GCC expression (GUSB level minus GCC level) or (GCC level minus GUSB level).

3.   The method according to claim 2, comprising the following steps:

- measuring the expression level of GCC in the sample by RT-qPCR to determine a

15   cycle threshold for GCC (Ct_{GCC});

- measuring beta-glucuronidase (GUSB) in the same sample by RT-qPCR to determine cycle threshold for GUSB (Ct_{GUSB}); and

wherein the detection of GCC uses relative quantification (delta-Ct) to determine the changes in mRNA level of GCC in a sample and expresses it relative to the mRNA levels

20   of beta-glucuronidase (GUSB) (delta-Ct= Ct_{GUSB} minus Ct_{GCC}).

4.   The method according to claim 3, wherein a delta-Ct of above about -12 is indicative of the presence of GCC positive cells in the sample.

5.   The method according to claim 3, wherein the detection of GCC uses the expression fold change (delta-delta-Ct) to determine the changes in mRNA level of GCC

25   in said sample and expresses it relative to the mRNA level of beta-glucuronidase (GUSB) in same said sample.

6.   The method of any one of claims 1 to 5 wherein said sample is an extra-intestinal/colorectal sample and said subject is a human.

7.   The method of claim 6, wherein said human is suspected of having a cancer selected from the group consisting of: colorectal, stomach, small intestine, esophageal and pancreatic cancer.

8.   The method of claim 7, wherein said extra-intestinal/colorectal sample is selected from the group consisting of: biopsy tissue, one or more lymph node, and blood.

9.   A method of diagnosing cancer in a patient suspected of having cancer, comprising the steps of quantifying GCC in an extra-intestinal/colorectal sample of said patient in accordance with any one of claims 1 to 5; and determining whether said sample harbors GCC positive cells, whereby the presence of GCC positive cells is indicative of colorectal, stomach, small intestine, esophageal or pancreatic cancer.

10.   A method of staging a human patient already diagnosed with cancer, comprising the steps of:

        a) detecting or measuring GCC in accordance with any one claims 1 to 5; and

        b) establishing a disease-stage based on the results of step a.

11.   The method according to claim 10, comprising the steps:

        •   measuring GCC in said sample by RT-qPCR to determine a cycle threshold for GCC (Ct_{GCC}) in an extra-intestinal/colorectal sample from said patient;

        •   measuring beta-glucuronidase (GUSB) in said sample by RT-qPCR to determine a cycle threshold for GUSB (Ct_{GUSB}) in said sample; and

        •   establishing relative quantification (delta-Ct) of $Ct_{GUSB} - Ct_{GCC}$,
wherein a delta-Ct of above about -12 is indicative of the presence of GCC positive cells in the sample, wherein the presence of GCC positive cells is indicative of metastasized colorectal, stomach, small intestine, pancreatic or esophageal cancer.

12.   The method of claim 11 wherein a delta-Ct higher than about -6 is indicative of the presence of GCC positive cells in the sample, whereby the presence of GCC positive cells is indicative that the patient has increased risk of recurrence of cancer.

13.   The method of claim 11 wherein the extra-intestinal/colorectal sample is at least one lymph node.

14.   A method of monitoring, or diagnosing metastasis in, a human already diagnosed with cancer, comprising the steps:

- measuring GCC in said sample by RT-qPCR to determine a cycle threshold for GCC (Ct_{GCC})) in an extra-intestianl/colorectal sample from said patient;

- measuring beta-glucuronidase (GUSB) in said sample by RT-qPCR to determine a cycle threshold for GUSB (Ct_{GUSB}) in said sample; and

5   - establishing relative quantification (delta-Ct) of $Ct_{GUSB} - Ct_{GCC}$,

wherein a delta-Ct of above about -12 is indicative of the presence of GCC positive cells in the sample, wherein the presence of GCC positive cells is indicative of metastasized colorectal, stomach, small intestine, pancreatic or esophageal cancer.

15.   The method of claim 14 wherein the extra-intestinal/colorectal sample is blood.

10   16.   A method to select among cancer patients having histopathologically negative lymph nodes those who can benefit from a course of treatment, comprising:

- carrying out the steps according to claim 13; and

- prescribing a course of treatment;

whereby cancer patients with GCC positive cells in at least one lymph node have a risk of
15   recurrence and survival rate comparable to that of patients considered of a higher risk by histopathology, thereby indicating that these patients might benefit from treatment with adjuvant chemotherapy, and whereby cancer patients with GCC negative lymph nodes are at a lower risk of disease recurrence and can avoid said treatment.

17.   The method of claim 16, wherein the presence of GCC positive cells is indicative
20   of a poor prognosis.

18.   The method according to claims 1 to 5, wherein the detection of the presence of GCC positive cells in tissues harboring metastases of an unknown origin (CUP) is a confirmation that the primary cancer is a colorectal, a stomach, a small intestine, a pancreatic or an esophageal cancer.

25   19.   The method according to claim 2, whereby if the relative GCC expression is higher than the limit of detection of GUSB, the absolute quantity of GCC in the sample is calculated and expressed in number of GCC copies in relation to an external standard.

20.   A kit for the detection, diagnosis, prognosis, monitoring and/or staging of a cancer in a patient, wherein the kit comprises:

- reagents for detecting GCC in an extra-intestinal/colorectal sample from the patient;

- reagents for detecting GUSB in the same sample; and

- instructions on how to measure GCC in relation to GUSB.

5   21.   A kit for the detection, diagnosis, prognosis, monitoring and/or staging of a cancer in a patient, wherein the kit comprises:

- PCR reagents for detecting GCC in an extra-intestinal/colorectal sample from the patient;

- instructions on how to determine a cycle threshold for GCC (Ct_{GCC});

10   - PCR reagents for detecting GUSB in the same sample;

- instructions on how to determine a cycle threshold for GUSB (Ct_{GUSB}); and

- instructions on how to calculate (delta-Ct) or (delta-delta-Ct) between Ct_{GCC} and Ct_{GUSB}.

22.   The kit according to claim 20 or 21, wherein said reagents comprises a
15   polynucleotide selected from the group consisting of: SEQ ID NO 17 to 43.

23.   The kit according to claim 20 or 21, wherein said reagents comprises a polynucleotide having 90% identity to: SEQ ID NO 17 to 43.

24.   The kit according to claim 20 or 21, wherein said reagents comprises a polynucleotide primer selected from the group consisting of: SEQ ID NO 17, 18, 20, 21,
20   23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41 and 42.

25.   The kit according to claim 20, 21 or 24, wherein said reagents comprises a polynucleotide probe selected from the group consisting of: SEQ ID NO 19, 22, 25, 28, 31, 34, 37, 40 and 43.

26.   The kit according to claim 24 or 25, wherein the primer comprises a polynucleotide
25   of SEQ ID NOs 20, 21, 38 and 39 and the probe comprises a polynucleotide of SEQ ID NOs 22 and 40.

27.   The method according to any one of claim 1 to 5, wherein said detecting or measuring is carried out with a polynucleotide selected from the group consisting of: SEQ ID NO 17 to 43.

28.   The method according to any one of claim 1 to 5, wherein said detecting or measuring is carried out with a polynucleotide having 90% identity to: SEQ ID NO 17 to 43.

29.   The method according according to claim 27, wherein said detecting or measuring of expression level of GCC or GUSB mRNA is carried out with the use of a polynucleotide primer selected from the group consisting of: SEQ ID NO 17, 18, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41 and 42.

30.   The method according to claim 27, wherein said detecting or measuring of expression level of GCC or GUSB mRNA is carried out with the use of a polynucleotide probe selected from the group consisting of: SEQ ID NO 19, 22, 25, 28, 31, 34, 37, 40 and 43.

31.   The method according to claim 27, wherein said polynucleotide is selected from the group consisting of: SEQ ID NOs 20, 21, 22, 38, 39 and 40.

32.   A polynucleotide selected from the group consisting of: SEQ ID NO 17 to 43.

33.   The polynucleotide according to claim 32, having 90% identity to: SEQ ID NO 17 to 43.

34.   The polynucleotide according to claim 32, wherein the polynucleotide primer is selected from the group consisting of: SEQ ID NO 17, 18, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33, 35, 36, 38, 39, 41 and 42.

35.   The polynucleotide according to claim 32, wherein the polynucleotide probe is selected from the group consisting of: SEQ ID NO 19, 22, 25, 28, 31, 34, 37, 40 and 43.

36.   The polynucleotide according to claim 32, selected from the group consisting of: SEQ ID NOs 20, 21, 22, 38, 39 and 40.

37.   A method of predicting the risk of cancer recurrence for a patient already diagnosed with cancer, comprising carrying the steps according to claim 5, wherein a delta-delta-Ct between -6 and -3 is indicative of the presence of GCC positive cells in the sample, whereby the presence of GCC positive cells is indicative that the patient has increased risk of recurrence of cancer.

38. The method of claim 37, wherein the patient is already diagnosed with a cancer selected from the group consisting of: colorectal, stomach, small intestine, pancreatic and esophageal.

39. The method of claim 38, wherein the patient is already diagnosed with colorectal cancer.

40. The method of claim 39 wherein the patient has a stage I or stage II colorectal cancer.

41. The method of claim 37, wherein the extra-intestinal/colorectal sample is a lymph node or blood.

42. The method of claim 41, wherein the extra-intestinal/colorectal sample is a lymph node.

43. The method of claim 42, wherein the extra-intestinal/colorectal sample is two or more lymph nodes.

44. The method of claim 42, wherein the extra-intestinal/colorectal sample is at least four lymph nodes.

45. The method of claim 42, wherein the extra-intestinal/colorectal sample is at least twelve lymph nodes.

46. The method according to claim 37, wherein a delta-Ct equal or higher than -6 represents a GCC positive result and a delta-Ct lower than -6 represents a GCC negative result, whereby said result allow discrimination for risk of recurrence and relapse-free survival (RFS) between GCC-negative and GCC-positive results.

47. The method according to claim 42 or 44, wherein the positive results are found in 1 to 3 lymph nodes of the same patient, whereby the relative risk of recurrence for the patient with 1 to 3 positive LNs according to the GCC/GUSB test is intermediate.

48. The method according to claim 42 or 44, wherein the positive results are found in 4 or more lymph nodes of the same patient, whereby the relative risk of recurrence for the patient with 4 positive LNs or more according to the GCC/GUSB test is high.

49. The method of claim 48, wherein the quantity of GCC detected is calculated for each individual lymph node.

50. The method of claim 48, wherein the quantity of GCC is the sum of the individual quantities of GCC in all lymph nodes of the patient.

51. The method according to claim 37, wherein a delta-Ct equal or higher than -5.9 represents a GCC positive result and a delta-delta-Ct lower than -5.9 represents a GCC negative result, whereby said result allow discrimination for risk of recurrence and relapse-free survival (RFS) between GCC-negative and GCC-positive results.

52. The method according to claim 51, wherein the positive results are found in 1 to 3 lymph nodes of the same patient, whereby the relative risk of recurrence for the patient with 1 to 3 positive LNs according to the GCC/GUSB test is intermediate.

53. The method according to claim 51, wherein the positive results are found in 4 or more lymph nodes of the same patient, whereby the relative risk of recurrence for the patient with 4 positive LNs or more according to the GCC/GUSB test is high.

54. A method of determining the GCC burden of a patient diagnosed with cancer, comprising carrying the steps according to claim 37, wherein if delta-Ct is equal or higher than about -12, the quantity of GCC mRNA is calculated in terms of number of copies in relation to an external standard, whereby the GCC burden is expressed in number of GCC copies in the sample.

55. The method of claim 54, wherein the GCC burden is established for each individual lymph node.

56. The method of claim 54, wherein the GCC burden is established on the basis of the total amount of GCC in all lymph nodes of the patient.

57. A method of predicting the risk of cancer recurrence of a patient diagnosed with cancer, comprising determining the GCC burden according to either claim 55 or 56, wherein a GCC burden above 10 copies is indicative of an increased likelihood of cancer recurrence.

58. A method of predicting the risk of cancer recurrence of a patient diagnosed with cancer, comprising determining the GCC burden according to claim 57, wherein a GCC burden above 25 copies is indicative of an increased likelihood of cancer recurrence.

59. A method of predicting the risk of cancer recurrence of a patient diagnosed with cancer, comprising the steps of:

- determining the quantity of GCC in one or more lymph nodes collected from the patient;
- classifying each of the one or more lymph nodes as GCC-negative or GCC-positive; and

5
- establishing the lymph node ratio, wherein the lymph node ratio is the number of GCC-positive nodes over the total of GCC-negative and GCC-positive lymph nodes;

whereby the larger the lymph node ratio, the greater the likelihood of cancer recurrence.
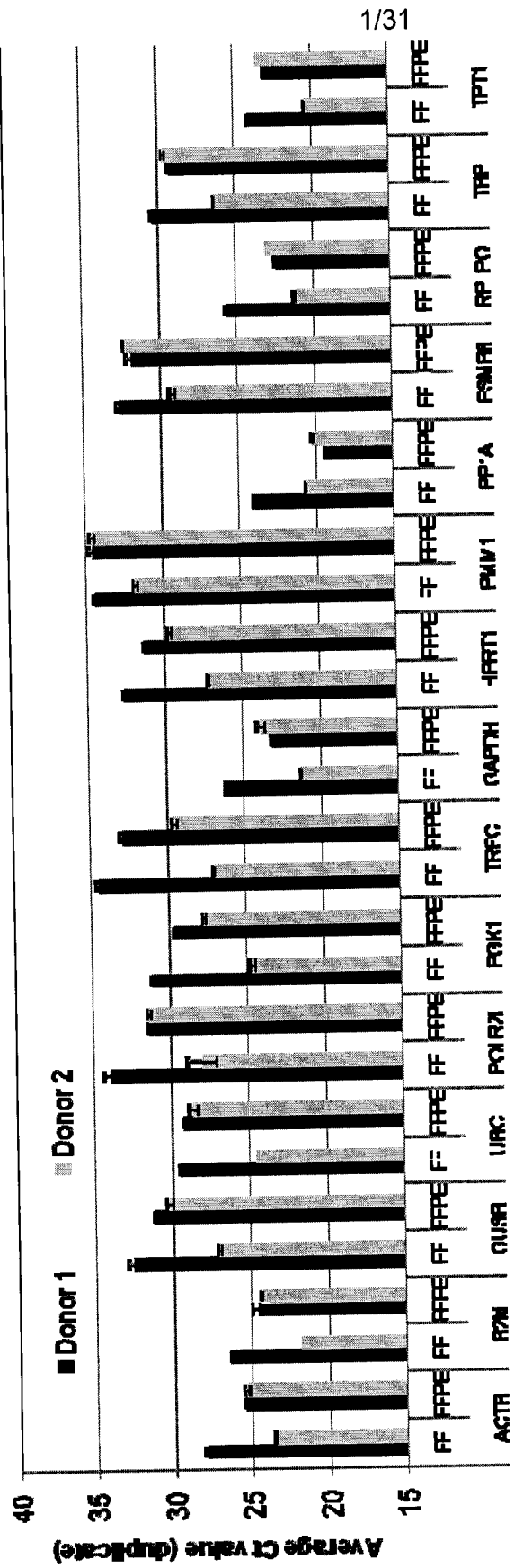
Figure 1. Expression levels of putative reference genes, presented as average Ct values in matched fresh frozen (FF) and FFPE colon cancer LNs.

2/31
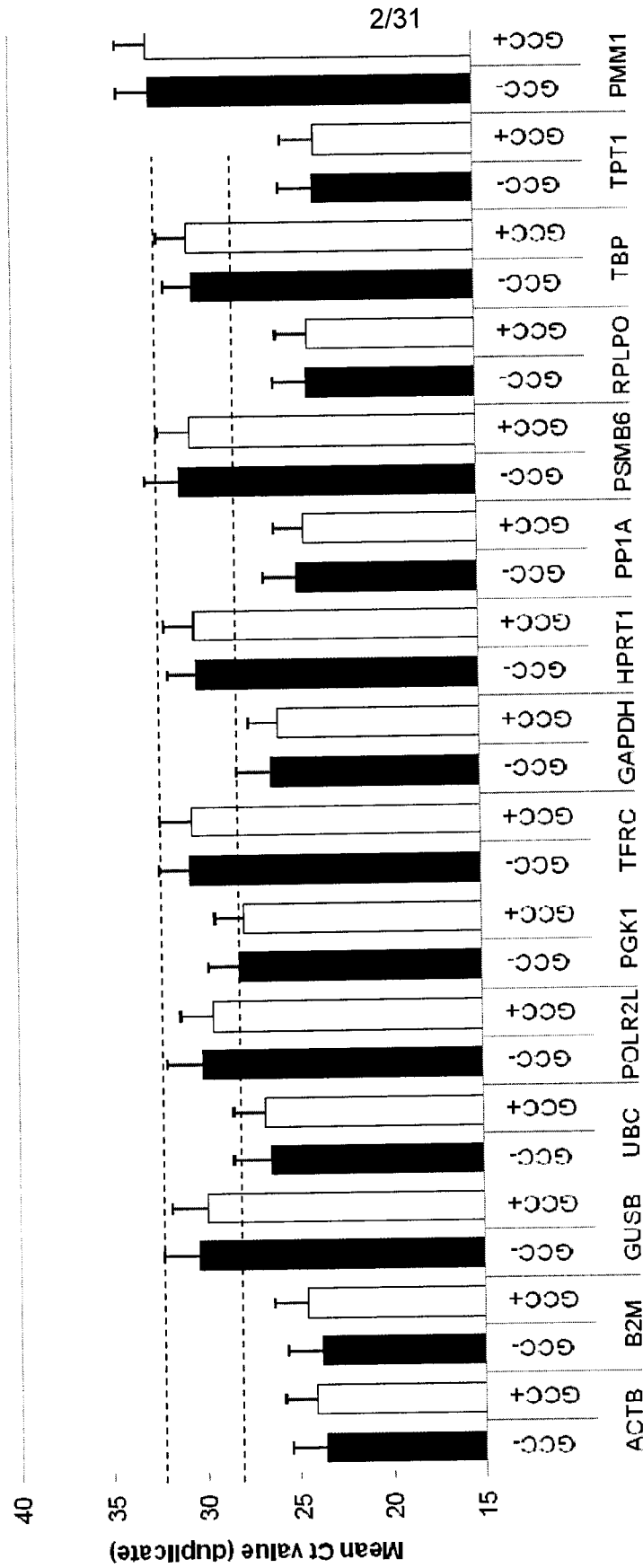
Figure 2. Expression levels of putative reference genes, presented as average Ct values in GCC negative and positive FFPE LNs. Targeted Ct range was delimited by the two dotted lines.
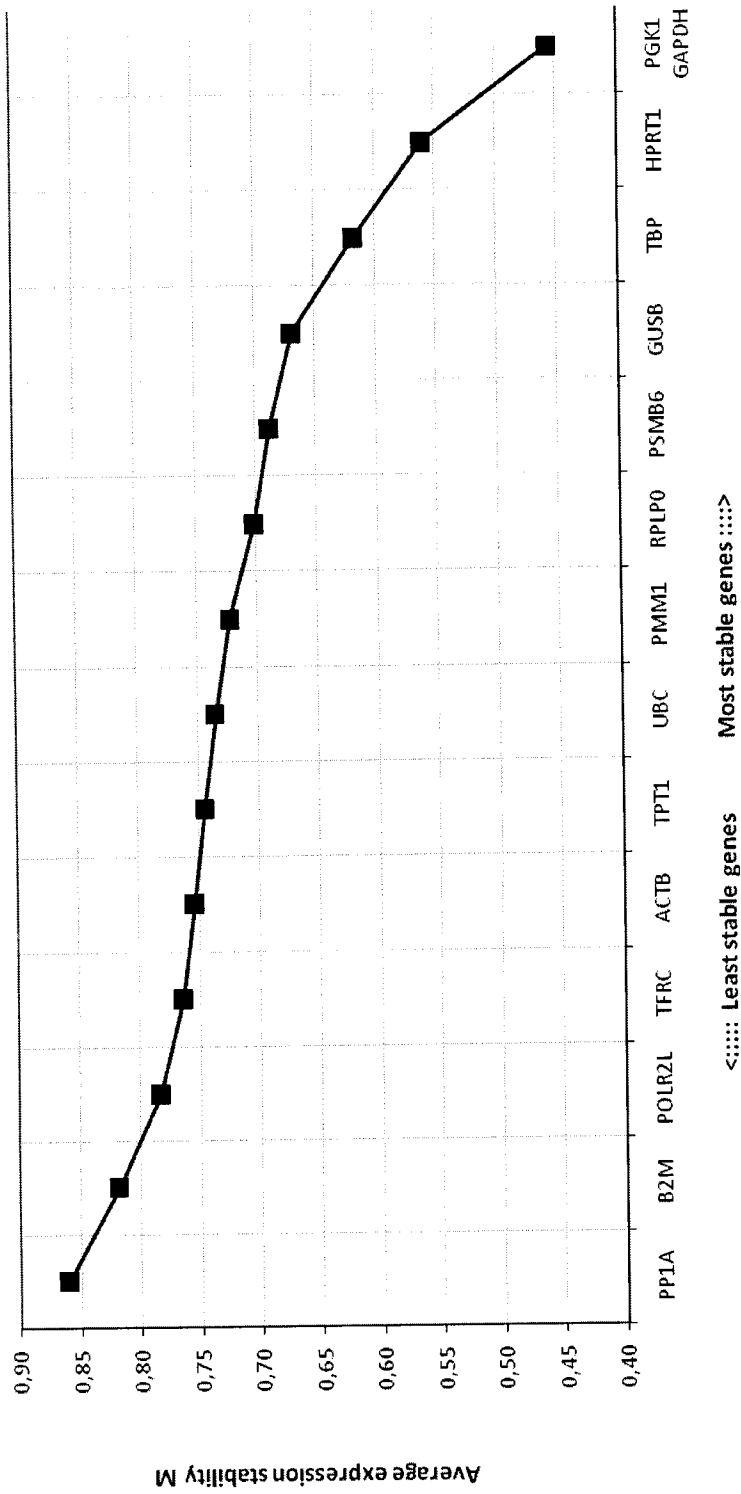
Figure 3. Average expression stability values of control genes.
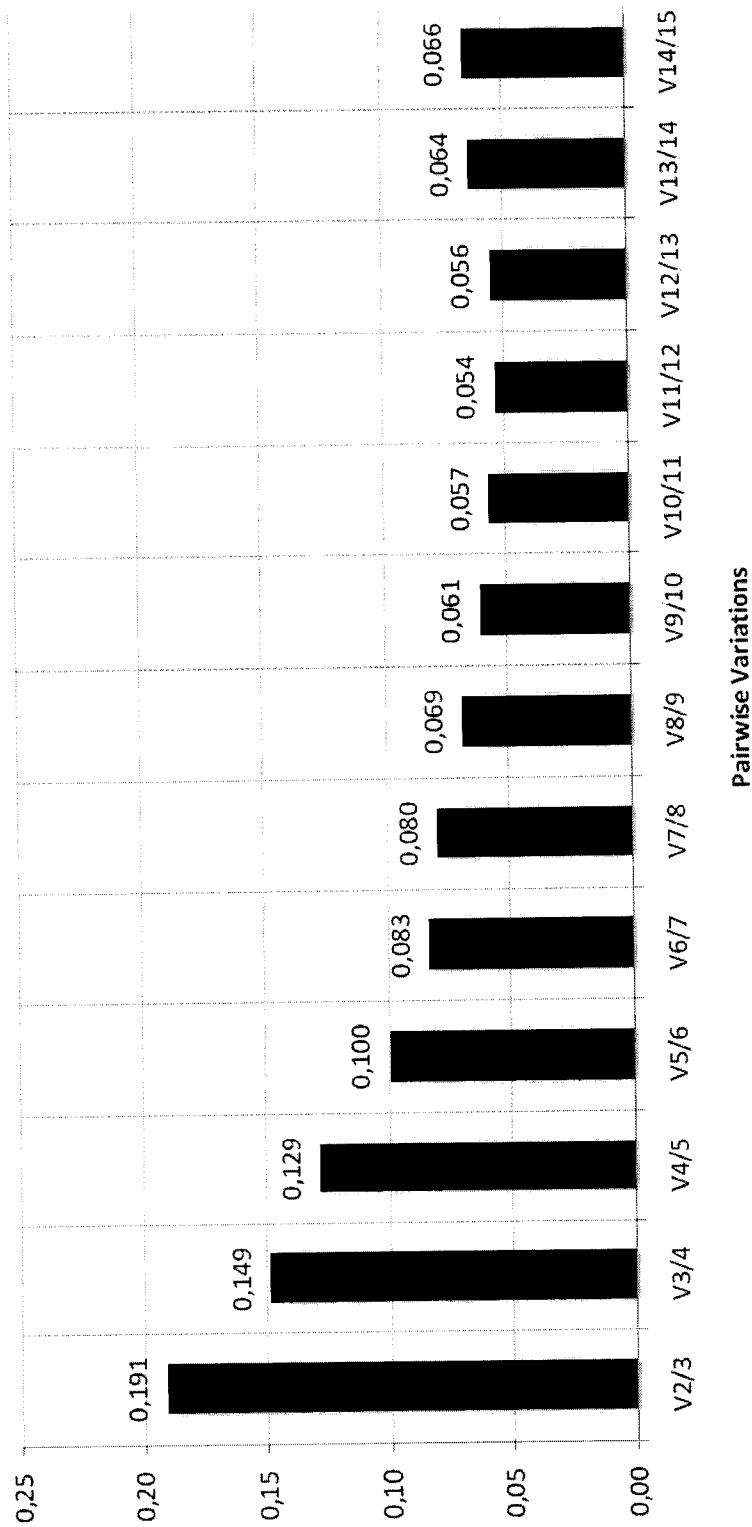
Figure 4. Determination of the optimal number of control genes for normalization.
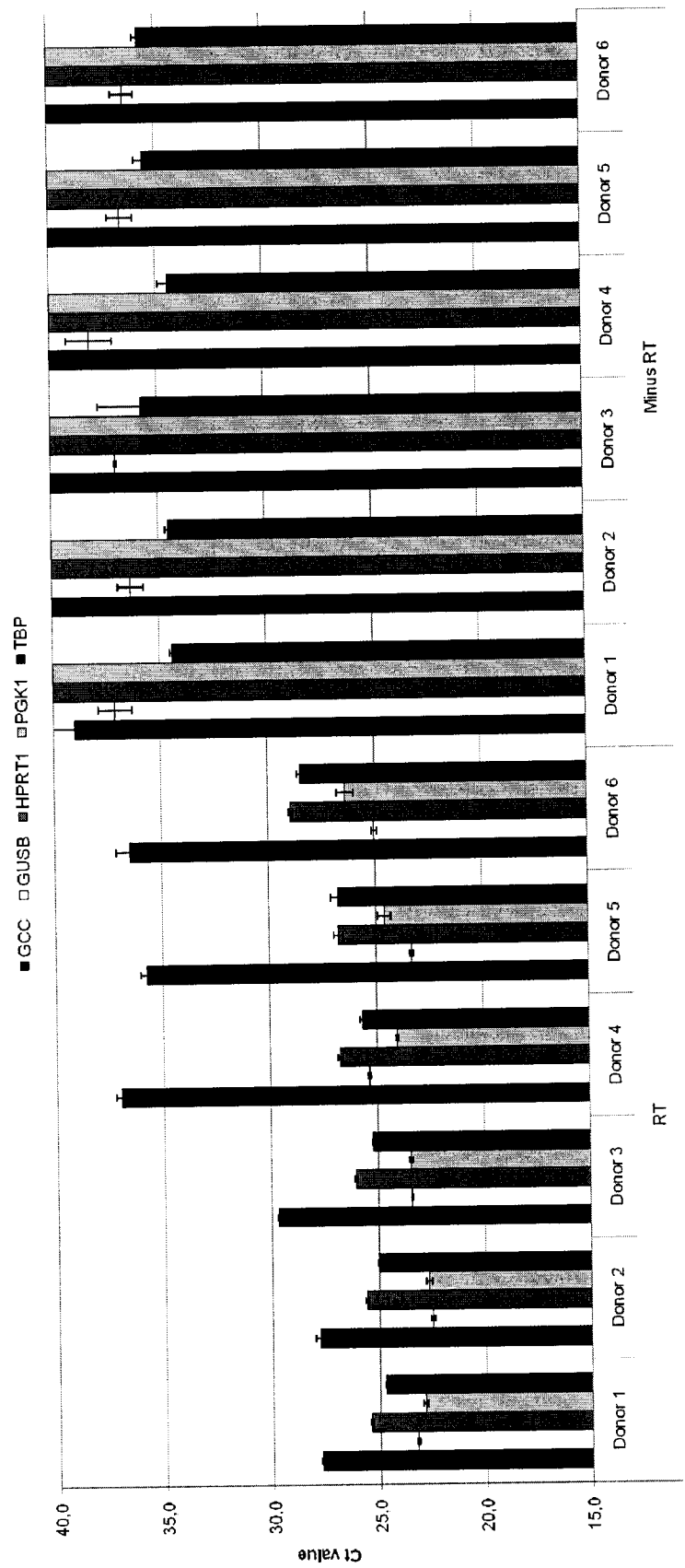
Figure 5. Expression profile of selected reference genes in GCC positive and negative LNs. Ct value for GCC, GUSB, HPRT1, PGK1 and TBP in FFPE samples tested in simplex reaction with 312.5 ng of cDNA. In minus RT experiments, no RT enzyme was added during the cDNA synthesis step.

A   **Time (hrs) of NaOH treatment at 60°C**

B   **Raw Ct for GCC and selected HKG in RNA treated with NaOH**
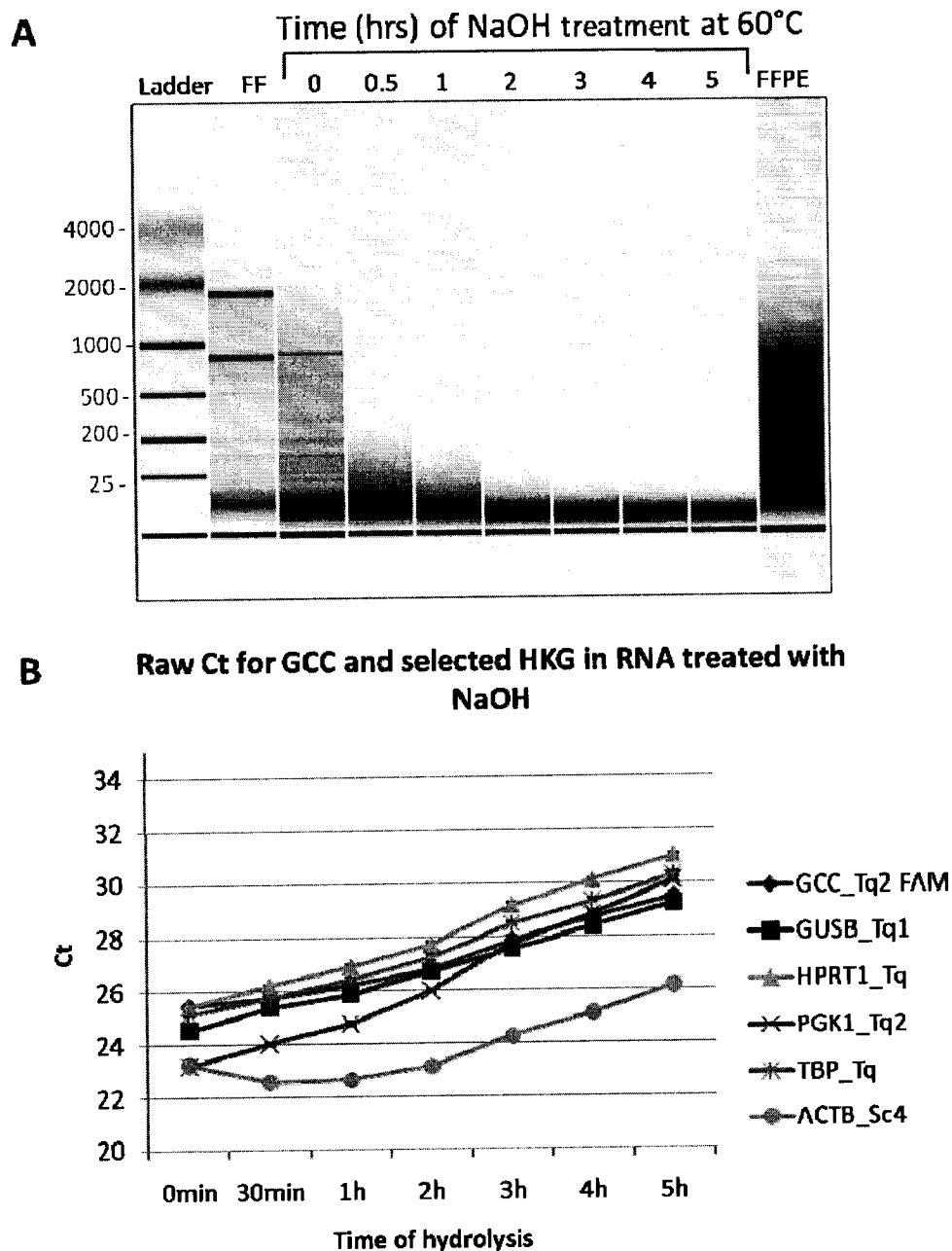
Figure 6. Effect of NaOH treatment on RNA quality and gene expression measures. The extent of RNA degradation following NaOH treatment was determined by capillary electrophoresis using the Agilent 2100 Bioanalyzer. Panel A) show a representative RNA fragmentation profile compared to intact RNA isolated from fresh frozen colon tissues and matched FFPE material. B) Ct values for GCC and 5 reference gene assays including ACTB Scorpions™ were determined at each time point of hydrolysis.
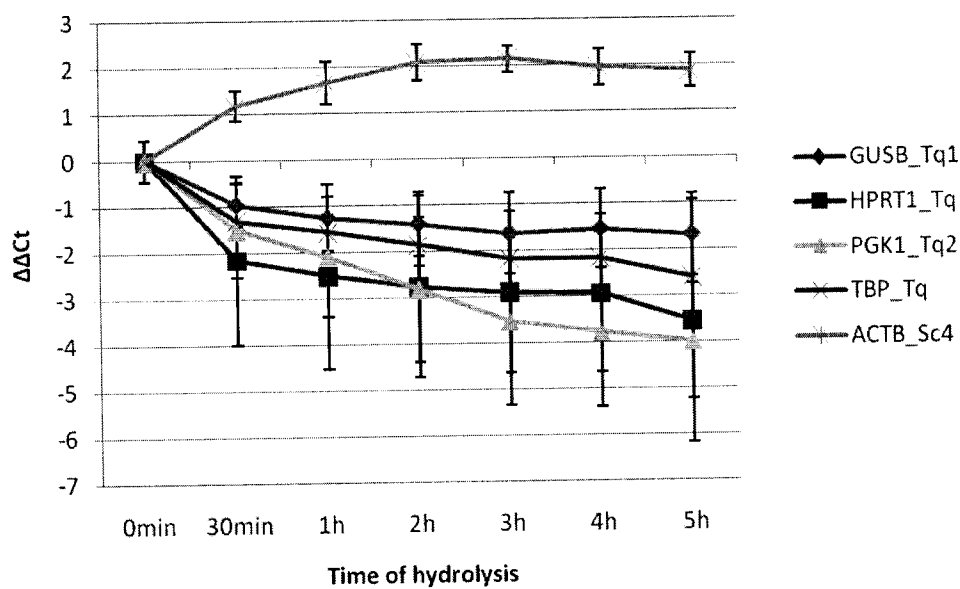
Figure 7. GCC expression variation ($\Delta\Delta Ct$) during NaOH degradation using five reference genes (GUSB, HPRT1, PGK1, TBP and ACTB) for normalization. Variations in delta Ct values (delta-delta-Ct = $[(Ct_{GCC}-Ct_{RG})t_x-(Ct_{GCC}-Ct_{RG})t_0]$ were determined at each time point of hydrolysis. Error bar represent SD from 3 independent RNA pools. To perform comparison between three experiments, the threshold for each gene was manually fixed to 0.10.
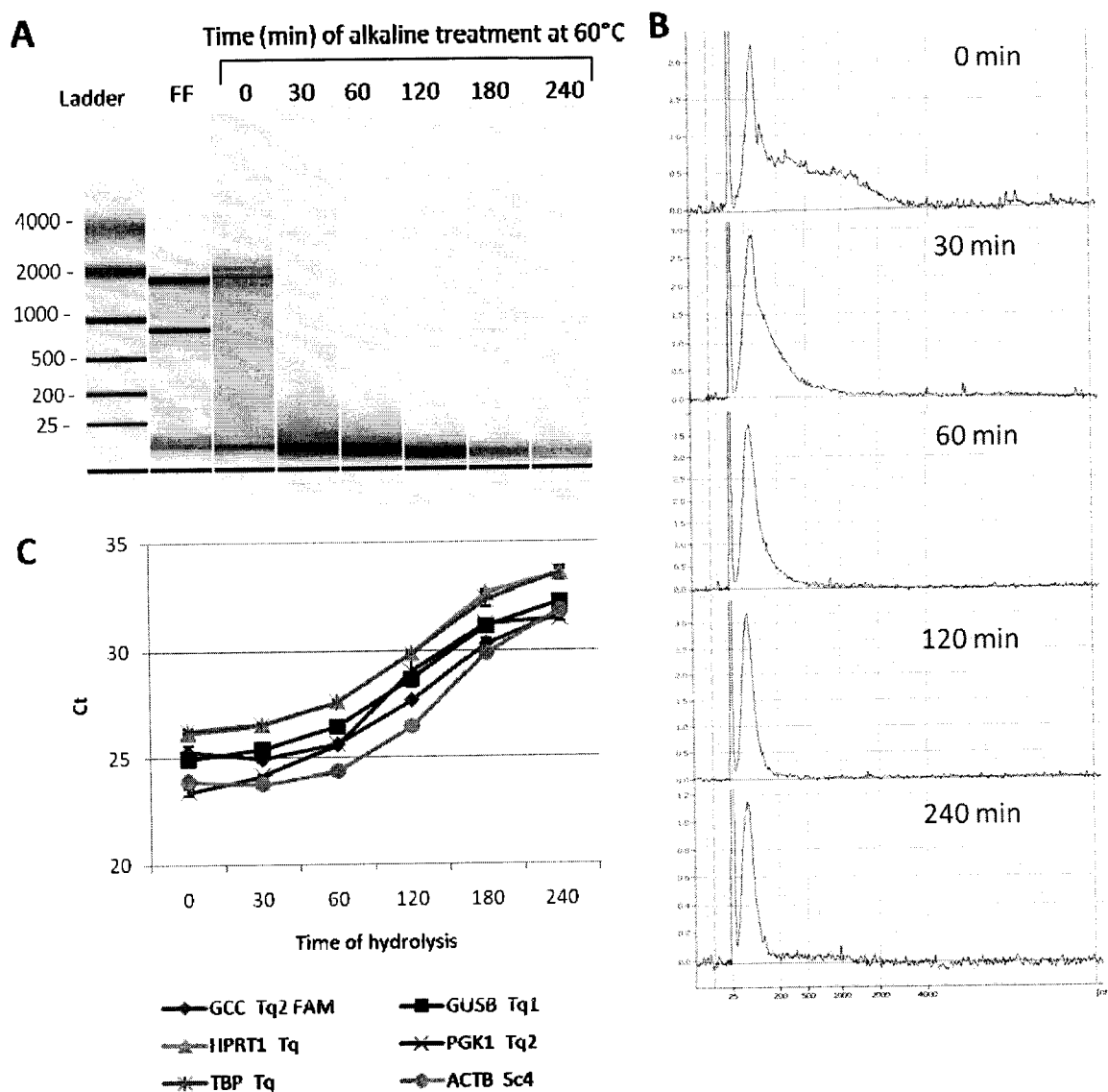
Figure 8. Effect of carbonate buffer alkaline treatment on RNA quality and gene expression pattern. The quality of RNA isolated at indicated time points was measured by capillary electrophoresis using the Agilent 2100 Bioanalyzer. A) Representative RNA fragmentation profile compared to intact RNA isolated from fresh frozen colon tissues. B) Electropherograms of RNA sample. Each time point contained an equal amount of material. C) Ct values for GCC and 5 reference gene assays, including the GCC/ACTB duplex assay.
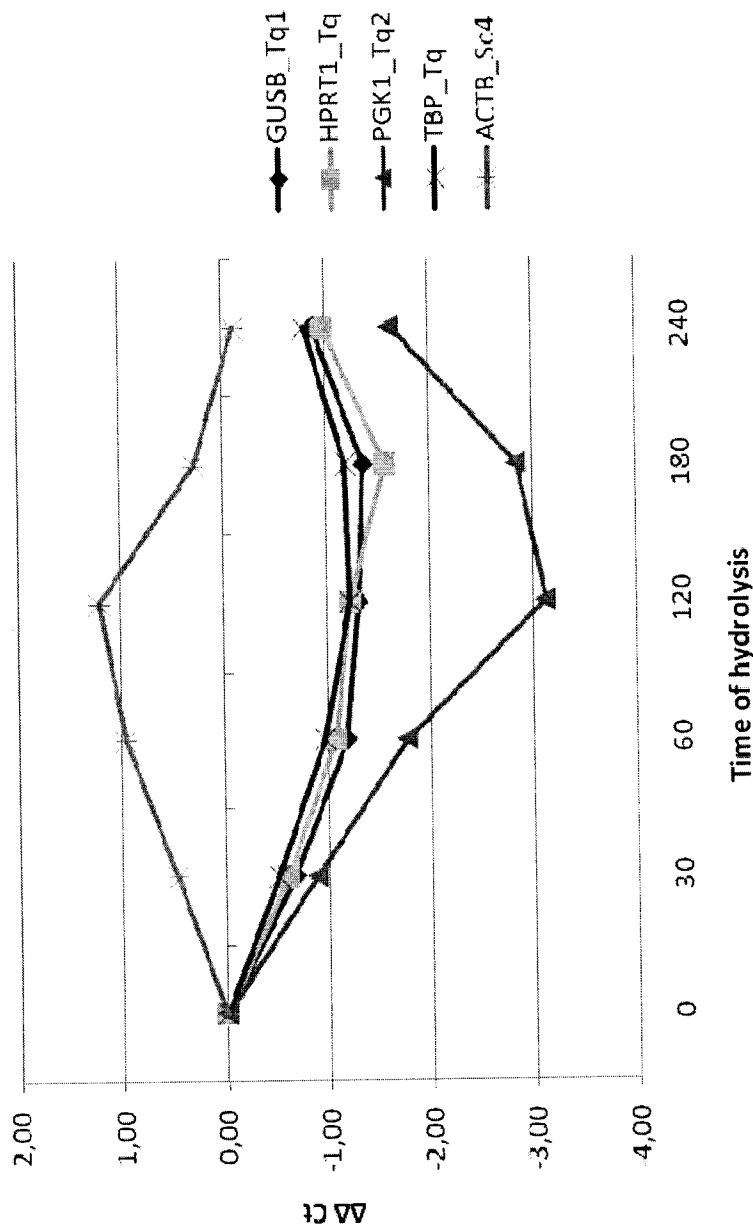
Figure 9. GCC expression variation (ΔΔCt) following controlled RNA degradation in carbonate buffer alkaline conditions. Variation in delta Ct values (delta-delta-Ct = $[(Ct_{GCC}-Ct_{RG})t_x-(Ct_{GCC}-Ct_{RG})t_0]$ were determined at each time point of hydrolysis.
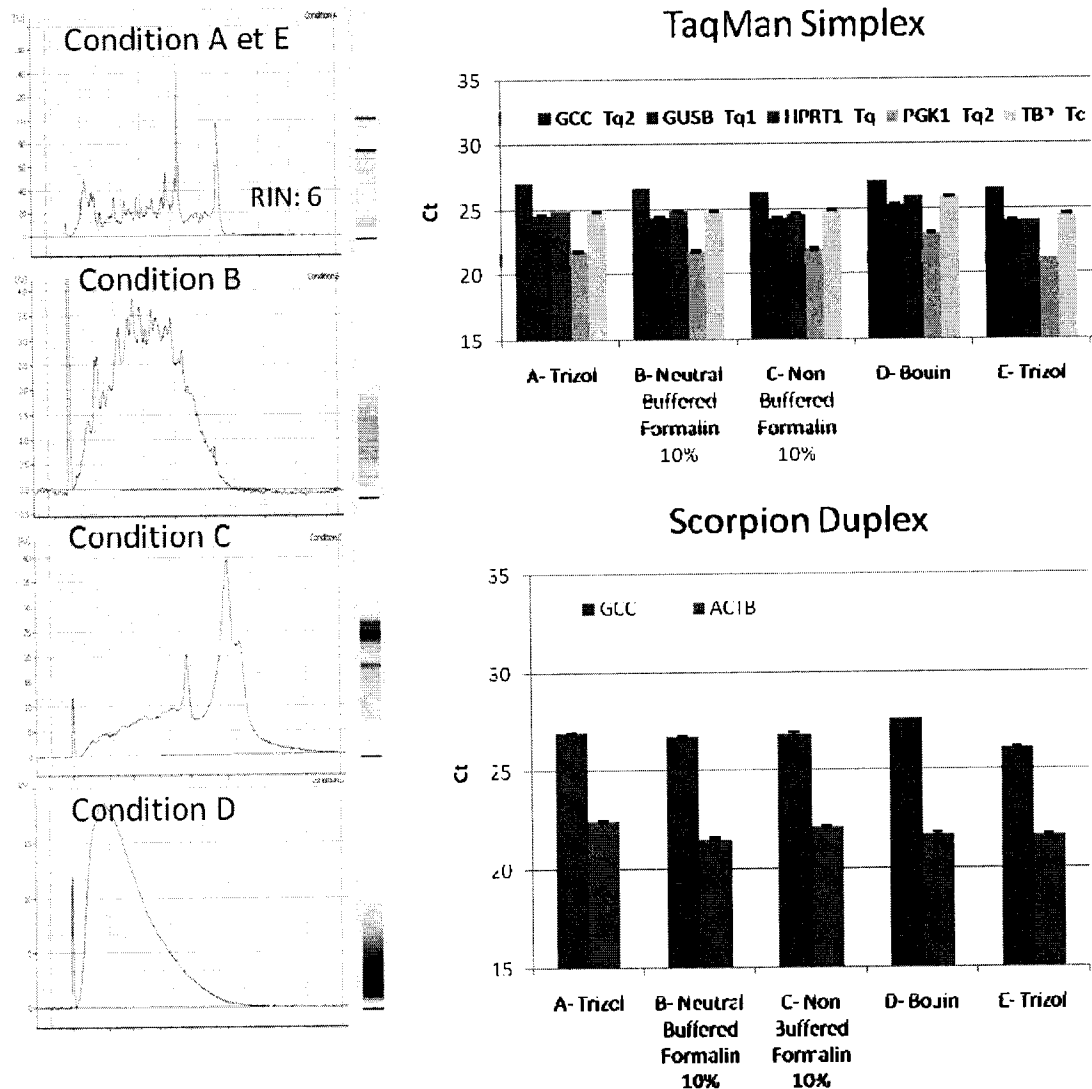
Figure 10. Gene expression profiling in frozen and fixed, paraffin-embedded (FPE) tissues using TaqMan simplex assays and the GCC/ACTB duplex assay. The quality of RNA isolated from each condition was measured by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Condition A and E are TRIzol™ extract, B: Neutral buffered formalin 10%, C: Non-buffered formalin 10%; D: Bouin's solution). Expression (Ct values) of GCC and 4 reference genes (GUSB, HPRT1, PGK1 and TBP) in TaqMan simplex reactions was compared to GCC/ACTB duplex assay.
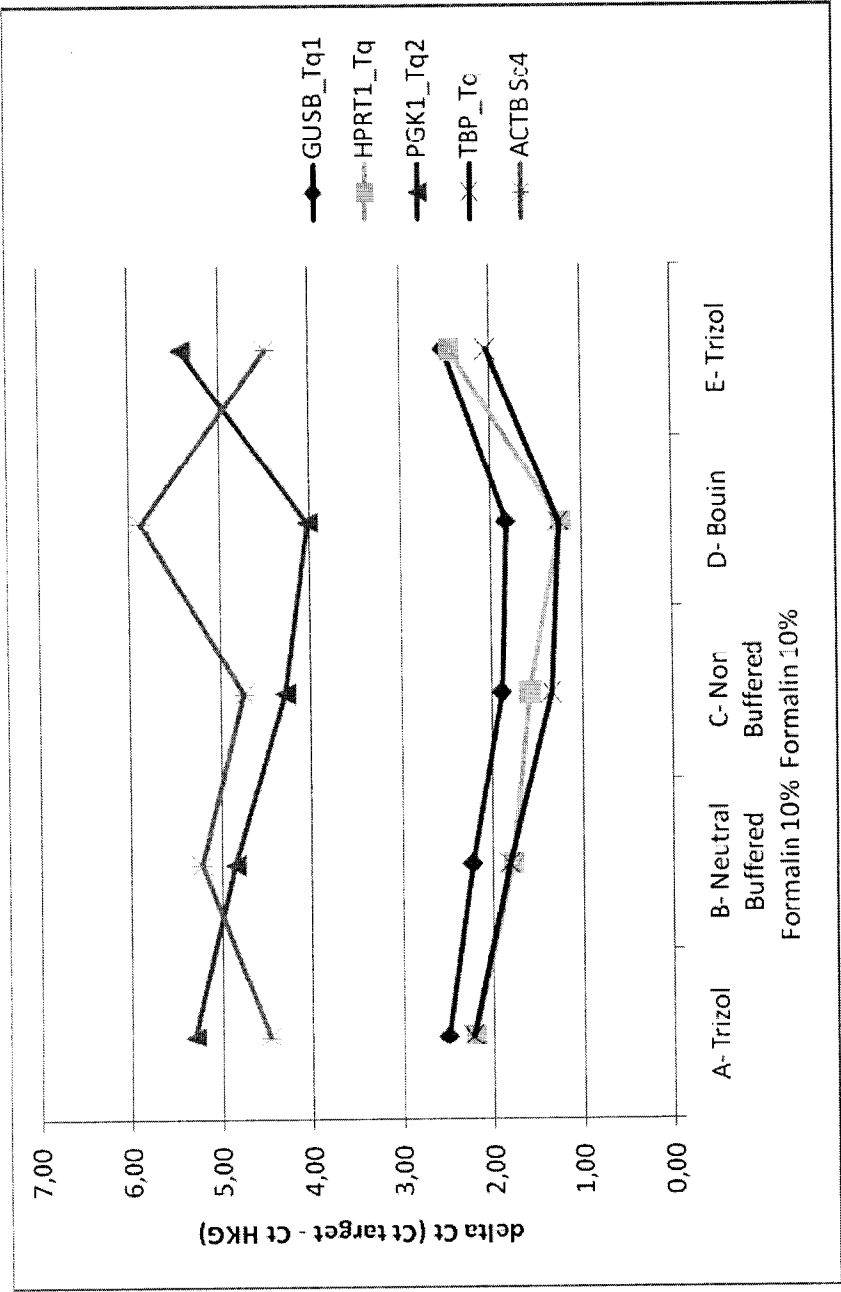
Figure 11. Comparison of GCC expression levels (delta-Ct) in cryo-sections of colon cancer tissue samples fixed or not. Variation in delta Ct values (delta-Ct = $(Ct_{GCC} - Ct_{RG})$) were determined for each RNA extract. The reverse transcription reactions were performed with the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's recommendation, using gene-specific primers (2 µM) and 500 ng of total RNA based on Quant-IT data. The real-time PCR were carried out in a 20-µl reaction volume with the Applied Biosystems 7900HT Fast Real-Time PCR Systems using either TaqMan simplex or the GCC/ACTB duplex assay. Primers and probes concentration for each simplex assays were 900 nM and 250 nM respectively. All reactions were performed in duplicate.

A
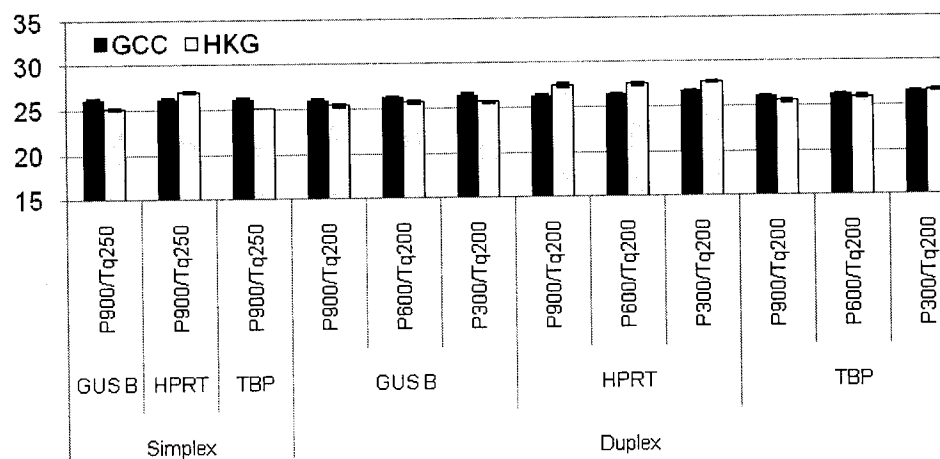
B
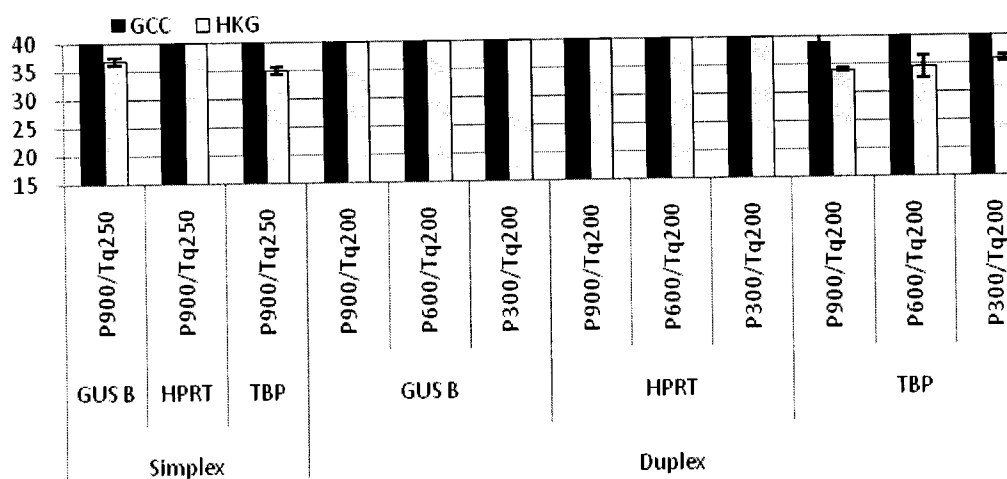
Figure 12. Comparison of GCC Taqman simplex and duplex amplifications in RNA extracted from a FFPE colon tissue. Upper panel shows GCC and various RG PCR amplifications in FFPE samples tested in simplex and duplex reactions using gene-specific reverse primers at 2 µM and 1.25 µg of RNA. Lower panel shows a comparison of minus RT-PCR amplification for GCC and various reference genes in simplex and duplex reactions.
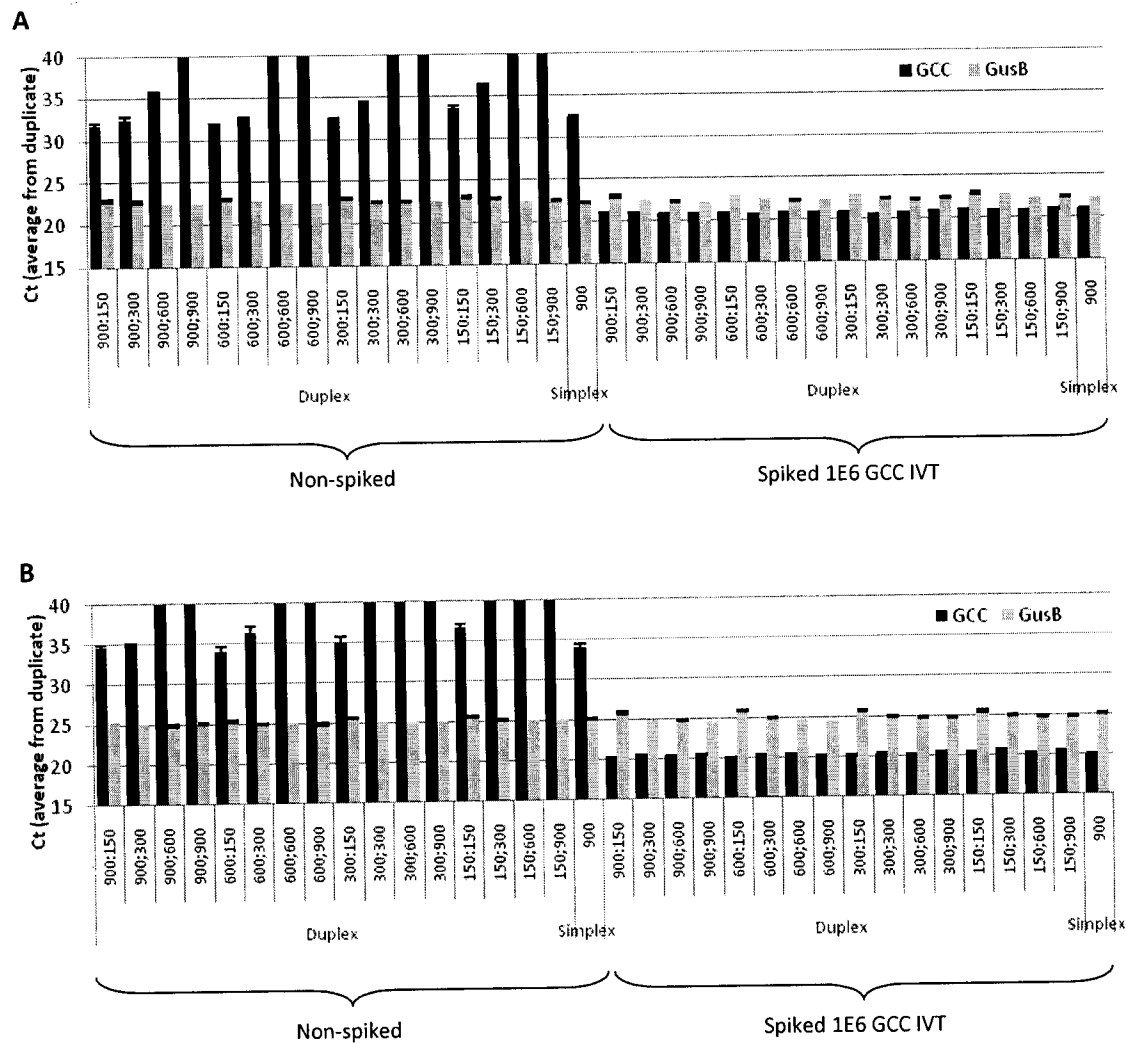
Figure 13. Comparison of GCC and GUSB Ct values between duplex and simplex reactions. The real-time PCR were carried out in duplex or simplex reactions of 20 µl with the Applied Biosystems 7900HT Fast Real-Time PCR Systems using TaqMan Fast Universal PCR Master Mix. Synthesis of cDNA was performed with 250 ng/µl (A) or 25 ng/µl (B) of uRNA spiked or not with 1x10^{6} GCC IVT. For real-time PCR reaction in simplex, primers concentration was 900 nM and FAM or VIC-labeled probes concentration was 250 nM. For duplex reactions, 4 concentrations of reverse and forward primers were tested while both FAM and VIC-labeled probes were fixed at 200 nM in a 20 µl PCR reaction.

A   GUSB

Variation in ΔCt calculation during NaOH degradation

Legend: Simplex 2;2, Duplex 2;2, Duplex 2;20, Duplex 20;2, Duplex 20;20

delta delta Ct vs Time of hydrolysis in NaOH at 60°C (hr)

B   HPRT1

Variation in ΔCt calculation during NaOH degradation

Legend: Simplex 2;2, Duplex 2;2, Duplex 2;20, Duplex 20;2, Duplex 20;20

delta delta Ct vs Time of hydrolysis in NaOH at 60°C (hr)

Figure 14. GCC and RG delta-Ct variation in duplex and simplex assays during NaOH degradation.

Expression of GCC was normalized with either GUSB or HPRT1. Variations in GCC relative quantification (delta-delta-Ct $= [(Ct_{GCC}-Ct_{RG})_{tx}-(Ct_{GCC}-Ct_{RG})_{t0}]$ were determined at indicated time points during NaOH hydrolysis. Variation between non-degraded and degraded samples should be lower than 1 delta-Ct to be considered not significant.
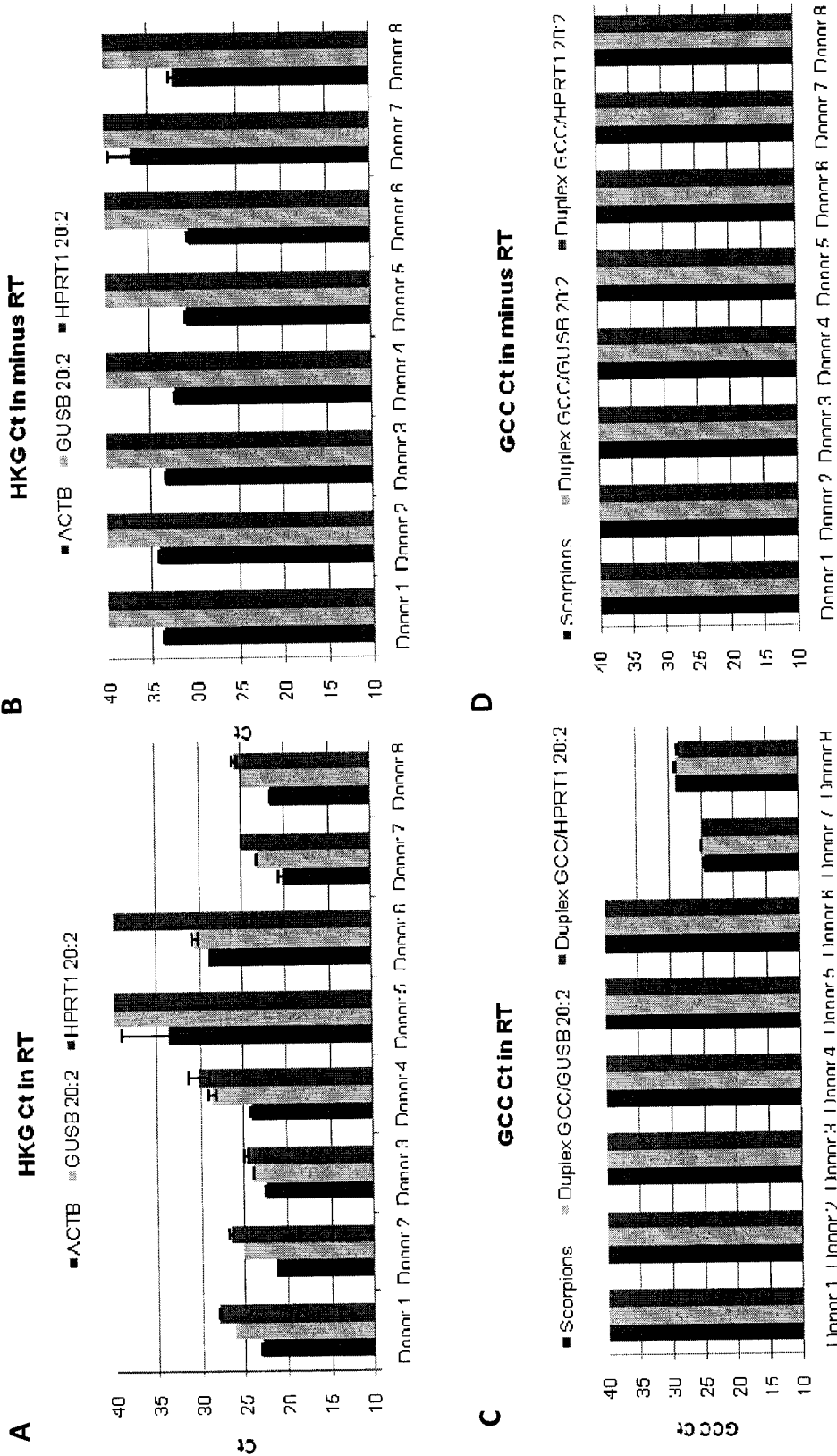
Figure 15. Comparison of FFPE colon cancer LNs tested with TaqMan and Scorpions™ duplex assays in minus RT condition. Panel A and C show Ct values for ACTB, GUSB, HPRT1 and GCC in 8 FFPE samples tested with 312.5 ng of cDNA in duplex reaction. In minus RT experiments (B and D), no RT enzyme was added during the cDNA synthesis step.
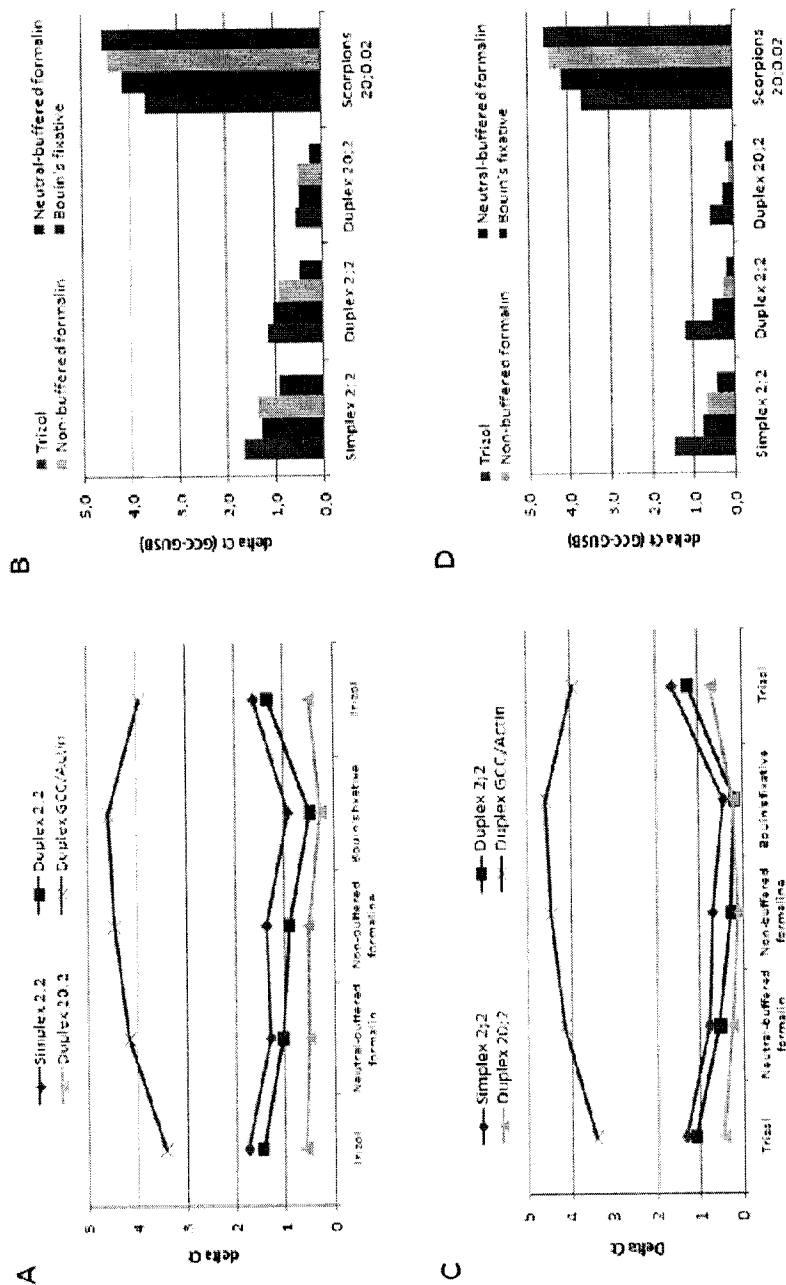
Figure 16. Comparison of GCC expression levels (delta-Ct) in cryo-sections of colon cancer tissue samples fixed or not. Delta Ct values $\Delta Ct = (Ct_{GCC} - Ct_{RG})$ were determined for each RNA extract using either GUSB (A and B) or HPRT1 (C and D). The reverse transcription reactions were performed in simplex using gene-specific primers at 2 µM and in duplex with indicated primers concentration. The real-time PCR were carried out in a 20µl reaction volume with the Applied Biosystems 7900HT Fast Real-Time PCR Systems using either TaqMan® simplex or duplex assay were compared to Scorpions™ duplex reaction. All reactions were performed in triplicate. Variation of less than 1 delta-Ct between frozen and fixed samples was considered not significant.

Figure 17. Amplification of reference genes in RNA extracted from 55 FPE pericolonic lymph node tissues with different archiving times from 1 month to 22 years. A) Capillary electrophoresis profiles of RNA extracted from archival FPE tissues. One µl of each RNA extract (150ng/µL) was analysed using the Agilent 2100 Bioanalyser and RNA Nano Chips. B) Ct values for ACTB and GUSB in FPE colon lymph node tissues. The real-time PCR were carried out in simplex reactions of 20 µl using the Applied Biosystems 7900HT Fast Real-Time PCR Systems and TaqMan Fast Universal PCR Master Mix.
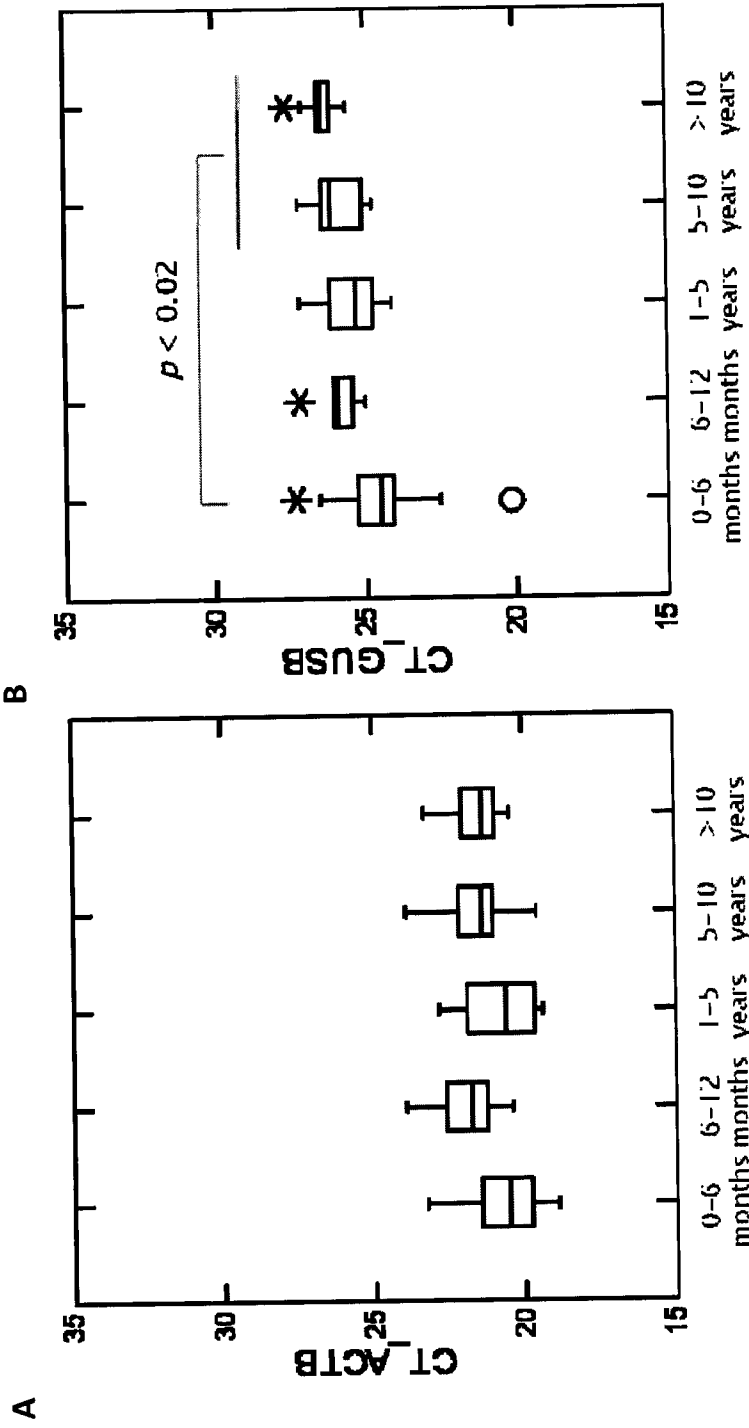
Figure 18. Comparison of ACTB and GUSB Ct value observed in group of blocks with different archiving time. Box-and-Whisker plots of the ACTB (A) and GUSB (B) mRNA expression (Ct value) in FPE colon lymph node tissues. For multiple comparisons, one-way ANOVA and post-hoc Turkey's test were used and $P < 0.05$ was considered statistically significant.

Figure 19. GCC relative expression levels in histopathology-negative and GCC/ACTB-negative (pN0(mol-)) and stage III GCC/ACTB-Positive (pN1-2(mol+)) LNs tested with the TaqMan GCC/GUSB assay. A receiver operating characteristic (ROC) analysis of the relative quantification using delta-Ct ($Ct_{GUSB} - Ct_{GCC}$) was used to determine a cut-off value for the GCC/GUSB duplex assay.
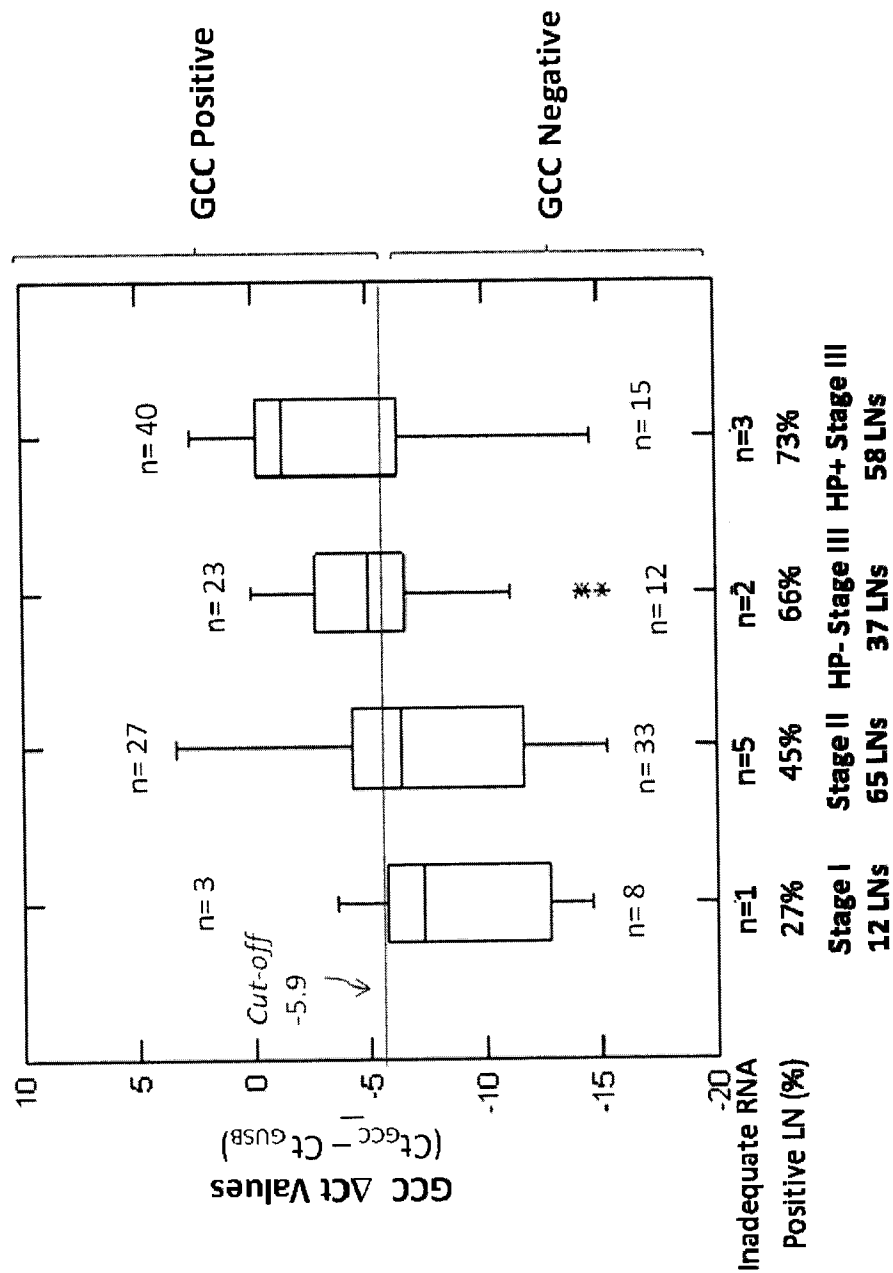
Figure 20. GCC relative expression levels evaluated with the TaqMan GCC/GUSB duplex assay. GCC relative expression delta-Ct ($Ct_{GUSB} - Ct_{GCC}$) in colon cancer stage I, II and III positive (HP+) LNs. GCC mRNA positive status was based on the analytical cut-off value of -5.9.

Figure 21. Average expression stability values of control genes in blood samples using geNorm.

Figure 22. Determination of the optimal number of reference genes for normalization using geNorm.
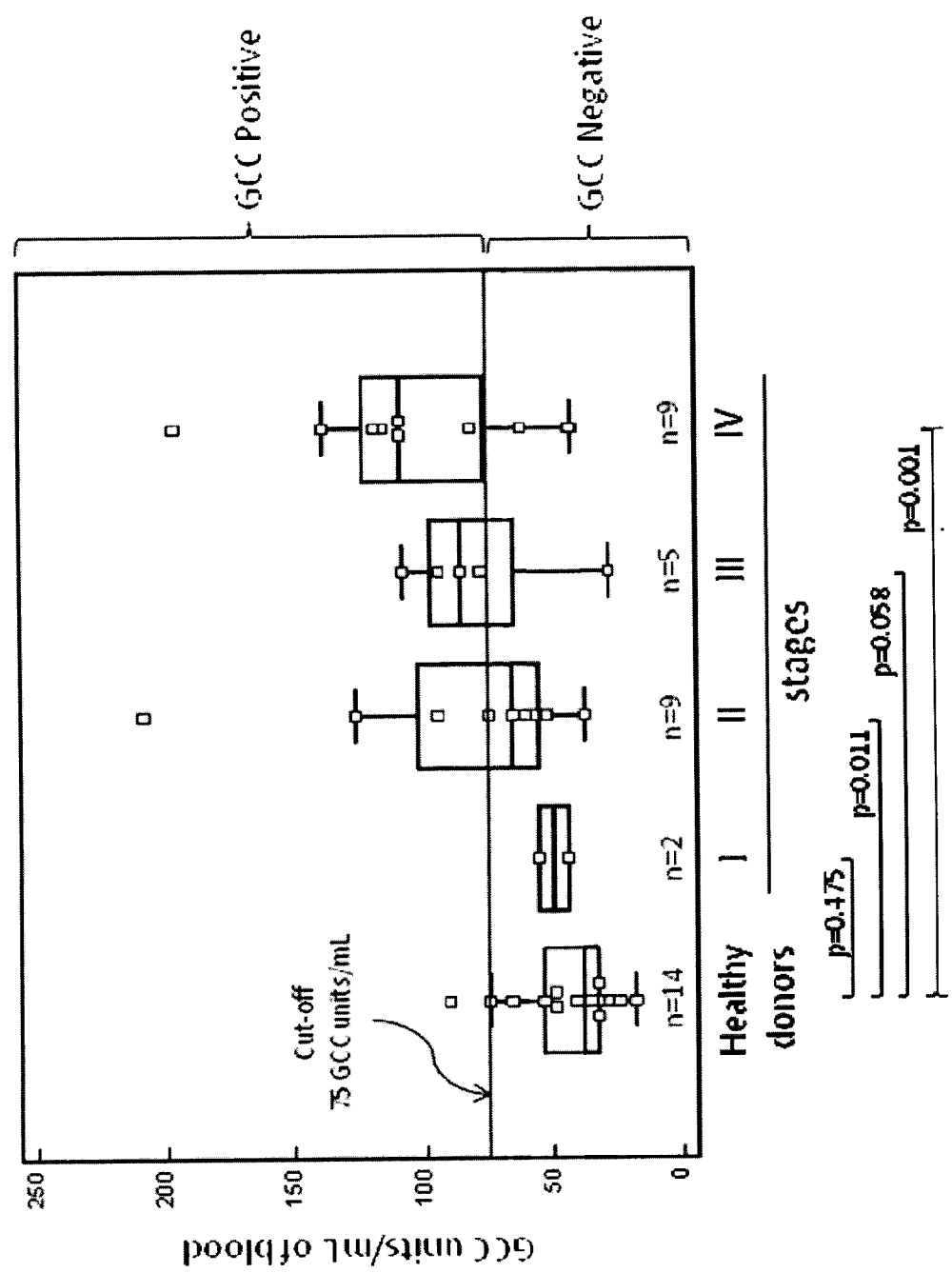
Figure 23. GCC expression level in blood samples. GCC mRNA positive status was based on the analytical cut-off value of 75 GCC units/mL.
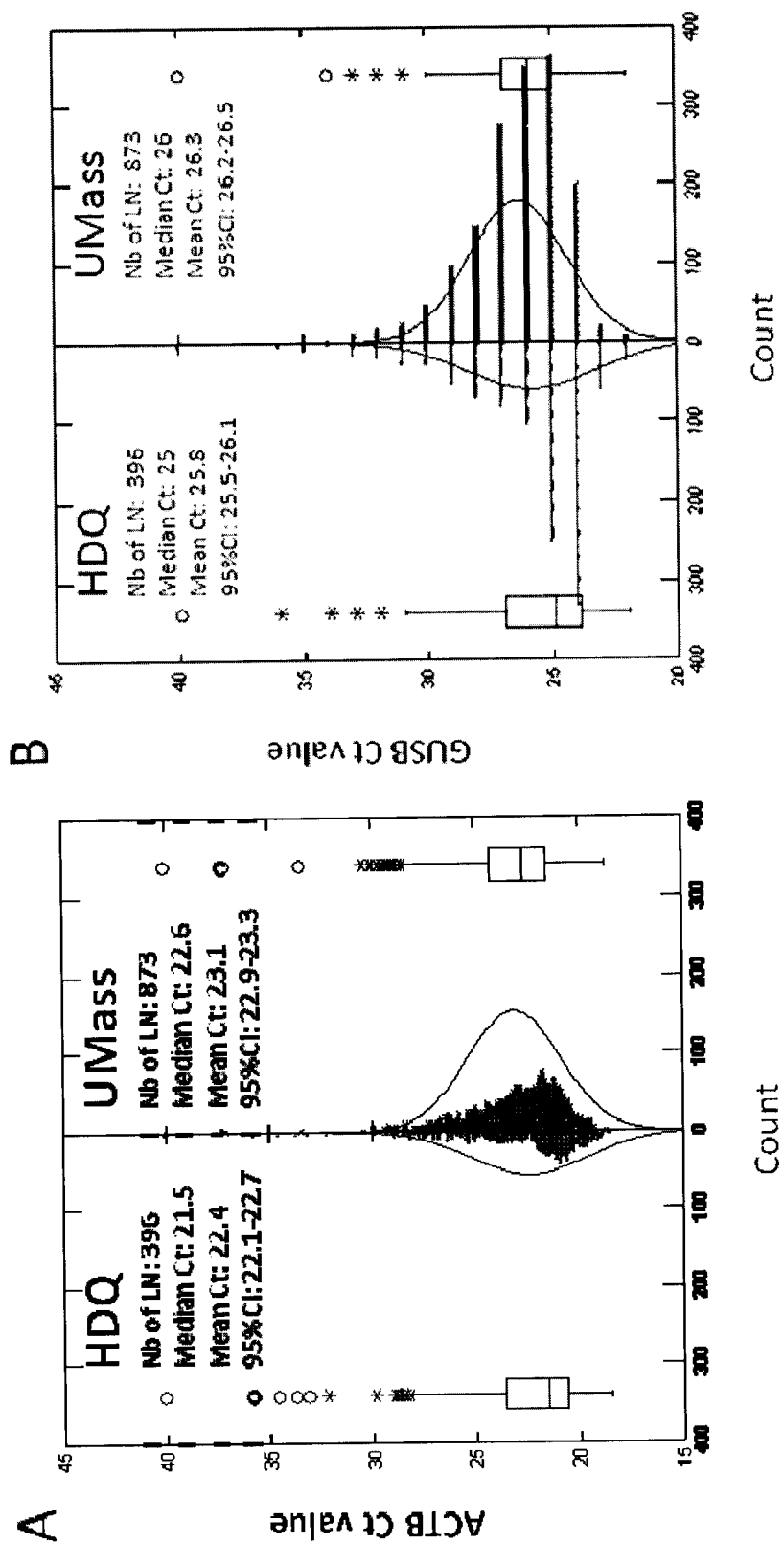
Figure 24. Distribution of A) ACTB Ct values and B) GUSB Ct values for two different patient cohorts. Wilcoxon rank test p-value: 0.0001.

Copies/Rx	Sensitivity (%)	Specificity (%)
0	100,0	0,0
1	70,8	23,2
2	54,2	38,4
25	54,2	65,7
50	41,7	74,8
100	33,3	86,9
200	29,2	89,9
400	16,7	92,9
800	12,5	93,9
1600	8,3	97,0

Copies/Rx	Sensitivity (%)	Specificity (%)
0	100,0	0,0
2	94,1	19,6
4	82,4	26,8
60	82,4	60,7
80	76,5	64,3
110	70,6	67,9
200	52,9	76,8
300	82,4	80,4
425	52,9	91,1
1100	11,8	91,1
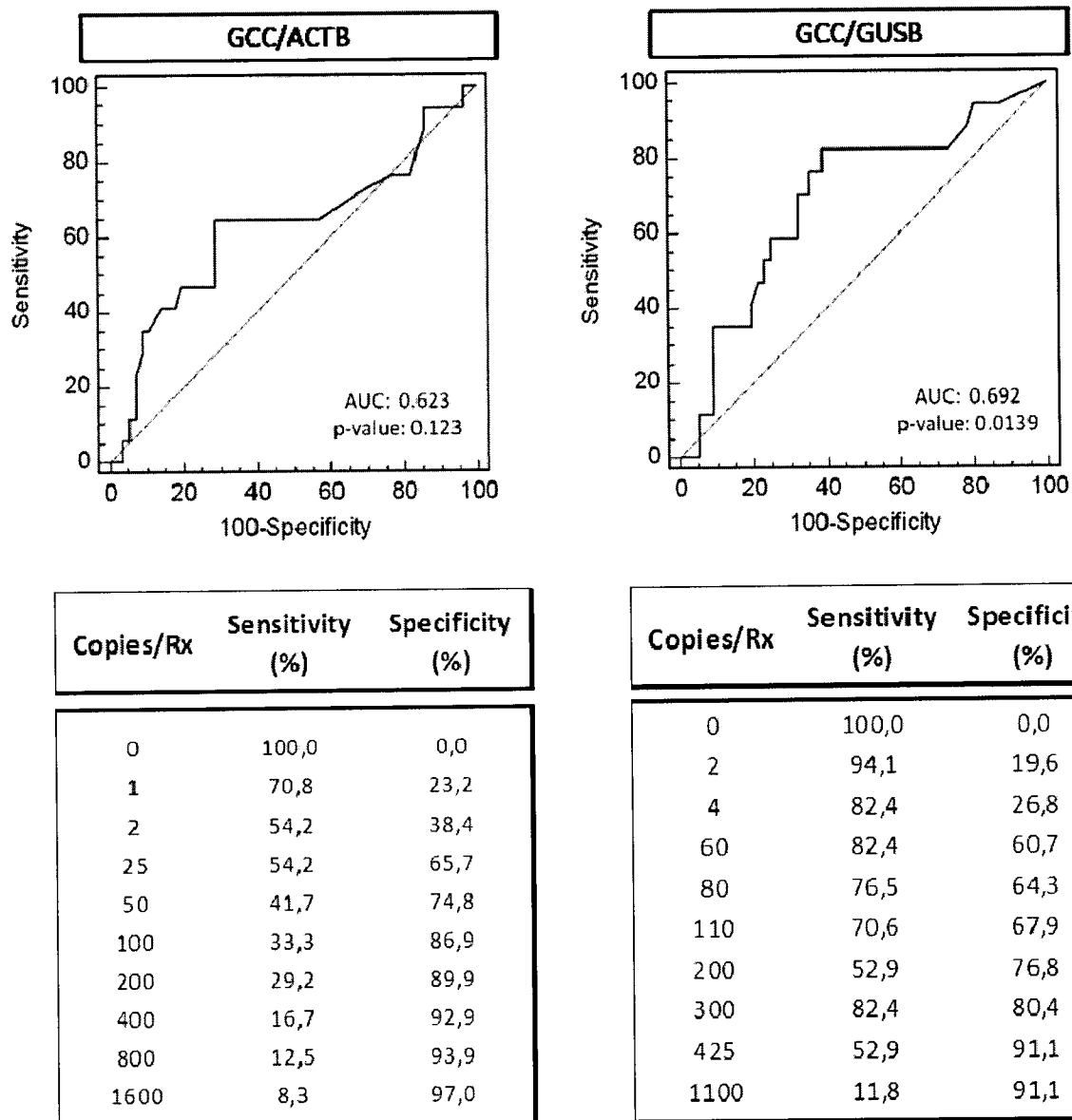
Figure 25. Combination of HDQ and UMass cohorts (n=73) ROC curve analysis for both GCC/ACTB and GCC/GUSB assays taking the highest GCC copies of any given LN of a case as the continuous variable. Sensitivity is defined as the detection rate of recurrent cases (after 36 months) while specificity is defined as the proportion of negative cases without recurrence.
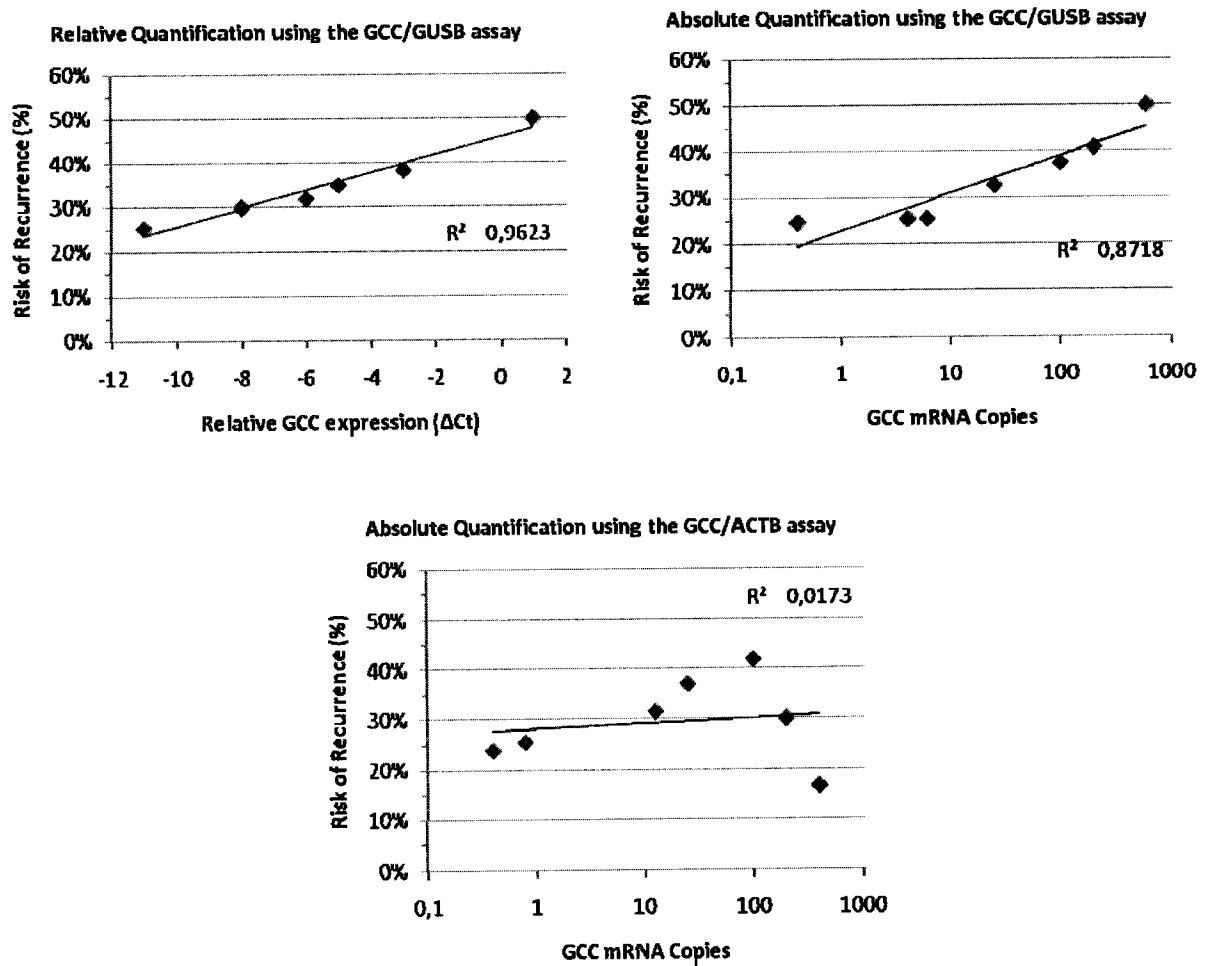
Figure 26. Relation between risk of recurrence for patients with a GCC positive test result and the GCC expression level used to determine test positivity.
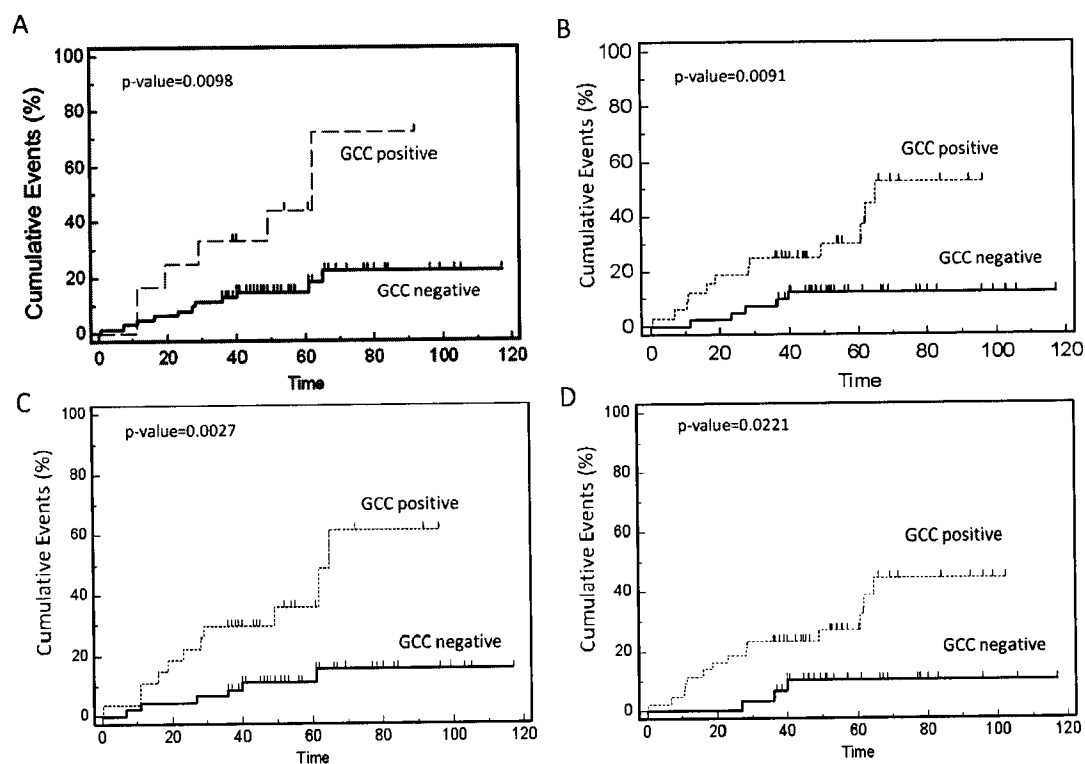
Figure 27. Kaplan-Meier graphical analysis of time to recurrence based on GCC positivity.
A) GCC/ACTB test with 100 copies cut-off; B) GCC/GUSB test with 100 copies cut-off; C) GCC/ACTB test with 25 copies cut-off; D) GCC/GUSB test with 25 copies. The GCC Negative cases are represented by the straight black line and the GCC Positive cases by the dashed gray line. Patients lost to follow-up were censored-out and are represented by straight up marks.
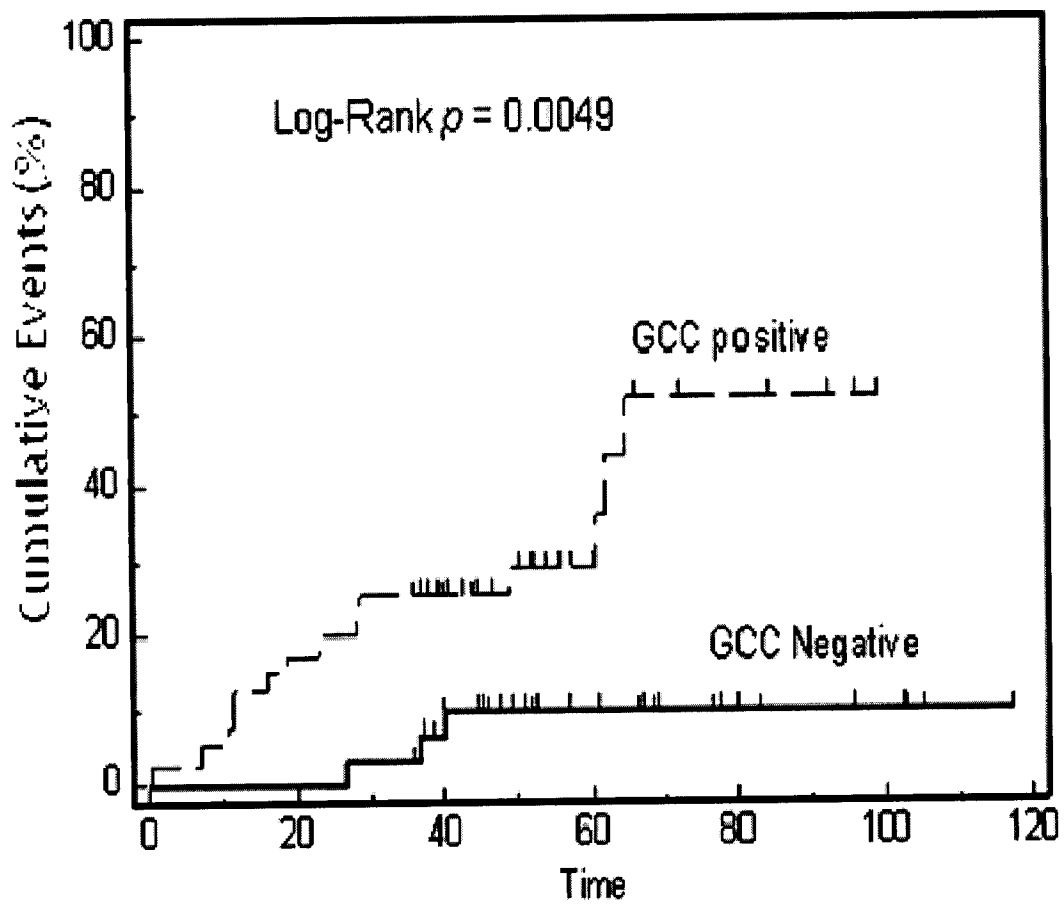
Figure 28. Kaplan-Meier graphical analysis of RFS based on the GCC positivity for the GCC/GUSB test with -
5.9 ΔCt for cut-off. The GCC Negative cases are represented by the straight line and the GCC Positive cases by
the dashed line. Censored-out cases are represented by straight up marks.
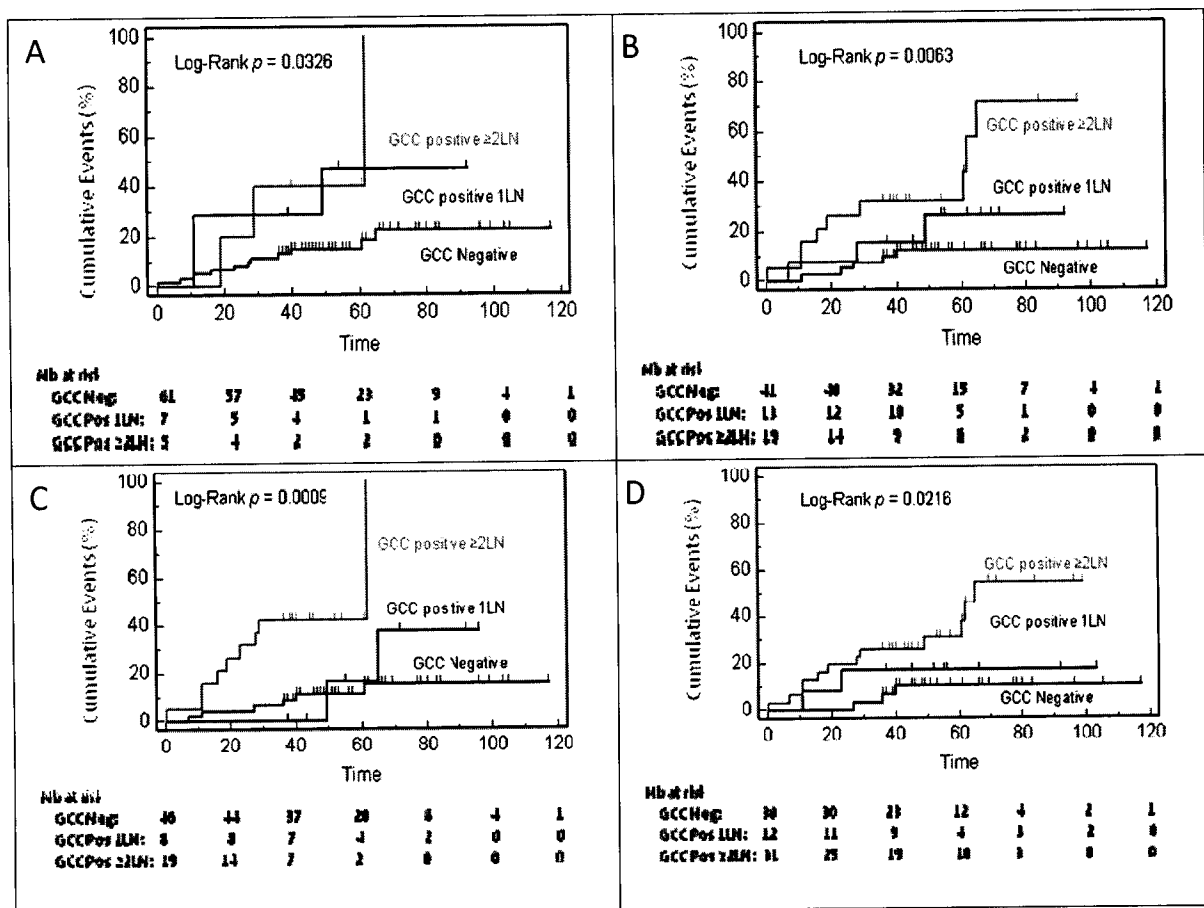
Figure 29. Kaplan-Meier graphical analysis of time to recurrence with 2 levels of stratification for GCC positive patients. A) GCC/ACTB test with 100 copies cut-off; B) GCC/GUSB test with 100 copies cut-off; C) GCC/ACTB test with 25 copies cut-off; D) GCC/GUSB test with 25 copies. Number of patients at risk is also indicated for each group.
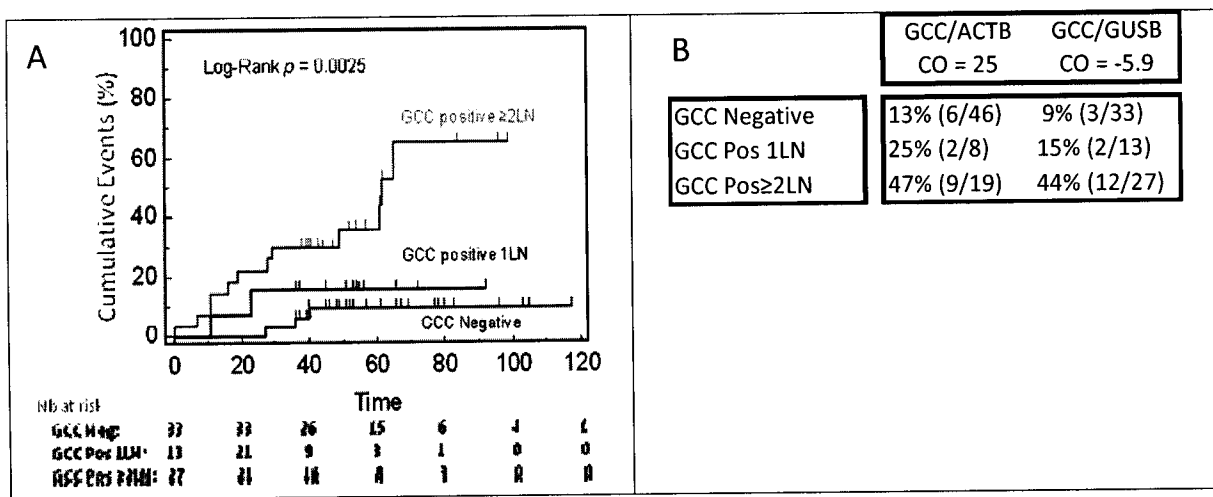
Figure 30. Kaplan-Meier graphical analysis of time to recurrence with 2 levels of stratification for GCC positive patients. A) GCC/GUSB test with -5.9 delta-Ct for cut-off. Number of patients at risk is also indicated for each group. B) Comparison of total recurrence rate between GCC/GUSB (delta-Ct = -5.9) and GCC/ACTB (GCC copies = 25)
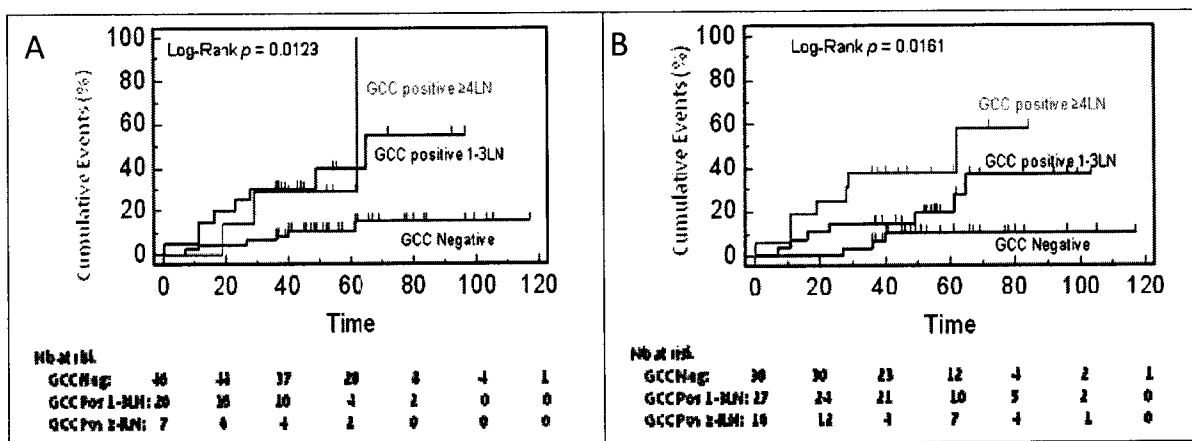
Figure 31. Kaplan-Meier graphical analysis of time to recurrence with stratification for the number of GCC positive LNs per patients. A) GCC/ACTB test with 25 copies cut-off; B) GCC/GUSB test with 25 copies cut-off. Number of patients at risk is also indicated for each group.

A.	CLASSIFICATION OF SUBJECT MATTER

IPC: *C12Q 1/527* (2006.01) , *C12Q 1/34* (2006.01) , *C12Q 1/68* (2006.01) , *G01N 33/573* (2006.01) , *G01N 33/574* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: *C12Q 1/527* (2006.01) , *C12Q 1/34* (2006.01) , *C12Q 1/68* (2006.01) , *G01N 33/573* (2006.01) , *G01N 33/574* (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
EPODOC, Canadian Patent Database, Scopus, Google (keywords: Guanylyl cyclase, glucuronidase, colorectal cancer, lymph, GUCY2C), GenomeQuest (SEQ ID NO:17-43)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BUSTIN, S. and MUELLER, R., "Real-time reverse transcription PCR and the detection of occult disease in colorectal cancer", Molecular Aspects of Medicine. June 2006 (06-2006), Vol. 27, pages 192-223. ISSN 0098-2997 *pp. 201, 209-210*	1-15, 18-21, 37-59
X	WO2008/045133 A2 (WANG, Y. et al.) 17 April 2008 (17-04-2008) *page 17, SEQ ID NO:44-46*	20-36
X	US2004/0018494 A1 (SHURTLIFF, R et al.) 29 January 2004 (29-01-2004) *page 5, SEQ ID NO:10*	20, 21, 23, 28, 33

[X] Further documents are listed in the continuation of Box C.	[X] See patent family annex.

*	Special categories of cited documents :	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 2010 (20-04-2010)	31 May 2010 (31-05-2010)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Jeremy Mclean (819) 994-9302

Box No. II	Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. :   16 and 17
    because they relate to subject matter not required to be searched by this Authority, namely :

    see supplemental sheet

2. [   ] Claim Nos. :
    because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [   ] Claim Nos. :
    because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III	Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. [   ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [   ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [   ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [   ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest   [   ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[   ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[   ] No protest accompanied the payment of additional search fees.

Box No. I	Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

    a.   (means)

      [X] on paper

      [X] in electronic form

    b.   (time)

      [X] in the international application as filed

      [   ] together with the international application in electronic form

      [   ] subsequently to this Authority for the purposes of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3.   Additional comments :

C (Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	GREEN, T. et al., "Validation of putative reference genes for normalization of Q-RT-PCR data from paraffin-embedded lymphoid tissue", Diagnostic Molecular Pathology. December 2009 (12-2009), Vol. 18, pages 243-249. ISSN 1052-9551 *whole document*	1-15, 18-59
A	WALDMAN, S. et al., "Association of GUCY2C expression in lymph nodes with time to recurrence and disease-free survival in pN0 colorectal cancer", Journal of the American Medical Association. 18 February 2009 (18-02-2009), Vol. 301, pages 745-752. ISSN 0098-7484 *whole document*	1-15, 18-59
A	CRONIN, M et al., "Measurement of gene expression in archival paraffin-embedded tissues: Development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay", American Journal of Pathology. January 2004 (01-2004), Vol. 164, pages 35-42. ISSN0002-9440 *page 36, Figure 3 legend*	1-15, 18-59

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2008045133A2	17-04-2008	CA2644586A1	17-04-2008
		CN101437962A	20-05-2009
		EP1996729A2	03-12-2008
		JP2009528825T	13-08-2009
		MX2008011356A	15-09-2008
		US2008058432A1	06-03-2008
US2004018494A1	29-01-2004	none	

Continuation of Box II:

Claims 16 and 17 are directed to a method with a step involving a purely mental act which the International Search Authority is not required to search. The subject matter involving prescribing a treatment accomplishes a result by means of a person's reasoning, in which the quality or character of the result may vary depending upon the skill, interpretation, or judgement of the person performing the method or process.