METHODS FOR THYROID CELL DETECTION

The present invention provides a sensitive assay for the detection of thyroid-specific gene transcripts in a biological sample, for example, a blood sample from a clinical patient, wherein a positive detection result indicates the presence of thyroid epithelial cells in that sample. The invention is useful in the detection of thyroid cancer recurrence in patients who have already undergone thyroid ablation as treatment for their disease. Unlike current assay methods, which result in suffering and risk to the patient by requiring removal of the patient from a post-surgical regimen of thyroid hormone replacement therapy, the technique disclosed herein retains sensitivity without such trauma to the patient and is unaffected by circulating anti-thyroglobulin antibodies that interfere with conventional assays in 10–25% of patients.
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Methods for Thyroid Cell Detection

This application claims the benefit of U.S. Provisional Application No. 60/049,144, filed June 10, 1997.

Field of the Invention

The present invention relates to the field of thyroid endocrinology, in particular thyroid cancer therapy and monitoring. The present invention relates to an assay useful for monitoring individuals for presence of viable thyroid tissue. The present invention relates to a RT-PCR assay for thyroid-specific mRNA transcripts in peripheral blood samples, the presence of which transcripts indicates the presence of circulating thyroid cells. In patients who have undergone thyroidectomy, for example as a treatment for thyroid malignancy, presence of functional thyroid cells indicates the persistence or recurrence of disease.

Background of the Invention

Approximately 13,900 patients are diagnosed with thyroid carcinoma yearly in the United States (Cancer Facts and Figures, American Cancer Society, Atlanta, 1995). The majority of these patients have differentiated thyroid carcinoma and are successfully treated by thyroidectomy using surgery and radiiodine (¹³¹I) ablation of residual thyroid tissue, followed by life-long suppression of thyroid-stimulating hormone (TSH or thyrotropin), a hormone that promotes growth and proliferation of thyroid cells.

Because TSH is released only when serum levels of thyroid hormone are low or normal, down-regulation of TSH secretion is achieved with administration of thyroid hormone (L-thyroxine), which both completes the negative feedback loop that inhibits release of TSH and prevents hypothyroidism in post-surgical thyroid cancer patients (see Figure 1). In general, thyroid cancer affects a relatively young population, with most patients in their 40’s and 50’s. Thus, in
this country an estimated 188,000 individuals are monitored for tumor
recurrence or progression of persistent disease, as either may occur many years
after initial treatment (National Cancer Institute Fact Book, U.S. Department of
Health and Human Services, Public Health Service, National Institutes of Health,
currently utilized to detect recurrent or residual thyroid cancer include periodic
total body iodine radioisotope ($^{131}$I) scanning and immunoassay of serum
concentrations of thyroglobulin protein. Remission is presumed in athyreotic
thyroid cancer patients in whom $^{131}$I uptake or serum thyroglobulin (Tg) values
are undetectable. To make $^{131}$I scanning and serum Tg measurement more
sensitive, thyroid hormone is withdrawn, inducing hypothyroidism, with
consequent hypersecretion of TSH. The elevated serum TSH causes any
remnant thyroid tissue to be maximally stimulated to proliferate and, inter alia, to
produce and secrete Tg and to concentrate $^{131}$I. Thus, adequate screening is
possible only following a period of withdrawal from thyroid hormone therapy

There are distinct drawbacks to this protocol for monitoring thyroid
function: In athyreotic patients, temporary withdrawal from thyroid hormone
results in severe, hypothyroidism that is associated with significant morbidity.
Occasionally, where residual malignant thyroid tissue is present, withdrawal of
thyroid hormone leads to rapid acceleration of tumor growth (Sfakianakis et al.,
1975, Ohio State Med. J., 71: 78-82.). Moreover, 15-30% of patients with
thyroid cancer have serum antibodies to Tg that distort Tg values in various
immunoassays. Similar artifacts are observed when excessive amounts of
circulating Tg in the sample exceed the binding capacity of the capture antibody
on the solid support in an immunometric assay (Torrens and Burch, 1996, The
Endocrinologist, 6: 125-144). Finally, some thyroid cancers produce variant
forms of Tg that escape detection by standard Tg immunoassays, and thus yield
falsely low values of serum Tg (Olivieri et al., 1991, Thyroidology, 3: 13-16).

Radioimmunoassay (RIA) of serum Tg and measurements of $^{131}$I indicate
the recurrence of cancer indirectly, because a positive result requires that the function of thyroid cells be observable; in addition, the cells must be differentiated in order to synthesize Tg and to concentrate $^{131}$I, as these cellular activities are typical only of mature, stimulated thyroid epithelial cells. As a result, the utility of such tests is limited by the functional status of the thyroid cells to be detected.

The drawbacks of current methodology would be overcome by an assay that more directly detects the presence of thyroid cells, that detects thyroid cells that are not fully differentiated or not normally functional, and that would be easily performed with widely available laboratory equipment, and without the necessity of exposing the patient to the dangers and discomfort of thyroid hormone withdrawal. A further advantage would be an assay that could be performed using an easily obtained peripheral blood sample and that would be more sensitive than currently available immunoassays and would not be susceptible to artifacts that confound standard assays.

These benefits and advantages are realized in an assay described herein for identifying the presence of thyroid cells in peripheral blood by detecting expression of the thyroid cell-specific mRNA for Tg or other thyroid-indicative proteins in circulating cells.

**Summary of the Invention**

The present invention provides a method for the detection of the presence of thyroid epithelial cells in a patient, comprising the step of detecting a transcript of a thyroid specific gene in a sample of reverse-transcribed total RNA of cells of blood, wherein detection is indicative of the presence of thyroid epithelial cells.

Another aspect of the invention is a method for monitoring in a recovering thyroid cancer patient the recurrence of disease, comprising the step of detecting in a sample of reverse-transcribed total RNA of cells of blood a transcript of a thyroid-cell-specific gene, wherein the cells of blood are obtained
from a recovering thyroid cancer patient and wherein detection of such a transcript is indicative of the recurrence of disease.

As used herein, the terms "reverse-transcribed RNA" and "reverse-transcribed total RNA" refer to DNA which is produced by the method in which an mRNA template is primed for second-strand nucleic acid synthesis by hybridization of an oligonucleotide primer, which synthesis is then carried out using the enzyme reverse transcriptase. Such DNA is referred to as complementary DNA (cDNA).

As used herein in reference to reverse-transcribed RNA, the term "total" refers to cDNA which is produced by a synthesis reaction in which hybridization of the oligonucleotide primer to the mRNA template is not limited by sequence specificity to a particular transcript, but instead may prime all, or nearly all (e.g. greater than 90%, preferably greater than 95%, and most preferably greater than 99%) of the mRNA.

As used herein, the term "oligonucleotide primer" refers to a nucleic acid molecule 6 to 100 nucleotides or ribonucleotides in length, preferably 10 to 40 nucleotides in length, most preferably 15 to 30 nucleotides in length, which is used to prime an enzymatic nucleic acid synthesis reaction. An oligonucleotide primer may anneal either to a sequence of a particular gene of interest, or to a sequence common to many genes; in the latter case, the term "oligonucleotide primer" may refer to a pool of nucleic acid molecules wherein each molecule has the same sequence (e.g., oligo dT or another sequence which is shared among many genes), or wherein the molecules of the pool differ in sequence from one another (e.g., a pool of random oligonucleotides). The sequence to which an oligonucleotide primer or oligonucleotide probe, as defined below, anneals is termed a "site", also as defined below.

As used herein in reference to a gene, mRNA transcript or protein, the term "thyroid-specific" preferably refers to that which is expressed or otherwise found or produced only in cells of thyroid tissue, particularly in thyroid epithelial cells, but may also include a gene, mRNA transcript or protein which is
expressed in thyroid cells or tissue and is additionally expressed in one or a plurality of other cell or tissue types, which plurality does not encompass all cell types of a human or other subject mammal. It is contemplated that when the term “thyroid-specific” refers to a gene, mRNA or protein which is expressed or otherwise found or produced in tissues in addition to thyroid tissue, the number of additional tissues typically will be small (e.g., 1 to 5).

As used herein, the term “gene” refers to a nucleic acid sequence of a human or other mammal which is transcribed, and includes exons and introns.

As used herein, the term “exon” refers to a portion of a gene, which portion is selected from the group that includes protein coding sequences, 5'-untranslated sequences and 3'-untranslated sequences of a gene.

As used herein, the term “intron” refers to a non-coding portion of a gene which is excised from between adjacent exons during post-transcriptional processing of an mRNA molecule. Such splicing occurs at the 5' and 3' ends of an intron, such that the 3' end of the exon upstream of an intron is joined to the 5' end of an exon downstream of the intron. The positions of the mRNA molecule at which cleavage and re-ligation (re-joining) occur are termed either intron- or intron/exon “splice sites” or “splice junctions”.

As used herein, the term “nucleic acid” refers to DNA and RNA, which may be either single- or double-stranded and may be either linear or circular.

Preferably, the reverse-transcribed total RNA is from a cell lysate of cells isolated from peripheral blood.

As used herein, the term “peripheral blood” refers to blood which is drawn from a vessel (e.g. a vein) that does not drain from the tissue of interest, in this case, thyroid tissue. Peripheral blood which is advantageously used according to the invention includes, but is not limited to, blood which is drawn from a brachial or femoral vein.

In preferred embodiments, the invention encompasses an assay for the presence of thyroid epithelial cells in a peripheral blood sample from a patient, performed by lysing the cells of the blood sample, isolating RNA from the lysed
cells of the sample and detecting thyroid-cell-specific transcripts in the RNA.

Thus, in one such preferred embodiment, thyroid cells are detected by the method comprising:

(a) collecting a sample of whole blood,

(b) lysing the cells in the sample to form a cell lysate,

(c) precipitating the total RNA in the cell lysate, e.g., with isopropanol,

and

(d) subjecting the resuspended RNA to analysis in order to detect thyroid-specific mRNA, the detection of thyroid-specific mRNA indicating the presence of living thyroid tissue.

Preferably, DNA and proteins are removed from the cell lysate prior to precipitating the said total RNA.

Preferably the RNA analysis comprises subjecting the RNA to reverse transcription and the polymerase chain reaction (RT-PCR), wherein a first strand cDNA transcript is prepared from mRNA, followed by PCR amplification of all or a specific fraction of the resulting cDNA. PCR is advantageously directed at amplification of thyroid-specific transcripts, that is, cDNA encoding protein products that are specific to living thyroid tissue, such as thyroglobulin (Tg), thyroid peroxidase (TPO), the sodium iodide symporter (NIS), Pax-8, thyroid transcription factor 1 (TTF-1) and thyroid transcription factor 2 (TTF-2). Most preferably, the analysis is directed at detecting transcripts of one or more of the group consisting of Tg, NIS, Pax-8, and TTF-1.

A most sensitive assay utilizes a reverse transcriptase reaction that is primed with oligo-dT or random oligonucleotides to optimally synthesize a pool of cDNA. Most preferably, the reverse transcription will be primed with random hexanucleotides so as to create a heterogeneous pool of cDNA transcripts that is representative of the population of mRNA molecules in thyroid cells, allowing for the subsequent PCR amplification of multiple thyroid-specific transcripts.

It is further preferred that a PCR amplification of the cDNA is primed with pairs of oligonucleotides that anneal to sequences unique to thyroid-cell-specific
gene exons and that each oligonucleotide of the pair of primer oligonucleotides anneals to sequences in a different exon of the thyroid-cell-specific gene than does the other, such that one or more introns are present between the exons. This protocol allows for the generation of DNA fragments after PCR of cDNA that are smaller than the size of DNA fragments that would be generated should contamination with genomic DNA occur.

Another aspect of the present invention is a method for the detection of the presence of thyroid epithelial cells in a patient, comprising providing a sample of reverse-transcribed RNA from cells of blood of a patient, performing a polymerase chain reaction (PCR) to amplify thyroid-cell-specific transcripts present in the reverse-transcribed RNA, wherein the PCR is primed with oligonucleotides that hybridize to a unique pair of sites comprising a first site and a second site in a thyroid-cell-specific gene and wherein the first and second sites are present in two different exons of the gene, such that their predicted PCR product spans one or a plurality of introns, and, performing a detection step to detect such a PCR product, wherein detection of the PCR product is indicative of the presence of thyroid epithelial cells.

Preferably, the sample comprises reverse-transcribed total RNA.

As used herein in reference to the priming of a nucleic acid synthesis reaction or nucleic acid detection procedure, the term “site” refers to a nucleic acid sequence present in a gene, mRNA transcript or cDNA, which sequence is long enough to permit specific hybridization of an oligonucleotide primer or probe (i.e., hybridization under stringent conditions) yet sufficiently short to allow for the exclusion of highly repetitive nucleic acid sequences; such a sequence is usefully from 6 to 100 nucleotides in length, preferably from 10 to 40 nucleotides in length, and most preferably from 15 to 30 nucleotides in length.

As used herein with regard to nucleic acid hybridization, the term “stringent conditions” refers to salt concentrations of less than about 1M, more usually less than about 500 mM and preferably less than about 200 mM.

Hybridization temperatures range from as low as 0°C to greater than 22°C,
greater than about 30°C, and (most often) in excess of about 37°C. Longer fragments may require higher hybridization temperatures for specific hybridization. As several factors affect the stringency of hybridization, the combination of parameters is more important than the absolute measure of any one alone.

As used herein in reference to sites to which the primers of a pair of PCR primers anneal, the term “unique” refers to the presence of such a pair of sites only in the thyroid-specific gene of interest, insofar as such information is known. Where the complete genomic sequence of an organism, such as a human, is unknown, a pair of primer sequences (and, hence, the sites to which they bind) are tested empirically for uniqueness in a biological sample (e.g., a blood sample) comprising the transcript from the organism prior to use of the primer pair in the methods of the invention. A single band of the size predicted from prior knowledge of the relevant mRNA sequence, either alone or accompanied by a single larger band resulting from amplification of the corresponding genomic DNA sequence comprising one or more intronic regions, is indicative of uniqueness of the sites to the gene of interest.

As used herein, the term “biological sample” refers to a whole organism or a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, serum, plasma, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). “Biological sample” further refers to a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof. Lastly, “biological sample” refers to a medium, such as a nutrient broth or gel in which an organism has been propagated, which contains cellular components, such as nucleic acid molecules. Most preferred in the present invention is a biological sample which comprises peripheral blood of a human.

The invention further encompasses a method for the detection of the presence of thyroid-cell-specific mRNA transcripts in a patient, comprising the
steps of providing a sample of reverse-transcribed total RNA from cells of blood, performing a polymerase chain reaction (PCR) to amplify thyroid-cell-specific transcripts present in the sample, wherein the PCR is primed with oligonucleotides that hybridize to a unique pair of sites comprising a first site and a second site in a thyroid-cell-specific gene and wherein the first and second sites are present in two different exons of the gene, such that their predicted PCR product spans one or a plurality of introns, and, performing a detection step to detect a PCR product which is thyroid-cell-specific, wherein detection of such a PCR product is indicative of the presence of thyroid-cell-specific mRNA transcripts in the patient.

The invention additionally provides a method for monitoring in a recovering thyroid cancer patient the recurrence of disease, comprising the steps of providing a sample of reverse transcribed RNA from cells of blood, wherein the cells are obtained from a recovering thyroid cancer patient, performing a polymerase chain reaction (PCR) to amplify thyroid-cell-specific transcripts present in the sample, wherein the PCR is primed with oligonucleotides that hybridize to a unique pair of sites comprising a first site and a second site in a thyroid-cell-specific gene and wherein the first and second sites are present in two different exons of the gene, such that their predicted PCR product spans one or a plurality of introns, and, performing a detection step to detect a PCR product, wherein detection of a PCR product is indicative of the recurrence of disease in the patient.

Preferably, in the above-described methods of the invention, the cells of blood are obtained from whole blood.

Alternatively, it is preferred that the cells of blood are obtained from a fraction of whole blood enriched for one or a plurality of cell types.

In preferred embodiments, such a fraction is isolated by centrifugation and is the erythrocyte fraction.

It is also preferred that the fraction is isolated by cell sorting.

Preferably, cell sorting is performed using an antibody or plurality of
antibodies recognizing an epitope or plurality of epitopes specific to epithelial cells, which become selectively enriched in the cell fraction so isolated.

It is preferred that the antibody is anti-cytokeratin.

Preferably, the epithelial cells are thyroid epithelial cells.

It is preferred that the antibody is selected from the pair including anti-thyroglobulin and anti-thyroid-stimulating-hormone-receptor, and highly preferred that the antibody is anti-thyroid-stimulating-hormone-receptor.

In the above-described methods, it is preferred that the RNA or the total RNA is tested to detect a thyroid-cell-specific transcript encoding a protein selected from the group that includes thyroglobulin (Tg) and the sodium iodide symporter (NIS) and the detection is effective to detect the presence of differentiated thyroid cells.

Preferably, the RNA or total RNA is tested to detect said thyroid-cell-specific transcripts selected from the group encoding Tg, Pax-8 and TTF-1, and said detection is effective to detect the presence of undifferentiated- or poorly-differentiated thyroid cells.

In a particularly preferred embodiment, providing of a reverse-transcribed sample comprises the step of performing a reverse transcription of mRNA of cells of blood from the patient.

It is preferred that the reverse transcription is primed with primers selected from the group that includes oligo-dT and random oligonucleotides.

It is also preferred that the oligonucleotides are hexanucleotides.

Preferably, the thyroid-cell-specific gene encodes a protein selected from the group comprising thyroglobulin (Tg), thyroid peroxidase (TPO), Pax-8, thyroid transcription factor 1 (TTF-1) and thyroid transcription factor 2 (TTF-2) and the sodium iodide symporter (NIS), more preferably, the thyroid-cell-specific gene encodes a protein selected from the group comprising Tg, Pax-8, TTF-1 and NIS, and, most preferably, the thyroid-cell-specific gene encodes Tg.

Highly preferred where the thyroid-cell-specific gene encoding Tg is to be amplified is the use of a PCR primer pair comprising:
mdr5: 5'-TGTGAGCTGCAGAGGAAACGCCC-3'  [SEQ ID NO: 1, nucleotides 141 through 164], and mdr7: 5'-ATACACCTCCATCCCCCTCTGCGTCCACACA-3'  [SEQ ID NO: 1, reverse complement of nucleotides 459 through 488], or, alternatively, a primer pair comprising:

5' GTGCCAACGCGAGTGAAGT 3'  [SEQ ID NO: 1, nucleotides 262 through 280], and
5' TCTGCTGTTTCTGTAGCTGACAAA 3'  [SEQ ID NO: 1, reverse complement of nucleotides 325 through 348].

Most preferred, when the thyroid-cell-specific gene encodes Pax-8, is use of a PCR primer pair comprising the following:

5' AAGGTGGTGGAGAAAGATTGGG 3'  [SEQ ID NO: 2, nucleotides 269 through 289], and
5' GAGGTGGTGCTGGCTGAAGGC 3'  [SEQ ID NO: 2, reverse complement of nucleotides 689 through 709].

Most preferred, when the thyroid-cell-specific gene encodes TTF-1, is use of a PCR primer pair comprising:

5' ACCAGGACACCATGAGGAAC 3'  [SEQ ID NO: 3, nucleotides 640 through 659], and
5' TGTCCTTGGCCTGGCGTTCA 3'  [SEQ ID NO: 3, reverse complement of nucleotides 988 through 1008].

Most preferred, when the thyroid-cell-specific gene encodes NIS, is use of the PCR primer pair comprising:

5' ACACGTGACGACCCTCTCTCT 3'  [SEQ ID NO: 4, nucleotides 1139-1160], and
5' TGCTGAGGTGCACACTGTA 3'  [SEQ ID NO: 4, reverse complement of nucleotides 1260 through 1279].

It is preferred that the above methods comprise the step, after the step of detecting a PCR product which is thyroid-cell-specific, of performing a measurement to quantitate the amount of the product so detected.
Preferably, the measurement comprises use of a fluorometric oligonucleotide probe specific for one or more of the PCR products produced from the recited thyroid-cell-specific transcripts.

As used herein, the term "oligonucleotide probe" refers to a nucleic acid molecule having the properties of an oligonucleotide primer, as defined above, with the exception that it is complexed to a label (e.g., a fluorescent, chemiluminescent, radioactive or chromogenic molecule) and hybridized to a target nucleic acid molecule for the purpose of detecting such a molecule. Most useful are oligonucleotide probes which hybridize to a sequence which is found only in the gene or gene product which is to be detected.

It is preferred that the fluorometric oligonucleotide is labeled at its 5' end with a dye selected from the group that includes 6-carboxy-fluorescein (FAM), tetrachloro-6-carboxy-fluorescein (TET), 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE) and hexachloro-6-carboxy-fluorescein (HEX).

In a particularly preferred embodiment, the thyroid-cell-specific transcript is that which encodes Tg.

Preferably, the fluorometric oligonucleotide probe comprises the following nucleic acid sequence:

5' CCCTTCGTCCCTGTGAGCTGCA 3'. [SEQ ID NO: 1, nucleotides 130 through 151], OR

5' ACAGACAAGCCACAGGCGTCCT 3'. [SEQ ID NO: 1, reverse complement of nucleotides 299 through 321].

In another preferred embodiment, the thyroid-cell-specific transcript is that which encodes NIS.

Preferably, the fluorometric oligonucleotide probe comprises the following nucleic acid sequence:

5' CGGGGACTCCAGGCGATCTTCG 3' [SEQ ID NO: 4, reverse complement of nucleotides 1218 through 1240].

A final aspect of the present invention is a method for the detection of the presence of thyroid epithelial cells in a human, comprising the steps of using a
cell-sorting procedure to separate a cell fraction comprising the thyroid epithelial cells from a sample of blood of the human, and performing a detection step to detect the thyroid epithelial cells in the fraction.

It is preferred that the human is a recovering thyroid cancer patient.

Preferably, the cell-sorting procedure comprises magnetic cell sorting (MACS).

It is highly preferred that the cell-sorting procedure employs an antibody directed against a thyroid-cell-specific antigen, and most preferred that the thyroid-cell-specific antigen is the human thyrotropin receptor.

The present invention is useful for monitoring the recovery of individuals treated for thyroid disorders, such as thyroid carcinoma, with complete thyroidectomy. The method is also useful in any situation where extremely sensitive detection of any residual living thyroid tissue in an individual or a biological sample is desired.

The method of the present invention is much more sensitive than current methods in which antibodies are used to detect thyroid cell proteins or which are based on iodine metabolism. The method can make use of a small sample of whole blood and avoids the necessity and danger to the patient of withdrawal of thyroid hormone to induce hypothyroidism and consequent release of TSH.

Furthermore, in preferred aspects, the method is able to detect recurrence of abnormal thyroid tissue, e.g., thyroid tumors having poorly differentiated or undifferentiated thyroid epithelial cells.

**Brief Description of the Drawings**

Figure 1 is a schematic diagram illustrating the effect of thyroid stimulating hormone (TSH) on thyroid functions, such as cell proliferation, iodine uptake, transcription of thyroid-cell-specific genes and the production and release of thyroid hormone, which subsequently inhibits TSH release in a negative feedback loop.

Figure 2 shows ethidium bromide staining of RT-PCR products derived
from two thyroid mRNA dilution series. Approximate numbers of thyroid cells per ml of blood are indicated. In Fig. 2A, a positive thyroid control (Thy) is included, as well as two reverse-transcription-negative PCR controls (Thy and Ly) derived from thyroid and lymphocyte cells. In both Figs. 2A and 2B, a negative RT-PCR control performed on water was run (H₂O).

Figure 3 presents Tg RT-PCR results from normal subjects and recovering thyroid cancer patients. Iodine uptake scan results are indicated (1, normal control patient; 2, uptake in thyroid bed; 3, no uptake in thyroid bed; 4, metastases; 5, no metastases). Reverse-transcribed samples (+) as well as negative controls in which samples were not reverse transcribed (-) are presented. Negative reverse transcription (RT) and PCR controls performed on water are indicated (H₂O). Ethidium bromide staining is shown above the corresponding Southern blot.

Figure 4 is a standard curve for Tg mRNA quantitation. Threshold cycle is shown on the y-axis, starting quantity of material is shown on the x-axis. Slope = -2.780. Y-intercept = 23.640. Correlation coefficient = 0.912. (Filled dots = standard; hatched dots = unknowns.)

Figure 5 is a standard curve for NIS mRNA quantitation. Threshold cycle is shown on the y-axis, starting quantity of material is shown on the x-axis. Slope = -2.869. Y-intercept = 28.766. Correlation coefficient = 0.981. (Filled dots = standard; hatched dots = unknowns.)

Figure 6 presents the direct detection of thyroid cells in human peripheral blood.

**Detailed Description of the Preferred Embodiments**

The present invention relates to a novel method for detecting living thyroid tissue in a patient or a biological fluid such as a blood sample or even a cell culture suspected of containing thyroid cells. The method is especially useful where sensitive detection of any living thyroid epithelial cells is critical.

Although the method of the present invention may be applied in any
circumstance where detection of thyroid cells is important, the most important application of the method is in detection of residual living thyroid tissue in individuals who have undergone therapeutic thyroidectomy, for instance as a treatment for thyroid cancer. The method will be described in more detail below with special reference to this therapeutic context, however it will be understood by persons skilled in this art that additional applications may be devised by following the principles described herein.

In basic terms, the method involves isolation of total RNA from peripheral blood drawn from a patient and reverse transcription of the RNA to create a pool of cDNA molecules, from which selected thyroid-cell-specific cDNA transcripts are amplified using PCR, followed by detection of the amplified thyroid-specific transcripts. A thyroid-specific transcript is one that is expressed in thyroid epithelial cells, but (generally) not in other cell types. The thyroid specificity of a gene can be assayed by conventional methods, for example by subtractive analysis: By this method, mRNA is prepared from a candidate tissue (say, the thyroid gland) and transcripts common to it and other cell types are removed by
hybridization to cDNA from the whole organism or other selected tissues followed by cleavage with a nuclease that preferentially cleaves the DNA/RNA heteroduplex. The remaining (unhybridized) material is enriched for transcripts found only in the tissue from which the mRNA was prepared, which can be cloned. Certain genes, such as thyroglobulin [SEQ ID NO: 1] and thyroid peroxidase (TPO; Genbank accession number M17755) are transcribed only in thyroid tissue, while others have a less stringently specific thyroid expression profile. These latter include the thyroid stimulatory hormone receptor gene (TSHr; Genbank accession number M32215), which is also expressed in lymphocytes, the sodium iodide symporter gene [SEQ ID NO: 4], expressed in salivary cells, gastric mucosa and other tissues at low levels, Pax-8 [SEQ ID NO: 2], the transcript of which is found in renal and pulmonary tissue, TTF-1 [SEQ ID NO: 3], which is transcribed in lungs and TTF-2, which is expressed in the anterior pituitary. It is advantageous to use sequences of the thyroglobulin and NIS genes according to the invention, since those genes are efficiently expressed in all differentiated thyroid cells; however, it is sometimes necessary to apply the methods of the invention to the detection of poorly-differentiated or undifferentiated thyroid cells. These cells are known to express Pax-8, TTF-1 and TTF-2 in many cases; of these, Pax-8 and TTF-1 are well characterized at the molecular level, and so can be utilized as an alternative to Tg. While the expression of neither gene is restricted to cells of the thyroid epithelium, expression of this combination of genes is uniquely thyroid-specific, which is sufficient to prevent false positive results that might result from the use of either alone.

As discussed below in Example 1, we have compared the accuracy of Tg detection using the present invention to that of conventional Tg immunoassays on a group of thyroid cancer patients both on and off of thyroid hormone therapy. The sensitivity achieved without trauma to the patient brought about by removal from thyroid hormone replacement therapy and requiring only a small blood sample, is comparable to the conventional methods in use in hospitals.
currently. We have additionally performed this RT-PCR method for detection of NIS and other thyroid-specific transcripts, specifically PAX-8 and TTF-1, that are expressed in some poorly-differentiated thyroid carcinomas, so that the invention can be adapted to diagnose recurrence of thyroid cells that do not express Tg and would, therefore, go undetected in a conventional Tg immunoassay. The thyroid-specific mRNA RT-PCR assay of the present invention provides a highly sensitive assay for monitoring the course of treatment and recovery of thyroid disease patients while advantageously limiting the number of patients who must unnecessarily undergo dangerous thyroid hormone withdrawal to monitor for disease recurrence.

The present invention uses RT-PCR as a means for producing a detectable amount of a thyroid-specific transcript from a blood sample wherein an mRNA produced by a small number of circulating cells would ordinarily be undetectable in the pool of total RNA due to the enormous dilution factor. Prior to RT-PCR, RNA is obtained from a patient or biological sample. In the examples presented below, RNA is prepared from a small sample of human blood. In the reverse transcription (RT) step of RT-PCR, the RNA is converted to first strand cDNA, which is relatively stable and is a suitable template for a PCR reaction. In the second step, the cDNA template of interest is amplified using PCR. This is accomplished by repeated rounds of annealing sequence-specific primers to either strand of the template and synthesizing new strands of complementary DNA from them using a thermostable DNA polymerase. While it is possible to use thyroid-specific gene primers in the RT step, it is advantageous to utilize random primers, such as random hexanucleotides, in the creation of cDNA. This converts the entire population of mRNA's in the sample to cDNA, and allows for the subsequent amplification of multiple transcripts of interest from the product of a single reaction, which is advantageous if a direct comparison of message distributions is desired. The same result can be achieved using oligo-dT as a primer, which primes all polyadenylated mRNA molecules. Selection of thyroid-specific primers for either the RT or PCR steps of the procedure is described in
detail in Example 1, below. Briefly, sequences are selected from within coding regions of the gene of interest such that they will anneal specifically to unique sites. Each of the two primers in a pair is designed such that it will anneal to a unique exon and such that one or more introns are present between the two exons. Therefore, the predicted PCR product will be larger if amplified from genomic DNA than it would be if amplified from cDNA, thereby providing a rigorous control to detect amplification of genomic DNA that might contaminate a PCR component. Commercially available software, such as “PrimerSelect” of the DNASTar™ software package (DNASTar, Inc.; Madison, WI) and OLIGO 4.0 (National Biosciences, Inc.) program, has been developed to provide computer assistance in the design of optimal primer pairs, and use of such programs greatly speeds up the primer selection process.

RT-PCR has been used to detect the presence of micro-metastases by amplification of various cell-type specific mRNA transcripts from peripheral blood. In the most extensively studied case, that of prostate specific antigen (PSA), detection of approximately 1 cancer cell in $10^5$ to $10^6$ white blood cells has been reported (Katz et al., 1994, Urology, 43(6): 765-775; Ghassein et al., 1995, J. Clin. Oncol., 13(5): 1195-1200; Jaakkola et al., 1995, Clin. Chem., 41(2): 182-186; Seiden et al., 1994, J. Clin. Oncol., 12(12): 2634-2639; Deguchi et al., 1993, Cancer Res., 53(22): 5350-5354; Moreno et al., 1992, Cancer Res., 52(21): 6110-6112; Galvan et al., 1995, Clin. Chem., 41(12): 1705-1709; Loric et al., 1995, Clin. Chem., 41(12): 1698-1704). The sensitivity and specificity of this test compared to clinically utilized immunometric tests is estimated to be 70-90%. Other cancers, including colon cancer, breast cancer, neuroblastoma and melanoma, but not thyroid cancer, have been assayed by similar methods.

One potential problem with the RT-PCR approach is “ectopic” or “illegitimate” expression of transcripts in lymphocytes and other circulating cells, particularly when using highly sensitive “nested” RT-PCR protocols. “Nested” PCR involves a second round of amplification performed on a PCR-amplified DNA
fragment using a second set of primers that are internal to or "nested" within the sequences amplified in the first PCR reaction. False positive results using nested protocols have been reported in the PSA RT-PCR literature (Loric et al., supra). In preliminary studies, we detected Tg expression in lymphoblast cultures prepared from patients with no prior history of thyroid disease using a nested protocol. To circumvent this potential problem, we designed PCR primer pairs that allowed us to attain a high degree of sensitivity without a second PCR reaction. We have not identified ectopic Tg expression in cultured lymphoblasts using this non-nested RT-PCR protocol.

We have identified several other methodological issues that can influence the sensitivity of RT-PCR of Tg mRNA from peripheral blood. Isolation of total RNA from whole blood appears to be more effective than RNA isolation from the mononuclear cell layer. This may be related to the density or "stickiness" of malignant thyroid epithelial cells. Although most epithelial cells appear to sediment with mononuclear cells in a polysucrose gradient, we performed RT-PCR on each layer of a fractionated blood sample and found that the majority of Tg mRNA is located in the erythrocyte fraction. Thus, isolation of RNA from the mononuclear cell fraction would be likely to decrease the sensitivity of the assay relative to the use of whole blood or the erythrocyte fraction. Furthermore, the use of random hexamers (or oligo dT) as primers for reverse transcription of RNA to complementary DNA (cDNA) appears to be more efficient than a Tg-specific primer, and also allows for the amplification of multiple transcripts by PCR.

Using the method described herein, we have been able to detect Tg mRNA in peripheral blood even in normal subjects (10/10) with (presumably) normal thyroid glands as well as in thyroid cancer patients with relatively minute amounts of residual thyroid tissue while they continue to take L-thyroxine (see Example 1). We believe that this is the desirable limit of sensitivity. Twenty-eight of 35 patients with no detectable thyroid tissue on most recent \(^{131}\)I scanning performed after withdrawal of thyroid hormone were negative on the assay when it was performed during thyroid hormone suppression therapy,
strongly suggesting that thyroid tissue must be present for a positive assay.

The sensitivity of our assay may be further improved by the application of cell-sorting technology to the enrichment of thyroid epithelial cell populations within blood samples, making earlier detection of cancer recurrence possible. Several new techniques have recently been utilized to enrich for circulating epithelial carcinoma cells such as colon cancer cells, breast cancer cells and prostate cancer cells, but not thyroid cancer cells, in peripheral blood (Wong et al., 1995, Br. J. Surg., 82: 1333-1337; Grewitz et al., 1995, J. Immunol. Meth., 183(2): 251-265; Dobrovic, 1997, BioTechniques, 22: 100-104).

Preferred among these techniques is magnetic cell sorting (MACS Magnetic Cell Sorting Systems; Milteneny Biotech, Auburn, CA). This technique utilizes ferromagnetic beads conjugated with cell-specific monoclonal antibodies to separate specific cell populations from whole blood. The enriched population can then be evaluated by RT-PCR following cell lysis. These systems allow for a 10,000 fold enrichment of cells so that as few as one cell in $10^7$ can be enriched to one in $10^3$ cells. Although this technique has been used to detect circulating prostate cancer cells and breast cancer cells in patients with documented metastatic disease, these enrichment techniques have not been previously applied to patients with thyroid cancer.

Magnetic cell sorting is carried out by first treating whole blood or buffy coat layers with saponin or other suitable detergents to lyse erythrocytes. Cells are fixed with formaldehyde, washed, and incubated with ferromagnetic beads to which a specific antibody has been bound. When the antibody binds to its target, that cell becomes labeled. The bound cells are enriched relative to other cells by passing the cells through columns containing powerful magnetic gradients. This yields a specific cell population that can be further analyzed by either immunological or RT-PCR techniques. A thyroid cell-specific antibody, such as an antibody recognizing the TSH receptor, may advantageously be used for this enrichment step.

Circulating thyroid cells may also be isolated selectively by incubating
microbeads conjugated, for example, to polyclonal goat-anti-mouse IgG (Millitenyi Biotec) with a monoclonal antibody to the human TSH receptor (e.g., NCL-TSH-R2; Novacastra, Burlingame, CA).

Several techniques for detecting PCR products quantitatively without electrophoresis may be advantageously used with the assay of the invention in order to make it more suitable for easy clinical use. One of these techniques, for which there are commercially available kits such as Taqman™ (Perkin Elmer, Foster City, CA), is performed with a transcript-specific antisense probe. This probe is specific for the PCR product (e.g. Tg DNA fragment) and is prepared with a quencher complexed to the 3' end of the oligonucleotide and fluorescent reporter probe complexed to the 5' end of the oligonucleotide. Different fluorescent markers can be attached to different reporters, allowing for measurement of two products in one reaction. Each time the primers anneal to their complementary sequence, Taq DNA polymerase is activated, it cleaves off the fluorescent reporters by its 5' nuclease activity and does not digest the free reporter. The reporters, now free of the quenchers, fluoresce. The color change is proportional to the amount of each specific product and is measured by fluorometer; therefore, the amount of each color can be measured and the RT-PCR product can be quantified at the end of each PCR cycle. Thus, the product amount at any particular PCR cycle and the cycle at which the specific product is identified (threshold cycle) can be determined for each sample. The PCR reactions can be performed in 96 well plates so that many patient samples can be processed and measured simultaneously. The Taqman™ system has the additional advantage of not requiring gel electrophoresis and allows for quantification when used with a standard curve.

For quantitation of Tg mRNA in peripheral blood, a synthetic DNA template can be created having a similar size and with sequences matching the thyroid-cell-specific gene primers on the ends, allowing for competitive RT-PCR. The DNA
competitor may be prepared using known methods, such as the PCR MIMIC™
construction kit (Clontech, Palo Alto, CA) to create a similar-sized DNA fragment
with Tg PCR primers on the 5' and 3' ends that can be separated from amplified Tg
cDNA by gel electrophoresis. The double stranded competitor DNA product is
made by PCR amplification of a heterologous DNA fragment using composite
oligonucleotide primers containing sequences specific to either end of the MIMIC
DNA sequence 3' to Tg 5'-specific sequences. This allows for amplification of the
competitor DNA with Tg sequences on the ends. The quantity of DNA is
determined by standard methods, and dilutions are made to use in competitive PCR.

A standard curve is created by addition of varying amounts of competitor
dNA template to a fixed amount of whole blood cDNA. Since the competitor and
Tg templates both contain sequences that match the Tg primers, they are
simultaneously amplified. PCR products may be electrophoresed on polyacrylamide
gels and quantified by laser densitometry following Southern blot or by performing
radio-labeled PCR using [α ³²P] dCTP. Alternatively, the different-sized products
may be quantified fluorometrically, if primers are labeled with fluorometric dyes,
using an automated DNA sequencer. The concentration of Tg cDNA reverse
transcribed from peripheral blood is equal to the amount of competitor DNA added
that results in equivalent Tg and competitor PCR products.

Another method by which to quantify the amount of thyroid-cell-specific
mRNA in blood is by “spiking” negative whole blood samples with known amounts
of human thyrocytes to create a standard curve of number of thyroid cells/ml of
whole blood. When using a fluorometric system, such as Taqman™, a template
standard curve can be linearized by using the threshold cycle rather than the final
amount of product as the data points. Such a quantitative assay is important for
the monitoring of disease progression and for uses in other thyroid disorders, such
as autoimmune thyroiditis or Graves' Disease (see end of Example 4, below). For
this procedure, estimation of the number of cells per ml of whole blood, standards
are prepared using primary cultures of thyroid epithelial cells and adding known numbers of cells to whole blood samples obtained from patients with no evidence of circulating thyroid cells. Normal human thyroid cells may be obtained via primary culture from patients undergoing thyroidectomy (Williams et al., 1987, Mol. Cell. Endocrinol., 51: 33-40). In a typical procedure, thyroid tissue is washed and minced in cold Hanks’ calcium and magnesium-free balanced salt solution (HBS) and digested with dispase (1 mg/ml) and collagenase (100 lu/ml; Boehringer Mannheim, Indianapolis, IN) in 10 ml HBS at 37°C for 1 hour with gentle pipette disruption every 15 minutes. The supernatant is collected, proteases are neutralized by adding 0.5% fetal calf serum, and it is then filtered though a 200 μm nylon mesh and washed with HBS. Seeding is carried out in RPMI 1640 medium containing 10% FCS onto 35 mm Petri dishes. Cells are isolated and added to the whole blood to create a standard curve. RNA isolation and RT-PCR are performed as above for each sample, and the reaction product is quantified either by laser densitometry after autoradiography, or by either of the techniques described above.

A method of constructing a standard curve which is particularly suited to clinical practice of the methods of the invention, relies on the use of in vitro transcription products. Once synthesized from thyroid RNA, double-stranded cDNA is ligated into a vector that contains sequences for RNA polymerase start sites (e.g., SP6, T3 or T7), which can be selected for transfection by ampicillin sensitivity, and is suitable for single-stranded DNA (e.g., pGEM®-3Z Vector; Promega, Madison, WI). After selection and growth of the cells, the plasmid DNA is prepared. The selected colonies are grown and provide an easily replenishable source of thyroglobulin cDNA. The correct identity and orientation of the cDNA in the plasmid is confirmed by direct sequencing. The plasmid is linearized by restriction digestion at a site near the 3' end of the cDNA, and sense RNA is synthesized using RNA polymerase (Riboprobe Combination System®; Promega). Specific amounts of Tg RNA, or another thyroid-specific RNA of interest, are added
to thyroid-cell-negative whole blood samples to create standard curves. Reactions
are performed in triplicate and precision of signal detected for cell amount of copies
of RNA added are examined with a 95% confidence interval. This method offers
good precision and the standard curves produced according to it are easily
recreated, as fresh patient material (such as thyroid cells or mRNA) is not required
for each new curve.

The method of the present invention may advantageously be performed in a
single tube reaction for reverse transcription of RNA and specific amplification of
thyroid-specific transcripts. This system utilizes two enzymes, AMV reverse
transcriptase to prepare first strand cDNA, and the thermostable Tfl DNA
polymerase for second strand cDNA synthesis and subsequent DNA amplification,
with an optimized single buffer system that permits RT-PCR to be performed in one
step. This simplifies the assay and minimizes the chance for contamination during
preparation of a separate PCR reaction. Commercial kits such as the Access™ RT-
PCR system (Promega; Madison, WI) conveniently assemble all materials (except
primers) necessary to carry out the method in this way. The single-tube RT-PCR
assay according to this technique has been used herein to amplify Tg mRNA from
thyroid tissue and has been optimized for peripheral blood samples.

Alternatively, it may be possible to use an enzyme such as rTth polymerase
(Perkin Elmer, Foster City, CA) that has reverse transcriptase activity in the
presence of Mn²⁺ and has DNA polymerase function at higher temperatures (Juhasz
et al., 1996, BioTechniques, 20: 592-600). Such an enzyme system allows for
single tube and single enzyme RT-PCR. PCR product detection has been performed
both by polyacrylamide gel electrophoresis and ethidium bromide staining and also
by performing the PCR reaction in a 96-well plate in combination with the
fluorescent detection system described above. Utilization of the fluorescent
detection system in the one-tube system allows for the simple addition of RNA to a
well containing the buffer, enzymes, dNTPs, primers and the detection probe
followed by RT-PCR and luminescent reading. The sensitivities of these systems
are equal or superior to standard two-tube methods (Chehadeh et al., 1995,
BioTechniques, 18: 26-28; Sellner et al., 1992, Nucleic Acids Res., 20: 1487-
1490; Juhasz et al., supra), although there is no excess cDNA available for
amplification of transcripts other than Tg.

In accordance with the foregoing disclosure, the method of this invention
provides a clinically useful assay utilizing RT-PCR to detect thyroid-specific cellular
functions. The method provides a useful diagnostic tool with which to monitor the
progression or recurrence of cancer of the thyroid without causing undue suffering
for the patient being evaluated. This assay can be adapted to yield quantitative
data on thyroid activity that does not require labor-intensive detection methods
such as gel electrophoresis and is, therefore, well-suited to use in hospital
laboratories. In addition, by employing PCR primers capable of amplifying
transcripts of abnormal, poorly differentiated or undifferentiated thyroid cells, such
as primers based on the coding sequences for PAX-8, TTF-1 or TTF-2, the method
can be made sensitive to rare forms of thyroid cancer. For the highest sensitivity
and widest clinical applicability, the method will be performed using primers for the
amplification of two or more thyroid-specific transcripts. Most preferably, the
method will be performed with primers to detect Tg, NIS and PAX-8. The method
can be performed with good sensitivity using whole blood; however, we have
identified in sedimented blood a fraction to which thyroid epithelial cells
preferentially segregate, namely, the erythrocyte layer, and accordingly sensitivity
of the method can be enhanced by preparing mRNA from this cell fraction and/or
through the application of other cell-sorting technologies. The specifics of the
inventive techniques are set forth in the following examples, which are meant to
illustrate, but in no way limit the scope of, the invention.
Example 1

An experiment was conducted to detect the presence of circulating thyroid cells in patients with metastatic disease. After obtaining informed consent, 3 ml of peripheral venous blood was removed by standard phlebotomy from each of 77 post-surgical thyroid cancer patients. Sixty-eight of the 77 patients were evaluated during thyroid hormone suppression therapy. Thirty five of these patients had no evidence of residual or recurrent disease on most recent radioiodine scan performed after thyroid hormone withdrawal. Another 19 displayed neck uptake of radioiodine within the thyroid bed, which is indicative of eutopie (normal) thyroid tissue or cancer. Fourteen patients had evidence of metastatic disease based upon radioiodine uptake at locations either in the neck, outside of the thyroid bed ($n = 3$) or outside of the neck ($n = 11$). Significantly, four of the 68 patients had circulating serum antibodies against Tg, which eliminated for them the possibility of immunoradiometric assay (IRMA) as a diagnostic tool. These patients highlight the need for an alternative clinical test, such as the test according to our invention.

Each blood sample was immediately placed in a 50 ml sterile conical polypropylene tube containing 18 ml of TRIzol LS, an RNA extraction buffer (BRL, Life Technologies, Gaithersburg, MD), and 3 ml of diethylprocarbazine (DEPC, Sigma, St. Louis, MO) treated water; care was taken to mix the blood thoroughly in the TRIzol after addition to the tube. After initial centrifugation at 3400 RPM at $4^\circ$C in a Beckman GPR centrifuge, the RNA was isolated as per manufacturer’s recommendations by chloroform extraction followed by isopropanol precipitation and 70% ethanol washing. Samples were dried in a vacuum centrifuge and resuspended in DEPC-treated water. RNA purity and concentration were determined by spectrophotometric analysis of UV absorption at 260 nm and 280 nm.

1 $\mu$g of total RNA and 75 pmol random hexamer primer (Pd(n)6, Pharmacia, Piscataway, NJ) were resuspended in a 10 $\mu$l volume with DEPC-treated water in
an RNase-free 0.5 μl tube. This mixture was incubated at 70°C for 10 minutes and
placed on ice for two minutes. The following reagents were added to the 10 μl
reaction; 1 μl (200U) MMLV-RT (Superscript® reverse transcriptase, BRL, Life
Technologies, Gaithersburg, MD), 4 μl 5x reaction buffer (BRL, Life Technologies,
Gaithersburg, MD), 2 μl 0.1M DTT, 1 μl 10 mM dNTP and 1 μl human placental
RNase inhibitor (10 to 50 units per μl; Boehringer Mannheim, Indianapolis, IN). In
addition, for each RNA sample a second reaction was prepared except that MMLV-
RT was omitted (RT negative control). The 19 μl reaction was incubated for 50
minutes at 42°C in a programmable thermal cycler (MJ Research, Watertown, MA)
and inactivated by heating to 90°C for 5 minutes. After cooling to 37°C, 1 μl
RNase H (3 units per μl; BRL, Life Technologies, Gaithersburg, MD) was added, the
reaction was incubated at 37°C for 20 minutes, then cooled to 4°C. RNA integrity
was confirmed by amplification of Interleukin-2 cDNA or Gαs cDNA; therefore, it
was ensured that any negative result subsequently observed on a test sample could
be ascribed to a lack of that specific mRNA and not to degradation of the pool of
mRNA or failure of the reverse transcription reaction.

Tg cDNA was amplified using oligonucleotide primers that span both the
second and third Tg introns to inhibit amplification of genomic DNA and to provide
a control by which amplification of genomic DNA, were it to occur, would be made
obvious due to the size increase in the PCR product. These primers were as
follows;

mdr5 (exon 2): 5'-TGTGAGCTGCAGAGGGAAACGGCC-3' [SEQ ID NO: 1,
nucleotides 141 through 164], and

mdr7 (exon 4): 5'-ATACACCTCCATCCCCCTCTGCTCCACACA-3' [SEQ ID
NO: 1, reverse complement of nucleotides 459 through 488].

Primer sequences were designed using the OLIGO™ Version 3.4 software
package, which selects candidate regions within a given sequence that are
optimized for annealing efficiency, for the likelihood that they will each prime only a
single site and to minimize the likelihood of primer-dimer formation. Oligonucleotide synthesis was performed on a Cyclone Plus DNA Synthesizer (Milligen/Biosearch, a division of Millipore; Bedford, MA). PCR was performed using 2\(\mu\)l of the cDNA, 25 pmol of each Tg oligonucleotide primer (mdr5 and mdr7), 2.5 \(\mu\)l of 10X PCR buffer 1 (Perkin-Elmer, Foster City, CA), 0.4 \(\mu\)l of 1.25 \(\mu\)M dNTP, 0.15 \(\mu\)l of Taq DNA polymerase (Perkin Elmer, Foster City, CA) and deionized water to a total volume of 25 \(\mu\)l. Mineral oil was overlaid and the PCR was performed using a programmable thermal cycler with the following program: initial denaturation at 94°C for 4 minutes, then 39 cycles consisting of denaturation (94°C for 1 minute), annealing (60°C for 1 minute), and extension (72°C for 1 minute). Final extension was for 4 minutes at 72°C. Appropriate reverse transcriptase negative controls were performed for RT-PCR of each RNA sample. These controls included the use of primers whose products span introns, such that the PCR product of genomic DNA would be larger than that of a cDNA transcript, and running a PCR reaction on the product of a reverse transcription that includes all components except reverse transcriptase, therefore detecting PCR products that were not derived from cDNA.

Ten \(\mu\)l of the RT-PCR product from the above reactions were mixed with DNA loading buffer and electrophoresed on an 8% polyacrylamide Tris-Borate-EDTA (TBE) minigel run at 200 volts in 1X TBE. DNA was visualized by UV transillumination after staining with ethidium bromide and documented by photography. Gels were soaked in 0.5X TBE for 20 minutes and transferred to positively-charged nylon transfer membranes (Nyttran Plus; Schleicher and Schuell, Keene, NH) by electroblotting in 0.5 x TBE buffer. DNA were immobilized by drying in a vacuum oven at 80°C for 2 hours.

After transfer and drying, membranes were prehybridized at 42°C for at least 3 hours in a shaking water bath in a buffer containing 48% formamide, 4.8X SSC, 20 mM Tris, pH 7.6, 1X Denhart’s solution, 1% SDS and 100 \(\mu\)g/ml heparin sulfate supplemented with 200 \(\mu\)g/ml denatured sheared salmon sperm DNA. 10
pmoles of a Tg internal oligonucleotide probe were labeled using $^{32}$P dATP (Amersham, Arlington Heights, IL) and T₄ polynucleotide kinase (BRL, Life Technologies, Gaithersburg, MD), and purified by chromatography using DE-52 (Whatman, Maidstone, England) Sephadex. Incorporated radioactivity was determined by analysis of an aliquot of the purified probe by liquid scintillation counting. The sequence of this probe, mdr6, is:

5' CTGCTGTGTTCTGAGTACAAAA 3' [SEQ ID NO: 1, reverse complement of nucleotides 324 through 347]. A second probe, mdr 12:

5' ATCCTCTGCACACTGGGCACGTAGTCTGCTTGTCCAGAAA 3' [SEQ ID NO: 1, reverse complement of nucleotides 165 through 206], has been synthesized and used in other experiments. After pre-hybridization, 1-5 x 10⁶ cpm of probe were added per ml of hybridization buffer. Following overnight hybridization at 42°C in a shaking water bath, blots were removed and washed in 5X SSC with 0.1% SDS at room temperature for 10 minutes three times before a final wash in 5X SSC and 0.1% SDS at 50°C for one hour. Membranes were dried and were subjected to autoradiography using intensifying screens at -80°C overnight.

No product was identified when reverse transcriptase was omitted from the reaction mixtures for each sample, indicating that PCR products seen in the actual test samples were derived from reverse-transcribed mRNA rather than genomic DNA. Identity of the Tg RT-PCR product detected by Southern blot using an internal Tg oligonucleotide probe was confirmed by restriction digest analysis and by direct sequencing of the RT-PCR product. TSH concentrations and serum Tg immunoassays were performed on blood samples obtained at the same time as the samples subjected to RT-PCR.

We initially determined the sensitivity of the assay by adding different amounts of thyroid gland RNA to 0.9 μg of RNA isolated from lymphoblasts. We were able to detect Tg-mRNA using a “non-nested” protocol with the addition of as little as 0.1 pg of thyroid RNA, which corresponds to 10 thyroid cells per ml of...
blood. Tg mRNA could not be detected from lymphoblast RNA alone. Figure 2A shows ethidium bromide staining of RT-PCR products amplified from a dilution series of thyroid mRNA added to lymphoblastoid RNA derived from normal subjects, while Figure 2B shows the results of a similar experiment in which thyroid mRNA was added to total RNA isolated from a athyreotic patient. Reverse transcriptase negative controls were performed for each sample and cDNA integrity was confirmed in parallel reactions in which either of the leukocyte-expressed transcripts Gαs or IL-2 were amplified.

After obtaining informed consent, 3 ml of blood were drawn from athyreotic recovering cancer patients or normal subjects. Investigators were blinded to the clinical status of the patients. Tg RT-PCR and Tg-IRMA (Optiquant assay; Kronus, San Juan Capistrano, CA) results were compared to most recent $^{131}$I scan results and serum Tg immunoassay values determined after thyroid hormone withdrawal. In this manner, the $^{131}$I scan results served as the “gold standard” against which the relative efficiencies of Tg RT-PCR and Tg-IRMA could be gauged, although in a clinical setting, such scan results are ordinarily assessed and used in combination with Tg immunoassay results. Serum TSH concentration was also measured in an ultrasensitive assay (3rd generation THS; Nichols, San Juan Capistrano, CA). Patients were considered to have residual normal or malignant thyroid tissue if the iodine uptake was greater than 0.01% within the neck or metastatic uptake was demonstrated.

Statistical analysis was performed using SAS 6.12 (SAS International, Inc.; Cary, NC). McNemar’s exact test was used to compare Tg RT-PCR and immunoassay results among the groups of patients studied. A $p$ value < 0.05 was considered significant.

Sample RT-PCR results, as shown by ethidium bromide staining and Southern analysis of the predicted 348 bp Tg product, are presented in Figure 3. All 10 normal control subjects were positive on the RT-PCR assay, indicating that the
assay detects the presence of normal and malignant cells and does not yield false negative results when significant thyroid tissue is present. In patients for whom radiographic scans following administration of labeled iodine yielded no evidence of thyroid cell activity, RT-PCR was negative in 80% of 35 patients tested on L-thyroxine therapy and in 75% of 8 patients tested after thyroid hormone withdrawal, while Tg-IRMA yielded negative results in 94% of 35 patients on L-T4 and in 88% of patients evaluated off of the drug. These may represent increased sensitivity of RT-PCR over radiographic scan, i.e. detection of thyroid activity in patients testing negative by current methods. Radiographic disease analysis detected thyroid tissue in 33 patients: 19 had residual eutopic (normal) or malignant thyroid tissue in the neck, while 14 had metastatic thyroid tissue. When assayed while taking L-thyroxine, 26 of these 33 patients, including all 14 with metastases, were positive on the Tg RT-PCR assay, as opposed to 12 of 33 (79% vs. 36%, p < 0.001) when Tg-IRMA was instead performed. Tg RT-PCR was positive in 3 of the 4 patients with neck or metastatic thyroid tissue in whom anti-Tg antibodies prevented determination of Tg-RIA. The results are presented in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Radioiodine Staging</th>
<th>on L-T4 (n = 68)</th>
<th>off L-T4 (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg IRMA</td>
<td>Tg RT-PCR</td>
</tr>
<tr>
<td>No Disease</td>
<td>2/35 (6%)</td>
<td>7/35 (20%)</td>
</tr>
<tr>
<td>Neck Disease (&gt;0.1%)</td>
<td>3/19 (16%)</td>
<td>12/19 (63%)</td>
</tr>
<tr>
<td>Metastasis</td>
<td>8/14 (57%)</td>
<td>14/14 (100%)</td>
</tr>
<tr>
<td>All Positive</td>
<td>11/33(33%)</td>
<td>25/33 (76%)</td>
</tr>
</tbody>
</table>
EXAMPLE 2

The purpose of this set of experiments was to further validate the RT-PCR assay for Tg mRNA and to compare its performance to clinical and biochemical tests currently in use.

We obtained blood samples from 7 patients, during thyroid hormone therapy and at the time of $^{131}\text{I}$ scanning after thyroid hormone withdrawal for clinically-indicated $^{131}\text{I}$ scanning. Four of 7 patients had metastases, one had neck uptake in the thyroid bed, and two had negative $^{131}\text{I}$ scans. All of the patients had simultaneous samples drawn for Tg mRNA RT-PCR and Tg-IRMA, and for measurement of TSH concentration. Three ml of whole blood was used for the Tg mRNA RT-PCR assay, meaning that no additional phlebotomy was needed for the patients, as they would routinely have serum TSH and Tg-IRMA measured at those times. There were no specific gender or race requirements for patients enrolled in this study. The efficacy of the RT-PCR assay was compared to that of serum Tg-IRMA in identifying positive patients. When patients were withdrawn from L-thyroxine therapy, both Tg-IRMA and RT-PCR detected the presence of thyroid cells in all four patients with metastases; however, during L-thyroxine therapy, Tg-IRMA failed to detect the presence of cancer cells in two of these same patients, whereas RT-PCR once again correctly identified all four as having evidence of disease. While a larger study will be needed to determine the whether the apparent advantage in sensitivity of RT-PCR over Tg-IRMA is clinically- or statistically meaningful, when combined with the data presented in Example 1, it is already clear that RT-PCR more sensitive than Tg-RIA and can be performed without subjecting patients to the suffering brought about by removal from L-thyroxine, as is required for an adequately sensitive Tg-immunoassay.

EXAMPLE 3

We are creating a mixed pool of reverse transcripts from cells of the peripheral blood and, in general, specifically amplifying Tg cDNA to detect thyroid
cells in that population; however, the expression of Tg transcripts is usually limited to differentiated thyroid cancers. Poorly-differentiated thyroid tumors that no longer express Tg often retain expression of three other relatively thyroid-specific transcripts, Pax-8 (a homeobox protein) and thyroid transcription factors 1 and 2 (TTF-1 and TTF-2) (Fabbro et al., 1994, Cancer Res., 54: 4744-4749). While Pax-8 is also expressed in renal and pulmonary tissue, TTF-1 in lungs and TTF-2 in the anterior pituitary gland, thyroid cells are the only known cells that express Pax-8 in combination with either TTF-1 or TTF-2 (Fabbro et al., supra). It is, therefore, desirable to examine these transcripts, which may be more useful than Tg in monitoring patients with poorly-differentiated thyroid carcinoma, and whose cDNA’s can be amplified readily from the pool resulting from our random primed-reverse transcription. As the sequence of TTF-2 is poorly defined and since expression of Pax-8 and TTF-1 are preserved in most thyroid tumors, including those with poorly-differentiated phenotypes (Fabbro et al., supra), we have used TTF-1 in conjunction with Pax-8.

Primer sequences were derived from the cDNA of Pax-8 and TTF-1 that span gene introns to exclude genomic DNA amplification. PCR conditions were optimized using RNA isolated from a normal thyroid gland and from two poorly-differentiated thyroid carcinoma cell lines (WRO and ARO, provided by Dr. R. Juillard, U.C.L.A., Los Angeles, CA) using TRizol LS as described above. Peripheral blood samples were obtained from patients with differentiated thyroid cancer as well as those with poorly-differentiated thyroid cancer and RT-PCR was performed as outlined above, including Southern blot analysis with internal oligonucleotide probes and sequencing of representative samples. ARO and WRO cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum in 150 cm² culture dishes kept in a humidified incubator at 37°C in 5% CO₂ for use as controls.

To start, the same pools of reverse transcripts used to calibrate the
efficiency and accuracy of Tg amplification (above, Example 1) were used in PCR reactions with the following primers specific for Pax-8 [SEQ ID NO: 2]:
mdr59 (exon 3): 5′ AAGGTGGTGGAGAGGATTGGG 3′ [SEQ ID NO: 2, nucleotides 269 through 289], and

5 mdr60 (exon 6): 5′ GAGGTGGTGCTGGCTGAAGGC 3′ [SEQ ID NO: 2, reverse complement of nucleotides 689 through 709].

These primers span three Pax-8 introns to enable identification of any amplification of genomic DNA. PCR was performed using 2μl of the cDNA, 25 pmol of each Tg oligonucleotide primer (mdr59 and mdr60), 2.5 μl of 10x PCR buffer 1 (Perkin-Elmer, Foster City, CA), 0.4 μl of 1.25 μM dNTP, 0.15 μl of Taq DNA polymerase (Perkin Elmer, Foster City, CA) and deionized water to a total volume of 25 μl. Mineral oil was overlaid and the reaction was placed in a programmable thermal cycler with the following program; initial denaturation at 94°C for 4 minutes, then 39 cycles consisting of denaturation (94°C for 1 minute), annealing (50°C for 1 minute), and extension (72°C for 1 minute). Final extension was for 4 minutes at 72°C. Appropriate reverse transcriptase negative controls were performed for RT-PCR of each RNA sample. These controls included the use of primers whose products span introns, such that the PCR product of genomic DNA would be larger than that of a cDNA transcript, and performing a PCR reaction on the product of a reverse transcription reaction that included all components except reverse transcriptase, therefore detecting PCR products that were not derived from mRNA.

Ten μl of the RT-PCR product from the above reactions were mixed with DNA loading buffer and electrophoresed on an 8% polyacrylamide Tris-Borate-EDTA (TBE) minigel run at 200 volts in 1x TBE. DNA was visualized by UV transillumination after staining with ethidium bromide and documented by photography. The size of the observed fragment was consistent with that predicted for the Pax-8 RT-PCR product, and this band was recognized by a labeled
Pax-8 probe in a diagnostic Southern analysis. No product was identified when reverse transcriptase was omitted from the reaction mixtures for each sample.

A similar attempt to selectively amplify TTF-1 cDNA [SEQ ID NO: 3] resulted in PCR products of aberrant electrophoretic mobility, indicating that either genomic DNA or a second, probably abundant, reverse transcript with sufficient homology to the primer sequences had been amplified in its stead. The primers used, which again hybridized to sequences of two different exons, were as follows:

**Pair 1**

mdr56 (exon 1): 5' GCAACGGCAACCTGGGCAACA 3' [SEQ ID NO: 3, nucleotides 601 through 621], and

mdr57 (exon 2): 5' TGTCCTTGGCCTGGCGCTTCA 3' [SEQ ID NO: 3, reverse complement of nucleotides 988 through 1008].

In this case, our results illustrate the importance of careful primer selection and of the need to evaluate every primer pair prior to the testing of patient blood samples.

Using the primers of pair 1, a band representing an RT-PCR product of the expected size was produced; however, supernumerary bands of other sizes were observed to accompany it. It is expected that, given the proper primer pair, TTF-1 will be amplified correctly and with efficiency comparable to that observed with Pax-8.

We have designed two additional sets of intron-spanning TTF-1 primers, which are as follows:

**Pair 2**

sense: 5' CGATGAGTCAAAGCACACG 3' [SEQ ID NO: 3, nucleotides 356 through 365], and

antisense: 5' TTTGCCGTCTTTCAACCAGGA 3' [SEQ ID NO: 3, reverse complement of nucleotides 1126 through 1145]

**Pair 3**

sense: 5' ACCAGGACACCATGAGGAAC 3' [SEQ ID NO: 3, nucleotides 640 through 659], and
antisense: 5' TGCTCTGGCCCTGGCGCTTCGA 3' [SEQ ID NO: 3, reverse complement of nucleotides 988 through 1008].

As in Example 1, we determined the sensitivity of the assay by adding different amounts of thyroid gland RNA to 0.9 µg of RNA isolated from of lymphoblasts. We were able to detect Pax-8 mRNA using a "non-nested" protocol with the addition of as little as 3 ng of thyroid RNA, which corresponds to approximately 300 thyroid cells per ml of blood; in some experiments, the sensitivity has been as high as 100 cells/ml. A single band of the correct size (0.44 kb) was observed when the product was electrophoresed, indicating good primer selection. The sensitivity of this assay is approximately 10-fold lower than RT-PCR of Tg transcripts under the same conditions; however, this correlates with the known ratio of Pax-8 to Tg transcript abundance in thyroid cells. We do not believe that this result reflects inefficient annealing of Pax-8 primers to their target sequences. Pax-8 mRNA could not be detected from lymphoblast RNA alone.

To compare directly the efficiency of Pax-8 amplification with that of Tg, 13 of the cDNA pools prepared from the RNA of patients' blood samples tested for the presence of Tg transcripts in Example 1 were selected for PCR with Pax-8 primers mdr59 and mdr60. Electrophoretic analysis of the Pax-8 RT-PCR product in these samples revealed a heterogeneous population of bands. These bands might represent alternative Pax-8 splicing variants; however, it is possible that they might indicate the need to adjust PCR buffer or annealing conditions for amplification of the Pax-8 transcript directly from patient blood or even that the primers used, while effective for use in with a purified RNA sample, are not suitable for use with raw clinical material.

Example 4

The detection of thyroid-cell-specific transcripts, as shown in the Examples above yields a positive or negative result regarding the recurrence of thyroid cancer in patients who have undergone thyroidectomy in the course of cancer treatment.
Based upon thyroid cell mass and the presence of metastases, this procedure indicates that mRNA is present above the lowest detection threshold for a given transcript and primer pair; however, it does not offer a reliable indication as to the extent to which the disease has recurred. This example demonstrates the application of quantitative PCR to the measurement of thyroid-cell-specific transcripts in the blood of recovering thyroid cancer patients. The results of quantitative PCR data for the Tg (15 patients) and NIS (13 of the same 15 patients) transcripts are provided herein. It is expected that results of monitoring of the NIS transcript level in a patient taking thyroid hormone may provide a clinical predictor of whether he or she is likely to have iodine-avid tissue on diagnostic thyroid hormone withdrawal $^{131}$I scan or respond to $^{131}$I therapy.

The quantitative RT-PCR assay system which is disclosed herein entailed amplification of reverse transcripts from peripheral blood of patients followed by amplification of thyroid-cell-specific transcripts, both as described above, followed by detection of the transcripts by hybridization of a fluorometrically-labeled oligonucleotide probe complementary to a site internal to (within) the amplified sequence and detection of the bound label with a PRISM™ 7700 Fluorometric Detection System (Applied Biosystems, Foster City, CA). PCR was performed using Amplitaq Gold (Applied Biosystems); after incubation at 50°C and an initial denaturation at 94°C for 10 minutes, 40 cycles of a two-step PCR were performed, each consisting of a denaturation step (1 minute, 94°C) and an annealing/extension step at 60°C for 1 minute. All samples Tg samples were run in triplicate, while NIS samples were run once.

For Tg, the intron-spanning PCR amplification primers employed were

5' GTGCCAACGGCAGTGAAAGT 3'  [SEQ ID NO: 1, nucleotides 262 through 280], and

5' TCTGCTGTTTCTGAGCTGACAA 3'  [SEQ ID NO: 1, reverse complement of nucleotides 325 through 348].
The PCR amplification primer pair used for NIS amplification comprised:

5' ACACTGACTGCGACCCCTCTCCT 3'   [SEQ ID NO: 4, nucleotides 1139-1160], and
5' TGCTGAGGGTGCCACTGTAA 3'   [SEQ ID NO: 4, reverse complement of nucleotides 1260 through 1279].

The Tg detection probe was 5' ACAGACAAAGCCACAGGCG GT CCT 3' [SEQ ID NO: 1, nucleotides 299 through 321]. This probe was labeled with the 6-carboxy-fluorescein (FAM) reporter dye. A second probe which is useful in Tg detection is 5' CCTTCCGTCCCTGTGAGCTGCA 3' [SEQ ID NO: 1, nucleotides 130 through 151], which may be labeled FAM, or with another reporter dye, as indicated below.

The NIS detection probe was
5' CGGGGACTCAGCCGATCTTCCG 3' [SEQ ID NO: 4, reverse complement of nucleotides 1218 through 1240], which was also labeled with FAM for these experiments. A second probe labeled with tetrachloro-6-carboxy-fluorescein (TET) is being tested for sensitivity. Four different fluorescent dyes are currently available for use, which additionally include 2,7-dimethoxy-r,5-dichloro-6-carboxyfluorescein (JOE) and hexachloro-6-carboxy-fluorescein (HEX). Since reverse transcription of mRNA in the invention may be performed using non-specific primers (e.g., random oligonucleotides, or oligo-dT), multiple thyroid-cell-specific transcripts may be amplified from the mixed pool of transcripts and detected. The use of different fluorescent dyes for different primer pairs enables the simultaneous monitoring of two or more transcripts; the number of different transcripts which may be detected at one time is limited only by the number of available dyes which fluoresce at different wavelengths. Simultaneous assay (i.e., in a single reaction) of multiple transcripts allows for monitoring of changes in the relative abundance of thyroid-cell-specific transcripts in a patient over time, which may provide information as to the progress of the disease.
Interpretation of raw data from such experiments requires comparison to a
standard, wherein fluorescence resulting from hybridization of a given probe to the
corresponding transcript can be converted into a value which represents an
absolute amount (e.g., in picograms) of the thyroid-cell-specific mRNA in the
starting sample. The conversion factor is dependent upon the efficiency with
which the probe anneals to its target sequence, which is directly proportional to the
$T_m$ of the probe/target duplex. A standard curve is derived as follows:

**Thyroid cell standard curves:**

Normal thyroid total RNA is mixed with whole blood total RNA from an
assay-negative athyreotic patient, as determined from background data. Relative
amounts of cells are determined using the assumptions that there are approximately
10 g of total RNA in a mammalian cell and approximately 5000 leukocytes/mm$^3$ in
whole blood (approximately $5 \times 10^6$/ml). After mixing the RNA, RT-PCR is
performed, and signal resulting from hybridization to a labeled probe is quantitated.

Alternatively, normal thyroid cells obtained by dispersion of cells from discarded
remnants of normal thyroid tissue obtained at the time of surgery (approved
protocol #98-187) are added to 1 ml of whole blood from an athyreotic patient and
total RNA is isolated and assayed as above. Reactions are, in either case,
performed in triplicate. The precision of signal detected for cell number or RNA
molecule copy number are examined with a 95% confidence interval.

**In vitro RNA copy number standard curves**

Because either of the above standard curves is limited by estimates of copies
of thyroglobulin, NIS or other target mRNA per cell, and by the requirement of
pathology samples, *in vitro* transcription using cDNA made with the 3' antisense
PCR primer is performed, as described above.

Standard curves for the Tg and NIS detection probes, prepared by the
addition of thyroid cell mRNA to blood, are shown in Figures 4 and 5, respectively.
Each represents the average of triplicate runs. The results of the Tg and NIS
transcript assays, which are already converted to pg of RNA using the Tg- and NIS standard curves, respectively, are shown in Table 2, along with information regarding the clinical status of each patient assayed. The threshold cycle is the number of PCR cycles necessary for the amount of thyroid-cell-specific product to generate a detectable signal (i.e., the detection threshold for the measuring device. Tg detection reactions were performed in triplicate, and an average of the three runs was calculated; the raw data from these experiments is presented in Table 3. The raw data for the NIS assays, of which only a single run was performed per patient, are shown in Table 4. In using the conversion factor, the background fluorescence (in the case of the NIS experiment summarized in Tables 2 and 4, background = 0.67) is subtracted, and the resulting number multiplied by a factor which is inversely proportional to the amount of RNA assayed, as per the manufacturer’s guidelines.

**Table 2**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Threshold cycle</th>
<th>NIS (pg RNA)</th>
<th>TG (pg RNA)</th>
<th>Clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>36.54</td>
<td>4.5</td>
<td>109</td>
<td>Neck Lymph Nodes</td>
</tr>
<tr>
<td>P2</td>
<td>31.23</td>
<td>930</td>
<td>142</td>
<td>No Disease, No scan since 1996, Soft Tissue Invasion</td>
</tr>
<tr>
<td>P3</td>
<td></td>
<td></td>
<td>28</td>
<td>Neck Recurrence, 1997</td>
</tr>
<tr>
<td>P4</td>
<td>36.75</td>
<td>6.2</td>
<td>25</td>
<td>Neck Adenopathy</td>
</tr>
<tr>
<td>P5</td>
<td>29.21</td>
<td>4700</td>
<td>385</td>
<td>Neck Nodes and Soft Tissue Extension</td>
</tr>
<tr>
<td>P6</td>
<td>30.28</td>
<td>2000</td>
<td>82</td>
<td>Normal control, No thyroidectomy</td>
</tr>
<tr>
<td>P10</td>
<td>36.78</td>
<td>3.6</td>
<td>4</td>
<td>Pulmonary Metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>--------</td>
</tr>
<tr>
<td>P11</td>
<td>35.72</td>
<td>11</td>
<td>0</td>
<td>No Evidence of Recurrence</td>
</tr>
<tr>
<td>P12</td>
<td>36.07</td>
<td>14</td>
<td>54</td>
<td>Neck Lymph Nodes</td>
</tr>
<tr>
<td>P13</td>
<td>36.95</td>
<td>2.7</td>
<td>0</td>
<td>1.2 cm node, no malignancy by pathology</td>
</tr>
<tr>
<td>P14</td>
<td>37.87</td>
<td>0</td>
<td>0</td>
<td>No Evidence of Recurrence</td>
</tr>
<tr>
<td>P15</td>
<td>36.31</td>
<td>5.9</td>
<td>0</td>
<td>No Evidence of Recurrence</td>
</tr>
<tr>
<td>P16</td>
<td>35.93</td>
<td>9.2</td>
<td>26</td>
<td>Neck Lymph Nodes</td>
</tr>
<tr>
<td>P17</td>
<td>36.24</td>
<td>6.7</td>
<td>24</td>
<td>No Recurrence</td>
</tr>
<tr>
<td>P18</td>
<td>0</td>
<td></td>
<td></td>
<td>0.4 x 1.0 cm lesion. No change in 2 years on CT</td>
</tr>
</tbody>
</table>

Total volume of sample + 42 μl cocktail = 52 μl; Volume assayed = 50 μl

These data demonstrate the sensitivity of the quantitative assay of the invention to differences in levels of two thyroid-cell-specific transcripts in different patients. That the assay is reliable is demonstrated by the data shown in Table 4, in which the raw data from the triplicate runs performed in the Tg mRNA quantitative assay are shown. The threshold cycle and raw fluorescence values within each group of three reactions display remarkable reproducibility, which indicates that this technique is well suited for use in the evaluation of clinical patients.
## Table 3

### Thermal Cycle Conditions

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
<th>Repeat</th>
<th>Ramp Time</th>
<th>Auto Increment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>50.00</td>
<td>2:00</td>
<td></td>
<td>Auto</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>95.00</td>
<td>10:00</td>
<td></td>
<td>Auto</td>
<td></td>
</tr>
<tr>
<td>10 Cycles</td>
<td>95.00</td>
<td>0:15</td>
<td>40</td>
<td>Auto</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60.00</td>
<td>1:00</td>
<td></td>
<td>Auto</td>
<td></td>
</tr>
</tbody>
</table>

### Standard Curve

**Slope:** -2.78  
**Intercept:** 23.64  
**Threshold:** 0.01  
**Fit R:** 0.91  
**Baseline Range:** (3, 15)

### Sample Information

<table>
<thead>
<tr>
<th>Well</th>
<th>Type</th>
<th>Sample_Name</th>
<th>Replicate</th>
<th>Ct</th>
<th>Quantity</th>
<th>Std. Dev</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>STND 1</td>
<td></td>
<td>3</td>
<td>15.01</td>
<td>1.2e+03</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>A2</td>
<td>STND 1</td>
<td></td>
<td>3</td>
<td>14.90</td>
<td>1.2e+03</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>A3</td>
<td>STND 1</td>
<td></td>
<td>3</td>
<td>14.97</td>
<td>1.2e+03</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>25 A4</td>
<td>STND 9</td>
<td></td>
<td>3</td>
<td>32.09</td>
<td>3.2e-03</td>
<td>68.22</td>
<td>24.67</td>
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<td>33.26</td>
<td>3.2e-03</td>
<td>68.22</td>
<td>24.67</td>
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<td>33.60</td>
<td>3.2e-03</td>
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<td>24.67</td>
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<td></td>
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<td>15.48</td>
<td>2.5e+02</td>
<td>68.22</td>
<td>24.67</td>
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<td>15.43</td>
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<td>68.22</td>
<td>24.67</td>
</tr>
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<td>30 B3</td>
<td>STND 2</td>
<td></td>
<td>3</td>
<td>15.51</td>
<td>2.5e+02</td>
<td>68.22</td>
<td>24.67</td>
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<td></td>
<td>3</td>
<td>35.80</td>
<td>6.4e-04</td>
<td>68.22</td>
<td>24.67</td>
</tr>
<tr>
<td>B5</td>
<td>STND 10</td>
<td></td>
<td>3</td>
<td>35.25</td>
<td>6.4e-04</td>
<td>68.22</td>
<td>24.67</td>
</tr>
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<td>STND 10</td>
<td></td>
<td>3</td>
<td>35.44</td>
<td>6.4e-04</td>
<td>68.22</td>
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<td>C1</td>
<td>STND 3</td>
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<td>18.02</td>
<td>5.0e+01</td>
<td>68.22</td>
<td>24.67</td>
</tr>
<tr>
<td>35 C2</td>
<td>STND 3</td>
<td></td>
<td>3</td>
<td>17.53</td>
<td>5.0e+01</td>
<td>68.22</td>
<td>24.67</td>
</tr>
<tr>
<td>C3</td>
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<td></td>
<td>3</td>
<td>17.24</td>
<td>5.0e+01</td>
<td>68.22</td>
<td>24.67</td>
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<td>C4</td>
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<td></td>
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<td>38.52</td>
<td>1.3e-04</td>
<td>68.22</td>
<td>24.67</td>
</tr>
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<td>3</td>
<td>38.25</td>
<td>1.3e-04</td>
<td>68.22</td>
<td>24.67</td>
</tr>
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<td>38.29</td>
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<td>24.67</td>
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<td>19.72</td>
<td>1.0e+01</td>
<td>68.22</td>
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Table 4

NIS quantitative assay
Thermal Cycle Conditions

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<th>Auto Increment</th>
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<td>95.00</td>
<td>10:00</td>
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<td></td>
<td>60.00</td>
<td>1:00</td>
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Standard Curve

Slope: -2.87
Intercept: 28.77
Fit R: 0.98
Threshold: 0.00
Baseline Range: (3, 15)

Sample Information

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<th>Quantity</th>
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<th>Mean</th>
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In addition to its utility in the monitoring of the recurrence of thyroid cancer,
the quantitative RT-PCR assay of the invention exemplified in this Example is of use in other clinical situations requiring precise quantitation of thyroid-specific products (e.g., thyroglobulin); at present, such assays are performed by conventional immunological methods. Tg immunoassays are used to differentiate between endogenous hyperthyroidism that is associated with elevated or normal serum thyroglobulin concentrations and exogenous (thyrotoxicosis factitia) hyperthyroidism that is associate with suppressed endogenous thyroid function and, thus, low serum Tg concentrations. The quantitative Tg RT-PCR described herein has potential utility in this type of a clinical scenario. In addition, in patients treated with radioiodine for Graves' disease or large multinodular goiters, alterations in detectable circulating Tg mRNA may correlate with the efficacy of therapy as measured by the cure rate from hyperthyroidism or by the reduction in thyroid gland size or volume.

Example 5

As stated above, the serum of certain individuals contains antibodies directed against thyroglobulin. When such individuals require monitoring for the recurrence of thyroid cancer, thyroglobulin radioimmune assay (Tg-RIA) is an unacceptable option, as its results are ambiguous; therefore, the discovery of an alternative monitoring method is an imperative, rather than a preferred means of avoiding the side-effects of hormone therapy withdrawal. In this Example, clinical data from recovering thyroid cancer patients having serum anti-Tg antibodies at the titers indicated are presented side-by-side in Table 5 with Tg mRNA levels (quantitated according to the invention as described above in Example 4) and the results of Tg-RIA.

These results demonstrate a striking correlation between the observed presence of thyroid tissue in Tg-antibody-positive clinical patients and their Tg mRNA levels. This is in sharp contrast to RIA results, which yielded false positives in at least three cases in which no Tg transcript was detected and there was no
clinical evidence of the recurrence of disease. In addition, in the four cases in which the highest levels of Tg mRNA are recorded, which instances correlate with the presence of radioiodine uptake in neck lymph nodes, as well as soft tissue invasion/extension of thyroid cancer cells, the Tg-RIA readings indicate disproportionately low levels of Tg, indicating the vulnerability of that technique to the confounding effect of the antibodies present in the patients. These results demonstrate that use of the present invention is advantageous over that of prior art methods, as it provides an improvement not only in the quality of life of patients but, additionally, a significant improvement in accuracy.
Table 5

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<th>Patient</th>
<th>Tg Ab (ng/ml)</th>
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<th>Tg mRNA (pg RNA)</th>
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<td>11</td>
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<td>82</td>
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<td>? 4 mm x 1.0 cm recurrence (no change in CT for 2 years)</td>
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Example 6

In the Examples above, the presence of living thyroid tissue in individuals who have undergone thyroidectomy has been demonstrated by the detection of thyroid-cell-specific mRNA transcripts in peripheral blood samples obtained from recovering thyroid cancer patients, suggesting the presence of circulating thyroid cells. To support this notion, an experiment was performed to detect thyroid cells.
directly in blood. Significantly, the results, which are presented herein in this
Example, provide a second means by which recovering thyroid cancer patients may
be monitored for the recurrence of disease without withdrawal from thyroid
hormone therapy and, additionally, represent the first direct observation of
circulating thyroid cells in the blood by any method.

In preliminary experiments, Ficoll-Hypaque gradients were used to fractionate
whole blood samples, and were able to amplify thyroglobulin mRNA from the
erthrocyte cell pellet, but not from either the mononuclear cell or plasma fractions
(data not shown). Consequently, we subjected whole blood from two normal
subjects to cell sorting using an anti-thyrotropin receptor antibody and magnetic
bead separation (MACS, as described above), a technique previously used to
identify circulating tumor cells in patients with other malignancies (Wong et al.,
1995, supra; G患儿t et al., 1995, supra).

One milliliter of venous blood from two normal subjects was collected in
EDTA tubes. Erythrocytes were lysed by addition of five milliliters of a solution
containing 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA. Erythrocyte ghosts
were removed by centrifugation at 300 x g at room temperature. The pellet was
washed twice by resuspension in 10 mls of buffer (5 mM EDTA, 0.5% BSA) and
centrifugation. Approximately 10⁷ cells were resuspended in 100 μl of a 1:100
dilution of monoclonal antibody directed against the human thyrotropin receptor
(NCL-TSH-R2; Novacastra, Burlingame, CA) for ten minutes at 4°C. After
incubation, the cells were washed in 10 ml of buffer and were resuspended in 100
μl of a 1:5 dilution of polyclonal goat-anti-mouse IgG conjugated to paramagnetic
microbeads (Miltenyi Biotec, Sunnyvale, CA). The bead/cell mixture was incubated
at 4°C for 15 minutes, and then applied to magnetic separating columns (mini-
MACS; Miltenyi Biotec), as recommended by the manufacturer.

Isolated cells were collected onto glass microscope slides by centrifugation,
airdried, and washed with Tris-buffered saline. Slides were incubated with a
polyclonal antiserum against human thyroglobulin (Immunotech, Westbrook, ME) at full-strength, and antibody binding was detected by immunoperoxidase staining using the avidin-biotin-peroxidase technique (Vector Laboratories, Burlingame, CA). Control reactions were performed using normal human thyroid tissue as a positive control and substitution of saline for thyroglobulin antiserum as the negative control.

The isolated cells were further characterized immunocytochemically using anti-thyroglobulin antiserum, and disclosed approximately three thyroglobulin-staining epithelioid cells per milliliter of blood (Fig. 6).

**Other Embodiments**

Other embodiments will be evident to those of skill in the art. The spirit and scope of the present invention are not limited to the above examples.
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(ii) TITLE OF INVENTION: METHODS FOR THYROID CELL DETECTION

(iii) NUMBER OF SEQUENCES: 4

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   (F) ZIP: 02109

(v) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Mb storage
   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: Windows 95
   (D) SOFTWARE: Wordperfect 6.1a

(vi) CURRENT APPLICATION DATA:
   (A) APPLICATION NUMBER:
   (B) FILING DATE: 9-JUN-1998

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/049,144
(B) FILING DATE: 10-JUN-1997
(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Williams, Kathleen M.
(B) REGISTRATION NUMBER: 34,380
(C) REFERENCE/DOCKET NUMBER: 1107/74857
(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-227-7111
(B) TELEFAX: 617-227-4399

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8448 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(ix) OTHER INFORMATION:
(A) NAME/KEY: HSTHYRR
(B) OTHER INFORMATION: Human thyroglobulin gene; Genbank accession number X05615.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
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GCTACATTCA CAGCAGCAGC ACCTCCTACC TCCTCTGCTG TCAGGATCAA GGCGAAGCTG 420
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TGCTTCAGAC CTACAGACG TTTCCGAGAG CATTGTGTCG CTTAGGTTCC TTCCAGAGGA 720
GGTTCCTGCA GGTTATCTGG TTAAGCCTTG GTGCTGACAG CCAAGGCGAG GAATGCTGCTG 780
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GATCGTGCTT GAGAAGAGAT CTCTATAGGC TCAGGAAAAC AGCTCTAAGA ACCTACAGA 8340
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(2) INFORMATION FOR SEQ ID NO: 2:
   (i)SEQUENCE CHARACTERISTICS:
      (A)LENGTH: 1372 nucleotides
      (B)TYPE: nucleic acid
      (C)STRAND: double
      (D)TOPOLOGY: linear
   (ii)MOLECULE TYPE: cDNA
   (ix)FEATURE:
      (A)NAME/KEY: HUMPA8A
      (D)OTHER INFORMATION: Human Pax8 gene; Genbank accession number L19606.
   (x)SEQUENCE DESCRIPTION: SEQ ID NO: 2:
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GTGCCCATTT GAGGAGGGCG ACTACCCAGA GCCCTATGCG TCACCCAGGC ACACCAAGG 780
CGAGCAGGGC CTCTACCCGG TGCCCTTGCT CAACAGCACC CTGGACGAGC GGAAGGCAC 840
CCTGACCCCT TCCAAACGGG CACTGGGGCG CAACCTCTCG ACTCACCAGA CCTACCCGG 900
GCTGCCAGAT CCTCAGAGAC CCTCCTGGAC AAAGGCAGGAA CCACCGCAGG TGTCAGTTT 960
TAGCTCCACC CCTCTCCCTT TATCGAGCTC CGCCTTTTTG GATCTCGACG AAGTCGGCTC 1020
CGGGCCTCCC CTTCTCAATG CTTTCCCCCA TGCTGCTCCCT GTGAAGGGCG AGTTCGGG 1080
CCAGGCCTCT CTCTCACGGG GAGGAGATGG GGGCCGGAG CTGCCTCCGG ACCACCCCA 1140
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GCCCTCCCC AACTCCAGCT TGCTGATTC CCGCATATTAT TACAGTTCGA CATCGAGGCC 1320
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2336 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: HSU33749

(D) OTHER INFORMATION: Human TTF-1 gene, Genbank accession number U33749.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
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55

SUBSTITUTE SHEET (RULE 26)
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CGCCGCGGGA GGGGCGAGAC TCAGGGAAGC TGCCGCGTCA CAGGAGCAAC AGTGAAGAA 660
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GAACCTCCTTG CAGCAGGATG GACTGCGGATG TAATACGAGA ATTTTTTTTTTT TTTTTTTTTTGA 1920
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CAAAGGAAAT GCCGGCGCTCC GCGCCGGCAG GAAGGTCCGCTA GGGCGAAGGC GGAGGACG 2040
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CCCCGACCTC TCACTTTTTT TATGCCGATT GACAAATCTG TGTATATTAT TGGCGAGATT 2220
GTATTTTGGG GCCGCTAGCA TTTTCTCTTG TAAACTATTG TATGATTTTG GCTAAATTACA 2280
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 2241 nucleotides
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
   (A) NAME/KEY: HSD87920.
   (D) OTHER INFORMATION: Human NIS gene, Genbank accession number D87920.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCGCGGGGAC AGGGAGGGCG ACAGCGGACAT CGACAGCCCAG TAGATTCCATA ACCCAGGGAG 60
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CCATGAGCTTT GGCTCTGAT T 2241
Claims

What is claimed is:

1. A method for the detection of the presence of thyroid epithelial cells in a patient, comprising the step of detecting a transcript of a thyroid specific gene in a sample of reverse-transcribed total RNA of cells of blood, wherein said detection is indicative of the presence of thyroid epithelial cells.

2. The method of claim 1, wherein said reverse-transcribed total RNA is from a cell lysate of cells isolated from peripheral blood.

3. A method for the detection of the presence of thyroid epithelial cells in a patient, comprising
   (a) providing a sample of reverse-transcribed RNA from cells of blood of said patient,
   (b) performing a polymerase chain reaction (PCR) to amplify thyroid-cell-specific transcripts present in said reverse-transcribed RNA, wherein said PCR is primed with oligonucleotides that hybridize to a unique pair of sites comprising a first site and a second site in a thyroid-cell-specific gene and wherein said first and second sites are present in two different exons of said gene, such that their predicted PCR product spans one or a plurality of introns, and,
   (c) performing a detection step to detect a said PCR product, wherein detection of a said PCR product is indicative of the presence of thyroid epithelial cells.

4. The method of claim 3, wherein said sample comprises reverse-transcribed total RNA.
5. A method for the detection of the presence of thyroid-cell-specific mRNA transcripts in a patient, comprising the steps:

   (a) providing a sample of reverse-transcribed total RNA from cells of blood,

   (b) performing a polymerase chain reaction (PCR) to amplify thyroid-cell-specific transcripts present in said sample, wherein said PCR is primed with oligonucleotides that hybridize to a unique pair of sites comprising a first site and a second site in a thyroid-cell-specific gene and wherein said first and second sites are present in two different exons of said gene, such that their predicted PCR product spans one or a plurality of introns, and,

   (c) performing a detection step to detect a said PCR product which is thyroid-cell-specific, wherein detection of a said PCR product is indicative of the presence of thyroid-cell-specific mRNA transcripts in said patient.

6. The method of claim 1, 3 or 5 wherein said cells of blood are obtained from whole blood.

7. The method of claim 1, 3 or 5 wherein said cells of blood are obtained from a fraction of whole blood which is enriched for one or a plurality of cell types.

8. The method of claim 7, wherein said fraction is isolated by centrifugation.

9. The method of claim 8, wherein said fraction so isolated and selected is the erythrocyte fraction.

10. The method of claim 7, wherein said fraction is isolated by cell-sorting.

11. The method of claim 10, wherein said cell sorting is performed using an antibody or plurality of antibodies recognizing an epitope or plurality of epitopes.
specific to epithelial cells, which become selectively enriched in the cell fraction so isolated.

12. The method of claim 11, wherein said antibody is anti-cytokeratin.

13. The method of claim 11, wherein said epithelial cells are thyroid epithelial cells.

14. The method of claim 13, wherein said antibody is selected from the pair including anti-thyroglobulin and anti-thyroid-stimulating-hormone-receptor.

15. The method of claim 14, wherein said antibody is anti-thyroid-stimulating-hormone-receptor.

16. The method of claim 3 or 5, wherein said providing of a reverse-transcribed sample comprises the step of performing a reverse transcription of mRNA of cells of blood from said patient.

17. The method of claim 16, wherein said reverse transcription is primed with primers selected from the group that includes oligo-dT and random oligonucleotides.

18. The method of claim 17, wherein said oligonucleotides are hexanucleotides.

19. The method of claim 1, 3 or 5, wherein said thyroid-cell-specific gene encodes a protein selected from the group comprising thyroglobulin (Tg), thyroid peroxidase (TPO), Pax-8, thyroid transcription factor 1 (TTF-1) and thyroid transcription factor 2 (TTF-2) and the sodium iodide symporter (NIS).
20. The method of claim 19, wherein said thyroid-cell-specific gene encodes a protein selected from the group comprising Tg, Pax-8, TTF-1 and NIS.

21. The method of claim 20, wherein said thyroid-cell-specific gene encodes Tg.

22. The method of claim 21, wherein said primer pair comprises the following:
   \[ 5' \text{TGTGAGCTGCAGAGGAAACGGCC} 3' \quad [\text{SEQ ID NO: 1, nucleotides 141 through 164}] \]
   and
   \[ 5' \text{ATACACCTCCATCCCCTCTCGTGTCACACA} 3' \quad [\text{SEQ ID NO: 1, reverse complement of nucleotides 459 through 488}] \]

23. The method of claim 21, wherein said primer pair comprises the following:
   \[ 5' \text{GTGCCAACGGCGAGTGAGT} 3' \quad [\text{SEQ ID NO: 1, nucleotides 262 THROUGH 280}] \]
   and
   \[ 5' \text{TCTGCTGTTTCTGTAGCTGACAAA} 3' \quad [\text{SEQ ID NO: 1, reverse complement of nucleotides 325 through 348}] \]


25. The method of claim 24, wherein said primer pair comprises the following:
   \[ 5' \text{AAGGTGGTGAGGCTGTTGAGATTGGG} 3' \quad [\text{SEQ ID NO: 2, nucleotides 269 through 289}] \]
   and
   \[ 5' \text{GAGGTGGTGCTGG CTGAAGGC} 3' \quad [\text{SEQ ID NO: 2, reverse complement of nucleotides 689 through 709}] \]

27. The method of claim 26, wherein said primer pair comprises the following:

5' ACCAGGACACCAGGAGGAAC 3' [SEQ ID NO: 3, nucleotides 640 through 659], and
5' TGTCCTTGCCGTTCGCTTCA 3' [SEQ ID NO: 3, reverse complement of nucleotides 988 through 1008].

28. The method of claim 20, wherein said thyroid-cell-specific gene encodes NIS.

29. The method of claim 28, wherein said primer pair comprises the following:

5' ACACTGACTGCGACCCCTCCT 3' [SEQ ID NO: 4, nucleotides 1139-1160], and
5' TGCTGAGGGTGCACCTGTAA 3' [SEQ ID NO: 4, reverse complement of nucleotides 1260 through 1279].

30. A method for monitoring in a recovering thyroid cancer patient the recurrence of disease, comprising the step of detecting in a sample of reverse-transcribed total RNA of cells of blood a transcript of a thyroid-cell-specific gene, wherein said cells of blood are obtained from a recovering thyroid cancer patient and wherein detection of a said transcript is indicative of the recurrence of disease.

31. A method for monitoring in a recovering thyroid cancer patient the recurrence of disease, comprising the steps of

(a) providing a sample of reverse transcribed RNA from cells of blood, wherein said cells are obtained from a recovering thyroid cancer patient,

(b) performing a polymerase chain reaction (PCR) to amplify thyroid-cell-specific transcripts present in said sample, wherein said PCR is primed with oligonucleotides that hybridize to a unique pair of sites comprising a first site and a
second site in a thyroid-cell-specific gene and wherein said first and second sites are present in two different exons of said gene, such that their predicted PCR product spans one or a plurality of introns, and,

(c) performing a detection step to detect a said PCR product, wherein detection of a said PCR product is indicative of the recurrence of disease in said patient.

32. The method of claim 31, wherein said sample comprises reverse-transcribed total RNA.

33. The method of claim 30 or 31, wherein said cells of blood are obtained from whole blood.

34. The method of claim 30 or 31, wherein said cells of blood are obtained from a fraction of whole blood enriched for one or a plurality of cell types.

35. The method of claim 34, wherein said fraction is isolated by centrifugation.

36. The method of claim 35, wherein said fraction so isolated is the erythrocyte fraction.

37. The method of claim 34, wherein said fraction is isolated by cell-sorting.

38. The method of claim 37, wherein said cell sorting is performed using an antibody or plurality of antibodies recognizing an epitope or plurality of epitopes specific to epithelial cells, which become selectively enriched in the cell fraction so isolated.
39. The method of claim 38, wherein said antibody is anti-cytokeratin.

40. The method of claim 38, wherein said epithelial cells are thyroid epithelial cells.

41. The method of claim 40, wherein said antibody is selected from the pair including anti-thyroglobulin and anti-thyroid-stimulating-hormone-receptor.

42. The method of claim 41, wherein said antibody is anti-thyroid-stimulating-hormone-receptor.

43. The method of claim 30, wherein said total RNA is tested to detect a said thyroid-cell-specific transcript encoding a protein selected from the group that includes thyroglobulin (Tg) and the sodium iodide symporter (NIS) and said detection is effective to detect the presence of differentiated thyroid cells.

44. The method of claim 30, wherein said total RNA is tested to detect said thyroid-cell-specific transcripts selected from the group encoding Tg, Pax-8 and TTF-1, and said detection is effective to detect the presence of undifferentiated- or poorly-differentiated thyroid cells.

45. The method of claim 30 or 31, wherein said providing of a reverse-transcribed sample comprises the step of performing a reverse transcription of mRNA of cells of blood from said patient.

46. The method of claim 45, wherein said reverse transcription is primed with primers selected from the group that includes oligo-dT and random oligonucleotides.
47. The method of claim 46, wherein said oligonucleotides are hexanucleotides.

48. The method of claim 31 wherein said thyroid-cell-specific gene encodes a protein selected from the group comprising thyroglobulin (Tg), thyroid peroxidase (TPO), Pax-8, thyroid transcription factor 1 (TTF-1) and thyroid transcription factor 2 (TTF-2) and the sodium iodide symporter (NIS).

49. The method of claim 48, wherein said thyroid-cell-specific gene encodes a protein selected from the group comprising Tg, Pax-8, TTF-1 and NIS.

50. The method of claim 49, wherein said thyroid-cell-specific gene encodes Tg.

51. The method of claim 50, wherein said primer pair comprises the following:

   5’ TGTGAGCTGAGGAGGAAACGGCC 3’ [SEQ ID NO: 1, nucleotides 141 through 164], and

   5’ ATACACCTCCATCTCCCTGCTGCACACA 3’ [SEQ ID NO: 1, reverse complement of nucleotides 459 through 488].

52. The method of claim 50, wherein said primer pair comprises the following:

   5’ GTGCCAACGCGCAGTGAGT 3’ [SEQ ID NO: 1, nucleotides 262 through 280], and

   5’ TCTGCCTTTCTGTAGCTGACAAA 3’ [SEQ ID NO: 1, reverse complement of nucleotides 325 through 348].


54. The method of claim 53, wherein said primer pair comprises the following:
5' AAGGTGGTGAGAAGATTGGG 3' [SEQ ID NO: 2, nucleotides 269 through 289], and
5' GAGGTGGTGCTGGCTGAAGGC 3' [SEQ ID NO: 2, reverse complement of nucleotides 689 through 709].

55. The method of claim 49, wherein said thyroid-cell-specific gene encodes TTF-1.

56. The method of claim 55, wherein said primer pair comprises the following:
5' ACCAGGACACCATTGAGGAAC 3' [SEQ ID NO: 3, nucleotides 640 through 659], and
5' TGTCCTTGGCCTGGCGCTTCA 3' [SEQ ID NO: 3, reverse complement of nucleotides 988 through 1008].

57. The method of claim 49, wherein said thyroid-cell-specific gene encodes NIS.

58. The method of claim 57, wherein said primer pair comprises the following:
5' AACTGACTGCGACCCCTCCTCCT 3' [SEQ ID NO: 4, nucleotides 1139-1160], and
5' TGCTGAGGTGGCACCCTGTAA 3' [SEQ ID NO: 4, reverse complement of nucleotides 1260 through 1279].

59. The method of claim 3, 5 or 31, further comprising the step, after the step of detecting a PCR product which is thyroid-cell-specific, of performing a measurement to quantitate the amount of said product so detected.

60. The method of claim 59, wherein said measurement comprises use of a fluorometric oligonucleotide probe specific for one or more of said PCR products
produced from said thyroid-cell-specific transcripts.

61. The method of claim 60, wherein said fluorometric oligonucleotide is labeled at its 5' end with a dye selected from the group that includes 6-carboxy-fluorescein (FAM), tetrachloro-6-carboxy-fluorescein (TET), 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein (JOE) and hexachloro-6-carboxy-fluorescein (HEX).

62. The method of claim 60, wherein said thyroid-cell-specific transcript is that which encodes Tg.

63. The method of claim 62, wherein said fluorometric oligonucleotide probe comprises the following nucleic acid sequence:

\[ 5' \text{CCCTTCGTCCCTGTGAGCTGCA} 3' \]

[SEQ ID NO: 1, nucleotides 130 through 151].

64. The method of claim 62, wherein said fluorometric oligonucleotide probe comprises the following nucleic acid sequence:

\[ 5' \text{ACAGACAAGCCACAGGCGTCCT} 3' \]

[SEQ ID NO: 1, reverse complement of nucleotides 299 through 321].

65. The method of claim 60, wherein said thyroid-cell-specific transcript is that which encodes NIS.

66. The method of claim 65, wherein said fluorometric oligonucleotide probe comprises the following nucleic acid sequence:

\[ 5' \text{CGGGGACTCCAGGCAGATCCTCG} 3' \]

[SEQ ID NO: 4, reverse complement of nucleotides 1218 through 1240].
67. A method for the detection of the presence of thyroid epithelial cells in a human, comprising the steps of:

(a) using a cell-sorting procedure to separate a cell fraction comprising said thyroid epithelial cells from a sample of blood of said human, and

(b) performing a detection step to detect said thyroid epithelial cells in said fraction.

68. The method of claim 67, wherein said human is a recovering thyroid cancer patient.

69. The method of claim 67, wherein said cell-sorting procedure comprises magnetic cell sorting (MACS).

70. The method of claim 67, wherein said cell-sorting procedure employs an antibody directed against a thyroid-cell-specific antigen.

71. The method of claim 70, wherein said thyroid-cell-specific antigen is the human thyrotropin receptor.
Fig. 3
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
   IPC(6) : Please See Extra Sheet.
   US CL : 435/6, 7.1, 91.21; 436/64, 503, 526; 536/24.31, 24.33
   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)
   U.S. : 435/6, 7.1, 91.21; 436/64, 503, 526; 536/24.31, 24.33

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

   Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
   Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT
   Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No.
   X            | DITKOFF et al., Detection of circulating thyroid cells in peripheral blood. Surgery. December 1996, Vol. 120, No. 6, pages 959-965, especially Abstract, pages 959-962. | 1-6, 16, 19-21, 31-33, 43-45, 48-50

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

   * Special categories of cited documents:
      "A" document defining the general state of the art which is not considered to be of particular relevance
      "B" earlier document published on or after the international filing date
      "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)
      "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
      "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
      "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
      "Y" document of particular relevance; the claimed invention cannot be considered novel or 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
      "A" document member of the same patent family

   Date of the actual completion of the international search
   12 AUGUST 1998

   Date of mailing of the international search report
   2 SEP 1998

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<table>
<thead>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>SMANIK et al., Cloning of the human sodium iodide symporter. Biochemical and Biophysical Research Communications. 1996, Vol. 226, No. 6, pages 339-345, especially Fig. 1, see entire document.</td>
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<td>Y</td>
<td>MALTHIERY, Y. et al., Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA. European Journal of Biochemistry. 1987, Vol. 165, pages 491-498, especially Fig. 2.</td>
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<td>SAIARDI et al., Cloning and sequence analysis of human thyroid transcription factor I. Biochimica et Biophysica Acta. 1995, Vol. 1261, pages 307-310, see entire document.</td>
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<td>Y</td>
<td>Database MEDLINE, US National Library of Medicine, (Bethesda, MD, USA), No. 94019277, embl54. KOZMIK et al. 'Alternative splicing of Pax-8 gene transcripts is developmentally regulated and generates isoforms with different transactivation properties.' Molecular and Cellular Biology. 1993, Vol. 13, pages 6024-6035, see entire document.</td>
<td>24,25,53, 54</td>
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):
C12Q 1/68; G01N 33/48, 33/53, 33/567, 33/553; C12P 19/34; C07H 21/04

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):
APS, STN, CAPLUS, WPIDS,INPADOC, EUROPATFUL, CANCERLIT, MEDLINE, BIOSIS, EMBASE search
terms: RT-PCR, peripheral blood, measure, quantitate, amplify, nucleic acid, cancer, cell sort, magnetic, thyroid, Pax-8,
thyroglobulin, NIS, sodium iodide symporter, fluoros#, TTF-1, antibodies, anti-cytokeratin, anti-TSH, 6-carboxy-
fluorescein