PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/68, C07K 16/18

A1

(11) International Publication Number: **WO 99/14372**

(43) International Publication Date: 25 March 1999 (25.03.99)

(21) International Application Number: PCT/US98/19362

(22) International Filing Date: 15 September 1998 (15.09.98)

(30) Priority Data:
60/058,925 15 September 1997 (15.09.97) US

(71) Applicant: ABBOTT LABORATORIES [US/US]; 100 Abbott Park Road, Abbott Park, IL 60064–3500 (US).

(72) Inventors: BILLING–MEDEL, Patricia, A.; 1427 Sherwood Court, Gurnee, IL 60031 (US). COHEN, Maurice; 2026 Deerfield Road, Highland Park, IL 60035 (US). FRIEDMAN, Paula, N.; 462 Cumnor Court, Deerfield, IL 60015 (US). GORDON, Julian; 207 East Sheridan Road, Lake Bluff, IL 60044 (US). HODGES, Steven, C.; 169 Stonegate Road, Buffalo Grove, IL 60089 (US). KLASS, Michael, R.; 1606 Mulberry Drive, Libertyville, IL 60048 (US). KRATOCHVIL, Jon, D.; 7101 Fifth Avenue, Kenosha, WI 53143 (US). RUSSELL, Eric; 16730 W. Apple Lane, Gurnee, IL 60031 (US). STROUPE, Stephen, D.; 945 Wilshire Drive, Libertyville, IL 60048 (US).

(74) Agents: WEINSTOCK, Steven, F.; Abbott Laboratories, D–377/AP6D, 100 Abbott Park Road, Abbott Park, IL 60064–3500 (US) et al.

(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: REAGENTS AND METHODS USEFUL FOR DETECTING DISEASES OF THE URINARY TRACT

(57) Abstract

Polypeptides and polynucleotides useful for detecting, diagnosing, staging, monitoring, prognosticating, *in vivo* imaging, preventing or treating, or determining the predisposition of an individual to diseases and conditions of the urinary tract, such as urinary cancer, are described. These sequences are derived from keratin/cytokeratin, CAS, or mat–8 polypeptides and polynucleotides. Also provided are antibodies which specifically bind to keratin/cytokeratin, CAS, or mat–8–encoded polypeptides or proteins, which molecules are useful for the therapeutic treatment of urinary tract diseases, tumors or metastases.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

REAGENTS AND METHODS USEFUL FOR DETECTING DISEASES OF THE URINARY TRACT

Background of the Invention

The invention relates generally to detecting diseases of the urinary tract. Furthermore, the invention also relates to reagents and methods for detecting diseases of the urinary tract. More particularly, the present invention relates to reagents such as polynucleotide sequences and the polypeptide sequences encoded thereby, as well as methods which utilize these sequences. The polynucleotide and polypeptide sequences are useful for detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating, or determining predisposition to diseases or conditions of the urinary tract such as urinary tract cancers.

The organs of the urinary tract include the bladder, kidneys, and ureter. The incidence of urinary tract cancers in the United States is projected to be 86,300 cases diagnosed and 24,700 related deaths to occur during 1998. The most prevalent of the urinary tract cancers is bladder cancer, with projections of 54,400 new cases diagnosed and 12,500 related deaths to occur during 1998 (American Cancer Society statistics). Bladder tumors are heterogeneous in their ability to progress and are characterized by a high rate of recurrence. Hence, bladder cancer patients are monitored closely following their initial treatment.

Diseases such as bladder cancer traditionally have been diagnosed by appearance of blood in urine (hematuria) and confirmed by more detailed visualization of cells in biopsy samples under a microscope by highly trained personnel. The standard surveillance technique to detect recurrent bladder cancer is cystoscopy. Flexible cystoscopes have made cystoscopy more acceptable to patients but the method remains invasive. Efforts to replace cystoscopy by examining voided urine for tumor cells have included cytopathology and flow cytometry but the sensitivities are not high enough to detect the majority of recurrence, particularly those that are well or moderately differentiated. Cost also limits their use as adjuncts to cystoscopy. Other urinary markers that might be useful for diagnosing recurrences are being developed, including nonspecific markers, such as various growth factors, immune complexes and tumor-related proteins. However, to date, no single test has proved reliable enough to gain widespread acceptance. In particular, no test to date has sufficient simplicity, sensitivity, specificity and cost effectiveness to warrant pre-symptomatic screening.

Following is a more detailed summary of methods and markers in urine that have been used for detection of bladder tumors, and their limitations: Hematuria is found in up to 20% of a target population (Mariani AJ, Mariani MC, Macchioni C, Stams UK, Hariharan A, Moriera A. J Urol 141:350-355, 1989; Messing EM, Young TB, Hunt VB, Roecker EB, Vailiancourt AM, Hisgen WJ, Greenberg EB, Kuglitsch ME, Wegenke, JD: J Urol 148:289-292, 1992; and Britton JP, Dowell AC, Whelan P, Harris CM. J Urol 148:788-790, 1992), and 4-9% of those will have malignancy. In one of the most recent studies, the BTA (basement membrane complexes) test showed sensitivity of 40%, 96% specificity for healthy volunteers and 80-90% specificity for patients with non-malignant genitourinary disease (Sarosdy MF, deVere White RW, Soloway MS, Sheinfeld J, Hudson MA, Schelihammer PF, Jarowenko MV, Adams G, Blumenstein BA: J Urol 154:379-384, 1995). Urinary basement membrane antigens have been found higher in invasive cancer, with sensitivity 58% and specificity 96% (Abou Farha KMM, Janknegt RA, Kester ADM, Arendt JW: Urol Int 50 133-140, 1993). The Aura-Tek FDP dipstick showed 69% sensitivity with a patient population and 96% specificity with a healthy population (Schmetter BS, Habicht KK, Lamm DL, Morales A, Grossman HB, Bander N, Hanna MG, Butman BR: J Urol 155:492A, 1996). Nuclear matrix protein NMP22 showed considerable overlap between patients with tumors and benign urological conditions (Carpinita GA, Stadler WM, Briggman JV, Chodak GW, Church PA, Lamm DL, Lange PH, Messing EM, Pasciak RM, Reservitz GB, Ross RN, Rukstalis DB, Sarosday MF, Soloway MS, Thiel RP, Vogelzang N, Hayden CL: J Urol 156:1280-1285, 1996). Carcinoembryonic antigen (CEA) has been shown to occur in urine of some patients with bladder cancer, but the potential diagnostic utility is unclear (Wahren B, Edsmyr F: Urol Res 6:221-224, 1978). High levels of autocrine motility factor (AMF) have been detected in urine of patients with bladder cancer, showing correlation with stage and grade, but the clinical utility has not been established (Guirguis R, Schiffman E, Liu B, Birbeck D, Engel J, Liotta L: J Nat Cancer Inst 80:1203-1211, 1988). Similarly, high levels of fibroblast growth factors (FGF) have been found in urine of some bladder cancer patients (O'Brien TS, Smith K, Cranston D, Fuggle S, Bicknell R, Harods AL: Brit J Urol 76:311-314, 1995; Nguyen M, Watanabe H, Budson E, Richie JP, Folkman J: J Nat Cancer Inst 85:241-242, 1993; Chopin DK, Caruelle J-P, Colmbel M, Pallcy S, Ravery V, Caruelle D, Abbout CC, Bardtault D: J Urol 160:1126-1130, 1991). Further, high urinary levels of Scatter Factor/Hepatocyte Growth Factor (SF/HGF) have been reported in patients with bladder cancer, but the data is as yet unconfirmed (Joseph A, Weiss GH, Lin L, Fuchs A, Chowdhury S, O'Shaugnessy P, Goldbert ID,

Rosen EM: J Nat Cancer Inst 87:372-377, 1995). In one unconfirmed report, urinary type IV collagenase has been found to be higher in urine from patients with invasive cancers (Margulies IMK, Hoyhtya M, Evans C, Stracke ML, Liotaa LA, Stetler-Stevenson WG: Cancer Epidemiol Biomarkers Prev 1:467-474, 1992). Hyaluronic acid has recently been proposed as a 93% specific and 92% sensitive marker (Lokeshwar VB, Öbeck C, Soloway MS, Block NL: Cancer res 57, 774-777, 1997), but the data remains to be confirmed by larger studies. Patent application EP 0678744A2 describes a urinary tumor associated protein which is characterized by being immunogenic in cancer patients. The antigen was not characterized in detail, and is unlikely to be a member of the keratin/cytokeratin family, since these are not known to be immunogenic in cancer patients. There is thus a great effort to discover specific urinary markers, but none have become routinely established.

Assays for urinary tumor markers based on proteins of the keratin/cytokeratin family have been described. Some of these assays use uncharacterized antibodies while others use antibodies with defined specificity. As suggested below, the basis of these assays appears to be selective release of soluble peptide fragments by tumorous cells. Furthermore, the literature suggests that keratins and cytokeratins are characteristic of the differentiated state of cells, and, where present, are indicative of the normal cellular origin of the tumor, not of the state of de-differentiation. Indeed, Nagle [Cancer & Metastasis Rev.15:473-482 (1996)] states that intermediate filaments in general, including the cytokeratins as a class, are valuable markers for distinguishing the cellular origin of various undifferentiated neoplasms.

Sundström and Stigbrand (International Journal of Biological markers, 9, 102-108, 1994) state that cytokeratin markers are characteristic of the expression patterns of normal epithelial cells and the retention of the pattern is a useful means for classifying tumors. These authors also describe Tissue Polypeptide Antigen (TPA) as a complex of cytokeratins 8, 18 and 19. Carbin, Eckman and Eneroth (Urol Res 17, 269-272, 1989) state that TPA in serum and urine is the result of increased turnover and autolysis of malignant cells. Because of the poly-specificity of the TPA assays, any selectivity due to de novo synthesis would not be apparent, and, should only be detectable with specificity using probes or antibodies to epitopes which are unique for the specific keratin/cytokeratins which are up-regulated in the cancerous tissue. Patent number US533832 describes a monoclonal antibody with reactivity to cytokeratins 8, 18 and 19, which they suggest might be useful for diagnosing cancer. There is no discussion of up-regulated proteins indicative of bladder cancer.

Attempts have been made to improve the specificity of assays by using TPA in combination with other markers (Halim AB, El-Amahdy O, Hamza S, Aboul-Ela M, Oehr P: International Journal of Biological markers, 7, 234-239, 1992; Casetta G, Piana P, Cavallini A, Vottero M, Tizzani A: Brit J Urol 72, 60-64, 1993). The specificity is still inadequate since false positives due to urinary tract infections may occur. Further, a diagnostic test based on a single marker is preferable to an assay using three markers. There have also been attempts to improve the TPA assay by the use of monoclonal antibodies (Sundström BE, d'Amico Y, Brundell J: Int J Biol Markers, 10, 166-173, 1995). However, these monoclonal antibodies were used only in an attempto mimic the oligo-specificity of the polyclonal antibody assay, and not to improve the specificity by looking at specific peptides up-regulated in the cancerous tissue.

Keratin 18 has been proposed for use as a target in more specific assays(Baker WC, White RD, Rossito PV, Min BH, Cardiff RD: J Urol 140, 436-439, 1988). However, the monoclonal antibodies used had cross-reactivity with keratin 8 (Rossito PV, Chan R, Strand MA, Miller CH, Baker WC, Deitsch AD, Devere White R, Cardiff RD: J Urol 140, 431-435, 1988). Furthermore, limited sensitivity and specificity was noted. Patent application PCT 95/31728 describes specific epitopes characteristic of cytokeratin 18 and detectable amounts of these epitopes in the serum of bladder cancer patients. However, the utility using these specific epitopes may be limited because of the specificity of the epitopes described. Further there is no suggestion of a general approach to look for keratins/cytokeratins up-regulated in bladder tumors. A commercial kit is available for cytokeratin 19, under the name Cyfra 21-1. With that kit, problems occur due to the occurrence of cells and cell debris in urine which must be first removed in order to determine soluble fragments (Dittado R, Bariolli P, Gion M, Mione R, Barichello M, Capitanio G, Cocco G, Cazzolato G, de Biasi F, Praturlon F, Antinozzi R, Gianneo E: Clin Chem 42, 1634-1638, 1996). False positives were obtained in patients with cystitis (Senga Y, Kimura G, Hattori T, Yoshida K: Urology 48, 703-710, 1996). None of the above works determined the merits of markers which are specifically up-regulated in bladder tumors. In a comprehensive study, Moll et al (Moll R, Achtstätter T, Balcarova-Ständer J, Ittensohn M and Franke WW: Amer J Pathol 132, 123-144, 1988) showed that cytokeratins 7, 8, 13, 19 are present in normal urothelium, and that 13 was greatly reduced in grade three transitional cell carcinoma. No mention was made of any species being present in increased amounts in the cancer.

Brinkman et al (Proc Natl Acad Sci U S A 1995 Oct 24;92(22):10427-10431) have discovered a gene referred to as CAS (cellular apoptosis

susceptibility). They have also observed that this gene is amplified and has increased expression in certain tumorous cell lines from leukemia, colon and breast cancer (Genome Res 1996 Mar;6(3):187-194). See, also, PCT Publication No. WO 9640713.

Morrison et al (Oncogene 1994, vol 9 pp 3417-3426, Journal of Biological Chemistry, vol 270, pp 2176-2182) have described a class of proteins which are expressed in tumors initiated by the oncogene neu but absent from those initiated by c-myc. One of these, a chloride channel protein, mat-8, was proposed as having diagnostic utility for breast cancer (PCT application WO96/05322).

However, to date, there has been no suggestion for using the above proteins as diagnostic markers for urinary tract diseases.

Summary of the Invention

The present invention is based on the surprising finding of keratin/cytokeratin and other markers up-regulated in urinary cancerous conditions but not expressed in the normal bladder. Thus, the present invention provides a method of detecting the presence of urinary tract disease in an individual which comprises providing a test sample from the individual and contacting the test sample with at least one keratin/cytokeratin, CAS, or mat-8-specific polynucleotide or complement thereof, wherein the keratin/cytokeratin, CAS, or mat-8-specific polynucleotide has at least 50% identity with a polynucleotide selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof; and detecting the presence of target keratin/cytokeratin, CAS, or mat-8 polynucleotides in the test sample which bind to the keratin/cytokeratin, CAS, or mat-8-specific polynucleotide, as an indication of urinary tract disease in the individual. The keratin/cytokeratin, CAS, or mat-8-specific polynucleotide may be attached to a solid phase prior to performing the method. Furthermore, in certain embodiments, the urinary tract disease is cancer.

The present invention also provides a method of detecting the presence of urinary tract disease in an individual which comprises (a) providing a test sample from the individual and performing reverse transcription on said sample using at least one primer in order to produce cDNA; (b) amplifying the cDNA obtained from step (a) using keratin/cytokeratin, CAS, or mat-8 oligonucleotides as sense and antisense primers to obtain keratin/cytokeratin, CAS, or mat-8 amplicon; and (c) detecting the presence of the keratin/cytokeratin, CAS, or mat-8 amplicon as an indication of urinary tract disease in the individual, wherein the keratin/cytokeratin, CAS, or mat-8 oligonucleotides utilized in steps (a) and (b) have at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID

NOS 1 through 13, and fragments or complements thereof. This reaction can be a direct or an indirect reaction. Further, the detection step can comprise utilizing a detectable label capable of generating a measurable signal. The detectable label can be attached to a solid phase. The test sample can also be reacted with a solid phase prior to performing one or more of the steps of the method. Further, the detection step may utilize a detectable label capable of generating a measurable signal. Additionally, in certain embodiments, the urinary tract disease is cancer.

The invention further provides a method of detecting the presence of urinary tract disease in an individual which comprises (a) providing a test sample from the individual and contacting the test sample with at least one keratin/cytokeratin, CAS, or mat-8 oligonucleotide as a sense primer and with at least one keratin/cytokeratin, CAS, or mat-8 oligonucleotide as an anti-sense primer and amplifying to obtain a first stage reaction product; (b) contacting the first stage reaction product with at least one other keratin/cytokeratin, CAS, or mat-8 oligonucleotide to obtain a second stage reaction product, with the proviso that the other keratin/cytokeratin, CAS, or mat-8 oligonucleotide is located 3' to the keratin/cytokeratin, CAS, or mat-8 oligonucleotides utilized in step (a) and is complementary to said first stage reaction product; and (c) detecting the second stage reaction product as an indication of urinary tract disease in the individual, wherein the keratin/cytokeratin, CAS, or mat-8 oligonucleotides utilized in steps (a) and (b) have at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof. The test sample can be reacted with a solid phase prior to performing one or more of the steps of the method. Further, the detection step may utilize a detectable label capable of generating a measurable signal. In certain embodiments, the urinary tract disease is cancer.

The invention also provides a test kit useful for detecting urinary tract disease in a test sample. The test kit comprises a container containing at least one keratin/cytokeratin, CAS, or mat-8 polynucleotide having at least 50% identity with a sequence selected from the group consisting SEQUENCE ID NOS 1 through 13, and fragments or complements thereof. Also provided is a test kit useful for detecting urinary tract cancer in a test sample which comprises a container containing a keratin/cytokeratin, CAS, or mat-8 polypeptide encoded by a nucleic acid sequence having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof. The polypeptides of the test kit may be attached to a solid phase. These test kits further comprise containers with tools useful for collecting test samples (such as, for example, blood, urine, saliva and stool). Such tools include lancets

and absorbent paper or cloth for collecting and stabilizing blood; swabs for collecting and stabilizing saliva; and cups for collecting and stabilizing urine or stool samples. Collection materials, such as, papers, cloths, swabs, cups, and the like, may optionally be treated to avoid denaturation or irreversible adsorption of the sample. The collection materials also may be treated with or contain preservatives, stabilizers or antimicrobial agents to help maintain the integrity of the specimens.

Also provided is a specific binding molecule which specifically binds to a keratin/cytokeratin, CAS, or mat-8 epitope. The keratin/cytokeratin, CAS, or mat-8 epitope is derived from a polypeptide encoded by a nucleic acid sequence having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof. The specific binding molecule may be an antibody molecule of fragment thereof.

Test kits useful for detecting urinary tract cancer in a test sample are also provided which comprise a container containing a specific binding molecule which specifically binds to a keratin/cytokeratin, CAS, or mat-8 antigen having a keratin/cytokeratin, CAS, or mat-8 epitope. The specific binding molecule may be an antibody or fragment thereof and may be attached to a solid phase. These test kits further comprise containers with tools useful for collecting test samples (such as, for example, blood, urine, saliva and stool). Such tools include lancets and absorbent paper or cloth for collecting and stabilizing blood; swabs for collecting and stabilizing saliva; and cups for collecting and stabilizing urine or stool samples. Collection materials, such as, papers, cloths, swabs, cups, and the like, may optionally be treated to avoid denaturation or irreversible adsorption of the sample. The collection materials also may be treated with or contain preservatives, stabilizers or antimicrobial agents to help maintain the integrity of the specimens.

Also provided is a method of detecting the presence of urinary tract cancer in an individual which comprises (a) providing a test sample from the individual and contacting the test sample with a specific binding molecule which specifically binds to an epitope of a keratin/cytokeratin, CAS, or mat-8 antigen selected from the group consisting of a polypeptide encoded by a nucleic acid sequence having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof. The contacting is performed for a time and under conditions sufficient for the formation of binding molecule/antigen complexes. Detection of the complexes is an indication of urinary tract cancer in the individual. The specific binding molecule may be an antibody molecule or a fragment thereof and may be attached to a solid phase.

The invention further provides a method of detecting the presence of urinary tract cancer in an individual which comprises (a) providing a test sample from the individual and contacting the test sample with a keratin/cytokeratin, CAS, or mat-8 polypeptide. The keratin/cytokeratin, CAS, or mat-8 polypeptide contains at least one keratin/cytokeratin, CAS, or mat-8 epitope of a polypeptide encoded by a nucleic acid molecule having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof. The contacting is performed for a time and under conditions sufficient to allow antigen/antibody complexes to form. Detection of the complexes is an indication of urinary tract cancer in the individual. The keratin/cytokeratin, CAS, or mat-8 polypeptide can be attached to a solid phase.

Also provided is a method for producing antibodies which specifically bind to keratin/cytokeratin, CAS, or mat-8 antigen, comprising administering to an individual an isolated immunogenic polypeptide or fragment thereof in an amount sufficient to elicit an immune response. The immunogenic polypeptide comprises at least one keratin/cytokeratin, CAS, or mat-8 epitope derived from a polypeptide encoded by a nucleic acid molecule having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof.

An additional method for producing antibodies which specifically bind to a keratin/cytokeratin, CAS, or mat-8 antigen is provided which comprises administering to an individual a plasmid comprising a sequence which encodes at least one keratin/cytokeratin, CAS, or mat-8 epitope derived from a polypeptide encoded by a nucleic acid molecule having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1through 13, and fragments or complements thereof,

In another embodiment of the invention, specific binding molecules, such as antibodies or fragments thereof against the keratin/cytokeratin, CAS, or mat-8 antigens, can be used to detect antigen, or for image localization of the antigen, in a patient for the purpose of detecting or diagnosing a disease or condition. Such antibodies can be polyclonal or monoclonal, or made by molecular biology techniques, and can be labeled with a variety of detectable labels, including but not limited to radioisotopes and paramagnetic metals. Furthermore, antibodies or fragments thereof, whether monoclonal, polyclonal, or made by molecular biology techniques, can be used as therapeutic agents for the treatment of diseases characterized by expression of the keratin/cytokeratin, CAS, or mat-8 antigen. In the case of therapeutic applications, the antibody may be used without

derivitization, or it may be derivitized with a cytotoxic agent such as a radioisotope, enzyme, toxin, drug, prodrug, or the like.

Brief Description of the Drawing

The following drawing is illustrative of embodiments of the invention and is not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 shows the results of a PCR fragment analysis using cytokeratin CK5-specific primers.

Detailed Description of the Invention

A general strategy for discovering genes and gene products which are specifically up-regulated in urinary tract cancers, is provided. A method for detecting a urinary tract cancer antigen in a test sample from an individual suspected of having a urinary tract cancer is provided, which comprises contacting the test sample with an antibody or fragment thereof which specifically binds to at least one urinary tract cancer antigen, for a time and under conditions sufficient for the formation of antibody/antigen complexes, and detecting the complex containing the antibody. The antibody can be attached to a solid phase and be either a monoclonal or polyclonal antibody. The antibody specifically binds to a polypeptide epitope characterized by being up-regulated in urinary tract cancers as detailed below. These polypeptides include a keratin/cytokeratin, CAS or mat-8 polypeptide, or fragments thereof.

A method for producing antibodies to urinary tract cancer gene products comprising administering to an individual an isolated immunogenic polypeptide or fragment thereof comprising at least one urinary tract cancer epitope in an amount sufficient to produce an immune response, also is provided.

The present invention provides methods for assaying a test sample for products of a gene characterized by being up-regulated in urinary tract cancers. Test samples which may be assayed by the methods provided herein include tissues, cells, body fluids and secretions. The present invention also provides reagents such as oligonucleotide primers and polypeptides which are useful in performing these methods.

In addition to urinary tract cancer, the markers represented by SEQUENCE ID NOS 1-13, and polypeptides encoded thereby, may also be up-regulated in other urinary tract diseases or conditions of the urinary tract including, but not limited to, cystitis, interstitial cystitis, urethritis, nephrosclerosis, and nephritis.

Portions of the nucleic acid sequences disclosed herein are useful as primers for the reverse transcription of RNA or for the amplification of cDNA; or as probes to determine the presence of certain mRNA sequences in test samples.

Also disclosed are nucleic acid sequences which permit the production of encoded polypeptide sequences which are useful as standards or reagents in diagnostic immunoassays, as targets for pharmaceutical screening assays and/or as components or as target sites for various therapies. Monoclonal and polyclonal antibodies directed against at least one epitope encoded by these polynucleotide sequences are useful as delivery agents for therapeutic agents as well as for diagnostic tests and for screening for diseases or conditions associated with urinary tract cancer. Isolation of sequences of other portions of the genes of interest can be accomplished utilizing probes or PCR primers derived from these nucleic acid sequences. This allows additional probes of the mRNA or cDNA of interest to be established, as well as the corresponding encoded polypeptide sequences. These additional molecules are useful in detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating, or determining the predisposition to diseases and conditions of the urinary tract, such as urinary tract cancer.

Techniques for determining amino acid sequence similarity are well-known in the art. In general, "similarity" means the exact amino acid to amino acid comparison of two or more polypeptides at the appropriate place, where amino acids are identical or possess similar chemical and/or physical properties such as charge or hydrophobicity. A so-termed "percent similarity" then can be determined between the compared polypeptide sequences. Techniques for determining nucleic acid and amino acid sequence identity also are well known in the art and include determining the nucleotide sequence of the mRNA for that gene (usually via a cDNA intermediate) and determining the amino acid sequence encoded thereby, and comparing this to a second amino acid sequence. In general, "identity" refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more polynucleotide sequences can be compared by determining their "percent identity." Two or more amino acid sequences likewise can be compared by determining their "percent identity." The percent identity of two sequences, whether nucleic acid or peptide sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be extended to use with peptide sequences using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M.O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by

Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An implementation of this algorithm for nucleic acid and peptide sequences is provided by the Genetics Computer Group (Madison, WI) in their BestFit utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package, Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, WI). Other equally suitable programs for calculating the percent identity or similarity between sequences are generally known in the art.

The compositions and methods described herein will enable the identification of certain markers as indicative of urinary tract cancer; the information obtained therefrom will aid in the detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating, or determining diseases or conditions associated with SEQUENCE ID NOS 1 through 13, and polypeptides encoded thereby, which have been found to be upregulated in urinary tract cancer. Test methods include, for example, probe assays which utilize the sequence(s) provided herein and which also may utilize nucleic acid amplification methods such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), and hybridization.

In addition, the nucleotide sequences provided herein contain open reading frames from which an immunogenic epitope may be found. This epitope is believed to be unique to urinary tract cancer associated with markers represented by SEQUENCE ID NOS 1 through 13. It also is thought that the polynucleotides or polypeptides and protein encoded by SEQUENCE ID NOS 1 through 13 are useful as markers. These markers are elevated in urinary tract cancer. The uniqueness of the epitope may be determined by (i) its immunological reactivity and specificity with antibodies directed against proteins and polypeptides encoded by SEQUENCE ID NOS 1 through 13, and (ii) its nonreactivity with other antibodies directed against other tissue markers. Methods for determining immunological reactivity are well-known and include, but are not limited to, for example, radioimmunoassay (RIA), enzyme-linked immunoabsorbent assay (ELISA), hemagglutination (HA), fluorescence polarization immunoassay (FPIA), chemiluminescent immunoassay (CLIA) and others. Several examples of suitable methods are described herein.

Unless otherwise stated, the following terms shall have the following meanings:

A polynucleotide "derived from" or "specific for" a designated sequence refers to a polynucleotide sequence which comprises a contiguous sequence of approximately at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 nucleotides, and even more preferably at least

about 15-20 nucleotides corresponding, i.e., identical or complementary to, a region of the designated nucleotide sequence. The sequence may be complementary or identical to a sequence which is unique to a particular polynucleotide sequence as determined by techniques known in the art. Comparisons to sequences in databanks, for example, can be used as a method to determine the uniqueness of a designated sequence. Regions from which sequences may be derived, include but are not limited to, regions encoding specific epitopes, as well as non-translated and/or non-transcribed regions.

The derived polynucleotide will not necessarily be derived physically from the nucleotide sequence of interest under study, but may be generated in any manner, including, but not limited to, chemical synthesis, replication, reverse transcription or transcription, which is based on the information provided by the sequence of bases in the region(s) from which the polynucleotide is derived. As such, it may represent either a sense or an antisense orientation of the original polynucleotide. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with the intended use.

A "fragment" of a specified polynucleotide refers to a polynucleotide sequence which comprises a contiguous sequence of approximately at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12 nucleotides, and even more preferably at least about 15-20 nucleotides corresponding, i.e., identical or complementary to, a region of the specified nucleotide sequence.

The term "primer" denotes a specific oligonucleotide sequence which is complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence. A primer serves as an initiation point for nucleotide polymerization catalyzed by either DNA polymerase, RNA polymerase or reverse transcriptase.

The term "probe" denotes a defined nucleic acid segment (or nucleotide analog segment, e.g., PNA as defined hereinbelow) which can be used to identify a specific polynucleotide present in samples bearing the complementary sequence.

"Encoded by" refers to a nucleic acid sequence which codes for a polypeptide sequence, wherein the polypeptide sequence or a portion thereof contains an amino acid sequence of at least 3 to 5 amino acids, more preferably at least 8 to 10 amino acids, and even more preferably at least 15 to 20 amino acids from a polypeptide encoded by the nucleic acid sequence. Also encompassed are polypeptide sequences which are immunologically identifiable with a polypeptide encoded by the sequence. Thus, a "polypeptide," "protein," or "amino acid"

sequence has at least about 50% identity, preferably about 60% identity, more preferably about 75-85% identity, and most preferably about 90-95% or more identity with an amino acid sequence. Further, the "polypeptide," "protein," or "amino acid" sequence may have at least about 60% similarity, preferably at least about 75% similarity, more preferably about 85% similarity, and most preferably about 95% or more similarity to a polypeptide or amino acid sequence. This amino acid sequence can be selected from the group consisting of polypeptides encoded by SEQUENCE ID NOS 1 through 13, and fragments thereof.

A "recombinant polypeptide," "recombinant protein," or "a polypeptide produced by recombinant techniques," which terms may be used interchangeably herein, describes a polypeptide which by virtue of its origin or manipulation is not associated with all or a portion of the polypeptide with which it is associated in nature and/or is linked to a polypeptide other than that to which it is linked in nature. A recombinant or encoded polypeptide or protein is not necessarily translated from a designated nucleic acid sequence. It also may be generated in any manner, including chemical synthesis or expression of a recombinant expression system.

The term "synthetic peptide" as used herein means a polymeric form of amino acids of any length, which may be chemically synthesized by methods well-known to the routineer. These synthetic peptides are useful in various applications.

The term "polynucleotide" as used herein means a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, the term includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modifications, such as methylation or capping and unmodified forms of the polynucleotide. The terms "polynucleotide," "oligomer," "oligonucleotide," and "oligo" are used interchangeably herein.

"A sequence corresponding to a cDNA" means that the sequence contains a polynucleotide sequence that is identical or complementary to a sequence in the designated DNA. The degree (or "percent") of identity or complementarity to the cDNA will be approximately 50% or greater, preferably at least about 70% or greater, and more preferably at least about 90% or greater. The sequence that corresponds to the identified cDNA will be at least about 50 nucleotides in length, preferably at least about 60 nucleotides in length, and more preferably at least about 70 nucleotides in length. The correspondence between the gene or gene fragment of interest and the cDNA can be determined by methods known in the art and include, for example, a direct comparison of the sequenced material with the

cDNAs described, or hybridization and digestion with single strand nucleases, followed by size determination of the digested fragments.

"Purified polynucleotide" refers to a polynucleotide of interest or fragment thereof which is essentially free, e.g., contains less than about 50%, preferably less than about 70%, and more preferably less than about 90%, of the protein with which the polynucleotide is naturally associated. Techniques for purifying polynucleotides of interest are well-known in the art and include, for example, disruption of the cell containing the polynucleotide with a chaotropic agent and separation of the polynucleotide(s) and proteins by ion-exchange chromatography, affinity chromatography and sedimentation according to density.

"Purified polypeptide" or "purified protein" means a polypeptide of interest or fragment thereof which is essentially free of, e.g., contains less than about 50%, preferably less than about 70%, and more preferably less than about 90%, cellular components with which the polypeptide of interest is naturally associated. Methods for purifying polypeptides of interest are known in the art.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, which is separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that the vector or composition is not part of its natural environment.

"Polypeptide" and "protein" are used interchangeably herein and indicate at least one molecular chain of amino acids linked through covalent and/or non-covalent bonds. The terms do not refer to a specific length of the product. Thus peptides, oligopeptides and proteins are included within the definition of polypeptide. The terms include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. In addition, protein fragments, analogs, mutated or variant proteins, fusion proteins and the like are included within the meaning of polypeptide.

A "fragment" of a specified polypeptide refers to an amino acid sequence which comprises at least about 3-5 amino acids, more preferably at least about 8-10 amino acids, and even more preferably at least about 15-20 amino acids derived from the specified polypeptide.

"Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as

recipients for recombinant vector or other transferred DNA, and include the original progeny of the original cell which has been transfected.

As used herein replicon" means any genetic element, such as a plasmid, a chromosome or a virus, that behaves as an autonomous unit of polynucleotide replication within a cell.

A "vector" is a replicon in which another polynucleotide segment is attached, such as to bring about the replication and/or expression of the attached segment.

The term "control sequence" refers to a polynucleotide sequence which is necessary to effect the expression of a coding sequence to which it is ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, such control sequences generally include a promoter, a ribosomal binding site and terminators; in eukaryotes, such control sequences generally include promoters, terminators and, in some instances, enhancers. The term "control sequence" thus is intended to include at a minimum all components whose presence is necessary for expression, and also may include additional components whose presence is advantageous, for example, leader sequences.

"Operably linked" refers to a situation wherein the components described are in a relationship permitting them to function in their intended manner. Thus, for example, a control sequence "operably linked" to a coding sequence is ligated in such a manner that expression of the coding sequence is achieved under conditions compatible with the control sequence.

The term "open reading frame" or "ORF" refers to a region of a polynucleotide sequence which encodes a polypeptide. This region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5' -terminus and a translation stop codon at the 3' -terminus. A coding sequence can include, but is not limited to, mRNA, cDNA and recombinant polynucleotide sequences.

The term "immunologically identifiable with/as" refers to the presence of epitope(s) and polypeptide(s) which also are present in and are unique to the designated polypeptide(s). Immunological identity may be determined by antibody binding and/or competition in binding. These techniques are known to the routineer and also are described herein. The uniqueness of an epitope also can be determined by computer searches of known data banks, such as GenBank, for the

polynucleotide sequence which encodes the epitope and by amino acid sequence comparisons with other known proteins.

As used herein, "epitope" means an antigenic determinant of a polypeptide or protein. Conceivably, an epitope can comprise three amino acids in a spatial conformation which is unique to the epitope. Generally, an epitope consists of at least five such amino acids, and more usually, it consists of at least eight to ten amino acids. An epitope can be derived from a polypeptide encoded by a nucleic acid molecule described herein. Methods of examining spatial conformation are known in the art and include, for example, x-ray crystallography and two-dimensional nuclear magnetic resonance.

A "conformational epitope" is an epitope that is comprised of a specific juxtaposition of amino acids in an immunologically recognizable structure, such amino acids being present on the same polypeptide in a contiguous or non-contiguous order or present on different polypeptides.

A polypeptide is "immunologically reactive" with an antibody when it binds to an antibody due to antibody recognition of a specific epitope contained within the polypeptide. Immunological reactivity may be determined by antibody binding, more particularly, by the kinetics of antibody binding, and/or by competition in binding using as competitor(s) a known polypeptide(s) containing an epitope against which the antibody is directed. The methods for determining whether a polypeptide is immunologically reactive with an antibody are known in the art.

As used herein, the term "immunogenic polypeptide containing an epitope of interest" means naturally occurring polypeptides of interest or fragments thereof, as well as polypeptides prepared by other means, for example, by chemical synthesis or the expression of the polypeptide in a recombinant organism.

The term "transfection" refers to the introduction of an exogenous polynucleotide into a prokaryotic or eukaryotic host cell, irrespective of the method used for the introduction. The term "transfection" refers to both stable and transient introduction of the polynucleotide, and encompasses direct uptake of polynucleotides, transformation, transduction, and f-mating. Once introduced into the host cell, the exogenous polynucleotide may be maintained as a non-integrated replicon, for example, a plasmid, or alternatively, may be integrated into the host genome.

"Treatment" refers to prophylaxis and/or therapy.

The term "individual" as used herein refers to vertebrates, particularly members of the mammalian species and includes, but is not limited to, domestic animals, sports animals, primates and humans; more particularly, the term refers to humans.

The term "sense strand" or "plus strand" (or "+") as used herein denotes a nucleic acid that contains the sequence that encodes the polypeptide. The term "antisense strand" or "minus strand" (or "-") denotes a nucleic acid that contains a sequence that is complementary to that of the "plus" strand.

The term "test sample" refers to a component of an individual's body which is the source of the analyte (such as antibodies of interest or antigens of interest). These components are well known in the art. A test sample is typically anything suspected of containing a target sequence. Test samples can be prepared using methodologies well known in the art such as by obtaining a specimen from an individual and, if necessary, disrupting any cells contained thereby to release target nucleic acids. These test samples include biological samples which can be tested by the methods of the present invention described herein and include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, sputum, bronchial washing, bronchial aspirates, urine, lymph fluids, and various external secretions of the respiratory, intestinal and genitourinary tracts, tears, saliva, milk, white blood cells, myelomas and the like; biological fluids such as cell culture supernatants; tissue specimens which may be fixed; and cell specimens which may be fixed.

"Purified product" refers to a preparation of the product which has been isolated from the cellular constituents with which the product is normally associated and from other types of cells which may be present in the sample of interest.

"PNA" denotes a "peptide nucleic acid analog" which may be utilized in a procedure such as an assay described herein to determine the presence of a target. "MA" denotes a "morpholino analog" which may be utilized in a procedure such as an assay described herein to determine the presence of a target. See, for example, U.S. Patent No. 5,378,841. PNAs are neutrally charged moieties which can be directed against RNA targets or DNA. PNA probes used in assays in place of, for example, the DNA probes of the present invention, offer advantages not achievable when DNA probes are used. These advantages include manufacturability, large scale labeling, reproducibility, stability, insensitivity to changes in ionic strength and resistance to enzymatic degradation which is present in methods utilizing DNA or RNA. These PNAs can be labeled with ("attached to") such signal generating compounds as fluorescein, radionucleotides, chemiluminescent compounds and the like. PNAs or other nucleic acid analogs such as MAs thus can be used in assay methods in place of DNA or RNA. Although assays are described herein utilizing DNA probes, it is within the scope of the routineer that PNAs or MAs can be

substituted for RNA or DNA with appropriate changes if and as needed in assay reagents.

"Analyte," as used herein, is the substance to be detected which may be present in the test sample. The analyte can be any substance for which there exists a naturally occurring specific binding member (such as an antibody), or for which a specific binding member can be prepared. Thus, an analyte is a substance that can bind to one or more specific binding members in an assay. "Analyte" also includes any antigenic substances, haptens, antibodies and combinations thereof. As a member of a specific binding pair, the analyte can be detected by means of naturally occurring specific binding partners (pairs) such as the use of intrinsic factor protein as a member of a specific binding pair for the determination of Vitamin B12, the use of folate-binding protein to determine folic acid, or the use of a lectin as a member of a specific binding pair for the determination of a carbohydrate. The analyte can include a protein, a polypeptide, an amino acid, a nucleotide target and the like. The analyte can be soluble in a body fluid such as blood, blood plasma or serum, urine or the like. The analyte can be in a tissue, either on a cell surface or within a cell. The analyte can be on or in a cell dispersed in a body fluid such as blood, urine, breast aspirate, or obtained as a biopsy sample.

"Urinary tract cancer," as used herein, refers to any malignant disease of the urinary tract including but not limited to, adenocarcinoma, transitional cell carcinoma, squamous cell carcinoma, carcinoma in situ, clear carcinoma, granular cell carcinoma and sarcomatoid carcinoma.

An "Expressed Sequence Tag" or "EST" refers to the partial sequence of a cDNA insert which has been made by reverse transcription of mRNA extracted from a tissue followed by insertion into a vector.

A "transcript image" refers to a table or list giving the quantitative distribution of ESTs in a library and represents the genes active in the tissue from which the library was made.

The present invention provides assays which utilize specific binding members. A "specific binding member," as used herein, is a member of a specific binding pair. That is, two different molecules where one of the molecules, through chemical or physical means, specifically binds to the second molecule. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors, and enzymes and the like. Furthermore, specific binding pairs can include members that are analogs

of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, antibodies and antibody fragments, both monoclonal and polyclonal and complexes thereof, including those formed by recombinant DNA molecules.

Specific binding members include "specific binding molecules." A "specific binding molecule" intends any specific binding member, particularly an immunoreactive specific binding member. As such, the term "specific binding molecule" encompasses antibody molecules (obtained from both polyclonal and monoclonal preparations), as well as, the following: hybrid (chimeric) antibody molecules (see, for example, Winter, et al., Nature 349:293-299 (1991), and U.S. Patent No. 4,816,567); $F(ab')_2$ and F(ab) fragments; Fv molecules (non-covalent heterodimers, see, for example, Inbar, et al., Proc. Natl. Acad. Sci. USA 69:2659-2662 (1972), and Ehrlich, et al., Biochem. 19:4091-4096 (1980)); single chain Fv molecules (sFv) (see, for example, Huston, et al., Proc. Natl. Acad. Sci. USA 85:5879-5883 (1988)); humanized antibody molecules (see, for example, Riechmann, et al., Nature 332:323-327 (1988), Verhoeyan, et al., Science 239:1534-1536 (1988), and UK Patent Publication No. GB 2,276,169, published 21 September 1994); and, any functional fragments obtained from such molecules, wherein such fragments retain immunological binding properties of the parent antibody molecule.

The term "hapten," as used herein, refers to a partial antigen or non-protein binding member which is capable of binding to an antibody, but which is not capable of eliciting antibody formation unless coupled to a carrier protein.

A "capture reagent," as used herein, refers to an unlabeled specific binding member which is specific either for the analyte as in a sandwich assay, for the indicator reagent or analyte as in a competitive assay, or for an ancillary specific binding member, which itself is specific for the analyte, as in an indirect assay. The capture reagent can be directly or indirectly bound to a solid phase material before the performance of the assay or during the performance of the assay, thereby enabling the separation of immobilized complexes from the test sample.

The "indicator reagent" comprises a "signal-generating compound" ("label") which is capable of generating and generates a measurable signal detectable by external means, conjugated ("attached") to a specific binding member. In addition to being an antibody member of a specific binding pair, the indicator reagent also can be a member of any specific binding pair, including either hapten-anti-hapten systems such as biotin or anti-biotin, avidin or biotin, a carbohydrate or a lectin, a complementary nucleotide sequence, an effector or a receptor molecule, an enzyme cofactor and an enzyme, an enzyme inhibitor or an

enzyme and the like. An immunoreactive specific binding member can be an antibody, an antigen, or an antibody/antigen complex that is capable of binding either to the polypeptide of interest as in a sandwich assay, to the capture reagent as in a competitive assay, or to the ancillary specific binding member as in an indirect assay. When describing probes and probe assays, the term "reporter molecule" may be used. A reporter molecule comprises a signal generating compound as described hereinabove conjugated to a specific binding member of a specific binding pair, such as carbazole or adamantane.

The various "signal-generating compounds" (labels) contemplated include chromagens, catalysts such as enzymes, luminescent compounds such as fluorescein and rhodamine, chemiluminescent compounds such as dioxetanes, acridiniums, phenanthridiniums and luminol, radioactive elements and direct visual labels. Examples of enzymes include alkaline phosphatase, horseradish peroxidase, beta-galactosidase and the like. The selection of a particular label is not critical, but it must be capable of producing a signal either by itself or in conjunction with one or more additional substances.

"Solid phases" ("solid supports") are known to those in the art and include the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic or non-magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, sheep (or other animal) red blood cells and Duracytes® (red blood cells "fixed" by pyruvic aldehyde and formaldehyde, available from Abbott Laboratories, Abbott Park, IL) and others. The "solid phase" is not critical and can be selected by one skilled in the art. Thus, latex particles, microparticles, magnetic or non-magnetic beads, membranes, plastic tubes, walls of microtiter wells, glass or silicon chips, sheep (or other suitable animal's) red blood cells and Duracytes® are all suitable examples. Suitable methods for immobilizing peptides on solid phases include ionic, hydrophobic, covalent interactions and the like. A "solid phase," as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon (attached to) the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the

performance of the assay or during the performance of the assay. The solid phase thus can be a plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface of a test tube, microtiter well, sheet, bead, microparticle, chip, sheep (or other suitable animal's) red blood cells, Duracytes® and other configurations known to those of ordinary skill in the art.

It is contemplated and within the scope of the present invention that the solid phase also can comprise any suitable porous material with sufficient porosity to allow access by detection antibodies and a suitable surface affinity to bind antigens. Microporous structures generally are preferred, but materials with a gel structure in the hydrated state may be used as well. Such useful solid supports include, but are not limited to, nitrocellulose and nylon. It is contemplated that such porous solid supports described herein preferably are in the form of sheets of thickness from about 0.01 to 0.5 mm, preferably about 0.1 mm. The pore size may vary within wide limits and preferably is from about 0.025 to 15 microns, especially from about 0.15 to 15 microns. The surface of such supports may be activated by chemical processes which cause covalent linkage of the antigen or antibody to the support. The irreversible binding of the antigen or antibody is obtained, however, in general, by adsorption on the porous material by poorly understood hydrophobic forces. Other suitable solid supports are known in the art.

Reagents.

The present invention provides reagents such as polynucleotide sequences represented by SEQUENCE ID NOS 1-13, polypeptides encoded thereby, and antibodies specific for these polypeptides. The present invention also provides reagents such as oligonucleotide fragments derived from the disclosed polynucleotides and nucleic acid sequences complementary to these polynucleotides. The polynucleotides, polypeptides, or antibodies of the present invention may be used to provide information leading to the detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating of, or determining the predisposition to, diseases and conditions of the urinary tract, such as urinary tract cancer. The sequences disclosed herein represent unique polynucleotides which can be used in assays or for producing a specific profile of gene transcription activity. Such assays are disclosed in European Patent Number 0373203B1 and International Publication No. WO 95/11995.

Selected polynucleotides represented by SEQUENCE ID NOS 1-13 can be used in the methods described herein for the detection of normal or altered gene expression. Such methods may employ polynucleotides or oligonucleotides

represented by SEQUENCE ID NOS 1-13, fragments or derivatives thereof, or nucleic acid sequences complementary thereto.

The polynucleotides disclosed herein, their complementary sequences, or fragments of either, can be used in assays to detect, amplify or quantify genes, nucleic acids, cDNAs or mRNAs relating to urinary tract disease and conditions associated therewith. They also can be used to identify an entire or partial coding region of a polypeptide encoded by SEQUENCE ID NOS 1-13. They further can be provided in individual containers in the form of a kit for assays, or provided as individual compositions. If provided in a kit for assays, other suitable reagents such as buffers, conjugates and the like may be included.

The polynucleotide may be in the form of RNA or DNA. Polynucleotides in the form of DNA, cDNA, genomic DNA, nucleic acid analogs and synthetic DNA are within the scope of the present invention. The DNA may be double-stranded or single-stranded, and if single stranded, may be the coding (sense) strand or non-coding (anti-sense) strand. The coding sequence which encodes the polypeptide may be identical to the coding sequence provided herein or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptide as the DNA provided herein.

This polynucleotide may include only the coding sequence for the polypeptide, or the coding sequence for the polypeptide and an additional coding sequence such as a leader or secretory sequence or a proprotein sequence, or the coding sequence for the polypeptide (and optionally an additional coding sequence) and non-coding sequence, such as a non-coding sequence 5' and/or 3' of the coding sequence for the polypeptide.

In addition, the invention includes variant polynucleotides containing modifications such as polynucleotide deletions, substitutions or additions; and any polypeptide modification resulting from the variant polynucleotide sequence. A polynucleotide of the present invention also may have a coding sequence which is a naturally occurring allelic variant of the coding sequence provided herein.

In addition, the coding sequence for the polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the polypeptide. The polynucleotides may also encode for a proprotein which is the protein plus additional 5' amino acid residues. A protein having a prosequence is a proprotein

and may, in some cases, be an inactive form of the protein. Once the prosequence is cleaved, an active protein remains. Thus, the polynucleotide of the present invention may encode for a protein, or for a protein having a prosequence, or for a protein having both a presequence (leader sequence) and a prosequence.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexa-histidine tag supplied by a pQE-9 vector to provide for purification of the polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. a COS-7 cell line, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein. See, for example, I. Wilson et al., Cell 37:767 (1984).

It is contemplated that polynucleotides will be considered to hybridize to the sequences provided herein if there is at least 50%, preferably at least 70%, and more preferably at least 90% identity between the polynucleotide and the sequence.

The degree of sequence identity between two nucleic acid molecules greatly affects the efficiency and strength of hybridization events between such molecules. A partially identical nucleic acid sequence is one that will at least partially inhibit a completely identical sequence from hybridizing to a target molecule. Inhibition of hybridization of the completely identical sequence can be assessed using hybridization assays that are well known in the art (e.g., Southern blot, Northern blot, solution hybridization, *in situ* hybridization, or the like, see Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.). Such assays can be conducted using varying degrees of selectivity, for example, using conditions varying from low to high stringency. If conditions of low stringency are employed, the absence of non-specific binding can be assessed using a secondary probe that lacks even a partial degree of sequence identity (for example, a probe having less than about 30% sequence identity with the target molecule), such that, in the absence of non-specific binding events, the secondary probe will not hybridize to the target.

When utilizing a hybridization-based detection system, a nucleic acid probe is chosen that is complementary to a target nucleic acid sequence, and then by selection of appropriate conditions the probe and the target sequence "selectively hybridize," or bind, to each other to form a hybrid molecule. In one embodiment of the present invention, a nucleic acid molecule is capable of hybridizing selectively to a target sequence under moderately stringent hybridization conditions. In the context of the present invention, moderately stringent hybridization conditions allow detection of a target nucleic acid sequence of at least

14 nucleotides in length having at least approximately 70% sequence identity with the sequence of the selected nucleic acid probe. In another embodiment, such selective hybridization is performed under stringent hybridization conditions. Stringent hybridization conditions allow detection of target nucleic acid sequences of at least 14 nucleotides in length having a sequence identity of greater than 90% with the sequence of the selected nucleic acid probe. Hybridization conditions useful for probe/target hybridization where the probe and target have a specific degree of sequence identity, can be determined as is known in the art (see, for example, Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press). Hybrid molecules can be formed, for example, on a solid support, in solution, and in tissue sections. The formation of hybrids can be monitored by inclusion of a reporter molecule, typically, in the probe. Such reporter molecules, or detectable elements include, but are not limited to, radioactive elements, fluorescent markers, and molecules to which an enzyme-conjugated ligand can bind.

With respect to stringency conditions for hybridization, it is well known in the art that numerous equivalent conditions can be employed to establish a particular stringency by varying, for example, the following factors: the length and nature of probe and target sequences, base composition of the various sequences, concentrations of salts and other hybridization solution components, the presence or absence of blocking agents in the hybridization solutions (e.g., formamide, dextran sulfate, and polyethylene glycol), hybridization reaction temperature and time parameters, as well as, varying wash conditions. The selection of a particular set of hybridization conditions is well within the skill of the routineer in the art (see, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.).

The present invention also provides an antibody produced by using a purified polypeptide encoded by a marker represented by SEQUENCE ID NOS 1-13 of which at least a portion of the polypeptide is encoded by a polynucleotide represented by SEQUENCE ID NOS 1-13. These antibodies may be used in the methods provided herein for the detection of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 in test samples. The presence of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 in the test samples is indicative of the presence of a urinary tract disease or condition, including urinary tract cancer. The antibody also may be used for therapeutic purposes, for example, in neutralizing the activity of polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 in conditions associated with altered or abnormal expression.

The present invention further relates to a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 which has the deduced amino acid sequence as provided herein, as well as fragments, analogs and derivatives of such polypeptide. The polypeptide of the present invention may be a recombinant polypeptide, a natural purified polypeptide or a synthetic polypeptide. The fragment, derivative or analog of the polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 may be one in which one or more of the amino acid residues is substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code; or it may be one in which one or more of the amino acid residues includes a substituent group; or it may be one in which the polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol); or it may be one in which the additional amino acids are fused to the polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are within the scope of the present invention. The polypeptides and polynucleotides of the present invention are provided preferably in an isolated form and preferably purified.

Thus, a polypeptide of the present invention may have an amino acid sequence that is identical to that of the naturally occurring polypeptide or that is different by minor variations due to one or more amino acid substitutions. The variation may be a "conservative change" typically in the range of about 1 to 5 amino acids, wherein the substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine or threonine with serine. In contrast, variations may include nonconservative changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without changing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR software (DNASTAR Inc., Madison WI).

Probes constructed according to the polynucleotide sequences of the present invention can be used in various assay methods to provide various types of analysis. For example, such probes can be used in fluorescent in situ hybridization (FISH) technology to perform chromosomal analysis, and used to identify cancer-specific structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR-

generated and/or allele specific oligonucleotides probes, allele specific amplification or by direct sequencing. Probes also can be labeled with radioisotopes, directly- or indirectly- detectable haptens, or fluorescent molecules, and utilized for in situ hybridization studies to evaluate the mRNA expression of the gene comprising the polynucleotide in tissue specimens or cells.

This invention also provides teachings as to the production of the polynucleotides and polypeptides provided herein.

Probe Assays

The sequences provided herein may be used to produce probes which can be used in assays for the detection of nucleic acids in test samples. The probes may be designed from conserved nucleotide regions of the polynucleotides of interest or from non-conserved nucleotide regions of the polynucleotide of interest. The design of such probes for optimization in assays is within the skill of the routineer. Generally, nucleic acid probes are developed from non-conserved or unique regions when maximum specificity is desired, and nucleic acid probes are developed from conserved regions when assaying for nucleotide regions that are closely related to, for example, different members of a multi-gene family or in related species like mouse and man.

The polymerase chain reaction (PCR) is a technique for amplifying a desired nucleic acid sequence (target) contained in a nucleic acid or mixture thereof. In PCR, a pair of primers are employed in excess to hybridize to the complementary strands of the target nucleic acid. The primers are each extended by a polymerase using the target nucleic acid as a template. The extension products become target sequences themselves, following dissociation from the original target strand. New primers then are hybridized and extended by a polymerase, and the cycle is repeated to geometrically increase the number of target sequence molecules. PCR is disclosed in U.S. Patents 4,683,195 and 4,683,202.

The Ligase Chain Reaction (LCR) is an alternate method for nucleic acid amplification. In LCR, probe pairs are used which include two primary (first and second) and two secondary (third and fourth) probes, all of which are employed in molar excess to target. The first probe hybridizes to a first segment of the target strand, and the second probe hybridizes to a second segment of the target strand, the first and second segments being contiguous so that the primary probes abut one another in 5'phosphate-3'hydroxyl relationship, and so that a ligase can covalently fuse or ligate the two probes into a fused product. In addition, a third (secondary) probe can hybridize to a portion of the first probe and a fourth (secondary) probe can hybridize to a portion of the second probe in a similar abutting fashion. Of course, if the target is initially double stranded, the secondary probes also will

hybridize to the target complement in the first instance. Once the ligated strand of primary probes is separated from the target strand, it will hybridize with the third and fourth probes which can be ligated to form a complementary, secondary ligated product. It is important to realize that the ligated products are functionally equivalent to either the target or its complement. By repeated cycles of hybridization and ligation, amplification of the target sequence is achieved. This technique is described more completely in EP-A- 320 308 to K. Backman published June 16, 1989 and EP-A-439 182 to K. Backman et al., published July 31, 1991.

For amplification of mRNAs, it is within the scope of the present invention to reverse transcribe mRNA into cDNA followed by polymerase chain reaction (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Patent No. 5,322,770; or reverse transcribe mRNA into cDNA followed by asymmetric gap ligase chain reaction (RT-AGLCR) as described by R. L. Marshall et al., PCR Methods and Applications 4:80-84 (1994).

Other known amplification methods which can be utilized herein include but are not limited to the so-called "NASBA" or "3SR" technique described by J. C. Guatelli et al., Proc. Natl. Acad. Sci. USA 87:1874-1878 (1990) and also described by J. Compton, Nature 350 (No. 6313):91-92 (1991); Q-beta amplification as described in published European Patent Application (EPA) No. 4544610; strand displacement amplification (as described in G. T. Walker et al., Clin. Chem. 42:9-13 [1996]) and European Patent Application No. 684315; and target mediated amplification, as described in International Publication No. WO 93/22461.

Detection of markers represented by SEQUENCE ID NOS 1-13 may be accomplished using any suitable detection method, including those detection methods which are currently well known in the art, as well as detection strategies which may evolve later. See, for example, Caskey et al., U.S. Patent No. 5,582,989, Gelfand et al., U.S. Patent No. 5,210,015. Examples of such detection methods include target amplification methods as well as signal amplification technologies. An example of presently known detection methods would include the nucleic acid amplification technologies referred to as PCR, LCR, NASBA, SDA, RCR and TMA. See, for example, Caskey et al., U.S. Patent No. 5,582,989, Gelfand et al., U.S. Patent No. 5,210,015. Detection may also be accomplished using signal amplification such as that disclosed in Snitman et al., U.S. Patent No. 5,273,882. While the amplification of target or signal is preferred at present, it is contemplated and within the scope of the present invention that

ultrasensitive detection methods which do not require amplification can be utilized herein.

Detection, both amplified and non-amplified, may be performed using a variety of heterogeneous and homogeneous detection formats. Examples of heterogeneous detection formats are disclosed in Snitman et al., U.S. Patent No. 5,273,882, Albarella et al in EP-84114441.9, Urdea et al., U.S. Patent No. 5,124,246, Ullman et al. U.S. Patent No. 5,185,243 and Kourilsky et al., U.S. Patent No. 4,581,333. Examples of homogeneous detection formats are disclosed in, Caskey et al., U.S. Patent No. 5,582,989, Gelfand et al., U.S. Patent No. 5,210,015. Also contemplated and within the scope of the present invention is the use of multiple probes in the hybridization assay, which use improves sensitivity and amplification of the signal. See, for example, Caskey et al., U.S. Patent No. 5,582,989, Gelfand et al., U.S. Patent No. 5,210,015.

In one embodiment, the present invention generally comprises the steps of contacting a test sample suspected of containing a target polynucleotide sequence with amplification reaction reagents comprising an amplification primer, and a detection probe that can hybridize with an internal region of the amplicon sequences. Probes and primers employed according to the method provided herein are labeled with capture and detection labels, wherein probes are labeled with one type of label and primers are labeled with another type of label. Additionally, the primers and probes are selected such that the probe sequence has a lower melt temperature than the primer sequences. The amplification reagents, detection reagents and test sample are placed under amplification conditions whereby, in the presence of target sequence, copies of the target sequence (an amplicon) are produced. In the usual case, the amplicon is double stranded because primers are provided to amplify a target sequence and its complementary strand. The double stranded amplicon then is thermally denatured to produce single stranded amplicon members. Upon formation of the single stranded amplicon members, the mixture is cooled to allow the formation of complexes between the probes and single stranded amplicon members.

As the single stranded amplicon sequences and probe sequences are cooled, the probe sequences preferentially bind the single stranded amplicon members. This finding is counterintuitive given that the probe sequences generally are selected to be shorter than the primer sequences and therefore have a lower melt temperature than the primers. Accordingly, the melt temperature of the amplicon produced by the primers should also have a higher melt temperature than the probes. Thus, as the mixture cools, the re-formation of the double stranded amplicon would be expected. As previously stated, however, this is not the case.

The probes are found to preferentially bind the single stranded amplicon members. Moreover, this preference of probe/single stranded amplicon binding exists even when the primer sequences are added in excess of the probes.

After the probe/single stranded amplicon member hybrids are formed, they are detected. Standard heterogeneous assay formats are suitable for detecting the hybrids using the detection labels and capture labels present on the primers and probes. The hybrids can be bound to a solid phase reagent by virtue of the capture label and detected by virtue of the detection label. In cases where the detection label is directly detectable, the presence of the hybrids on the solid phase can be detected by causing the label to produce a detectable signal, if necessary, and detecting the signal. In cases where the label is not directly detectable, the captured hybrids can be contacted with a conjugate, which generally comprises a binding member attached to a directly detectable label. The conjugate becomes bound to the complexes and the conjugate's presence on the complexes can be detected with the directly detectable label. Thus, the presence of the hybrids on the solid phase reagent can be determined. Those skilled in the art will recognize that wash steps may be employed to wash away unhybridized amplicon or probe as well as unbound conjugate.

In one embodiment, the heterogeneous assays can be conveniently performed using a solid phase support that carries an array of nucleic acid molecules. Such arrays are useful for high-throughput and/or multiplexed assay formats. Various methods for forming such arrays from pre-formed nucleic acid molecules, or methods for generating the array using *in situ* synthesis techniques, are generally known in the art. (See, for example, Dattagupta, et al., EP Publication No. 0 234, 726A3; Southern, U.S. Patent No. 5,700,637; Pirrung, et al., U.S. Patent No. 5,143,854; PCT International Publication No. WO 92/10092; and, Fodor, et al., Science 251:767-777 (1991)).

Although the target sequence is described as single stranded, it also is contemplated to include the case where the target sequence is actually double stranded but is merely separated from its complement prior to hybridization with the amplification primer sequences. In the case where PCR is employed in this method, the ends of the target sequences are usually known. In cases where LCR or a modification thereof is employed in the preferred method, the entire target sequence is usually known. Typically, the target sequence is a nucleic acid sequence such as, for example, RNA or DNA.

The method provided herein can be used in well-known amplification reactions that include thermal cycle reaction mixtures, particularly in PCR and gap LCR (GLCR). Amplification reactions typically employ primers to repeatedly

generate copies of a target nucleic acid sequence, which target sequence is usually a small region of a much larger nucleic acid sequence. Primers are themselves nucleic acid sequences that are complementary to regions of a target sequence. Under amplification conditions, these primers hybridize or bind to the complementary regions of the target sequence. Copies of the target sequence typically are generated by the process of primer extension and/or ligation which utilizes enzymes with polymerase or ligase activity, separately or in combination, to add nucleotides to the hybridized primers and/or ligate adjacent probe pairs. The nucleotides that are added to the primers or probes, as monomers or preformed oligomers, are also complementary to the target sequence. Once the primers or probes have been sufficiently extended and/or ligated, they are separated from the target sequence, for example, by heating the reaction mixture to a "melt temperature" which is one in which complementary nucleic acid strands dissociate. Thus, a sequence complementary to the target sequence is formed.

A new amplification cycle then can take place to further amplify the number of target sequences by separating any double stranded sequences, allowing primers or probes to hybridize to their respective targets, extending and/or ligating the hybridized primers or probes and re-separating. The complementary sequences that are generated by amplification cycles can serve as templates for primer extension or filling the gap of two probes to further amplify the number of target sequences. Typically, a reaction mixture is cycled between 20 and 100 times, more typically, a reaction mixture is cycled between 25 and 50 times. The numbers of cycles can be determined by the routineer. In this manner, multiple copies of the target sequence and its complementary sequence are produced. Thus, primers initiate amplification of the target sequence when it is present under amplification conditions.

Generally, two primers which are complementary to a portion of a target strand and its complement are employed in PCR. For LCR, four probes, two of which are complementary to a target sequence and two of which are similarly complementary to the target's complement, are generally employed. In addition to the primer sets and enzymes previously mentioned, a nucleic acid amplification reaction mixture may also comprise other reagents which are well known and include but are not limited to: enzyme cofactors such as manganese; magnesium; salts; nicotinamide adenine dinucleotide (NAD); and deoxynucleotide triphosphates (dNTPs) such as, for example, deoxyadenine triphosphate, deoxyguanine triphosphate, deoxycytosine triphosphate and deoxythymine triphosphate.

While the amplification primers initiate amplification of the target sequence, the detection (or hybridization) probe is not involved in amplification. Detection

probes are generally nucleic acid sequences or uncharged nucleic acid analogs such as, for example, peptide nucleic acids which are disclosed in International Publication No. WO 92/20702; morpholino analogs which are described in U.S. Patents Nos 5,185,444, 5,034,506 and 5,142,047; and the like. Depending upon the type of label carried by the probe, the probe is employed to capture or detect the amplicon generated by the amplification reaction. The probe is not involved in amplification of the target sequence and therefore may have to be rendered "non-extendible" in that additional dNTPs cannot be added to the probe. In and of themselves, analogs usually are non-extendible and nucleic acid probes can be rendered non-extendible by modifying the 3' end of the probe such that the hydroxyl group is no longer capable of participating in elongation. For example, the 3' end of the probe can be functionalized with the capture or detection label to thereby consume or otherwise block the hydroxyl group. Alternatively, the 3' hydroxyl group simply can be cleaved, replaced or modified. U.S. Patent Application Serial No. 07/049,061 filed April 19, 1993 describes modifications which can be used to render a probe non-extendible.

The ratio of primers to probes is not important. Thus, either the probes or primers can be added to the reaction mixture in excess whereby the concentration of one would be greater than the concentration of the other. Alternatively, primers and probes can be employed in equivalent concentrations. Preferably, however, the primers are added to the reaction mixture in excess of the probes. Thus, primer to probe ratios of, for example, 5:1 and 20:1, are preferred.

While the length of the primers and probes can vary, the probe sequences are selected such that they have a lower melt temperature than the primer sequences. Hence, the primer sequences are generally longer than the probe sequences. Typically, the primer sequences are in the range of between 20 and 50 nucleotides long, more typically in the range of between 20 and 30 nucleotides long. The typical probe is in the range of between 10 and 25 nucleotides long.

Various methods for synthesizing primers and probes are well known in the art. Similarly, methods for attaching labels to primers or probes are also well known in the art. For example, it is a matter of routine to synthesize desired nucleic acid primers or probes using conventional nucleotide phosphoramidite chemistry and instruments available from Applied Biosystems, Inc., (Foster City, CA), DuPont (Wilmington, DE), or Milligen (Bedford MA). Many methods have been described for labeling oligonucleotides such as the primers or probes of the present invention. Enzo Biochemical (New York, NY) and Clontech (Palo Alto, CA) both have described and commercialized probe labeling techniques. For example, a primary amine can be attached to a 3' oligo terminus using 3'-Amine-

ON CPG™ (Clontech, Palo Alto, CA). Similarly, a primary amine can be attached to a 5' oligo terminus using Aminomodifier II® (Clontech). The amines can be reacted to various haptens using conventional activation and linking chemistries. In addition, copending applications U.S. Serial Nos. 625,566, filed December 11, 1990 and 630,908, filed December 20, 1990, teach methods for labeling probes at their 5' and 3' termini, respectively. International Publication Nos WO 92/10505, published 25 June 1992, and WO 92/11388, published 9 July 1992, teach methods for labeling probes at their 5' and 3' ends, respectively. According to one known method for labeling an oligonucleotide, a label-phosphoramidite reagent is prepared and used to add the label to the oligonucleotide during its synthesis. See, for example, N.T. Thuong et al., Tet. Letters 29(46):5905-5908 (1988); or J.S. Cohen et al., published U.S. Patent Application 07/246,688 (NTIS ORDER No. PAT-APPL-7-246,688) (1989). Preferably, probes are labeled at their 3' and 5' ends.

A capture label is attached to the primers or probes and can be a specific binding member which forms a binding pair with the solid phase reagent's specific binding member. It will be understood that the primer or probe itself may serve as the capture label. For example, in the case where a solid phase reagent's binding member is a nucleic acid sequence, it may be selected such that it binds a complementary portion of the primer or probe to thereby immobilize the primer or probe to the solid phase. In cases where the probe itself serves as the binding member, those skilled in the art will recognize that the probe will contain a sequence or "tail" that is not complementary to the single stranded amplicon members. In the case where the primer itself serves as the capture label, at least a portion of the primer will be free to hybridize with a nucleic acid on a solid phase because the probe is selected such that it is not fully complementary to the primer sequence.

Generally, probe/single stranded amplicon member complexes can be detected using techniques commonly employed to perform heterogeneous immunoassays. Preferably, in this embodiment, detection is performed according to the protocols used by the commercially available Abbott LCx® instrumentation (Abbott Laboratories, Abbott Park, IL).

The primers and probes disclosed herein are useful in typical PCR assays, wherein the test sample is contacted with a pair of primers, amplification is performed, the hybridization probe is added, and detection is performed.

Another method provided by the present invention comprises contacting a test sample with a plurality of polynucleotides, wherein at least one polynucleotide is a molecule represented by SEQUENCE ID NOS 1-13 as described herein,

hybridizing the test sample with the plurality of polynucleotides and detecting hybridization complexes. Hybridization complexes are identified and quantitated to compile a profile which is indicative of urinary tract disease, such as urinary tract cancer. Expressed RNA sequences may further be detected by reverse transcription and amplification of the DNA product by procedures well-known in the art, including polymerase chain reaction (PCR).

Drug Screening and Gene Therapy.

The present invention also encompasses the use of gene therapy methods for the introduction of anti-sense molecules represented by SEQUENCE ID NOS 1-13 derived molecules, such as polynucleotides or oligonucleotides of the present invention, into patients with conditions associated with abnormal expression of polynucleotides related to a urinary tract disease or condition especially urinary tract cancer. These molecules, including antisense RNA and DNA fragments and ribozymes, are designed to inhibit the translation of mRNA represented by SEQUENCE ID NOS 1-13, and may be used therapeutically in the treatment of conditions associated with altered or abnormal expression of polynucleotide represented by SEQUENCE ID NOS 1-13.

Alternatively, the oligonucleotides described above can be delivered to cells by procedures known in the art such that the anti-sense RNA or DNA may be expressed in vivo to inhibit production of a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 in the manner described above. Antisense constructs to a polynucleotide represented by SEQUENCE ID NOS 1-13, therefore, reverse the action of transcripts represented by SEQUENCE ID NOS 1-13 and may be used for treating urinary tract disease conditions, such as urinary tract cancer. These antisense constructs may also be used to treat tumor metastases.

The present invention also provides a method of screening a plurality of compounds for specific binding to polypeptide(s) encoded by markers represented by SEQUENCE ID NOS 1-13, or any fragment thereof, to identify at least one compound which specifically binds the polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13. Such a method comprises the steps of providing at least one compound; combining the polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 with each compound under suitable conditions for a time sufficient to allow binding; and detecting the polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 binding to each compound.

The polypeptide or peptide fragment employed in such a test may either be free in solution, affixed to a solid support, borne on a cell surface or located

intracellularly. One method of screening utilizes eukaryotic or prokaryotic host cells which are stably transfected with recombinant nucleic acids which can express the polypeptide or peptide fragment. A drug, compound, or any other agent may be screened against such transfected cells in competitive binding assays. For example, the formation of complexes between a polypeptide and the agent being tested can be measured in either viable or fixed cells.

The present invention thus provides methods of screening for drugs, compounds, or any other agent which can be used to treat diseases associated with markers represented by SEQUENCE ID NOS 1-13. These methods comprise contacting the agent with a polypeptide or fragment thereof and assaying for either the presence of a complex between the agent and the polypeptide, or for the presence of a complex between the polypeptide and the cell. In competitive binding assays, the polypeptide typically is labeled. After suitable incubation, free (or uncomplexed) polypeptide or fragment thereof is separated from that present in bound form, and the amount of free or uncomplexed label is used as a measure of the ability of the particular agent to bind to the polypeptide or to interfere with the polypeptide/cell complex.

The present invention also encompasses the use of competitive screening assays in which neutralizing antibodies capable of binding polypeptide specifically compete with a test agent for binding to the polypeptide or fragment thereof. In this manner, the antibodies can be used to detect the presence of any polypeptide in the test sample which shares one or more antigenic determinants with a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 as provided herein.

Another technique for screening provides high throughput screening for compounds having suitable binding affinity to at least one polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 disclosed herein. Briefly, large numbers of different small peptide test compounds are synthesized on a solid phase, such as plastic pins or some other surface. The peptide test compounds are reacted with polypeptide and washed. Polypeptide thus bound to the solid phase is detected by methods well-known in the art. Purified polypeptide can also be coated directly onto plates for use in the screening techniques described herein. In addition, non-neutralizing antibodies can be used to capture the polypeptide and immobilize it on the solid support. See, for example, EP 84/03564, published on September 13, 1984.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of the small molecules including agonists, antagonists, or inhibitors with which they interact. Such structural analogs can be used to design drugs which are more active or stable forms of the

polypeptide or which enhance or interfere with the function of a polypeptide in vivo. J. Hodgson, Bio/Technology 9:19-21 (1991).

For example, in one approach, the three-dimensional structure of a polypeptide, or of a polypeptide-inhibitor complex, is determined by x-ray crystallography, by computer modeling or, most typically, by a combination of the two approaches. Both the shape and charges of the polypeptide must be ascertained to elucidate the structure and to determine active site(s) of the molecule. Less often, useful information regarding the structure of a polypeptide may be gained by modeling based on the structure of homologous proteins. In both cases, relevant structural information is used to design analogous polypeptide-like molecules or to identify efficient inhibitors

Useful examples of rational drug design may include molecules which have improved activity or stability as shown by S. Braxton et al., Biochemistry 31:7796-7801 (1992), or which act as inhibitors, agonists, or antagonists of native peptides as shown by S.B.P. Athauda et al., J Biochem. (Tokyo) 113 (6):742-746 (1993).

It also is possible to isolate a target-specific antibody selected by an assay as described hereinabove, and then to determine its crystal structure. In principle this approach yields a pharmacophore upon which subsequent drug design can be based. It further is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies ("anti-ids") to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-id is an analog of the original receptor. The anti-id then can be used to identify and isolate peptides from banks of chemically or biologically produced peptides. The isolated peptides then can act as the pharmacophore (that is, a prototype pharmaceutical drug).

A sufficient amount of a recombinant polypeptide of the present invention may be made available to perform analytical studies such as X-ray crystallography. In addition, knowledge of the polypeptide amino acid sequence which is derivable from the nucleic acid sequence provided herein will provide guidance to those employing computer modeling techniques in place of, or in addition to, x-ray crystallography.

Antibodies specific to a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 (e.g., anti-marker antibodies) further may be used to inhibit the biological action of the polypeptide by binding to the polypeptide. In this manner, the antibodies may be used in therapy, for example, to treat urinary tract diseases including urinary tract cancer and its metastases.

Further, such antibodies can detect the presence or absence of a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 in a test sample and, therefore, are useful as diagnostic markers for the diagnosis of a urinary tract disease or condition especially urinary tract cancer. Such antibodies may also function as a diagnostic marker for urinary tract disease conditions, such as urinary tract cancer.

The present invention also is directed to antagonists and inhibitors of the polypeptides of the present invention. The antagonists and inhibitors are those which inhibit or eliminate the function of the polypeptide. Thus, for example, an antagonist may bind to a polypeptide of the present invention and inhibit or eliminate its function. The antagonist, for example, could be an antibody against the polypeptide which eliminates the activity of a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 by binding a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13, or in some cases the antagonist may be an oligonucleotide. Examples of small molecule inhibitors include, but are not limited to, small peptides or peptide-like molecules.

The antagonists and inhibitors may be employed as a composition with a pharmaceutically acceptable carrier including, but not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof. Administration of polypeptide inhibitors is preferably systemic. The present invention also provides an antibody which inhibits the action of such a polypeptide.

Antisense technology can be used to reduce gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the polypeptide of the present invention, is used to design an antisense RNA oligonucleotide of from 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription, thereby preventing transcription and the production of the polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13. For triple helix, see, for example, Lee et al., Nuc. Acids Res. 6:3073 (1979); Cooney et al., Science 241:456 (1988); and Dervan et al., Science 251:1360 (1991) The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of a mRNA molecule into the polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13. For antisense, see, for example, Okano, J. Neurochem. 56:560 (1991); and Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988). Antisense oligonucleotides act with greater efficacy when modified to contain

artificial internucleotide linkages which render the molecule resistant to nucleolytic cleavage. Such artificial internucleotide linkages include, but are not limited to, methylphosphonate, phosphorothiolate and phosphoroamydate internucleotide linkages.

Recombinant Technology.

The present invention provides host cells and expression vectors comprising polynucleotides represented by SEQUENCE ID NOS 1-13 of the present invention and methods for the production of the polypeptide(s) they encode. Such methods comprise culturing the host cells under conditions suitable for the expression of the polynucleotide represented by SEQUENCE ID NOS 1-13 and recovering the encoded polypeptide from the cell culture.

The present invention also provides vectors which include polynucleotides represented by SEQUENCE ID NOS 1-13 of the present invention, host cells which are genetically engineered with vectors of the present invention and the production of polypeptides of the present invention by recombinant techniques.

Host cells are genetically engineered (transfected, transduced or transformed) with the vectors of this invention which may be cloning vectors or expression vectors. The vector may be in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transfected cells, or amplifying gene(s) represented by SEQUENCE ID NOS 1-13. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing a polypeptide by recombinant techniques. Thus, the polynucleotide sequence may be included in any one of a variety of expression vehicles, in particular, vectors or plasmids for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus and pseudorabies. However, any other plasmid or vector may be used so long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into appropriate restriction endonuclease sites by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art. The DNA sequence in the expression vector is operatively linked to an appropriate expression

control sequence(s) (promoter) to direct mRNA synthesis. Representative examples of such promoters include, but are not limited to, the LTR or the SV40 promoter, the E. coli lac or trp, the phage lambda P sub L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. In addition, the expression vectors preferably contain a gene to provide a phenotypic trait for selection of transfected host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transfect an appropriate host to permit the host to express the protein. As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Salmonella typhimurium; Streptomyces sp.; fungal cells, such as yeast; insect cells, such as Drosophila and Sf9; animal cells, such as CHO, COS or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings provided herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art and are commercially available. The following vectors are provided by way of example. Bacterial: pINCY (Incyte Pharmaceuticals Inc., Palo Alto, CA), pSPORT1 (Life Technologies, Gaithersburg, MD), pQE70, pQE60, pQE-9 (Qiagen) pBs, phagescript, psiX174, pBluescript SK, pBsKS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); Eukaryotic: pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

Plasmid pINCY is generally identical to the plasmid pSPORT1 (available from Life Technologies, Gaithersburg, MD) with the exception that it has two modifications in the polylinker (multiple cloning site). These modifications are (1) it lacks a HindIII restriction site and (2) its EcoRI restriction site lies at a different

location. pINCY is created from pSPORT1 by cleaving pSPORT1 with both HindIII and EcoRI and replacing the excised fragment of the polylinker with synthetic DNA fragments. This replacement may be made in any manner known to those of ordinary skill in the art. For example, the two nucleotide sequences, may be generated synthetically with 5' terminal phosphates, mixed together, and then ligated under standard conditions for performing staggered end ligations into the pSPORT1 plasmid cut with HindIII and EcoRI. Suitable host cells (such as E. coli DH5μ cells) then are transfected with the ligated DNA and recombinant clones are selected for ampicillin resistance. Plasmid DNA then is prepared from individual clones and subjected to restriction enzyme analysis or DNA sequencing in order to confirm the presence of insert sequences in the proper orientation. Other cloning strategies known to the ordinary artisan also may be employed.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, SP6, T7, gpt, lambda P sub R, P sub L and trp. Eukaryotic promoters include cytomegalovirus (CMV) immediate early, herpes simplex virus (HSV) thymidine kinase, early and late SV40, LTRs from retroviruses and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention provides host cells containing the above-described construct. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation [L. Davis et al., Basic Methods in Molecular Biology, 2nd edition, Appleton and Lang, Paramount Publishing, East Norwalk, CT (1994)].

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Recombinant proteins can be expressed in mammalian cells, yeast, bacteria, or other cells, under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook

et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor, NY, 1989).

Transcription of a DNA encoding the polypeptide(s) of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transfection of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), alpha factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transfection include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces and Staphylococcus, although others may also be employed as a routine matter of choice.

Useful expression vectors for bacterial use comprise a selectable marker and bacterial origin of replication derived from plasmids comprising genetic elements of the well-known cloning vector pBR322 (ATCC 37017). Other vectors include but are not limited to PKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transfection of a suitable host and growth of the host to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction), and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well-known to the ordinary artisan.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts described by Gluzman, Cell 23:175 (1981), and other cell lines capable of expressing a compatible vector, such as the C127, HEK-293, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Representative, useful vectors include pRc/CMV and pcDNA3 (available from Invitrogen, San Diego, CA).

Polypeptides encoded by markers represented by SEQUENCE ID NOS 1-13 are recovered and purified from recombinant cell cultures by known methods including affinity chromatography, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography or lectin chromatography. It is preferred to have low concentrations (approximately 0.1-5 mM) of calcium ion present during purification [Price, et al., J. Biol. Chem. 244:917 (1969)]. Protein refolding steps can be used, as necessary, in completing configuration of the polypeptide. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

Thus, polypeptides of the present invention may be naturally purified products expressed from a high expressing cell line, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant

production procedure, the polypeptides of the present invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated. The polypeptides of the invention may also include an initial methionine amino acid residue.

The starting plasmids can be constructed from available plasmids in accord with published, known procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to one of ordinary skill in the art.

Methods for DNA sequencing are well known in the art. Conventional enzymatic methods employ DNA polymerase, Klenow fragment, Sequenase (US Biochemical Corp, Cleveland, OH) or Taq polymerase to extend DNA chains from an oligonucleotide primer annealed to the DNA template of interest. Methods have been developed for the use of both single-stranded and double-stranded templates. The chain termination reaction products may be electrophoresed on urea/polyacrylamide gels and detected either by autoradiography (for radionucleotide labeled precursors) or by fluorescence (for fluorescent-labeled precursors). Recent improvements in mechanized reaction preparation, sequencing and analysis using the fluorescent detection method have permitted expansion in the number of sequences that can be determined per day using machines such as the Applied Biosystems 377 DNA Sequencers (Applied Biosystems, Foster City, CA).

The reading frame of the nucleotide sequence can be ascertained by several types of analyses. First, reading frames contained within the coding sequence can be analyzed for the presence of start codon ATG and stop codons TGA, TAA or TAG. Typically, one reading frame will continue throughout the major portion of a cDNA sequence while other reading frames tend to contain numerous stop codons. In such cases, reading frame determination is straightforward. In other more difficult cases, further analysis is required.

Algorithms have been created to analyze the occurrence of individual nucleotide bases at each putative codon triplet. See, for example J.W. Fickett, Nuc. Acids Res. 10:5303 (1982). Coding DNA for particular organisms (bacteria, plants and animals) tends to contain certain nucleotides within certain triplet periodicities, such as a significant preference for pyrimidines in the third codon position. These preferences have been incorporated into widely available software which can be used to determine coding potential (and frame) of a given stretch of DNA. The algorithm-derived information combined with start/stop codon information can be used to determine proper frame with a high degree of certainty.

This, in turn, readily permits cloning of the sequence in the correct reading frame into appropriate expression vectors.

The nucleic acid sequences disclosed herein may be joined to a variety of other polynucleotide sequences and vectors of interest by means of well-established recombinant DNA techniques. See J. Sambrook et al., supra. Vectors of interest include cloning vectors, such as plasmids, cosmids, phage derivatives, phagemids, as well as sequencing, replication and expression vectors, and the like. In general, such vectors contain an origin of replication functional in at least one organism, convenient restriction endonuclease digestion sites and selectable markers appropriate for particular host cells. The vectors can be transferred by a variety of means known to those of skill in the art into suitable host cells which then produce the desired DNA, RNA or polypeptides.

Occasionally, sequencing or random reverse transcription errors will mask the presence of the appropriate open reading frame or regulatory element. In such cases, it is possible to determine the correct reading frame by attempting to express the polypeptide and determining the amino acid sequence by standard peptide mapping and sequencing techniques. See, F.M. Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY (1989). Additionally, the actual reading frame of a given nucleotide sequence may be determined by transfection of host cells with vectors containing all three potential reading frames. Only those cells with the nucleotide sequence in the correct reading frame will produce a peptide of the predicted length.

The nucleotide sequences provided herein have been prepared by current, state-of-the-art, automated methods and, as such, may contain unidentified nucleotides. These will not present a problem to those skilled in the art who wish to practice the invention. Several methods employing standard recombinant techniques, described in J. Sambrook (supra) or periodic updates thereof, may be used to complete the missing sequence information. The same techniques used for obtaining a full length sequence, as described herein, may be used to obtain nucleotide sequences.

Expression of a particular cDNA may be accomplished by subcloning the cDNA into an appropriate expression vector and transfecting this vector into an appropriate expression host. The cloning vector used for the generation of the urinary tract tissue cDNA library can be used for transcribing mRNA of a particular cDNA and contains a promoter for beta-galactosidase, an amino-terminal met and the subsequent seven amino acid residues of beta-galactosidase. Immediately following these eight residues is an engineered bacteriophage promoter useful for artificial priming and transcription, as well as a number of unique restriction sites,

including EcoRI, for cloning. The vector can be transfected into an appropriate host strain of E. coli.

Induction of the isolated bacterial strain with isopropylthiogalactoside (IPTG) using standard methods will produce a fusion protein which contains the first seven residues of beta-galactosidase, about 15 residues of linker and the peptide encoded within the cDNA. Since cDNA clone inserts are generated by an essentially random process, there is one chance in three that the included cDNA will lie in the correct frame for proper translation. If the cDNA is not in the proper reading frame, the correct frame can be obtained by deletion or insertion of an appropriate number of bases by well known methods including in vitro mutagenesis, digestion with exonuclease III or mung bean nuclease, or oligonucleotide linker inclusion.

The cDNA can be shuttled into other vectors known to be useful for expression of protein in specific hosts. Oligonucleotide primers containing cloning sites and segments of DNA sufficient to hybridize to stretches at both ends of the target cDNA can be synthesized chemically by standard methods. These primers can then be used to amplify the desired gene segments by PCR. The resulting new gene segments can be digested with appropriate restriction enzymes under standard conditions and isolated by gel electrophoresis. Alternately, similar gene segments can be produced by digestion of the cDNA with appropriate restriction enzymes and filling in the missing gene segments with chemically synthesized oligonucleotides. Segments of the coding sequence from more than one gene can be ligated together and cloned in appropriate vectors to optimize expression of recombinant sequence.

Suitable expression hosts for such chimeric molecules include, but are not limited to, mammalian cells, such as Chinese Hamster Ovary (CHO) and human embryonic kidney (HEK) 293 cells, insect cells, such as Sf9 cells, yeast cells, such as Saccharomyces cerevisiae and bacteria, such as E. coli. For each of these cell systems, a useful expression vector may also include an origin of replication to allow propagation in bacteria and a selectable marker such as the beta-lactamase antibiotic resistance gene to allow selection in bacteria. In addition, the vectors may include a second selectable marker, such as the neomycin phosphotransferase gene, to allow selection in transfected eukaryotic host cells. Vectors for use in eukaryotic expression hosts may require the addition of 3' poly A tail if the sequence of interest lacks poly A.

Additionally, the vector may contain promoters or enhancers which increase gene expression. Such promoters are host specific and include, but are not limited to, MMTV, SV40, or metallothionine promoters for CHO cells; trp, lac,

tac or T7 promoters for bacterial hosts; or alpha factor, alcohol oxidase or PGH promoters for yeast. Adenoviral vectors with or without transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to drive protein expression in mammalian cell lines. Once homogeneous cultures of recombinant cells are obtained, large quantities of recombinantly produced protein can be recovered from the conditioned medium and analyzed using chromatographic methods well known in the art. An alternative method for the production of large amounts of secreted protein involves the transfection of mammalian embryos and the recovery of the recombinant protein from milk produced by transgenic cows, goats, sheep, etc. Polypeptides and closely related molecules may be expressed recombinantly in such a way as to facilitate protein purification. One approach involves expression of a chimeric protein which includes one or more additional polypeptide domains not naturally present on human polypeptides. Such purification-facilitating domains include, but are not limited to, metal-chelating peptides such as histidine-tryptophan domains that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase from Invitrogen (San Diego, CA) between the polypeptide sequence and the purification domain may be useful for recovering the polypeptide.

Immunoassays.

Polypeptides encoded by markers represented by SEQUENCE ID NOS 1-13, including fragments, derivatives, and analogs thereof, or cells expressing such polypeptides, can be utilized in a variety of assays, many of which are described herein, for the detection of antibodies to urinary tract tissue. They also can be used as immunogens to produce antibodies. These antibodies can be, for example, polyclonal or monoclonal antibodies, chimeric, single chain and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

For example, antibodies generated against a polypeptide comprising a sequence of the present invention can be obtained by direct injection of the polypeptide into an animal or by administering the polypeptide to an animal such as a mouse, rabbit, goat or human. A mouse, rabbit or goat is preferred. The polypeptide is encoded by markers represented by SEQUENCE ID NOS 1-13, and fragments thereof. The antibody so obtained then will bind the polypeptide itself. In this manner, even a sequence encoding only a fragment of the polypeptide can

be used to generate antibodies that bind the native polypeptide. Such antibodies then can be used to isolate the polypeptide from test samples such as tissue suspected of containing that polypeptide. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique as described by Kohler and Milstein, Nature 256:495-497 (1975), the trioma technique, the human B-cell hybridoma technique as described by Kozbor et al., Immun. Today 4:72 (1983) and the EBV-hybridoma technique to produce human monoclonal antibodies as described by Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc, New York, NY, pp. 77-96 (1985). Techniques described for the production of single chain antibodies can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. See, for example, U.S. Patent No. 4,946,778.

Various assay formats may utilize the antibodies of the present invention, including "sandwich" immunoassays and probe assays. For example, the antibodies of the present invention, or fragments thereof, can be employed in various assay systems to determine the presence, if any, of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 in a test sample. For example, in a first assay format, a polyclonal or monoclonal antibody or fragment thereof, or a combination of these antibodies, which has been coated on a solid phase, is contacted with a test sample, to form a first mixture. This first mixture is incubated for a time and under conditions sufficient to form antigen/antibody complexes. Then, an indicator reagent comprising a monoclonal or a polyclonal antibody or a fragment thereof, or a combination of these antibodies, to which a signal generating compound has been attached, is contacted with the antigen/antibody complexes to form a second mixture. This second mixture then is incubated for a time and under conditions sufficient to form antibody/antigen/antibody complexes. The presence of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 in the test sample and captured on the solid phase, if any, is determined by detecting the measurable signal generated by the signal generating compound. The amount of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 present in the test sample is proportional to the signal generated.

In an alternative assay format, a mixture is formed by contacting: (1) a polyclonal antibody, monoclonal antibody, or fragment thereof, which specifically binds to antigen encoded by markers represented by SEQUENCE ID NOS 1-13, or a combination of such antibodies bound to a solid support; (2) the test sample; and (3) an indicator reagent comprising a monoclonal antibody, polyclonal antibody, or fragment thereof, which specifically binds to a different antigen

encoded by markers represented by SEQUENCE ID NOS 1-13 (or a combination of these antibodies) to which a signal generating compound is attached. This mixture is incubated for a time and under conditions sufficient to form antibody/antigen/antibody complexes. The presence, if any, of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 present in the test sample and captured on the solid phase is determined by detecting the measurable signal generated by the signal generating compound. The amount of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 present in the test sample is proportional to the signal generated.

In another assay format, one or a combination of at least two monoclonal antibodies of the invention can be employed as a competitive probe for the detection of antibodies to antigen encoded by markers represented by SEQUENCE ID NOS 1-13. For example, polypeptides such as the recombinant antigens disclosed herein, either alone or in combination, are coated on a solid phase. A test sample suspected of containing antibody to antigen encoded by markers represented by SEQUENCE ID NOS 1-13 then is incubated with an indicator reagent comprising a signal generating compound and at least one monoclonal antibody of the invention for a time and under conditions sufficient to form antigen/antibody complexes of either the test sample and indicator reagent bound to the solid phase or the indicator reagent bound to the solid phase. The reduction in binding of the monoclonal antibody to the solid phase can be quantitatively measured.

In yet another detection method, each of the monoclonal or polyclonal antibodies of the present invention can be employed in the detection of antigens encoded by markers represented by SEQUENCE ID NOS 1-13 in tissue sections, as well as in cells, by immunohistochemical analysis. The tissue sections can be cut from either frozen or chemically fixed samples of tissue. If the antigens are to be detected in cells, the cells can be isolated from blood, urine, breast aspirates, or other bodily fluids. The cells may be obtained by biopsy, either surgical or by needle. The cells can be isolated by centrifugation or magnetic attraction after labeling with magnetic particles or ferrofluids so as to enrich a particular fraction of cells for staining with the antibodies of the present invention. Cytochemical analysis wherein these antibodies are labeled directly (with, for example, fluorescein, colloidal gold, horseradish peroxidase, alkaline phosphatase, etc.) or are labeled by using secondary labeled anti-species antibodies (with various labels as exemplified herein) to track the histopathology of disease also are within the scope of the present invention.

In addition, these monoclonal antibodies can be bound to matrices similar to CNBr-activated Sepharose and used for the affinity purification of specific polypeptides encoded by markers represented by SEQUENCE ID NOS 1-13 from cell cultures or biological tissues such as to purify recombinant and native proteins encoded by markers represented by SEQUENCE ID NOS 1-13.

The monoclonal antibodies of the invention also can be used for the generation of chimeric antibodies for therapeutic use, or other similar applications.

The monoclonal antibodies or fragments thereof can be provided individually to detect antigens encoded by markers represented by SEQUENCE ID NOS 1-13. Combinations of the monoclonal antibodies (and fragments thereof) provided herein also may be used together as components in a mixture or "cocktail" of at least one antibody of the invention, along with antibodies which specifically bind to other regions, each antibody having different binding specificities. Thus, this cocktail can include the monoclonal antibodies of the invention which are directed to polypeptides encoded by markers represented by SEQUENCE ID NOS 1-13 disclosed herein and other monoclonal antibodies specific to other antigenic determinants of antigens encoded by markers represented by SEQUENCE ID NOS 1-13 or other related proteins.

The polyclonal antibody or fragment thereof which can be used in the assay formats should specifically bind to a polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13 or other polypeptides encoded by markers represented by SEQUENCE ID NOS 1-13additionally used in the assay. The polyclonal antibody used preferably is of mammalian origin such as, human, goat, rabbit or sheep polyclonal antibody which binds polypeptide encoded by markers represented by SEQUENCE ID NOS 1-13. Most preferably, the polyclonal antibody is of rabbit origin. The polyclonal antibodies used in the assays can be used either alone or as a cocktail of polyclonal antibodies. Since the cocktails used in the assay formats are comprised of either monoclonal antibodies or polyclonal antibodies having different binding specificity to polypeptides encoded by markers represented by SEQUENCE ID NOS 1-13, they are useful for the detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating, or determining the predisposition to, diseases and conditions of the urinary tract, such as urinary tract cancer.

It is contemplated and within the scope of the present invention that antigen encoded by markers represented by SEQUENCE ID NOS 1-13 may be detectable in assays by use of a recombinant antigen as well as by use of a synthetic peptide or purified peptide, which peptide comprises an amino acid sequence of markers represented by SEQUENCE ID NOS 1-13. The amino acid sequence of such a

polypeptide is selected from the group consisting of those encoded by markers represented by SEQUENCE ID NOS 1-13, and fragments thereof. It also is within the scope of the present invention that different synthetic, recombinant or purified peptides, identifying different epitopes of markers represented by SEQUENCE ID NOS 1-13, can be used in combination in an assay for the detecting, diagnosing, staging, monitoring, prognosticating, in vivo imaging, preventing or treating, or determining the predisposition to diseases and conditions of the urinary tract, such as urinary tract cancer. In this case, all of these peptides can be coated onto one solid phase; or each separate peptide may be coated onto separate solid phases, such as microparticles, and then combined to form a mixture of peptides which can be later used in assays. Furthermore, it is contemplated that multiple peptides which define epitopes from different antigens may be used for the detection, diagnosis, staging, monitoring, prognosis, prevention or treatment of, or determining the predisposition to, diseases and conditions of the urinary tract, such as urinary tract cancer. Peptides coated on solid phases or labeled with detectable labels are then allowed to compete with those present in a patient sample (if any) for a limited amount of antibody. A reduction in binding of the synthetic, recombinant, or purified peptides to the antibody (or antibodies) is an indication of the presence of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 in the patient sample. The presence of antigen encoded by markers represented by SEQUENCE ID NOS 1-13 indicates the presence of urinary tract disease, especially urinary tract cancer, in the patient. Variations of assay formats are known to those of ordinary skill in the art and many are discussed herein below.

In another assay format, the presence of antibodies against markers represented by SEQUENCE ID NOS 1-13 and/or antigen encoded by markers represented by SEQUENCE ID NOS 1-13 can be detected in a simultaneous assay, as follows. A test sample is simultaneously contacted with a capture reagent of a first analyte, wherein said capture reagent comprises a first binding member specific for a first analyte attached to a solid phase and a capture reagent for a second analyte, wherein said capture reagent comprises a first binding member for a second analyte attached to a second solid phase, to thereby form a mixture. This mixture is incubated for a time and under conditions sufficient to form capture reagent/first analyte and capture reagent/second analyte complexes. These so-formed complexes then are contacted with an indicator reagent comprising a member of a binding pair specific for the first analyte labeled with a signal generating compound and an indicator reagent comprising a member of a binding pair specific for the second analyte labeled with a signal generating compound to

form a second mixture. This second mixture is incubated for a time and under conditions sufficient to form capture reagent/first analyte/indicator reagent complexes and capture reagent/second analyte/indicator reagent complexes. The presence of one or more analytes is determined by detecting a signal generated in connection with the complexes formed on either or both solid phases as an indication of the presence of one or more analytes in the test sample. In this assay format, recombinant antigens derived from the expression systems disclosed herein may be utilized, as well as monoclonal antibodies produced from the proteins derived from the expression systems as disclosed herein. For example, in this assay system, antigen encoded by markers represented by SEQUENCE ID NOS 1-13 can be the first analyte. Such assay systems are described in greater detail in EP Publication No. 0473065.

In yet other assay formats, the polypeptides disclosed herein may be utilized to detect the presence of antibody against antigen encoded by markers represented by SEQUENCE ID NOS 1-13 in test samples. For example, a test sample is incubated with a solid phase to which at least one polypeptide such as a recombinant protein or synthetic peptide has been attached. The polypeptide is selected from the group consisting of those encoded by markers represented by SEQUENCE ID NOS 1-13, and fragments thereof. These are reacted for a time and under conditions sufficient to form antigen/antibody complexes. Following incubation, the antigen/antibody complex is detected. Indicator reagents may be used to facilitate detection, depending upon the assay system chosen. In another assay format, a test sample is contacted with a solid phase to which a recombinant protein produced as described herein is attached, and also is contacted with a monoclonal or polyclonal antibody specific for the protein, which preferably has been labeled with an indicator reagent. After incubation for a time and under conditions sufficient for antibody/antigen complexes to form, the solid phase is separated from the free phase, and the label is detected in either the solid or free phase as an indication of the presence of antibody against antigen encoded by markers represented by SEQUENCE ID NOS 1-13. Other assay formats utilizing the recombinant antigens disclosed herein are contemplated. These include contacting a test sample with a solid phase to which at least one antigen from a first source has been attached, incubating the solid phase and test sample for a time and under conditions sufficient to form antigen/antibody complexes, and then contacting the solid phase with a labeled antigen, which antigen is derived from a second source different from the first source. For example, a recombinant protein derived from a first source such as E. coli is used as a capture antigen on a solid phase, a test sample is added to the so-prepared solid phase, and following

standard incubation and washing steps as deemed or required, a recombinant protein derived from a different source (i.e., non-E. coli) is utilized as a part of an indicator reagent which subsequently is detected. Likewise, combinations of a recombinant antigen on a solid phase and synthetic peptide in the indicator phase also are possible. Any assay format which utilizes an antigen specific for markers represented by SEQUENCE ID NOS 1-13 produced or derived from a first source as the capture antigen and an antigen specific for markers represented by SEQUENCE ID NOS 1-13 from a different second source is contemplated. Thus, various combinations of recombinant antigens, as well as the use of synthetic peptides, purified proteins and the like, are within the scope of this invention. Assays such as this and others are described in U.S. Patent No. 5,254,458.

Other embodiments which utilize various other solid phases also are contemplated and are within the scope of this invention. For example, ion capture procedures for immobilizing an immobilizable reaction complex with a negatively charged polymer (described in EP publication 0326100 and EP publication No. 0406473), can be employed according to the present invention to effect a fast solution-phase immunochemical reaction. An immobilizable immune complex is separated from the rest of the reaction mixture by ionic interactions between the negatively charged poly-anion/immune complex and the previously treated, positively charged porous matrix and detected by using various signal generating systems previously described, including those described in chemiluminescent signal measurements as described in EPO Publication No. 0 273,115.

Also, the methods of the present invention can be adapted for use in systems which utilize microparticle technology including automated and semi-automated systems wherein the solid phase comprises a microparticle (magnetic or non-magnetic). Such systems include those described in, for example, published EPO applications Nos. EP 0 425 633 and EP 0 424 634, respectively.

The use of scanning probe microscopy (SPM) for immunoassays also is a technology to which the monoclonal antibodies of the present invention are easily adaptable. In scanning probe microscopy, particularly in atomic force microscopy, the capture phase, for example, at least one of the monoclonal antibodies of the invention, is adhered to a solid phase and a scanning probe microscope is utilized to detect antigen/antibody complexes which may be present on the surface of the solid phase. The use of scanning tunneling microscopy eliminates the need for labels which normally must be utilized in many immunoassay systems to detect antigen/antibody complexes. The use of SPM to monitor specific binding reactions can occur in many ways. In one embodiment, one member of a specific binding partner (analyte specific substance which is the monoclonal antibody of the

invention) is attached to a surface suitable for scanning. The attachment of the analyte specific substance may be by adsorption to a test piece which comprises a solid phase of a plastic or metal surface, following methods known to those of ordinary skill in the art. Or, covalent attachment of a specific binding partner (analyte specific substance) to a test piece which test piece comprises a solid phase of derivatized plastic, metal, silicon, or glass may be utilized. Covalent attachment methods are known to those skilled in the art and include a variety of means to irreversibly link specific binding partners to the test piece. If the test piece is silicon or glass, the surface must be activated prior to attaching the specific binding partner. Also, polyelectrolyte interactions may be used to immobilize a specific binding partner on a surface of a test piece by using techniques and chemistries. The preferred method of attachment is by covalent means. Following attachment of a specific binding member, the surface may be further treated with materials such as serum, proteins, or other blocking agents to minimize non-specific binding. The surface also may be scanned either at the site of manufacture or point of use to verify its suitability for assay purposes. The scanning process is not anticipated to alter the specific binding properties of the test piece.

While the present invention discloses the preference for the use of solid phases, it is contemplated that the reagents such as antibodies, proteins and peptides of the present invention can be utilized in non-solid phase assay systems. These assay systems are known to those skilled in the art, and are considered to be within the scope of the present invention.

It is contemplated that the reagent employed for the assay can be provided in the form of a test kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a probe, primer, monoclonal antibody or a cocktail of monoclonal antibodies, or a polypeptide (e.g. recombinantly, synthetically produced or purified) employed in the assay. The polypeptide is selected from the group consisting of those encoded by markers represented by SEQUENCE ID NOS 1-13, and fragments thereof. Other components such as buffers, controls and the like, known to those of ordinary skill in art, may be included in such test kits. It also is contemplated to provide test kits which have means for collecting test samples comprising accessible body fluids, e.g., blood, urine, saliva and stool. Such tools useful for collection ("collection materials") include lancets and absorbent paper or cloth for collecting and stabilizing blood; swabs for collecting and stabilizing saliva; cups for collecting and stabilizing urine or stool samples. Collection materials, papers, cloths, swabs, cups and the like, may optionally be treated to avoid denaturation or irreversible adsorption of the sample. The collection materials also may be treated with or

contain preservatives, stabilizers or antimicrobial agents to help maintain the integrity of the specimens. Test kits designed for the collection, stabilization and preservation of test specimens obtained by surgery or needle biopsy are also useful. It is contemplated that all kits may be configured in two components which can be provided separately; one component for collection and transport of the specimen and the other component for the analysis of the specimen. The collection component, for example, can be provided to the open market user while the components for analysis can be provided to others such as laboratory personnel for determination of the presence, absence or amount of analyte. Further, kits for the collection, stabilization and preservation of test specimens may be configured for use by untrained personnel and may be available in the open market for use at home with subsequent transportation to a laboratory for analysis of the test sample.

In Vivo Antibody Use.

Antibodies of the present invention can be used in vivo; that is, they can be injected into patients suspected of having diseases of the urinary tract for diagnostic or therapeutic uses. The use of antibodies for in vivo diagnosis is well known in the art. Sumerdon et al., Nucl. Med. Biol 17:247-254 (1990) have described an optimized antibody-chelator for the radioimmunoscintographic imaging of carcinoembryonic antigen (CEA) expressing tumors using Indium-111 as the label. Griffin et al., J Clin Onc 9:631-640 (1991) have described the use of this agent in detecting tumors in patients suspected of having recurrent colorectal cancer. The use of similar agents with paramagnetic ions as labels for magnetic resonance imaging is known in the art (R. B. Lauffer, Magnetic Resonance in Medicine 22:339-342 (1991). It is anticipated that antibodies directed against antigen encoded by markers represented by SEQUENCE ID NOS 1-13 can be injected into patients suspected of having a disease of the urinary tract such as bladder cancer for the purpose of diagnosing or staging the disease status of the patient. The label used will depend on the imaging modality chosen. Radioactive labels such as Indium-111, Technetium-99m, or Iodine-131 can be used for planar scans or single photon emission computed tomography (SPECT). Positron emitting labels such as Fluorine-19 can also be used for positron emission tomography (PET). For MRI, paramagnetic ions such as Gadolinium (III) or Manganese (II) can be used. Localization of the label within the urinary tract or external to the urinary tract may allow determination of spread of the disease. The amount of label within the urinary tract may allow determination of the presence or absence of cancer of the urinary tract.

For patients known to have a disease of the urinary tract, injection of an antibody directed against antigen encoded by markers represented by SEQUENCE

ID NOS 1-13 may have therapeutic benefit. The antibody may exert its effect without the use of attached agents by binding to antigen encoded by markers represented by SEQUENCE ID NOS 1-13 expressed on or in the tissue or organ. Alternatively, the antibody may be conjugated to cytotoxic agents such as drugs, toxins, or radionuclides to enhance its therapeutic effect. Garnett and Baldwin, Cancer Research 46:2407-2412 (1986) have described the preparation of a drug-monoclonal antibody conjugate. Pastan et al., Cell 47:641-648 (1986) have reviewed the use of toxins conjugated to monoclonal antibodies for the therapy of various cancers. Goodwin and Meares, Cancer Supplement 80:2675-2680 (1997) have described the use of Yittrium-90 labelled monoclonal antibodies in various strategies to maximize the dose to tumor while limiting normal tissue toxicity. Other known cytotoxic radionuclides include Copper-67, Iodine-131, and Rhenium-186 all of which can be used to label monoclonal antibodies directed against antigen encoded by markers represented by SEQUENCE ID NOS 1-13 for the treatment of cancer of the bladder.

The present invention will now be described by way of examples, which are meant to illustrate, but not to limit, the scope of the present invention.

EXAMPLES

Example 1: Identification of Bladder Tumor Library EST Clones

Experiments performed in support of the present invention suggested that a multiplicity of cytokeratins may be up-regulated in bladder tumors. A search was conducted in Genbank for title entries with the keywords keratin or cytokeratin. This search resulted in a collection of 57 Genbank entries which were annotated in the LifeSeq™ database. The number of EST's occurring in tumor, normal and other libraries, categorized by keratin or cytokeratin type, were classified according to type utilizing all available published information from literature and sequences in GenBank. Because the cytokeratin type designated 50 kDa was not obviously categorized it was treated as a separate class.

The above search data are summarized in Table 1, which further includes the ratios of the numbers of EST's in "bladder tumor" to "bladder normal" libraries. The table has been sorted according to this ratio with the highest ratio at the top of the table. In the case where there are no occurrences in the normal libraries, the ratio is expressed as greater than (>) a minimal value, rather than infinity.

Table 1

	Number of Clones in Libraries					
	Bladder			Other		
Keratin/ cyto-keratin type	Normal	Tumor	Tumor to Normal Ratio		Sequence ID No.	Genbank ID No.
50 kDa	1	31	31	231	1	187604
5	0	27	>27	550	2	186697
16	0	20	>20	120	3	186676
17	3	53	18	736	4	30378
6	2	31	16	337	5	consen-sus*
18	1	15	15	785	6	34036
7	2	26	13	275	7	186729
8	5	25	5	839	8	191399
19	8	31	4	570	9	184568
13	6	15	3	83	10	30376
15	0	3	>3	144	11	34070
14	0	1	>1	85		
10	0	1	>1	18		
20	0	0		115		
4	2	0		16		
3	0	0		1		
2	0	0		14		
Hair type 1	0	1		3		

* consensus sequence derived from keratin/cytokeratin type 6a, 6c, 6e, and 6f, respectively, Genbank Index Nos. 908769, 186699, 908602, and 908804.

In Table 1, the number of clones in each of the libraries are summed for each keratin or cytokeratin type. Sequence ID No. refers to the prototypic sequence that is presented in the Sequence Listing of the present application (the corresponding Genbank ID No. is the source of the sequence). In the case of cytokeratin type 6, due to the published variant forms, a consensus sequence was derived for Seq ID No. 5, based on the individual sequences as shown in the footnote to Table 1.

Representative sequences corresponding to the keratin or cytokeratin types are presented as the SEQUENCE ID Nos. listed herein, and, further, each representative sequence is referenced to a Genbank Index number. Together, these data permit the selection of those sequences that are highly expressed in different bladder tumors compared with the normal state. These representative sequences can be used to define translation open reading frames by standard methods. Further, analysis of the protein structure can be used to define epitopes that will be unique for the preferentially expressed species and minimize cross-reactivity with the non- or low-expressed species.

Example 2: CAS gene expression in bladder tumor libraries.

A cellular apoptosis susceptibility (CAS) gene (PCT application WO 9640713) was found to be represented in EST libraries from bladder tumors but not in libraries derived from normal bladders. Table 2 presents the occurrences of clones with sequences matching CAS mRNA in the LifeSeq™ database.

Table 2

Tissue Type	Normal		Tumor	
	No. of Clones	Represented in number of libraries	No. of Clones	Represented in number of libraries
Bladder	0	0	5	5
Brain	11	7	9	5
Breast	5	4	8	5
Colon	3	3	2	2
Lung	5	4	8	5
Prostate	4	3	1	1
Total Others	73			

While this gene is expressed in libraries from a variety of normal and tumor tissues, it can be seen that it is present in 5 independent bladder tumor libraries but it is not present in normal libraries. The expression in bladder tumor indicates that the sequence provides useful probes for bladder tumors, for example, when test urine is probed for the presence of (1) an mRNA having sequences complementary to SEQUENCE ID NO. 12, or (2) a protein, or fragments thereof, encoded by the sequence.

Example 3: mat-8 gene expression in bladder tumor libraries

A chloride channel protein found in human breast cancer cells has been designated mat-8 (mammary tumor 8 kD: PCT application WO 96/05322, Morrison and Leder). Clones in the LifeSeq™ database carrying the sequence coding for this protein were investigated -- the results of an analysis similar to that of Example 2 are presented in Table 3.

Table 3

Tissue Type	Normal		Tumor	
	No. of Clones	Represented in number of libraries	No. of Clones	Represented in number of libraries
Bladder	0	0	7	4
Breast	6	3	10	3
Colon	56	12	5	2
Lung	12	6	1	1
Prostate	29	10	4	2
Total Others	37			

The gene appears to be expressed in a variety of organs and tissues, but 7 clones were found in 5 bladder tumor libraries and none from any normal bladder. The expression in bladder tumor indicates that the sequence provides useful probes for bladder tumors, for example, when test urine is probed for the presence of (1) an mRNA having sequences complementary to SEQUENCE ID NO. 13, or (2) a protein, or fragments thereof, encoded by the sequence.

It is also noted that this gene appears to be down-regulated in tumors in certain organs: 98 occurrences in normal colon compared with 10 in colon tumors; 41 occurrences in normal prostates compared with 7 in prostate tumors.

Example 4: Nucleic Acid Preparation

A. RNA Extraction from Tissue. Total RNA is isolated from urinary tract tissues and from non-urinary tract tissues. Various methods are utilized, including but not limited to the lithium chloride/urea technique, known in the art and described by Kato et al., (J. Virol. 61:2182-2191, 1987), and TRIzol™ (Gibco-BRL, Grand Island, NY).

Briefly, tissue is placed in a sterile conical tube on ice and 10-15 volumes of 3 M LiCl, 6 M urea, 5 mM EDTA, 0.1 M β-mercaptoethanol, 50 mM Tris-HCl (pH 7.5) are added. The tissue is homogenized with a Polytron® homogenizer (Brinkman Instruments, Inc., Westbury, NY) for 30-50 sec on ice. The solution is transferred to a 15 ml plastic centrifuge tube and placed overnight at -20°C. The tube is centrifuged for 90 min at 9,000 x g at 0-4°C and the supernatant is immediately decanted. Ten ml of 3 M LiCl are added and the tube is vortexed for 5 sec. The tube is centrifuged for 45 min at 11,000 x g at 0-4°C. The decanting, resuspension in LiCl, and centrifugation is repeated and the final pellet is air dried

and suspended in 2 ml of 1 mM EDTA, 0.5% SDS, 10 mM Tris (pH 7.5). Twenty microliters (20 µl) of Proteinase K (20 mg/ml) are added, and the solution is incubated for 30 min at 37°C with occasional mixing. One-tenth volume (0.22-0.25 ml) of 3 M NaCl is added and the solution is vortexed before transfer into another tube containing 2 ml of phenol/chloroform/isoamyl alcohol (PCI). The tube is vortexed for 1-3 sec and centrifuged for 20 min at 3,000 x g at 10°C. The PCI extraction is repeated and followed by two similar extractions with chloroform/isoamyl alcohol (CI). The final aqueous solution is transferred to a chilled 15 ml Corex glass tube containing 6 ml of absolute ethanol, the tube is covered with parafilm, and placed at -20°C overnight. The tube is centrifuged for 30 min at 10,000 x g at 0-4°C and the ethanol supernatant is decanted immediately. The RNA pellet is washed four times with 10 ml of 75% ice-cold ethanol and the final pellet is air dried for 15 min at room temperature. The RNA is suspended in 0.5 ml of 10 mM TE (pH 7.6, 1 mM EDTA) and its concentration is determined spectrophotometrically. RNA samples are aliquoted and stored at -70°C as ethanol precipitates.

The quality of the RNA is determined by agarose gel electrophoresis (see Example 6, Northern Blot Analysis) and staining with 0.5 µg/ml ethidium bromide for one hour. RNA samples that do not contain intact rRNAs are excluded from the study.

Alternatively, for RT-PCR analysis, 1 ml of Ultraspec RNA reagent is added to 120 mg of pulverized tissue in a 2.0 ml polypropylene microfuge tube, homogenized with a Polytron® homogenizer (Brinkman Instruments, Inc., Westbury, NY) for 50 sec and placed on ice for 5 min. Then, 0.2 ml of chloroform is added to each sample, followed by vortexing for 15 sec. The sample is placed on ice for another 5 min, followed by centrifugation at 12,000 x g for 15 min at 4°C. The upper layer is collected and transferred to another RNase-free 2.0 ml microfuge tube. An equal volume of isopropanol is added to each sample, and the solution is placed on ice for 10 min. The sample is centrifuged at 12,000 x g for 10 min at 4°C, and the supernatant is discarded. The remaining pellet is washed twice with cold 75% ethanol, resuspended by vortexing, and the resuspended material is then pelleted by centrifugation at 7500 x g for 5 min at 4°C. Finally, the RNA pellet is dried in a Speedvac (Savant, Farmingdale, NY) for 5 min and reconstituted in RNase-free water.

B. RNA Extraction from Blood Mononuclear Cells. Mononuclear cells are isolated from blood samples from patients by centrifugation using Ficoll-Hypaque as follows. A 10 ml volume of whole blood is mixed with an equal volume of

RPMI Medium (Gibco-BRL, Grand Island, NY). This mixture is then underlayed with 10 ml of Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and centrifuged for 30 minutes at 200 x g. The buffy coat containing the mononuclear cells is removed, diluted to 50 ml with Dulbecco's PBS (Gibco-BRL, Grand Island, NY) and the mixture centrifuged for 10 minutes at 200 x g. After two washes, the resulting pellet is resuspended in Dulbecco's PBS to a final volume of 1 ml.

RNA is prepared from the isolated mononuclear cells as described by N. Kato et al., J. Virology 61: 2182-2191 (1987). Briefly, the pelleted mononuclear cells are brought to a final volume of 1 ml and then are resuspended in 250 µL of PBS and mixed with 2.5 ml of 3M LiCl, 6M urea, 5mM EDTA, 0.1M 2-mercaptoethanol, 50mM Tris-HCl (pH 7.5). The resulting mixture is homogenized and incubated at -20°C overnight. The homogenate is centrifuged at 8,000 RPM in a Beckman J2-21M rotor for 90 minutes at 0-4°C. The pellet is resuspended in 10 ml of 3M LiCl by vortexing and then centrifuged at 10,000 RPM in a Beckman J2-21M rotor centrifuge for 45 minutes at 0-4°C. The resuspending and pelleting steps then are repeated. The pellet is resuspended in 2 ml of 1 mM EDTA, 0.5% SDS, 10 mM Tris (pH 7.5) and 400 µg Proteinase K with vortexing and then it is incubated at 37°C for 30 minutes with shaking. One tenth volume of 3M NaCl then is added and the mixture is vortexed. Proteins are removed by two cycles of extraction with phenol/ chloroform/ isoamyl alcohol (PCI) followed by one extraction with chloroform/ isoamyl alcohol (CI). RNA is precipitated by the addition of 6 ml of absolute ethanol followed by overnight incubation at -20°C. After the precipitated RNA is collected by centrifugation, the pellet is washed 4 times in 75% ethanol. The pelleted RNA is then dissolved in solution containing 1mM EDTA, 10mM Tris-HCl (pH 7.5).

Non-urinary tract tissues are used as negative controls. The mRNA can be further purified from total RNA by using commercially available kits such as oligo dT cellulose spin columns (RediCol™ from Pharmacia, Uppsala, Sweden) for the isolation of poly-adenylated RNA. Total RNA or mRNA can be dissolved in lysis buffer (5M guanidine thiocyanate, 0.1M EDTA, pH 7.0) for analysis in the ribonuclease protection assay.

C. RNA Extraction from polysomes. Tissue is minced in saline at 4°C and mixed with 2.5 volumes of 0.8 M sucrose in a $TK_{150}M$ (150 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) solution containing 6 mM 2-mercaptoethanol. The tissue is homogenized in a Teflon-glass Potter homogenizer with five strokes at 100-200 rpm followed by six strokes in a Dounce homogenizer, as described by B. Mechler, Methods in Enzymology 152:241-248 (1987). The homogenate then is centrifuged at 12,000 x g for 15 min at 4°C to sediment the nuclei. The

polysomes are isolated by mixing 2 ml of the supernatant with 6 ml of 2.5 M sucrose in $TK_{150}M$ and layering this mixture over 4 ml of 2.5 M sucrose in $TK_{150}M$ in a 38 ml polyallomer tube. Two additional sucrose $TK_{150}M$ solutions are successively layered onto the extract fraction; a first layer of 13 ml 2.05 M sucrose followed by a second layer of 6 ml of 1.3 M sucrose. The polysomes are isolated by centrifuging the gradient at 90,000 x g for 5 hr at 4°C. The fraction then is taken from the 1.3 M sucrose/2.05 M sucrose interface with a siliconized pasteur pipette and diluted in an equal volume of TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). An equal volume of 90°C SDS buffer (1% SDS, 200 mM NaCl, 20 mM Tris-HCl, pH 7.4) is added and the solution is incubated in a boiling water bath for 2 min. Proteins next are digested with a Proteinase K digestion (50 mg/ml) for 15 min at 37°C. The mRNA is purified with 3 equal volumes of phenol-chloroform extractions followed by precipitation with 0.1 volume of 2 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol at -20°C overnight. The precipitated RNA is recovered by centrifugation at 12,000 x g for 10 min at 4°C. The RNA is dried and resuspended in TE (pH 7.4) or distilled water. The resuspended RNA then can be used in a slot blot or dot blot hybridization assay to check for the presence of cytokeratin mRNA (see Example 6).

The quality of nucleic acid and proteins is dependent on the method of preparation used. Each sample may require a different preparation technique to maximize isolation efficiency of the target molecule. These preparation techniques are within the skill of the ordinary artisan.

Example 5: Ribonuclease Protection Assay

A. Synthesis of Labeled Complementary RNA (cRNA) Hybridization Probe and Unlabeled Sense Strand. Labeled antisense and unlabeled sense riboprobes are transcribed from the cytokeratin gene cDNA sequence which contains a 5' RNA polymerase promoter such as SP6 or T7. The sequence may be from a vector containing the appropriate cytokeratin cDNA insert, or from a PCR-generated product of the insert using PCR primers which incorporate a 5' RNA polymerase promoter sequence. For example, the described plasmid, clone 1890382 or another comparable clone, containing the cytokeratin gene cDNA sequence, flanked by opposed SP6 and T7 or other RNA polymerase promoters, is purified using a Qiagen Plasmid Purification Kit (Qiagen, Chatsworth, CA). Then 10 μg of the plasmid DNA are linearized by cutting with an appropriate restriction enzyme such as Dde I for 1 hr at 37°C. The linearized plasmid DNA is purified using the QIAprep Kit (Qiagen, Chatsworth, CA) and used for the synthesis of antisense transcript from the appropriate promoter using the Riboprobe® in vitro

Transcription System (Promega Corporation, Madison, WI), as described by the supplier's instructions, incorporating either (alpha^{32}P) CTP (Amersham Life Sciences, Inc. Arlington Heights, IL) or biotinylated CTP as a label. To generate the sense strand, 10 μg of the purified plasmid DNA are cut with restriction enzymes, such as Xba I and Not I, and transcribed as above from the appropriate promoter. Both sense and antisense strands are isolated by spin column chromatography. Unlabeled sense strand is quantitated by UV absorption at 260 nm.

B. Hybridization of Labeled Probe to Target. Frozen tissue is pulverized to powder under liquid nitrogen and 100-500 mg are dissolved in 1 ml of lysis buffer, available as a component of the Direct Protect™ Lysate RNase Protection Kit (Ambion, Inc., Austin, TX). Further dissolution can be achieved using a tissue homogenizer. In addition, a dilution series of a known amount of sense strand in mouse liver lysate is made for use as a positive control. Finally, 45 μl of solubilized tissue or diluted sense strand are mixed directly with either ; 1) 1 x10^5 cpm of radioactively labeled probe, or 2) 250 pg of non-isotopically labeled probe in 5 μl of lysis buffer. Hybridization is allowed to proceed overnight at 37°C. See, T. Kaabache et al., Anal. Biochem. 232:225-230 (1995).

C. RNase Digestion. RNA that is not hybridized to probe is removed from the reaction as per the Direct Protect™ protocol using a solution of RNase A and RNase T1 for 30 min at 37°C, followed by removal of RNase by Proteinase K digestion in the presence of sodium sarcosyl. Hybridized fragments protected from digestion are then precipitated by the addition of an equal volume of isopropanol and placed at -70°C for 3 hr. The precipitates are collected by centrifugation at 12,000 x g for 20 min.

D. Fragment Analysis. The precipitates are dissolved in denaturing gel loading dye (80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue), heat denatured, and electrophoresed in 6% polyacrylamide TBE, 8 M urea denaturing gels. The gels are imaged and analyzed using the STORM™ storage phosphor autoradiography system (Molecular Dynamics, Sunnyvale, CA). Quantitation of protected fragment bands, expressed in femtograms (fg), is achieved by comparing the peak areas obtained from the test samples to those from the known dilutions of the positive control sense strand (see Section B, supra). The results are expressed in molecules of cytokeratin RNA/cell and as a image rating score. In cases where non-isotopic labels are used, hybrids are transferred from the gels to membranes (nylon or nitrocellulose) by blotting and then analyzed using detection systems that employ streptavidin alkaline phosphatase conjugates and chemiluminesence or chemifluoresence reagents.

Detection of a product in urinary tract tissue, in urine, or in cells exfoliated into urine comprising a sequence selected from the group consisting of SEQUENCE ID NOS 1-13, and fragments or complements thereof suggests a diagnosis of urinary tract cancer.

5

Example 6: Northern Blotting

The Northern blot technique is used to identify a specific size RNA fragment from a complex population of RNA using gel electrophoresis and nucleic
10 acid hybridization. Northern blotting is well-known technique in the art. Briefly, 5-10 µg of total RNA (see Example 3) are incubated in 15 µl of a solution containing 40 mM morphilinopropanesulfonic acid (MOPS) (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde, 50% v/v formamide for 15 min at 65°C. The denatured RNA is mixed with 2 µl of loading buffer (50%
15 glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) and loaded into a denaturing 1.0% agarose gel containing 40 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA and 2.2 M formaldehyde. The gel is electrophoresed at 60 V for 1.5 hr and rinsed in RNAse free water. RNA is transferred from the gel onto nylon membranes (Brightstar-Plus, Ambion, Inc., Austin, TX) for 1.5
20 hours using the downward alkaline capillary transfer method (Chomczynski, Anal. Biochem. 201:134-139, 1992). The filter is rinsed with 1X SSC, and RNA is crosslinked to the filter using a Stratalinker™ (Stratagene, Inc., La Jolla, CA) on the autocrosslinking mode and dried for 15 min. The membrane is then placed into a hybridization tube containing 20 ml of preheated prehybridization solution (5X
25 SSC, 50% formamide, 5X Denhardt's solution, 100 µg/ml denatured salmon sperm DNA) and incubated in a 42°C hybridization oven for at least 3 hr. While the blot is prehybridizing, a ^{32}P-labeled random-primed probe is generated using the CYTOKERATIN insert fragment (obtained, for example, by digesting clone 1890382 with XbaI and NotI, or another comparable clone with appropriate
30 restriction enzymes) using Random Primer DNA Labeling System (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Half of the probe is boiled for 10 min, quick chilled on ice and added to the hybridization tube. Hybridization is performed at 42°C for at least 12 hr. The hybridization solution is discarded and the filter is washed in 30 ml of 3X
35 SSC, 0.1% SDS at 42°C for 15 min, followed by 30 ml of 3X SSC, 0.1% SDS at 42°C for 15 min. The filter is wrapped in Saran Wrap, exposed to Kodak XAR-Omat film for 8-96 hr, and the film is developed for analysis. Detection of a product in urinary tract tissue, in urine, or in cells exfoliated into urine comprising

a sequence selected from the group consisting of SEQUENCE ID NOS 1-13, and fragments or complements thereof suggests a diagnosis of urinary tract cancer.

Example 7: Blot/Slot Blot

Dot and slot blot assays are quick methods to evaluate the presence of a specific nucleic acid sequence in a complex mix of nucleic acid. To perform such assays, up to 50 µg of RNA are mixed in 50 µl of 50% formamide, 7% formaldehyde, 1X SSC, incubated 15 min at 68°C, and then cooled on ice. Then, 100 µl of 20X SSC are added to the RNA mixture and loaded under vacuum onto a manifold apparatus that has a prepared nitrocellulose or nylon membrane. The membrane is soaked in water, 20X SSC for 1 hour, placed on two sheets of 20X SSC prewet Whatman #3 Dot filter paper, and loaded into a slot blot or dot blot vacuum manifold apparatus. The slot blot is analyzed with probes prepared and labeled as described in Example 4, supra. Detection of a product in urinary tract tissue, in urine, or in cells exfoliated into urine comprising a sequence selected from the group consisting of SEQUENCE ID NOS 1-13, and fragments or complements thereof suggests a diagnosis of urinary tract cancer.

Other methods and buffers which can be utilized in the methods described in Examples 5, 6, and 7 but not specifically detailed herein, are known in the art and are described in J. Sambrook et al., supra.

Example 8: In Situ Hybridization

This method is useful to directly detect specific target nucleic acid sequences in cells using detectable nucleic acid hybridization probes.

Tissues are prepared with cross-linking fixative agents such as paraformaldehyde or glutaraldehyde for maximum cellular RNA retention. See, L. Angerer et al., Methods in Cell Biol. 35:37-71 (1991). Briefly, the tissue is placed in greater than 5 volumes of 1% glutaraldehyde in 50 mM sodium phosphate, pH 7.5 at 4°C for 30 min. The solution is changed with fresh glutaraldehyde solution (1% glutaraldehyde in 50mM sodium phosphate, pH 7.5) for a further 30 min fixing. The fixing solution should have an osmolality of approximately 0.375% NaCl. The tissue is washed once in isotonic NaCl to remove the phosphate.

The fixed tissues then are embedded in paraffin as follows. The tissue is dehydrated though a series of increasing ethanol concentrations for 15 min each: 50% (twice), 70% (twice), 85%, 90% and then 100% (twice). Next, the tissue is soaked in two changes of xylene for 20 min each at room temperature. The tissue

is then soaked in two changes of a 1:1 mixture of xylene and paraffin for 20 min each at 60°C; and then in three final changes of paraffin for 15 min each.

Next, the tissue is cut in 5 μm sections using a standard microtome and placed on a slide previously treated with a tissue adhesive such as 3-aminopropyltriethoxysilane.

Paraffin is removed from the tissue by two 10 min xylene soaks and rehydrated in a series of decreasing ethanol concentrations: 99% twice, 95%, 85%, 70%, 50%, 30%, and then distilled water twice. The sections are pre-treated with 0.2 M HCl for 10 min and permeabilized with 2 μg/ml Proteinase K at 37°C for 15 min.

Labeled riboprobes transcribed from the plasmid (see Example 4) are hybridized to the prepared tissue sections and incubated overnight at 56°C in 3X standard saline extract and 50% formamide. Excess probe is removed by washing in 2X standard saline citrate and 50% formamide followed by digestion with 100 μg/ml RNase A at 37°C for 30 min. Fluorescence probe is visualized by illumination with ultraviolet (UV) light under a microscope. Fluorescence in the cytoplasm is indicative of specific mRNA. Alternatively, the sections can be visualized by autoradiography. Detection of a product in urinary tract tissue, in urine, or in cells exfoliated into urine comprising a sequence selected from the group consisting of SEQUENCE ID NOS 1-13, and fragments or complements thereof suggests a diagnosis of urinary tract cancer.

Example 9: Reverse Transcription PCR

A. One Step RT-PCR Assay. Target-specific primers are designed to detect the above-described target sequences by reverse transcription PCR using methods known in the art. One step RT-PCR is a sequential procedure that performs both RT and PCR in a single reaction mixture. The procedure is performed in a 200 μl reaction mixture containing 50 mM (N,N,-bis[2-Hydroxyethyl]glycine), pH 8.15, 81.7 mM KOAc, 33.33 mM KOH, 0.01 mg/ml bovine serum albumin, 0.1 mM ethylene diaminetetraacetic acid, 0.02 mg/ml NaN_3, 8% w/v glycerol, 150 μM each of dNTP, 0.25 μM each primer, 5U rT' polymerase, 3.25 mM $Mn(OAc)_2$ and 5 μl of target RNA (see Example 3). RNA and the rTth polymerase enzyme are unstable in the presence of M the $Mn(OAc)_2$ should be added just before target addition. Optimal c cDNA synthesis and thermal cycling readily can be determined b the art. The reaction is incubated in a Perkin-Elmer Thermal C Conditions which may be found useful include cDNA synt

45 min and 30-45 amplification cycles at 94°C, 1 min; 55°-70°C, 1 min; 72°C, 2 min. One step RT-PCR also may be performed by using a dual enzyme procedure with Taq polymerase and a reverse transcriptase enzyme, such as MMLV (Moloney murine leukemia virus) or AMV (avian myeloblastosis virus) RT (reverse transcriptase) enzymes.

B. Traditional RT-PCR. Cytokeratin genes share many homologous regions. Therefore, care is required in the design of RT-PCR primers to ensure the desired specificity. For example, to guide the primer design, all 100 bp strings of the cytokeratin ck5 mRNA sequence (SEQUENCE ID NO 2) were compared with the sequences of all other available cytokeratin mRNAs. A ck5 substring was flagged as a possible region for a primer provided that it was no more than 70% identical to any other 100 bp substring in any other cytokeratin. Then, using primer analysis software, for example, Oligo™ (Version 4.0), RT-PCR primers (SEQUENCE ID NO 14 and SEQUENCE ID NO 15) were designed. A traditional two-step RT-PCR reaction was performed, as described by K. Q. Hu et al., Virology 181:721-726 (1991). Briefly, 0.5 μg of extracted mRNA (see Example 3) was reverse transcribed in a 20 μl reaction mixture containing 1X PCR II buffer (Perkin-Elmer), 5 mM MgCl2, 1 mM each dNTP, 20 U RNasin, 2.5 μM random hexamers, and 50 U MMLV RT. Reverse transcription was performed at room temperature for 10 min, 42°C for 30 min in a PE-480 thermal cycler (Perkin-Elmer), followed by further incubation at 95°C for 5 min to inactivate the RT. PCR was performed using 2 μl of the cDNA reaction in a final PCR reaction volume of 50 μl containing 1X PCR II buffer (Perkin-Elmer), 50 mM KCl, 1.5 mM MgCl2, 200 μM dNTPs, 0.5 μM of each sense and antisense primer, (SEQUENCE ID NO 14) and (SEQUENCE ID NO 15), respectively, and 2.5 U of Taq polymerase. The reaction was incubated in a MJ Research Model PTC-200, as follows: 35 cycles of amplification (94°C, 45 sec; 62°C, 45 sec; 72°C, 2 min.); a final extension (72°C, 5 min); and a soak at 4°C.

C. PCR Fragment Analysis. The correct products were verified by size determination using gel electrophoresis with a SYBR® Green I nucleic acid gel stain (Molecular Probes, Eugene, OR). Gels were stained with SYBR® Green I at a 1:10,000 dilution in 1X TBE for 45 min. The gel, Figure 1, was imaged using a STORM™ imaging system (Molecular Dynamics, Sunnyvale, CA). In Figure 1, the lane contents were as follows: lane 1, 100 bp-ladder molecular weight markers; lane 2, placental DNA; lane 3, bladder cancer RNA; lane 4, normal bladder RNA; lane 5, normal bladder RNA; lane 6, normal bladder RNA; lane 7, bladder cancer RNA; lane 8, prostate BPH RNA; lane 9, normal colon RNA; lane 10, breast cancer RNA; and lane 11, colon cancer RNA. Figure 1 shows a 607 bp

cytokeratin ck5-specific PCR amplification product in lanes 3, 7, 8 and 9, indicating that cytokeratin ck5 mRNA was present in 2 of 2 bladder cancer samples and 0 of 3 normal bladder samples tested. In Figure 1, the 607 bp cytokeratin ck5-specific PCR amplification product is also observed (faintly) in one normal colon sample (lane 9), and in one prostate BPH sample (lane 8). These data suggest that cytokeratin ck5 mRNA expression is bladder cancer specific. Therefore, detection of a product in urinary tract tissue, in urine, or in cells exfoliated into urine comprising SEQUENCE ID NO 2, and fragments or complements thereof indicates the presence of ck5 mRNA suggesting a diagnosis of urinary tract cancer.

Similarly, detection of a product in urinary tract tissue, in urine, or in cells exfoliated into urine comprising a sequence selected from the group consisting of SEQUENCE ID NO 1 and SEQUENCE ID NOS 3-13, and fragments or complements thereof suggests a diagnosis of urinary tract cancer.

Example 10: OH-PCR

A. Probe selection and Labeling. Target-specific primers and probes are designed to detect the above-described target sequences by oligonucleotide hybridization PCR. International Publication Nos WO 92/10505, published June 25, 1992, and WO 92/11388, published July 9, 1992, teach methods for labeling oligonucleotides at their 5' and 3' ends, respectively. According to one known method for labeling an oligonucleotide, a label-phosphoramidite reagent is prepared and used to add the label to the oligonucleotide during its synthesis. For example, see N. T. Thuong et al., Tet. Letters 29(46):5905-5908 (1988); or J. S. Cohen et al., published U.S. Patent Application 07/246,688 (NTIS ORDER No. PAT-APPL-7-246,688) (1989). Preferably, probes are labeled at their 3' end to prevent participation in PCR and the formation of undesired extension products. For one step OH-PCR, the probe should have a T_M at least 15°C below the T_M of the primers. The primers and probes are utilized as specific binding members, with or without detectable labels, using standard phosphoramidite chemistry and/or post-synthetic labeling methods which are well-known to one skilled in the art.

B. One Step Oligo Hybridization PCR. OH-PCR is performed on a 200 µl reaction containing 50 mM (N,N,-bis[2-Hydroxyethyl]glycine), pH 8.15, 81.7 mM KOAc, 33.33 mM KOH, 0.01 mg/ml bovine serum albumin, 0.1 mM ethylene diaminetetraacetic acid, 0.02 mg/ml NaN_3, 8% w/v glycerol, 150 µM each of dNTP, 0.25 µM each primer, 3.75 nM probe, 5U rTth polymerase, 3.25 mM $Mn(OAc)_2$ and 5 µl blood equivalents of target (see Example 3). Since RNA and the rTth polymerase enzyme are unstable in the presence of $Mn(OAc)_2$, the $Mn(OAc)_2$ should be added just before target addition. The reaction is incubated in

a Perkin-Elmer Thermal Cycler 480. Optimal conditions for cDNA synthesis and thermal cycling can be readily determined by those skilled in the art. Conditions which may be found useful include cDNA synthesis (60°C, 30 min), 30-45 amplification cycles (94°C, 40 sec; 55-70°C, 60 sec), oligo-hybridization (97°C, 5 min; 15°C, 5 min; 15°C soak). The correct reaction product contains at least one of the strands of the PCR product and an internally hybridized probe.

C. OH-PCR Product Analysis. Amplified reaction products are detected on an LCx® Analyzer system (available from Abbott Laboratories, Abbott Park, IL). Briefly, the correct reaction product is captured by an antibody labeled microparticle at a capturable site on either the PCR product strand or the hybridization probe, and the complex is detected by binding of a detectable antibody conjugate to either a detectable site on the probe or the PCR strand. Only a complex containing a PCR strand hybridized with the internal probe is detectable. Detection of a product in urinary tract tissue, in urine, or in cells exfoliated into urine comprising a sequence selected from the group consisting of SEQUENCE ID NOS 1-13, and fragments or complements thereof suggests a diagnosis of urinary tract cancer.

Many other detection formats exist which can be used and/or modified by those skilled in the art to detect the presence of amplified or non-amplified nucleic acid sequences including, but not limited to, ligase chain reaction (LCR, Abbott Laboratories, Abbott Park, IL); Q-beta replicase (Gene-Trak™, Naperville, Illinois), branched chain reaction (Chiron, Emeryville, CA) and strand displacement assays (Becton Dickinson, Research Triangle Park, NC).

Example 11: Synthetic Peptide Production

Synthetic peptides are modeled and then prepared based upon the predicted amino acid sequences encoded by the mRNAs of SEQUENCE ID NOS 1-13 (see Example 1). In particular, a number of peptides are prepared. All peptides are synthesized on a Symphony Peptide Synthesizer (available from Rainin Instrument Co, Emeryville, CA) or similar instrument, using FMOC chemistry, standard cycles and in-situ HBTU activation. Cleavage and deprotection conditions are as follows: a volume of 2.5 ml of cleavage reagent (77.5% v/v trifluoroacetic acid, 15% v/v ethanedithiol, 2.5% v/v water, 5% v/v thioanisole, 1-2% w/v phenol) is added to the resin, and agitated at room temperature for 2-4 hours. The filtrate is then removed and the peptide is precipitated from the cleavage reagent with cold diethyl ether. Each peptide is filtered, purified via reverse-phase preparative HPLC using a water/acetonitrile/0.1% TFA gradient, and lyophilized. The product is confirmed by mass spectrometry (see Example 12).

Disulfide bond formation is accomplished using auto-oxidation conditions, as follows: the peptide is dissolved in a minimum amount of DMSO (approximately 10 ml) before adding buffer (0.1 M Tris-HCl, pH 6.2) to a concentration of 0.3 - 0.8 mg/ml. The reaction is monitored by HPLC until complete formation of the disulfide bond, followed by reverse-phase preparative HPLC using a water/acetonitrile/0.1% TFA gradient and lyophilization. The product then is confirmed by mass spectrometry (see Example 12).

The purified peptides can be conjugated to Keyhole Limpet Hemocyanin or other immunoreactive molecule with glutaraldehyde, mixed with adjuvant, and injected into animals.

Example 12a: Expression of Protein in a Cell Line Using Plasmid 577

A. Construction of Expression Plasmids. Plasmid 577, described in U.S. patent application Serial No. 08/478,073, filed June 7, 1995, has been constructed for the expression of secreted antigens in a permanent cell line. This plasmid contains the following DNA segments: (a) a 2.3 kb fragment of pBR322 containing bacterial beta-lactamase and origin of DNA replication; (b) a 1.8 kb cassette directing expression of a neomycin resistance gene under control of HSV-1 thymidine kinase promoter and poly-A addition signals; (c) a 1.9 kb cassette directing expression of a dihydrofolate reductase gene under the control of an Simian Virus 40 (SV40) promoter and poly-A addition signals; (d) a 3.5 kb cassette directing expression of a rabbit immunoglobulin heavy chain signal sequence fused to a modified hepatitis C virus (HCV) E2 protein under the control of the Simian Virus 40 T-Ag promoter and transcription enhancer, the hepatitis B virus surface antigen (HBsAg) enhancer I followed by a fragment of Herpes Simplex Virus-1 (HSV-1) genome providing poly-A addition signals; and (e) a residual 0.7 kb fragment of SV40 genome late region of no function in this plasmid. All of the segments of the vector were assembled by standard methods known to those skilled in the art of molecular biology.

Plasmids for the expression of secretable proteins are constructed by replacing the hepatitis C virus E2 protein coding sequence in plasmid 577 with that of a polynucleotide sequence as follows. Digestion of plasmid 577 with XbaI releases the hepatitis C virus E2 gene fragment. The resulting plasmid backbone allows insertion of the cDNA insert downstream of the rabbit immunoglobulin heavy chain signal sequence which directs the expressed proteins into the secretory pathway of the cell. The cDNA fragment is generated by PCR using standard procedures. Encoded in the sense PCR primer sequence is an XbaI site, immediately followed by a 12 nucleotide sequence that encodes the amino acid

sequence Ser-Asn-Glu-Leu ("SNEL") to promote signal protease processing, efficient secretion and final product stability in culture fluids. Immediately following this 12 nucleotide sequence the primer contains nucleotides complementary to template sequences encoding amino acids of the gene . The antisense primer incorporates a sequence encoding the following eight amino acids just before the stop codons: Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (SEQUENCE ID NO 16). Within this sequence is incorporated a recognition site to aid in analysis and purification of the protein product. A recognition site (termed "FLAG") that is recognized by a commercially available monoclonal antibody designated anti-FLAG M2 (Eastman Kodak, Co., New Haven, CT) can be utilized, as well as other comparable sequences and their corresponding antibodies. For example, PCR is performed using GeneAmp® reagents obtained from Perkin-Elmer-Cetus, as directed by the supplier's instructions. PCR primers are used at a final concentration of 0.5 μM. PCR is performed on the plasmid template in a 100 μl reaction for 35 cycles (94°C, 30 seconds; 55°C, 30 seconds; 72°C, 90 seconds) followed by an extension cycle of 72°C for 10 min.

B. Transfection of Dihydrofolate Reductase Deficient Chinese Hamster Ovary Cells. The plasmid described supra is transfected into CHO/dhfr- cells [DXB-111, Uriacio et al., Proc Natl Acad Sci U S A 77:4451-4466 (1980)]. These cells are available from the A.T.C.C., 12301 Parklawn Drive, Rockville, MD 20852, under Accession No. CRL 9096. Transfection is performed using the cationic liposome-mediated procedure described by P. L. Felgner et al., Proc Natl Acad Sci U S A 84:7413-7417 (1987). Particularly, CHO/dhfr- cells are cultured in Ham's F-12 media supplemented with 10% fetal calf serum, L-glutamine (1 mM) and freshly seeded into a flask at a density of 5 - 8 x 10^5 cells per flask. The cells are grown to a confluency of between 60 and 80% for transfection. Twenty micrograms (20μg) of plasmid DNA are added to 1.5 ml of Opti-MEM I medium and 100 μl of Lipofectin Reagent (Gibco-BRL; Grand Island, NY) are added to a second 1.5 ml portion of Opti-MEM I media. The two solutions are mixed and incubated at room temperature for 20 min. After the culture medium is removed from the cells, the cells are rinsed 3 times with 5 ml of Opti-MEM I medium. The Opti-MEM I-Lipofection-plasmid DNA solution then is overlaid onto the cells. The cells are incubated for 3 hr at 37°C, after which time the Opti-MEM I-Lipofectin-DNA solution is replaced with culture medium for an additional 24 hr prior to selection.

C. Selection and Amplification. One day after transfection, cells are passaged 1:3 and incubated with dhfr/G418 selection medium (hereafter, "F-12 minus medium G"). Selection medium is Ham's F-12 with L-glutamine and

without hypoxanthine, thymidine and glycine (JRH Biosciences, Lenexa, Kansas) and 300 μg per ml G418 (Gibco-BRL; Grand Island, NY). Media volume-to-surface area ratios of 5 ml per 25 cm^2 are maintained. After approximately two weeks, DHFR/G418 cells are expanded to allow passage and continuous maintenance in F-12 minus medium G.

Amplification of each of the transfected cDNA sequences is achieved by stepwise selection of $DHFR^+$, $G418^+$ cells with methotrexate (reviewed by R. Schimke, Cell 37:705-713 [1984]). Cells are incubated with F-12 minus medium G containing 150 nM methotrexate (MTX) (Sigma, St. Louis, MO) for approximately two weeks until resistant colonies appear. Further gene amplification is achieved by selection of 150 nM adapted cells with 5 μM MTX.

D. Antigen Production. F-12 minus medium G supplemented with 5 μM MTX is overlaid onto just confluent monolayers for 12 to 24 hr at 37°C in 5% CO_2. The growth medium is removed and the cells are rinsed 3 times with Dulbecco's phosphate buffered saline (PBS) (with calcium and magnesium) (Gibco-BRL; Grand Island, NY) to remove the remaining media/serum which may be present. Cells then are incubated with VAS custom medium (VAS custom formulation with L-glutamine with HEPES without phenol red, available from JRH Bioscience; Lenexa, KS, product number 52-08678P), for 1 hr at 37°C in 5% CO_2. Cells then are overlaid with VAS for production at 5 ml per T flask. Medium is removed after seven days of incubation, retained, and then frozen to await purification with harvests 2, 3 and 4. The monolayers are overlaid with VAS for 3 more seven day harvests.

E. Analysis of Antigen Expression. Aliquots of VAS supernatants from the cells expressing the protein constructs are analyzed, either by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using standard methods and reagents known in the art (Laemmli discontinuous gels), or by mass spectrometry.

F. Purification. Purification of the protein containing the FLAG sequence is performed by immunoaffinity chromatography using an affinity matrix comprising anti-FLAG M2 monoclonal antibody covalently attached to agarose by hydrazide linkage (Eastman Kodak Co., New Haven, CT). Prior to affinity purification, protein in pooled VAS medium harvests from roller bottles is exchanged into 50 mM Tris-HCl (pH 7.5), 150 mM NaCl buffer using a Sephadex G-25 (Pharmacia Biotech Inc., Uppsala, Sweden) column. Protein in this buffer is applied to the anti-FLAG M2 antibody affinity column. Non-binding protein is eluted by washing the column with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl buffer. Bound protein is eluted using an excess of FLAG peptide in 50 mM Tris-

HCl (pH 7.5), 150 mM NaCl. The excess FLAG peptide can be removed from the purified protein by gel electrophoresis or HPLC.

Although plasmid 577 is utilized in this example, it is known to those skilled in the art that other comparable expression systems, such as CMV, can be utilized herein with appropriate modifications in reagent and/or techniques and are within the skill of the ordinary artisan.

The largest cloned insert containing the coding region of the gene is then sub-cloned into either (i) a eukaryotic expression vector which may contain, for example, a cytomegalovirus (CMV) promoter and/or protein fusible sequences which aid in protein expression and detection, or (ii) a bacterial expression vector containing a superoxide-dismutase (SOD) and CMP-KDO synthetase (CKS) or other protein fusion gene for expression of the protein sequence. Methods and vectors which are useful for the production of polypeptides which contain fusion sequences of SOD are described in EPO 0196056, published October 1, 1986, and those containing fusion sequences of CKS are described in EPO Publication No. 0331961, published September 13, 1989. This SOD-purified protein can be used in a variety of techniques, including, but not limited to animal immunization studies, solid phase immunoassays, etc.

Example 12b: Expression of Protein in a Cell Line Using pcDNA3.1/Myc-His

A. Construction of Expression Plasmids. Plasmid pcDNA3.1/Myc-His (Cat.# V855-20, Invitrogen, Carlsbad, CA) has been constructed, in the past, for the expression of secreted antigens by most mammalian cell lines. Expressed protein inserts are fused to a myc-his peptide tag. The myc-his tag (SEQUENCE ID NO 17) comprises a c-myc oncoprotein epitope and a polyhistidine sequence which are useful for the purification of an expressed fusion protein by using either anti-myc or anti-his affinity columns, or metalloprotein binding columns.

Plasmids for the expression of secretable proteins are constructed by inserting a polynucleotide sequence selected from the group consisting of SEQUENCE ID NOS 1-13, and fragments or complements thereof. Prior to construction of an expression plasmid, the cDNA sequence is first cloned into a pCR®-Blunt vector as follows:

The cDNA fragment is generated by PCR using standard procedures. For example, PCR is performed procedures and reagents from Stratagene®, Inc. (La Jolla, CA), as directed by the manufacturer. PCR primers are used at a final concentration of 0.5 μM. PCR using 5 U of pfu polymerase (Stratagene, La Jolla, CA) is performed on the plasmid template (see Example 2) in a 50 μl reaction for 30 cycles (94°C, 1 min; 65°C, 1.5 min; 72°C, 3 min) followed by an extension

cycle of 72°C for 8 min. (The sense PCR primer sequence comprises nucleotides which are either complementary to the pINCY vector directly upstream of the gene insert or which incorporate a 5' EcoRI restriction site, an adjacent downstream protein translation consensus initiator, and a 3' nucleic acid sequence which is the same sense as the 5'-most end of the cDNA insert. The antisense PCR primer incorporates a 5' NotI restriction sequence and a sequence complementary to the 3' end of the cDNA insert just upstream of the 3'-most, in-frame stop codon.) Five microliters (5 µl) of the resulting blunted-ended PCR product are ligated into 25 ng of linearized pCR®-Blunt vector (Invitrogen, Carlsbad, CA) interrupting the lethal ccdB gene of the vector. The resulting ligated vector is transformed into TOP10 E. coli (Invitrogen, Carlsbad, CA) using a One Shot™ Transformation Kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The transformed cells are grown on LB-Kan (50 µg/ml kanamycin) selection plates at 37°C. Only cells containing a plasmid with an interrupted ccdB gene will grow after transformation [Grant, S.G.N., Proc Natl Acad Sci U S A 87:4645-4649 (1990)]. Transformed colonies are picked and grown up in 3 ml of LB-Kan broth at 37°C. Plasmid DNA is isolated by using a QIAprep® (Qiagen Inc., Santa Clarita, CA) procedure, as directed by the manufacturer. The DNA is cut with EcoRI or SnaBI, and NotI restriction enzymes to release the insert fragment. The fragment is run on 1% Seakem® LE agarose/0.5 µg/ml ethidium bromide/TE gel, visualized by UV irradiation, excised and purified using QIAquick™ (Qiagen Inc., Santa Clarita, CA) procedures, as directed by the supplier's instructions.

The pcDNA3.1/Myc-His plasmid DNA is linearized by digestion with EcoRI or SnaBI, and NotI in the polylinker region of the plasmid DNA. The resulting plasmid DNA backbone allows insertion of the purified cDNA fragment, supra, downstream of a CMV promoter which directs expression of the proteins in mammalian cells. The ligated plasmid is transformed into DH5 alpha™ cells (GibcoBRL Grand Island, NY), as directed by the manufacturer. Briefly, 10 ng of pcDNA3.1/Myc-His containing an insert are added to 50 µl of competent DH5 alpha cells, and the contents are mixed gently. The mixture is incubated on ice for 30 min, heat shocked for 20 sec at 37°C, and placed on ice for an additional 2 min. Upon addition of 0.95 ml of LB medium, the mixture is incubated for 1 hr at 37°C while shaking at 225 rpm. The transformed cells then are plated onto 100 mm LB/Amp (50µg/ml ampicillin) plates and grown at 37°C. Colonies are picked and grown in 3 ml of LB/Amp broth. Plasmid DNA is purified using a QIAprep Kit. The presence of the insert is confirmed using techniques known to those skilled in the art, including, but not limited to restriction digestion and gel analysis. (J. Sambrook et al., supra.)

B. Transfection of Human Embryonic Kidney Cell 293 Cells. The expression plasmid described in section A, supra, is retransformed into DH5 alpha cells, plated onto LB/ampicillin agar, and grown up in 10 ml of LB/ampicillin broth, as described hereinabove. The plasmid is purified using a QIAfilter™ Maxi Kit (Qiagen, Chatsworth, CA) and is transfected into HEK293 cells [F.L. Graham et al., J. Gen. Vir. 36:59-72 (1977)]. These cells are available from the A.T.C.C., 12301 Parklawn Drive, Rockville, MD 20852, under Accession No. CRL 1573. Transfection is performed using the cationic lipofectamine-mediated procedure described by P. Hawley-Nelson et al., Focus 15.73 (1993). Particularly, HEK293 cells are cultured in 10 ml DMEM media supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM) and freshly seeded into 100 mm culture plates at a density of 9 x 10^6 cells per plate. The cells are grown at 37 °C to a confluency of between 70% and 80% for transfection. Eight micrograms (8 µg) of plasmid DNA are added to 800 µl of Opti-MEM I® medium (Gibco-BRL, Grand Island, NY), and 48-96 µl of Lipofectamine™ Reagent (Gibco-BRL, Grand Island, NY) are added to a second 800 µl portion of Opti-MEM I media. The two solutions are mixed and incubated at room temperature for 15-30 min. After the culture medium is removed from the cells, the cells are washed once with 10 ml of serum-free DMEM. The Opti-MEM I-Lipofectamine-plasmid DNA solution is diluted with 6.4 ml of serum-free DMEM and then overlaid onto the cells. The cells are incubated for 5 hr at 37°C, after which time, an additional 8 ml of DMEM with 20% FBS are added. After 18-24 hr, the old medium is aspirated, and the cells are overlaid with 5 ml of fresh DMEM with 5% FBS. Supernatants and cell extracts are analyzed for gene activity 72 hr after transfection.

C. Analysis of Antigen Expression. The culture supernatant, supra, is transferred to cryotubes and stored on ice. HEK293 cells are harvested by washing twice with 10 ml of cold Dulbecco's PBS and lysing by addition of 1.5 ml of CAT lysis buffer (Boehringer Mannheim, Indianapolis, IN), followed by incubation for 30 min at room temperature. Lysate is transferred to 1.7 ml polypropylene microfuge tubes and centrifuged at 1000 x g for 10 min. The supernatant is transferred to new cryotubes and stored on ice. Aliquots of supernatants from the cells and the lysate of the cells expressing the protein construct are analyzed for the presence of recombinant protein. The aliquots can be run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using standard methods and reagents known in the art. (J. Sambrook et al., supra) These gels can then be blotted onto a solid medium such as nitrocellulose, nytran, etc., and the

protein band can be visualized using Western blotting techniques with anti-myc epitope or anti-histidine monoclonal antibodies (Invitrogen, Carlsbad, CA) or polyclonal serum (see Example 15). Alternatively, the expressed recombinant protein can be analyzed by mass spectrometry (see Example 13).

D. Purification. Purification of the recombinant protein containing the myc-his sequence is performed using the Xpress® affinity chromatography system (Invitrogen, Carlsbad, CA) containing a nickel-charged agarose resin which specifically binds polyhistidine residues. Supernatants from 10 x 100 mm plates, prepared as described supra, are pooled and passed over the nickel-charged column. Non-binding protein is eluted by washing the column with 50 mM Tris-HCl (pH 7.5)/150 mM NaCl buffer, leaving only the myc-his fusion proteins. Bound recombinant protein then is eluted from the column using either an excess of imidazole or histidine, or a low pH buffer. Alternatively, the recombinant protein can also be purified by binding at the myc-his sequence to an affinity column consisting of either anti-myc or anti-histidine monoclonal antibodies conjugated through a hydrazide or other linkage to an agarose resin and eluting with an excess of myc peptide or histidine, respectively.

The purified recombinant protein can then be covalently cross-linked to a solid phase, such as N-hydroxysuccinimide-activated sepharose columns (Pharmacia Biotech, Piscataway, NJ), as directed by supplier's instructions. These columns containing covalently linked recombinant protein, can then be used to purify anti- antibodies from rabbit or mouse sera.

E. Coating Microtiter Plates with Expressed Proteins. Supernatant from a 100 mm plate, as described supra, is diluted in an appropriate volume of PBS. Then, 100 µl of the resulting mixture is placed into each well of a Reacti-Bind™ metal chelate microtiter plate (Pierce, Rockford, IL), incubated at room temperature while shaking, and followed by three washes with 200 µl each of PBS with 0.05% Tween® 20. The prepared microtiter plate can then be used to screen polyclonal antisera for the presence of antibodies (see Example 17).

Although pcDNA3.1/Myc-His is utilized in this example, it is known to those skilled in the art that other comparable expression systems can be utilized herein with appropriate modifications in reagent and/or techniques and are within the skill of one of ordinary skill in the art. The largest cloned insert containing the coding region of the gene is sub-cloned into either (i) a eukaryotic expression vector which may contain, for example, a cytomegalovirus (CMV) promoter and/or protein fusible sequences which aid in protein expression and detection, or (ii) a

bacterial expression vector containing a superoxide-dismutase (SOD) and CMP-KDO synthetase (CKS) or other protein fusion gene for expression of the protein sequence. Methods and vectors which are useful for the production of polypeptides which contain fusion sequences of SOD are described in published EPO application No. EP 0 196 056, published October 1, 1986, and vectors containing fusion sequences of CKS are described in published EPO application No. EP 0 331 961, published September 13, 1989. The purified protein can be used in a variety of techniques, including, but not limited to animal immunization studies, solid phase immunoassays, etc.

Example 13: Chemical Analysis of Proteins

A. Analysis of Tryptic Peptide Fragments Using MS. Sera from patients with urinary tract disease, such as urinary tract cancer, sera from patients with no urinary tract disease, extracts of urinary tract tissues or cells from patients with urinary tract disease, such as urinary tract cancer, extracts of urinary tract tissues or cells from patients with no urinary tract disease, exfoliated urinary tract cells in urine, and extracts of tissues or cells from other non-diseased or diseased organs of patients are run on a polyacrylamide gel using standard procedures and stained with Coomassie Blue. Sections of the gel suspected of containing a target polypeptide are excised and subjected to an in-gel reduction, acetamidation and tryptic digestion. P. Jeno et al., Anal. Bio. 224:451-455 (1995) and J. Rosenfeld et al., Anal. Bio. 203:173-179 (1992). The gel sections are washed with 100 mM NH_4HCO_3 and acetonitrile. The shrunken gel pieces are swollen in digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 μg/ml trypsin) at 4°C for 45 min. The supernatant is aspirated and replaced with 5 to 10 μl of digestion buffer without trypsin and allowed to incubate overnight at 37°C. Peptides are extracted with 3 changes of 5% formic acid and acetonitrile and evaporated to dryness. The peptides are adsorbed to approximately 0.1 μl of POROS R2 sorbent (Perseptive Biosystems, Framingham, Massachusetts) trapped in the tip of a drawn gas chromatography capillary tube by dissolving them in 10 μl of 5% formic acid and passing it through the capillary. The adsorbed peptides are washed with water and eluted with 5% formic acid in 60% methanol. The eluant is passed directly into the spraying capillary of an API III mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) for analysis by nano-electrospray mass spectrometry. M. Wilm et al., Int. J. Mass Spectrom. Ion Process 136:167-180 (1994) and M. Wilm et al., Anal. Chem. 66:1-8 (1994). The masses of the tryptic peptides are determined from the mass spectrum obtained off the first quadrupole. Masses

corresponding to predicted peptides can be further analyzed in MS/MS mode to give the amino acid sequence of the peptide.

B. Peptide Fragment Analysis Using LC/MS. The presence of polypeptides predicted from mRNA sequences found in hyperplastic disease tissues also can be confirmed using liquid chromatography/tandem mass spectrometry (LC/MS/MS). D. Hess et al., METHODS, A Companion to Methods in Enzymology 6:227-238 (1994). The serum specimen or tumor extract from the patient is denatured with SDS and reduced with dithiothreitol (1.5 mg/ml) for 30 min at 90°C followed by alkylation with iodoacetamide (4 mg/ml) for 15 min at 25°C. Following acrylamide electrophoresis, the polypeptides are electroblotted to a cationic membrane and stained with Coomassie Blue. Following staining, the membranes are washed and sections thought to contain the unknown polypeptides are cut out and dissected into small pieces. The membranes are placed in 500 µl microcentrifuge tubes and immersed in 10 to 20 µl of proteolytic digestion buffer (100 mM Tris-HCl, pH 8.2, containing 0.1 M NaCl, 10% acetonitrile, 2 mM $CaCl_2$ and 5 µg/ml trypsin) (Sigma, St. Louis, MO). After 15 hr at 37°C, 3 µl of saturated urea and 1 µl of 100 µg/ml trypsin are added and incubated for an additional 5 hr at 37°C. The digestion mixture is acidified with 3 µl of 10% trifluoroacetic acid and centrifuged to separate supernatant from membrane. The supernatant is injected directly onto a microbore, reverse phase HPLC column and eluted with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid. The eluate is fed directly into an electrospray mass spectrometer, after passing though a stream splitter if necessary to adjust the volume of material. The data is analyzed following the procedures set forth in Example 13, Section A.

Example 14: Gene Immunization Protocol

A. In vivo Antigen Expression. Gene immunization circumvents protein purification steps by directly expressing an antigen in vivo after inoculation of the appropriate expression vector. Also, production of antigen by this method may allow correct protein folding and glycosylation since the protein is produced in mammalian tissue. The method utilizes insertion of the gene sequence into a plasmid which contains a CMV promoter, expansion and purification of the plasmid and injection of the plasmid DNA into the muscle tissue of an animal. Preferred animals include mice and rabbits. See, for example, H. Davis et al., Human Molecular Genetics 2:1847-1851 (1993). After one or two booster immunizations, the animal can then be bled, ascites fluid collected, or the animal's spleen can be harvested for production of hybridomas.

B. Plasmid Preparation and Purification. cDNA sequences are generated from the cDNA-containing vector using appropriate PCR primers containing suitable 5' restriction sites. The PCR product is cut with appropriate restriction enzymes and inserted into a vector which contains the CMV promoter (for example, pRc/CMV or pcDNA3 vectors from Invitrogen, San Diego, CA). This plasmid then is expanded in the appropriate bacterial strain and purified from the cell lysate using a CsCl gradient or a Qiagen plasmid DNA purification column. All these techniques are familiar to one of ordinary skill in the art of molecular biology.

C. Immunization Protocol. Anesthetized animals are immunized intramuscularly with 0.1-100 μg of the purified plasmid diluted in PBS or other DNA uptake enhancers (Cardiotoxin, 25% sucrose). See, for example, H. Davis et al., Human Gene Therapy 4:733-740 (1993); and P. W. Wolff et al., Biotechniques 11:474-485 (1991). One to two booster injections are given at monthly intervals.

D. Testing and Use of Antiserum. Animals are bled and the resultant sera tested for antibody using peptides synthesized from the known gene sequence (see Example 16) using techniques known in the art, such as Western blotting or EIA techniques. Antisera produced by this method can then be used to detect the presence of the antigen in a patient's tissue or cell extract or in a patient's serum by ELISA or Western blotting techniques, such as those described in Examples 15 through 18.

Example 15: Production of Antibodies

A. Production of Polyclonal Antisera. Antiserum against polypeptides is prepared by injecting appropriate animals with peptides whose sequences are derived from that of the predicted amino acid sequence of the nucleotide sequence. The synthesis of peptides is described in Example 11. Peptides used as immunogen either can be conjugated to a carrier such as keyhole limpet hemocyanine (KLH), prepared as described hereinbelow, or unconjugated (i.e., not conjugated to a carrier such as KLH).

1. Peptide Conjugation. Peptide is conjugated to maleimide activated keyhole limpet hemocyanine (KLH, commercially available as Imject®, available from Pierce Chemical Company, Rockford, IL). Imject® contains about 250 moles of reactive maleimide groups per mole of hemocyanine. The activated KLH is dissolved in phosphate buffered saline (PBS, pH 8.4) at a concentration of about 7.7 mg/ml. The peptide is conjugated through cysteines occurring in the peptide sequence, or to a cysteine previously added to the synthesized peptide in

order to provide a point of attachment. The peptide is dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Company, St. Louis, MO) and reacted with the activated KLH at a mole ratio of about 1.5 moles of peptide per mole of reactive maleimide attached to the KLH. A procedure for the conjugation of peptide is provided hereinbelow. It is known to the ordinary artisan that the amounts, times and conditions of such a procedure can be varied to optimize peptide conjugation.

The conjugation reaction described hereinbelow is based on obtaining 3 mg of KLH peptide conjugate ("conjugated peptide"), which contains about 0.77 μmoles of reactive maleimide groups. This quantity of peptide conjugate usually is adequate for one primary injection and four booster injections for production of polyclonal antisera in a rabbit. Briefly, peptide is dissolved in DMSO at a concentration of 1.16 μmoles/100 μl of DMSO. One hundred microliters (100 μl) of the DMSO solution are added to 380 μl of the activated KLH solution prepared as described hereinabove, and 20 μl of PBS (pH 8.4) are added to bring the volume to 500 μl. The reaction is incubated overnight at room temperature with stirring. The extent of reaction is determined by measuring the amount of unreacted thiol in the reaction mixture. The difference between the starting concentration of thiol and the final concentration is assumed to be the concentration of peptide which has coupled to the activated KLH. The amount of remaining thiol is measured using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), Pierce Chemical Company, Rockford, IL). Cysteine standards are made at a concentration of 0, 0.1, 0.5, 2, 5 and 20 mM by dissolving 35 mg of cysteine HCl (Pierce Chemical Company, Rockford, IL) in 10 ml of PBS (pH 7.2) and diluting the stock solution to the desired concentration(s). The photometric determination of the concentration of thiol is accomplished by placing 200 μl of PBS (pH 8.4) in each well of an Immulon 2® microwell plate (Dynex Technologies, Chantilly, VA). Next, 10 μl of standard or reaction mixture is added to each well. Finally, 20 μl of Ellman's reagent at a concentration of 1 mg/ml in PBS (pH 8.4) is added to each well. The wells are incubated for 10 minutes at room temperature, and the absorbance of all wells is read at 415 nm with a microplate reader (such as the BioRad Model 3550, BioRad, Richmond, CA). The absorbance of the standards is used to construct a standard curve and the thiol concentration of the reaction mixture is determined from the standard curve. A decrease in the concentration of free thiol is indicative of a successful conjugation reaction. Unreacted peptide is removed by dialysis against PBS (pH 7.2) at room temperature for 6 hours. The conjugate is stored at 2-8°C if it is to be used immediately; otherwise, it is stored at -20°C or colder.

2. Animal Immunization. Female white New Zealand rabbits weighing 2 kg or more are used for raising polyclonal antiserum. Generally, one animal is immunized per unconjugated or conjugated peptide (prepared as described hereinabove). One week prior to the first immunization, 5 to 10 ml of blood is obtained from the animal to serve as a non-immune prebleed sample.

Unconjugated or conjugated peptide is used to prepare the primary immunogen by emulsifying 0.5 ml of the peptide at a concentration of 2 mg/ml in PBS (pH 7.2) which contains 0.5 ml of complete Freund's adjuvant (CFA) (Difco, Detroit, MI). The immunogen is injected into several sites of the animal via subcutaneous, intraperitoneal, and/or intramuscular routes of administration. Four weeks following the primary immunization, a booster immunization is administered. The immunogen used for the booster immunization dose is prepared by emulsifying 0.5 ml of the same unconjugated or conjugated peptide used for the primary immunogen, except that the peptide now is diluted to 1 mg/ml with 0.5 ml of incomplete Freund's adjuvant (IFA) (Difco, Detroit, MI). Again, the booster dose is administered into several sites and can utilize subcutaneous, intraperitoneal and intramuscular types of injections. The animal is bled (5 ml) two weeks after the booster immunization and the serum is tested for immunoreactivity to the peptide, as described below. The booster and bleed schedule is repeated at 4 week intervals until an adequate titer is obtained. The titer or concentration of antiserum is determined by microtiter EIA as described in Example 18, below. An antibody titer of 1:500 or greater is considered an adequate titer for further use and study.

B. Production of Monoclonal Antibody.

1. Immunization Protocol. Mice are immunized using immunogens prepared as described hereinabove, except that the amount of the unconjugated or conjugated peptide for monoclonal antibody production in mice is one-tenth the amount used to produce polyclonal antisera in rabbits. Thus, the primary immunogen consists of 100 μg of unconjugated or conjugated peptide in 0.1 ml of CFA emulsion; while the immunogen used for booster immunizations consists of 50 μg of unconjugated or conjugated peptide in 0.1 ml of IFA. Hybridomas for the generation of monoclonal antibodies are prepared and screened using standard techniques. The methods used for monoclonal antibody development follow procedures known in the art such as those detailed in Kohler and Milstein, Nature 256:494 (1975) and reviewed in J.G.R. Hurrel, ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL (1982). Another method of monoclonal antibody development which is based on the Kohler and Milstein method is that of L.T. Mimms et al., Virology 176:604-619 (1990).

The immunization regimen (per mouse) consists of a primary immunization with additional booster immunizations. The primary immunogen used for the primary immunization consists of 100 μg of unconjugated or conjugated peptide in 50 μl of PBS (pH 7.2) previously emulsified in 50 μl of CFA. Booster immunizations performed at approximately two weeks and four weeks post primary immunization consist of 50 μg of unconjugated or conjugated peptide in 50 μl of PBS (pH 7.2) emulsified with 50 μl IFA. A total of 100 μl of this immunogen is inoculated intraperitoneally and subcutaneously into each mouse. Individual mice are screened for immune response by microtiter plate enzyme immunoassay (EIA) as described in Example 18 approximately four weeks after the third immunization. Mice are inoculated either intravenously, intrasplenically or intraperitoneally with 50 μg of unconjugated or conjugated peptide in PBS (pH 7.2) approximately fifteen weeks after the third immunization..

Three days after this intravenous boost, splenocytes are fused with, for example, Sp2/0-Ag14 myeloma cells (Milstein Laboratories, England) using the polyethylene glycol (PEG) method. The fusions are cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal calf serum (FCS), plus 1% hypoxanthine, aminopterin and thymidine (HAT). Bulk cultures are screened by microtiter plate EIA following the protocol in Example 18. Clones reactive with the peptide used an immunogen and non-reactive with other peptides (i.e., peptides of a protein not used as the immunogen) are selected for final expansion. Clones thus selected are expanded, aliquoted and frozen in IMDM containing 10% FCS and 10% dimethyl-sulfoxide.

2. Production of Ascites Fluid Containing Monoclonal Antibodies. Frozen hybridoma cells prepared as described hereinabove are thawed and placed into expansion culture. Viable hybridoma cells are inoculated intraperitoneally into Pristane treated mice. Ascitic fluid is removed from the mice, pooled, filtered through a 0.2 μ filter and subjected to an immunoglobulin class G (IgG) analysis to determine the volume of the Protein A column required for the purification.

3. Purification of Monoclonal Antibodies From Ascites Fluid. Briefly, filtered and thawed ascites fluid is mixed with an equal volume of Protein A sepharose binding buffer (1.5 M glycine, 3.0 M NaCl, pH 8.9) and refiltered through a 0.2 μ filter. The volume of the Protein A column is determined by the quantity of IgG present in the ascites fluid. The eluate then is dialyzed against PBS (pH 7.2) overnight at 2-8°C. The dialyzed monoclonal antibody is sterile filtered and dispensed in aliquots. The immunoreactivity of the purified monoclonal antibody is confirmed by determining its ability to specifically bind to the peptide used as the immunogen by use of the EIA microtiter plate assay procedure of

Example 18. The specificity of the purified monoclonal antibody is confirmed by determining its lack of binding to irrelevant peptides such as peptides not used as the immunogen. The purified anti- monoclonal thus prepared and characterized is placed at either 2-8°C for short term storage or at -80°C for long term storage.

4. Further Characterization of Monoclonal Antibody. The isotype and subtype of the monoclonal antibody produced as described hereinabove can be determined using commercially available kits (available from Amersham. Inc., Arlington Heights, IL). Stability testing also can be performed on the monoclonal antibody by placing an aliquot of the monoclonal antibody in continuous storage at 2-8°C and assaying optical density (OD) readings throughout the course of a given period of time.

C. Use of Recombinant Proteins as Immunogens. It is within the scope of the present invention that recombinant proteins made as described herein can be utilized as immunogens in the production of polyclonal and monoclonal antibodies, with corresponding changes in reagents and techniques known to those skilled in the art.

Example 16: Purification of Serum Antibodies Which Specifically Bind to Synthetic Peptides

Immune sera, obtained, for example, as described hereinabove in Examples 14 and/or 15, is affinity purified using immobilized synthetic peptides prepared as described in Example 11, or recombinant proteins prepared as described in Example 12. An IgG fraction of the antiserum is obtained by passing the diluted, crude antiserum over a Protein A column (Affi-Gel protein A, Bio-Rad, Hercules, CA). Elution with a buffer (Binding Buffer, supplied by the manufacturer) removes substantially all proteins that are not immunoglobulins. Elution with 0.1M buffered glycine (pH 3) gives an immunoglobulin preparation that is substantially free of albumin and other serum proteins.

Immunoaffinity chromatography is performed to obtain a preparation with a higher fraction of specific antigen-binding antibody. The peptide used to raise the antiserum is immobilized on a chromatography resin, and the specific antibodies directed against its epitopes are adsorbed to the resin. After washing away non-binding components, the specific antibodies are eluted with 0.1 M glycine buffer, pH 2.3. Antibody fractions are immediately neutralized with 1.0M Tris buffer (pH 8.0) to preserve immunoreactivity. The chromatography resin chosen depends on the reactive groups present in the peptide. If the peptide has an amino group, a resin such as Affi-Gel 10 or Affi-Gel 15 is used (Bio-Rad, Hercules, CA). If coupling through a carboxy group on the peptide is desired, Affi-Gel 102 can be

used (Bio-Rad, Hercules, CA). If the peptide has a free sulfhydryl group, an organomercurial resin such as Affi-Gel 501 can be used (Bio-Rad, Hercules, CA).

Alternatively, spleens can be harvested and used in the production of hybridomas to produce monoclonal antibodies following routine methods known in the art as described hereinabove.

Example 17: Western Blotting of Tissue Samples

Protein extracts are prepared by homogenizing tissue samples in 0.1M Tris-HCl (pH 7.5), 15% (w/v) glycerol, 0.2mM EDTA, 1.0 mM 1,4-dithiothreitol, 10 µg/ml leupeptin and 1.0 mM phenylmethylsulfonylfluoride [Kain et al., Biotechniques,17:982 (1994)]. Following homogenization, the homogenates are centrifuged at 4°C for 5 minutes to separate supernatant from debris. Debris is reextracted by homogenization with a buffer that is similar to above also contains 0.1M Tricine and 0.1% SDS. The supernatant from the second extraction is used for Western blotting. For protein quantitation, 2-5 µl of supernatant are added to 1.5 ml of Coomassie Protein Reagent (Pierce, Rockford, IL), and the resulting absorbance at 595 nm is measured.

For SDS-PAGE, samples are adjusted to desired protein concentration with Tricine Buffer (Novex, San Diego,CA), mixed with an equal volume of 2X Tricine sample buffer (Novex, San Diego,CA), and heated for 5 minutes at 100°C in a thermal cycler. Samples are then applied to a Novex 10-20% Precast Tricine Gel for electrophoresis. Following electrophoresis, samples are transferred from the gels to nitrocellulose membranes in Novex Tris-Glycine Transfer buffer. Membranes are then probed with specific anti-peptide antibodies using the reagents and procedures provided in the Western Lights or Western Lights Plus (Tropix, Bedford, MA) chemiluminesence detection kits. Chemiluminesent bands are visualized by exposing the developed membranes to Hyperfilm ECL (Amersham, Arlington Heights, IL).

Competition experiments are performed in an analogous manner as above, with the following exception; the primary antibodies (anti-peptide polyclonal antisera) are pre-incubated for 30 minutes at room temperature with varying concentrations of peptide immunogen prior to exposure to the nitrocellulose filter. Development of the Western is performed as above.

After visualization of the bands on film, the bands can also be visualized directly on the membranes by the addition and development of a chromogenic substrate such as 5-bromo-4-chloro-3-indolyl phosphate (BCIP). This chromogenic solution contains 0.016% BCIP in a solution containing 100 mM NaCl, 5 mM $MgCl_2$ and 100 mM Tris-HCl (pH 9.5). The filter is incubated in the

solution at room temperature until the bands develop to the desired intensity. Molecular mass determination is made based upon the mobility of pre-stained molecular weight standards (Novex, San Diego, CA) or biotinylated molecular weight standards (Tropix, Bedford, MA).

Example 18: EIA Microtiter Plate Assay

The immunoreactivity of antiserum preferably obtained from rabbits or mice as described in Examples 14, 15, or 16 is determined by means of a microtiter plate EIA, as follows. Briefly, synthetic peptides prepared as described in Example 10, are dissolved in 50 mM carbonate buffer (pH 9.6) to a final concentration of 2 μg/ml. Next, 100 μl of the peptide or protein solution are placed in each well of an Immulon 2® microtiter plate (Dynex Technologies, Chantilly, VA). The plate is incubated overnight at room temperature and then washed four times with deionized water. The wells are blocked by adding 125 μl of a suitable protein blocking agent, such as Superblock® (Pierce Chemical Company, Rockford, IL), in phosphate buffered saline (PBS, pH 7.4) to each well and then immediately discarding the solution. This blocking procedure is performed three times. Antiserum obtained from immunized rabbits or mice prepared as previously described is diluted in a protein blocking agent (e.g., a 3% Superblock® solution) in PBS containing 0.05% Tween-20® (monolaurate polyoxyethylene ether) (Sigma Chemical Company, St. Louis, MO) and 0.05% sodium azide at dilutions of 1:100, 1:500, 1:2500, 1:12,500, and 1:62,500 and placed in each well of the coated microtiter plate. The wells then are incubated for three hours at room temperature. Each well is washed four times with deionized water. One hundred μl of alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG antiserum (Southern Biotech, Birmingham, AB), diluted 1:2000 in 3% Superblock® solution in phosphate buffered saline containing 0.05% Tween 20® and 0.05% sodium azide, is added to each well . The wells are incubated for two hours at room temperature. Next, each well is washed four times with deionized water. One hundred microliters (100 μl) of paranitrophenyl phosphate substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) then are added to each well. The wells are incubated for thirty minutes at room temperature. The absorbance at 405 nm is read of each well. Positive reactions are identified by an increase in absorbance at 405 nm in the test well above that absorbance given by a non-immune serum (negative control). A positive reaction is indicative of the presence of detectable antibodies. Titers of the anti-peptide antisera are calculated from the previously described dilutions of antisera and defined as the calculated dilution, where A_{405nm}=0.5 OD.

In addition to titers, apparent affinities [K_d(app)] may also be determined for some of the anti-peptide antisera. EIA microtiter plate assay results can be used to derive the apparent dissociation constants (K_d) based on an analog of the Michaelis-Menten equation [V. Van Heyningen, Methods in Enzymology, Vol.121, p. 472 (1986) and further described in X. Qiu, et al., Journal of Immunology, Vol. 156, p. 3350 (1996)]:

$$[\text{Ag-Ab}] = [\text{Ag-Ab}]_{max} \quad X \quad \frac{[Ab]}{[Ab] = K_d}$$

Where [Ag-Ab] is the antigen-antibody complex concentration, $[\text{Ag-Ab}]_{max}$ is the maximum complex concentration, [Ab] is the antibody concentration, and K_d is the dissociation constant. During the curve fitting, the [Ag-Ab] is replaced with the background subtracted value of the OD_{405nm} at the given concentration of Ab. Both K_d and $[OD_{405nm}]_{max}$, which corresponds to the $[\text{Ag-Ab}]_{max}$, are treated as fitted parameters. A software program, for example, Origin™, can be used for the curve fitting.

Example 19: Coating of Solid Phase Particles

A. Coating of Microparticles with Antibodies Which Specifically Bind to Antigen. Affinity purified antibodies which specifically bind to protein (see Example 15) are coated onto microparticles of polystyrene, carboxylated polystyrene, polymethylacrylate or similar particles having a radius in the range of about 0.1 to 20 μm. Microparticles may be either passively or actively coated. One coating method comprises coating EDAC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich Chemical Co., Milwaukee, WI) activated carboxylated latex microparticles with antibodies which specifically bind to protein, as follows. Briefly, a final 0.375% solid suspension of resin washed carboxylated latex microparticles (available from Bangs Laboratories, Carmel, IN or Serodyn, Indianapolis, IN) are mixed in a solution containing 50 mM MES buffer, pH 4.0 and 150 mg/l of affinity purified anti-protein antibody (see Example 14) for 15 min in an appropriate container. EDAC coupling agent is added to a final concentration of 5.5 μg/ml to the mixture and mixed for 2.5 hr at room temperature.

The microparticles then are washed with 8 volumes of a Tween 20®/sodium phosphate wash buffer (pH 7.2) by tangential flow filtration using a 0.2 μm Microgon Filtration module. Washed microparticles are stored in an appropriate

buffer which usually contains a dilute surfactant and irrelevant protein as a blocking agent, until needed.

B. Coating of 1/4 Inch Beads. Antibodies which specifically bind to the specific antigen also may be coated on the surface of 1/4 inch polystyrene beads by routine methods known in the art (Snitman et al., US Patent 5,273,882) and used in competitive binding or EIA sandwich assays.

Polystyrene beads first are cleaned by ultrasonicating them for about 15 seconds in 10 mM $NaHCO_3$ buffer at pH 8.0. The beads then are washed in deionized water until all fines are removed. Beads then are immersed in an antibody solution in 10 mM carbonate buffer, pH 8 to 9.5. The antibody solution can be as dilute as 1 µg/ml in the case of high affinity monoclonal antibodies or as concentrated as about 500 µg/ml for polyclonal antibodies which have not been affinity purified. Beads are coated for at least 12 hours at room temperature, and then they are washed with deionized water. Beads may be air dried or stored wet (in PBS, pH 7.4). They also may be overcoated with protein stabilizers (such as sucrose) or protein blocking agents used as non-specific binding blockers (such as irrelevant proteins, Carnation skim milk, Superblock®, or the like).

Example 20: Microparticle Enzyme Immunoassay (MEIA)

Specific antigens are detected in patient test samples by performing a standard antigen competition EIA or antibody sandwich EIA and utilizing a solid phase such as microparticles (MEIA). The assay can be performed on an automated analyzer such as the IMx® Analyzer (Abbott Laboratories, Abbott Park, IL).

A. Antibody Sandwich EIA. Briefly, samples suspected of containing antigen are incubated in the presence of specific antibody-coated microparticles (prepared as described in Example 19) in order to form antigen/antibody complexes. The microparticles then are washed and an indicator reagent comprising an antibody conjugated to a signal generating compound (i.e., enzymes such as alkaline phosphatase or horseradish peroxide) is added to the antigen/antibody complexes or the microparticles and incubated. The microparticles are washed and the bound antibody/antigen/antibody complexes are detected by adding a substrate (e.g., 4-methyl umbelliferyl phosphate (MUP), or OPD/peroxide, respectively), that reacts with the signal generating compound to generate a measurable signal. An elevated signal in the test sample, compared to the signal generated by a negative control, detects the presence of antigen. The presence of antigen in the test sample is indicative of a diagnosis of bladder cancer.

B. Competitive Binding Assay. The competitive binding assay uses a peptide or protein that generates a measurable signal when the labeled peptide is contacted with an anti-peptide antibody coated microparticle. This assay can be performed on the IMx® Analyzer (available from Abbott Laboratories, Abbott Park, IL). The labeled peptide is added to the specific antibody-coated microparticles (prepared as described in Example 17) in the presence of a test sample suspected of containing the antigen, and incubated for a time and under conditions sufficient to form labeled peptide (or labeled protein) / bound antibody complexes and/or patient antigen / bound antibody complexes. The antigen in the test sample competes with the labeled peptide (or protein) for binding sites on the microparticle, specific antigen in the test sample results in a lowered binding of labeled peptide and antibody coated microparticles in the assay since antigen in the test sample and the peptide or protein compete for antibody binding sites. A lowered signal (compared to a control) indicates the presence of specific antigen in the test sample. The presence of antigen derived from a polypeptide encoded by the polynucleotides comprising SEQUENCE ID NOS 1-13, and fragments or complements, thereof in bladder tissue suggests the diagnosis of bladder cancer.

The polynucleotides and the proteins encoded thereby which are provided and discussed hereinabove are useful as markers of urinary tract cancer. Tests based upon the appearance of this marker in a test sample such as exfoliated cells in urine or micrometastases in blood can provide low cost, non-invasive, diagnostic information to aid the physician to make a diagnosis of cancer, to help select a therapy protocol, or to monitor the success of a chosen therapy. This marker may appear in readily accessible body fluids such as blood or urine as antigens derived from the diseased tissue which are detectable by immunological methods.

Example 21: Immunohistochemical Detection of Protein

Antiserum against a synthetic peptide described in Example 15, above, is used to immunohistochemically stain a variety of normal and diseased tissues using standard proceedures. Briefly, frozen blocks of tissue are cut into 6 micron sections, and placed on microscope slides. After fixation in cold acetone, the sections are dried at room temperature, then washed with phosphate buffered saline and blocked. The slides are incubated with the antiserum against a synthetic peptide derived from the peptide sequence at a dilution of 1:500, washed, incubated with biotinylated goat anti-rabbit antibody, washed again, and incubated with avidin labeled with horseradish peroxidase. After a final wash, the slides are incubated with 3-amino-9-ethylcarbazole substrate which gives a red stain. The

slides are counterstained with hematoxylin, mounted, and examined under a microscope by a pathologist.

We Claim:

1. A method of detecting the presence of urinary tract disease in an individual, said method comprising:

(a) providing a test sample from the individual and contacting the test sample with at least one keratin/cytokeratin, CAS, or mat-8-specific polynucleotide or complement thereof, wherein said keratin/cytokeratin, CAS, or mat-8-specific polynucleotide has at least 50% identity with a polynucleotide selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof; and

(b) detecting the presence of target keratin/cytokeratin, CAS, or mat-8 polynucleotides in the test sample which bind to said keratin/cytokeratin, CAS, or mat-8-specific polynucleotide as an indication of urinary tract disease in the individual.

2. The method of claim 1, wherein said target keratin/cytokeratin, CAS, or mat-8 polynucleotide is attached to a solid phase prior to performing step (a).

3. The method of claim 1, wherein said keratin/cytokeratin, CAS, or mat-8-specific polynucleotide is attached to a solid phase prior to performing step (a).

4. A method of detecting the presence of urinary tract disease in an individual, said method comprising:

(a) providing a test sample from the individual and performing reverse transcription on said sample using at least one primer in order to produce cDNA;

(b) amplifying the cDNA obtained from step (a) using keratin/cytokeratin, CAS, or mat-8 oligonucleotides as sense and antisense primers to obtain keratin/cytokeratin, CAS, or mat-8 amplicon; and

(c) detecting the presence of said keratin/cytokeratin, CAS, or mat-8 amplicon as an indication of urinary tract disease in the individual, wherein the keratin/cytokeratin, CAS, or mat-8 oligonucleotides utilized in steps (a) and (b) have at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof.

5. The method of claim 4, wherein said test sample is reacted with a solid phase prior to performing one of steps (a), (b), or (c).

6. The method of claim 4, wherein said detection step comprises utilizing a detectable label capable of generating a measurable signal.

7. A method of detecting the presence of urinary tract disease in an individual, said method comprising:

(a) providing a test sample from the individual and contacting the test sample with at least one keratin/cytokeratin, CAS, or mat-8 oligonucleotide as a sense primer and with at least one keratin/cytokeratin, CAS, or mat-8 oligonucleotide as an anti-sense primer and amplifying to obtain a first stage reaction product;

(b) contacting said first stage reaction product with at least one other keratin/cytokeratin, CAS, or mat-8 oligonucleotide to obtain a second stage reaction product, with the proviso that the other keratin/cytokeratin, CAS, or mat-8 oligonucleotide is located 3' to the keratin/cytokeratin, CAS, or mat-8 oligonucleotides utilized in step (a) and is complementary to said first stage reaction product; and

(c) detecting said second stage reaction product as an indication of urinary tract disease in the individual, wherein the keratin/cytokeratin, CAS, or mat-8 oligonucleotides utilized in steps (a) and (b) have at least 50% identity with a sequence selected from the group consisting SEQUENCE ID NOS 1 through13, and fragments or complements thereof.

8. The method of claim 7, wherein said test sample is reacted with a solid phase prior to performing one of steps (a), (b), or (c).

9. The method of claim 7, wherein said detection step comprises utilizing a detectable label capable of generating a measurable signal.

10. The method of claim 9, wherein said detectable label is reacted to a solid phase.

11. A test kit useful for detecting urinary tract disease, said test kit comprising a container containing at least one keratin/cytokeratin, CAS, or mat-8 polynucleotide having at least 50% identity with a sequence selected from the group consisting SEQUENCE ID NOS 1 through 13, and fragments or complements thereof.

12. A specific binding molecule which specifically binds to a keratin/cytokeratin, CAS, or mat-8 epitope, wherein said keratin/cytokeratin, CAS, or mat-8 epitope is derived from a polypeptide encoded by a nucleic acid sequence having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof.

13. The specific binding molecule of claim 12, wherein said molecule is an antibody molecule.

14. A test kit useful for detecting urinary tract disease, said kit comprising a container containing a keratin/cytokeratin, CAS, or mat-8 polypeptide encoded by a nucleic acid sequence having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof.

15. The test kit of claim 14, wherein said keratin/cytokeratin, CAS, or mat-8 polypeptide is attached to a solid phase.

16. A test kit useful for detecting urinary tract disease in a test sample, said kit comprising a container containing a specific binding molecule which specifically binds to an epitope from a keratin/cytokeratin, CAS, or mat-8 polypeptide encoded by a nucleic acid sequence having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof.

17. The kit of claim 16, wherein said specific binding molecule is attached to a solid phase.

18. A method of detecting the presence of antigen indicative of urinary tract disease in an individual, said method comprising:

(a) providing a test sample from the individual and contacting the test sample with a specific binding molecule which specifically binds to an epitope of a keratin/cytokeratin, CAS, or mat-8 antigen selected from the group consisting of a polypeptide encoded by a nucleic acid sequence having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof, wherein said contacting is performed for a time and under conditions sufficient for the formation of binding molecule/antigen complexes; and

(b) detecting the presence of said complexes as an indication of urinary tract disease in the individual.

19. The method of claim 18, wherein said specific binding molecule is an antibody molecule or a fragment thereof.

20. The method of claim 18, wherein said specific binding molecule is attached to a solid phase.

21. A method of detecting the presence of antibody indicative of urinary tract disease in an individual, said method comprising:

(a) providing a test sample from the individual and contacting the test sample with a keratin/cytokeratin, CAS, or mat-8 polypeptide, wherein said keratin/cytokeratin, CAS, or mat-8 polypeptide contains at least one keratin/cytokeratin, CAS, or mat-8 epitope of a polypeptide encoded by a nucleic acid molecule having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof, and further wherein said contacting is performed for a time and under conditions sufficient to allow antigen/antibody complexes to form; and

(b) detecting the presence of said complexes as an indication of urinary tract disease in the individual.

22. The method of claim 21, wherein said keratin/cytokeratin, CAS, or mat-8 polypeptide is attached to a solid phase.

23. A method for producing antibodies which specifically bind to keratin/cytokeratin, CAS, or mat-8 antigen, comprising administering to an individual an isolated immunogenic polypeptide or fragment thereof in an amount sufficient to elicit an immune response, wherein said immunogenic polypeptide comprises at least one keratin/cytokeratin, CAS, or mat-8 epitope derived from a polypeptide encoded by a nucleic acid molecule having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1 through 13, and fragments or complements thereof.

24. A method for producing antibodies which specifically bind to keratin/cytokeratin, CAS, or mat-8 antigen, comprising administering to an individual a plasmid comprising a sequence which encodes at least one keratin/cytokeratin, CAS, or mat-8 epitope derived from a polypeptide encoded by

a nucleic acid molecule having at least 50% identity with a sequence selected from the group consisting of SEQUENCE ID NOS 1through 13, and fragments or complements thereof

25. The test kit of claim 11 further comprising a container with tools useful for collection of said sample, wherein the tools are selected from the group consisting of lancets, absorbent paper, cloth, swabs and cups.

26. The test kit of claim 14 further comprising a container with tools useful for collection of said sample, wherein the tools are selected from the group consisting of lancets, absorbent paper, cloth, swabs and cups.

27. The test kit of claim 16 further comprising a container with tools useful for collection of said sample, wherein the tools are selected from the group consisting of lancets, absorbent paper, cloth, swabs and cups.

28. The test kit of claim 16, wherein said specific binding molecule is an antibody or fragment thereof.

29. The method of claim 1, wherein the presence of said target keratin/cytokeratin, CAS, or mat-8 polynucleotide in the test sample is indicative of urinary tract cancer.

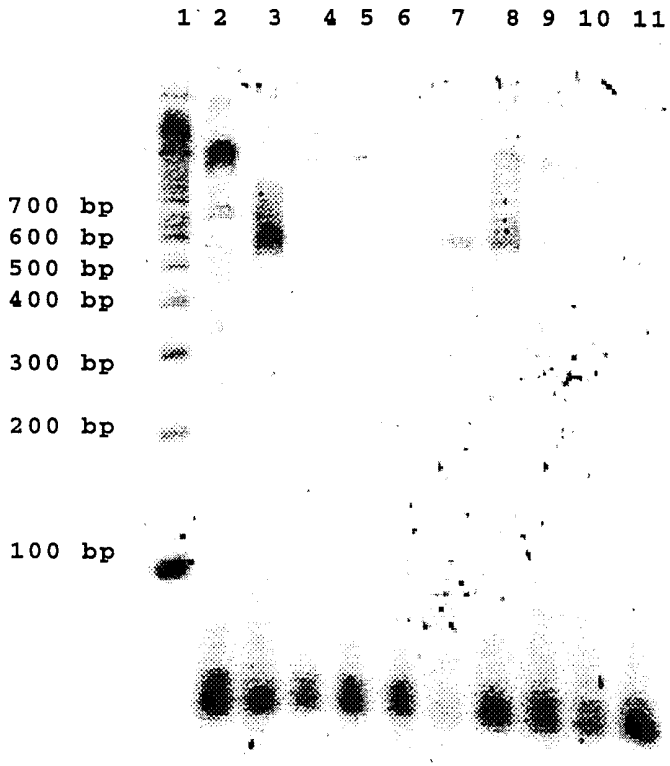
30. The method of claim 4, wherein the presence of said amplicon is indicative of prostrate disease.

31. The method of claim 7, wherein the presence of said second stage reaction product is indicative of urinary tract cancer.

32. The method of claim 18, wherein detection of said complexes is indicative of urinary tract cancer.

33. The method of claim 21, wherein detection of said complexes is indicative of urinary tract cancer.

CK5 Figure 1



LANE	SAMPLE
1	100 bp Molecular Weight Marker
2	Placental DNA
3	Bladder cancer
4	Normal bladder
5	Normal bladder
6	Normal bladder
7	Bladder cancer
8	Prostate BPH
9	Normal colon
10	Breast cancer
11	Colon cancer

SEQUENCE LISTING

<110> Abbott Laboratories

<120> Reagents and Methods Useful for Detecting Diseases of the Urinary Tract

<130> 6180.PC.01

<160> 17

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 1048
<212> DNA
<213> Homo sapiens

<400> 1
attctcacag ccacagtgga caatgccaat gtccttctgc agattgacaa tgcccgtctg 60
gccgcggatg acttccgcac caagtatgag acagagttga acctgcgcat gagtgtggaa 120
gccgacatca atggcctgcg cagggtgctg gacgaactga ccctggccag agctgacctg 180
gagatgcaga ttgagagcct gaaggaggag ctggcctacc tgaagaagaa ccacgaggag 240
gagatgaatg ccctgagagg ccaggtgggt ggagatgtca atgtggagat ggacgctgca 300
cctggcgtgg acctgagccg cattctgaac gagatgcgtg accagtatga gaagatggca 360
gagaagaacc gcaaggatgc cgaggaatgg ttcttcacca agacagagga gctgaaccgc 420
gaggtggcca ccaacagcga gctggtgcag agcggcaaga gcgagatctc ggagctccgg 480
cgcaccatgc agaacctgga gattgagctg cagtcccagc tcagcatgaa agcatccctg 540
gagaacagcc tggaggagac caaaggtcgc tactgcatgc agctggccca gatccaggag 600
atgattggca gcgtggagga gcagctggcc cagctccgct gcgagatgga gcagcagaac 660
caggagtaca agatcctgct ggacgtgaag acgcggctgg agcaggagat cgccacctac 720
cgccgcctgc tggagggcga ggacgcccac ctctcctcct cccagttctc ctctggatcg 780
cagtcatcca gagatgtgac ctcctccagc cgccaaatcc gcaccaaggt catggatgtg 840
cacgatggca aggtggtgtc cacccacgag caggtccttc gcaccaagaa ctgaggctgc 900
ccagccccgc tcaggcctag gaggcccccc gtgtggacac agatcccact ggaagatccc 960
ctctcctgcc caagcacttc acagctggac cctgcttcac cctcaccccc tcctggcaat 1020
caatacagct tcattatctg agttgcat 1048

<210> 2
<211> 2235
<212> DNA
<213> Homo sapiens

<400> 2
acagagccac cttctgcgtc ctgctgagct ctgttctctc cagcacctcc caacccacta 60
gtgcctggtt ctcttgctcc accaggaaca agccaccatg tctcgccagt caagtgtgtc 120
cttccggagc gggggcagtc gtagcttcag caccgcctct gccatcaccc cgtctgtctc 180
ccgcaccagc ttcacctccg tgtcccggtc cgggggtggc ggtggtggtg gcttcggcag 240
ggtcagcctt gcgggtgctt gtggagtggg tggctatggc agccggagcc tctacaacct 300
gggggggctcc aagaggatat ccatcagcac tagaggaggc agcttcagga accggtttgg 360
tgctggtgct ggaggcggct atggctttgg aggtggtgcc ggtagtggat ttggtttcgg 420
cggtggagct ggtggtggct ttgggctcgg tggcggagct ggctttggag gtggcttcgg 480
tggccctggc tttcctgtct gccctcctgg aggtatccaa gaggtcactg tcaaccagag 540
tctcctgact cccctcaacc tgcaaatcga ccccagcatc cagagggtga ggaccgagga 600
gcgcgagcag atcaagaccc tcaacaataa gtttgcctcc ttcatcgaca aggtgcggtt 660
cctggagcag cagaacaagg ttctggacac caagtggacc ctgctgcagg agcagggcac 720
caagactgtg aggcagaacc tggagccgtt gttcgagcag tacatcaaca acctcaggag 780
gcagctggac agcatcgtgg gggaacgggg ccgcctggac tcagagctga gaaacatgca 840
ggacctggtg gaagacttca agaacaagta tgaggatgaa atcaacaagc gtaccactgc 900
tgagaatgag tttgtgatgc tgaagaagga tgtagatgct gcctacatga acaaggtgga 960
gctggaggcc aaggttgatg cactgatgga tgagattaac ttcatgaaga tgttctttga 1020
tgcggagctg tcccagatgc agacgcatgt ctctgacacc tcagtggtcc tctccatgga 1080
caacaaccgc aacctggacc tggatagcat catcgctgag gtcaaggccc agtatgagga 1140
gattgccaac cgcagccgga cagaagccga gtcctggtat cagaccaagt atgaggagct 1200
gcagcagaca gctggccggc atggcgatga cctccgcaac accaagcatg agatcacaga 1260
gatgaaccgg atgatccaga ggctgagagc cgagattgac aatgtcaaga aacagtgcgc 1320
caatctgcag aacgccattg cggatgccga gcagcgtggg gagctggccc tcaaggatgc 1380

caggaacaag ctggccgagc tggaggaggc cctgcagaag gccaagcagg acatggcccg 1440
gctgctgcgt gagtaccagg agctcatgaa caccaagctg gccctggacg tggagatcgc 1500
cacttaccgc aagctgctgg agggcgagga atgcagactc agtggagaag gagttggacc 1560
agtcaacatc tctgttgtca caagcagtgt ttcctctgga tatggcagtg gcagtggcta 1620
tggcggtggc ctcggtggag gtcttggcgg cggcctcggt ggaggtcttg ccggaggtag 1680
cagtggaagc tactactcca gcagcagtgg gggtgtcggc ctaggtggtg ggctcagtgt 1740
ggggggctct ggcttcagtg caagcagtgg ccgagggctg ggggtgggct ttggcagtgg 1800
cgggggtagc agctccagcg tcaaatttgt ctccaccacc tcctcctccc ggaagagctt 1860
caagagctaa gaacctgctg caagtcactg ccttccaagt gcagcaaccc agcccatgga 1920
gattgcctct tctaggcagt tgctcaagcc atgttttatc cttttctgga gagtagtcta 1980
gaccaagcca attgcagaac cacattcttt ggttcccagg agagccccat tcccagcccc 2040
tggtctcccg tgccgcagtt ctatattctg cttcaaatca gccttcaggt ttcccacagc 2100
atggcccctg ctgacacgag aacccaaagt tttcccaaat ctaaatcatc aaaacagaat 2160
ccccacccca atcccaaatt ttgttttggt tctaactacc tccagaatgt gttcaataaa 2220
atgcttttat aatat 2235

<210> 3
<211> 1828
<212> DNA
<213> Homo sapiens

<400> 3
ggatccccac aactgctccc caagacagcc caggatggca tcactgagct ctctttcagc 60
caaggctgtc actgtggggc agggagttct tctgaagggc tgactcactg cctggggacg 120
cagttgccac aaagccacct gtgccaaggc ccgactggcc ccgagcggtc caggaaaggg 180
agcctgattc cccacgccta gcctgagcat cacaagctgc atttctgtgt tttctctggc 240
cccacacccc caaagctggt ggaactctag ccggcacaca gcagagttga tcctgggcta 300
ataatccaga gtgaggagtt ggacgggacc gggagtgatg aaatccagag gggaacctgg 360
agtaccagcc agttagaggg ccccgccttc cccagctgct ataaaggtct ctggggttgg 420
aggcagccac agcacgctct cagccttcct gagcaccttt ccttctttca gccaactgct 480
cactcgctga cctccctcct tggcaccatg accacctgca gccgccagtt cacctcctcc 540
agctccatga agggctcctg cggcatcgga ggcggcatcg gggcgggctc cagccgcatc 600
tcctccgtcc tggccggggc ctcctgccct gccagcacct acgggggcgc ctctgtctcc 660
tctcgcttct cctctggggg agcctgcggg ctgggggcgg gctatggcgg tggcttcagc 720
agcagcagca gctttggtag tggcttcggg ggaggatatg gtggtggcct tggtgctggc 780
ttcggtggtg gcttgggtgc tggctttggt ggtggttttg ctggtggtga tgggcttctg 840
gtgggcagtg agaaggtgac catgcagaac ctcaatgacc gcctggcctc ctacctggac 900
aaggtgcgtg ctctggagga ggccaacgcc gacctggaag tgaagatccg tgactggtac 960
cagaggcagc ggcccagtga gatcaaagac tacagtccct acttcaagac catcgaggac 1020
ctgaggaaca agatcattgc ggccaccatt gagaatgcgc acgccctttt gcagattgac 1080
aatgccaggc tggcagccga tgacttcagg accaagtatg aggcacgaac tggcctgcgg 1140
cagactgtgg aggccgacgt caatggcctg cgccgggtgt tggatgagct gaccctggcc 1200
aggactgacc tggagatgca gatcgaaggc ctgaaggagg agctggccta cctgaggaag 1260
aaccacgagg aggagatgct tgctctgaga ggtcagaccg gcggagatgt gaacgtggag 1320
atggatgctg cacctggcgt ggacctgagc cgcatcctga atgagatgcg tgaccagtac 1380
gagcagatgg cagagaaaaa ccgcagagac gctgagacct ggttcctgag caagaccgag 1440
gagctgaaca aagaagtggc ctccaacagc gaactggtac agagcagccg cagtgaggtg 1500
acggagctcc ggagggtgct ccagggcctg gagattgagc tgcagtccca gctcaggatg 1560
aaagcatccc tggagaacag cctggaggag accaaaggcc gctactgcat gcagctgtcc 1620
cagatccagg gactgattgg cagtgtggag gagcagctgg cccagctacg ctgtgagatg 1680
gagcagcaga gccaggagta ccagatcttg ctggatgtga agacgcggct ggagcaggag 1740
attgccacct accgccgcct gctggagggc gaggatgccc acctttcctc ccagcaagca 1800
tctggccaat cctattcttc ccgcgagg 1828

<210> 4
<211> 1435
<212> DNA
<213> Homo sapiens

<400> 4
acaacttggg gcccctctcc tctccagccc ttctcctgtg tgcctgcctc ctgccgccgc 60
caccatgacc acctccatcc gccagttcac ctcctccagc tccatcaagg gctcctccgg 120
cctgggggggc ggctcgtccc gcacctcctg ccggctgtct ggcggcctgg gtgccggctc 180
ctgcaggctg ggatctgctg gcggcctggg cagcaccctc gggggtagca gctactccag 240
ctgctacagc tttggctctg gtggtggcta tggcagcagc tttgggggtg ttgatgggct 300
gctggctgga ggtgagaagg ccaccatgca gaacctcaat gaccgcctgg cctcctacct 360
ggacaaggtg cgtgccctgg aggaggccaa cactgagctg gaggtgaaga tccgtgactg 420

gtaccagagg caggccccgg ggcccgcccg tgactacagc cagtactaca ggacaattga 480
ggagctgcag aacaaggttt gagacagagc aggccctgcg cctgagtgtg gaggccgaca 540
tcaatggcct gcgcagggtg ctggatgagc tgaccctggc cagagccgac ctggagatgc 600
agattgagaa cctcaaggag gagctggcct acctgaagaa gaaccacgag gaggagatga 660
acgccctgcg aggccaggtg ggtggtgaga tcaatgtgga gatggacgct gccccaggcg 720
tggacctgag ccgcatcctc aacgagatgc gtgaccagta tgagaagatg gcagagaaga 780
accgcaagga tgccgaggat tggttcttca gcaagacaga ggaactgaac cgcgaggtgg 840
ccaccaacag tgagctggtg cagagtggca agagtgagat ctcggagctc cggcgcacca 900
tgcaggcctt ggagatagag ctgcagtccc agctcagcat gaaagcatcc ctggagggca 960
acctggcgga gacagagaac cgctactgcg tgcagctgtc ccagatccag gggctgattg 1020
gcagcgtgga ggagcagctg gcccagcttc gctgcgagat ggagcagcag aaccaggaat 1080
acaaaatcct gctggatgtg aagacgcggc tggagcagga gattgccacc taccgccgcc 1140
tgctggaggg agaggatgcc cacctgactc agtacaagaa agaaccggtg accacccgtc 1200
aggtgcgtac cattgtggaa gaggtccagg atggcaaggt catctcctcc cgcgagcagg 1260
tccaccagac cacccgctga ggactcagct accccggccg gccacccagg aggcagggag 1320
gcagccgccc catctgcccc acagtctccg gcctctccag cctcagcccc ctgcttcagt 1380
cccttcccca tgcttccttg cctgatgaca ataaagcttg ttgactcagc tatga 1435

<210> 5
<211> 2283
<212> DNA
<213> Homo sapiens

<400> 5
ctccagcctc tcacactctc ctcagctctc tcatctcctg gaaccatggc cagcacatcc 60
accaccatca ggagccacag cagcagccgc cggggtttca gtgccaactc agccaggctc 120
cctggggtca gccgctctgg cttcagcagc atctccgtgt cccgctccag gggcagtggt 180
ggcctgggtg gtgcatgtgg aggagctggc tttggcagcc gcagtctgta tggcctgggg 240
ggctccaaga ggatctccat tggagggggc agctgtgcca tcagtggcgg ctatggcagc 300
agagccggag gcagctatgg ctttggtggc gccgggagtg gatttggttt cggtggtgga 360
gccggcattg gctttggtct gggtggtgga gccggccttg ctggtggctt tgggggccct 420
ggcttccctg tgtgcccccc tggaggcatc caagaggtca ctgtcaacca gagtctcctg 480
actcccctca acctgcaaat tgaccccgcc atccagcggg tgcgggccga ggagcgtgag 540
cagatcaaga ccctcaacaa caagtttgcc tccttcatcg acaaggtgcg gttcctagag 600
cagcagaaca aggttctgga caccaagtgg accctgctgc aggagcaggg caccaagact 660
gtgaggcaga acctggagcc gttgttcgag cagtacatca acaacctcag gaggcagctg 720
gacaacatcg tgggggaacg gggccgcctg gactcggagc tgagaaacat gcaggacctg 780
gtggaggacc tcaagaacaa atatgaggat gaaatcaaca agcgcacagc agcagagaat 840
gaatttgtga ctctgaagaa ggatgtggat gctgcctaca tgaacaaggt tgaactgcaa 900
gccaaggcag acactctcac agatgagatc aacttcctga gagccttgta tgatgcagag 960
ctgtcccaga tgcagaccca catctcagac acatccgtgg tgctatccat ggacaacaac 1020
cgcaacctgg acctggacag catcatcgct gaggtcaagg cccaatatga ggagattgct 1080
cagaggagcc gggctgaggc tgagtcctgg taccagacca agtacgagga gctgcaggtc 1140
acagcaggca gacatgggga cgacctgcgc aacaccaagc aggagattgc tgagatcaac 1200
cgcatgatcc agaggctgag atctgagatc gaccacgtca agaagcagtg tgccaacctg 1260
caggccgcca ttgctgatgc tgagcagcgt ggggagatgg ccctcaagga tgctaagaac 1320
aagctggaag ggctggagga tgccctgcag aaggccaagc aggacctggc ccggctgctg 1380
aaggagtacc aggagctgat gaatgtcaag ctggccctgg acgtggagat cgccacctac 1440
cgcaagctgc tggagggcga ggagtgcagg ctgaatggcg aaggcgttgg acaagtcaac 1500
atctctgtag tgcagtccac cgtctccagt ggctatggcg gtgccagtgg tgtcggcagt 1560
ggcttaggcc tgggtggagg aagcagctac tcctatggca gtggtcttgg cgttggaggt 1620
ggcttcagtt ccagcagtgg cagagccatt gggggtggcc tcagctctgt tggaggcggc 1680
agttccacca tcaagtacac caccacctcc tcctccagca ggaagagcta caagcactaa 1740
agtgcgtctg ctagctctcg gtcccacagt cctcaggccc ctctctggct gcagagccct 1800
ctcctcaggt tgcctgtcct ctcctggcct ccagtctccc ctgctgtccc aggtagagct 1860
ggggatgaat gcttagtgcc ctcacttctt ctctctctct ctataccatc tgagcaccca 1920
ttgctcacca tcagatcaac ctctgatttt acatcatgat gtaatcacca ctggagcttc 1980
actgttacta aattattaat ttcttgcctc cagtgttcta tctctgaggc tgagcattat 2040
aagaaaatga cctctgctcc ttttcattgc agaaaattgc caggggctta tttcagaaca 2100
acttccactt actttccact ggctctcaaa ctctctaact tataagtgtt gtgaaccccc 2160
acccaggcag tatccatgaa agcacaagtg actagtccta tgatgtacaa agcctgtatc 2220
tctgtgatga tttctgtgct cttcactctt tgcaattgct aaataaagca gatttataat 2280
aca 2283

<210> 6
<211> 1407
<212> DNA

<213> Homo sapiens

<400> 6
tcgtccgcaa agcctgagtc ctgtcctttc tctctccccg gacagcatga gcttcaccac 60
tcgctccacc ttctccacca actaccggtc cctgggctct gtccaggcgc ccagctacgg 120
cgcccggccg gtcagcagcg cggccagcgt ctatgcaggc gctgggggct ctggttcccg 180
gatctccgtg tcccgctcca ccagcttcag gggcggcatg gggtccgggg gcctggccac 240
cgggatagcc gggggtctgg caggaatggg aggcatccag aacgagaagg agaccatgca 300
aagcctgaac gaccgcctgg cctcttacct ggacagagtg aggagcctgg agaccgagaa 360
ccggaggctg gagagcaaaa tccgggagca cttggagaag aagggacccc aggtcagaga 420
ctggagccat tacttcaaga tcatcgagga cctgagggct cagatcttcg caaatactgt 480
ggacaatgcc cgcatcgttc tgcagattga caatgcccgt cttgctgctg atgactttag 540
agtcaagtat gagacagagc tggccatgcg ccagtctgtg gagaacgaca tccatgggct 600
ccgcaaggtc attgatgaca ccaatatcac acgactgcag ctggagacag agatcgaggc 660
tctcaaggag gagctgctct tcatgaagaa gaaccacgaa gaggaagtaa aaggcctaca 720
agcccagatt gccagctctg ggttgaccgt ggaggtagat gcccccaaat ctcaggacct 780
cgccaagatc atggcagaca tccgggccca atatgacgag ctggctcgga agaaccgaga 840
ggagctagac aagtactggt ctcagcagat tgaggagagc accacagtgg tcaccacaca 900
gtctgctgag gttggagctg ctgagacgac gctcacagag ctgagacgta cagtccagtc 960
cttggagatc gacctggact ccatgagaaa tctgaaggcc agcttggaga acagcctgag 1020
ggaggtggag gcccgctacg ccctacagat ggagcagctc aacgggatcc tgctgcacct 1080
tgagtcagag ctggcacaga cccgggcaga gggacagcgc caggcccagg agtatgaggc 1140
cctgctgaac atcaaggtca agctggaggc tgagatcgcc acctaccgcc gcctgctgga 1200
agatggcgag gactttaatc ttggtgatgc cttggacagc agcaactcca tgcaaaccat 1260
ccaaaagacc accacccgcc ggatagtgga tggcaaagtg gtgtctgaga ccaatgacac 1320
caaagttctg aggcattaag ccagcagaag cagggtaccc tttggggagc aggaggccaa 1380
taaaaagttc agagttcatt ggatgtc 1407

<210> 7
<211> 1493
<212> DNA
<213> Homo sapiens

<400> 7
gaattccggc gagtgcgcgc tcctcctcgc ccgccgctag gtccatcccg gcccagccac 60
catgtccatc cacttcagct ccccggtatt cacctcgcgc tcagccgcct tctcgggccg 120
cggcgccagg tgcgcctgag ctccgctcgc cccggcggcc ttggcagcag cagcctctac 180
ggcctcggcg cctcgcggcc gcgcgtggcc gtgcgctctg cctatggggg cccggtgggc 240
gccggcatcc gcgaggtcac cattaaccag agcctgctgg ccccgctgcg gctggacgcc 300
gacccctccc tccagcgggt gcgccaggag gagagcgagc agatcaaagc cctcaacaac 360
aagtttgcct ccttcatcga caaggtgggg tttctggagc agcagaacaa gctgctggag 420
accaagtgga cgctgctgca ggagcagaag tcggccaaga gcagccgcct cccagacatc 480
tttgaggccc agattgctgg ccttcggggt cagcttgagg cactgcaggt ggatgggggc 540
cgcctggagc aggggctgcg gacgatgcag gatgtggtgg aggacttcaa gaataagtac 600
gaagatgaaa ttaaccgccg cacagctgct gagaatgagt ttgtggtcct gaagaaggat 660
gtggatgctg cctacatgag caaggtggag ctggaggcca aggtggatgc cctgaatgat 720
gagatcaact tcctcaggac cctcaatgag acggagttga cagagctgca gtcccagatc 780
tccgacacat ctgtggtgct gtccatggac aacagtcgct ccctggacct ggacggcatc 840
atcgctgagg tcaaggcaca gtatgaggag atggccaaat gcagccgggc tgaggctgaa 900
gcctggtacc agaccaagtt tgagaccctc caggcccagg ctgggaagca tggggacgac 960
ctccggaata cccggaatga gatttcagag atgaaccggg ccatccagag gctgcaggct 1020
gagatcgaca acatcaagaa ccagcgtgcc aagttggagg ccgccattgc cgaggctgag 1080
gagtgtgggg agctggcgct caaggatgct cgtgccaagc aggaggagct ggaagccgcc 1140
ctgcagcggg ccaagcagga tatggcacgg cagctgcgtg agtaccagga actcatgagc 1200
gtgaagctgg ccctggacat cgagatcgcc acctaccgca agctgctgga gggcgaggag 1260
agccggttgg ctggagatgg agtgggagcc gtgaatatct ctgtgatgaa ttccactggt 1320
ggcagtagca gtggcggtgg cattgggctg accctcgggg gaaccatggg cagcaatgcc 1380
ctgagcttct ccagcagtgc gggtcctggg ctcctgaagg cttattccat ccggaccgca 1440
tccgccagtc gcaggagtgc ccgcgactga gccgcctccc accactccac tcc 1493

<210> 8
<211> 1724
<212> DNA
<213> Homo sapiens

<400> 8
ttcggcaatt cctacctcca ctcctgcctc caccatgtcc atcagggtga cccagaagtc 60

ctacaaggtg tccacctctg gcccccgggc cttcagcagc cgctcctaca cgagtgggcc 120
cggttcccgc atcagctcct cgagcttctc ccgagtgggc agcagcaact ttcgcggtgg 180
cctgggcggc ggctatggtg gggccagcgg catgggaggc atcaccgcag ttacggtcaa 240
ccagagcctg ctgagcccct tgtccctgga ggtggacccc aacatccagg ccgtgcgcac 300
ccaggagaag gagcagatca agaccctgaa caacaagttt gcctccttca tagacaaggt 360
acggttcctg gagcagcaga acaagatgct ggagaccaag tggagcctcc tgcagcagca 420
gaagacggct cgaagcaaca tggacaacat gttcgagagc tacatcaaca accttaggcg 480
gcagctggag actctgggcc aggagaagct gaagctggag gcggagcttg gcaacatgca 540
ggggctggtg gaggacttca agaacaagta tgaggatgag atcaataagc gtacagagat 600
ggagaacgaa tttgtcctca tcaagaagga tgtggatgaa gcatacatga acaaggtaga 660
gctggagtct cgcctggaag ggctgaccga cgagatcaac ttcctcaggc agctgtatga 720
agaggagatc cgggagctgc agtcccagat ctcggacaca tctgtggtgc tgtccatgga 780
caacagccgc tccctggaca tggagagcat cattgctgag gtcaaggcac agtacgagga 840
tattgccaac cgcagccggg ctgaggctga gagcatgtac cagatcaagt atgaggagct 900
gcagagcctg gctgggaagc acggggatga cctgcggcgc acaaagactg agatctcaga 960
gatgaaccgg aacatcagcc ggctccaggc tgagattgag ggcctcaaag gccagagggc 1020
ttccctggag gccgccattg cagatgccga gcagcgtgga gagctggcca ttaaggatgc 1080
caacgccaag ttgtccgagc tggaggccgc cctgcagcgg gccaagcagg acatggcccg 1140
gcagctgcgt gagtaccagg agctgatgaa cgtcaagctg gccctggaca tcgacatcgc 1200
cacctacagg aagctgctgg agggcgagga gagcccgctg gagtctggga tgcagaacat 1260
gagtattcat acgaagacca ccggcggcta tgcgggtggt ttgagctcgg cctatgggga 1320
cctcacagac cccggcctca gctacagcct gggctccagc tttggctctg gcgcgggctc 1380
cagctccttc agccgcacca gctcctccag ggccgtggtt gtgaagaaga tcgagacacg 1440
tgatgggaag ctggtgtctg agtcctctga cgtcctgccc aagtgaacag ctgcggcagc 1500
ccctcccagc ctacccctcc tgcgctgccc cagagcctgg gaaggaggcc gctatgcagg 1560
gtagcactgg gaacaggaga cccacctgag gctcagccct agccctcagc ccacctgggg 1620
agtttactac ctggggaccc cccttgccca tgcctccagc tacaaaacaa ttcaattgct 1680
ttttttttt ttggtcccaa aataaaacct cagctagctc tgcc 1724

<210> 9
<211> 1602
<212> DNA
<213> Homo sapiens

<400> 9
cgcggaccgg ggcggggcac ctctggaggg caggggcctc tggtctctgg gaggggaggg 60
aattgaccaa tggggagaga gcccatattt gctctcagga gcctgcaaat tcctcagggc 120
tcagatatcc gcccctgaca ccattcctcc cttccccccc ccaccggccg cgggcataaa 180
aggcgccagg tgagggcctc gccgctcctc ccgcgaatcg cagcttctga gaccagggtt 240
gctccgtccg tgctccgcct cgccatgact tcctacagct atcgccagtc gtcggccacg 300
tcgtccttcg gaggcctggg cggcggctcc gtgcgttttg ggccgggggt cgcttttcgc 360
gcgcccagca ttcacggggg ctccggcggc cgcggcgtat ccgtgtcctc cgcccgcttt 420
gtgtcctcgt cctcctcggg gggctacggc ggcggctacg gcggcgtcct gaccgcgtcc 480
gacgggctgc tggcgggcaa cgagaagcta accatgcaga acctcaacga ccgcctggcc 540
tcctacctgg acaaggtgcg cgccctggag gcggccaacg gcgagctaga ggtgaagatc 600
cgcgactggt accagaagca ggggcctggg ccctcccgcg actacagcca ctactacacg 660
accatccagg acctgcggga caagattctt ggtgccacca ttgagaactc caggattgtc 720
ctgcagatcg acaatgcccg tctggctgca gatgacttcc gaaccaagtt tgagacggaa 780
caggctctgc gcatgagcgt ggaggccgac atcaacggcc tgcgcagggt gctggatgag 840
ctgaccctgg ccaggaccga cctggagatg cagatcgaag gcctgaagga agagctggcc 900
tacctgaaga agaaccatga ggaggaaatc agtacgctga ggggccaagt gggaggccag 960
gtcagtgtgg aggtggattc cgctccgggc accgatctcg ccaagatcct gagtgacatg 1020
cgaagccaat atgaggtcat ggccgagcag aaccggaagg atgctgaagc ctggttcacc 1080
agccggactg aagaattgaa ccgggaggtc gctggccaca cggagcagct ccagatgagc 1140
aggtccgagg ttactgacct gcggcgcacc cttcagggtc ttgagattga gctgcagtca 1200
cagctgagca tgaaagctgc cttggaagac acactggcag aaacggaggc gcgctttgga 1260
gcccagctgg cgcatatcca ggcgctgatc agcggtattg aagcccagct gggcgatgtg 1320
cgagctgata gtgagcggca gaatcaggag taccagcggc tcatggacat caagtcgcgg 1380
ctggagcagg agattgccac ctaccgcagc ctgctcgagg gacaggaaga tcactacaac 1440
aatttgtctg cctccaaggt cctctgaggc agcaggctct ggggcttctg ctgtcctttg 1500
gagggtgtct tctgggtaga gggatgggaa ggaagggacc cttacccccg gctcttctcc 1560
tgacctgcca ataaaaattt atggtccaag ggaaaaaaaa aa 1602

<210> 10
<211> 1677
<212> DNA
<213> Homo sapiens

<400> 10
ggccaagcaa gcttctatct gcacctgctc tcaatcctgc tctcaccatg agcctccgcc 60
tgcagagctc ctctgccagc tatggaggtg gtttcggggg tggctcttgc cagctgggag 120
gaggccgtgg tgtctctacc tgttcaactc ggtttgtgtc tgggggatca gctgggggct 180
atggaggcgg cgtgagctgt ggttttggtg gaggggctgg tagtggcttt ggaggtggct 240
atggaggtgg ccttggaggt ggctatggag gtggccttgg aggtggcttt ggtgggggtt 300
ttgctggtgg ctttgttgac tttggtgctt gtgatggcgg cctcctcact ggcaatgaga 360
agatcaccat gcagaacctc aacgaccgcc tggcttccta cctggagaag gtgcgcgccc 420
tggaggaggc caacgctgac ctggaggtga agatccgtga ctggcacctg aagcagagcc 480
cagctagccc tgagcgggac tacagcccct actacaagac cattgaagag ctccgggaca 540
agatcctgac cgccaccatt gaaaacaacc gggtcatcct ggagattgac aatgccaggc 600
tggctgtgga cgacttcagg ctcaagtatg agaatgagct ggccctgcgc cagagcgtgg 660
aggccgacat caacggcctg cgccgggtgc tggatgagct cactctgtct aagactgacc 720
tggagatgca gatcgagagc ctgaatgaag agctagccta catgaagaag aaccatgaag 780
aggagatgaa ggaatttagc aaccaggtgg tcggccaggt caacgtggag atggatgcca 840
ccccaggcat tgacctgacc cgcgtgctgg cagagatgag ggagcagtac gaggccatgg 900
cagagaggaa ccgccgggat gctgaggaat ggttccacgc caagagtgca gagctgaaca 960
aggaggtgtc taccaacact gccatgattc agaccagcaa gacagagatc acggagctca 1020
ggcgcacgct ccaaggcctg gagattgagc tgcagtccca gctgagcatg aaagcggggc 1080
tggagaacac ggtggcagag acggagtgcc gctatgccct gcagctgcag cagatccagg 1140
gactcatcag cagcatcgag gcccagctga gcgagctccg cagtgagatg gagtgccaga 1200
accaagagta caagatgctg ctggacatca agacacgtct ggagcaggag atcgccacct 1260
accgcagcct gctcgagggc caggacgcca agaagcgtca gcccccgtag cacctctgtt 1320
accacgactt ctagtgcctc tgttaccacc acctctaatg cctctggtcg ccgcacttct 1380
gatgtccgta ggccttaaat ctgcctggcg tcccctccct ctgtcttcag cacccagagg 1440
aggagagagc cggcagttcc ctgcaggaga gaggaggggc tgctggaccc aaggctcagt 1500
ccctctgctc tcaggacccc ctgtcctgac tctctcctga tggtgggccc tctgtgctct 1560
tctcttccgg tcggatctct ctcctctctg acctggatac gctttggttt ctcaacttct 1620
ctaccccaaa gaaaagatta ttcaataaag tttcctgcct ttctgcaaac ataaaaa 1677

<210> 11
<211> 1709
<212> DNA
<213> Homo sapiens

<400> 11
ggtacctcct gccagcacct cttgggtttg ctgagaactc acgggctcca gctacctggc 60
catgaccacc acatttctgc aaacttcttc ctccaccttt gggggtggct caacccgagg 120
gggttccctc ctggctgggg gaggtggctt tggtgggggg agtctctctg ggggaggtgg 180
aagccgaagt atctcagctt cttctgctag gtttgtctct tcagggtcag gaggaggata 240
tgggggtggc atgagggtct gtggctttgg tggaggggct ggtagtgttt tcggtggagg 300
ctttggaggg ggcgttggtg ggggttttgg tggtggcttt ggtggtggcg atggtggtct 360
cctctctggc aatgagaaaa ttaccatgca gaacctcaat gaccgcctgg cctcctacct 420
ggacaaggta cgtgccctgg aggaggccaa tgctgacctg gaggtgaaga tccatgactg 480
gtaccagaag cagaccccag ccagcccaga atgcgactac agccaatact tcaagaccat 540
tgaagagctc cgggacaaga tcatggccac caccatcgac aactcccggg tcatcctgga 600
gatcgacaat gccaggctgg ctgcggacga cttcaggctc aagtatgaga atgagctggc 660
cctgcgccag ggcgttgagg ctgacatcaa cggcttgcgc cgagtcctgg atgagctgac 720
cctggccagg actgacctgg agatgcagat cgagggcctg aatgaggagc tagcctacct 780
gaagaagaac cacgaagagg agatgaagga gttcagcagc cagctggccg gccaggtcaa 840
tgtggagatg gacgcagcac cgggtgtgga cctgacccgt gtgctggcag agatgaggga 900
gcagtacgag gccatggcgg agaagaaccg ccgggatgtc gaggcctggt tcttcagcaa 960
gactgaggag ctgaacaaag aggtggcctc caacacagaa atgatccaga ccagcaagac 1020
ggagatcaca gacctgagac gcacgatgca ggagctggag atcgagctgc agtcccagct 1080
cagcatgaaa gctgggctgg agaactcact ggccgagaca gagtgccgct atgccacgca 1140
gctgcagcag atccaggggc tcattggtgg cctggaggcc cagctgagtg agctccgatg 1200
cgagatggag gctcagaacc aggagtacaa gatgctgctt gacataaaga cacggctgga 1260
gcaggagatc gctacttacc gcagcctgct cgagggccag gatgccaaga tggctggcat 1320
tggcatcagg gaagcctctt caggaggtgg tggtagcagc agcaatttcc acatcaatgt 1380
agaagagtca gtggatggac aggtggtttc ttcccacaag agagaaatct aagtgtctat 1440
tgcaggagaa acgtcccttg ccactcccca ctctcatcag gccaagtgga ggactggcca 1500
gagggcctgc acatgcaaac tccagtccct gccttcagag agctgaaaag ggtccctcgg 1560
tcttttattt cagggctttg catgcgctct attccccctc tgcctctccc caccttcttt 1620
ggagcaagga gatgcagctg tattgtgtaa caagctcatt tgtacagtgt ctgttcatgt 1680
aataaagaat tacttttcct tttgcaaat 1709

<210> 12

<211> 3147
<212> DNA
<213> Homo sapiens

<400> 12
gtcgcgccat tttgccgggg tttgaatgtg aggcggagcg gcggcaggag cggatagtgc 60
cagctacggt ccgcggctgg ggttccctcc tccgtttctg tatccccacg agatcctata 120
gcaatggaac tcagcgatgc aaatctgcaa acactaacag aatatttaaa gaaaacactt 180
gatcctgatc ctgccatccg acgtccagct gagaaatttc ttgaatctgt tgaaggaaat 240
cagaattatc cactgttgct tttgacatta ctggagaagt cccaggataa tgttatcaaa 300
gtatgtgctt cagtaacatt caaaaactat attaaaagga actggagaat tgttgaagat 360
gaaccaaaca aaatttgtga agccgatcga gtggccatta aagccaacat agtgcacttg 420
atgcttagca gcccagagca aattcagaag cagttaagtg atgcaattag cattattggc 480
agagaagatt ttccacagaa atggcctgac ttgctgacag aaatggtgaa tcgctttcag 540
agtggagatt tccatgttat taatggagtc ctccgtacag cacattcatt atttaaaaga 600
taccgtcatg aatttaagtc aaacgagtta tggactgaaa ttaagcttgt tctggatgcc 660
tttgctttgc ctttgactaa tctttttaag gccactattg aactctgcag tacccatgca 720
aatgatgcct ctgccctgag gattctgttt tcttccctga tcctgatctc aaaattgttc 780
tatagtttaa actttcagga tctccctgaa ttttgggaag gtaatatgga aacttggatg 840
aataatttcc atactctctt aacattggat aataagcttt tacaaactga tgatgaagag 900
gaagccggct tattggagct cttaaaatcc cagatttgtg ataatgccgc actctatgca 960
caaaagtacg atgaagaatt ccagcgatac ctgcctcgtt ttgttacagc catctggaat 1020
ttactagtta caacgggtca agaggttaaa tatgatttgt tggtaagtaa tgcaattcaa 1080
tttctggctt cagtttgtga gagacctcat tataagaatc tatttgagga ccagaacacg 1140
ctgacaagta tctgtgaaaa ggttattgtg cctaacatgg aatttagagc tgctgatgaa 1200
gaagcatttg aagataattc tgaggagtac ataaggagag atttggaagg atctgatatt 1260
gatactagac gcagggctgc ttgtgatctg gtacgaggat tatgcaagtt ttttgaggga 1320
cctgtgacag gaatcttctc tggttatgtt aattccatgc tgcaggaata cgcaaaaaat 1380
ccatctgtca actggaaaca caaagatgca gccatctacc tagtgacatc tttggcatca 1440
aaagcccaaa cacagaagca tggaattaca caagcaaatg aacttgtaaa cctaactgag 1500
ttctttgtga atcacatcct ccctgattta aaatcagcta atgtgaatga atttcctgtc 1560
cttaaagctg acggtatcaa atatattatg atttttagaa atcaagtgcc aaaagaacat 1620
cttttagtct cgattcctct cttgattaat catcttcaag ctggaagtat tgttgttcat 1680
acttacgcag ctcatgctct tgaacggctc tttactatgc gagggcctaa caatgccact 1740
ctctttacag ctgcagaaat cgcaccgttt gttgagattc tgctaacaaa ccttttcaaa 1800
gctctcacac ttcctggctc ttcagaaaat gaatatatta tgaaagctat catgagaagt 1860
ttttctctcc tacaagaagc cataatcccc tacatcccta ctctcatcac tcagcttaca 1920
cagaagctat tagctgttag taagaaccca agcaaacctc actttaatca ctacatgttt 1980
gaagcaatat gtttatccat aagaataact tgcaaagcta accctgctgc tgttgtaaat 2040
tttgaggagg ctttgttttt ggtgtttact gaaatcttac aaaatgatgt gcaagaattt 2100
attccatacg tctttcaagt gatgtctttg cttctggaaa cacacaaaaa tgacatcccg 2160
tcttcctata tggccttatt tcctcatctc cttcagccag tgctttggga aagaacagga 2220
aatattcctg ctctagtgag gcttcttcaa gcattcttag aacgcggttc aaacacaata 2280
gcaagtgctg cagctgacaa aattcctggg ttactaggtg tctttcagaa gctgattgca 2340
tccaaagcaa atgaccacca aggtttttat cttctaaaca gtataataga gcacatgcct 2400
cctgaatcag ttgaccaata taggaaacaa atcttcattc tgctattcca gagacttcag 2460
aattccaaaa caaccaagtt tatcaagagt tttttagtct ttattaattt gtattgcata 2520
aaatatgggg cactagcact acaagaaata tttgatggta tacaaccaaa aatgtttgga 2580
atggttttgg aaaaaattat tattcctgaa attcagaagg tatctggaaa tgtagagaaa 2640
aagatctgtg cggttggcat aaccaactta ctaacagaat gtcccccaat gatggacact 2700
gagtatacca aactgtggac tccattatta cagtctttga ttggtctttt tgagttaccc 2760
gaagatgata ccattcctga tgaggaacat tttattgaca tagaagatac accaggatat 2820
cagactgcct tctcacagtt ggcatttgct gggaaaaaag agcatgatcc tgtaggtcaa 2880
atggtgaata accccaaaat tcacctggca cagtcacttc acatgttgtc taccgcctgt 2940
ccaggaaggg ttccatcaat ggtgagcacc agcctgaatg cagaagcgct ccagtatctc 3000
caagggtacc ttcaggcagc cagtgtgaca ctgctttaaa ctgcattttt ctaatgggct 3060
aaacccagat ggtttcctag gaaatcacag gcttctgagc acagctgcat taaaacaaag 3120
gaagttttcc ttttgaactt gtcacga 3147

<210> 13
<211> 510
<212> DNA
<213> Homo sapiens

<400> 13
cccgatttct cccggaacct ctgctcagcc tggtgaacca cacaggccag cgctctgaca 60
tgcagaaggt gaccctgggc ctgcttgtgt tcctggcagg ctttcctgtc ctggacgcca 120

atgacctaga agataaaaac agtcctttct actatgactg gcacagcctc caggttggcg 180
ggctcatctg cgctggggtt ctgtgcgcca tgggcatcat catcgtcatg agtgcaaaat 240
gcaaatgcaa gtttggccag aagtccggtc accatccagg ggagactcca cctctcatca 300
ccccaggctc agcccaaagc tgatgaggac agaccagctg aaattgggtg gaggaccgtt 360
ctctgtcccc aggtcctgtc tctgcacaga aacttgaact ccaggatgga attcttcctc 420
ctctgctggg actcctttgc atggcagggc ctcatctcac ctctcgcaag agggtctctt 480
tgttcaattt tttttaatct aaaatgatta 510

<210> 14
<211> 20
<212> DNA
<213> Homo sapiens

<400> 14
caccatgtct cgccagtcaa 20

<210> 15
<211> 20
<212> DNA
<213> Homo sapiens

<400> 15
ggtccacttg gtgtccagaa 20

<210> 16
<211> 8
<212> PRT
<213> Artificial Sequence
<220>
<223> Affinity purification system recognition site

<400> 16
Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

<210> 17
<211> 21
<212> PRT
<213> Artificial Sequence
<220>
<223> Affinity purification system recognition site

<400> 17
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Glu His
1 5 10 15
His His His His His
20

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 98/19362

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07K16/18

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 31728 A (BEKI AB ;BJOERKLUND PETER (SE); RYDLANDER LARS (SE)) 23 November 1995 see whole doc. esp. claims and table 1, apge 13 ---	1-20, 22-32
X	WO 96 05322 A (GEN HOSPITAL CORP ;HARVARD COLLEGE (US)) 22 February 1996 cited in the application see whole doc. esp. claims and p. 2,line 3 ff ---	1-33
X	WO 96 40713 A (US HEALTH ;PASTAN IRA (US); BRINKMANN ULRICH (US)) 19 December 1996 cited in the application see whole doc esp. claims and example 13 --- -/--	1-20,23, 25-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but 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 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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1998	13/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DE 39 42 999 A (BOEHRINGER MANNHEIM GMBH) 4 July 1991 see claims and example 1 ---	12,13, 16-20, 23,24
A	DATABASE EMBL embl heidelberg AC:J00124, 13 June 1985 "homo sapiens 50 kda typeI epidermal keratin gene" XP002088177 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:S79867, 19 February 1996 "type I keratin 16" XP002088178 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:X62751, 27 May 1992 "H. sapiens mRNA for keratin related protein" XP002088179 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:L42611, 27 July 1995 "homo sapiens keratin 6 isoform K6e" XP002088180 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:M21389, 22 April 1989 "Human keratin type II mRNA" XP002088181 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:X12881, 3 July 1989 "human mRNA for cytokeratin 18" XP002088182 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:M13955, 1 October 1996 "human mesothelial keratin k7" XP002088183 see abstract ---	
	-/--	

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMBL embl heidelberg AC:M34225, 3 July 1990 "human cytokeratin 8 mRNA" XP002088184 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:J03607, 16 July 1988 "human 40-kDa keratin intermediate" XP002088185 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:X52426, 30 November 1992 "h. sapiens mRNA for cytokeratin 13" XP002088186 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:X07696, 12 July 1988 "Human mRNA for cytokeratin 15" XP002088187 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:T61223, 12 May 1997 "human cellular apoptosis susceptibility cas protein cDNA" XP002088188 see abstract ---	
A	DATABASE EMBL embl heidelberg AC:X93036, 28 November 1995 "H.sapiens mRNA for mat8 protein" XP002088189 see abstract ---	
P,X	KLEIN A. ET AL.,: "expression of cytokeratin 20 in urinary cytology of patients with bladder carcinoma" CANCER, vol. 82, no. 2, - 15 January 1998 pages 349-354, XP002088176 see the whole document --- -/--	1,3,4,7, 9,11,25, 31

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 98/19362

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MOLL R ET AL: "CYTOKERATIN 20 IN HUMAN CARCINOMAS A NEW HISTODIAGNOSTIC MARKER DETECTED BY MONOCLONAL ANTIBODIES" AMERICAN JOURNAL OF PATHOLOGY, vol. 140, no. 2, 1 January 1992, pages 427-447, XP000562928 see whole doc. esp table 2 and table 3 -----	12,13, 16-20, 23,27,28

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 19362

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

Remark: Although claims 23 and 24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

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

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

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

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

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

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

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir ational Application No
PCT/US 98/19362

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531728 A	23-11-1995	AU 2582195 A	05-12-1995
		EP 0765479 A	02-04-1997
		JP 10500408 T	13-01-1998
		SE 9500023 A	18-11-1995
WO 9605322 A	22-02-1996	US 5728579 A	17-03-1998
WO 9640713 A	19-12-1996	US 5759782 A	02-06-1998
		AU 6269896 A	30-12-1996
DE 3942999 A	04-07-1991	AT 120550 T	15-04-1995
		CA 2046888 A	28-06-1991
		DE 59008810 D	04-05-1995
		DK 460190 T	28-08-1995
		WO 9110139 A	11-07-1991
		EP 0460190 A	11-12-1991
		ES 2070488 T	01-06-1995
		JP 8002919 B	17-01-1996
		JP 4504063 T	23-07-1992
		US 5288614 A	22-02-1994