(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 August 2017 (03.08.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/132276 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/US2017/014969

(22) International Filing Date:
25 January 2017 (25.01.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/287,182 26 January 2016 (26.01.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,

[Continued on next page]

(54) Title: PREDICTIVE DIAGNOSTIC WORKFLOW FOR TUMORS USNIG AUTOMATED DISSECTION, NEXT GENERATION SEQUENCING, AND AUTOMATED SLIDE STAINERS

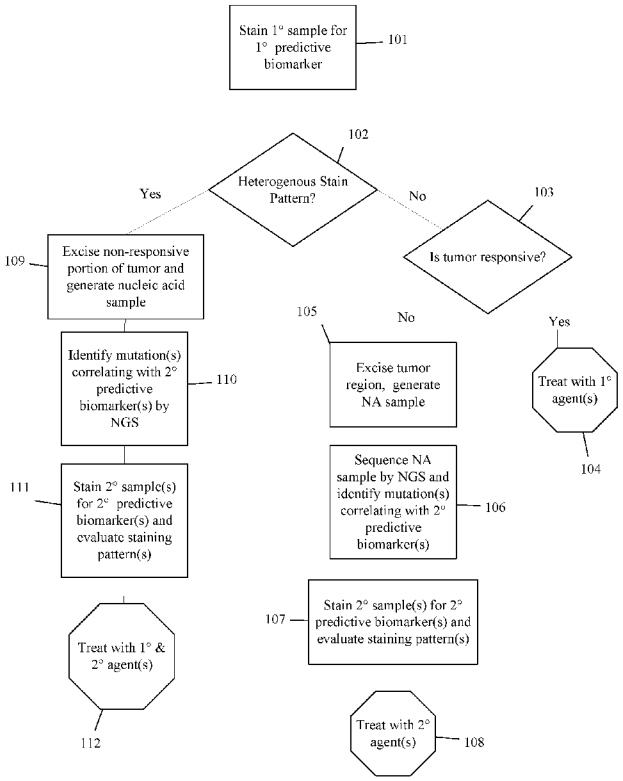

Fig. 1

(57) Abstract: Systems and methods for selecting therapeutic agents for cancers using next generation sequencing, automated dissection, and/or automated slide stainers are disclosed. Non-responsive regions of a tumor sample having a heterogenous staining pattern for a predictive biomarker are excised using an automated dissection tool. Mutations linked to additional predictive biomarkers are identified in the excised portion of the sample by next generation sequencing. The relevance of the additional predictive biomarker(s) is confirmed by histochemical staining. Therapeutic courses may then be selected on the basis of the staining patterns of the predictive biomarkers.

WO 2017/132276 A1

DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

PREDICTIVE DIAGNOSTIC WORKFLOW FOR TUMORS USING AUTOMATED DISSECTION, NEXT GENERATION SEQUENCING, AND AUTOMATED SLIDE STAINERS

CROSS-REFERENCE TO RELATED APPLICATIONS

The benefit of US Provisional Patent Application Number US 62/287,182, filed January 26, 2016, the content of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to use of automated slide stainers, automated dissection tools, and/or next generation sequencers to assay predictive biomarkers and/or select therapies for cancers.

Description of Related Art

In the last 20 years, cancer research and treatment has undergone a sea change. Whereas the first sequencing of the human genome cost on the order of $500 million to $1 billion, next generation sequencing (NGS) strategies have shrunk that cost to less than $2000, thereby permitting large-scale and relatively inexpensive evaluation of cancer genetics. *See* Reuter *et al.* These advances have accelerated the rate at which new therapeutic and diagnostic targets are identified and developed: in 2014 alone, the FDA approved a total of 51 new molecular entities and new biological products, many of which are targeted therapies. *See* FDA White Paper.

While such developments have certainly improved the treatment landscape, cancer remains remarkably resilient. Owing to their natural heterogeneity, tumors still frequently become resistant to previously effective therapeutics. *See* Sun & Yu. Diagnostic workflows to effectively and efficiently address this problem have yet to be realized.

Some diagnostic workflows have been identified using laser capture microdissection (LCM) and NGS. *See* Amemiya *et al.*; AB Brochure; Zhang *et al.* These procedures typically involve separately collecting tumor cells and non-tumor cells from the tumor using a LCM system, extracting genomic DNA (gDNA) or RNA from the collected cells, and sequencing specific loci to identify cancer-related mutations in the tumor cells and the normal cells. The mutations in the tumor cells are compared to the mutations in the non-tumor cells

to identify mutations over-represented in the tumor cells. While these methods may provide a high-level view of mutations represented in the tumor, they do not provide any information about the spatial relationship of the mutations or how the mutations are inter-related from a functional standpoint.

BRIEF SUMMARY OF THE INVENTION

This disclosure relates generally to use of automated dissection tools and/or next generation sequencing (NGS) platforms in cancer diagnostics and selection of therapeutics.

Methods are provided in which regions of a tumor sample predicted not to respond to a first therapeutic agent are excised from the sample with an automated dissection tool, mutations correlated with predictive biomarkers are detected in the excised region using NGS, and additional samples of the tumor are stained for one or more predictive biomarker(s) identified by NGS.

Systems for performing the methods described herein are provided comprising various combinations of cellular samples, nucleic acid samples, automated dissection tools, NGS systems, automated slide stainers, image scanners and analysis systems, and laboratory information systems.

Sets of diagnostic samples for use in the methods and systems disclosed herein are provided comprising slides containing cellular samples of a tumor and nucleic acid samples obtained from specific regions of such slides.

Other inventions and embodiments of the foregoing are set forth herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flowchart demonstrating an exemplary workflow for the processes disclosed herein. Rectangular boxes indicate processes performed on samples. Diamond boxes indicate evaluation steps. Octagonal boxes indicate treatment decisions or treatment steps.

Fig. 2 is an exemplary system including an image analysis component as disclosed herein.

Fig. 3 is an exemplary system including a laboratory information system (LIS) and optional image analysis component as disclosed herein. Circles and ovals indicate potential sample components of the system. Squares and rectangles indicate sample manipulation, data

analysis, and data storage components of the systems. Pentagonal shapes indicate data output from the system. Dashed lines indicate alternate workflows.

Fig. 4 is a series of IHC images from a prostate tumor evaluated according to a methods and system as disclosed herein. A primary sample was stained for PTEN. ROIs for excision and mutation analysis are outlined in the PTEN-stained image with the "o" symbol. Secondary samples were stained for EGFR and HER2.

Fig. 5 is a series of IHC images from a prostate tumor evaluated according to a methods and system as disclosed herein. A primary sample was stained for PTEN. ROIs for excision and mutation analysis are outlined in the PTEN-stained image with the "o" symbol. Secondary samples were stained for EZH2.

Fig. 6 is a series of IHC images from a lung tumor evaluated according to a methods and system as disclosed herein. A primary sample was stained for EGFR L858R. ROIs for excision and mutation analysis are outlined in the EGFR L858R-stained image with the "o" symbol. Secondary samples were stained for p53.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art. See, *e.g.*, Lackie, DICTIONARY OF CELL AND MOLECULAR BIOLOGY, Elsevier (4th ed. 2007); Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL, Cold Springs Harbor Press (Cold Springs Harbor, N.Y. 1989). The term "a" or "an" is intended to mean "one or more." The terms "comprise," "comprises," and "comprising," when preceding the recitation of a step or an element, are intended to mean that the addition of further steps or elements is optional and not excluded.

Antibody: The term "antibody" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

Antibody fragment: An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab,

Fab', Fab'-SH, F(ab')2; diabodies; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

Biomarker: As used herein, the term "biomarker" shall refer to any molecule or group of molecules found in a biological sample that can be used to characterize the biological sample or a subject from which the biological sample is obtained. For example, a biomarker may be a molecule or group of molecules whose presence, absence, or relative abundance is:

- characteristic of a particular cell or tissue type or state;
- characteristic of a particular pathological condition or state; or
- indicative of the severity of a disease state, the likelihood of progression or regression of the disease state, and/or the likelihood that the disease state will respond to a particular treatment.

As another example, the biomarker may be a cell type or a microorganism (such as a bacteria, mycobacteria, fungi, viruses, and the like), or a substituent molecule or group of molecules thereof.

Biomarker-specific reagent: A specific detection reagent that is capable of specifically binding directly to one or more biomarkers in the cellular sample, such as a primary antibody.

Cellular sample: As used herein, the term "cellular sample" refers to any sample containing intact cells, such as cell cultures, bodily fluid samples or surgical specimens taken for pathological, histological, or cytological interpretation.

Detection reagent: A "detection reagent" is any reagent that is used to deposit a stain in proximity to a biomarker-specific reagent in a cellular sample. Non-limiting examples include biomarker-specific reagents (such as primary antibodies), secondary detection reagents (such as secondary antibodies capable of binding to a primary antibody), tertiary detection reagents (such as tertiary antibodies capable of binding to secondary antibodies), enzymes directly or indirectly associated with the biomarker specific reagent, chemicals reactive with such enzymes to effect deposition of a fluorescent or chromogenic stain, and the like.

Detectable moiety: A molecule or material that can produce a detectable signal (such as visually, electronically or otherwise) that indicates the presence (i.e. qualitative analysis) and/or concentration (i.e. quantitative analysis) of the detectable moiety deposited on a sample. A detectable signal can be generated by any known or yet to be discovered

mechanism including absorption, emission and/or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons). The term "detectable moiety" includes chromogenic, fluorescent, phosphorescent, and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity). In some examples, the detectable moiety is a fluorophore, which belongs to several common chemical classes including coumarins, fluoresceins (or fluorescein derivatives and analogs), rhodamines, resorufins, luminophores and cyanines. Additional examples of fluorescent molecules can be found in Molecular Probes Handbook — A Guide to Fluorescent Probes and Labeling Technologies, Molecular Probes, Eugene, OR, TheroFisher Scientific, 11th Edition. In other embodiments, the detectable moiety is a molecule detectable via brightfield microscopy, such as dyes including diaminobenzidine (DAB), 4-(dimethylamino) azobenzene-4'-sulfonamide (DABSYL), tetramethylrhodamine (DISCOVERY Purple), N,N'-biscarboxypentyl-5,5'-disulfonato-indo-dicarbocyanine (Cy5), and Rhodamine 110 (Rhodamine).

Histochemical detection: A process involving staining a biomarker or other structures in a tissue sample with detection reagents in a manner that permits in a manner that permits microscopic detection of the biomarker or other structures in the context of the cross-sectional relationship between the structures of the tissue sample. Examples include immunohistochemistry (IHC), chromogenic *in situ* hybridization (CISH), fluorescent *in situ* hybridization (FISH), silver *in situ* hybridization (SISH), and hematoxylin and eosin (H&E) staining of formalin-fixed, paraffin-embedded tissue sections.

Monoclonal antibody: An antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring

production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, or a combination thereof.

Predictive biomarker: A biomarker whose staining pattern in a cellular sample is indicative of the likelihood that a particular treatment course will be effective or that other courses of treatment will not be effective.

Sample: As used herein, the term "sample" shall refer to any material obtained from a subject capable of being tested for the presence or absence of a biomarker.

Secondary detection reagent: A specific detection reagent capable of specifically binding to a biomarker-specific reagent.

Section: When used as a noun, a thin slice of a tissue sample suitable for microscopic analysis, typically cut using a microtome. When used as a verb, the process of generating a section.

Serial section: As used herein, the term "serial section" shall refer to any one of a series of sections cut in sequence by a microtome from a tissue sample. For two sections to be considered a "serial section" of one another, they do not necessarily need to be consecutive sections from the tissue, but they should generally contain the same tissue structures in the same cross-sectional relationship, such that the structures can be matched to one another via morphology.

Specific detection reagent: Any composition of matter that is capable of specifically binding to a target chemical structure in the context of a cellular sample. As used herein, the phrase "specific binding," "specifically binds to," or "specific for" or other similar iterations refers to measurable and reproducible interactions between a target and a specific detection reagent, which is determinative of the presence of the target in the presence of a heterogeneous population of molecules including biological molecules. For example, an antibody that specifically binds to a target is an antibody that binds this target with greater affinity, avidity, more readily, and/or with greater duration than it binds to other targets. In one embodiment, the extent of binding of a specific detection reagent to an unrelated target is less than about 10% of the binding of the antibody to the target as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, a biomarker-specific reagent that specifically binds to a target has a dissociation constant (Kd) of $\leq$1 μM, $\leq$100 nM, $\leq$10 nM,

≤1 nM, or ≤0.1 nM. In another embodiment, specific binding can include, but does not require exclusive binding. Exemplary specific detection reagents include nucleic acid probes specific for particular nucleotide sequences; antibodies and antigen binding fragments thereof; and engineered specific binding compositions, including ADNECTINs (scaffold based on 10th FN3 fibronectin; Bristol-Myers-Squibb Co.), AFFIBODYs (scaffold based on Z domain of protein A from S. aureus; Affibody AB, Solna, Sweden), AVIMERs (scaffold based on domain A/LDL receptor; Amgen, Thousand Oaks, CA), dAbs (scaffold based on VH or VL antibody domain; GlaxoSmithKline PLC, Cambridge, UK), DARPins (scaffold based on Ankyrin repeat proteins; Molecular Partners AG, Zürich, CH), ANTICALINs (scaffold based on lipocalins; Pieris AG, Freising, DE), NANOBODYs (scaffold based on VHH (camelid Ig); Ablynx N/V, Ghent, BE), TRANS-BODYs (scaffold based on Transferrin; Pfizer Inc., New York, NY), SMIPs (Emergent Biosolutions, Inc., Rockville, MD), and TETRANECTINs (scaffold based on C-type lectin domain (CTLD), tetranectin; Borean Pharma A/S, Aarhus, DK). Descriptions of such engineered specific binding structures are reviewed by Wurch et al., *Development of Novel Protein Scaffolds as Alternatives to Whole Antibodies for Imaging and Therapy: Status on Discovery Research and Clinical Validation*, Current Pharmaceutical Biotechnology, Vol. 9, pp. 502-509 (2008), the content of which is incorporated by reference.

Stain: When used as a noun, the term "stain" shall refer to any substance that can be used to visualize specific molecules or structures in a cellular sample for microscopic analysis, including brightfield microscopy, fluorescent microscopy, electron microscopy, and the like. When used as a verb, the term "stain" shall refer to any process that results in deposition of a stain on a cellular sample.

Subject: As used herein, the term "subject" or "individual" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g., cows, sheep, cats, dogs, and horses), primates (e.g., humans and non-human primates such as monkeys), rabbits, and rodents (e.g., mice and rats). In certain embodiments, the individual or subject is a human.

Tissue sample: As used herein, the term "tissue sample" shall refer to a cellular sample that preserves the cross-sectional spatial relationship between the cells as they existed within the subject from which the sample was obtained.

Tumor sample: A cellular sample obtained from a tumor.

II. Abbreviations

1°: Primary

2°: Secondary

5-FU: 5-fluorouracil

CISH: Chromogenic *in situ* hybridization

FFPE: Formalin-fixed, paraffin-embedded

FISH: Fluorescent *in situ* hybridization

gDNA: Genomic DNA

H&E: hematoxylin and eosin

IHC: Immunohistochemistry

ISH: In situ hybridization

LCM: Laser capture microdissection

LIS: Laboratory information system

MPS: Massively parallel sequencing

NA: Nucleic acid

NGS: Next generation sequencing

PCR: Polymerase chain reaction

ROI: Region of interest

SISH: Silver *in situ* hybridization

SNP: Single nucleotide polymorphism

III. Methods of selecting cancer treatments and treating cancers.

In an embodiment, a treatment selection process is provided comprising histochemical staining, automated dissection, and next generation sequencing steps. The typical workflow for selecting a therapeutic course is illustrated at Fig. 1.

A. Samples and sample staining

A tumor sample is obtained and a first portion of the tumor sample (hereafter termed a "primary sample" or 1° sample") is stained for a first predictive biomarker (also referred to as a "primary predictive biomarker" and a "1° predictive biomarker") for a first therapeutic agent 101.

Typically, the tumor sample is a tissue sample. In a specific embodiment, the tumor sample is a formalin-fixed, paraffin-embedded (FFPE) tissue samples. Any predictive biomarker may be used, whether now known or known in the future. For example, the

predictive biomarker may be predictive of a response to a chemotherapy, to a targeted therapy, to a radiation therapy, or to a combination thereof. Exemplary predictive biomarkers are disclosed in Table 1:

Biomarker	Biomarker Description	Therapeutic Agent
ALK & ALK fusions	In certain tumors, Anaplastic Lymphoma Kinase (ALK) can be aberrantly expressed, often in the form of fusion proteins resulting from translocation events involving the *ALK* gene.	ALK inhibitor (crizotinib, alectinib) HSP90 inhibitors (luminespib) EGFR inhibitor (osimertinib, erlotinib, gefitinib)
AREG	Amphiregulin (AREG) is a secrete peptide hormone that acts as an activating ligand of EGFR.	anti-EGFR antibodies EGFR inhibitor (osimertinib, erlotinib, gefitinib)
ATM	ATM is serine/threonine kinase involved in double stranded DNA damage repair response. Mutations in ATM leading to reduced function or loss of function are associated with several cancers.	DNA-damaging agents (platinum-based drugs, nucleoside analogs) PARP inhibitor (olaparib) ATR inhibitors (AZD6738) CHK1 inhibitors (MK-8776)
BCL2	BCL2 is an anti-apoptotic protein. Aberrant expression of BCL-2 (often due to translocations involving the *BCL2* gene) are associated with many cancers.	Bcl-2 inhibitor (venetoclax)
BRAF	BRAF is a serine/threonine kinase that plays a role in EGFR-mediated signal transduction. Constitutively active mutants of BRAF (such as V600E) are found in ~15% of all known human cancers.	anti-EGFR antibodies (cetuximab, panitumumab) MEK inhibitor (tramatenib), B-Raf inhibitor (dabrafenib, vemurafenib) Dasatinib
BRCA1	BRCA1 is involved in many cellular functions, including DNA repair and cell-cycle checkpoint control. Cancer-associated mutations often result in loss of function.	PARP inhibitor (olaparib, rucaparib) Chemotherapy (anthracyclines, CMF, taxanes)

c-KIT	c-KIT is a receptor tyrosine kinase that is involved in cell survival, proliferation, and differentiation. Cancer-associated mutations are typically activating or gain-of-function mutations.	tyrosine kinase inhibitor (imatinib mesylate, sunitinib)
CAIX	Carbonic anhydrase-IX (CAIX) is a zinc metalloenzymes that catalyzes the reversible hydration of carbon dioxide. CAIX is upregulated in some cancers.	IL-2
CCR4	C-C chemokine receptor type 4 (CCR4) is a G protein-coupled receptor family that is critical to T_{reg} cell migration.	anti-CCR4 (mogamulizumab)
CD30	CD30 is a cell surface receptor expressed by activated, but not resting, T-cells and B-cells. CD30 expression is associated with some lymphomas.	anti-CD30 antibody-drug conjugate (brentuximab vedotin)
Claudin18.2	Isoform 2 of claudin 18 (Claudin 18.2) is claudin-family protein overexpressed in many gastric tumors	Anti-claudin-18.2 antibody (IMAB362)
17p13.1	Chromosome 17, region 17p13.1, encompasses several tumor suppressor genes, including TP53. Deletions in this region are associated with several cancers.	Bcl-2 inhibitor (venetoclax)
DLL3	Delta-like 3 (DLL3) is a Notch-ligand that is predominantly expressed in fetal brain. Aberrant DLL3 expression is associated with some neuroendocrine tumors.	Anti-DLL3 antibody-drug conjugate (rovalpituzumab tesirine)
EGFR1	Epidermal Growth Factor Receptor 1 (EGFR1) is an EGFR-family receptor involved in development and regulation of cellular proliferation, survival, and migration. EGFR1 amplification and over-expression is often observed in many cancers.	anti-EGFR antibodies (cetuximab, panitumumab) EGFR inhibitor (osimertinib, erlotinib, gefitinib) HER2/EGFR tki (afatinib)

Estrogen Receptor	Estrogen receptor (ER) is a nuclear receptor for estrogen. Two ER proteins are expressed by humans: ERα (encoded by *ESR1* gene) and ERβ (encoded by *ESR2* gene). ER is a dimer in activated form (which may be an αα, ββ, or αβ dimer). ER over-expression is associated with many cancers, including breast cancer, ovarian cancer, colon cancer, prostate cancer, and endometrial cancer	aromatase inhibitors (anastrozole, exemestane, letrozole) selective ER modulators (tamoxifen, raloxifene, bazedoxifene) selective estrogen receptor degrader (fulvestrant)
EREG	Epiregulin (EREG) is a secrete peptide hormone that acts as an activating ligand of EGFR.	anti-EGFR antibodies EGFR inhibitor (osimertinib, erlotinib, gefitinib)
ERCC1	The *ERCC1* gene encodes DNA excision repair protein ERCC-1, which is involved in DNA repair and recombination. ERCC1 is often under-expressed or not expressed in cancer, frequently due to epigenetic changes, such as promoter methylation.	platinum-based chemotherapy (cisplatin)
FGF19	Fibroblast growth factor 19 (FGF19) is a protein hormone that functions as a heparin-dependent ligand of FGF4. FGF19 is over-expressed in many cancers, including primary human hepatocellular carcinomas, lung squamous cell carcinomas, and colon adenocarcinomas.	FGFR4 inhibitor (BLU-554)
FGFR2	Fibroblast growth factor receptor 2 is encoded by the *FGFR2* gene, consisting of 21 exons and encoding multiple splice variants. FGFR2b and FGFR2c isoforms are representative, each having extracellular three Ig-like domains, transmembrane domain, and cytoplasmic tyrosine kinase domain. FGFR2b differs from FGFR2c only in the latter half of the third Ig-like domain. FGFR2s function as a trans-membrane receptor for FGF-family proteins. Missense mutations, amplifications, and intronic SNPs are associated with many cancer types. As used herein, "FGFR2" refers to the *FGFR2* gene, and any gene products thereof.	anti-FGFR2 (FPA144) FGFR inhibitor (ARQ 087, Lucitanib, AZD4547, BGJ398, LY2874455, JNJ-42756493)

FGFR3	Fibroblast growth factor receptor 3 is encoded by the *FGFR3* gene. FGFR3 is over-exprssed in many cancers	FGFR3 inhibitor (dovitinib, AZD4547) TORC 1/2 inhibitor (CC-223)
FOLR1	Folate receptor alpha is encoded by the *FOLR1* gene, and has a high affinity for folate. FOLR1 gene products are over-expressed in many epithelial-derived tumors.	FOLR1 Ab-drug conjugate (IMGN853)
HER2	Human epidermal growth factor receptor 2 (HER2) is encoded by the *ERBB2* gene, and proto-oncogene. *ERBB2* is amplified or over-expressed in some cancers.	anti-HER2 (trastuzumab, pertuzumab, ado-trastuzumab emtansine)
KRAS	KRAS is a proto-oncogene GTPase encoded by the *KRAS* gene. Activating mutations have been identified in many cancers.	anti-EGFR (cetuzimab, panitumumab)
MGMT	O^6-Methylguanine-DNA-methyltransferase (MGMT) is a perotein encoded by the *MGMT* gene on chromosome 10. It is involved in a single-enzymatic DNA repair pathway. Loss of MGMT expression is associated with many cancers, including glioma, lymphoma, breast, and prostate cancer, and retinoblastoma.	5-FU-based adjuvant therapy
MSLN	Mesothelin (MSLN) is encoded by the *MSLN* gene on chromosome 16. Its biological functionis unknown, but it isover-expressed in several tumors, including mesothelioma, and ovarian and pancreatic adenocarcinoma.	anti-MSLN antibody-drug conjugate (anetumab ravtansine)
p53	p53 is a tumor suppressor encoded by the *TP53* gene on chromosome 17. Inactivating mutations of p53 are associated with severl cancers.	cisplatin-based chemotherapy MDM2 antagonist neoadjuvant radiation
MDM2	E3 ubiquitin-protein ligase Mdm2 (MDM2) is encoded by the *MDM2* geneon chromosome 12. Increased expression of MDM2is associated with many tumors.	MDM2 antagonist

Progesterone receptor	Progesterone receptor (PR) is a nuclear receptor for the steroid hormone progesterone, encoded by the *PGR* gene residing on chromosome 11q22. PR over-expression is associated with many cancers, including breast cancer, ovarian cancer, colon cancer, prostate cancer, and endometrial cancer	progesterone anatagonists (mifepristone)
PD-L1	Programmed death-ligand 1 (PD-L1), which is encoded by the *CD274* gene, induces suppression of T-cells via interaction with the PD-1 protein. Over-expression or aberrant expression of PD-L1 is frequently observed in numerous cancers and plays a role in immune avoidance by the tumor.	anti-PD-L1 (atezolizumab, durvalumab) anti-PD-1 (nivolumab, pembrolizumab)
PDGFRB	Beta-type platelet-derived growth factor receptor (PDGFRB) is a protein that in humans is encoded by the *PDGFRB* gene. PDGFRB is over-expressed in many cancers.	tyrosine kinase inhibitor (imatinib mesylate)
PTEN	Phosphatase and tensin homolog (PTEN) is a protein encoded by the *PTEN* gene. Inactivating mutations are associated with development of many cancers.	anti-HER2 (trastuzumab, pertuzumab, ado-trastuzumab emtansine)
TP	Thymidine phosphorylase (TP) is a pentosyltransferases that plays a key role in pyrimidine salvage to recover nucleosides after DNA/RNA degradation and is also involved in angiogenesis. TP is unregulated in many cancers.	5-FU- and capcetabine-based chemotherapy

Table 1

Many resources are available for identifying predictive biomarkers and their associated therapeutics. One example is the website "mycancergenome.org," which is maintained by the Vanderbilt-Ingram Cancer Center. Additionally, the FDA maintains a website with updated approvals of companion and complementary diagnostics. In a specific embodiment, at least the first predictive biomarker is predictive for a targeted therapy. In another embodiment, the predictive biomarker is a companion diagnostic for a targeted therapeutic.

The tumor samples are typically divided into several portions and affixed to a medium for microscopic analysis, such as a microscope slide. Where the sample is a tissue sample,

the several portions may be tissue sections. In some embodiments, serial sections are taken from FFPE tissue samples. In some embodiments, serial sections are taken from a plurality of different sites of a FFPE block, which can be done to capture both intra-section heterogeneity and intra-block heterogeneity. In some embodiments, serial sections are taken from a plurality of different biopsy samples taken from different locations in the same tumor, which can be done to capture both intra-section heterogeneity and intra-tumor heterogeneity.

Staining is typically histochemical staining. Histochemical staining techniques typically involve contacting the sample with a biomarker-specific reagent under conditions sufficient to permit specific binding between the biomarker-specific reagent and the biomarker of interest. Binding of the biomarker-specific reagent to the biomarker facilitates deposition of a detectable moiety on the sample in proximity to locations containing the biomarker. The detectable moiety can be used to locate and/or quantify the biomarker to which the specific detection reagent is directed. Thereby, the presence and/or concentration of the target in a sample can be detected by detecting the signal produced by the detectable moiety.

In some embodiments, the detectable moiety is directly conjugated to the biomarker-specific reagent, and thus is deposited on the sample upon binding of the biomarker-specific reagent to its target (generally referred to as a direct labeling method). Direct labeling methods are often more directly quantifiable, but often suffer from a lack of sensitivity. In other embodiments, deposition of the detectable moiety is effected by the use of a detection reagent associated with the biomarker-specific reagent (generally referred to as an indirect labeling method). Indirect labeling methods have the increase the number of detectable moieties that can be deposited in proximity to the biomarker-specific reagent, and thus are often more sensitive than direct labeling methods, particularly when used in combination with dyes.

In some embodiments, an indirect method is used, wherein the detectable moiety is deposited via an enzymatic reaction localized to the biomarker-specific reagent. Suitable enzymes for such reactions are well-known and include, but are not limited to, oxidoreductases, hydrolases, and peroxidases. Specific enzymes explicitly included are horseradish peroxidase (HRP), alkaline phosphatase (AP), acid phosphatase, glucose oxidase, β-galactosidase, β-glucuronidase, and β-lactamase. The enzyme may be directly conjugated to the biomarker-specific reagent, or may be indirectly associated with the biomarker-specific reagent via a labeling conjugate. As used herein, a "labeling conjugate" comprises:

(a) a specific detection reagent; and

(b) an enzyme conjugated to the specific detection reagent, wherein the enzyme is reactive with the chromogenic substrate, signaling conjugate, or enzyme-reactive dye under appropriate reaction conditions to effect *in situ* generation of the dye and/or deposition of the dye on the tissue sample.

In non-limiting examples, the specific detection reagent of the labeling conjugate may be a secondary detection reagent (such as a species-specific secondary antibody bound to a primary antibody, an anti-hapten antibody bound to a hapten-conjugated biomarker-specific reagent, or a biotin-binding protein bound to a biotinylated biomarker-specific reagent), a tertiary detection reagent (such as a species-specific tertiary antibody bound to a secondary antibody, an anti-hapten antibody bound to a hapten-conjugated secondary biomarker-specific reagent, or a biotin-binding protein bound to a biotinylated secondary biomarker-specific reagent),or other such arrangements. An enzyme thus localized to the sample-bound biomarker-specific reagent can then be used in a number of schemes to deposit a detectable moiety.

In some cases, the enzyme reacts with a chromogenic compound/substrate. Particular non-limiting examples of chromogenic compounds/substrates include 4-nitrophenylphospate (pNPP), fast red, bromochloroindolyl phosphate (BCIP), nitro blue tetrazolium (NBT), BCIP/NBT, fast red, AP Orange, AP blue, tetramethylbenzidine (TMB), 2,2'-azino-di-[3-ethylbenzothiazoline sulphonate] (ABTS), o –dianisidine, 4-chloronaphthol (4-CN), nitrophenyl-β-D-galactopyranoside (ONPG), o-phenylenediamine (OPD), 5-bromo-4-chloro-3-indolyl-β–galactopyranoside (X-Gal), methylumbelliferyl-β-D-galactopyranoside (MU-Gal), p-nitrophenyl-α-D-galactopyranoside (PNP), 5-bromo-4-chloro-3-indolyl- β –D-glucuronide (X-Gluc), 3-amino-9-ethyl carbazol (AEC), fuchsin, iodonitrotetrazolium (INT), tetrazolium blue, or tetrazolium violet.

In some embodiments, the enzyme can be used in a metallographic detection scheme. Metallographic detection methods include using an enzyme such as alkaline phosphatase in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. In some embodiments, the substrate is converted to a redox-active agent by the enzyme, and the redox-active agent reduces the metal ion, causing it to form a detectable precipitate. (see, for example, U.S. Patent Application No. 11/015,646, filed December 20, 2004, PCT Publication No. 2005/003777 and U.S. Patent Application Publication No. 2004/0265922; each of which is incorporated by reference herein in its entirety). Metallographic detection methods include

using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to for form a detectable precipitate. (See, for example, U.S. Patent No. 6,670,113, which is incorporated by reference herein in its entirety).

In some embodiments, the enzymatic action occurs between the enzyme and the dye itself, wherein the reaction converts the dye from a non-binding species to a species deposited on the sample. For example, reaction of DAB with a peroxidase (such as horseradish peroxidase) oxidizes the DAB, causing it to precipitate.

In yet other embodiments, the detectable moiety is deposited via a signaling conjugate comprising a latent reactive moiety configured to react with the enzyme to form a reactive species that can bind to the sample or to other detection components. These reactive species are capable of reacting with the sample proximal to their generation, i.e. near the enzyme, but rapidly convert to a non-reactive species so that the signaling conjugate is not deposited at sites distal from the site at which the enzyme is deposited. Examples of latent reactive moieties include: quinone methide (QM) analogs, such as those described at WO2015124703A1, and tyramide conjugates, such as those described at, WO2012003476A2, each of which is hereby incorporated by reference herein in its entirety. In some examples, the latent reactive moiety is directly conjugated to a dye, such as N,N'-biscarboxypentyl-5,5'-disulfonato-indo-dicarbocyanine (Cy5), 4-(dimethylamino) azobenzene-4'-sulfonamide (DABSYL), tetramethylrhodamine (DISCO Purple), and Rhodamine 110 (Rhodamine). In other examples, the latent reactive moiety is conjugated to one member of a specific binding pair, and the dye is linked to the other member of the specific binding pair. In other examples, the latent reactive moiety is linked to one member of a specific binding pair, and an enzyme is linked to the other member of the specific binding pair, wherein the enzyme is (a) reactive with a chromogenic substrate to effect generation of the dye, or (b) reactive with a dye to effect deposition of the dye (such as DAB). Examples of specific binding pairs include:

(1) a biotin or a biotin derivative (such as desthiobiotin) linked to the latent reactive moiety, and a biotin-binding entity (such as avidin, streptavidin, deglycosylated avidin (such as NEUTRAVIDIN), or a biotin binding protein having a nitrated tyrosine at its biotin binding site (such as CAPTAVIDIN)) linked to a dye or to an enzyme reactive with a chromogenic substrate or

reactive with a dye (for example, a peroxidase linked to the biotin-binding protein when the dye is DAB); and

(2) a hapten linked to the latent reactive moiety, and an anti-hapten antibody linked to a dye or to an enzyme reactive with a chromogenic substrate or reactive with a dye (for example, a peroxidase linked to the biotin-binding protein when the dye is DAB).

In a specific embodiment, the primary predictive biomarker is a protein and the staining method comprises a histochemical protein staining procedure (such as immunohistochemistry or an analogous procedure using other entities specific for the protein biomarker). In some embodiments, the primary predictive is a nucleic acid, and the staining method comprises a histochemical staining procedure using a nucleic acid probe (such as *in situ* hybridization (ISH)). In other embodiments, other types of biomarkers may be detected using specific binding agents for those biomarkers. For example, hyaluronan (HA) is an anionic, nonsulfated glycosaminoglycan that commonly accumulates in certain tumor types. HA typically is detected using an affinity histochemistry technique, wherein the specific binding agent is a fusion protein between an HA binding protein (such as hyaluronan binding protein (uniprot no. Q07021) or TNF-stimulated gene 6 (uniprot no. P98066)) and an immunoglobulin Fc region or a member of a specific binding pair (such as biotin).

Non-limiting examples of commercially available detection reagents or kits comprising detection reagents suitable for use with present methods include: VENTANA ultraView detection systems (secondary antibodies conjugated to enzymes, including HRP and AP); VENTANA iVIEW detection systems (biotinylated anti-species secondary antibodies and streptavidin-conjugated enzymes); VENTANA OptiView detection systems (OptiView) (anti-species secondary antibody conjugated to a hapten and an anti-hapten tertiary antibody conjugated to an enzyme multimer); VENTANA Amplification kit (unconjugated secondary antibodies, which can be used with any of the foregoing VENTANA detection systems to amplify the number of enzymes deposited at the site of primary antibody binding); VENTANA OptiView Amplification system (Anti-species secondary antibody conjugated to a hapten, an anti-hapten tertiary antibody conjugated to an enzyme multimer, and a tyramide conjugated to the same hapten. In use, the secondary antibody is contacted with the sample to effect binding to the primary antibody. Then the sample is incubated with the anti-hapten antibody to effect association of the enzyme to the secondary antibody. The sample is then incubated with the tyramide to effect deposition of

additional hapten molecules. The sample is then incubated again with the anti-hapten antibody to effect deposition of additional enzyme molecules. The sample is then incubated with the detectable moiety to effect dye deposition); VENTANA *ultra*View ISH detection systems (for use with haptenated nucleic acid probes and anti-hapten primary antibodies; kit includes secondary antibodies conjugated to enzymes, including HRP and AP) ; VENTANA ISH iVIEW detection systems (for use with haptenated nucleic acid probes and anti-hapten primary antibodies; biotinylated anti-species secondary antibodies and streptavidin-conjugated enzymes); VENTANA DISCOVERY, DISCOVERY OmniMap, DISCOVERY UltraMap anti-hapten antibody, secondary antibody, chromogen, fluorophore, and dye kits, each of which are available from Ventana Medical Systems, Inc. (Tucson, Arizona); PowerVision and PowerVision+ IHC Detection Systems (secondary antibodies directly polymerized with HRP or AP into compact polymers bearing a high ratio of enzymes to antibodies); and DAKO EnVision™+ System (enzyme labeled polymer that is conjugated to secondary antibodies).

B. Heterogeneity analysis and treatment selection

Turning back to Fig. 1, once the primary sample is stained for a first predictive biomarker 101, it is evaluated for heterogeneity 102. In this context, heterogeneity is evaluated on the basis of whether different portions of the stained sample have staining patterns that indicate a difference in response to the primary agent. Where multiple sections from different locations in the tumor or different locations in a FFPE tissue block are evaluated for the first predictive biomarker, the sample shall be considered "heterogenous" if any section demonstrates a heterogenous staining pattern. In this context the term "primary agent" or "1° agent" shall refer to a therapeutic course for which the first predictive biomarker is predictive.

If the sample is not heterogenous (that is, the entire first portion of the tumor has a staining pattern indicting the same response), then the user evaluates the staining pattern to determine whether the tumor is likely to respond to the primary agent 103. If the answer is yes, then the subject is treated with the primary agent 104. If the answer is no, then a tumor area is marked as a region of interest (ROI) in the primary sample, the ROI is transferred to an automated dissection tool, the ROI is excised from a sufficient number of unstained serial sections of the primary sample with an automated dissection tool, a nucleic acid sample is generated from the excised sample 105. In this context, "sufficient number" shall mean at

least enough sections to provide sufficient material to perform a next generation sequencing (NGS) process. The nucleic acid sample is evaluated by NGS for the presence of mutations correlating with additional predictive biomarkers (termed "secondary predictive biomarkers" or "2° predictive biomarkers") 106. A user (such as a pathologist) selects secondary predictive biomarkers based on the mutation analysis, additional portions of the tumor (termed "secondary samples" or "2° samples) are then stained for the secondary predictive biomarkers, and the staining pattern(s) are analyzed to determine whether the tumor is likely to respond to the secondary agent(s) 107. In this context the term "secondary agent" or "2° agent" shall refer to a therapeutic course for which the secondary predictive biomarker is predictive. The secondary agent(s) to which the tumor is likely to respond are selected as treatment candidates 108. In an embodiment, the secondary samples are serial sections of the first portion of the samples.

If the sample is heterogenous (that is, the first portion of the tumor has a staining pattern in at least one region that indicates the different response from the rest of the tumor), then the regions of the primary sample having a staining pattern indicating a lack of response are marked as an ROI, the ROI is transferred to an automated dissection tool, the ROI is excised from a sufficient number of unstained serial sections of the primary sample with an automated dissection tool, and a nucleic acid sample is generated from the excised sample 109. The nucleic acid sample is evaluated by NGS for the presence of mutations correlating with additional predictive biomarkers (termed "secondary predictive biomarkers" or "2° predictive biomarkers") 110. A user (such as a pathologist) selects secondary predictive biomarkers based on the mutation analysis, additional portions of the tumor (termed "secondary samples" or "2° samples) are stained for the secondary predictive biomarkers, and the staining pattern(s) are analyzed to determine whether the tumor is likely to respond to the secondary agent(s) 111. The primary agent and any secondary agent(s) to which the tumor is likely to respond are selected as treatment candidates 112. In an embodiment, the secondary samples are serial sections of the first portion of the samples.

IV. Systems

In an embodiment, systems are provided for performing the methods described herein. In an embodiment, a system is provided including on one or more of an automated dissection apparatus, an NGS platform, and/or an automated slide staining platform, the system being adapted to perform the methods as described herein. The systems may also include an image

analysis system for assessing staining patterns of stained slides and for capturing and storing images thereof, and/or LIS for tracking samples and workflows, storing diagnostic information about the samples, and/or tracking or providing instructions for assays to be performed on samples.

A. Automated dissection tools

Automated dissection tools are devices that automatically excise tissue from slides. Typical automated dissection tools have two main components: (1) a tissue removal component that interacts with the tissue on the slide in a manner that precisely excises ROIs without substantially removing non-interested areas of the tissue; and (2) a computer-implemented guidance system that allows the user to select regions for excision in an image of the slide and guides the tissue removal component. Automated dissection tools generally fall into two categories: laser microdissection and mesodissection.

Laser microdissection tools typically comprise a microscope and a laser beam (with wavelengths in the infrared and/or ultraviolet range). A review of various laser microdissection technologies can be found at Legres *et al.*. The user selects cells for excision from the guidance system, the laser cuts the area surrounding the ROI, and the cells of the ROI are removed. In an embodiment, the automated dissection tool is a laser microdissection tool.

Mesodissection tools essentially are tissue mills. In the typical design, a slide is placed on a stage that controls X and Y axis. The tissue is forced against a rotating cutting bit to cut the desired sections from the slide, and the cut sections are removed from the slide. An example of a mesodissection tool is described by Adey *et al.* In the example described by Adey, a cutting bit is used that simultaneously dispenses a liquid on the slide and aspirates the liquid from the slide. As the tissue is cut, it is suspended in the liquid and aspirated along with the aspirated liquid. A software system is provided that allows the user to digitally annotate the tissue sections for excision. In an embodiment, the automated dissection tool is a mesodissection tool.

B. Next generation sequencing systems

As used herein, a "next generation sequencing platform" is any nucleic acid sequencing platform based on massively parallel sequencing (MPS): sequencing millions to billions of short read fragments (from 10s to 100s of bases in length) simultaneously. MPS

typically can achieve an output of at least 10 Mbp per 18 hour cycle. NGS platforms can be broadly separated into categories on the basis of (1) template preparation method; and (2) process for performing MPS. Table 2 includes some examples of template preparation methods:

Template preparation methods	**Commercial Sequencers**
Emulsion PCR	ROCHE 454; Life Technologies Ion Proton; Life Technologies Ion Torrent
Clonal Bridge Amplification	Illumina MiSeq; Illumina HiSeq; Illumina Genome Analyzer IIX
Rolling circle amplification	Complete Genomics
Single molecule	Helicos Biosciences Heliscope; Pacific Biosciences SMRT

Table 2

Table 3 includes some examples of strategies for MPS:

MPS Strategy		**Commercial Sequencers**
Pyrosequencing	**Description**	Templates are amplified using sequential addition of a single dNTPs. Pyrophosphate produced from the reaction is enzymatically converted proportionally to a visible signal, which is detected and recorded as a flowgram
	Sequencers	ROCHE 454
Cyclic reversible termination	**Description**	"Bases are read using a cyclic reversible termination strategy, which sequences the template strand one nucleotide at a time through progressive rounds of base incorporation, washing, imaging and cleavage. In this strategy, fluorescently-labeled 3´-O-azidomethyldNTPs are used to pause the polymerization reaction, enabling removal of unincorporated bases and fluorescent imaging to determine the added nucleotide. Following scanning of the flow cell with a coupled-charge device (CCD) camera, the fluorescent moiety and the 3´ block are removed, and the process is repeated." Reuter *et al.*
	Sequencers	Illumina MiSeq; Illumina HiSeq; Illumina Genome Analyzer IIX; Helicos Biosciences Heliscope

semiconductor sequencing technology	**Description**	"[Sequencing occurs by] a sequencing-by-synthesis reaction. . . . pH changes induced by the release of hydrogen ions during DNA extension. These pH changes are detected by a sensor . . . and converted into a voltage signal. The voltage signal is proportional to the number of bases incorporated, and the sequential addition of individual nucleotides during each sequencing cycle allows base discrimination." Reuter *et al.*
	Sequencers	Life Technologies Ion Proton; Life Technologies Ion Torrent
Phospholinked Fluorescent Nucleotides	**Description**	"DNA synthesis occurs in zeptoliter-sized chambers, called zero-mode waveguides (ZMW), in which a single polymerase is immobilized at the bottom of the chamber. The physics of these chambers reduces background noise such that phosphate-labeled versions of all 4 nucleotides can be present simultaneously. Thus, polymerization occurs continuously, and the DNA sequence can be read in real-time from the fluorescent signals recorded in a video." Reuter *et al.*
	Sequencers	Pacific Biosciences SMRT

Table 3

In an embodiment, the NGS method includes one or more technologies from Table 2 or Table 3.

C. Automated slide stainers

In some embodiments, the system includes an automated slide staining platform. Automated slide stainers typically include at least: reservoirs of the various reagents used in the staining protocols, a reagent dispense unit in fluid communication with the reservoirs for dispensing reagent to onto a slide, a waste removal system for removing used reagents and other waste from the slide, and a control system that coordinates the actions of the reagent dispense unit and waste removal system. In addition to performing staining steps, many automated slide stainers can also perform steps ancillary to staining (or are compatible with separate systems that perform such ancillary steps), including: slide baking (for adhering the sample to the slide), dewaxing (also referred to as deparaffinization), antigen retrieval, counterstaining, dehydration and clearing, and coverslipping. The Prichard reference describes several specific examples of automated IHC/ISH slide stainers and their various features, including the intelliPATH (Biocare Medical), WAVE (Celerus Diagnostics), DAKO OMNIS and DAKO AUTOSTAINER LINK 48 (Agilent Technologies), BENCHMARK

(Ventana Medical Systems, Inc.), Leica BOND, and Lab Vision Autostainer (Thermo Scientific) automated slide stainers. Additionally, Ventana Medical Systems, Inc. is the assignee of a number of United States patents disclosing systems and methods for performing automated analyses, including U.S. Pat. Nos. 5,650,327, 5,654,200, 6,296,809, 6,352,861, 6,827,901 and 6,943,029, and U.S. Published Patent Application Nos. 20030211630 and 20040052685, each of which is incorporated herein by reference in its entirety.

Commercially-available staining units typically operate on one of the following principles: (1) open individual slide staining, in which slides are positioned horizontally and reagents are dispensed as a puddle on the surface of the slide containing a tissue sample (such as implemented on the DAKO AUTOSTAINER Link 48 (Agilent Technologies) and intelliPATH (Biocare Medical) stainers); (2) liquid overlay technology, in which reagents are either covered with or dispensed through an inert fluid layer deposited over the sample (such as implemented on VENTANA BenchMark and DISCOVERY stainers); (3) capillary gap staining, in which the slide surface is placed in proximity to another surface (which may be another slide or a coverplate) to create a narrow gap, through which capillary forces draw up and keep liquid reagents in contact with the samples (such as the staining principles used by DAKO TECHMATE, Leica BOND, and DAKO OMNIS stainers). Some iterations of capillary gap staining do not mix the fluids in the gap (such as on the DAKO TECHMATE and the Leica BOND). In variations of capillary gap staining termed dynamic gap staining, capillary forces are used to apply sample to the slide, and then the parallel surfaces are translated relative to one another to agitate the reagents during incubation to effect reagent mixing (such as the staining principles implemented on DAKO OMNIS slide stainers (Agilent)). In translating gap staining, a translatable head is positioned over the slide. A lower surface of the head is spaced apart from the slide by a first gap sufficiently small to allow a meniscus of liquid to form from liquid on the slide during translation of the slide. A mixing extension having a lateral dimension less than the width of a slide extends from the lower surface of the translatable head to define a second gap smaller than the first gap between the mixing extension and the slide. During translation of the head, the lateral dimension of the mixing extension is sufficient to generate lateral movement in the liquid on the slide in a direction generally extending from the second gap to the first gap. *See* WO 2011-139978 A1. It has recently been proposed to use inkjet technology to deposit reagents on slides. *See* WO 2016-170008 A1. This list of automated staining technologies is not

intended to be comprehensive, and any fully or semi-automated system for performing biomarker staining may be used.

D. Image analysis systems

In some embodiments, digital images of the stained slides are analyzed instead of (or in addition to) live reading on a microscope. In such embodiments, the stained slides can be imaged on a imager slide scanner. At a basic level, slide scanners generate a representative digital image of the stained sample. The typical slide scanner includes at least: (1) a microscope with lens objectives, (2) a light source (such as halogen, light emitting diode, white light, and/or multispectral light sources), (3) robotics to move glass slides around (or to move the optics around the slide), (4) one or more digital cameras for image capture, (5) a computer and associated software to control the robotics and to manipulate, manage, and view digital slides. Digital data at a number of different X-Y locations (and in some cases, at multiple Z planes) on the slide are captured by the camera's charge-coupled device (CCD), and the images are joined together to form a composite image of the entire scanned surface. Common methods to accomplish include:

(1) Tile based scanning, in which the slide stage or the optics are moved in very small increments to capture square image frames, which overlap adjacent squares to a slight degree. The captured squares are then automatically matched to one another to build the composite image; and

(2) Line-based scanning, in which the slide stage moves in a single axis during acquisition to capture a number of composite image "strips." The image strips can then be matched with one another to form the larger composite image. In some cases,

A detailed overview of various slide scanners can be found at Farahani *et al.* Examples of slide scanners include: 3DHistech PANNORAMIC SCAN II; DigiPath PATHSCOPE; Hamamatsu NANOZOOMER RS, HT, and XR; Huron TISSUESCOPE 4000, 4000XT, and HS; Leica SCANSCOPE AT, AT2, CS, FL, and SCN400; Mikroscan D2; Olympus VS120-SL; Omnyx VL4, and VL120; PerkinElmer LAMINA; Philips ULTRA-FAST SCANNER; Sakura Finetek VISIONTEK; Unic PRECICE 500, and PRECICE 600x; VENTANA ISCAN COREO and ISCAN HT; and Zeiss AXIO SCAN.Z1. Other exemplary systems and features can be found in, for example, International Patent Application No.: PCT/US2010/002772

(Patent Publication No.: WO/2011/049608) entitled IMAGING SYSTEM AND TECHNIQUES or disclosed in U.S. Patent Application No. 61/533,114, filed on Sep. 9, 2011, entitled IMAGING SYSTEMS, CASSETTES, AND METHODS OF USING THE SAME. International Patent Application No. PCT/US2010/002772 and U.S. Patent Application No. 61/533,114 are incorporated by reference in their entities.

In some embodiments, the image analysis system is integrated with the automated dissection tool, such that ROIs identified by the pathologist in the image analysis system may be transferred directly to the automated dissection tool for identification of regions to be excised from the slide. In other embodiments, the image analysis system is adapted only for diagnostic evaluation of the microscope slides, and a separate imaging system is integrated with the automated dissection tool for identifying ROIs and directing the excision thereof.

An exemplary system including an image analysis system is illustrated at Fig. 2. An automated slide stainer 201 is provided to stain the primary slide of a set of slides from a single sample 202. The stained slide is scanned by the image analysis system 203, and the pathologist reviews the staining pattern and determines (a) whether the sample is heterogenous, and (b) whether any portion of the sample is likely to respond to the drug for which the primary biomarker is diagnostic. The pathologist manually marks any non-responsive regions as an ROI in the image analysis system 203, which is transferred to an automated dissection tool 204. Unstained slides from the set of slides containing serial sections of the primary slide are transferred to the automated dissection tool 204, the ROI is matched to the unstained sections, and the ROI is excised. The excised portion of the sample is processed to obtain a nucleic acid sample and the nucleic acid sample is sequenced on a NGS sequencer 205. A software suite is used to identify mutations associated with the sample, and the user selects one or more of the mutations that is associated with a predictive biomarker. Unstained slides from the set of slides 202 are passed to the automated slide stainer 201 (which may be the same or different from the automated slide stainer that stained the primary slide). The automated slide stainer 201 stains the unstained slides with biomarker-specific reagents for the predictive biomarkers selected by the user. If desired, the slides stained with the secondary predictive biomarkers may be evaluated on the image analysis system 203. When all slides have been evaluated, a diagnostic report 206 including the prediction for each predictive biomarker is generated, from which the treating physician may make diagnostic and therapeutic decisions.

E. Laboratory information systems

The system may further include a LIS. LIS typically performs one or more functions selected from: recording and tracking processes performed on samples and on slides and images derived from the samples, instructing different components of the system to perform specific processes on the samples, slides, and/or images, and track information about specific reagents applied to samples and or slides (such as lot numbers, expiration dates, volumes dispensed, etc.). LIS usually comprises at least a database containing information about samples; labels associated with samples, slides, and/or image files (such as barcodes (including 1-dimensional barcodes and 2-dimensional barcodes), radio frequency identification (RFID) tags, alpha-numeric codes affixed to the sample, and the like); and a communication device that reads the label on the sample or slide and/or communicates information about the slide between the LIS and the other components of the immune context scoring system. Thus, for example, a communication device could be placed at each of a sample processing station, automated slide stainer, automated dissection tool, and NGS system. When the sample is initially processed into sections, information about the sample (such as patient ID, sample type, processes to be performed on the section(s)) may be entered into the communication device, and a label is created for each section generated from the sample. At each subsequent station, the label is entered into the communication device (such as by scanning a barcode or RFID tag or by manually entering the alpha-numeric code), and the station electronically communicates with the database to, for example, instruct the station or station operator to perform a specific process on the section and/or to record processes being performed on the section. At a scanning platform, the scanning platform may also encode each image with a computer-readable label or code that correlates back to the section or sample from which the image is derived, such that when the image is sent to the image analysis system, image processing steps to be performed may be sent from the database of LIS to the image analysis system and/or image processing steps performed on the image by image analysis system are recorded by database of LIS. Commercially available LIS systems useful in the present methods and systems include, for example, VENTANA Vantage Workflow system (Roche).

An exemplary system including an LIS and an optional image analysis system is illustrated at Fig. 3. A tissue sample 301 is divided into a plurality of samples, including at least one primary sample 302 mounted on a microscope slide and a set of secondary samples 303, also mounted on microscope slides. Instructions to stain the primary sample 302 for a

first predictive biomarker are recorded into an LIS 304 and input into an automated slide stainer 305. In some embodiments, the instructions are sent from the LIS 304 to the automated slide stainer 305 and automatically implemented on the automated slide stainer 305. In some embodiments, the LIS 304 generates a label associated with the primary sample 302. In some embodiments, the label bears the instructions imprinted on the label, which the user may manually enter into the automated slide stainer 305. In other embodiments, the label includes a designation (such as a barcodes (including 1-dimensional barcodes and 2-dimensional barcodes), radio frequency identification (RFID) tags, alpha-numeric codes, and the like) readable by a station at the automated slides stainer to either to directly instruct the automated slide stainer 305 of the program to implement on the slide, or to inform the user of how to program the automated slide stainer 305. The slide is stained by the automated slide stainer 305 for the first predictive biomarker to obtain the primary stained slide 306. If evaluation on an image analysis system is desired, the primary stained slide 306 is scanned by the image analysis system 307, and the pathologist reviews the staining pattern and determines (a) whether the sample is heterogenous, and (b) whether any portion of the sample is likely to respond to the drug for which the primary biomarker is diagnostic. If image analysis is not desired, the pathologist performs the same analysis on a microscope. In either case, the pathologist's analysis is recorded in the LIS 304. Where image analysis is performed, digital images of the primary stained slide 306 may also be recorded in the LIS 304, which may also be manually annotated to identify the ROI. The primary stained slide 306 is then transferred to the automated dissection tool 308, and the ROI is identified in a sufficient number of unstained serial sections of the primary sample 302 and excised. In some embodiments, the ROI is identified *de novo* on the automated dissection tool 308, for example, by a technician or pathologist identifying morphological structures in the unstained section that correlate to the ROI from the primary slide. In other embodiments, the ROI is transferred to the automated dissection tool 308 from the image analysis system 307 or the LIS 304, which may then be modified or accepted by the user. The precise portion which is excised may be recorded in the LIS 304. The excised portion of the sample is processed to obtain a nucleic acid sample 309 and the nucleic acid sample is sequenced on a NGS sequencer 310. A software suite associated with the NGS identifies mutations associated with the sample, and the user selects one or more of the mutations that is associated with a predictive biomarker. The mutations identified by the NGS 310 and the secondary predictive biomarkers selected by the user may be stored in the LIS 304. Instructions to stain the

secondary sample(s) 303 for additional predictive biomarker(s) are recorded into the LIS 304 and input into an automated slide stainer 305 (which may be the same or different from the automated slide stainer that stained the primary slide). In some embodiments, the instructions are sent from the LIS 304 to the automated slide stainer 305 and automatically implemented on the automated slide stainer 305. In some embodiments, the LIS 304 generates a label associated with the primary sample 302. In some embodiments, the label bears the instructions imprinted on the label, which the user may manually enter into the automated slide stainer 305. In other embodiments, the label includes a designation (such as a barcodes (including 1-dimensional barcodes and 2-dimensional barcodes), radio frequency identification (RFID) tags, alpha-numeric codes, and the like) readable by a station at the automated slides stainer to either to directly instruct the automated slide stainer 305 of the program to implement on the slide, or to inform the user of how to program the automated slide stainer 305. The slide is stained by the automated slide stainer 305 for the additional predictive biomarker(s) to obtain the secondary stained slides 311. If image analysis is desired, the secondary stained slide(s) 306 is/are scanned by the image analysis system 307, and the pathologist reviews the staining pattern and determines whether any portion of the sample is likely to respond to the drug(s) for which the secondary biomarker(s) is/are diagnostic. If image analysis is not desired, the pathologist performs the same analysis on a microscope. In either case, the pathologist's analysis is recorded in the LIS 304. Where image analysis is performed, digital images of the secondary stained slide(s) 311 may also be recorded in the LIS 304. When all slides have been analyzed, a diagnostic report 312 including the evaluation for each predictive biomarker is generated, from which the treating physician may make diagnostic and therapeutic decisions.

V. Examples

To test feasibility of the described methods and workflows, and to determine if it could be used to gain predictive diagnostic information about a case, a model system was developed using PTEN or EGFR as a primary predictive biomarker. All histochemical stains were performed on a VENTANA BenchMark ULTRA IHC/ISH slide stainer. ROI identification and excision was performed using a ROCHE Automated Dissection Tool mesodissection instrument. DNA was isolated using Roche MagNA Pure96, and quality control of the thus obtained DNA sample was performed by qPCR utilizing a Roche Lightcycler 480. 10ng of DNA was obtained for highest library prep success rate (1.6ng/μL

preferred). A total number of slides necessary for each ROI was calculated based on the assumption that ~250mm^2 of tissue would be required. Targeted sequencing was performed on a Life Technologies ION TORRENT PGM NGS platform utilizing Life Technology Cancer HotSpot Panel v2 and the on-system variant analysis.

In a first example, a prostate tumor sample was stained for PTEN via IHC. Positively-stained tumor regions were isolated and sequenced using only a filter for coverage and non-synonymous mutations. Results are shown at Table 4:

Table 4

Mutation	Allele Called	Frequency (>500 coverage)
FGFR3	Novel	100%
PDGFRA	Hotspot	47.2%
KDR	Novel	49.2%
CSF1R	Novel	98.8%
EGFR	Novel	36.4%
RET	Novel	100%
TP53	Novel	27.25%
ERBB2	Novel	4.3%
SMAD4	Novel	12.4%

*not all SNPs listed

For quality purposes only mutations that had coverage great than 500X (meaning that SNP was sequenced a least 500 times) were recorded. From this list, IHC tests are commercially available for EGFR expression and HER2 which is encoded by the ERBB2 gene. Using two more slides for these tests, over-expression of EGFR and unexpected expression of HER2 in a prostate cancer case were observed, both of which could identify additional treatment targets.

A second prostate case was again stained for PTEN by IHC, the image of which can be seen at Fig. 5. A section of positively-staining sample was sequenced, the results of which are displayed at Table 5:

Table 5

Mutation	Allele Called	Frequency (>500 coverage)
FGFR3	Novel	100%
PDGFRA	Novel	100%
KIT	Novel	13.7%
APC	Novel	47.1%
JAK2	Novel	28.2%
RET	Novel	100%
PTEN	Deletion	6.3%
HRAS	Hotspot	91.3%
EZH2	Novel	4.3%

*not all SNPs listed

Additional sections were stained for EZH2, results of which can be seen at Fig. 5. c-Kit was additionally recognized as a potential predictive biomarker. However, IHC stain revealed no staining of interest. This illustrates the point that the presence of a mutation correlating with a predictive biomarker does not necessary provide a clinically actionable course of treatment.

A lung case was stained via IHC for a single nucleotide polymorphism of EGFR: EGFR L858R. Image is displayed at Fig. 6. A region negative for the mutation was excised and sequenced. Results are shown at Table 6. A p53 IHC stain was performed, an image of which can be seen at Fig. 6. The stained slide demonstrated a loss of p53 expression throughout, which is predictive for cisplatin-based chemotherapy and MDM2 antagonists.

The lung case was also used to test the necessity of the dissection tool as opposed to sequencing the whole sample. As can be seen at Table 7, some mutations that are detectable in excised portions of the sample can be lost if the whole sample is sequenced. This shows the importance of sequencing annotated tumor regions rather than the whole tissue.

Table 6

Mutation	Allele Called	Frequency (>500 coverage)
KIT	Hotspot	40.8%
EGFR	Hotspot	16.5%
PTEN	Hotspot	7.6%
HRAS	Hotspot	36.1%
TP53 (7)	Hotspot	14.4 – 23.4%
FGFR3	Novel	100%
CSF1R	Novel	100%
RET	Novel	54.9%
SMAD4	Deletion	69.8%

*not all SNPs listed

Table 7

Excised Tumor

Mutation	Allele Called	Frequency (>500 coverage)
KIT	Hotspot	40.8%
EGFR	Hotspot	16.5%
PTEN	Hotspot	7.6%
HRAS	Hotspot	36.1%
PT53 (7)	Hotspot	14.4 – 23.4%
FGFR3	Novel	100%
CSF1R	Novel	100%
RET	Novel	54.9%
SMAD4	Deletion	69.8%

*not all SNPs listed

Whole Specimen

Mutation	Allele Called	Frequency (>500 coverage)
KIT	Hotspot	46.1%
EGFR	Hotspot	8.5%
PTEN	Hotspot	Lost sequence
HRAS	Hotspot	44.8%
PT53 (7)	Hotspot	Lost sequence
FGFR3	Novel	100%
CSF1R	Novel	100%
RET	Novel	52.3%
SMAD4	Deletion	Lost sequence

*not all SNPs listed

VII. ADDITIONAL EXEMPLARY EMBODIMENTS

The following additional embodiments are also specifically disclosed. This is not intended to be an exhaustive list.

1. A method comprising:

obtaining a first sample of a tumor, wherein the first sample is histochemically stained for a first predictive biomarker for a first therapeutic agent;

excising one or more region(s) from the first sample with a automated dissection tool, wherein the excised region has a staining pattern for the first predictive biomarker indicating that the region is unlikely to respond to the first therapeutic agent;

detecting with a next generation sequencer one or more one or more mutations predictive of a response to one or more additional therapeutic agents in a nucleic acid sample derived from the excised region(s) of the first sample;

staining one or more additional samples of the tumor for one or more additional predictive biomarker(s) correlating to the one or more mutations identified in the samples, the one or more additional predictive biomarkers being predictive of a response to one or more of the additional therapeutic agent(s).

2. A method comprising:

obtaining:

a first sample of a tumor stained for a first predictive biomarker for a first therapeutic agent, wherein a staining pattern of the first predictive biomarker indicates that at least a first region of the first sample is unlikely to respond to the first therapeutic agent; and

a nucleic acid sample derived from the first region of the first sample, wherein the nucleic acid sample is derived from the first region of the first portion of the tumor by excising the first region of the first sample with a automated dissection tool, and extracting genomic DNA from the excised portion of the sample;

detecting with a next generation sequencer one or more mutations in the nucleic acid sample predictive of a response to one or more additional therapeutic agents;

staining one or more additional samples of the tumor for one or more additional predictive biomarker(s) correlating to the one or more mutations identified in the samples, the one or more additional predictive biomarkers being predictive of a response to one or more of the additional therapeutic agent(s).

3. A method comprising:

obtaining:

a first sample of a tumor stained for a first predictive biomarker for a first therapeutic compound, wherein a staining pattern of the first predictive biomarker indicates that at least a first region of the first sample is unlikely to respond to the first therapeutic agent; and

one or more additional samples of the tumor;

staining the one or more additional samples for one or more additional predictive biomarkers for additional therapeutic agents, wherein the additional predictive biomarkers correspond to one or more mutations identified by a next generation sequencer in a nucleic acid sample excised with a automated dissection tool from the first region of the first sample, wherein the one or more mutations are predictive of a response to one or more additional therapeutic agents.

4. A method comprising:

staining a first sample of a tumor with:

a first specific binding agent that is specific for a first predictive biomarker for a first therapeutic agent, and

a set of detection reagents for visualizing the specific binding agent when bound to the first section; and

staining one or more additional sample of the tumor with:

one or more additional specific binding agent specific for one or more predictive biomarker(s) for at least one additional therapeutic agent, wherein the additional predictive biomarker(s), wherein the additional predictive biomarker(s) correspond to nucleic acids identified in a region extracted from the first section with a automated dissection tool, wherein the region extracted with the automated dissection tool has a staining pattern for the first biomarker indicating that at least a portion of the tumor is unlikely to respond to the first therapeutic agent, and

a set of detection reagents for visualizing the specific binding agent when bound to the second section.

5. The method of any of embodiments 1–4, further comprising:

generating a report identifying a therapeutic course for the subject, said therapeutic course comprising administering to the subject:

the first therapeutic agent if at least one region of the first sample has a staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is likely to respond to the first therapeutic agent; and

one or more of the additional therapeutic agent(s) if at least one region of the additional sample(s) has a staining pattern for the corresponding additional predictive biomarker indicating that at least a portion of the tumor is likely to respond to the additional therapeutic agent.

6. The method of any of embodiments 1–4, further comprising:

administering a therapeutic course for the subject, said therapeutic course comprising:

the first therapeutic agent if at least one region of the first sample has a staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is likely to respond to the first therapeutic agent; and/or

one or more of the additional therapeutic agent(s) if at least one region of the additional sample(s) has a staining pattern for the corresponding additional predictive biomarker indicating that at least a portion of the tumor is likely to respond to the additional therapeutic agent.

7. The method of any of embodiments 1–6, wherein the tumor is a solid tumor.

8. The method of embodiment 7, wherein the solid tumor is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, and the first sample and the additional sample(s) are microtome sections of the FFPE tissue sample.

9. The method of embodiment 8, wherein first sample and the additional sample(s) are serial sections.

10. The method of any of embodiments 1–9, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

11. The method of any of embodiments 1–10, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1,

estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

12. A system comprising:

(a) a set of microscope slides comprising:

(a1) a first microscope slide having deposited thereon a first sample of a tumor, wherein the first sample is histochemically stained for a first predictive biomarker for a first therapeutic agent;

(a2) one or more additional unstained microscope slides having deposited thereon an additional sample of the tumor;

(b) an image analysis system for identifying one or more regions of the first sample having a staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is unlikely to respond to the first therapeutic agent;

(c) an automated dissection tool programmed to excise the one or more regions of the first sample having a staining pattern for the first predictive biomarker characteristic of a lack of response to the first therapeutic agent from the first sample;

(d) a next generation sequencer programmed to identify the presence or absence of mutations correlated with one or more additional predictive biomarkers in a nucleic acid sample derived from the regions of the first sample excised by the automated dissection tool; and

(e) an automated slide stainer programmed to stain the additional slide(s) with one or more of the additional predictive biomarker(s).

13. The system of embodiment 12, further comprising:

(f) a laboratory information system (LIS) comprising a database, the database containing:

(f1) a mutation analysis of the nucleic acid sample by the next generation sequencer, wherein the mutation analysis indicates at least the presence or absence of mutations in the nucleic acid sample correlating to one or

more additional predictive biomarker(s) for one or more additional therapeutic agent(s); and

(f2) instructions for directing the automated slide stainer to stain the second sample of the tumor with the one or more additional predictive biomarkers identified by the mutation analysis.

14. The system of embodiment 12, wherein at least one of the unstained microscope slides has affixed thereto a label generated by the LIS and readable by the automated slide stainer, wherein the label identifies the slide as being appropriate for execution of the instructions of (f2) by the automated slide stainer.

15. The system of embodiment 14, wherein the label automatically directs the automated slide stainer to execute the instructions on the second sample.

16. The system of embodiment 14, wherein the label generates a report for an operator of the automated slide stainer, the report instructing the manual operator to program the automated slide stainer to execute the instructions on the second sample.

17. The system of any of embodiments 12–16, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

18. The system of any of embodiments 12–17, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

19. A system comprising:

(a) a nucleic acid sample derived from one or more regions excised from a first sample of a tumor, wherein the first sample of the tumor is stained for a first

predictive biomarker, and further wherein the one or more regions excised from the section have a staining pattern of the first predictive biomarker indicating that at least a portion of the tumor is unlikely to respond to a first therapeutic agent;

(b) a next generation sequencer adapted to identify the presence or absence of mutations correlating to one or more additional predictive biomarkers;

(c) a laboratory information system (LIS) comprising a database, the database containing:

(c1) mutation analysis of a nucleic acid sample by next generation sequencing, wherein the mutation analysis indicates at least the presence or absence of mutations in the nucleic acid sample, the mutations correlating to one or more additional predictive biomarker(s) for one or more additional therapeutic agent(s); and

(c2) instructions for directing an automated slide stainer to stain a second sample of the tumor with the one or more additional predictive biomarkers identified by the mutation analysis.

20. The system of embodiment 19, further comprising:

(d) an unstained microscope slide having deposited thereon the second sample of the tumor; and

(e) an automated slide stainer adapted to stain the second sample according to the instructions of (c2).

21. The system of embodiment 20, wherein the unstained microscope slide has affixed thereto a label generated by the LIS and readable by the automated slide stainer, wherein the label identifies the slide as being appropriate for execution of the instructions of (c2) by the automated slide stainer.

22. The system of embodiment 21, wherein the label automatically directs the automated slide stainer to execute the instructions on the second sample.

23. The system of embodiment 21, wherein the label generates a report for an operator of the automated slide stainer, the report instructing the manual operator to program the automated slide stainer to execute the instructions on the second sample.

24. The system of any of embodiments 19–23, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

25. The system of any of embodiments 19–24, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

26. A system comprising:

(a) an unstained microscope slide having deposited thereon a first sample of a tumor;

(b) an automated slide stainer; and

(c) a laboratory information system (LIS) comprising a database, the database containing:

(c1) diagnostic information of a second sample of the tumor, wherein the second sample is stained for a first predictive biomarker for a first therapeutic agent;

(c2) mutation analysis of a nucleic acid sample by next generation sequencing, wherein the nucleic acid sample is obtained from a portion of the second sample having a staining pattern for the first predictive biomarker indicating that at least a portion of the second sample is unlikely to respond to the first therapeutic agent, and wherein the mutation analysis indicates at least the presence or absence of mutations in the nucleic acid sample correlating, the mutations

correlating to one or more additional predictive biomarker(s) for one or more additional therapeutic agent(s); and

(c3) instructions for directing the automated slide stainer to stain the first sample of the tumor with the one or more additional predictive biomarkers identified by the mutation analysis.

27. The system of embodiment 26, wherein the unstained microscope slide has affixed thereto a label generated by the LIS and readable by the automated slide stainer, wherein the label identifies the slide as being appropriate for execution of the instructions of (c2) by the automated slide stainer.

28. The system of embodiment 27, wherein the label automatically directs the automated slide stainer to execute the instructions on the second sample.

29. The system of embodiment 27, wherein the label generates a report for an operator of the automated slide stainer, the report instructing the manual operator to program the automated slide stainer to execute the instructions on the second sample.

30. The system of any of embodiments 26–29, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

31. The system of any of embodiments 26–30, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

32. The system of any of embodiments 12–31, wherein the tumor is a solid tumor.

33. The system of embodiment 32, wherein the solid tumor is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, and the samples of the tumor are microtome sections of the FFPE tissue sample.

34. The system of embodiment 33, wherein sample stained for the additional predictive biomarker(s) is a serial section of the sample stained for the first predictive biomarker.

35. A set of diagnostic samples derived from a tumor, said set of diagnostic samples comprising:

(a) a first sample of a tumor, wherein the first sample is stained for a first predictive biomarker for a first therapeutic agent, wherein at least a portion of the first sample has a first staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is unlikely to respond to the first therapeutic agent;

(b) a nucleic acid sample obtained by a method comprising:

(b1) excising with a automated dissection tool the portion of the first sample having the staining pattern indicating that the portion of the tumor is unlikely to respond to the first therapeutic agent; and

(b2) extracting the nucleic acid sample from the excised portion of the first sample in a manner compatible with use of the nucleic acid sample in a next generation sequencer; and

(c) one or more additional samples of the tumor, wherein the additional sample(s) are stained for one or more additional predictive biomarker(s) for one or more additional therapeutic agent(s), wherein the additional predictive biomarker(s) correspond(s) to a mutation identified in the nucleic acid sample.

36. The set of diagnostic samples of embodiment 35, wherein the tumor is a solid tumor.

37. The set of diagnostic samples of embodiment 36, wherein the solid tumor is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, and the samples of the tumor are microtome sections of the FFPE tissue sample.

38. The set of diagnostic samples of embodiment 37, wherein sample stained for the additional predictive biomarker(s) is a serial section of the sample stained for the first predictive biomarker.

39. The set of diagnostic samples of any of embodiments 35–38, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

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40. The set of diagnostic samples of any of embodiments 35–39, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

41. A method comprising histochemically staining a first tissue section of a tumor with a biomarker-specific reagent for a first predictive biomarker, the first predictive biomarker being associated with a mutation identified in a region of a serial section of the first tissue section, wherein the region has a staining pattern for a second predictive biomarker predicted not to respond to a therapeutic agent for which the second predictive biomarker is predictive.

42. A method of embodiment 41, wherein the mutation is identified by:
excising the region from the tissue section using an automated dissection tool; and
sequencing a nucleic acid sample derived from the excised region by a next generation sequencing technique.

43. The method of embodiment 42, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

44. The method of any of embodiments 41–43, wherein the tumor is a solid tumor.

45. The method of embodiment 44, wherein the solid tumor is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, and the first sample and the additional sample(s) are microtome sections of the FFPE tissue sample.

46. The method of any of embodiments 41–45, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

47. Any of embodiments 1-46, wherein the predictive biomarker(s) is/are stained by immunohistochemistry (IHC) or *in situ* hybridization (ISH).

VIII. References

The content of each of the following references is hereby incorporated by reference in its entirety.

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Applied Biosystems, Inc., *Uncovering tumor heterogeneity in FFPE samples by laser capture microdissection and next-generation sequencing* (2015), https://tools.thermofisher.com/content/sfs/brochures/PG1395-PJ6555-CO29666-LCM-App-note-Focus-on-LCM-for-tumor-heterogeneity-Global-FHR.pdf ("AB Brochure").

Farahani *et al.*, *Whole slide imaging in pathology: advantages, limitations, and emerging perspectives*, Pathology and Laboratory Medicine Int'l, Vol. 7, p. 23–33 (June 2015), the content of which is incorporated by reference in its entirety.

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CLAIMS

What is claimed is:

1. A method comprising:

obtaining a first sample of a tumor, wherein the first sample is histochemically stained for a first predictive biomarker for a first therapeutic agent;

excising one or more region(s) from the first sample with a automated dissection tool, wherein the excised region has a staining pattern for the first predictive biomarker indicating that the region is unlikely to respond to the first therapeutic agent;

detecting with a next generation sequencer one or more one or more mutations predictive of a response to one or more additional therapeutic agents in a nucleic acid sample derived from the excised region(s) of the first sample;

staining one or more additional samples of the tumor for one or more additional predictive biomarker(s) correlating to the one or more mutations identified in the samples, the one or more additional predictive biomarkers being predictive of a response to one or more of the additional therapeutic agent(s).

2. A method comprising:

obtaining:

a first sample of a tumor stained for a first predictive biomarker for a first therapeutic agent, wherein a staining pattern of the first predictive biomarker indicates that at least a first region of the first sample is unlikely to respond to the first therapeutic agent; and

a nucleic acid sample derived from the first region of the first sample, wherein the nucleic acid sample is derived from the first region of the first portion of the tumor by excising the first region of the first sample with a automated dissection tool, and extracting genomic DNA from the excised portion of the sample;

detecting with a next generation sequencer one or more mutations in the nucleic acid sample predictive of a response to one or more additional therapeutic agents;

staining one or more additional samples of the tumor for one or more additional predictive biomarker(s) correlating to the one or more mutations identified in

the samples, the one or more additional predictive biomarkers being predictive of a response to one or more of the additional therapeutic agent(s).

3. A method comprising:

obtaining:

a first sample of a tumor stained for a first predictive biomarker for a first therapeutic compound, wherein a staining pattern of the first predictive biomarker indicates that at least a first region of the first sample is unlikely to respond to the first therapeutic agent; and

one or more additional samples of the tumor;

staining the one or more additional samples for one or more additional predictive biomarkers for additional therapeutic agents, wherein the additional predictive biomarkers correspond to one or more mutations identified by a next generation sequencer in a nucleic acid sample excised with a automated dissection tool from the first region of the first sample, wherein the one or more mutations are predictive of a response to one or more additional therapeutic agents.

4. A method comprising:

staining a first sample of a tumor with:

a first specific binding agent that is specific for a first predictive biomarker for a first therapeutic agent, and

a set of detection reagents for visualizing the specific binding agent when bound to the first section; and

staining one or more additional sample of the tumor with:

one or more additional specific binding agent specific for one or more predictive biomarker(s) for at least one additional therapeutic agent, wherein the additional predictive biomarker(s), wherein the additional predictive biomarker(s) correspond to nucleic acids identified in a region extracted from the first section with a automated dissection tool, wherein the region extracted with the automated dissection tool has a staining pattern for the first biomarker indicating that at least a portion of the tumor is unlikely to respond to the first therapeutic agent, and

a set of detection reagents for visualizing the specific binding agent when bound to the second section.

5. The method of any of claims 1–4, further comprising:

generating a report identifying a therapeutic course for the subject, said therapeutic course comprising administering to the subject:

the first therapeutic agent if at least one region of the first sample has a staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is likely to respond to the first therapeutic agent; and

one or more of the additional therapeutic agent(s) if at least one region of the additional sample(s) has a staining pattern for the corresponding additional predictive biomarker indicating that at least a portion of the tumor is likely to respond to the additional therapeutic agent.

6. The method of any of claims 1–4, further comprising:

administering a therapeutic course for the subject, said therapeutic course comprising:

the first therapeutic agent if at least one region of the first sample has a staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is likely to respond to the first therapeutic agent; and/or

one or more of the additional therapeutic agent(s) if at least one region of the additional sample(s) has a staining pattern for the corresponding additional predictive biomarker indicating that at least a portion of the tumor is likely to respond to the additional therapeutic agent.

7. The method of any of claims 1–6, wherein the tumor is a solid tumor.

8. The method of claim 7, wherein the solid tumor is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, and the first sample and the additional sample(s) are microtome sections of the FFPE tissue sample.

9. The method of claim 8, wherein first sample and the additional sample(s) are serial sections.

10. The method of any of claims 1–9, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

11. The method of any of claims 1–10, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

12. A system comprising:

(a) a set of microscope slides comprising:

(a1) a first microscope slide having deposited thereon a first sample of a tumor, wherein the first sample is histochemically stained for a first predictive biomarker for a first therapeutic agent;

(a2) one or more additional unstained microscope slides having deposited thereon an additional sample of the tumor;

(b) an image analysis system for identifying one or more regions of the first sample having a staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is unlikely to respond to the first therapeutic agent;

(c) an automated dissection tool programmed to excise the one or more regions of the first sample having a staining pattern for the first predictive biomarker characteristic of a lack of response to the first therapeutic agent from the first sample;

(d) a next generation sequencer programmed to identify the presence or absence of mutations correlated with one or more additional predictive biomarkers in a

nucleic acid sample derived from the regions of the first sample excised by the automated dissection tool; and

(e) an automated slide stainer programmed to stain the additional slide(s) with one or more of the additional predictive biomarker(s).

13. The system of claim 12, further comprising:

(f) a laboratory information system (LIS) comprising a database, the database containing:

(f1) a mutation analysis of the nucleic acid sample by the next generation sequencer, wherein the mutation analysis indicates at least the presence or absence of mutations in the nucleic acid sample correlating to one or more additional predictive biomarker(s) for one or more additional therapeutic agent(s); and

(f2) instructions for directing the automated slide stainer to stain the second sample of the tumor with the one or more additional predictive biomarkers identified by the mutation analysis.

14. The system of claim 12, wherein at least one of the unstained microscope slides has affixed thereto a label generated by the LIS and readable by the automated slide stainer, wherein the label identifies the slide as being appropriate for execution of the instructions of (f2) by the automated slide stainer.

15. The system of claim 14, wherein the label automatically directs the automated slide stainer to execute the instructions on the second sample.

16. The system of claim 14, wherein the label generates a report for an operator of the automated slide stainer, the report instructing the manual operator to program the automated slide stainer to execute the instructions on the second sample.

17. The system of any of claims 12–16, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

18. The system of any of claims 12–17, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

19. A system comprising:

(a) a nucleic acid sample derived from one or more regions excised from a first sample of a tumor, wherein the first sample of the tumor is stained for a first predictive biomarker, and further wherein the one or more regions excised from the section have a staining pattern of the first predictive biomarker indicating that at least a portion of the tumor is unlikely to respond to a first therapeutic agent;

(b) a next generation sequencer adapted to identify the presence or absence of mutations correlating to one or more additional predictive biomarkers;

(c) a laboratory information system (LIS) comprising a database, the database containing:

(c1) mutation analysis of a nucleic acid sample by next generation sequencing, wherein the mutation analysis indicates at least the presence or absence of mutations in the nucleic acid sample, the mutations correlating to one or more additional predictive biomarker(s) for one or more additional therapeutic agent(s); and

(c2) instructions for directing an automated slide stainer to stain a second sample of the tumor with the one or more additional predictive biomarkers identified by the mutation analysis.

20. The system of claim 19, further comprising:

(d) an unstained microscope slide having deposited thereon the second sample of the tumor; and

(e) an automated slide stainer adapted to stain the second sample according to the instructions of (c2).

21. The system of claim 20, wherein the unstained microscope slide has affixed thereto a label generated by the LIS and readable by the automated slide stainer, wherein the label identifies the slide as being appropriate for execution of the instructions of (c2) by the automated slide stainer.

22. The system of claim 21, wherein the label automatically directs the automated slide stainer to execute the instructions on the second sample.

23. The system of claim 21, wherein the label generates a report for an operator of the automated slide stainer, the report instructing the manual operator to program the automated slide stainer to execute the instructions on the second sample.

24. The system of any of claims 19–23, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

25. The system of any of claims 19–24, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

26. A system comprising:

(a) an unstained microscope slide having deposited thereon a first sample of a tumor;

(b) an automated slide stainer; and

(c) a laboratory information system (LIS) comprising a database, the database containing:

(c1) diagnostic information of a second sample of the tumor, wherein the second sample is stained for a first predictive biomarker for a first therapeutic agent;

(c2) mutation analysis of a nucleic acid sample by next generation sequencing, wherein the nucleic acid sample is obtained from a portion of the second sample having a staining pattern for the first predictive biomarker indicating that at least a portion of the second sample is unlikely to respond to the first therapeutic agent, and wherein the mutation analysis indicates at least the presence or absence of mutations in the nucleic acid sample correlating, the mutations correlating to one or more additional predictive biomarker(s) for one or more additional therapeutic agent(s); and

(c3) instructions for directing the automated slide stainer to stain the first sample of the tumor with the one or more additional predictive biomarkers identified by the mutation analysis.

27. The system of claim 26, wherein the unstained microscope slide has affixed thereto a label generated by the LIS and readable by the automated slide stainer, wherein the label identifies the slide as being appropriate for execution of the instructions of (c2) by the automated slide stainer.

28. The system of claim 27, wherein the label automatically directs the automated slide stainer to execute the instructions on the second sample.

29. The system of claim 27, wherein the label generates a report for an operator of the automated slide stainer, the report instructing the manual operator to program the automated slide stainer to execute the instructions on the second sample.

30. The system of any of claims 26–29, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

31. The system of any of claims 26–30, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

32. The system of any of claims 12–31, wherein the tumor is a solid tumor.

33. The system of claim 32, wherein the solid tumor is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, and the samples of the tumor are microtome sections of the FFPE tissue sample.

34. The system of claim 33, wherein sample stained for the additional predictive biomarker(s) is a serial section of the sample stained for the first predictive biomarker.

35. A set of diagnostic samples derived from a tumor, said set of diagnostic samples comprising:

(a) a first sample of a tumor, wherein the first sample is stained for a first predictive biomarker for a first therapeutic agent, wherein at least a portion of the first sample has a first staining pattern for the first predictive biomarker indicating that at least a portion of the tumor is unlikely to respond to the first therapeutic agent;

(b) a nucleic acid sample obtained by a method comprising:

(b1) excising with a automated dissection tool the portion of the first sample having the staining pattern indicating that the portion of the tumor is unlikely to respond to the first therapeutic agent; and

(b2) extracting the nucleic acid sample from the excised portion of the first sample in a manner compatible with use of the nucleic acid sample in a next generation sequencer; and

(c) one or more additional samples of the tumor, wherein the additional sample(s) are stained for one or more additional predictive biomarker(s) for one or more

additional therapeutic agent(s), wherein the additional predictive biomarker(s) correspond(s) to a mutation identified in the nucleic acid sample.

36. The set of diagnostic samples of claim 35, wherein the tumor is a solid tumor.

37. The set of diagnostic samples of claim 36, wherein the solid tumor is a formalin-fixed, paraffin-embedded (FFPE) tissue sample, and the samples of the tumor are microtome sections of the FFPE tissue sample.

38. The set of diagnostic samples of claim 37, wherein sample stained for the additional predictive biomarker(s) is a serial section of the sample stained for the first predictive biomarker.

39. The set of diagnostic samples of any of claims 35–38, wherein the next generation sequencer operates on a principle selected from the group consisting of pyrosequencing, cyclic reversible termination, semiconductor sequencing technology, and phospholinked fluorescent nucleotides.

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40. The set of diagnostic samples of any of claims 35–39, wherein the first predictive biomarker and the additional predictive biomarker(s) are selected from the group consisting of ALK, ATM, BCL2, BRAF, BRCA1, c-KIT, CAIX, CCR4, CD30, Claudin, 17p13.1, DLL3, EGFR1, estrogen receptor, EREG, ERCC1, FGF19, FGFR2b, FGFR3, FOLR1, hyaluronan, HER2/NEU, K-ras, MGMT, MSLN, p53, MDM2, progesterone receptor, PD-L1, PDGFRB, PTEN, and thymidine phosphorylase.

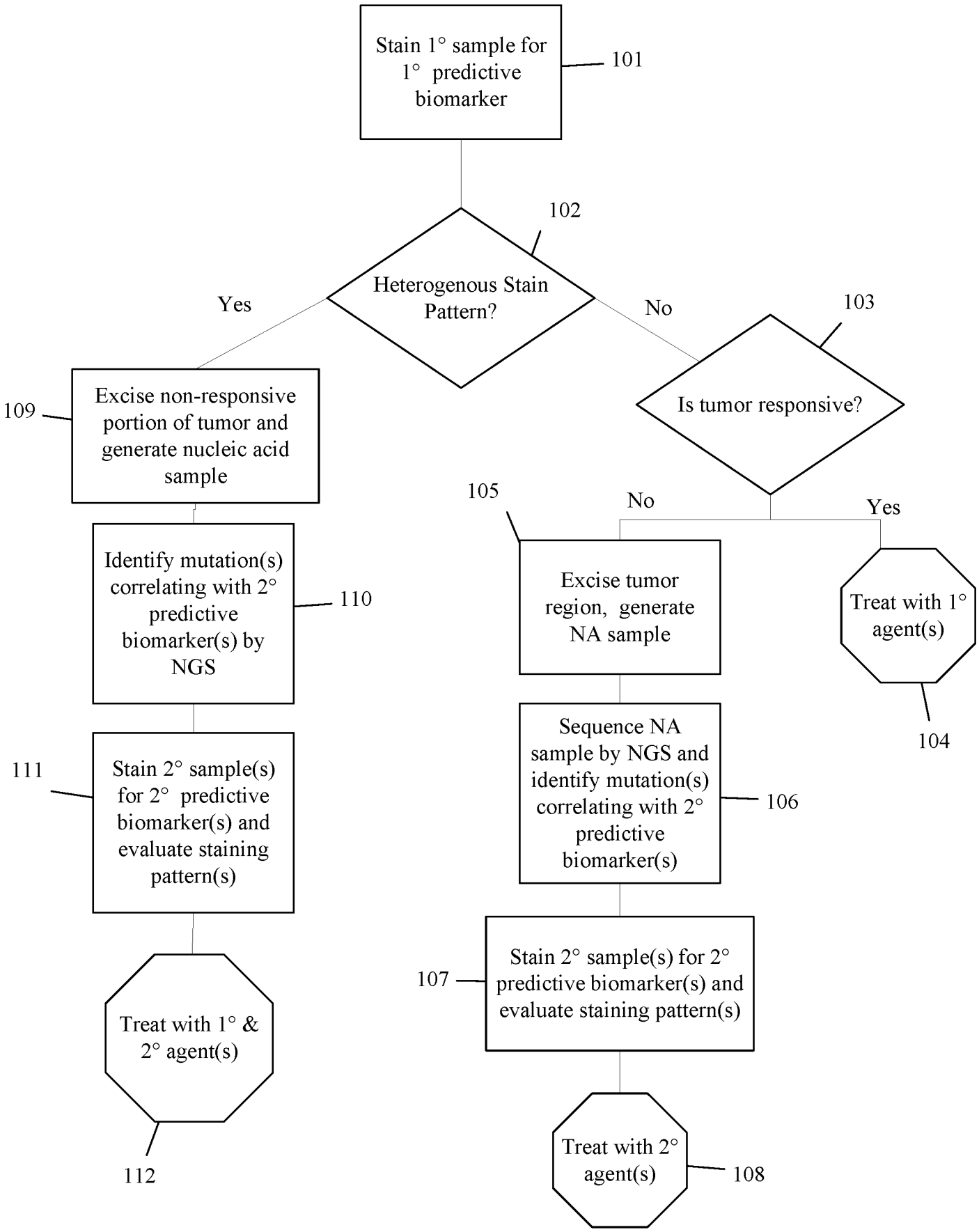

Stain 1° sample for 1° predictive biomarker
101
Heterogenous Stain Pattern?
102
Yes
No
Excise non-responsive portion of tumor and generate nucleic acid sample
109
Identify mutation(s) correlating with 2° predictive biomarker(s) by NGS
110
Stain 2° sample(s) for 2° predictive biomarker(s) and evaluate staining pattern(s)
111
Treat with 1° & 2° agent(s)
112
Is tumor responsive?
103
No
Yes
Excise tumor region, generate NA sample
105
Treat with 1° agent(s)
104
Sequence NA sample by NGS and identify mutation(s) correlating with 2° predictive biomarker(s)
106
Stain 2° sample(s) for 2° predictive biomarker(s) and evaluate staining pattern(s)
107
Treat with 2° agent(s)
108

Fig. 1

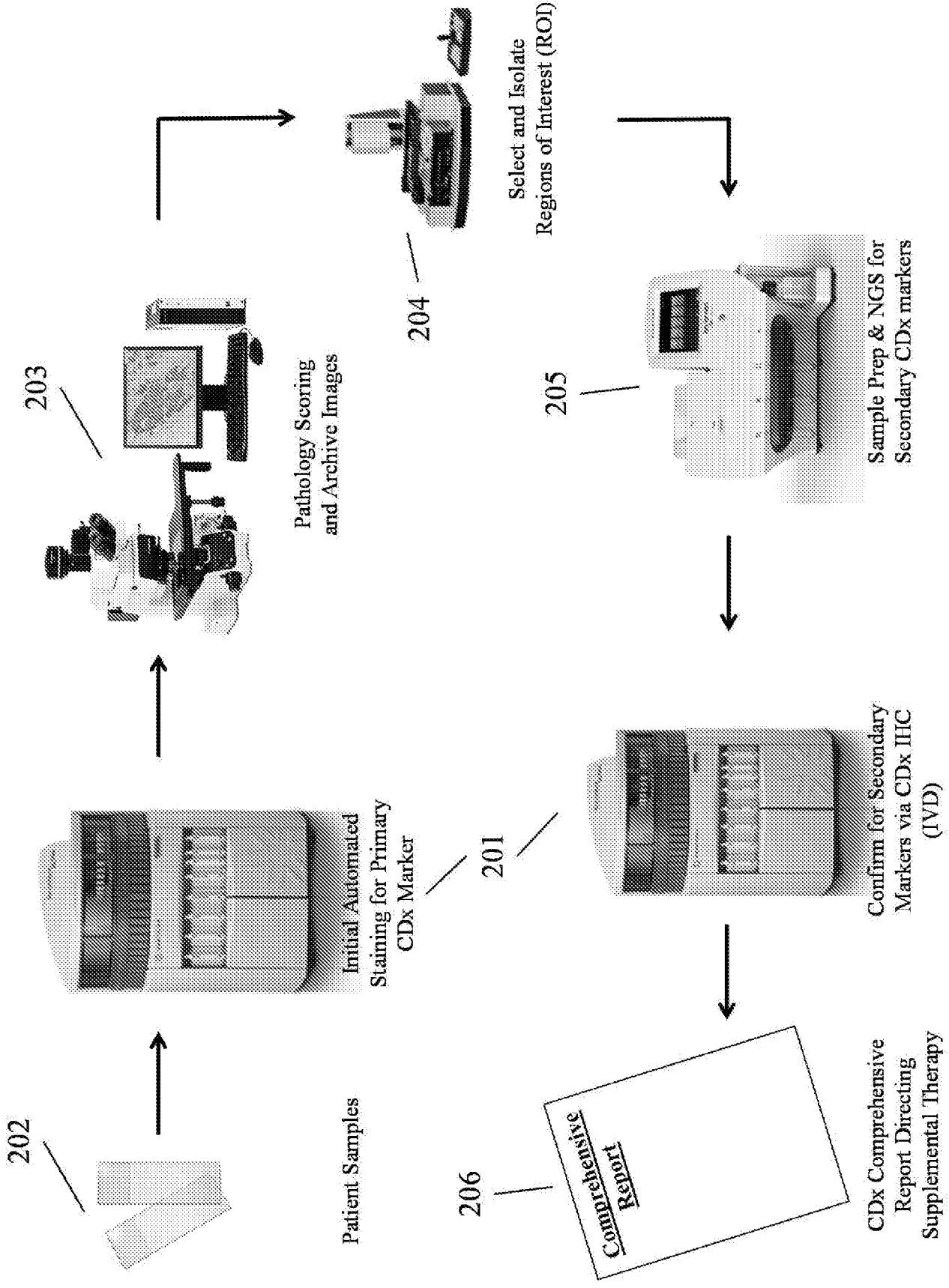

202
Patient Samples
201
Initial Automated Staining for Primary CDx Marker
203
Pathology Scoring and Archive Images
204
Select and Isolate Regions of Interest (ROI)
205
Sample Prep & NGS for Secondary CDx markers
Confirm for Secondary Markers via CDx IHC (IVD)
206
Comprehensive Report
CDx Comprehensive Report Directing Supplemental Therapy

Fig. 2

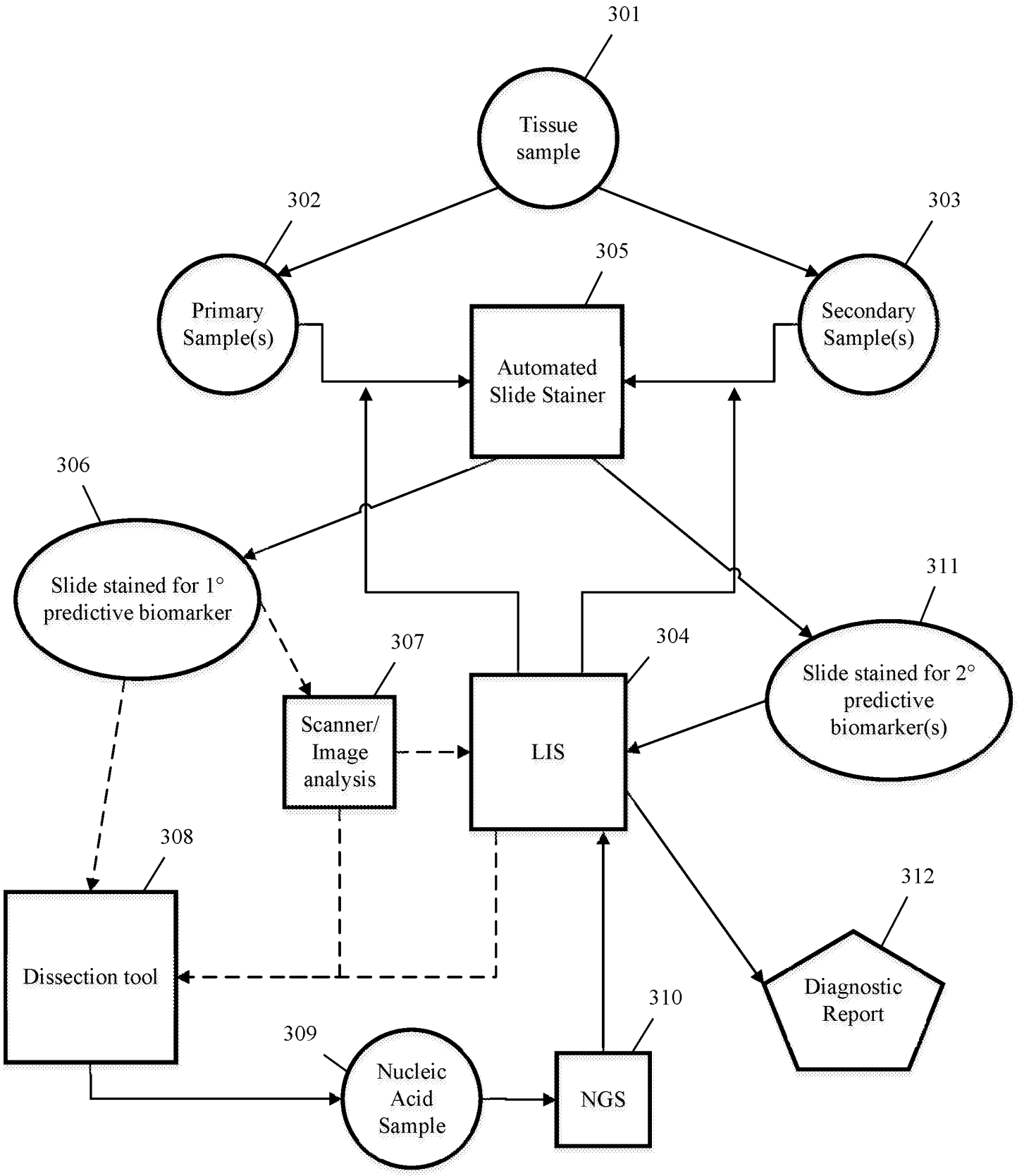
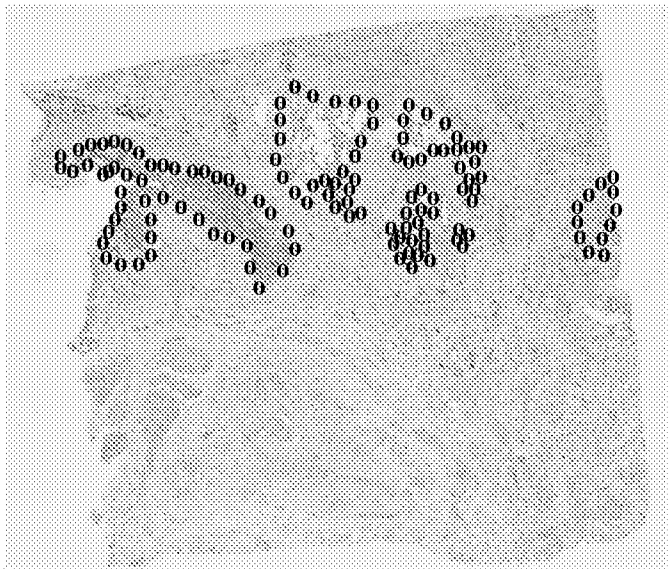
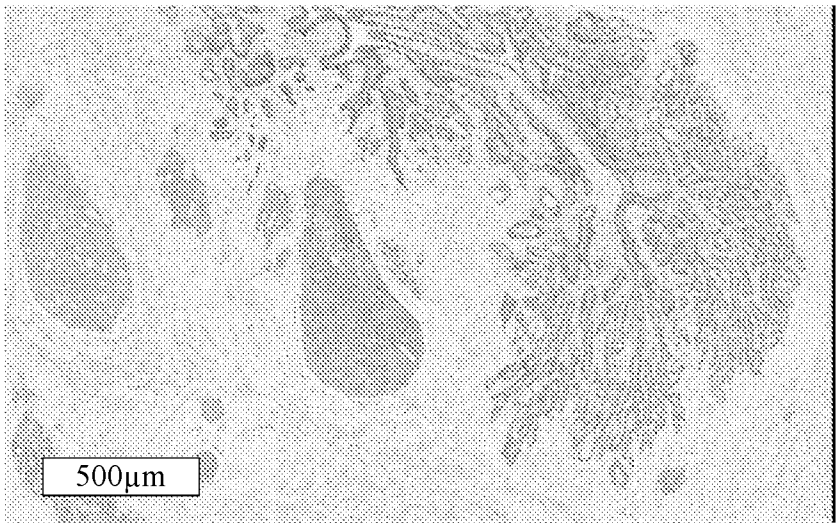

301
Tissue sample
302
303
Primary Sample(s)
305
Automated Slide Stainer
Secondary Sample(s)
306
Slide stained for 1° predictive biomarker
311
307
304
Slide stained for 2° predictive biomarker(s)
Scanner/ Image analysis
LIS
308
312
Dissection tool
Diagnostic Report
310
309
Nucleic Acid Sample
NGS

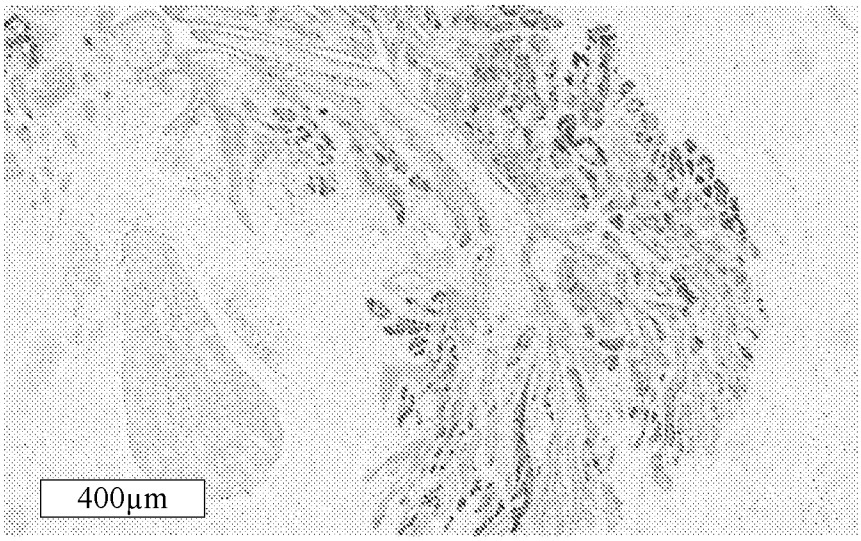
Fig. 3



PTEN

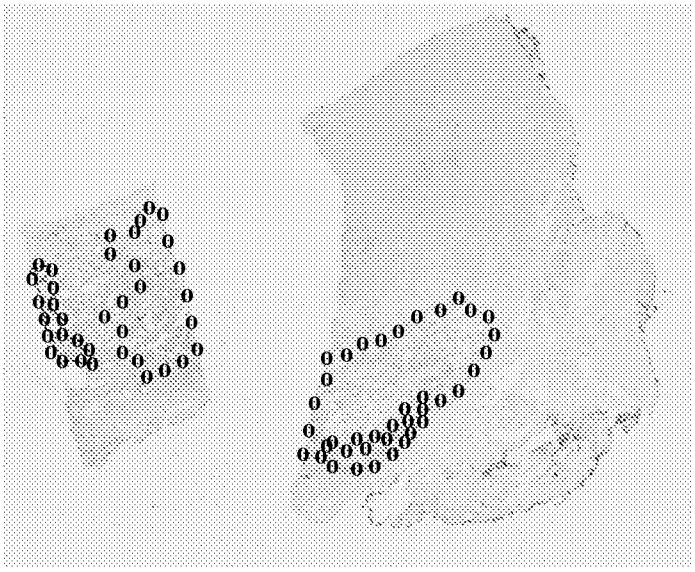


EGFR

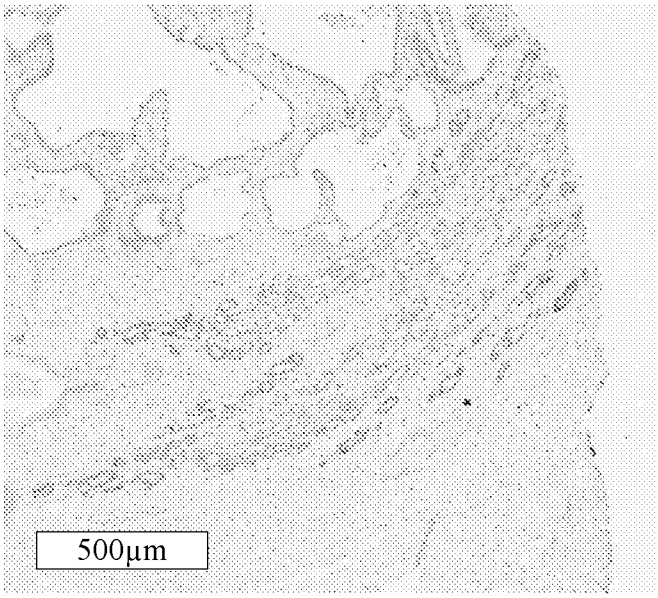


HER2/ ERBB2

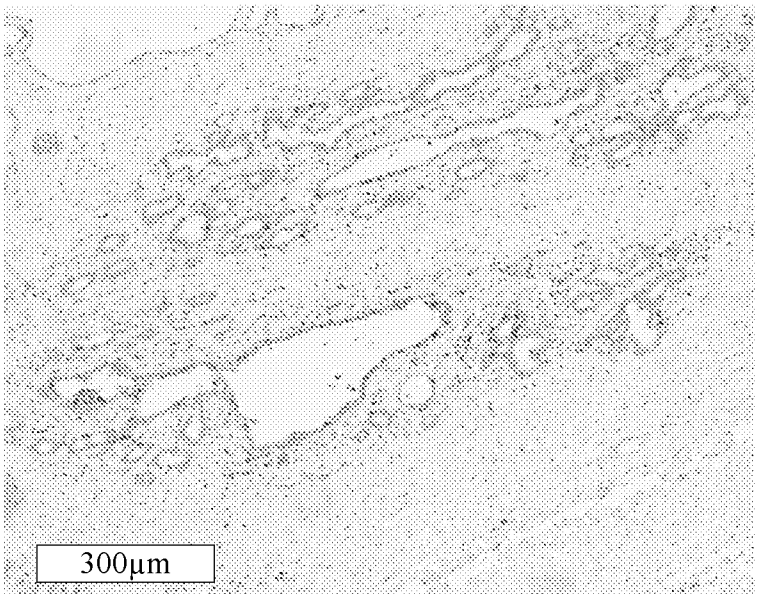
Fig. 4



PTEN



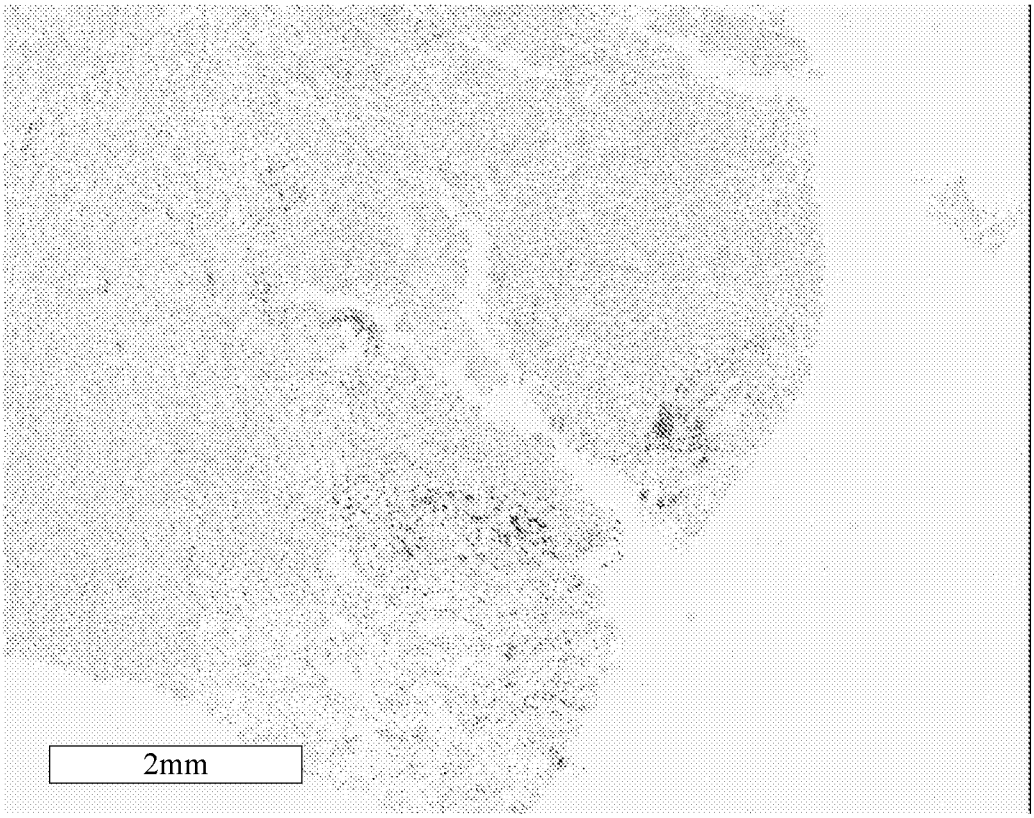
EZH2



EZH2

Fig. 5

EGFR L858R



p53

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/014969

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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)
C12Q

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NIR PELED ET AL: "Next-Generation Sequencing Identifies and Immunohistochemistry Confirms a Novel Crizotinib-Sensitive ALK Rearrangement in a Patient with Metastatic Non-Small-Cell Lung Cancer", JOURNAL OF THORACIC ONCOLOGY, vol. 7, no. 9, 1 September 2012 (2012-09-01), pages e14-e16, XP055063267, ISSN: 1556-0864, DOI: 10.1097/JTO.0b013e3182614ab5 page e14; figure 1 abstract	1-40

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent 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
28 March 2017	05/04/2017

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, Fax: (+31-70) 340-3016	Reuter, Uwe

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANDREA MAFFICINI ET AL: "Reporting Tumor Molecular Heterogeneity in Histopathological Diagnosis", PLOS ONE, vol. 9, no. 8, 15 August 2014 (2014-08-15) , page 1, XP055357016, DOI: 10.1371/journal.pone.0104979 pages 2,4,8; table 2	1-40
A	Thermofisher: "Uncovering tumor heterogeneity in FFPE samples by laser capture microdissection and next-generation sequencing", , 31 December 2015 (2015-12-31), XP055356504, Retrieved from the Internet: URL:https://tools.thermofisher.com/content/sfs/brochures/PG1395-PJ6555-CO29666-LCM-App-note-Focus-on-LCM-for-tumor-heterogeneity-Global-FHR.pdf [retrieved on 2017-03-20] page 2 page 4	1-40
A	US 2015/361508 A1 (VON HOFF DANIEL D [US] ET AL) 17 December 2015 (2015-12-17) pages 27,29	1-40
A	Soma Datta ET AL: "Laser capture microdissection: Big data from small samples", Histology and histopathology, 1 November 2015 (2015-11-01), page 1255, XP055356531, Spain DOI: 10.14670/HH-11-622 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4665617/pdf/nihms707886.pdf figure 3	1-40
A	M.-T. LIN ET AL: "Clinical Validation of KRAS, BRAF, and EGFR Mutation Detection Using Next-Generation Sequencing", AMERICAN JOURNAL OF CLINICAL PATHOLOGY, vol. 141, no. 6, 16 May 2014 (2014-05-16), pages 856-866, XP055358532, US ISSN: 0002-9173, DOI: 10.1309/AJCPMWGWGO34EGOD page 9, paragraph 3	1-40

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/014969

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